DISCUSSION

Antiepileptic Property of Marsilea quadrifolia Linn.: 

Epilepsy is a chronic disease and continues for many years to life time, if not treated adequately. Recent reports suggest that, epilepsy occurs due to an imbalance between inhibitory and excitatory influences in the brain (Scharfman & Schwarcz, 2007). Although there is a large number of models available for screening the anticonvulsant activity of a drug, the maximal electroshock model (MES) and the pentylenetetrazol model (PTZ) remain the ‘gold standards’ in early stages of testing (Schmidt & Rogawski, 2002). Findings from the experiment –I demonstrated that, M. quadrifolia Linn. has significant antiepileptic effects on the MES and PTZ induced epileptic models. Both the doses of MQ extracts effectively attenuated the seizures in MQ pre-treated rats. The percentage of animals showing hind limb extension was decreased to 50% in MQ treated rats. MQ also prolonged the HLE time in MES induced epileptic rats in comparison to control rats. In other words, the seizure protection rate was 50% in both the doses of MQ pre-treated group compared to control, which received CMC as an intervention. Several reports suggest the involvement of voltage dependent Na+ channels and N-methyl-D-aspartate (NMDA) receptor in the development of MES induced tonic hind limb extension in rats. These can be prevented either by drugs that inhibit voltage-dependent Na+ channels, such as Phenytoin, Valproate, Felbamate and Lamotrigine or by drugs that block glutaminergic excitation mediated by the N-methyl-D-aspartate (NMDA) receptor, such as Felbamate (Mac Namara, 1994 and Rogawski & Porter, 1990). The effectiveness of MQ extract in MES induced model, indicates that certain compound/s in the MQ extract might be modulating the above mentioned channels or receptors.

Pre-treatment with MQ extract significantly attenuated the PTZ induced seizures in rats. PTZ is a tetrazol derivative that has been shown to have convulsion actions in mice, rats, cats, and primates. PTZ induced epilepsy model is an effective model for screening the efficacy of antiepileptic drugs. The behavioral and EEG manifestations of PTZ induced seizures in rodents suggest that the drug is a model of generalized tonic clonic seizures (Asla et al., 2006). In the current study it was found that, the time elapsed before the appearance of myoclonic jerk and generalized seizures was significantly increased in rats treated with various doses of MQ extracts (400 mg kg⁻¹ b.w. and 600 mg kg⁻¹ b.w.). The higher dose had a significant effect compared to the low dose studied. Additionally, both doses of MQ extract
significantly decreased the amplitude and increased the EEG frequency when compared to
PTZ challenge (Figure 31, 32 & 33) but higher dose (MQ 600 mg kg\(^{-1}\) b.w.) received group
showed a significant result in comparison to the low dose group (Figure 31, 32 & 33). Reports suggest that, the cause of PTZ induced seizures is presumably due to the impairing
gamma-amino-butyric acid (GABA)-mediated inhibition by an action at the GABA\(_A\) receptors (Ramanjaneyulu & Ticku, 1984). GABA is one of the important endogenous
inhibitory neurotransmitters widely distributed in the central nervous system (CNS). Its
reduction in the brain is associated with numerous of neurological disorders (such as anxiety,
depression, and epilepsy) (Krystal et al., 2005; Nutt, 2006 & Landmark, 2007). The post
synaptic GABA\(_A\) receptors are implicated in the inhibitory mechanism. Drugs that enhance
GABA\(_A\) receptor-mediated inhibition, such as benzodiazepines, phenobarbital, valproate and
felbamate can be used to prevent the convulsions induced by PTZ (MacDonald & Kelly, 1995; White et al., 1995 and Sahu et al., 2012). MQ extract received group showed a
comparable result to sodium valproate treated group in PTZ model. This was evident even in
the EEG study.

