BIOASSAY GUIDED FRACTIONATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITOR COMPOUND FROM *HYPERICUM PERFORATUM*

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Pharmacy in the School of Pharmacy, University of the Western Cape

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DECLARATION

I declare that the thesis, bioassay guided fractionation of Angiotensin converting enzyme inhibitor compound from *Hypericum perforatum*, has not been submitted before for any degree examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete reference.

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ABSTRACT

Due to the contribution of hypertension to various cardiovascular diseases, many studies are currently focused on identifying efficient bioactive compounds with antihypertensive activity and thus reducing the levels of cardiovascular disease. ACE inhibitors are an important component of the therapeutic regimen for treating hypertension, but due to the increase in the prevalence of side effects of synthetic compounds, alternative and complementary medicines which may consist of pure bioactive compound or a combination of various compounds from natural sources are gaining importance in overcoming hypertension. *Hypericum perforatum* has been studied for various activities including anti-bacterial, anti-depressant, anti-oxidant properties, but studies on its cardiovascular effects specifically ACE inhibitory activity have not yet been explored. In this study, ACEI assay-guided fractionation of the ethanol extract of *Hypericum perforatum* was carried out other to isolate a compound with ACE inhibition. A compound – (3-hydroxy 4, 4 dimethyl-4-butyrolactone) was isolated from an active fraction of the plant extract and was tested for ACE inhibition and its chemical structure elucidated using 1HNMR and C^{13}NMR spectrometry and further characterized using mass spectrometry and FTIR.

The pure compound: 3-hydroxy 4, 4 dimethyl-4-butyrolactone eluted from ethyl acetate: hexane solvent ratio (80:20) with a molecular formula of $C_6H_{10}O_3$ and molecular mass of 130. The ACE inhibitory activity of the active compound was compared to that of the standard ACEI

drug, captopril, with 1 mg/ml of the compound inhibiting ACE by 70% compared to 98.58% with captopril. IC50 was calculated as 63.88 µg/ml for the active compound compared to 0.0165 µg/ml for captopril.

3-hydroxy 4, 4 dimethyl-4-butyrolactone may be wholly or partly responsible for the ACE inhibitory activity of *Hypericum perforatum*, and has promise as a lead compound for development of ACEI for the treatment of cardiovascular disease.

DEDICATION

I dedicate this work to God Almighty who has made it possible for me to complete this research.

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I appreciate God Almighty for his endless grace and mercies which has accompanied me throughout the duration of my studies. Without him with me, this work will not have been made possible.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

TABLE OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
C-GMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
CHD	Coronary heart disease
COMT	Catechol-o-methyltransferase
^{13}C NMR	Carbon nuclear magnetic resonance
DAD	Diode array detector
EGF	Epidermal Growth Factor
ELISA	Enzyme linked immunosorbet assay
ESI	Electro spray ionization
FAPGG	Furanacryloyl-L-phenylalany lglycyl glycine
GC-MS	Gas chromatography-mass spectroscopy
GCI	Global Impression Index
GABA	Gamma-Aminobutyric acid, or γ-aminobutyric acid

GIT	Gastrointestinal tract
HAM-D	Hamilton Depression Scale
HA	Hippuric acid
HHL	Hippuryl-L-histidyl-L-leucine
HL	L-histidyl-L-leucine
HPLC	High performance liquid chromatography
HNMR	Proton Nuclear Magnetic Resonance
IR	Infrared Radiation
L-NAME	N-nitro l-arginine methyl ester
MAO	Monoamino-oxidase
MI	Myocardial infarction
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NMR	Nuclear Magnetic Resonance
PTK	Protein tyrosine kinase
RF	Retardation factor

ROS	Reactive oxygen species
SEM	Standard error of mean
SJW	St John's Wort
TSP	Thermo spray interface
TLC	Thin layer chromatography

KEYWORDS

3-hydroxy 4, 4 dimethyl-4-butyrolactone

Angiotensin converting enzyme inhibitor

Bioassay

Cardiovascular disease

Hypericum perforatum

Hypertension

St John's Wort

CHAPTER 1

INTRODUCTION

1.1 Epidemiology of cardiovascular disease

The epidemiological transition was first explained by Abdel Omran in the 1970s as a change in predominant forms of disease and mortality that affects a population negatively as its economy and health system develops (Omran, 1970). Chronic diseases are now the main dominant cause of the global burden of disease, and cardiovascular disease (CVD) is the largest contributor to chronic diseases. An estimated 29.6% of all deaths were caused by CVD in 2010 of which Ischaemic heart disease alone accounted for 7 million deaths worldwide (Wong, 2014). In 2013 the Global Burden of Disease Study estimated that almost 30% of all deaths worldwide were caused by CVD (Wong, 2014).

The rate of CVD deaths are decreasing in most high-income countries but increasing in most low and middle-income countries (Bhatnagar *et al.*, 2015). CVDs consists of chronic heart diseases (CHD) including: stable, unstable and non-fatal myocardial infarction (MI), coronary death, heart failure, cardiac arrest, ventricular arrhythmias, sudden cardiac death, rheumatic heart disease, transient ischaemic attack, ischaemic stroke, subarachnoid, intra-cerebral haemorrhage, abdominal aortic aneurysm, peripheral artery disease, and congenital heart

disease (Wong, 2014). Ischaemic heart disease is the biggest manifestation of CVD followed by cerebrovascular disease causing the second highest mortality rate (Valentin Fuster, 2010).

According to the WHO, cardiovascular disease is today the main cause of global mortality, with expected increases in the mortality rate in the future (WHO, 2008). The number of CVD deaths mostly from stroke, heart disease, and rheumatic heart disease increased from 14.4 million in 1990 to 17, 5 million in 2005, and most of these deaths occurred in low and middle-income countries. Researchers believe that by 2030 non-communicable diseases will be responsible for three-quarters of deaths worldwide of which CVD will be responsible for more deaths in low-income countries than infectious disease, maternal, perinatal conditions and nutritional disorders combined (Valentin Fuster, 2010).

In South Africa, about 6.3 million people are hypertensive, and with poor blood pressure control (Valentin Fuster, 2010). The National Demographic Health Survey found that of all individuals living with hypertension only 39% of cases were documented and less than a third (29%) of the hypertensive patients were diagnosed and are on medication, whilst only 14% was well controlled (Wright, 2007). In South Africa, the standard care for hypertension is prescription medication, alongside a stepwise lifestyle modification programme. There are several classes of drugs that have been used in the treatment of hypertension, and these include diuretics, beta-blockers (β-blockers), calcium channel blockers (CCB‘s) and angiotensin-converting enzyme (ACE) inhibitors (Wright, 2007)

The proportion of all deaths caused by CVD is greater in women (51%) than men (42%) as compared to 19% and 23% respectively for cancer. Coronary heart disease (CHD), when considered separately, accounts for 1.8 million deaths on an annual basis. CHD causes one in five of all deaths in patients suffering from CVD (Nichols *et al*, 2014; Wong, 2014). The risk factors such as hypertension, smoking, diabetes mellitus, high cholesterol levels and obesity are the top six causes of death globally (Wong, 2014). Prevention of CHD focuses on identifying and managing these risk factors at both the population and individual levels through primordial, primary, and secondary prevention (Wong, 2014). Minimizing obesity, sedentary lifestyle and an unhealthy diet can reduce the incidence of heart disease, stroke, and diabetes mellitus by 80% (Valentin Fuster, 2010).

Recent epidemiological studies have provided valuable insights into the natural history and risk factors associated with the development and prognosis of CVD. These provide the foundation for intervention studies as well as clinical trials that are aimed at the primordial, primary and secondary prevention of CVD (Wong, 2014). Primordial prevention is mainly focused at preventing CVD risk factors such as hypertension, obesity, and dyslipidemia, whereas primary prevention focuses on the modification of these factors and the prevention of clinical manifestations of CVD such as myocardial infarction and stroke. Secondary prevention is

aimed at patients who have already have the manifestation of CVD but where extensive control of risk factors will have a major effect on preventing the relapse of the disease (Wong, 2014).

1.2 Traditional medicines as drugs for the treatment of diseases

Traditional medicine is the sum total of knowledge, skills, and practices that are based on theories, beliefs, and experiences which are unique to different cultures. These beliefs and knowledge are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (Mahomoodally, 2013). Complementary or alternative medicines are traditional medicines that has been adopted by other populations. The extensive use of traditional medicines mainly in Africa has been linked to cultural and economic reasons. Hence, African leaders have been encouraged to promote and integrate traditional medicine with their health systems (Li *et al.*, 2005). Knowledge about medicines which has been passed down by oral tradition from one generation to the next by an elderly priest, witch doctors or medicine men as written records are almost none existent (Nakamura *et al.*, 2005).

Medicines derived from plants occupy a vital place in disease treatment worldwide with their greatest attribute being their tendency to cause low side effects as compared to modern synthetic chemotherapeutic, immunomodulation and cytotoxic medicines (Ingle *et al.*, 2017). According to the WHO, due to poverty and lack of access to medical care, about 65% to 85%

of the world population living in developing countries depend mainly on medicinal plants as source of primary health care. The utilization of herbal medicinal preparations are very popular in developing countries as they have a long-lasting tradition for their use (Calixto J.B, 2000).

One of the weaknesses of herbal preparations is the fact that they do not have an immediate or strong pharmacological action and for this reason, they cannot be used for emergency treatments. Herbal medicines are generally associated with the widely believed myth that they are very safe and free from side effects, but the presence of many toxic constituents such as digitalis, pyrrolizidine alkaloids, ephedrine, and phorbol esters proves otherwise (Calixto J.B, 2000). Herbal plants do however have less frequent adverse effects as compared to synthetic drugs. Two kinds of side effects for herbal medicines have been identified. The first kind of adverse effects are intrinsic to herbal drugs, meaning they are mainly linked to the predictable toxicity, overdose and contra-interaction with other conventional drugs. The second kind of adverse effects are extrinsic to the preparation which is related to several manufacturing problems like the miss-identification of the plant, lack of standardization, poor good manufacturing practice, contamination, substitution, adulteration, and incorrect preparation and dosage (Calixto J.B, 2000). Over the years there has been a lot of success with herbal medicines as over 4000 herbal medicines since 1978 have been submitted to pharmacovigilance in Germany and many have been withdrawn from the market because of their toxic effect and risk for human use (Calixto J.B, 2000).

1.3 Medicinal plants as the source of drug

Herbal medicine is based on the principle that plants contain natural substances that can promote health and alleviate illness. Recent researches have focused their interest on herbal plants possessing various medicinal properties. A wide variety of phytochemicals like flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, ceramics, saponins, plant sterols, curcumins, and phthalides has been identified and extracted from many herbal plants. Many western drugs have their origins in plant extracts including ephedrine which was extracted from the Chinese plant Ephedra and used for asthma treatment, salicylic acid was obtained from a willow tree bark and was used to relieve fevers, and Paclitaxel was isolated from the bark of Pacific yew and used for cancer treatment (Craig, 1999). Natural products are widely sold over the counter for their use in the treatment and management of common ailments and conditions such as arthritis, anxiety, cough, cold, constipation, fever, headaches, stress, ulcers, weakness and pre-menstrual syndrome. Some of the common herbs used today are Echinacea, garlic, ginseng, gold-ensal, gingko, saw palmetto, aloe vera, and feverfew. Researchers have proven the usefulness of ginger for motion sickness, licorice (glycyrrhizin) for ulcers, and Echinacea for immune support (Craig, 1999).

1.4 Medicinal plants used in CVD

Natural medicines with cardiovascular properties have been used to manage and treat people with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, venous insufficiency, and arrhythmias (Mashour and George, 2015). Some of these natural medicines with known cardiovascular effects include: digitoxin which was extracted from *D. pupurea* or *D. lanata* and digoxin which was extracted from *D. lanata* alone. Adonis, black Indian hemp, red-headed cotton hemp, balloon cotton, king's crown, wintersweet, sea mango, wall-flower, lily of the alley, rubber vine, black hellebore, oleander, frangipani, strophanthus, yellow oleander, and squill all contain potent cardio-active glycosides which have a positive inotropic action on the heart for the treatment of congestive heart failure (Mashour and George, 2015). The roots of *R. serpentina* (snakeroot) and its alkaloid- reserpine lowers blood pressure by decreasing cardiac output, peripheral vascular resistance, heart rate and renin secretion. *Stephania tetrandra* has been shown to have a calcium channel blocking effect. The root of *Lingusticum wallichii, Evodia rutaecarpa, Uncaria rhynchophylla* are all Chinese plant used as either circulatory stimulant, hypertensive, hypotensive drug or sedative. The seeds of horse chestnut, *Aesculus hippocastanum* have long been used to treat venous disorder like varicose veins as they inhibit the activity of lysosomal enzymes thought to contribute to the varicose vein by weakening vessel walls and increasing permeability which causes dilated veins and edema. (Mashour and George, 2015).

1.5 Modern drug therapy

The variety and use of cardiovascular drugs have greatly increased over the past decades, starting from the 1950s when an effective oral diuretic became available, to the 1960s when a class of beta blockers was discovered, all through to the 1980s when calcium channel blockers and angiotensin converting enzymes inhibitors became widely used. All these drug discoveries made it possible to dramatically change the treatment of heart failure and hypertension and brought about major changes in the doctors' ability to treat patients with hypertension or angina pectoris, as well as allowing patients with hypertension, heart failure, and coronary artery disease to be treated more effectively (Weisfeldt and Zieman, 2007). The development and use of thrombolytics have revolutionized the ability to treat patients with heart attacks. There are many other different classes of drugs used for various cardiovascular diseases. These drug classes include: Statins, antihypertensive, antiarrhythmic, diuretics, anticoagulants, antiplatelet and thrombolytics (Weisfeldt and Zieman, 2007).

The efficacy of these treatments to reduce cardiovascular morbidity and mortality in stroke, heart attack, and heart failure was established principally through large-scale clinical trials. These large scale clinical trials may also include studying phytochemicals of various herbal extracts in order to determine their biological activity. Phytochemicals found in variety of herbal extracts have shown to reduce high blood pressure, cholesterol concentrations, provide some protection against cancer, and stimulate the immune system. Therefore the need for new

drugs from these phytochemical that will be more affordable, more available and safe is increasing (Weisfeldt and Zieman, 2007).

There is on-going research to prove the pharmacological actions of many of these cardio-protective herbal medicines and to encourage the future pharmaceutical development of many of these herbal drugs that could be therapeutically beneficial. Studies on cardiovascular effects of *Hypericum perforatum* have not yet been documented but its phytochemical profile has been reported. This study will determine the ACE inhibitory effect of compound(s) isolated from *Hypericum perforatum*.

1.6 Aims

The aim of this study is to isolate a biologically active compound from *Hypericum perforatum* that has ACE inhibitory activity using the bioassay-guided fractionation method. The study will also aim to characterize the isolated compound(s) using NMR, MS and IR spectroscopy.

1.7 Objectives

The objectives of this is study include:

- To determine the ACE inhibitory activity of fractions of the organic extracts of *Hypericum perforatum* leaves.
- To isolate compound(s) with ACE inhibitory activity from the ACE inhibitory-active fraction(s) of the extracts of *Hypericum perforatum* leaves.
- Characterisation of the isolated active compound(s) using NMR and IR spectroscopy, and high-resolution mass spectrometry.

1.8 Hypothesis

To guide this research the following hypothesis was formulated:

Hypericum perforatum contains compound(s) with potent ACE inhibitor activity comparable to that of captopril.

1.9 Research question

This research was designed in other to answer this question below:

Does *Hypericum perforatum* contain compound(s) with ACE inhibitory effect?

1.10 Justification of study

Investigating the ACE-inhibitory activity of *Hypericum perforatum* will further contribute to studies towards alleviating hypertension in SA. At the end of this study, the isolated compound thus represents a new lead compound for the possible development of a new ACE inhibitors.

CHAPTER 2.

LITERATURE REVIEW

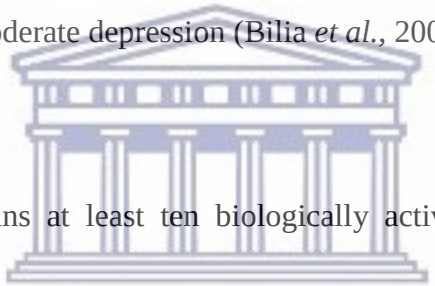
INTRODUCTION

2.1 Botanical profile of *Hypericum perforatum*

The genus hypericum Linn belongs to the family hypericaceae, comprising of about 450 species with *Hypericum perforatum* being the most representative. *Hypericum perforatum* L. (hypericaceae), is a low evergreen shrub with attractive bright orange-yellow flowers. It produces a slender, erect stem up to 1m high in summer and spreading prostrate stems in winter, with heights up to 60cm. It has light green leaves with translucent oil glands and bright yellow star flowers with black oil glands on the margin of the petals from October to January. The seeds are small, elongated and with rounded ends. They germinate after some days if exposed to soil at high temperatures. The brownish yellow roots have a woody and resistant consistency from the seedling. *Hypericum perforatum* is a herbaceous perennial herb which is indigenous to North America, Europe, the Canary Island and western Asia. Common names for *Hypericum `perforatum* include St John's Wort, goat weed, touch and heal, penny-john, Klamath weed, gammock and rosin rose (Kopleman *et al.*, 2001; Ganzera, Zhao and Khan, 2002). The name "*hypericum"* is derived from Greek with hyper meaning over and eikon meaning image and

the name St John's Wort was given as the flowers bloom around Saint John's day, 24th of June (Barnes, Anderson and Phillipson, 2001).

