Papaverine Derivatives, a Potent Inhibitor of Neuroinflammation in Epileptic Seizures, A Pharmacological and *In-Silico* Approach Against TNF-α as a Targeted Receptor

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Summary: Epilepsies are a heterogeneous group of disorders expressed in terms of propensity to experience spontaneous recurrent seizures attack. Verapamil, a synthetic amide derivative containing papaverine, is a calcium channel blocker that significantly reduces the frequency of seizures. Based on this, the current work was planned to concentrate on the synthesis of more recent papaverine derivatives that contain amides. Z1 and Z2 were created using synthesis. Following synthesis, docking analysis was performed, and bond energies were computed to determine their binding affinities against the TNF- α protein in particular because it upregulates neuroexcitation, enables excessive calcium absorption, and ultimately results in neurotoxicity. Later, compounds were examined for their antioxidant and antiepileptic properties. Elisa and H&E staining were also carried out, and the results demonstrated that treatment with papaverine derivatives reduced the biochemical and immunohistochemical changes brought on by pentylenetetrazol that were related to TNF- α.

Keywords: Papaverine derivatives, Anti-epileptic, Anti-oxidant, PTZ Induced epilepsy, The role of TNF- α

Introduction

The most striking feature of epilepsy, a chronic neurological disorder, is recurrent seizures. There are 50 to 70 million affected persons worldwide, thus safer profile drugs with better anti-convulsant efficacy are still required [1]. Antiepileptic drugs (AEDs) are used to treat epilepsy and are categorized in a number of ways. But only approximately a third of epileptic patients have complete remission [2]. Additionally, these medications' clinical applications have been connected to dependence, tolerance, and severe side effects include glaucoma, paresthesia, and the teratogenicity of agranulocytosis. Therefore, the development of innovative antiepileptic medicines that effectively and safely reduce epileptogenesis is urgently needed [3].

Over the past 20 years, research on the basic mechanisms of epileptogenesis has been conducted [4]. The involvement of TNF- in oxidative stress and apoptosis in the aetiology of epilepsy is well-known. TNF can control neuron activity and lead to epilepsy by increasing glutamate, lowering the production of -aminobutyric acid, promoting neuroinflammation, and altering the synaptic function in astrocytes [5]. According to recent studies, when TNF- was released by microglial cells as opposed to astrocytes, which induced inflammation and degeneration, it had a more tissue-repair and remyelination-focused effect [6]. Additionally, brain injury and neurological abnormalities were more common in animals with high levels of TNF- upregulation in their neurons and astrocytes. Consequently, being crucial in epilepsy [7].

The use of anti-TNF medications to treat epileptic disorders has received minimal attention [8]. However, a reduction in seizure frequency was the noted as the main outcome of a prospective, open-label study by Lagarde and colleagues, with "responders" being those who had a reduction in seizure frequency of at least 50%. None of the patients were seizure-free, but five of them responded to the medicine, and one more had a short decrease in the number of seizures. Overall, these results encourage further investigation into the potential efficacy of alternative TNF-inhibitors in the treatment of specific forms of epilepsy. Patients with illnesses that are slowly getting worse can be good candidates for anti-TNF-a medication [9].

Papaverine undergoes tetra-O-methylation at positions C6, C7, C3′, and C4′. In addition to O-linked methyl groups, the 1-benzylisoquinoline scaffold of papaverine also possesses a 3′-hydroxyl, 1, 2-, and 3, 4-desaturations, resulting in three fully conjugated rings [10]. A common drug for its anti-spasmodic and muscle-relaxing effects, it is an opium alkaloid. Additionally, it widens coronary arteries and brain blood vessels. Currently, it is also used to treat erectile dysfunction [11].

