

Effects of Aqueous and Ethanolic Extracts of *Nicotiana tabacum* on Neurobehaviour and Hippocampus of Wistar Rats

MI Auza^{1*}, AO Ibegbu², B Danborn¹, AY Solomon³ and EE Oguche⁴

¹Department of Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences Ahmadu Bello University, Zaria, Nigeria

²Department of Anatomy, Faculty of Basic Medical Sciences, Alex Ekwueme University, Ndufu-Alikwo, Ebonyi State, Nigeria

³Department of Anatomy, Faculty of Basic Medical Science, College of Health Science, Bingham University, Karu, Nigeria

⁴Department of Anatomy, Ecwa College of Health Technology, Kagoro, Kaduna, Nigeria

***Corresponding Author:** MI Auza, Department of Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences Ahmadu Bello University, Zaria, Nigeria.

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Abstract

Nicotine, the primary component of tobacco produces reinforcing effects both in humans and animals. The aim of the present study was to investigate some of the effects of both ethanolic and aqueous extracts of tobacco on the behaviour and histomorphology of hippocampus of adult Wistar rats. Forty two (42) healthy adult Wistar rats were used for the study and, the animals were randomly divided into seven groups of six rats each with an average weight of 150g. Group 1 served as the Control and was administered distilled water (1 ml/kg bw). Groups 2, 3 and 4 received 1000 mg/kg bwt (30% LD₅₀), 500 mg/kg bwt (20% LD₅₀) and 250 mg/kg bwt (10% LD₅₀) of aqueous extract of *Nicotiana tabacum* respectively, while Groups 5, 6 and 7 received 1000 mg/kg bwt (30% LD₅₀), 500 mg/kg bwt (20% LD₅₀) and 250 mg/kg bwt (10% LD₅₀) of ethanolic extract of *Nicotiana tabacum* respectively. The rats were humanely sacrificed after 21 days of treatments and the brains excised, blotted, weighed and fixed in Bouins fluid for neurohistological study, using Haematoxylin and Eosin, and Cresyl Fast Violet methods. The result showed that there was a significant decrease in the body weight in Groups 2 (139.50 ± 69.59), 6 (145.67 ± 19.40) and 7 (135.00 ± 69.48) when compared to the Control Group (217.33 ± 15.87) (P < 0.05), average brain weight decreased in all treated Groups with Groups 2 (1.30 ± 0.64), 5 (1.42 ± 0.12) and 7 (1.30 ± 0.65) having the least decrease when compared to the control Group (1.73 ± 0.15) though the decrease was not significant. The result for MWM showed that the mean time taken by the rats to locate the platform after the administration of the extracts was increased in groups 6 (12.10 ± 11.33) and 7 (12.65 ± 8.83) when compared with the Control Group (11.69 ± 11.08) though the increase observed was not significant. Histological and histochemical examinations of Wistar rats hippocampi treated with aqueous and ethanolic revealed mild histoarchitectural distortion of the hippocampal regions CA3, neurodegenerative changes, such as, irregular arrangement of CA3 hippocampal neurones, indistinct neurones (reduced staining intensity), neuronal degeneration: degenerated pyramidal cells when compared with the Control Group. The results showed significant decreases in the body weight between tobacco administered groups compared to the Control group (p < 0.05). Therefore, the results suggested that the consumption of the aqueous and ethanolic extracts of *Nicotiana tabacum* leaves may reduce body weight gain, reduce brain weight, and may lead to some level of neurohistoarchitectural alterations which may alter the normal functions of the brain such as learning and memory which may lead to brain dysfunction, despite its "pleasant" effects.

