



# Histomorphological Study of the Effect of Methanol Leaf Extract of *Nicotiana tabacum* (Tobacco) on Substantia Nigra Par Reticularis in Wistar Rats

<sup>1</sup>Wahab, A.O., <sup>1</sup>Hamman, W.O., <sup>1</sup>Agbon, A.N., <sup>2</sup>Akpulu, P.S. and <sup>3</sup>Sadam, A.A.

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<sup>1</sup>Neuroanatomy and Neuroscience Research Unit, Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria; <sup>2</sup>Department of Medical Laboratory Science, Federal University Lafia, Nassarawa, Nigeria; <sup>3</sup>Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria.

#### Address for Correspondence:

**Wahab, A.O.**  
Neuroanatomy and Neuroscience Research Unit, Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria.  
[wahab.ahmed3579@gmail.com](mailto:wahab.ahmed3579@gmail.com)  
+2348106725366

#### Abstract

**BACKGROUND AND AIM:** Smokeless or “Spit” are products of tobacco without combustion or pyrolysis at the time of use and the consumption of smokeless tobacco products continue to grow globally in form of dipping, snuffing, chewing and sniffing. The neurotoxic effect of tobacco is well documented. The Substantia nigra par reticularis, an important basal nuclei structure sets the pattern for facilitation of voluntary movements of the body. This study assessed the effect of methanol leaf extract of *Nicotiana tabacum* (MLNT) on histomorphological studies of the substantia nigra par reticularis in Wistar rats.

**METHODOLOGY:** Twenty male Wistar rats were divided into four groups (A-D, n=5). Group A was the control group treated with Tween 80 and Olive oil which served as vehicle for dissolution of MLNT. Groups B-D was treated with 200, 400 and 600 mg/kg MLNT respectively. Treatment was done for orally for four weeks. At the end of the experiment, the harvested brain tissue was weight before been processed for histological analysis. The body weight and the brain organosomatic index was computed and analyzes statistically using Statistical Package for social Science (IBM SPSS) Version 25.0.

**RESULTS:** Results obtained for the body weight show no statistical significant difference between the initial and the final weight when compared to the control and also, the brain organosomatic index significant increase ( $p < 0.05$ ) in all treated groups when compared to the control, especially with MLNT (200 mg/kg). Histological and histochemical observations showed neurodegeneration in all treated groups when compared to the control.

**CONCLUSION:** Finding from this studies revealed that MLNT could act as a potential neurotoxicant that can cause the acceleration of neurodegeneration in the substantia nigra par reticularis of Wistar rats thereby altered the coordination of motor activity.

#### Keywords:

substance abuse, substantia nigra par reticularis, organosomatic, neurotoxic and neurodegeneration

#### INTRODUCTION

Smokeless or “Spit” are products of tobacco without combustion or pyrolysis at the time of use (USNIH, 2008) and its products have been in existence for thousands of years among different populations of the world. Over time, these products have gained popularity globally (such as Tombak in Sudan, Snus in Sweden and Khaini in India) with mass marketing of new forms sold under different brand names (Idris *et al.*, 1995; Foulds *et al.*, 2003; Kumar *et al.*, 2006). The consumption of smokeless tobacco products

continue to grow globally in form of dipping, snuffing, chewing and sniffing (Idris *et al.*, 1995; Pereira *et al.*, 2001; Adeniyi and Musa, 2011). One of the chemicals delivered to tobacco users in through consumption of tobacco products is nicotine. Tobacco contains an average of 1.5% nicotine by weight (Benowitz *et al.*, 1983; Ebbert *et al.*, 2004). This chemical affects behavioral and physiological activities, and has in fact, been implicated as being responsible for majority of the psychological actions of tobacco (Russel,

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1997). Research has shown that nicotine is very well absorbed from tobacco; it is very well distributed rapidly and in biologically active concentration to organ-system including the brain. Sustained exposure to nicotine leads to tolerance, driven by an increase in the number of nicotinic cholinergic receptors and other changes within the brain (Benowitz, 2010). Tobacco use is the leading cause of preventable death and is responsible for about 6 million deaths each year worldwide (Ng *et al.*, 2014; WHO, 2011). The death toll is expected to rise to 8 million by 2030, if current trends continue (WHO, 2011). Thus, urgent action to curb the consumption of tobacco products is imperative, particularly in developing countries where tobacco use is becoming alarmingly popular (Mbulo *et al.*, 2016). In 2010, annual tobacco-related deaths in low and middle income countries were estimated at 4.3 million, corresponding to about 25% more deaths than tuberculosis, malaria and HIV/AIDS (Eriksen *et al.*, 2015).

