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Stereological Estimation of *Mus musculus* (Mice) Hippocampal Volumes in Alcohol and *Cannabis sativa* (Marijuana) Co-Administration

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ABSTRACT

Substance abuse especially amongst young adults constitutes a global problem, with some studies indicating volumetric reductions in neuroanatomical structures among these abusers. In the present study, 24 apparently healthy mice were divided into 4 groups (n=6): Group one was administered 1 ml/kg body weight (bwt) of distilled water; group two had 145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa*; group three had 20% v/kg bwt of alcohol and group four had 145 mg/kg bwt of ethyl acetate fraction of *Cannabis sativa* and 20% v/kg bwt of alcohol for 21 days with feeding. Neurobehavioural assessment was done via Y-maze and Novel Object Recognition (NOR) paradigms. Hippocampi were fixed in Bouin's fluid and isotropic uniform random samples were obtained via orientator method. Serial sections were systematically obtained, processed and stained with haematoxylin and eosin. Hippocampal volumes were estimated using the Cavalieri estimator and data were expressed as mean±SEM. One way analysis of variance and Kruskal Wallis tests were used to compare the means at $p<0.05$. Y-maze result showed a significant increase ($p<0.05$) in the mean rank, while NOR result showed a significant decrease ($p<0.05$) in discrimination and retention indexes in the co-administered group. However, hippocampal volume estimation showed no significant difference ($p>0.05$) in the groups. Histopathological evaluation revealed scattering, disorganization and focal necrosis of neural cells of the hippocampus in the co-administered group. In conclusion, the combined use of *Cannabis sativa* and alcohol can over-time lead to detrimental effects in the function, structure and volume of the hippocampus in users.

Key words: *Cannabis sativa, Alcohol, Volume Estimation, Hippocampus*

INTRODUCTION

The World Drug Report (2014) had scored Nigeria higher than any other African country in terms of drug seizure, especially *Cannabis* and its use is increasing especially amongst the youths and university students (Owoaje and Bello 2010). Amresh et al. (2011) reported that alcohol and *Cannabis* are the most popularly abused substances amongst adolescents. During adolescence, the brain undergoes substantial developmental changes as it transits into adulthood and therefore the abuse of these type of substances can alter neural structure and function.

The main psychoactive constituent of *Cannabis sativa* is tetra-hydro-cannabinol (THC). Some of its effects are neurotoxic and cause impaired short-term

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memory, attention and even reduced motor skills (Chan et al. 1998; Bartholomew 2011; Rochetti et al. 2013). *Cannabis sativa* functions by interacting with specific endogenous receptors, cannabinoid receptors 1 and 2 (CB1 and CB2) (AIHW 2002). These receptors normally modulate neuronal activity by affecting second messenger and ion transport systems. CB1 receptors are found in the hippocampus, cerebral cortex, limbic areas, basal ganglia, cerebellum and thalamic areas (Glass et al. 1997; AIHW 2002). The hippocampus may be particularly vulnerable to the effects of cannabinoid exposure (Ranganathan and D'Souza 2006; Lorenzetti et al. 2013). A post-mortem study in human brains has shown that reduction in CB1 receptor density in the caudate nucleus, putamen, mesencephalon and hippocampus was associated with chronic abuse of *Cannabis* (Villares 2007). Tapert et al. (2002b) showed that chronic abuse of marijuana is associated with poorer attention function. Likewise, Tait et al. (2011) found that heavy marijuana use in adolescents over an 8 year period was associated with decrement in verbal memory. Similarly, others have reported cognitive deficits in adolescents that regularly abuse *Cannabis* (Tapert et al. 2002a; Harvey et al. 2007; Solowij and Battisti 2008; Mathias et al. 2011; Tait et al. 2011). Heather (2001) and Hall and Degenhard (2009) both reported that *Cannabis* impairs cognitive and psychomotor performance in adolescents. Hippocampal volumetric reductions have been reported in *Cannabis* users (Lorenzetti et al. 2013; Rochetti et al. 2013), as such the neurobehavioural anomalies that accompany *Cannabis* users. However, Koenders et al. (2016) did not find any significant change in hippocampal volumes of heavy *Cannabis* users after considering the dosage and onset of *Cannabis* use.

