



# THERAPEUTIC POTENTIALS OF VERNONIA AMYGDALINA IN THIOACETAMIDE-INDUCED NEUROTOXICITY IN THE CA1 HIPPOCAMPAL SUBFIELD VIA INHIBITION OF MICROGLIAL ACTIVATION

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## ABSTRACT

Thioacetamide (TAA)-induced hepatic encephalopathy (HE) has been reported in several studies. Sequel to acute or chronic liver failure, there is impairment in the hippocampal structure and hippocampal-mediated functions. The precise pathophysiological mechanism leading to neurological alterations remains elusive. The hippocampus also has rarely been examined in HE, hence this study. *Vernonia amygdalina* (VA) which possesses diverse biological activities has been studied and has promising therapeutic potential in several neurodegenerative diseases. Here, we investigated the neuroprotective activities of *Vernonia amygdalina* against thioacetamide-induced neuronal damage in the CA1 region of the hippocampus in the rat model. Thirty male Wistar rats were divided into five groups A-E (n=6). Group A (control) was the placebo and was administered 2mL/kg distilled water, group B was administered 100mg/kg thioacetamide only. Groups C-E were administered 100mg/kg of thioacetamide and were subsequently treated with 100mg/kg VA, 200mg/kg VA, and 3mg/kg vitamin C respectively. All administration was carried out for 14 days duration orally. Neurobehavioral tests were conducted. Required brain tissues for biochemical analyses were harvested following rat sacrifice. Glucose-6-phosphate dehydrogenase activity and hippocampal Glutamate levels were evaluated. Histological stains (H&E and cresyl violet) and immunohistochemical expression of Iba-1 protein (microglial marker) were used to assess neuroinflammation and neurodegeneration in the CA1 subfield. Statistical analyses were carried out using Graph pad prism 5 and all results were expressed as Mean±SEM. Means differences were identified by Tukey's *post hoc* test. Statistical significance was set at ( $p < 0.05$ ). The neurobehavioral test results showed an observable increase in anxiety and impaired spatial working memory of the Wistar rats while glutamate ( $p = 0.6187$ ;  $F = 0.6844$ ) and G6PDH activity ( $p = 0.6187$ ;  $F = 0.9486$ ) were not significantly different across groups. Thioacetamide treatment also resulted in neuroinflammation *vis-à-vis* microglial activation, causing neurodegeneration in the CA1 subfield. *Vernonia amygdalina* treatment ameliorated these neurotoxic changes. Altogether, *Vernonia amygdalina* treatment ameliorated thioacetamide-induced neurotoxicity in the CA1 region of the hippocampus via anti-neuroinflammatory and neuroprotective mechanisms.

**Keywords:** Neurotoxicity, Thioacetamide, *Vernonia amygdalina*, Microglial Activation, Therapeutic, Spatial Memory, CA1

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## INTRODUCTION

Hepatic encephalopathy (HE) is a neurological complication resulting from acute or chronic liver failure (Sepehrinezha *et al.*, 2021). Over time, several pharmacological agents like acetaminophen, galactosamine, and lipopolysaccharide have been employed in the induction of HE. However, these agents are not reproducible and repeatable because the pathological features manifested in these models do not closely mimic those seen in HE patients and there is also some variability in the neuropathological changes in different species (Butterworth *et al.*, 2009). Thioacetamide (TAA) is a sulfur-containing chemical that has extensive use in the agriculture, rubber, paper manufacturing, and metallurgy sectors (Sepehrinezha *et al.*, 2021). TAA has been frequently utilized in the HE animal model because it is a reproducible model in many animal species, including mice, rats, and guinea pigs, and may generate liver damage that matches acute liver failure (ALF) and HE observed in patients (Sepehrinezha *et al.*, 2021). Furthermore, TAA, like human ALF, provides a timeframe that is ideal for conducting HE experiments. Unarguably, one of the reported neurotoxic mechanisms of TAA in humans and animals is oxidative stress which is the generation of reactive oxygen species (ROS) leading to an overwhelming antioxidant defense mechanism and damage to cellular components such as lipids, proteins, and DNA; this, in turn, can impair cellular structures and functions (Mladenovic *et al.*, 2012). The mechanisms of its neurotoxic effects remain elusive and its effects on the brain are linked to the induction of liver failure, a condition which has been termed to be hepatic encephalopathy.

According to the study by Mendez *et al.*, (2008), the brain tissue of patients diagnosed with hepatic encephalopathy exhibits cellular morphological changes that

could be associated with memory impairment. Also, TAA neurotoxicity has been known to result in spatial memory impairment which is linked to astrocytes and neuronal impairment in the mammillary nuclei and the hippocampus (Mendez *et al.*, 2008).

The CA1 hippocampal region area receives major input connections from several extrinsic sources including CA3 pyramidal cells via their ipsilateral Schaffer collaterals and contralateral commissural fibers, and layer three excitatory cells of the entorhinal cortex (EC) through the temporal-ammonic pathway, as well as the medial septum and diagonal. Behavioral evidence indicates functionally separable roles of CA3 and entorhinal inputs to CA1 in hippocampus-dependent learning and memory (Brun *et al.*, 2002; Nakashiba *et al.*, 2008; Remondes and Schuman, 2004; Suhel *et al.*, 2011). A wide array of phytochemicals (including anti-nutritional factors) is present in *Vernonia amygdalina* (VA). Phytochemicals such as Saponin, Alkaloids, Lignans, Edotides, Flavonoids, Tannin, steroids, Terpenes, Oxalates, Phytates, and Tannins have been reported (Udensi *et al.*, 2002; Ejoh *et al.*, 2007). Vitamin C (ascorbic acid) is a potent antioxidant that could offer potential neuroprotection via oxidative stress-induced cellular damage (Al-Attar, 2011, Robea *et al.*, 2020). Since oxidative stress is one of the reported mechanisms of TAA, the inclusion of vitamin C is necessary in this study as a standard experimental drug. VA offers neuroprotective effects through the inhibition of acetylcholinesterase and butyrylcholinesterase activities, as well as through enhancement of learning and memory in rats (Reginald, 2010; Oladele *et al.*, 2020). Akani *et al.*, (2017) also reported an enhancing effect of VA on memory through the modulation of the cholinergic activities in the brain. Undisputedly, TAA-induced HE had been documented. But

