EVALUATION OF THE EFFECT OF COMBRETUM MICRANTHUM FRACTION ON ALUMINIUM-INDUCED OXIDATIVE STRESS IN MICE

BY

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MSc. BIOCHEMISTRY

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, BAYERO UNIVERSITY, KANO, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MSc. IN BIOCHEMISTRY.

June, 2021

DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Aminu Ibrahim and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write-up (Zainab Isah Abdullahi: SPS/16/MBC/00036) were carried out under my supervision.

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ACKNOWLEDGEMENT

I am really grateful to Allah (SWT) WHO blessed me with the optimum ability to conduct this research. My special gratitude to my Supervisor "Dr. Aminu Ibrahim that untirelessly provided me with all the supports needed from a supervisor, which leads to the successful accomplishment of this work. May Allah reward him with endless blessings.

A boundless gratitude to my Husband, Mal. Nasir Shehu, who has motivated me towards and throughout the studies; and provided me with all the supports needed as a rightful partner. May Allah blessed us all.

My special bulk of gratitudes to my well-deserved dedicated FATHER, Alhaji Isah Abdullahi and his family, who is always showering his blessful prayers upon my studies and my entire life with my family. May Allah blessed his family, and reward him with Jannat.

I will never forget the support of my Lecturers in the Department; Dr. Mustapha Huguma, Mal. Aliyu Yunus, and their supporting staffs at the Pharmacology Department of Aminu Kano Teaching Hospital. May Allah's blessings always be with you all.

My gratitudes also to my entire family, friends, neighbours, Lab. Technicians, Lab. Assistants, and to all that provides me with support. May Allah bless us all.

DEDICATION

I dedicated this research work to my own family, my Father in person of Alhaji Isah Abdullahi, and my Late mother Hajiya Maryam Muhammad. May her soul be resting in jannatul firdaus. Really meant more than everything to my life.

TABLE OF CONTENTS

Table of Contents

DECLARATION ii
CERTIFICATION iii
APPROVAL iv
ACKNOWLEDGEMENT v
DEDICATION vi
TABLE OF CONTENTS vii
List of Tables x
List of Figures xi
ABSTRACT xii
CHAPTER ONE 1
1.0 Introduction 1
1.1 Statement of Research Problem 3
1.2 Justification 4
1.3 Aim and Objectives 4
1.3.1 Aim 4
1.3.2 Objectives 5
CHAPTER TWO 6
2.0 Literature Review 6
2.1.0 Oxidative stress 6
2.2 Oxidants and Free Radical Production 6
2.3 Physiological Activities of Free Radicals 7
2.4 Detrimental Effects of Free Radicals on Human Health 8
2.4.1 Cancer and Oxidative Stress 9
2.4.2. Cardiovascular Disease and Oxidative Stress. 10
2.4.3. Neurological Disease and Oxidative Stress. 10
2.4.4. Respiratory Disease and Oxidative Stress. 11
2.4.5. Rheumatoid Arthritis and Oxidative Stress. 11
2.4.6. Kidney Diseases and Oxidative Stress. 11
2.5.0 Terpenoids: 12
2.6.0 Combretum micranthum G. Don 12
2.7.0 Aluminium and oxidative stress: 14
2.7.1 Sources of Aluminium 14
2.7.2 Symptoms of Aluminium Toxicity 14

2.7.3 The effect of Short-term exposure to Aluminium 15
2.7.4 Neurotoxic effect of Aluminium 15
2.7.5 Aluminium and its role in neurodegeneration 17
2.7.6 Effect of Aluminium Toxicity 19
2.7.7 Neurodegenerative diseases: 20
2.7.8 Neurodegenerative Diseases and their effect on Brain 20
2.8 Neuroprotection /Neuroprevention via phytotheraphy 25
2.4.1 Phytochemicals for neuroprotection and regeneration 25
2.4.2 Summary on how antioxidants regulate neurodegeneration 27
CHAPTER THREE 28
3.0 Materials and Methods 28
3.1 Materials 28
3.1.1 Chemicals and reagents 28
3.11 Plant Material Collection and Identification 29
3.12 Animals acquisition and care 29
3.2.0 Methods 29
3.2.1 Preparation of *C. micranthum* extract 29
3.2.2 Extractions 30
3.22.1 Microwave Assisted Extraction (MAE) 30
3.2.3 Qualitative test for terpenoids: 30
3.3 Determination of total Terpenoids: 31
3.4 Fractionation of crude extract (chloroform, methanolic, & ethylacetate fractions): 31
3.5 Invitro antioxidant assays for the chloroform, ethylacetate and methanolic fractions: 31
3.51 DPPH scavenging activity (Monzocco et al 1998 method). 31
3.52 Reducing Power Method (RP) (Oyaizu, 1998 and Jayaprakash et al, 2001 methods). 32
3.6 Experimental design 33
3.7 Tissue collection and preparation of tissue homogenates 34
3.8 Biochemical assay and histopathology of brain homogenate 35
3.81 Determination of Gamma-Aminobutyric (GABA): 35
3.8.2 Super oxide dismutase (SOD): 35
3.8.3 Lipid peroxidation / Malondialdehyde (MDA) assay 36
3.8.4 Histopathology: 36
3.8.5 Phytochemical screening by GC-MS 37
3.9 Statistical analysis 37
3.91 Ethical clearance 37
CHAPTER FOUR 38

4.0 Results and discussion 38
4.1 Results 38
4.1.1 Phytochemical screening of the leaves extract 38
4.2 In vitro antioxidant assays of the 3 of the fractions. 39
4.3 The in vivo assay of some of the brain biochemical markers 43
4.4 GC-MS Analysis of the extract 44
4.5 Results of Histopathology assay: 45
4.6 Discussion 47
CHAPTER FIVE 50
5.0 Summary, Conclusion, and Recommendations 50
5.1 Summary of the results/ findings 50
5.2 Conclusion 50
5.3 Recommendations 51
APPENDIX 52
REFERENCES 53

List of Tables

Table 1 Result of Quantitative Analysis - - - - - - 35

Table 2 Result of IC50 Activity - - - - - - - 36

Table 3 Result of Reducing Power Activity - - - - - 37

Table 4 Result of Reducing Power Assay - - - - - - 38

Table 5 Result of IC50 Values for Reducing Power Assay - - - 39

Table 6 Result of Invivo Assay of Brain Biochemical Markers - - - - 40

Table 7 Result of GCMS Analysis - - - - - - - 41

List of Figures

Figure A-F Results of brain Histopathology - - - - - 42-43

List of Appendix - - - - - - - - 49

ABSTRACT

C. micranthum leaves were dried, and extracted using microwave assisted extraction, and Qualitative phytochemical screening of the extract revealed the presence of Terpenoid, and 206.72mg/g of linolool of Terpenoid was found in the quantitative test. Fractionation of the crude extract by Solvent-solvent extraction was performed using chloroform, methanol, and ethylacetate. In-vitro assay on the fractions detected that the chloroform fraction have higher antioxidant activity by having a high DPPH and reducing power activities by a lower IC50 of -4.19and 25.19 respectively. Invivo assay of SOD, GSH, and GABA levels showed that their levels were significantly increased with a significant decrease in MDA level all in the 100mg/kg + ALCL3 treated mices. The brain histopathology does not show any sign of oxidative stresss or Neurodegeneration in the groups treated with 100mg/kg extract + ALCL3. C. micranthum have a neuroprotective property.

CHAPTER ONE

1.0 Introduction

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. ROS can play, and in fact they do it, several physiological roles (i.e., cell signaling), and they are normally generated as by-products of oxygen metabolism; despite this, environmental stressors (i.e., UV, ionizing radiations, pollutants, and heavy metals) and xenobiotics (i.e antiblastic drugs) contribute to greatly increased ROS production, therefore, causing an imbalance that leads to cell and tissue damage (oxidative stress). However, if not strictly controlled, oxidative stress can be responsible for the induction of several diseases, both chronic and degenerative, which involves diseases like neurological; cardiovascular; respiratory; renal; and cancer, as well as speeding up body aging process and cause acute pathologies (i.e., trauma and stroke).

Several antioxidants have been exploited in recent years for their actual or supposed beneficial effect against oxidative stress, such as vitamin E, flavonoids, and polyphenols. While describing oxidative stress just as harmful for human body, it is true as well that it is exploited as a therapeutic approach to treat clinical conditions such as cancer, with a certain degree of clinical success.

Combretum micranthum G. Don. (C. micranthum) commonly called *kinkeliba* belongs to the family *Combretacaea*. It is a shrubby or tree plant that has other common names in the current African languages:Talli in Fulani, Sereo or Sexo or Keseu in Wolof, Patakaro in Mandingo, kokobe in Bambara, Geza in Hausa, Sesed in Serere, and Ngolobe' in Malinke.' It is widely distributed in savannah regions and some places near coastal areas such as West

Africa, South Africa, and Central Africa particularly in the following countries: Senegal, Mali, Sierra Leone, Guinea, Gambia, Niger, Ghana, Nigeria and Benin. It may grow up to 10 metres in height and has acuminate leaves, its flowers are borne as auxiliary clusters on scaly stalks with small, scaly and four winged fruits. The antioxidant constituents of the *C. micranthum* include flavonoids, tannins, carbohydrates, saponins, terpenoids, and alkaloids (www.phytomania.com).

It is used traditionally in Senegal and Mali for fatigue, liver ailments, headache, convalescence, blood disease, weight loss, cancer, sleep problems, and *C. micranthum* is beneficial for the management of pain and inflammation, diarrhea, cure many ailments in Africa and Asia and has been used as potent antibacterial agent in traditional medicine (Muttaka et al, 2016). It is especially used for fasting by Mourides in Senegal due to its glucose lowering activity. In Nigeria, the leaves are used to treat fever, colic, vomiting, inflammation, diarrhea, skin disease, and to stop bleeding (Abdullahi et al, 2015). Kinkeliba means the "health tree" and the French import kinkeliba and call it "tisane de longue vie" or infusion of long life. The leaves extract of the plants has been demonstrated to contain a range of polyphenol compounds and many antioxidants. These compounds are known for antioxidative activities that may offer protection from free radical damage (Welch et al; 2017).

