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EFFECTS OF ORAL INGESTION OF HYOSCYAMINE FROM *Daturastramonium* SEEDS ON THE HIPPOCAMPUS IN ADULT WISTAR RATS (*Rattusnorvegicus*)

Idris, A. T.,[1*] Sunday, A. M.,[2] Ibrahim, A. I.,[3] James, O. N.,[4] Musa, A. K.,[1]and Abdullahi, G.[1]

[1]Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Science, Bayero University Kano, Nigeria.
[2]Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Science, Ahmadu Bello University Zaria, Kaduna State, Nigeria.
[3]Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Science, Federal University, Dutse, Jigawa State, Nigeria.
[4]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Kaduna State, Nigeria.
***Corresponding author: gwtel4u@gmail.com**

ABSTRACT
The study aimed to evaluate the effects of oral ingestion of hyoscyamine fraction of Daturastramonium seeds on the hippocampus in adult Wistar rats. Fresh seeds of D. stramonium were procured and fractionated using high-performance liquid chromatography (HPLC). Twenty-four healthy adult Wistar rats weighed 230±0.50 grams, were procured and divided equally into four groups for the experiment. The group one received an equivalent bodyweight of normal saline, while three other groups received 200, 400 and 800 mg/kgbwt of hyoscyamine fraction of D. stramonium respectively for three weeks. At the end of the experiment, the animals were subjected to memory test using Morris water maze (MWM) and Novel object recognition test (NORT) test paradigms. The data obtained were expressed as mean ± SEM and repeated measures ANOVA with Fisher's multiple comparisons post-hoc tests were used to obtain mean differences using Minitab 17 (LLC., U.K.) statistical package software. P < 0.05 was considered statistically significant. There was a statistically significant increase in the exploration time (p = 0.031) and escape latency period (p <0.001) in the novel object recognition and Morris water maze test between the groups in the treated compared to the control group. The CA3 region of the treated group showed significant neuronal lesions, cytoplasmic vacuolations, pyknosis and necrosis. In conclusion, exposure to hyoscyamine fraction of D. stramonium at adulthood impaired memory in Wistar rats.
Keywords: Daturastramonium, hippocampus, hyoscyamine, learning, memory.

INTRODUCTION

Daturastramonium is a hallucinogenic plant that belongs to the Solanaceae family. It has potential medicinal values owing to its pharmacological properties. Some of these values include anti-inflammatory, anticholinergic, antihistaminic, acaricidal, antimicrobial, and anticancer activities (Soni*et al*., 2012). It was also reported to be used in the treatment of depression, madness and epilepsy (Mueser*et al*., 1998). Although all parts of *D. stramonium* are toxic, its ripened seeds contain the highest concentration of its active principles (Miraldi*et al*., 2001; Joshia and Prakashb, 2015; Benouadah*et al*., 2016; Jonasson and Afshari, 2016; Mishra, 2018). The active principles include scopolamine, atropine and other alkaloids which are classified as deliriants, or anticholinergics (Diker*et al*., 2007). Its frequent poisoning may be linked to its ubiquitous nature, ease of contaminating foodstuffs and potable water and its high toxicity (Kurzbaum*et al*., 2001; Oberndorfer*et al*., 2002; Arouko*et al*., 2003; Steenkamp *et al*., 2004; Al-Shaikh and Sablay, 2005; Boumba*et al*., 2005; Ertekin*et al*., 2005; Forrester, 2006; Montcriol*et al*., 2007; Dubey and Sanjeev, 2017; Trancă*et al*., 2017; Başaran*et al*., 2018; Korkmaz*et al*., 2019). Overdose of *D. stramonium* produces a classic anticholinergic syndrome which leads to severe and fatal complications (Melvin and Hourani, 2014). Intoxication typically produces both central and peripheral symptoms which include; delirium, hyperthermia, tachycardia, bizarre behaviour, and severe mydriasis with resultant painful photophobia that can last several days(Roblot, 1995; Vearrier and Greenberg, 2010). Regardless of the part ingested, *D.*

stramonium may cause complications whose diagnosis may be difficult to unravel (Diker*et al*., 2007; Jonasson and Afshari, 2016; Uddin *et al*., 2017). Several cases of suicide and murder in India and Europe have been traced to *Daturastramonium*, hence, strict legislation prohibiting its cultivation has been implemented in several places (Preissel and Hans-George, 2002; Bontoyan, 2010; Jonasson and Afshari, 2016; Mishra, 2018).

