Some Bioactivity of Triterpenes from Stem Bark of *Protorhus longifolia* and their Derivatives

Rebamang Anthony Mosa

January 2014

UNIVERSITY OF ZULULAND

Some Bioactivity of Triterpenes from Stem Bark of *Protorhus longifolia* and their Derivatives

Rebamang Anthony Mosa

20055704

A thesis submitted in fulfillment of the requirement for the Degree of Doctorate of Philosophy in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, South Africa

Supervisor: Prof. A.R. Opoku

January 2014

DECLARATION

The experimental work presented in this thesis was conducted in the Department of Biochemistry and Microbiology, University of Zululand (UZ), Pharmacology Division, University of Cape Town (UCT) and Diabetes Discovery Platform, Medical Research Council (MRC), Tygerberg, from May 2011 - November 2013, under the supervision of Prof. A.R. Opoku

This study presents original work by the author. Where use was made of outside sources, proper attributions have been made in the text.

I declare the above statement to be true.

Mosa Rebamang Anthony

Prof. A.R. Opoku

DEDICATION

I wish to dedicate this piece of work to the Mosa family, they have sown and this is the fruit thereof.

ACKNOWLEDGEMENTS

It seems impossible until it is done. When the tough gets going look up to the Lord.

GENERAL ABSTRACT

Despite availability of the current clinical drugs, metabolic disorders and microbial infections continue to be serious threats to human health. The diverse significant pharmacological effects possessed by plant-derived triterpenes have made these compounds new targets for drug development against an array of diseases resistant to conventional medicines. This work investigated some bioactivity of two lanostane triterpenes and their derivatives.

Two lanostane triterpenes (3β-hydroxylanosta-9,24-dien-21-oic acid, **RA5** and methyl-3β-hydroxylanosta-9,24-dien-21-oate, **RA3**) were isolated from stem bark of *Protorhus longifolia* (Benrh) Engl and their acetyl (3β-acetyloxylanosta-9,24-dien-21-oic acid, **R51**, methyl-3β-acetyloxylanosta-9,24-dien-21-oate, **R31**) and oxo (3β-oxolanosta-9,24-dien-21-oic acid, **R52**, methyl-3β-oxylanosta-9,24-dien-21-oate, **R32**) derivatives were prepared. Structures of the compounds were established based on spectroscopic (IR, NMR, HRMS) data analysis.

The *in vitro* anti-hyperlipidemic activity of the compounds was evaluated on selected lipid and carbohydrate digestive enzymes. The effect of the compounds on glucose uptake in C2C12 muscle cells and 3T3-L1 adipocytes, and triglyceride accumulation in 3T3-L1 adipocytes was also investigated.

The anticoagulant and anti-inflammatory activity was investigated using tail bleeding time assay and cotton pellet-induced granuloma model in *Sprague-Dawley* rats, respectively. The effect of the triterpenes on the expression of heat shock protein 70 (Hsp70) and their anti-protein aggregation activity using malate dehydrogenase (MDH) aggregation suppression assay were studied.

The broth micro dilution assay was also used to determine the antimicrobial and antiplasmodial activity of the isolated compounds.

The triterpenes effectively inhibited the activities of lipid digestive enzymes (pancreatic lipase, cholesterol esterase) and HSL with IC_{50} values ranging from 60.1 to 677.8 µM; except for the weak activity of **R52** on C2C12 cells, the other compounds (50 µg/ml) effectively stimulated glucose uptake in both 3T3-L1 and C2C12 cells. A significant reduction (34.8%) of intracellular lipid accumulation was also observed following a 48 h exposure of the 3T3-L1 adipocytes to **RA5** (25 µM). The compounds did not show any cytotoxic effects on the 3T3-L1 and C2C12 cells.

The triterpenes exhibited anti-inflammatory activity and a reduction of granuloma formation by up to 40.3% after treatment with **RA5** (250 mg/kg) was observed.

Both triterpenes significantly ($p < 0.05$) increased bleeding time with up to 7.3 min recorded post treatment with **RA3** (250 mg/kg). The triterpenes improved the activity of Hsp70 on MDH aggregation suppression. They also effectively stimulated expression of Hsp70 *in E. coli* cells and in the plasma of inflamed rats.

