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Parkia clappertoniana Mitigated Neuronal Damage in the Hippocampus in Lithium Chloride-Pilocarpine-induced Epilepsy in Wistar rat Model

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ABSTRACT
This study investigated the effects of *Parkia clappertoniana* (PC) on the hippocampal structure and functions in Lithium Chloride-Pilocarpine-induced Epilepsy in Wistar rat Model. A total of 36 adult male Wistar rats were used for this study and divided into groups A to F, group A (Control), B (200mg/kg of PC), C (127mg/kg of Lithium chloride + 30mg/kg of Pilocarpine), D (127mg/kg of Lithium chloride + 30mg/kg of Pilocarpine + 100mg/kg of PC), E (127mg/kg of Lithium chloride + 30mg/kg of Pilocarpine + 200mg/kg of PC), F(127mg/kg of Lithium chloride + 30mg/kg of Pilocarpine +10mg/kg Sodium valproate). Treatment lasted for 28 days and the neurobehavioural studies carried out were Open Field and Radial Arm Maze tests. Animals were sacrificed and fixed using perfusion method. The brain specimens were grossed and subjected to morphological analysis, neurotransmitter test for glutamate, gamma aminobutyric acid (GABA) and enzyme test for Glutamate decarboxylase. Results showed significant decrease in the level of anxiety, exploratory activities and memory across the groups induced with epilepsy when compared with the control and observable increase in anxiety, exploratory activities and memory of the groups treated with PC and sodium valproate. The neurotransmitter glutamate showed significant increase in group C when compared with control and the rest of the treated groups. Histological result showed evidences of neuronal degeneration, vacuolation, and pyramidal cell dispersion in group C when compared with the control and rest of the groups. The pyramidal cells appeared better organized in group administered with low dose of PC when compared with the groups treated with high dose. Also, the GFAP test showed reactive astrocytosis in the groups induced with epilepsy and observable decrease in astrogliosis in groups treated with PC and sodium valproate. In conclusion, this study shows possible anticonvulsant properties of *Parkia clappertoniana* as seen in amelioration of glutamate levels following administration of PC and removed on its effects on hippocampal CA1 neuron and reduction in astrocytic reactivity and this is in agreement with previous study that reported its used in folk medicine to treat epilepsy especially in the northern part of Nigeria.

Keywords: Epilepsy, Parkia clappertoniana, Hippocampus, Lithium Chloride, Pilocarpine

INTRODUCTION
Epilepsy is a brain disorder characterized by occurrence of seizures, resulting from a brief episode of abnormal excessive or synchronous neuronal activity in the brain[1]. Epilepsy is the third leading contributor to the global burden of disease for neurological disorders and affects 65 million people worldwide. According to a meta-analysis of international studies, the prevalence of epilepsy is 6.4 cases per 1,000 persons and the annual incidence is 67.8 cases per 100,000 person-year[2]. Some cases of epilepsy are induced by genetic factors, but it can also result from brain injuries caused by blows to the head, stroke, infections, high fever or tumors[2]. It has been observed that heredity (genetics) plays an important role in many causes of epilepsy in very young children, but it can be a factor for people of any age [3]. Epilepsy could also be caused by structural, metabolic and infectious causes, in some cases, however, the cause is unknown[3]. The hippocampus is part of the limbic system and plays a major role in long-term memory and spatial navigation. Hippocampus is a paired structure, with mirror-image halves in the left and right sides of the brain. It is located inside the medial temporal lobe, beneath the cortical surface[4] . Hippocampal dysfunction is an overlapping feature of temporal lope epilepsy. The function and connectivity of human brain is disrupted in epilepsy[5]. The hippocampus is the largest structure of the mesial temporal lobe and believed to be the primary brain structure underlying the pathophysiology of hallucinations and disturbance of cognition, both of which is a symptom common to TLE. In translational studies on individuals with TLE, electroencephalogram recordings as well as electrophysiological characterizations showed synchronous hyperactivity and the presence of spontaneously occurring interictal spike discharge in the hippocampus [6]. Similar to the molecular changes observed, a number of studies using hippocampal autopsies from patients with TLE demonstrated increase in brain-derived neurotrophic factor (BDNF) mRNA and

protein levels of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and NMDA (N-Methyl- d-aspartate) receptor subunits, implicating enhanced excitatory synaptic transmission[7].

