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Effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats

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Abstract

Cognitive impairment in epileptics may be a consequence of the epileptogenic process as well as antiepileptic medication. Thus, there is a need for drugs, which can suppress epileptogenesis as well as prevent cognitive impairment. In the present study, the effect of aqueous extract of *Centella asiatica* (CA) (100 and 300 mg/kg), an Indian medicinal plant known to possess antiepileptic, cognitive-enhancing and antioxidant property, was evaluated on the course of kindling development, kindling-induced learning deficit and oxidative stress markers in pentylenetetrazole (PTZ) kindled rats. Male Wistar rats were injected PTZ (30 mg/kg ip) once every alternate day (48 ± 2 h) until the development of the kindling. Passive avoidance test and spontaneous locomotor activity were carried out 24 and 48 h after the last administration of PTZ, while the oxidative stress parameters (malondialdehyde [MDA] and glutathione) were carried out in the whole brain upon completion of the behavioral assessment. The administration of CA (300 mg/kg orally) decreased the PTZ-kindled seizures and showed improvement in the learning deficit induced by PTZ kindling as evidenced by decreased seizure score and increased latencies in passive avoidance behavior. However, low dose of the CA (100 mg/kg) showed improvement only in the learning deficit due to the kindling and failed to improve the seizure score. The findings suggest the potential of aqueous extract of CA as adjuvant to antiepileptic drugs with an added advantage of preventing cognitive impairment.

Keywords: Epilepsy; Kindling; PTZ; Learning; Passive avoidance; Centella asiatica; Rats

1. Introduction

Epilepsy is a common neurological condition associated with the alteration in psychological, emotional and educational parameters. More than half of the epileptics had some sort of cognitive problems with abnormal behavioral manifestations (Rodin et al., 1977). These abnormalities are related to multiple factors including seizure type, age of onset, location of the focus, seizure frequency and type of EEG pattern (Bornstein et al., 1988). Another factor that may affect cognition is antiepileptic drug (AED) medication. Although it is understood that the beneficial results of seizure suppression are of great clinical importance, there are indications of cognitive side effects of the drugs administered at therapeutic doses especially with the polytherapy (Aldenkamp et al., 1993; Galham et al., 1990; May et al., 1992; Nichols et al., 1983). Thus, there is a need for drugs, which can suppress epileptogenesis and contain cognitive-improving property.

Many laboratory models simulate human epilepsy as well as provide a system for studying epileptogenesis (Temkin et al., 2001). The kindling model has become a widely employed technique for studying seizure mechanisms and considered to be a useful experimental model for human epilepsy (Mason and Cooper, 1972). Pentylenetetrazole (PTZ) kindling is an acknowledged model for epilepsy and refers to a phenomenon in which repeated injection of a convulsant causes gradual seizure development culminating in generalized tonic–clonic seizures associated with a cognitive deficit (Becker et al., 1992). Examination of PTZ-kindled rat brains also revealed a significant neuronal cell loss in hippocampal CA1 and CA3 structures and the hilus, possibly the cause of observed cognitive deficits (Pohle et al., 1997). It is reported that 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 rats (Rocha et al., 1996; Schroder et al., 1993; Sechi et al.,

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1997; Sejima et al., 1997; Rauca et al., 1999; Becker et al., 1997).

In the Indian system of medicine, Ayurveda, *Centella asiatica* (Umbelliferae, CA) has been used in various parts of India for different ailments like headache, body aches, insanity, asthma, leprosy, ulcers, eczemas and wound healing (Chatterjee et al., 1992; Chopra et al., 1956; Shukla et al., 1999; Suguna et al., 1996). In the course of pharmacological studies, the plant showed CNS depressant activity (Sakina and Dandiya, 1990), antitumor activity (Babu et al., 1995; Qian et al., 1982) and an inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai et al., 1984). The whole plant of CA has been shown to be beneficial in improving memory (Mukerji, 1953; Vaidyaratnam, 1994) and is also reported to improve the general mental ability of mentally retarded children (Apparao et al., 1973; Kakkar, 1990). Nalini et al. (1992) have shown that fresh leaf juice improves passive avoidance task in rats. We have earlier demonstrated that the aqueous extract of CA has cognition-enhancing properties with an associated decrease in the brain oxidative stress parameters of the normal rats (Veerendra Kumar and Gupta, 2002). Katare and Ganachari (2001) have reported that the CA has an anticonvulsant effect with an associated decrease in the oxidative stress in the lithium pilocarpine model of status epilepticus. Therefore, the present study was aimed to evaluate the effect of aqueous extract of CA on the course of kindling development, kindling-induced learning deficit and oxidative stress markers in PTZ-kindled rats.

