THE EFFECTS OF AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF *Nicotiana tabacum* ON THE CEREBELLAR AND CEREBRAL CORTICES AND HIPPOCAMPUS OF ADULT WISTAR RATS

BY

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AHMADU BELLO UNIVERSITY,

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December, 2017.

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (M.SC) DEGREE IN HUMAN ANATOMY

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ZARIA, NIGERIA

December, 2017

DECLARATION

I, Moses Ibrahim AUZA hereby declare that the work in this dissertation titled "The Effect of Aqueous and Ethanolic Extracts of *Nicotiana tabacum* on the Cerebellar and Cerebral Cortices and Hippocampus of Adult Wistar Rats" was performed by me in the Department of Human Anatomy under the supervision of Dr. A.O. Ibegbu and Dr. B. Danborno.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at any university.

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Signature Date

CERTIFICATION

This dissertation "The Effect of Aqueous and Ethanolic Extracts of *Nicotiana tabacum* on the Cerebellar and Cerebral Cortices and Hippocampus of Adult Wistar Rats" by Moses Ibrahim Auza meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to God Almighty who made this work a reality that gave me the strength and wisdom in the course of this work

ACKNOWLEDGEMENTS

Conducting and writing this dissertation has been an incredible learning experience and I have received considerable support from several sources. First, to God almighty, in whom I live, move and have my being, for all his benefits and the multitude of great things he has done for me.

My profound gratitude goes to my supervisors, Dr. A. O Ibegbu and Dr. B. Danborno for their immense guidance, supervision and professional advice without which this present work could have never been completed.

My sincere appreciation goes to the H.O.D, Dr. (Mrs) Z. M. Bauchi and the Post graduate Coordinator, Dr S. A. Musa and my lecturers: Dr. U. E Umana, Dr. W. O Hamman, Dr J. A. Timbuak, Dr. I.A. Iliya. I say thanks for impacting and building up my knowledge of the complex creation of God. My thanks also go out to the technical staff Mr. P. S. Akpulu, Mr B. Adetiba who contributed in the preparation of the tissue sections.

I acknowledge and an appreciate Dr. A. N Agbon and Dr. A. A. Sadeeq for their insightful comments. My sincere appreciation goes to brethren in the Chapel of Redemption, postgraduate Fellowship (PGF), Fellowship of Christian Students (FCS) for their encouragement and prayers without which my stay here would have been a serious struggle.

I will not fail to mention special friends like Pastor Misheal Tomizayi, Prophet Jerry Koce, Matthew Habila, Budaye Michael, Usman Ibe, Solomon Abel, Rachael Henry, Elijah Adeigbe, and my brothers and roommates Stephen Lazarus and Kehinde Balogun, for your time, assistance and understanding throughout the development of this dissertation. Your

words, concerns, attention and prayers really helped to make this feat an easy possibility. God bless you all, Amen.

My heartfelt gratitude goes to my parents, Mr. and Mrs. Pmayigna Auza Ibrahim whose love, encouragement, support and advice has kept me on course. I owe a great debt of gratitude to my siblings, Mrs Patience Felix, Mrs Rachael Lamech, Dr Victor Ibrahim and Mrs Mary Ephraim who had always understood why I had to be absent or fail in some of my obligations in the course of my work, their loving supports and prayers made this work exciting, God bless you all, Amen.

ABSTRACT

Nicotiana tabacum is the scientific name of the tobacco plant grown in several countries of the world. It has been used in variables ways such as smoking, snuffing, even chewing. The present study was to investigate the possible effects of aqueous and ethanolic leaf extracts of *Nicotiana tabacum* on the hippocampus, cerebral and cerebellar cortices of adult Wistar rats for a period of 21 days. Forty two adult Wistar rats of average weight of 150 g were used and were divided into 7 groups of 6 rats each (n = 6). The animals in Group 1 served as the Control and were given 0.2 ml of distilled water, Groups 2, 3 and 4 received 1000 mg/kg body weight, 500 mg/kg body weight and 250 mg/kg body weight respectively of aqueous extract of *Nicotiana tabacum* while, Groups 5, 6 and 7 received 1000 mg/kg body weight, 500 mg/kg body weight and 250 mg/kg body weight of ethanolic extract of *Nicotiana tabacum* respectively for 21 days via oral route once daily. At the end of the administration, the animals were then subjected to Morris Water Maze (MWM) and Beam Walking Test (BWT) and were humanely sacrificed on day 22 of the experiment and the brains were excised, fixed in Bouin's fluid and processed using Haematoxylin and Eosin (H&E) and Cresyl Fast Violet (CFV) staining technique. The result showed a significant decrease in the body weight in Groups 2 (139.50±69.59 g), 6 (145.67±19.40 g) and 7 (135.00±69.48 g) when compared to the Control Group (217.33± 15.87 g) ($P< 0.05$). The result showed that the mean latency time by rats to locate the platform after the administration of the extracts was increased in groups 6 (12.10±11.33 s) and 7 (12.65±8.83 s) when compared with the Control Group (11.69±11.08 s) though the increase observed was not significant. Oxidative stress markers were accessed in the brain and the result showed a significant decrease in CAT level in 2 (43.50±2.12 µg/ml), 4 (41.50±0.70 µg/ml),

5 (43.00±1.41 µg/ml), 6 (39.00±1.41 µg/ml) and 7 (36.00±1.41 µg/ml) ($p<0.05$) when compared with the Control (48.00±1.41 µg/ml). The result also showed a significant decrease in SOD level in 3 (2.10±0.14 nmol/ml), 5 (1.75±0.07 nmol/ml), 6 (1.90±0.01 nmol/ml) and 7 (1.70±0.14 nmol/ml) ($p<0.05$) when compared with the Control (2.45±0.21 nmol/ml). The result of the histological and histochemical examination of the hippocampus, cerebellar and cerebral cortices revealed degeneration in the brain. Cerebral and cerebellar cortices of treated rats with both extracts of *Nicotiana tabacum* revealed distortions such as pyknosis, karyorrhesis, vacuolations compared to the Control. Hippocampi of rats treated with both extracts revealed changes such as, irregular arrangement of CA3 hippocampal neurones and pyknosis when compared with the Control Group. The result from this study affirms that tobacco has effects on the brain and may induces neurotoxicity at various doses in Wistar rats. Therefore, suggests that the consumption of both form of *Nicotiana tabacum* may reduce body weight and alter the normal functions of the brain which may lead to brain dysfunction.

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LIST OF ABBREVIATIONS

ANTH	Aqueous leaf extract of *Nicotiana tabacum* High dose
ANTL	Aqueous leaf extract of *Nicotiana tabacum* Low dose
ANTM	Aqueous leaf extract of *Nicotiana tabacum* Medium dose
µl	Microliter
bwt	body weight
BWT	Beam Walking Test
CAT	Catalase
CNS	Central Nervous System
CTRL	Control
Cu	Copper
ENTH	Ethanolic leaf extract of *Nicotiana tabacum* High dose
ENTL	Ethanolic leaf extract of *Nicotiana tabacum* Low dose
ENTM	Ethanolic leaf extract of *Nicotiana tabacum* Medium dose
Fe	Iron
g	Gram
GP	Group
GPx	Glutathione peroxidase
kg	Kilogram
LD_{50}	Lethal Dose
LPO	Lipid peroxidation
MDA	Malonaldehyde
mg	milligram
Mn	Manganese
ml	milliliters
MWM	Morris water maze

RBW	Relative Brain Weight
ROS	Reactive oxygen species
SEM	Standard error of Mean
SOD	Superoxide dismutase
WHO	World Health Organization
Zn	Zinc

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Smokeless tobacco is a product of tobacco without combustion or pyrolysis at the time of use (IARC, 2007). One of the forms of smokeless tobacco, is chewing and sniffing tobacco. Snuff is a form of smokeless tobacco that has been processed to fine powder and packaged either in cans or pouches (Emmanuel *et al.*, 2012). Its users take it in pinch or dip or quid and places it between the lower lip or cheek and gum and suck it. Another route for the use of snuff, though rare about 9.6% is by sniffing or nasal use and this is the most common among Nigerian users (Emmanuel *et al.*, 2012). Users of smokeless tobacco believe it is safer than the smoking tobacco. However, it still has lots of effects. Addiction to nicotine is one of the side effects of its use (USNIH, 2012). Another is cancer of the mouth and pharynx, for which there are inconclusive reports. Other side effects includes leukoplakia, gum recession, bone

loss around the teeth, abrasion of teeth and bad breath (Zhang *et al.*, 2001).

One of the chemicals delivered to tobacco users in cigarette smoking or other forms of tobacco like the chewed or snuffed form is nicotine. When tobacco is smoked or chewed, nicotine is absorbed by the lungs and oral cavity and quickly moved into the bloodstream where it is distributed rapidly through the circulatory system to reach the brain and peripheral nervous system (King *et al.*, 2004). Nicotine is an alkaloid organic compound, found naturally throughout the tobacco plant, with a high concentration in the leaves. It constitutes 0.3 to 5% of the plant by dry weight. The biosynthesis takes place in the roots and it is accumulated in the leaves (Kishore, 2014). This chemical negatively affects behavioral, physiological and histological activities of the organs of the body especially the brain (Benowitz *et al.*, 1983; Katzung, 2005; NIH, 2009). Tobacco is consumed in every parts of the world, most especially in the developing countries (Aghaji, 2008). Nicotine is the highest and most toxic compound of tobacco leave and of neurotoxic importance (Penton and Lester, 2009).

Exposure to tobacco nicotine either from smoking, snuffing or chewing has been frequently associated with alteration in the normal function of the brain and the nervous system (NIH, 2009). Nicotine, in medical products such as nicotine patches, nicotine gums and quit smoking drugs, are used to aid in smoking sensation and other nicotine addiction. Using a controlled amount of nicotine help to reduce nicotine withdrawal symptoms when one quits from the use of tobacco products (Penton and Lester, 2009; NIH, 2009). 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 (WHO, 2015).

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. Also, an annual 5 million death is attributed to tobacco smoking and it is the second leading cause of mortality among adults worldwide (Aghaji, 2008; NIH, 2009).

Nasal or dry snuff, by contrast, offers tobacco consumer an efficient absorption of nicotine as cigarette smoke inhalation whereas the use of oral or wet snuff is similar to that of pipe and cigar smoking and chewing tobacco (Russell-Wood, 1987). Research has shown that nicotine is very well absorbed from tobacco, very well distributed rapidly and in biologically active concentration to body organs especially the brain. Nicotine has also been implicated as a major cause of the predominant behavioral effects of tobacco and some of its physiologic consequences (Adeniyi and Musa, 2011). Nicotine has been shown to induce a dose-dependent increase in neuronal activity in a distributed system of brain regions, including the nucleus accumbens, amygdala, cingulate cortex, and frontal lobes (Stein *et al.,* 1998; D'Souza and Markou, 2011).

Nicotine is a powerful and complex drug which reacts with excitable cells in many parts of the body and brain (Adeniyi and Musa, 2011). One of the reasons for this is that nicotine is structurally similar to acetylcholine, a vital neurotransmitter, which acts to bridge the synaptic gap between nerve endings. It is structurally similar to acetylcholine and can bind to acetylcholine receptors throughout the body (Ashton and Stepney 1982; Ishibashia, *et al.,* 2014). The effect of nicotine is biphasic in that different dosage levels have differential impacts: a small dose produces a stimulant effect while a large dose acts as a depressant

and an overdose blocks neurotransmission altogether leading to instant death (Rassoulpour, *et al.*, 2005). Besides its interaction and relationship with acetylcholine, nicotine has been shown to release many other types of nerve transmitters, including norepinephrine, epinephrine, serotonin and dopamine, some of which have been shown to be related to hallucinogens (Benowitz, 2010; Ishibashia, *et al.*, 2014) decreasing skin temperature; producing feelings of well-being, arousal, alertness and many others (Matta, 2007; Benowitz, 2009). Nicotine seems to act in such a critical way in the body that there is more than a suspicion that it acts to release primary drives similar to hunger pangs (West and Grunberg 1991; Wilbert 1987). All of these chemical changes in the body result in physiological and psychological changes including changes in blood pressure and pulse rate; increasing and decreasing respiration (Wilbert 1987).

Nicotine at low dose directly stimulates the CNS, especially the brainstem resulting in sympathetic neural discharge, which increases blood pressure and heart rate among other behavioral stimulations (Asvold *et al*, 2014). Nicotine, at high doses, directly stimulates the peripheral nervous system, producing ganglionic stimulation and the release of adrenal catecholamine (Ishibashia, *et al.*, 2014). With very high dose administration of nicotine, hypotension and decreased heart rate result which is mediated by peripheral vagal activation or by direct CNS depressor effects (Tseng *et al*, 1993). Nicotine induced a dose-dependent increase in several behavioral parameters, including feelings of "rush" and "high" and drug liking (Tseng *et al*, 1993).

One of the effects of nicotine is development of tolerance to its own actions; a likely mechanism by which it produces addiction like other addictive drugs. After repeated use of

nicotine, the responsiveness to the drug becomes decreased and increasingly larger doses will be required to produce the same effect (Stein *et al.*, 1998)

Although nicotine is a major chemical constituent of tobacco, which may affects neurobehavioral, histological, hematological, and biochemical activities of some of the organs or systems of the body (Adeniyi, *et al.*, 2010a; Adeniyi, *et al.*, 2010b; Adeniyi and Musa, 2011; Adias *et al.*, 2014), other alkaloids are also present. The other alkaloids include; nornicotine, anabasine, myosmine, nicotyrine and anatabine. These make up 8 to 12 percent of the total alkaloid content of tobacco products. Some of the alkaloids apart from having a direct effect may influence the effect of nicotine e.g. nicotyrine inhibits metabolism of nicotine in animals thereby prolonging the effect (Stahlandske and Slanina, 1982).

An inverse relationship between tobacco product usage and body weight has been documented in many studies. Tobacco users weigh less than non-tobacco users of the same age and gender, and anorexia often occurs with tobacco users (Huot *et al.*, 2004; Adeniyi and Musa, 2011). Cessation of tobacco usage without nicotine replacement therapy is usually accompanied by weight gain (Williamson *et al.*, 1991; O'Hara *et al.*, 1998). As a consequence, some people are willing to use tobacco in order to lose or maintain their body weight, and are reluctant to stop using tobacco due to concerns about weight gain on cessation, especially among youths (Crisp *et al.*, 1999). The effects of tobacco usage on appetite which is known to inhibit appetite may also contribute to the morbidity and mortality of users with chronic lung disease and wasting (Bullo-Bonet *et al.*, 1999; Benowitz, 2010).

Tobacco exerts an inflammatory stimulus on macrophages, which brings about the production of inflammatory cytokines, such as tumor necrosis factor (TNF) α, which might be an important early event in the development of disease states associated with tobacco (Fernandez-Real *et al.*, 2003). Increased TNF α activity (Fernandez-Real *et al.*, 2003) and decreased actions have been observed in nicotine administration studies (Ouyang *et al.*, 2000). TNF α activity is produced by both immunocompetent cells and adipocytes, it can regulate lipid metabolism, adipocyte differentiation, inhibit appetite, and is a mediator of cachexia (Torti *et al.*, 1989; Ventre *et al.*, 1997).

1.2 Statement of Research Problem

Tobacco consumption continues to grow all over the world. Exposure to tobacco nicotine, either from smoking, snuffing or chewing with its numerous toxic and mutagenic substances may have toxic effect on the brain which may alter normal functions of the brain and the whole nervous system. The reason behind the frightening statistical data that reveals the annual mortality rate in Nigeria could be as a result of the scarcity of research done and reported on this subject matter. Neurologic and behavioral changes on cerebrum, cerebellum and hippocampus may alter brain functions and may result to neurological disorder. Hence the need to carry out this research, to study its effects on brain regions.

1.3 Justification/Significance of the Study

The World Health Organization (WHO) has urged government across the world to ban tobacco advertisement, promotion and sponsorship, as part of measures to protect the world's 1.8 billon young people. According to WHO, there are about 2.4 billion people in the world today that consume the tobacco products either in form of snuff, chewing,

smoking or snuff dipping. This representing almost one third of the world population; about 50 – 55 % of men and less than 20 % of women are estimated to be smoking tobacco globally, while 50 % of men and less than 25 % of women are estimated to be using smokeless tobacco globally. Also, annually 5 million deaths is attributed to tobacco smoking and it is 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. These findings and reports suggest the need for further experimental and clinical studies of the effect of tobacco intake on the brain and brain functions.

The result of this study will provide evidence of the effect of tobacco use and enable legislation of laws and public policies with the aim of warning, educating and creating awareness on the dangers of tobacco usage hence may suggest abstinence or decline from the use of tobacco which is detrimental to human health. The result will also add to the few literatures available on the subject matter.

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1 Aim of the Study

The aim of the study was to investigate the effects of aqueous and ethanolic leaf extracts of *Nicotiana tabacum* on the cerebellum, cerebral cortex and hippocampus in adult Wistar rats.

1.4.2 Objectives of the Study

The objectives of the study were to investigate:

i. the neurobehavioral changes using Morris Water Maze test for spartial learning and memory and beam walking test for motor coordination in adult Wistar rats

following oral administration of aqueous and ethanolic leaf extract of *Nicotiana tabacum*.

ii. the effects of aqueous and ethanolic leaf extract of *Nicotiana tabacum* on trace elements in the brain namely Zn, Mn, Fe and Cu in adult Wistar rats using Atomic Absorption Spectrometry (AAS).

iii. the effect on oxidative stress markers namely Catalase (CAT), Superoxide dismutase (SOD), Malondialdehyde (MDA) and Glutathione Peroxidase (GPx) following oral administration of aqueous and ethanolic leaf extract of *Nicotiana tabacum* in adult Wistar rats.

iv. the effect on the histology of the cerebellum, cerebral cortex and hippocampus following administration of aqueous and ethanolic leaf extract of *Nicotiana tabacum* in adult Wistar rats.

1.5 SCOPE OF THE STUDY

The study will be limited to the behavioural, histological, histochemical, neurochemical and biochemical changes in the cerebral and cerebellar cortices, hippocampus and neurobehavioral assessments with administration of aqueous and ethanolic leaf extracts of *Nicotiana tabacum*.

1.6 STUDY HYPOTHESES

Aqueous and ethanolic extracts of *Nicotiania tabacum* will:

i. induce neurotoxicity in the cerebellum, cerebral cortex and hippocampus

ii. induce neurobehavioral changes and depreciation in fine motor movement.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.*1 Nicotiana tabacum*

Nicotiana tabacum is a plant grown throughout the world, and its manufactured products are known to virtually everyone (Ren and Timko, 2001; Kishore, 2014). Tobacco exists in four principal dimensions: botany, chemistry and pharmacology, economics—production and consumption—and history. The tobacco plant is of the genus .*Nicotiana*, one of the larger divisions of the family Solanaceae, otherwise known as nightshades.

There are seventy species in the genus *Nicotiana* alone, 60 per cent of which are native to South America, 25 per cent to Australia and the South Pacific, and 15 per cent to North America (Johannes *et al.,* 2002; Chase *et al.,* 2003; Knapp *et al.,* 2004). According to Thomas Goodspeed, the origin of the genus lies in the South American continent from where it was dispersed to all other continents including Australia. Most authorities in the field are in broad agreement with Good-speed, though some dispute his interpretation of the intercontinental transfer of the genus (Feinhandler *et al.,* 1979). There is further agreement that of all the species in existence, only two, *tabacum* and *rustica*, have been cultivated, and it was these two that generally supplanted the wild species, in the Americas. It is commonly known as Taba in Hausa, Utaba in Igbo and Ewe taba in Yoruba (Aiyeloja and Bello, 2006).

2.1.1 Botanical description

Nicotiana tabacum is a stout viscid annual or limited perennial herb found in South America, North America, Australia and in tropical zones such as Nigeria (Kishore, 2014). This plant is also known as *Nicotiana rustica* which is one of the varieties of tobacco

species that is used in Western North America (Kishore, 2014). *Tabacum* is a coarse, erect, viscidly-hairy annual or perennial herb growing to a height of 1 to 3 meters. Leaves are large, elliptic-ovate to oblong or obovate, 10 to 30 centimeters long or longer, narrowed by the base, and sessile or short-stalked. Flowers usually in terminal panicles. Calyx is green, ovoid or tubular, 1 to 1.5 centimeters long, with five triangular lanceolate teeth. Corolla is white and pink, linear, funnel-shaped and about 5 centimeters long as seen in Figure 2.1. Capsule is ovoid, 1.5 to 2 centimeters long, with very numerous white seeds (Kishore, 2014).

2.1.2 Taxonomy

Kingdom:	Plantae	-	Plant
Subkingdom:	Tracheobiorta	-	Vascular plant
Superdivision:	Spermatophyte	-	Seed plants
Division:	Magnoliophyta	-	Flowering plants
Class:	Magnoliopsida	-	Dicotyledons
Subclass:	Asteridae		
Order:	Solanales		
Family:	Solanaceae	-	Potato family
Genus:	*Nicotiana*	-	Tobacco plant
Specie:	*Nicotiana tabaccum*		

Source: (Kishore, 2014,)



Figure 2.1: *Nicotiana tabacum* leaves (Mali and Rawat, 2013)

2.1.3 Uses of Tobacco

The cured leaf provides a pleasurable and habit-forming narcotic used for smoking, chewing and snuffing.

2.1.3.1 Smoking

Smoking is now practiced throughout the entire world. This use was observed by Columbus in the West Indies in 1492. At this time *Nicotiania tabacum* was grown for this purpose in Central America and Northern and Eastern South America. The dried leaves remain effective for 6 months after drying and used as snuff or are smoked (Grieve, 1995).

2.1.3.2 Edible Uses

The leaves can be dried and chewed as an intoxicant (Charlton, 2004). Protein can be extracted from the leaves. It is an odourless, tasteless white powder and can be added to cereal grains, vegetables, soft drinks and other foods (Facciola, *1990)*. It can be whipped like egg whites, liquefied or gelled and can take on the flavour and texture of a variety of foods. It is 99.5% protein, contains no salt, fat or cholesterol and it is currently being tested as a low calorie substitute for mayonnaise and whipped cream (Facciola, *1990*).

2.1.3.3 Medicinal Uses

Traditionally, this plant had a wide range of application, in Europe where it was first grown as an ornamental it was used medicinally for the treatment of sores and ulcers, snuff was taken for headaches, and was used to scare snakes from the environment. Also plant extract of *Nicotiana tabacum* contribute to the sympathomimetic response to nicotine in the activation of chemoreceptors of the aortic and carotid bodies, these reflex results in vasoconstriction, tachycardia, and elevated blood pressure (Francis *et al.*, 1999). The leaves are antispasmodic, discutient, diuretic, emetic, expectorant, irritant, narcotic, sedative and

sialagogue (Grieve, 1995; Charlton, 2004). They are used externally in the treatment of rheumatic swelling, skin diseases and scorpion stings (Chopra *et al.,* 1986). The plant should be used with great caution (Grieve, 1995), when taken internally it is an addictive narcotic. Aqueous and ethanolic extract of *Nicotiana tobacum* used as medicinal plant extract in treatment of tuberculosis (Adeleye *et al.,* 2008). A homeopathic remedy is made from the dried leaves and it is used in the treatment of nausea and travel sickness (Castro, 1990). *Nicotiana tabacum* plants extract show anti-Alzheimer's activity and improved memory (Scerri, 2005). Nicotine, isolated from leaves of *Nicotiana tabacum* when complexed with zinc and studied for their antibacterial activities revealed antibacterial activity against different types of bacterial strains (Zaidi *et al.,* 2012). Nicotine also possesses a biphasic action on the adrenal medulla: small doses evoke the discharge of catecholamines, and larger doses prevent their release in response to splanchnic nerve stimulation. Nicotine, like acetylcholine is known to stimulate a number of sensory receptors (Cheeta *et al.,* 2000). *Nicotiana tabacum* possess dose-dependent anthelmintic activity hence justifiies its use in traditional system of medicine (Anita *et al.,* 2008). Externally tobacco leaves can be applied to stings in order to relieve the pain, they are also cure for painful piles (Grieve, 1995). In case of bites of poisonous reptiles and insects; hysteria; pain, neuralgia; laryngeal spasm; gout; growth of hair; tetanus; ringworm; rodent ulcer; ulcers; wounds and as respiratory stimulant tobacco leaves are used (Stewart, 1967).

2.1.3.4 Other Uses

All parts of the plant contain nicotine and this has been extracted and used as an insecticide (Grieve, 1995; Charlton, 2004). The juice of the leaves can be rubbed on the body as an insect repellent (Grieve, 1995). The leaves have been dried and chewed as an intoxicant.

The dried leaves are also used as snuff or smoked. This is the main species that is used to make cigarettes and cigars. A drying oil is obtained from the seed (Hill, 1952; Grieve, 1995).

2.1.4 Chemical Composition of Tobacco

The tobacco plant has a general composition which can be found in most other plants. A tobacco leaf contains a complex mixture of chemical components: cellulose products, starches, proteins, sugars, alkaloids, pectic substances, hydrocarbons, phenols, fatty acids, isoprenoids, sterols and inorganic minerals.

i. **Water:** the water content of the leaf decrease during ripening; freshly harvested leaf contains 85-95% and the rest is made up of mineral matter and organic compounds (Seeman *et al.,* 1999). The 85-95% is reduced on wilting and 10-25% on the completion of curing.

ii. **Carbohydrates:** the proportion of carbohydrates to nitrogenous matter increase during ripening and because of the high content, the tissue, particularly in heavy-bodied leaf, becomes brittle and cracks readily when folded between the fingers. Carbohydrate comprises of 20-50 % of the total dry matter of the mature leaf, of which starch and invert sugars are the most important constituents.

iii. **Nitrogenous compound**: Nicotine is the most important nitrogenous compound in tobacco (Wilbert, 1987). In the green leaf most of nitrogen is in the protein fraction, with nicotine second in importance. Green cigar-wrapper leaf contains 3-15% of protein weight and is low in carbohydrates, heavy nitrogenous manuring increase the nicotine content.

iv. **Organic acid**: non-volatile polybasic acids occur in the leaves. During curing citric acid tends to increase; oxalic acid undergo no change; malic acid shows a decided loss.

v. **Polyphenols:** tannin-like occur in important quantities in the leaf and affect and properties of the cured product on the death of the cells in air and fire-curing they are oxidized giving red and brown colours to leaf.

vi. **Minerals:** tobacco has a high content of ash, the quantity in dry leaf ranging from 12-25% the quantity and composition of the mineral components affect the combustibility and other elements of quality in the leaf.

vii. **Ethereal oil and resins:** these occur in the capitate glandular hairs of the leaf and furnish the aromatic principles of tobacco.

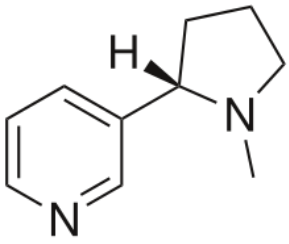
viii. **Chlorophyll and other pigments:** during the early phase of curing the green pigments, chlorophyll are destroyed by oxidation and yellow pigments, carotene and xanthophylls, become evident completion of the yellowing process marks the approximate end of starvation period.

ix. **Alkaloids:** nicotine is the primary alkaloid present in tobacco, it is a plant substance of basic reaction. Other related alkaloids present in tobacco includes nicoteine, nicotimine, anatalline, nor-nicotine, anabasine, myosmine, nicotyrine and anatabine as shown in Figure 2.2 (Wilbert, 1987).

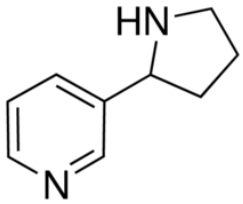
2.1.5 Chemical Composition of Smokless Tobacco

The chemical composition of tobacco undergoes substantial changes during growing, curing, processing and storing (Peele *et al*., 2001; Bush *et al*., 2001). The purpose of curing

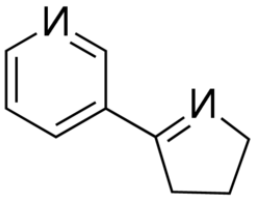
is to produce a dried leaf of suitable physical properties and chemical composition. At the beginning of curing, a tobacco leaf is metabolically active and continues to live until biochemical processes are arrested by thermal effects or desiccation. In curing, the starch content of the leaves declines drastically, while the amount of reducing sugars increases by 100% (Walton *et al.*, 1995). Protein and nicotine contents decrease slightly. The bulk of the processed tobacco leaf before fermentation consists of carbohydrates of about 50% and proteins (wiernik *et al.*, 1995).



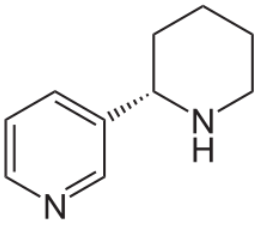
Nicotine



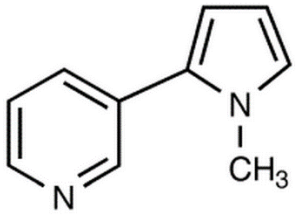
Nornicotine



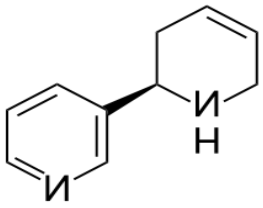
Myosmine



Anabisin



Nicotyrine



Anatabine

(Bush *et al*., 2001)

Figure 2. 2: The chemical structures of some alkaloids present in *Nicotiana tabacum*.

Fermentation of cured tobacco causes the contents of carbohydrates and polyphenols in the leaves to diminish. Other major components are alkaloids about 0.5–5.0%, which include nicotine as the predominant compound of about 85–95% of total alkaloids, terpenes of about 0.1–3.0%, polyphenols of 0.5–4.5%, phytosterols of 0.1–2.5%, carboxylic acids of 0.1–0.7%, alkanes of 0.1–0.4%, aromatic hydrocarbons, aldehydes, ketones, amines, nitriles, *N*- and *O*-heterocyclic hydrocarbons, pesticides, alkali nitrates of 0.01–5%) and at least 30 metallic compounds (Brunnemann and Hoffmann, 1992; IARC, 2004).

Because of the disappearance of carbohydrates and polyphenols during fermentation, heavy casings and additives applied during processing such as molasses, liquorice and fruit extracts are added to tobacco to meet the consumer's requirements which improve taste, flavour and aroma, and prolong shelf-life. Many smokeless tobacco formulations use plant extracts or chemicals as flavouring agents (Mookherjee and Wilson, 1988; Roberts, 1988; Sharma *et al*., 1991). Tobacco additives may include glycerol triacetate, cellulose acetate (Filter bonding Agent); titanium dioxide (Whitening Agent); guar gum (Binder); polyvinyl

acetate (Adhesive); sugar; phenolic resin; glycerol (Humectant); Hexanal (Flavour), lemon Oil (Flavour); propyl-PHB, potassium sorbate (preservative); cocoa, glycyrrhizin and theobromine (Bronchodilators); methyl or ethyl salicylate, β-citronellol, 1,8-cineole, menthol, benzyl benzoate, eugenol and possibly coumarin, among others (LaVoie *et al.*, 1989; Stanfill *et al.*, 2006). Eugenol which is a creamy Snuff sold in India. Gupta, 2004) and menthol are used to numb the throat and facilitate tobacco use (Ahijevych and Garrett, 2004; Wayne & Connolly, 2004).

2.1.6 Pytochemical Properties of *Nicotiana tabaccum*

Nicotiana tabaccum contain biologically active constituents that includes alkaloids, flavonoids, cardiac glycosides, tannins, saponins, steroids, terpenoid, carbohydrates and glycosides, depending on the solvent (aqueous or organic) used in the phyto chemical extraction (Mensah *et al.*, 2009; Ojo *et al.*, 2012).

2.1.7 Adverse Health Effects of Smokeless Tobacco

There are many health problems associated with the use of tobacco, these include diseases affecting the heart (circulatory diseases) and lung, excess saliva production and drooling, bad breath; bad taste in mouth, sores, patches, and lumps in mouth or neck, difficulty in chewing, swallowing and moving tongue or jaw, Receding gums; permanent gum loss, Stained and sensitive teeth or tooth decay, stains on clothing, smelly hair and clothes, damage to the respiratory system, decreased lung capacity, coughing, bronchitis and asthma, addiction to nicotine, increased likelihood of drug use and risky behaviour (Sivanandam, 2010). The main harmful effects of tobacco are;

I. Cardiac complications

Tobacco use has many adverse effects on the heart, including hypertension, blocked blood vessels, weakened pumping of the heart, narrow arteries leading to heart attack and death. In addition, weakened blood flow to the brain can cause strokes.

II. Lung disorders

Smoking causes chronic bronchitis, changing the size and shape of the airways of the lungs, enlarging the mucous glands, and causing coughing and production of excess phlegm. It is also the leading cause of emphysema, a lung condition marked by an abnormal increase in the size of the air spaces, resulting in labored breathing and an increased susceptibility to infection (Sivnandam, 2010).

III. Thrombosis

Cigarette smoking is a major risk factor for acute coronary thrombosis. Indeed, a majority of sudden cardiac deaths attributable to acute thrombosis are in cigarette smokers. It causes the majority of sudden cardiac deaths and myocardial infarctions. Both active and passive cigarette smoke exposure seem to increase the risk of coronary thrombosis and myocardial infarctions. Cigarette smoke exposure seems to alter the hemostatic process via multiple mechanisms, which include alteration of the function of endothelial cells, Platelets, fibrinogen, and coagulation factors. This creates an imbalance of antithrombotic/prothrombotic factors and profibrinolytic/anti-fibrinolytic factors that support the initiation and propagation of thrombosis (Barua and Ambrose, 2013).

IV. Anti-fertility Effects

Tobacco use can cause reproductive damage, including abnormal sperm cells and impotence, decrease in sperm motility and concentration as well as decreased

spermatogenic cells in the histology of the testis in men and menstrual disorders, early menopause, and difficulty maintaining pregnancy in women (Gambo *et al.,* 2013; Messi *et al.,* 2017).

V. Cancer

Lung, upper respiratory tract, and cervical cancers are primarily found in smokers while stomach cancer is mainly found in chewing tobacco users. Other cancers that can attack users of both forms of tobacco include cancers of the larynx, mouth, throat, pancreas, kidney, and bladder (Sivanandam, 2010). People who indulge in tobacco chewing have higher risk of oral cancer to people who take alcohol. The most infected area in oral cancer is the tongue and the area below the tongue (Ebbert *et al.,* 2004). The release of these harmful chemicals from tobacco may explain why tobacco use has been strongly associated with oral cancer (Johnson and Bain 2000, Reibel 2003, WHO 2003a). The cancer slowly spreads to cheeks and throat. Though it can attack any part lips, tongue, upper and lower mouth, the cheeks, or gums and oesophagus. It is very important to go for early diagnosis as soon as one feels suspicious. Lung cancer: chewing tobacco leads to oral cancer but it is not the end of it can spread the disease in lungs and linings of stomach. Reports show that 90% of lung cancers are cases of people who either smoke or chew tobacco. Destructive agents termed as carcinogens in tobacco injure the cells in the lungs. Over a period of time, these damage cells may develop into lung cancer (Sivanandam, 2010).

VI. Caries: The ingredients of tobacco consist of gravels, sand, and other harmful chemicals that erode the enamel of tooth. Continuous chewing leads to early loss of tooth. Early Decay of Tooth: Chewing leaves small particles in tooth that forms

bacteria and plaque; it harms enamel and gums, which leads to decay of tooth. Gum slump: Chewing leads to decomposing of gums, the gums get infected and the grip on tooth loosens which exposes the sensitive area of tooth (sham *et al.,* 2003).

VII. **Halitosis:** There is nothing as bad as bad breadth of a person; they are major turn off for people around them. The long-term habit of chewing and spitting is unacceptable and looks indecent (Purseglove, 1951).

VIII. **Thesaurus**: Eating habit of people who chews tobacco tends to be unhealthy, continuous chewing affects the taste bud and the sensitivity of them decreases. This leads to an increase in intake of more salt, sugar and spices in food as he feels a bland taste in his mouth. (Pirie, 1961).

IX. **Other health complications**

The use of tobacco during pregnancy can lead to miscarriage, stillbirth, low birth weight, premature birth, or sudden infant death syndrome. Children born to women who use tobacco during pregnancy can develop upper respiratory problems, ear complications, asthma, and learning and behaviour problems. Other damage that long-term tobacco use can cause includes prematurely wrinkled skin, gum and tooth loss, lost or weakened sense of taste and smell, weakened immune system, stomach ulcers, and unwanted weight fluctuation (Sivanandam, 2010).

2.1.8 Historical Overview

The tobacco plant is thought to have originated on the mainland between North and South America. Its cultivation probably dates back at least 5000 years; tobacco seeds were discovered during archaeological excavations in both Mexico and Peru around 3500 BC, which shows that tobacco was an article of value to the inhabitants (Voges, 1984).

American Indians were probably the first people to smoke, chew and snuff tobacco, as early as the 1400s (Christen *et al.,* 1982).

2.1.8.1 Tobacco chewing

In 1499, Amerigo Vespucci found Indians on Margarita Island, off the coast of Venezuela, who chewed a green herb known as tobacco in order to quench their thirst, since it produced an increase in salivation; he also reported that the Indians chewed tobacco leaves to whiten their teeth and to alleviate hunger (Heimann, 1960; Stewart, 1967; Voges, 1984).

The practice of tobacco chewing was widespread in parts of Central and South America in the late 1500s (Voges, 1984). Columbus, in 1571, observed men in Veragua, later known as Costa Rica, who put a dry herb in their mouths and chewed it (Heimann, 1960). Tobacco chewing seems to have been a common practice among the American Indians, especially when long distances had to be covered; it has been reported that tobacco was the support against hunger, thirst and fatigue when an American Indian would trek for 2 or 3 days with no other support. Several American tribes mixed either lime or finely-powdered and burned, fresh- or saltwater molluscs with their chewing tobacco (Curtis, 1935).

Among Native Americans, chewing tobacco was thought to have several medicinal uses, such as to alleviate toothache, to disinfect cuts by spitting the tobacco juice and saliva mixture onto the wound, and to relieve the effects of snake, spider and insect bites (Axton, 1975). Tobacco was soon grown in Europe as both a decorative and medicinal plant. In 1559, Jean Nicot, in whose honour the genus *Nicotiana* was named, was ambassador to Sebastian, King of Portugal. He grew tobacco and promoted the product in Europe for its magic ‘cure-all’ properties (Christen *et al.,* 1982). Portuguese and Spanish sailing crews

who were addicted to tobacco carried seeds, and planted them at ports. When smoking was forbidden on British naval vessels because of the fire hazard, sailors turned to chewing tobacco and snuff. In Europe, tobacco was regarded as a prophylactic during the plague and, for those who did not like smoking, chewing was an alternative. Tobacco chewing was recommended for cleaning the teeth of women and children (Brooks, 1952). During the 1860s, tobacco was chewed in the form of either a plug or a twist. Of the 348 tobacco factories listed in the 1860 Census for Virginia and North Carolina, only seven manufactured smoking products (Heimann, 1960).

