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1 **Tetramethylpyrazine contributes to the neuroprotection in a rodent epileptic model of**
2 **pentylentetrazole-induced kindling**

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20 **Running title: Neuroprotection against PTZ induced kindling**

21

22

23 **Abstract:**

24 **Objectives:** In the present study, TMP was evaluated for its therapeutic potential as an
25 alternative therapy for epileptogenesis and its associated comorbidities in rats.

26 **Methods:** The sub-convulsant dose of Pentylentetrazole (PTZ) (35 mg/kg, i.p) was injected on
27 alternative days to produce kindling for 32 days and observed for seizure score percent of
28 kindled animals in each group. After kindling, the animals were evaluated in models of anxiety,
29 memory, and predictive of depression. The neuroprotective effect of TMP was assessed by
30 estimating the biochemical parameters in the cortex and hippocampus of the brain.
31 Histopathological alterations were also observed in the cortex and hippocampus (CA1, CA3, and
32 DG).

33 **Key findings:** The administration of TMP reduced the seizure score and percentage of kindled
34 animals dose-dependently. Furthermore, TMP significantly improved the behavioural parameters
35 measured in the predictive models of depression but not in the anxiety and cognitive
36 performances of the animals. The oxidative-nitrosative stress, excitotoxicity, neuroinflammation,
37 and histological alterations in the brain induced by PTZ were significantly mitigated by
38 administering the TMP high dose of 60 mg/kg.

39 **Conclusion:** In conclusion, the TMP attenuated the depression behaviour in the PTZ induced
40 kindled rats, and reduced the oxidative-nitrosative stress, excitotoxicity, neuroinflammation, and
41 histological alterations of the brain.

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44

45 **Introduction**

46 Epilepsy is a chronic neurological disorder characterized by synchronizing abnormal
47 electrical discharge of a group of excitable neurons, manifested as seizures.^[1] Epilepsy is known
48 for its behavioural abnormalities, which are estimated to affect 70 million population
49 worldwide.^[2] The ultimate goal in treating epilepsy is to offer a good quality of life without
50 seizures and associated comorbidities. Epilepsy and its associated neuropsychiatric
51 manifestations like depression, anxiety, and cognitive impairments have been recognized for
52 decades. Despite having several new antiepileptic agents, the management of epilepsy was still
53 inadequate due to the side effects and high rate of refractoriness to the existing drugs.^[3,4] So, the
54 present study was intended to investigate alternative medicine, which can provide a good quality
55 of life without seizures.

56 Kindling is a widely used chronic animal model for investigating epileptogenesis and
57 helps in evaluating the novel antiepileptic agents in the drug discovery process. The repeated
58 administration of the sub-convulsive Pentylentetrazole (PTZ) dose as a GABA_A receptor
59 antagonist is commonly used for kindling the animals in experimental studies.^[5] Furthermore, the
60 PTZ induced kindling exhibits behavioural alterations, revealing that this model also mimics the
61 comorbidities of epilepsy in animal models.^[6] Researchers proposed various pathophysiological
62 mechanisms like oxidative stress, mitochondrial dysfunction,^[7] neuroinflammation,^[8] and
63 imbalance in the excitatory and inhibitory neurotransmitters^[9] as the underlying cause of the
64 seizures and its comorbidities.^[4] Therefore, targeting the neurochemical balance, mitigating
65 reactive oxygen species (ROS) and reactive nitrogen species (RNS), and attenuation of
66 neuroinflammation may be a useful preventive or treatment approach in managing epilepsy and
67 related comorbidities.

68 Tetramethylpyrazine (TMP) is one of the principal active compounds isolated from
69 *Ligusticum wallichii* (Chuan Xiong), a Chinese herbal medicine.^[10] TMP has been demonstrated
70 in the treatment of several neurovascular and cardiovascular diseases and is popularly known to
71 exhibit anti-oxidant, anti-apoptotic and anti-inflammatory properties. Previous experimental
72 studies have reported that TMP exerts significant neuroprotection in models of global and focal
73 cerebral ischemia,^[11,12] Parkinson's disease (PD),^[13] Alzheimer's disease,^[14] and traumatic brain
74 injury (TBI).^[15] In addition, TMP has been proven for its neuroprotective activity against various
75 neurotoxic agents like 3-nitropropionic acid,^[16] and also kainate-induced excitotoxicity
76 models.^[17] Literature supports that TMP's protective potential may be due to its high antioxidant
77 potential and downregulation of pro-inflammatory cytokine production.^[18,19] TMP is reported to
78 have an ameliorating effect on mitochondrial dysfunction by promoting the biogenesis of
79 mitochondria.^[20] Furthermore, TMP showed an anti-depressant effect^[21] and attenuates memory
80 impairment in animal models. Since these reports of central nervous system effects are strongly
81 supported that the TMP may effectively penetrate the blood brain barrier. The continuous
82 monitoring of TMP concentrations in the blood and brain samples of rats with the help of micro-
83 dialysis indicates that the unbound TMP best fit to a two compartment model and the elimination
84 half-life were found to be 82.1 and 184.6 min in rat's blood and brain, respectively.^[22] So the
85 pharmacokinetic data, brain/blood concentration ratios of TMP suggested that the effective
86 penetration of TMP through the blood brain barrier. However, whether TMP could prevent the
87 PTZ induced kindling associated neurodegeneration and behavioural alterations is not yet
88 known. To our knowledge, no study has explained the effect of TMP on PTZ induced kindling.
89 Hence, keeping the above literature in mind, the present study was designed to evaluate TMP's
90 effect on PTZ-kindling induced seizures by evaluating the behavioural, bio-chemical, pro-

91 inflammatory cytokines, and histopathological studies in rats. The results might implicate a new
92 therapeutic agent with lower side effects.