The increase in tobacco use in developing countries is a cause for concern. Tobacco in any form is lethal; tobacco use is a major risk factor for chronic health conditions including cancer and cardiovascular disease (WHO, 2008). The economic costs of tobacco related diseases and deaths are huge, currently costing hundreds of billions of dollars each year (WHO, 2008).<sup>15</sup> Future impact of tobacco use is expected to be considerably severe in developing countries with unprecedented mortality rates due to the fragile, poorly resourced health care systems in these countries.

The substantia nigra, located in the midbrain region plays an important role in coordination of motor activities. It has two subdivisions namely: substantia nigra pars compacta and substantia nigra pars reticularis. The substantia nigra is a part of dopaminergic systems that received so much concern and has been linked to Parkinson's Disease (Julian *et al.* 2003). The substantia nigra pars compacta is composed of a thin band of cells that overlies the substantia nigra pars reticularis located laterally to the dopaminergic neurons in the ventral tegmental area (VTA) that forms the mesolimbic dopamine pathway. Despite a lot of research has been done in illuminating the neuroanatomical and neuropharmacological effect of *Nicotiana tabacum*, there is no research on the substantia nigra pars reticularis hence, the study aims to demonstrate the effect of methanol leaf extract of *Nicotiana tabacum* using morphology, histological and histochemical assessment.

## MATERIALS AND METHODS

**Ethical Approval:** The approval for this research was given by Ahmadu Bello University Ethical Review Committee with approval number ABUCAUC/2021/120. This research was conducted in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NRC, 2011).

**Plant Material:** Fresh *Nicotiana tabacum* (tobacco) leaves were obtained from a local farm in Zaria, Kaduna State, Nigeria and authenticated and deposited in the Herbarium Unit of Department of Biological Sciences, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria with a Voucher Specimen Number of ABU 054.

**Plant Extract Preparation:** The preparation of methanol leaf extract of *Nicotiana tabacum* was conducted in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The method of maceration as reported by Wahab *et al.*, (2023) was adopted.

The leaves were air dried in an enclosed environment and pulverized using laboratory mortar and pestle. 600 g of the powdered material was soaked in absolute methanol and was allowed to stand for a period of eight days after which the solutions were filtered and funneled. The filtrate was poured into an evaporating dish which was allowed to stand for three days so as to allow the solvent to evaporate. The percentage yield obtained was 16.66%.

**Experimental Animals:** A total of twenty apparently healthy male Wistar rats (150 to 200 g) were obtained from Animal House of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria and housed in plastic cages (40cm x 35 cm) of five Wistar rats each. The Wistar rats were acclimatized for two (2) weeks prior to the commencement of the experiments. They were fed with grower pelletized feed and the rats were provided with tap water *ad libitum*.

**Drugs:** Olive Oil (Goya extra virgin cooking oil) 250ml manufactured by Evans Industry Limited, Nigeria was purchased at Samaru market, Zaria and was used in combination with Tween 80 to dissolve MLNT.

**Tween 80:** Tween 80 manufactured by Haihang Industry, China was obtained from Department of Human Anatomy, Ahmadu Bello University, Zaria.

**Ketamine:** Ketamine manufactured by Tag pharmaceutical limited, India was obtained from reputable pharmaceutical shop at Zaria and was used as an anesthesia.

