Ameliorative Effect of Ethanolic Extract of Moringa oleifera Leaves in Combination with Curcumin against PTZ Induced Kindled Epilepsy in rats; In Vivo and In Silico Study

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Article

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Abstract: The ameliorative effect ethanolic extract of *M. oleifera (MO)* leaves in combination of curcumin against seizures, cognitive impairment & oxidative stress PTZ-induced kindled rats. Molecular docking was performed to predict the potential phytochemical of MO and curcumin against Epilepsy. The effect of pretreatment with leaves of *M. oleifera* ethanolic extracts (MOEE) (250, 500mg/kg, orally), curcumin (200 and 300 mg/kg, orally), valproic acid used as standards (100mg/kg) and combined effect of MOEE (250mg/kg) and curcumin (200mg/kg) at low dose, on pentylenetetrazole (PTZ)-induced kindling. For the development of kindling the individual Wistar rats (Male) were injected pentyletetrazole (40 mg/kg, i.p.) on every alternate day. Molecular Docking has been done by AutoDock4.2 tool to merge the ligand orientations in the binding cavity. From the RCSB website, the crystal structure of Human Glutathione reductase (PDB ID: 3DK9) was obtained. Curcumin and MOEE showed dose-dependent effect. The combined effects of leaves of MOEE & curcumin significantly improved the seizure score and decreased the number of myoclonic jerks in compare with standard dose of valproic acid. PTZ kindling induced a significant oxidative stress and cognitive impairment which was reversed by pretreatment with MOEE & curcumin. Glutathione reductase (GR) is an enzyme, which plays key role in the cellular control of reactive oxygen species (ROS). Therefore, activating GR can uplift the antioxidant property, which leads to the inhibition of ROS induced cell death in brain. The combination of ethanolic extract of *M. oleifera* (MOEE) leaves and curcumin have shown better result than any other combination for antiepileptic effect by virtue of antioxidant effects. As per docking study showed that the chlorogenic acid and quercetin is treated with the combination of curcumin can be much more potential.

Keywords: *Moringa oleifera;* curcumin; neuroprotective; pentylenetetrazole; oxidative stress

1. Introduction

Epilepsy is a persistent neurological brain disorder characterised by abnormal electrical activity in the cerebral neurons. Epilepsy affects approximately 50 million people worldwide. Epilepsy affects all the age groups, especially young people in first two decades of life and elderly [1]. The importance of oxidative stress being an essential mechanism for understanding seizures caused by epilepsy has

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been extensively acknowledged. However, there is a lack of clear evidence that free radicals are actively involved in physiological processes during oxidative stress induced by convulsants [2]. Oxidative stress results a functional cellular disruption and cause cell death via oxidation of bio molecules such as proteins, lipids and nucleotides [3]. As a result, treating epilepsy through the use of non-pharmacological and antioxidant methods which target oxidative stress may be effective. The commonly used anticonulsant drugs like, sodium valproate, Phenytoin, phenobarbitone and carbamazepine for symptomatic effects not underlying pathological state of epilepsy. About 80 of epileptic patients are adequately controlled with currently available anticonvulsant drugs, while 20 % increase restorative failure and want adjoin on treatment [4]. The fundamental disadvantage of antiepileptic medications is that they cause undesired side effects and require long-term adherence during the duration of treatment. Both epilepsy and antiepileptic treatments have negative effects on learning and memory. In past few years large number of newer antiepileptic drugs have been has been approved or last phase of development as add on therapy for poorly controlled epilepsy, but safety and tolerability of these drugs needs to be proven [5,6]. Although there is no experimental model that could faithfully reproduce all human TLE features, some models are selected to ask a specific set of questions, the pentylenetetrazole (PTZ) kindling model is widely accepted as an experimental animal model for estimating the effectiveness of antiepileptic drugs or studying the pathogenesis of epilepsy [7].

Kindled seizures have been shown to cause a neuronal loss in limbic systems CA1, CA3, dentate gyrus of hippocampus, amygdala and entorhinal cortex [8]. The Memory impairment has been attributed to the hippocampus's neurological damage. An increased activity of the glutamatergic transmission has also been found to play a crucial role in neuronal cell death of the PTZ kindling in rats due to free radicals generation [9]. In order to control epilepsy and its consequences, exogenous dietary supplementation (antioxidants) could serve as a beneficial strategy. The origins of modern medications may be explored in traditional medicine. we have chosen two herbal drugs on the basis of antioxidant properties, one was leaves of *M. oleifera* Lam (Moringaceae) and another one is curcumin (isolated compounds) both of the plants is used in African and Indian traditional medicine to treat not only seizures but also leprosy, stroke, anemia, and mental disorders [10,11].

M. oleifera Lam. leaves are a good source of nutrition and have anti-tumor, anti-inflammatory, anti-ulcer, anti-atherosclerotic, and anticonvulsant properties due to the presence of polyphenolic and flavonoids compounds such as Querectin, chlorogenic acid, kaempferol, beta-carotene, and amino acids, which contribute to their antioxidant property [12].

One such medicine is curcumin, which has been shown to ameliorate or even prevent further progression of diseases [13]. Turmeric has been used for decades in India for its health advantages, as well as a spice and colourant. Curcumin, a principal curcuminoid in turmeric, is obtained from dried rhizomes of the plant *Curcuma longa* [14]. Curcumin has been reported to possess antioxidant, anti-inflammatory, anti-proliferative, anti-apoptotic properties anti-inflammatory, anticancerous, antiepileptic, antidepressant, immunomodulatory, neuroprotective, antiapoptotic and antiproliferative effects [15]. Curcumin is also an effective scavenger of reactive oxygen species and reactive nitrogen species [16]. The aforementioned properties of curcumin suggest a potential of using it to treat PTZ-induced kindling while preventing of seizures and memory loss.

Therefore, the combined effect of curcumin and *M. oleifera* ethanolic extract (MOEE) supplementation on epileptic seizure, cognitive impairment & oxidative stress in PTZ-induced kindling in rats was assessed in the current investigation. The molecular docking investigations are going to provide novel viewpoints on the creation of anti-epileptic drugs.

2. Results

2.1. Effect of M. oleifera & curcumin on the seizure severity score in Pentyletetrazole treated rats

Higher the seizure score lesser is the seizure protective effect and vice versa. After the challenge dose in the kindling, all rats in a group had lived without any problem. When compare wth PTZ-group, the combined low dose (250 mg/kg) of MOEE and low dose (200 mg/kg) of curcumin treated

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groups demonstrated a significantly difference in the seizure score. When compare with PTZ groups to standard (valproic acid) groups shows a significantly difference in the seizure score. The combined Low dose MOEE & Low dose of curcumin treated group compare with valproic acid did not significantly vary in the seizure score, which suggest that the combination treatment exerted excellent antiepileptic activity in reducing the seizure activity (Figure 1).

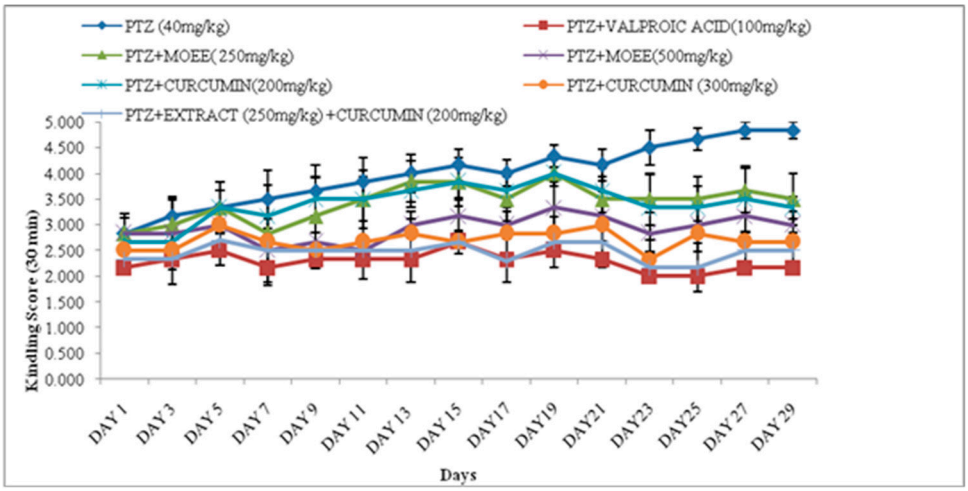

Figure 1. Effect of ethanolic extract of *M. oleifera* and curcumin on the seizure severity score in PTZ treated Wistar albino rats. Each value expressed as mean ± SEM; n=6.