In 1797, Adam Clarke, a famous Methodist minister, appealed to all tobacco consumers and religious followers to avoid the use of tobacco for the sake of their health and their souls. This plea was also due to the fact that it had become unsafe to kneel when praying because chewers had made the floors unsanitary (Brooks, 1952). By the 1890s, public outcry made tobacco chewing socially unacceptable behaviour and unlawful in most public places (Christen *et al.,* 1982). Nevertheless, chewing remained the dominant form of tobacco use in America until the expansion of the cigarette industry in 1918 (Maxwell, 1980).

2.1.8.2 Snuff taking

The native populations of Brazil were the first people known to use snuff. Using a cup and a pestle made from rosewood, the tobacco leaves were ground into a powder and acquired the delicate aroma of the wood. The resulting snuff was placed in ornately decorated bone tubes, one end of which was plugged to preserve the fragrance (Curtis, 1935). The American Indians inhaled powdered tobacco through a hollow Y-shaped piece of pipe by

placing the forked ends into each nostril and the other end near the powdered tobacco (Christen *et al.*, 1982).

In Haiti, snuff powder was used by medicine men for clearing nasal passages and as an analgesic (Stewart, 1967). The Dutch, who named the powdered tobacco 'snuff', were using the product by 1560 (Christen *et al.*, 1982). By the early 1600s, snuff had become an expensive commodity and its use had spread throughout South America, China, Japan and Africa.

In 1620, the Royal Snuff Factory was established in Seville, and this became the centre of the manufacture and development of this product (Voges, 1984). Snuff use expanded through Japan to China (Ching Dynasty) in the 1650s: The Chinese believed that snuff cured pains in the eyes and teeth, alleviated throat ailments, constipation and cold symptoms, and promoted sweating (Christen *et al.*, 1982).

Snuff use reached a peak in England during the reign of Queen Anne (1702–14), and was called the 'final reason for the human nose'. It was at this time that ready-made snuff became available in England. It continued to be popular during the reign of George III, and his wife, Charlotte (1760–1820), referred to as 'Snuffy Charlotte', had an entire room in Windsor Castle devoted to her snuff stock. The first snuff mills in America were constructed in Virginia in about 1730 (Heimann, 1960). The snuff was made from New England tobacco and its quality was said to equal that of the native Scottish varieties (Robert, 1949).

The use of smokeless tobacco products in the USA was widespread throughout the nineteenth century, Dental snuff was advertised to relieve toothache; to cure neuralgia,

bleeding gums and scurvy; and to preserve and whiten teeth and prevent decay (Christen *et al.*, 1982).

Tobacco was introduced into South Asia in the 1600s as a product to be smoked and was gradually used in many different forms (Bhonsle *et al.*, 1992; Gupta and Ray, 2003). The chewing of betel quid (pan) was a popular practice that existed for over 2000 years and extended eastwards as far as the South Pacific Islands. After its introduction, tobacco soon became a new ingredient in betel quid, which has become the most commonly used form of smokeless tobacco in South Asia (Gupta and Ray, 2003; IARC, 2004a).

2.1.8.3 Attitudes and beliefs regarding smokeless tobacco use

The use of tobacco, including smokeless tobacco, has been controversial since its introduction. Therefore, a history of smokeless tobacco use is not complete without a discussion of the attacks on tobacco by various groups. In 1590 in Japan, tobacco was prohibited, and users lost their property or were jailed. James VI of Scotland, who became King James I of England and Ireland in 1603, was a strong anti-smoking advocate and increased taxes on tobacco by 4000% in an attempt to reduce the quantity imported into England. In 1633, the Sultan Murad IV of Turkey made any use of tobacco a capital offence, punishable by death from hanging, beheading or starvation, and maintained that tobacco caused infertility and reduced the fighting capabilities of his soldiers. The Russian Czar Michael Fedorovich, the first Romanov (1613–45), prohibited the sale of tobacco, and stated that users would be subject to physical punishment; persistent users would be killed. A Chinese law in 1638 threatened that anyone who possessed tobacco would be beheaded (Christen *et al.*, 1982).

During the mid-1600s, Pope Urban VIII banned the use of snuff in churches, and Pope Innocent X antagonised its use by priests in the Catholic Church. Other religious groups banned snuff use: John Wesley (1703–91), the founder of Methodism, antagonised its use in Ireland; similarly, the Mormons, Seventh-Day Adventists, Parsees and Sikhs of India, Buddhist monks of Korea, members of the Tsai Li sect of China, and some Ethiopian Christian sects forbade the use of tobacco (Christen *et al.*, 1982). In Bavaria, Germany, in 1652, tobacco was available only on a doctor's prescription; Frederick the Great, King of Prussia, prevented his mother, the Dowager Queen of Prussia, from using snuff at his coronation in 1790. Louis XV, ruler of France from 1723 to 1774, banned the use of snuff from the Court of France (Christen *et al.*, 1982).

In 1761, John Hill, a London physician and botanist, concluded that nasal cancer could develop as a consequence of snuff use. He reported five cases of 'polypusses, a swelling in the nostril that was hard, black and adherent with the symptoms of an open cancer' (Redmond, 1970).

2.2 THE CENTRAL NERVOUS SYSTEM

The central nervous system CNS is made up of the brain and the spinal cord. The vertebrae of the spine encase and protect the soft neural tissue of the spinal cord, just like the skull protects the brain. The motor and sensory nerves running throughout the body make up the peripheral Nervous System.

2.2.1 Brain

The brain is a spongy, three pound mass of tissue, it is the most complex living structure in the universe. With the capacity to create a network of connections that far surpasses any social network and stores more information than a supercomputer. This single organ

controls every aspect of our body, ranging from heart rate and sexual activity to emotion, learning and memory. The brain controls the immune system's response to disease, and determines, in part, how well people respond to medical treatments. Ultimately, it shapes our thoughts, hope, dreams and imaginations. It is the ability of the brain to perform all of these functions that makes us human (Pelvig *et al.*, 2008).

2.2.2 Gross Anatomy of the Brain

The brain is composed of the cerebellum, cerebrum and brainstem. When the calvaria and dura are removed, gyri (folds), sulci (grooves) and fissures (clefts) of the cerebral cortex are visible through the delicate arachnoid-pia layer. Whereas the gyri and the sulci demonstrate much variation, the other features of the brain, including overall brain size, are remarkably consistent from individual to individual.

2.2.2.1 Cerebrum

The cerebrum includes the cerebral hemispheres and basal ganglia. The cerebral hemisphere, separated by the falx cerebri within the longitudinal cerebral fissure, are the dominant features of the brain. Each cerebral hemisphere is divided for descriptive purposes into four lobes, each of which is related to, but the boundaries of which do not correspond to, the overlying bones of the same name from a superior view, the cerebrum is essentially divided into quarters by the median longitudinal cerebral fissure and the coronal central sulcus. The central sulcus separates the frontal lobes from the parietal lobes. In a lateral view, these lobes lies superior to the transverse lateral sulcus and the temporal lobe inferior to it. The posteriorly placed occipital lobes are separated from the parietal and temporal lobes by the plane of the parieto-occipital sulcus, visible on the medial surface of the cerebrum in a hemisected brain. The anterior most points of the anteriorly projecting

frontal and temporal lobes are the frontal and temporal poles. The posterior most point of the posteriorly projecting occipital lobe is the occipital pole. The hemisphere occupy the entire supratentorial cranial cavity. The frontal lobes occupy the anterior cranial fossae, the temporal lobes occupy the lateral parts of the middle cranial fossae, and the occipital lobes extend posteriorly over the tentorium cereblli.

i. The diencephalon is composed of the epithalamus, dorsal thalamus, and hypothalamus and forms the central core of the brain.
ii. The midbrain, the rostral part of the brainstem, lies at the junction of the middle and posterior cranial fossae. CN III and IV are associated with the midbrain.
iii. The pons is the part of the brainstem between the midbrain rostrally and the medulla oblongata caudally; it lies in the anterior part of the posterior cranial fossa. CN V is associated with the pons
iv. The medulla oblongata (medulla) is the most caudal subdivision of the brainstem that is continuous with the spinal cord; it lies in the posterior cranial fossa. CN IX, X, and XII are associated with the medulla, whereas CN VI-VIII are associated with the junction of pons and medulla.
v. The cerebellum is the large brain mass lying posterior to the pons and medulla and inferior to the posterior part of the cerebrum. It lies beneath the tentorium cerebelli in the posterior cranial fossa. It consist of two lateral hemispheres that are united by a narrow middle part, the vermis.

2.2.2.2 Cerebellum

The cerebellum or small brain lies in the posterior cranial fossa. In adult the weights of the cerebellum is about 150 g. It has a superficial layer of grey matter, the cerebellar cortex. It lies behind the pons and the medulla. It separated from the cerebrum by a fold of dura matter called tentorium cerebelli (Singh, 2002). Its function include posture and coordination of movements of eyes, limbs as well as that of the head. Further it is involved in motion that has been learned and perfected though practice, and will adapt to new learned movements (Kandel *et al.,* 2000). Despite its previous classification as a motor structure, the cerebellum also displays connections to areas of the cerebral cortex involved in language as well as cognitive functions.

2.2.2.3 Hippocampus

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. A likely pathway by which the prefrontal cortex controls memory retrieval in the dorsal hippocampus is via prefrontal projections to the perirhinal and lateral entorhinal cortex, where object representations are processed and sent unto the hippocampus. Furthermore, recent evidence suggests there is a bidirectional flow of information between the medial prefrontal cortex and hippocampus, where in the events that initiate prefrontal control over memory retrieval in the hippocampus may arise in the ventral part of the hippocampus (Komorowski *et al.,* 2013). In humans and other primates, the hippocampus is located inside the medial temporal lobe, beneath cortical surface (Amaral *et al.,* 2006).

2.2.3 Histology of the Brain

2.2.3.1 Cerebrum

The cerebral hemispheres consist of a convoluted cortex of grey matter overlying the central medullary mass of white matter which conveys fibres between different parts of the cortex and to and from other parts of the CNS.

Histologically the neurons in the neocortex are arranged into six layers as shown in Figure 2.7, the layers differing in neuron morphology, size and population density. The layers merge with one another rather than being highly demarcated and vary somewhat from one region of the cortex to another depending on cortical thickness and function, characteristics of each layer being as follows:

i. ***Plexiform (molecular) layer***: This most superficial layer mainly contains dendrites axons of the cortical neurons making synapses with one another; the sparse nuclei are those of neuroglia and occasional horizontal cells of cajal.

ii. ***Outer granular layer****:* A dense population of small pyramidal cells and stallate cells make up this thin layer which also contains various axons and dendritic connections from deeper layers.

iii. ***Pyramidal cell layer****:* Pyramidal cells of moderate size predominate in this broad layer, the cells increasing in size deeper in the layer. Martinotti cells are also present.

iv. ***Inner granular layer****:* This layer consist mainly of densely packed stallate cells. Forms found in this layer. It contains numerous small pyramidal cells

and cells of martinotti, as well as stallate cells, especially superficially and fursifrom cells in the deepest layer.

v. ***Ganglionic layer****:* Large pyramidal cells and smaller numbers of stallate cells of Martinotti make up this layer originating from the huge pyramidal (ganglion) Betz cells of the motor cortex.

vi. ***Multiform cell layer****:* This is so named for the wide variety of differing morphological forms found in this layer.it contains numerous small pyramidal cells and cells of Martinotti, as well as stellate cells, especially superficially and fusiform cells in the deep part.

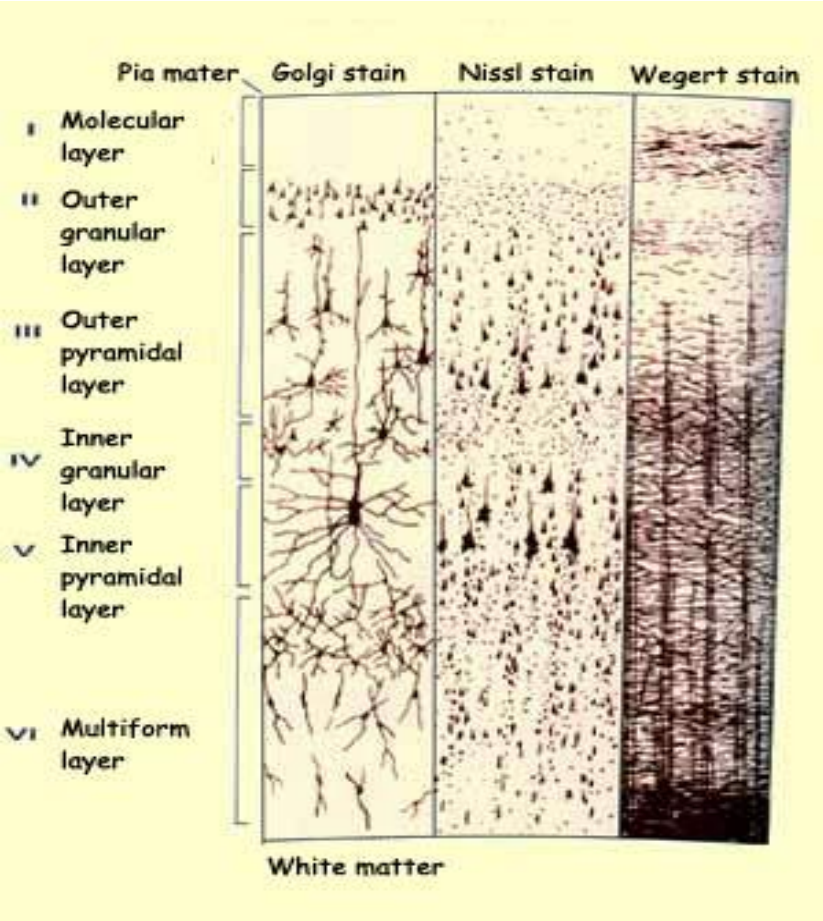


Figure 2.3: Histological characteristics of the cerebreal cortex (Kandel *et al.*, 2000)

2.2.3.2 Cerebellum

The cerebellum, which coordinates muscular activity and maintains posture and equilibrium, consists of a cortex of grey matter and with a central core of white matter containing four pairs of nuclei. Afferent and efferent fibres pass to and from the brain stem via inferior, middle and superior cerebellar peduncles linking medulla, pons and midbrain respectively.

2.2.3.3 Cerebellar Cortex Organization

The cerebellar cortex forms a series of deeply convoluted folds or folia supported by a branching central medulla of white, the cortex is seen to consist of three layers:

i. The outer molecular layer
ii. The inner granular layer
iii. Purkinje cells layer

i. ***Molecular layer***: The outer molecular layer is made up of few neurons and large numbers of unmyelinated fibres, basket cells, Stellate cells and as well parallel fibres, which are the axons of the granule cells. The extensive dendritic tree of the Purkinje cell extends into the molecular layer (Young *et al.,* 2007).

ii. ***Purkinje layer***: Purkinje layer of cerebellar cortex is the middle and most important layer of the cerebellar cortex. Purkinje cells have very large cell bodies, a relatively fine axon extending down through the granular cell layer, and extensively branching dendritic system which arborizes into the outer molecular layer. All of the inputs to the cerebellum are directed toward influencing the firing Purkinje cells, and only axons of Purkinje cells leave the cerebellar cortex (Young *et al.,* 2007).

iii. ***The inner granular cell layer***; it is the innermost layer of the cerebellar cortex and it is deep and contains Golgi cells, granule cells, and glomeruli cells. The non-myelinated axons of the numerous small neurons present in the granular layer ascends outwards to the molecular layer where they bifurcate to run parallel to the surface to synapse with the dendrites of the Purkinje cells (Young *et al.,* 2007). Each glomerulus is surrounded by a glial capsule and contains a granule cell and axons of Golgi cells, which synapse with granule cells. The granule is the only excitatory neuron within the cerebellar cortex.

2.2.3.4 Hippocampus

This relative small structure of the limbic system is absolutely vital for learning and memory in all mammals. These brain regions have a highly complex innervation patterns and takes on the shape of a “sea horse”, i.e. hippocampus as shown in figure 2.4.

The trisynaptic circuit is a relay of synaptic transmission in the hippocampus, which is made up of three major cell groups: granule cells, CA3 ("Cornu Ammonis" area 3) pyramidal neurons, and CA1 ("Cornu Ammonis" area 1) pyramidal cells (Andersen, 1975). The hippocampal relay involves 3 main regions within the hippocampus which are classified according to their cell type and projection fibers. The first projection of the hippocampus occurs between the entorhinal cortex and the dentate gyrus. The entorhinal cortex transmits its signals from the parahippocampal gyrus to the dentate gyrus via granule cell fibers known collectively as the perforant path. The dentate gyrus then synapses on pyramidal cells in CA3 via mossy cell fibers. CA3 then fires to CA1 via Schaffer collaterals which synapse in the subiculum and are carried out through the fornix. Collectively the

dentate gyrus, CA1 and CA3 of the hippocampus compose the trisynaptic loop (Amaral, 1995).

EC → DG via the perforant path (synapse 1), DG → CA3 via mossy fibres(synapse 2), CA3 → CA1 via schaffer collaterals(synapse 3) (Amaral, 1995).

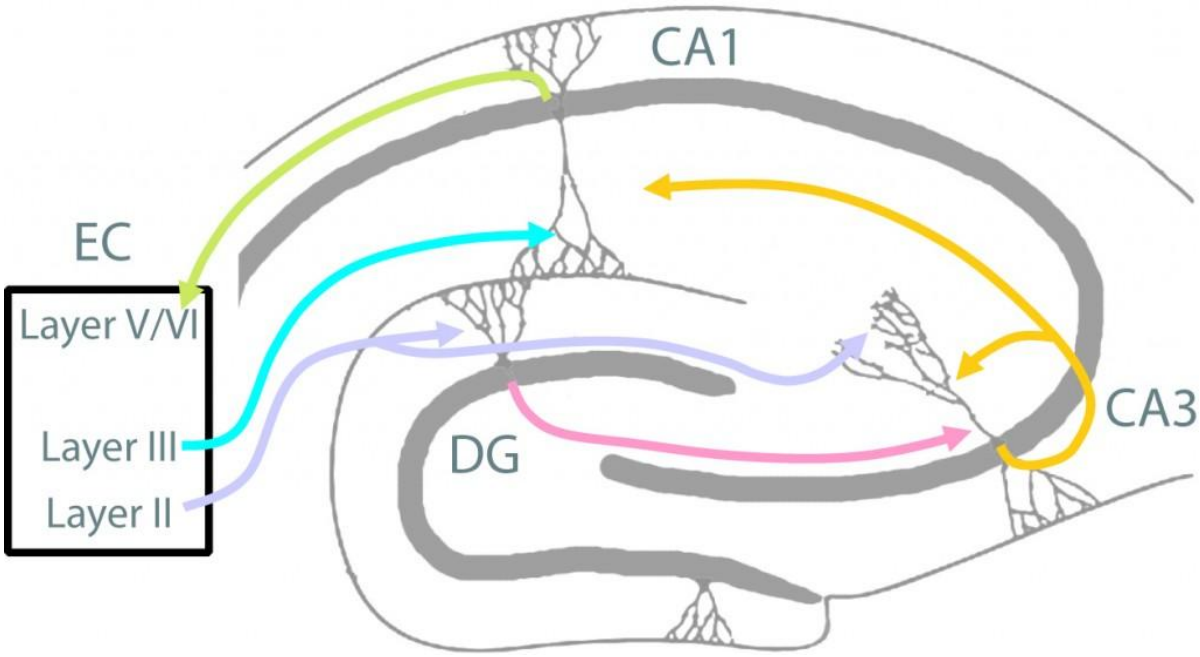


Figure 2.4 Histological regions of the Hippocampus (Masurkar *et al.*, 2017)

2.2.4 Functions of the Brain

2.2.4.1 Cerebrum

The cerebrum is the largest and "newest" part of the human brain and is made up of the cortex. Major regions of the cortex are responsible for the processing of our sensation, how we receive the world. The frontal lobe is implicated in motor control, complex thoughts, associations, and social thinking. The parietal lobe is the key for all our perception. The occipital lobe processes all visual input while the temporal lobe processes both auditory (hearing) and olfactory (smell) input (Ganong, 2005).

2.2.5.2 Cerebellum

Flocculonodular lobe, or vestibulocerebellum, is formed by the nudulus in the vermis and the flanking flocculus in the hemisphere on each. The lobe, which is phylogenetically the oldest part of the cerebellum, has vestibular connection and is concerned with equilibrium and learning-induced changes in the vestibulocerebellum. Spinocerebellum; form by the vermis and the adjacent medial portions of the hemispheres receives proprioceptive input from the motor cortex. By comparing plan with performance, it smoothes and coordinates movements that are proximal limb muscles, whereas the hemisphere project the brainstem areas concerned with control of distal limb muscles.

Cerebrocerebellum, or neocerebellum. The lateral portions of the cerebellar hemispheres. They are the newest from a phylogenetic point of view, reaching their greatest development in humans. They interact with the motor cortex in planning and programming movements. The cerebellum is concerned with learned adjustments that make coordination easier when a given task is performed over and over. As a motor task is learned, activity in the brain shifts from the prefrontal areas to the parietal and motor cortex and the cerebellum. The

basis of the learning in the cerebellum is probably the input via olivary nuclei. It is worth noting in this regard that each Purkinje cell receives input from 250,000 to 1 million mossy fibres, but each has only a single climbing fibre from the inferior olive, and the fibres makes 2000- 3000 synapses on the Purkinje cell. Climbing fibre activation produces a large, complex spike in the Purkinje cell; and this spike in some way produces long term modification of the pattern of mossy fibre input to the particular Purkinje cell. Climbing fibre activity is increased when a new movement is being learned, and selective lessions of the olivary complex abolish the ability to produce long term adjustment in certain motor responses (Ganong, 2005).

2.2.5.3 Hippocampus

Hippocampus (and its adjacent temporal and parietal lobe structures, all together called the hippocampal formation) has numerous but mainly indirect connections with many portions of the cerebral cortex as well as with the basal structures of the limbic system- the amygdala, the hypothalamus. The septum, and the mammillary bodies. Almost any type of sensory experience causes activation of at least some part of the hippocampus, and the hippocampus in turn distributes many outgoing signals to the anterior thalamus, hypothalamus, and other parts of the limbic system, especially through the fornix, a major communicating pathway. Thus, the hippocampus is an additional channel through which incoming sensory signals can initiate behavioural reactions for different purposes. As in other limbic structures, stimulation of different areas in the hippocampus can cause almost any of the different behavioural patterns such as pleasure, rage, passively, or excess sex drive.

Portions of the hippocampus have been surgically removed bilaterally in a few human beings for treatment of epilepsy. These people can recall most previously learned memories satisfactorily. However, they often can learn essentially no new information that is based on verbal symbolism. In fact, they often cannot even learn the names of the people with whom they come in contact every day, yet they can remember for a moment or so what transpires during the course of their activities. Thus they are capable of short- term memory for seconds up to a minute or two, although their ability to establish memories lasting longer than a few minutes is either completely or almost completely abolished. This suggests that the hippocampus is associated with memory and learning. It has been suggested that the hippocampus provides the drive that causes that cause translation of short-term memory into long term memory- that is, the hippocampus transmits some signal or signals that seem to make the mind rehearse over and over the new information until permanent storage takes place (Guyton and Hall, 2006)

2.3 NEUROBEHAVIOURAL STUDIES

There are a wide variety of neurobehavioral tests available for laboratory rodents, from tests of basic locomotor and function, to analyses of more complex behaviour related to cognition and emotionality.

2.3.1 Beam Walking Test

Motor coordination and balance is assessed by measuring the ability of the experimental animals to traverse a narrow beam to reach an enclosed safety platform (home cage) (Carter *et al.*, 1999; Fan *et al.*, 2008). The motor coordination accessed is particularly of the hind

limbs, using the beam walking test. The difficulty of this task can be varied by using beams with different shapes and widths (Jover *et al.*, 2006).

2.3.2 Morris Water Maze Test

The Morris water navigation task, also known as the Morris water maze, is a behavioural procedure widely used in behavioural neuroscience to study spatial learning and memory. It was developed by a neuroscientist, Richard G. Morris in 1981, who used it to show that lesions of the hippocampus impaired spatial learning (Morris, 1981)

In the typical paradigm, a rat is placed into a small pool of water-back-end and first to avoid stress, and facing the pool-side to avoid bias-which contains an escape platform hidden a few millimetres below the water surface. Visual cues, such as colored shapes, are placed around the pool in plain sight of the animal. The pool is usually 1.2 to 1.8 meter in diameter and 60 centimetres deep. The pool can also be half-filled with water to 30 centimetres in depth. A sidewall above the waterline prevents the rat or mouse from being distracted by laboratory activity; and from climbing out of from the pool. When released, the subject swims around the pool in search of an exit while various parameters are recorded, including the time spent in each quadrant of the pool, the time taken to reach the platform (latency), and total distance travelled. Escape from the water reinforces a desire to quickly find the platform, and on subsequent trials (with the platform in the same position) subjects are able to locate the platform increasingly rapidly. This improvement in performance occurs presumably as a result of learning and memory for where the hidden platform is located relative to the conspicuous visual cues. After enough practice, a capable rat or mouse can swim directly from any point to the platform. There are a variety of paradigms for the water maze that can be used to examine different cognitive functions

(D'Hooge and Deyn, 2001), in particular, cognitive flexibility can be assessed using a water maze paradigm in which the hidden platform is continually re-located (Saab *et al.*, 2011)

CHAPTER THREE

3.0 MATERIAS AND METHODS

3.1 Plant Materials

3.1.1 Collection and Identification of Plant

The leaves of *Nicotiana tabacum* were collected February 14th 2016 from Zaria town, Zaria, Kaduna State, Nigeria. The leaves were taken to the Herbarium Unit of the Department of Biological Science, Faculty of Life Sciences, Ahmadu Bello University, Zaria for identification and authentication, and a voucher specimen number was provided as 540**.**

3.2 Experimental Animals

For the purpose of this research, forty two apparently healthy Wistar rats were obtained from the Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences Ahmadu Bello University Zaria. The animals were housed in clean environment in the animal House of the Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, 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 clean water. The animals were categorized into control and treatment groups. The animals were weighed before the commencement of the experiment, with an average weight 150 g.

3.3 Animal Feed

Pelletized growers feed manufactured by The Grand Cereals and Oil Mills Limited (GCOML) Km 17, Zawan roundabout, Plateau State, Nigeria. It was obtained and used to feed the animals for the experiment.

Composition of feed:

i. Crude protein (13 %)
ii. Crude fibre (8 %)
iii. Fat (15 %)
iv. Calcium (0.80 %) and phosphorus (0.40 %)
v. Others constituents include: cereals, essential amino acids/proteins, vegetables, animal protein, minerals, antibiotics, salt, antioxidant and Vitamins

3.4 Set Up of Neurobehavioral Studies

For the purpose of this study; two neurobehavioral setups were constructed for the neurobehavioral test. These were:

i. Morris Water Maze (MWM) for testing learning and memory
ii. Beam Walking Maze (BWM) for testing motor coordination and balance

3.5 Methods

3.5.1 Preparation of *Nicotiana Tabacum* Leaf Extract from tobacco leaves

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 Sarojini *et al*. (2011), for the preparation of aqueous and ethanolic extracts were employed. The procedure of preparation was as follows:

The leaves were air dried in an enclosed environment and pulverized using laboratory mortar and pestle 450 g of the powder was soaked in sufficient solvents of distilled water

and 100 % ethanol and allowed to stand for 24 hours and 72 hours respectively, after which the solutions were filtered using whatman filter paper 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 ^{0}C and 50 ^{0}C respectively until the solvent has evaporated. The residue (extracts) were scraped into a suitable container. %Yields of tobacco extract were calculated in order to find out the effective solvent of extraction.

3.5.2 Phytochemical screening

Phytochemical screening of the extract for the presence of secondary metabolites was performed with standard methods using the following reagents and chemicals: alkaloids with Mayer's reagent and Dragendorff's reagent (Farnsworth, 1966; Harborne, 1998), flavonoids with the use of 10% lead acetate and 20% sodium hydroxide (Sofowora 1993, Trease and Evans 1996), phenols and tannins with 2% ferric chloride solution (Yadav and Agarwala, 2011) and saponins with ability to produce suds (Houghton and Raman, 1998). Steroid with Liebermann-Buchard test consisting of a mixture of glacial acetic acid and sulphuric acid (Shoppee, 1964**).** Anthraquinones with Borntragers test consisting of filtrate from the mixture of the extract and benzene with 10% ammonia solution (Sofowora, 1993).

3.5.3 Dose Preparation of Aqueous and Ethanolic Extracts of *Nicotiana Tabacum*

The extracts were measured and dissolved in distilled water. This made up the working solution. From freshly prepared working solution, little portion was measured out for animal administration (Appendix III).

Acute toxicity (LD_{50}) study: Acute toxicity study was carried out using the method of Lorke, (1983). 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- phase 1 and phase 2. The animals in each phase were further sub-divided, randomly, into three groups of three rats each. In the first phase, the three groups (A, B and C) were administered aqueous extract of *Nicotiana tabaccum* (10, 100 and 1000 mg/kg body weight) by oral gavages respectively. In the second phase, the three groups (D, E and F) consisting of one rat each were administered aqueous extract of *Nicotiana tabaccum* (1600, 2900 and 5000 mg/kg body weight) by oral gavages, respectively. The rats were observed for signs of toxicity and mortality for 24 hours. At the end of the LD_{50} study, the LD_{50} for ethanolic extract was above 5000 mg/kg body weight as no mortality was recorded for ethanolic treated group at 5000 g/kg body weight, however for the aqueous treated group, one case of mortality was observed in the group treated with 5000 mg/kg body weight of the second phase and the LD_{50} was calculated to be 3807 mg/kg body weight. The LD_{50} of aqueous extract was adopted for the ethanolic extract.

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

3.6 Experimental Design

In this study, a total number of 42 Wistar rats were obtained and distributed into seven groups with 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 bwt) 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 as shown in Table 3.1.

Table 3.1 Experimental design table of oral administration of Aqueous and Ethanolic leaf extracts of *Nicotiana tabacum* in adult Wistar Rats.

Groups	Treatment and daily dose	Duration (days)	Day of Sacrifice
1	Control(normal saline) 0.2 ml/kg body weight)	21	22nd
2	Aqueous extract HD (1000 mg/kg body weight)	21	22nd
3	Aqueous extract MD (500 mg/kg body weight)	21	22nd
4	Aqueous extract LD (250 mg/kg body weight)	21	22nd
5	Ethanol extract HD (1000 mg/kg body weight)	21	22nd
6	Ethanol extract MD (500 mg/kg body weight)	21	22nd
7	Ethanol extract LD (250 mg/kg body weight)	21	22nd

LD= low Dose, MD= Medium Dose, HD= High Dose

3.7 Amount of Food Intake

The amount of feed intake was determined measuring the same amount of feed in (grams) given to all the groups. The remaining feed was then subtracted from the total feed given to get the amount the amount of feed consumed by each group.

Food consumed (g) = Total Feed (g) – Feed remain (g)

3.8 Measurement of Animal Weights

The weights of the animals were measured using electronic compact scale KERRO BL20001. Records of the weights were carried out weekly in grams (g).

3.9 Neurobehavioral Studies

All experimental procedures were done in the Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The following neurobehavioral tests were conducted:

3.9.1 Beam Walking Test

Motor coordination and balance was assessed by measuring the ability of the animals to traverse a narrow beam to reach an enclosed safety platform (Carter *et al.,* 1999; Fan *et al.,* 2008). In this test three parameters were recorded which include; (A) Time spent on platform TP in seconds, (B) Latency: which is the time (in seconds) taken by the animals to traverse the beam TW from point A (starting point) to B (safety Box) as shown in plate I, it was used to assess learning and memory (C) Number of Foot slips (NFS) which was used to assess the level of balance and coordination.

Procedure: the test consisted of elevated platforms connected by a 100 cm long wooden beam with a width of 3 cm. The beam was placed horizontally, with one end mounted on a narrow support and the other end attached to a goal box (20 x 20 x 20 cm).

Beam-walking ability was measured using a 6-point scale described by (Puurunen, 2001): the rat that was not able to stay on the beam were assigned *0 point*; the rat that did not move, but was able to stay on the beam was assigned *1 point*; the rat that tried to traverse the beam , but fell was assigned *2 points*; the rat traversed the beam with more than 50% (foot slips > 4) foot slip of hind limb were assigned *3 points*; the rat traversed the beam with more than one foot slip, but less than 50%, were assigned *4 points;* the rat that had only one foot slip of the hind limb, were assigned *5 points*; the rat that traversed the rat that traversed the beam without any hind limb slips, were assigned *6 points*. Beam walking ability was recorded in seconds; the time the animal spent on the platform, the time spent to traverse the beam. Number of foot slips (hind limbs) for each rat was determined by scoring each rat on the platform based on the number of foot slip made by the hind limb as shown plate I.

In addition, as described by Abou-Donia *et al*. (2008), the time taken (latency) until the animal's nose enter the goal box (up to 60 s) was recorded. Rats that fell off the beam or did not entered the goal box were assigned latencies of 60 s. Beam walk scores were based on an average of 3 trials, each separated by 30 minutes per rats.

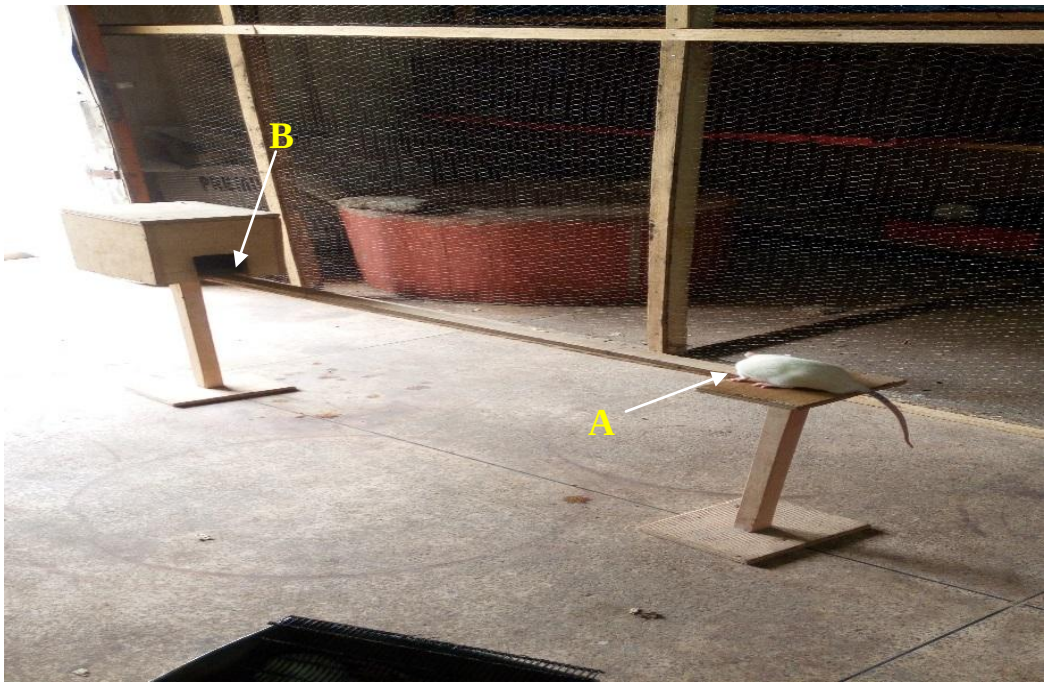


Plate I: Beam Walking Test setup

3.9.2 Morris Water Maze

The Morris Water Maze (MWM) which was first established by neuroscientist Richard G. Morris in 1981 was used in order to test hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory (Morris, 1981). 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 (A) as shown in plate II were assessed to test for spatial memory.

3.9.2.1 Equipment Preparation

i. A circular pool with a diameter of 150 cm and a depth of 50 cm was obtained with gray coated interior.

ii. 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).

iii. The room was arranged in such a way that the animals being tested cannot see the interior of the experimental apparatus during testing.

iv. The bottom of the pool was divided into four equal parts with a vertical and horizontal marking, using a black paint.

3.9.2.2 Testing Procedure

i. 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.

ii. To begin testing, the rat were lifted from the home cage at the tail, supported around the neck, and gently place into the water, facing the edge of the pool. The researchers quickly moved away from the testing pool.

iii. 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 sec before returning it to its home cage.

iv. The process was repeated for all rats in the trail. Each subsequent trial was started with a different platform location and starting direction of north, east and west.

v. 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.



Plate II: Morris water maze setup. (A) = safety platform

3.10 Animal Sacrifice

At the end of the administration (day 21), animals in both control and experimental groups were humanely sacrificed using chloroform anaesthesia and brain tissues from the animals were collected by opening through the mid-sagittal suture of the skull. 1 g of brain tissues were homogenized, transferred to a centrifuge tubes for the estimation of neurotrace elements (Zn, Cu, Fe and Mn) and superoxide dismutase (SOD), catalase (CAT), glutathione and malondaldehyde (MDA) for oxidative stress makers to determine damage to brain tissues by reactive oxygen species, see Appendix IV. The remaining brain tissues in various groups were then transferred into the specimen bottles containing Bouin's fluid for fast fixation.

3.11 Morphological Assessment

3.11.1 Change in Weight

All experimental animals' body weights were taken; at the onset of treatment, weekly throughout the study and at the end of the study before sacrifice. Twelve hours after the last administration, the Wistar rats were humanely sacrificed using chloroform anaesthesia.

3.11.2 Brain Weight Changes

The brain weight were recorded for analysis after sacrifice. The ratio of the body weights to the brain weights were taken, and the brain tissues were fixed in Bouin's fluid.

3.11.3 Relative Brain weight

Relative brain weights were determined by recording the weights of the brain and body weight of the animal. Then the percentage of the ratio of brain to body weight was calculated.

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

3.12 Biochemical and Neurochemical Analysis

3.12.1 Sample collection and preparation of tissue homogenates

Animals were sacrificed and the brains were quickly removed. The brain tissues containing cerebellum, cerebral cortex and hippocampus were collected, and then 1 g of tissues were immediately homogenized. The tissue homogenates were centrifuged at appropriate centrifugal force for 10 min and the supernatant were utilized for different estimations according to the method of Bradford (1976).

3.12.2 Malondialdehyde assay

MDA levels in brain samples were measured by the method of Uchiyama and Mihara (1978). This method depends on the formation of MDA as an indicator of lipid peroxidation, which reacts with thiobarbituric acid producing thiobarbituric acid reactive substances (TBARS), measured spectrophotometrically at 532 nm.

3.12.3 Superoxide dismutase activity assay

Activity of SOD enzyme was evaluated by the method of Sun *et al. (*1988). The analysis of SOD was based upon the principle in which xanthine reacts with xanthine oxidase to produce superoxide radicals. The SOD activity was measured by the level of suppression of this reaction. Results were expressed as U/mg protein.