Alcohol is a multiple action depressor of the central nervous system and the depression caused by it is dose-dependent. Ingestion of exceeding doses can disrupt the integrative control of the cerebral cortex and under its influence, confused and disorganized thinking results, even motor coordination may be disrupted (Martin et al. 2003). Alcohol has the ability to alter human consciousness and act as a 'gateway' drug to the use of other abused substances like hallucinogens and *Cannabis* (Makanjoula et al. 2014). At higher doses, alcohol significantly inhibits neuronal activity in the Cornu Ammonis (CA1 and CA3) regions of the hippocampus impairing memory and memory encoding (Ryabinin et. al. 1997; Weiner and Dunwiddie 1997; Ryabinin 1998; White 2003; Rose and Grant, 2010). However, even after such neurobehavioural anomaly reports, studies on the effect of heavy alcohol drinking on hippocampal volume is still a subject of controversy. Some reports in the literatures point to a volumetric reductions in alcohol-dependent subjects (Bleich et al. 2003a), others have disputed such claims (Bleich et al. 2003b) and yet, others are less conclusive (Agartz et al. 1999).

The study therefore proposed that, both substances, when used singly or together may have a detrimental effect on the hippocampus in adolescents by hampering the normal developmental increase (Goddings et al. 2014) in hippocampal volume but users may not be aware of this.

MATERIALS AND METHODS

Substance Procurement, Extraction and Fractionation

Leaves of *Cannabis sativa* (1 kg) were obtained from the National Drug Law Enforcement Agency, Kaduna State Command (Ref. No. NDLEA/KDSC/CUN/94/Vol.II/8). The leaves were authenticated at the Department of Botany, Ahmadu Bello University, Zaria and assigned a voucher number (2438). 20% v/v was prepared from absolute alcohol manufactured by May and Baker™, Poole-England (Nagy 2008).

Methanolic extraction of leaves of *Cannabis sativa* was done with the aid of a Soxhlet apparatus. Fractionation of *Cannabis sativa* leaves was done in the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The fractionation process was carried out by passing the methanolic extract through different solvents: N-hexane (non-polar), N-butanol (polar), Ethyl acetate (moderately polar) and aqueous (polar). The idea behind the different solvents with different polarity is to separate the different compounds in the *Cannabis*. THC was measured in each fraction via a gas chromatography mass spectrometer (GC-MS) technique and ethyl acetate fraction had the highest concentration of ∆-9-tetra-hydro-cannabinol (THC) and was therefore used for the study.

Animal Procurement, Handling and Experimental Design

The use of the animals in the research was approved by the Animal Use and Care Committee (No. ABUCAUC/2015/01), and animals were handled as stipulated by the guidelines for the use of animals for scientific research purposes.

Twenty four apparently healthy 56 days old mice and weighing between 20-28 g were procured from the Animal Resource Center in the Department of Pharmacology, Ahmadu Bello University, Zaria, and then transferred to the Laboratory Animal Holding Facility in the Department of Human Physiology, Ahmadu Bello University. Animals were allowed to acclimatize for two weeks before the commencement of the study and were fed with water and animal feed (growers mash) *ad libitum*. Animals were randomly divided into 4 groups with 6 mice per group. A control group was administered 1 ml/kg bwt of distilled water; a second group (*Cannabis* group) was administered

145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa*; a third group (alcohol group) was administered 20% v/kg bwt of alcohol, while the last group was co-administered 145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa* and 20% v/kg bwt of alcohol for 21 days via oral gavage.