2.2. Neurobehavioral Observations

2.2.1. Effects of MOEE & curcumin on elevated plus maze test

There was not a noticeable variance in the initial transfer latency between open arm vs close arm. However, when the retention transfer latency was examined after 24 hours the original transfer latency, a significant difference was found. When compare control group with PTZ treated groups were significantly increased the retention transfer latency (***p <0.0001) (Figure 2). When compare with control group, the combined effect of MOEE and curcumin at doses of 250 mg/kg & 200 mg/kg wasn't reflected in a significant difference (***p<0.0001) in the retention transfer latency. When compare with PTZ groups, the *per se* groups have shown a significantly difference (***p<0.0001) was observed.

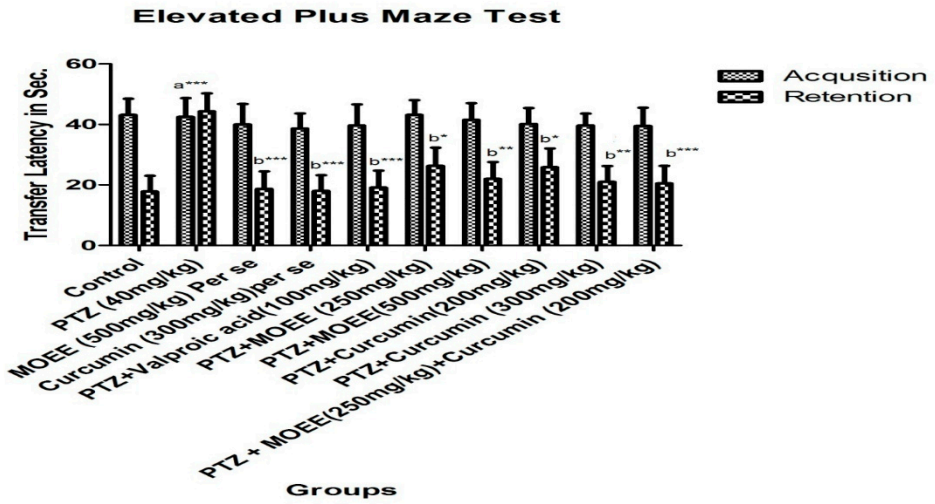

Figure 2. Effect of MOEE and curcumin on elevated plus maze test in PTZ induced kindled rats. Values were expressed as mean ± standard error of the mean (SEM).*$^{*}p<0.01$, $^{**}p<0.001$ & $^{***}p<0.0001$. ns= non-significant, a-control Vs PTZ, b-PTZ Vs all groups.

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2.2.2. Effects of MOEE and curcumin on step through latency test

The passive avoidance test measures the animals' memory & remembers skill. The initially transfer latency does not differ significant among the groups, however PTZ treated groups considerably delayed the passive avoidance paradigm's retention latency when compare with PTZ-treated group, according to Tukey's post hoc analysis. Whencompare with control group, the retention latency significant decreased in the PTZ group (***p<0.0001). As an MOEE and curcumin were combined with PTZ as a pretreatment, the retention delay was significantly increased (**p <0.001) as compare with PTZ group. When compare with PTZ-treated groups, the combined effect of MOEE and Curcumin induced a very significant change (***p<0.0001) in the retention transfer latency (Figure 3).

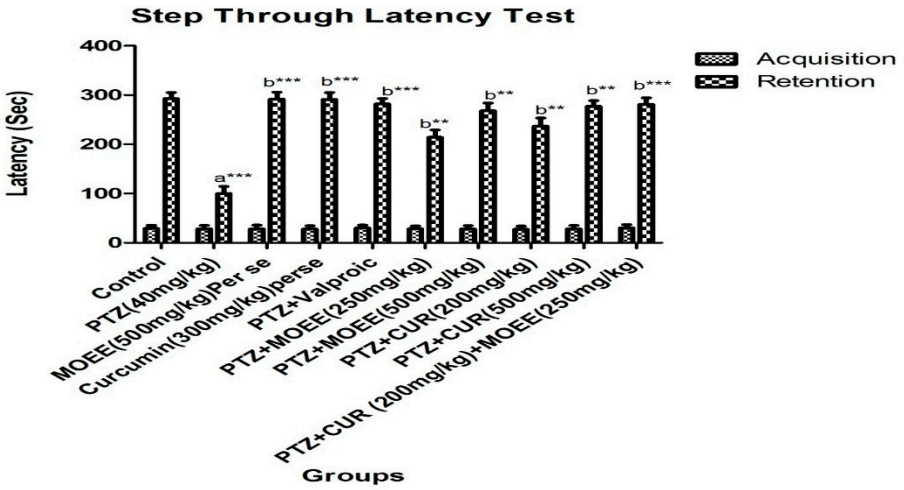

Figure 3. Effect of MOEE and Curcumin on step through latency test in PTZ induced kindled Rats. Value was expressed as mean ± standard error of the mean. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ns= non-significant, **a**-control Vs PTZ, **b**-PTZ Vs all groups.

2.2.3. Effect of MOEE & curcumin in open field apparatus test

The stimulation of the CNS by PTZ is revealed by the increase in locomotor activity, which may be triggered by a decrease in the brain's GABA neurotransmitter. However, there was little difference in the locomotor activity of PTZ rats given with valproic acid, MOEE at 500 mg/kg, or curcumin at 300 mg/kg. Moreover, there is no any significant difference in combination of MOEE & curcumin supplement on the locomotors activity of the animals as compare to PTZ groups (Figure 4).

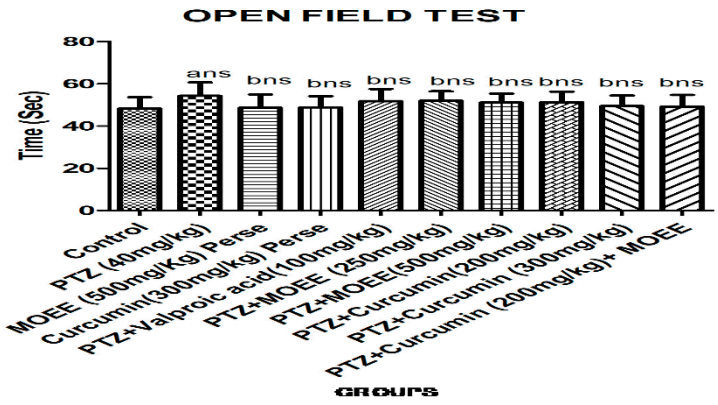

Figure 4. Effect of MOEE and Curcumin on Open Field Test in PTZ induced kindled Rats. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ns= non-significant, **a**-control Vs PTZ, **b**-PTZ Vs all groups.

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2.3. In-vivo antioxidant activity

Evidence suggests that antioxidants may reduce the lesions induced by oxidative free radicals in experimental models of epilepsy.

