1-Triacontanol Cerotate Isolated from *Marsilea quadrifolia* Linn. Safeguards Hippocampal CA3 Neurons and Augments Special Memory Deficit in Chronic Epileptic rats

Cerotato de 1-Triacontanol Aislado de *Marsilea quadrifolia* Linn. Protege las Neuronas CA3 del Hipocampo y Mejora el Déficit de Memoria Especial en Ratas Epilépticas Crónicas

Adhikari Snehunsu¹; Satheesha B. Nayak²; Mamta Kandwal³; Adhikari Piyali⁴; Murali Adiga⁵; Pabitra Sahoo⁶; Medabala T.⁷; K. Raghavenra Rao⁷ & Alex Joseph⁸


SUMMARY: Currently many people with epilepsy do not have seizure control even with the best available medications. Moreover various antiepileptics have adverse cognitive impact with other side effect. Thus, need for new antiepileptic drugs still remains challenge. However, many of the natural components have antiepileptic action and this fact remains scientifically unexplored. This study was designed to check the behavioral and neuro-pathological outcome of 1-Triacontanol cerotate (1TAC), isolated from *Marsilea quadrifolia* Linn. (MQ) on chronic Pentylenetetrazol (PTZ) kindling model of epilepsy in rats. Two-month-old adult male Wistar rats (n=60) were randomly divided into six groups; Group I (Cage Control), II (Vehicle Control), III (Positive Control), IV (Standard drug treated), V (1TAC: 40 mg/kg) & VI (1TAC: 80 mg/kg). To induce kindling a 35 mg/kg dose of PTZ was injected i.p. in every 48 hrs for 30 days in Group III to VI. Spatial memory performance was tested using Morris water maze, following which brains were further processed for histopathological investigations. Interestingly, 1TAC was able to minimize the loss of pyramidal cells in hippocampal CA3 region. These cellular changes were behaviorally responded as improved special learning and memory, a better spatial navigation and object place configuration. The current study strongly implicates that 1TAC from MQ has potent neuroprotective role and augments special memory deficit in chronic epileptic rats. The isolated component which attenuates spatial memory performance could be beneficial outcome to retain cognitive blunting in chronic epilepsy.

KEY WORDS: 1-Triacontanol cerotate; *Marsilea quadrifolia* Linn.; Pentylenetetrazol, Epilepsy; Hippocampal CA3 zone; Pyramidal cells; Brain antioxidants; Cresyl Violet.

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by seizures, current estimates between 0.5-2 % of the global population being affected (Naseer *et al.*, 2009). More than half of the epileptics had some sort of cognitive problems with abnormal behavioural manifestations (Rodin *et al.*, 1977). Common side effects of epilepsy include slowed motor and psychomotor speed, poor attention and mild memory impairment (Meador *et al.*, 2005). Over 30 % of people with epilepsy do not have seizure control even with the best available medications (Kwan *et al.*, 2010). Above all, various antiepileptics have adverse cognitive impact with other side effects. The major cognitive impacts of these side-effects in human are language and working memory which are negatively affected (Bittigau *et al.*, 2003; Yang *et al.*, 2016). Thus, new antiepileptic drugs with least cognitive side effects or beneficiary role in cognitive enhancement till remains a challenge.
However, many of the natural components exert antiepileptic action and this fact remains scientifically unexplored. In the traditional Indian Ayurvedic medicine and also in folk medicine *Marsilea quadrifolia* Linn. (MQ) is used for the treatment of behavioural and epileptic disorders since Vedic days. Furthermore, we are able to isolate 1-Triacontanol cerotate (1TAC) also known as marsilin from M. quadrifolia Linn., having potent scavenging activity in different brain regions against reactive oxidative mutilation on lingering PTZ induced kindled epilepsy, as described in one of the earlier studies (Snehunsu et al., 2015). The current study was designed to explore the behavioural and neuro-pathological outcome of 1TAC on chronic epilepsy model induced by PTZ.

