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Anxiety Reaction and Astrocyte Expression to Pentylenetetrazol Induced Kindling Model in Wistar Rats Following Pretreatment with Plant Tannin

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Abstract:- Tannins are water-soluble phenolic compounds found in plants which have the ability to form complexes with nutritionally important nutrients such as proteins and minerals. In view of the recent findings of the health benefits and classification of tannin, this study focused on assessing how plant tannin can affect or change behavioural patterns using animals exposed to pentylenetetrazol (PTZ) –induced convulsion and also compare the inhibitory role of tannin in astrocytosis. Forty nine adult male wistar rats in seven groups were used for this study. Group 1,the normal control group was given growers feed and appropriate volume of Normal saline, group 2 was given 5mg/kg of PTZ only, Groups 3, 4,5 & 6 were given tannin (100mg/kg,200mg.kg, 400mg.kg & 400mg/kg respectively) and 5mg/kg (single dose) of PTZ while group 7 (positive control group) was given 2.4mg/kg of tegretol (a control drug for convulsion) and 8.2mg/kg of PTZ. Tannin was administered orally to animals in groups 3, 4, 5 (Pre-treatment groups) and 6 for 20 days. On the 18th day of administration, PTZ administration commenced to the 20th day. Findings from this study showed that 100mg/kg and 400mg/kg of tannin had anti-convulsive properties in response to PTZ –induced convulsion, and also reduced the rate of astrocytosis in the temporal lobe. Tannin also elicited anxiolytic traits as animals pre-treated with tannin spent some time in the open arm of the EPM. Thus, tannin inhibited astrocytosis, protected neurons, reduced convulsion duration and has the potential of reducing rate of anxiety in rodents.

Keywords:- *Pentylenetetrazol, anxiety, astrocyte, tannin, convulsion.*

I. INTRODUCTION

Anxiety is a physiological and psychological response that occurs when the mind and body encounters stressful, dangerous or unfamiliar situations. It manifests as a feeling of discomfort, distress or apprehension before a significant event[1]. It's a common emotional experience that everyone experiences to some gradations throughout their lives. Fear and anxiety serve important functions, allowing us to identify and respond to threats, ensure our safety and enable adaptation to our environment. However, when anxiety becomes overwhelming and significantly interferes with our daily functioning in important areas such as school, work or even inter-personal relationships, it may indicate the setting in of an anxiety disorder[2].

These anxiety disorders are becoming increasingly prevalent in our present day society due to several occupational and environmental factors associated with personal wellbeing and growth and it has contributed to cases of mental illness [3].

Interestingly, research over the years have consistently been carried out across the globe on ways of managing and treating anxiety – related disorders including providing local treatment measures from countries including Africa. In the field of behavioral sciences, models have been used with laboratory animals to proffer or point to solutions that may be evidently useful in the treatment and management of anxiety.

The Elevated Plus Maze (EPM) model has extensively been used over many decades as a reliable tool for establishing anxiogenic and anxiolytic substances using rodents as a screening test as a general research tool in neurobiological anxiety research [4]. The elevated plus maze (EPM) is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and also help to suggest or define brain regions and mechanisms underlying anxiety-related behavior [5]. The EPM has been described as a simple method for assessing anxiety responses of rodents [6]. Initially, it was a task, using a Y-shaped apparatus that included an elevated open alley, which produced a strong approach–avoidance conflict, and an enclosed alley, which did not, was first described by Montgomery [7].This task was later modified into an EPM apparatus with four arms (two open and two enclosed) that are arranged to form a plus shape and this was designated by Handley and Mithani [8]. These authors described the assessment of anxiety behavior of rodents by using the ratio of time spent on the open arms to the time spent on the closed arms. According to standard practice, behavior on the EPM is evaluated during a single trial to avoid the possibility of habituation to the apparatus that would result in lost sensitivity to key outcome measures[9].

Thus, in research to test for levels of anxiety, anxiogenic compounds are given to rodents' overtime and a complementary substance is also administered thereafter to test for its anxiolytic ability prior to the rodents' exposure to the EPM apparatus. It is pertinent to note that an anxiogenic substance is one that causes anxiety and the effect is in contrast to anxiolytic agents, which inhibits anxiety. Together these categories of psychoactive compounds may be referred to as anxiotropic compounds. Pentetrazol (pentylenetetrazol) is a central and respiratory stimulant, similar to doxapram hydrochloride. It is a GABAreceptor antagonist and is anxiogenic [10][11]. In this study, PTZ was administered in low doses as an anxiogenic drug and also as a convulsant after tannin were given to the rodents as a protective substance. PTZ is characterized by high bioavailability due to easy penetration through biological membranes, rapid distribution to organs after intraperitoneal (i.p.) injection, a very short latency of action, uniform distribution in the brain, and ability to stimulate epileptogenic activity by blocking g-aminobutyric acid (GABA)-mediated transmission [12][13].

As an anxiogenic substance was given to the rodents used for this study, tannin, a secondary metabolite found in plants was used as an anti-convulsant and anxiolytic compound. Tannins are polyphenol compounds, with many health-related beneficial effects [14]. Their distribution and concentration are highly different in the different parts of a plant, such as in the leaves, roots, seeds, and fruits [15]. Tannins are biologically potent compounds in relation to the regulation of health-associated aspects of all living beings, including humans. They can be effective in the treatment of various diseases by virtue of the multitude of their beneficial activities [16]. They have anti-inflammatory [17][18], anti-oxidative [19], anti-convulsant [20] and anti-tumour potentials [21].

