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TANNIN AND FLAVONOID ATTENUATES PENTYLENETETRAZOL-INDUCED WORKING MEMORY DEFICITS AND TNF-A LEVELS IN SELECTED LIMBIC AREAS OF ADULT WISTAR RATS

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ABSTRACT

Polyphenols influence on the modulation of cytokines, pro-inflammatory genes expression and neuroprotection has been supported by different studies. This study used tannin and flavonoid polyphenols from phyllanthus amarus leaves. Spatial working memory deficits has been reportedly associated with chronic neurodegenerative diseases in the long term and this study accessed how tannin and flavonoid synergy can ameliorate such deficits and proffer protective effects in few limbic system areas (amygdala and hippocampus). 49 wistar rats were used for this study in seven groups. Group 1 was given growers feed and water, group 2 was given 10.3mg/kg of PTZ only for 4 days, group 3,100mg/kg of tannin + flavonoid (Marc) for 20 days and 2.6mg/kg of PTZ for 4 days, group 4 was given 200mg/kg of Marc for 20 days and 5.1mg/kg of PTZ for 4 days, group 5, 400mg/kg of Marc for 20 days and 10.3mg/kg of PTZ for 4 days, group 6,400mg/kg of Marc only for 20 days while group 7 was given 3.2mg/kg of tegretol (a control drug) for 20 days and 10.3mg/kg of PTZ for 4 days. Results indicate that PTZ elicited memory deficits which was enhanced by tannin + flavonoid (12.98±15.91) and PTZ also initiated degranulation in the hippocampus and mild glial cells activation in the Amygdala with no significant effects in change in Tnf-α blood levels. In conclusion, Marc improved spatial working memory, had neuropretective effects in the amygdala and hippocampus microstructure and did not inhibit Tnf-α blood levels.

KEYWORDS: Pentylenetetrazol, neuroprotective, tannin, flavonoid, memory.

INTRODUCTION

Inflammatory responses and depressive symptoms were part of an integrated adaptive response to pathogens.[1] Certain bioactive substances or lifestyle pressure changes may trigger proinflammatory actions in the body that may resultantly affect the cognitive abilities of the subject which is indicated by an increase in inflammatory mediators. Inflammation occurs as a result of an imbalance between pro-inflammatory mediators such as cytokines, chemokines and acute phase proteins and anti-inflammatory mediators. This makes the cytokines and chemokines to be excessively activated.[2,3] Low working memory (WM) functions in the brain has been linked as common features with depression[4] and only in individuals with low Brain Derived Neurotropic Factor (BDNF), low WM capacity are associated with increased symptoms of Mild Depressive Disorder (MDD) because it has been reported that BDNF play a role in the effects of psychotherapy that involves changing cognitions and behaviors.[5] These WM deficits have been seen to affect certain brain areas with decreased activity and also increase activity in other brain areas. For example, experiments conducted show that low WM significantly decreased activity in the right precentral gyrus, right precuneus and right insula with consistent functional abnormalities in the cortical-limbic-subcortical circuitry during WM processing.[4] In view of these decline in working memory functions, it is pertinent to also note that there is a relationship between low WM levels and activation of pathophysiological mechanisms like proinflammatory cytokines and chemokines because these behavioural decline are linked to low neuronal and neurotransmitter functions as well as activation of glial cells in brain areas involved[6] and if these neuroinflammatory processes are sustained can disrupt many molecular and cellular pathways in the Central Nervous System (CNS) and Treatment-resistant WM deficits are often accompanied by an elevated systemic and central neuroinflammatory response.[7,8]

Treatment and management of low WM conditions involves many approaches and this study focused on portentous plant metabolites like polyphenols of potent-proven African herbs. The polyphenols (tannin and flavonoid) used for this study were extracted from Phyllanthus Amarus (PA) leaves which have been reported to possess cytoprotective abilities in the brain.[9,10] Thus, findings from this work serves as a way to recommend the initiation of large-scale clinical investigations to determine the potential for the oral synergistic use of tannin and flavonoid.

Polyphenols are a category of compounds naturally found in plant foods and are also thought to reduce inflammation, which is thought to be the root cause of many chronic illnesses.[11] There are many research reports about the therapeutic and health benefits of plant polyphenols.[12-15] Polyphenols have been reported to help improve focus and memory[16] including cocoa flavonoids which help improve blood flow to the brain.[17,18] Tannins, water-soluble phenolic compounds, have been reported to have the ability to form complexes with nutritionally important nutrients such as protein and mineral elements. Toxicity of tannin has been demonstrated in experimental animals although no deleterious effect of ingestion of tannin on human physiology has been reported.[19]

MATERIALS AND METHOD

Purchase and Preparation of Pentylenetetrazol

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 Pentylenetetrazol (98%), $C_6H_{10}N_4$; 138.17MW. Storage (2 – 8^oC), 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 to the 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.

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.

