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NEUROPROTECTIVE EFFICACY OF CURCUMIN AND BERBERINE WITH LAMOTRIGINE PRETREATMENT AGAINST CHEMICALLY INDUCED INTRACTABLE EPILEPSY IN ALBINO MICE

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ABSTRACT
Epilepsy is a chronic neurological disorder defined by recurrent unprovoked seizures, which is attributed to abnormal neuronal discharges in the brain. A large number of epileptic patients are reported to suffer from the drug resistant or refractory epilepsy, which are often associated with cognitive dysfunction, oxidative stress and damage of neuron. A large number of antiepileptic drugs have been developed, however there are still a number of drug resistant epilepsy patients. Therefore, the aim of the present study was to investigate the neuroprotective and anticonvulsant effects of curcumin and berberine on PTZ (40 mg/kg) + LTG induced drug-resistant epilepsy or intractable epilepsy in albino mice.
Healthy adult male albino mice (25-30g) were housed under standard laboratory conditions. Mice were randomly assigned to nine experimental groups. PTZ (40 mg/kg) induced epilepsy was treated using the drug-resistant epilepsy model created by treating mice with lamotrigine and berberine and curcumin were given in low and high doses individually and in combination. Seizure severity, onset time and duration, oxidative stress markers and brain histopathology were investigated.
It is hypothesized that, the treatment with both curcumin and berberine will have significant effects in lowering the seizures frequency and severity by antioxidant and neuroprotective actions. Furthermore, the combined administration of both agents in high doses will provide significant neuroprotection than low doses. Histopathological examination will be expected to show less damage to neuron and brain architecture.
It is concluded that, phytochemicals like curcumin and berberine could play a significant role as a potential therapeutic agent along with conventional drugs for the treatment of intractable epilepsy due to their multifarious beneficial effects like antioxidant, anti-inflammatory and neuroprotective properties.

KEYWORDS: PTZ kindling, Intractable epilepsy, Histopathology, Curcumin, Berberine, lamotrigine

1. INTRODUCTION

Epilepsy is among the most prevalent neurological diseases of the brain in the world and influences the lives of millions. Epilepsy is defined as recurrent seizures resulting from the excessive synchronized neuronal electrical discharge of neurons in the brain.[1] Although several antiepileptic drugs (AEDs) are available for seizure management, approximately one-third of patients develop drug-resistant or intractable epilepsy, where seizures remain uncontrolled despite adequate pharmacological therapy. This condition is associated with increased morbidity, cognitive dysfunction, emotional disturbances, and poor quality of life.[2]
Pentylenetetrazole (PTZ) is a well-established chemoconvulsant widely used in experimental models for inducing seizures and studying epilepto-genesis. PTZ primarily functions through the antagonism of GABA-mediated inhibitory neurotransmission, leading to neuronal hyperexcitability and recurrent seizures. Chronically administered PTZ evokes changes which mimic intractable human epilepsy and has been utilized as a useful experimental model in screening antiepileptic drugs and neuroprotective agents.[3]
It is already well established that oxidative stress is a very important role in the pathogenesis of epilepsy. Generation of free radicals during repeated seizures attack induce lipid peroxidation, mitochondrial dysfunction, neuroinflammation and neuronal apoptosis.[4] Constant accumulation of oxidation induces seizure state further enhances neurodegeneration and increasing neuronal vulnerability. Henceforth, compounds with both antioxidants and neuroprotective effect could be potentially used in epilepsy management. [5]
Polyphenolic compound curcumin which is extracted from rhizome of turmeric (Curcuma longa) have attracted scientist due to their anti-oxidant, anti-inflammatory and neuroprotective activities. .[6] The substance curcumin has been studied to have effects on neurotransmitter release, suppressionof oxidative stress and reduction of neuronal cell death in certain neurological disorder.

Berberine is an isoquinoline alkaloid that is found in several traditional medicinal plants like Berberis vulgaris and shows multiple pharmacological properties such as antioxidant, anti-inflammatory, anticonvulsant, neuroprotective effects. Moreover, berberine can modulate signaling pathways in neurons, inhibit neuroinflammation and protect neuronal mitochondria. Hence, berberine might have a therapeutic role to limit seizure induced damage in neurons.[8]
The main objective of the present investigation is to assess the neuroprotective activity of curcumin and berberine against PTZ (40 mg/kg) with Lamotrigine (5 mg/kg) induced intractable epilepsy in albino mice. Moreover, we analyze whether low and high dose treatment has better neuroprotection and anticonvulsant action as compared to individual treatment.[9]
The experimental design includes normal control, PTZ-induced epileptic control, standard antiepileptic treatment groups, and various treatment groups receiving berberine and curcumin in different doses and combinations. Assessment parameters include seizure behavior, oxidative stress markers, and histopathological examination of brain tissue.[10]

2. MATERIALS AND METHODS

2.1. Experimental Animals

Animals and Ethics Statement Swiss albino mice (male; 25 to 30 g) were obtained from the Subharti University Meerut, INDIA, and sustained in a standard laboratory environment consistent pelleted diet and water ad libitum with appropriate ventilation and proper hygiene. The protocol was approved by Institutional Animals Ethics Committee (IAEC) (1024/PO/ReBiBt/S/2008/CCSEA/25-7). IAEC guidelines were obeyed to avoid any suffering to experimental animals during the entire procedure.

2.2. Chemical and Reagents

The ethanolic extract of curcumin and berberine was collected from Vital Herbs, Add; - Z-26/27 Commercial Enclave, Mohan Garden, Uttam Nagar, Delhi, 110059 (GSTN: 07CIRPD7950H1ZV). The extract was stored in a cool and dry place and kept away from strong light and heat.

2.3. Experimental Design

Animal randomly divided into groups, which each group number of 6 animals

Group	Treatment
Group I	Normal control
Group II	PTZ (40 mg/kg) only
Group III	Lamotrigine(5 mg/kg) + PTZ(40 mg/kg)
Group IV	Lamotrigine (5 mg/kg) + PTZ (40 mg/kg) + Valproic Acid(400 mg/kg)
Group V	PTZ (40 mg/kg) + Lamotrigine (5 mg/kg) + Valproic Acid(400 mg/kg) + Berberine(50 mg/kg)
Group VI	PTZ (40 mg/kg) + Lamotrigine (5 mg/kg) + Valproic Acid(400 mg/kg) + Berberine(100 mg/kg)
Group VII	PTZ (40 mg/kg) + Lamotrigine (5 mg/kg) + Valproic Acid(400 mg/kg) + Curcumin (50 mg/kg)
Group VIII	PTZ (40 mg/kg) + Lamotrigine (5 mg/kg) + Valproic Acid(400 mg/kg) + Curcumin (100 mg/kg)
Group IX	PTZ (40 mg/kg) + Lamotrigine (5 mg/kg) + Valproic Acid(400 mg/kg) + Curcumin (100 mg/kg) + Berberine(100 mg/kg)

2.4. PTZ-induced Kindling Seizures

Pentylenetetrazole Induced Kindled and refractory/intractable Seizures PTZ (40 mg/kg, i.p.) was given in sub-convulsive doses on alternate days for 37 days until the animal seemed to have complete motor seizures. Animals were kept in a plexiglass chamber (40 × 23 × 23 cm) with partitions in between after each injection of PTZ, and seizure intensity was monitored for thirty (30) minutes.19-20 The frequency of convulsion was evaluate using a 6 point scoring scale: score 0 = no response, score1 = myoclonic jerks, score 2 = Straub's tail with an upright position, score 3 = clonus with head jerks, total responses of mean were assesed for the period of treatment.[11]

2.5. Pretreatment with LTG and Pentylenetetrazol-induced kindling or Drug-resistant epilepsy

Drug-resistant epilepsy was induced in animals by LTG, pre-treatment (5 mg/kg) 45 minutes before pentylenetetrazol administration (40mg/kg, i.p.)[12] on every alternate day till the stable kindled state (three consecutive stages and five seizures) or till 19 injections of PTZ, based on whichever procedure was achieved prior in the mice (37 days). The seizure score was evaluate using a modified Racine scale [13] described as Stage 0: no response; Stage 1: hyperactivity, restlessness, and vibrissae twitching; Stage 2: head nodding, head clonus, and myoclonic jerks; Stage 3: unilateral or bilateral limb clonus; Stage 4: forelimb clonic seizures; Stage 5: generalized tonic-clonic seizures with falling; Stage 6: hind limb extension. The mice were observed for 30 minutes in a plexiglass chamber (40 × 23 × 23 cm) after every PTZ injection.

