Isolation and Bioassay evaluation of Angiotensin Converting Enzyme Inhibitory compounds of *Centella asiatica.*

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A thesis submitted in partial fulfilment of the requirement for the degree of Master of Pharmacy in Pharmacology in the Faculty of Natural Sciences,

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DECEMBER 2019

ABSTRACT

Hypertension is by far the most common risk factor for cardiovascular disease (CVD), which has been identified as the leading cause of death worldwide and a major economic burden in developing countries. Over the years, there has been an increased interest in isolating and identifying bioactive compounds from medicinal plant, with the aim of finding alternative sources of therapy to some of the problematic synthetic drugs and to validate the therapeutic use of some traditional plants. The renin angiotensin aldosterone system is a key regulator of blood pressure, on which ACE (Angiotensin Converting Enzyme) inhibitors act and have been at the forefront of therapeutic strategy for treatment and management of hypertension and CVD. But despite the success of ACEI's, their long term use has been associated with side-effects coupled with its contraindication in pregnancy. The plant of interest, *Centella asiatica* is a widely known medicinal plant, used in treatment of a variety of conditions including hypertension. There is currently no scientific evidence validating its claimed use in hypertension. This study therefore, investigated the ACE inhibitory effects of *Centella asiatica.*

Crude methanol, ethanol and aqueous extracts of *Centella asiatica* were assayed for ACE inhibition activity. Methanol and ethanol crude extract(s) was subjected to a bioassay guided fractionation process to isolate and identify the active compounds. A fluorescence based ACE assay was utilized at various stages of the process including HPLC purification stage to screen the fractions and compounds for ACE inhibition activity.

Bioassay guided fractionation of the methanol extract yielded three known compounds namely **3,4′,5,7-tetrahydroxyflavone (M_{4B}) (Kaempferol)**, 3**,5,7,3′,4′- pentahydroxyflavone**(**M_{5B})**

(Quercetin) and 4 Hydroxybenzoic acid. Of the three compounds 3, 4′,5,7-tetrahydroxyflavone (M_{4B}) $_{Kaempferol}$, 3,5,7,3′,4′- pentahydroxyflavone(M_{5B}) $_{Quercetin}$ inhibited 50% concentration of ACE at 542.39µg/ml and 302.72µg/ml respectively whereas 4 Hydroxybenzoic acid showed no activity. The activity of the compounds was compared to its reference standard Captopril which inhibited 50% ACE inhibition at 18.63ng/ml. Compound 3, 4′,5,7-tetrahydroxyflavone (M_{4B}) $_{Kaempferol}$, 3,5,7,3′,4′- pentahydroxyflavone(M_{5B}) $_{Quercetin}$ showed 53% and 73% ACE inhibition at 1mg/ml compared to Captopril (98.5%). In addition to the ACE inhibition activity, both compound showed good antioxidant activity comparable to the reference standard ascorbic acid.

In conclusion, *Centella asiatica* showed considerable ACE inhibition effect. Its antihypertensive effect could be enhanced by the synergistic effect of ACE inhibition and antioxidant activity.

Keywords: Cardiovascular disease, Hypertension, ACE inhibitors, Renin Angiotensin System Medicinal plants, *Centella asiatica*, Bioassay guided fractionation, Bioassay, Oxidative stress, Antioxidant activity.

DECLARATION

I declare that the thesis **Isolation and Bioassay Evaluation of Angiotensin Converting Enzyme Inhibitory compounds of *Centella asiatica*** is my own work, and has not been submitted before for any degree or examination in any other University and that all the resources I have used or quoted have been indicated and acknowledged by means of complete referencing.

Emmanuel Ichoku.

Signature: …….

31st December,2019.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to God almighty who has guided me and given me the strength and wisdom to pursue this Master's Degree

A very special thanks and my sincere gratitude to my supervisor Dr Kenechukwu Obikeze for accepting me as his master's student and for his great support, patience and guidance throughout this research.

I would like to specially thank Professor Denzil Beukes for his assistance with the chemistry aspect of the research and for his guidance and willingness to always assist

Finally, I wish to express my utmost gratitude to the following people who contributed to making this journey a more enjoyable one:

- My parents for continuous encouragement and moral support
- My wife Mrs. Amilomo Wendy Ichoku for her patience and love.
- Prof Edith Beukes for her assistance with NMR training and Analysis.
- Mrs. Eloise Kuhn for her great support and assistance.
- Mr. Yunus Kippie, Mr. Adeyemi Adegoke, and Miss Lerata Mookho, for their technical assistance in making this research less time consuming and enjoyable.
- Mr. Yves Tchakounte for his encouragement
- National Research Fund South Africa for funding the research
- My fellow master's students in the School of Pharmacy especially the pharmacology research group.
- UWC School of Pharmacy.

DEDICATION

To my Parents Mr and Mrs Fidelis. S. Ichoku and My wife Mrs Amilomo. W. Ichoku

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACEi's	Angiotensin Converting Enzyme inhibitors
AT_1	Angiotensin Receptor 1
BP	Blood Pressure
BP	Blood Pressure
CV	Cardiovascular
CVD	Cardiovascular diseases
d	Doublet
DBP	Diastolic blood pressure
dd	Doublet of a doublet
DMSO	Dimethylsulphoxide
EC50	Half Maximal Effective Concentration
ECG	Electrocardiogram
ESI	Electrospray Ionization
ET-1	Endothelin One
EtOAc	Ethyl acetate

FTIR	Fourier Transform Infrared
HHL	Hippuryl-Histidyl-Leucine
HOPE	Health Outcomes Prevention Evaluation
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
HRMS	High Resolution Mass Spectroscopy
IC50	Half Maximal Inhibitory concentration
IR	Infra-Red
LMICs	Low and Middle Income Countries.
m	Multiplet
MS	Mass Spectroscopy
Multi	Multiplicity
NCE's	New Chemical Entities
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OPA	Opthaldialdehyde
PTLC	Preparative Thin Layer Chromatography
q	Quartet
RAAS	Renin Angiotensin Aldosterone System
s	Singlet

SBP	Systolic Blood Pressure
TLC	Thin Layer Chromatography
UPLC	Ultra Pressure Liquid Chromatography
UV-Vis	Ultra Violet Visible
WHO	World Health Organization
δ	Chemical shift (ppm)

Chapter 1

Introduction

1.1 Background on Cardiovascular Disease: Epidemiology and Risk factors

Globalization, economic transition and industrialization has advanced non-communicable diseases such as cardiovascular diseases, cancer, diabetes, and chronic lung disease to the position of world leading cause of mortality (**Zimmet and Alberti, 2006)**. According to WHO, cardiovascular diseases are the leading cause of death in the world and a major barrier to sustainable human development **(Horton, 2013).** In 2011, the United Nations formally recognized non-communicable diseases, including CVDs, as a major concern for global health and set out an ambitious plan to dramatically reduce the effect of these diseases in all regions **(Alwan *et al.*, 2010)**. In 2008, it accounted for 30% of worldwide deaths, which is an estimated 17 million deaths from an annual total of 57 million **(Paolo *et al*, 2016).** CVD comprises a group of disorders of the heart and blood vessels namely hypertension, coronary heart disease, cerebrovascular disease, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure.

The **WHO Global status report 2010** for non-communicable diseases highlighted the steady rise of CVD mortality in LMICs (Low and Middle Income Countries) with rates of up to 300 – 600 deaths attributed to CVD per 100,000 populations, this was projected to increase causing preventable loss of live **(WHO, 2010)**. According to **Paolo *et al* (2016)** an alarming 80% of these deaths are identified to occur in low- and middle-income countries (LMICs).

CVD is attributed to traditional risk factors such as high blood pressure, tobacco use, excessive alcohol use, high blood glucose, high blood cholesterol, high body mass index, physical inactivity and poor dietary choices. All these factors cumulatively account for 61% of CVD

deaths globally. Hypertension is considered the leading risk factor for CVD, accounting for 9.4 million deaths globally with an estimated 1 billion people with uncontrolled hypertension as of 2008 **(WHO, 2013).** It has been predicted that adults with hypertension will increase by about 60% to a total of 1.56 billion in 2025, with disproportionate prevalence in developing countries including those in sub-Saharan Africa (Kearney *et al*., 2005)**.**

According to a study by **Msenburi *et al* (2014),** more South Africans die of CVD than all of the cancers combined. Though distribution of risk factors for CVD differ amongst various populations**,** hypertension remains the most significant of the risk factors (**Fatema, Zwar, Milton, and Ali, 2016)**. It is also a major comorbidity for type 2 diabetes and end stage renal disease. According to a review by the European Heart Journal based on the INTERHERT study, hypertension and obesity are more important problem (risk) factors in South Africa as compared to elevated cholesterol in countries like China (Gersh, Sliwa, Mayosi, and Yusuf, 2010). A Soweto hospital-based study by **Sliwa *et al* (2008)** showed that 50% of all CVD patients present with hypertension, half of whom are obese.

Dietary modification, weight loss, exercise, and pharmacological interventions such as the use of angiotensin converting enzyme inhibitors (ACEIs), beta-blockers, calcium channel antagonists, and diuretics are utilized as a combination therapeutic strategy for treatment of hypertension and cardiovascular disease. Angiotensin converting enzyme (ACE) inhibitors are an established therapy widely used for the treatment of cardiovascular disease since they improve blood pressure, and prolong survival in patients with acute myocardial infarction, asymptomatic left ventricular dysfunction, congestive heart failure and renal dysfunction (Loizzo *et al*., 2006)**.** Pharmacological interventions that exist to treat hypertension have been successful. However, the prolonged use of these drugs could initiate negative side effects such as cough, dry throat, allergic reactions, dizziness, angioedema and kidney failure **(Israili and**

Hall, 1992, Sica, 2007). Moreover, contraindication of ACEIs in pregnancy does preclude its therapeutic use in some situations **(Oh, Kang, and Lee, 2002).**

Over the years, there has been an increased exploration of new alternatives as replacements for existing problematic drugs, the search for more effective and safer drugs has been increasingly directed and targeted at bioactive compounds from natural sources such as medicinal plants. Various classes of plant compounds play a role in their therapeutic property. Flavonoids, anthocyanidins, triterpenes are some examples of bioactive compounds identified from medicinal plants for their various therapeutic properties **(Somova *et al.*, 2003; Actis-Goretta *et al.*, 2006; Ojeda *et al.*, 2010).**

1.2 Use of Traditional medicine

Medicinal plants remain a popular source of therapy in various societies, thus playing a significant role in meeting the primary healthcare needs of the population **(Ibrahim and Damasceno 2012)**. According to the WHO, 60% of the world's population rely on medicinal plants for their primary health care needs, with that rising to 80% in the developing countries. The historic use of traditional medicines initially took the form of crude drugs such as tinctures, poultices, decoctions, solution, and powders before the advent of synthetic drugs (**Balunas and Kinghorn 2005**). The method of use, preparation and application of these traditional medicines were developed by trial and error over centuries, and documented in ancient literature of Chinese, Indian and Egyptian societies. Twentieth century advances in medicine such as the discovery of penicillin and morphine initiated the decline of the use of traditional medicines and the disappearance of many of the traditional indigenous medicinal practices. As a consequence, a great number of these remedies have disappeared with time, although some still presently find use for the treatment of diseases by traditional medicine practitioners of all culture **(Obikeze, 2009).** The investigation of original traditional medicines and phytotherapy

is once again receiving scientific attention, the aim being to develop new effective drugs that are non-toxic and inexpensive, with the latter being most important to developing countries. Additionally, the use of phytomedicines is becoming more scientifically based, with increasing emphasis placed on proven product safety and efficacy (Taylor, Rabe, Mcgaw, Jäger, and Van Staden, 2001)

1.3 The South African Perspective

South Africa boasts an amazing floral diversity, with an estimated 30000 species of higher plants, many of which are endemic to the region. In the developing parts of the world such as South Africa, traditional medicine still forms the backbone of rural and increasingly urban healthcare. A large number of plant species are used for medicinal purposes in South Africa, with more than 20 000 tons of plant materials harvested, processed and sold annually as traditional medicine **(Mander *et al*., 1997)**. Medicinal plants in KwaZulu-Natal alone support traditional health services with a value of more than $30 million annually, with Zulu traditional healers using 1032 species from 147 families (approximately 25% of the flora of KwaZulu – Natal) (Taylor *et al*., 2001; Light, Sparg, Stafford, and Staden, 2005**).** An estimated three million people in South Africa are currently using indigenous traditional plant medicines for primary healthcare purposes (Dyubeni and Buwa, 2012).

Traditional medicine is an integral part of South African culture. It is estimated that about 12 – 15 million people still depend on traditional herbal medicines for their healthcare needs. In most societies, traditional medicine is currently used concomitantly with conventional medicine, with no regulation to that effect therefore raising concerns over safety, and potential toxicity that could result from such use (Fennell *et al*., 2004). Efforts are being made towards investigating and documenting the traditional medicinal use of a number of southern African plants **(Bhat, 2014)** this has been evidenced by an increased contribution of research

on South African medicinal plants over the last 10 years—a trend that is clearly reflected in publications in the *Journal of Ethnopharmacology* (Light *et al*., 2005). There is greater emphasis on ethnopharmacological research as a way of establishing the scientific rationale of traditional medicine and to validate their use. Researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value **(Fennell *et al.*, 2004).**

1.4 Medicinal plants as a source of drug

Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world and higher plants contribute no less than 25% of the total **(Pan *et al*., 2013)**. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century. Early drugs such as cocaine, codeine, digoxin and quinine were all products of drug discovery from medicinal plants. According to **Newman *et al* (2003),** approximately 28% of NCEs (New Chemical Entities) were natural products or natural product-derived. Of the natural products, medicinal plants present a variety of options for drug discovery either as bioactive compounds for direct use as drug e.g. digoxin, or bioactive compounds with structures which themselves may act as lead compounds for more potent compounds.

The advent of synthetic and combinatorial chemistry provided new hope for higher success rate of new chemical entities, but despite its success it has failed to improve the success rate of new drug discovery **(Katiyar *et al*,, 2012).** This scenario has then prompted the adoption of the integrated drug discovery approach, where Ayuverdic wisdom can synergize with drug discovery from plant sources. The initial step in drug discovery is considered the identification of new chemical entities (NCE), which can either be through chemical synthesis or isolation from natural products through bioassay guided fractionation.

Bioassay guided fractionation is a common approach for drug discovery from plants by the pharmacological assessment of crude plant extracts and their fractions. This approach allows for fractions to be screened in pursuit of those that contain biologically active compounds. The active compound is then purified and identified.

1.5 Justification for the study

The World Health Organization estimates that 80% of the world population relies (wholly or partially) on traditional medicine for primary health care. Consequently, they encouraged and championed the movement for countries to integrate traditional medicine into their primary healthcare systems in order to achieve universal health coverage (WHO 2010). Despite the increased acceptance and use of traditional medicine in healthcare, there are concerns relating to lack of authentication, and toxicity emanating from concomitant administration with conventional medicines coupled with the lack of scientific proof of effectiveness and safety. Hence, the increasing research into medicinal plants to validate their claims of traditional medicinal use as well as to discover new drugs and drug leads. Research in the area of medicinal drug discovery has increased over the years, with the increasing need to discover new drugs to replenish the therapeutic armoury for some of the world's chronic conditions. This need is further strengthened by economic cost and side effects of conventional medicines.

The plant of interest is *Centella asiatica,* a plant of the Apiaceae family native to South Africa, Madagascar, India, China, Sri lanka and Indonesia. It *is* one of the most versatile and widely used plants in the world, known to be consumed as green leafy vegetables in countries like Sri Lanka and Philippines **(Orhan, 2012)**. In the South African Market, products such as Gotu Kola Capsules (100% *Centella asiatica*) by Flora Force® and Mentat Syrup (70mg *Centella asiatica*) by Himalaya® contain *Centella asiatica*. Previous studies have shown that the primary active constituents of *Centella asiatica* are saponins, pentacyclic triterpenes and their

glycosides (asiaticosides, asiatic acid, madecassoside and madesiatic acid), alkaloids, flavonoids, amino acids, fatty acids, glycosides, and steroids (Subban, Veerakumar, Manimaran, Hashim, and Balachandran, 2008)**.** This specie is widely used to treat a variety of skin conditions which includes psoriasis, eczema, ulcer, bronchitis and venous hypertension. Several biological activities have been reported for *C. asiatica*, this includes wound healing, antiulcer, antidepressant, antimicrobial, and anti-inflammatory. In addition to these properties, *C. asiatica* has been claimed to be used in the treatment of hypertension and as a diuretic, claims which are yet to be scientifically validated. Review of previous studies have shown *C. asiatica* to contain various classes of bioactive compounds known to exhibit ACE inhibition including triterpenes, flavonoids, amino acids and alkaloids **(Morigwa *et al*, 1986; Hansen *et al.*, 1995; Oh *et al.*, 2003, 2004; Loizzo *et al.*, 2008).**

1.6 Hypothesis

The Hypothesis is that *Centella asiatica* mediates its antihypertensive effect through compounds that inhibit the activity of angiotensin converting enzyme.

1.7 Aim of study

This study therefore, aims to investigate the angiotensin converting enzyme inhibition activity of the methanol, ethanol and aqueous extracts of *Centella asiatica* leaves using a fluorescence based *in vitro* bioassay, and to isolate and identify the ACE inhibitory compound/s from the most active extract or fractions using the bioassay guided fractionation process.

1.8 Objectives of the study

The overall intention of the study is to provide a scientific basis for the use of *Centella asiatica* in the treatment and management of hypertension. The proposed objectives were:

- To assess the ACE inhibitory activity of the methanolic, ethanolic and aqueous extract of *Centella asiatica* leaves extract.
- To isolate bioactive compound/s from the extract exhibiting good ACE inhibitory activity.
- To identify and characterize the bioactive compound/s using spectroscopic techniques.

1.9 Scope of the Thesis

In addition to the **Introduction** Chapter, the thesis presents four more chapters. A brief description of what is discussed in each chapter is outlined below:

Chapters 2 expands more on cardiovascular disease and its main risk factor - hypertension. It reviews the renin angiotensin aldosterone system (RAAS) and its role in cardiovascular disease and hypertension. The role of medicinal plants as source of ACE inhibitors and drug discovery is explored and presented. Finally, it looks at the various processes and steps involved in drug discovery from medicinal plants (Extraction, Isolation, Purification and Identification) which includes types of bioassay utilized in drug discovery process.

Chapter 3 focuses on materials, techniques, procedures and all experimental methodology used to achieve the objectives of the study, as well as the in vitro ACE assay.

Chapter 4 presents and discusses in detail, the ACE inhibitory activity of plant extracts/fractions and isolated compounds, the outcome of the bioassay guided fractionation

process and the spectroscopic isolation and identification of the bioactive compound as well as the structure activity relationship of the isolated compounds.

Chapter 5 is the conclusion, limitation and recommendation drawn from the study outcomes.

Chapter 2

Literature Review

2.1 Hypertension as a major risk factor for CVD

Hypertension is a major driver of CVD in Africa especially stroke and hypertensive heart disease. It is commonly defined as systolic blood pressure (SBP) above 140 mmHg and/or a diastolic blood pressure (DBP) above 90 mmHg. This level of blood pressure can induce heart disease, congestive heart failure, impaired vision and diabetic nephropathy. It is generally described as a silent killer due to its asymptomatic nature and can therefore go undetected for years, affecting organ systems and shortening life expectancy by 10 – 20 years (Xie and Zhang, 2012). Epidemiologic data demonstrates a strong correlation between blood pressure (BP) and cardiovascular (CV) morbidity and mortality **(Kannel *et al*,, 2003).**

2.2. Treatment and management of Hypertension

Blood pressure is controlled by neural mechanisms carried out by the autonomic nervous system and humoral mechanisms involving substances such as nitric oxide (NO) and endothelin-1 (ET-1) that are released by different cell types **(Larson, Symons, & Jalili, 2012)**. Decreased vasodilation, increased vasoconstriction, and greater vascular peripheral resistance characterize hypertension. Treatment for hypertension is a life-long commitment that requires drug therapy in combination with lifestyle changes such as weight reduction if overweight, limitation of alcohol intake and a reduction in salt and fat intake. Treating hypertensive patients with antihypertensive drug therapy provides significant clinical benefits. Epidemiological studies have shown that normalization of blood pressure is associated with a significant reduction in major cardiovascular events. Evidence from large-scaled placebo controlled clinical trials have shown that the increased risks of CV events and death associated with

elevated BP are reduced substantially by antihypertensive therapy (Ettehad *et al*., 2016)**.** CVD and primarily hypertension can be treated with one or a combination of many therapeutic agents namely angiotensin converting enzyme inhibitors, angiotensin receptor blockers, diuretics, beta blockers, calcium channel blockers, vasodilators and centrally acting agents (**Aronow *et al,* 2012)**. ACE inhibitors are established as one of the main therapeutic agents for treatment of hypertension. It has favourable metabolic, renal, cardiovascular, and quality-of-life effects as compared with other regimens **(Niskanen *et al*., 2001).** ACE as a component of the renin angiotensin aldosterone system plays a key role in the homeostatic mechanism of mammals, by contributing to the maintenance of normal blood arterial pressure, cell function and water-electrolyte balance, **(Chen *et al.*, 2009).**

2.3 Role of Renin Angiotensin Aldosterone system and Angiotensin converting enzyme II in Hypertension

The renin angiotensin aldosterone system plays an integral role in the control of blood pressure and preservation of hemodynamic stability through the regulation of extracellular fluid volume, sodium balance, cardiac and vascular trophic effects. One of the key components of RAAS is angiotensin II, a main effector molecule and a potent vasoconstrictor that acts as a systemic hormone (endocrine) or as a locally generated factor (paracrine, autocrine). The over activity of RAAS has been associated with the development of atherosclerosis, hypertension, left ventricular hypertrophy and cardiovascular events such as myocardial infarction, stroke, congestive heart failure, and nephrosclerosis **(Atlas, 2007).**

For the past 20 years, efforts have been focused on pharmacotherapy that attenuates the activity of the renin-angiotensin-aldosterone system (RAAS). Three major classes of drugs that fulfils this purpose are the angiotensin-converting enzyme (ACE) inhibitors, β-blockers, and angiotensin receptor blockers. Each of these therapeutic classes exerts its inhibitory effect in different ways. β-Blockers primarily inhibit renin production, thus dampening the systemic

RAAS. ACE inhibitors inhibit the angiotensin converting enzyme responsible for the cleavage of peptide fragments off angiotensin I to create the active moiety angiotensin II. Angiotensin receptor blockers rather than inhibiting the formation of angiotensin II occupies its high-affinity type 1 (AT_1) receptor, thus inhibiting its ability to exert biological activity **(Weir and Dzau,1999).**

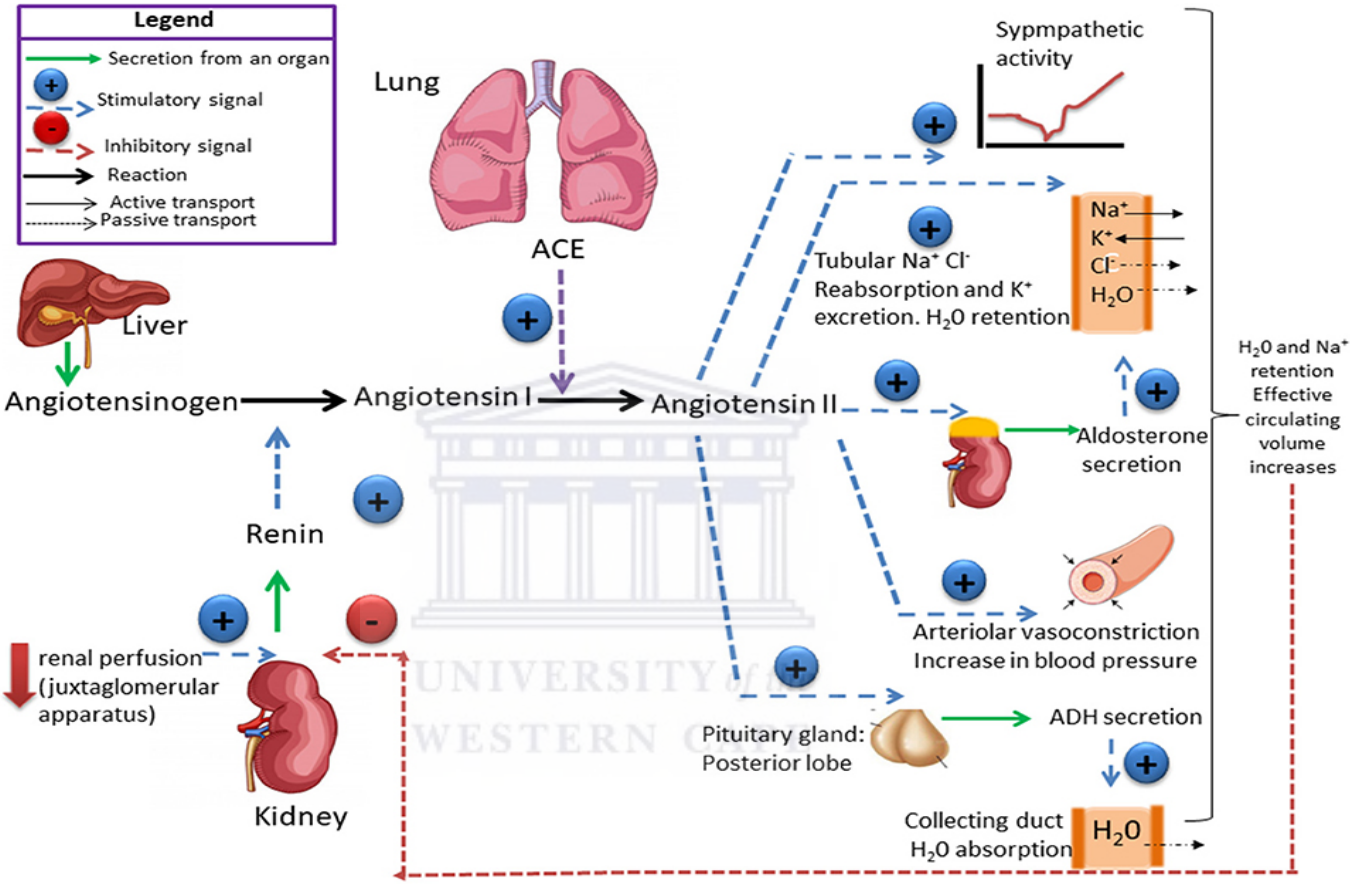

Figure 2. 1 The Renin Angiotensin Aldosterone System (Almeida & Coimbra, 2019)

Angiotensin II, the principal effector peptide of the RAAS, has far reaching effects on vascular structure, growth and fibrosis, and is a key regulator of vascular remodelling and inflammation. Besides its vasoconstrictive effects, angiotensin II through a variety of different mechanisms, whether direct or indirect, can substantially affect the structure, function, and atherosclerotic risk of blood vessels. It stimulates growth, inhibits apoptosis, and promotes smooth muscle cell

growth and migration through a variety of different mechanisms, predominantly by causing oxidative stress and altering the redox potential of the blood vessel. Oxidative stress due to angiotensin II and free radical generation has been implicated in hypertension. ACE inhibitors such as captopril are believed to possess antioxidant activity against these factors. **(Malgorzataí *et al.*, 1997)**. The generation of Angiotensin II by ACE promotes platelet aggregation, thrombosis and endothelial dysfunction. All these factors are known to increase vascular tone, promote remodelling and restructuring and augment atherosclerotic risk(Weir and Dzau, 1999).

ACE is a dipeptidyl carboxypeptidase with a zinc atom. It consists of a single polypeptide chain containing two domains: N and C. Each domain consists of two catalytic sites. The lung capillaries boast the highest concentration of ACE, organs such as renal proximal tubules, gastrointestinal tract, brain tissues and cardiac tissue contain ACE which exist as a membrane bound enzyme as well as a circulatory or globular enzyme **(Ortiz-Salmeróna, Baron, and García-Fuentes, 1998) .** The pivotal role of ACE and subsequently Angiotensin II in the sequence of events constituting the cardiovascular continuum make it a logical target in therapeutic strategies aimed at reducing the overall cardiovascular risk factor profile of an individual. According to Heart Outcomes Prevention Evaluation (HOPE) study, ACE inhibition with Ramipril reduces the risk of a primary cardiovascular event (cardiovascular death, stroke, or acute myocardial infarction) in high risk patients by 22% **(Sleight, 2000)**. ACE activity is inhibited by ACE inhibitors which has three distinct chemical structures namely sulfhydryl containing agents (Captopril), the non-sulfhydryl or carboxylic acid containing agents(Enalapril) and the phosphoric acid derivatives (Fosinopril). These inhibitors bind to the zinc ion in the ACE molecule through their sulfhydryl and phosphoric acid moieties whereas other inhibitory agents bind to the zinc through carboxyl residues **(Duncan, 1998).** ACE inhibition has become a promising approach for drug targeting in the treatment of

cardiovascular diseases such as hypertension, heart failure, and diabetic nephropathy. Despite the benefits associated with ACE inhibitors, they exhibit adverse effects such as bronchospasm, rashes, cough **(Wood, 1995)** as well as contraindication in pregnancy.**(Lip *et al.*, 1997; Steffensen *et al.*,1998).** These side effects coupled with other factors such as high cost of conventional medicine and cultural practices point to the increasing interest in medicinal plants and the need to discover leads for novel drug discovery.

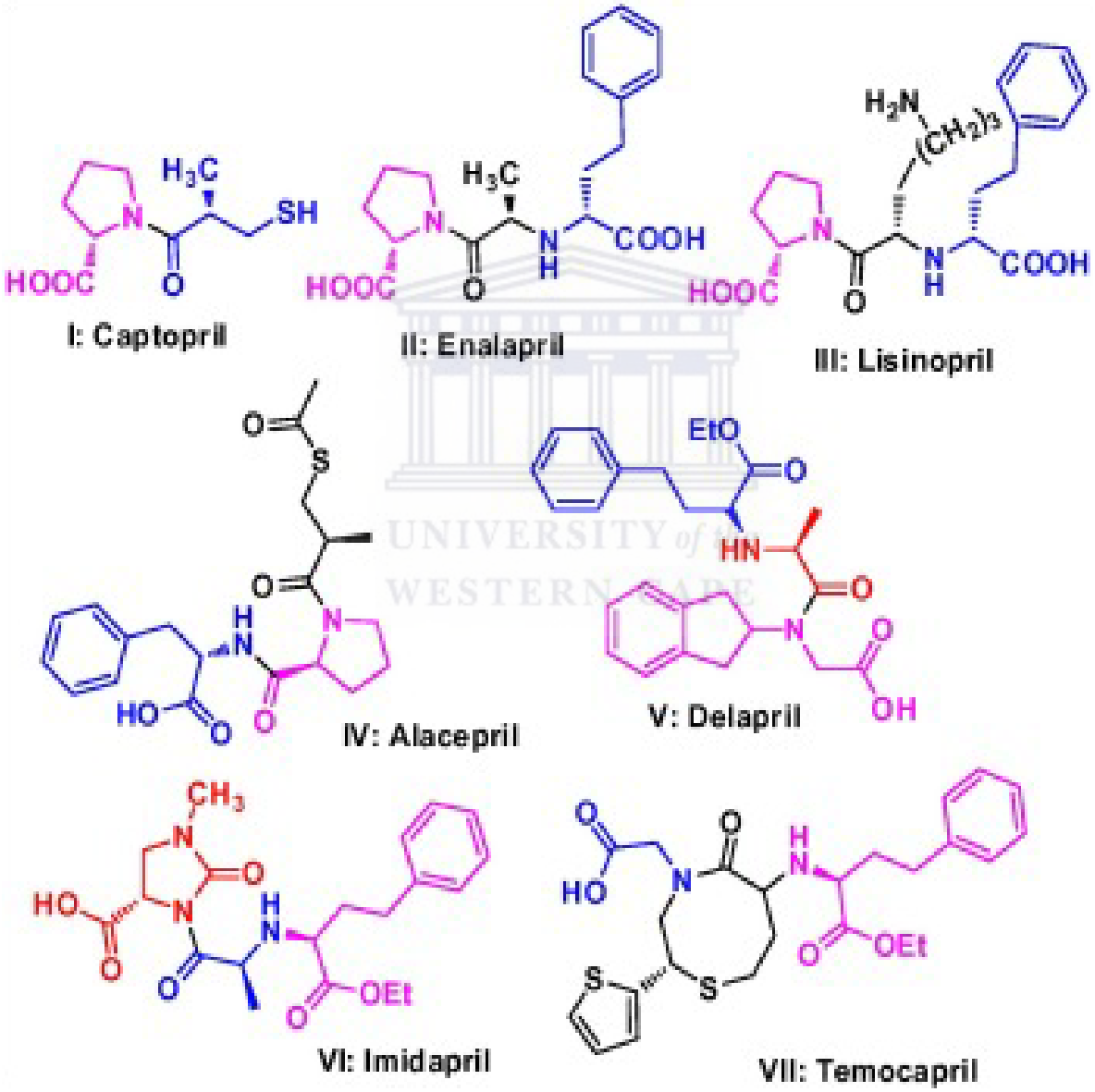

Figure 2. 2 Structures of synthetic ACE Inhibitors (Jallapally *et al.*, 2015).

