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Protective Effects of Diosgenin in Pentylenetetrazole Induced Kindling Model of Epilepsy in Mice

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Abstract

The present study was aimed at investigating the effects of Diosgenin, a plant derived steroidal sapogenin on the development and acquisition of pentylenetetrazole (PTZ) kindling along with altered biochemical parameters. Diosgenin (5 and 10 mg/kg, i.p.) was evaluated on the course of kindling development and its ability to suppress the PTZ induced oxidative damage in the brain tissue when given as a pretreatment prior to each PTZ injection. Various oxidative stress parameters such as superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT) and lipid peroxidation (MDA) were assessed at the end of the study. Neuronal damage was studied by hematoxylin and eosin (H&E) staining technique. The neuroinflammatory marker glial fibrillary acidic protein (GFAP) was also evaluated at the end of the study using immunohistochemistry. Diosgenin significantly prevented the process of epileptogenesis in PTZ induced kindling model as exhibited by lower seizure score. The biochemical alterations induced by PTZ were ameliorated in diosgenin treated animals which was indicated by decreased MDA and increased SOD, GSH, CAT levels. Diosgenin treatment protected the neurons from seizure induced damage. It also alleviated the levels of GFAP which was manifested by reduced immunostaining. The above results are suggestive of the neuroprotective potential of Diosgenin which can be correlated with its ability to not only suppress oxidative damage but also to reduce seizure generation and further damage associated with the same. Diosgenin, thus could be a promising candidate in mitigating the sequel of events implicated in the progression of epileptic disorder/seizures.

Keywords: Kindling; Diosgenin; Oxidative stress; Epileptogenesis; Neuroinflammatory; Neuroprotective

Abbreviations: PTZ: Pentylenetetrazole; GFAP: Glial fibrillary acidic protein; MES: Maximal electroshock; ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; LPO: Lipid peroxidation; MDA: Malonaldialdehyde; CPCSEA: Control and Supervision of Experiments on Animals

Introduction

Epilepsy is a very common neurological disorder characterised by spontaneous recurrent seizures affecting more than 50 million people across the globe [1]. Inspite the availability of an expanded array of anti-epileptic drugs, one third patients remain refractory to the treatment [2]. Furthermore, the available treatment provides symptomatic relief and does not alter the progression of the disorder. There is an unmet need of novel drugs with anti convulsant and anti-epileptogenic profile. Kindling is a very well acknowledged model to study the process of epileptogenesis. It is a phenomenon characterized by repeated administration of a subconvulsive chemical or electrical stimuli causing gradual seizure development culminating in generalized tonic-clonic seizures [3]. Epileptic seizures are associated with altered excitatory and inhibitory neurotransmitter levels. Seizure induced brain insult is a dynamic process involving various factors like excitotoxicity, neuroinflammation, mitochondrial dysfunction and oxidative stress. The phenomenon denoted by excitotoxicity has been linked to the generation of reactive free radicals [4,5]. The free radicals dramatically alters the neuronal function leading to increased oxidative stress [6]. Brain is more vulnerable to oxidative stress because it consumes highest amount of oxygen as compared to other organs. It is very rich in poly unsaturated fatty acids that are susceptible to lipid peroxidation, is rich in iron which catalyzes hydroxyl radical formation and has comparatively fewer antioxidant mechanism [7]. Accumulating evidences have revealed the role of reactive radical species in the progression of epileptogenesis culminating in neuronal death [5]. Kindling induced seizures results in hypertrophy of astrocytes. Reactive astrogliosis is a prominent feature observed in the epileptic foci and may have causal role in the development and propagation of seizures. The reactive astrocytes express glial fibrillary acidic protein (GFAP) as a marker at the insultedsite of brain [8]. The strategies/therapeutic interventions exhibiting neuroprotective, anti oxidant and anti inflammatory effects would be of utmost significance to alter/ forestall the progression of the disorder. Diosgenin is a steroidal sapogenin found in various plants with predominance in Trigonella foenum. It is reported that in ayurvedic and unani system of medicine, Trigonella foenum has been used to treat epilepsy, paralysis, gout and dropsy [9]. It has diverse biological properties such as anti cancer, hypolipidemic, anti oxidant, anti inflammatory, etc. [10]. Diosgenin elicited anticarcinogenic activity via reducing peroxidation reaction and marker enzymes by enhancing the intrinsic antioxidant defense mechanism [11]. Moreover, Diosgenin was found to inhibit up-regulation of adhesion molecules induced by TNF-α through the inhibition of MAPK, Akt and NF-κB signalling pathways and ROS production [12]. The biodistribution studies have demonstrated a significant concentration of Diosgenin in brain indicating its blood brain barrier permeability [13]. It has also ameliorated the cognitive