2.3.1. Effects of MOEE & curcumin on brain MDA level

Malondialdehyde (MDA) level was significantly to be increase (***p < 0.0001) in the brain with PTZ treated groups as compare with control groups. The combined effect of MOEE & Curcumin supplement significantly lower (**p<0.001) in PTZ induced Peroxidation of lipids in the brain. The levels of MDA in MOEE and curcumin *per se* groups differ significantly (***p<0.0001) compare with the levels of PTZ groups. The MOEE treated groups, both low dose and high dose have shown less significantly (*p<0.01) difference in MDA level as compare with PTZ treated groups. The combined effect of MOEE and curcumin showed that the level of MDA was significant decrease (**p<0.001) when compare with PTZ groups (Figure 5A).

2.3.2. Effects of MOEE & curcumin on brain of glutathione level

The brain GSH level showed highly significant difference in PTZ treated groups as compare with control groups (***p<0.0001). When compare to PTZ group with *per se* groups, standard valproic acid groups & the combination of curcumin & MOEE treated groups have shown highly significant (***p<0.0001) elevation of GSH level in the brain. Low dose MOEE treated group showed non-significant (ns) difference while high dose MOEE and curcumin showed significant difference (**p<0.001) as compared with PTZ treated group. Hence, the combination of MOEE & curcumin was more effective to restoring the depleted GSH level in the brain & it is equally efficacious as compared to standard valproic acid **(**Figure 5B).

2.3.3. Effect of MOEE & curcumin on brain of SOD levels

In SOD levels, When compare the control group with PTZ group, the brain superoxide dismutase significant decrease (**p<0.001). The *per se* groups & the combination of MOEE & curcumin treated groups showed significantly showed better significance difference (**p<0.001) in the level of SOD compared with PTZ treated group. The standard valproic acid, high dose MOEE and curcumin groups had less significant difference (*p<0.01) in the levels of brain GSH levels compare with PTZ group. However, the MOEE low dose did not show significant difference (ns) in the level of superoxide dismutase in brain as compare with PTZ control groups (Figure 5 C).

2.3.4. Effect of MOEE & curcumin on brain catalase levels

In PTZ treated groups a severe depletion of catalase level in the brain, the level of catalase with PTZ-treated group has showed a very significantly difference (***p<0.0001) with control group. When compare to PTZ treated group and *per se* groups, standard valproic acid group and with combined (curcumin+ MOEE) treated groups have shown highly significant (***p<0.0001) elevation in the catalase level of brain. The curcumin treated group (both low dose and high dose) as well as high dose MOEE have sown significant elevation (**p<0.001) in the catalase level of animals as compare with PTZ treated groups (Figure 5 D).

2.3.5. Effects of MOEE & curcumin on brain of NO levels

The NO levels was observed to be increase remarkably and highly significant (***p<0.0001) in compare with PTZ treated groups as well as control groups. The low dose treatments with MOEE or curcumin showed only significant difference (**p<0.001) whereas the standard valproic acid group, *per se* groups, high dose MOEE or curcumin groups and the combined MOEE and curcumin treated groups have shown highly significant (***p<0.0001) difference in lowering the nitric oxide levels as compare to PTZ treated groups **(**Figure 5 E**)**.

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2.3.6. Effects of MOEE & curcumin in brain of AChE levels

The level of acetylcholine in the brain is a good indicator of memory, and acetylcholinesterase activity has been used as a substitute for acetylcholine. The current study's findings are consistent with the PTZ group's raised AChE activity, which showed that elevated AChE was one of the causes of the memory loss. The PTZ treated group showed highly significantly difference (***p<0.0001) in elevated AChE level as compare with control groups. However, AChE activity in all the remaining groups have shown highly significant difference (***p<0.0001) in decrease AChE level as compare with PTZ groups (Figure 5 F).

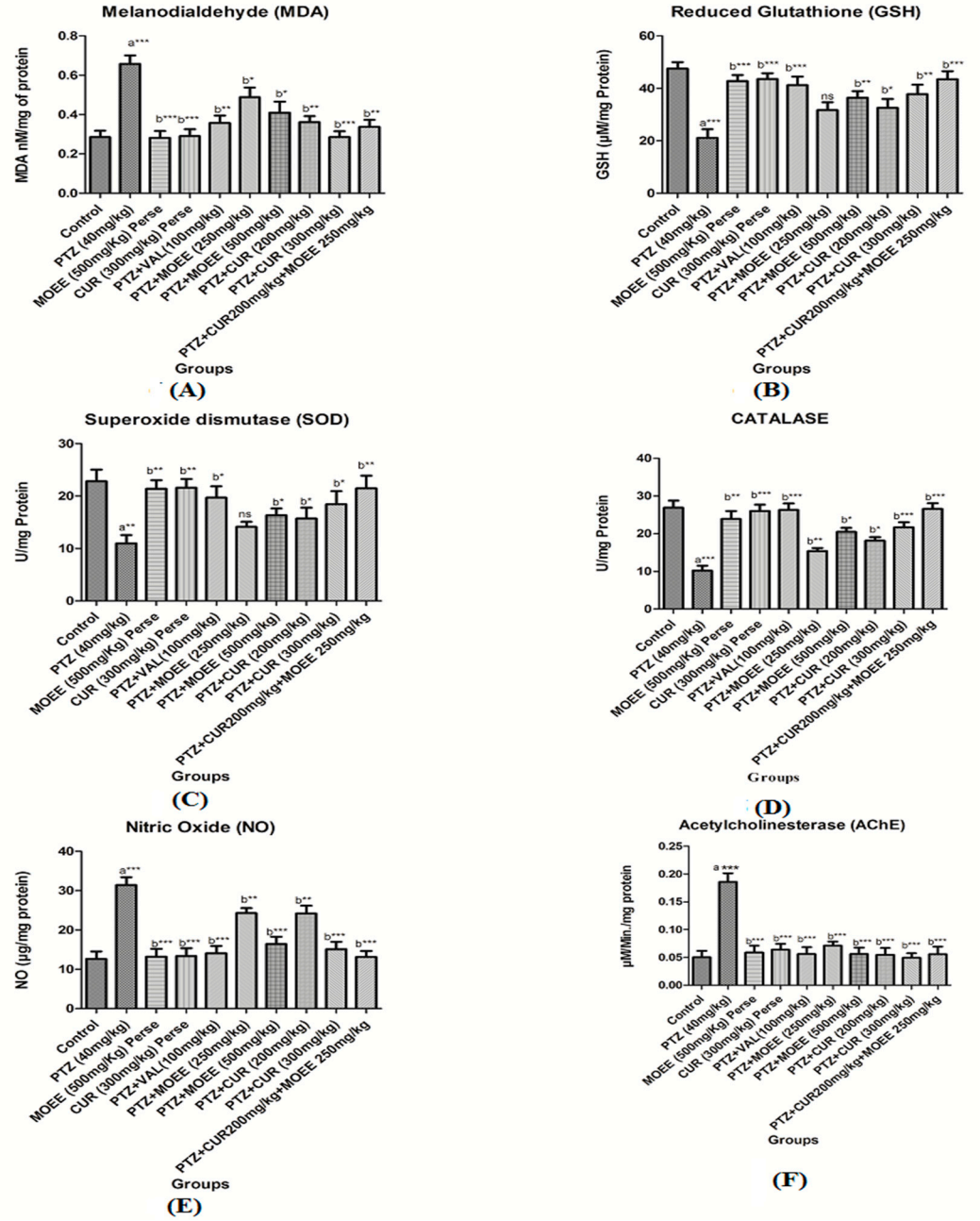

Figure 5. Effect of MOEE & curcumin on brain of (A) MDA levels (B) glutathione levels (C) SOD levels (D) catalase levels (E) Nitric oxide levels (F) AChE levels in PTZ induced kindled Rats. Value was expressed as mean ± standard error of mean (SEM). $^{*}p<0.01$, $^{**}p<0.001$, $^{***}p<0.0001$ ns= non significant, **a**-control Vs PTZ, **b**-PTZ Vs all groups.

