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Conessine alleviates PTZ-induced epilepsy in rat model via attenuating neuroinflammation and oxidative stress

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ABSTRACT

Epilepsy is a complex neurological disorder which affects the quality life of individual and also hinders the economic status of both the individuals and the country since it requires prolonged medications. The etiology of epilepsy is varied and the neuroinflammation has been considered to be the prime initiator and the stimulator of the disease progression. Therefore anti-inflammatory drugs are suggested to treat seizure and also to prevent epilepsy. The currently available antiepileptic drugs cause's adverse effects which obstructs the usage of these drugs and also these drugs are not cost effective. Phytochemicals are effective alternative to these drugs and it is prescribed in the traditional medicine. Conessine, a steroidal alkaloid present in bark extracts of Holarrhena sps possess antibiotic, anti-diarrheal, antimalarial and anti-inflammatory properties. We evaluated the antiepileptic potency of conessine in PTZ challenged animal model treated with 10 and 20 mg/kg of conessine respectively. The seizure induction, severity and the behavioral changes were observed in the rats. The impact of conessine on neurochemical signaling in PTZ challenged rats were assessed via quantifying the neurotransmitter and ATPase levels in the brain tissue. Antioxidants and MDA levels were measured to evaluate the antioxidant potency of conessine in epilepsy induced rats. iNOS and nNOS which are inducers of NO during epileptic conditions were analyzed in the test rats. The stimulators and inflammatory cytokines levels were quantified in the brain to analyze the anti-neuroinflammatory efficacy of the conessine in PTZ challenged rats. AKT/mTOR prime signaling proteins in initiation and progression of epilepsy was quantified in the brain tissue of experimental animals. Neurodegeneration inducers cytochrome *c*, NF-κB, COX-2 and Caspase 3 were quantified to evaluate the ameliorative potency of conessine against PTZ induced neuronal damage. The neuroprotective and antiepileptic potency of conessine was confirmed with the histopathological scoring of brain tissue in PTZ challenged rats. Conessine treatment effectively inhibited the seizure induction and regulated the neurochemical signaling in epileptic induced rats. It also attenuated the neuroinflammation and oxidative stress induction induced by PTZ treatment. The prime signaling proteins nitric oxide synthatase and AKT/mTOR signaling proteins were effectively inhibited by the conessine treatment which eventually prevented the neurodegeneration inducers. Conessine treatment significantly enhanced the levels of Caspase 3. The histopathological analysis confirms the neuroprotective, anti-inflammatory and antiepileptic potency of conessine. Conessine treatment also doesn't caused any behavioral alterations in the rats hence it is safe and potent alternative for currently available antiepileptic drugs.

1. Introduction

Epilepsy, foremost chronic neurological disorder marked by periodic, unprovoked convulsions, which may be partial or generalized (Walton et al., 2021; Paz and Huguenard, 2015). This condition represents 1 % of world disease encumbrance out which the low and middle income countries contribute 80 % (Zhang et al., 2023). Although epilepsy affects both sexes and all age groups, the incidence is notably high

Abbreviations: PTZ, Pentylenetetrazole; MDA, Malondialdehyde; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; AKT, Protein Kinase B; mTOR, Mammalian target of rapamycin; COX-2, Cyclooxygenase 2; NF-κB, Nuclear factor kappa B; TNF-α, Tumor Necorsis Factor alpha; IL-R-1, Interleukin receptor 1; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; HMGB1, High mobility group box 1; TLR-4, Toll like receptor 4; Na^+ K^+-ATPase, Sodium Potassium ATPase; Ca^{2+} ATPase, Calcium ATPase; GABA, gamma-aminobutyric acid.

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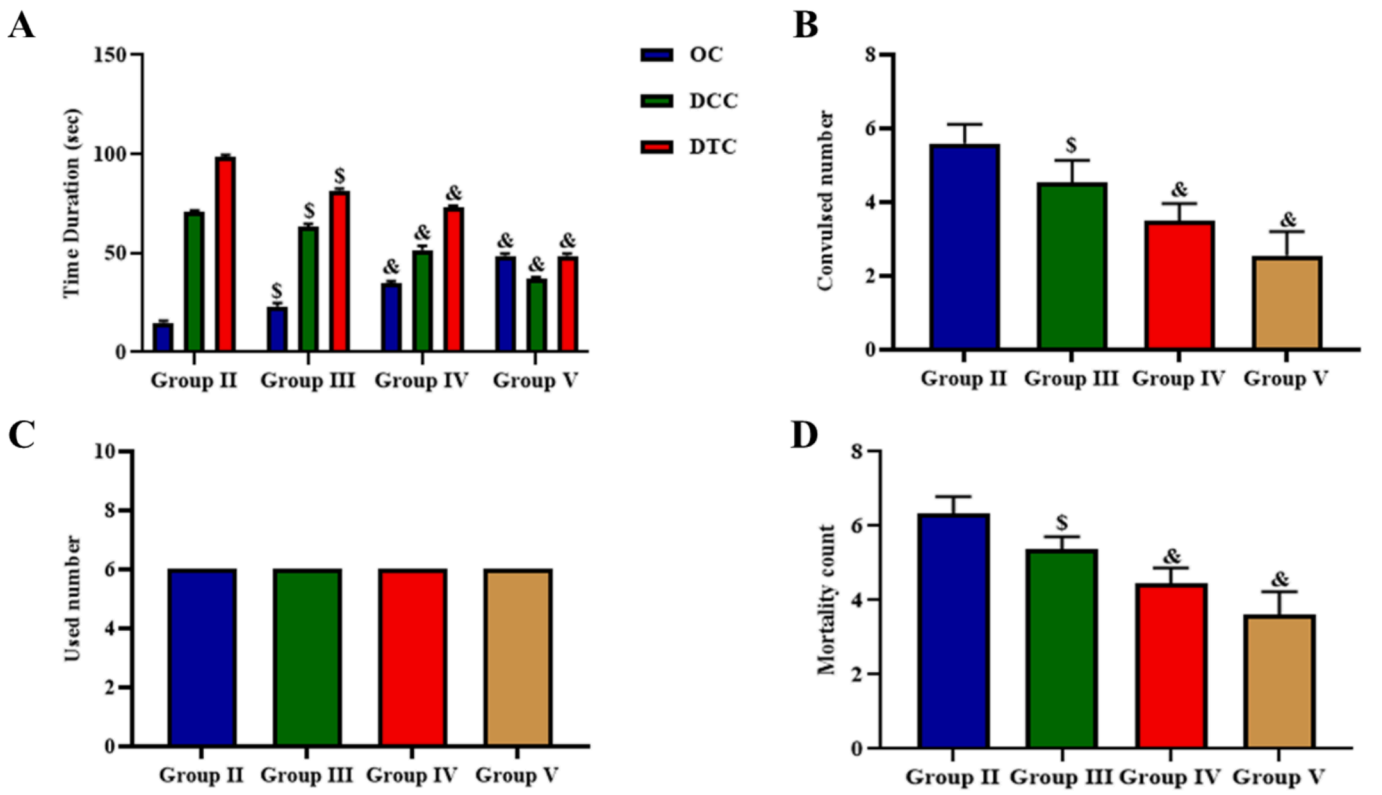

Fig. 1. Impact of conessine against seizure induction by PTZ. A) Epileptic duration, B) Number of convulsed rats, C) Number of rats used, D) Mortality count. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant. Note: OC: Onset of convulsion; DCC: Duration of clonic convulsion; DTC: Duration of tonic convulsion.

among children and adolescents. Each year, 5 million epilepsy incidence were reported worldwide (Biset et al., 2024). Individuals with uncontrolled epilepsy frequently experience additional health issues, increased healthcare expenses, and greater threats of damages, status epilepticus, and sudden unexpected death in epilepsy. The etiology of epilepsy can be diverse, stemming from various diseases and injuries, though in about one in two cases, the exact reason remains unidentified (Chouchi et al., 2019; Neligan and Hauser, 2012). Memory issues are prevalent among those with epilepsy, contributing to the disease's significant impact on patients' quality life (Solati et al., 2019).