**MATERIAL AND METHOD**

**Study design:** Adult male albino rats (n=60) of two months old, weighing between 140 to 180 g were used in the study. Rats were obtained from the central animal house stock of Kasturba Medical College, Manipal, India. They were retained at a precise temperature room at 25°C, were daily exposed to light and dark cycle of 12 hours duration and provided with food and water ad libitum. The entire research protocol was scrutinized and permitted by the Institutional Animal Ethics Committee (IAEC) of Kasturba Medical College, Manipal, India.

A total of 60 rats were indiscriminately separated into six groups (n=10). The study groups were as follows:

- **Group I (Cage Control):** Rats of this group remained untouched in the cage throughout the trial date.
- **Group II (Vehicle Control):** Rats of this group received normal saline (0.9 g% NaCl) i.p and distilled water orally for 30 days.
- **Group III (Distilled water + PTZ):** Rats of this group received 30 mg/kg b.w. of PTZ i.p. in every 48 hrs and equal volume of distilled water orally for 30 days.
- **Group IV (Sodium Valproate 200 mg / kg + PTZ):** Rats of this group received 200 mg/kg Sodium Valproate 30 minute prior to the PTZ challenge (30 mg/kg b.w. of PTZ i.p.) in each 48 hours for 30 days.
- **Group V (1TAC 40 mg/kg + PTZ):** Rats of this group received 40 mg/kg 1TAC (Marsilin) 30 minute prior to the PTZ challenge (35 mg/kg b.w. of PTZ i.p.) in each 48 hours for 30 days.
- **Group VI (1TAC 80 mg/kg + PTZ):** Rats of this group received 80 mg/kg 1TAC (Marsilin) 30 minute prior to the PTZ challenge (35 mg/kg b.w. of PTZ i.p.) in each 48 hours for 30 days.

**PTZ-Kindling for chronic chemical induced generalized epilepsy:** Kindling is progressive increase in susceptibility to evoked seizures. Kindling model, induced by PTZ, is one of the unique and well-established models to create chronic epilepsy in experimental conditions. Kindling is progressive increase in vulnerability to evoked seizures. PTZ, inhibitor of chloride channel associated with GABA A, is one of the most preferred substances to induce chemical kindling. A 35 mg/kg dose of PTZ (Sigma, St. Louis, MO, USA) was injected intraperitoneally in each 48 hours for 30 days to induce kindled (Goddard, 1967; Chen et al., 2002; Wu et al., 2006; Szyndler et al., 2010; Snehunsu et al., 2015; Erkeç & Arihan, 2015).

**Water Maze Task:** Spatial memory performance was tested using Morris water maze task. A water maze is a round pool (diameter 180 cm, height 75 cm), filled with water to a depth of 50 cm. One escape stage (“4” × “4”) was submerged below the water surface (1 cm) and kept constantly in the target quadrant of the tank. The water was made opaque by adding milk. The pool was situated in a 3.6 X 3.3 m² room. Four points on the rim of the arena were designated as north (N), south (S), east (E) and west (W), thus dividing the pool into four quadrants. Two black and white pictures were hung on the walls to provide extra-maze indications for allowing the rats to learn a spatial atlas approach. Throughout the experimental period, room cues were kept in the same position (Morris, 1984; Abel & Reddy, 1997; Kumar et al., 2009).

**Acquisition of the task:** All the experimental trials were performed at the evening time of the day. All the rats were given four trials per day from four different quadrants with an inter trial interval of 5 min. The trials were carried out for consecutive 5 days to train the rats in the water maze acquisition performance to locate the unseen stage using reference memory. Rats were free on the water surface with the face towards the wall. All the rats were allowed to swim until the escape platform or for a maximum of 120 s in case the rat was unable to navigate to reach the platform within 120 s; it was lightly guided to the platform and allowed to remain there for 15 s during the inter trial interval rats were kept warm by tungsten lamps. All the events were captured using a colour video camera (Morris; Abel & Reddy; Kumar et al.).