The aim of this study is to investigate the anxiolytic and anti-convulsant abilities of tannin as a measure of suggesting to new pharmacologic remedies towards the management of both anxiety and convulsion cases. The ultimate goal is to identify the anxiolytic ability of tannin and also access the reactive astrocyte population following PTZ induced convulsions that are associated with elevated anxiety patterns in an aid to developing more traditional and effective ways of managing anxiety.

II. MATERIALS AND METHODS

A. Purchase and Preparation of PTZ

5g of Pentelenetetrazol (PTZ) was purchased from the Physiology Department of the University of Port Harcourt, River State Nigeria and kept in a refrigerator, prior to use. MACKUN Pentylenetetrazole (98%), $C_6H_{10}N_4$; 138.17MW. Storage (2 – 8°C), P815563-5G. Lot#: C13139738. CAS: 54 – 95 – 5.

2.4g of PTZ was immersed in 232.8mls of distilled water to get the stock solution for administration. According tothe Cayman chemical safety and toxicity data sheet, the $LD_{50}$of pentylenetetrazol (i.p) for rats is 82mg/kg. This was observed as the lethal dose and adhered to.

B. Collection of plant material

Fresh Phyllanthus Amarus (PA) leaves were collected from Agwu and Aninri Local Government Areas, Enugu state of Nigeria and were authenticated in the Department of plant science and Biotechnology of the University of Nigeria, Nsukka, Enugu State, Nigeria.

C. Preparation of plant material

Percolation method was used to prepare the crude extract[22][23]. The PA leaves were washed with tap water to remove dirt, dried under room temperature until leaves became dry, crispy. The leaves were then finely pulverized using a manual blender into powder, and the powdered material was passed through a sieve of suitable mesh size to separate the smaller powdered particles from the larger ones. The larger powdered particles were then returned to the blender for further grinding. The plant extract solution was obtained by percolating 515g of the powdered leaf sample in 70% methanol [24] [25].

- ***Phytochemical screening of Crude extract:*** Phytochemical screening was carried out on the crude extract of the leaf sample to identify qualitative and quantitative metabolites. The following metabolites were found: carbohydrates (Molisch's test*)*, Aromatic amino acid (Xantheoprotein protein test), saponnins (frothing test), tannin (ferric chloride test [26], potassium iodate test [27], alkaloids (wagners's test & Dragindroff Test), terpenoids (Salkowski test) [28] , phenol [29] [30]. Tannins contents were quantified using colorimetric assays.
- **Partitioning/fractionating of crude plant extract**: 49.3g of the crude extract was removed from the refrigerator and exposed to fractionate and isolate tannin using the separating funnel method [31] [23] with n-hexane and n-butanol solvents.
- **Identification of tannin:** After the isolation of tannin, it was pertinent to perform a test to ascertain the presence of tannin in the final fractionate extracted. Thus, two methods were used for this study:

✓ All solutions were prepared from analytical grade reagents and distilled–deionized water was used throughout. The reagent KIO_3 solution (2.5% w/v) was prepared by dissolution of 25 g of KIO_3 in 1000 mL of water. The extraction solution, used for sample preparation, was acetone 70% (v/v), which was prepared by diluting 70 mL of pure acetone in 100 mL of water. The stock analytical solution of 5000 mg L^{-1} was prepared by solubilization of 0.25 g of tannic acid in 50 mL of extraction solution [27].

✓ A quantity (50 mg) of extract was boiled in 20 ml of distilled H_2O and filtered. A few drops of 0.1%. $FeCl_3$ was added in filtrate and observed for colour change; brownish green or a blue-black colouration was taken as evidence for the presence of tannins [29] [30].

To get stock solution for administration, 1000mg of Tannin fractionate was dissolved in 100mls of 3% tween 80 solution and kept in a sample bottle ready for use.

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D. Acute Toxicity Test

Pilot study for dose response trial was carried out to ascertain the appropriate dose for tannin using Lorke's method [32]. The acute toxicity of the extract was done using Lorke's method [32] with modifications by dividing it in to two phases.

- **Phase 1**: Nine wistar rats were divided into three equal groups. The three equal groups were administered orally with graded doses (10, 100 and 1000 mg/kg respectively) of fractionate. The animals are placed under observation for 24 hours to monitor their behavior as well as if mortality will occur.
- **Phase 2:** Another nine mice were divided into three equal groups, which received graded doses (1600, 2900 and 5000 mg/kg) of the extract respectively. The number of deaths in each group within 24 h was recorded and the final LD_{50} values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) and the lowest lethal dose (where deaths occurred). [32]:

Then the LD_{50} is calculated by the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,
D_{100} = Lowest dose that produced mortality.
Result: No fatality was recorded
Elevated Plus Maze (Anxiety)Test

The elevated "plus" maze used in this study was made of wood, and consisted of two open arms, 50 × 10 cm (length × width), and two enclosed arms 50 × 10 × 50 cm (length × width × height), arranged such that the two arms of each type were opposite to each other. The maze was elevated to a height of 50 cm above the ground[5].

Each rat was placed in the center of the elevated plus maze facing an open arm, and allowed 5 min free exploration. The following parameters were measured: number of open arm entries, time spent in open arms, number of closed arm entries, time spent in open arm and total arm entries. Arm entry is defined as when the hind paws of the rats are completely within the arm. The apparatus is cleaned with 5% ethanol before testing a new animal to eliminate possible bias due to odours left by previous animal [33] [34] [35].

