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Co-administration of *Zingiber officinale* Extract and Sodium Valproate Ameliorates Seizure Severity, Cognitive Deficit, and Neuronal Cell Loss in Pentylenetetrazole-kindled Mice

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Abstract

Preparations of *Zingiber officinale* are used in Nigerian folk medicine to manage colds, pain, arthritis, nausea, and epilepsy. The ameliorative effects of co-administering aqueous *Zingiber officinale* extract (GE) and sodium valproate (SDV) on pentylenetetrazole-kindled mice were evaluated regarding cognitive deficits, neuronal cell loss, and seizure severity. GFAP was also quantified. Male mice were pretreated with GE (50 mg/kg), SDV (100 and 200 mg/kg), and GE + SDV before kindling. After kindling, the mice underwent a learning performance test. The animals received a challenge dose of pentylenetetrazole one week after kindling. The brains were excised one day after the challenge test and were processed for GFAP

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immunohistochemistry and histopathology. GE alone did not affect PTZ-kindled seizures. However, treatment with GE and SDV significantly improved learning performance and protected against neuronal cell loss in pentylenetetrazole-kindled mice. The level of astrocyte activation was reduced in the kindled group pretreated with the extract. The results obtained suggested that co-administration of GE and a low dose of SDV significantly ameliorated learning deficits and protected against neuronal cell loss, astrogliosis, and neuroinflammation, suggesting that GE might be a beneficial co-medication in the management of epilepsy.

Keywords
Epilepsy; kindling; learning; *Zingiber officinale;* sodium valproate

1. Introduction

Epilepsy is a prevalent neurological disorder that affects people of all ages. Globally, about 2.4 million people are diagnosed with epilepsy annually [1]. This debilitating neurological disorder is often accompanied by cognitive deficits affecting attention, language, memory, and executive functions and adversely affects the quality of life of the patients and their families [2-4]. Although epilepsy is a treatable condition, over 75% of the patients from low-income populations around the world do not receive any treatment [5]. Therefore, new antiepileptic medications with high efficacy and a more tolerable side-effect profile need to be developed and made available in low-income countries.

Medicinal plants with high efficacy may be used as supplements to antiepileptic drugs (AEDs) to potentiate their effectiveness with tolerable side effects [6]. In a recent review, the antiepileptic efficacy of 18 herbal agents, including *Zingiber officinale*, was discussed [7]. *Zingiber officinale* Rosc. (Zingiberaceae), or ginger is a widely used herb and condiment [8]. It is traditionally used in the management of colds, pain, arthritis, nausea, and vomiting [9-13]. Recent preclinical studies showed that Zingiber extract might also be useful in the treatment of Alzheimer's disease and cognitive impairment [14]. The bioactive components of *Zingiber officinale* are well-characterized [15]. Specifically, [6]-gingerol, [8]-gingerol, and [6]-shogaol can penetrate the blood-brain barrier via passive diffusion, suggesting that these substances might contribute to the effects of ginger extracts on the central nervous system [16]. Moreover, ginger extract (GE) or its constituents might exert anticonvulsant effects on different models of epilepsy. Using the Li-pilocarpine model of status epilepticus, Rashid et al. [17] found that zingerone diminished the severity of seizures. Moreover, it restored cognitive functions and prevented histopathological damage in the hippocampus [17]. A hydroethanolic extract of ginger was reported to have anticonvulsant effects against acutely induced seizures in mice [18, 19]. The authors suggested that GE interacted with inhibitory and excitatory systems, antioxidant mechanisms, by inhibiting oxidative stress and inflammation, and obstructing the mitochondrial pathway of apoptosis. Since epilepsy is a chronic disease, potential antiepileptic agents must also be tested in chronic epilepsy models. Pentylenetetrazole (PTZ) kindling is a validated chronic epilepsy model used widely to study epileptogenesis and epilepsy-related comorbidities and to discover novel antiepileptic compounds [19, 20].

Sodium valproate (SDV) is a commonly used AED. Its different antiepileptic mechanisms contribute to its broad antiepileptic spectrum [19, 21]. In this study, we determined whether chronic administration of aqueous extract of ginger alone (GE) or in combination with SDV can ameliorate seizure severity, cognitive impairments, and neuronal cell loss in PTZ-kindled mice. The effects of GE on the astrocyte marker glial fibrillary acid protein (GFAP) were examined by immunostaining.