**Histopathological analysis:** Histological study was carried out to detect structural organization of pyramidal neurons in hippocampal CA3 zone, and their survival upon chronic epilepsy. Followed by transcardial perfusion, animals were decapitated, brains were carefully removed and processed further for paraffin embedding. The coronal brain sections (6 mm) were obtained using rotary microtome (Leica RM2155, Germany). Each sixth section of the hippocampus...
were stained using Cresyl violet (CV) staining and rest all with Haematoxylin and Eosin staining.

**Quantification of pyramidal cells hippocampal CA3 region:** Pyramidal cells were quantified by direct visual counting of viable neurons using a light microscope (Motic Image Plus 2.0, China) under the total magnification of 400X. Cells were counted in each section out of every sixth section (30 μm apart from each other). Such types of three sections were considered from each animal. In each section, both right and left hippocampus were considered. The cell counts were expressed as the number of cells in unit length of the cell turf (cells/ 120 μm²). For unbiased cell counting, slides were made double-blinded after coded by a person who was unaware about the experimental conditions. Only cells with evident nucleus and nucleolus were included in the counts. A photograph of each section was captured in 5X, 10X and 40X magnifications using Motic Image Plus 2.0. The cell densities were assessed offline using the same software (Govindaiah et al., 1997; Veena et al., 2009; Shafri et al., 2012).

**Statistical Analysis:** The entire test results were expressed as mean ± standard error of mean (SEM). One-way of analysis of variance (ANOVA), followed by post-hoc Tukey’s test was done to find out the significance of differences among the groups. The ‘p’ values less than 0.05 were considered as significant.

**RESULTS**

Latency to find the platform by rats in various groups during consecutive trials of Morris water maze

**Effect of 1TAC on Water maze performance during the training:** During the five successive days (four trials/ day) over twenty trial sessions learning deficits were analysed. On the 2nd trial chronic untreated epileptic animals in Group III had longer latency to find out the unseen stage, compared to the normal control animals in Groups II (Fig. 1, Group III vs Group II qqqp<0.001 using one-way ANOVA, followed by post-hoc Tukey’s test). It was found that Group VI showed better response than other treatment groups (Fig. 1, Group III vs Group V and VI were respectively ££p<0.01, ***p<0.001; one-way ANOVA, followed by post-hoc Tukey’s test).

Next, upon 3rd trial chronic untreated epileptic animals in Group III had longer latencies to find out the hidden stage compared to the normal control animals in Groups II (Fig. 1, Group III vs Group II qqqp<0.001; one-way ANOVA, followed by post-hoc Tukey’s test). Our result clearly revealed that Group VI showed better response than other treatment groups (Fig. 1, Group III vs Group V and VI were respectively ££p<0.01, ***p<0.001; one-way ANOVA, followed by post-hoc Tukey’s test).

![Fig. 1. Learning curves during twenty successive trials (four trials/ day) of training sessions in the Morris water maze, denotes spatial learning of rats in different groups. Data were represented as mean ± SEM. qqq, £££ and ***p<0.001; qq, ££ and **p<0.01; q, £ and *p<0.05; where q, £ and * respectively represented comparison of any group with Group II, V and VI.](image-url)
Fig. 2. Photomicrographs showing hippocampal pyramidal cells in CA3 region with Haematoxylin and Eosin staining, magnification (10X) under the low power microscope.

Fig. 3. Photomicrographs showing hippocampal pyramidal cells in CA3 region with Haematoxylin and Eosin staining, magnification under the low power (10X) microscope.
Consecutively, learning curves (Fig. 1) obtained during the entire twenty successive trials revealed deficit of progressive spatial learning in Group III rats. They had longer latencies to find out the hidden platform compared to the normal control animals in Groups II (Figs. 1-4, Group III vs Group II \( qqqp<0.001 \); one-way ANOVA, followed by post-hoc Tukey’s test). However, rats of Groups I and II originated to find out the secreted stage from 8th trial onwards, and continued to retain their learning ability throughout the training period. Results suggest that treatment with 1TAC (both the doses) in the Group V and VI facilitated progressive learning and exerted protective effect in rats.