1.1 Statement of Research Problem

Oxidative stress has been very common nowadays, it affects body organs (including the brain), causing diseases, hence, becoming detrimental to human health. Aluminium is the most abundant element in the atmosphere in our foods and environment to the extent that exposure to it cannot be prevented, and it was found by many researches to be an agent that produces free radicals, leading to the oxidative stress. However, life is really stressful nowadays, curing oxidative stress using pharmaceutical drugs is expensive and associated with many side effects, which made many people to avoid it including those that can afford, so, making life miserable. This rises the urge of conducting this research, to detect whether the leaves of the selected plant of known antioxidant activity, harmless/not toxic can be effective in protecting the body from oxidative stress (Aluminium induced).

1.2 Justification

Oxidative stress and free radicals are generally known to be detrimental to human health. A large amount of studies demonstrates that in fact free radicals contribute to initiation and progression of several pathologies, ranging from CVD to cancer. Antioxidants, as class of compounds able to counteract oxidative stress and mitigate its effects on individuals' health, gained enormous attention from the biomedical research community, because these compounds not only showed a good degree of efficacy in terms of disease prevention and/ or treatment but also because of the general perception that they are free from important side effects. If it is true that antioxidants can be very useful in preventing, managing, or treating human pathologies, it is true as well that they are not immune to generating adverse effects (Gabriel P. et al, 2017).

Combrethum micranthum leaves extract are known for antioxidant activities that may offer protection from free radical damage (Welch et al, 2017).

With these, this research intended to study whether methanolic terpenoid extract of combrethum micranthum leaves have a powerful antioxidant effect that can provide a protective effect against Aluminium-induced oxidative stress (neurotoxic effect) on the brain of laboratory mice.

1.3 Aim and Objectives

1.3.1 Aim

The present study is aimed to investigate the effect of *Combretum micranthum* leaves terpenoid extract on aluminium induced oxidative stress in brain tissue of laboratory mice.

1.3.2 Objectives

a. To perform phytochemical screening on methanolic extract of *C. miranthum* leaves.
b. To fractionate the methanolic leaves extract using solvent-solvent extraction, and determine the fraction with the best antioxidant activity via invitro antioxidant assay (DPPH and Reducing power assays).
c. To determine the effect of the best antioxidant activity fraction (invivo assay) on some biochemical parameters and oxidative stress markers (GABA, SOD, MDA, and GSH) of the brain tissue; and morphology of the brain.
d. To characterize the bioactive compounds in the best activity fraction using GCMS (Gas Chromatography Mass Spectrometry).

CHAPTER TWO

2.0 Literature Review

2.1.0 Oxidative stress

ROS are mainly produced by mitochondria, during both physiological and pathological conditions, that is, O_2 •− can be formed by cellular respiration, by lipoxygenases (LOX) and cyclooxygenases (COX) during the arachidonic acid metabolism, and by endothelial and inflammatory cells (Al-Gubory K. H. A., 2012). Despite the fact that these organelles have an intrinsic ROS scavenging capacity, it is worth to note that this is not enough to address the cellular need to clear the amount of ROS produced by mitochondria (Glasauer and Chandel, 2014). Cells deploy an antioxidant defensive system based mainly on enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to protect themselves from ROS-induced cellular damage. (Deponte M., 2013).

Superoxide radicals (O2 •−), hydrogen peroxide (H2O2), hydroxyl radicals (•OH), and singlet oxygen (1O2) are commonly defined reactive oxygen species (ROS); they are generated as metabolic by-products by biological systems (Sato H., 2013). Processes, like protein phosphorylation, activation of several transcriptional factors, apoptosis, immunity, and differentiation, are all dependent on a proper ROS production and presence inside cells that need to be kept at a low level. When ROS production increases, they start showing harmful effects on important cellular structures like proteins, lipids, and nucleic acids (Wu Q. J. et al, 2013).

2.2 Oxidants and Free Radical Production

ROS production basically relies on enzymatic and nonenzymatic reactions. Enzymatic reactions able to generate ROS are those involved in respiratory chain, prostaglandin synthesis,

phagocytosis, and cytochrome P450 system (Kumar and Pandey, 2015).

Superoxide radical (O_2 •−) is generated by NADPH oxidase, xanthine oxidase, and peroxidases. Once formed, it is involved in several reactions that in turn generate hydrogen peroxide, hydroxyl radical (OH•), peroxynitrite (ONOO−), hypochlorous acid (HOCl), and so on. H_2O_2 (a nonradical) is produced by multiple oxidase enzymes, that is, amino acid oxidase and xanthine oxidase. Hydroxyl radical (OH•), the most reactive among all the free radical species in vivo, is generated by reaction of O_2 •− with H_2O_2, with Fe^{2+} or Cu^{+} as a reaction catalyst (Fenton reaction). Nitric oxide radical (NO•), which plays some important physiological roles, is synthesized from arginine-to-citrulline oxidation by nitric oxide synthase (NOS) (Kumar S and A. K. Pandey, 2013). Even nonenzymatic reactions can be responsible for free radical production, that is, when oxygen reacts with organic compounds or when cells are exposed to ionizing radiations. Nonenzymatic free radical production can occur as well during mitochondrial respiration (Droge W., 2002). Free radicals are generated from both endogenous and exogenous sources. Immune cell activation, inflammation, ischemia, infection, cancer, excessive exercise, mental stress, and aging are all responsible for endogenous free radical production. Exogenous free radical production can occur as a result from exposure to environmental pollutants, heavy metals (Cd, Hg, Pb, Fe, and As), certain drugs (cyclosporine, tacrolimus, gentamycin, and bleomycin), chemical solvents, cooking (smoked meat, used oil, and fat), cigarette smoke, alcohol, and radiations. When these exogenous compounds penetrate the body, they are degraded or metabolized, and free radicals are generated as by-products (Valko M., 2006).

2.3 Physiological Activities of Free Radicals

When maintained at low or moderate concentrations, free radicals play several beneficial roles for the organism. For example, they are needed to synthesize some cellular structures and to be used by the host defense system to fight pathogens. In fact, phagocytes synthesize and store free radicals, in order to be able to release them when invading pathogenic microbes have to be destroyed (Valko M., 2006). The pivotal role of ROS for the immune system is well exemplified by patients with granulomatous disease. These individuals are unable to produce O2 •− because of a defective NADPH oxidase system, so they are prone to multiple and in most of the cases persistent infections (Droge W., 2002). Free radicals are also involved in a number of cellular signaling pathways. They can be produced by non-phagocytic NADPH oxidase isoforms; in this case, free radicals play a key regulatory role in intracellular signaling cascades, in several cell types such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue. Probably, the most well-known free radical acting as a signaling molecule is nitric oxide (NO). It is an important cell-to-cell messenger required for a proper blood flow modulation, involved in thrombosis, and is crucial for the normal neural activity (Patcher P., 2007). NO is also involved in nonspecific host defense, required to eliminate intracellular pathogens and tumor cells. Another physiological activity of free radicals is the induction of a mitogenic response.

2.4 Detrimental Effects of Free Radicals on Human Health

As stated before, if in excess, free radicals and oxidants give rise to a phenomenon known as oxidative stress; this is a harmful process that can negatively affect several cellular structures, such as membranes, lipids, proteins, lipoproteins, and deoxyribonucleic acid (DNA) (Valko M., 2006). Oxidative stress emerges when an imbalance exists between free radical formation and the capability of cells to clear them. For instance, an excess of hydroxyl radical and peroxynitrite can cause lipid peroxidation, thus, damaging cell membranes and lipoproteins. This in turn will lead to malondialdehyde (MDA) and conjugated diene compound formation, which are known to be cytotoxic as well as mutagenic. Being a radical chain reaction, lipid peroxidation spreads very quickly affecting a large amount of lipid molecules. Proteins may as well be damaged by oxidative stress, undergoing to conformational modifications that could determine a loss, or an impairment, of their enzymatic activity (Halliwell B., 2007). Even DNA is prone to oxidative stress-related lesions, the most representative of which is the 8-oxo-2′-deoxyguanosine (8-OHdG) formation; this is a particularly pernicious DNA lesion, which can be responsible for both mutagenesis, as pointed out by Nishida et al, 2013. It can also cause a loss in the epigenetic information, probably due to an impairment in CpG island methylation asset in gene promoters. It is worth to note that Valavanidis T. et al, 2013, have already proposed 8-OHdG levels in a tissue as biomarker of oxidative stress. Of course cells can put in place several mechanisms, such as the base excision repair (BER) or antioxidants, as defense response against DNA lesions. If not strictly controlled, oxidative stress can be responsible for the induction of several diseases, both chronic and degenerative, as well as speeding up body aging process and cause acute pathologies (i.e., trauma and stroke).

2.4.1 Cancer and Oxidative Stress.

Cancer onset in humans is a complex process, which requires both cellular and molecular alterations mediated by endogenous and/or exogenous triggers. It is already well known that oxidative DNA damage is one of those stimuli responsible for cancer development (Droge W., 2002). Cancer can be driven and/or promoted by chromosomal abnormalities and oncogene activation determined by oxidative stress. Hydrolyzed DNA bases are common byproducts of DNA oxidation and are considered one of the most relevant events in chemical carcinogenesis. The formation of such kind of adducts impairs normal cell growth by altering the physiological transcriptomic profile and causing gene mutations. Oxidative stress can also cause a variegated amount of modifications against DNA structure, for example, base and sugar lesions, DNA-protein crosslinks, strand breaks, and base-free sites. For instance, tobacco smoking, environmental pollutants, and chronic inflammation are sources of oxidative DNA damage that could contribute to tumor onset (Pizzino G. et al, 2014). Oxidative stress resulting from lifestyle reasons can also play an important role in cancer development, as suggested by the strong correlation between dietary fat consumption (a factor that exposes the organism at greater risk of lipid peroxidation) and death rates from different types of cancer (Droge W., 2002).