2. Materials and Methods

2.1 Collection and Identification of Plant Material

Rhizomes of *Zingiber officinale* were collected in November 2017 from Kachia, Kaduna State, Nigeria. It was identified and authenticated by a taxonomist, Namadi Sanusi of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria, by comparing it with an existing Voucher specimen (Number 2099).

2.2 Experimental Animals

CD-1 [CRL: CD1 (ICR)] male mice (24-30 g) were purchased from Charles River, Sulzfeld, Germany, housed in groups of 8-10 in standard cages (Macrolon III) containing heat-treated, dust-free bedding (LTE E-001, abedd, Wien, Austria), and were exposed to controlled temperature conditions (20 ± 2 °C) and a cycle of 12 h light and 12 h dark. The animals were fed standard laboratory food (ssniff R/M H, ssniff Spezialdiäten GmbH, Soest, Germany), and offered tap water ad libitum. All substances were dissolved in isotonic saline solution (sal) and injected intraperitoneally (i.p.) at 10 mL/kg body weight. The solvent was given for control. Ethical approval was sought and obtained from the Saxony-Anhalt Committee on Animal Care (42502-2-1491). The experiments were conducted following the regulations of the European Commission and those of the German Act on the Use of Experimental Animals.

2.3 Preparation of the Ginger Extract

The rhizomes of *Zingiber officinale* were washed, thinly sliced, dried under shade, powdered, and extracted with water using cold maceration for 24 h with occasional agitation. The extract was filtered using Whatman filter paper. The filtrate was dried using a water bath (45-50 °C), and the dry extract (yield 19.5% w/w) was stored in desiccators.

2.4 Pentylenetetrazole (PTZ)-induced Kindling in Mice

Mice were randomly assigned to the various groups and kindled with repetitive intraperitoneal (i.p.) injections of an initially subconvulsant PTZ dose of 42.5 mg/kg (ED 16 related to clonic seizures was established in a separate group of animals) on alternate days for four weeks with 12 PTZ injections. In all the PTZ treatment groups, PTZ was administered 30 min after pretreatment with ginger extract (50.0 mg/kg), sodium valproate (100, 200 mg/kg), and their co-administrations (Table 1). In previous experiments (data not shown), this dose of GE was the highest tolerable dose. Control animals received the same number of sal injections. Mice were observed in their home cage for 30 min after PTZ injections for behavioral seizures. The seizures were classified according to a modified Racine scale as follows [22]:

Stage 0: no response
Stage 1: facial and ear twitching
Stage 2: rearing without myoclonic jerks
Stage 3: rearing with myoclonic jerks
Stage 4: tonic-clonic seizures and turning over onto side position
Stage 5: generalized tonic-clonic seizures and turning over onto back position

Twenty-four hours after the completion of kindling, the learning performance of the animals was tested in a two-way shuttle box. The animals were challenged with another subconvulsant dose of PTZ below the kindling dose (37.5 mg/kg, i.p.) on day 7 after the completion of kindling. Twenty-four hours after the challenge test, the brains were excised for histomorphological and immunohistochemical studies.

Table 1 Experimental groups for studying the effects of Ginger extract (GE), sodium valproate (SDV), and its combinations on pentylenetetrazole (PTZ)-kindling in mice. PTZ (42.5 mg/kg) was administered 30 min after pretreatment; n = 10-14 per group.

Sal	Sal + PTZ		
GE50 + Sal	GE 50 + PTZ	GE50 + SDV 100 + Sal	GE50 + SDV 100 + PTZ
SDV 100 + Sal	SDV 100 + PTZ		
SDV 200 + Sal	SDV 200 + PTZ		