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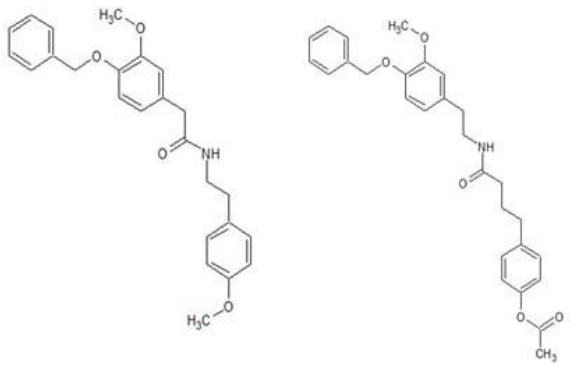
Verapamil, a papaverine derivative, is a potent anti-convulsant that lessens seizures by acting as a calcium channel blocker [12]. Numerous papaverine-containing products have been found to have anti-inflammatory and antioxidant effects. Additionally, papaverine has been shown to suppress the release of TNF-α and IL-1, making this research even more crucial [13].

The goal of the current work was to synthesis a number of new papaverine derivatives and examine their TNF-inhibitory potential for anti-epileptic activity in the PTZ-induced epilepsy model. The findings will not only aid in our understanding of the cascade mechanisms that result in cell death, but they will also hint at the possibility of papaverine derivatives serving as a future treatment.

Experimental

General procedure for the synthesis of papaverine derivative

By using the Bischler-Napicralski isoquinoline synthesis, papaverine can be produced from vanilline with a yield of about 15%. It can also be produced by charging a hydrogenator with a solution of acetic acid and methanol (10.0 mL and 2.0 mL, respectively) with a catalyst made of 10% Pd/C and papaveranol (1.0 g, 0.0027mol). Until the consumption of the hydrogen gas slows down, this is hydrogenated at 10 psi of hydrogen pressure at 25 to 35 OC. In the next steps, the catalyst can be removed, the solution concentrated, the leftover treated with 10% sodium carbonate solution (20 mL, 20 vol), and the solid removed. Column chromatography is used to further purify the crude product (silicagel, hexane-EtOAc, 8:2). The yield from this procedure is 0.86g (66%) of. Papaverine derivative [14].



Z1: 3-benzyloxy-4-methoxy-β-phenylethylamide of 3-methoxy-4-benzyloxy acetic acid
Z2: 3-benzyloxy-4-acetoxy-3-phenylpropanoic acid amide of β-3-methoxy-4-benzyloxy-phenylethylamine

Fig. 1: Structure of newly synthesized papaverine derivative.

Anti-oxidant activity of Papaverine derivatives

Utilizing the stated approach with a few minor modifications, 2,2-diphenylpicrylhydrazyl (DPPH) was used to test the anti-oxidant activity of the compounds. All compounds were produced as stock solutions with concentrations of 1 g/ml, 3 g/ml, 10 g/ml, 100 g/ml, 300 g/ml, 700 g/ml, and 1000 g/ml. Each test tube included 2 ml of the test samples, which were extracted from the stock solution. In a dark cabinet, 8 ml of the already-made DPPH solution was then added to the test tubes to bring the total amount to 10 ml. To stop light from oxidizing DPPH, all test tubes were wrapped in aluminum foil and placed in an incubator at room temperature for 30 minutes. When the test tubes' color changed from violet to yellow, it meant that the substances inside had the ability to oxidize. A UV visible spectrophotometer set to a wavelength of 517 nm was used to test the compounds' absorbance [15]. The following formula was used to compute the percentage of scavenging activity:

$$Percentage\ scavenging\ activity = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Finally, the results obtained were compared with the antioxidant potential of ascorbic acid as a standard.