2.6. Behavioural Study
The Racine scale was used to observe the severity of behavioural response in PTZ-induced seizures: score 0: No response, score 1: Facial & ear twitching, score 2: Myoclonic involuntary jerks, score 3: Myoclonic sudden jerks, score 4: Tonic-clonic seizure, score 5: Generalized clonic-tonic convulsion.[14]

2.7. Elevated plus-maze model
Elevated model is made up of two (2) uplifted long open arms size (18 cm × 7 cm) and, two long-closed arms size (18 cm × 7 cm) joined by a main rectangular approx. size - 6 cm × 5 cm, which is another model for assessing cognitive dysfunction. The model is assessed to a height of 28 cm above from the ground, and each arms has 12-cm-high sidewalls. The individual animals were keep on the edge of one open arm, facing a distance from the mid center, & the period it took the mice to run from the open arm to either of the closed (elevated) arms on the apparatus was observed to determine the initial transfer latency (ITL). The mice were lightly pushed in the direction of a closed arm if they did not reach either of the closed arms within one hundred twenty (120) seconds (cut-off time).[15] The mice were permitted to observe the response for 20 second before finally being returned to their main cage. After 24 hr, time spent by the mice to reached in the closed arm was observed [retention transfer latency] (RTL).

2.8. Actophotometer Test
The Actophotometer is a widely used instrument for assessing locomotor activity and exploratory behavior in experimental animals. The apparatus consists of a square chamber measuring approximately 30 cm × 30 cm × 30 cm, fitted with photoelectric cells and infrared light beams connected to a digital activity counter. The model works on the principle that interruption of light beams by the movement of the animal produces activity counts automatically. Individual mice were placed separately in the Actophotometer chamber, and their spontaneous locomotor activity was recorded for a period of 5 min. In order to avoid a stressed induced response on animal's activity the animals were habituated for a short period in the apparatus before the real experiment. Increased activity scores denote an augmented locomotor and exploratory behavior while the decrease in activity shows sedation, muscle relaxation, functional damage to the brain or seizure-mediated behavior. [16] The present study employed the Actophotometer test for assessing the effects of curcumin and berberine treatment on motor activity and central nervous system functions in PTZ-induced epileptic mice.

2.9. Morris Water Maze (MWM)
The Morris Water Maze test was employed to evaluate spatial learning and memory functions. Mice were trained to locate a hidden platform submerged in water. Escape latency time and time spent in the target quadrant were recorded to assess cognitive impairment and neuroprotective effects of the treatments. [18]

3. Isolation of Brain and Homogenization
After the completed behaviours studies, mice in their respective groups (n=84) were sacrificed, and isolation of the brain tissue and preparation of the homogenate were done as per formerly documented reports [35]. The supernatants were used for bio-chemical assessments.

4. Biochemical Assay
Animals were sacrificed in order by the action of cutting off the head of animal after behavioural assessments; The brains were washed with isotonic saline, then noted the weight, and cut into two similar halves. The other half part was used to make biochemical calculations. For biochemical analysis, 10% (w/v) homogenates tissues were added to 0.1 M phosphate (pH 7.4) buffer. After that centrifugated homogenates at 10,000 grams for 15 min at 4°C. Supernatants were split into aliquots & were used for biochemical assessment [18-19] For the biochemical analyses, a UV spectrophotometer 1800 [Shimadzu] was used.

4.1. Estimation of Protein Level
The protein was also determined and followed by the biuret technique and used with bovine serum albumin used as the standard. [27]

4.2. Estimation of nitrite level
The amount of nitrite in the precipitate was measured as a marker of nitric oxide (NO) synthesis in which the colorimetric method was used with Greiss reagent (0.2% N-[1-naphthyl] ethylenediamine di-hydrochloride, 2.6% phosphoric acid, 1% sulfanilamide). The Greiss reagent and supernatant were combined in equal proportions and reserved for 10 min. The absorbance was noted by using a double beam UV–VIS spectrophotometer [UV spectrophotometer 1800 (Shimadzu)] at 560 nm.[21] The sodium nitrite standard calibration curve was used to evaluate the unknown concentration amount of nitrite in the supernatant, which was quantified as µg/mg of Protein**.**

Estimation of reduced glutathione (GSH) level
Glutathione levels in the animal brain were usually find out using Ellman and his collaborators' approach. The 1 ml of supernatant was crystallised by cold digested at 4°C for 60 min with one (1) ml [5% sulfosalicylic acid]. The homogenate was centrifuged at 1000 RPM (rotations per minute) for 15 min at 4°C. 2.7 ml of [0.1 M phosphate buffer] at pH 8 and 0.3 ml of 5,5 [dithiobis-2-nitrobenzoic acid] were used for 1 ml of the precipitate. The finding was determined by using the chromophore's molar extinction coefficient (1.38 × l04 M-1 cm-1) and was represented as micromoles of GSH per

mg of protein. The yellow colour formed was instantly detected at 430 nm using a double beam UV–VIS spectrophotometer. [22] [UV spectrophotometer 1800 (Shimadzu)] was used.

4.3. Estimation of Lipid Peroxidation Level (MDA)

The Wills model was followed to calculate the quantitative level of lipid peroxidation in the Albino mice's brains. The values were determined using the chromophore's molar extinction coefficient (1.58 × 107 M-1 cm-1) & reported in the result as malondialdehyde nanomole per milligram of proteins. Whereas the level of malondialdehyde (MDA), which is a main content of lipid peroxidation, was also determined using a double beam UV–VIS spectrophotometer. [23] [UV spectrophotometer 1800 (Shimadzu)] in a reaction with thio-barbituric acid at 540 nm.25.

4.4. Estimation of Catalase Level

The Luck method was used to evaluate catalase level, which accurately measures the decomposition of hydrogen peroxides (H_2O_2) at 240 nm. The combination comprised 0.04 ml of homogenate tissue supernatant (10%) and 3 ml of hydrogen peroxides with phosphate buffer.25 The variation in the find value of absorbance was noted at 240 nm by using a [UV spectrophotometer 2202 (systronics)]. [24]

4.5. Estimation of Superoxide Dismutase (SOD) Activity

The activity of superoxide dismutase measure used by the the Kono method, which inhibited the reduction of nitro blue tetrazolium (NBT), which was determined by double beam UV–VIS spectrophotometer at 540 nm.[24] [UV spectrophotometer 1800 (Shimadzu) (Japan)]. The results were reported in micromoles of hydrogen peroxides ($H_2 O_2$) disintegrated per mg of protein/minutes. In a sense, the reaction was started by the pouring of hydroxylamine hydrochloride into a solution of Nitro-blue tetrazolium chloride. The findings were represented as units per mg of protein, with one part of the enzyme matching the amount of enzymes that completely inhibits the rate of the reaction.

4.6. Estimation of Acetylcholinesterase (AChE) Activity

The Ellman method was followed to estimate acetylcholinesterase (AChE) activity in the brain tissue of experimental mice. The assay is based on the hydrolysis of acetylthiocholine iodide by AChE, producing thiocholine, which reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a yellow-colored chromogen. The absorbance was measured using a double beam UV–VIS spectrophotometer [UV spectrophotometer 1800 (Shimadzu)] at 412 nm.[26] The enzyme activity was expressed as micromoles of acetylthiocholine hydrolyzed per minute per milligram of protein. Increased AChE activity indicates cholinergic dysfunction and neuronal damage associated with epileptic seizures.