While both compounds exhibited strong antibacterial activity against the tested bacteria with MIC and MBC values ranging from 0.16 to 5.00 mg/ml and 0.63 to 5.00 mg/ml, respectively, the compounds did not show any antifungal and antiplasmodial activity.

The various bioactivity exhibited by the compounds suggest potential new approaches towards development of pharmacologically active agents with beneficial multiple effects.

LIST OF ABBREVIATIONS USED

4-NPB	4-Nitrophenyl butyrate
4-NPP	4-Nitrophenyl palmitate
AA	Arachidonic acid
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADA	Acid-dextrose-anticoagulant
ADM	Adipogenic differentiation medium
ADP	Adenosine diphosphate
AMP	Adenosine-3′,5′-cyclic monophosphate
AMPK	AMP-activated protein kinase
ANOVA	One way analysis of variance
ASA	Acetylsalicylic acid
ATCC	American type culture collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CA	Cholic acid
CDCl$_3$	Deuterated chloroform
COSY	Correlation spectroscopy
COX	Cyclooxygenase
CQ	Chloroquine
CS	Citrate synthase
DCA	Deoxycholic acid
DEPT	Distortionless enhancement by polarisation transfer
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPPH	1,1′-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol tetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
FBS	Foetal bovine serum
FCS	Foetal calf serum

GAST	Glycerol-alanine-salts-Tween
GCA	Glycocholic acid
GLUT	Glucose transporter
HMBC	Heteronuclear multiple bond correlation
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HRMS	High resolution mass spectrometry
HSL	Hormone sensitive lipase
HSP	Heat shock protein
HSQC	Heteronuclear multiple quantum coherence
IBMX	Dexamethasone, 3-isobutyl-1-methylxanthine
IC_{50}	Inhibitory concentration with 50%
INT	Iodonitrotetrazolium chloride
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IR	Infra-red
KRB	Krebs-Ringer bicarbonate buffer
KZN	KwaZulu-Natal
LCA	Lithocholic acid
LDH	Lactate dehydrogenase
MBC	Minimum bacterial concentration
MDH	Malate dehydrogenase
MFC	Minimum fungal concentration
MIC	Minimum inhibitory concentration
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
NSAIDs	Non steroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PfHsp70	*Plasmodium falciparum* heat shock protein 70
PG	Prostaglandin
PI3-K	Phosphatidylinositol 3-kinase

PPAR-α	Peroxisome proliferator-activated receptor-alpha
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBA	Total bile acid
TBS	Tris-buffered saline
TCA	Taurocholic acid
TG	Triglyceride
TLC	Thin-layer chromatography
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
UV	Ultraviolet light
WHO	World Health Organisation

RESEARCH OUTPUT

1. Manuscripts submitted for editorial consideration

   a. **Rebamang A**. **Mosa**, Thabo Ndwandwe, Nontando F. Cele and Andy R. Opoku. Anticoagulant and anti-inflammatory activity of a triterpene from *Protorhus longifolia* stem bark. *African Journal of Pharmacy and Pharmacology*.

   b. **Rebamang A**. **Mosa**, Mandlakayise L. Nhleko, Thandeka V. Dladla, and Andy R. Opoku. Antibacterial activity of two triterpenes from stem bark of *Protorhus longifolia* (Benrh) Engl. *Journal of Medicinal Plants Research.*

2. Manuscripts in preparation

   a. **Mosa**, **R.A**. Naidoo, J.J. Nkomo, F.S. Opoku, A.R. Muller, C.J.F. Mazibuko, S.E.. *In vitro* anti-hyperlipidemic activity of triterpenes from stem bark of *Protorhus longifolia* (Benrh) Engl.