Neurobehavioural Paradigms Assessment

Y-maze

The Y-maze discriminates learning, spatial reference memory and spatial working memory, which are related to the hippocampus and prefrontal brain regions in rodents (Xu et al. 2013). The evaluation of spontaneous alternation was used to investigate short term spatial working memory in mice.

The Y-maze apparatus was made of black polyvinyl chloride (PVC), and consisted of three equal arms (length 50cm, height 20cm and width 10cm), interconnected at 120 degrees. The arms were labelled A, B and C. The mice were first pre-trained in the Y-maze apparatus followed by the actual test. The test measured working memory in a mouse by scoring the number of alternations the mouse spent in Y-maze when it enters all three arms without going into the same arm twice in a row. Each mouse was allowed to explore the apparatus for 5 minutes and then the apparatus was cleaned with 5% ethanol before the next mouse was introduced. The sequence of arm entries was manually recorded. An alternation was defined as entry into all three arms consecutively (i.e. ACB, ACB, BAC, etc). A mouse was considered inside a specific arm when it had all the four paws inside that arm. The number of maximum spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation was calculated as (actual alternations/maximum alternations) x 100). For instance, if a mouse performed CABBCACBACCBACB, the number of arm entries would be 15 - 2 = 13, and spontaneous alternations: CAB, BCA, ACB, CBA, BAC, CBA, BAC, ACB. Therefore the percentage alternation was calculated as [8/(13)]x100= 61.5%] (Olakunle et al. 2012).

The Novel Object Recognition Test (NORT)

The NOR task evaluates the rodent's ability to recognize a novel object in the environment. This test accesses the natural preference for novel objects displayed by rodents (Antunes and Biala 2012). The test was conducted in the open field box (72×72 cm). All animal test was conducted under dim lighting conditions via a 60-Watt red light bulb. The task procedure consisted of three phases; habituation, familiarization and test phase. In the habituation phase, the animals were habituated by allowing each animal explore freely the open field arena in the absence of objects. The animal was then removed from the arena and placed in the holding cage. During the familiarization phase, a single animal was placed in the open-field arena containing two identical sample objects (A+A), for 5 minutes. To prevent coercion to explore the objects, the animal was released against the center of the opposite wall with its back to the objects. After a retention interval, during the test phase (60 seconds), the animal was returned to the open-field arena with the two objects, one was identical to the sample and the other is novel (A+B) for 5 minuites. The new object was similar in size but different to the familiar object in order to reduce preference for either object. All objects and the apparatus were cleaned using 70% alcohol to eliminate olfactory stimuli (Ennaceur 2010).

Discriminatory Index (DI)

This index quantifies discrimination between the novel object and familiar objects. It was computed as follows:

$$\mathbf{DI = T_n - T_f \div T_n + T_f}$$

Where $\mathbf{T_n}$ = Time(s) spent with new object, $\mathbf{T_f}$ = Time(s) spent with familiar object.

Recognition Index (RI)

This is the time spent exploring the novel object relative to the total time spent exploring both objects. It is the main index of retention. It was calculated thus:

$$\mathbf{RI = T_n \div T_n + T_f}$$

Where T_n and T_f are same as above.

After the neurobehavioural paradigms, the hippocampus tissues were collected after anaesthesia with ketamine hydrochloride (0.5 ml/kg bwt), decapitation and dissection. Tissues were fixed in bouin's fluid.

Sampling and Tissue Processing

Isotropic uniform random samples of the tissues were obtained via the orientator method (Ali et al. 2012). Tissue samples were then routinely processed for histological analysis via dehydration in graded changes of alcohol, then cleared in xylene, infiltrated and embedded in paraffin wax. Serial sections (coronal) were cut with a rotatory microtome (LEICA®) at 10µ. A random number 5 was selected from the random number table and 10 sections were randomly picked (1, 6, 11, 16, 21, 26, 31, 36, 41, 46) from each group and floated out in warm water bath, mounted on charged slides, left to air dry and stained with haematoxylin and eosin. The remaining sections were also stained with haematoxylin, bluing was done in tap water, differentiated in 70% ethanol and then stained with eosin. Sections were washed and used for histological analysis of the hippocampus.