3.12.4 Catalase activity assay

Catalase enzyme activity was evaluated spectrophotometrically by the method of Goth, 1991. Using a coupled assay system. The assay include 7 mM H_20_2 as the substrate for

catalase and followed loss H_20_2 over time as a measure of the initial rate of catalase activity (expressed as μmol H_20_2 min^{-1}.mg protein-1).

3.12.5 Glutathione assay

Glutathione peroxidase concentration was measured by calculating the content of non-protein sulfhydryl groups by technique described by Sedlak *et al.,* 1968. 400 uL of the homogenate was added to 320 uL of distilled water and 80 uL of trichloroacetic acid, mixture was centrifuge for 15 min at 3000 rpm and then 400 uL of supernatant was taken and added to 800 uL of Tris-HCl buffer (0.4 M, pH 8.9) and 20 uL of 5, 5'- dithiobis (2-nitrobenzoic acid) 0.1 M and after 1 min the reading was made at 412 nm.

3.13 Neurochemical Studies

Neurochemical analysis for metals (Cu, Fe Mn and Zn) estimation was done using the brain tissue. Rats' brain were dissected out, weighed and homogenized in 0.1 M Phosphate Buffer Solution PBS (pH 7.4), 1 g of brain tissue/ 4 ml of PBS (Bouras *et al.,* 1996). The homogenate were then centrifuged and aliquots of the supernatant were obtained for neurochemical analysis. Estimation of the metals (Cu, Fe Mn and Zn) in the brain tissue homogenate was done using Atomic Absorption Spectrophotometer (AAS-AA240SF, Varian) at the Multiuser Laboratory, Department of Chemistry, Faculty of Physical Sciences, Ahmadu Bello University Zaria (Appendix IV).

3.14 Histological Studies

Harvested brain tissues were fixed in Bouin's fluid and tissues processed for routine histological examination, stained with haematoxylin and eosin (H&E), cresyl fast Violet staining techniques and examined under the light microscope.

3.14.1 Tissue Processing

This was done by passing the tissue through ascending grades of alcohol of 70%, 90% and 95% sand 100% for dehydration for two changes of (50 minutes each changes respectively). It was then passed into xylene for 40 minutes to clear the alcohol and make it mixable with molten paraffin wax. After this, tissue was transferred into molten paraffin wax for infiltration (Drury and Wallington, 1973). Embedding commenced with the use of molten paraffin as the embedding media at a temperature of 60^0C for 40 minutes. After the tissue were embedded in paraffin wax, they were mounted on wooden blocks and then sectioned as follows, the blocked tissue were affixed to a rotating microtome and trimmed to get an even surface prior to sectioning. Thin section 8 - 10 microns each were then cut and the sections were floated on warm water bath to spread the tissue properly prior to mounting on a glass slide. The tissue were then passed through changes of xylene for dewaxing and then taken to water by passing the slide with the tissue intact through descending grades of alcohol-absolute, 100%, 95%, 90%, and 70%, two changes each for each and finally water. The tissues were stained with Crysel fast violet stain and haematoxylin for twenty-five minutes, the excess of which was rinsed off under running tap water. Counter staining for Haematoxylin was then done using eosin, the excess of which was also rinsed off under tap water (Appendix II).

After proper staining, the sections were dehydrated through ascending grades of alcohol, followed by clearing with xylene. DPX was used as mountant and the slide were covered with cover slip (Appendix I). Slides were viewed under light microscope and photomicrograph obtained using scope photo digital camera.

3.15 Statistical Data Analysis

Results obtained were analysed using Statistical Package for Social Scientist (SPSS version 20.0) and results were expressed as mean ± 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 $p<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical Analysis

The extraction of tobacco leaves from two (2) solvents such as Water and ethanol gave different percentage yield of extractions. Table 4.1 shows the % yield of the aqueous and ethanolic extract of *Nicotiana tabacum* leaves. It was observed that the ethanolic extract has the highest percentage yield (13.7%) when compared to aqueous extract which had a yield of 8.8%. A screening of the phytochemical composition as presented in Table 4.2 showed the presence of carbohydrates, cardiac glycosides, saponnins, flavonoids, tannins, triterpenes and Alkaloids in all the extracts while none of the extracts possess anthraquinones. Steroids were detected in only ethanolic extract and absent in aqueous extract.

4. 2 Acute Toxicity Study

In the acute toxicity study (Table 4.3a&b), there was no death among the rats that received 10-

1000mg/kg body weight of both extracts. No death recorded in the rats that received 1600 mg/kg bwt and 2900 mg/kg bwt of aqueous and ethanolic extracts. The rat that received 5000 mg/kg in the aqueous group died within 24hrs, however the rat that received 5000 mg/kg body weight of the ethanolic extract did not die. The oral LD_{50} of the aqueous extract of *Nicotiana tabacum* was calculated to be 3807 mg/kg body weight and was adopted as LD_{50} for the ethanolic extract in adult Wistar rats.

Table 4.1: The percentage yield ofaqueous and ethanolic extracts of *Nicotiana tabacum*

	Weight (g)		
Solvent	Tobacco leaves	Crude extract	% Yield
Water	450	39.6	8.8
Ethanol	450	62.0	13.7

Table 4. 2 Phytochemical Constituents of the leaf extracts of *Nicotiana tabacum*

	Aqueous extract	**Ethanol extract**
Constituents	Inference	Inference
Carbohydrates	+	+
Athraquininones	-	-
Cardiac glycosides	+	+
Glycosides	+	+
Saponnins	+	+
Flavonoids	+	+
Tannins	+	+
Steroids	-	+
Triterpenes	+	+
Alkaloids	+	+

+ = Positive (present)
- = Negative (absent)

Table 4.3a: Oral acute toxicity of the leaf extracts of *Nicotiana tabacum* in adult Wistar rats.

Groups	Dosage mg/kg	No of Death		Percentage of Death (%)	
		Aqueous	Ethanolic	Aqueous	Ethanolic
A	10	0	0	0	0
B	100	0	0	0	0
C	1000	0	0	0	0

Table 4.3b: Oral acute toxicity of the leaf extracts of *Nicotiana tabacum* in adult Wistar rats.

Groups	Dosage mg/kg	No of Death		Percentage of Death (%)	
		Aqueous	Ethanolic	Aqueous	Ethanolic
D	1600	0	0	0	0
E	2900	0	0	0	0
F	5000	1	0	100	0

4.3 Physical Observation

During the period of administration of the aqueous and ethanol extracts of *Nicotiana tabacum* to the experimental animals, the animals were observed for any change in physical activity and behavioural pattern. Physical activity of the present study revealed normal movement in all groups, altered feeding patterns as food and water intake in the tobacco treated groups was reduced when compared to the control group although it was not significant. Also, animals in the experimental group displayed unusual level of restlessness and aggression when compared to the control groups. There were no significant changes in the skin colour and arrangement, the colour of their eyes was normal compared to the control groups. Also, the gross anatomy of the brain of the tobacco administered groups appeared normal when compared to the control groups

4.3.1 Body Weights

The weights of the Wistar rats before and after the experiments were observed and subjected to statistical analysis. The result showed that body weight of the Wistar rats in the control group which had access to distilled water and normal chow was significantly increased, $P<0.05$. While body weights in all the treated groups decreased when compared with the control group with a level of significance observed in Groups 2, 6 and 7, $P<0.05$ as shown in Figure 4.1. The result also showed that body weight of rats treated with ethanolic extract decreased more when compared with the aqueous extract treated group.

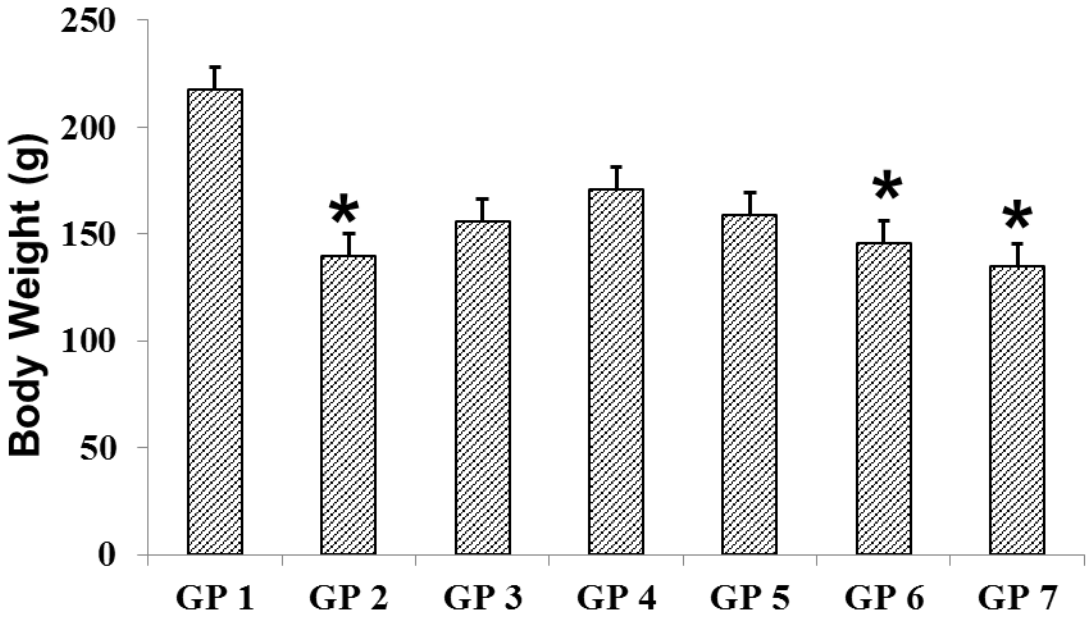


Figure 4. 1 Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on body weight of Wistar rats at day 21 *P<0.05 one way ANOVA followed by Tukey's post hoc test, n= 6

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

4.3.2 Amount of Food Intake

Over the whole experimental period, daily food intake of Control group was normal. However food intake level in the treated Groups was altered when compared to the Control as shown in Figure 4.2 and Table 4.4. The result showed that the amount of food intake in the tobacco treated Groups; 2, 3, 4, 6 and 7 were lower when compared to the control. Also in comparing between the aqueous and ethanolic treated Groups, the amount of food consumed in the aqueous treated Groups were less when compared to the ethanolic treated Groups. However the amount of food consumed of rats exposed to tobacco was reduced after 21 days of treatment as shown in Table 4.4. There was a time effect on the weight of Control rats, reflecting weight gain, which was opposite in all the tobacco treated groups with significant difference observed in Groups 2, 6 and 7 as shown in Figure 4.1. Body weight of the tobacco treated group was reduced within 21 days of exposure to tobacco.

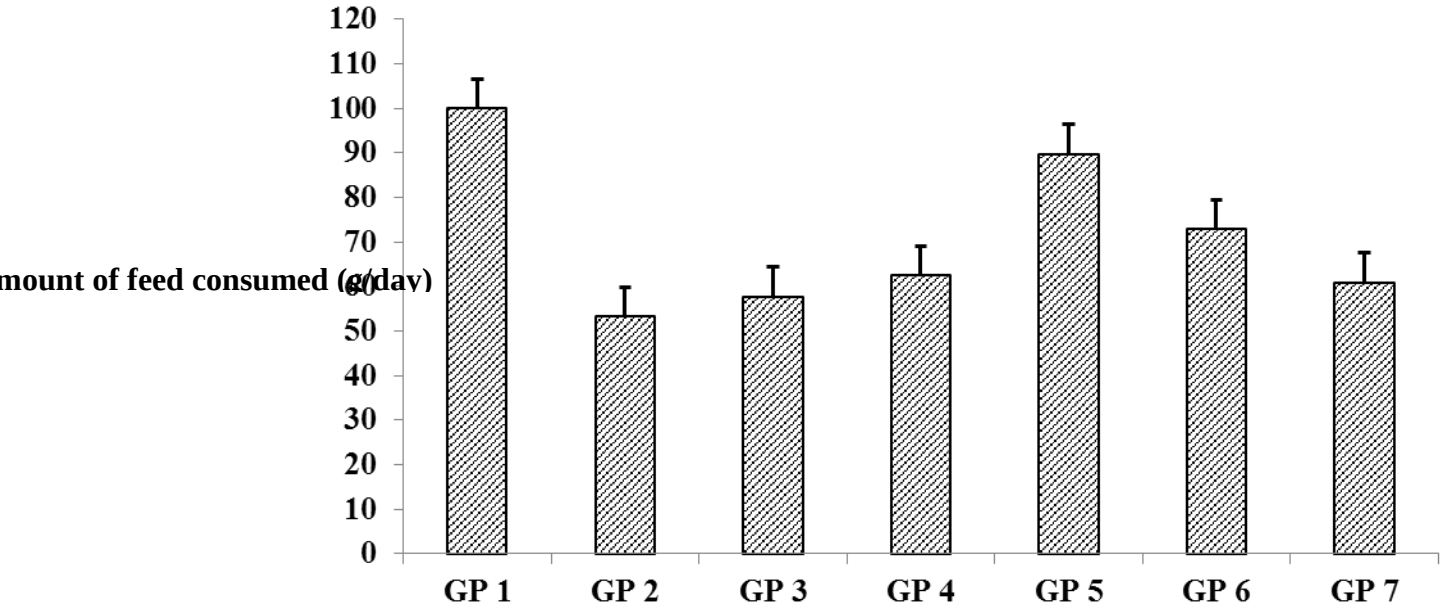


Figure 4.2: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on the Amount of feed consumed. P>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

Table 4.4: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on the Amount of food consumed.

Groups	Day 16 (g)	Day 19 (g)	Day 21(g)
1 Control	100.0	100.0	100.0
2 ANT HD	52.3	50.9	86.9
3 ANT MD	55.7	55.6	61.8
4 ANT LD	51.0	65.2	71.4
5 ENT HD	92.2	88.2	89.2
6 ENT MD	81.9	78.2	58.4
7 ENT LD	64.0	48.8	70.0

ANT = Aqueous extract of *Nicotiana tabacum*, ENT = Ethanolic extract of *Nicotiana tabacum*, HD = High Dose, MD = Medium Dose, LD = Low Dose. P>0.05

4.3.3 Brain Weight in Relation to body Weight.

The average brain weight for treatment groups after the experiment reduced. As shown in Figure 4.3 and Table 4.5. The result showed lower brain weight in the treatment Groups when compared to the Control Group, although the difference was not significant P>0.05.

4.3.4 Relative Brain Weight (RBW)

The result showed that the RBW of the rats in all the experimental Groups were higher than the control though not significant P>0.05. 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 other experimental Groups as shown in Table 4.5.

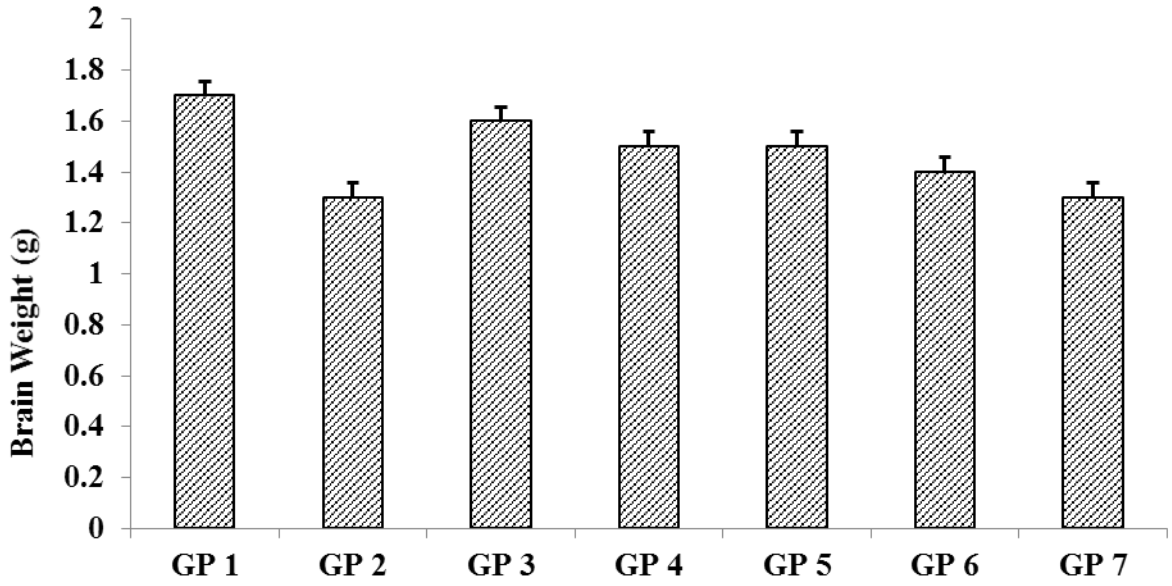


Figure 4.3: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Brain weight of Wistar rats. P>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

Table 4. 5 The Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Relative brain weight in Wistar rats.

Groups	Brain weight (g) Mean ± SEM	Body weight (g) Mean ± SEM	RBW (%)
GP 1 Control	1.73±0.15	217.33± 15.87	0.79
GP 2 ANT HD	1.30±0.64	139.50±69.59	0.94
GP 3 ANT MD	1.60±0.11	155.83±16.67	1.03
GP 4 ANT LD	1.54±0.55	170.80±24.43	0.90
GP 5 ENT HD	1.52±0.75	159.00±16.06	0.96
GP 6 ENT MD	1.42±0.12	145.67±19.40	0.95
GP 7 ENT LD	1.30±0.65	135.00±69.48	0.96

P>0.05**;** ANT = Aqueous extract of *Nicotiana tabacum,* ENT = Ethanolic extract of *Nicotiana tabacum,* HD = High Dose, MD = Medium Dose, LD = Low Dose.

4.3 **Neurobehavioral Studies**

4.3.1 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) shown in Table 4.4. The difference in the mean latency time observed following treatment with aqueous and ethanolic extracts of *Nicotiana tabacum* increased in all groups except groups 2, 4 and 5 as shown in Figure 4.4. At the end of the administration the result showed increase in the mean latency time of ethanolic treated groups when compared with groups treated with aqueous extracts of *Nicotiana tabacum* though the increase was not significant $P>0.05$.

Table 4.6: Mean training and test time before and during administration of *ANT* and *ENT* in Morris Water Maze (MWM).

GROUPS	Training time (sec)	Week 1 (sec)	Week 2 (sec)	Week 3 (sec)
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
GP 1	10.86 ± 2.29	11.41±5.45	7.53 ± 2.97	11.69±11.08
GP 2	8.11 ± 2.29	8.45±5.58	9.50±9.46	6.55±5.02
GP 3	9.12 ± 2.28	7.25±3.14	13.12±5.27	10.23±4.16
GP 4	11.94 ± 2.95	8.32±6.80	9.98±4.35	10.83±6.20
GP 5	10.40 ± 2.63	13.52±11.24	10.23±8.53	8.36±3.74
GP 6	10.66 ± 2.63	11.88±9.95	7.04±2.46	12.10±11.33
GP 7	9.58 ± 1.56	8.27±4.30	8.86±3.44	12.65±8.83

P>0.05**;** ANT = Aqueous extract of *Nicotiana tabacum*, ENT = Ethanolic extract of *Nicotiana tabacum.*

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

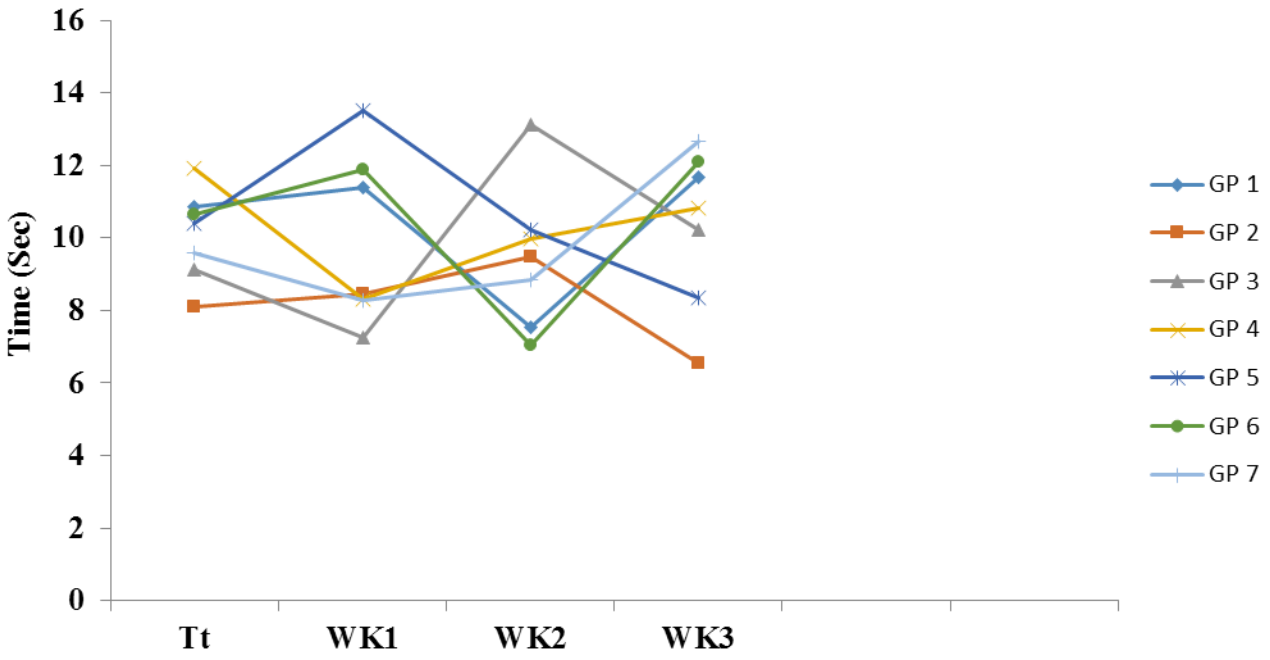


Figure 4.4: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Mean latency time in MWM.

n = 6; Tt = Mean Training time (Before Administration); WK = Week.

4.3.2 Beam walking Test

The result of the effects of *Nicotiana tabacum* administration on motor coordination and balance following 60 seconds Beam walking in tobacco treated groups of adult Wistar rats was observed and recorded.

The results showed that all the groups had increased time spent on the platform (TP) after administration with groups 2, 3, 4, and 7 having higher time when compared to the mean training time Tt and Control group though the increase was not significant as shown in Figure 4.5.

The result also showed that all the groups had increased latency time (LT) following administration of the extracts though the increase was not significance when compared to the mean training time (Tt) and Control Group as shown in Figure 4.6. The results showed that the Number of Foot Slip (NFS) was decreased in Groups 2, 3, 6 and 7 following administration of the extracts when compared to the Control Group though the decrease was not significant as shown in Figure 4.7.

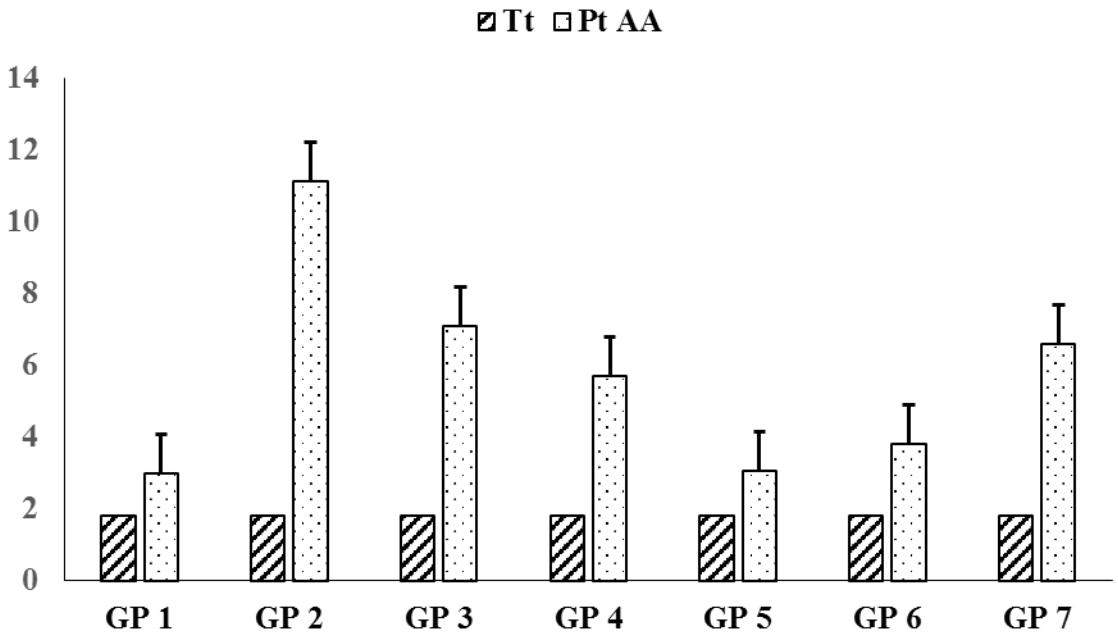


Figure 4.5: Effect of oral administration of aqueous and ethanolic extracts of *Nicotiana tabacum* on Beam walking test (latency on Platform). P>0.05

Tt = Mean Training time on platform (Before Administration); Pt AA = Time on platform After Administration.

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

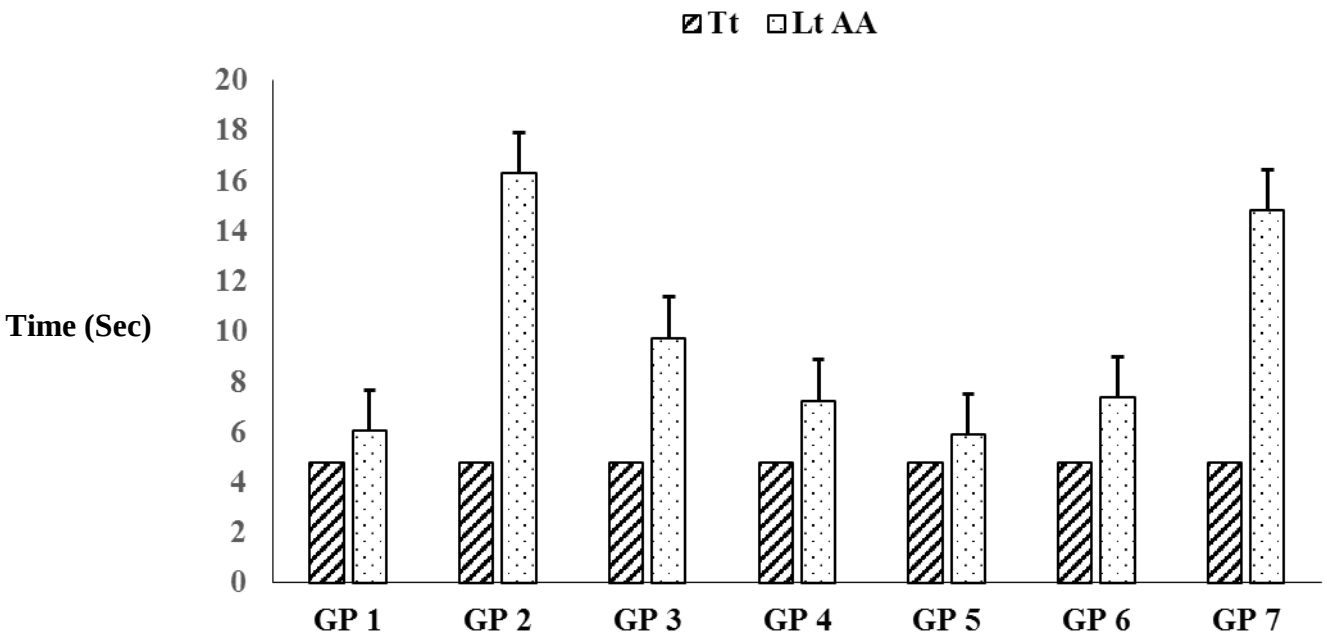


Figure 4.6: Effect of oral administration of aqueous and ethanolic extracts of *Nicotiana tabacum* on Beam walking test (Latency time) in adult Wistar rats.

Tt = Mean Training time (Before Administration); Lt AA = latency Time After Administration. p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

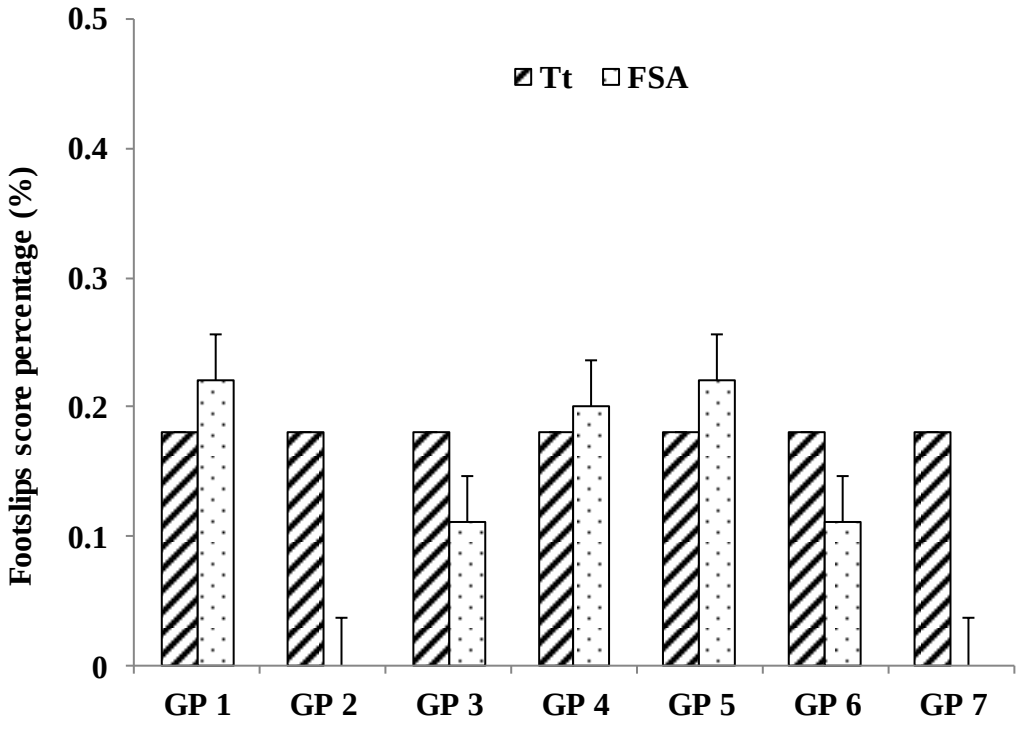


Figure 4.7: Effect of oral administration of aqueous and ethanolic extracts of *Nicotiana tabacum* on Beam walking test (Number of Footslip) in adult Wistar rats.

Tt = Mean Training time (Before Administration); FSA = Footslip After Administration. p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg)

4.4 Biochemical Studies

Biochemical analyses for lipid peroxide levels and antioxidant enzyme activity namely Malondialdehyde, (MDA); superoxide dismutase, (SOD); catalase, (CAT) and glutathione peroxidase, (GPx) were assessed in brain tissue homogenate of Wistar rats. The result showed that there was increased MDA activity in all the treated Groups especially with the ethanolic extract treated groups, though the increase was not significant $P>0.05$ when compared to the Control Group. The result also showed that the level of CAT in all the treated groups was decreased when compared to the Control Group, the decrease was significant in Groups 2, 4, 5, 6 and 7 ($P<0.05$). The result of SOD level decreased in the treated Groups when compared to Control Group. The decrease in SOD activity was significant in the Groups 3, 5, 6 and 7 ($P<0.05$) when compared to the Control Group as shown in Table 4.4. There was increase in GPx level in Groups 2 and 3 when compared to the Control Group though the increase was not significant ($P>0.05$) while on the other hand GPx activity decreased in Groups 4, 5, 6 and 7 respectively. However the decrease in GPx level in Groups 4 and 5 were not significant but were significant in Groups 6 and 7 ($P<0.05$) as shown in table 4.4. In comparing the effects of the aqueous and ethanolic extracts of *Nicotiana tabacum* on the oxidative stress markers, the level CAT, SOD and GPx decreased more in the ethanolic treated groups ($P<0.05$) than the aqueous treated group.

Table 4.7: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on brain tissue oxidative stress markers in adult Wistar rats.

GROUPS	CAT(µg/ml) Mean ± SEM	MDA(µg/ml) Mean ± SEM	SOD(nmol/ml) Mean ± SEM	GPx (µg/ml) Mean ± SEM
1	48.00±1.41	1.00±0.14	2.45±0.21	47.50±2.12
2	43.50±2.12*	1.20±0.14	2.25±0.21	49.00±2.82
3	45.50±2.12	1.15±0.21	2.10±0.14*	49.00±2.82
4	41.50±0.70*	1.25±0.21	2.15±0.07	46.50±2.12
5	43.00±1.41*	1.40±0.14	1.75±0.07*	45.50±0.70
6	39.00±1.41*	1.30±0.14	1.90±0.01*	43.00±1.41*
7	36.00±1.41*	1.30±0.28	1.70±0.14*	40.00±0.00*

CAT = Catalase, MDA = Malondaldehyde, GPx =Glutathione Peroxidase, SOD = Superoxide Dismutase, *P<0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

4.5 Neurochemical Studies

Four neurotrace elements were analysed following oral administration of aqueous and ehanolic extracts of *Nicotiana tabacum*. They include Manganese (Mn), Iron (Fe), Zinc (Zn) and Copper (Cu). This study assessed the concentration of neuro trace elements in brain tissue homogenates of adult Wistar rats. The results showed Manganese (Mn) level was slightly increased in Groups 3, 4, 5 and 6 respectively but was not significant as shown in Figure 4.8. The level of Iron (Fe) when compared to the Control was increased in Groups 3, 6 and 7 respectively and decreased in Groups 4 and 5 though the difference was not significant ($P>0.05$) as shown in Figure 4.9. The result of Zn level showed slight increased across the treated groups, with Group 3 showing more higher level of increase when compare to the Control group though the increase was not significant ($P>0.05$) as shown in Figure 4.10. The result also showed a slight decrease in the level of Cu in Groups 6 and 7 though the decrease was not significant ($P>0.05$) as shown in Figure 4.11.