3. Conference presentations (See Appendix F for details)

   a. Opoku, A.R. **Mosa**, **R.A**. Mathenjwa, M.S. Qalase, M.S. "The effect of the triterpenes from the stem bark of *Protorhus longifolia (Berhn) Engl.* on the expression of heat shock protein 70 in *E. coli* and rats". 5th World Congress on Medicinal & Aromatic Plants. 17-24 August 2014. Brisbane, Australia.

   b. **Rebamang A**. **Mosa**, Naidoo, J.J. Nkomo, F.S. Opoku, A.R. Muller, C.J.F. "*In vitro* antihyperlipidemic activity of triterpenes from stem bark of *Protorhus longifolia* (Benrh) Engl. 61st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA)". 01-05 September 2013. Münster, Germany.

   c. Opoku, A.R. **Mosa**, **R.A**. Cobongela, S.Z.Z. Machaba, K.E. *"In Vivo* Antihyperlipidemic activity of a triterpene from stem bark of *Protorhus longifolia* (Benrh.) Engl". International Conference on Advances in Medical Sciences. 16-19 April 2013. Kuala Lumpur, Malaysia.

d. Opoku, A. **Mosa**, **R**. Naidoo, J. Nkomo, F. "The *in vitro* effects of a triterpene from the stem bark of *Protorhus longifolia* (Benrh) Engl. on some lipid and carbohydrate disgestive enzymes". III International Symposium on Medicinal and Nutraceutical Plants. 14-19 October 2012. Aracaju, Brazil.

TABLE OF CONTENTS

CHAPTER FIVE

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

Abnormal metabolism is the precursor to a wide range of serious human health problems such as obesity, diabetes, and thromboembolic disorders. Metabolic disorders do not only reduce life expectancy, but also affect socioeconomic aspects. Discovery and development of drugs against metabolically related disorders is of importance and a good approach to sustain human health. Despite the continuous progress made so far to find effective pharmacological agents, infectious diseases and metabolically related diseases and disorders are still a major public health threat. The alarming increase in the incidence of these health problems indicates a need to discover new active therapeutic drugs with diverse chemical structures and novel mechanisms of action.

The concerns about conventional drugs' efficacy and safety have raised interest levels on natural products and their derivatives as alternative potential medicine. Natural products identified from traditional medicinal plants have become very important in the development of pharmacologically active agents. Traditional healers with their invaluable knowledge on medicinal plants play an important role in providing leads to the discovery of new pharmacologically active plant-derived compounds. Over the years, medicinal plants as primary sources have substantially contributed to the discovery and development of pharmacologically active drugs in clinical use today.

The diverse significant pharmacological effects possessed by plant-derived triterpenes have made these compounds new targets for drug development against an array of diseases resistant to conventional medicines. Thus the aim of this study was to investigate bioactivity of some triterpenes from stem bark of *Protorhus longifolia* (Benrh) Engl*,* and their derivatives.

1.1 Structure of the Thesis

This thesis consists of five chapters and appendices:

Chapter One gives a brief background and motivation for the study.

Chapter Two gives the literature review of the study. It also describes the aim and objectives of the study conducted.

Chapter Three describes the materials and methods that were used to conduct all the experiments involved in the study. Results and discussion are also presented in this chapter.

Chapter Four gives the overall discussion of the results obtained in the study.

Chapter Five comprises the conclusions drawn from results obtained in the study. Also presented in this chapter are limitations of the study and suggestions for further studies.

CHAPTER TWO

2.0 GENERAL LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal plants are those plants that possess medicinal properties to cure various ailments. These plants are untapped rich sources of biologically active compounds (phytochemicals) vital to human health. The phytochemicals, usually referred to as secondary metabolites of the plants, have no nutritive value in plants. These are the compounds that possess the diverse medicinal properties of the plants. The common phytochemicals include saponins, alkaloids, tannins, terpenoids, and flavonoids. These compounds are widely distributed in the plant kingdom and they are usually found at varying concentrations in different parts of plants (Kee *et al.*, 2008).

Medicinal plants-based treatment of various ailments has a long and extensive history. In recent years, there has been an increased interest in natural products and their derivatives due to concerns about conventional drug costs, efficacy and safety. The continuous effort to improve the well being and prevalence of chronic illnesses resistant to conventional medicines, have added more impetus in relying on plant-derived drugs. Medicinal plant-derived formulations are no longer only for rural communities. Even some western medical practitioners are now recommending biopharmaceuticals (Motlhanka *et al.*, 2010). Integration of a multi-target action approach in treating ailments is considered highly possible with the use of plant extracts (Appendino and Pollastro, 2010). Plants, due to their wide chemical diversity, play a very important role in the introduction of new therapeutic drugs. Their extracts provide a useful source of bioactive ingredients which can be developed as drugs directly or serve as novel structural templates (Kee *et al.,* 2008).

