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Proanthocyanidin-Rich-Fraction of *Vitis vinifera* Seed Abrogates Convulsion Indices: Glutamatergic/ NMDA Inhibition, Enhancement of Anti-Neu N, and NRF2 Expression

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ABSTRACT

Proanthocyanidins are oligomeric flavonoids with central nervous system activities. This study investigated the mechanisms of anticonvulsant and neuroprotective effects of proanthocyanidin-rich-fraction (PRF) obtained from *Vitis vinifera* seed. A total of 90 male Albino mice were challenged with convulsion using picrotoxin, strychnine, or pilocarpine-hydrochloride at 30th minutes of intraperitoneal injection of 0.9% NaCl (0.2 mL), PRF (200, 100, 50 mg/kg), reference drug, diazepam (7.5 mg/kg), ketamine (0.5 mg/kg), or haloperidol (5 mg/kg) post-treatment. Thereafter, the onset of convulsion and percentage mortality was recorded. The histomorphological and immunohistochemical (glial fibrillary acid protein [GFAP], neuronal nuclear protein [NeuN], and nuclear factor erythroid 2-related factor 2 [NRF2) profiles of the hippocampus were analyzed. Besides marked delay in the onset of convulsion and percentage mortality in the PRF treatment, especially, there was attenuation in the hippocampal (CA 1) morphological aberrations due to picrotoxin, and pilocarpine-induced convulsion. The GFAP expression was inhibited in the PRF treatment groups. There was an increase in the intensity score of Anti-Neu N in the PRF treatment groups, while a decrease in expression of the hippocampal neurons was observed across the convulsed mice. The Nrf2 count was decreased due to convulsion but increased significantly in the PRF-treated mice. The delay in seizure onset reduced hippocampal impairment, and mortality due to the convulsion could be traceable to the inhibition of glutamatergic/NMDA transmission and enhancement of Anti-Neu N, and NRF2 expression.

Keywords: Convulsion, Proanthocyanidin-rich-fraction, *Vitis vinifera* seed, Neuroprotection

Introduction

One of the most debilitating, chronic, non-communicable diseases of the brain is epilepsy. [1] This central nervous system (CNS) disorder is often distinguished by repeated seizures.[1] Epilepsy seizure affects more than 70 million people worldwide. Although epilepsy is a global disease, it has an unequal distribution, and about 80% of the affected individuals reside in low and middle-income countries.[2] Proanthocyanidins, also called condensed tannins, are oligomers and polymers of monomeric flavonoids.[3] Proanthocyanidin is found in leaves, fruits, bark, seeds, flowers, and roots of many plants, including the *Vitis vinifera* (red grape) plant.[4] Accumulating evidence has shown numerous biological importance of proanthocyanidin such as antioxidant, hypnotic, anticonvulsant.[4,5] Despite potential pharmacological effects in the management of diverse forms of CNS disorders including epilepsy seizures, the scientific community has given little attention to their therapeutic importance[5]. Besides gamma-aminobutyric acid ($GABA_{A)}$ receptors, findings from experimental studies have shown that the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor is another potential target during refractory convulsive SE.[6]

One of the most important reasons for the treatment of SE patients is to ensure stability in the normal systemic functions in a bid to minimize brain injury until seizure control is achieved eventually.[7] However, about 60% of patients with epilepsy receive no antiepileptic treatment, largely for economic and social reasons.[8], while 33% of the patients who receive antiepileptic treatment are poorly managed.[9] Findings from our previous study showed the sedative and anticonvulsant activities of proanthocyanidin-rich-fraction (PRF) obtained from *Vitis vinifera* seed in electrically convulsed mice (Accepted by epilepsy and behavior but yet to be published). However, this present study was designed to evaluate the mechanism of anticonvulsant effects of PRF obtained from *Vitis vinifera seed* in male Albino mice.

Materials and Methods

Plant material

The air-dried powdered seed of *Vitis vinifera* was obtained from the Wuhan Venz Pharm., China. The method of extraction, purification, quantification, and identification used as High-Performance Liquid Chromatography. [10]

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Ethical approval
Ethical approval was obtained from the Health Research Ethics Committee, Institute of Public Health, Obafemi Awolowo University, Ile –Ife, with identification number IPH/OAU/12/1061 which is in line with the National Institute of Health (NIH) in the "Guild to the care and use of animals in Research and Teaching" (National Institute of Health, USA, 2011).

