Ursolic acid along with Caprylic acid Ameliorates Pentylenetetrazole Induced Seizures Like Behavior in Adult Zebrafish

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Research Article

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Abstract

Background: Epilepsy is a neurological disorder characterized by recurrent unprovoked seizures. Despite the existence of more than 20 anti-epileptic drugs there is still a need for new treatments which could not only treat symptoms but also cure epileptogenesis. PTZ is a Chemoconvulsant that impair GABAergic and glutamatergic neurotransmission, promoting excitotoxicity and seizures. Therefore PTZ exposure has been considered a suitable protocol to assess seizure-like behaviours. UA is a triterpenoid compound having anti-inflammatory, anti-oxidant and neuro protective action. CA is one of the component of ketogenic diet and it supresses the inflammation.

Objective: The objective of the present study was to investigate the effect of combination therapy of UA and CA on seizures, neuronal damage and inflammation induced by PTZ in adult zebrafish.

Materials and method: Zebrafishes were pretreated with Diazepam (1.25 mg/kg), UA(50 and 150 mg/kg), CA (60 mg/kg) and combination of UA (50 mg/kg) and CA (60 mg/kg) followed by PTZ (170 mg/kg) *i.p* administration. Various neurobehavioral, biochemical parameters like lipid peroxidase (LPO), catalase, superoxide dismutase, glutathione-s-transferase (GSH) and acetylcholinesterase (AChEs), molecular parameters such as TNF-α, IL-10, Nrf-2 and IL-1β and mitochondrial parameters like complex I, II, IV and MTT assay, histopathological study were performed to determine the effect of therapy.

Result: UA of both doses and CA decreased mean seizure score, mean seizure time . Importantly, combination of UA 50 mg/kg and CA 60 mg/kg attenuated seizure-like behavioral scores, decreased mean seizure time, mean seizure score and reduced the frequency of clonic-like seizures (score 4). Combination of UA 50 mg/kg and CA 60 mg/kg also prevented oxidative stress in PTZ-challenged fish by decreasing lipid peroxidation, acetyl cholinesterase activity and increasing catalase, glutathione-s-transferase and superoxide dismutase levels. Additionally, the combination therapy prevented inflammatory response by declining TNF-α and IL-1β levels and raising IL-10 and Nrf-2 levels. Moreover combination of UA 50 mg/kg and CA 60 mg/kg significantly improved mitochondrial complex I, II and IV activity as well as increase MTT assay. Furthermore, morphology of neuronal cell was prevented in combination of UA 50 mg/kg and CA 60 mg/kg when seen in histopathology. Similarly as observed in DZP group, combination of UA 50 mg/kg and CA 60 mg/kg affect the overall swimming activity of fish, suggesting different mechanisms of action. Collectively, we show that combination of UA 50 mg/kg and CA 60 mg/kg attenuates PTZ-induced seizure-like behaviours, brain oxidative stress, mitochondrial and morphological damage of neuronal cell in zebrafish, suggesting the involvement of antioxidant mechanisms in neuroprotection.

Conclusion: The present study shows that combination of UA 50 mg/kg and CA 60 mg/kg ameliorates the seizures completely and have neuroprotective action via their anti-inflammatory, anti-oxidant properties.

Highlights

- A single injection of PTZ 170 mg/kg produces acute seizures like behavior in adult zebrafish.

- UA and CA exhibit anti-seizure activity.
- UA and CA were reported to reduce inflammation and oxidative stress.
- Combination of UA and CA demonstrated an anti-inflammatory effect via downregulating several inflammatory markers (TNF- α and IL-1β) in the epileptic brain.
- UA and CA in combination prevents mitochondrial enzymes activity dysfunction.

1. Introduction

Epilepsy is a most common central nervous system disorder affecting 70 million people characterized by recurrent seizures, abnormal brain activity, periods of unusual behavior disorder (1). Loss of awareness or consciousness along with disturbances in movement, mood, sensation (including taste, hearing and vision) or other cognitive functions are temporary symptoms (2). Etiologies behind epilepsy are environmental factors, lesions in the brain, traumatic brain injury, brain tumors, brain infection like meningitis, heart stroke, biochemical and physiological defects like the imbalance between excitatory and inhibitory neurotransmitters. Despite tremendous progress in understanding the molecular processes of epileptogenesis and the launch of more than 20 novel antiseizure medicines (ASDs) since 1993, 30% of patients remain resistant to existing treatment choices. The significance of neuroinflammation blood brain barrier (BBB) breakdown, oxidative stress, and neuronal death in epileptic seizures is becoming more well accepted. Inflammatory events that result in the release of proinflammatory cytokines have a role in activating apoptotic events that lead to neuronal death, which contributes to the occurrence of seizures. Experimental findings reveal that during seizures, reactive oxygen species (ROS) are overproduced in brain tissue, while natural antioxidant molecules are inactivated. As an outcome, anticonvulsant therapy techniques that can arrest illness development and overcome the limitations of currently existing AEDs are urgently needed.

Despite the fact that there are now a plethora of rodent models of various epilepsies and epilepsy syndromes, the high expense of breeding and regulatory restrictions on rodent experiments limit their usage in drug screening (3).The zebrafish (Danio rerio) has a number of characteristics that make it a useful model for neuroscience. Simple breeding and maintenance requirements, high fertility, quick outward development, and a variety of approaches for cheaply creating genetically modified strains are all aspects that make zebrafish an appealing study model (4). Zebrafish can be studied at all stages of development and several methods are available for the manipulation of genes in zebrafish(5). Another reason is that 70% of zebrafish genes have one or more human orthologues, but only 47% of human genes have one-to-one orthologues, a zebrafish gene that has a similar (6). The zebrafish model has the benefits of similarity to seizure-like behaviors as humans making it ideal for preclinical epilepsy research (7).

One of the earliest proconvulsant medications used in animal models to elicit seizure activity was pentylenetetrazole (PTZ). PTZ interfere with GABAergic neurotransmission. The interaction of Gaba-aminobutyric acid (GABA) with both ionotropic and metabotropic membrane receptors mediates a rapid inhibitory synaptic transmission (8). This interaction occurs in the inflow of negatively charged chloride ions, which contributes to quick neuronal hyperpolarization in the case of the ligand-gated ion channel

GABA-A receptor. This characteristic might explain the proconvulsant action that results by increasing neuronal excitation (9).

Plants and their extracts have been utilised as treatments for a variety of ailments throughout history. In our study we have used combination therapy of UA and CA. UA is a triterpenoid compound, 3β-hydroxy-12-ursen-28-ic acid. It is widespread distributed in nature, especially in herbs like basil, rosemary, and sage, as well as common fruits like apples and pears, UA has a wide range of pharmacological effects such as anticancer, anti-obesity (10), prevent neurodegeneration (11), and anti-inflammatory properties. CA is a small component of coconut oil and palm kernel oil and is found naturally in the milk of several animals (12). It is the primary fatty acid component of medium-chain triglycerides in ketogenic diets, as it is a medium-chain saturated fatty acid (MCFA) (13). Additionally, CA a straight chain isomer of a well-known antiepileptic medication Valproic acid (14). For the first time we are investigating the effect of UA and CA combination as antiepileptic drug in zebrafish.

2. Materials And Method

2.1 Animals

Adult zebrafish (Danio rerio) 3 to 6 months old of either sex was purchased from Aquarts, 26B K Komedanbagan alley, Kolkata, India. The fishes were housed in aquariums at 28°C with a 10/14-hour dark/light cycle. The pH of the system water between 6.8 and 7.1 was maintained. Tetrabits total bioactive formula fish food Flakes were given to the fish twice daily.

2.2 Drugs and Chemicals

TCI provided PTZ and CA Hydrate. UA was purchased from Yarrow Pharma. Diazepam is bought from TCI. DMSO was purchased from HI-Media. AChI, DTNB and were purchased from Sigma Aldrich. All additional chemicals for biochemical estimation were acquired from Sigma Aldrich, however only the analytical grade chemicals were used and they were all freshly prepared before use. Protocol has been approved by Institutional Biosafety Committee (IBSC) with protocol no. ISFCP/IBSC/M1/2021/14.

2.3 Study Design

Zebrafish were separated in a 1L tank with appropriate aeration and temperature before the procedure takes place. Zebrafishes were employed and the animals were separated into different groups as indicated in Table 1. Before PTZ exposure, animals were pretreated with UA (150 mg/kg i.e. high dose and 50 mg/kg i.e. low dose) (15) ; CA (60mg/kg) (16) these concentrations were chosen on the basis of previous reports which shows positive effects. To investigate the effects of combination therapy, UA 50 mg/kg and CA were administered in zebrafish within the interval of 20 mins. As a positive control, another cohort was exposed to 1.25 mg/kg DZP for 30 min, while the vehicle control group received 10% DMSO v/v. The induction of seizure-like behaviours was further performed by i.p injection of PTZ 170 mg/kg. Detailed experimental protocol is shown in figureure 1.

Table 1
Animal grouping

S. no	Groups	Dose and route	No. of animals
1	Vehicle control	DMSO 10% v/v	20
2	Per-se	1. DZP (1.25 mg/kg) 2. UA (150 mg/kg) 3. CA (60 mg/kg)	20*3
3	Toxin	PTZ (170 mg/kg)	20
4	Treatment	1. DZP + PTZ 2. UA (50 mg/kg) + PTZ 3. UA (150 mg/kg) + PTZ 4. CA (60 mg/kg) + PTZ	20*4
5	Combination	UA (50 mg/kg) + CA (60 mg/kg) + PTZ	20
	TOTAL		200

2.4 Procedure for a Zebrafish Anaesthesia and Intraperitoneal Injection

PTZ and UA were injected intraperitoneally to the zebrafish according to the Kundap et al. technique. Various intraperitoneal injections were delivered at alternating lateral ends instead of the midline between the pelvic fins when different intraperitoneal infusions were necessary. Each zebrafish was trapped separately with a fish holding net and then immersed in a tricaine MS-222 solution of 100 mg/L for anaesthetic preparation. The fish were submerged in anaesthetic water for 30 seconds or until they stopped moving. Once anaesthetized, the zebrafish were removed and weighed to determine the dosage and, ultimately, the infusion volume. A fragile sponge, about 20 mm tall, was soaked in water and placed in a 60 mm Petri plate. To regulate and keep the fish for the intraperitoneal infusion, an incision between 10 and 15 mm was created in the sponge. The intraperitoneal infusion was delivered under a dissecting microscope by inserting the needle between the pelvic fins in the midline. After that, an adequate volume was injected into the zebrafish, taking into account the zebrafish's body weight. Using a 10µl Hamilton syringe, all intraperitoneal infusions were delivered into the stomach pit, midline to the pelvic fins (700 series, Hamilton 80400). The experiment was conducted in a separate behaviour room with a temperature of 26 to 30 degrees Celsius and a humidity of 50 to 60%. To minimise any novel tank reaction, all zebrafish were acclimatised in the mentioned behaviour room for 2 hours before to the experiment. In addition, a tiny injection volume of 10L per gramme of fish was used, as well as a 35-gauge needle. In zebrafish, this intraperitoneal injection approach was proven to be efficacious and did not result in any death during the experiment. The zebrafish were immediately transported to an observation tank after receiving the intraperitoneal injection (6).