2.5 Cognitive and Learning Performance Experiment

A two-way shuttle box was used to evaluate cognitive and learning deficits associated with PTZ-induced kindling 24 h after the last kindling injection [23]. That was when the effects of the drugs administered in the last kindling injection were expected to have been cleared. The shuttle box was a computer-controlled automatic device, divided into two compartments (13 × 15 × 10 cm) separated by a hurdle (4 cm) and located in a sound-attenuating enclosure ventilated by an extractor fan. The central ceiling of each compartment was illuminated with bulbs (40 W), and a sound was generated by a buzzer (90 dB) that served as the conditioned stimulus (CS). An unconditioned stimulus (UCS) was also produced by an electric foot shock of 0.1-0.4 mA (50 Hz, impulse width of 10 ms, pulsatile direct current, rectangular pulses), delivered through stainless steel rods on the floor of the apparatus. Each trial began with the presentation of a conditioned stimulus (light and sound) and was followed immediately by the simultaneous presentation of a conditioned stimulus and an unconditioned stimulus (foot shock). There was an interval of 4 s between the conditioned stimulus and the unconditioned stimulus, and each trial was limited to 20 s for the animal to react. Inter-trial intervals lasted for randomized periods of 15-45 s. If the animal did not react by avoiding or escaping from the compartment where the shock was applied, the trial was repeated after a 30 s interval. Each session of the experiment consisted of 30 trials and was repeated on four consecutive days. Before the first session, the mice were allowed to explore the box for 5 min. In the subsequent sessions, the mice were allowed 1 min to explore. The parameters recorded included the number of escapes, represented by the crossings into the opposite compartment when the CS and the UCS were presented simultaneously, with a reaction time of 4-20 s, and the number of conditioned avoidance reactions when the mice moved to the opposite compartment during the presentation of CS alone, with a reaction time <4 s. The number of

conditioned avoidance reactions recorded represented actual learning success. Inter-trial responses were also recorded for evaluation [23, 24].

2.6 Histological Analysis of Brain Tissue

Animals from all treatment groups were divided randomly into two groups and were subjected either to histopathological analysis or immunohistochemistry 24 h after the challenge experiment. The mice were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.), transcardially perfused with 40 mL isotonic saline, and decapitated. The brains were removed, frozen in 2-methylbutane (-45 °C), and placed in a -70 °C freezer until they were cut. Before cutting, the brains were embedded in the OCT compound. Using a cryomicrotome, sections of 10 µm were cut in the plane of the nucleus habenulae. The sections were stained with toluidine blue and embedded in DPX mounting medium. Cells in the CA1 and CA3 region of the hippocampus were counted in squares of 500 µm x 500 µm using a counting net. The average of five fields from the left and right hippocampus per animal was used for statistical evaluation [23].

2.7 Immunohistochemistry

The mice were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with physiological saline followed by ice-cold 4% (w/v) paraformaldehyde (PFA) and 0.1 M phosphate-buffered saline (PBS) solution. After perfusion, their brains were carefully removed and placed in vials containing 4% PFA. The hippocampi were isolated from the brains, cut into 450 µm slices using a tissue chopper, and post-fixed overnight in 4% paraformaldehyde in phosphate-buffered solution (pH = 7.4). The fixed isolated hippocampi were transferred to a 20% sucrose/PBS solution until they were completely embedded for cryoprotection. The isolated hippocampus tissues were then frozen in a cryostat using a tissue freezing medium. After tissue processing and embedding in the tissue freezing medium, the hippocampus tissues were further cut into 40 µm slices using a cryomicrotome.

The sections were transferred onto a plate containing PBS and kept at 4 °C. Subsequently, the hippocampus sections were rinsed with PBS (pH = 7.4) and washed in 0.1% Triton X-100/PBS (PBST) for 10 min, three times at room temperature. The sections were blocked by adding B-Block + 0.5% Triton (45 mL + 225 µL Triton 100%) solution. The sections were incubated with anti-GFAP chicken antibody (ab32454, 1:1.000) and kept at 4 °C overnight in a dark, humid room with slight agitation. After incubation, the sections were washed three times with Tris-Phosphate-Buffered Saline (TPBS) for 5 min at room temperature. After washing, the sections were incubated with the secondary antibodies in the TPBS solution with slight agitation for 4 h at 4 °C in a dark, humid room. The secondary antibody (Cy3-conjugated AffiniPure Donkey Anti-Rabbit IgG) was kept in the TPBS solution with slight agitation for 4 h at 4 °C in a dark, humid room. The secondary antibodies were diluted with a TPBS-TS-1% solution in the ratio of 1:2000. All the sections were washed with PBS three times for 3 min and treated with DAB solution at room temperature for 30 min in the dark. The sections were then washed with running water, counterstained with a solution of hematoxylin for 1 min, and rinsed in tap water for 10 min. The sections were dehydrated in increasingly graded alcohols and treated with xylene. Using a tissue brush, the sections were transferred from the well-plates to the slides and covered with 5 µL of Mowiol and round cover glass. The immunostaining sections were then evaluated and photographed with a binocular fluorescence microscope (Leica

