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SHORT COMMUNICATION

Alleviating Potential of *Zingiber officinale* and Cow Urine Distillate Co-administered with Levetiracetam in Epileptic Rats: A Pharmacokinetic and Pharmacodynamics Approach

Nilay Solanki[1*], Harshal Patel[1], Milap Patel[1], Yamini Patel[1], Preetu Shukla[2], Jagdish Kakadiya[2], Rajesh Maheshwari[3] and Priyal Chauhan[1]

[1]Department of Pharmacology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT-Campus, Changa – 388421, Gujarat, India; nivyrx@gmail.com
[2]Department of Pharmacology, Parul Institute of Pharmacy and Research, Parul University, Vadodara – 391350, Gujarat, India
[3]Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Piparia, Vadodara – 391760, Gujarat, India

Abstract

Background: Epilepsy is a severe neurological condition that affects all ages of people. Complex pathways involved in pathogenesis make it complicated to treat; selected antiepileptic drug options are available for Epilepsy. There is a need for an hour to develop novel treatment approaches for epilepsy with lesser side effects. This research aimed to evaluate the alleviating role of bio-enhancers co-administered with levetiracetam for pilocarpine-induced epilepsy. **Methodology:** Pilocarpine (250 mg/kg) was used to develop epilepsy in rats. Levetiracetam (LEV) (140 mg/kg) was administered with *Zingiber officinale* Extract (ZOE) (15 mg/kg and 30 mg/kg) and Cow Urine Distillate (CUD) (1.5 ml/kg and 3 ml/kg). HPLC was used to evaluate drug concentration in blood. Serum nitrate, catalase, CRP, calcium level and calcium level of the brain, behavioural markers in rats were assessed and compared with the Leviteracetam group only. **Result and Discussion:** The present study showed that combining ZOE and CUD with levetiracetam was advantageous through substantial reduction ($p < 0.05$) in serum nitrate, CRP and increased catalase ($p < 0.05$), while reduced serum calcium compared to LEV alone. Combination of ZOE and CUD with levetiracetam treatment also reduced seizure behaviour and duration in rats. The bioavailability of LEV in plasma and brain was increased when epileptic rats were treated with LEV plus ZOE and CUD compared to disease control. **Conclusion:** Utilization of *Zingiber officinale* and CUD in combination with LEV was proven therapeutically effective in the epileptic model and used to lower the dose of LEV along with reducing seizure behaviour and time with the potential for the treatment of epilepsy.

Keywords: Catalase and Nitrate, Cow Urine Distillate, Epilepsy, Levetiracetam, *Zingiber officinale* Extract

1. Introduction

Epilepsy is a central nervous system disease characterized by recurrent seizures, where partial or generalized involuntary movements can cause loss of consciousness and bowel or bladder control. Epilepsy is believed to be one of the conditions closely linked to dramatic lifestyle behavioural and social outcomes. The last ten years of the 20th century are named the "decade of the brain" in neuroscience. This neurological illness

*Author for correspondence

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affects 50 million individuals worldwide. Nearly 80% of epilepsy patients live in low-income nations. If properly diagnosed and treated, 70% of epileptics may live seizure-free[1]. Seizure management therapy has been provided by traditional Anti-Epileptic Medicines (AEDs) to epileptic patients but these medicines don't improve the well-being of all patients because of their absence of efficacy and side effects. This downside leads to pragmatic treatment for the use of natural bio-enhancers with traditional AEDs. The increased bioavailability approach eventually leads to increased drug concentrations at the site of action, improving its effectiveness and thereby sequentially reducing the medicinal dose required to achieve a therapeutic effect. As bio-enhancers, several natural products such as aloe, quercetin, glycyrrhizin, *Zingiber officinale* Extract, caraway, curcumin etc., have been demonstrated. Herbal products are the most effective to increase different medicines' bioavailability as they are safe, non-toxic, easily available, non-addictive, inert pharmacologically and non-allergenic, etc[2]. Few studies indicated that *Zingiber officinale* extract and cow urine distillate have substantial bio-enhancement potential with many drugs[3,4]. The present study explores the effectiveness of a combination treatment of natural bio-enhancers with traditional AED (Levetiracetam) induced by pilocarpine. Status Epilepticus (SE) is a most prevalent, life-threatening neurologic condition associated with abnormal and prolonged seizure activity (at least 5 min). If SE persists for more than 30 minutes, significant long-term repercussions may arise[5]. This model has provided details concerning the neurochemical and behavioural characteristics of the seizure activity. The rationale for selecting *Zingiber officinale* was due to its significance in enhancing brain cell protection when combined with antiepileptics, while simultaneously reducing its dosage[6]. In numerous epilepsy models, cow urine combined with antiepileptic drugs demonstrated potentially beneficial effects[7].