2.5 PTZ mediated seizures and other behaviours in adult zebrafish

2.5.1 Seizures in zebrafish

All the animals were pretreated with UA 50, UA 150 and UA 50 + CA and then injected with PTZ. The seizure intensity were evaluated using a specific scoring system according to a technique previously described by Kundap et al 2017 and are as Score 1 - Short swim mainly at the bottom of the tank Score 2 - Increased swimming activity and high frequency of opercular movement Score 3 - Burst swimming, left and right movements as well as erratic movements Score 4- Circular movements (17, 18). The mean seizure time, mean seizure score and frequency of score 4 were recorded for 10 minutes and analysed by ANY-maze™ software (Stoelting CO, USA) at 30 frames/s. After evaluating the behaviour in observational tank the fishes were transferred in novel tank diving apparatus followed by open field test for other behavioral parameter assessment.

2.5.2 Novel tank diving test

The 1.5 L trapezoidal tank was utilised to assess anxiety and depression forms of behaviour in zebrafish. Using a marker on the exterior walls the tank was split into two equal virtual horizontal sections. The top part of tank is favourable zone for fish thus the fish tends to spend maximum time there. The test lasted 5 minutes. We looked at time spent in the top zone (TSTZ) and time spent in bottom zone (TSBZ) and the number of entries in the top zone during the experiment (19).

2.5.3 Open Field Test

The open field test is used to evaluate zebrafish swimming patterns and locomotion. The open field arena was a 5 litre cuboidal tank. This test is also used to assess zebrafish exploratory behaviour; typically, fish prefer to swim and explore the entire apparatus that is filled with water, but unwell zebrafish do not swim properly and vary from their stereotype behaviour (20). The test lasts a total of 5 minutes. In this we measured the distance travelled (m) by zebrafish in the test arena and average swimming speed (m/sec).

2.6 Biochemical Estimation

2.6.1 Protein Estimation

The biuret technique was employed to estimate protein, and bovine serum albumin was utilised as a standard. 2.9 mL NaCl and 3 mL working biuret reagent were added to 0.1 mL homogenate, and absorbance was measured at 540 nm using a Shimadzu spectrophotometer. The amount of protein in each sample was then estimated using a standard and represented in milligramme (21).

2.6.2 LPO assay

LPO levels were determined using TBARS and the MDA colour reaction, as described by Wills et al 1965. Tissues were homogenised, 0.1M Tris HCl was added to the homogenate then test tubes containing samples were set for 2 hours incubation at 37□ C. Ice cold 10%w/v TCA was added and then centrifuged at

12000 rpm for 10 mins, supernatant was pipette out then 0.067% TBA was added the centrifuged tubes were kept in hot water for 10 minutes, and a Shimadzu UV Spectrophotometer was used to measure the amount of malondialdehyde (MDA) through a reaction with TBA at 532 nm. The results were measured as moles of MDA/mg protein (22).

2.6.3 AChEs assay

According to Ellman's method 0.1 M Sodium phosphate buffer, Ellman's reagent and 14.9 Mm AChI were added to supernatant and change in absorbance was measured immediately at 412 nm for 2 mins. The results were expressed as µ mol substrate hydrolysed /min/mg protein (23).

2.6.4 SOD assay

The method is described by Kono (1978) according to him 0.1 ml homogenate was added to nitro blue tetrazolium and hydroxylamine hydrochloride solution and absorbance was measured at 560 nm. The activity is measured in units per milligramme of protein (24).

2.6.5 Catalase assay

According to Luck, 1971, the tissue was homogenized with 50 mM Phosphate buffer and 3ml Hydrogen peroxide then it was set for centrifugation at 4000 rpm for 15 minutes at 4 C. Change in absorbance was measured at 240 nm for 2 mins with 30/60 sec interval. The activity is measured as micro moles of hydrogen peroxides decomposed/min/mg protein (25).

2.6.6 Reduced GSH assay

Ellman et al 1959 described the GSH method, according to him homogenate was added to 4% Sulphosalycylic acid and then centrifuged at 1200g for 5 min at 4 C. Supernatant was pipette out and 0.1 Mm DTNB was added. The absorbance was measured at 412 nm. The activity is measured in micro moles of GSH/mg protein (26).

2.7 Molecular estimation

2.7.1 TNF-α, IL-10 and IL-1β

The effect of treatment on the cytokines TNF-α, IL-10, IL-1β in brain tissues was assessed using the standard protocol described by ELK Biotechnology Fish TNF-α, IL10 and IL1β ELISA Kit. The process is composed of a 5-hour sandwich immunoassay in which samples, standards of various concentrations, and a blank were put to a 96-well plate before the primary antibody was added (monoclonal). Following incubation, secondary antibody (polyclonal), enzyme, substrate, and stop reagent were added, followed by washing with wash buffer to remove unbound at various time intervals as specified in the procedure. In an ELISA reader, the absorbance was measured at 450nm, and the findings were expressed in pg/mg protein (27).

2.7.2 Nrf-2

The levels of Nrf-2 in nuclear fractions isolated from the brain tissue were evaluated using an ELK Biotechnology Inc. Fish Nrf-2 ELISA Kit. The process consist of total 5-hours. Sandwich immunoassay had been used, in which samples, standard of different concentrations and a blank were placed in a 96-well plate before the primary antibody was added (monoclonal). Following incubation, secondary antibody (polyclonal), enzyme, substrate, and stop reagent were added, followed by washing with wash buffer at various time intervals as described in the procedure to remove unbound. The absorbance was measured at 450nm in an ELISA reader. The data are given in ng / mg of protein (28).

2.8 Mitochondrial Parameters

2.8.1 Complex-I assay (NADH dehydrogenase activity)

The activity of mitochondrial complex-I was measured using King and Howard's (1967) spectrophotometric technique. Catalytic oxidation of NADH to NAD + is followed by cytochrome C reduction in this mechanism (cyt-C). The reaction mixture consists of 350 ml of 0.2 M glycyl glycine buffer, 100 ml of 1.05 mM cyt-C, 100 ml of 6 mM NADH, 2.4 ml of distilled water, 10 ml of sample, and 20 ml of 0.02 M sodium bicarbonate. Over the course of 180 seconds, the change in optical density at 550 nm was observed (29).

2.8.2 Complex-II assay (SDH activity)

King's spectrophotometrically (1967) method was used to measure SDH activity. Succinate is oxidised with potassium ferricyanide, an artificial electron acceptor, in this method. The procedure employed 0.2 M phosphate buffer pH 7.8, 1 percent BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. To begin the reaction, 200 µL of succinate dehydrogenase were added to 25 µL of potassium ferricyanide. After that, 300 µL of BSA was added to the reaction mixture, followed by 25 µl of mitochondrial sample, and the change in absorbance was measured at 420 nm for 180 seconds (30).

2.8.3 Complex-IV assay (Cytochrome oxidase activity)

The Sottocasa technique was used to determine the activity of cytochrome oxidase (complex-IV) in brain mitochondria. 75 mM phosphate buffer, 0.3 mM reduced cytochrome C, and mitochondrial sample were used in the assay. The procedure began with the addition of a solubilized mitochondrial sample to phosphate buffer, followed by the addition of reduced cytochrome C to complete the reaction. At 550 nm, the absorbance change was observed for 180 seconds. nmol of cyt-C oxidised per mg of protein per minute were used to illustrate the results (31).

2.8.4 MTT Assay

The MTT assay was performed by conversion intracellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan by mitochondrial succinate dehydrogenases, which is a common procedure for predicting cell viability (32, 33). Whole zebrafish brains were submerged in 0.5 mg/mL MTT solution for 20 minutes at 37° after euthanasia. Following the incubation time, 300 mL DMSO was added to each sample and held overnight under continual homogenization to allow the solubility of formazan crystals to be measured. Finally, 200 µL of extracted formazan were poured onto 96-well plates, and cell

viability was measured at 560 and 650 nm. The percentage of vehicle control was used to express the results (34).

2.9 Histopathological examination

The brain was dissected out and immersed in formalin (10% v/v). With the use of a microtome, the brain tissues were fixed in paraffin blocks and sectioned into 3 mm thicknesses. Brain segments (5–10 m) were de-waxed and stained with H&E. Under a fluorescent microscope, the stained slices were inspected at 40X (27). (Model: 102 M, Motic microscope, China).

2.10 Statistical Analysis

GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego, CA, USA), was used to analyse all data, which were expressed as the mean ± SD. The behavioural, biochemical and neuroinflammatory, mitochondrial parameter assessment data was evaluated using one-way analysis of variance. Post hoc comparisons between groups were made by using Tukey's test. The value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Behavioral Study

3.1.2 Seizure related phenotypes

The mean seizure score, mean seizure time and number of score 4 events were assessed in a 10-min PTZ exposure period as shown in Fig. 2. PTZ elicited seizure-like behaviours and zebrafish displayed erratic swimming and circular movements, which closely resemble tonic/ clonic seizures (scores 3 and 4). DZP attenuated PTZ-induced changes in behavior. **(A)** represents the track plots of swimming pattern. **(B)** shows mean seizure score where, DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg, CA 60 mg/kg and combination of UA 50 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) decrease the mean seizure score when compared with PTZ 170 mg/kg. Moreover UA 150 mg/kg and CA 60 mg/kg have more potential to reverse the PTZ induced affect as they significantly decrease the mean seizure score when compared to UA 50 mg/kg. Also combination of UA 50 mg/kg and CA 60 mg/kg shows most ameliorating effect as it significantly reduce mean seizure score when compared to UA 50 mg/kg ($p < 0.001$), UA 150 mg/kg ($p > 0.05$) and CA 60 mg/kg ($p < 0.001$). **(C)** shows mean seizure time in which DZP 1.25 mg/kg UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly reverse the PTZ induced effect on mean seizure time, as it significantly ($p < 0.001$) decreased the mean seizure time when compared with PTZ 170 mg/kg, But combination of UA 50 mg/kg and CA 60 mg/kg was found to be significantly greater in ameliorating this effect when compared to UA 50 mg/kg ($p < 0.001$), UA 150mg/kg ($p > 0.05$) and CA 60 mg/kg ($p < 0.01$). **(D)** shows the results of no. of score 4 events occurred. DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) reverse the affect caused by PTZ 170 mg/kg. Moreover UA 150 mg/kg as well as CA 60 mg/kg have more ameliorating effect on the seizure like behavior than UA 50 mg/kg as no. of score 4 event is significantly decrease ($p < 0.001$) in UA 150 mg/kg and CA 60 mg/kg as compared to

UA 50 mg/kg. But combination of UA50 mg/kg and CA 60 mg/kg was found to be more effective in ameliorating seizures as there is no seizure score seen in combination therapy and also the effects were similar to DZP 1.25 mg/kg.

