STUDIES ON THE EFFECT OF N-BUTANOL EXTRACT OF *Cannabis sativa* L. ON THE FRONTAL CORTEX OF ADULT WISTAR RATS.

BY

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BY

Richard ZACHARIAH, B.Sc. (ABU, 2014)

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DEPARTMENT OF HUMAN ANATOMY, FACULTY OF BASIC MEDICAL SCIENCES, COLLEGE OF MEDICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA NIGERIA.

November, 2019.

DECLARATION

I declare that the work in this dissertation entitled, "**STUDIES ON THE EFFECT OF N-BUTANOL EXTRACT OF *Cannabis sativa* L. ON THE FRONTAL CORTEX OF ADULT WISTAR RATS.**" has been carried out by me in the Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, Ahmadu Bello University, Zaria under the supervision of Prof. S.S. Adebisi and Dr. U.E Umana

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

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CERTIFICATION

This dissertation entitled; "**STUDIES ON THE EFFECT OF N-BUTANOL EXTRACT OF Cannabis sativa L. ON THE FRONTAL CORTEX OF ADULT WISTAR RATS**." by Richard ZACHARIAH, meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

THIS DISSERTATION IS DEDICATED

To

MY LOVING FATHER AND MOTHER

And to

MY DEARLY BELOVED SISTERS AND BROTHER

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ABSTRACT

Cannabis (marijuana) abuse has become a source of concern globally affecting almost every nation for decades. Medical use of marijuana is also on the increase being legalized in some countries. Despite extensive research on smoked Cannabis, little is known about the psychotropic effects of marijuana wax (similar to *n*-butanol extract), a high potency form of Cannabis that is gaining in popularity. The present study examined the effect of n-butanol extract of *Cannabis sativa* L. on the frontal cortex of adult Wistar rats which involved the following investigations: acute toxicity study, routine histological and histochemical studies using haematoxylin & eosin and cresyl fast violet staining techniques; neurobehavioral studies using ladder rung walking (LRW) to assess motor function and acetylcholinesterase (AChE) activity using brain tissue homogenate. Thirty (30) Wistar rats (male and female) were divided into two experimental categories: twelve (12) rats for acute toxicity studies using Lorke's method and eighteen (18) rats for the main experiment were divided into three (3) groups. Group I served as the control group and was administered 1 ml of distilled water, while group II and III were administered 250 mg/kg and 500 mg/kg of n-butanol extract of *Cannabis sativa* L. respectively for 21 days via oral route. The result for acute toxicity study showed no mortality within 24 hours after oral administration of the extract. Reduced distribution of Nissl substance and distortion in cytoarchitecture of frontal cortex (layer V) with vacuolation, pyknosis and dissolution of nucleolus was observed in the treatment groups. The result for neurobehavioral study also showed a significant decrease ($p<0.05$) in foot fault scoring, increase in latency and foot placement accuracy of Wistar rats administered 250 mg/kg and 500 mg/kg n-butanol extract of *Cannabis sativa* L. when compared to the control. Neurotoxicity of cannabis exposure was also evident as it increased acetylcholinesterase activity in the treatment group. In conclusion, findings suggest that, n-butanol extract of *Cannabis sativa* L. at 250 mg/kg and 500 mg/kg is capable of causing neurotoxicity in the frontal lobe of adult Wistar rats which may leads to motor deficit.

TABLE OF CONTENTS

Cover page i

Fly leaf ii

Title page iii

Declaration iv

Certification v

Dedication vi

Acknowledgments vii

Abstract ix

List of figures xvi

List of tables xvii

List of plates xviii

List of appendices xx

List of abbreviations xxi

CHAPTER ONE

1.0 Introduction 1

1.1 Background of Study 1

1.2 Statement of Research Problem 2

1.3 Justification/Significance of the Study 3

1.4 Aim and Objective of the Study 4

1.4.1 Aim of the study 4

1.4.2 Objectives of the study 4

1.5 Research Hypothesis 4

CHAPTER TWO

2.0 Literature Review **5**

2.1 Description of Plant **5**

2.1.1 Brief history 8

2.1.2 Cannabis strains 8

2.1.3 Epidemiology 12

2.1.4 Legal Status 12

2.1.5 Phytochemical properties of *Cannabis sativa* L. 13

2.1.6 Pharmacokinetics 15

2.1.7 Pharmacodynamics 17

2.1.8 Metabolism and elimination of Δ9-THC 19

2.2 Endocannabinoid system **20**

2.2.1 Function of the endocannabinoid system in the brain 20

2.2.2 Function of the endocannabinoid system in peripheral tissues 23

2.3 Cannabinoid Receptor **24**

2.4 Neurotransmitter **25**

2.4.1 Acetylcholine 25

2.5 Cannabis and motor function **29**

2.6 The Nervous System **29**

2.6.1 The central nervous system 30

2.6.2 The brain 31

2.6.3 Cerebral cortex 31

2.6.4 Areas of the cerebral cortex 34

2.6.5 Layers of the cerebral cortex 35

2.6.6 The Frontal Lobe 38

2.7 Substance Abuse 41

2.8 Medical use of Cannabis 41

CHAPTER THREE

3.0 Materials and Methods 42

3.1 Materials 42

3.1.1 Ethical approval 42

3.1.2 Experimental animals 42

3.1.3 Equipment 43

3.1.4 Reagents 43

3.2 Methods 43

3.2.1 Plant Material 43

3.2.2 Preparation of *Cannabis sativa* L. extract and fractionation 43

3.2.3 Qualitative phytochemical analysis of *Cannabis sativa* L. extract 46

3.2.4. Quantitative phytochemical determination of *Cannabis sativa* L. using spectrophotometric method 48

3.2.6 Acute toxicity test 53

Figure 3.2:Schematic diagram of experimental design for Acute Toxicity study 54

3.3 Dose Preparation of *Cannabis sativa* L. Extract 55

3.4 Experimental Design 55

3.5 Neuro Behavioral Studies 57

3.5.1 The ladder rung walking task 57

3.6 Morphological Assessment 63

3.7 Animal Sacrifice 63

3.8 Histological and Histochemical Evaluation 64

3.8.1 Fixation 64

3.8.2 Tissue processing 64

3.8.3 Embedding 65

3.8.4 Sectioning 65

3.8.5 Staining of slides 65

3.8.6 Haematoxylin and eosin staining techniques 65

3.8.7 Cresyl fast violet (Nissl) stain 66

3.9 Neurotransmitter Analysis 66

3.10 Statistical Analysis 67

CHAPTER FOUR

4.0 Result 68

4.1 Extraction of Plant 68

4.2 Phytochemical screening, Quantitative Estimation and Thin Layer Chromatography (TLC) of *Cannabis sativa* L. Extract 70

4.3 Acute Toxicity (LD_{50}) Study 74

4.4 Physical Observation 76

4.5 Neurobehavioural Studies 78

4.5.1 The ladder rung walking task..... 78

4.6 Histological and Histochemical Studies..... 88

4.6.1 Histological and Histochemical Features of the Cerebral Cortex of Control (Untreated) Wistar Rat. 88

4.7 Acetylcholinesterase Activity..... 95

CHAPTER FIVE

5.0 Discussion..... 97

5.1 Plant Extraction..... 97

5.2 Phytochemical screening, Thin Layer Chromatography and Quantitative estimation of *Cannabis sativa* L. extracts 97

5.3 Acute Toxicity (LD_{50}) Study 98

5.4 Morphological Studies 99

5.5 Neurobehavioural Studies 100

5.6 Histological and Histochemical Studies..... 102

5.7 Acetylcholinesterase Activity..... 105

CHAPTER SIX

6.0 Summary, Conclusion, Recommendations and Contribution to Knowledge 106

6.1 Summary 106

6.2 Conclusion..... 107

6.3 Recommendations 107

6.4 Contribution to Scientific knowledge 107

References **109**

Appendices **131**

LIST OF FIGURES

Figure 2.1: Leaves of Cannabis strains 10

Figure 2.2: Strains of Cannabis plants 11

Figure 2.3: Schematic representation of cholinergic neurotransmission mediated by the neurotransmitter acetylcholine and boosted by reversible inhibitors of acetylcholinesterase (AChE). 27

Figure 2.4: Acetylcholine pathway 28

Figure 2.5: General area of the brain showing the cerebral cortex 33

Figure 2.6: Schematic illustration of the six horizontal layers characteristic of the cerebral neocortex. 37

Figure 3.1: Liquid- Liquid Partition Method 45

Figure 4.1 Effect of n-butanol extract of *Cannabis sativa* L. on the activity of Acetylcholinesterase in the cerebrum of Control and Experimental rats 96

LIST OF TABLES

Table 3.1: Treatments of Experimental Animals 56

Table 4.1: Yield and appearances of the *Cannabis sativa* L. plant 69

Table 4.2a: Phytochemical Screening of *Cannabis sativa* L. Extract 71

Table 4.2b: Quantitative Analysis of the crude Extract of *Cannabis sativa* L. 72

Table 4.3: Result of Acute Toxicity (LD_{50}) Study using Lorke's method 75

Table 4.4: Weight comparison, weight change of Wistar rats *initial* (day 1) and *final* (day 21) of treatment and Brain Somatic Index. 77

Table 4.5a: Foot fault scoring 79

Table 4.5b: Foot fault scoring 80

Table 4.5c: Foot placement accuracy analysis (steps) 82

Table 4.5d: Foot placement accuracy analysis (number of errors) 84

Table 4.5e: Effect of *Cannabis sativa* L. Extract on time taken to cross the Ladder Rung Apparatus 86

Table 4.5f: Effect of *Cannabis sativa* L. Extract on time taken to cross the Ladder Rung Apparatus 87

LIST OF PLATES

Plate I: *Cannabis sativa* L. plant 6

Plate II: Dried Leaves of *Cannabis sativa* L. plant 7

Plate III: Thin layer chromatography plates with crude, n-butanol and aqueous spot 51

Plate IV: Thin layer chromatography chamber 52

Plate V: The skilled ladder rung walking test apparatus in the fronto-lateral view 61

Plate VI: The skilled ladder rung walking test apparatus in the Ventro-lateral view 62

Plate VII: The cannabis extracts gave more than ten spots, with the yellow and violet spot corresponding to CBD and THC respectively. 73

Plate VIII: Section of Wistar rat frontal cortex (layer V) of the control (untreated) group with normal histology of the frontal cortex. H and E stain (Mag x 250). 89

Plate IX: Section of Wistar rat frontal cortex (layer V) of the group administered *Cannabis sativa* L. (250 mg/kg) with distortion in the histology of the frontal cortex Pyramidal cell. H and E stain (Mag x 250). 90

Plate X: Section of Wistar rat frontal cortex (layer V) of the group administered *Cannabis sativa* L. (500 mg/kg) with distortion in the histology of the frontal cortex Pyramidal cell layer. H and E stain (Mag x 250). 91

Plate XI: Section of Wistar rat frontal cortex (layer V) of the control (Group I) group with normal histology of the frontal cortex; P=Pyramidal cell intensely stained. Cresyl Fast Violet Stain (CFV) stain (Mag x 250). 92

Plate XII: Section of Wistar rat frontal cortex (layer V) of Group II (250 mg/kg) with distortion in the histology of the frontal cortex lightly stained. CFV stain (Mag x 250). 93

Plate XIII: Section of Wistar rat frontal cortex (layer V) of Group III (500 mg/kg) showing distortion in the histology of the frontal cortex lightly stained. CFV stain (Mag x 250). ... 94

LIST OF APPENDICES

Appendix I: sample of letter to the H.O.D requesting for an introductory letter to N.D.L.E.A office, Kaduna State Command **131**

Appendix II: Letter of approval to obtain Cannabis sativa plant **132**

Appendix III: Ethical clearance **133**

Appendix IV: Assay of acetylcholinesterase activity in the brain **134**

Appendix V: Quantitative phytochemical determination of c*annabis sativa* l. using spectrophotometric method **136**

Appendix VI: Dosage calculation **139**

Appendix VII: Dosage calculation for ketamin intraperitonial (IP) injection **141**

LIST OF ABBREVIATIONS

11-OH-THC	11-hydroxy Δ9–Tetra hydrocannabinol
ABUCAUC	Ahmadu Bello University ethical committee on animal use and care
ABUTH	Ahmadu Bello University Teaching Hospital
Ach	Acetylcholine
AChE	Acetylcholinesterase
AChRs	Activates acetylcholine receptors
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
B.C	before Christ
BCE	before Common Era
BCG	bromocresol green
BHO	Butane Hash Oil
CB	Cannabinoids receptors
CBD	Cannabidiol
CFV	Cresly Fast Violet
ChAT	choline acetyltransferase
CNS	Central Nervous System
CYP	cytochrome P450
DE	diosgenin equivalents
DW	dry weight
eCBs	Endocannabinoids
ECS	Endocannabinoid System
$FeCl_3$	Iron (III) chloride
GABA	gamma - Aminobutyric acid or γ-aminobutyric acid
GAE	Gallic acid equivalent

H&E	Hematoxylin and Eosin
H_2SO_4	Sulphuric acid
HAChT or ChT	high affinity choline transporter
IP	intraperitoneally
L.	Linn
LD_{50}	Median Lethal Dose
LFL	Left Forelimb
LHL	Left Hind Limb
LRWA	Ladder Rung Walking Apparatus
LSD	Fisher's Least Significant Difference
MCDP	Ministerial Committee on Drug Policy
mL	millilitre
Na_2CO_3	Sodium carbonate
NaOH	sodium hydroxide
NAS	National Association of Science
NDLEA	National Drug Law Enforcement Agency
ng	nanograms
RFL	Right Forelimb
RHL	Right Hind Limb
SC	Saponins Content
SEM	standard error of mean
SPSS	Statistical Package for the Social Sciences
TAC	Total Alkaloid Content
TBI	Traumatic Brain Injury
TC	Tannin Content
TFC	Total Flavonoid Content
THCCOOH	11-nor-9-carboxy- Δ9-tetrahydrocannbinol

TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
U.S	United State
UV	Ultraviolet
VAChT	vesicular acetylcholine transporter
Vd	venereal disease
W.H.O	World Health Organization
Δ9-THC	Delta-9-tetrahydrocannabinol

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Cannabis among other names is also known as marijuana, is a genus of flowering plant in the family Cannabaceae, originated in Central Asia and is now cultivated worldwide, including in Africa, Europe, southern Asia, the Middle East, India, and the Americas (ElSohly and Slade, 2007). Cannabis is mostly used as a medicinal drug or for recreation purpose, although it may also be used for religious or spiritual purposes, this substance is majorly abuse by adolescence and young adult (ElSohly and Slade, 2007; WHO, 2017). The species *Cannabis sativa* L. was first classified by Carl Linnaeus in 1953 (Greg, 2005). Among 483 known compounds in the plant, the main psychoactive part of cannabis is delta-9-tetrahydrocannabinol (THC) including at least 65 other cannabinoids (Ethan, 2013; Newton, 2013; Gloss 2015). Cannabis is the preferred designation of the plant cannabis sativa, cannabis indica and of minor significance, cannabis ruderalis (Gloss, 2015).

Substance abuse refers to the harmful or hazardous use of psychoactive substances, including illicit drugs and alcohol (Gloss, 2015). Substance abuse, also known as drug abuse, is a patterned use of a substance (drug) in which the user consumes the substance in amounts or with methods which are harmful to themselves or others. The drugs used are often associated with levels of intoxication that alter judgment, perception, attention and physical control, not related with medical or therapeutic effects (Leikin, 2007). In some cases criminal or anti-social behaviours occur when the person is under the influence of a drug, and long term personality changes in individuals may occur as well (Ksir *et al.*, 2002). Drugs most often associated with this term include: cannabis, opioids, alcohol, cocaine, methaqualone, and some substituted amphetamines

(Zammit, 2002). The use of psychoactive substances causes significant health and social problems for the people who use them, and also for others in their families and communities. WHO estimated that 7% of the global burden of disease in 2004 was due to cannabis, opioid and cocaine use (WHO, 2017).

Cerebral cortex is the largest region of the cerebrum in the mammalian brain and plays a key role in motor function. Delta-9-tetrahydrocannabinol (THC) is the main active chemical found in marijuana, the THC in marijuana interferes with the functioning of the cerebral cortex, resulting in impaired motor function (Miller *et al.,* 2001; Nicholson *et al.,* 2004; Murillo *et al.,* 2006).

1.2 Statement of Research Problem

Cannabis (marijuana) abuse has been in the increase and has become a source of concern globally affecting almost every nation for decades. The United Nations has found that cannabis is the most used illicit drug in the world and medical use of marijuana is also on the increase being legalized in some countries.

In 2015, it was estimated that 255 million people used illicit drugs, such as cannabis, amphetamines, opiods, and cocaine, which translates into an annual prevalence of illicit drug use of 5.3%, cannabis is most used with 183 million users. Also, statistics obtained by the NDLEA has indicated that between 2014 and 2015, North Western Nigeria comprising Kaduna, Kano, Jigawa, Zamfara, and Kebbi had the highest number of people arrested in Nigeria with various cases related to cannabis.

Unlike other substances, such as alcohol or tobacco, whose use may confer risk, no accepted standards exist to help guide individuals as they make choices regarding the issues of how to use cannabis safely and, in regard to therapeutic uses effectively.

Despite extensive research on smoked Cannabis, little is known about the psychotropic effects of marijuana wax (similar to *n*-butanol extract), a high potency form of Cannabis that is gaining in popularity, hence the need for this study.

Therefore, there is an urgent need to educate the public, health professionals, and policymakers about the risks involved with using high potency Butane Hash Oil (BHO), also known as "marijuana wax". This present studies sought to determine the effect of n-butanol extract of *cannabis sativa* L. administration on Wistar rats following oral administration.

1.3 Justification/Significance of the Study

Cannabis is the most used illicit drug in the world (WHO, 2010; Mbadugha *et al.,* 2015). There are evidences that exposure to cannabis can lead to health challenges and consumption of cannabis has been reported to impair motor skills (Kano *et al.,* 2009; Imam *et al.,* 2017).

This study will give a further insight which might turns out to be imperative owing to the wide spread misuse of *Cannabis sativa* L. among the teenagers and young adults and to create more awareness on the health effects of abusing these substance.

Also, the result of this study generated will serve to inform all agencies interested in addressing substance abuse globally about the magnitude of the problem and its effects in the frontal lobe of adult Wistar rats.

1.4 Aim and Objective of the Study

1.4.1 Aim of the study

The aim of the study was to;

evaluate the effect of oral administration of *n*-butanol extract of *Cannabis sativa* L. on the cytoarchitecture, motor and neurochemical activity in the frontal cortex of adult Wistar rats.

1.4.2 Objectives of the study

The objectives of the study were to;

I. Assess the motor function of adult Wistar rats administered with *Cannabis sativa* L. extract.
II. Examined the neuronal cells of frontal cortex (Layer V) of adult Wistar rats histologically.
III. assess the activity of acetylcholinesterase in the cerebrum following oral administration of *Cannabis sativa* L. extract on adult Wistar rat.

1.5 Research Hypothesis

Oral administration of *n*-butanol extract of *Cannabis sativa* L. will alter the cytoarchitecture, impair motor and neurochemical activity in the frontal cortex of adult Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of Plant

Cannabis is a tall plant with a stiff upright stem, divided serrated leaves, and glandular hairs. It is used to produce hemp fibre and as a drug (Oxford Dictionary, 2018).

Cannabis is a generic term used to denote the several psychoactive preparations of the plant Cannabis sativa, it is by far the most widely cultivated, trafficked and abused illicit drug and half of all drug seizures worldwide are cannabis seizures (WHO, 2018).

Cannabis is mostly used as a medicinal drug or for recreation purpose, although it may also be used for religious or spiritual purposes, this substance is majorly abuse by adolescence and young adult (ElSohly, 2007; WHO, 2017). The species Cannabis sativa was first classified by Carl Linnaeus in 1953 (Greg, 2005).

Among 483 known compounds in the plant, the main psychoactive part of cannabis is delta-9-tetrahydrocannabinol (THC) including at least 65 other cannabinoids (Ethan, 2013; Newton, 2013 & Gloss, 2015). Cannabis is the preferred designation of the plant cannabis sativa, cannabis indica and of minor significance, cannabis ruderalis (Gloss, 2015).

Today, more than 560 constituents of cannabis have been identified in cannabis (Gould, 2015; ElSohly, 2017).



(Ethan, 2013)

Plate I: *Cannabis sativa* L. plant



Plate II: Dried *Cannabis sativa* L. plant

Photographed by researcher

2.1.1 Brief history

Cannabis Sativa (cannabis) is among the earliest plants cultivated by man. The first evidence of the use of cannabis was found in China, where archeological and historical findings indicate that that plant was cultivated for fibers since 4.000 B.C. (Li *et al.*, 1974). With the fibers obtained from the cannabis stems the Chinese manufactured strings, ropes, textiles, and even paper. Textiles and paper made from cannabis were found in the tomb of Emperor Wu (104-87 B.C.), of the Han dynasty (Li *et al*., 1974).

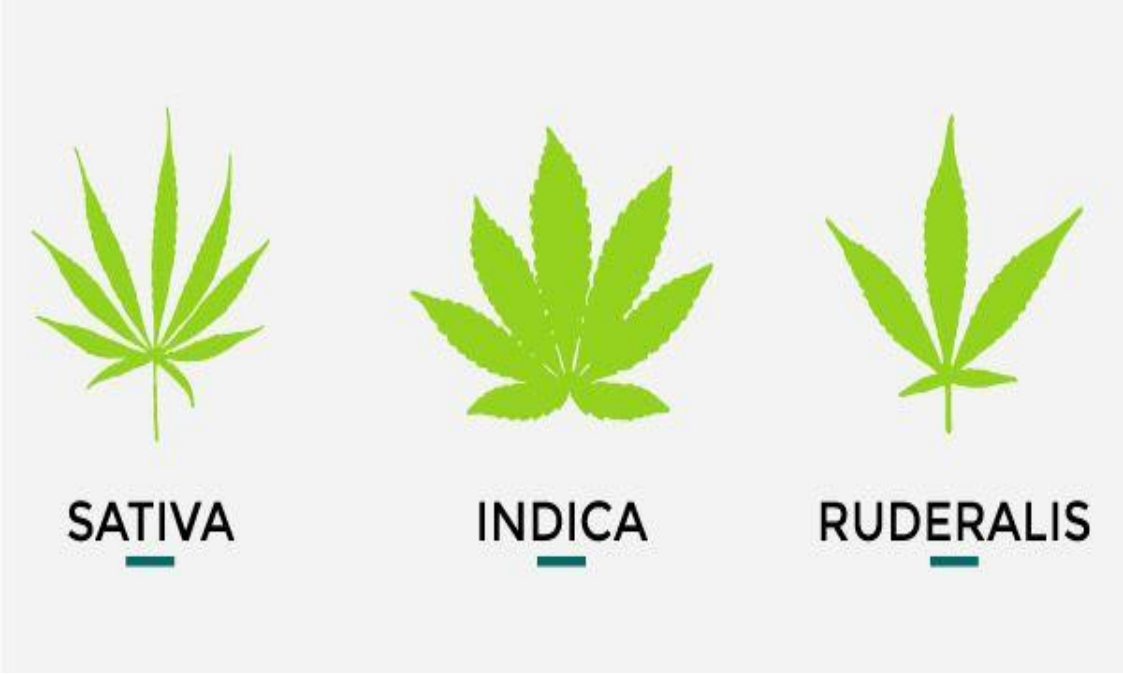
Cannabis is known in Africa at least since the 15th century, and its use was, possibly, introduced by Arab traders, somehow connected to India. This is evidenced by the similarity of the terms used for preparing the plant in Africa and India. In Africa, the plant was used for snake bite, to facilitate childbirth, malaria, fever, blood poisoning, anthrax, asthma, and dysentery (Du Toit, 1980).