Hippocampus is an extension of temporal part of the cerebral cortex (Gilbert and Brushfield, 2009) which is essential for the rapid formation and consolidation of memories into in the neocortex (Corkin, 1984; Milner *et al*., 1998; Squire and Alvarez, 1995). Therefore, stress or chemical substances can damage the hippocampus.

In Nigeria, *Daturastramonium* is intentionally abused by the youth for recreational purposes or accidentally ingested as food contaminant following the harvest of farm produce. This leads to several cases of medical emergencies and health problems which sometimes results in death. Despite the widely reported toxicity of the plant, literature is scanty on the role of repeated abuse of the toxic phytoconstituents such as hyoscyamine fraction of *D. stramonium* on the hippocampus. Hence, the present study aimed to evaluate the effects of hyoscyamine fraction of *D.stramonium* seeds on the hippocampus of in adult Wistar rats. The study may serve as a tool to strengthen the existing legislation governing the prohibition of drugs and substances abuse.

MATERIALS AND METHODS

Plant Materials, Extraction and Fractionation

Ethical approval was obtained from the Ahmadu Bello University Committee on Animal Use and Care(ABUCAUC/2018/042). Fresh *D. stramonium*seedswere procured from Sharada residential area of Kano Municipal Local Government, Kano State, Nigeria. The seeds were identified and a voucher number (VN108) was issued at the herbarium of the Botany Department, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria. The seeds were extracted and partition-fractioned according to Kamada*et al.* (1986) and Djilani*et al*., (2006). Two thousand grams of the dried seeds were weighed using a digital weighing machine, grounded to a pulp using an electronic blender. The pulverized seeds powder was cold macerated with 70% ethanol and fractioned with a 5 ml portion of 10% sulphuric acid (H_2SO_4). The quantity of the fraction was obtained by the high-performance liquid chromatography (HPLC). All analyses were carried out at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria.

Experimental Animals and Design

Forty (40) healthy adolescent Wistar rats at postnatal day (PND) 21-42 comprised of equal genders were procured from the Animal House of the Anatomy Department, Faculty of Basic Medical Sciences, Bayero University Kano. The animals were housed and allowed to acclimatize for two weeks at ambient temperature, with alternate day and night cycles natural condition at the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria. Rat chow (*Vital feeds*®) and tap water were made available to the animal's*ad libitum*. The median lethal dose (LD_{50}) of hyoscyamine fraction was determined using Lorke's (1983) method. The animals were randomly selected and divided into four. The first group received a single daily dose of equivalent bodyweight of normal saline, while the remaining groups received 200, 400 and 800 mg/kgbwt of hyoscyamine fraction of *D. stramonium*seeds as control and treated groups respectively orally for three weeks. All the procedures carried out were in concert with the approval of Ahmadu Bello University (ABU) Zaria Animal's Right and Ethics Committee for Scientific Research Purposes.

Novel Object Recognition Test

This was to test short-term memory after administration of the hyoscyamine fraction of the *D. stramonium* seeds. The test consisted of three phases i.e. (habituation, sampling and test) which was completed in two days. For each phase of this test, the open field arena was thoroughly cleaned with an unscented bleach germicidal wipe, 70% Ethanol followed by distilled water before initial use. A day before object exposure, the rats were habituated to the open field arena in a 50 x 50 cm wooden box. Before habituation session, a SONY® (Model DCR – PJ5E) digital and a video camera was used for a proper video covering of the rats in the maze. A rat at a time was gently removed from the home cage and placed in the centre of the arena. The video covering system was turned on and the rat was allowed to freely explore the arena for 10 minutes. At the end of every session, the arena was thoroughly sanitized before the next session began. This was repeated for all the rats until all got habituated the arena. The same protocol was observed during the sampling and test phases only that, two identical objects (A_1 and A_2) were used and two unidentical objects (A and B) objects were used for 15 minutes respectively.