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2.4. Binding mode analysis of curcumin, quercitin, chlorogenic acid & valproic acid

To evaluate the dependability and repeatability of the docking process for our investigation, the internal ligand (FAD) was docked into the PDB ID: 1RT2 in the current study (Figure 6). The Compound "FAD" has a 3.35 root mean square deviation (RMSD) between its anticipated conformation and its actual X-ray crystallographic conformation.

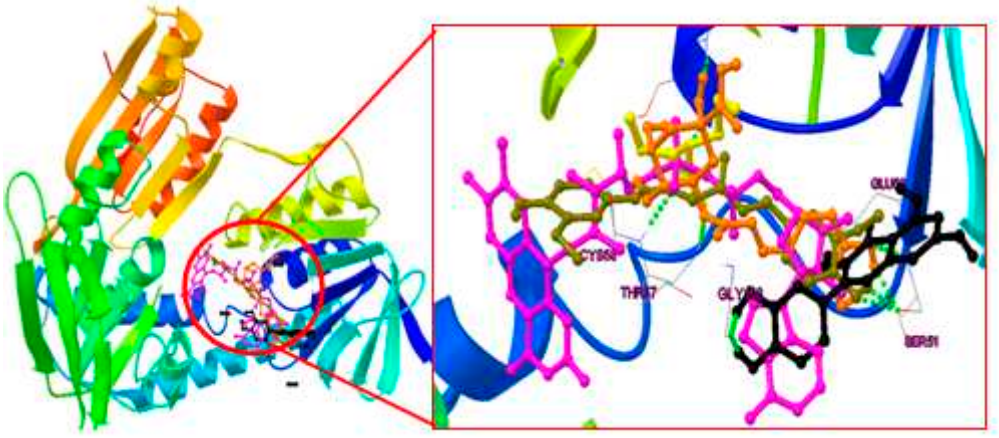
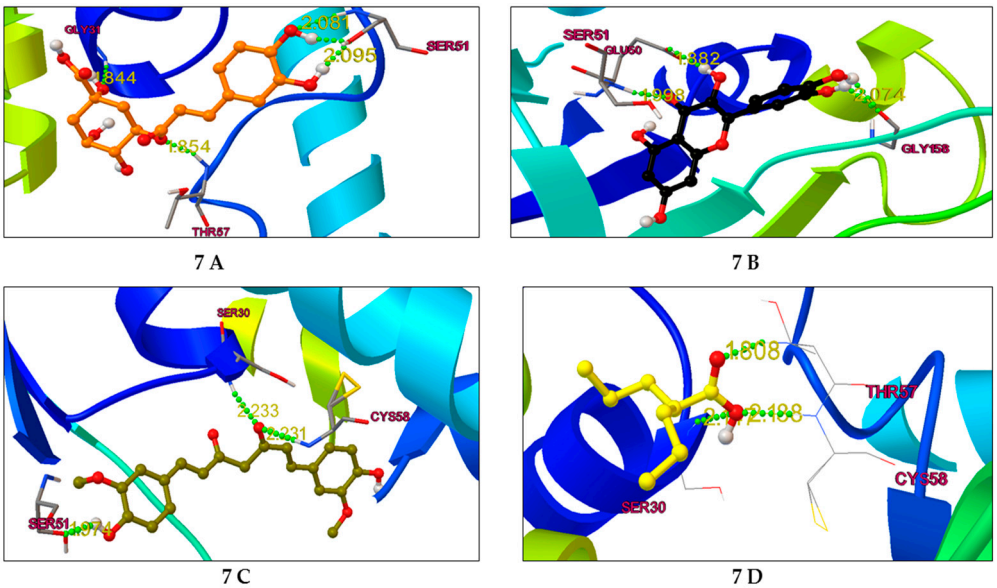


Figure 6. Overlapping of all the ligand [Chlorogenic acid (Saffron color); Quercetin (black); Curcumin (olive green); internal ligand (pink); Valproic Acid (Yellow)] in the active site of Human Glutathione Reductase (PDB ID: 3DK9).

2.4.1. Post-Docking Analysis

The docking analysis was performed on each naturally isolated molecule in the active site of HGR (PDB ID: 3DK9) and Valproic Acid was docked as a standard ligand. The binding energy (Kcal/mole), inhibitory constant (Ki) and interactions (H-bond) for these compounds are given in Table 1. All the individual interactions of the molecules for 3DK9 are shown in Figure 7A-7D respectively. Through docking experiments, it was shown that curcumin is most effective in the active part of human glutathione reductase (PDB ID: 3DK9).



7 A 7 B

7 C 7 D

Figure 7. A. Binding interaction of Chlorogenic acid (Saffron color) in the active site of human glutathione reductase (PDB ID: 3DK9); **7 B-** Binding interaction of Quercetin (black); **7 C-** Curcumin (olive green); **7 D-** Valproic Acid (yellow) in the active site of human glutathione reductase.

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Table 1. List of extracted molecules with their binding affinity in the active site of Human Glutathione Reductase (PDB ID: 3DK9).

Molecule	KI value	Docking Score (Kcl/mol)	Hydrogen Bond Interaction
Curcumin	0.264 μM	-8.97	SER 51, GLY29, CYS 58
Quercetin	2.33 μM	-7.68	GLU 50, SER 51, GLY 158
Chlorogenic acid	3.29 μM	-7.48	GLY 31, THR 57, SER51
Valproic Acid	190.91uM	-5.07	SER 30, THR 57,CYS 58

2.4.2. Binding mode analysis

Chlorogenic acid has formed five hydrogen bonds with three different amino acids (Figure 7 A). The Catechol moiety has formed three hydrogen bonds with Ser51 at distance range of 2.05 to 2.10 Å, the carboxylic acid group has formed a hydrogen bond with Gly31 at 1.844Å. The linker oxygen atom completely filled the hydrophobic pocket of the human glutathione reductase active site by forming a hydrogen bond with Thr57 at a distance of 1.85. At a distance of 2.074, the catechol moiety of quercetin has a hydrogen bond established with the gly158.The carbonyl and hydroxyl group have formed two hydrogen bonds with Glu 50 and Ser 51 at a distance of 1.8 and 1.9 Å and completely blocked the catalytic binding site of the receptor that can be the reason that quercetin is more potent than chlorogenic acid (Figure 7 B).

At a distance of 1.9, Ser51 and the salicylic acid moiety of curcumin had formed a hydrogen bond. At distances of 2.2 and 2.3, respectively, the linker carbonyl group has made two hydrogen interactions with Ser30 and Cys58.Due the structural nature of the curcumin, which consists of the two bulky groups connected by conjugated bonds makes it super flexible to completely occupy the hydrophobic and catalytic binding site and that can be the reason that Curcumin has shown to most potent among others (Figure 7 C). Valproic Acid has formed three hydrogen bonds with three different amino acids (Figure 7 D). At a distance of 1.808, the carbonyl (= O) group has a hydrogen connection established with the Thr57. The hydroxyl (-OH) group has formed two hydrogen bonds with the Cys 58 and Ser 30 at a distance of 2.18Å and 2.11Å respectively and occupying the hydrophobic pockets. As, Valproic acid is small structure and not able to occupy the whole binding pocket. This can be the reason; valproic acid is lacking to provide good docking score. The possible mechanism of antiepileptic effects or neuroprotection against PTZ by binding mode analysis of Curcumin, quercitin and chlorogenic acid. ROS generated on by oxidative stress are the primary cause of epilepsy, which is an abnormal interruption of nerve cell activity in the brain. ROS are a particular class of oxygen-containing unstable molecule that easily interacts with other molecules in a cell. Reactive oxygen species in cells have the potential to harm proteins, RNA, and DNA, which results in cell death. A crucial enzyme in the cellular regulation of reactive oxygen species (ROS) is glutathione reductase (GR). Therefore, activating GR can uplift the antioxidant property, which leads to the inhibition of ROS induced cell death in brain, thus Epilepsy can be prevented after the molecular modeling studies, it has been observed that, the active pocket or, site of human glutathione reductase is “Y” shaped and to get a good a binding activity, it needs long and “Y” shaped ligands and it can also be concluded that if any of the compounds between chlorogenic acid and quercetin is treated with the combination of curcumin can be much more potential.

