ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITY AND ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACT OF *COMBRETUM MICRANTHUM* LEAVES

BY

Rukaiyat Lawal MASHI

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(P13SCBC8031)

A DESSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE DEGREE (M.Sc.) IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY
ZARIA-NIGERIA

JULY, 2018

DECLARATION

I declare that the work in this dissertation entitled "**Antioxidant Activity and Angiotensin Converting Enzyme Inhibition by Aqueous Extract of *Combretum micranthum* Leaves** was performed by me in the Department of Biochemistry, Ahmadu Bello University Zaria under the supervision of Prof. Sani Ibrahim and Prof. B.D. James. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project was previously presented for another degree or diploma at any university.

Rukaiyat Lawal MASHI _____ _____
(P13SCBC8031) Signature Date

CERTIFICATION

This dissertation entitled "**Antioxidant Activity and Angiotensin Converting Enzyme Inhibition by *Combretum micranthum* Leaves**" by Rukaiyat Lawal MASHI meets the regulations governing the award of the degree of M.Sc.(Biochemistry) of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Professor S. Ibrahim Chairman, Supervisory Committee	(Signature)	(Date)
Professor B.D. James Member, Supervisory Committee	(Signature)	(Date)
Professor M. N. Shuaibu Head of Department	(Signature)	(Date)
Professor S.Z. Abubakar Dean, School of Postgraduate Studies	(Signature)	(Date)

DEDICATION

This work is dedicated to Almighty Allah who bestowed his abundant mercy upon me before and during the period of this work.

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ABSTRACT

Angiotensin I Converting Enzyme (ACE) is a glycoprotein with peptidyl dipeptide hydrolase activity which cleaves Angiotensin I to produce Angiotensin II. Angiotensin II is a powerful vasoconstrictor that as well regulates the synthesis of aldosterone that consequently interferes with blood pressure regulation. This study evaluates the effect of aqueous extract of *Combretum micranthum* leaves and its fractions on the *in vitro* activity of rabbit ACE and the possible antioxidant potentials fractions. *C. micranthum* leaves were extracted using aqueous as solvent. Consequently, the *in vitro* ACE inhibitory activity of the extract was conducted using cushman and cheng method and the aqueous extract was further subjected to chromatographic fractionation where the fractions obtained were assayed for *in vitro* inhibitory activity against rabbit ACE and their possible antioxidant potentials. Aqueous extract of *C. micranthum* leaves produced 59.43±4.00 % inhibitory activity comparable to captopril which produced 83.02±2.67% activity. The chromatographic fractions A, B, C and D were able to inhibit the *in vitro* activity of rabbit ACE with the inhibitory percentages of 8.21±41.19, 97.69±8.57, 78.32±7.14 and 98.32±2.66 % respectively. Phenolic compounds were found in the various fractions of the aqueous extract of *C. micranthum* with fraction B having the highest amount of these phenolics. The antioxidant potency of these fractions was evident in their free radical scavenging $IC_{50,}$ reducing power ability and total antioxidant capacity which all showed that fraction B may possibly exhibit the highest antioxidant potency as compared to fractions C and D. The bioactive phytochemical constituents from the GC-MS analysis of fraction B of the aqueous extract of *C. micranthum* where found to be Megastigmatrienone, 3,5-Dimethoxy-4-hydroxyphenylacetic acid and Estra-1,3,5(10)-trien-17β-ol. Hence, the results demonstrated that aqueous extract of *C. micranthum* has shown the tendency to inhibit the *in vitro* activity of ACE which could be attributed to its antioxidant activity demonstrated by the reduction power and the total antioxidant capacity of the various fractions.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The word hypertension is defined as a persistence increase in systemic arterial blood pressure (Sembulingam and Sembuligam, 2006). Clinically, when the systolic pressure remains elevated above 140mmHg and diastolic pressure remains elevated above 90mmHg, it is considered as hypertension. The prevalence varies with age, race, education, occupation and many other variables (Benowitz, 2009). In Nigeria for example, the true incidence of hypertension remains unknown but its prevalence among male and female is estimated to be 11.2% with age adjusted figure of 9.3% (Nurudeen *et al.,* 2013). This translates into approximately 13.4 million Nigerians becoming hypertensive at the age of 15years and above, using the projected national population census figure of 120million (Akinkungbe, 1998). In fact, hypertension is reported to be next to malaria as most serious health problems in developing tropical countries (Agunwa, 1988).

The global dimension of hypertension is immense, as it ranks the most common cardiovascular ailment afflicting about one billion people in the world and causing roughly 7.1 million deaths annually (Brundtland, 2002). Hypertension is said to be the most common cardiovascular disease among Africans and congestive cardiac failure its commonest complication (Akinkungbe 1972, 1985). Earlier studies suggested that hypertension was rare in African population (Sharper *et al.,* 1969, Pobee *et al.,* 1977), however, epidemiological transition, urbanization, adoption of urban and foreign lifestyles and improved case findings, among others, have made hypertension more prevalent as shown in some studies (Cooper *et al.,* 1998). The last Nigerian National Non-communicable Disease Survey (NNCDS) conducted in 1997 reported 11.4% prevalence of adult hypertension, varying from 14.8% in urban to 9.8% in rural residences respectively. However, a report on Nigeria from the World

Health Organization says that, in 2008, the probability of dying between the ages of 30 and 70 years from any of the 4 main NCD is 20% while adult risk factors for raised blood pressure was 34.8% – 33.5% for males and 36.1% for females (WHO, 2008). Another report estimated 38.6% of males and 41.2% of females in the country suffered from hypertension in 2012, figures that were above the regional average for both genders (WHO, 2012). The high prevalence of hypertension together with its deleterious effect on health makes it a major public health problem.

Angiotensin I Converting Enzyme (ACE) is a glycoprotein with peptidyl dipeptide hydrolase activity which cleaves Angiotensin I to produce Angiotensin II in the blood. The powerful vasoconstrictive action of Angiotensin II and its stimulatory action on the synthesis and release of aldosterone favours retention of sodium and water. It also hydrolyzes and inactivates bradykinin, a peptide with a powerful vasodilatory action (Hernandez-Ledesma *et al.,* 2003; Wong *et al.,* 2004). The utilization of synthetic ACE inhibitors, such as the well-known captopril, provides definitive positive health effects and is considered an important therapeutic approach in the treatment of high blood pressure, though the use of these pharmacological drugs is not advisable in healthy or low-risk populations (Carretero and Oparils, 2000).

The evidence that certain flavonoid-rich natural products can induce reductions in blood pressure and inhibit ACE activity opens the possibility that their consumption may mimic synthetic ACE inhibitors and provide preventive health benefits probably avoiding adverse side effects associated with the synthetic ones in current usage (Actis-Goretta*et al*., 2006). If the formation of angiotensin II and the activation of vasodilatorykinins are suppressed by selective ACE inhibitors, there will be a lowering of blood pressure. Some plant products and substances isolated from plants show inhibitory effects on ACE (Farzamirad and Aluko, 2008).

There are 11 ACE inhibitors approved for therapeutic use which can be sub-classified into three groups based on their chemical composition (Jimshenna and Gowda, 2011).

i. Sulfhydryl - containing inhibitors e.g Captopril, Zofenopril.

ii. Dicarboxylate - containing inhibitors e.g Enalapril, Lisinopril, Ramipril.

iii. Phosphate - containing inhibitors e.g Fosinopril

Due to the adverse effect observed with the use of synthetic ACE inhibitors, there is a need to look into natural ACE inhibitors from plants.

Combretum micranthum has a number of uses, traditionally it is used as, antihypertensive, diuretic, anti-diarrhoeal, anti-syphilis, antimalarial agent, and to treat hepatitis, jaundice and bronchitis (Uduma*et al.,* 2010). Stefano *et al* (2014) reported the antimicrobial potency of the leaf extract. Phytochemical studies carried out in the genus *Combretum* including *Combretum micranthum* have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans and non-protein amino acids, among others (Toua *et al.,* 2015; Pietrovski *et al.,* 2006).

1.2 Statement of the Problem

Hypertension is a risk factor for cardiovascular disease. It is the most common and persistent serious health problem, It affects 20-45% of the active population and carries a high risk factor of arteriosclerosis, stroke, myocardial infarction and end stage renal diseases (Jung *et al.,* 2006). It affects 15-25% of adult and 50-60% of elderly people (Gokce, 2004). It is predicted that the rate of hypertension would increase by 60% in 2025 (Kearney *et al.*, 2005). More than 40 million people worldwide are currently receiving synthetic Angiotensin converting enzyme inhibitors which are associated with the development of adverse

consequences such as; Kidney failure, angioedema, hypotension, decrease in white blood cells etc.

1.3 Justification of the Study

Several plants have been reported to have been used in the management of hypertension with no or less side effects. *Combretum micranthum* has numerous medicinal applications and it is used traditionally for the treatment of hypertension among the local people. Recently it has been hypothesized that oxidative stress is a key player in the pathogenesis of human hypertension (Rodrigo *et al.,* 2011; Montezano and Touyz, 2012). Hence there is a need to simultaneously evaluate the antioxidant potency and Angiotensin converting enzyme inhibitory activity with the aim of identifying hypertensive drug candidate from plants.

1.4 Aim of the Study

The aim of the study is to investigate the effect of aqueous extract of *Combretum micranthum* leaves on angiotensin converting enzyme and its Antioxidant properties.

1.4.1 Specific objectives

The specific objectives of the present study are to;

i. Evaluate the ACE inhibitory activity of aqueous extract of *Combretum micranthum* leaves.

ii. Subject the aqueous extract of the leaves of *Combretum micranthum* to column chromatography

iii. Determine the antioxidant potency and ACE inhibitory activity of fractions from the column chromatography of the aqueous extract.

iv. Subject the fraction with the best ACE inhibitory activity to GC-MS.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Combretum micranthum*

2.1.1 Habitat

The *Combretaceae* is a large family of herbs, shrubs and trees, comprising about 20 genera and 600 species with tropical distribution around the globe and centres of diversity in Africa and Asia (Masoko *et al.,* 2005). *Combretum micranthum*is an undomesticated shrub species that is commonly found in the Tiger bush region of western Africa; a bushy shrub or creeper that can reach up to 20m in height by twining around the branches of nearby trees (Le Fever and Le Jeune, 1997).*Combretum micranthum*is common on cultivated and fallow ground, throughout the continent, but it appears to be dominant in sub-Saharan Africa, from Sudan to Nigeria, from Gambia to Congo, with higher concentrations in Senegal, Mali, and Burkina Faso (Iwu, 1993).

2.1.2 General description

The bark is grey and fibrous with orange to brown red slash while the stems are red- brown, hairy and scaly. The leaves are opposite and the flowers are born as axillary cluster on scaly stalks, with a whitish corolla and ferruginous scales covering the calyces while the flowers typically produce nectar and attract insects, birds, and small mammals (Iwu, 1993; Le Fever and Le Jeune, 1997).

2.1.3 Common names

Combretum micranthum belongs to the family of plant called *Combretaceae* and is commonly called ‘farargeza’ in Hausa, ‘okan’ in Yoruba and ‘nzatego’ in Igbo (Burkill, 1985).