The EPM tests were conducted in the light phase were the rats were brought out of the animal room to the behavioral testing room.

E. Research Design

Forty nine adult male wistar rats in seven groups were used for this study. Group 1,the normal control group was given growers feed and appropriate volume of water, group 2 was given 5mg/kg (single dose) of PTZ for 3 days, group 3 was given 100mg/kg of tannin for 20 days and 5mg/kg (single dose) of PTZ for 3 days, group 4 was given 200mg/kg of tannin for 20 days and 5mg/kg (single dose) of PTZ for 3 days, group 5 was given 400mg/kg of tannin for 20 days and 5mg/kg (single dose) of PTZ for 3 days, group 6 was given 400mg/kg of tannin only for 20 days while group 7 was given 2.4mg/kg of tegretol (a control drug for convulsion) for 20 days and 8.2mg/kg of PTZ for 3 days.

Tannin was administered via oral route to animals in groups 3,4,5 and 6 for 20 days, tegretol was administered orally to animals in group 7 only via oral route for 20 days. On the 18th day of administration, PTZ was given i.p (intraperitoneally) once for 3 consecutive days one hour after administration of tannin. The animals were maintained on a regular light cycle. Basal Y maze test was carried out prior to commencement of administration and also at the end of administration on day 20 to compare possible differentials in cognitive levels across groups. Immediately after the administration of PTZ on the 20th day. Glial Fibrillary Acidic Protein (GFAP) encoded for astrocyte expression in the temporal lobe microstructure across all groups was carried out[36].

III. RESULTS

Data collected from this research was analyzed with SPSS data software (Version 23) with P value at 0.05 (level of significance) using the one way ANOVA. Post Hoc test (multiple comparison test) was carried out were there wasa statistically significant difference between the means of three or more independent groups.

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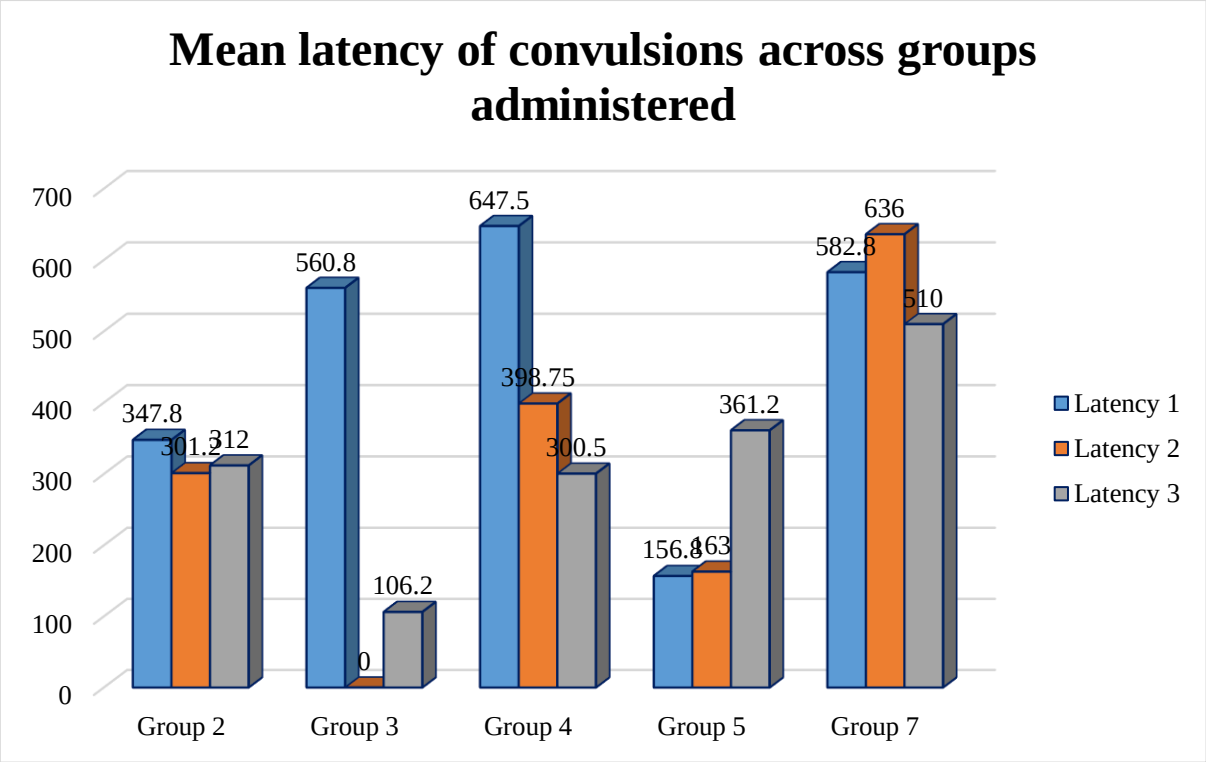

Fig. 1: Bar graph showing mean comparison of duration of convulsion amongst groups administered with PTZ within three days. One way ANOVA. The mean difference is significant at the 0.05 level. Latency 2 was significant at 0.015*. Values are mean ± SD; n = 7