3. Discussion

The current pharmacotherapy of epilepsy has the limitations of a chronic course, involving unavoidable adverse effects, economical burden, and falls short of the therapeutic goal of a seizure free status in nearly one third of the patients [17]. Use of plant based products for therapy of convulsions has been a part of long-standing tradition in Asia, Africa, and South America [18]. Many

plant extracts have shown the presence of anticonvulsant activity in animal seizure models, which has been attributed to the action of flavonoids, furanocoumarins, phenylpropanoids, and terpenoids on gamma amino butyric acid (GABA) receptors and voltage gated ion channels [19]. These phytochemicals facilitate the maintenance of normal physiological function of the major inhibitory neurotransmitters [20]. Modern laboratory methods and the emphasis on evidence-based medicine have renewed interest in research on herbal items in an effort to discover a safe and effective antiepileptic compound. The present understanding of epilepsy indicates that it may be useful to develop antiepileptic medications with added antioxidant action, and medicinal plants are an excellent source for such an endeavour. Traditional medicinal plants provide a wealth of information for the creation of contemporary pharmaceuticals. The herbal medicine drawn extensive appreciation from the research bases and enterprises lately at the national and international levels. Hence there has been extreme focused with respect to potential phytochemical to protect the neuronal activity and defensive component against epilepsy.

Epilepsy is one of the most common neurological disorders estimated to affect around 50 million people worldwide characterized by epileptic seizures associated with complex molecular, biochemical, physiological and anatomical changes in the brain [21]. The potential cause of epilepsy includes brain injury, brain tumor, stroke, or inflammation in brain [22]. Epileptic seizures occur due to abnormal discharge or excessive firing activity of neurons in the brain [23]. The neuronal death in epilepsy could be attributed to the oxidative stress induced free radical generation in the brain due to lipid peroxidation, protein oxidation and DNA damage [24]. Both epilepsy and antiepileptic medications have a negative impact on an epileptic patient's ability to learn and remember things. Currently available antiepileptic drugs target only the symptoms but do not prevent the underlying pathology of epilepsy or its associated comorbidities [25]. Still more than 30% patients experience epileptic seizures after therapy with AED [26,27]. It is therefore necessary to find alternative natural remedies (phytoconstituents or nutraceuticals) to the traditional AED that might offer beneficial clinical effectiveness and tolerance with low side effects.

M. oleifera Lam. (Family: Moringaceae) is common culinary plant known as drumstick tree. *M. oleifera* Lam is a widely available plant in Southeast Asia which has been evaluated for the presence of antioxidant activity in a few earlier studied [28]. The most fascinating characteristics of this species are antioxidant [29], and anti-inflammatory characteristics [28]. Traditional system of medicine claims to suggest that the leaves of *M. oleifera* have the potential in the treatment of epilepsy [30]. The leaves of *M. oleifera* have been reported to contain a number of phytoconstituents including alkaloids, carotenoids, flavonoid, polyphenol, phenolic acids, tannins, saponins & vitamins. The leaves are also said to be rich in Vitamin A and C, beta-carotene, chlorogenic acid, kaempferol, and quercetin which contribute to their antioxidant property [31,32]. Curcumin, a principal curcuminoid present in turmeric, is obtained from the dried rhizomes of *Curcuma longa* [33]. Curcumin has been reported to possess antioxidant, anti-inflammatory, antiepileptic, immunomodulatory, and neuroprotective activity [34]. Curcumin is also believed to be an effective scavenger of reactive oxygen and nitrogen species [35]. Therefore the characteristics of curcumin propose to enormous prospective as a medicine for treating PTZ-induced kindling that has seizures and cognitive impairment. Due to presence of phenolic group, curcumin acts as a strong antioxidant and inhibit the generation of reactive oxygen species such as superoxide anions and nitrite radical generation both in vitro and in vivo [36]. The presence of hydroxyl (OH) groups in phenolic compounds may contribute directly to their antioxidant activity and be a significant predictor of their radical scavenging ability [37]. Pentylenetetrazole (PTZ) kindling model is widely accepted as an experimental animal model for evaluating the effectiveness of antiepileptic drugs or studying the pathogenesis of epilepsy [38]. Kindling is the process of repeatedly decreasing the seizure threshold in the brain by electrical or chemical stimulation, which results in repetitive seizures. It causes development of seizure gradually that transcribes in generalized tonic-clonic seizures often associated with cognitive impairments [39]. Pentylenetetrazol on repeated administration with a subconvulsant dose determines the nature and intensity of convulsant activity. Seizure activity of drugs in animals is evaluated on the basis of seizure score gained by the administered drug [40]. Higher the seizure score lesser is the seizure

protective effect and vice versa. As compared to PTZ treated (4.83±0.17) animals, the low dose curcumin (3.33±0.21) and low dose MOEE (3.50±0.50) group animals has significant difference in the seizure score value. The high dose curcumin treated (2.66±0.33) groups has shown highly significant difference whereas high dose MOEE treated (3.00±0.26) group has shown significant difference in their seizure score as compare to PTZ treated groups. The combined low dose MOEE and low dose curcumin treated group has shown highly significant difference in the seizure score (2.50 ± 0.34) as compare to PTZ treated group. The standard (valproic acid) treated groups has highly significant difference in the seizure score (2.16 ± 0.31) as compare to PTZ groups. There were significantly differences initiate in the convulsion score between valproic acid groups and combined Low dose MOEE and Low dose of curcumin, which suggest that the combination treatment exerted excellent antiepileptic activity in reducing the seizure activity. Neurobehavioral assessments were performed to assess cognitive functions such as learning and memory by elevated plus maze (EPM) and passive avoidance (PA) as described by (Sarangi et al., 2017) [41]. The biological processes in the brain that contribute to impairment in cognitive function have been reported to be influenced by ongoing seizure activity and antiepileptic drugs treatment [42]. The reduction in cognitive functions in kindled rats might be caused by an assortment of circumstances. One of several explanation refers to the degenerative processes in the brain structures, secondary to seizure related ischemia and hypoxia [43]. It has been shown that kindled seizures are associated with a selective degeneration of cortical and limbic structures including hippocampus areas, involving loss of neurons, glial and neuronal growth, and astrocytes hypertrophy [44]. In addition, the seizure activity and antiepileptic activity have causes increased the level of free radicals & reduced antioxidant scavenging defense activity [45]. This imbalance in the body's oxidant and antioxidant defence mechanisms may result in seizures and cognitive impairment. Elevated plus maze apparatus were used for evaluating memory in rodents, there was no significantly difference in initial transfer latency between open & closed arms. On the other hand, a significant difference was seen in the retention transfer latency, which was assessed twenty four hour following the initial transfer latency. PTZ kindling induced a highly significantly increase (***$p<0.0001$) in retention transfer latency as compare with control group, according to post hoc analysis. The dose-dependent effect of Curcumin & MOEE inverted the effect of PTZ-induced kindling on retention transfer latency was observed. The retention transfer latency decrease less significantly (*$p<0.01$) in the low dosage curcumin and low dose MOEE groups compare with PTZ group. However, when significant amounts of curcumin and MOEE were used, the transfer latency was significantly reduced (**$p<0.001$) as compare with PTZ groups. When compare with control group, the combined impact of MOEE and Curcumin at doses of 250 mg/kg and 200mg/kg did not showed a highly significant difference (***$p<0.0001$) in retention transfer latency on EMP tests. When compare with PTZ group, the *per se* groups showed a significantly significantly difference (***$p<0.0001$).The previous study also reported that the 200mg/kg and 300mg/kg dose did not cause any significant in the transfer latency [46]. When the Pentylenetetrazole groups was compare with control group, the retention latency decreased significantly (***$p<0.0001$). However, the MOEE & curcumin was combined with PTZ result, substantial dose-dependent increase (**$p<0.001$) in retention latency as compare with PTZ groups. When compare with PTZ-treated groups, the combined effects of MOEE and Curcumin resulted in a highly significant difference (***$p<0.0001$) in retention transfer latency of Passive avoidance test. The increase in the locomotor activity reveals the stimulant effect of PTZ on CNS, due to decreased GABA neurotransmitter in brain [47]. Moreover, kindling process is known to increase the strength of excitatory synaptic connections and decreases the strength of connectivity between inhibitory synapses which could be the reason for an increase in the locomotor activity of PTZ groups [48]. However, PTZ groups with 300 mg/kg curcumin, 500 mg/kg MOEE, or standard (valproic acid) showed no variation in locomotor activity. In addition, there is no significant change in locomotor activity of the rats treated with MOEE and curcumin compare with PTZ groups. Free radicals are frequently created in the body as a result of aerobic metabolism. Overproduction of these free radicals (ROS/RNS) results in damage to lipids, proteins, DNA in the cells and eventually leading to various neurological disorders [49]. Moreover, brain is quite susceptible to oxidative damage as it contains high amount of polyunsaturated fatty acids

