

## Anticonvulsant And Antioxidant Actions Of Some Dibenzo-a-Pyrone Derivatives In Pentylene –Induced Kindling Model In Mice

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### ABSTRACT

**Objective:** The present study was carried out to investigate the effect of Dibenzo- $\alpha$ -pyrone derivatives on the course of pentylenetetrazole (PTZ)-induced chemical kindling and oxidative stress markers in PTZ-kindled mice. **Methods:** Kindling was induced by repeated injections of a subconvulsive dose of PTZ (25mg/Kg, i.p.) on alternate days for 5 weeks or until stage 5 of the seizure score was evoked on three consecutive administrations. Butylamine, Diethylamine and Pyrrolidine Derivatives of Dibenzo- $\alpha$ -pyrone were administered daily in three doses (10, 20 and 40mg/kg) per orally (p.o.) along with alternate day PTZ. Following PTZ kindling, oxidative stress parameters, i.e. levels of malondialdehyde (MDA) and reduced glutathione (GSH), were assessed in isolated homogenized whole brain tissue. **Results:** PTZ treatment progressively increased the seizure score in control mice. Biochemical analysis revealed a significant increase in MDA levels and decreased GSH levels in the brain homogenate of PTZ-kindled mice. Daily treatment with Butylamine, Diethylamine and Pyrrolidine Derivatives of Dibenzo- $\alpha$ -pyrone in doses of 20 and 40mg/kg significantly decreased the PTZ-induced seizure score. However, a low dose (10mg/kg) failed to improve the seizure score. Pretreatment of derivatives in all doses showed an ameliorating effect on biochemical alteration induced by PTZ treatment. **Conclusion:** The present study indicate the potential anticonvulsant activity of Dibenzo- $\alpha$ -pyrone derivatives against PTZ-induced kindling in mice.

**KEY WORDS:** Epilepsy, Dibenzo- $\alpha$ -pyrone, pentylenetetrazole, kindling, oxidative stress

### 1. INTRODUCTION

Dibenzo- $\alpha$ -pyrone (DBP) have been found to be the active constituent of a very important & multifaceted herbo-mineral drug called SHILAJIT. Shilajit having various biological activities has always provoked the research scholars to inquire about its active constituents. Over sixty years of clinical research have shown that Shilajit has positive effects on humans. It increases longevity, improves memory and cognitive ability, reduces allergies and respiratory problems, reduces stress, and relieves digestive troubles. It is anti-inflammatory, antioxidant, and eliminates free radicals. The research proves that Shilajit increases immunity, strength, and endurance, and lives up to its ancient reputation as the “destroyer of weakness”.<sup>1,2</sup> Researchers propose that the physiological properties of shilajit are due to compounds such as the Dibenzo- $\alpha$ -pyrone, along with triterpenes and phenolic lipids. Fulvic acids may also have a physiological role, acting as carrier molecules for the more bioactive smaller compounds.<sup>3</sup>

We have synthesized few mannich derivatives of the oxygenated Dibenzo- $\alpha$ -pyrone. For the synthesis of the oxygenated DBP moiety, the mostly adopted method has been developed by Hurtley W.R.H et al (1870, 1929). The Hurtley condensation involved copper catalyzed condensation of 2-halobenzoic acid with various  $\beta$ -dicarbonyls (1,3-diketone) in water, alcohol/  $\beta$ -carbonyl itself in the presence of a strong base.<sup>4</sup>

Epilepsy is a neurological disorder which effects 50 million of worlds population.<sup>5</sup> Many anticonvulsant agents are available to tackle this neurological disorder. Despite treatment with available antiepileptic drugs (AED), epilepsy remains refractory in one third of patients. Further, adverse effects associated with AED and recurrent seizures limit their use. Increasing data from experimental and clinical reports suggest the involvement of oxidative stress in pathophysiology of epilepsy.<sup>6,7</sup> Excessive oxidative stress contributes to neuronal degradation through lipid peroxidation and decreased glutathione concentrations in the epileptic focus.<sup>8</sup> Therefore there is a need for the development of newer AEDs with fewer adverse effects and higher efficacy.