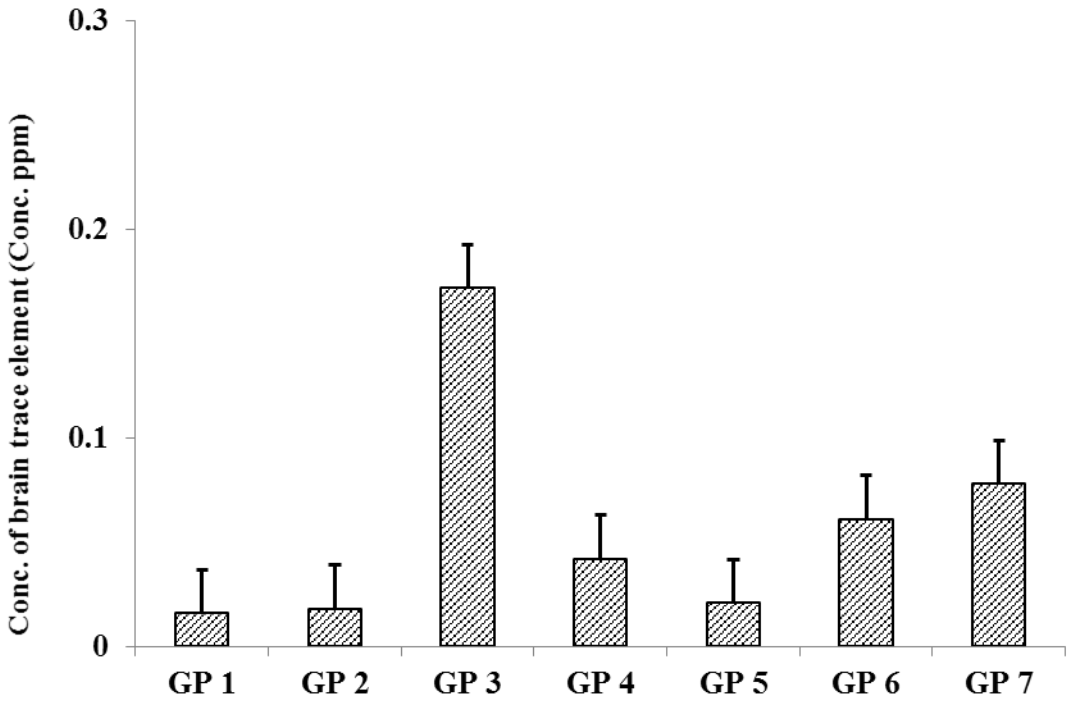


Figure 4.8: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Neuro trace elements (Mn) in adult Wistar rats. p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

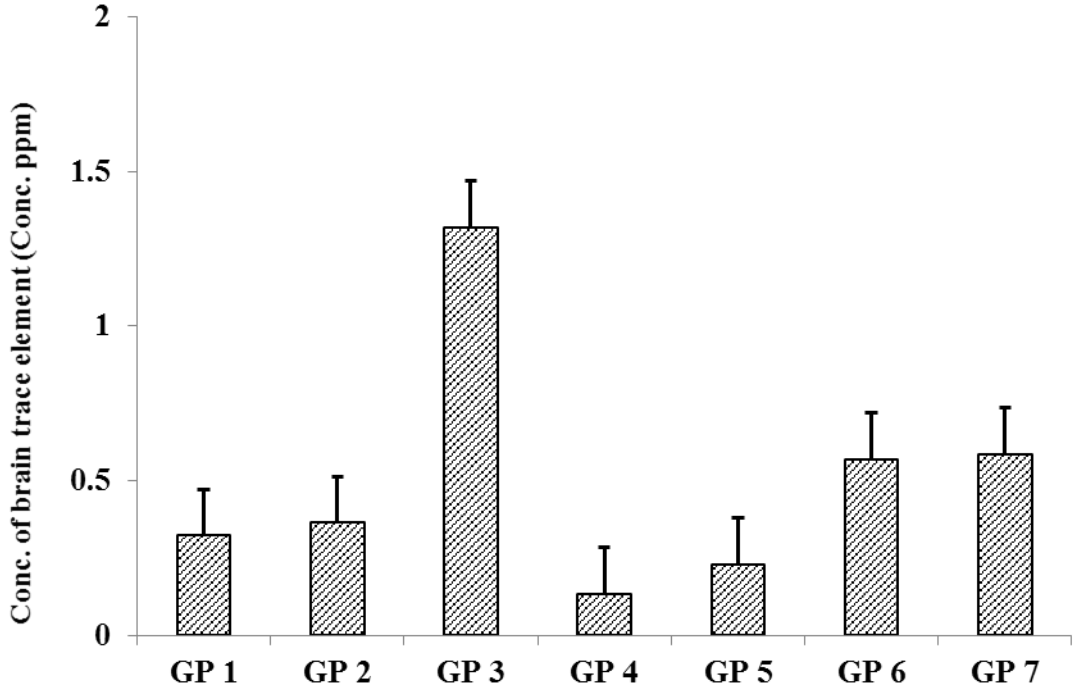


Figure 4.9: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Neuro trace elements (Fe) in adult Wistar rats. p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose(1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose(500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

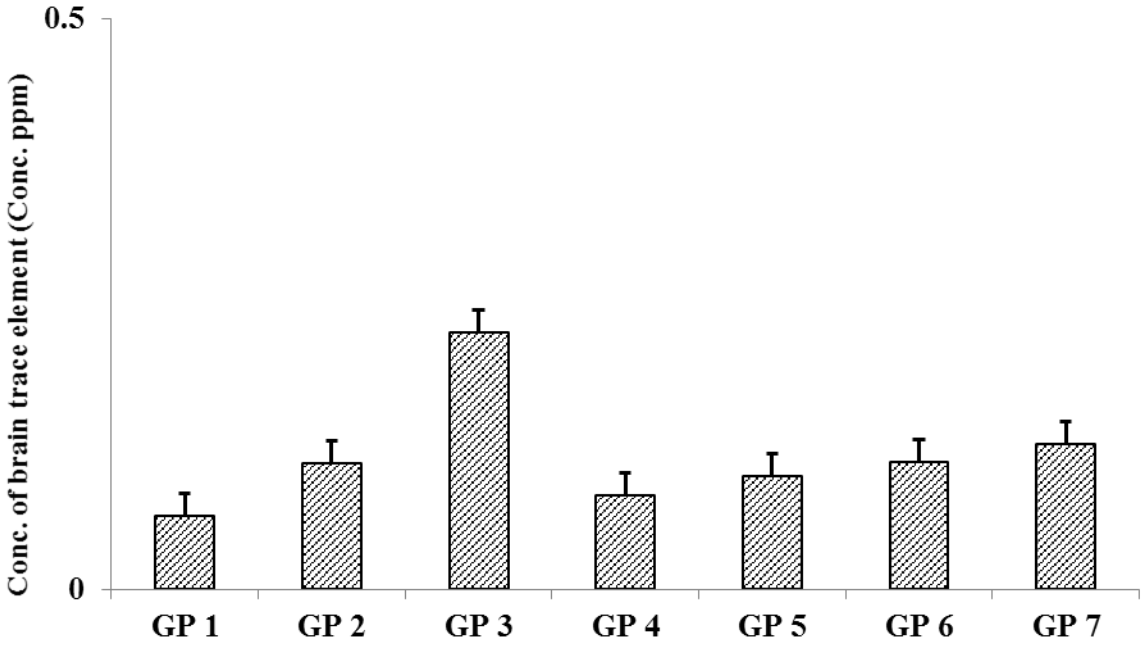


Figure 4.10: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Neuro trace elements (Zn) in adult Wistar rats. p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

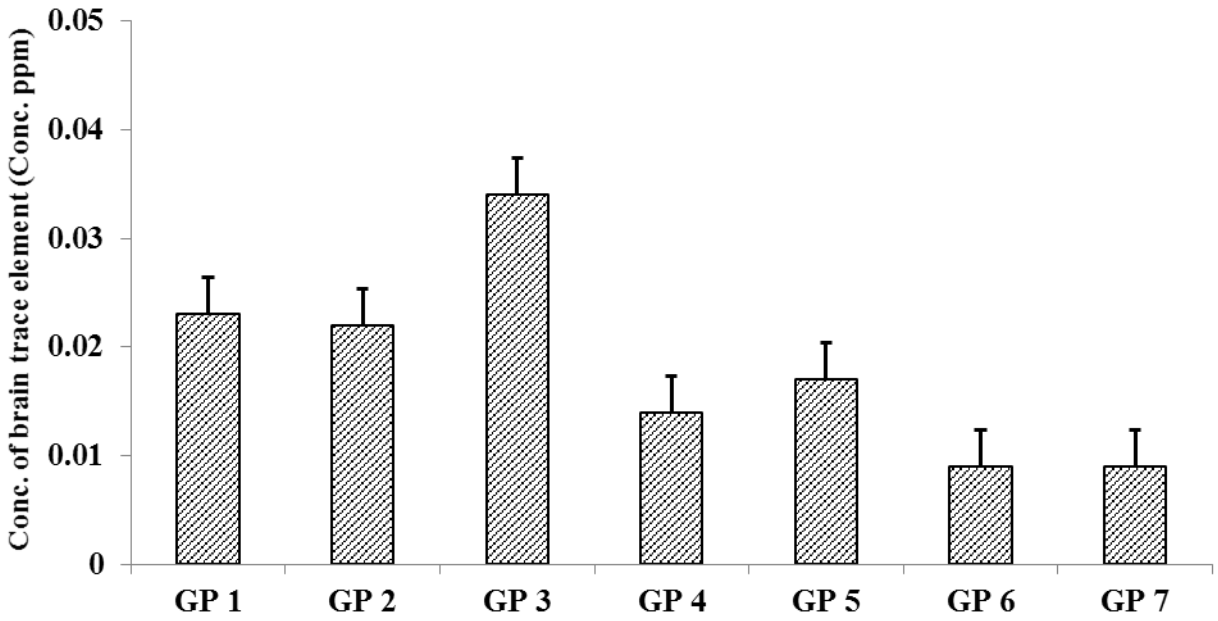


Figure 4.11: Effect of aqueous and ethanolic extracts of *Nicotiana tabacum* on Neuro trace elements (Cu). p>0.05

GP 1= control, GP 2 = Aqueous leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 3 = Aqueous leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 4 = Aqueous leaf extract of *Nicotiana tabacum* Low dose (250 mg/kg), GP 5 = Ethanol leaf extract of *Nicotiana tabacum* High dose (1000 mg/kg), GP 6 = Ethanol leaf extract of *Nicotiana tabacum* Medium dose (500 mg/kg), GP 7 = Ethanol leaf extract of *Nicotiana tabacum* High dose (250 mg/kg).

4.6 Histological and Histochemical Studies

The study of the Histological sections of the brain regions namely; cerebral cortex – layer V, cerebellar cortex and hippocampus) stained with routine Haematoxylin and Eosin, (H & E) (Cresly Fast Violet stains were processed and examined under the light microscope.

4.6.1 Histological and histochemical features of the cerebral cortex

The cerebral cortical sections of adult Wistar rats in the Control Group showed normal histoarchitecture of the cerebral cortex; the characteristic appearance of neurons arranged. The ganglionic layer (Layer V) revealed neuron morphology, size and population density, large pyramidal cells called ganglion or Betz cells and stallate cells as shown in Plate III and IV.

The result of the histological and histochemical examination of the Cerebral cortices of adult Wistar rats treated with aqueous and ethanolic extracts of *Nicotiana tabacum* also revealed mild distortions in the histoarchitecture of the layer V of the cerebral cortex, showing neuronal degeneration changes such as neurons with eosinophilic neurons (En), pyknotic nuclei (P), karyorrhectic nuclei (K), vacuolations (V) compared to the Control as shown in Plates V to XVI

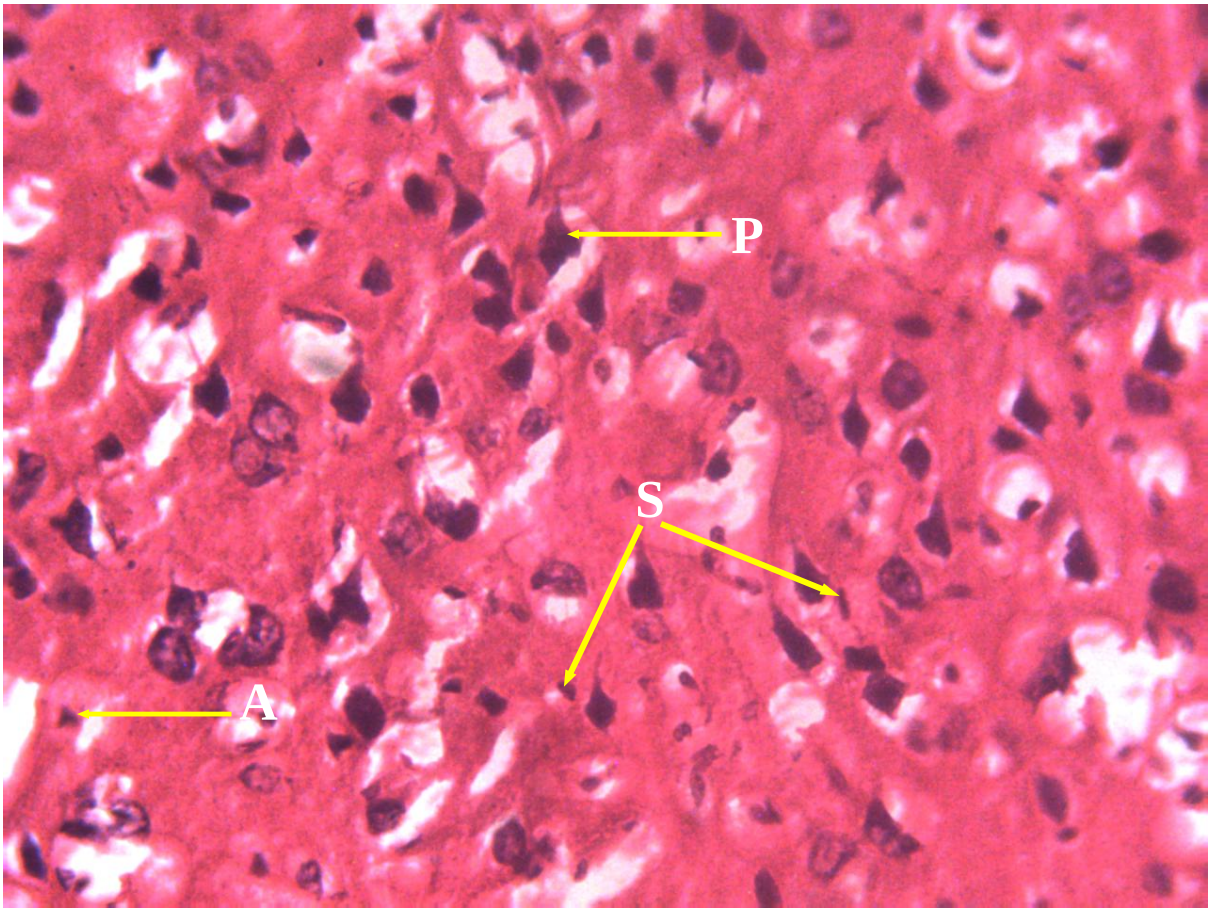


Plate III: Section of Wistar rat cerebral cortex showing layer V of the control group with normal histoarchitecture of the cerebral cortex with pyramidal cell (P); stellate cells (S); Astrocytes (A). H and E stain (x 250).

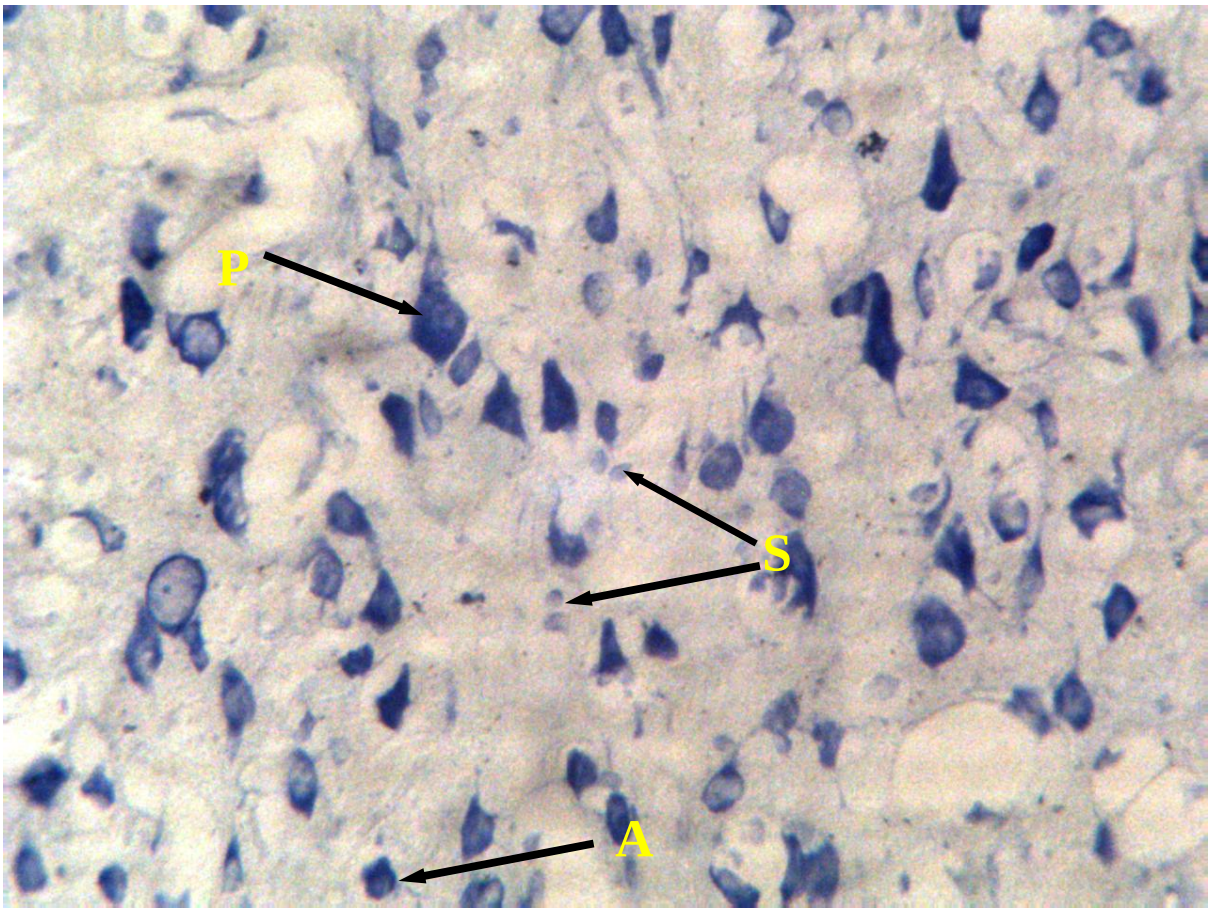


Plate IV: Section of Wistar rat cerebral cortex showing layer V of the control group with normal histoarchitecture of the cerebral cortex with pyramidal cell (P); stellate cells (S); Astrocytes (A). CFV stain (x 250).

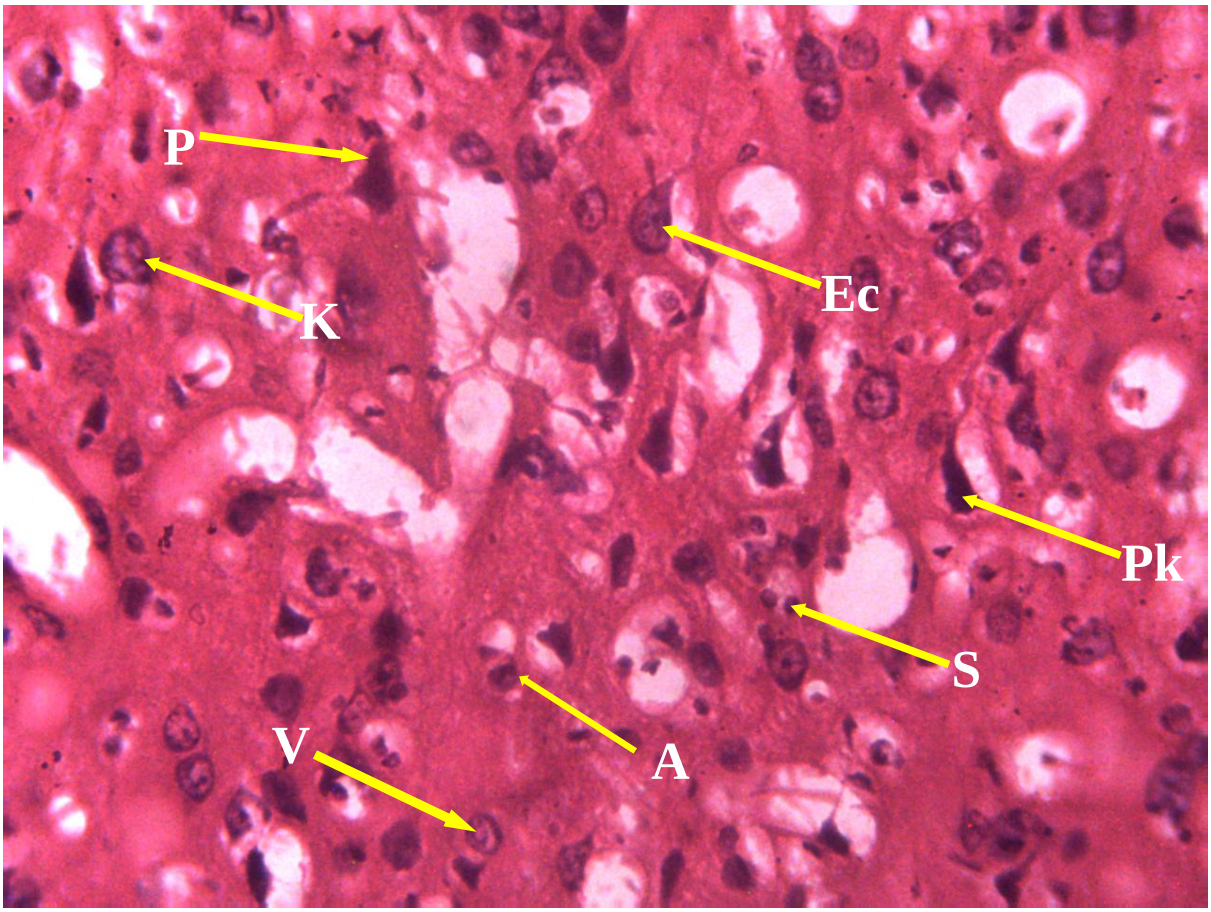


Plate V: Section of layer V of cerebral cortex of the group 2 animals administered High dose of (1000 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); neurons with eosinophilic cytoplasm (Ec); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). H and E Stain (x 250).

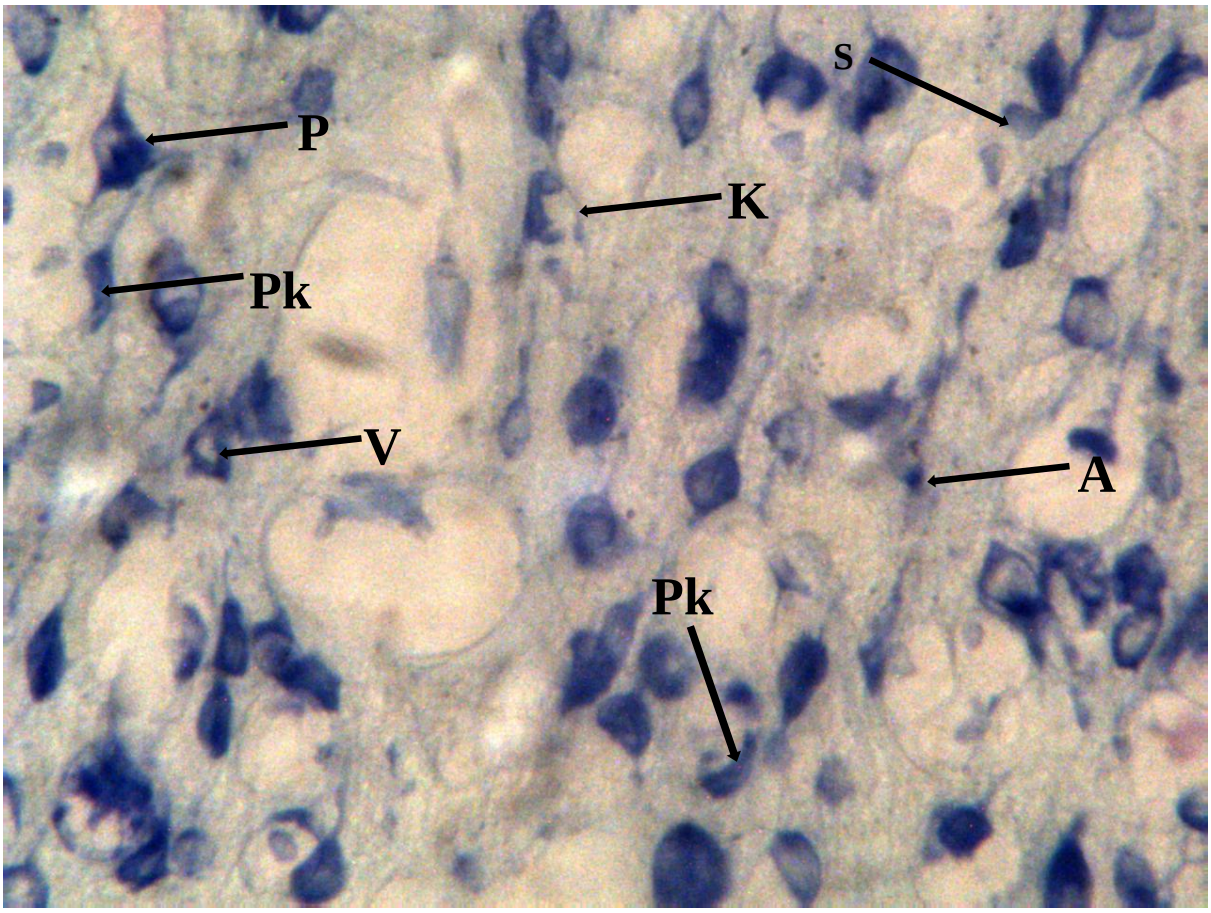


Plate VI: Section of layer V of cerebral cortex of the group 2 animals administered High dose of (1000 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); neurons with vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). CFV Stain (x 250).

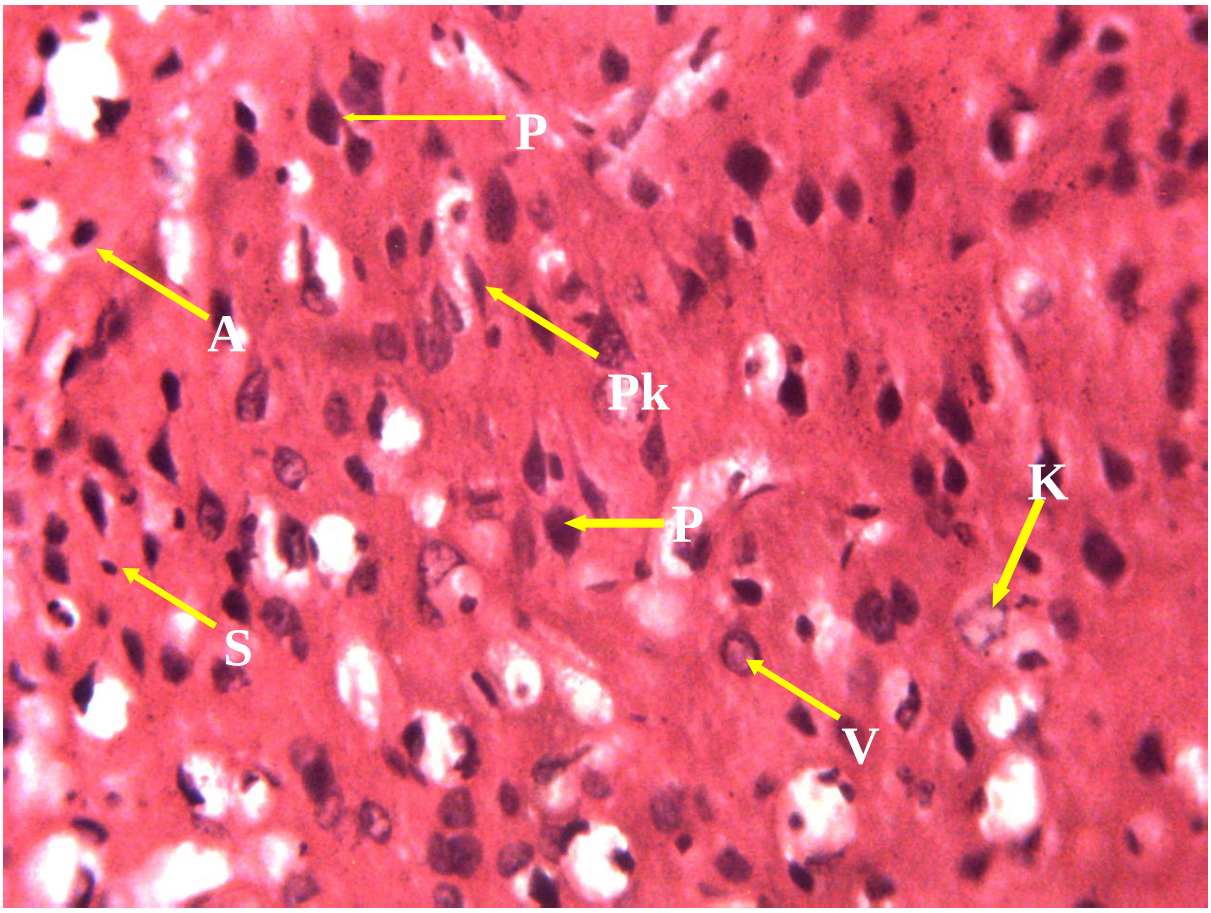


Plate VII: Section of layer V of cerebral cortex of group 3 animals administered Medium dose of (500 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); neurons with eosinophilic cytoplasm (Ec); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). H and E Stain (x 250).

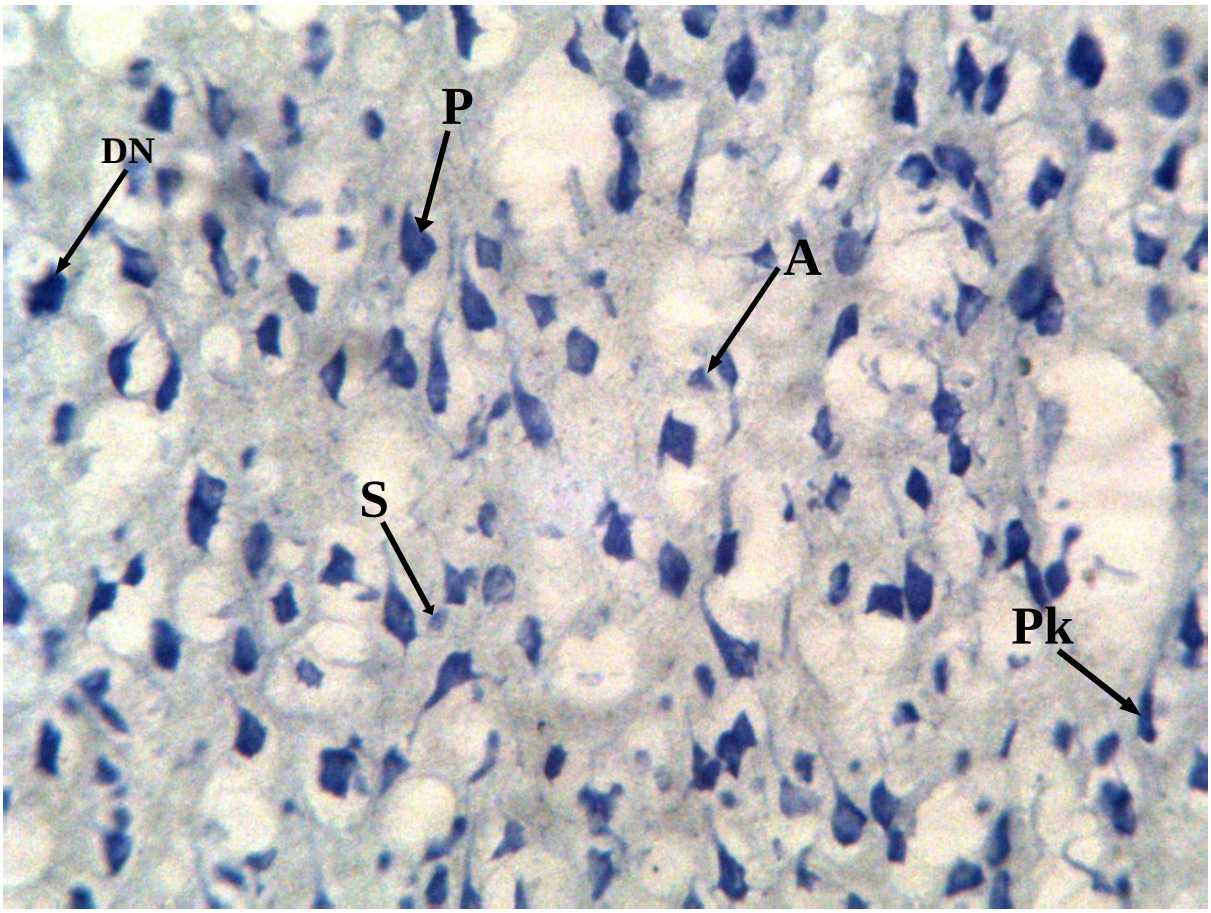


Plate VIII Section of layer V of cerebral cortex of group 3 animals administered Medium dose of ANT (500 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); Dark neurons (DN); neuronal degeneration depicting Pyknosis (Pk). CFV Stain (x 250).

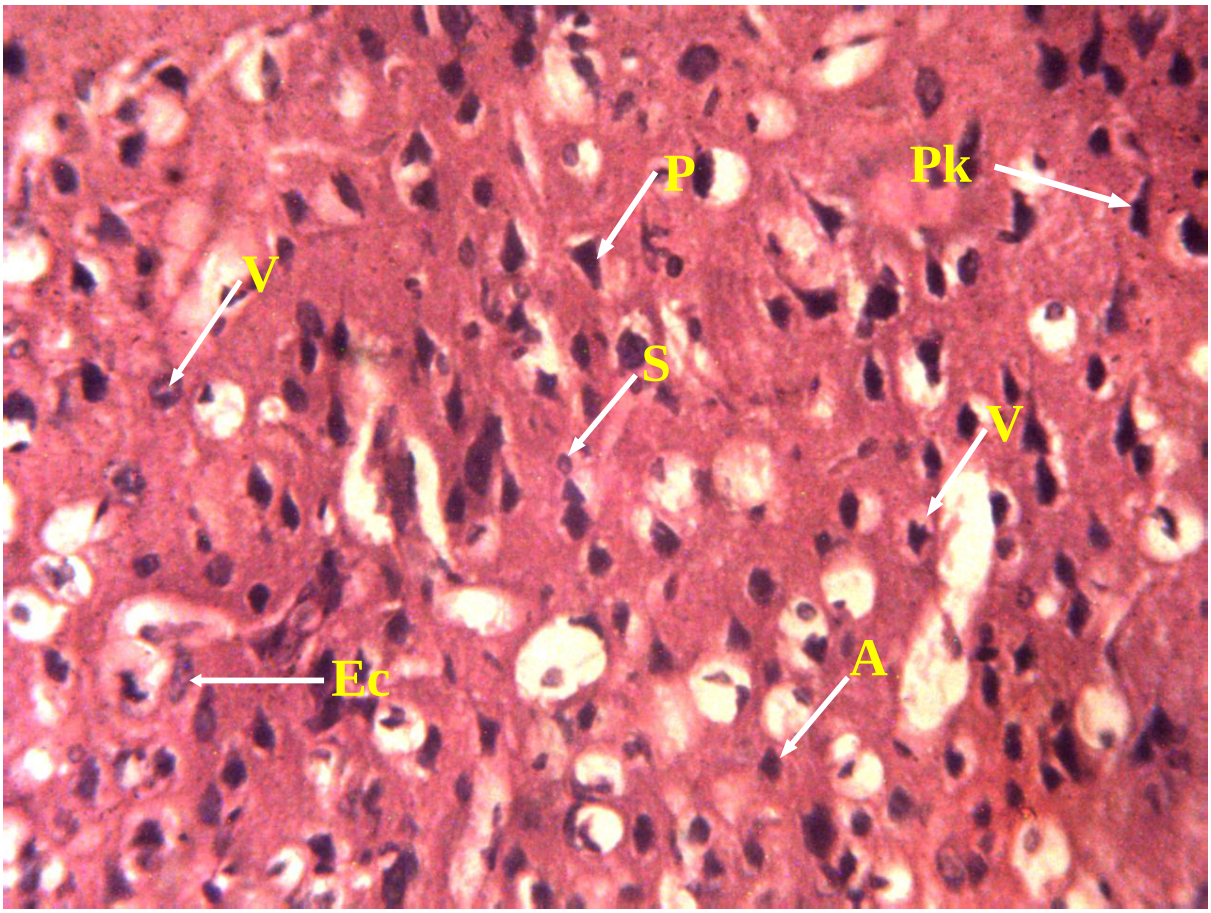


Plate IX: Section of layer V of cerebral cortex of the group 4 animals administered Low dose of ANT (250 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); neurons with eosinophilic cytoplasm (Ec); vacuolation (V); neuronal degeneration depicting Pyknosis (Pk). H and E Stain (x 250).

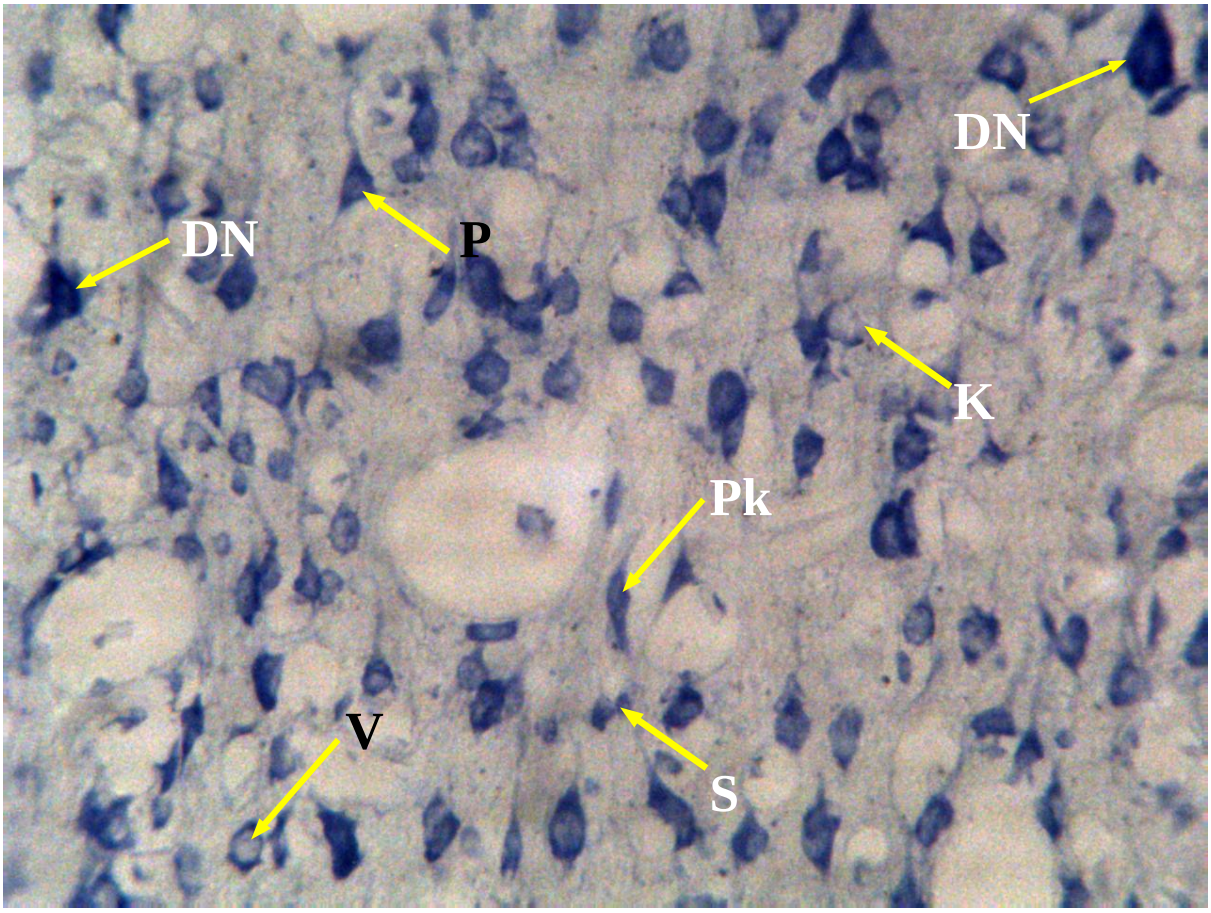


Plate X: Section of layer V of cerebral cortex of group 4 animals administered Low dose of ANT (250 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); Dark neurons (DN) neurons with eosinophilic cytoplasm (Ec); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). CFV (x 250).

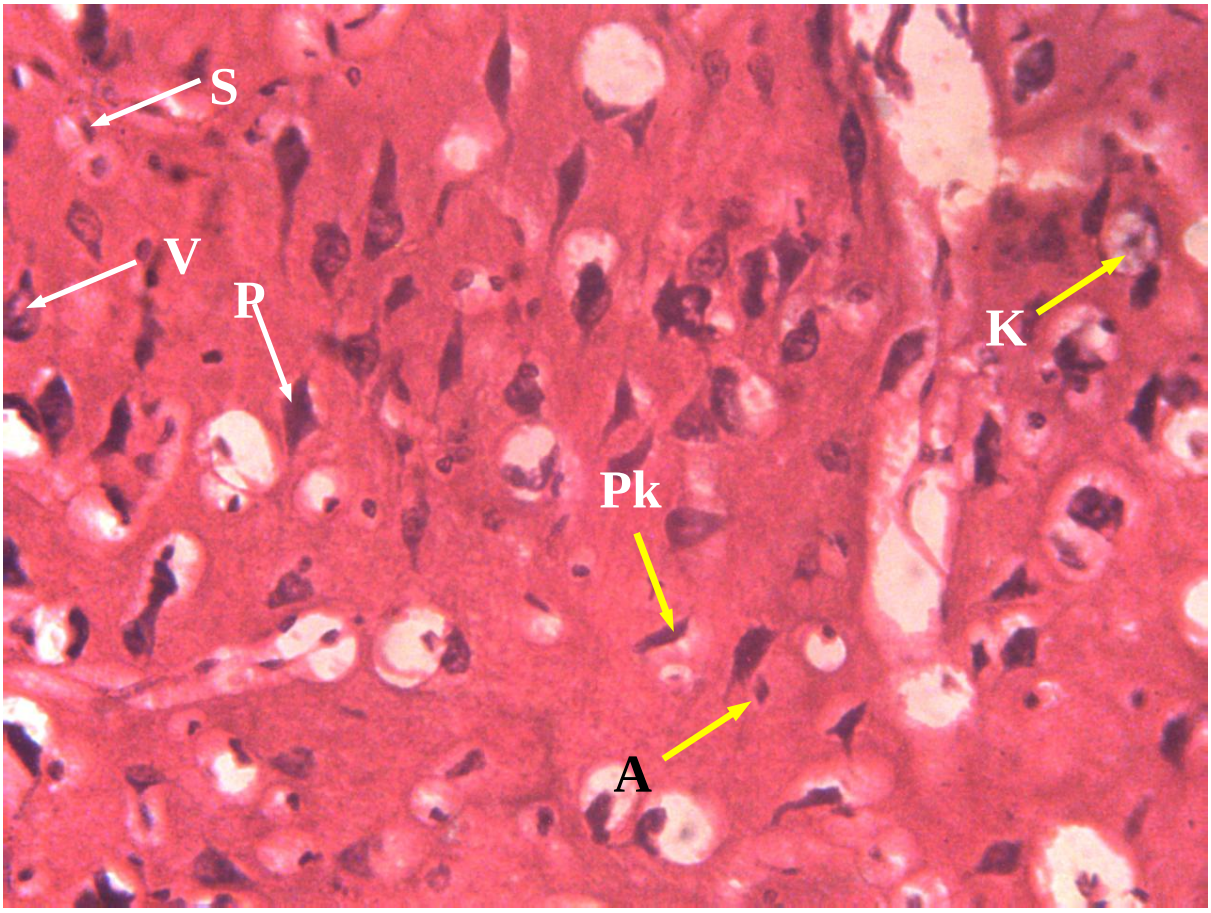


Plate XI: Section of layer V of cerebral cortex of group 5 animals administered High dose of ENT (1000 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). H and E (x 250).