Animals
Ninety male Albino mice (3 months old, 23-25 g) were procured from the animal house of the College of Health Sciences, Osun State University, Osogbo, Nigeria. The Animals were kept in propylene cages in groups of 6 mice per group under a natural 12-h light-dark cycle and were given free access to feed and water *ad-libitum.* In addition, the animals were allowed to acclimatize to the laboratory conditions for seven days before the commencement of the behavioral study.

Drugs
Pilocarpine hydrochloride, diazepam, propylene glycol, picrotoxin, carbamazepine, and strychnine (Sigma-Aldrich Co. USA) were dissolved in normal saline.

Assessment of anticonvulsant activities
Three individual experiments were carried out using various antagonists/agonists.
In experiment I, mice were grouped into 5 (n = 6) and pre-treated with normal saline (0.2ml), proanthocyanidin-rich- fraction (PRF) (200. 100, 50 mg/kg, i.p), and diazepam (5 mg/kg). Thirty minutes later, each mouse received a single intraperitoneal (i.p) injection of picrotoxin (7.5 mg/kg, i.p.), [11] and the indices of behavioral manifestations of convulsion (latency to first clonic or tonic convulsion; development of convulsion; latency to death; and percentage mortality) were recorded. However, strychnine (4 mg/kg)[11], and pilocarpine-hydrochloride (350 mg/kg)[12] were used in the second and third experiments to induce the convulsion while ketamine, and carbamazepine (reference drugs) were administered in groups experiment 3 & 4 respectively.
The progression in the behavioral manifestation of convulsion in each experiment was monitored and video recorded (DS126311; Canon Inc., Tokyo Japan), in a crystal-clear glass box (15cm X 15cm X 10cm). [12]

Discetion and hippocampal histomorphology
The dead mice as a result of convulsion were decapitated immediately, while the remaining live mice in each group were euthanized by cervical dislocation 5 hours after the induction of convulsion. The cranium was crack-opened, while the brains were fixed in 10% neutral-buffered formalin, while the tissue samples were subjected to routine histological processing.
Histomorphological and morphometrical assessments of the hippocampus. This was carried out according to the previous method of Osuntokun *et al.*[13]

Immunohistomistry of the hippocampus and photomicrograph
The glial fibrillary acid protein (GFAP) expression was determined according to the principle described in the previous study of Osuntokun *et al* .[12], and the expression of a neuronal nuclear protein of the hippocampus was carried out according to the method of Gusel'nikova and Korzhevskiy.[14] Photomicrographs sections of the gross structure and sub-regions (CA 1) were taken at X 100 and 400 magnifications.

Statistical analysis
A one-way analysis of variance (ANOVA) and Student Newman-Keul posthoc test were used to analyze the data expressed as mean ± SEM, using. A significant difference was set at $p < 0.05$.

Results and Discussion

The seizure onset was delayed significantly ($p = 0.0004$) across the PRF-treatment groups. However, there was a marked ($p = 0.0003$) delay in the onset of convulsion in the PRF 50 mg/kg treatment group (just as it was found in the reference drug DZP relative to PRF (200 mg/kg). Development of convulsion was attenuated across the treatment groups but more noticeable in the PRF (50 mg/kg) and DZP treated mice. The latency to death was delayed significantly ($p = 0.0035$) in the PRF treated mice compared with the control. Similar to what is obtainable in the reference drug DZP, PRF (50 mg/kg) delayed the latency to death significantly (0.0028) among the PRF-treated mice. There was 60 % mortality in the PRF (200, and 100 mg/kg) treated group, 20% in PRF (50 mg/kg), while DZP pre-treated mice had 0% (Table 1).The latency to convulsion was delayed ($p = 0.0001$) in the PRF (50 and 100 mg/kg) like it was also found in the reference drug ketamine-treated mice. Among the PRF treatment groups, there was a marked delay in seizure onset (0.0061) in the PRF (50 mg/kg) among the PRF-treated groups. In this study, the development of convulsion was not hindered by the PRF (200 mg/kg) but abrogated marginally by the PRF 50 mg/kg, and ketamine reference drug. However, the latency to death was delayed significantly in the PRF (100 mg/kg) and PRF (50 mg/kg) compared with the control, while PRF (200 mg/kg) had no significant effect. In addition, neither PRF nor ketamine treatment had a significant effect on the percentage mortality (Table 2).