Parkia clappertoniana (PC) known locally as Dorawa belongs to the genus parkia and parkia is a pantropical genus of trees containing about 30 species [8] . The common species of the Parkia genus are *Parkia biglobosa*, P. *filicoidea and P.clappertoniana*. *Parkia clappertoniana* is a tree of about 18meters high and 3.6meters in width with spreading branches. The plant tends to occur in the savanna country and has been recorded from Gold Coast (Ghana), Togoland (Ghana and Togo), Dahomey (Benin) and Northern Nigeria[8]. Parkia species have been reportedly used in folk medicine for the treatment of various diseases especially infections. The roots and leaves of *Parkia clappertoniana* are pounded with water and used as an eye wash; the roots and the leaves were also reported to be active against dental caries, conjunctivitis[8]. It was also reported that an infusion of the stem bark was successfully used for the treatment of many infectious diseases such as diarrhoea, orchitis, dental caries, pneumonia, bronchitis, violent stomachaches, severe cough, infected wounds, otitis, dermatosis, amoebiasis, bilharziosis, leprosis, ankylosis, tracheitis, and conjunctivitis[8]. Currently available antiepileptic drugs have a limited efficacy and their negative properties limit use and cause difficulties in patient management. Numerous traditional therapeutic approach with the use *Parkia clappertoniana* to treat epilepsy has been reported especially in the Northern side of Nigeria[9]. However, there is very little experimental report on the anticonvulsant properties of Parkia clappertoniana.

MATERIALS AND METHODS

Thirty-six (36) mature male Wistar rats (160-250g) were procured from the animal house of Babcock University, Ogun State, Nigeria and ethical approval was obtained from Babcock University Health Research Ethical Committee (BUHREC) with the ethical code BUHREC048/19. They were kept in clean cages under 12/12 hours normal light/dark cycle and allowed to acclimatize for a period of seven days before the commencement of the experiment. They were fed on standard pellet diet and water *ad libitum*.

Animal groupings: The 36 Wistar rats were grouped into 6 groups of n = 6 (see table 1) Group A was the control and received a single dose of 0.5 ml of normal saline intraperitoneally and 0.5ml of nomal saline orally throughout the period of administration. Group B received a single dose of 0.5 ml of normal saline intraperitoneally and received 200mg/kg of PC extract (orally) daily. Epilepsy was induced in groups C, D, E and F rats with a single dose of pilocarpine (30 mg/kg, i.p) 24 hours after a single dose of lithium chloride administration (127 mg/kg, i.p). Seizures (status epilepticus) were allowed to last for 45 minutes and then were terminated by the administration of diazepam (10 mg/kg, i.m.) to reduce rate of animal death. After the first seizure was achieved in all the groups, rats in groups D and E were administered orally 100 mg/kg and 200 mg/kg of PC respectively while group F was administered 10mg/kg of sodium valproate a standard antiepileptic drug and group C was left untreated. Administration of PC and sodium valproate for groups B, D, E and F started on the second day post induction of epilepsy and ended on the 28th day (26days).

All rats were handled in accordance with the guidelines for animal research as detailed in the guidelines for the care and use of laboratory animals. Lithium chloride was obtained from Sigma-Aldrich Co., St. Louis, USA). Pilocarpine hydrochloride was obtained from Sigma-Aldrich Co., St. Louis, USA). Diazepam (Made for F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Collection and Preparation of *Parkia Clappertoniana*: Fresh stem bark were obtained from Maiduguri, Borno State, Nigeria and was taken to Forest Herbarium Ibadan (FHI) for identification with a Voucher number (FHI 112895). Samples were dried at room temperature for 30 days after which it was milled into coarse powder with an electric blender; the milled samples were kept in tight bottles.

A known weight of the powder was extracted in 1000 ml of ethanol for 72 hours at room temperature. The extract was filtered with Whatman No. 1 filter paper and the resulting filtrate concentrated in a Rotary Evaporator. The mixture was further transferred into steam bath where evaporation took place to give the required brownish-black residue. After which it was reconstituted in distilled water to give the required doses to be used throughout the experimental period. For the preparation of *Parkia clappertoniana*, 10g of extracted *Parkia clappertoniana* measured using a Pocket Digital Weighing Scale 5g/0.01g ACL-PDS002 poured into a beaker, 200mL of normal saline was measured using measuring cylinder, the 200mL of normal saline was added to 10g of extracted *Parkia clappertoniana*, and then mixed properly.

Table 1: Experimental Design

GROUPS	NO OF ANIMALS	DOSES	RATIONALE
A	6	0.5ml single dose of normal saline intraperitoneally and 0.5ml daily dose of normal saline orally	Control group
B	6	0.5ml single dose of normal saline intraperitoneally and 200mg/kg of PC extract orally	To observe the sole effect of PC
C	6	A single dose of Lithium chloride (127mg/kg) and Pilocarpine(30mg/kg) intraperitoneally	To induce epilepsy
D	6	A single dose of Lithium chloride (127mg/kg) and Pilocarpine(30mg/kg) intraperitoneally and 100mg/kg of PC extract orally	To observe possible anticonvulsant effects at lower dose
E	6	A single dose of Lithium chloride (127mg/kg) and Pilocarpine(30mg/kg) intraperitoneally and 200mg/kg of PC extract orally)	To observe possible anticonvulsant effects at higher dose
F	6	A single dose of Lithium chloride (127mg/kg) Pilocarpine (30mg/kg) (IP) + 10mg/kg of Sodium Valproate (orally)	To compare the anticonvulsant effect of PC extract with a standard antiepileptic drug, Sodium Valproate