3.1.3 Novel Tank Diving Test

Figure 3 shows that PTZ 170 mg/kg significantly altered the zebrafish behavior in NTD test diving test. In **(A)** it was found that zebrafish treated with PTZ 170 mg/kg spent more time at the bottom of the tank in comparison with vehicle group. However combination of UA 50 mg/kg and CA 60 mg/kg treated displayed a preference for the both zones equally. In **(B)** TSTZ is significantly ($p < 0.001$) reduced in PTZ 170 mg/kg treated group as compared to vehicle control. DZP 1.25 mg/kg, UA 50 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) reversed the altered behavior of zebrafish as fish spent more time in top zone when compared with the PTZ 170 mg/kg treated group. Moreover pre-treatment with UA 150 mg/kg and CA 60 mg/ kg showed more significant ($p < 0.001$) effect in ameliorating altered behavior caused by PTZ 170 mg/kg when compared with UA 50 mg/kg. Since pre- treatment with UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg were capable of ameliorating PTZ 170 mg/kg induced altered behavior but combination of UA 50 mg /kg and CA 60 mg /kg showed best effect among all pre-treatment as TSTZ was significantly ($p < 0.001$) increased in combination therapy when compared with UA 50 mg/kg, UA 150 mg/kg and CA60 mg/kg. **(C)** shows that PTZ 170 mg/kg significantly ($p < 0.001$) increased the TSBZ when compared with the vehicle group. DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly decreases the TSBZ when compared with PTZ 170 mg/kg. Pre-treatment with UA 150 mg/kg and CA 60 mg/kg showed more ameliorating effect on PTZ induced behavioral alterations, as TSBZ was significantly ($p < 0.001$) reduced when compared with UA 50 mg/kg. Moreover combination therapy showed best effect among all pre-treatment groups as TSBZ was significantly decreased in combination therapy of UA 50 mg/kg and CA 60 mg/kg when compared with UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg. **(D)** showed that PTZ 170 mg/kg significantly ($p < 0.001$) reduced the no. of entries in top zone when compared with vehicle group. Whereas, DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) increased the no. of entries in top zone when compared with PTZ 170 mg/kg. Moreover UA 150 mg/kg and CA 60 mg/kg showed more ameliorating effect on PTZ induced behavioral alterations, as no. of entries in top zone were significantly ($p < 0.001$) increased when compared with UA 50 mg/kg. Combination therapy with UA 50 mg/kg and CA 60 mg/kg was found to be significantly ($p < 0.001$) greater in ameliorating this effect when compared to UA 50 mg/kg and CA 60 mg/kg. Also, combination therapy of UA 50 mg/kg and CA 60 mg/kg showed that no of entries in top zone was significantly ($p < 0.01$) increased in this group when compared with UA 150 mg/kg. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in NTD test.

3.1.4 Open Field Test

Figure 4 shows that PTZ 170 mg/kg significantly altered the zebrafish behavior in OF test. **(A)** shows that PTZ 170 mg/kg significantly ($p < 0.001$) decreased the distance travelled by animal when compared to

vehicle group. While, DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly increased the distance travelled by animal when compared to PTZ 170 mg/kg. Moreover combination of UA 50 mg/kg and CA 60 mg/kg was found to be more effective in ameliorating the PTZ 170 mg/kg induced effect on distance travelled as distance travelled by animal was significantly ($p < 0.001$) increased in this group when compared with UA 50 mg/kg. Also distance travelled by animal in combination therapy of UA 50mg/kg and CA 60 mg/kg was significantly ($P < 0.05$) increased as compared with UA 150 mg/kg and CA 60 mg/kg. **(B)** shows that PTZ 170 mg/kg significantly ($p < 0.001$) increases the speed of animal when compared with vehicle group. Whereas, pre -treatment with DZP 1.25 mg/kg, UA 50 mg/kg, UA150 mg/kg and CA 60 mg/kg significantly reversed the PTZ 170 mg/kg induced alteration on speed of animal as speed of zebrafish was significantly ($p < 0.001$) reduced when compared with PTZ 170 mg/kg. Moreover pre-treatment with combination therapy of UA 50 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) improves this effect as compared with UA 50 mg/kg. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in OF test.

3.2 Oxidative Stress

Table 2 represent that PTZ 170 mg/kg administration caused significant ($p < 0.001$) rise in LPO, AChEs concentration and depletion of reduced GSH, SOD and catalase levels in the brain of zebrafish as compared to vehicle group. Whereas, DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg administration significantly ($p < 0.001$) attenuated oxidative stress (reduced the elevated MDA, and AChEs concentration and restored SOD, catalase and GSH levels), as compared to PTZ 170 mg/kg treated animals. Further, treatment of UA 50 mg/kg in combination with CA 60 mg/kg significantly ($p < 0.05$) potentiated their protective effect as compared to their effects alone in PTZ 170 mg/kg treated animals. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in oxidative stress biomarkers. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in oxidative stress parameters.

Table 2

Effect of combination of UA (50mg/kg) and CA(60mg/kg) on LPO, AChE, SOD, Catalase and GSH in different groups (n = 3). Results were expressed as mean ± SD and analyzed by one way ANOVA followed by tukey's post hoc test. [a]P < 0.001 vs vehicle treated group, [b]P < 0.001vs PTZ(170mg/kg) treated group, [c]P < 0.05 vs PTZ + UA(50 mg/kg) treated group, [d]P < 0.05 vs PTZ + UA (150mg/kg), [e]P < 0.05 vs PTZ + CA(60mg/kg). [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid, LPO: lipid per oxidase, AChE : acetyl cholinesterase, SOD: superoxide dismutase, GSH: glutathione s transferase].

S.no	Groups	LPO (n mole /mg)	AChEs (n mole/mg)	SOD (µg /mg)	CATALASE(µm of H⊠O⊠/min/mg)	GSH (n mole/mg)
1	Vehicle	0.04355687± 0.0130	41.666670 ± 4.2452	40.123460 [ns] ± 0.8800	40.123460 [ns] ± 0.8800	134.243900 [ns] ± 3.0560
2	DZP (125 mg/kg) *per se*	0.04884005 [ns] ± 0.0013	41.666670 [ns] ± 4.245	40.360370 [ns] ± 2.5371	1.871730 [ns] ± 0.0613	140.097600 [ns] ± 0.4224
3	UA (150 mg/kg) *per se*	0.0501813 [ns] ± 0.0024	41.666670 [ns] ± 4.2452	39.920460 [ns] ± 5.2768	1.779058 [ns] ± 0.0751	141.235800 [ns] ± 7.5425
4	CA (60 mg/kg) *per se*	0.03879223 [ns] ± 0.0044	39.215690 [ns] ± 4.2452	41.218480 [ns] ± 5.7035	1.852553 [ns] ± 0.0465	134.731700 [ns] ± 1.0631
5	PTZ (170 mg/kg)	0.1899335[a]± 0.0077	134.731700[a]± 1.0631	12.761830 [a] ± 0.2312	0.1352374 [a] ± 0.0137	2.536585[a]± 1.1177
6	PTZ + DZP	0.04433761[b] ± 0.00180	41.666670 [b] ± 4.2452	42.844910 [b] ± 3.0030	1.542899 [b] ± 0.0434	81.560970[b]± 0.2439
7	PTZ + UA 50	0.1085157 [b] ± 0.0002	75.980390 [b] ± 4.2452	23.372350 [b] ± 1.5934	0.7871838 [b] ± 0.0112	30.829270[b]± 1.357
8	PTZ + UA 150	0.06561591[b,c] ± 0.0007	55.147060 [b,c] ± 3.6764	35.059510 [b,c] ± 2.0316	1.014154 [b] ± 0.0516	53.268290[b,c]± 0.7317
9	PTZ + CA	0.06537444 [b,c] ± 0.0034	53.921570 [b,c] ± 4.2452	35.657480 [b,c] ± 1.6336	1.050078 [b,c] ± 0.1198	53.918700[b,c]± 2.1028

S.no	Groups	LPO (n mole /mg)	AChEs (n mole/mg)	SOD (µg /mg)	CATALASE(µm of H⍰O⍰/min/mg)	GSH (n mole/mg)
10	PTZ + UA 50 + CA	0.0445414 ^{b,c,d,e} ± 0.0007	34.313720 ^{b,c,d,e} ± 4.2452	46.613680 ^{b,c,d,e} ± 6.2490	1.878884 ^{b,c,d,e} ± 0.0341	77.333340^{b,c,d,e}± 4.0397

3.3 Inflammatory Markers

Figure 5 shows the results of inflammatory markers in different groups. **(A)** shows, in zebrafish PTZ 170 mg/kg administration significantly elevated the expression of TNF-α level when compared to the vehicle group ($p < 0.001$). Whereas, DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) decreased the concentration of TNF-α when compared to PTZ 170 mg/kg treated group. Further, pre-treatment with UA 150 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) ameliorated the level of TNF-α as compared to the UA 50 mg/kg treated group. Moreover, combination of UA 50 mg/kg and CA 60 mg/kg was found to be significantly ($p < 0.001$) more effective in showing anti-inflammatory action as compared to alone pre- treated groups. **(B)** showed PTZ 170mg/kg administration in zebrafish brain showed a significant increased level of IL-1β in adult zebrafish brain as compared with the vehicle group ($p < 0.001$). However, treatment with DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg has significantly reduced the level of IL-1β as compared with the PTZ 170 mg/kg treated group ($p < 0.001$). Moreover, UA 150 mg/kg and CA 60 mg kg has shown more ameliorating effect than UA 50 mg/kg ($p < 0.001$). Among UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg combination of UA 50 mg/kg and CA 60 mg/kg was found to be more significant in decreasing the IL-1β level when compared with UA 50 mg/kg ($p < 0.001$), UA 150 mg/kg and CA 60 mg/kg ($p < 0.01$). However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in inflammatory markers estimation.

3.4 Anti-inflammatory Markers

Figure 6 shows the results of anti-inflammatory markers in different groups. **(A)** showed, PTZ 170 mg/kg administration significantly declined the IL-10 level in zebrafish brain when compared with vehicle group ($p < 0.001$). However, Pre-treatment with DZP 1.25 mg/kg, UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg significantly restores the IL-10 level when compared with PTZ 170 mg/kg treated group ($p < 0.001$). Moreover, UA 150 mg/kg and CA 60 mg/kg showed more capacity to restore the IL-10 level as there is a significant rise in IL-10 level when compared with UA 50 mg/kg ($p < 0.001$). Although all the pre-treatment have ameliorating effect but combination therapy of UA 50 mg/kg and CA 60 mg/kg was found to be best among all these groups as it showed similar effect like DZP 1.25 mg/kg and also raised the IL- 10 level significantly when compared with UA 50 mg/kg ($p < 0.001$), UA 150 mg/kg and CA 60 mg/kg ($p < 0.05$). **(B)** portray the results of NRF-2 level, PTZ 170 mg/kg administration significantly dropped the NRF-2 level in zebrafish brain, But DZP1.25 mg/kg, UA 50 mg/kg ,UA 150 mg/kg and CA 60 mg/kg elevate the level significantly ($p < 0.001$) as compared to the PTZ 170 mg/kg treated group. Furthermore, UA 150 mg/kg and CA 60 mg/kg was found to be more effective in restoring the NRF-2 level as it shows the significant ($p < 0.01$) raise in NRF-2 level when compared with UA 50 mg/kg. Although all pre-treatments were capable of

ameliorating PTZ induced alterations yet combination of UA 50 mg/kg and CA 60 mg/kg was more potential than all alone pre-treated groups as it significantly ($p < 0.001$) restores the level when compared with UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg. As expected, their protective effect was similar to that of DZP 1.25 mg/kg treatment in PTZ treated animals. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in anti-inflammatory markers estimation.