**Acute toxicity Studies and Dose Preparation of MLNT:** The acute toxicity study on MLNT was done following up-and-down method. Up-and-down method which requires fewer animals to achieve similar accuracy as the LD<sub>50</sub> test because animals are dosed once at a time (Bruce, 1985; Lipnick *et al.*, 1995; Lichman, 1998). This study was conducted in two (first and second) stages. In the first stage, a dose of 5000mg/kg was administered to three rats and signs of toxicity and mortality observed within 24 hours. In between fifteen to thirty minutes of observation, the rats experienced force breathing, loss of balance and posture and were inactive as a result of the sedative effects of the extract. 100% mortality was recorded that is, all the rats die within twenty-four hours

of observation. In the second stage, a dose of 2000mg/kg was administered to three rats and equally observed for signs of toxicity and mortality within 24 hours. After 24 hours of observation, there was no mortality even after seven days of observation.

Thus, oral median lethal dose (LD<sub>50</sub>) of MLNT used for the experiment was taken to be 2000mg/kg. Varying percentage of LD<sub>50</sub> was selected (10%, 20% and 30% representing 200 mg/kg, 400 mg/kg and 600 mg/kg respectively) for the study.

**Experimental Design:** Twenty male rats were divided randomly into four groups of five rats each. Group A was the control and was administered with vehicle (Olive oil and Tween 80/ Oil). Group B was administered with 200 mg/kg MLNT, group C was administered with 400 mg/kg MLNT and group D was administered with 600 mg/kg MNLNT..

**Animal sacrifice:** Twenty-four hours following the termination of the experiment, the Wistar rats were sacrificed using ketamine (75 mg/kg intraperitoneal (i.p)). The Wistar rats were decapitated and the brain tissues were carefully excised and weight using weighting scale for morphological evaluation. The harvested brain was fixed in Bouin's fluid for histological (Haematoxylin and Eosin) and histochemical assessment.

**Morphological Assessments:** The body weights of the Wistar rats were obtained on the first and the last day of treatment as the initial and final weights respectively. The absolute body weights (BW) of the rats were measured using digital electronic scale KERRO BL20001. Records of the weights were obtained at the beginning, during (Initial) and at the end (Final) of the study. Initial body weight (IW) and Final body weight (FW) was compared and analyzed statistically. Additionally, the body weight changes were compared and analyzed statistically using the formula below:

$$\text{Body Weight Change} = \frac{\text{FW} - \text{IW}}{\text{IW}} \times 100\%$$

Organ somatic Index (OI) was determined from two factors, the brain weight and absolute body weight of the rat and analyzed statistically. OI was computed by the percentage of the ratio of brain to body weight using the formula below:

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

**Histological and Histochemical Assessment:** The substantia nigra par reticularis was excised and processed for histological and histochemical assessment.

**Tissue Processing:** The fixed brain tissues were removed from the Bouin's fluid and dehydrated using ascending grades of alcohol. The dehydrated tissues were then cleared in two changes chloroform for two hours, the clearing helped in removing opacity from the dehydrated tissues thereby making them transparent. The cleared tissues were then infiltrated by immersion in molten paraffin wax and allowed

to solidify. The embedded tissues were blocked in a rectangular block and then sagittal sections were cut using rotary microtome at five micrometer per section. The tissue sections were allowed to float in water bath at three hundred degree celsius to help the spreading of the paraffin ribbons. The clean slides were used to pick the tissue from warm water bath (Drury *et al.*, 1967).

**Haematoxylin and Eosin (H and E) Staining:** The slides with brain sections were left to dry and the methods of haematoxylin and eosin staining technique was carried by de-waxing the tissue in two changes of xylene for three minutes each, hydrated by passing them through descending grades of alcohol (100%, 95%, 90% and 70%) for three minutes each, then stained in Harris haematoxylin for ten minutes, and wash in tap water to removed excess stain. The slides were then flooded with acid alcohol for few seconds for differentiation and then washes in tap water again. The slides were then blued in Scott's tap water for five minutes and counter-stained with eosin for three minutes. The sections were rinsed in tap water, and then dehydrated in ascending grades of alcohol and cleared in xylene (Drury *et al.*, 1967). Tissues were then mounted with cover slips using a mounting media (egg white). Sections photomicrographs were taking using digital camera Amscope (MD 900) fitted to light microscope (Leica Microsystem Inc. Tokyo, Japan).