This study may provide important evidence regarding the therapeutic potential of naturally derived compounds as adjunctive treatments for drug-resistant epilepsy. The findings could contribute to the development of safer and more effective neuroprotective strategies for the management of chronic epileptic disorders.

4.7. Histopathological Examination

Brain tissue, especially the hippocampus and cerebral cortex, was fixed in 10% neutral formalin buffered, sectioned, and stained with H&E. They were examined histologically for degeneration of neuron, pyknosis, vacuolization, or other neuropathology.

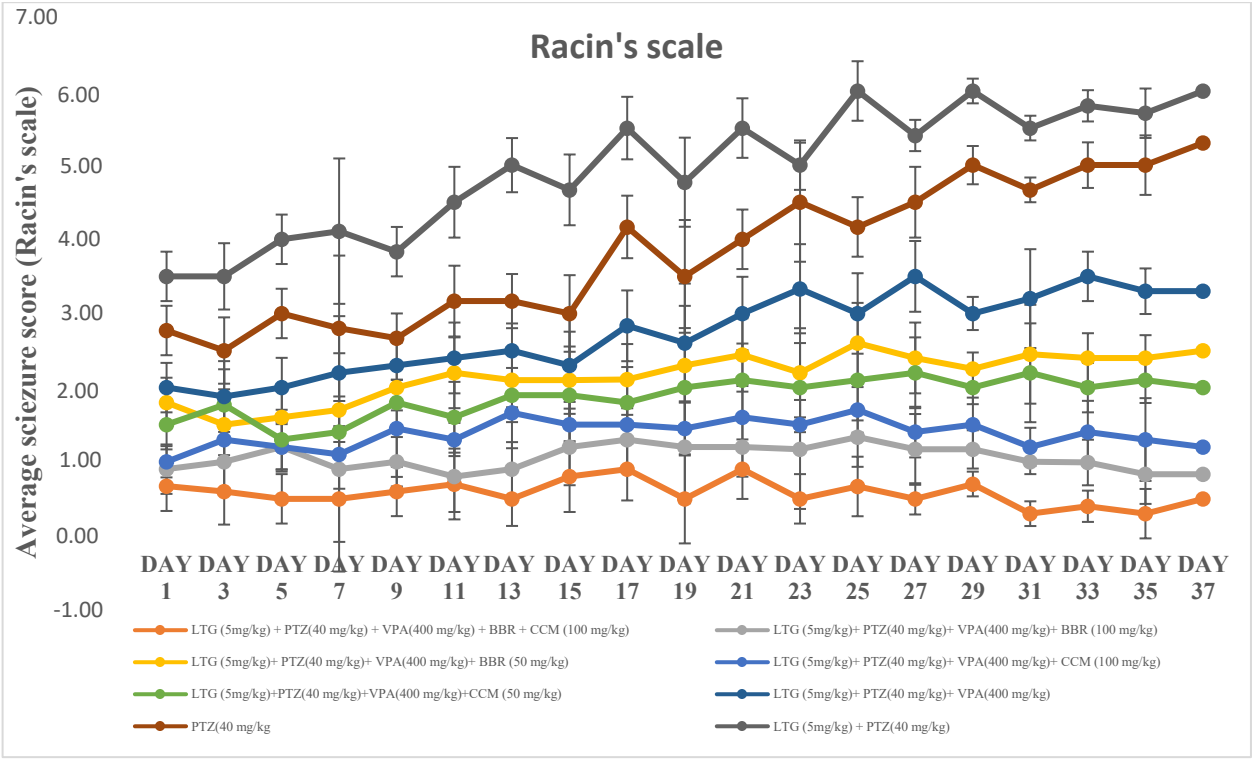
4.8. Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison test, which was done by Graph-Pad Prism software. Values of $p < 0.05$ were considered statistically significant.

5. RESULTS AND DISCUSSION

5.1. Influence Curcumin and Berberine on PTZ-induced intractable Seizures Score

The administration of repeated PTZ (40 mg kg; i.p) on alternating days and LTG pre-treatment (5 mg/kg) 45 minutes before pentylenetetrazol administration (40mg/kg, i.p.) on every alternate day till the stable kindled state (three consecutive stages and seizures) or till 19 injections of PTZ (for 37 days) led to a continual rise in convulsive properties, exhibited by a significant increase in the score (average score of 4-6). Curcumin, Berberine and valproic acid [$F (8, 45) = 44.64$, $^{ab}p< 0.05$ score as compared to PTZ-treated animals. A high dose of Curcumin and Berberine significantly reduced seizures score and offered neuroprotection compared to a low dose(50mg/kg).



Effect of Curcumin and Berberine on PTZ-induced intractable seizures score.
Intractable seizures/epilepsy in mice were provoked by recurrent injection of the PTZ (40 mg kg, i.p), and pre-treatment LTG (5 mg/kg, i.p) 45 minutes before pentylenetetrazol administration (40mg/kg, i.p.) on every alternate day till the stable kindled state seizure severity was noticed for ½ hour after every PTZ administration. Administration of Curcumin and Berberine (50 and 100 mg kg; p.o) and valproic acid (200 mg kg; i.p) significantly ($p<0.05$) diminished seizures score in mice. Outcomes are represented as mean ± SEM; one-way ANOVA preceded by Tukey's multiple Comparison Tests. VPA (Valproic Acid), PTZ (Pentylene Tetrazole), LTG(Lamotrigine), CCM (Curcumin), BBR (Berberine)

5.2. Behavioural Parameter

5.2.1. Elevated Plus Maze

The effect of the treated on learning and memory performance in the Elevated Plus Maze (EPM) test was evaluated by measuring the Transfer Latency (TL) significantly increase the acquisition and retention time, when the compare to control group [$F(8,45) = 11.023$, $p < 0.05$; $^{ab}p<0.50$ vs control. However, compared treatment group with PTZ and LTG+PTZ groups significantly decreased [$F(8,45) = 11.023$, $p < 0.05$, $^{c}p<0.50$ vs PTZ and LTG +PTZ], The dose dependent effect has been observed.