2.4.2. Cardiovascular Disease and Oxidative Stress.

Cardiovascular diseases (CVDs) are clinical entities with a multifactorial etiology, generally associated with a very large amount of risk factors, the most broadly recognized of which are hypercholesterolaemia, hypertension, smoking habit, diabetes, unbalanced diet, stress, and sedentary life. During the last years, research data pointed out that oxidative stress should be considered either a primary or a secondary cause for many CVDs. Oxidative stress acts mainly as a trigger of atherosclerosis. It is well known that atheromatous plaque formation results from an early endothelial inflammation, which in turn leads to ROS generation by macrophages recruited in situ. Circulating LDL are then oxidized by reactive oxygen species, thus leading to foam cell formation and lipid accumulation. The result of these events is the formation of an atherosclerotic plaque. Both in vivo and ex vivo studies provided evidences supporting the role of oxidative stress in atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure (Chatterjee M. et al, 2007)

2.4.3. Neurological Disease and Oxidative Stress.

Oxidative stress has been linked to several neurological diseases (i.e., Parkinson's disease, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis, depression, and memory loss). In AD, several experimental and clinical researches showed that oxidative damage plays a pivotal role in neuron loss and progression to dementia. *β*-

amyloid, a toxic peptide often found present in AD patients' brain, is produced by free radical action and it is known to be at least in part responsible for neurodegeneration observed during AD onset and progression (Navarro J. et al, 2014).

2.4.4. Respiratory Disease and Oxidative Stress.

Several researches pointed out that lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), determined by systemic and local chronic inflammation, are linked to oxidative stress (Caramori and Papi, 2004). Oxidants are known to enhance inflammation via the activation of different kinases involving pathways and transcription factors like NF-kappa B and AP-1.

2.4.5. Rheumatoid Arthritis and Oxidative Stress.

Rheumatoid arthritis is a chronic inflammatory disorder affecting the joints and surrounding tissues, characterized by macrophages and activated T cell infiltration (Valko M. et al 2007). Free radicals at the site of inflammation play a relevant role in both initiation and progression of this syndrome, as demonstrated by the increased isoprostane and prostaglandin levels in synovial fluid of affected patients.

2.4.6. Kidney Diseases and Oxidative Stress.

Oxidative stress is involved in a plethora of diseases affecting renal apparatus such as glomerulo- and tubule-interstitial nephritis, renal failure, proteinuria, and uremia (Droge W., 2002). The kidneys are negatively affected by oxidative stress mainly because of the fact that ROS production induces the recruitment of inflammatory cells and proinflammatory cytokine production, leading to an initial inflammatory stage. In this early phase, a predominant role is played by TNF-alpha and IL-1b, as proinflammatory mediators, as well as by NF-κB as transcriptional factor required to sustain the inflammatory process. The latter stage is characterized by an increase in TGF-beta production, which orchestrates the extracellular matrix synthesis. So, when the oxidative stress stimuli act chronically on kidney tissues, the results will be an initial stage of inflammation and later the formation of abundant fibrotic tissue that impairs organ function potentially leading to renal failure. Certain drugs, such as cyclosporine, tacrolimus, gentamycin, and bleomycin, are known to be nephrotoxic mainly because of the fact that they increase free radical levels and oxidative stress via lipid peroxidation (Galle J., 2001). Heavy (Cd, Hg, Pb, and As) and transition metals (Fe, Cu, Co, and Cr), acting as powerful oxidative stress inducers, are responsible for various forms of nephropathy, as well as for some types of cancers (Valko M. et al, 2005 and Valko M. et al, 2006).

Generally, these all can affirm that oxidative stress and free radicals are confirmed to be responsible for several pathological conditions affecting different tissues and systems, thus being one of the most important and pervasive harms to human health.

2.5.0 Terpenoids:

Terpenoids are organic compounds that are produced from the oxidation of terpenes. Plant terpenoids are used for their aromatic qualities and play a role in traditional herbal remedies. They contribute to the scent of eucalyptus, the flavours of cinnamon, cloves, and ginger, the yellow in sun flowers, and the red colour of tomatoes.

Plant terpenoids (isoprenoids) are biosynthesized from C-5 precursors (isoprene units) by the action of prenyl transferases and terpenoid synthase, and oxidation by cytochrome P450.

2.6.0 Combretum micranthum G. Don

2.6.1 Antioxidant Capacity of *C. micranthum G. Don*:

Ethanol extract of *C. micranthum G. Don* leaves is reported to be rich in polyphenols (tannins, flavonoids and other components) constituents known to possess various beneficial pharmacological properties such as antioxidant, neuromodulator, anti-mutagenic, anticarcinogenic, hypolipidemic and cardioproprotective activities that may offer protection from free radical damage (Chika and Bello, 2010). Mira et al (2002) study revealed that polyphenolic compounds in the *C. micranthum G. Don* function as effective antioxidants by quenching the free radicals of biological systems with their phenolic ring and multiple hydroxyl moieties; phenolic activity covers a wide range of reactive oxygen, nitrogen, and chlorine species such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. Polyphenols can also chelate metal ions leading to a decrease in metal ion pro-oxidant activity. Free radicals cause oxidative damage to nucleic acids, proteins, and lipids and this oxidation of biological macromolecules has now been strongly associated with the development of many physiological diseases: Alzheimer's, Parkinson's, diabetes, atherosclerosis, and carcinogenesis (Paya et al, 1992; and Pham-Huy, 2008). The attack of free radicals against the body is known as oxidative stress and while the human body does generate its own enzymatic antioxidants, such as superoxide dismutase, catalase, and peroxidase, it does not provide enough protection against oxidative stress. Many studies have shown that consuming proper quantities of antioxidants can slow oxidative stress and subsequently prevent the diseases that may develop from the excessive oxidation (*Soory, 2009*).

The main physiological symptoms of degenerative diseases include elevated oxidative/nitrosative stress, mitochondrial dysfunction, protein misfolding/aggregation, synapse loss, and decreased neuronal survival. When neurons and immune cells are exposed to toxic proteins, a large amount of energy is needed to defend against the accumulated oxygen and nitrogen species that induce stress in the surrounding environment. This results in mitochondrial malfunction with the release of cytochrome C and other mitochondrial proteins, which pave the way towards apoptosis. This overabundance of protein aggregation affects cellular signalling and neuronal function and is a key cause of neuronal loss (Ramu et al, 2015).

Traditional herbs and phytochemicals may delay the onset of neurodegenerative diseases and slow its progression and also allow recovery by targeting multiple pathological causes by antioxidative, anti-inflammatory, and antiamyloidogenic properties. These properties also enabled the phytochemicals to regulate mitochondrial stress, apoptotic factors, free radical scavenging system, and neurotrophic factors (Ramu et al, 2015).

2.7.0 Aluminium and oxidative stress:

2.7.1 Sources of Aluminium

Food related uses of Aluminium compounds include preservatives, fillers, coloring agents, anti-caking agents, emulsifiers and baking powders. Soy-based infant formula which contain aluminium-based food additives is also high in aluminum (WHO, 1997). Aluminum can be found in such everyday items as: aluminum foil and tin foil, animal feed, antacids, anti-perspirants, baking, powders, beverages in aluminum cans (soda, beer, juice), bleached flour, ceramic plates (leaded glazes), cigarette filters, color additives, construction materials, cookware, cosmetics, dental amalgams, deodorant stones and crystals, fluoridated water increases leaching of aluminium from aluminium pots and pans, insulated wiring, milk products, nasal spray, pesticides, vaccines (it is used as a preservative in many vaccines), tap water (Aluminum is added to many municipal water supplies), tobacco smoke, and toothpaste, (ASTDR, 2008).

2.7.2 Symptoms of Aluminium Toxicity

Aluminum toxicity presents itself in stages. Early symptoms include flatulence, headaches, colic, dryness of skin and mucous membranes, a tendency for colds, burning pain in the head relieved by food, heartburn and an aversion to meat. Later symptoms include paralytic muscular conditions, loss of memory, confusion and various forms of dementia.

2.7.3 The effect of Short-term exposure to Aluminium

Wistar rats exposed to aluminium chloride in their drinking-water at reported aluminium doses of 5 and 20 mg/kg of body weight for 6 months showed reduced body weight and reduced erythrocyte counts and associated parameters, but there were no clear dose–response relationships (Somova & Khan, 1996). Results of histopathological examinations indicated spongiform changes and neurofibrillary degeneration in the hippocampus and atrophy and fibrosis in the kidney at 20 mg/kg of body weight (Somova, Missankov & Khan, 1997).

2.7.4 Neurotoxic effect of Aluminium

Behavioural impairment has been reported in laboratory animals exposed to soluble aluminium salts (e.g. lactate, chloride) in the diet or drinking-water in the absence of overt encephalopathy or neuro-histopathology. Both rats (Commissaris et al, 1982; Thorne et al., 1987; Connor et al, 1988) and mice (Yen-Koo, 1992) have demonstrated such impairments at doses exceeding 200 mg of aluminium per kilogram of body weight per day. Although significant alterations in acquisition and retention of learned behaviour were documented, the possible role of organ damage (kidney, liver, immunological) due to aluminium was incompletely evaluated in these studies (WHO, 1997).

Following the observation that high levels of aluminium in dialysis fluid could cause a form of dementia in dialysis patients, a number of studies were carried out to determine if aluminium could cause dementia or cognitive impairment as a consequence of environmental exposure over long periods. Aluminium was identified, along with other elements, in the amyloid plaques that are one of the diagnostic lesions in the brain for Alzheimer disease, a common form of senile and pre-senile dementia. Numerous epidemiological studies have been carried out to try to determine the validity of this hypothesis. These have been reviewed in detail by several authorities, including JECFA (FAO/WHO, 2007; WHO, 2007), the United Kingdom Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT, 2005), the United States Agency for Toxic Substances and Disease Registry (ATSDR, 2008) and Environment Canada & Health Canada (2010).