surprisingly, the neurologic effect(s) of TAA is yet to receive deserving attention, particularly with a focus on the CA1 subfield of the hippocampus and its mediated

functions. Here, we hypothesized that VA would be able to ameliorate or mitigate TAA-induced hippocampal neurological impairment.

## MATERIALS AND METHODS

### ***Ethical Consideration***

The experimental rats were housed at Babcock University's animal holding following the Institute of Laboratory Animal Resources, National Research Council, DHHS, pub no. NIH86-23, 1885 guidelines for the care and use of animals in research and teaching. In addition, institutional ethical permission was acquired from the Babcock University Health Research Ethical Committee (BUHREC), Babcock University, Ilishan-Remo, Ogun state, with the ethical number 428/21.

### ***Chemicals and Drugs***

TAA was procured from Sigma Aldrich (St Louis, MO, USA). 1g of TAA was weighed using a weighing scale and dissolved in 50 mL of distilled water (concentration= 20 mg/mL) and kept in a refrigerator.

Vitamin C was procured from Babcock University Teaching Hospital (BUTH) in tablet form and weighed with a weighing scale. It was dissolved in 100 mL of distilled water and kept in a refrigerator.

All reagents and Iba-1 antibodies used in this experimental study were obtained from reputable sources and were of analytical grade and purity.

### ***VA extract preparation***

Fresh VA leaves were acquired from a vegetable garden in Ilishan-remo town (Latitude: 6.8932 East and Longitude: 3.7105 North) in the early hours of the morning. Identification of the leaves was carried out by a taxonomist. Preparation of the ethanol extract of VA was by the methods of Abdumalik *et al.*, (2016) and Omisore *et al.*, (2022). The leaves after detachment from the stem were washed, air-dried, then pulverized into a coarse powder and percolated in 20 mL dichloromethane (DCM), then vigorously agitated and left to

stand for five days in an air-tight bottle. The crude extract was gathered by sieving into a quartz beaker and repeated until the extract was particle-free. The combined aliquot collected was concentrated in a steam bath to about 5 ml and purified by passing through a pasture pipette on a membrane and air-dried to about 2 mL. The aqueous extract was stored in an air-tight bottle pending administration.

### ***VA dose determination***

The LD<sub>50</sub> of aqueous extract VA has been determined to be greater than 5000mg/kg using Lorke's method (Lorke, 1983). Therefore, justification for the dose regimen used in this study was by picking 1/50<sup>th</sup> (low dose) and 1/25<sup>th</sup> (high dose) of 5000mg/kg which corresponds to 100 mg/kg and 200 mg/kg respectively.

### ***Animal Procurement, Grouping, and Drug/Extract Administration***

The summary of grouping and experimental protocol is presented in Table 1. A total of thirty healthy male Wistar rats (*Rattus norvegicus*) were used for the study. The rats weighing between (120-150g) were purchased from Babcock University animal house and housed in the animal holding facility in well-ventilated plastic cages under standard environmental conditions of 12 hours of light/dark cycles with adequate access to rat chow and clean water. The rats were randomly assigned into groups A-E (n=6 each). Group A, the control group received 2 mLs/Kg distilled water as a placebo for 24 days; Group B received 100 mg/kg TAA only; Group C received 100 mg/kg TAA and 100 mg/kg VA; Group D received 100 mg/kg TAA and 200 mg/kg VA; Group E 200 mg/kg AlCl<sub>3</sub> 100 mg/kg TAA and 3 mg/kg vitamin C (standard drug). TAA

administration was done orally with the aid of a cannula for 14 days while *V/A* was for 10

days. TAA dosage was based on previous literature (Qin *et al.*, 2014).

**Table 1: Grouping and Experimental Design**

Groups	Drug and Extract	Dosing	Duration	Administration Route
Control (A)	Distilled water as placebo	2 mLs/Kg	24 days	Orally
B (Negative control)	Thioacetamide (TAA) only	100 mg/kg	14 days	Orally
C	TAA and <i>Vernonia amygdalina</i>	100 mg/kg and 10 mg/kg respectively	14 days + 10 days respectively each; drug first, treatment after	Orally
D	TAA and <i>Vernonia amygdalina</i>	100 mg/kg and 200 mg/kg respectively	14 days + 10 days respectively each; drug first, treatment after	Orally
E (standard anti-oxidant drug)	TAA and and Vitamin C	100 mg/kg and 3 mg/kg	14 days + 10 days respectively each; drug first, treatment after	Orally

### Neurobehavioral assessment

The method used for neurobehavioural assessment was that of Olatunji *et al.*, (2021). Behavioral evaluations to observe motor deficits encompassing locomotion, anxiety, and memory (working and reference) were carried out preceding sacrifice with the use of the open field box and radial arm maze respectively. For locomotion and anxiety assessment, rats from each group were placed in the center of the apparatus, a white open box (72 × 72 × 36 cm) (Fig 1b) with black dividing lines. For memory assessment, a radial arm maze (Fig 1a) whose alternate arms were baited was used. Ethanol (70%) was used to clean both apparatuses in between tests to prevent olfactory cues. The behaviors in the open field box scored included line crossing, and the center square entries and duration. In the radial arm maze test, the time taken for the rat to identify the baited arm was noted. While exploring, the animals were recorded with a camcorder (DNE webcam, Porto Alegre, Brazil) placed at the top, and the video was analyzed by independent observers blind to the procedure.



