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Histomorphological and Biochemical Analyses Following Adminstration of Methanol Leaf Extract of *Nicotiana tabacum* (Tobacco) on the Cerebellum of Wistar Rats

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Abstract

BACKGROUND: The cerebellum, an essential brain region, coordinates voluntary movements, balance, and posture. While both beneficial and toxic effects of tobacco on various bodily systems have been reported, there's limited data on its neurotoxic effect on the cerebellum and motor functions.
AIM: This study aimed to histomorphologically and biochemically assess the effects of methanol leaf extract of *Nicotiana tabacum* (MLNT) on the brain and cerebellum of Wistar rats.
METHODOLOGY: Twenty male Wistar rats were divided into four groups (A-D) of five animals each, with A as control (Tween 80/Oil; 1:4) and B-D receiving MLNT at 200, 400, and 600 mg/kg, respectively, for four weeks with concurrent measurement of body weight.
RESULTS: Significant percentage weight changes were observed in all treatment groups compared to controls. Histological analysis revealed neurodegeneration in the cerebellar histoarchitecture of MLNT-treated groups compared with controls. Although no significant difference was observed in glutathione (GSH) levels, a significant increase in Malondialdehyde (MDA) level was observed at 200 mg/kg MLNT dosage compared to control ($P<0.05$).
CONCLUSION: This study indicates that methanolic leaf extract of *Nicotiana tabacum* induces neurotoxicity in Wistar rat cerebellum, potentially via heightened oxidative stress, particularly at 200 mg/kg dosage.

Keywords:
Cerebellum, Histological, Biochemical, Neurodegeneration, Neurotoxic

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Introduction

The cerebellum is basically known for its coordination of somatic motor function, muscle tone, balance and equilibrium. However, elaborate investigation into the cerebellum has shown that it also plays a role in non-motor activities of the brain such as cognition, emotions, behavior, and memory processes through its connections with other areas of the brain (Schmahmann, 2000; Konarski *et al*., 2005). Cerebellum contains more neurons than the rest of the brain because of the presence of large number of tiny granule cells in it (Llinas *et al*., 2004). Cerebellum takes up ten percent (10%) of the bulk of the structure of the cerebellum is made up of a very tightly folded layer of gray mater (cerebellar cortex), underneath the gray matter of the cortex lies the white matter which is composed of largely myelinated nerve fibers running to and from the cortex (Ghez *et al*., 1985).

Nicotiana tabacum generally called tobacco is a plant with an extraordinary history of use. It commenced with a history of sacred worship in the Native American Pipe ritual, when smoking tobacco would support and clear the mind as the smoke was believed to carry one's prayers to the Great Spirit (Kishore, 2004). In addition, it had a wide variety of uses for physical complaints, such as venomous bites and stings, internal and external parasites, and the symptomatic relief of pain, which justifies its wide use and appreciation by traditional practitioners all over the world (Haber, 1994). Even though tobacco has long been removed from

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pharmacopoeias and from medical practice historically, it has been an essential element in the ceremonial aspects of many communities and has taken on many sacred roles throughout the culture. Tobacco was, and is, a sacred plant used for spiritual, emotional, mental and physical guidance. (Kishore, 2004). The smoking and chewing products derived from this plant, few people realize its many other uses. Tobacco has medicinal values, makes an extremely valuable ornamental plant and flower garden specimen and is used to make one of nature's finest biodegradable natural pesticides (Nan-Ren and Michael, 2001).

The tobacco products are manufactured from the leaves, cured & dried and used in cigars and cigarettes, snuff, and pipe and chewing tobacco (Gilman *et al.*, 2004; Kishore, 2004). The pharmacological activities of *Nicotiana tabacum* is mostly due to its content of nicotine which stimulates the nicotine receptors leading to release of substances such as acetylcholine, norepinephrine, dopamine, serotonin, vasopressin and growth hormone. Nicotine has been demonstrated to accelerate angiogenesis and wound healing in genetically diabetic mice (Johannes *et al*., 2002). The ethnomedicinal uses of *Nicotiana tabacum* plant include the use of decoction of the leaves as antispasmodics, diuretics, emetics, expectorants, sedatives, and in rheumatic swellings, anesthetics, antibacterial, antimicrobial, anthelmintic, anticonvulsants and for anti-fungal activities. Other uses includes treatment of asthma by Indians, treatment of worms in Africa, treatment of wounds in Columbia and treatment of dysmenorrheal in Cuba among others (Kishore, 2004). The plant *Nicotiana tabacum* also have the great activities on peripheral nervous system, central nervous system, cardiovascular system, gastrointestinal tract, exocrine glands, Hematopoietic system, algesia, Alzheimer and on body weight (Kishore, 2004).

