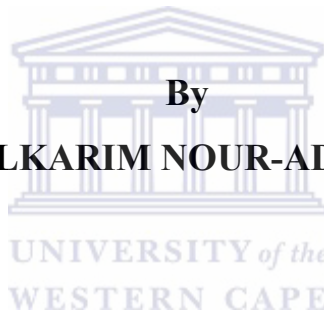# UNIVERSITY *of the* WESTERN CAPE

CARDIOVASCULAR EFFECTS, MOLECULAR DOCKING AND CHEMOINFORMATICS ANALYSIS OF COMPOUNDS ISOLATED FROM *LEONOTIS LEONURUS*.

By

ABD-ALKARIM NOUR-ADDIN SASI

A thesis submitted in fulfilment of the requirements for the degree of Magister Pharmaceuticiae, School of Pharmacy, in the Department of Pharmacology, University of the Western Cape.

Supervisor: **Dr Obikeze Kenechukwu**
Co supervisor: **Prof. Alan Christoffels**

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ABSTRACT

Cardiovascular effects, molecular docking and Chemoinformatics analysis of compounds isolated from *Leonotis leonurus.*

Abd-Alkarim Sasi

Leonotis leonurus (*L. Leonurus*) has relatively abundant diterpenes and has been used as a traditional herbal medicine for treating several ailments including influenza, muscular cramps, skin related diseases, menstrual, antilipidemic, hyperglycaemia and hypertension. In this study, diterpenoid compounds such as; Dubiin, Saponified-Dubiin, Hispanol, Marrubiin and DC9 were isolated from *L. Leonurus* plant. The cardiovascular effects of these isolated compounds were investigated in order to determine the response of anaesthetised normotensive Wistar rats (*in-vivo*) to the compounds. Also, the drug-likeness of the isolated diterpenoid compounds and their binding interaction with β1 adrenoceptor (PDB: 2Y04), angiotensin II receptor (Ang II) (PDB: 3R8A), Angiotensin converting enzyme (ACE) (PDB: 4XX3), and renin receptor (PDB: 2X8Z) by using molecular docking methods and Chemoinformatics analysis was performed (*in-silico*). Important molecular descriptors and molecular docking were used in our Chemoinformatics (*in-silico*) analysis to study the drug-likeness and the binding affinity for of each molecule (Dubiin, Saponified-Dubiin, Hispanol, Marrubiin and DC9). The molecular descriptors and the binding energy were calculated by using the molecular operating environment software (MOE 2013). The lowest energy and highest cluster conformations of the molecules were further analysed. All the five (5) diterpenoids were predicted to have good oral bioavailability after oral administration and passed the Blood-Brain Barrier (BBB) rules. Also, the compounds were predicted to have high probability of being good Drug-like candidates, except for DC9, which is predicted to have lower possibilities of being Drug-like candidate than the other diterpenoids. Furthermore, these compounds (Dubiin, Saponified-Dubiin, Hispanol, Marrubiin and DC9) were shown to interact with β1 adrenoceptors *in-silico*, an interaction that was confirmed *in-vivo* by increases in Blood pressure (SP, DP and MAP) and Heart rate (HR). In anaesthetized

normotensive male Wistar rats (*in-vivo*), Dubiin (0.5 - 40mg/kg; IV), Saponified-Dubiin (0.5 - 60mg/kg; IV) Hispanol (0.5 - 40mg/kg; IV), DC9 (0.5 - 40mg/kg; IV) and Marrubiin (0.5 - 40mg/kg; IV) produced dose dependent increase in Systolic pressure (SP), Diastolic pressure (DP), and Mean arterial pressure (MAP) at all doses. Also, the compounds produced dose dependent increase in Heart rate (HR). From the *in-vivo* and *in-silico* studies it can be concluded that all the five (5) isolated diterpenoid compounds showed cardiovascular effects on Blood pressure (BP) and Heart rate (HR) by acting as $\beta 1$ adrenoceptor agonists. Also, these diterpenoids compounds could be responsible for the cardiovascular effect observed in the methanol extracts from previous studies. These cardio-active compounds are prototype or "lead compounds" for designing and developing new non-toxic and effective drugs for cardiovascular disease (CVD) treatment.

DECLARATION

I declare that, *Cardiovascular effects, molecular docking and Chemoinformatics analysis of compounds isolated from Leonotis leonurus*, is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: **Abd-Alkarim Nour-Addin Sasi**

Signed: _____

Date: **04/12/15**

DEDICATION

To those who gave me full support and guidance; my father, mother and family…
whom I call my small world.

To my dear friends; who shared with me my dreams.

To my supporting teachers, through all my school life.

I say:

All that it takes to fulfil your dreams and aspiration:

A true prayer from the beloved ones …

A true support from a dear friend …

Guidance from a dedicated teacher …

A little bit of luck, hard work, and Grace from God …

To you…

…………… I dedicate the fruit of my effort.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

2D	Two- dimensional
3D	Three-dimensional
Å	Angstrom (unit of length)
ACE	Angiotensin converting enzyme
ADME	Absorption, distribution, metabolism, and excretion
ANG II	Angiotensin II receptor
ANOVA	Analyses of variance
AROM	Number of aromatic rings
AT1	Angiotensin II receptor type I
BBB	Blood brain barrier
BP	Blood pressure
BV	Blood vessel
Ca^{2+}	Calcium channels
CMC	Comprehensive medicinal chemistry
DC	Diterpenoid compound
DP	Diastolic pressure
DSS	Dahl salt sensitive rats
HBA	Hydrogen bond acceptors
HBD	Hydrogen bond donors
HIV	Human immunodeficiency virus
HR	Heart rate
K^{+}	Potassium channels
L. Leonurus	*Leonotis Leonurus*

LOGP	Octanol-water partition coefficient
MAP	Mean arterial pressure
MDDR	Mdl drug data report
MOE	Molecular operating environment
MORF	Molar refractivity
MW	Molecular weight
NAC	No acids
NaCl	Sodium chloride
NAT	Total number of atoms
PDB	Worldwide protein data bank
PSA	Polar surface area
QSAR	Quantitative structure-activity relationship
RAAS	Renin Angiotensin Aldosterone System
RIGB	Rigid bonds
RMSD	Root mean square deviation
ROTB	Rotatable bonds
SHR	Spontaneously hypertensive rats
SP	Systolic pressure
TOHB	Total number of hydrogen bonds
WHO	World health organization
Zn^{2+}	Zinc ion
ΔG_b	Binding free energy

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CHAPTER ONE

This chapter will provide background information on traditional medicinal plants and their uses including the treatment of cardiovascular diseases. This chapter will also discuss the phytochemical composition of traditional medicinal plants and their pharmacological effects especially on the cardiovascular system. Emphasis would be placed on the cardiovascular effects of *L. Leonurus* and plant-derived diterpenes. In addition, computational modelling as a tool for drug discovery will be discussed. In conclusion, the significance of the study and the problem statement will be presented and the chapter layout for the rest of the thesis presented.

1.1   INTRODUCTION

In recent decades, there has been an increase in the incidence of diseases of lifestyle such as diabetes and cardiovascular disorders especially in the developing countries (Adeyi *et al*., 2007). Some of the most prominent diseases of lifestyle are cardiovascular diseases (CVD), which incidentally are the leading cause of morbidity and mortality worldwide (Kreatsoulas and Anand, 2010; Patil *et al*., 2010). According to reports (Pieters and Vorster, 2008), Africa accounts for about 1.3 million people affected by cardiovascular diseases every year. Several studies have shown that there are various factors associated with CVD in humans including gender, age, dyslipidemia, obesity, tobacco smoking, diet and lifestyle (Appel *et al*., 1997; Chalmers *et al*., 1999; Dahl and Heine, 1975; Liu *et al*., 2013; Seedat, 2007). For instance, it has been established that there is a relationship between mean blood pressure (BP) and daily salt consumption by humans which results in cardiovascular diseases (Schmieder *et al*., 1987). The treatment and management of cardiovascular diseases can be achieved with the use of several commercially available medicines. However, the need for better treatment regimens for cardiovascular diseases has resulted in continuous research and drug design with a view to manufacturing new and more effective drugs. In addition to this, the use of pharmaceuticals for the treatment of cardiovascular disease is known to be expensive and thus inaccessible to the

majority of the global population (Ubani, 2011). As a result, it has been suggested that an extensive research on medicinal herbs is imperative in order to obtain better, affordable and effective drugs. This is because medicinal plants are potential sources of cheap starting materials for the synthesis of new drugs in the drug discovery process (Henkel *et al*., 1999). The traditional routes of drug discovery and design approaches are known to be a time-consuming and expensive process. Also, these procedures are associated with a high failure rate (DiMasi et al., 1991; Tufts CSDD, 2014). Consequently, in recent years, one of the promising areas that have been identified, and is attracting a lot of attention is the use of computer-based techniques and molecular modelling to design and evaluate novel potential drugs on a computer prior to laboratory preparation in order to reduce the time and costs involved (Zonta *et al*., 2010). These new computer-based drug design techniques will continue to undergo several modifications and improvements thereby leading to new and more powerful drugs (Wlodawer and Vondrasek, 1998).

Southern Africa is a region with rich abundant and pharmaceutically important plants species. This geographical region represents more than 10 % of the global vascular plant flora, on less than 2.5 % of the earth's land surface area with 24, 000 of higher plant species from 368 families (Leistner, 2005). Traditional medicine practice is common in Southern Africa with approximately 70% of the South African population depending on traditional medicines for their primary health care needs (Bannerman, 1983). Recent studies have also shown that natural products along with their derivatives represent more than 50% of all the drugs used in clinical treatment. This represents approximately 7,000 medical compounds in the modern pharmacopoeia (Gurib-Fakim, 2006; Lin *et al*., 1999). Natural products are known to have pharmacological or biological activity that have been used to treat diseases (Prasad and Aggarwal, 2011). Generally, natural plant products can be broadly classified into two categories i.e. primary and secondary metabolites. The primary metabolites (e.g. amino acids, nucleotides, sugars, acyl lipids) are characterized by their broad distribution in all living things, while the secondary metabolites (e.g. phenols, quinones, terpenes, and alkaloids) are attributed to give specific species some characteristic features such as colour (Hanson, 2003; Harrison, 1983). A large class of

secondary products are known as diterpenoids (terpenes) and these are categorized according to the number of ring systems (acyclic, bicyclic, tetracyclic, macrocyclic) present in their structure. These compounds have unique characteristics and are known for their cardiovascular effects, with several *in-vivo* and *in-vitro* studies reporting significant cardiovascular effects in the treatment of cardiovascular diseases (CVD) with diterpenoids extracted from plants (Baccelli *et al*., 2005; El Bardai *et al*., 2003, 2001; Silva *et al*., 2005; Tirapelli *et al*., 2008). The ability of diterpenoids to treat cardiovascular related diseases have made them a source of new prototypes for the discovery and development of novel cardiovascular therapeutic agents. There are several medicinal plants from South Africa that are known to possess cardiovascular activity and not limited to *L. Leonurus* which include *Croton Zambesicus* (Baccelli *et al*., 2005), *Croton Cajucara* Benth (Guerrero *et al*., 2004), *Andrographis Paniculata* (Zhang *et al*., 1998), *Marrubium vulgare* and *Orthosiphon aristatus* (Kaplan and Rivett, 1968). These medicinal plants have been chemically investigated, with diterpenoids identified as one of their major constituents.

Leonotis leonurus (Lamiaceae) is a traditional medicinal plant indigenous to southern Africa. It is commonly referred to locally as wild dagga due to its use as a substitute for *Cannabis sativa*. This plant has long been used in traditional herbal medicine for treating dermatological infections, muscular cramps, female menstrual problems, hyperlipidemia, hyperglycaemia and hypertension (Wyk *et al*., 2012). Previous studies on the extracts from *L. Leonurus* have shown that the nature of solvents used in the extraction, and the dosage administered determine the cardiovascular effects produced. For instance, Obikeze *et al*., (2013) reported an increase in BP and Heart rate (HR) using a methanol extract of *L. Leonurus* leaves. In another study, Obikeze, (2004) reported that the administered dosage of the aqueous extract in anaesthetized normotensive rats caused an increase in the BP and a decrease in HR. The result from the Obikeze (2004) study was different from a similar study reported by Ojewole (2003), who observed that the aqueous extract resulted in a decrease in BP and HR in anaesthetized, normotensive and spontaneously hypertensive rats (SHR). Also, *in-vitro* studies by Mugabo *et al*., (2002) reported a positive chronotropic and inotropic effect with an aqueous extract of *L. Leonurus* on isolated Langendorff perfused male

Wistar rat hearts. Generally, the compounds in *L. Leonurus* that are responsible for its cardiovascular effect are unknown but with the abundance of diterpenes isolated from the plant, it has been postulated that these are the likely compounds with cardiovascular effects characteristic of the plants extracts. The diterpenoid compounds that have so far been isolated from *L. Leonurus* belong to Labdane-type diterpenoids i.e. bicyclic (Mazimba, 2015; Nsuala *et al*., 2015). Only one diterpenoid, -9, 13-epoxylabda-6(19), 15(14) diol dilactone (EDD) isolated from the methanol extracts of the leaves of *L. Leonurus* has been reported to produce cardiovascular effects (Obikeze *et al*., 2008). EDD was found to exhibit a dual effect on the cardiovascular system in isolated arteries as well as in anesthetized rats. However, there are many diterpenoids isolated from *L. Leonurus* for which no cardiovascular studies have been carried out. These include; Dubiin, Sponified-Dubiin, Hispanol, DC9 and Marrubiin (Kaplan *et al*., 1970; Popoola *et al*., 2013; Rivett, 1964; Savona *et al*., 1978). Marrubiin was one of the first diterpenoids isolated from *Marrubium vulgare* extract and subsequent studies on this diterpenoid have reported a vasorelaxant activity on the isolated rat aorta (*in-vitro*) (El Bardai *et al*., 2003; Khan *et al*., 2012). Marrubiin was also the primary diterpene isolated from a *L. Leonurus* extract (Mnonopi *et al*., 2011; Rivett, 1964). In this study we investigate the cardiovascular activity of five (5) diterpenoid compounds labelled DC1, DC2, DC8, DC9 and DC15 isolated from *L. Leonurus* in anaesthetized normotensive Wistar rats. The study will also determine the binding affinity of these isolated diterpenoid compounds to different cardiovascular receptors as well as predicting their oral bioavailability and drug likeness by using Chemoinformatics techniques (*In-silico*).

1.2 PROBLEM STATEMENT

Leonotis leonurus extracts has been widely studied especially for their cardiovascular effects due to their traditional use in the treatment of cardiovascular diseases (CVD), but the compounds responsible for the different cardiovascular effects observed with these extracts are yet largely unknown. Although *L. Leonurus* contains an abundance of diterpenes, none of these compounds has so far any current clinical use for the

treatment of cardiovascular related diseases. This is mainly due to limited studies on the specific diterpenoid compounds with cardiovascular effects found in *L. Leonurus*. This study seeks to determine the different cardiovascular effects of previously isolated diterpenoids extracted from *L. Leonurus*.

1.3   SIGNIFICANCE OF STUDY

The isolation of these diterpenoid compounds from the extracts of *L. Leonurus* is the first step in determining the possible cardiovascular effects of these compounds and possible further development as drugs. This study will serve to determine the specific cardiovascular activities unique to each of the isolated diterpenoids compounds, as such this study will provide information necessary to drug discovery that would determine the usefulness of each compound as a lead compound for the design and development of new drugs for the treatment of cardiovascular related diseases (CVD).

1.4   THESIS LAYOUT

In this thesis, chapter one will provide general background information on *L. Leonurus*, its cardiovascular properties and progress in the development and improvement of their cardiovascular activities. Also in chapter one, the benefits of computational tools in drug discovery will be discussed. The significance and problem statement of this study will be presented in chapter one.

In chapter two, a brief overview of the evidence of the use of traditional medicines for the treatment of cardiovascular disease will be presented. Also, chapter two will discuss some of the existing medicinal plant species and their use in treatment of different ailments, with emphasis on the plants that are used for the treatment of cardiovascular diseases. A description of *Leonotis leonurus*, its use in traditional medicine as well as the results of various studies into its composition and pharmacologic effects will be described. The chapter also highlights the benefits and successful application of Computer aided softwares (*in-silico*) in drug discovery.

Chapter three of this thesis will describe the material used and the various analytical techniques such as Chemoinformatics methods that were used to study the drug-likeness of the isolated diterpenoids. Also, chapter three will provide detailed experimental procedures and preparations for both the drug-likeness and the molecular docking studies. Chapter three also presents the results of the drug-likeness and the molecular docking studies and discusses their implications on *in-vivo* studies.

Chapter four of this thesis will present the materials and methods used for the *in-vivo* study. A full description of the cardiovascular model and experimental protocol used to determine the cardiovascular effects of the isolated compounds would be presented, and the results obtained from the studies presented. A discussion of the results obtained would also be contained in chapter four.

Chapter five presents an analysis of the *in-vivo* and *in-silico* results with respect to predictions and provides conclusions derived from the *in-silico* and the *in-vivo* study. Recommendations on further research work to be carried out is also presented in this chapter.

CHAPTER TWO

2   LITRATURE REVIEW

In this chapter, the previous studies that have been conducted on the use of available natural products from plants and their various applications will be summarized. Various benefits of medicinal and herbal plants and how they are used to treat different ailments especially cardiovascular diseases in human beings are discussed. The chapter also highlights the traditional use of *Leonotis leonurus* including previous studies that have been reported on the extracts and various compounds that have been isolated from the plant. Finally, the benefit and successful application of Computer aided software (*in-silico*) in drug discovery is summarized within this chapter.

2.1   NATURAL PRODUCTS

Natural products can be described as purified organic compounds that have been isolated from natural sources which are mostly plants. The composition of natural products from plants depends on several factors such as plant species, plant part, and other ecological factors (Muhizi, 2002). Natural products are known to have active components and these active components have pharmacological or biological activity that have been explored and used in drug discovery to treat diseases (Prasad and Aggarwal, 2011). Consequently, the use of natural products from medicinal plants is a potential source of cheap starting materials for the synthesis of drugs especially for developing new drugs in the drug discovery process (Henkel *et al*., 1999; Muhizi, 2002). According to Newman and Cragg, (2012), natural products consist of approximately one-half of U.S. Food and Drug Administration-approved drugs.

The synthesis pathway of natural plant products is often either by primary or secondary metabolism (Hanson, 2003). Generally, the classification of natural plant products can be broadly considered in two categories i.e. primary and secondary metabolites (Harrison, 1983). Primary metabolites are essential because they facilitate plant

growth, development and survival. Unlike primary metabolites, plants are known to produce a large variety of secondary compounds but they have not been shown to have any direct function on growth and development of the plants. Another major difference between primary and secondary metabolites is that secondary metabolites have a limited distribution within the plant kingdom. For instance, a specific secondary metabolite is often found in only one plant species or related group of species, whereas primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 2010). In addition, the use of secondary metabolites has been extensively studied and forms a major area of research for organic chemists (Harrison, 1983).

2.2   TERPENES

Terpenes are the largest class of natural products in plants (Ahuja, 2006). All terpenes consist of five-carbon elements with branched carbon skeleton of isopentane and their basic structural elements are referred to as isoprene units. Generally, terpenes decompose at high temperatures to give isoprene (Figure 2.1) and are classified according to the number of isoprene units. The various isoprene units present in terpenes include; monoterpenes (2 isoprene units), sesquiterpenes (3 isoprene units), diterpenes (4 isoprene units), sesterpenes (5 isoprene units), triterpenes (6 isoprene units), carotenes (8 isoprene units), and polyisoprenes (n isoprene units) (Taiz and Zeiger, 2010). The diverse substances of this class of natural products are known to be insoluble in water and consist of different aromatic compounds, vitamins and steroids (Muhizi, 2002). When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. The difference between terpenes and terpenoids is that terpenes are hydrocarbons, whereas terpenoids contain additional functional groups (Schrader and Bohlmann, 2015).
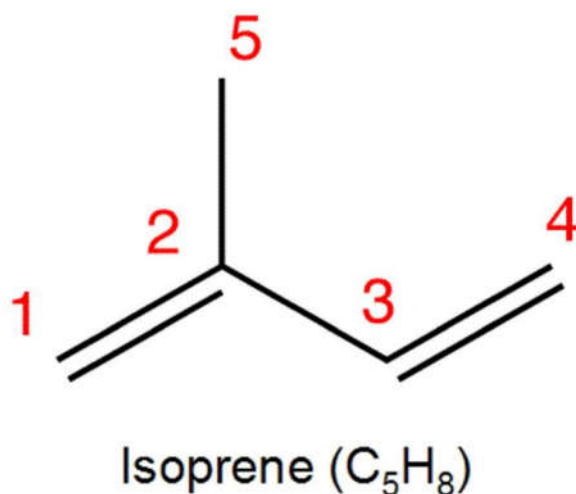
Figure 2.1: The basic structural elements of terpenes (Isoprene unit).

Diterpenoids are known to be one of the classes of the isoprene units with unique characteristics such as cardiovascular effects and are considered as a large class of secondary products. The molecular structure of diterpenoids is derived from four (4) isoprene units which are joined in a head-to–tail. Diterpenoids are classified according to the number of ring systems present (acyclic, bicyclic, tetracyclic, macrocyclic). Diterpenoids are known to possess a wide spectrum of important biological activities, (Dewick, 2011; Hoffmann, 2003). For instance, several *in-vivo* and *in-vitro* studies have shown that diterpenoids extracted from plants have significant cardiovascular effects. The ability of diterpenoids to treat cardiovascular related diseases have made them a source of new prototypes for the discovery and development of novel cardiovascular therapeutic agents such as Ca^{2+} channel blockers (Baccelli *et al*., 2005; El Bardai *et al*., 2003, 2001; Silva *et al*., 2005; Tirapelli *et al*., 2008).

2.3   GENERAL VIEW ON TRADITIONAL MEDICINE

The world health organization (WHO) defined traditional medicine as the comprehensive knowledge, skills, and practices based on theories, beliefs, and

experiences which are unique and indigenous to different cultures. In addition, traditional medicine, whether explicable or not, can be used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (Zhang, 2000). In specific terms, traditional medicine or alternative medicine describes the use of plants and animals in medicinal treatment (De Smet, 1991). Consequently, plants that are used to prevent or treat diseases, improve and promote health and also with the ability to exhibit curative properties are defined as herbal medicine. As a result, several herbs and minerals are known to be the basis of ancient medicinal treatments as described by many historical documents (Hoffmann, 2003; Wyk *et al*., 2012). The compendium of traditional medicines is global and some of the examples include traditional Chinese medicine, the Ayurveda (Dubey *et al*., 2004; Patwardhan *et al*., 2005), and traditional African medicine (Okpako, 1999).

Herbal medicine is a prominent form of medicine with ancient applications and has been in use for centuries. However, herbal medicines have some unique disadvantages which include; poor hygiene and sanitation that can lead to other health problems due to microorganisms, as well as the presence of dangerous chemical impurities such as pesticides and other chemical contaminants from agricultural activities (Pearson, 1995; Sofowora, 1982). The lack of a comprehensive database and the absence of convincing scientific evidence supporting the efficacy of herbal medicines and the lack of a toxicity profile is another disadvantage of herbal medicine (Sofowora, 1982). This is because the distribution of naturally occurring toxic chemicals such as arsenic and selenium can contaminate medicinal plants resulting in other serious health complications hence the toxicity profile of herbal plants is crucial to its usage (Pearson, 1995). In addition, there is insufficient knowledge and lack of precise diagnosis by traditional healers before administering these herbal drugs. The lack of precision in correct dosage mainly because the traditional healers do not know or understand the pathology of certain diseases thereby end up treating the symptoms rather than the disease which can sometimes lead to other health complications (Sofowora, 1982).

In traditional herbal medicine, the herbal remedies can be prepared in several ways and administered or dispensed in different dosage forms. The preparation and

administration of herbal medicines often depend on the use of the plant (Aulton and Taylor, 2013). The dosage forms of herbal medicines vary in types and include semi-solids (e.g. pastes, creams, ointments), solids (e.g. whole or powdered plant parts, pill, tablets), liquid dosage forms (e.g. Infusions, decoctions, elixirs, tinctures) and gases (e.g. Incense, fumigants, inhalants). The liquid dosage form is the most prominent and in this form medicines are administered orally or applied externally on the affected parts of the body (Germishuizen and Meyer, 2003). It is important to emphasize that the method of preparation of herbal medicines as well as the concentration of the herbs in preparations could be critical. This is because different preparations may result in different effects (Jager *et al*., 2011). For example, different cardiovascular activities were observed when different methods were used to prepare decoctions of the leaves of *L. Leonurus* (Mugabo et al., 2002; Obikeze, 2004; Ojewole, 2003).

2.3.1 TRADITINAL MEDICINAL PLANTS IN SOUTH AFRICA

The use of pharmaceutical drugs for the treatment of some ailments such as high Blood pressure (BP) are expensive and unaffordable to majority of the global population (Ubani, 2011). Bearing this in mind, it has been suggested that extensive research on herbs is imperative in order to obtain better, more affordable and effective drugs. Recent studies have shown that natural products along with their derivatives represent more than 50 % of all the drugs used in clinical treatment (Gurib-Fakim, 2006). Also, it has been estimated that more than two-thirds (35,000) of the global plant species are found in developing countries and they have medicinal values which is beneficial to human health and survival. Approximately 7,000 medical compounds in the modern pharmacopoeia are derived from plants (Hefferon, 2012). The relative abundance of medicinal plant species is crucial to research and development especially in the treatment of ailments that are unique to geographical areas. South Africa has abundant herbal medicines that have been used in the traditional practice for the treatment of several illnesses (Lin *et al*., 1999). Herbal medicines are an essential part of the culture and folklore of the African populace. These herbal medicines are found in approximately 24, 000 of higher plant species of 368 families. This represents more

than 10 % of the world's vascular plant flora on less than 2.5 % of the earth's land surface area (Leistner, 2005). Recent studies have shown that most of the South African communities depend on herbal medicines to meet their health care needs (Afolayan and Sunmonu, 2010; Fennell *et al*., 2004). Bannerman, (1983) stated that approximately 70 % of the South African population depends on traditional medicines, including plants such as *L. Leonurus* for the treatment of a large number of diseases. The next section (2.4) will briefly discuss some of the existing medicinal plant species and their use in the treatment of different ailments.

2.4   MEDICINAL PLANTS WITH CARDIOVASCULAR EFFECTS

2.4.1   *Tulbaghia violacea* (Alliaceae)

Tulbaghia violacea plant belongs to Alliaceae family of herbs. The crude methanol leaf extract of *Tulbaghia violacea* has been reported to reduce BP and HR in spontaneously hypertensive male rats (SHR). The BP and HR- reducing effect of the methanol leaf extract has been suggested to involve several mechanisms via the inhibition of angiotensin converting enzymes (ACE) and β1 adrenoceptors. Also, the extract can act as a stimulant for muscarinic receptors and reduces the level of aldosterone in plasma (Raji *et al*., 2012; Ramesar *et al*., 2008). In a related study, Mackraj *et al*., (2008) reported a reduction in systemic arterial BP that is associated with decrease in renal angiotensin II receptor in Dahl salt sensitive (DSS) rats.

2.4.2   *Allium sativum* (Liliaceae)

Garlic and its derivatives have been described to considerably reduce the Diastolic (DP) and Systolic pressure (SP) in humans with high BP (McMahon and Vargas, 1993; Preuss *et al*., 2001). The cardio protective effects of dietary Garlic have been attributed to its ability to produce hydrogen sulfide (H_2S) which has been reported to relax vascular smooth muscle and also induce vasodilatation of isolated blood vessels (Zahid

Ashraf *et al*., 2005). In addition, *Allium sativum* has the potential to reduce the risk of cardiovascular diseases in humans by lowering both lipids and circulating angiotensin II levels in the blood (Augusti *et al*., 2005; Mohamadi *et al*., 2000). This plant species have the ability to perform several other functions such as the inhibition of blood coagulation, platelet aggregation, thrombus formation, angiotensin converting enzyme (Sendl A *et al*., 1992) and increased fibrinolysis (Rahman and Lowe, 2006).

2.4.3 *Rauwolfia serpentina* (Apocynaceae)

Rauwolfia serpentina is a small shrub with snake shaped woody roots. This plant species is best known as insanity herb and is often used to treat and cure snake bites and scorpion stings (Srivastava *et al*., 2006). Besides the curative activity of *Rauwolfia serpentina,* the alkaloids from this plant species have been reported to induce antihypertensive activity. The induced antihypertensive activity of *Rauwolfia serpentina* is achieved by controlling nerve impulses along certain nerve pathways via the depletion of catecholamines from peripheral sites that can act on the heart and blood vessels. The main constituent of *Rauwolfia serpentina* is Reserpine which is used as an antihypertensive and antipsychotic drug (Gurib-Fakim, 2006).

2.4.4 *Crataegus monogyna* (Rosaceae)

Crataegus monogyna is also known as hawthorn or single-seeded hawthorn. The flowers and leaves from this plant have been used as traditional remedy in the treatment of various cardiovascular diseases such as hypertension, myocardial dysfunction, angina, tachycardia and cardiac failure. In addition, *Crataegus monogyna* has been shown to cause a significant group difference in mean diastolic blood pressure (DP) reductions in comparison with the placebo group (Walker *et al*., 2006).

2.4.5 *Ocimum gratissimum* (Labiatae)

Ocimum gratissimum was first reported to show physiological evidence with hypotensive effect after intravenous administration of the essential oil of *Ocimum gratissimum* on anesthetized and conscious rats (Lahlou *et al*., 2004). Also, Patil *et al*., (2010) reported that the alcoholic extract of the leaves from *Ocimum gratissimum* showed significant antihypertensive activity in conscious albino hypertensive rats when compared to standard drug enalapril maleate.

2.4.6 *Cissus assamica* (Vitaceae)

Cissus assamica (Laws) Craib, has been used mainly to treat snakebite in China. According to a study reported by Yang *et al*., (1998), dried extracts from *Cissus assamica* root has the ability to cause vasodilation of blood vessels on isolated aortic rings of rats by antagonizing the vasoconstriction induced by endothelin-1.
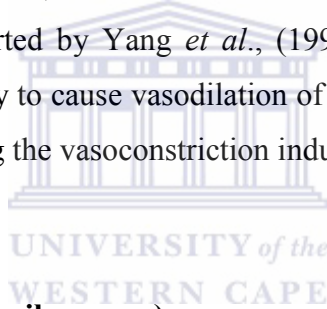
2.4.7 *Curcuma longa* (Zingiberaceae)

Turmeric (*Curcuma longa*) is a rhizome and an Indian traditional medicine which is mostly used as a spices. Studies have shown that turmeric exhibits antidiabetic and antihypertensive activities in humans (Chattopadhyay *et al*., 2004). The extract of *Curcuma longa* has the ability to cause vasodilatation by inhibiting the conversion of inactive angiotensin-I to the potent vasoconstrictor angiotensin II. (Lekshmi *et al*., 2013; Zahid Ashraf *et al*., 2005).

2.4.8 *Salvia miltiorrhiza* (Lamiaceae)

Salvia miltiorrhiza (Red sage) plant has been studied with reports showing that it can induce vasodilatation and reduce BP in the two-kidney, one-clip (2KIC) endovascular

hypertension model in hamsters. This was achieved by stimulating the synthase production of endothelial nitric oxide (Kim *et al*., 2007).

2.4.9 *Leonotis leonurus* (Lamiaceae)

Leonotis leonurus (*L. Leonurus*) is an ancient traditional plant that is relatively abundant in South Africa and has been used in herbal medicine to control and manage hypertension (Wyk *et al*., 2012). For instance, previous studies on *L. Leonurus* have shown that extracts from this plant are known to have cardiovascular effects and these effects are have unique differences as observed with other different extracting solvents. In a study by Obikeze *et al*., (2013), it was reported that the methanol extracts of *L. Leonurus* leaves have both β1 agonist and direct vasoconstrictive effects and cause increase in all cardiac parameters of both the *in-vitro* and *in-vivo* conditions. This study was in contrast to earlier studies where *L. Leonurus* aqueous leaf extract was reported to show hypotensive activity (Mugabo *et al*., 2012; Ojewole, 2003).

2.5 DITERPENOIDS AS ANTIHYPERTENSIVE COMPOUNDS IN MEDICINAL PLANTS

There are several medicinal plants that are known to possess antihypertensive activity. These medicinal plants have been chemically investigated and diterpenoids have been identified as one of the major constituents. As a result, there have been several studies that have been directed on the cardiovascular activity of these unique compounds (Tirapelli *et al*., 2008). Diterpenoids can be extracted from several plants and used for treating various ailments. The subsequent section will briefly describe some of the plants containing diterpenoids as reported in literature and their respective uses.

2.5.1 *Croton Zambesicus* **(Euphorbiaceae)**

In traditional African medicinal practice, *Croton Zambesicus* plant is widely used to treat hypertension, urinary infections and malaria (Adjanohoun et al., 1989). Two diterpenoid compounds were isolated from the dichloromethane extract of *Croton zambesicus* leaves and the mixture of both diterpenoids was reported to induce vascular relaxation via blockage of extracellular Ca^{2+} influx. Also, each purified diterpenoid was observed to show lower activity than the mixture (Baccelli *et al.*, 2005).

2.5.2 *Croton Cajucara Benth* **(Euphorbiaceae)**

This plant is commonly known as Sacaca or Cajuçara and it is obtained from the extracts of the stem, bark and leaves of *Croton Cajucara Benth*. *Croton Cajucara Benth* is used as a traditional herbal medicine for the treatment of hypertension, diabetes, diarrhoea, malaria, fever, gastrointestinal, renal and hepatic disorders, as well as in the control of cholesterolemia (Costa *et al.*, 1999; Hiruma-Lima *et al.*, 2002, 2000). The various diterpenoids that have been isolated from the *Croton Cajucara Benth* plant include; trans-dehydrocrotonin, and cis-dehydrocrotonin (Guerrero *et al.*, 2004). Silva *et al* (2005) reported that the diterpene trans-dehydrocrotonin produced its hypotensive activity as well as bradycardiac effects in humans due to a vasorelaxant effect on aortic rings and a direct negative chronotropic effect on the right atria of rats.

2.5.3 *Andrographis Paniculata* **(Acanthaceae)**

Andrographis Paniculata is one of the well-known plants in Malaysia and is used to treat diabetes, hypertension and other diseases (Asmawi, 2012). The aqueous extracts of *Andrographis Paniculata* are known to decrease the Systolic Blood pressure (SP) in both normotensive and spontaneously hypertensive rats (Zhang and Tan, 1997, 1996). In another study by Zhang *et al*., (1998), it was reported that the diterpenoid

lactones isolated from *A. Paniculata*, namely deoxyandrographolide and 14-deoxy-11, 12 di-dehydroandrographolide, can cause a significant decrease in Mean arterial blood pressure (MAP) and HR. In addition, these diterpenoids have been scientifically proven to non-competitively and dose-dependently antagonize, isoproterenol induced positive chronotropic actions in rat atria (Zhang *et al*., 1998). Yoopan *et al* (2007) identified the main site of action in humans for the hypotensive effects of *A. paniculata* extracts as the vascular smooth muscle. In a recent study conducted by Sriramaneni *et al* (2012), it was shown that chronic treatment using *A. Paniculata* preserved vascular endothelial, functions in spontaneously hypertensive rats (SHR).