In-Silico studies

Using Autodock Vina version 4.2.6, in-silico tests of recently synthesised papaverine derivatives were conducted to determine their possible binding affinities at the active binding sites of the target proteins (San Diego, CA, USA). The PDB files of the chosen TNF- α receptor's X-ray crystal structures (PDB ID: 2AZ5) were downloaded from the website for the RCSB protein database. We obtained the targets' active binding pockets from https://bio.tools/dogsitescorer (DoG Site Scorer) [16]. For molecular docking, ligand-protein complexes were prepared. The target proteins were cleaned using Biovia Discovery Studio Visualizer (DSV), which removed water and cocrystallized ligands before saving the data in PDB format. By tracing the structures of ligands in ChemSketch, mol files for those compounds were created. Using Open Babel, all papaverine derivative and ligand file formats were converted to PDB. Furthermore, AutoDock Tools version 1.5.6 was used to create PDBQT files for targets and ligands. Additionally, PyRx, a computer programme for molecular docking, was employed for docking purposes. The optimum conformational poses and ligand-target molecular

interactions were interpreted using Biovia DSV [17]. By contrasting the best poses of the co-crystallized ligands with the best poses of re-docked configurations, the docking technique was validated.

Animals and experimental groups

Swiss albino mice (25-30g) of either gender was kept in climate-controlled housing at a constant temperature of 22–25 °C and on a 12–12–12 cycle of light and darkness, with free access to food and water. Throughout the trial period, mice were housed in groups. five groups, each with five animals, were created at random from the entire animal population: I saline-treated control group with 2.5% DMSO (10 mL/kg, i.p); (ii) illness group treated with PTZ (90 mg/kg, i.p); (iii) positive control group treated with diazepam (1 mg/kg, i.p) plus PTZ (90 mg/kg, i.p); (iv) compound Z1 (20 mg/kg, i.p) plus PTZ (90 mg/ All substances were initially dissolved in 2.5% dimethyl sulfoxide (DMSO), and saline was used to adjust the volume. The Riphah Institute of Pharmaceutical Science (RIPS), Riphah International University, Islamabad, Pakistan, conducted all studies in compliance with the authorized protocols of its research and ethical commission (REC) (REC/RIPS/2018/17). Animals were sacrificed using the accepted methodology of CO_2 euthanasia after behavioral investigations. Brain tissues from one cohort were removed and kept at 80 °C before being homogenized to collect the supernatant for additional research (n = 5). For a different cohort, brain tissues were embedded in paraffin and preserved in 4% formalin before being cut into 4 m-thin coronal sections using a rotary microtome.

Anti-epileptic activity of Papaverine derivatives

In this study, 30 min after intraperitoneal injections of vehicle in the vehicle control group I diazepam in the positive control group (iii), and freshly synthesised derivatives in the sample groups, intraperitoneal PTZ (90 mg/kg) that was dissolved in saline was administered (iv and v). Doses have already been mentioned. Myoclonus and a generalised tonic-clonic seizure brought on by PTZ dosage were similar to tonic-clonic seizures in people. Animals were watched for the onset of myoclonus and generalised clonus immediately after receiving i.p. PTZ, and the latency to this phase was measured before the duration. The length of time between an i.p. PTZ injection and the start of a seizure was thought to be the latency. There were 5 groups of animals, each with n=5. Anti-convulsant drugs are those that slow the development of these seizures and/or lessen the duration of tonic-clonic seizures. Additionally, the animals' mortality was monitored [18].

$$Percentage\ mortality = \frac{Number\ of\ mice\ dead\ after\ convulsion}{Total\ number\ of\ mice\ used} \times 100$$

Hematoxylin and Eosin (H&E) Staining

Absolute xylene was used to deparaffinize tissue slides, and a gradient of ethyl alcohol (100–70%) was used as a rehydrating agent. All slides were cleaned with distilled water before being submerged in hematoxylin for at least ten minutes. Slides were washed in 1% HCl water and 1% ammonia before being dipped once more in water. The slides were also exposed to the eosin solution for ten minutes. These slides were then dried and cleaned with water. These slides were dehydrated using ethyl alcohol in various concentrations (70, 95, and 100). Glass cover slips were added as per the stated method after the slides had been cleaned with xylene. The photos were captured using a light microscope from Olympus in Tokyo, Japan. For each slide, three pictures were captured. To focus primarily on the inflammatory-infiltrated cells, all pictures were stained. Effects of neuron cell shapes on vacuolation and cell size. All TIF images' threshold intensities were changed equally.