2.1.2 Cannabis strains

Cannabis strains are either pure or hybrid varieties of the plant genus Cannabis, which encompasses the species C. sativa, C. indica and C. ruderalis. Cannabis sativa plant has its origins in Asia, America and Africa, while Cannabis indica has its origins in Pakistan and India and Cannabis ruderalis has its origin in Siberia and Kazakhstan (Stafford, 1992; Karl *et al.*, 2004; Fischedick, 2010).

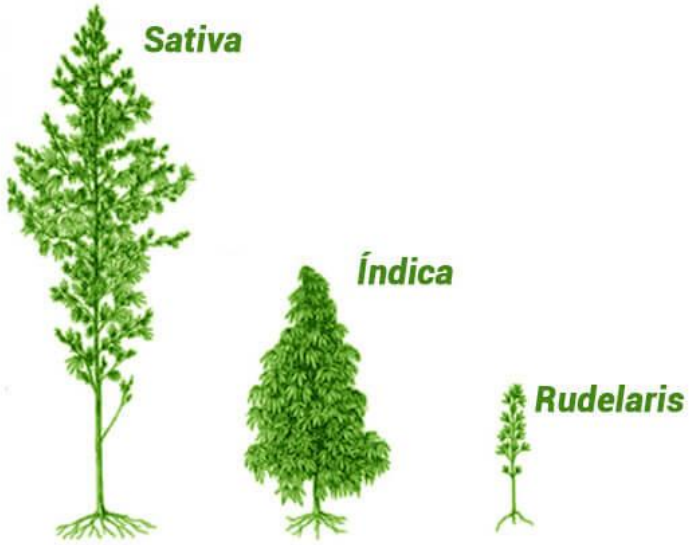
The two species of the Cannabis genus that are most commonly grown are Cannabis indica and Cannabis sativa (Small, 1976). A third species, Cannabis ruderalis, is very short and produces only trace amounts of tetrahydrocannabinol (THC), and thus is not commonly grown for industrial, recreational or medicinal use (Greg, 2001).

Pure sativas are relatively tall (reaching as high as 4.5 meters), with long internodes and branches, and large, narrow-bladed leaves. Pure indica varieties are shorter and bushier, have wider leaflets. They are often favored by indoor growers for their size. Sativas bloom later than indicas, often taking a month or two longer to mature. The subjective effects of sativas and indicas are said to differ, but the ratio of tetrahydrocannabinol (THC) to cannabidiol (CBD) in most named drug varieties of both types is similar (averaging about 200:1). Unlike most commercially developed strains, indica landraces exhibit plants with varying THC/CBD ratios (Hillig, 2004).



(Elzinga *et al.*, 2015)

Figure 2.1: Leaves of Cannabis strains



(Eggan *et al.*, 2010)

Figure 2.2: Strains of Cannabis plants

2.1.3 Epidemiology

In 2015, it is estimated that 255 million people used illicit drugs, such as cannabis, amphetamines, opiods, and cocaine, which translates into an annual prevalence of illicit drug use of 5.3%, cannabis is most used with 183 million users (WHO, 2017). Also, statistics obtained by the NDLEA has indicated that between 2014 and 2015, North Western Nigeria comprising Kaduna, Kano, Jigawa, Zamfara, and Kebbi had the highest number of people arrested in Nigeria with various cases related to cannabis (Shehu, 2017).

2.1.4 Legal Status

The legality of cannabis for medical and recreational use varies by country (Bewley-Taylor, 2016; Habibi, 2018). The use of cannabis for recreational purposes is prohibited in most countries. Other countries have much more severe penalties such as some Asian and Middle Eastern countries where possession of even small amounts is punished by imprisonment for several years (Powell, 2018).

Countries that have legalized recreational cannabis are Canada and Uruguay, along with nine states and the District of Columbia in the United States. A policy of limited enforcement has also been adopted in many countries, in particular Spain and the Netherlands where the sale of cannabis is tolerated at licensed establishments (Haines, 2017; Smith, 2018). Countries that have legalized the medical use of cannabis include Australia, Canada, Chile, Colombia, Germany, Greece, Israel, Italy, the Netherlands, Peru, Poland, and the United Kingdom. Others have more restrictive laws that only allow the use of certain cannabinoid drugs, such as Sativex or Marinol. In the United States, 31 states and the District of Columbia have legalized the medical use of cannabis, but at the federal level its use remains prohibited for any purpose.

In Africa, Lesotho and Zimbabwe are the first country to legalize cannabis in 2016 and 2017 respectively (Kieron, 2018). Cannabis in Nigeria is illegal yet the country is a major source of West African-grown cannabis, and ranked world's eight highest consumer of cannabis (Ministerial Committee on Drug Policy-MCDP, 2007). Cannabis is widely grown across the States of Nigeria including Ondo State, Edo State, Delta State, Osun State, Oyo State and Ogun State (Seshata , 2013). Cannabis use and other related drug abuse cases are controlled by the National Drug Law Enforcement Agency in collaboration with the U.S government to combat narcotic trafficking in West Africa. The possession of cannabis is illegal and is punishable by a minimum sentence of 12 years in prison. In serious trafficking cases, life imprisonment may be imposed (MCDP, 2007).

2.1.5 Phytochemical properties of *Cannabis sativa* L.

Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant body; bark, leaves, stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components (Tiwari *et al.*, 2011; Choudhary *et al.*, 2014).

Numerous chemicals are produced in cannabis through the secondary metabolism. They include cannabinoids, terpenes and phenolic compounds (Flores-Sanchez *et al.*, 2008; Andre, 2016).

The phytocannabinoids, cannabidiol (CBD), and delta-9-tetrahydrocannabinol (Δ9-THC) are the most studied extracts from cannabis sativa subspecies hemp and marijuana. CBD and Δ9-THC interact uniquely with the endocannabinoid system (ECS). Through direct and indirect actions, intrinsic endocannabinoids and plant-based phytocannabinoids modulate and influence a variety of physiological systems influenced by the ECS (Maroon *et al.*, 2018).

Terpenes form the largest group of phytochemicals, with more than 100 molecules identified in Cannabis (Rothschild *et al.,* 2005; Brenneisen, 2007). Terpenes are responsible for the odor and flavor of the different Cannabis strains. They have therefore likely contributed to the selection of Cannabis narcotic strains under human domestication (Small, 2015).

Terpenes, along with cannabinoids, have successfully been used as chemotaxonomic markers in Cannabis, as they are both considered as the main physiologically active secondary metabolites (Fischedick *et al.,* 2010; Elzinga *et al.,* 2015).

Phenolic compounds, also known as phenylpropanoids, constitute one of the most widely distributed groups of secondary metabolites in the plant kingdom. They present more than 10,000 different structures, including phenolic acids, benzoic and hydroxycinnamic acids, flavonoids such as flavonols and flavones, stilbenes and lignans (Andre *et al.,* 2010). In Cannabis, about 20 flavonoids have been identified, mainly belonging to the flavone and flavonol subclasses (Flores-Sanchez & Verpoorte, 2008; Andre *et al.,* 2016).

According to Zeenat *et al.* (2018), phytochemicals compound perform a large range of actions, alkaloids defend against chronic diseases (Yue-Zhong, 1998). Phenolic compounds such as Flavonoid and Tannins perform important antioxidants activity and possess anti-inflammatory properties, Tannins revealed antitumor, antibacterial, antidiabetic and anti-inflammatory activities, tannins are also indicated to hinder HIV replication (Covello, 2008). Saponins possess antibiotic properties and defend against hypercholesterolemia and hyperglycemia, saponins also possess anti-inflammatory, antioxidant, anticancer and central nervous system activities (Covello, 2008). Steroids and triterpenoids show analgesic properties. Plant steroids are

significant for their cardio tonic and central nervous system activities, analgesic, anti-inflammatory, insecticidal and antimicrobial properties (Raskin, 2010).

2.1.6 Pharmacokinetics

Δ9-THC which is highly lipophilic get distributed in adipose tissue, liver, lung and spleen (McBurney *et al.,* 1986, Chiarotti *et al.,* 2000; Musshoff *et al.,* 2006; Sharma *et al.,* 2012). Hydroxylation of Δ9-THC generates the psychoactive compound 11-hydroxy Δ9–Tetra hydrocannabinol (11-OH-THC) and further oxidation generates the inactive 11-nor-9-carboxy-Δ9-tetrahydrocannbinol (THCCOOH). THCCOOH is the compound of interest for diagnostic purposes. It is excreted in urine mainly as a glucuronic acid conjugate (Musshoff *et al.,* 2006; Sharma *et al.,* 2012). Δ9-THC is rapidly absorbed through lungs after inhalation. It quickly reaches high concentration in blood (Vandevenne *et al.,* 2000; Sharma *et al.,* 2012). Approximately 90% of THC in blood is circulated in plasma and rest in red blood cells. Following inhalation, Δ9-THC is detectable in plasma within seconds after the first puff and the peak plasma concentration is attained within 3-10 minutes (Wahlqvist *et al.,* 1970; Widman *et., al* 1974; Owens *et al.*, 1981; Law *et al.,* 1984; Vandevenne *et al.,* 2000; Sharma *et al.*, 2012). However, the bioavailability of Δ9-THC varies according to the depth of inhalation, puff duration and breath-hold. Considering that approximately 30% of THC is assumed to be destroyed by pyrolysis, the systemic bioavailability of THC is ~23-27% for heavy users (Pertwee, 2009; Hollister *et al.,* 1981) and 10-14% for occasional users (McBurney *et al.,* 1986; Castle *et al.*, 2004; Sharma *et al.*, 2012). Maximum Δ9-THC plasma concentration was observed approximately 8 minutes after onset of smoking, while 11-OH-THC peaked at 15 minutes and THC-COOH at 81 minutes. This Δ9-THC concentration rapidly decreases to 1-4 ng/mL within 3-4 hour (Huestis *et al.*, 1992; Sharma *et al.*, 2012).

In comparison to smoking and inhalation, after oral ingestion, systemic absorption is relatively slow resulting in maximum Δ9-THC plasma concentration within 1-2 hours which could be delayed by few hours in certain cases (Lemberger *et al.,* 1971; Hollister *et al.,* 1981; Sharma *et al.,* 2012). In some subjects, more than one plasma peak was observed (Law *et al.,* 1984; Karschner *et al.,* 2009; Sharma *et al.,* 2012). Extensive liver metabolism probably reduces the oral bioavailability of Δ9-THC by 4-12% (Owens *et al.,* 1981). After oral administration, maximum Δ9-THC plasma concentration was 4.4-11 ng/mL for 20 mg (Chiarotti *et al.,* 2000) and 2.7-6.3 ng/mL for 15 mg (Lemberger *et al.,* 1971; Kogan *et al.,* 2007; Sharma *et al.,* 2012). Much higher concentration of 11-OH THC was produced after ingestion than inhalation (Lemberger *et al.,* 1971; Hollister *et al.,* 1981; Sharma *et al.,* 2012). Following assimilation via the blood, Δ9-THC rapidly penetrates in to fat tissues and highly vascularized tissues including brain and muscle resulting in rapid decrease in plasma concentration (Haggerty *et al.,* 1986; Huestis, 2005; Sharma *et al.,* 2012).This tissue distribution is followed by slow redistribution of it from the deep fat deposits back into the blood stream.

It should be noted that the residual Δ9-THC levels are maintained in the body for a long time following abuse. The half- life of it for an infrequent user is 1.3 days and for frequent users 5-13 days (Smith-Kielland *et al.,* 1999; Sharma *et al.,* 2012). After smoking a cigarette containing 16-34 mg of Δ9-THC, THC-COOH is detectable in plasma for 2-7 days (Reiter *et al.,* 2001; Huestis, 2005; Sharma *et al.,* 2012). A clinical study carried out among 52 volunteers showed that THC-COOH was detectable in serum from 3.5 to 74.3 hours. Initial concentration was between 14-49 ng/mL (Reiter *et al.,* 2001; Sharma *et al.,* 2012). This was considerably less than the THC-COOH detection time of 25 days in a single chronic user (Lowe *et al.,* 2009; Sharma *et al.,* 2012).

2.1.7 Pharmacodynamics

Cannabis produces sedation, and significant pharmacodynamics interactions may occur if it is administered with other CNS depressant drugs (such as sedatives or hypnotics), via potentiation of central effects (Arellano *et al.,* 2017; Lucas *et al.,* 2018). In human volunteers, ethanol was found to increase plasma THC levels and the subjective effect of smoked cannabis (Lukas *et al.,* 2001; Lucas *et al.,* 2018). Cannabis use is associated with both pathological and behavioural toxicity (Andreasson, 1989; Thomas *et al.,* 2014; Schoeler, *et al.,* 2016; Lucas *et al.,* 2018). Contraindications to cannabinoid therapies include significant psychiatric, cardiovascular, renal or hepatic illness (Lucas *et al.,* 2018). THC produces dose-dependent performance impairment (Toennes *et al.,* 2008; Lucas *et al.,* 2018). Following a single inhaled dose of THC, impairment was greatest during the first hour post dose and declined over the following 2–4 hours (Johansson *et al.,* 1989; Lucas *et al.,* 2018) Substantial cognitive and psychomotor impairment is associated with blood THC concentrations in excess of 5 ng ml–1 (Hartman *et al.,* 2013; Lucas *et al.,* 2018).

In healthy volunteers, administration of THC produced psychotic symptoms, altered perception, increased anxiety and cognitive deficits (D'Souza *et al.,* 2004; Lucas *et al.,* 2018). Cannabinoids may induce tachycardia (Karschner *et al.,* 2011; Lucas *et al.,* 2018) probably via direct agonism of CB1 receptors in cardiac tissue (Dumont *et al.,* 2009; Lucas *et al.,* 2018). Cardiac toxicity may occur via additive hypertension and tachycardia with amphetamines, cocaine, atropine or other sympathomimetic agents (Benowitz *et al.,* 1977; Foltin *et al.,* 1987; Lucas *et al.,* 2018).

Co-administration of CBD has been reported to reduce THC-associated adverse psychotropic and cardiovascular effects (tachycardia) (Russo *et al.,* 2006). CBD has been reportedly associated with fatigue and somnolence (Devinsky *et al.,* 2017) potentially compounded by co-

administration with CNS active medications. Cannabis with high THC content is associated with a greater severity of addiction relative to cannabis with low THC content (Freeman *et al.,* 2015).

A large, nationally representative sample of US adults determined that the lifetime cumulative probability of transitioning from cannabis use to dependence was 8.9%, with increased risk of transition to dependence conferred by history of psychiatric or substance dependence comorbidity (Lopez-Quintero *et al.,* 2011). In general, currently available pharmacokinetic and pharmacodynamics data were obtained from studies in healthy volunteers, or cannabis users. Pharmacokinetic data derived from such studies cannot simply be extrapolated to more vulnerable patient groups or the cannabis naive population. Patient specific variables influencing cannabinoid pharmacokinetics may include history of cannabis use, pharmacogenetics, body size and composition, disease state, diet, micro biome and additional unknown factors (Martin *et al.,* 2018).

There are limited data regarding the efficacy and safety of cannabis use in older subjects (van den Elsen *et al.,* 2014). This population may benefit from its potential symptomatic and palliative benefits but, in the context of comorbidity, polypharmacy and increased cognitive vulnerability, is predisposed to more severe manifestations of adverse effects such as sedation, with a resultant increased risk of falls (van den Elsen *et al.,* 2014). Pharmacokinetic parameters influenced by age, such as reduced hepatic and renal clearance, and relative increases in body fat (Clegg *et al.* 2013*;* Lucas *et al.,* 2018) and, consequently, Vd, can result in an increased bioavailability of THC and prolongation of half-life (van den Elsen *et al.,* 2014). For most cannabinoid formulations, there are limited data pertaining to their pharmacokinetic profiles, which are likely to demonstrate both inter and intra patient variability (Martin *et al.,* 2018).

Caution must be exercised in extrapolating data between different routes of administration and formulations, the selection of which should be tailored depending on individual patient requirements. The limited availability of applicable pharmacokinetic and pharmacodynamics information highlights the need to initiate the prescription of cannabis medicines using a start low and go slow approach, carefully observing the patient for desired and adverse effects. It is only through further clinical studies, collecting pharmacokinetic and pharmacodynamics data in the actual patient population for whom prescribing may be considered , that a better understanding of these drugs will be achieved, enhancing safe and optimal prescribing (Lucas *et al.,* 2018).

2.1.8 Metabolism and elimination of Δ9-THC

Δ9-THC is metabolized in the liver by microsomal hydroxylation and oxidation catalyzed by enzymes of cytochrome P450 (CYP) complex. The average plasma clearance rates have been reported to be 11.8 ± 3 L/hour for women and 14.9 ±3.7 L/hour for men (Karschner *et al.,* 2009; Sharma *et al.,* 2012). Others have determined approximately 36 L/hour for naïve cannabis users and 60 L/hour for regular cannabis users (Musshoff *et al.,* 2006).

More than 65% of cannabis is excreted in the feces and approximately 20% is excreted in urine (Lemberger *et al.,* 1971). Most of the cannabis (80-90%) is excreted within 5 days as hydroxylated and carboxylated metabolites (Goulle *et al.,* 2008). There are eighteen acidic metabolites of cannabis identified in urine (Halldin *et al.,* 1982) and most of these metabolites form a conjugate with glucuronic acid, which increases its water solubility. Among the major metabolites (Δ9-THC, 11-OH-THC, and THCCOOH), THCCOOH is the primary glucuronide conjugate in urine, while 11-OH-THC is the predominant form in feces (Vandevenne, *et al.,* 2000; Huestis, 2005). Since Δ9-THC is extremely soluble in lipids, it results in tubular re-

absorption, leading to low renal excretion of unchanged drug. Urinary excretion half-life of THCCOOH was observed to be approximately 30 hours after seven days and 44-60 hours after twelve days of monitoring (Huestis, 2005; Kelly *et al.,* 1992). After smoking approximately 27 mg of Δ9-THC in a cigarette, 11-OH-THC peak concentration was observed in the urine within two hours in the range of 3.2-53.3 ng/mL, peaking at 77.0±329.7 ng/mL after 3 hours and THCCOOH peaking at 179.4ng/mL ± 146.9 after 4 hours (Johansson *et al.,* 1989; Manno *et al.,* 2001).

2.2 Endocannabinoid system

The Endocannabinoid System in the CNS: The ECS has emerged as one of the key regulatory mechanisms in the brain controlling multiple events such as mood, pain perception, learning and memory among others (Marsicano and Lutz, 2006; Kano *et al.,* 2009). It is also thought to provide a neuroprotective role during traumatic brain injury (TBI) and may be part of the brain's natural compensatory repair mechanism during neurodegeneration (Pryce *et al.,* 2003; Klein, 2005; Campbell and Gowran, 2007; Bilkei-Gorzo, 2012). New roles for the ECS in drug abuse and dependence are identified almost continuously, further strengthening the relevance of this system not only during cannabis abuse but also other illicit drugs as well (Maldonado *et al.,* 2006; Xi *et al.,* 2011; Parsons and Hurd, 2015). In the CNS, eCBs act as retrograde messengers mediating feedback inhibition modulating synaptic plasticity (Howlett, 2005; Chevaleyre *et al.,* 2006; Katona and Freund, 2012).

2.2.1 Function of the endocannabinoid system in the brain

Understanding the multiple functions of endocannabinoid signaling in the brain offers insight into the pharmacological effects of cannabis and other exogenous cannabinoids, their therapeutic

potential and undesirable adverse effects. An overview by Kalant (Kalant, 2014) describes in depth "on demand" endocannabinoid modulation of excitatory and inhibitory synaptic transmission and regulatory functions in the brain (lger and Kim, 2011).

Brain development, neurogenesis, psychiatric disorders: Endocannabinoid signaling is crucial for brain development, and guides neural stem cell survival and proliferation, cell fate decisions and the motility and differentiation of ensuing neuronal and glial cells (Maccarrone *et al.,* 2014)

Developmental endocannabinoid signaling, from fetus to young adult, may be susceptible to cannabis use during pregnancy and adolescence, possibly affecting brain structure and function. Endocannabinoids and cannabis-altered endocannabinoid signaling may contribute to neuropsychiatric diseases that are of developmental origins and in which modifications to signaling have been observed: autism (Foldy *et al.,* 2013), schizophrenia (Eggan *et al.,* 2010), bipolar disorder (Minocci, 2011) and depression (Monteleone *et al.,* 2010). The central role of the cannabinoid system in promoting adult neurogenesis in the hippocampus and the lateral ventricles provides insight into the processes underlying post-developmental neurogenesis in the mammalian brain. Both THC (Steel *et al.,* 2014) and CBD (Schiavon *et al.,* 2016) inhibit neurogenesis in adolescent or adult rodent brain, a process of potential relevance to a wide range of cannabis-induced adverse events (Prenderville *et al.*, 2015).

Neuroprotection: Cannabinoids and CB1, CB2 receptors display neuroprotective effects in the brain by preventing or decreasing the severity of damage resulting from mechanical, blood flow, or other forms of injury. Genetic ablation of the CB1 receptor exacerbates ischemic stroke (Parmentier-Batteur *et al.,* 2002), with CB2 agonists providing anti-inflammatory properties and CB1 activation promoting hypothermia. The use of cannabis for this purpose is compromised by psychoactive effects and the development of tolerance to its neuroprotective effects.

Cannabinoids and sensory function (olfaction, auditory, pain): The endocannabinoid system contributes to olfactory, auditory and pain sensations. A review of these functions is beyond the scope of this summary but readers are referred to an excellent overview (Kalant, 2014). There is extensive anatomical overlap of the opioid and cannabinoid receptor systems, and it appears probable that functional interactions between them occur in the production of analgesia.

Appetite and nausea: A number of nuclei in the medulla are involved in the regulation of appetite and nausea. These nuclei coordinate sensory input from the brainstem, vagal complex, vestibular organs, and peripheral organs. Endocannabinoids and CB1 agonists inhibit vagal fibers to promote eating and CB1 antagonists to decrease or inhibit food intake (Izzo *et al.*, 2009).

Sleep: Endogenous and exogenous cannabinoids, including cannabis and THC, affect sleep patterns (Murillo-Rodríguez, 2008). There is poor quality evidence that cannabis or cannabinoids have therapeutic benefit in sleep disorders (Whiting *et al.*, 2015).