**Cresyl Fast Violet Staining:** The tissue sections were deparaffinized and hydrated to distilled water and then stained for 5 minutes in Cresyl violet solution. The stained sections were rinsed in two changes of distilled water and placed in 95% alcohol for 30 seconds. Sections were transferred to absolute alcohol for 30 seconds and then placed in xylene for 1 minute and 2 minutes sequentially. Differentiations were made in absolute alcohol, two changes for 10 and 30 seconds each. The sections were then taken through several change of xylene and mounted with synthetic resin (Drury *et al.*, 1967). Sections photomicrographs were taking using digital camera Amscope (MD 900) fitted to light microscope (Leica Microsystem Inc. Tokyo, Japan).

**Data Analysis:** Results obtained from the experiment were analyzed using Statistical Packaging for Social Science (IBM SPSS version 25.0) and the results expressed as mean  $\pm$  SEM (standard error of mean). One-way ANOVA (analysis of variance) was used to compare the mean difference among the mean group followed by Least Significant Difference *post hoc* test. T- test was used were appropriate. p- value < 0.05 was considered statistically significant.

## RESULTS

**Morphological Assessments:** The body weights of the Wistar rats were obtained on the first and the last day of treatment as the initial and final weights respectively. The result for the body weight revealed that there is no significant difference in the treatment group when compared with the control and

the brain organosomatic index significantly increased ( $p < 0.05$ ) in all treated groups compared to the control.

**Histological Findings:** Section of the substantia nigra par reticularis of the control group as represented by A in Figure 3 show normal histoarchitectural feature of neuronal cell and glia cell. Section of the substantia nigra par reticularis of the Wistar rats administered with 200 mg/kg MLNT as represented by B in Figure 3 show neurodegenerative changes in the histoarchitectural distortion as gliosis, pyknosis and satellitogiosis. Section of the substantia nigra par reticularis of the Wistar rats administered with 400 mg/kg MLNT as represented by C in Figure 3 show neurodegenerative changes in the histoarchitectural distortion as chromatolysis, gliosis, pyknosis and vacuolation. Section of the substantia nigra par reticularis of the Wistar rats administered with 600 mg/kg MLNT as represented by D in Figure 3 show neurodegenerative changes in the histoarchitectural distortion such as pyknosis, Karyorrhexis, chromatolysis and vacuolation.

**Histochemical Findings:** Section of the substantia nigra par reticularis of the control group as represented by A in Figure 4 show normal histoarchitectural feature of neuronal cell and glia cell. Section of the substantia nigra par reticularis of the Wistar rats administered with 200 mg/kg MLNT as represented by B in Figure 4 show neurodegenerative changes in the histoarchitectural distortion as Pyknosis, chromatolysis and vacuolation. Section of the substantia nigra par reticularis of the Wistar rats administered with 400 mg/kg MLNT as represented by C in Figure 4 show neurodegenerative changes in the histoarchitectural distortion as chromatolysis, pyknosis and vacuolation. Section of the substantia nigra par reticularis of the Wistar rats administered with 600 mg/kg MLNT as represented by D in Figure 4 show neurodegenerative changes in the histoarchitectural distortion such as chromatolysis and vacuolation.

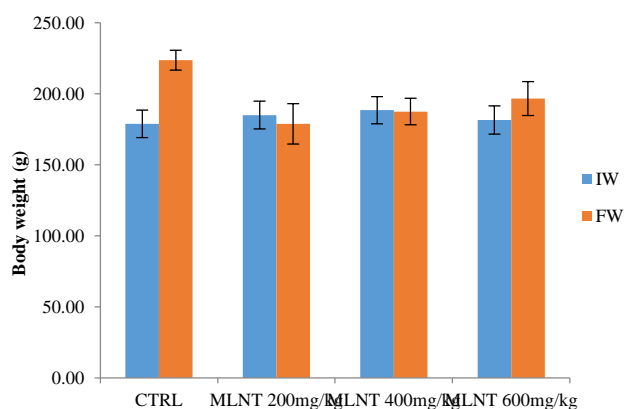


Figure 1: Effect of MLNT on Body Weight Changes in Wistar Rats = 5; mean  $\pm$  SEM. CTRL= Control (2 ml/kg; 4:1 oil: tween 80, p.o); MLNT= Methanol Leaf Extract of *Nicotiana tabacum*, IW= Initial Weight; FW= Final Weight