3.5 Mitochondrial Enzyme Complexes and MTT Assay

Figure 7 depicts Mitochondrial enzyme complex (NADH dehydrogenase, succinate dehydrogenase cytochrome oxidase) and MTT assay. **(A)** portrays the findings of complex I and in these we observed that animals pre-treated with PTZ (170 mg/kg) were significantly ($P < 0.001$) depleted as compared to vehicle group. Although, DZP 1.25 mg/kg, UA 50 mg/kg ,UA 150 mg/kg and CA 60 mg/kg restored mitochondrial complex I activity significantly ($p < 0.001$) as compared to the PTZ 170 mg/kg treated group. Moreover, UA 150 mg/kg and CA 60 mg/kg was found to be more effective in restoring the complex I activity as it shows the significant ($p < 0.05$) raise when compared with UA 50 mg/kg. Furthermore, combination of UA 50 mg/kg and CA 60 mg/kg was more potential than all alone pre-treated groups as it significantly ($p < 0.05$) restores the parameters when compared with UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg. **(B)** results reveal the activity of complex II, in this PTZ 170 mg/kg treated fishes showed significant($p < 0.001$) decline in this activity as compared to vehicle group. However, fishes pre-treated with DZP 1.25 mg/kg, UA 50 mg/kg, CA 60 mg/kg significantly restored this enzymatic activity. Furthermore, UA 150 mg/kg and CA 60 mg/kg found to be more effective than low dose of UA i.e. 50 mg/kg as our results showed significant ($p < 0.001$) rise in these groups as compared to UA 50 mg/kg. Interestingly, combination of UA 50 mg/kg and CA 60 mg/kg resulted in significant ($p < 0.01$) rise in Complex II enzymes activity as compared to UA 50 mg/kg, CA 60 mg/kg and UA 150 mg/kg, this dramatic rise in enzymes activity proved that combination therapy was more potent than alone one. **(C)** depicts the result of Complex IV enzymes activity, and the results evident that fishes pretreated with PTZ 170mg/kg significantly ($p < 0.001$) dropped the enzymatic activity when compared with vehicle group. However, groups pre-treated with DZP 1.25 mg/kg, UA 50 mg/kg, UA150 mg/kg and CA 60 mg/kg significantly ($p < 0.01$) restore the enzymatic activity as compared to PTZ 170 mg/kg. Furthermore, UA 150 mg/kg and CA 60 mg/kg found to be more potential to restore enzymatic activity in the results, as they are significantly ($p < 0.001$) increases the activity when compared with UA 50 mg/kg. Additionally, combination therapy have more potential among all pre-treatments as it significantly ($p < 0.05$) restore the enzymatic activity as compared to UA 50 mg/kg and UA 150 mg/kg.(**D**) displays the results of MTT assay, In the results PTZ 170 mg/kg treated fish shows significant decrease in MTT assay activity when compared to vehicle control, however DZP 1.25 mg/kg, UA 50 mg/kg, UA150 mg/kg and CA 60 mg/kg significantly ($p < 0.01$) restore the activity as compared to PTZ 170 mg/kg. Additionally, UA 150 mg/kg and CA 60 mg/kg found to be more potential to restore enzymatic activity in the results, as they are significantly ($p < 0.001$) increases the activity when compared with UA 50 mg/kg. Importantly our results showed that combination therapy showed best results among all pre-treatment as MTT assay activity is significantly improved in combination therapy when compared with UA 50 mg/kg, CA

60 mg/kg and UA 150 mg/kg. However there is no significant effect was found in vehicle group i.e. DMSO, and *per se* group of DZP 1.25 mg/kg, UA 150 mg/kg and CA 60 mg/kg in mitochondrial parameters.

3.6 Histopathological Analysis

Figure 8 represents morphological structure of neuronal cells **(A)** and **(B)** portrays the histopathological evaluation of brain tissue. It was carried under fluorescent microscopy. The data was quantified by Image J software as shown in **(C)** the brains of vehicle and per se treated groups showed undamaged neuronal cells. However, treatment with PTZ 170 mg/kg causes disarrangement of various cell layers as well as the pyramidal neuronal cell loss was found which was significant ($p < 0.001$) as compared to the vehicle group. Furthermore, pre-treatment with DZP 1.25 mg/kg, UA 50 mg/kg ,UA 150 mg/kg and CA 60 mg/kg significantly ($p < 0.001$) attenuated the loss of neuronal cell density as compared to PTZ treated animals. Moreover pre-treatment with UA 150 mg/kg and CA 60 mg/kg was found to be more effective as it significantly ($p < 0.001$) displayed less neuroinflammation as compared to UA 50mg/kg. Furthermore combination of UA 50 mg/kg and CA 60 mg/kg exhibit significantly ($p < 0.01$) least neuroinflammation as compared to UA 50 mg/kg, UA 150 mg/kg and CA 60 mg/kg.

4. Discussion

In this study, we reported for the first time a preventive effect of combination therapy of UA 50 mg/kg and CA 60 mg/kg in PTZ induced seizures.

Our research revealed similarly to DZP, combination of UA and CA cures seizure like behaviours. PTZ 170 mg/kg administration elicits all seizure-like behavioral phenotypes, in which 95% animals reached score 4 and frequently exhibited tonic/clonic seizure scores (35). The outcomes demonstrated that combined therapy entirely eradicates seizures in animals given PTZ. Since combination therapy pre-treatment reduces the mean seizure score, mean seizure time, and number of episodes of score 4, the combination of UA 50 mg/kg and CA 60 mg/kg attenuates seizure-like behaviours (clonic-like behaviours). The reason behind this effect could be that UA may have modulatory effect on the central nervous system (CNS) since it inhibits neuronal firing and encourages hyperpolarization (36). Additionally, combination therapy pre-treated fish displayed typical swimming behaviours, including repeated brief swims. The antioxidant properties of UA, which further limit seizure induction through oxidative stress, as well as the fact that CA is an isomer of valproic acid which exhibits anti-seizure properties, may be the reason for this action.

The combination of UA 50 mg/kg and CA 60 mg/kg restored the exploratory nature of zebrafish. The track plot of open field test clearly shows that animals pretreated with UA and CA covers the whole field when compared with the PTZ treated animals. Also, the distance covered by combination therapy treated group was more than the PTZ treated zebrafish which ensures the exploratory behavior of zebrafish.. When compared to the PTZ treated groups also the combination treated group similarly showed a decrease in swimming speed. Since About 40–50% of children and adolescents with epilepsy are thought to have mental and behavioural comorbidities (37). Therefore testing for anxiety like behavior is an essential factor. In novel tank diving test PTZ treated fish displayed anxiety-like behavior as they mainly resided at the

bottom half of their tank, in contrast to control fish which proportionately spent time in both halves (38). As our data in novel tank diving test shows that time spent in upper zone of the tank and number of entries in upper zone is more in groups pretreated with combination therapy when compared with the PTZ treated groups, this implies that combination therapy is able to reduces the anxiety like behavior in zebrafish that may be induced by PTZ administration.Free radicals are typical by-products of aerobic metabolism within cells. Consequently, they result in cellular malfunction when free radical generation rises or the body's defence mechanisms weaken (39). According to the results of the current investigation, administration of PTZ at a dose of 170 mg/kg led to a significantly higher level of LPO, AChEs and depleted the reduced glutathione, SOD, GSH and catalase activity in PTZ-treated animals, which supports the previous investigation that PTZ causes oxidative stress. Whereas UA and CA alone are able to abolish the effects of PTZ on SOD, catalase, lipid peroxidation, Acetylcholinesterase and GSH but combination therapy significantly restores all the parameters which could be clearly seen as catalase, SOD and GSH activity is increased significantly in both the UA and CA combination treated groups and LPO decreases significantly in combination of UA50 mg/kg and CA 60 mg/kg treated group, indicating that UA and CA has a complex modulatory influence on enzymatic antioxidant defences. Additionally, UA is a very effective scavenger of reactive species such as singlet oxygen (O2) and others. One potential mechanism for the neuroprotective effect of UA and CA against PTZ-induced oxidative damage appears to be their antioxidant properties.

Numerous inflammatory mediators, including soluble polypeptides known as cytokines, play a crucial role in both the activation of innate immunity and the transition to adaptive immunity. Ils and TNFs are two of these molecules (40).Through previous studies, it was concluded that PTZ increased the level of inflammatory cytokines such as TNF-α and IL-1β in the brain via activation of microglia and astrocytes (39) (41). Our findings were consistent with previous research. Here in our study PTZ- treated group had higher levels of TNF-α and IL-1β than the vehicle group. However, when compared to the PTZ-treated group, UA and CA combination treated groups reduced TNF- α and IL-1β levels.

When cells improve their antioxidant defences by activating the nuclear factor erythroid 2-related factor, defensive mechanisms against the production of reactive oxygen and nitrogen species are activated in the brain (Nrf2). It has been established that the Nrf2 protein is one of the key regulators of the cellular antioxidant system among the numerous neuroinflammatory pathways. (42). Besides, it boost the expression of several endogenous antioxidant enzymes by binding to antioxidant response elements, Nrf2 is a crucial regulator of redox balance and intracellular signalling. In animals treated with PTZ, we have observed an increase in the expression of Nrf2. However, reversal effect on this parameter was seen in combination therapy of UA50 mg/kg and CA 60 mg/kg. As our results displayed significant reduction I Nrf2 level when compared with the PTZ treated group. These results validated our hypothesis that CA and UA contain antioxidant potential and free-radical scavenging activity.

IL-10 is an important immunoregulatory cytokine with multiple biologic effects on different cell types (43). IL-10 promotes survival of neurons and glia, halting, thus the deleterious effects of TNF-α in epilepsy. Interestingly, IL 10 activity increased in combination of UA50 mg/kg and CA 60 mg/kg. PTZ groups, reflecting a complex modulatory effect of UA and CA on enzymatic antioxidant defences. In our results animals receiving PTZ 170 mg/kg showed significant rise in IL 10 level when compared with vehicle

whereas, combination therapy significantly reduce the level of IL 10 when compared with PTZ treated group.