Preparation of plant material

Percolation method was used to prepare the crude extract.[20] 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.[21]

Partitioning/fractionating of crude plant extract: 49.3g of the crude extract was removed from the refrigerator and exposed to fractionate and isolate tannin and flavonoid using the separating funnel method with n-hexane and n-butanol solvents.[22]

Identification of tannin and Flavonoid: After the isolation of tannin and flavonoid, it was pertinent to perform a test to ascertain the presence of both polyphenols in the final fractionate extracted.

Test for tannin: 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; greenish-black colouration was taken as evidence for the presence of tannins.[23]

Test for flavonoid: One to five drops of concentrated hydrochloric acid (HCl) were added to little amount of ethanolic extract of the plant material. Immediate development of a red colour indicates the presence of flavonoids.[23]

Acute Toxicity Test for tannin and flavonoid

Pilot study for dose response trial was carried out to ascertain the appropriate dose for tannin and flavonoid using Lorke's method. The acute toxicity of the extract was done using Lorke's method 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 wistar 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).

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.[24]

Behavioural (Y- Maze) test for spatial recognition memory

The Y maze measures spatial working and recognition memory by making use of a rodent's natural exploratory

instincts. The Y-maze consists of three arms of equal length interconnected at 120^{O}. **Trial one** (Testing Phase): Animals were habituated to the room sixty (60) minutes prior to testing, without the y-maze being visible in light-dimmed room. One arm of the Y-Maze was closed and the animals were introduced to the maze to explore two arms for eight (8) minutes.[25,26] The closed arm was labelled "the novel arm" while the other two opened arms were labelled "familiar arms". **Trial two**: After thirty (30) minutes, all three arms were opened for the animal to explore for another five (5) minutes. This trial two takes advantage of the innate tendency of rats to explore novel unexplored areas i.e the labelled "novel arm" which was previously blocked in trial one. Rats with intact memory will recognize the novel arm which was previously blocked and explore the novel and familiar arms for spontaneous alternation scores whereas rats with impaired memory will explore less of the three arms including the novel arm of the Y maze. Thus, trial two represents a classic test for spatial recognition memory. In this way, comparison of exploratory behaviors of control versus experimental animals are carried out in order to determine if working memory is impaired as the total number of arm entries and the number of alternations were recorded.[27] Generally, after each animal test, the animal is placed in a holding cage and the y-maze was cleaned/disinfected with 70% alcohol and dried prior to placing the next rat.[28] After the whole trial, animals were returned to their original cages. All trials were recorded with a video camera set up above the Y maze apparatus and a timer.

Research Design

Forty nine (49) 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 10.3mg/kg of PTZ only (single dose) for 4 days, group 3 was given 100mg/kg of tannin + flavonoid (Marc) for 20 days and 2.6mg/kg (single dose) of PTZ for 4 days, group 4 was given 200mg/kg of Marc for 20 days and 5.1mg/kg (single dose) of PTZ for 4 days, group 5 was given 400mg/kg of Marc for 20 days and 10.3mg/kg (single dose) of PTZ for 4 days, group 6 was given 400mg/kg of Marc only for 20 days while group 7 was given 3.2mg/kg of tegretol (a control drug for convulsion) for 20 days and 10.3mg/kg of PTZ for 4 days.

The Marc (tannin + flavonoid) 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. From the 17^{th} day of administration, PTZ was given i.p (intraperitoneally) once for 4 consecutive days one hour after administration of Marc. 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 on the 20^{th} day, blood was collected from five animals from each group for assessment of TNF-α levels. Routine paraffin method was used to perform Hematoxylin and Eosin counterstaining procedure for comparison of microscopic morphology some limbic system brain areas across groups.

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 was a statistically significant difference between the means of three or more independent groups.

Y maze test results

Table 1 showing mean comparison of total entry and percentage spontaneous alternation (PSA) values for Y maze test before (day 0) and after administration (day 20) of administration of Marc and PTZ. P Value for total arm entries showed P>0.05 (0.174 & 0.662) while that of percentage spontaneous alterations showed P< 0.05 which is statistically significant at 0.024 in the second test.

BEFORE ADMINISTRATION(1st Test)			POST ADMINISTRATION(2nd Test)		
TOTAL ARM ENTRIES					
GROUP	MEAN±SD	P VALUE	GROUP	MEAN±SD	P VALUE
1	9.50±0.577	0.174	1	6.25±5.058	0.662
2	3.50±2.082		2	2.00±1.000	
3	10.00±7.789		3	5.25±9.215	
4	4.75±2.630		4	2.50±2.121	
5	6.25±3.862		5	0.67±0.577	
6	7.00±4.546		6	6.00±6.245	
7	3.50±3.109		7	1.50±1.732	
BEFORE ADMINISTRATION(1st Test)			POST ADMINISTRATION(2nd Test)		
PERCENTAGE SPONTNEOUS ALTERATION (%)					
GROUP	MEAN±SD	P VALUE	GROUP	MEAN±SD	P VALUE
1	39.75±9.025	0.265	1	34.03±22.945	0.024*
2	22.92±15.773		2	0.00±0.000	

3	46.97±16.725		3	5.28±10.550	
4	19.05±5.057		4	12.50±17.678	
5	33.75±13.769		5	0.00±0.000	
6	24.85±16.964		6	21.15±19.516	
7	50.00±40.825		7	0.00±0.000	