2.4. Traditional Use of Medicinal plants

Traditional medicine encompasses knowledge systems that have been established over generations based on the concepts, beliefs and practices native to diverse cultures prior to the modern medicine era. Since the beginning of human civilization, medicinal plants have been the backbone of traditional systems of healing throughout the globe and an integral part of history and culture. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. The earliest records dating from around 2600 BC, documents the use of some of the plant derived substances in Mesopotamia. These include oils of the *Cedrus species* (Cedar), *Glycyrrhiza glabra* (Licorice), and *Papaver somniferum* (Poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation. **(Gurib-Fakim, 2006)**

The use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for the treatment of various health challenges in different national health care settings. This past decade has obviously witnessed a tremendous surge in acceptance and public interest in natural therapies both in developing and developed countries. It is estimated that more than 60% of the world's population utilize plants as their primary source of medicinal agents and moreover traditional medicine is still the only health resource available to about 60% of the world population. WHO estimates that between 60% and 90% of Africa's population rely on medicinal plants to totally or partially meet their healthcare needs **(Taylor, 1999).** This is valid considering 60% of South

Africa's population consult one of an estimated 200000 traditional healers. **(Van wyk *et al*, 1997)**. Over 5000 plants are known to be used for medicinal purposes in Africa but only a few have been studied and scientifically validated.

The extensive use of traditional medicine in Africa, which composed mainly of medicinal plants, has been argued to be linked to cultural and economic reasons, this is why WHO encourages African member states to promote and integrate traditional medicinal practices into their health systems. WHO traditional medicine strategy 2014 – 2023 was developed and launched specifically for this purpose. As the use of medicinal plants grows, public health concerns surrounding their safety is increasingly recognized by regulatory authorities and further research is needed to ascertain the efficacy and safety of several of the practices and medicinal plants used by traditional medicine systems **(WHO, 2003).**

2.5. Plant Compounds and their biological activity

Plants consist of a wide array of biologically active compounds which can act singly or synergistically together to confer therapeutic benefit. In medicinal plant study, it is of utmost importance to understand the various classes of chemical components present in a plant. Below are some of these plants compound and their biological activity.

2.5.1. Alkaloids

Alkaloids are a large class of nitrogen containing secondary metabolites of plants, microbes or mammals. They exist as primary, secondary, tertiary or quartenary amines and are famous for their manifold pharmacological activities. *Papavier somniferum* from opium poppy is one of first alkaloids discovered. Alkaloids possess various remarkable biological activities such as antibacterial (berberine), antihypertensive (reserpine), vasodilation (vincamine) and anticancer (vincristine) properties.

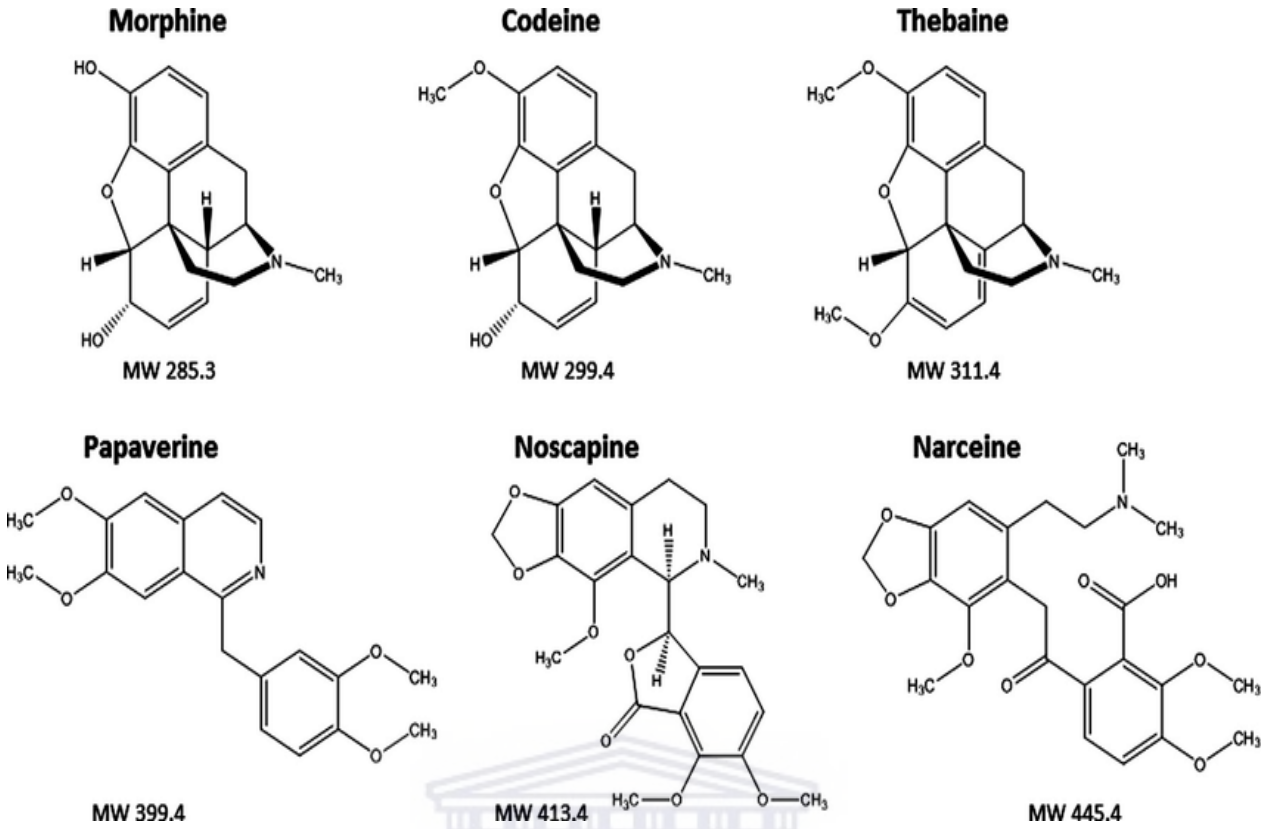

Figure 2. 3 Structure of Alkaloids (López *et al*., 2018).

2.5.2. Terpenes or Terpenoids

Terpenoids make up the largest class of secondary metabolites and are united by their common biosynthetic origin from acetyl-CoA or glycolytic intermediates. Terpenoids compounds are made up of five carbon units often called isoprene units that assemble in a regular pattern, usually head to tail in terpenes up to 25 carbons. They are classified on the basis of their isoprene units into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40). They play different roles in plants such as in defence, thermotolerance, wound healing and pollination of seed crops.

Table 2. 1 Different Classes of Terpenoids and their Examples.

Class of Terpenoids	No of Carbons	No. of isoprene units	Example
Monoterpenoids	10	2	Thymol, Menthol
Sesquiterpenoids	15	3	Ginger, mints, Farnesol
Diterpenoids	20	4	Taxol
Sesterterpenoids	25	5	
Triterpenoids and steroids	30	6	Oleanolic acid
Tetraterpenoids	40	8	B-Carotene

2.5.2.1 Triterpenoids and steroids

The biological activities of triterpenoids have attracted much interest. They are known as cancer chemo-preventive, antiulcer, antidiabetic agents, and inhibitors of angiogenesis. They are the biological active components of several famous herbal medicines, such as ginseng, licorice and bupleurum.

Steroids are triterpene derivatives that are based on the cyclopentane perhydrophenanthrene ring system. They are well known for their biological role as the hormones - testosterone and progesterone. Recent chemical investigation and pharmacological studies reveal various biological roles such as anti-inflammation, anti-cancer, anti-cardiovascular, hypoglycaemic and antifungal activities for steroidal compounds in plants **(Liu, 2011).**

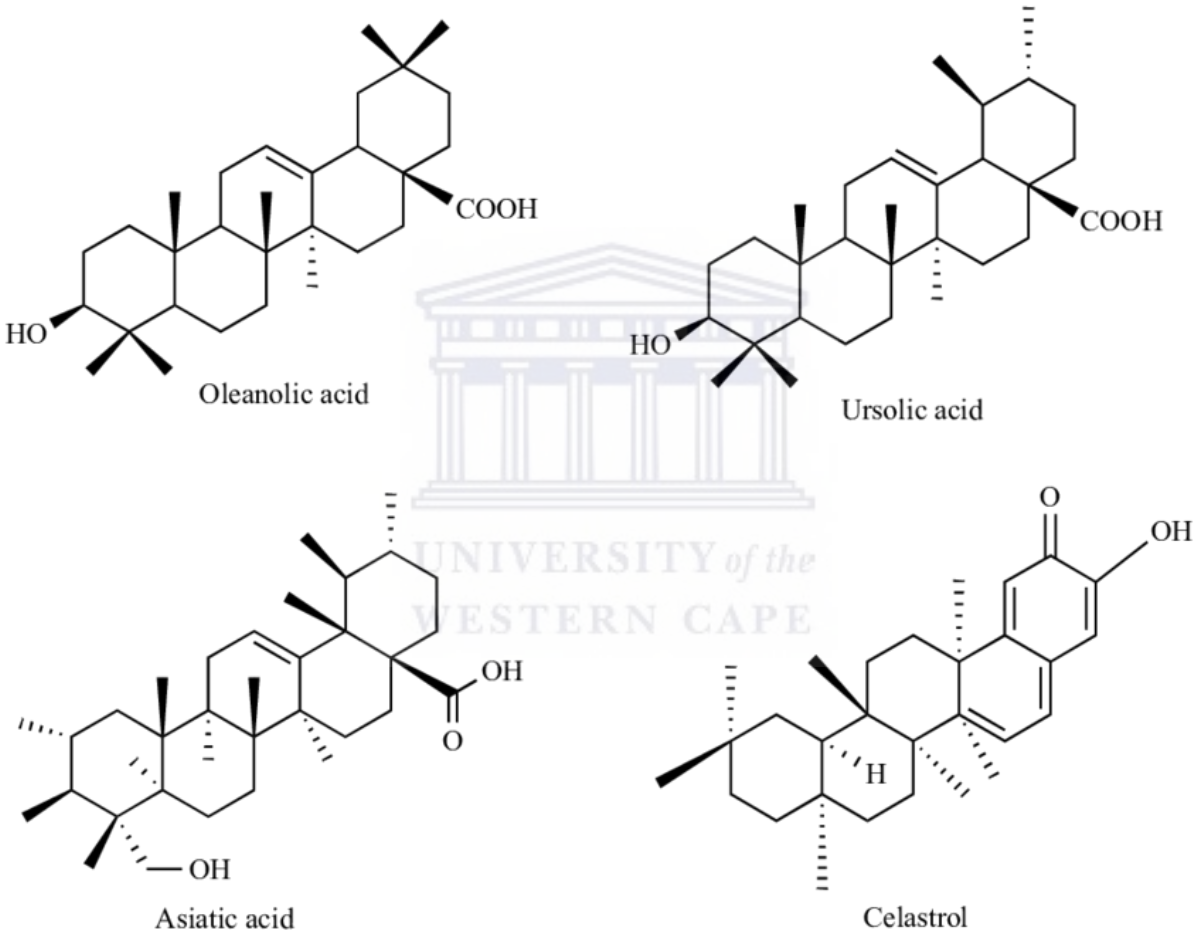

Figure 2. 4 Structures of some Triterpenoids (Ruszkowski & Bobkiewicz-kozlowska, 2014)

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2.5.3 Phenols

Phenols are a group of secondary metabolites characterized by the presence of one or several hydroxyl (OH) groups attached to an aromatic ring. Structures of phenolic compounds are very diverse and occur either as simple compounds with one aromatic ring or as complex polymers (polyphenols) with different functional groups attached. **(Mann J *et al,* 1994).**

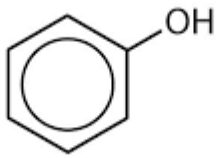

Figure 2. 5 Structure of Phenol

Table 2. 2 Different classes of Phenolic compounds

Class of Phenols	Basic carbon skeleton	No. of carbons
Simple phenols and benzoquinones	C6	6
Naphthaquinones	C6-C4	10
Anthraquinones	C6-C2-C6	14
Flavonoids	C6-C3-C6	15
Tannins	$(C6-C3-C6)_n$	N

2.5.3.1: Flavonoids

Flavonoids are the largest and most diversified group of polyphenolic compounds structurally based on a C_{15} skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4. Flavonoids constitute one of the most characteristic classes of compounds in higher plants, and are responsible for many of the plants colours that dazzle us with their brilliant shades of yellow , orange, or red. Classification of flavonoids is commonly based on the oxidation or saturation of the intermediate C ring. Major groups include the flavonols, flavones, isoflavonoids, and cyanidins.

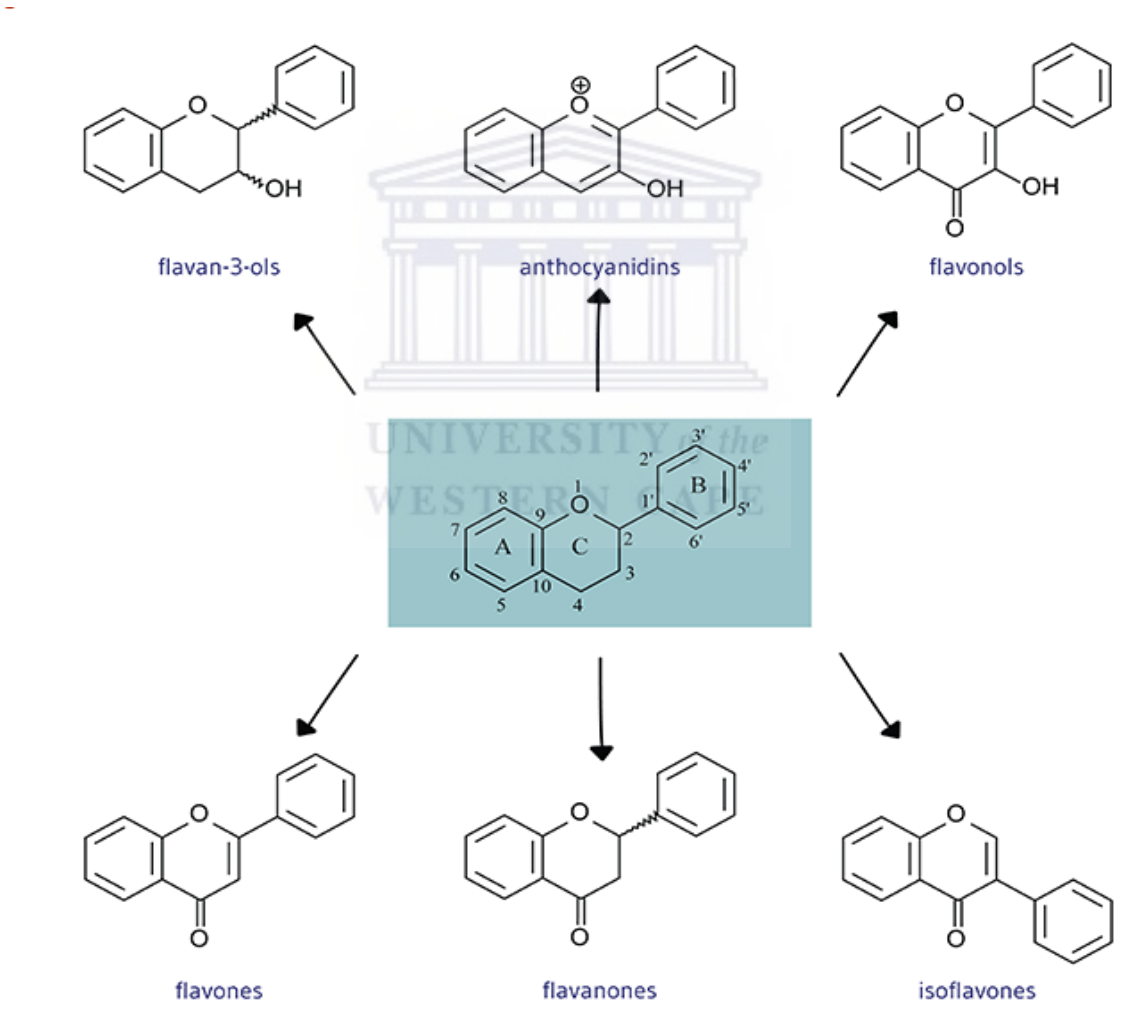

Figure 2. 6 Basic structure of Flavonoid classes (Higdon, 2005)

Flavonoids are most commonly known for their antioxidant activity **(Balasuriya and Rupasinghe, 2011)** The capacity of flavonoids acting as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. It has been discovered that flavonoids also provide other important biological activities such as antibacterial, antiviral, anti-allergic, antiviral, antihypertensive, anti-allergic, antiplatelet, anti-inflammatory and anti-tumour activities **(Kumar and Pandey, 2013).**

2.6. Plants as Sources of ACE Inhibitors

Plants produce and contain a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances or phytochemicals are divided into primary and secondary metabolites. The primary metabolites such as carbohydrates, lipids, and proteins are found in all plants and perform metabolic roles that are essential whereas secondary metabolites, which often are differentially distributed among limited taxonomic groups within the plant kingdom have an ecological role in regulating the interactions between plants and their environment. A number of these secondary metabolites in plants play defensive roles and serve as chemical messengers **(Liu, 2011).**

Secondary metabolites are the main source of pharmaceutical drugs and serve as templates for many medicinal derivatives currently in use. There are three broad categories of plant secondary metabolites; (i) Terpenes and terpenoids, (ii) alkaloids, (iii) phenolic compounds.

A number of compounds from plants have been identified to possess *in vitro* ACE inhibitory activity. These include alkaloids **(Oh *et al.*, 2004),** phenylpropanes, proanthocyanin **(Anderson *et al.*, 2004)**, flavonoids (Oh *et al*., 2004; Loizzo *et al*., 2006), triterpenes and

terpenoids**(Morigwa *et al.*, 1986; Hansen *et al*., 1995)**, xanthones (C. H. Chen, Lin, Lin, and Hsu, 1992), fatty acids and peptides/amino acids (D. H. Lee, Kim, Park, Choi, and Lee, 2004). Recent studies indicate that phenolic rich foods and plants have the ability to inhibit ACE activity, both *in vitro* and *in vivo*. This was confirmed by **Actis-Goretta *et al* (2006)** whose study confirmed that flavanol and procyanidins exhibit ACE inhibitory activity. In that study, significant inhibition was observed for epigallocatechin which would support the idea that the extent of ACE inhibition could be associated with the number of hydroxyl groups in the flavanol/procyanidin available to establish hydrogen bonds with the ACE protein. This study will investigate *Centella asiatica* for ACE inhibition.

2.7 *Centella asiatica*

Centella asiatica, is a tropical medicinal plant from the Apiaceae family native to South Africa, Madasgascar, and the Southeast Asian countries such as India, Sri lanka, China, Indonesia and Malaysia. It is commonly known as gotu kola, asiatic pennywort, Indian pennywort, wild violet, and tiger herb. *Centella asiatica* is described as a hungry feeder with a penchant for a lot of water. It is found in abundance on moist, sandy or clayey soils, often in large clumps forming dense green carpets. In South Africa, *Centella asiatica* is found along the moist eastern parts, widely distributed from the Cape Peninsula northwards. The species has been identified to vary considerably in different parts of the world and is sometimes treated as several distinct species. (**Van Wyk *et al*, 2002**).

2.7.1. Botanical description

The plant is propagated from seeds or stolon's. The stems of the plant are green to reddish green in colour and have long stalked, green leaves with rounded apices. The edible leaves are kidney shaped, borne on pericladial petioles, smooth surfaced, thin and soft, with palmate

nerves and can measure from 1 – 5 cm in width. Nodes found on the stem give rise to long petioles (5 -15cm) which hold the leaves. The fresh or dried aerial parts consisting of leaves and stems are used for medicinal purposes (Bandara, Lee, and Thomas, 2011).

Figure 2. 7 *Centella asiatica* (L.) Urban (Apiaceae).

Table 2. 3 Taxonomy of the Gotu Kola Plant

Classification	Name
Kingdom	Plantae
Division	Magonoliaphyta
Class	Magnolipsida
Order	Apiales
Family	Apiaceae
Genus	*Centella*
Species	*Centella asiatica* (L.) Urban

Ref: (Bandara *et al*., 2011).

2.7.2. Therapeutic Uses of *Centella asiatica*

C. asiatica has been used for medicinal purposes since prehistoric times as an adaptogenic for enhancing cognitive function by revitalizing the brain and nerve cells. The plant possesses a wide range of health benefits that includes cardio-protective, anti-inflammatory, antiepileptic, sedative, antiviral, antibacterial, anti-ulcer, anti-psoriatic, anti-cytotoxic and anti-tumour properties (**Orhan, 2012; Gohil *et al*, 2010)**. *C. asiatica* is currently known as Gotu Kola in South Africa (Marketed by Flora force®). It is sold as a herbal product for treatment of variety of conditions such as wound healing, anxiety, depression and venous insufficiency. *C. asiatica* has been reported to act on the connective tissues of vascular walls, being effective in hypertensive micro-angiopathy and venous insufficiency and decreasing capillary filtration rate by improving microcirculatory parameters (**Cesaron *et al*, 1992**). Studies by **Sanctis *et al* (2001)** showed that the total triterpene fraction of *Centella asiatica* at 180mg/day improved venous hypertension together with capillary filtration rate and ankle oedema.

According to the **WHO (1999),**the medicinal uses of *Centella asiatica* supported by clinical data include the treatment of wounds, burns, ulcerous skin diseases and the prevention of hypertrophic scars. Extracts of the plant have been used to treat second and third degree burns. Topical application of asiaticoside, a major constituent of *Centella asiatica*, significantly enhanced the rate of wound healing in normal and diabetic animals as assessed by an increase in collagen synthesis and tensile strength of the wound tissues (Shukla *et al*., 1999)**.** Oral administration of extracts is used to treat stress induced stomach and duodenal ulcers **(Sairam, Rao, and Goel, 2001).** Uses reported in folk medicine but not supported by experimental or clinical data, include treatment for hypertension, anaemia, asthma, albinism, measles, cholera, bronchitis, cellulite, constipation, nephritis, nervous disorders, epilepsy, hepatitis, dysuria, haematemesis, jaundice, toothache and as an antipyretic, analgesic, anti-inflammatory and brain tonic. (**WHO 1999)**

2.7.3 Phytochemical Composition of *C. asiatica*

Centella asiatica have been reported to contain a lot of constituents belonging to various chemical classes. The chemical constituents identified include terpenes, phenols, alkaloids, polyacetylenes, carbohydrates, amino acids. Triterpene saponosides such as asiatic acid, madecassic acid, asiaticoside, madecassoside, betulunic acid, and thankunic acid are the major chemical classes contained in *C. asiatica*. The plant contains other triterpenes such as brahmic acid, centellin, centellicin, asiaticin, terminolic acid, centellasaponins A-D, ursolic acid, and pomolic acid **(Inamdar *et al.*, 1996).**

Several flavonoid derivatives such as quercetin, kaempferol, patuletin, rutin, apigenin, castilliferol, castillicetin and myricetin has been reported to be present in *Centella asiatica* (Subban *et al*., 2008)**.** Other classes of compounds present in *Centella asiatica* include polyacetylenes (e.g. cadinol, centellinol, centellin, centellicin, and asiaticin), Sterols (e.g., 11-oxoheneicosanil-cyclohexane, dotriacont-8- en-1-oic acid, sitosterol 3-O-β-glucoside, stigmasterol 3-Oβ-glucoside, and castasterone), and phenolic acids (e.g., rosmarinic acid, 3,5-di-O-caffeoil quinic acid, 1, 5-di-O-caffeoil quinic acid, 3,4-di-O-caffeoil quinic acid, 4, 5-di-O-caffeoil quinic acid, ettacrynic acid, chlorogenic acid, and isochlorogenic acid **(Govindan *et al*., 2007; Siddiqui *et al*., 2007; Sondhi *et al*., 2010).**

2.8 Drug discovery from Medicinal Plants

Plants have been used as a source of therapy for thousands of years. Medicinal use initially took the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations. In more recent history, the use of plants as medicines has involved the isolation of active compounds that began with the isolation of morphine from opium in the early 19th

century**(Kinghorn, 2001).** Drug discovery from medicinal plants led to the isolation of early drugs such as codeine, morphine, digitoxin, and quinine.

Roughly 49% of the new chemical entities introduced between 1981 and 2002 where natural products, semi synthetic natural product analogues or synthetic compounds based on natural product pharmacophore. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world today. Medicinal plants have continued to play an essential role in healthcare and their use by different cultures has been extensively documented. An estimated 80% of the world population continue to rely on traditional medicine for their basic healthcare needs **(Ekor, 2014**). According to **Farnsworth *et al* (1985),** analysis of the data on prescriptions dispensed from community pharmacies in the US from 1959 to 1980, indicates that 25% contained plant extracts or active principles derived from higher plants and at least 119 chemical substances derived from 90 plant species can be considered as important drugs currently in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine. Indeed, today many pharmacological classes of drugs include a natural prototype **(Gilani *et al* 1992).**

The recent discovery of taxol, camptothecin, vincristine and vinblastine as anticancer drugs further demonstrates the great pharmaceutical potential of plants. Further evidence of plant derived compound/s is the Digitalis and the cardiac glycoside derived from the foxglove (*Digitalis purpurea*), one of the most important drug in cardiovascular medicine.

The natural product drug discovery process generally involves the testing of extracts of source organisms of plant, marine or microbial origin in appropriate *in vitro* assays (cell or enzyme/target based), followed by bioassay-guided fractionation of the active extracts and isolation and purification of active constituents. Those constituents showing significant *in vivo*

activity in appropriate animal models are considered as lead molecules which may be selected as candidates for preclinical development. Initially, such leads may be structurally modified through use of medicinal or combinatorial chemistry techniques to provide agents having superior activity or decreased toxicity (optimization of the therapeutic index) and acceptable pharmacological properties.

2.9 Bioassay guided fractionation/Drug discovery process

Bioassay-guided fractionation is a process whereby extracts are chromatographically fractionated and re-fractionated until an active biological compound is isolated. Each of the fractions produced during the process is evaluated in a bioassay system and then only the active fractions re-fractionated. This process is often employed in drug discovery because of its effectiveness in directly linking the analysed extract or compound isolated using the fractionation process with a certain biological activity

Drug discovery from medicinal plants follows a step wise approach and involves a multi-disciplinary approach. Initial steps in drug discovery involve identification of NCEs, which can be either sourced through chemical synthesis or can be isolated from natural products through bioassay guided fractionation. **(Katiya *et al*, 2012).** The starting point for plant-based new drug discovery should be identification of the right candidate plant by applying several approaches, with the choice of approach dependent on the aim. Plant selection for screening can be based on a randomized approach, which involves the collection of all plants found in the study area; phytochemical targeting, which entails the collection of all members of a plant family known to be rich in bioactive compounds; the ethno-directed sampling approach, based on traditional medicinal uses of a plant; phytochemical constituents; chemotaxonomic approach; and a method based on specific plant parts, such as seeds. In the ethno-

pharmacological approach, the main requirement is the knowledge of the plant parts traditionally employed as remedies. The process of drug discovery follows different stages as illustrated below.

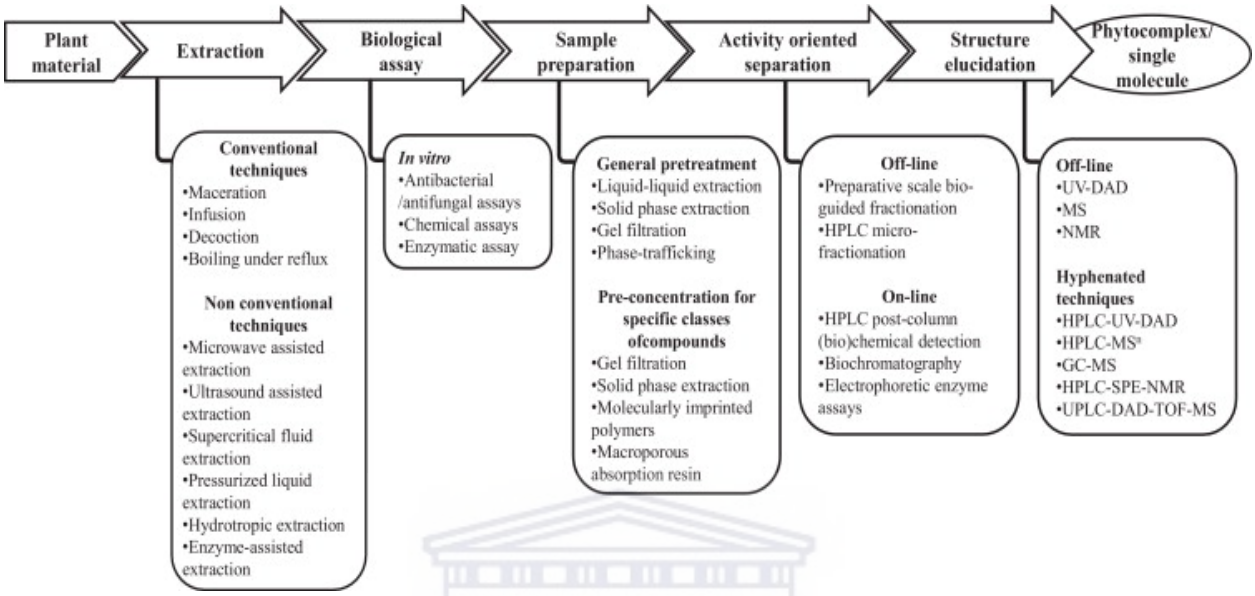

Figure 2. 8 Process of drug discovery from medicinal plant.

First is the Choice of plant material which is succeeded by the preferred extraction technique based on plant properties and desired goal. Extracted plant material are biologically assayed, followed by sample preparation and activity oriented separation/purification using chromatographic techniques such as column, thin layer and high performance liquid chromatography. The final process involves spectroscopic identification and elucidation of isolated bioactive compounds.

2.9.1 Plant Extraction Techniques

Plants contain a wide variety of chemical compounds belonging to the different phytochemical classes with different solubility profiles in aqueous and non-aqueous solvents. Extraction is the first crucial step in the analysis of medicinal plants, because it is necessary to extract the desired

components from the plant materials for further separation and characterization. The basic steps prior to extraction include pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous mixture thus increasing contact of sample surface with solvent system (Sasidharan, Chen, Saravanan, Sundram, and Yoga Latha, 2011). Selection of the solvent system is dependent on the specific nature of the bioactive compounds being targeted. Isolation of bioactive compounds, starts with immersion of plants into a suitable solvent or alternatively drying and grinding the plant into a homogenous powder and extracting using a suitable solvent. Methods of extraction include maceration, percolation, extraction under reflux, Soxhlet extraction, sonication assisted solvent extraction (SAE), and supercritical fluid extraction.

2.9.1.1 Maceration

Maceration is a technique used in wine making and has been adopted and widely used for medicinal plants research. Maceration involves soaking plant material in a stoppered or closed container with a preferred solvent and allowing to stand at room temperature for a certain period, from a few hours to days or several weeks depending on properties of the material, solvent and purpose of the experiment. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing. The process is intended to soften and break the plant's cell wall to release the soluble phytochemicals. This technique is used owing to its simple approach, low cost and suitability for thermolabile compounds **(Zhang *et al*, 2018).**

2.9.1.2 Percolation

Percolation is a process that involves the slow descent of a solvent through a powdered substance until it absorbs certain constituents and drips out through the filtered bottom of the container. This is the procedure used most frequently to extract active ingredients in the

preparation of tinctures and fluid extracts. Fresh solvent is continuously added on top of the plant material until the recovered residue of the extraction solution turns out to be too little**.** The percolator is a cylindrical or conical container made from glass or metal with a tap at the bottom. The percolation is usually done at moderate rate (e.g 6 drops/min) until the extraction is completed before evaporation to get a concentrated extract.

2.9.1.3 Extraction under Reflux

Reflux involves the condensation of vapours and the return of the condensate to the system from which it originated. This method is suitable for heat stable compounds. It can be used for both small and large extraction for herbal study. During the extraction process, the material is immersed in an appropriate solvent in a round-bottomed flask, which is vertically connected to a condenser. The solvent is heated by water bath and electric heater to boiling. The evaporated solvent goes up into the condenser, and then is condensed into liquid form and drops down to the flask. Increasing temperature in the process generates heat that help the solvent penetrate into the plant cells thus increasing the solubility of compounds in the solvent. The extraction efficiency is higher and is one of the most popular method employed for herbal study.

2.9.1.4 Sonication assisted solvent extraction

Sonication assisted extraction is a modified maceration in which ultrasound is utilized to improve the extraction efficiency. The plant material is placed in a closed container like in maceration. The container is then placed in an ultrasonic bath. In such a condition, ultrasound transfers the mechanical power onto the plant cells, leading to breakdown of cell walls and increased solubilization of extracts in the solvent. Factors affecting extraction yields are frequency, length, and temperature. The benefit of SAE is mainly due to reduction in extraction

time and solvent consumption, therefore it is an easy and efficient method commonly used in the lab **(Liu, 2011).** However, use of ultrasound energy more than 20kHz may have an effect on the active phytochemicals through the formation of free radicals(Handa, Khanuja, Longo, and Rakesh, 2008)

2.9.1.5 Soxhlet extraction

Soxhlet extraction is a convenient method for the extraction of herbal materials of small to medium volumes. The commercially available soxhlet instrument is composed of an extraction chamber with reflux condenser and collecting flask. The chamber is placed between the collection flask and refluxing condenser. The plant powder or pieces are kept in a cellulose thimble in the extraction chamber. A suitable solvent is added to the flask and heated under refluxing. The solvent will first be evaporated up into the condenser, then liquefied into the chamber. When the condensed solvent in the chamber reaches a certain height, it is siphoned into the flask, and the next extraction is initiated. Usually, 50 -60 times of recycling are necessary for an extraction. Because of the repeated extraction, this method is usually more efficient than refluxing and produces a higher yield of extract with less volume of solvent.