While the precise molecular mechanisms behind acquired epileptogenesis remain unclear, evidence from the last twenty years indicates that inflammation initiated by damage to brain may lead to epilepsy (Chen et al., 2023). Neuroinflammation has been implicated in various neurological disorders can initiate epileptogenic processes via glia cells activation, brain infiltration of immune cells which consequently disrupts the balance among glutamatergic and GABAergic signaling, lowers the seizure threshold, and contributes to the life time epileptic episodes (Marchi et al., 2014; Pracucci et al., 2021). Extensive preclinical studies in animal models indicate that anti-inflammatory therapies targeting specific inflammatory components are effective in attenuating convulsions and preventing epilepsy (Vezzani et al., 2019; Li et al., 2023; Rabidas et al., 2023).

Chronic epilepsy patients entail persistent treatment with antiepileptic drugs (AEDs), and those experiencing refractory epilepsy requires polytherapy (Cavalleri et al., 2011). Approximately 40 % of epilepsy patients are resistant to AEDs and other currently prescribed treatments (Galovic et al., 2021). Patients on AEDs often report side effects such as memory impairment, cognitive decline, retinal dysfunction, anemia, lymphoma, and hormonal imbalances (Srivastav et al., 2023). The need for prolonged medication also imposes financial strain on healthcare systems, particularly in middle and low-income countries (WHO, 2024). Despite advancements in seizure control, effective epilepsy treatment remains challenging. Comprehensive therapy must extend beyond seizure management to address cognitive impairments and enhance the overall quality of life for patients.

Despite advancements in conventional medicine, around 70 % of people in developing countries continue to depend on complementary and alternative medicines (Muhammad et al., 2022). Phytotherapy is widely used around the world as a complementary treatment for epilepsy. Medicinal plants are often regarded as a gentler and safer option compared to chemical antiepileptic drugs (Kaur et al., 2021). In traditional medicine such as Chinese, Thai and Ayurveda the Holarrhena sps plant stem and bark extracts were utilized to treat various diseases (Chakraborty and Brantner, 1999; Kumar et al., 2007). The extracts consist of steroid alkaloids which tends to render antibiotic, antifeedant, antidiarrheal, astringent properties (Siriyong et al., 2018). In this study we examined the antiepileptic potency of the one of steroidal alkaloid conessine present in the extracts of Holarrhena sps plant belonging to the Apocynaceae family.

2. Materials and methods

2.1. Chemicals

The conessine and other chemicals were procured from Sigma Aldrich, USA. All the assay kits to evaluate the biochemical markers were acquired from Cusabio, RayBiotech, and Abcam, USA, respectively.

2.2. Animals

Eight weeks aged healthy albino Wistar rats (200–240 g) were housed in the sterile polyacrylic cages with free access to food and

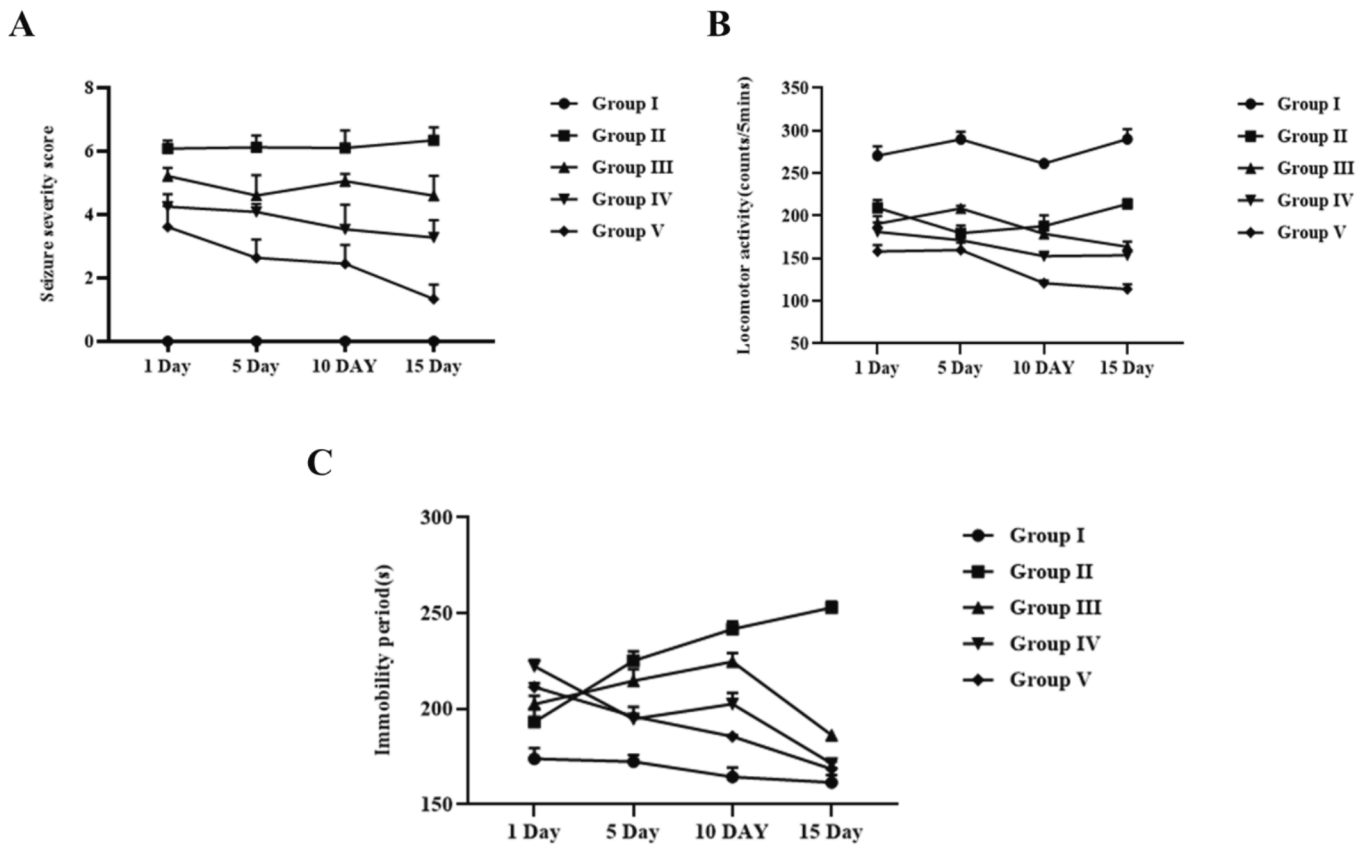

Fig. 2. Ameliorative effect of conessine against seizure and behavioural changes induced by PTZ. A) Seizure severity score, B) Locmotor activity score, C) Immobility period assessed by Open Field test. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

water. The international standard laboratory conditions prescribed by the animal ethical committee for housing rats was strictly maintained in the animal housing laboratory. All the procedures performed on the test animals were approved by the members of ethical committee. The animals were handled with care and concern all efforts to minimize the trauma in animals were followed.

2.3. Treatment regimen

A total of 30 rats were grouped into five for treatment groups with six animals in each group (n = 6). Group I received a 0.9 % NaCl solution as a placebo. To induce epileptic episodes, Group II rats were administered 45 mg/kg of PTZ dissolved in 0.9 % saline intraperitoneally. Group III, IV rats received respectively 10 and 20 mg/kg of conessine orally 30 min before the PTZ treatment. Group V rats were given 2 mg/kg of the standard drug diazepam 30 min prior to the PTZ challenge. Following the PTZ challenge, the rats were carefully observed for 30 min to identify the onset and duration of seizure. The number of rats convulsed and the mortalities were recorded. Blood was collected from the experimental animals before subjecting to euthanization for biochemical analysis. The brain was excised from the euthanized and stored at −20 °C for further analysis.

2.4. Seizure scaling

Following the PTZ infusion the rats were placed in a plexiglass enclosure and observed 30 min to identify any convulsion symptoms. The intense of convulsion was categorized per the criteria outlined by Pourmotabbed et al. (2011). The delay in the onset of minimal clonic seizure (MCS) and generalized tonic-clonic seizure (GTCS) was measured as an indicator of seizure activity.