93 **Materials and methods**

94 **Animals:** Adult male Wistar rats weighing 150-200 g were procured from Mahaveer Enterprises,
95 Hyderabad, India. All the animals were maintained at standard temperature ($25^{\circ}\text{C} \pm 5^{\circ}\text{C}$) and
96 humidity ($55 \pm 10\%$), with a 12:12 h light-dark cycle. The rats were allowed to have free access
97 to food and water ad libitum. The experimental protocol was approved by the Institutional
98 Animal Ethical Committee (1725/GO/Re/S/13/CPCSEA) of Acharya Nagarjuna University
99 College of Pharmaceutical Sciences, dated 29/01/2019 with an approval number
100 ANUCPS/IAEC/AH/P/9/2019. All the experiments were performed following the committee for
101 the purpose of control and supervision of experiments on animals (CPCSEA), India.

102 **Drugs:** Tetramethylpyrazine (Sigma-Aldrich, USA), PTZ (Alfa aesar, India), Valproic acid (Sun
103 Pharmaceutical Industries Ltd, India), TBA (Otto Chemical, India). 5,5' dithiobis-(2-
104 Nitrobenzoic acid) DTMB (Loba Chemie, India) and all other chemicals were purchased from
105 Hi-Media Laboratories Pvt, Ltd., Mumbai.

106 **Dose selection:** The dose of PTZ^[23] and TMP^[16] was selected from the previous literature.

107 **Experimental design:** Animals were randomly divided into five groups, and each group
108 consisted of 15 animals. The treatment schedule was summarized as follows.

109 Group I: normal saline (i.p)

110 Group II: PTZ (35 mg/kg, i.p)

111 Group III: Valproic acid (150 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

112 Group IV: TMP (30 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

113 Group V: TMP (60 mg/kg, i.p) + PTZ (35 mg/kg, i.p)

114 The first group of animals served as a normal control group and was administered with
115 normal saline intraperitoneally (i.p). The second group of animals was administered with PTZ
116 (35 mg/kg, i.p) and served as a seizure control group. The third group was administered with a
117 standard drug, valproic acid (150 mg/kg, i.p), along with PTZ (i.p), and served as standard
118 treatment. The fourth and fifth groups were administered with TMP (30 mg/kg and 60 mg/kg,
119 i.p) suspended in normal saline, respectively, along with PTZ (35 mg/kg, i.p). In all the
120 experimental groups, the treatments were given 30 min before the administration of PTZ. The
121 respective treatments were given for 32 days, with 16 alternative injections of PTZ.^[24] After 24
122 hours of the last PTZ injection, behavioural performances were assessed in all the animals, but
123 the forced swim test was performed 48 hours after kindling. After 48 hours, the animals were
124 sacrificed to estimate biochemical and histological alterations in the brain.

125 **Kindling procedure:** The sub-convulsant dose of PTZ (35 mg/kg, i.p) was dissolved in saline
126 and injected on alternative days to all the animals except the normal control group. The animals
127 were observed for 30 min for seizure scores by placing the animals in individual boxes after the
128 PTZ injection. The intensity of the seizure was recorded according to Racine's seizure score^[25]
129 as follows:

130 Score 0- No response

131 Score 1- Hyperactivity, restlessness, and vibrissae twitching

132 Score 2- Head nodding, head clonus, and

133 Score 3- Myoclonic jerks

134 Score 4- Forelimb clonic seizure with rearing

135 Score 5- Generalized tonic-clonic seizures with falling.

136 Animals were considered kindled when the seizure control group presented a seizure score of 4
137 or 5 in three consecutive PTZ injections.

138 **Behavioural parameters:** Each group of animals was divided into two subgroups with an equal
139 probability of having kindled and non-kindled animals randomly to assess the behavioural
140 parameters. The first subgroup of animals was used to determine the open field test,^[26] novel
141 object recognition test (NOR),^[27] and novel place recognition test (NPR)^[27], elevated plus maze
142 test for anxiety.^[24] The second subgroup of animals from each group was assessed for the Y
143 maze test,^[16] elevated plus maze test for memory,^[16] and forced swim test.^[28] The tests were
144 performed in the same order as described in the previous literature.

145 **Biochemical estimations:** All the subgroups of animals were euthanized with ketamine 60
146 mg/kg/i.p and decapitated for harvesting the brain samples. Six brain samples from the first
147 subgroup were used to estimate oxidative stress parameters, nitrite levels, and
148 acetylcholinesterase activity (AChE) in the half of the cerebral hemisphere. The remaining
149 cerebral hemisphere was used for the estimations of GABA and glutamate. The biochemical
150 oxidative stress parameters like malondialdehyde (MDA),^[29] nitrite,^[30] reduced glutathione
151 (GSH),^[31] catalase (CAT)^[32] and superoxide dismutase (SOD),^[32] along with AChE^[33] were
152 estimated in the brain homogenate of the hippocampus and cortex, as described in the previous
153 literature. The amino acid neurotransmitters like GABA and glutamate were evaluated in all the
154 hippocampus and cortex of the brain by using paper chromatography, as described in the
155 previous literature.^[34]

156 **Estimation of pro-inflammatory cytokines:** The remaining six animals from the second
157 subgroup were euthanized with ketamine 60 mg/kg/i.p and decapitated for harvesting the brain
158 samples. The brain samples were sectioned into the hippocampus and cortex from each cerebral

159 hemisphere, and used to estimate pro-inflammatory cytokines. The remaining half of the cerebral
160 hemisphere from each animal was used for the histological studies.