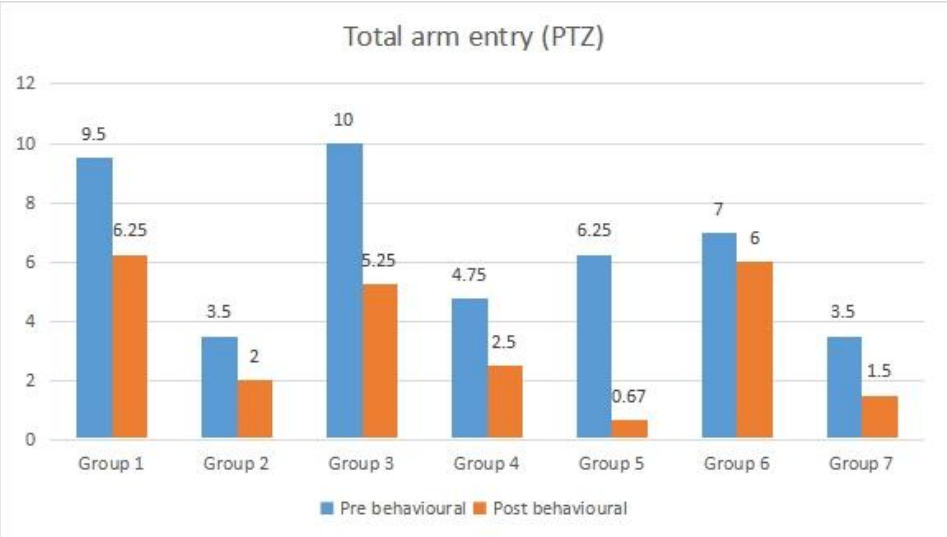

Fig. 1: Showing the comparative values for pre and post administration periods for total arm entries scores for y maze tests. P=0.662. n = 7.

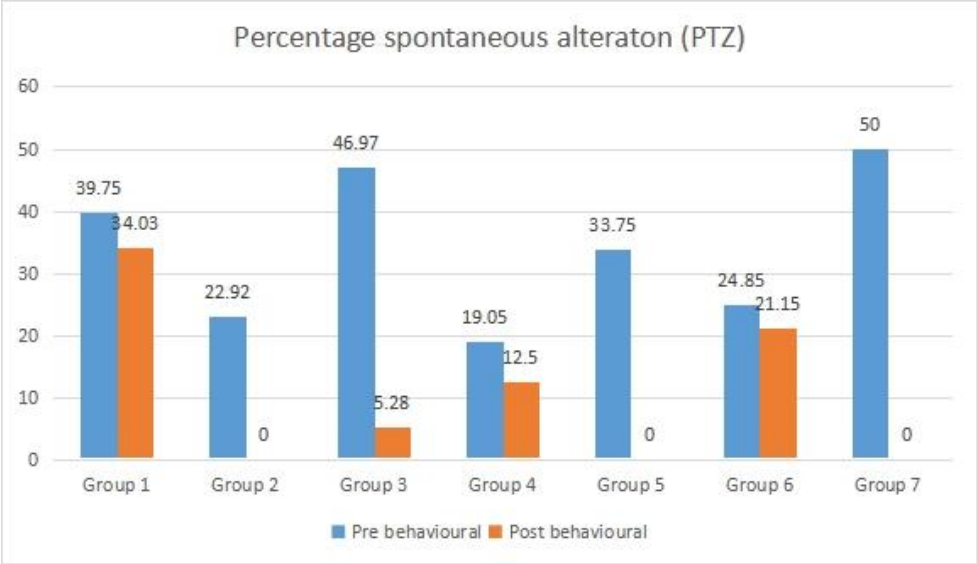

Fig. 2: Showing comparative values for pre and post administration percentage spontaneous alteration (PSA) scores for y maze tests. P=0.024(statistically significant). n = 7.

Result of TNF-α level test

Table 3: showing summary values for tnf-α levels among groups. P=0.027(statistically significant).

Group	Mean±SD	p-value
Group 1	7.57±0.876	0.027*
Group 2	9.835±0.629	
Group 3	10.22±1.633	
Group 4	10.345±0.219	
Group 5	10.385±0.120	
Group 6	11.38±0.566	
Group 7	9.805±0.127	