Racine's Scale: Classification of seizure severity were confirmed according to the modified Racine's scale [10] (1) mouth and facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing with forelimb clonus, (5) rearing and falling

Video Recording: An observational system utilizing webcam fitted into a laptop was used to monitor the behaviour of the animals after lithium chloride pilocarpine and PC administration. Input from webcam was managed on a PC and recorded by VCL software. Video of seizure behaviour was scored offline according to modified seizure severity scales (Racine's scale). The observation was done during the 12 hours day cycle in order to avoid disruption in natural day/night cycle of the experimental animals.

Neurobehavioral Studies: Neurobehavioral tests were carried out on the animal following lithium chloride pilocarpine and PC administration. The following tests were performed: Anxiety and motor associated behavioral tests with the use of Open-field test,
Learning and memory test with the use of multiple Radial arm-mazes,
All neurobehavioral tests were recorded using a digital camcorder and later scored manually by at least two independent trained observers.

Animal Sacrifice: At the end of administration, rats in each group for histological and immunohistochemistry were anesthetized with diether and perfused intra-cardially with 100 mL phosphate buffered saline (PBS, 0.1 M pH 7.4), followed by 250 mL of neutral buffered formalin. The brains were excised and fixed in 10% neutral buffered formalin. One millimeter thick coronal brain slice was obtained at the level of the optic chiasma and processed via paraffin embedding method. Also, rats for neurotransmitters were sacrificed by cervical dislocation, hippocampus was dissected, and homogenized and its supernatant preserved at -200C for assay of brain glutamate and gamma aminobutyric acid and GAD were determined with standard test kits

Histological demonstration: The brains were fixed in 10% Neutral Buffered Formalin (NBF) by immersion. One mm thick coronal brain sections of hippocampus was obtained and processed for routine paraffin embedding. Sections were stained with H&E stain for general histoarchitectural demonstration of the hippocampus.

Immunohistochemical Studies: Hipocampal serial sections (5 µm) were taken from paraffin blocks to glass slides and placed on the hot plate at 700c for at 1hour. Sections were brought to water and subjected to 2 changes of xylene, and subsequently to 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the tissue sections by heating them on citric acid solution (PH 6.0) for 25 minutes. Tissue sections were equilibrated gradually with cold water to displace the hot citric acid solution for at least 5 minutes. Peroxidase blocking was done on the sections by simply covering section with 3% hydrogen

peroxide (H2O2) for 15 minutes. Sections were washed with Phosphate Buffered Saline (PBS) and protein blocking were performed using Avidin for 15 minutes.

Sections were washed with PBS and endogenous biotin in tissues was blocked using biotin for 15 minutes. After washing with PBS, sections were incubated with the respective diluted primary antibody (GFAP) at a dilution factor of 1:100. Excess antibody was washed off with PBS and a secondary antibody were applied on section for 15minutes. Sections were washed and the LABEL which is the horseradish peroxidase (HRP) was applied on the sections for 15minutes. A working DAB (3'3 Diaminobenzidine) solution was made up by mixing 1 drop of the DAB chromogen to 1mL of the DAB substrate. This working solution was applied on sections after washing off the HRP with PBS for at least 5 min. The brown reactions begin to appear at this moment especially for a positive target. Excess DAB solution and precipitate were washed off with water. Sections were counterstained with Haematoxylin solution for 2 minutes, dehydrated in alcohol, cleared in xylene and mounted in (Distyrene Plasticizer Xylene) DPX. IHC images were captured with a Leica DM750 microscope interfaced with Leica ICC50 camera and digital photomicrographs were archived.

Statistical Analysis: The results obtained were expressed as Mean ± SEM for each group. All grouped data were evaluated statistically with the use of Graphpad prism 5 software using one-way analysis of variance (ANOVA). Student-Newman-Kleus post Hoc test was used to identify differences between individual means. Confidence interval was placed at 95% so that in all cases a value of P< 0.05 was considered significant.

RESULTS

Grooming: Figure 1A shows the result of the frequency of grooming on the open field test of both the control and the treated. The result showed a significant decrease in group C (EP ONLY) (2.8±0.583) when compared with group A (CONTROL) (7.6±0.812). There was also a significant increase in groups E (EP+PC HD) and F (EP+SV) (7.6±1.69), higher in group F when compared with group C.

Rearing: Figure 1B showed the result of the frequency of rearing of both the control and treated groups of an open field test. The result showed no significant difference across all the groups, but there was observable decrease in groups C(EP ONLY) (5.0±1.26), D(EP+PC LD) (3.8±1.02), E(EP+PC HD) (4.6±0.98) and F(EP+SV) (6.2±1.24) when compared with group A (CONTROL) (6.8±1.46), lowest in group D(EP+PC LD) (3.8±1.02).