Effect of 1TAC on Neuro-pathological Evaluation of Hippocampal CA3 Hematoxylin and Eosin Staining: The distribution of pyramidal neuronal cells in hippocampal CA3 regions were examined under low (X10) power objective. As shown in Figs. 2-4, in the control group I (Cage) and II (Vehicle), pyramidal cells were typically layered, and were having large, round, transparent, intact nuclei. From the result of our current study it was found that chronic untreated epileptic animals in group III, the pyramidal cells neurons in the CA3 region were severely damaged, in disorderly arrays, significantly reduced in number, and characterized by pyknotic and indistinct nuclei (Fig. 2-4). In group IV (received treatment with Sodium Valproate of 200 mg/kg b.w.), less number of apoptotic cells were found compared to group III, but the total number of normal pyramidal cells were markedly decreased. The pyramidal cells in CA3 were found to be normal with significantly decreased cell death and least apoptosis in the group VI, which received treatment with 1TAC in high dose. In the group V, which received treatment with 1TAC in low dose, retention of normal cells was under challenge, but shown a better effect compared to the group IV.

**Effect of 1TAC on Neuropathological Evaluation of Hippocampal CA3 regions by Cresyl Violet Staining:** Round, clear, medium or large neurons with distinct nucleus and cytoplasm is evenly filled with Nissl substance were counted. Cells with darkly stained cytoplasm, shrunken cells and cells with fragmented nuclei were excluded from the count. The normal neuron cell bodies appeared with distinct nucleus and nucleoli. Degenerating cell bodies were having pyknotic nuclei and vacuolar spaces.
Effect of 1TAC on neuronal cell count in the Hippocampal CA3 regions: Effect of 1TAC on Pyramidal cell count in the Hippocampal CA3 regions: Neuronal cell count in the hippocampal CA3 regions in square mm area was found to be $223.80 \pm 7.79$ and $211.20 \pm 7.05$ respectively in groups I and II. This cell count was significantly decreased by ~ 2-times, in group III animals (Fig. 5, Group III vs Group II $\text{EEp}<0.001$; one-way ANOVA, followed by post-hoc Tukey’s test). Form the result, it is clear that after various drug treatment cell count was not reduced as seen in group III (Fig. 5, Group III vs Group IV, V and VI $\text{EEp}<0.001$; one-way ANOVA, followed by post-hoc Tukey’s test). Also denotes, Group VI has got highest efficacy to prevent the reduction of cell loss in the hippocampal CA3 zones compared to other treatment groups (Fig. 5, Group VI vs Group V $***p<0.001$and IV $\Delta\Delta p<0.01$; one-way ANOVA, followed by post-hoc Tukey’s test).

Fig. 5. Effect of 1TAC on Pyramidal cell count in Hippocampal CA3 regions of chronic epileptic rats. Sodium valproate and 1TAC (both doses) were significantly shield to retention of neuronal cell count, but the high dose of 1TAC shows a significantly improved effect compared to low dose. $\text{qqq, EE, ***} p<0.001$; $\Delta\Delta p<0.01$ and $\Delta p<0.05$.