Medicinal plants as primary sources, contribute more than 50% of all drugs currently in clinical use and higher plants contribute no less than 25% of the total (Van Wyk *et al.*, 2009). Well known examples of plant-derived drugs include aspirin, morphine, cocane, reserpine, berberine, atropine, taxol and vincristine. Traditional healers play an integral role in providing leads to the discovery of new bioactive plant-derived

compounds. The process of selecting plants with desired compounds and/or bioactivity would be very difficult and time consuming without assistance from the traditional healers (Rajadurai *et al.* 2009; Van Wyk *et al.*, 2009).

South Africa has a very rich flora with over 30 000 species of plants and of this, 3 000 species are used as medicines (Neilsen *et al.*, 2012; Vashka, 2013). An ethnobotanical survey (conducted by this researcher in 2009) revealed that *Protorhus longifolia* (Figure 2.1*)* as one of the plants commonly used by Zulu traditional healers to cure various diseases including blood-clotting related diseases. This plant is highly rated and recommended for its traditional medicinal uses.

2.1.1 *Protorhus longifolia* (Benrh.) Engl.

Figure 2.1: A picture of *Protorhus longifolia* showing its bark and leaves (A) along with its ripe fruits (B) (Google pictures, visited: 20.01.2014)

Protorhus longifolia (Benrh.) Engl. from Anacardiaceae family is commonly known as *unhlangothi* (Zulu), *Uzintlwa* (Xhosa) and red beech (English). It is an evergreen tree (up to 15 m in height), very resistant to drought. It has dark green, glossy leaves which are normally crowded near the branch tips. The old leaves usually develop some yellow or bright red colour. During flowering, it has very small greenish white flowers. Its fruits are purple when ripe in summer. The tree is indigenous to Southern Africa as it is the only species in the genus *Protorhus* that is found in Southern Africa (Archer, 2000). It grows on river banks, open woodlands and in the forests. In South

Africa, it is mostly found in the Northern Province, Eastern Cape, KwaZulu-Natal and Mpumalanga.

The tree is ranked highly for its commercial value as its bark is sold in most *muthi* markets in South Africa (Keirugni and Fabricius, 2005; Dold and Cocks, 2002). The stem bark of *P. longifolia* has been traditionally used to cure various diseases such as heartwater and diarrhoea in cows (Dold and Cocks, 2001) and also to "thin" blood. Leaf extracts of the plant have previously been reported to possess antimicrobial activity (Suleiman *et al.*, 2010) while Mosa *et al.* (2011a) reported anti-platelet aggregation, cytotoxicity and antioxidant activity of the stem bark extracts. Hutchings *et al.* (2006) quantified tannin content (7% tannins and 10.2 – 18% tanning material) from the bark of *P. longifolia.* Lupane triterpenoids such as lupinone, lupinine, lupeol, luteolin and lupulon have been isolated from the leaves of the plant (Ntuli, 2006). Lanostane-type of triterpenes (Figure 2.2) have also been isolated from the stem bark of the plant and their anti-platelet and anti-inflammatory activity has recently been reported (Mosa *et al.*, 2011b). This was the first time these compounds were isolated from a higher plant. Lanosteryl triterpenes have commonly been isolated from mushroom (Keller *et al.*, 1996; Ko *et al.*, 2007; Zhou *et al.*, 2008).