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Knowing the literature reports shown by DBP an active constituent of herbal drug Shilajit, the present work aims to investigate the effect of Dibenzo- $\alpha$ -pyrone derivatives on the course of pentylenetetrazole (PTZ)-induced chemical kindling and oxidative stress markers in PTZ-kindled mice. PTZ-kindling is a well established animal model which stimulate clinical epilepsy. In this model repeated injection of sub-convulsive dose of PTZ causes gradual development of seizure culminating to generalized-tonic-clonic seizures. Further malondialdehyde and glutathione levels were also measured to determine if oxidative stress was involved in epilepsy. Reports indicate that the free radical generation due to the increased activity of the glutamatergic transmitter plays a crucial role in neuronal cell death of the PTZ kindling in rodents.

## **2. MATERIAL AND METHODS**

### **2.1. Animals**

Healthy Swiss albino mice of either sex weighing 24-30 g (n=8/group) were used in the study. Animals were housed in groups of six mice per cage (43x28.6x15.5cm) with a natural light/dark cycle and provided with free access to pellet diet and water. Procedures adopted during experiments on animals and their care were conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals, India, and the study was approved by Institutional Animal Ethics Committee.

### **2.2. Methodology**

The present study was undertaken on PTZ-kindling in mice. PTZ – kindling is a well established animal model which stimulate clinical epilepsy. In this model repeated injection of sub-convulsive dose of PTZ causes gradual development of seizure culminating to generalised -tonic-clonic seizures. Further reports indicate that the free radical generation due to the increased activity of the glutamatergic transmitter plays a crucial role in neuronal cell death of the PTZ kindling in rodents.<sup>9-14</sup>

### **2.3. Drugs And Dosing Schedules**

Butylamine, Diethylamine and Pyrrolidine Derivatives of Dibenzo- $\alpha$ -pyrone were used in the present study. Derivatives were administered in doses of 10, 20 and 40mg/Kg per orally (p.o.)., Pentylenetetrazole was dissolved in saline and injected i.p. in dose 25mg/kg. Distilled water was given to control groups. Diazepam was suspended in distilled water and administered orally in a dose of 3mg/Kg. All drugs were administered in a volume of 10ml /kg/dose and the animals were tested 1 hour after drug administration.

### **2.4. Pentylenetetrazole Induced Kindling**

For PTZ kindling, a subconvulsant dose of PTZ 25mg/Kg body weight was injected intraperitoneally on every second day (i.e. Day 1, Day 3, Day 5, etc.) The PTZ injections were stopped when the animals showed adequate kindling, i.e. seizure score of 5 on three consecutive injections. The first incidence of seizure with score 5 was ob-

served between Day 27 and Day 31. Thus in no case did the PTZ schedule exceed Day 35. In the groups treated with antiepileptic drugs, the PTZ doses were tested up to Day 35 or up to seizure of score 5 on three consecutive injections, whichever was earlier. After each PTZ injection, the convulsive behavior was observed for 30min. The resultant seizure was scored as follows : Stage 0(no response); Stage1(hyperactivity, restlessness and vibrissae twitching); Stage2(head nodding, head clonus and myoclonic jerks); stage 3(unilateral or bilateral limb clonus); stage4 (forelimb clonic seizures); stage5(generalized clonic seizures with falling); Number of animals in each group was eight.

### **2.5. Measurement of Oxidative Stress Parameters**

Further malondialdehyde and glutathione levels were also measured to determine if oxidative stress was involved in epilepsy.

#### **2.5.1. Tissue Preparation**

At the end of study period, the animals were sacrificed by ether anesthesia ; the brain was quickly dissected out in toto, washed with ice-cold sodium phosphate buffer, weight and stored over ice. The brains were further processed within half an hour of dissection and the estimation of oxidative stress were done in the same working day. Brain tissue was homogenized with 10times (w/v) sodium phosphate buffer. The homogenate was centrifuged at 3000 rpm for 15min. The supernatant was used for the estimation of MDA and glutathione levels.