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impairment and exhibited memory enhancing effect which was partly due to enhanced endogenous antioxidant enzymatic activities [14]. Another finding revealed the potential of Diosgenin to recover the memory deficit in 5XFAD mice and restored the axonal and presynaptic degeneration in the cerebral cortex and hippocampus [15]. Diosgenin was evaluated for its anticonvulsant activity in acute pentylenetetrazole (PTZ) and maximal electroshock model (MES) model in our lab and was found active in these models. (Data not shown) In context to the above mentioned findings, the present study was undertaken to evaluate the antiepileptogenic potential of Diosgenin in chronic PTZ induced kindling model of epilepsy in mice along with associated biochemical alterations. Kindling was induced by PTZ which is a widely accepted model of human epilepsy [16]. Diosgenin was assessed on the course of PTZ induced kindling along with oxidative stress in kindled mice. The oxidative stress markers such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), lipid peroxidation (LPO) were evaluated in brain at the end of the study. Furthermore, the effect of Diosgenin on neuroinflammatory marker glial fibrillary acidic protein (GFAP) and neuronal damage induced by PTZ kindling was assessed by immunohistochemistry and histopathological studies respectively.

Materials and Methods

Diosgenin was procured from Total Herb Solutions, Mumbai and pentylenetetrazole (PTZ) was purchased from Sigma-Aldrich (USA). Diazepam was obtained as valium® from Roche pharmaceuticals. All other chemicals and reagents used in the experiments were of analytical grade. Primary monoclonal mouse antibody to glial fibrillary acidic protein was procured from Abcam, USA.

Animals

Adult male Swiss-albino mice (20-25 g) were obtained from Bharat serums Pvt. Ltd., Thane, Mumbai and were allowed to acclimatize in the animal house of Institute of Chemical Technology (ICT). They were maintained at a controlled temperature (23 ± 2°C) and relative humidity (50-70%) under 12-12 hr light-dark cycle with free access to rodent chow and water ad libitum. All the experimental procedures and protocols used in the study were approved by the Institutional Animal Ethical Committee registered under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (ICT/IAEC/2014/P21).

Experimental design

Animals were randomly divided into five groups each containing 10 animals.

Group I: Normal control, **Group II**: PTZ control (vehicle+PTZ) **Group III:** Diosgenin 5 mg/kg+PTZ, **Group IV**: Diosgenin 10 mg/kg+PTZ **Group V**: Diazepam 1 mg/kg+PTZ. All the treatments were done by intraperitoneal (i.p.) route. PTZ was dissolved in sterile salin (0.9% w/v). Diosgenin was suspended using tween 80 in sterile saline and the later was used as vehicle control. Animals were pretreated with Diosgenin and Diazepam before PTZ injection (35 mg/kg).

Induction of kindling by PTZ induced seizures

All the animals except the normal control were injected with a subconvulsive dose of PTZ (35 mg/kg) on every alternate day to induce kindling. Diosgenin and Diazepam were injected 30 mins prior to each PTZ injection. Mice were observed for 30 mins after PTZ injection and seizure scores were recorded according to ref. [16] with slight modification which is as follows: stage 0 (No response); stage 1 (Myoclonic jerk); 2 (Straub tail); 3 (clonic jerk without loss of righting reflex); 4 (Clonic seizures with loss of righting reflex); 5 (clonic-Tonic seizures). The animals were considered as kindled after attaining a seizure score of 4 on three consecutive days. In the present study, 14 injections of PTZ were required to acquire kindling. At the end of the study, animals were sacrificed and their brains were isolated for further evaluation of biochemical parameters, histopathology and immunohistochemistry studies.