2. Aim

The objective of this research was to evaluate the effect of bio-enhancers in the pilocarpine-induced epilepsy model co-administered with levetiracetam.

3. Materials and Methods

3.1 Drug and Chemicals

Pilocarpine hydrochloride and *Zingiber officinale* Extract were purchased from Sigma (St. Louis, MO, USA). The gratis sample of levetiracetam was provided by Shashun Pharmaceutical for research purposes. The C-reactive protein assay kits and serum calcium content were purchased from Spanish Diagnostics. HPLC-grade acetonitrile and methanol were used for bioavailability tests.

3.2 Preclinical Protocol

In the present study, Wistar rats of both sexes weighing 200-350 grams were used. Rat acclimatization duration was permitted for 2 weeks at the animal house facility of the Ramanbhai Patel College of Pharmacy. They were kept in polypropylene cages with the regular laboratory conditions as per Committee for the Control and Supervision of Experiments on Animal (CCSEA) guidelines, with a light-dark cycle of 12-12 hours (21°C ± 1°C and 50% humidity) as well as free access to a standard rat pellet diet and *ad libitum* water. The experimental protocol has been approved by the Institutional Animals Ethics Committee (RPCP/IAEC/2014-2015/MPH-CT-002) with CPCSEA registered animal house facility (940/PO/Re/S/06/CPCSEA).

3.2.1 Induction of Pilocarpine Seizures

A freshly formulated solution of pilocarpine hydrochloride (200 mg/kg i.p.) was administered to animals. After which behavioural seizures typically started in 10-20 minutes. Levetiracetam (LEV) dissolved in saline (140mg/kg oral) was administered 30 minutes before pilocarpine injection for the treatment of seizures[8]. *Zingiber officinale* Extract and cow urine distillate were used as bio enhancers along with low and high-dose therapy.

3.2.2 Experimental Groups

Forty-two animals were divided into 7 classes (n = 6). Group I: (normal control): saline-treated; Group II (disease control): saline and pilocarpine; Group III: LEV treated; Group IV: LEV + *Zingiber officinale* Extract (15 mg/kg) (GE-15); Group V: LEV + *Zingiber officinale*

Extract (30 mg/kg) treated (GE-30); Group VI: LEV + cow Urine distillate (1.5 ml/kg) treated (CUD-1.5), Group VII: LEV + Cow Urine Distillate treated (3 ml/kg) (CUD-3). Upon administration of pilocarpine, the activity of rats has been closely monitored for about 5 hours to determine initial seizure duration, epileptic status, frequency and mortality. Scale 0-face clonus, behavioural detention, lifting of ears, rapid respiration; Scale 1-head nodding, mouth movement (lips and tongue), salivation; Scale 2-head and eye clonus; Scale 3-front clone, wet dog shakes; Scale 4-clone rearing; Scale 5-clonic rearing, with body function losses. In addition, the seizure time and length of each animal are calculated for animals[9]. Once the seizures were started care was taken for 30 minutes period, the animals were anaesthetized and up to 1 ml of blood was collected from a retro-orbital plexus, and the serum was separated and stored in deep freeze.