Fig 1a: Radial arm maze apparatus Fig 1b: Open field box apparatus

### Biochemical assay

#### Glucose-6-phosphate dehydrogenase (G6PDH)

The protocol used by Owolabi *et al.*, (2017) was followed. G6PDH has dual coenzyme specificity. When assayed under conditions that are optimal for the particular coenzyme, the ratio of observed catalytic activity is NAD/NADP = 1.8. The reaction velocity is 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.

**Assay Procedure:** The spectrophotometer was adjusted 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<sub>2</sub>, 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 a spectrophotometer at 30°C for 7–8 min to achieve temperature equilibration and establish a blank rate if any. This was followed by adding 0.1 mL diluted enzyme and recording an increase in A 340 /min for 4–5 min. The A 340 /min was calculated from the initial linear portion of the curve.

### **Neurotransmitter assay**

#### **Glutamate**

The method of Adelodun *et al.*, (2021) was used for the determination of hippocampal glutamate levels. Estimation of glutamate levels was done by preparing brain homogenates in phosphate buffer saline (PBS). The homogenates were centrifuged at 13000 g for 10 minutes to remove all forms of insoluble materials. Supernatants were collected for measurement of glutamate using Enzyme-linked immunosorbent Assay (ELISA) kits by a microplate reader at 450nm. The samples were brought to a final volume of 50µL with glutamate assay buffer. Glutamate levels were determined using a standard curve as described by the manufacturer (ab83389, Abcam).

### **Histology and Immunohistochemistry**

The rats utilized for histology and immunohistochemistry assessments were sacrificed via intracardiac perfusion following the evaluation of their neurobehavioral performance. Precise measurements were taken using an AB204 Mettler Toledo weighing balance following the meticulous removal of the brains using bone forceps. The brains were blotted dry with filter paper and weighed. The brains were preserved by immersing them in a solution of Neutral Buffered Formalin (NBF) at a concentration of 10%. The Hippocampal slices as 1 mm thick were prepared for conventional paraffin embedding for H and E staining and cresyl fast violet using a modified method (Adelodun *et al.*, 2016 and Alvarez *et al.*,

1990) and immunohistochemistry (microglia morphology with anti-iba-1 antibody) according to the protocol below.

Paraffin-embedded sections were deparaffinized with xylene, rehydrated through descending grades of ethanol (100%, 95%, 70 % ethanol), and taken to water. Heat-mediated antigen retrieval was then performed using a citrate-based antigen unmasking solution, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300) in a steamer for 30 mins. Sections were washed in PBS for 2 min followed by endogenous peroxidase blocking in 0.3 % hydrogen peroxide solution in PBS for 10 ins. Sections were washed in PBS for 2 min again and were incubated in 2.5% normal horse serum for 20 min. for protein blocking. Following the blocking, sections were incubated in rabbit polyclonal anti-iba1 (Novus Biologicals, USA) at 1:1000 at 2 hrs at room temperature and washed in PBS for 5 min. Another incubation was done in ImmPRESS™ HRP Anti-Rabbit IgG (Peroxidase) Polymer Reagent, (i.e. horse radish peroxidase) for 30 min. Sections were washed in PBS for 5 min, 2 times and color was developed with DAB peroxidase (HRP) Substrate Kit (Vector® Labs, USA). Sections were rinsed well in tap water and then counter-stained in hematoxylin. Finally, sections were dehydrated through ascending grades of ethanol (70%, 95%, 100%), cleared in xylene, and mounted with permount (Fischer Scientific, USA).

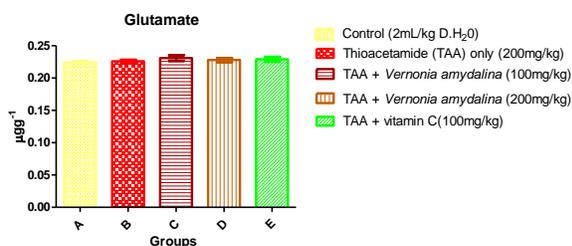
### **Statistics**

Results obtained from the study were analyzed using Graph Pad Prism® software (Version 6.1) Data was analyzed by analysis of variance (ANOVA) and Tukey's *post hoc* test was used to identify differences between group means. Data are presented as mean ± standard error of the mean (mean ± SEM). *p*-value less than 0.05 (*p*<0.05) was considered statistically significant and the confidence interval was set at 95%.

## RESULTS

Glutamate concentration was unaltered following Thioacetamide and Vernonia amygdalina treatments

Fig 2 shows glutamate concentration across the groups. Thioacetamide administration and Vernonia amygdalina treatments did not cause any significant ( $p = 0.6187$ ;  $F = 0.6844$ ) change in hippocampal glutamate concentration in the thioacetamide-only group ( $0.23 \pm 0.002$ ) and treatment groups TAA and Vernonia amygdalina 100mg/kg ( $0.23 \pm 0.004$ ), TAA and Vernonia amygdalina 200mg/kg ( $0.23 \pm 0.003$ ), and TAA and Vernonia amygdalina vitamin C ( $0.23 \pm 0.004$ ) when compared to the control group ( $0.23 \pm 0.002$ ). This showed that thioacetamide administration and treatments with Vernonia amygdalina did not exert any modulating influence on hippocampal glutamate concentrations.