Water extract of \textit{M. quadrifolia} is reported to have anti-convulsive effect in rats (Sahu et al., 2012). The CNS depressant and sedative effects of MQ were also documented earlier (Reddy et al., 2012). The active component in the MQ extract would have modulated various
channels (Na\(^+\), Ca\(^{++}\)) or receptors (NMDA, GABA\(_A\)) in the brain, and this could be one of the
reasons for the attenuation of seizures and EEG alterations in MQ pre-treated rats. The
interesting aspect from the experiment-I is that the treatment with MQ extract ameliorated the
epileptic seizures both in MES and in PTZ induced models but as indicated in the results a
better effect was observed on PTZ induced epileptic model compared to MES model. Further
studies may be carried out to demonstrate the modulatory action of various active
component/s on GABAergic, NMDA receptor or Na\(^+\) channels in the brain. In this regards, if
the active component/s present in MQ can minimize PTZ induced seizure development could
be helpful as an antiepileptic drug. Reports suggest that, GABA\(_A\) receptor agonists (natural or
synthetic compounds) as well as drugs, which allosterically modulate the GABA\(_A\) receptor
channel complex, would be the future therapeutically effective anticonvulsant agents
(Johnston, 2005).
**Antiepileptic Property of 1-Triacontanol cerotate isolated from Marsilea quadrifolia Linn.:**

Bioassay guided fractionation from MQ extract revealed that one component to be most potent against PTZ induced epilepsy, was named initially as MS1. A critical analysis from all the physical and spectral data i.e. IR, MS MS, 1H and 13C NMR from MS1 confirmed that it is 1-Triacontanol cerotate (Marsilin), as reported earlier (Iyer & Shah, 1973).

Study to investigate the detail bioactivity using 1TAC was conducted on standard PTZ induced kindling rat model of epilepsy which is a typical chemical induced chronic epilepsy model. Various researches believe that, this model is exclusively use not only to check the antiepileptic property, but also to investigate epilepsy induced cognitive dampening (Gupta et al., 2003; Tian et al., 2009 and Erkeç & Arihan, 2015). Result of this current study from experiment- IV reviled that 1TAC (in both the doses) was able to decrease the epileptiform activity in a better way compared to sodium valproate. Never the less, the higher dose of 1TAC i.e. 80 mg kg\(^{-1}\) b.w. gave an enhanced outcome in comparison to 200 mg kg\(^{-1}\) b.w. of sodium valproate.

The overall epoch to epoch analysis of EEG recorded from frontal cortex and hippocampal CA3 zone revealed that spontaneous occurrence of spike-wave discharges was noticeably present in the untreated chronic epileptic rats of group III. The EEG amplitudes were increased and frequencies were decreased typically in this group compared to the normal control animals. This is exclusively because of synchronous neuronal discharges during epilepsy. Also the powers of lower frequency EEG waves were increased progressively due to kindling in untreated animals typically depicts the severity to the epilepsy. On the other hand frontal cortical as well as hippocampal EEG amplitudes were significantly attenuated in rats pre-treated with SV and 1TAC. Sodium valproate at a dose of 200 mg kg\(^{-1}\) b.w. was able to significantly reduce the EEG amplitudes compared to group III animals. The high dose of 1TAC showed significantly better effect than Sodium valproate to attenuate the EEG amplitude upon PTZ challenges. That effect can be correlated with the behavioural responses obtain from the results of antiepileptic study. During the kindling process of one month the latency for the seizures and the grade of seizures in each group were assessed. Result of the current study clearly indicates that during the generation of kindling process latency for the onset of seizures was tapered down markedly in animals received only PTZ (in Group III), but it was significantly prolonged for the PTZ with drugs (sodium valproate & 1TAC) pre-treated animals. Results also indicate the high dose of 1TAC (80 mg kg\(^{-1}\) b.w.) was shown to
be most effective to prolong the latency time for the generation of seizures, compared to other groups. Another crucial finding from the current study is that, there is a steep increase of seizure stages with progressive PTZ dosing. This clearly indicates the susceptibility of untreated animals to the vigorous epilepsy. That response was markedly dampened by different doses of ITAC. Result obtained from the current study also revealed the remarkable effect of ITAC in 80 mg kg\(^{-1}\)b.w. diminished the generation of kindling effect.