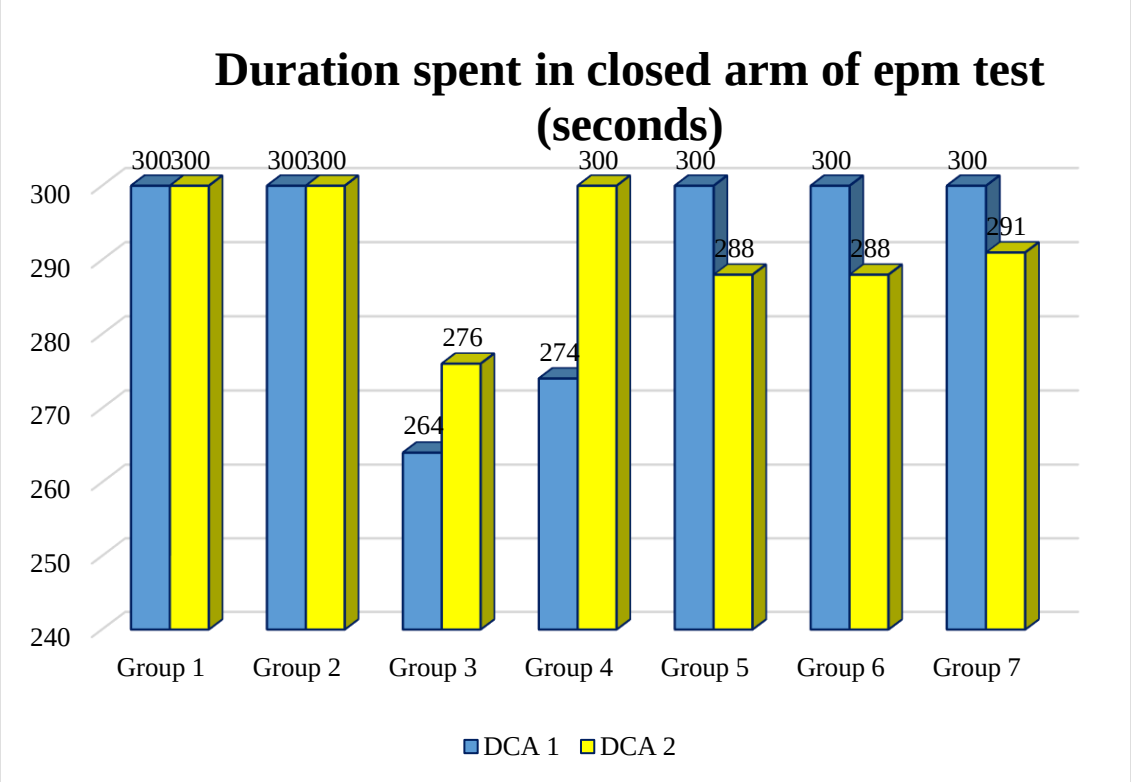

Fig 2: Bar graph showing the mean time animals in all groups spent in the closed arm of the elevated plus maze apparatus. One way ANOVA. The mean difference is significant at the 0.05 level. Values are mean ± SD; n = 7.

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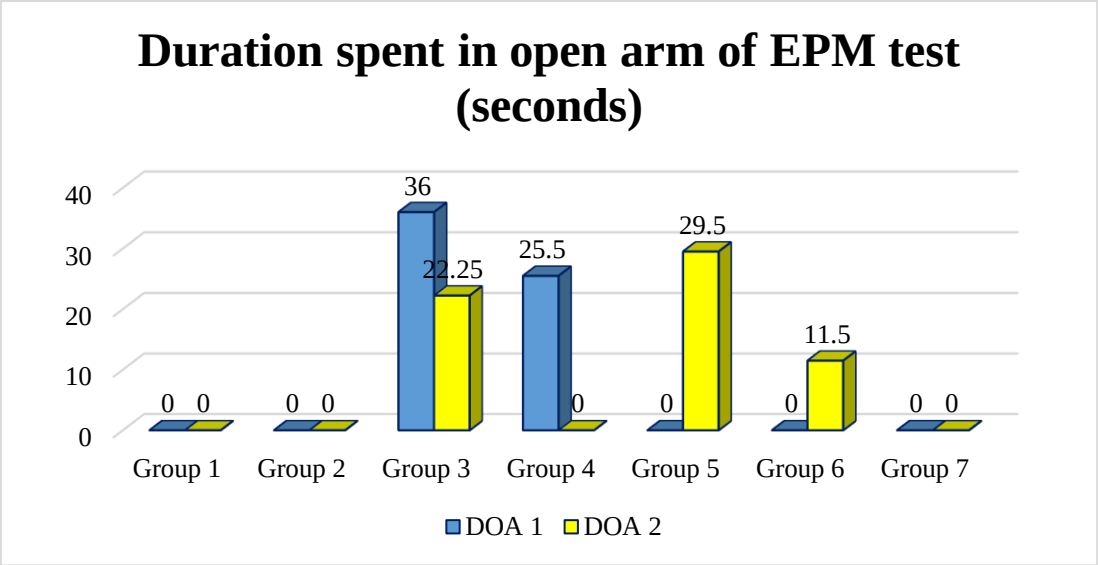

Fig 3: Bar graph showing the mean time animals in all groups spent in the open arm of the elevated plus maze apparatus. One way ANOVA. The mean difference is significant at the 0.05 level.Values are mean ± SD; n = 7.

Table 1: Mean astrocyte cell count across groups with Image J software across all groups

Astrocyte cell count	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	P-value
	52.00 **β ϒm**	198.00 # **ϒacdm**	135.00 # **βac**	74.00 **β ϒ**	69.00 **β ϒm**	99.00 **β**	117.00 # **βc**	0.001*

Values are mean ± SD; n = 7. The mean difference is significant at the 0.05 level.