Sample preparation and biochemical estimations

The animals were sacrificed and subjected to intracardial perfusion with 0.9% saline solution (37°C) for biochemical estimations. The animals in the immunohistochemistry and histopathological group were further perfused with 4% paraformaldehyde. Brains isolated for biochemical analysis were rinsed in ice-cold isotonic saline and were homogenized with ice-cold 0.1 M phosphate buffer saline (pH 7.4). Further, the homogenate was centrifuged at -4°C (10,000 rpm; R-248M of CPR-24 plus Instrument, Remi, India) for 15 min, and aliquots of the same were used for estimation of biochemical parameters.

Assessment of SOD, CAT and GSH

The antioxidant parameters (SOD, CAT and GSH) were determined according to ref. [17] with minor modifications. The activity of SOD in the brain homogenate was assayed by assessing its ability to scavenge superoxide radicals generated by auto-oxidation of pyrogallol in the alkaline medium. One unit of SOD represents the amount of enzyme required for 50% inhibition of pyrogallol autoxidation. CAT activity was evaluated on the basis of its ability to scavenge hydrogenperoxide radicals. The results were expressed as units of CAT activity/mg of protein. GSH levels were estimated by using 5,5-Dithiobis (2-nitrobenzoic acid) that binds to the thiol group to give a coloured compound detected at 412 nm. The results were expressed as nanomoles/mg of protein.

Determination of LPO

The LPO content in the brain homogenate was determined spectrophotometrically as per the method described by ref. [17]. It was detrmined by measuring the MDA content at the end of reaction. LPO was expressed as nmol of MDA/mg of protein.

Estimation of protein concentration

The protein content of the brain homogenate was analysed by the dye binding method of Bradford. Bovine serum albumin (BSA) was used as a standard. Brain homogenate (5 µl) was added to 200 µl of Bradford reagent (Sigma Aldrich) and incubated at 37°C for 15 min. The absorbance was recorded at 596 nm with the help of microplate spectrophotometer (Epoch, Biotek, USA) (Data not shown).

Histopathology

After fixation with 4% paraformaldehyde, the brain samples were routinely processed and subjected to paraffin embedding. The coronal sections of 10 µm passing through hippocampus were sliced, mounted and stained by hematoxylin and eosin (H&E) and observed under microscopes at different magnifications. The sections were assessed for the microscopical alterations pertaining to neuronal damage like pyknotic nuclei, distorted morphology of cell, etc.

Immunohistochemistry

Immunohistochemistry was performed on 4% paraformaldehyde-fixed, 10 µm -thick frozen brain sections (consisting 14-15 sections)

passing through the hippocampus region of brain. Further, the sections were fixed on poly-L-lysine coated slide, transferred through three changes of xylene for 30 min and then rehydrated with decreasing grades of absolute alcohol, 95%, 70%, 50%. Peroxidase activity was blocked by incubating with 3% hydrogen peroxide in methanol for 5 min. Primary monoclonal antibody to GFAP was incubated for 30 min at room temperature and then washed with Tris buffer solution pH 7.4 for 10 min. Sections were incubated with Poly-Horseradish peroxidise (Poly-HRP) for 30 mins and washed in Tris buffer solution pH 7.4 for 10 min. Further, they were incubated with substrate and examined for the colour change to brown which appeared within 5-10 min [18,19].

Statistical analysis

Data of all the results were expressed as mean ± SEM. The analysis of all the studies were done with the help of analysis of variance (ANOVA) followed by Dunnett's test. For immunohistochemistry quantification and for oxidative stress biomarkers *p<0.05, **p<0.01, ***p<0.001 were considered to be statistically significant when compared with PTZ control group and $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 when compared to normal control group.

Results

Effect of Diosgenin on PTZ induced kindling

The repeated administration of PTZ (35 mg/kg) on every alternate day (for 28 days, 14 injections) resulted in kindling, as indicated by progressive increase in the seizure score (Figure 1). Diosgenin at the dose of 5 mg/kg and Diazepam at the dose of 1 mg/kg significantly (p<0.001) prevented the intensification of kindling. There was a dose dependent effect observed in inhibiting seizure score but the protection afforded at 5 mg/kg dose was commendable as compared to 10 mg/kg of diosgenin.