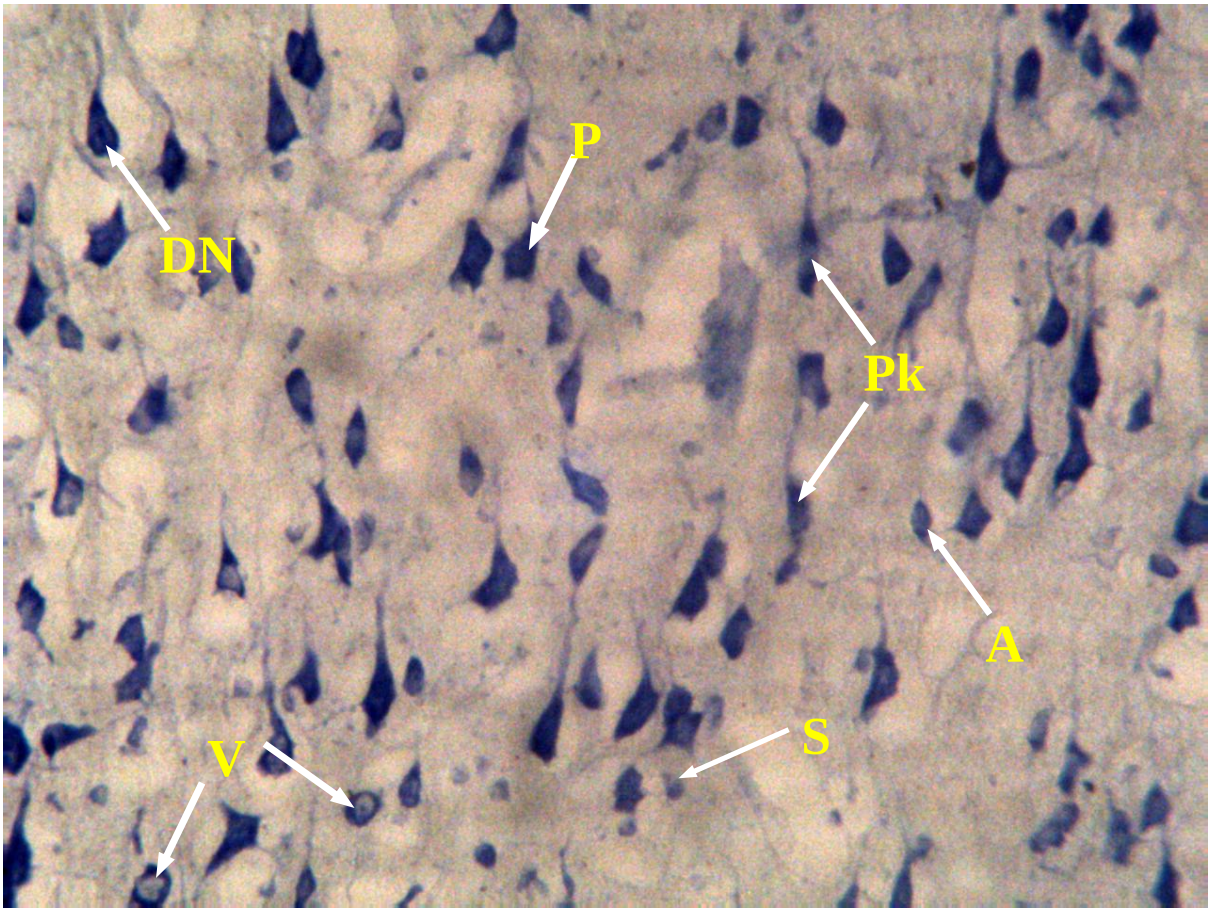


Plate XII: Section of layer V of cerebral cortex of group 5 animals administered High dose of ENT (1000 mg/kg showing pyramidal cell (P); stellate cells (S); Astrocytes (A); Dark neurons (DN); vacuolation (V); neuronal degeneration depicting Pyknosis (Pk). CFV (x 250).

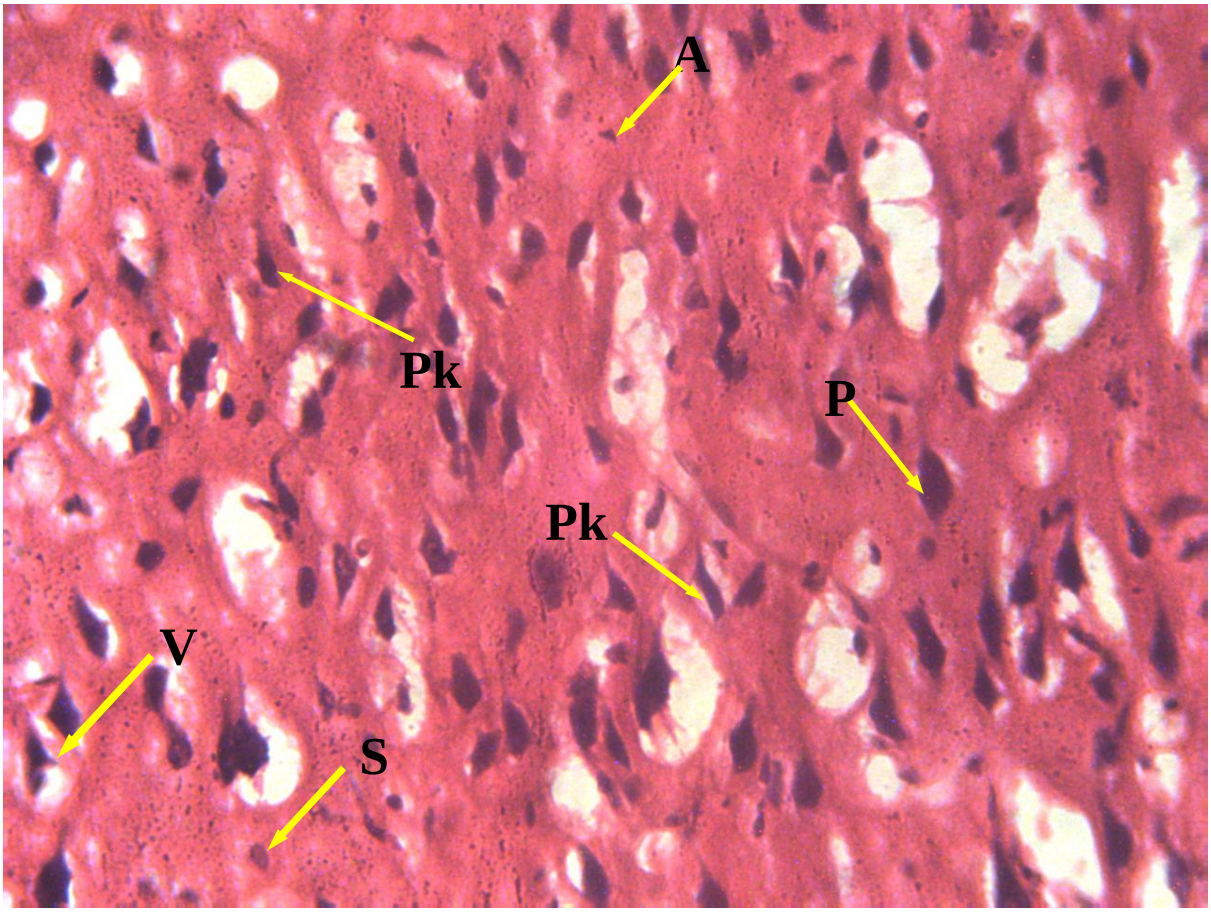


Plate XIII: Section of layer V of cerebral cortex of group 6 animals administered Medium dose of ENT (500 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); vacuolation (V); neuronal degeneration depicting Pyknosis (Pk). H and E Stain (x 250).

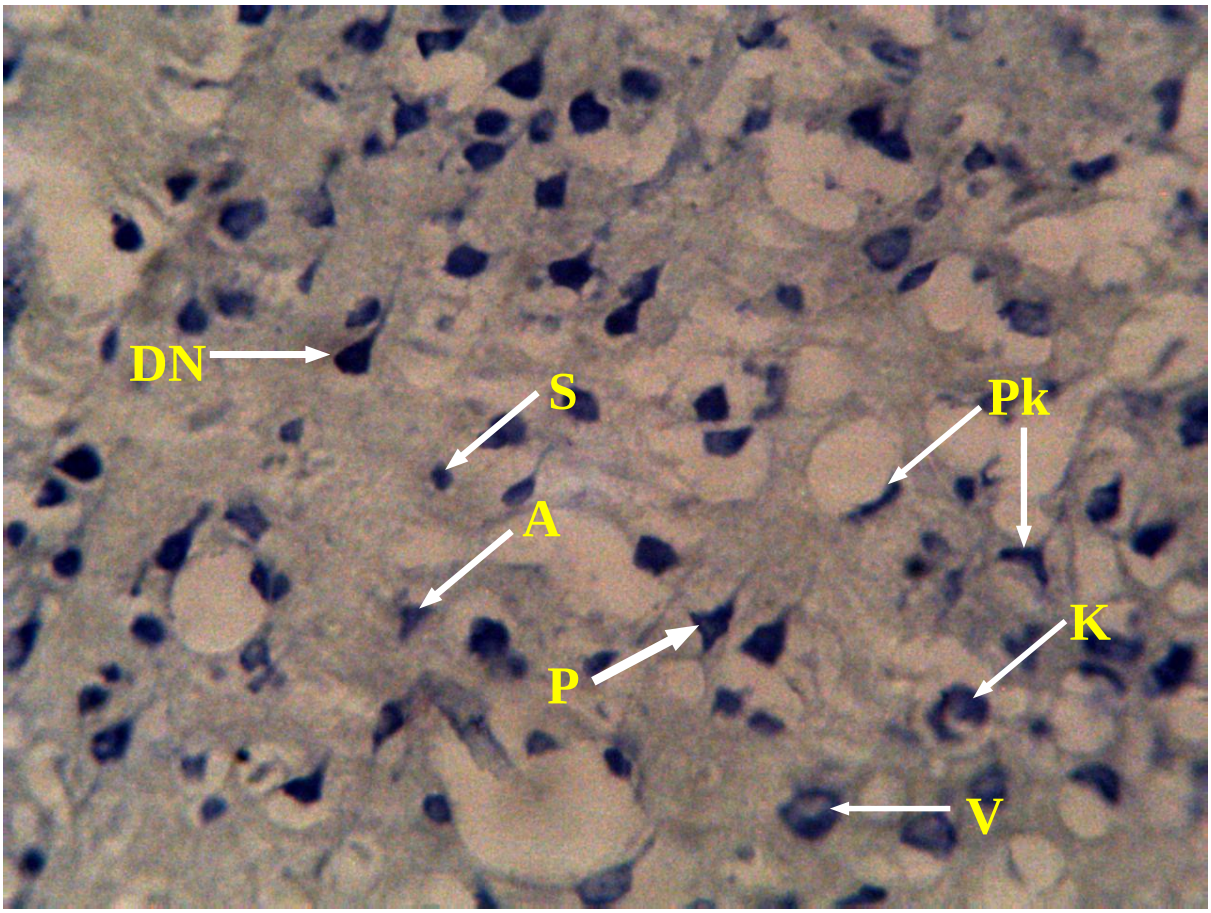


Plate XIV: Tranverse section of layer V of cerebral cortex of group 6 animals administered Medium dose of ENT (500 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). CFV Stain (x 250).

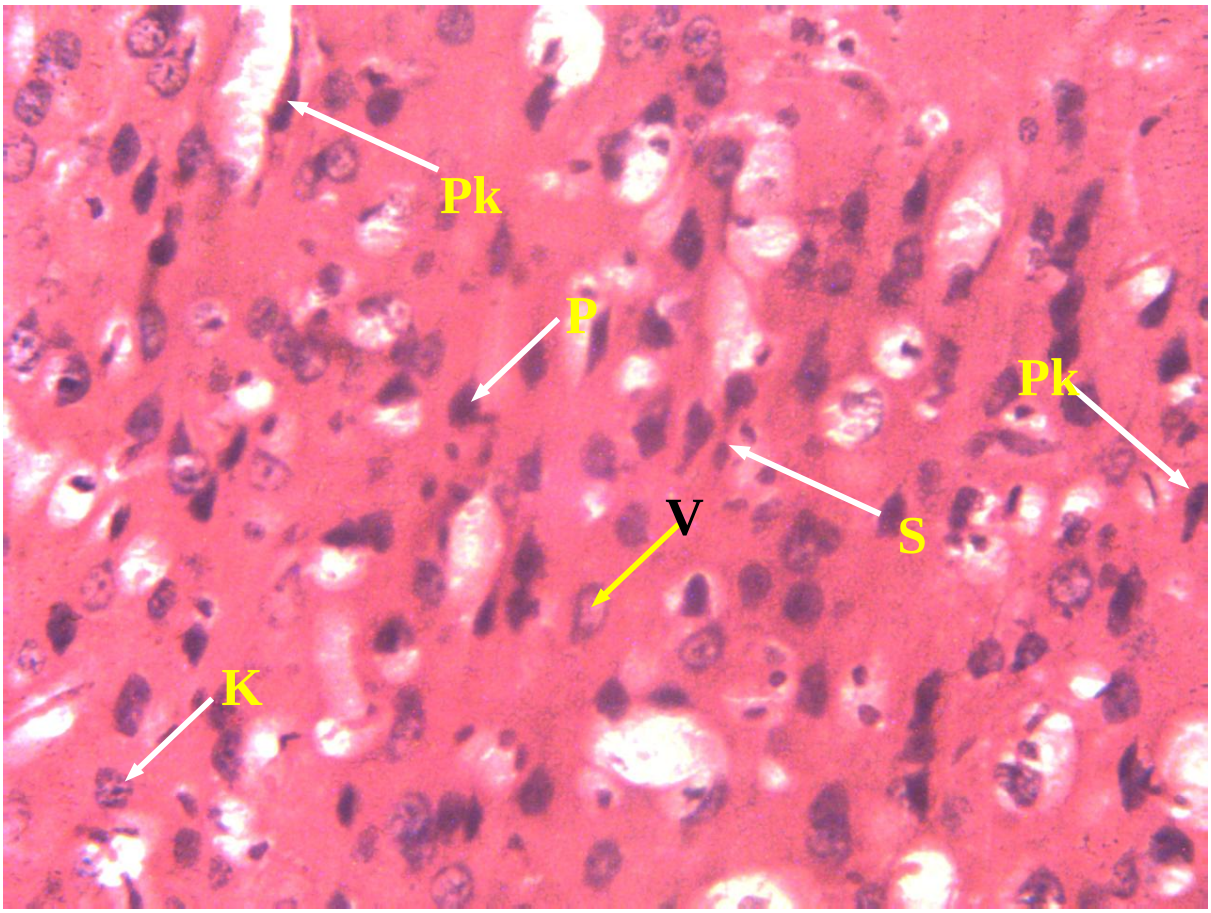


Plate XV: Section of layer V of cerebral cortex of group 7 animals administered Low dose of ENT (250 mg/kg) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). H and E Stain (x 250).

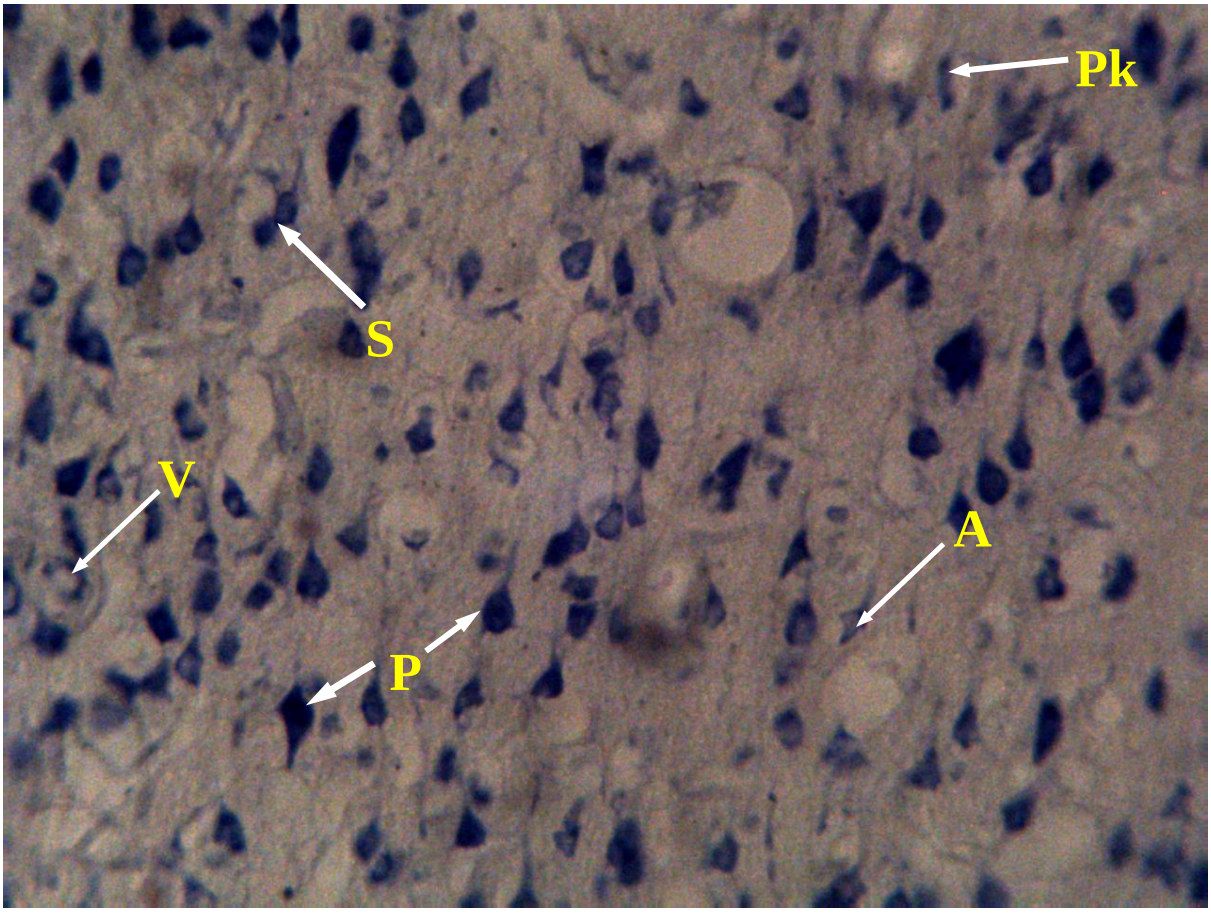


Plate XVI: Section of layer V of cerebral cortex of Group 7 animals administered Low dose of ENT (250 mg/kg)) showing pyramidal cell (P); stellate cells (S); Astrocytes (A); vacuolation (V); neuronal degeneration depicting Karyorrhesis (K); Pyknosis (Pk). CFV Stain (x 250).

4.6.2 Histological and Histochemical studies of the Cerebellar Cortex of Wistar rats

The studies cerebellar sections of the Wistar rats in the Control Group showed normal histoarchitecture of the cerebellar cortex. The characteristic appearance of the three cortical layers: an outter molecular layer with distinct neurons and inner granular layer. Between these layers is a layer of flasked shaped Purkinje cells as shown in Plates XVII and XVIII.

The cerebellar sections of Groups 2, 3, 4, 5, 6 and 7 (1000 mg/kg, 500 mg/kg and 250 mg/kg) treated Wistar rats revealed histoarchitectural distortion of the cerebellar cortex; neurodegenerative changes, such as cytoplasmic shrinkage of Purkinje cells and vacuolations in internal granule cell layer as shown in Plates XIX-XXX.

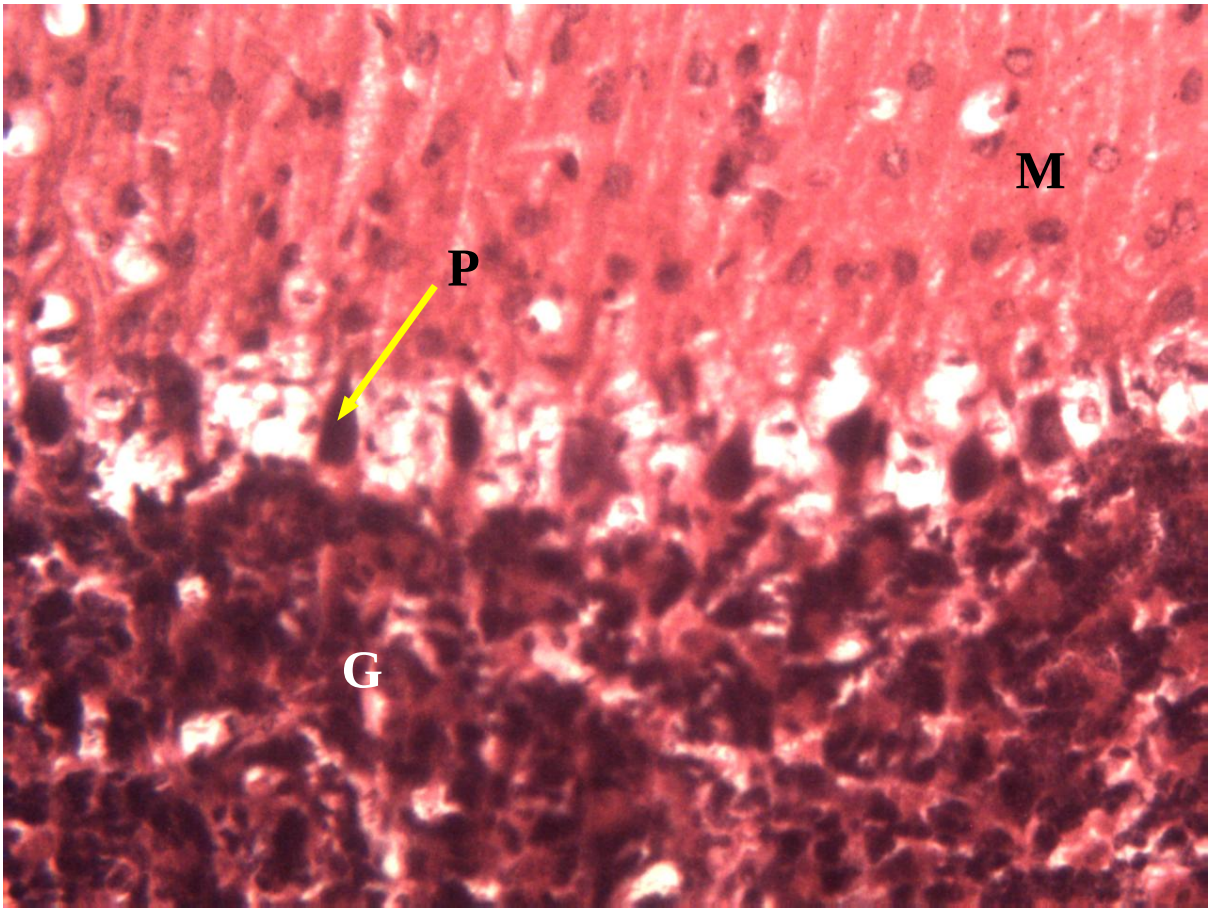


Plate XVII: Section of cerebellar cortex of the control group showing normal histology of the cerebellar cortex with Purkinje cells (P); granular layer (G); molecular layer (M). H and E stain. (x 250).

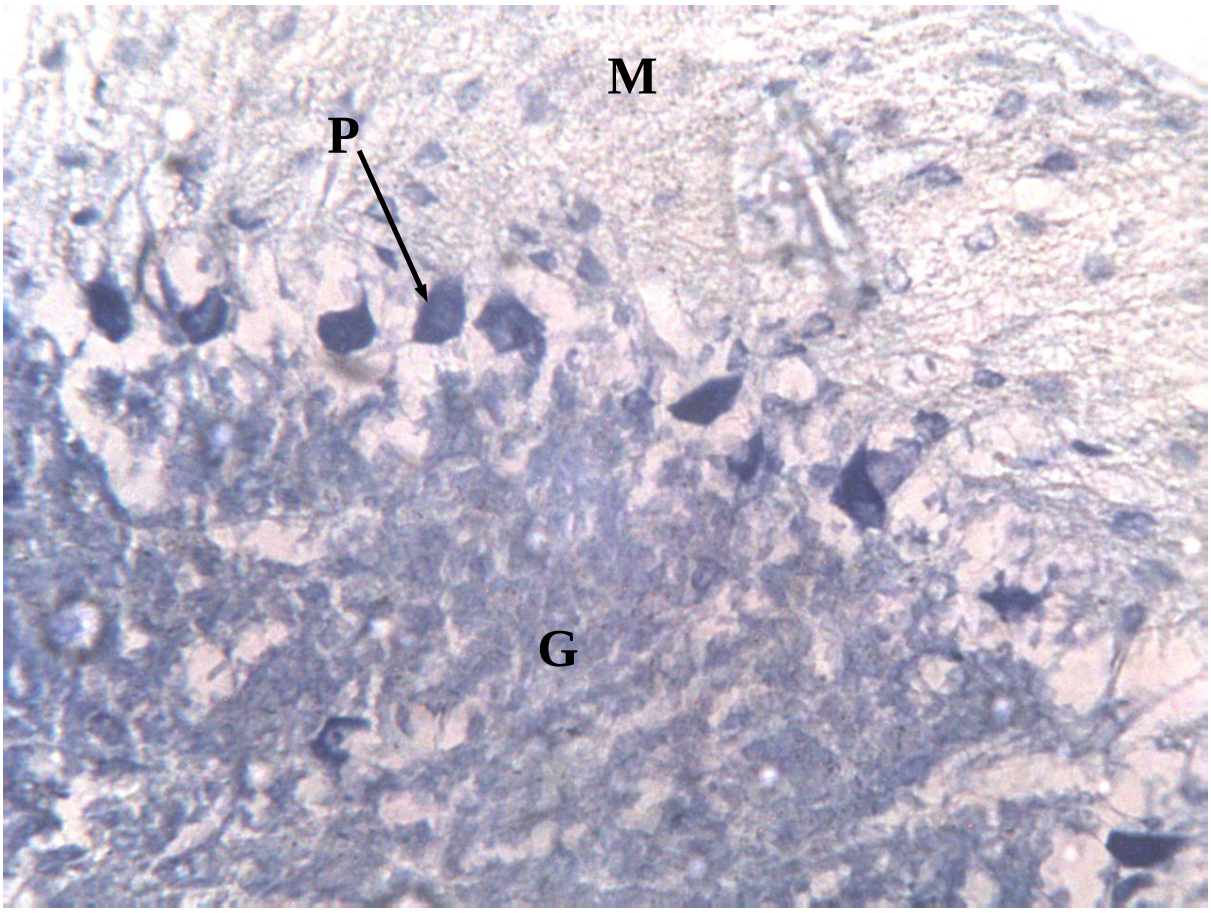


Plate XVIII: Section of cerebellar cortex of the control Group showing normal histology of the cerebellar cortex with Purkinje cells (P); granular layer (G); molecular layer (M). CFV stain (x 250).

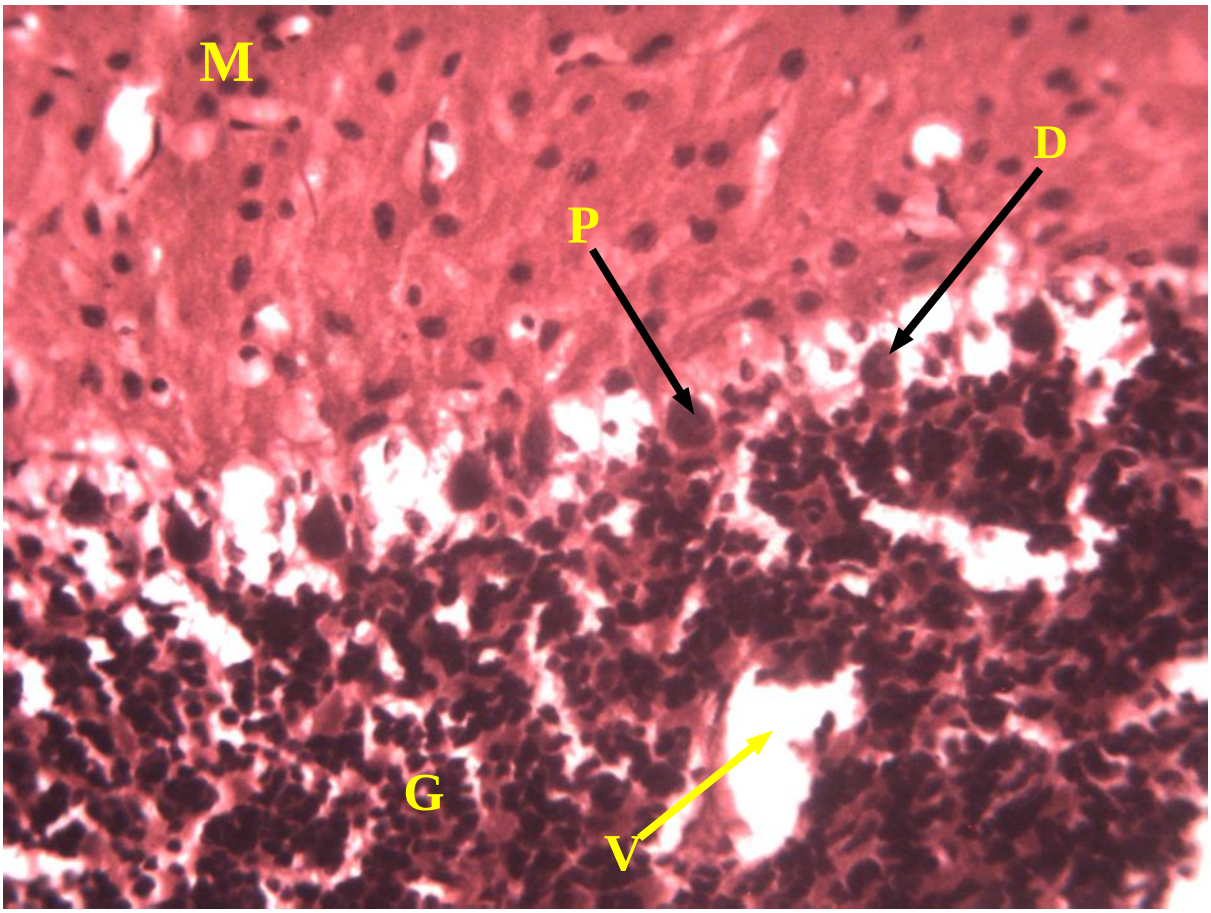


Plate XIX: Section of Wistar rat cerebellar cortex of the group 2 administered aqueous leave extract of *Nicotiana tabacum* High dose (1000 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

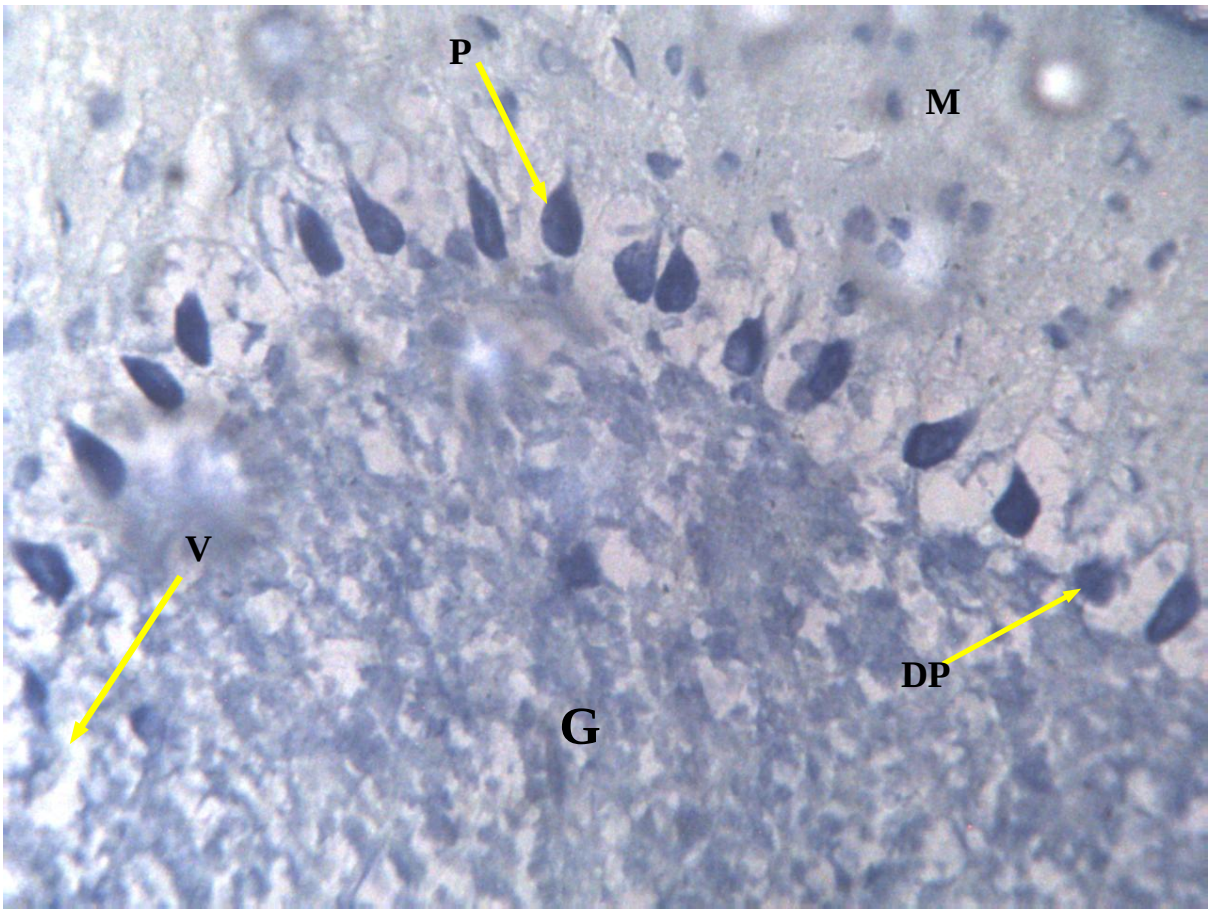


Plate XX: Section of Wistar rat cerebellar cortex of the group 2 administered aqueous leave extract of *Nicotiana tabacum* High dose (1000 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

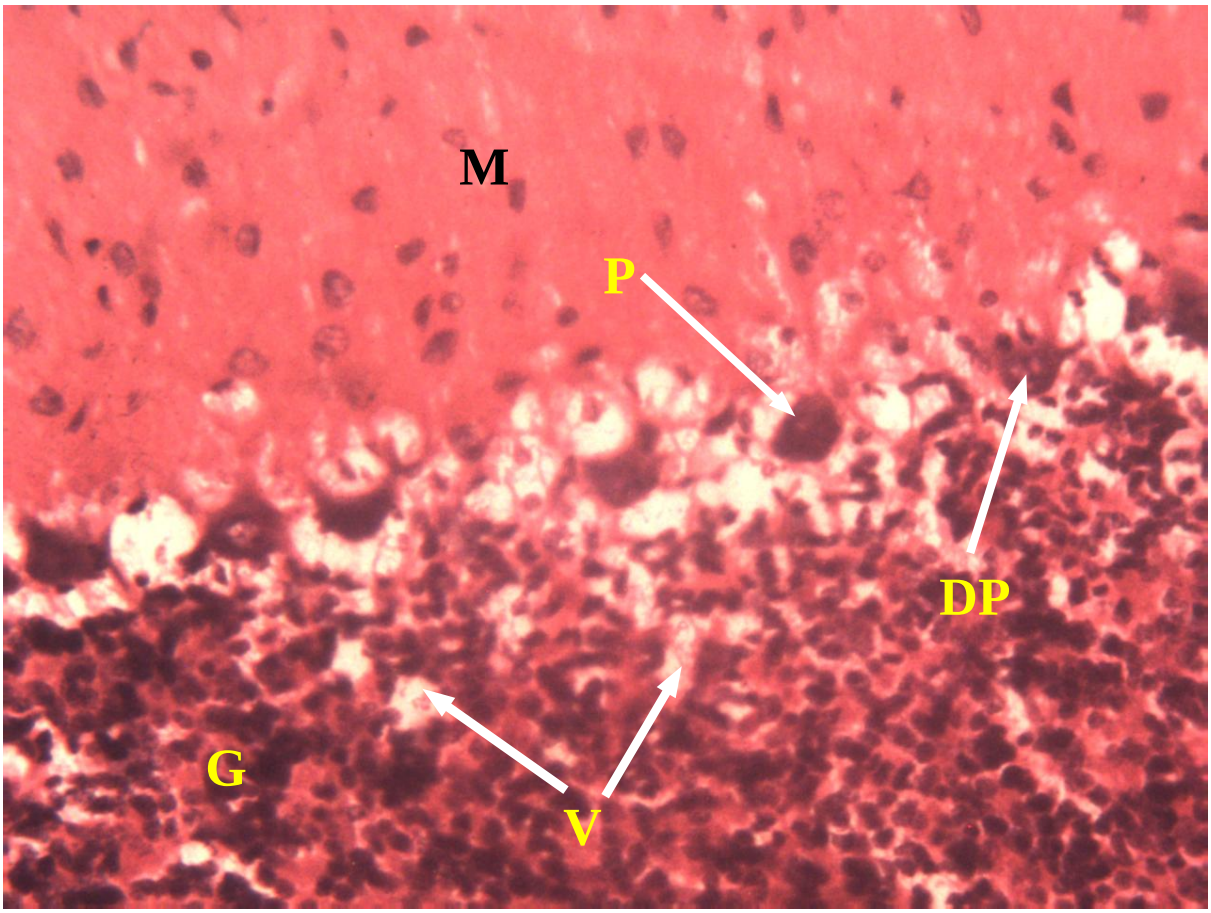


Plate XXI: Section of Wistar rat cerebellar cortex of the group 3 administered aqueous leave extract of *Nicotiana tabacum* Medium dose (500 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