#### **2.5.2. Measurement Of Lipid Peroxidation**

Malondialdehyde (MDA), which is a measure of lipid peroxidation, was measured spectrophotometrically.<sup>15</sup>

#### **2.5.3. Measurement Of Reduced Glutathione**

Reduced glutathione was estimated spectrophotometrically.<sup>16</sup>

### **2.6. Statistical Analysis**

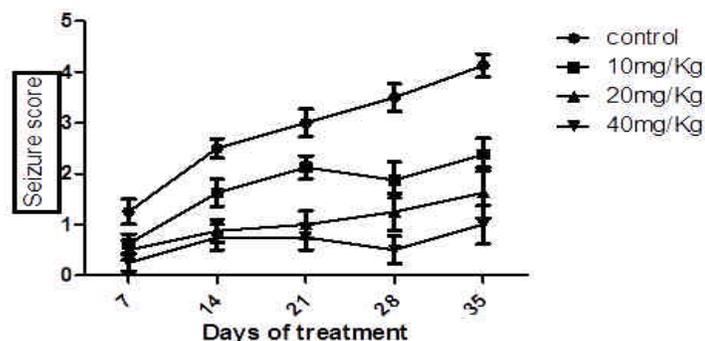
The results were expressed as mean  $\pm$  standard error of mean. Statistical analysis of the data was performed using one –way analysis of variance followed by post hoc Tukeys test. The p values less than 0.05 were considered significance.

## **3. RESULTS AND DISCUSSION**

### **3.1. Effect Of Various Doses On PTZ-Kindling In Mice**

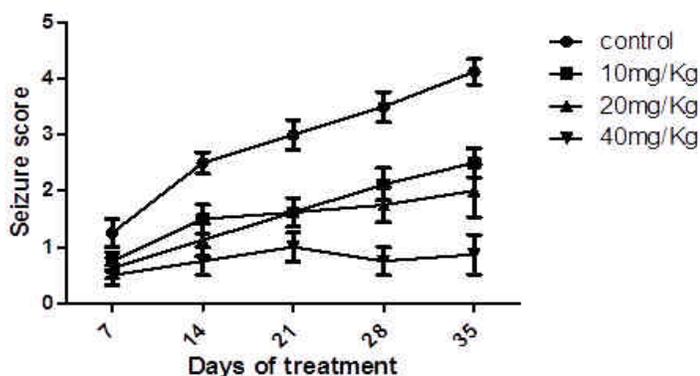
In vehicle treated group repeated administration of PTZ led to the development of kindling in 5 weeks. Butylamine, Diethylamine and Pyrrolidine Derivatives of Dibenzo- $\alpha$ -pyrone (10, 20 and 40mg/Kg, p.o.) treatment dose dependently decreased incidence and seizure score (p<0.01). The treatment with diazepam (3mg/Kg) in kindled animals showed significant reduction in incidence and severity. The results of protection offered by 40mg/Kg were comparable to those of diazepam treated group (Figure1, 2 and3)

**Figure 1** The effect of Butylamine derivative of Dibenzo-a-pyrone on pentylenetetrazole-induced kindling in mice.



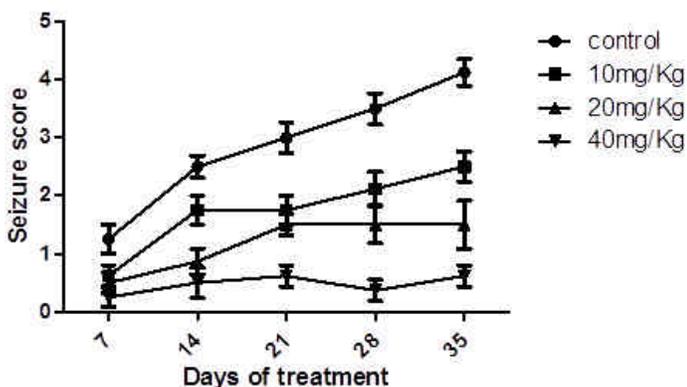
Data are expressed as mean  $\pm$  SEM; n =8 in each group. ANOVA followed by Tukeys test.  $p < 0.01$  control versus drug (10mg/Kg).  $p < 0.01$  control versus drug (20mg/Kg).  $p < 0.01$  control versus drug (40mg/Kg)