Plate I: *Combretum micranthum* (Snapshot from Malumfashi Local Government Area of Kastina State, Nigeria)

2.1.4 Chemical components

Phytochemical studies carried out on the genus *Combretum* have demonstrated the occurrence of many classes of primary and secondary metabolites, including triterpenes, flavonoids, lignans, stilbenoids and non-protein amino acids among other several unusual compounds such as 9,10-dihydrophenanthrenes and a substituted bibenzyl (Sudipta *et al*., 2014). Ethanol root extract of *C. micranthum* has been previously reported to have contained flavonoids, saponins, carbohydrates, anthraquinones, tannins and cardiac glycosides while sterols, alkaloids and terpenes were found in its aqueous leaves extract (Abdallahi, 2008; Welch, 2010). Consequently, Welch (2010) reported the presence of many polyphenolic compounds including catechins, glycosyl flavones, flavans, galloylated-C-glycoflavone and

epicatechins as well as gallic acid, betaine, choline, combretine, vitexin and isovitexin, m-inositol, sorbitol, myricetin-3-O-glucoside and myricetin-3-O-rutinoside in n-butanol and ethylacetate fractions of aqueous-ethanolic leaves extract of *C. micranthum*.

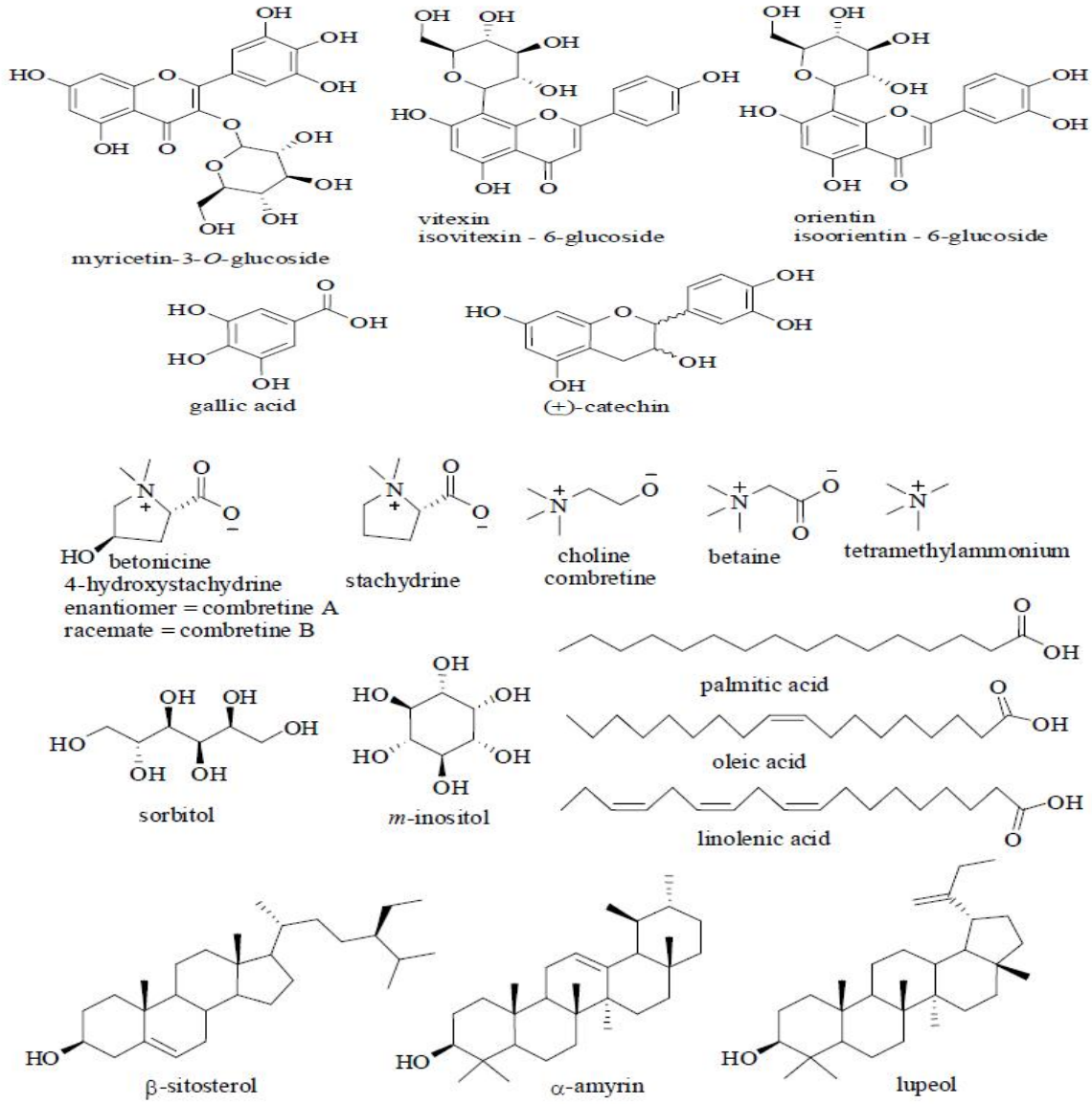


Figure 2.1: Some Compounds Previously isolated from *C.micranthum* (Welch, 2010)

2.1.5 Traditional uses

Combretum genus are well known in African traditional medicine, and used for the treatment of a variety of ailments and diseases ranging from scorpion and snake bites, mental problems, heart diseases and worm infestation to fever and microbial infections (Atindehou *et al.,* 2004). The leaves are used to treat hypertension. The root of *C. micranthum* is used in the treatment

of biliary fever, kidney infection,naso-pharyngeal infection, vomiting, malaria, microbial infection and used as diuretic and anti-inflammatory agent also, the root decoction is vermifugal and is said to have been used for sore as antiseptic for washing open wounds and drunk for treatment of guinea worm infestation (Yaouba *et al.,* 2012). The leaves decoction or fresh leaves have been used to treat cough, bronchitis, hepatobiliary diseases, hepatitis, and malaria (Ancolio *et al.,* 2002). It is also used in combination with other plant parts for the treatment of beri-beri, haemorrhage, leprosy, enuresis, diarrhoea and blenorrhagia (Kola and Benjamin, 2002). The dried powder root and fruit when mixed with palm oil is used in treatment of sprains, bruises, suppurating swelling (Burkil, 1985).

2.1.6 Some reported biological activities

The aqueous ethanol root extract had been reported to possess dose-dependent anti-convulsant activity in both electroshock and chemically induced convulsion (Danmallam *et al.,* 2011) while, aqueous ethanol leaf and stem-bark extracts were also reported to have analgesic, anti-inflammatory and anti-diarrhoea activities (Abdullahi, 2008). Similarly, aqueous leaves extract showed antipyretic, analgesic and anti-inflammatory properties (Olajide *et al.,* 2003) as well as significant anti-diabetic activity for type II diabetes mellitus (Aminu and Oricha, 2010). Equally, the aqueous fresh leaf extract of *C. micranthum* showed anti-bacterial activity (Uduma *et al.,* 2012) while methanol leaf extract demonstrated inhibitory action against herpes virus types I and II (Ferrea*et al.,* 1993).Karou *et al*. (2005) in an attempt to establish a link between antioxidant capacity and antimicrobial activity of *C. micranthum* leaves, demonstrated that the leaves showed microbicide activity against *Shigella dysenteriae*, *Salmonella parathyphi B*. and *Staphylococcus aureus* as well as microbiostatic activity against *Shigellaflexneri, Shigellaboydii, Salmonella typhi, Klebsiella ozenae*and *K. pneumonia.*

2.2 The Angiotensin-Converting Enzyme (ACE)

Angiotensin converting enzyme (also known as ACE, kininase II, or dipeptidylcarboxypeptidase) is an essential component of renin-angiotensin system (RAS) that regulates blood pressure by controlling the level of fluids in the body (Denis, 1999). ACE is a zinc-containing enzyme that cleaves dipeptide units at the C-terminus from peptide substrates (Sebastien *et al.,* 2008). Likewise it converts the inactive decapeptide angiotensin I to octapeptide angiotensin II by removing the dipeptide His-Leu (Denis, 1999; Inoue *et al*., 2011). Consequently, angiotensin II binds to the type I angiotensin II receptor (AT1) which triggers physiological actions that result in vasoconstriction and therefore increase blood pressure and also, it degrades bradykinin (a potent vasodilator) and other peptides, including substance P, gonadotropin-releasing hormone, the tetrapeptide *N*-acetyl-Ser-Asp-Lys-Pro, and angiotensin 1–7 (Sebastien *et al.,* 2008). The localization of ACE on endothelial cell membranes generates angiotensin II in close proximity to the vascular smooth muscle, a critical target organ in the regulation of blood pressure and electrolyte balance. ACE has been considered as a target for research on antihypertensive agents and a therapeutic approach for blood pressure management (Inoue *et al*., 2011).

2.3 Angiotensin-Converting Enzyme Inhibitors

Blood pressure drugs, especially inhibitors of the angiotensin-I-converting enzyme are generally used to regulate blood pressure in the renin-angiotensin system (Boschin *et al.,* 2014). The first ACE inhibitors were isolated from the snake venom of Bothropsjararaca (Ferreira, 1965). The drugs captopril, lisinopril, and enalapril were developed based on a snake venom peptide scaffold (Jimshenna and Gowda, 2011). It has been reported that the effectiveness of the inhibitors on hypertensives were 40%–50% when used as a mono-therapy, and reached up to 80%–90% when used in combination with diuretics (Cheung *et al.,* 2009). However, some side effects of these inhibitors such as dry cough, taste disturbances and skin

rashes from long term usage were reported (Cheung *et al.,* 2009; Gu and Wu, 2013). Consequent to these numerous adverse effects of synthetic ACE inhibitors, research has turned towards biological sources like plant extracts.

ACE-inhibitory activity of citrus leaves extracts were reported in rats fed with palm oil heated five times (Siti *et al.,* 2017), while methanol extracts of *Musa X paradisiaca* inhibited ACE by 68.63%–98.3% (Acharya *et al.,* 2016). Caren *et al.* (2017) reported that different types of plants such as wheat, peas, mushrooms, soybeans, walnuts, date seed flour, bitter melon seeds and spinach have been used to obtain ACE-inhibitory peptides. ACE inhibition by the peptides can be competitive or non-competitive enzyme inhibition (Guang and Philips 2009; Maestri *et al.,* 2015). Jang *et al.* (2011) reported that all of the purified ACE inhibitors from the mushroom *Pleurotuscornucopiae* were non-competitive inhibitors whereas, Shi *et al.* (2014) reported that the peptides from peanut bound competitively with the substrate at the active site of ACE and showed a competitive inhibiton pattern.

2.4 Oxidative Stress

Oxidative stress is a phenomenon associated with pathogenetic mechanisms of several diseases including atherosclerosis, ulcerative colitis, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases or aging processes (Hamouda *et al.,* 2011; Rana *et al.,* 2014) Oxidative stress is defined as an imbalance between production of free radicals or reactive metabolites (oxidants) and their elimination by protective mechanisms, referred to as antioxidative systems (Patil *et al.,* 2007). This imbalance leads to damage of important biomolecules and organs with potential impact on the whole organism. Oxidative and antioxidative processes are associated with electron transfer influencing the redox state of cells and the organism (Rana *et al.,* 2014).