Keywords: *Nicotiana tabacum*; Hippocampus; Nicotine; Neurohistoarchitecture; Neurobehaviour

Introduction

Smokeless tobacco is a product of tobacco without combustion or pyrolysis at the time of use. One of the forms of smokeless tobacco is the chewing and sniffing form of tobacco. Snuff is a form of tobacco that is processed to fine powder and packaged either in cans or pouches. Its user takes a “pinch”, “dip”, or “quid” and places it between the lower lip or cheek and gum and suck on it. Another route for the use of snuff, though rare is by sniffing, i.e. nasal use. This route is common among Nigerian users [1]. For thousands of years smokeless tobacco products have been in existence among different populations. Over time, these products have gained popularity throughout the world (such as Tombak in Sudan, Snus in Sweden and Khaini in India) with mass marketing of new forms sold under different brand [2-4]. Research has shown that smokeless tobacco prepared from *Nicotiana tabacum* leaves contain low amounts of nicotine (1.17%), nornicotine (0.04%), and 0.06% anabasine. International Agency for Research on Cancer (IARC) reported that moist snuff contains aliphatic and aromatic hydrocarbons, formaldehyde, ketones, alcohols, phenols, amines, amides, alkaloids, metals, radio elements such as polonium-210, uranium-235, 238 [5]. Carcinogens in tobacco, the most abundant and strongest being tobacco-specific N-nitrosamines (TSNA), such as N-nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are formed by N-nitrosation of nicotine [6]. Tobacco consumption continues to grow all over the world. The hippocampus is a major component of the brains of humans and other mammals. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation. Like the cerebral cortex, with which it is closely associated, it is a paired structure with mirror-image halves in the left and right sides of the brain. In humans and other primates, the hippocampus is located inside the medial temporal lobe, beneath cortical surface [7].

Exposure to tobacco nicotine either in smoking or smokeless forms includes: cigars, pipe tobacco, snuff, and chewing tobacco, has been reported to be associated with alteration in the normal functions of the brain and the whole nervous system [8-11]. Nicotine has been reported to be the highest and most toxic compound of aqueous extract of tobacco leaves [12-14]. Nicotine is used to other nicotine addictions [9,10]. Using a controlled amount of nicotine helps to reduce nicotine withdrawal symptoms when one attempts to quit the use of tobacco products [9,11,15]. According to data acquired from WHO, there are about 2.4 billion people in the world today that consume tobacco products either in form of snuff, chewing, smoking or snuff dipping [16]. This represents about one third of the world; population of which about 50-55% of men and less than 20% of women are estimated to be smoking globally, while 50% of men and less than 25% of women are estimated to be using smokeless tobacco globally. Annually, about 5 million deaths are attributed to tobacco smoking contributing the second leading cause of mortality among adults worldwide. This frightening data attests to the death of about three million people in the year 2007 alone [17,18], these findings and reports suggest the need for thorough experimental and clinical studies of the effects of tobacco intake on the body systems, most especially the brain. The aim of this study therefore, was to investigate some of the effects of both aqueous and ethanolic leave extracts of *Nicotiana tabacum* on the hippocampus of adult Wistar rats.

Materials and Methods

Animal

Forty two apparently healthy adult Wistar rats of both sexes were obtained from the Department of Human Anatomy Bello University, Zaria. The experiment was approved by the animal Ethical committee of Ahmadu Bello University Zaria. The animals were housed in clean environment in the animal House of the Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria and were acclimatized for 2 weeks before the commencement of the experiment.

All animals were maintained on standard animal diet and water was allowed *ad libitum*. The animals were categorized into control and treatment groups and were weighed before the commencement of the experiment, with an average weight of 150g.

Extract preparation

The leaves of *Nicotiana tabacum* were collected from Zaria town, Zaria, Kaduna State, Nigeria. The leaves were taken to the Herbarium Unit of the Department of Biological Science, Ahmadu Bello University, Zaria for identification and authentication, and a voucher specimen number was provided as 540.

Preparation of *Nicotiana tabacum* leaves extract was done in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The method of maceration as reported by Kolawole [19], for the preparation of aqueous and ethanolic extracts were employed.

The leaves were air dried in an enclosed environment and pulverized using laboratory mortar and pestle and 450g of the powder was soaked in sufficient solvents, water and ethanol and were allowed to stand for 24 hours and 72 hours respectively, after which the solutions were filtered using filter and funnel. The filtrate was then poured into the evaporating dishes and evaporated on the water bath (Digital thermostatic water bath Mc Donald Scientific international - 220 volt, 50 Hz and 10A) at a temperature of 80°C and 50°C respectively until the solvent has evaporated. The extracts were then emptied into a suitable container stored.