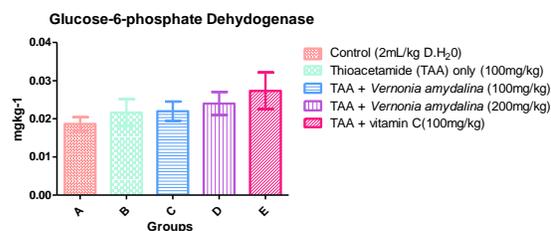


**Figure 2: Glutamate concentrations in experimental and control rats treated with thioacetamide and Vernonia amygdalina and Vit. C across the groups. No significant difference was observed in means across groups ( $p > 0.05$ ).**

### Thioacetamide administration did not alter Glucose-6-phosphate dehydrogenase activity

Fig 3 shows Glucose-6-phosphate dehydrogenase activity across the groups. Thioacetamide administration for 14 days did not significantly ( $p = 0.6187$ ;  $F = 0.9486$ ) result in any change in hippocampal Glucose-6-phosphate dehydrogenase activity in thioacetamide-only group ( $0.02 \pm 0.003$ ) spatial memory, the radial arm maze apparatus was used to assess spatial

Vernonia amygdalina 100mg/kg ( $0.23 \pm 0.003$ ), TAA and Vernonia amygdalina 200mg/kg ( $0.02 \pm 0.003$ ), and TAA and vitamin C ( $0.03 \pm 0.004$ ) when compared to the control group ( $0.23 \pm 0.002$ ).



**Figure 3: Bar chart shows Glucose-6-phosphate dehydrogenase activity in experimental and control rats treated with thioacetamide and Vernonia amygdalina.**

### Neurobehavioral Assessment

In this experiment, the open field and radial arm maze behavioural paradigms were employed for the assessment of spatial memory and anxiogenic response (Fig. 4a-e). Our data showed that with the open field test, although not statistically significant, there was an observable reduction in the rearing frequency across the groups compared to the control which is a pointer to the anxiogenic ability of thioacetamide. In the thioacetamide-treated groups, the frequency of grooming was observably higher when compared to the control group, with group E being an exception grooming frequency was observably lower when compared to other groups. This is a pointer to the fact that vitamin C was able to ameliorate anxiety-like responses in thioacetamide-treated rats. Total locomotor activity and the total number of line crossings decrease observably in thioacetamide-treated groups with increases in total locomotor activity and the total number of line crossings in the 100 mg/kg Vernonia amygdalina treated group. In this study, because hippocampal-dependent function is memory. It was observed that there was observable impairment in spatial working

memory in the thioacetamide-only groups with observable reversal in impaired spatial memory demonstrated by the 100 mg/kg Vernonia amygdalina group. 100 mg/kg **Thioacetamide Treatment Resulted in CA1 Hippocampal Neuronal Degeneration and Activation of Microglial Cells**

From the cytoarchitectural observations made in the CA1 region using the H&E and cresyl violet stains (plates 1-4) following thioacetamide treatment, there was evidence of pyramidal neuronal degeneration characterized by pyramidal cellular dispersion, hypereosinophilia, pyramidal neuronal vacuolations, and karyorrhectic changes. The Vernonia amygdalina-treated groups (groups C and D) and the vitamin C-treated group (group E) showed CA1 cytoarchitectural preservation comparable to the control group (group A) (plates 1-4).

treatment Vernonia amygdalina has demonstrated memory-improving potential following thioacetamide-induced CA1 region hippocampal neurotoxicity.

Assessment of microglial morphology was by the IHC using anti-iba 1 antibody for detection of iba-1(ionized calcium binding adaptor molecule-1) protein. The control group depicts immunoreexpression of Iba-1 microglial cells with intact morphology, whereas the thioacetamide-only group (group B) revealed enhanced immunoreactivity to anti-iba 1 as depicted by activated microglial cells with altered morphology (plate 6). Albeit, the microglial cells in the Vernonia amygdalina and vitamin C-treated groups particularly 200 mg/kg Vernonia amygdalina-treated groups demonstrated a comparable morphology to the control.