2.9.2 Cardiovascular bioassay models for screening of medicinal plant

In modern medicine, drug discovery has become more focused on ligands, substrates and inhibitors of specific target (enzyme and receptors) that play an important role in disease regulation. CVD models such as *in vivo* (laboratory animal model), *in vitro*, and *in silico*/computational models are used to assess the efficacy and safety of new drugs earlier in the drug development pipeline. These models are also utilized during pre- clinical evaluation to assess the risk of cardiac adverse effects of new drugs in humans. They remain a key tool for investigating the action of drugs on biological systems.

2.9.2.1 in vivo models for cardiovascular activity

In vivo assays assesses drug effects on the heart in its natural physiological environment. They are suited for observing the overall effect of an administered substance on a living subject, and measure endpoints such as electrocardiogram (ECG), blood pressure (BP) and heart rate (HR) via implantable telemetry. **(Hanton, 2007)**. Some of the *in vivo* cardiovascular models commonly used include BP measurement in pithed rats, tail cuff method, direct measurement of BP in conscious rats with indwelling catheter, telemetric monitoring of CVD parameters in rats, hemodynamic evaluation in anesthetized dogs, the salt-induced hypertensive rat model, the salt-loaded hypertensive model, and the anaesthetized normotensive rat model.

Most of these *in vivo* cardiovascular models in experimental animals involve blood pressure measurement and are classified under direct and indirect methods of blood pressure measurement. The tail cuff method is an example of indirect method of measurement, it is considered a non-invasive method, technically non demanding and suitable for chronic studies considering that serious risk to animal health is minimal. However, one of the disadvantages is the need to restrain the animal and apply heat to achieve tail blood flow, these applications could negatively influence blood pressure reading. The use of an indwelling catheter is a direct method considered the most accurate method of measurement. It eliminates the need for restraining and heating the animal, but requires expertise to use **(Kamadyaapa, 2008).**

2..9.2.2 in vitro models for cardiovascular activity

in vitro assays refer to the technique of performing a given procedure in a controlled environment outside a living organism. It involves the use of isolated organs or parts of organs in a simulated physiological environment. *in vitro* models using isolated cells, cardiac tissues or whole perfused heart, provide simple, rapid and sensitive methods with a moderate to high

throughput, for screening drug candidates for biological activity. Some of the *in vitro* models used in CVD include the rabbit lung derived ACE inhibition assay, the Langendorff perfused heart model, working heart perfused heart model, isolated cardiac myocyte models. (**Hanton, 2007)**.

The application of the assay method for screening for ACE inhibitory activity in natural product research is a very helpful method owing to its rapidity, accuracy, and simplicity. The ACE inhibitory activity has become an effective screening method in the search for new antihypertensive agents from herbal plants. Some of the *in vitro* assay method used to examine the activity of ACE inhibitors are based on substrate usage, with the various methods distinguished by their substrates and measurement methods for their enzymatic reaction and product separation. These include Cushman and Cheung's (1971) method using hippuryl-histidyl-leucine (HHL) as a substrate, Holmquist, Bünning, and Riordan's (1979) method using furanacryloyl-tripeptide as a substrate, Elbl and Wagner's (1991) method using tripeptide dansyltriglycine as a substrate, Amos and Arieh's (1978) method using o-aminobenzoylglycyl-p-nitrophenylalanilproline as a substrate, and Lam, Himamura, Anabe, Shiyama, and Keda's (2008) method using 3-hydroxybutyrylglycyl-glycyl-glycine as a substrate. For the various methods, measurement of the enzymatic activity is carried out using either spectrophotometry, fluorometry, high-performance liquid chromatography, electrophoresis, or radiochemistry.

For this study, we utilized a fluorimetric ACE assay using HHL as the substrate as this assay is rapid, extremely sensitive and uncomplicated in terms of procedure, and has the advantage of greater efficiency with respect to both time and reagents over others **(Schwager *et al*, 2006).** In this assay, the terminus of the ACE cleaved substrate binds to OPA which allows for enzyme activity to be measured based on a decrease or increase in fluorescence intensity. The presence of an inhibitor in the enzyme-substrate mix results in a decrease in fluorescence intensity. The choice of fluoroscopy as a mode of detection was due to its simplicity, sensitivity and rapidity,

and is particularly applicable for the screening of large numbers of ACE inhibitors and biological samples. The substrate and all the reagents are commercially available and relatively inexpensive. In addition, assay procedure required no solvent extraction, and has the advantage of greater efficiency with respect to both time and reagent. (Schwager *et al*, 2006).

2.10 Isolation and Purification techniques

Selection of methods in the isolation and purification of herbal extract depends on the properties of the compounds and purposes of the study. Isolation and Purification techniques serves to remove interferences from the analyte as well as fractionate extracts into smaller fractions. In general, crude extracts are initially separated into several fractions by isolation methods such as liquid-liquid extraction.

Isolation and purification of compounds from complex herbal extracts depend on successful application of several isolation methods. Column chromatography and thin layer chromatography (TLC) are usually necessary, and mostly used due to their convenience, economy, and availability in various stationary phases. Many bioactive molecules have been isolated and purified by using thin layer and column chromatographic methods.

2.10.1 Thin layer chromatography

TLC is an easy, economic, and quick method that can be used for qualitative and quantitative analysis as well as purification of natural products. Practically, TLC is used in identifying unknown compounds and detecting the presence of secondary metabolites in herbs. As an auxiliary tool, TLC is used to help optimize conditions of column chromatography, including selecting the mobile and solid phases, identifying compounds isolated from column as well as confirming purity of the isolates **(Liu, 2011).**

When applying TLC for isolation, the solution of herbal extract on a suitable solvent (ethanol or methanol) is spotted as a single round spot or thin line on a TLC plate near the edge of one side of the plate. The TLC plate is then obliquely placed into a well-closed tank containing sufficient volume of solvent but maintaining the sample spot or line above the surface of the solvent. The developing solvent will slowly migrate up the plate due adsorption of the solvent on the plate.

An important qualitative parameter in TLC of a particular sorbent and solvent system is the $R_{f.}$ value, which is defined as:

$R_{f.}$ = Distance of analyte / Distance of solvent front

In this study, TLC was used in the preliminary screening of crude plant extracts, monitoring columns during isolation and purification. TLC coupled with UV detection and spray reagents provide valuable information on type of compound in an extract. It is a simple, rapid and cost effective technique allowing for analysis of several samples.

2.10.2 Preparative Thin Layer Chromatography (PTLC)

PTLC is generally applied for purification of compounds in a relatively simple fraction that have been separated by several chromatographic columns. Preparative thin layer chromatography (PTLC) is used to separate and isolate amounts of material larger than normal for analytical TLC. The quantities processed range from 10 mg to greater than 1 gram. In preparative TLC, materials to be separated are often applied as long streaks, rather than spots, in the sample application zone. The main difference from analytical TLC is in the thickness of the plates. It uses preparative plates of about 1 – 2 mm in thickness allowing the application of larger volume of sample. After development the plates are visualized under UV, and the band

with analyte of interest is scraped off together with the adsorbent using a spatula. The analyte is separated from the adsorbent by filtration and concentrated. **(Sherma *et al* 1987)**

2.10.3 Adsorption or Open Column Chromatography

The mechanism of this chromatography lies on the physical and chemical interaction between the stationary phase and solute molecules**.** The separation depends on the difference between the adsorption strength of the material in stationary phase to solute molecules in mobile phase. The solvent of the mobile phase competes with the solute molecules for adsorption sites. Normal silica gel is the commonly used in terms of stationary phase and is regarded as a typical polar sorbent. Due to differences in chemistry, analytes separate as they move down the column. These are continuously monitored by TLC and similar fractions are combined and concentrated. The column can be eluted in two ways, either using a single solvent or gradient elution - a series of solvents with increasing polarity/ elution strength are used to elute the column. This method is however considered very tedious and labour intensive.

2.10.4 High Performance Liquid Chromatography

HPLC is a popular method for chemical analysis and separation that is applied in many fields such as the food, pharmaceutical, and agricultural industries. HPLC achieves separation by utilizing the fact that different compounds have different migration rates given a particular stationary and mobile phase **(Liu, 2011).** HPLC fulfils different objectives; HPLC uses various separation mechanisms such as adsorption chromatography, partition chromatography (normal and reverse phase partition), ion exchange chromatography, ion pairing chromatography, and size exclusion chromatography. There is Analytical and Preparative HPLC. Analytical HPLC is used to determine the existence and possibly the concentration of analyte in a sample. It is often utilized in quality control of medicinal products. Preparative HPLC refers to the process

of isolation and purification of small quantitative compounds using HPLC. It has been applied to the isolation of most classes of compounds in herbal medicine, especially minor bioactive compounds. The HPLC instrument is an automated closed system consisting of a pump system to generate pressure, narrow columns packed with small particle size adsorbents and a detector**.** In this study, HPLC was used for further purification and separation of the fractions of the plant extracts. The detector emits a response to the eluting sample compound and subsequently signals a peak on the chromatogram. This is interpreted to guide the next step in the purification and identification process.

2.11 Compound Identification and Elucidation techniques

Compound identification or structural elucidation involves the determination of factors such as atom number, number of bonds, configuration, and conformation of pure compounds. The development and improvement of analytical instrument to determine structure has been one of the biggest advancements in organic chemistry during the past 60 years. Today almost any structure can be determined with these instruments. Several techniques are available for elucidating and identifying the structure of a pure compound, these include NMR spectroscopy, Mass spectrometry, UV-vis Spectroscopy, and IR Spectroscopy.

2.11.1. Nuclear Magnetic Resonance (NMR)

Structure elucidation and identification of novel compounds from natural and/or synthetic sources depends heavily on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) as complementary tools. Techniques such NMR spectroscopy are one of the important characterization tools in chemistry, often utilized to provide insight into the

chemical components or profile of samples such as crude extracts and their fractions, they also serve as a guide in the purification process of crude samples. NMR essentially provides simultaneous access to both qualitative (chemical structure) and quantitative information of samples and has the advantage of accuracy, reproducibility, and flexibility with respect to the nature of the analyte **(Dayrit and Dios, 2017).** In practice, the acquisition of a ^{1}H NMR spectrum is the first step in NMR-based structure elucidation and metabolomics analysis. Especially for sample-limited natural products, 1D ^{1}H NMR and its 2D counterparts are typically the first-line structural tools employed for identification and de-replication purposes. Three key parameters are important in the interpretation of NMR spectra, these include chemical shift, spin-spin coupling (splitting) and peak intensity. The chemical shift is the resonant frequency of a nucleus relative to a standard in a magnetic field. One factor that affects chemical shift is the changing of electron density from around a nucleus, such as a bond to an electronegative group. Hydrogen bonding also changes the electron density in ^{1}H NMR, causing a larger shift. Chemical shifts can be used to identify structural properties in a molecule based on our understanding of different chemical environments. Another useful parameter that allows NMR spectra to give structural information is spin-spin coupling. Different spin states interact through chemical bonds in a molecule to give rise to this coupling. In NMR spectra, this effect is shown through peak splitting that can give direct information concerning the connectivity of atoms in a molecule. Nuclei which share the same chemical shift do not form splitting peaks in an NMR spectra. Peak intensity otherwise known as size of the peaks, the size of the peaks in the NMR spectra can give information concerning the number of nuclei that gave rise to that peak. This is done by measuring the peak's area using integration. Yet even without using integration the size of different peaks can still give relative information about the number of nuclei.

2-D NMR in itself is an effective tool for identification and validation of structure, especially for understanding the stereochemistry of the molecule. The commonly used 2D-NMR techniques are COSY (used to determine the chemical shift, coupling relationship and connecting sequence of protons), NOESY(provides important information about stereochemistry and solution conformation by analysing the connectivity through the space of atoms in a molecule), TOCSY (allows for easy recognition of complete families of coupled spins), HMQC and HSQC (allows for determination of carbon to hydrogen connectivity), and HMBC (used to identify proton nuclei that are separated by more than one bond) (Liu, 2011).

2.11.2 IR Spectroscopy

Infrared spectroscopy is useful in the identification of functional groups present in a compound. This technique is based on the absorption of electromagnetic radiation at wavelengths ranging between 4000 and 400 cm^{-1}. At this range of wavelengths specific functional groups give characteristics vibration, bending and stretching. This is recorded by the IR instrument and gives basic information on the structure **(Liu, 2011).**

2.11.3 Mass Spectroscopy

Mass spectroscopy measures the molecular masses of individual compounds and atoms precisely, by converting them into charged ion. It is widely used for structural elucidation because of its unique ability to accurately measure molecular mass and to provide structural related information. The evolution of MS is mainly characterized by the development of an ion source and a mass analyser **(Liu, 2011).**

In mass spectroscopy, a compound is ionized, the ions are separated on the basis of their mass/charge ratio, and the number of ions representing each mass/charge unit is recorded as a spectrum. The recorded spectrum is analysed to reveal the molecular weight of the compound

and the fragmentation patterns of the parent compound. For unknown compounds, the molecular ion, the fragmentation pattern, and complementary forms of spectrometry such as IR and NMR put together give a molecular skeleton of the compound **(Silverstein *et al*, 2005).**

2.11.4 UV Spectroscopy

Ultra violet spectroscopy is mainly concerned with electron transitions in conjugated systems, it uses UV-VIS light to measure the transmittance of the light that passes through a sample. the positions and intensities of the absorption band maxima depend to a large extent on the particular system under construction. The electron transitions are very sensitive to structural changes and reflect the strains imposed on the system by steric and electronic interactions. The absorption wavelength is in the range of 200 – 800 nm; it is usually possible to correlate changes in spectra with changes in structure with a fair degree of success. One of the strengths of UV spectroscopy is the relative simplicity and inexpensiveness of the instrumentation coupled with great sensitivity. The ease with which quantitative measurement can be made is also a great advantage **(Scheinmann, 2013).**

In this project, *Centella asiatica* will be subjected to bioassay guided fractionation following the drug discovery processes described above. The subsequent chapters present a further discussion of these processes and their outcomes.

Chapter 3

Materials and Method

Introduction

Bioassay guided fractionation is an important strategy in the scientific validation of the claimed traditional uses of medicinal plants as well as the discovery of novel compounds. The successful isolation of bioactive compounds from indigenous medicinal plants often validates indigenous knowledge, adds value to plants and supports the knowledge of their medicinal use. It may also contribute to the research and development of new pharmaceutical drugs for the treatment of various diseases. In this study, bioassay guided fractionation was used to isolate compounds exhibiting ACE inhibition from extracts of *Centella asiatica*.

The process starts with the extraction of the powdered plant parts, followed by sequential fractionation of the active crude extract using gravity column chromatography and solvents of increasing polarity. Fractionation and isolation was guided by ACE activity assay of the various fractions. Active fractions were further purified using high performance liquid chromatography (HPLC), and the isolated compounds characterized by NMR, HRMS, IR and UV spectral data and by comparison to literature data.

This chapter reports on the materials and equipment used in the isolation of active compounds and the bioassay used to guide the isolation of active compounds. The chapter also describes the methods applied and the protocols followed for the extraction, fractionation, isolation, purification and identification of the active compounds from *Centella asiatica*, as well as the ACE inhibition assay used to determine bioactivity of the extracts/fractions/compounds.

3.1 Equipment and Materials

The equipment and materials used in the study are as follows:

Table 3. 1 List of chemicals

Chemical	Supplier
Methanol (HPLC grade)	Sigma Aldrich Co.
Deuterated methanol	"
Chloroform	"
Hydrochloric acid (10M)	"
2,2-diphenyl-1-picrylhydrazyl (DPPH)	"
Sodium Hydroxide Pellets	"
Sodium Chloride	"
Ascorbic acid	"
Boric acid powder	"
o-pthaldialdehyde	"
N-Hippuryl-His- Leucine (HHL)	"
Rabbit lung derived Angiotensin-converting enzyme	"
Acetic acid (HPLC grade)	"
Hexane(Analytical reagent Grade	Lab Chem.
Ethyl acetate (Analytical reagent Grade)	Lab Chem.
Ethanol(Analytical reagent Grade	Lab Chem.

Table 3. 2 List of equipment

Equipment	Model and Manufacturer
-86°C ultralow freezer	(*Nuaire, Model NU 9668E, Nuaire, Japan)*
Oven	*(Labotech, Model LDO-080F, DaihanLabotech Co Ltd, Korea)*
Analytical balance	*(Ohaus, Model PA413, Ohaus Corporation, USA),*
Vortex mixer	*(KK, Model VM-300, Germany Industrial Corp, Taiwan),*
Vacuum pump	*(Buchi, Model V-500, BuchiLarbotechnik AG, Switzerland*
Rota vapor	*(Buchi, Model R-11, BuchiLarbotechnik AG, Switzerland*
Freeze drier	*(Virtis TM mobile freeze-dryer, model 125L),*
Buchner funnel	
Whatman No. 1 paper filter, nylon syringe filters ***(25 mm diameter, 0.45 µm pore size)***	
M**icropipettes, HPLC filter unit**	(*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA),*
M**embrane filters** ***Durapore, 0.45 µm HV, Millipore***	
FTIR instrument fitted with UATR and controlled with Spectrum® software version 6.3.5.0176	*(Perkin-Elmer 100)*

3.1.1 Equipment for the isolation, purification, and identification of bioactive compounds

3.1.1.1 *Column Chromatography*

Waters Sep. Pak® column (2.5 x 10cm) with silica gel 60 (0.063-0.0200 mm) (Merck, Germany), was used for column chromatography.

3.1.1.2 *Thin Layer Chromatography*

Thin layer chromatography was carried out on pre-coated silica gel F_{254} plates (Merck) with a 0.2 mm layer thickness. Visualization of the TLC spots was carried out under UV light at 254nm and/or 366nm, and further detection of compounds was achieved by spraying with DPPH solution.

3.1.1.3 *High-Performance Liquid Chromatography*

Sample purification was carried out using an Agilent Technologies 1260 Infinity (Made in Germany), a PC with Chemstation® software (Agilent, OpenLAB CDS) and a normal phase column (Whatmann 10uM silica prep column 50cm).

3.1.1.4 *Nuclear Magnetic Resonance (NMR) Spectroscopy*

NMR spectra were recorded on an NMR Spectrometer (Bruker Avance 400 MHz Rheinstetten, Germany**).** All spectra were referenced to residual solvent signals (δ_H of 7.2600 ppm and δC of 77.00 ppm) of deuterated chloroform (d_{CDCl3}) and (δ_H of 4.87, 3.31 ppm and δ_C of 49.10 ppm) Methanol. Chemical shifts were measured in parts per million (ppm) and coupling constants (*J*) in Hz**.**

3.1.1.5 *Fourier Transform – Infra-Red Spectroscopy*

Fourier transform infrared spectroscopy (FTIR) measurements were carried out using an IR spectrometer (Perkin Elmer spectrum 400 FT-IR/FT-NIR spectrometer, Waltham, USA) controlled with Spectrum® software version 6.3.5.0176 Spectrum 100 (PerkinElmer).

3.1.1.6 *UV Visible Spectroscopy*

UV spectrum of the isolated compounds were obtained using a UV-Visible spectrophotometer (GBC CINTRA 202 New Hampshire, USA). All sample for analysis were dissolved in methanol.

3.1.1.7 *Mass Spectroscopy*

High-resolution mass spectroscopy (HRMS) analysis was conducted using a Waters ultra-pressure liquid chromatograph (UPLC) (Waters, Midford, USA) using electrospray ionization (ESI) interface working in positive mode.

3.2 Plant Material

Dried *Centella asiatica* leaf powder was purchased from Warren Chem. Specialties LTD (Cape Town, South Africa). The powdered plant came in sealed, opaque, plastic bags and was stored in a dark place away from light in a temperature controlled (20^{o}C) laboratory room. The Certificate of Analysis of purchased plant powder is included in Appendix XXXIV of the thesis.

3.3. Experimental methods

3.3.1. Preparation of crude extracts of *C. asiatica*

The dried leaf powder of *C. asiatica* (500 g) was macerated in 2.5 L of methanol, ethanol or distilled water for 24 hrs, with occasional shaking. The resulting methanol or ethanol extract was filtered under vacuum using a Buchner funnel with Whatman No 1 filter paper, and the residual plant material subjected to further maceration with fresh solvent up to three times to ensure exhaustive extraction. Individual methanol or ethanol filtrate were combined and

concentrated under reduced pressure at 40℃ using the rotary evaporator (Buchi Rota vapour), the obtained extract was transferred to a pre-weighed beaker and stored at -8 ºC. Aqueous extracts were filtered using the Buchi vacuum pump, the filtrate was transferred to 250 ml round bottom flasks, flash frozen with liquid nitrogen and thereafter freeze dried under vacuum for 96 hours. The resultant freeze-dried extract was stored in airtight amber bottles and put in a desiccator. The percentage yield of the organic and aqueous extracts were then determined and recorded.

3.3.2. ACE Inhibition assay

3.3.2.1 *Preparation of sodium borate buffer solution*

To prepare sodium borate buffer solution (1 L) with a final concentration of 0.4 M Boric acid, 0.3 M NaCl and a pH of 8.3, NaCl (17.532 g) and Boric acid (24.732 g) were weighed into a 1 L bottle, and 900 ml of distilled water added. The mixture was stirred using a magnetic stirrer for 25minutes to ensure complete dissolution, and the pH determined, and adjusted to 8.3 using 0.1 M NaOH solution. The final volume of buffer was made up to 1 L and the pH was again measured and confirmed as 8.3.

3.3.2.2 ***Preparation*** *of Hip-His-Leu (HHL) solution*

HHL stock solution (116.4 mM) was prepared by dissolving 50 mg of HHL in 1 ml acetic acid in a 10 ml glass vial and stored at -5 ºC. To prepare the 3.5 mM HHL required for the ACE assay, 300 µL of the HHL stock solution (116.4mM) was pipetted into a glass vial, followed by 570 µL of 10M NaOH. Previously prepared sodium borate buffer solution (section 3.3.2.1) was then added up to the 8 ml mark on the glass vial, and the pH of the solution determined and adjusted to 8.3 using 0.1M NaOH. The solution was made up to a final volume of 10 ml using sodium borate buffer solution and stored at -5 ºC.

3.3.2.3 *Preparation of ACE solution*

ACE solution was prepared by adding 200 µL of NaB buffer solution to the ACE vial containing 0.25 Units of Purified rabbit lung Enzyme. The ACE solution was stored at a temperature of -20℃.

3.3.2.4 *Preparation of plant extracts for ACEI assay*

Crude organic extracts of *C. asiatica* were dissolved in DMSO in NaB buffer (10% DMSO for the ethanol extract and 5% DMSO for the methanol extract). A preliminary ACE assay using DMSO in NaB buffer (5% and 10%) as enzyme inhibitors did not produce any inhibition of the angiotensin converting enzyme activity. The aqueous extracts were freely soluble in the buffer solution.

3.3.2.5 *ACE inhibitory assay protocol*

ACE inhibition activity of the crude extracts/fractions/compounds of the plant was performed according to the method of Balasuriya and Rupasinghe (2012) with some modifications. In this assay, ACE cleaves the substrate HHL to expose a free N-terminus which is then labelled with o-pthaldialdehyde (OPA), a fluorescence agent.

Preliminary ACEI assay of the crude extracts was carried out at concentrations of 1 mg/ml, 6 mg/ml, and 10 mg/ml of the methanol, ethanol and aqueous extracts. ACE enzyme (5.6 µL) was pre-incubated with 5 µL of either the various concentrations of the plant extracts (enzyme inhibitor), deuterated water (negative control) or captopril (positive control) for 20 minutes in sodium borate buffer making a final volume of 25 µL. After incubation, 10 µL of HHL (3.5 mM) was added to the solution to give the reaction mixture a final volume of 35 µL. The reaction mixture was incubated at 37°C for 60 minutes and the reaction terminated by adding 150 µL of NaOH (0.34M). OPA (20 µL of a 20 mg/ml) was added, the mixture incubated for 10 minutes at room temperature, and the reaction terminated by adding 50 µL of 3M HCl. This

final assay solution was pipetted in 150 µL aliquots into a 96 well microtitre plate and fluorescence read on a fluorescent plate reader (Synermix, Biotek) at 355nm excitation and 535nm emission wavelengths. All experiments were carried out in triplicates.

Percentage inhibition of ACE activity was then calculated from fluorescence intensity according to the following equation:

$$\text{ACE Inhibition (\%)} = (F_{control} - F_{sample}) / (F_{control} - F_{blank}) \times 100$$

Where:

$F_{control}$ = Fluorescence intensity of the control (Inhibitor replaced with D_2O)

F_{sample} = Fluorescence intensity of the enzyme inhibitor (captopril, crude extract, or fractions).

F_{blank} = Fluorescence intensity of blank (enzyme and inhibitor replaced with distilled water)

The inhibitory concentration 50 % (IC_{50}) was calculated from a dose-response curve obtained by plotting the percentage inhibition versus the concentrations of sample with the use of Microsoft excel and Graph Pad Prism 6 Software.

The ACE assay of fractions of the crude extracts and compounds isolated during the fractionation and purification process was carried out according the ACE assay protocol above.

3.3.2.6 *IC50 determination of captopril*

The half maximal inhibitory concentration (IC_{50}) value of the reference inhibitor captopril was determined from a concentration - response curve using Graph Pad Prism software version 5 and Microsoft excel. The IC_{50} value obtained was then compared with the value reported in

literature. A Stock solution of 1 mg/1ml captopril in buffer solution was initially prepared, and dilutions done to obtain various assay concentrations of 200, 100, 50, 25, 12.5 and 6.25 ng/ml. The Percentage inhibition of each of the captopril working solution was determined using the ACE assay protocol from section 3.3.2.5.

3.3.3. Bioassay-guided fractionation of methanol crude extracts

Gravity column chromatography, thin layer chromatography and high performance column chromatography were utilized to isolate the bioactive compounds. Silica gel (70 – 230 mesh) was used as the stationary phase and ethyl acetate/hexane in varying proportions as mobile phase for the column chromatography. Thin layer chromatography was used to determine and monitor the purity of fractions collected.

3.3.3.1 *Preparation of methanol sample for fractionation and isolation*

Methanol extract (1g) was dissolved in methanol, and the solution adsorbed unto 1.5 g of silica gel (35 -70 Mesh) in a round bottom flask. Excess methanol was evaporated on a rotary evaporator at 40^{o}C leaving behind dried granules.

3.3.3.2 *Fractionation of Methanol extract by Column Chromatography*

Fractionation was carried out using the Waters Sep Pak® 35cc Vac Cartridges (2.5 x 10cm), of which the tip was plugged with a small cotton wool. Silica gel 60 -230 Mesh (10g) in hexane solvent was loaded unto the column. The adsorbed sample of the methanol crude extract was added on top of the loaded silica gel column followed by a cotton wool plug to prevent the impact of the eluting solvent disturbing the sample. The column was eluted with a stepwise gradient consisting of 50 ml volumes of hexane (100%), hexane: ethyl acetate [(80:20), (60:40), (40:60), and (20:80)], ethyl acetate (100%), methanol: ethyl acetate (50:50) and methanol (100%). Each of the fractions collected were dried and transferred to a pre-weighed glass vial.

The weight and % yield of each fraction was determined and subsequently screened for ACE inhibition activity using the protocol described in section 3.3.2.5.

The decision to fractionate 1g at a time (up to 15 times), was to avoid the loss of important components that might emanate from upscaling to a larger glass column. A total of 15g of crude extract was fractionated using 750 ml of solvent in the various ratios as described above.

Table 3. 3 Solvent System used for Fractionation of *Centella asiatica* methanol Crude Extract.

Solvent (% v/v)	Volume (mL)	Fraction	Yield(mg)	% Percentage Yield
Hexane 100	750	M_1	-	-
Hex: EtoAc 80:20	750	M_2	35	0.23
Hex: EtoAc 60:40	750	M_3	240	1.6
Hex: EtoAc 40:60	750	M_4	150	1.0
Hex: EtoAc 20:80	750	M_5	118.5	0.79
Ethyl acetate 100	750	M_6	57	0.38
EtoAc: Methanol 50:50	750	M_7	8005.5	53.37
Methanol 100	750	M_8	4455	29.79

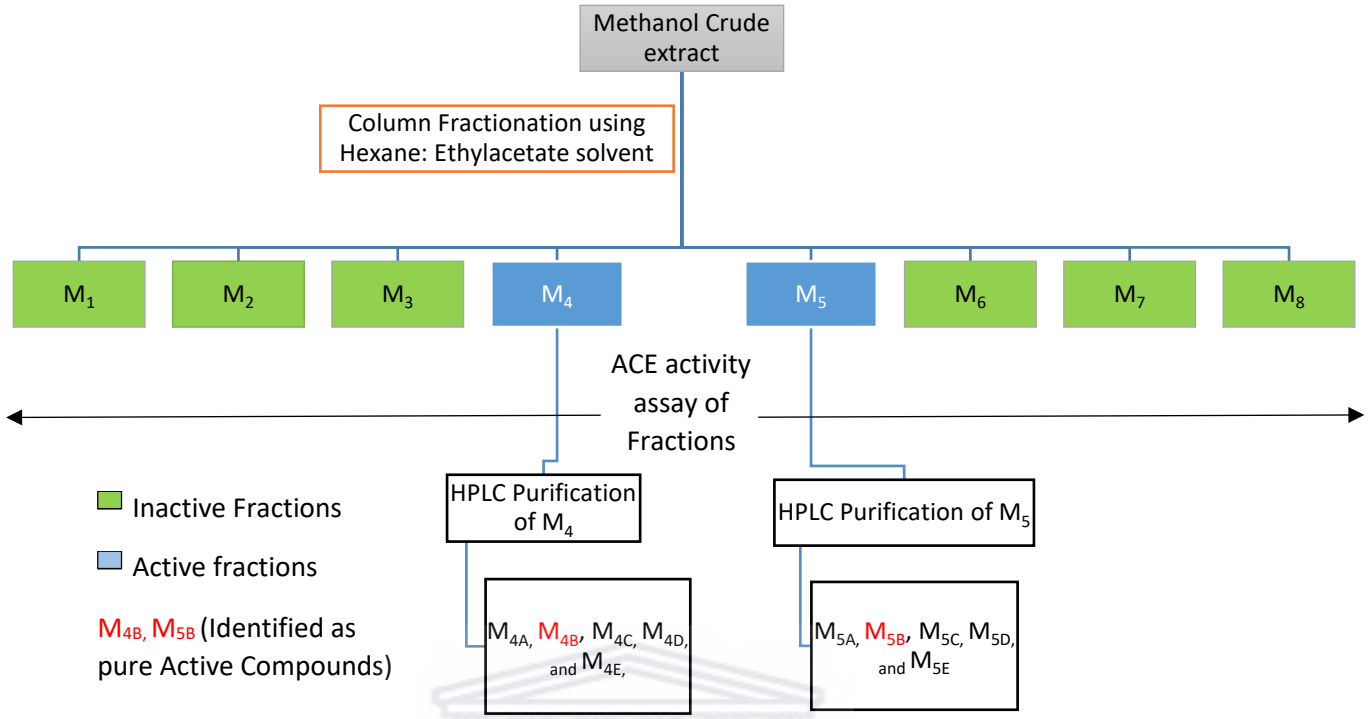

Figure 3. 1 Schematic Diagram for Bioassay guided fractionation of Methanol Extract

3.3.3.3 *ACE activity screening of fractions*

Column fractions of the methanol extracts of *C. asiatica* were screened for ACE activity using the ACE assay method described in section 3.3.2.5. Fractions M_4 and M_5 were determined to be the most active inhibitors of ACE, and were analysed by means of a thin layer chromatography and ^{1}H NMR spectroscopy to establish their purity.

3.3.3.4 *HPLC purification of active fractions*

Fractions M_4 and M_5 were purified on Agilent HPLC equipped with RI detector, and Whatman 10um Silica Prep column 50cm as stationary phase. Samples were run at a flow rate of 3ml/min, UV wavelength; 254nm, at Temperature of 30℃ with a runtime of 30 - 50 minutes.

3.3.3.4.1 Purification of Fraction M_4

Fraction M_4 150 mg (H4E6) was purified using normal phase HPLC [Whatman 10um Silica Prep column (50cm), isocratic elution using 1000 ml Hexane (40%): Ethyl acetate (60%) over 30 min, 3ml/min, detection at 254nm]. The sample was dissolved in 2.5ml of Hexane: Ethyl acetate (40:60), and 100 uL was first injected into the HPLC and eluted for 50 minutes, to establish the runtime. Subsequent injections of 200 uL each were ran for 30 minutes, with a total of 10 injections. All distinct peaks, ethyl acetate wash and fractions with no distinct peaks were collected.

3.3.3.4.2 Purification of fraction M_5

Fraction M_5 118.5 mg (H2E8) was purified using normal phase HPLC [Whatman 10um Silica Prep column (50 cm), isocratic elution using 1000 ml Hexane (20%): Ethyl acetate (80%) over 30 min, 3ml/min, detection at 254nm]. Prior to injection onto the column, fraction M_5 (118.5 mg) was dissolved in 2.0 ml hexane: Ethyl acetate (20:80), filtered, and 100 µl injected into the HPLC and eluted for 30 minutes. A total of 8 injections were carried out. Similar peaks were collected for each injection and combined together. All prominent peaks, ethyl acetate wash and fractions without distinct peaks were collected. Collected sample were dried by rotary evaporation, and sent for NMR analysis.

3.3.4 Bioassay-guided fractionation of ethanol crude extract

The ethanol extract of *C. asiatica* was chemically profiled by NMR spectroscopy, which revealed the presence of fatty acids. The ethanol extract was therefore defatted by dissolving the ethanol extract (2.3g) in methanol (100 ml) and transferred to a 500 ml separating funnel,

100 ml hexane was added followed by 20 ml distilled water. The separating funnel was stoppered, shaken and left to stand for 20 minutes. Fatty acid, hexane and aqueous methanol layers were separately collected, dried and assayed for ACE activity.