2.5. Free Radicals

Free radicals are species (atoms, molecules, ions) that have one or more unpaired electrons in their outer orbital that make them very unstable and quite reactive with other molecules by pairing up their electron(s) to generate a more stable compound (Salwa *et al.,* 2011). Reactive oxygen species (ROS) or free radicals, formed during physiological and pathological conditions in the body are extremely reactive and react with proteins, lipids, carbohydrates and nucleic acids (Carmen *et al.,* 2012). Free radicals are derived from oxygen, nitrogen and sulphur to form reactive oxygen species, reactive nitrogen species and reactive sulphur species respectively. The nitrogen derived free radicals are nitric oxide (NO), peroxy nitrite anion (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3); the thiol derived free radicals include sulphite (SO_3^{2-}), disulfide S oxide (DSSO), sulfenic acid (RSOH) and sulfenyl (RS.) radicals (Lu *et al*., 2010). When the body is overloaded with free radicals that cannot be gradually reduced, their accumulation in the body generates a phenomenon called oxidative stress (Carmen *et al.,* 2012).

2.5.1. Oxidative damage to lipids,proteins and the DNA by free radicals

All of the most important classes of biomolecules may be attacked by free radicals but lipids are probably the most sensitive. Cell membranes are rich with polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidising radicals (Vasudevan *et al.,* 2011). The oxidative destruction of PUFAs is known as lipid peroxidation which involves pulling out of hydrogen atom(s) from the vicinity of polyunsaturated fatty acid double bond which is particularly damaging because it proceeds as a self-perpetuating chain-reaction (Laetitia *et al.,* 2012).

Oxidative attack on proteins results in site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross linked reaction products, altered electrical charges and increased susceptibility to proteolysis (Carmen *et al.,* 2012).

Activated oxygen species and agents that generate oxygen free radicals, such as ionizing radiations, induce numerous lesions in DNA that causes deletion, mutations and other lethal genetic effects (Craft *et al.,* 2012; Lu *et al.,* 2010). Characterization of this damage to DNA has indicated that both sugar and base moieties are susceptible to oxidation, causing base degradation, single strand breakage and cross links to proteins.

2.6 Antioxidant

Antioxidants are exogenous or endogenous compounds which either prevent or delay the generation of toxic oxidants or intercept those that are already generated to inactivate them, thereby blocking the chain of propagation reaction by these oxidants (Halliwell 2010). They can also initiate repair processes (e.g damaged DNA repaired by sulphaoxidereductase) which remove damaged macromolecules to prevent their accumulation that may further hinders cellular process and viability (Subash *et al.,* 2010). They have the ability to protect human body cells from the damages caused by unstable free radicals (highly reactive chemicals that play part in generating oxidative stress in biological system) by stabilizing them.

2.6.1 Classification of antioxidants according to the source

According to the source, antioxidants could be classified into: (a) endogenous (b) exogenous

2.6.1.1 Endogenous antioxidants

The endogenous group includes metallo-enzymes superoxide dismutase (zinc, manganese, and copper), glutathione peroxidase (selenium) and catalase, and proteins like albumin, transferrin, ceruloplasmin, metallothionein and haptoglobin.

2.6.1.2 Exogenous antioxidants

The most important exogenous antioxidants are dietary phytochemicals (such as polyphenols, quinones, flavonoids, catechins, coumarins, terpenoids) and the smaller molecules like

ascorbic acid (Vitamin C), alpha-tocopherol, beta-carotene, Vitamin-E and their supplements (Prochazkova *et al.,* 2010).

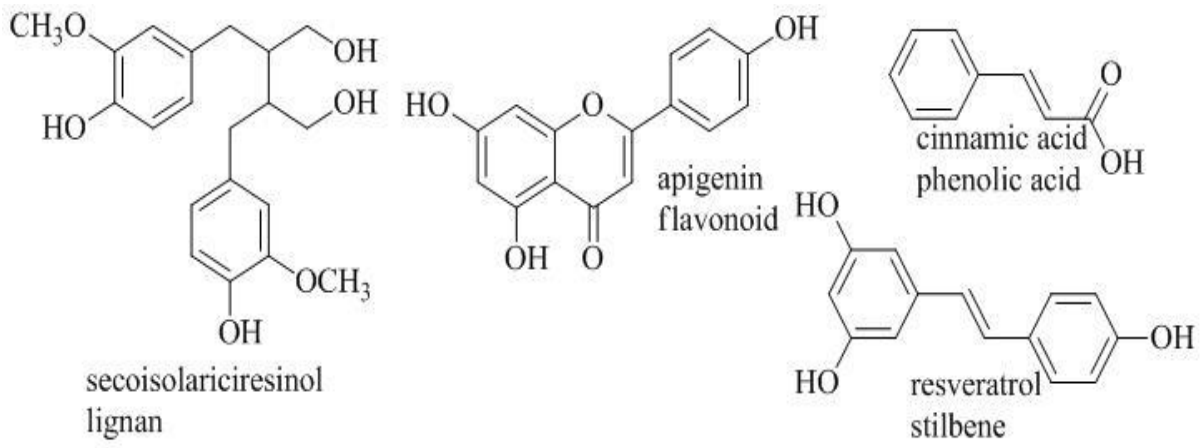


Figure 2.2: Example of Four Classes of Polyphenols: Phenolic acids, lignin, flavonoids and stilbenes

2.7Hypertension and Oxidative Stress

There is accumulating evidence that oxidative stress plays a role in the progression of hypertension (Dhalla *et al.*, 2000).Increased vascular oxidative stress could be involved in the pathogenesis of hypertension (Rodrigo *et al.*, 2011). In human, essential hypertension caused by ROS may increase due to a diminution of the activity of antioxidant enzymes (Pedro *et al.,* 2000). It is known that superoxide rapidly inactivates endothelium-derived nitric oxide (NO), the most important endogenous vasodilator, thereby promoting vasoconstriction (Zicha *et al.,* 2001). Thus oxidative stress may account for endothelial dysfunction, but it is unknown whether this abnormality is a primary event or a consequence of increased blood pressure (John and Schmieder, 2003). It has been reported that antioxidants such as vitamin E, glutathione peroxidase and superoxide dismutase (SOD) were decreased in essential hypertensive subject whereas; administration of SOD to hypertensive rats reduces blood pressure remarkably (Nakazono *et al.,* 1991). Increased ROS in human heart is associated with aortic valve stenosis (Peria-Silver *et al*., 2009).

Several lines of evidence demonstrate that oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases, including hypertension, dyslipidemia, atherosclerosis, myocardial infarction, angina pectoris, and heart failure (Linke *et al.*, 2005). The condition of ischemia and reperfusion occurring after stroke has been shown to be associated with free radical-mediated reactions potentially leading to cell death (Alexandrova *et al.*, 2004).

Pulmonary fibrosis is the end result of a diverse group of lung disorders. Several studies have suggested that oxidant- antioxidant imbalance in the airways plays a critical role in the pathogenesis of idiopathic pulmonary fibrosis (IPF) (Montuschi *et al*, 1998). In addition, oxidants may contribute to the development of pulmonary fibrosis due to their effects on the production of cytokines and growth factors.

Oxidative damage to DNA, proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, most notably heart disease and cancer (Halliwell, 1994). A growing body of animal and epidemiological studies as well as clinical intervention trials suggest that antioxidants may play a pivotal role in preventing or slowing the progression of both heart disease and some forms of cancer (Hennekens, 1994).

Several factors, such as high cholesterol levels, hypertension, cigarette smoking, and diabetes, are believed to promote atherosclerosis; a growing body of evidence suggests a critical step in its development is the oxidation of low-density lipoprotein (LDL) within the arterial wall (Jialal and Fuller, 1993). This theory is supported by several epidemiological studies which link low intakes of dietary antioxidants to an increased frequency of heart disease (Hennekens, 1994).

In the past two decades, a number of preclinical studies have been published implicating enhanced production of reactive oxygen and nitrogen species (ROS/RNS) in the development

and progression of hypertension (Harrison and Gongora, 2009). Increased vascular oxidative stress is also thought to play a key role in the pathophysiological consequences of hypertension (including vascular remodelling, inflammation, endothelial dysfunction, atherosclerosis, vascular cognitive impairment, stroke, and aorta aneurysm formation) (Burwell *et al.*,2006). Accordingly, animal studies showed that attenuation of cellular oxidative stress by over expression of superoxide dismutase (SOD) or treatment with antioxidants scavenging superoxide attenuated hypertension, whereas depletion of SOD expression exacerbates hypertension (Fukai and Usio-fukai,2011). Similarly, attenuation of cellular oxidative stress by molecular or pharmacological methods was shown to confer multifaceted cardiac and vascular protective effects, preventing/delaying the development of complications of hypertension in animal models (Wilcox, 2010). Duffy *et al*. (2001) demonstrated a beneficial effect of vitamin C on blood pressure. Equally, many antihypertensive drugs were shown to inhibit ROS production and reduce vascular oxidative stress (Harrison and Gongora, 2009).

Major sources of vascular ROS production include NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthase, and mitochondria (Dikalov, 2011). In recent years, a number of studies have been published on the role of NADPH oxidases, xanthine oxidase, and uncoupled nitric oxide synthase in hypertension-related cardiovascular pathologies (Weseler and Bast, 2010). Although mitochondria represent one of the most significant sources of cellular ROS generation (mitochondrial ROS production can reach up to 2% of the electron flow), the regulation of mitochondrial ROS generation and its pathophysiological role in hypertension are much less understood.

In cultured vascular smooth muscle cells (VSMCs) and isolated arteries from hypertensive rats and humans, ROS production is enhanced, redox-dependent signalling is amplified, and antioxidant bioactivity is reduced(Wilcox, 2003). Accordingly, evidence at multiple levels

supports a role for oxidative stress in the pathogenesis of hypertension. ROS in other organ systems, such as the heart, nervous system, and kidneys, have also been implicated in the pathophysiology of hypertension.

Clinical studies demonstrated increased ROS production in patients with essential hypertension, renovascular hypertension, malignant hypertension, and preeclampsia (Ward *et al.,* 2004). These findings are based, in general, on increased levels of oxidative stress (Minuz *et al.*, 2004). Accumulation of ROS by-products from oxidised genomic and mitochondrial DNA have also been demonstrated in hypertensive individuals. Polymorphonuclear leukocytes and platelets, rich O_2^- sources, also participate in vascular oxidative stress and inflammation in hypertensive patients (Cracowski *et al.*, 2003). Decreased antioxidant activity (SOD, catalase) and reduced levels of ROS scavengers (vitamin E, glutathione) may contribute to oxidative stress (Minuz *et al.*, 2004).