Motor function: The endocannabinoid system plays a complex role in regulating motor pathways, which conceivably are relevant to symptomatic relief, or to addressing the underlying pathology in a wide range of neurological diseases characterized by motor impairment (El Manira and Kyriakatos, 2010). CB1 receptors are abundant in brain regions that regulate motor function and coordination, including the basal ganglia, cerebellum. CB1 receptors are down-regulated in several neurological conditions (Fernández-Ruiz, 2009).

Cognitive functions: Cannabinoids can both facilitate and degrade learning processes dependent upon the process involved. Endocannabinoids apparently facilitate various forms of learning and memory processes in a number of brain regions. The endogenous cannabinoid system is also

implicated in extinguishing learning of aversive situations. On the other hand, THC and cannabis decrease working memory, apparently by actions in the hippocampus, a brain region critical for learning and memory. The memory decrements induced by THC or cannabis resemble hippocampal lesions. These impairments may result from suppression of glutamate release in the hippocampus, which is responsible for the establishment of synaptic plasticity (Mechoulam and Parker, 2013; Zanettini *et al.,* 2011; Morena and Campolongo, 2014).

2.2.2 Function of the endocannabinoid system in peripheral tissues

Gastrointestinal (GI) tract: CB1 and CB2 receptors are highly expressed on enteric nerves and on enteroendocrine cells (CB2) throughout the intestinal mucosa, on immune cells (CB1 and CB2), and enterocytes (CB1 and CB2). Many gut functions are regulated by endocannabinoids critical for central nervous system (CNS) control of its metabolic and homeostatic functions (Maccarrone *et al.*, 2015).

Cardiovascular system: CB1, CB2, endocannabinoids and their enzymes are present in cardiovascular tissues and may contribute to the development of common cardiovascular disorders. An acute action of cannabis is mild tachycardia, with increases in cardiac output and increased myocardial oxygen requirement (Maccarrone *et al.,* 2015).

Liver: Cannabinoid receptor expression is normally low in liver, with CB1 and CB2 receptors acting in opposite directions. CB2 receptors mediate several biological functions in various types of liver cells, and CB1 blockade contributes to beneficial metabolic effects. CB1 expression increases in pathological states, promoting fibrogenesis, steatosis, and cardiovascular complications of liver disease. In contrast, CB2 is protective, reducing these indices of liver dysfunction (Maccarrone *et al.,* 2015).

Immune System: Endocannabinoids modulate the functional activities of immune cells, largely though CB2 receptors, providing novel targets for therapeutic manipulation (Henry *et al.,* 2016)

Muscle: Endocannabinoid signaling (largely through CB2 receptors) contributes to regulating energy metabolism in muscle and the formation of new muscle fibers (Maccarrone *et al.,* 2015).

Reproductive System: Endocannabinoid signaling, primarily mediated by the CB2 receptor regulates all critical stages of pregnancy and affects pregnancy events. Signaling is also involved in the preservation of normal sperm function, and thus male fertility (Maccarrone *et al.,* 2015).

Skin: Endocannabinoid signaling, through both CB1 and CB2, is involved in regulating skin functions such as proliferation, differentiation, cell survival, immune responses and suppressing cutaneous inflammation. Exogenous modulators of the receptors could clarify the role of the endocannabinoid system in hyperproliferative skin conditions, allergic and inflammatory skin diseases (Maccarrone *et al.,* 2015).

Other organs: The role of endocannabinoid signaling in respiratory tract and urinary system remains unclear, but there is preliminary evidence that CB1 and CB2 receptors may contribute to kidney disease (Maccarroner*et al.,* 2015).

2.3 Cannabinoid Receptor

The cannabinoid receptors are G protein-coupled receptors that are activated by endocannabinoids or exogenous agonists such as tetrahydrocannabinol (Tung, 2010).

CB1 Receptors: The CB1 receptor is one of the most abundant G protein-coupled receptors (GPCRs) in the CNS and is found in particularly high levels in the neocortex, hippocampus, basal ganglia, cerebellum and brainstem (Herkenham *et al.,* 1991; Marsicano and Kuner, 2008). CB1 receptors are also found on peripheral nerve terminals and some extra-neural sites such as

the testis, eye, vascular endothelium and spleen. Interestingly, CB1 receptors are highly enriched at presynaptic and axonal compartments, restricting their function to sites of synaptic activity (Straiker and Mackie, 2005; Wu *et al.,* 2008). The CB1 receptor binds the main active ingredient of Cannabis sativa (marijuana), Δ9-tetrahydrocannabinol (Δ9-THC) and mediates most of the CNS effects of Δ9-THC (Zimmer *et al.,* 1999).

CB2 Receptors: The CB2 receptor exhibits a more defined pattern of expression in the brain than CB1 receptors, and is found predominantly in cells and tissues of the immune system (Klein, 2005; Mackie, 2006). In the CNS, CB2 receptor expression is associated with inflammation and it is primarily localized to microglia, resident macrophages of the CNS (Mackie, 2008; Palazuelos *et al.,* 2009). This selective localization together with the modulatory effect of the CB2 receptor on microglia function is particularly relevant since microglial cells have a significant role in Alzheimer's disease (AD) and other diseases associated with the basal ganglia (Ramírez *et al.,* 2005; Sagredo *et al.,* 2007; Fernández-Ruiz *et al.,* 2011; Yeh *et al.,* 2016). Interestingly, recent work also indicates that CB2 receptors expressed in neurons can control synaptic function and are involved in drug abuse and synaptic plasticity (Xi *et al.,* 2011).

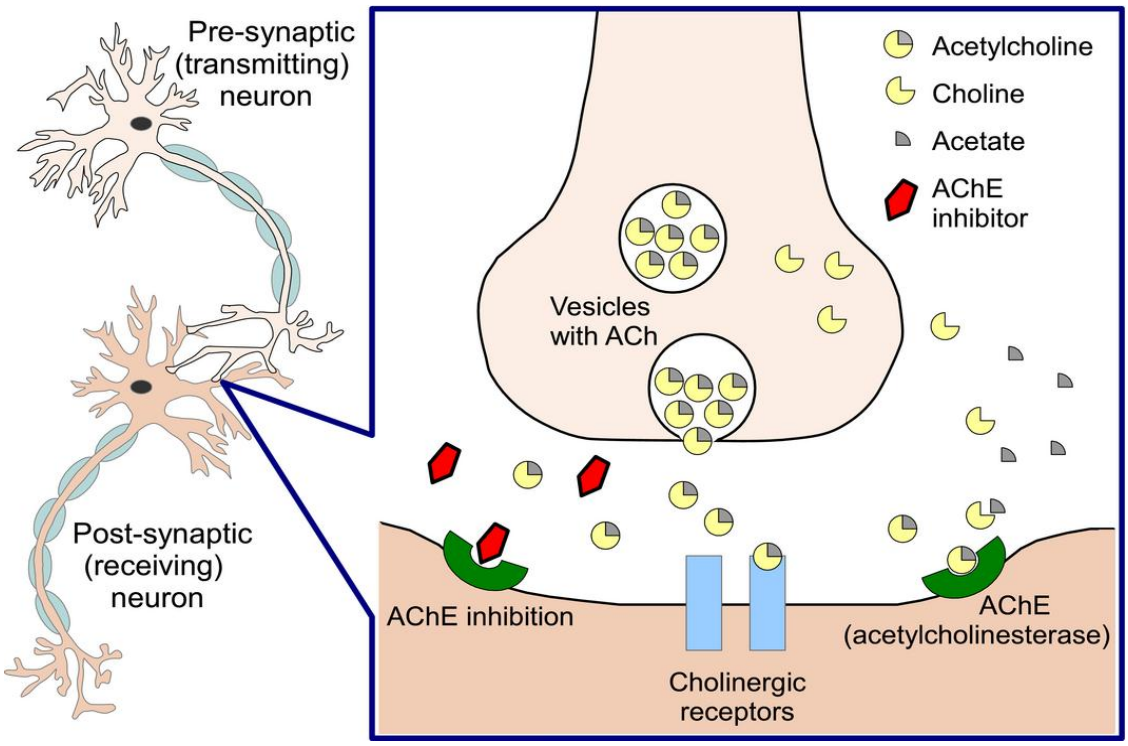
2.4 Neurotransmitter

2.4.1 Acetylcholine

Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolytic metabolism of the neurotransmitter acetylcholine (ACh) into choline and acetate (Brett and Andrew, 2012). It is the neurotransmitter used at the neuromuscular junction in other words, it is the chemical that motor neurons of the nervous system release in order to activate muscles (Tiwari, 2017). This property means that drugs that affect cholinergic systems can have very dangerous effects ranging from paralysis to convulsions (Tiwari, 2017).

Acetylcholine (ACh) was the first substance proven to be a neurotransmitter (Loewi, 1921). It was identified in Ascaris and other nematodes in 1955 by Helen Mellanby (Mellanby, 1955), and was subsequently shown to be an excitatory transmitter at nematode neuromuscular junctions (del Castillo *et al.,* 1963; del Castillo *et al.*, 1967).

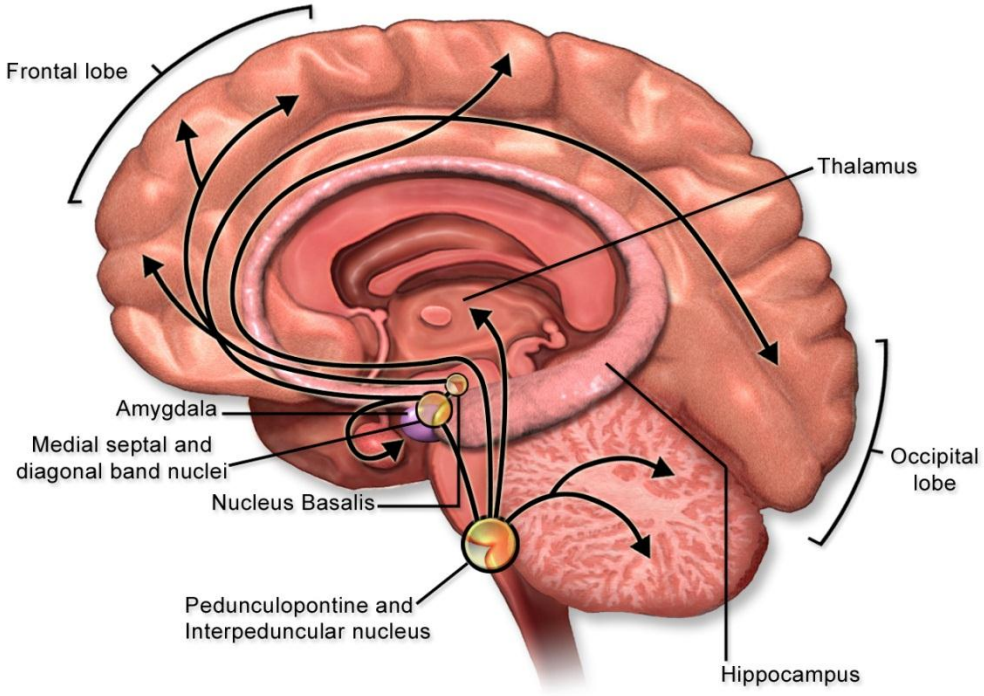
ACh is synthesized by choline acetyltransferase (ChAT), and is loaded into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). The synaptic vesicle lumen is acidified by the action of an ATP-dependent proton pump located in the synaptic vesicle membrane. The pH gradient between the vesicle lumen and the cytoplasm provides the driving force for ACh transport; the VAChT essentially "exchanges" ACh for protons. The docking and priming of synaptic vesicles, and their calcium-stimulated fusion with the cell membrane are all general processes that are independent of the neurotransmitter contained in the vesicles. Following synaptic vesicle fusion and transmitter release, the ACh diffuses within the synaptic cleft and activates acetylcholine receptors (AChRs), usually located on post-synaptic cells. For most other neurotransmitters (e.g., GABA, dopamine, serotonin), the action of the transmitter is terminated by transporter- mediated removal of the transmitter from the synaptic cleft. The action of acetylcholine, however, is terminated by direct enzymatic hydrolysis of the neurotransmitter in the synaptic cleft by acetylcholinesterase (AChE). The resulting choline is then transported back into the presynaptic neuron by a high affinity choline transporter (HAChT, or ChT); this choline is then available for the synthesis of additional Ach (Rand, 2007).



(Maciej, and Frederick, 2018)

Figure 2.3: Schematic representation of cholinergic neurotransmission mediated by the neurotransmitter acetylcholine and boosted by reversible inhibitors of acetylcholinesterase (AChE).

Alkaloids that inhibit acetylcholinesterase enhance the cholinergic neurotransmission by preventing the acetylcholine breakdown by AChE.



(Bruce, 2014)

Figure 2.4: Acetylcholine pathway

2.5 Cannabis and motor function

Learning new motor skills is critical for adapting to constantly changing environment, organism, and task constraints throughout the lifespan (Clark and Metcalfe 2002). Importantly, complex motor behaviors comprise of simpler actions produced in a specific order at a specific time. Playing the piano, speaking, writing, driving, and playing sports are examples of intricate motor skills composed of a sequence of simple actions with important ordinal and temporal components. These important skills are obtained through the process of motor learning, which is integral for conducting activities of daily living, interacting with others and the environment, and having a fulfilling life. It is interesting to note that learning and adaptation via synaptic plasticity is a requirement of living in a dynamic environment and is also the basis of drug addiction. The evidence for impairments in cognition and certain motor tasks in cannabis users taken together with the cognitive resources required in order to learn and perform motor skills suggests a potential for impairments in users of cannabis in motor learning as well (Prashad and Filbey, 2016).

2.6 The Nervous System

The nervous system is a complex collection of nerves and specialized cells known as neurons that transmit signals between different parts of the body. It is essentially the body's electrical wiring (Kim, 2018).

The nervous system derives its name from nerves, which are cylindrical bundles of fibers (the axons of neurons), that emanate from the brain and spinal cord, and branch repeatedly to innervate every part of the body (Kandel *et al.,* 2000). Nerves are large enough to have been recognized by the ancient Egyptians, Greeks, and Romans (Finger, 2001).

Structurally, the nervous system has two components: the central nervous system and the peripheral nervous system. According to the National Institutes of Health, the central nervous system is made up of the brain, spinal cord and nerves. The peripheral nervous system consists of sensory neurons, ganglia (clusters of neurons) and nerves that connect to one another and to the central nervous system (Kim, 2018).

Functionally, the nervous system has two main subdivisions: the somatic, or voluntary, component; and the autonomic, or involuntary, component. The autonomic nervous system regulates certain body processes, such as blood pressure and the rate of breathing that work without conscious effort, according to Merck Manuals. The somatic system consists of nerves that connect the brain and spinal cord with muscles and sensory receptors in the skin (Kim, 2018).

Sensory neurons react to physical stimuli such as light, sound and touch and send feedback to the central nervous system about the body's surrounding environment, according to the American Psychological Association. Motor neurons, located in the central nervous system or in peripheral ganglia, transmit signals to activate the muscles or glands (Kim, 2018).

Glial cells, derived from the Greek word for "glue," are specialized cells that support, protect or nourish nerve cells, according to the Oregon Institute of Health and Science University (Kim, 2018).

2.6.1 The central nervous system

The central nervous system consists of the two major structures: the brain and spinal cord. The brain is encased in the skull, and protected by the cranium (Arthure *et al.*, 2010). The spinal cord

is continuous with the brain and lies caudally to the brain (Kandel *et al.*, 2012) and is protected by the vertebrae (Arthure *et al.*, 2010).

2.6.2 The brain

The brain is an organ that serves as the center of the nervous system in all vertebrate and most invertebrate animals. The brain is located in the head, usually close to the sensory organs for senses such as vision. The brain is the most complex organ in a vertebrate's body. In a human, the cerebral cortex contains approximately 10–20 billion neurons (von Bartheld *et al.*, 2016).

2.6.3 Cerebral cortex

The cerebral cortex is the largest region of the cerebrum in the mammalian brain and plays a key role in memory, attention, perception, cognition, awareness, thought, language, and consciousness (Kandel *et al.*, 2000).

The cerebral cortex, the outer layer of gray matter of the cerebrum, is found only in mammals. In larger mammals, including humans, the surface of the cerebral cortex folds to create gyri (ridges) and sulci (furrows) which increase the surface area (Angevine *et al.*, 1981).

The cerebral cortex is generally classified into four lobes: the frontal, parietal, occipital and temporal lobes. The lobes are classified based on their overlying neurocranial bones (Rosdahl *et al.*, 2008).

The frontal lobe is located at the front of each cerebral hemisphere and positioned in front of the parietal lobe and above and in front of the temporal lobe. It is separated from parietal lobe by a space between tissues called the central sulcus, and from the temporal lobe by a deep fold called the lateral sulcus also called the Sylvian fissure. The precentral gyrus, forming the posterior

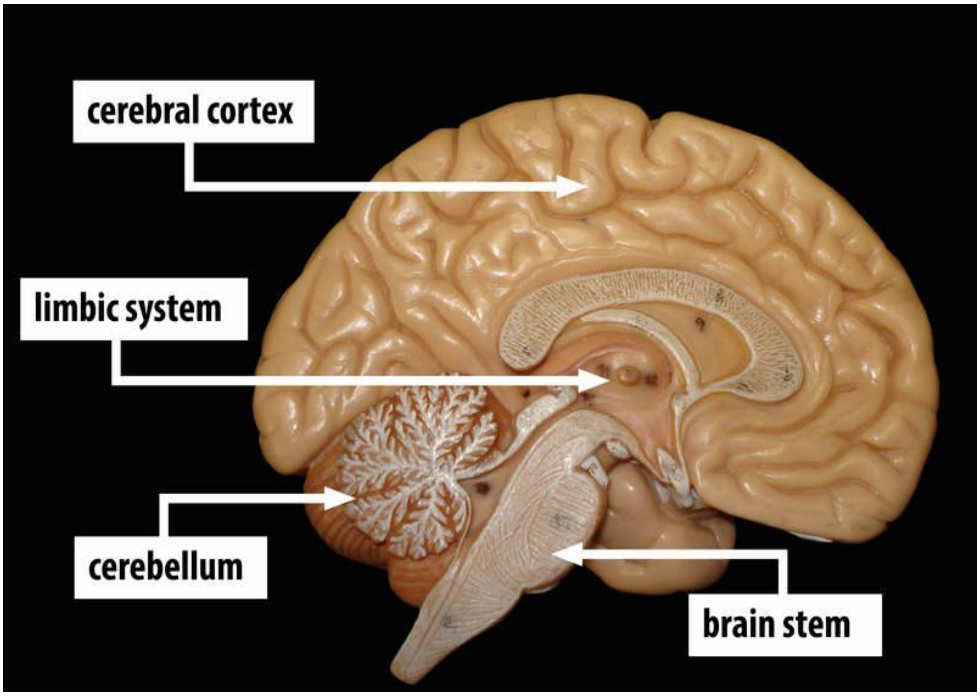
border of the frontal lobe, contains the primary motor cortex, which controls voluntary movements of specific body parts (Fix, 2008).

The parietal lobe is positioned above the occipital lobe and behind the frontal lobe and central sulcus. The parietal lobe integrates sensory information among various modalities, including spatial sense and navigation (proprioception), the main sensory receptive area for the sense of touch (mechanoreception) in the somatosensory cortex which is just posterior to the central sulcus in the postcentral gyrus, and the dorsal stream of the visual system. The major sensory inputs from the skin (touch, temperature, and pain receptors), relay through the thalamus to the parietal lobe (Schacter *et al.*, 2009).

The occipital lobe is the visual processing center of the mammalian brain containing most of the anatomical region of the visual cortex. The primary visual cortex is Brodmann area 17, commonly called V1 (visual one). Human V1 is located on the medial side of the occipital lobe within the calcarine sulcus; the full extent of V1 often continues onto the posterior pole of the occipital lobe. V1 is often also called striate cortex because it can be identified by a large stripe of myelin, the Stria of Gennari. Visually driven regions outside V1 are called extrastriate cortex. There are many extrastriate regions, and these are specialized for different visual tasks, such as visuospatial processing, color differentiation, and motion perception (Schacter *et al.*, 2009).

The temporal lobe is located beneath the lateral fissure on both cerebral hemispheres of the mammalian brain (Guilherme, 2010).

The temporal lobe is involved in processing sensory input into derived meanings for the appropriate retention of visual memories, language comprehension, and emotion association (Smith, 2007).



(Biswas-Diener, 2018)

Figure 2.5: General area of the brain showing the cerebral cortex

2.6.4 Areas of the cerebral cortex

The Frontal Lobe contains areas that Brodmann identified as involved in cognitive functioning and in speech and language. Area 4 corresponds to the precentral gyrus or primary motor area. Area 6 is the premotor or supplemental motor area. Area 8 is anterior of the premotor cortex. It facilitates eye movements and is involved in visual reflexes as well as pupil dilation and constriction. Areas 9, 10, and 11 are anterior to area 8. They are involved in cognitive processes like reasoning and judgment which may be collectively called biological intelligence including executive function. Areas 44 and 45 are Broca's area (McCaffrey, 2014).

Areas in the Parietal Lobe play a role in somatosensory processes. Areas 3, 2, and 1 are located on the primary sensory strip, with area 3 being above the other two. These are somasthetic areas, meaning that they are the primary sensory areas for touch and proprioception including kinesthesia. Areas 5, 7, and 40 are found posterior to the primary sensory strip and are considered presensory association areas where somatosensory processing occurs. Area 39 is the angular gyrus. Areas involved in the processing of auditory information and semantics as well as the appreciation of smell are found in the Temporal Lobe. Area 41 is Heschl's gyrus, the primary auditory area. Area 42 immediately inferior to area 41 and is also involved in the detection and recognition of speech. The processing done in this area of the cortex provides a more detailed analysis than that done in area 41. Areas 21 and 22 are the auditory association areas. Both areas are divided into two parts; one half of each area lies on either side of area 42. Collectively they can be called Wernicke's area. Area 37 is found on the posterior-inferior part of the temporal lobe. Lesions here can cause anomia (McCaffrey, 2014).

The Occipital Lobe contains areas that process visual stimuli. Area 17 is the primary visual area. Areas 18 and 19 are the secondary visual (association) areas where visual processing occurs (McCaffrey, 2014).

2.6.5 Layers of the cerebral cortex

After the work of Korbinian Brodmann (1909) the neurons of the cerebral cortex are grouped into six main layers, from outside (pial surface) to inside (white matter):

Layer I, the molecular layer, contains few scattered neurons and consists mainly of extensions of apical dendritic tufts of pyramidal neurons and horizontally oriented axons, as well as glial cells (Shipp, 2007). During development Cajal-Retzius (Meyer *et al.*, 1999) and subpial granular layer cells (Judaš *et al.*, 2010) are present in this layer. Also, some spiny stellate cells can be found here. Inputs to the apical tufts are thought to be crucial for the feedback interactions in the cerebral cortex involved in associative learning and attention (Gilbert *et al.*, 2007). While it was once thought that the input to layer I came from the cortex itself (Cauller, 1995), it is now realized that layer I across the cerebral cortex mantle receives substantial input from matrix or M-type thalamus cells (Rubio-Garrido *et al.*, 2009).