161 The dissected hippocampus and cortex were rinsed with 0.9% cold saline and
162 homogenized (10% w/v) individually in ice-cold phosphate buffer (0.1 M, pH 7.4), centrifuged
163 at 10,000 RPM to collect the supernatant for the estimations of IL- β and TNF- α in the
164 hippocampus and cortex. The analysis of IL- β and TNF- α was done using ELISA kits and the
165 protocol of the (ELAB-sciences, China).^[35]

166 **Histopathological study:** The remaining half of each group's cerebral hemispheres were stored
167 in the formalin (10 % v/v) solution. After 24 hours, the brain samples were embedded in the
168 paraffin wax, dehydrated with a series of alcohol with different concentrations, and cleaned the
169 samples with xylene. Three coronal sections from each brain sample were dissected into 5 μ m
170 thickness and stained with hematoxylin and eosin (H & E) to observe the viable neuronal cell
171 count in the hippocampus and cortex.^[34]

172 **Statistical analysis**

173 All the values were expressed as Mean \pm standard error mean (SEM). The seizure score
174 was analyzed using two-way ANOVA, followed by Bonferroni's post hoc test for multiple
175 comparisons. The behavioural parameters were analyzed by Kruskal-Wallis test followed by
176 Dunn's Multiple Comparison test. All the other parameters were analyzed by one-way ANOVA
177 followed by Tukey's test by using Graph pad prism. The significance was set at $P \leq 0.05$.

178 **Results**

179 **Effect of TMP on seizure score:** The sub-convulsive dose of PTZ for 32 days gradually
180 increased the seizure score in 1st, 2nd, 3rd and 4th week significantly ($P < 0.01$, $P < 0.001$, $P < 0.001$)

181 and $P < 0.001$ respectively) in the PTZ group of animals compared to the control group of animals
182 [$F(4, 55) = 46.7$]. TMP 60 mg/kg showed a significant reduction in seizure score and % kindling
183 in the 3rd ($P < 0.001$) and 4th ($P < 0.001$) week of the study period compared to the PTZ treatment
184 group. However, the low dose of TMP 30 mg/kg decreased the seizure score and % kindling, but
185 the results were not significant compared to the PTZ treatment group at the end of the treatment
186 schedule. The valproate treatment group showed efficient protection against PTZ induced
187 kindling. It also showed a significant difference in seizure scores at 3rd ($P < 0.001$) and 4th
188 ($P < 0.001$) weeks than the PTZ treatment group of animals (**Table 1 and Figure 1**).

189
190 **Effect of TMP on open field test:** The number of crossings indicates the exploratory behaviour
191 of the animals in the open field test. In this test, we observed a significant ($P < 0.05$) decrease in
192 the number of crossings in the PTZ control group of animals compared to the normal control
193 group of animals. The treatment groups valproate and TMP did not present any significant
194 increase in the number of crossings compared to the PTZ control group of animals (**Figure 2A**).

195 The anxiety-like behaviour of animals was assessed by the % of time spent in the central
196 square of the open field. The % time spent in the central square represents the anxiolytic
197 behaviour of the animals. In the present study, the PTZ control group of animals reduced the %
198 time spent in the central square than the control group of animals, but not significantly. The VPA
199 and TMP treatment groups were also not significantly different from the PTZ control group of
200 animals in % of the time spent in the central square of the open field test. (**Figure 2B**).

201
202 **Effect of TMP on the elevated plus-maze test:** The elevated plus-maze test was performed to
203 evaluate the anxiety-like behaviour of the animals. In our study, the PTZ control group of

204 animals showed a significant ($P<0.05$) decrease in the % of time spent in the open arm and also a
205 significantly ($P<0.01$) decrease in the number of open arm entries. The treatment groups
206 valproate, TMP 30 mg/kg, and 60 mg/kg did not significantly increase the % of time spent in the
207 open arm and the number of open arm entries. The anxiety index showed a significant increase
208 ($P<0.01$) in the PTZ control group of animals compared to the normal control group of animals.
209 The treatment group, valproate showed a significant decrease ($P<0.05$) in the anxiety index than
210 the PTZ control group of animals. In contrast, TMP 30 & 60 mg/kg did not significantly decrease
211 the anxiety index than the PTZ control group of animals (**Figures 3A, 3B & 3C**).

212
213 The elevated plus-maze test was also performed to evaluate the memory dysfunction in animal
214 models. In the present study, the PTZ control group of animals showed a significant ($P<0.001$)
215 increase in the retention transfer latency compared to the control group of animals. The treatment
216 group valproate, showed a significant ($P<0.01$) amelioration of memory dysfunction by
217 decreasing the retention transfer latency as compared to the PTZ control group of animals. The
218 TMP treatment at both doses also noticeably decreased the retention transfer latency (**Figure 4**).

219
220 **Effect of TMP on Y-maze test:** The spatial memory was significantly ($P<0.001$) decreased in
221 the PTZ control group of animals by reducing the % spontaneous alternations compared to the
222 control group of animals. The treatment group valproate showed a significant ($P<0.01$) increase
223 in the % spontaneous alternations when compared to the PTZ control group of animals, which
224 indicates the improvement of spatial memory. The TMP treatment also showed the fairly
225 substantial increase in the % spontaneous alternations when compared to the PTZ control group
226 of animals (**Figure 5**).

227

228 **Effect of TMP on novel object recognition test:** In the memory phase of the NOR test, almost
229 all groups of animals except PTZ group showed a considerable (but not significant) increase in
230 exploration behaviour at the novel object rather than the familiar object. The PTZ control group
231 of animals not at all significantly explored the novel and familiar objects. Accordingly, the
232 exploratory ratio of the PTZ control group of animals was significantly ($P<0.01$) decreased when
233 compared to the control group of animals. However, the treatment group's valproate and TMP 30
234 & 60 mg/kg didn't show a significant increase in exploratory ratios compared to the PTZ control
235 group of animals (**Figures 6A & 6B**).

236

237 **Effect of TMP on novel place recognition test:** The PTZ control group of animals relatively
238 spent less time at the novel place than in the familiar place compared to the control animals
239 ($P<0.05$). The treatment group valproate alone showed a considerable (but not significant)
240 increase in novel place exploration rather than the familiar place. The PTZ control group of
241 animals showed a significant ($P<0.01$) inhibition of the exploratory ratio compared to the control
242 group of animals. The treatment group valproate showed a significant ($P<0.05$) increase in the
243 exploratory ratio compared to the PTZ control group of animals. The TMP treatment did not
244 show a significant increase in the exploration ratio compared to the PTZ control group of
245 animals (**Figures 7A & 7B**).