Morris Water Maze

The aim was to test spatial learning and memory after administration of the hyoscyamine fraction of the *D. stramonium* seeds. This was carried out for six consecutive days using a modified Morris method (1984). The apparatus consisted of a circular Aluminium tank of 100 cm diameter and 60 cm depth with an escape platform of 20 cm long and 12 cm diameter, filled with a pool of clean water of about two-third of the tank at 22 – 25^{o}C, deep enough to expose 2.54 cm (1 inch) of the platform above the water surface. A digital video device, SONY® (Model DCR – PJ5E) was suspended directly over the pool to capture the entire setup. The rats were trained for 5 days with methylene-blue coloured water that submerged the platform about 1 inch under. A latency period of 60 sec was allowed for each to find the platform. This was repeated for all the rats at five different locations by changing the positions of the platform in the pool within the N, E, S, and W directions following Qing *et al.*(2008) protocol. The same protocol was observed during the test day 6, however, 30 seconds for trial and the escape platform was removed. The time taken for each rat to identify the usual position of the platform was recorded and while all videos recorded for the trials were analyzed for the escape latency.

Animal Sacrifice and Tissue Preparation

The animals were euthanized using 75% Ketamine (10 mg/ml USP) anaesthesia, the brains were dissected, removed and fixed in Bouin's fluid. The tissues were processed in the Department of Pathology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika, Zaria, Kaduna state, Nigeria. The brain tissues were dehydrated in different grades of alcohol and cleared in xylene using an automatic processing machine (Shandon Southern Duplex Processor). The tissues were then infiltrated with paraffin wax and blocked in the coronal plane. Serial sections of the blocks were taken at 8 µm with a (LeitzWetzlar) microtome, mounted on glass slides and allowed to dry overnight. The staining technique employed was hematoxylin and eosin in paraffin sections (Lillie and Fullmer, 1976). Sections were then observed under a light Olympus Binocular Microscope (Ch-20i, Uttar Pradesh, India) high magnifications (x 40) and micrographs were taken with the help of Celestron® eyepiece digital camera (EC 3.0 MP, China). Coronal sections of the hippocampus were observed in the treated rats and compared to the controls.

Statistical Analyses

The data were expressed as mean ± SEM. Repeated measures ANOVA followed with Fisher's multiple comparisons post-hoc was carried out to find the mean differences in the escape latency, exploration, discrimination and novelty preference time between groups using *Minitab* 17 (LLC., U.K.) statistical package software. $P< 0.05$ was considered statistically significant. All figures and charts were constructed using *GraphPad* Prism 8.

RESULTS

No mortality symptoms were observed in the first phase when the animals received 10, 100 and 1,000 mg/kgbwt. However, muscarinic symptoms such as restlessness (hyperactivity), laboured breathing, piloerection, abdominal cramps, stooling (diarrhoea), and urination, were observed especially at the 1,000 mg/kgbwt. The symptoms later disappeared and the animals became calm, weak and quiet. During the second phase, the symptoms persisted with high intensity in all the groups treated with 1,600, 2,900 and 5,000mg/kgbwt. Neurotoxicity symptoms were observed but, no mortality was recorded even at the highest dose. The *D. stramonium*fraction was therefore considered safe and 5000 mg/kgbwt was taken as the LD_{50}.

