

Original Research Article

Kainic Acid Epilepsy Model in Wistar rats: The Comparative Ameliorate use of *Bryophyllum pinnatum*, Ketogenic diet and Carbamazepine on the frontal lobe cortex with selected behavioral parameters

Abstract

Introduction: Frontal lobe Epilepsy is a neurological disorder characterized by brief, recurrent seizures which arise from the frontal lobes of the brain. *Bryophyllum pinnatum*, commonly known as life plant, is a natural herb which has been known for its anticonvulsant potential in experimental animals. The ketogenic diet is a form of treatment for epilepsy in children created due to the observation that fasting had anti-seizure properties. This comparative study investigated the potential ameliorative use of *Bryophyllum pinnatum*, Ketogenic diet and Carbamazepine, on the frontal lobe cortex in kainic acid induced epilepsy wistar rat models.

Materials and Methods: Twenty-eight (n=28) adult male Wistar rats with an average weight of 150g were randomly distributed into five groups labelled A, B, C, D & E. Group A served as normal control; group B was treated with kainic acid only, group C was treated with Kainic acid + *Bryophyllum pinnatum*, group D was treated with Kainic acid + ketogenic diet and group E was treated with Kainic acid + carbamazepine. Rats were sacrificed after 29 days treatment period and their brains excised. The precentral cortices were cut and phosphate buffer preserved for enzymes and hormonal assay and the rest brain samples were stored in formal saline for histological demonstrations.

Results: kainic acid caused extensive damage to the cortical structures. *Bryophyllum pinnatum* stimulated regeneration of damaged cells and restoration of myelination and cellular integrity.

Discussion: Although, there was extensive damage done by kainic acid to the cortical structures in group

Comment [s1]: Can this be changed?
Difficult to understand

Comment [LA2]: Expunge complete sentence: 'commonly known as life plant'

Comment [s3]: While only males?

Comment [LA4]: In order to give room for consistency in results, putting into cognizance, female hormones, which could alter or affect the bio-chemical results/analysis

Comment [s5]: Which type of control? Should be clearly stated

Comment [LA6]: Positive control

Comment [LA7]: Neurobehavioural studies which included Elevated Plus Maze and Barnes Maze tests were carried out before the rats were sacrificed.

B, group C, group D and group E, *Bryophyllum pinnatum* stimulated the restoration of neurotransmission and massive production of astrocytes which aided in neuronal regeneration.

Key words: kainic acid, epilepsy, *bryophyllum pinnatum*, ketogenic diet, carbamazepine

1.0 Introduction

The most common epilepsy subdivision is symptomatic partial epilepsy which gives rise to simple partial seizures and can be divided into temporal and frontal lobe epilepsy. Frontal lobe epilepsy is the less common type of partial epilepsy which accounts for 20-30% of operative procedures involved in intractable epilepsy (2). Frontal lobe epilepsy accounts for 1-2% of all epilepsies (1). Frontal lobe epilepsy affects the **prefrontal cortex** (PFC) which comprises the anterior part of the frontal lobe, located anterior to the premotor cortex and whose primary functions are regulation and control of **attention** and **memory** (3;4). Predisposing factors are: tumors, head trauma, vascular malformations, malformations of cortical development and genetic causes. Kainic acid, commonly referred to as 'Kainate' is a natural marine acid and has its presence in some seaweed (*DIGENEA SIMPLEX*). It is an active neuroexcitatory amino acid which acts by activating receptors for glutamate and a direct agonist of the glutamic kainite receptors. High administration of concentrated solutions will result in immediate neuronal assault by excess stimulation of neurons, which leads to neuronal injury and finally, neuronal death (5). Thus, in high, concentrated doses kainic acid can be referred to as a neurotoxin, and in small doses of dilute solution, kainic acid will chemically stimulate neurons. The agent is known to have an excitatory effect on the central nervous system which is used in research of epilepsy to induce seizures in experimental animal, but at typical dose of 10-30 mg/kg in mice. It also has an epileptogenic property (6). *Bryophyllum pinnatum* commonly known as life plant, miracle leaf and air plant is a fresh shrub, which grows 2-4 feet tall. It belongs to the kingdom plantae, Crassulaceae family and has ornamental and medicinal uses (7). It is astringent, sour in taste, sweet in the post digestive effect and has hot potency. It is found in many parts of the world mostly and is used in folkloric medicine in tropical America,

Comment [s8]: Why some words in bold?

Comment [LA9]: Just an oversight, as this was actually corrected before submission, but probably correction was not saved

Australia, tropical Africa, china and southern part of Nigeria (8; 9). It contains the following active compounds: flavoids, bufadienolides glycosides, organic acids and steroids (10; 11). In 2015, (12) studied the anticonvulsant potential of *bryophyllum pinnatum* in experimental animals. Carbamazepine on the other hand, known by the trade otherwise known as TEGRETOL is a use-dependent blocker of voltage-gated sodium channels, used primarily in the treatment of epilepsy and neuropathic pain. It is ionized within intracellular fluid, the drug is able to bind to activate voltage-gated sodium channels, which prevent prolonged and sustained firing of an action potential. This then leaves the affected cells less excitable until the drugs dissolve. It is also a GABA receptor agonist as it has also been shown to potentiate GABA receptors made up of alpha 1, beta 2, and gamma 2 subunits (13). The ketogenic diet is another form of treatment for epilepsy in children that was created initially in the 1920s due to the observation that fasting had anti-seizure properties (12). During fasting, the body metabolizes the fat stores via lipolysis and then the fatty acids undergo beta-oxidation into acetoacetate, β -hydroxybutyrate, and acetone ketone bodies. The cell then uses it as precursors to generate adenosine triphosphate (ATP). The ketogenic diet is always high in fat and low in carbohydrates, so as to stimulate the metabolic effects of starvation in the body by enforcing the body to use fat as the primary fuel source (14).

Comment [LA10]: Replace words with: 'sometimes known by the trade name, Tegretol

Comment [s11]: The writing should be uniform

Comment [LA12]: Just another oversight, normal font used for the whole article shall apply

Comment [LA13]: gamma-aminobutyric acid (GABA)

2.0 Material and Methods

2.1 Animal Treatment and Tissue Processing

Twenty-eight [n=28] adult male Wistar rats, with the average body weight of 150g, were procured for the study from the institutional animal holding facility. The standard procedures for animal use and handling were followed according to Babcock University's Health Research Ethical Committee which approved the project with BUHREC no: 648/17. The rats were divided into five groups after the period of acclimatization as follows: Group A: the control group; animals were treated with only normal saline orally as a placebo, Group B: This group was treated with kainic acid only(negative control group), Group C: This group was treated with Kainic acid + *Bryophyllum pinnatum*, Group D: This group was

Comment [s14]: While only adults?