Antioxidants occurnaturally in the diet, and may be taken as dietary supplements, and some are produced endogenously. They are necessary components of the body's metabolic processes and are essential to quench ROS. They are also part of the electron transport chain of respiratory metabolism. Antioxidants work together to maintain adequate total antioxidant capacity by acting in the place of another or by regenerating each other (Wilson *et al.*, 2001). Antioxidants of higher electro negativity will regenerate those of lower electro negativity. Current therapies for hypertension normalise blood pressure by various means without removing the cause. Many of these treatments are prone to side-effects which may result in poor compliance. The ideal treatment would be a natural compound which would address the cause of the disease and could control blood pressure without side-effect

2.7.1 Classification and management of hypertension

Table 2.1: Classification of hypertension

Diagnostic category	Systolic (mmHg)		Diastolic (mmHg)
Optimal	<120	And	<80
Normal	120-129	and/or	80-84
High normal	130-139	and/or	85-89
Grade 1 (mild) hypertension	140-159	and/or	90-99
Grade 2 (moderate) hypertension	160-170	and/0r	100-109
Grade 3 (severe) hypertension	>180	and/or	>110

ESH/ESC: European Society of Hypertension/European Society of Cardiology (2007)

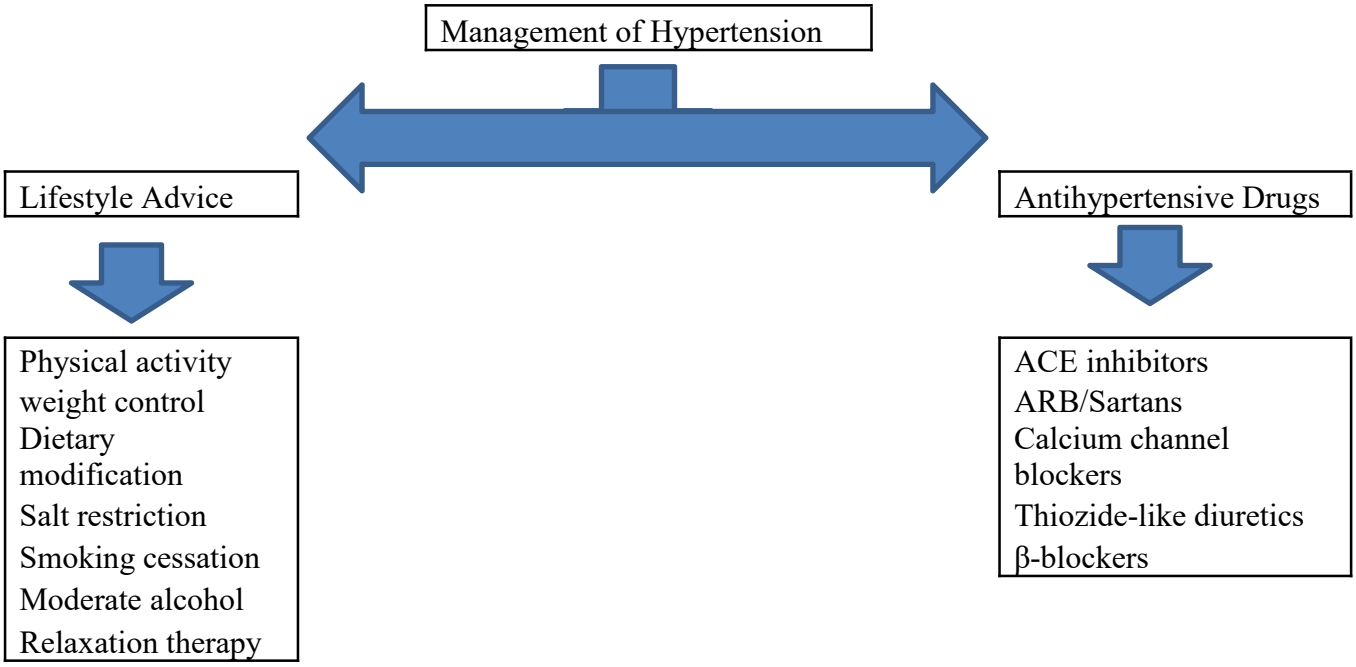
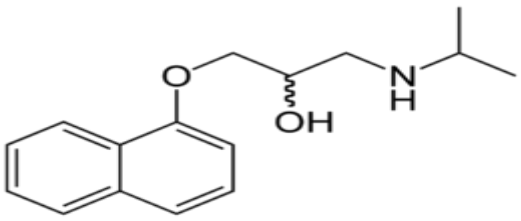


Figure 2.3: Management of Hypertension

2.7.2 Mechanism of action of other classes of antihypertensive drugs other than ACEIs

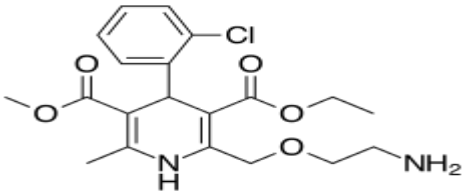
Beta blockers- Beta blockers interfere with the binding to the receptor of epinephrine and other stress hormones, and weaken the effects of stress hormones. They are competitive antagonists that block the receptor sites for the endogenous catecholamines epinephrine (adrenaline) and norepinephrine (noradrenalin) on adrenergic beta receptors, of the

sympathetic nervous system, which mediates the fight-or-flight response (Frishman, 2005). Beta blockers inhibit sympathetic outflow centrally, decrease cardiac contractility, and reduce renin-angiotensin-aldosterone system activity by inhibiting renin release which results in reduction of arteriole blood pressure (Samuel, 2017). Examples are atenolol, carvediol, propranolol, metaprolol and pindolol.



Propranolol- beta blocker

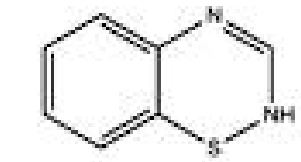
Calcium channel blockers-are several medications that disrupt the movement of calcium (Ca^{2+}) through calcium channels. By blocking the calcium signal on adrenal cortex cells, they directly reduce aldosterone production, which correlates to lower blood pressure (Olson, 2011). Examples are amlodipine, felodipine, lercanidipine and nifedipine.



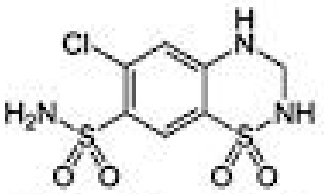
Amlodipine- CCB

Thiazides-like diuretics achieve their diuretic action via inhibition of the Na+/Cl− cotransporter (NCC) in the renal distal convoluted tubule (Ellison *et al.,* 1985). The NCC facilitates the absorption of sodium from the distal tubules back to the interstitium and accounts for approximately 7% of total sodium reabsorption (Adrogue and Madias, 2007). By decreasing sodium reabsorption, thiazide use acutely results in an increase in fluid loss to

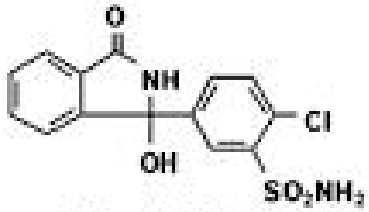
urine, which leads to decreased extracellular fluid (ECF) and plasma volume. This volume loss results in diminished venous return, increased renin release, reduced cardiac output and decreased blood pressure (Conway and Lauwers 1960). Examples are shown below:



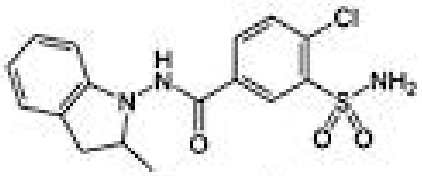
Benzothiadiazine ring



Hydrochlorothiazide

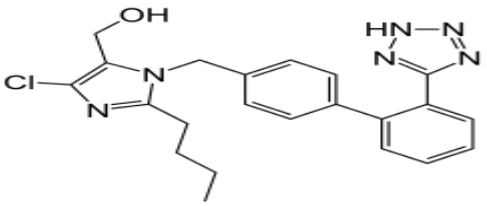


Chlorthalidone



Indapamide

ARBs/sartans-These substances are AT_1-receptor antagonists; that is, they block the activation of angiotensin II AT_1 receptors. Blockage of AT_1 receptors directly causes vasodilation, reduces secretion of vasopressin, and reduces production and secretion of aldosterone, among other actions. The combined effect reduces blood pressure (Domenic, 2001). Examples are candesartan, losartan, eprosartan and valsartan.



Losartan- ARB

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical and reagents

Angiotensin converting enzyme as a lyophilized powder from rabbit lung and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemical Co. (Germany). All solvents and other chemicals were purchased from Haddis international Samaru Zaria.

3.1.2 Equipment

Jenway 6405 UV/V Spectrophotometer made by Jenway limited, Beacon road, Staffordshire ST15 05A, UK. Water Bath made by Grant Instruments, Shepreth Road, Cambridgeshire SG8 6GB, UK. HeraeusLabofuge 300 Centrifuge made by Thermo Fisher Scientific Company, 81 Wyman Street, Massachusette, USA. RS-232C Electronic Weighing Balance made by Itin Scale Company, 4802 Glenwood Road, New York, USA. Thomas-Wile Laboratory Mill Model 4 made by Thomas Scientific, 1654 High Hill Road, NJ 08085, USA.

3.1.3 Plant Sample collection and identification

Combretum micranthum plant was collected during the dry season from Malumfashi Local Government Area of Kastina State, Nigeria. The Plant sample was identified and authenticated in Herbarium unit of the Department of Botany, Ahmadu Bello University, Zaria where a voucher sample was deposited (Voucher Number 900257).

3.2 Methods

3.2.1 Preparation of aqueous extract

The *Combretum micranthum* leaf was washed and air dried at room temperature. Dried samples was pulverised using pestle and mortar. Exactly 1 Litre of distilled water was added to 500g powdered leaves and soaked for 24 hours. The filtrate was then concentrated by

evaporation using a water bath at 40^0C. After which the aqueous extract obtained was then stored inside a container and kept until required at room temperature.

3.2.2 Determination of ACE inhibitory effect of aqueous extract of *Combretum micranthum* leaves and its fractions

The assay for ACE inhibitory activity was carried out using the Cushman and Cheung method (1971) with some modifications on the assay conditions. Briefly, 50µl of ACE solution (100mU/ml) was added to 50µl of sample solution (0.5mg/ml) and incubated at 37^{o}Cfor 10 minutes. Substrate (150µl) solution (8.3mM Hip-His-Leu in Borate buffer) was then added to the reaction mixture and then incubated for 1hr 20minutes at 37^{o}C. The reaction was terminated by adding 250µl of 1M HCl and then 1.5 ml ethyl acetate was added to extract the hippuric acid formed by the action of ACE. Ethyl acetate was then evaporated under air flow at 37^{o}C; the residual Hippuric Acid (HA) was then dissolved in 1 ml of deionized water and absorbance of the solution taken at 228 nm to determine the hippuric acid concentration. The sample blank was prepared in the same way above, with change in the order in which the reagents were added, HCl was added before enzyme. The reaction blank was prepared in the same way as the sample blank, replacing the volume of tested sample with buffer. Captopril was used as the standard drug. The percentage inhibition was then calculated from the equation:

$$\% \text{ IACE} = \frac{100[\ (A-B) - (C-D)]}{A - B}$$

A represents absorbance in the presence of ACE, B absorbance of the reaction blank, C absorbance in the presence of ACE and inhibitor, and D absorbance of the sample blank. All determinations were carried out in triplicate.