Figure 1 comparison for the test – phase in novel object recognition test experiment between the control and 200, 400, and 800 mg/kgbwt adult treated Wistar rats' groups using two non-identical objects, familiar object (A) and non-familiar object (B) There was a significant difference in the exploration time between the group [F (3, 40): = 9.80, p = 0.003]. The control group spent statistically significant time (p = 0.031) exploring the unknown object (B) compared to the known object (A) The exploration time between the two objects for all the treated groups was, however, not significant ($p > 0.05$): For the low dose treated group p = 0.191, medium dose, p = 0.406 and for the high dose, p = 0.071 respectively.

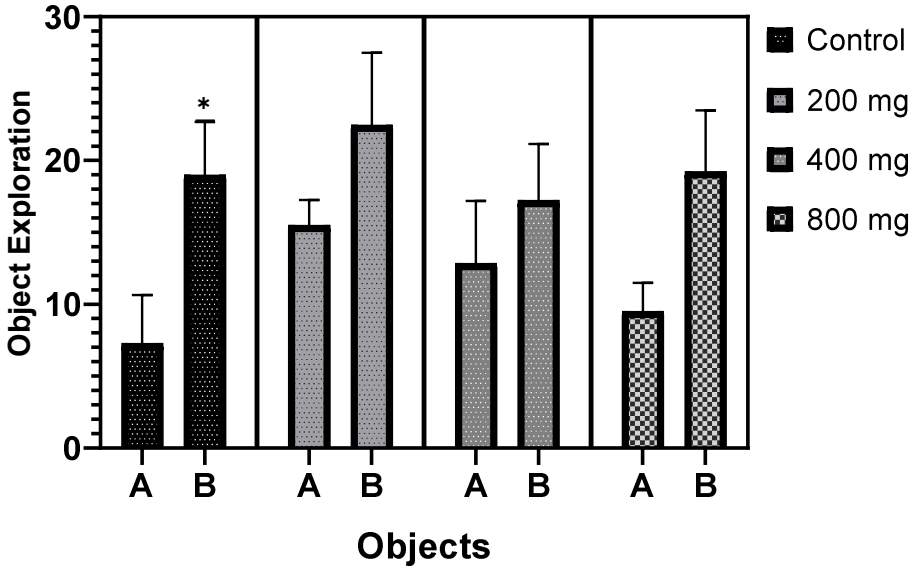

Figure 1. The test phase of Novel object recognition in Adult Wistar rats (A= familiar object, B= Novel object):

Figure 2 shows the trend of escape latencies using repeated-measures ANOVA with Fisher's pair-wise comparisons post-hoc test for the spatial learning and memory test between the control, 200, 400 and 800 mg/kgbwthyoscyamine fraction treated adult Wistar rat groups. There was a statistically significant difference between the groups [F (3, 120) = 3.01, p = 0.033] and between days [5 (5, 120) = 14.80, p< 0.001] for the group treated with 200 mg/kgbwt. The post-hoc test shows that in the first three days of the five days training, control groups showed consistent shorter duration of escape latencies which were statistically not significant (p > 0.05) when compared to the treated groups in the same days. Conversely, the escape latencies of the fourth and fifth days among the treated groups were slightly shorter, however, not significant statistically (p > 0.05) when compared with their respective controls. On the sixth day which was the test – day, escape latency of the control was significantly lower statistically (p = 0.008) when compared to the treated groups. For the group treated with 400 mg/kgbwt also, a statistically significant difference between the groups [F (3, 120) = 3.01, p = 0.033] and days [5 (5, 120) = 14.80, p< 0.001] was observed. There was a consistent flow pattern of escape latencies between the two groups with the controls taking the leads in the first five training days. Although the difference in the latency time was prominent in the fourth day, however, no statistically significant (p = 0.628) difference was observed between the groups. It took the treated group a longer duration in the sixth day to arrive the platform (p = 0.023) when compared to the control, hence statistically significant. Finally, the figure also showed a comparison of 800 mg/kgbwthyoscyamine fraction treated adult Wistar rats. There was statistically significant difference between the treated groups [F (3, 120) = 3.01, p = 0.033] and between days [5 (5, 120) = 14.80, p< 0.001]. The control group took lesser escape latency time during the first and third training days compared to the control. Although the difference in the escape latency time was prominent on the third day, however, no statistically significant difference (p> 0.05) was observed between the groups. The control group showed higher escape latency on the fifth day but lower and statistically significant escape time (p = 0.041) on the sixth day.