2.5.4   *Marrubium vulgare* (Lamiaceae)

Marrubium vulgare is another plant that is frequently used in traditional medicine to cure various diseases. For example, *Marrubium vulgare* is helpful for the treatment of bronchial asthma, and non-productive cough. It also has hypoglycemic, vasorelaxant, analgesic, antiinflammatory, antioxidant, anti dermatogenic and antibacterial activity (Bokaeian *et al*., 2014). *Marrubium vulgare* extract is also extensively used as traditional medicine to treat hypertension, and has been shown to produce vascular relaxation and decrease SP in SHRs (El Bardai *et al*., 2001). The diterpenoid compounds marrubiin and marrubenol have been isolated from *Marrubium vulgare,* and have shown vasorelaxant activity on the rat aorta. The mechanism of this relaxant activity is due to the interaction with L-type Ca^{2+} channels (El Bardai *et al*., 2004, 2003).

2.5.5   *Orthosiphon aristatus* (Lamiaceae)

The leaves of *Orthosiphon aristatus* have been prescribed in traditional Indonesian medicine especially for the treatment of hypertension (Awale *et al*., 2002). There are four (4) diterpenoids that have been isolated from the leaves of *O. aristatus* and they have shown vasorelaxant activity on the rat aorta (Ohashi *et al*., 2000).

2.5.6 *Leonotis Leonurus* (Lamiaceae)

Several pure compounds have been isolated from *Leonotis leonurus* (*L. Leonurus*) extracts including diterpenoids. So far only one diterpenoid isolated from *L. Leonurus*, 9, 13-epoxylabda-6(19), 15(14) diol dilactone (EDD)has been reported to have cardiovascular effect (Obikeze *et al*., 2008). It was found that EDD exhibits a dual effect on the cardiovascular system in isolated arteries as well as in anesthetized rats. At low doses, EDD produced significant dose-dependent decreases in BP, while at higher doses it produced significant dose-dependent increases in BP. All doses induced significant dose-dependent decreases in HR (Obikeze *et al*., 2008). No cardiovascular studies have been done on the other diterpenes isolated from *L. Leonurus*.

2.6 *LEONOTIS LEONURUS*: DESCRIPTION AND USES

Leonotis leonurus (*L. Leonurus*) is shrub indigenous to Southern Africa and commonly referred to as wild dagga. Other indigenous names of *L. Leonurus* include "umunyane" "lebake" and "umfincafincane" (Dyson, 1988). This plant is one of the more prominently used traditional medicinal plants in South Africa and has been documented for use in treating several diseases in both humans and animals (Hutchings, 1996; Oyedemi and Afolayan, 2011; Watt and Breyer-Brandwijk, 1962; Wyk *et al*., 2012). *L. Leonurus* grows mostly along forest margins, rocky hillsides, riverbanks as well as grasslands of the Eastern, Western Cape, Kwazulu-Natal and Mpumalanga Provinces of South Africa (Wyk *et al*., 2012). The generic name *Leonotis* stands for lion's ear which is due to the shape and texture of the flower (Pienaar, 1994). The features of *L. Leonurus* plant include tubular orange red flowers which are grouped in dense clusters along the stems. The fruit consists of four little nutlets situated at the base of calyx tube (Figure 2.2). Naturally, *L. Leonurus* dies after flowering and new growth starts in spring. The propagation of *L. Leonurus* plant is by cuttings, division of the rootstock or seeds (Adamson and Salter, 1950; Hutchinson, 1973). Plants grow between 1-3m (Nichols, 2002).

Common names

English: Wild dagga, red dagga, wild hemp, lion's ear/tail, minaret flower.
Zulu: Imunyane, umunyane.
Sotho: Lebake, levake.
Xhosa: Umfincafincane.

(Dyson, 1988)

Figure 2.2: *Leonotis Leonurus* **(Lamiaceae) Common names.**

As indicated earlier, *L. Leonurus* has been used for several medicinal purposes in both animals and humans(Mazimba, 2015; Nsuala *et al*., 2015). For instance, dry leaves of *L. Leonurus* can be smoked alone or mixed with tobacco and has been reported to show narcotic or marijuana-like effects in humans (Watt and Breyer-Brandwijk, 1962). The aqueous extracts from *L. Leonurus* plant are used internally for the treatment of colds, dysentery, coughs, amenorrhea, influenza, bronchitis, high blood pressure, headaches, dyslipidemia and a hypoglycemic effect (McGaw and Eloff, 2008; Oyedemi and Afolayan, 2011).The aqueous extract of *L. Leonurus* has been applied to treat skin-related infections such as boils, eczema, itching and muscular cramps (Bienvenu *et al*., 2002; Hutchings, 1996; Wyk *et al*., 2012). Aqueous extracts are also used for the treatment of internal parasites in animals (Scott *et al*., 2004).

L. Leonurus is a versatile plant species is relatively abundant hence it is imperative to conduct an extensive laboratory testing of its extract and the isolated pure compounds. This is to provide a scientific basis and pharmacological understanding in order to support and expand the existing folkloric uses in the treatment of emerging ailments. The extracts from *L. Leonurus* plant are reported to possess an anticonvulsant effect (Bienvenu *et al*., 2002). In a study by Qi *et al* (2010), *leonurine* which is a compound isolated from a *L. Leonurus* extract was reported to show antioxidant and cardio-protective properties with significant improvement in myocardial function (Liu *et al*., 2010). The aqueous extracts from the leaves of *L. Leonurus* hav been reported to prevent many free-radical-related diseases as a result of the presence of compounds such as phenolics, flavonoids, flavonols and proanthocyanidins in the extract (Oyedemi and Afolayan, 2011). Ojewole (2003) reported that the aqueous leaf extract of *L. Leonurus* possessed antinociceptive, anti-inflammatory, as well as hypoglycemic activity. Similarly Oyedemi and Afolayan (2011) demonstrated that oral administration of aqueous extract of *L. Leonurus* leaves has antilipidemic and also antihyperglycemic effect which is capable of reducing the blood glucose levels by potentiating insulin secretion. Also, the decoctions from the leaves of *L. Leonurus* have been shown to have antihelmintic effect on gastrointestinal helminths in animals such as goats (Maphosa *et al*., 2010). Further study by Maphosa *et al* (2012) revealed that the extract exhibits anti-inflammatory and analgesic activities in rats. Previous studies have shown that the leaf extracts from *L. Leonurus* plant possesses hypotensive activity (Mugabo *et al*., 2012; Ojewole, 2003). This study did not agree with a recent study reported by Obikeze *et al*., (2013). In this recent study, it was reported that the extracts from *L. Leonurus* leaf showed an increase in all cardiac parameters for both the *in-vitro* and *in-vivo* studies with methanol extracts of the plant. The differences in the effects observed by Mugabo *et al*., (2012), Ojewole, (2003) and Obikeze, (2004) when they used different solvents to extract the plant could be due to the difference in solubility of the cardio-active compounds. The phytochemical studies performed on *L. Leonurus* extracts shows that it contains tannins, flavonoids, sterols, diterpenoids, triterpenoids, alkaloids, quinines and saponins (Bienvenu *et al*., 2002; Mazimba, 2015; Nsuala *et al*., 2015).

Previous studies on marrubiin, first isolated from *Marrubium vulgare* extracts, have reported a vasorelaxant activity on the isolated rat aorta (El Bardai *et al*., 2003; Khan *et al*., 2012). Marrubiin is also one of the primary diterpenoids found in *L. Leonurus* (Mnonopi *et al*., 2011; Rivett, 1964) and has been suggested to be responsible for the anticoagulant, antiplatelet, antidiabetic, and cardio protective effects of the plant extracts (Mnonopi *et al*., 2011). Other diterpenoids previously extracted from *L. Leonurus* include *Leoleorin A* (or Compound Y), *Leoleorin B*, *Leoleorin C, Leoleorin D, Leoleorin E, Leoleorin F, Leoleorin G* (or Leonurenone B), *Leoleorin H* (or Leonurenone C), *Leoleorin I, Leoleorin J* (or Leonurenone A), *Leoleorin L, Leoleorin M, Leoleorin N* and 16-epi-*Leoleorin F* (Bienvenu *et al*., 2002; He *et al*., 2012; Kaplan and Rivett, 1968; Naidoo *et al*., 2011; Wu *et al*., 2011). Compound X, premarrubiin, Dubiin, Saponified-Dubiin, Hispanol, DC9 and leonurun have also been isolated from the plant (Henderson and Mccrindle, 1969; Kaplan *et al*., 1970; Kaplan and Rivett, 1968; McKenzie *et al*., 2006; Savona *et al*., 1978). Recently, Narukawa *et al* (2015) reported two new diterpenoids 14α-hydroxy-9α, 13α-epoxylabd-5(6)-en-7-on-16, 15-olide and 13ξ-hydroxylabd-5(6), 8(9)-dien-7-on-16, 15-olide from the plant.
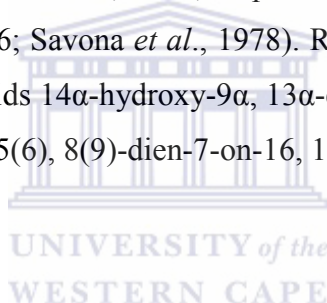
2.7 ANIMAL MODELS IN CARDIOVASCULAR RESEARCH

The use of animal models can present useful information about genes as well as pathways to understanding the complex pathophysiologic characteristics within humans without the added risk of harming an actual human through the procedure (Pravenec and Kurtz, 2010). From the available experimental data based on several studies with over 4000 Medline references in the last 10 years, it was shown that the most commonly studied animal model of hypertension development is the use of spontaneously hypertensive rat (SHR) (Okamoto and Aoki, 1963). In comparative studies that were performed on nine genetic hypertensive rat strains, done by Horie *et al* (1986), SHR showed markedly higher BP levels and earlier blood pressure rises in comparison with other hypertensive strains. This is because of the similarity between SHR and human anatomy. These similarities are known to range from a genetic susceptibility to high BP without having specific aetiology. The responses to drug

treatment between SHR and humans have been similar especially with the development of many features of human hypertensive end-organ damage such as cardiac hypertrophy, cardiac failure and renal dysfunction (Pinto *et al*., 1998). As in the case of humans, hypertension is known to develop more rapidly and become severe in male than female SHR (Barrett-Connor, 1997; Iams and Wexler, 1979). Another advantage of using the SHR model is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing and sustained hypertensive phases, with each phase characterized to last at least several weeks (Folkow and Svanborg, 1993). One of the characteristics of SHR that differs from human hypertension is that SHRs reproducibly develop hypertension in young adults compared to the middle age in humans (Kauser and Rubanyi, 1995). Although the SHR is the animal of choice for the screening of antihypertensive agents (Leong *et al*., 2015; Okamoto and Aoki, 1963; Trippodo and Frohlich, 1981), the use of normotensive rats are considered to be more appropriate especially for pharmacologic screening in cases where the cardiovascular effect of the compounds is unknown as it allows for the study of both hypertensive and hypotensive effects and also due to economic reasons (Obikeze *et al*., 2013, 2008). In addition, normotensive and SHRs have comfortably short life spans. 2.5–3 years and 1.5–2.5 years respectively. The short life span of these species make them a suitable candidate as it is relatively less time-consuming and easier to follow the changes during such a short life span (Folkow and Svanborg, 1993).

Generally, there are three (3) methods that are used for BP measurement in rats; tail cuff plethysmography, intra-arterial catheters and radio telemetry. The tail cuff plethysmography is a simple, indirect method, surgically non-invasive and suitable especially for a large population of animals, short and long experiments. However, this method is known to be imprecise when compared with other methods. The continuous monitoring of direct arterial BP using intra-arterial catheters is more precise and with high fidelity especially when measuring mean arterial blood pressure and heart hemodynamics. Unlike the tail cuff method, the intra-arterial method requires surgery. The third method used in the measurement of BP is radio telemetry. This method

allows for the study of BP in conscious and freely moving animals. However, it is extremely expensive (Vogel, 2007).

2.8   DRUG DESIGN AND DISCOVERY

The traditional drug discovery and design methods are time-consuming, capital intensive processes and synonymous with a high failure rates. For instance, the average cost for discovering and developing a new drug has increased from $4 million in 1962 to over $350 million in 1996 with the current estimated cost to be approximately $2.6 Billion (DiMasi *et al*., 1991; Tufts CSDD, 2014). Consequently, new approaches for drug design and methodologies are constantly being developed in order to reduce the time and costs. One of the most recent approaches is the increase in the use of computer-based techniques and molecular modelling to design and virtually evaluate novel potential drugs before laboratory preparation (Zonta *et al*., 2010). Essentially, these newly designed drug approaches will continue to undergo several modifications and improvements thereby leading to new and more powerful drugs (Wlodawer and Vondrasek, 1998).

The computer-aided techniques *(in-silico)* used in drug design and discovery are automated processes. It is a powerful, versatile technique that is used to identify a lead compound on computer before it is synthesized in the laboratory. This method is quicker and economical especially on a per compound in comparison with laboratory test (Blundell, 1996). This is mainly because of the fact that *(in-silico)* tools and techniques are capable of reducing both cost and time that are associated with the development of drug-like molecules (Singh *et al*., 2006). Another important benefit of these techniques is the ability to reduce failure during clinical trials. This is because at least 41 % of the failures in new drug development are attributed to poor pharmacokinetics and drug-drug interactions (Aulton and Taylor, 2013; Prentis *et al*., 1988). Hence the ability to predict drug properties in earlier phases of development would increase safety and reduce the number of *(in-vivo)* tests and ultimately accelerate the drug discovery process. In addition, the computational methods could

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help to predict the modifications needed to improve some of the identified poor pharmacokinetic parameters (Vass, 2011). These advantages make computational techniques an integral part of a successful and profitable drug design process (Blundell, 1996). In addition, large databases such as the ZINC library of compounds are often tested in software and are freely available for other users. (Zonta *et al*., 2010). Computer-aided drug design techniques are important in drug discovery and historically assist with providing useful insights and suggestions on the synthesis of new molecular structures and also experimental analysis prior to synthesis (Lybrand, 1995). One of the successful applications of computer-aided drug design is the recent development of human immunodeficiency virus (HIV-1) protease inhibitors via three-dimensional (3D) structures of protein target molecules in structure-based drug design (Wlodawer and Vondrasek, 1998).

To date there have been several successful computer-assisted molecular design attempts to involve the use of lead optimization in order to improve the activity, specificity, and pharmacokinetics of lead compounds (Young, 2009). Examples of drugs that have been developed using computer-aided drug design include captopril (antihypertensive), indinavir sulphate (anti HIV) (Figure 2.3), teveten (antihypertensive), donepezil hydrochloride (Alzheimers disease), dorzolamide (Glaucoma), zolmitriptan (migraine), NVP-AUY922 (anticancer), and LY 517717 (factor Xa inhibitor) (Glen *et al*., 1995; Greer *et al*., 1994; Kawakami *et al*., 1996; Keenan *et al*., 1993; Talele *et al*., 2010).
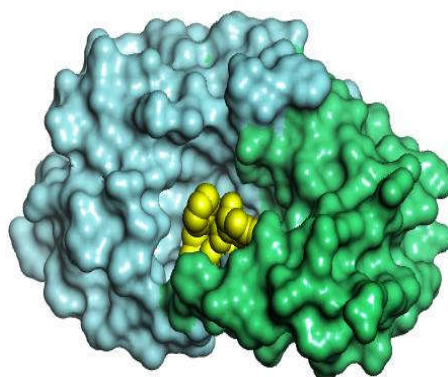
Figure 2.3: 3D structure of Indinavir, HIV-1 protease inhibitors bound to the HIV-1 protease receptor.

Indinavir (small yellow docked molecule towards the centre of the figure) bound to HIV-1 protease receptor pocket (PDB: 1HSG). The drug molecule fits tightly in the binding site and blocks the normal protein function (Wlodawer and Vondrasek, 1998).
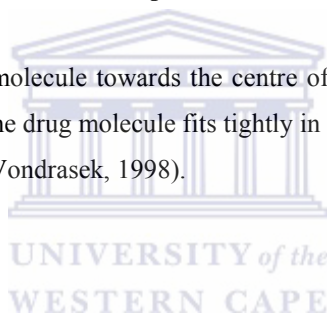
2.9 MOLECULAR DOCKING

Molecular docking (MD) is defined as a computational process of identifying a ligand that is capable of fitting both geometrically and energetically within the binding site (active site) of a protein (target) (Figure 2.3) (Teodoro *et al.*, 2001). Molecular docking (MD) can assist to predict the binding modes (interactions) and binding energy (ΔG_b) of a ligand with a known 3D structure of a protein and has been widely used for drug hit identification and lead optimization (Kitchen *et al.*, 2004). The binding mode (interactions) and binding energy (ΔG_b) are easy to visualize and analyse computationally. The more the negative value of the binding energy (ΔGb), the stronger the interactions between the ligand and the target (Lim *et al.*, 2011; Temirak *et al.*, 2014; Thakur and Thakur, 2015). The primary tool for this analysis is the "Protein-ligand docking" (molecular docking) technique. This technique provides different information on the strength of the interaction between the compound (drug)

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and the biological system (target) (Kitchen *et al.*, 2004). Generally, the application of computational methods to study the formation of intermolecular complexes has been the focus for researchers as it is widely accepted that drug activity is obtained through the binding mode (interactions) of a ligand to the pocket of a protein. For example, during the binding conformations of the complex of a protein with a therapeutic drug, the molecules are known to exhibit geometric and chemical complementarities which are both essential for successful drug activity (Teodoro *et al.*, 2001).

Historically, drug discovery did not emerge until the first structures of the targets (receptors) were solved. One of the pioneer studies by Ehrlich in 1897 suggested a theory that the side chain with specific groups on the cells can combine with the toxin. Ehrlich coined these side chains as receptors (Klebe, 2000). Several drugs are known to have protein targets and understanding their interaction is crucial to designing a potent compound (Bruce, 2010). This is because most of the targets for computer-aided drug design are protein based such as enzymes or cell surface receptors (Overington *et al.*, 2006). In particular, specific amino acid sequences in the binding site (active site) are necessary for the receptor to bind ligands (Roy and Luck, 2007). The main process involved for a drug to bind to a receptor is such that the drug should be in the correct shape to fit into the active site. This mechanism is referred to as the lock-and-key theory of drug action (Jorgensen, 1991). Receptor active sites are described as having specificity pockets which are empty and available areas of the active site where the drug can bind (Alberts *et al.*, 2013). Another requirement is that the drug should have the right functional group to bind to the active site (Lodish *et al.*, 2000). For example, if the active site of the receptor contains a hydrogen bond donor, then the drug should have a hydrogen bond acceptor in order to be positioned and give a hydrogen bond binding the drug to the active site. Also, other important drug interactions with active sites include π-system stacking, positioning of charged groups to form ionic bonds, van der Waals interactions and steric hindrance (Young, 2009).

The positioning (pose) of the drug in the active site can be obtained only with great effort and by using appropriate techniques such as X-ray crystallography to generate crystal structures of the protein with the drug soaked into the crystal before starting a

drug design project (MartÃ--Renom *et al.*, 2000). The protein structure used in drug design is most often obtained from high resolution X-ray crystallographic experiments, while the structures are rarely determined by NMR (Vass, 2011). If there is no experimentally determined structure available, a homology model has been suggested as an alternative to be used for drug design and drug discovery (Enyedy *et al.*, 2001a, 2001b). It is important for the proteins and ligand to undergo preparation procedure before docking which is simply the generation of a chemically correct three-dimensional (3D) structure for both. This may involve converting from 2D to 3D, adding atoms missing in the database files, defining the correct topology and finding the possible tautomer and protonation states at physiological pH (MartÃ--Renom *et al.*, 2000).

The initial steps in new drug discovery involve the identification of new chemical entities and this procedure can be via chemical synthesis or direct isolation of compounds from natural products (Koehn and Carter, 2009; Rishton, 2008). For instance, more than 80% of drug compounds were pure natural products or derived from natural sources and almost half of the drugs approved since 1994 are based on natural products. According to Harvey (2008), 13 new drugs related to natural products have been approved between 2005 and 2007 and are obtained from natural sources. The starting point for plant-based new drug discovery should be the identification of a suitable candidate plant with a well-documented traditional use, as well as the isolation of the pure compounds that are responsible for the activity. This is an integrated approach with the benefits of saving costs and time, and when coupled with molecular docking enhances the success rate of drug discovery (Katiyar *et al.*, 2012).

2.10 DRUG-LIKENESS

Drug-likeness is a qualitative concept used in drug design to define the potential of a drug to produce *in-vivo* biological activity that is based on pharmacokinetic factors like bioavailability. Drug-likeness is estimated from the molecular descriptors (Physicochemical properties) before the substance is even synthesized and tested. One

of the traditional methods used to evaluate Drug-likeness is to check if it complies with the physicochemical properties based on the Lipinski's rule. However, many alternative rules have been developed from Lipinski's rule such as Ghose filter, Veber rule, Blood brain barrier likeness (BBB) and MDL Drug Data Report (MDDR) like rule (Bickerton *et al*., 2012; Ghose *et al*., 1999; Lipinski *et al*., 1997; Oprea, 2000; Veber *et al*., 2002; Yusof and Segall, 2013).

2.10.1 Lipinski's rule

This is a prominent rule that is used to predict the drug-likeness. The Lipinski's Rule of Five (Ro5) by Lipinski in 1997 was based on the observation that most orally administered drugs are relatively small and moderately lipophilic molecules. This rule is also known as the Pfizer's rule of five or simply the rule of five (Lipinski, 2004; Lipinski *et al*., 1997). The rule states that a compound is more likely to exhibit poor absorption or permeation when two or more of the following physicochemical criteria are violated. A drug-like molecule has a molecular weight (MW) between (160-480 g/mol), An octanol-water partition coefficient (LOGP) not greater than five (5), not more than five (5) hydrogen bond donors (HBD) or not more than ten (10) hydrogen bond acceptors (HBA) (Table 2.1). The rule describes molecular properties as an important parameter for a drugs pharmacokinetics especially in the human body including other mechanisms such as their absorption, distribution, metabolism, and excretion ("ADME"). However, the rule does not predict if a compound is pharmacologically active (Schneider., 2000).

Table 2.1: Important descriptors and values to satisfy Lipinski's rule.

Name of the rule	(MW)	(LOGP)	(HBD)	(HBA)
Lipinskies Rule of Five (ROF)	160 - 480	≤ 5	≤ 5	≤ 10

2.10.2 Ghose filter

Ghose *et al* (1999) characterized the Comprehensive Medicinal Chemistry (CMC) data base by establishing qualifying ranges which accounts for more than 80 % of the compounds. Based on the filter, a drug-like molecule has to have an octanol-water partition coefficient (LOGP) between 0.4 and 5.6, a molecular weight (MW) between 160 and 480, a molar refractivity (MORF) between 40 and 130, and the total number of atoms (NAT) between 20 and 70 (Table 2.2).

Table 2.2: Important descriptors and values to satisfy Ghose filter.

Name of the rule	(MW)	(LOGP)	(MORF)	(NAT)
Ghose filter	160 - 480	-0.4 - 5.6	40 - 130	20 - 70

2.10.3 MDDR like rule

MDDR-like rule (MDL Drug Data Report) was defined and published by Oprea (2000), who concluded that the rule of five test "Lipinski's Rule" could not be applied to discriminate between drugs and non-drugs. Oprea (2000) suggested that the probability of finding a drug-like compound is higher in those compounds with six (6) or more rotatable bonds (ROTB $\geq$ 6), eighteen (18) or more rigid bonds (RIGB $\geq$ 18), and three (3) or more aromatic rings (AROM $\geq$ 3). The probability of finding a 'nondrug-like' compound is higher in the ranges of those with five (5) rotatable bonds or less (ROTB $\leq$ 5), seventeen (17) or less rigid bonds (RIGB $\leq$ 17), and two (2) or less aromatic rings (AROM $\leq$ 2).

Table 2.3: Important descriptors and values to satisfy MDDR like rule.

MDDR like rule	(ROTB)	(RIGB)	(AROM)
Drug-like	$\geq$ 6	$\geq$ 18	$\geq$ 3
Non drug-like	$\leq$ 5	$\leq$ 17	$\leq$ 2

2.10.4 Vebers rule

One of the notable contributions was made by Veber *et al*., (2002) in which two additional criteria were proposed for predicting rat oral bioavailability after investigating a dataset of 1100 compounds with rat oral bioavailability data. It was observed that those with fewer than ten (10) rotatable bonds (ROTB) and polar surface area (PSA) less than 140 Å^2 had a better probability of achieving a good oral bioavailability after oral administration, as presented in (table 2.4).

Table 2.4: Important descriptors and values to satisfy Vebers rule.

Name of the rule	(ROTB)	(PSA)
Vebers rule	≤ 10	≤ 140

2.10.5 BBB likeness rule

This filter defines and predicts the Blood-Brain Barrier likeness (BBB) of the molecule as presented in (table 2.5). According to this rule, the calculated molecular weight (MW) should be 400 or less, with eighth (8) or less hydrogen bonds (TOHB) (sum of hydrogen bond donors (HBD) and the hydrogen bond acceptors) (Kerns and Di, 2010; Pardridge, 2012, 2005), and no acid groups in the structure for it to cross the BBB, with the absence of an acidic group in particular increasing BBB permeation (Ducharme *et al*., 2005).

Table 2.5: Important descriptors and values to satisfy BBB likeness rule.

Name of the rule	(MW)	(NAC)	(TOHB)
BBB likeness rule	≤ 400	No Acids	≤ 8

2.11 PHYSICOCHEMICAL PROPERTIES (MOLECULAR DESCRIPTORS)

It is now possible to establish reliable physicochemical properties (molecule descriptors) for a fairly wide variety of molecules. The effects of a chemical species on a living organism or its distribution in the environment are controlled by the

physicochemical properties of the chemical species. The important physicochemical properties include; partition coefficient, aqueous solubility, vapours pressure and dissociation constant. Although all of these properties can be measured, the use of appropriate software tools saves time and resources, also by using quantitative structure permeability relationships (Dearden, 2012; Moss *et al*., 2002). The molecular descriptors are often derived mathematically from either the 2D or the 3D molecular structure or their associated physicochemical properties. There are thousands of such descriptors available, and numerous software programs are available for their calculation. Experimental descriptor values for many known molecules are also available in many books and compendia (Dearden, 2012).

Chemical molecular descriptors can be used to establish a mathematical relationship with quantitative biological activity. This is because certain responses and mathematical expressions can be used to predict the biological response from other chemical descriptors. This mathematical expression is referred to as a quantitative structure-activity relationship (QSAR). The resulting mathematical relationship that is established between the chemical molecular descriptors and physicochemical properties is referred to as a quantitative structure-property relationship (QSPR). The physicochemical properties of chemical compounds between different phases can be successfully described by the modelling of the structure information (Shankar *et al*., 2014).

2.12 AIM OF THE STUDY

Previous studies have shown that *L. Leonurus* extracts have cardiovascular effects with differences in the effects observed with different extracting solvents. However the compounds that are responsible for the cardiovascular activity observed with the plant extracts remain largely unknown. The aim of this study was therefore to investigate the cardiovascular activity of five (5) diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) isolated from *L. Leonurus* extracts in anaesthetized normotensive Wistar rats. The study will also attempt to predict their receptor binding affinity to different

receptors, as well as predicting their oral bioavailability and Drug-likeness by using Chemoinformatics techniques (*In-silico*).

2.13 HYPOTHESES

Considering the abundance of diterpenoids in the plant it is hypothesized that;

- More than one diterpenoids is responsible for the different cardiovascular effects observed with *Leonotis Leonurus* extracts.

- There is a correlation between the predicted cardiovascular effects of the compounds following Chemoinformatics studies and actual cardiovascular effects obtained from *in-vivo* studies in normotensive Wistar rats.

2.14 OBJECTIVES

The objectives of this study were to:

1. Perform Chemoinformatics studies (*in-silico*) on five (5) diterpenoid compounds isolated from *Leonotis Leonurus*.

    a. Calculate the molecular descriptors (physicochemical properties).
    b. Predict the Drug-likeness for the isolated compounds.
    c. Study each of the five (5) isolated diterpenoid compounds within the active site of a receptor by using Molecular Docking (MD) software(s), in order to predict their binding mode (interactions) and affinity with available three-dimensional structures of different receptors.

2. Evaluate the cardiovascular properties of each of the five (5) isolated diterpenoid compounds on normotensive rats.

3. Compare the results from both studies, to investigate the correlation between Chemoinformatics studies (*In-silico*) for those compounds and their cardiovascular effects in SHR (*In-vivo*).

CHAPTER THREE

3   *IN-SILICO* STUDIES

This chapter will list the material and software used in the *in-silico* studies. The chapter will also describe the various Chemoinformatics techniques that were used in the Drug-likeness and the binding affinity studies of the diterpenoid compounds. This chapter will also provide details of the experimental preparation and procedure for both Drug-Likeness and Molecular Docking (MD) experiments.
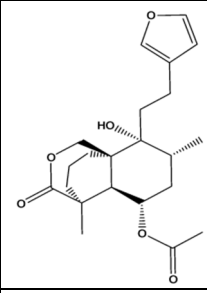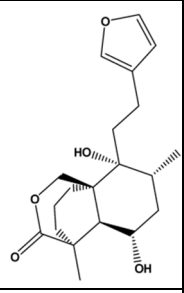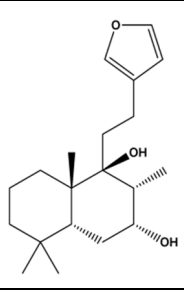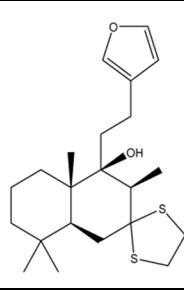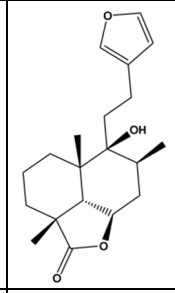
3.1   MATERIALS

The following software were used in this study: The ChemDraw® Ultra 13.0 (Cambridgesoft, USA) software was used to draw the various chemical structures, while the Chem3D® Ultra 13.0 (Cambridgesoft, USA) was used to convert the sketched structures to three dimension (3D) structures. Molecular operating environment (MOE) 2013 software (Chemical Computing Group Inc, US) was used to visualize, prepare ligands, prepare receptors, perform molecular docking, calculate molecular descriptors and visualize and analyse the docking result. Discovery Studio® 0.4software (Client, USA) was also used to visualize molecular docking. Lastly, 3D crystal structure models of the targets (receptors) were downloaded from worldwide protein data bank (PDB) (http://www.rcsb.org).

3.1.1   Ligand preparation for Drug-likeness and docking

The diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) isolated from *L. Leonurus* were sketched and saved as .MDL (Mol file format) using ChemBioDraw® Ultra 13.0 (Cambridgesoft, USA). The structures of the ligands prepared and used for this study are presented in Table 3.1 below.

Table 3.1: Structures of five (5) Diterpenoid compounds found in *L. Leonurus*.

Code	DC1	DC2	DC8	DC9	DC15
Structure					
Name	Dubiin	Saponified-Dubiin	Hispanol	DC9	Marrubiin

The sketched structures were then converted to three-dimensional (3D) structures (Figure 3.1). The energy of the 3D structures were then minimized with the use of MM2 Force field in ChemBio3D® Ultra 13.0 (Cambridgesoft, USA) software. The main goal of 3D structure preparation was to correct and fix the structural data to be ready for molecular descriptor calculations and docking.
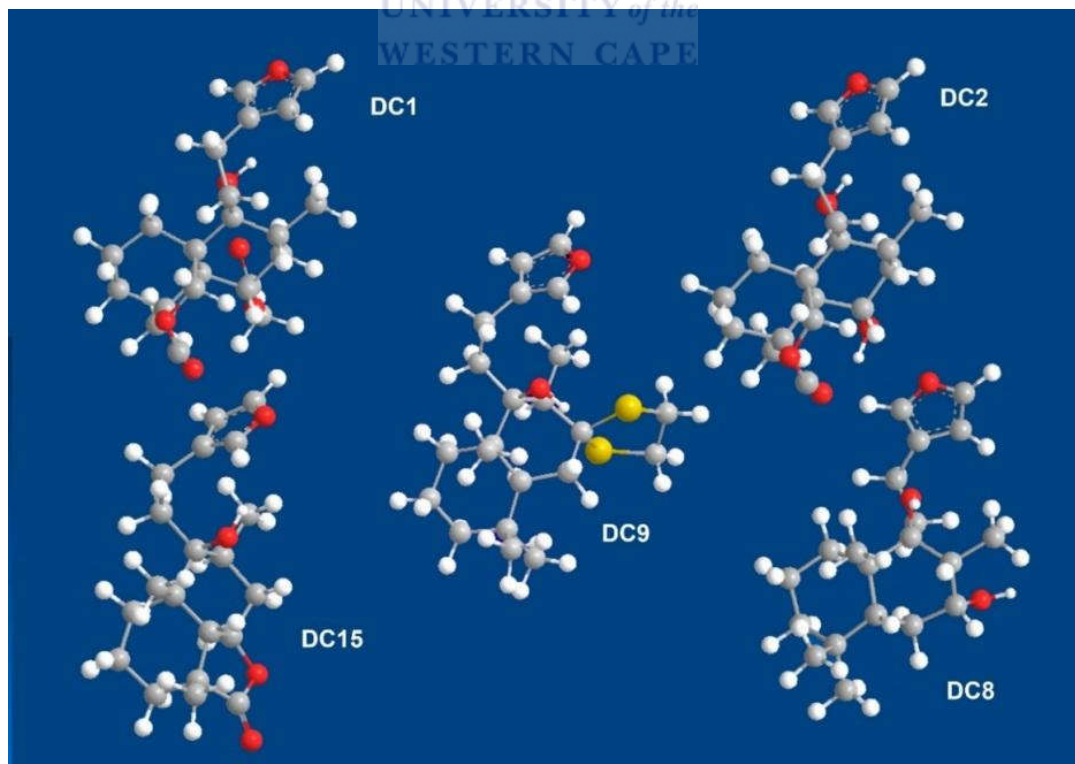
Figure 3.1: 3D structures of five (5) diterpenoid compounds found in *L. Leonurus*.

3.1.2   Receptor preparation for docking

Three-dimensional (3D) experimentally determined X-ray crystal structures of target receptors in complex with their ligands were downloaded from the protein data bank. Receptors used in this study were Renin (PDB: 4XX3), Angiotensin converting enzyme (ACE) (PDB: 2X8Z), Angiotensin II receptor (AT_1) (PDB: 3R8A) and β1 adrenoceptor (PDB: 2Y04) (Figure 3.2).