Historically, many Ayurveda philosophers and healers have recognized the properties of this plant and utilized it in various disorders. Although tobacco is still used traditionally in its historic manner by many tribes and by many native people, the continued abuse of the commercial tobacco is much more frequent and has taken its toll on the native people's health (Landoni, 1990). The purpose of this study was to evaluate histomorphological and biochemical effects of methanol leaf extract of *Nicotiana tabacum* (MLNT) on the cerebellum of Wistar rats.

Materials and Methods

Plant Material

Fresh *Nicotiana tabacum* (tobacco) leaves were obtained from a local farm in Zaria, Kaduna State, Nigeria. The plant was 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. Briefly, the leaves were washed and air dried in an enclosed environment and pulverized using laboratory mortar and pestle. 600 g of the powdered leaves was soaked in absolute methanol and was allowed to stand for a period of eight days with gentle shaking, after which the solutions was filtered with Whatman paper No.1. The filtrate was poured into 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%.

Acute toxicity study

The acute toxicity study of MLNT was done following up-and-down method. Up-and-down method which requires fewer animals to achieve similar accuracy to the classical procedure 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 5000 mg/kg body weight was administered to three rats which were then observed within 24 hours for signs of toxicity and mortality. In between fifteen to thirty minutes of observation, the rats experienced breathing difficulties, loss of balance and posture and were inactive. All the rats died within twenty-four hours of observation (% mortality).

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

Thus, oral median lethal dose (LD50) of MLNT used for the experiment was estimated to be 2000 mg/kg. Varying percentage of LD50 was selected (10%, 20% and 30%) for the study.

Experimental Design

A total of twenty apparently healthy male Wistar rats (150 to 200 g) were obtained from Animal House of the Department of Pharmacology and Therapeutic, Faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria and housed in plastic cages (40 cm x 35 cm). Animal feed and water were provided ad libitum. The animals were divided randomly into four groups of five rats each. Group A was the control and was administered with Olive oil (Tween 80/Oil; 1:4; v/v). Group B was administered with 200 mg/kg body weight of MLNT, group C was administered with 400 mg/kg body weight of MLNT and

group D was administered with 600 mg/kg body weight of MNLT.

Animal sacrifice and Tissue Processing

Twenty-four hours following the termination of the experiment, the Wistar rats were injected with ketamine (75 mg/kg) and Diazepam (5 mg/kg), then, decapitated and the whole brain tissues were carefully excised and weighted. The harvested cerebellum was halved; the cerebellum in part was cut sagittally which was used for histological analysis (Haematoxylin and Eosin) while the other half was homogenized for oxidative stress analysis; malondialdehyde (MDA) and glutathione (GSH). The cerebellar cortex was fixed in Bouin's fluid. Histological staining was carried out in paraffin embedded sections following method of Canene-Adams (2013). Thin sections from paraffin embedded cerebellar tissues were processed for light microscopy and stained using Hematoxylin and Eosin. The other half of the brain was pulverized in 0.25 M sucrose with the aid of an automated homogenizer at 4oC. Lysates from the cerebellum were centrifuged for 10 minutes at 12,000 and the supernatants were aspirated into plain labelled glass cuvette placed on ice. Both MDA and GSH activity were assayed using ELISA kit following manufacturer's procedure.

Data Analysis

Results obtained from the experiment were analyzed using Statistical Package for Social Sciences (IBM SPSS version 25.0) and the results expressed as mean ± SEM. T-test was used to compare the mean difference between the groups followed by LSD post hoc test , p value < 0.05 was considered statistically significant.