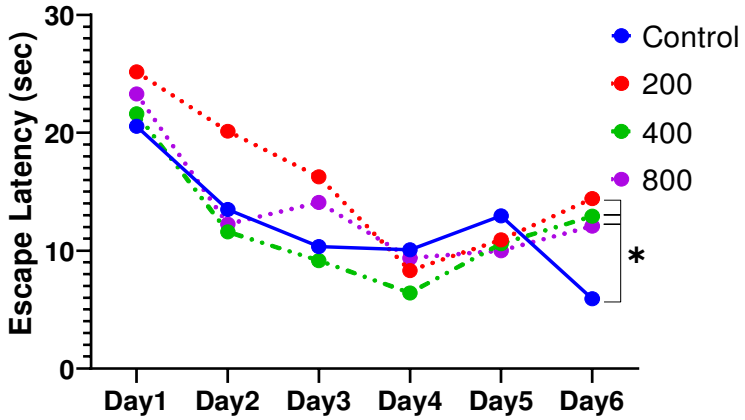

Figure 2. Morris water maze test in Wistar rats treated with hyoscyamine at adulthood

Plate 1 showed a photomicrograph of CA3 region of the hippocampus in control and treated groups of adult Wistar rats at 12th week after being treated orally with an equivalent bodyweight of normal saline (a), 200 (b), 400 (c) and 800 (d) mg/kgbwt of hyoscyamine fraction of *D.stramonium* respectively for three weeks, from PND 56 – 77. Plate Ia showed normal histology of the CA3 region featuring SO, SR and PC in the region with traces of mossy fibres within the neuropil. In plate Ib, which received the least dose, scanty, hypochromic, pyknotic (black arrowheads) cytoplasmic vacuolation (black asterisk) and necrotic (black arrowheads) pyramidal cells were observed. The CA3 in plate Ic regions of the group treated with moderate-dose a scanty, hypochromic, cytoplasmic vacuolation (black asterisk) and necrotic (black arrowheads) cells. For the group that received the highest dose, plate Id, also scanty, hypochromic, hypertrophied pyramidal cells (black arrows): cytoplasmic vacuolation (black asterisk) and necrotic (black arrowheads) cells were also observed.