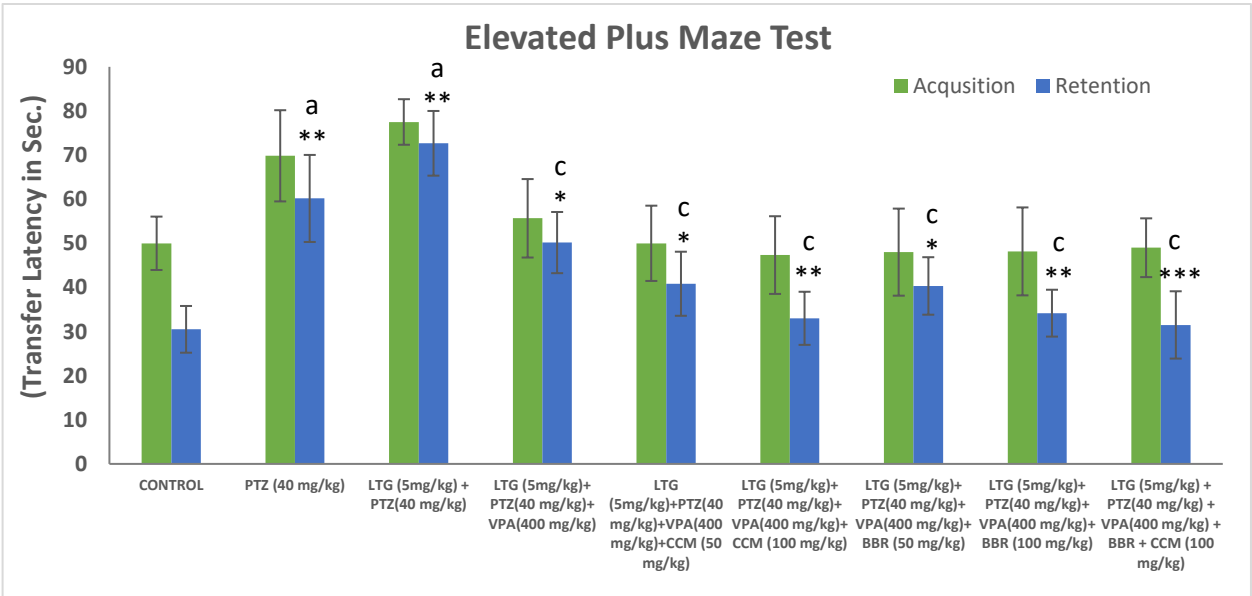


Figure 2:- Effect of Berberine and Curcumin on TL in PTZ and LTG+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Transfer Latency (TL) [F (8,45) = 11.023, $p<0.05$, $^{ab}p<0.50$, vs control; $^{c}p<0.50$ vs PTZ and PTZ+LTG] was considered statistically significant. EPM (Elevated Plus Maze Test), VPA (Valproic Acid), PTZ (Pentylene Tetrazole), LTG(Lamotrigine), CCM (Curcumin), BBR (Berberine)

5.2.2. Actophotometer

The effect of the treatment on learning and memory performance in the Actophotometer test was evaluated by measuring the locomotor activit significantly deccrease the lovomoter activity time, when the compare to control group [F (8,45) = 6.023, $p < 0.05$, $^{ab}p<0.50$, vs control], However the compared treatment group with PTZ and LTG+PTZ groups significantly increased ELT [F (8,45) = 8.230, P<0.05); $^{ab}p<0.05$ vs control: $^{c}p<0.05$ vs PTZ and PTZ+LTG) The dose dependent effect has been observed.

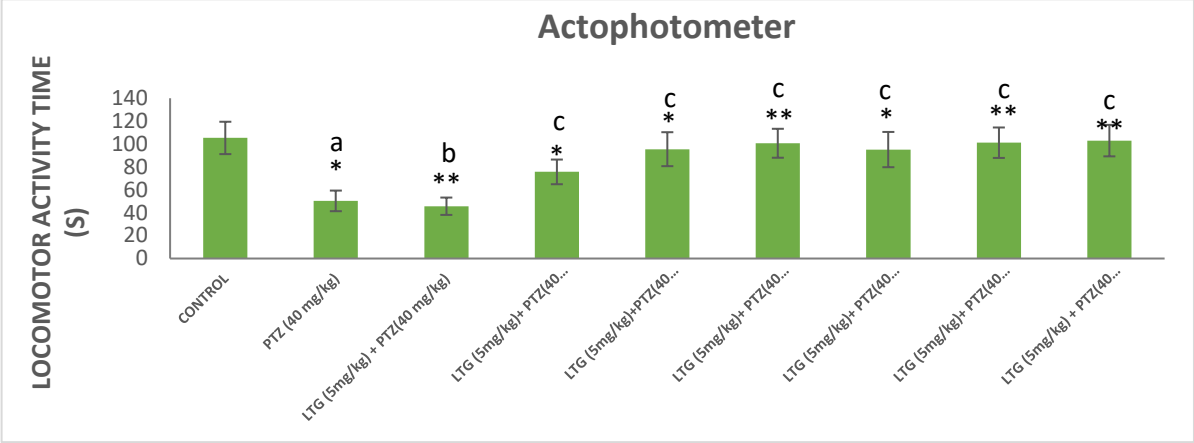


Figure 3:- Effect of Berberine and Curcumin on locomotor activity time in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Locomoter activity time [F (8,45) = 6.023, $p<0.05$, $^{ab}p<0.50$, vs control, $^{c}p<0.50$ vs PTZ and PTZ+LTG was considered statistically significant. Actophotometer, VPA (Valproic Acid), PTZ (Pentylene Tetrazole), LTG(Lamotrigine), CCM (Curcumin), BBR (Berberine)

5.2.3. Morris water maze

The effect of the treatment on learning and memory performance or acquisition is indicated by the higher day 4 ELT in MWM trails compared to day 1st escape latency time (ELT). However, a decreased TSTQ on the fifth day indicate memory loss. The control group showed a remarkable (p<0.050) drop-in day 4 ELT compared to the first day of ELT Fig4 and a significant increase in the fifth day TSTQ (in the Q4 quadrant) compared to the other Q1, Q2, Q3 quadrants, which indicate typical learning and memory. On day 4th, PTZ and PTZ+LTG showed that a significant increase in ELT [F (8,45) = 10.230, p<0.05) $^{ab}p<0.05$ vs control] and a significant decrease in TSTQ [F (8,45) = 10.230, p<0.05) $^{c}p<0.05$ vs PTZ and PTZ+LTG)] on day five. Fig4, However, the effect of PTZ and PTZ+LTG were considerably reversed in mice administered by the Valproic acid, Curcumin and Berberine. The dose dependent effect has been observed.

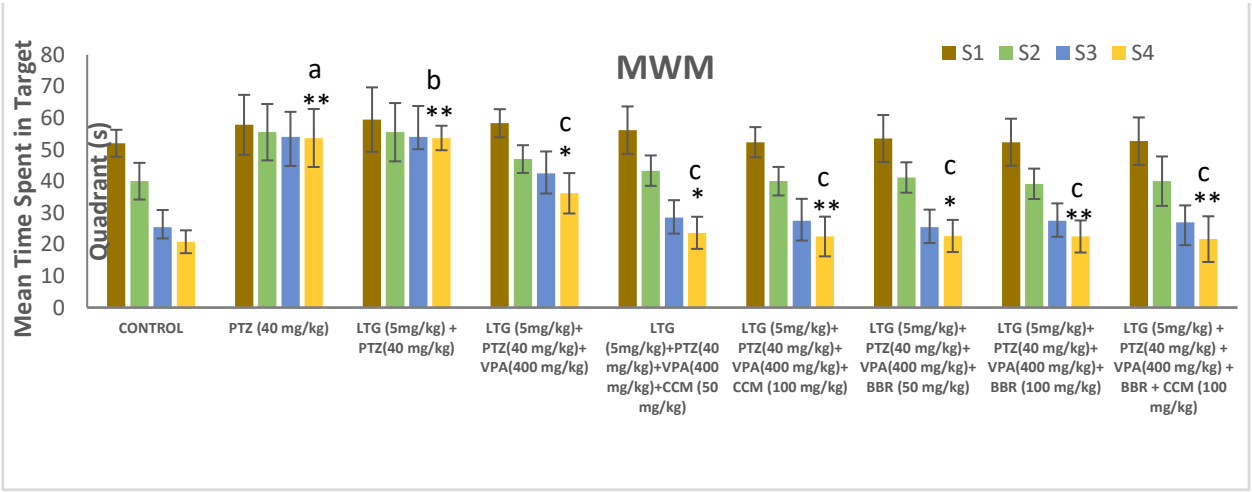


Figure 4:- Effect of Berberine and Curcumin on locomotor activity time in PTZ and PTZ+LTG induced experimental animals

Results are expressed as Mean ± SEM (n=6), one-way ANNOVA followed by Tukey's multiple comparison test. ELT [F (8,45) = 10.230, P<0.05; $^{ab}p<0.05$ vs control and $^{c}p<0.05$ vs ELT in PTZ and PTZ+LTG). MWM (Morris Water Maze),

ELT (Escape latency test), VPA (Valproic Acid), PTZ (Pentylene Tetrazole), LTG(Lamotrigine), CCM (Curcumin), BBR (Berberine)