3.3.4.1 *Fractionation of the hexane fraction of the ethanol extract.*

The hexane fraction was chosen for fractionation following ACE activity assay screening. The hexane fraction (300 mg) was dissolved in 1ml of hexane: ethyl acetate (80:20) and was loaded unto the waters sepak column (2.5x 10cm) containing 10g silica gel. The column was eluted using a total of 400 ml of hexane: ethyl acetate (80:20). Fractions were collected in small volumes using glass vials to a total of 37 fractions. Collected fractions were immediately analysed on silica gel TLC plates to identify and pool similar fractions together. F2, F3 and F4 were pooled together as fraction A; F5, F6, F7, F8, and F9 as fraction B; F10, F11, F12, F13, F14, F15, F16, F17, F18 as fraction C; F19.F20, F21 as fraction D; F22, F23, F24, F25, F26, F27, F28 as fraction E; and F29, F30, F31, F32, F33, F34, F35, F36, F37 as fraction F. All pooled fractions were assayed for ACEI activity using the assay protocol in section 3.3.2.5.

3.3.4.1.1 HPLC Purification of Fraction A

Fraction A was chosen for further purification by virtue of the NMR chemical profile and ACE inhibition activity. Fraction A (80mg) was purified using normal phase HPLC [(Whatman 10um Silica Prep column (50cm); isocratic elution using 1000ml hexane (80%): ethyl acetate (20%) over 45 min; 3ml/min; detection at 254nm]. The sample was dissolved in 1ml of hexane (80%): ethyl acetate (20%), followed by an initial 100 µl injection to establish the separation time, then subsequently four 200 µl injections were carried out. A total run time of 30 minutes was established for the purification. Pronounced peaks were collected, dried in a glass vial and assayed for ACE activity. A total of 5 peaks and ethyl acetate wash were collected.

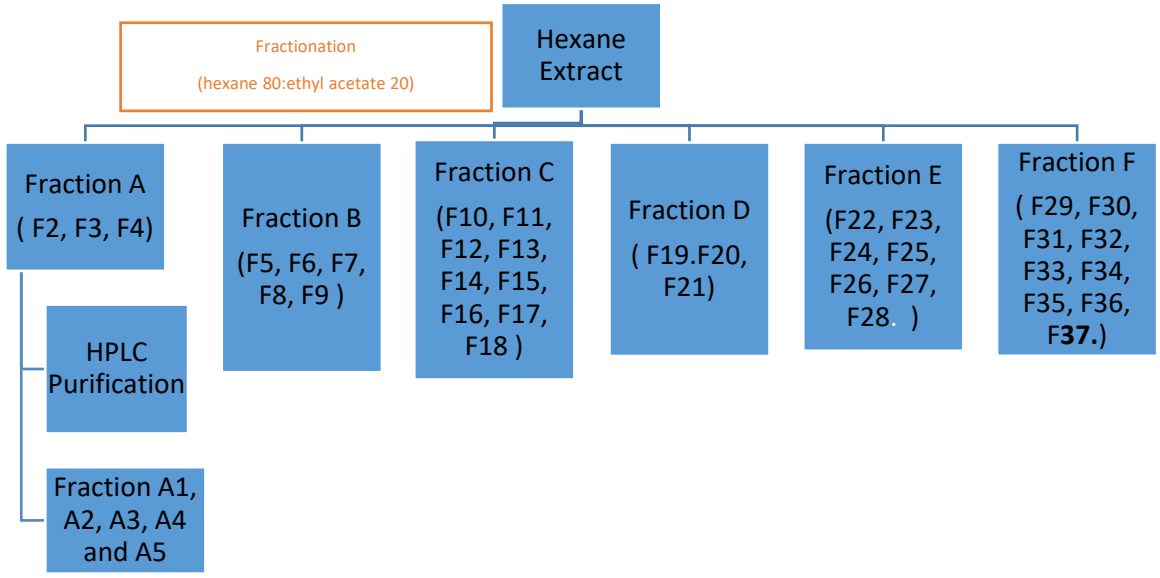

Figure 3. 2 Schematic Diagram for Bioassay guided fractionation of Hexane extract from Defatted Ethanol extract.

3.3.4.1.2 NMR Spectroscopy of fraction A

NMR spectral profile of sub-fraction A_3 (fraction of interest) revealed the presence of a coumaric acid structure. However, due to the difficulty in obtaining a clear elucidation of the full structure of the compound of interest as well as the insufficient quantity obtained from the purification process, methyl, ethyl and butyl esters of the synthetic coumaric acid were synthesized and compared to the proposed structure of the compound from fraction A_3. This was to facilitate the confirmation of the proposed structure.

Synthesis of the compounds were done by reflux, to synthesize the methyl ester of coumaric acid, 1.0014g of coumaric acid was added to a three neck round bottom flask, followed by 10ml Methanol and 0.5ml Conc. H_2SO_4. The sample was spotted on a TLC at intervals during the reflux process to monitor the progress of the reaction. After 2 hours, the sample was pipetted into a 250ml round bottom flask and dried on a rotary evaporator. Once drying was completed, 10ml $CHCl_3$ (ethyl acetate) was added to the flask, swirled and transferred to a separating

funnel. Distilled water was added leading to formation of layers, the aqueous layer was removed and saturated aqueous $NaHCO_3$ added. The aqueous layer was again removed, and the remaining sample washed with brine and allowed to dry in a desiccator.

For ethyl and propyl ester of coumaric acid, the process above was repeated but replacing Methanol with ethanol and propan-1-ol respectively.

3.3.5 NMR Spectroscopy of extracts/fractions/compounds

In this study, NMR was easily available and cost-effective, therefore was utilized for profiling of extracts/fractions/compounds and to monitor their purity at various steps. The preliminary analysis of the crude extracts and fractions were based on their 1D ^{1}H and 2D ^{13}C NMR and only the final pure compounds required a full data set (^{1}H, ^{13}C, DEPT, COSY, HMBC, HSQC). The methanol and ethanol crude extracts were initially profiled to get an overview of their chemical profile. As the fractionation process progressed, the resultant fractions at each stage were also profiled to determine the effect of solvent elution on the resolution of various components of the sample as well as to establish the purity of the fractions.

For the NMR analysis, Crude extracts were dissolved in 0.5ml $CDCl_3$, whereas some fractions and compounds were dissolved in 0.5ml CD_3OD due to poor solubility in $CDCl_3$. Samples were subsequently transferred into a 5mm NMR tube, which were sealed, appropriately labelled and sent for analysis. The generated spectra were analysed using Topspin® Software 4.0.6. Analysed samples were retrieved by evaporation

3.3.6 Spectroscopic identification of bioactive compound

3.3.6.1 *Infrared (IR) spectroscopy*

Infrared spectra of the isolated compounds was measured using Perkin Elmer Spectrum 400 Fourier transform infrared (FTIR) spectrometer (Perkin Elmer Scientific, Massachusetts, United States). For measurement, a small dried sample of the isolated compounds were transferred to the crystal of the IR equipment. Some Pressure (50 - 60%) was gradually applied using the pressure gauge. Measurement was done at a scanning range of 500 to 4000 $cm^{-}1$. Generated IR spectra was identified and analysed by OMNIC Spectra software provided in the spectrometer system.

3.3.6.2 *UV Spectroscopy*

UV spectrum of the compounds were measured using GBC CINTRA 202 UV-VIS Spectrometer. Samples were dissolved in methanol. The UV spectra was analysed using the CINTRA Software.

3.3.6.3 *NMR spectrometry*

NMR experiments were performed using Bruker AV400 NMR spectrometer in the NMR laboratory of University of the Western Cape. Samples were dissolved in deuterated methanol (CD_3OD) and $CDCl_3$, filtered through a cotton wool plugged Pasteur pipette and placed in 5mm NMR tube (Norell, United States). All chemical shifts were referenced relative to corresponding residual solvent signals. The NMR experiments were analysed using "Topspin" software. The 1D ^{1}H and ^{13}C NMR experiments were carried out. In addition, homo and Heteronuclear 2D experiments, Correlated Spectroscopy with gradients (g-COSY) and Heteronuclear Multiple-Quantum Correlation (HMQC) spectra were also acquired.

3.3.6.4 *Mass spectrometry*

Mass Spectroscopy analysis of compounds was carried on Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer at Stellenbosch University Central Analytical Facility. 1 mg of each sample was dissolved in 1 ml methanol (Romil), followed by a further 10-fold dilution into methanol. 2 µL of sample was injected into a stream of methanol flowing at 0.3 ml/min, using a Waters ultra-pressure liquid chromatograph (UPLC) (Waters, Midford, USA) which conveyed the sample to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer used for high-resolution accurate mass analysis.

Data was acquired in scan mode, the mass spectrometer was optimized for best sensitivity, a cone voltage of 15 V, desolvation gas was nitrogen at 650 L/hr and desolvation temperature 275 ºC. The instrument was operated with an electrospray ionization probe in the positive mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determinations

3.3.7 Antioxidant assay of compounds

3.3.7.1 *DPPH radical scavenging assay*

Radical scavenging activity of isolated active compounds against stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich, Germany) was determined using spectrophotometry. When DPPH (a stable free radical) reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in form of absorbance were measured at 517 nm on a UV/visible light spectrophotometer (GBC CINTRA 202 New Hampshire, USA).

The radical scavenging activity of the extracts were estimated according to the procedure described by (Shen *et al*., 2010) with some modification. Briefly, a 0.1 mM solution of DPPH

radical solution in methanol was prepared and 1 ml of this solution was mixed vigorously with 3ml of different concentrations (31.25,62.5, 125, 250, 500 µg/ml in methanol) of each extract. After 30 min incubation in the dark and at room temperature, absorbance (*A*) was measured at 517 nm using a UV/VIS spectrometer. The percentage of the radical scavenging activity (RSA) was calculated based on the following equation:

$$\textbf{DPPH Scavenged (\%)} = (A_{Control} - A_{Sample})/A_{Control} \times 100$$

$A_{control}$ **and** A_{sample} are the absorbance values (at 517 nm) for the control and sample, respectively.

Sample = 1ml DPPH solution + 3ml Sample Solution

Negative Control = 1ml DPPH solution + 3ml Distilled water

Positive Control = 1mm DPPH + 3ml various concentration of Ascorbic acid in methanol

EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was obtained from the linear regression of plots of mean percentage of the DPPH radical scavenging activity against the concentration of the test compounds (µg/ml) obtained from three replicate assays.

3.4 Statistical Analysis

All experiments/ analyses in the present study were performed in triplicates. The results were expressed as mean. EC_{50} and IC_{50} calculation were determined from non-linear regression plot using Microsoft Excel.

The next chapter will report and discuss the results of the methods, protocols and procedures followed above.

Chapter 4

Results

Introduction

ACE Inhibitors remain crucial in the therapeutic strategy for treatment of cardiovascular disease and its risk factor hypertension. Increasing use and interest in plants highlights the need for increased research towards their quality control to ensure safe use, validate their claimed therapeutic efficacy and to identify bioactive/novel compounds that could replenish already depleting therapeutic armory and provide alternatives to ineffective and problematic drugs. The aim of the study was to isolate a bioactive ACE inhibitory compound, thereby validating the claimed use of *Centella asiatica* in the treatment of hypertension.

This chapter presents the results of ACE inhibition activity screening of crude extracts, fractions and compounds derived from the bioassay guided fractionation of *Centella asiatica*, and the identification, elucidation and antioxidant activity of the isolated bioactive compounds.

4.1 Extraction and preliminary bioassay of plant extracts

The extraction of 500 g of *Centella asiatica* using methanol, ethanol and water as solvents resulted in methanol, ethanol and freeze dried aqueous extracts with percentage yields of 15% w/w (75 g), 11.2% w/w (56 g) and 4.6% w/w (23 g) respectively. The greater yields with methanol and ethanol as compared to water suggests that the plant's constituent compounds were more likely to exhibit greater solubility in polar than non-polar solvents.

The ACE inhibition assay of the crude organic and aqueous extracts was conducted according to modified method of Balasuriya and Rupasinghe (2012) as detailed in section 3.3.2.5. Concentrations of 1 mg/ml, 6 mg/ml and 10 mg/ml of the methanol, ethanol and aqueous extracts of *Centella asiatica* inhibited ACE activity in a dose dependent manner (table 4.1).

Inhibition of ACE was greatest with the methanol extract (85% at 10 mg/ml concentration), while the aqueous extract exhibited the least inhibition of enzyme activity (36.2% at 10 mg/ml concentration). The methanol and ethanol extracts exhibited inhibition significantly greater than that obtained with similar concentrations of the aqueous extract for all concentrations assessed. Although both methanol and ethanol extracts exhibited greater than 50% enzyme inhibition at the 1mg/ml concentration, inhibition was significantly lower than that observed with the same concentration of the standard ACEI captopril (98.5%) (table 4.1). Of the three extracts tested, the methanol and ethanol extracts demonstrated inhibition of ACE above 50% at all concentrations, and these extracts were subsequently subjected to the bioassay-guided fractionation process as described in section 3.3.2.5 of **Chapter 3**.

Table 4. 1 Percentage inhibition of ACE activity by the methanol, ethanol and aqueous crude extracts of *Centella asiatica.*

Plant Extract	**% Inhibition**		
	(1mg/ml)	(6mg/ml)	(10mg/ml)
Methanol Extract	51.36	63.0	85.0
Ethanol Extract	55.2	66.4	78.6
Aqueous Extract	18.2	25.7	36.2
Captopril	98.5	-	-

4.2 Bioassay guided fractionation of the methanol crude extract

The fractionation of the methanol extract using silica gel column chromatography, yielded 8 fractions (M_1– M_8), which were assayed for ACE inhibition activity using the assay protocol described in section 3.3.2.5. Fractions M_1 and M_2 produced no inhibition of enzyme activity when tested for inhibition of ACE activity at the 1 mg/ml concentration, while fractions M_3, M_6, M_7 and M_8 all produced inhibition of enzyme activity below 50% (table 4.2). Fraction M_4

was the most active (60.6 % inhibition), followed by fraction M_5 (53.3 % inhibition) at the 1 mg/ml concentration (table 4.2). There was an observed increase in the percentage inhibition of ACE by both fractions (M_4 and M_5) compared to that obtained with the crude methanol extract (51.36% Inhibition) at a similar concentration. This increase could be attributed to concentration of active components as inactive diluents are fractionated out. Due to the greater inhibitory activity observed with fractions M_4 and M_5, they were chosen for NMR analysis and further HPLC purification to isolate active compound.

Table 4. 2 Percentage inhibition of ACE activity by fractions of the methanol extract of *Centella asiatica.*

Column Fractions	% ACE Inhibition (1 mg/ml)	Percentage Yield
M_1	-	-
M_2	-	0.23
M_3	0.5	1.6
M_4	60.6*	1.0
M_5	53.3*	0.79
M_6	20.1	0.38
M_7	28.3	53.37
M_8	30..4	29.79

* Active fractions subjected for further purification.

4.2.1. HPLC purification of active fractions (M_4 and M_5)

Fractions M_4 and M_5 were purified using a normal phase preparative HPLC according to the method described in sections 3.3.3.4.1 and 3.3.3.4.2.

HPLC fractionation of fraction M_4 (150 mg) resulted in 5 fractions (M_{4A}, M_{4B}, M_{4C}, M_{4D} and, M_{4E}) (see appendix XXXII for chromatogram), which were subjected to the ACEI bioassay at a concentration of 1mg/ml. Fraction M_{4B} exhibited the greatest ACE inhibition of 65% followed by M_{4C} at 20.5%. The other fractions showed ACE inhibition less than 15% (table 4.3). Apart from the ethyl acetate wash, M_{4B} and M_{4C} showed the highest yield of 12.67% and 12.4% respectively in relation to the 150mg starting material (M_4). Further bioassays and NMR spectroscopy focused on fraction M_{4B} as the fraction with the greatest ACE inhibition (see appendix II for NMR spectra).

Table 4. 3 Percentage inhibition of ACE activity by sub-fractions of fraction M_4 of the methanol extract of *Centella asiatica.*

Fractions	Retention time	% Yield	% ACE inhibition (1mg/ml)
M_{4A}	**11.383**	**8.53**	**3.2**
M_{4B}*	**13.386**	**12.67**	**65**
M_{4C}	**15.241**	**6.6**	**20.5**
M_{4D}	**17.182**	**5.4**	**10**
(M_{4E}) Ethyl acetate wash	**-**	**22.23**	**15**
Fractions with no peak	**-**	**37.47**	**12.36**

***Most Active Fraction identified as pure compound

HPLC fractionation of fraction M_5 (115mg) yielded 5 fractions (M_{5A}, M_{5B}, M_{5C}, M_{5D} and M_{5E}) (see appendix XXXIII for chromatogram). ACEI bioassay at a concentration of 1mg/ml revealed fraction M_{5B} to exhibit the highest ACE inhibition of 70.5% followed by M_{5C} (23.2%). Fraction M_{5A} exhibited no inhibition of enzyme activity, while all other fractions showed ACE

inhibition less than 15% (table 4.4). Further bioassays and NMR spectroscopy focused on M_{5B} as the fraction with the greatest ACE inhibition.

Table 4. 4 Percentage inhibition of ACE activity by sub-fractions of fraction M_5 of the methanol extract of *Centella asiatica.*

Purified Fractions	Retention time	% Yield	% ACE inhibition (1mg/ml)
M_{5A}	9.150	5.04	-
M_{5B} *	10.804	11.02	70.5
M_{5C}	11.453	4.2	23.2
M_{5D}	13.713	8.17	10.4
M_{5E} (Ethyl acetate Wash)	-	20.57	7
Fractions with no peak	-	40.26	5

* Most active Fraction identified as pure compound

4.3. Bioassay guided fractionation of the ethanol extract

NMR profiling of the ethanol extract of *Centella asiatica* revealed the presence of fatty acids. Prior to the fractionation process, the extract was defatted according to the method described in section 3.3.3.5.1.

Defatting yielded methanol (550.6mg) and hexane (848.4mg) extracts which were subjected to ACE inhibition activity assay. The hexane extract exhibited a greater inhibition of ACE activity (51%) compared to the methanol extract (45 %), and so was subjected to further fractionation to isolate active compound/s.

Fractionation of the hexane extract yielded 5 fractions (F_A - F_E), with Fraction F_A exhibiting the highest ACE inhibition (49 %) (Table 4.5). Fraction A was subjected to further purification to isolate active compound(s)

Table 4. 5 Percentage Inhibition of ACE activity by Hexane extract fractions

Fractions	% ACE Inhibition
F_A	49
F_B	12.5
F_C	21
F_D	8.1
F_E	5.2

4.3.1. Purification of fraction F_A

Fraction F_A was purified using normal phase HPLC (see section 3.3.3.5.2). Four peaks eluting at 9.456, 11.076, 14.856, and 18.440 minutes, and the ethyl acetate wash were collected, yielding sub-fractions F_{A1} - $F_{A5.}$ ACE inhibition assay at a concentration of 1mg/ml produced the greatest inhibition (39%) with F_{A3} (see table 4.6). Fraction F_{A1} and F_{A5} exhibited no ACE inhibition activity while F_{A2} and F_{A4} showed less than 20% inhibition.

Table 4. 6 Percentage Inhibition of ACE activity by HPLC fractions of Hexane extract.

HPLC Fractions	Retention Time	% Yield	% ACE inhibition
F_{A1}	**9.456**	**5.2**	**-**
F_{A2}	**11.076**	**8.5**	**4.2**
F_{A3}	14.856	15.6	39
F_{A4}	18.440	18.1	15.8
F_{A5} (ethyl acetate wash)	-	43.8	-

NMR analysis of F_{A3} indicated that it consisted of one compound. However, some difficulty was experienced in elucidating the complete structure. The compound was suspected to be an

ester of coumaric acid, hence the decision to synthesize the esters of a synthetic coumaric acid to enable the identification of the purified compound through the comparison of its NMR spectra to that of the synthesized compounds.

4.3.1.1 *Synthesis of methyl, ethyl and propyl ester of coumaric acid*

Three esters of coumaric acid were successfully synthesized with yields of 95.75% (methyl hydroxycinnamate), 85.2% (ethyl hydroxycinnamate) and 51.67% (propyl hydroxycinnamate) (see appendix I for synthesis pathway). The NMR profile of the three compounds were compared to isolated compound F_{A3} to fully elucidate the structure (see appendix XXVIII – XXX for NMR data), and the isolated compound identified as ethyl hydroxycinnamic acid.

ACE activity was conducted on the synthesized ethyl hydroxycinnamic acid due to insufficient quantities of the isolated compound. The synthesized ethyl hydroxycinnamate (1 mg/ml) exhibited low ACE inhibitory activity of 15.2%, significantly lower that the inhibition produced by the compounds isolated from the methanol extract. The synthesized compound was not pursued further owing to its low ACE inhibition and as well as insufficient quantities of the isolated compound.

4.4 Structural elucidation and characterization of the isolated compounds

Column chromatographic separation of the methanol extract yielded fractions M_4 and M_5 with significant ACE inhibition activity. The fractions were further purified using HPLC to produce M_{4B}, from fraction M_4, and two compounds M_{5B} and M_{5C} from fraction M_5. NMR, MS, IR and UV Spectroscopy were used as tools for elucidation and characterization of the isolated compounds, comparable literature data of the isolated compounds were thoroughly searched using Scifinder, Molbase and PubChem. The results are presented below.

4.4.1. Structural elucidation and characterization of compound M_{4B} (Kaempferol)

M_{4B} (19 mg, 12.7% w/w yield) was a yellow powder with good solubility in ethanol, methanol, dimethyl sulfoxide, and poor solubility in water, and a melting point of 277 °C. The polar solubility of the isolated compound could be related to the initial solvent of extraction (methanol). The UV spectrum showed λmax at 265 and 366 nm suggestive of the presence of flavonol bond (see appendix VIII). The ^{1}H-NMR (400MHz, CD_3OD,) showed 2 peaks at δ 6.11 (^{1}H, d, J = 1.87 Hz) and 6.30 ppm (^{1}H, d, J = 2.34 Hz) consistent with the meta protons H-6 and H-8 on A-ring and an AA′BB′ system at 7.99 (2H, d, J = 8.9 Hz, H-2′, 6′) and 6.79 (2H, d, J = 8.87 Hz, H-3′, 5′) corresponding to the protons on B-ring. ^{13}C NMR (100 MHz, CD_3OD,) ppm 146.47 (s, C-2), 135.1 (s, C-3), 176.37 (s, C-4), 161.26 (s, C-5), 97.86 (d, C-6), 164.17 (s, C-7), 93.1 (d, C-8), 156.86 (s, C-9), 122.33 (s, C-1'), 129.28 (d, C-2'), 115.48 (d, C-3'), 159.16 (C-4'), 121.9 (d, C-5'), 134.7(d, C-6'), 109.9 (d, C-l''), 83.5 (d, C-2''), 78.8 (d, C-3''), 88.6 (d, C-4''), 62.4 (t, C-5'') (see appendix III and IV). The HRMS yielded a quasi-molecular ion peak $[M-H]^-$ at *m/z* 285 and $[M+H]^+$ at *m/z* 287 and corresponded with the molecular formulae $C_{15}H_{10}O_6$, and a molecular mass of 286.04 g/mol (See appendix X). The compound was elucidated **as 3,4′,5,7-Tetrahydroxyflavone** (kaempferol) (see figure 4.1) by analysis of the various spectral data (MS, ^{1}H-, ^{13}C-NMR), and by comparison to literature values (Aisyah *et al*., 2017)**(table 4.7).**

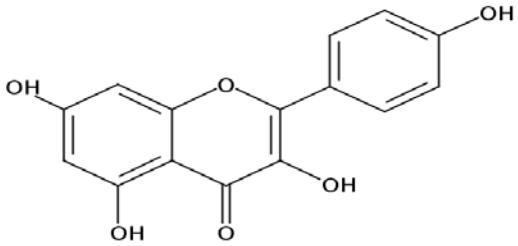

Figure 4. 1 Structure of 3,4′,5,7-Tetrahydroxyflavone (kaempferol)

Table 4.7 NMR Spectroscopic data of 3,4′,5,7-Tetrahydroxyflavone (kaempferol)

Carbon Position	**δ_H (ppm)**	**Mult.**	***J* (Hz)**	**δ_C (ppm)**	**Type**	**HMBC**	**Literature Values (δ_H)** (Aisyah *et al*., 2017)	**Literature Values (δ_C)** (Aisyah *et al*., 2017)
2				**146.64**	C			**146.8**
3					C			
4				**176.37**	C			**176.6**
5				**161.26**	C			**162.3**
6	**6.11**	**d**	**1.87**	**97.86**	CH	C8	**6.28**	**99.2**
7				**164.17**	C			**164.9**
8	**6.30**	**d**	**2.34**	**93.1**	CH	C6	**6.52**	**94.4**
9				**156.86**	C			**157.7**
10				**103.15**	C			**104.1**
1’				**122.33**	C			**123.3**
2’	**7.99**	**d**	**8.90**	**129.28**	CH	C6’	**8.04**	**125.9**
3’	**6.79**	**d**	**8.87**	**115.48**	C		**6.95**	**116.3**
4’	**-----**			**159.16**	C			**160.1**
5’	**6.79**	**d**	**8.87**	**115.48**	C	C1’	**6.95**	**116.3**
6’	**7.99**	**d**	**8.90**	**129.28**	CH	C2’,C4’, C2	**8.04**	**125.9**

4.4.2 Structural elucidation and characterization of compound M_{5B} (quercetin)

M_{5B} (5.5mg 4.8% w/w Yield) was a yellow powder with good solubility in ethanol, methanol, dimethyl sulfoxide, and poor solubility in water, and with a melting point of 313 °C. The UV spectrum showed λ max at 256 and 370 nm, suggesting the presence of a flavonol bond (see appendix XVII). The IR spectrum, recorded in the 4000 to 600 cm^{-1} range, was typical of a flavonoid structure. The spectra showed absorption bands with maxima at the following frequencies (V_{max}) 3400.79, 2925, 1607.46, 1520.49, 1362.89. The peaks at 3400.79 and 2925 are characteristic of the aromatic ring structure and absorption maxima at 1607 and 1520 indicating the presence of quinoid structure and –C=C- bonds, respectively. The absorption maximum at 1362 cm^{-1} is due to phenolic OH groups (see appendix XIX). The 1H-NMR spectrum showed 2 characteristic peaks at δ6.20 (1H, d, *J* = 2.0 Hz) and 6.41 ppm (1H, d, *J* = 2.0 Hz) consistent with the meta protons H-6 and H-8 on A-ring and implying the presence of a 5,7-dihydroflavonol in an A ring, which connects to no glycosides. Peaks at 7.55 (1H, d, *J* = 2.0 Hz, 8.4 Hz, H-6′), 7.68(1H, d, *J* = 2.2 Hz, H-2′), and 6.88 (1H, d, *J* =8.6 Hz, H-5′) corresponding to the catechol protons on B ring. The ^{13}C-NMR spectrum revealed ^{l3}CNMR (100MHz CD_3OD) 8 92.99 (C-8), 97.82 (C-6), 103.11 (C-l0), 115.48 (C-3', C-5'), 122.73 (C-1 '), 129.28 (C-2', C-6’), 135.38 (C-3), 147.37 (C-2), 156.83(C-9), 147.7 (C-4’), 161.12 (C-5), 164.17 (C-7), 175.93 (C-4) (see appendix XII- XIII). The HRMS yielded a molecular peak $[M-H]^-$ at *m/z* 301 and $[M+H]^+$ at *m/z* 303 and corresponded to the molecular formulae $C_{15}H_{10}O_7$,and a molecular mass 302.05g/mol (see appendix XVIII).The compound was elucidated as **3,5,7,3′,4′- pentahydroxyflavone** (quercetin) by analysis of the various spectral data (MS, 1H-, 13C-NMR), and by comparing to literature values(Aisyah *et al*., 2017; Cao, Wan, Yu, Zhou, and Tian, 2011) (figure 4.8)**.**

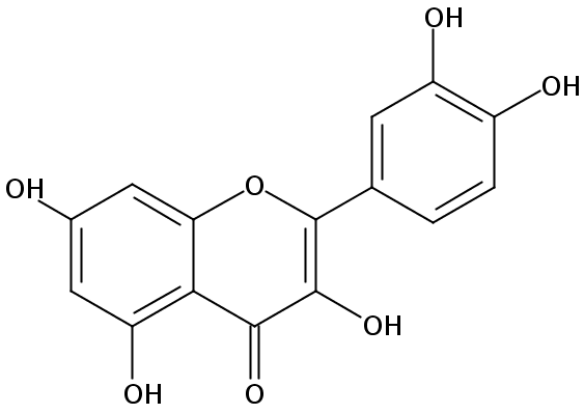

Figure 4. 2 Structure of 3,5,7,3′,4′- pentahydroxyflavone (quercetin)

Table 4. 8 NMR Spectroscopic data of 3,5,7,3′,4′- pentahydroxyflavone

Carbon Position	**δ_H(ppm)**	**Mult**	***J* (Hz)**	**δ_C(ppm)**	**Type**	**HMBC**	**Literature Values(δ_H)** (Aisyah *et al*., 2017)	**Literature Values(δ_C)** (Aisyah *et al*., 2017)
2				**147.37**	C			147.7
3				**135.83**	C			135.7
4				**175.93**	C			176.8
5				**161.12**	C			160.7
6	**6.20**	**d**	**2.0**	**97.82**	CH	C8	6.20	94.5
7				**164.17**	C			163.9
8	**6.41**	**d**	**2.0**	**92.99**	CH	C6	6.40	94.5
9				**156.83**	C			156.1
10				**103.11**	C			103.0
1’				**122.73**	C			121.9
2’	**7.68**	**d**	**2.2**	**114.60**	CH	C6’	7.65	115.0
3’				**145.20**	C			145.0
4’				**147.7**	C			145.8
5’	**6.88**	**d**	**8.6**	**116.05**	C	C1’ , C3’	6.88	115.6
6’	**7.55**	**d**	**8.4**	**129.68**	CH	C2’,C4’, C2	7.50	124.5

4.4.3 Structural elucidation and characterization of compound M_{5C} (4 Hydroxybenzoic acid)

M_{5C} was a white powder soluble in ethanol and methanol, and slightly soluble in water, with a melting point of 214.5^{o}C. The UV spectrum showed λ max at 256 and 369 (see appendix XXVI). The NMR spectrum shows 1H-NMR (MeOH-d, 400 MHz) δ 7.78 (2H, dd, J = 8.75,1.96 Hz, H-2,6), 6.72 (2H, dd, J = 8.78,1.9.2 Hz, H-3,5). 13C-NMR (CD3OD, 100 MHz) δC: 168.18 (COOH), 163.35 (C-4), 131.59 (C-2,6), 121.29 (C-1), 114.91(C-3,5) (see appendix XXI - XXII). The HRMS yielded a molecular peak $[M-H]^-$ at *m/z* 137 and $[M+H]^+$ at *m/z* 139 and corresponded to the molecular formulae $C_7H_6O_3$, and a molecular mass 138.02 g/mol (see appendix XXVII). The compound was elucidated as 4-Hydroxybenzoic acid (figure 4.13) by analysis of the spectral data and comparison to literature data**(Lee *et al.*, 2011)**

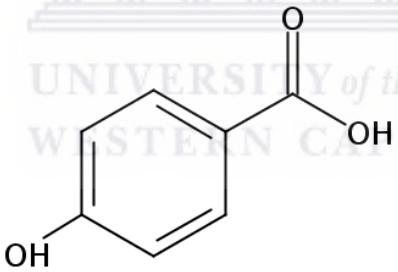

Figure 4. 3 Structure of 4-Hydroxybenzoic acid

Table 4. 9 NMR Spectroscopic data of 4-Hydroxybenzoic acid

Carbon atom	**δ_H(ppm)**	**Mult**	***J*(Hz)**	**Type**	**δ_C(ppm)**	**Literature Values(δH)** (Lee *et al.*, 2011)	**Literature Values(δC)** (Lee ***et al.***, 2011)
1				C 1	121.29		122.9
2	7.78	dd	8.75	C 2	131.59	7.88	133.2
3	6.72	dd	8.78	C 3	114.91	6.82	116.2
4				C 4	163.35		163.5
5	6.72	dd	8.78	C 5	114.91	6.82	116.2
6	7.78	dd	8.75	C 6	131.59	7.88	133.2
7				- COOH	168.18		170.2

4.5 Determining the IC_{50} of captopril

The IC_{50} value of an inhibitor is dependent upon inhibition mechanisms and assay conditions used and may vary from one laboratory to another, but is useful for comparing activities within a set of determinations **(Bush, 1983).**

IC_{50} of captopril was determined and compared to the literature value of 23nM (**Ondetti, 1988).** Table 4.5 shows the percentage ACE inhibition by captopril at various concentrations. Captopril in this study exhibited a higher IC_{50} of 85.76nM in comparison to the literature value of 23nM, but was still significant in relation the isolated compounds.