DM 6B, Leica, Wetzlar, Germany); the number of positive cells in an area of 100 μm^2 was counted under a light microscope at a magnification of 40x [25]. Three areas per animal were evaluated, and the average was used for statistical evaluation.

2.8 Statistical Analysis

The SPSS 26 software was used for statistical analysis. Results were expressed as the mean ±standard error (mean ±SEM). The differences between the control and test groups were analyzed for statistical differences by performing one-way ANOVA for the challenge test, shuttle-box learning performance, and cell counts. Two-way ANOVA with the factors pretreatment (sal vs. GE) and treatment (sal vs. PTZ) was performed for kindling studies and immunostaining, followed by Bonferroni's post hoc test for multiple comparisons. The differences were considered to be statistically significant at $p \leq 0.05$.

3. Results

3.1 Effect of the Extract on Seizure Intensity

As shown in Figure 1, repeated administration of an initially subconvulsant dose of PTZ (42.5 mg/kg) on every alternate day produced kindling in most mice in the sal + PTZ (negative control) group. Pretreatment with sodium valproate at 100 and 200 mg/kg significantly ($p \leq 0.05$) reduced the seizure severity score compared to the score of the sal-treated group. GE alone did not affect kindled seizures ($p > 0.05$). However, the co-administration of GE with sodium valproate did not significantly enhance the effect of sodium valproate in reducing the seizure severity score (Figure 2). The kindled mice responded to the PTZ-challenged dose with a higher seizure severity score than the non-kindled control mice (Figure 3). This suggested that the treatment suppressed acute kindled seizures, but there was no effect on the kindling process, i.e., the gradual increase in brain excitability.

Yakubu et al., Fig. 1

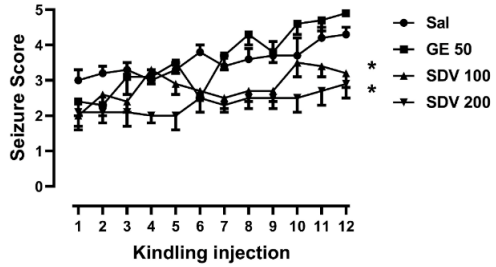

Figure 1 The effect of an aqueous extract of *Zingiber officinale* (GE) and sodium valproate (SDV) on pentylenetetrazole (PTZ)-induced seizures in mice. Pretreatment was given 30 min before PTZ administration. The doses are presented in mg/kg. The data are presented as the mean ±SEM, n = 9-11; *$p \leq 0.001$, no significant differences between sal/PTZ vs. GE50; sal/PTZ vs. SDV100/PTZ, $p \leq 0.05$; sal/PTZ vs. SDV200, $p \leq 0.001$.

Yakubu et al., Fig 2

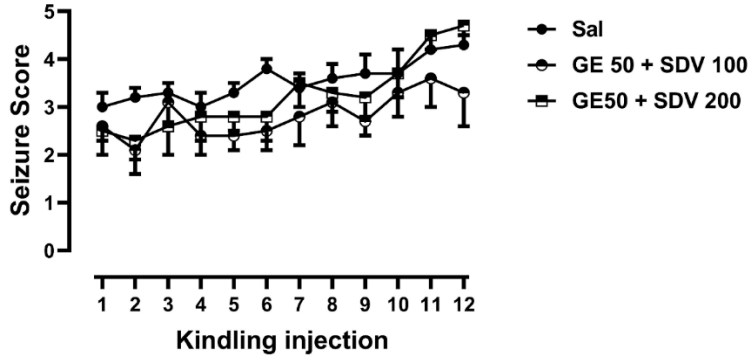

Figure 2 The effect of aqueous extract of *Zingiber officinale* (GE) and sodium valproate (SDV) combinations on PTZ-induced seizures in mice. Pretreatment was given 30 min before PTZ administration. The doses are presented in mg/kg, and the data are presented as the mean ±SEM, n = 5-11.