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which can be readily per-oxidized [50]. To counteract excess free radical generation, there are several endogenous antioxidants (catalase, superoxide dismutase, glutathione) they produce protective effect against free radicals geneneration in the brain.

Lipid peroxidation is a process in which free radicals formed react with lipids present in cell membranes leading to cell damage [51]. Reactive aldehydes such as malondialdehyde (MDA) are the end products of lipid peroxidation. MDA is an end product of free radical generation and is used as an indicator of oxidative stress in biological system [52]. Malondialdehyde levels were found to be significantly higher (***p<0.0001) in the brains with PTZ treated groups as compare with controls groups. MOEE and Curcumin supplementation substantially reduced PTZ-induced lipid peroxidation in the brain (**p<0.001). The *per se* groups of MOEE & curcumin was significantly difference (***p<0.0001) in MDA levels when compared with PTZ treated groups. The MOEE groups, both low dose and high dose have shown less significantly (*p<0.01) difference in MDA level as compare with PTZ treated groups. The combined effect of MOEE + Curcumin showed that the level of MDA was significantly decrease (**p< 0.001) when compare with PTZ treated groups. The combination supplement has shown similar effect to standard valproic acid (**p<0.001) in reducing the MDA level and hence lipid peroxidation in the brain. This antioxidant effect of curcumin in PTZ kindled rats is supported by the finding of recent study [53] where in oral supplement of curcumin decreases the catalase, MDA & glutathione in rat cerebellum and cerebrum. By scavenging free radicals, GSH serves a crucial function in protecting cells from oxidative damage. It is utilised as an indicator of oxidative stress. The GSH level was significantly decrease in PTZ groups in compare with control group (***p<0.0001). When PTZ-treated group compare with, the *per se* groups, standard valproic acid group, and combination of curcumin & MOEE groups all showed a highly significantly (***p<0.0001) increase in brain GSH levels. Low dose MOEE treated group showed non-significant (ns) difference while high dose MOEE and curcumin showed significant difference (**p<0.001) as compare with PTZ treated group. Hence, the combination treatment group was more effective to restoring the depleted GSH level in the brain and it was equally efficacious as standard valproic acid treatment.

The PTZ group had significantly lower brain superoxide dismutase levels in compare with control group (**p<0.001). The *per se* groups and the combination of MOEE +Curcumin treated group far better than the standard valproic acid groups & have shown significantly difference (**p<0.001) in the level of SOD as compare with PTZ groups. The standard valproic acid, high dose MOEE and curcumin groups were less significantly difference (*p<0.01) in the GSH levels as compare with PTZ groups. The brain catalase level of PTZ treated group have shown highly significantly difference (***p<0.0001) as compare with control group as PTZ causes severe depletion of catalase level in brain. When compare to PTZ treated groups, the *per se* groups, standard valproic acid group and the combined (curcumin+ MOEE) treated groups have shown highly significantly (***p<0.0001) elevation in the catalase level of brain. The Nitric oxide levels were observed to be increased remarkably and is highly significantly (***p<0.0001) in the brain with PTZ treated groups in comparison with control. The low dose treatments with MOEE or curcumin showed only significantly difference (**p<0.001) whereas the standard valproic acid group, *per se* groups, high dose MOEE or curcumin groups and the combined MOEE & curcumin treated groups have shown highly significant (***p<0.0001) difference in lowering the nitric oxide levels as compared to PTZ treated group. The present research's results are consistent with the enhanced AChE activity in the PTZ group, suggesting that increased AChE was an additional factor in memory loss. When compared to the normal control group, the PTZ-treated group displays a highly significant difference (***p<0.0001) in higher AChE levels. However, when compare with PTZ-treated groups, AChE activity in all of the other groups showed a highly significant difference (***p<0.0001).

On the above mention result indicate that the combined effect of *M. oleifera* leaves of ethanolic extract and curcumin has significant protection against PTZ induced kindled epilepsy in Rats. This probably may due to presence of falvonoid and phenolic content. Reduction of free radical as increased expression of antioxidant enzymes in the reduction in lipid peroxidation. The majority of plant extract decreases the significant amount of free radicals. Phenolic compounds are unique

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secondary metabolites present in the plants and exhibit a number of therapeutic applications such as antioxidant, anticancer and neuroprotective activity etc. The presence of hydroxyl groups contributes significantly to the phenolic compound's scavenging capacity. According to the results, ethanolic extracts of *M. oleifera* leaves and curcumin have strong antioxidant, free radical scavenging, and antiepileptic actions. The possible mechanism of antiepileptic effects or neuroprotection against PTZ by binding mode analysis of Curcumin, Quercitin and Chlorogenic Acid. Epilepsy is characterized as an aberrant interruption of nerve cell activity in the brain produced by oxidative stress-induced reactive oxygen species (ROS). ROS are unstable molecules containing oxygen and easily interact with other molecules in a cell. Reactive oxygen species in cells can damage genetic materials and proteins that it can lead to cell death. Glutathione Reductase (GR) is an enzyme that regulates reactive oxygen species (ROS) in the cell.

Therefore, activating GR can uplift the antioxidant property, which leads to the inhibition of ROS induced cell death in brain, thus Epilepsy can be prevented after the molecular modeling studies, it has been observed that, the active pocket or, site of Human Glutathione Reductase is "Y" shaped and to get a good a binding activity, it needs long and "Y" shaped ligands and it can also be concluded that if any of the compounds between Chlorogenic acid and Quercetin is treated with the combination of Curcumin can be much more potential.

4. Materials and Methods

4.1. Reagents and Chemicals

All the reagents and chemicals have been purchased from authentic resources (Sigma Aldrich, St. Louis, MO). The curcumin was purchased from sigma chemicals Co. (St. Louis, MO, USA). High performance chromatography (HPLC) analysis of curcumin powder revealed that it contains 95.02% curcuminoids.The Pentylenetetrazole, reduced glutathione, DMSO (dimethyl sulphoxide) DTNB, thiobarbituric acid, tetra ethoxy propane,Trichloro acetic acid, pyridine, n-batanol, and sodium dodecyl sulphate were purchased from sigma Aldrich.