Current study, as well as various other studies revealed that chronic exposure to PTZ at dose of 35 mg kg\(^{-1}\) b.w. once in 48 hrs. induced chronic kindling to the epilepsy(Chen et al., 2002; Xiao et al., 2006 and Erkeç & Arihan, 2015). This is a well-established model to mimic chronic epilepsy induced cognitive deficits. PTZ typically shuts down the GABA\(_A\) mediated influx of chloride ions (Cl\(^-\)) into the post synaptic cell. GABA is one of the important inhibitory neurotransmitter in the central nervous system to develop inhibitory post synaptic potential (IPSP). It plays a critical role in the balancing of excitatory and inhibitory processes exclusively in the cortical and subcortical regions. Subconvulsive dose of PTZ over a period of one month gradually blocks GABA\(_A\) receptors on the post synaptic membrane, predominantly causes epilepsy with higher grade in the next progressive dose of this type of model (Ramanjaneyulu & Ticku, 1984).

Antiepileptic drugs generally act by: (Michael et al., 2004)

1. Modifying excitatory and inhibitory neurotransmission through effects on voltage gated ion channels, GABA\(_A\) receptors and glutamate mediated excitatory neurotransmission,
2. Normalizing biochemical and morphological changes involved in the normal function of neuronal networks such as increased excitatory activity in the presynaptic neuron, calcium influx, phosphatases or protein kinase activation, phosphorylation and dephosphorylation of receptors, ion channels and other proteins, cytoskeletal changes, and mobilisation of receptors and ion channels,
3. Potentiating GABA neurotransmission through inhibiting calcineurin (protein phosphatase 2B),
4. Diminishing activation of NMDA receptors and
5. Inhibiting activation of extracellular regulated kinase and p38\(\alpha\) mitogen activated protein kinase pathways.
1TAC possess noticeable anti-epileptic activity. This could be due to modulation of any one or more path ways as that of the above mentioned antiepileptics.

Acetyl choline (ACh) is one of the excitatory neurotransmitter present diffusely in the brain. It develops excitatory post synaptic potential (EPSP) upon binding on its receptors by influxing mainly sodium ions (Na⁺) into the cell. Acetylcholine esterase by cleaving ACh re-establishes resting membrane potential (RMP).

Result from our current study revealed that the AChE activity was significantly reduced by ~2 fold in the PTZ kindled positive control rats, compared to the normal control rats. This caused secondary increase of ACh concentration in the postsynaptic membranes, might be responsible for the excitotoxicity during the development of status epilepticus as found in other studies (Jope et al., 1991 & Bertrand, 2002).

The AChE activity was reestablished back almost to the normal after treatment with SV and 1TAC (both doses). This might balance the GABA mediated less inhabitation (in presence of PTZ as it blocks GABAₐ receptor) by dampening down the ACh mediated excitation. That could have (almost) reestablished back the balance by modulating the overall excitatory and inhibitory inputs. Though 1- Triacontanol cerotate (1TAC); Marsilin has been previously reported to be active against convulsion (Chatterjee et al., 1963), but the exact cause for such antiepileptic action was not elucidated earlier. The main effect of 1TAC was found to be annulling of metrazole induced convulsions and lethality, increase in electroshock seizure threshold. Earlier evidence on rabbits suggests that pretreatment with 1TAC caused general decrease in fast waves in electroencephalography. It also markedly potentiated of sodium thiopental induced sleep indicating some of its GABAergic action. Earlier study rationalized clinically when it has been found to be very much effective for those epileptic patients associated with aggressiveness, restlessness, anxiety and weariness (Mukherjee et al., 1963).

**Effect of 1-Triacontanol cerotate on oxidative stress induced by chronic epilepsy in frontal cortex and hippocampus:**

Epilepsy is a chronic neurological disorder. Recent reports suggest that excitotoxicity is the leading cause for neuronal cell deaths in epilepsy (Choi, 1988 & Lipton et al., 1994). Studies also reported that oxidative stress due to production of ROS exacerbates epilepsy. Several studies pointed out a significant increase in ROS production in the brain regions of animal caused by recurrent seizures (Sudha et al., 2001 & Waldbaum & Patel, 2010). These ROS causes a cascade of neurochemical events leading to neurodegeneration and cell death (Vesna
et al., 2003). These are the primary causes for blunting of cognitive function in chronic epilepsy.