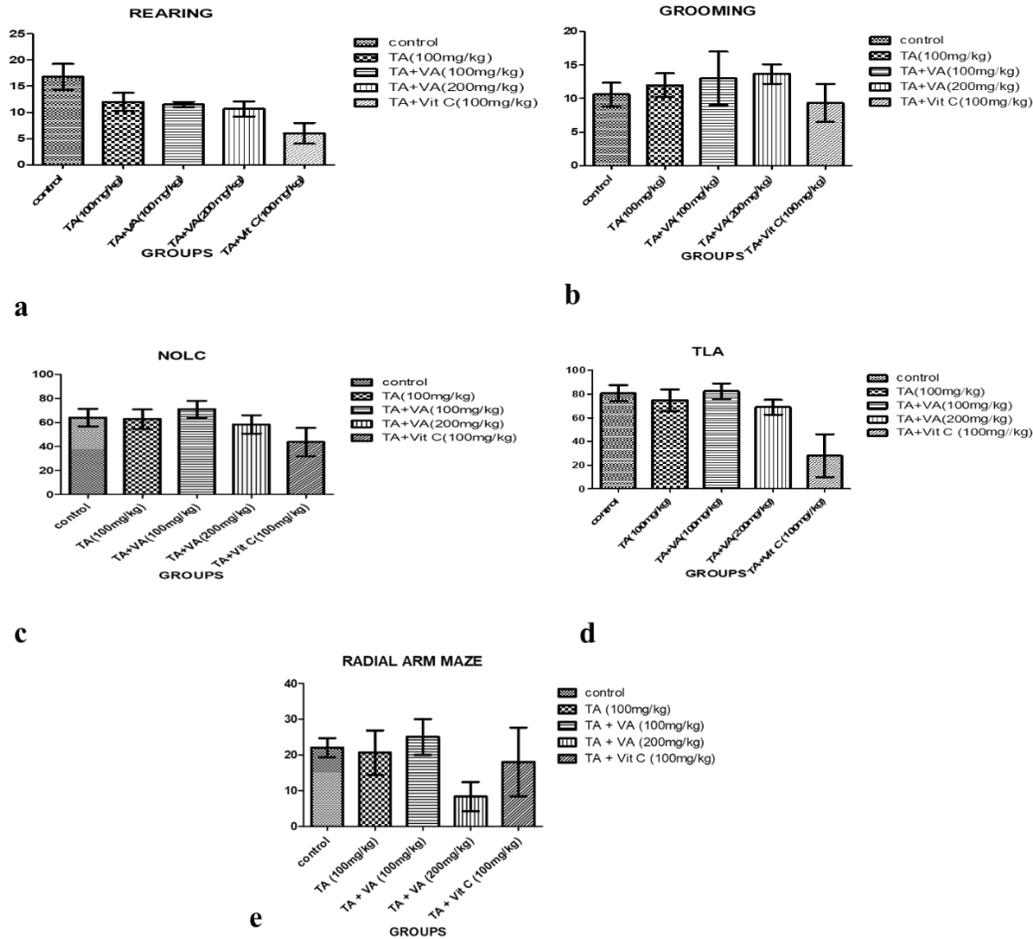


Figure 4: Bar charts represent the Rearing frequency, grooming frequency, number of lines crossed (NOLC), and total locomotor activity (TLA) in thioacetamide, Vernonia amygdalina and Vit. C treated rats across the groups. No significant difference was observed in means across groups ( $p > 0.05$ ).

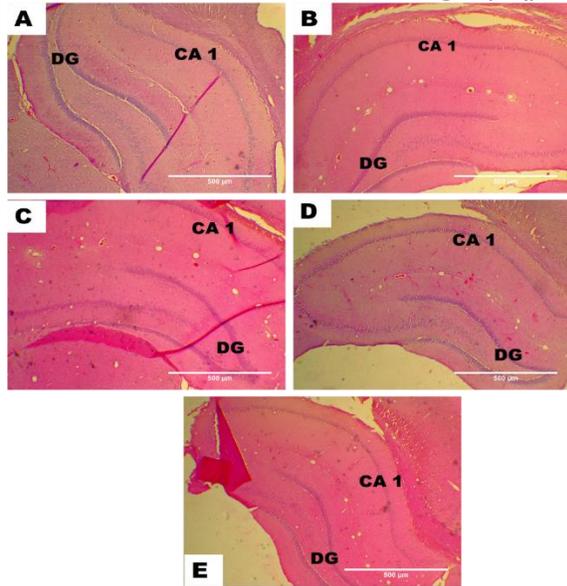


PLATE 1: Representative Light photomicrograph showing the panoramic view of the hippocampal formation consisting of the Cornu ammonis (CA1) and the Dentate Gyrus (DG). Stain: Haematoxylin and Eosin stain. Scale bar: 500µm

evidence of pyramidal neuronal degeneration characterized by pyramidal cellular dispersion, hyper eosinophilia, pyramidal neuronal vacuolations, and karyorrhectic changes (red arrowhead). The Vernonia amygdalina-treated groups (groups C and D) and the vitamin C-treated group (group E) showed CA1 cytoarchitectural preservation comparable to the control group (group A). Stain: Haematoxylin and Eosin stain. Scale bar: 500µm.

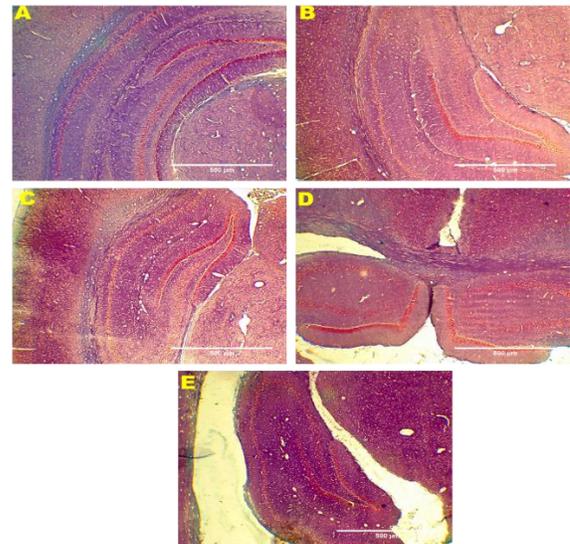


PLATE 3: Panoramic view of rat hippocampal formation subjected to Cresyl violet stain. Photomicrographs show the outline of Nissl expressions in cells of the hippocampal formation. Scale bar: 500 µm; CA – Cornu Ammonis; DG – Dentate gyrus