Post HOC test was done using (turkey HSD alpha) multiple comparison test. #=Significant when compared with group 1(normal control), β = Significant when compared with group 2 (negative control), ϒ= Significant when compared with group 3(low dose treatment), A= Significant when compared with group 4 (middle dose treatment), C= Significant when compared with group 5(high dose treatment), d= Significant when compared with group 6, m= Significant when compared with group 7 (positive control group).

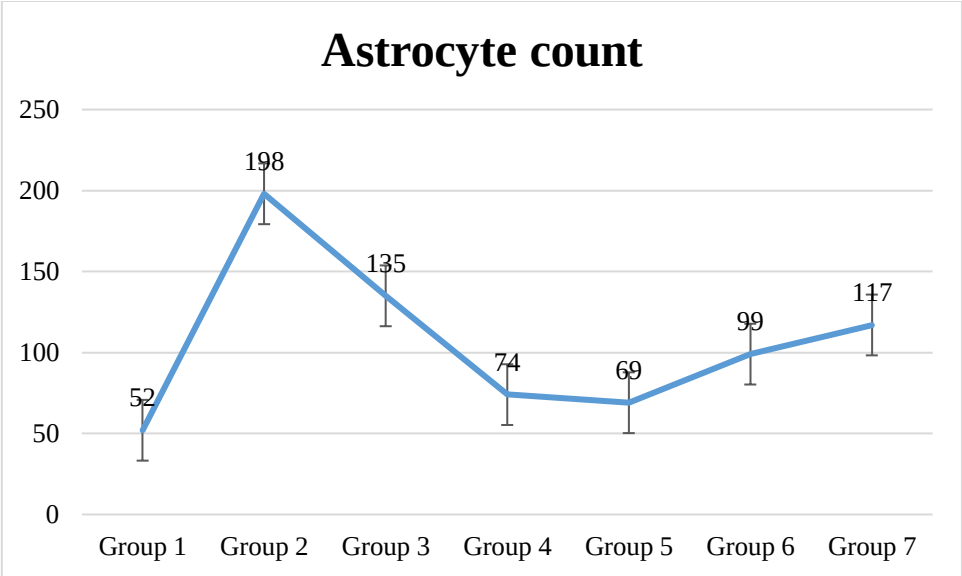

Fig. 4: Showing the mean astrocyte cell count in the temporal lobe across all groups using image j software (one way ANOVA).P value was significant at 0.05. (P = 0.001*). n =7 and values are mean ± SD

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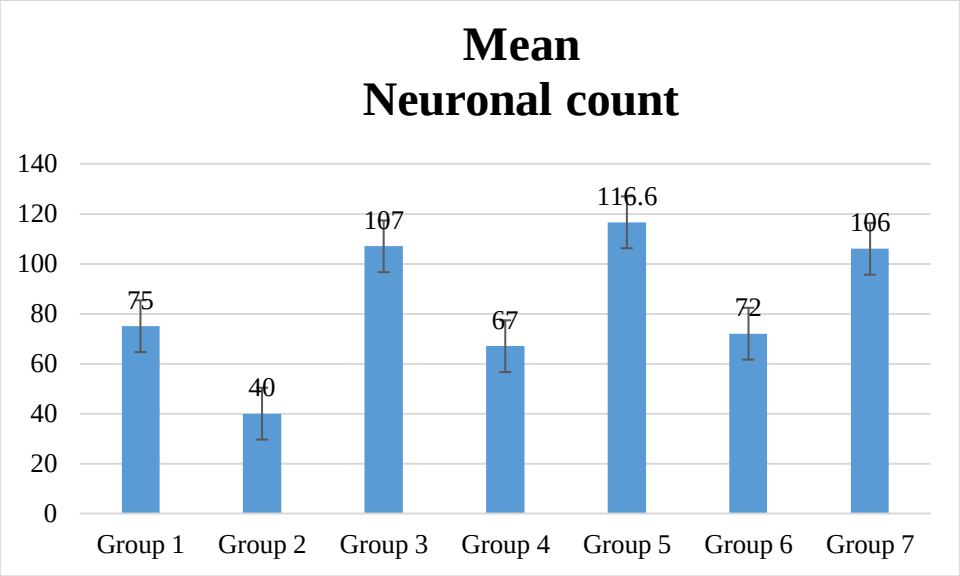

Fig. 5: Showing the mean neuron cell count in the temporal lobe across all groups using image j software (one way ANOVA). P value was significant at 0.05 (P = 0.048*). N = 7 and values are mean ± SD

IV. PHOTOMICROGRAPH OF GFAP STAIN FOR ASTROCYTES

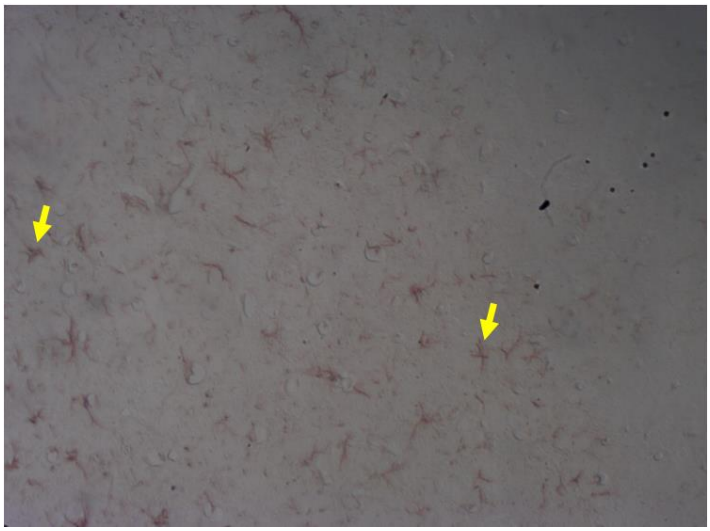

Fig. 6: GROUP 1: SHOWING MILD ASTROCYTE (ARROW) EXPRESSION IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS
GFAP. X300

