

RESEARCH PAPER

Title: Isoliquiritigenin Attenuates PTZ-Induced Seizures and Neuronal Injury Associated with Reduced Brain mTOR Protein Levels

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ABSTRACT:

Background: Epilepsy is a chronic neurological disorder characterized by recurrent seizures and neuronal damage. Despite the availability of antiepileptic drugs, treatment resistance and adverse effects remain major challenges. Isoliquiritigenin (ILQ), a natural flavonoid with reported neuroprotective properties, has emerged as a potential therapeutic candidate for epilepsy.

Methods: Male *Swiss albino* mice were administered PTZ (40 mg/kg, i.p.) on alternate days for 13 days to induce kindling. ILQ (20 and 40 mg/kg, p.o.) and sodium valproate (200 mg/kg, i.p.) were evaluated for their effects on seizure latency, seizure duration, seizure severity, and behavioural performance. Brain mTOR protein levels were assessed following treatment using ELISA. In parallel, PTZ-induced neurotoxicity was established in human SH-SY5Y neuroblastoma cells, and the neuroprotective effect of ILQ (10 μ M and 20 μ M) was evaluated using the MTT cell viability assay.

Results: ILQ produced dose-dependent antiepileptic effects in PTZ-kindled mice by increasing seizure latency and reducing seizure duration and seizure frequency, with the 40 mg/kg dose showing greater efficacy than the 20 mg/kg dose and approaching the effects of sodium valproate. These improvements were accompanied by reduced brain mTOR protein levels, and enhanced viability of PTZ-treated SH-SY5Y human neuroblastoma cells, demonstrating its neuroprotective potential.

Conclusion: ILQ exhibited antiepileptic and neuroprotective effects in both PTZ-induced animal and cellular models. These effects were associated with reduced brain mTOR protein levels and improved neuronal survival, supporting the potential of ILQ for the management of epilepsy.

Keywords: Epileptogenesis, Isoliquiritigenin, Neuroprotective, Human SH-SY5Y cell lines, mTOR.

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1. INTRODUCTION

Epilepsy is a chronic, noncommunicable neurological disorder characterized by recurrent, unprovoked seizures resulting from abnormal and excessive neuronal discharges in the brain. It affects approximately 50 million people worldwide, with an estimated prevalence of 4–10 per 1,000 individuals and a higher incidence in developing countries, particularly in rural populations [1,2]. Despite the availability of several antiseizure medications targeting voltage-gated sodium channels, calcium channels, and γ -aminobutyric acid (GABA) receptors, nearly one-third of patients remain drug-resistant. Moreover, current therapies are primarily symptomatic and are often associated with adverse effects such as sedation and hypersensitivity reactions. These limitations highlight the need to explore novel molecular targets involved in epileptogenesis [3,4].

The mammalian target of rapamycin (mTOR) plays an important role in regulating neuronal growth, metabolism, and survival, and its dysregulation has been implicated in epileptogenesis. Consequently, agents capable of modulating mTOR-associated processes and protecting neurons have attracted considerable interest as potential therapeutic candidates for epilepsy [5,6,7,8]. Among these, naturally occurring phytochemicals have gained increasing attention because of their diverse pharmacological activities.

Isoliquiritigenin (ILQ), a naturally occurring flavonoid isolated from *Glycyrrhiza* species. Recent studies have also demonstrated that ILQ suppresses mTOR signalling and its downstream effectors in several cancer models, contributing to its antiproliferative and pro-apoptotic activities [9]. Given the emerging role of mTOR in epileptogenesis, these findings suggest that ILQ may offer therapeutic benefits in epilepsy. However, its antiepileptic efficacy and its association with brain mTOR protein levels remain largely unexplored. The present study evaluated the antiepileptic and neuroprotective potential of ILQ using PTZ-induced in vivo and in vitro models.