Renin in complex with 70X (PDB: 4XX3)

Angiotensin converting enzymes (ACE) in complex with Captopril (PDB: 2X8Z)

Angiotensin II (ANG II) Receptor in complex with HIG (PDB: 3R8A)

β1 receptor in complex with Salbutamol (PDB: 2Y04)
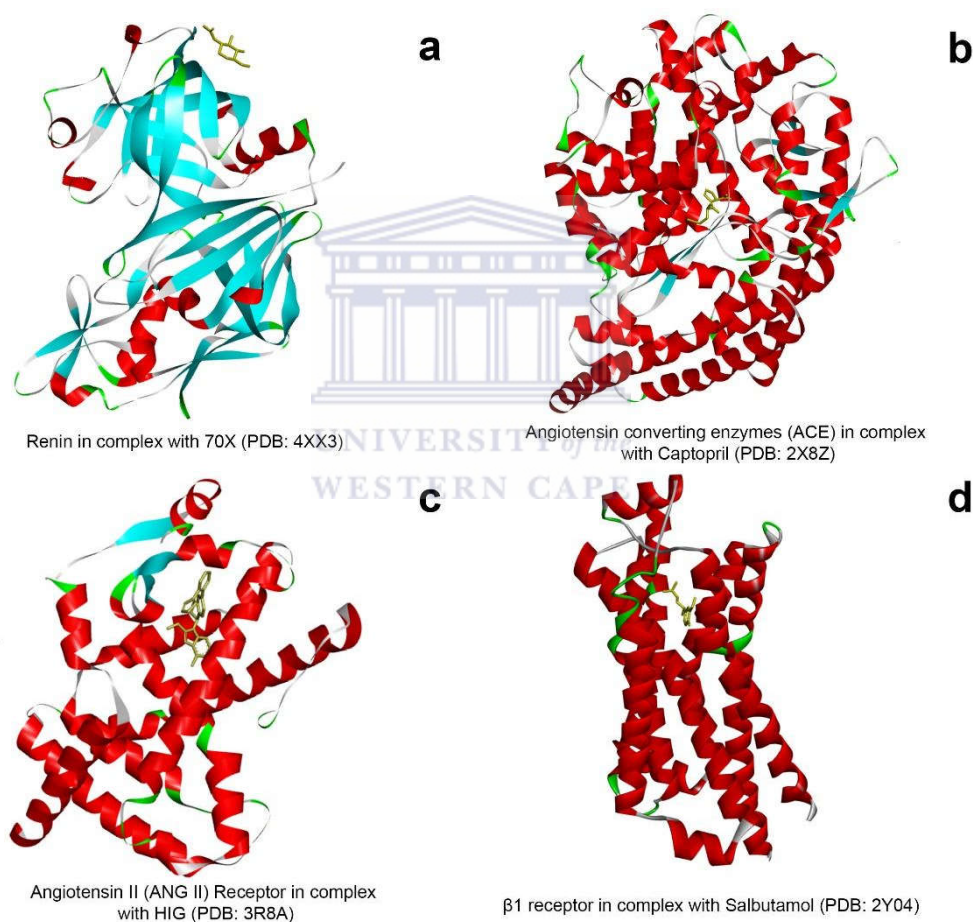
Figure 3.2: 3D structures of receptors in complex with their native ligands

Figure shows 3D structures of a) renin (PDB: 4XX3) in complex with 70X, b) Angiotensin converting enzyme (ACE) in complex with Captopril (PDB: 2X8Z), c) Angiotensin II receptor (AT_1) receptor in complex with HIG (PDB: 3R8A) and, d) β1 adrenoceptor with in complex with Salbutamol (PDB: 2Y04).

To prepare the receptors for docking, the (3D) receptor structures were downloaded from the protein data bank (http://www.rcsb.org), and then loaded to MOE. The preparation of the receptors involved the following processes; removal of all water molecules, addition of hydrogen atoms, completion of residues with missing atoms, selection of appropriate alternate locations and calculation of partial charges. In MOE these structural issues were automatically corrected using the structure preparation application.

3.2   METHODS

Chemoinformatics analysis and docking study was performed on DC1, DC2, DC8, DC9 and DC15, diterpenoid compounds isolated from *L. leonurus*. Computational software was used to predict the oral bioavailability and Drug-likeness for each compound, as well as the binding affinity for each compound with available receptors (Renin, Angiotensin converting enzyme, Angiotensin II receptor and β1 adrenoceptor).
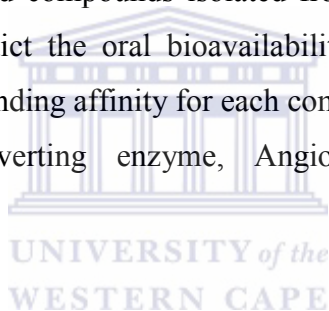
3.2.1   DRUG-LIKENESS STUDY

The data analysis was performed using twelve widely used molecular descriptors (Physicochemical properties), including molecular weight (MW), number of atoms (NAT), number of hydrogen bond donors (HBD), number of hydrogen bond acceptors (HBA), total number of hydrogen bonds (TOHB), molecular polar surface area (PSA), molar refractivity (MORF), number of rotatable bonds (ROTB), number of rings (NRING), partition coefficient (LOGP), number of rigid bonds (RIGB), and number of acids (NAC). These molecular descriptors were chosen on the basis that they have all been shown to influence the Drug-likeness of molecules. The data pre-processing and descriptor calculations were done with Molecular Operating Environment software 2013 (MOE). Based on molecular descriptor calculations , five (5) different Drug-likeness rules (Lipinski's rule of five, Ghose filter, Veber filter, Blood-Brain

Barrier likeness (BBB) and MDDR-like rule) were employed to predict the oral bioavailability of the compounds (see Chapter two).

3.2.2   MOLECULAR DOCKING STUDY

To identify the molecular binding mode (interactions) of the molecules within the receptor, all the five (5) optimized molecules (DC1, DC2, DC8, DC9 and DC15) were docked against the 3D structures of β1 adrenoceptor (PDB: 2Y04), renin receptor (PDB: 4XX3), Angiotensin converting enzyme (ACE) (PDB: 2X8Z) and Angiotensin II type I (AT$_1$) receptor (PDB: 3R8A). The Docking procedure was performed in MOE by default parameters. The binding site was identified by specifying the atoms of a co-crystallized ligand (native ligand) presented in the pocket while the native ligand atoms were ignored by the software during the docking procedure. For each molecular species, a number of placements called poses were generated and scored. The scores were then calculated as a free energy of binding (ΔG$_b$) and the final ten (10) highest scoring poses (conformations) for each molecule along with their scores and binding energies (ΔG$_b$) were collated into a database. The database file generated from the docking procedure was further analysed, with the binding mode (interactions) of the highest ten (10) conformations for each docked molecule in the active site visualized and studied with the help of MOE visualization window. Green stick rendering was added to the native ligands (70X, Captopril, HIG, and Salbutamol) obtained from the PDB files, while the isolated diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) were marked in yellow stick style for better contrast and to enable the study of the interactions of these docked compounds within the receptor active site. Among the visualization of the conformation generated from the docking for each molecule the conformation with the best binding mode (interactions) with the lowest binding energy (ΔG$_b$) was selected for further analysis.

Before docking the isolated diterpenoid compounds the molecular docking (MD) with MOE was performed between the receptors and their native ligands (70X, Captopril, HIG, and Salbutamol) to validate the docking protocol by calculating the root mean

square deviation (RMSD). The binding energy (ΔG_b) and the binding mode (interaction) for the native ligands were also calculated and analysed.

3.3   RESULTS AND DISCUSSION

3.3.1   Drug-Likeness

Molecular physicochemical properties (molecular descriptors) and the Drug-Likeness are the two properties that are significant for considering a compound to become a successful drug candidate. Twelve important molecular descriptors for five (5) diterpenoid compounds found in *L. Leonurus* (DC 1, DC 2, DC 8, DC 9, and DC 15) were calculated and summarized in Table 3.2. The molecular descriptors were used to determine if the molecules satisfied the Drug-Likeness criteria based on existing rules. In order to determine the numerical and structural identity associated with each molecular descriptor, MOE software was used to calculate the molecular descriptors (physicochemical properties) for each compound. The 2D structure for the compounds has been presented in (Table 3.1) and the 3D structure presented in (Figure 3.1).

Table 3.2: Physicochemical properties (Molecular descriptors) calculation for five compounds isolated from *L. Leonurus* isolated diterpenoid compounds.

Code	MW	MF	TOHB	HBD	HBA	LOGP	NAT	MORF	NRING	RIGB	ROTB	PSA
DC1	391.48	$C_{22}H_{30}O_6$	4	1	3	3.26	58	83.22	4	20	7	85.9
DC2	349.44	$C_{20}H_{28}O_5$	5	2	3	2.69	53	74.07	4	20	6	79.9
DC8	320.47	$C_{20}H_{32}O_3$	4	2	2	4.17	55	90.82	3	16	5	53.6
DC9	394.64	$C_{22}H_{34}O_2S_2$	2	1	1	5.99	60	94.27	4	21	5	33.9
DC15	332.44	$C_{20}H_{28}O_4$	3	1	2	3.72	52	71.15	4	19	3	59.6

3.3.1.1   Drug-likeness - compound DC1

In Table 3.2, the molecular descriptors for DC1 ($C_{22}H_{30}O_6$) showed that the compound has a molecular weight of 391.484 g/mol. The results also indicate the presence of aromatic moieties with four (4) rings and twenty (20) rigid bonds, which have seven

(7) rotatable origins (Table 3.2). DC1 can form 4 (four) hydrogen bonds. The calculated partition coefficient of DC1 predicted value (LOGP) was 3.2644 while its molar refractivity value (MORF) was calculated to be 83.22. Also, the polar surface value (PSA) indicated 85.97 and the total number of atoms (NAT) was 58.

The Lipinski's rule can be used to investigate the oral bioavailability and Drug-Likeness of a compound. In Table 3.2, the LOGP, MW, and TOHB values for DC1are 3.2644, 391.484 g/mol and 4 respectively. According the Lipinski's rule as described in Table 2.1, it was observed that the selected molecular descriptors fulfilled the Lipinski's rule. As a result, DC1 complied with Lipinski's rule and exhibited nil violation of rule.

In order to further validate the Drug-Likeness of DC1 (Table 3.2), the Ghose filler rule as described in Table 2.2, was used to establish the molecular descriptor within specific qualifying ranges. According to the Ghose rule, DC1 partition coefficient (LOGP = 3.2644) was within the preferred range (0.4 - 5.6). The molecular weight (MW) was found to be 391.484 g/mol while the molar refractivity (MORF) was 83.22. The Ghose range for MW is between 160 and 480 g/mol and the corresponding range for MORF is between 40 and 130. This implies that DC1 satisfies the Ghose rule. The total number of atoms (NAT) for DC1 was 58 and lies within the Ghose preferred range (20 - 70). In summary, DC1 complied with the Ghose rule.

Veber rule was also used to investigate the Drug-Likeness of DC1 as described in Table 2.4. The most important molecular descriptor parameters used in this rule are ROTB and PSA. In Table 3.2, the ROTB and PSA values for DC1 are seven (7) rotatable bonds and 85.97 respectively. The Veber rule stipulates that the preferred range of rotatable bonds must not be more than ten (10) rotatable bonds and the PSA value must be in the range of ≤140 Å2. The calculated ROTB and PSA for DC1s showed that the Veber rule was obeyed.

Oprea (2000) suggested that the probability of finding a Drug-Like compound is higher provided the compounds passed all the criteria in MDDR-like rule. DC1 contains

seven (7) rotatable bonds (ROTB), while the preferred value is six (6) or more rotatable bonds (ROTB ≥ 6). DC1 also contains twenty (20) rigid bonds which lies within the preferred range (RIGB ≥ 18), and four (4) aromatic rings (AROM) which also lies within the preferred range (AROM ≥ 3). The MDDR-like rule was obeyed by compound DC1.

With respect to the Blood-Brain Barrier (BBB) rule, as shown in Table 3.2, the calculated values for the MW and TOHB are 391.484 g/mol and 4 hydrogen bonds respectively. These values are within the accepted range for MW (≤ 400) and TOHB (≤ 8) (Pardridge, 2005, Pardridge, 2012, Kerns and Di, 2010). The BBB result indicates that there are no acid groups present in the DC1 structure. This is because the absence of an acidic group has been reported to increase BBB permeation of compounds (Ducharme *et al*., 2005). The Drug-Likeness for DC1 according to the rules (Lipinski's rule, Ghose filter, Veber filter, Blood-Brain Barrier (BBB) likeness and MDDR-like rule) are summarized in Table 3.3.

As presented in Table 3.3, the molecular descriptors analysis for DC1 showed that DC1 passed all the five (5) rules. This indicates that DC1 has a high probability of being a good Drug-like candidate, with good oral bioavailability after oral administration, and penetration of the Blood-brain barrier (BBB).

3.3.1.2 Drug-likeness - compound DC2

As presented in Table 3.2, the molecular descriptors for DC2 ($C_{20}H_{28}O_5$) indicated that the compound has a molecular weight of 349.447 g/mol. The MOE software was used to determine detailed information on the structural configuration of DC2. DC2 has a structural configuration that contains four (4) aromatic rings, twenty (20) rigid bonds with six (6) rotatable origins and five (5) hydrogen bonds. The calculated partition coefficient (LOGP) of DC2 was 2.6936, while its molar refractivity value (MORF) was calculated to be 74.07. The polar surface value (PSA) was 75.99 and the total number of atoms (NAT) was 53.

The oral bioavailability and Drug-Likeness of DC2 was determined using the Lipinski's rule. In Table 3.2, the LOGP, MW, and TOHB values for DC2 were 2.6936, 349.447 g/mol and five (5) respectively. The values are within the range of the Lipinski's rule as described in (Table 2.1). It was observed that the selected molecular descriptors fulfilled the Lipinski's rule.

The Ghose rule was used to validate the Drug-likeness of DC2 (Table 3.2) and to determine if the molecular descriptors are within specific qualifying ranges. According to the Ghose rule as described in (Table 2.2), the partition coefficient (LOGP = 2.6936) was within the preferred range (0.4 - 5.6). Molecular weight (MW) was found to be 349.447 g/mol and the molar refractivity (MORF) was 74.07, all well within the preferred ranges (MW; 160 - 480 g/mol: MORF; 40 - 130). The total number of atoms (NAT) in DC2 was 53, and also within the reference range (20 - 70). The use of Ghose rule to investigate DC2 showed that it agreed with all the conditions (LOGP, MORF, MW and NAT) of the rule.

Veber rule was also used to investigate the Drug-Likeness of DC2 by investigating its ROTB and PSA values which are two important parameters that are considered using this rule as described in (Table 2.4). The ROTB and PSA values for DC2 as presented in (Table 3.2) are six (6) rotatable bonds and 75.99 respectively. According to this rule, the preferred range of rotatable bonds must not exceed ten (10) rotatable bonds and the PSA value must be less than or equal to 140 Å2. DC2 thus obeyed the Veber rule.

Oprea (2000), suggested that the probability of finding a Drug-like compound is higher especially if such the compounds fulfilled all the criteria in MDDR-like rule as described in Table 2.3. In the case of DC2, the Lipinski's, Ghose and Veber rules were all obeyed (Table 3.2). DC2 contains 6 rotatable bonds (ROTB) (preferred range: ROTB ≥ 6). DC2 contains twenty (20) rigid bonds (preferred range: RIGB ≥ 18), and has four (4) rings (AROM) (acceptable range: AROM ≥ 3).The MDDR-like rule was obeyed by compound DC2.

DC2 was investigated to predict its Blood-Brain Barrier (BBB) permeation as described in Table 2.5. It was observed that DC2 satisfied the BBB rule for its MW and TOBH values. The calculated values for the MW and TOHB were 349.447 g/mol and five (5) hydrogen bonds respectively. These values were within the accepted range for MW and TOHB ($\leq$ 400 g/mol and $\leq$ 8 hydrogen bonds respectively) (Pardridge, 2005, Pardridge, 2012, Kerns and Di, 2010). From the BBB result, it can be deduced that DC2 does not contain any acid group in its structural configuration. This is because the absence of an acidic group has been reported to increase BBB permeation of compounds (Ducharme *et al*., 2005). The Drug-Likeness for DC2 according to the rules (Lipinski's rule, Ghose filter, Veber filter, Blood-Brain Barrier likeness (BBB) and MDDR-like rule) are summarized in Table 3.3.

As presented in Table 3.3, the molecular descriptors analysis for DC2 showed that DC2 passed all the five (5) rules. This indicates that DC2 has a high probability of being a good Drug-like candidate, with good oral bioavailability after oral administration, and permeation through the Blood-Brain Barrier (BBB).
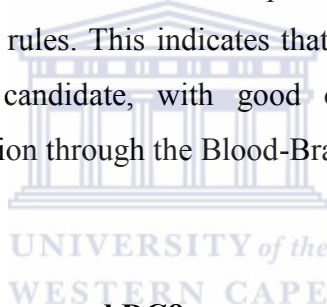
3.3.1.3 Drug-likeness - compound DC8

As presented in Table 3.2, compound DC8 has the following molecular descriptors values; MW (320.473 g/mol), TOHB four (4), three (3) rings and sixteen (16) rigid bonds, LOGP (4.1766), MORF (90.82), PSA (53.69) and NAT (55). The structure for DC8 was determined to be $C_{20}H_{28}O_5$. It was observed that all the molecular descriptors values for DC8 satisfied the Lipinski's rule as described in Table 2.1. With respect to the Ghose rule, the Drug-likeness indicators of DC8 were within the accepted range of the rule as described in Table 2.2. According to this rule, the molecular descriptors values for DC8 which include partition coefficient i.e. LOGP, MW, MORF, NAT all obeyed the rule (Table 3.2). With respect to the Veber rule (Table 2.4), the ROTB and PSA values for DC8 compound were five (5) rotatable bonds and 53.69 respectively. These values are within the limits of the Veber rule thus DC8 satisfied the rule.

DC8 was investigated to predict its Blood-Brain Barrier (BBB) permeation as described in Table 2.5. The calculated values (Table 3.2) for MW and TOHB were 320.473 g/mol and four (4) hydrogen bonds respectively. These values were within the accepted range for MW and TOHB (Pardridge, 2005, Pardridge, 2012, kerns and Di, 2010). DC8 does not contain acid group thus DC8 satisfied the BBB rule (Ducharme *et al.*, 2005).

Unlike DC1 and DC2, it was observed that DC8 did not satisfy the MDDR-Like rule as it violated two (2) of this rule's criteria. According to Oprea (2000), DC8 is more likely to be non-Drug-like because it contains five (5) rotatable bonds (ROTB) which is out the preferred range of ≥ 6 ROTB. DC8 also contains sixteen (16) rigid bonds which was out of the preferred range as the minimum value for rigid bonds is eighteen (18) (RIGB ≥ 18). The structural configuration results for DC8 showed that it contained three (3) aromatic rings (AROM) which lies within the preferred range (AROM ≥ 3). The Drug-Likeness for DC8 according to the rules are summarized in Table 3.3.

As presented in Table 3.3, the molecular descriptors analysis for DC8 showed that the molecule (DC8) passed four (4) rules i.e. Lipinski's rule, Ghose filter, Veber filter, and Blood-Brain Barrier likeness. However, DC8 violated two of the three criteria for the MDDR-Like rule. The result suggests that DC8 can be predicted to have a good probability of being a good Drug-Like candidate, good oral bioavailability after oral administration, and pass the Blood-Brain Barrier (BBB).

3.3.1.4   Drug-likeness - Compound DC9

From Table 3.2, the molecular descriptors for DC9 showed that the compound has a molecular formula and weight as $C_{22}H_{34}O_2S_2$ and 349.447 g/mol respectively. DC9 contains aromatics moieties of four (4) rings, 21 rigid bonds (Table 3.1) and five (5) rotatable origins. As a result, DC9 can form two (2) hydrogen bonds. The calculated

partition coefficient value (LOGP) of DC9 was 5.992 and its MORF value was calculated to be 94.27. The PSA and NAT values were 33.97 and 60 respectively.

The application of Lipinski's rule to determine the oral bioavailability and Drug-Likeness of DC9 (Table 3.2) showed that the compound violated one of the four criteria for Lipinski's as described in Table 2.1. However, the Lipinski's rule states that if a compound violates one criteria, its Drug-likeness properties may not be affected (Lipinski *et al*., 1997; Lipinski, 2004). The LOGP value of DC9 was out of the Lipinski's rule preferred range because it was higher than 5 whereas the rule stipulates that a compound must be equal to or less than five (5). The MW of DC9 (349.447 g/mol) showed that it was within the preferred range (160 to 480 g/mol). DC9 has the possibility to form two (2) hydrogen bonds hence it agreed with Lipinski's rule that the total hydrogen bond (TOHB) of a compound must not exceed five (5). DC9 passed three (3) of four (4) criteria and according to this rule, a compound is more likely to exhibit poor absorption or permeation if the compound violated two or more of the mentioned physicochemical criteria in Table 2.1. This therefore means that DC9 complied with the Lipinski's rule for oral bioavailability and Drug-Likeness.

The Ghose filter was used to analyse the Drug-likeness of DC9 and the molecular descriptor within the specific qualifying ranges of Ghose rule as described in Table 2.2. The molecular weight (MW) was found to be 394.644 g/mol, while the molar refractivity (MORF) was 94.27. The Ghose filter range for MW is between (160 and 480 g/mol) and the corresponding range for MORF is between 40 and 130. The partition coefficient (LOGP) for DC9 (5.992) was out of the Ghose filter preferred range (20 - 70). The result showed that the molecular descriptors for DC9 violated one of the Ghose filter criteria which is the LOGP value.

The Veber rule (Table 2.4) was used to investigate the molecular descriptor parameter for DC9 for its ROTB and PSA values. In Table 3.2, the ROTB and PSA values for DC9 were five (5) rotatable bonds and 53.69 respectively. The Veber rule requires the preferred range of rotatable bonds to be no more than ten (10) while the PSA value

must be ≤140 Å2. The calculated ROTB and PSA for DC9 was five (5) and 53.99 respectively hence the Veber rule was obeyed for this compound.

As described in Table 2.3, DC9 was investigated in order to determine if it obeyed MDDR-like rule. The structure of the compound indicated that it contained five (5) rotatable bonds (ROTB), with the preferred range of at least six (6) rotatable bonds or more (ROTB ≥ 6). DC9 also contained 21 rigid bonds and this agreed with the preferred range i.e. minimum of 18 or more (RIGB ≥ 18). DC9 contained four (4) rings (AROM) which also lies within the preferred range i.e. equal to three (3) or more (AROM ≥ 3). In summary, molecular descriptors for DC9 violated the MDDR-like rule criteria as it contained a lower number of rotatable bonds ROTB.

DC9 was investigated to predict its Blood-Brain Barrier (BBB) permeation as described in Table 2.5. It was observed that DC9 satisfied the BBB rule. For instance, in Table 3.2, the calculated values for the MW and TOHB were 394.644 g/mol and two (2) hydrogen bonds respectively. These values are within the accepted range for MW and TOHB (≤ 400 and ≤ 8 respectively) (Pardridge, 2005, Pardridge, 2012, Kerns and Di, 2010). The BBB result is significant as it serves as an indicator to show that DC9 does not contain acid groups in its structure which has been attributed to decrease permeation capacity of compound (Ducharme *et al*., 2005).

The Drug-Likeness for DC9 according to the rules i.e. Lipinski's rule, Ghose filter, Veber filter, Blood-Brain Barrier likeness (BBB) and MDDR-like rule are summarized in Table 3.3.

As presented in Table 3.3, the molecular descriptor analysis for DC9 showed that the compound passed three (3) rules which include Lipinski's rule of five, Veber filter, and Blood Brain Barrier likeness, while it violated the MDDR-Like rule and Ghose filter. Although the Lipinski's rule of five, Veber filter, and Blood Brain Barrier likeness results predict that DC9 would have a good oral bioavailability after oral administration and pass the Blood-Brain Barrier (BBB), the compound has a lower

possibility of being a Drug-Like molecule due to failure of the Ghose filter and MDDR-Like rule.

3.3.1.5   Drug-likeness - compound DC15

The following molecular descriptors for DC15 are shown in Table 3.2, with the molecular formula $C_{20}H_{28}O_4$ and the MW i332.440 g/mol. The molecular formula implies that the structural configuration of DC15 contains aromatic moieties with four (4) rings, nineteen (19) rigid bonds (Table 3.1) and three (3) rotatable origins. Therefore, DC15 is capable of forming three (3) hydrogen bonds. The calculated partition coefficient value (LOGP) for DC15 was 3.721, while its molar refractivity (MORF) value was calculated to be 71.15. The polar surface (PSA) value was 59.67 and the total number of atoms (NAT) was 52.

DC15 was analysed using the Lipinski's rule to investigate its oral bioavailability and Drug-Likeness. In Table 3.1, DC15 values for LOGP, MW, and TOHB were 3.721, 332.440 g/mol and 3 respectively. These values were observed to agree with the Lipinski's rule as described in Table 2.1 hence DC15 did not violate any of the rule criteria.

With respect to the Ghose filter rule for its Drug-likeness as described in Table 2.2, the partition coefficient for DC15 (3.721)was within the preferred range (0.4 - 5.6). The molecular weight (MW) (332.440 g/mol) and molar refractivity (MORF) (71.15) were within the ranges (160 - 480 g/mol for MW and 40 - 130 for MORF). This implies that DC15 satisfied the Ghose rule. The total number of atoms (NAT) for DC15 was 52 and lies within the Ghose preferred range (20 - 70). Therefore, DC15 complied with the Ghose rule.

The Veber rule was used to investigate the ROTB and PSA for DC15 which are the two most important molecular descriptor parameters in the twelve (12) descriptors as described in Table 2.4. The ROTB and PSA values for DC15 were three (3) rotatable

bonds and 59.67 respectively. The Veber rule stipulates that the preferred range of rotatable bonds must not be more than ten (10) rotatable bonds and the PSA value must be ≤140 Å2. The calculated ROTB and PSA for DC15 showed that the Veber rule was obeyed.

DC15 was investigated in order to determine if it obeyed the MDDR-like rule as described in Table 2.3. DC15 contained three (3) rotatable bonds (ROTB) which was less than the minimum six (6) rotatable bonds required. DC15 contained 19 rigid bonds and this agreed with the preferred range i.e. minimum of 18 or more (RIGB ≥ 18). The structure of this compound showed that it contained four (4) rings (AROM) which also lies within the preferred range (AROM ≥ 3). However, DC15 violated one of criteria for MDDR-Likeness i.e. RTOB, hence DC15 is more likely to be a non-Drug-like molecule (Table 2.3).

DC15 was investigated to predict its Blood-Brain Barrier (BBB) permeation as described in Table 2.5. It was observed that DC15 satisfied the BBB rule for MW and TOBH which are 332.440 g/mol and 3 respectively. These values are within the accepted range for MW (≤ 400) and TOHB (≤ 8) (Pardridge, 2005, Pardridge, 2012, Kerns and Di, 2010). The BBB result indicates that no acid groups are present in DC15 and as a result will increase the BBB permeation of compounds (Ducharme *et al*., 2005). The Drug-Likeness for DC15 according to the rules i.e. Lipinski's rule, Ghose filter, Veber filter, Blood-Brain Barrier likeness (BBB) and MDDR-like rule are summarized in Table 3.3.

As presented in Table 3.3, the molecular descriptor analysis for DC15 showed that the compound passed four (4) rules i.e. Lipinski's rule of five, Ghose filter, Veber filter, and Blood Brain Barrier likeness but violated one of three criteria of the MDDR-Like rule. The result showed that DC15 has a good probability of being a good Drug-Like candidate with good oral bioavailability after oral administration as it passed the Blood-Brain Barrier (BBB).

Table 3.3: Result of Drug-likeness prediction of *L. Leonurus* compounds.

Filters	DC 1	DC 2	DC 8	DC 9	DC 15
Lipinski's rule	Pass	Pass	Pass	Pass	Pass
Ghose filter	Pass	Pass	Pass	Fail	Pass
MDDR-like rule	Pass	Pass	Fail	Fail	Fail
Veber filter	Pass	Pass	Pass	Pass	Pass
BBB likeness	Pass	Pass	Pass	Pass	Pass

3.3.2   MOLECULAR DOCKING

Molecular docking (MD) is a method used to understand the binding interactions (binding modes and binding affinity) between the active site of the targeted receptor and the test compound. In this study, MD analysis was performed on DC1, DC2, DC8, DC9 and DC15 (Table 3.1). The MD analysis was used to understand the binding modes and estimate the binding affinity of DC1, DC2, DC8, DC9 and DC15 against the 3D structure of receptors downloaded from the protein data bank (PDB) coded as PDB: 2Y04, PDB: 4XX3, PDB: 3R8A and PDB: 2X8Z. The receptors have been presented and described in Figure 3.2.

3.3.2.1   Accuracy of the docking protocol

Molecular docking (MD) with MOE was performed between the receptor and their native ligands to validate the docking protocol. This approach is often done by calculating the root mean square deviation (RMSD) (Chen *et al.*, 2007; Temirak *et al.*, 2014; Wang *et al.*, 2003). The RMSDs values between the re-docked poses and the original poses of the native ligands are indicative of whether the docking protocol is accurate, with values under 2 Å indicative of an accurate protocol. If the RMSD of the best docked conformation of the native ligand is 2.0 Å or less from the experimental one (native ligand), the used scoring function (protocol) is successful (Wang *et al.*, 2003). The obtained pose from re-docking the native ligands with their receptors were well correlated with the original poses, with small RMSD values (Figure 3.3).
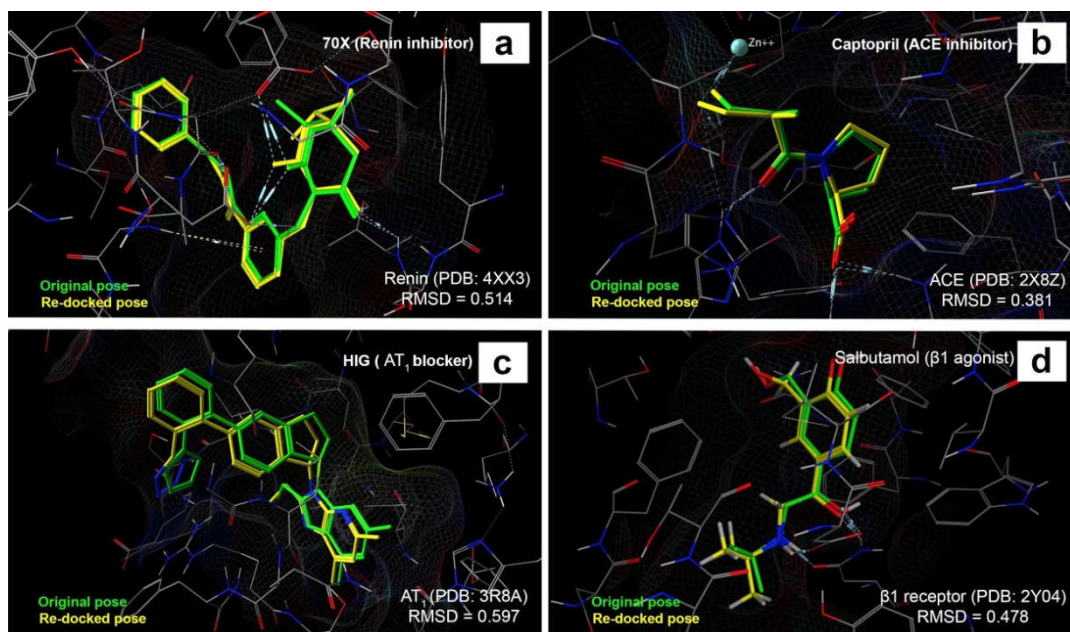
Figure 3.3: Root-mean-square deviation between the original poses and the re-docked poses

Figure shows the Root-mean-square deviation (RMSD) between the original poses (Green) and the re-docked poses (Yellow). a) RMSD value (0.514 Å) between the re-docked pose (Yellow) and the original pose (Green) of the native ligand (70X) in renin active site (PDB: 4XX3). b) Shows RMSD value (0.381 Å) between the re-docked pose (Yellow) and the original pose (Green) of the native ligand (Captopril) in ACE (PDB: 2X8Z) active site. c) RMSD value (0.597 Å) between the re-docked pose (Yellow) and the original pose (Green) of the native ligand (HIG) in AT_1 receptor (PDB: 3R8A) active site. d) RMSD value (0.478Å) between the re-docked pose (Yellow) and the original pose (Green) of the native ligands (Salbutamol) in β1 adrenoceptor (PDB: 2Y04) active site.

Molecular docking (MD) was performed between renin and it is native inhibitor (70X) which was obtained from the protein data bank (PDB: 4XX3). The RMSD value for the re-docked native ligand (Figure 3.3a) was 0.514 Å. The re-docked 70X was superimposed and this inhibitor showed that it perfectly aligns with is original position in the active site of the receptor i.e. renin. Also, the angiotensin converting enzymes (ACE) receptor was re-docked with its native inhibitor (Captopril) which was obtained from protein data bank (PDB: 2X8Z). It was observed that the RMSD value for the re-docked Captopril in the ACE active site as shown in Figure 3.3b was 0.381 Å and the active site of the re-docked Captopril which was superimposed on the receptor indicated similarity in position and binding mode as the original docking site of inhibitor. The MD procedure that was performed between Angiotensin II receptor (ANG II) and its native antagonist (HIG) (PDB: 3R8A) showed an RMSD value of

0.59 Å for the re-docked native ligand (Figure 3.3b) with the re-docked HIG perfectly superimposed with it is original position in the active site of the inhibitor. As for the β1 adrenoceptor, the molecular docking of this receptor and its native partial agonist (salbutamol) which was obtained from protein data bank (PDB: 2Y04) resulted in an RMSD value of 0.478 Å for the re-docked native ligand between salbutamol in the β1 adrenoceptor active site (Figure 3.3c). All the re-docked inhibitors showed perfect alignment (site positions) with their respective native ligands while the RMSD values of the re-docked receptors and native ligands showed close proximity. The obtained RMSDs values were summarised in Table 3.4.

Table 3.4: RMSDs values between the original poses and the re-docked poses of the native ligands.

	Target	PDB Code	Native ligand	RMSD
1	Renin	4XX3	70X	0.514 Å
2	ACE	2X8Z	Captopril	0.381 Å
3	ANG II receptor	3R8A	HIG	0.597 Å
4	β1 adrenoceptor	2Y04	Salbutamol	0.478 Å

RMSD - root mean square deviation between the original position of native ligand in the active site and the re-docked position of the native ligand. PDB - protein data bank. ACE - angiotensin converting enzymes. ANG II - Angiotensin II receptor, Å - Angstrom.

These RMSDs values obtained after re-docking the native ligands into the receptor active site (Table 3.4) indicated that the docking protocol involving MOE under our experimental conditions seems to be accurate (Wang *et al*., 2003). This is because the lower the RMSD values, the higher the resemblance to the biological co-crystallization. After the docking protocol was validated, the isolated diterpenoid compounds from *L. Leonurus* were then docked to each receptor (PDB: 4XX3, 2X8Z, 3R8A and 2Y04) and results will be presented and discussed in the following sections.

### 3.3.2.2 Docking of isolated diterpenoid compounds into Renin

The molecular docking technique was used to understand the binding modes and also estimate the binding affinity of the isolated diterpenoids i.e. DC1, DC2, DC8, DC9 and DC15s into the renin active site. The 3D structure of renin in complex with the

native inhibitor (70X) was obtained from protein data bank (PDB) and coded as PDB: 4XX3 (Figure 3.1a). Renin is an enzyme that participates in the body's renin angiotensin aldosterone system pathway (RAAS). The RAAS pathway is known to be involved in the regulation of blood volume, vascular resistance and also responsible for cardiovascular pathology. As a result, RAAS is often the target in the treatment of cardiovascular diseases (De Mello, 2014). Renin acts as an enzyme, hydrolysing angiotensinogen to angiotensin I (ANG I), which is further hydrolysed by ACE to angiotensin II a potent vasoconstrictor. For this reason, drugs known as renin inhibitors are used to lower BP. The native ligand (70X) has been reported to act as a subnanomolar inhibitor of renin (McKittrick *et al.*, 2015).

From the database file generated by the MOE software as docking result, the RMSD value between the original pose of 70X and the pose resulting from the re-docking was 0.514 Å (Table 3.4), with the generated pose from docking a close fit when superimposed on the originally embedded pose (Figure 3.3a). The binding energy (ΔG_b) value obtained was - 8.207 kcal/mol. Isolated diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) (Table 3.1) were docked into renin at similar position which was previously occupied by the native ligand (70X) (Figure 3.2a). From the database file generated by the MOE docking software, DC9 was observed to have the highest binding affinity with the lowest binding energy (ΔG_b) of -5.501 kcal/mol. The binding energy for DC1, DC2 and DC15s were -5.273, -4.335 and -4.365 kcal/mol respectively. DC8 exhibited the lowest binding affinity with the highest binding energy (ΔG_b) of -3.830 kcal/mol respectively.