Traditionally St John's Wort has been used for the treatment of excitability, neuralgia, sciatica, menopause neurosis, anxiety, depression, malaria, viral infections, bacterial infections, burns, wounds, insect bites, as an astringent, stress reduction, immune support and as a nerve tonic (Choudhuri and Valerio, 2005). The plant has also been indicated for gout, hepatic disorders and gastric ulcers. Today it is commercially available in tablet, capsule, tea, and tincture form for the treatment of mild to moderate depression (Bilia *et al.*, 2001).



Hypericum perforatum contains at least ten biologically active compounds belonging to various phytochemical classes including: Flavonoids (rutin, hyperoside, isoquercitrin, quercitrin, and quercetin); naphthodianthrones (hypericin, pseudohypericin) and phloroglucinols (hyperforin and adhyperforin); and to some lesser extent phenylpropanes, flavonol derivatives, flavones, proanthocyanidins, xanthones, some amino acids, and essential oils ((Tatsis *et al.*, 2007). The qualitative and quantitative variation of secondary metabolites in *Hypericum perforatum* is affected by the ecological, environmental, physiological and genetic factors. When the plant extract is exposed to light, protohypericin and protopseudohypericin are converted into hypericin and pseudohypericin respectively and this

also leads to the degradation of phloroglucinol which is very sensitive to oxidation and is unstable in solution when exposed to air (Tatsis *et al.*, 2007).

This chapter presents a review of *Hypericum perforatum*; phytochemical constituents and pharmacological and clinical studies done on the plant. The use of bioassays to identify bioactive compounds in the plants as well as an overview of the actions of angiotensin converting enzyme and angiotensin converting enzyme inhibitors will also be covered.

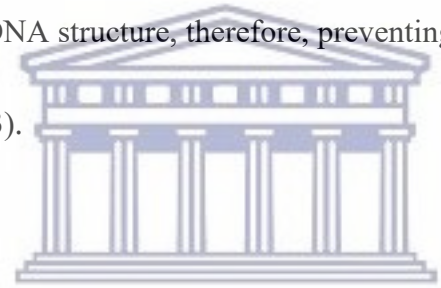
2.2 Phytochemical profile

2.2.1 Naphthodianthrones

These compounds mainly attracted biochemists at first because of their intense red colour and their phototoxic properties. Total hypericin content is used to standardize St John's wort with the total content varying from 0.05% to 3%(Choudhuri and Valerio, 2005). Initially, two photo-derivatives namely protohypericin and pseudoprotohypericin were isolated from the plant but they proved to be unstable, but were later converted to their stable form, hypericin and pseudohypericin due to the effect of light. Pseudohypericin is the main naphthodianthrone in St John's Wort and is mainly present in two to four folds higher than hypericin (Patočka, 2003). These phytochemicals have a selective solubility in almost all solvents but they are insoluble in water at ambient temperature, although their solubility increases at higher temperatures.

Hypericin is documented to be the main photosensitizing constituent of *Hypericum perforatum* and is believed to cause hypericism. It is used in photodynamic cancer therapy as a photosensitizer whereas, pseudohypericin has no photo toxicity (Patočka, 2003).

Clinical data has suggested that the antiretroviral effect of hypericin and pseudohypericin can be due to the inhibition of some phosphorylation influenced by protein kinase C during the cell's viral infection (Patočka, 2003). Further findings indicate that hypericin inhibits the DNA relaxation activity of topoisomerase II alpha. This antagonism is thought to be due to hypericin's ability to distort DNA structure, therefore, preventing topoisomerase II binding or DNA cleavage.(Patočka, 2003).



Hypericin is thought to be derived from anthranoid metabolism and emodinanthrone is thought to be the precursor. The protonaphthodianthrones, protohypericin, and protopseudohypericin represent partially cyclic precursors of the naphthodianthrones, which upon irradiation with visible light, is converted into their naphthodianthrone analogs. The ratio of protonaphthodianthrones to naphthodianthrones in the fresh plant and in the extract may vary significantly between 1:5 and 5:1 respectively, depending on the extent of the photo-conversion that took place during the preparation of the extract. Cyclopseudohypericin is thought to be an oxidation product of pseudohypericin (Wild, 2003).

Hypericin and its analogs are slightly acidic compounds. They are freely soluble in methanol as well as pyridine and other organic bases, yielding cherry red solutions with red fluorescence. Pseudohypericin and hypericin both absorb visible light with a maximum absorption at 588nm. They are highly fluorescent in methanol when exposed to ultraviolet (UV) light. Hypericin and pseudohypericin are soluble in alkaline solutions, with the solutions red below pH 11.5, and green with red fluorescence above pH 11.5. The naphthodianthrones, especially hypericin are almost insoluble in water at ambient temperature. However, it has been found that more than 40% of hypericin and psuedohypericin present in the herb is extractable when using water heated between 60^{o}C and 80^{o}C. Hypericin forms monobasic salts with inorganic bases between a pH of 4 and 11 and are soluble in polar solvents. However, pseudohypercin and hypericin occur mainly as potassium salts (Wild, 2003).

2.2.2 Phloroglucinols

Two phloroglucinol compounds have been found in *Hypericum perforatum* namely hyperforin as the main compound and adhyperforin containing one additional methyl group. Both compounds occur in the reproductive part of the plant and to a lesser extent in flowers and greater extent in ripe fruit. Hyperforin is lipophilic and unstable towards light and heat. Although the pharmacological activities of hyperforin are little known, it has been shown to inhibit or modulate various neurotransmitter systems *in vitro* as it is a potent reuptake inhibitor of serotonin, dopamine, noradrenaline, and GABA (Patočka, 2003). It has been demonstrated

as a modulator of various neuronal ion channels as well as the inhibition of smooth muscle contraction induced by many neurotransmitters. It is believed that hyperforin is the major active phytochemical responsible for the antidepressant effects of the plant, with its mechanism of action considered to be due to synaptic reuptake of neurotransmitters (Patočka, 2003). It has been reported that the amount of both compounds increases from about 2% hyperforin and 0.2% adhyperforin in the unripe fruits to 5% hyperforin and 2% adhyperforin in the ripe fruits. Hyperforin and adhyperforin are acylphloroglucinol-type compounds, which consist of a phloroglucinol skeleton substituted with lipophilic isoprene chains. The phloroglucinols are lipophilic, unstable towards heat and light either in storage or in solution, and are very susceptible to oxidation (Wild, 2003). The stability of isolated hyperforin in solution and solid form was investigated in several antioxidant systems, and hyperforin was reported to show greater stability in polar solvents than in non-polar solvents. It was shown to be much more stable in a pH 2.0 methanol solution than in a pH 12 solution. It was also reported that degradation under light exposure can be diminished in methanol by acidification at a pH of 2.0, and degradation under light exposure at pH 12.0 results in total decomposition within 30 days. However, when kept in the dark, hyperforin is stable in acid and alkaline methanolic solutions (Wild, 2003).

2.2.3. Flavonoids

This group of compounds includes the flavonol, the flavonol glycosides, and the flavones. Flavonols are the aglycone forms of the flavonol glycosides and include quercetin, kaempferol, luteolin, and myricetin. Of these four, quercetin is by far the most common (Wild, 2003). Quercetin is the major biologically active compound of *Hypericum perforatum* constituting between 2% to 4%. Hyperin and rutin mainly dominate among the glycosides followed by quercitrin then isoquercitrin. These flavonoid glycosides have spasmolytic activity and are inhibitors of monoamine oxidase A and catechol-O-methyltransferase enzymes, but the levels of the flavonoids are too low to be responsible for the therapeutic efficacy of the crude drug. The flavonoids are also believed to be capillary protectants. (Patočka, 2003).

2.2.4 Proanthocyanidins

These are present in the plant in the form of tannins and they are measured by their conversion to red colour cyanidins with their concentration highest at the pre-flowering stage. They also possess biological effect including antioxidant, antiviral and antimicrobial effects (Patočka, 2003). Phenylpropanoids, mainly present as esters of hydroxycinnamic acid and chlorogenic acid have been detected, but at concentrations below 1%. Phenylpropanoids have been reported to have central stimulating activity. Caffeoylquinic acid, a chlorogenic acid has been shown to exhibit weak spasmolytic activity *in vitro* and choleretic activity *in vivo* (Nahrstedt and Butterweck, 2013).

2.3 Pharmacological activities of *H. perforatum*

The plant has been assessed pharmacologically for a broad spectrum of effects that include: anti-anxiety, antibiotic, anti-spasmodic, anti-oxidant, calcium channel blocker, gene expression induction, wound healing, smooth muscle relaxant, sleep potentiator, anti-inflammatory and anti-microbial activity.

The essential oils in the plant have been reported to possess anti-microbial activity, with the essential oils and water-soluble part of the alcoholic extracts showing minor anti-fungal and significant antibacterial properties. Hyperforin and adhyperforin has been reported to have an antibiotic effect three times greater than that of sulphanilamide through keloid inhibition (Singh *et al.*, 1999).

Hypericin has shown significant antidepressant activity by inhibiting monoamine oxidase (MAO) and β-hydroxylase enzymes *in vitro*. Hypericin also plays a role in the suppression of interleukin-6 in the blood and also modulates the expression of serotonin receptors that inhibit the neuronal uptake of serotonin, norepinephrine, and dopamine. Hypericin, hyperforin, and xanthones are the main constituents that exhibit the antidepressant activity ((Vattikuti and Ciddi, 2005).

Hypericin was found to be active against human leukaemia, squamous carcinoma, nasopharyngeal carcinoma, mouse mammary carcinoma, and fibroblast cells. It also inhibits the Epidermal Growth Factor (EGF) receptor and Protein Tyrosine Kinase (PTK) activity. Hyperforin inhibits MT-450 breast carcinoma in immune-competent Wistar rats to a similar extent as that of paclitaxel without any sign of acute toxicity (Vattikuti and Ciddi, 2005).

Hypericin is well-known as a photosensitizing agent for photodynamic therapy of cancer and viral infections. The antiviral activity of Hypericin and pseudohypericin against retrovirus like HIV involves the combination of its photodynamic and lipophilic properties while hypericin alone binds the cell membrane and cross-links the viral protein leading to its inability to retrieve the reverse transcriptase activity (Singh *et al.*, 1999). Also, activity has been reported against murine cytomegalovirus, sindbis virus and equine infectious anaemia virus (Singh *et al.*, 1999). The mechanism of action of this activity seems to involve the photo activation process that forms singlet oxygen, rendering viral fusion inactive. Although it is seen that hypericin has some antiviral activity *in vivo*, the photodynamic properties do limit its usefulness as an antiretroviral agent. However, hypericin can photo reduce oxygen to superoxide radicals, forming semi-quinone in the absence of light. Both hypericin and pseudohypericin have been shown to inhibit regulatory enzymes, proteins kinase C as well as inhibit receptor tyrosine kinase activity of epidermal growth factor (Singh *et al.*, 1999). The procyanidin fraction

enhances coronary flow by antagonizing histamine or prostaglandin $F_{2\alpha}$-induced arterial contractions (Singh *et al.*, 1999).

2.4 Clinical Studies on *H. perforatum*

Hypericum perforatum has been tested clinically for anti-retroviral, antibacterial, anticancer, anti-mutagenic, antiviral activities and as an Immuno-stimulant, but the most documented clinical studies are on its anti-depressant activity.

Miller and Rossel (1994) reported that St. John's Wort extracts inhibited serotonin receptor expression at 50 µM (~25ug/ml), while Perovic and Miller (1995) reported inhibition of serotonin uptake at 6.2 µg/ml of the extracts. The Inhibition of catechol-O-methyl transferase (COMT) was also reported with various ethanolic St. John's Wort fractions at supra-physiologic concentrations of up to 500 µg/ml. St. John's Wort extracts also reduced cytokine expression and were hypothesized to reduce depression in susceptible individuals by reducing interleukin concentrations. Although this hypothesis hasn't been proven, the link between depression and the immune system is gaining greater attention (Singh *et al.*, 1999).

During a bioassay-guided fractionation of a methanolic extract of St John's Wort, hypericin and pseudohypercin were detected as the compounds that showed greatest antidepressant activity in a forced swimming test (Nahrstedt and Butterweck, 2013).. In a tail suspension test in mice, pure hyperforin reduced immobility time with 4 mg/kg and 8 mg/kg doses, but was

inactive below or above these dosages. In the same study, it was also shown that step by step removal of hyperforin and hypericin didn't result in the loss of the whole plant pharmacological activity, meaning that other constituents of the plant may contribute to the antidepressant activity (Nahrstedt and Butterweck, 2013)..

Amentoflavone was shown to cross the blood-brain barrier *in vitro* by passive diffusion, and in forced swimming tests, confirmed the antidepressant activity of flavonoids with an activity that was comparable to imipramine. Further evidence of the antidepressant activity of the plant's flavonoids was provided by Butterweck (1997), who reported that isoquercitrin, hyperoside and miquelianin down-regulated plasma adrenocorticotropic and corticosterone hormone level after two weeks of daily treatment (Nahrstedt and Butterweck, 1997).

2.5. Methods for the isolation of pure compounds with specific activity from medicinal plants.

There are several steps to be taken before the utilization of a biologically active compound from herbal plant in humans, and these steps include: extraction, pharmacological screening, isolation, characterization of the bioactive compound, toxicological evaluation and clinical evaluation.

2.5.1 Extraction techniques

Extraction is the process of separating a medicinally active agent or compound(s) from a solid or liquid mixture by using a suitable liquid solvent. Several methods for extracting compounds from a plant matrix exist and include: maceration, percolation, soxhlet extraction, decoction, hydro-distillation, ultrasound assisted extraction, microwave assisted extraction and supercritical fluid extraction.(Sasidharan *et al.*, 2011).

Maceration involves leaving the powdered plant soaked in a suitable solvent in a closed container at room temperature with occasional shaking. The solvent is filtered, leaving behind a concentrated extract. This process is inexpensive, non-complicated and limits compound degradation when used with cold solvents. The set back to this process is that the extraction takes longer and uses large amounts of solvent. Percolation extraction is similar to maceration, but hot solvent is continuously passed through the plant material. This process is much quicker but could produce decomposition because of the hot solvents used (Sharma *et al* ., 2015). Decoction process is used for extraction of compounds that are soluble in water and less heat sensitive. This process involves the addition of distilled water to the dried extract with continuous heating for a period of time at 100^0C. It is cooled down to room temperature, solvent is filtered to obtain the filtrate. This filtrate is concentrated to obtain the extract. The advantage

of this method is that it does not require expensive equipment and it is very easy to perform, but it cannot be used on heat sensitive constituents (Lumpur, 2017).

Soxhlet extraction is a continuous form of percolation with fresh solvent until complete exhaustion of the solute in the raw material (Lumpur, 2017). The material is separated from the extract by covering it in a paper thimble underneath the dropping condensed solvent, and when full the solvent in the thimble goes into the main vessel containing the extractant. The separation of the solvent is made using rotary evaporator, whereby a vacuum evaporation is carried out using a vacuum pump with a check valve (Lumpur, 2017). The advantage of this process is that fresh solvent continually extracts the plant material more effectively with little solvent. This method does not require filtration after extraction. Its disadvantage is that it brings about most decomposition with the high temperature and continuous heating. Long duration and high extraction amount of solvent consumed can lead to economic loss and environmental problems (Lumpur, 2017).

Hydro-distillation is mainly used for the extraction of plant material that doesn't use organic solvents. In this process, water is added in sufficient amount to plant materials that are packed in a still container and then the plant sample get heated either by boiling or by means of direct steam. The hot water and steam acts as an important factors to free bioactive compounds from plant tissue. Indirect cooling by water condenses the vapour mixture of water and oil, and this

mixture flows from the condenser to a separator where oil and bioactive compounds separates automatically from water (Lumpur, 2017). The main drawback to this method is that the high temperatures can cause loss of some volatile components, hence limiting its use for heat sensitive compound extraction (Lumpur, 2017).

Ultrasound assisted extraction is an inexpensive, fast, simple, less energy consuming and efficient extraction technique that produces acoustic cavitation in the solvent by the passage of an ultrasound wave. Ultrasound also exhibits an automated effect by allowing greater diffusion of solvent into the tissue and increasing the contact surface area to allow the solute to quickly diffuse from solid phase to the solvent (Lumpur, 2017). Its advantages include a reduction in extraction time and solvents used. There is more effective mixing, faster energy transfer and reduced thermal gradient and extraction temperatures (Wen *et al.*, 2019). The disadvantage is mainly based on the fact that this technique have lower efficiency when compared to the other new extraction techniques (Lumpur, 2017).