Enzyme linked immunosorbent assay (ELISA)

TNF-α expressions were observed using rat ELISA in accordance with the manufacturer's published methodology, with a few minor variations. TNF- α ELISA kit from Shanghai Yuchun Biotechnologies, China (CAT# E-ELR0674). This protocol's method was broken down into two phases. In the first step, brain tissue supernatant was homogenized in phosphate buffer solution and kept at -80 0C. In the second phase, particular protein concentrates were treated with indicated antibodies in 96-well plates following centrifugation (13,500X g for 1 hour) to measure TNF-α. After the interaction of the substrate with the enzyme, the concentration of the required antibody was determined using an Elisa microplate reader (BioTek ELx808, Biotek, Winooski, VT, USA). Values were represented as cytokines per milliliter (pg/ml) pictograms. In triplets, the procedure was repeated.

Statistical Analysis

Results were presented as the mean SEM, and one-way analysis of variance (ANOVA) was used for analysis. Post hoc Tuckey's test was then performed using Graph Pad Prism 6. (San Diego, CA, USA). The behavioural data was also subjected to a two-way grouping analysis. ImageJ software was used to examine histological data. Against the saline group, the symbol # indicates relevance, and against the scopolamine group,

the symbol * indicates significance. At a p value of 0.05, data were deemed statistically significant.

Results and Discussion

Evaluation of In-silico studies

Two recently created papaverine derivatives were molecularly docked against the active TNF-α binding sites. **Table 1** provides an illustration of the outcomes. In order to confirm the molecular docking process, co-crystallized ligands were re-docked to the same target protein. Fig 2 shows a comparison of the optimal binding postures produced for cocrystallized compounds against TNF- α before and after re-docking. The lowest-energy conformer was compound Z1 was rigidly docked in separate experiment (**Fig. 2**). These findings contain the Kcal/mol values for the protein-ligand binding affinities as well as the RMSDs (root mean square deviations) for each binding mode. Leu 55, which forms the typical Pi-sigma bonds, is one of the protein residues that the docking studies have identified as being crucial for binding. Both substances failed to create a hydrogen bond. Similar to this, compound Z2's greatest energy conformer revealed specific amino acids that are essential for binding, such as Tyr 59, which forms a pi-sigma bond with the protein. The next step in this research was to carry out in vitro and in vivo tests to see if our chemicals have the potential to be anti-oxidant and anti-epileptic.

Table-1: Binding energy values obtained from docking compound Z1 and Z2 with TNF-α.

Compound	Protein	PDB ID	H Bond	Amino acid	Binding Energy (kcal/mol)
Z1			-	-	**-8.2**
Z2	**TNF-α**	**2AZ5**	-	-	**-8.4**