3.2.3 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was carried out to determine the best solvent system for the column chromatography. A thin layer chromatographic plate pre-coated with silica gel was used. The crude extract was dissolved in solvent and applied to the plate. The plates were placed in chromatographic tanks with a mixture of different solvent systems. The different solvent systems used include ethylacetate and methanol 9:1, 8:2, 7:3, ethylacetate 100%, chloroform and methanol 9:1 and chloroform 100%. Thereafter, the plates were removed, sprayed with p-anisaldehyde and followed by heating at 110 °C for 5 minutes, the solvent system ethylacetate and methanol 8:2 gave the best TLC profile.

3.2.4 Column chromatography of aqueous extract of C. *micranthum*

The column was packed with slurry of 150g of silica gel (60-120mesh) in 350ml of ethyl acetate. After the column has settled, 5g of the crude aqueous extract was loaded and eluted with 500ml ethyl acetate 100%,ethyl acetate and methanol (3:2,2:3,1:4,each 500ml) and 500ml 100% methanol. After collecting 75 Aliquots(40ml each),TLC was carried out and those aliquots with similar TLC profile were pooled together to give four pooled fractions(A-D).The fractions were tested against Angiotensin converting enzyme in order to ascertain their inhibitory potentials.

3.2.5 Determination of total phenolic content of the aqueous extract fractions of *Combretum micranthum* leavess

Total phenolic content of the fractions were determined using the method of Mc Donald *et al* (2001) with slight modifications.

Principle: Polyphenol in fractions react with Folin-ciocalteu to form a blue complex that can be quantified by visible-light spectrophotometry.

Procedure: Calibration curve was prepared by mixing ethanol solution of Garlic acid (1ml; 0.025-0.400mg/ml) with 5ml Folin-ciocalteu reagent (diluted tenfold) and sodium carbonate

(4ml, 0.7M). Absorbance values were measured at 765nm using a UV-VIS spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan) and the standard curve was plotted. One millilitre (1ml) of each of the solution of fraction in methanol (5g/L) was also mixed with the reagents above and after 30min the absorbance was measured. All determinations were carried out in triplicate. The total phenolics components in the fractions in Garlic Acid Equivalent (GAE) were calculated by the formula;

T= C.V/M; where T is the total phenolic contents, milligram per gram of sample fraction in Gallic Acid Equivalent ; C is the concentration of Garlic acid established from the calibration curve, mg/ml; V is the volume of fraction, millilitre; M is the weight of sample fraction (g).

3.2.6 Determination of antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl free radicalactivity of the aqueous extract fractions of *Combretum micranthum* leaves

The antioxidant activity of fractions of aqueous extract of the plant was assayed by the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method described by Karadag *et al.,* (2009).

Principle**:** 1,1-Diphenyl-2-picrylhydrazyl (DPPH) contains an odd electron in its structure. Its deep purple colour is reduced to a colourless compound, 2,2-diphenyl-1-picryhydrazine when it reacts with an antioxidant, which can donate a hydrogen atom or an electron to it. The change in colour was measured spectrophotometrically at 517 nm using a UV/Visible light spectrophotometer.

Procedure: The assay mixture contained 2ml of 1.0 mM DPPH radical solution prepared in methanol and 1 ml of standard or extract solution of different concentrations (10 – 500 µg/ml). The solution was rapidly mixed and incubated in dark at 37^{o}C for 20 minutes. The decrease in absorbance of each solution was measured at 517 nm using spectrophotometer.

Ascorbic acid was used as positive control while 2 ml of 1.0 mM DPPH radical solution with 1 ml ethanol was taken as blank.

The percentage of radical scavenging (%) was calculated by:

% Free Radical Scavenging Activity = $A_c - A_s/A_c$ x 100

Where, A_c = Absorbance of control at 517 nm

A_s = Absorbance of sample at 517 nm

The concentration of sample required to scavenge 50% of DPPH free radical (IC_{50}) was determined from the curve of percentage inhibitions plotted against the respective concentrations.

3.2.7 Estimation of reducing power of the aqueous extract fractions of *Combretum micranthum* leaves

This was determined according to the method of Oyaizu (1986).

Principle: Substances which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex. Procedure: The fractions and standard (1ml) of various concentrations (100, 200, 300ug/ml) were mixed with phosphate buffer (pH 6.6, 0.2M, 2.5ml) and potassium ferricyanide (1%, 2.5ml).The mixture was incubated at 50^0C for 20minutes.Trichloroacetic acid (10%, 2.5ml) was added to the mixture. A portion of the resulting mixture was mixed with $FeCl_3$ (0.1%, 0.5ml) and the absorbance was measured at 700nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated reductive potential of the fractions.

3.2.8 Determination of total antioxidant capacity of the aqueous extract fractions of *Combretum micranthum* leaves

The total antioxidant capacity of the fractions was evaluated by the phosphor-molybdenum method according to the procedure described by Prieto *et al.* (1999).

Principle: The assay is based on the reduction of Mo (VI) to Mo (V) by the fractions and subsequent formation of green phosphate Mo (V) complex at acid pH.

Procedure: 0. 3mililitre of various concentrations of fractions (100, 200, 300ug/ml) were combined with 3ml reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4Mm ammonium molybdate).The absorbance of the reaction mixture was measured at 695nm using a spectrophotometer against a blank after cooling to room temperature. Methanol (0.3ml) in place of fractions was used as blank. The total antioxidant activity was expressed as the number gram equivalents of ascorbic acid.

fraction.

3.2.9 GC-MS (Gas chromatography- Mass spectroscopy) analysis of fraction B

Fraction B was further subjected to GC-MS analysis. The analysis was conducted with an Agilent Technologies 68 90 GC coupled with an Agilent 5973 mass selective detector and driven by Agilent Chemstation software (Agilent Technologies, USA). A DB-5SIL MS capillary column was used (30 m x 0.25 mm i.d., x 0.25 μm film thickness). The carrier gas was ultra-pure helium at a flow rate of 0.7 mL min^{-1} and a linear velocity of 37 cm s^{-1}. The injector temperature was set at 250 °C. The initial oven temperature was 60 °C, which was programmed to 280 °C at the rate of 10 °C min^{-1} with a hold time of 3 min. Injections of 2 μL were made in the split less mode with a manual split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 min and scan range 50-700 amu. Compounds were identified by direct comparison of the retention times and mass fragmentation pattern with those from the National Institute of Standards and Technology (NIST) library.

3.3 Statistical Analysis

The data were analysed by the one way analysis of variance (one-way ANOVA) using SPSS program (version 20 SPSS Inc., Chicago, IL, USA). The differences in parameters were compared using Bonferroni multiple comparison test (a post-hoc test). The results were expressed as mean ± standard deviation (SD). P value less than 0.05 was considered as significant ($P< 0.05$). Results were presented in table, charts and graphs using MICROSOFT WORD and EXCEL.

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Inhibition of Aqueous Extracts of *C.micranthum* Leaves against ACE

Table 4.1 indicates the results of ACE inhibitory activity of aqueous extract of *C.micranthum* leaves and a standard antihypertensive synthetic drug of the class of ACEI (captopril). A given concentration (500µg/ml) of the sample was used and the result reported was a reflection of the mean values of triplicate performances. It showed that, the aqueous extract of *C. micranthum* leaves possessed inhibitory activity of 59.43±4.00 %whereas the standard drug (captopril) exhibited an activity of 83.02±2.67 % against ACE.

4.2 Result of thin layer chromatography.

The chromatographic profile of Aqueous extract is shown in plate II

4.3 Fractions from Column Chromatography of Aqueous Extract of *C. micranthum* Leaves

A total of seventy five fractions, 40ml each were collected. The fractions were pooled based on their TLC profile to give four major fractions (Table 4.2). Plate III and IV show the TLC profile of column chromatographic fractions of aqueous extract of C. *micranthum*.

4.4 Percentage Inhibition of Column Chromatographic Fractions of Aqueous Extract of *C.micranthum* Leaves against Standard Rabbit ACE

The four fractions of the extract obtained from the column chromatographic process were evaluated for ACE inhibitory activity using standard rabbit ACE. Samples were prepared in a concentration of 500 µg/ml each and repeated three times to obtain the mean value as reported. The result in Table 4.3 shows the inhibitory activity against ACE of the various column chromatographic fractions obtained from the aqueous extract of *C. micranthum*. Fraction A showed a significantly ($P<0.05$) lower inhibitory activity against standard rabbit

ACE as compared to fractions B, C and D. However, there was no significant difference ($P>0.05$) among B, C and D fractions.

4.5 Total Phenolic Content and DPPH Free Radical Scavenging Activity IC_{50} of Column Chromatographic Fractions of *C. micranthum* Leaves

The result is presented in Table 4.4 and it showed that all the fractions are significantly ($P<0.05$) different from one another with fraction B having the highest phenolic content (252.50±5.62 mg/g GAE) as compared to fractions C (118.30 ± 1.27 mg/g GAE) and D (55.73 ±2.56 mg/g GAE).

There was an inverse variation between the amount of phenolic content and the free radical scavenging activity IC_{50} as shown in Table 4.4 and depicted in Figure 4.1. The Table 4.4 clearly showed the significant difference ($P<0.05$) among the IC_{50} of the fractions with fraction B having the lowest IC_{50} (121.51±5.23 µg/ml) indicating its high free radical scavenging activity as compared to fractions C and D.

Table 4.1: Percentage Inhibition of ACE activity by the Aqueous Extract of *C. micranthum* Leaves

Sample	% Inhibition
Aqueous	59.43±4.00[b]
Captopril	83.02±2.67[b]

One-way ANOVA, Values with different superscript down the column differs significantly at P<0.05.Data are expressed in mean ±Standard deviation

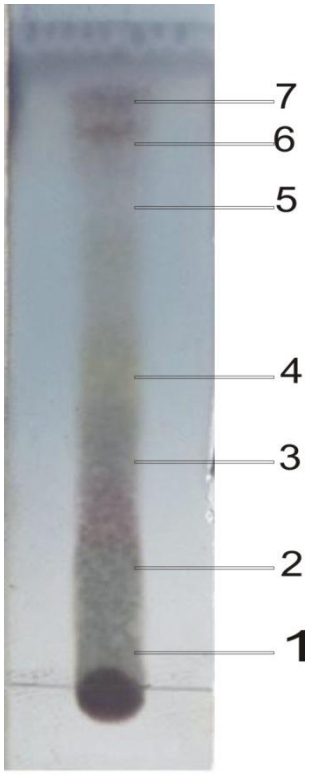


Plate II: Thin Layer Plate (TLC) Profile of the Aqueous Crude Extract using EA: Methanol (8:2).