Photomicrographs were taken with a light microscope (Leitz Wetzlar, Germany) and a digital microscope camera (DCM 510 pixel, ScopePhoto® China) at ×40 and ×250.

Stereological Analysis

A test point counting grid (Cavalieri estimator) was superimposed on the hippocampus tissue sections and single test points hitting the hippocampus areas (CA1, CA2, CA3, and subiculum) were counted and summed. The volume changes of the hippocampus were calculated as previously described by Gundersen et al. (1998) by the following imputations:

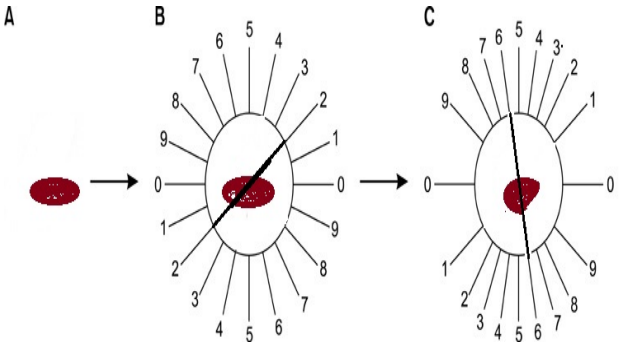

Figure 1: The Orientator Grid. This grid was used to obtain isotropic uniform random samples of the hippocampus. First, the tissues were placed in a circle with equal divisions, a random number 2 was selected from the random number table and the tissues were cut with a sharp blade along this axis; Secondly, the cut tissues were again placed in a second circle with unequal divisions and another random number 6 was selected from the random number table and tissues were again cut at this axis and then processed.

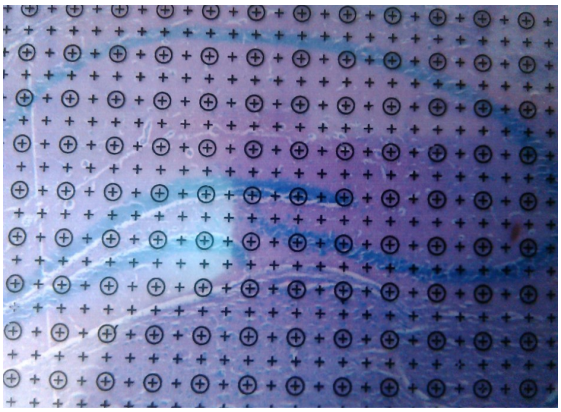

Figure 2: The Test-Point Counting Grid (Cavalieri Estimator). The grid was superimposed on a section of the hippocampus after staining with H&E and single test points hitting the hippocampus were analysed.

V (mm^3) = $\bar{T}$ x a/p x $\sum$Pi ($\bar{T}$= distance from the 1st section to the 46th section = 50mm; a/p = area per point on the counting = $1mm^2$ (already standardized on the grid).; $\sum$Pi = sum of test points; Pi=test points hitting the section), (Figure 1 and 2).

Noise due to Errors in the Sampling

Noise = 0.0724 x B/$\sqrt{A}$ x $\sqrt{}$ n x $\sum$Pi ($\sum$Pi = sum of test points; Pi=test points hitting the section).

Variations due to the systematic random sampling of the serial sections was calculated:

VAR_{SURS} = 3 (A-Noise) – 4(B + C) + C (A=$\sum$ Pi x Pi; B=$\sum$ Pi x Pi+1; C= $\sum$ Pi x Pi+2)

Total variance (TVAR) = Noise + VAR_{SURS}

Coefficient of error due to the entire sampling process (CE) was calculated: CE= $\sqrt{}$TVAR/$\sum$Pi.