The position of the native inhibitor (70X), the isolated diterpenoid compounds and their binding mode (interactions) within the active site of renin were visualized in three-dimension (3D) and analysed with the help of MOE visualization window as shown in Figure 3.4.
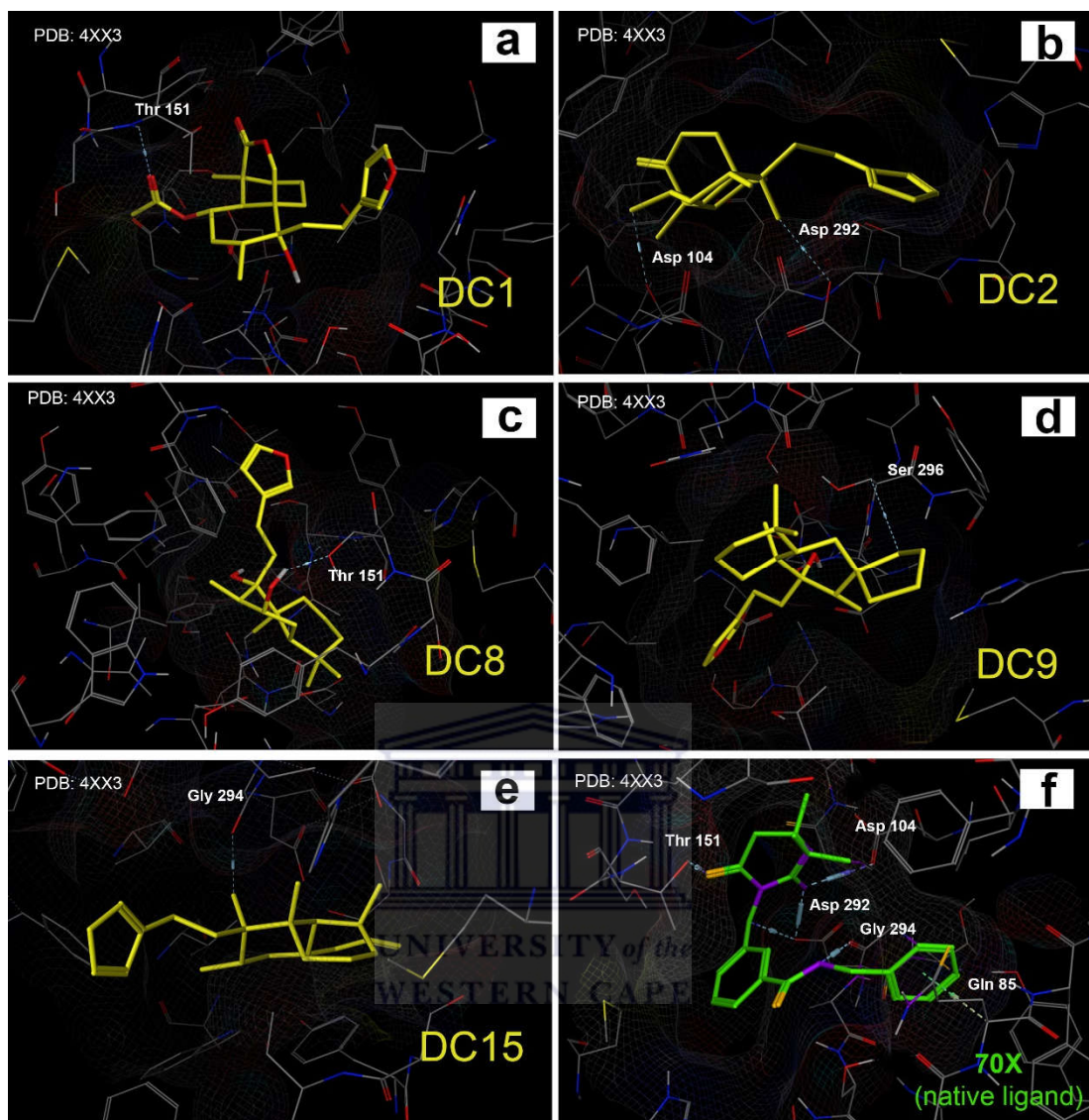
Figure 3.4: The binding mode (interactions) of *L. Leonurus* diterpenoid compounds with renin
a) DC1, b) DC2, c) DC8, d) DC9, e) DC15 (in yellow colour) and f) native renin inhibitor (70X) (in green colour) with renin (PDB: 4XX3) active site residues. The blue dash line presents the hydrogen bonds between the molecule and the receptor amino acids residues, while the yellow dash line presents the π interaction between the molecule and the receptor amino acids residues.

The visual inspection of the binding mode (interaction) of renin inhibitor (70X) within the active site of renin (Figure 3.4f; Table 3.5) showed five (5) hydrogen bond interactions with four (4) amino acids residues - Gly 294, Asp 292, Asp 104 and Thr 151. The Asp 292 residue constitutes of two (2) hydrogen bonds which are involved in the interaction with 70X and the Gln 85 residue was responsible for the π interaction with 70X.

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The binding mode (interaction) analysis of the isolated diterpenoids showed that DC1 (Figure 3.4a; Table 3.5) interacted with renin through one (1) hydrogen bonds with Thr 151 residues. In the case of DC2 (Figure 3.4b; Table 3.5), it was observed that the compound interacted with renin through two (2) hydrogen bonds with the residues Asp 292 and Asp. DC8 (Figure 3.4c; Table 3.5) it was observed to interact with renin through one (1) hydrogen bond with Thr 151 residue. The interaction between DC9 (Figure 3.4d; Table 3.5) and renin was observed to occur with one (1) hydrogen bond with Gly 294 residue. DC15 (Figure 3.4e; Table 3.5) interacted with renin through one (1) hydrogen bond with the residue Gly 294.

The isolated diterpenoids showed interactions with different amino acid residues, forming different numbers of hydrogen bonds and π interactions with renin (Table 3.5). This may be due to differences in their physicochemical properties (Table 3.2). Differences in the types and numbers of functional groups also affects the availability of atoms in the active site for these interactions to occur (Jorgensen, 1991; Lodish *et al*., 2000).

Table 3.5: List of binding energy, Hydrogen bonds and π interaction between *L. leonurus* diterpenoids and renin.

Compound	(ΔGb)	Hydrogen bond interaction		π interaction	
		Amino acid residue	TOHB	Amino acid residue	Number of π interaction
DC1	-5.273	Thr 151	1	-	0
DC2	-4.335	Asp 292 and Asp 104	2	-	0
DC8	-3.830	Thr 151.	1	-	0
DC9	-5.501	Ser 296	1	-	0
DC15	-4.365	Gly 294	1	-	0
70X	-8.207	Gly 294, Asp 292, Asp 104 and Thr 151	5	Gln 85	1

The binding energy (ΔG_b) and the binding mode (interactions) analysis of the diterpenoids were compared with the binding energy and the binding mode property of renin inhibitor (70X) in the active site of renin (Figure 3.4; Table 3.5).

The binding mode (interaction) of DC1 (Figure 3.4a) and DC8 (Figure 3.4c) in the active site of renin was similar to the interactions between 70X (Figure 3.4f) with

renin. The similarity in binding mode is such that the isolated diterpenoids (DC1 and DC8) and 70X all interacted with renin at the Thr 151 residue. DC2 (Figure 3.4b), the compound interacted with Asp 104 and Asp 292 residue which was also similar to that observed for 70X binding mode. The binding mode result of DC15 (Figure 3.4e) indicated that the interaction with renin was similar to the observed interaction for 70X in which both DC15 and 70X (renin inhibitor) interacted with Gly 294 residue. In the case of DC9 interactions with renin (Figure 3.4c) did not indicate any similarity with the binding mode (interactions) of 70X (renin inhibitor). Although the diterpenoids (DC1, DC2, DC8, DC9 and DC15) exhibited binding energy (ΔGb) with renin, the binding energy (ΔG_b) values of the diterpenoids were higher in comparison with the binding energy observed between the renin inhibitor (70X) and renin. For instance the binding energy values obtained between DC1, DC2, DC8, DC9 and DC15 and renin were -5.273, -4.335, -3.830, -5.501 and -4.365 kcal/mol which indicated that DC9 has the lowest binding energy (ΔG_b) with renin. The more the negative binding energy (ΔG_b) value, the stronger the interactions (Lim *et al*., 2011; Temirak *et al*., 2014; Thakur and Thakur, 2015). Also, the isolated diterpenoid compounds showed differences in their binding mode (interaction) with renin in comparison with the interaction between 70X (renin inhibitor) and renin. This implies that from the obtained binding energy (ΔG_b) values and the binding mode (interactions) for the isolated diterpenoid compounds, these compounds (DC1, DC2, DC8, DC9 and DC15) are not expected to have renin inhibition activity or at best to exhibit a weak renin inhibition activity when tested *in-vivo* (De Mello, 2014; McKittrick *et al*., 2015).

3.3.2.3   Docking of isolated diterpenoid compounds into ACE

The molecular docking (MD) technique was used to understand the binding modes and also estimate the binding affinity of the isolated diterpenoids with the angiotensin converting enzyme (ACE) active site. The 3D structure of ACE in complex with the native inhibitor (Captopril) was obtained from protein data bank (PDB) and coded as PDB: 2X8Z (Figure 3.1b). ACE is an enzyme that participates in the body's renin angiotensin aldosterone system (RAAS) pathway. The RAAS pathway is known to be

involved in the regulation of blood volume, vascular resistance and plays an important role in cardiovascular pathology. RAAS is often the target in the treatment of cardiovascular diseases (De Mello, 2014). ACE indirectly increases BP by converting angiotensin I (ANG I) to angiotensin II (ANG II), which constricts the vessels. For this reason, drugs known as ACE inhibitors, which exhibit inhibitory activity on the ACE, such as Captopril, are used to lower BP. Captopril is widely used clinically for the treatment of hypertension (Akif *et al.*, 2012, 2010; Amery *et al.*, 2012).

The first approach in the docking technique that was used in this study was to re-dock the native inhibitor (Captopril) into ACE (PDB: 2X8Z) and thus to calculate its RMSD and (ΔG_b). From the database file generated by the MOE software as docking result, the RMSD value between the original pose of Captopril and the pose resulting from the re-docking was 0.381 Å (Table 3.4). It is found that the generated pose from docking was a perfect fit when superimposed with the originally embedded pose (Figure 3.3b), while the binding energy (ΔG_b) value obtained was -7.958 kcal/mol. Thereafter, the compounds DC1, DC2, DC8, DC9 and DC15 (Table 3.1) were docked into ACE at a similar position which was previously occupied by the native ligand (Captopril) (Figure 3.2b).

From the database file generated by the MOE docking software, docking of DC1 into the ACE active site (PDB; 2X8Z) was observed to have the highest binding affinity with the lowest binding energy (ΔG_b) of -6.612 kcal/mol. DC9, DC15 and DC8 followed closely with binding free energy score (ΔG_b) of -6.498, -6.340 and -6.148 kcal/mol respectively. DC2 exhibited lowest binding affinity with a binding energy (ΔG_b) of -5.776 kcal/mol when compared the binding energy with the other isolated diterpenoids.

The position of the native inhibitor (Captopril), isolated diterpenoid compounds and their binding mode (interactions) within the active site of ACE were visualized in three-dimension (3D) and analysed with the help of MOE visualization window as shown in Figure 3.6.
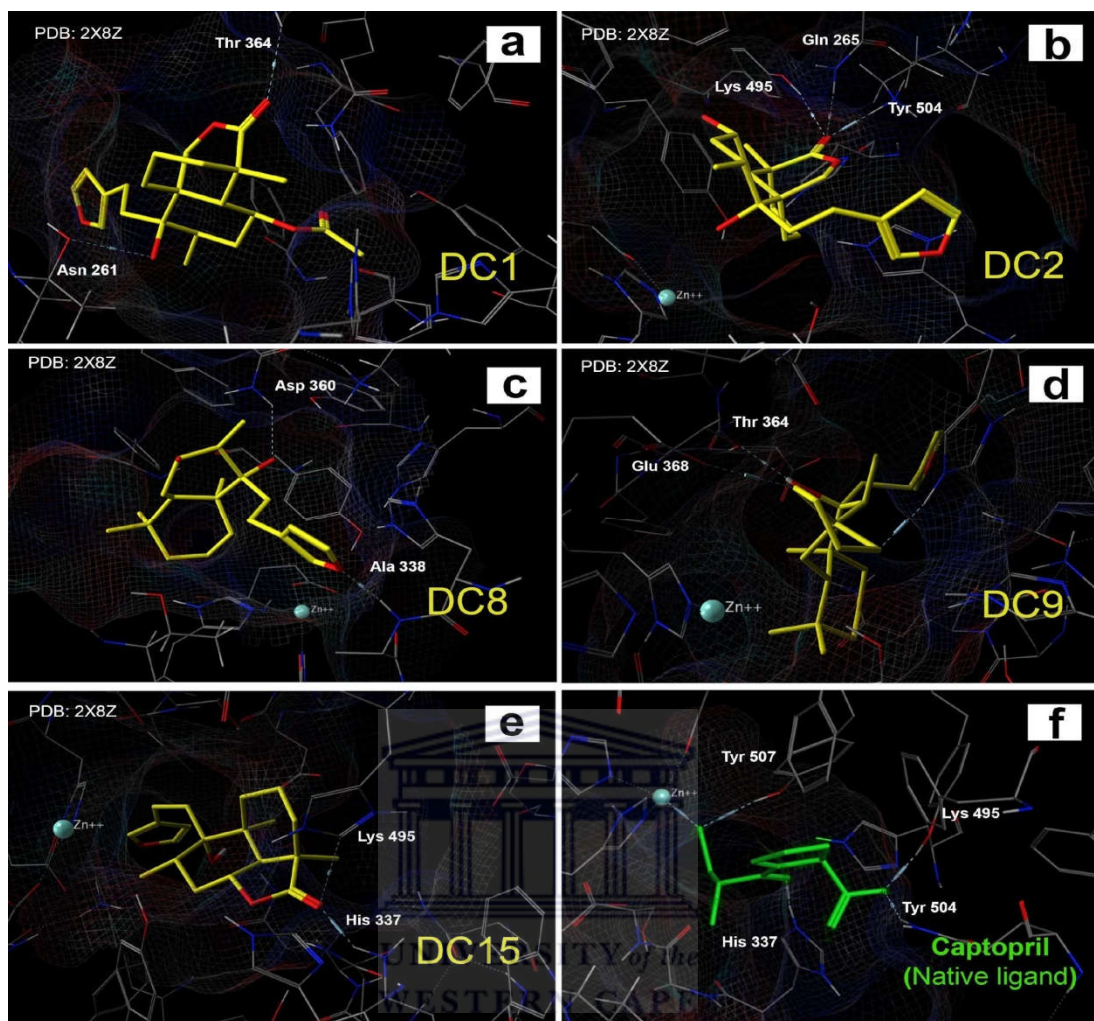
Figure 3.5: The binding mode (interactions) of *L. Leonurus* diterpenoid compounds with ACE
a) DC1, b) DC2, c) DC8, d) DC9, e) DC15 (in yellow colour) and f) native ACE inhibitor (Captopril) (in green colour) with ACE (PDB: 2X8Z) active site residues. The blue dash line presents the hydrogen bonds between the molecule and the receptor amino acids residues, while the yellow dash line presents the π interaction between the molecule and the receptor amino acids residues.

From Figure 3.5e the visualization of the binding mode (interaction) of ACE inhibitor (Captopril) within the active site of ACE, showed that the inhibitor had four (4) hydrogen bonds with four (4) amino acids (Lys 495, Tyr 504, His 337, and Tyr 507). Also the Captopril (ACE inhibitor) makes a direct interaction with the Zn^{2+} ion. The binding mode (interactions) visualization analysis of isolated diterpenoids showed that DC1 (Figure 3.5a; Table 3.6) interacted with ACE through two (2) amino acids residues (Thr 364 and Asn 261), it interacted with these residues by forming two (2) hydrogen bonds. DC2 (Figure 3.5b; Table 3.6) was observed to interact with ACE

through three (3) amino acid residues (Tyr 504, Lys 495 and Gln 265) by forming three (3) hydrogen bonds. DC9 (Figure 3.5d; Table 3.6) was observed to interact with ACE through two (2) amino acid residues (Glu 368 and Thr 364) by forming two (2) hydrogen bonds. DC15 (Figure 3.5e; Table 3.6) showed interactions with two (2) amino acid residues (His 337 and Lys 495) by forming two (2) hydrogen bonds with these residues. In the case of DC8 (Figure 3.5c; Table 3.6) it was observed to interact with ACE by forming one (2) hydrogen bonds with the residue Asp 360 and Ala 338.

The following Table (Table 3.6) presents summary of results obtained from visualizing the active site and the results obtained from the database file generated by MOE software after the docking procedures. These results include the binding energy (ΔG_b) and the binding mode (interactions) i.e. number and the name of amino acids residues responsible for the hydrogen bond as well as π interaction that were formed between the docked compounds and the ACE active site. Also the number of the hydrogen bond and number of π interactions formed are presented and discussed.

Table 3.6: List of binding energy, and list of Hydrogen bonds and π interaction formed between *L. leonurus* diterpenoid compounds and ACE.

Compound	(ΔG_b)	Hydrogen bond interaction		π interaction	
		Amino acid residue	TOHB	Amino acid residue	Number of π interaction
DC1	-6.612	Thr 364 and Asn 261	2	-	0
DC2	-5.776	Tyr 504, Lys 495 and Gln 265	3	-	0
DC8	-6.148	Asp 360 and Ala 338	1	-	0
DC9	-6.498	Glu 368 and Thr 364	2	-	0
DC15	-6.340	His 337 and Lys 495	2	-	0
Captopril	-7.958	Lys 495, Tyr 504, His 337, Tyr 507 and direct interaction with Zn2+	4	-	0

The binding energy and the binding mode (interactions) analysis of DC1, DC2, DC8, DC9 and DC15 were compared with the binding energy and the binding mode property of ACE inhibitor (Captopril) in the active site of ACE (Figure 3.5f; Table 3.6). As presented in Table 3.6 the binding mode (interaction) of DC15 (Figure 3.5e) in the active site of ACE were similar with what observed from Captopril interaction. Both

DC15 and Captopril (ACE inhibitor) interacted with His 337 and Lys 495 residues through hydrogen bonding. Unlike DC15, Captopril (Figure 3.5f) showed direct interaction with the Zn^{2+} ion, while DC15 showed no interaction with Zn^{2+}. The binding mode (interaction) of DC2 (Figure 3.5b) in the active site of ACE was similar to that observed from Captopril interaction, with both DC2 and Captopril (ACE inhibitor) interacting with Tyr 504 and Lys 495 residues through hydrogen bonding. Unlike DC2, Captopril (Figure 3.5f) showed direct interaction with the Zn^{2+} ion, while DC2 showed no interaction with Zn^{2+}. DC1, DC8 and DC9 did not show any similarity with Captopril binding mode (interactions). The isolated compounds (DC1, DC2, DC8, DC9 and DC15) exhibited binding energy (ΔG_b) with ACE, with the binding energy similar to that observed with the ACE inhibitor (Captopril), this could suggest that these isolated diterpenoids compounds could have similar affinity to the ACE receptor to that observed with Captopril (Lim *et al*., 2011; Temirak *et al*., 2014; Thakur and Thakur, 2015). On other hand, the isolated diterpenoids showed a difference in binding mode (interaction) to that observed with Captopril (ACE inhibitor). None of the isolated diterpenoid compounds interacted with the Zn^{2+} ion, while the ACE inhibitor (Captopril) makes a direct interaction with the catalytic Zn^{2+} with ACE, the success of clinically used ACE inhibitors, such as Captopril and lisinopril depend on their ability to interact directly with the zinc ion (Akif *et al*., 2010; Akif *et al*., 2012). The difference in binding mode (interaction) and the absence of direct interaction with the Zn^{2+} ion with the isolated diterpenoids may suggest that these compounds would not exhibit ACE inhibition when tested *in-vivo*.

3.3.2.4   Docking of isolated diterpenoid compounds into AT₁ receptor

The Molecular Docking technique was used to understand the binding modes and also estimate the binding affinity of the isolated diterpenoids into the Angiotensin II receptor type I (AT₁) active site. The 3D structure of AT₁ in complex with the native blocker (HIG) was obtained from protein data bank (PDB) and coded as PDB: 3R8A (Figure 3.1c). The AT₁ receptor is known to be part of renin-angiotensin-aldosterone system pathway (RAAS). These receptors are found in blood vessels and mediate the

major cardiovascular effects of angiotensin II (ANG II). It has vasopressor effects and regulates aldosterone secretion. The RAAS pathway is known to be involved in the regulation of blood volume, vascular resistance and plays an important role in cardiovascular pathology. RAAS is often the target in the treatment of cardiovascular diseases (De Mello, 2014). For this reason, drugs known as angiotensin II receptor (AT_1) blockers are used to lower BP (Casimiro-Garcia *et al*., 2011).

The native ligand HIG was re-docked to its receptor (AT_1 receptor PDB: 3R8A) to calculate the RMSD and (ΔG_b). The RMSD value for the re-docked ligand was 0.597 Å (Table 3.4), and the generated pose from docking when superimposed on the originally embedded pose showed a perfect fit (Figure 3.3c). The binding energy (ΔG_b) value obtained after re-docking HIG (AT_1 blocker) was -9.129 kcal/mol. DC1, DC2, DC8, DC9 and DC15 (Table 3.1) were docked into AT_1 receptor at a similar position previously occupied by the native ligand (HIG) (Figure 3.1c). From the database file generated by the MOE docking software, docking of DC9 into AT_1 active site (PDB: 3R8A) was observed to have the highest binding affinity with the lowest binding free energy (ΔG_b) of -5.671 kcal/mol. DC1 and DC8 followed closely with binding free energy score (ΔG_b) of -5.306 and -5.214 kcal/mol respectively. DC2 and DC15 exhibited lowest binding affinity with binding free energy scores of (ΔG_b) of -3.509 and -3.029 kcal/mol respectively.

The position of the native blocker (HIG), isolated diterpenoids and their binding mode (interactions) within the active site of AT_1 receptor were visualized in three-dimension (3D) and analysed with the help of MOE visualization window as shown in Figure 3.6.
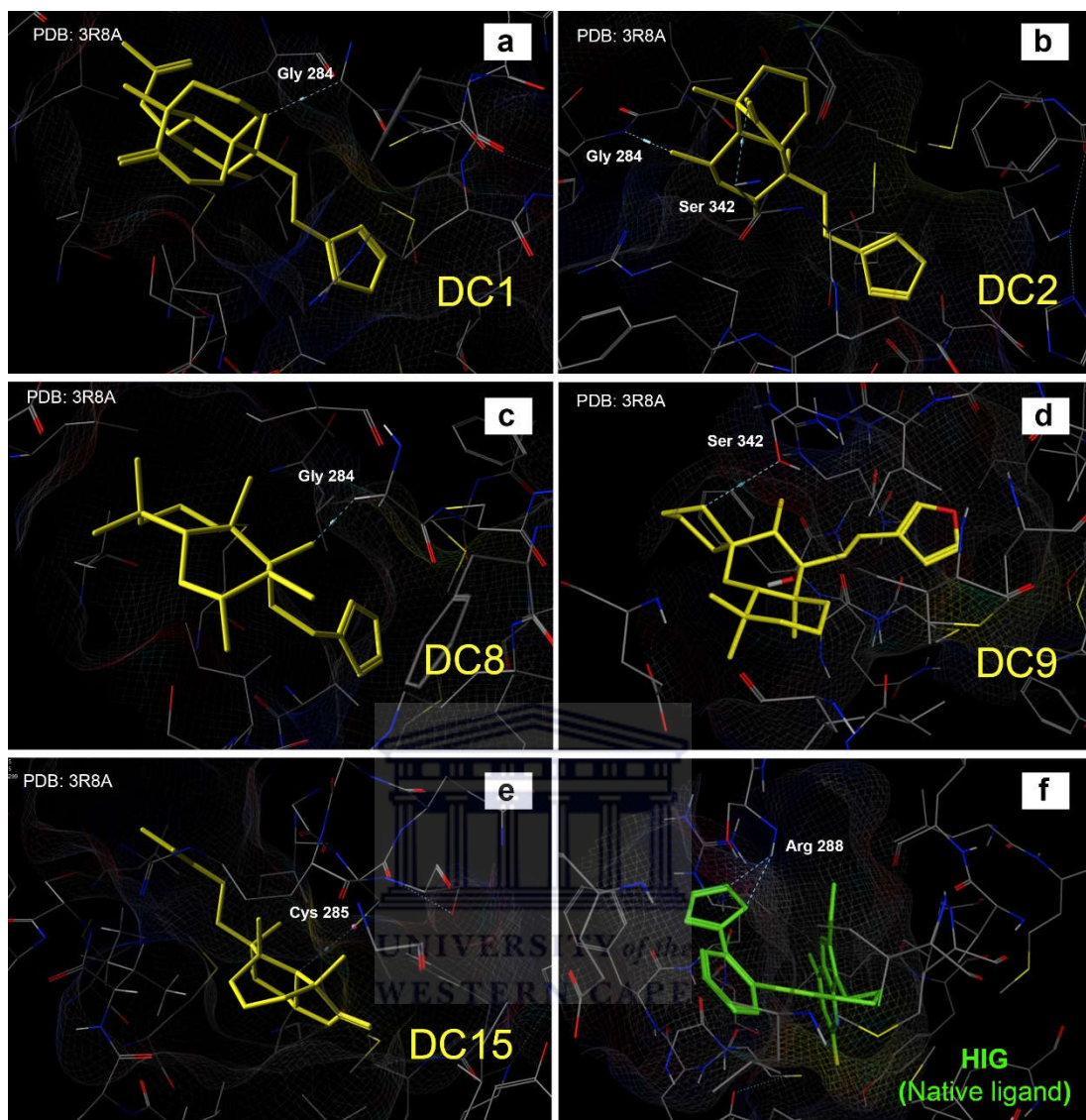
Figure 3.6: The binding mode (interactions) of *L. leonurus* diterpenoid compounds with AT$_1$ receptor

a) DC1, b) DC2, c) DC8, d) DC9, e) DC15 (yellow coloured) and f) native AT$_1$ blocker (HIG) (in green colour) with angiotensin II (AT$_1$) receptor (PDB: 3R8A) active site residues. The blue dash line presents the hydrogen bonds between the molecule and the receptor amino acids residues, while the yellow dash line presents the π interaction between the molecule and the receptor amino acids residues.

From Figure 3.6e the visualization of the binding mode (interaction) of AT$_1$ blocker (HIG) within the active site of AT$_1$ receptor, showed two (2) hydrogen bonds with one (1) amino acid residue (Arg 288). While the binding mode (interactions) visualization analysis of isolated diterpenoids showed that DC1 (Figure 3.6a; Table 3.7) and DC8 (Figure 3-6c; Table 3.7) interacted with AT$_1$ receptor through one (1) hydrogen bonding with the residues Gly 284. DC2 (Figure 3.6b; Table 3.7) was observed to

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interact with the receptor through two (2) hydrogen bonds with the residues Ser 342 and Gly 284. DC9 (Figure 3.6d; Table 3.7) was observed to interact with the receptor through one (1) hydrogen bonds with the residues Ser 342. DC15 (Figure 3-6e; Table 3.7) was observed to interact with the receptor through one (1) hydrogen bonding with the residue Cys 285.

The following Table (Table 3.7) presents a summary of results obtained from visualizing the active site and the results obtained from the database file generated by MOE software after the docking procedures. These results include the binding energy (ΔG_b) and the binding mode (interactions) i.e. number and the name of amino acids residues responsible for the hydrogen bond as well as π interaction that were formed between the docked compounds and AT_1 receptor active site. Also the number of the hydrogen bond and number of π interactions formed are presented and discussed.

Table 3.7: List of binding energy, and list of hydrogen bonds and π interaction formed between *L. leonurus* diterpenoid compounds and AT_1 receptor.

Compound	(ΔG_b)	Hydrogen bond interaction		π interaction	
		Amino acid residue	Number of H-B	Amino acid residue	Number of π interaction
DC1	-5.306	Gly 284	1	-	0
DC2	-3.509	Ser 342 and Gly 284	2	-	0
DC8	-5.214	Gly 284	1	-	0
DC9	-5.671	Ser 342	1	-	0
DC15	-3.029	Cys 285	1	-	0
HIG	-9.129	Arg 288	2	-	0

The binding energy (ΔG_b) and the binding mode (interactions) analysis of DC1, DC2, DC8, DC9 and DC15 (Table 3.7) were compared with the binding energy and the binding mode property of the AT_1 blocker (HIG) in the active site of AT_1 receptor as shown in Figure 3.6f.

As presented in Table 3.7, the binding mode (interactions) analysis of DC1, DC2, DC8, DC9 and DC15s did not show any similarity with HIG (AT_1 blocker) interactions. Although these isolated compounds (DC1, DC2, DC8, DC9 and DC15) exhibited binding energies (ΔG_b) with the AT_1 receptor, the binding energy (ΔG_b) of the diterpenoids were higher than to that observed with HIG (AT_1 blocker), this could

suggest that these isolated diterpenoid compounds could have low affinity to the AT_1 receptor, as the more negative binding energy (ΔG_b) value indicates stronger interactions (Lim *et al.*, 2011; Temirak *et al.*, 2014; Thakur and Thakur, 2015). The difference in binding mode (interaction) and the binding energy (ΔG_b) to that observed with AT_1 blocker (HIG) suggests that these compounds (DC1, DC2, DC8, DC9 and DC15) would not produce the same pharmacological effect observed with AT_1 blocking drugs or may have weak AT_1 blocking activity when tested *in-vivo*.

3.3.2.5 Docking of isolated diterpenoid compounds into β1 adrenoceptor

The molecular docking (MD) technique was used to understand the binding modes and also estimate the binding affinity of the isolated diterpenoids DC1, DC2, DC8, DC9 and DC15 to the β1 adrenoceptor active site. The 3D structure of the β1 adrenoceptor in complex with the native agonist (salbutamol) was obtained from protein data bank (PDB) and coded as PDB: 2Y04 (Figure 3.1d) (Warne *et al.*, 2011).

The first step in the docking technique was to re-dock the native agonist (Salbutamol) to its receptor active site (β1 adrenoceptor) and thus calculate its RMSD and binding energy (ΔG_b). From the database file generated by the MOE software, the RMSD value between the original pose of Salbutamol and the pose resulting from the re-docking was 0.478 Å (Table 3.3), and the generated pose from docking when superimposed on the originally embedded pose showed a perfect fit. The binding energy (ΔG_b) value obtained was -5.654 kcal/mol. Thereafter, the *L. Leonurus* diterpenoids (Table 3.1) were docked into the β1 adrenoceptor at a similar position to that previously occupied by the native ligand (Salbutamol) (Figure 3.2c). From the database file generated by the MOE docking software, docking of the isolated diterpenoids DC1, DC2, DC8, DC9 and DC15 into the β1 adrenoceptor active site (PDB; 2Y04) resulted in binding energies (ΔG_b) of -5.450, -5.315, -5.264, -5.270, and -5.144 kcal/mol respectively. This result showed that the binding energy for these compounds were similar, with DC1 having the highest (ΔG_b) values and DC15 the lowest (ΔG_b) value.

The position of the native agonist (Salbutamol), *L. Leonurus* diterpenoids and their binding mode (interactions) within the active site of β1 adrenoceptor were visualized in three-dimension (3D) and analysed with the help of MOE visualization window as shown in Figure 3.7.
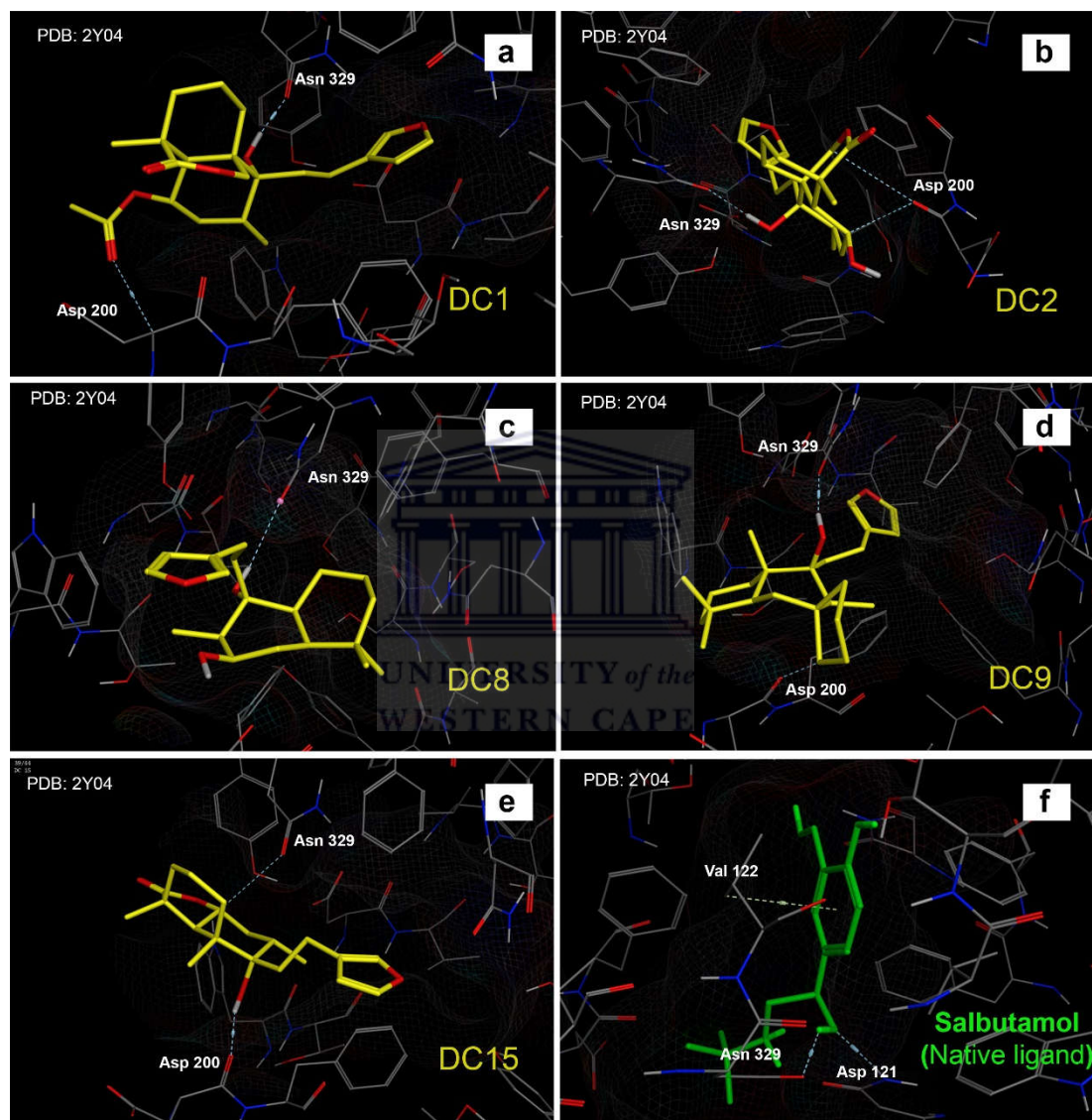
Figure 3.7: The binding mode (interactions) of *L. Leonurus* diterpenoid compounds with β1 adrenoceptor

a) DC1, b) DC2, c) DC8, d) DC9 and e) DC15 (in yellow colour) and f) native β1 adrenoceptor agonist (Salbutamol) (in green colour) with the β1 adrenoceptor active site residues. The blue dash line presents the hydrogen bonds between the molecule and the receptor amino acids residues, while the yellow dash line presents the π interaction between the molecule and the receptor amino acids residues.