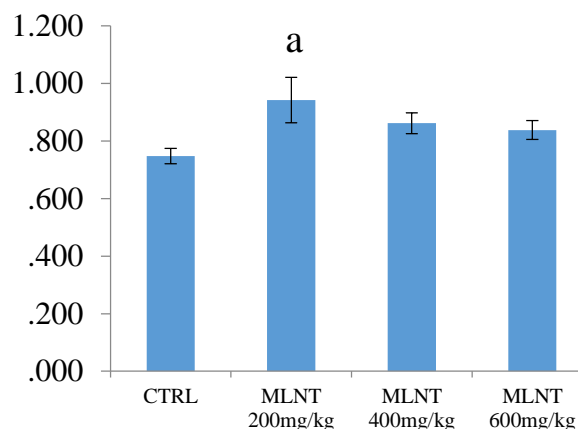


Figure 2: Effect of MLNT on the Organosomatic Index in Wistar Rat; n= 5; mean  $\pm$  SEM, one-way ANOVA LSD post hoc test; a=  $p < 0.05$  when compared with Control. CTRL= Control (2 ml/kg; 4:1 oil: tween 80, p.o); MLNT= Methanol Leaf Extract of *Nicotiana tabacum*.

## DISCUSSION

In this study, histomorphological assessment of substantia nigra par reticularis were carried out to evaluate the neurotoxic effect of MLNT. The result for the body weight showed no significant difference in the treatment group when compared with the control. Our results for the body weight was in consistent to Auza *et al.* (2017) and this is due to the fact that the action of tobacco on body weight appears to be nicotine mediated which act on autonomic ganglia thereby releasing neurotransmitters that suppresses eating and increase metabolic rate. Also, finding from this study was against the work done by Adeniyi and Ghazal, (2012) who reported a significant decrease in the body weight between groups A and B when compared to group C. The observed retarded increase in body weight in groups A and B could be due to the negative effects of the extracts on the normal metabolism as reported by West and Russell (1985).

Neuropathological changes are associated with neurodegeneration triggered by neurotoxin in difference region of the brain (He *et al.*, 2020). Neurodegenerative change such as Karyorrhexis, gliosis, pyknosis, satellitogiosis, vacuolations, and chromatolysis were observed in the rat treated with MLNT. Neuronal degenerative changes are indicative of neurotoxicity in the central nervous system (Majid *et al.*, 2008; Nahla *et al.*, 2011; Kalantaripour *et al.*, 2012). This is in agreement with previous studies that *Nicotiana tabacum* can cause neurotoxicity in mice and rats (Adeniyi *et al.*, 2010a, Adeniyi *et al.*, 2010b, Adeniyi and Musa, 2011, Adeniyi and Ogundele, 2014, Auza *et al.*, 2019) Thus, tobacco crosses membrane and affects the cellular integrity of tissue.