Acute toxicity study

Acute toxicity (LD_{50}) study was carried out using the method of Lorke [19]. Twenty-two Wistar rats were divided into two groups- Aqueous group (eleven rats) and Ethanolic group treatment (eleven rats). The animals in each groups were further divided into two phases- 1 and 2. The animals in each phase were observed for signs of toxicity and mortality after 24 hours. At the end of the LD_{50} study, the LD_{50} for ethanolic extract was above 5000 mg/kg bwt as no mortality was observed for ethanolic treated group at 5000 g/kg bwt, however for the aqueous treated group, one case of mortality was observed in the group treated with 5000 mg/kg bwt in the second phase and the LD_{50} was then calculated to be 3807 mg/kg bwt. The LD_{50} of aqueous extract was adopted for the ethanolic extract.

Different doses of 30%, 20% and 10% of the LD_{50} of aqueous and ethanolic extracts of *Nicotiana tabacum* were administered to the Wistar rats by oral gavages using orogastric tube for the experimental period.

Experimental design

In the present study, a total number of 42 adult Wistar rats were obtained and divided into seven groups of six rats per group with an average weight of 150 g. Group 1 served as the Control and was administered distilled water (1 ml/kg bw) and Groups 2-7 were the treatment Groups. Groups 2, 3 and 4 received 1000 mg/kg bwt (30% LD_{50}), 500 mg/kg bwt (20% LD_{50}) and 250 mg/kg bwt (10% LD_{50}) of aqueous extract of *Nicotiana tabacum* respectively, while Groups 5, 6 and 7 received 1000 mg/kg bwt (30% LD_{50}), 500 mg/kg bwt (20% LD_{50}) and 250 mg/kg bwt (10% LD_{50}) of ethanolic extract of *Nicotiana tabacum* respectively. All administration were given orally and lasted for a period of 21 days

Neurobehavioural observations

Neurobehavioural studies were carried out at 10:00 h on the day of the day of the study using Morris Water maze (MWM) to study spatial memory and learning in both the treated and control animals. The MWM is a relatively simple procedure typically consisting of six day trials before treatment, the main advantage being the differentiation between the spatial (hidden-platform) and non-spatial (visible platform) conditions (Eichenbaum., *et al.* 1990). In this setup experiment, latency which is the time (in seconds) taken for the animals to locate the safety platform.

Equipment preparation

A circular pool with a diameter of 150 cm and a depth of 50 cm was obtained with gray coated interior. A 10 cm diameter gray coated platform was placed in the pool. The pool was filled with water until the platform was 1cm above the water surface; the water equilibrated to about room temperature (25°C). The room was arranged in such a way that the animals being tested cannot see the interior of the experimental apparatus during testing. The bottom of the pool was divided into four equal parts with a vertical and horizontal marking, using a black paint.

Testing procedure

- The rats were transferred from their housing facility to the point of the behavioural test. They were kept in an area where they cannot see the pool or spatial cues to adjust to the new environment for some time before testing.

- To begin testing, the rat was lifted from the home cage at the tail, supported around the neck, and gently placed into the water, facing the edge of the pool. The researchers quickly moved away from the testing pool.
- As soon as the rat was placed in the water, it finds the platform before the 90 sec cut-off, it was allowed to stay on the platform for 5 seconds then return to its home cage. In situations where the rat could not find the platform, it was placed on the platform and allowed to stay there for 20 seconds before returning it to its home cage.
- The process was repeated for all rats in the trial. Each subsequent trial was started with a different platform location and starting direction of north, east and west.
- When testing was completed, the rats were returned to their housing facility. Rats were dried off and normothermia is assured prior to returning to animal facility.
- For each rat, the direction of the platform was changed as well as the starting point for the trial. The time it took the rat to locate the platform was recorded for each of the 3 trials and the average taken to give single escape time latency for each test object. The rats were trained for 7 days before treatment. During the treatment and at the end of the extracts administration, the rats were tested.

Animal sacrifice

After administration, the rats from each group were humanly sacrificed on day 22 and their brains were excised and the weights were taken and recorded. The brains were quickly transferred to specimen bottles containing Bouin's fluid and were fixed. Thereafter, the hippocampi were excised and processed for histological studies.