From Figure 3.7e, the visualization of the binding mode (interaction) of β1 agonist (Salbutamol) within the active site of β1 adrenoceptor, showed four (4) hydrogen bond interactions with three (3) amino acids residues Asn 329, Asn 310 and Asp 121. Salbutamol also interacted with the receptor through the formation of one (1) π interaction with the Val 122 residue. The binding mode (interactions) visualization analysis of the isolated diterpenoids showed that DC1 (Figure 3.7a; Table 3.8), DC9 (Figure 3.7d; Table 3.8) and DC15 (Figure 3.7e; Table 3.8) interacted with the β1 adrenoceptor by forming two (2) hydrogen bonds with the amino acids residues Asp 200 and Asn 329. Unlike DC1, DC9 and DC15, DC2 (Figure 3.7b; Table 3.8) was observed to interact with the receptor through three (3) hydrogen bonds; two bonds with the Asp 200 residue and one (1) hydrogen bond with Asn 329 residue. The interaction between DC8 (Figure 3.7c; Table 3.8), and the receptor occurred through only one (1) hydrogen bond with the Asn 329 residue. These compounds (DC1, DC2, DC9 and DC15 (Figure 3.7e; Table 3.8) showed identical interactions to each other with the amino acid residues Asp 200 and Asn 329. There were no major difference in their (ΔG_b) values suggesting that these compounds have similar affinity to the β1 adrenoceptor.
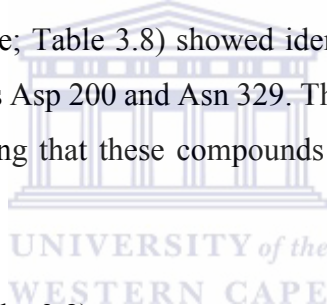
The following Table (Table 3.8) presents a summary of results obtained from visualizing the active site and the results obtained from the database file generated by MOE software after the docking procedures of the *L. leonurus* diterpenoids and native agonist (Salbutamol) with the β1 adrenoceptor. These results include the binding energy (ΔG_b) and the binding mode (interactions) i.e. number and the name of amino acids residues responsible for the hydrogen bond as well as π interactions that were formed between the docked compounds and the β1 adrenoceptor. Also the number of the hydrogen bond and number of π interactions formed are presented.

Table 3.8: List of binding energy, and list of hydrogen bonds and π interaction formed between *L. leonurus* **diterpenoid compounds and β1 adrenoceptor.**

Compound	(ΔG_b)	Hydrogen bond interaction		π interaction	
		Amino acid residue	Number of H-B	Amino acid residue	Number of π interaction
DC1	-5.450	Asp 200 and Asn 329	2	-	0
DC2	-5.315	Asp 200 and Asn 329	3	-	0
DC8	-5.264	Asn 329	1	-	0
DC9	-5.270	Asp 200 and Asn 329	2	-	0
DC15	-5.144	Asp 200 and Asn 329	2	-	0
Salbutamol	-5.654	Asp 121 and Asn 329	2	Val 122	1

The binding energy and the binding mode (interactions) analysis of the isolated diterpenoids DC1, DC2, DC8, DC9 and DC15 (Table 3.8) were compared with the binding energy and the binding mode property of the agonist salbutamol in the active site of β1 adrenoceptor as shown in Figure 3.7f.

As presented in Table 3.8 the binding mode (interaction) of DC1, DC2, DC8, DC9 and DC15 in the active site of the β1 adrenoceptor were similar to those of salbutamol. DC1, DC2, DC8, DC9, DC15 and Salbutamol (β1 agonist) all interacted with the same amino acid residues (Asp 329). Also, the binding energy (ΔG_b) of DC1, DC2, DC9 and DC15 were close to that observed with salbutamol, which could indicate that these compounds may have similar affinity as salbutamol to the β1 adrenoceptor (see Table 3.8 and Figure 3.7). All the five (5) diterpenoid compounds exhibited binding energy value (ΔG_b) close to that observed with salbutamol, indicating the possibility of strong interactions, as the more negative the binding energy (ΔG_b) value, the stronger the interactions (Lim *et al*., 2011; Temirak *et al*., 2014; Thakur and Thakur, 2015). The binding mode (interactions) and the binding energy (ΔG_b) for DC1, DC2, DC8 DC9 and DC15 were similar to that observed with salbutamol, which could indicate that these compounds may produce similar pharmacological effects to that observed with salbutamol (β1 agonist activity) when tested *in-vivo*.

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CHAPTER FOUR

4   *IN-VIVO* STUDIES METHODOLOGY

This chapter lists the materials, reagents, and equipment, and describes the methods and procedures used in this study to investigate the *in-vivo* cardiovascular activity of five (5) diterpenoid compounds isolated from *L. leonurus*.

4.1   EQUIPMENT AND REAGENTS

The equipment used in the *in-vivo* experiments included the small animal operating table (BioScience, Cape Town, SA), Lab Chart 4 for windows software, BP transducer, PowerLab® 4/20T unit, and BP amplifier (AD instruments, Bella Vista, Australia), and double-syringe pump (AP22, ASCOR, Poland). The surgical tools included; syringes (1 ml, 5ml and 10 ml), forceps, polyethylene cannulae, bulldog clamp, respiratory tubing, surgical blades, sterile gauze, sterile pads, Blunt-nosed scissor, vanna micro-dissecting scissors, catgut sutures and adhesive tape (See Figure 4.1). The drugs and chemicals used include, NaCl 0.9% saline solution, Sodium pentobarbital (1ml/200mg) (Kyron Laboratories, South Africa), Heparin (1ml/5000 unit) (Fresenius Kabi, South Africa). The five (5) diterpenoids (DC1, DC2, DC8, DC9 and DC15) compounds tested were suspended in normal saline with a 0.1 ml Tween 80.
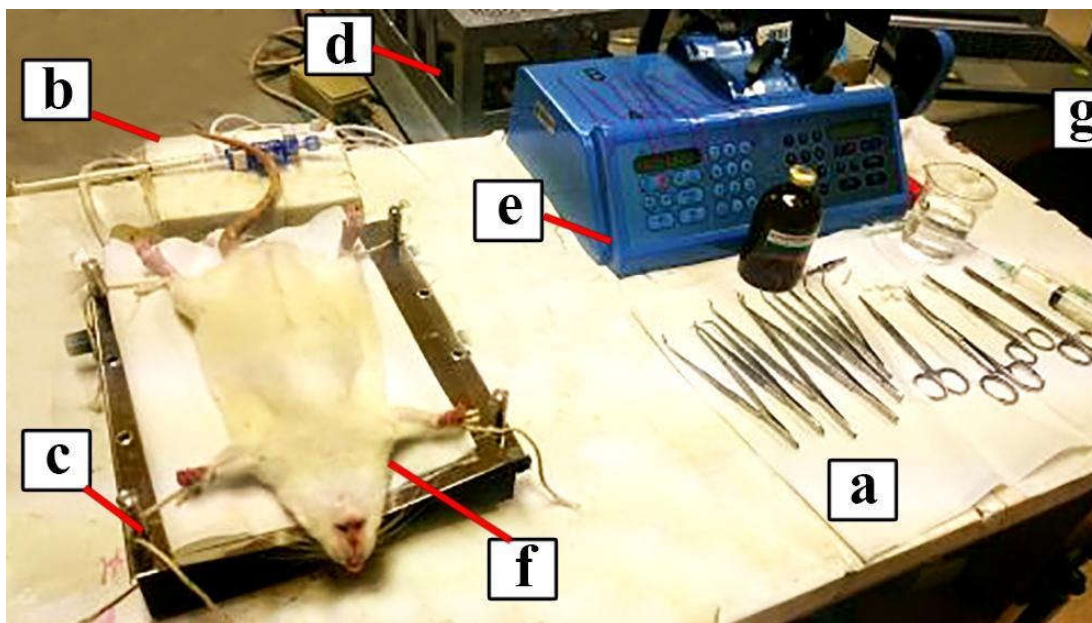
Figure 4.1: equipment and reagents used in the *in-vivo* study

Figure Shows a) Surgical tools, b) Blood pressure transducer, c) Rat-operating table, d) PowerLab®
4/20T unit, and Blood pressure amplifier, e) Double-syringe pump, f) Normotensive rat, g) Computer.

4.2   ANIMALS

Healthy male normotensive Wistar rats weighing 250–350 g, and less than 5 months
old were obtained from the animal unit of the school of Pharmacy, at University of the
Western Cape. Animals were housed in standard rat cages and given free access to
both food and water throughout the study period. The room temperature was kept at
24°C, with a 12:12-h light dark cycle.

4.3   ETHICAL CONSIDERATIONS

The animals were allowed free access to food and water before the commencement of
the experiments. The methods used in this study were approved by the Ethics
Committee of the University of the Western Cape, and the registration number
obtained was 13/1/17. All experiments were carried out in accordance with the ethical
care of animals.

4.4 ANIMAL PREPARATION

Animals were prepared according to the method described by (Raji *et al.*, 2013, 2012). Rats were anesthetized with 6% sodium pentobarbital at a dose of (40mg/kg) intraperitoneally, and placed in dorsal recumbence position (Figure 4.2) on a heated rat-operating table, with the temperature monitored and maintained at $37 \pm 0.5°$ C throughout each experiment via a rectal thermometer. Three preparatory surgical procedures a) tracheotomy, b) jugular vein catheterization, and c) femoral artery catheterization were performed.

4.4.1 TRACHEOTOMY

In order to maintain airflow during the experiment, the anesthetized animal was placed in dorsal recumbence position on the small animal operation table. An incision was made in the neck of the rat for tracheostomy and jugular vein cannulation. The longitudinal neck muscles (sternohyoid and longus colli) were dissected along the midline through the small skin incision 2-3 mm rostral to the manubrium, and the trachea was visualized. The trachea was incised transversely 2-3 tracheal rings caudal to the thyroid isthmus but 1 tracheal ring rostral to the underlying suture. The tracheotomy tube was inserted 8 mm into the trachea and secured in place using the suture (Figure 4.2).

4.4.2 JUGULAR VEIN CANNULATION

The right external jugular vein was cannulated with a small polyethylene cannula filled with heparinized saline (10% heparin in 0.9% NaCl) during the experiment (Figure 4.2), to allow intravenous infusion of drugs via a syringe and the syringe pump. The external jugular vein was located via the tracheotomy incision bilaterally in the incised region, and visible just below the dermis. The vein was isolated from surrounding

tissues using artery forceps or curved, blunt forceps, and tied off proximal to the brain. A small cut was made on the vein to insert a catheter up to 1″ towards the heart, and secured with a thread.
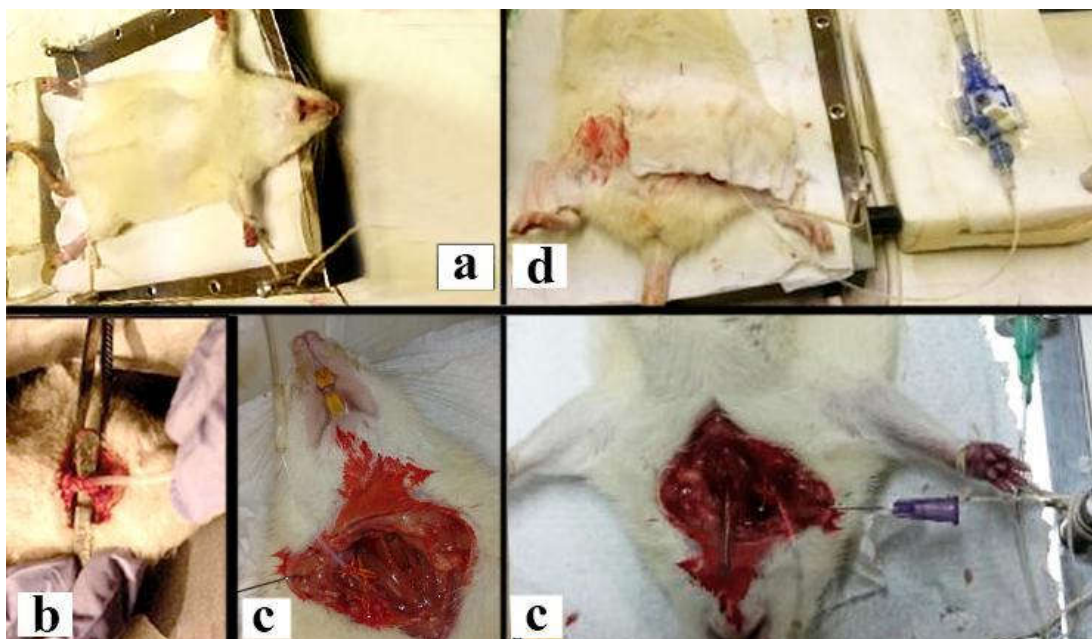
4.4.3   FEMORAL ARTERY CANNULATION

The left femoral artery was cannulated with a small polyethylene cannula filled with heparinized saline (10% heparin in 0.9% NaCl) (Figure 4.2), for continuous BP monitoring during the experiment via an incision is made in the medial surface of the leg. Using blunt dissection, the femoral artery was located, isolated from the femoral vein and nerve and tied off distal to the heart. A micro-vascular clamp was placed on the artery proximal to the heart from the ligature, a small cut made into the femoral artery and the catheter inserted and secured to prevent dislodgement. The femoral cannula was connected to a BP transducer attached to a BP amplifier and PowerLab® (4/20T unit) for recordings of the BP and HR on the Chart 4.0® for Windows software (all AD Instruments, Australia).

Rats were given oxygen throughout the experiments through an oxygen mask, and allowed a 30min stabilization period to ensure that BP and HR parameters were stable before any further procedures. Drugs were infused at a constant rate (0.3 ml/min) via a double-syringe pump (AP22, ASCOR, Poland), and the cannula flushed with 0.4 ml of normal saline after each infusion. Changes to parameters were recorded within 3 min of infusion. Blood pressures and HR were allowed to return to baseline values (between 10–15 minutes) before further doses were infused.

Figure 4.2: Surgical procedures involved in the *in-vivo* study

Figure shows a) Rat-operating table, b) Tracheotomy Cannulation, c) Jugular Vein Cannulation, d) Femoral Artery Cannulation.

4.5   PREPARATION OF TEST COMPOUNDS

All compounds tested (DC1, DC2, DC8, DC9, and DC15) were sparingly soluble in normal saline and tween 80 was used to stabilise fine suspensions of the test compounds in normal saline. Two drops of tween 80 was added to a paste of the compound in normal saline, and then made up to the required volume with normal saline. Fresh solutions for the different concentrations of test compounds were prepared for each day's experiments.
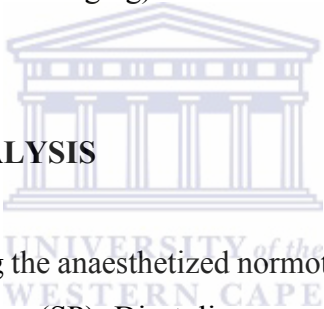
4.6   EXPERIMENTAL PROTOCOL

Animals were randomly divided into five (5) groups, each group receiving different doses of test compounds (DC1, DC2, DC8, DC9, and DC15) infused through the venous cannula at a constant rate (0.3 ml/min) via a double-syringe pump. Blood pressures and HR were allowed to return to baseline values (between 10–15 minutes)

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before further doses were infused. Dose-response experiments for each compound was repeated in six animals to validate an observation of the results.

a) Group I. DC1 – Dose response curve (0.5mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, and 40 mg/kg).

b) Group II. DC2 – Dose response curve (0.5mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg).

c) Group III. DC8 - Dose response curve (0.5mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg).

d) Group IV. DC9 - Dose response curve (0.5mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, and 40 mg/kg).

e) Group V. DC15 - Dose response curve (0.5mg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, and 40 mg/kg).

4.7   STATISTICAL ANALYSIS

Data from experiments using the anaesthetized normotensive rat model was expressed as change in Systolic pressure (SP), Diastolic pressure (DP), Mean arterial pressure (MAP) and Heart rate (HR). This change was calculated as the difference between the value of the parameter just before the administration of the test compounds and the value at the peak of effect of the test compound. Mean change (Δ mean $\pm$ S.E.M) was calculated and statistically analysed using the student's t test for significant difference ($p < 0.05$). The Microsoft Excel 2013 software was used for statistical analysis, and the Graphpad prism 6 software was used to illustrate the results as graphs.

4.8   RESULTS

Five (5) compounds (DC 1, DC 2, DC 8, DC 9, and DC 15) isolated from *L. leonurus* were tested for cardiovascular effect on the anaesthetised normotensive rat model. All the rats died after administration of doses above 80mg of the compounds. The

following results were obtained for the different doses of the compounds administered. Tables and graphs would be used to present the observed effects.

4.8.1 EFFECTS ON SYSTOLIC BLOOD PRESSURE (SP)

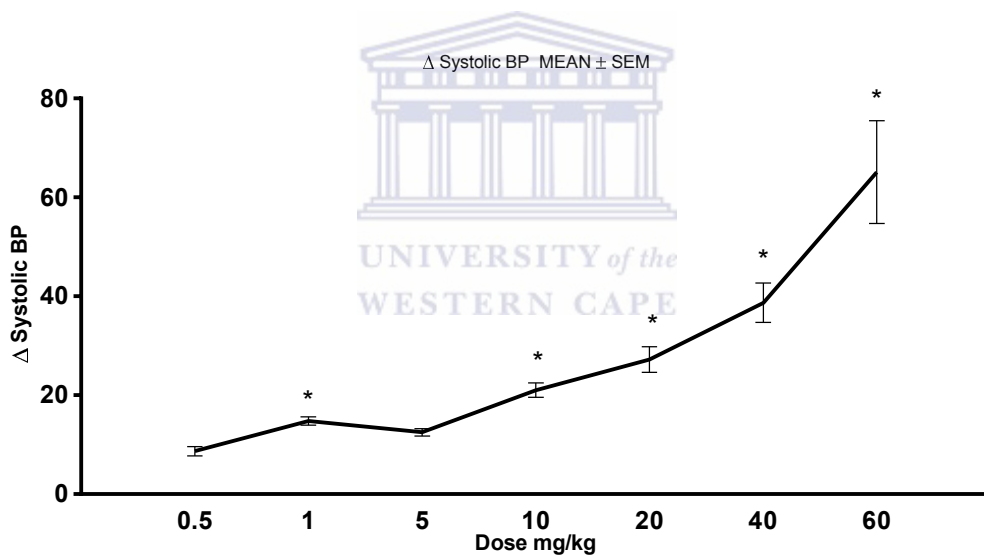### 4.8.1.1 Effects of DC1 on Systolic blood pressure (SP)

Figure 4.3 shows the effect of DC1 administered in a dose range of (0.5 mg/kg - 40 mg/kg) on the systolic blood pressure in anaesthetised normotensive Wistar rats. DC1 produced dose dependent increases in systolic blood pressure for all doses administered, the increases were shown to be statistically significant at the 10 mg/kg, 20 mg/kg, and 40 mg/kg doses (see Figure 4.3; Appendix V). At the lowest dose (0.5 mg/kg), it produced a 2.933 mmHg $\pm$ 0.6677 increase in systolic pressure, while the highest dose administered (40 mg/kg), produced a 42.360 mmHg $\pm$ 5.803 change in systolic pressure. Changes to SP were deemed statistically significant compared to the solvent (normal saline).
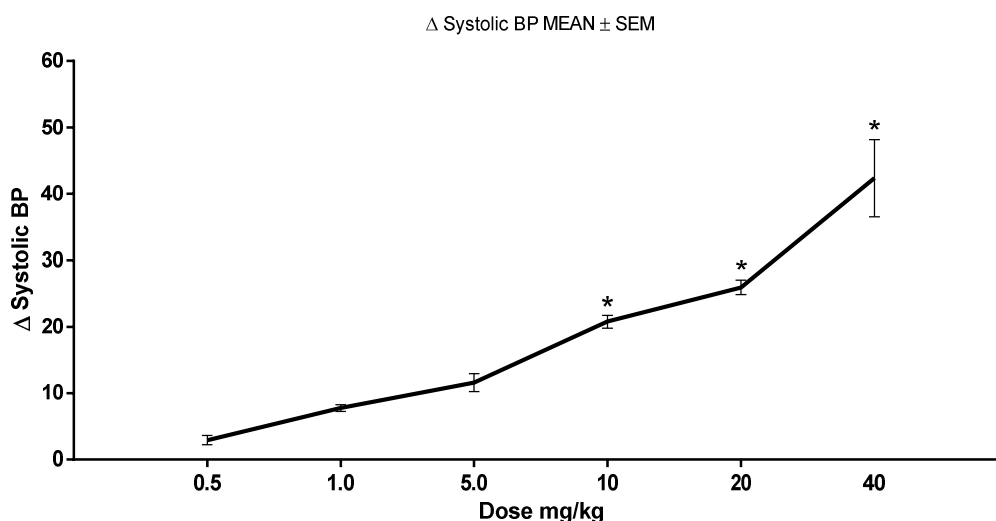
Figure 4.3: Effects of DC1 (0.5 mg/kg - 40 mg/kg) on systolic pressure in normotensive Wistar rats. * indicates statistical significant change.

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4.8.1.2 Effects of DC2 on Systolic blood pressure (SP)

Figure 4.4 shows the effect of DC2 administered in a dose range of (0.5 mg/kg, - 60 mg/kg) on the systolic blood pressure (SP) in anaesthetised normotensive rats. DC2 had a dose dependent effect on systolic pressure (see Figure 4.4; Appendix V), with the change in pressure increasing as the dose was increased, the increases were shown to be statistically significant at the higher (10, 20, 40 and 60 mg/kg) doses. At the lowest dose (0.5 mg/kg), it produced 8.675 mmHg ± 0.9162 increase in systolic pressure, while the highest dose administered (60 mg/kg), produced a 65.110 mmHg ± 10.380 increase in systolic pressure.

Figure 4.4: Effects of DC2 (0.5 mg/kg - 60 mg/kg) on systolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

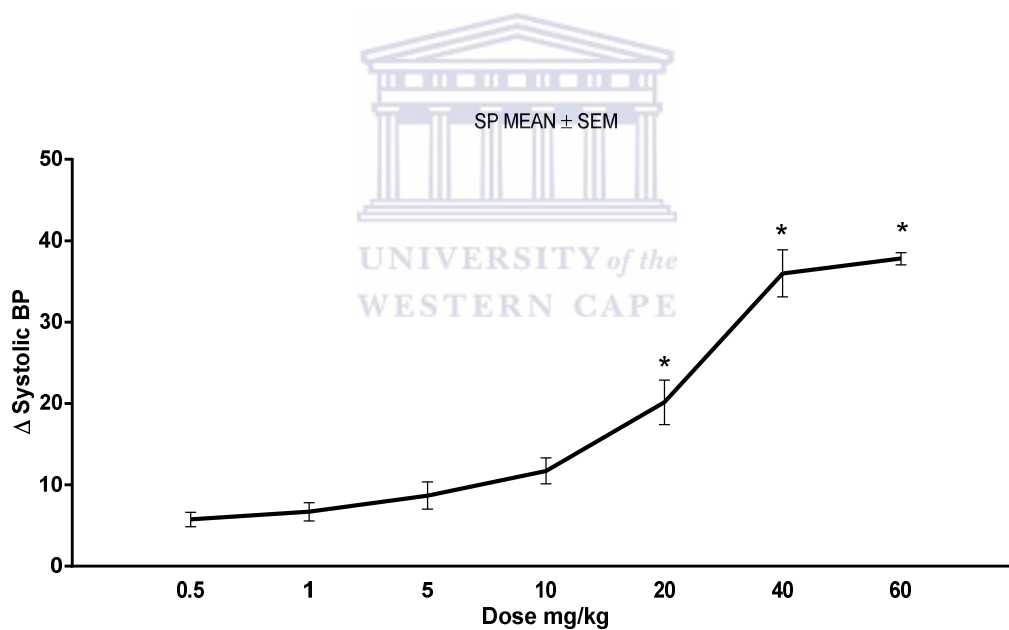### 4.8.1.3   Effects of DC8 on Systolic blood pressure (SP)

Figure 4.5 shows the effect of DC8 administered in a dose range of (0.5 mg/kg - 60 mg/kg) on the systolic blood pressure (SP) in anaesthetised normotensive rats. DC8 had a dose dependent effect on the systolic pressure (see Figure 4.5; Appendix V), with the change in pressure increasing as the dose was increased, the increases were shown to be statistically significant at the higher (20, 40 and 60 mg/kg) doses. At the lowest dose (0.5 mg/kg), it produced a non-significant 5.732 mmHg ± 0.8752 increase in systolic pressure, while the highest dose administered (60 mg/kg), produced a 37.790 mmHg ± 0.7398 change in systolic pressure.

Figure 4.5: Effects of DC8 (0.5 mg/kg - 60 mg/kg) on systolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

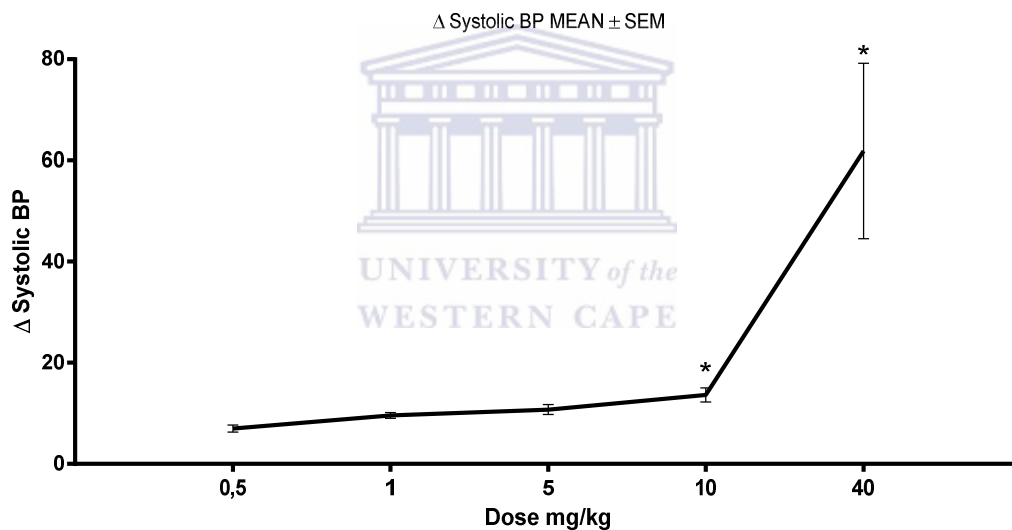### 4.8.1.4   Effects of DC9 on Systolic blood pressure (SP)

Figure 4.6 shows the effect of DC9 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the systolic blood pressure in anaesthetised normotensive rats. DC9 produced dose dependent increases in systolic pressure, the increases were shown to be statistically significant at the 10 mg/kg and 40 mg/kg doses only (see Figure 4.6; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 6.993 mmHg $\pm$ 0.7063 increase in systolic pressure, while the highest dose administered (40 mg/kg), produced a 61.850 mmHg $\pm$ 17.340 change in systolic pressure.

Figure 4.6: Effects of DC9 (0.5 mg/kg - 40 mg/kg) on systolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.1.5   Effects of DC15 on Systolic blood pressure (SP)

Figure 4.7 shows the effect of DC15 administered in a dose range of (0.5 mg/kg - 40 mg/kg) on the systolic blood pressure (SP) in anaesthetised normotensive rats. DC15 produced dose dependent increases in systolic pressure, the increases were shown to be statistically significant at the 5 mg/kg, 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.7; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-statistically significant 5.845 mmHg ± 0.9175 increase in systolic pressure, while the highest dose administered (40 mg/kg), produced a 22.630 mmHg ± 0.9431 change in systolic pressure.
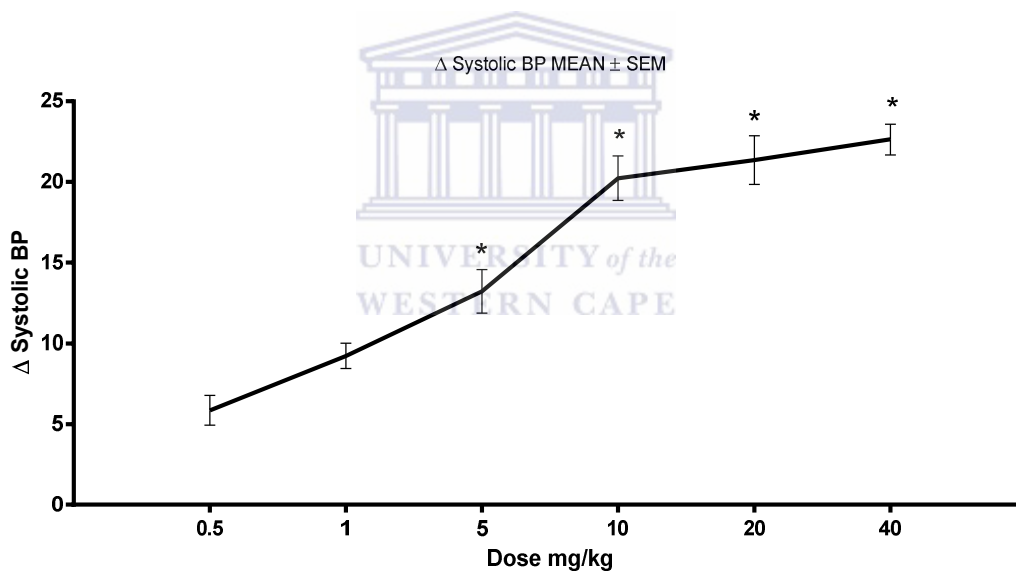

Figure 4.7: Effects of DC15 (0.5 mg/kg - 40 mg/kg) on systolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.2   EFFECT ON DIASTOLIC PRESSURE (DP)

4.8.2.1   Effects of DC1 on diastolic blood pressure (DP)

Figure 4.8 shows the effect of DC1 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the diastolic blood pressure (DP) in anaesthetised normotensive rats. DC1 produced dose dependent increases in diastolic pressure, the increases were shown to be statistically significant at the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.8; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 2.1180 mmHg $\pm$ 0.6047 increase in systolic pressure, while the highest dose administered (40 mg/kg), produced a 41.0600 mmHg $\pm$ 5.3820 change in systolic pressure.
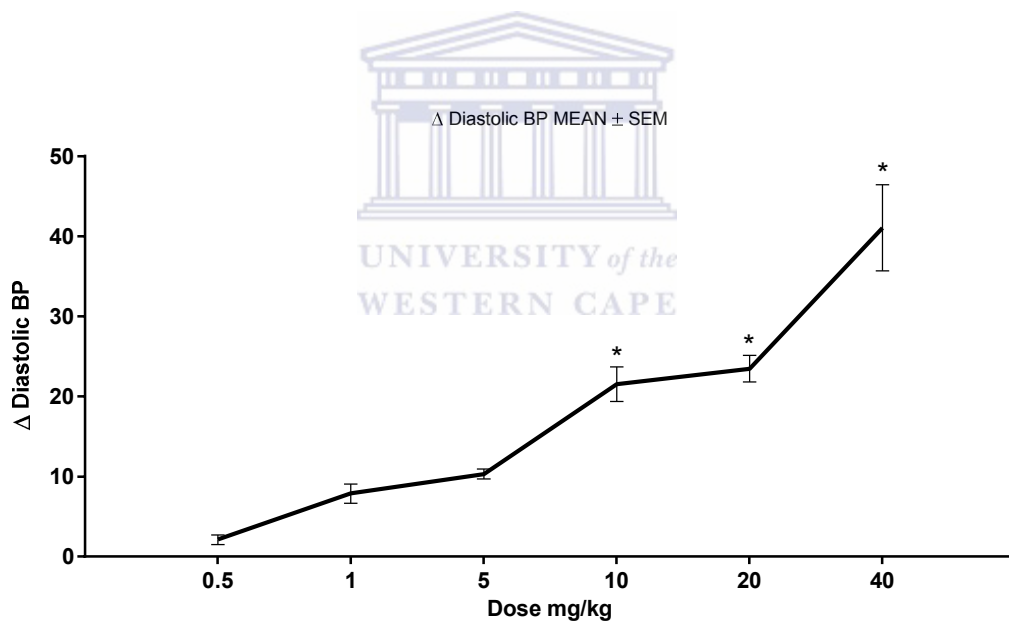

Figure 4.8: Effects of DC1 (0.5 mg/kg - 40 mg/kg) on diastolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

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4.8.2.2 Effects of DC2 on Diastolic blood pressure (DP)

Figure 4.9 shows the effect of DC2 administered in a dose range of (0.5 mg/kg - 60 mg/kg) on the DP in anaesthetised normotensive rats. DC2 produced dose dependent increases in diastolic pressure, the increases were shown to be statistically significant at the 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.9; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 4.749 mmHg $\pm$ 0.5796 increase in diastolic pressure, while the highest dose administered (60 mg/kg), produced a 58.200 mmHg $\pm$ 12.990 change in diastolic pressure.

Figure 4.9: Effects of DC2 (0.5 mg/kg - 60 mg/kg) on diastolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.2.3 Effects of DC8 on Diastolic blood pressure (DP)

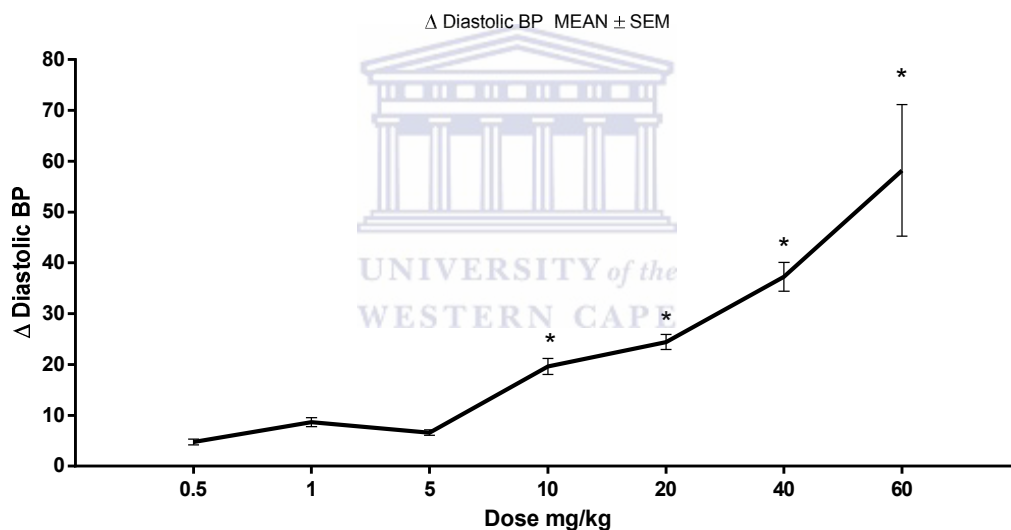Figure 4.10 shows the effect of DC8 administered in a dose range of (0.5 mg/kg - 60 mg/kg) on the DP in anaesthetised normotensive Wistar rats. DC8 produced dose dependent increases in diastolic pressure, with the increases shown to be statistically significant at the 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.10; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 2.1710 mmHg ± 1.1690 increase in diastolic pressure, while the highest dose administered (60 mg/kg), produced a 23.7900 mmHg ± 3.0520 change in diastolic pressure.