Table 4.10 IC_{50} of Reference standard (Captopril)

Concentration of Captopril in assay (ng/ml)	% ACE Inhibition
6,25	27
12,5	45
25	60
50	65,8
100	78
200	91,3

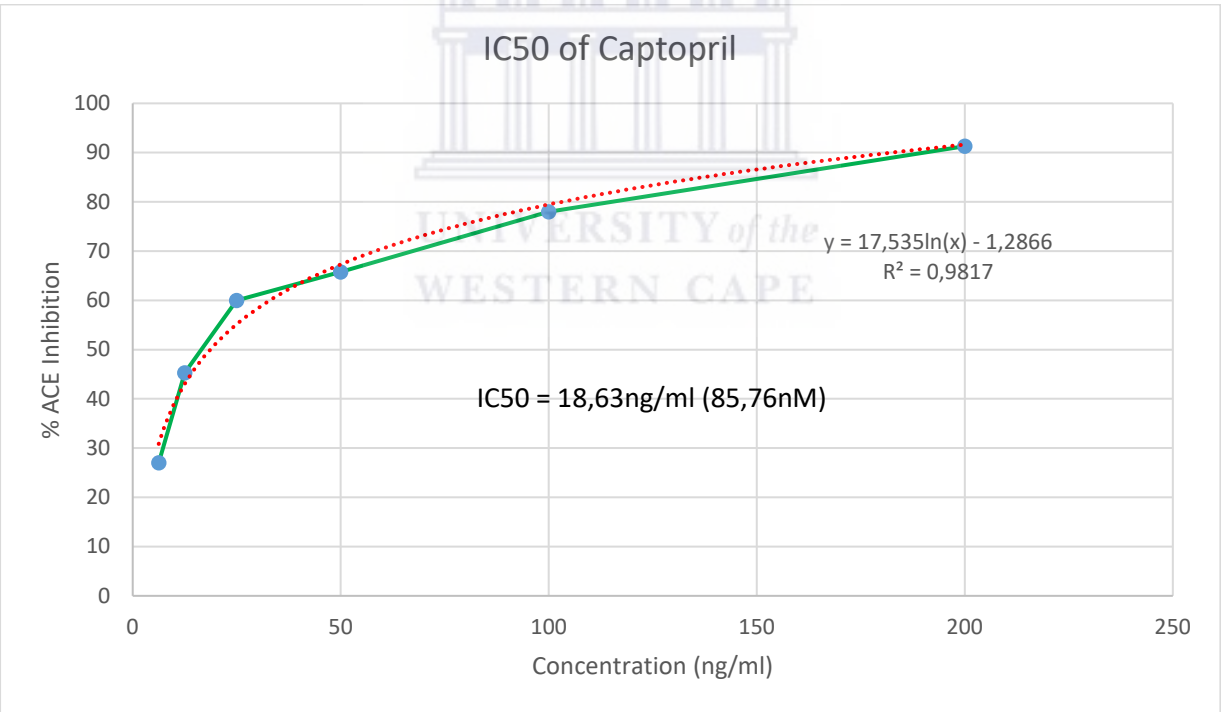

Figure 4. 4 IC_{50} and percentage inhibition of ACE by captopril

4.6 ACE inhibition and IC_{50} of the isolated compounds

The ACE activity of the compounds were assayed based on the fluorimetric determination of the amount of histidyl-leucine (His-Leu) cleaved from the enzyme substrate, hippuryl-histidyl-leucine (Hip-His-Leu). Detection of fluorescence intensity was aided by a fluorescence compound o-pthaldialdehyde (OPA) that binds to His-Leu. The ACE bioassay was conducted on the isolated compounds **(**3,4′,5,7-Tetrahydroxyflavone and 3,5,7,3′,4′-Pentahydroxyflavone**)** that exhibited significant inhibitory effect on ACE activity.

Both compounds exhibited a concentration dependent inhibition of ACE activity greater than 50% at 500 ug/ml concentration. 3,5,7,3′,4′- pentahydroxyflavone expressed a greater ACE inhibition at all concentrations compared to 3,4′,5,7-Tetrahydroxyflavone. 3,5,7,3′,4′-pentahydroxyflavone at the highest dose of 1000 µg/ml, showed ACE inhibition of 73% compared to 3,4′,5,7-Tetrahydroxyflavone (59.9%), and the 50% inhibitory concentration of 3,5,7,3′,4′- pentahydroxyflavone (302.72 µg/ml) was 1.8 fold lower than that obtained with 3,4′,5,7-Tetrahydroxyflavone (542.39 µg/ml), indicating that 3,5,7,3′,4′- pentahydroxyflavone was a better ACE inhibitor than 3,4′,5,7-Tetrahydroxyflavone (table 4.11 and figure 4.15). The IC50 of the reference inhibitor (captopril) as determined in this study was 18.63 ng/ml

Table 4. 11 Percentage inhibition of ACE activity by kaempferol and quercetin isolated from *C. asiatica*.

% ACE Inhibition activity of isolated compounds		
Concentration (µg/ml)	**3,4′,5,7-tetrahydroxyflavone (M_{4B}) Kaempferol**	**3,5,7,3′,4′- pentahydroxyflavone (M_{5B}) Quercetin**
62.5	2	11.4
125	14.0	21.5
250	34.6	47.7
500	53.0	68.3
1000	59.9	73.0
IC 50	542.39 µg/ml	302.72 µg/ml

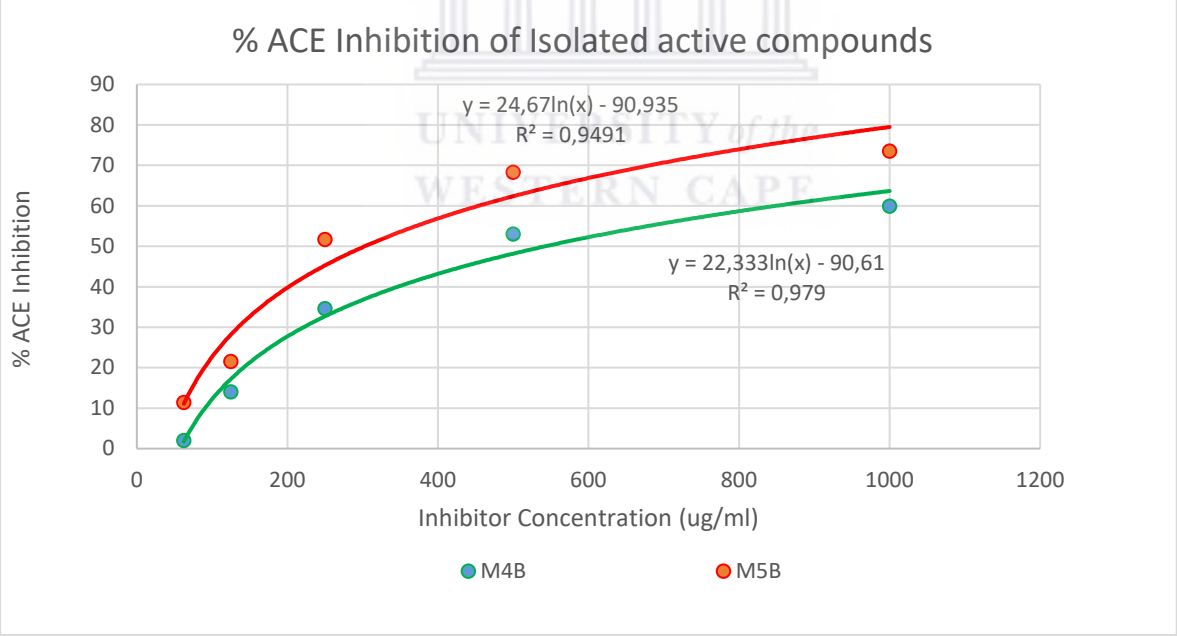

Figure 4. 5 ACE inhibitory activity of 3,4′,5,7-tetrahydroxyflavone (M_{4B}) *Kaempferol* and 3,5,7,3′,4′- pentahydroxyflavone (M_{5B}) *Quercetin*

4.7 DPPH radical scavenging assay

The DPPH assay method is a rapid, simple and inexpensive method to measure antioxidant capacity using DPPH (a stable free radical). The DPPH radical scavenging activity indicates hydrogen donating ability of compounds. The degree of reduction in absorbance measurement is indicative of scavenging potential of compounds. In this study, the DPPH radical scavenging capacity of the active compounds (3,4′,5,7-Tetrahydroxyflavone and 3,5,7,3′,4′-Pentahydroxyflavone) was evaluated.

All the test compounds exhibited dose dependent scavenging of DPPH radical. Compounds **(**3,4′,5,7-Tetrahydroxyflavone and 3,5,7,3′,4′-Pentahydroxyflavone**)** showed comparable antioxidant activity to the positive control (ascorbic acid). At a concentration of 125 µg/ml, 3,4′,5,7-Tetrahydroxyflavone, 3,5,7,3′,4′-Pentahydroxyflavoneand ascorbic acid scavenged more than 50% of the free radicals. At double the concentration i.e. 250 µg/ml, 3,5,7,3′,4′-Pentahydroxyflavone and ascorbic acid showed greater than 85% scavenging capacity compared to 3,4′,5,7-Tetrahydroxyflavone at 75.4%. The highest scavenging activity of 91.2% was exhibited by 3,5,7,3′,4′-Pentahydroxyflavone followed by 3,4′,5,7-Tetrahydroxyflavone (81%) at the highest concentration of 500 ug/ml. The EC50 value for 3,5,7,3′,4′-Pentahydroxyflavone was determined as 84.83 µg/ml, which was 1.3 fold more potent than 3,4′,5,7-Tetrahydroxyflavone (109.99 µg/ml) and 2.2 fold less potent than ascorbic acid (38.77 µg/ml). Figure 4.16 below depicts the scavenging activity of the isolated compounds and ascorbic acid.

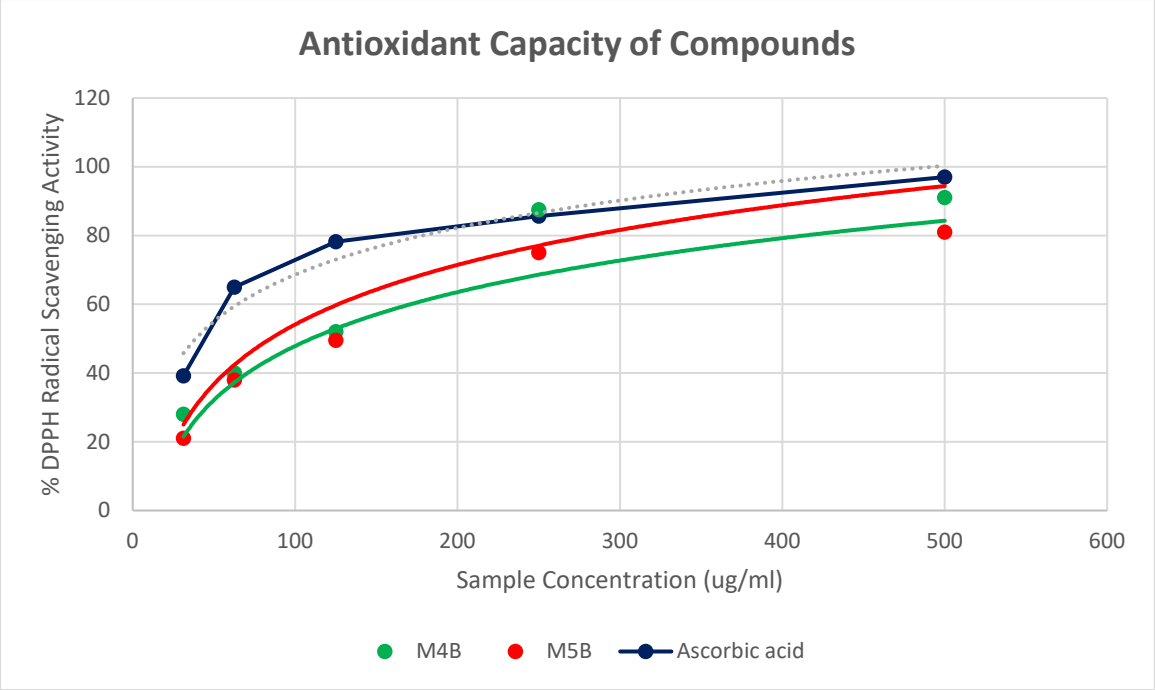

Figure 4. 6 DPPH radical scavenging activity of the isolated active compounds.

Table 4. 12 EC_{50} of compounds

Compound	**EC_{50} (µg/ml)**	**Regression Equation**	**Correlation coefficient (R^2)**
3,4′,5,7-Tetrahydroxyflavone(M_{4B})	109.99	y = 22,65ln(x) - 56,463	0,9746
3,5,7,3′,4′-Pentahydroxyflavone(M_{5B})	84.83	y = 25,031ln(x) - 61,156	0,9393
Ascorbic acid	38.77	y = 19,65ln(x) - 21,874	0,9425

Chapter 5
Discussion and Conclusion

5.1 Discussion

As part of our search for an alternative therapeutic approach for treatment of high blood pressure, as well as scientific validation of claimed therapeutic use of medicinal plants in hypertension. The methanol, ethanol and aqueous extracts of *Centella asiatica* were screened for their inhibitory effects on ACE. Amongst the extracts screened, methanol and ethanol extracts exhibited significant ACE inhibitory activity of 51.36% and 55.2% at 1mg/ml concentration respectively and 85% and 76.8% at 10mg/ml concentration respectively.

The extraction yield of the methanol and ethanol extracts in this study were 15% and 11.7% respectively compared to a much lower yield of 4.6% for the aqueous extract. This reflected an order of decreasing solvent polarity from water (lowest yield) to methanol (highest yield). Similar observation was made with regards to the ACE inhibitory effects of the crude extracts of methanol and ethanol which was greater compared to the aqueous extract. These differences in ACE inhibition by various extracts could be attributed to the solvent polarity and its ability to extract specific types of compound. In this study, less polar solvents such as methanol and ethanol seemed to extract ACE inhibitory compounds compared to the more polar aqueous solvent. A study by **Truong *et al* (2019)** showed methanol solvent extracted the highest level of phenolics, flavonoids, alkaloids and terpenoids compared to aqueous and ethanol solvent, aqueous solvent was said to show high efficiency in extraction of phenolic but not flavonoids, alkaloids and terpenoids. For this study, the aqueous extract exhibited a low yield and poor ACEI activity possibly due to poor solubility of the ACE inhibitory compounds in aqueous solvent.

The bioassay guided fractionation of the methanol and ethanol extracts afforded three pure compounds. These compounds were identified as 3,4′,5,7-tetrahydroxyflavone (Kaempferol), 3,5,7,3′,4′- pentahydroxyflavone (Quercetin), and the inactive 4 Hydroxybenzoic acid. The structures of the compounds were determined on the basis of their spectral data and compared to literature data as previously described. The two active compounds, 3,4′,5,7-tetrahydroxyflavone, and 3,5,7,3′,4′- pentahydroxyflavone, isolated from the methanol extract of *Centella asiatica* exhibited a dose dependent inhibition of ACE Activity, the 50% inhibitory concentration were determined as 542.39 µg/ml and 302.72 µg/ml respectively. This is the first report of ACE inhibitory effect of these compounds in *Centella asiatica.*

The isolated active compounds 3,4′,5,7-tetrahydroxyflavone (Kaempferol), and 3,5,7,3′,4′-pentahydroxyflavone (Quercetin) both consist of a flavonoid structure which is comprised of two phenyl rings (A and C rings) joined to a catechol group (B ring). Both compounds have a primary flavanone structure but 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) has an additional OH group at position 3′ of the catechol group and therefore exhibited a 1.8-fold decrease in IC50 relative to 3,4′,5,7-tetrahydroxyflavone (Kaempferol). **Quin *et al* (2012)** in a structure activity relationship study of the ability of different flavonoids to inhibit ACE activity confirmed that the presence of the catechol group in the B-ring (3′,4′- dihydroxy) appears to be fundamental to achieving an increased ACE inhibitory activity, the study showed that the absence of the 3′ hydroxyl group in apigenin caused a 57% reduction of ACEI activity at 100 mM relative to the luteolin. This presence of additional OH group on C3′ possibly explain the lower IC50 exhibited by 3,5,7,3′,4′- pentahydroxyflavone. In addition, the study identified that the double bond between C2 and C3 at C-ring and keto group at the C4 carbon on the C=ring to be essential for inhibiting ACE activity. The two active compounds isolated in this study comprised of these key functional groups. The importance of these functional groups were further confirmed in a study by **Tsutsumi *et al* (1998),** where docking results of both quercetin

and epicatechin showed that the absence of the keto group and the C2−C3 double bond shifts the zinc binding site to the 7-OH moiety resulting in a reduced ACEI activity. Both 3,4′,5,7-tetrahydroxyflavone (Kaempferol), and 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) isolated in this study contained these key functional group and double bond. **Shukor *et al.*, (2013)** further confirmed the role of the catechol B ring group , and C2=C3 double bond in ACEI activity . In contrast to the active compounds, the inactive 4 Hydroxybenzoic acid lacked these basic functional structures/groups which could possibly explain the reason for its lack of ACE inhibitory effect. The 4 Hydroxybenzoic acid comprises a benzoic acid structure carrying a hydroxyl substituent at the C-4 of the benzene ring, and is considered a phenolic derivative of the benzoic acid. Another important component of structure – ACE activity relationship is ACE, a zinc-containing peptidyl dipeptide hydrolase **(Becker and Scholkens, 1987)**. Binding to ACE sub site is considered essential for ACEI activity. The active site of ACE consists of three parts; a carboxylate binding functionality such as the guanidium group of Arg, a pocket that accommodates the side chain of c-terminal amino acid residues, and a zinc ion. The zinc ion coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack(Oh *et al*., 2004). Some flavonoids are suggested to show *in vitro* activity via the generation of chelate complexes within the active centre of ACE. Hydroxyl groups are suggested to be structural moieties that chelates zinc ions thus inactivating ACE activity**(Chen and Lin, 1992).** The hydroxyl group on B ring catechol group of 3,4′,5,7-tetrahydroxyflavone (Kaempferol), and 3,5,7,3′,4′-pentahydroxyflavone (Quercetin) can make charge-charge interactions with the active site Zn^{2+} of ACE thus exhibiting ACE inhibitory effect. Interestingly, **Quin *et al.*, 2012** showed that charge-charge interaction with 3' hydroxyl is stronger than that of with the 4' interaction. Which could explain why kaempferol showed a lower ACE inhibition effect relative to

quercetin. Therefore, these structural features and hydroxyl groups are considered a putative zinc-chelating sites.

The renin angiotensin system has been implicated in inducing oxidative stress in vascular and endothelial smooth muscle cells and may be an important mechanism also participating in the pathophysiology of hypertension. Studies have suggested that free radicals might mediate the pathogenesis of vascular hyper permeability and cellular damage associated with oxidative stress **(Dhalla *et al.*, 2000**). Oxidative stress has also been implicated as a risk factor for cardiovascular disease through its triggering of the atherogenic processes that hardens and narrows arteries thus impeding the flow of blood around the body. In a study by **Wilson** (1990), rats made acutely hypertensive by administering angiotensin II showed severe and extensive endothelial and smooth muscle lesions that were inhibited upon antioxidant treatment. It could be said that an important synergistic role exists between antioxidant and ACE inhibition in the treatment of hypertension and cardiovascular disease. Flavonols act as prominent antioxidants in biological systems. The antioxidant capacity of the isolated compounds from *Centella asiatica* was determined to be comparable to the reference compound (Ascorbic acid). 3,4′,5,7-tetrahydroxyflavone (Kaempferol), and 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) scavenged 50% of the DPPH radical at 84.83 and 109.99 µg/ml compared to the reference standard (38.77 µg/ml). A study by **Okawa *et al*** (2001) showed quercetin (8 µM) to exhibit stronger DPPH radical scavenging activity than kaempferol (41.2 µM). The structural properties once more play a role in the biological activity of these compounds, the presence of a dihydroxy group at C-3ʿand 4ʿ(catechol) position in the B-ring of quercetin flavanol skeleton is suggested to enhance the DPPH radical scavenging activity.

In conclusion, the structure− function relationships can be useful for designing new ACE inhibitors based on phenolic compounds and further strategies such as glycosylation of C3 and C7 could confer greater ACE inhibitory activity. Although the ACEI activity of these

compounds do not reach the potency of drugs commonly used in the treatment of hypertension, the synergistic role of antioxidant and ACE inhibition properties can play a role in mild to moderate treatment of hypertension and a plant like *Centella asiatica,* serve as a source of dietary supplementation or considered as a naturally functional food.

5.2 Conclusion

The use of ACE inhibitors to treat hypertension, which is considered a major risk factor for cardiovascular disease remains a very important therapeutic strategy, but the manifestation of side effects has stirred up interest in alternative source of therapy hence the increasing interest in medicinal plant as source of safe and effective treatment. The aim of this study was to investigate the antihypertensive effect of *Centella asiatica* using an *in vitro* bioassay. The objectives were to evaluate the inhibition of ACE activity by crude extracts of *Centella asiatica* and to isolate and identify the ACE inhibitory compounds using bioassay guided fractionation. It was therefore hypothesized that *Centella asiatica* mediates its anti-hypertensive activity by means of ACE inhibition.

In this study, methanol, ethanol and aqueous extract were assayed for ACE activity. The active methanol and ethanol extract were subjected to a bioassay guided fractionation. Active fractions were further purified on a high performance liquid chromatography to yield the final pure compounds which were identified by means of NMR, MS, UV and IR spectroscopy. ACE Inhibition and antioxidant activity were conducted on the active compounds.

From the results obtained methanol extract yielded three compounds, 3,4′,5,7-tetrahydroxyflavone (kaempferol), 3,5,7,3′,4′- pentahydroxyflavone (quercetin), and 4 Hydroxybenzoic acid. Quercetin and kaempferol were determined to exhibit significant ACE inhibition as well as antioxidant activity.

These compounds have been previously reported to be present in *Centella asiatica*, but this is the first report of their ACE inhibitory effect in this plant. Though the compounds show less inhibition than the reference standard captopril, the synergistic effect of ACE inhibition and antioxidant activity is believed to play an important role in treatment of hypertension and cardiovascular disease. Furthermore, these compounds could be utilized as leads by synthesising glycosylated conjugates which has been shown to exhibit significant ACE inhibition activity. It is noteworthy that quercetin and kaempferol are commercialized products, therefore the results of this study highlights the need to be mindful of drug-drug interactions that might arise in hypertensive patients who are also on antihypertensive drugs. In other words, there is a possibility of an increased antihypertensive effect in patients concomitantly taking quercetin and kaempferol containing herbal products together with an antihypertensive drug

5.3 Limitations

An *in vitro* assay was the only method used in this study, due to lack of access to animals, in vivo assay was not conducted. It is worthy of note that *in vitro* conditions/results does not necessarily reflect or translate to *in vivo* outcome.

5.4 Recommendation

Future studies are required to investigate the *in vivo* effect of the isolated compounds and to synthesize conjugates of the compounds which can be examined for activity using *in vitro* and *in vivo* experiments.

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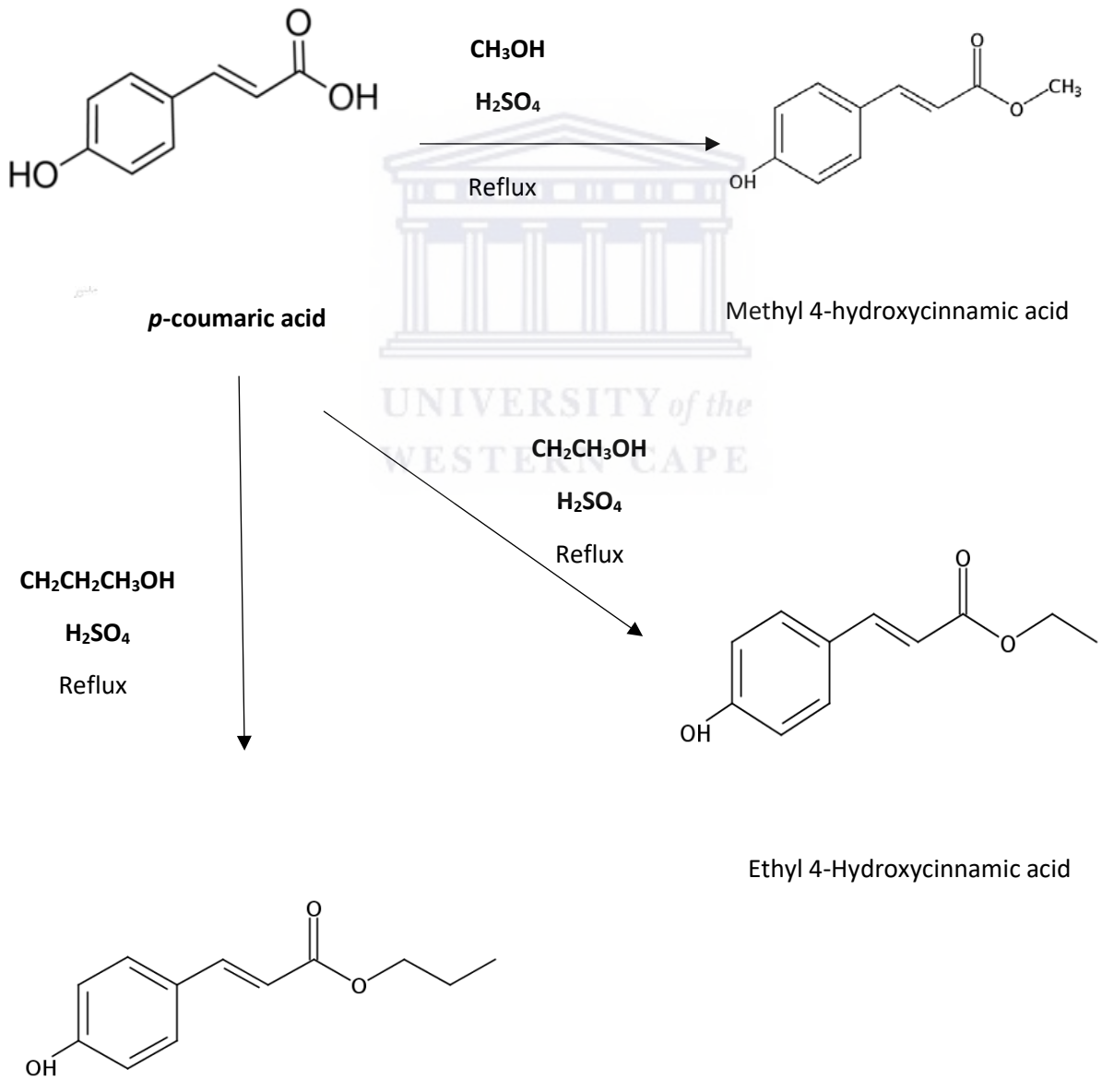
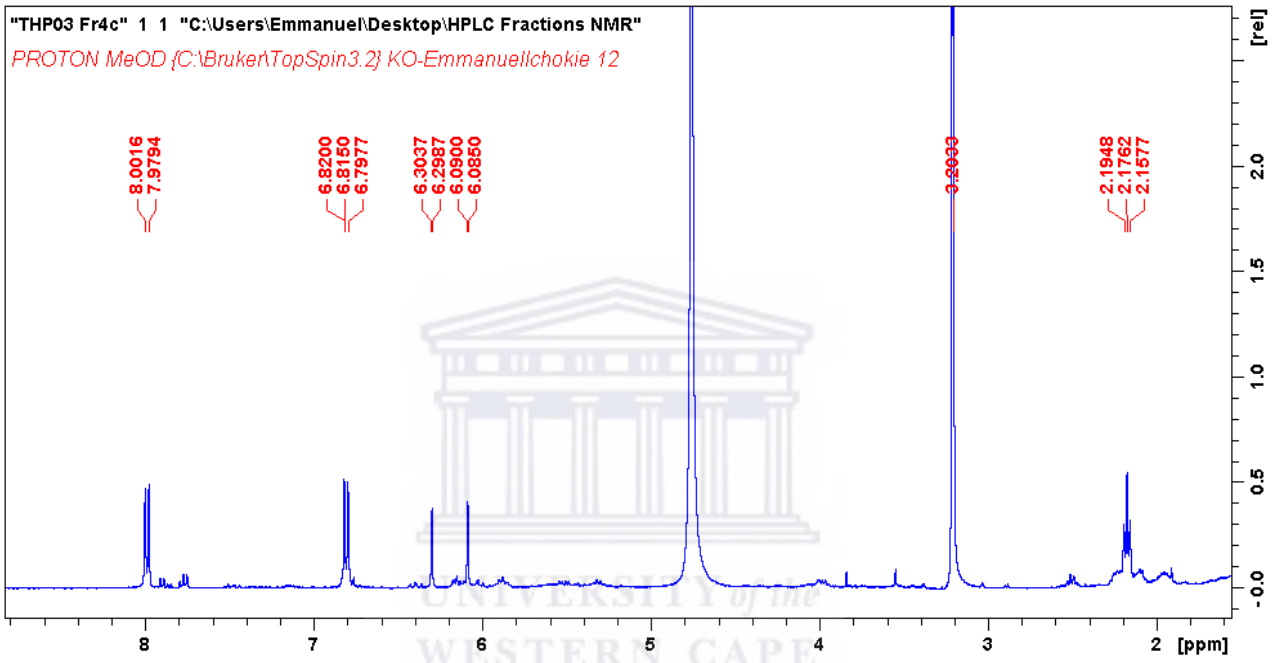
APPENDIX

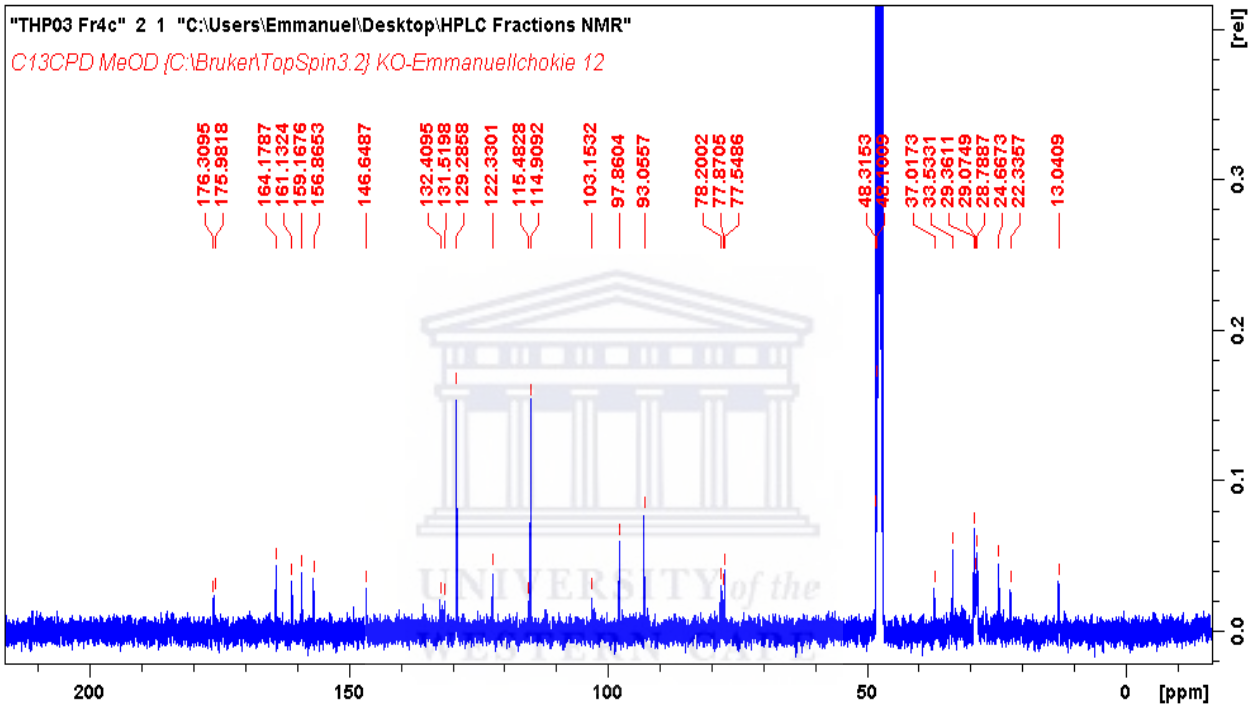
Appendix I: Synthetic Pathway for *p*-Coumaric acid derivatives



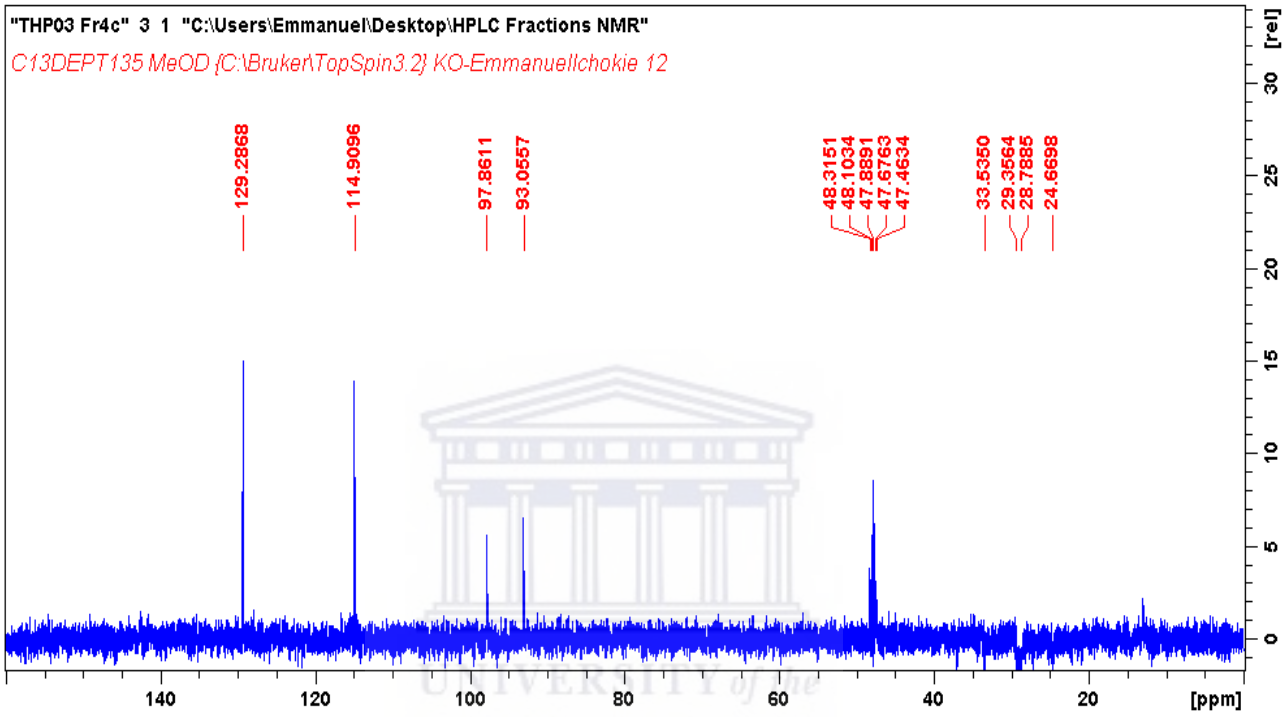
Appendix II :1H-NMR spectrum of 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD,400 MHz)