Total locomotory activities: Figure 1C show the result of total locomotion activities of both the control and treated group. No significant difference was observed across all the groups, but there is an observable decrease without any significance was observed in groups B (PC ONLY) (26.0±1.18) and E (EP+PC HD) (27.2±4.81), lower in group B compared with all other groups.

Number of line crossing: Figure 1D showed the result of total number of line crossing of both the control and treated group. There was no significant difference across all groups.

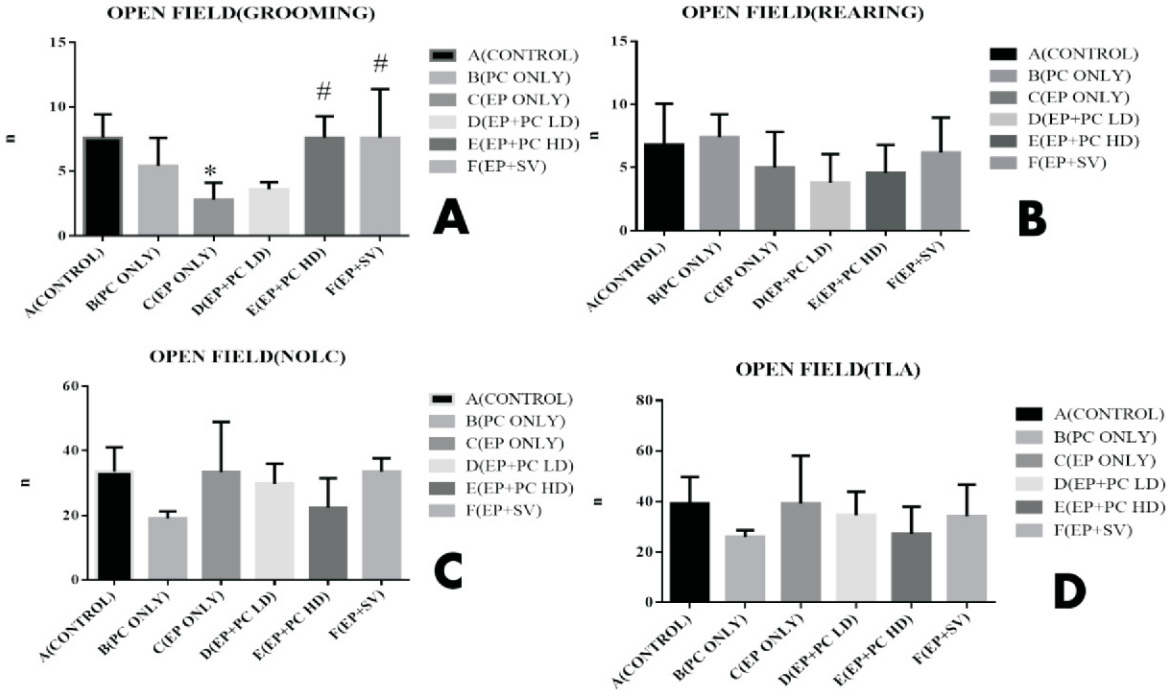

Figure 1: Chart Indicating Number of line crossing

Latency of first arm entry: Figure 2A showed the result of latency of the first arm entry on the radial arm maze test for both the control and the treated groups. The result showed no significance difference across all the groups

Number of re-entry: Figure 2B showed the result of re-entry on the radial arm maze test of both the control and the treated. The result showed a significant increase in group C when compared to the control group and the rest of the treated group and group B

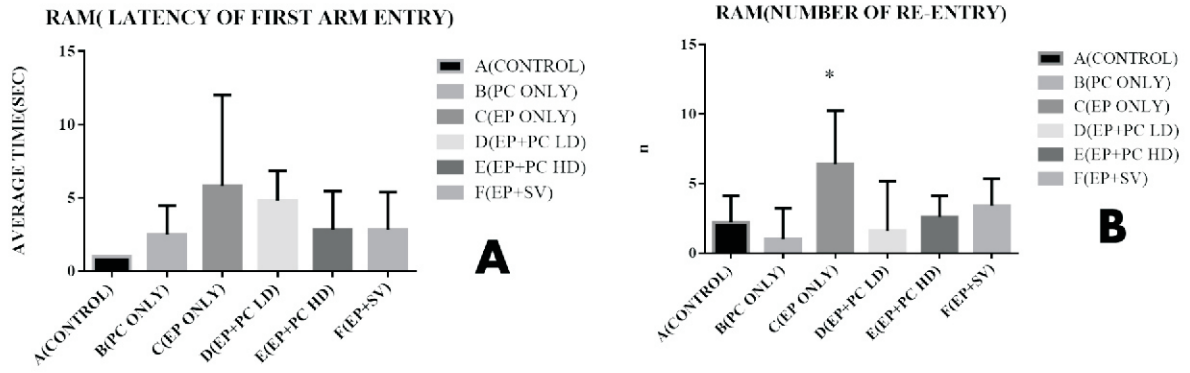

Figure 2: Chart Indicating Latency of first arm entry and Number of re-entry.