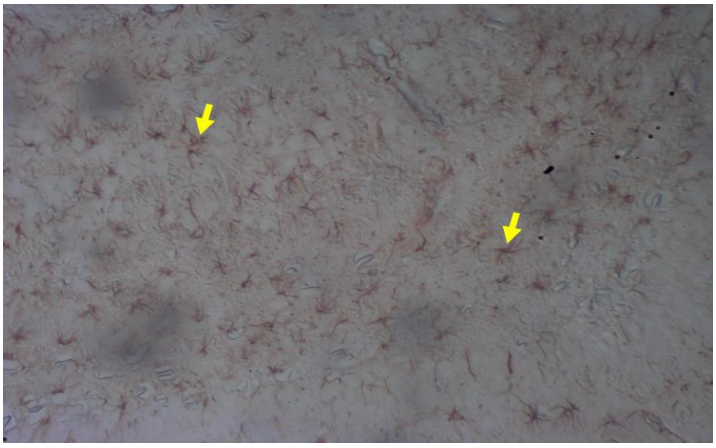

Fig. 7: GROUP 2: SHOWING GREAT REACTIVE ASTROCYTE (ARROW) EXPRESSION (ASTROCYTOSIS) IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS.
GFAP. X300

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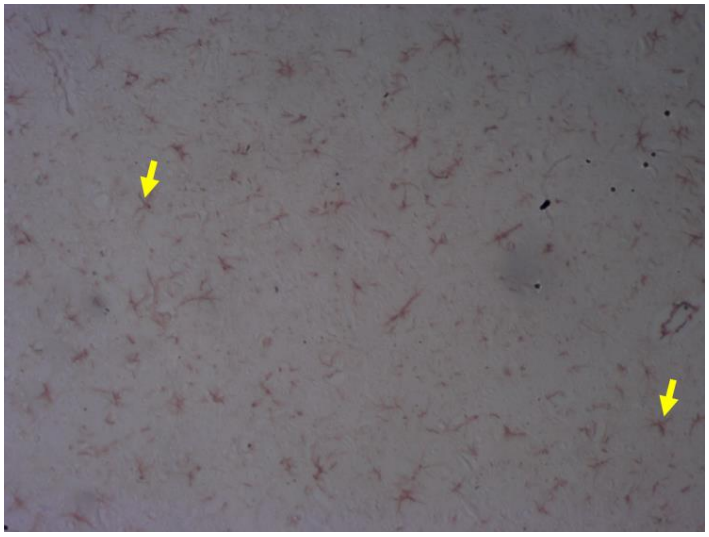

Fig. 8: GROUP 3: SHOWING PROMINENT REACTIVE ASTROCYTE (ARROW) EXPRESSION IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS. A PROGNOSIS TO ASTROCYTOSIS.
GFAP. X300

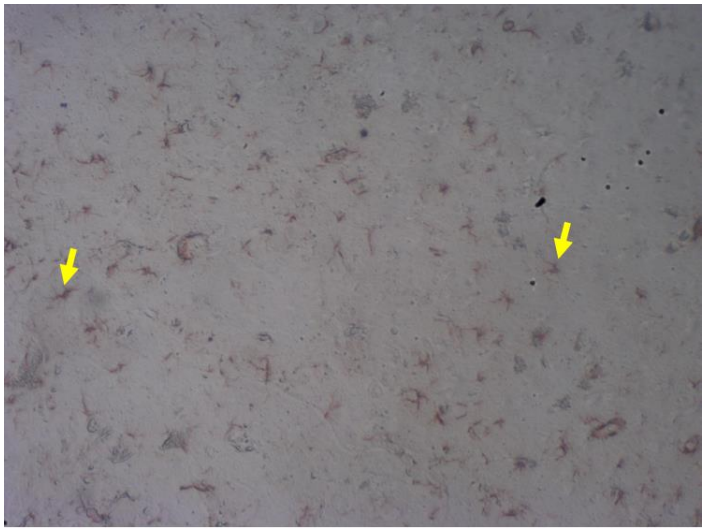

Fig. 9: GROUP 4: SHOWING PROMINENT ASTROCYTE (ARROW) EXPRESSION IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS
GFAP. X300

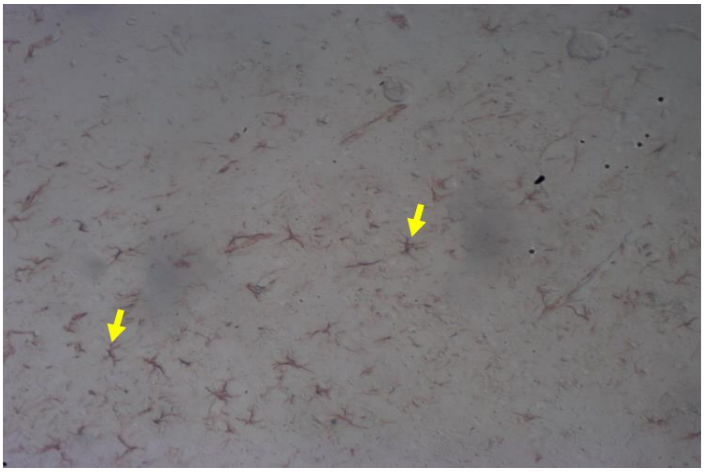

Fig 10: GROUP 5: SHOWING AVERAGE ASTROCYTE (ARROW) EXPRESSION IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS
GFAP. X300