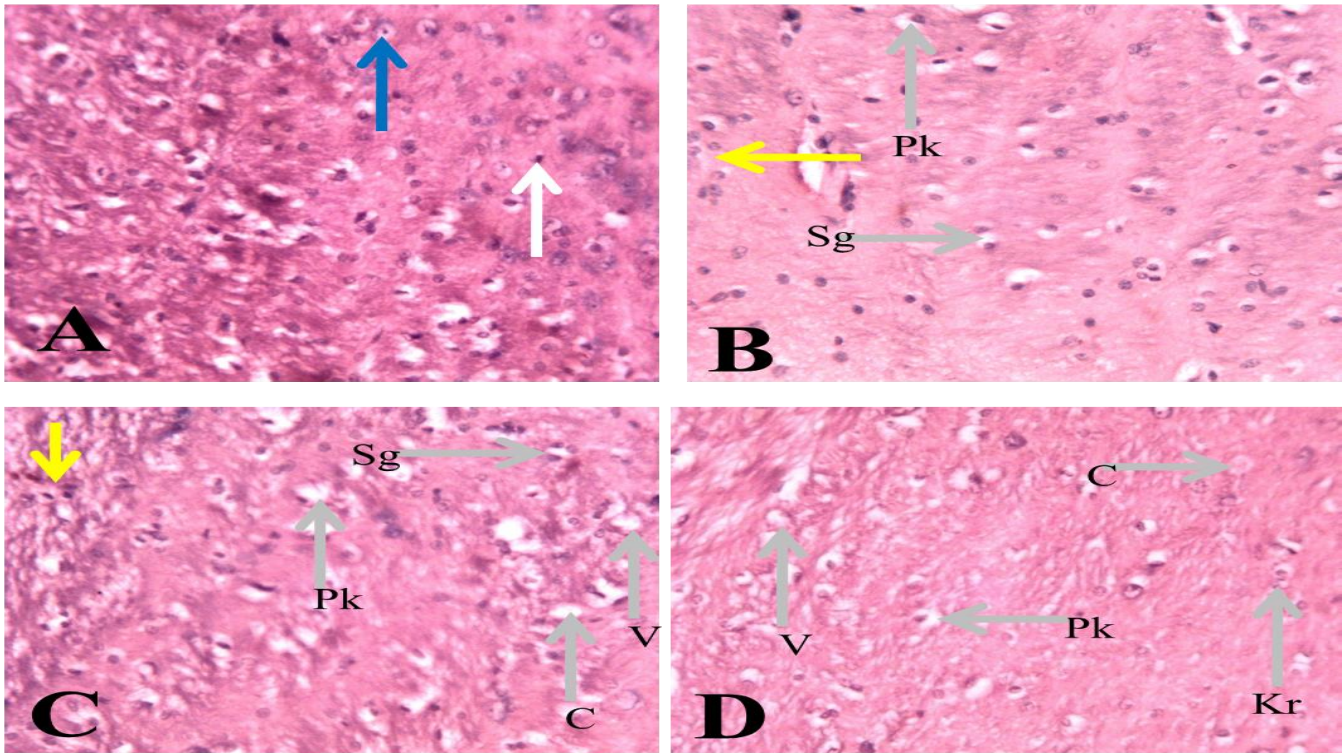


Figure 3: Section of substantianigra of Wistar rat of the control group showing normal cytoarchitecture of the substantianigra) H and E stain (Mag x 250); Blue arrow (Neuron); White arrow (Glia cell); Yellow arrow (Gliosis); V (Vacuolation) Kr(Karyorrhexis); Pk (Pyknosis) C (Chromatolysis) and Sg (Satellogliosis)