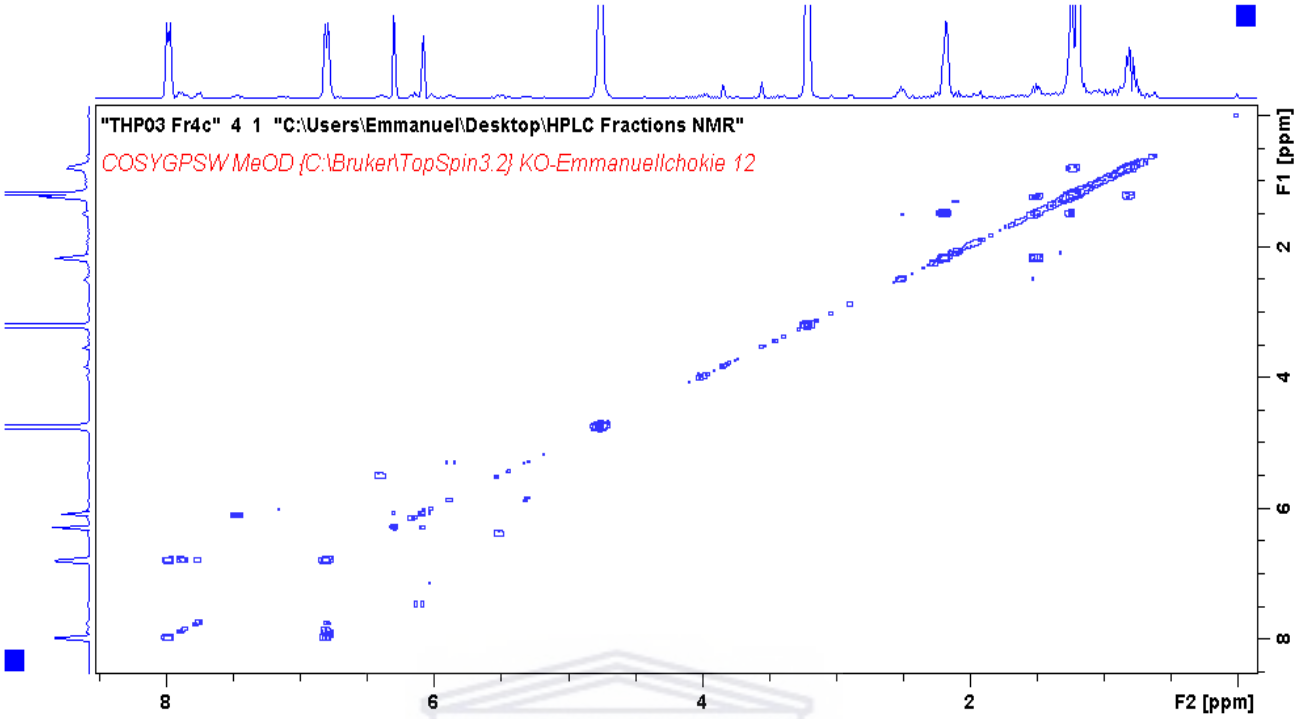
Appendix III: ^{13}C-NMR data of 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD, 400 MHz)



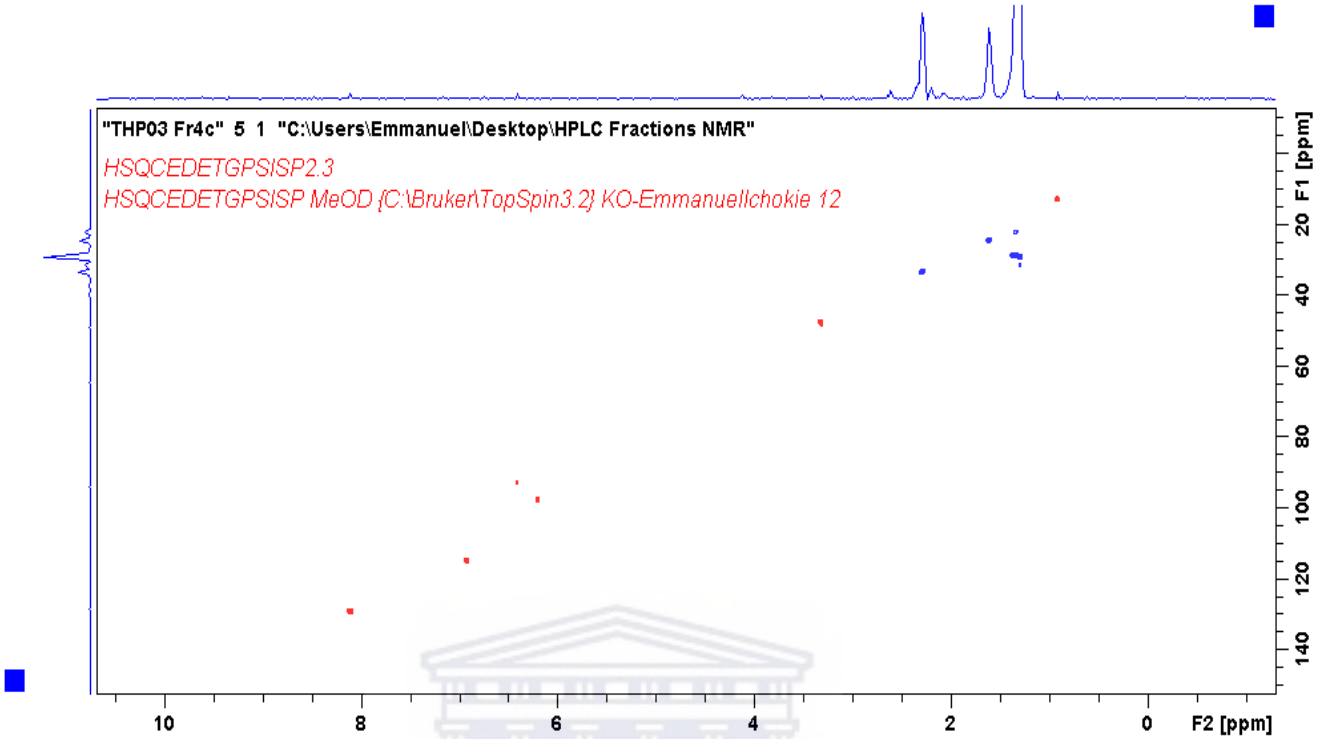
Appendix IV: C13 Dept. Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD, 400 MHz)



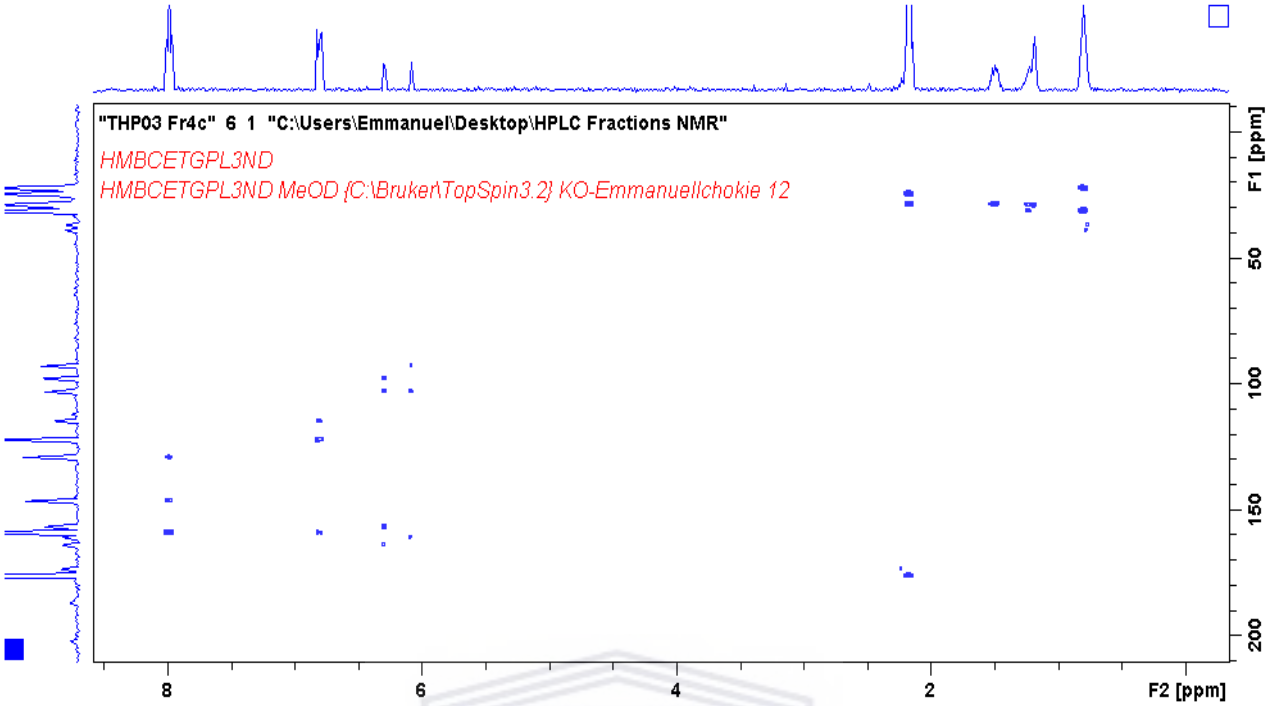
Appendix V: COSY Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD, 400 MHz)



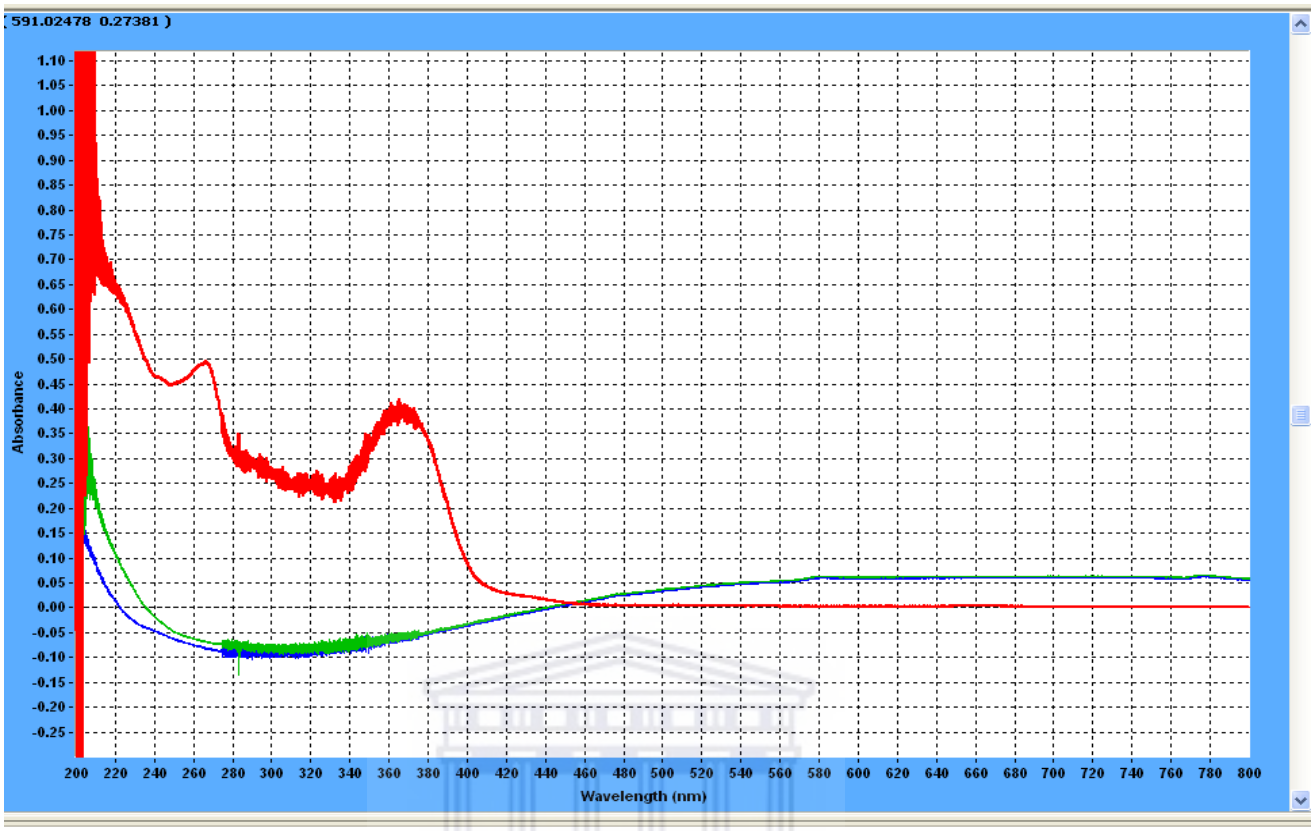
Appendix VI: HSQC Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD, 400 MHz)



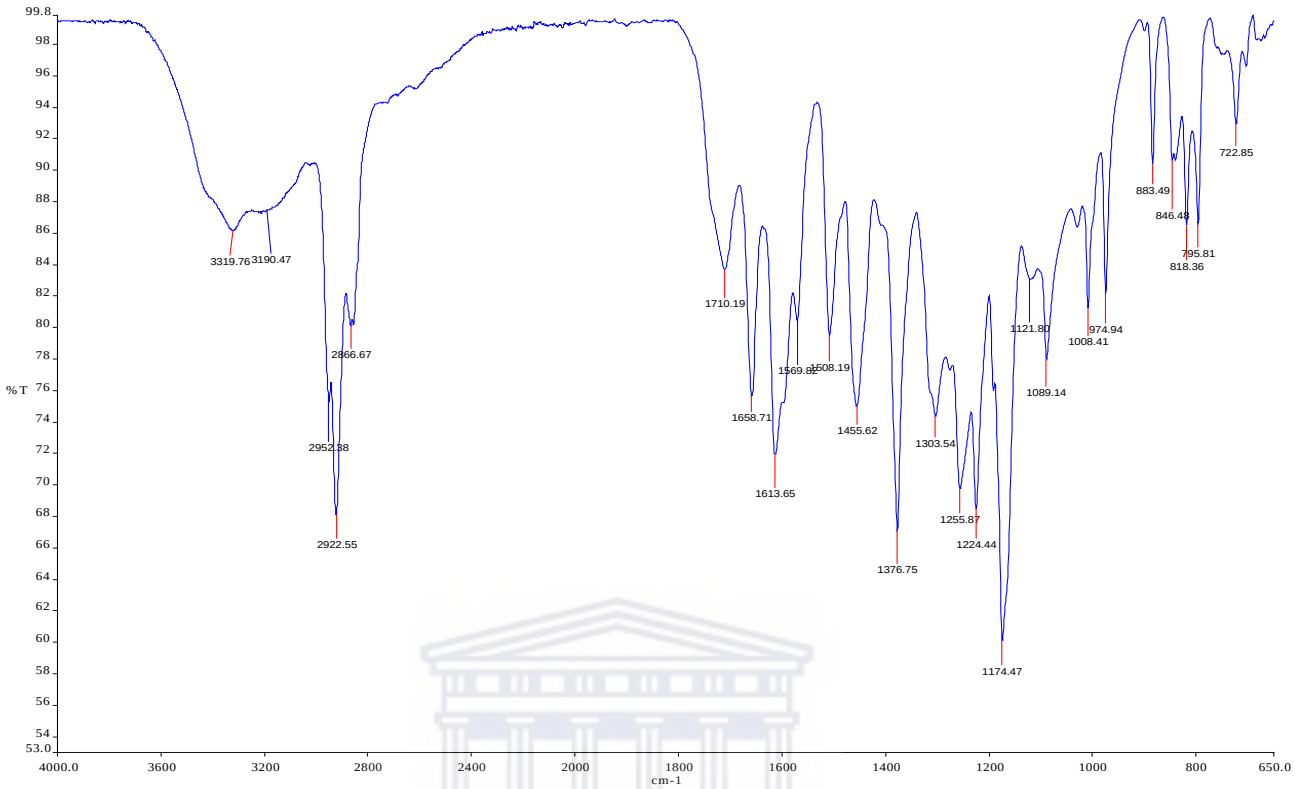
Appendix VII: HMBC Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol) (CD_3OD, 400 MHz)



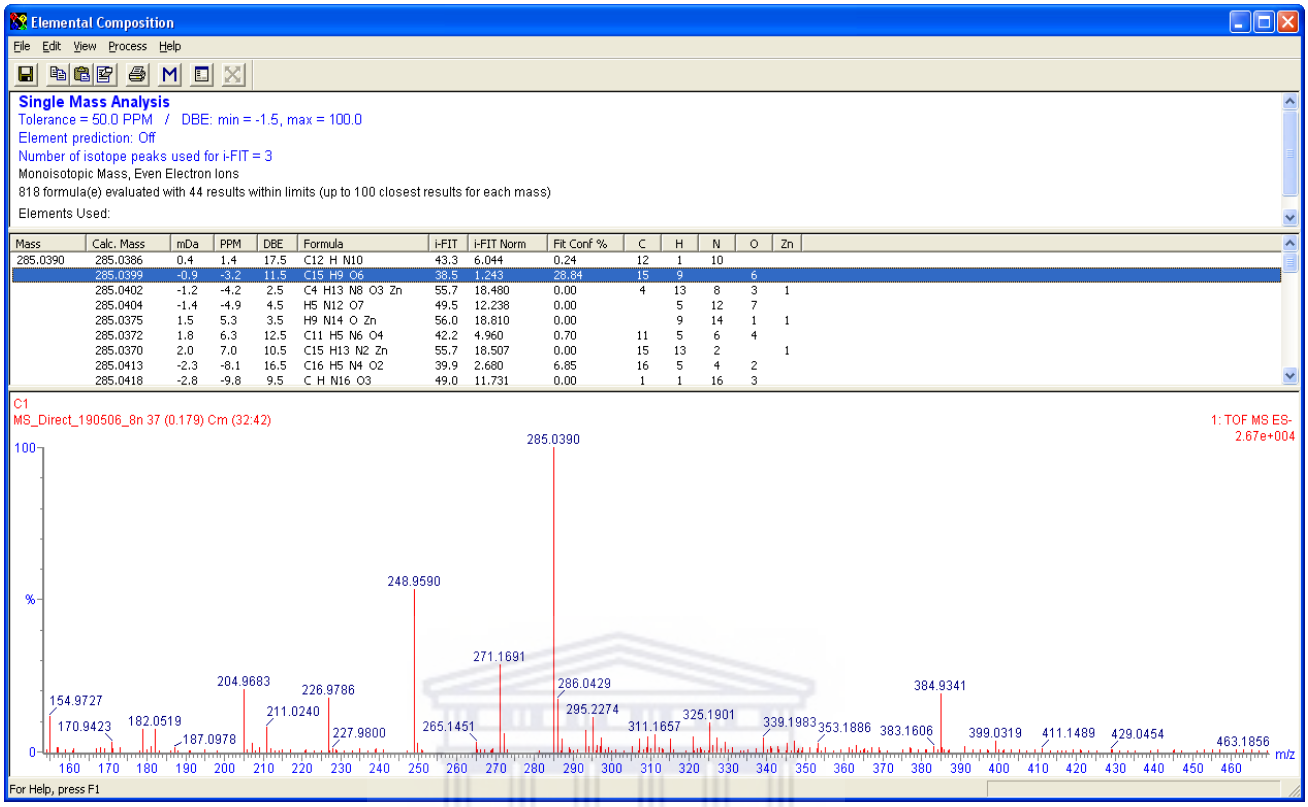
Appendix VIII: UV Spectrum of 3,4′,5,7-Tetrahydroxyflavone (kaempferol).

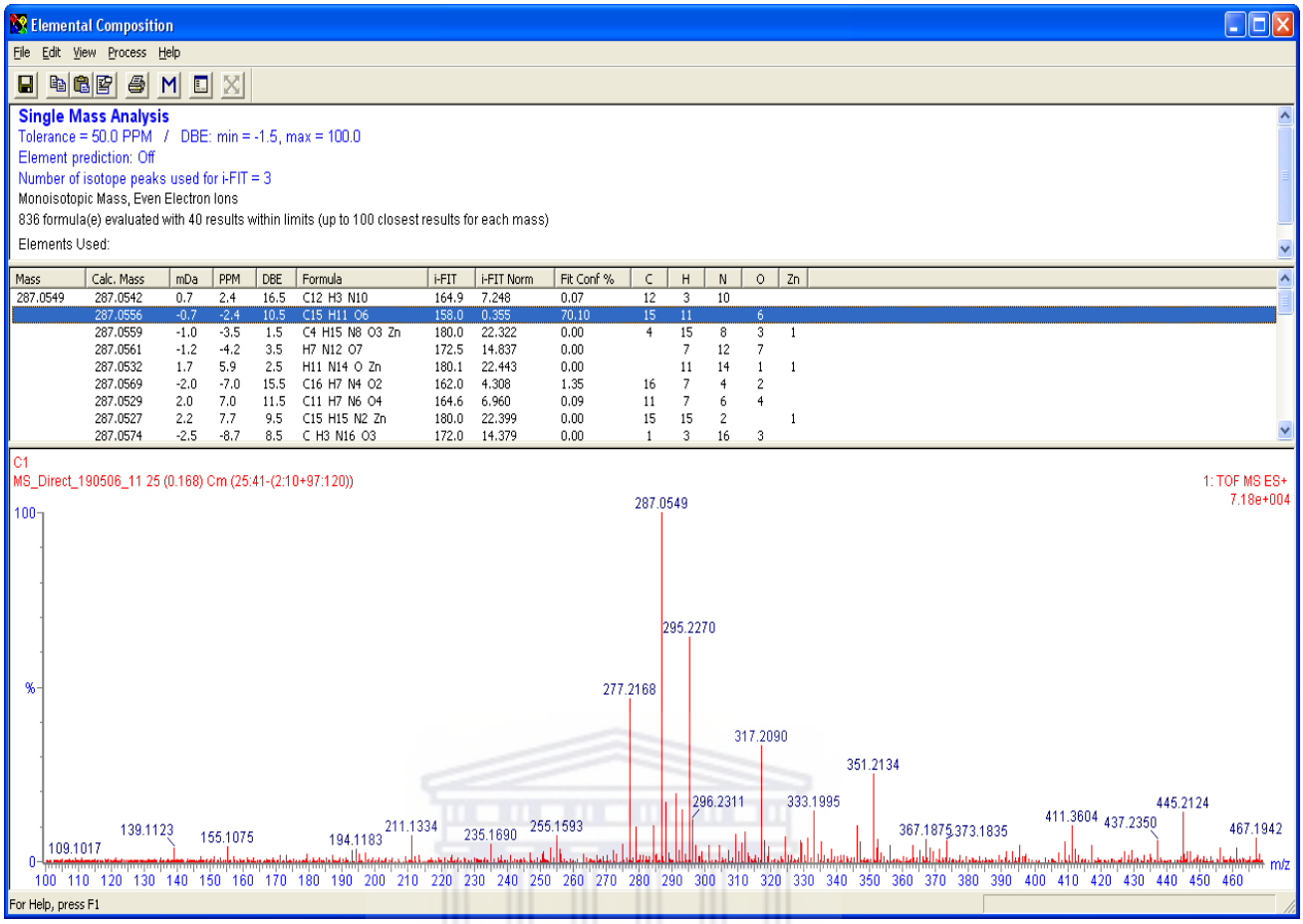


Appendix IX: IR Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol).

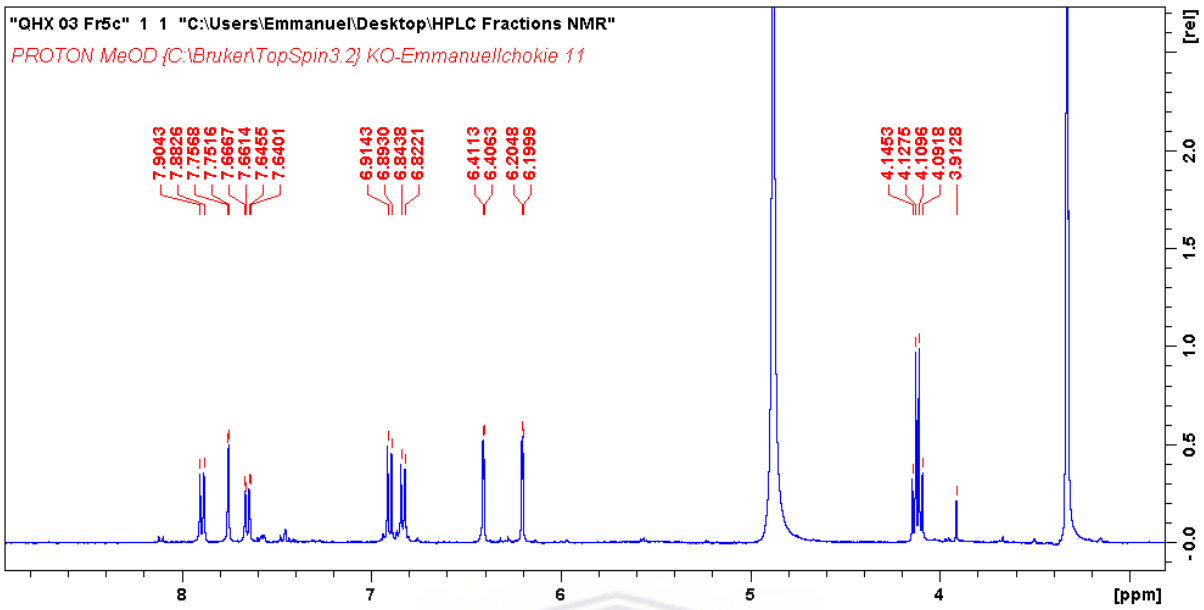


Appendix X: HRMS Spectra for 3,4′,5,7-Tetrahydroxyflavone (kaempferol).

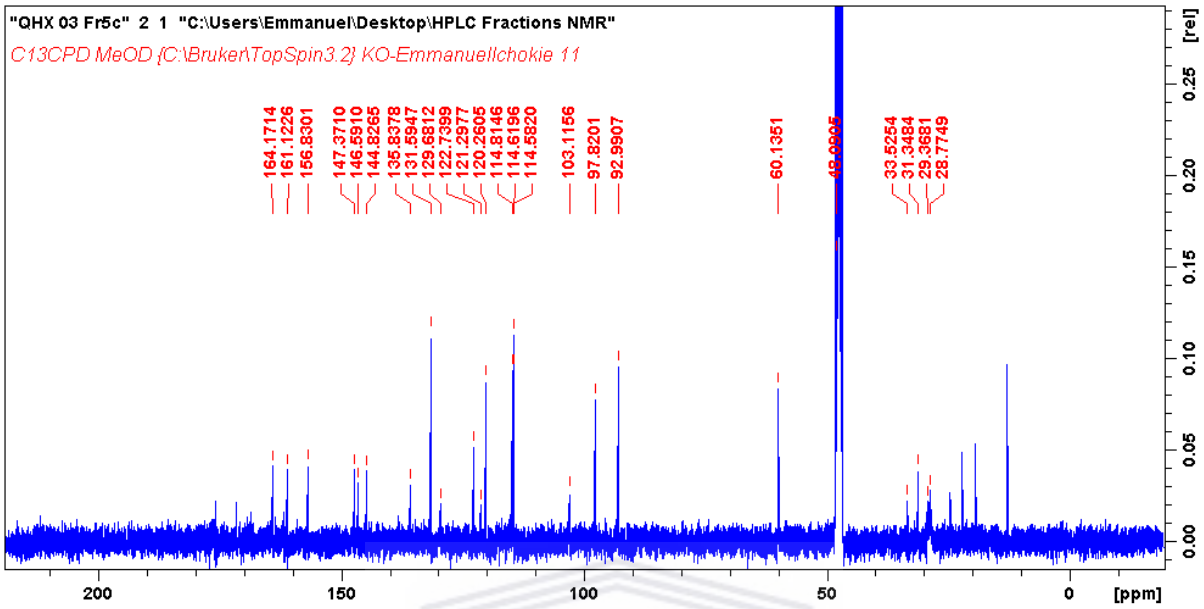




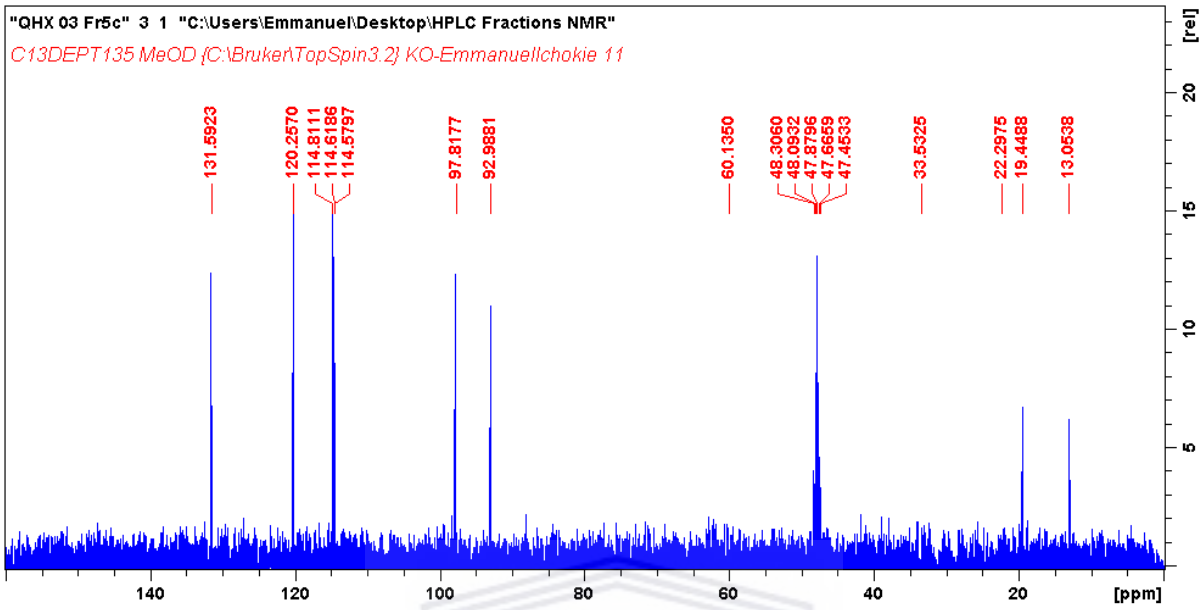
Appendix XI: ^{1}H-NMR spectra of 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) (CD_3OD, 400 MHz)



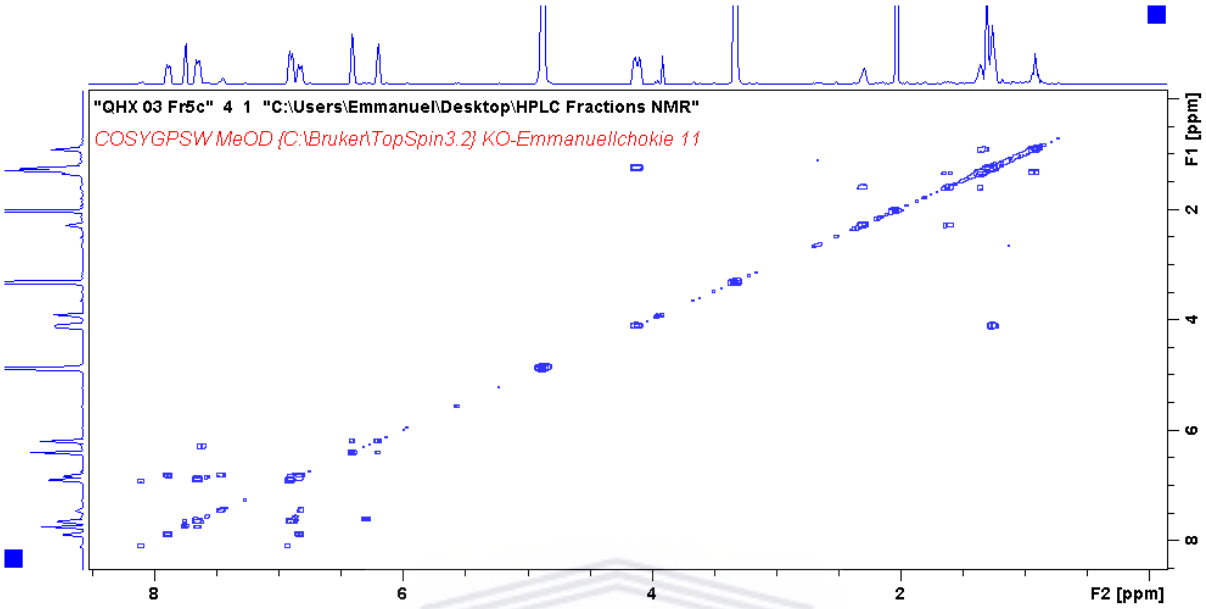
Appendix XII: ^{13} C-NMR spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) (CD_3OD, 400 MHz)