Results

Physical observations: The brain of the Wistar rats were observed to be milky in color presenting with two major sulcal depressions on the dorsal surface, separating the cerebrum from cerebellum. The skin color, the color of the eyes, and the gross morphology of the brain of the tobacco treated groups were observed to be normal similar to the control group. The body weights of the Wistar rats were obtained on the first and the last day of treatment as the initial weight (IW) and final weight (FW) respectively using digital weighing balance. The percentage weight change was estimated using the formula below:

Percentage Weight Change = (FW−IW)/IW ×100%

The percentage weight was significantly lower in all the treatment groups when compared to the control (Figure 1).

Histological findings: The general histological presentation of the cortical cerebellar structure revealed the molecular layer, Purkinje cell layer and the granular layer which were examined using the microscope. Section of the cerebellar region of the control group as represented by 'a' in Figure 2 shows normal histoarchitectural region of the cerebellum with a well-defined molecular layer, Purkinje's layer and granular layer. Section of the cerebellar region of the Wistar rats administered with 200 mg/kg body weight of MLNT as represented by 'b' in Figure 2 shows neurodegenerative changes such as as karyorehexis , gliosis, perineuronal vacuolation, chromatolysis and necrosis of the Purkinje's cell of the cerebellar cortex of the Wistar rat. Section of the cerebellar region of the Wistar rats administered with 400 mg/kg body weight of MLNT as represented by 'c' in Figure 2 show neurodegenerative changes such as karyorehexis , karyolysis and satelliogliosis in the cerebellar cortex of Wistar rat. Section of the cerebellar region of the Wistar rats administered with 600 mg/kg body weight of MLNT as represented by d in Figure 2 show neurodegenerative changes such as karyolysis and necrosis in the cerebellar cortex of Wistar rat.

Biochemical findings: GSH and MDA were assessed as makers of oxidative stress using brain homogenate. The malondialdehyde level was significantly elevated (p 0.01)in the Wistar rats administered with 200 mg/kg body weight of MLNT (Figure 3) while the GSH was decreased in all treatment groups when compared with the control through not significant (Figure 4).

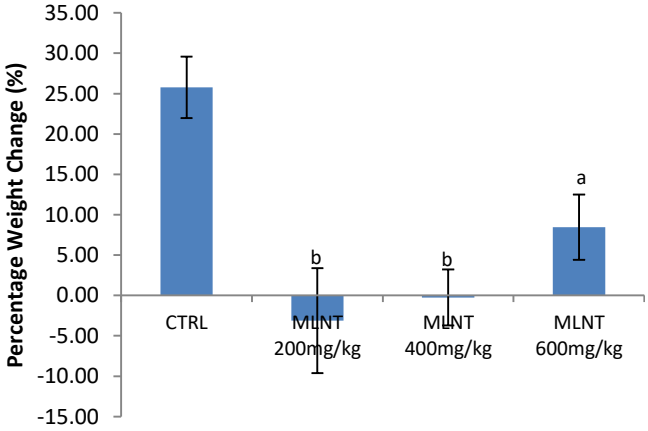

Figure 1: Effect of Methanol Leaf Extract of *Nicotiana tabacum on Weight of the Rats*

Results presented as Mean ± SEM, one-way ANOVA LSD post hoc test; a= p<0.05, b = p <0.001 when compared with control. CTRL= Control (2 ml/kg; 4:1 oil: tween 80, p.o); MLNT= Methanol Leaf Extract of *Nicotiana tabacum*.