2.5. Open field test (OFT)

OFT was conducted to assess the effects of long-lasting stress on rats. OFT was designed to analyze the impact of PTZ-induced stress on the motivational activity of the animals on the 1st, 5th, 10th and 15th days of experiment. Rats were placed in the central area of the open field and allowed to roam freely for 5 min. During this test, two criteria were quantified: the rate of locomotion via exploring the squares and the immobility expressed by the freeze bouts in rats were recorded. The animals were acclimated to the testing environment for at least 2 h before the start of the test. The OFT was conducted in a soundproof area with no human intervention, using a 5 % ethanol solution the OFT maze was sterilized to eliminate any bias due to odors left by previous rats. The motivational activity of the mice was assessed randomly by impartial observer, and the mean outcomes were statistically evaluated.

2.6. Quantification of neurotransmitters

A 5 mg of brain tissues was pulverized into small fragments and homogenized in 500 μL of PBS using a glass homogenizer on ice. The resulting suspension was subjected to ultra sonication to further disrupt the cell membranes. Subsequently, the homogenates were centrifuged at 1500*g* for 15 min. The supernatant was used for the quantification of neurotransmitters. Dopamine, Glutamate, and GABA levels were quantified using the colorimetric assay kits procured form MyBiosource.com. The test was done according to the kit manual and the final absorbance was measured at 450 nm. The concentration of samples were calculated corresponding to the mean absorbance from the standard curve.

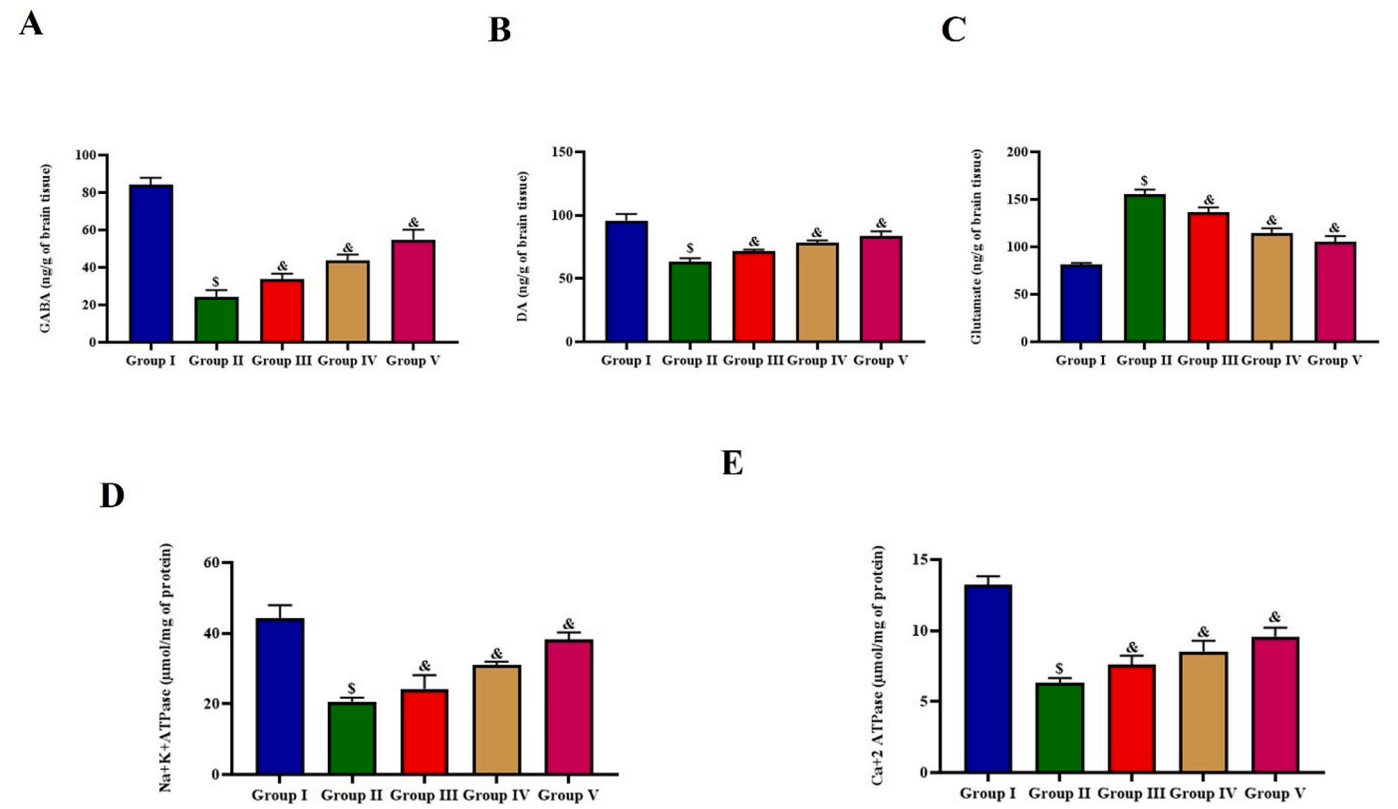

Fig. 3. Impact of conessine on neurochemical signaling in PTZ challenged rats. A) Gamma-aminobutyric acid (GABA), B) Dopamine, C) Glutamate, D) Na^+ K^+ ATPase, E) Ca^{2+}–ATPase. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

2.7. Quantification of ATPases

Na^+ K^+ ATPase activity homogenate of brain was quantified using the protocol described by Daemen et al. (1970) and the Ca^{2+}–ATPase activity was quantified with protocol prescribed by Hjerten and Pan 1983. The ATPase activities were quantified based on the levels of inorganic phosphorous which is generated during the conversion of ATP to ADP in the existence of Na^+ K^+ and Ca^{2+} ions correspondingly. The blue coloration produced due to reduction of hexavalent molybdenum of phosphomolybdate by the ANSA was measured at 620 nm using microplate reader. The test was conducted in triplicates.

2.8. Analysis of SOD and MDA

Superoxide dismutase and the reduced glutathione levels were quantified in the brain tissue homogenate using the protocol of Marklund and Marklund (1974) and Moron et al. (1979) correspondingly. The capacity of superoxide dismutase inhibiting the pyrogallol oxidation was measured to quantify the levels of SOD and the final absorbance was measured at 570 nm. Oxidative damage induced by PTZ was assessed by quantifying malondialdehyde levels in the brain tissue of conessine treated PTZ kindled rats. MDA was estimated by quantifying the levels of thiobarbituric acid reactive substance (TBARS) using the method prescribed Ohkawa et al. (1979). Nitric oxide levels were measured using the nitrate assay kit procured form Sigma Aldrich. The reaction nitrogen oxide with Griess reagent was measured at 540 nm.

2.9. Quantification of NOS

The interplay between NO, iNOS, and nNOS highlights the complex role of NO in epilepsy conditions hence we quantified the levels of NO, iNOS, and nNOS in the brain regions of the conessine treated convulsed rats. The NO, iNOS, and nNOS levels were quantified using the ELISA kits purchased from Cusabio, USA. The assay were performed in triplicates and the levels of NO, iNOS, and nNOS with standard curve.

2.10. Quantification of inflammation stimulating cytokines

Inflammation suggestively involved in the pathophysiology of epileptic episodes through various mechanisms. Targeting inflammation presents a potential therapeutic strategy for epilepsy therefore we evaluated the inflammatory inducing cytokines IL-1β, IL-6, TNF-α and IL-1-R1 using the ELISA kits procured from Abcam, USA. CUSABIO ELISA kits were used to quantify HMGB1, TLR-4 in the cortex and hippocampus of the experimental animals. The reagents, working standards, and samples were prepared freshly prepared as directed by the kit manual. The test was performed in triplicates and the final absorbance was read at 450 nm.

2.11. Quantification of AKT/mTOR signaling proteins

The AKT/mTOR signaling pathway acts a significant role in the pathophysiology of epileptic episodes via its role on cell growth, survival, synaptic plasticity, and neuroinflammation. The total AKT, mTOR and phosphorylated AKT, mTOR protein in the brain regions of the experiment rats were quantified with ELISA kits purchased from Ray-Biotech, USA. The assay was performed in triplicates and the final absorbance was measured 450 nm.