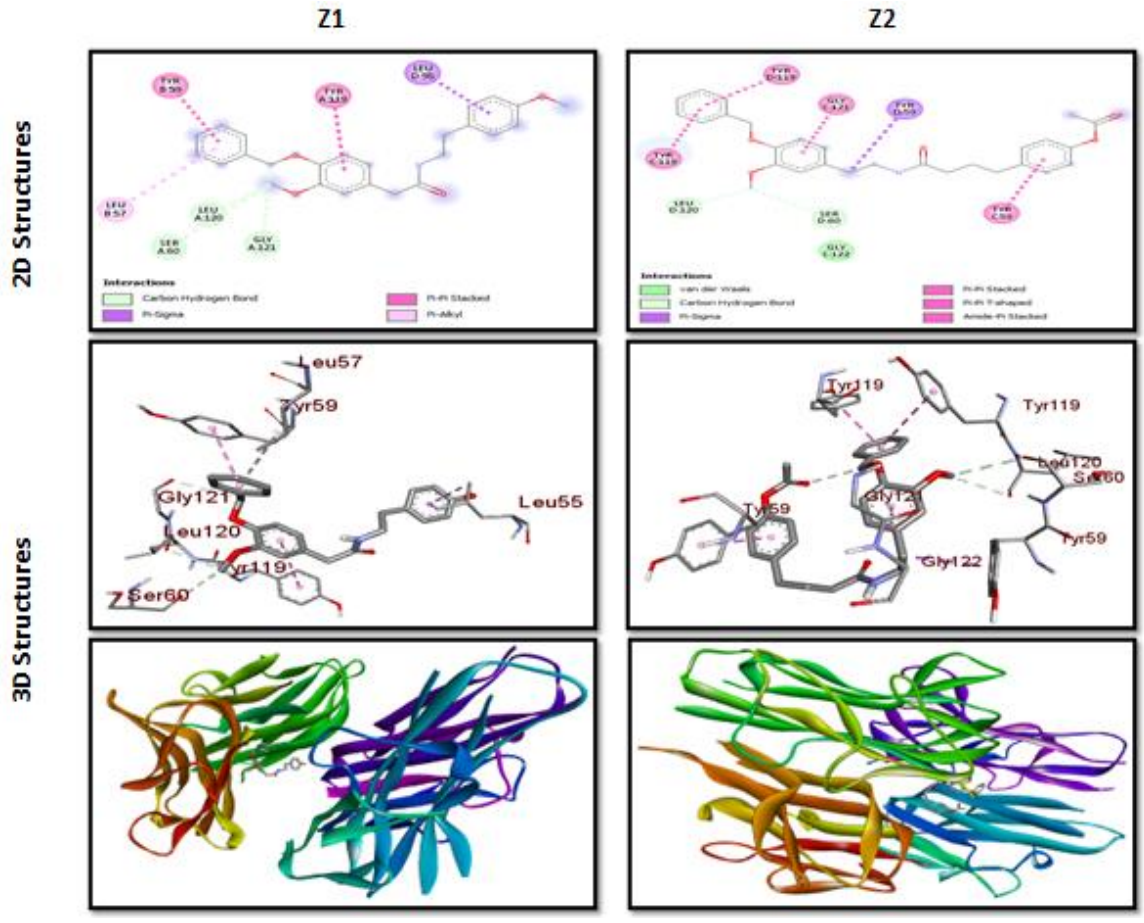


Fig. 2: Best binding pose for newly synthesized papaverine derivative Z1 and Z2 in the binding pockets of and tumor necrotic factor (TNF-α) respectively. A shows 2-D conformation whereas b and c shows 3-D conformation.

Anti-oxidant potential of Papaverine derivatives Z1 & Z2

The anti-oxidant activity of ascorbic acid (standard), Z1 and Z2 is shown as percentage SEM (Standard error of mean) in table no. 2. Z1 shows percentage SEM of 10.8% ± 2.8, 19.19% ± 3.76, 24.8% ± 4.04, 33.39% ± 1.47, 51.8% ± 2.61, 57.45% ± 3.60 and 60.89% ± 3.43 at concentration of 1µg/mL, 3µg/mL, 5µg/mL, 10µg/mL, 100µg/mL, 300µg/mL and 1000µg/mL respectively. Z2 shows percentage SEM of 12% ± 3.13, 19.71% ± 2.69, 37.32% ± 3.35, 44.55% ± 3.66, 62.14% ± 2.15, 65.73% ± 3.68 and 68.77% ± 3.23 at concentration of 1µg/mL, 3µg/mL, 5µg/mL, 10µg/mL, 100µg/mL, 300µg/mL and 1000µg/mL respectively. As shown in the Table no.2 and

Table-2: Table presents percent free radical scavenging of novel Papaverine derivatives (Z1-Z2) and ascorbic acid against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Values are expressed as mean ± SEM.