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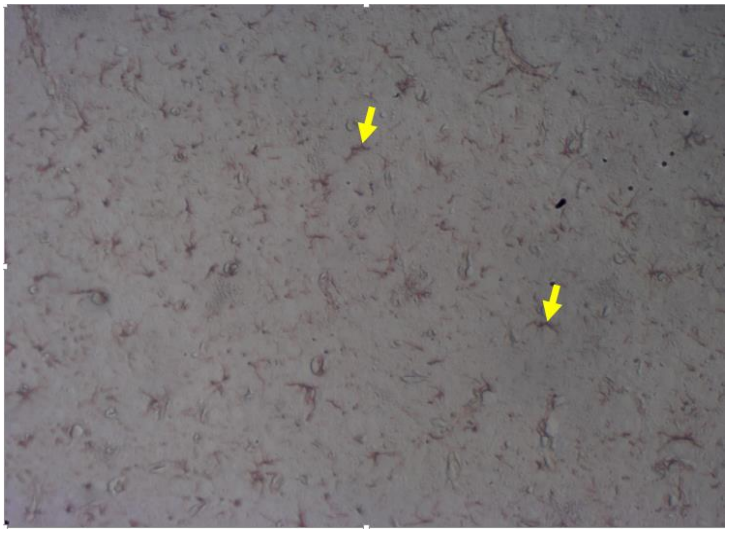

Fig. 11: GROUP 6: PROMINENT ASTROCYTE (ARROW) EXPRESSION IN THE CYTOARCHITECTURE OF THE TEMPORAL CEREBRAL CORTEX OF ADULT WISTAR RATS. A PROGNOSIS TO ASTROCYTOSIS.
GFAP. X300

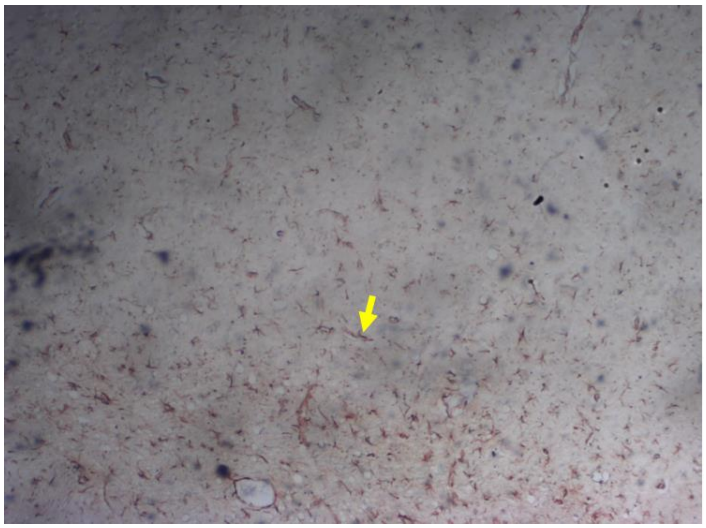

Fig 12: Group 7: Showing Prominent Astrocyte (Arrow) Expression In The Cytoarchitecture Of The Temporal Cerebral Cortex Of Adult Wistar Rats. A Prognosis To Astrocytosis
GFAP. X300

V. DISCUSSION OF FINDINGS

This study focused on assessing the anti-convulsant and anxiogenic property of tannin with a resultant assay on astrocyte population's reaction in the microstructure of the temporal lobe using adult wistar rats. Results gotten from this study showed that tannin had a mild anticonvulsant property especially for animals in groups three (low dose treatment), group four (middle dose treatment) and group five (high dose treatment) (Fig. 1). Animals in the pretreatment groups generally had a high convulsion duration in the first day of PTZ administration and subsequently showed reduced convulsion duration from the second to the third day. One of the main advantages of PTZ kindling as a model of the latent period of the temporal lobe is that an initially nonconvulsive dose of PTZ induces a low mortality of experimental animals and at the same time most of the animals are involved in seizure activity [37].

Animals in the low dose pretreatment group (group 3) which were pretreated with 100mg/kg of tannin however showed no signs of convulsion after the second day of PTZ administration and also showed the lowest convulsion duration in the third day of PTZ administration; this is a strong indication of the anti-convulsant effect of tannin. The animals in group two which were given 5mg/kg.ip of PTZ only showed an almost consistent duration in the response to convulsion for three days while the animals in group seven (the positive control group) which were pretreated with 2.4mg.kg of tegretol (carbamazepine) showed no signs of abating the convulsion duration (Fig. 1). This may be as result of GABA mediated inhibition because repeated PTZ induced seizures alter the GABA-mediated inhibition and glutamate-mediated excitation, which may contribute to increased seizure susceptibility [37]. But available reports shows that carbamazepine has not yet been fully tested in rodents as an effective agent against suppression of full one time-PTZ kindling model but have good anti-kindling effects in suppression of PTZ kindling

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development [38] [39] [40]. However, the control of convulsion has primarily focused on suppressing seizure activity by antiepileptic drugs after convulsion has developed.

In a similar research, 100mg.kg of tannin also reduced duration and activity of kainic acid (15mg/kg.ip) induced seizures using mice and also reduced inflammation levels[41]. A study using aqueous extracts of citrus aurantium proved its anti—convulsant effects in a PTZ kindling model in a mechanism involving NMDA and mGluR`s I and II [42].

