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HISTOLOGICAL STUDY OF CYCLOPHOSPHAMIDE ON HIPPOCAMPUS OF WISTAR RATS FOLLOWING THE ADMINSTRATION OF ALKALOIDS ISOLATED FROM DISCOREA BULBIFERA BULBIS

ENYE LINUS ANDERSON *

Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria. *Corresponding Author Email: enyelinus@abuad.edu.ng

SAKA OLUSOLA STEPHEN

Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria.

FAURE ADEDAMOLA A

Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria.

EDEM E EDEM

Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria.

AYEGBUSI OLATEJU S

Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria.

OGUNSANYA SANMI

Department of Anatomy, Babacock University Ilishan-Remo, Ogun State, Nigeria.

SAKA A. A

Department of Public Health, College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Nigeria. Department of Anatomy, Ajayi Crowther University Oyo, Nigeria.

Abstract

Cyclophosphamide (CYC) is commonly used in cancer chemotherapy, which causes immunosuppression and tissue oxidative stress at high doses. To enhance the anti-cancer efficacy and reduce the toxicity of CTC, investigating potential adjuvant treatments that can attenuate the adverse effects has been demonstrated. This study explored potential effects of CYC on the hippocampal architechture following the administration of alkaloid isolated from *Dioscorea bulbifera.* Group A was made the control and given 0.14 ml distilled water for 14 days. Group B were be administered with Cyclophosphamide 150mg / kg only through intraperitoneal route (IP) for 7 days. Group C was administered with Cyclophosphamide 150 mg/ kg and 50 mg / kg of Alkaloid concomitantly for 7 days and Group D was administered Alkaloid for 7 days and then Cyclophosphamide of 150 mg / kg for 7 days. The Alkaloid compound was administered via oral gavage and cyclophosphamide was administered through IP. Administration of Cyclophosphamide (150 g/kg) induced major alterations and has promoted general alteration in the hippocampus and dentate gyrus; furthermore, we can conclude that the Alkaloids (50mg/kg) from *Discorea Bulbifera* treated the toxicity very minimally but a higher dose could have been more effective. Therefore, the administration of Cyclophosphamide should be strictly monitored and regularly evaluated in individuals treating cancer using this drugs in order to alleviate or prevent some of these alterations.

Keywords: *Cyclophosphamide, Alkaloid compound, Discorea Bulbifera, Hippocampus.*

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INTRODUCTION

Cyclophosphamide (CYC) is an ancient alkylating drug commonly used in cancer as a chemotherapeutic treatment [1]. Cyclophosphamide is an inactive prodrug that is rapidly absorbed and has a high bioavailability following oral treatment [2]. It is transformed to 4-hydroxycyclophosphamide by the cytochrome P450 system in the liver, which is in equilibrium with its tautomer aldophosphamide [3].

Aldehyde dehydrogenase converts this to inactive carboxycyclophosphamide, although some aldophosphamide escapes dehydrogenase's actions and is cleaved into two hazardous metabolites: alkylating phosphoramide mustard and inactive Acrolein. [4].

Discorea bulbifera has a subtle odor and a bitter-salty flavor, as well as a higher nutritional content than other Discorea species [5]. Discorea bulbifera formulations have been used to treat memory enhancement, anti-aging, constipation, and fever [6]. Discorea bulbifera bulbi have also been shown to contain antifertility steroids, flavonoids, alkaloids, and saponin, as well as other therapeutic properties for contraception, sexual vigor, ulcers, syphilis, tuberculosis, leprosy, cough, and diabetes [5].

The impact of D. bulbifera on a variety of disorders has been well documented. It has been discovered that it causes hepatotoxicity [7], Nephrohtoxicity [8], Thyroid toxicity [9] and Gastro Intestinal Tract problems [10]. Hepatotoxicity is the most common type of plant toxicity, and it is characterized by nausea, vomiting, liver malfunction, or jaundice in humans [7]. For their mutagenic or carcinogenic activity, antibacterial, antifungal, and antiviral capabilities, alkaloids have been employed in both ancient and modern medicine [11].