Yakubu et al., Fig. 3

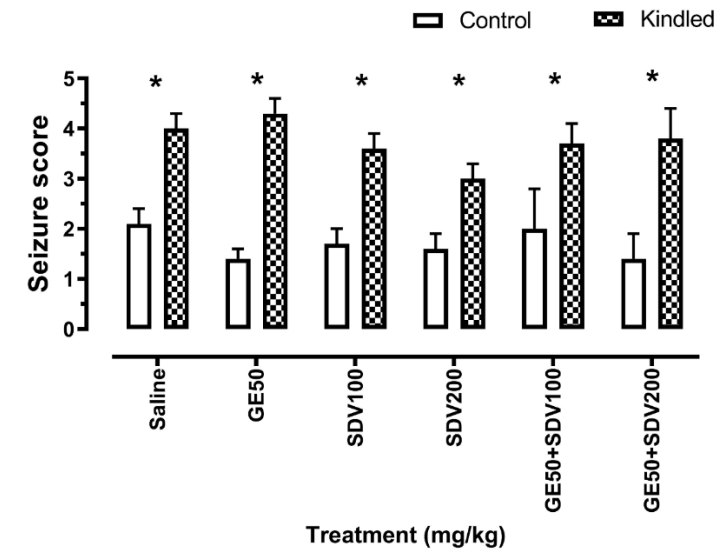

Figure 3 The seizure score of mice (control vs. kindled) pretreated with saline, aqueous extract of *Zingiber officinale* (GE), sodium valproate (SDV), and its combinations to a challenge dose of 37.5 mg/kg pentylenetetrazole injected one week after the completion of kindling; mean ±SEM, n = 5-11; *p ≤ 0.05.

3.2 Effect of the Extract on Learning Performance after PTZ-induced Kindling in Mice

In Figure 4A, no significant differences were observed in the number of conditioned reactions between the mice in the control groups when GE or SDV was administered alone or in combination (Figure 4B). As shown in Figure 4C, the number of conditioned reactions in saline-treated kindled mice was significantly lower compared to that in the saline-treated control group. Pretreatment

with ginger extract (50 mg/kg) and sodium valproate (100, 200 mg/kg) significantly ($p \leq 0.05$) increased the number of conditioned reactions (Figure 4C). Similarly, co-administration of the extract with sodium valproate significantly ($p \leq 0.05$) increased the number of conditioned reactions compared to the number of reactions in the saline-PTZ treated group (Figure 4D), suggesting that the pretreatment improved learning performance in the kindled groups.