2. MATERIAL AND METHOD

2.1 Animals and experimental conditions

Male Swiss albino mice (25–30g) were procured from Global Bioresearch Solutions Pvt Ltd. Pune. They were housed in groups of 8 in plastic perspex cages under standard conditions (ambient temperature of 20–22°C 12h:12h light/dark cycle). Chow pellets and water were

freely accessible. Animals were allowed to adapt themselves to the new environment for one week prior to the start of the experimental model. Experiments were approved by an Institutional Animal Ethics Committee for the use of animal subjects (Approval number-IAEC/VIP/2023/July/P01).

2.2 Procurement and Preparation of Drug Solution

PTZ, Sodium Valproate, ILQ were purchased from TCI Chemicals (India). Sodium Valproate and PTZ were dissolved in saline and administered by intraperitoneal route [10]. ILQ was dissolved in 0.5 % CMC w/v in saline and administered by oral route [11].

2.4 PTZ Induced Kindling in Mice

2.4.1 Induction of epilepsy:

For standardization of model three Swiss Albino mice were selected. All three animals were given different doses of PTZ i.e. 60 mg/kg, 50 mg/kg and 40 mg/kg intraperitoneally respectively [10,12]. The animals treated with doses of 60 mg/kg and 50mg/kg resulted in immediate tonic clonic seizures in mice whereas no immediate seizures were observed with dose of 40 mg/kg. For the purpose of conducting kindling with PTZ the dose of 40 mg/kg was selected for the study.

2.4.2 Experimental design and drug treatment:

Experimental animals were weighed and randomly divided into 6 groups (n=6): Group I: Control; Group II: Vehicle control; Group III: Disease Control; Group IV: Standard group (sodium valproate, 200mg/kg per *i.p.*); Group V: ILQ treatment group (20mg/kg per oral); Group VI: ILQ treatment group (40mg/kg per oral). Mice were given 40 mg/kg/*i.p.* of PTZ on alternate days for 13 days i.e. each animal from all groups except control, vehicle control group received 7 doses of PTZ. Animals were fully kindled after receiving 3rd dose of PTZ. After each injection of PTZ, mice were placed individually in plastic Perspex cages and convulsive behaviour was observed for 30 mins. The intensity of seizures was rated according to Racine's scale as follows: 0 = No response; 1 = Urination, salivation, defecation, scratching, sniffing; 2 = Ear and facial twitching; 3 = Myoclonic jerks; 4 = Turning into side position, clonic-tonic seizures; 5 = Turning to back position, tonic-clonic seizures. Mice were considered to be fully kindled when they exhibited score of 4 or 5. Mice from standard treatment group as well as ILQ treated group IV (20mg/kg) and V (40mg/kg) were given respective treatment from 3rd dose of PTZ daily till the last dose of PTZ. Sodium valproate and ILQ were

administered 30 minutes and 60 minutes prior injection of PTZ respectively [10,11,13].

2.5 Seizure Analysis

On 13th day after mice received the last dose of PTZ they were observed individually for 30 mins to check the latency for the occurrence of tonic-clonic seizures, duration of seizure persistence and the counts of tonic and clonic seizures [13]. Except for the counts of tonic clonic seizures, scores were recorded in secs for latency and duration of seizures.

2.6 Behavioral Parameters

Rotarod performance and chimney tests were performed to evaluate motor coordination, neuromuscular function, and possible sedative effects of the treatment in experimental animals.

2.6.1 Rotarod performance

On 13th day after mice received the last dose of PTZ and after checking for occurrence of seizures, muscle coordination and grip was evaluated within 1 hr after giving PTZ injection using rota rod (Dolphin, India). Rota rod was rotated at a constant speed of 25 rpm. During the test mice were allowed to accelerate freely on rod for 3 mins and the time required for the mice to fall from the rod was noted [14].

2.6.2 Chimney test

On 13th day after receiving the last dose of PTZ and after checking for occurrence of seizures mice were subjected to chimney test within 1 hr after giving PTZ injection to test for motor function. They had to climb backward up a plastic tube (inner diameter 3 cm, length 25 cm). Motor impairment was indicated by the inability of mice to climb backward up the tube within 60 sec, and the results were shown as the percentage of animals that failed to perform the test [15].