Table 4.2: Column Chromatography of Aqueous Extract of *C. micranthum*

Fraction	Collection	Number of Spot
A	1-5	6
B	6-13	9
C	14-41	4
D	42-75	0

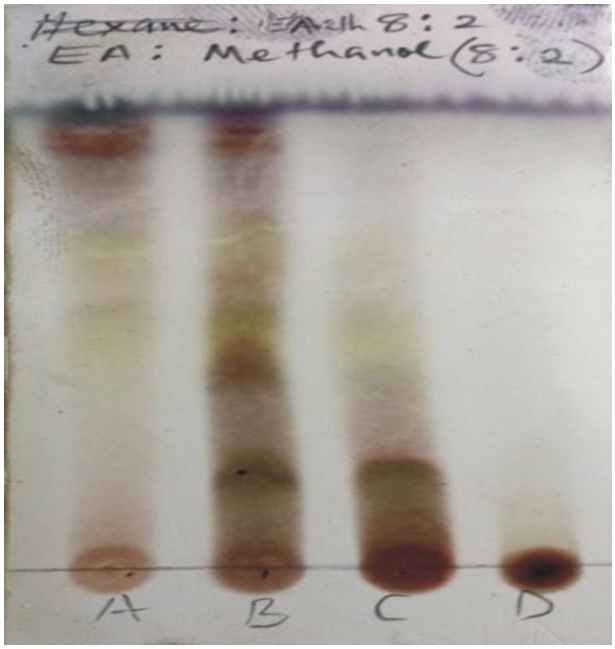
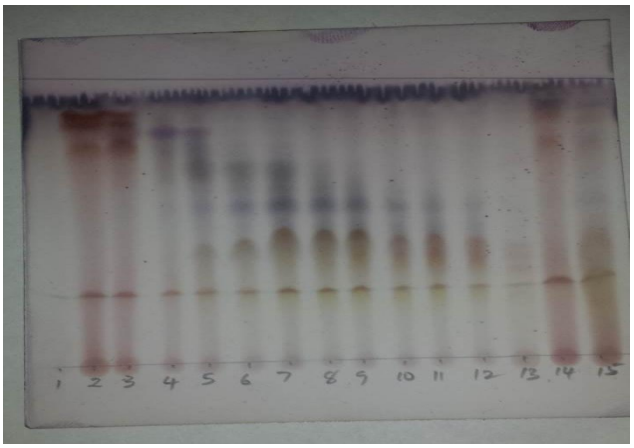
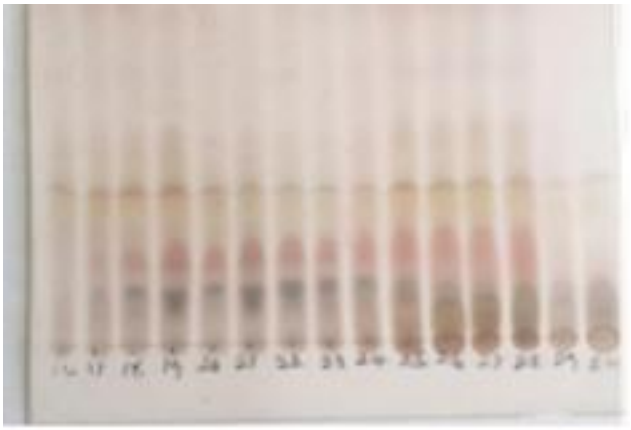


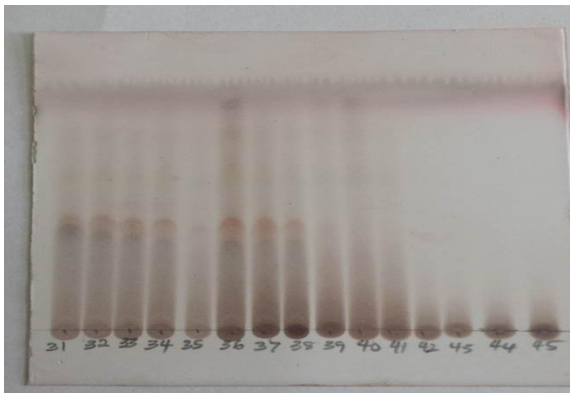
Plate III: Thin Layer Plate (TLC) Profile of the Fractions (A-D)



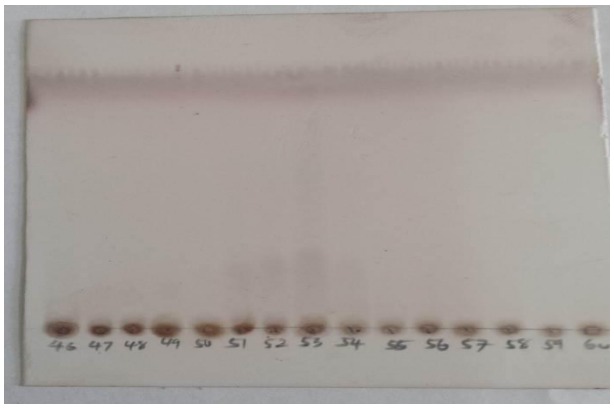
100 % EA (1-15)



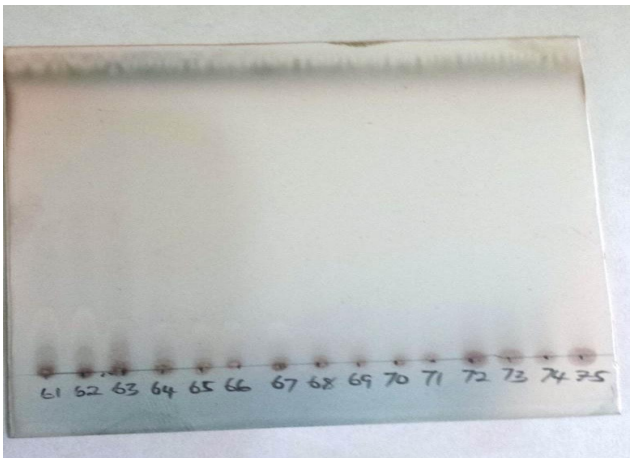
EA:Methanol 3:2 (16-30)



EA:Methanol 2:3 (31-45)



EA:Methanol 1:4 (46-60)



100 % Methanol (61-75)

Plate IV: TLC Profiles of Column Chromatographic Fractions of Aqueous extract

Table 4.3: Percentage Inhibition of ACE activity by the Column Chromatographic Fractions of Aqueous extract of *C. micranthum* Leaves

Fraction	% Inhibition
A	8.21± 41.19[a]
B	97.69± 8.57[b]
C	78.32 ±7.14[b]
D	98.32 ±2.66[b]
Captopril	86.16±5.76[b]

One-way ANOVA, Data are expressed as mean±standard n=3. Values with different superscript down the column differ significantly at P≤0.05.

Table 4.4: Total Phenolic Content and Free Radical Scavenging Activity IC_{50} of Column Chromatographic Fractions of *C .micranthum* Leaves

Fraction	TP (mg/g) GAE	IC_{50} (µg/ml)
B	252.50±5.62[a]	121.51±5.23[d]
C	118.30±1.27[b]	255.55±19.59[e]
D	55.73±2.56[c]	308.83±14.60[f]

One-way ANOVA, Data are expressed as mean±standard n=3. Values with different superscript down the column differ significantly at $P \leq 0.05$. IC_{50} = Inhibitory Concentration at 50%.GAE = Gallic Acid Equivalent

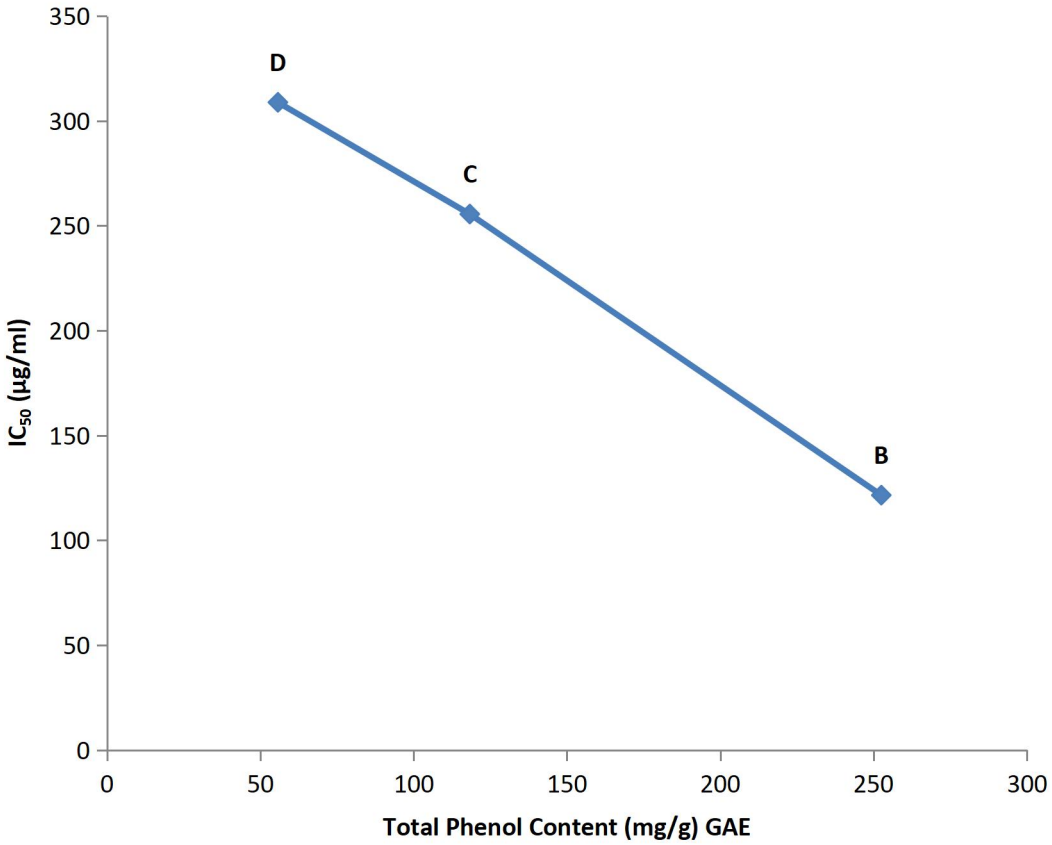


Figure 4.1: Relationship between the IC_{50} and the TPC of the Fractions

As Total phenol increases, the IC_{50} decreases which implies positive correlation between the total phenol and free radical scavenging activity.

4.6 Reducing power of Colomn chromatographic Fractions of *C. Micranthum* Leaves

Reducing Power of Aqueous Extract Column Chromatographic Fractions is shown in Table 4.5, fraction D shows significant ($P<0.05$) lower reducing power when compared with fraction B and C at 100µg/ml, 200 µg/ml and 300µg/ml. The result has shown clearly that fraction B possesses the highest reducing power as compared to fraction C and D at the various concentrations.

4.7 Total Antioxidant Capacity of Column Chromatographic Fractions of *C. Micranthum* Leaves

The aqueous extract fractions of *C. micranthum* leaves showed very potent total antioxidant capacity. The results is presented inTable 4.6 where fraction B shows a significantly ($P>0.05$) higher total antioxidant capacity as compared to fractions C and D.