1　　　　　　　　**2**
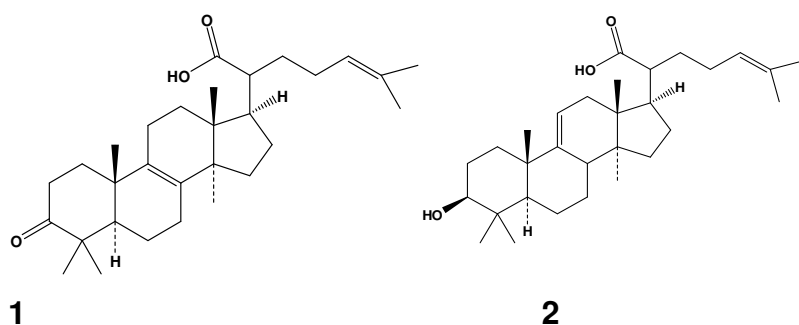
Figure 2.2: Chemical structures of 3-Oxo-5α-lanosta-8,24-dien-21-oic acid (**1**) and 3β-Hydroxylanosta-9,24-dien-21-oic acid (**2**).

2.1.2 Triterpenes

Triterpenes are part of the largest group of biologically active plant products known as terpenes. Terpenes are subdivided based on the number of isoprene subunits, $(C_5H_8)_n$ (where *n* = number of isoprene units). Therefore, this group consists of monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}),

triterpenes (C_{30}), and tetraterpenes (C_{40}). Reports have shown that despite the wide distribution of terpenes in plant kingdom, most of the bioactive terpenes have been found in higher plants (Muffler *et al.*, 2011, Parmar *et al.*, 2013).

Triterpenes are mainly found on plant surfaces such as stem bark or leaf and fruit waxes (Jäger *et al.*, 2009), and more than 30 000 have already been identified (Dzubak *et al.*, 2006). Their content in different plants is greatly influenced by various factors such as species, season and geographical distribution (Parmar *et al.*, 2013). Triterpenes are formed through the mevalonate pathway from two molecules of farnesyl pyrophosphate (FPP) joined tail-to-tail to yield squalene. Squalene is derived biosynthetically by cyclisation of a number of isoprene units (Figure 2.3). The cyclisation of sqaulene is via 2,3-oxidosqualene produced upon the action of squalene epoxidase (Dewick, 2009; Yadav *et al.*, 2010). Many triterpenes occur free, but others occur as esters. Triterpenes contain six isoprene units (C_{30}) and can exist as monocyclic, dicyclic, tricyclic, tetracyclic, or pentacyclic derivatives.
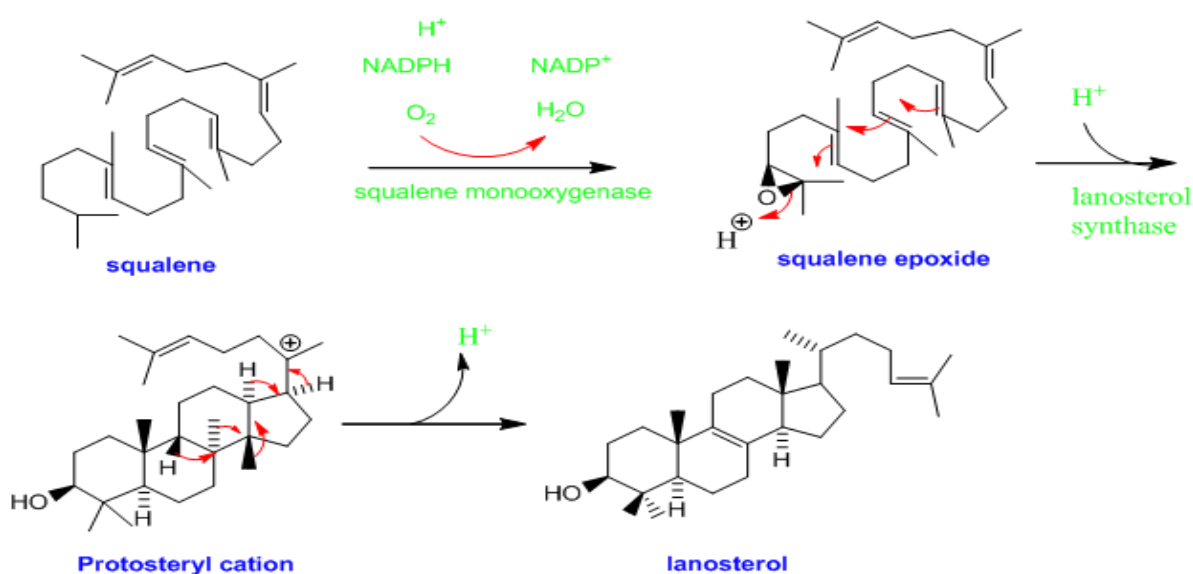
Figure 2.3: Simplified sketch showing cyclization of squalene to form triterpenes (http://pms.iitk.ernet.in/wiki/index.php/Terpenes, 20.01.2014).