Strength of solution (µg/mL)	Ascorbic acid		Z1		Z2	
	Mean	SEM	Mean	SEM	Mean	SEM
1	32.91	2.59	10.83	2.80	12.00	3.13
3	36.63	3.28	19.19	3.76	19.71	2.69
5	39.53	2.80	24.80	4.04	37.32	3.35
10	42.97	2.68	33.39	1.47	44.55	3.66
100	60.04	2.11	51.80	2.61	62.14	2.15
300	78.54	3.32	57.45	3.60	65.73	3.68
1000	91.13	3.59	60.89	3.43	68.77	3.23

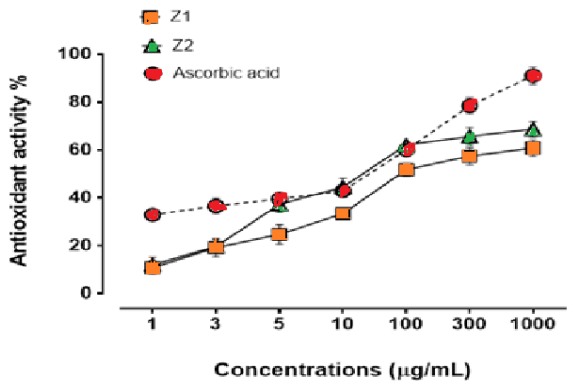


Fig. 3: Graph shows a comparison of percentage anti-oxidant activity of the standard Ascorbic acid with percentage anti-oxidant activity of newly synthesized compound Z1 and Z2. Values are expressed as mean ± SEM.

Antiepileptic activity potential of Papaverine derivatives Z1 & Z2

Group ii that was injected with normal saline solution, exhibited a 100% mortality rate within 5 minutes of injecting the PTZ dose, and the onset time of seizures was as short as 20- 40 seconds in all mice of this group. On the other hand, all other groups i.e., group iii, group iv and group v, showed 0% mortality throughout the span of 30 minutes following the PTZ dose and later. The mean SEM was 65.00 ± 2.1, for onset of myoclonic seizures, 98.00 ± 2.5 for onset of tonic-clonic seizures and 30.0 ± 2.1 for duration of tonic-clonic seizure after injecting Z1, at a dose of 20 mg/Kg dose while Z2 showed the mean ±SEM to be 76.20 ± 3.4 for onset of myoclonic seizures, 120.00 ± 2.7 for onset of tonic-clonic seizures and 20.0 ± 2.0 for duration of tonic-clonic seizure after injecting Z2, also at a dose of 20mg/kg. Diazepam being the standard for anti-epileptic activity showed a mean SEM of 78.60 ± 2.5 for myoclonic seizure, 125.80 ± 4.6 for onset of tonic-clonic seizures and 15.6 ± 3.6 for duration of tonic-clonic seizures at a dose of 1 mg/Kg. It was observed that compound Z1 and Z2 did vividly prolong the onset time of myoclonic and tonic-clonic seizures. However, duration of tonic-clonic seizure was not drastically reduced although a significant reduction in the time duration can still be observed. The results also indicate that the synthesized compounds did not turn out to be toxic at the mentioned doses as the mortality was seen to be 0.00%.

$$Percentage\ mortality = \frac{Number\ of\ mice\ dead\ after\ convulsion}{Total\ number\ of\ mice\ used} \times 100$$