Alkaloids are central nervous system stimulants with a wide range of pharmacological characteristics (brucine) [12], anticholinergic agents (tropane) [13], oxytocic and vasoconstrictor activity (ergometrine) [14], and antimalarial activity [15], local anaesthetic properties (Morphine). Morphine is a well-known alkaloid that has been utilized in medicine for many years. Because of its addictive qualities, this alkaloid is utilized as a potent narcotic to treat pain [16].

Many alkaloids are poisonous to animals and can even kill them [17]. Pyrrolizidine alkaloids are hepatotoxic, while Tropane alkaloids are cardiotoxic and can disrupt respiration and central nervous system function [18]. D. bulbifera contains only one alkaloid, dihydrodioscorine, which was discovered by Adetoun Adeleye and Dr. T. Ikotun, according to the Chinese Materia Medica. [9].

Throughout the years, three basic principles in hippocampal function have dominated the literature: inhibition, memory, and space. The behavioural inhibition theory explains why people behave in particular ways [19]. Both psychologists and neuroscientists believe the hippocampus plays a key role in the formation of new memories of previously experienced events (episodic or autobiographical memory) [20].

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MATERIALS AND METHODS

Animal Care and Management

Twenty-four (24) healthy Adult Male wistar rats weighing between 132g to 168g of the same species of rattus Norvegicus were purchased from the Animal Holdings of the Department of Anatomy, Ekiti State University, Ado-Ekiti and were used for this research. The animals were housed in the Animal Holdings of Afe Babalola University, Ado-Ekiti. They were placed on standard laboratory pellet and clean water was provided. The rats were maintained in natural day and night cycle and temperature and allowed to acclimatize for a period of one week, after which they were then arranged into 4 groups of animals according to their weights.

Animal treatment

Group A was made the control and given 0.14 ml distilled water for 14 days. **Group B** were be administered with Cyclophosphamide 150 mg/kg only through intraperitoneal route for 7 days. **Group C** was administered with Cyclophosphamide 150 mg/kg and 50 mg/kg of Alkaloid fraction of Discorea Bulbifera concomitantly for 7 days and Group **D** was administered Alkaloid for 7 days and then Cyclophosphamide of 150 mg/kg for 7 days. The Alkaloid compound was administered orally, using an oral cannula and cyclophosphamide was administered through intraperitoneal route. The duration of experiment was 14 days.

Experimental Design

S/N	Groups	Agents	Doses	Route	Duration
1	A	Distilled water	0.14 ml	Oral	14 days
2	B	Cyclophosphamide only	150 mg/kg	Intraperitoneal	7 days
3	C	Cyclophosphamide + Alkaloid	150 mg/kg + 50 mg/kg	Intraperitoneal + Oral	7 days
4	D	Alkaloid + Cyclophosphamide	50 mg/kg + 150 mg/kg	Oral + Intraperitoneal	7 days + 7 days

Plant Material and Preparation of Extract

Freshly harvested bulbils of *Dioscorea bulbifera* will be procured from our local market. The bulbils will be washed, peeled, cut into thin slices, chopped into pieces and air-dried. The chopped dried bulbils will finally be pounded into fine powder using mortar and pestle. 500g of sample were weighed into a 2 litres beaker. 2.5L of 10% acetic acid in ethanol was added and placed in a water bath at 50c for 4 hours with continuous stirring. This was later filtered and concentrated on water bath to one quarter of the original volume at 50c. 100ml of concentrated ammonia hydroxide was added drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with 2M of ammonia hydroxide and then filtered, the residue is then alkaloid was dried and collected.