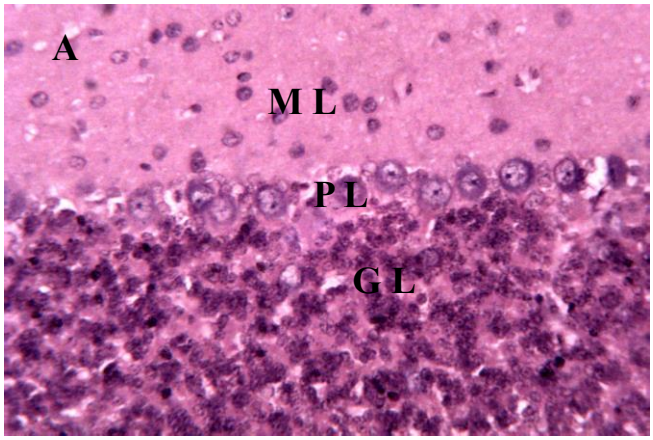

Figure 2a: Section of cerebellar region of the control group. (H & E 250X). (M L = Molecular Layer; P L= Purkinje's Layer; G L= Granular Layer)

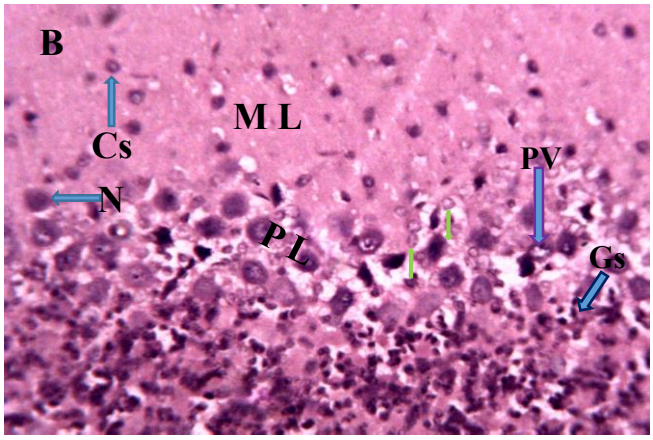

Figure 2b: Section of cerebellar region of the group administered with 200 mg/kg body weight of MLNT. (H & E 250X). (M L = Molecular Layer; P L= Purkinje's Layer; G L= Granular Layer; Cs= Chromatolysis; N= Necrosis of Purkinje's cell (Degenerating Purkinje's Layer); PVC= Perineuronal Vacuolation; Gs=Gliosis; green arrow= Karyorrhexis)

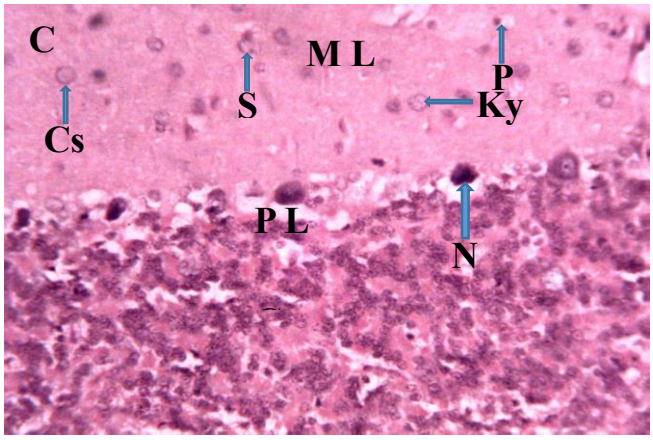

Figure 2c : Section of cerebellar region of the group administered with 400 mg/kg body weight of MLNT. (H & E 250X). (M L = Molecular Layer; P L= Purkinje's Layer; G L= Granular Layer; Cs= Chromatolysis; N= Necrosis of Purkinje's cell (Degenerating Purkinje's Layer); Ky= Karyolysis of molecular layer; Pk= Pyknosis; Sg= Satelliogliosis (abnormal condition where by a neuronal cell fused with a glial cell)