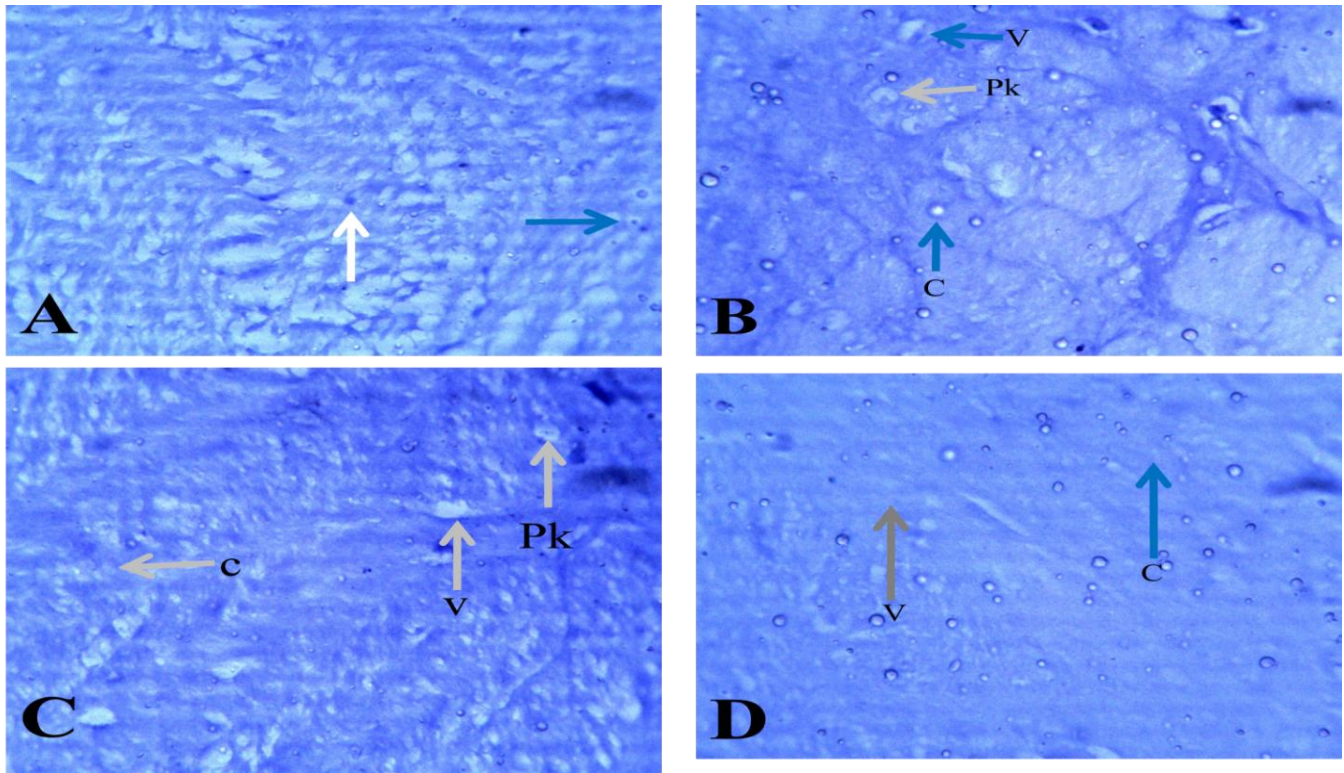


Figure 4: Section of substantianigra of Wistar rat of the control group showing normal cytoarchitecture of the substantianigra) CFV stain (Mag x 250); White arrow (Neuron); Blue arrow (Glia cell); V(Vacuolation); C (chromatolysis), Pk (Pyknosis)

Ribosomes, which are intensely basophilic owing to the presence of numerous phosphate groups made up of ribosomal ribonucleic acid acting as polyanions, react with basic stains such as methylene blue, toluidine blue and haematoxylin. The sites in the cytoplasm that are rich in ribosomes stained intensely with these dyes. These basophilic regions are called Nissl bodies in neurons. The number of Nissl bodies varies according to neuronal types and functional state (Ajibade et al 2011). Histochemical observation revealed a reduced staining intensity of the Nissl bodies in rat treated with MLNT when compared with the control. Injury to axons or neuronal exhaustion, resulting from strong or prolonged stimuli, causes a reduction in the number of Nissl bodies. This alteration called chromatolysis, occurs simultaneously with nuclear migration to the periphery of the perikaryon (Luis et al., 1986). Chemical and toxic substances affect the Nissl bodies thereby influencing their metabolic activity (Davis *et al.*, 1991) thus, MLNT had an irreversible effect on the Nissl substances resulting in histochemical changes in the neurons which manifested as neurodegeneration and loss of Nissl bodies may consequently affect the synthesis of both transport and structural protein responsible for the neuronal functions.

## Conclusion

Our laboratory studies have demonstrated the possibility that MLNT could act as a potential neurotoxicant that can cause the acceleration of neurodegeneration in the substantia nigra par reticularis of Wistar rats thereby altered the coordination of motor activity.

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