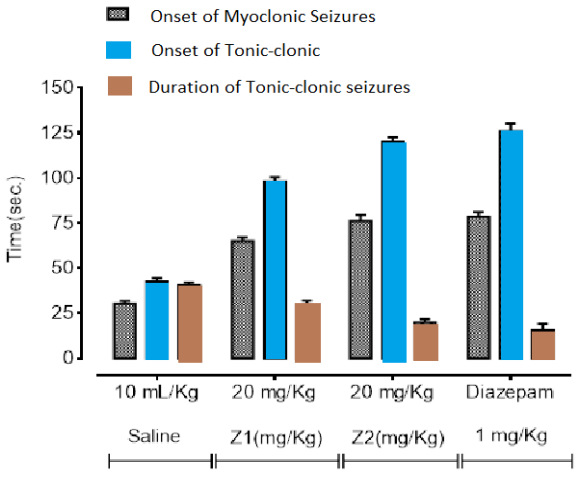


Fig. 4: Graph shows a comparison between newly synthesized derivatives with diazepam treated group on onset time of myoclonic, onset time of tonic-clonic seizures and the duration of tonic-clonic seizures.

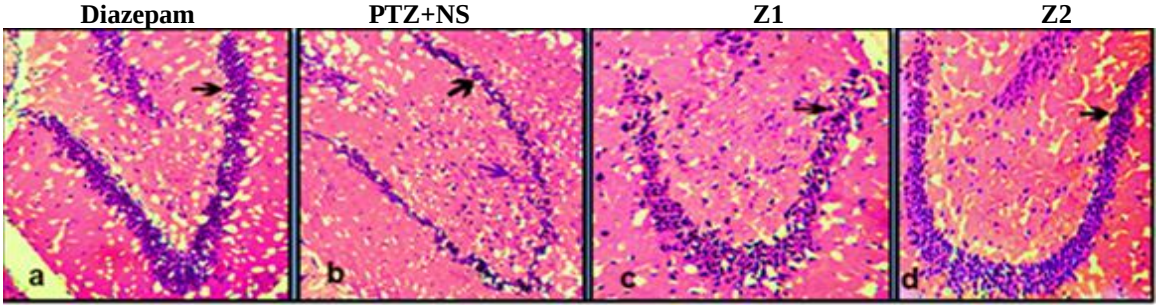
Effect of papaverine derivatives Z1 & Z2 on PTZ-induced neuroinflammation

By using hematoxylin and eosin staining, Papaverine derivatives' neuroprotective potential was further clarified. Compared to the saline control group, the PTZ-treated group of mice showed significant histological changes in the granular cell layer of the dentate gyrus of the brain (Fig 5A). PTZ treatment, as shown, caused aberrant histological characteristics such as altered neuronal morphology linked to cytoplasmic eosinophilia and nuclear basophilia. Administration of papaverine derivatives showed a significant reduction in PTZ-induced neurotoxicity. As a result, groups treated with Papaverine derivatives showed higher levels of cellular intactness and integrity (p 0.05, Fig 5B).

Table-3: Effect of newly synthesized Papaverine derivatives on pentylenetetrazole (PTZ)-induced epilepsy. # Denotes a significant difference against the saline group; *, **, *** show significant difference against the PTZ group. $p < 0.05$ is considered to be significant.

Groups	Mean SEM (myoclonic jerks)	Mean SEM (Onset of tonic-clonic seizures)	Mean SEM (Duration of tonic-clonic seizures)
Group ii **NS (10ml/kg, ip.) + PTZ (90mg/Kg ip)**	**30.45 ± 1.5$^{\#}$**	**42.67 ± 1.8$^{\#}$**	**40.6 ± 1.5$^{\#}$**
Group ii **Diazepam (1 mg/kg, ip.) + PTZ (90mg/Kg ip)**	**78.60 ± 2.5****	**125.80 ± 4.6*****	**15.6 ± 3.6*****
Group iv **Z1 (20 mg/kg, ip.) + PTZ (90mg/Kg ip)**	**65.00 ± 2.1****	**98.00 ± 2.5****	**30.0 ± 2.1***
Group v **Z2 (20 mg/kg, ip.) + PTZ (90mg/Kg ip)**	**76.20 ± 3.4*****	**120.00 ± 2.7****	**20.0 ± 2.0*****