DISCUSSION AND CONCLUSION

The results of the Morris water maze place navigation test in this study indicate that PTZ-induced epilepsy led to impairment in spatial learning ability and reference memory in the rats. Many of the experimental and clinical researches have confirmed that a number of factors lead to cognitive dysfunction after seizures. Chronic epilepsy affected the acquisition of learnt responses in MWM test. The chronic untreated epileptic animals exhibited longer latency to reach the hidden platform in the learning sessions compared to normal control animals. Our study revealed that 1TAC ameliorated the spatial memory impairment in PTZ-induced rat model. The beneficial effects of 40 mg/kg b.w. of 1TAC on spatial learning was found to be beneficial than SV 200 mg/kg b.w. It has been shown that the hippocampus is closely involved in learning and memory, and especially spatial cognitive function. Hippocampal long-term potentiation facilitates synaptic activity and is an important molecular mechanism of synaptic plasticity. Changes in the synapses have a direct impact on the performance of rats in Morris water maze learning and memory tests (Malone et al., 2008).

Several experimental studies in rodents demonstrated that inflammatory reactions in the brain can increase neuronal excitability, damage neuronal cell survival, and increase the permeability of the blood-brain barrier to blood-borne components and cells (Jankowsky & Patterson, 2001; Vezzani & Granata, 2005). Experimentally developed seizures in rodent produce marked inflammatory response in brain areas involved in the commencement and propagation of epileptic activity. Some of the clinical studies clearly indicated that patients with tonic-clonic seizures induce a pro-inflammatory marker cytokines in plasma and CSF, comprising of greater IL-6 levels and lesser IL-1Ra–to–IL-1a ratio (Peltola et al., 1998, 2000, 2002; Hulkkonen et al.; Vezzani & Granata; Vezzani et al.). Numerous researchers have reported that oxidative stress lead to over production of ROS which aggravates epilepsy (Waldbaum & Patel, 2010; Shin et al., 2011; Rowley & Patel, 2013). One of the previous studies has clearly revealed that upsurge in malondialdehyde and diminution GSH concentration in frontal cortical as well as hippocampal areas of the brain regions of PTZ kindled rats. Furthermore, 1TAC, isolated from MQ attenuated reactive oxidative damage in those brain regions (Snehunsu et al., 2015). Number of current studies from the crude extracts of MQ claimed to have marked antioxidant effects (Zahan et al., 2011) and free radicle scavenging activity (Jagadeesan et al., 2011). Some earlier findings have clearly shown that methanolic extract of MQ effectively improved PTZ-induced EEG changes in rat. This was further evident by one of the earlier research where methanolic extract of MQ was proofed to have antiepileptic potency when...
experimentally treated upon maximal electroshock and PTZ induced epileptic animals (Snehunsu et al., 2013). It is clear from the result of the current study that 1TAC has a variety of neurological effects that could have played crucial role in minimizing cognitive blunting due to chronic epilepsy.

The hippocampus exhibits an extremely explicit role in the pathogenesis of epilepsy (Sendrowski & Sobaniec, 2013). Wide range of the patients with drug resistant temporal lobe epilepsy have a characteristic pathology of the brain structure – hippocampus sclerosis, characterized by loss of pyramidal neurons, severe glial reaction and remodelling of neuronal networks (Sendrowski & Sobaniec). Observations from the morphological analysis clearly indicated pyramidal cells in CA3 region were severely challenged in chronic untreated epileptic animals of group III. Neuronal cell count was significantly reduced, characterized by cells with pyknotic and indistinct nuclei compared to control group. In group IV (received treatment with Sodium Valproate with 200 mg/kg b.w. doses), less number of apoptotic cells were found compared to group III, but the total number of normal pyramidal cells were markedly decreased. The pyramidal cells in CA3 were found to be normal with significantly decreased cell death and least apoptosis in the group VI which received treatment with 1TAC with high dose. Though the retention of neuronal integrity was under challenge in the animals received treatment with 1TAC in low dose in the group V, but showed relatively better neuroprotective action compared to the group IV.