Comment [LA15]: To allow for consistency of results, with respect to the development and maturation of the rat brains. There is always room for further studies on juvenile rats

Comment [s16]: Specify the type of control

Comment [LA17]: Normal control, as noted previously,

treated with Kainic acid + ketogenic diet and Group E (positive control group): This group was treated with Kainic acid + carbamazepine (positive control group). Kainic acid was injected into the Wistar rats in groups B-E and this was done using the intraperitoneal method. Once the animals showed signs of the Racine's scale, they were injected with diazepam for relaxation of the rats to avoid death (15). The treatment lasted 29 days. The rats were sacrificed through the cervical dislocation after the treatment period and their brains excised. The precentral cortices were cut and phosphate buffer preserved for enzymes and hormonal assay. The rest of brain samples were stored in formal saline for histological demonstrations. The fixed tissues were processed following specific histological and histochemical protocols. Basic tissue processing included dehydration [using graded concentration of alcohol], clearing [using xylene], impregnation, and embedding [using molten wax]. The tissue samples were sectioned with a rotary microtome (~20 microns). The sections were mounted on glass slides for staining. Which method of staining?

Comment [s18]: Will this not affect the study?

Comment [LA19]: It didn't, as it was damage control, aimed at stopping Status Epilepticus (SE), to avoid neuronal death and eventual death of the rats; and was expected to have dissipated before the next administration of kainic acid

2.1.1 Racine's Scale: Is a tool used to determine the intensity of a seizure in rodent models of experimental epilepsy. The motor symptoms include: Mouth and facial movement (intensity stage1), Head nodding (stage 2), Forelimb clonus (stage 3), Seizures characterized by rearing (stage 4), Seizures characterized by rearing and falling (stage 5) (16).

2.1.2 Preparation of *Bryophyllum pinnatum* Extract: Extract of *bryophyllum pinnatum* dose 400mg/kg per day. The extract was prepared by blending and macerating 500g of the fresh leaves with 100ml of distilled water and kept at 40°C for 24 hours. The resulting mixture was filtrated (Whatmann size). The filtrate evaporated to dryness in water bath at 60 degrees Celsius and the result was a greenish residue.

Comment [s20]: Only one dose?

Comment [LA21]: 400mg/kg per day, for 29 days (Reference to the abstract)

2.2 Neurobehavioral Tests

The neurobehavioral tests were carried out on the 16th day of the study. The neurobehavioral tests included the elevated plus maze (EPM) and Barnes mazes. The tests were conducted in a large quiet room between the hours of 9 am and 1 pm and all the vents were recorded with a camera and observed

Comment [s22]: This would have appeared in the introduction

Comment [LA23]: Neurobehavioural studies which included Elevated Plus Maze and Barnes Maze tests were carried out before the rats were sacrificed.

Comment [LA24]: The above statement has been included in the introduction as observed

critically.

2.2.1 The Elevated Plus Maze: This assessed the anti-anxiety effects of pharmacological agents and steroid hormones, and mechanisms underlying anxiety-related behaviour (17). The maze was prepared by cleaning, drying and setting the video-tracking device. Data sheets preparation was appropriately based on grouping and other experimental design parameters. Rats were introduced into the behavioural testing room and placed at the junction of the open and closed arms, facing the open arm opposite to where the experimenter is. Then rat monitoring and data collection was done while video-tracking device and timer was set for 5 minutes. All open arm entries and close arm entries were recorded with time spent per entry. Each rat was removed from maze, after the 5-min test and returned to the holding cage. Maze was cleaned properly and dried with paper towels before introducing another rat. Procedure was repeated for other animals under similar conditions. Data analysis and interpretation was done using the following major parameters: (a) open arm time (b) closed arm time (c) open arm entries made (d) closed arm entries made (e) total entries made (f) The ratio of open or closed arm entries/time to the total arm entries/time.

2.2.2 Barnes Maze: This was used to assess spatial learning and memory in the treated rats. It was developed by Dr. Carol Barnes (18). The animals interacted with the Barnes maze in three phases: habituation (1 day), training (2–4 days in the short training) and probe (1 day). Before starting each experiment, rats were acclimated to the testing room for 1 h. Then all rats (n=2–4) from one cage were placed in individual holding cages where they remained until the end of their testing sessions. On the habituation day, the rats were placed in the centre of the maze underneath a clear 3,500-ml glass beaker for 30 s while white noise was played through a sound system. Then, the rats were guided slowly by moving the glass beaker, over 10–15 s to the target hole that leads to the escape cage. The rats were then given 3 min to independently enter through the target hole into the escape cage. If they did not enter on their own during that time, they were nudged with the beaker to enter. The rats were allowed to stay in the escape cage for 1 min before being returned to the holding cage. Once all animals had completed the 1-session habituation, they were all returned to their home cage. In the training phase, rats were placed

Comment [s25]: Which steroid hormone?

Comment [LA26]: Corticotropin Releasing Hormone/Glucocorticoids (E.g., cortisol)

inside an opaque cardboard cylinder, 10" tall and 7" in diameter, in the center of the Barnes maze for 15 s. This allowed the rats to face directions randomly when the cylinder was lifted and the trial began. At the end of the holding period, a buzzer was turned on, the cylinder was removed, and the rats were allowed to explore the maze for 2 min. If a rat found the target hole and entered the escape cage during that time, the end-point of the trial, it was allowed to stay in the escape cage for 1 min before being returned to the holding cage. If it did not find the target hole, the rat was guided to the escape hole using the glass beaker and allowed to enter the escape cage independently. If it did not enter the escape cage within 3 mins, it was nudged with the beaker until it did. If the rat still did not enter the escape cage after 1 min of nudging, it was picked up and manually put on the platform in the escape cage. Then it was allowed 1 min inside the escape cage before being returned to the holding cage. In all cases, the buzzer was turned off once the rat entered the escape cage. The total number of trials used was 5 for short training, 3 trials on training day 1 and 2 trials on training day. During the training phase, measures of primary latency and primary habituation session were recorded. On the probe day, 48 h after the last training day, the escape cage was removed, rats were placed inside the opaque cylinder in the center of the maze for 15 s, the buzzer was turned on and the cylinder removed. Each rat was given 2 min to explore the maze, at the end of which, the buzzer was turned off and the mouse was returned to its holding cage.

During the probe phase, measures of time spent per quadrant and habituation session per quadrant were recorded. For these analyses, the maze was divided into quadrants consisting of 5 holes with the target hole in the center of the target quadrant. The other quadrants going clockwise from the target quadrant were labeled: positive, opposite, and negative.