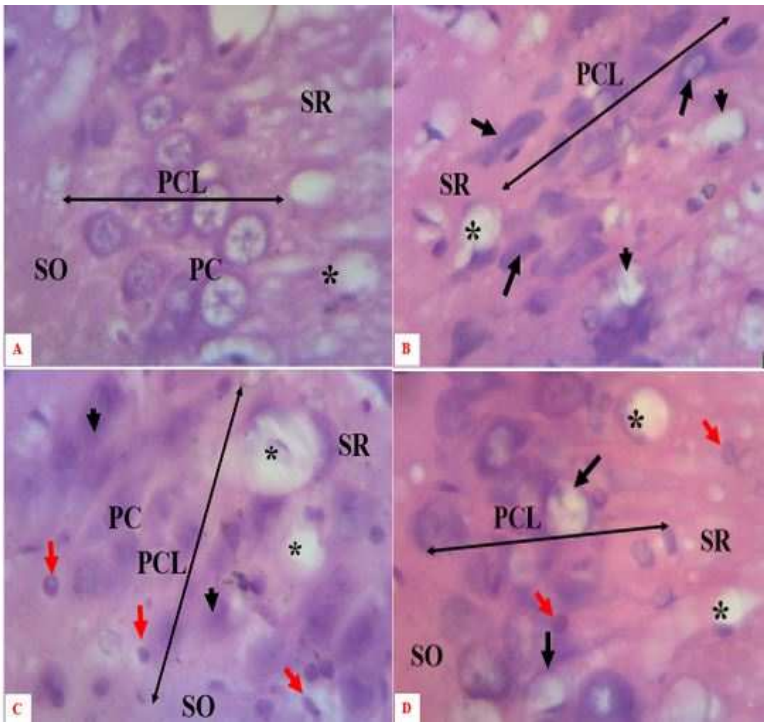

Plate 1. Photomicrographs of Cornu Ammonis (CA3) of the hippocampus in adult Wistar rat at 12th week after being treated with an equivalent bodyweight of normal saline (**A**), 200 (**B**), 400 (**C**) and 800 (**D**) mg/kgbwt hyoscyamine fraction of *D. stramonium* L. seeds from the PND 56 – 77 (H&E, x400). **PCL** = pyramidal cell layer, SO = stratum oriens, **SR** = stratum radiatum, , **PC** = pyramidal cells, * = cytoplasmic vacuolation. **SLM** = stratum lacunosum moleculare, ⟶ = necrotic neuronal cells, ◀ = pyknotic neuronal cells, ➔ = oligodendrocytes.

DISCUSSION

The toxicity of *Daturastramonium* is attributed to present of tropane alkaloids obtainable in all its parts especially the seeds. The commonest alkaloids include; hyoscine, atropine, scopolamine and hyoscyamine (Arnett, 1995). Ingestion of hyoscyamine fraction resulted in both central and peripheral muscarinic symptoms in the rats fed with graded doses of the fraction. However, no mortality was recorded as a result of ingestion both during toxicity testing and experiment itself. In a related study by Babalola*et al.* (2014) reported that the median toxic dose of *D. stramonium*fed orally in dogs was at the safety margin as considered Centre for Disease Control (CDC) the United State of America, states. However, there was no published literature to make a comparison with regard to the present study in Wistar rats. Considering the foregoing it could be assumed that oral ingestion of*D. stramonium* seeds might equally have high safety margin in Wistar rats. The clinical symptoms observed might probably not to be unconnected with the anticholinergic properties of tropane alkaloids which competes and irreversibly inhibits acetylcholine on muscarinic receptors, thereby causing both central and peripheral nervous system manifestations (Hanna *et al.,* 1992). The central nervous system features include restlessness (hyperactivity), laboured breathing and delirium, while the peripheral symptoms observed include breathing, piloerection, abdominal cramps, stooling (diarrhoea), and urination. Similar observations were reported in patients involved in *D. stramonium*poisoning (Ramirez *et al.,* 1999).

The curent study observed a statistically significant difference in the exploration time across the groups when compared to the control except for the group that received the highest dose. Both discrimination index and novelty preference showed a statistically significant decrease in the affinity to the novel object in the treated group when compared to control, surprisingly in a slight J-shaped response curve. Also, a statistically significant decrease in the Morris water maze task was equally observed in the cognitive function test. This has indicated a memory loss, a neurodegenerative symptom similarly viewed (Langa*et al*., 2004; Schneider *et al*., 2007). Studies have shown that *D. stramonium* contains tropane alkaloids which induce hallucinations and metabolic disorder in rats (Abubakar*et al.,* 2010; Damilare*et al.,* 2010; Richard *et al.,* 2011; Tijani *et al.,* 2012) and also implicated in the disorders of hippocampal development in rats (Ishola and Adeniyi, 2013). Atropine, tropane alkaloid of similar action was reported to cause a significant deficit in recognition memory in intraperitoneally treated rats when compared with the control from the novel object recognition result (Olawepo*et al*., 2017). This was also supported by the fact that *D.stramonium* has been documented to cause permanent short-term memory loss, and impairs learning because it contains a compound known as gamma-l-glutamyl-l-aspartate (Schmitz-Bourgeois *et al*., 1988). Studies have also reported impairment in short-term memory, disorientation, confusion, hallucinations, psychosis, agitated delirium, seizures, coma, respiratory failure and cardiovascular collapse (Alberto *et al*., 2001). following accidental poisoning or intentional ingestionof*D.stramonium*. The slight J-shaped response curve observed has indicated that ingestion of low-dose of hyoscyamine fraction of *D.stramonium* could equally impair memory as or even more than the higher dose repeatedly over time. This could be attributed to so many factors such as the presence of phenolic compounds (Nweke*et al.,* 2015), perfluorinated carboxylic acids (Mulkiewicz*et al.,* 2007), mycotoxins (Wang *et al.,* 2014), bacteriocins (Murado and Vázquez, 2010), antibiotics (Migliore*et al.,* 2013), herbicides (Nweke*et al.,* 2016), heavy metals (Shen *et al.,* 2009) and ionic liquids (Wang *et al.,* 2011): either as an individual or as mixtures. Similarly, the poor response in the higher dose observed could probably result from desensitization of the muscarinic receptors, thus leading to addiction.