4.8 Phytochemical Constituents of Column Chromatographic Fraction B of *C. Micranthum* Leaves

Considering the ACE inhibitory activity, total phenolic content, free radical scavenging activity and total antioxidant capacity assessment of the various fractions, it has been adjudged that fraction B is the most active fraction of all in terms of ACE inhibitory and the antioxidant activity of the aqueous extract of C. *micranthum*. Hence, fraction B was selected for further analysis using GC-MS to identify the possible active components that were responsible for such better performance noticed as compared to fraction C and D. The GC-MS phytochemical screening of fraction B of the aqueous extract of *C. micranthum* as shown on Table 4.7 revealed the presence of Megastigmatrienone, 3,5-Dimethoxy-4-hydroxyphenylacetic acid and Estra-1,3,5(10)-trien-17β-ol with retention time of 32.745, 38.629 and 39.139 minutes respectively.

Table 4. 5: Reducing power of column chromatographic Fractions of Aqueous extract of *C. micranthum* leaves

Fraction	100ug/ml	200ug/ml	300ug/ml
B	0.75 ± 0.04^{a}	1.26 ± 0.056^{a}	1.72 ± 0.11^{a}
C	0.68 ± 0.05^{a}	1.11 ± 0.03^{b}	1.42 ± 0.08^{b}
D	0.39 ± 0.04^{b}	0.52 ± 0.00^{c}	0.67 ± 0.03^{c}

One-way ANOVA, Data are expressed as mean±standard n=3. Values with different superscript down the column differ significantly at $P \leq 0.05$.

Table 4. 6: Total Antioxidant Capacity of Aqueous Extract Column Chromatographic Fractions of *C. Micranthum* Leaves

Fraction	TAC (µg AA/mg of Extract)
B	16.40 ± 0.89^{b}
C	1.10 ± 0.14^{a}
D	2.08 ± 0.32^{a}

One-way ANOVA, Data are expressed as mean±standard n=3. Values with different superscript down the column differ significantly at P≤0.05.

TAC=Total Antioxidant Capacity; AA= Ascorbic Acid

Table 4. 7: Identified Compounds of the Fraction B of the Aqueous Extract of *C. Micranthum* Leaves by GC-MS

S/No	Compound	Retention Time (min)	% Similarity
1	Megastigmatrienone	32.745	94
2	3,5-Dimethoxy-4-hydroxyphenylacetic acid	38.629	64
3	Estra-1,3,5(10)-trien-17β-ol	39.139	99

CHAPTER FIVE

5.0 DISCUSSION

High blood pressure is a silent killer, causing several serious diseases such as heart failure, kidney failure and stroke (Maghrani *et al*, 2005). There are a number of choices for the treatment of hypertension among population ranging from lifestyle, diet and the use of synthetic drugs and traditional herbs. Some of the treatment options using synthetic drugs include diuretics, β-blockers, calcium channel blockers and angiotensin II receptor blockers as well as angiotensin converting enzyme inhibitors (Niusha *et al.,* 2013). Angiotensin converting enzyme is a zinc metallopeptidase that converts the angiotensin I (inactive decapeptide) to angiotensin II (a potent vasoconstrictor) and bradykinin (a hypotensive peptide) to inactive components and consequently, high ACE activity leads to increased concentration of angiotensin II and decreased concentration of bradykinin thereby leading to hypertension (Salah *et al.,* 2001). Therefore, development of agents that inhibit the conversion of angiotensin I to angiotensin II and the breakdown of bradykinin to inactive components began as a therapeutic strategy to treat hypertension. Natural products and active substances derived from medicinal plants could as well be important sources of ACE inhibitors such as captopril, a synthetic antihypertensive drug, which was developed by changing and optimizing the structure of the venom of the Brazilian viper (Cushman and Ondetti, 1991).

In this study, aqueous extract and its partially purified fractions of C. *micranthum* leaves (a plant whose leaves have been previously used for treatment of hypertension in traditional medicine in Africa for many decades) were found to exhibit ACE inhibitory activity. Similarly, ACE inhibitory activity was previously reported of plants such as *Rubus Sp, Crataegus microphylla and Onopordon acanthium* that were all used in management of hypertension in traditional medicine without any scientific verification in the past (Niusha *et*

al., 2013). In addition, antihypertensive activity owe to ACE inhibitory activity is reported in other traditional medicinal plants including *Crataegus oxyacantha* (Lacaille *et al.,* 2001), *Onopordon leptolepis* and *Onopordon carmanicum* (Esmaeili and Saremnia, 2012). Hence, this research revealed that the reported ACE inhibitory activity of C. *micranthum* leaves extract could be one of the possible mechanisms (since other hypotensive mechanisms such as beta receptor blockers and calcium antagonists that were not assessed could be present) while this plant has been effectively utilized for the treatment/management of hypertension in the folklore. However, the result did not show any significant difference ($P<0.05$) between the ACE inhibitory activity of the aqueous extract and that of the standard drug captopril. Equally, the ACE inhibitory performance of the various fractions were comparable to that of the standard drug at $P<0.05$ except for fraction A.

Reactive oxygen species (ROS) play a significant role in cardiovascular diseases such as hypertension and congestive heart failure. In hypertensive patients, angiotensin II increases chronically and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated which causes a rise in ROS (Griendling *et al*., 1994). Angiotensin II also stimulates the production of superoxide anion and hydrogen peoxide in polymorphonuclear leukocytes which inactivate the vasodilatory endothelial derived vascular relaxing factor (nitric oxide-NO) and proatacyclins (PGI2) (Elbl & Wagner, 1991) and as a result, it is more beneficial for an antihypertensive drug to have antioxidant effect. This study also investigated C. *micranthum* leaves for indicators of antioxidant potential such as total phenolic content and antioxidant activity itself in addition to its ACE inhibitory activity.

Plant phenolics, flavonoids and ascorbic acid constitute major groups of phytochemicals acting as primary *in vitro* antioxidants or free radical terminators (El-Sayed *et al*., 2012). Therefore, it was reasonable to determine their concentrations in the various fractions with the aim of utilising the fraction with the highest concentration of *in vitro* antioxidants

(Kumbhare *et al*., 2012). Also, plants with high phenolic contentshave shown high flavonoid content and antioxidant activity as reported for other plant species (Makepeace *et al*., 1985); that is to say, polyphenols, flavonoids and ascorbic acid scavenging potentials is (Wang *et al*., 2008) dependent upon their unique structure, the number and position of the hydroxyl groups (Pazos *et al*., 2005). The potential health benefits associated with these phytochemicals has generated great interest among scientists for the development of natural *in vitro* antioxidant compounds from plants (Rohman *et al*., 2010; Masoumeh *et al*., 2011).

The aqueous C. *micranthum* leaves extract fractions were found to contained phenol and consequently showed free radical scavenging activity as well as total antioxidant activity. These findings from this present study are totally in agreement with the earlier study carried out by Niusha *et al.* (2013) where *RubusSp, Crataegus microphylla and Onopordon acanthium* were investigated for both ACE inhibitory and antioxidant activities and were equally found to possess both activities. However, fraction B showed a significantly ($P<0.05$) good performance as compared to fraction C and D in terms of the phenolic content, free radical scavenging activity expressed as $IC5_0$ and the total antioxidant capacity. Furthermore, it was also found out that there was an inverse relationship between the free radical scavenging activity and the phenolic content indicating that the higher the phenolic content, the lower the IC_{50} but, the better the total antioxidant performance which corresponds to the findings of Giri *et al.* (2013) where extracts from *Terminali achebula,Terminali abellirica* and *Bergenia ciliate* demonstrated a similar positive correlation between the total phenolic content and the free radical scavenging activity IC_{50}. Presence of reductones in medium such as plant extracts caused reduction of Fe^{3+}/Ferric cyanide complex to ferrous form when monitored spectrophotometrically (Jamuna *et al.*, 2014). Consequently, the three selected fractions (B, C and D) of aqueous extract of *C. micrathum* were confirmed for their reducing capacity with the fraction B showing the highest reducing tendency. Hence, the overall

performance of the antioxidant activity of the various fractions of the aqueous extract of *C. micranthum* leaves may be as well among other antioxidant mechanisms possibly connected to presence reductones in them.

The larger molecules like Megastigmatrienone, 3,5-Dimethoxy-4-hydroxyphenylacetic acid and Estra-1,3,5(10)-trien-17β-ol found in *C. micranthum* leaves extract may provide more hydroxyl and heterocyclic oxygen groups for the ACE inhibition as it was experienced with some flavonoids (Ojeda *et al.,* 2010), anthocyanins (Kwon *et al.,* 2010), isoflavones (Montenegro *et al.,* 2009) that have proved to be effective in decreasing the ACE activity.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMMNDATIONS

6.1 Summary

The findings from this present study can be summarised as follows:

i. Aqueous extract and fract ions of *C. micranthum* leaves were able to inhibit the *in vitro* activity of ACE.

ii. Fraction B has the highest phenolic content and the total antioxidant capacity as compared to fraction C and D.

iii. The phytochemical constituents from the GC-MS analysis of fraction B of the aqueous extract of *C. micranthum* leaves revealed the presence of Megastigmatrienone,

3,5-Dimethoxy-4-hydroxyphenylacetic acid and Estra-1,3,5(10)-trien-17β-ol.

6.2 Conclusion

Aqueous extract of *C. micranthum* leaves and its fractions have shown the tendency to inhibit the *in vitro* activity of ACE which may be the mechanism while it has been used in the past for the treatment of hypertension in the traditional medicine. Similarly, the various fractions demonstrated antioxidant activity that would be helpful in the management of hypertension that is accompanied with the generation of free radical species.

6.3 Recommendations

From the study, we recommend that the aqueous fraction of *C. micranthum* leaves possess *in vitro* antioxidant and ACE inhibitory effect.