Effect on SOD, CAT and GSH activity

The oxidative stress parameters assessed in the study were SOD, CAT and GSH which are the major players of natural antioxidant defence system (Figure 2). PTZ induced kindling resulted in elevated oxidative stress leading to decreased SOD (p<0.01), CAT (p<0.05) and GSH (p<0.01) activities as depicted in PTZ control mice in comparison to normal mice. Diosgenin at 5 mg/kg (p<0.01) and 10 mg/kg (p<0.05) significantly increased the SOD activity as compared to PTZ control. Interestingly, it was observed that Diosgenin improved the SOD activity in comparison to normal control mice. Diazepam exhibited elevated levels of SOD (p<0.05) as compared to PTZ group. Diosgenin treatment at the dose 5 and 10 mg/kg (p<0.01 and p<0.05 respectively) significantly improved the activity of CAT. PTZ kindled mice exhibited a remarkable decrease in the levels of GSH (p<0.01) as compared to normal control. Treatment with Diosgenin at 5 and 10 mg/kg (p<0.01, p<0.01) significantly elevated GSH levels as compared to PTZ group. Diazepam also significantly restored the levels of reduced glutathione (p<0.05). Nevertheless, the improvement elicited by Diosgenin in enhancing antioxidant defence mechanism was better than Diazepam.

Evaluation of LPO

Repeated administration of PTZ resulted in increased free radicals and in turn oxidative stress as reflected by elevated levels of MDA, a marker of lipid peroxidation in PTZ kindled mice (p<0.01) (Figure 3). However, administration of Diosgenin at 5 and 10 mg/kg significantly lowered the MDA levels (p<0.05, p<0.05) as compared to PTZ group. Furthermore, diazepam, too, reduced the levels of MDA (p<0.05) in comparison to PTZ group.

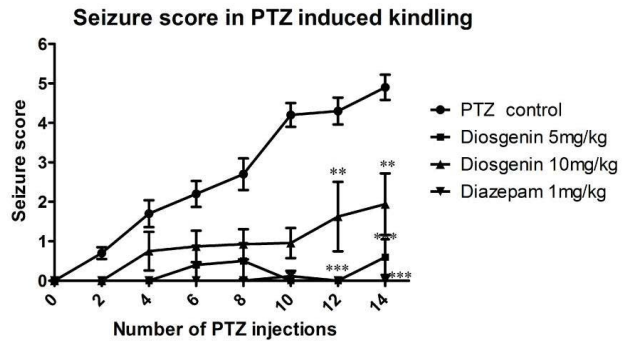

Figure 1: Seizure score in PTZ kindling model. Effect of Diosgenin pretreatment on the development of PTZ-induced kindling. Seizure scores are expressed as mean ± SEM. n=10. *p<0.05, **p<0.01, ***p<0.001 compared with PTZ group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

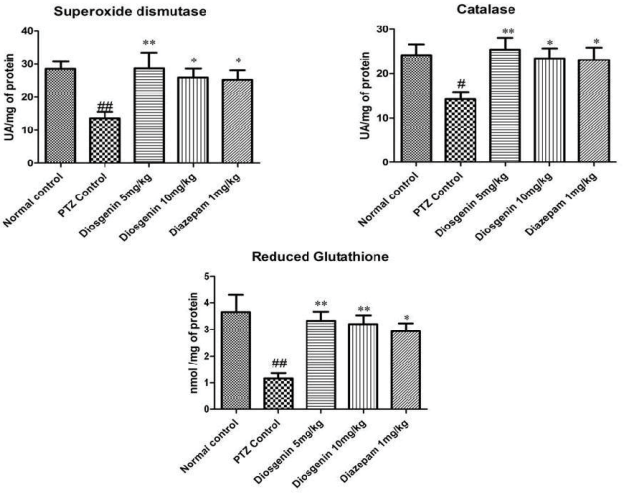

Figure 2: Effect of Diosgenin on SOD, CAT and GSH activities in brain. Data expressed as mean ± SEM. n=6. ##p<0.01, #p<0.05 compared with normal group and *p<0.05, **p<0.01 compared with PTZ group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