Effects of PRF obtained from Vitis vinifera on the pilocarpine-induced convulsion
In this study, PRF, pre-treatment delayed the on-set of seizure activity ($p = 0.0001$). However, there was significant ($p = 0.0002$) delay in the on-set of convulsions in the PRF (100 mg/kg) treatment group among the PRF treatments. In addition, there was 100% mortality in the normal saline-treated mice, 60% in the PRF (200 mg/kg), 20 % mortality in the PRF (100 mg/kg), and 40 % in the PRF (50 mg/kg) treated mice, and 10 % in the carbamazepine treated mice (Table 3).
Figure 1 below shows the photomicrographs from the representative animals in each of the experimental groups. The histological profile of the control group was well preserved as it was devoid of histological derangement. In the NS + convulsion, there are several forms of neuronal fragmentation and chromatolysis (white circular ring) in addition to a morphological heterogeneous pattern stemming from diminished eosinophilic neurons with either pyknotic or karyorrhectic nuclei (red arrows), neuronal bulging (white arrow), and an assiduous microglial cell (blue arrow) (Figure 1).
Photomicrograph of the hippocampus of normal saline-treated without a seizure (negative control) showed CA regions, dentate gyrus (DG), and blood vessels. The fibrous astrocytes processes are stained brown and appear unremarkable, while the section shows non-reactive astrocytes with no evidence of glial scarring. The hippocampus of the positive control mice (normal saline-treated + convulsion) showed classical CA regions, dentate gyrus DG, and blood vessels. The fibrous astrocytes processes are stained brown (arrowhead) appear week, very close to the pyramidal neuron and with evidence of glial scarring (redpointed arrow). Photomicrograph of the hippocampus of the graded doses of PRF, and DZP treatment groups showed classical CA regions, DG, and blood vessels (arrowhead) (Figure 2).
The hippocampal section of the control mice showed an essentially normal expression of Anti-Neu N within the giant pyramidal cells and granular cells of the hippocampus (CA 1) tissue (+++). In the positive control section, however, there was reduced expression of Anti-Neu N within the hippocampal neurons with giant pyramidal cells and Dentate Gyrus (DG) containing the granular cells and blood capillaries (arrow) disposed within the neuropil (NP) (intensity score +). Sections of the hippocampus of the mice treated with graded doses of PRF showed composed CA regions and the Dentate gyrus (DG) made of the giant pyramidal cells and the DG containing the granular cells and blood capillaries (black arrow) disposed within the neuropil (NP). The section shows a moderate expression of Anti-Neu N within the hippocampal neurons compared with the control (intensity score ++).

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Table 1: Effects of proanthocyanidins-rich fraction obtained from *Vitis vinifera* on picrotoxin-induced generalized model of convulsion

Treatment	Latency to Convulsion (s)	Development of convulsion	Latency to death	Percentage Mortality
Normal saline	585.00 ± 0.95	5/5	182.00 ± 123.50	100
PRF (200 mg/kg)	645.00 ± 1.65	5/5	460.00 ± 72.11 $^{\beta}$	80
PRF (100 mg/kg)	915.00 ± 1.87$^{\beta}$	5/5	780.00 ± 91.65 $^{\beta}$	80
PRF (50 mg/kg)	1200.00 ± 1.78 $^{\beta\delta}$	2/5	620.00 ± 121.70 $^{\beta}$	40
DZP	1140.00 ± 0.91 $^{\beta\delta}$	0/5	---	0

β: increase compared with control (p = 0.0004); δ: increase compared with PRF (200 mg/kg) (p = 0.0478); α: decrease compared with control (p = 0.0006); ϒ: decrease compared with PRF (200 mg/kg) (p = 0.0023); μ: decrease compared with PRF (100 mg/kg) (p = 0.0005); π: decrease compared with PRF (50 mg/kg) (p = 0.0349).