2. Materials and methods

2.1. Plant material and preparation of the extract

Plants were procured from the commercial market at Khari Baoli, New Delhi. The samples were then authenticated for their correct botanical identity by the Chief Botanist, Department of Drug Standardization, Dabur Research Foundation, Ghaziabad, India. The whole plant was dried and coarsely ground with a grinder. For the preparation of the aqueous extract, the coarse powder of the plant was extracted with 8 parts of water under boiling for 5 h and was filtered through a 400-mesh cloth to collect the extract. The extract was concentrated and finally spray dried to get a powder of greenish brown. The percentage w/w yield of aqueous extract was 41. The loss on drying at 105 °C and the ash content of the powder were 5.0% and 8.0% w/w, respectively. The concentration of the known active component, asiaticoside, was determined by HPLC and was found to be 3% w/w.

2.2. Animals

Study was carried out using male Wistar rats weighing 200–250 g. They were obtained from the central animal house facility of All India Institute of Medical Sciences, New Delhi and stock bred in the Departmental animal house. The rats were group housed in polyacrylic cages (38 × 23 × 10 cm) with not more than four animals per cage and were maintained under standard laboratory conditions with natural dark and light cycles (approximately 14 h light–10 h dark cycle) and a room temperature of 25 ± 1 °C. They were allowed free access to standard dry diet (Golden Feeds, India) and tap water ad libitum. All the behavioral procedures were carried out between 0900 and 1300 h. All procedures described were reviewed and approved by the Institutional Committee for Ethical Use of Animals.

2.3. Experimental design

Animals were randomly divided into four groups of eight animals each. The first group received saline intraperitoneally while the second, third and fourth groups were administered PTZ (30 mg/kg) dissolved in saline on every second day (48 ± 2 h). One hour before administration of PTZ, the first and second groups received vehicle (Tween 80/water 1:5), the third and fourth groups were administered aqueous extract of CA (100 and 300 mg/kg, dissolved in Tween 80 + water) orally through an intragastric feeding tube. PTZ was administered until the PTZ + vehicle-treated group showed the seizure score of 5. Passive avoidance test and spontaneous locomotor activity were assessed 24 (initial latency) and 48 h (retention latency) after the last administration of PTZ. Following the behavioral test, the animals were sacrificed and the whole brain was dissected for estimation of markers of oxidative stress (malondialdehyde [MDA] and glutathione).

2.4. Kindling induction

For PTZ kindling, a subconvulsant dose of PTZ 30 mg/kg body weight was injected intraperitoneally on every second day (i.e. Day 1, Day 3, Day 5...). 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 five was observed between Day 35 (i.e. 18th injection) and Day 39 (i.e. 20th injection). Thus, in no case did the PTZ schedule exceed Day 43 (22nd injection). In the groups treated with CA, the PTZ doses were tested up to Day 43 or up to seizure of score 5 on three consecutive injections, whichever was earlier.

After each PTZ injection, the convulsive behavior was observed for 30 min. The resultant seizures were scored as follows: 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 clonic seizures with falling*) (Malhotra and Gupta, 1997).