2.7 Biochemical Estimation:

2.7.1 Estimation of mTOR levels in mice brain by Elisa test

For brain mTOR estimation, mice were euthanized by carbon dioxide (CO₂) inhalation, followed by decapitation. The brains were immediately removed, rinsed with ice-cold normal saline to remove residual blood, and kept in PBS (pH 7.4). Brains were then homogenized with tissue homogenizer (Polytron, India), homogenate was centrifuged at 10000 g at 4°C for 1 hr in refrigerated centrifuge (Eltrec, India) and the supernatant obtained was used for the assay. mTOR levels were assayed by using a kit which uses enzyme-linked immunosorbent assay based on the Biotin double antibody sandwich technology (Krishgen Biosystems, India) [16]. Assay was performed using Elisa microplate reader (Epoch, India) as per the instructions mentioned in kit insert provided by vendor. Concentration of mTOR was then obtained by

plotting standard curve using the standard concentration and standard absorbance.

2.8 In-vitro Cell line study

2.8.1 Cell Line and Culture Conditions

The human neuroblastoma cell line SH-SY5Y was procured from NCCS (National Centre for Cell Science), Pune, India. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen) and 1% antibiotic-antimycotic solution under standard culture conditions (37°C, 5% CO₂, and humidified atmosphere). Cells were sub-cultured every 2 days, and passage number 46 was used for the present study.

2.8.2 Experimental Groups

The study consisted of the following experimental groups:

1. Control
2. PTZ-treated group (30 mM)
3. PTZ + Sodium valproate (1 mM)
4. PTZ + Test drug-1 (10 µM)
5. PTZ + Test drug-2 (20 µM)

2.8.3 MTT Cytotoxicity Assay

Cell viability was assessed using the MTT assay based on the reduction of yellow tetrazolium salt (MTT) (Invitrogen) into purple formazan crystals by metabolically active cells. SH-SY5Y cells suspension of 200 µl were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. After incubation, cells were treated with the Sodium valproate 1mM, ILQ 10µM and 20µM for 1 h, followed by exposure to 30 mM pentylenetetrazol (PTZ) except in the untreated control group. The cells were further incubated for 24 h at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific) [17].

Following treatment, the spent medium was removed and MTT reagent was added to a final concentration of 0.5 mg/mL of total volume. Plates were incubated for 3 h in the dark to allow formation of formazan crystals. Subsequently, the MTT solution was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Absorbance was recorded at 570 nm using a microplate reader (xMark, BioRad, USA). Cell viability was calculated using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

Microscopic evaluation of cells morphology was performed under the inverted biological microscope (CKX415F, Olympus, Japan) at the

20x magnification and recorded with the help of Digital Camera by MICAM software.

3 RESULT

3.1 Seizure Analysis

3.1.1 Seizure latency

Seizure latency (min) following PTZ administration was measured and compared among the ILQ-treated groups, the sodium valproate-treated group. Seizure latency was recorded as the time interval between PTZ administration and the onset of the first seizure. In Fig.1, PTZ-induced kindling group showed

seizure latency 65.6 ± 7.745 , whereas seizure latency was increased dose dependently in groups receiving ILQ at doses of 20 mg/kg (132.3 ± 17.648) and 40 mg/kg (164.78 ± 22.521) orally respectively. Group treated with sodium valproate also showed increased seizure latency (212.6 ± 24.842) as compared to group receiving only PTZ. Animals in control group did not show any seizures, however statistical significance was seen in treatment groups as compared to diseased control group.

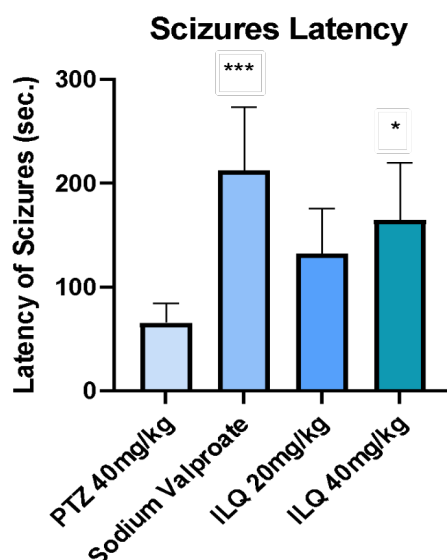


Fig. 1 Effect of ILQ on seizure latency in groups treated with pentylenetetrazol. The values are expressed as mean $\pm$ SEM (n=6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, * $p < 0.05$, *** $p < 0.001$ compared with PTZ induced epileptic group