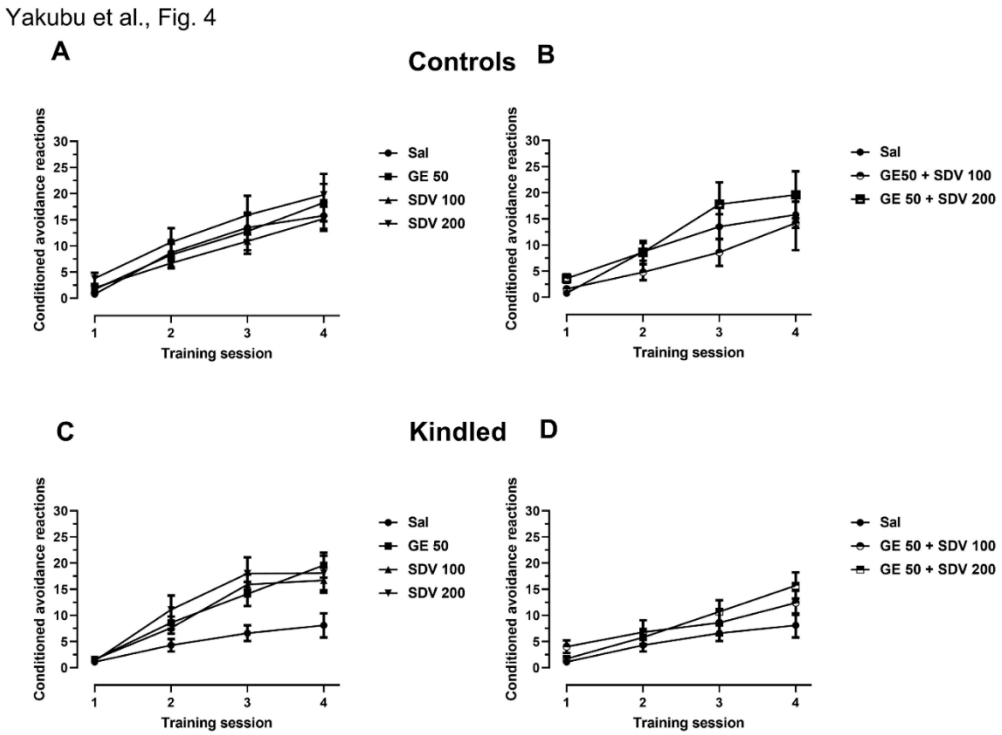

Figure 4 The effect of the aqueous extract of *Zingiber officinale* (GE) and sodium valproate (SDV) or its combinations on shuttle-box performance in pentylenetetrazole-kindled and control mice. A: Learning performance in the kindling control groups treated with either saline (Sal), GE, or SDV alone. B: Learning performance in the control groups treated with either Sal or a combination of GE + SDV. C: Learning performance in kindled mice treated with either Sal, GE, or SDV alone. D: Learning performance in kindled mice treated with either Sal or a combination of GE + SDV. The doses are presented in mg/kg, n = 5-11; *$p \leq 0.05$.

3.3 Histology

As shown in Figure 5, the number of neurons in the hippocampal CA1 region of the brain decreased significantly ($p \leq 0.05$) after repeated PTZ treatment (kindling) in mice receiving saline. However, pretreatment with ginger extract (50 mg/kg), sodium valproate (50, 100 mg/kg), and co-administration of the extract with sodium valproate (50 and 100 mg/kg) counteracted this neuronal cell loss in the CA1 region of the hippocampus. In the CA3 region, no significant differences between the treatment groups were found. Therefore, the data were not presented. Thus, CA1 is a specific target for kindling-induced cell loss.

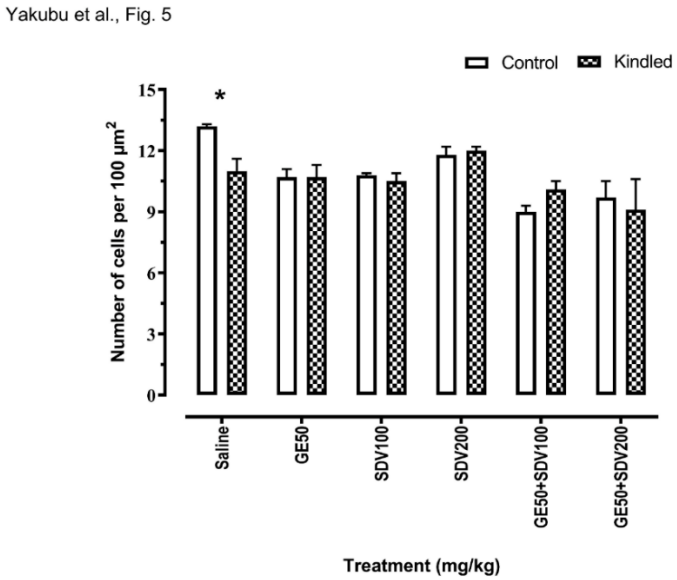

Figure 5 The effect of an aqueous extract of *Zingiber officinale* (GE), sodium valproate (SDV) in doses of 50, 100, and 200 mg/kg, or its combinations on the number of neurons in the mouse hippocampal CA1; n = 5-11, *p ≤ 0.05.

3.4 Immunohistochemistry

The number of GFAP+ cells in the CA1 region of the hippocampus differed depending on pretreatment and treatment (Figure 6). There was a significant ($p < 0.05$) effect of pretreatment (sal vs. GE) and treatment (sal vs. PTZ), but not the interaction. The results of one-way ANOVA revealed a significant ($p < 0.01$) difference across the four groups. A post hoc analysis revealed a significantly higher number of GFAP+ cells compared to the other three groups ($p < 0.05$).