Damage to the various brain regions like the hippocampus, striatum, basal forebrain, cerebral ventricle and frontal cortex have shown impaired MWM performance in rats (D’Hooge & De Deyn, 2001). Moreover, evidence indicates that, the hippocampus is the major brain structure involved in the acquisition and storage, as well as retrieval of spatial information of information (Riedel et al., 1992). Hippocampus has a specific role in spatial aspect of MWM learning (Clark et al., 2007; Talpos et al., 2008). The hippocampus is associated with the spatial navigation and with hippocampal lesion animal will not be able to form the object-place configurations that are important in spatial memory (Broadbent et al., 2006).

Current study clearly indicates that treatment with 1TAC has a substantial beneficial effect on chronic animal model of epilepsy. Hence, the isolated component 1TAC (marsilin) from MQ which attenuates spatial memory performance in PTZ kindled rats; it could be beneficial even in humans in the future as an antiepileptic drug, with less cognitive knock-on effect.

ACKNOWLEDGEMENTS

This work was supported by a research grant for the faculty of Kasturba Medical College, Manipal University, Manipal, India. Authors are thankful to Dr. Raju Suresh Kumar, Assistant Professor, Department of Physiology, King Saud bin Abdulaziz University for Health Sciences, Saudi Arabia for the installation of the setup of Morris Water Maze at Kasturba Medical College, Manipal which was exclusively used for this research.


RESUMEN: Actualmente, muchas personas con epilepsia no cuentan con un control adecuado de las convulsiones, incluso con los mejores medicamentos disponibles. Además, varios antiepilépticos tienen un impacto cognitivo adverso además de efectos secundarios. Por lo tanto, la necesidad de nuevos fármacos antiepilépticos sigue siendo un desafío. Sin embargo, muchos de los componentes naturales tienen acción antiepiléptica y este hecho permanece científicamente inexplorado. Este estudio se diseñó para verificar el resultado conductual y neuro-patológico del cerotato de 1-triacontanol (1TAC), aislado de Marsilea quadrifolia Linn. (MQ) en el modelo de epilepsia en ratas del pentilenetetrazol (PTZ) crónico (PTZ). Ratas Wistar adultas de dos meses de edad (n = 60) se dividieron aleatoriamente en seis grupos; Grupo I (Control de jaula); II (Control de vehículo); III (Control positivo), IV (Medicamento estándar de tratamiento), V (1TAC: 40 mg / kg) y VI (1TAC: 80 mg / kg). Para inducir la inflamación se inyectó una dosis de 35 mg / kg de PTZ i.p. en cada 48 horas durante 30 días en los grupos III a VI. El rendimiento de la memoria espacial se probó utilizando el laberinto de agua de Morris, después de lo cual se procesaron los cerebros para investigaciones histopatológicas. Curiosamente, 1TAC pudo minimizar la pérdida de células piramidales en la región CA3 del hipocampo. Estos cambios celulares respondieron de manera conductual como una mejora del aprendizaje especial y la memoria, una mejor navegación espacial y la configuración del lugar del objeto. El estudio actual implica fuertemente que 1TAC de MQ tiene un potente papel neuroprotector y mejora el déficit de memoria especial en ratas epilépticas crónicas. El componente aislado que atenúa el rendimiento de la memoria espacial podría ser un resultado beneficioso para retener la reducción cognitiva en la epilepsia crónica.

PALABRAS CLAVE: 1-Triacontanol cerotate; Marsilea quadrifolia Linn; Pentilenetetrazol; Epilepsia; Zona de hipocampo CA3; Células piramidales; Antioxidantes cerebrales; Violeta de cresilo.
REFERENCES


Kwan, P.; Arzimanoglou, A.; Berg, A. T.; Brodie, M. J.; Allen Hauser, W.; Mathern, G.; O ’[...](continued in next line)


Corresponding Author:
Dr. Satheesha Nayak B.
Professor of Anatomy
Melaka Manipal Medical College (Manipal Campus)
Manipal Academy of Higher Education
Madhav Nagar, Manipal
Karnataka
INDIA

Email: satish.nayak@manipal.edu

Received: 17-05-2018
Accepted: 31-08-2018