Frontal cortex and hippocampus are the key structures for the cognition. Normally hippocampal neurogenesis plays an important role to maintain the normal cognitive functions (Gisele et al., 2014). Brain cells are very much susceptible to free radical damage because of their high content of iron and polyunsaturated fatty acids (PUFA). PUFA is a substrate for lipid peroxidation (Halliwell et al., 1989).

PTZ kindling model is one of the best studied model to induce chronic epilepsy in experimental animals (Fatih et al., 2012). The result from the current study indicate significant increase of lipid peroxidation levels and significant decrease of GSH concentration in frontal cortical as well as hippocampal areas of rat brain in PTZ kindled epilepsy of group III which did not receive any drag treatment. These altered levels were brought back significantly to the normal upon drug treatments (both SV & higher doses of 1TAC). However, our findings clearly indicated that even the lower dose of 1TAC could attenuate ROS production but best result could be obtained with higher dose.

An earlier in vitro study revealed that crude methanolic extract of MQ had a good potential as an antioxidant (Zahan et al., 2011). This was also supported by a previous study, in which the methanolic extract of MQ was proved to have anticonvulsive property when tested upon maximal electroshock and PTZ induced epilepsy in rats (Adhikari et al., 2013). An isolated component marselin (1TAC) is now proved to be responsible for antiepileptic action of MQ. Further studies are needed to determine the status of other antioxidant enzymes in different brain regions, as well as other metabolic factors that may predispose to reactive oxidative damage in chronic epilepsy.

Effect of 1-Triacontanol cerotate on emotional learning and memory:

In the passive avoidance form of learning an animal learns to stop its innate behavioural responses when it results punishment. Passive avoidance tests are also known as conditioned avoidance test; have been used in several studies to assay memory retention and also retrieval after various treatments (Glick et al., 1975; Bartus et al., 1980 & Gupta et al., 2003). Usually rats select dim area over illuminated one when placed in a bright illumination space connected with a dark enclosure. They rapidly enter the dark compartment and remain there. After an aversive consequence of foot shock in the dark compartment, the animals modify their behaviour by inhibiting their innate response to stay in the dark and remain in the bright compartment (Bures et al., 1983). Hence in this task the animals learned to suppress a particular behaviour (Patterson, 1987).
In this current study, chronic epilepsy affected the passive avoidance behaviour of rats. Moreover, the memory retention and retrieval were affected remarkably due to chronic PTZ kindled epilepsy. It is apparent from the result that, when the chronic untreated epileptic animals were exposed to passive avoidance paradigm during the initial three trials (for the habituation) to detect the latency to enter into dark compartment, they displayed an altered activity. Perhaps, during this learning process obtained latency was slightly slower in this group of animals, compared to the other groups. That was indicated by their increased latency to enter the dark compartment. In other word, a minor anxiety like behaviour was found due to chronic epilepsy, which was not that obvious in the epilepsy with drug treated animals.

Followed by the learning phase foot shock, retention was tested after 24 hrs and 48 hrs. The retention latency to enter in the dark compartment was obtained least in chronic epileptic untreated (Group III) compared to normal control animals, which is ~ 3 times higher. But it was significantly prolonged by ~2.6 and 2.8 fold, respectively in Group IV and Group VI, received treatment with 200 mg kg\(^{-1}\) b.w. of sodium valproate and 80mg kg\(^{-1}\) b.w. of 1TAC.

In 48 hrs retention testing, it was noted that there was a significantly less time to enter the dark compartment in Group III (chronic untreated epileptic rats) compared to the chronic epileptics treated with 80mg kg\(^{-1}\) b.w. of 1TAC.