2.5. Behavioral tests

2.5.1. Single-trial passive avoidance learning

Memory retention deficit was evaluated by step through passive avoidance apparatus. The apparatus consist of equal size light and dark compartments (30 × 20 × 30 cm). A 40-W lamp was fixed 30 cm above its floor in the center of the light compartment. The floor consisted of metal grid connected to a shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. Twenty-four hours after the last administration of PTZ, rats were placed in the light compartment and the time lapse before each animal entered the dark compartment and had all four paws inside it was measured in seconds and termed as "initial latency" (IL). Immediately after the rat entered the dark chamber with all the four paws inside the dark chamber, the trap door was closed and an electric foot shock (50 V a.c.) was delivered for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. Twenty-four hours after the IL, the latency time was again measured in the same way as in the acquisition trial and was termed as the retention latency (RL). However, during the retention trial, no foot shock was delivered, and the latency time was recorded to a maximum of 600 s. To improve the reliability and validity of the foot shock avoidance test, the grid as well as the rat paw were moistened with water before foot shock as this is known to reduce the wide interanimal variability in paw skin resistance of the rats (Mayer et al., 1990).

2.5.2. Closed-field activity

Each animal was observed over a period of 5 min in a square closed arena (30 cm) equipped with infrared light sensitive photocells using a digital photoactometer (Techno India) after the passive avoidance test and values expressed as counts/5 min. The apparatus was placed in a darkened, light and sound attenuated, and ventilated testing room with other behavioral testing apparatus (Lannert and Hoyer, 1998).

2.6. Biochemical tests

Following the behavioral testing, the animals were decapitated under ether anesthesia and the brains were quickly removed, cleaned with ice-cold saline and stored at − 80 °C.

2.6.1. Tissue preparation

Brain tissue samples were thawed and homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from rat brain were used to determine lipid peroxidation and glutathione.

2.6.2. Measurement of lipid peroxidation

MDA, a measure of lipid peroxidation, was measured as described by Jainkang et al. (1990). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples, then heated at 100 °C for 60 min. The mixture was cooled with tap water and 5 ml of *n*-butanol/pyridine (15:1), 1 ml of distilled water was added. The mixture was vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer. The concentration of MDA is expressed as nmol/g tissue.

2.6.3. Measurement of reduced glutathione

Glutathione was measured according to the method of Ellman (1959). The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′5-dithiobis(2-nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as μg/g tissue.

2.7. Statistical analysis

To evaluate the development of seizures in the course of kindling, the repeated-measures analysis was applied. The data of behavioral and biochemical tests are represented as mean ± S.E.M. in the figures and were processed by one-way ANOVA followed by posttest Tukey's HSD.

3. Results

3.1. Effect of CA (100 and 300 mg/kg) on induction of kindling by PTZ

In PTZ + vehicle-treated group, repeated administration of subconvulsant dose of PTZ (30 mg/kg) on every alternate day (for 43 days, 22 injections) resulted in increasing convulsive activity leading to generalized clonic–tonic seizures score of 5 [$F(1,13) = 419.71$, $P < .001$]. Administration of CA in the dose of 100 mg/kg did not modify the course of kindling induced by PTZ. However, a higher dose of CA (300 mg/kg) suppressed the kindled seizure significantly [$F(1,12) = 142.619$, $P < .001$], as none of the animal could achieve Stage 5 with 22 injections of PTZ (30 mg/kg) (Fig. 1).

3.2. Effect of CA (100 and 300 mg/kg) on step through latency in single-trial passive avoidance learning

The mean IL 24 h after the last administration of PTZ did not differ significantly [$F(3,26) = 0.082$, $P < .969$], between the saline + vehicle, PTZ + vehicle and CA + PTZ-treated groups. Retention latency 48 h after the last administration of PTZ in the PTZ + vehicle-treated group was significantly less [$F(3,26) = 22.606$, $P < .001$], compared with the sali-