Figure 4.10: Effects of DC8 (0.5 mg/kg - 60 mg/kg) on diastolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

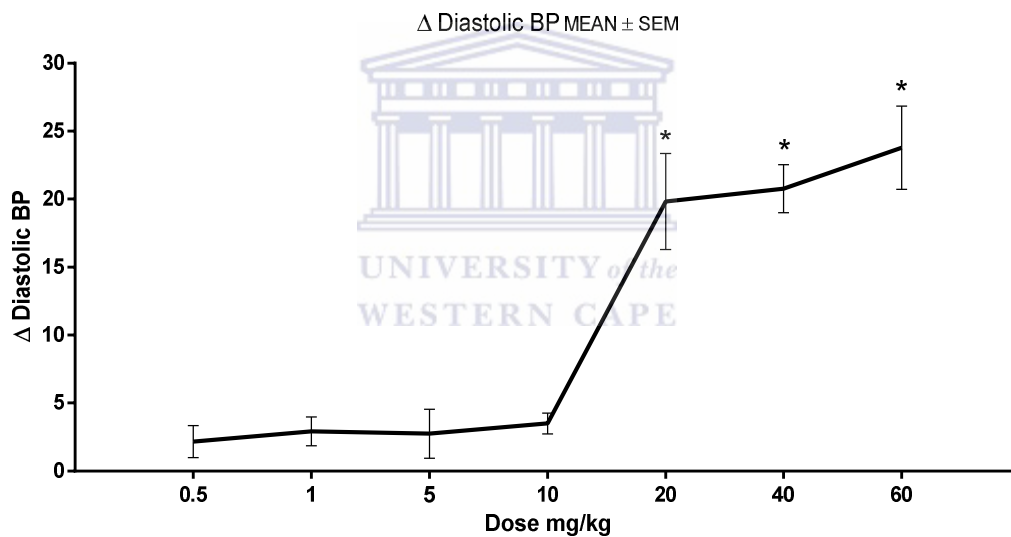### 4.8.2.4   Effects of DC9 on Diastolic blood pressure (DP)

Figure 4.11 shows the effect of DC9 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the DP in anaesthetised normotensive rats. DC9 produced dose dependent increases in diastolic pressure, with the increase statistically significant at the highest (40 mg/kg) dose (see Figure 4.11; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 5.454 mmHg ± 0.4553 increase in diastolic pressure, while the highest dose administered (40 mg/kg), produced a 65.540 mmHg ± 13.320 change in diastolic pressure.

Figure 4.11: Effects of DC9 (0.5 mg/kg - 60 mg/kg) on diastolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

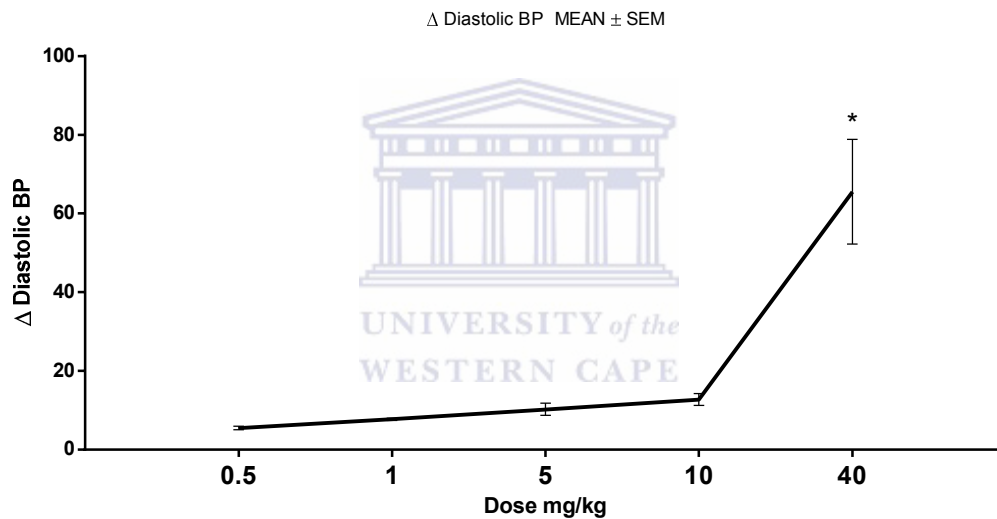### 4.8.2.5 Effects of DC15 on Diastolic blood pressure (DP)

Figure 4.12 shows the effect of DC15 administered in a dose range of (0.5 mg/kg - 40 mg/kg) on the DP in anaesthetised normotensive rats. DC15 produced dose dependent increases in diastolic pressure, with the increases shown to be statistically significant at the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.12; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 5.4460 mmHg ± 0.3822 increase in diastolic pressure, while the highest dose administered (40 mg/kg), produced a 22.5500 mmHg ± 0.9463 change in diastolic pressure.

Figure 4.12: Effects of DC15 (0.5 mg/kg - 40 mg/kg) on diastolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.3   EFFECT ON MEAN ARTERIAL PRESSURE (MAP)

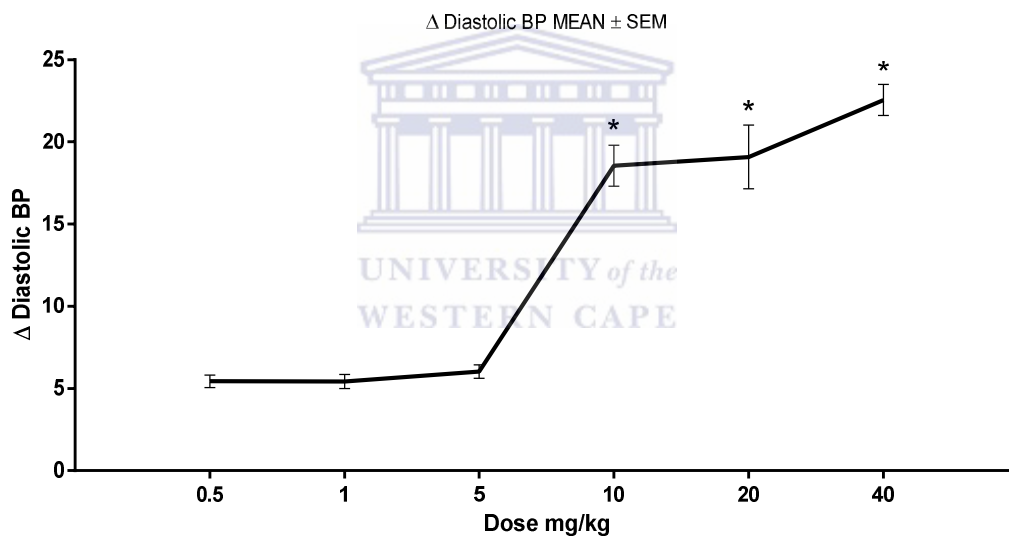#### 4.8.3.1   Effects of DC1 on mean arterial pressure (MAP)

Figure 4.13 shows the effect of DC1 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the MAP of anaesthetised normotensive Wistar rats.   DC1 produced dose dependent increases in MAP for all doses administered, with the increases statistically significant with the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.13; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 2.390 mmHg $\pm$ 0.6006 increase in MAP, while the highest dose administered (40 mg/kg), produced a 41.490 mmHg $\pm$ 5.346 change in MAP.

Figure 4.13: Effects of DC1 (0.5 mg/kg - 40 mg/kg) on mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change**.**

4.8.3.2 Effects of DC2 on Mean arterial pressure (MAP)

Figure 4.14 shows the effect of DC2 administered in a dose range of (0.5 mg/kg, - 60 mg/kg) on the MAP of anaesthetised normotensive Wistar rats. DC2 produced dose dependent increases in MAP for all doses administered, with the increases statistically significant at the 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.14; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 6.058 mmHg ± 0.5361 increase in MAP, while the highest dose administered (60 mg/kg), produced a 60.500 mmHg ± 12.120 change in MAP.
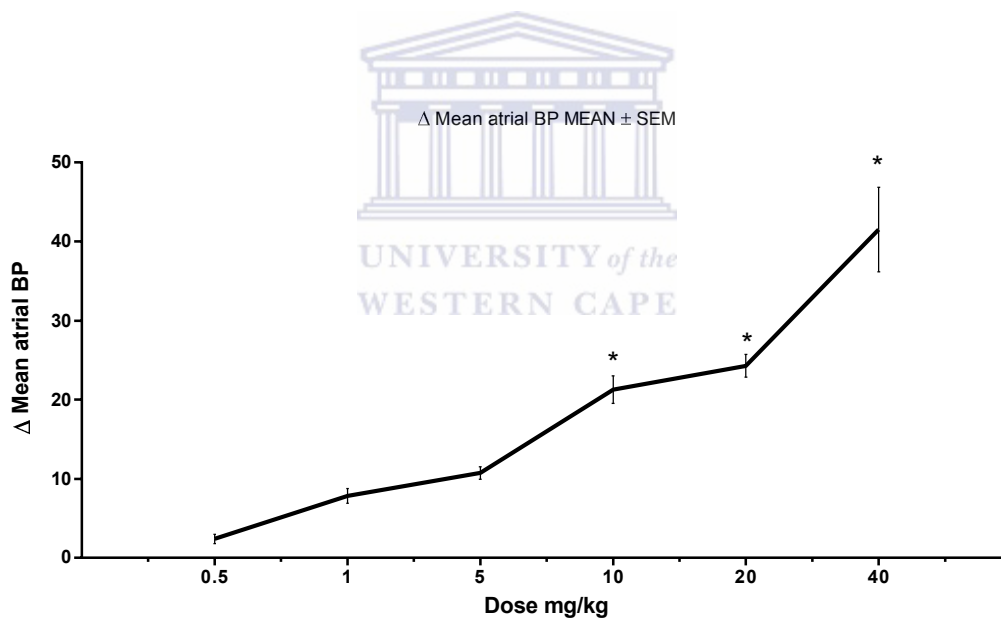

Figure 4.14: Effects of DC2 (0.5 mg/kg - 60 mg/kg) on mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.3.3 Effects of DC8 on Mean arterial pressure (MAP)

Figure 4.15 shows the effect of DC8 administered in a dose range of (0.5 mg/kg, - 60 mg/kg) on the MAP of anaesthetised normotensive Wistar rats. DC8 produced dose dependent increases in MAP, with the increases statistically significant at the 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.15; Appendix V). At the lowest dose (0.5 mg/kg), it produced a non-significant 3.3580 mmHg ± 0.6708 increase in MAP, while the highest dose administered (60 mg/kg), produced a 28.4500 mmHg ± 2.2820 change in MAP.

Figure 4.15: Effects of DC8 (0.5 mg/kg - 60 mg/kg) on mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

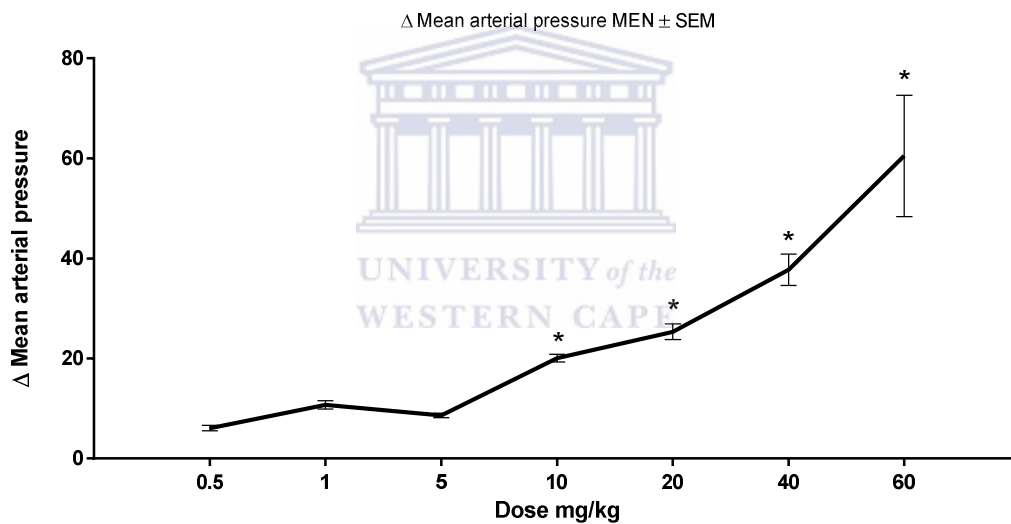### 4.8.3.4   Effects of DC9 on Mean arterial pressure (MAP)

Figure 4.16 shows the effect of DC9 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the MAP of anaesthetised normotensive Wistar rats. DC9 produced dose dependent increases in MAP, with the increases statistically significant at the 10 mg/kg and 40 mg/kg doses only (See Figure 4.16; Appendix V). At the lowest dose (0.5 mg/kg), it produced a 5.967 mmHg ± 0.2071 increase in MAP, while the highest dose administered (40 mg/kg), produced a 64.310 mmHg ± 14.550 change in MAP.
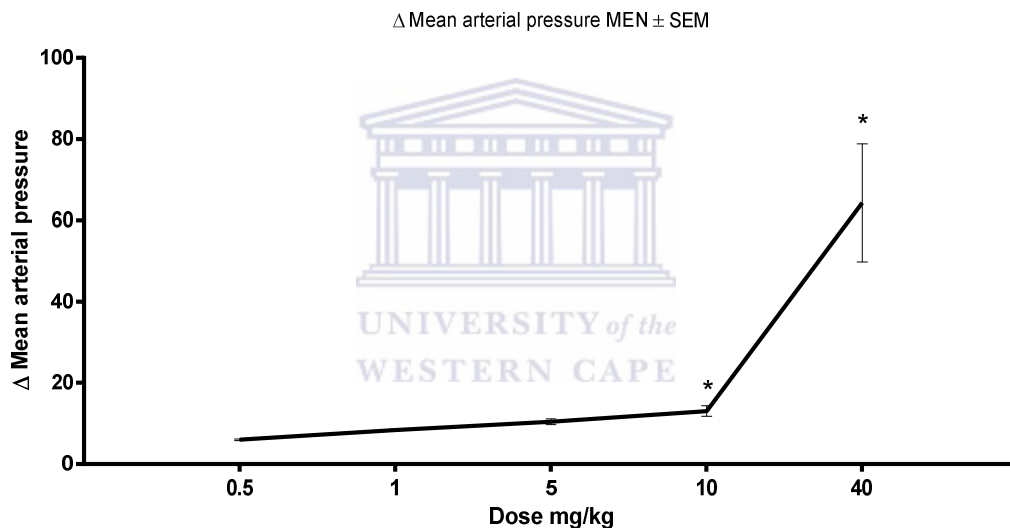
Figure 4.16: Effects of DC9 (0.5 mg/kg - 40 mg/kg) on mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.3.5   Effects of DC15 on Mean arterial pressure (MAP)

Figure 4.17 shows the effect of DC15 (0.5 mg/kg - 40 mg/kg) on the MAP of anaesthetised normotensive Wistar rats. DC15 produced dose dependent, increases in MAP, with the increases statistically significant at the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.17; Appendix V). At the lowest dose (0.5 mg/kg), it produced a 5.579 mmHg ± 0.5124 increase in, while the highest dose administered (40 mg/kg), produced a 22.570 mmHg ± 0.7373 change in MAP.
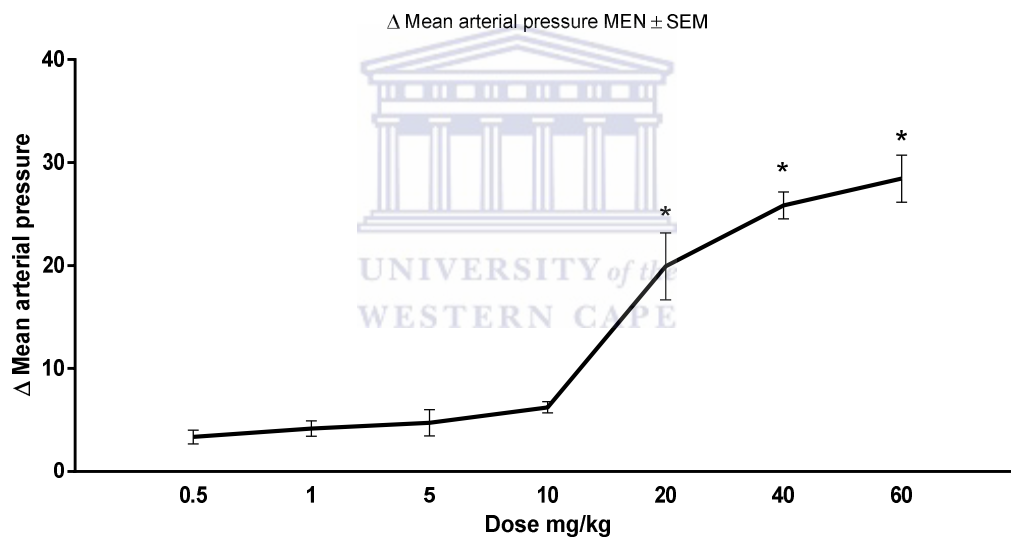

Figure 4.17: Effects of DC15 (0.5 mg/kg - 40 mg/kg) on mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.4   EFFECT ON HEART RATE (HR)

4.8.4.1   Effects of DC1 on Heart rate (HR)

Figure 4.18 shows the effect of DC1 administered in a dose range of (0.5 mg/kg - 40 mg/kg) on the HR of anaesthetised normotensive Wistar rats. DC1 produced dose dependent increases in HR, with the increases statistically significant at the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.18; Appendix V). The lowest dose (0.5 mg/kg), produced a 4.4880 bpm ± 2.4930 increase in HR, while the highest response (29.1300 bpm ± 4.9160) was observed with the 20mg/kg dose administered. Further increases in the dose of DC1 not produce any further increase in the in heart rate beyond the 20 mg/kg dose.

Figure 4.18: Effects of DC1 (0.5 mg/kg - 40 mg/kg) on Heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change.
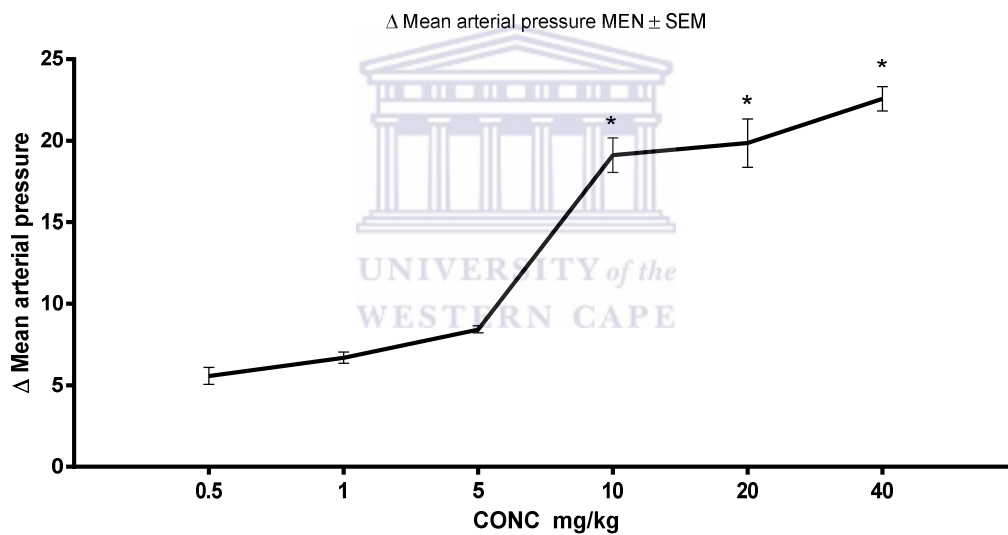
4.8.4.2 Effects of DC2 on Heart rate (HR)

Figure 4.19 shows the effect of DC2 administered in a dose range of (0.5 mg/kg, - 60 mg/kg) on the HR of anaesthetised normotensive Wistar rats. DC2 produced dose dependent increases in HR, with the increases statistically significant at the 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.19; Appendix V). The lowest dose (0.5 mg/kg), produced a 6.122 bpm ± 0.3989 increase in heart rate, while the highest dose administered (60 mg/kg), produced a 46.800 bpm ± 2,739 increase in HR.

Figure 4.19: Effects of DC2 (0.5 mg/kg - 40 mg/kg) on Heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.4.3 Effects of DC8 on Heart rate (HR)

Figure 4.20 shows the effect of DC8 administered in a dose range of (0.5 mg/kg, - 60 mg/kg) on the HR of anaesthetised normotensive Wistar rats. DC8 produced dose dependent increases in HR, with the increases statistically significant at the 5 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg and 60 mg/kg doses (see Figure 4.20; Appendix V). The lowest dose (0.5 mg/kg), produced a 5.4510 bpm ± 0.8787 increase in HR, while the highest dose administered (60 mg/kg), produced a 95.6000 bpm ± 0.8771 change in HR.
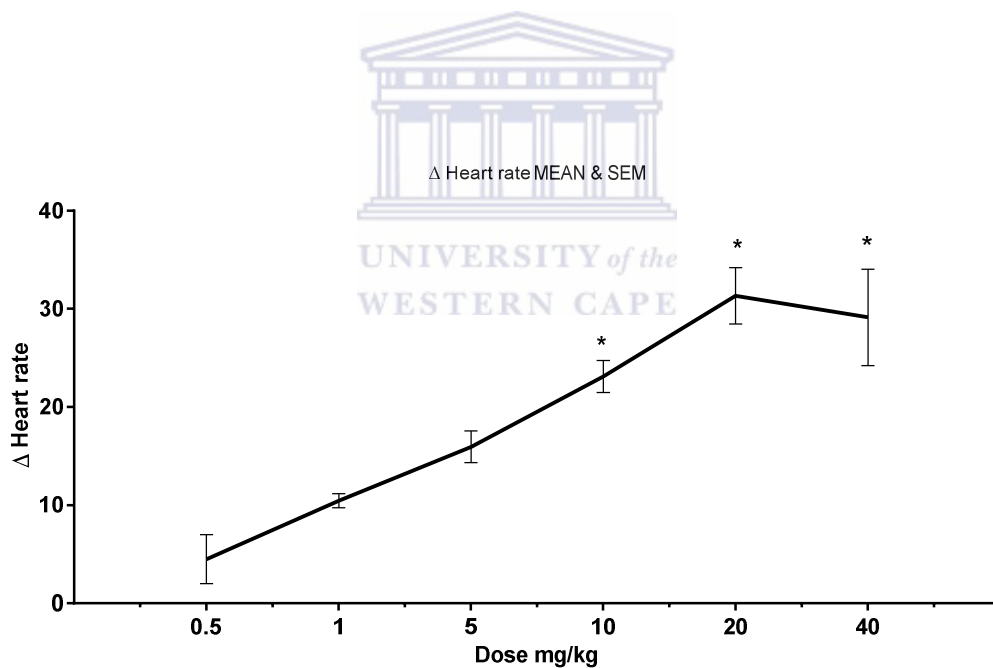

Figure 4.20: Effects of DC8 (0.5 mg/kg - 60 mg/kg) on Heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change.

4.8.4.4 Effects of DC9 on Heart rate (HR)

Figure 4.21 shows the effect of DC9 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the HR of anaesthetised normotensive Wistar rats. DC9 produced dose dependent increases in HR, with the increases statistically significant at the 5 mg/kg, 10 mg/kg and 50 mg/kg doses (see Figure 4.21; Appendix V). The lowest dose (0.5 mg/kg), produced a 6.102 bpm ± 0.9101 increase in HR, while the highest dose administered (40 mg/kg), produced a 35.110 bpm ± 6.909 change in HR.
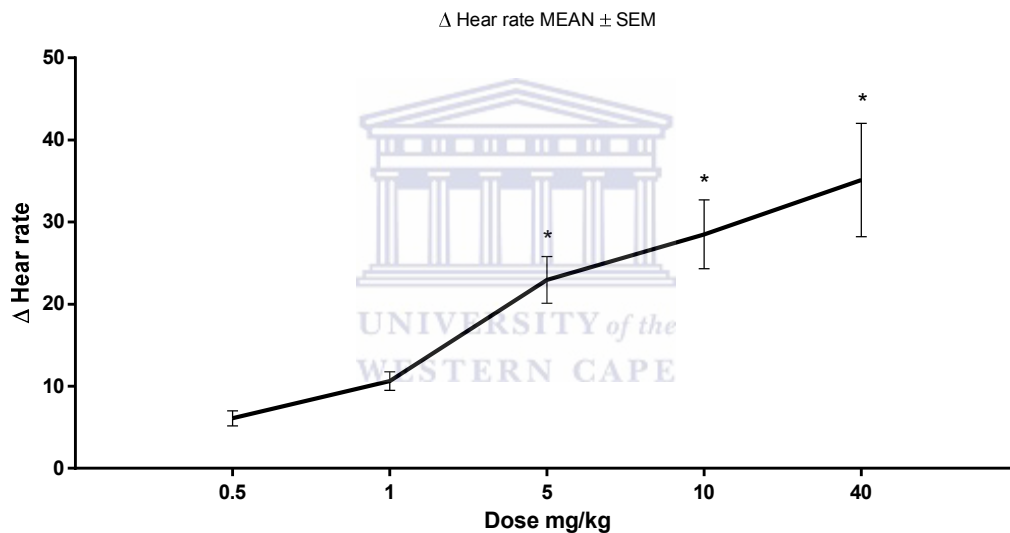
Figure 4.21: Effects of DC9 (0.5 mg/kg - 40 mg/kg) on Heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change.
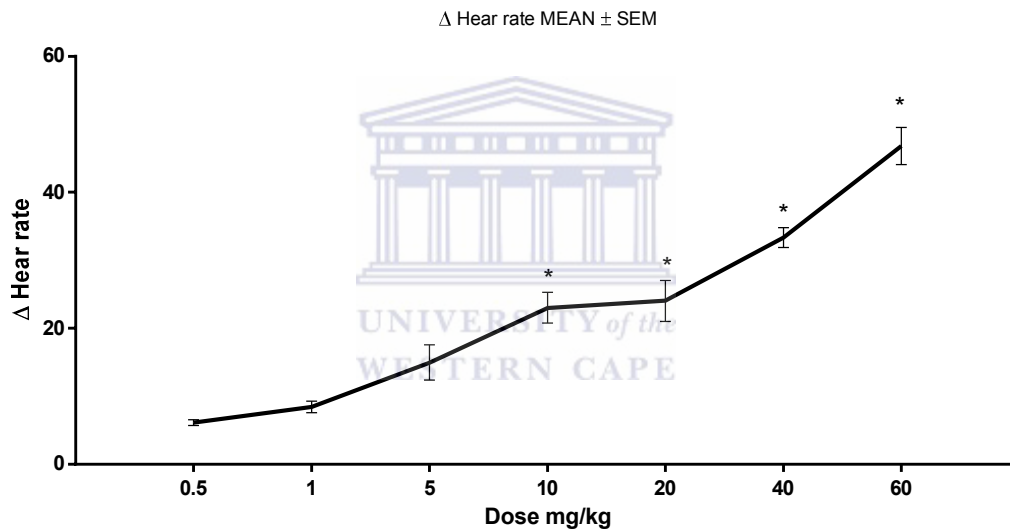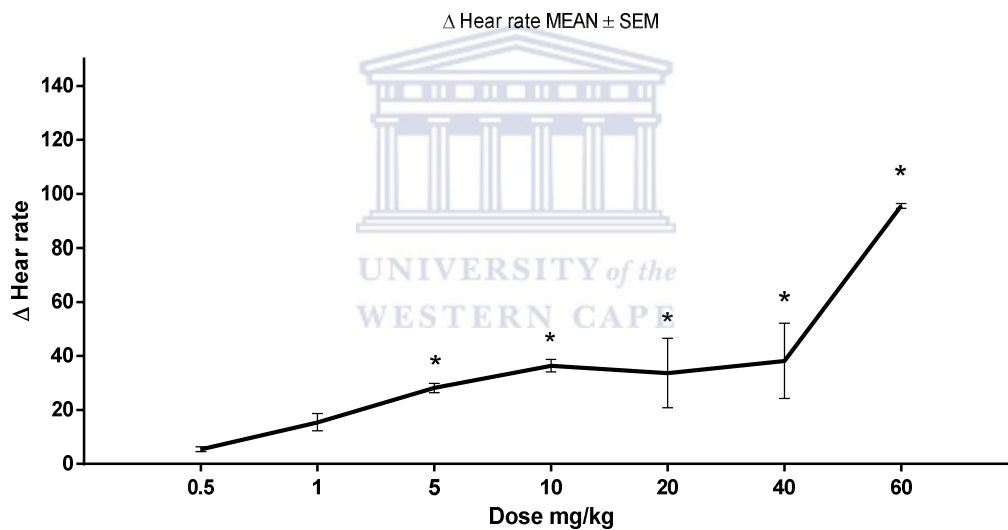
4.8.4.5 Effects of DC15 on Heart rate (HR)

Figure 4.22 shows the effect of DC15 administered in a dose range of (0.5 mg/kg, - 40 mg/kg) on the HR of anaesthetised normotensive Wistar rats. DC15 produced dose dependent increases in HR, with the increases statistically significant at the 10 mg/kg, 20 mg/kg and 40 mg/kg doses (see Figure 4.22; Appendix V). The lowest dose (0.5 mg/kg) produced a 7.127 bpm ± 1.047 increase in HR, while the highest dose administered (40 mg/kg), produced a 31.590 bpm ± 2.423 increase in HR.

Figure 4.22: Effects of DC15 (0.5 mg/kg - 40 mg/kg) on Heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change.
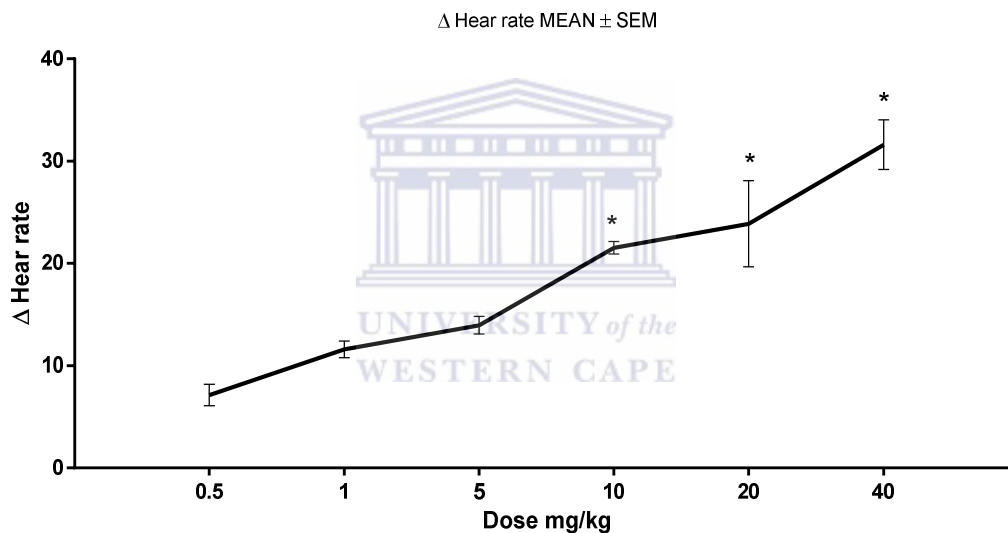
4.9 DISCUSSION

In this study all five (5) diterpenoids isolated from *L. leonurus* (DC 1, DC 2, DC 8, DC 9, and DC 15) (Table 3.1) were evaluated for their cardiovascular effect on anaesthetised normotensive Wistar rat models. The cardiovascular effects of a drug can be deciphered by its effect on measurable cardiovascular parameters like systolic pressure, diastolic pressure, mean arterial pressure, and heart rate (Obikeze, 2004; Raji *et al*., 2012). With respect to the five diterpenoids tested, all produced dose dependent and significant increases in systolic, diastolic, and mean arterial pressures, and heart rate (Appendix V).

The increases in Blood pressure (BP) observed was the opposite of the effects reported by Njagi and Ojewole in anaesthetized normotensive animals using the crude aqueous extracts of the plant (Njagi *et al*., 2001; Ojewole, 2003). However this effect was similar to that noted in earlier experiments using crude aqueous extracts of the leaves only and methanol extracts of the leaves (Mugabo *et al*., 2002; Obikeze, 2004; Obikeze *et al*., 2013). Also, the increase in BP was similar to that produced by a novel diterpene - EDD as reported by Obikeze and co-workers (2008), with EDD inducing dose-dependent statistically significant increases in BP with high doses(Obikeze *et al*., 2008).

The increase in HR with the isolated diterpenoids was also similar to that observed by Raji *et al*., (2013) with methanol extracts, but was once more the opposite of the decrease in HR reported by Obikeze, (2004) and Ojewole, (2003) with the crude aqueous extract. EDD however showed dose-dependent statistically significant decreases in HR with all doses, the opposite of the effect observed with the five compounds evaluated in this study (Obikeze *et al*., 2008).

DC15 (Marrubiin) is a diterpenoid found in different plants such as *Marrubium vulgare* and *Phlomis bracteosa*, and has been reported to exhibit vasorelaxant activity *in-vitro* on the isolated rat aorta. The mechanism of its relaxant activity is due to Ca^{2+} channel blockade (El Bardai *et al*., 2003; Khan *et al*., 2012). Vasorelaxant activity by

Calcium channel Ca^{2+} blockade has been identified as the mechanism of action of the cardiovascular effects of many plant compounds including diterpenoids (Ambrosio *et al*., 2006; Baccelli *et al*., 2005; El Bardai *et al*., 2004, 2003; Khan *et al*., 2012; Somova *et al*., 2001). A cardio selective Ca^{2+} channel blocker would produce decreases in Heart rate (HR) and blockade of Ca^{2+} channels in the contractile tissues of arteries would produce vasorelaxation which will cause a decrease in blood pressure (BP) (Fozzard, 2002; Wakabayashi *et al*., 1995). This was the opposite of the effect observed with the five (5) diterpenoids tested in this study. Furthermore, the dose-dependent increases in blood pressure (SP, DP and MAP) and HR observed with these compounds (Table 3.1) as shown in Appendix V could be indicative of positive chronotropic and inotropic effect in the Heart. Interestingly a positive chronotropic and inotropic effect was similarly reported by (Mugabo *et al*., 2002) with the crude aqueous extract of *L. leonurus* in Langendorff perfused isolated rat hearts and by Obikeze (2013) with a fraction of the methanol extract of *L. leonurus* administered to anaesthetized normotensive rats. Suggesting the presence of DC15 in these extracts. The effects observed in this study were different from those observed by Khan *et al* (2012) and El Bardai *et al* (2003), possibly because the hypotensive effect of Marrubiin (DC15) as a result of vasorelaxant effect on the arteries masked by its effect on the Heart (positive chronotropic and inotropic effect) when tested *in-vivo*.

Neural and hormonal regulation of the Heart is mediated by β1 adrenoceptors in the Heart, stimulation of these receptors with an agonist drug would lead to a positive chronotropic and inotropic effect. The activation of β1 adrenoceptors on the myocardial cell surface by an agonist leads to an increase in Heart rate (HR) (a positive chronotropic effect) and an increase force of contraction (a positive inotropic effect). Inhibition of these receptors with an antagonist would lead to a decrease in Heart rate (HR) (a negative chronotropic effect) and decrease in force of contraction (a negative inotropic effect) (Warne *et al*., 2011). Moreover, β1 adrenoceptor agonists produce a positive chronotropic and inotropic effect by increasing intracellular Ca^{2+} in cardiac cells, and β1 adrenoceptors blockers would prevent the increase of intracellular Ca^{2+}, an effect similar to that produced by Ca^{2+} channel agonists and blockers (Opie, 2004).

Also, the peripheral vascular resistance (PVR) plays an important role in determining Blood pressure (BP). Certain agents called vasoactive agents could cause the vasoconstriction or vasodilatation of Blood vessels. Increases in Blood pressure (BP) could occur either through a direct vasoconstrictor effect or an indirect vasoconstrictor effect. For instance, a direct vasoconstrictor effect is mediated by an agonistic effect on α1 receptors or agonistic effect on Angiotensin II receptors (AT_1) in the contractile tissue of the arteries, while an indirect vasoconstrictor effect is mediated via the release of catecholamines into the synaptic space (Moini, 2010; Ruffolo and Hieble, 1994).