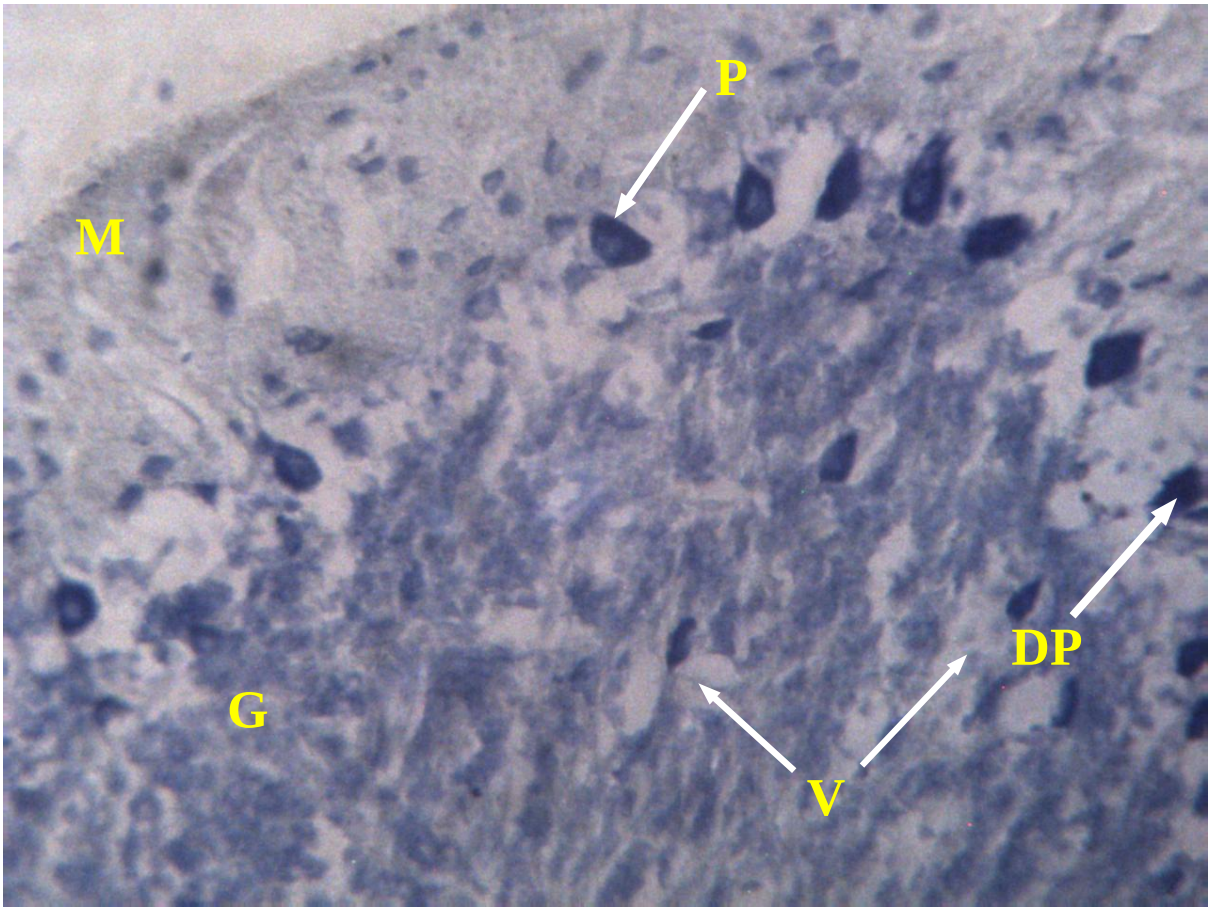


Plate XXII: Section of Wistar rat cerebellar cortex of the group 3 administered aqueous leave extract of *Nicotiana tabacum* Medium dose (500 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

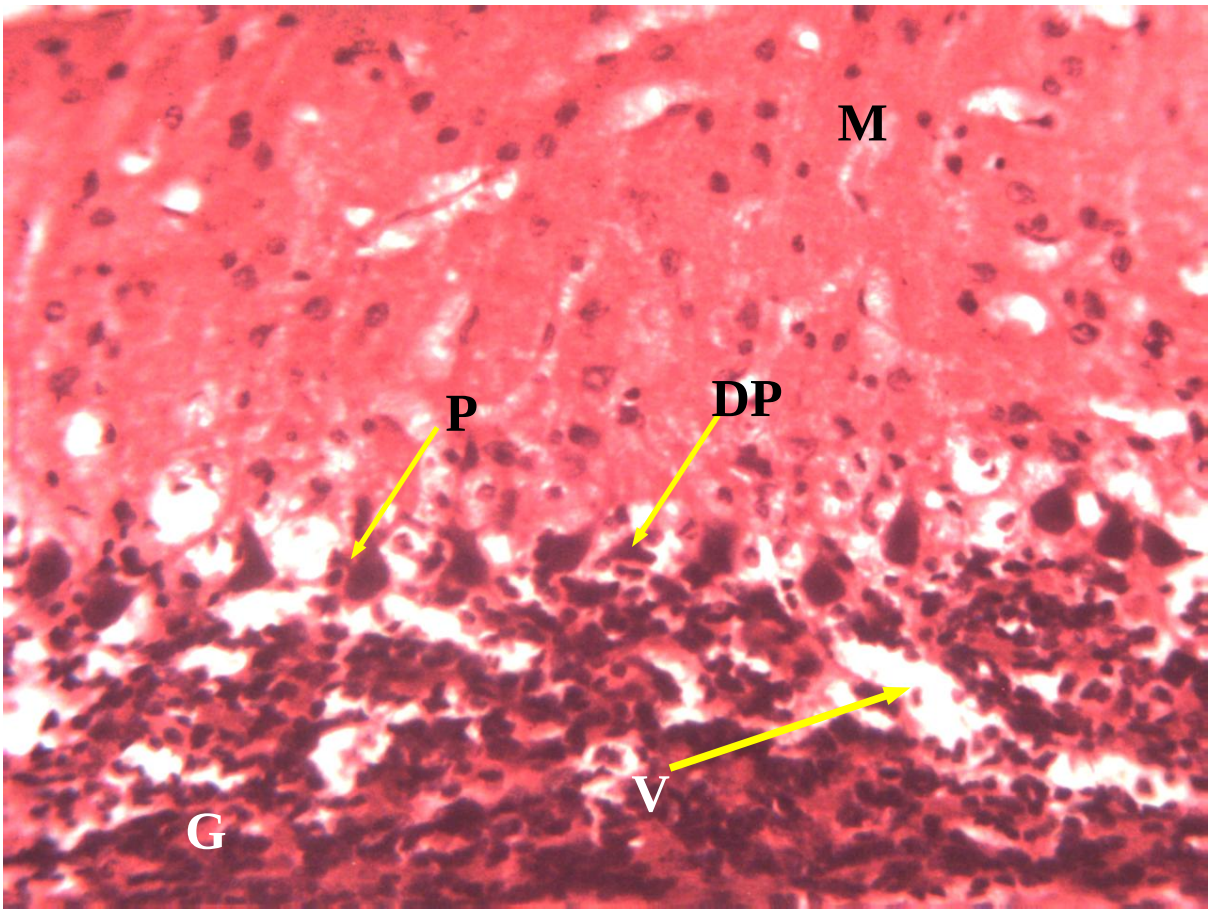


Plate XXIII: Section of Wistar rat cerebellar cortex of the group 4 administered aqueous leave extract of *Nicotiana tabacum* Low dose (250 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

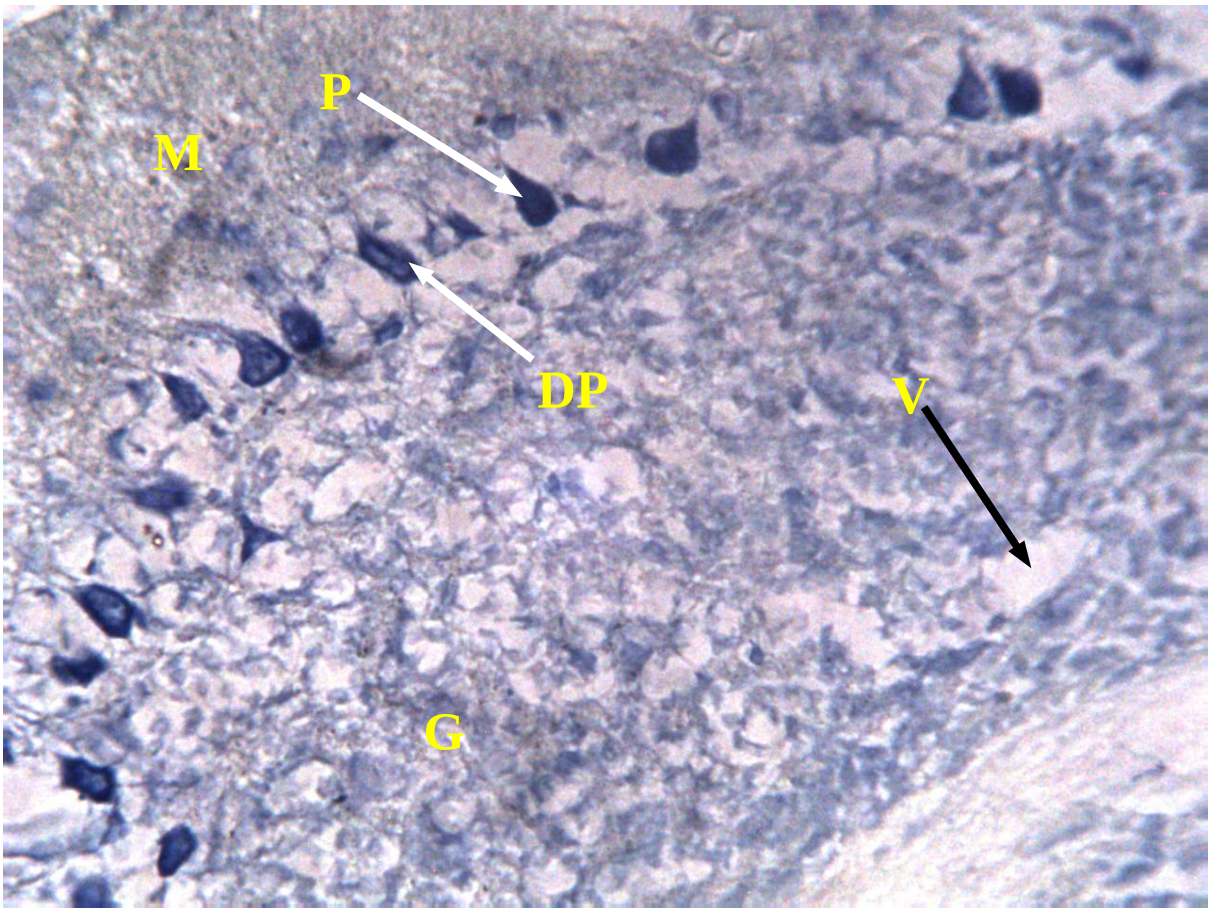


Plate XXIV: Section of Wistar rat cerebellar cortex of the group 4 administered aqueous leave extract of *Nicotiana tabacum* Low dose (250 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

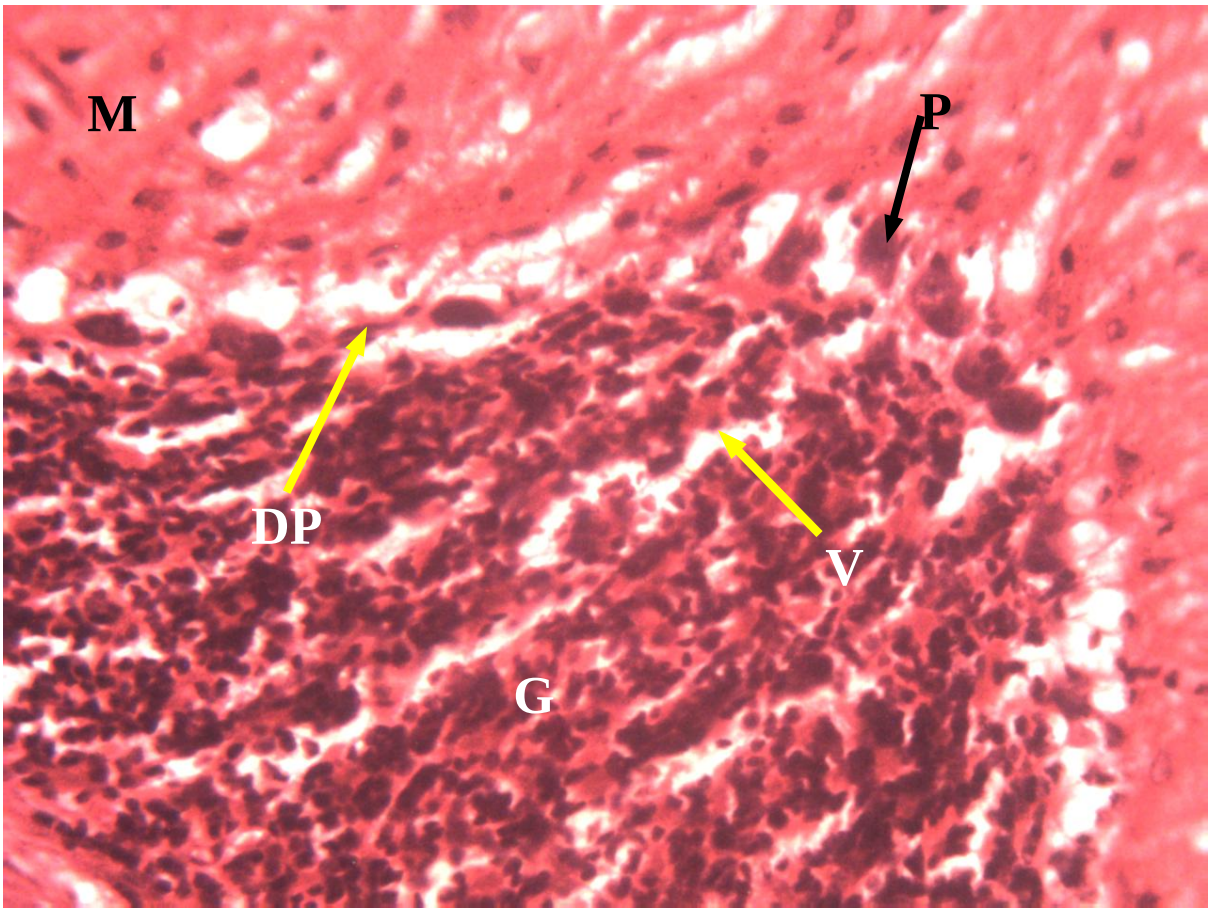


Plate XXV: Section of Wistar rat cerebellar cortex of the group 5 administered ethanolic leave extract of *Nicotiana tabacum* high dose (1000 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

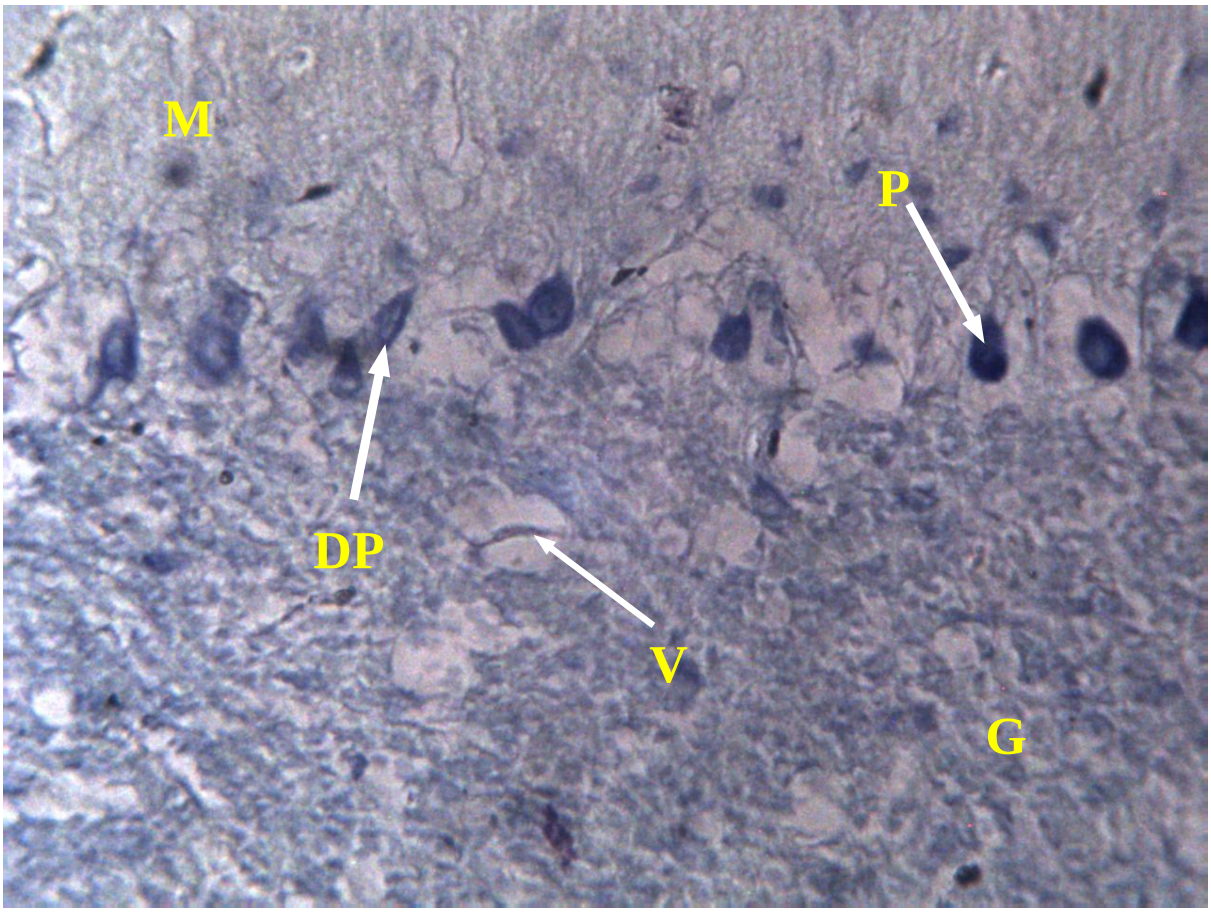


Plate XXVI: Section of Wistar rat cerebellar cortex of the group 5 administered ethanolic leave extract of *Nicotiana tabacum* high dose (1000 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

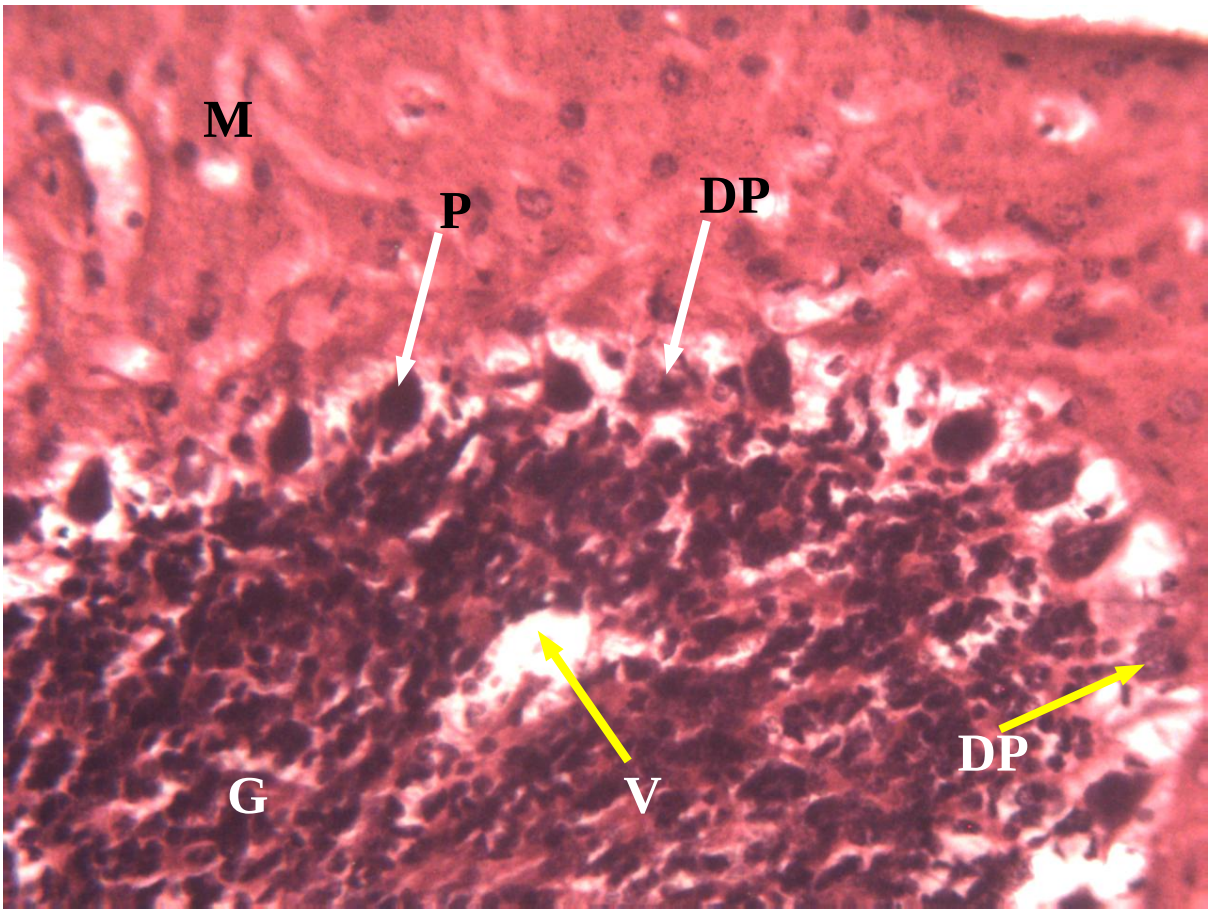


Plate XXVII: Section of Wistar rat cerebellar cortex of the group 6 administered ethanolic leave extract of *Nicotiana tabacum* medium dose (500 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

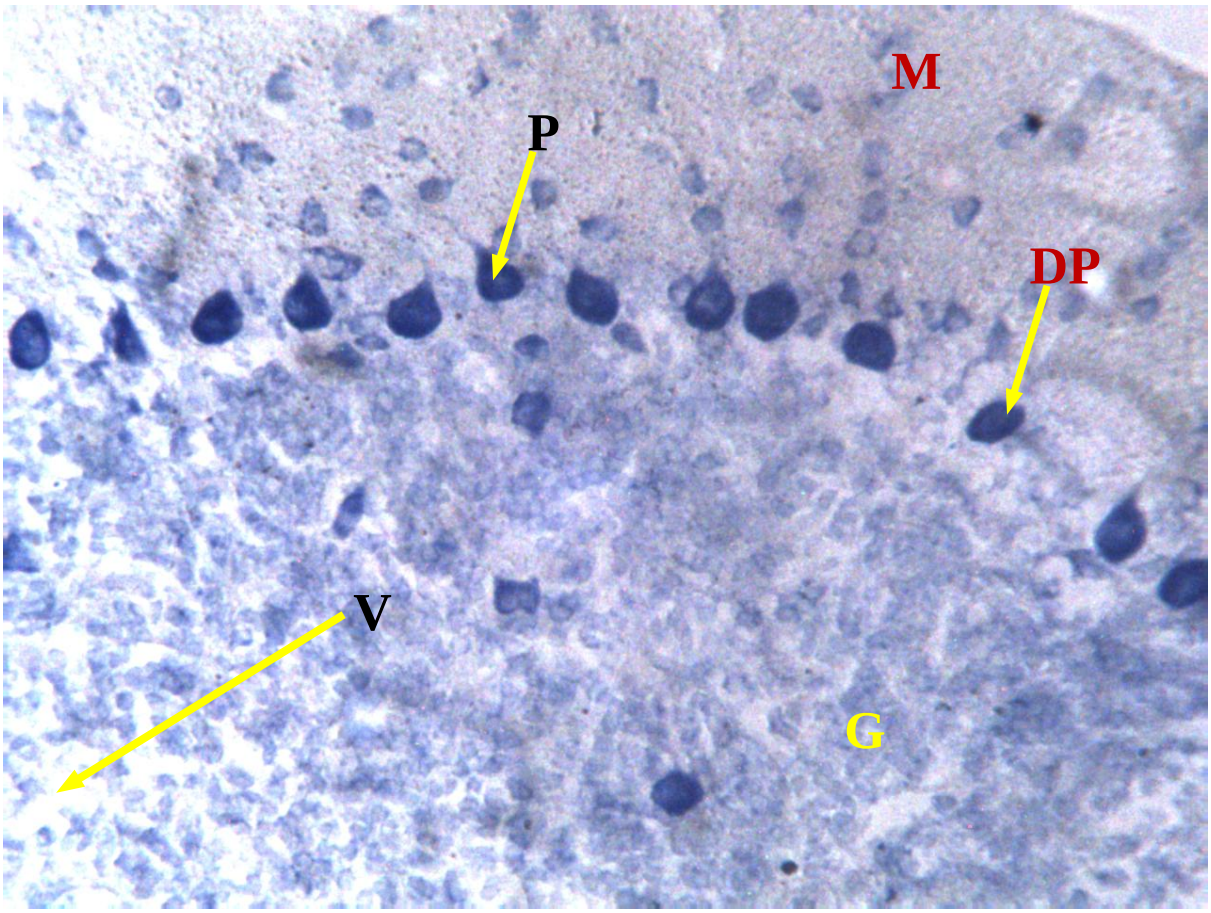


Plate XXVIII: Section of Wistar rat cerebellar cortex of group 6 administered ethanolic leave extract of *Nicotiana tabacum* medium dose (500 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

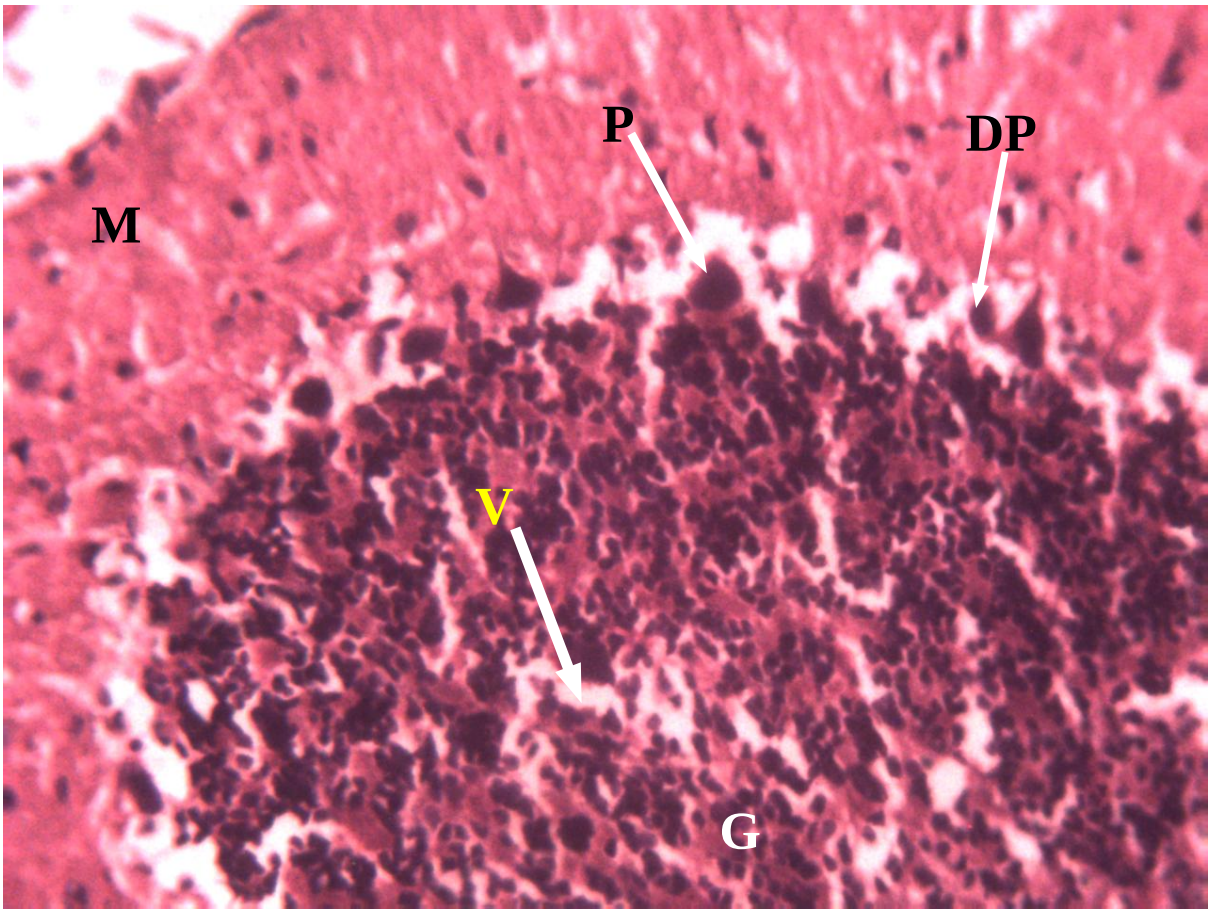


Plate XXIX: Section of Wistar rat cerebellar cortex of group 7 administered ethanolic leave extract of *Nicotiana tabacum* low dose (250 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). H and E stain (x 250).

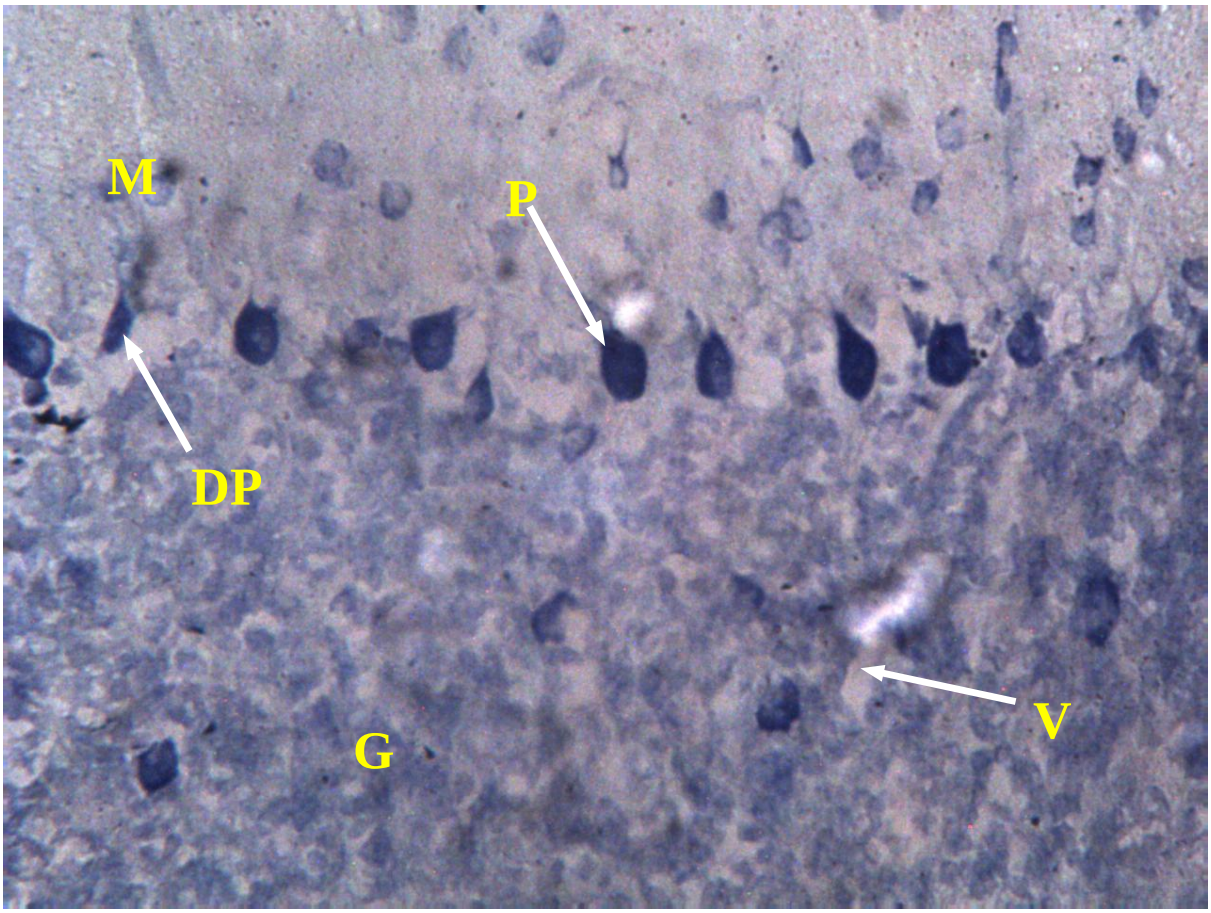


Plate XXX: Section of Wistar rat cerebellar cortex of the group 7 administered ethanolic leave extract of *Nicotiana tabacum* low dose (250 mg/kg) showing classical appearance of eosinophilic (degenerating) Purkinje neuron (DP); granular layer (G) with vacuolations suggesting degeneration of Purkinje neuron dendrite; molecular layer (M); Purkinje cell (P). CFV stain (x 250).

4.6.3 Histological and Histochemical Studies of the Hippocampus

The result of the histological and histochemical study of the Wistar rat hippocampus showed that it is subdivided into four regions (CA1 – CA4, CA which means Cornu Ammonis) according to density, size and branching of axons and dentrites of the pyramidal cells. Each of these regions consists of three layers: stratum molecular (molecular layer), stratum pyramidale (pyramidale layer) which contains bodies of the pyramidal cells and stratum multiforme (multiform layer). The continuation of CA3 in the concavity of dendate gyrus (fascia dentate) is the CA4. CA1 is characterized by densely packed medium sized cells; CA3 is the region with large less densely packed cells while CA2 is a transition field between CA3 and CA1.

Histological examination of Wistar rats hippocampi, particularly CA3 regions, in the Control Group showed 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. Histochemical staining for Nissil substance revealed normal appearance of hippocampal neurones and other interneurones, such as stallate, and basket cells of Cajal, which differ from the pyramidal granule cells, observed most clearly in CA3 as shown in Plates XXXI and XXXII.

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 as shown in Plates XXXIII to XLIV.

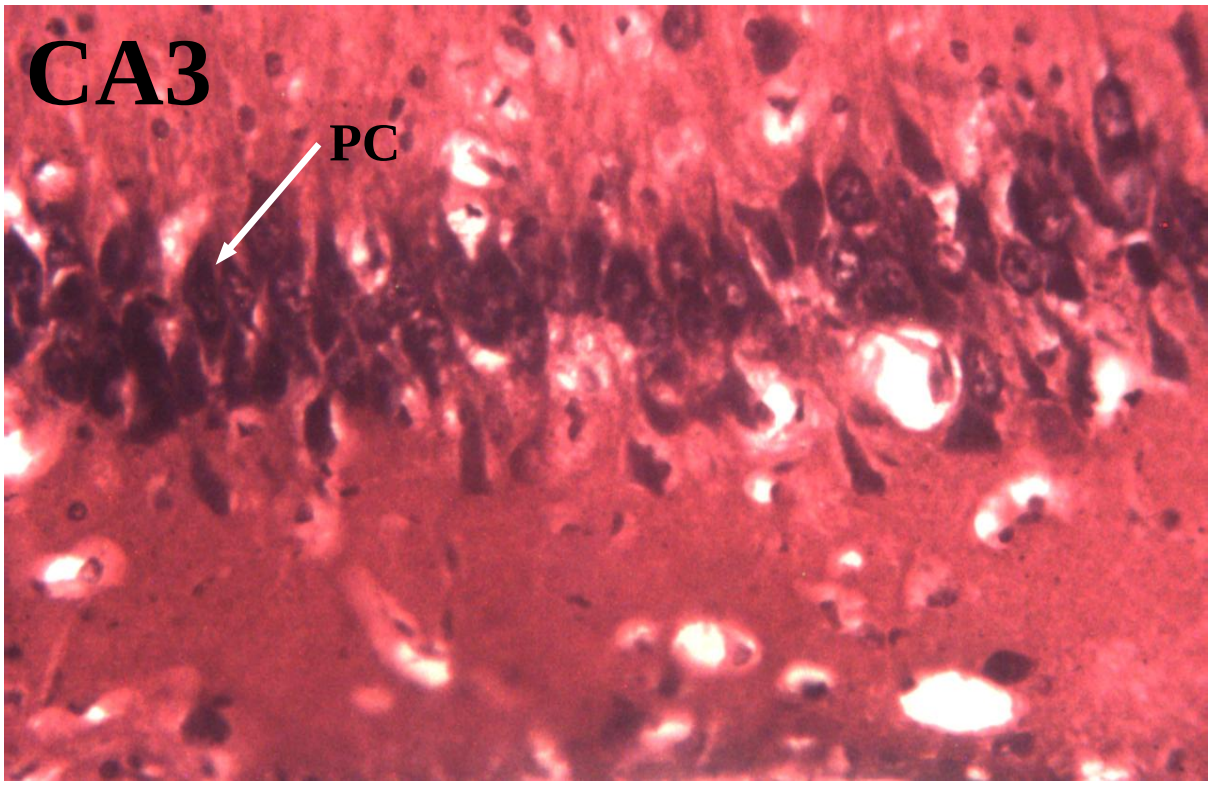


Plate XXXI: Tranverse section of CA3 region of the hippocampus of the control group showing well defined and packed pyramidal cells (PC). H & E (x250).