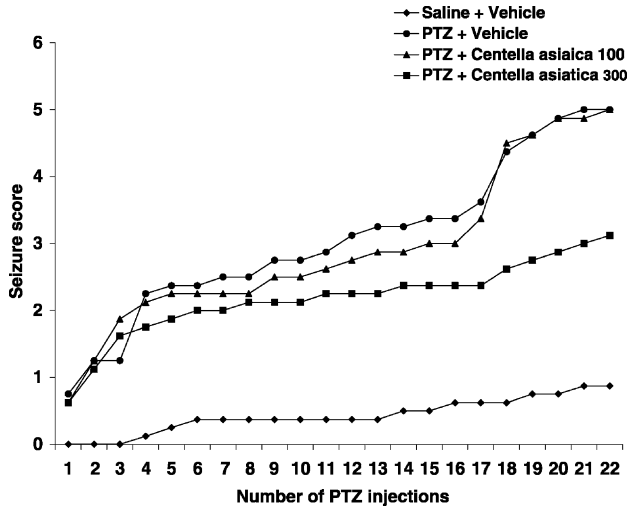

Fig. 1. Effect of aqueous extract 100 and 300 mg/kg of CA pretreatment on the development of PTZ-kindled seizures. The CA was injected 1 h prior to PTZ challenge. On the ordinate: seizure score (mean ± S.E.M.). On the abscissa: number of PTZ injections.

ne + vehicle-treated group. The CA 100 [$F(3,26) = 22.606$, $P < .001$] and 300 mg/kg [$F(3,26) = 22.606$, $P < .001$] treated PTZ groups showed significant reversal of PTZ-induced cognitive deficit. The results have been summarized in Fig. 2.

3.3. Effect of CA (100 and 300 mg/kg) on spontaneous locomotor activity

The spontaneous locomotor activity did not differ significantly between the saline + vehicle, PTZ + vehicle and

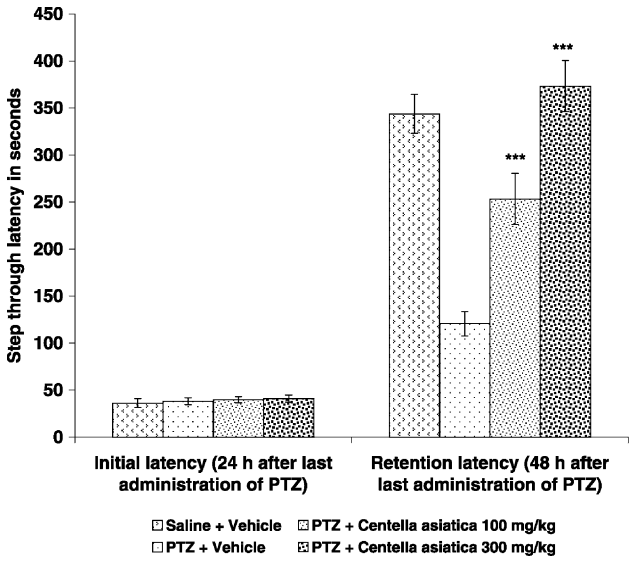

Fig. 2. Effect of aqueous extract 100 and 300 mg/kg of CA on step through latency in PTZ-kindled rats. On the ordinate: step through latency (s) in mean ± S.E.M. On the abscissa: IL and RL 24 and 48 h after the last injection of PTZ, respectively. *** Significance of difference vs. PTZ-treated rats at $P < .001$.

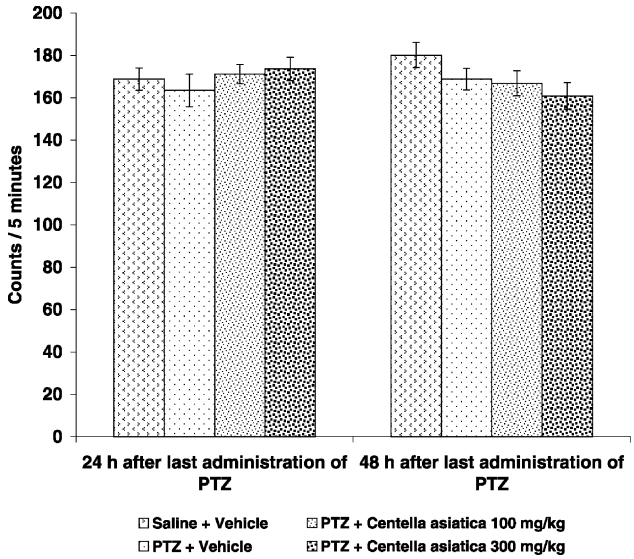

Fig. 3. Effect of aqueous extract 100 and 300 mg/kg of CA on spontaneous locomotor activity in PTZ-kindled rats. On the ordinate: counts/5 min (mean ± S.E.M.). On the abscissa: 24 and 48 h after the last administration of PTZ.