Neurotoxicity of Al is known to result in impairment of learning memory and cognitive functions both from clinical observations and from animal experiments (Ashall and Goate, 1994). Aluminum has been implicated in human neurodegenerative diseases and it has been linked etiologically and epidemiologically to several neurological conditions including Alzheimer's disease (Solfrizzi *et al.*, 2006; Santibanez *et al*, 2007). Various investigations have suggested that Alzheimer's disease is more common in areas where Al content of water supplies is the highest (Christen, 2000). Indeed, the brain is a target of Al toxicity which can alter blood brain barrier (BBB) mediating Al transport to the brain and gets deposited in the cortex and hippocampus by altering the physiological ligands present at these barriers in states (Yokel, 2000). Aluminium also forms stable complexes with aspartic and glutamic acids, which cross the blood brain barrier, and then are deposited in the brain (Deloncle *et al.*, 1999). As a consequence, glutamic acid is not sufficiently available to form glutamine, thereby, ammonia is accumulated in the brain and exerts toxic effects leading to neuronal death and may affect each and every neurotransmitter system (El-Rahman, 2003).

Al is known to interfere with cholinergic neurotransmission (Amador *et al.*, 2001), glutamatergic neurotransmission (Platt *etal.*, 1994) and gamma-aminobutyric acid neurotransmission (Cordeiro *et al.*, 2003). In the mammalian brain, Al affects the synthesis, storage and transport of central neurotransmitter systems such as dopamine, serotonin, adrenalin, gamma amino butyric acid (GABA) and glutamate (Palmer and Dekosky, 1993). The molecular mechanisms behind Al neurotoxicity are not clear and many hypotheses have been suggested. These hypotheses include exacerbation of oxidative stress (Kaneko *et al*., 2007), disruption of calcium homeostasis (Kaur and Gill, 2005) and impairment of intracellular signal transduction pathways (Shafer and Mundy, 1995).In the central nervous system, neurotransmission is coupled to learning and memory, therefore changes in the neurotransmission would certainly affect the behavioural responses (Bhalla *et al*., 2010). The metabolism of neurotransmitters is altered in response to Al exposure; these alterations seem to be brain region specific and dependent on homeostasis of ion and energy conduction and on protein expression.

In another investigation, glutamate receptors are putative sites of action in Al neurotoxicity (Platt *et al*., 1994). Oxidative stress resulted from Al exposure elevates CNS glutamate levels by stimulating the activity of N-methyl-D-aspartate (NMDA) receptors and the pro-oxidant effects of Al alters the physical properties of membrane interfering with the functioning of voltage-activated ionic channels and alters the secretion of neurotransmitters (Donald *et al*., 1989). As an inter-relationship between glutamate and oxidative stress the elevated glutamate release can lower intracellular GSH levels (Kowluru *et al*., 2001). GSH depletion has been found to occur during apoptosis mediated glutamate neurotoxicity (Almeida *et al*., 1998).

In point of fact, neurotransmitters balance is a multifaceted process that may involve contributions from other cells rather than from presynaptic cells alone. In the presence of Al, the uptake of neurotransmitters was found to be below control levels, with some exceptions (Goncalves and Silva, 2007).

2.7.5 Aluminium and its role in neurodegeneration

Aluminium (aluminium) is the most abundant metallic element and constitutes about 8% of Earth’s crust. It occurs naturally in the environment as silicates, oxides and hydroxides, combined with other elements, such as sodium, chloride, and fluoride, and as complexes with organic matter. It is a highly neurotoxic element and has been suggested to play a role in degeneration of nerve cells in the brain of human and experimental animals (Yumoto *et al*., 2001).

2.7.6 Effect of Aluminium Toxicity

Aluminum toxicity can cause a wide array of disorders spanning multiple health systems. It is important to recognize how greatly our bodies can be affected by this one toxic metal. Some of which include:

(i) *Nervous system:*

In animal studies, aluminum blocks the action potential or electrical discharge of nerve cells, reducing nervous system activity. Aluminum also inhibits enzymes in the brain (Na-K-ATPase and hexokinase). It may also inhibit uptake of important chemicals by nerve cells (dopamine, norepinephrine, and 5-hydroxytryptamine). (Palmer and Dekosky, 1993).

(ii) *Behavioral effects*:

Dementia resulting from kidney dialysis related to aluminum toxicity causes memory loss, loss of coordination, confusion, and disorientation.

Some of diseases and health issues that can be caused by aluminum toxicity:

Aluminum toxicity causes memory loss, loss of coordination, confusion, and disorientation; Alzheimer's disease, amyotrophic lateral sclerosis, anemia and other blood disorders, appetite loss, autism spectrum disorders, behavioral problems, breast cancer, learning delays and disabilities, colds, colic, colitis, confusion, constipation, dementia, dental cavities, dental caries, dry mouth, dry skin, energy loss, excessive perspiration, fatigue, flatulence, headaches, heartburn, hyperactivity, hypoparathyroidism, inhibition of enzyme systems, kidney and liver dysfunctions, lowered immune function, memory loss, neuromuscular disorders, numbness, osteomalacia, osteoporosis, paralysis, parkinson's disease, peptic ulcers, psychosis, reduced intestinal activity, senility, skin problems, spleen pain stomach pain, ulcers, weak and aching muscles.(Wendy and Greenfield, 2018).

2.7.7 Neurodegenerative diseases:

These are disorders in which nerve cells from brain and spinal cord are lost leading to either functional loss (ataxia) or sensory dysfunction (dementia), mitochondrial dysfunctions and excitotoxicity and finally apoptosis. They are progressive disorders of central nervous system that affect the central and peripheral nervous systems. Gradual and progressive loss of neural cells may lead to neurodegeneration. Many factors are known to play a direct role in the initiation of neurodegeneration; free radical formation by the Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is one of the main causative factors. Neuro-inflammatory process is known to play a crucial role in the progress of several neurodegenerative diseases. According to the reports of the National Institute of Neurological Disorders and Stroke, there were more than 600 neurological disorders recorded worldwide (Meek et al, 1998).

Neurodegenerative diseases are estimated to be the second most common cause of death among elderly by the 2040s (Ansari *et al.,* 2010). Many neuropsychiatric and neurodegenerative disorders, such as Alzheimer's disease, anxiety, cerebrovascular impairment, depression, seizures, Parkinson's disease and organs defects or dysfunction are appearing in the current era due to stressfull lifestyle and oxidative stress. Among the strategies for protection against these disorders, phytochemicals may represent a valuable remedy in preventing neurodegenerative diseases. Treatment of these disorders with prolonged administration of synthetic drugs will lead to severe side effects. (Phani et al, 2015).

2.7.8 Neurodegenerative Diseases and their effect on Brain

Neurodegenerative diseases are progressive disorders of central nervous system that affect the central and peripheral nervous systems. Gradual and progressive loss of neural cells may lead to neurodegeneration, and during the neurodegenerative state, there would be decreased activities of antioxidant enzymes such as SOD, CAT, GPx, and GSH, which signify the role of antioxidants in neuroprotection.

Many factors are known to play a direct role in the initiation of neurodegeneration, free radical formation by the Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is the main causative factor. Neuro-inflammatory process is known to play a crucial role in the progress of several neurodegenerative diseases.

According to the reports of the National Institute of Neurological Disorders and Stroke, there were more than 600 neurological disorders recorded worldwide (Meek et al; 1998). Some of them were briefly discussed in this review at length.

(i) Anxiety

Anxiety is characterised by psychological and physiological state in which cognitive, somatic, emotional, and behavioral components are involved. It can increase to an extent that may interfere with even normal routine of life and person may feel apprehensive regarding happenings of normal things in life. Anxiety disorders comprise seven clinical conditions (Pathak et al; 2011) such as (a) Generalized Anxiety Disorder (GAD): In this condition person will have persistent fear and worry and become overly concerned with everyday matters. (b) Panic disorder: Person suffers from brief attacks of intense terror and apprehension, often marked by confusion, dizziness, trembling, shaking, nausea, difficulty breathing. (c) Phobias: In which fear and anxiety is triggered by a specific stimulus or situation. Agoraphobia: It is specific anxiety about being in a place or situation where escape is difficult or embarrassing or where help may be unavailable. (d)Social Anxiety Disorder

(SAD): It is an intense fear of negative public scrutiny or of public embarrassment or humiliation. (e) Obsessive-Compulsive Disorder (OCD): It is primarily characterized by repetitive obsessions and compulsions. (f) Post-Traumatic Stress Disorder (PTSD): It resulted from an extreme situation, such as major accident, child abuse, war situation, natural disaster, rape, hostage situations, etc. (g) Separation anxiety disorder: It is the feeling of inappropriate or excessive levels of anxiety over being separated from a place or person. Monoamines (dopamine, noradrenaline and serotonin), neuropeptides (galanin, neuropeptide Y, arginine vasopressin, tackykinin), neurosteroids and cytokines have been observed to play a modulators role in anxiety states.

(ii) Attention Deficit/ Hyperactivity Disorder

Attention Deficit/Hyperactivity Disorder (ADHD) is a loosely defined assemblage of neuropsychiatric symptom clusters that emerge in childhood and often persist into adulthood. ADHD is observed with increasingly employed as a diagnostic label for individuals who display a wide range of symptoms, such as inability to stay focused, restlessness, mood swings, temperament, problems in completing tasks, disorganization and inability to cope with stress (Brue et al; 2002).

(iii) Depression

The term depression indicates a dampened mood and pervasive unhappiness. Depression is one of the most common emotional disorders, it may be manifested in varying degrees from feelings of slight sadness to utter misery and dejection. Changes in mood and the occurrence of depressive symptoms are determined by the biological as well as an environmental factor. According to World Health Organization (WHO), approximately 450 million people are suffering from a mental or behavioural disorder, and is predicted to increase significantly by 2020. Depression is one of the top ten causes of morbidity and mortality worldwide based on a survey by the WHO. In depressed conditions, there are several structural alterations of neurons that will take place such as, a decreased volume of the frontal cortex and hippocampus, dysfunctions of HPA axis, abnormalities in 5-HT and its receptors (Brue et al; 2002). It is well established that the 5-HT is a monoaminergic neurotransmitter which is also dysregulated in other neurological disorder conditions such as, anxiety and schizophrenia (Brue et al; 2002).