Effects of Seizure on the levels of Neurotransmitters

Glutamate level: Figure 3A showed the result of neurotransmitter glutamate across the group. Group C C(EP ONLY) showed significant increase when compared with the control (group A) and group B(PC ONLY). Groups D (EP+PC LD), E(EP+PC HD) and F were significantly lower when compared with epilepsy only group

Gamma aminobutyric acid (GABA) level: Figure 3B showed the result of neurotransmitter gamma aminobutyric acid (GABA) across the group. The results showed no Significant deference when the control was compared with the rest of the treated groups

Glutamate de-carboxylase (GAD) level: Figure 3C showed the result of Glutamate de-carboxylase (GAD) . Significant increase was observed in group E (EP+PC HD) when compared to groups A, B, C and D and , there was also a significant increase in group F(EP+SV) when compared with groups B(PC ONLY), C(EP ONLY) and D(EP+PC LD).

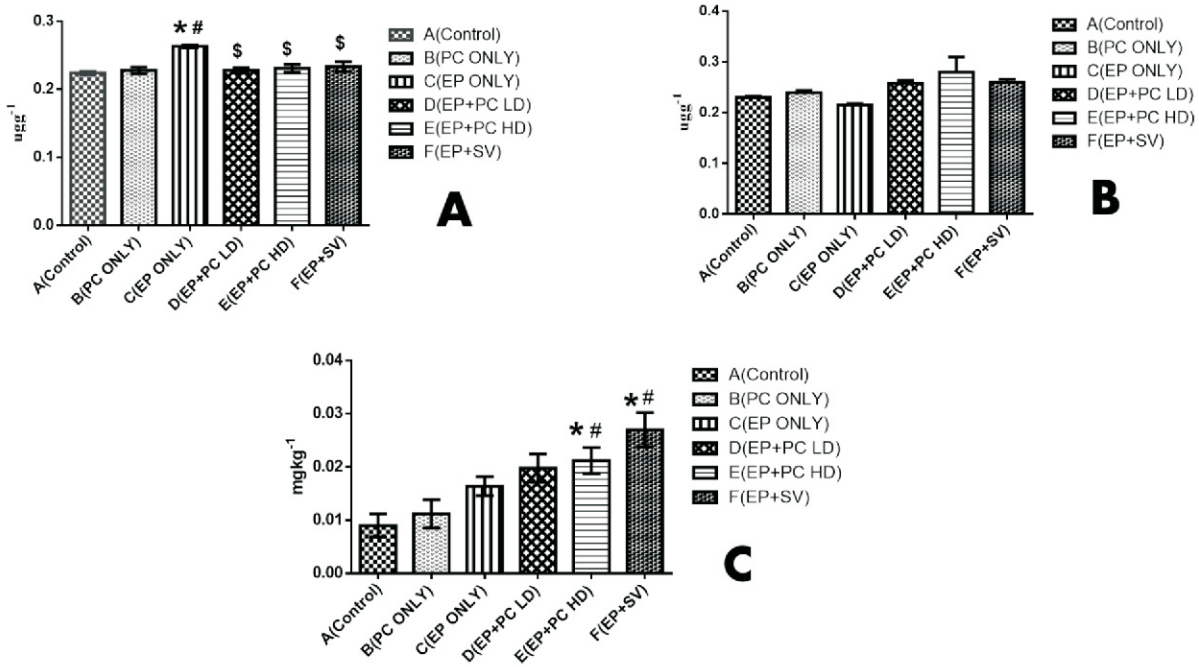