CA (100 and 300 mg/kg) + PTZ-treated groups after 24 h [$F(3,26) = 0.525$, $P < .669$] and 48 h [$F(3,26) = 1.898$, $P < .155$] of the last administration of PTZ (Fig. 3).

3.4. Effect of CA (100 and 300 mg/kg) on MDA level in PTZ-kindled rats

The MDA level in the PTZ + vehicle-treated group was significantly higher than that seen in saline + vehicle-treated group [$F(3,26) = 14.893$, $P < .001$]. CA 100 [$F(3,26) = 14.893$, $P < .001$] and 300 mg/kg [$F(3,26) = 14.893$, $P < .001$] treated PTZ groups showed significant lower levels of MDA as compared with the PTZ + vehicle-treated group (Fig. 4).

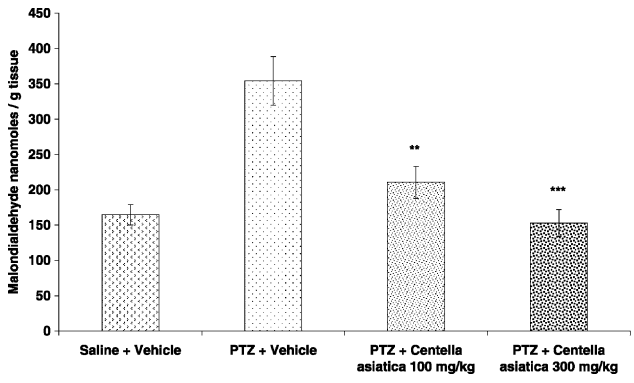

Fig. 4. Effect of aqueous extract 100 and 300 mg/kg of CA on MDA levels in nmol/g tissue in PTZ-kindled rats. On the ordinate: MDA nmol/g tissue (mean ± S.E.M.) after the 48 h of last administration of PTZ. **, *** Significance of difference vs. PTZ-treated rats at $P < .01$, .001, respectively.

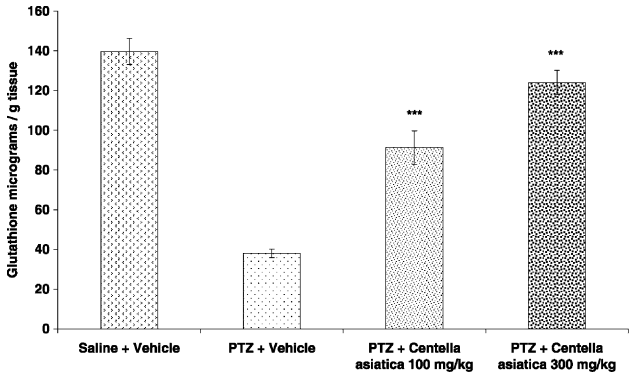

Fig. 5. Effect of aqueous extract 100 and 300 mg/kg of CA on glutathione levels in μg/g tissue in PTZ-kindled rats. On the ordinate: glutathione μg/g tissue (mean ± S.E.M.) after the 48 h of last administration of PTZ. * * * Significance of difference vs. PTZ-treated rats at $P < .001$.

3.5. Effect of CA (100 and 300 mg/kg) on glutathione level in PTZ-kindled rats

There was a significant [$F(3,26) = 44.307$, $P < .001$] lower level of glutathione in the PTZ + vehicle-treated group as compared with the saline + vehicle-treated group. The CA 100 [$F(3,26) = 44.307$, $P < .001$] and 300 mg/kg [$F(3,26) = 44.307$, $P < .001$] treated PTZ groups showed significant higher levels of glutathione compared with the PTZ + vehicle-treated group (Fig. 5).