We observe a relatively variable degrees of neuronal lesion ranging from neuronal hyperchromasia, pyknosis, necrosis, slight hypertrophy and cytoplasmic vacuolation when compared to the control group. This has indicated that oral ingestion of hyoscyamine fraction of *D. stramonium* has a profound toxic effect that could lead to neurodegeneration on the hippocampus of the Wistar rats. This was in line with Ekanem*et al*. (2016), which reported that oral ingestion of *D. stramonium* extract caused atrophy of the axons and fibres, vacuolation, cell necrosis and cell losses. Bihaqi*et al*. (2012) also observed neuronal loss, ghost cells, haemorrhage and vacuolated cytoplasm on treated rats that received daily intraperitoneal administration of scopolamine in the histology of the cerebral cortex examined under a light microscope. Although the cause of these changes is unknown, however, evidence can be supported by the fact that various mediators can contribute to excitotoxin action, which include the production of reactive oxygen

intermediates, nitric oxide, p53 and cytokines which could lead to a series of cascading events leading to cell losses in the hippocampus (Coyle and Puttfarcken, 1993; Epstein *et al*., 1994; Ankarcrona*et al*., 1995; Morrison *et al*., 1996). *D. stramonium* tropane alkaloids all have a long duration of effect, cross the blood-brain barrier and have central anticholinergic effects (Bania*et al*., 2004): which induce strong hypnosis and can also induce neuronal degeneration (Hughes and Clark, 1939).

CONCLUSION

In conclusion, oral ingestion of hyoscyamine fraction of *D. stramonium* at adulthood caused alteration in the histoarchitectural patterns of the cornuammonis (CA3) region of hippocampus neurons, which impaired cognitive learning and spatial memory. It is therefore recommended that repeated ingestion of hyoscyamine fraction of *D. stramonium* should be avoided.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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APPENDIX – I

AHMADU BELLO UNIVERSITY, ZARIA
DIRECTORATE OF ACADEMIC PLANNING & MONITORING
Vice Chancellor
Director:
Tel: Zaria +
Fax:
Telegram:
e-mail:

Appl No.: ABUCAUC/2018/Human Anatomy/042
Approval No: ABUCAUC/2018/042

3rd October, 2018

Dr. S. A. Musa
Department of Human Anatomy,
Faculty of Basic Medical Sciences,
College of Health Sciences,
Ahmadu Bello University,
Zaria.

Sir,

APPROVAL OF RESEARCH STUDY 'EVALUATION OF INGESTION OF HYOSCYAMINE FRACTIONATE OF DATURA STRAMONIUM SEEDS ON CEREBRUM DEVELOPMENT IN WISTAR RATS *(RATTUS NORVEGICUS)*

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Anatomy. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

U.D. Abdullahi
For: Chairman, ABUCAUC.

Cc. Director, DAPM
Director, IC &ICT
Provost, College of Health Sciences
Dean, Faculty of Basic Medical Sciences,
HOD, Human Anatomy,
Prof. Aliyu Mohammed