2.12. Analysis of neurodegeneration

Caspase-3, cytochrome *c*, NF-κB, and COX-2 play significant roles in the mechanisms underlying epilepsy, particularly through their contributions to inflammation, apoptosis, and oxidative stress. Apoptosis

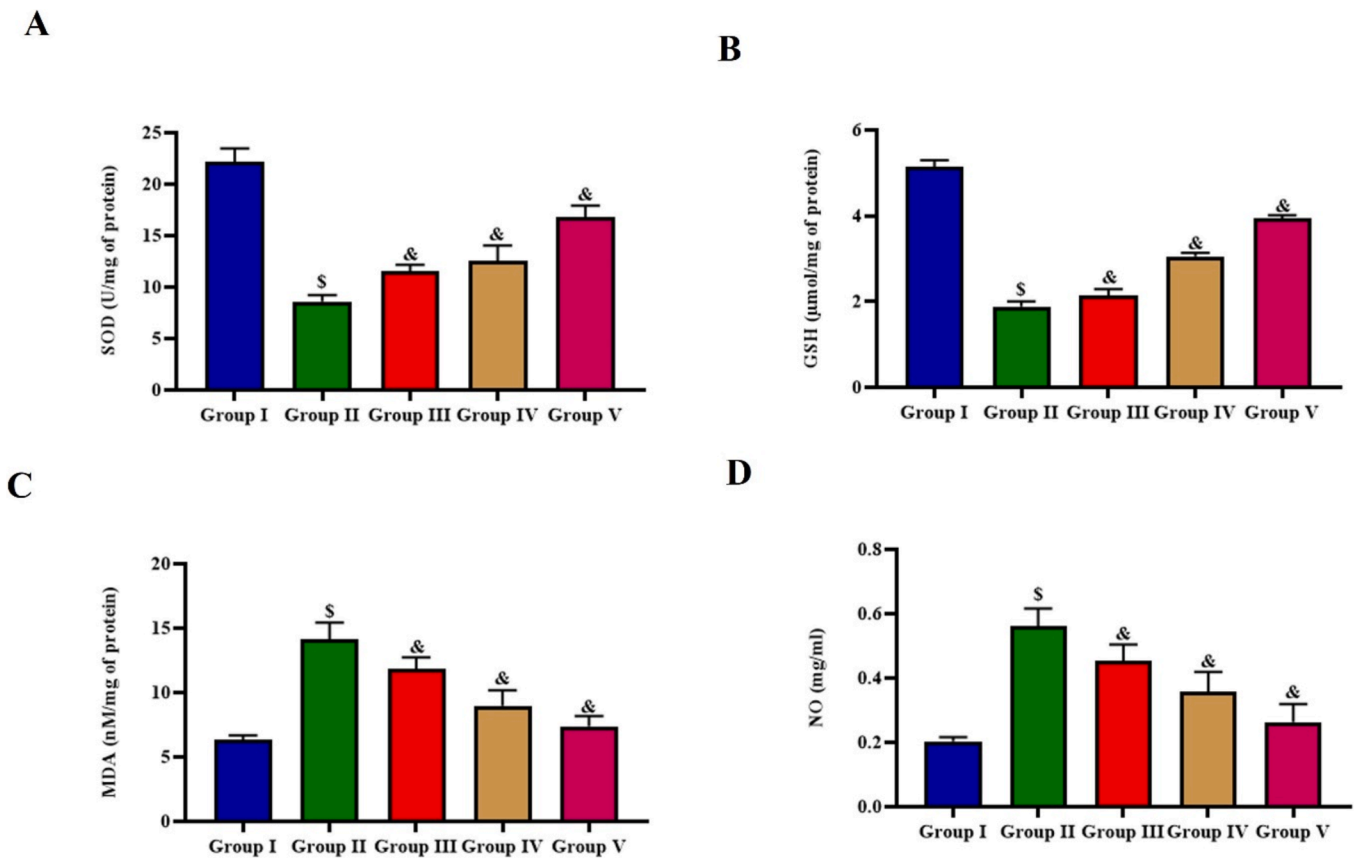

Fig. 4. ROS species neutralizing potency of conessine in PTZ challenged rats. A) Enzymatic antioxidant SOD, B) Non-enzymatic antioxidant GSH C) Lipid peroxidation level MDA, D) Nitric oxide in the hippocampus and cortex region of the experimental rats. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

inducers Caspase-3, cytochrome *c* were in the brain region of the test animals with the ELISA kits from Abcam. USA. ELISA kits were used for the quantification of inflammation inducers NF-κB, and COX-2.

2.13. Histopathological analysis and scoring

10 % neutral formalin fixed brain tissue were dehydrates for 12 h. The rinsed samples were subjected to ethanol dehydration, xylene treatment and paraffinization. Sections of 4–5 µm thickness were cut from the paraffin blocks using a microtome. All samples underwent hematoxylin and eosin staining for histopathological examination. The histopathological assessment concentrated on inflammatory influx, blood vessel congestion, pyknosis, and neuronal necrosis. Various areas of each section were examined under a 40x objective lens. The severity of the findings was graded on a scale of 0–10. Evaluation and imaging of the samples were performed using a binocular light microscope by a histopathologist who was blinded to the treatment groups.

2.14. Statistical analysis

Results obtained from various experiments were presented as mean ± SEM. One-way ANOVA and Tukey's multiple comparison test were performed to detect the intergroup and intragroup statistical difference respectively. The data analyzed were performed with SPSS version 20.0 software, and Significance level was considered at $p < 0.05$.

3. Results

3.1. Impact of conessine against seizure induction by PTZ

Fig. 1(A–D) depicts the epileptic action of PTZ in rats and the ameliorative effect of conessine against the seizure. Control group rats doesn't exhibited any sign of convulsion and nil mortalities was observed whereas in the PTZ challenged rats both convulsions and mortalities were observed (Fig. 1D). All the six number of rats were convulsed in the PTZ challenged rats whereas the number of rats convulsed were decreased in the conessine and the standard epileptic drug diazepam treated rats (Fig. 1B). The onset of convulsion was observed within few seconds in the PTZ challenged rats whereas it is delayed in the conessine and diazepam injected rats. The duration of clonic and tonic convulsions where also decreased in conessine treated rats compared to PTZ challenged rats (Fig. 1A). Compared to 10 mg conessine treated rats the 20 mg conessine rats shown decreased convulsion episodes.

3.2. Ameliorative effect of conessine against seizure and behavioral changes induced by PTZ

Fig. 2A illustrates the seizures severity score of PTZ challenged and the drugs treated groups. The severity score remained to be high in the PTZ challenged rats throughout the treatment period whereas the seizure severity scores tends to decline in conessine treated rats from day 5 onwards. On 15th day of treatment a considerable decrease in the seizure severity was observed in both doses of conessine treated rats. Diazepam injected animals shown suggestively decreased intensities of