2.3 Staining Techniques

2.3.1 The H&E staining technique was done following the methods of (19). The slides, after dewaxing and rehydration, were placed in hematoxylin for 8–15 minutes for staining. The slides were then rinsed in tap water. The slides were further dipped in a bluing agent for 3–5 long dips. They were stained with

Comment [LA27]: 10cm tall and 7cm in diameter

Comment [s28]: This test should be clearly explained

Comment [LA29]: There is need to expunge the whole sentence and replace with the new:

"The total number of trials documented for the Barnes maze was 5, which covered 5 days (short training). There was 1 trial on habituation day, 3 trials on training day and 1 or 2 trials on probe day"

Comment [s30]: This sentence is not clear

Comment [LA31]: In reference to the above comment, sentence needs to be completely expunged and replaced with the new.

Also, there is need for space between the 'period' and the next sentence starting with 'During'

Eosin for a period of 30 seconds and hematoxylin for ~2 minutes. They were also dehydrated in 95% and 100% alcohol, with 3 changes each for 2 minutes. They were then cleared in 3 changes of xylene for 2 minutes each and after which the cover glass was mounted.

2.3.2 Luxol Fast Blue technique was done following the methods of (20). The slides were placed in 2 changes of xylene, 3 minutes each, then placed in 2 changes of 100% ethanol, 3 minutes each, after which, they were then placed in 95% ethanol for 3 minutes. They were also placed in 75% ethanol for 3 minutes, then rinsed in 2 changes of distilled water, 3 minutes each. The slides were placed in luxol fast blue solution in a plastic coplin jar and microwaved at the lowest power setting for 1 minute. Care was taken avoid boiling the solution. The slides were allowed to remain in the hot solution for an additional 20-30 minutes, then rinsed in tap water, 2 times, 3 minutes each, after which, they were briefly rinsed in 3 dips of distilled water. The sections were differentiated in 0.05% lithium carbonate, 5-10 dips and differentiation was continued in 70% ethanol, 5-10 dips, after which they were rinsed in 2 changes of distilled water, 2 minutes each and then sections were counterstained in Cresyl Fast Violet solution after which they were dehydrated in 4 changes of 100% ethanol, 2 minutes each. The slides were cleared in 3 changes of xylene, 3 minutes each, after differentiation, checked under the microscope and then slides were mounted.

2.3.3 GFAP Immunohistochemical Staining was done using IHC standard procedure credited to (21). Tissues were deparaffinised using standard techniques and pre-treated with citrate buffer at pH 6.0 and high heat epitope retrieval techniques. Blocking reagent was added to the tissue specimens and incubated in an enclosed chamber for 5 minutes. Specimens were gently rinsed with 1X Rinse Buffer for a minimum of 15 seconds while holding the slides at 45° angle.

End of the slides were tapped onto a paper towel to remove excess. Buffer was rinsed after. A dilution of primary antibody was applied over the entire tissue specimen, incubated in an enclosed chamber at room temperature for 60 minutes and specimens were rinsed. Biotinylated secondary antibody was applied to the tissue specimens, incubated in an enclosed chamber for 10 minutes and specimens were rinsed. Streptavidin-HRP solution was applied to the tissue specimens, incubated in an enclosed chamber for 10

minutes and rinsed. DAB (chromogen reagent) was applied to the tissue specimens and incubated in an enclosed chamber for 10 minutes and specimens were rinsed again. Tissue specimens were counterstained with Hematoxylin solution and incubated in an enclosed chamber for 1 minute, after which specimens were rinsed. Tissue slides were placed directly into a container filled with deionized water and dehydrated through graded series of alcohols, immersed in xylene, before xylene-based mounting media was applied and coverslips were applied for permanent mounting.

2.4 Neurotransmitter Assay

Phosphate buffer saline (PBS) was decanted from each tissue sample and the tissue was taken out from the bottle and placed in the mortar. 2ml of sucrose was then added to the tissue in the mortar and each tissue was homogenized with a pestle, after which it was poured into new sample bottles. After homogenizing, the bottles containing the samples were kept in a centrifuge and left for 15 minutes at 4000G. The homogenate was then centrifuged and the supernatant was decanted and used for the neurotransmitter assay. The neurotransmitters assayed in this study are dopamine, glutamate and serotonin.

Solution A: 2.27G OF KH_2PO_4 +250 ml of distilled water (Shake well to help the dissolving of the KH_2PO_4). **Solution B:** 2.365g of Na_3HPO_4 +250 ml of distilled water (Shake well to help the dissolving of the Na_3HPO_4). Mix solution A and solution B together.

Materials/Solutions used for the Assay: Standard solution of known volume (360pmol/L) was provided as part of the ELISA kit for the neurotransmitter assay, at 0.5ml x 1bottle, with the standard diluent at 1.5ml x 1bottle. Horseradish peroxidase Conjugate reagent (HRP -conjugate reagent) was provided at 3ml x 1bottle for signal amplification, Sample diluent at 6ml x 1bottle, Chromogen A and B solutions (tracers) at 6ml x 1bottle each, Stop solution at 6ml x 1bottle and Wash solution at (20ml x 30fold) x 1bottle.

Dilution of Standard: 10 Standard well on the ELISA plates were coated and 100 μl standard was added to the first and second wells 50 μl standard dilution was added to the first and second well and properly

Comment [s32]: What are the used for?

Comment [LA33]: Refer to the comment below

Comment [s34]: Doesn't understand the preparation of these solutions

Comment [LA35]: Refer to the comment below

Comment [LA36]: Expunge the whole paragraph and replace with the new procedure below

mixed. 100µl was taken out of the first and second well and was added to the third and fourth well separately. 50µl Standard dilution was added to the third and fourth well, after which, they were properly mixed. 50µl was taken out of the third and fourth well and discarded, while 50µl was added to the fifth and sixth well. 50µl Standard dilution was added to the fifth and sixth well, and was properly mixed. 50µl was taken out of the fifth and sixth well and was added to the seventh and eighth well, after which 50µl Standard dilution was added to the seventh and eighth well and was properly mixed. 50µl was taken out of the seventh and eighth well and was added to the ninth and tenth well, 50µl Standard dilution was added to the ninth and tenth well and was properly mixed. 50µl was taken out of the ninth and tenth well and discarded. A well was left empty as blank control.

2.4.1 Dopamine: All reagents, sample and standards were well prepared. 50µl standard was added to each well. Detection Reagent A was immediately prepared, shaken, mixed and incubated for 1 hour at 37°C, after which it was aspirated and washed 3 times. 100µl Detection Reagent B was prepared and incubated for 30 minutes at 37°C, after which it was aspirated and washed 5 times. 90µl substrate solution was added and incubated for 10-20 minutes at 37°C. 50µl stop solution was added and read at 450nm immediately (22).