Figure 3: Chart Indicating Effects of Seizure on the levels of Neurotransmitters

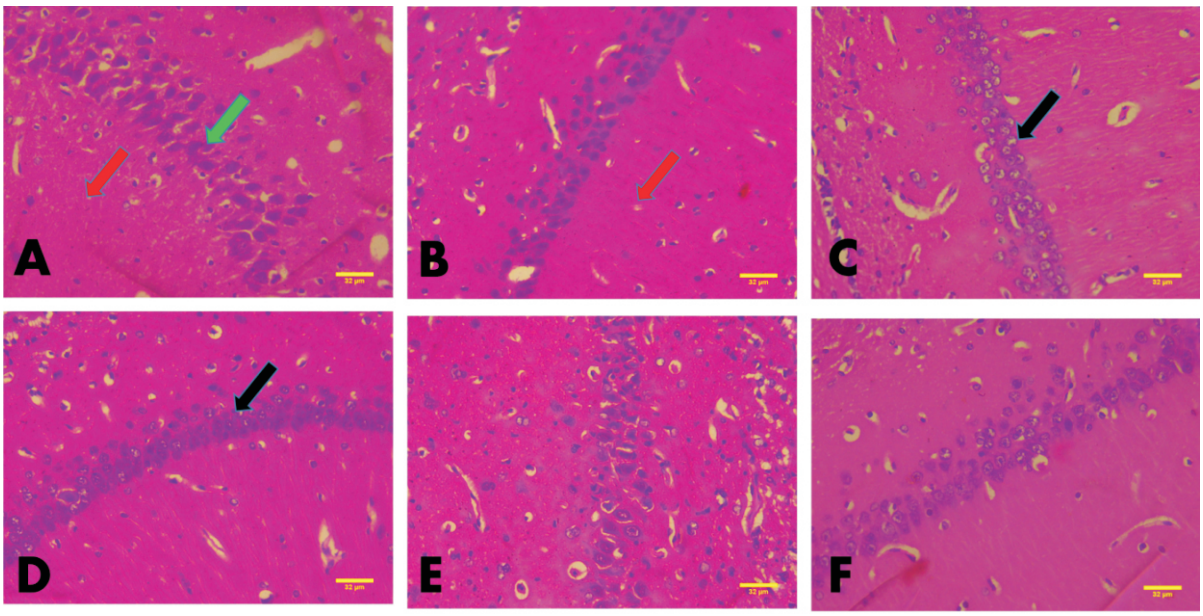

Figure 4: Photomicrographs of the CA 1 of the hippocampus showing the general histoarchitecture of the Cornu Ammonis 1 region. This plate represent the A= Control), B= PC ONLY, C= EP ONLY, D= EP+PC LD (100mg/kg) , E=EP + PC HD (200mg/kg), F =EP + SV(10mg/kg). Green arrow= Intact neuron (pyramidal neuron), black arrow= degenerating neuron (pyknotic neuron), Red Arrow= Neuropil (H&E x1000) Scale bar = 13 µm.

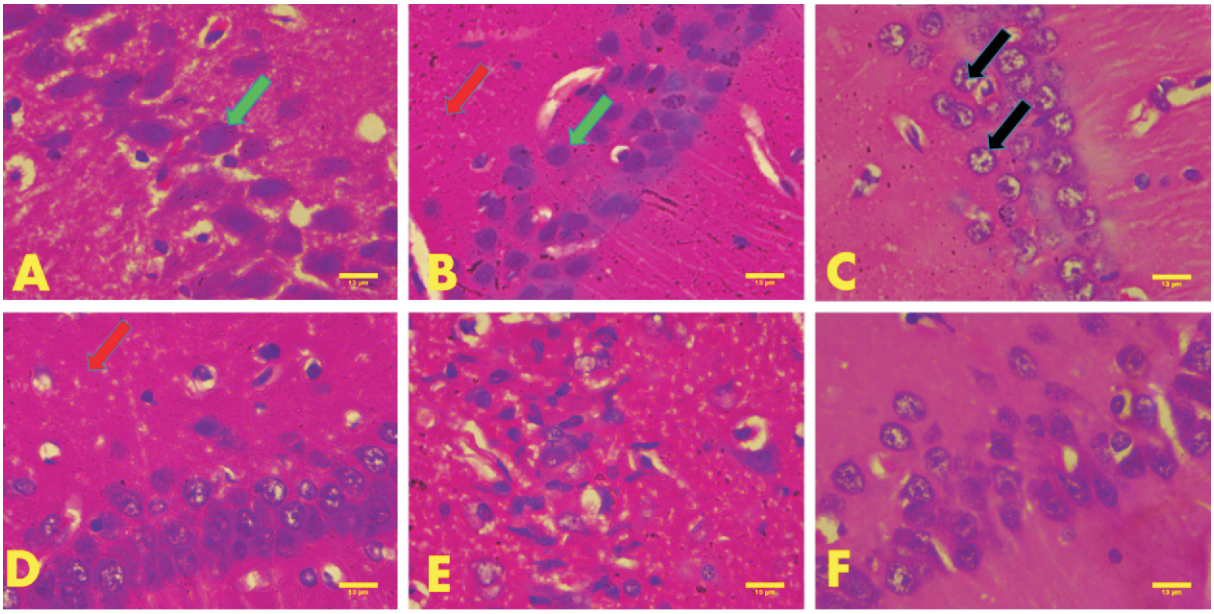

Figure 5: Photomicrographs of the CA 1 of the hippocampus showing the general histoarchitecture of the Cornu Ammonis 1 region. This plate represent the A= Control), B= PC ONLY , C= EP ONLY , D= EP+PC LD (100mg/kg), E=EP + PC HD (200mg/kg), F =EP + SV(10mg/kg) Green arrow= Intact neuron (pyramidal neuron), black arrow= degenerating neuron (pyknotic neuron), Red Arrow= Neuropil (H&E x1000) Scale bar = 13 µm.