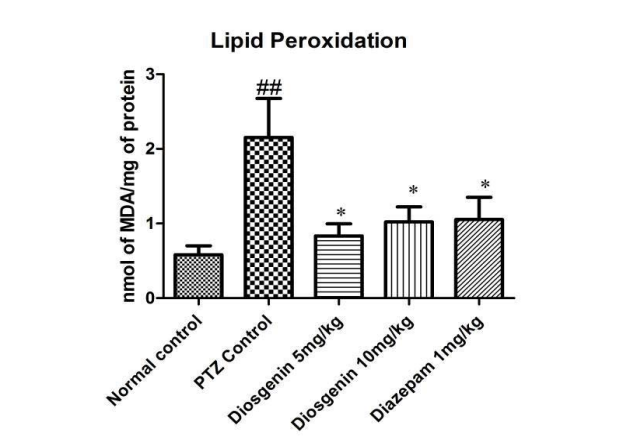

Figure 3: Effect of Diosgenin on lipid peroxidation in brain. Data expressed as mean ± SEM. n=6. ##p<0.01 compared with normal group and *p<0.05, compared with PTZ group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

Histopathology

H&E staining exhibited severe degeneration of nerve cells, necrotic neurons in the hippocampus along with loss of dendrites in the PTZ kindled group (Figure 4). Moreover, the dead neurons in the hippocampal region with pyknotic
from surviving cell which show rou
without any distorted morphology. Diosgenin at the dose of 5 mg/kg significantly ameliorated the neuronal damage induced by seizures. A slight degeneration of neurons was observed in 10 mg/kg Diosgenin treated group. Diazepam, too mitigated the neuronal death during the progression of kindling.

Glial fibrillary acidic protein

The immunostaining for GFAP is shown below in Figure 5 which exhibited brown colored staining indicating reactive GFAP-positive astrocytes. There was a significant increase in the GFAP levels (76%) in the PTZ kindled group as compared to normal animals ($p<0.001$) (Figure 6). Diosgenin at 5 mg/kg ($P<0.001$) and 10 mg/kg ($P<0.01$) significantly decreased GFAP level in comparison to PTZ group. Diosgenin exhibited better improvement in reducing GFAP levels as compared to diazepam.

Discussion

The present study demonstrates the protective role of Diosgenin, a plant derived steroidal saponin on the course of PTZ induced kindling in mice. The findings of the current study also illustrated the beneficial role of diosgenin in attenuating oxidative stress, neuroinflammation and prevented neuronal damage. In recent times, there has been an upsurge in the screening of phytoconstituents for their medicinal properties, owing to their low toxicity, potent pharmacological effects and economic availability. Diosgenin is a free radical scavenger exhibiting antioxidant effect against the damage caused by free radicals. It can thus, be implicated in diseases involving free radicals as they are the likely candidates responsible for neuronal damage. It has many pharmacological activities but there have not been any reports related to its anti epileptogenic effect. Therefore, we screened Diosgenin for its protective effect on the course of PTZ kindling and associated biochemical alterations. The doses were selected on the basis of preliminary study performed in acute models wherein the higher doses showcased ceiling effect. In the present study, lower doses of diosgenin (5 and 10 mg/kg) were used as it was a chronic model and diosgenin was administered for longer period of time. Treatment was done by intraperitoneal route as there are reports stating poor absorption of Diosgenin by oral route. In addition, there might be a probability to overlook on activity of a poorly bioavailable compound. In the current study, PTZ was used to induce kindling which is a very well acknowledged model for studying the process of epileptogenesis. The repeated administration of a subconvulsive dose of PTZ, a selective blocker of chloride channel specific to GABA-A receptor results in progressive intensification of convulsive effect, culminating in generalized seizures [20]. In the aforementioned study, we required 14 injections of PTZ (35 mg/kg) to establish kindling in animals. PTZ kindling resulted in increased oxidative stress and further neuronal damage. Diosgenin at 5 mg/kg exhibited significant effect in suppressing the process of epileptogenesis which was comparable to standard Diazepam. The efficacy of diosgenin in kindling model might be ascribed to its GABA mediated effect or it might prevent the spread of seizure through the neural tissue. Diosgenin might be exhibiting its effect through GABAergic mechanism or might be preventing seizure spread through the neural tissue by the blockade of voltage gated