(iv) Dementia

Parkinson'sDisease (PD) is a serious motor disorder and the most common brain degenerative disease in humans. The loss of dopaminergic neurons in the substantia nigra is the most pervasive factor to contribute the characteristic symptoms of PD. It is characterized by four main symptoms: tremor, rigidity, bradykinesia and impairment of balance. The classic pathological findings are the presence of Lewy bodies in the substantia nigra, and loss of nerve cells in portions of its ventral tier (Drevets et al 1999). Besides the neuro-pathologic symptoms, PD can be caused neuro-chemically by a consistent deficit in cholinergic neurotransmission. Alzheimer's disease (AD) is a neurodegenerative disorder, which is characterized by deficit, learning and memory loss followed by cognitive disorders like depression, agitation and psychosis.

(v) Epilepsy

Epilepsy is the most common neurological disorder, which affects almost 50 million worldwide (Scheuer and Pedley, 1990). It is a characterized by unpredictable occurrence of periodic epileptic seizures, i.e. involuntary contraction of striated muscle repeatedly. Seizures take place due to excessive and rapid discharge of cerebral neurons in the gray matter of the brain. In epilepsy, impaired voltage-sensitive sodium and calcium-channel functioning, impaired GABA-mediated inhibition and excessive glutamate-mediated neurotransmission may trigger a cascade of events leading to neuronal damage and cell death.

(vi) Excitotoxicity

Excitotoxicity plays a key role in intoxication of some poisons like domoic acid (Amnesic shellfish poison by marine algae), kainic acid, etc. Excitotoxicity is the pathological process by which nerve cells are damaged and killed by excessive glutamate stimulation. Excitotoxicity may also express in different pathological conditions like, spinal cord injury, traumatic brain injury, multiple sclerosis, stroke, amyotrophic lateral sclerosis, and other neuronal disorders (Doble, 1999). It is also involved in most aspects of normal brain functions including cognition, memory, and learning. Excitotoxicity can occur through over-activation of the NMDA receptor with the subsequent influx of Ca2+, activation of both noise and iNOS, and excess generation of NO (Albin and Greenamyre, 1992).

(vii) Schizophrenia

It is one of the most important forms of psychiatric illness. In this condition the patients don't know what is happening at present. The symptoms of this disease are 2 types:

1. Positive symptoms: delusions, hallucination, thought disorders, abnormal behavior.
2. Negative symptoms: withdrawal from social contact, flattening of emotional responses. The level of neurotransmitters such as dopamine, 5-HT, acetylcholine and norepinephrine levels upregulate in the brain in this condition (Kumar et al; 2006).

2.8 Neuroprotection /Neuroprevention via phytotheraphy

Neuroprotection refers to the strategies and relative mechanisms able to struggle down the Central Nervous System (CNS) against neuronal damage caused by various neuropsychiatric and neurodegenerative disorders such as Alzheimer’s disease, anxiety, cerebrovascular impairment, seizures, Parkinson’s disease, etc. Neurodegenerative diseases are estimated to be the second most common cause of death among elderly by the 2040s. Among the strategies for neuroprotection, phytochemicals may represent a valuable remedy in preventing neurodegenerative diseases. Many categories of natural and synthetic neuroprotective agents have been reported, however, synthetic neuroprotective agents are believed to have certain side effects such as dry mouth, tiredness, drowsiness, sleepiness, anxiety or nervousness, difficulty with balance, etc (Phani et al, 2015). Herb based medicated products have drawn considerable awareness from research bases and industries in recent years at the national and international levels. Hence, there has been intense interest focused on the part of potential phytochemicals to modulate neuronal function and protective mechanism against neurodegeneration. As complementary and alternative therapy, herbal medicine or phytotherapy, refers to the medical utilization of plant components (leaves, stems, roots, flowers, fruits and seeds) for their curative properties. Generally, herbal products contain a variety of bioactive phytochemicals, including alkaloids, steroids, terpenoids, saponins, phenolics, flavonoids, etc. and it is difficult to specify which part of the herb has biological activity for special discourse. Researchers around the globe are searching for bioactive phytochemicals from herbs which are being used as neuroprotective in traditional medicine like Chinese medicinal system, Indian Ayurvedic medicine system, Korean system of medicine, Mediterranean system of medicine, etc. (Phani et al, 2015).

2.4.1 Phytochemicals for neuroprotection and regeneration

Many neuropsychiatric and neurodegenerative disorders, such as Alzheimer's disease, anxiety, cerebrovascular impairment, depression, seizures, Parkinson's disease, etc. are appearing in the current era due to the stressfull lifestyle. Treatment of these disorders with prolonged administration of synthetic drugs will lead to severe side effects. In recent years, scientists have focused the attention of research towards phytochemicals to cure neurological disorders. Nootropic herb refers to the medicinal role of various plants/parts for their neuroprotective properties by the active phytochemicals including alkaloids, steroids, terpenoids, saponins, phenolics, flavonoids, etc. Phytocompounds from medicinal plants play a major part in maintaining the brain's chemical balance by acting upon the function of receptors for the major inhibitory neurotransmitters. (Phani et al 2015).

Phytochemical based antioxidants may have neuro-protective and neuro-regenerative roles by reducing or reversing cellular damage and by slowing progression of neuronal cell loss (Moosmann and Behl; 2002). In nature, antioxidants are grouped as endogenous or exogenous, the endogenous group includes enzymes like Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and several proteins like albumin, ceruloplasmin, haptoglobin and transferrin. The most important exogenous antioxidants are dietary phytochemicals (polyphenols, phenolic acids, flavonoids, terpenoids, saponins etc.) and vitamins (ascorbic acid, alpha-tocopherol, and beta-carotene) (Berger et al; 2005). Antioxidants offer a promising approach in the control or slowing down progression of several neurodegenerative disorders leading to changes in cerebrovascular blood flow such as, Alzheimer’s disease, amyotrophic lateral sclerosis, Huntington’s disease, ischaemic stroke, haemorrhagic stroke and Parkinson’s disease (Berger et al; 2005). In normal condition, a steady-state exists between pro-oxidants and antioxidants. However, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress promotes consequential severe damage to cellular machinery. Oxidative stress is associated with dysfunction in mitochondrial and endoplasmic reticulum, which includes apoptosis and protein misfolding in neurons. During neurodegenerative state, there would be decreased activities of antioxidant enzymes such as SOD, CAT, GPx, and GSH, which signify the role of antioxidants in neuroprotection (Phani et al, 2015).

2.4.2 Summary on how antioxidants regulate neurodegeneration

Overall, Phani et al (2015), revealed that phytochemicals provide an effective way of halting neurodegenerative disease. Phytochemicals and derivatives such as 3,7-dihydroxy-2,4,6-trimethoxy-phenanthrene, diosniposide B, lignan derivatives, ginkgolide B, 4,6-dimethoxyphenanthrene-2,3,7-triol, spicatoside A, ginsenoside Rg3, limonoid derivatives, quercetin, cyanidin-3-*O*-β-glucopyranoside, clerodane diterpenoids, apigenin derivatives, and quinic acid derivatives induce neuronal cell differentiation and upregulate neurotrophic factors such as nerve growth factor(NGF) and brain derived neurotrophic factor (BDNF). These compounds may have the potential to prevent and arrest neurodegeneration by inducing neurotrophic factors and by boosting the activity of certain components of the antioxidant system, such as superoxide dismutase (SOD) and catalase. They may also inhibit the production of reactive oxygen species (ROS) and inflammatory mediators such as nitric oxide (NO), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF- κB), interleukin (IL)-1β, intrinsic nitric oxide synthase (iNOS), and prostaglandin (PG)E . NGF triggers the tropomyosin-related kinase A (TrkA) signaling pathway by inhibiting caspase protein expression and via degradation of beta amyloid oligomers in the brain. In general, polyphenols activate neurotrophins and have antioxidative and antiapoptotic activities in neurons.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

All chemicals and reagents used in this work were of analytical grade and purchased from Ado Jones laboratory chemicals and equipments Ltd. The laboratory equipments were also of standard quality, and were obtained from the Bayero University Kano Biochemistry and Biotechnology laboratory; and Multi-user science research Laboratory (Ahmadu Bello University, Zaria) These are: HiNaRi Microwave Appliance (Model No. : MX 120BTC, 240V, 2450MHz, 1350Watts) made in Korea, water bath, separating funnel, beakers (250ml, 500ml, 1000ml), retort and tripod stands, hand gloves, filter paper, siever, measuring cylinders, glass test tubes, foil paper, spectrophotometer, rotary evaporator, pipettes, test tube racks, mini weighing scale, methanol, chloroform, Conc. H_2SO_4, litmus paper, PH paper, ethyl acetate, 1-diphenyl-2-picrylhydrazyl (2, 2-diphenyl-1-picrylhydrazyl; DPPH), potassium ferricyanide, trichloroacetic acid, ferric chloride, phosphate buffer, iron (iii) chloride, aluminium trichloride, GABA ELISA kit (Shanghai Koon Co. Ltd, China), formalin, phosphate buffer saline, distilled water, carbonate buffer, adrenaline, thiobarbituric acid, and Gas chromatography mass spectrometer instrument (GCMS).

3.11 Plant Material Collection and Identification

Combretum micranthum leaves from plants of same age group from a single population were collected from Shira Local Government Area of Bauchi State, where it is readily available. The leaves were identified and deposited at the herbarium unit of Department of Biological Sciences, Bayero University, Kano (B.U.K.), having a voucher number BUKHAN 0272.

3.12 Animals acquisition and care

Thirty six (36) Swiss albino mice (19 - 22g body weight) were used for this study. The animals were obtained from the animals` facility in the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were housed in standard metal animal cages at room temperature in the animal house of Pharmacology Department, Aminu Kano Teaching Hospital, AKTH. They were allowed to acclimatize for a week prior to use.