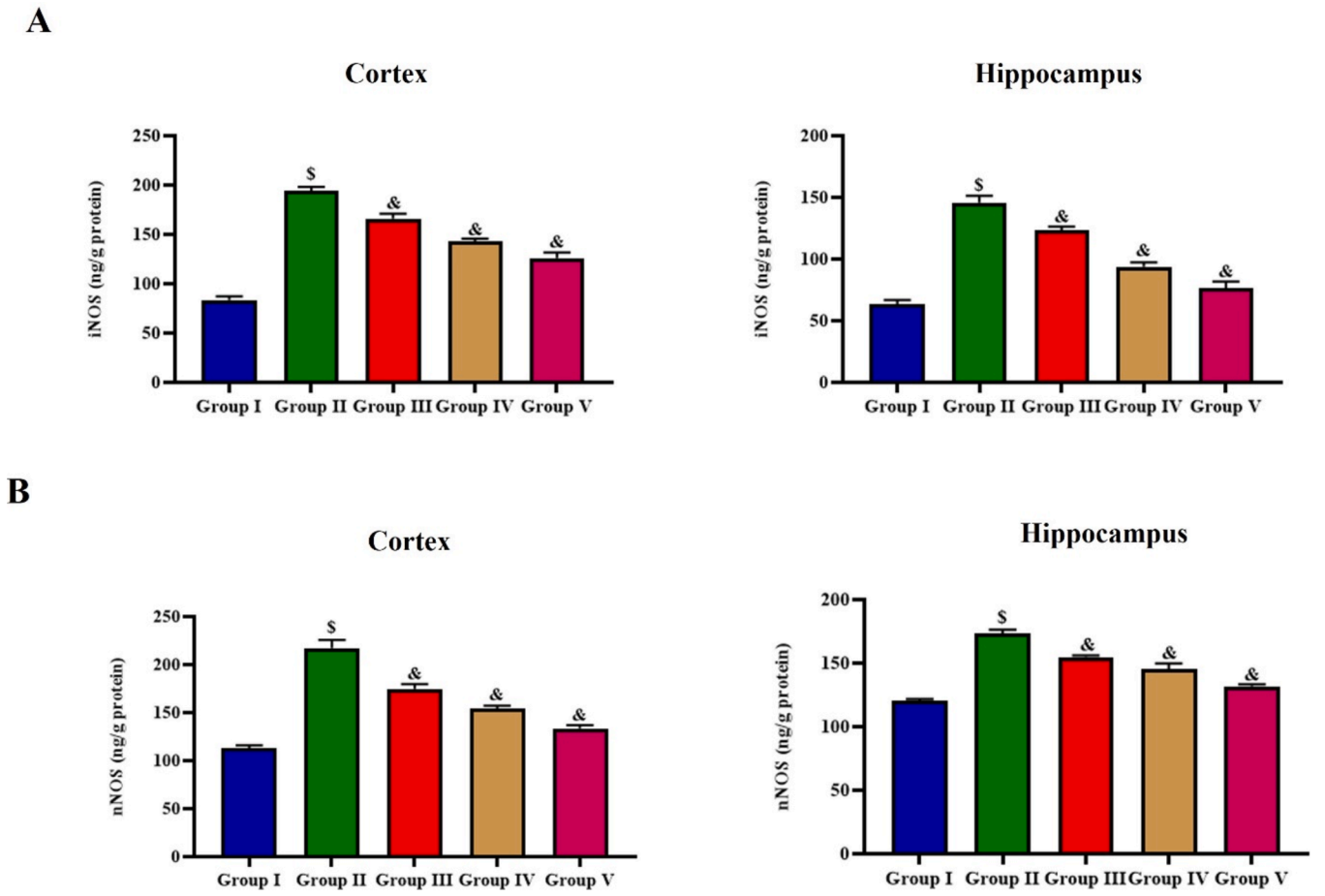

Fig. 5. Impact of conessine on nitric oxide synthase in PTZ challenged rats. A) Inducible Nitric Oxide Synthase (iNOS), B) neuronal Nitric Oxide Synthase (nNOS) in the hippocampus and cortex region of the experimental rats. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

seizure severity than the other groups.

Fig. 2B exhibits the results of locomotor activity of the rats observed in the OFT analysis. On day 1 all the rats exhibited decreased exploratory behavior and on final day of the treatment the exploratory behavior of control rats was increased compared to the other groups. On final day of treatment the exploratory behavior was decreased in both conessine and diazepam injected animals than the PTZ challenged rats.

The freeze bouts exhibited by the experimental rats were depicted in Fig. 2C. On the initial day of treatment than the other groups control group rats exhibited decreased duration of freeze bouts and it is further decreased at the termination of the treatment period. Whereas the PTZ challenged rats shown increased duration of freeze bouts at the end of the treatment. A 20 mg/kg of conessine treatment suggestively reduced the duration of freeze bouts in the rats compared to the PTZ challenged rats.

3.3. Impact of conessine on neurochemical signaling in PTZ challenged rats

Fig. 3(A–E) represents neurotransmitters levels in the brain tissue homogenate of the experimental animals. PTZ treatment considerably decreased inhibitory neurotransmitter GABA and increased excitatory neurotransmitter glutamate. It also decreased the levels of dopamine a crucial neurotransmitter responsible for the coordination of movement. Both the doses of conessine treatment considerably increased the levels of GABA (Fig. 3A), dopamine (Fig. 3B) and decreased the levels of glutamate (Fig. 3C) in the PTZ challenged rats. Conessine treatment in PTZ challenged rats also increased the levels of Na^+ K^+ ATPase (Fig. 3D) and Ca^{2+}–ATPase (Fig. 3E) which are responsible for maintaining the cellular homeostasis and neuronal function. The levels of Na^+ K^+ ATPase and Ca^{2+}–ATPase was significantly decreased in the PTZ challenged rats compared to the other group.

3.4. ROS neutralizing potency of conessine in PTZ challenged rats

The potency of conessine to scavenge free radicals and the antioxidant generation in PTZ challenged rats were assessed and the results were represented in the Fig. 4. PTZ treatment decreased the levels of both enzymatic antioxidant SOD and non-enzymatic antioxidant GSH whereas treatment with conessine increased the antioxidant levels in dose dependent manner. Conessine treatment significantly inhibited the lipid peroxidation in the PTZ challenged rats which was evidenced with the decreased levels of MDA. The nitric oxide which is the also contributes to oxidative stress along with other ROS was also decreased by the conessine treatment in the PTZ challenged rats.

3.5. Impact of conessine on nitric oxide synthase in PTZ challenged rats

Fig. 5 represents iNOS and nNOS levels in the brain regions of PTZ challenged rats. iNOS which is primarily associated with inflammation, neurodegeneration in epilepsy and nNOS which is involved in the excitotoxicity, seizure propagation were considerably increased in PTZ challenged rats whereas the conessine treatment decreased both the levels of iNOS and nNOS in PTZ challenged rats. Compared to the