Relative brain weight

Relative brain weights were determined by dividing the brain weights over the body weights of the animal expressed as the percentage of the ratio of brain to body weight.

$$RBW = \frac{\text{Brain weight}}{\text{Body weight}} \times 100\%$$

Neurohistology

The hippocampi were excised and processed for Haematoxylin and Eosin (H and E) and Cresyl Fast Violet (CFV) staining techniques. The tissues were processed and embedded in paraffin wax for routine histologic studies. The brain tissues of 5 μ were sectioned with the Letiz rotary microtome. The sections were mounted, stained with H and E and Cresyl fast Violet methods and examined with the light microscope and the photomicrographs were taken.

Statistical analysis

Results obtained were analysed using Statistical Package for Social Scientist (SPSS version 20.0) and results were expressed as mean \pm SEM and the presence of significant difference among means of the group were determined using one way analysis of variance (ANOVA) with Tukey's post hoc test for significance. Values were considered significant when $p < 0.05$.

Results

Physical observations

The skin colour, the colour of the eyes, and the gross morphology of the brain of the tobacco treated groups were observed to be normal as that of the Control Group. Unusual level of restlessness, aggression, head deeding (HD) and stretching were displayed in the experimental when compared to the Control Group.

The weight changes

The result showed that body weight of the Wistar rats in the control group was significantly increased when compared to the experimental Groups ($P < 0.05$). However the body weights in all the treated Groups were decreased when compared with the Control group but

with a level of significance observed in Groups 2, 6 and 7 ($P < 0.05$) respectively as shown in table 1. The results also showed that body weight of rats treated with the ethanolic extract were significantly decreased when compared with the aqueous extract treated group ($P < 0.05$) as shown in table 1.

Groups	Initial (I) weight (g) (Day 1)	Final (F) weight (g) (Day 22)	Weight change(g) (F-I)
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
1	173.64 \pm 3.78	217.33 \pm 6.46*	43.66 \pm 3.66*
2	161.67 \pm 8.99	139.50 \pm 28.41*	-22.16 \pm 22.40*
3	157.33 \pm 9.94	155.83 \pm 6.80	-1.50 \pm 5.70
4	161.60 \pm 11.14	170.80 \pm 10.92	9.20 \pm 4.16
5	160.50 \pm 8.26	159.00 \pm 6.55	-1.50 \pm 2.30
6	151.00 \pm 7.06	145.67 \pm 7.92*	-5.33 \pm 4.88*
7	154.33 \pm 9.30	135.00 \pm 28.36*	-19.33 \pm 25.42*

Table 1: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on body weight of Wistar rats. $n = 6$; mean SEM; * $P < 0.05$.

Brain weight in relation to body weight

The result showed lower brain weight in the treatment Groups when compared to the Control Group, although the difference was not significant as shown in table 1. The result showed that the RBW of the rats in all the experimental Groups were higher than the Control though the increase was not significant. The result showed that Group 3 that received 500 mg/kg bwt of aqueous extract of *Nicotiana tabacum* leave extract had the highest RBW when compared to the other experimental Groups as shown in table 2.

Groups	Brain weight (g) Mean \pm SEM	Body weight (g) Mean \pm SEM	RBW (%)
1	1.73 \pm 0.15	217.33 \pm 6.46	0.79
2	1.30 \pm 0.64	139.50 \pm 28.41	0.94
3	1.60 \pm 0.11	155.83 \pm 6.80	1.03
4	1.54 \pm 0.55	170.80 \pm 10.92	0.90
5	1.52 \pm 0.75	159.00 \pm 6.55	0.96
6	1.42 \pm 0.12	145.67 \pm 7.92	0.95
7	1.30 \pm 0.65	135.00 \pm 28.36	0.96

Table 2: The Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Relative brain weight in Wistar rats.

Neurobehavioral studies

Morris water maze analyses

At the end of the training, all the animals learnt how to locate the hidden platform, as it was reflected in their ability to locate the platform within a short time as seen in the mean training time (Tt) as shown in figure 1. At week 3, the difference in the mean latency time observed following treatment with aqueous and ethanolic extracts of *Nicotiana tabacum* was increased in all groups except Groups 2, 4 and 5 as shown in figure 1. At the end of the administration the result showed increase in the mean latency time of ethanolic extract treated groups when compared with groups treated with aqueous extracts of *Nicotiana tabacum* though the increase was not significant as shown in figure 1.