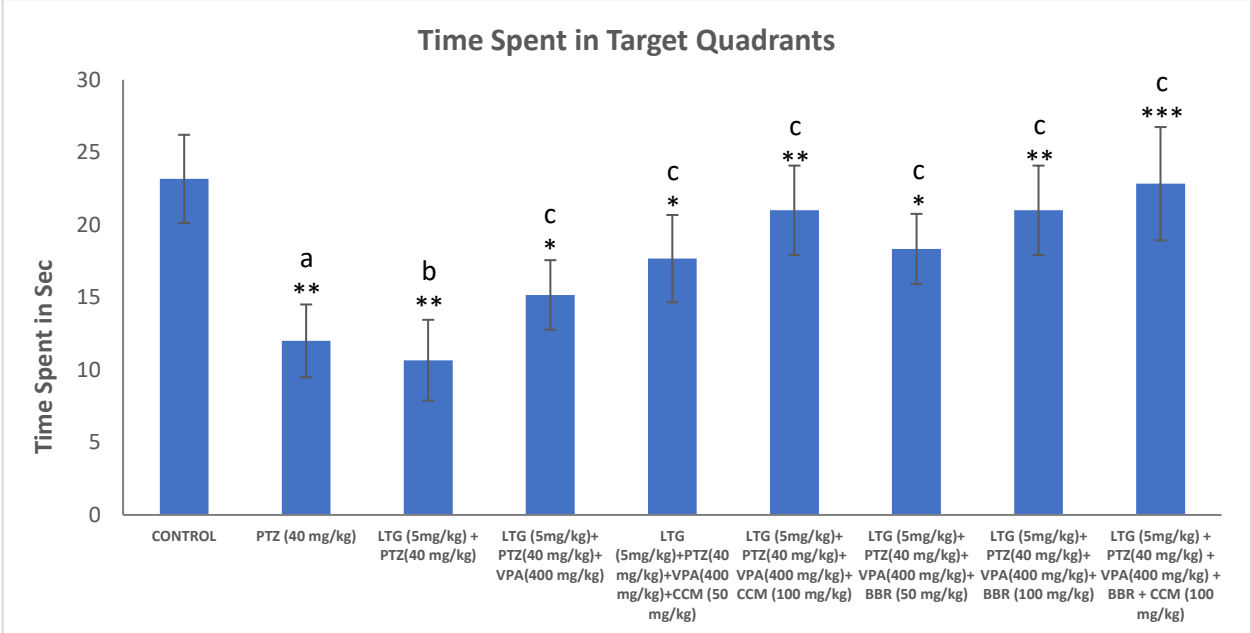


Figure 5:- Effect of Berberine and Curcumin on locomotor activity time in PTZ and PTZ+LTG induced experimental animals

Results are expressed as Mean ± SEM (n=6), one-way ANNOVA followed by Tukey's multiple comparison test. ELT [F (8,45) = 10.232, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ and PTZ+LTG]. MWM (Morris Water Maze), ELT (Escapelatency test), VPA (Valproic Acid), PTZ (Pentylene Tetrazole), LTG(Lamotrigine), CCM (Curcumin), BBR (Berberine)

5.3. Biochemical Parameters

5.3.1. Estimation of Reduced Glutathione (GSH)

When compared to the control group, the level of GSH was significantly decreased in the PTZ and GPT+PTZ treated group [F (8,45) = 12.012, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ and LTG+PTZ groups significantly increased the level of GSH [F (8,45) = 12.012, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the dose dependent effect has been observed.

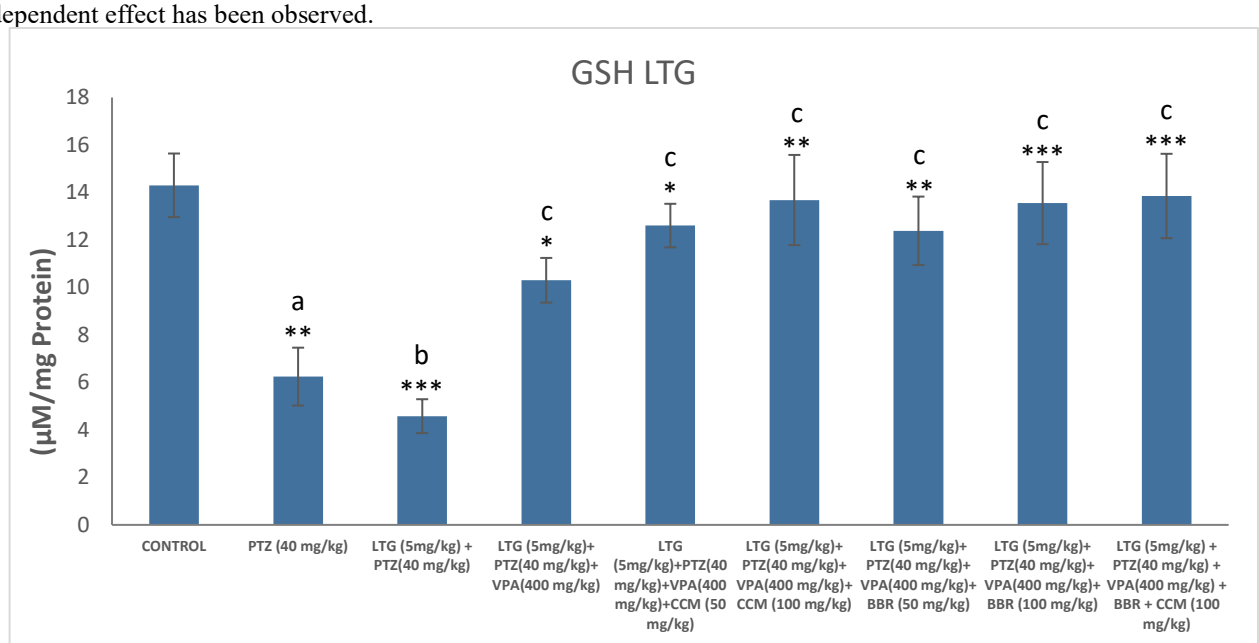


Figure 6:- Effect of Berberine and Curcumin on reduced glutathione (GSH) levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 12.02, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ PTZ+LTG] was

considered statistically significant. GSH (Reduced Glutathione), VPA (Valproic Acid), PTZ(Pentylenetetrazole), LTG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

5.3.2. Estimation of Malondialdehyde (MDA)

When compared to the control group, the level of **Malondialdehyde (MDA)** was significantly increased in the PTZ and LTG+PTZ treated group the level of **MDA** [F (8,45) = 11.11, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ and LTG+PTZ groups significantly decreased [F (8,45) = 11.11, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the dose dependent effect has been observed.

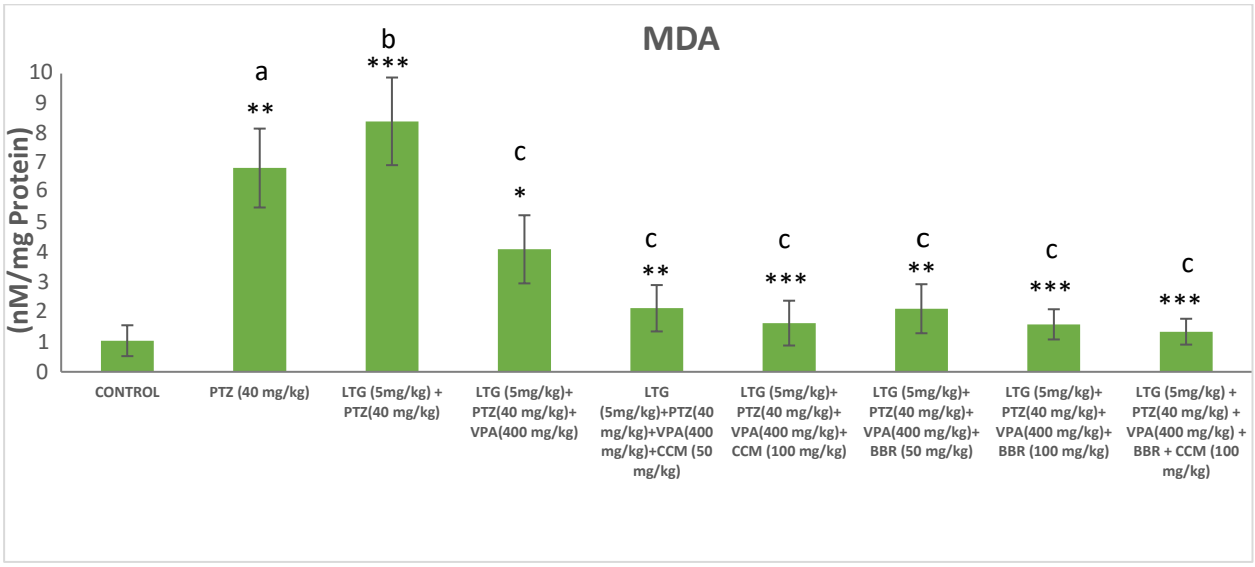


Figure 7:- Effect of Berberine and Curcumin on MDA (levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 11.11, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ PTZ+LTG] was considered statistically significant. MDA(Malondialdehyde), VPA(Valproic Acid), PTZ(Pentylene Tetrazole), LTG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