3.3 Parameter Estimation

3.3.1 Determination of Serum Nitrite (Oxidative Stress)

The nitric acid content was deviously determined by Griess reaction-based methodology as a nitrite/nitrate concentration. The serum and reagent mixture were incubated at 370 and absorption was taken with UV at 540 nm.

3.3.2 Serum Calcium Determination

As described in the serum calcium package, calcium was estimated. Absorbance with spectrophotometer UV was measured at 578 nm.

3.3.3 Serum C-Reactive Protein (CRP) Determination (Inflammation)

Inflammation due to epilepsy was indirectly quantified with the C-Reactive Protein method listed in the package. A UV was used to assess the complex formed by the reagents and the absorbance at a 550 nm spectrophotometric meter. Absorbance at 60 and 120 seconds was calculated.

3.3.4 Serum Catalase Determination (Antioxidant Activity)

The degree of CAT was calculated by kinetic UV Spectroscopy. Blanking 100 µl DDW and 2.9 ml PB of the analyzer, adding 100 µl serum sample and 2.9 ml PB to the test. After this stage, set the wavelength of 240 nm, then add 50 µl H_2O_2 and autozero for the test reading, add 50 µl H_2O_2 just before observing the absorbance at 240 nm for the test sample, continuously read absorbance for up to 3 minutes every 30 seconds. Rate of catalase expressed in µmol H_2O_2 used per protein min/mg[10].

3.4 Pharmacokinetic Analysis

Thirty animals were divided into five groups: (I) Levetiracetam (LEV) - 140 mg/kg; (II) *Zingiber officinale* Extract (GE-15) - 15 mg/kg; (III) *Zingiber officinale* Extract (GE-30) - 30 mg/kg; (IV) Cow Urine Distillation (CUD- 1.5) - 1.5 ml/kg; (V) Cow Urine Distillation (CUD- 3) - 3 ml/kg.

A blood sample from the retro-orbital vein was obtained at 0, 1 and 2 hours to determine the concentration of the drug in plasma. After 2 hours, rats were euthanized and the brain tissue was removed. 10% homogenous brain was prepared in ice cold saline with a tissue homogenizer. The concentration of the drug was determined by the injection in HPLC of plasma and brain homogenate.

3.4.1 HPLC Method for LEV

HPLC method for measurement of LEV concentration was performed as per the established method under optimized conditions (Table 1)[11,12].

Table 1. Optimized HPLC condition

Mobile Phase	Solution A: Acetonitrile (96:04)
Pump Mode	Binary
Diluent	Solution A
Column	Chromosil C18 column (250 X 4.6 mm, 5µ)
Column Temp	Ambient
Wavelength	205 nm
Injection Volume`	10µl
Flow rate	0.9 ml/min
Run time	10 min
Retention time	7.99 min

3.5 Statistical Analysis

The data were analyzed by MS Excel with ANOVA-single factor analysis. All the results were expressed as means ± SEM and the significant difference between the groups were considered as $p < 0.05$, 0.01.

4. Results

This research demonstrated that after 5 min of Pilocarpine (PC) injection into animals, cholinergic symptoms occur, including piloerection, diarrhoea, and mild tremors followed by seizures. Also, behavioural alteration was developed by disease control animals, inclusive of masticatory movements, bradykinesia, salivation, tremor, and SE seizure[13]. SE developed after 30 min of PC injection and caused signs that include intermittent forelimb and hind limb clonus, hyperextension of the tail, head bobbing, and hind limb along with loss of pose that was continuous for over 50 min throughout the 24 h observation period. Bioenhancers with LEV-treated animals showed a lesser number of scores compared to LEV-treated animals alone. Particularly high doses of GE and COD have a lesser prevalence of chronic seizure. The duration of seizure activity in the disease control group was 382 min, which was longer compared to the treated groups.