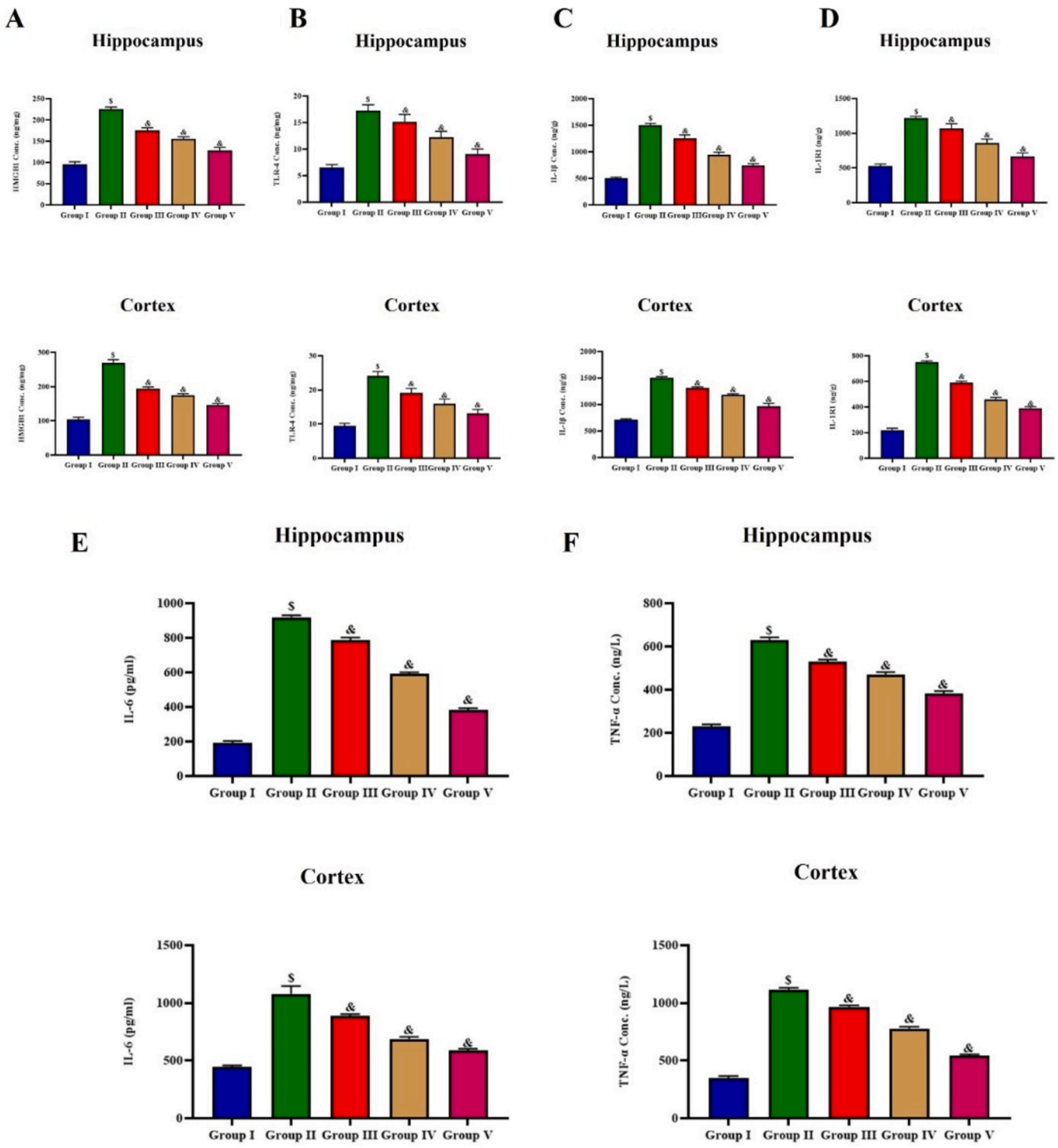

Fig. 6. Inhibitory effect of conessine against inflammation stimulating cytokines in PTZ challenged rats. A) High Mobility Group Box 1 (HMGB1), B) Toll-Like Receptor 4 (TLR-4), C) Interleukin 1β (IL-1β), D) Interleukin 1 Receptor (IL-1-R1), E) Interleukin 6 (IL-6), F) Tumor Necrosis Factor α (TNF-α) in the hippocampus and cortex region of the experimental rats. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

hippocampus region the cortex of the PTZ challenged rats produced significant levels of iNOS and nNOS. Conessine treatment decreased the levels NOS in both hippocampus and cortex region in a dose dependent manner.

3.6. Inhibitory effect of conessine against inflammation stimulating cytokines in PTZ challenged rats

Fig. 6 depicts the levels of inflammation stimulating cytokines in PTZ challenged rats brains. High Mobility Group Box 1 protein that plays a crucial role in DNA repair and inflammation. Toll-Like Receptor 4 stimulates production of inflammatory cytokines and the initiation of an immune response in the presence of pathogen. Both HMGB1 and TLR4

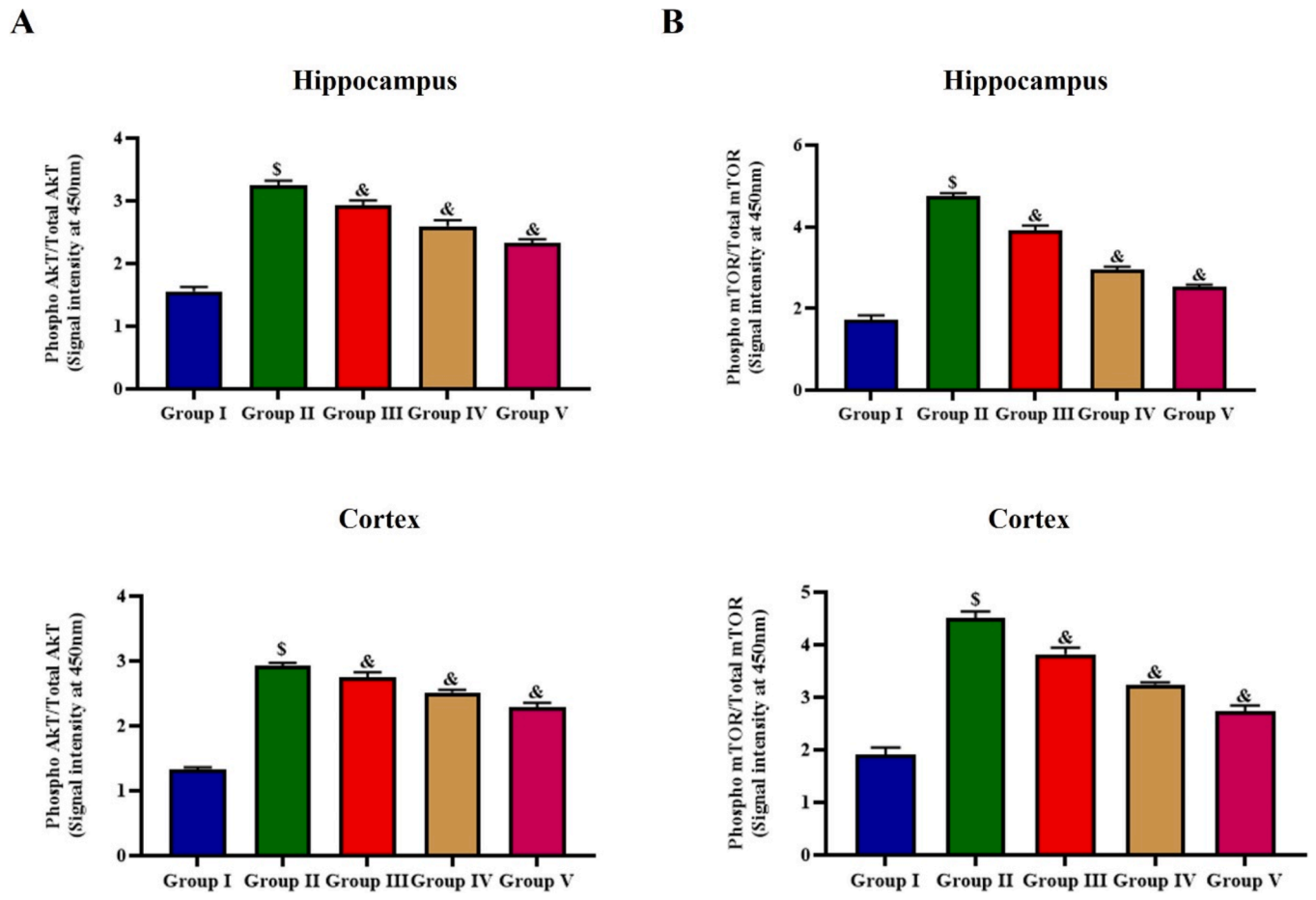

Fig. 7. Attenuating effect of conessine against AKT/mTOR signaling proteins in PTZ challenged rats. A) PhosphoAKT/Total AKT B) PhosphomTOR/mTOR levels in the hippocampus and cortex region of the experimental rats. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

were increased in the PTZ challenged rats. PTZ injection suggestively decreased the inflammation inducing cytokines IL-1β, IL-6, TNF-α in the hippocampus and cortex regions. It also increased the levels of IL-1binding receptors in both the brain regions analyzed. Conessine treatment significantly decreased the in inflammatory cytokines stimulators HMGB1, TLR4 and the inflammation stimulating cytokines in the hippocampus and cortex regions of the PTZ challenged rats. The levels of IL-R1 was also considerably reduced in the conessine injected animals the PTZ challenged rats.

3.7. Attenuating effect of conessine against AKT/mTOR signaling proteins in PTZ challenged rats

Deregulation of AKT/mTOR signaling pathway, particularly the phosphorylation states of AKT and mTOR, are associated in the epilepsy pathogenesis and progression. The levels of total and phosphorylated AKT, mTOR protein were quantified in the brain regions of experimental rats were illustrated in the Fig. 7. The PTZ treatment considerably enhanced the phosphorylated AKT, mTOR protein in both the brain regions whereas compared to the cortex regions phosphorylated AKT, mTOR proteins were enhanced in the hippocampus region. The treatment with conessine in the PTZ challenged rats significantly inhibited the AKT and mTOR proteins phosphorylation in both hippocampus and the cortex regions.