5.3.3. Estimation of Nitric Oxide (NO)

When compared to the control group, the level of Nitric Oxide (NO) was significantly increased in the PTZ and LTG+PTZ treated group [F (8,45) = 11.32, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ and LTG+PTZ groups significantly decreased [F (8,45) = 11.32, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the level of Nitric Oxide, dose dependent effect has been observed.

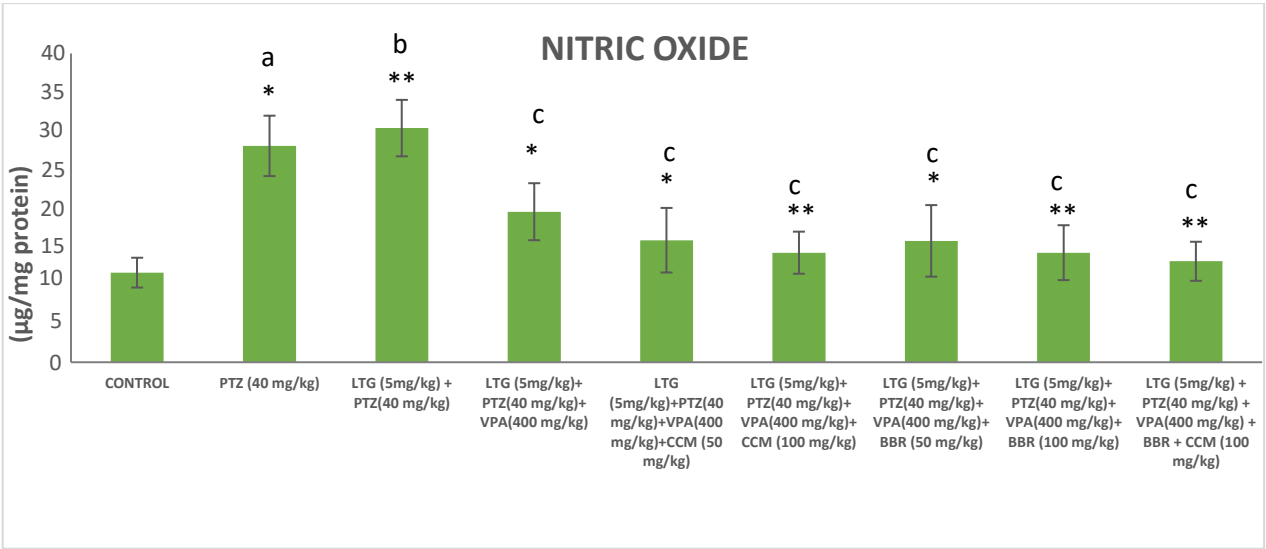


Figure 8:- Effect of Berberine and Curcumin on Nitric Oxide levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 11.32, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ and PTZ+LTG] was

considered statistically significant. NO (Nitric Oxide), VPA(Valproic Acid), PTZ(Pentylene Tetrazole), TG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

5.3.4. Estimation of Catalase (CAT)

When compared to the control group, the level of Catalase (CAT)was significantly decreased in the PTZ and LTG+PTZ treated group [F (8,45) = 9.999, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ and LTG+PTZ groups significantly increased the level of Catalase [F (8,45) = 9.999, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the dose dependent effect has been observed.

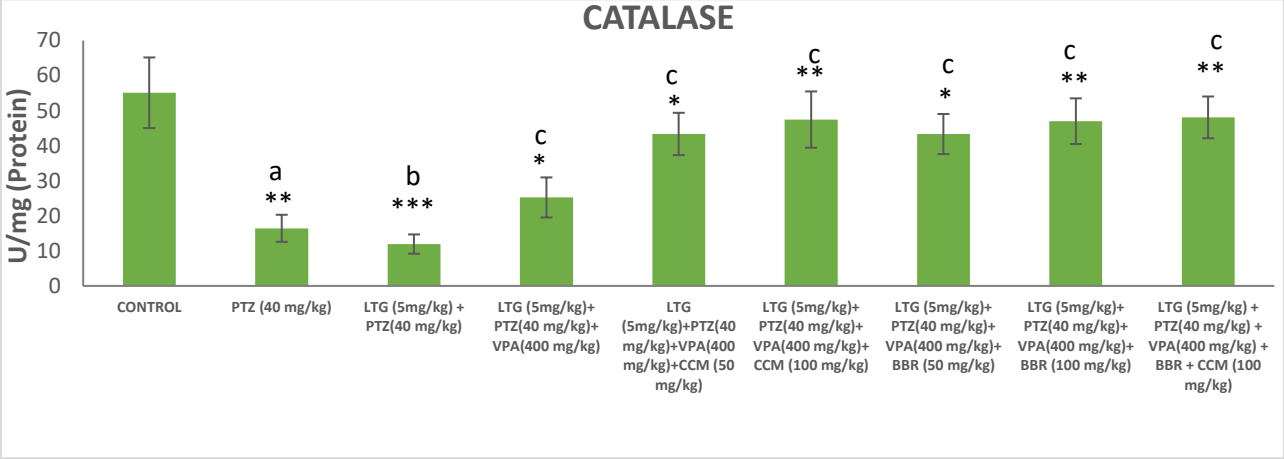


Figure 9:- Effect of Berberine and Curcumin on Catalase (CAT) levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 9.999, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ PTZ+LTG] was considered statistically significant. CAT(Catalase), VPA(Valproic Acid), PTZ(Pentylene Tetrazole), LTG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

5.3.5. Estimation of Superoxide Dismutase (SOD)

When compared to the control group, the level of Superoxide Dismutase (SOD) was significantly decreased in the PTZ and LTG+PTZ treated group [F (8,45) = 10.99, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ and LTG+PTZ groups significantly increased level of Superoxide Dismutase [F (8,45) = 10.99, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the dose dependent effect has been observed.