Among the triterpenes, tetracyclic triterpenes include lanostane and dammarane structures. This subtype of triterpenes is characterised by the presence of a ketone or hydroxyl group at carbon atom-3 and of a side chain bearing a double bond. Some other possible functional groups are also usually present. Lanostane type compounds

can be distinguished from the dammaranes by the presence of a secondary double bond usually at position 7 or 8 (Colombini and Modugno, 2009), but other positions are also possible.

The occurrence of the so-called incurable diseases has fuelled so much interest on plant-derived triterpenoids due to their potential pharmacological effects (Stiti and Hartmann, 2012). These compounds reportedly exhibit diverse potentially significant pharmacological effects that include anti-platelet aggregation (Mosa *et al*., 2010b, Sankaranarayanan *et al*., 2010), anti-hyperglycaemic (Ghosh *et al*., 2011), anti-hyperlipidemic (Liu *et al*., 2007, Lee *et al*., 2010), anticancer (Zhou *et al*., 2008) and anti-inflammatory activity (Yadav *et al*., 2010). With this continuously growing evidence that supports therapeutic potential of plant-derived triterpenes, these compounds have now become new targets for drug development.

2.2 Human Metabolic Disorder

Abnormal metabolism is the precursor to a wide range of serious human health problems such as obesity, hyperlipidemia, diabetes, cardiovascular diseases, stroke, hypertension and cancer, collectively being referred to as metabolic syndrome. The debilitating effects of these pathophisiological conditions are commonly linked to oxidative stress (Furukawa *et al*., 2004; Bandeira *et al*., 2013). Metabolic disorders do not only reduce life expectancy, but also affect socioeconomic aspects. Thus a clear understanding of the biochemical basis of metabolism is vital in the management of metabolic disorders and related diseases. Furthermore, the discovery and development of drugs against metabolically related disorders is of importance and a good approach to sustain human health. Researchers (Panchal *et al*., 2012; Prathapan *et al*., 2012; Zhang *et al*., 2013) are continuously focusing their attention towards finding solutions to alleviate incidences of metabolic disorders and their related diseases.

2.3 Obesity

Obesity is a common chronic metabolic disorder of lipids and carbohydrate which is characterised by excessive deposition of fat in adipose tissue and other internal organs. This leads to an increase in the number and size of adipocytes (Liu *et al*., 2012). The metabolic features of obesity include abnormally elevated blood lipids as

a result of enhanced lypolytic response (Zhang *et al.*, 2013). Physiological, genetical (inherited low basal metabolic rate), behavioural and environmental factors such as excessive intake of high caloric food, predominantly high-fat diet and sedentary lifestyle, all contribute to the development of overweight and obesity (Little *et al.*, 2007).

Obesity has reached epidemic levels with about 300 million adults clinically obese (WHO, 2009). It has also been projected that if not controlled, the number will reach 1.12 billion by 2030 (Kelly *et al.*, 2008). Thus, obesity is a global concern and the major contributor to increased prevalence of various pathophysiological disorders such as hyperlipidemia, diabetes mellitus, certain types of cancer, inflammation, cardiovascular diseases and other atherothrombotic related diseases (Gurevich-Panigrahi *et al.*, 2009; Yun, 2010; Roberts *et al.*, 2010). Therefore, prevention and treatment of obesity and the related co-morbidities are considered a public health priority (Kelly *et al.*, 2008).

Understanding of the biology of obesity phenomenon is very important in the search for its intervention. Changes in lifestyle, especially exercising and dieting have so far been the most commonly practised approaches (Tucci *et al.*, 2010). Other various therapeutic approaches to curb obesity have been proposed. These include control of lipid metabolism, inhibition of lipases and adipocyte differentiation (Yun, 2010). The combined effect of these approaches could result in most effective anti-obesity treatment (Ahn *et al.*, 2012).