3.1.2 Duration of seizures

The duration of seizures (seconds) following PTZ administration was measured and compared among the ILQ-treated groups, the sodium valproate-treated group. Seizure duration was recorded as the time interval between the onset and cessation of seizure activity (Fig.2). PTZ-induced kindling group presented increased

duration of seizures (414.5 ± 47.162) whereas duration was significantly decreased dose dependently in groups receiving ILQ at doses of 20 mg/kg (292.7 ± 24.097) and 40 mg/kg (233.2 ± 27.461) orally respectively. Group treated with sodium valproate also showed decreased duration of seizures (225.7 ± 31.961) as compared to group receiving only PTZ.

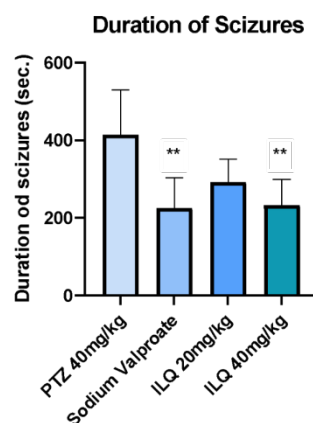


Fig.2 Effect of ILQ on duration of seizures in groups treated with PTZ. The values are expressed as mean $\pm$ SEM (n=6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, **p<0.01 compared with PTZ induced epileptic group

3.1.3 Counts of clonic-tonic seizures

The number of clonic-tonic seizures induced by PTZ was recorded in the ILQ-treated groups, the sodium valproate-treated group. Seizure counts represent the total number of clonic-tonic seizure episodes observed during the experimental period. In **Fig. 3**, PTZ- induced kindling group presented increased counts of seizures

(18.333 $\pm$ 2.092) whereas counts were significantly decreased dose dependently in groups receiving ILQ at doses of 20 mg/kg (7.5 $\pm$ 1.522) and 40 mg/kg (6.166 $\pm$ 1.249) orally respectively. Group treated with sodium valproate also showed decreased counts of seizures (4.833 $\pm$ 0.909) as compared to group receiving only PTZ.

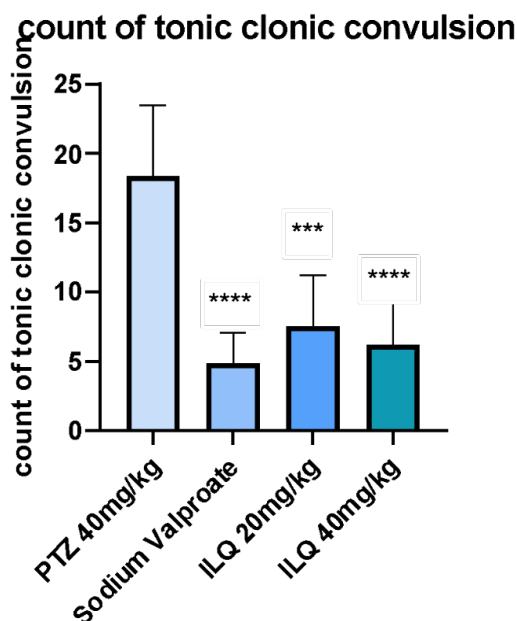


Fig. 3 Effect of ILQ on counts of seizures in groups treated with PTZ. The values are expressed as mean $\pm$ SEM (n=6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, ***p<0.001, ****p<0.0001 compared with PTZ induced epileptic group

3.2 Behavioral Parameters

3.2.1 Rotarod performance

Themotor coordination and muscle grip effect in groups treated with PTZ, sodium valproate, ILQ are shown in **Fig. 4** Group treated with only PTZ showed decreased grip force with decrease in fall time (secs) (5.000 ± 0.577). Fall time was significantly increased dose dependently with

ILQ treatment at doses of 20mg/kg (42.833 ± 2.441) and 40mg/kg (62.833 ± 4.778) respectively. Group treated with standard drug sodium valproate also showed increased grip strength with increased fall time (75.333 ± 5.761) as compared to group treated with PTZ. Maximum grip strength and increased fall time was seen in control animals (92.333 ± 4.862).