Comparing the effects of the five (5) diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) to that of methanol extracts on the cardiovascular system, both diterpenoids and methanol extracts had similar effects on Systolic pressure (SP), Diastolic pressure (DP), Mean arterial pressure (MAP) and Heart rate (HR). A fraction of the methanol extracts of the leaves of *L. leonurus* were reported to exhibit a positive chronotropic and inotropic effect both *in-vivo* and *in-vitro*, indicative of a β1 adrenoceptor agonist (Obikeze, 2005; Obikeze *et al.*, 2013). This suggests that these five (5) isolated diterpenoids compounds may have produced their cardiovascular effects by acting on the same receptors and they could also be the compounds responsible for the cardiovascular effect observed with the methanol extract of the plant.

CHAPTER FIVE

5   DISCUSSES AND CONCLUSION

5.1   DISCUSSION

This chapter further discusses the results obtained from *in-silico* and *in-vivo* studies done on the five *Leonotis leonurus* diterpenoids. Conclusions derived from the study and recommendations are also given.

L. Leonurus was chosen for this study because of its use in traditional medicine for the treatment of a wide range of diseases including cardiovascular disease (CVD) and also conflicting results from previous studies of its effects on the cardiovascular system. The purpose of this study was to investigate the Drug-likeness and to investigate the cardiovascular activity of five (5) diterpenoid compounds isolated from *L. Leonurus* extract, by performing Chemoinformatics analysis (*in-silico*) and invasive Blood pressure measurement technique (*in-vivo*).

In the Chemoinformatics (*in-silico*) study, twelve (12) important molecular descriptors (physicochemical properties) for five (5) diterpenoid compounds found in *L. Leonurus* were calculated. Based on these calculated molecular descriptors, the Drug-Likeness of these isolated diterpenoid compounds were predicted. Also, the binding modes (interactions) and the binding energy were calculated by Molecular Docking (MD) of these isolated diterpenoids compounds against the 3D structure of renin receptor, Angiotensin converting enzyme (ACE), Angiotensin II receptor (AT_1) and $\beta 1$ adrenoceptor. While in the *in-vivo* study, the cardiovascular activity of the isolated diterpenoids compounds were investigated by infusing these compounds intravenously (IV) into anaesthetized normotensive Wistar rats. Continuous monitoring of direct arterial Blood pressure (BP) using intra-arterial catheter inserted in the rat femoral arterial while infusing the compounds was carried out. At the end of the study, all the objectives of the study were achieved.

In the Renin-Angiotensin-Aldosterone System (RAAS). Renin acts as an enzyme which is involved in regulation of Blood volume, vascular resistance by hydrolysing Angiotensinogen to Angiotensin I (ANG I), which is further hydrolysed by ACE to Angiotensin II (ANG II) a potent vasoconstrictor (De Mello, 2014). The major cardiovascular effects of angiotensin II (ANG II) is mediated by Angiotensin II receptors (AT_1), these receptors are found in Blood vessels. The RAAS pathway is often the target in the treatment of cardiovascular diseases (CVD) for example hypertension (De Mello, 2014). For this reason drugs known as renin inhibitors (70X), ACE inhibitors (Captopril) and Angiotensin II receptor (AT_1) blockers (HIG) are used to lower Blood pressure (BP) (Akif *et al*., 2010; Casimiro-Garcia *et al*., 2011; Warne *et al*., 2011; Akif *et al*., 2012; Amery *et al*., 2012; McKittrick *et al*., 2015).

From the molecular docking (MD) the isolated diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) exhibited high binding energy (ΔG_b) values with renin in comparison to binding energy of 70X (renin inhibitor) with renin. Also, these compounds showed differences in their binding mode (interaction) with renin in comparison to the interaction between 70X (renin inhibitor) and renin, the more negative binding energy (ΔG_b) value indicates stronger interactions (Lim *et al*., 2011; Temirak *et al*., 2014; Thakur and Thakur, 2015). This implies that these isolated diterpenoid compounds are not expected to have renin inhibition activity or at best to exhibit a weak renin inhibition activity when tested *in-vivo*. The result obtained from *in-vivo* studies, showed that all the five (5) diterpenoid compounds caused an increase in Blood pressure (BP) and Heart rate (HR) which was the opposite to that expected with renin inhibitors (De Mello, 2014; McKittrick *et al*., 2015). For instance, the correlation between both studies (*in-silico* and *in-vivo*) indicate that these diterpenoid compounds do not exhibit any renin inhibition (antihypertensive) activity. DC1, DC2, DC8, DC9 and DC15 exhibited binding energy (ΔG_b) with ACE, similar to that observed with the ACE inhibitor - Captopril, and this could suggest that these isolated diterpenoids compounds could have similar affinity to the ACE receptor as that observed with Captopril. (Lim *et al*., 2011; Temirak *et al*., 2014; Thakur and Thakur, 2015). On other hand, none of the isolated diterpenoid compounds interacted with the

Zn^{2+} ion. The success of clinically used ACE inhibitors, such as Captopril and lisinopril depend on their ability to interact directly with the zinc ion (Akif *et al*., 2010; Akif *et al*., 2012). The difference in binding mode (interaction) and the absence of a direct interaction with the Zn^{2+} ion with the isolated diterpenoids may suggest that these compounds would not exhibit ACEI-dependent antihypertensive activity when tested *in-vivo*. The result obtained from *in-vivo* studies, showed that all the five (5) isolated diterpenoid compounds caused an increase in Blood pressure (BP) and Heart rate (HR) which was the opposite to that expected with ACE inhibitors. For instance, the correlation between both studies (*in-silico* and *in-vivo*) indicate that these diterpenoid compounds do not exhibit any ACE inhibition (antihypertensive) activity.

The binding mode (interactions) analysis of DC1, DC2, DC8, DC9 and DC15s did not show any similarity with HIG (AT$_1$ blocker) binding mode. Although DC1, DC2, DC8, DC9 and DC15 exhibited binding energies (ΔG_b) with the AT$_1$ receptor, the binding energy (ΔG_b) of the diterpenoids were higher than that observed with HIG (AT$_1$ blocker), suggesting that these compounds would have low affinity for the AT$_1$ receptor. The differences in the binding energy (ΔG_b) and binding mode (interactions) between the diterpenoids and HIG with AT$_1$ receptor suggests that these compounds would not exhibit antihypertensive activity as a result of AT$_1$ blocking activity when tested *in-vivo*. The result obtained from *in-vivo* studies, showed that all the five (5) isolated diterpenoid compounds (DC1, DC2, DC8, DC9 and DC15) caused an increase in Blood pressure (BP) and Heart rate (HR) which was the opposite to the antihypertensive effect of AT$_1$ blockers. For instance, the correlation between both studies (*in-silico* and *in-vivo*) indicate that these diterpenoid compounds do not exhibit any AT$_1$ blocking (antihypertensive) activity (De Mello, 2014).

The binding mode (interactions) and the binding energy (ΔG_b) for DC1, DC2, DC9 and DC15 were similar to that observed with Salbutamol, which could indicate that these compounds expected to produce similar pharmacological effects to that observed with the activation of β1 adrenoceptor. The result obtained from our *in-vivo* studies, showed that all the five (5) isolated diterpenoid compounds (DC1, DC2, DC8, DC9

and DC15) caused an increase in Blood pressure (BP) and Heart rate (HR) which was similar to that exhibited with β1 agonist drugs (Warne *et al*., 2011).

In anaesthetized normotensive male Wistar rats, each of the isolated diterpenoid compounds produced significant dose-dependent increases in Blood pressure (SP, DP and MAP) and Heart rate (HR). Furthermore, the positive chronotropic and positive inotropic effects that were observed in the *in-vivo* study were similar to that observed with β1 agonist drugs (Warne *et al*., 2011) and different to that observed with renin inhibitors, ACE inhibitors and Angiotensin II (AT$_1$) blockers (De Mello, 2014). The result obtained from *in-vivo* studies correlated with the *in-silico* studies.

5.2   CONCLUSION

From the study results, all the five (5) diterpenoid compounds - DC1, DC2, DC8, DC9 and DC15 were predicted to have a good oral bioavailability and pass through the Blood-Brain Barrier (BBB). Also, the compounds were predicted to have high probability of being good Drug-like candidates, except for DC9, which is predicted to have lower possibilities of being Drug-like candidate than the other diterpenoids (DC1, DC2, DC8 and DC15). Furthermore, these compounds (DC1, DC2, DC8, DC9 and DC15) were shown to interact with β1 adrenoceptors *in-silico*, an interaction that was confirmed *in-vivo* by increases in Blood pressure (BP) and Heart rate (HR). These interactions were similar to that observed with the known β1agonist (Salbutamol). From the *in-vivo* and *in-silico* studies it can be concluded that all the five (5) isolated diterpenoids compounds showed cardiovascular effects on Blood pressure (BP) and Heart rate (HR) by acting as β1 adrenoceptor agonists. Also, these diterpenoids compounds could be responsible for the cardiovascular effect observed in the methanol extracts from previous studies. These cardio-active compounds are prototype or "lead compounds" for design and developing new non-toxic and effective drugs for cardiovascular disease (CVD) treatment.

5.3 RECOMENDATION

The results from this study are not however conclusive on the mechanism of the action of the *L. leonurus* diterpenoids. Further studies using known drugs, isolated organs and homology modelling are required to confirm the mechanism of action through which these compounds produce their cardiovascular effect and also confirm their oral bioavailability and Blood-Brain Barrier permeability. The scope of this study was limited by the lack of sufficient funds and availability of the isolated compounds. Also limited access to equipment, materials and software needed to carry out some of the additional experiments contributed to limiting the scope of this study.

REFERENCE

Adamson, R.S., Salter, T.M., 1950. Flora of the Cape Peninsula. Juta & Co., Cape Town, p. 889.

Adeyi, O., Smith, O., Robles, S., 2007. Public Policy and the Challenge of Chronic Noncommunicable Diseases. World Bank, Washington, D.C.

Adjanohoun, E., Adjakide, V., Souza, de, 1989. Contribution to Ethnobotanical and Floristic Studies in Republic of Benin. Agency Cult. Tech. Coop 1, 245.

Afolayan, A.J., Sunmonu, T.O., 2010. In Vivo Studies on Antidiabetic Plants Used in South African Herbal Medicine. J. Clin. Biochem. Nutr. 47, 98–106.

Ahuja, M., 2006. Life Sciences. Gyan Publishing House, Delhi, p. 88.

Akif, M., Georgiadis, D., Mahajan, A., Dive, V., Sturrock, E.D., Isaac, R.E., Acharya, K.R., 2010. High Resolution Crystal Structures of Drosophila Melanogaster Angiotensin Converting Enzyme in Complex with Novel Inhibitors and Anti-Hypertensive Drugs. J. Mol. Biol. 400, 502–517.

Akif, M., Masuyer, G., Bingham, R.J., Sturrock, E.D., Isaac, R.E., Acharya, K.R., 2012. Structural Basis of Peptide Recognition by the Angiotensin-1 Converting Enzyme Homologue AnCE from Drosophila Melanogaster. Fed. Eur. Biochem. Soc. 279, 4525–4534.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2013. Essential Cell Biology, 4th ed. Garland Science, New York, pp. 364-397

Ambrosio, S.R., Tirapelli, C.R., da Costa, F.B., de Oliveira, A.M., 2006. Kaurane and Pimarane-Type Diterpenes From the Viguiera Species Inhibit Vascular Smooth Muscle Contractility. Life Sci. 79, 925–933.

Amery, A., Fagard, R., Lijnen, P., Staessen, J., 2012. Hypertensive Cardiovascular Disease: Pathophysiology and Treatment, 1st ed. Springer Netherlands, The Hague, pp. 117-147.

Appel, L.J., Moore, T.J., Obarzanek, E., Vollmer, W.M., Svetkey, L.P., Sacks, F.M., Bray, G.A., Vogt, T.M., Cutler, J.A., Windhauser, M.M., Lin, P.H., Karanja, N., 1997. A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure. DASH Collaborative Research Group. N. Engl. J. Med. 336, 1117–1124.

Asmawi, M.Z., 2012. Chronic Effects of *Andrographis paniculata* Chloroform Extract in Spontaneously Hypertensive Rats. J. Pharm. Sci. Res. 4, 1924–1928.

Augusti, K.T., Chackery, J., Jacob, J., Kuriakose, S., George, S., Nair, S.S., 2005. Beneficial Effects of a Polar Fraction of Garlic (*Allium Sativum Linn*) Oil in Rats Fed With Two Different High Fat Diets. Indian J. Exp. Biol. 43, 76–83.

Aulton, M.E., Taylor, K.M.G., 2013. Aulton's Pharmaceutics: The Design and Manufacture of Medicines, 4th ed. Elsevier Health Sciences, Edinburgh, pp. 3-75.

Awale, S., Tezuka, Y., Banskota, A.H., Shimoji, S., Taira, K., Kadota, S., 2002. Norstaminane- and Isopimarane-Type Diterpenes of *Orthosiphon stamineus* from Okinawa. Tetrahedron 58, 5503–5512.

Baccelli, C., Block, S., Van Holle, B., Schanck, A., Chapon, D., Tinant, B., Meervelt, L.V., Morel, N., Quetin-Leclercq, J., 2005. Diterpenes Isolated from *Croton zambesicus* inhibit KCl-induced contraction. Planta Med. 71, 1036–1039.

Bannerman, R.H., 1983. Traditional Medicine and Health Care Coverage. A Reader for Health Administrators and Practitioners. World Health Organisation, Geneva.

Barrett-Connor, E., 1997. Sex Differences in Coronary Heart Disease Why Are Women So Superior? The 1995 Ancel Keys Lecture. Circulation 95, 252–264.

Bickerton, G.R., Paolini, G.V., Besnard, J., Muresan, S., Hopkins, A.L., 2012. Quantifying the Chemical Beauty of Drugs. Nat. Chem. 4, 90–98.

Bienvenu, E., Amabeoku, G.J., Eagles, P.K., Scott, G., Springfield, E.P., 2002. Anticonvulsant Activity of Aqueous Extract of *Leonotis leonurus*. Phytomedicine Int. J. Phytother. Phytopharm. 9, 217–223.

Blundell, T.L., 1996. Structure-Based Drug Design. Nature 384, 23–26.

Bokaeian, M., Saboori, E., Saeidi, S., Niazi, A.A., Amini-Borojeni, N., Khaje, H., Bazi, S., 2014. Phytochemical Analysis, Antibacterial Activity of *Marrubium vulgare* L against Staphylococcus aureus in vitro. Zahedan J. Res. Med. Sci. 16, 60–64.

Bruce, C.L., 2010. Classification and Interpretation in Quantitative Structure-Activity Relationships (eThesis).

Casimiro-Garcia, A., Filzen, G.F., Flynn, D., Bigge, C.F., Chen, J., Davis, J.A., Dudley, D.A., Edmunds, J.J., Esmaeil, N., Geyer, A., Heemstra, R.J., Jalaie, M., Ohren, J.F., Ostroski, R., Ellis, T., Schaum, R.P., Stoner, C., 2011. Discovery of a Series of Imidazo[4,5-b] pyridines with Dual Activity at Angiotensin II Type 1 Receptor and Peroxisome Proliferator-Activated Receptor-γ. J. Med. Chem. 54, 4219–4233.

Chalmers, J., MacMahon, S., Mancia, G., Whitworth, J., Beilin, L., Hansson, L., Neal, B., Rodgers, A., Ni Mhurchu, C., Clark, T., 1999. 1999 World Health Organization-International Society of Hypertension Guidelines for the management of hypertension. Guidelines sub-committee of the World Health Organization. Clin. Exp. Hypertens. 21, 1009–1060.

Chattopadhyay, I., Biswas, K., Bandyopadhyay, U., Banerjee, R.K., 2004. Turmeric and curcumin: Biological Actions and Medicinal Applications. Curr. Sci. 87, 44–53.

Chen, J.J., Swope, D.M., Dashtipour, K., 2007. Comprehensive Review of Rasagiline, A Second-Generation Monoamine Oxidase Inhibitor, for the Treatment Of Parkinson's Disease. Clin. Ther. 29, 1825–1849.

Costa, A.M.L., Silva, J.C.R., Campos, A.R., Rao, V.S.N., Maciel, M. a. M., Pinto, A.C., 1999. Antioestrogenic Effect of Trans-Dehydrocrotonin, A Nor-Clerodane Diterpene From *Croton Cajucara Benth* in Rats. Phytother. Res. 13, 689–691.

Dahl, L.K., Heine, M., 1975. Primary Role of Renal Homografts in Setting Chronic Blood Pressure Levels in Rats. Circ. Res. 36, 692–696.

Dearden, J.C., 2012. Prediction of Physicochemical Properties, in: Computational Toxicology, Methods in Molecular Biology. pp. 93–138.

De Mello, W.C., 2014. Beyond the Circulating Renin–Angiotensin Aldosterone System. Front. Endocrinol, pp. 9-53

De Smet, P.A., 1991. Is There Any Danger in Using Traditional Remedies? J. Ethnopharmacol. 32, 43–50.

Dewick, P.M., 2011. Medicinal Natural Products: A Biosynthetic Approach. John Wiley & Sons, pp. 4-154.

DiMasi, J.A., Hansen, R.W., Grabowski, H.G., Lasagna, L., 1991. Cost of Innovation in the Pharmaceutical Industry. J. Health Econ. 10, 107–142.

Dubey, N.K., Kumar, R., Tripathi, P., 2004. Global Promotion of Herbal Medicine: India's Opportunity. Curr. Sci. 86, 37–41.

Ducharme, Y., Blouin, M., Carrière, M.-C., Chateauneuf, A., Côté, B., Denis, D., Frenette, R., Greig, G., Kargman, S., Lamontagne, S., Martins, E., Nantel, F., O'Neill, G., Sawyer, N., Metters, K.M., Friesen, R.W., 2005. 2,3-Diarylthiophenes as Selective EP1 Receptor Antagonists. Bioorg. Med. Chem. Lett. 15, 1155–1160.

Dyson, A., 1988. Indigenous Healing Plants of the Herb and Fragrance Gardens: Getting To Know Kirstenbosch National Botanical Garden. National Botanical Institute, Cape Town, pp. 41-57.

El Bardai, S., Hamaide, M.-C., Lyoussi, B., Quetin-Leclercq, J., Morel, N., Wibo, M., 2004. Marrubenol Interacts With the Phenylalkylamine Binding Site of The L-Type Calcium Channel. Eur. J. Pharmacol. 492, 269–272.

El Bardai, S., Lyoussi, B., Wibo, M., Morel, N., 2001. Pharmacological Evidence of Hypotensive Activity of *Marrubium Vulgare* and *Foeniculum Vulgare* in Spontaneously Hypertensive Rat. Clin. Exp. Hypertens. 23, 329–343.

El Bardai, S., Morel, N., Wibo, M., Fabre, N., Llabres, G., Lyoussi, B., Quetin-Leclercq, J., 2003. The Vasorelaxant Activity of Marrubenol and Marrubiin from *Marrubium Vulgare*. Planta Med. 69, 75–77.

Enyedy, I.J., Lee, S.L., Kuo, A.H., Dickson, R.B., Lin, C.Y., Wang, S., 2001a. Structure-Based Approach for the Discovery of Bis-Benzamidines as Novel Inhibitors of Matriptase. J. Med. Chem. 44, 1349–1355.

Enyedy, I.J., Ling, Y., Nacro, K., Tomita, Y., Wu, X., Cao, Y., Guo, R., Li, B., Zhu, X., Huang, Y., Long, Y.Q., Roller, P.P., Yang, D., Wang, S., 2001b. Discovery of Small-Molecule Inhibitors of Bcl-2 through Structure-Based Computer Screening. J. Med. Chem. 44, 4313–4324.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J., 2004. Assessing African Medicinal Plants for Efficacy and Safety: Pharmacological Screening and Toxicology. J. Ethnopharmacol. 94, 205–217.

Folkow, B., Svanborg, A., 1993. Physiology of Cardiovascular Aging. Physiol. Rev. 73, 725–764.

Fozzard, H.A., 2002. Cardiac Sodium and Calcium Channels: A history of excitatory Currents. Cardiovasc. Res. 55, 1–8.

Germishuizen, G., Meyer, N.L., 2003. Plants of southern Africa: An Annotated Checklist. National Botanical Institute, Pretoria, pp. 1-53.

Ghose, A.K., Viswanadhan, V.N., Wendoloski, J.J., 1999. A Knowledge-Based Approach in Designing Combinatorial or Medicinal Chemistry Libraries for Drug Discovery. 1. A Qualitative and Quantitative Characterization of Known Drug Databases. J. Comb. Chem. 1, 55–68.

Glen, R.C., Martin, G.R., Hill, A.P., Hyde, R.M., Woollard, P.M., Salmon, J.A., Buckingham, J., Robertson, A.D., 1995. Computer-Aided Design and Synthesis of 5-Substituted Tryptamines and Their Pharmacology at the 5-HT1D Receptor: Discovery of Compounds with Potential Anti-Migraine Properties. J. Med. Chem. 38, 3566–3580.

Greer, J., Erickson, J.W., Baldwin, J.J., Varney, M.D., 1994. Application of the Three-Dimensional Structures of Protein Target Molecules in Structure-Based Drug Design. J. Med. Chem. 37, 1035–1054.

Guerrero, M.F., Puebla, P., Carrón, R., Martín, M.L., San Román, L., 2004. Vasorelaxant Effect of New Neo-Clerodane Diterpenoids Isolated From Croton Schiedeanus. J. Ethnopharmacol. 94, 185–189.

Gurib-Fakim, A., 2006. Medicinal Plants: Traditions of Yesterday and Drugs of Tomorrow. Mol. Aspects Med. 27, 1–93.

Hanson, J.R., 2003. Natural Products: The Secondary Metabolites. Royal Society of Chemistry, pp. 2.

Harrison, L.J., 1983. Secondary metabolites of the Hepaticae (PhD). University of Glasgow, Scotland.

Harvey, A.L., 2008. Natural Products in Drug Discovery. Drug Discov. Today 13, 894–901.

Hefferon, K., 2012. The Lure of Herbal Medicine, in: Let Thy Food Be Thy Medicine: Plants and Modern Medicine. Oxford University Press.

He, F., Lindqvist, C., Harding, W.W., 2012. Leonurenones A–C: Labdane diterpenes from *Leonotis leonurus*. Phytochemistry 83, 168–172.

Henderson, M.S., Mccrindle, R., 1969. Premarrubiin. A Diterpenoid from *Marrubium Vulgare* L. J. Chem. Soc. C Org. 2014–2015.

Henkel, T., Brunne, R.M., Müller, H., Reichel, F., 1999. Statistical Investigation into the Structural Complementarity of Natural Products and Synthetic Compounds. Angew. Chem. Int. Ed. 38, 643–647.

Hiruma-Lima, C.A., Gracioso, J.S., Bighetti, E.J.B., Grassi-Kassisse, D.M., Nunes, D.S., Brito, A.R.M.S., 2002. Effect of Essential Oil Obtained from Croton Cajucara Benth on Gastric Ulcer Healing And Protective Factors of the Gastric Mucosa. Phytomedicine Int. J. Phytother. Phytopharm. 9, 523–529.

Hiruma-Lima, C.A., Gracioso, J.S., Rodríguez, J.A., Haun, M., Nunes, D.S., Souza Brito, A.R.M., 2000. Gastroprotective Effect of Essential Oil from *Croton Cajucara Benth*. (Euphorbiaceae). J. Ethnopharmacol. 69, 229–234.

Hoffmann, D., 2003. Medical Herbalism: The Science and Practice of Herbal Medicine. Healing Arts Press, Rochester, pp. 678.

Horie, R., Kihara, M., Lovenberg, W., Ben-Ishay, D., Bianchi, G., Iwai, J., Nagaoka, A., Rapp, J.P., Sassard, J., Simpson, F.O., 1986. Comparison of Various Genetic Hypertensive Rat Strains. J. Hypertens. Suppl. Off. J. Int. Soc. Hypertens. 4, S11–14.

Hutchings, A., 1996. Zulu Medicinal Plants: An Inventory, 1st ed. University of Natal Press, Pietenmaritzburg, pp. 450.

Hutchinson, J., 1973. The Families of Flowering Plants: Arranged According To A New System Based on Their Probable Phylogeny, 3rd ed. Clarendon Press, Oxford, p. 698.

Iams, S.G., Wexler, B.C., 1979. Inhibition of the Development of Spontaneous Hypertension in SH Rats by Gonadectomy or Estradiol. J. Lab. Clin. Med. 94, 608–616.

Jager, S., Beffert, M., Hoppe, K., Nadberezny, D., Frank, B., Scheffler, A., 2011. Preparation of Herbal Tea as Infusion or by Maceration at Room Temperature Using *Mistletoe Tea* as an Example. Sci. Pharm. 79, 145–155.

Jorgensen, W.L., 1991. Rusting of the Lock and Key Model for Protein-Ligand Binding. Science 254, 954–955.

Kaplan, E.R., Naidu, K., Rivett, D.E.A., 1970. Diterpenoids of *Leonotis* Species. Part III. 8Î2-Hydroxymarrubiin from *L. Dysophylla Benth*. J. Chem. Soc. C Org. 1656–1658.

Kaplan, E.R., Rivett, D.E.A., 1968. The Structures of Compounds X and Y, Two Labdane Diterpenoids, from *Leonotis Leonurus*. J. Chem. Soc. C Org. 262–266.

Katiyar, C., Gupta, A., Kanjilal, S., Katiyar, S., 2012. Drug Discovery from Plant Sources: An Integrated Approach. Int. Q. J. Res. Ayurveda 33, 10–19.

Kauser, K., Rubanyi, G.M., 1995. Gender Difference in Endothelial Dysfunction in the Aorta of Spontaneously Hypertensive Rats. Hypertension 25, 517–523.

Kawakami, Y., Inoue, A., Kawai, T., Wakita, M., Sugimoto, H., Hopfinger, A.J., 1996. The Rationale for E2020 as a Potent Acetylcholinesterase Inhibitor. Bioorg. Med. Chem. 4, 1429–1446.

Keenan, R.M., Weinstock, J., Finkelstein, J.A., Franz, R.G., Gaitanopoulos, D.E., Girard, G.R., Hill, D.T., Morgan, T.M., Samanen, J.M., Peishoff, C.E., 1993. Potent Nonpeptide Angiotensin II Receptor Antagonists. 2. 1-(Carboxybenzyl) Imidazole-5-Acrylic Acids. J. Med. Chem. 36, 1880–1892.

Kerns, L., Di, L., 2010. Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization. Academic Press, Connecticut, p. 130.

Khan, A.-U., Ullah, R., Khan, A., Mustafa, M.R., Hussain, J., Murugan, D.D., Hadi, A.H.B.A., 2012. Vasodilator Effect of Phlomis Bracteosa Constituents is mediated through Dual Endothelium-Dependent and Endothelium-Independent Pathways. Clin. Exp. Hypertens. 34, 132–139.

Kim, D.D., Sánchez, F.A., Durán, R.G., Kanetaka, T., Durán, W.N., 2007. Endothelial Nitric Oxide Synthase is A Molecular Vascular Target for The Chinese Herb Danshen in Hypertension. Am. J. Physiol. - Heart Circ. Physiol. 292, H2131–H2137.

Kitchen, D.B., Decornez, H., Furr, J.R., Bajorath, J., 2004. Docking and Scoring In Virtual Screening for Drug Discovery: Methods and Applications. Nat. Rev. Drug Discov. 3, 935–949.

Klebe, G., 2000. Recent Developments in Structure-Based Drug Design. J. Mol. Med. Berl. Ger. 78, 269–281.

Koehn, F.E., Carter, G.T., 2009. Rediscovering Natural Products as a Source of New Drugs. Discov. Med. 5, 159–164.

Kreatsoulas, C., Anand, S.S., 2010. The Impact of Social Determinants on Cardiovascular Disease. Can. J. Cardiol. 26, 8C–13C.

Lahlou, S., Interaminense, L. de F.L., Leal-Cardoso, J.H., Morais, S.M., Duarte, G.P., 2004. Cardiovascular Effects of the Essential Oil of *Ocimum Gratissimum* Leaves in

Rats: Role of the Autonomic Nervous System. Clin. Exp. Pharmacol. Physiol. 31, 219–225.

Leistner, O.A., 2005. Seed Plants of Southern Tropical Africa: Families and Genera. sabonet, Pretoria, p. 125.

Lekshmi, P.C., Arimboor, R., Nisha, V.M., Menon, A.N., Raghu, K.G., 2013. In Vitro Antidiabetic and Inhibitory Potential of Turmeric (*Curcuma Longa L*) Rhizome against Cellular and LDL Oxidation and Angiotensin Converting Enzyme. J. Food Sci. Technol. 51, 3910–3917.

Leong, X.-F., Ng, C.-Y., Jaarin, K., Leong, X.-F., Ng, C.-Y., Jaarin, K., 2015. Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis. BioMed Res. Int. 2015, 1–11.

Lim, S.V., Rahman, M.B.A., Tejo, B.A., 2011. Structure-Based and Ligand-Based Virtual Screening of Novel Methyltransferase Inhibitors of the Dengue Virus. BMC Bioinformatics 12, S24.

Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, A.D., Terblanche, S.E., JaÂ¨ger, A.K., Staden, J., 1999. Preliminary Screening of Some Traditional Zulu Medicinal Plants for Anti-Inflammatory and Anti-Microbial Activities. 1999 15, 267–274.

Lipinski, C.A., 2004. Lead- and Drug-Like Compounds: The Rule-of-Five Revolution. Drug Discov. Today Technol. 1, 337–341.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental And Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. Adv. Drug Deliv. Rev., In Vitro Models for Selection of Development Candidates 23, 3–25.

Liu, J., Wang, W., Liu, J., Qi, Y., Sun, J.-Y., Zhao, D., 2013. Clustering of Cardiovascular Risk Factors and Hypertension Control Status among Hypertensive Patients in the Outpatient Setting. Zhonghua Xin Xue Guan Bing Za Zhi 41, 1050–1054.

Liu, X., Pan, L., Gong, Q., Zhu, Y., 2010. Leonurine (SCM-198) Improves Cardiac Recovery in Rat During Chronic Infarction. Eur. J. Pharmacol. 649, 236–241.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000. Functional Design of Proteins, in: Molecular Cell Biology. W. H. Freeman, United States, p. 1300.

Lybrand, T.P., 1995. Ligand-Protein Docking and Rational Drug Design. Curr. Opin. Struct. Biol. 5, 224–228.

Mackraj, I., Ramesar, S., Singh, M., Govender, T., Baijnath, H., Singh, R., Gathiram, P., 2008. The In Vivo Effects of *Tulbhagia Violacea* on Blood Pressure in a Salt-Sensitive Rat Model. J. Ethnopharmacol. 117, 263–269.

Maphosa, V., Adedapo, A.A., Moyo, B., Masika, P.J., 2012. Anti-Inflammatory and Analgesic Activities of the Aqueous Extract of *Leonotis Leonurus* Leaves in Rats. Afr. J. Biotechnol. 11, 6878–6883.

Maphosa, V., Masika, P.J., Bizimenyera, E.S., Eloff, J.N., 2010. In-Vitro Anthelminthic Activity of Crude Aqueous Extracts of Aloe Ferox, *Leonotis Leonurus* and Elephantorrhiza Elephantina Against Haemonchus Contortus. Trop. Anim. Health Prod. 42, 301–307.

MartÃ--Renom, M.A., Stuart, A.C., Fiser, A., SÃ¡nchez, R., Melo, F., \vSali, A., 2000. Comparative Protein Structure Modeling of Genes and Genomes. Annu. Rev. Biophys. Biomol. Struct. 29, 291–325.

Mazimba, O., 2015. *Leonotis Leonurus*: a Herbal Medicine Review. J. Pharmacogn. Phytochem. 3, 74–82.

McGaw, L.J., Eloff, J.N., 2008. Ethnoveterinary Use of Southern African Plants and Scientific Evaluation of Their Medicinal Properties. J. Ethnopharmacol. 119, 559–574.

McKenzie, J.M., Green, I.R., Mugabo, P., 2006. Leonurun, a Novel Labdane Diterpenoid from *Leonotis Leonurus*. South Afr. J. Chem. 59, p114.

McKittrick, B.A., Caldwell, J.P., Bara, T., Boykow, G., Chintala, M., Clader, J., Czarniecki, M., Courneya, B., Duffy, R., Fleming, L., Giessert, R., Greenlee, W.J., Heap, C., Hong, L., Huang, Y., Iserloh, U., Josien, H., Khan, T., Korfmacher, W., Liang, X., Mazzola, R., Mitra, S., Moore, K., Orth, P., Rajagopalan, M., Roy, S., Sakwa, S., Strickland, C., Vaccaro, H., Voigt, J., Wang, H., Wong, J., Zhang, R., Zych, A., 2015. Iminopyrimidinones: A Novel Pharmacophore for the Development of Orally Active Renin Inhibitors. Bioorg. Med. Chem. Lett. 25, 1592–1596.

McMahon, F.G., Vargas, R., 1993. Can Garlic Lower Blood Pressure? A Pilot Study. Pharmacother. J. Hum. Pharmacol. Drug Ther. 13, 406–407.

Mnonopi, N., Levendal, R.-A., Davies-Coleman, M., Frost, C.L., 2011. The Cardioprotective Effects of Marrubiin, a Diterpenoid Found in *Leonotis Leonurus* Extracts. J. Ethnopharmacol. 138, 67–75.

Mohamadi A, Jarrell St, Shi Sj, Andrawis Ns, Myers A, Clouatre D, Preuss Hg, 2000. Effects of Wild versus Cultivated Garlic on Blood Pressure and Other Parameters in Hypertensive Rats. Heart Dis. Hagerstown Md 2, 3–9.

Moini, J., 2010. Cardiopulmonary Pharmacology for Respiratory Care. Jones & Bartlett Learning, Singapore, p. 314

Moss, G.P., Dearden, J.C., Patel, H., Cronin, M.T.D., 2002. Quantitative Structure-Permeability Relationships (Qsprs) for Percutaneous Absorption. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 16, 299–317.

Mugabo, P., Khan, F., Burger, A., 2012. Effects of *Leonotis Leonurus* Aqueous Extract on the Isolated Perfused Rat Heart. Int. J. Med. Aromat. Plants 2, 281–292.

Mugabo, p, Njagi, A., Dietrich, D.., Syce, J., 2002. Cardiovascular Effects of *Leonotis Leonurus* in the Normotensive Rat. Rev. Fitoter. 2.

Muhizi, T., 2002. The Extraction, Purification and Evaluation of Compounds from the Leaves of *Leonotis Leonorus* for Anticonvulsant Activity (Thesis). University of the Western Cape.

Naidoo, D., Maharaj, V., Crouch, N.R., Ngwane, A., 2011. New Labdane-Type Diterpenoids from *Leonotis Leonurus* Support Circumscription of Lamiaceae s.l. Biochem. Syst. Ecol. 39, 216–219.

Narukawa, Y., Komori, M., Niimura, A., Noguchi, H., Kiuchi, F., 2015. Two New Diterpenoids from *Leonotis Leonurus* R. Br. J. Nat. Med. 69, 130–134.

Newman, D.J., Cragg, G.M., 2012. Natural Products as Sources of New Drugs over the 30 Years from 1981 to 2010. J. Nat. Prod. 75, 311–335.

Nichols, G., 2002. Down to Earth: Gardening with Indigenous Shrubs. Struik, Cape Town, p. 67.

Njagi, A., Mugabo, P., Dietrich, D., 2001. *Leonotis Leonurus* Effect on Blood Pressure and Cardiac Function. (Unpublished Honor's report. UWC.). Cape Town.

Nsuala, B.N., Enslin, G., Viljoen, A., 2015. "Wild cannabis": A Review of the Traditional Use and Phytochemistry of *Leonotis Leonurus*. J. Ethnopharmacol. 174, 520–539.

Obikeze, K., 2004. Cardiovascular Effects of *Leonotis Leonurus* Extracts in Normotensive Rats and in Isolated Perfused Rat Heart (Thesis). University of the Western Cape.