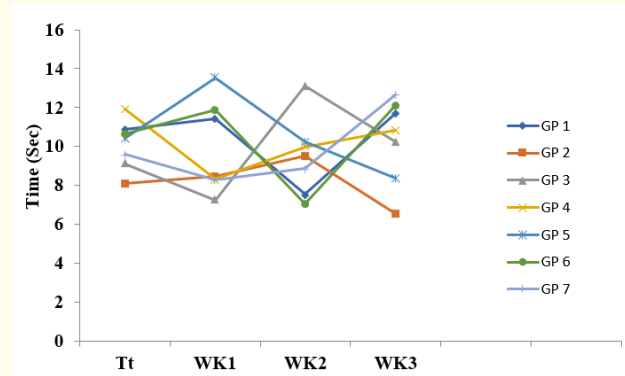


Figure 1: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Morris water maze test. $n=6$; mean \pm SEM; Tt = Mean Training time (Before Administration); WK = Week; GP = Group.

Hippocampal histology

Light microscopic examination of the hippocampal CA3 region stained with routine H&E histological and histochemical (CFV) stains revealed in the Control Group normal histoarchitecture of these regions the basic pattern of an ordered sheet of large neurones (pyramidal and granule cells) whose cell bodies are all packed together. The large neurones are the giant pyramids of CA3 as shown in plate Ia. Histochemical staining for Nissl substance revealed normal appearance of hippocampal neurones and other interneurones, such as stallate, fusiform and basket cells of cajal, which differ from the pyramidal granule cells, observed most clearly in CA3 as shown in plate Ib.

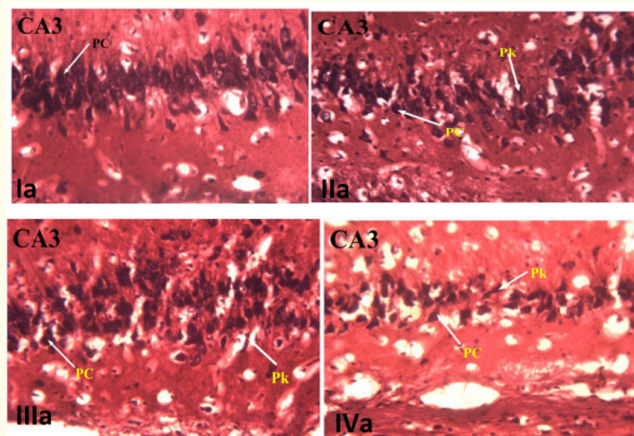


Plate I: Showing section of CA3 regions of the Hippocampus of the Control Group (Ia) and treated Groups 2 (IIa), 3 (IIIa), 4 (IVa) of Aqueous extract of *Nicotiana tabacum*, Ia showing well defined and packed pyramidal cells (P); granule cell (G) while Groups 2 (IIa), 3 (IIIa) and 4 (IVa) showed irregular arrangement of pyramidal cells, Pyramidal cells (PC); Pyknotic cell (Pk). H and E stain (Mag x250).

Histological and histochemical examinations of Wistar rats hippocampi treated with aqueous and ethanolic extracts revealed mild histoarchitectural distortion of the hippocampal regions CA3, neurodegenerative changes, such as, irregular arrangement of CA3 hip-

pocampal neurones, indistinct neurones reduced staining intensity, neuronal degeneration: irregular arrangement of pyramidal cells, Pyknotic cells (Pk) as shown in (Plates II-IV).