**Figure 2** The effect of Diethylamine derivative of Dibenzo-a-pyrone on pentylenetetrazole-induced kindling in mice.



Data are expressed as mean  $\pm$  SEM; n =8 in each group. ANOVA followed by Tukeys test.  $p < 0.01$  control versus drug (10mg/Kg).  $p < 0.01$  control versus drug (20mg/Kg).  $p < 0.01$  control versus drug (40mg/Kg)

**Figure 3** The effect of Pyrrolidine Derivative of Dibenzo-a-pyrone on pentylenetetrazole-induced kindling in mice.

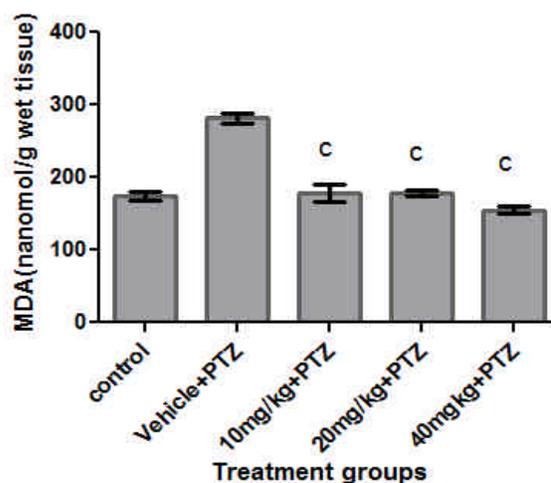


Data are expressed as mean  $\pm$  SEM; n =8 in each group. ANOVA followed by Tukeys test.  $p < 0.01$  control versus drug (10mg/Kg).  $p < 0.01$  control versus drug (20mg/Kg).  $p < 0.01$  control versus drug (40mg/Kg)

### 3.2. Measurement Of Malondialdehyde And Glutathione

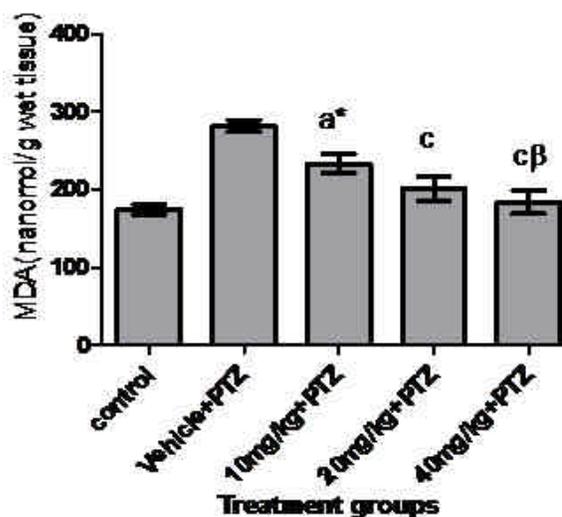
In the vehicle treated PTZ-kindled group there was significant increase in MDA ( $p < 0.001$ ) and decrease in glutathione levels ( $p < 0.001$ ) when compared to values of control group. Derivatives (10, 20 and 40mg/Kg, p.o.) administration in all doses significantly decreases MDA and increase glutathione levels (Figure 4, 5, 6, 7, 8, 9). (Groups: A-control, B-vehicle +PTZ, C-drug dose 10mg/kg +PTZ, D-drug dose 20mg/kg +PTZ, E-drug dose 40mg/kg +PTZ)