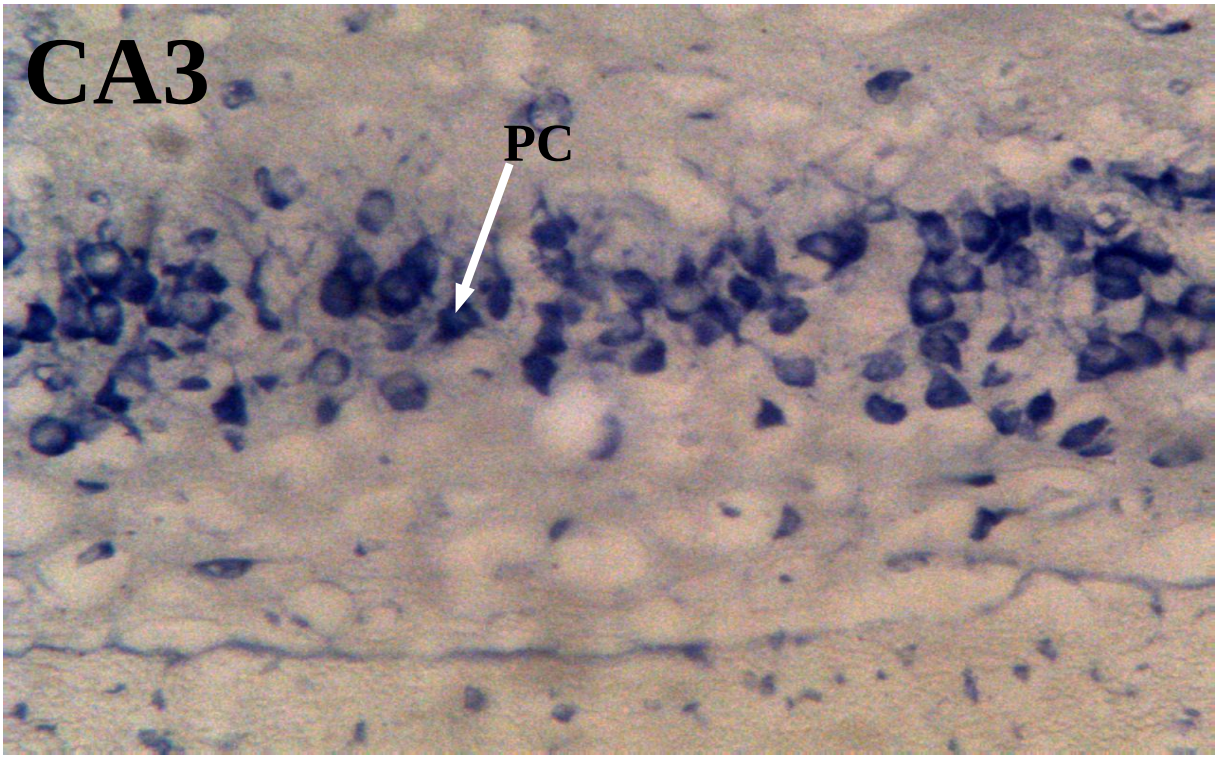


Plate XXXII: Section of CA3 region of the hippocampus of the control group showing well defined and packed pyramidal cells (P). CFV (x250).

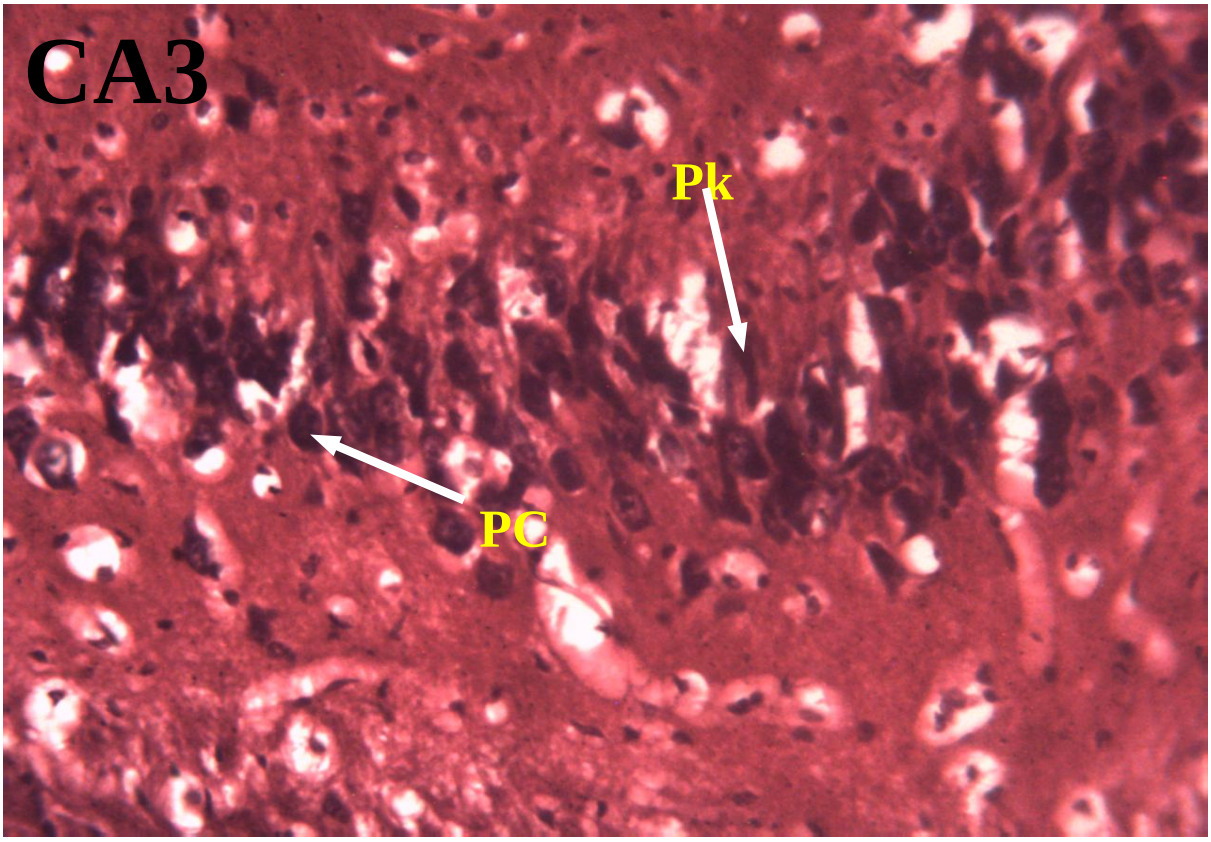


Plate XXXIII: Section of CA3 Region of the Hippocampus of the group 2 administered High dose of ANT (1000 mg/kg) showing irregular arrangement of pyramidal cells, Pyramidal cells (PC); Pyknosis (Pk). H & E (x250).

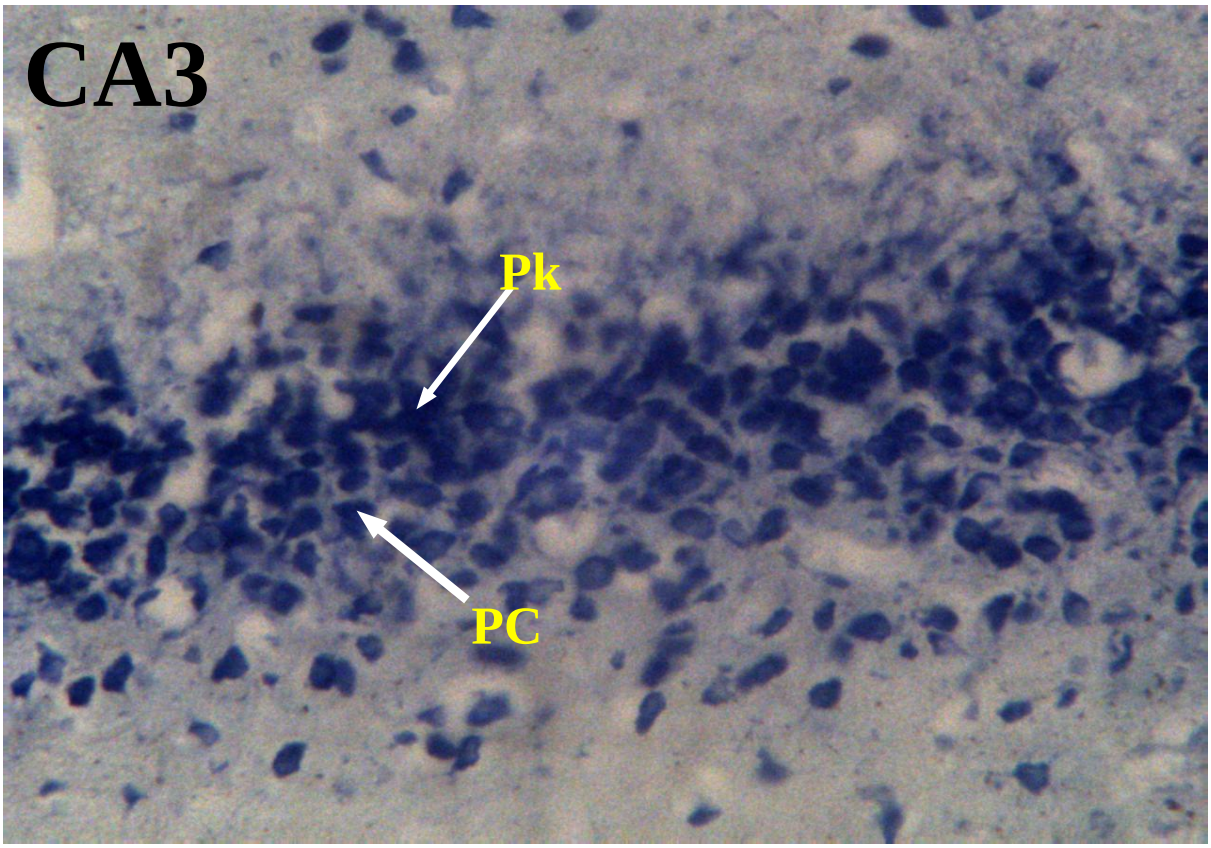


Plate XXXIV: Section of CA3 Region of the Hippocampus of the group 2 administered High dose of ANT (1000 mg/kg) showing irregular arrangement of pyramidal cells, Pyramidal cells (PC); Pyknosis (Pk). CFV (x250).

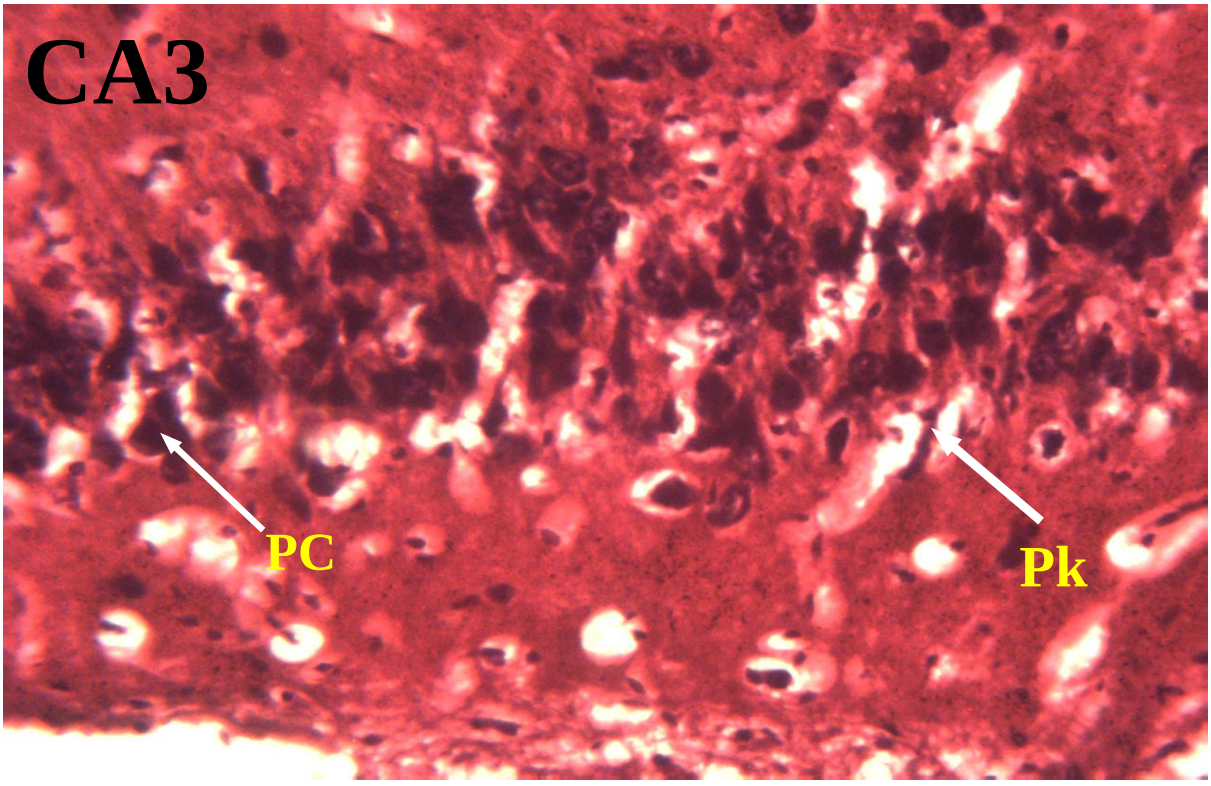


Plate XXXV: Section of CA3 Region of the Hippocampus of the group 3 administered Medium dose of ANT (500 mg/kg) showing less compactly arranged pyramidal cells (P); Pyramidal cells (PC); Pyknosis (Pk). H and E (x250).

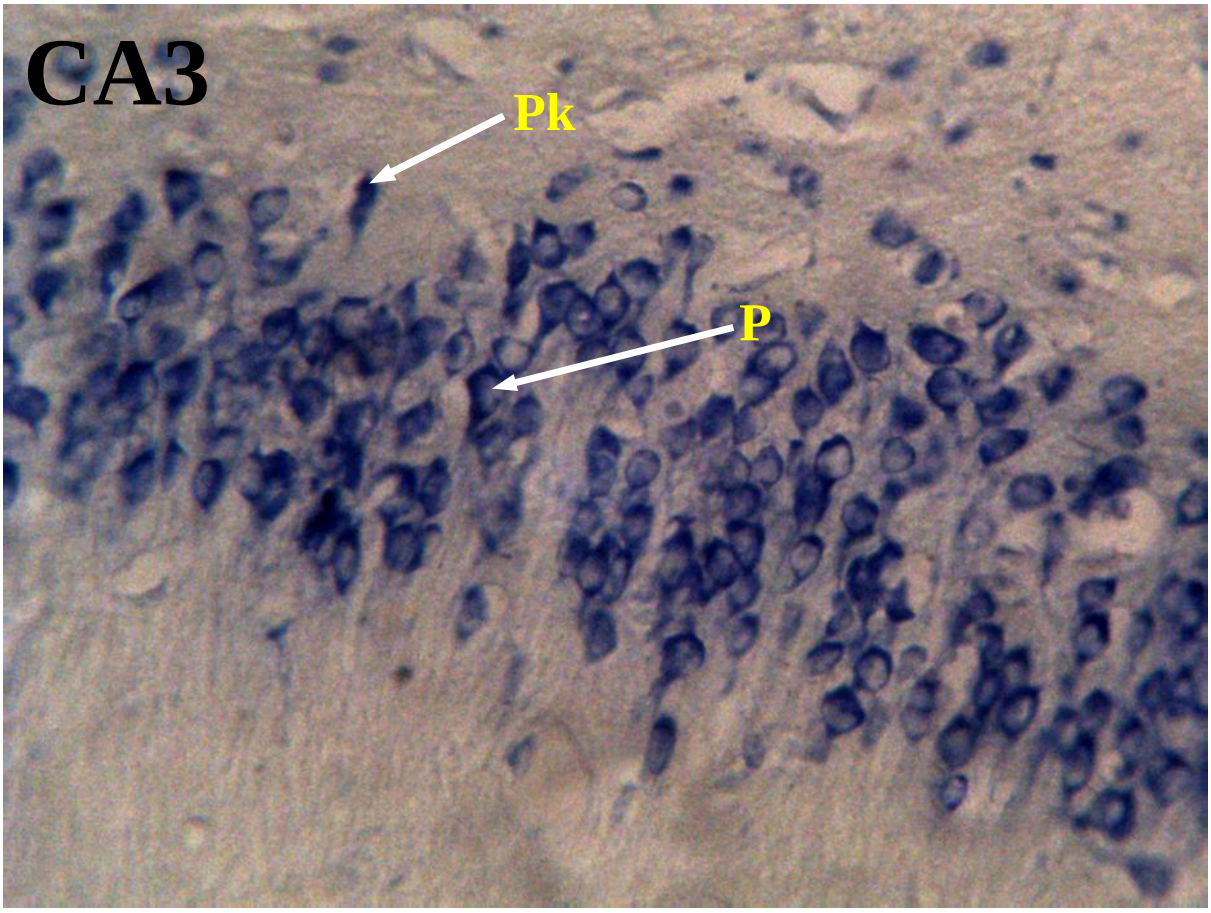


Plate XXXVI: Section of CA3 Region of the Hippocampus of the group 3 administered Medium dose of ANT (500 mg/kg showing less compactly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). CFV (x250).

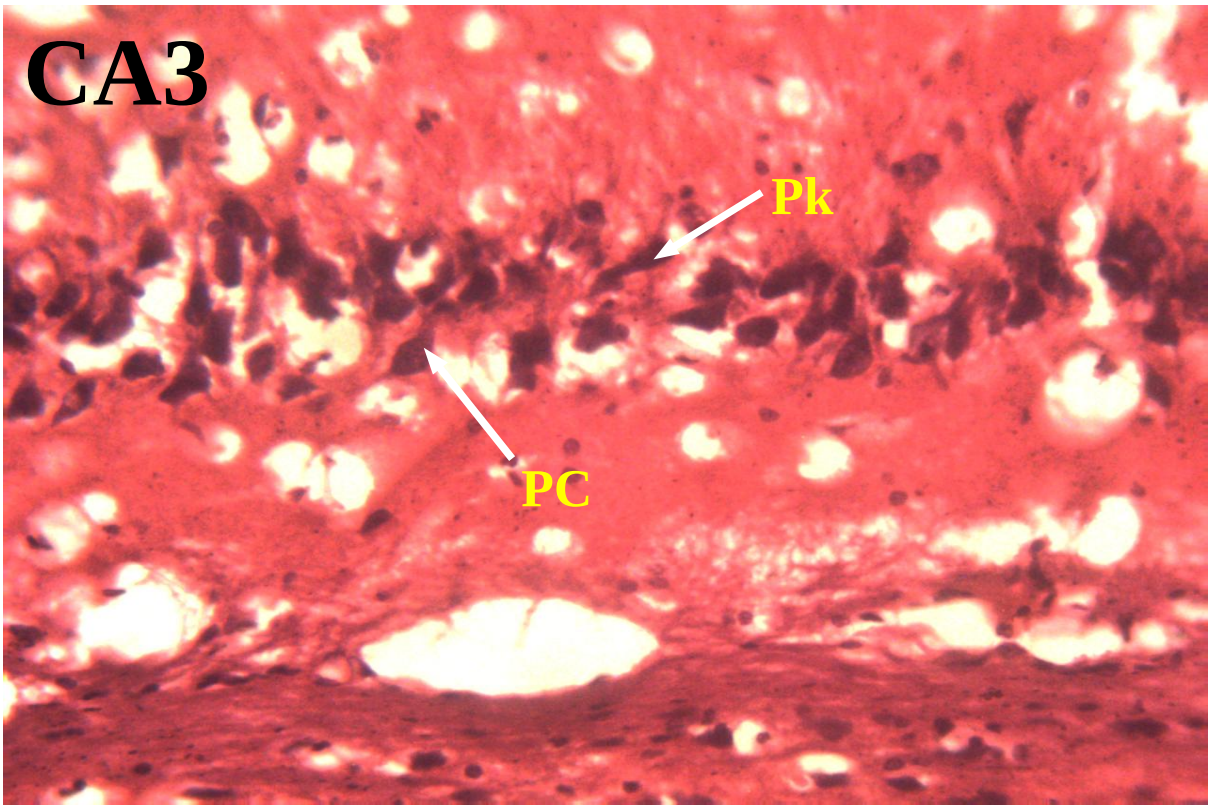


Plate XXXVII: Section of CA3 Region of the Hippocampus of the group 4 administered Low dose of ANT (250 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). H and E (x250).

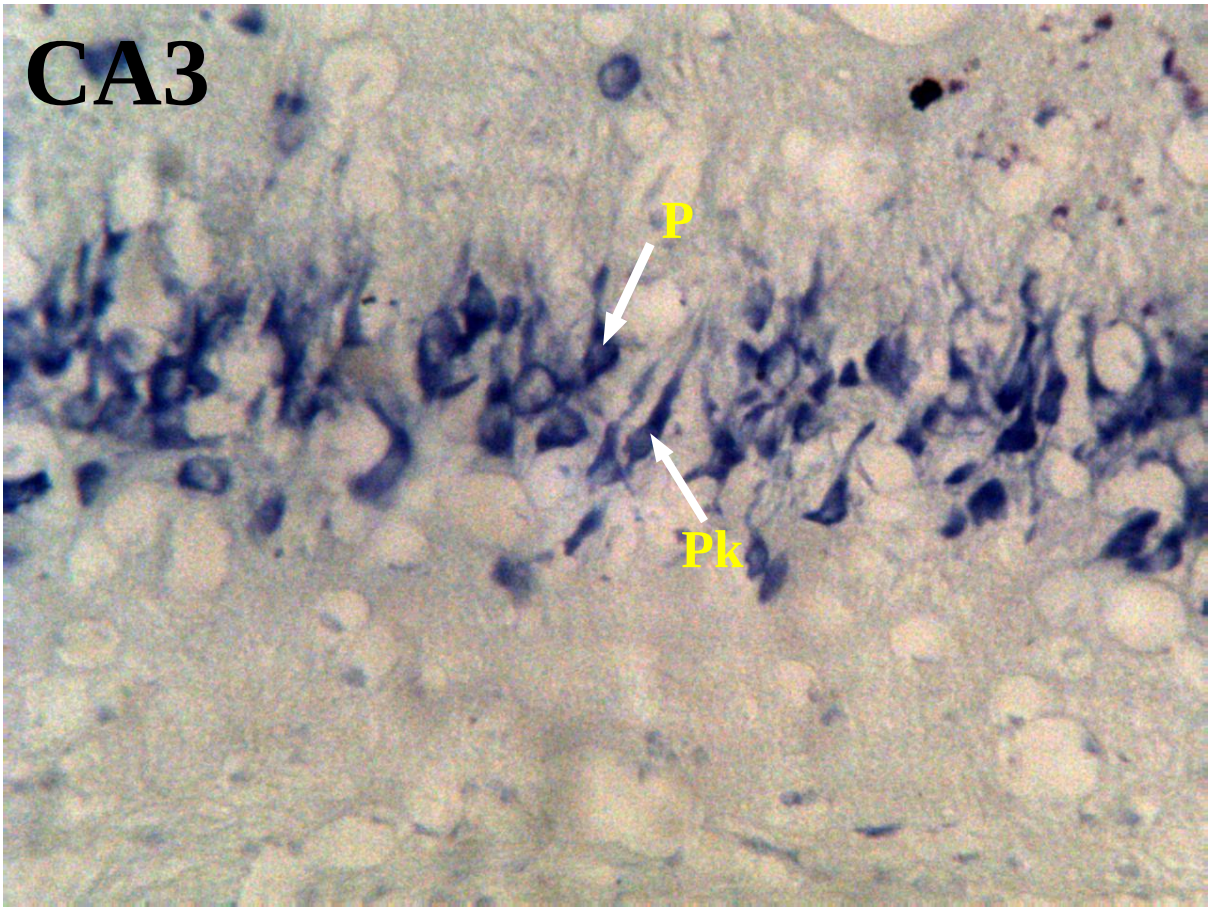


Plate XXXVIII: Section of CA3 Region of the Hippocampus of the group 4 administered Low dose of ANT (250 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). CFV (x250).

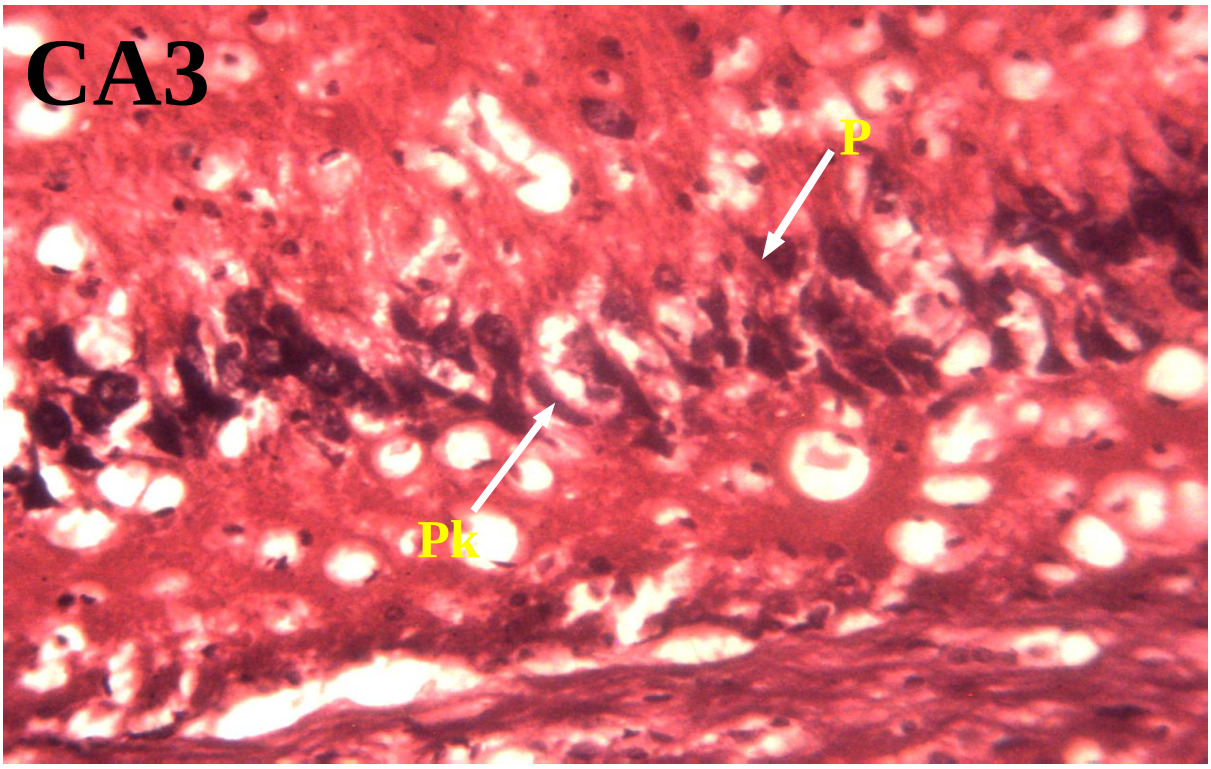


Plate XXXIX: Tranverse section of CA3 region of the hippocampus of the group 5 administered High dose of ENT (1000 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). H & E (x250).

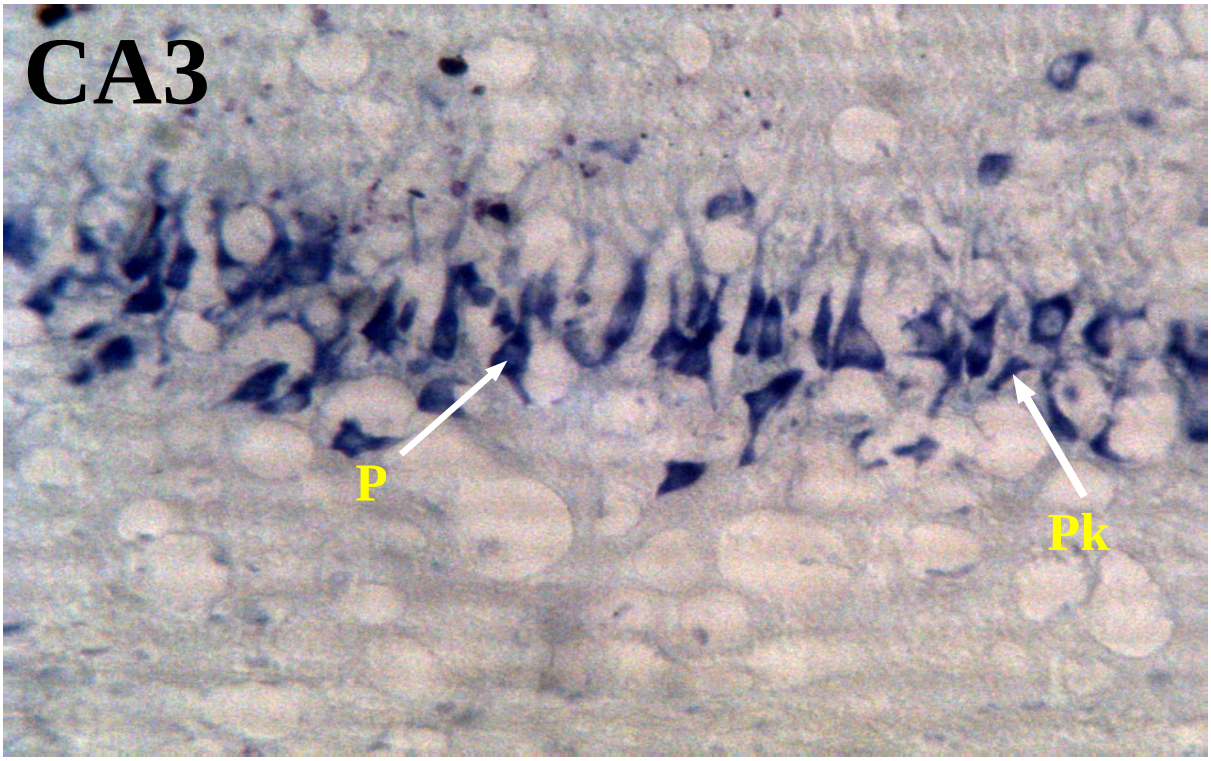


Plate XL: Tranverse section of CA3 region of the hippocampus of the group 5 administered High dose of ENT (1000 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). CFV (x250).

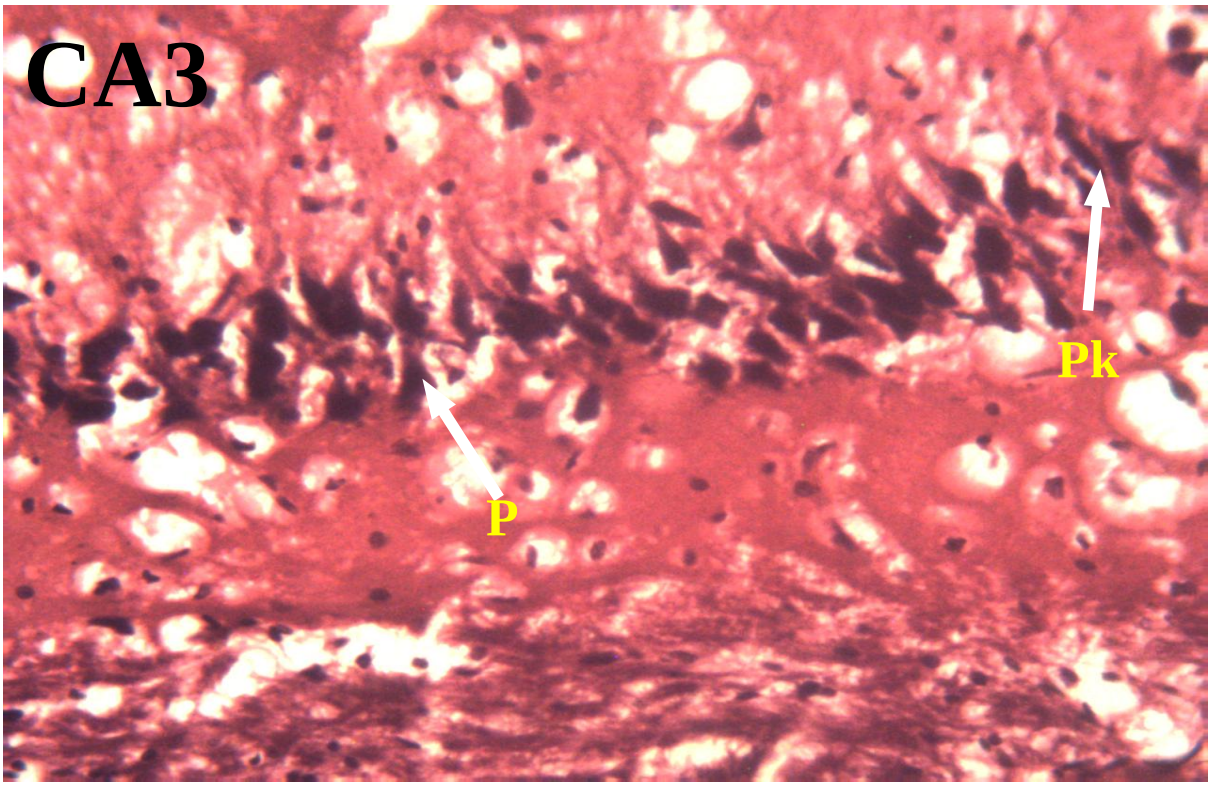


Plate XLI: Section of CA3 Region of the Hippocampus of the group 6 administered Medium dose of ENT (500 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). Hand E (x250).

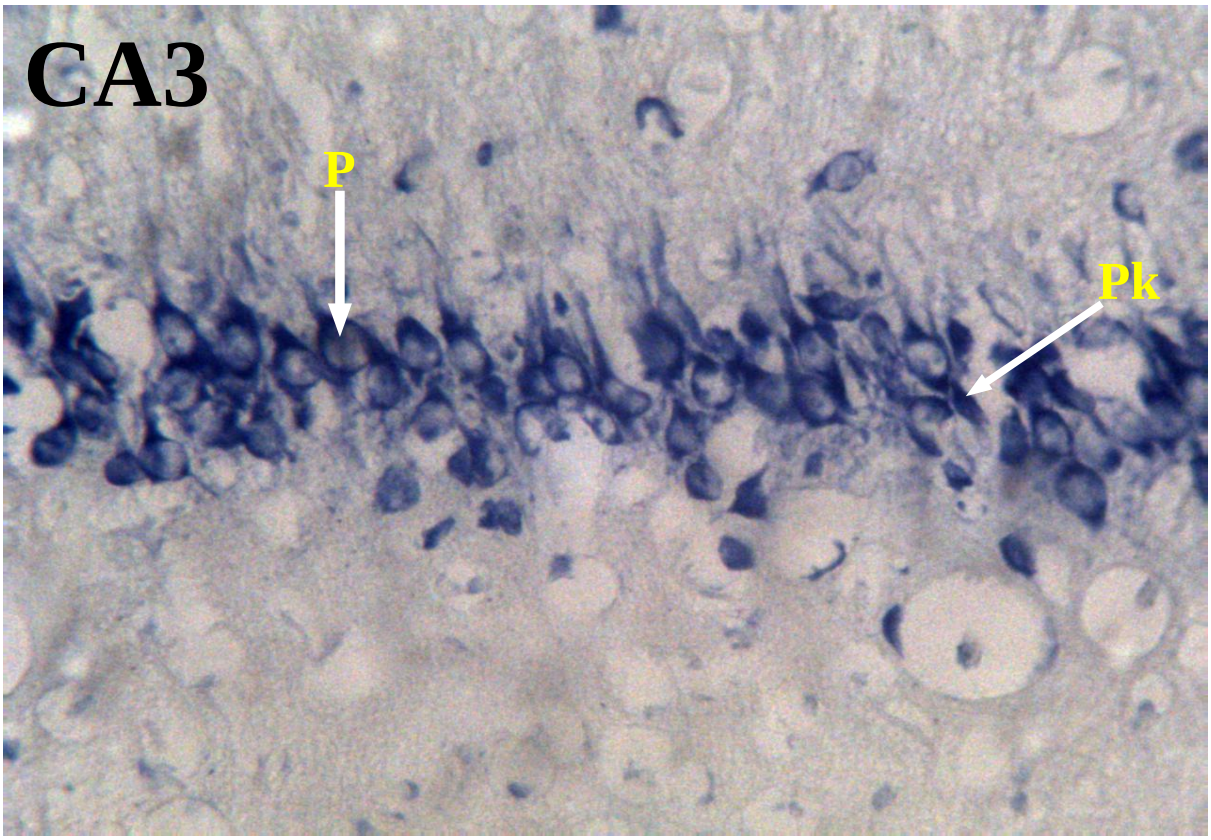


Plate XLII: Section of CA3 Region of the Hippocampus of the group 6 administered Medium dose of ENT (500 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). CFV (x250).

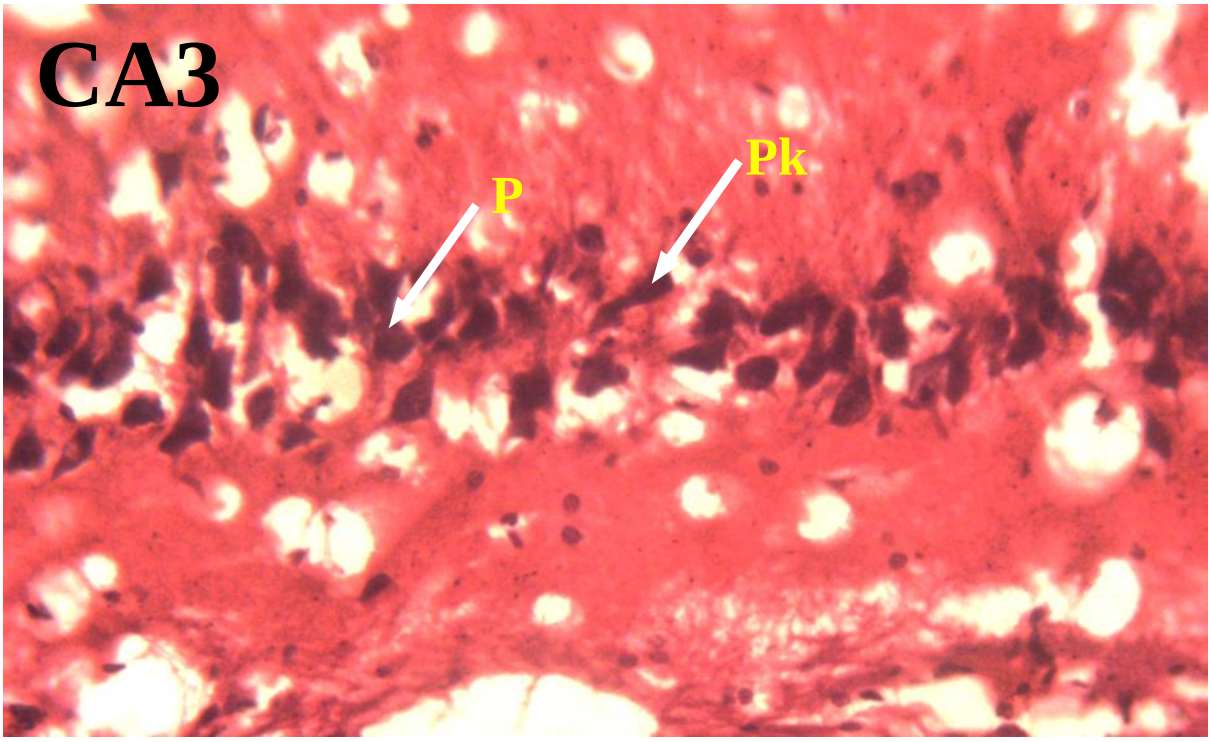


Plate XLIII: Section of CA3 Region of the Hippocampus of the group 7 administered Low dose of ENT (250 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). Hand E (x250).