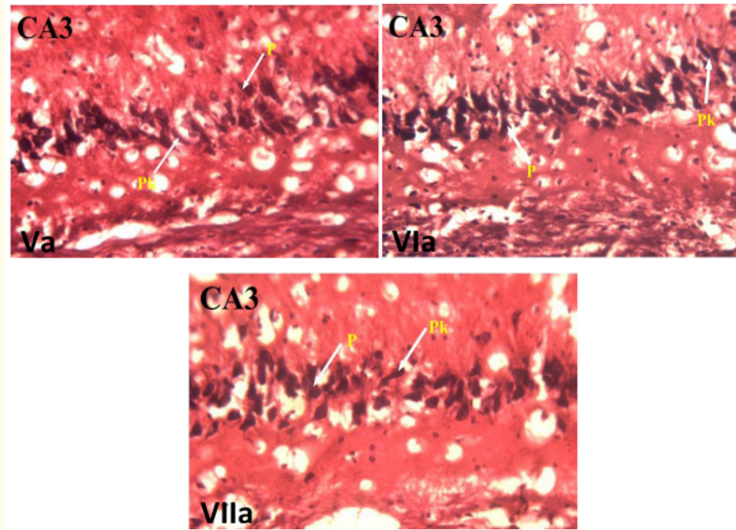


Plate II: Showing section of CA3 region of the Hippocampus of the treated Groups 5 (Va), 6 (VIa), 7 (VIIa) of Ethanolic extract of *Nicotiana tabacum*, showing irregular arrangement of pyramidal cells, Pyramidal cells (P); pyknotic cell (Pk). H and E stain (Mag x250).

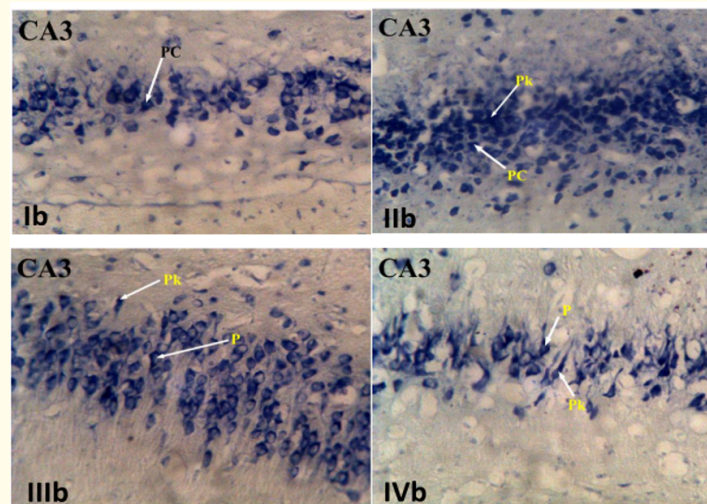


Plate III: Showing section of CA3 regions of the Hippocampus of the Control Group (Ib) and treated Groups 2 (IIb), 3 (IIIb), 4(IVb) of Aqueous extract of *Nicotiana tabacum*, Ia showing well defined and packed pyramidal cells (PC); while Groups 2 (IIb), 3 (IIIb) and 4 (IVb) showed irregular arrangement of pyramidal cells, Pyramidal cells (P); Pkynotic cell (Pk). CFV stain (Mag x250).

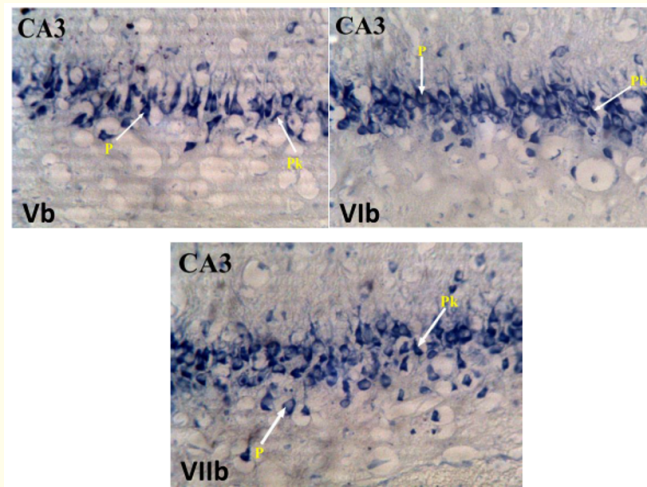


Plate IV: Showing section of CA3 region of the Hippocampus of the treated Groups 5 (Vb), 6 (VIb) and 7 (VIIb) of Ethanolic extract of *Nicotiana tabacum*, showing irregular arrangement of pyramidal cells, Pyramidal cells (P); Pyknotic cell (Pk) CFVstain (Mag x250).