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Drug (Cyclophosphamide injection)

Highly active anti-cancer drug (Cyclophosphamide injection) Cyclophosphamide (Biochem pharmaceutical Industries Ltd. Ackruti Star, Unit NO. 103, MIDC, Andheri (E), Mumbai - 400 093) was obtained from the Department of Pharmacy, Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria.

Determination of Body Weight and Sacrifice of Animals

Body weights were taken at the beginning, 3rd week and last day of the experiment. The animals were sacrificed on the last day of the experiment by cervical dislocation, blood samples were collected through the cardiac puncture and then centrifuged, the plasma was decanted from the blood into another sample bottle for biochemical analysis.

The hippocampus were harvested and weighed using the gallenhamp electronic balance (MP 10001) weighing balance and fixed in 10% formal-saline for histological studies.

Percentage weight change was calculated using:

$$\frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Tissue was stained with: Haematoxylin and Eosin method was used to demonstrate the general histoarchitecture of the hippocampus and Cresyl Fast Violet (Vogts') Method for Nissl Substance

Biochemical Analysis

The whole blood via cardiac puncture collected was centrifuged for biochemical estimation of acetylcholinesterase and glutamate concentration. In the bench centrifuge, each compartment is balanced at the opposite compartment.

The machine spun for 15 minutes at 3000 RPM separating the blood cells at the bottom and serum atop the blood. The acetylcholinesterase and glutamate were analyzed by ELISA method using rat ELISA kits.

Acetylcholinesterase (AChE) Assay procedure

Acetylcholinesterase activity was determined spectrophotometrically by measuring the rate of hydrolysis of the substrate, ATCI according to the method of Ellman *et al*., [21].

Aliquots of the brain homogenates were diluted with 2.6 ml 0.1 M phosphate buffer (pH 7.4) to a total volume of 3 ml, which contained 100 mM DTNB.

The enzyme activity was calculated by extinction co-efficient (E412= 1.36 x 104 M-1 cm-1) for the yellow anion 5-thio-2 nitrobenzoic acid.

The AChE and activity was expressed as nmole of ATCI hydrolyzed per min per milligram of protein and for serum ATCI/BuTCI hydrolyzed/ml serum/min

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Glutamate Assay Procedure

Estimation of glutamate level was done by preparing brain homogenates in phosphate buffer saline (PBS) according to the method of Adelodun *et al*., [22]. The brain 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 kit manufacturer

Photomicrography

Olympus binocular microscope was used. A 5.1-megapixel MV550 research camera for microscopes was mounted in one of the oculars. This was connected to a computer running on image capture and analysis software. The system was adjusted to obtain clarity and resolution. The image was captured and saved on the computer.

Statistical Analysis

One-way ANOVA was used to analyse data, followed by Student Newman-keuls (SNK) test for multiple comparisons. Graph Pad Prism 5 (Version 5.03, Graphpad Inc.) was the statistical package used for data analysis. Significant difference was set at $p<0.05$.

Results

Figure 2: A) Effect of Alkaloid on Body Weight of Rats Administered with Cyclophosphamide**:** One-way ANOVA revealed that, there was significant difference in weight change across all experimental groups ($F = 175.9$; $p < 0.001$). Post hoc analysis showed that weight of cyclophosphamide group was significantly lower than the vehicle treated group. The weight reduction induced by cyclophosphamide was a little bit reversed by pre-treatment of alkaloid administration in group D ($p<0.05$);

B) The Effect of Alkaloid on Activity of Glutamate in Wistar Rats Administered with Cyclophosphamide**:** There was significant difference in glutamate activity in group B (cyclophosphamide only) ($F = 11.89$; $p = 0.0026$) compared to control group A;

C) The Effect of Alkaloid on Activity of Ache in Wistar Rats Administered with Cyclophosphamide**:** There was significant difference in Ache activity in group B (cyclophosphamide only) ($F = 46.56$; $p < 0.0001$) compared to control group A.