All experimental procedures were approved by the Bayero University, College of Health Science Research Ethics Committee (CHS-HREC). Ethical clearance was provided with the number BUK/CHS/HREC/VII/62. (A copy attached).

3.2.0 Methods

3.2.1 Preparation of *C. micranthum* extract

The leaves of the plants *C. micranthum* used for the study were plucked from the stem, washed and then dried under shade. The dried samples were then ground into powder in a laboratory mortar using a pestle. The powdered samples were then kept in a closed air tight container and used in the experiment. The residue obtained after removing the solvent, were dried to give free flowing powder.

3.2.2 Extractions

3.22.1 Microwave Assisted Extraction (MAE)

HiNaRi Microwave Appliance (Model No.: MX 120BTC, 240V, 2450MHz, 1350Watts) was used in this study. Powdered leaves were extracted with methanol in the microwave apparatus. The powdered leaves samples and the methanol were mixed thoroughly, then sufficient time were allowed for the powdered leaves to absorb the methanol and get saturated (infused). The saturated powder was then placed into the extraction vessel, and 3L of the extracting solvent (methanol) was then added. Different time of irradiation at 40^0C with the microwave extractor operating at 850watts power level is needed for MAE. The sample was then treated in an intermittent way (3 times), i.e irradiation–cooling–irradiation under the microwave, maintaining a particular ratio of 5 minutes irradiation and cooling for 3 cycles. (Waghmare et al., 2015).

The sample was then filtered, the supernatant was concentrated using rotary evaporator at 40 ^{0}C to 1/10 of its volume. It was then acidified with 2M H_2SO_4, followed by exhaustive extraction with chloroform ($CHCl_3$) which results in the formation of 2-layers: the neutral layer (lower layer) which contains Terpenoids & Phenolics mostly; and an acidic layer (the upper layer) which contains alkaloids, N-oxides, and flavonoids. However, these 2-layers were identified/differentiated using litmus paper and pH paper (Harbone, 1983).

The neutral layer was then obtained/separated using separating funnel, and then further extracted with chloroform. It was then qualitatively tested using Salkowski's test to confirm the presence of terpenoids, and a positive result was obtained. This is considered as the crude extract.

3.2.3 Qualitative test for terpenoids:

This was done by the use of Salwoki's test (Das et al, 2014). To 2ml of the crude extract, 1ml of chloroform was added, followed by few drops of concentrated H_2SO_4. A reddish brown precipitate was formed, which indicates the presence of terpenoids.

3.3 Determination of total Terpenoids:

Total terpenoid content was determined by the method of Ghorai *et al* (2012). To 1 mL of the plant (crude) extract, 3 mL of chloroform was added. The sample mixture was thoroughly vortexed and left for 3 min and then 200 μL of concentrated sulfuric acid (H_2SO_4) was added. Then it was incubated at room temperature for 1.5h-2h in dark condition and during incubation a reddish brown precipitate was formed. Then carefully and gently, all supernatant of reaction mixture was decanted without disturbing the precipitation. 3 ml of 95% (v/v) methanol was added and vortexed thoroughly until all the precipitant dissolved in methanol completely. The absorbance was read at 538 nm using UV/visible spectrophotometer. The total terpenoid content was calculated by calibration curve of Linalool (monoterpenoid) and the results were expressed as Linalool equivalent (mg/g). (Geetha et al, 2015).

3.4 Fractionation of crude extract (chloroform, methanolic, & ethylacetate fractions):

The crude extract was then subjected to fractionation using chloroform, methanol, and ethyl acetate as solvents. The fractions from each solvent were obtained using a separating funnel, and it was based on their polarity i.e from the least polar chloroform, then ethyl acetate and lastly the methanol. These 3 fractions were then subjected to 2 in vitro assays**:** DPPH scavenging activity and reducing power activity, to determine the fraction with the best antioxidant activity among them, that will be orally administered to the rats.

3.5 Invitro antioxidant assays for the chloroform, ethyl acetate and methanolic fractions:

3.51 DPPH scavenging activity (Monzocco et al 1998 method).

The molecule 1, 1-diphenyl-2-picrylhydrazyl (2, 2-diphenyl-1-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour.

In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored. According to Manzocco et al (1998), the sample extract (0.2 ml) was diluted with methanol and 2 ml of DPPH solution (0.5 mM) was added. After 30 min, the absorbance was measured at 517 nm in a spectrophotometer. The percentage of the DPPH radical scavenging was calculated using the equation as given below:

$$\% \text{ inhibition of DPPH radical} = [(A_{br} - A_{ar})/A_{br}] \text{ X } 100$$

Where A_{br} is the absorbance before reaction (or absorbance of blank) and A_{ar} is the absorbance after reaction has taken place (or absorbance of the test sample).

3.52 Reducing Power Method (RP) (Oyaizu, 1998 and Jayaprakash et al, 2001 methods).

This method is based on **the principle that** 'substances which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then react with ferric chloride to form ferric-ferrous complex that has maximum absorption at 700nm'.

In this method, antioxidant compound forms a coloured complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples

(Jayaprakash *et al*., 2001). In the method described by Oyaizu (1986) 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) are added to 1.0 ml of sample dissolved in distilled water. The resulting mixture is incubated at 50 C for 20 min, followed by the addition of 2.5 ml of Trichloro acetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700nm against blank sample using spectrophotometer.

3.6 Experimental design

Animals were equally randomized to six groups of 6 animals each:

- Group-I: Control: administered orally with distilled water only.
- Group-II: 40mg/kg b.w $AlCl_3$ were administered orally, simultaneously with 100mg/kg body weight (b.w) of the extract (chloroform fraction).
- Group-III: 40mg/kg b.w $AlCl_3$ was administered orally simultaneously with 50mg/kg b.w extract.
- Group-IV: 40mg/kg b.w $AlCl_3$ was administered orally simultaneously with 25mg/kg b.w extract.
- Group V: Negative control: 40mg/kg b.w $AlCl_3$ only was administered orally without the extract.
- Group VI: Positive control: 40mg/kg b.w of $ALCL_3$ and a standard drug 0.5mg /kg bw diazepam orally. (Wolfman et al, 1994).

The treatment period was for 2 weeks (Wang et al, 2007) and the mice were then slaughtered by neck and the brain tissue was collected for histopathology and GABA, GSH, SOD, and MDA (lipid peroxidation) tests. The brain tissue was selected inplace of serum because, Aluminium chloride was used for inducing the oxidative stress, and its effect is highly on the brain.

3.7 Tissue collection and preparation of tissue homogenates

The mice from each group were slaughtered and dissected after the treatment period. Brains of the mice were quickly taken out, divided into two portions: those to be used for histopathology; to determine the morphology of the brains were inserted into formalin to preserve their integrity, while the other portions that were used for Biochemical Analyses were put into phosphate buffer saline. All procedures were carried out in ice cold conditions

3.8 Biochemical assay and histopathology of brain homogenate

3.81 Determination of Gamma-Aminobutyric (GABA):

Gamma-aminobutyric acid (GABA- a major neurotransmitter) levels was determined using Rat Gamma-aminobutyric acid (GABA) ELISA kit (Shanghai Koon Biotech Co., Ltd., China), based on the principle of biotin antibody sandwich technology enzyme linked immunosorbent assay (ELISA). Prepared Standard and Sample were added to the wells that were pre-coated with objective antibody, which was followed by adding streptavilin HPR to form an immune complex, and incubated at 37^0C for 60minutes, then the plate was washed 5 times to remove unbounded enzyme. The substrate, chromogen A and B, were then added and allowed for 15 minutes, the solution develops colour (the solution turns blue), this was then followed by adding the stop solution (the blue colour change into yellow immediately at that moment). The absorbance (OD value) was read at 450nm within 15 minutes, then the concentration of the corresponding samples were calculated. The colour depth or light was positively correlated with the concentration of Gamma-aminobutyric acid (GABA).

3.8.2 Super oxide dismutase (SOD):

The level of SOD activity was assayed by the method described by Mccord and Fridovich (1969). The principle works on the basis of superoxide dismutase to inhibit auto oxidation of adrenaline at pH 10.2. 0.1ml of the serum was diluted in 0.9ml of distilled water to make 1:10 dilution of serum. An aliquant mixture of 0.2ml of the diluted serum was added to 2.5ml of 0.05M Carbonate buffer. The reaction was started with the addition of 0.3mM Adrenaline. The reference mixture contained 2.5ml of 0.05M Carbonate buffer, 0.3ml of 0.3mM of distilled water. Absorbance was measured over 30s up to 150s at 450nm in a spectrophotometer.

3.8.3 Lipid peroxidation / Malondialdehyde (MDA) assay

Lipid peroxidation (LPO) assay Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation.

Lipid peroxidation or oxidative damage was determined according to Okhawa *et al.,* (1979) with slight modification by Atawodi *et al.,* (2011). Oxidative damage was assessed spectrophotometrically by measuring the levels of MDA by lipid peroxidation as thiobarbituric acid reactive substances (TBARS) when reacted with 2-thiobarbituric acid solution. 100uL of the supernatant was deproteinized by adding 2ml of 14% trichloroacetic acid (TCA) and 2ml of 0.67% TBA solution. The mixture was heated in a water bath at 80^0C for 30 minutes to complete the reaction and then cooled rapidly on ice for 5 minutes. After centrifugation at 2000g for 10 minutes, the absorbance of the coloured product (TBARS) was measured at 532nm with a UV spectrophotometer. The concentrations of TBARS were determined in triplicate and calculated using the molar extinction coefficient of malondealdehyde -$1.56x10^5$mol/L/cm. All TBARS concentrations are expressed in umol/g tissue protein.

3.8.4 Histopathology:

The tissues were immediately fixed in 10 % formal saline after removal from the animal for 48 hours. After the fixing process was completed, the tissues were processed by passing them through ascending grades of methanol from 70% to 90% and 100% methanol for a period of 12 hours to properly dehydrate them after which they were cleared in xylene for 2 hours and then infiltrated and embedded in liquid paraffin wax. The tissues were then cut using rotary microtome at 5 micron thickness and the sections were stained using haematoxylin and eosin staining technique. After dehydration and cleaning, sections were finally viewed under light microscope (Harris, 1900).