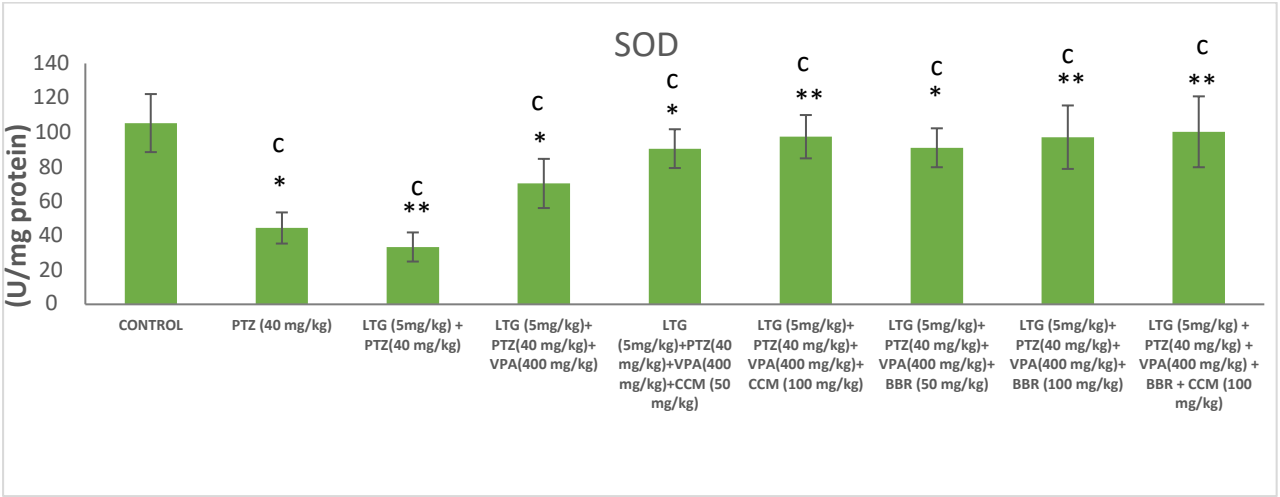


Figure 10:- Effect of Berberine and Curcumin on Superoxide Dismutase (SOD) levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 10.99, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ PTZ+LTG] was considered statistically significant. SOD(Superoxide Dismutase),VPA(Valproic Acid), PTZ(Pentylene Tetrazole), LTG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

5.3.6. Estimation of Acetylcholinesterase (AChE)

When compared to the control group, the level of Acetylcholinesterase was significantly increased in the PTZ and LTG+PTZ treated group [F (8,45) = 11.33, P<0.05; ^{ab}p<0.05 vs control]. When the compared treatment group with PTZ

and LTG+PTZ groups significantly decreased the level of Acetylcholinesterase [F (8,45) = 11.33, P<0.05; ^{c}p<0.05 vs PTZ and LTG+PTZ], the dose dependent effect has been observed.

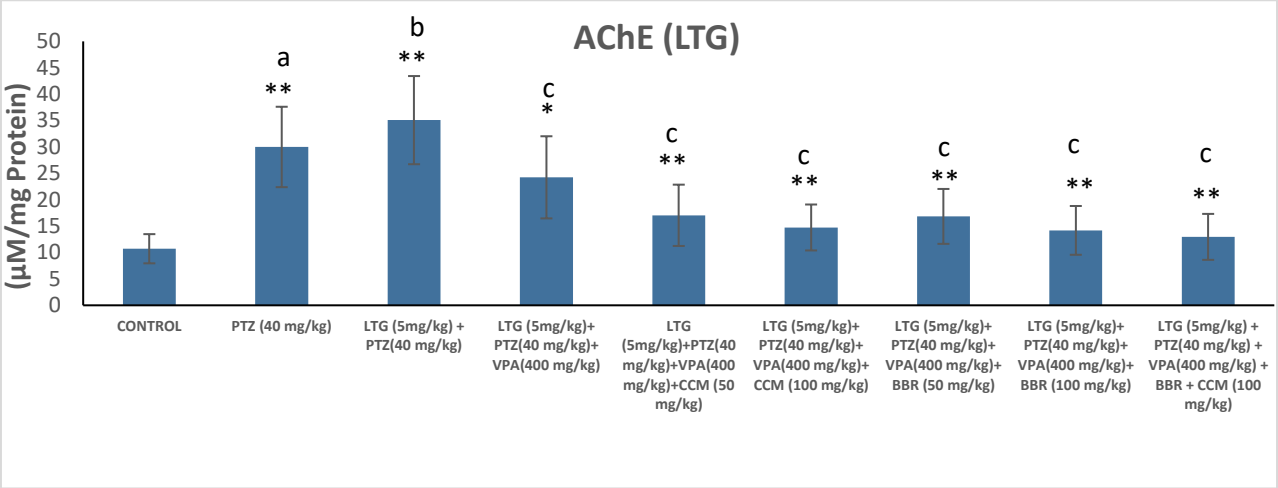
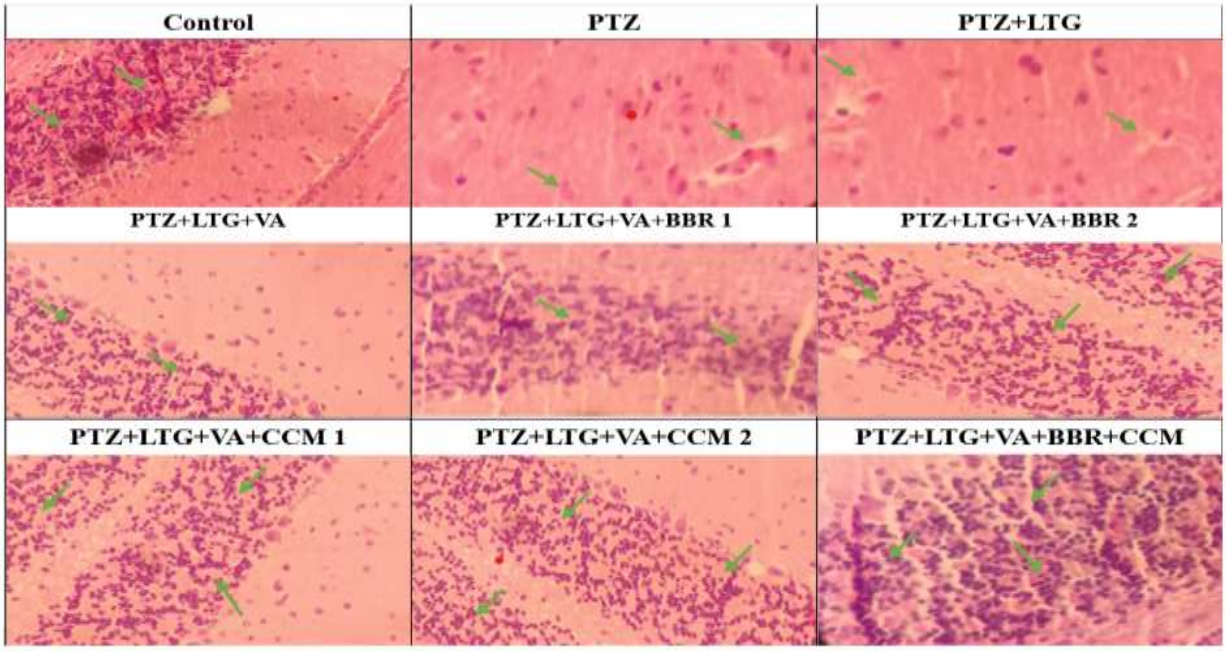


Figure 11:- Effect of Berberine and Curcumin on Acetylcholinesterase (AChE) levels in PTZ and PTZ+LTG induced experimental animals

Results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test [F (8,45) = 11.33, P<0.05; ^{ab}p<0.05 vs control and ^{c}p<0.05 vs PTZ PTZ+LTG] was considered statistically significant. AChE(Acetylcholinesterase), VPA(Valproic Acid), PTZ(Pentylene Tetrazole), LTG(Lamotrigine), CCM(Curcumin), BBR(Berberine).

6. Histopathological

Histopathological: Histopathological examination of sections from the hippocampi of control group showed normal neuronal architecture with normal cellular organization. However, PTZ treated groups show neuronal degeneration, pyknotic nucleus, vacuolation and gliosis. With Lamotrigine + PTZ neuronal damage persisted and develops drug resistance epilepsy, whereas valproic acid provides some improvement in neuronal architecture, while berberine or curcumin with PTZ treatment decreases neurodegeneration dose dependent manner, whereas the combination therapy of both curcumin (100mg/kg) and berberine (100mg/kg) has significant effect with normal neuronal architecture with little degeneration and gliosis.



Effect of Berberine and Curcumin on histopathology**,** Illustrative images of H&E stained (magnification 40x) in the cortex area. The neurons as ordinary displayed regulatory round and bright pink nucli. However, dead cells exhibited pyknotic nuclei & shrunken bodies of cells. Arrow indicate shrunken cells the pyknosis, as well as the unevenly shaped and tangled cells.