2.4.1 Dopamine:

Principle of the Assay: Purified antigen was used to coat Microelisa strip plate to make a solid-phase antigen. Dopamine was added to the wells after washing and removing on-combinative antibodies and other components. Horseradish peroxidase (HRP) is added to each of the Microelisa strip plates and combined with Dopamine antigen to form an antibody-antigen-enzyme-antibody complex. After washing unbound antibodies and other components completely, TMB (3,3',5,5'-Tetramethylbenzidine) -TMB substrate solution was added to each well. Only the wells that contained the complex appeared blue in colour. The HRP enzyme-catalyzed reaction was terminated by the addition of sulphuric acid solution, which, gave a yellow colour. The absorbance was measured spectrophotometrically at a wavelength of 450nm. The Optical Density (OD) value was read as proportional to the concentration of Dopamine.

Comment [LA37]: New insertion

Comment [s38]: Which reagents

Comment [LA39]: Expunge the whole paragraph for dopamine assay'

Comment [s40]: Should be clearly explained

Comment [LA41]: Expunge the whole paragraph and replace with the one below, as most of the questions asked about standard and reagent are already taken care of in the preceding paragraphs.

Comment [s42]: What is the standard used?

Comment [LA43]: Please refer to the previous comment

Comment [s44]: Which reagent? Since solution A and B were mixed

Comment [LA45]: Please refer to the previous comment

Comment [s46]: Which reagent?

Comment [LA47]: Please refer to the previous comment

Comment [LA48]: Expunge the whole paragraph and replace with the new procedure below

Procedure: 40µl Sample dilution buffer and 10µl sample were added to sample wells. Samples were carefully loaded to the bottom of the well, without touching the well wall, after which they were properly mixed and incubated for 30 mins at 37°C, after being sealed with closure plate membrane. 30-fold wash solution was diluted with 30-fold distill water and was reserved. Closure plate membrane was carefully peeled off and the plates were aspirated and refilled with wash solution. Plates were left to rest for 30seconds after which wash solutions were discarded. Washing procedure was repeated for 5 times. 50µl HRP-Conjugate reagent was added to each well except the blank control well, after which they were incubated and washed. 50µl Chromogen solution A and 50µl Chromogen solution B were added to each well, while mixing and shaking gently, and incubated at 37°C for 15mins (Note that: light was avoided during coloring). 50µl stop solution was added to each well to terminate the reaction and absorbance (OD) was read at 450nm, using Microtiter Plate Reader (Note that: the OD value of the blank control was set at zero) (22).

Comment [LA49]: Expunge the whole paragraph and replace with this new procedure

2.4.2 Serotonin: 20µL of samples were pipetted into glass test tubes. 100µL of diluted assay buffer was pipetted into each tube and vortexed. 25µL of Acetylcholine reagent was also pipetted into each tube and vortexed immediately after pipetting. Tubes were covered and incubated for 15 mins at 37°C in a water bath. 2mL of diluted assay buffer was pipetted into each tube and vortexed. All tubes were centrifuged for 10mins at $1500 \times g$. 50µL of each sample and the standard was pipetted into the respective wells of the Microtiter plate. 50µL of serotonin biotin and serotonin antiserum were pipetted into each well. Plates were covered with adhesive foils and incubated for 90 minutes at RT 18-25°C on an orbital shaker (50rpm). Adhesive foils were removed and incubation solution was discarded. Excess solution was removed by tapping, with the inverted plate on a paper towel. 150µL of freshly prepared enzyme conjugate was pipetted into each wall and plates were covered with adhesive foil and incubated for 90 minutes at RT 18-25°C on an orbital shaker (500rpm). 200µL of PNPP (p-Nitrophenyl Phosphate) substrate solution was pipetted into each well and incubated for 60 minutes at RT 18-25°C on an orbital shaker (500rpm). Substrate reaction was stopped by adding 50µL of PNPP stop solution into each well.

Comment [LA50]: Please replace acetylcholine with Serotonin

Comment [s51]: Which standard?

Comment [LA52]: Please refer to the previous comment on Materials/Solutions for the assay and Standard dilution

Contents were briefly mixed by gently shaking the plate. Optical density was measured with a photometer at 405nm (reference wavelength: 600-650 nm) within 60 minutes after pipetting of the stop solution (23).

2.4.3 Glutamate: Supernatants were cell cultured to remove insoluble particles and centrifuged at 10,000 rpm for 5 min. Glutamate standard curve was prepared in the same non-conditioned media. PH was maintained between 7 and 8 for optimal working conditions as the Fluorometric Probe is unstable at high pH (>8.5). Cells were re-suspended at $1-2 \times 10^6$ cells/mL in PBS or 1X Assay Buffer for cell lysates, homogenized on ice and centrifuged to remove debris. Cell lysates were assayed in 1X Assay Buffer and centrifuged at 10,000 rpm for 5 min to remove insoluble particles. The supernatants were assayed in 1X Assay Buffer (24).

2.4.3 Glutamate:

Principle of the Assay: Purified antigen was used to coat Microelisa strip plate to make a solid-phase antigen. Glutamate was added to the wells after washing and removing onco-combinative antibodies and other components. Horseradish peroxidase (HRP) is added to each of the Microelisa strip plates and combined with Glutamate antigen to form an antibody-antigen-enzyme-antibody complex. After washing unbound antibodies and other components completely, TMB (3,3',5,5'-Tetramethylbenzidine) -TMB substrate solution was added to each well. Only the wells that contained the complex appeared blue in colour. The HRP enzyme-catalyzed reaction was terminated by the addition of sulphuric acid solution, which, gave a yellow colour. The absorbance was measured spectrophotometrically at a wavelength of 450nm. The Optical Density (OD) value was read as proportional to the concentration of Glutamate.

Procedure: 40µl Sample dilution buffer and 10µl sample were added to sample wells. Samples were carefully loaded to the bottom of the well, without touching the well wall, after which they were properly mixed and incubated for 30 mins at 37°C, after being sealed with closure plate membrane. 30-fold wash solution was diluted with 30-fold distilled water and was reserved. Closure plate membrane was carefully peeled off and the plates were aspirated and refilled with wash solution. Plates were left to rest for

Comment [s53]: This is not within the range

Comment [LA54]: Please note: typographical error. The OD was measured at 450nm wavelength

Comment [LA55]: The whole procedure will be replaced by the properly written and detailed one below

Comment [s56]: Which supernatant?