3.8.5 Phytochemical screening by GC-MS

Methanolic extract (0.5g) was dissolved in 95% methanol. The extract was filtered through microfilter 0.45 µm, then 2 µl of this solution was employed for GC-MS screening. GC-MS screening was carried out on a Shimadzu GCMS-QP2010 Ultra system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), helium (99.999%) was used as carrier gas at a Flow Control Mode :Pressure:100.0 kPa, Total Flow :17.6 mL/min, Column Flow :1.33 mL/min, Linear Velocity :43.0 cm/sec, Purge Flow :3.0 mL/min, Split Ratio :10.0, injector temperature 220°C; ion-source temperature 200°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 220°C, ending with a 9 min isothermal at 220°C. Mass spectra were taken at 70 eV, then the time required for sample chromatography was 20 minutes (Poaranthaman *et al*., 2012). Compound were identified using MassHunter\Library\NIST14.L at Multi-User Science Research Laboratory, Ahmed Bello University Zaria.

3.9 Statistical analysis

Results were expressed as mean ± S.D (standard deviation of the mean). Data was analyzed using one way analysis of variance (ANOVA) and Tukey's multiple comparison test. 0.05 level of probability was used as the criterion of significance in all cases.

3.91 Ethical clearance

- Ethical approval for this research proposal was approved by The College Research Ethics Committee of Bayero University, Kano. A Copy of the ethical clearance is attached to this project.

CHAPTER FOUR

4.0 Results and discussion

4.1 Results

4.1.1 Phytochemical screening of the leaves extract

Qualitative test on the crude leaves extract revealed the presence of alkaloids (Wagners test, terpenoids (Salkowski test), flavonoids (Shinoda test), and phenolics (Lead acetate test and Iron (iii) chloride test).

Table 1: Quantitative Phytochemical estimation of total Terpenoids.

Sample	Terpenoid content (mg/g of linalool equivalent)
C. micranthum leaves crude extract	206.72

4.2 In vitro antioxidant assays of the 3 of the fractions.

Table 2: Results of the DPPH scavenging activity (% inhibition) of the fractions

Conc. mg/ml	Ascorbic acid std (%)	$CHCL_3$ Fraction (%)	Ethyl acetate fraction (%)	MeoTH Fraction (%)
0.2	83.69	78.70	68.29	64.76
0.4	86.96	80.87	72.47	66.57
0.6	86.89	83.58	76.70	66.79
0.8	89.16	83.69	78.42	69.40
1.0	89.37	84.00	82.47	70.11

For each of the fractions and the standard (the ascorbic acid), the test was performed 3 times and the average/mean of the absorbances was calculated and used to determine the percentage inhibition (using formula), these table represents the percentage inhibition values for the standard and the fractions.

Table 3: IC50 values of the DPPH scavenging activity (using Microsoft Excel)

Ascorbic acid	CHCL3 fraction	Ethylacetate fraction	Methanol
0.416	0.441	0.470	0.536

Table 4: Reducing Power Assay Result.

Conc. (mg/ml)	EthAct Fraction	$CHCL_3$ Fraction	MeoTH Fraction	Ascorbic acid Standard
0.2	0.605	0.773	0.443	1.388
0.4	0.765	0.888	0.833	2.576
0.6	1.224	1.278	1.018	4.883
0.8	1.210	1.585	1.221	7.218
1.0	1.745	1.822	1.334	7.386

Table 5: Results of the IC50 values of the Reducing power activity (using Microsoft Excel)

Ascorbic acid std.	CHCL3 fraction	Ethylacetate fraction	Methanol fraction
6.32	25.19	28.39	32.89

These results indicated that the chloroform fraction have a lower IC50 value than the other fractions when compared to that of the ascorbic acid standard.

4.3 The in vivo assay of some of the brain biochemical markers

Table 6: Results of the brain tissue lipid peroxidation (MDA), GABA, and the antioxidants (GSH & SOD) levels after the 2 weeks administration of the treatments.

Treatment	MDA (µmol/g protein)	GABA (mM)	GSH (µg/ml)	SOD (unit/ml)
Distilled water 10ml/kg	19.03± 1.54[a]	431.05±79.81 [a]	22.28±4.61[a]	15.18±3.15[a]
40mg/kg $ALCL_3$	31.08±1.53[b]	259.28±37.54[b]	19.71±2.71[a]	11.06±1.52[b]
25mg/kg extract+ 40mg/kg $ALCL_3$	26.93± 5.28[c]	331.68±42.44[b]	21.58±1.05[a]	11.20±0.65[b]
50mg/kg extract + 40mg/kg $ALCL_3$	23.58± 3.26[a]	356.83±73.42[d]	22.46±4.18[a]	11.95±0.97[b]
100mg/kg extract + 40mg/kg $ALCL_3$	20.83 ± 3.6[a]	400.34±116.40[c]	31.46±0.76[b]	14.68±2.97[a]
40mg/kg $ALCL_3$ + 0.5mg/kg Diazepam	21.10± 4.16[a]	377.03±38.76[c]	26.25±0.42[b]	13.48±2.92[a]

Results with different superscripts along the vertical columns (within the individual groups) are statistically significant ($p<0.05$).

4.4 GC-MS Analysis of the extract

Table 7: Results obtained from the GCMS Analysis

S/N	Name	RT	Mwt(g/mol)	Chemical Formular	Uses
1	Isoxazolidine	82.73	73.09	C_3H_7NO	It serves as anti-inflammatory and anti-lipid peroxidation agent.
2	N-ethyl formamide	85.33	73.05	C_3H_7NO	NIL
3	2,4-dimethyl hept-1-ene	66.61	126.24	C_9H_{18}	It is used as a fragrance.
4	1-propanol	66.61	60.10	C_3H_8O	Used in making cosmetics, perfumes, soaps, and antifreeze.
5	1-hexene	66.61	84.16	C_6H_{12}	Used in the production of flavours, lubricants perfumes, and dyes.

These table revealed the compounds that are found present in the extract with their respective uses from the characterization of the extract by the GCMS.

4.5 Results of Histopathology assay:

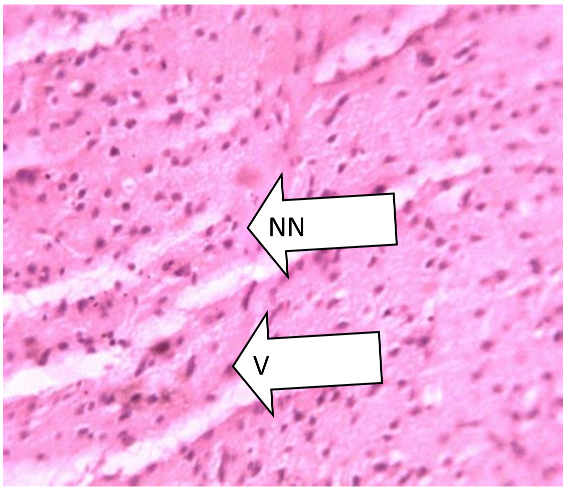


Fig A: Showing Neuronal necrosis (NN) and Vacoulation (V) in the

brain after 2weeks administration of 40mg/kg $ALCL_3$.

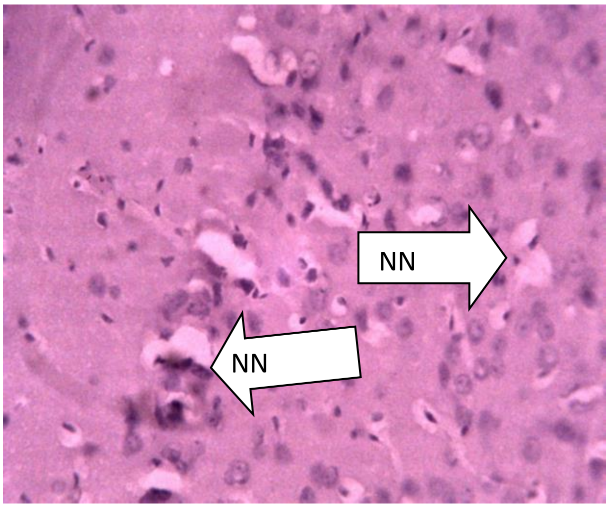
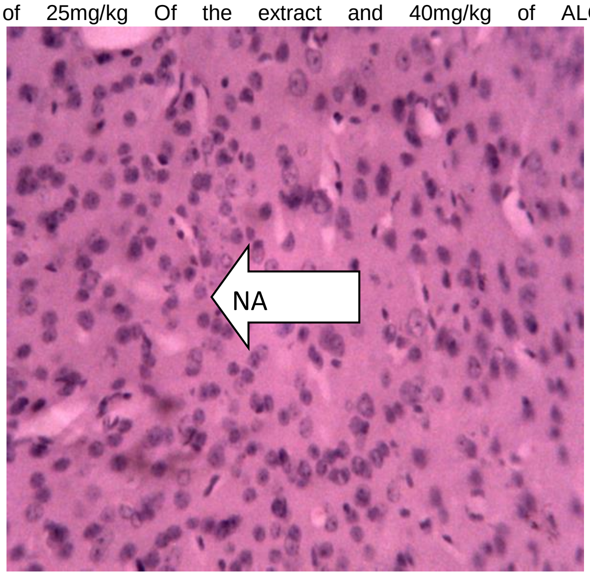


Figure B: Showing slight Neuronal necrosis (NN) after 2weeks

Administration of 50mg/kg extract concurrently with 40mg/kg $ALCL_3$.

Figure C: Showing neuronal atrophy (NA) after 2weeks administration

of 25mg/kg Of the extract and 40mg/kg of $ALCL_3$.