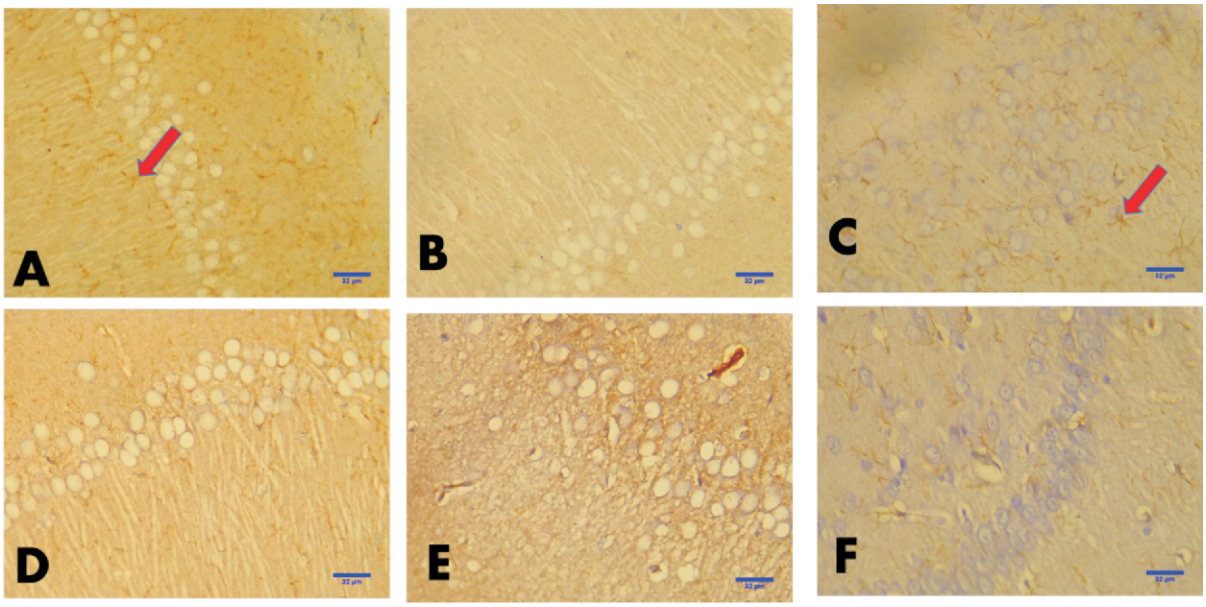

Figure 6: Photomicrographs of the CA 1 of the hippocampus showing the expression of astrocytes in the Glial Fibrillary acidic Protein (GFAP) stained slides. This plate represent the A= Control), B= PC ONLY , C= EP ONLY , D= EP+PC LD (100mg/kg), , E=EP + PC HD (200mg/kg), F =EP + SV(10mg/kg).Red arrow shows astrocytes (GFAP x400) Scale bar = 32 µm.

DISCUSSION

The study was aimed at investigating the antiepileptic potentials of PC on glutamate, gamma aminobutyric acid glutamate de-carboxylase and on rat's hippocampal neurons and astrocytes in lithium chloride pilocarpine induced epilepsy. Parkia species have been reportedly used in folk medicine for the treatment, control, prevention, & improvement of infectious disease such as bacterial infections, diarrhoea and so on [8] . Also, its use with some other traditional plants locally for the treatment of epilepsy amongst the Hausa/Fulani tribes of Northern Nigeria was reported [9].

PC was reported to contain the following chemical components such as, saponins, tannins, alkaloids, glycosides, flavonoids, triterpenoids, steroids and anthroquinones[11]. Epileptic activity has been proposed to be associated with an imbalance in the levels of inhibitory and excitatory neurotransmitters. A distortion in the normal GABAergic axons of the dentate gyrus could have resulted to the increase in GABA neurotransmitter than usual, distortion in the normal level of this neurotransmitter could have resulted in excessive excitability. Glutamate plays a major role in synaptic plasticity and is involved in cognitive functions such as learning and memory [12]. In rodent models, altering glutamate receptor or glutamate transporter expression by knockout or knockdown procedures can induce or suppress epileptic seizures. Regardless of the primary cause, synaptically released glutamate acting on ionotropic and metabotropic receptors appears to play a major role in the initiation and spread of seizure activity [13] and this is in accordance with David Y Ko [14] that the release of glutamate could result in epilepsy by activating the glutameric receptor. Gamma-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter which maintains the inhibitory tone that counterbalances neuronal excitation and when this balance is perturbed, seizures may ensue. GABA is formed within GABAergic axon terminals are released into the synapse, where it acts at one of two types of receptor: GABAA, which controls chloride entry into the cell, and GABAB, which increases potassium conductance, decreases calcium entry, and inhibits the presynaptic release of other transmitters[15]. GAD is the principal enzyme that catalyses the decarboxylation of the neurotransmitter glutamic acid to gamma-aminotobutyric acid (GABA). Antibodies directed against GAD have been found in patients with a number of neurological conditions including stiff person syndrome, cerebellar ataxia, and in patients with epilepsy alone [16]. In the present study, the result showed a significant increase in the glutamate de-carboxylase level of groups E when compared with the B group, a significant increase in group F when compared with groups B and D . This is in accordance with [16] that antibodies directed against GAD have been found in patients with a number of neurological conditions including patients with epilepsy alone, this means PC could have some anticonvulsant effect because there is increased GAD in group E, if the duration of PC is at a higher dose and a longer duration, there would be more GAD released which would result to the synthesis of GABA. As shown in figure4, there is appearance of pyramidal cell dispersion within the CA1 region in group E(EP+PC HD), which could be as a result of lithium chloride pilocarpine induced and this is in accordance