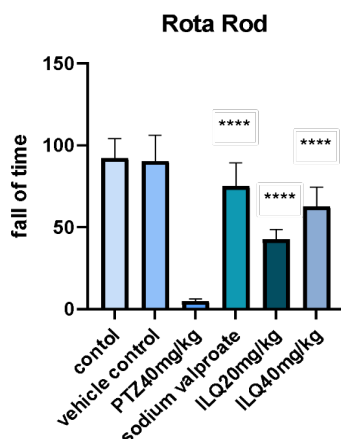


Fig.4 Effect of ILQ on in various groups treated with PTZ. The values are expressed as mean $\pm$ SEM (n=6). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test ****p<0.0001 vs PTZ induced epileptic group

3.2.2 Chimney test

Theeffect of ILQ in chimney test in which the results are shown (**Fig.5**) as the number of animals failing to perform the test. 100% of animals receiving only PTZ failed to perform the test. ILQ treatment reduced time to escape chimney at doses of 20 mg/kg (27.5 ± 6.254) and

40 mg/kg (33.166 ± 3.071) respectively. Animals receiving standard drug sodium valproate also showed reduced time to escape chimney (20.167 ± 3.646). All animals of control (13 ± 1.966) and vehicle control (18 ± 3.891) group were able to successfully perform the test.

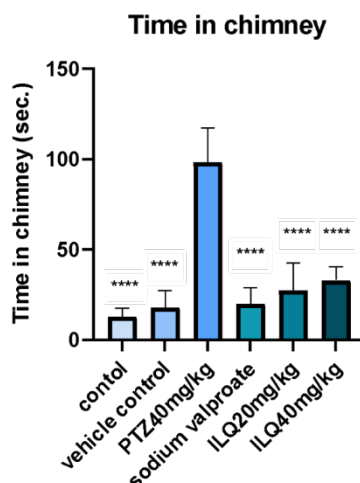


Fig. 5 Chimney test. Data was analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (n=6). ****p<0.0001 vs PTZ induced epileptic group

3.3 Biochemical estimation

3.3.1 Estimation of mTOR levels in mice brain by Elisa test

Fig.6 shows the levels of mTOR in mice brain (ng/ml). PTZ kindling markedly increased brain mTOR protein levels compared with the normal control group (5.62 ± 0.46 vs. 1.30 ± 0.10 ng/mL, $p < 0.001$). Treatment with ILQ significantly

reduced the elevated mTOR protein levels to 3.31 ± 0.35 ng/mL and 2.91 ± 0.08 ng/mL at doses of 20 and 40 mg/kg, respectively ($p < 0.01$ vs. PTZ). Although sodium valproate reduced seizure severity, it did not produce a statistically significant change in brain mTOR protein levels compared with the PTZ group.

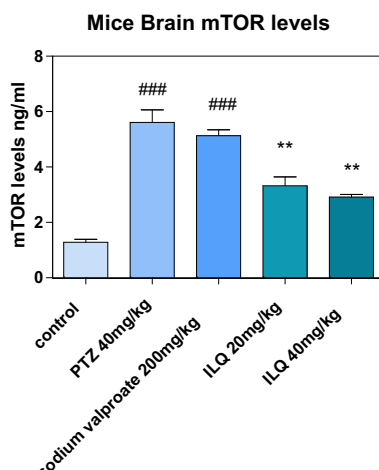


Fig. 6 mTOR levels in mice brain in groups treated with PTZ. The values are expressed as mean $\pm$ SEM (n=4). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, *p<0.05, **p<0.01 vs PTZ induced epileptic group; ###p<0.001 vs control group

3.4 In-vitro Cell line study

3.4.1 MTT cytotoxicity assay:

In the previous studies, the IC₅₀ value of PTZ in the SHSY5Y cell was found as 30 mM and this value was used in this study [17]. Exposure of SH-SY5Y cells to PTZ (30 mM) significantly reduced cell viability (**Fig.7**) to 42.20% compared

with the untreated control group (100%), indicating marked cytotoxicity. Treatment with sodium valproate (1 mM) significantly improved cell viability to 84.95%. Similarly, Test ILQ (10 μ M) and ILQ (20 μ M) increased cell viability to 54.55% and 72.30%, respectively.