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Biochemical Assay

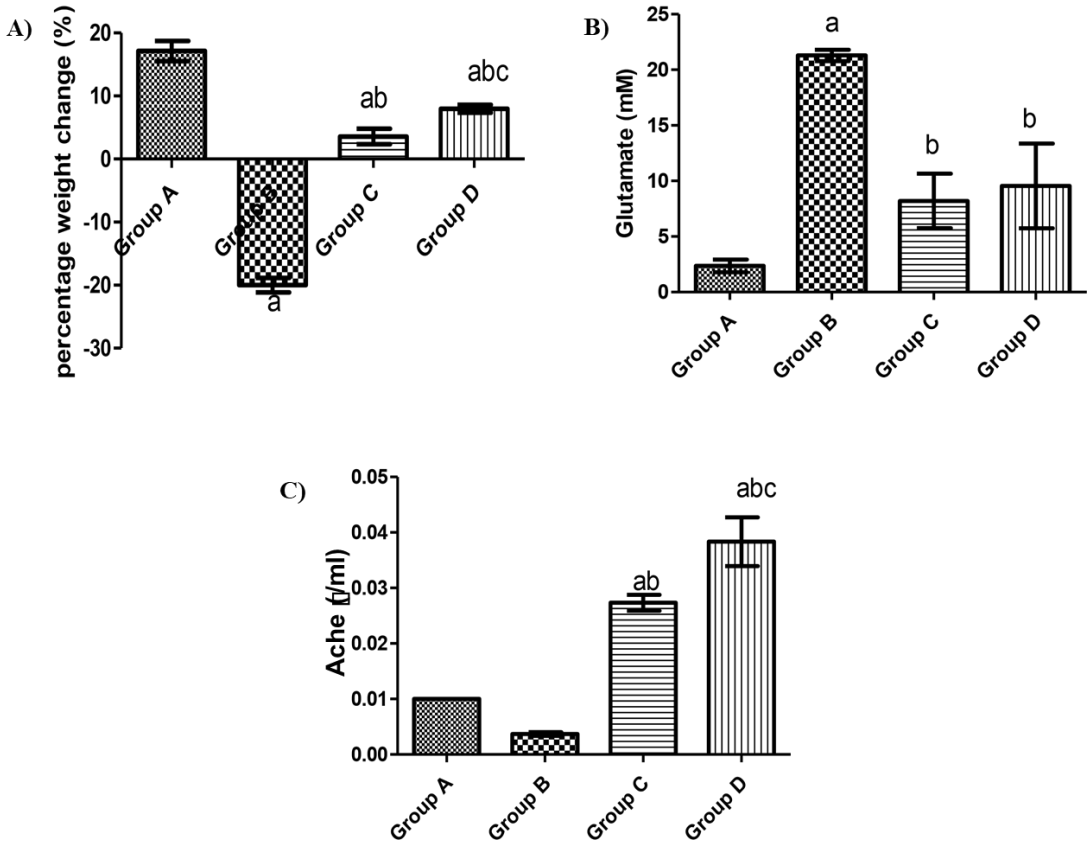


Figure 2

Figure 2: A) Effect of alkaloid on Body Weight of Rat fed with Cyclophosphamide. n = 7, values are expressed as % weight change ± SEM. a = relative to control at $p<0.05$; b= relative to group B (cyclophosphamide only) at $p<0.05$. Group A = control, Group B = cyclophosphamide only, Group C = 150 mg/ kg cyclophosphamide + 50 mg/kg concomitant administration, Group D = pretreatment of 50 mg/kg of alkaloid + 150 mg/kg cyclophosphamide. **B)** The Effect of Alkaloid on Activity of Serum Glutamate in Wistar Rats Administered with Cyclophosphamide. n = 7, Values are expressed as mean of glutamate ± SEM. a = relative to control at $p<0.05$; b= relative to group B (cyclophosphamide only) at $p<0.05$. Group A = control, Group B = cyclophosphamide only, Group C = 150 mg/ kg cyclophosphamide + 50 mg/kg concomitant administration, Group D = pre-treatment of 50 mg/kg of alkaloid + 150 mg/kg cyclophosphamide **C)** The Effect of Alkaloid on Activity of Serum Ache in Wistar Rats Administered with Cyclophosphamide. n = 7, Values are expressed as mean of Ache ± SEM. a = relative to control at $p<0.05$; b= relative to group B (cyclophosphamide only) at $p<0.05$. Group A = control, Group B = cyclophosphamide only, Group C = 150 mg/ kg cyclophosphamide + 50 mg/kg concomitant administration, Group D = pre-treatment of 50 mg/kg of alkaloid + 150 mg/kg cyclophosphamide.