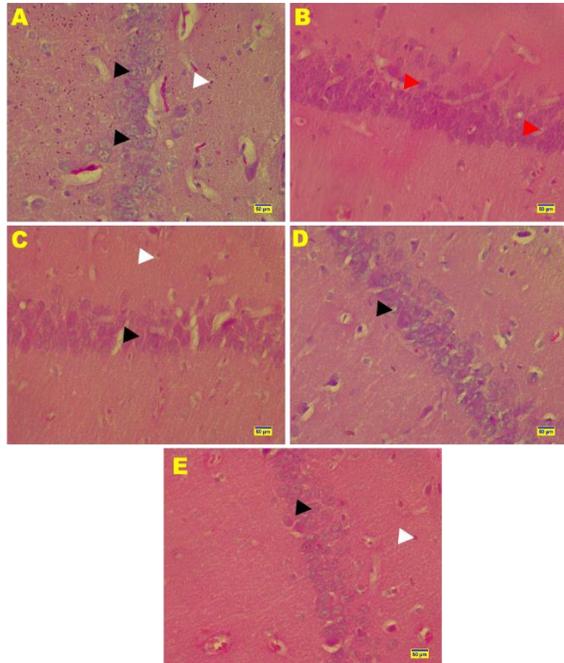


PLATE 2: Representative photomicrographs of the hippocampal sections of Wistar rats showing the cytoarchitecture of the CA1 hippocampal region of the control group (A) and rats treated with Thioacetamide, TAA and Vernonia amygdalina and + Vitamin C (B-E). Thioacetamide-only treated group (group B) showed

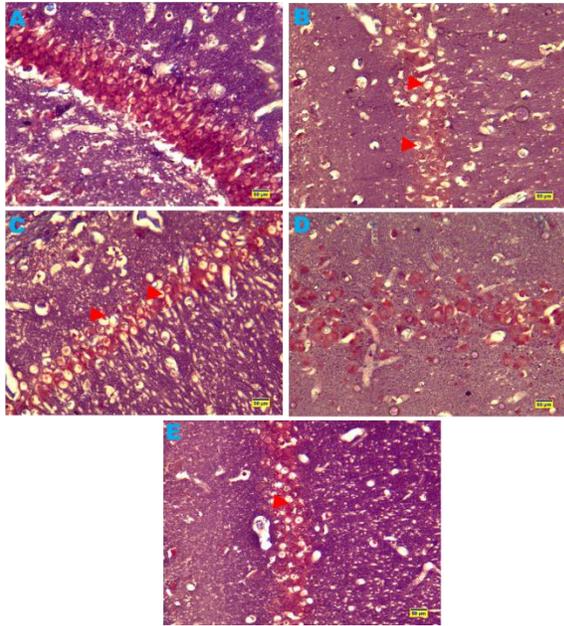


PLATE 4: Representative photomicrographs of the hippocampal sections of Wistar rats showing the cytoarchitecture of the CA1 hippocampal region of the control group (A) and rats treated with Thioacetamide, TAA and Vernonia amygdalina and + Vitamin C (B-E). Thioacetamide only, TAA and Vernonia amygdalina and TAA and vitamin c (group B, C and E) treated groups showed loss of Pyramidal neuronal Nissl substance expression and features of chromatolysis (red arrow head) while group D (200mg/kg Vernonia amygdalina) showed CA1 cytoarchitectural preservation that is near normal to the control group (group A). Stain: Cresyl violet stain. Scale bar: 500µm.

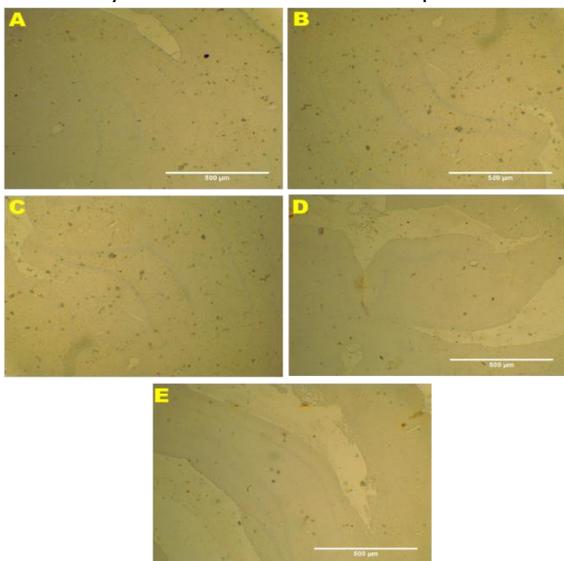


PLATE 5: Representative photomicrographs of the hippocampal sections of Wistar rats depicting the immunoeexpression of Microglia (anti-Iba-1) in the CA1 hippocampal region of the control group (A) rats treated with Thioacetamide, TAA and Vernonia amygdalina and + Vitamin C (B-E). Scale bar: 500 µm

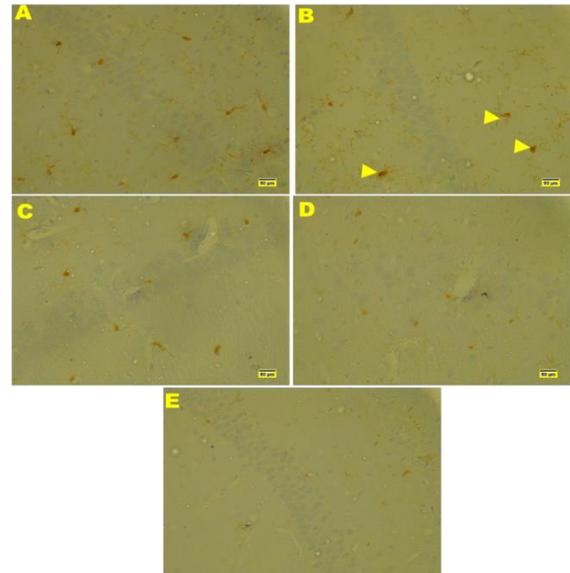


PLATE 6: Representative photomicrographs of the hippocampal sections of Wistar rats depicting the immunoeexpression of Microglia (anti-Iba-1) in the CA1 hippocampal region of the control group (A) and rats treated with Thioacetamide, TAA, and Vernonia amygdalina and + Vitamin C (B-E). The control group showed expression of microglial cells with intact morphology, while the Thioacetamide-only group (group B) and 100mg/kg Vernonia amygdalina treated group demonstrated activated microglial cells with altered morphology (yellow arrowhead). While in the Vernonia amygdalina high dose treated; group D (200mg/kg) and the Vitamin C treated group showed microglial cells with morphologies comparable to the control. Scale bar: 50µm.