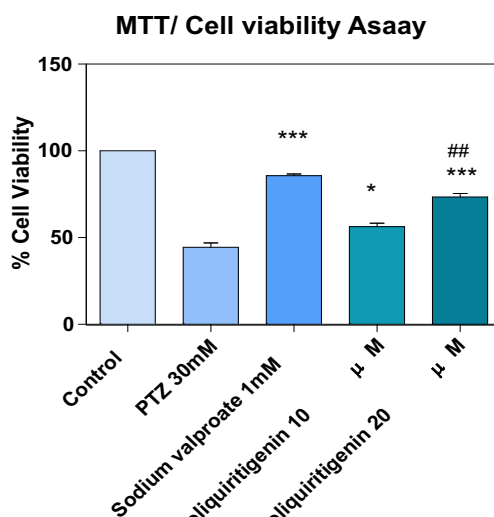


Fig. 7 MTT assay; Cell Viability in PTZ-Induced SH-SY5Y Cell, the values are expressed as mean $\pm$ SEM (n=4). Data was analysed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test * $p < 0.05$, *** $p < 0.001$ vs PTZ induced epileptic group; ## $p < 0.01$ vs standard (sodium valproate) treated group

3.4.2 Microscopic examination:

Microscopic examination of SH-SY5Y cells revealed distinct morphological alterations following PTZ exposure. Control cells exhibited normal neuronal morphology with adherent, elongated, and with long neurites possessing intact cellular structure (**Fig. 8a**). In contrast, PTZ-treated cells demonstrated pronounced cytotoxic changes characterized by reduced cell density, cellular shrinkage, neurite shortening and rounding of cells, indicating severe neuronal injury (**Fig. 8 b**).

Treatment with sodium valproate markedly attenuated PTZ-induced morphological damage and restored cellular integrity, as evidenced by increased cell density and preservation of normal cellular morphology (**Fig. 8 c**). Similarly, ILQ (10 μ M) treatment showed considerable neuroprotective effects with partial restoration of neurites restoration (**Fig. 8 d**) compared with the PTZ-treated group. ILQ (20 μ M) also demonstrated protective effects (**Fig. 8 e**); however, the degree of morphological recovery was comparatively more than that observed with ILQ (10 μ M).