Obikeze, K.C., McKenzie, J.M., Green, I.R., Mugabo, P., 2008. Characterization And Cardiovascular Effects Of (13S)-9α, 13α-Epoxylabda-6β2 (19), 15 (14) Diol Dilactone, a Diterpenoid Isolated from *Leonotis Leonurus*: Short Communication. South Afr. J. Chem. 61, 119–122.

Obikeze, k., Mugabo, P., Green, I., Dietrich, D., Burger, A., 2013. Effects of a Methanol Fraction of the Leaves of *Leonotis leonurus* on the Blood Pressure and Heart Rate of Normotensive Male Wistar Rats. World Acad. Sci. Eng. Technol. 81, 113–116.

Ohashi, K., Bohgaki, T., Matsubara, T., Shibuya, H., 2000. Indonesian Medicinal Plants. XXIII. Chemical Structures of Two New Migrated Pimarane-Type Diterpenes, Neoorthosiphols A and B, and Suppressive Effects on Rat Thoracic Aorta of Chemical Constituents Isolated From the Leaves of *Orthosiphon Aristatus* (Lamiaceae). Chem. Pharm. Bull. (Tokyo) 48, 433–435.

Ojewole, J.A.O., 2003. P-2: Hypotensive Effect of *Leonotis Leonurus* Aqueous Leaf Extract in Rats. Am. J. Hypertens. 16, 40A–40A.

Okamoto, K., Aoki, K., 1963. Development of a Strain of Spontaneously Hypertensive Rats. Jpn. Circ. J. 27, 282–293.

Okpako, D.T., 1999. Traditional African Medicine: Theory and Pharmacology Explored. Trends Pharmacol. Sci. 20, 482–485.

Opie, L.H., 2004. Heart Physiology: From Cell to Circulation, 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 182-232.

Oprea, T.I., 2000. Property Distribution of Drug-Related Chemical Databases. J. Comput. Aided Mol. Des. 14, 251–264.

Overington, J.P., Al-Lazikani, B., Hopkins, A.L., 2006. How Many Drug Targets are there? Nat. Rev. Drug Discov. 5, 993–996.

Oyedemi, S.O., Afolayan, A.J., 2011. In vitro and in vivo Antioxidant Activity of Aqueous Leaves Extract of *Leonotis leonurus* (L.) R. Br. Int. J. Pharmacol. 7, 248–256.

Pardridge, W.M., 2012. Drug Transport across the Blood–Brain Barrier. J. Cereb. Blood Flow Metab. 32, 1959–1972.

Pardridge, W.M., 2005. The Blood-Brain Barrier: Bottleneck in Brain Drug Development. NeuroRx 2, 3–14.

Patil, R., Raju, A., Chimkode, Tripathi, R., Tripathi, A., Agrawal, N., 2010. Antihypertensive Activity of Leaves Extracts of *Ocimum Gratissimum Linn*. Adv. Pharmacol. Toxicol. 11, 103–107.

Patwardhan, B., Warude, D., Pushpangadan, P., Bhatt, N., 2005. Ayurveda and Traditional Chinese Medicine: A Comparative Overview. Evid. Based Complement. Alternat. Med. 2, 465–473.

Pearson, L.C., 1995. The Diversity and Evolution of Plants, 1st ed. CRC Press, New York, pp. 348-571.

Pienaar, K., 1994. Gardening With Indigenous Plants. Struik, p. 96.

Pieters, M., Vorster, H.H., 2008. Nutrition and Hemostasis: A Focus on Urbanization in South Africa. Mol. Nutr. Food Res. 52, 164–172.

Pinto, Y.M., Paul, M., Ganten, D., 1998. Lessons from Rat Models of Hypertension: from Gold blatt to Genetic Engineering. Cardiovasc. Res. 39, 77–88.

Popoola, O.K., Elbagory, A.M., Ameer, F., Hussein, A.A., 2013. Marrubiin. Molecules 18, 9049–9060.

Prasad, S., Aggarwal, B.B., 2011. Turmeric, the Golden Spice: From Traditional Medicine to Modern Medicine, in: Benzie, I.F.F., Wachtel-Galor, S. (Eds.), Herbal

Medicine: Biomolecular and Clinical Aspects. CRC Press/Taylor & Francis, Boca Raton, pp. 263-288.

Pravenec, M., Kurtz, T.W., 2010. Recent Advances in Genetics of the Spontaneously Hypertensive Rat. Curr. Hypertens. Rep. 12, 5–9.

Prentis, R.A., Lis, Y., Walker, S.R., 1988. Pharmaceutical Innovation by the Seven UK-Owned Pharmaceutical Companies (1964-1985). Br. J. Clin. Pharmacol. 25, 387–396.

Preuss, H.G., Clouatre, D., Mohamadi, A., Jarrell, S.T., 2001. Wild Garlic Has a Greater Effect than Regular Garlic on Blood Pressure and Blood Chemistries Of Rats. Int. Urol. Nephrol. 32, 525–530.

Qi, J., Hong, Z.Y., Xin, H., Zhu, Y.Z., 2010. Neuroprotective Effects of Leonurine on Ischemia/Reperfusion-Induced Mitochondrial Dysfunctions in Rat Cerebral Cortex. Biol. Pharm. Bull. 33, 1958–1964.

Rahman, K., Lowe, G.M., 2006. Garlic and Cardiovascular Disease: A Critical Review. J. Nutr. 136, 736S–740S.

Raji, I.A., Mugabo, P., Obikeze, K., 2012. Effect of Tulbaghia Violacea on the Blood Pressure and Heart Rate in Male Spontaneously Hypertensive Wistar Rats. J. Ethnopharmacol. 140, 98–106.

Raji, I., Mugabo, P., Obikeze, K., 2013. The Contributions of Muscarinic Receptors and Changes in Plasma Aldosterone Levels to the Anti-Hypertensive Effect of *Tulbaghia Violacea*. BMC Complement. Altern. Med. 13.

Ramesar, S., Baijnath, H., Govender, T., Mackraj, I., 2008. Angiotensin I-Converting Enzyme Inhibitor Activity of Nutritive Plants in KwaZulu-Natal. J. Med. Food 11, 331–336.

Rishton, G.M., 2008. Natural Products as a Robust Source of New Drugs and Drug Leads: Past Successes and Present Day Issues. Am. J. Cardiol. 101, 43D–49D.

Rivett, D.E.., 1964. The Isolation of Marrubiin from *Leonotis Leonurus* R.Br. J. Chem. Soc. 1857–1858.

Roy, U., Luck, L.A., 2007. Molecular modeling of estrogen receptor using molecular operating environment. Biochem. Mol. Biol. Educ. 35, 238–243.

Ruffolo, R.R., Hieble, J.P., 1994. Alpha-Adrenoceptors. Pharmacol. Ther. 61, 1–64.

Savona, G., Piozzi, F., Rodriguez, B., 1978. Hispanolone, a New Furanoditerpene. Heterocycles. 9, 257.

Schmieder, R.E., Messerli, F.H., Garavaglia, G.E., Nunez, B.D., 1987. Cardiovascular Effects of Verapamil in Patients with Essential Hypertension. Circulation. 75, 1030–1036.

Schneider, G., 2000. Prediction of Drug-Like Properties. Landes Bioscience, Austin.

Schrader, J., Bohlmann, J., 2015. Biotechnology of Isoprenoids. Springer Science & Business Media, Swizerland, p. 217.

Scott, G., Springfield, E.P., Coldrey, N., 2004. A Pharmacognostical Study of 26 South African Plant Species Used as Traditional Medicines. Pharm. Biol. 42, 186–213.

Seedat, Y.K., 2007. Impact of Poverty on Hypertension and Cardiovascular Disease in Sub-Saharan Africa. Cardiovasc. J. Afr. 18, 316–320.

Sendl A, Elbl G, Steinke B, Redl K, Breu W, Wagner H, 1992. Comparative Pharmacological Investigations of *Allium Ursinum* and *Allium Sativum. Planta* Med. 58, 1–7.

Shankar, M., Srividya, B.K., Hemachandar, K., Sivanna, P., Babu, M.N., 2014. Importance of Structural Activity Relationship in Computer Aided Drug Design-A Review. Eur. J. Pharm. Sci. Res. 1, 13–16.

Silva, R.M., Oliveira, F.A., Cunha, K.M.A., Maia, J.L., Maciel, M.A.M., Pinto, A.C., Nascimento, N.R.F., Santos, F.A., Rao, V.S.N., 2005. Cardiovascular Effects of Trans-Dehydrocrotonin, a Diterpene from Croton Cajucara in Rats. Vascul. Pharmacol. 43, 11–18.

Singh, S., Malik, B.K., Sharma, D.K., 2006. Molecular Drug Targets and Structure Based Drug Design: A holistic approach. Bioinformation 1, 314–320.

Sofowora, A., 1982. Medicinal Plants and Traditional Medicine in Africa. Wiley, New York, p. 286.

Somova, L.I., Shode, F.O., Moodley, K., Govender, Y., 2001. Cardiovascular and Diuretic Activity of Kaurene Derivatives of *Xylopia Aethiopica* and *Alepidea Amatymbica*. J. Ethnopharmacol. 77, 165–174.

Sriramaneni, R.N., Y, P.R., Nair, R., Ameer, O.Z., Salman, I.M., Sadikun, A., Asmawi, M.Z., 2012. Chronic Effects of Andrographis paniculata Chloroform Extract in Spontaneously Hypertensive Rats. J Pharm Sci Res 4, 1924–1928.

Srivastava, A., Tripathi, A.K., Pandey, R., Verma, R.K., Gupta, M.M., 2006. Quantitative Determination of Reserpine, Ajmaline, and Ajmalicine in Rauvolfia Serpentina by Reversed-Phase High-Performance Liquid Chromatography. J. Chromatogr. Sci. 44, 557–560.

Taiz, L., Zeiger, E., 2010. Plant Physiology. Sinauer Associates, p. 565.

Talele, T.T., Khedkar, S.A., Rigby, A.C., 2010. Successful Applications of Computer Aided Drug Discovery: Moving Drugs from Concept to the Clinic. Curr. Top. Med. Chem. 10, 127–141.

Temirak, A., Shaker, Y.M., Ragab, F.A.F., Ali, M.M., Ali, H.I., El Diwani, H.I., 2014. Part I. Synthesis, Biological Evaluation and Docking Studies of New 2-Furylbenzimidazoles as Antiangiogenic Agents. Eur. J. Med. Chem. 87, 868–880.

Teodoro, M., Phillips, G., Kavraki, L., 2001. Molecular Docking: A Problem with Thousands of Degrees of Freedom, in: Robotics and Automation. Presented at the IEEE International Conference, Institute of Electrical and Electronics Engineers, New York, pp. 960–965.

Thakur, V.K., Thakur, M.K., 2015. Handbook of Polymers for Pharmaceutical Technologies, Structure and Chemistry. John Wiley & Sons, p. 672.

Tirapelli, C.R., Ambrosio, S.R., da Costa, F., B., de Oliveira, A., M., 2008. Diterpenes: A Therapeutic Promise for Cardiovascular Diseases. Recent Patents Cardiovasc. Drug Discov. 3, 1–8.

Trippodo, N.C., Frohlich, E.D., 1981. Similarities of Genetic (Spontaneous) Hypertension. Man and Rat. Circ. Res. 48, 309–319.

Tufts CSDD, 2014. Tufts Center for the Study of Drug Development; Cost Study [WWW Document]. PR Tufts CSDD 2014 Cost Study. URL http://csdd.tufts.edu/news/complete_story/pr_tufts_csdd_2014_cost_study (accessed 2.8.15).

Ubani, L.U., 2011. Preventive Therapy in Complimentary Medicine. Xlibris Corporation, Bloomington, pp. 31–305.

Vass, M., 2011. In Silico Modelling of Cooperative Ligand Binding (Degree of Magister). Eötvös Loránd University, Budapest.

Veber, D.F., Johnson, S.R., Cheng, H.-Y., Smith, B.R., Ward, K.W., Kopple, K.D., 2002. Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. J. Med. Chem. 45, 2615–2623.

Vogel, H., 2007. Drug Discovery and Evaluation: Pharmacological Assays, 3rd ed. Springer Science & Business Media, New York, pp. 187-198

Wakabayashi, I., Sakamoto, K., Hatake, K., 1995. Inhibitory Effects of Cadmium Ion on Extracellular Ca^{2+}-Independent Contraction of Rat Aorta. Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. 293, 133–140.

Walker, A.F., Marakis, G., Simpson, E., Hope, J.L., Robinson, P.A., Hassanein, M., Simpson, H.C., 2006. Hypotensive Effects of Hawthorn for Patients with Diabetes Taking Prescription Drugs: A Randomised Controlled Trial. Br. J. Gen. Pract. 56, 437–443.

Wang, R., Lu, Y., Wang, S., 2003. Comparative Evaluation of 11 Scoring Functions for Molecular Docking. J. Med. Chem. 46, 2287–2303.

Warne, T., Moukhametzianov, R., Baker, J.G., Nehmé, R., Edwards, P.C., Leslie, A.G.W., Schertler, G.F.X., Tate, C.G., 2011. The Structural Basis for Agonist and Partial Agonist Action on A B (1)-Adrenergic Receptor. Nature 469, 241–244.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa: Being an Account of Their Medicinal and Other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal, 2nd ed. E. & S. Livingstone, Pretoria, p. 517.

Wlodawer, A., Vondrasek, J., 1998. Inhibitors f HIV-1 Protease: A Major Success of Structure-Assisted Drug Design. Annu. Rev. Biophys. Biomol. Struct. 27, 249–284.

Wu, H., Fronczek, F.R., Ferreira, D., Burandt, C.L., Zjawiony, J.K., 2011. Labdane Diterpenoids from *Leonurus sibiricus*. J. Nat. Prod. 74, 831–836.

Wyk, B.-E.V., Oudtshoom, B.V., Gericke, N., 2012. Medicinal Plants of South Africa, 2nd ed. Briza Publications, Pretoria, pp. 74–188.

Yang, L.C., Wang, F., Liu, M., 1998. A Study of an Endothelin Antagonist from A Chinese Anti-Snake Venom Medicinal Herb. J. Cardiovasc. Pharmacol. 31 Suppl 1, S249–250.

Yoopan, N., Thisoda, P., Rangkadilok, N., Sahasitiwat, S., Pholphana, N., Ruchirawat, S., Satayavivad, J., 2007. Cardiovascular Effects of 14-deoxy-11,12-Didehydroandrographolide and *Andrographis paniculata* Extracts. Planta Med. 73, 503–511.

Young, D.C., 2009. Computational Drug Design: A Guide for Computational and Medicinal Chemists. John Wiley & Sons, New Jersey, pp. 17 -239.

Yusof, I., Segall, M.D., 2013. Considering the Impact Drug-Like Properties Have On the Chance of Success. Drug Discov. Today 18, 659–666.

Zahid Ashraf, M., Hussain, M.E., Fahim, M., 2005. Antiatherosclerotic Effects of Dietary Supplementations of Garlic and Turmeric: Restoration of Endothelial Function in Rats. Life Sci. 77, 837–857.

Zhang, C., Kuroyangi, M., Tan, B.K., 1998. Cardiovascular Activity of 14-Deoxy-11, 12-Didehydroandrographolide in the Anaesthetised Rat and Isolated Right Atria. Pharmacol. Res. Off. J. Ital. Pharmacol. Soc. 38, 413–417.

Zhang, C.Y., Tan, B.K., 1997. Mechanisms of Cardiovascular Activity of *Andrographis Paniculata* in the Anaesthetized Rat. J. Ethnopharmacol. 56, 97–101.

Zhang, C.Y., Tan, B.K., 1996. Hypotensive Activity of Aqueous Extract of *Andrographis Paniculata* in Rats. Clin. Exp. Pharmacol. Physiol. 23, 675–678.
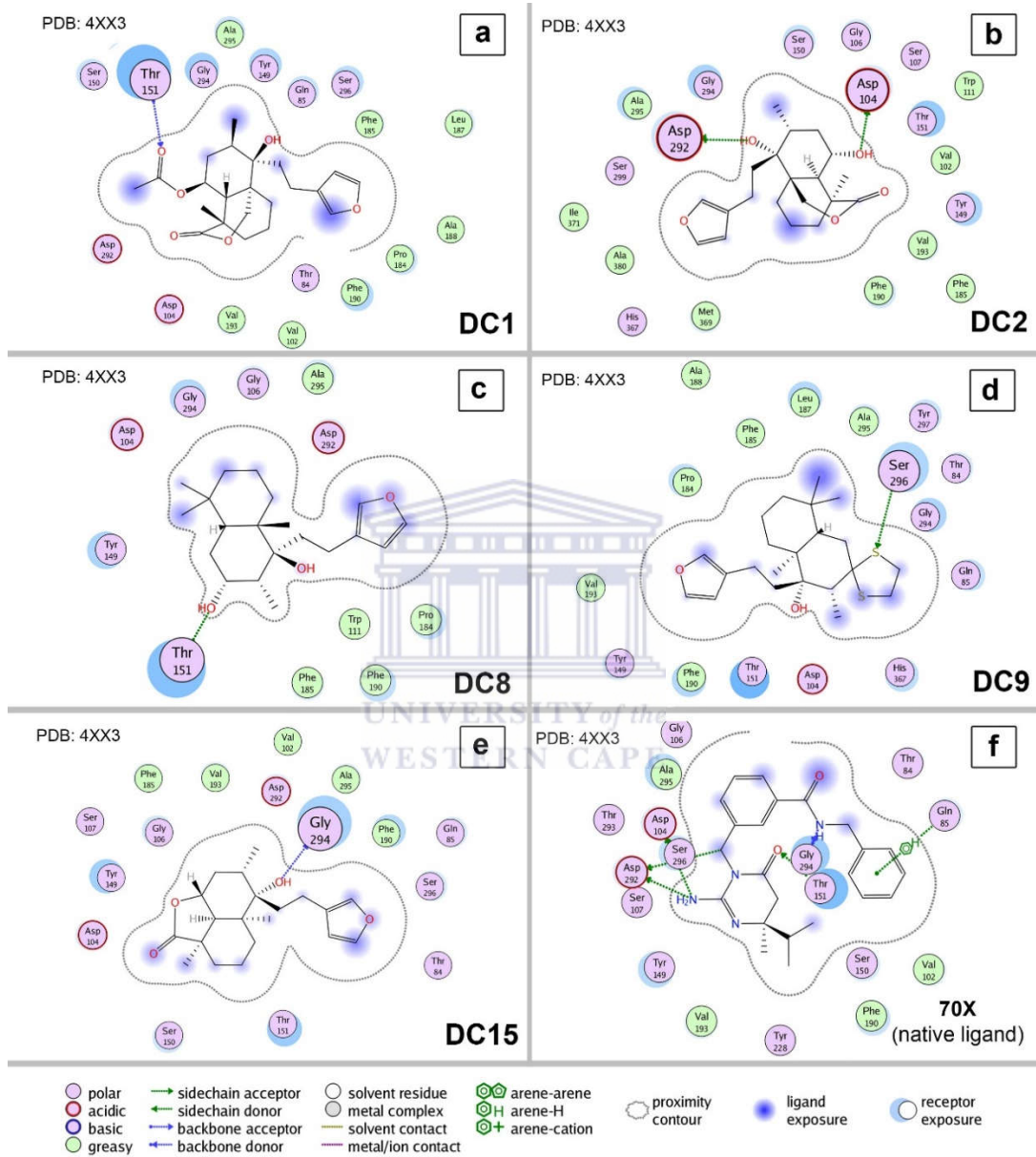
Zhang, X., 2000. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. World Health Organ. 1–71.

Zonta, N., Coluccia, A., Brancale, A., 2010. Advanced In Silico Approaches in Antiviral Research. Antivir. Chem. Chemother. 20, 147–151.
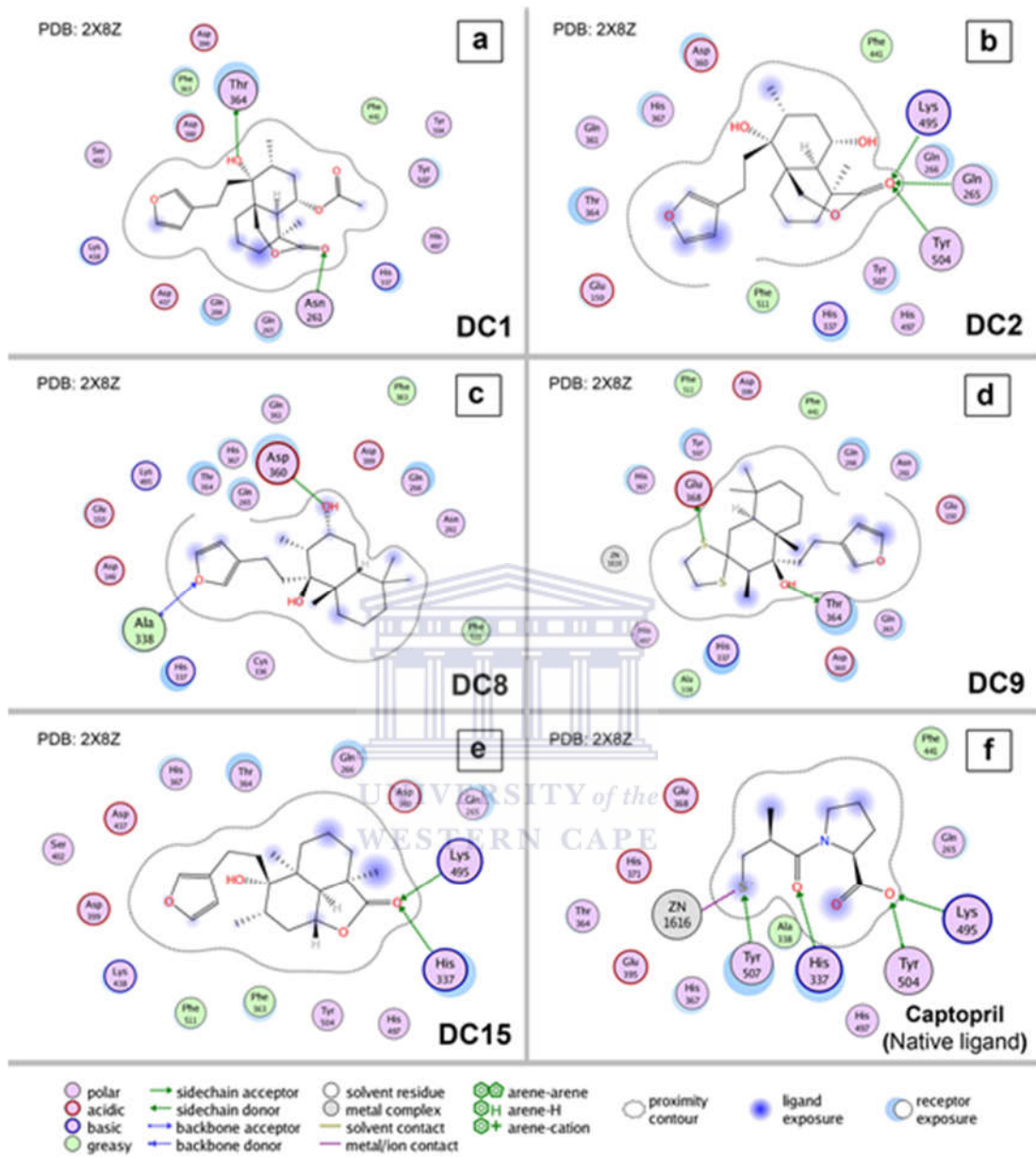
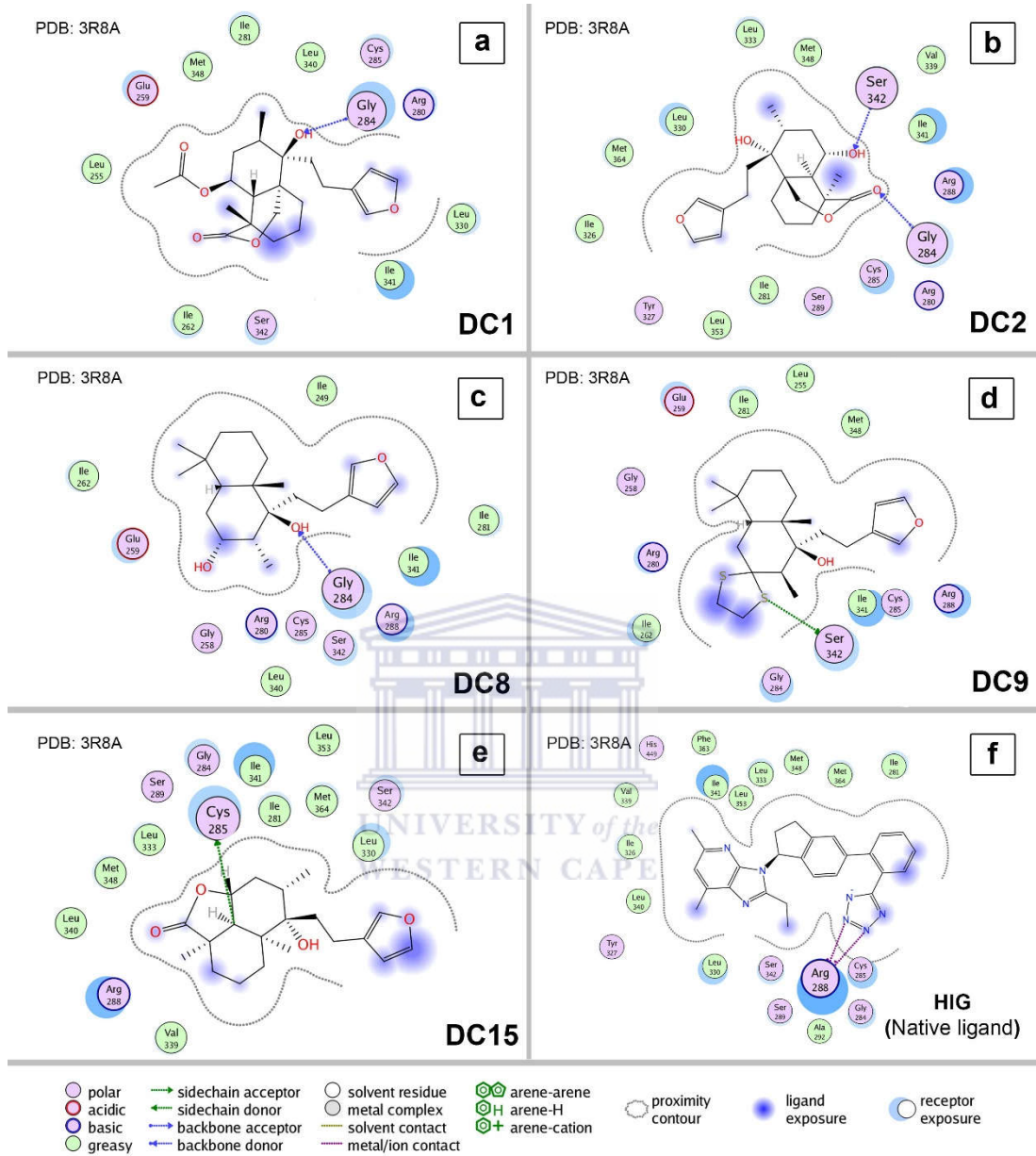**APPENDIX I: Interactions of L. Leonurus diterpenoids compounds with renin**

APPENDIX II: Interactions of *L. Leonurus* diterpenoids compounds with ACE
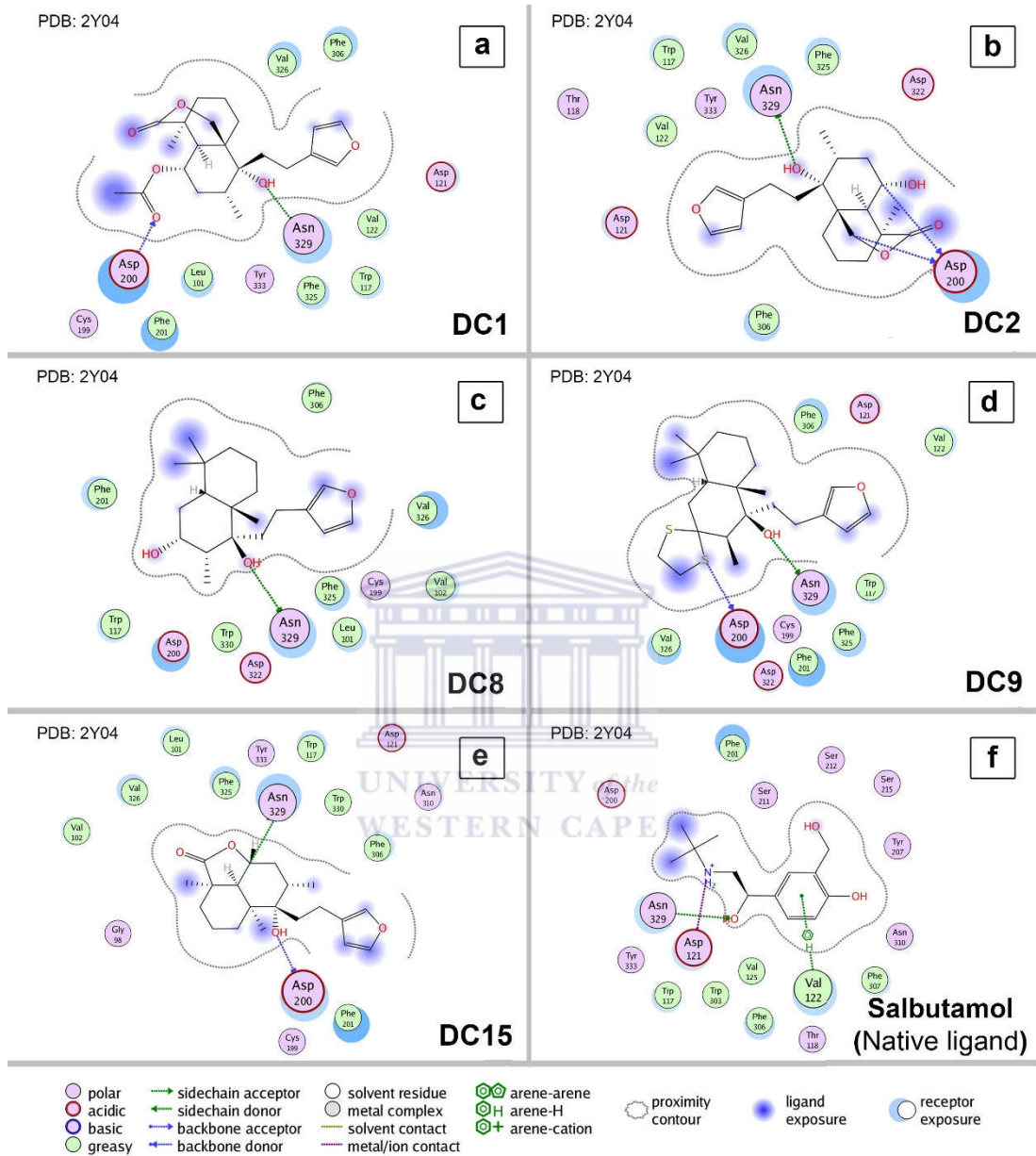
APPENDIX III: Interactions of *L. Leonurus* diterpenoids compounds with AT₁ receptor

APPENDIX V: Effects of diterpenoid compounds on Blood pressure and Heart rate

DC1	Conc	SP	DP	MAP	HR
	mg/kg	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM
	0,5	2,933 ± 0,6677	2,1180 ± 0,6047	2,390 ± 0,6006	4,4880 ± 2,4930
	1	7,766 ± 0,5164	7,8740 ± 1,1960	7,838 ± 0,9533	10,4600 ± 0,7133
	5	11,600 ± 1,350	10,3100 ± 0,6398	10,740 ± 0,7938	15,9500 ± 1,6260
	10	20,760 ± 0,9731*	21,5200 ± 2,1700*	21,270 ± 1,739*	23,1000 ± 1,6370*
	20	25,920 ± 1,095*	23,4600 ± 1,6460*	24,280 ± 1,430*	31,3200 ± 2,8700*
	40	42,360 ± 5,803*	41,0600 ± 5,3820*	41,490 ± 5,346*	29,1300 ± 4,9160*
DC2	Conc	SP	DP	MAP	HR
	mg/kg	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM
	0,5	8,675 ± 0,9162	4,749 ± 0,5796	6,058 ± 0,5361	6,122 ± 0,3989
	1	14,810 ± 0,8572*	8,641 ± 0,8372	10,700 ± 0,8374	8,440 ± 0,8545
	5	12,500 ± 0,7503	6,597 ± 0,5378	8,564 ± 0,4541	14,960 ± 2,604
	10	21,030 ± 1,460*	19,600 ± 1,558*	20,080 ± 0,7594*	23,020 ± 2,255*
	20	27,200 ± 2,576*	24,390 ± 1,471*	25,320 ± 1,586*	24,030 ± 3,003*
	40	38,680 ± 3,978*	37,260 ± 2,846*	37,730 ± 3,138*	33,350 ± 1,470*
	60	65,110 ± 10,380*	58,200 ± 12,990*	60,500 ± 12,120*	46,800 ± 2,739*
DC8	Conc	SP	DP	MAP	HR
	mg/kg	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM
	0,5	5,732 ± 0,8752	2,1710 ± 1,1690	3,3580 ± 0,6708	5,4510 ± 0,8787
	1	6,683 ± 1,099	2,9160 ± 1,0690	4,1720 ± 0,7508	15,4600 ± 3,2080
	5	8,702 ± 1,683	2,7470 ± 1,8120	4,7320 ± 1,2580	28,1400 ± 1,7910*
	10	11,700 ± 1,585	3,4940 ± 0,7695	6,2310 ± 0,5266	36,4500 ± 2,3040*
	20	20,150 ± 2,723*	19,8200 ± 3,5360*	19,9300 ± 3,2560*	33,6800 ± 12,8700*
	40	35,990 ± 2,887*	20,7700 ± 1,7690*	25,8400 ± 1,2980*	38,2000 ± 13,9100*
	60	37,790 ± 0,7398*	23,7900 ± 3,0520*	28,4500 ± 2,2820*	95,6000 ± 0,8771*
DC9	Conc	SP	DP	MAP	HR
	mg/kg	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM
	0,5	6,993 ± 0,7063	5,454 ± 0,4553	5,967 ± 0,2071	6,102 ± 0,9101
	1	9,612 ± 0,566	7,709 ± 0,249	8,343 ± 0,2532	10,640 ± 1,132
	5	10,770 ± 0,9866	10,210 ± 1,530	10,400 ± 0,6947	22,950 ± 2,834*
	10	13,650 ± 1,359*	12,710 ± 1,518	13,030 ± 1,306*	28,510 ± 4,201*
	40	61,850 ± 17,340*	65,540 ± 13,320*	64,310 ± 14,550*	35,110 ± 6,909*
DC15	Conc	SP	DP	MAP	HR
	mg/kg	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM	ΔMEAN ± SEM
	0,5	5,845 ± 0,9175	5,4460 ± 0,3822	5,579 ± 0,5124	7,127 ± 1,047
	1	9,224 ± 0,7923	5,4230 ± 0,4334	6,690 ± 0,3393	11,590 ± 0,8099
	5	13,220 ± 1,350*	6,0310 ± 0,4127	8,427 ± 0,2163	13,960 ± 0,8717
	10	20,230 ± 1,378*	18,5600 ± 1,2450*	19,120 ± 1,051*	21,520 ± 0,6188*
	20	21,360 ± 1,497*	19,0900 ± 1,9410*	19,850 ± 1,484*	23,870 ± 4,215*
	40	22,630 ± 0,9431*	22,5500 ± 0,9463*	22,570 ± 0,7373*	31,590 ± 2,423*