Recent approaches have focused on inhibition of nutrients digestive enzymes and adipogenesis as a plausible means towards the treatment of obesity (Ahn *et al.*, 2012; Park *et al.*, 2013). The currently approved clinical anti-obesity drugs include orlistat and sibutramine. Orlistat is an irreversible inhibitor of pancreatic lipase, therefore limiting intestinal digestion and absorption of dietary triglycerides (TGs). It covalently binds to the serine in the active site of the lipase (Hadváry *et al.*, 1991). Sibutramine on the other hand is an oral anorectic, a drug that suppresses appetite and, therefore, reduces food consumption. It is a centrally acting serotonin-norepinephrine reuptake inhibitor, thus, accumulation of these neurotransmitters in synaptic clefts enhances satiety (Bray and Greenway, 2007).

Despite their efficacy, both drugs have been associated with adverse side effects. The use of sibutramine has been associated with dry mouth, headache, dizziness, constipation, hypertension, and insomnia (Díaz and Folgueras, 2011). Administration of orlistat is associated with gastrointestinal effects such as steatorrhea, hyper-defecation, abdominal pain, fecal urgency, flatulence and nausea (Chaput *et al.*, 2007; Díaz and Folgueras, 2011). A search for new effective antiobesity drugs with an improved safety profile is appropriate.

A growing body of evidence continues to support the importance of plants as rich sources of anti-obesity compounds. Reduction of triglycerides accumulation in 3T3-L1 cells as a measure of anti-adipogenic activity by a number of plant-derived compounds (Liu *et al.*, 2012) including lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* (Lee *et al.*, 2010) has previously been reported. The reduction of lipid accumulation in adipose tissue and other insulin target tissues is vital for the prevention of obesity and insulin resistance in type 2 diabetes (Zeng *et al.*, 2012). Furthermore, the effects of *Curcuma longa* extracts on adipogenesis and glucose uptake were investigated in 3T3L1 and L6 cell lines (Prathapan *et al.*, 2012). The results indicated that the plant had no significant effect on cellular glucose uptake but effectively inhibited adipocytes differentiation.

2.4 Hyperlipidemia

Hyperlipidemia is a disorder of lipids metabolism and is characterised by abnormally elevated levels of lipids (fatty acids, total cholesterol and TGs) in blood circulation. Blood lipid levels are influenced by exogenous lipid absorption, endogenous lipid synthesis and metabolism in the body (Xie *et al.*, 2007). The increase in plasma levels of free fatty acids are the result of increased mobilisation from adipose tissue. The increased plasma levels of fatty acids inhibit glucose uptake and utilisation, thus, crucial to the development of insulin resistance and type 2 diabetes.

Hyperlipidemia has also become a global concern since its prevalence is currently increasing at an alarming rate worldwide (Zhang *et al.*, 2013). Chronic hyperlipidemia is crucial in causing micro and macrovascular complications such as cerebrovascular, cardiovascular, and metabolic syndrome diseases (Adisakwattana *et al.*, 2010).

Regulation and decreasing elevated blood lipids levels is vital for the prevention and treatment of hyperlipidemia and its related diseases (Derosa *et al.*, 2006; Karalis *et al.*, 2012).

Hyperlipidemia can be familial (primary) as a result of specific inherited genetic defects or acquired (secondary) when it results from some other underlying disorders such as obesity, diabetes and hypothyroidism (Ahmed *et al.*, 1998). It may also occur without a known cause (Ibrahim *et al.,* 2013). Most commonly, like obesity, excessive intake of high caloric diet, particularly high-fat diet, and lack of physical activity are the major contributors to the disorder. High cholesterol diet is regarded the main culprit in the development of hypercholesterolemia, atherosclerosis and ischemic heart disease (Kamesh and Sumathi, 2012, Kumar and Sivashanmugam, 2012).