Comment [LA57]: The whole procedure will be expunged and replaced with a properly written and detailed procedure. Supernatant has been explained in details in previous paragraphs/comments

Comment [LA58]: Please, expunge the whole procedure and replace with the new below

30seconds after which wash solutions were discarded. Washing procedure was repeated for 5 times. 50µl HRP-Conjugate reagent was added to each well except the blank control well, after which they were incubated and washed. 50µl Chromogen solution A and 50µl Chromogen solution B were added to each well, while mixing and shaking gently, and incubated at 37°C for 15mins (Note that: light was avoided during coloring). 50µl stop solution was added to each well to terminate the reaction and absorbance (OD) was read at 450nm, using Microtiter Plate Reader (Note that: the OD value of the blank control was set at zero) (24).

2.5 Enzyme Assay

2.5.1 Cytochrome C-Oxidase Assay: The absorption of cytochrome C at 550 nm would change with its oxidation state. This property was the basis for the assay. Cytochrome C was reduced with dithiothreitol and then reoxidized by the cytochrome C oxidase. The difference in extinction coefficients (De **mM**) between reduced and oxidized cytochrome C was 21.84 at 550 nm. The oxidation of cytochrome C by cytochrome C oxidase is a biphasic reaction with a fast initial burst of activity followed by a slower reaction rate. In this assay, the initial reaction rate was measured during the first 45 s of the reaction. Total volume of the reaction was 1.1 mL. Spectrophotometer settings following the decrease in absorption at 550 nm at room temperature (25 ° C) using a kinetic program was 5 s delay, 10 s interval, and 6 readings. The instrument was set up prior to starting any reaction. The wavelength setting was considered critical and could deviate by no more than 2 nm. No signal was observed with a deviation of 10 nm (25).

Assay procedure included the addition of 0.95 mL of 1 × assay buffer to a cuvette and zeros the spectrophotometer. Also, a suitable volume of enzyme solution or mitochondrial suspension was added to the cuvette, bringing the reaction volume to 1.05 mL with 1 × enzyme dilution buffer. Mixing was by inversion. This was followed by starting the reaction by the addition of 50 mL of ferrocytochrome C substrate solution and mixing by inversion.

The A550/min was read immediately due to the rapid reaction rate of this enzyme. Background values were expected between 0.001 and 0.005 A550/min. The activity of the sample was calculated (25).

Comment [s59]: ???

Comment [LA60]: Typographical error.
The correct unit is (η per meter)

2.5.2 Glucose 6-Phosphate Dehydrogenase: This dehydrogenase has dual coenzyme specificity. When assayed under conditions that are optimal for the particular coenzyme, the ratio of observed catalytic activity is $\text{NAD/NADP} = 1.8$. The reaction velocity was determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD or NADP. One unit reduces 1 μmol of pyridine nucleotide per minute at 30 ° C and pH 7.8 under the specified conditions (26).

Assay procedure involved adjusting the spectrophotometer to 340 and 30 ° C. Reagents were pipetted into each cuvette as follows: 0.055 M Tris HCl buffer, pH 7.8 with 0.0033 M MgCl_2 , 2.7 mL; 0.006 M NADP (or 0.06 M NAD), 0.1 mL; 0.1 M glucose-6-phosphate, 0.1 mL. Incubation was done in spectrophotometer at 30 ° C for 7–8 min to achieve temperature equilibration and establish blank rate, if any. This was followed by adding 0.1 mL diluted enzyme and recording increase in A_{340}/min for 4–5 min. The A_{340}/min was calculated from the initial linear portion of the curve (26).

2.5.3 Lactate Dehydrogenase Assay Procedure

Reagent Preparation: The plate reader was turned on to allow light source to warm up, and absorbance wavelength was set to 340 nm. Kit reagents were warmed to room temperature for 30 minutes and reagent mix was reconstituted by adding exactly 27 mL of deionized water to the LDH reagent mix powder. Reagents were mixed by swirling the bottle 10 times. Contents were allowed to dissolve for 10 minutes at room temperature.

Procedure: Six clean microcentrifuge tubes were labelled 1, 2, 3, 4, 5 and 6 (Neg). Contents of Standard vial were dissolved in 920 L of Standard Dilution Buffer, properly mixed and 150 L of dissolved Standard was transferred to Tube 1. Standard was serially diluted by adding the appropriate volumes of Standard and Standard Dilution Buffer. 5 μL of each sample or was added to microplate wells. 250 μL reconstituted LDH Reagent Mix was also added to the wells. Absorbance of the wells was measured at 340 nm (= initial reading) and exactly 5 minutes later, the absorbance was again read. The initial absorbance was subtracted from the 5 min absorbance for each of the samples. Average of the values were taken to obtain the average absorbance increase in 5 minutes for each sample. The average 5 min

absorbance increase was multiplied by 2,187 (conversion factor) to obtain LDH activity (IU/L), (27).

2.5.4 Creatinine Kinase Assay Procedure: This Creatinine Kinase Assay procedure followed the standardized method of (28). Creatine kinase (CK) catalyzed the reaction between creatine phosphate (CP) and adenosine 5'-diphosphate (ADP) with formation of creatine and adenosine 5'-triphosphate (ATP). The latter phosphorylated glucose to glucose-6-phosphate (G6P) in the presence of hexokinase (HK). G6P is oxidized to Gluconate-6P in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADP) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6P-DH). The conversion was monitored kinetically at 340 nm by the rate of increase in absorbance resulting from the reduction of NADP to NADPH proportional to the activity of CK present in the sample. This working principle was used for the assay.

Reagent 1: Buffer/Glucose/NAC. Imidazol buffer 100 mmol/L pH 6.7, glucose 20 mmol/L, NAC 20 mmol/L, magnesium acetate 10 mmol/L, NADP 2.5 mmol/L, HK \square 4 KU/L, EDTA 2 mmol/L.