To enable ATP generation from ADP and phosphate, the ETC components in mitochondria catalyse a sequence of redox processes connected to a proton gradient across the inner mitochondrial membrane (37). Furthermore, since mitochondria are the main sites for producing reactive oxygen species, these are particularly prone to oxidative stress and damage. This has an impact on both the function of cellular macromolecules and the ability of the electron transport chain to produce ATP. Damage from oxidation to the mitochondria may have an impact on neural excitability and make people more susceptible to seizures (44). Different neurological illnesses may potentially be influenced by mitochondrial oxidative stress and malfunction. Important cellular processes carried out by mitochondria affect the excitability of neurons. Evidence for the connection between epilepsy and mitochondrial oxidative stress is growing recently (38). Intense seizure activity creates a severe opening of N-methyl-D-aspartate receptor (NMDA)-dependent ion channels, which leads to an increase in intracellular and intramitochondrial Ca2+, which in turn causes the mitochondrial membrane to depolarize and produce superoxide (39). In this research, it was observed that PTZ-treated zebrafish brains had decreased activity of the ETC enzymes NADH: cytochrome C reductase, succinate dehydrogenase, and cytochrome C oxidase supporting mitochondrial dysfunction hypothesis during epileptogenesis. UA and CA in combination reduces oxidative stress and prevent complex-I, II and IV activity from deterioration. Additionally, it was discovered that PTZ-treated zebrafish had considerably lower MTT reduction, which indicates lower mitochondrial respiration. When UA and CA were administered in combination to PTZ-treated zebrafish, the MTT decrease was dramatically increased, indicating an improvement in mitochondrial activities. Combination of UA and CA significantly restored mitochondrial enzyme complex activities suggesting its therapeutic potential against mitochondrial dysfunction. Since caprylic acid increased the ATP/ADP ratios in both the cytosol and the mitochondria, along with a considerable rise in glucose synthesis and a decrease in the lactate + pyruvate flux (45) which is one of the mechanism of CA's mitochondrial protective property. Therefore, the present study demonstrates the neuroprotective potential of UA and CA against PTZ-induced.

Histopathological analysis revealed damaged morphology in the group administered with PTZ which was confirmed by the imaging software that quantifies the percentage covered by healthy neuronal cells. Moreover combination of UA and CA attenuates the above given morphological damage.

The results obtained so far suggest that PTZ-induced alterations in behaviour, oxidative stress biomarkers, inflammatory markers, anti-inflammatory markers, mitochondrial parameters and morphological damage were cured by the combination therapy of UA and CA. Overall, we suggest a protective role of multi drug approach against PTZ-induced behavioral and neurochemical changes in zebrafish. Figure 9 shows the mechanistic representation of the study.

5. Conclusion

Herein, we conclude that combination of UA 50 mg/kg and CA 60 mg/kg suppresses seizure-like behavior and it is a potential approach to be a novel treatment for seizures. Pre-treatment with the combination of

UA 50 mg/kg and CA 60 mg/kg also counteracts the upregulation and rise of TNF-α, IL-1β, LPO and AChEs as a result of a PTZ induced seizure. Moreover, behavioral observation, biochemical analysis, inflammatory cytokines, anti-inflammatory markers, mitochondrial parameters and histopathology analysis results suggest that combination of UA 50 mg/kg and CA 60 mg/kg can reverse seizures like symptoms in PTZ induced seizure zebrafish model.

The anti-seizure effect of this treatment is also comparable to that of diazepam. Moreover, overall findings suggest that combination of UA 50 mg/kg and CA 60 mg/kg has potential therapeutic value for the management of seizures. However, further work is highly recommended to evaluate the therapeutic effects of combination of UA 50 mg/kg and CA 60 mg/kg against genetic models and to compare its efficacy with current AEDs.

Abbreviations

AChEs: Acetylcholinesterase

CA: Caprylic acid

DMSO: Dimethyl sulfoxide

DZP: Diazepam

GSH: Glutathione

IL 10: Interleukin 10

IL-1β : Interleukin 1 beta

LPO: Lipid peroxidation

MTT assay:((3-4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)

NBT: Nitrobluetetrazole

NDT: Novel diving test

Nrf2: Nuclear factor-erythroid factor2-related factor 2

OFT: Open field test

PTZ: Pentylenetetrazole

ROS: Reactive oxygen species

SOD: Superoxide dismutase

TBA :Thiobarbituric acid

TCA :Trichloroacetic acid

TNF- α :Tumour necrosis factor

TSBZ: Time spent in bottom zone

TSTZ: Time spent in top zone

UA: Ursolic acid

Declarations

Author contribution

AS: Supervised and conceptualized the work and critically reviewed the manuscript;

CS: Thoroughly checked the manuscript;

DS: Performed the experiments and wrote the manuscript and draw the figures and tables.

SK: Helped in performing experiments

LK : Helped in writing manuscript

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Conflict of interest

All authors confirm and declare no competing financial interests

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Figures

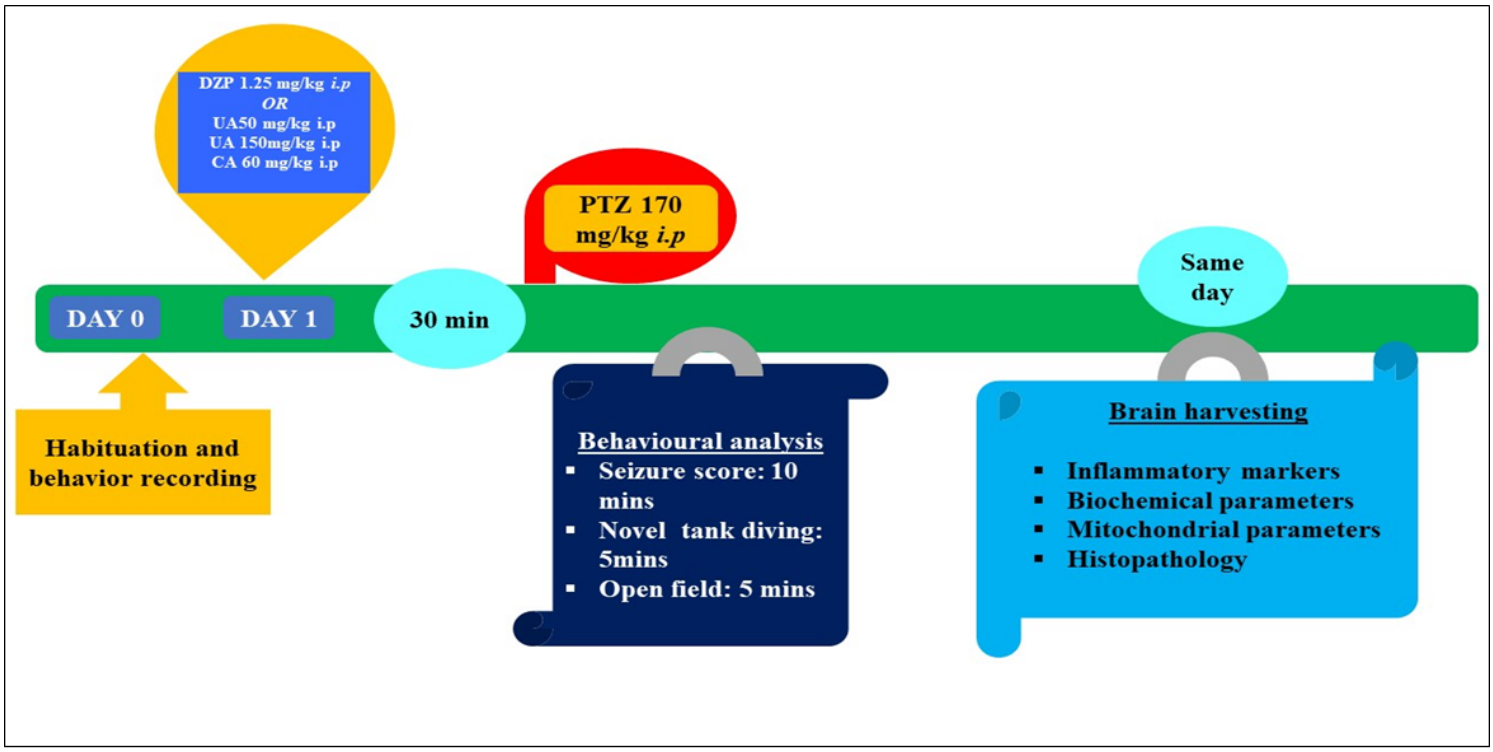

Figure 1

Figure 1

Study design

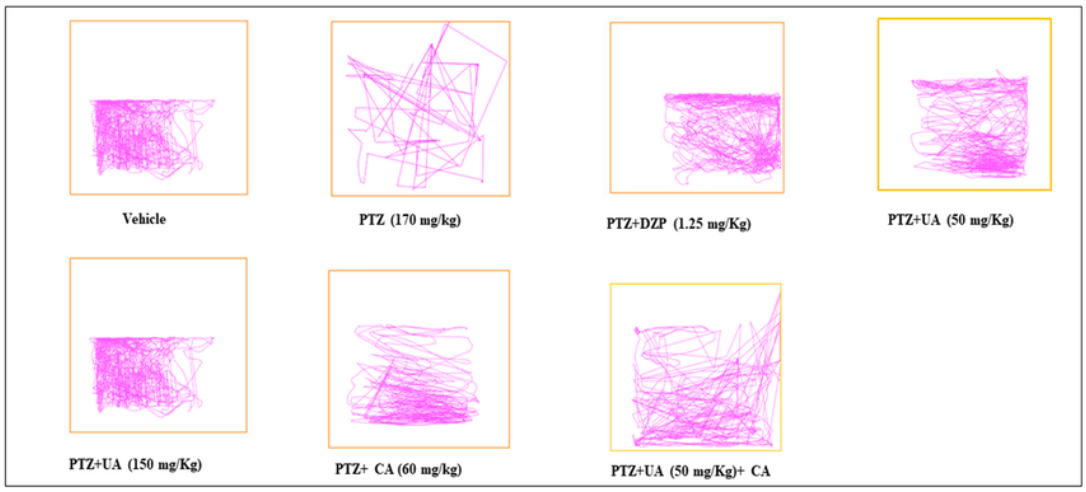

Figure 2A

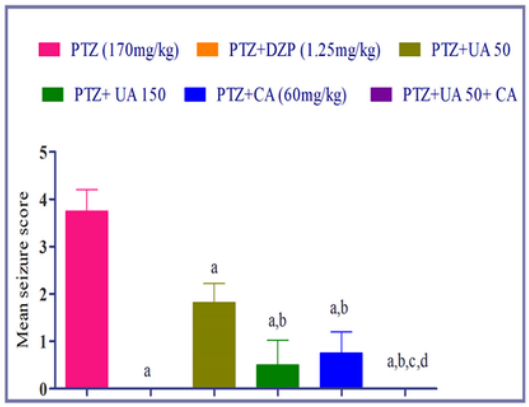

Figure 2B

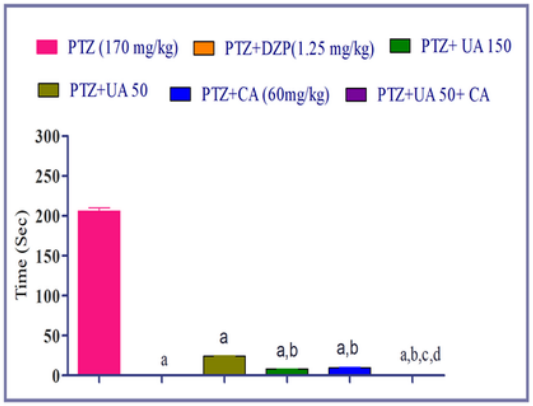

Figure 2C

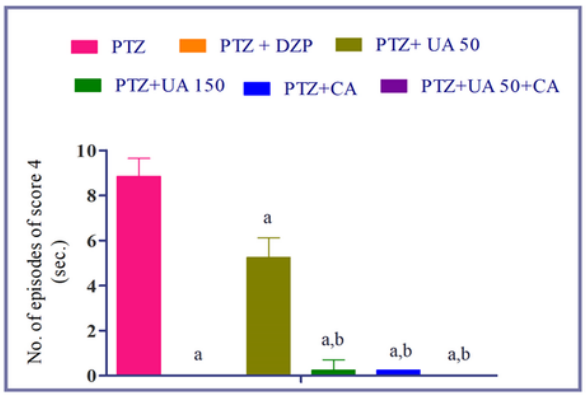

Figure 2D

Figure 2

Effect of combination of UA (50 mg/kg) and CA(60 mg/kg) on seizures in different groups (n=12). Results were expressed as mean ± SD and analyzed by one way ANOVA followed by Tukey's post hoc test. **(A)** Track Plots **(B)** represents **mean seizure score** where $^{a}p < 0.001$ vs PTZ (170 mg/kg) treated group, $^{b}p < 0.001$vs PTZ+UA (50 mg/kg) treated group, $^{c}p < 0.05$ vs PTZ+ UA (150 mg/kg) treated group, $^{d}p < 0.001$ vs PTZ+CA (60 mg/kg) and **(C)** represents **total time of seizures** where $^{a}p < 0.001$ vs PTZ (170 mg/kg) treated

group, [b]p <0.001vs PTZ+UA (50 mg/kg) treated group, [c]p < 0.05vs PTZ+ UA (150 mg/kg) treated group, [d]p < 0.01 vs PTZ+CA (60mg/kg). **(D)** represents **no. of episodes of score 4** where [a]p < 0.001 vs PTZ (170 mg/kg) treated group, [b]p < 0.001vs PTZ+UA (50 mg/kg) treated group. [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid].