4. Discussion

Free radicals have been suggested to be the most likely candidate responsible for producing the neuronal changes mediating the behavioral deficits in neurodegenerative disorders (Cantuti et al., 2000; Maxwell, 1995). We have demonstrated in our laboratory that antioxidants are effective in the rodent models of epilepsy, stroke and Alzheimer's disease (Gupta et al., 2002; Srivastava et al., 2002; Sinha et al., 2002; Sharma and Gupta, 2001). Currently, there has been an increasing interest in the biochemical effects of medicinal plants with antioxidant property as they could be candidates for the prevention of oxidative damage and memory deficit associated with epilepsy (Noda et al., 1997). CA has recently been reported to have an antilipid peroxidative and antiepileptic activity in the lithium pilocarpine model of status epilepticus (Katare and Ganachari, 2001) and was shown to be beneficial in improving memory in normal rats (Veerendra Kumar and Gupta, 2002). It is also reported to improve general mental ability of mentally retarded children (Apparao et al., 1973; Kakkar, 1990). Therefore, we tested if the aqueous extract of CA improves the kindling-induced learning deficit as well as the effect on the course of kindling development in rats.

In the present study, it was observed that the administration of subconvulsant dose of PTZ (30 mg/kg) for 22 times resulted in Stage 5 seizures, which was associated with cognitive impairment as evidenced by reduction of retention latency in passive avoidance behavior. These results are in conformity with other workers who also demonstrated cognitive impairment after administration of the subconvulsant dose of PTZ (Grecksch et al., 1991; Becker et al., 1995; Papazova and Bakarova, 1995; Homayoun et al., 2001).

The administration of CA (300 mg/kg) orally decreased the PTZ-kindled seizures and showed improvement in the learning deficit induced by PTZ kindling as evidenced by decreased seizure score and increased latency in passive avoidance behavior. The lower dose of CA (100 mg/kg) ameliorated the learning deficits caused by kindling, but failed to reduce the seizure score. The reason for the above finding may be explained by the fact that the measurement of seizure score is rather a widely used and simple method of assessing seizure activity. However, it is possible that smaller changes in the brain activity go unmeasured in this method. Thus, the possibility of detecting improvement in seizure activity with additional measures such as EEG may not be ruled out.

The general stimulant or depressant activity of a CNS active drug may affect the animal response on behavioral paradigms. Therefore, the effect of CA was also studied on spontaneous locomotor activity. There was insignificant difference between the locomotor activities of saline, vehicle-treated PTZ and CA (100 and 300 mg/kg)-treated PTZ groups. This makes it unlikely that the changes in passive avoidance attention observed in the CA-treated rats would have been due to any CNS depressant/stimulant activity of CA extract.

Free radicals have been implicated in the development of seizures (Sejima et al., 1997). However, when the production of free radicals increases or the defense mechanism of the body decreases, they cause cellular dysfunction by attacking at the polyunsaturated sites of the biological membranes leading to lipid peroxidation (Gupta and Sharma, 1999). Some antioxidants have been shown to be effective in reducing the oxidative stress in the models of epilepsy (Willimore and Rubin, 1981; Kabuto et al., 1998). Therefore, we also evaluated the effect of CA on oxidative stress in PTZ kindling.

The increase in the levels of MDA, a marker of lipid peroxidation in our study, indicates increased free radical generation in the vehicle-treated PTZ rats. The significantly lower levels of MDA in the brain of the 100- and 300-mg/kg CA-treated PTZ rats as compared with the vehicle-treated PTZ rats indicate attenuation of lipid peroxidation. There was a simultaneous significant decrease in the reduced glutathione levels in vehicle-treated PTZ rats. Glutathione is an endogenous antioxidant present mainly in the reduced form within the cells. It reacts with the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals. During this defensive process, reduced glutathione gets converted to its oxidized form with the help of the enzyme glutathione peroxidase. The decreased level

of reduced glutathione in vehicle-treated PTZ seen in our study indicates that there was an increased generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress (Schulz et al., 2000). The decrease in MDA levels and increase in the glutathione levels in CA-treated PTZ groups may be due to its antioxidant property.

In conclusion, the present study demonstrates that *Centella asiatica* significantly prevented the cognitive impairment and attenuated the oxidative stress induced by PTZ kindling. Therefore, it could offer a useful support to the basic antiepileptic therapy in preventing the development of cognitive impairment reported with several AEDs.

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