3.8. Alleviating effect of conessine against neurodegeneration induced in PTZ challenged rats

The neurodegeneration inducers cytochrome *c*, NF-κB, and COX-2 and proteolytic Caspase 3 were quantified in the hippocampus and the cortex regions of the test rats and the levels are presented in Fig. 8. Inflammation, apoptosis and oxidative stress plays a key role in the induction and development of neurodegeneration during epileptic episodes, the PTZ treatment considerably enhanced the levels apoptotic inducer cytochrome *c* and decreased Caspase-3. It enhanced the inflammation and oxidative stress stimulators NF-κB, and COX-2 in the hippocampus and cortex region. Conessine treatment significantly inhibited the levels of cytochrome *c*, NF-κB, and COX-2 whereas increased Caspase 3 activation which involves in alteration of the neurite structure and doesn't induce neurodegeneration.

3.9. Ameliorative effect of conessine against brain tissue damage induced by PTZ treatment

The neurodegeneration induced by PTZ and the ameliorative effect of conessine against PTZ were examined with histopathological analysis and the severity of tissue damage was scored based on the severity of damage (Fig. 9). The severity of PTZ induced neuronal damage was assessed by examining the inflammatory influx, congestion of blood vessel, pyknosis and necrosis of the neuronal cells. PTZ treatment considerably increased the influx of inflammatory cells and occurrence of pyknosis and necrosis in the neuronal cells. It also stimulated the

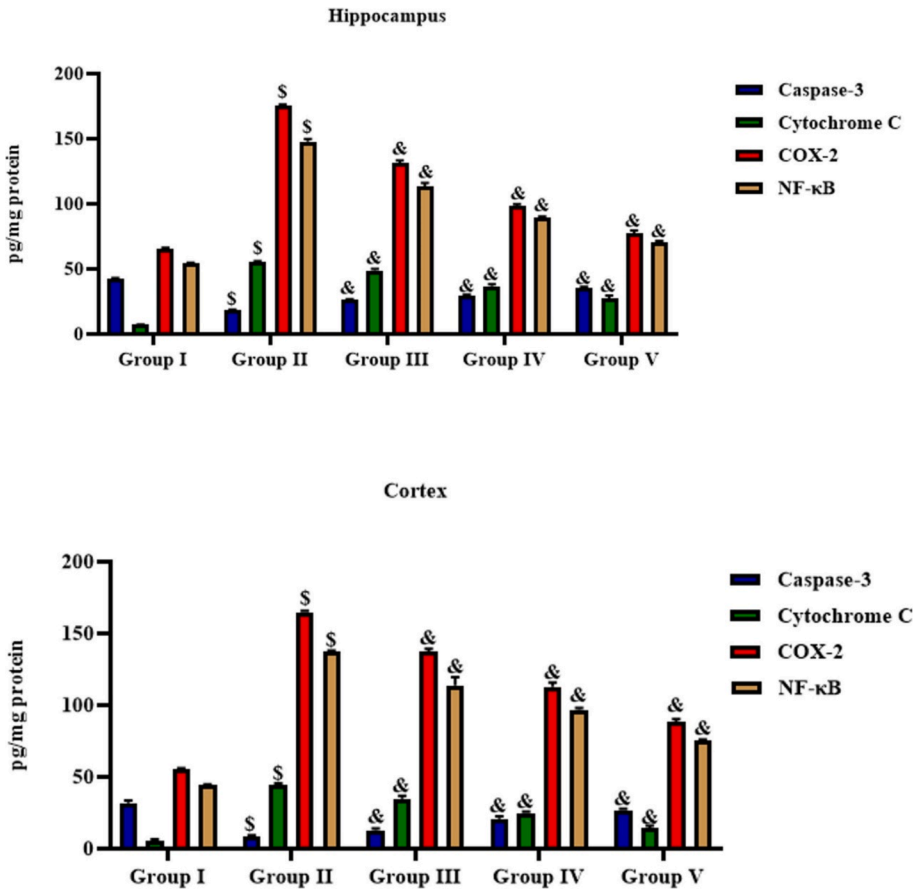

Fig. 8. Alleviating effect of conessine against neurodegeneration induced in PTZ challenged rats. Caspase 3, Cytochrome *C*, Nuclear Factor κB (NF-κB), Cyclooxygenase −2 (COX-2) levels in the hippocampus and cortex region of the experimental rats. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

congestion of blood vessels in the rats brain tissue treated PTZ. Conessine injection significantly inhibited the induction of pykonsis and necrosis in the neuronal cells of PTZ challenged rats. It attenuated the influx of inflammatory cells and the congestion of blood vessels in the PTZ challenged rats.

4. Discussion

Globally more than 50 million individuals were reported with epilepsy and it stands as the most predominant and recurrently arising neurological disease (Zhang et al., 2023; Klein et al., 2022). Even though multiple number of anti-seizure drugs were marketed within the past few years, the percentage of patients attaining seizure freedom remains questionable (Golyala and Kwan, 2017; Hauser, 2018). Hence to develop a potent, cost effective and a drug which doesn't causing any side effects is the need of the day. Conessine is a steroidal alkaloid present in the barks of Holarrhena sps (Dua et al., 2013). The ameliorative potency of conessine against epilepsy was not yet elucidated. Hence we analyzed the antiepileptic action of conessine in the rats convulsed with PTZ treatment. The cerebral cortex and the hippocampus are the two sensitive regions of brain which is provoked during the epileptic episodes (Bromfield et al., 2006). Localized malformations and development of cortical lesions were reported as the cause of epilepsy in most of the cases (Guerrini et al., 2003). Abnormal neurogenesis in the hippocampal regions are the result of seizure which causes circuits faults consequently changes action of the neuronal cells (Blümcke et al., 2012). Cognitive decline is often found in the patients reported with sclerosis in the hippocampal region (Wieser, 2004; Chatzikonstantinou, 2014). Hence assessing the potency of a drug to prevent epileptic induction in these two areas is necessary to formulate effective antiepileptic drug.

PTZ-Induced Kindling animal model is best, cost effective and easily reproducible type of animal model which mimics the pathology of human epileptic condition (Shimada and Yamagata, 2018). PTZ antagonizes the GABA receptors thereby attenuates the inhibitory synapse signaling and causes hyperexcitability causing generalized convulsions (Squires et al.,1984; Tourov et al., 1996). Repeated seizures were produced with the chronic administration of PTZ (Schmidt, 1987). Treatment with antiepileptic such as valproate, retigabine, phenobarbital etc. alleviates the seizure induced by PTZ (White, 2003). Conessine treatment also correlates with these reports, the rats treated with conessine significantly reduced the seizure induction and severity in the PTZ challenged rats. It also prevented the mortality in the PTZ challenged epileptic induced rats.

The swift and transitory neuronal synchronization activity triggered by the neurotransmitters cause's epileptic seizures. Neurotransmitters are key inducers of epileptogenesis the deregulated and non-coordinated activation of excitatory and inhibitors neurotransmitters causes imbalanced electrical stimulation which eventually leads to epilepsy (Danbolt et al., 2016). The research studies with various animal and cell models had proven the disruption in the levels of excitatory neurotransmitter glutamate and inhibitory neurotransmitter GABA induces neuronal excitability leads to seizures (Akyuz et al., 2021). Both animal and preclinical studies have evidenced the role of dopamine in seizure propagation (Bameri et al., 2018). In our study the PTZ treatment decreased inhibitory neurotransmitter GABA and dopamine, increased