Microwave assisted extraction is a method used to extract soluble products into a fluid from a wide range of material using a microwave (non-ionizing electromagnetic fields in the frequency range from 300 MHz to 300 GHz) (Wen *et al.*, 2019). Heating with a microwave causes a direct impact on polar materials. Microwaves penetrate into biomaterials by interacting with polar molecules such as water inside the materials through generation of heat.

The infiltration of microwaves into plant matrix depends on factors such as: dielectric constant, moisture content, temperature, and the frequency of the electrical field. The water contained in a plant material makes it possible for the absorption of microwave energy which leads to the internal superheating and cell structure disruption. This action facilitates the diffusion of bioactive compounds from the plant matrix (Lumpur, 2017). Microwave assisted extraction is less time consuming, inexpensive, with less solvent is used and has a special heating mechanism. The drawback to this technique is that the operating temperature is high which causes problems for the extraction of antioxidants. This process also produces low yields when solutes or solvents are non-polar and it also needs a filtration or centrifugation stage to remove residue of the extract (Lumpur, 2017).

Supercritical fluid extraction is the process of separating the extractant from the medium using supercritical fluids such as carbon dioxide (CO_2) as the extracting solvent. This process mostly use carbon dioxide at high pressure to extract the high value products from natural materials. Unlike other processes, the extraction process leaves no solvent residue behind. The CO_2 is non-toxic, non-flammable, odourless, tasteless, inert, and inexpensive. Due to its low critical temperature of 31°C, carbon dioxide is known to be perfectly adapted in food, aromas, essential oils and nutraceutical companies (Sapkale *et al*., 2010). The major disadvantage is mostly on the economic aspect as this method is considered more expensive than traditional extraction process. The technology requires a non-negligible energy consumption to establish

the pressure and temperature during the different extraction, separation, solvent recycling stages (Lumpur, 2017).

2.5.2 Identification and characterization methods

Chromatographic techniques

Column chromatography is an important separation technique that has a huge impact in the field of separation science. In Chromatography, separation depends on the compound distributing between the stationary and mobile phase. Stationary phases may include an adsorbent, a paper, or a thin layer of adsorbent on a glass plate, and the mobile phase may include a liquid or a gas. When the solid stationary phase is in the form of a column, this is known as column chromatography (Sjögren, 2005). A column is the most useful method for separating and purifying both liquid and solids and the principle of chromatography is based on the adsorption of substance by the adsorbent. The most common adsorbents used are silica, alumina, calcium carbonate, calcium phosphate, magnesia and starch and the selection of solvent is based on the nature of both solvent and adsorbent. The column chromatography will allow the separation and collection of compounds individually. The main disadvantage of this technique is that it is tedious, time consuming and complicated when it comes to larger samples (Weller, 2012).

Thin layer chromatography (TLC) and bio-autographic techniques, are quick and inexpensive techniques that give fast answers as to the number of components that are in a mixture. Bio-autography technique is used specifically for determination of antimicrobial bioactive compound. Identification tests include spraying of phytochemical screening reagents which will cause colour change according to the phytochemical pre-existing in the plant extract or by viewing the plate under UV light as this confirms the purity and identity of the isolated compound (Sasidharan *et al.*, 2011). The major advantage is that it provides light images and fluorescence images, and it also gives different levels of profile and corresponding integral data with chromatography screening and digital processing. The main disadvantage is that is has, low resolution, low sensitivity and difficulty detecting trace components.(Lumpur *et al*., 2017).

High-performance liquid chromatography (HPLC) is very versatile, robust and widely used. This is the technique of choice for fingerprint study in the quality control of the herbal plant. HPLC can separate a mixture of compounds. It is used in phytochemical and analytical chemistry to identify, qualify and purify the individual compounds of the mixture. HPLC technique is based on the principal that certain compounds have different migration rates in a given column and mobile phase. The extent and degree of separation is determined by the choice of stationary phase and mobile phase (Lumpur, 2017). Purification by HPLC is a process whereby the target compound is separated or extracted from other compounds as each compound must have a distinctive peak under certain chromatographic conditions. In order to

get optimum separation of each compound with HPLC, a suitable detector must be selected and set to right detection point, separation assay must be developed, and the flow rate, a proper mobile phase and columns must be selected. HPLC is extremely quick and efficient method that is very versatile and precise when it comes to identifying and quantifying chemical components. Despite its advantages, HPLC can be very costly, requiring large amount of expensive solvents, the method can be complex to troubleshoot problems or develop new methods because of its range of different modules, column and mobile phase (Sasidharan *et al.*, 2011).

Gas chromatography (GC) is a highly sensitive powerful separation technique that is used for discovery of volatile organic compounds. This technique allows for more precise, quantitative determination of difficult mixtures, such as traces of compounds in the parts per trillions range(Siddiqui, 2017). The main advantage is based on its use in the study of pharmaceutical products with high molecular mass including polypeptides or thermally unstable antibiotics. It is an important tool for the analysis of impurities in pharmaceutical products. Its main constraint is based on its inability to detect non-volatile drug substances and it is not suitable for thermally liable substances (Varghese *et al* ., 2017).

Non-chromatography techniques

Immunoassay uses monoclonal antibodies against medicines as it shows for receptor binding analysis, enzyme assays, quantitative and qualitative analytical techniques. Enzyme-linked immune-sorbent assay (ELISA) that are based on monoclonal antibodies are documented to be more sensitive than HPLC techniques. Major disadvantages to this technique is that results obtained by immunoassay needs to be confirmed using another methods such as chromatography coupled with mass spectrometry (Sasidharan *et al.*, 2011).

Phytochemical screening assay are a simple and inexpensive method that gives quick answers to the different types of phytochemicals in the mixture, it helps to give an idea regarding the type of phytochemical present in the extract mixture and is an important tool in the analyses of bioactive compounds (Sasidharan *et al.*, 2011).

2.5.3 Structural elucidation methods (spectroscopic techniques)

Nuclear Magnetic Resonance Spectroscopy (NMR) is a nuclear specific spectroscopic technique that uses a large magnet to analyse the intrinsic spin properties of atomic nuclei. NMR uses electromagnetic radiation to promote shifts between nuclear energy levels (Resonance). In analytical chemistry it is used in quality control and in research it is used in determining the content and purity of a sample as well as its molecular structure. It gives the

physical, chemical and biological properties of a compound (Ingle *et al.*, 2017). One dimensional technique is routinely used for less complicated structures, but for more complicated structures of a molecule, two dimensional NMR techniques are used. ^{13}C NMR is used to identify the number and types of carbon bonds that are present in a compound. ^{1}H NMR is used to identify the number of hydrogen molecules that are present in the compound and also to identify how the hydrogen atoms are connected. (Ingle *et al*., 2017). The main advantage of NMR is its ability to rapidly screen and identify samples. The main disadvantage of this method is that the equipment is very costly (Ashok, 2014).

Mass spectrometry (MS) is a powerful analytical technique that is known for its use in the identification of unknown compounds. It is also used for the quantification of known compounds and to elucidate structure and chemical properties of molecules. This method is able to determine the molecular weight of a compound (Ingle *et al.*, 2017). MS uses a technique that allows small molecules to be ionized by high energy electrons. Electrostatic acceleration and magnetic field fluctuations accurately measures mass to charge ratios of these ions and therefore, provide a precise molecular weight of a compound. The disadvantage of MS is that it requires a pure compound and is difficult to identify volatile compounds (Ingle *et al.*, 2017; Varghese *et al*., 2017). Liquid chromatography coupled with mass spectrometry (LC/MS) is another powerful technique that is used in the analysis of complex botanical extracts. It is important as it provides abundant information for structural elucidation of the compounds when

tandem mass spectrometry (MSn) is applied. To facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable, the combination of HPLC and MS are normally applied (Sasidharan *et al.*, 2011).

Spectrophotometry is a quantitative measurement of the reflection and transmission properties of a material as a function of a wavelength (Siddiqui, 2017). Spectrophotometry is based on natural UV absorption and chemical reactions. The advantages of these method are low time and labour consumption as well as very precise when used in the analysis of pharmaceutical dosage form. The disadvantages of this method is that spectrometers requires frequent calibration to retain accuracy and precision of the instrument, therefore choosing what type of material to be used as calibrator requires the knowledge of the type of sample being analysed (Siddiqui, 2017).

Ultraviolet-Visible spectroscopy uses UV-Vis light to measure the transmittance of the light that passes through a sample. The chemical structure of the molecule is measured as the amount of absorbance at any wavelength. UV-Vis is used in a qualitative manner to identify functional groups or to confirm the identity of a compound by matching the absorbance spectrum. It is also used in a quantitative manner by determining the concentration of the compound in relation to its absorbance. This is a very popular analytical technique because it is versatile

and is able to detect nearly every molecule. The main set back of this method is that it is not sensitive enough because not a lot of light is absorbed over a short path length (Pearson *et al*., 2002; Sasidharan *et al*., 2011).

Fourier-transform infrared spectroscopy (FTIR) is a tool used for the identification of functional groups that are present in the unknown plant extract. It helps for the identification and structure determination of a molecule (Ingle *et al*., 2017). The FTIR of pure compounds are as unique as a fingerprint and for most compounds the spectrum of an unknown compound can be identified by comparison to a library of known compounds. This technique is very versatile, highly sensitive, non-invasive and non-destructive. It can be used to analyse samples having low transmission and weak spectra and samples can either be organic, inorganic, polymers or any combination of these. The disadvantage is that they often expensive, have lower throughput, requires more sample, labour and training as well as it being difficult to automate based on the fact that data can easily be manipulated (Bekele *et al.*, 2017).

Hyphenated technique is the coupling of a separation technique and on-line identification technique. A variety of hyphenated techniques such as LC-MS, GC-MS and LC-NMR have been applied in the analysis of pharmaceuticals (Saddiqe *et al*., 2010). The availability of methods permitting the analysis of the entire plant extract is a challenge because the efficacy of the plant of interest is not based on a single constituent but the whole mixture of constituents

instead(Tatsis *et al.*, 2007). Therefore, the coupling of liquid chromatography with spectroscopic techniques like UV, NMR, and MS provides a useful way for quick data collection and structure elucidation. LC/DAD, diode array detector is effective for rapid screening of mixtures but the light absorbance data collection proves to be insufficient for structure elucidation (Tatsis *et al.*, 2007). Hyphenated LC/UV/MS instrumentation is fast and it limits the exposure of samples to light and air therefore, minimizing degradation. NMR, on the other hand, is a very useful technique for structural elucidation of organic molecules and therefore, the incorporation of LC and NMR could lead to the complete separation and structure determination of compounds. LC/NMR has also become very popular and important in the biomedical, pharmaceutical, environmental, food, and natural food analysis as well as in drug metabolites identification (Tatsis *et al.*, 2007). However one of the disadvantages of LC/NMR is that the amount of fraction that is eluted from the column is not sufficient to conduct further studies such as biological activity assays. Recent studies have documented a more hyphenated technique - SPE/NMR, which was used to analyse oregano plant extract. A solid phase extraction (SPE) unit was inserted between the LC–UV unit and NMR spectrometer so to trap the eluting compounds onto the SPE cartridges. Each one of the trapped compounds was eluted into the NMR probe with deuterated solvent (Tatsis *et al.*, 2007).

For the purpose of this study, maceration, column chromatography, TLC, HPLC, NMR, MS and IR will be employed in the extraction, isolation, purifying and characterization of active compound(s) of *Hypericum perforatum.*

2.7 Assays to determine cardiovascular effects of medicinal plants

In the drug development process, new drug entity needs to be evaluated for its efficacy against the target disease. In cardiovascular disease, preclinical evaluation for efficacy would include *in vivo* and *in vitro* studies on the effect of the drugs on the heart, vasculature and enzymes involved in homeostasis. The efficacy of new pharmaceuticals on cardiac structures are based on the reliable and accurate evaluation results of *in vitro* and *in vivo* experiments that asses the effects of the drug or extract in its physiological environment (Hanton, 2007).

The *in vivo* methods are concerned with measuring the effect of the drug on electrical or mechanical performance of the heart and cardiovascular system. The effect of the mechanical performance of the heart *in vivo* can be evaluated in terms of the blood pressure and heart rate whereas the actions on electrical properties are characterized by an electrocardiogram (EKG) (Pugsley, 2003). Some of the *in vivo* methods include: direct measurement of blood pressure in conscious rat with indwelling catheter, blood pressure measurements in the pithed rat, tail cuff methods and hemodynamic screening in anesthetized rats (Pugsley, 2003). The

measurement of blood pressure in conscious rat is restricted to two methods. The direct and indirect methods (Kamadyaapa, 2008).

Indirect methods are non-invasive methods of measuring blood pressure, the most common indirect method used in rodent studies is the use of a tail cuff device. Advantages of indirect methods is that they are less demanding technically and they are suitable for chronic studies since serious risks to animal health are minimal (Vliet *et al.*, 2001). The main limitation of this method is that it is indirect, discontinuous and requires restraint of animals. In some cases heating of the animal is used in order to enable sufficient blood flow to ensure easy measurement. But this is a problem as the combination of warming and restraint can lead to significant increases in core body temperature, and then affect blood pressure. Therefore the result of blood pressures obtained with the tail-cuff method may not only reflect the animal blood pressure, but also the reactivity of blood pressure to the stress of the procedure (Vliet *et al.*, 2001). Due to these limitations, the direct method is use to verify blood pressure measurement results from the tail-cuff method (Kamadyaapa. ,2008).

Direct methods use techniques by which arterial blood pressure is measured directly with the aid of a sensor device (catheter) that is implanted invasively within a suitable artery. The sensor is connected to a calibrated pressure transducer for blood pressure measurement (Vliet *et al.*, 2001). This is the most accurate method of blood pressure measurement. The major advantages

of this method are: the cost of materials needed for the procedure are inexpensive and precise calibration is easier as the whole procedure can be performed under normal physiological conditions without involving the warming and restraining of the animals. This procedure allows for continuous long-term blood pressure recordings under conditions of relatively low stress. Major disadvantages is that the catheter implantation surgery and anaesthesia causes disturbance to the animal, its blood pressure and heart rate (Vliet *et al.*, 2001). Another limitation is based on the fact that if the procedure is not done properly it can create potentials for infection and potential for loss of catheter patency. This may lead to a degradation or loss of blood pressure signal resulting in limited dynamic response, which makes detection of the true systolic and diastolic pressures challenging in small animals with high heart rates (Vliet *et al.*, 2001).

Many types of *in vitro* cardiac preparations are used to study cardiac function and the electrophysiological effects of drugs on myocardial cells. Methods include: isolated cardiac tissue preparation, single myocardial cells, the Langendorff isolated rat heart, heterologous expression system, inhibition of ACE inhibitors *in vitro*, and renin inhibitory activity using human kidney renin and a synthetic substrate (Pugsley, 2003).

Perfused organ preparations such as the modified Langendorff technique and the working heart preparation have been used to assess integrative myocardial function *in vitro* upon exposure to

different drugs and toxins (Partridge, Johnson and Ramos, 2005). The isolated heart model such as the Langendorff heart makes it possible for a broad range of physiological and pharmacological studies to be done, as well as removing false effects of other organ systems. The working preparation is perfused through the left atrium to generate a left-sided working preparation. The perfusion fluid reaching the ventricle is ejected via the aorta into a chamber against hydrostatic pressure to mimic physiological resistance to flow (Partridge, Johnson and Ramos, 2005) . The advantage of perfused preparation in toxicological studies is that the level of structural organization is similar to that encountered *in vivo,* and changes in physiological or pharmacological sensitivity, excitability and contractility can be easily be monitored. The most major limitations of perfused preparations are the small number of duplicate preparations that can be handled at any one time, and the time available for isolation and placement of the tissue under physiological conditions is short (Partridge, Johnson and Ramos, 2005).

Isolated muscle preparations include isolated atrial or ventricular tissue, papillary muscle and isolated coronary right ventricular wall. The small size of the cardiac tissue allows for the rapid and continuous diffusion of oxygen and nutrient to subcellular layers resulting in viable stable preparation. Advantage of this approach provides more to the study of cardiac drugs on the mechanical, electrical and biochemical properties of the heart. It is also a sensitive indicator of chronotropic and inotropic action on the heart. (Pugsley, 2003).

Drug discovery studies on anti-hypertensive drugs have developed rapidly since the discovery of ACE. The ACE inhibitory activity has become an effective screening method in the search for new anti-hypertensive drug from herbal plants. The activity of ACE inhibition by *in vitro* methods have become an effective assay method in identification of active anti-hypertensive molecule (Ahmad *et al*., 2017). The *in vitro* assay methods used to determine the activity of ACE inhibitors are based on the substrate usage by the ACE enzyme. These assays includes: Cushman and Cheung Method which uses a substrate hippuryl-histidyl-leucine (HHL); Holmquist method uses furanacryloyl-tripeptide as a substrate; Elbl and Wagner method uses a substrate benzoil-[l-14C] glicyl-L-histidine-L-leucine; Carmel and Yaron method uses o-aminobenzoylglycyl-p-nitrophenylalanilproline as a substrate; and Lam method uses 3-hydroxybutyrylglycyl-glycyl-glycine as substrate. There are several different methods that are used to measure the results of enzymatic reactions or separation of the substrate with products. These methods includes spectrophotometric, fluorometric, high-performance liquid chromatography, electrophoresis, and radiochemistry. The application of these methods for screening the ACE inhibitors activity and identification of active compounds from natural products provides results that are simple, accurate and rapid. The most common ACE inhibitory activity tests is the Cushman and Cheung method and Elbl and Wagner methods (Ahmad *et al*., 2017).