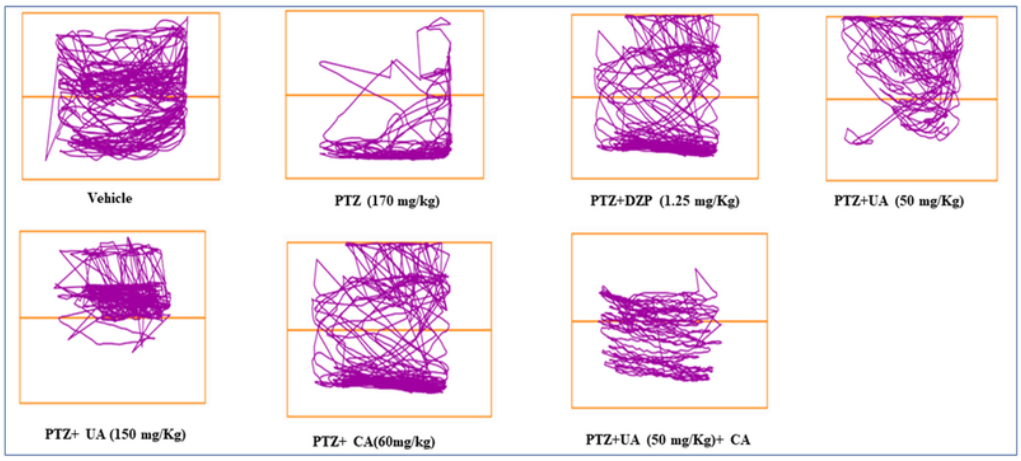

Figure 3A

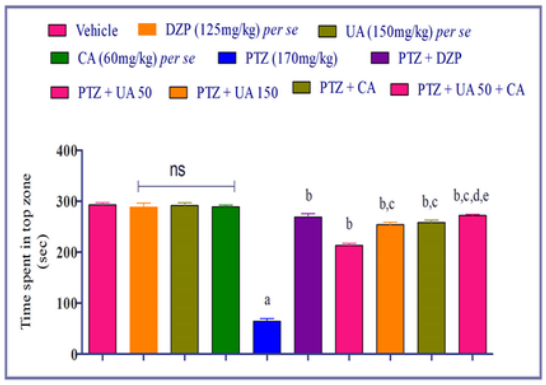

Figure 3B

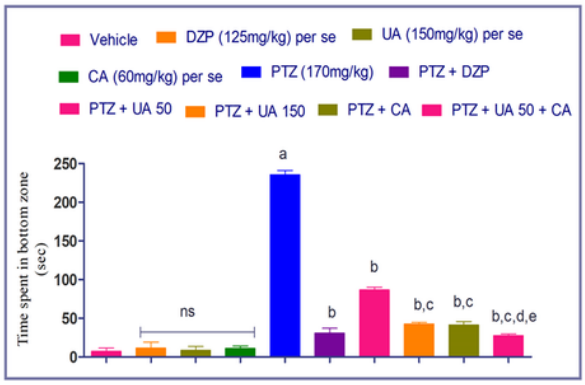

Figure 3C

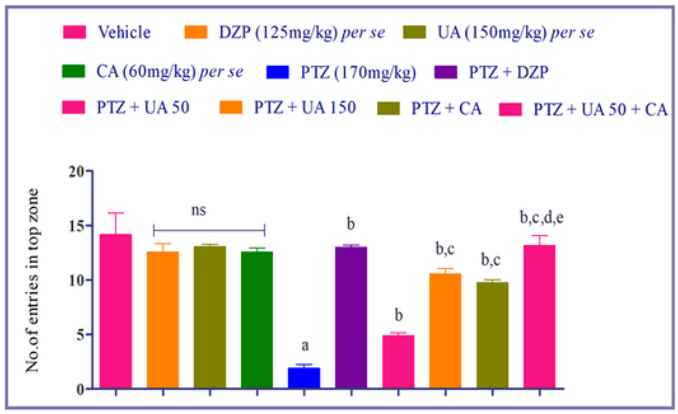

Figure 3D

Figure 3

Effect of combination of UA (50 mg/kg) and CA(60 mg/kg) on Novel Tank Diving test in different groups (n=12). Results were expressed as mean ± SD and analysed by one way ANOVA followed by Tukey's post hoc test. **(A)** Represent the **swimming pattern in NTD test** for each experimental group **(B)** represents **TSTZ** **(C)** represents **TSBZ** where, $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p < 0.01$ vs PTZ+UA (150mg/kg) , $^{e}p < 0.001$ vs PTZ+CA(60mg/kg). $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p < 0.001$ vs PTZ+UA (150 mg/kg) , $^{e}p < 0.001$ vs PTZ+CA (60 mg/kg). **(D)** represents **no. of entries in top zone** where, $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p < 0.01$ vs PTZ+UA (150mg/kg) , $^{e}p < 0.001$ vs PTZ+CA(60mg/kg). . [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid, TSTZ : Time spent in top zone, TSBZ : time spent in bottom zone].

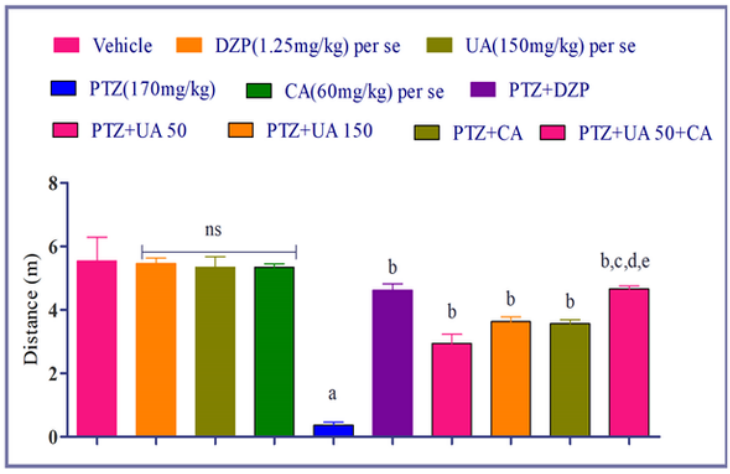

Figure 4A

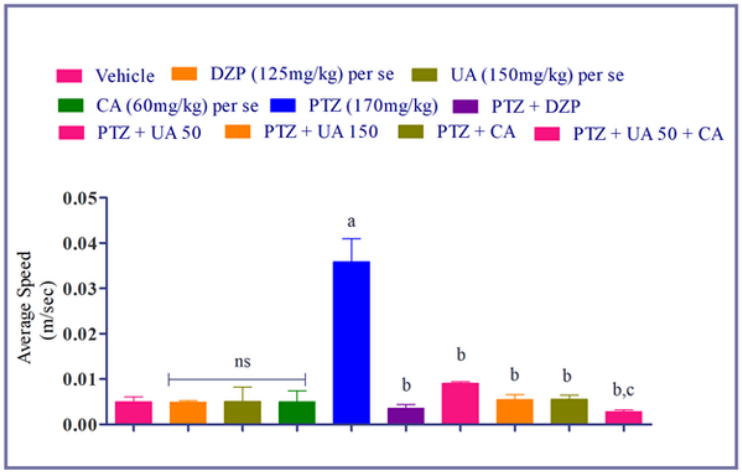

Figure 4B

Figure 4

Effect of combination of UA (50 mg/kg) and CA (60 mg/kg) on open field test in different groups (n=12). Results were expressed as mean ± SD and analyzed by one way ANOVA followed by Tukey's post hoc test. **(A)** represents **distance travelled** where $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p < 0.05$ vs PTZ+UA (150mg/kg) , $^{e}p < 0.05$ vs PTZ+CA(60mg/kg). and **(B)** represents **average speed** where $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group.