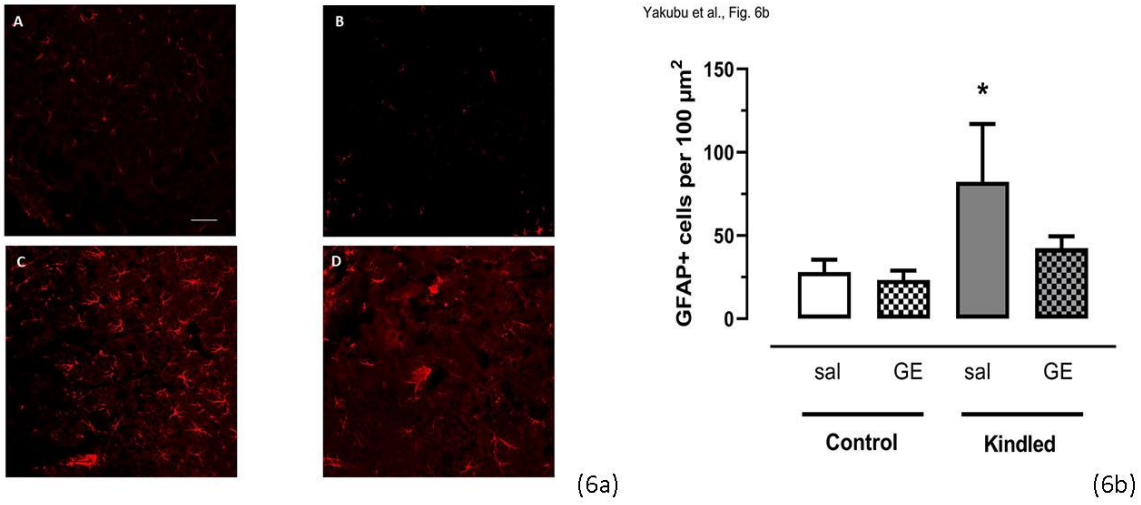

Figure 6 (a) GFAP immunostaining is shown for the hippocampal CA1 of mice treated with saline/saline (A), Ginger extract/saline (B), saline/pentylenentetrazole (C), and Ginger extract/saline (D); bar: 10 µm. **(b)** The number of GFAP+ cells in the hippocampal CA1 per 100 µm^2. Five consecutive slices from 4-6 animals were evaluated per group. Sal = saline, GE = Ginger extract; mean ±SEM; *p < 0.05 compared to all other groups.

4. Discussion

In this study, we evaluated the effects of GE alone and in combination with SDV on seizure severity, kindling-associated impairments in shuttle-box performance, and histopathological alterations in the hippocampus of PTZ-kindled mice. GE alone did not affect seizure severity (Figure 1, Figure 2, and Figure 3) but counteracted kindling-induced learning deficits (Figure 4) and kindling-induced histopathological damage in the hippocampus (Figure 5). Recently it was reported that hydroethanolic GE ameliorates acute PTZ seizures via antioxidant mechanisms, oxidative stress inhibition, and by simultaneously influencing different kinds of calcium channels [18, 19, 25]. Although the aqueous extract did not affect seizure severity, similar mechanisms might induce neuroprotection which was responsible for the reduction in the severity of kindling-related learning deficits, diminished cell loss in the hippocampus, and a reduction in GFAP expression in this study (Figure 6).

Combination therapy of lycopene along with SDV can potentiate the antiepileptic effect of the compound by attenuating the seizure score and oxidative stress against pentylenetetrazole-induced kindling in mice [26]. Our results of the kindling experiment were consistent with this mode of action, given that the antioxidative effects of GE have been described [19, 25, 27]. An interaction between the anticonvulsant mechanisms of GE and SDV did not enhance the effectiveness. Rather, the combinations reduced the anticonvulsant activity of sodium valproate. However, the combination of GE and SDV significantly counteracted kindling-induced learning deficits.