## DISCUSSION

Several investigations have reported thioacetamide (TAA)-induced hepatic encephalopathy (HE). There is an impairment in hippocampus structure and hippocampal-mediated function following acute or chronic liver failure in humans. The particular pathophysiological process that causes neurological changes remains elusive. The hippocampus has also been studied infrequently in HE, which is why this study was conducted.

The importance of several neurotransmitter systems in hepatic encephalopathy cannot be overstated, especially given the centrality of cognitive and executive brain functions in hepatic encephalopathy and the modulating roles of these neurotransmitter systems on these brain functions. The impact of thioacetamide treatments on hippocampus glutamate concentration was investigated in this study, as well as treatments with *Vernonia amygdalina* and the anti-oxidant medication (Vitamin C). The glutamate concentration (Fig.2) after thioacetamide administration exhibited no significant difference when compared to the control group. The principal excitatory neurotransmitter of the central nervous system is glutamate (Palomero-Gallagher and Zilles, 2013). According to Simplicio *et al.* (2019), two significant glutamatergic routes have been implicated in the pathogenesis of hepatic encephalopathy: one is a hypothetic pathway assumed to start from the frontal cortex, and the other is the perforant pathway originating in the entorhinal cortex. The frontal descending pathway is thought to originate in layer V pyramidal neurons, which project to the centers of other neurotransmitters in the brainstem, synapsing with dopaminergic neurons of the midbrain, raphe nuclei serotonergic neurons, and locus coeruleus noradrenergic neurons and influencing their activity (Stahl, 2013). Hypothetically, glutamatergic hyperactivity would act as a final pathway common to the changes induced by hyperammonemia and

neuroinflammation, disturbing other neurotransmission systems, in steps that would invoke neuropsychiatric symptoms, and, in more severe cases, cause coma (Montana *et al.*, 2014). Excessive glutamate activity is also capable of microglia activation (Su *et al.*, 2015). The perforant pathway originates in the medial portion of the temporal cortex, called the entorhinal cortex, and projects to the granular cells of the dentate gyrus. The axons of these cells form a pathway of mossy fibers, which goes to the Cornu Ammonis (CA) or Ammon's horn, more precisely to the pyramidal cells of the CA3 region. Then, the pyramidal cells emit excitatory collaterals, the Schaffer collaterals that project to the pyramidal cells of the CA1 region. Because of the projections of the Schaffers collateral fibers to the CA1 region of the hippocampus, and given its role in memory, the neurons in this region are highly vulnerable to neurotoxins. A brief discharge of high-frequency stimuli in any of these three components of the perforant pathway increases the excitatory postsynaptic potentials in hippocampal neurons, which can last for hours, days, or even weeks. This facilitation is called long-term potentiation and, in addition to the hippocampus, also occurs in the amygdala, striatum (putamen and caudate nucleus), and cerebellar Purkinje cells, being essential for the formation of new traces of memory and learning (Cabrera-Pastor *et al.*, 2016; Hernández-Rabaza *et al.*, 2016a).

The picture here also corroborates the result obtained from the cytoarchitectural assessment of the CA1 region using the H&E and cresyl violet stains (plates 1-4) following thioacetamide treatment where there was evidence of pyramidal neuronal degeneration characterized by pyramidal cellular dispersion, hypereosinophilia, pyramidal neuronal vacuolations, and karyorrhectic changes. The *Vernonia amygdalina* treated groups (groups C and D) and the vitamin C treated group (group E) showed CA1 cytoarchitectural preservation comparable to

the control group (group A). This further shows the neuroprotective ability of *Vernonia amygdalina* and the anti-oxidant role of vitamin C in ameliorating thioacetamide-induced hippocampal neuronal damage. Research into the involvement of other mechanisms leading to neuronal toxicity other than glutamate-mediated excitotoxicity is necessary.

The neuroinflammatory marker (Iba-1) produced by microglial cells was used to assess neuroinflammation in this investigation (plates 5 and 6). Most central nervous system pathophysiological abnormalities are accompanied by central inflammatory processes and microglia activation. Glial cell dysfunction has also been shown to precede and even drive the development of a variety of neurodegenerative disorders (Jurga *et al.*, 2020). The control group exhibited immunoexpression of Iba-1 by intact microglial cells, whereas the thioacetamide-only group (group B) revealed enhanced immunoreactivity to Iba-1 and activated microglial cells with altered morphology (plate 6). Albeit, the *Vernonia amygdalina* and vitamin C treated groups particularly the 200 mg/kg *Vernonia amygdalina* treatment group following thioacetamide administration demonstrated microglial cells with morphology comparable to the control. Microglia have been shown to influence the activity of astrocytes and neurons. Microglial cells are immune cells that have a phagocytic function and actively monitor the brain parenchyma. When there are no inflammatory stimuli, microglia cells stay dormant and have ramified processes (resting phenotype). However, in the presence of an inflammatory stimulus, they become reactive and acquire an amoeboid shape (active phenotype), correlating with increased production of cytokines and chemokines (Hickman *et al.*, 2018). The mechanism of neuronal degeneration observed in this study may be due to microglial activation. It has also been