Table 2: Effects of proanthocyanidins-rich fraction obtained from *Vitis vinifera* on strychnine-induced model of convulsion

Treatment	Latency to Convulsion (s)	Development of convulsion	Latency to death	Percentage Mortality
Normal saline	216.00 ± 0.40	5/5	18.50 ± 1.56	100
PRF (200 mg/kg)	768.00 ± 2.20 $^{\beta}$	3/5	1476 ± 299.80 $^{\beta}$	40
PRF (100 mg/kg)	720.00 ± 1.09 $^{\beta}$	3/5	1095 ± 368.50 $^{\beta}$	40
PRF (50 mg/kg)	192.00 ± 0.49$^{\alpha}$	5/5	24.00 ± 14.70 $^{\alpha}$	100
Ketamine	1320.00 ± 1.08 $^{\beta\Upsilon}$	5/5	150.00 ± 15.50 $^{\alpha}$	100

β: increase compared with control (p = 0.0001); α: decrease compared with PRF (200 mg/kg) (p = 0.0008); ϒ: increase compared with PRF treated mice (p = 0.0001)

Table 3: Effects of proanthocynidins-rich-fraction obtained from *Vitis vinifera seed* on the percentage mortality following seizure-induced-pilocarpine-hydrochloride

Treatment	Latency to Convulsion (s)	Development of convulsion	Latency to death	Percentage Mortality
Normal saline	552.0 ± 69.46	6/6	17.20 ± 1.77	100
PRF (200 mg/kg)	1068 ± 81.14$^{\beta}$	5/6	24.00 ± 14.70	60
PRF (100 mg/kg)	1092 ± 176.40 $^{\beta}$	5/6	172.00 ± 36.11 $^{\beta\delta}$	40
PRF (50 mg/kg)	1670 ± 102.50 $^{\beta\delta\Upsilon}$	4/6	1136 ± 152.20 $^{\beta\delta\Upsilon}$	20
CBZ (25 mg/kg)	2436 ± 135.60 $^{\beta\delta}$	3/6	1476 ± 183.00 $^{\beta\delta\Upsilon}$	10

β: increase compared with the control (p = 0.0001); δ: increase compared with the PRF (200 mg/kg) (p = 0.0001); ϒ: increase compared with the PRF (100 mg/kg) (p = 0.0001)

Moreover, there was a significant (p = 0.0018) decrease in the number of Nrf2 counts in the hippocampus of the positive control mice, compared with the control. However, hippocampal Nrf2 count increased significantly (p = 0.0001) in the PRF-treated mice, even more than the reference drug DZP when compared with their positive control counterpart (Figure 3).

There are overwhelming reports from both clinical and experimental studies on the consequences of seizures, most especially convulsive status epilepticus; this stems from hypoxia, hyperthermia, hypotension, hypoglycemia, acidosis, with eventual brain injury and/or mortality.[12, 15, 16] Moreover, it is a known fact that about one-third of epilepsy patients suffer from uncontrolled seizures despite pharmacotherapy.[17]; therefore, anticonvulsant and neuroprotective assessment of plant extract with acclaimed CNS effects is not just necessary but a norm in a bit to delineate its potential mechanism of action, hence this study.

In this present study, we demonstrated that a lower dose (50 mg/kg) of PRF isolated from *Vitis vinifera* seed delayed the onset of convulsion, and latency to death, and also decreased the percentage of mortality. This is a piece of suggestive evidence that PRF possesses an anticonvulsant property. It is a known fact that picrotoxin exerts its action by antagonizing the $GABA_A$ receptor.[18] Schuler and his co-worker[19] implicated the influx of extracellular Ca++ to a prolonged depolarization with a resultant influx of Na+. However, data from this study revealed that anticonvulsant indices of PRF are more pronounced in the picrotoxin, and pilocarpine-induced convulsion (table 1 and 2) than the strychnine-induced (table 3).