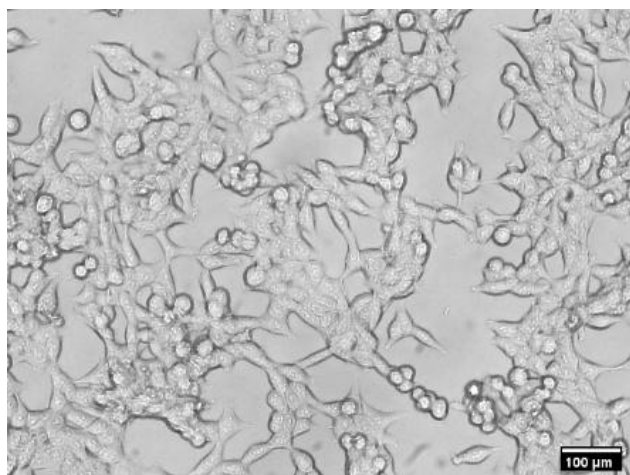


Fig. 8 a: Control

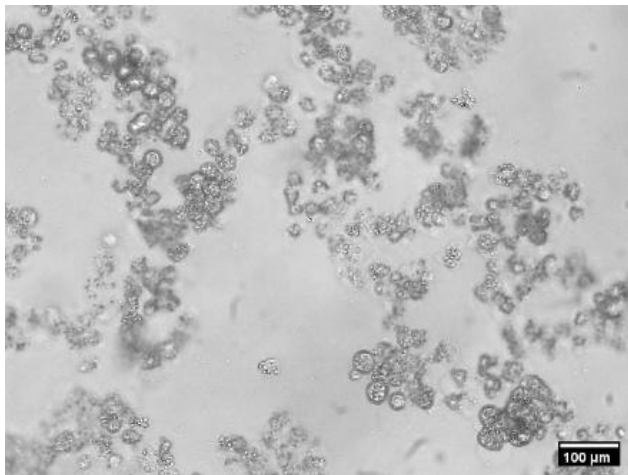


Fig.8 b: PTZ (30mM)

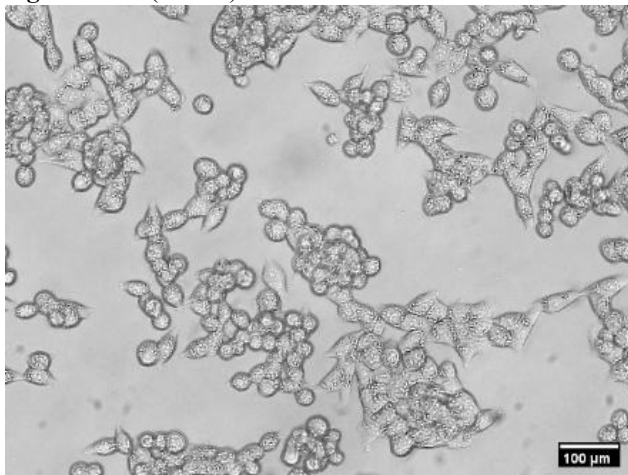


Fig.8 c: Sodium Valproate (1mM)

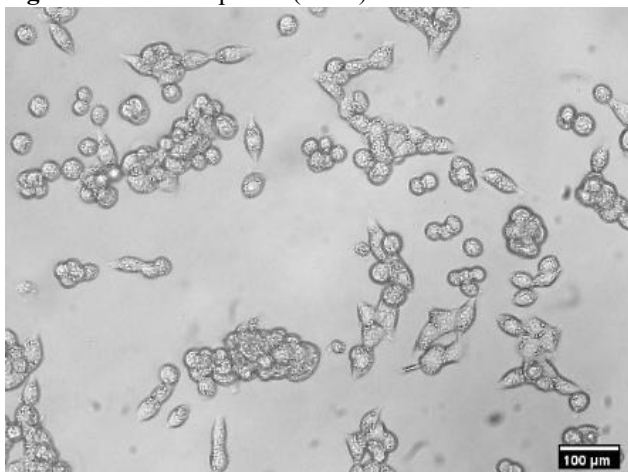


Fig.8 d: ILQ (10μM)

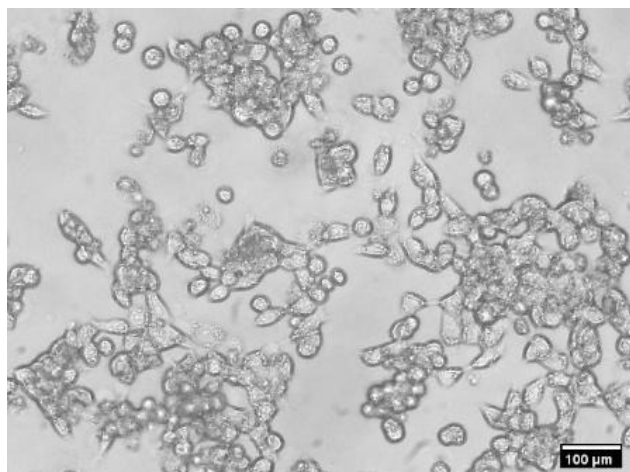


Fig.8 c: ILQ (20 μ M)

Fig. 8 Morphological evaluation of PTZ-induced SH-SY5Y cells following treatment with sodium valproate and test compounds using phase-contrast microscopy (20 $\times$)

A1: Untreated control: SH-SY5Y cells showing normal neuronal morphology with long neurites and intact cellular structure