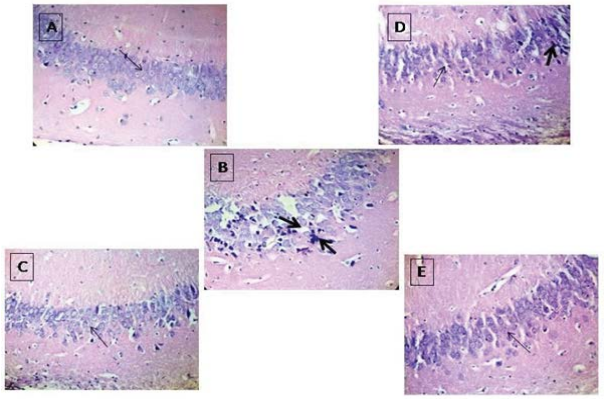

Figure 4: H&E staining of coronal sections of hippocampal region in PTZ kindling model. A-Normal control, B-Negative control, C-PTZ + Diosgenin 5 mg/kg, D-PTZ + Diosgenin 10 mg/kg, E-PTZ + Diazepam 1 mg/kg. Photomicrographs (40X) showing changes in staining of damaged neurons (dark stained neurons with severe degeneration indicated by thick arrow (B) in PTZ kindled group) as compared to normal neurons indicated by thin arrow.

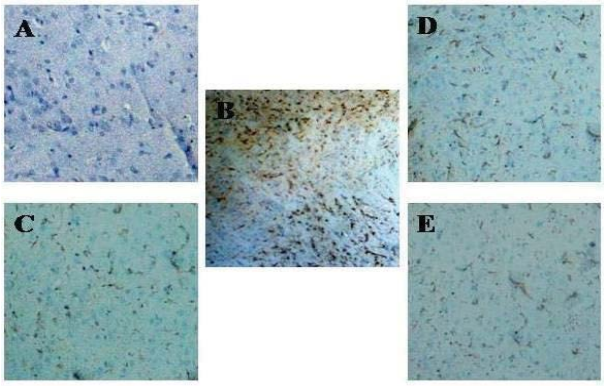

Figure 5: GFAP immunohistochemistry. n=4. Representative photomicrographs of GFAP-immunoreactive astroglial cells in the hippocampus. A-Normal control, B-Negative control, C-PTZ + Diosgenin 5 mg/kg, D-PTZ+Diosgenin 10 mg/kg, E-PTZ + Diazepam 1 mg/kg.

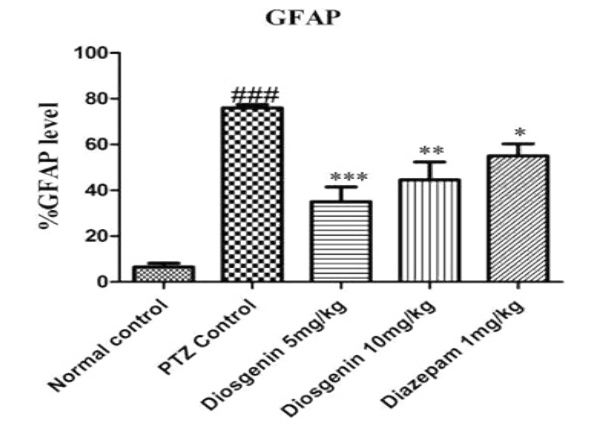

Figure 6: Level of GFAP. Data expressed as mean ± SEM. n=4. ###$p<0.001$ compared with normal group and *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with PTZ group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