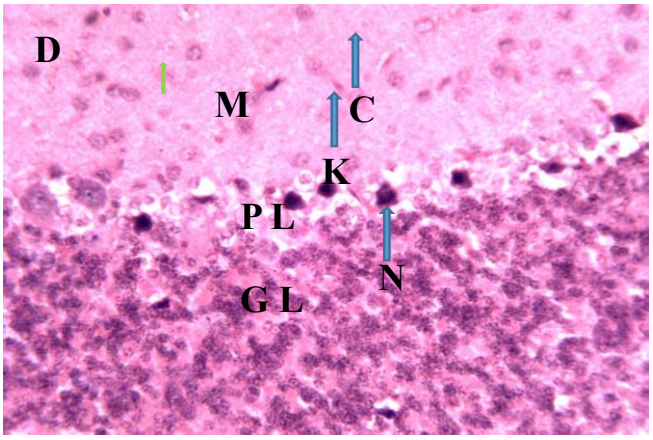

Figure 2d : Section of cerebellar region of the group administered with 200 mg/kg body weight of MLNT. (H & E 250X). (M L = Molecular Layer; P L= Purkinje's Layer; G L= Granular Layer; Cs= Chromatolysis; N= Necrosis of Purkinje's cell (Degenerating Purkinje's Layer); Ky= karyolysis; green arrow= Karyorrhexis)

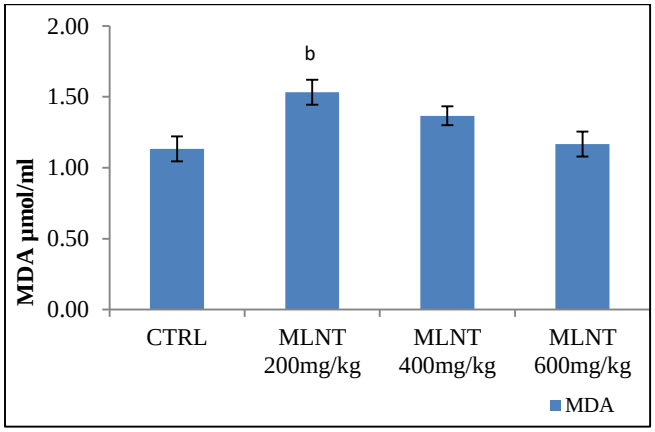

Figure 3: Effect of MLNT on Cerebellar Tissue MDA level in Wistar rats. Results presented as mean ± SEM (n= 5) using ANOVA, LSD post hoc test. b= p<0.01 when compared with control. CTRL= Control (2 ml/kg; 8:2 oil: tween 80, p.o); MLNT= Methanol Leaf Extract of *Nicotiana tabacum*, MDA= Malondialdehyde

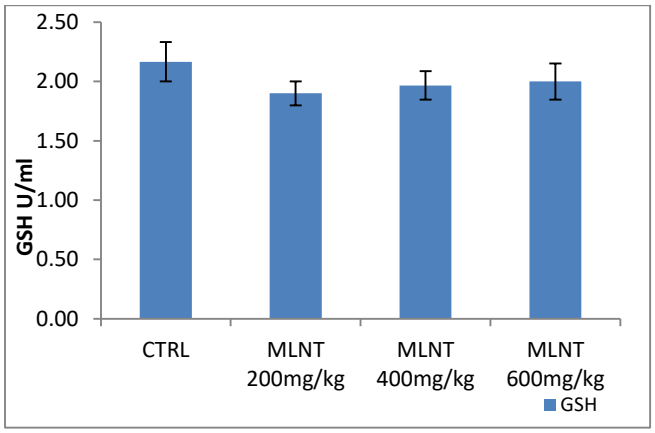

Figure 4: Effect of MLNT on Cerebellar Tissue GSH levels in Wistar rats. Results presented as mean ± SEM (n= 5) using ANOVA, LSD post hoc test; CTRL= Control (2 ml/kg; 8:2 oil: tween 80, p.o); MLNT= Methanol Leaf Extract of *Nicotiana tabacum*, GSH= Glutathione

Discussion

The cerebellum is a region of the brain that plays an important role in integration and regulation of well-coordinated muscular activities, regulation of tone, posture and equilibrium by regulating impulses from tactile, proprioception, visual and auditory receptors. The cerebellar hemisphere is the major channel that connects sensory areas of the brain to motor areas of the brain. It forms a relationship during coordinated movement which is involved in motor learning and modifications of reflexes (Tewari *et al*., 2010). Atrophy to the cerebellum is usually accompanied by different ataxia and an unstable gait (Fonnum and Lock, 2000). The cerebellum has been reported to be susceptible to toxic chemical compounds and drugs at higher doses most especially, the granular layer and purkinje neurons are usually more vulnerable compared to the molecular layer which is least affected (Manto, 2012).