Cushman and Cheung assay method measures the activity of ACE inhibitors by using hippuryl-histidyl-leucine (HHL) subtract. In the presence of the ACE, the HHL is hydrolysed into Hippuric acid (HA) which can be measured at a wavelength of 228 nm using an ultraviolet-visible (UV-Vis) spectrophotometer instrument. When there is ACE inhibition, the concentration of HA formed will be reduced. This reaction is visualized by the means of fluorescence which occurs when o-Phthaldialdehyde (OPA) is added to the assay solution. OPA reacts with the cleaved substrate and is measured by a fluoro-colorimeter instrument at a wavelength of 495 nm emission and 365 nm excitation. Elbl and Wagner assay uses the chromophore- and fluorophore-labeled tripeptide dansyl triglycine as substrate. The substrate is cleaved by the ACE into dansylglycine and diglycine and this is separated by RP-HPLC. These techniques have limitations, such as being very laborious and having low sensitivity in terms of determining if inhibition is due to any external factors (Ahmad *et al*., 2017).

2.8 The renin-angiotensin-aldosterone system in cardiovascular disease

Angiotensinogen is converted to angiotensin I by renin after it has been released from the liver into the circulation. Renin is a proteolytic enzyme which synthesized, stored and released largely from the juxtaglomerular apparatus in the kidneys when there is a decrease in blood pressure, low sodium concentration and increased sympathetic activity. Angiotensin 1 has a

mild vasoconstrictor effect, but that's not enough to achieve a significant physiological effect. Angiotensin I is converted to angiotensin II by angiotensin converting enzyme as well as other enzymes such as chymase, cathepsin, and tonin. Angiotensin II inhibits renin through the process of negative feedback, thereby acting in a self-regulatory manner. Angiotensin II is a multi-functional peptide that acts on different tissues; in the adrenal gland it stimulates the release of aldosterone and causes the constriction of renal arterioles, consequently, increasing salt and water retention in the kidneys. It is involved in the regulation of salt and fluid homeostasis in the brain by influencing the autonomic nervous system, vasopressin release. In the blood vessels, it acts as a powerful vasoconstrictor as well as remodelling the vascular wall through stimulation of smooth muscle cell growth, up-regulation of growth factors and by affecting the synthesis of extracellular matrix proteins. In the heart, it has inotropic and hypertrophic effects and promotes cardiac fibrosis. Also, it is involved in inflammation by enhancing up regulation of endothelial adhesion molecules, pro-inflammatory cytokines and production of reactive oxygen species (Ljungberg, 2011).

2.9 Angiotensin converting enzyme and its inhibitors

ACE is a glycoprotein with peptidyl dipeptide hydrolase that breaks up angiotensin I to produce angiotensin II in the blood. The vasoconstrictive action of angiotensin II, its simulation of the synthesis and release of aldosterone is so powerful that it has a consequent increase in arterial blood pressure. ACE also hydrolyses and inactivates a peptide bradykinin which has

vasodilatory actions (Afonso *et al.*, 2013a). ACE inhibitors work by inhibiting the conversion of angiotensin 1 to the potent vasoconstrictor angiotensin II, therefore improving blood flow and blood pressure. Angiotensin II is a hormone that circulates in the bloodstream which causes constriction of the blood vessels and increases the work required by the heart to pump blood into the body's main arteries. It also acts as growth hormone that increases the size or thickness of many cardiovascular structures, hence high levels of circulating angiotensin II eventually leads to a condition known as hypertrophy characterised by the thickening and stiffening of the heart muscles (Sweitzer, 2003). This can be a problem for the heart muscle if it has already been weakened by a heart attack or heart failure. In many cardiovascular diseases, angiotensin II is present in abnormally high quantities. Therefore, ACEIs' are prescribed to reverse the hypertrophy of the heart and vessel walls (Sweitzer, 2003). The use of synthetic modern ACE inhibitors such as captopril, enalapril and lisinopril provide definitive positive health effects and is considered as an important therapeutic approach in the treatment of high blood pressure (Simaratanamongkol *et al.*, 2014; Vijayan, Chitra and Penislusshiyan, 2018). ACEIs has three distinct chemical structures namely: sulfhydryl containing agents (captopril), the non-sulfhydryl containing agents 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. ACEIs are still considered effective and safe for the treatment of hypertension as their anti-hypertensive effect are enhanced through low salt diet and their pharmacological actions including vasodilation,

increased sodium excretion, diuresis and lowering blood pressure. ACEIs are well tolerated and has a high safety profile, although they are not safe in pregnancy.(Duncan A C, 1998).

Certain herbal preparations have been found to inhibit ACE, and the evidence that certain flavonoid-rich natural products can induce reductions in blood pressure and inhibit ACE activity opens the possibility that their consumption may mimic synthetic ACE inhibitors and provide preventive health benefits while avoiding the associated adverse effects. (Afonso *et al.*, 2013a). Tannins are polyphenol plant compounds that precipitate proteins and interfere with the activities of ACE macromolecules. Any plant extract with ACE inhibition ≥50% needs to be tested for the presence of tannins so to eliminate false positive results. (Afonso *et al.*, 2013b).

2.10 Bioassay-guided isolation

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 (Sowers, Epstein and Frohlich, 2001).

Bioassays are divided into two methods namely: diffusion and dilution methods. In diffusion methods, the active compounds have to diffuse into a medium containing the target organism whereas, in dilution methods, the metabolites and the target organism dissolve in the medium. The disadvantage of diffusion bioassays is that the medium may interfere with inhibitory action of the active compounds thereby giving false results, whereas with dilution bioassays, finding solvents in which the active compound is soluble in might be difficult (Panuganti, 2015).

Isolation of biologically active metabolites from plant materials is achieved following the steps of sample preparation, separation, isolation and enrichment, accompanied by a suitable bioassay. In this study, extracts of *Hypericum perforatum* and fractions obtained from chromatography were screened to determine the compound with ACE inhibition activity using an *in vitro* fluorometric ACE inhibition assay-guided fractionation. HPLC was employed for purification, and NMR, IR and MS were used for identification and structural elucidation of the isolated bioactive compound.

CHAPTER 3

MATERIALS AND METHODS

This chapter describes the techniques used in the extraction, isolation and purification of the active compound from *Hypericum perforatum* crude extract. It also describes in detail the assay used in evaluating the ACE inhibitory activity of the extract and the compound isolated from *Hypericum perforatum*. The methodology used for structural elucidation and identification of the pure active compound by NMR, FTIR and MS are also discussed below.

3.1 Material

Chemicals and consumables

Dried and ground *Hypericum perforatum* was purchased from Warren Chem Specialities PTY LTD (Cape Town, South Africa) and authenticated and certificate of analysis was provided by DIXA AG Company and deposited to the University of the Western Cape, School of Pharmacy lab where it was stored in an air tight container until needed. See appendix VI for certificate of analysis.

The solvents (ethanol, ethyl-acetate, hexane, and methanol) used in the extraction and isolation process were purchased from KIMIX chemical and Laboratory suppliers (Cape Town, South Africa).

ACE enzyme, O-phthaldialdehyde (OPA), L-hippuryl-L-histidyl-L-leucine (L-hip-Lhis-L-lue) (HHL), Boric acid, sodium chloride, enalapril, and captopril used in the bioassay were all purchased from Sigma Aldrich (Cape Town, South Africa).

Equipment

The TLC tank, TLC aluminium sheets (20 cm x 20 cm; F_{254}), Whatman filter paper and silica gel (60 mesh) were purchased from Sigma Aldrich (Cape Town, South Africa). Evaporation of excess organic solvent was carried out using a Buchi Rotavapor R-200, Buchi heating bath B-400, Buchi Vac V-500 (all Buchi Labor Technik, Flawil, Switzerland). Aqueous samples were frozen using a minus -86 ultra-freeze (NuAire, Plymouth, USA.), and freeze-dried using a Sentry 2.0 freeze dryer (Virtis SP scientific, Pennsylvania, USA). Fluorometric visualization of the ACEI bioassay was carried out using a GBC CINTRA 2020 UV-Vis spectrophotometer (Hampshire, USA). HPLC separation was carried out on an Agilent HPLC system (Bruker, Germany), and NMR experiments were run on a Bruker Avance 400 MHz spectrometer (Bruker, Rheinstetten, Germany). IR spectrum was acquired using a Perkin Elmer spectrum 400 FT-IR/FT-NIR spectrometer (Perkin Elmer Inc. Waltham, USA) and Mass spectroscopic analysis was carried out on Leco Pegasus HRT, GC×GC-high resolution MS (LECO cooperation, St Joseph, USA).

3.2. Plant preparation and extraction

To prepare organic and aqueous extracts of the plant, 1 kg of finely grounded powder of *Hypericum perforatum* was macerated in 1.5 L of ethanol (99.9%) and distilled water respectively for 48 hours with frequent agitation. Both extracts were filtered using Whatman No.1 filter paper and the solvent removed under vacuum by rotary evaporator at 40^{o}C (ethanol extract) or freeze-dried (aqueous extract) to yield ethanol and aqueous extracts, respectively. The extracts were weighed 245.29 g w/w for ethanol extract and 24.96 g w/w (~25% yield for ethanol extract and 2% for aqueous extract) and stored at 4^{o}C in air-tight containers until required.

Both ethanol and aqueous extracts were tested for ACEI activity using the ACEI assay as described by Cushman and Cheung (1971) with some modifications (see section 3.6). The extract that exhibited the active greater inhibition of ACE was used further for the purpose of this study.

3.3 Fractionation of ethanol extract

3.3.1 Column chromatography parameters

A 1.2 meter, 5 cm diameter glass column was used in the fractionation of extracts. The stationary phase comprised of 100 g silica gel (70-230 mesh) tightly packed into the column,

while the mobile phase comprised of a mixture of ethyl acetate and hexane , using a gradient elution method running from non-polar to polar solvent (see table 3.1).

TABLE 3. 1 COLUMN GRADIENT ELUTION PARAMETER

Fraction	Volume	Solvent
1	500 ml	Hexane (100%)
2	500 ml	Hexane: Ethyl acetate (80%:20%)
3	500 ml	Hexane: Ethyl acetate (60%:40%)
4	500 ml	Hexane: Ethyl acetate (40%:60%)
5	500 ml	Hexane: Ethyl acetate (20%:60%)
6	500 ml	Methanol: Ethyl acetate (50%: 50%)
7	500 ml	Methanol (100%)

3.3.2 Column fractionation

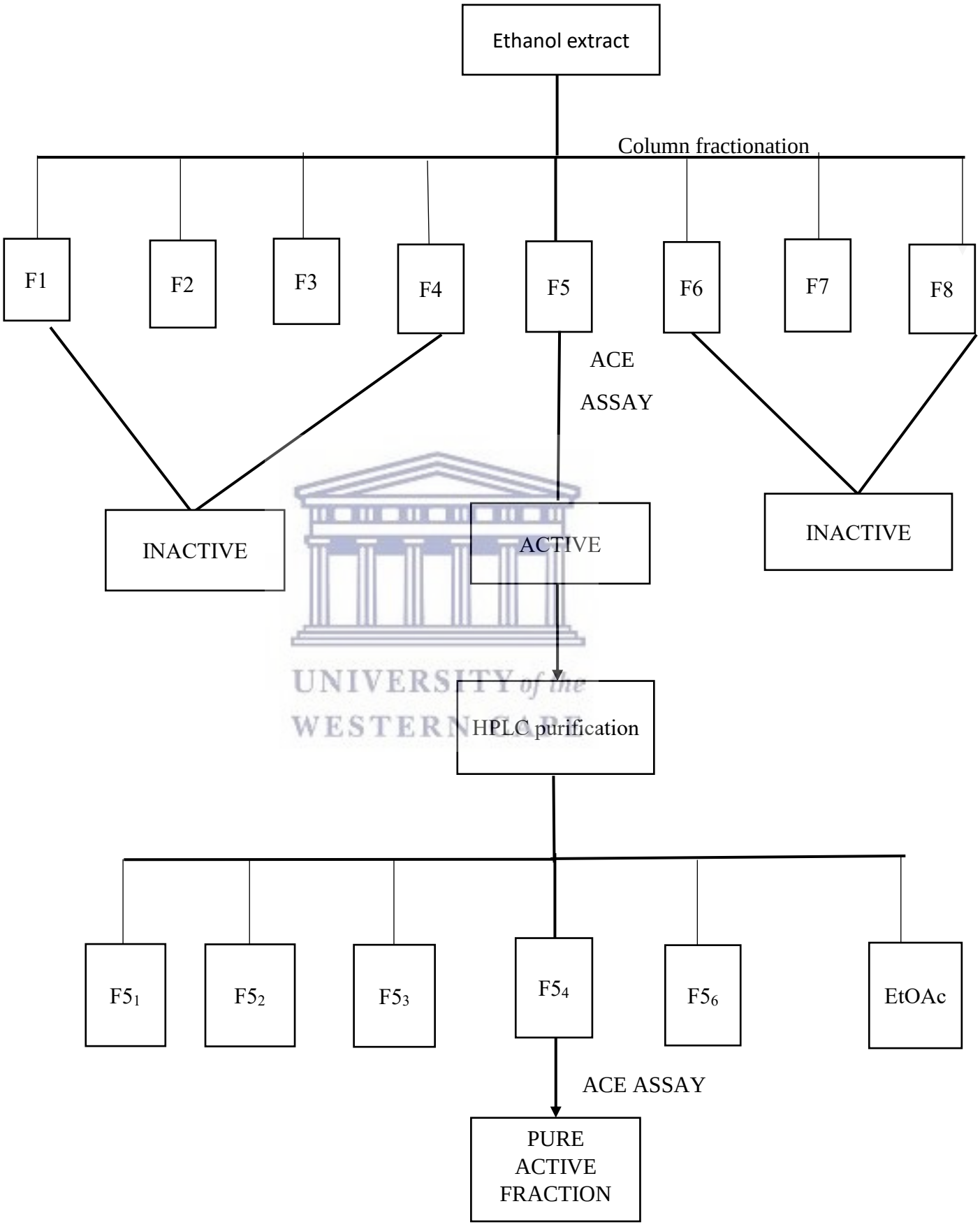
Hypericum perforatum ethanol extract (10 g) was dissolved in 25 ml of ethanol, the solution adsorbed on a small amount of silica gel (35-60 mesh) to make a slurry and excess solvent

evaporated using a rotary evaporator to yield ethanol extract granules. The granules were applied on the top of the stationary phase bed, covered with absorbent cotton to avoid disturbance in the sample and eluting solvent run on a gravity feed using the protocol stated in table 3.1. Collected fractions were spotted on TLC plates to visualize the compounds contained in each fraction. Fractions containing similar profile of compounds were combined, the excess solvent removed and the fractions tested for bioactivity using the ACEI bioassay (see section 3.6). Figure 3.1 shows systemic flow of how fractions were obtained.

3.4 Purification of active fraction

The HPLC separation was performed using an Agilent standard cell 1260 infinity HPLC system. (Agilent technologies, Germany), coupled with variable UV wavelength detection at 254 nm and refractive index detector (RID). The partially pure active compound (F5) (100 mg) eluted from column fractionation was subjected to normal phase preparative HPLC for purification. In this method, ethyl acetate (80%) and hexane (20%) were used as mobile phase at a flow rate of 3 ml/min, and injection volume of 200 µl for a total time of 35 minutes for each injection. All analyses were carried out at a temperature of 30^{o}C and bar pressure of 50.73 psi.

FIGURE 3. 1: ISOLATION PROCESS FROM ETHANOL EXTRACT OF HYPERICUM PERFOATUM



3.5 Structural determination of bioactive compounds

3.5.1 NMR spectroscopy

NMR spectrometer was equipped with a 5 mm BBO probe at 298 K using standard 1D and 2D NMR pulse sequences. All spectra were referenced to residual undeuterated solvent peaks (Ahmed *et al.*, 2004). 1D NMR including both ^{1}H NMR and ^{13}C NMR experiments were used to locate atom positions and fragment units. 2D NMR including COSY, HSQC, and HMBC was carried out to obtain a more accurate assignment of proton and carbon chemical shift. About 5 mg of purified compound was dissolved in 0, 5 ml of deuterated chloroform ($CDCl_3$). Signals were recorded in chemical shifts δ and expressed in parts per million (ppm).

3.5.2 Fourier Transform infrared (FTIR) spectroscopy

Fourier Transform infrared spectroscopy spectra were recorded for *Hypericum perforatum* active pure compound on Perkin Elmer spectrum 400 FT-IR/FT-NIR spectrometer version 6.3.50176 software. A small amount of properly triturated sample was placed on the crystal of a PerkinElmer FTIR Spectrometer (Spectrum 400, PerkinElmer, USA). Pressure was applied gradually using the pressure gauge between 50 and 60%. A resolution of 4 cm^{-1} and a scanning range of 500 to 4000 cm^{-1} were used. Resulting peaks were compared to published data of functional groups.