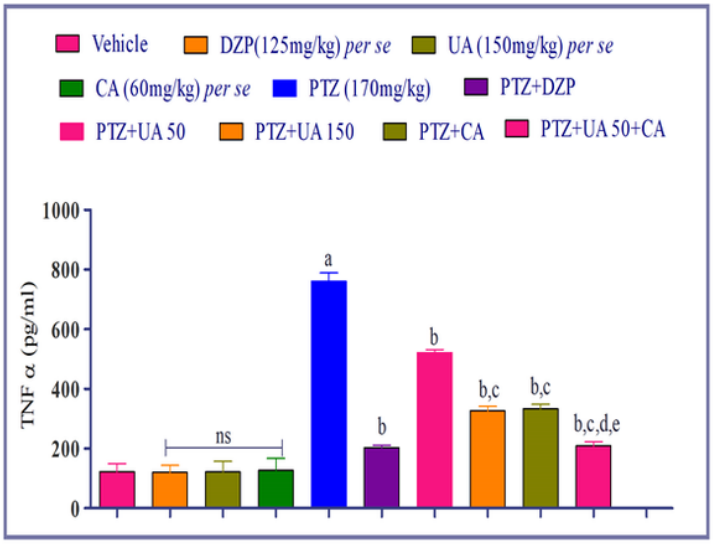

Figure 5A

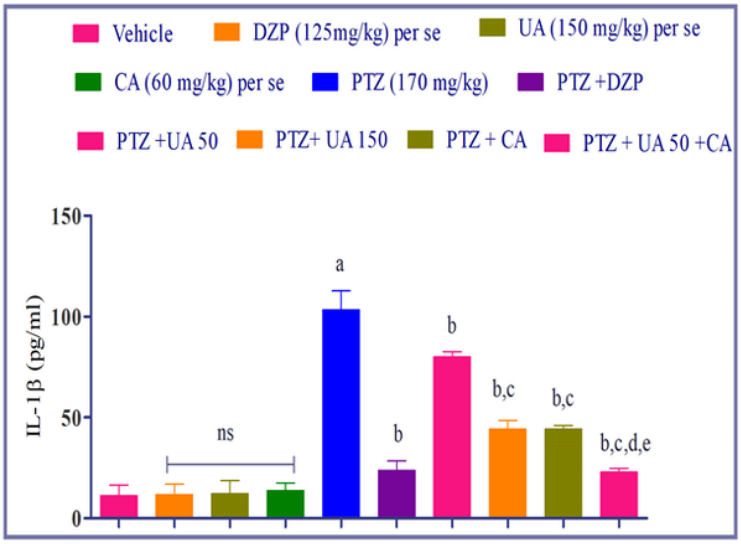

Figure 5B

Figure 5

Effect of combination of UA (50 mg/kg) and CA(60 mg/kg) on inflammatory markers in different groups (n=3). Results were expressed as mean ± SD and analysed by one way ANOVA followed by Tukey's post hoc test **(A) TNF-α** where,. $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p < 0.001$ vs PTZ+UA (150mg/kg) , $^{e}p < 0.001$ vs PTZ+CA(60 mg/kg). **(B) IL-**[illegible] where $^{a}P < 0.001$ vs vehicle treated group, $^{b}P < 0.001$vs PTZ(170 mg/kg) treated group, $^{c}P < 0.001$ vs PTZ+ UA (50 mg/kg) treated group, $^{d}P < 0.01$ vs PTZ+UA (150 mg/kg) , $^{e}P < 0.01$ vs PTZ+CA(60mg/kg). [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid].

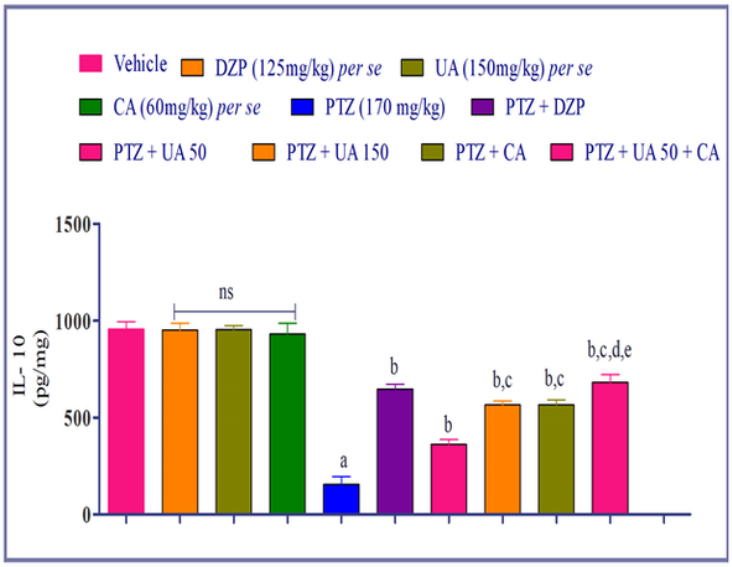

Figure 6A

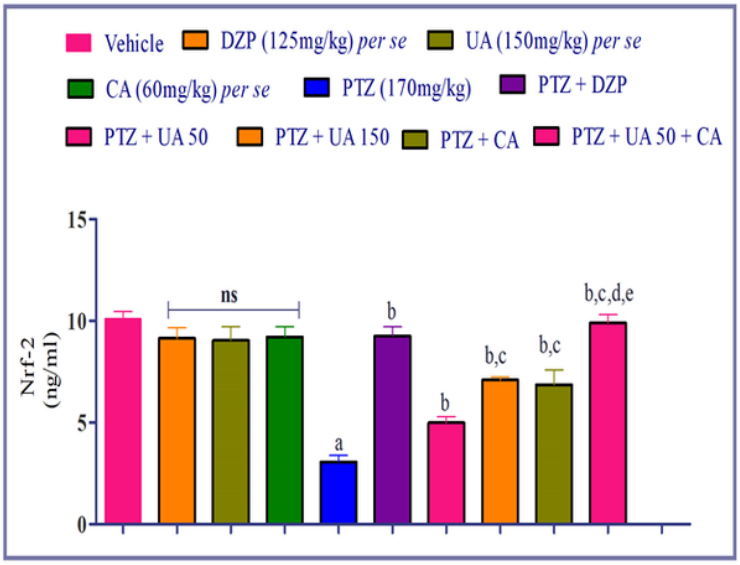

Figure 6B

Figure 6

Effect of combination of UA (50 mg/kg) and CA(60 mg/kg) on anti-inflammatory markers in different groups (n=3). Results were expressed as mean ± SD and analysed by one way ANOVA followed by Tukey's post hoc test. **(A) IL-10** where, $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA(50 mg/kg) treated group, $^{d}p > 0.05$ vs PTZ+UA (150 mg/kg) , $^{e}p < 0.05$ vs PTZ+CA(60 mg/kg). **(B) Nrf-2** where, $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$,0.01 vs PTZ(170 mg/kg) treated group, $^{c}p < 0.001$, 0.01 vs PTZ + UA (50 mg/kg) treated group, $^{d}p < 0.001$ vs PTZ+UA (150 mg/kg) , $^{e}p < 0.001$ vs PTZ+ CA(60 mg/kg). [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid].

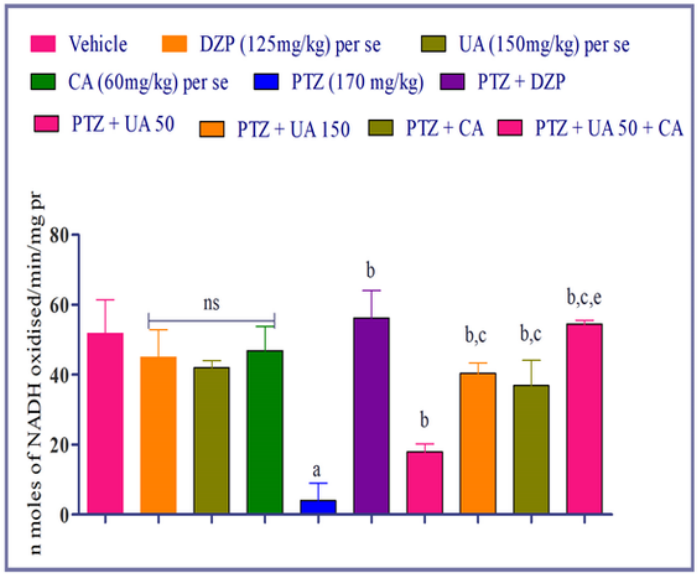

Figure 7A

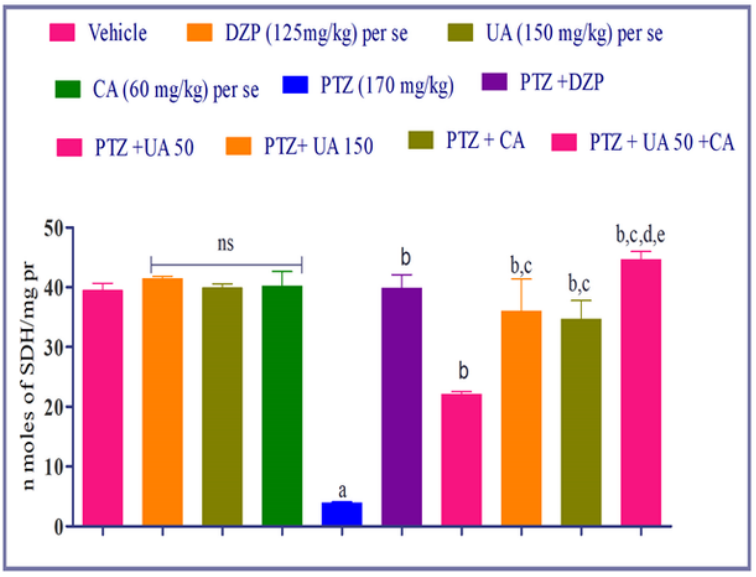

Figure 7B

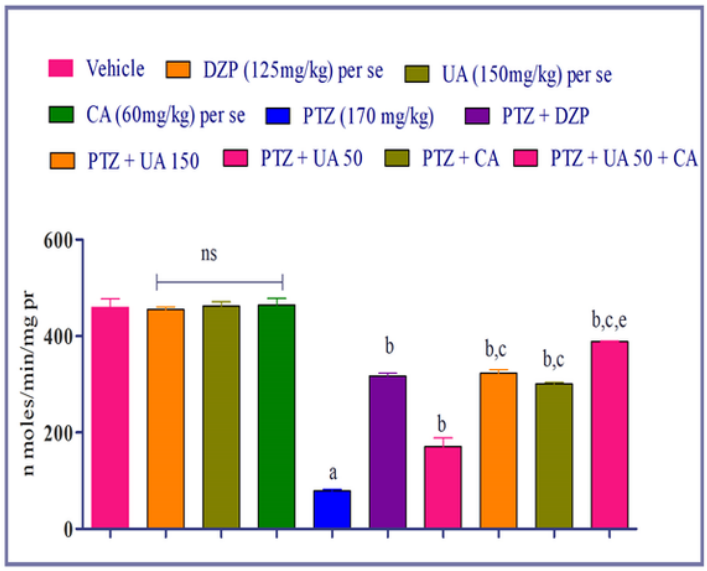

Figure 7C

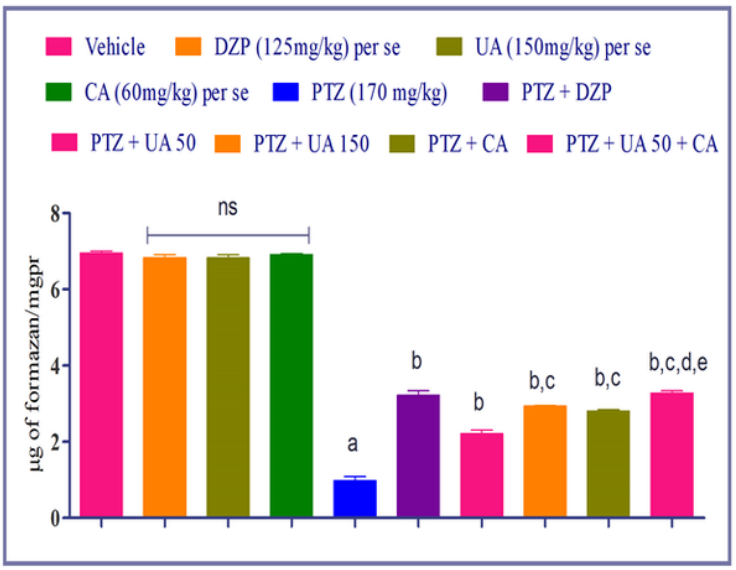

Figure 7D

Figure 7

Effect of combination of UA (50mg/kg) and CA(60mg/kg) on mitochondrial parameters in different groups (n=3). Results were expressed as mean ± SD and analysed by one way ANOVA followed by Tukey's post hoc test. **(A) complex I** where, ^{a}P <0.001 vs vehicle treated group, ^{b}P <0.001vs PTZ(170mg/kg) treated group, ^{c}P <0.05 vs PTZ+ UA(50 mg/kg) treated group, ^{e}P <0.05 vs PTZ+CA (60mg/kg). **(B) complex II**, where . ^{a}P <0.001 vs vehicle treated group, ^{b}P <0.001vs PTZ(170mg/kg) treated group, ^{c}P <0.001 vs PTZ+ UA(50 mg/kg) treated group, ^{d}P <0.01 vs PTZ+UA (150mg/kg) , ^{e}P <0.001 vs PTZ+CA (60mg/kg). **(C) Complex IV** where, . ^{a}p < 0.001 vs vehicle treated group, ^{b}p < 0.01vs PTZ (170mg/kg) treated group, ^{c}p < 0.001 vs PTZ+ UA(50 mg/kg) treated group, ^{e}p < 0.05 vs PTZ+CA (60 mg/kg**). (D) MTT assay** where ^{a}p < 0.001 vs vehicle treated group, ^{b}p < 0.001vs PTZ (170mg/kg) treated group, ^{c}p < 0.001 vs PTZ+ UA(50 mg/kg) treated

group, [d]p < 0.001 vs PTZ+UA (150 mg/kg), [e]p < 0.001 vs PTZ+CA (60 mg/kg). [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid].