6.1. Group I - Normal Control
Sections from hippocampi and cerebral cortex of this group displayed normal histoarchitecture. Neurons showed intact, well defined nucleus and normal cellular arrangement with lack of neuronal degeneration, gliosis, edema or inflammation infiltration cell.

6.2. Group II - PTZ (40mg/kg) Only
Sections displayed remarkable pathological alterations including widespread neuronal degeneration, neuronal loss, pyknotic nucleus, neuronal vacuolation and edema with prominent gliosis and infiltration inflammatory cells of hippocampi (CA1, CA3 and dentate gyrus). Seizure induces significant neuronal damage.

6.3. Group III - Lamotrigine (5mg/kg) + PTZ (40mg/kg)
Sections demonstrated sustained neuronal injury with moderate to severe neuronal loss and neuronal degeneration in comparison to control with large number of pyknotic neuronal nucleus with darkly stained nucleus, vacuolation area with limited reduction in histological injury in compared to PTZ group.Lamotrigine resistance epilepsy might be induced with inadequately neuroprotective role of lamotrigine.

6.4. Group IV - Lamotrigine (5mg/kg) + PTZ (40mg/kg) + Valproic Acid (400mg/kg)
Sections revealed moderate improvement in the neuronal architecture; the loss of neurons and inflammatory infiltration was decreased compared with Group III, however, several pyknotic neuronal, mild edema and local gliosis were evident in hippocampus

6.5. Group V - PTZ + Lamotrigine + Valproic Acid + Berberine (50mg/kg)
Sections showed marked neuroprotective role and revealed neuronal improvement, decreased neuronal loss and vacuolation and gliosis. Small proportion of neuronal degenerations was observed and better tissue organization was achieved than group IV**.**

6.6. Group VI - PTZ + Lamotrigine + Valproic Acid + Berberine (100mg/kg)
Sections showed the almost complete recovery of normal brain architecture. Mostly the neuronal structures of hippocampi are well-formed with retained cellular architecture. Neuronal degenerations, edema and inflammatory infiltration are almost absent in the sections showing dose dependent neuroprotection role of berberine.

6.7. Group VII - PTZ + Lamotrigine + Valproic Acid + Curcumin (50mg/kg)
Sections showed moderate neuroprotection against PTZ mediated damage, with neuronal organization being somewhat preserved; number of pyknotic neurons and gliosis reduced than group IV with a few pyknotic neuronal, inflammatory infiltration and degenerations still present in the hippocampi and cerebral cortex sections.

6.8. Group VIII - PTZ + Lamotrigine + Valproic Acid + Curcumin (100mg/kg)
Sections revealed substantial neuroprotective effect and showed marked recovery of neuronal structures. Neurons from hippocampus and cortex appear almost normal with no significant neuronal degenerations or vacuolations and no gliosis or inflammation is noted compared to lower dose of curcumin.

6.9. Group IX - PTZ + Lamotrigine + Valproic Acid + Curcumin (100mg/kg) + Berberine (100mg/kg)
Sections revealed the maximum neuroprotection with normal cellular arrangement of neuronal architecture with well-preserved neuronal layers, without any neuronal loss or degeneration, edema and inflammation infiltration as seen with the comparison between other groups.

7. DISCUSSION
On the basis of behavior, it is evident that chronic PTZ treatment, which established intractable epilepsy characterized with severe seizures along with high anxiety level, loss of locomotion and behavior impairment [27], was normalized in a much more prominent way with Lamotrigine alone. However, addition of Valproic acid into Lamotrigine resulted in better cure with higher efficacy than Lamotrigine alone and the effects were additionally much enhanced by using Berberine and Curcumin as add-on therapies with Lamotrigine and Valproic Acid in a dose-dependent manner. Among all experimental group Group IX (PTZ + Lamotrigine + Valproic Acid + Curcumin 100 mg/kg + Berberine 100 mg/kg) show greater protection against neurobehavior and convulsion in all parameters tested [28].
From all the results of above mention behaviour, we may hope this combined therapy may become a novel drug target against epilepsy.
The addition of Berberine and Curcumin to conventional therapies of intractable epilepsy may bring significant clinical implication [29]. As it comes out of biochemistry report that, repetitive treatment with PTZ led to an extensive damage through extensive oxidative and nitrosative stress, i.e. A deficiency of antioxidants, including non-enzymatic(GSH) and enzymic ones such as catalase (CAT) and superoxide dismutase (SOD) activities. It caused a marked increase in lipids peroxidative product Malondialdehyde (MDA) concentration and enhanced the concentration of nitric oxide, along with modulation of acetylcholinesterase activity [30]. Lamotrigine significantly attenuated the biochemical alterations, while combination therapy with valproic acid provided enhanced antioxidant protection.[31]

More importantly, adjunct administration of berberine and curcumin with traditional AEDs produced an additional improvement on each marker of oxidative stress in a dose dependent fashion.[32] Higher therapeutic efficacy found in Group IX (PTZ + Lamotrigine + Valproic Acid + Curcumin 100 mg/kg + Berberine 100 mg/kg) supports the possible synergistic interaction between conventional antiepileptic drugs and naturally sourced phytoconstituents[33]. It seems that combination treatment could prevent or delay progress of oxidative damages by re-activating endogenous antioxidant activities, blocking the production of lipid peroxidation and nitrosative stress and normalization of cholinergic function and preservation of neuronal tissues from PTZ evoked oxidative injury[34]. The neuroprotective role of berberine and curcumin as adjuvant therapy has become more evident in the treatment of refractory epilepsy.

CONCLUSION

Our results show that the chronic administration of PTZ resulted in a resistant epilepsy model associated with recurring seizures, behavioural deficits, oxidative stress and neuronal impairment. The administration of lamotrigine considerably minimized the occurrence of seizures as well as improving the behavioural and biochemical results, which was furthermore potentiated with the addition of valproic acid. Moreover, co-administration with berberine and curcumin induced dose-dependent neuroprotective effects through attenuation of antioxidant status (GSH, CAT and SOD), diminishing of oxidative and nitrosative stress (MDA and NO), normalizing acetylcholinesterase (AChE) activity, improving the cognitive and motor behaviour, and preserving neurons.

Group IX (PTZ + Lamotrigine + Valproic Acid + Curcumin 100 mg/kg + Berberine 100 mg/kg) among all groups shows maximal anticonvulsant and neuroprotective effects implying a synergism between standard anti-epileptic drugs and natural phytoconstituents.

In conclusion, it is strongly suggested that, the combinatorial administration of curcumin and berberine as additional therapy can potentiate the effect of standard anti-epileptic therapy and prevent from neurodegeneration caused by epilepsy which should be further validated by clinical trials and additional mechanism studies.

AUTHORS' CONTRIBUTIONS

Each author actively contributed to the idea and design, collection of the data, or the analysis and interpretation of the findings. All authors also made substantial contributions in drafting the article and reviewing it.

LIST OFABBREVIATION

AChE = Acetylcholinesterase
EDTA = Ethylene Diamine-tetra-acetic-acid
EPM = Elevated Plus Maze
FST = Forced Swim Task
GABA = Gamma-aminobutyric Acid
GSH = Glutathione
GTCS = Generalized Tonic-clonic Seizures
NBT = Nitro-blue Tetrazolium-chloride
PA = Passive Avoidance
PTZ = Pentylenetetrazol
SOD = Super oxidase dismutase
TL = Transfer Latency
BBR = Berberine
CCM = Curcumin
LTG = Lamotrigine

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol was approved by Institutional Animals Ethics Committee (IAEC) (1024/PO/Re/S/08/CPCSEA).

HUMAN AND ANIMAL RIGHTS

No humans were used in this research. All animal experimentation was done according to IAEC guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information is available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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