246

247 **Effect of TMP on forced swim test:** The PTZ control group of animals showed a significant
248 ($P<0.001$) increase in the immobility time compared to the control group of animals. The

249 treatment groups valproate, and TMP 60 mg/kg showed significant ($P<0.05$) decrease in the
250 immobility time when compared to the PTZ control group of animals (**Figure 8**).

251
252 **Effect of TMP on oxidative stress parameters:** The MDA and nitrite levels were significantly
253 increased in the PTZ control group of animals compared to the normal control group of animals
254 in the hippocampus [$F(4, 25) = 5.26, P<0.01$ & $F(4, 25) = 15.2, P<0.001$] and cortex [$F(4, 25)$
255 $= 4.76, P<0.01$ & $F(4, 25) = 7.86, P<0.001$] respectively [$F(4, 25) = 5.26$. The standard VPA
256 treatment group showed significant alleviation in MDA ($P<0.01$ & $P<0.05$) and nitrite levels
257 ($P<0.01$) in the hippocampus and cortex when compared to the PTZ control group of animals.
258 The TMP 60 mg/kg showed a significant ($P<0.01$) decrease in MDA and nitrite levels in the
259 hippocampus and the cortex. The TMP 30 mg/kg did not significantly decrease MDA and nitrite
260 levels compared to the PTZ control group of animals.

261 The endogenous antioxidant parameters like GSH [$F(4, 25) = 10.3, P<0.001$ & $F(4, 25)$
262 $= 7.21, P<0.01$], CAT [$F(4, 25) = P<0.001$ & $F(4, 25) = 19.3, P<0.001$] and SOD [$F(4, 25) =$
263 $5.34, P<0.01$ & $F(4, 25) = 5.29, P<0.01$] were significantly diminished in the PTZ control group
264 of animals in the hippocampus and cortex, respectively as compared to the normal control group
265 of animals. The VPA treatment group significantly attenuated the PTZ induced alteration in the
266 levels of GSH ($P<0.01$), CAT ($P<0.01$ & $P<0.001$), and SOD ($P<0.05$) in the hippocampus and
267 cortex, respectively, as compared to the PTZ control group of animals. The TMP 60 mg/kg
268 significantly improved the levels of GSH ($P<0.01$), CAT ($P<0.01$) and SOD ($P<0.05$) in the
269 hippocampus and cortex, respectively, as compared to the PTZ control group of animals. The
270 TMP 30 mg/kg showed a significant elevation in the CAT ($P<0.05$) levels in the cortex alone as
271 compared to the PTZ control group of animals (**Table 2**).

272 **Effect of TMP on AChE activity:** The administration of a chronic sub-convulsive dose of PTZ
273 significantly raised the activity of AChE [$F(4, 25) = 12.6, P < 0.001$ & $F(4, 25) = 13.4, P < 0.01$]
274 in the hippocampus and cortex, respectively, compared to the normal control group of animals.
275 The VPA treatment significantly inhibited the AChE ($P < 0.001$ & $P < 0.01$) activity in the
276 hippocampus and cortex, respectively, compared to the PTZ control group of animals. The TMP
277 treatment groups 30 mg/kg ($P < 0.05$) and 60 mg/kg ($P < 0.01$ & $P < 0.05$) attenuated the activity of
278 AChE in the hippocampus and cortex, respectively when compared to the PTZ control group of
279 animals, whereas significance was not found in the cortex of TMP 30 mg/kg treatment group
280 **(Figure 9)**

281

282 **Effect of TMP on GABA and glutamate:** The administration of chronic sub-convulsive doses
283 of PTZ significantly ($P < 0.01$) altered the neurotransmitters in the brain by escalating the
284 glutamate levels and declining the GABA levels in the hippocampus [$F(4, 25) = 5.32$ & $F(4,$
285 $25) = 5.19$] and cortex [$F(4, 25) = 5.58$ & $F(4, 25) = 4.13$] when compared to the control group
286 of animals respectively. The VPA treatment significantly ($P < 0.05$) diminished the
287 neurotransmitter alterations in the hippocampus and cortex induced by the PTZ compared to the
288 PTZ control group of animals. The TMP 60 mg/kg showed a significant ($P < 0.05$) decrease in the
289 glutamate levels in the hippocampus and cortex when compared to the PTZ control group of
290 animals. GABA levels were also significantly ($P < 0.05$) elevated in the hippocampus but not in
291 the cortex than in the PTZ control group of animals. At the same time, the low dose of TMP 30
292 mg/kg did not significantly differ from the PTZ control group of animals in both hippocampus
293 and cortex **(Figures 10A & 10B)**.

294

295 **Effect of TMP on pro-inflammatory cytokines:** The administration of PTZ significantly
296 ($P<0.001$) elevated the pro-inflammatory cytokines (IL-1 β & TNF- α) in the hippocampus [$F(4,$
297 $25) = 30.80$ & $F(4, 25) = 9.864$] and cortex [$F(4, 25) = 41.51$ & $F(4, 25) = 14.73$] when
298 compared to the control group of animals. The standard VPA treatment group significantly
299 reduced the levels of IL-1 β and TNF- α in both the hippocampus ($P<0.001$ and $P<0.01$) and
300 cortex ($P<0.001$ and $P<0.05$), respectively when compared to the PTZ control group of animals.
301 The TMP 60 mg/kg treatment group was significantly decreased the IL-1 β and TNF- α levels in
302 both the hippocampus ($P<0.001$) and cortex ($P<0.05$), whereas the TMP 30 mg/kg has not
303 significantly decreased the levels of pro-inflammatory cytokines, except IL-1 β ($P<0.05$) in the
304 hippocampus when compared to the PTZ control group of animals (**Figures 11A & 11B**).