It was observed that pilocarpine-induced seizures trigger the stimulation of the metabolism of nitric oxide, which evidenced interaction with glutamate receptors to create part of its stimulatory action on the CNS. Significant differences in the serum nitrite levels were observed and illustrated in Table 2 for diseased rats compared to normal ($p < 0.05$), while treatment with LEV and bio-enhancers showed a reduction in serum nitrite levels ($p < 0.05$) in epileptic rats compared to disease control.

In our study, a high level of calcium (excitotoxicity) was found in disease control, while the treatment with LEV and bio-enhancers showed a decrease in the level of calcium (serum and brain) as compared to disease control (Table 2).

There is a significant reduction in catalase enzyme level ($p < 0.05$) in disease rats. The use of *Zingiber officinale* Extract and CUD with levetiracetam significantly

Table 2. Serum parameters different experimental groups

Groups	Normal Control	Disease control	LEV STD	LEV + GE-15	LEV + GE-30	LEV + CUD-1.5	LEV + CUD-3
Serum Nitrite (mmol/l)	2.247±0.22	5.57±0.44 ##	1.7±0.13	1.266±0.17 *	1.567±0.09*	1.059±0.08*	1.277±0.19*
Serum calcium (mg/dl)	7.777±3.31	31.49±0.89 ##	11.532±1.43	9.755±1.28 *	10.537±1.0 *	8.178±0.94 *	11.135±1.09 *
Serum CRP (mg/dl)	7.910±0.42	21.382±0.75 ##	18.397±0.30	16.277±0.96 **	11.293±0.29 **	14.874±1.26 **	11.065±0.39 **
Serum catalase (unit/ml)	4.77±0.23	2.89±0.58 ##	4.333±0.33	5.333±0.66 *	5.666±0.33*	5.333±0.33*	5.666±0.57*

(Results were analyzed using One way ANOVA method for statistical significance)

* P<0.05 LEV+GE and LEV + CUD treatment is significantly different from Standard (LEV) only for serum nitrite levels

P<0.0013 highly significant difference between disease control and normal control groups for serum nitrite levels

* P>0.05 LEV+GE and LEV + CUD treatment is non-significantly different from Standard (LEV) only for serum calcium levels

# P<0.0005 significant difference between disease control and normal control groups for serum Calcium levels

** P<0.00001 LEV+GE and LEV + CUD treatment is highly significantly different from Standard (LEV) only for serum CRP levels

P<0.0013 highly significant difference between disease control and normal control groups for serum CRP levels

* P<0.05 LEV+GE and LEV + CUD treatment is significantly different from Standard (LEV) only for serum Catalase levels

# P<0.05 significant different between disease control and normal control groups for serum Catalase levels

increased the catalase level; here, CUD was found to be more effective than other treatments (Table 2).

In our study, we observed that the serum CRP concentration ($p < 0.05$) was increased in the disease state, while the *Zingiber officinale* Extract and CUD with LEV treatment showed a significant decrease in the level of CRP. In this study, *Zingiber officinale* Extract proved better for reducing the symptoms of epilepsy (Table 2).

The concentration of LEV in plasma was decreasing concerning time (Figure 3), and after 2 hr, brain homogenate was evaluated for drug content, which showed a higher concentration of LEV in rat brains compared to rat plasma (Figure 4). The brain concentration of LEV was highest in animals treated with high-dose *Zingiber officinale* Extract (30 mg/kg) compared to animals treated with LEV alone or with CUD. Bioavailability was improved in cases of treatment with the use of bio-enhancers, where the concentration of the drug in plasma and brain homogenates was found to be high as compared to LEV (Figure 4).