The EPM basal test carried out showed that in the first test, animals in groups one, two, five, six and seven spent all the time in the closed arm and in the second test, made a minor reduction in the time spent in the closed arm (Fig. 2). Animals in groups three and four showed some marked open arm entry exploration in the basal test but showed a decline in the time spent in the open arm in the second test. Wistar rats usually often avoid the open arm of the maze due to their natural aversion for open spaces [5] but these natural behavioral tendencies can change in response to prior intake of either anxiogenic or anxiolytic biochemical substances. Anxiogenic drugs reduce time spent on the open arms and anxiolytic drugs increase the time spent on the open arms of the elevated plus maze [43]. Recent reports suggest that there is some evidence of test decay effects, that is, there are differences in elevated plus maze behavior when rodents are exposed to the plus maze on more than one occasion. For instance, decreased activity on the open arms of the maze is typical on the second exposure to this task compared to the first exposure[44] [45].

Findings from this study indicate that animals in the low dose (100mg/kg of tannin) and high dose (400mg/kg of tannin) treatment displayed open arm activity after administration of PTZ. The critical measure for anxiety is the time spent in the open arms of the maze i.e increase in open arm time [46]. Anxiety score is usually an emotional shift in the animals and it has been suggested as a probable explanation where the rodent switches from initial exploration of the maze to learned avoidance of the open arms even during the last minutes of the first trial, and shifts into an acquired phobic-like state [47]. These above scores suggests the anxiolytic effects of tannin in this study and also agrees with reports suggesting that plants in Nigeria, with high tannin concentration have anxiolytic properties[48].

Astrocyte expression rate in the temporal lobe microstructure was assessed using the Glial Fibrillary Acidic Protein (GFAP) biomarker. This was in order to highlight the potential ability of tannin in inhibiting reactive astrocyte response in the cerebral, temporal lobe as a result of pre-inflammatory actions caused by PTZ administration. Polyphenols in plants has been reported to have a high brain bioavailability [49] and it is crucial that the biodistribution of a natural or a synthetic product is known in order to anticipate its therapeutic efficacy and limit adverse effects, particularly when a bioactivity in the CNS is considered essential[49]. Tannin is a polyphenol used in this study in PTZ kindling model as an antagonist in the physiological response to intraperitoneal administration of PTZ which elicited convulsive actions. Reports on the therapeupetic effects of tannin showed that it improves the availability of protein and mineral elements in the body [50].

Plant nutrients can pass into the bloodstream through dietary digestion, but due to the blood–brain barrier, substances present in the bloodstream are not fully absorbed and used by the brain. The neuro-glial cell-vascular unit, is formed around the blood vessels of the brain however, through connexin (Cx) channels allow nutrients to enter the brain and leave harmful substances in the blood vessels [51].

Glial cells including astrocytes play a vital role in the recovery of brain functions [52]. Thus, Findings from this study showed that reactive astrocytes were expressed greatly in animals in group two (given PTZ only) (table 1 & Fig 7). This is a prognosis of astrocytosis that also suggests destruction of surrounding neurons in the temporal lobe as these astrocytes are activated in neuroinflammatory reactions as neurotoxic reactive astrocytes, neuroinflammatory astrocytes of A1 astrocytes [53]. Animals that received tannin intervention (groups 3, 4 and 5) had moderate astrocyte populations (table 1; figs 8, 9 & 10). However, animals which received the lowest dose pretreatment of tannin (100mg/kg) had a high reactive astrocyte expression (Fig 8) perhaps due to the low dose response to the 5mg/kg daily of PTZ. The normal control group astrocyte population (table 1 & Fig 6) when compared to those of the middle and high dose pretreatment groups justifies that 200mg/kg and 400mg/kg of tannin inhibited reactive astrocyte response to the agonistic, excitatory agitations of PTZ.

Reports suggests that after traumatic brain injury in mouse, astrocytes react within 24 hours and reach a peak of approximately 3–7 days post injury, showing a continuous reactive state [54] [55]. These astrocytes (called reactive astrocytes) undergo molecular, morphological, and functional changes in response to pathological stimuli, such as CNS disease, injury, and deleterious experimental manipulation, among others[56]. Thus, PTZ kindling model used in this study may have elicited traumatic brain actions which was quelled by the inhibitory action of tannin derived from phyllanthus amarus leaves.

Neurological findings from this study showed that animals in group two had depletion in the mean neuronal cell population compared to the control group. Also, the animals pre-treated with tannin (groups 3,4, 5) had more neuronal cell population compared to those in group two (Fig. 5). This neuronal cell depletion represented in animals in group two justifies the high number of astrocytes activated for that same group because inflammatory astrocytes are activated when there is neuronal depletion [57]

Tannin have been reported to protect the hippocampal neurons from damage in extremely low electromagnetic fields by regulating the activation of Bcl-2 and Bcl-xl proteins and increasing Ca2+ levels [58].

VI. CONCLUSION

Findings from this study showed that 100mg/kg and 400mg/kg of tannin had anti-convulsive properties in response to PTZ –induced convulsion, and also reduced the rate of astrocytosis in the cerebral, temporal lobe. Tannin also elicited anxiolytic traits as animals pre-treated with tannin spent some time in the open arm of the EPM. Thus, tannin inhibited astrocytosis, had protective effects on temporal lobe neurons, reduced convulsion duration and has the potential of reducing rate of anxiety in rodents.

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