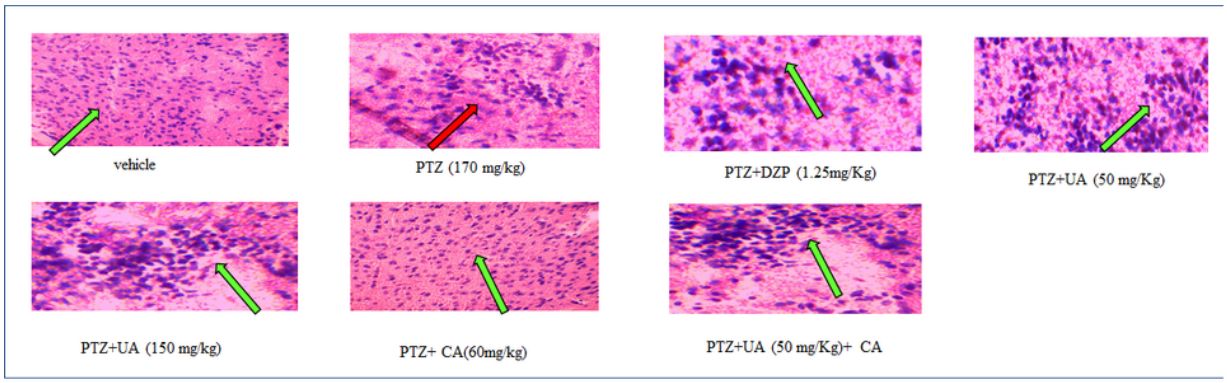

Figure 8A

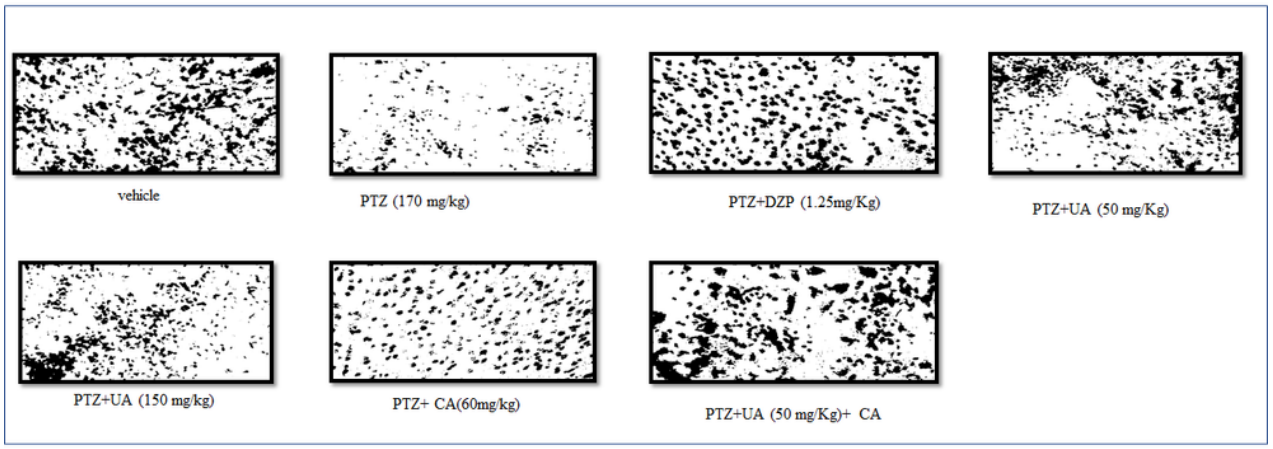

Figure 8B

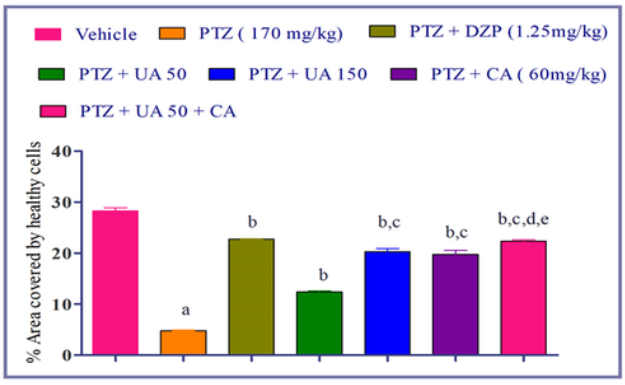

Figure 8C

Figure 8

Effect of UA and CA on Neuromorphological study of zebrafish brain by Haematoxylin and Eosin staining. **(A)** Representative image is of whole brain cross sectional. Red arrows depict pyknotic cells in PTZ (170 mg/kg) treated group and green arrow depicts normal histological structures of cells in vehicle treated group and restored histological degeneration in DZP (1.25 mg/kg), UA 50 mg/kg),UA (150 mg/kg), CA (60 mg/kg) and combination of UA (50 mg/kg) + CA (60 mg/kg) pretreated groups. magnification 40X, scale 20µm**. (B) Quantification of healthy cells (C)** Effect of combination of UA (50 mg/kg) and CA(60 mg/kg) on **% area covered by healthy cells** in different groups (n=3). Results were expressed as mean ± SD and analysed by one way ANOVA followed by Tukey's post hoc test. $^{a}p < 0.001$ vs vehicle treated group, $^{b}p < 0.001$vs PTZ(170 mg/kg) treated group, $^{c}p < 0.001$ vs PTZ+ UA (50 mg/kg) treated group, $^{d}p < 0.01$ vs PTZ+UA (150 mg/kg) , $^{e}p < 0.001$ vs PTZ+CA (60 mg/kg). [Abbreviations – PTZ: pentylenetetrazole, DZP : diazepam, UA : ursolic acid, CA: caprylic acid].

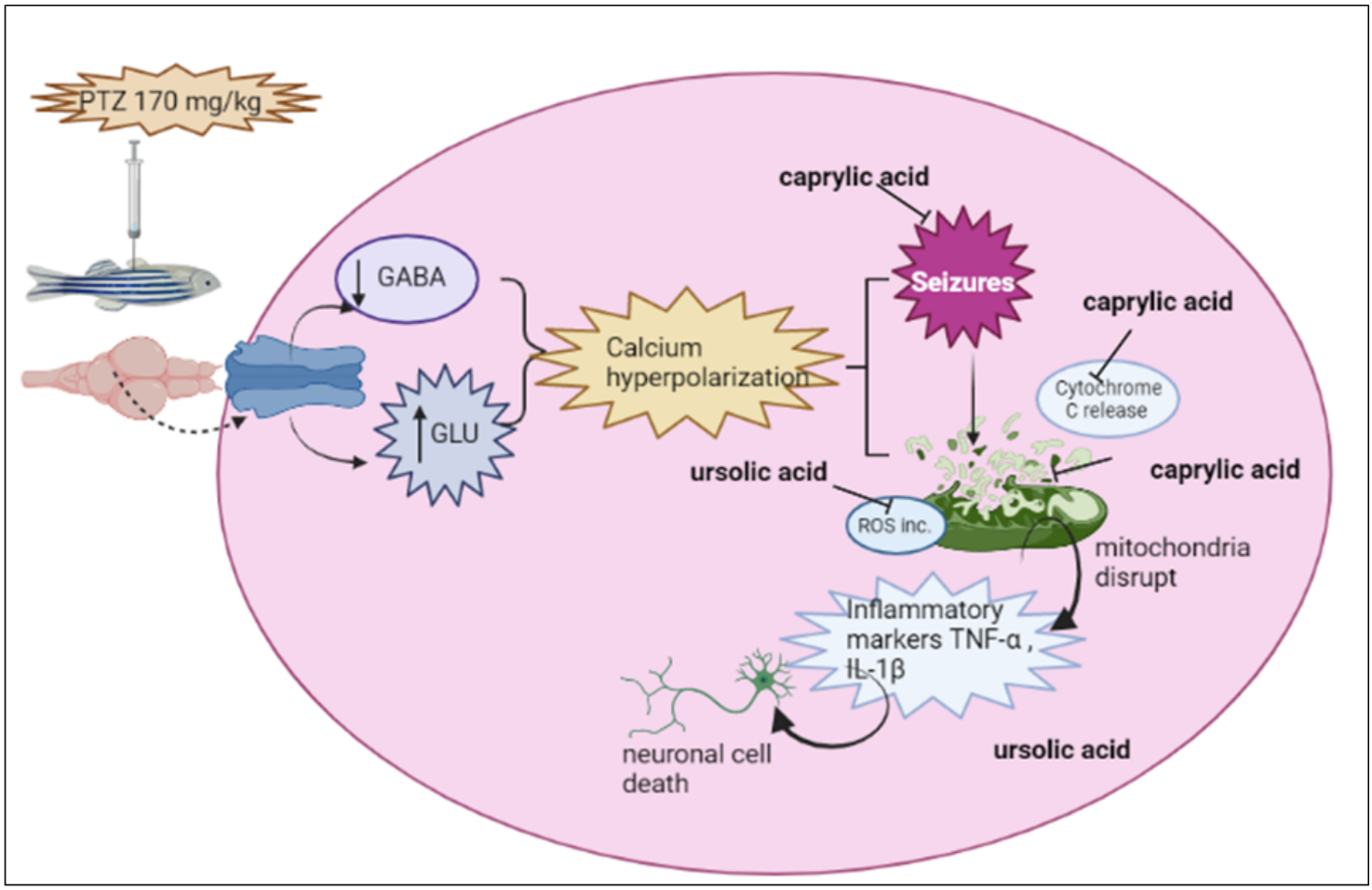

Figure 9

Figure 9

Mechanistic representation of UA and CA therapy on PTZ induced seizures. [PTZ: pentylenetetrazole, CA: Caprylic acid, UA: ursolic acid, TNF-α: Tumor necrosis factor, IL-1β: interleukin 1β, GABA: Gamma-aminobutyric acid. GLU: Glutamate].