5. Discussion

The score for seizure behaviour was high in the disease state but went down after treatment (Figure 1). This shows that seizure behaviour was common in the disease state. As was already said, the Racine scale was used to describe the steps of a seizure[13]. Our results showed that a high amount of *Zingiber officinale* Extract cut the length of seizures (Figure 2). This means that, in general, seizures were worse and more common in diseases than in other treatments. Death of neurons and seizures are both caused by oxidative stress and problems with the mitochondria. Recent research has shown that mitochondrial failure and chronic reactive stress, both of which cause the death of neurons and seizures, are linked[14]. The fact that the level of nitrate in the adult rats went up after seizures and SE caused by pilocarpine suggested that the level of ROS may have gone up, which could be part of how seizures damage neurons. Nitric oxide level controls some important biological signals in several bodily processes, such as neurotransmission, blood pressure regulation, body defence mechanisms, smooth muscle relaxation, and immune regulation[15]. It was found that seizures caused by pilocarpine cause the breakdown of nitric oxide to speed up. This showed that nitric oxide interacts with glutamate receptors to make part of its stimulating effect on the CNS. The amounts of nitrite and nitrate in the blood, which are both made from nitric oxide, were very different. Treatment with LEV and bio-enhancers was better than disease control at lowering blood nitrite levels in epileptic animals. This suggests that nitro active stress may have been reduced since nitro active stress causes neural damage, which causes seizures. In this case, it was found that the extract of *Zingiber officinale* worked better as a bioenhancer. Some NO inhibitors, like NG-nitro-L-arginine and NG-nitro-L-arginine methyl ester, which are good for treating epilepsy, may be released during treatment[16]. A high level of calcium was found in epileptic rats, which triggers a sequence of events that include the activation of nitric oxide synthase enzyme, ultimately interfering with oxidative metabolism and generating free radicals that finally damage the neuronal membrane. Excitotoxic neuronal death is correlated with status epilepticus. Depolarisation and action potential generation depend on Ca^{2+}, Na^{+}, and K^{+} ion channels array. An increase in K^{+} influx (repolarisation), leads to control in an influx of calcium and thence reduces glutamate release[17,18].

Significant lowering in catalase enzyme in epilepsy was observed in the present study, due to which there is the rise of oxidative stress. The utilization of bio-enhancers significantly increased the catalase level which ultimately reduces oxidative stress, which is one of the mechanisms of seizure development in epileptic models[19]. Catalase is a very important enzyme in protecting the cell from oxidative damage by counteracting the negative effects of oxygen stress in the status epilepticus[20].

Serum inflammation markers like interleukin-6, fibrinogen, and CRP rise in people who have seizures caused by Pilocarpine. This causes secondary damage to the brain and makes it more likely that the person will have more seizures[21]. Higher levels of CRP in the brains of rats with epilepsy cause early inflammation, while CRP levels dropped significantly in the treatment groups. This is likely because CRP is broken down by a CYP450-dependent process[22].

Figure 3 showed that the amount of LEV in plasma is going down with time. Figure 4 showed that the amount of LEV in rat brain is higher than in rat plasma.

The most LEV was found in the brain tissue in case of high dose levels of GE (30mg/kg) compared to animals given LEV alone or CUD. When bio-enhancers were used