3.5.3 Gas chromatography- mass spectroscopy (GC-MS)

The pure active extract was subjected to GC-MS analysis. The GC-MS spectroscopic study was carried out using Leco Pegasus HRT, GC×GC-high resolution MS. A 1D: 30 m × 0.25 mm, 0.25 μm df Rxi-5MS (Restek) column was used. The carrier gas was helium at a constant flow rate of 1 mL/min. The injector temperature was set at 40°C for 2 minutes, then increased by 20°C/min to 220°C and held for 15 min. Injections of 1 μl were made in a split mode with a split ratio of (1:20). The mass spectrometer was operated in the electron ionization mode of (70eV); HR TOF-MS, m/z 5-520, 13 Hz.

3.6 ACE inhibitory assay

Inhibition of ACE activity by the fractions and isolated compound was assessed according the method of Cushman and Cheung (1971), modified by Liberman (1975). In this method, L-hippuryl-L-histidyl-L-leucine (L-hip-Lhis-L-lue) (HHL) acts as the enzyme substrate and enzyme inhibition is measured as the reduction in fluorescent produced when the cleaved portion of HHL (histidyl-leucine) binds to O-phthaldialdehyde (OPA). The following steps describe ACE inhibitory assay procedure that was used in this study.

3.6.1 Angiotensin converting enzyme inhibitory assay

This assay was carried out in a 25 µl incubation mixture containing 4 µl of inhibitor solution (plant extract/fractions/purified compound) at different concentrations, 15.4 µl sodium borate buffer containing a mixture of 0.4M Boric acid and 0.3M sodium chloride (pH 8.3) and 5.6 µl of the enzyme, pre-incubated for 20 minutes. . The blank sample was prepared by replacing the inhibitor solution with the sodium borate buffer. The control sample was prepared by replacing the inhibitor with captopril. The reaction was then initiated by the addition of 10 µl of 116.4 mM hippuryl histidyl-leucine (HHL) substrate and the reaction mixture was incubated at 37ºC for 60 minutes. The reaction was then terminated by adding 150µl of 0.34M NaOH, and then 20 µl of 20 mg/ml OPA added to the reaction mixture and incubated for 10 minutes. The reaction was stopped after incubation by adding 50µl of 3M HCl. Black 96 well-plates containing the various inhibitor concentrations in the reaction mixture was introduced to the fluorescent plate reader (make) with parameters set at 355 nm excitation and 535 nm emission frequency. The ACE activity or inhibition of ACE activity was obtained by fluorometric determination of the amount of histidyl-leucine (His-Leu) cleaved by the ACE enzyme of which was made visible with o-Phthaldialdehyde (OPA) through the use of UV-VIS spectrophotometer. The percentage inhibition of the sample was calculated using the following equation

$$\%perecentage\ inhibition = \frac{\text{fluroscent enzyme control} - \text{fluroscent sample}}{\text{fluroscent enzyme control} - \text{fluroscent blank}} X\ 100$$

CHAPTER 4

RESULTS AND DISCUSSION

Due to the contribution of hypertension to various cardiovascular diseases, many studies are currently focused on identifying efficient bioactive compounds with antihypertensive activity and thus reducing the levels of cardiovascular disease. ACE inhibitors are an important component to the therapeutic regimen for treating hypertension, but due to the increase in the prevalence of side effects of synthetic compounds, alternative and complementary medicines which may consist of pure bioactive compound or a combination of various compounds from natural sources are gaining importance in overcoming hypertension. *Hypericum perforatum* has been studied for various activities including anti-bacterial, anti-depressant, anti-oxidant properties, but studies on its cardiovascular effects specifically ACE inhibitory activity have not yet been explored. In this study, ACEI assay-guided fractionation of the ethanol extract of *Hypericum perforatum* was carried in other to isolate a compound with ACE inhibition. A compound isolated from an active fraction of the plant extract was tested for ACE inhibition and its chemical structure elucidated. The results of the structure elucidation of the compound isolated from *Hypericum perforatum* as well as the ACE inhibition experiments is presented in this chapter.

4.1.0 Structure Elucidation of the compounded isolated

The ethanol crude extract of *Hypericum perforatum* was subjected to column fractionation, and re-fractionation guided by ACE inhibition activity of the fractions. Fraction (F5) was assumed to contain the main ACE inhibiting compound because it showed higher percentage of ACE inhibition. Therefore, fraction (F5) was subjected to normal phase HPLC purification, and the pure active fraction - $F5_4$ identified using NMR spectroscopic techniques and literature data. The pure compound was isolated as a colourless oil and a final weight of 10 mg of pure compound was recovered from 100 mg of ethanol extract (F5) and its percentage yield was calculated to be 0.001% (w/w). The low percentage yield could be due practical loss of material during the re-fractionation process. The structure of compound were determined using ^{1}H and ^{13}NMR, DEPT, HSQC, HMBC and by comparison of data from literature. The pure active compound was previously reported as a hydroxylactone, and the molecular formula $C_6H_{10}O_3$ was confirmed by C-NMR, DEPT and GC-MS analysis and the structure is presented in figure 4.6. The isolated hydroxylactone was identified as 3-hydroxy 4, 4 dimethyl-4-butyrolactone and this is the first time it has been isolated from *Hypericum perforatum.*

4.1.1 NMR

The ^{1}H-NMR spectrum of compound 1 showed typical signals of two methyl groups: two singlets at δ 1.48 (H-5) and 1.41 (H-6) and their carbon signals at δ 21.1 (q) and 26.1 (q),

respectively. The downfield proton signal which appeared as a doublet of doublets at d 4.20 (J = 3.5, 6.5 Hz) correlated with an oxygenated carbon signal at δ 73.6 in the HSQC spectrum. The limited quantity of 1 gave only poor ^{13}C and DEPT-135 NMR spectra; therefore ^{13}C NMR chemical shifts were obtained from edited-HSQC and HMBC spectra. The presence of downfield quaternary carbon signal at d 87.7 in the ^{13}C-NMR suggested the presence of a second oxygenated carbon. ^{13}C NMR chemical shifts at 173 ppm indicated the presence of a carbonyl moiety. The assignment of all proton signals and their connectivity to carbons was established from the results of the 1D and 2D NMR, COSY, HSQC and the long range coupling HMBC experiments. Data from the ^{1}H NMR and ^{13}C NMR are summarized below. Full set NMR spectra are presented below and also in appendix I-III.

TABLE 4. 1:^{1}H NMR (400 MHz) AND ^{13}C NMR (100 MHz) DATA FOR PURE COMPOUND IN $CDCl_3$ (Δ IN PPM AND J IN HZ) AND BY COMPARISON OF DATA WITH LITERATURE ((Ahmed *et al.*, 2004)

Atom no	δ_H	δ_{C*}	Type	HMBC to	Literature values (δH) (Ahmed *et al.*, 2004)	Literature values (δC) (Ahmed *et al.*, 2004)
1	-	173.0	C			174.2
2a	2.55, dd, J = 17.9, 3.3	38.1	CH2	C-1	2.52	38.2
2b	2.95, dd, J = 17.9, 6.6	38.1		C-1	2.91	38.2
3	4.21, J = 6.3, 3.3,	73.6	CH	C-2	4.20	73.8
4	-	87.7	C			87.6
5	1.41, s	26.1	CH3	C-3, C-4, C-6	1.38	21.0
6	1.43, s	21.1	CH3	C-3, C-4, C-6,	1.44	26.1
*** ^{13}C NMR chemical shifts were obtained from edited-HSQC and HMBC NMR spectra**						

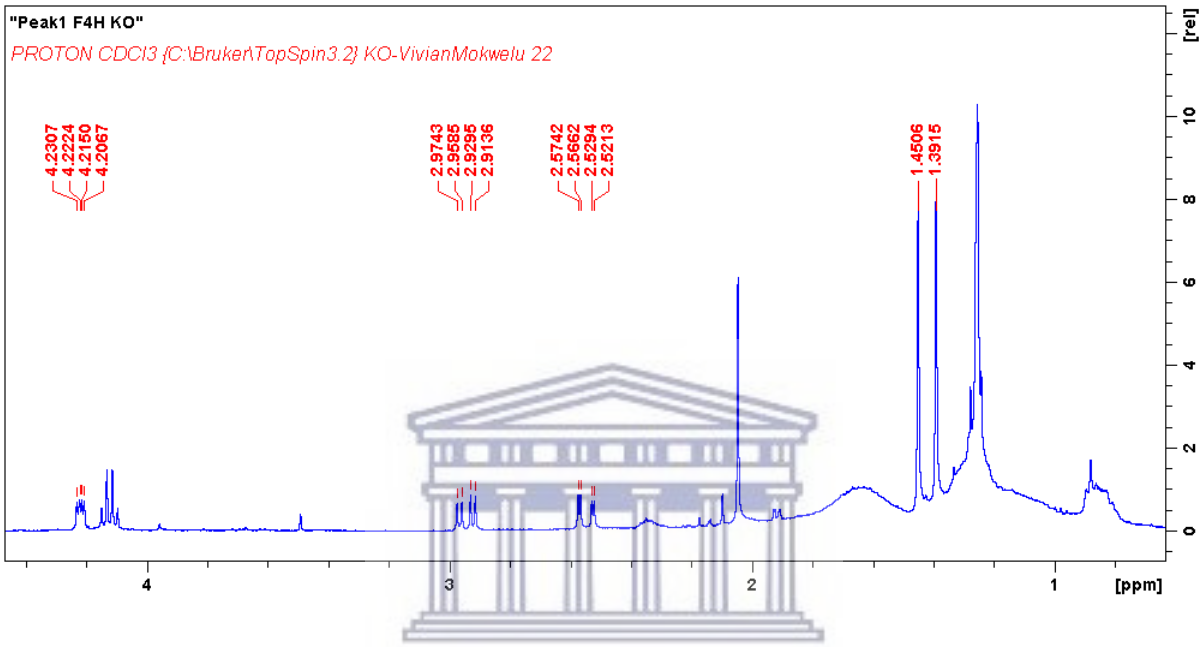


FIGURE 4. 1 : ^{1}H SPECTRUM OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

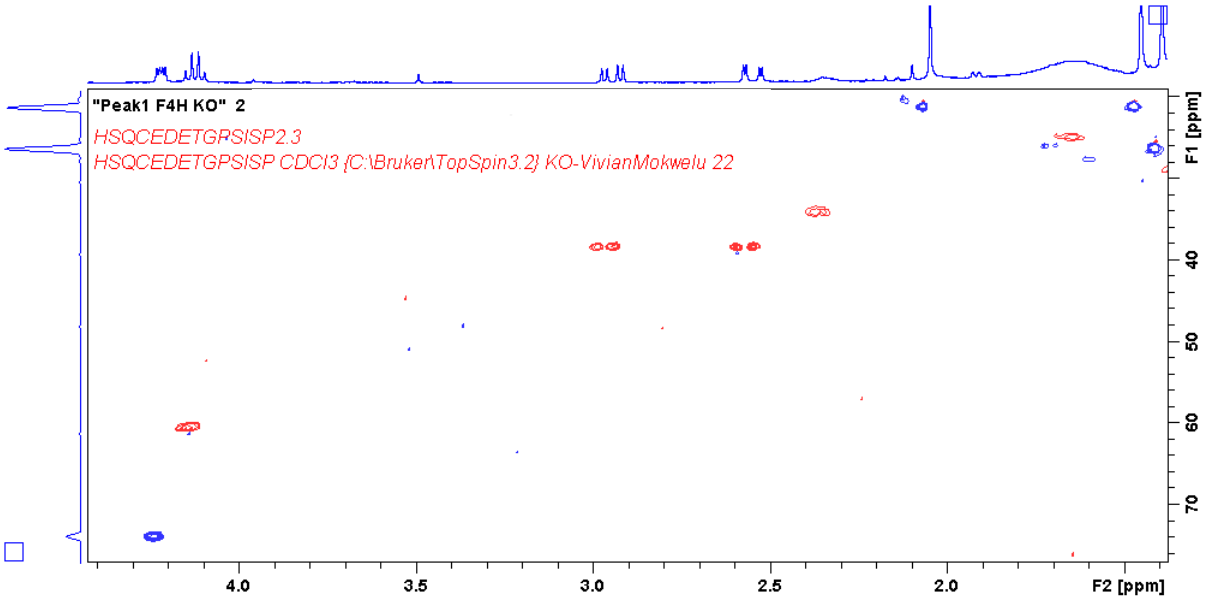
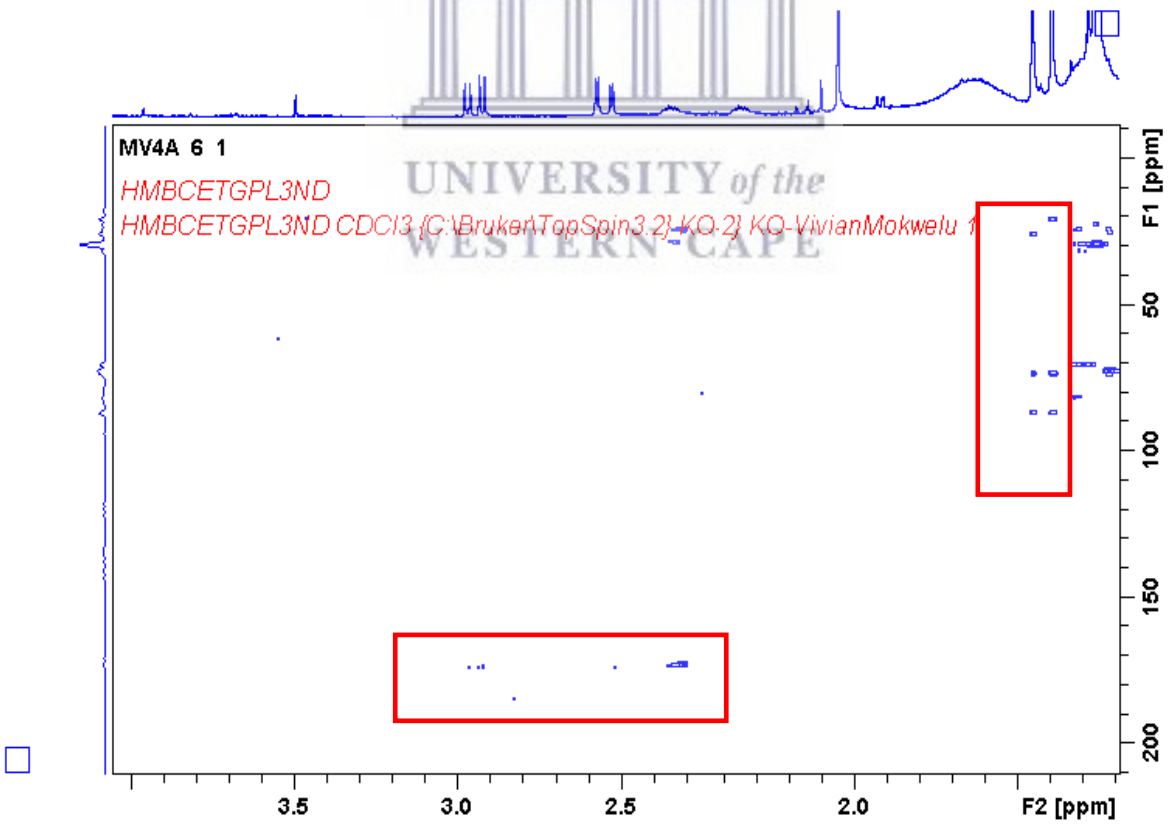


FIGURE 4. 2: HSQC SPECTRUM OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE



FIGUR E 4. 3 : HMBC SPECTRUM OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

4.1.2 FTIR

The IR spectroscopy was used to confirm any functional groups present in the isolated compound and the spectra were recorded with Perkin Elmer spectrum 400 FT-IR/FT-NIR spectrometer version 6.3.50176. The infrared spectrum of the isolated compound indicated strong bands at 3408.44 (O-H), 2926.03 (C-H), and 1748.86 (C=O) cm^{-1}