**Figure 4.** Effect of Butylamine derivative of Dibenzo-a-pyrone on malondialdehyde levels in mice brain tissues.



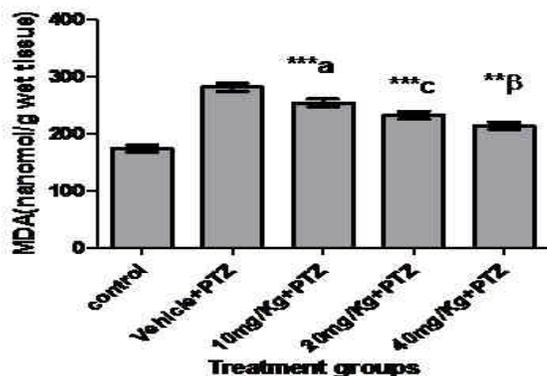
Data are expressed as mean  $\pm$  SEM; n =8 in each group. ANOVA followed by Tukeys test.  $^*p < 0.05$  : A vs. C,  $^*p < 0.05$  : B vs. C,  $^*p < 0.05$  : C vs. D,  $^{**}p < 0.01$  : A vs. D,  $^b p < 0.01$  : B vs. D,  $^b p < 0.01$  : C vs. E,  $^{***}p < 0.001$  : A vs. E,  $^c p < 0.001$  : B vs. E,  $^d p < 0.001$  : D vs. E

**Figure 5.** Effect of Diethylamine derivative of Dibenzo-a-pyrone on malondialdehyde levels in mice brain tissues.



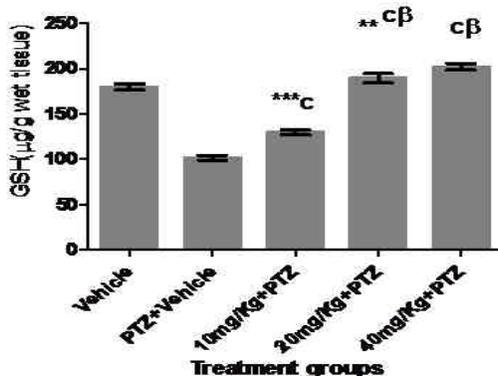
Data are expressed as mean  $\pm$  SEM; n =8 in each group. ANOVA followed by Tukeys test.  $^*p < 0.05$  : A vs. C,  $^*p < 0.05$  : B vs. C,  $^*p < 0.05$  : C vs. D,  $^{**}p < 0.01$  : A vs. D,  $^b p < 0.01$  : B vs. D,  $^b p < 0.01$  : C vs. E,  $^{***}p < 0.001$  : A vs. E,  $^c p < 0.001$  : B vs. E,  $^d p < 0.001$  : D vs. E

Figure 6. Effect of Pyrrolidine Derivative of Dibenzo-a-pyrone on malondialdehyde levels in mice brain tissues.



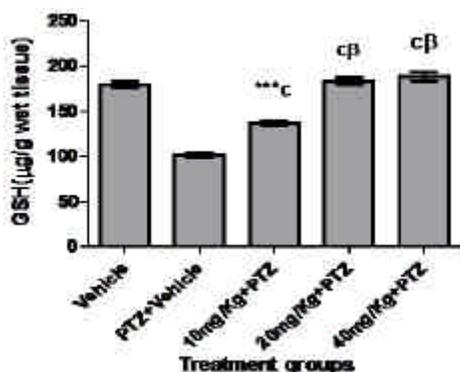
Data are expressed as mean  $\pm$  SEM; n=8 in each group. ANOVA followed by Tukeys test. \* $p < 0.05$  : A vs. C, <sup>a</sup> $p < 0.05$  : B vs. C, <sup>a</sup> $p < 0.05$  : C vs. D, \*\* $p < 0.01$  : A vs. D, <sup>b</sup> $p < 0.01$  : B vs. D, <sup>b</sup> $p < 0.01$  : C vs. E, \*\*\* $p < 0.001$  : A vs. E, <sup>c</sup> $p < 0.001$  : B vs. E, <sup>c</sup> $p < 0.001$  : D vs. E

Figure 7. Effect of Butylamine derivative of Dibenzo-a-pyrone on glutathione levels in mice brain tissues. mcg/g wet tissue.