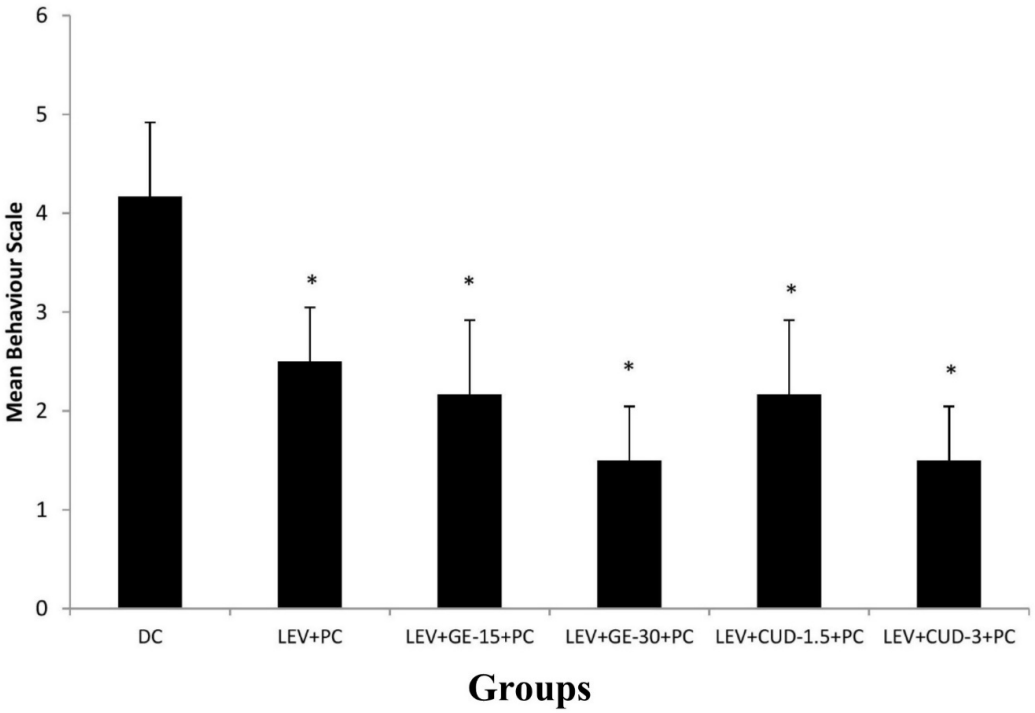


Figure 1. Mean seizure behaviour scale; *p < 0.05 compared to disease control group.