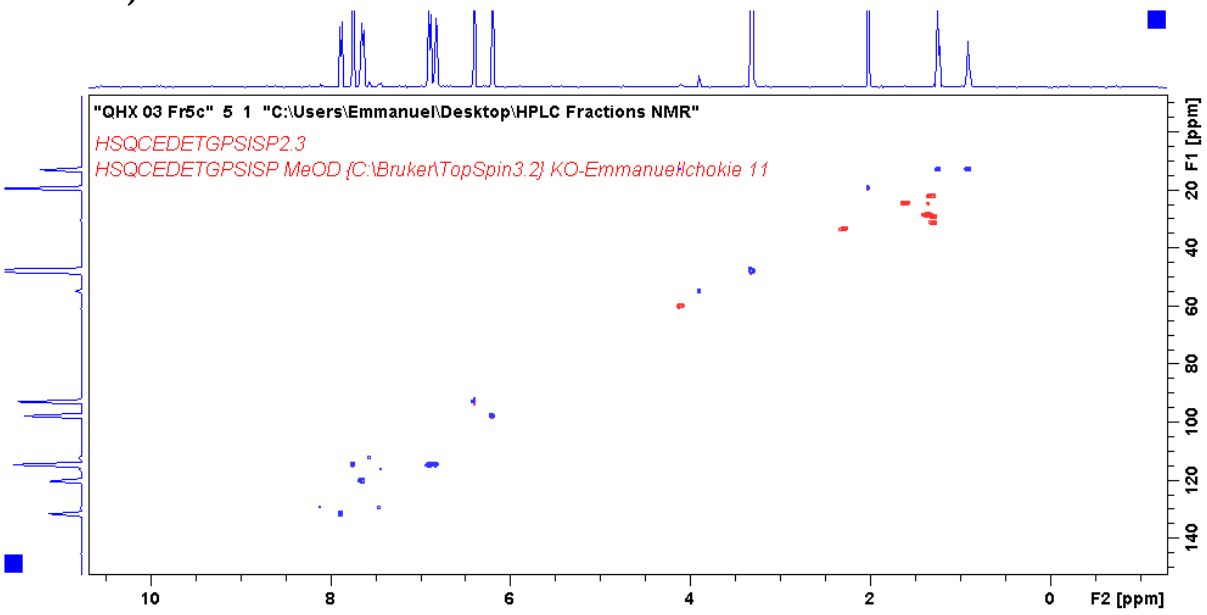
Appendix XIII: C13 Dept. spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) (CD_3OD, 400 MHz)



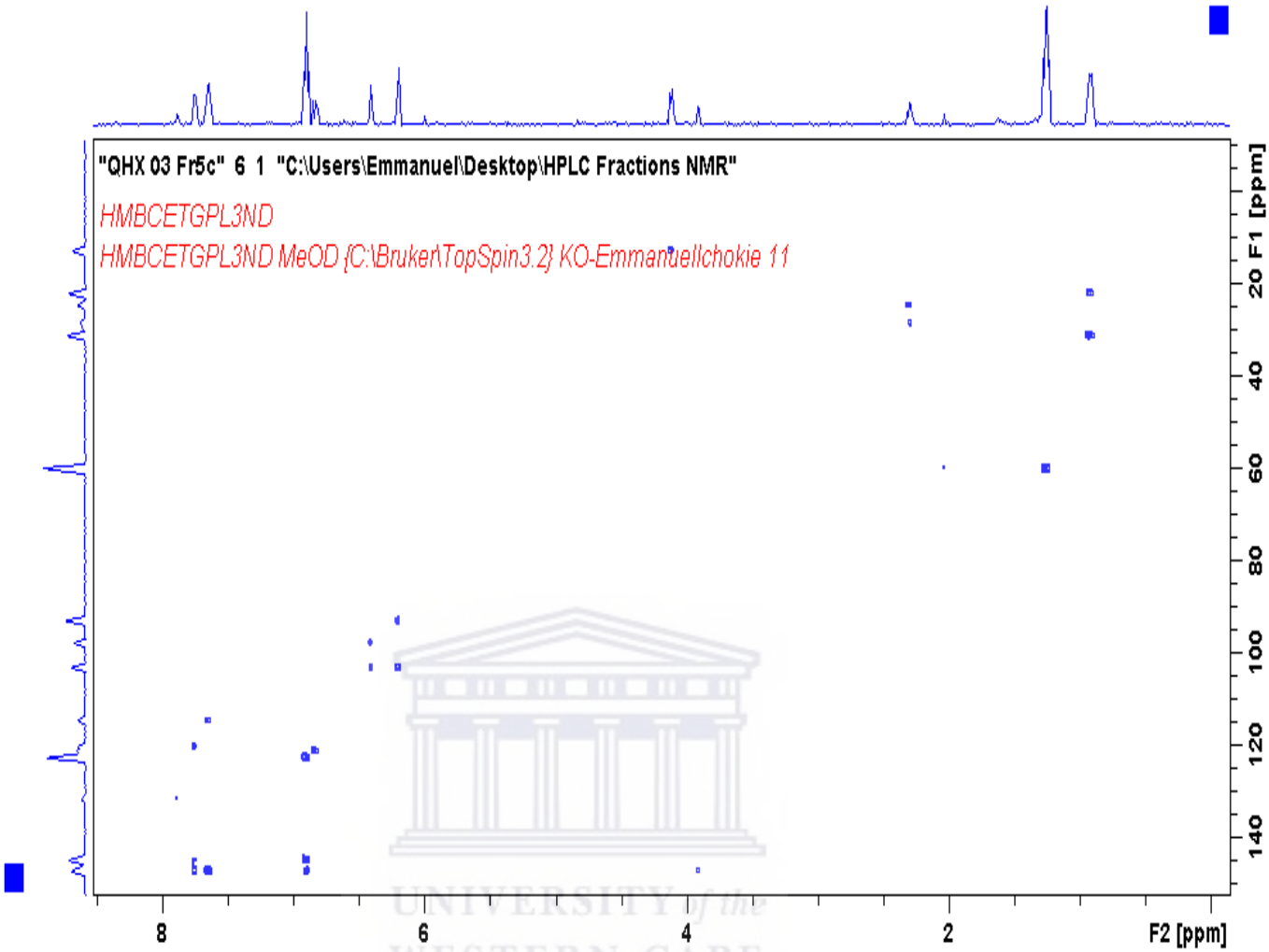
Appendix XIV: COSY Spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) (CD_3OD, 400 MHz)



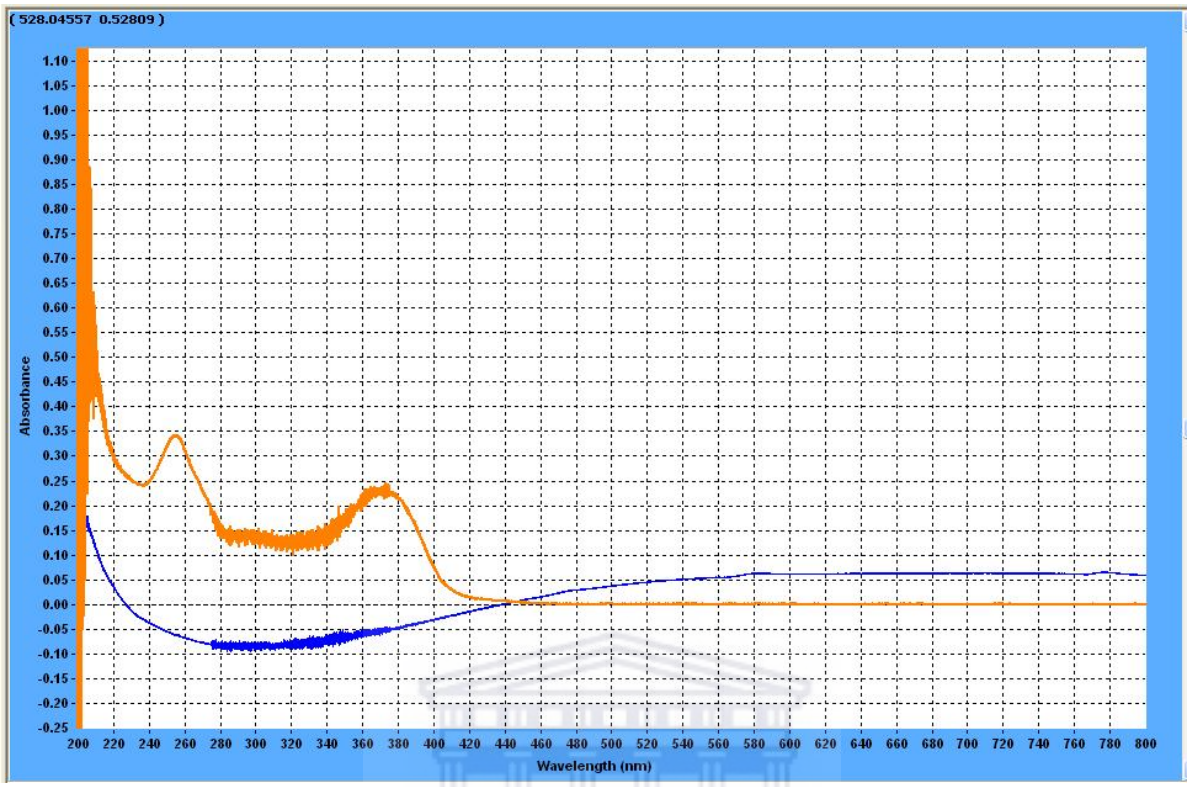
Appendix XV: HSQC Spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin) (CD_3OD, 400 MHz)



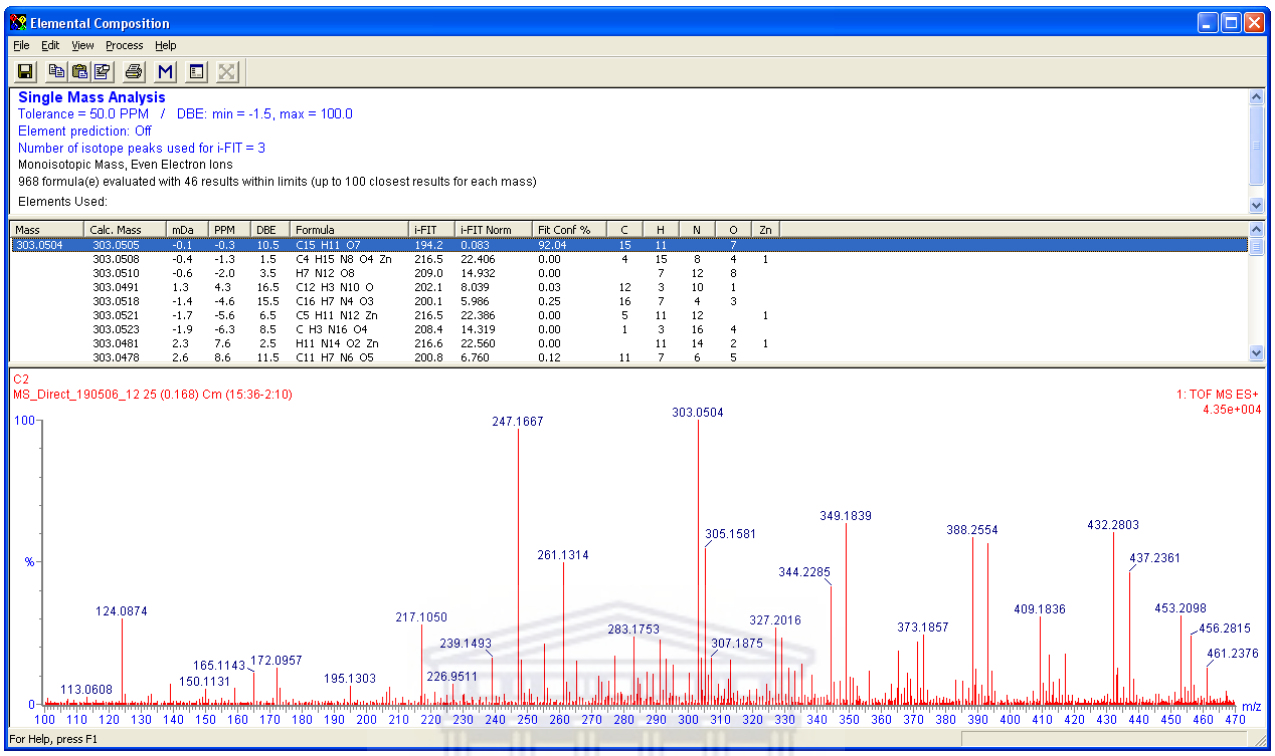
Appendix XVI: HMBC Spectra for 3,5,7,3′,4′- pentahydroxyflavone(Quercetin) (CD_3OD, 400 MHz)

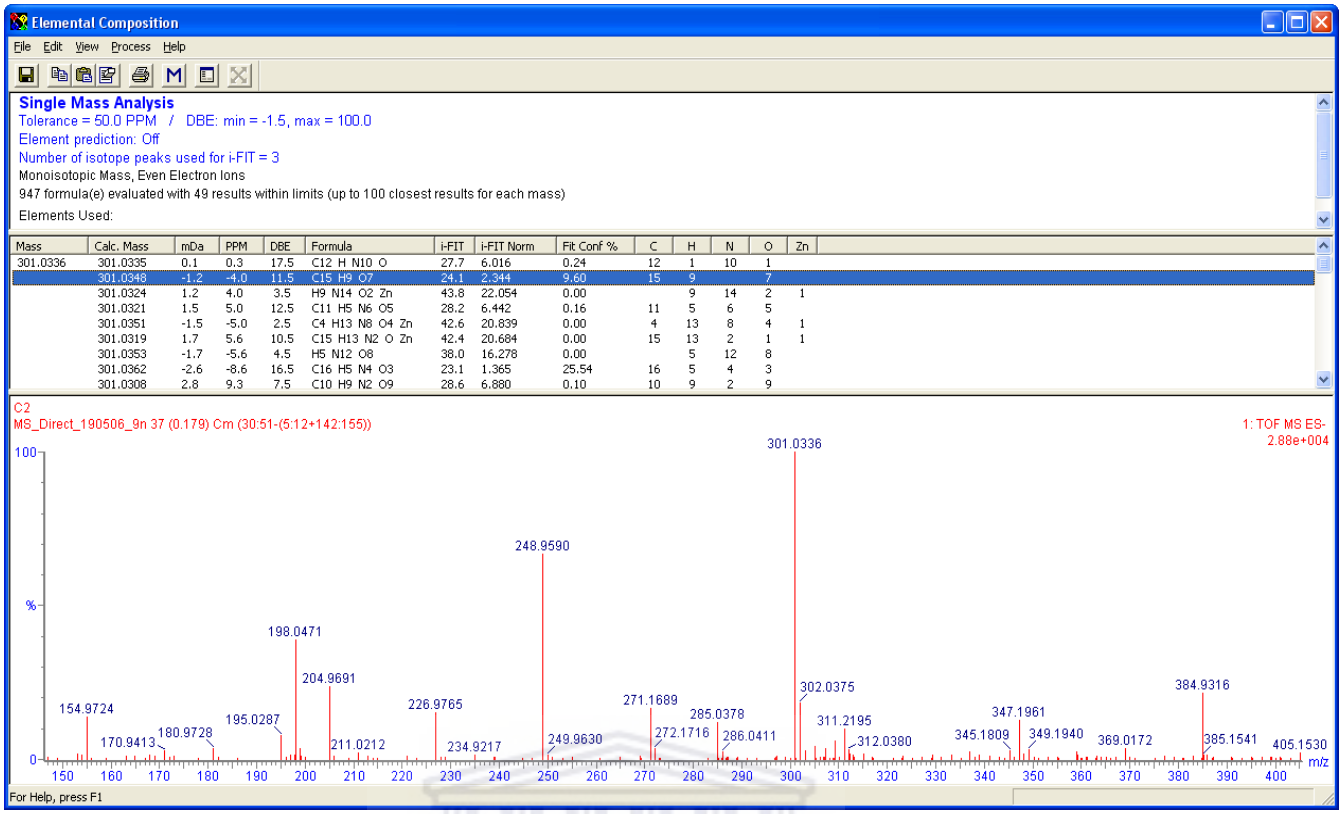


Appendix XVII: UV Spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin)



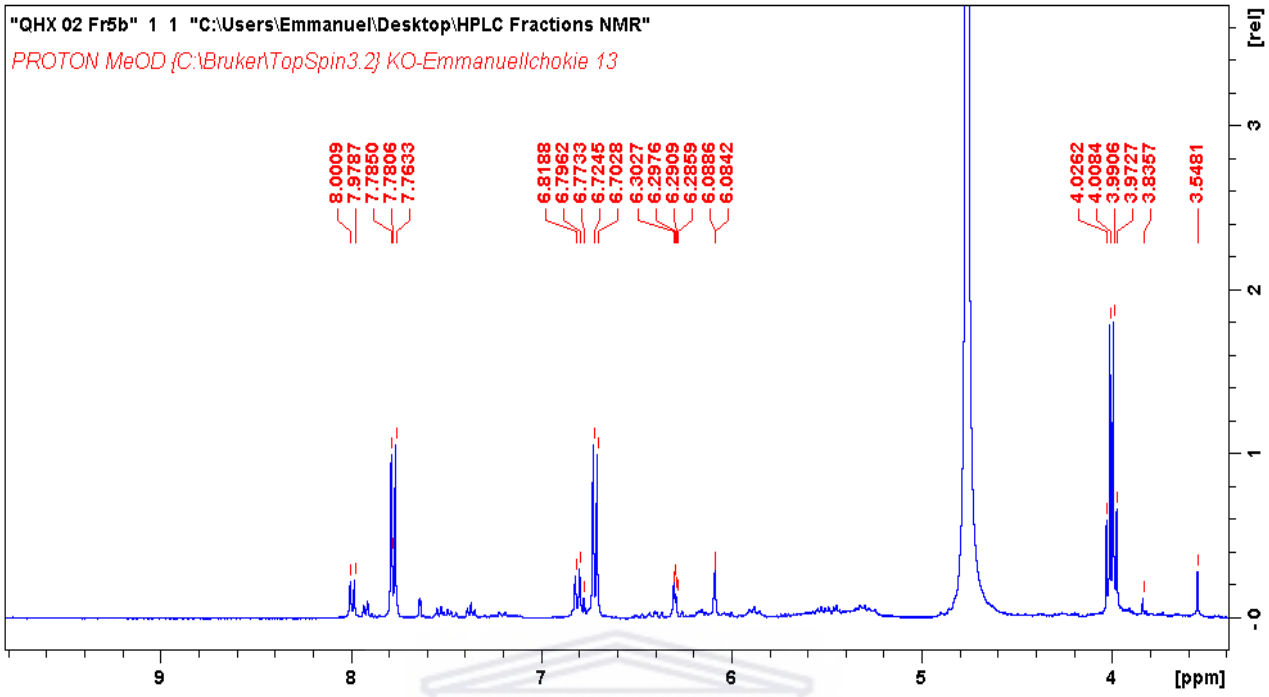
Appendix XVIII: HRMS Spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin)



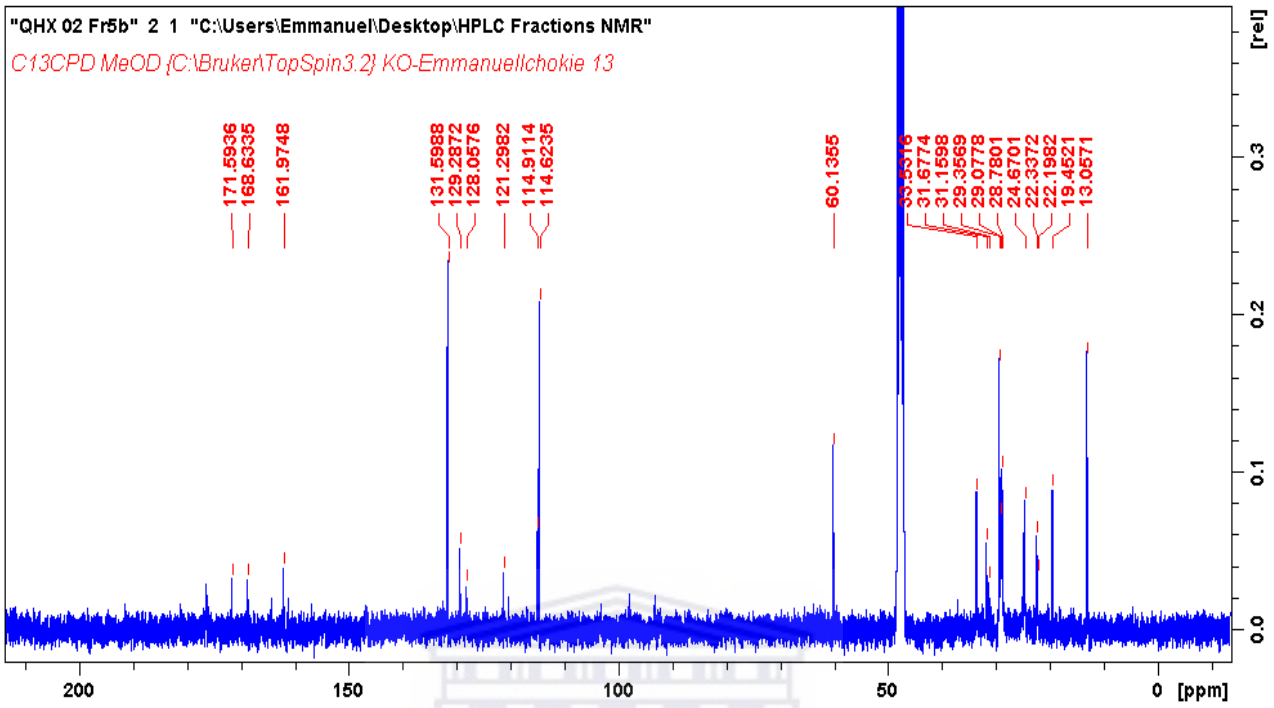


Appendix XIX: IR Spectra for 3,5,7,3′,4′- pentahydroxyflavone (Quercetin)

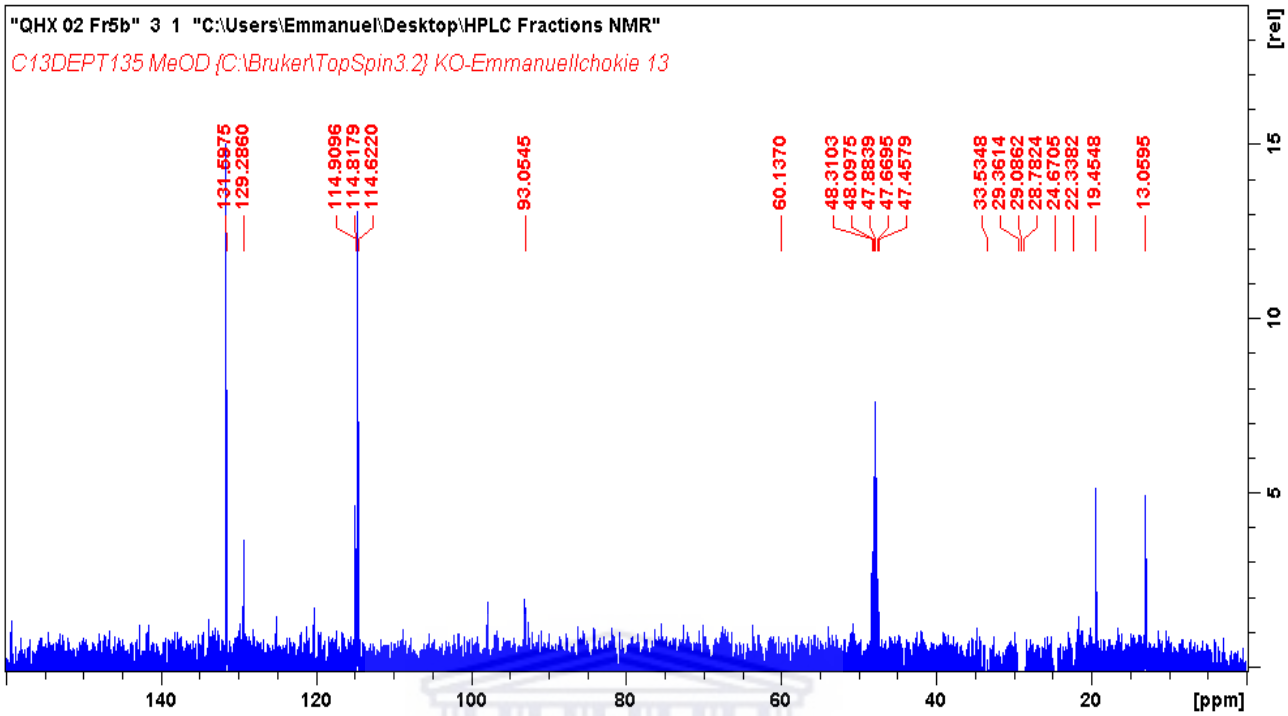
Appendix XX: ^{1}H-NMR spectrum of 4-Hydroxybenzoic acid (CD_3OD, 400 MHz)



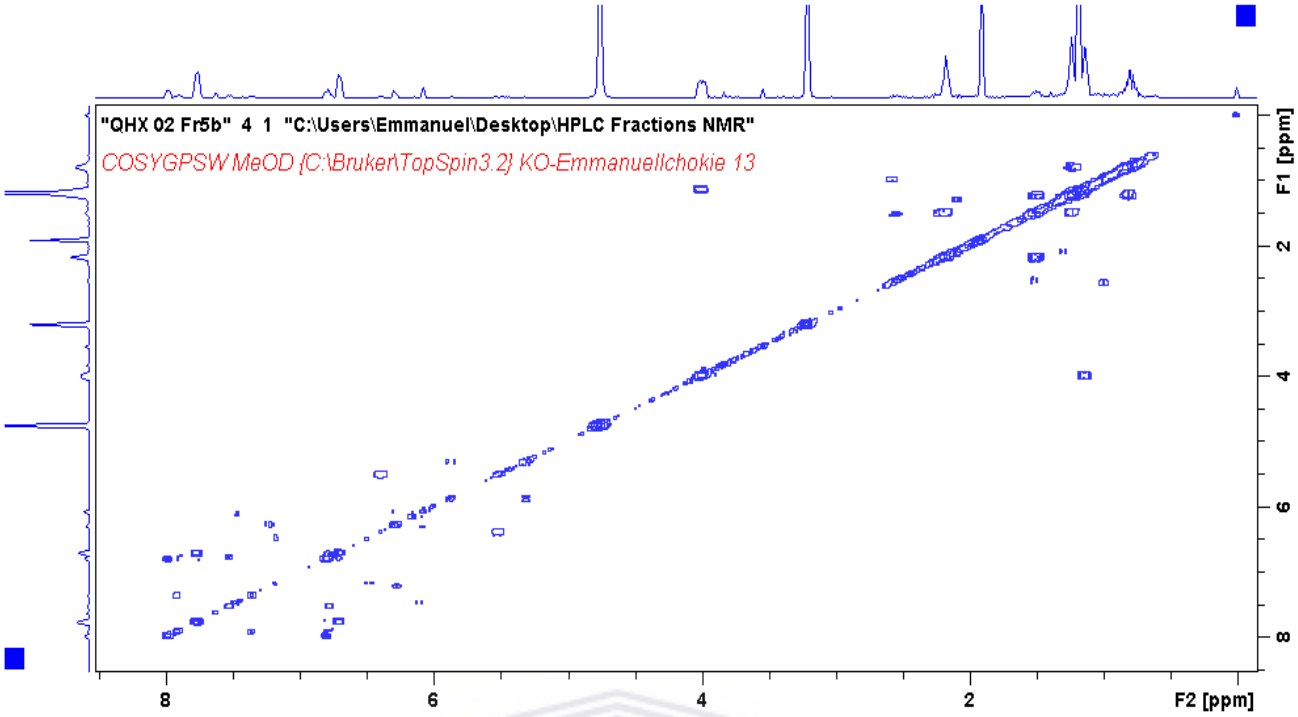
Appendix XXI: ^{13}C-NMR data of 4-Hydroxybenzoic acid (CD_3OD, 100 MHz)



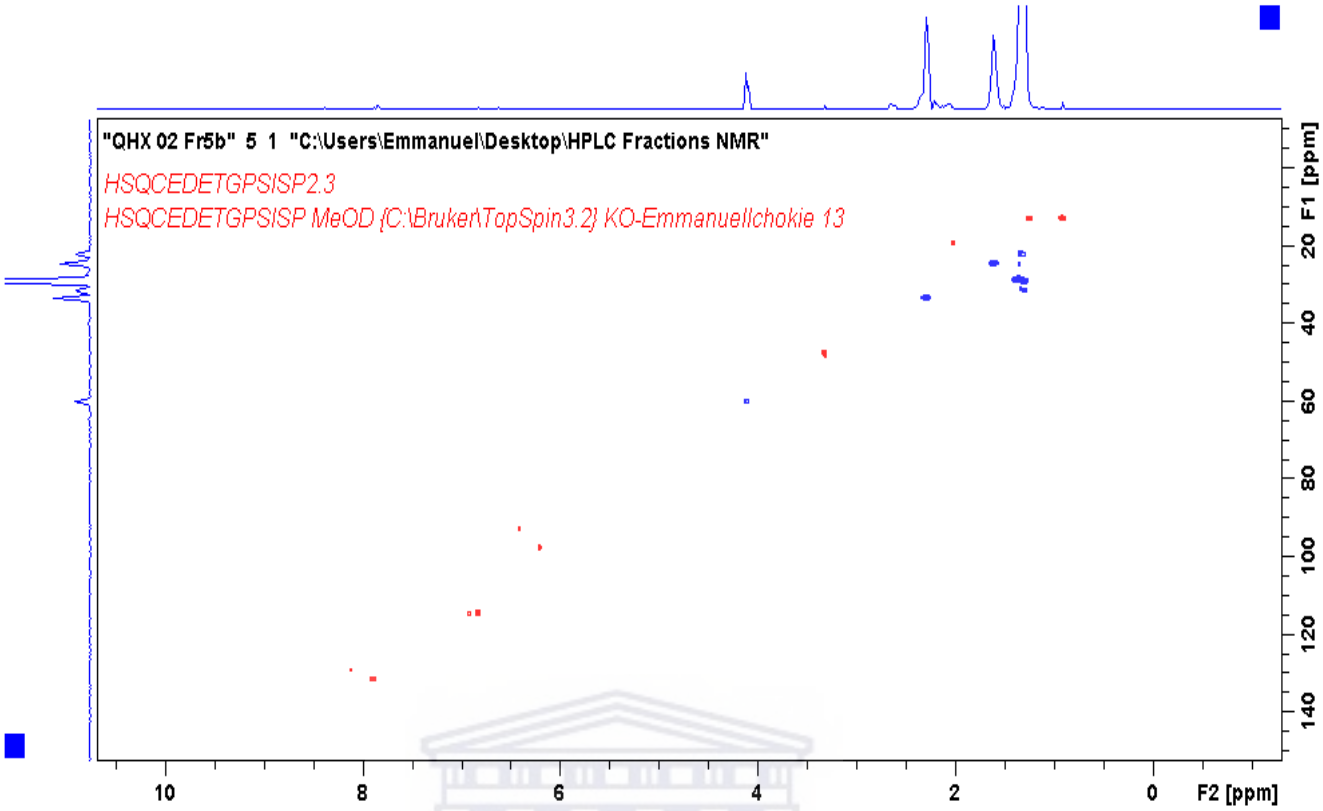
Appendix XXII: C13 Dept. Spectra for 4 Hydroxybenzoic acid (CD_3OD, 400 MHz)