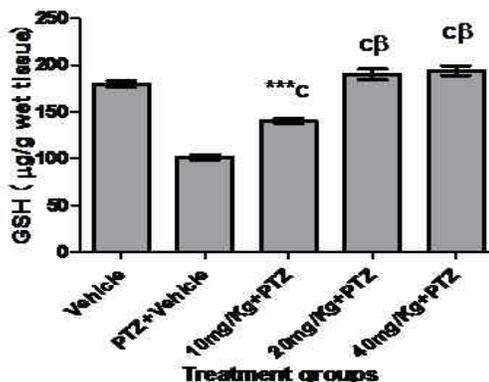
Data are expressed as mean  $\pm$  SEM; n=8 in each group. ANOVA followed by Tukeys test. \* $p < 0.05$  : A vs. C, <sup>a</sup> $p < 0.05$  : B vs. C, <sup>a</sup> $p < 0.05$  : C vs. D, \*\* $p < 0.01$  : A vs. D, <sup>b</sup> $p < 0.01$  : B vs. D, <sup>b</sup> $p < 0.01$  : C vs. E, \*\*\* $p < 0.001$  : A vs. E, <sup>c</sup> $p < 0.001$  : B vs. E, <sup>c</sup> $p < 0.001$  : D vs. E

Figure 8. Effect of Diethylamine derivative of Dibenzo-a-pyrone on glutathione levels in mice brain tissues. mcg/g wet tissue.



Data are expressed as mean  $\pm$  SEM; n=8 in each group. ANOVA followed by Tukeys test. \* $p < 0.05$  : A vs. C, <sup>a</sup> $p < 0.05$  : B vs. C, <sup>a</sup> $p < 0.05$  : C vs. D, \*\* $p < 0.01$  : A vs. D, <sup>b</sup> $p < 0.01$  : B vs. D, <sup>b</sup> $p < 0.01$  : C vs. E, \*\*\* $p < 0.001$  : A vs. E, <sup>c</sup> $p < 0.001$  : B vs. E, <sup>c</sup> $p < 0.001$  : D vs. E

Figure 9. Effect of Pyrrolidine Derivative of Dibenzo-a-pyrone on glutathione levels in mice brain tissues. mcg/g wet tissue.



Data are expressed as mean  $\pm$  SEM; n=8 in each group. ANOVA followed by Tukeys test. \* $p < 0.05$  : A vs. C, <sup>a</sup> $p < 0.05$  : B vs. C, <sup>a</sup> $p < 0.05$  : C vs. D, \*\* $p < 0.01$  : A vs. D, <sup>b</sup> $p < 0.01$  : B vs. D, <sup>b</sup> $p < 0.01$  : C vs. E, \*\*\* $p < 0.001$  : A vs. E, <sup>c</sup> $p < 0.001$  : B vs. E, <sup>c</sup> $p < 0.001$  : D vs. E

The results of the present study indicate that administration of Dibenzo- $\alpha$ -pyrone derivatives has a protective effect on PTZ-induced kindling in mice. It was observed that derivatives (20 and 40mg/kg, p.o.) significantly reduced the seizure score in mice as compared to the PTZ+ vehicle-treated group. However in a dose of 10 mg/kg, failed to reduce the seizure score.

Clinical reports suggest that not only epilepsy but drugs used in the treatment of epilepsy also exert negative effect on cognition which effects quality of life of epileptic patients. Ayurvedic literature recommends various plants possessing neuroprotective activity. Dibenzo- $\alpha$ -pyrone which is an active component of Shilajit has been found to possess memory improving and antioxidant activity. It is an effective antioxidant which could be responsible for its anticonvulsive activity.