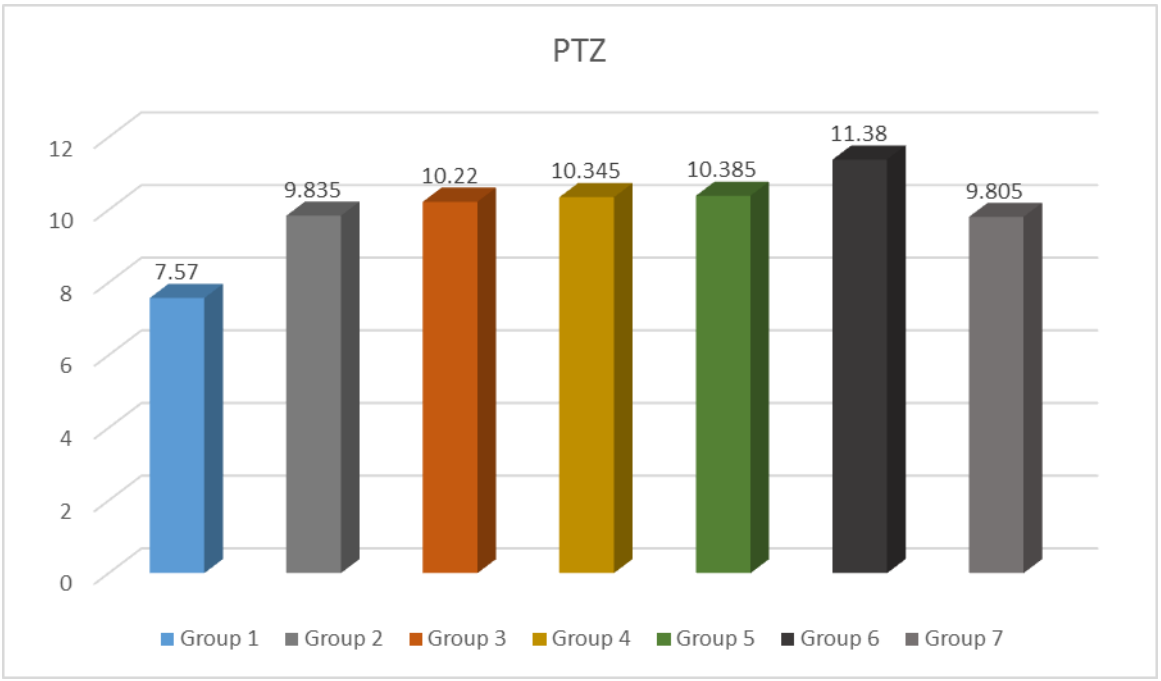

Fig. 3: Showing mean scores for tnf-α biomarker test carried out across all groups. P=0.027. n = 7.

Histological findings
AMYGDALA

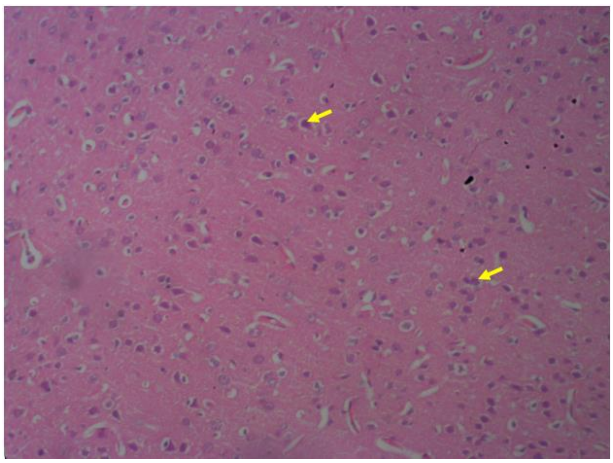

Fig. 4: Group 1: Amygdala showing neuronal cells (arrow). H & E. X300.

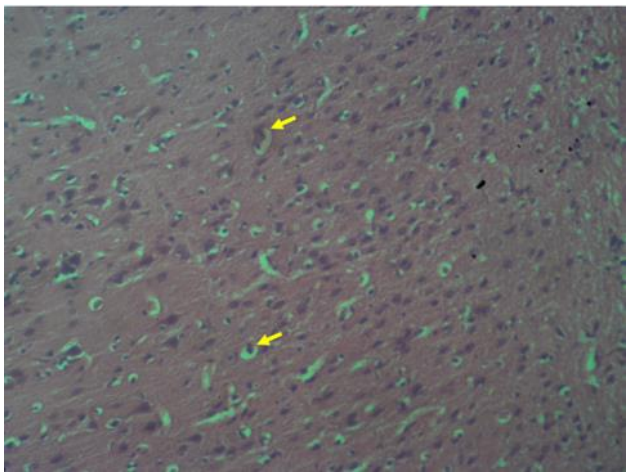

Fig. 5: Group 2: Amygdala showing neuronal cells (arrow) & glial cell activation. H & E. X300.

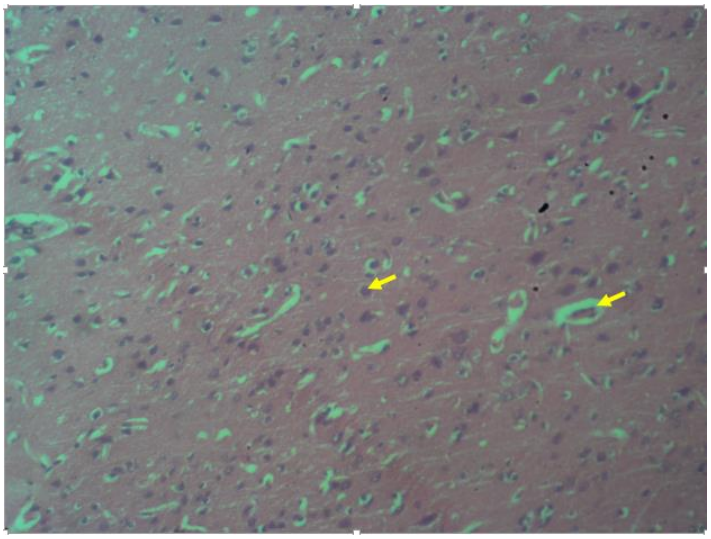

Fig. 6: Group 3: Amygdala showing neuronal cells (arrow). H & E. X300.