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Hippocampal Histopathology

Results obtained from histological studies of H and E stained sections revealed distinct cellular arrangement of pyramidal neurons in the *Cornus ammonis* (CA1, 2, and 3) regions and likewise the granule cells of the dentate gyrus in the control group (Figure 3A) and the group administered with Cyclophosphamide and Alkaloid (group C and D) (Figure 3C & 3D). Pyramidal neurons in the CA1, 2, and 3 regions of group administered with Cyclophosphamide only (Figure 3B) showed distortion in the cellular arrangement with narrowing of the hippocampal fissure. The hippocampal sections were also stained in Cresyl violet to demonstrate the presence of Nissl substances and the result obtained revealed normal granule neurons histomorphology in groups A (Figure 4A) with distinct cell body and well-delineated cytoarchitecture, while groups B (Figure 4B) showed granule neurons with degenerative features evident by intensely stained eosinophilic cytoplasm, cytoplasmic fragmentation, and intracellular nuclear material aggregation. Group C and D revealed a marked decrease in Nissl's substance (Figure 4C & 4D).

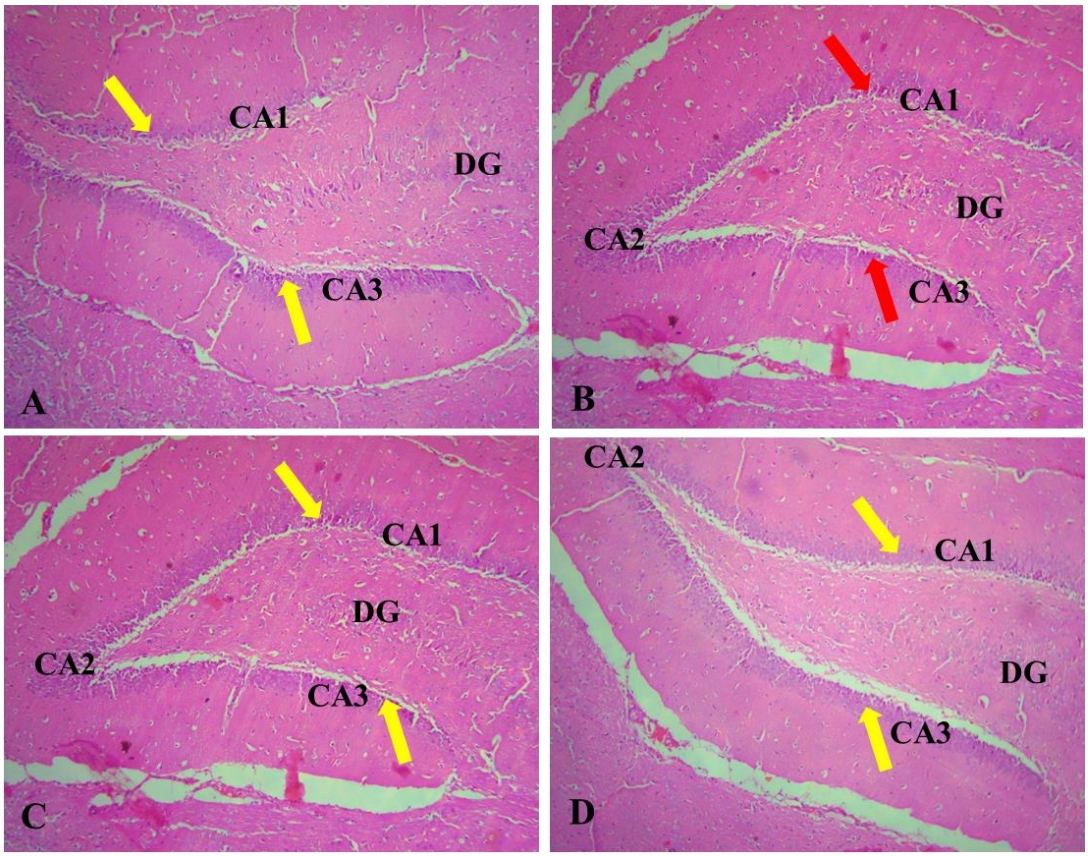


Figure 3

Figure 3: Representative photomicrographs of the hippocampus showing different subfields CA1, CA2, and CA3, and the dentate gyrus (DG) in group A (Control), group B (Cyclophosphamide only), group C (Cyclophosphamide + Alkaloid) and group D (Alkaloid + Cyclophosphamide). Cells in the control group (A), groups C and D are in a normal

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array (yellow arrow) and are characterized by the distinct cellular arrangement of pyramidal neurons in the *Cornus ammonis* region and the granule cells of the dentate gyrus. While cells in group B presented with several pyknotic changes and disorderliness in pyramidal and granular neuronal arrangement (red arrow). Stain: H&E.