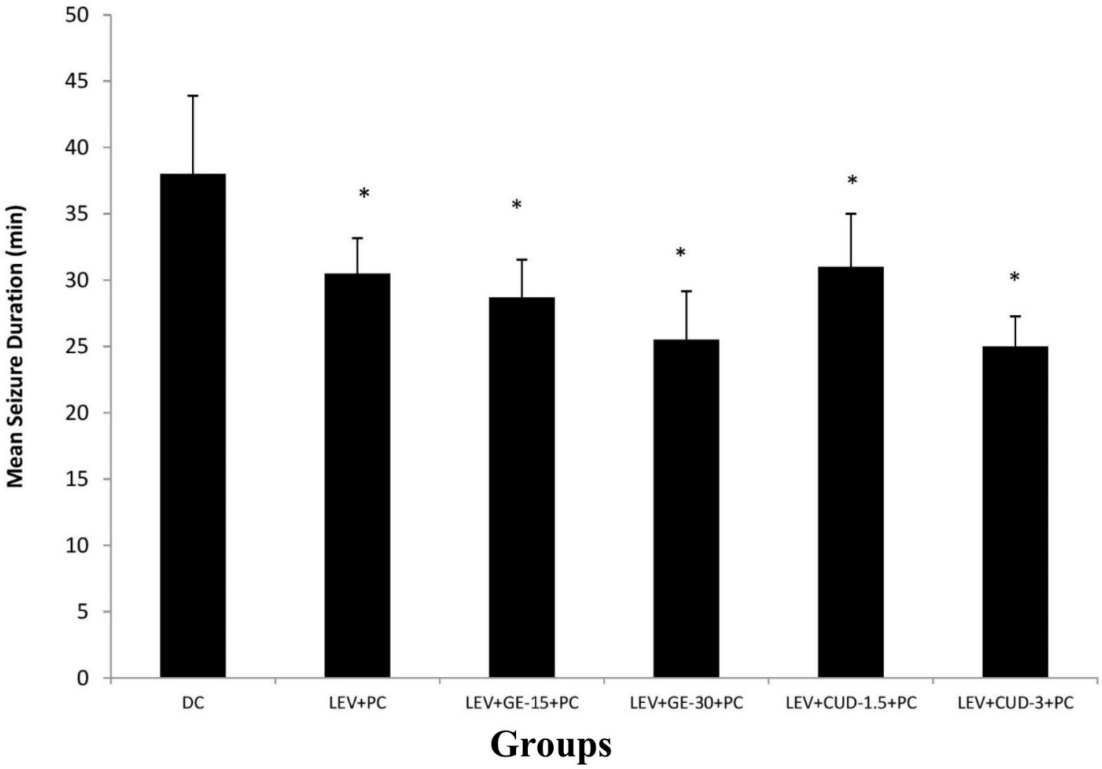


Figure 2. Mean seizure duration (min); *p < 0.05 compared to disease control group.

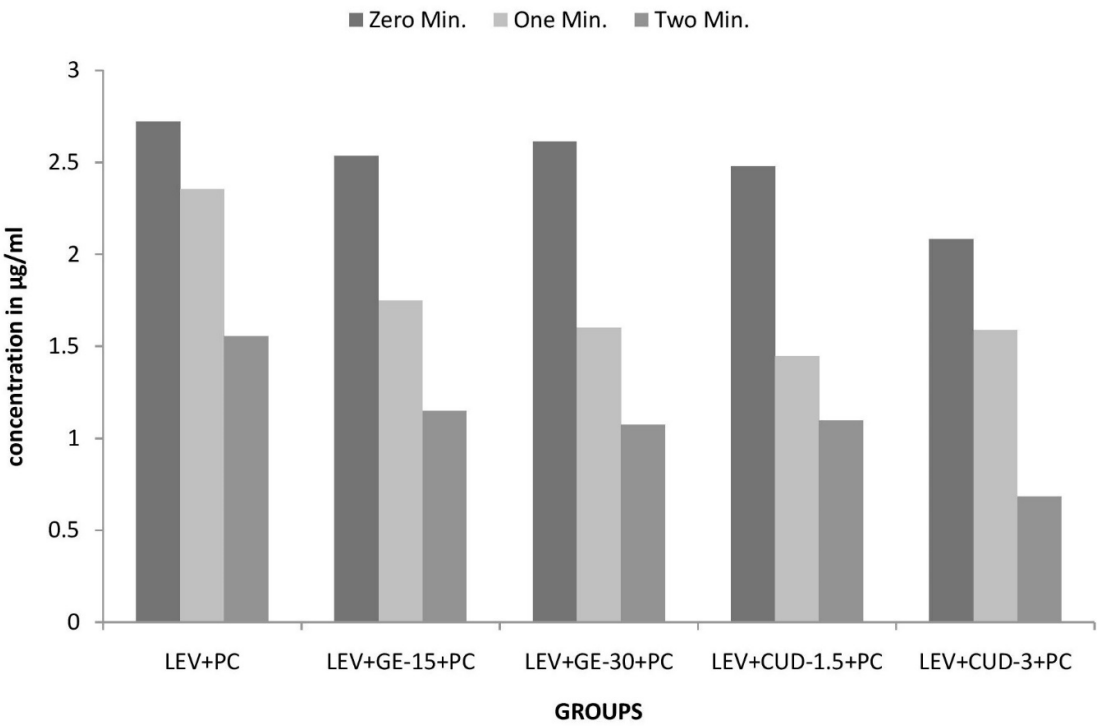
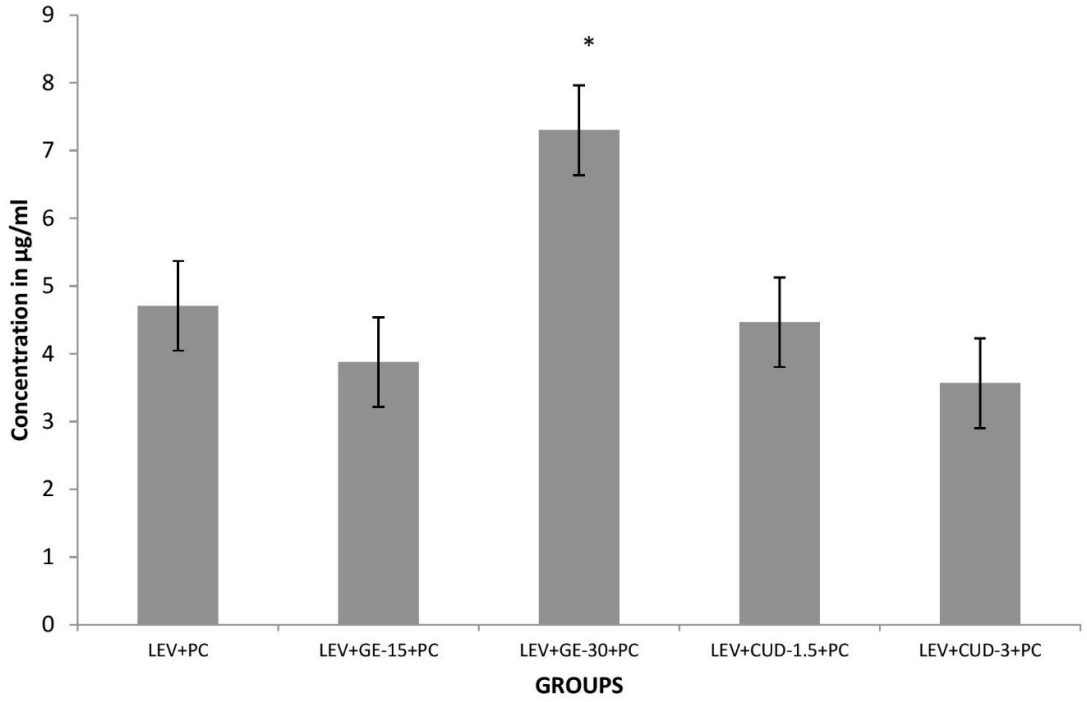