More work should be done on the *in vivo* inhibitory effect of aqueous extract of *C. micranthum.*

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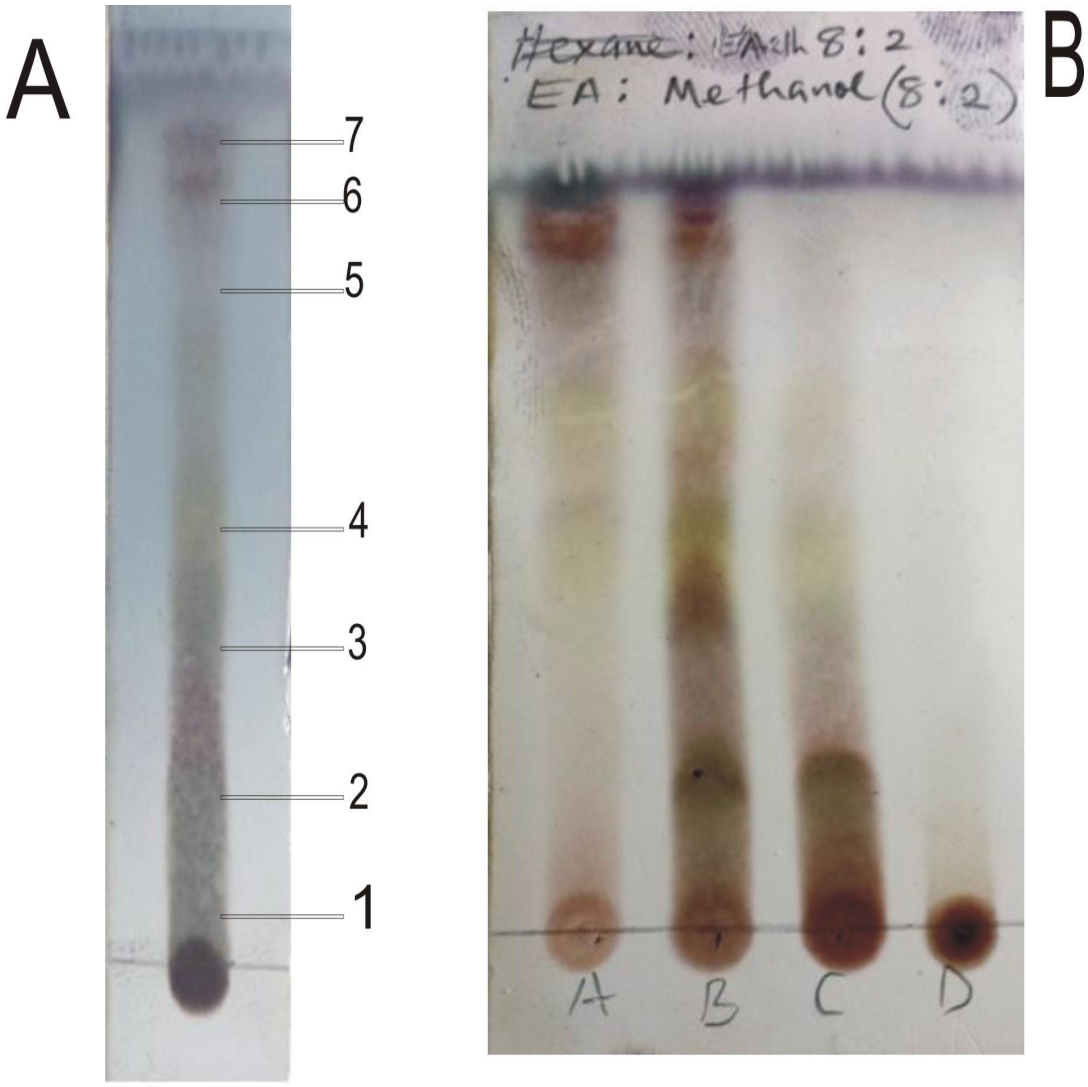
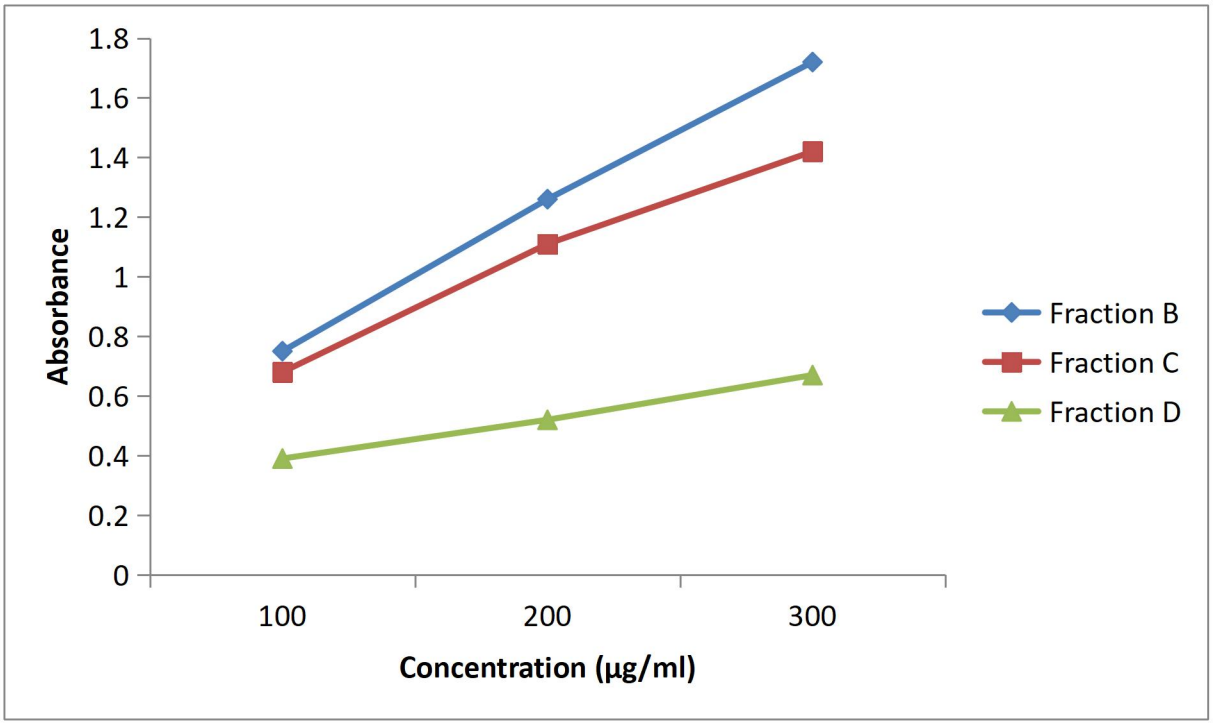
APPENDICES

Appendix 1.0



Appendix 2.0

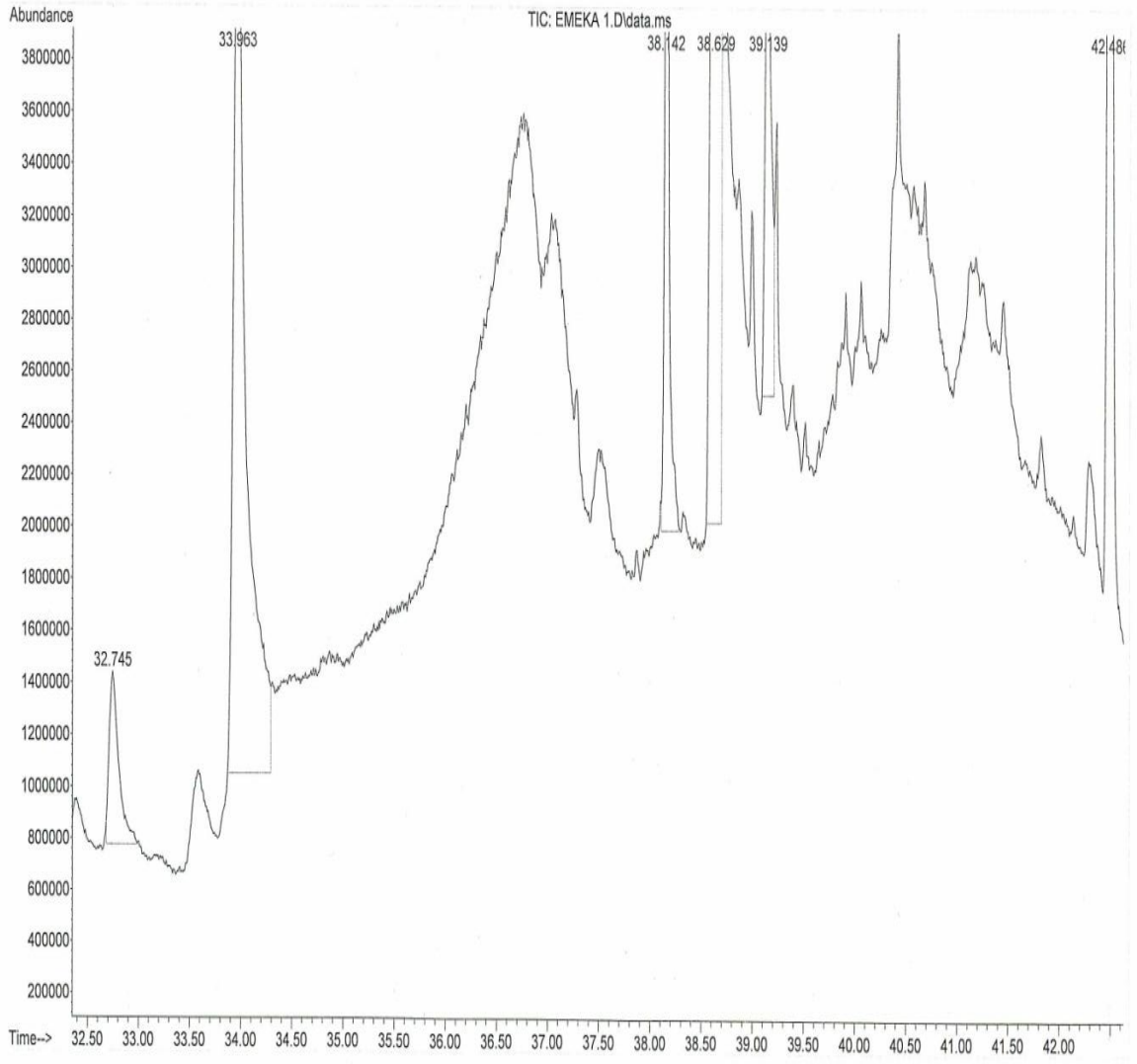
APPENDIX 3.0



Reducing Power of Column Chromatographic Fractions of *C.micranthum*Leaves

APPENDIX
4.0

File :C:\msdchem\1\Essential Oils\EMEKA 7\EMEKA 1.D
Operator : EMEKA
Acquired : 13 Aug 2016 12:02 using AcqMethod ESSENTIAL OILS_SCAN.M
Instrument : MSD
Sample Name: RUKAYA LAWAL
Misc Info :
Vial Number: 2



APPENDIX

5.0

CENTRAL RESEARCH LAB.ILORIN. Area Percent Report

Data Path : C:\msdchem\1\Essential Oils\EMEKA 7\
Data File : EMEKA 1.D
Acq On : 13 Aug 2016 12:02
Operator : EMEKA
Sample : RUKAYA LAWAL
Misc :
ALS Vial : 2 Sample Multiplier: 1

Integration Parameters: rteint.p
Integrator: RTE
Smoothing : ON Filtering: 5
Sampling : 1 Min Area: 3 % of largest Peak
Start Thrs: 0.2 Max Peaks: 100
Stop Thrs : 0 Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method : C:\Users\admin\Desktop\METHODS\ESSENTIAL OILS_SCAN2.M
Title :

Signal : TIC: EMEKA 1.D\data.ms

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	32.745	3513	3521	3552	rVB6	663883	4376392	12.76%	3.740%
2	33.963	3664	3676	3718	rBV	3660809	31616730	92.19%	27.016%
3	38.142	4203	4208	4227	rVB	3765234	10444944	30.46%	8.925%
4	38.629	4259	4270	4278	rBV4	4267340	28180155	82.17%	24.080%
5	39.139	4328	4335	4343	rBV2	1894533	8116304	23.67%	6.935%
6	42.486	4753	4761	4784	rVB	11946691	34294297	100.00%	29.304%

Sum of corrected areas: 117028822

ESSENTIAL OILS_SCAN2.M Sat Aug 13 19:39:11 2016