These findings clearly suggest that, after being exposed to aversive stimulation as foot shock in the passive avoidance task, chronic epileptic animals did not remember this task on the following days and this clearly indicates impairment of fear memory. That specific chronic suffering from seizure had a strong influence on the associative memory which had built up through repetition over many trials of retention in rats.

The change in the behaviour of animals (the shorter latency to enter the dark compartment) in the passive avoidance task could be due to altered function of hippocampus and frontal cortex. The exact mechanisms that underlie the possible harmful effects of chronic epilepsy on learning and memory are still uncertain (Christian et al., 2004; Parichehr et al., 2014 and Kaur et al., 2014).

The altered functioning could be due to either neuronal loss because of cell death or altered morphology of the neurons in the key brain structures. In this current study, haematoxylin and eosin staining of the hippocampal CA3 region revealed that the pyramidal neurons were severely damaged and were disorderly arranged, because of the chronic PTZ induced untreated epilepsy. This was later confirmed with cresyl violet staining which showed marked reduction of pyramidal cell number, and characterized by pyknotic and indistinct nuclei. Resent reports suggest that, due to anatomical and physiological characteristics,
lesions of basolateral amygdala and hippocampus interaction plays a crucial role in the fear condition (Cahill & McGaugh, 1995 and Maren et al., 1998).

Other than involvement of amygdala, a number of experimental and clinical studies have shown the role of hippocampal formation and related structures in medial prefrontal cortex in regulation of fear learning and memory (Squire, 1996 & Jin, 2015). In rats bilateral lesion of specific area of the hippocampal CA1 and CA3 produced greater impairments in the performance of Passive avoidance task (Sandi et al., 1992 & Azadbakht et al., 2015). These findings suggest the involvement of the hippocampus in the associative learning process.

In the current study the haematoxylin and eosin and cresyl violet staining of the hippocampal CA3 region clearly showed significant decrease of pyramidal cells which clearly indicates the degenerative changes in PTZ kindled rats. These degenerative changes were prevented with 1TAC treatment. In brief, it could be suggested that, PTZ kindling rats induced structural changes in these brain regions, which in turn could have altered the passive avoidance behaviour in rats.

**Effect of 1-Triacontanol cerotate on spatial memory performance deficits induced by chronic epilepsy:**

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\(^{-1}\) b.w. of 1TAC on spatial learning was found to be beneficial than SV 200 mg kg\(^{-1}\) 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 (LTP) 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).

It is clear from the result that 1TAC has a variety of neurological effects that could have played crucial role in minimizing cognitive blunting due to chronic epilepsy. Several studies have reported that oxidative stress due to production of ROS exacerbates epilepsy (Waldbaum & Patel, 2010; Shin et al., 2011 and Rowley & Patel, 2013). We found
significant increase in lipid peroxidation and decrease GSH level in frontal cortical as well as hippocampal areas of rat brain in PTZ kindled epilepsy of group III, compared to normal control. These altered levels were brought back significantly to the normal upon drug treatments (both SV & higher doses of 1TAC). Number of current studies from the crude extracts of MQ claimed to have marked antioxidant effects (Zahan et al., 2011) and free radical scavenging activity (Jagadeesan, 2011). However, our findings clearly indicated that even the lower dose of 1TAC attenuated reactive oxidative damage, but best result was obtained with higher dose.

Observation from the morphological analysis indicated that chronic untreated epileptic animals in group III, the neurons in the CA3 region were severely damaged, significantly reduced in number, and characterized by pyknotic and indistinct nuclei. In group IV (received treatment with Sodium Valproate 200 mg kg⁻¹ b.w.), less number of apoptotic cells was 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.

Damage to the various brain regions like the hippocampus, striatum, basal forebrain, cerebellum and frontal cortex have shown impaired MWM performance in rats (D’Hooge & 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., 1999). Hippocampus has a specific role in spatial aspect of MWM learning (Robert 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 (Nicola et al., 2006).

Hence, the isolated component 1TAC (marsilin) 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. Current study clearly indicates that treatment with 1TAC has a substantial beneficial effect on chronic animal model of epilepsy.