305

306 **Effect of TMP on histological alterations in the hippocampus and cortex:** The present study
307 observed histological alterations in the hippocampus (CA1, CA3, and DG) and cortex. The PTZ
308 treatment group significantly ($P<0.001$) decreased the viable cell count in the different regions of
309 the hippocampus (CA1, CA2, and DG) [$F(4, 25) = 18.7$, $F(4, 25) = 23.8$, and $F(4, 25) = 14.1$]
310 and cortex [$F(4, 25) = 14.1$]. The treatment group VPA significantly attenuated the changes
311 induced by PTZ in the hippocampus CA1 ($P<0.01$), CA3 ($P<0.001$), and DG ($P<0.01$) along
312 with the cortex ($P<0.01$) as compared to the normal control group of animals. The TMP of 30
313 mg/kg increased the viable neuronal cell count in all the regions, but significance ($P<0.05$) was
314 only found in the CA3 and DG regions of the brain compared to the PTZ control group of
315 animals. The high dose of TMP 60 mg/kg attenuated PTZ induced neuronal loss significantly
316 ($P<0.05$) in the hippocampus (CA1, CA3, and DG) and cortex as compared to the PTZ control
317 group of animals (**Figure 12 & Table 3**).

318

319 **Discussion:**

320 The PTZ induced model was well established to explore the epileptogenesis pattern and
321 the behavioural, biochemical, and neurochemical alterations affected by epilepsy.^[36] Further, the
322 kindling model is a pivotal and indistinguishable model of pharmacoresistant epilepsy,
323 interfering with the current antiepileptic drug therapy.^[23] So, in the present study, we
324 investigated the effect of TMP on the development of PTZ induced kindling and its associated
325 behavioural despair in rats.

326 According to the previous reports,^[5,23,37] repetitive administration of sub-convulsive
327 doses of PTZ on alternative days results in kindling, as evidenced by the seizure score adapted
328 from Racine's scale and % of animals kindled. Our finding in the present study revealed that the
329 treatment with TMP 60 mg/kg diminished seizure score and the % of animals kindled at the end
330 of the 4th week of kindling with PTZ. A recent study demonstrated that the TMP 20 mg/kg and
331 50 mg/kg significantly reduced the seizure score in electrical kindled mice. In contrast, TMP did
332 not reverse the generalized seizures induced by both maximal electroshock (MES) and
333 pentylenetetrazole (PTZ) models in the same study.^[38] Our study results are consistent with their
334 research in the kindling model, but the significant reversal of seizure score was observed only in
335 the late stage of the treatment protocol (3rd and 4th week). We hypothesized that the chronic
336 treatment with TMP reduced the progression of seizure score in the late stage of PTZ kindling
337 instead of the initial stages of the kindling process.

338 Recurrent seizures are associated with emotional imbalance,^[39] psychological
339 problems,^[40] and cognitive impairment.^[41] Many patients with epilepsy have been diagnosed

340 with emotional disorders like anxiety and depression.^[42] In the present study, the open field test
341 and elevated plus maze test were performed to evaluate the anxiety-like behaviour in the PTZ
342 induced kindled animals. There were no significant differences between the groups in the open
343 field test. In line with the previous literature,^[43] in the present study, PTZ induced kindling did
344 not increase the time spent in the periphery of the open field test, which is not anxiogenic. VPA
345 and TMP treatment groups were also not significantly different from the PTZ alone treatment
346 group. In contrast, in the elevated plus-maze test, the PTZ control group increased the anxiety
347 index more than the control animals. In rodents showing repugnance towards the open and
348 elevated spaces, they spent more time in the dark and enclosed areas, indicating an anxiogenic
349 nature of the animals. In the present study, the PTZ induced kindling associated anxiety in the
350 elevated plus-maze test is in line with the previous reports.^[6,44] Our study results showed that the
351 treatment group VPA showed a significant decrease in the anxiety index in the elevated plus-
352 maze test. Additionally, TMP 60 mg/kg treated animals showed a fairly substantial decrease in
353 the anxiety index. A recent study^[45] has demonstrated that the TMP treatment decreased the
354 anxiety index and reduced the grooming behaviour in the single prolonged stress (SPS) animal
355 model. Further, in the same study, it was also proved that TMP administration reduced anxiety-
356 like behaviour, which is indicated by an increase in the central zone exploration during the open
357 field test. Lee et al. (2018)^[45] hypothesized that the anti-anxiety effect of TMP is related to its
358 inhibitory effect on serotonergic dysregulation. Our results of the elevated plus-maze test are in
359 line with the previous literature.^[45]

360 Experimental and clinical evidence has proved that epileptic patients suffer from
361 cognitive impairment.^[46] It has been shown that the memory deficit caused by chronic
362 administration of PTZ is a result of excessive generation of free radicals and subsequent neuronal

363 damage in several regions of the brain.^[47] Some other factors may contribute to kindling-induced
364 cognitive impairments, such as a decline in the acetylcholine levels and increased neuronal death
365 in the hippocampal regions of the brain.^[23] In the present study, we found that the PTZ induced
366 kindling results in cognitive impairment, which is evident in the elevated plus-maze test, Y-maze
367 test, novel object recognition test, and novel place recognition test. The PTZ kindling-induced
368 cognitive impairment results are in good agreement with the previous literature.^[23,43] With the
369 exception of the novel object recognition test, treatment with VPA in the current investigation
370 alleviated cognitive deficits. The TMP treatment demonstrated dose-dependent therapeutic
371 effectiveness even though it did not significantly address cognitive impairments. Several reports
372 supported the protective effect of TMP on memory impairment. TMP proved to be a potent
373 pharmacological agent in improving cognitive performance by restoring cAMP/PKA/CREB
374 signalling pathway deficit against scopolamine-induced memory impairment.^[48] In another
375 study, TMP mitigated the short-term and long-term memory impairment induced by intracerebral
376 administration of streptozotocin by inhibiting the GSK-3 β and restoring the cholinergic
377 function.^[49] Our recent study on the effect of TMP against 3-nitropropionic acid-induced
378 neurotoxicity showed a significant improvement in cognitive performance by protecting the
379 neuronal cells in the hippocampus and restoring the cholinergic neurotransmission in the
380 brain.^[16] The present study results are in good agreement with the previous literature.^[16,23]