Statistical Analysis

Data was expressed as mean ± SEM and one way analysis of variance was used to compare the means at $p < 0.05$. SPSS (version 20) was used for the statistical analysis.

RESULTS

Neurobehavioural Results

Figure 3 showed the results of the mean ranks, discrimination and recognition indexes from the Y-maze and novel object recognition tests (NORT) assessments. The mean ranking which is a measure of percentage alternation from the Y-maze assessment showed a statistical significant increase ($p < 0.05$) in the co-administered and *Cannabis* administered groups compared to the control. An increase in mean ranks of the percentage alternation signifies memory impairment. Discrimination index (DI) result from the NORT assessment showed a statistically significant with the substances compared to the control. The decrease signified impairment in discrimination between the novel and familiar objects. While, the result of the recognition index (RI) NORT assessment showed a statistically significant decrease ($p < 0.05$) in the group treated with coadministration of the abused substances compared to the control. This signified impairment in the measure of the novel object recognition.

Histopathological Evaluation Results

Figure 4: Showed the plates of photomicrographs of the hippocampus from mice in the control (A) and substance-administered groups (B, C, D). A represents the normal cyto-architecture of the hippocampus from the control group. The neural cells are seen to be closely arranged and in a row. Photomicrographs B and C represent hippocampus tissues from the *Cannabis* and alcohol administered groups. No significant histopathological changes were seen. However, photomicrograph D is from the co-administered group. The neural cells in the CA2 region and subiculum appeared scattered and disorganized. Focal necrosis of some neural cells was also observed in photomicrographs E and F from the co-administered group at a higher magnification.

Volume Estimation Results

Table 1 showed the calculated estimated volumes of the hippocampus in the control and substance-fed

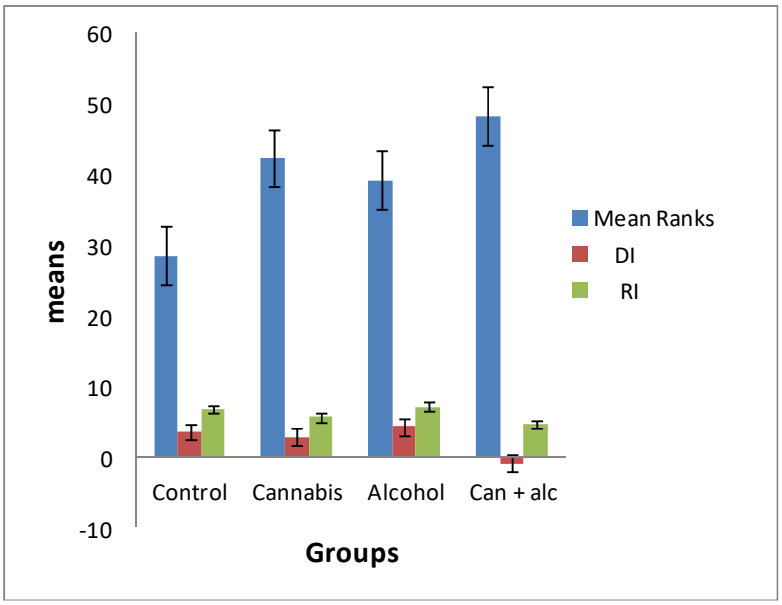

Figure 3: Mean Ranks and Means of Discrimination, Recognition Indexes in Mice administered 1 ml/kg bwt distilled water (Control), 145 mg/kg bwt ethyl acetate fraction of *Cannabis sativa*, 20% v/kg bwt of alcohol and 145 mg/kg bwt of ethyl acetate fraction of *Cannabis sativa* plus 20% v/kg bwt of alcohol for 21 days. * indicate statistical significant difference compared to the control.

mice. The results showed decrease in the hippocampal volumes of the substance-fed mice when compared to the control. However, these volume changes were not statistically significant ($p > 0.05$).