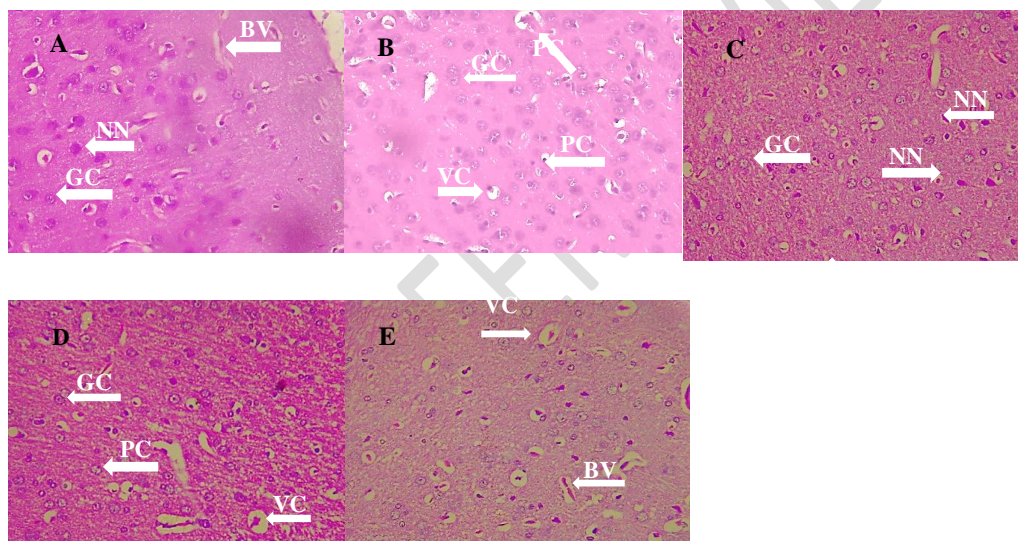
Reagent 2: 2 Substrate/Coenzymes. CP 30 mmol/L, AMP 5 mmol/L, ADP 2 mmol/L, di(adenosine-5') pentaphosphate 10 μ mol/L, G6PDH μ 1.5 KU/L Stored at 2-8°C. (The two reagents were mixed 4 mL of R1 + 1 mL of R2). The mixture (working reagent), samples and controls were pre-incubated to reaction temperature. Photometer was set to 0 absorbance with distilled water. Working reagent 1.0mL and sample 40 μ L were pipetted into cuvette at 25°C and gently mixed by inversion. Cuvette was inserted into the cell holder and stopwatch was set to monitor. Incubation was done for 3 minutes and initial absorbance reading was recorded. Absorbance readings were repeated exactly after 1, 2 and 3 minutes. Difference between absorbances and mean of the results were calculated to obtain the average change in absorbance per minute (μ A/min) (28).

3.0 Results

3.1 Histology and Immunohistochemistry (Figures 1-3)

Results of the study presented (below) are photomicrograph sections showing the cellular/cortical integrity and the degree of assault/recovery across the groups, as demonstrated using the H and E (Figure 1), Cresyl Fast Violet (Figure 2) and GFAP (Figure 3).

Epilepsy, as induced by kainic acid in this study, caused alterations in the cortical histoarchitecture of the experimental animals. Nissl bodies expression was also altered in the epileptic groups. Aberrations in the morphologies of Neurons [Group B], as well as the observable changes in the activities of RER or Nissl bodies demonstration were observed. Glia astrocytes were generally demonstrated across the groups. However, when compared with others, Group B clearly showed evidence of amelioration.



Comment [s61]: This is suprising, since group B was treated with kainic acid alone
Contradiction from the previous sentence

Comment [LA62]:

Comment [LA63]: This is a typographical error. There is no contradiction. Group B will be replaced by: Group D, Hence, Group D showed evidence of amelioration.

Figure 1: showing photomicrographs of the Frontal Lobe of rats in Groups A-E, demonstrating the frontal cortex stained with H&E [H&E A-E X400]. There was neuronal and glial cell degeneration in Groups B, D & E. Group B shows signs of changes in cortical histoarchitecture and neuronal distribution in a pattern that is attributable to the effects of kainic acid induced neurodegeneration.

Note: Arrows point to specific features that are denoted by letters.

Legends: NN, normal neuron; GC, glial cells; VC, vacuolated cell; PC, pyknotic cell; BV, blood vessel,

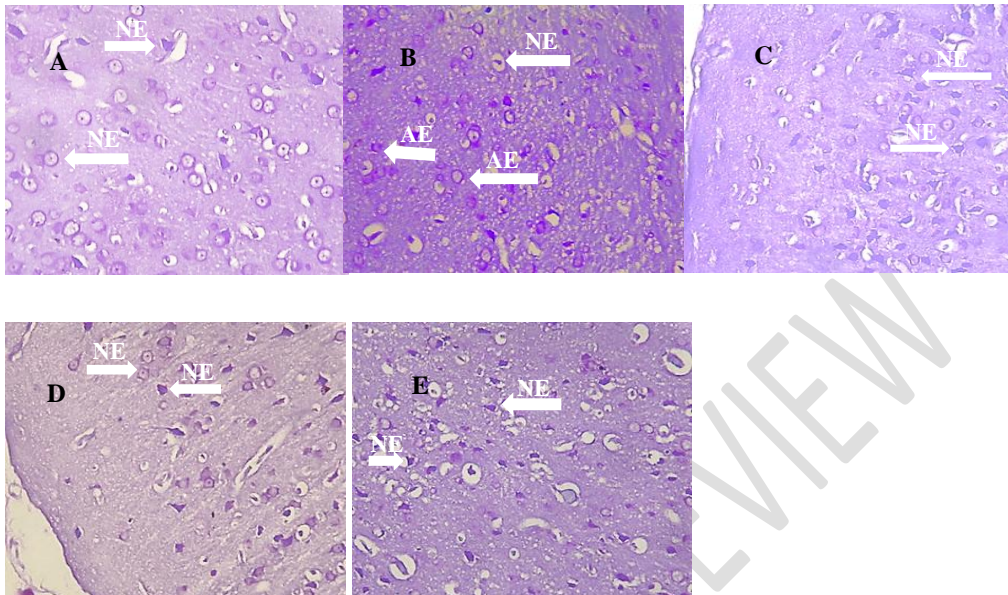
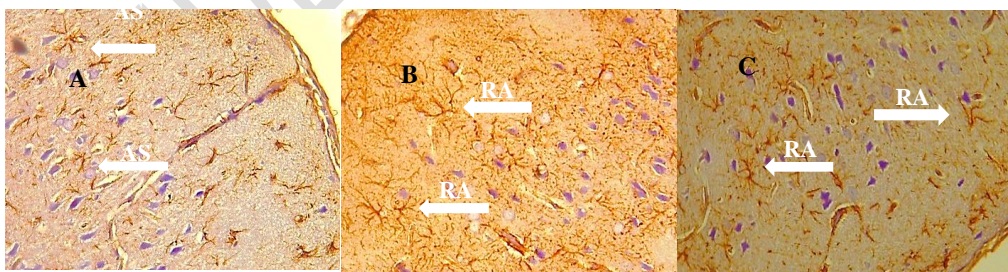


Figure 2: showing photomicrographs of the Frontal Lobe of rats in Groups A-E, demonstrating the frontal cortex stained with Cresyl fast violet [CFV A-E, X400]. There was differential expression of Nissl bodies, which was used as a marker of neuronal intracellular endoplasmic reticulum synthesizing activities. Alteration in Nissl expression was observable in the Group B [Blue arrows], following kainic acid induced epilepsy; relative amelioration is observable in all intention groups (C-D).

Legends: NE, normal neuronal nissl expression; AE, aberrations in neuronal Nissl expression.