Animals in the sal/PTZ-treated group exhibited a significantly higher seizure severity score than the animals in the control group in the challenge test, suggesting a lowered seizure threshold in the kindled animals with an increased seizure susceptibility (Figure 1 and Figure 2). Pretreatment with GE, SVD, or its combinations did not reduce seizure scores in the challenge test, suggesting that GE, SDV, and its combinations reduced the severity of acute kindled seizures but did not interfere with the developmental component of kindling (Figure 3).

Kindling development is a process of epileptogenesis and is often associated with neuronal cell loss, cognitive impairment, and learning deficit [28]. This characterizes kindling as a relevant animal model of human epilepsy. Neuronal cell loss, one of the pathophysiological consequences of epileptogenic brain injuries that induce spontaneous recurrent seizures, is implicated in epileptogenesis [27, 29]. Amelioration of neuronal cell loss and cognitive deficits in pentylenetetrazole-kindled mice suggests there might be a protective potential against epileptogenesis and epilepsy-related alterations in different domains. In this study, co-administration of GE and SDV significantly improved learning performance in kindled mice in a two-way shuttle box experiment (Figure 4) and protected against neuronal cell loss in the hippocampus of the kindled mice (Figure 5) via an undetermined mechanism.

Sodium valproate exerts its broad-spectrum antiepileptic effect via multiple mechanisms, including the inhibition of sodium ion channels or by increasing the turnover of gamma-aminobutyric acid (GABA) thereby potentiating GABAergic functions in specific brain regions probably involved in the control of seizure generation and propagation [30]. Moreover, preclinical and clinical studies have shown that this substance interferes with GFAP pathways in various conditions. In a model of traumatic brain injury, sodium valproate significantly decreased serum GAFP levels [31]. A similar effect was found in kindling experiments [32]. Even in clinical investigations, sodium valproate, and especially its combination with the antiepileptic drug

levetiracetam, exhibited superior effectiveness [33]. The combination resulted in higher efficacy, fewer adverse reactions, and better quality of life [33].

Astrocytes are modulators of different brain diseases and can contribute to altered neuronal activity in several frontal cortex pathologies, such as ischemic stroke and epilepsy [34]. Our results showed an increase in GFAP immunoreactivity in the hippocampus (Figure 6), which was consistent with the results found in a previous study [35], which showed that PTZ-kindling can induce astrogliosis. In the mice that received GE before each kindling stimulation, the number of GFAP+ cells was significantly lower than that in the animals that received sal before kindling.

This decrease in the expression of GFAP immunoreactivity in the hippocampus of kindled mice pretreated with the extract suggested a level of protection against astrogliosis and neuroinflammation and might be interpreted as further evidence of the neuroprotective and antiepileptic effect of GE. Subsequent studies should consider the effect of GE in combination with SDV and other antiepileptic drugs on their antiepileptic and anti-inflammatory actions.

In conclusion, co-administration of GE and a low dose of SDV significantly ameliorated learning deficits and protected against neuronal cell loss, astrogliosis, and neuroinflammation in pentylenetetrazole-kindled mice. This indicated that GE contains psychoactive compounds with antiepileptic and neuroprotective properties and, thus, may be beneficial in combination therapy of epilepsy.

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Author Contributions

Musa Itopa Yakubu and Axel Becker performed the experiments, Nuhu Mohammed Danjuma, Mohammed Garba Magaji and Sani Malami were involved in the conceptualization, original draft preparation and supervision of the project, Ben Ahmed Chindo and Medinat Yakubu Abbas participated in the investigation and data analysis, and Musa Itopa Yakubu, Ben Ahmed Chindo and Axel Becker wrote and edited the manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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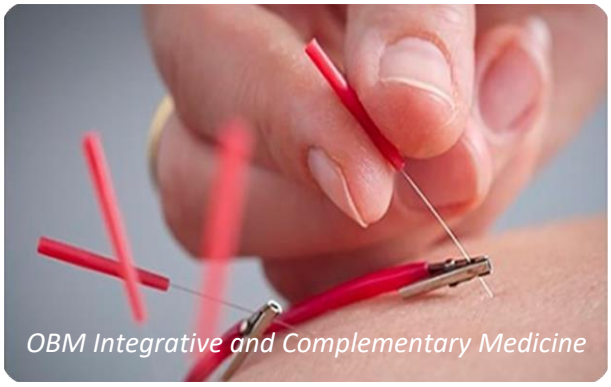

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