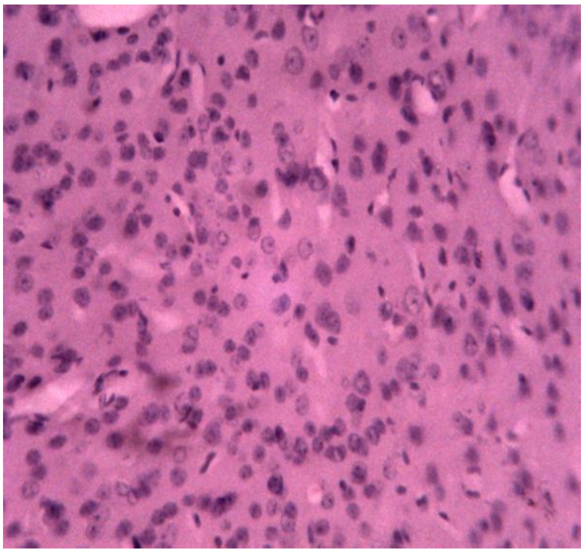


Figure D: Showing normal neurons after 2weeks

Administration of 100mg/kg extract followed by 40mg/kg $ALCL_3$

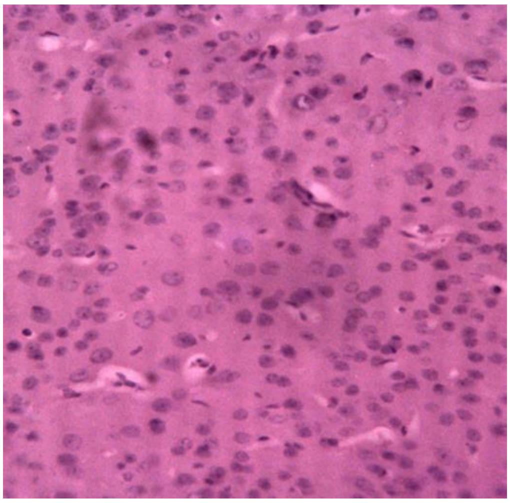


Figure E: Showing normal neurons after 2weeks of administration of10ml/kg distilled water only.

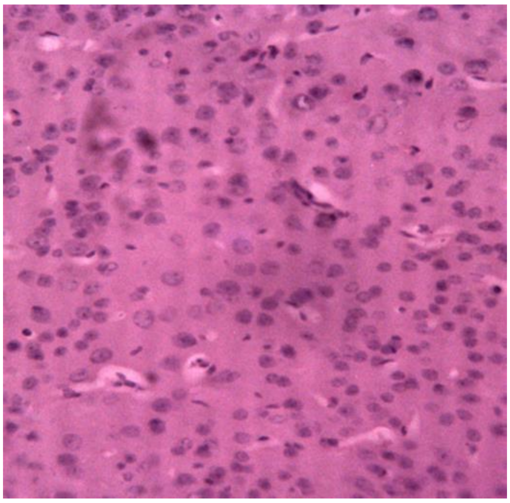


Figure F: Showing normal neurons after 2weeks administration of 40mg/kg $ALCL_3$ and 0.5mg/kg Diazepam.

4.6 Discussion

In this research, the effect of terpenoid extract of *C. micranthum* leaves extract in protecting neurons against oxidative stress (Al-induced) was analysed. $AlCl_3$ was used to induce oxidative stress, and the toxicity was detected in mice group that only $AlCl_3$ was administered without the extract, while protective effect of the extract against the induced stress was observed in the mice group treated with 100mg/kg extract + 40mg $AlCl_3$.

The research started by conducting a preliminary phytochemical screening of the *C. micranthum* leaves crude extract, where, presence of terpenoids, alkaloids, flavonoids, and phenolics were detected using Salkowski, Wagner, Shinoda, and Lead acetate tests respectively. Also, the Quantitative Phytochemical estimation of total Terpenoids on the crude leaves extract by the use of Ghorai et al, 2012 method, revealed 206.72mg/g Linalool equivalent of Terpenoid content. However, the fractionation of the crude extract using solvent-solvent extraction with the use of Methanol, Ethylacetate, and Chloroform gave 3-fractions viz: Methanol fraction, Ethylacetate fraction, and Chloroform fraction.

Evaluation of the fraction with best antioxidant capacity among these 3-fractions was done using the in vitro antioxidant assay by using the DPPH and reducing power assays. The results of the Reducing power assay from table (3) has shown that the chloroform fraction had higher significant antioxidant activity ($p<0.05$) due to its high optical density with lower IC50 of 25.19 (Tables 5) that is lower than that of the remaining fractions (Jayaprakash et al, 2001). Also, the DPPH Assay table confirmed that chloroform fraction had the highest antioxidant capacity due to higher values of DPPH % inhibition when compared with that of the remaining fractions table (2) and a lower IC50 value of 0.441 than that of the methanol and ethylacetate fractions, -1.99 and -0.90 respectively, (Table 3) when compared to that of the standard.

From the histopathology result, significant increase was observed in the MDA level with decrease in the SOD, GSH and GABA levels in the groups treated with 40mg/kg $ALCl_3$ (Table 6), which indicates the occurrence of lipid peroxidation and neurodegeneration (oxidative stress caused by $ALCl_3$ toxicity), but in the groups administered with 100mg/kg extract+ 40mg/kg $ALCl_3$ and 40mg/kg $ALCl_3$ +0.5mg/kg diazepam, a significant increase ($p<0.05$) in the SOD, GSH, and GABA levels with an insignificant increase in the MDA levels when compared to the control. This revealed the occurrence of degeneration or neurodegeneration (oxidative stress) in the brain tissue in the 40mg/kg $ALCl_3$, which was induced by the $ALCLl_3$ and neuroprotection in the groups treated with 100mg/kg extract+$ALCL_3$. This is in-line with the research findings by Phani et al, 2015, ‘that during neurodegenerative state, there would be decreased activities of antioxidant enzymes such as SOD, CAT, GPx, and GSH.

The result of the GC-MS analysis of the extract had identified the presence of isoxazolidine, (found in all the peaks), N-etyhl formamide, 2,4-dimethyl hept-1-ene, 1-propanol, and 1-hexene. The 2,4-dimethyl hept-1-ene is used in manufacturing industries as fragrance, 1-propanol is used in making cosmetics, perfumes, soaps, and antifreeze. 1-hexene is used in the production of lubricants, perfumes, and dyes, however, the isoxazolidine was found to have an anti-inflammatory and anti-lipid peroxidation property. It was discovered by Sadashiva et al 2005. that isoxazolidine possesses these properties by inhibiting the activity of phospholiphase A_2 (PLA_2) by binding to the active site of the enzyme, there by quenching its fluorescence, and the quenching increases with increase in the length of the alkyl or aryl group property (PLA_2 E), but the inhibition is substrate dependent. As PLA_2 become inhibited, the release of arachidonic acid will also become inhibited, therefore, inhibiting inflammation and hydrolysis of phospholipids. Hence, this really contributes to the antioxidant effect of the extract. Hence, making *C. micranthum* to have the capacity of protecting/preventing oxidative stress. This supports the findings that the *C. micranthum* leaves extract are known for antioxidant activities that may offer protection from free radical damage (Welch et al; 2017).

Thus, isoxazolidine when isolated from the *c. miramthum* extract can now be used in place of the synthetic drugs which have side effects to treat against oxidative stress and neurodegenerative diseases, cancer, and their prevention as well being natural and free from side effects.

CHAPTER FIVE

5.0 Summary, Conclusion, and Recommendations

5.1 Summary of the results/ findings

Extraction was done using microwave assisted extraction, and qualitative phytochemical screening of the extract revealed the presence of terpenoid, alkaloids, flavonoids, and phenolics; and 206.72mg/g of terpenoid was found in the quantitative test. Fractionation of the crude extract by solvent-solvent extraction was performed using chloroform, methanol, and ethylacetate. In-vitro assay detected that the chloroform fraction among the 3-fractions have higher antioxidant activity via a high DPPH and reducing power activities by a lower IC50 of 0.441 and 25.19 respectively. Invivo assay of SOD, GSH, and GABA levels showed that their levels were significantly increased with a significant decrease in MDA level all in the 100mg/kg + $ALCl_3$ treated mices. The brain histopathology showed neuroprevention in the groups treated with 100mg/kg extract+$ALCl_3$. C. micranthum have neuroprotective property.

Combretum micranthum is beneficial for the management of pain and inflammation, diarrhea, cure many ailments in Africa and Asia and has been used as potent antibacterial agent in traditional medicine (Muttaka, Jamilu, and Sule, 2016).

Isoxazolidine was found predominant in the GC-MS analysis of the extract, which was found to have anti-inflammatory, anti-tumour, and antilipid peroxidation properties as itt was discovered by Sadashiva et al 2005.

5.2 Conclusion

The findings of this study revealed that *C. micranthum G. Don Terpenoid leaves* extract can be used to protect against oxidative stress due to its richness in antioxidants and the important compound “isoxazolidine” which have anti-inflammatory, anti-tumour, and anti-lipid peroxidation properties.

These findings have also proven the findings which revealed that “Traditional herbs and phytochemicals may delay oxidative stress and neurodegeneration onset and slow its progression and also allow recovery by targeting multiple pathological causes by antioxidative, anti-inflammatory, and antiamyloidogenic properties. They also regulate mitochondrial stress, apoptotic factors, free radical scavenging system, and neurotrophic factors (Ramu et al, 2015).

These findings will help in controlling drug abuse withdrawal effect induced by stressful life style that is now an endemic deleterious event, since the use of phytotherapy will decrease the use of synthetic drugs, hence, their rate of production and supply will decrease, and their abuse will become alleviated.

Also, the use of chemotheraphy in treating tumours and cancer which is painful with side effects to body organs will decline.

5.3 Recommendations

1. A research should be conducted on the isolation of ‘isoxazolidine’ compound from the *C. micranthum* extract and determine its effect on some important biochemical markers and lipid peroxidation of brain tissue and its morphology using laboratory animals.

2. The same research using different plant that is also well known in having high antioxidant capacity should be carried out in order to have more alternative plants in the phytotherapy for the protection against brain oxidative stress and tissue degeneration.

APPENDIX

Combretum Micranthum

Figure 1: Leaves of *Combretum micranthum*

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