381 Another most frequent comorbidity associated with epilepsy is depression, with a
382 prevalence of 25-55% in epileptic patients.^[50] The present study assessed the depression in
383 kindled animals by the forced swim test. A considerable increase in the immobility time
384 indicates depression in animal studies. PTZ control group of animals showed depression-like
385 behaviour, as revealed by longer immobility time in the forced swim test. Imbalance in the

386 central monoaminergic levels has been considered the major contributing factor in the
387 development of depression.^[51] Studies showed that a decline in the monoamines in PTZ induced
388 kindled rats results in a longer immobility time in the forced swim test.^[1] Our study also showed
389 a significant increase in the immobility time in the PTZ control group of animals. Whereas
390 treatment with VPA and TMP (60 mg/kg) reduced the immobility time significantly, suggesting
391 the potential of TMP in mitigating the PTZ induced depression. The results are in tune with the
392 earlier reports, demonstrated in several animal models. Alteration in the hippocampal neuronal
393 cells is the hallmark in the animal models of depression. In addition, scientific reports also
394 indicated the role of oxidative stress in depression-like symptoms and the therapeutic benefits of
395 several antioxidants in dealing with depression.^[52] Our study results proved that the treatment
396 with TMP restored the antioxidant defence in different brain regions, which may be the
397 contributing factor in decreasing the immobility time in kindled rats. Some studies stated that the
398 TMP treatment promoted the BDNF signalling pathway, and phosphorylation of CREB proteins
399 in the hippocampus may be attributed to its anti-depressant activity.^[21] The present study results
400 showed the anti-depressant effect of TMP, which may be attributed to its neuronal protection in
401 the hippocampus.

402 In the previous literature, it was reported that a significant increase in the brain/blood
403 concentration ratio of TMP^[22] and a lower brain/blood concentration ratio to the VPA indicates a
404 higher penetration of TMP into the brain rather than the VPA.^[53] Contrary to the
405 pharmacokinetic data, in the present study, the behavioural alterations induced by the PTZ
406 kindling were significantly mitigated by the standard drug VPA rather than the TMP
407 administration. However, treatment with the TMP substantially reduced the behavioural
408 alterations dose dependently. Hence, the possible reason for an insignificant improvement in

409 behavioural alterations with the TMP might be due to its modest dose administration than the
410 VPA.

411 Studies have demonstrated that redox homeostasis is essential for the brains' normal
412 functioning. The excessive generation of ROS and RNS contributes to impairment in the brain's
413 redox state, which appears to be involved in the pathogenesis of epilepsy.^[54,55] Accumulating
414 evidence indicated that the administration of PTZ increases the generation of ROS and RNS,
415 which may play an essential role in neuronal damage.^[56] Moreover, currently using conventional
416 antiepileptic drugs disrupt the redox homeostasis by increasing the oxidative stress, thereby
417 worsening the brain's antioxidant status that may hinder the antiepileptic activity.^[2,55] So, it may
418 prove worthwhile to use an alternative antiepileptic agent with potent antioxidant properties in
419 modulating the process of epileptogenesis. MDA is an end product of lipid peroxidation, an
420 indicator of oxidative damage. The innate antioxidant defence system like SOD, CAT, and GSH
421 acts as scavengers against oxidative stress.^[57] Our study results confirm with the earlier studies
422 that the PTZ kindled rats showed a significant increase in the MDA levels and a significant
423 decline in the SOD, CAT, and GSH levels in different regions of the brain. The treatment with
424 VPA and TMP restored the antioxidant defence system and reduced MDA levels in the brain.
425 The study findings confirm the antioxidant potential of TMP and its neuronal protection. Further,
426 TMP and its derivatives proved to be a potent activator of the Nrf2 signalling pathway. They
427 were responsible for enhancing the antioxidant defence system, inhibiting the excitotoxicity, and
428 inhibiting the apoptotic process in the neuronal cells.^[58,59]

429 Maintaining a balance between the excitatory and inhibitory neurotransmitters in the
430 CNS plays a crucial role in preventing neuronal disorders, especially epilepsy. It has been
431 reported that the elevated levels of NO in the striatum modulate the release of the excitatory

432 neurotransmitter glutamate in the chemically induced neurotoxic models.^[60] On the other hand,
433 studies proved that the elevation of nitrotyrosine promoted the peroxidation of lipids.
434 Observations in the PTZ kindled animals lend additional confirmation to the involvement of
435 nitrosative stress in the seizure-mediated hippocampal neurodegeneration.^[56] In the present
436 study, NO levels were indirectly measured by estimating the nitrite levels in the brain. The PTZ
437 control group showed elevated nitrite levels in the hippocampus and cortex in tune with the
438 literature. The treatment with VPA and TMP reduced the levels of nitrite induced by PTZ in the
439 hippocampus and cortex. TMP proved its inhibitory response on iNOS to reduce the nitrosative
440 stress and inflammatory response in the retinal capillary endothelial cells.^[61] Thus, reducing
441 nitrosative stress might be a prominent therapeutic approach to mitigating epileptogenesis in
442 kindles rats.