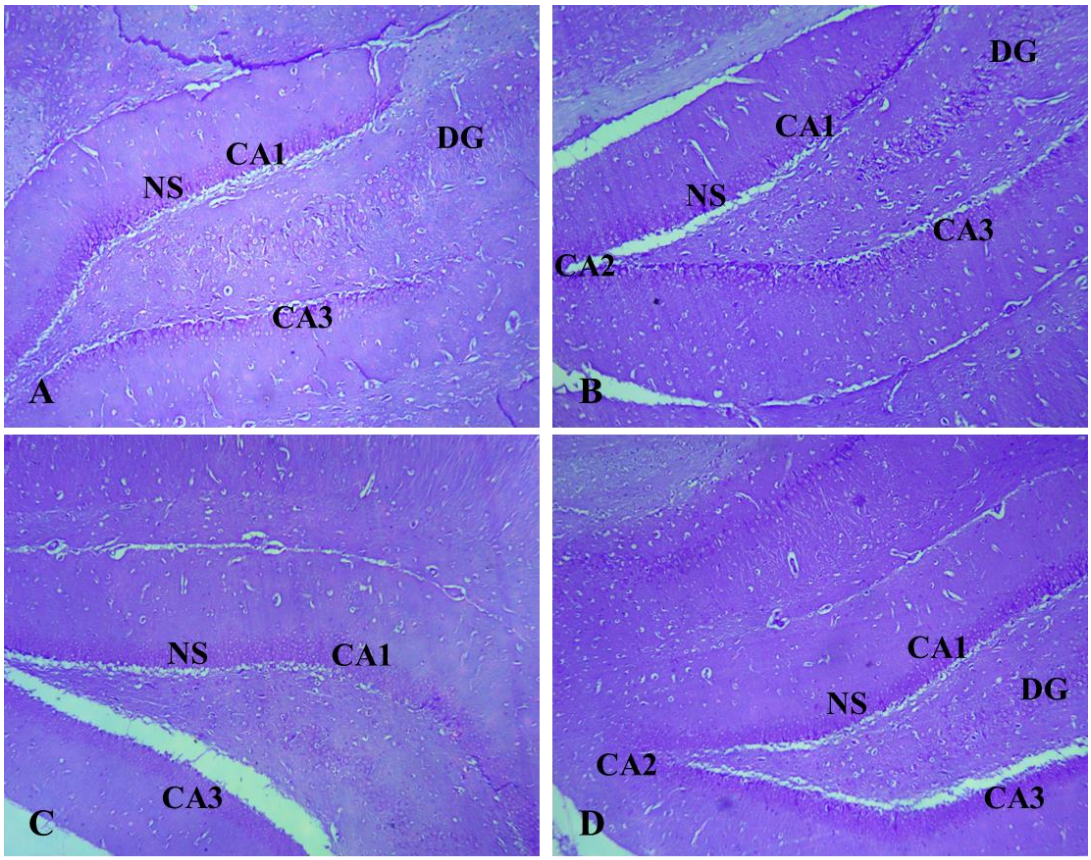


Figure 4

Figure 4: Representative photomicrographs of the hippocampus showing different subfields CA1, CA2, and CA3, and the dentate gyrus (DG) in group A (Control), group B (Cyclophosphamide only), group C (Cyclophosphamide + Alkaloid) and group D (Alkaloid + Cyclophosphamide). Stain: (CFV X200).

DISCUSSION

This study investigated the effects of the treatment of the Cyclophosphamide induced toxicity on the hippocampus of adult male Wistar rats with the use of alkaloids fraction of *Discorea Bulbifera Bulbis* extract. It was observed that there was significant reduction in the weight of group B animals which were administered 150 mg/kg of cyclophosphamide (**Fig 2**). The reduction in percentage body weight of the rats would have been as a result of the high level of Cyclophosphamide toxicity when compared to others groups. This present research was in accordance with [23] reported that significant weight gain reduction in pregnant rats. Glutamate is an abundant excitatory neurotransmitter in the

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brain and plays an important role in both physiological and pathological processes. Glutamate may act as a potent neurotoxin in excessive amounts through increasing the excitability and by activating the proteolytic enzymes [24]. Glutamate elicits synaptic responses via metabotropic mGLU and ionotropic NMDA receptors, causing neuronal excitement and Ca^{2+} release from intracellular reserves. On the other hand, activation of ionotropic receptors increases cellular permeability to Ca^{2+} [25]. The amino acid transporters found in neurons and glial cells are critical for glutamatergic transmission to work normally and for keeping extracellular glutamate levels below