Results from previous studies have suggested that PTZ-induced kindling is associated with enhanced activity of a subpopulation of glutamatergic synapses using N-methyl-D-aspartate (NMDA) receptors, increase in extracellular glutamate levels and subsequent free radical species generation in neurons.<sup>17, 18</sup> In the present study we have assessed the effect of chronic administration of derivatives in doses 10, 20 and 40mg/Kg, p.o. on PTZ-induced kindling and oxidative stress in mice. The result of the present study showed that subconvulsant dose of PTZ (25mg/Kg, i.p.) has induced kindling in 5 weeks. Administration of derivatives dose dependently protected against kindling as indicated by decreased seizure score. The seizure protection offered by 40mg/Kg, p.o. was comparable to standard epileptic drug diazepam. The protection offered by diazepam on PTZ kindling is well established and is known to occur via interaction between benzodiazepine-binding site at the GABA-benzodiazepine receptor ionophores complex (GABA-A receptor).<sup>19</sup>

Free radicals are the normal product of cellular aerobic metabolism involved in the development of seizures.<sup>20</sup> However, when the production of free radicals increases or defense mechanism of the body decreases, they cause cellular dysfunction by attacking at the polyunsaturated sites of the biological membranes causing lipid peroxidation. The increase in levels of malondialdehyde (MDA) is a marker of lipid peroxidation. In the present study we have measured oxidative stress parameters viz. malondialdehyde (MDA) and glutathione in kindled brain tissues to ascertain the involvement of oxidative stress in epileptogenesis and its modulation by the derivatives synthesized. Repeated PTZ administration has significantly increased the free radical generation as indicated by increased MDA in the vehicle treated PTZ-kindled mice. Synthesised derivatives (10,20 and 40mg/Kg, p.o.) dose dependently decreased the MDA levels in the brain tissue of PTZ-kindled mice.

In the present study the decreased level of glutathione were observed in the vehicle treated PTZ-kindled mice. Synthesised derivatives (10, 20 and 40mg/Kg, p.o.) administration in all doses demonstrated increase in glutathione level in kindled mice brain tissue. Glutathione (GSH) is an endogenous antioxidant which gets converted to oxidized form. This oxidized form of GSH reacts with free radicals and prevent generation of most toxic hydroxyl radical. Increased lipid peroxidation during kindling is independent of iron salts and excitotoxin. These result indicate that during kindling there was excessive oxidative stress pertaining to depleted glutathione levels while combating oxidative stress.<sup>21,22</sup> However, synthesized derivative treatment has restored the reduced glutathione level in the brain tissues of PTZ-kindled mice. Thus, in the present study the observed protective effect against PTZ-induced kindling could be due to the inhibition of oxidative injury.

The anticonvulsant effect of various antiepileptic drugs like phenytoin, valproate, phenobarbitone and carbamazepine has been studied in the kindling model of epilepsy. Among these valproate and phenobarbitone have been found to inhibit the development of kindling in experimental animals; however, phenytoin and carbamazepine failed to inhibit kindling development.<sup>23-26</sup> Phenytoin, carbamazepine and phenobarbitone have been reported cause an imbalance between the oxidative and antioxidant statuses. In experimental studies, treatments with Phenytoin, carbamazepine and phenobarbitone have resulted in an increased level of MDA and decreased GSH level in the brain, while valproate has shown antioxidant action.<sup>27-31</sup> In the present study Dibenzo- $\alpha$ -pyrone derivatives not only suppress seizures but also reduce oxidative stress. In the background of the above mentioned findings, it appears that Dibenzo- $\alpha$ -pyrone derivatives has anticonvulsant and antioxidant effects against PTZ-induced kindled seizure similar to sodium valproate.

#### 4. CONCLUSION

The present data support that synthesized derivatives of Dibenzo- $\alpha$ -pyrone showed anticonvulsant effect against PTZ kindled seizure in mice. Pretreatment also significantly decreased MDA and increased GSH levels. These data support the role of Dibenzo- $\alpha$ -pyrone derivatives in epilepsy as they significantly reduced the progression of kindling and attenuated the oxidative stress in mice. Our study is a preliminary study about the anticonvulsant and antioxidant effects of Dibenzo- $\alpha$ -pyrone derivatives on PTZ kindled seizure. Therefore it could be a promising candidate to control both development of seizure and oxidative stress during epilepsy.

However, further experimental, biochemical and clinical studies are required to determine the exact mode of action of Dibenzo- $\alpha$ -pyrone derivatives and to identify its possible interaction with other neurotransmission systems involved in epilepsy.

#### Conflicts Of Interest

All authors have none to declare.

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