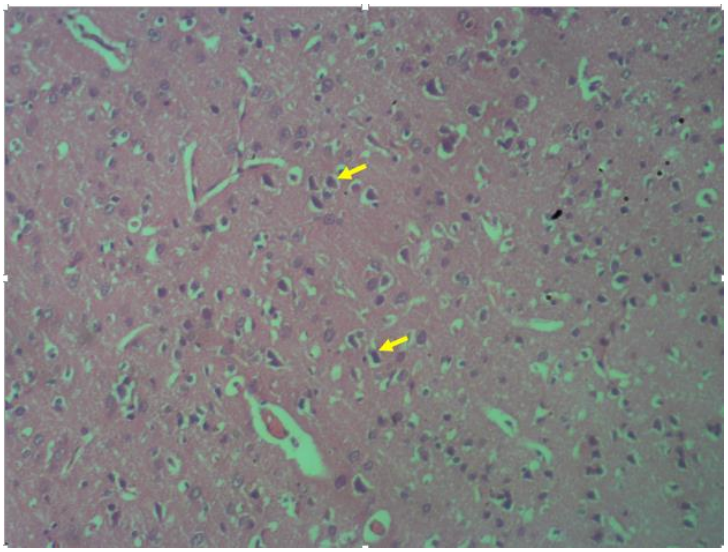

Fig. 7: Group 4: Amygdala showing neuronal cells (arrow). H & E. X300.

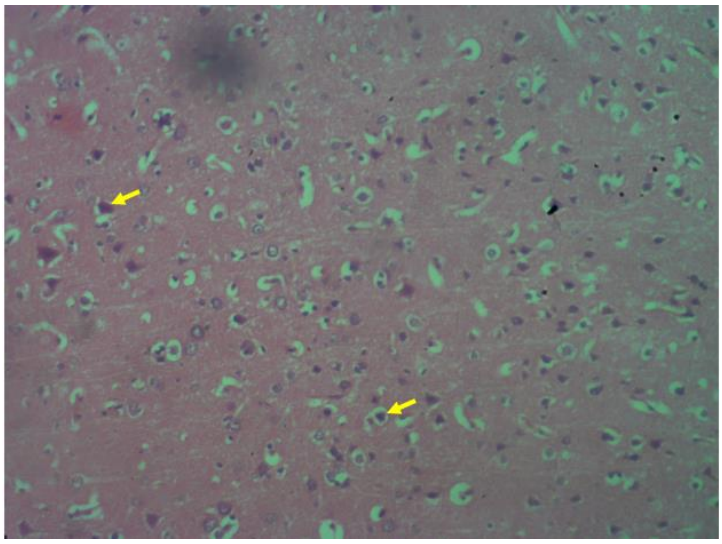

Fig. 8: Group 5: Amygdala showing neuronal cells (arrow). H & E. X300.

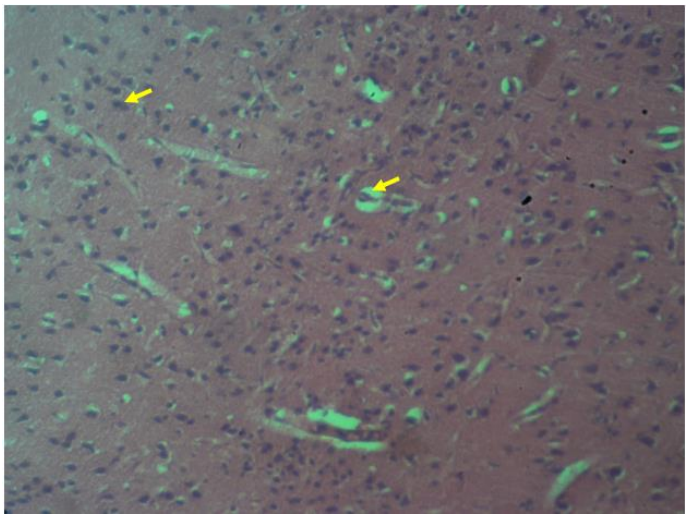

Fig. 9: Group 6: Amygdala showing neuronal cells (arrow) & glial cell acivation. H & E. X300