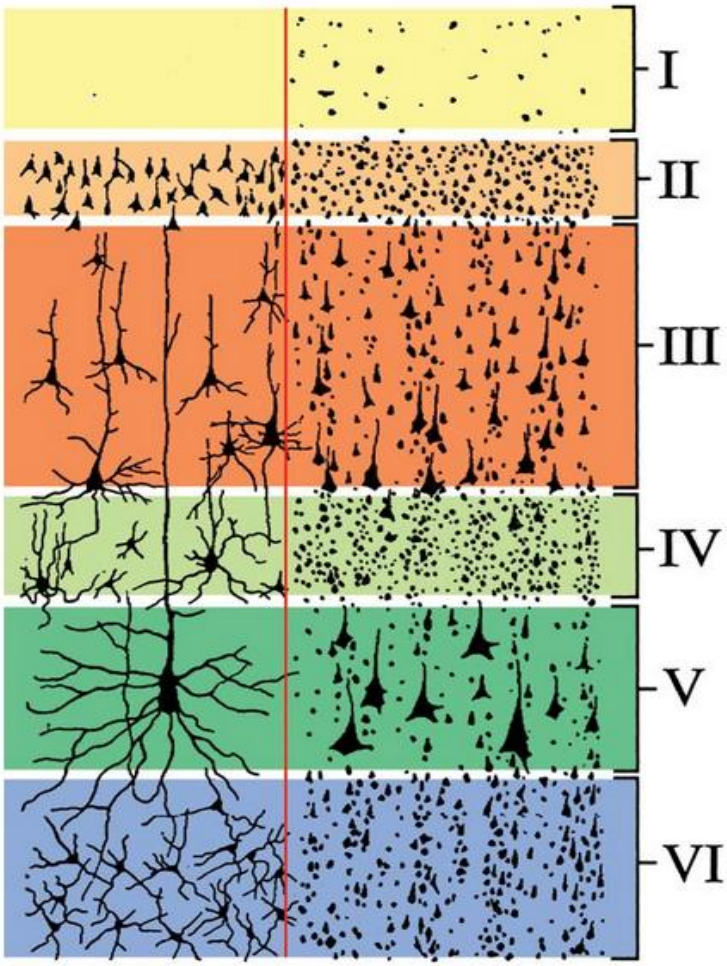
Layer II, the external granular layer, contains small pyramidal neurons and numerous stellate neurons (Lam *et al.*, 2010).

Layer III, the external pyramidal layer, contains predominantly small and medium-size pyramidal neurons, as well as non-pyramidal neurons with vertically oriented intracortical axons; layers I through III are the main target of interhemispheric corticocortical afferents, and layer III is the principal source of corticocortical efferents (Lam *et al.*, 2010).

Layer IV, the internal granular layer, contains different types of stellate and pyramidal neurons, and is the main target of thalamocortical afferents from thalamus type C neurons as well as intra-hemispheric corticocortical afferents. The layers above layer IV are also referred to as supragranular layers (layers I-III), whereas the layers below are referred to as infragranular layers (layers V and VI) (Lam *et al.,* 2010).

Layer V, the internal pyramidal layer, contains large pyramidal neurons which give rise to axons leaving the cortex and running down to subcortical structures (such as the basal ganglia). In the primary motor cortex of the frontal lobe, layer V contains Betz cells, whose axons travel through the internal capsule, the brain stem and the spinal cord forming the corticospinal tract, which is the main pathway for voluntary motor control (Lam *et al.,* 2010).

Layer VI, the polymorphic or multiform layer, contains few large pyramidal neurons and many small spindle-like pyramidal and multiform neurons; layer VI sends efferent fibers to the thalamus, establishing a very precise reciprocal interconnection between the cortex and the thalamus (Creutzfeldt, 1995). That is, layer VI neurons from one cortical column connect with thalamus neurons that provide input to the same cortical column. These connections are both excitatory and inhibitory. Neurons send excitatory fibers to neurons in the thalamus and also send collaterals to the thalamic reticular nucleus that inhibit these same thalamus neurons or ones adjacent to them (Lam *et al.,* 2010). One theory is that because the inhibitory output is reduced by cholinergic input to the cerebral cortex, this provides the brainstem with adjustable "gain control for the relay of lemniscal inputs" (Lam *et al.,* 2010).



(Ranson and Clark, 1959)

Figure 2.6: Schematic illustration of the six horizontal layers characteristic of the cerebral neocortex.

2.6.6 The Frontal Lobe

The frontal lobe is the largest region of the four major lobes of the brain in mammals, is located at the front of the parietal lobe and the temporal lobe. It is separated from the parietal lobe by a groove between tissues called the central sulcus, and from the temporal lobe by a deeper groove called the lateral sulcus (Sylvian fissure). The most anterior rounded part of the frontal lobe (though not well-defined) is known as the frontal pole, one of the three poles of the cerebrum (Chayer and Freedman, 2001).

The frontal lobe is covered by the frontal cortex. The frontal cortex includes the premotor cortex, and the primary motor cortex – cortical parts of the motor cortex. The front part of the frontal lobe is covered by the prefrontal cortex (Chayer and Freedman, 2001). The frontal lobe is the largest lobe of the brain and makes up about a third of the surface area of each hemisphere (Malcolm, 1985).

In humans, the frontal lobe reaches full maturity around the late 20s (Giedd *et al.*, 1999) marking the cognitive maturity associated with adulthood. A small amount of atrophy, however, is normal in the aging person's frontal lobe. Fjell, in 2009, studied atrophy of the brain in people aged 60–91 years. The 142 healthy participants were scanned using MRI. Their results were compared to those of 122 participants with Alzheimer's disease. A follow-up one year later showed there to have been a marked volumetric decline in those with Alzheimer's and a much smaller decline (averaging 0.5%) in the healthy group (Fjell, 2009). These findings corroborate those of Coffey, who in 1992 indicated that the frontal lobe decreases in volume approximately 0.5%–1% per year (Coffey, 1992). The entirety of the frontal cortex is the action cortex. It is devoted to action

of one kind or another: skeletal movement, ocular movement, speech control, the expression of emotions.

Psychological tests that measure frontal lobe function include finger tapping (as the frontal lobe controls voluntary movement), the Wisconsin Card Sorting Test, and measures of language, numeracy skills and decision making which are all controlled by frontal lobe (Kimberg, and Farah, 1993; Yang *et al.*, 2017).

2.6.6.1 The motor cortex

The motor cortex is the region of the cerebral cortex involved in the planning, control, and execution of voluntary movements (Martin *et al.*, 2005).

The motor cortex can be divided into three areas:

Primary motor cortex is the main contributor to generating neural impulses that pass down to the spinal cord and controls the execution of movement. However, some of the other motor areas in the brain also play a role in this function. It is located on the anterior paracentral lobule on the medial surface.

Premotor cortex is responsible for some aspects of motor control, possibly including the preparation for movement, the sensory guidance of movement, the spatial guidance of reaching, or the direct control of some movements with an emphasis on control of proximal and trunk muscles of the body located anterior to the primary motor cortex.

supplementary motor area (or SMA), has many proposed functions including the internally generated planning of movement, the planning of sequences of movement, and the coordination

of the two sides of the body such as in bi-manual coordination located on the midline surface of the hemisphere anterior to the primary motor cortex (Martin et al., 2005).

2.6.6.2 The primary motor cortex

The primary motor cortex (Brodmann area 4) is a brain region that in humans is located in the dorsal portion of the frontal lobe. It is the primary region of the motor system and works in association with other motor areas including premotor cortex, the supplementary motor area, posterior parietal cortex, and several subcortical brain regions, to plan and execute movements. Primary motor cortex is defined anatomically as the region of cortex that contains large neurons known as Betz cells. Betz cells, along with other cortical neurons, send long axons down the spinal cord to synapse onto the interneuron circuitry of the spinal cord and also directly onto the alpha motor neurons in the spinal cord which connect to the muscles. These axons form a part of the corticospinal tract (Binkofski, 2002)

2.6.6.3 The corticospinal tract

The corticospinal tract is a white matter motor pathway starting at the cerebral cortex that terminates on lower motor neurons and interneurons in the spinal cord, controlling movements of the limbs and trunk. There are more than one million neurons in the corticospinal tract, and they become myelinated usually in the first two years of life. The corticospinal tract is one of the pyramidal tracts, the other being the corticobulbar tract (Kolb, and Whishaw, 2009).

The primary purpose of the corticospinal tract is for voluntary motor control of the body and limbs. After patients are lesioned in some part of the pyramidal tracts, they are paralyzed on the corresponding side of the body. However, they can re-learn some crude, basic motions, just no

fine movements. This implies that the connections to these tracts are crucial for fine movement, and only partial recovery is possible if they are damaged (Hall and Guyton, 2005).

2.7 Substance Abuse

Substance abuse refers to the harmful or hazardous use of psychoactive substances, including illicit drugs and alcohol (Gloss, 2015). Substance abuse, also known as drug abuse, is a patterned use of a substance (drug) in which the user consumes the substance in amounts or with methods which are harmful to themselves or others. The drugs used are often associated with levels of intoxication that alter judgment, perception, attention and physical control, not related with medical or therapeutic effects (Leikin, 2007). In some cases criminal or anti-social behaviors occur when the person is under the influence of a drug, and long term personality changes in individuals may occur as well (Ksir *et al.*, 2002). Drugs most often associated with this term include: cannabis, opioids, alcohol, cocaine, methaqualone, and some substituted amphetamines (Zammit, 2002). The use of psychoactive substances causes significant health and social problems for the people who use them, and also for others in their families and communities. WHO estimated that 7% of the global burden of disease in 2004 was due to cannabis, opioid and cocaine use (WHO 2010).

2.8 Medical use of Cannabis

Cannabis is believed to have been used for medical purposes for thousands of years. The earliest well documented evidence of use has been dated to 4,000 BCE in China, and textual evidence indicates it was used medicinally in various locations (including Greece, China, India, Egypt and the Middle East) up to 4,000 years ago. Cannabis has been used to treat many conditions, including pain, anxiety, gout, burns, dandruff, jaundice, depression, insomnia, appetite loss, and asthma, amongst others (Russo, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Ethical approval

Ethical approval was obtained from Ahmadu Bello University ethical committee on animal use and care for the conduct of this research with approval number **ABUCAUC/2018/046 (Appendix III).**

Letter of introduction to NDLEA command office Kaduna State was also obtained from the Head of Human Anatomy Department, Ahmadu Bello University Zaria through the supervisory committee **(Appendix II).**

3.1.2 Experimental animals

Thirty (30) Wistar rats of both sexes with average weight of 132 g were procured from the Department of Preventive Medicine, Faculty of Veterinary medicine, Ahmadu Bello University, Zaria, Kaduna, Nigeria.

The Wistar rats were transferred and housed in wired cages in the Animal House of the Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, and allowed to acclimatize for two (2) weeks prior to the start of the experiments.

All animals were given pelletized feed (Grand Cereals and Oil Mills Limited, Plateau State, Nigeria) and water *ad libitum*. Rats were weighed at the beginning, during and at the end of the study.

3.1.3 Equipment

A Ladder Rung Walking Apparatus (LRWA) of 100 cm long with adjustable rungs at distance of 1 – 5 cm was locally constructed in the Mechanical Workshop of Human Anatomy Department, Ahmadu Bello University, Zaria, Nigeria.

Other materials used in the course of the study include: digital weighing balance, cages, water bottles, sawdust for bedding, microscope, glass slides, cover slips, fixative, bottles for fixing tissues, dissecting set, beakers, ketamine, sacrificing chamber, orogastric tube, syringes, and microtome.

3.1.4 Reagents

Reagents used includes eosin, haematoxylin, graded absolute alcohol, 95% alcohol, 70% alcohol, Xylene, paraffin wax, Bouin's fluid, Stains, Absolute Ethanol (Sigma Aidrich- Missouri, U.S.A.), n-butanol (Sigma Aidrich Missouri, U.S.A.), and Tween 80 (BDH).

3.2 Methods

3.2.1 Plant Material

The plant, *Cannabis sativa* L. was obtained from NDLEA Command office, Kaduna State. It was taken to the Herbarium section in the Department of Botany, Faculty of Life Science, Ahmadu Bello University, Zaria for identification and authentication and was given a Voucher Number **V/N: 2438** by a Botanist, Mal. Namadi Sunusi

3.2.2 Preparation of *Cannabis sativa* L. extract and fractionation

The plant obtained was taken to the Department of Pharmacognosy and Drug development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria for extraction and fractionation. Solvent-solvent partitioning was done using the protocol designed by Kupchan and Tsou (1973) (Kupchan *et al.*, 1973).

400 g of the grounded powdered leaves of *Cannabis sativa* L. was extracted using sohxlet apparatus in 2.5 L ethanol solvent for 24 hours. The ethanol crude extract was evaporated to atmosphere using water bath. The extract was further extracted by partitioning between Aqueous and n-butanol using a separating funnel. The n-butanolic portion was used for the studies.



Dried *Cannabis sativa* L. plant

↓

Ethanol Extract

↓

n-butanol/ H_20

↓

Aqueous Extract | *n*-butanol Extract

Figure 3.1: Liquid- Liquid Partition Method

3.2.3 Qualitative phytochemical analysis of *Cannabis sativa* L. extract

Preliminary qualitative phytochemical screening was carried out following standard protocols (Harborne, 1973; Trease and Evans, 1989; Soforawa, 1993)

The phytochemical analysis is for the determination of Carbohydrates, Anthraquinones, Cardiac glycosides, Alkaloids, Tannins, Flavonoids, Saponins glycoside, Steroids and Triterpenes in ethanol, n-butanol and Aqueous extracts of *Cannabis sativa* L.

3.2.3.1 Test for carbohydrates

Molisch test: Two drops of alcoholic ά- naphthol solution was treated with filtrate in a test tube. Carefully, using a dropper along with side of test tube, disposed tubes and pour drop wise conc. Sulphuric acid. At junction or interface of two liquids, the presence of carbohydrates indicates by the formation of violet color.

3.2.3.2 Test for anthraquinones

Bonfragers test: To the extract solution (1 mL), 5% H_2SO_4 (1 mL) was added. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with equal volume of chloroform and kept to stand for 5 min. Then lower layer of chloroform was shaken with half of its volume with dilute ammonia. The formation of rose pink to red color of the ammoniacal layer gives indication of anthraquinone glycosides.

3.2.3.3 Test for cardiac glycoside

Keller-Kilani test: Extract was mixed with 2 ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2 ml of concentrated H_2SO_4. A brown ring at the interface indicated the presence of cardiac glycosides.

3.2.3.4 Test for alkaloids

Wagner's test: Potassium iodide (2 g) and iodine (1.27 g) were dissolved in distilled water (5 mL) and the solution was diluted to 100 mL with distilled water. Few drops of this solution were added to the filtrate; a brown colored precipitate indicates the presence of alkaloids.

3.2.3.5 Test for tannins

Ferric chloride test: Extract was mixed with 2 ml of 2% solution of $FeCl_3$. A blue-green or blue-black coloration indicated the presence of polyphenols and tannins.

3.2.3.6 Test for flavonoids

Sodium hydroxide test: The plant extract is treated with 2-3 drops of sodium hydroxide solution. Acute yellow color formation, that indicates presence of the flavonoids, by the addition of some drops of sulphuric acid that changed to colourless.

3.2.3.7 Test for saponins glycosides

Frothing Test: Extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously for 30 seconds. The formation of stable foam (1 cm height) after 30 minutes was taken as an indication for the presence of saponins.

3.2.3.8 Test for steroids and triterpenes

Lieberman Burchard test: Extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2 ml of chloroform. Then 2 ml of each of concentrated H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

3.2.4. Quantitative phytochemical determination of *Cannabis sativa* L. using spectrophotometric method

The extract of *Cannabis sativa* L. was quantified by spectrophotometric method using standard procedure.

3.2.4.1 Determination of total phenolic content (TPC)

Estimation of total phenol content in *Cannabis sativa* L. extract was measured spectrophotometrically by Folin-Ciocalteu colorimetric method, using Gallic acid as the standard and expressing results as Gallic acid equivalent (GAE) per gram of sample. Different concentrations (0.01–0.1 mg/ml) of Gallic acid were prepared in methanol. Aliquots of 0.5 ml of the test sample and each sample of the standard solution was taken, mixed with 2 ml of Folin-Ciocalteu reagent (1:10 in deionized water) and 4 ml of saturated solution of sodium of sodium carbonate (7.5% w/v). The tubes were covered with silver foils and incubated at room temperature for 30 minutes with intermittent shaking. The absorbance was taken at 765 nm using methanol as blank. All the samples was analysed in three replications. The total Phenol was determine with the help of standard cure prepared from pure phenolic standard (Gallic acid) (Ainsworth and Gillespie, 2007; Alhakmani *et al.*, 2013).

3.2.4.2 Determination of total flavonoid content (TFC)

The TFC of *Cannabis sativa* L. extract was determined by aluminium chloride colorimetric assay (Zhishen *et al.*, 1999). Briefly, 0.5 ml aliquots of the samples (ethanol extract) and standard solution solution (0.01-1.0 mg/ml) of rutin was added with 2 ml of distilled water and subsequently with o.15 ml of sodium nitrite (5% $NaNO_2$, w/v) solution was added. After 6 minutes, 0.15 ml of (10% $AlCl_3$, w/v) solution was added. The solution was allowed to stand for 6 minutes and after that 2 ml of sodium hydroxide (4% NaOH, w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with immediate addition of distilled water,

mixed thoroughly and allowed to stand for another 15 min. the absorbance of each mixture was determined at 510 nm against the same mixtures. TFC was determining as mg rutin equivalent per gram of sample with the help of calibration curve of rutin. All determinations were performed in triplicate (Khodaie *et al.,* 2012).

3.2.4.3 Determination of total alkaloid content (TAC)

TAC was quantified by spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green (BCG). The plant extract and fractions (1 mg/ml) was dissolved in 2 N HCL and then filtered. The pH of phosphate buffer solution will be adjusted to neutral with o.1 ml of this solution will be transferred to a separating funnel, and then 5 ml of BCG solution along with 5 ml of phosphate buffer was added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The whole experiment was conducted in three replicated (Shamsa *et al.,* 2008; Sharief *et al.,* 2011 and Ajanal, 2012).

3.2.4.4 Determination of tannin content (TC)

The tannin was determined by Folin – Ciocalteu method. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteuphenol reagent, 1 ml of 35% Na_2CO_3 solution and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of reference standard solutions of Gallic acid (20, 40, 60, 80 and 100 µg/ml) was prepared in the same manner as described earlier. Absorbance for test and standard solutions was measured against the blank at 725nm with a UV/Visible spectrophotometer. The tannin content was expressed in terms

of mg of GAE/g of extract (Marinova *et al*., 2005; Rajeev *et al*., 2012; AfifyAel-M *et al.,* 2012; Joneshia, 2016).

3.2.4.5 Determination of saponins content (SC)

Total saponin was determined according to method described by Makkar *et al.,* (2007). A known quantity of freeze-dried extract was dissolved in aqueous 50% methanol and a suitable aliquot (5 mg/ml) was taken. Vanillin reagent (0.25 ml; 8%) was added followed by sulphuric acid (2.5 ml; 72% v/v). The reaction mixtures was mixed well and incubated at 60^{o}C in a water bath for 10 min, after incubation, the reaction mixtures was cooled on ice and absorbance at 544 nm (UV visible spectrophotometer) was read against a blank that does not contain extract. The standard calibration curve was be obtained from suitable aliquots of diosgenin (0.5 mg/ml in 50% aqueous methanol). The total saponin concentration is express as mg diosgenin equivalents (DE) per g dry weight (DW) (Rajat, 2015).

3.2.5 Thin Layer Chromatography (TLC)

Thin layer chromatography was used in identifying the chemical constituents present in n-butanol, aqueous and ethanol extracts. The TLC of the extracts was carried out on pre-coated silica plates using the ascending technique with suitable solvent system. TLC pre-coated plates were cut into suitable sizes. A line was drawn horizontally at about 0.5cm from the bottom to serve as the origin. The plant extract was dissolved in its solvent of extraction for spotting. A suitable solvent system was chosen and poured into the developing chamber and allowed to saturate. A micro capillary tube was dipped into the prepared extract sample and gently spotted at the marked origin from the base of the plate. The plate was then transferred into the chamber and allowed to elute until it develops to the solvent front of about 1 cm before the plate apex: at which it will be removed. The plates was sprayed with Dragendorff reagent and heated in an

oven at 105 ^{o}C for 5 minutes. The plate was visualized under daylight to detect specific compound (Alina and Danuta, 2009; Elke, 2007).

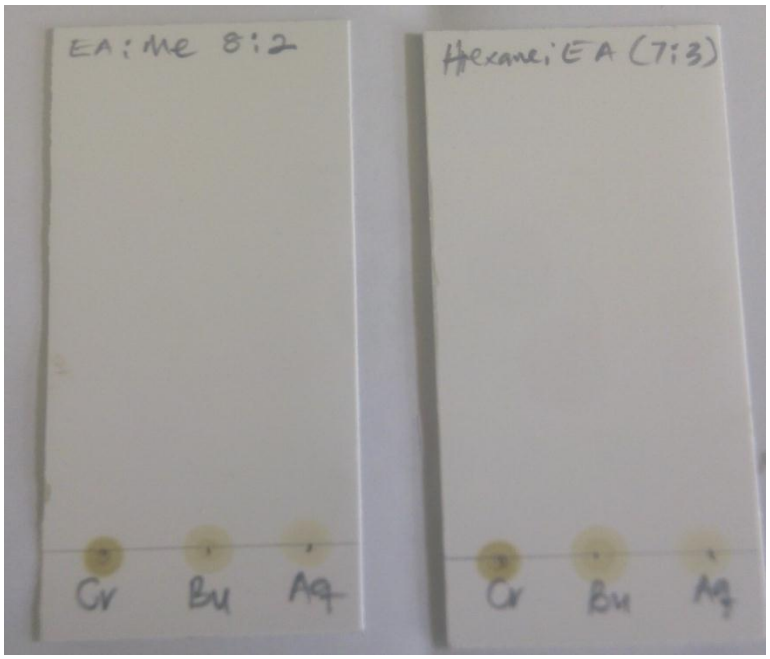


Plate III: Thin layer chromatography plates with crude, n-butanol and aqueous spot

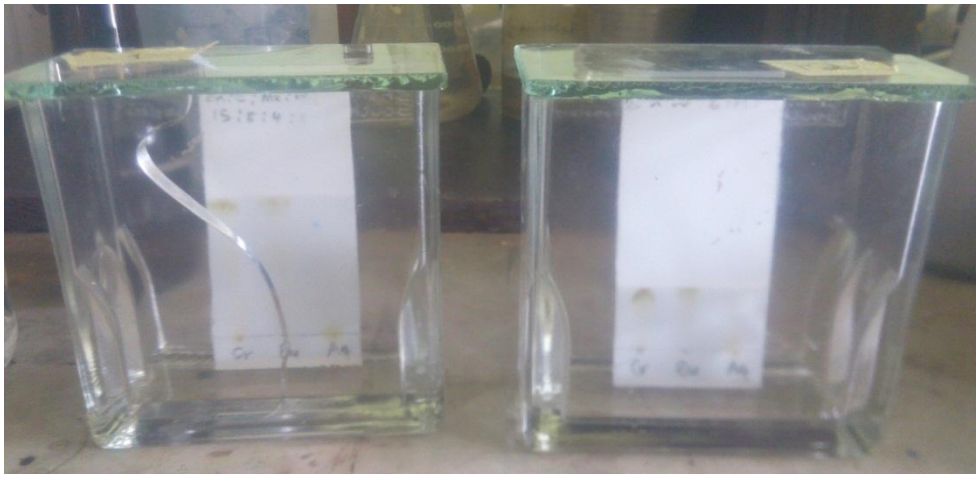


Plate IV: Thin layer chromatography chamber

3.2.6 Acute toxicity test

Acute toxicity study was carried out using the method of (Lorke, 1983). This method has two phases which are phases 1 and 2 respectively. Twelve females Wistar rats weighing between 130g - 140g were used for this test, nine rats and three rats were used in phase 1 and 2 respectively.