potentially excitotoxic levels [24]. In the present study, glutamate concentration was significant across the groups but most significant in Group B (**Fig 2**). This might be that; the effect of Cyclophosphamide was not significant to the brain. Acetyl choline is a neurotransmitter that plays a crucial function in the central nervous system, and it has been linked to behavioural, learning, and memory problems, as well as neurodegenerative disorders [26]. Our results showed that administration of Cyclophosphamide in rats was significant in AChE activity in the hippocampus but the Group B was very low (**Fig. 2**).

The control group shows normal hippocampal formation composed of the subiculum, Ammon's horn and the dentate gyrus, also numerous the Nissl substance (**Fig. 3**). The cyclophosphamide group only shows slight atrophy of hippocampal tissue and narrowing of the hippocampal fissure is noted. The cellular layers appear normal in dentate gyrus (**Fig. 3**). Group C and D, showed moderately changes in the hippocampal tissue and the hippocampal fissure appears normal. The cellular layers appear normal in dentate gyrus (**Fig. 3**).

CONCLUSION

Administration of Cyclophosphamide (150 mg/kg) induced major alterations and has promoted general alteration in the hippocampus and dentate gyrus; furthermore, we can conclude that the Alkaloids (50 mg/kg) from *Discorea Bulbifera* treated the toxicity very minimally. Therefore, there is need for proper monitoring and regular evaluation of Cyclophosphamide using it for chemotherapy in order to prevent the observed toxicity effect.

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Conflict of interest

The authors have no conflict of interest to declare.

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