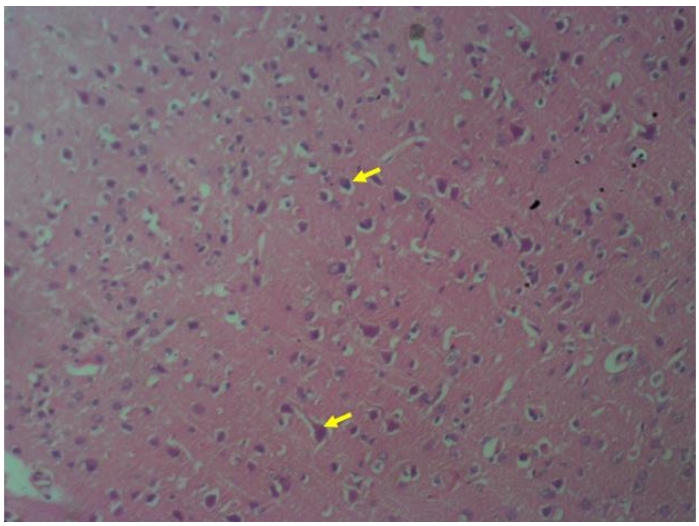

Fig. 10: Group 7: Amygdala showing neuronal cells (arrow). H & E. X300.

HIPPOCAMPUS

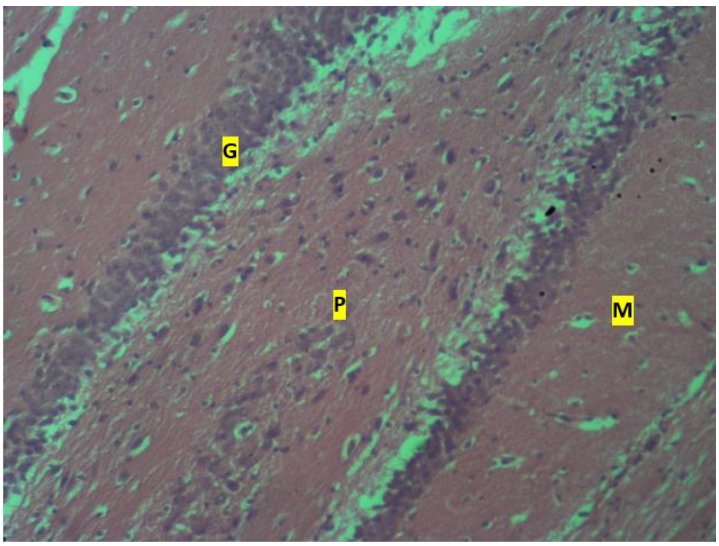

Fig. 11: Group 1: Hippocampal microstructure showing normal dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). H & E. X300

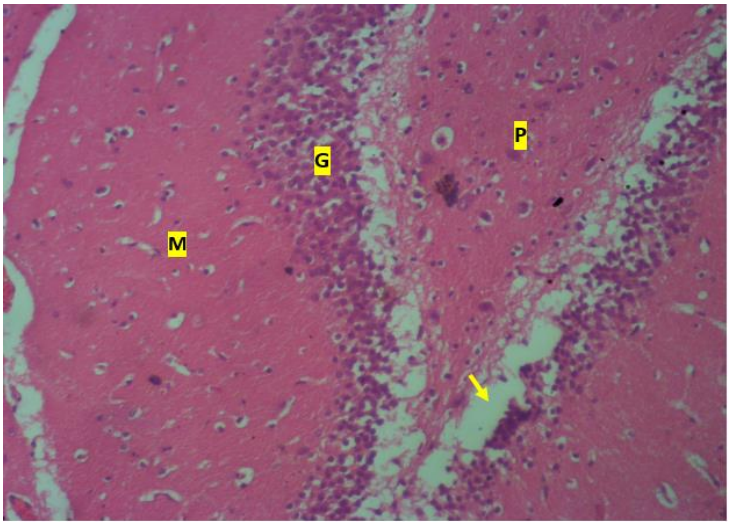

Fig. 12: Group 2: Hippocampal microstructure showing neuronal cells in the dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). Cyto-architecture shows focal area of degranulation in the granular layer (arrow). H & E. X300

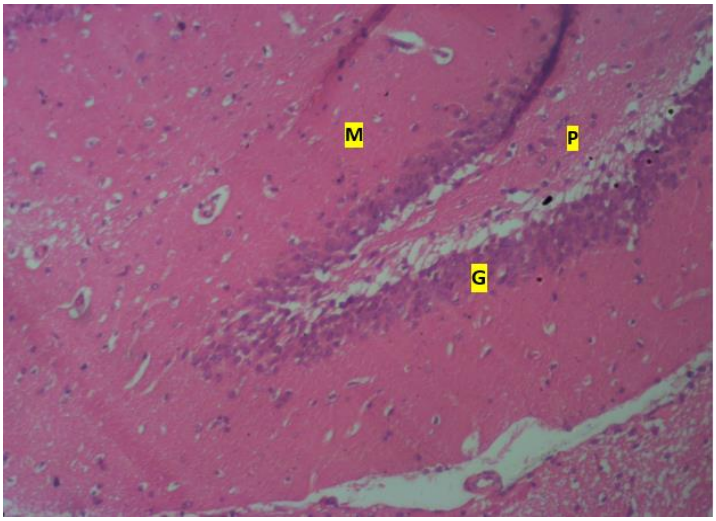

Fig. 13: Group 3: Hippocampal microstructure showing normal dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). H & E. X300