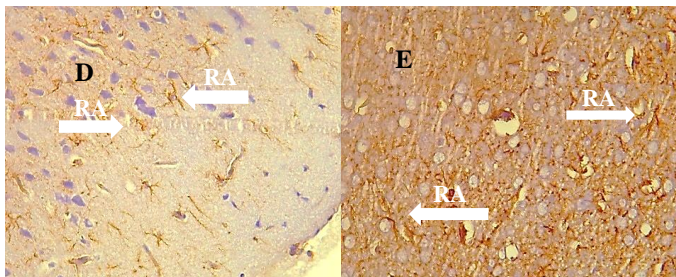


Figure 3: showing photomicrographs of the Frontal Lobe of rats in Groups A-E, demonstrating the frontal cortex stained with GFAP [GFAP A-E X400]. There was an increase in the area percentage of the cerebral cortex astrocytes across the groups, especially, groups B & E.

Legends: AS, astrocytes; RA, reactive astrocytes.

3.2 Neurochemical changes (Figure 4 and Figure 5)

Quantitative Results of the study are presented (below) are graphs representing Enzymes (lactate dehydrogenase, creatinine kinase, Glucose 6-Phosphate Dehydrogenase & cytochrome c-oxidase) assay as demonstrated in Figure 4 and Neurotransmitters (glutamate, serotonin & dopamine) assay as demonstrated in Figure 5.

Enzyme activities were generally altered. With respect to neurotransmitters, the activity levels were not significantly altered except in the case of serotonin, when intervention was done with carbamazepine. Generally, alternations in neurotransmitter levels or activities were not significantly altered, but the enzymes were markedly altered

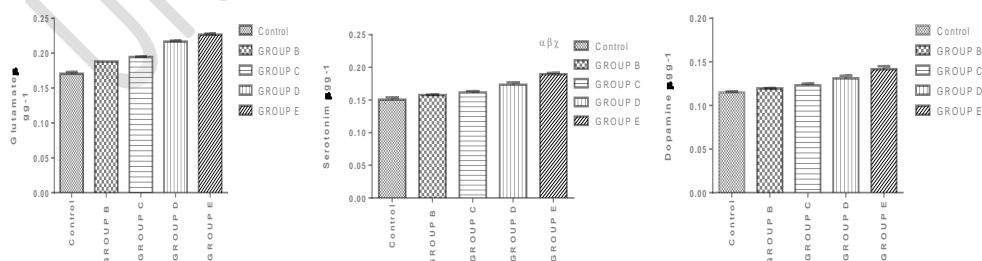


Figure 4: Bar chart, showing neurotransmitter levels in the treated and control groups after 21 days of treatment. The glutamate levels of groups D-E were higher than other groups, while serotonin levels increased significantly in group E when compared to the control group and groups B-D.

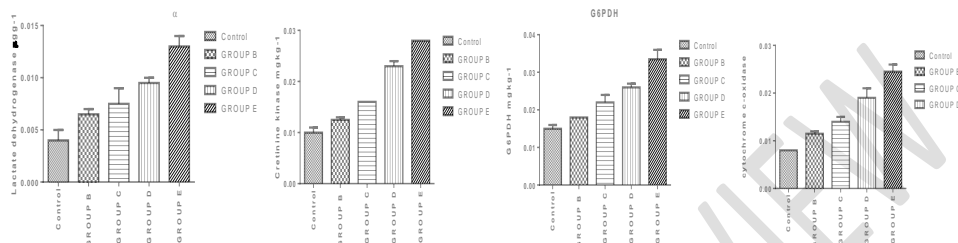


Figure 5: Bar chart, showing enzyme levels in treated and control groups after 21 days of treatment. Creatinine kinase, (G6PDH) and cytochrome c-oxidase enzyme levels were higher in the treated groups when compared to the control group. Lactate dehydrogenase treated groups were significantly higher when compared to the control.

3.3 Behavior (Figure 6 and Figure 7)

Represented below are graphs showing the Neurobehavioral Assessment as demonstrated in Figure 6 and Figure 7. Latency was used in the current study as a marker of memory quality. All the treated animals exhibited shorter latency duration. However, anxiety was negatively implicated when epilepsy was induced and untreated in Group B.

3.3.1 Barnes Maze (Primary and Total Latency)

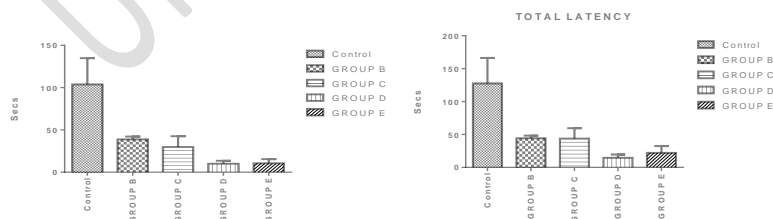


Figure 6: Bar chart, showing learning and memory levels in the treated and control groups after 21 days of treatment. Latency results show that memory was severely impaired in all treated groups relative to the control group.

Comment [s64]: Or 29 days?

Comment [LA65]: after 29 days:
Typographical error

3.3.2 Elevated Plus Maze: (Open and Closed Arm Duration/Entry)

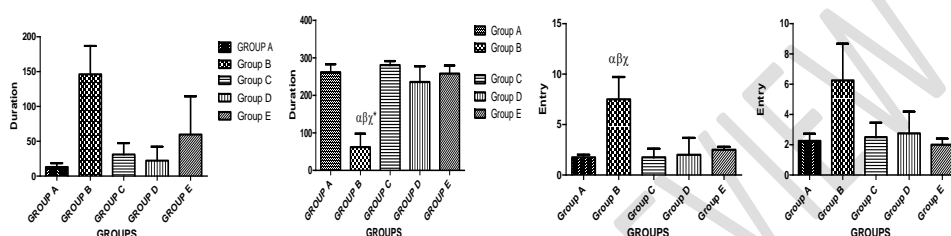


Figure 7: Bar chart, showing anxiety levels in treated and control groups after 21 days of treatment. There was significant difference in anxiety level of group B as compared to other treated groups and the control.

4.0 Discussion

Neuronal and glial cell degeneration is attributable to kainic acid and the resultant epileptic seizures

The histological findings on the cerebral cortex of the animals in group B revealed neuronal and glial cell death and pyknosis, which may have resulted from the epileptic activity of the Wistar rats. Gross degeneration was observed in the neuroglia in group E, as it appeared less intact relatively. Immunohistochemical findings showed there was an increase in the area percentage of reactive astrocytes in the cerebral cortex, across the treated groups relative to the control group. This further suggests there was assault to the cerebral cortex caused by the epileptic activities of the rats, and is in consonance with the properties attributed to kainic acid as an agent, which has excitatory effect on the central nervous system and also has epileptogenic property (6).