3.2.6.1 Phase 1

In this phase, nine rats were divided into three groups of three animals each. Each group of animals was administered different doses (10, 100 and 1000 mg/kg) of n-butanol extract of *Cannabis sativa* L. The animals were placed under observation for 24 hours to monitor their behavior as well as if mortality will occur.

3.2.6.2 Phase 2

In the second phase of the study, the procedure was repeated using three rats, which were distributed into three groups of one animal each. The animals were administered higher doses (1600, 2900 and 5000 mg/kg) of n-butanol extract of *Cannabis sativa* L. and then observed for 24 hours for behavior as well as mortality.

Then formula for LD_{50} Calculation:

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality (Lorke, 1983).

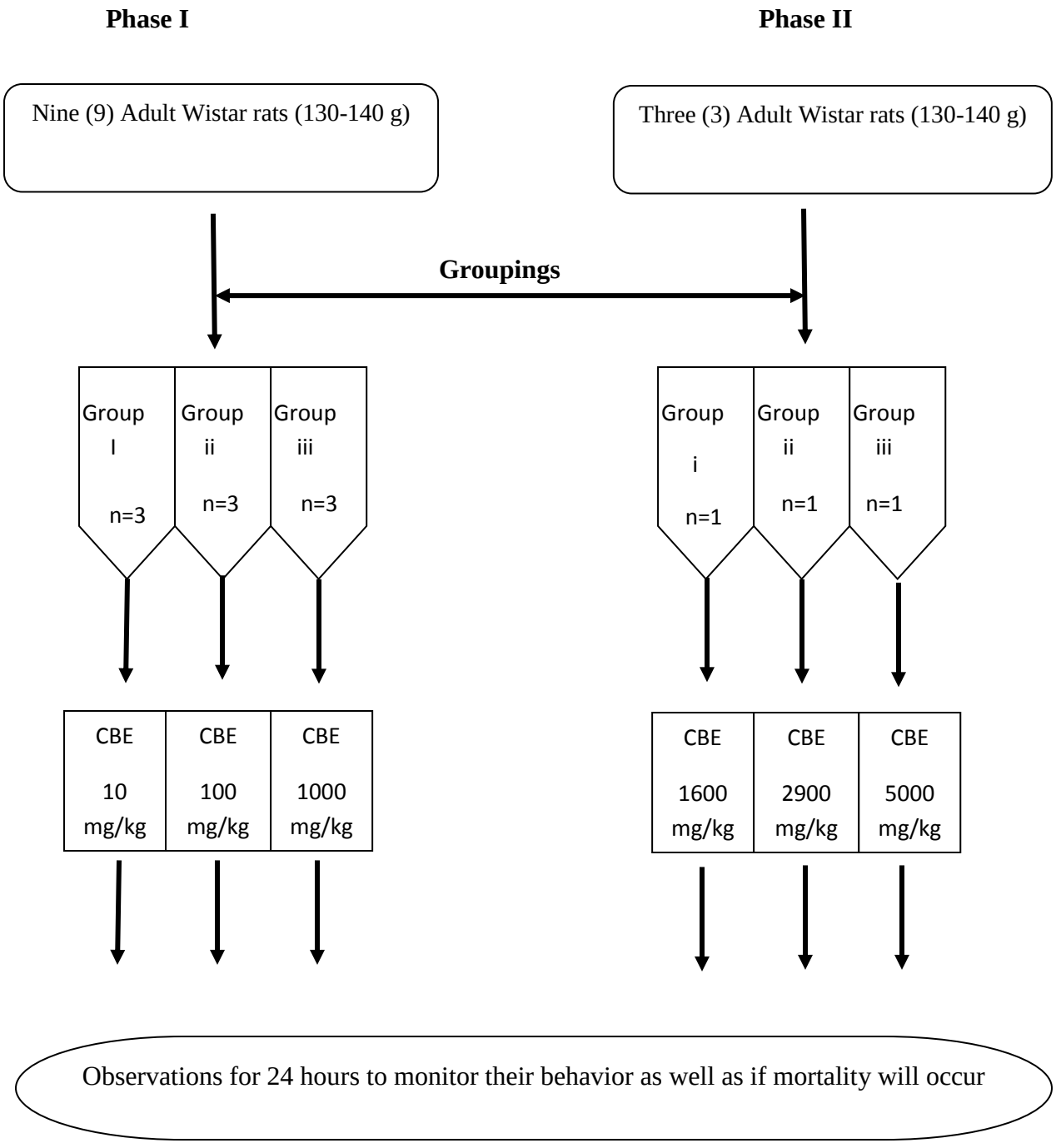


Figure 3.2: Schematic diagram of experimental design for Acute Toxicity study

3.3 Dose Preparation of *Cannabis sativa* L. Extract

10.8 g of the n-butanol extract of *Cannabis sativa* L. was measured and dissolved in six (6) drops of tween 80 before diluting the dissolved extract in 200 ml of distilled water. That is, 3.4 g and 7.4 g of the extract for group II and III representing 250 mg and 500mg respectively were dissolved in 100 ml each after being dissolved in Tween 80. This makes up the working solution. From freshly prepared working solution, little portion was measured out for animal administration. Different doses of extract were administered to experimental animals based on their individual body weight by oral gavages using orogastric cannula for the treatment period. The method for computing the dosage is indicated in **appendix VI.**

3.4 Experimental Design

Eighteen (18) Wistar rats (male and female) were divided into three experimental groups consisting of six (6) animals per group. Prior to the administration day the animals were fasted overnight (12 hours), the neurobehavioral test was done early in the morning before administration. Group I served as control and was administered distilled water (1 ml) while, groups II and III were treatment groups. Group II and III were administered n-butanol extract of *Cannabis sativa* L. 250 mg/kg, 500mg/kg, respectively (that is, 5% and 10%) of LD_{50} respectively for a period of three (3) weeks. All administrations were via oral route.

Table 3.1: Treatments of Experimental Animals

Group (n)	Concentration	Duration
I	Distilled water (1 ml)	21 days
II	250 mg/kg *n*-butanol ECS (5% of LD_{50})	21 days
III	500 mg/kg *n*-butanol ECS (10% of LD_{50})	21 days

n= 6: number of rats, administration was via oral route; Extract of *Cannabis sativa* L. (ECS). LD_{50} of n-butanol extract of *Cannabis sativa* L.: > 5000 mg/kg based on Lorke's Method.

3.5 Neuro Behavioral Studies

3.5.1 The ladder rung walking task

The ladder rung walking test was adopted from the ladder rung walking task (Metz and Whishaw 2002).

The present study describes a new task to assess skilled walking in the rat to measure both forelimb and hind limb function at the same time. Animals are required to walk along a horizontal ladder on which the spacing of the rungs is variable and is periodically changed. Changes in rung spacing prevent animals from learning the absolute and relative location of the rungs and so minimize the ability of the animals to compensate for impairments through learning. In addition, changing the spacing between the rungs allowed the test to be used repeatedly in long-term studies. Methods are described for both quantitative and qualitative description of both fore- and hind limb performance, including limb placing, stepping, co-ordination. Furthermore, use of compensatory strategies is indicated by missteps or compensatory steps in response to another limb's misplacement (Metz and Whishaw, 2002)

3.5.1.1 Protocol

The horizontal ladder rung walking test apparatus consisted of side walls made of clear Plexiglas and metal rungs (3 mm diameter), which was inserted to create a floor with a minimum distance of 1 cm between rungs (Metz and Whishaw, 2009). The side walls are 1 m long and 19 cm high measured from the height of the rungs. The ladder was elevated 30 cm above the ground with a neutral start cage and a refuge (home cage) at the end. Because animals were habituated during training, the elevation of the apparatus was unlikely to cause anxiety. The width of the alley was adjusted to the size of the animal, so that it was about 1 cm wider than an animal to prevent the animal from turning around.

The difficulty of the task was modified by varying the position of the metal rungs. A regular pattern of the rungs allowed the animals to learn the pattern over several training sessions and to anticipate the position of the rungs. An irregular pattern was changed from trial to trial prevented the animal from learning the pattern. For the regular arrangement, the rungs were spaced at 2 cm intervals. For the irregular pattern, the distance of the rungs varied systematically from 1 to 5 cm. Five templates of irregular rung patterns was used, so that the same patterns will applied to all animals to standardize the difficulty of the test and enhance comparability of the outcome.

3.5.1.2 Video recording

A camera (Canovision, Canon Inc.) and a phone Camera (Tecno Hot 6) were positioned at a slight ventral angle, so that positions of all four limbs could be recorded simultaneously. The shutter speed was set at 500 - 2000 s. The video recordings were analyzed using frame-by-frame analysis at 30 f/sec.

3.5.1.3 Behavioral training and test analysis

The animals were trained to cross the ladder from a neutral cage to reach their home cage, so the home cage with littermates provided the positive reinforcement for walking. All animals crossed the ladder in the same direction. No further reinforcement was given to motivate the animals to cross the ladder. All animals were trained and tested five times per session.

3.5.1.4 Foot fault scoring

The qualitative evaluation of forelimb and hind limb placement was performed using a foot fault scoring system as described earlier (Metz and Whishaw, 2002). Analysis was made by inspection of the video recordings frame-by-frame. Only consecutive steps of each limb were analyzed. Therefore, the last step before a gait interruption, such as a stop or a foot fault, and the first step

after an interruption was not scored. The last stepping cycle performed at the end of the ladder was excluded from scoring. Limb placement was scored in terms of limb placement on a rung and limb protrusion between rungs when a miss occurred.

The types of foot or paw placement on the rungs was rated using a 7-category scale. Foot or paw placement on the rung was rated according to their position and errors that occurred in placement accuracy.

(0) Total miss. 0 points were given when the limb completely missed a rung i.e. did not touch it, and a fall occurred.

A fall will be defined as a limb deeply falling in-between rungs and body posture and balance were disturbed. (1) Deep slip. The limb was initially placed on a rung, then slipped off when weight-bearing and caused a fall. (2) Slight slip. The limb was placed on a rung, slipped off when weight bearing, but did not result in a fall nor interrupt the gait cycle. In this case, the animal was able to maintain balance and continue a coordinated gait. (3) Replacement. The limb was placed on a rung, but before it was weight bearing it was quickly lifted and placed on another rung. (4) Correction. The limb aimed for one rung, but was then placed on another rung without touching the first one. Alternatively, a score of 4 was recorded if a limb was placed on a rung and was quickly repositioned while remaining on the same rung. (5) Partial placement. The limb was placed on a rung with either wrist or digits of the forelimb or heel or toes of the hind limb. (6) Correct placement. The mid-portion of the palm of a limb was placed on the rung with full weight support.

When different errors occurred at the same time, the lowest of the scores was recorded. For instance, if a foot was first placed on a rung and then placed on another one in the same step

(score 3), and then slipped and fell in-between rungs (score 1), a score of 1 was recorded. When a fall occurred, only the limb initiating the error was rated and none of the other limbs was scored until the animal had repositioned all limbs. Error scores of five trials were averaged for analysis.

3.5.1.5 Foot placement accuracy analysis (number of errors)

The number of errors in each crossing will be counted. Errors will be determined based on the foot fault scoring system. An error will be defined as each limb placement that received a score of 0, 1 or 2 points, i.e. an error represents any kind of foot slip or total miss. The number of errors and the number of steps will be recorded for each limb separately. From these data, the mean number of errors per step will be calculated and averaged for five trials. The second quantitative parameter analyzed will be the average time needed to cross the entire length of the ladder task. Time measurement will start after the animal is placed on the ladder and began walking the entire length of it. The time an animal spent in a stop will not be included in the measurement.

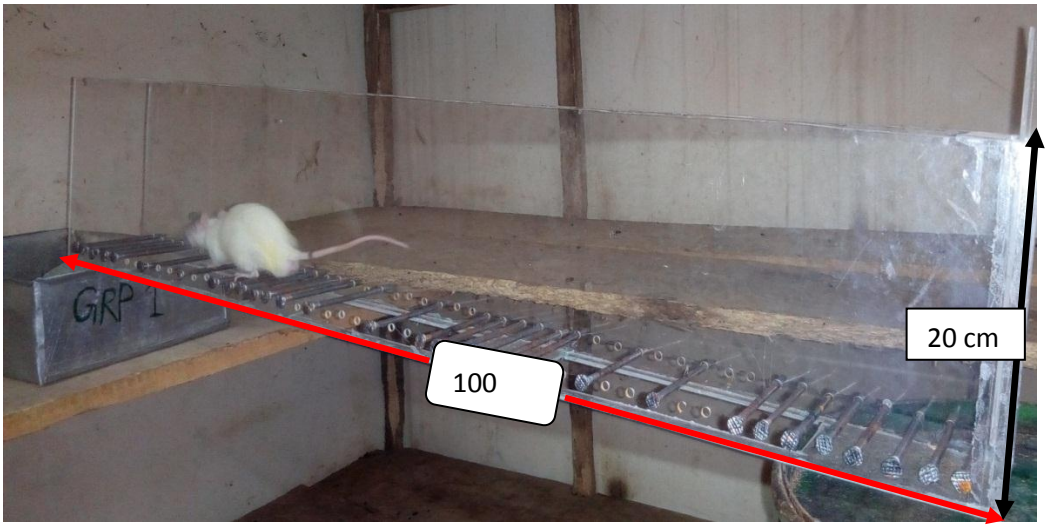


Plate V: The skilled ladder rung walking test apparatus in the fronto-lateral view

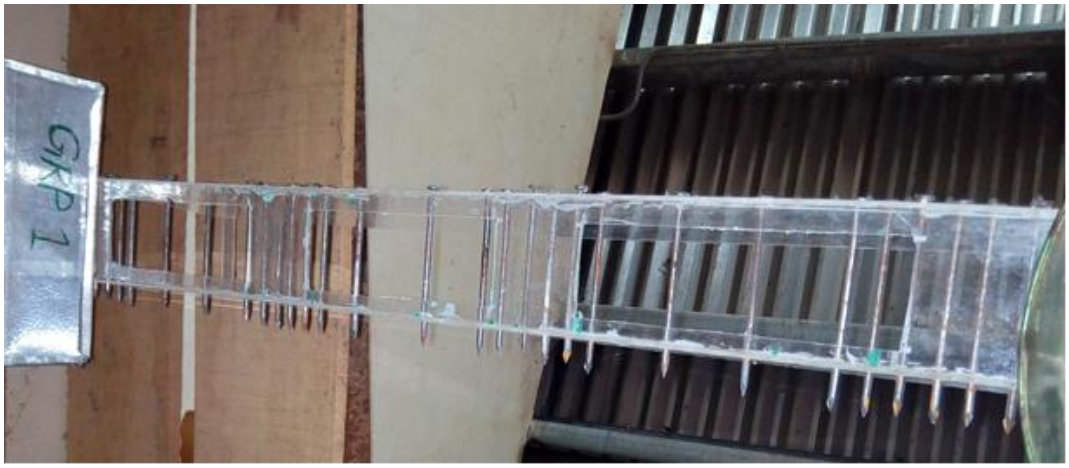


Plate VI: The skilled ladder rung walking test apparatus in the Ventro-lateral view

3.6 Morphological Assessment

All Experimental animals' body weight, that is, Group 1, 2 and 3 were taken before commencement of study and weekly throughout the study period using Electronic Compact scale (KERRO BL2001) in the Department of Human Anatomy, Ahmadu Bello University Zaria, Nigeria.

3.7 Animal Sacrifice

Upon completion of administration and behavioral testing, the weights of the animals were taken. Rats were deeply anaesthetized with Ketamine at a dose of 75 mg/kg intraperitoneally (IP) (Wellington *et al.*, 2013), and euthanized. The skull was opened by incision of the skin and muscle of skull through mid-sagittal suture to harvest the brain.

The harvested brains were weighed and divided into two halves, one half for homogenate and the other for histological demonstration.

Half of the harvested brain organs were weighed and fixed in Bouin's fluid and tissues processed for light microscopic examination at Histopathology Department of Ahmadu Bello University Teaching Hospital, Shika.

Half of the other brain dissected out, was also weighed, minced and homogenized in cold phosphate buffer (pH 7.5) five times its weight in mls. The homogenate were then centrifuged at 3000 rpm for 10 minutes and aliquots of the supernatant were obtained for AChE analysis. The study was carried out at Chemical Pathology Department of Ahmadu Bello University Teaching Hospital, Shika. Protocol is indicated in **Appendix IV**

3.8 Histological and Histochemical Evaluation

The other half of the harvested brain was weighed and immediately fixed in Bouin's fluid. It was then processed routinely, sectioned at 10µm thickness, and stained using H and E and Cresyl fast violet method.

3.8.1 Fixation

The isolated cerebrum (frontal lobe) from the rats were fixed rapidly in freshly prepared Bouin's fluid solution to forestall bacteria decomposition, tissue decay and maintained the structure of the cell and its subcellular components (Cellular organelles).

3.8.2 Tissue processing

The fixed cerebrum was taken through the routine process of tissue processing (dehydration, clearing and infiltration) to remove the water from the tissues and replace with a medium that solidifies to allowthin sections to be cut (Winsor, 1994).

Dehydration – the fixed cerebrum (frontal lobe) samples were passed through increasing grades of alcohol (from 70% to 90% and to absolute or 100%) to remove the water present – either bound or free in the tissue

Clearing – after dehydration , the samples of the cerebrum (frontal lobe) were placed in different changes of Xylene to clear the alcohol content in the tissue thereby making the tissue transparent and clear (Winsor, 1994).

Infiltration – after clearing, the cerebrum (frontal lobe) samples were transferred and immersed into a paraffin molyen wax which replaces the Xylene and infiltrates the tissue sample.

3.8.3 Embedding

After the cerebrum (frontal lobe) samples have been processed successfully, they were transferred into flat metal mould with the molten paraffin wax. The samples in the metal moulds with the molten paraffin wax were allowed to cool and solidify for 24 – 48 hours prior to formation of tissue blocks (Winsor, 1994).

3.8.4 Sectioning

The tissue blocks formed after external embedding were cut and trimmed into thin sections at 10µm using a rotary microtome. The sections were introduced into a water bath (at 50^0C to spread the ribbons) and the placed on microscopic slides and finally dried briefly in the oven (Winsor, 1994).

3.8.5 Staining of slides

The tissue sections on each microscopic slide were finally stained for histological study (using the procedures of the Haematoxylin and Eosin staining techniques), histochemical study (using the procedures of Cresyl fast violet technique).

3.8.6 Haematoxylin and eosin staining techniques

The architecture of the cerebrum (frontal lobe) was observed by using procedure of Ehrlich's (Ehrlich, 1886).

a) Sections was dewax in Xylene for 15 minutes
b) Sections were then hydrated by treating with absolute alcohol (100%), 90% alcohol and 70% alcohol three minutes each.
c) The sections were now stained in haematoxylin for 10 minutes
d) Sections were washed well in running water for 2-3 minutes

e) The sections were differentiated in 1% acid alcohol (1% Hcl in 70% alcohol) for 1 minute (This makes the blue staining turn red due to the action of the acid).

f) To regain the blue colour, the sections were washed in alkaline running tap water (Scoh's tap water) for 10 – 20 minutes.

g) Sections rinsed in water

h) Stained in 1% of aqueous eosin for 3 minutes

i) Rinsed in water (to wash excess stain).

j) Sections were dehydrated in alcohol (70%, 90%, and absolute for two minutes each).

k) Sections were cleared in xylene for 2 minutes.

l) Sections were mounted in synthetic resin medium

3.8.7 Cresyl fast violet (Nissl) stain

a) Sections were dewax sections and brought to water

b) The sections were covered with filtered cresyl fast violet; stain for 10-20 minutes

c) The sections were rinsed in distilled water

d) Sections were differentiated in 0.25% acetic alcohol until most of the stain has been removed (5s).

e) Sections were briefly passed through absolute alcohol into xylene and check microscopically

f) The sections were now rinsed well in xylene

g) Mounted in Canada balsam (Bancroft and Gamble, 2008)

3.9 Neurotransmitter Analysis

Assessment of brain Acetylcholinesterase (AChE) activity was carried out using brain homogenate and Ellman's Technique was used to assess the level of AChE at Chemical

Pathology Department, Ahmadu Bello University Teaching Hospital (ABUTH) Shika Protocol is indicated in **Appendix IV.**

3.10 Statistical Analysis

Data obtained was expressed as mean ± SEM (standard error of mean). One-way analysis of variance (ANOVA) was used to compare the mean differences followed by LSD post-hoc test and a P - value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS Version 20.

CHAPTER FOUR

4.0 RESULT

4.1 Extraction of Plant

Cannabis sativa plant was extracted sequentially in ethanol, n-butanol and water. A total of three extracts were obtained from the plants and the typical yields and appearances of each of the extracts following evaporation to dryness under reduced pressure are shown in **Table 4.1.** Results showed that the ethanol extracts gave more yields than the n-butanol and aqueous extracts. Also the appearances of the extracts were observed. All the extracts were sticky. Colour differences were also observed with the dark green color seen in the ethanol and deep green color in the n-butanol extracts while aqueous were lightly colored.

Table 4.1: Yield and appearances of the *Cannabis sativa* L. plant

Extract	Weight (g)	%Yield	Appearances
Ethanol	27.55	6.89%	Dark green
n-butanol	17.45	4.36%	Deep green
Aqueous	3.75	0.86%	Light green

4.2 Phytochemical screening, Quantitative Estimation and Thin Layer Chromatography (TLC) of *Cannabis sativa* L. Extract

The phytochemical screening of ethanol, n-butanol and Aqueous extracts of *Cannabis sativa* L. revealed the presence of some secondary metabolites such as Carbohydrates, Cardiac glycosides, Alkaloids, Tannins, Flavonoids, Saponins glycoside, Steroids and Triterpenes. However, Anthraquinone was absent in ethanol, n-butanol and Aqueous extracts of Cannabis sativa L. and Tannins was absent in n-butanol extract as shown in **Table 4.2a.**

Quantitative estimation of secondary metabolite of the crude extract of *Cannabis sativa* L. showed the presence of total Alkaloid (5.2%), Flavonoid (9.01 %), Phenol (22.09 %), Tannins (7.38%) and Saponins (3.67%) **(Table 4.2b).** The Thin Layer Chromatography of cannabis extracts gave more than ten spots, with the yellow and violet spot corresponding to CBD and THC respectively as shown in **(Plate VII)**

Table 4.2a: Phytochemical Screening of *Cannabis sativa* L. Extract

S/N	PHYTOCONSTITUENTS AND TEST	INFERENCES		
		Ethanol extract	n-Butanol extract	Aqueous extract
1	Carbohydrates -Molisch test	+	+	+
2	Anthraquinones -Bonfragers test	–	–	–
3	Cardiac glycosides -Keller Kiliani test	+	+	+
4	Alkaloids -Wagner test	+	+	+
5	Tannins -Ferric chloride test	+	–	+
6	Flavonoids -Sodium hydroxide test	+	+	+
7	Saponins glycoside -Frothing Test	+	+	+
8	Steroids and Triterpenes -Lieberman Burchard test	+	+	+

KEYS

+ = (Positive) Present − = (Negative) Absent

Table 4.2b: Quantitative Analysis of the crude Extract of *Cannabis sativa* L.