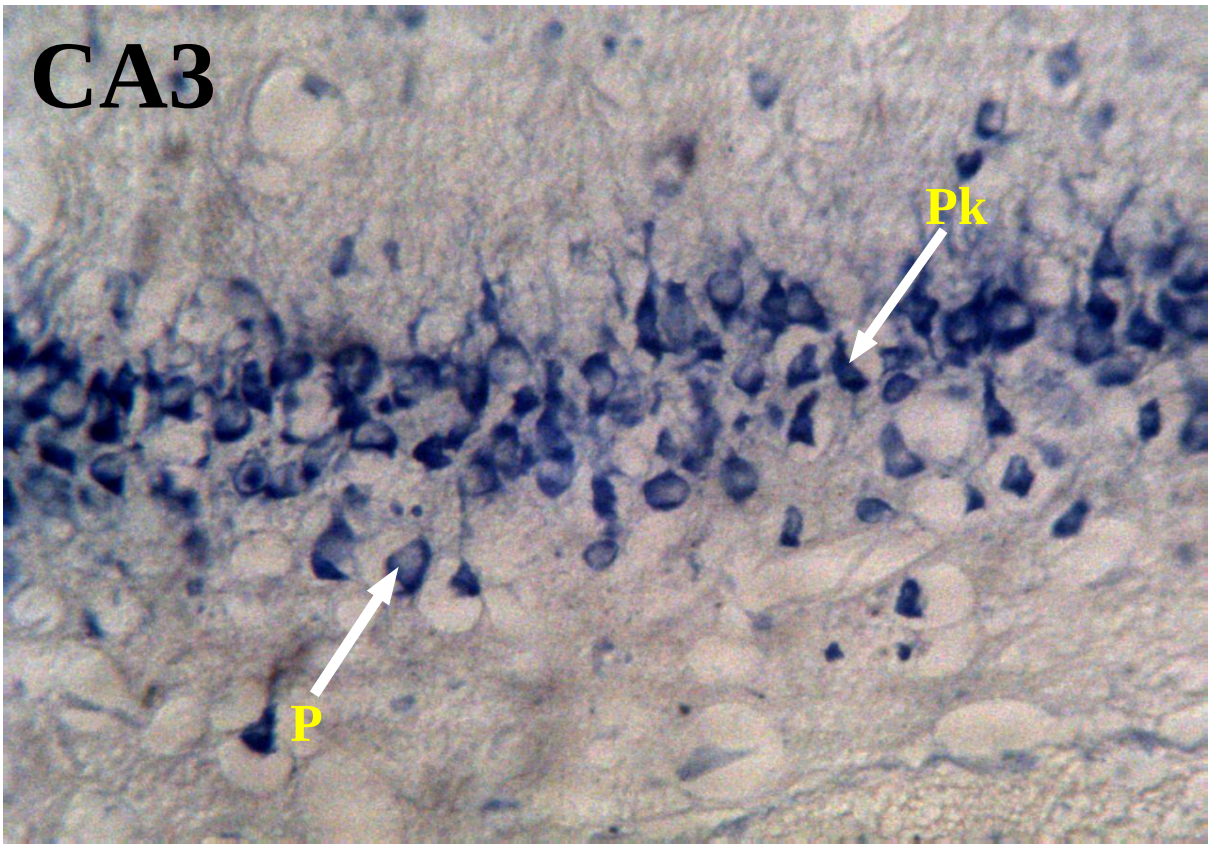


Plate XLIV: Section of CA3 Region of the Hippocampus of the group 7 administered Low dose of ENT (250 mg/kg) showing scantly arranged pyramidal cells (PC); Pyramidal cells (PC); Pyknosis (Pk). CFV (x 250).

CHAPTER FIVE

5.0 Discussion

The present study, preliminary phytochemical screening was carried out, and the effects of the aqueous and ethanolic leaves extracts of *Nicotiana tabacum* on the cerebral, cerebellar and hippocampal toxicity in Wistar rats was assessed using morphologic, histochemical, neurobehavioral, neurochemical and biochemical methods.

5.1 Phytochemical studies

The results of the Phytochemical screening of the plant *Nicotiana tabacum* leaves extracts namely aqueous and ethanolic, in the present study, revealed the presence of carbohydrates, glycosides, flavonoids, tannins, triterpenes, saponins and alkaloids in both extracts which has been reported as alternative medications to attenuate oxidative stress and inflammation associated with degenerative diseases. However the results of this study showed that the ethanolic extract of *Nicotiana tabacum* displayed the most effective decrease of CAT, SOD and GPx activity. This pointed to the fact that ethanolic extract of *Nicotiana tabacum* inhibits CAT, SOD and GPx, which is in conformity with earlier reports that mild inhibitors of antioxidant enzymes inhibits CAT, SOD, MDA and GPx (Azevedo *et al.,* 2001). The inhibitory potential displayed by both aqueous and ethanolic extracts of *Nicotiana tabacum* towards SOD, CAT and GPx implies that the pythochemical components anthraquininones which are absent in both extracts could be the reason behind the decrease of CAT, SOD and GPx in the treated group, possibly interfering with the action of antioxidants enzymes. Studies have shown that their presence possess multiple biological activities including antioxidant, anticancer and antimicrobial activities (Azevedo *et al.,* 2001; Kaushik Sunil, 2011).

5.2 Physical observation

The result of the physical observation showed increased physical activity exhibited by the treated groups which reflected toxicity. Altered physical activity manifesting as restlessness, hyperactivity and loss of appetite indicates toxicity (Perkins *et al.,* 1994; Willis, *et al.,* 2014). Also the result obtained from this study reveals that the appetite of the tobacco administered Groups were suppressed hence resulting to reduced food intake in Groups 2, 3, 4, 5, 6 and 7 as seen in Figure 4.2. The decrease in body weight change served as a sensitive indication of the general health status of animals (Salawu *et al.,* 2009). This action of tobacco on body weight appears to be nicotine mediated as indicated by Hajek *et al,* (1988). Previous studies in both humans and animals have reported that nicotine administration decreases body weight and caloric intake (Adeniyi *et al.,* 2010). Thus, the effects of nicotine to suppress appetite and decrease food intake leading to reduced body weight in Wistar rats. The present study reveals a decrease in body weight following tobacco administration. The observed reduction in weight of the animals in the experiment may implicate nicotine in the tobacco plant use as reported by Chen *et al.,* (2005) and Adeniyi *et al.* (2010a).

Weight loss is a commonly cited reason for tobacco users, especially among young females (reviewed in Perkins, 1993), and fear of weight gain has been an obstacle to successful cessation (Froom *et al.,* 1998). The physiologic effect of nicotine has been described as a combination of a reduction in caloric intake and an increase in caloric expenditure. Investigators have found that weight changes upon initiation and cessation of tobacco usage are nicotine associated (Grunberg *et al.,* 1986). Consistent with many previous studies, Doherty *et al.* (1996) found that nicotine replacement during tobacco cessation suppresses

weight gain. The mechanisms through which nicotine decreases body weight are complex and incompletely understood. 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 (Benowitz, 2010). 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 catecholamines in the central nervous system, dopamine, norepinephrine, serotonin, acetylcholine, glutamate, γ -aminobutyric acid, and other neurotransmitters. The release of this neurotransmitters suppress eating and increase metabolic rate (Benowitz, 2010).

Body weight is determined by the balance of caloric intake and daily energy expenditure. Daily energy expenditure is determined by resting metabolic rate, physical activity and the thermic effects of food. Nicotine reduces body weight by raising the resting metabolic rate while blunting the expected increase in food intake in response to the increase in metabolic rate. Like many antiobesity drugs, nicotine is a sympathomimetic agent. Sympathomimetic drugs increase energy expenditure via action on peripheral tissue and through regulation of metabolism in the brain. Nicotine promotes the local release of norepinephrine within body tissues and systemic release of epinephrine from the adrenal glands. Nicotine increases thermogenesis in adipose tissue, partly by increasing lipolysis and subsequent recycling of fatty acids into triglycerides (Hellerstein *et al.*, 1994; Andersson *et al.*, 2001).

Nicotine has many potential effects on central nervous system regulation of eating and energy expenditure. The regulation of eating behaviour and metabolic rate by the brain occurs in the hypothalamus, which integrates peripheral signals of satiety and adiposity as well as central motivational and emotional influences. Leptin is released from adipose

tissue in proportion to the amount of adipose and acts centrally to suppress food intake and increase metabolic rate. However, it has been suggested that nicotine may increase the effects of leptin in the brain by enhancing leptin binding or increasing the sensitivity of downstream transduction cascades (Jo *et al.,* 2002).

The release of hormones such as norepinephrine, dopamine, serotonin and γ-aminobutyric acid by the central nervous system influences brain chemicals that suppress eating and increase metabolic rate (such as pro-opiomelanocortin and cocaine-amphetamine-regulated transcript) as well as those that suppress eating and decrease metabolic rate (such as neuropeptide Y, Agouti-related peptide, melanin-concentrating hormone, and orexin) (Valassi *et al.,* 2008).

Nicotine has complex effects on these hormones; the acute response is consistent with activation of systems that decrease appetite and increase body metabolism, whereas the chronic changes are consistent with activation of systems that increase appetite and decrease metabolic rate (Jo *et al.,* 2002). The findings of this study stands in agreement with the works of Doherty *et al.* (1996); Chen *et al.,* (2005) and Adeniyi *et al.,* (2010a) seen with reduction in body weight after tobacco intake.

5.3 Neurobehavioral Studies

In the present study, two neurobehavioral tests were conducted to assess a variety of endpoints, such as cognition, sensory-motor activity and motor coordination and balance in Wistar rats. Morris water maze (MWM) was used to evaluate learning and memory and beam walking test (BWT) to monitor motor coordination and balance of Wistar rats after the administration of aqueous and ethanolic extracts of *Nicotiana tabacum* for 21 days.

The Morris water maze is one of the most widely used tasks in behavioural neuroscience for studying the psychological processes and neural mechanisms of spatial learning and memory (Brandeis *et al.,* 1989). 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. Increased latency was observed with tobacco treated Groups 2 and 3, at day 14 of treatment which indicated learning and memory impairment. Larry *et al.* (1998) elucidated that distinct forms of memory are mediated by different CNS regions, such as the prefrontal cortex and limbic structures including the Hippocampus. Memory forms can be classified as declarative or explicit meaning the ability to recall past events deliberately and are hippocampus-dependant; and non-declarative or procedural also called implicit, defined by unconsciously performed skilled motor or cognitive actions that are mainly dependant on the cerebellum (Packard and McGaugh, 1992; McDonald and White, 1993. Memory is an organism's mental ability to store, retain and recall information, and the hippocampus plays an important role in learning and memory (Chakravartthi *et al.,* 2012). The findings from the present study suggested that tobacco administration, showed variation in learning and memory.

In the study beam walking test was used to assess motor coordination and balance in the Wistar rats, the control of movement, motor coordination and balance is linked to the cerebellum. The hippocampus coordinates the different muscle groups so that the muscle exerts movements fluently and precisely. It also receives feedback information about intended and actual movements, hence it is seen as a stabilizing control system which receives forewarning of each motor impulse. Atrophy to the cerebellum is usually accompanied by different ataxia and an unstable gait (Fonnum and Lock, 2000). Beam

walking ability was measured using foot-slip score and latency (time) until the Wistar rat's nose entered the goal box of the beam walking apparatus.

Across the period of the study, the administration of extracts had no effect on foot-slip score and beam walking ability when compared with the Control. However, the administration of both extracts revealed normal walking performance in groups 2, 3, 6 and 7 when compared with the Control, thus indicating a smooth motor coordination and balance. Excitotoxicity characterized by a cascade of reactions resulting in a rise in intra-cellular calcium and free radical formation play an important role in cerebellar toxicity involving granular cells or Purkinje cells since both cells have glutamatergic receptors. Excitotoxicity can be initiated by glutamate agonist and by inhibitors of neuronal metabolism (Fonnum and Lock, 2000). Also increased latency was observed with all tobacco treated Groups after 21 days of treatment, their ability to recall past events deliberately was affected which indicated learning and memory impairment. The findings from the present study also suggested that tobacco administration, showed variation in learning and memory.

5.4 Morphological Studies

Morphological studies involves studying shapes and the detection of possible shape changes between groups. Evaluation of shape change in the brain is a key step in the development of new rodent models, monitoring of different pathologies and the measuring of environmental influences (Scheenstra *et al.,* 2010). No gross changes were observed between the experimental Groups and Control Group till the completion of the experimental procedure. The brain with its component parts of the rats in both the Control and the experimental Groups appeared morphologically normal. In this study, brain weights

and the relative brain weights were computed and compared with the Control. Brain weight decreased in treated groups relative to Control. However Relative organ weight increased in treated Groups relative to 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 been reported with intoxication of heavy metals (Amal and Mona, 2009).

5.5 Neurochemical/Biochemical Studies

Trace metals are of neurological importance and have critical actions in neurodevelopment, neurotransmitter synthesis, energy metabolism and antioxidant defence. They are important for cognitive function (Sujuan *et al.*, 2008).

In the present study we determined the levels of the antioxidants MDA, CAT, GPx and SOD, as well as the neurotrace elements Zn, Mn, Fe and Cu to ascertain the level of damage in Cerebellar and cerebral cortices and hippocampus of tobacco treated groups. We found decreased levels of superoxide dismutase, catalase and glutathione peroxidase in tobacco treated groups compared to controls. It reportedly contains toxic levels of arsenic, lead, cadmium, and nitrates, which could potentially be converted to nitrites. The presence of nitrates, nitrites, a high nicotine content and an average alkaline pH of 8.5, all potentially favor the formation of tobacco specific nitrosamines (Zakiullah *et al*., 2011).

Many trace elements are known to cause abnormal mental function when either a deficiency or excess exists. When a nutritional mineral accumulates excessively in the body, it can become toxic (Watts, 1990). Some recent works suggest that the optimal

concentrations of Cu, Zn and Fe exist for optimal cognitive function in adult (Lam *et al.*, 2008). In the brain, zinc plays a key role in synaptic plasticity and so in learning (Hambidge and Krebs, 2007; Bitanihirwe *et al.,* 2009; Nakashima and Dyck, 2009). It also can be a neurotoxin, suggesting zinc homeostasis plays a critical role in normal functioning of the brain and central nervous system (Bitanihirwe *et al.,* 2009). The hippocampus is particularly rich in zinc (Dreosti, 1984).

The result obtained from the study showed elevated levels of Mn, Zn and Fe in the treated Groups compared to the Control. Elevated levels of Fe was observed in the Groups; 3, 6 and 7 and decreased in the groups; 4 and 5 when compared to the Control, although the difference observed in both increases and decreased Groups were not significant. Among the numerous biological effects of iron, there is considerable evidence that iron is also important for neurological functioning and development (Lozoff *et al.,* 1987; Beard *et al.,* 1993; Roncagliolo *et al.,* 1998). The biological basis of the behavioral and cognitive developmental delays observed in iron-deficient infants is not completely understood but possibilities include: that abnormalities in the neurotransmitter metabolism (Erikson,et *al 2001*), decreased myelin formation (Larkin and Rao (1990) and alterations in brain energy metabolism (Rao and Georgieff 2001). Excess accumulation of Fe affects the CNS in that increased Fe has been observed in the lesion site of a number of neurodegenerative disorders and models of neurodegeneration (Olanow *et al.,* 1990). Increased Fe deposition in regions of the brain has been implicated in Parkinson's disease (Watts, 1990). Fe levels are also increased at site of degeneration in Alzheimer disease (AD); which have been detected in the hippocampus, nucleus basalis, frontal cortex and motor cortex of AD patients (Conner *et al.,* 1992; Richardson., 1993). The findings from the present study

suggested that tobacco administration, showed increased level of Fe which resulted to impaired learning and memory.

Copper is one of the many metal ions that are required for essential body functions but are toxic in excess (Cox and Moore, 2002; Desai and Kaler, 2008). Copper is present throughout the brain and is most prominent in the basal ganglia, hippocampus, cerebellum and in cell bodies of cortical pyramidal and cerebellar granular neurones (Madsen and Gitlin, 2007). Excess copper has been implicated either directly or indirectly in the pathogenesis of numerous neurological diseases, such as depression, AD, Parkinson's disease PD and Wilson disease (Scheinberg, 1976; Desai and Kaler, 2008). Copper is a neuromodulator for dopamine which is a neurotransmitter associated with motor activities. In this present study there was alteration in the level of copper indirectly affected dopamine that plays a role in motor coordination. Hence increase in the mean latency time in beam walking test could be associated with copper alteration.

Exposure of Wistar rats to *Nicotiana tabacum* has been shown to induce changes in the cerebral and cerebellar content of Fe, Zn and Cu in rats. In the present study no significant change was observed in the treated Groups when compared to Control.

Oxidative stress which is a common pathology which occurs due to imbalance between production and detoxification of reactive oxygen species (ROS) has also been implicated in many neurodegenerative disease such as Alzheimer's disease and Parkinson's disease (Gilgun-Sherki *et al.,* 2001; Trushina and MeMurray, 2007). The brain contains antioxidant mechanisms which are likely to quench these reactive oxygen species. However during complications these antioxidant mechanisms may downplay and create a situation which is

called Oxidative stress. The brain has a large potential oxidative capacity but a limited ability to counteract oxidative stress. Oxidative stress has been implicated in mechanisms leading to neuronal cell injury in various state of the brain including neurodegenerative disorders. Although the brain accounts for less than 2 % of the body weight, it consumes about 20 % of oxygen available through respiration. Therefore, because of its high oxygen demand, the brain is the most susceptible organ to oxidative damage (Weiss and Fintelmann, 2000). Due to high oxygen consumption of the brain, it is more susceptible to oxidative stress. Halliwell *et al.*, (1999) had reported that increased oxidative stress leads to Lipid peroxidation, protein damage and induction of apoptosis.

In the present study, malondialdhyde (MDA) were assayed for the estimation of lipid peroxidation levels, while antioxidant enzymatic activity was estimated by assaying for superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in the brain tissues.

Tissue MDA levels were increased slightly in all treated groups when compared with the Control, although not significant. Elevation of MDA levels suggest enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defence mechanism to prevent formation of excessive radicals (Himakar *et al.*, 2010). Lipid peroxidation is a free radical-related process, which is potentially harmful because if uncontrolled, self-enhancing process causes disruption of membrane, lipids and other cell components. It is also involve in oxidative stress, which plays a role in neural-degeneration (Rao and Purohit, 2011). High level of lipid peroxidation (LPO) which is not normal and the simultaneous decline of antioxidants defence mechanism can lead to cellular organelles damage and oxidative stress

(Mahboob et al., 2005). Treatment of rats with *Nicotiana tabacum* in the present study revealed a slight increase in MDA levels, indicative of generation of LPO. Increased production of many ROS such as superoxide and hydrogen peroxide results in lipid peroxidation and oxidative stress tissue damage. Slight increase in the level of ROS and oxidative stress, depletes glutathione and thiols causing increased neurotoxicity from interaction of ROS, glutamate and dopamine. However, a Turkish study on smokeless tobacco product reported an increased state of oxidative stress via raised levels of malondialdehyde and reduction in the levels of SOD and Catalase in its users (Kilinc *et al*., 2004). Another study conducted on tobacco chewers has also reported oxidative stress and decreased concentrations of antioxidant enzymes in its users (Samal *et al*., 2006).

SOD is an antioxidant that protect the cell against oxidative stress, it is a sensitive index in oxidative damage as it scavenges the superoxide anion from hydrogen peroxide leading to the reduction in the toxic effects (Khan *et al.*, 2012). Our observations indicating decreased SOD levels indicate that aqueous and ethanolic extracts of *Nicotiana tabacum* usage may result in oxidative stress, several studies have confirmed that tobacco leaves contain high arsenic and cadmium concentrations which are known to be potential candidates for oxidative stress (Zakiullah *et al.* 2011; Borgerding *et al.*, 2012; Khilifi *et al*., 2014).

Catalase is a key component of the antioxidant defence system. Inhibition of these protective mechanism enhanced sensitivity to free radical induced cellular damage (Young and Woodside, 2001). It is widely distributed in all animal tissues decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Fang *et al.*, 2000). GPx is equally an important antioxidant enzyme, which reacts with hydrogen peroxide thus preventing intracellular damage caused by the free radicals (Lee *et al.*, 2007)

In the present study, tissue CAT activity levels in all treated Groups were decreased and was significant in Groups 2, 4, 5, 6 and 7. Tissue GPx activity levels were decreased in Groups 4, 5, 6 and 7 especially in the ethanolic extract treated groups. Our observations indicating decreased CAT and GPx levels indicate that aqueous and ethanolic extracts of *Nicotiana tabacum* usage may increase the level of reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, which provoke lipid, protein oxidation (Clarkson, 1997; Li *et al.,* 2006) hence resulting in oxidative stress, studies done by Borgerding *et al.,* 2012 have confirmed that tobacco leaves contain high arsenic and cadmium concentrations which are known to be potential candidates for oxidative stress. This effect on GPx agrees with the reports of Sreekala and Indira (2009), who suggest that nicotine alone increases lipid peroxidation by the inhibition of antioxidant enzymes. Also, the decrease could be due to the interaction with nitric oxide (Benuck, 1995), which is produced by the inhibition of mitochondrial electron transport chain (Mito- ETC) complexes (I, II, III), in different brain regions (Das, 2009).

The GPx enzymes catalyse the reduction of H_2O_2 and organic Peroxide (R-O-O-H) to water and the corresponding stable alcohol thus inhibiting the formation of free radicals. Thus, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide, linked with neurodegenerative diseases (Khan *et al.,* 2012). The extent of injury is generally related to an increase or decrease of one or more free radical scavenging enzymes of which GPx is one (Pippenger *et al.,* 1998).

5.6 Histological and Histochemical Studies

In the present study, light microscopic examination of routinely Haematoxylin and Eosin, (H&E) and histochemically Cresly Fast Violet stained sections of Wistar rats brain regions namely: cerebral cortex-layer V, cerebellar cortex and hippocampus were undertaken.

The result from the present study showed mild histoarchitectural distortions such as degeneration of neuronal cells, shrinkage of cell body, vacuolations, pkynosis and karyorrhesis.

Neurodegeneration is a process involved in both neuropathological conditions and brain ageing (Kumar and Khanum, 2012). Histoarchitectural distortion of neural tissues manifesting as, neuronal degenerative changes are indicative of neurotoxicity in the central nervous system (Kalantaripour *et al.,* 2012). Degenerative changes observed as cortical neuronal shrinkage and neuropil vacuolations, loss of pyramidal neuronal processes and necrosis in cerebral section of tobacco treated Wistar rats when compared with the cerebral section of the Control indicated treatment related neurotoxicity. This is in concordance to previous studies on tobacco that reports that *Nicotiana tabacum* has the capacity to induce neuronal damage (Adeniyi *et al.,* 2014). Neuronal injury may result in reversible or irreversible cell damage or cell death (Kumar *et al.,* 2006). Cell death has been reported to result from neuronal degeneration (Waters *et al.,* 1994; Agbon *et al.,* 2014). Cell death may result from necrosis, a pathologic type of cell death that occurs from extrinsic insults to the cells, such as chemical injury, toxins, thermal, traumatic and mechanical factors (Wyllie *et al.,* 1980; Kumar *et al.,* 2006). Histoarchitectural distortion such as pyknosis and karyorrhesis of neurones when compared to the Control Groups observed from the cerebral sections of Wistar rats treated with both aqueous and ethanolic extracts induced

neurotoxicity (Holland, 1996). Necrosis, pkynosis and karyorrhesis is an indication of treatment-related toxicity (Kumar *et al.,* 2006). Degenerating neurones stain very poorly rather than darker with CFV as a result of disassociation of ribosomes from the RER which occurs in the early stage of cell degeneration (German, 2011).

Treatment of Wistar rats with aqueous and ethanolic extracts showed distortion in the histoarchitecture of the cerebral cortical paranchyma. This implies that the extracts has neurotoxic effects. Results from this study are consistent with reports on the effect of ethanolic extract of *Nicotiana tabacum* on the hippocampus which showed pyknosis of pyramidal cell (Adeniyi *et al.,* 2010b; Aghaji 2008). In the present study, the cerebellum, cerebral cortex and hippocampus interestingly revealed susceptibility and more damage in the aqueous treated Groups of *Nicotiana tabacum* when compared with the Ethanolic treated Groups of *Nicotiana tabacum*. However mild damage in both treated groups were observed in groups with doses 250 mg/kg and this is consistent with the findings reported by Adeniyi (2010a) on the effect of ethanolic extract of nicotiana tabacum altered hippocampal histology and behaviour in mice.

Purkinje cells and granule cells were the most important targets in the cerebellum for toxic substances (Fonnum and Lock, 2000). Neurodegenerative changes such as perineuronal vacuolations, neuronal cytoplasmic shrinkage, Purkinje cell and internal granule cell necrosis, and indistinct neurones due to reduced staining intensity observed in tobacco treated Wistar rats implied treatement related toxicity. Findings were in consonance to studies related to *Nicotiana tabccum* induced cell injury on cerebellar structures by Omotoso, (2014). *Nicotiana tabccum* disturbs neuronal network structure and induces cell

apoptosis in cortical neurones. Mild histoarchitectural distortion such as perineuronal vacuolations, outer molecular cell and internal granule cell necrosis when compared to the Control was observed from cerebellar sections of Wistar rats treated with aqueous and ethanolic extracts of *Nicotiana tabacum* suggested that tobacco induced cerebellar damage. The brain is made up of neurones and neuroglia, the neurons being responsible for sending and receiving nerve impulses or signals. The microglia and astrocytes are essential for ensuring proper functioning of neurones and, are quick to intervene when neurones become injured or stressed. As they are sentinels of neurone well-being, pathological impairment of microglia or astrocytes could have a devastating consequences for brain function (Manickam *et al.,* 2009). Acute injury causes neurones to generate signals that inform neuroglia about the neuronal status. Depending on how severe a degree of neuronal injury, neuroglia will either nurse the injured neurones regeneration or kill them if they are not viable. These types of neuroglia responses are considered to represent normal physiological and neuroprotective responses (Kumar and Khanum, 2012).

Adeniyi (2010) reported that, neuronal damage in the form of shrinkage, atrophy and neuronal necrosis was greatly increased in the brain of rats treated with *Nicotiana tabcum*. Aqueous extract treated groups showed a dose-dependent distortion of the histoarchitecture of cerebellum compared to the Control. However, ethanolic extract of *nicotiana tabacum* treated groups revealed lower distortion or damage with dose 1000 mg/kg. This is consistent with the findings of Rezwani *et al.*, (2001); Chen *et al.* (2003). The results thus support an emerging pattern wherein tobacco (nicotine) exposure elicits cerebellar damage leading to abnormality of cellular pattern.

Leaves extract of *Nicotiana tabacum* at dose 1000 mg/kg showed negative effect on the rate of neuronal death in CA3 hippocampus, which may be due to high concentration of antioxidants that may be harmful (Kuhn, 2003). Observed histoarchitectural distortion of the hippocampus region CA3, such as, irregular arrangement of CA3 hippocampal neurones and degenerating pyramidal cells, was indicative of treatment (tobacco) 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 apoptosis demonstrating the capacity of nicotine in promoting apoptosis. Taken together, these finding suggested that nicotine which is a major constituent of tobacco is a neurotoxic agent regardless of its protective property in many other experimental paradims (Prendergas *et al.,* 2001; Fonnum *et al.,* 2000). 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 (Seidman, 2011). Irregular arrangement of CA3 neurones in the present study implies treatment related degenerative changes. Towfighi *et al.,* (2004) reported a preferential degeneration of deep CA3 pyramidal cells was observed during early postnatal development in rabbits as a consequence of pilocarpine-induced seizures. Basophlia of cytoplasm and pkynosis 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 (Seilhean *et al.,* 2004)

Mild neurodegenerative changes in CA3 regions of the hippocampus, such as, irregular arrangement of CA3 hippocampal neurones, degenerating pyramidal cells, pkynotic

necrosis, and vacuolations were observed in Wistar rats treated with both extracts (Aqueous and ethanolic). Neurodegenerative changes have been reported as direct toxic effect of tobacco (Omotoso, 2014). 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 (Mena *et al.*, 2004)

CHAPTER SIX

6.0 Conclusion and Recommendation

6.1 Conclusion

In the present study, evaluation of the effect of aqueous and ethanolic extracts of *Nicotiana tabacum* in Wistar rats. The result of the phytochemical screening revealed the presence of antioxidants, flavonoids, saponins, tannins and alkaloids which displayed inhibitory potential towards SOD, CAT and GPx. There decrease have been known to play a role in neuronal degeneration and necrosis on the neurons. Exposure to tobacco resulted to aggression related responses, altered the levels of biochemical, with memory impairments, no motor deficits in neurobehavioral assessment.

Findings from the present study revealed that the aqueous and ethanol leave extracts of *Nicotiana tabccum* as a neurotoxic agent following;

i. Tobacco induced aggression-related responses, with no motor coordination and balance deficits in neurobehavioural assessment and decrease body weights after administration of tobacco for 21 days.
ii. Morphologic features of the brain regions namely cerebellar and cerebral cortices and hippocampus were preserved in relation to the control.
iii. *Nicotiana tabacum* alters histologic and histochemical features in cerebral cortex (pyramidal cells) and cerebellar cortex (Purkinje cells) and hippocampus regions (CA3) , it shows that tobacco can do more harm than good
iv. Imbalance in the concentration levels of biochemical parameters CAT, SOD, GPx was remarkably observed.

v. Ethanolic leave extract of *Nicotiana tabacum* has more effect on body weight, histological features and biochemical parameters in relation to the aqueous extract.

The results of this study revealed that the aqueous and ethanol extracts of *Nicotiana tabacum* contains substance(s) that are neurotoxic to the brain. These neurotoxic properties explain the rationale for the effect on the brain as a neurotoxic agent. The result of the present study suggests that the aqueous and ethanol leave extracts of *Nicotiana tabacum* may prove unsafe in causing alteration in the brain cerebral, cerebellar cortices and hippocampus of Wistar rats

Neurodegenerative activities observed was dose dependant 250, 500 and 1000 mg/kg doses possessing maximal activity for both extracts aqueous and ethanol leave extracts of *Nicotiana tabacum*. In relation to extracts, neurotoxic activity, ethanolic leave extract of *Nicotiana tabacum* is more hazardous. This could be that ethanolic extract possess certain additional neurotoxic properties or may involve different mechanisms of constituent neurotic activities. It can therefore be concluded that high consumption of *Nicotiana tabacum*, resulted in the alteration in the histological profile and antioxidant enzymes, thus may comprise risk factors for the development of neurodegenerative disease, which could signify a large public health

Problem.

6.2 Recommendation

From the study carried out, the consumption of *Nicotiana tabacum* revealed decrease in body weight, imbalance in oxidative stress markers and neurodegeneration in the histology

of brain tissue, I hereby recommend that the intake of tobacco be restricted from its users to deliver them from its effects.

Further studies on the exact compound(s) or active ingredients of *Nicotiana tabacum* are required to fully investigate the mechanisms responsible for the observed neurotoxic activity of aqueous and ethanol of leave extracts of *Nicotiana tabacum*.

Further studies are required to investigate the most appropriate and unsafe or hazardous extract dose with neurotoxic effect and in depth studies (such as, frozen sections, electron-microscopy, steorology and immunohistochemistry) are required to confirm the findings of the study.

Further studies are required to investigate the possible neurotoxic activity of other solvents (e.g organic solvents, such as methanol and chloroform) extract of *Nicotiana tabacum* leave extract and other parts (such as seed) of the plant.

6.3 Contribution to Knowledge

i. Phytochemical constituents in both extracts (aqueous and ethanolic) of *Nicotian tabacum* were all similar except steroids which revealed to be negative in the aqueous extract and positive in ethanolic extract of *Nicotiana tabacum*.

ii. Administration of both extracts revealed decrease in body and brain weight in groups 2 (139.50±69.59 g), 6 (145.67±19.40 g) and 7 (135.00±69.48 g) when compared to the Control group (217.33± 15.87 g) $P< 0.05$.

iii. Administration of both extracts revealed a significant decrease in CAT level in groups 2 (43.50±2.12 µg/ml), 4 (41.50±0.70 µg/ml), 5 (43.00±1.41 µg/ml), 6 (39.00±1.41 µg/ml) and 7 (36.00±1.41 µg/ml) $p<0.05$ when compared with the

Control (48.00±1.41). Also it revealed a significant decrease in SOD level in 3 (2.10±0.14 nmol/ml), 5 (1.75±0.07 nmol/ml), 6 (1.90±0.01 nmol/ml) and 7 (1.70±0.14 nmol/ml) $p<0.05$ when compared with the control (2.45±0.21 nmol/ml) in Wistar rats which may affect brain function such as memory.

iv. Histologically and histochemically, administration of extracts remarkably revealed neuronal damage, cytoplasmic vacuolations, degeneration of pyramidal cells in Wistar rats and are potential candidates for inducing memory impairment.

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APPENDIX I

TISSUE PROCESSING- THE ROUTINE AUTOMATIC PROCESS

1. **Fixation** in formal saline/ Bouin's fluid lasted 48 hours
2. **Washing** in running water if necessary
3. **Dehydration** in graded alcohol
    - 70% alcohol for 3 hours
    - 90% alcohol for 3 hours
    - First change absolute (100%) alcohol for 1 hours
    - Second change absolute (100%) alcohol for 1 hours
    - Third change absolute (100%) alcohol for 2 hours
    - Fourth change absolute (100%) alcohol for 2 hours
4. **Clearing**
    - Put in pure xylene first change for 1.5 hour
    - Put in pure xylene second change for 2.5 hour
5. **Infiltration**
    - First change pure paraffin (wax bath) 3 hours
    - Second change pure paraffin (wax bath) 3 hours
6. **Embed** in paraffin
7. **Microtomy**

(Drury *et al.*, 1967)

APPENDIX II

PREPARATION OF STAINING SOLUTION AND STAINING

1. Haematoxylin and Eosin Stain

A. **Haematoxylin** (Harris (1990) Haematoxylin)

- Haematoxylin - 1 g
- Absolute alcohol - 10 ml
- Ammonium or potassium alum - 20 g
- Distilled water - 200 ml
- Mercuric oxide - 0.5 g

Haematoxylin was dissolved in alcohol and alum which was dissolved in water was added. The mixture was then boiled and mercuric acid added when solution turn dark purple. It was then cooled rapidly.

B. Eosin

- Distilled water
- Crystal of thymol

5 % eosin was made by dissolving 5 g of eosin in 100 mlof distilled water. To prevent growth of mould in the stock solution, a crystal of thymol was added to obtain 1% staining solution, 20 ml of the stock solution was diluted by making it up to 100 ml.

C. Staining

- **Removal of wax with xylene**
  - Dewaxing I, 3 minutes
  - Dewaxing II, 3 minutes
- **Hydration with graded alcohol**
  - Absolute alcohol, 1 minute
  - 95% alcohol, 1 minute
  - 70% alcohol, 2 minutes
  - 50% alcohol, 2 minutes

- 30% alcohol, 1 minute

- **Staining Proper**
  - Haematoxylin, 10 – 15 minutes
  - Distilled water (washing)
  - 35 % alcohol, 1 minute
  - Alcohol, 30 seconds (for differentiation between nucleus and cytoplasm)
  - Distilled water, 1 minute
  - Alkaline – alcohol, 2 minutes (for bluing of the nucleus)
  - Distilled water, 1 minute
  - Staining in 1% eosin, 2 minutes
- **Dehydration in alcohol**
  - 95% alcohol, 10 -15 seconds
  - Absolute alcohol, 1 minute
- **Clearing**
  - Xylene – alcohol, 2 minutes
  - Pure xylene I, 3 minutes
  - Pure xylene II, 3 minutes
- **Mounted** in DPX

(Drury *et al.,* 1967)

2. CRESYL FAST VIOLET STAINING

- **Fixation** in 10% formal saline
- **Section**
- **Technique**
  - Dewax using xylene and take section to water
  - Staining for 1 – 6 hours 0.2% cresyl fast violet
  - Rinse in distilled water and different in 95% alcohol
  - Dehydrate, clear and mount in mountant.

(Drury et al., 1967)

APPENDIX III

METHOD OF DETERMINING THE VARIOUS DOSES OF *Nicotiana tabacum* LEAF EXTRACT SOLUTION.

1. **Dosage calculation of leave extracts of *Nicotiana tabacum* (aqueous and ethanolic) solutions**

   To determine the equivalent dosage of volume in ml to mg/kg body weight (bw), the following steps were observed:

A) **Dosage in mg/kg bw** (1000, 500, and 250 mg/kg bw of extract)

Taking 1000 mg/kg bw for instance, the dosage is calculated as follows:

1000 mg (of extract solute) …….. 1 kg (body weight of rat)

1000 mg (of extract solute) ……. 1000 g (body weight of rat)

X mg (of extract solute) ……. Y g (measured body weight of rat)

$$\textbf{X mg (of extract solute)} = \frac{\textbf{Y g (measured body weight of rat)}}{\textbf{1000 g (1 kg bw)}}$$

B) Dosage in ml:

Calculating the dosage in ml equivalent to mg/kg bw, it is important to consider the steps taken in constituting the working solution

- Working Solution of Extract:

  100 g (of extract solute) ……. dissolved in 800 ml (distilled water)

  100,000 mg (of extract solute) ……dissolved in 800 ml (distilled water)

  X mg (of extract calculated from (A) above) …….. Dissolved in X ml

$$\textbf{X ml (of extract solution)} = \frac{\textbf{X mg (of extract) x 800 ml}}{\textbf{100,000 mg}}$$

APPENDIX IV

ANALYTICAL METHOD FOR DETERMINING METALS IN BIOLOGICAL TISSUES

1. **Tissue preparation/ digestion** (EMMI, 1997)
    - Take 1 ml (0.25 g) of sample (homogenized tissue; 1 g in 4 ml of phosphate buffer) into boiling tube and add 2 ml of concentrated HNO_3
    - Heat (100oC) for 2 hours and allow to cool
    - Add 0.3 ml of 30% H_20_2
    - Heat (100^oC) for 1-2 hours and allow to cool
    - Filter using (Whatmann filter paper)
2. **Dilution/ deionization**
    - Make volume (of digested sample) into 20 ml using deionized water
3. **Analytical method:** Atomic Absorption Spectrophotometry (AS)
4. **Computation of element concentration**

    Assuming the result obtained from AAS analysis was X conc.ppm for sample 1. To determine the concentration of element in parts per million (ppm) or mg (of element)/kg (of tissue), the following steps were observed:

Sample 1 = $\frac{\textbf{\textit{X} x 20ml} \text{ (dissolution factor)}}{\textbf{0.25 g} \text{ (sample weight in ml of homogenized tissue)}}$ = **A**

Blank sample = $\frac{\textbf{\textit{Y} x 20ml} \text{ (dissolution factor)}}{\textbf{2.3 ml} \text{ (2 ml of } HNO_3 \text{ and 0.3 ml } HNO_3\text{)}}$ = **B**

Total concentration of element in tissue *(ppm or mg/kg***) = A-B**