Figure 3. Concentration of LEV in plasma at different time interval.



* p<0.05 significant difference in LEV+GE-30+PC compared to LEV+PC

Figure 4. Concentration of LEV in brain homogenate.

to treat a patient, it was found that the percentage of the drug in plasma and brain homogenates was higher than in LEV.

Based on the results, a combination of *Zingiber officinale* extract with LEV showed potential anticonvulsant effects of LEV with the reduced dose, probable mechanistic view with a significant increase in catalase and thus reduced oxidative stress level and decline in nitric oxide and calcium with indirect calcium channel inhibition, declined seizure behaviour and duration in high dose of GE; while CUD combined with LEV showed better seizure reducing potential by reducing nitrosative oxidative stress and reduced calcium signalling and reduced seizure behaviour and duration in high dose of CUD in epileptic animals with improved bioavailability.

6. Conclusion

The combination of bio-enhancers with LEV facilitated the protection against status epilepticus, reduced the seizure duration and provided better therapeutic efficacy. From this *in vivo* pharmacokinetic study, it was concluded that LEV concentration in the brain was found to be higher than the plasma level. The results of this study suggested a high dose of *Zingiber officinale* Extract produced higher bioavailability of LEV in the brain. The overall therapeutic effect of LEV in combination with *Zingiber officinale* Extract and CUD was observed therapeutically beneficial by significantly reducing oxidative, nitrosative stress, and inflammation markers and showed improvement in behavioural score compared to LEV alone on status epilepticus. The effectiveness of *Zingiber officinale* Extract and CUD in combination with LEV indicated its synergistic role in the modern medicine era for the treatment and management of epilepsy. This study was conducted in animals, further clinical study may give more insight into the utilization of these bio enhancers in combination with modern medicine for the treatment of Epilepsy.

7. Acknowledgement

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