Phytochemical Constituents	Yield (%)
Alkaloid	5.28
Flavonoid	9.01
Phenol	22.09
Tannins	7.38
Saponin	3.67

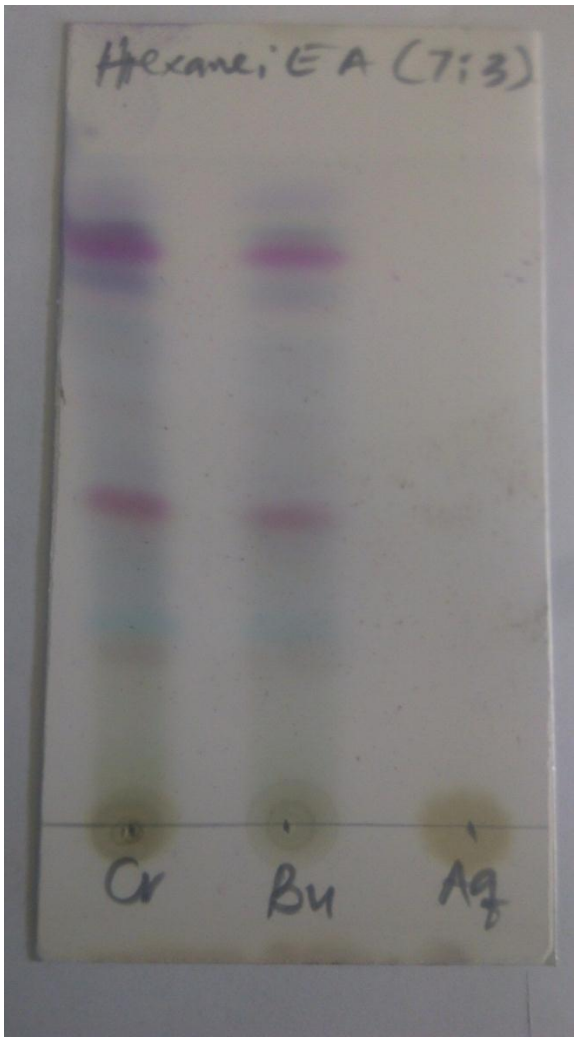


Plate VII: The cannabis extracts gave more than ten spots, with the yellow and violet spot corresponding to CBD and THC respectively.

4.3 Acute Toxicity (LD_{50}) Study

The acute lethal study of n-butanol extract of *Cannabis sativa* L. on Wistar rats showed no mortality within 24 hours after oral administration of the extract both in phase I and phase II, and the LD_{50} was greater than 5000 mg/kg. The major sign of toxicity noticed within 24 hours was sluggishness. The LD_{50}, being greater than 5000 mg/kg, is suggested to be safe by Lorke **(Table 4.3).**

Table 4.3: Result of Acute Toxicity (LD_{50}) Study using Lorke's method

	Groups (n)=3	Concentrations mg/kg	Mortality
	I	10	No
PHASE I	II	100	No
	III	1000	No
	Groups (n)=1		
	I	1900	No
PHASE II	II	2600	No
	III	5000	No

4.4 Physical Observation

During the period of administration, the physical activities of experimental animals (Wistar rats) were observed. Rats in the control group were observed to exhibit normal physical activities, such as movement and playfulness, whereas animals in the treatment groups exhibited decreased activity, hind limb ataxia and their feces have an unusually strong, putrid smell especially in group three (3) administered with 5000 mg/kg.

The weights of the rats, in all groups (Group 1, 2 and 3), were observed to have increased when initial and final weights were compared. However, there were no significant differences ($p>0.05$) in weight changes (difference in initial and final weights) when treated groups were compared with the control **(Table 4.4).**

The relative organ weights (brain weight/ body weight x 100) were computed and compared with the control. Relative to the control, there was increase in relative organ weight in all the groups. However, observed increases were not statistically significant ($p>0.05$) **(Table 4.4).**

Table 4.4: Weight comparison, weight change of Wistar rats *initial* (day 1) and *final* (day 21) of treatment and Brain Somatic Index.

	Initial Weight (g) (Mean ± SEM)	Final Weight (g) (Mean ± SEM)	Weight Change (g) (Mean ± SEM)	Brain Weight (g) (Mean ± SEM)	Brain SI (g) (Mean ± SEM)
G1	133.00±5.51	153.67±5.37	20.67±1.43	1.70±0.04	1.11±0.05
G2	128.17±2.82	154.17±4.83	26.00±5.30	1.67±0.03	1.09±0.04
G3	133.50±4.88	143.00±9.75	9.50±7.58	1.65±0.02	1.23±0.07
F	0.419	0.810	2.427	0.679	2.042
p	0.665	0.463	0.122	0.523	0.167

n = 6; Paired sample t- test; One way ANOVA. $p>0.05$. Brain Somatic Index= Brain weight/Body weight x 100, Group 1 (G1), Group 2 (G2), and Group 3 (G3)

4.5 Neurobehavioural Studies

4.5.1 The ladder rung walking task

In the Ladder Rung Walking Task, specific variables (foot fault scoring, time taken to move from neutral cage to home cage) were used to assess motor function in Wistar rats.

4.5.1.1 Foot fault scoring

The result revealed that foot fault scoring of Left Hind Limb (LHL) in Group 2 at day 7 after administration (2.67±1.02) was significantly lower compared to day 0 of administration (6.00±0.0) and day 14 after administration (6.00±0.00), also Right Hind Limb (RHL) in Group 3 at day 0 (5.83±0.17) was significantly higher compared to Day 7 after administration (3.33±0.84) and at Day 21 of administration (4.17±0.70) while at after day 7 of administration was significantly lower compared to after 14 of administration (5.50±0.34), also LHL after day 7 of administration (2.00±1.0) was significantly lower compared to Day 0 and after day 14 of administration respectively **(Table 4.5a)**

Table 4.5a: Foot fault scoring

		Day 0 (Mean ± SEM)	Day 7 (Mean ± SEM)	Day 14 (Mean ± SEM)	Day 21 (Mean ± SEM)	*p*
Group 1	RFL	6.00±0.0	6.00±0.0	6.00±0.0	6.00±0.0	-
	LFL	6.00±0.0	6.00±0.0	6.00±0.0	6.00±0.0	-
	RHL	5.83±0.17	5.83±0.17	6.00±0.0	6.00±0.0	0.252
	LHL	5.33±0.21	5.83±0.17	5.33±0.67	6.00±0.0	0.336
Group 2	RFL	6.00±0.00	6.00±0.00	5.17±0.54	5.00±3.63	0.236
	LFL	6.00±0.0	6.00±0.0	6.00±0.0	5.00±0.63	0.175
	RHL	6.00±0.0	3.67±1.17	5.50±0.50	3.83±0.60	0.16
	LHL	6.00±0.0[a]	2.67±1.02[ab]	6.00±0.0[b]	3.33±1.05	0.025
Group 3	RFL	5.83±0.17	5.83±0.17	5.50±0.50	5.33±0.49	0.518
	LFL	5.83±0.17	6.00±0.00	5.33±0.67	5.33±0.49	0.498
	RHL	5.83±0.17[ab]	3.33±0.84[ac]	5.50±0.34[c]	4.17±0.70[b]	0.023
	LHL	5.83±0.17[a]	2.00±1.0[ab]	4.67±0.72[b]	3.83±0.95	0.031

Values in Mean ± SEM. One way ANOVA followed by LSD post hoc test. Cells carrying the same superscript ([a,b,c]) on each column are significantly different ($p<0.05$) when compared with the control. Number of rats (n)=6; RFL= Right forelimb; LFL=Left Forelimb; RHL= Right Hind Limb; LHL= Left Hind Limb

Table 4.5b: Foot fault scoring

		Group 1	Group 2	Group 3	F	*p*
Day 0	RFL	6.00±0.0	6.00±0.00	5.83±0.17	1	0.391
	LFL	6.00±0.0	6.00±0.0	5.83±0.17	1	0.391
	RHL	5.83±0.17	6.00±0.0	5.83±0.17	0.5	0.616
	LHL	5.33±0.21^{ab}	6.00±0.00^{a}	5.83±0.17^{b}	5	**0.022**
Day7	RFL	6.00±0.0	6.00±0.00	5.83±0.17	1	0.391
	LFL	6.00±0.0	6.00±0.0	6.00±0.00	-	-
	RHL	5.83±0.17	3.67±1.17	3.33±0.84	2.612	0.106
	LHL	5.83±0.17 ^{ab}	2.67±1.02^{a}	2.00±1.00^{b}	6.072	**0.012**
Day 14	RFL	5.83±0.17	5.17±0.54	5.50±0.50	0.969	0.402
	LFL	6.00±0.00	6.00±0.0	5.33±0.67	1	0.391
	RHL	6.00±0.0	5.50±0.50	5.50±0.34	0.682	0.521
	LHL	6.00±0.0	6.00±0.0	4.67±0.72	1.395	0.278
Day 21	RFL	6.00±0.0	5.00±3.63	5.33±0.49	1.207	0.327
	LFL	6.00±0.0	5.00±0.63	5.33±0.49	1.207	0.327
	RHL	6.00±0.0 ^{ab}	3.83±0.60^{a}	4.17±0.70^{b}	4.773	**0.025**
	LHL	6.00±0.0	3.33±1.05	3.83±0.95	3.006	0.080

Values in Mean ± SEM. One way ANOVA followed by LSD post hoc test. Cells carrying the same superscript (^{a,b}) on each column are significantly different ($p<0.05$) when compared with the control. Number of rats (n)=6; RFL= Right forelimb; LFL=Left Forelimb; RHL= Right Hind Limb; LHL= Left Hind Limb

4.5.1.2 Foot placement accuracy analysis (steps)

The result revealed that the footsteps of RFL in Group 1 at day 7, 14 and 21 after administration (9.67±0.67), (9.67±0.21) (9.33±0.21) respectively was significantly lower compared to RFL in Group 2 and 3 at day 7, 14 and 21 after administration (12.17±0.17), (11±0.37), (12.67±0.21) and (11.00±0.0), (10.67±0.21), (12.83±0.31), also LFL in Group 1 at day 7, 14 and 21 after administration (9.17±0.31), (9.83±0.17), (9.33±0.21) respectively was significantly lower compared to LFL in Group 2 and 3 at day 7, 14 and 21 after administration (10.83±0.60), (11.33±0.21), (12.67±0.21) and (11.50±0.22), (10.5±0.22) (12.83±0.31) respectively. Also RHL in Group 1 at day 7, 14 and 21 after administration (8.00±0.26), (7.50±0.345) and (8.0±0.26) respectively was significantly lower compared to RHL in Group 2 and 3 at day 7, 14 and 21 after administration (9.33±0.33), (8.83±0.31), (9.33±0.42) and (10.17±0.60), (8.83±0.48) (9.83±0.17), also LHL in Group 1 at day 14 and 21 after administration (7.33±0.52) and (8.00±0.26) respectively was significantly lower compared to LFL in Group 2 and 3 at day 14 and 21 after administration (9.33±0.21), (9.33±0.42) and (8.83±0.48), (9.83±0.17) respectively. **(Table 4.5c)**

Table 4.5c: Foot placement accuracy analysis (steps)

		Day 0	Day 7	Day 14	Day 21
RFL	Grp 1	6.33±0.33	9.67±0.67^{ab}	9.67±0.21^{ab}	9.33±0.21^{ab}
	Grp 2	6.67±0.21	12.17±0.17^{a}	11±0.37^{a}	12.67±0.21^{a}
	Grp 3	6.17±0.17	11.00±0.0^{b}	10.67±0.21^{b}	12.83±0.31^{b}
F		1.061	9.941	6.5	63.788
p		0.371	0.002	0.009	<0.001
LFL	Grp 1	6.33±0.33	9.17±0.31^{ab}	9.83±0.17^{ab}	9.33±0.21^{ab}
	Grp 2	6.67±0.21	10.83±0.60^{a}	11.33±0.21^{a}	12.67±0.21^{a}
	Grp 3	6.00±0.00	11.50±0.22^{b}	10.5±0.22^{b}	12.83±0.31^{b}
F		2.143	8.571	13.864	63.788
p		0.152	0.003	<0.001	<0.001
RHL	Grp 1	6.17±0.40	8.00±0.26^{ab}	7.50±0.345^{ab}	8.0±0.26^{ab}
	Grp 2	5.83±0.31	9.33±0.33^{a}	8.83±0.31^{a}	9.33±0.42^{a}
	Grp 3	6.50±0.43	10.17±0.60^{b}	8.83±0.48^{b}	9.83±0.17^{b}
F		0.759	6.649	4.051	9.898
p		0.485	0.009	0.039	0.002
LHL	Grp 1	6.17±0.17	8.83±0.17	7.33±0.52^{ab}	8.00±0.26^{ab}
	Grp 2	5.83±0.31	10.17±0.40	9.33±0.21^{a}	9.33±0.42^{a}
	Grp 3	6.50±0.34	9.50±0.50	8.83±0.48^{b}	9.83±0.17^{b}
F		1.395	3.038	10.263	9.898
p		0.278	0.078	0.002	0.002

Values in Mean ± SEM. One way ANOVA followed by LSD post hoc test. Cells carrying the same superscript (^{a,b}) on each column are significantly different ($p<0.05$) when compared with the control. Number of rats (n)=6; *RFL= Right forelimb; LFL=Left Forelimb; RHL= Right Hind Limb; LHL= Left Hind Limb*

4.5.1.3 Foot placement accuracy analysis (number of errors)

The result revealed that, there is no errors in Group 1 at day 0, 7, 14 and 21 while group 2 and 3 showed errors at day 7, 14 and 21 (0.833±0.31, 0.50±0.50, 1.167±0.65 and 1.17±0.40, 0.67±0.33, 1.00±0.68) respectively, though not statistically significant **(Table 4.5d)**

Table 4.5d: Foot placement accuracy analysis (number of errors)

	Day 0	Day 7	Day 14	Day 21	*p*
Group 1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	-
Group 2	0.0±0.0	0.83±0.31	0.50±0.50	1.17±0.65	0.349
Group 3	0.0±0.0	1.17±0.40	0.67±0.33	1.00±0.68	0.278

Values in Mean ± SEM. One way ANOVA followed by LSD post hoc test. $p>0.05$. Number of rats (n) =6

4.5.1.4 Time score

The result of the time score for each group of animal to move from the neutral cage to the home cage is reported in Table 7. The result revealed that the time taken to move from the neutral cage in group 3 and 2 to the home cage is higher compare to group 1 (control). Also, the time score at Day 7 is statistically significant with a p – value (0.029) with result of Group 3 at day 7 after administration (8.00±0.82) showing a significantly higher value compared to Group 1 at day 7 (4.83±0.87). However, the treated groups in day 0, 14 and 21 did not show statistically significant difference **(Table 4.5e)**

Table 4.5e: Effect of *Cannabis sativa* L. Extract on time taken to cross the Ladder Rung Apparatus

	Day 0 (Mean ± SEM)	Day 7 (Mean ± SEM)	Day 14 (Mean ± SEM)	Day 21 (Mean ± SEM)
Group 1	6.33±0.67	4.83±0.87[a]	4.67±0.80	4.00±0.52
Group 2	6.50±0.76	6.33±0.33	8.33±1.05	11.33±4.23
Group 3	7.17±0.70	8.00±0.82[b]	7.67±1.38	12.50±3.25
F	0.38	4.89	3.12	2.22
P	0.688	**0.029**	0.074	0.162

Values in Mean ± SEM. One way ANOVA followed by LSD post hoc test. Cells carrying different superscript (^{a,b}) on each column are significantly different ($p<0.05$). Number of rats (n)=6

Table 4.5f: Effect of *Cannabis sativa* L. Extract on time taken to cross the Ladder Rung Apparatus

	Day 0	Day 7	Day 14	Day 21	F	*p*
Grp 1	6.33±0.67	4.83±0.87	4.67±0.80	4.00±0.52	3.83	0.032
Grp 2	6.50±0.76	6.33±0.33	8.33±1.05	11.33±4.23	1.11	0.343
Grp 3	7.17±0.70	8.00±0.82	7.67±1.38	12.50±3.25	1.85	0.223

Values in Mean ± SEM. Repeated ANOVA followed by LSD post hoc test; $p>0.05$ when compared with the control. Number of rats (n) =6

4.6 Histological and Histochemical Studies

Histological sections of Wistar rats' brain regions (frontal cortex – layer V) stained with routine (Haematoxylin and Eosin, H&E) histological and histochemical (Cresly Fast Violet) stains and examined under the light microscope revealed the following:

4.6.1 Histological and Histochemical Features of the Frontal Cortex of Control (Untreated) Wistar Rat.

The cerebral sections of Wistar rats in the control group showed normal histoarchitecture of the frontal cortex. Particularly, the ganglionic layer (layer V) revealed large pyramidal (ganglion/ Betz) cells of the motor cortex. Histochemical (Cresly fast violet, CFV) staining for Nissl substances revealed normal appearance of distinctly stained large pyramidal neurons. **Plate VIII and Plate XI**

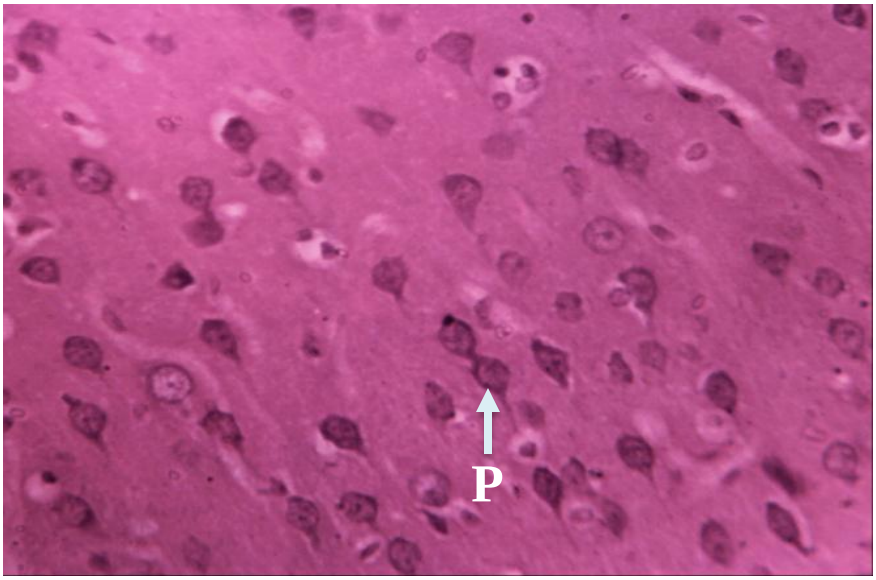


Plate VIII: Section of Wistar rat frontal cortex (layer V) of the control (untreated) group with normal histology of the frontal cortex: P=Pyramidal cell. H and E stain (Mag x 250).

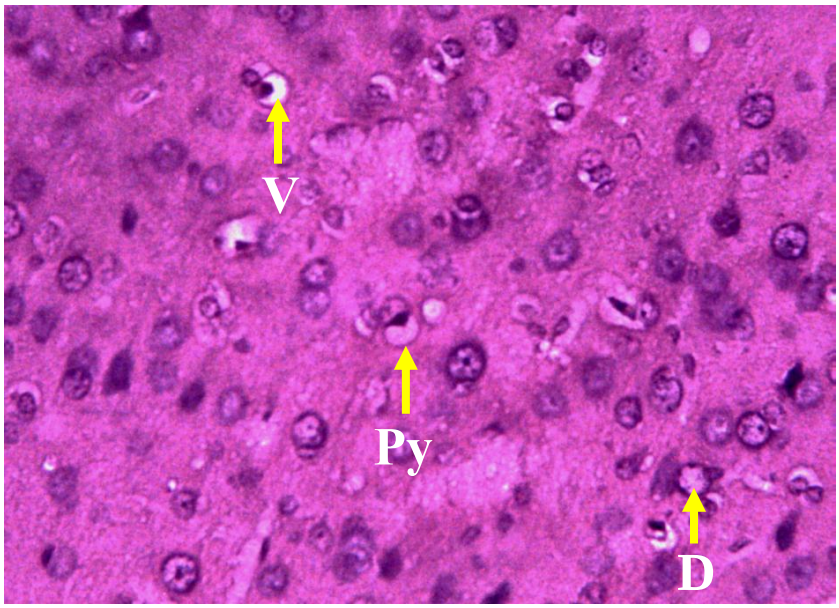


Plate IX: Section of Wistar rat frontal cortex (layer V) of the group administered *Cannabis sativa* L. (250 mg/kg) with distortion in the histology of the frontal cortex Pyramidal cell, D= Dissolution of nucleolus, V= Vacuolation, Py= Pyknosis. H and E stain (Mag x 250).

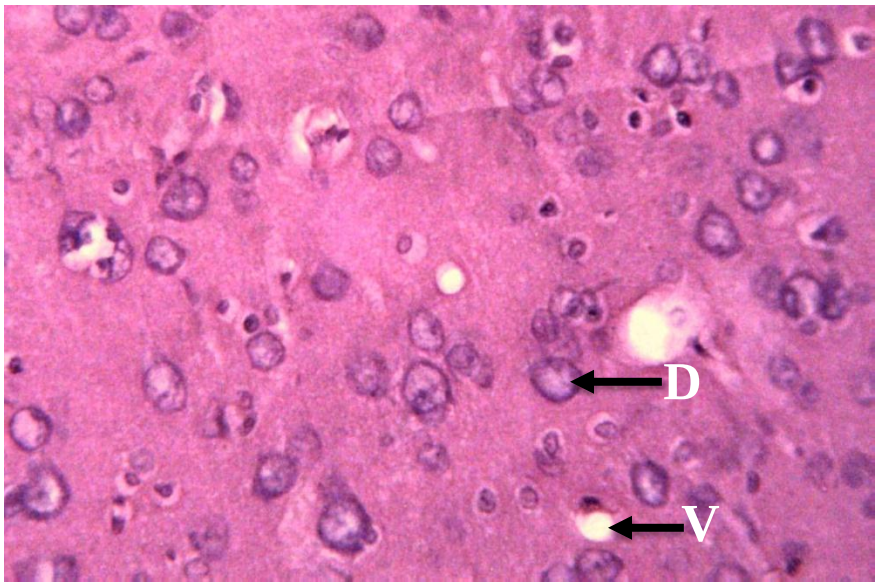


Plate X: Section of Wistar rat frontal cortex (layer V) of the group administered *Cannabis sativa* L. (500 mg/kg) with distortion in the histology of the frontal cortex Pyramidal cell layer; D = Dissolution of nucleulus, V= Vacuolation. H and E stain (Mag x 250).