Kainic acid is implicated in loss of cortical integrity

Groups B-E showed neuroglia which appeared to be less intact relative to the control group within the cortex. Neuronal morphologies changed when animals were exposed to the agent without intervention [Group B]. Nissl bodies expression as a marker of rough endoplasmic reticulum activities, otherwise known as Nissl bodies in the neurons was also altered in the epileptic groups. Aberrations in the morphologies of Neurons [Group B], as well as the observable changes in the activities of RER or Nissl bodies demonstration, are a marker of neurocortical changes that are attributable to the effects of the changes in the brain cortex of the epileptic animal. The effects were however largely ameliorated in the groups that were treated with the extract as well as the orthodox drug as these effects were either absent or greatly mitigated. The used extract treatment, therefore, had ameliorative effects in the epileptic animal brains.

***Bryophyllum pinnatum* showed potential to ameliorate neuronal damage**

Observations from group D showed that there was degeneration of neuroglia in the cerebral cortex, though it was observed to be mild. Pyknotic and vacuolated neuronal cells were also observed. There was no significant repair seen, attributable to the ketogenic diet regimen. This agrees with Todorova et al, (30) work: 'the ketogenic diet inhibits epileptogenesis in EL mice: A genetic model for idiopathic epilepsy'. According to him, seizure protection was transient, in as much as the treated and control mice experienced a similar number and intensity of seizures after 6 weeks on the diet. Plasma β -hydroxybutyrate levels were said to be significantly higher in mice on ketogenic diet, however, the level of ketosis was not predictive of seizure protection in EL mice.

Glial fibrillary acidic protein expression is an indication of cortical neuroinflammation to which the glia astrocyte would react. Glia astrocytes were generally demonstrated across the groups. However, Group B showed that GFAP expression was enhanced. In this group, the experimental animals' brains showed

enhanced GFAP expression that was indicative of astrocyte reaction. When compared with others, Group B clearly showed evidence of amelioration. Results therefore would suggest that the extract had effects similar to the therapeutic effects of carbamazepine in terms of ameliorating extensive neuroinflammation as indicated by the enhanced astrocyte reaction. There was an indication of ongoing neuronal regeneration aided by the presence of abundant astrocytes in group C with a few vacuolated cells seen. The ongoing repair could be attributed to the aqueous extract, *bryophyllum pinnatum* treatment administered to the group. This supports (29) in 'the evaluation of anticonvulsant potentials of *bryophyllum pinnatum*' that the herb has anticonvulsant properties.

The Neurotransmitters

The neurotransmitter results revealed that the glutamate levels of groups D-E were high when compared to the control group and groups B & C. This suggests excitotoxicity. The increased glutamate level in group D (ketogenic diet) is in accordance with (31), where it was reported that, in the occurrence of ketosis in the brain, there is an activation of astrocytic metabolism resulting in enhanced conversion of glutamate to glutamine, thereby providing more glutamine to serve as a GABA precursor. In epilepsy, both Gamma y-aminobutyric acid (GABA) hypoactivity and glutamate hyperactivity have been recorded, while dopamine shows hyperactivity and serotonin shows hypoactivity (32). The concentration level of serotonin in group E increased significantly when compared to the control group and groups B, C, D. This supports (33) who reported that Carbamazepine administration causes large increase in extracellular serotonin concentration and dose-related anticonvulsant effects. (34) also reported that disturbance of the serotonergic systems is involved in the following mental disorders: schizophrenia, depression, infantile autism, and obsessive-compulsive disorder. Dopamine concentration in the treated groups showed little difference when compared to the control group, except for group E (Carbamazepine) where the concentration was highest. This finding supports the view proposed by (35) that valproate and carbamazepine increase prefrontal dopamine release by 5-HT 1A receptor activation.

Comment [s66]: The negative control group?

Comment [LA67]: No, Group D: Typographical error.

'When compared with others, Group D clearly showed evidence of amelioration'

Increased Creatinine kinase (G6PDH) and Cytochrome c-oxidase could be attributable to the antiepileptic effect of *Bryophyllum pinnatum*, ketogenic diet and carbamazepine

According to this study, the results for the level of the enzymes Creatinine kinase (G6PDH) and cytochrome c-oxidase in the treated groups were higher when compared to the control group. However, lactate dehydrogenase treated group was significantly higher when compared to the control group. The increase in lactate dehydrogenase could be as a result of seizures and epileptic activity which tend to be reduced by inhibition of the metabolic pathway via the lactate dehydrogenase which is a component of the astrocyte-neuron lactate shuttle. While the increase in Creatinine kinase, (G6PDH) and cytochrome c-oxidase could be as a result of antiepileptic effect *bryophyllum pinnatum*, ketogenic diet and carbamazepine which tend to increase metabolic activities generally in the body, this increase in activity would increase in the use of utility of ATP and hydrolysis of glucose, therefore the increase in Creatinine kinase, (G6PDH) and cytochrome c-oxidase was observed.

Kainic Acid Induces Recurrent Seizures and Behavioral Changes in Wistar rats

The Barnes maze tests were used to investigate spatial learning and memory which are two vital expressional functions of the frontal cortex (18). Neurobehavioral findings at the latency phase showed that memory was severely impaired in all treated groups relative to the control group which correlates with the previous report by (36) who reported impaired spatial memory acquisition in GAD65 mice.

For anxiety level investigations, the Wistar rat models, were subjected to Elevated Plus Maze test. Results from the elevated plus maze study indicated that there was significant difference in anxiety levels of the group B, which was induced with kainic acid only, relative to the control groups. This is in contrast with (37), who reported that anxiety was higher in rats who had experienced KA-induced status epilepticus.

Conclusion and Recommendation

The results obtained from this study showed that, kainic acid caused extensive damage to the cortical and glial structures of the frontal cortex, and caused neuronal death, induced by increased glutamate levels and massive release of the excitatory amino acid l-glutamate into the extracellular space. *Bryophyllum pinnatum* administered at 400mg/kg body weight, ameliorated the effects of kainic acid on the cortical structures and aided glial and consequentially, neuronal regeneration. The neurobehavioral results revealed that *bryophyllum pinnatum*, ketogenic diet and Carbamazepine may cause impairment of memory and cognition. There was no significant elevation in the anxiety levels of the treated rats, especially those who experienced KA-induced status epilepticus. This is not in agreement with the existing notion that, rats who experienced KA-induced status epilepticus had elevated anxiety levels, though there was no significant repair seen in rats treated with ketogenic diet. Further study is recommended to confirm the results of this research. Pharmacological study of the antiepileptic effect of *bryophyllum pinnatum* on the PFC of the frontal lobes, structure and function should be done.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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