sodium channels as it had shown activity in acute PTZ and MES model respectively. (Unpublished data) Astrogliosis, characterised by the hypertrophy of the cell bodies and processes is an important feature of the epileptic foci. There is an increased expression of glial fibrillary acidic protein (GFAP), a neuro inflammatory marker assessing the reactive state of astrocytes in response to various insults [21]. PTZ induced seizures resulted in reactive astrogliosis and in turn increased GFAP immunoreactivity. The kindled animals treated with Diosgenin exhibited significantly lower GFAP levels better than diazepam. The plausible reason for this would be attributed to the anti-inflammatory activity of Diosgenin along with the seizure inhibitory effect during the process of kindling. Oxidative stress is characterized by an imbalance between the increased cellular reactive oxygen species and natural antioxidant defense of the body. Various rodent experimental models of epilepsy such as PTZ kindling have shown to increase oxidative stress [4]. PTZ induced seizures trigger a variety of biochemical changes such as activation of membrane phospholipases, proteases and nucleases [3]. Furthermore, any alteration in membrane phospholipid metabolism would result in release of free fatty acids (FFAs), diacyl glycerols, lipid peroxide, free radicals etc. The elevated free radicals perturb the defense mechanism or causes cellular dysfunction by attacking the polyunsaturated sites of biological membrane resulting in lipid peroxidation [22]. The current study supports this hypothesis as indicated by increased oxidative stress. In addition, seizure generation causes impairment of the endogenous anti-oxidant levels. PTZ induced kindling model was preferred in this study over single dose of PTZ as the former model leads to impaired ant oxidative mechanism in the brain of mice whereas the latter does not cause any such alterations. The increased levels of MDA which is a marker of lipid peroxidation indicated elevated free radicals production in PTZ kindled mice. This increment in the levels of MDA was significantly prevented by Diosgenin treatment. The lower levels of MDA in disogenin treated group as compared to PTZ control indicates attenuation in lipid peroxidation. Diosgenin is a very effective free radical scavenger and thus protects against the damage due to free radicals. There was a concomitant decrease in the SOD and CAT activities in PTZ kindled mice. SOD and CAT are the major enzymes involved in the exclusion of two deleterious reactive species, superoxide and hydrogen peroxide respectively [23]. Diosgenin treatment increased the levels of both SOD and CAT thus exhibiting a protective effect against PTZ induced oxidative stress. GSH is a natural antioxidant involved in the cellular detoxification of reactive oxygen species [24]. It prevents the formation of the most toxic hydroxyl radical and gets converted into its oxidized form during this defensive process. The level of GSH is depleted in chronic models of epilepsy as well as the brains of the patients with chronic epilepsy [25]. PTZ treatment reduced the levels of GSH which was restored to normal by Diosgenin treatment. Overall, Diosgenin ameliorated oxidative stress by decreasing the lipid peroxidation, restoring the GSH levels as well as increasing the SOD and CAT levels by virtue of its anti-oxidant property. PTZ kindling results in altered glutamatergic function. The increased liberation of glutamate causes an excessive flux of intracellular calcium leading to cell death [26]. Over production of free radicals due to excitotoxicity has a significant role in causing neuronal death in PTZ kindled animals [2]. In the current study, PTZ kindled group exhibited neuronal death as manifested by pyknotic nuclei, degeneration and necrotic nuclei in the hippocampus region of the brain. There have been conflicting reports pertaining to neuronal death and related changes as art factual in kindling model but our data is in line with those studies which have demonstrated neuronal damage in the hippocampal area of the brain [27,28]. Additionally, excessive generation of free radicals due to seizures causes neuronal death [5] which was evinced in the present study. Diosgenin treatment prevented neuronal death very effectively which could be attributed to its seizure inhibitory effect as well as anti oxidant effect forestalling sequel of events causing damage. In conclusion, the present findings showed that Diosgenin has an inhibitory effect on the progression of PTZ kindling which indicates that it possess anti epileptogenic activity along with attenuation of oxidative stress and neuroinflammation. Our study is a preliminary finding about the antiepileptogenic and antioxidant effect of Diosgenin against PTZ induced kindling and thus it might be a prospective candidate in the treatment of epilepsy. Furthermore, neurochemical and behavioral studies are needed to elicit the exact mechanism of the same.

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Conflict of Interest

The authors declare no conflict of interest.

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