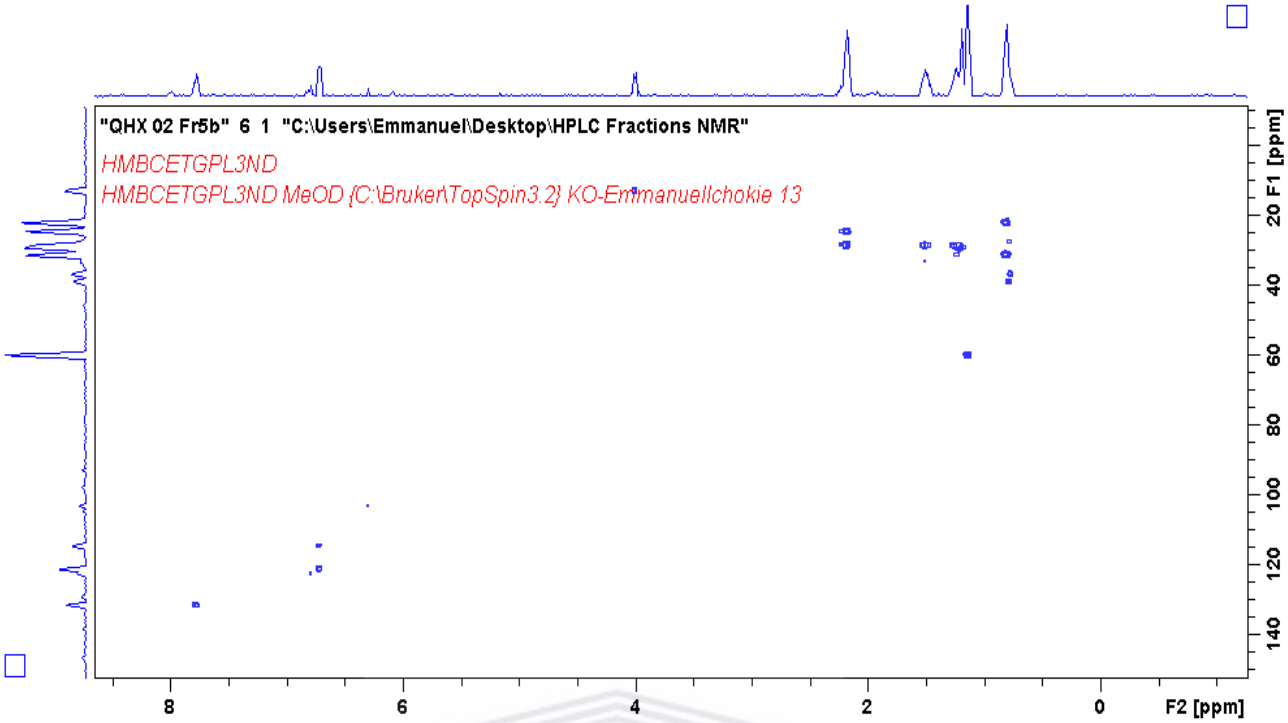
Appendix XXIII: COSY Spectra for 4 Hydroxybenzoic acid (CD_3OD, 400 MHz)



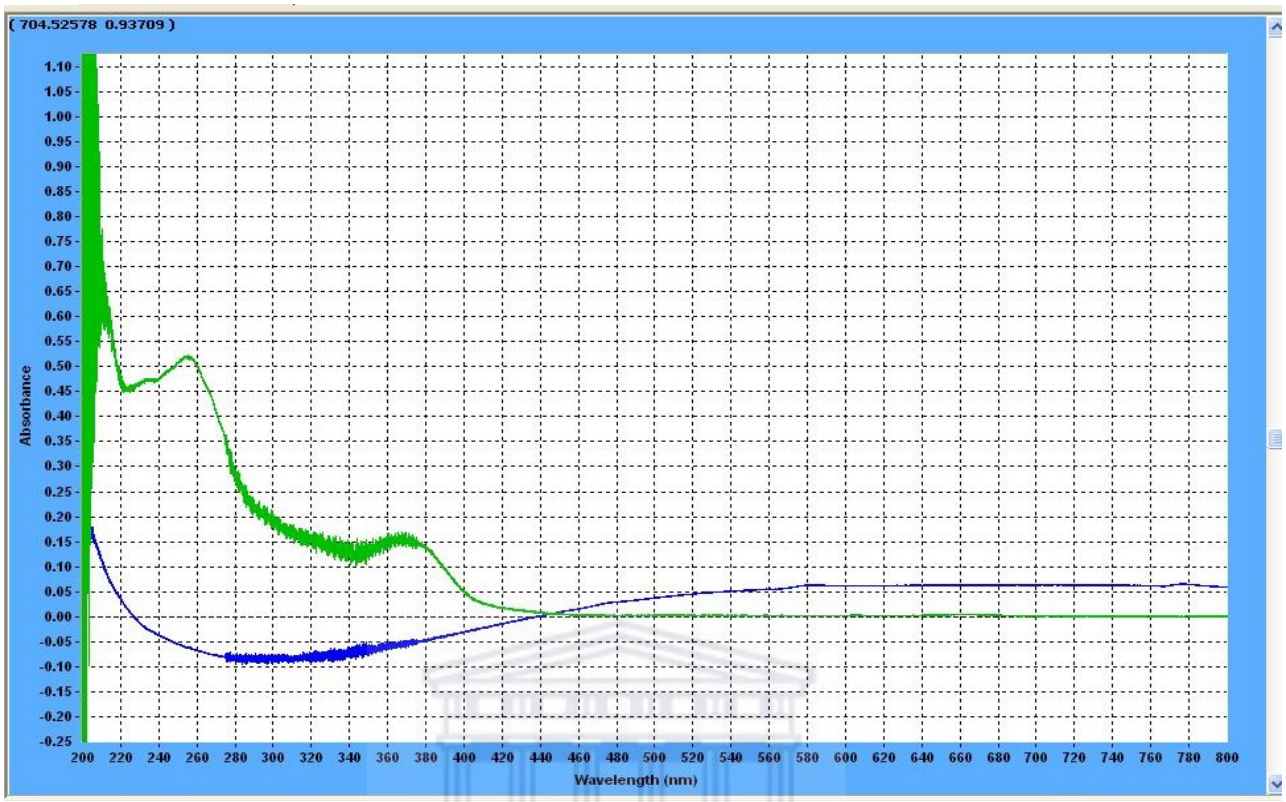
Appendix XXIV: HSQC Spectra for 4 Hydroxybenzoic acid (CD_3OD, 400 MHz)



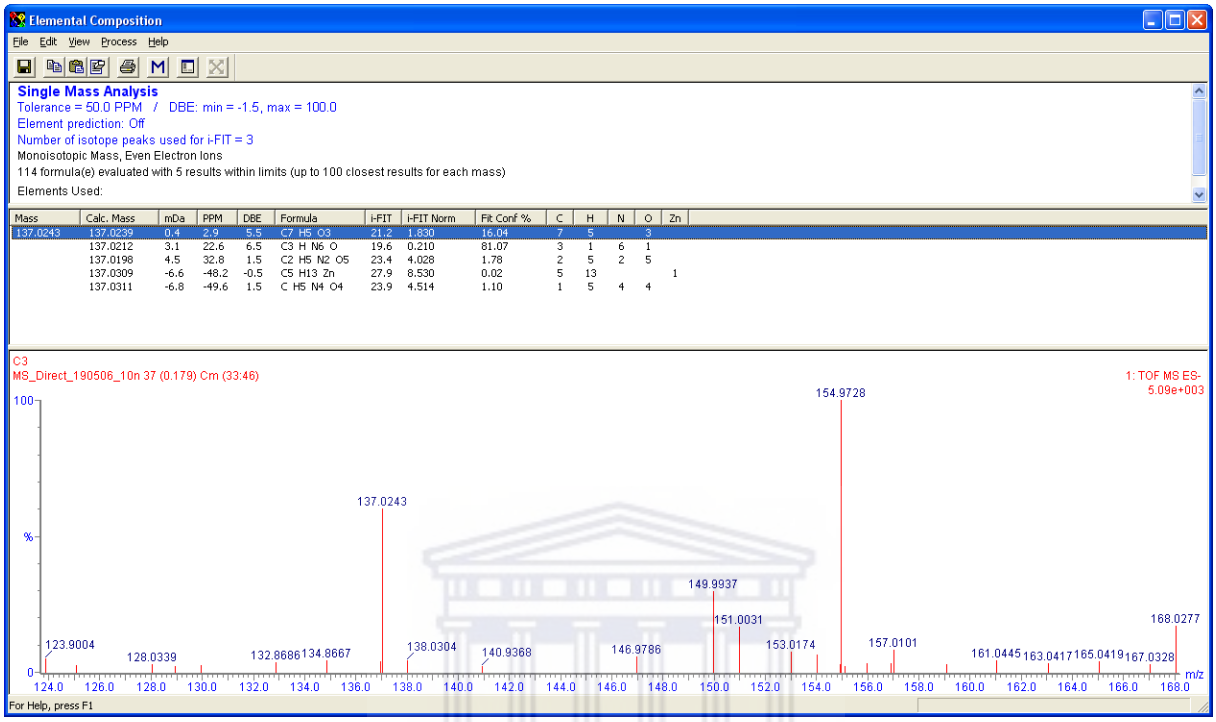
Appendix XXV: HMBC Spectra for 4 Hydroxybenzoic acid (CD_3OD, 400 MHz)

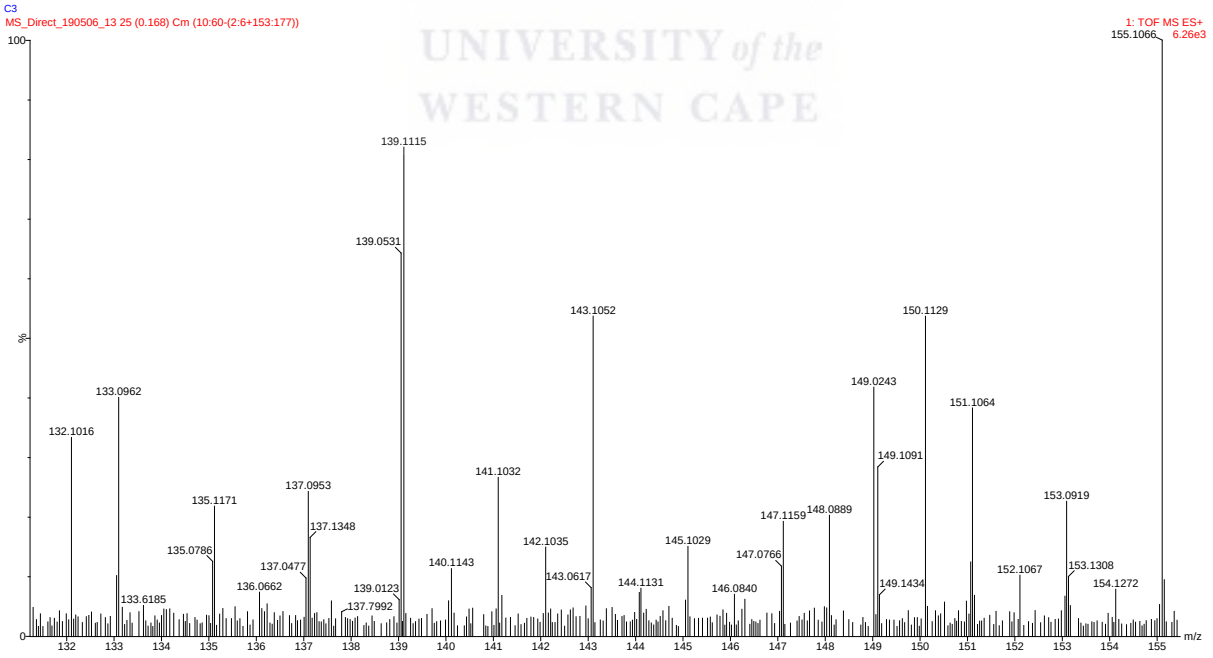


Appendix XXVI: UV Spectra for 4 Hydroxybenzoic acid

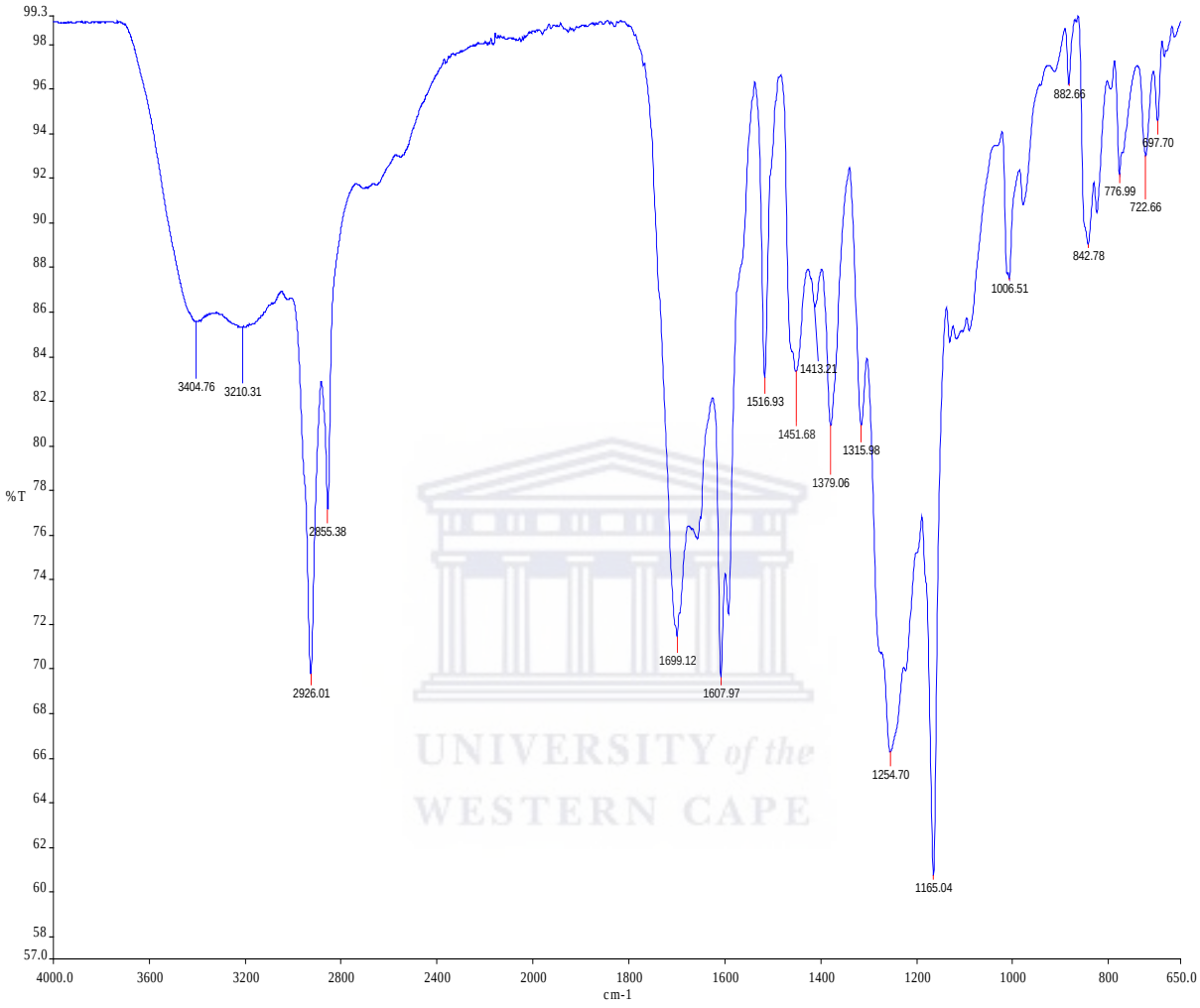


Appendix XXVII: HRMS Spectra for 4 Hydroxybenzoic acid

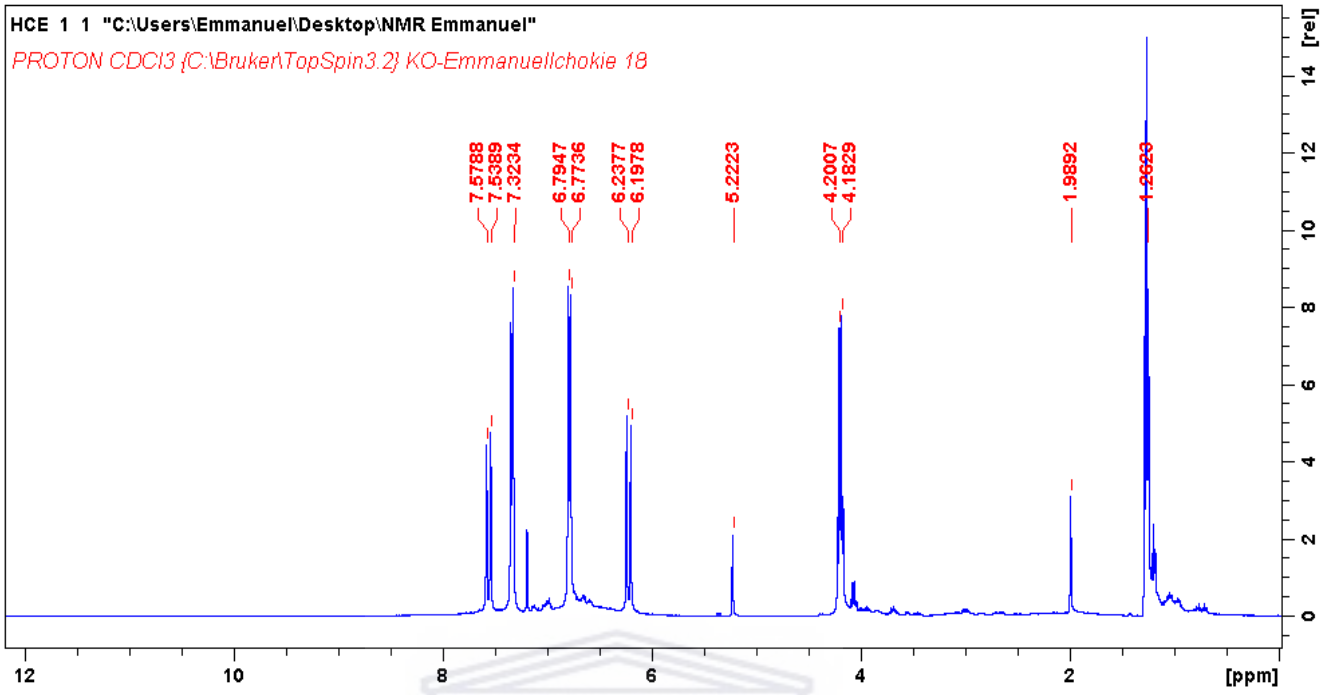




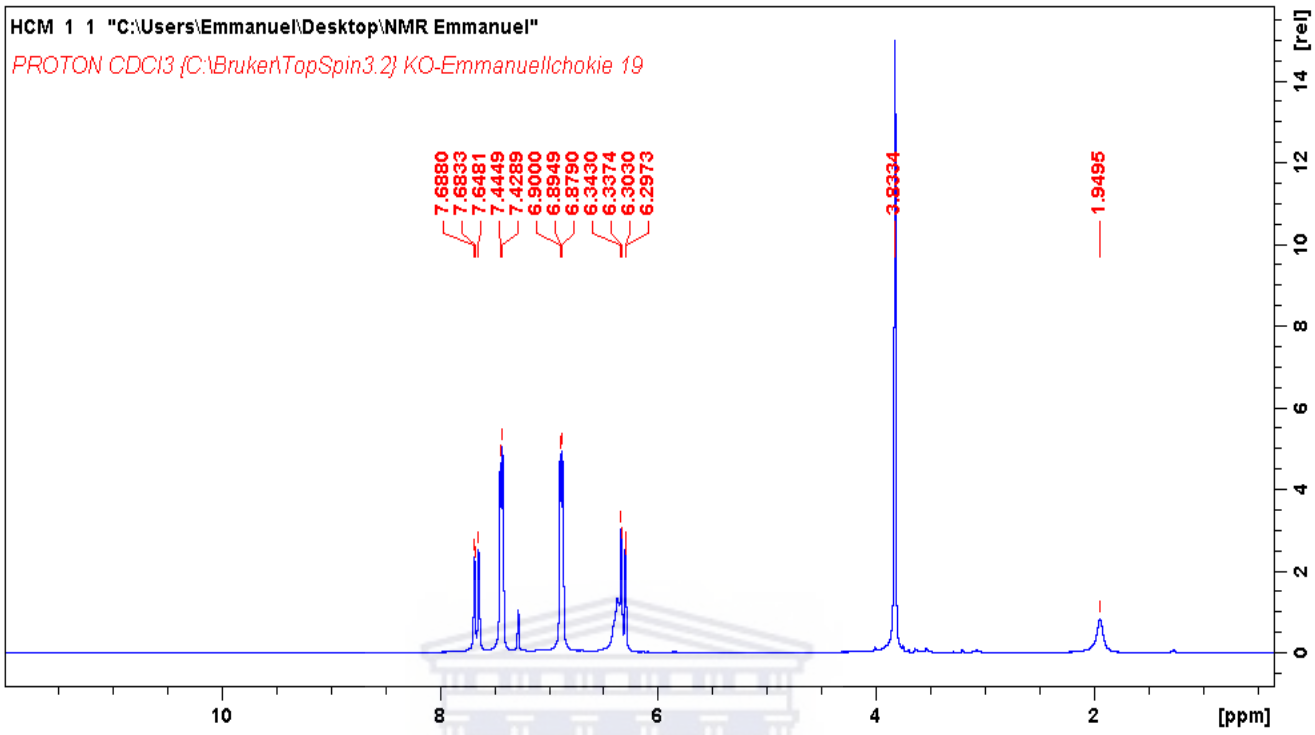
Appendix XXVIII: IR Spectra for 4 Hydroxybenzoic acid



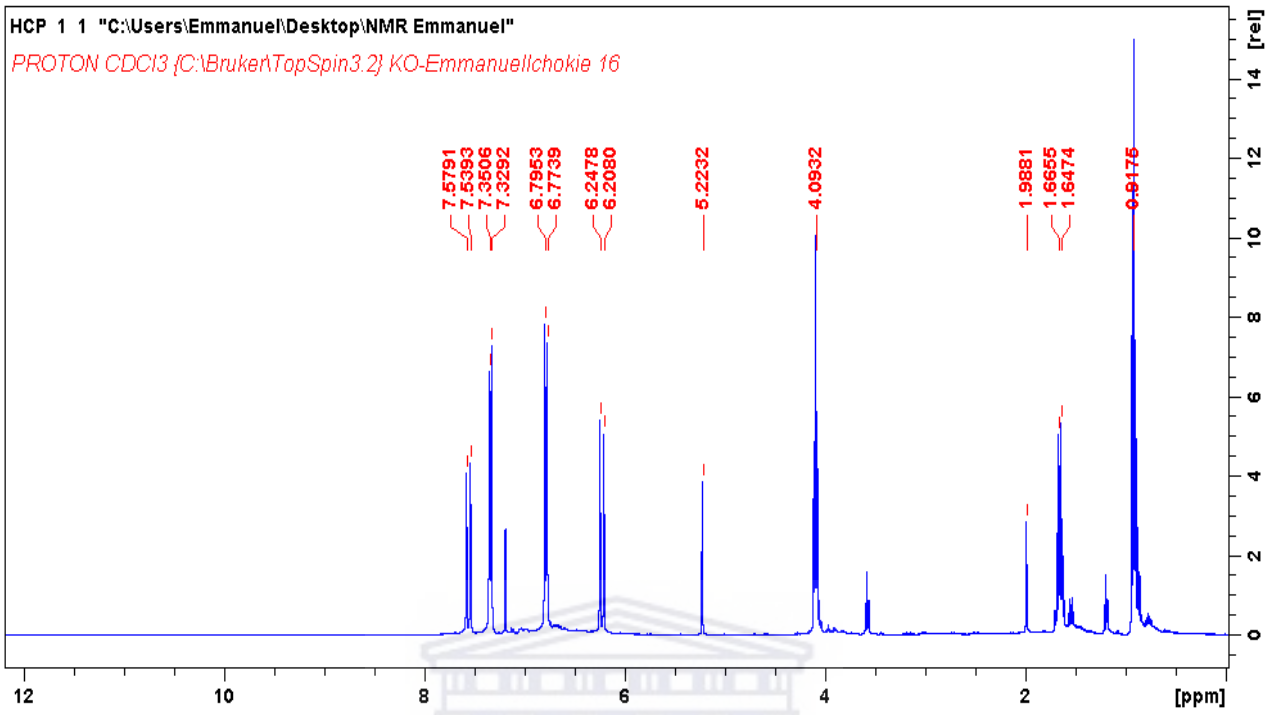
Appendix XXIX: ^{1}H NMR of Ethyl Hydroxycinnamic acid



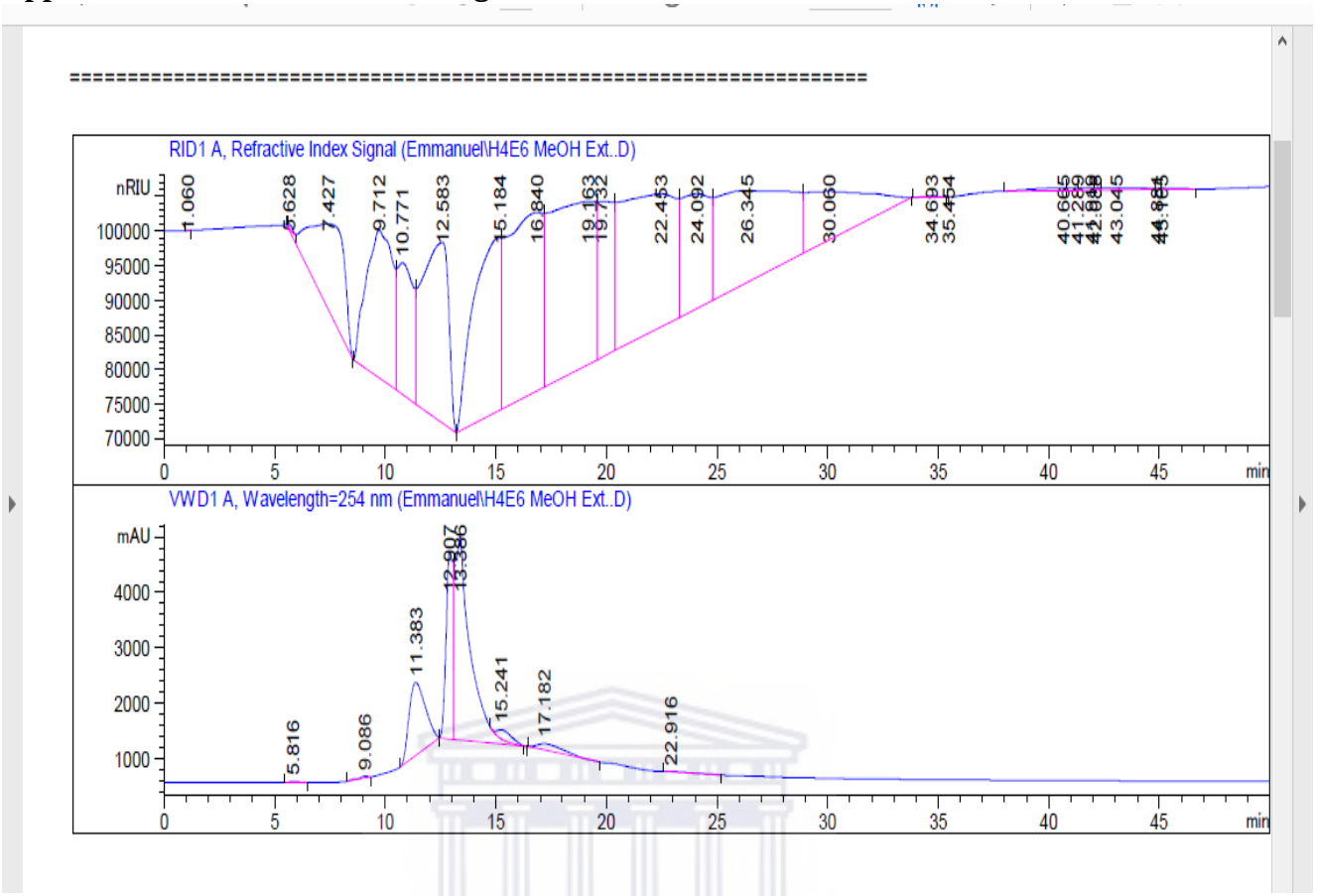
Appendix XXX: ^{1}H NMR of Methyl Hydroxycinnamic acid



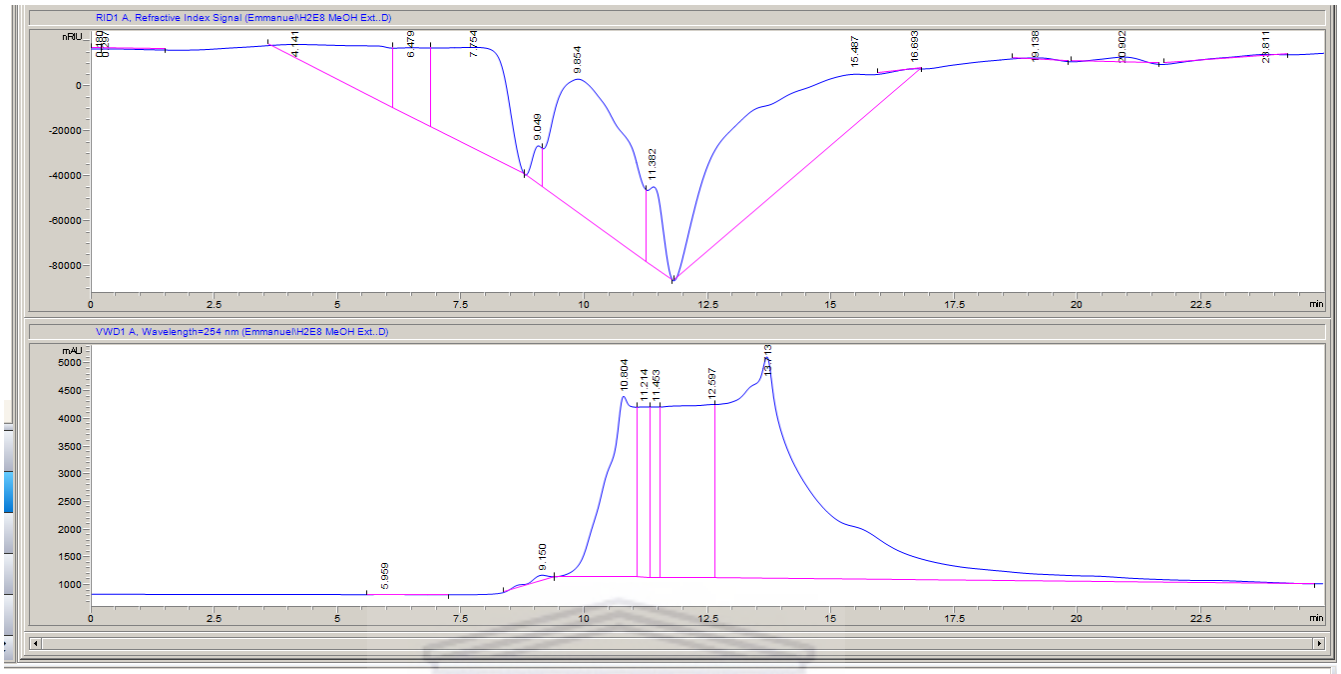
Appendix XXXI: ^{1}H NMR of Propyl Hydroxycinnamic acid



Appendix XXXII: HPLC Chromtogram of Fraction M_4



Appendix XXXIII: HPLC Chromatogram of Fraction M5



Appendix XXXIV: Certificate of Analysis of *Centella asiatica* (Gotu Kola)

JIAHERB® Phytochem

Shaanxi Jiahe Phytochem Co., Ltd. Tel:0086-29-88344265 Fax:0086-29-88325519
A-6th Floor, No.66 Jinye 1st Road,Xi'an, China 710077
http://www.jiaherb.com

CERTIFICATE OF ANALYSIS

Gotu Kola Extract 4:1

Batch No.: CJXC-A-822919 Manufacture Date: 20180413 Expiry Date: 20200412

General Information			
Part Used	Grass	Solvents Used	Water & Ethanol
Botanical Source	*Centella asiatica(L.)Urb.*	Country Of Origin	China
ITEMS	**SPECIFICATION**	**METHOD**	**TEST RESULTS**
Physical& Chemical Data			
Color	Greenish Brown	Organoleptic	Conform
Odour	Characteristic	Organoleptic	Conform
Appearance	Fine Powder	Organoleptic	Conform
Analytical Quality			
Identification	Identical to *R.S.* sample	HPTLC	Identical
Extract Ratio	4:1		Conform
Sieve analysis	100 % through 80 mesh	USP <786>	Conform
Loss on Drying	≤5.0 %	Eur.Ph.7.0 [2.8.17]	3.75%
Total Ash	≤10.0 %	Eur.Ph. <2.4.16>	2.95%
Bulk density	40-60 g/100mL	Eur.Ph. <2.9.34>	47 g/100mL
Tap density	60-90g/100mL	Eur.Ph. <2.9.34>	69 g/100mL
Contaminants			
Lead (Pb)	≤3.0 mg/kg	Eur.Ph. <2.2.58>ICP-MS	0.0845 mg/kg
Arsenic (As)	≤2.0 mg/kg	Eur.Ph. <2.2.58>ICP-MS	0.1789 mg/kg
Cadmium (Cd)	≤1.0 mg/kg	Eur.Ph. <2.2.58>ICP-MS	0.0784 mg/kg
Mercury (Hg)	≤0.1 mg/kg	Eur.Ph. <2.2.58>ICP-MS	0.0372 mg/kg
Solvents Residue	Meet Eur.Ph.7.0 <5.4>	Eur.Ph. <2.4.24>	Conform
Pesticides Residue	Meet USP Requirements	USP36 <561>	Conform
Microbiological			
Total Plate Count	≤10000 cfu/g	USP36 <2021>	70 cfu/g
Yeast &Mold	≤1000 cfu/g	USP36 <2021>	10 cfu/g
E.Coli.	Negative	USP36 <2022>	Conform
Salmonella	Negative	USP36 <2022>	Conform
General Status			
Non-Irradiation	≤700	EN13751:2002<PSL>	241
Packing&Storage	Packed in paper-drums and two plastic-bags inside. N.W.25kgs .I.D.35×H51cm; Store in a well-closed container Away from moisture,light, oxygen.		
Shelf life	24 months under the conditions above and in its original packaging.		
Manufacturer	**Shaanxi Jiahe Phytochem Co., Ltd. Xi'an, P.R China.**		

Quality Assurance Officer 崔鹏

Corrector 质检专用章

Analyst 锋惠印战

No：STP-QCP-<2>（818）

75292314
28153
ZA10007434

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Killarney Gardens, Cape Town
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APPROVED

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