443 Studies have documented the cholinergic dysfunction in the PTZ kindled animals.^[23] In
444 tune with the literature, the present study also showed a significant decline in the acetylcholine
445 levels in the hippocampus and cortex, which was evident by an indirect measure of increased
446 AChE activity. Furthermore, elevated AChE may be a significant contributing factor in kindling-
447 induced cognitive impairment.^[23] Since the dysfunction in the cholinergic system of the epileptic
448 brain has been reported in the literature, we hypothesized that the enhancement of cholinergic
449 activity in the brain is a potential target in mitigating epilepsy and its related comorbidities.^[62]
450 Treatment with the VPA and TMP showed significant mitigation in cholinergic dysfunction. A
451 recent study on the effect of TMP against 3-NP induced Huntington's disease-like symptoms in
452 rodents also proved the role of TMP in increasing the Ach levels in the brain.^[16] So, the results
453 proposed TMP's role in ameliorating epilepsy and its associated comorbidities by declining the
454 AChE in the hippocampus and cortex.

455 The alterations of excitatory and inhibitory neurotransmission are implicated in the
456 pathophysiology of epileptic patients and animal models.^[60] The previous literature proved that
457 the administration of PTZ induced kindling elevated the levels of glutamate and decreased
458 GABA levels in the hippocampus and cortex of the brain.^[23] Studies showed that elevated levels
459 of glutamate and aspartate were observed in the brain's extracellular regions following the
460 administration of PTZ in rats.^[36] Further, the PTZ- induced kindled rats showed decreased
461 neuronal uptake of glutamate, which results in neuronal excitotoxicity.^[63] Indeed, the present
462 study results showed elevated levels of glutamate and reduced GABA levels in the PTZ kindled
463 rats. The cell cultures of glioma treated with the TMP reduced the glutamate levels in the culture
464 media. It was speculated that it might be due to inhibition of glutamate biosynthesis and
465 enhancement of glutamate uptake.^[64] The TMP also proved its neuroprotective activity against 3-
466 nitropropionic acid-induced neurotoxicity by elevating the GABA levels in the brain.^[16] In line
467 with the previous literature, the TMP 60 mg/kg treatment significantly opposed the kindling-
468 induced neurochemical alteration by decreasing the glutamate levels and elevating the GABA
469 levels in both hippocampus and cortex. Thus, the present study results suggest a firm link
470 between the neurochemical balance in treating epilepsy and its associated comorbidities with
471 TMP.

472 In addition, the experimental and clinical evidence indicates that the inflammation in the
473 brain might be a consequence of epilepsy or its cause.^[65] The animal models of epilepsy also
474 showed increased pro-inflammatory cytokines like IL-1 β , IL-6, and TNF- α in the brain
475 tissue.^[2,66] Similar observations were also found in the serum and cerebrospinal fluid samples of
476 epileptic patients.^[67] The overexpression of inflammatory cytokines like IL-6 and TNF- α in the
477 astrocytes demonstrated the decreased seizure threshold, increased frequency of spontaneous

478 seizures, and neuronal loss in animal models.^[68,69] On the other hand, IL-1 β regulates neuronal
479 excitability by decreasing the production of the inhibitory neurotransmitter GABA^[70] and may
480 promote other cytokines IL-6 TNF- α .^[71] In the present study, the pro-inflammatory cytokines
481 were significantly elevated in the PTZ control groups of animals, consistent with the previous
482 literature.^[2,66] TMP attenuated the production of pro-inflammatory cytokines in the activated
483 microglial cells and effectively reduced NF-kB activation.^[35] In another study, TMP attenuated
484 the neuroinflammation via the miR-150/AKT3 pathway regulation and mitigated the cognitive
485 impairment induced by anaesthetics.^[72] TMP treatment groups decreased the pro-inflammatory
486 cytokines dose-dependently in the current study in parallel with the literature. So, the
487 neuroprotective potential of TMP might be strongly associated with the inhibition of
488 neuroinflammation in the brain.

489 Further, the neuroprotective role of TMP against PTZ induced kindling was assessed by
490 histological studies in the hippocampus (CA1, CA3, and DG) and cortex. Recurrent seizures lead
491 to neuronal death.^[73] Studies have shown that the PTZ kindling induces neuronal damage in the
492 hippocampus and cortex, resulting in cognitive impairment.^[23,74] Literature proved that oxidative
493 stress, mitochondrial dysfunction, and excitotoxicity promote the neuronal cells to
494 neurodegeneration.^[57] In line with the literature, study results showed a significant decrease in
495 the viable neuronal count in the hippocampus (CA1, CA3, and DG) and cortex. VPA and TMP's
496 treatment groups significantly protected the neuronal cells from neurodegeneration and increased
497 the viable neuronal count in the hippocampus (CA1, CA3, and DG) and cortex. These results are
498 in good agreement with the earlier reports, stating that the attenuation of 3-NP induced
499 Huntington's like symptoms and cerebral ischemic conditions by the administration of TMP in
500 rodents.^[16,75]

501 As a multi-target product, TMP is promising and deserves more intensive research to
502 establish an appropriate dose for its therapeutic benefit. Several studies were done to examine its
503 clinical effectiveness; the findings are too good to be true. There is no definitive information on
504 the average dosage, therapeutic duration, or adverse event reporting, all of which are crucial for
505 clinical trials. The establishment of standard TMP dose use would be more beneficial. The
506 outcomes of a clinical study would thus be more convincing and provide trustworthy support for
507 practical decision-making. Academics still face several challenges, such as toxicity, efficacy, and
508 pharmacological effects, despite the overwhelming interest in therapeutic usage.

509

510 **Conclusion and future perspectives:**

511 The current study results revealed that TMP's administration attenuated the development
512 of PTZ induced kindling, but it was not significantly mitigated the behavioural alterations except
513 depression behaviour. Whereas, VPA administration significantly attenuated the behavioral
514 alterations when compared to the TMP treatment. However, treatment with the TMP
515 substantially reduced the behavioural alterations dose dependently. The observed effects may be
516 attributed to decreasing oxidative-nitrosative stress, excitotoxicity, neuroinflammation, and
517 increasing the AChE levels in the brain. The neuroprotection against PTZ-induced kindling was
518 confirmed by the histological observation in the hippocampus and cortex. Further
519 pharmacokinetic and dynamic studies are warranted in preclinical and clinical scenarios to
520 establish an appropriate dose and the development of TMP as a therapeutic regimen for the
521 management of epilepsy and associated behavioural alterations. In conclusion, the

522 multifunctionality of TMP make it a good option for future investigation in the quest to attain the
523 highest possible therapeutic effectiveness with the lowest possible toxicity.