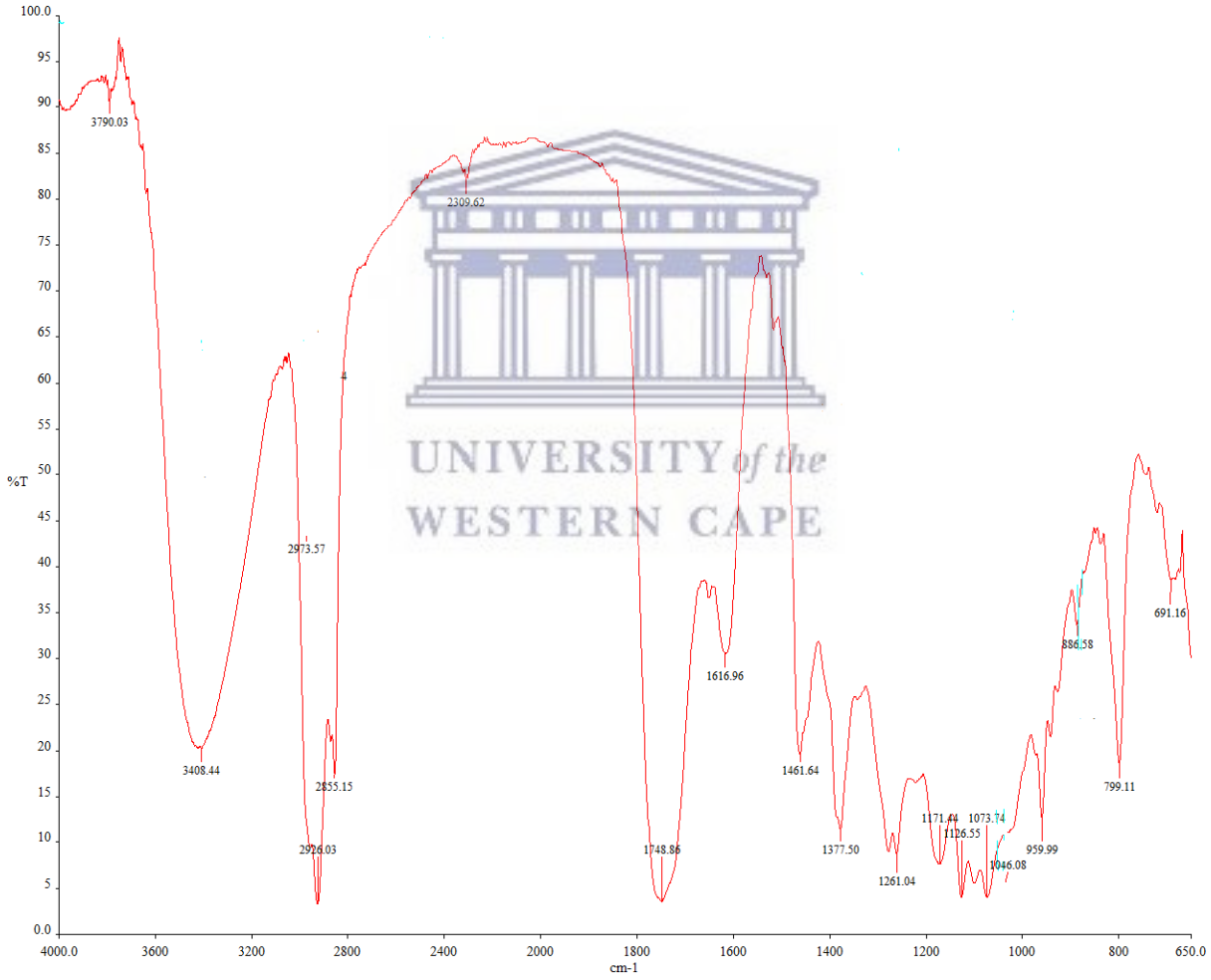


FIGURE 4. 4: FTIR SPECTRUM OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

4.1.3 MS

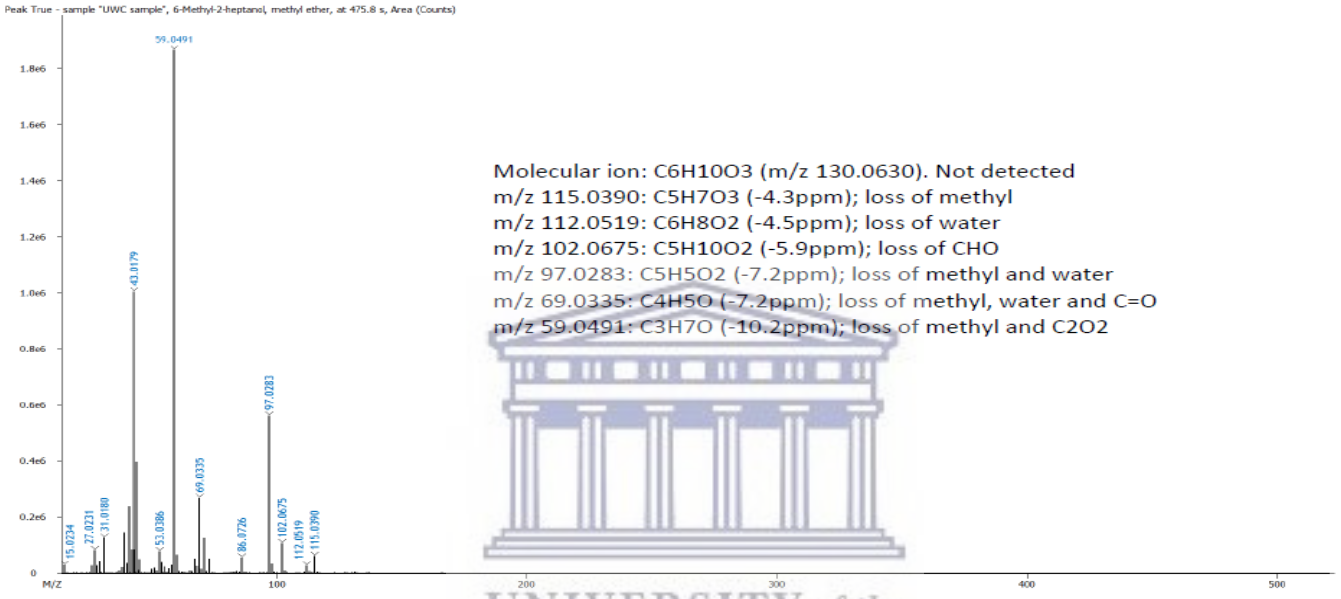
GC-MS was used to confirm the molecular formula of the compound as predicted by NMR and IR data. The GC-MS experiments could not observe the molecular ion (m/z 130) of the compound however, a peak corresponding to the loss of H2O from the parent molecule (m/z 112) was observed. Loss of H2O from secondary alcohols with alpha protons are quite common. Computing the molecular weight from the molecular weight of the fragment ions resulted in a molecular formula - $C_6H_{10}O_3$ and a molecular weight - 130, which was consistent with the proposed structure of the compound as predicted by NMR

CAF Central Analytical Facilities
STELLENBOSCH UNIVERSITY

Requested: Onyinye Vivian Mokwelu
Report No: ST_UWC__HRMS_190121
Instrument: Leco Pegasus HRT, GC×GC-high resolution MS

Experimental conditions

Separation mode:	1D GC
Column(s):	^{1}D: 30 m × 0.25 mm, 0.25 µm d_f Rxi-5MS (Restek)
Temperature programme:	40°C (2 min), 20°C/min to 220°C (15 min)
Carrier gas:	Helium, 1 mL/min (constant flow)
Injection:	Liquid injection, 1 µL split (1:20)
Detection:	EI ionization (70eV); HRT TOF-MS, m/z 5-520, 13 Hz; mass accuracy 0.32 ppm (m/z 218.9)



Molecular ion: C6H10O3 (m/z 130.0630). Not detected
m/z 115.0390: C5H7O3 (-4.3ppm); loss of methyl
m/z 112.0519: C6H8O2 (-4.5ppm); loss of water
m/z 102.0675: C5H10O2 (-5.9ppm); loss of CHO
m/z 97.0283: C5H5O2 (-7.2ppm); loss of methyl and water
m/z 69.0335: C4H5O (-7.2ppm); loss of methyl, water and C=O
m/z 59.0491: C3H7O (-10.2ppm); loss of methyl and C2O2

GC–HRT Unit
De Beers building, Private Bag X1, Matieland, 7602, South Africa
Tel: +27 21 808 3351 Fax: +27 21 808 3360 ajdevill@sun.ac.za www.sun.ac.za/caf

FIGURE 4. 5: GC-MS DATA OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

A thorough search of literature and database including SciFinder®, ChEMBL®, chemsearch®, Molbase® and Pubchem® of chemical structures indicated that this is the first time this compound has been isolated from *Hypericum perforatum.* The isolated pure compound was identified and confirmed by the NMR spectra in literature to be 3 hydroxy 4, 4-dimethyl-4-butyrolactone, with a molecular weight of 130 and molecular formula of $C_6H_{10}O_3$. (figure 4.6) (Ahmed *et al.*, 2004). The compound belongs to a class of organic compounds known as lactones, and contains a gamma butyrolactone moiety which consists of an aliphatic five member ring with four carbon atoms, one oxygen atom and a ketone group on the carbon adjacent to the oxygen atom. The oxygen atom is double bonded to a carbon atom. The carbon atoms are attached to another oxygen atom that forms part of a closed ring (fig 4.6).

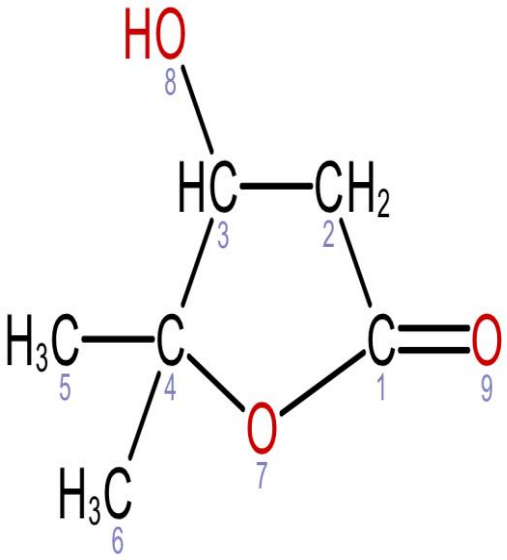


FIGURE 4. 6: 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

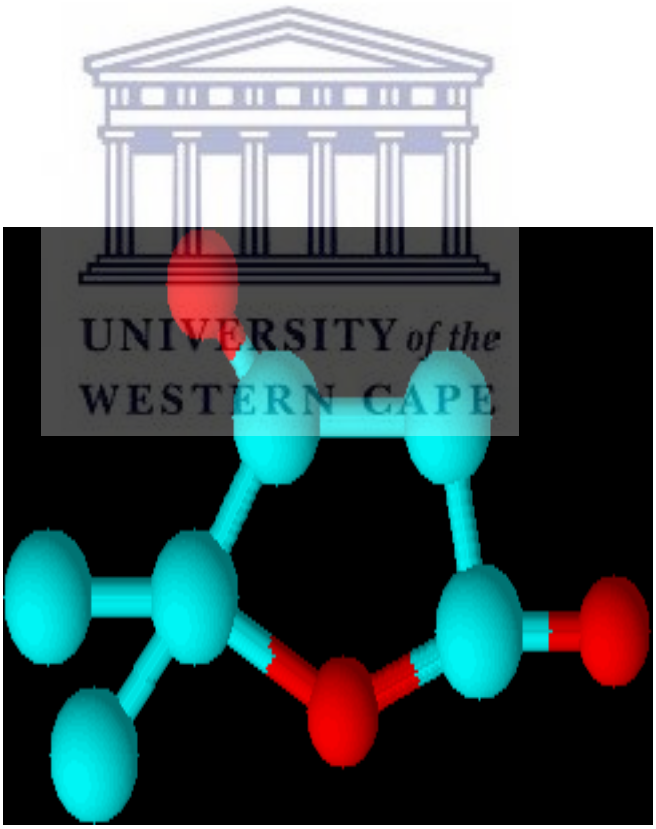


FIGURE 4. 7: 3D REPRESENTATION OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

4.2 *In vitro* bioassay-guided isolation

Both aqueous and ethanol crude extracts of *Hypericum perforatum* were first tested for ACE inhibition as various concentrations (200 mg/ml, 100 mg/ml, 10 mg/ml and 1 mg/ml) using a modified version of the method as described by Cushman and Cheung (1971) (see section 3.6 of chapter 3). Dose-dependent ACE-inhibition was observed with both extracts, with the highest ACE-inhibition observed with the highest concentration (200 mg/ml) of both extracts (99.53% with the ethanol extract and 98.83% with the aqueous extract). Enzyme inhibition observed with the highest concentrations of both extracts was comparable to that obtained with the standard drug captopril (98.58% inhibition with a 1 mg/ml concentration) (see table 4.2). Both ethanol and aqueous extract showed good inhibitory activity, with a slightly greater activity from the organic extract.

TABLE 4. 2: ACEI BIOASSAY ACTIVITY OF THE ORGANIC AND AQUEOUS EXTRACTS OF HYPERICUM *PERFORATUM*

Concentration	1 mg/ml	10 mg/ml	100 mg/ml	200 mg/ml
Ethanol crude extract (%inhibition)	87.11%	97.09%	98.31%	99.53%
Aqueous crude extract (% inhibition)	86.46%	96.15%	97.64%	98.83%
Captopril (% inhibition)	98.58%			

.

Due to this greater ACE-inhibitory and a greater yield (25% yield for organic extract and 2% yield for aqueous extract), the organic extract was selected for further bioassay guided fractionation according to the method described in section 3.3 of chapter 3.

The ACE inhibitory activity of each fraction (1 mg/ml) from the gradient elution of the organic extract was tested for angiotensin converting enzyme inhibition using the bioactivity assay (see section 3.6; Chapter 3). Fraction F5 that eluted with ethyl acetate: hexane (80:20) mobile phase produced the highest inhibition of enzyme activity with a percentage inhibition of 70.03% at a concentration of 1mg/ml, with all other fractions producing enzyme inhibition below 45% (Table 4.3).

TABLE 4. 3: ACE BIOASSAY ACTIVITY OF FRACTIONS (1MG/ML) FROM GRADIENT ELUTION OF ETHANOL EXTRACT OF *HYPERICUM PERFORATUM*.

FRACTIONS	F2 (H:E) 80:20	F3 (H:E) 60:40	F4 (E:H) 60:40	F5 (E:H) 80:20	F6 100% EtOAc	F7 (M:E) 1:1	F8 100% CH_3OH
% INHIBITION	3.5%	4.4%	32.83%	70.03%	16.76%	44.70%	7.3%

The most active fraction (F5) was then assumed to contain the main ACE inhibiting compound, and was subjected to further fractionation using HPLC (see section 3.4; Chapter 3) and ACE bioactivity assay (see section 3.6; Chapter 3).

HPLC fractionation of fraction F5 yielded five fraction peaks ($F5_1$ – $F5_5$) eluting at different times (8, 9, 10, and 20 and 24 minutes for fractions $F5_1$ to $F5_5$ respectively). All eluted fractions were tested for activity using the ACE assay (see section 3.6; Chapter 3), and produced enzyme inhibition (-1.86%, 43.77%, 49%, 71%, 17.83% respectively for fractions $F5_1$ to $F5_5$) at a concentration of 1 mg/ml (table 4.4). The ethyl acetate wash and a combination of all fractions with no distinct HPLC peaks produced 26.36% and 25.07% inhibition of enzyme activity respectively at a concentration of 1 mg/ml (table 4.4).

TABLE 4. 4: ACE BIOASSAY ACTIVITY OF FRACTIONS (1MG/ML) FROM HPLC FRACTIONATION OF FRACTION F5 FROM THE ETHANOL EXTRACT OF *HYPERICUM PERFORATUM*.

Fractions	$F5_1$	$F5_2$	$F5_3$	$F5_4$	$F5_5$	Ethyl acetate	No peaks fraction
% inhibition	-1.8%	43.77%	49%	71%	17.83%	26.36%	25.07%

The most active fraction ($F5_4$) was characterized using NMR, indicating it was a pure compound and further structure elucidation as described in section 3.5; chapter 3 was carried

out to identify the compound as 3-hydroxy 4, 4 dimethyl-4-butyrolactone (see section 4.1 and figure 4.6).

The IC50 for 3-hydroxy 4, 4 dimethyl-4-butyrolactone was determined using different concentrations (40, 80, 160 and 480 µg/ml) (see table 4.5). The isolated compound had an IC50 value of 63.88 µg/ml, comparable to the IC50 of captopril (0.0165 µg/ml). Non- linear regression analysis indicated the R^2 value for 3-hydroxy 4, 4 dimethyl-4-butyrolactone and captopril as 0.9947 and 0.8291 respectively (see figure 4.8 and 4.9)

TABLE 4. 5: ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE, ISOLATED FROM THE ORGANIC EXTRACT OF *HYPERICUM PERFORATUM* AND CAPTOPRIL, THE STANDARD ACE INHIBITOR.