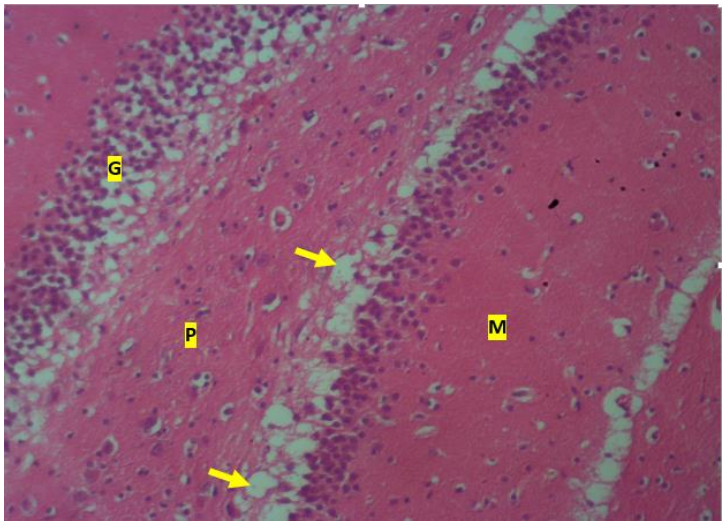

Fig. 14: Group 4: Hippocampal microstructure showing the dentate polymorphic layer (P), dentate granular layer (G) and the dentate molecular layer (M). Cyto-architecture shows focal area of degranulation in the granular layer (arrow). H & E. X300.

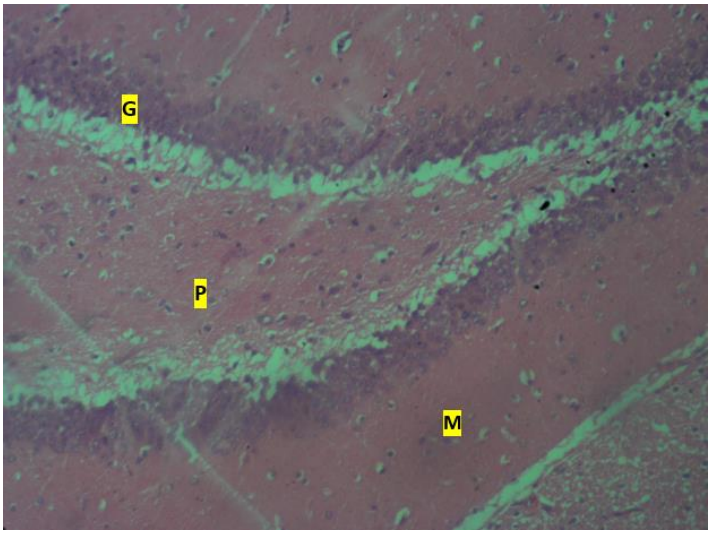

Fig. 15: Group 5: Hippocampal microstructure showing normal dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). H & E. X300.

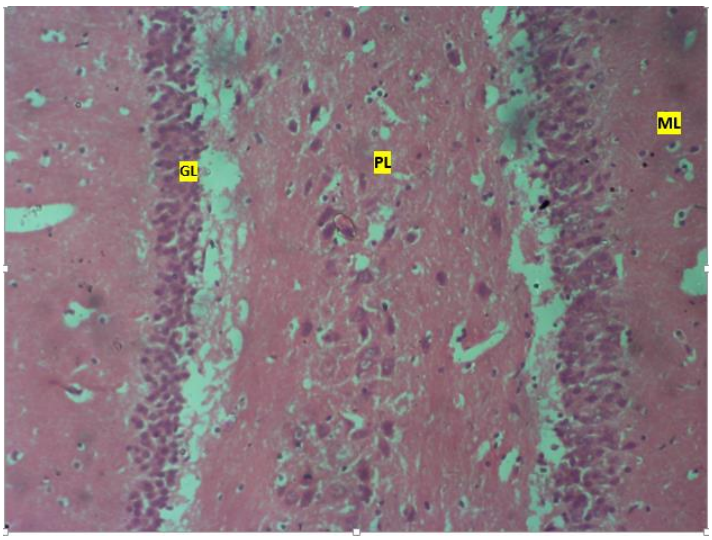

Fig. 16: Group 6: Hippocampal microstructure showing normal dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). H & E. X300.

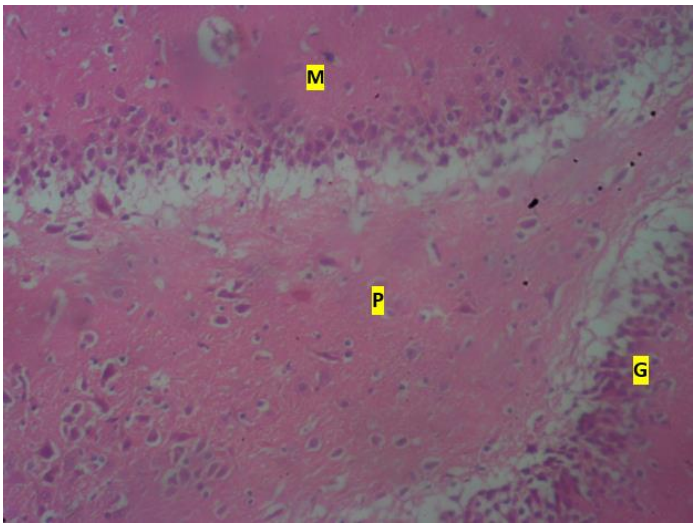

Fig. 17: Group 7: Hippocampal microstructure showing normal dentate polymorphic layer (P), dentate granular layer (G) and in the dentate molecular layer (M). H & E. X300.