DISCUSSION

This study investigated the impact of *Cannabis sativa* (marijuana) and alcohol co-administration on the structure of the hippocampus. From the findings, we observed that after 21-days exposure of the mice to the substances of abuse there was a significant increase ($p < 0.05$) in the mean ranks of the percentage alternation from the Y-maze assessment in the combined and *Cannabis* administered groups compared to the control. This increase in mean rank signified memory impairment in the affected mice. This could be due to the fact that delta-9 THC acts via the endocannabinoid receptor 1(CB1), which is densely concentrated at the hippocampus. Disruption of the structure of this CB1 receptor can decrease glutamate release at the synapse and interrupt the process of long-term potentiation, which ultimately impaired memory. Similarly, Young et al. (2006) reported an impairment in spatial and non-spatial memory, following administration of delta-9 THC for 21 days in Wistar rats. Later, Bartholomew (2011) and Rochetti et al. (2013) also reported impairment in memory function due to THC in abusers of *Cannabis*. Earlier, Ryabinin et al. (1997) and Ryabinin et al. (1998) reported inhibition of neuronal activity in the CA1 and CA3 regions of the hippocampus in chronic *Cannabis* misuse and abuse. Consequently, the increase in the mean rank of the percentage alternation caused by the alcohol administration was not statistically significant. Similarly, Yusuf and Youssef (2016), reported that alcohol administered alone did not cause any significant changes in the spartial memory of Wistar rats, which could have been due to alcohol tolerance in the rats after administration. The results from the second neurobehavioural paradigm assessment showed significant decreases ($p < 0.05$) in the discrimination and recognition indexes (DI and RI) for the group that was co-administered with the abused substances compared to the control. These decrease in both DI and RI indicated impairment in memory. Now, alcohol has been found to potentiate the effects of other drugs when it is co-administered (Althobaiti and Sari 2016; Yusuf and Youssef 2016). Alcohol metabolism might have increased the blood concentration of delta-9 tetrahydrocannabinol, thereby potentiating the effects of *Cannabis* on memory impairment. This is in line with the work of Johannes et al. (2011) who reported greater impairment on memory when alcohol was co-administered with *Cannabis sativa*. However, it is in contrast to the work carried out by Ortiz et al. (2004) who reported enhancement in memory when alcohol was co-administered with *Cannabis sativa*.

Our results from the hippocampal volume estimations showed decreases in the volumes of hippocampus. The co-administered group had the highest decrease in hippocampal volume followed by the *Cannabis* administered group and lastly, the alcohol administered group, even though the volume changes were not statistically significant ($p > 0.05$). Similarly, Bleich et al. (2003a), Lorenzetti et al. (2013) and Rochetti et al. (2013), all reported reductions in hippocampal volumes in heavy *Cannabis* and alcohol misuse and/or abuse. Such a decrease in volume may reflect loss of hippocampal cells that could impair cognitive performance. While still others have reported no reduction in the volumes of such a neuroanatomical structure after studying the onset and dosage of these substances abused (Bleich et al. 2003b; Ashtari et al. 2011; Demirakca et al., 2011; Koenders et al. 2016).

Visual observations of the hippocampus sections revealed scattering and disorganization in the arrangement of the neural cells of the hippocampus. Focal necrosis of some neural cells was also observed. This may reflect the decrease in the hippocampal volumes as seen in our volume estimations from the different substance administered and co-administered groups.

CONCLUSION

In this study the impact of *Cannabis sativa* and alcohol abuse on the structural integrity of the hippocampus was investigated and the results showed that the combined use of *Cannabis* and alcohol caused impairment in memory, decreased in hippocampal volume with consequent disorganization of the normal arrangement and focal necrosis of neural cells of the hippocampus in abusers (mice). This may over-time have detrimental effect on function of the hippocampus in users especially young adults.

Acknowledgement
Our gratitude goes to Dr. Ibrahim Abdullahi Iliya for his technical contributions during the methodology for the stereological estimation of the hippocampal volumes.