Extract/ drug (concentration)	**% inhibition of ACE activity**
$F5_4$ (40 µg/ml)	28.89%
$F5_4$(80 µg/ml)	70.72%
$F5_4$(180 µg/ml)	71.53%
$F5_4$(480 µg/ml)	92.76%
Captopril (1 ng/ml)	17.7%
Captopril (10 ng/ml)	42.4%
Captopril (100 ng/ml)	74.3%
Captopril (1000 ng/ml)	95.6%

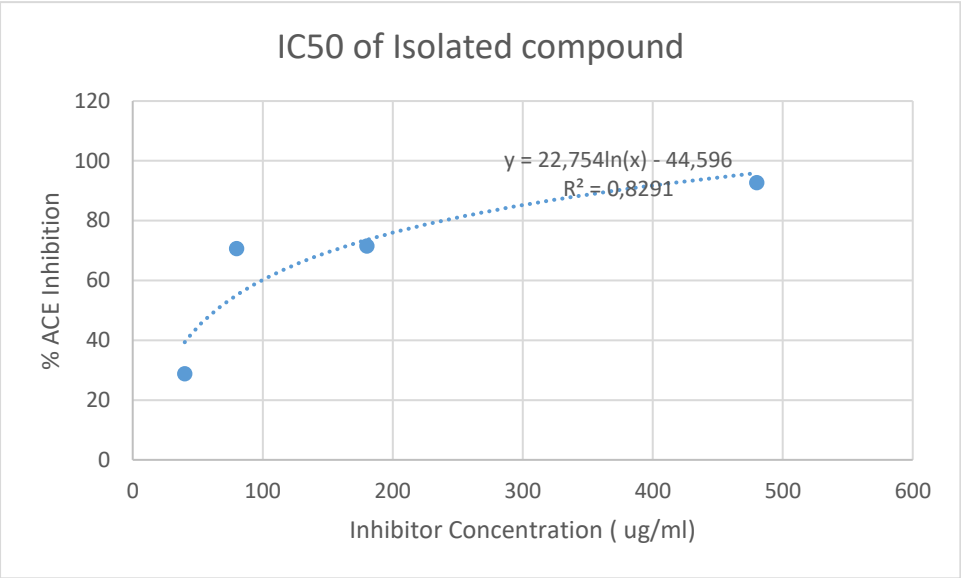


FIGURE 4. 8: IC50 PLOT FOR THE ACE INHIBITORY EFFECT OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE, ISOLATED FROM THE ORGANIC EXTRACTS OF *HYPERICUM PERFORATUM*.

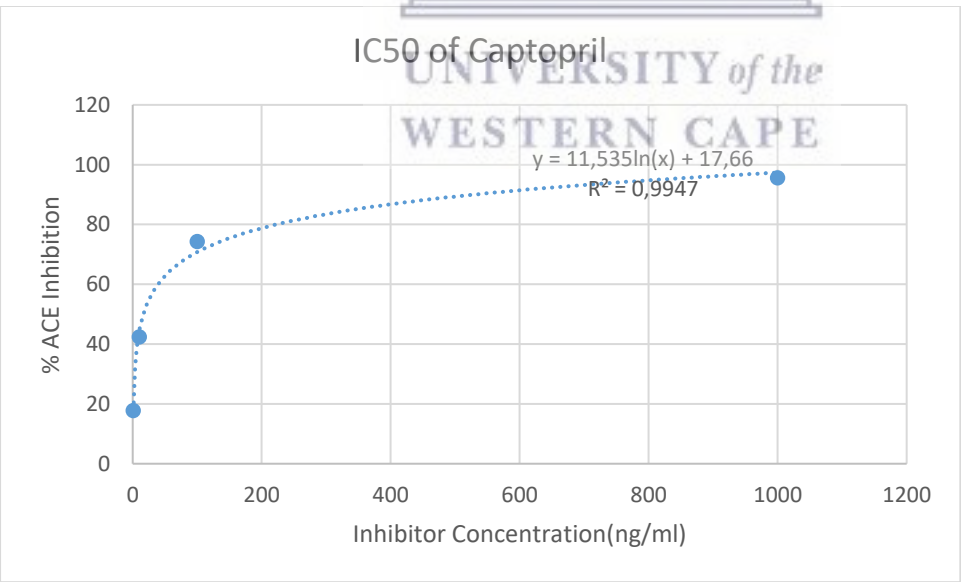


FIGURE 4. 9: IC50 PLOT FOR THE ACE INHIBITORY ACTIVITY OF CAPTOPRIL

4.3 Discussion

This study investigated the potential of *Hypericum perforatum as* a new source of compound(s) with inhibitory activity against ACE. About 1 kg of crude powdered *Hypericum perforatum* was macerated in ethanol to obtain a gummy red ethanol extract with a 25% yield. The resulting ethanolic extract was subsequently subjected to fractionation via gradient elution on a silica gel column using different ratios of ethyl acetate and hexane as solvent system. The ACEI assay-guided fractionation of the organic extract of *Hypericum perforatum* yielded fraction 5 with percentage inhibition of 70.03%, with further HPLC fractionation yielding the most active fraction ($F5_4$), which was characterized using NMR and GC-MS to identify the structure (3-hydroxy 4, 4 dimethyl-4-butyrolactone), molecular weight (130) and molecular formula ($C_6H_{10}O_3$). An IC50 value of 63.88 µg/ml and an R^2 value of 0.8291 was calculated.

3-hydroxy 4, 4 dimethyl-4-butyrolactone is a hydroxylactone which are subclass of lactones. Lactones have a broad pharmacological profile making them compounds of interest as lead structures for the development of new drug entities. Pharmacological activities reported with lactones include: antibacterial, antimalarial, anti-viral, antifungal, anti-helminthic, anticancer, anti-inflammatory and cytostatic properties (Kozioł *et al.*, 2017). Hydroxylactone are classified as natural lactones and have been isolated from a number of African traditional

medicinal plants in a very small percentages and normally appear as essential oils (Seitz and Reiser, 2005; Buckle, 2015).

In this study 3-hydroxy 4, 4 dimethyl-4-butyrolactone was isolated from *Hypericum perforatum*. 3-hydroxy 4, 4 dimethyl-4-butyrolactone has been previously isolated from other plants including some hypericum spp, with documentation of its various pharmacological activities in literature. It was extracted as a natural product for the first time from *Hypericum hircinum* used as a monoamine oxidase inhibitor (Chimenti *et al.*, 2006). It was also extracted from *Anaphalis margaritacea (L.*), a plant widely used by many Native American tribes across North America to treat respiratory problems and rheumatism and evaluated for its antibacterial activities, although no activity was reported on the compound (Ahmed *et al.*, 2004). It was one of the compounds isolated from the leaves of *Clusia burlemarxii* and tested for antimicrobial activity against *Streptococcus mutans, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Salmonella choleraesuis, Pseudomonas aeruginosa, Aspergillus Niger and Cladosporium cladosporioides,* but showed no activity against gram negative bacteria and fungi (Ribeiro *et al.*, 2011). In a phytochemical study of the biological chemical constituents of *Platanus orientalis* and *Platanus acerifolia* buds for its use as an estrogen and antiestrogen like activities, this compound was isolated in a very small amount and tested inactive for the purposed activity (Dang *et al.*, 2016). A butyrolactone extracted from Moraceae plant; *Ficus Carica* was found to have a weak miticidal activity (Yatagai., *et al*

1994). A short review from 2017 from journal of cardiology and cardiovascular therapy found that butyrolactone derived coumarins exhibited a positive cardiovascular effect mainly due to their antioxidant activity (Padte, Pednekar and Shejwalkar, 2017). This is the first time 3-hydroxy 4, 4 dimethyl-4-butyrolactone has been isolated from *Hypericum perforatum.*

Gilani *et al* (2005; 2011) reported a combination of vasoconstrictor and vasodilatatory effects with different extracts of *Hypericum perforatum* in isolated aorta preparations, and a cardio-suppressant effect similar to that of papaverine at concentration 20 times higher than those required to produce spasmolytic effect in smooth muscle preparations when tested on isolated guinea-pig atria (Gilani *et al.*, 2005; Khan, Gilani and Najeeb-Ur-Rehman, 2011). This is the first report of ACEI effect from either an extract or a compound isolated from *Hypericum perforatum*. The ACEI effect of 3-hydroxy 4, 4 dimethyl-4-butyrolactone have never been studied or documented. 3-hydroxy 4, 4 dimethyl-4-butyrolactone was less active than captopril in inhibiting the actions of ACE (IC50 of 63.88 µg/ml, compared to 0.0165 ug/ml), it however is comparable to the ACE inhibitory activity reported with various compounds including Delphinidin-3-O-sambubioside, Quercetin glucuronide ,cyanidin-3-O- sambubioside, Luteolin-7-O-glucopyranoside, Epicatechin - tetramer and various plant extracts including *Hibiscus sabdariffa, Camelia synensis, Vaccinium ashei reade, Senecio inaequidens, S. ambiguous subsp. Ambigus ,S. ambiguous subsp. Ambigus, Cryptomeria japonica*, and *Malus domestica* (Balasuriya and Rupasinghe, 2011). Although 3-hydroxy 4, 4 dimethyl-4-

butyrolactone was less active than captopril in inhibiting the actions of ACE, it was shown to possess a significant ACEI activity and has potential as a lead compound for the synthesis of more active ACEIs.

The structure of the isolated compound is dissimilar to that of compounds previously reported as inhibitors of ACE like flavonoids, flavanol, flavones, isoflavones and anthocyanin. It has been suggested that the inhibition of ACE activity identified with these secondary metabolites maybe due to their rigid structure and presence of hydroxylated aromatic ring.(Bonesi *et al.*, 2010). Although 3-hydroxy 4, 4 dimethyl-4-butyrolactone has a structure that is much simpler than these compounds, the presence of a hydroxy (-OH) group attached to position 3 of the identified structure might have contributed greatly to ACE inhibitory effect of 3-hydroxy 4, 4 dimethyl-4-butyrolactone.

CHAPTER 5

CONCLUSION

A compound - 3-hydroxy 4, 4 dimethyl-4-butyrolactone was isolated from the ethanol extracts of the powdered stem and leaves of *Hypericum perforatum* using ACEI assay-guided fractionation techniques. The chemical formula as well as the structure of the compound was determined using NMR, MS and IR techniques. In this study, the isolated compound, 3-hydroxy 4, 4 dimethyl-4-butyrolactone was found to possess significant ACE inhibition activity which was compared to that of standard drug captopril (control).

This is the first report of 3-hydroxy 4, 4 dimethyl-4-butyrolactone being isolated *Hypericum perforatum* and also the first report of its ACE inhibitory effect. Although 3-hydroxy 4, 4 dimethyl-4-butyrolactone showed ACE inhibitory effect less than that of captopril, has potential as a lead compound for the synthesis of more active ACEIs for the treatment of hypertension.

Further studies especially, *in vivo* experiments are however needed to fully characterise the antihypertensive effect of 3-hydroxy 4, 4 dimethyl-4-butyrolactone isolated from *Hypericum perforatum*.

Limitations

The ACE *in vitro* bioassay was the only approach used to access inhibitory effect of *Hypericum perforatum* extracts and due to time constraints, and the lack of access to animals, in *vivo* assays could not be conducted to confirm this activity.

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APPENDICES

APPENDIX I : ^{1}H SPECTRA OF 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

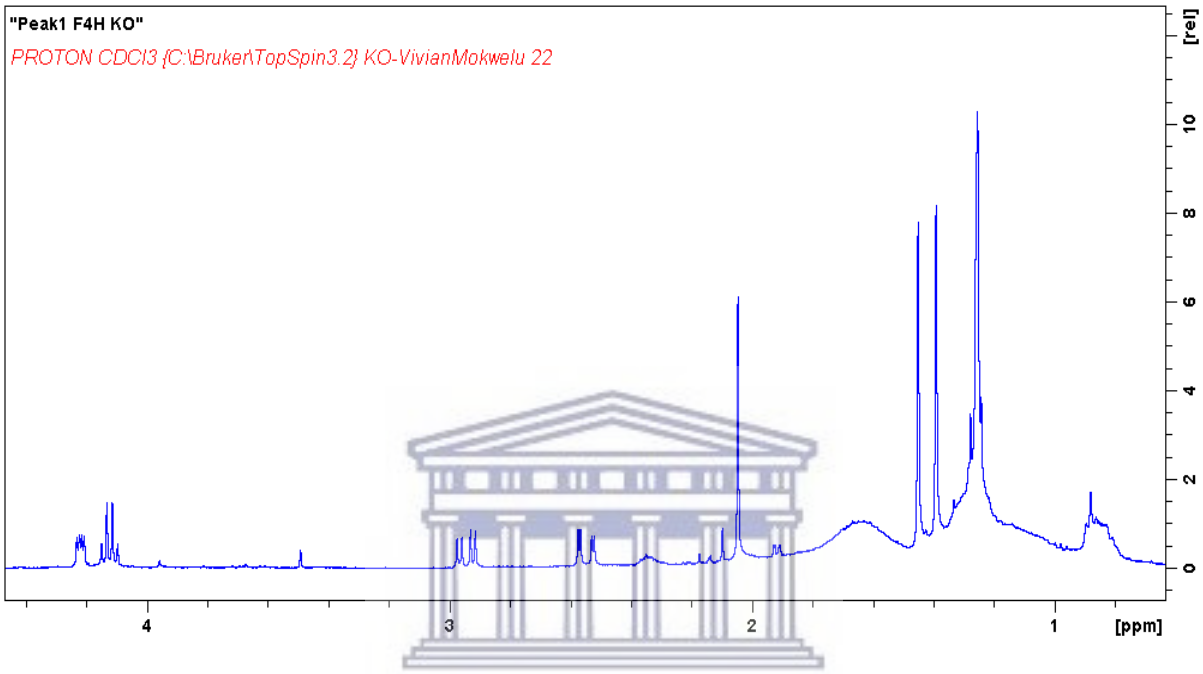

Figure A1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 3-hydroxy 4, 4 dimethyl-4-butyrolactone

APPENDIX II HSQC SPECTRUM FOR 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