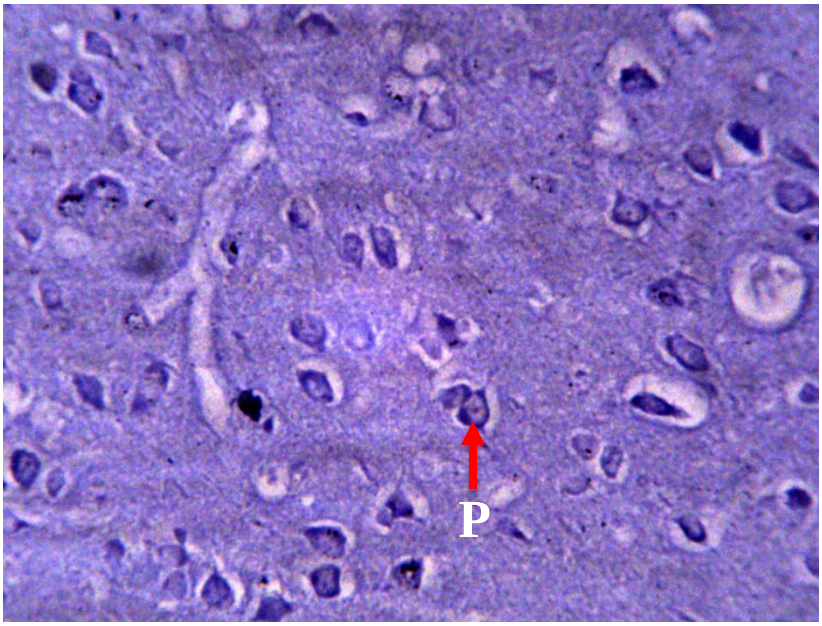


Plate XI: Section of Wistar rat frontal cortex (layer V) of the control (Group I) group with normal histology of the frontal cortex; P=Pyramidal cell with Nissl substance intensely stained at the soma. Cresyl Fast Violet Stain (CFV) stains (Mag x 250).

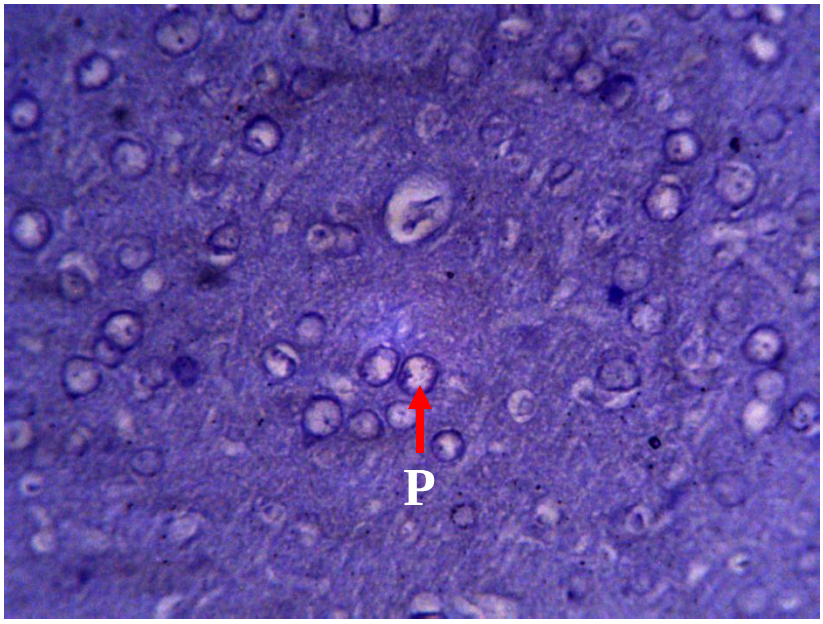


Plate XII: Section of Wistar rat frontal cortex (layer V) of Group II (250 mg/kg) with distortion in the histology of the frontal cortex lightly stained. P= Pyramidal cell with dissolution of Nissl Substance (Chromatolysis). Cresyl Fast Violet Stain (CFV) stains (Mag x 250).

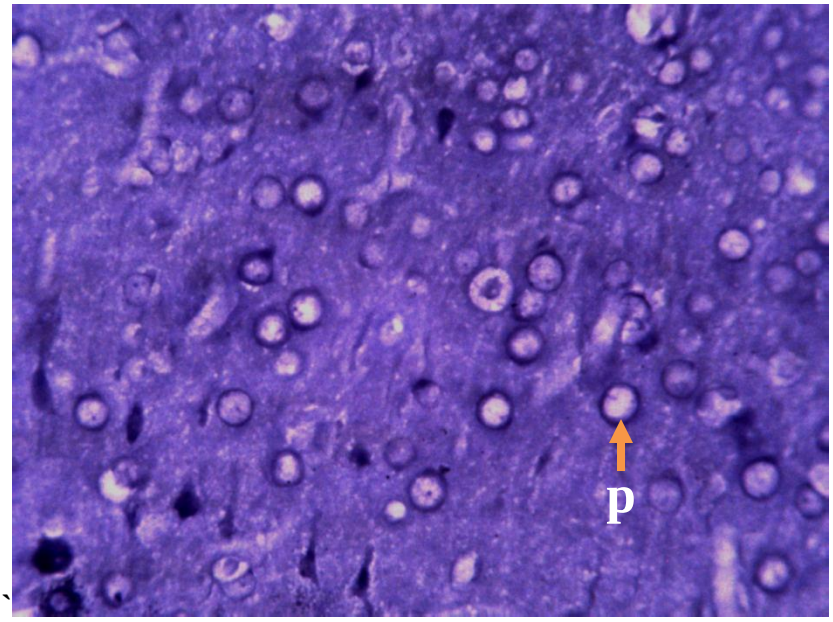


Plate XIII: Section of Wistar rat frontal cortex (layer V) of Group III (500 mg/kg) showing distortion in the histology of the frontal cortex lightly stained. P= Pyramidal cell with dissolution of Nissl Substance (Chromatolysis). Cresyl Fast Violet Stain (CFV) stains (Mag x 250).

4.7 Acetylcholinesterase Activity

Increase in brain AChE activity was observed in animal treated with n-butanol extract of *Cannabis sativa* L. compared to the control group. AChE activity in brain increased in group II and III in comparison with the control. **Figure 4.1**

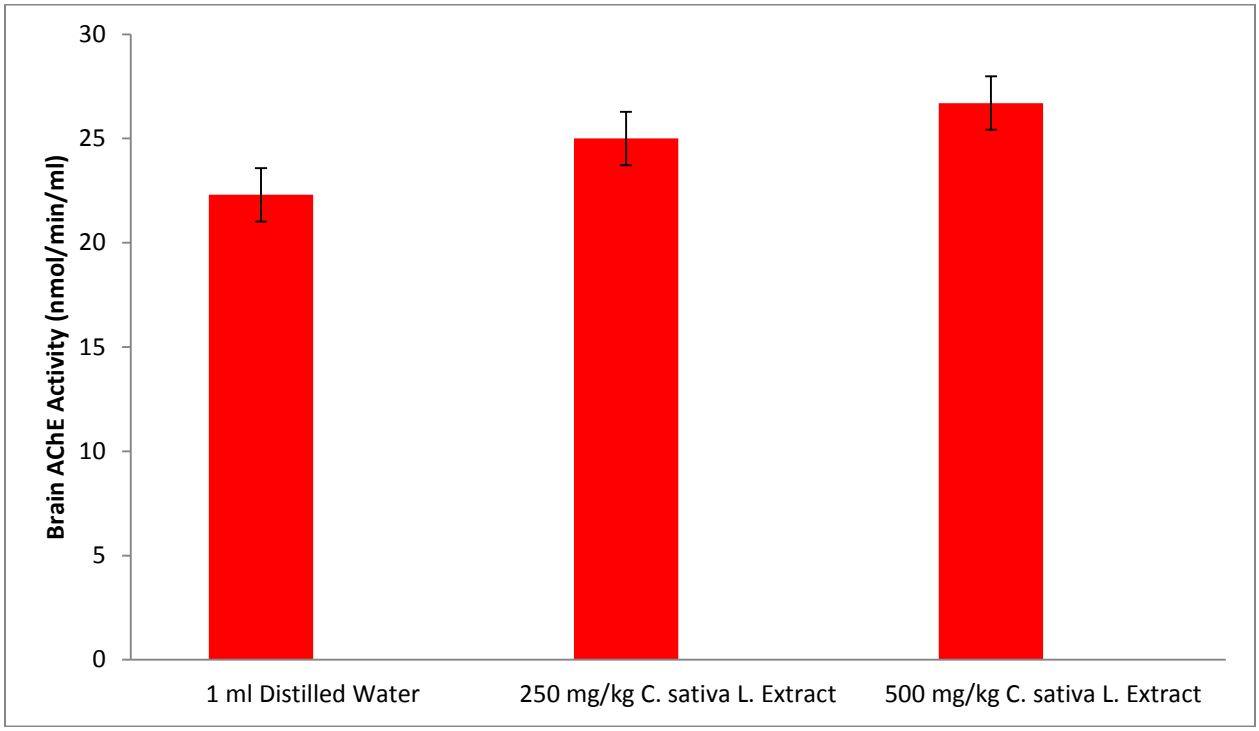


Figure 4.1 Effect of n-butanol extract of *Cannabis sativa* L. on the activity of Acetylcholinesterase in the cerebrum of control and experimental rats. n=6; mean ± SEM; One way ANOVA; *p*=0.068

CHAPTER FIVE

5.0 DISCUSSION

In this study, plant extraction, phytochemical screening and acute toxicity study was carried out, and effect of n-butanol extract of *cannabis sativa* L. on the frontal lobe of adult Wistar rats was assessed using physical observation, neurobehavioural studies, histological and histochemical studies and also assessing acetylcholinesterase activity.

5.1 Plant Extraction

Results demonstrated that the ethanol and n-butanol extract gave the highest yields while the aqueous extracts had the lowest yields. This is in line with past works in which ethanol and n-butanol extracts are preferred due to their high yield in extraction processes. Another finding discovered that the biggest benefits of this method of extraction is that there is no risk of leaving toxic residual chemicals in the final cannabis extract, and it also enables the extraction of all compounds of interest, chiefly cannabinoids (Lukhele and Motadi, 2016; Mike, 2018; Frances *et al.,* 2019). The strong odor of the extract might be as a result of the presence of terpenes which is in line with other studies that cannabis has terpenes which has a unique aroma and flavor (Langenheim, 1994). **Table 4.2b**

5.2 Phytochemical screening, Thin Layer Chromatography and Quantitative estimation of *Cannabis sativa* L. extracts

The phytochemical screening result is similar to a study that found that numerous compounds are formed in cannabis through the secondary metabolism. They include cannabinoids, terpenes and phenolic compounds (Flores-Sanchez and Verpoorte, 2008). According to a study by Waris *et al*., (2018) they also observed that phytochemical screening of cannabis sativa plant revealed the presence of alkaloids, flavonoids, saponins, steroids, tannins, triterpenoids and glycosides which

is in consistence with this findings. Kulkarni *et al.,* (2018) reported a work on cannabis TLC and found out that the plant showed many spots with violet and yellow color indicating presence of THC and CBD respectively which is similar with this finding. Therefore, this study showed that cannabis sativa plant have numerous compound including phytocannabinoid which is also indicative of the presence of THC, CBD and other cannabinoid which is confirmed by Thin Layer Chromatography. With the presence of this compounds especially THC, it means that this plant has the capacity to cause psychoactive effect by binding with the cannabinoid receptors which are well endowed in the cerebral cortex and other brain regions, therefore eliciting it effects. However, the combination of cannabinoids together with non-cannabinoid constituents could enhance the beneficial effects of THC and could reduce undesirable side effects (Pollastro *et al.,* 2018). High amount of Phenol constituent found in the quantitative screening of this plant could also be indicative of it pharmacological values.

5.3 Acute Toxicity (LD_{50}) Study

The acute lethal study of n-butanol extract of *Cannabis sativa* L. on Wistar rats showed no mortality within 24 hours after oral administration of the extract. However, the major signs of toxicity noticed within 24 hours include ataxia, lethargy and asthenia as the doses increased towards 5000 mg/kg b.w. Turner *et al.,* (2018) have also recorded that oral doses of ethanol extract from 5 to 300 mg produced severe symptoms such as hypotension, panic, anxiety, myoclonic jerking/hyperkinesis, delirium, respiratory depression, and ataxia. Therefore, the LD_{50}, being greater than 5000 mg/kg b.w., is thought to be safe as recommended by Lorke's method. Similar result was observed by Beaulieu (2005), that the administration of cannabis sativa yields a reaction characterized by hypolocomotion, hypothermia, catalepsia and antinociception. Also, from studies in knockout animals, it has been revealed that the

cannabinoid receptor (CB1) is responsible for these effects (Ledent *et al.,* 1999). The LD_{50} of oral administration of cannabis in rats is 800 mg/kg to 1900 mg/kg depending on sex and strain (Thompson *et al.,* 1973) which is different with this work stating that the acute toxicity is greater than 5000mg/kg.

The acute oral toxicity of cannabis as reported by O'Shaughnessy (1842) and Moreau (1845) who administered large amounts of hashish extracts to rats did not produce any lethal effect which also agrees with this work. However, toxic signs preceding death that were studied were ataxia, hyperexcitability, depression, loss of righting reflex and dyspnea progressing to respiratory arrest Gabriel (1972). According to Loewe, the LD_{50} of cannabis extracts administered to rat orally was 21.6 mg/kg which is not in line with this finding. Another study reported diarrhea in mice, tremor and lacrimation in rats after oral administration of cannabis. All toxic signs disappeared within 24 hours in the surviving animals. With the same compound Scheckel *et al.,* (1968) observed a 50% mortality in the squirrel monkey after a dosage varying from 36 to 64 mg/kg administered intraperitoneally though not related but similar to this work.

5.4 Morphological Studies

Decreased physical activity was observed in group II and III which reflects treatment related toxicity. This is similar with reports on cannabis related toxicity which alter physical activities manifesting as sluggishness (Pacher *et al.,* 2017). Changes in body weight of group III was observed to reduce while group II increases compared to the control group which could be as a result of the cannabis administered at different dosages which is in agreement with the work of Henrike *et al*., (2018) which they reported that caloric intake and body weight are significantly lower than in control group after been administered with cannabis (Ravinet *et al.,* 2004; Henrike *et al.,* 2018). It may be that administration of high dose of cannabis directly suppresses appetite,

thereby preventing weight gain (Schefflera, 2018). However, other indirect effects such as food neglect may be influential and could explain these findings. Also, in this study, relative organ weights were computed and compared with the control. Relative organ weight increase in group III and decrease in group II relative to the control, though not statistically significant.

5.5 Neurobehavioural Studies

Horizontal rung ladder walking task with an irregular rung pattern has previously been described as a sensitive test to evaluate both forelimb and hind limb use in rats after lesions of the motor system (Metz and Whishaw, 2002).

The result in this study showed forelimb and hind limb impairment in the treatment groups which could be as a result of motor impairment in the cerebral cortex of the Wistar rat due to the extract administered since both animals and humans frontal region of the cerebral cortex contains high concentrations of CB1 receptors which the active compound in cannabis (Delta -9-tetrahydrocannabinol) binds, thus eliciting its effect (Iversen, 2003), similar work was reported by Metz *et al.,* (2005) and Knieling *et al.,* (2009) in which their data showed impaired forelimb and hind limb. Motor impairments, particularly in hindlimb use, were revealed by a reduced foot fault score and higher error rate that was persistent for a period of 21 days. Impairment of motor performance of the hindlimbs predominates in this study which might be as a result of motor disabilities (Stroemer *et al.,* 1995; Metz *et al.,* 1998; Muir and Whishaw, 1999 and Liebigt *et al.,* 2012). Our results revealed that the treatment group (II and III) showed worse walking adaptability (lower skilled walking score) and longer duration of time to cross the ladder from the neutral cage to the home cage, when compared to the control group.

Motor deficits shown in ladder walking clearly indicate that cannabis has a strong influence on the accuracy of limb placement for both affected limbs. This study also showed that, the high

sensitivity of ladder rung walking task is capable of detecting motor impairment in treated groups which is similar with a work which also reported high sensitivity of ladder rung walking task which aid the detection and observation of motor impairments in animal experiments (Metz and Whishaw, 2009). Other studies carried out using cannabis show that low dose (0.1 mg/kg) of cannabinoids increase motor activity, while high doses (1 mg/kg) decrease motor activity and produce catalepsy (Katsidoni *et al.,* 2013). Another finding using different model by Ahmed *et al.,* (2018) indicate that embryos exposed to cannabis during a short-term but critical period of gastrulation showed alterations in motor neuronal morphology and also affect locomotion. Also, cannabis has been shown to have deleterious effect on the brain altering corticostriatal connectivity and locomotor activity (Tomas-Roig, 2018), but the effect of cannabis on motor activity is dose-dependent (Boggs *et al.,* 2018). Psychoactive compound found in cannabis (Delta-9-tetrahydrocannabinol) has also been observed to reduce motor activity in mice (Lloyd, 2018).

A study by Kasten *et al.,* (2019) reported decrease in locomotor activity in rats administered 20mg of cannabis sativa, this could be as a result of Cannabis sativa having high amount of delta-9-tetrahydrocannabinol which binds to cannabinoid receptors and may interrupt well maintained inhibitory signaling regulated by endogenous cannabinoids (Freund and Katona, 2007; Svizenksa *et al.,* 2008; Chevaleyre and Piskorowski, 2014). However, another studies investigated the effects of cannabis smoke exposure on exploratory behavior. The studies showed that cannabis smoke increased locomotor activity when the rats were tested immediately after smoke exposure, but not when tested 4 h, 24 h or 48 h after cannabis smoke exposure, the rapid action of cannabis by increasing locomotor activity might be because of the route of

administration, other studies also revealed that smoke cannabis act faster for a short period of time.

Another study similar to this work was also reported by Malyshevskaya *et al.,* (2017) they observed behavioral changes after administration of Cannabis, including suppression of locomotor activity, extensor rigidity in hind limbs ataxia, impaired walking, and muscular jerks. All these changes observed could be as a result of the presence of psychoactive compound in the plants.

Okon *et al.,* (2014) also reported a similar result to this study, at dose of 10mg/kg and 20mg/kg body weight of ethanol extract of *Cannabis sativa* L. for 28 days, they observed decrease locomotor activity compared with the control that received normal saline. Therefore, since a lower dose (10 mg/kg and 20 mg/kg) of the extract is liable of causing motor impairment it is not doubt that 250 mg/kg and 500 mg/kg of n-butanol extract of *cannabis sativa* L. would cause motor deficit as seen in the foot fault scoring. These studies therefore suggest that, n-butanol extract of *cannabis sativa* L. could have high content of psychoactive compound thereby, causing motor deficit by binding with the cannabinoid receptors in the cerebral cortex and causing damage at the corticospinal tract linking the Cerebral cortex and the spinal cord.

5.6 Histological and Histochemical Studies

Histological assessments also verified evidence of alterations in the structure of layer V (pyramidal neurons), degenerative alterations was detected as, dissolution of nucleolus, vacuolation, Pyknosis and less intense staining of nissl substance (chromatolysis) in frontal cortex section of cannabis treated Wistar rats when compared with the frontal cortex section of the control group, it indicates treatment related neurotoxicity.

The animals treated with cannabis extract especially in group III of both H and E and Cresyl Fast Violet stain showed vacuolation and chromatolysis as seen in **Plate X and Plate XIII** respectively compared with the control. Also, there are mark irregularities in the distributions of nissl granulation in the perikaryon and increased dark pyknotic nuclei, presence of dark neuron suggesting protein denaturation and neural degeneration as seen in group III of Cresyl Fast Violet stain.

This agrees with the findings of previous studies on cannabis who reported that cannabis has the capacity to induce nervous tissues damage (Arman, 2011; Rapp *et al*. 2013; Jacobus *et al.,* 2016 and Orr *et al.,* 2016). Administration of cannabis as reported by Imam *et al.,* (2017) observed that, the pyramidal cells at the internal pyramidal cell layer show some degree of retraction of processes, vacuolation of the surrounding neuropil of the pyramidal cells, and hyperchromatic and shrunken perikarya which is also similar to this study.

The histopathological changes detected in this study are similar to those recorded in the study of Solanke, *et al.* (2016) and Imam *et al.,* (2017) in which the animals treated with Cannabis sativa showed neuro-degeneration of neurons and stroma. This could be as a result of the toxic effect of Cannabis sativa which has been attributed to the tetrahydrocannabinoids (Solanke, *et al*. 2016). Another study by Odokuma *et al.,* (2015) showed that the histomorphologic changes induced by Cannabis sativa in short- and long-term studies caused extensive cerebral gliosis in the brains of adult Wistar rats and concluded that there were both dose- and time-dependent toxic effects of *Cannabis sativa* L.on the experimental animals.

In another study by Adams *et al.,* (2017) observed degeneration in the brain histology administered with 700 mg/kg methanol extract of C. sativa leaves for 21 days which suggest

that the extract possess functional and structural toxicity in male rats. Findings from this study therefore support the assumption that consumption of Cannabis sativa may contribute to increasing incidence of brain damage. In treated male albino rats, Heba *et al.,* (2010) observed irregular shrunken cells with dense nuclei and cytoplasm. The cells are surrounded by irregular wide spaces which are also in line with this study.

Amaza *et al.,* (2013) also reported that administration of 500 mg/kg, 750 mg/kg and 900 mg/kg of aqueous extract of Cannabis sativa showed perivascular congestion as well as lymphatic infiltration in the cerebral coetex of adult Wistar rat. This study is also in agreement with study conducted by Una *et al.,* (1997) who also reported that there were changes in the neurons in selected brain regions most especially the cerebral cortex as to the administration of the same substance.

Neurodegeneration is the progressive loss of structure or function of neurons. Also, is a process involved in both neuropathological conditions and brain ageing (Arman, 2011; Kumar and Khanum, 2012). Also, this study is in consistence with the findings of Ebuehi and Abey (2016) who reported altered brain structure of neuronal and glial cells, as increased cellularity, hypertrophy of glial cells and hyperplasia in the brain following administration of Cannabis sativa compared to the control group.

The results of this study suggest that cannabis use may be associated with altered brain structure, especially in particular regions rich in CB1 receptors like the cerebral cortex. Therefore, this altered architecture explains the lowered score of the foot fault task activity exhibited by the experimental rats.