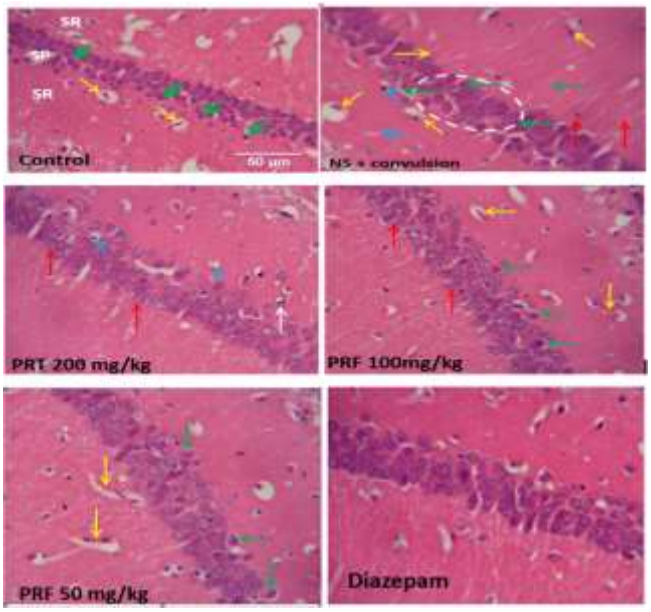

Figure 1: Effects of PRF on the histomorphological profile of hippocampus of mice subjected to picrotoxin-induced convulsion

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An indication that PRF exerts its anticonvulsant effect by blocking the GABA antagonist, picrotoxin, and pilocarpine-hydrochloride similar to what is obtainable in the reference drug, DZP. There were several forms of neuronal fragmentation and chromatolysis in addition to a morphological heterogeneous pattern stemming from diminished eosinophilic nerve cells with either pyknotic or karyorrhectic nuclei, neuronal bulging, and an assiduous microglial cell (reactive gliosis) due to picrotoxin-induced convulsion (Figures 1 & 2). This is a suggestive pointer to the excitotoxicity sequela to uncontrolled convulsion. PRF isolated from *Vitis vinifera* seed demonstrated hypothermic effects in our preliminary investigation (yet to be published). It is therefore logical to link the attenuation of hippocampal degeneration following picrotoxin-induced convulsion in this present study to the beneficial effect of hypothermia induced by PRF treatment. Proanthocyanidin is a positive modulator of GABAA receptors, with a higher affinity for the a2 subtype.[20] This is not unconnected to their permeability to cross the blood-brain barrier.[21] It is well known that agonists of N-methyl-D-aspartic acid (NMDA) or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors can elicit seizures, while antagonists have been shown to inhibit seizures.[22] Based on the data in this present study, however, where PRF delayed the onset of the seizure, latency to death, and percentage mortality (Table 2), it may be logical to look beyond the box of the GABAergic system as the only mechanism of action of PRF. Therefore, the anticonvulsant activities of PRF, especially at the lower dose (50 mg/kg) in pilocarpine-induced convulsion suggest a potential role for NMDA and AMPA receptor antagonists in anti-seizure properties. Wang *et al*[22]. reported that some catechins do interact with the glutamatergic system.

In this study, the fibrous astrocytes processes in the hippocampus of the untreated convulsed mice appear week, very close to the pyramidal neuron and with evidence of glial scarring. This is consistent with the conclusion of the previous study of Osuntokun *et al.* [12], *that astrocytes will not but* respond to any form of CNS insults through a process referred to as *reactive astrogliosis and often time manifested through* the spreading of the cytoskeleton to the neighboring pyramidal neurons. However, in this present study, this anomaly was decimated in the PRF treatment group.

Another consequential effect of convulsion in this study was demonstrated by a reduced expression of Anti-Neu N within the hippocampal neurons (Figure 3). However, the sections of the hippocampus of the mice treated with graded doses of PRF showed a moderate expression of Anti-Neu N within the hippocampal neurons as obtainable in the control group(Figure 3).

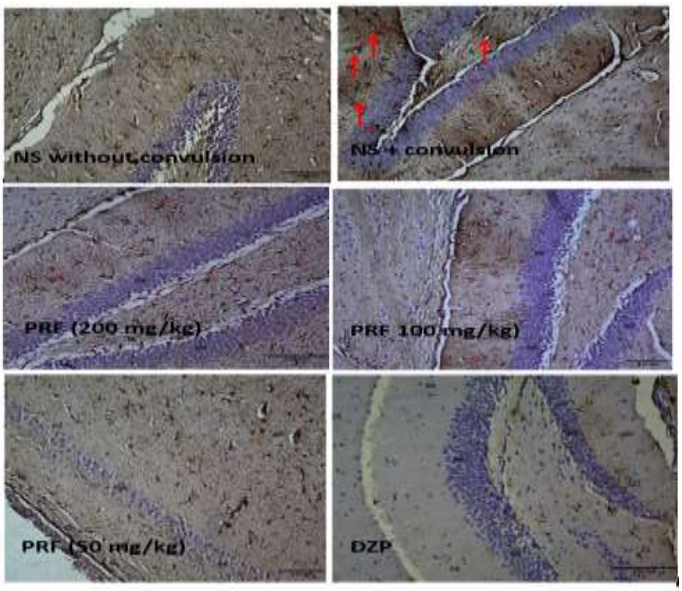

Figure 2: Effects of PRF on the immunoexpression of astrocytic reaction in the hippocampus of mice subjected to pilocarpine-hydrochloride-induced convulsion