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750

751

752 **Table 1**

753 *Effect of TMP on seizure score and seizure latency*

Groups	Week 1	Week 2	Week 3	Week 4	% of animals Kindled	Number of animals died/used
PTZ	1.33 ± 0.211 ^{*b}	1.67 ± 0.333 ^{*c}	3.17 ± 0.477 ^{*c}	4.00 ± 0.365 ^{*c}	66.66	2/15
Valproic acid +PTZ	0.500 ± 0.224	0.833 ± 0.307	1.17 ± 0.167 ^{@c}	1.67 ± 0.333 ^{@c}	0	0/15
TMP 30 mg/kg + PTZ	0.667 ± 0.333	1.17 ± 0.167	1.67 ± 0.494 ^{#c}	3.00 ± 0.365	33.33	1/15
TMP 60 mg/kg + PTZ	0.500 ± 0.224	1.17 ± 0.401	1.33 ± 0.211 ^{\$c}	2.00 ± 0.447 ^{\$c}	16.66	0/15

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755 Results are expressed as mean ± SEM (n=12); *b, *c indicates P<0.01, P<0.001, respectively
756 PTZ Vs control; @c indicates P<0.001 PTZ Vs VPA; #b, #c indicates P<0.01, P<0.001,
757 respectively PTZ Vs TMP 30 mg; \$c indicates P<0.001 PTZ Vs TMP 60 mg. Data were
758 analyzed by two-way ANOVA, followed by Bonferroni's post hock test using Graph pad prism
759 software. Statistical significance was set at P ≤ 0.05.

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762

763 **Table 2**764 *Effect of TMP on PTZ induced oxidative stress parameters in the hippocampus and cortex.*

Treatment	Hippocampus					Cortex				
	MDA	Nitrite	GSH	CAT	SOD	MDA	Nitrite	GSH	CAT	SOD
	nmol/g m wet weight tissue	Nmol/g m wet weight tissue	µmol/gm wet weight tissue	µmol/min/ gm wet weight tissue	Units/gm wet weight tissue	nmol/g m wet weight tissue	Nmol/g m wet weight tissue	µmol/g m wet weight tissue	µmol/min/ gm wet weight tissue	Units/gm wet weight tissue
Normal control	132 ± 38.60	47.4 ± 6.08	2.02 ± 0.28	16.2 ± 1.69	19.9 ± 2.36	Normal control	123 ± 20.4	82.8 ± 4.46	2.76 ± 0.53	18.9 ± 1.45
PTZ	371 ± 58.50**	100 ± 4.97***	0.56 ± 0.08***	6.15 ± 0.96***	8.60 ± 1.38**	PTZ	328 ± 67.0**	132 ± 4.91***	0.37 ± 0.05**	6.21 ± 1.05***
VPA	149 ± 32.10##	68.5 ± 3.91##	1.72 ± 0.21##	13.4 ± 1.24##	18.0 ± 1.41#	VPA	133 ± 15.1#	96.1 ± 7.93##	2.77 ± 0.61##	14.7 ± 0.97###
TMP 30 mg/kg	202 ± 45.60	87.3 ± 5.11	0.91 ± 0.07	9.98 ± 0.67	13.7 ± 2.17	TMP 30 mg/kg	178 ± 35.0	112 ± 6.27	1.16 ± 0.23	11.0 ± 0.94#
TMP 60 mg/kg	174 ± 28.40#	74.1 ± 5.25#	1.53 ± 0.18##	12.9 ± 1.27##	16.7 ± 2.00#	TMP 60 mg/kg	164 ± 28.6#	103 ± 8.54#	2.48 ± 0.30##	12.9 ± 0.77##

765

766 Results are expressed as mean \pm SEM (n=6); **, *** indicates P<0.01, P<0.001, respectively, Vs
 767 control; #, ## and ### indicates P<0.05, P<0.01, and P<0.001 respectively, Vs PTZ. Data were
 768 analyzed by one-way ANOVA followed by Tukey's test using Graph pad prism software.
 769 Statistical significance was set at $P \leq 0.05$.

770

771 **Table 3**

772 *Effect of TMP on the hippocampus (CA1, CA3, and DG) and cortex neuronal count in PTZ*
 773 *induced kindled rats.*

Treatment	Hippocampus			Cortex
	CA1	CA3	DG	
Normal control	33.5 \pm 2.64	29.8 \pm 2.26	52.5 \pm 3.43	30.5 \pm 2.88
PTZ	11.8 \pm 1.58***	9.83 \pm 1.25***	22.5 \pm 2.47***	11.7 \pm 1.89***
VPA	23 \pm 1.81##	21.5 \pm 1.26###	43.0 \pm 4.11##	22.3 \pm 1.73##
TMP 30 mg/kg	15.3 \pm 1.41	14.8 \pm 1.76#	27.0 \pm 2.78	16.3 \pm 1.28
TMP 60 mg/kg	20.5 \pm 1.93#	17.5 \pm 0.76#	38.5 \pm 3.10#	20.5 \pm 1.06#

774

775 Results are expressed as mean \pm SEM (n=6); *** indicates P<0.001, Vs control; #, ##, and ###
 776 indicate P<0.05, P<0.01, and P<0.001, respectively, Vs PTZ. Data were analyzed by one-way
 777 ANOVA followed by Tukey's test using Graph pad prism software. Statistical significance was
 778 set at $P \leq 0.05$.

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