A



B

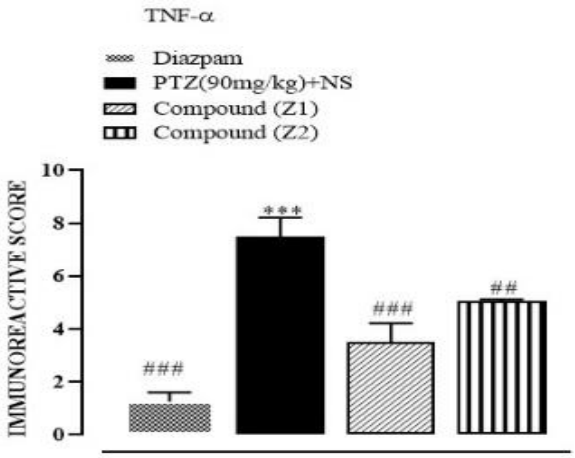


Fig. 5: (A) Effect of pretreatment with test compound on H & E staining demonstrating histopathological changes in the granular cell layer of dentate gyrus. Photomicrographs are 20X original magnifications. control group showing normal histopathology. PTZ group represented remarkable histopathological deformations. Diazepam and treated compound Z1 & Z2 groups having normal pathology. (B) # shows significant difference relative to relative to disease group *. Data presented as means ± SEM. ** $p < 0.01$, * $p < 0.05$.

Effect of papaverine derivatives Z1 & Z2 on attenuation of PTZ-induced neuroinflammation

The upregulation of TNF-α in the PTZ-treated brain was supported by ELISA data (Fig 6, p 0.01). TNF- α overexpression was significantly reduced by papaverine derivatives (p 0.05). Through additional confirmation using immunohistochemical analysis, it was discovered that the PTZ-treated brain displayed a similar pattern of TNF-α expression, although papaverine derivatives drastically reduced the levels of TNF-α.

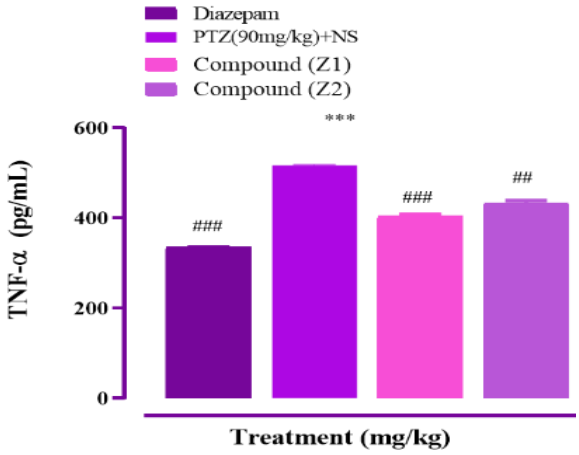


Fig. 6: Fig represents the protein expression of TNF-α. Test compound Z 1& Z2 lessen the release of neuroinflammatory mediators shown by ELISA. All data were presented in the form of mean ± SEM (n=5). The data is presented as mean ± SEM. * $p < 0.05$ relative to scopolamine, while $p < 0.05$ and $p < 0.01$ relative to saline control group ($n = 5$).

Conclusion

Novel papaverine derivatives were created and synthesized in the current work. To rank and reveal the specifics of their binding and atom interacting interactions, molecular docking data against TNF-α were obtained. Free radical scavenging assays performed in vitro revealed that each of these substances has dose-dependent antioxidant properties. Additional in vivo findings revealed that novel derivatives significantly decreased the oxidative stress caused by PTZ that causes seizures, possibly by controlling the TNF-α pathway, ultimately providing neuroprotection against neuronal inflammation. Further neuroinflammatory diseases including Alzheimer's and Parkinson's may benefit from the usage of these compounds.

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