DISCUSSION

This study focused on examining the ability of polyphenols (tannin and flavonoid) to moderate PTZ-induced working memory deficits and tnf-α levels in some selected limbic areas using adult wistar rats. In the 1st trial of Y maze test was carried out prior to commencement of administration of marc (tannin + flavonoid) in order to compare and make references with the 2nd trial on behavioural scores that may have been stimulated by the marc and PTZ. Generally, psychoactive substances usually change behavior of animals towards a positive or negative trend and rodents in the experimental groups (groups 2 to 7) showed lower total arm entries and percentage spontaneous alternations (PSA) compared to the normal control group (table 1). Total arm entries made by rodents used for Y maze studies indicate an advantage to measure various parameters of behavior related to spatial exploration.[29] Marc used in this study increased the levels of exploration compared to rodents in the negative control which was given PTZ only. However, the rodents in group 7 pre-treated with 3.2mg/kg of tegretol showed low exploratory traits compared to rodents pre-treated with marc and this is an indication of the spatial exploratory stimulus of the marc. More arm entries is an indication that the animals tend to enter the arm that was previously blocked and is able to detect is as a less frequently visited arm of the maze. Spontaneous alternation (SA) is a measure of spatial working memory and can be evaluated by allowing rats to explore all three arms of the maze which is driven by an innate curiosity of rodents to explore previously unvisited arms.[30] Alternation indicates sequential entries into all three arms (when an animal visits all three arms clockwise or counterclockwise sequentially) alternation is achieved.[31] In this study, the percentage of alternation was observed and rodents in groups 2, 5 and 7 did not display any alternation in the 2nd trial which was done after pre-treatment with the marc and administration of PTZ. This is an indication of low levels of spatial working memory while rodents in groups 3, 4 and 6 pre-treated with the marc showed improved working memory (Table 1). Comparatively, animals which were given PTZ only showed no alternations while those in groups 3 and 4 showed marked improvements in spatial working memory at the end of the study. Flavonoids help improve cognitive abilities in the brain and also remedy brain dysfunctions in cases like dementia and Alzheimer's disease (AD). The mechanisms of flavonoids are mediated via inhibition of cholinesterase (AChE), butytycholinesterase (BChE) and modulation of signaling pathways that are implicated in cognitive and neuroprotective functions.[32]

Tnf-α inflammatory cytokine levels examined across all groups indicate a significant difference between that of the normal control (7.57 ± 0.876) and group 2 (9.835 ± 0.629) which was given 10.3mg/kg of PTZ. However, the tnf-α levels across the experimental groups pre-treated with the marc showed were higher and spread with minimal differences than those in group 2 given PTZ only except for those pre-treated with 3.2mg/kg of tegretol whose tnf-α levels were equivalent to those in group 2 (Table 3). Although there are few research works that have reported the immunomodulatory and anti-inflammatory role of polyphenols.[33]

Histopathological findings in the limbic areas (amygdala and hippocampus) indicate that PTZ –induced low working memory activated glial cells and caused degranulation in the hippocampal dentate gyrus in animals in group 2 which were given PTZ only. Polyphenol interventions with the marc (tannin + flavonoid) proved its neuroprotective abilities and this has been stated in research reports.[33][14,34]

CONCLUSION

Polyphenols (tannin and flavonoids) used in this study showed its ability to improve spatial working memory and spatial exploration behaviours and also protect the neuronal tissue in the limbic areas of the amygdala and the hippocampal dentate gyrus. Tnf-α levels did not change with the pre-treatment of tannin and flavonoid but PTZ did cause a spike in these proinflammatory cytokine in the blood when compared to the control group. This findings in the behavioural alterations also correlate with few studies carried out to suggest the neuropreotective potential of polyphenols in pathophysiological cases especially in AD and dementia. Thus, this study joins other related research reports in calling for more clinical trials in proffering ways into the management of neurodegenerative diseases via the production of polyphenol-rich supplements.

CONFLICT OF INTEREST: There was no stated conflict of interest by the authors.

ACKNOWLEDGEMENT: I acknowledge the fatherly guide and contribution of Professor Godson Anyanwu in the design and supervision of this research work.

AUTHOR'S CONTRIBUTIONS: Christian Mba initiated and carried out this study. Professor Godson Emeka Anyanwu supervised the research, analyzed the data and also provided guide to the research design. Dr. Finbarrs-Bello guided the analytical insight into the behavioural parameters and its interpretation.

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