A2: PTZ (30mM) treated cells exhibiting marked cytotoxicity, neurite shortening, cell shrinkage and reduced cellular density

A3: Sodium Valproate (1mM) treated cells showing restoration of cellular morphology and improved cell survival

A4: ILQ (10 μ M) treated cells demonstrating partial recovery of neuronal morphology and increased viable cell population

A5: ILQ (20 μ M) treated cells showing preserved neuronal morphology and interconnected neurites against PTZ-induced cytotoxicity

4. DISCUSSION

The present investigation demonstrates that ILQ exhibits significant antiepileptic activity in the PTZ-induced kindling model in mice, as evidenced by prolonged seizure latency, reduced seizure duration, decreased frequency of clonic-tonic seizures, and improvement in behavioral and motor coordination parameters. The observed effects were dose-dependent and comparable to the standard antiepileptic drug sodium valproate, supporting the therapeutic potential of ILQ in epilepsy management.

PTZ-induced kindling is a widely accepted experimental model that mimics progressive neuronal hyperexcitability and epileptogenesis observed in human epilepsy[18]. The pronounced reduction in seizure severity and frequency following ILQ treatment indicates its ability to suppress neuronal hyperexcitability and interrupt

epileptogenic progression. These findings align with previous studies reporting the neuroprotective and anti-inflammatory properties of ILQ in various neurological disorders [11].

It has been observed the significant elevation of brain mTOR levels in the PTZ-induced epileptic group and dose-dependent reduction following ILQ treatment. Thus, the anticonvulsant activity observed in the present study may be attributed to modulation of the mTOR signalling pathway, a critical regulator of neuronal growth, synaptic plasticity, and excitability. Dysregulation of mTOR signalling has been strongly implicated in epileptogenesis, and inhibition of this pathway has been shown to attenuate seizure development and progression [19]. Previous studies have demonstrated that ILQ suppresses the PI3K/Akt/mTOR pathway in several cancer models [9], suggesting that modulation of mTOR-associated processes may contribute to its pharmacological actions. Although the present findings demonstrate reduced brain mTOR protein levels following isoliquiritigenin treatment, the study evaluated total mTOR protein by ELISA and therefore does not establish direct inhibition of mTOR signaling. Additional mechanistic studies involving pathway-specific markers, such as phosphorylated mTOR and downstream signaling proteins, are required to clarify the molecular basis of these effects. Behavioural assessments further supported the neuroprotective profile of ILQ [20, 21]. Improvement in motor coordination and reduced motor impairment observed in the rotarod and

chimney tests indicate that the compound does not induce sedative or motor-depressant effects, which are commonly associated with several conventional antiepileptic drugs. This favourable behavioural profile highlights its potential advantage in long-term epilepsy management.

Furthermore, in cell line study, PTZ exposure markedly decreased SH-SY5Y cell viability, suggesting induction of neuronal toxicity and epileptiform cellular damage. Treatment with sodium valproate effectively restored cell viability, validating the experimental model [17]. Both dose of ILQ (10 μ M and 20 μ M) demonstrated dose dependent protective effects against PTZ-induced cytotoxicity, possibly through mechanisms associated with enhanced neuronal survival and attenuation of oxidative stress-mediated cellular injury.

Collectively, these findings suggest that ILQ exerts multifaceted neuroprotective and antiepileptic effects through modulation of mTOR signalling and stabilization of neuronal excitability. The present study provides strong preclinical evidence supporting ILQ as a promising therapeutic candidate for epilepsy. In conclusion, the findings of this study provide compelling preclinical evidence supporting the potential of ILQ as an effective antiepileptic agent. Further investigations involving molecular pathway validation are needed to establish its therapeutic applicability and translational value.

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Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval:

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of VIVA Institute of Pharmacy, Virar, Mumbai (IAEC/VIP/2023/July/P01) under Committee for Control and Supervision of Experiments on animals, Government of India. (Registration number: 2001/PO/Re/S/18/CCSEA).

Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Statements and Declarations:

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript. The authors have no relevant financial or non-financial interests to disclose.

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