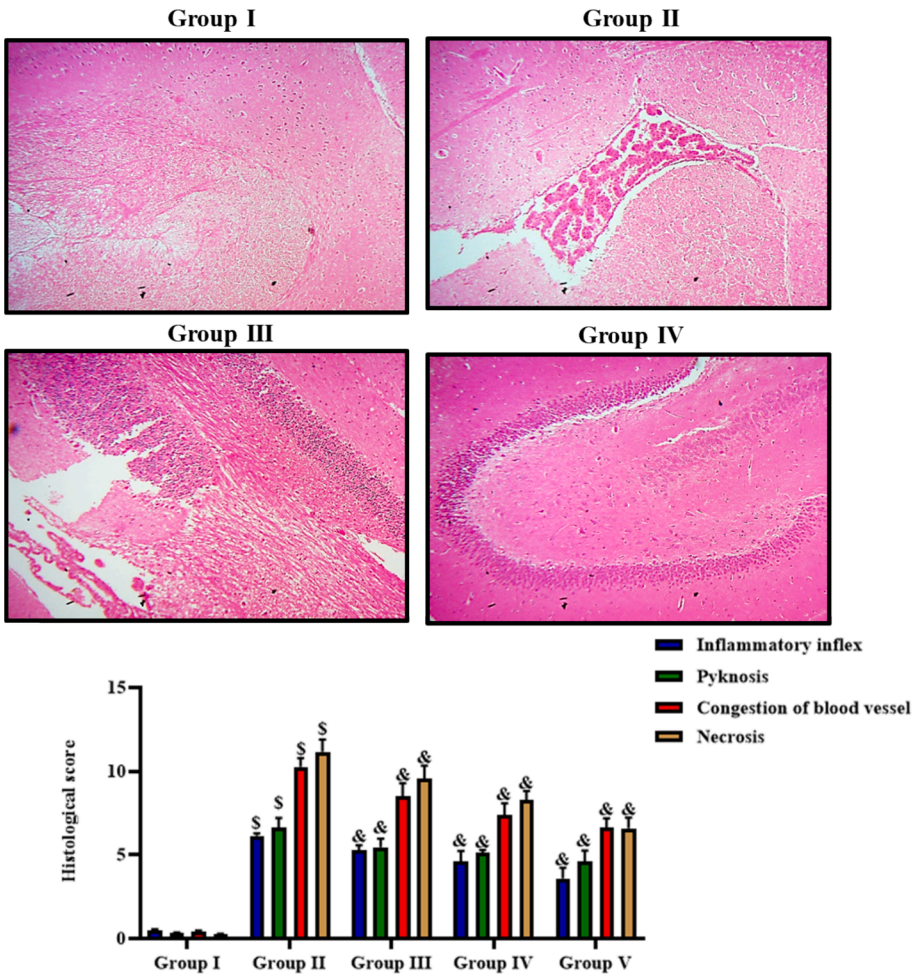

Fig. 9. Ameliorative effect of conessine against brain tissue damage induced by PTZ treatment. Severity of PTZ induced neuronal damage assessed by inflammatory inflex, congestion of blood vessel, pyknosis and necrosis of the neuronal cells. The data obtained from various experiments were presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was used to analyze the intergroup and intragroup statistical difference respectively. n = 6, p-value of <0.05 was perceived statistically significant.

the excitatory neurotransmitter glutamate confirming the induction of hyperexcitability of neurons whereas the conessine treatment significantly decreased the glutamate levels and increased the GABA and dopamine levels. It also significantly increased the levels of Na^{+}-K^{+}-ATPase and Ca^{2+} ATPase which was tend to be decreased in epileptic conditions. The inhibition of Na^{+}-K^{+}-ATPase and Ca^{2+} ATPase with drugs induced epileptic seizure whereas the activation with antibody alleviated epilepsy (Sun et al., 2022) this results correlated with our conessine data.

The multifactorial effects of ROS, nitrogen species and pro-inflammatory cytokines orchestrates the epilepsy progression (Puttachary et al., 2015). Reports suggests treatment with PTZ elevates the ROS and NOS leading to lipid peroxidation and it also decrease the levels of antioxidants (Erakovic et al., 2003). These findings correlates with our study were the PTZ challenged exhibited decreased levels of antioxidants and increased levels of lipid peroxidation. Increased NO levels associated with oxidative stress were evidenced in neurological pathologies such as epilepsy (Sadaf et al., 2020). Disruption of nNOS is reported with neuronal apoptosis and neurotoxicity (Wang et al., 2019). Epileptic models induced with kanic acid reported increased levels of nNOS which involves in epileptogenesis (Cosgrave et al., 2008). Conessine injection considerably attenuated the oxidative stress and enhanced the antioxidants in the PTZ challenged rats. It also decreased the levels of NO and both nNOS and iNOS levels in the hippocampus and cortex region of PTZ challenged rats suggesting the antiepileptic potency of conessine.

Neuroinflammation and recurrent seizures can both initiate and result from one another, leading together to severe clinical consequences. IL-1R/TLR signaling is one of the pathway was extensively studied in the pathophysiology of neuroinflammation and seizures. It exerts innate immunity activated IL-1R1 releases inflammatory stimulating cytokine IL-1β which infiltrates into the brain causing neuroinflammation (De Simoni et al., 2000). Activation of TLR-4-HMGB1 pathway triggers calcium release leading to neuronal excitability and neurodegeneration (Walker et al., 2017). In rodents the status epilepticus stimulated by pilocarpine, overexpression of IL-1β, IL-6, COX-2, TNFα, and the inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) has been observed in the hippocampus and piriform cortex (Singh et al., 2018). The excitability of neuronal cells were regulated by IL-1β and TNFα they both enhance the NMDA receptor and affects the AMPA receptor respectively which results in glutamate receptors stimulation causes increased influx of calcium and hyperexcitability of neurons (Amini et al., 2015). The oscillation in the concentration of ROS, mitochondrial membrane damage and apoptosis induction eventually triggers inflammatory response (McElroy et al., 2017). Conessine treatment both IL-R1 and TLR-4-HMGB1 signaling proteins and attenuated the inflammatory inducing cytokines thereby prevented neuroexcitability in the hippocampus and cortex region of the PTZ challenged rats. PTZ challenged rats enhanced the levels of NFκB and COX2 expression which was

decreased with the conessine treatment. The decrease in glutamate levels in conessine treated rats may be due to the inhibition of IL-1β and TNFα levels.

Research suggests that inhibiting the Akt/mTOR signaling pathway will be a potential target for treating inflammation-related disorders (Wang et al., 2018). Abnormal activation of the mTOR signaling pathway significantly disrupts cortical brain development and is linked to epilepsy. Clinical trials have demonstrated the effectiveness of mTOR inhibitors in treating seizures (Crino, 2015). Conessine treatment significantly attenuated AKT and mTOR phosphorylation in analyzed brain regions suggesting anti-seizure activity. Further studies have revealed that extended seizures induce nitrosative and oxidative stress, resulting in the release of cytochrome *C* from the mitochondria into the cytoplasm. This process stimulates caspase-3 activation, initiating apoptotic signaling and causing neuronal cell death in the epileptic brain (Chuang et al., 2007; 2019). In our study PTZ treatment induced oxidative stress, increased the levels of cytochrome *C* and COX2 thereby induced neurodegeneration which was evidenced with our histopathological analysis. Whereas conessine decreased the levels of cytochrome *C* and COX2 thereby prevented neurodegeneration. Conessine also increased the levels of caspase 3 which generally involved in apoptosis but it also involves synaptic plasticity regulation, glial cells differentiation and remodeling of cytoskeleton (Tzeng et al., 2013) hence the role of caspase 3 in epileptogenesis is unclear.

5. Conclusion

Our findings conclude conessine, a steroidal alkaloid scavenges oxidative stress, regulates neurotransmitter signaling thereby prevents neuroinflammation in PTZ challenged rats. It effectively inhibits the TLR-4-HMGB1, ILR-1 and AKT/mTOR signaling which was targeted by the researchers to develop potent antiepileptic drugs. Inflammatory stimulating cytokines, NFκβ, COX-2 and nitric oxide synthases levels were significantly reduced in the conessine treated rats confirms the anti-inflammatory potency of conessine in epilepsy induced rats. It also decreased the levels of cytochrome *C* and COX-2 proving its antioxidant effect and the histopathological scoring confirms the anti-inflammatory, anti-oxidant and antiepileptic potency of conessine in PTZ challenged rats. The open field analysis confirms conessine doesn't render any behavioral changes in the animals hence altogether conessine is potent safe antiepileptic drug.

Ethics approval

All work has been done under the guidelines of Institutional Ethics Committee.

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CRediT authorship contribution statement

Fang Chen: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision. **Tingting Peng:** Data curation, Conceptualization. **Mengjiao Gou:** Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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