4.2. Preparation of M. oleifera leaves extract

In the month of January 2020, Moringa oleifera Lam. fresh leaves (Moringaceae) were collect from Moradabad, U.P, India & the specimen were verified (voucher no: NICAIR/ RHMD/Consult/2020/3600-01) and examined by Dr. Sunita Garg, Emeritus Scientist, RHMD, NISCAIR, PUSA Institute, New Delhi 110012, India. The dehydrated leaves of M. oleifera material were then grind with a multifunction grinder (Huangdai, China) and fed through a 60-mesh sieve number. Using a soxhlet extractor, 100 gram of coarsely crushed Moringa oleifera lam leaves were treated with continuous hot extraction by elevated temperature with ethanol. The ethanolic extract of Moringa oleifera leaves was separately filtered by whatman filter paper & it dried by a rotatory evaporator to get dry extracts. The dry extracts were kept in the freezer (0–4°C) until they were required. According to dried leaf weight, the extraction yield was 1.36% (w/w).

4.3. Inducing of kindled seizures & experiment design

Albino Wistar male adult (150–200 g) rats were used & kept in 25°C ± 5 °C temperature, humidity of 50 ± 10%, & 12 hour dark cycle & 12 hour light cycle. The study was approval from the institutional animal ethical committee (Reg. No. (Reg. No.837/PO/ ReBiBt /S/O4/CPCSEA) & was carried out in compliance with the Indian government's standards on animal guideline, the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

After acclimatization in one week, animal (Wistar albino male rat) were categories into ten groups and each groups i.e six animals. (Table 2; Figure 1). The selection of the Dose of Ethanolic extracts of Moringa Oleifera leaves (MOEE) by previous reported study as well as literature survey [54]. PTZ was dissolved in 0.9% saline and administered intraperitoneally (i.p.) on alternate days in a dosage of 40 mg/kg for a duration of 29 days, whereas curcumin and MOEE were freshly prepared throughout the duration of the study [55]. The curcumin (200mg/kg & 300mg/kg, p.o) (Mehla et al.,

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2010) and MOEE (250mg/kg & 500mg/kg, p.o.) [54], and sodium valproate (100mg/kg, i.p.) was given daily orally (p.o.) for a period of 29 days, 30 minutes before to the PTZ treatment on alternate days, and suspended in 1% carboxymethyl cellulose [56,57]. Animals were housed in a plexiglass chamber (30X24X22 centimeter) after each PTZ dose administered and seizure activity was monitor & recorded after 30 min. In accordance to the Racine scale [40], the seizure response's strength was measured as follows: There are five possible responses: 0 for no response, 1 for mouth and facial jerks, 2 for nodding or myoclonic body jerks, 3 for forelimb clonus, 4 for rearing, falling down, forelimb clonus, and 5 for status epilepticus. Figure 8 and Table 2 shows the precise experimental layout.

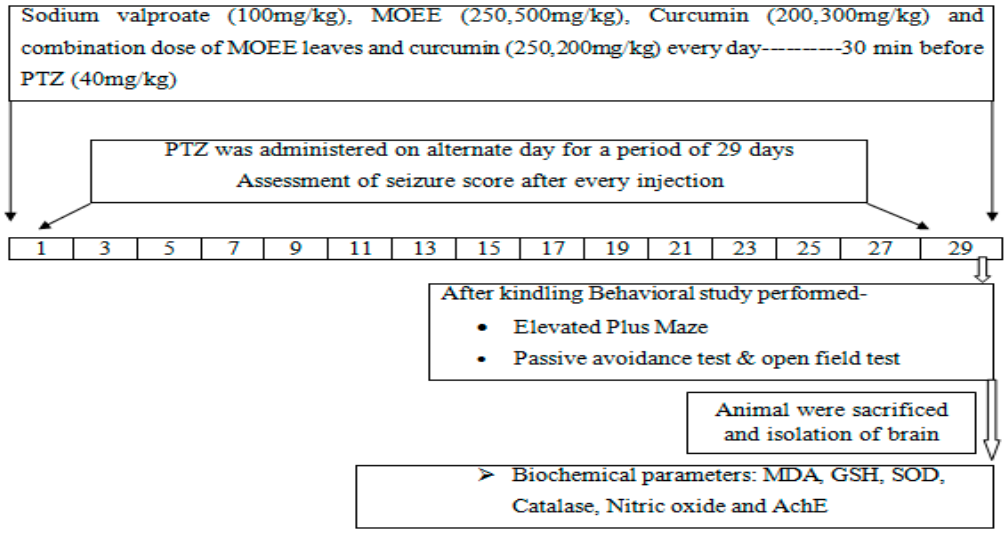

Figure 8. Experimental protocol of pentylenetetrazole (PTZ)-induced kindling.

Table 2. Animal grouping and treatments for Extracts of *Moringa olifera* leaves and curcumin in PTZ induced kindled epilepsy.

Groups (n = 10)	Treatments
I	Normal control with vehicle
II	PTZ (40mg/kg, i.p.) –PTZ was administered on alternate day for a period of 29 days
III	PTZ treated (40mg/kg, i.p.) + MOEE (250mg/kg, p.o.) - for a period of 29 days and 30 min. before PTZ treatment.
IV	PTZ treated (40mg/kg, i.p.) + MOEE (500mg/kg, p.o.) - for a period of 29 days and 30 min. before PTZ treatment.
V	PTZ treated (40mg/kg, i.p.) + Curcumin (200mg/kg, p.o.) - for a period of 29 days and 30 min. before PTZ treatment.
VI	PTZ treated (40mg/kg, i.p.) + Curcumin (300mg/kg, p.o.) - for a period of 29 days and 30 min. before PTZ treatment
VII	PTZ treated (40mg/kg, i.p.) + curcumin (200mg/kg, p.o.) + MOEE (250mg/kg, p.o) –for a period of 29 days and 30 min. before PTZ treatment.
VIII	PTZ treated (40mg/kg, i.p.) + valproic acid (100 mg/kg, i.p.) - for a period of 29 days and 30 min. before PTZ treatment.
XI	MOEE (500mg/kg, p.o.) *per se* - for a period of 29 days
X	Curcumin (300mg/kg, p.o.) *per se*- given daily by oral route for a period of 29 days

MOEE- *Moringa oleifera* ethanolic extracts.

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4.3.1. Neurobehavioral Assessment

These behavior studied were performed at 24 hour after completion of PTZ challenge dose. The elevated plus maze was used to test cognitive impairment after the completion of PTZ challenge dose. Only one animal tested at a time for the behavioral study.

4.3.2. Elevated plus maze test

EPM apparatus consists of up of two open arms, two closed arms and a centre region. The elevated plus maze was used to evaluate cognitive impairment in rats, as previously described. (Holmes and Rodgers, 2003; Mehla et al., 2010) [57,58]. The initial transfer latency in the first trial as the time needed the animal enters in a closed arm while looking away from the central platform with all four limbs. The cutoff time was set at sixty seconds. The rat was then given another 10 seconds to roam freely through the maze with both open and closed arms. The retention transfer latency test was performing in same manner as the acquisition trial 24 hours later. The elevated plus maze was once more used to confine the rats. On the second trial, the transfer latency was set to sixty second.

4.3.3. Step-through passive avoidance Test

Using the previously reported step-through passive avoidance apparatus, memory retention impairment was examined [59]. The device has two distinct chambers, each with a steel grid floor. A guillotine door that joined the compartments was present. The black room was maintained completely dark, while the white chamber was illuminated by a bulb. For the acquisition trial each animal was kept inside illuminated chamber. After 60 seconds the door of guillotine between light and dark chambers was opened for animal habituation & the initial time of latency was noted when the animal enter in the dark chamber. When the initial time of more than 60s the Rats were not allowed to participate in subsequent experiments. The guillotine door was closed the moment the rat entered in dark room, after 3 second electric foot shock (75 V, 0.2 mA, 50 Hz) was administer through the grid area. Just underneath five seconds later, the rat was taken out of the darkened space & put back into the cage. In the acquisition trial the Retention latency was evaluated after 24 hours in the same manner. The delay time was recorded up to 300 seconds.