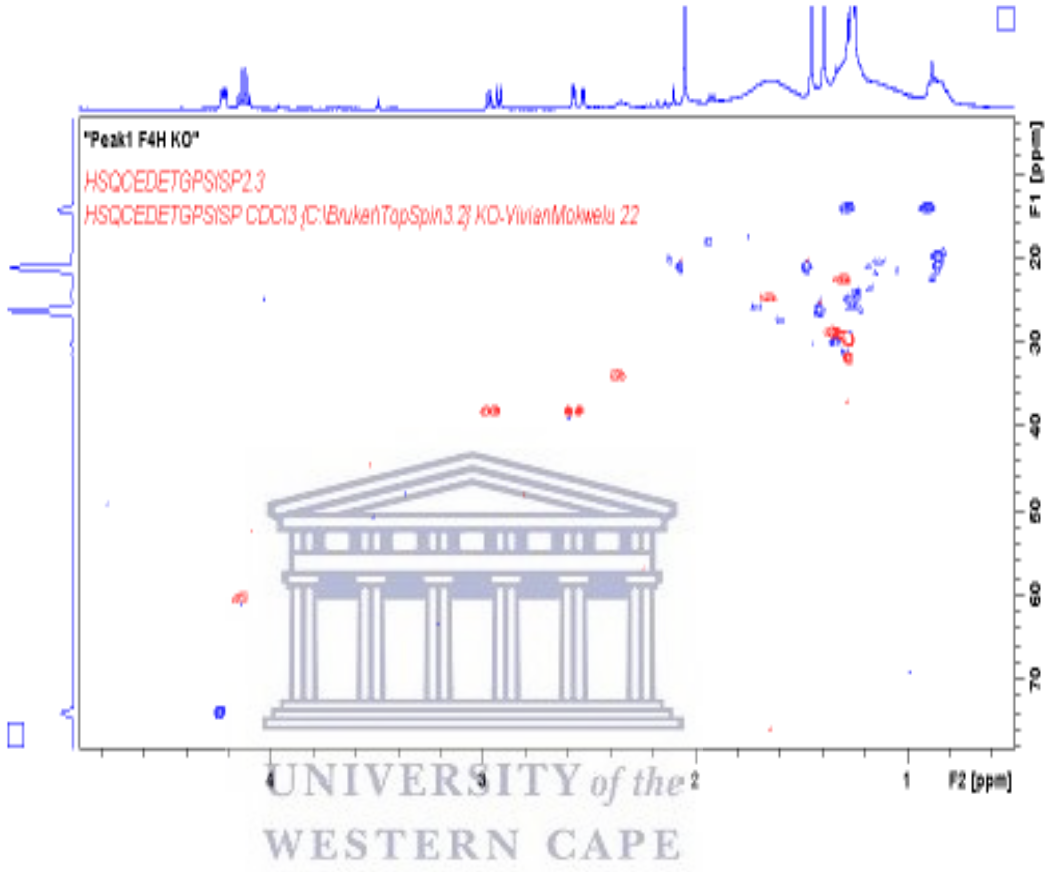

Figure A2. HSQC spectrum of 3-hydroxy 4, 4 dimethyl-4-butyrolactone

APPENDIX III HMBC SPECTRUM FOR 3-HYDROXYL 4, 4 DIMETHYL-4-BUTYROLACTONE

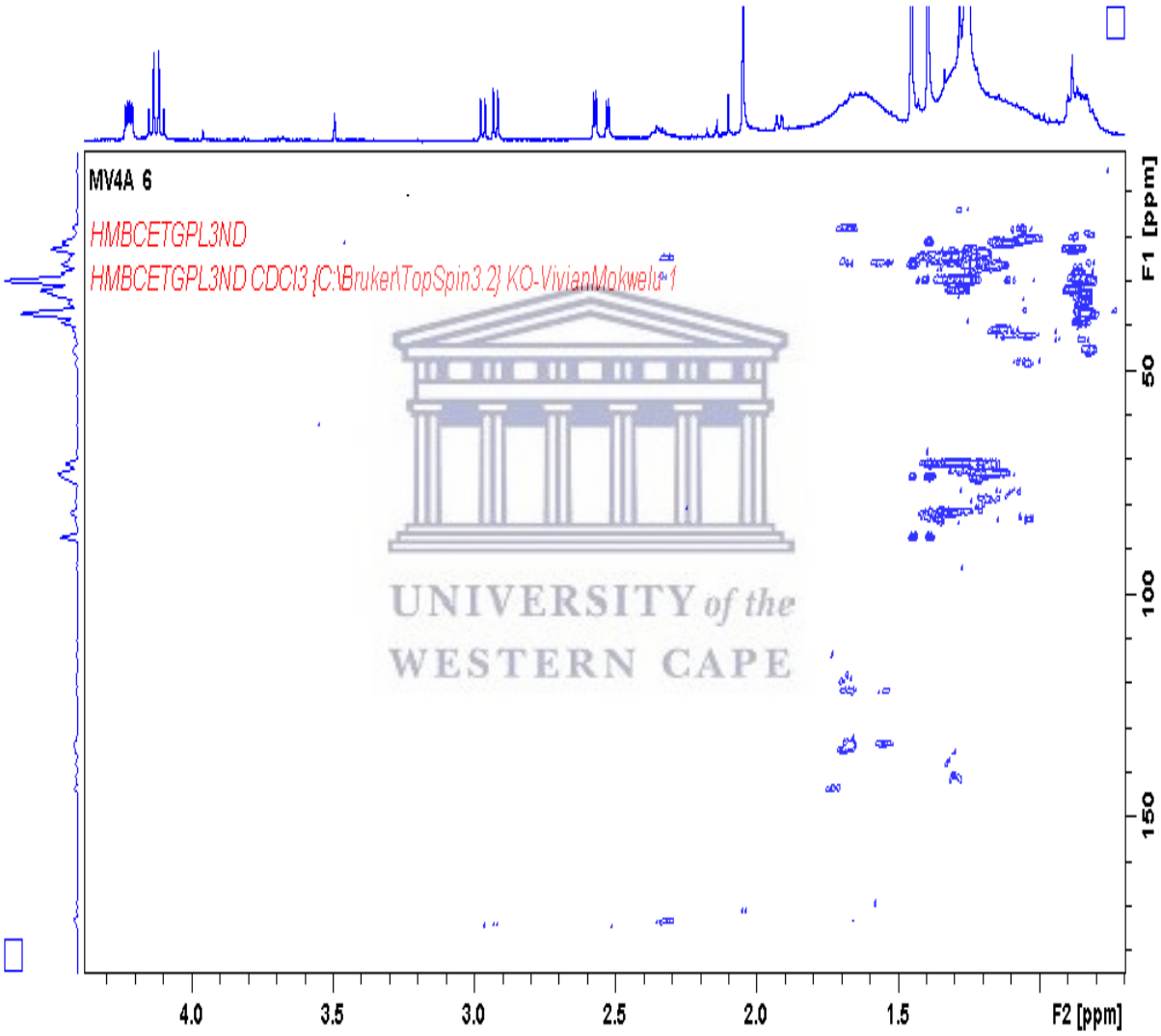

Figure A3. HMBC spectrum of 3-hydroxy 4, 4 dimethyl-4-butyrolactone

APPENDIX IV :FTIR SPECTRUM FOR 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE (TRIPLCATE)

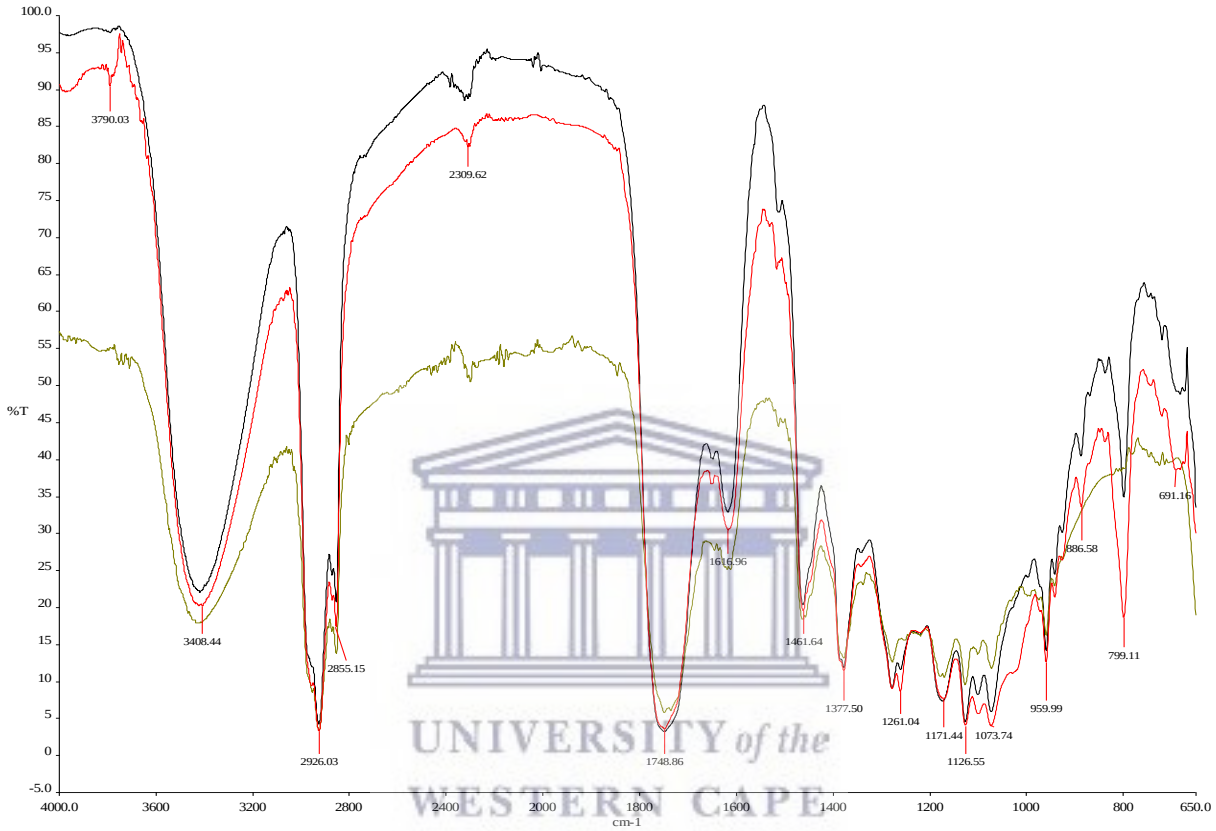

Figure A4: FTIR spectrum for 3-hydroxy 4, 4 dimethyl-4-butyrolactone acquired thrice

APPENDIX V :GC-MS SPECTRA FOR 3-HYDROXY 4, 4 DIMETHYL-4-BUTYROLACTONE

CAF Central Analytical Facilities
STELLENBOSCH UNIVERSITY

Requested: Onyinye Vivian Mokwelu
Report No: ST_UWC__HRMS_190121
Instrument: Leco Pegasus HRT, GC×GC-high resolution MS

Experimental conditions

Separation mode:	1D GC
Column(s):	^{1}D: 30 m × 0.25 mm, 0.25 μm d_f Rxi-5MS (Restek)
Temperature programme:	40°C (2 min), 20°C/min to 220°C (15 min)
Carrier gas:	Helium, 1 mL/min (constant flow)
Injection:	Liquid injection, 1 μL split (1:20)
Detection:	EI ionization (70eV); HRT TOF-MS, m/z 5-520, 13 Hz; mass accuracy 0.32 ppm (m/z 218.9)

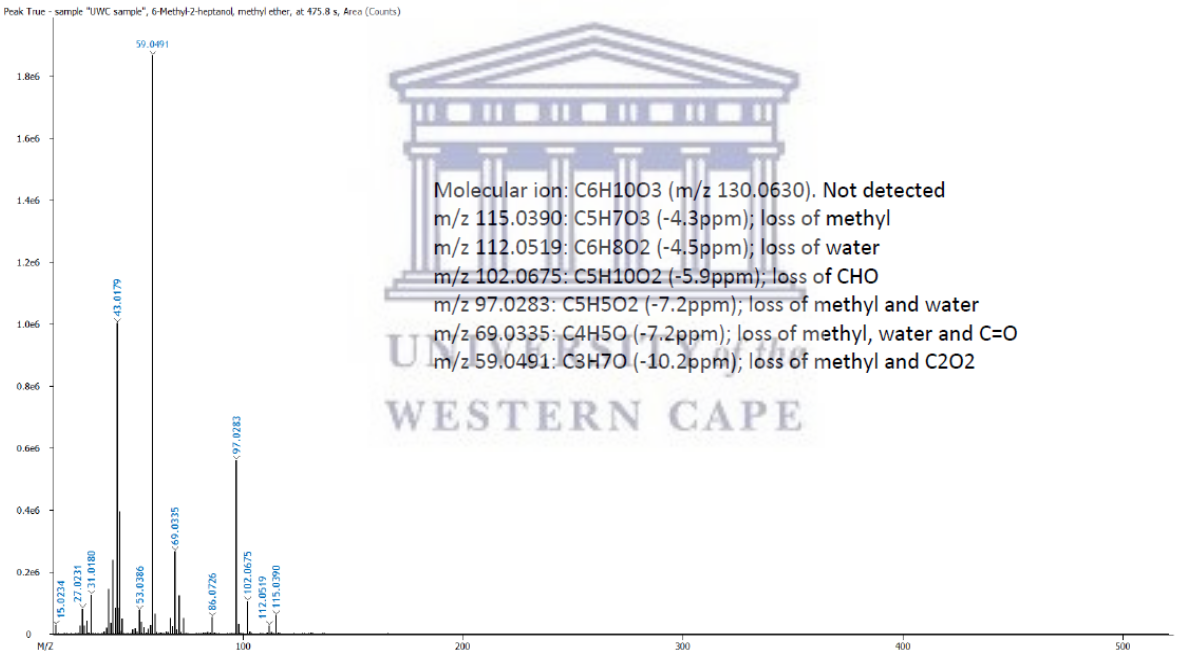

Figure A5: GC-MS spectra for 3-hydroxy 4, 4 dimethyl-4-butyrolactone

APPENDIX VI : CERTIFICATE OF ANALYSIS OF HYPERICUM PEFORATUM

HEILKRÄUTER & GEWÜRZE

Brenntag South Africa (Pty) Ltd
Killarney Gardens
11 Mansell Road
ZA-7441 Cape Town

Seite 1 from 4

Certificate of Analysis

Hyperici PhEur
Herba pulv

St John's wort herb PhEur
powdered

Date	14.01.2019
Analysis No.	AZ96143
Order No./Date	707091/11.01.19
External Document No.	75313516
Item No.	2363
Your Item No.	29260000
Batch	173435
Origin	BG
Release Date	19.09.2017
Date expiration	15.08.2020
According to	Ph.Eur. 8.0

Description	Method	Specifications	Result
Ph.Eur. Monogr. Nr. 1438			
DEFINITION Die blühenden, getrockneten, ganzen oder geschnittenen Triebspitzen von Hypericum perforatum L.			
IDENTIFICATION			
A. Appearance	Ph.Eur. Monographie	Entspricht	Complies
B. Microscopical analysis	Ph.Eur. Monographie	Entspricht	Complies
C. Thin-layer chromatography (TLC)	Ph.Eur. 2.2.27	Entspricht	Complies
TESTS FOR PURITY			
Foreign matter:			
Stems with a diameter greater than 5 mm	Ph.Eur. 2.8.2	max. 3.0 %	0.0 %
Other foreign matter	Ph.Eur. 2.8.2	max. 2.0 %	0.0 %
Loss on drying	Ph.Eur. 2.2.32	max. 10.0 %	7.1 %
Total Ash	Ph.Eur. 2.4.16	max. 7.0 %	4.0 %
ASSAY			
Total hypericins expressed as hypericine (dried drug).	Ph.Eur. Monographie	min. 0.08 %	0.11 %
MICROBIOLOGY			
Aerobic Plate count	Ph.Eur. 2.6.12	max. 50'000'000 KBE/g	540'000 KBE/g
Yeasts and moulds (TYMC)	Ph.Eur. 2.6.12	max. 500'000 KBE/g	200'000 KBE/g
Escherichia Coli	Ph.Eur. 2.6.31	max. 1'000 KBE/g	450 KBE/g
Salmonella	Ph.Eur. 2.6.31	Abwesend/25g	Absent/25g

This certificate of analysis has been produced by our quality testing program. The results of the analysis originate in our own laboratory, one of our contractors' laboratories, or have been taken from those of our suppliers. This does not imply any legally-binding warranty of definite qualities or appropriateness for a specific application. The certificate does not exonerate the user from carrying out their own tests of the product's identity and properties and its suitability for the required use. This certificate has been computer-generated and is valid without signature.

Dixa
HEILKRÄUTER & GEWÜRZE

Certificate of Analysis

Hyperici PhEur
Herba pulv

St John's wort herb PhEur
powdered

Date	14.01.2019
Analysis No.	AZ96143
Order No./Date	707091/11.01.19
External Document No.	75313516
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According to	Ph.Eur. 8.0

Description	Method	Specifications	Result
HEAVY METALS			
Lead	AAS	max. 5.0 mg/kg	0.593 mg/kg
Cadmium	AAS	max. 1.0 mg/kg	0.719 mg/kg
Mercury	AAS	max. 0.1 mg/kg	< 0.015 mg/kg
AFLATOXINE			
Aflatoxin B1	HPLC	max. 2.0 µg/kg	< 0.25 µg/kg
Aflatoxin B2	HPLC		< 0.15 µg/kg
Aflatoxin G1	HPLC		< 0.30 µg/kg
Aflatoxin G2	HPLC		< 0.25 µg/kg
Aflatoxins B1+B2+G1+G2	Berechnet	max. 4.0 µg/kg	Complies
Ochratoxin A	HPLC		< 1.0 µg/kg

Pesticide

Hyperici PhEur
Herba pulv

St John's wort herb PhEur
powdered

Date	14.01.2019
Analysis No.	AZ96143
Order No./Date	707091/11.01.
External Document No.	75313516
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Your Item No.	29260000
Batch	173435
Origin	BG
Release Date	19.09.2017
Date expiration	15.08.2020
According to	Ph.Eur. 8.0

Substance active	Result [mg/kg]	DG
Org. Chlorpestizide:		
Nicht nachweisbar		
Org. Phosphorpestizide:		
Nicht nachweisbar		
Org. Stickstoffpestizide:		
Nicht nachweisbar		
Pyrethroide:		
Nicht nachweisbar		
Weitere Pestizide, Synergisten		
Nicht nachweisbar		
DITHIOCARBAMATE		
Dithiocarbamate, berechnet als CS2	< 0.05	
Bemerkung:		
Detaillierte Liste auf Anfrage		
.		
.		
.		
.		
Pyrrolizidinalkaloide:		
Echimidin	< BG	
Echimidin N-Oxid	< BG	
Erucifolin	< BG	
Erucifolin N-Oxid	< BG	
Europin	< BG	
Europin N-Oxid	< BG	
Heliotrin	< BG	

Substance active	Result [mg/kg]	DG
Jacobin	< BG	
Jacobin N-Oxid	< BG	
Lasiocarpin	< BG	
Lasiocarpin-N-Oxid	< BG	
Monocrotalin	< BG	
Monocrotalin-N-Oxid	< BG	
Retrorsin	< BG	
Retrorsin-N-Oxid	< BG	
Senecionin	< BG	
Senecionin-N-Oxid	< BG	
Seneciphyllin	< BG	
Seneciphyllin-N-Oxid	< BG	
Senecivernin	< BG	
Senecivernin N-Oxid	< BG	
Senkirkin	< BG	
Trichodesmin	< BG	
Summe Intermedin, Lycopsamin, Indicin	< BG	
Intermedin N-Oxid	< BG	
Indicin N-Oxid, Lycopsamin N-Oxid	< BG	
Summe Pyrrolizidinalkaloide (PA)	< BG	
Summe BfR (28 PA)	< BG	

B E M E R K U N G E N
Für Heilmittel gilt laut Swissmedic
ein Höchstwert von
1.0 µg Pyrrolizidinalkaloide
bezogen auf die maximale Tagesdosis.

Dixa
HEILKRÄUTER & GEWÜRZE

Pesticide

Hyperici PhEur
Herba pulv

St John's wort herb PhEur
powdered

Date	14.01.2019
Analysis No.	AZ96143
Order No./Date	707091/11.01.
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Item No.	2363
Your Item No.	29260000
Batch	173435
Origin	BG
Release Date	19.09.2017
Date expiration	15.08.2020
According to	Ph.Eur. 8.0

Substance active	Result [mg/kg]	DG	Substance active	Result [mg/kg]	DG
Heliotrin-N-Oxid	< BG		Stand Februar 2017.		

KRÄUTER EXPERTEN 1616

DIE SCHWEIZER NATURMANUFAKTUR FÜR HEILKRÄUTER UND GEWÜRZE
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