Conflict of Interest
None declared.

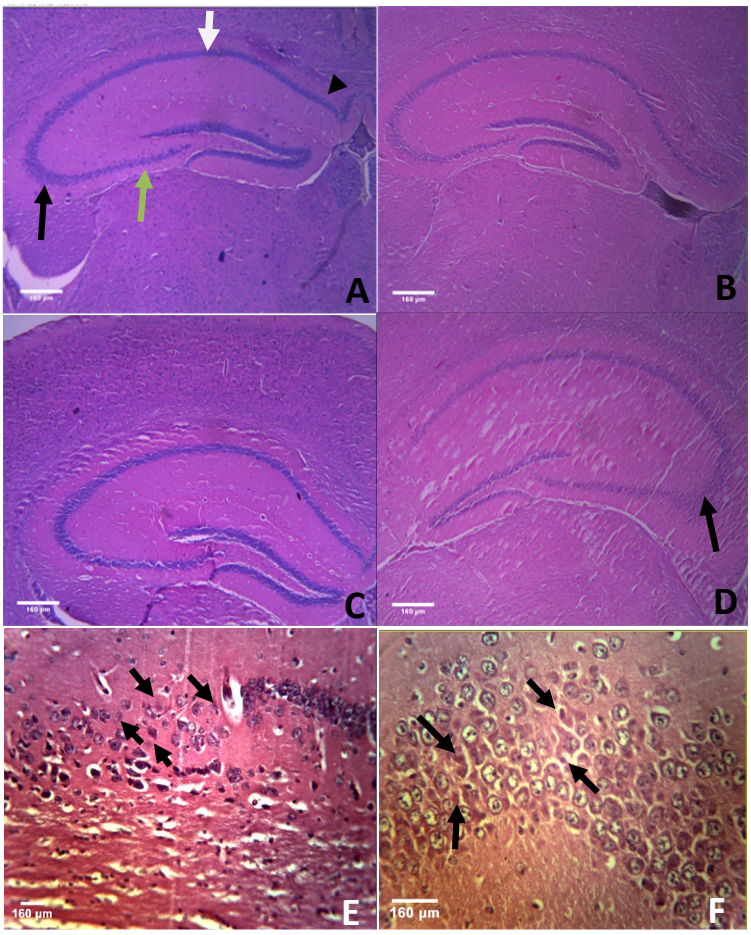

Figure 4: Photomicrographs of hippocampus from the different treated groups. A. The normal cyto-architectural arrangement of cells in the different regions of the hippocampus (white arrow-CA1 region; black arrow-CA2 region; green arrow-CA3 region and black arrow head-subiculum). Scattering and disorganization of neural cells in subiculum (black arrow head) and CA2 region observed in Photomicrograph D. Focal necrosis of neural cells seen at higher magnification in subiculum (E) and CA2 (F) regions of hippocampus from co-administered group. Legend: Control group (A, ×40); Cannabis group (B ×40), Alcohol group (C, ×40); Co-administered group (D, ×40); Co-administered group (E & F, ×250); H & E stain.

Table 1: Volume estimation of hippocampus in normal control and substance-fed mice

Groups	Volume (mm^3)	Noise	VAR_{SURS}	TVAR	CE	Mean ± SEM	P
NC	11100	215.56	-14873.68	-14658.12	0.54	22.20±1.21	0.053
CS	10250	188.34	-13211.02	-13022.68	0.55	20.50±1.13	
ALC	10400	196.10	-13906.30	-13710.19	0.56	20.50±1.31	
COM	9750	173.28	-10989.82	-10816.55	0.53	19.50±1.33	

VAR_{SURS}= Variance of the systematic uniform random sampling, TVAR = Total variance. CE = Coefficient of error; NC-normal control; CS-*Cannabis sativa*; ALC-alcohol; COM-combined or co-administered group.

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