5.7 Acetylcholinesterase Activity

In this study, treating rat with n-butanol extract of *Cannabis sativa* L. resulted in an increase in brain acetylcholinesterase (AChE) activity. Increase in the level of acetylcholinesterase activity in the treatment group could be as a result of delta -9- tetrahydrocannabinol chemical structure which is similar to the brain chemical anandamide (endogenous cannabinoids) and because of the similarity in structure, it allows the body to recognize THC and to alter normal brain communication. Anandamide function as neurotransmitters, they send chemical messages between nerve cells (neurons) throughout the nervous system. They affect brain areas that influence pleasure, movement, coordination, sensory and time perception. Because of this similarity, THC is able to attach to molecules called cannabinoid receptors on neurons in these brain areas and activate them, disrupting various mental and physical functions. Abdel-Salam *et al.,* (2016) and Murillo-Rodríguez (2018) also reported increase in the level of brain AChE activity in Wistar Rats treated with cannabis. As a result, this could contribute to motor deficits, memory problems and the decline in cognitive function in people abusing this substance. This study also agrees with the findings of Khadrawy *et al.,* (2017) and Johnston *et al.,* (2016) who also reported increase in acetylcholinesterase (AChE) activity in Cannabis treatment group.

The neural communication network that uses these cannabinoid neurotransmitters, known as the endocannabinoid system, plays a critical role in the nervous system's normal functioning, so interfering with it can have profound effects as reported by Mehmedic (2010). **Figure 4.1**

CHAPTER SIX

6.0 Summary, Conclusion, Recommendations and Contribution to Knowledge

6.1 Summary

In this study, evaluation of the effect of n-butanol extracts of *Cannabis sativa* L. in the frontal cortex of adult Wistar rats, the results of phytochemical screening, revealed the presence of Carbohydrates, Cardiac glycosides, Alkaloids, Flavonoids, Saponins glycoside, Steroids and Triterpenes, quantitative Analysis revealed the percentage yield of Alkaloid (5.28%), Flavonoid (9.01%), Phenol (22.09%), Tannins (7.38%) and Saponin (3.67%) and Thin Layer Chromatography gave more than ten spots, with the yellow and violet spot corresponding to CBD and THC respectively. Exposure to n-butanol extracts of *Cannabis sativa* L. resulted in neurotoxicity, which was evident from morphologic, histologic and histochemical alterations in the brain of the rats and motor deficits in neurobehavioural assessment, and altered the levels of acetylcholinesterase activity

Findings of the present study revealed the potentials of the n-butanol extracts of *Cannabis sativa* L. as neurotoxic agent in the following studies:

- Histologic and histochemical alterations in the cytoarchitecture of the frontal cortex (Layer V) of adult Wistar rats
- Impaired motor activity in neurobehavioural assessment relative to the control
- Increased the level of Acetylcholinesterase activity

The results of this study revealed that, the n-butanol extracts of *Cannabis sativa* L. Contains pharmacologically active substances with psychoactive properties. These psychoactive properties explain the rational for the abuse of this plant by teenagers and young adult.

6.2 Conclusion

According to this study, n-butanol *Cannabis sativa* L. had degenerative effects at certain levels on the frontal lobe of adult Wistar rat and has the ability to cause immediate toxic effect. Therefore, oral administration of *n*-butanol extract of *Cannabis sativa* L. altered cytoarchitecture, impaired motor and neurochemical activity in the cerebral cortex of adult Wistar rats.

6.3 Recommendations

This study recommends that;

i. *Cannabis sativa* L. abuse should be avoided due to its harmful effects.
ii. Further studies such as stereology, should be carried out to determine the effect of n-butanol ECS on the number of pyramidal cells and volume of the frontal cortex.
iii. Further studies on the effect of n-butanol extract of *Cannabis sativa* L. on acetylcholine level at the neuromuscular junction should be carried out employing different neurobehavioural paradigms.

6.4 Contribution to Scientific knowledge

- Oral administration of n-butanol extract of *cannabis sativa* L. at dose of 250 mg/kg daily on the 7^{th} day caused a significant decrease in the foot fault of left hind limb (2.67±1.02) and at dose of 500 mg/kg daily on the 7^{th} day caused a significant decrease in the foot fault of right hind limb (3.33±0.84) and left hind limb (2.00±1.0) of adult Wistar rats.

- Also, at dose of 250 mg/kg daily significantly increased foot placement accuracy in the right fore limb and left forelimb respectively at day 7 (12.17±0.17) (10.83±0.60), 14 (11±0.37) (11.33±0.21) and 21 (12.67±0.21) (12.67±0.21), in the right hind limb and left hind limb

respectively at day 7 (9.33±0.33) (10.17±0.40), 14 (8.83±0.31) (9.33±0.21) and 21 (9.33±0.42) (9.33±0.42)

- Oral administration of n-butanol extract of *cannabis sativa* L. at dose of 500 mg/kg daily significantly increased foot placement accuracy in the right fore limb and left forelimb respectively at day 7 (11.00±0.0) (11.50±0.22), 14 (10.67±0.21) (10.5±0.22) and 21 (12.83±0.31) (12.83±0.31), in the right hind limb and left hind limb respectively at day 7 (10.17±0.60) (9.50±0.50), 14 (8.83±0.48) (8.83±0.48) and 21 (9.83±0.17) (9.83±0.17).

- At doses of 250 mg/kg.bw and 500 mg/kg.bw, n-butanol extract of *Cannabis sativa* L. caused neurotoxicity such as dissolution of the nucleolus and less intense staining of the nissl substance and has no significant effect on AchE activity.

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APENDIX I

SAMPLE OF LETTER TO THE H.O.D REQUESTING FOR AN INTRODUCTORY LETTER TO N.D.L.E.A OFFICE, KADUNA STATE COMMAND

Department of Human Anatomy,
Faculty of Basic Medical Science,
College of Health Sciences,
ABU, Zaria.
26th February, 2018.

The Head of Department,
Department of Human Anatomy,
Faculty of Basic Medical Sciences,
College of Health Sciences,
ABU Zaria.

Through:
The Chairman Supervisory Committee
Department of Human Anatomy
ABU Zaria, Zaria.

Through:
The Member Supervisory Committee
Department of Human Anatomy
ABU, Zaria.
Dear Ma,

APPLICATION SEEKING FOR AN INTRODUCTORY LETTER TO NDLEA COMMAND OFFICE KADUNA IN ORDER TO GET FRESH CANNABIS

I Zachariah Richard, an M.Sc student with registration number **P16MDHA8031**, humbly wish to request for an introductory letter to National Drug Law Enforcement Agency (NDLEA) command office in Kaduna in order to get fresh *Cannabis sativa* for my research work, titled;
EFFECT OF *Cannabis sativa* ADMINISTRATION ON THE CEREBELLAR CORTEX OF ADULT MALE WISTAR RATS

Ma, I will be glad if my request is given due consideration, thank you very much for going through my letter. Looking forward to hearing from you.

Yours faithfully,



Zachariah Richard.
(08031521783)

APPENDIX II

LETTER OF APPROVAL TO OBTAIN CANNABIS SATIVA PLANT

DEPARTMENT OF HUMAN ANATOMY
FACULTY OF BASIC MEDICAL SCIENCES
COLLEGE OF HEALTH SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA

Nulli Secundus

Vice-Chancellor: Professor Ibrahim Garba, B.Sc (Geology). M.Sc (Mineral Expl), ABU, PhD (Geology) Lond, D.I.C, FNMGS
Head of Department: Dr. Z.M. Bauchi B.Sc, M.Sc., Ph.D (ABU)

ABU/HA/SA.6 — 12th March, 2018

The State Commander,
National Drug Law Enforcement Agency (NDLEA),
Kaduna State Command,
Kaduna

REQUEST FOR CANNABIS SATIVA FOR RESEARCH PURPOSES: ZACHARIAH RICHARD, REG. NO (P16MDHA8031)

Zachariah Richard is an M.Sc student in this Department and had requested for introductory letter to your office to enable him obtain 500g of cannabis sativa for use in his research work for an M.Sc degree in Human Anatomy titled "Effect of *Cannabis sativa* administration on the cerebellar cortex of adult male wistar rats".

We anticipate your kind assistance in this regard.

Thank you very much.

Yours faithfully,

Dr. Z.M. Bauchi
Head of Department
08028543774

13 MAR 2018

NATIONAL DRUG LAW ENFORCEMENT AGENCY
DATE 13/03/2018
RECEIVED

The above mentin exhibit cannabis sativa 500 grams was issued to Zachariah Richard for the purpose of his researdh at the above mentined institution from NDLEA office Kwargwaje Zaria today the 22nd day of March 2018.

APPENDIX III

ETHICAL CLEARANCE

AHMADU BELLO UNIVERSITY, ZARIA
DIRECTORATE OF ACADEMIC PLANNING & MONITORING
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Approval No: ABUCAUC/2018/046

3rd October, 2018

Prof. S.S. Adebisi
Department of Human Anatomy,
Faculty of Basic Medical Sciences,
College of Health Sciences,
Ahmadu Bello University,
Zaria.

Dear Sir,

APPROVAL OF RESEARCH STUDY 'STUDIES ON THE EFFECT OF N-BUTANOL EXTRACT OF *CANNABIS SATIVA* L. ON CEREBRAL CORTEX OF ADULT WISTAR RATS'

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Anatomy. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

U.D. Abdullahi
For: Chairman, ABUCAUC.

Cc. Director, DAPM
Director, IC &ICT
Provost, College of Health Sciences
Dean, Faculty of Basic Medical Sciences,
HOD, Human Anatomy,
Prof. Aliyu Mohammed

APPENDIX IV

ASSAY OF ACETYLCHOLINESTERASE ACTIVITY IN THE BRAIN

METHODOLOGY

Reagents

1. 0.1M Phosphate buffer Solution A: 5.22g of K_2HPO_4 and 4.68g of NaH_2PO_4 are dissolved in 150 ml of distilled water. Solution B: 6.2g NaOH is dissolved in 150ml of distilled water. Solution B is added to solution A to get the desired pH (pH 8.0 or 7.0) and then finally the volume is made up to 300ml with distilled water.

2. DTNB Reagent 39.6 mg of DTNB with 15 mg $NaHCO_3$ is dissolved in 10 ml of 0.1M phosphate buffer (pH 7.0).

3. Acetylthiocholine (ATC) 21.67 mg of acetylthiocholine is dissolved in 1 ml of distilled water.

ASSAY PROCEDURE

1. Dissection: Adult Male Wistar rats (111-140g body weight) were used for the experiment. The rats were decapitated; brains are removed quickly and placed in ice-cold saline. Cerebral cortex were quickly dissected out on a petri dish chilled on crushed ice.

2. The tissues were weighed and homogenized in 0.1M Phosphate buffer (pH 7.5).

3. 0.4ml aliquot of the homogenate is added to a cuvette containing 2.6 ml phosphate buffer (0.1M, pH 7.5) and 100µl of DTNB.

4. The contents of the cuvette are mixed thoroughly by bubbling air and absorbance is measured at 412 nm in a LKB spectrophotometer. When absorbance reaches a stable value, it was recorded as the basal reading.

5. 20µl of substrate i.e., acetylthiocholine is added and change in absorbance is recorded for a period of 10 mins at intervals of 2 mins. Change in the absorbance per minute is thus determined.

Calculations: The enzyme activity is calculated using the following formula;

R = 5.74x 10-4 x A/CO

Where,

R = Rate in moles of substrate hydrolyzed / minute / gm tissue

A = Change in absorbance / min

CO = Original concentration of the tissue (mg / ml).

(Srikumar, 2014)

APPENDIX V

QUANTITATIVE PHYTOCHEMICAL DETERMINATION OF C*ANNABIS SATIVA* L. USING SPECTROPHOTOMETRIC METHOD

S/N	Concentration µg/ml	Alkaloid (Atropine) 470nm	Flavonoids (Quarcetin) 510nm	Phenol (Gallic acid) 765nm	Tannins (Gallic acid) 725nm	Saponins (Saponins) 544nm
1	20	0.082	0.071	0.008	0.004	0.062
2	40	0.201	0.126	0.016	0.012	0.104
3	60	0.354	0.210	0.021	0.017	0.155
4	80	0.408	0.273	0.033	0.025	0.248
5	100	0.531	0.334	0.044	0.032	0.316
6	**Gradient Factor (GF)**	0.0055	0.00325	0.0043	0.00035	0.0031
7	**Weight of Sample (W)**	0.1mg	0.1mg	0.1mg	0.1mg	0.1mg
8	**Sample**	0.099	0.281	0.181	2.111	0.121
		0.094	0.273	1.182	2.104	0.116
		0.095	0.278	0.178	2.108	0.118

Calculations

Formula

$$\text{Alkaloid/Flavonoid/Phenol/Tannins/Saponins (mg)} = \frac{A \times GF}{W}$$

$$\% \text{ A/F/P/T/S} = \frac{\left(\frac{A \times GF}{W} \right)}{W} \times 100\%$$

For;

Alkaloid

i. $\frac{0.099 \text{ x } 0.0055}{0.1} = \frac{0.005445}{0.1}$ X 100 = 5.445%

ii. $\frac{0.094 \text{ x } 0.0055}{0.1} = \frac{0.00517}{0.1}$ x 100 = 5.17%

iii. $\frac{0.095 \text{ x } 0.0055}{0.1} = \frac{0.005225}{0.1}$ x 100 = 5.225%

AVERAGE = $\frac{5.445 + 5.17 + 5.225}{3}$ **= 5.28%**

Flavonoid

i. $\frac{0.281 \text{ x } 0.00325}{0.1} = \frac{0.0091325}{0.1}$ X 100 = 9.1325%

ii. $\frac{0.273 \text{ x } 0.00325}{0.1} = \frac{0.0088725}{0.1}$ x 100 = 8.8725%

iii. $\frac{0.278 \text{ x } 0.00325}{0.1} = \frac{0.009035}{0.1}$ x 100 = 9.035%

AVERAGE = $\frac{9.1325 + 8.8725 + 9.035}{3}$ **= 9.013%**

Phenol

i. $\frac{0.181 \text{ x } 0.0043}{0.1} = \frac{0.007783}{0.1}$ X 100 = 7.783%

ii. $\frac{1.182 \text{ x } 0.0043}{0.1} = \frac{0.050826}{0.1}$ x 100 = 50.826%

iii. $\frac{0.178 \text{ x } 0.0043}{0.1} = \frac{0.007654}{0.1}$ x 100 = 7.654%

AVERAGE = $\frac{7.783 + 50.826 + 7.654}{3}$ **= 22.088%**

Tannin

i. $\frac{2.11 \text{ x } 0.00035}{0.1} = \frac{0.007385}{0.1}$ X 100 = 7.385%

ii. $\frac{2.104 \text{ x } 0.00035}{0.1} = \frac{0.007364}{0.1}$ x 100 = 7.364%

iii. $\frac{2.108 \text{ x } 0.00035}{0.1} = \frac{0.007378}{0.1}$ x 100 = 7.378%

AVERAGE = $\frac{7.385 + 7.364 + 7.378}{3}$ **= 7.376%**

Saponin

i. $\frac{0.121 \text{ x } 0.0031}{0.1} = \frac{0.003751}{0.1}$ X 100 = 3.751%

ii. $\frac{0.116 \text{ x } 0.0031}{0.1} = \frac{0.003596}{0.1}$ x 100 = 3.596%

iii. $\frac{0.118 \text{ x } 0.0031}{0.1} = \frac{0.003658}{0.1}$ x 100 = 3.658%

AVERAGE = $\frac{3.751 + 3.596 + 3.658}{3}$ **= 3.668%**

APPENDIX VI

DOSAGE CALCULATION

For Group III (500mg)

148g--------- Rat with the highest weight

500 mg ------ 1000 g

X mg---------148 g

X= 74 mg

74 mg-------1 ml

7400 mg------100 ml

Working solution = 7.4 g------ 100 ml

Rat 1- 136

148g ------ 1ml

136g --------- x

X= 0.92

Rat 2- 128

148g ------ 1ml

128g --------- x

X= 0.87

Rat 3- 128

148g ------ 1ml

128g --------- x

X= 0.87

Rat 4- 145

148g ------ 1ml

145g --------- x

X= 0.98

Rat 5- 148

148g ------ 1ml

148g --------- x

X= 1ml

Rat 6- 116

148g ------ 1ml

116g --------- x

X= 0.78

Summary

S/n	Weight (g)	Dosage (ml)
1	136	0.92
2	128	0.87
3	128	0.87
4	145	0.98
5	148	1
6	116	0.78

131g --------- x

X= 0.97 ml

For Group II (250mg)

135g--------- Rat with the highest weight

250 mg ------ 1000 g

X mg---------135 g

X= 33.75 mg

33.75mg-------1 ml

3375 mg------100 ml

Working solution = 3.4 g------ 100 ml

Rat 1- 132

135 g ------ 1 ml

132g --------- x

X= 0.98 ml

Rat 2- 121

135 g ------ 1 ml

121g --------- x

X= 0.90 ml

Rat 3- 132

135 g ------ 1 ml

132g --------- x

X= 0.98 ml

Rat 4- 131

135 g ------ 1 ml

Rat 5- 118

135 g ------ 1 ml

118g --------- x

X= 0.87 ml

Rat 6- 135

135 g ------ 1 ml

135g --------- x

X= 1 ml

Summary

S/n	Weight (g)	Dosage (ml)
1	132	0.98
2	121	0.90
3	132	0.98
4	131	0.97
5	118	0.87
6	135	1

APPENDIX VII

DOSAGE CALCULATION FOR KETAMIN INTRAPERITONIAL (IP) INJECTION

50mg ----------1ml
75mg ---------- x
x = 75 x 1
$x = \frac{75 \text{ x } 1}{50}$
x =1.5 ml
75mg/kg---------1.5ml

GROUP I

Rat 1- 155
75mg --------- 1000g
x mg ---------- 155g
$x = \frac{75 \text{ x } 155}{1000}$
=11.63mg
75mg/kg--------- 1.5ml
11.625---------- x
$x = \frac{11.63 \text{ x } 1.5}{75}$
x = 0.23 ml

Rat 2- 144
75mg --------- 1000g
x mg ---------- 144g
$x = \frac{75 \text{ x } 144}{1000}$
=10.8 mg
75mg/kg--------- 1.5ml
10.80---------- x
$x = \frac{10.80 \text{ x } 1.5}{75}$
x = 0.22 ml

Rat 3- 161
75mg --------- 1000g
x mg ---------- 161g
$x = \frac{75 \text{ x } 161}{1000}$
=12.08 mg
75mg/kg--------- 1.5ml
12.08---------- x
$x = \frac{12.08 \text{ x } 1.5}{75}$
x = 0.24 ml

Rat 4- 163
75mg --------- 1000g
x mg ---------- 163g
$x = \frac{75 \text{ x } 163}{1000}$
=12.23 mg
75mg/kg--------- 1.5ml
12.23---------- x
$x = \frac{12.23 \text{ x } 1.5}{75}$
x = 0.25 ml

Rat 5- 152
75mg --------- 1000g
x mg ---------- 152g
$x = \frac{75 \text{ x } 152}{1000}$
=11.40 mg
75 mg/kg--------- 1.5 ml
11.40---------- x
$x = \frac{11.40 \text{ x } 1.5}{75}$
x = 0.23 ml

Rat 6- 137

75mg --------- 1000g
x mg ---------- 137g
$x = \frac{75 \text{ x } 137}{1000}$
=10.28 mg
75 mg/kg--------- 1.5 ml
10.28---------- x
$x = \frac{10.28 \text{ x } 1.5}{75}$
x = 0.21 ml

GROUP II
Rat 1- 165
75mg --------- 1000g
x mg ---------- 165g
$x = \frac{75 \text{ x } 165}{1000}$
=12.38mg
75mg/kg--------- 1.5ml
12.38---------- x
$x = \frac{12.38 \text{ x } 1.5}{75}$
x = 0.25 ml

Rat 2- 142
75mg --------- 1000g
x mg ---------- 142g
$x = \frac{75 \text{ x } 142}{1000}$
=10.65 mg
75mg/kg--------- 1.5ml
10.65---------- x
$x = \frac{10.65 \text{ x } 1.5}{75}$
x = 0.21 ml

Rat 3- 159
75mg --------- 1000g
x mg ---------- 159g
$x = \frac{75 \text{ x } 159}{1000}$
=11.93 mg
75mg/kg--------- 1.5ml
11.93---------- x
$x = \frac{11.93 \text{ x } 1.5}{75}$
x = 0.24 ml

Rat 4- 150
75mg --------- 1000g
x mg ---------- 150g
$x = \frac{75 \text{ x } 150}{1000}$
=11.25 mg
75mg/kg--------- 1.5ml
11.25---------- x
$x = \frac{11.25 \text{ x } 1.5}{75}$
x = 0.23 ml

Rat 5- 120
75mg --------- 1000g
x mg ---------- 120g
$x = \frac{75 \text{ x } 152}{1000}$
=9.0 mg
75 mg/kg--------- 1.5 ml
9.0---------- x
$x = \frac{9.0 \text{ x } 1.5}{75}$
x = 0.18 ml

Rat 6- 144
75mg --------- 1000g
x mg ---------- 144g
$x = \frac{75 \text{ x } 144}{1000}$
=10.8 mg
75mg/kg--------- 1.5ml
10.80---------- x
$x = \frac{10.80 \text{ x } 1.5}{75}$
x = 0.22 ml

GROUP III
Rat 1- 157
75mg --------- 1000g
x mg ---------- 157g

$x = \frac{75 \text{ x } 157}{1000}$

=11.78mg

75mg/kg--------- 1.5ml

11.78---------- x

$x = \frac{11.78 \text{ x } 1.5}{75}$

x = 0.24 ml

Rat 2- 128

75mg --------- 1000g

x mg ---------- 128g

$x = \frac{75 \text{ x } 128}{1000}$

=9.60 mg

75mg/kg--------- 1.5ml

9.6---------- x

$x = \frac{9.6 \text{ x } 1.5}{75}$

x = 0.19 ml

Rat 3- 137

75mg --------- 1000g

x mg ---------- 137g

$x = \frac{75 \text{ x } 137}{1000}$

=10.28 mg

75mg/kg--------- 1.5ml

10.28---------- x

$x = \frac{10.28 \text{ x } 1.5}{75}$

x = 0.21 ml

Rat 4- 118

75mg --------- 1000g

x mg ---------- 118g

$x = \frac{75 \text{ x } 118}{1000}$

=8.85 mg

75mg/kg--------- 1.5ml

8.85---------- x

$x = \frac{8.85 \text{ x } 1.5}{75}$

x = 0.18 ml

Rat 6- 117

75mg --------- 1000g

x mg ---------- 117g

$x = \frac{75 \text{ x } 117}{1000}$

=8.78 mg

75 mg/kg--------- 1.5 ml

8.78---------- x

$x = \frac{8.78 \text{ x } 1.5}{75}$

x = 0.18 ml

SUMMARY

Group I (n)	Weight (g)	Dosage (ml)
1	155	0.23
2	144	0.22
3	161	0.24
4	163	0.25
5	152	0.23
6	137	0.21
GROUP II		
1	165	0.25
2	142	0.22
3	159	0.24
4	150	0.23
5	120	0.18
6	144	0.22
GROUP III		
1	157	0.24
2	128	0.19
3	137	0.21
4	118	0.18
5	-	-
6	117	0.18