Discussion

The result showed a decrease in the change in body weight and this may serve as a sensitive indication of the general health status of animals [20]. This action of tobacco on body weight appears to be nicotine mediated [21]. Previous studies in both humans and animals have reported that nicotine administration decreases body weight and caloric intake [22]. Most of the effects of tobacco on body weight are mediated by nicotine. Nicotine acts on nicotinic cholinergic receptors in the brain and autonomic ganglia [23]. The binding of nicotine to the nicotinic receptors opens ion channels, allowing entry of sodium and calcium, which, in turn, augment the release of various neurotransmitters such as catecholamine's in the central nervous system, dopamine, norepinephrine, serotonin, acetylcholine, glutamate, γ -aminobutyric acid, and other neurotransmitters. The release of these neurotransmitters suppresses eating and increase metabolic rate [23].

The result also show decrease in the brain weight in the treated groups relative to the Control. However, the relative organ weight were increased in the treated Groups relative to the Control with Group 3 having the highest RBW. This may account for the shift in the carbohydrate metabolic pathway due to stress induced by the activities of nicotine in the brain of the animals and may be indicative of treatment related toxicity. Increased brain weight has also been reported with the intoxication of heavy metals [24,25]. Memory is an organism's mental ability to store, retain and recall information, and the hippocampus plays an important role in learning and memory [26]. Learning and memory of Wistar rats is reflected by the time it takes the rats to escape to the safety platform after administration when compared to the performance at the training session. The observed increase in latency with tobacco treated Groups 2 and 3, may reflect the possibilities of learning and memory impairment. Larry elucidated that distinct forms of memory are mediated by different CNS regions, such as the prefrontal cortex and limbic structures including the Hippocampus [27]. Memory forms can be classified as declarative or explicit meaning the ability to recall past events deliberately and are hippocampus-dependant. It has been reported that exposure of rats to *Nicotiana tabacum* showed induced memory impairments [28,29]. The findings from the present study suggested that tobacco administration, showed variation in learning and memory. Observed histoarchitectural distortion of the hippocampus region CA3, such as, irregular arrangement of CA3 hippocampal neurones, pyknosis and degenerating pyramidal cells, was indicative of treatment tobacco treatment, induced neurodegenerative changes. A recent report has indicated that the activation of nicotine receptors by

low doses of nicotine resulted in apoptotic cell death in primary hippocampal progenitor cell demonstrating the capacity of nicotine in promoting apoptosis. Taken together, these findings suggested that nicotine which is a major constituent of tobacco is a neurotoxic agent regardless of its protective property in many other experimental paradigms [30,31]. Hippocampal CA3 neurones, characteristically, are packed together; arranged in few very dense rows. Histoarchitecture of CA3 region is important, because of the particular vulnerability of the neurones in the pyramidal layer to toxic events [32]. Irregular arrangement of CA3 neurones in the present study implies treatment related degenerative changes. Towfighi had reported a preferential degeneration of deep CA3 pyramidal cells was observed during early postnatal development in rabbits as a consequence of pilocarpine-induced seizures [33]. Basophilia of cytoplasm and pyknosis of neurones, which histologically characterizes neuronal atrophy, a descriptive term given to a wide variety of irreversible neuronal injuries resulting to slow cell death, occurs in many degenerative disorders [34].

Mild neurodegenerative changes in CA3 regions of the hippocampus, such as, irregular arrangement of CA3 hippocampal neurones, degenerating pyramidal cells, pyknotic necrosis, and vacuolations were observed in Wistar rats treated with both extracts of tobacco, aqueous and ethanolic. Neurodegenerative changes have been reported as direct toxic effect of tobacco [35]. Acute neuronal necrosis involves the cytoplasmic organelles and the cell membrane, which ruptures, leading to cell death. Features include neuronal cytoplasmic shrinkage, disappearance of Nissl bodies and intense eosinophilia [36].

Conclusion

Tobacco has been reported to cause different neurological effects in both human and experimental animal, from all the changes observed between the treated and Control Groups, it is worthy to conclude that the administration of tobacco aqueous and ethanolic leaves extracts, can result in body and brain weight changes, distorted neurohistoarchitecture and alterations in memory and learning.

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