During the period of administration of MLNT the animal were observed for any changes in physical activities and behavioral pattern. Physical activity of the present study revealed normal movement in the control group but the treatment groups exhibited difficulty in breathing, loss of posture and balanced, aggressive and convulsive like-behavior, body and limbs shaking and disorientated movement within two to five minutes of administration at a dose of 400 mg/kg and 600 mg/kg respectively. Reduced a change in the observed physical activity and change in behavioral pattern in MLNT treatment groups is suggestive of treatment related toxicity.

Determination of shape, colour and size of the brain are importance step in the monitoring of different pathologies and determination of environmental influences (Scheenstra *et al*., 2010). The milky coloration of the brain was observed in both the control and treatment groups. The coloration is a common manifestation of the structure of the central nervous system linked with the presence of an integral biochemical component and lipid moieties (Aschner *et al*., 2010; Poon *et al*., 2018). Also, the skin color of MLNT treated groups appear to be normal when compared with the control. The overall gross features of the MLNT treated groups appeared normal when compared to the control group.

The result for the body weight revealed that there is no significant difference in the treatment group when compared with the control. The percentage weight change was significantly decreased in all treatment groups when compared to the control.

Histopathological changes in the neural tissue manifesting as neurodegenerative changes are indicative of neurotoxicity in the central nervous system (Majid *et al*., 2008; Nahla *et al*., 2011; Kalantaripour *et al*., 2012). Degenerative changes observed include cerebellar neuronal degeneration and vacuolations and loss of cellular components with reduced population of Purkinje cells in cerebellar section of rats treated with MLNT when compared with the control implies treatment related neurotoxicity. This is in concordance with the studies related to *Nicotiana tabacum* caused cell injury on the cerebellar structures by Omotoso (2014).

Auza *et al*. (2017) also reported degenerated pyramidal cells, shrinkage of cell body, cytoplasmic and neuropil vacuolations and distorted Purkinje cells. Purkinje and granule cells were the most important targets in the cerebellum for toxic substances (Fonnum and Lock, 2000). MLNT crosses membrane and affects the cellular integrity of tissue. In this study, MLNT possibly acted as a neurotoxin to the cerebellar cortex, as such, distorting neuronal integrity and causing disruption in membrane permeability and tissue homeostasis. The degenerative effect of MLNT on the Purkinje layers of the cerebellum observed in this study might be responsible for the cerebellar degeneration.

Oxidative stress, a common pathology which occurs due to imbalance between production and detoxification of reactive oxygen species (ROS), has been implicated in many neurodegenerative diseases (Gilgun-Sherki *et al*., 2001; Trushina and Memurray, 2007). In this study, biochemical analysis for lipid peroxidation level and antioxidant enzyme activity (Glutathione, GSH) were assessed in the brain tissue homogenate of Wistar rats. Tissue MDA levels were significantly increased in Wistar rats administered with 200 mg/kg body weight of MLNT when compared with the control group, which suggests enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive radicals (Himakar *et al*., 2010). Finding from this study was in agreement with Auza *et al*. (2017) who reported a slightly increased in MDA level in Wistar rats administered with aqueous extract of *Nicotiana tabacum*. GSH is a powerful antioxidant of cellular defensive mechanisms against reactive oxygen species (Babich *et al*., 2011) present within the cytosol of cells and is the major intracellular non-protein thiol compound. The principal function of GSH is to scavenge free radicals and peroxide produced during normal cellular respiration that would otherwise cause oxidative damage to lipids, protein and nucleic acids (Schuck *et al*., 2008). Oxidative stress is characterized by depletion of intracellular GSH especially in case of cancer cells (Plelicano *et al*., 2004). Finding from this study revealed that tissue GSH activity showed no significant difference in all treatment groups when compared with the control which is an indication of enhance oxidative stress. The results thus support an emerging pattern wherein tobacco exposure elicits oxidative stress causes tissue damage as a result of production of free radicals. In conclusion, findings from this study indicated that methanol leaf extract of *Nicotiana tabacum* could be a neurotoxin acting through histoarchitectural distortion and induction of oxidation stress in the cerebellum of Wistar rats.

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