with Blumcke *et al* [17] , that cell dispersion is a common histopathological condition seen in an epileptic brain. Figure 5C showed dispersion in the arrangement in the normal orientation of the CA1 pyramidal cell in groups C (EP ONLY) and high degree of pyramidal cell dispersion within the CA1 region in group E (EP+PC HD) is also seen and this is accordance with Blumcke *et al* [17]. Also, in figure 5C, there is appearance of vacuolated cytoplasm, and scattered cells and this is in accordance with the features found in hippocampal sclerosis, a common feature in experimental models of convulsions and epilepsy as well as in patients with drug-resistant epilepsy[18].

Astrocytes are important glial cells that performs complex functions which includes the uptake and recycling of neurotransmitters and the buffering of extracellular potassium [19]. As seen in figure 6, there were expressions of GFAP immunoreactive astrocytes seen across groups A to F, there is presence of structural modified non-overlapping astrocytes in groups A and B characterized by classical reactivity of many short processes filled with the intermediate filament, the astrocytes make contact with the neurons and other astrocytes, this tallies with the characteristics of astrocytes explained by [20] . Mild gliosis, a reactive change of glial cells in response to damage of the astrocyte was observed in group C when compared with groups A and B, this result is in concordance with Francesco *et al* [21] when he observed gliosis in rat hippocampal brain treated with lithium chloride pilocarpine. It was as well explained by Cohen-Gadol *et al* [22] that reactive changes in astrocytes are frequently encountered in the hippocampus associating with temporal lobe epilepsy (TLE) in humans. This reactive changes are called reactive astrocytosis and often occur with neuronal loss and synaptic rearrangement [23]. Reactive astrocytes exhibit increased expression of glial cytoskeletal proteins, glial fibrillary acidic protein (GFAP) which are used to assess the development of reactive astrocytosis [24] .

As explained by Clasadonte and Haydon [25] changes in expression of two astrocyte-specific enzymes, glutaminesynthetase (GS) and adenosine kinase (ADK),could promote seizures in the epileptic brain. This statement could as well be one of the reasons seizures were generated in the animals as a result of lithium-chloride pilocarpine administration.

There was reduction in asrtroctic reactivity in groups D, E, F when compared to group C, this could be the result of the action of PC admistered to animals initially induced with epilepsy which appear to mitigates astrocytic reactivity in the treated groups.

As stated by [21] gliosis could have generated to epileptogenesis by facilitating the susceptibility to seizure generation once epileptic state had been installed. There is a degree of morphological arrangement of the astrocyte in groups D, E, F and this agrees with the possible antiepileptic properties of *parkia clappertoniana* more effectively at a lower dose and antiepileptic property of sodium valproate as pertaining to group F.

Astrocytes are considered nonexcitable cells, because unlike neurons they do not fire action potentials [25] but, they form an integral and active part of excitatory and inhibitory synaptic transmission and communicate back to synapses [26] also, astrocytes display a form of excitability that is based on variations of the intracellular Ca2+ concentration. In relation with epilepsy, emerging evidence has suggested a critical role for these glial cells in the pathogenesis of neurological disorders such as epilepsy[27]. Astrocytes become reactive in the epileptic brain and show changes in the expression of metabolic enzymes such as glutamine synthetase and adenosine kinase leading to modification of neuronal excitability. Astrocytes also release glutamate through a Ca2+-dependent mechanism that can synchronize neuronal firing and modulate neuronal excitability and synaptic transmission.

In conclusion, this study shows possible anticonvulsant properties of *Parkia clappertoniana* as seen in amelioration of glutamate levels following administration of PC and its effects on hippocampal CA 1 neuron and reduction in astrocytic reactivity and this is in agreement with previous study that reported its use in folk medicine to treat epileptic patients especially in the northern part of Nigeria

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