demonstrated in this study that thioacetamide is capable of causing microglial activation and increased Iba-1 expression. Also as seen in hepatic encephalopathy, molecules such as ammonia, glutamate, and some locally produced neuroactive steroids (neurosteroids) may trigger the transition of microglial from the resting phenotype to the active phenotype (Su *et al.*, 2015). Neuroinflammation influences glutamatergic activity. Microglial activation in the cerebellum of rats subjected to chronic hyperammonemia stimulates the production of proinflammatory cytokines such as TNF and IL-1b, as well as the expression of TNF receptors. TNF receptors are also present on the surface of astrocytes, and their activation generates increased glutaminase, which contributes to an increase in glutamate production (Cabrera-Pastor *et al.*, 2018). There is also evidence that excess glutamate activates microglia, resulting in an intercellular vicious loop (Su *et al.*, 2015). The activity of Glucose-6-phosphate dehydrogenase (G6PDH) (Fig. 3) following treatment with thioacetamide alone, thioacetamide and *Vernonia amygdalina*, and vitamin C was not significantly different from the control and also when comparisons were done across the groups. Following exposure to thioacetamide, its bioactivation leads to the production of its metabolites; thioacetamide S-oxide which in turn generates peroxide radicals capable of liberating reactive oxygen species (Ghosh *et al.*, 2016). The liberated reactive oxygen species possesses the potential to be distributed in various tissues, the brain included. The metabolites generated are then distributed in the various tissues, the brain included. This shows the possible mechanism by which thioacetamide could induce neurotoxicity.

Several studies have proven the protective effect of the enzyme G6PDH in the brain (Jeng *et al.*, 2013; No'brega-Pereira *et al.*, 2016; Adelodun *et al.*, 2021). G6PDH is one

of the most critical enzymes required for NADPH generation. G6PDH catalyzes the rate-limiting step in the pentose phosphate pathway (PPP), which provides nucleotide precursors for DNA replication as well as NADPH reductive capacity for ROS detoxification and de novo lipid synthesis (Adelodun *et al.*, 2021). Also, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is influenced by  $[NADP^+]/[NADPH]$ . During oxidative stress, there could be a drop in the GSH/GSSG (Chai *et al.*, 1994). The protective ability of cells against oxidative stress is dependent on the reduced ability of NADPH. A balanced state of G6PDH is essential for optimal brain function. The increases in the activities of G6PDH even though not significant suggest the modulating role of thioacetamide on hippocampal G6PDH activity. This area needs to be further explored.

Assessment of neurobehaviour (Fig. 4a-e) in this study using the open field test showed that there was an observable reduction in the rearing frequency across the groups compared to the control which points to the ability of thioacetamide to initiate anxiogenic responses. Also, the frequency of grooming was observably higher in the thioacetamide-treated groups when compared to the control group, with group E being an exception grooming frequency was observably lower when compared to other groups. This is a pointer to the fact that vitamin C was able to ameliorate anxiety-like responses in thioacetamide-treated rats. An increase in the frequency of grooming depicts increased anxiety (Reimer *et al.*, 2015). For rearing, there is an observable increase in the frequency of rearing in the control group when compared to other groups. Total locomotor activity and total number of line crossings decrease observably in thioacetamide-treated groups with increases in total locomotor activity and total number of line crossings in the 100 mg/kg *Vernonia amygdalina* treated group showing that the mechanism of action of

which *Vernonia amygdalina* may be dose-dependent. In this study, because hippocampal-dependent function is spatial memory, the radial arm maze apparatus was used to assess spatial memory. It was observed that there was observable impairment in spatial working memory in the thioacetamide-only groups with observable reversal in impaired spatial memory demonstrated by the 100 mg/kg *Vernonia amygdalina* group. 100 mg/kg treatment *Vernonia amygdalina* has demonstrated memory-improving potential following thioacetamide-induced CA1 region hippocampal neurotoxicity.

### Conclusion

Thioacetamide caused neuroinflammation *vis-à-vis* microglial activation, resulted in neurodegeneration in the CA1 subfield, and also impaired spatial working memory while *Vernonia amygdalina* treatment ameliorated these neurotoxic changes. *Vernonia amygdalina* has proven to possess probable therapeutic potential against thioacetamide-induced neuronal damage in CA1 of the hippocampus of the Wistar rats.

### Study Limitations and Recommendations

The active constituents of *Vernonia amygdalina* which may be attributed to the observed neuroprotective effects were not explored in this study. We therefore recommend *in vivo* and *in vitro* assays be done following isolation of the active plant constituents in order to make an informed decision on the constituent(s) that possesses this neuroprotective property. Additionally, ultramicroscopic studies could be employed to assess the hippocampal cellular organelles.

### Author Contributions/ Credit Author Statement

**Taiye S. Adelodun**- Conceptualization; Data curation; Formal analysis; Investigation; Methodology, Supervision;

Validation; **Ayodeji Z. Abijo**- Writing original draft; review & editing, Data Analysis Software; **Osuoha O. Maryjane**- Data Curation and analysis, Investigation; **Sunday Y. Olatunji**, Writing - review & editing; **Arayombo B. Elijah**- Writing - review & editing

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#### **Compliance with ethical standards**

Institutional ethical permission was acquired from the Babcock University Health Research Ethical Committee (BUHREC), Babcock University, Ilishan-Remo, Ogun state, with the ethical number **428/21**.

#### **Declaration of competing interest**

The authors declare no conflicts of interest.

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