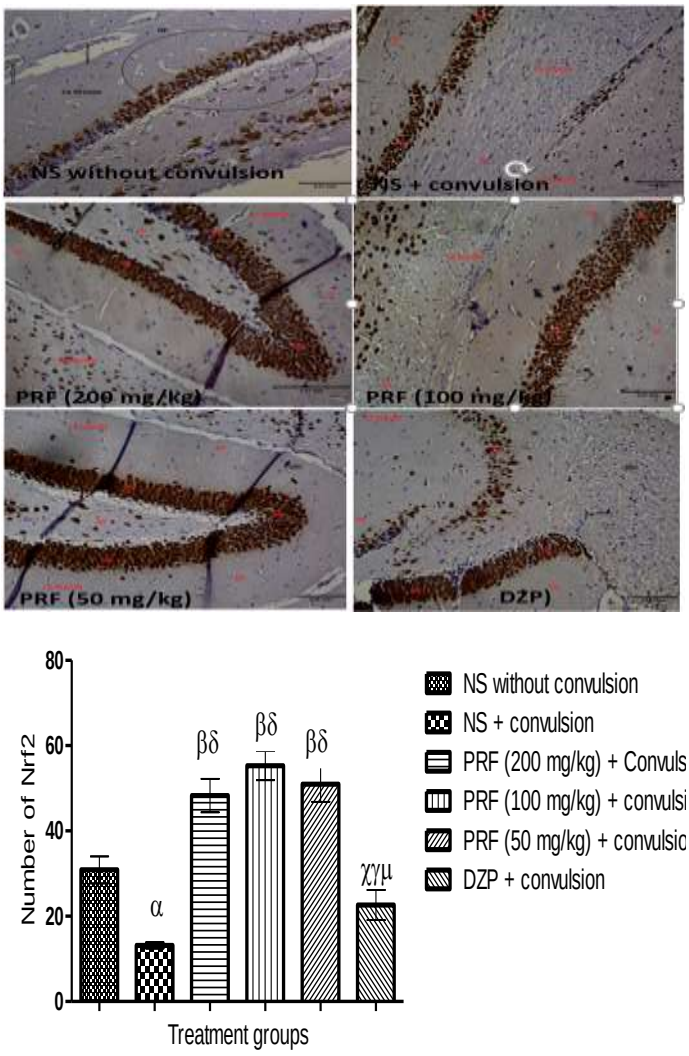

Figure 3: Effects of PRF on the immunohistochemistry Neu N in the hippocampus of mice subjected to pilocarpine-induced convulsion.

α: decrease compared with the negative control (p = 0.0018)
β: increase compared with the negative control (p = 0.0001)
δ: increase compared with the positive control (p = 0.0001)
x: decrease compared with PRF (200 mg/kg) (p = 0.0001)
ϒ: decrease compared with PRF (100 mg/kg) (p = 0.0001)
µ: increase compared with PRF (50 mg/kg) (p = 0.0001)

Therefore, it is worthy of note that, one of the probable ways by which PRF protects the CNS during convulsion is the protection of hippocampal neurons.

Conclusion

In conclusion, our data demonstrated the excitotoxic effects of convulsion manifested with diverse hippocampal abnormalities such as morphological impairment, reactive astrogliosis, and a reduced expression of Anti-Neu N and Nrf2. However, PRF treatment abrogated the seizure indices by enhancement of GABAergic activities, inhibiting NMDA/glutamatergic transmission, and also protected the hippocampal neurons with evidence of Anti-Neu N and Nrf2 immunohistochemical expression. The quest for electroencephalography during seizure and western blot analysis of the hippocampal tissue after seizure activities may also shed more light on the anticonvulsant and neuroprotective effects of the PRF isolated from *Vitis vinifera*.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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