4.3.4. Open field test (OFT)

The Open Field Test is an experiment used in scientific research to measure rodents' levels of general motor activity and fear [60]. It is a widely used quantitative and qualitative assessment of rodent's general locomotors activity and exploratory desires. The frequency to which behaviour in open spaces aligns with common locomotor activity in other contexts.The Open Field Test is an experiment used in scientific research to measure rodents' levels of general motor activity and fear [60]. It is a widely used quantitative and qualitative assessment of rodent's general loco motor activity and exploratory desire.

Put a rat in the middle of each compartment. Once the test session has begun, care should be made to remain as far away and still as possible if the experimenter plans to stay in the testing room. Exploratory activity can be dramatically impacted by sudden motion or loudness. Throughout the testing procedure, rodents are free to roam the chamber. Each line crossed or photocell beam break results in one activity point being awarded. The average test time for evaluating uneasy environment exploration is five minutes. A 30-minute test session is recommended if the researcher wants to look at habituation to an environment that has become more and more familiar. After the test is over, put the rodent back in its cage. Rearing behaviors', faeces, and grooming activity can all be assessed in addition to horizontal units of activity.

4.4. Measurement of oxidative stress

The animals were decapitated complying with the conclusion of the treatment paradigm and the neurobehavioral assessment; Animals were decapitate under anaesthesia (ketamine), brains were rinsed, after decapitate the brains was immediately removed, cleaned with ice-cold saline & stored

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at 80 °C until biochemical analysis was done within seven days. For biochemical estimation 10% (w/v) tissue homogenates in mix with 0.1-M phosphate buffer (pH 7.4). The homogenates were centrifuge for 15 minutes at 10,000 xg at 4 °C. Supernatants were divided into aliquots, which were then used to make biochemical estimates.

4.4.1. Lipid peroxidation (MDA)

Malondialdehyde (MDA) was estimated as described previously [61]. The 0.1 ml of the sample (homogenate tissue) was mixed with 1.5 ml thiobarbituric acid (0.8% w/v), 1.5 ml of acetic acid (20% v/v) & 0.2 ml of sodium dodecyl sulphate (8.1% w/v) & being heated at 95 °C for 60 min. After the addition of 5 ml of n-butanol/pyridine (15:1) and 1 ml of distilled water, the mixture was cooled using tap water. Following a vortex, the mixture went through a centrifuge at 4000 rpm for a period of ten minutes. The organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer (Specord 200, Analytic Jena AG, Germany).

4.4.2. Reduced glutathione estimation

Reduced glutathione (GSH) was measured according to the method of Ellman 1959 [62]. The equal amount of homogenated mixture were mixed with 10% trichloroacetic acid & centrifuged to separation of proteins., 0.5 ml of 5′5-dithiobis (2-nitrobenzoic acid), 2 ml of 0.3 M phosphate buffer (pH 8.4) & then add 0.4 ml of distilled water to the 0.1 ml of supernatant. Within 15 minutes of vortexing the solution, the absorbance was obtained at 412 nm.

4.4.3. Superoxide dismutase estimation

Superoxide dismutase activity was accessed according to the method described by Kono, wherein the reduction of nitrobluetetrazolium was inhibited by the superoxide dismutase and measured at 560nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). In a nutshell, the hydroxylamine hydrochloride was added to the combination of the sample and nitrobluetetrazolium to initiate the reaction and the results was expressed as unit/milligramme protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 100% [63].

4.4.4. Catalase estimation

Catalase activity was assayed by the method of Luck, where in breakdown of hydrogen peroxides (H_2O_2) is measured at 240 nm [64]. The test Solution contains 0.05 mL of homogenate (10%) supernatant tissue & 3 mL of Hydrogen peroxide , phosphate buffer solution, and the absorbance have taken at 240 nm. Mic.mole of H_2O_2 decomposed per mg of protein/min. was used.

4.4.5. Nitrite Estimation

Green and his coworkers' Greiss reagent, which contains 2.5% phosphoric acid 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride &1% sulfanilamide, were used in a colorimetric test to determine the development of nitrite in supernatant, a sign of nitrite generation. Greiss reagent and supernatant were mixed in equal parts, & entire mixture was incubating for 10 min. at room temp. The absorbance was taken at 540 nm. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and was expressed as micromole per litre [65].

4.4.6. Acetyl cholinesterase activity

According to Gorun et al., the basic idea underlying the strategy is to determine the amount of thiocholine that is formed during the hydrolysis of acetylthiocholine. The color was read immediately at 412nm [66]. A sufficient quantity of ingredient was added to a cuvette containing Ellman's reagent & 0.1 M sodium phosphate buffer (pH 8.0). 14.9 mM of acetylthiocholine iodide was added to start the reaction & the rate of change in absorbance was monitore after two min. at 412 nm. The results

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were represent as nmoles substrate hydrolyzed/min/mg protein using the 5-mercapto-2-nitrobenzoate's (13.6 x 103 M"1 cm'1) molar absorption value.

4.5. In Silco docking analysis of curcumin, quercitin & chlorogenic acid in compare with valproic acid as anti-epileptic drug

During last couple of decades, the research on anti-epileptic studies has taken an impetus in the field of medicinal chemistry. Due to lack to crystal structure of receptors and selected molecule (Curcumin, Quercitin, Chlorogenic Acid) in complex form, it was challenging to build the 3D coordinate for farther computational analysis. To this end, and to generate more precise and reasonable active site coordinates, Molecular Docking has been done by AutoDock 4.2 tool to merge the ligand orientations in the binding cavity. The crystal structure of Human Glutathione Reductase (PDB ID: 3DK9) [67] was retrieved from the RCSB website (https://www.rcsb.org/) [68] and was used to generate initial 3D coordinates, because of its high resolution with 1 Å and a ligand in the active site. The macromolecular protein was prepared via few steps, firstly co-crystallized water molecules were deleted along with addition of polar Hydrogen and compute gasteiger charge [69]**.** The first conformation of a newly designed molecule Human Glutathione Reductase as active site was produced by superimposing structure of chosen molecules against a pre-docked ligand in the PDB. Grid box was then determined by the native ligand (FAD) position on the binding site (Gly31, Gly157, Gly158, Glu50, Ala155, and Asp331) [70,71] with XYZ Grid points of 60×60×60 and grid spacing of 0.375Å. Finally for docking, grid parameter files (gpf) and docking parameter files (dpf) were written using MGL Tools-1.5.6 and both were carried out with following parameters, number of runs: 50, population size: 150, number of evaluations: 2,500,000 and number of generations: 27,000, using Lamarckian algorithm [72]**.** All the ligands were optimized by Avogadro **(**Hanwell et al., 2012**)** suite program before the docking studies.

4.6. Statistical analysis

Using Graph Pad Prism 8 (CA, USA), data were examined. The mean and standard errors mean (SEM) were used to express the experimental results. A one-way analysis of variance (ANOVA) Tukey multiple comparison test was used

5. Conclusions

These results indicate that ethanolic extracts of Moringa oleifera leaves and curcumin have substantial anticonvulsant effect against PTZ-induced convulsions. MOEE and curcumin were additionally shown to be protective against PTZ-induced kindling. These effects might be attributed due to antioxidant activity, as evidenced by a decrease in MDA, Nitrite, and AChE levels and an increase in GSH, SOD, and Catalase levels in the brains of PTZ-treated rat. The study indicates that the combined impact of MOEE and curcumin might be a promising natural molecule for use in epilepsy; however, more research is needed to identify the extract's active ingredients and determine its pharmacodynamic profile. According to docking study, it has been observed that, the active pocket or, site of human glutathione reductase is “Y” shaped and to get a good a binding activity, it needs long and “Y” shaped ligands and it can also be concluded that if any of the compounds between chlorogenic acid and quercetin is treated with the combination of curcumin can be much more potential.

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