Since obesity and hyperlipidemia often coexist, almost the same therapeutic approaches are used to manage this metabolic disorder. Preventive measures of hyperlipidemic incidences include consumption of low-fat diet and regular exercise. Treatment of hyperlipidemia aims at decreasing the atherogenic lipoproteins (very low density lipoprotein and low density lipoproteins) and increasing high density lipoproteins levels. Its treatment or management is always directed at the underlying cause.

Since dietary lipids have to be emulsified and digested for their efficient absorption, inhibition of enzymes which digest dietary lipids and therefore limiting their intestinal absorption is considered a plausible approach to reduce incidences of hyperlipidemia and its related diseases. Heidrich *et al.* (2004) has demonstrated using high cholesterol-diet fed hamsters that inhibitors of pancreatic cholesterol esterase may provide a method to limit the absorption and bioavailability of dietary cholesterol. Carbohydrates consumption should also be controlled since the body converts excess calories into fats.

A variety of drugs are currently used to lower plasma TGs and cholesterol levels. These include orlistat, the famous pancreatic lipase inhibitor; 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (statins), bile acid sequestrant (resin), and fibrates. Statins reduce intracellular cholesterol levels by

inhibiting HMG-CoA reductase, the rate-limiting step enzyme in cholesterol biosynthesis. The resins form insoluble complexes with bile acids in the intestines, limiting their availability as physiologic detergents and also limit their reabsorption. This in turn shifts a greater amount of cholesterol towards synthesis of bile acids to maintain homeostasis (Kobayashi *et al.*, 2007). Fibrates exert their anti-hypertriglyceridemic effect mainly through binding to peroxisome proliferator-activated receptor-alpha (PPAR-α) which leads to increased lipoprotein activity and beta oxidation of fatty acids (Staels *et al.*, 1995).

Despite their efficacy, the adverse effects of the current anti-hyperlipidemic drugs (Chaput *et al.*, 2007; Díaz and Folgueras, 2011) stimulate the search for alternative medicine with an improved efficacy and safety profile. Plants have always been a rich reservoir of bioactive compounds with diverse therapeutic properties including hypolipidemic effect. Consumption of plant sterols and their esters has been reported to not only lower intestinal cholesterol absorption but decrease blood levels of the atherogenic low density lipoprotein cholesterol as well (Sudhahar *et al.,* 2007; Brown *et al.*, 2009). Thus, a search for new lead molecules with anti-hyperlipidemic properties from plants could be an appropriate strategy.

A large number of plants and plant-derived bioactive compounds have been reported to possess or exhibit hypolipidemic properties. Extracts of *Rosmarinus officinalis* extract and selected phenolic constituents were reported to exhibit anti-hyperlipidemic activity through inhibition of pancreatic lipase and hormone sensitive lipase (Bustanji *et al.*, 2011). Inhibition of hormone sensitive lipase (HSL) is also good for the control of plasma glucose levels. Thus, inhibitors of HSL are important drug targets in the prevention of hyperlipidemia and consequent peripheral insulin resistance (Ali *et al.*, 2012). Lee *et al.* (2010) reported the pancreatic lipase inhibitory activity of *C*-glycosidic flavones from *Eremochloa ophiuroides.* The hypolipidemic effect of two triterpenes (oleanolic acid and maslinic acid) was also reported in high-fat diet induced hyperlipidemia in rats. Their effect was reported to be through inhibition of intestinal absorption and storage of cholesterol (Liu *et al.,* 2007). Zhang *et al.* (2013) have recently demonstrated that the plasma lipid lowering effect of *Pandanus tectorius* fruit extract in hamsters fed a high fat-diet was through

upregulation of the expression of PPAR-α and activation of AMP-activated protein kinase (AMPK) in the liver.

Despite lipids being such an important component of human nutrition, their metabolism needs to be well controlled. Dietary fats consist mostly of TGs and these are the carriers of energy as well as important fatty acids (Mu and Høy, 2004). The main digestion of lipids occurs in the lumen of small intestines (duodenum). Several lipid digestive enzymes (serine hydrolases); colipase-dependent pancreatic lipase and cholesterol esterase together with bile salts are essential in the digestion and then absorption of lipids. Bile salts play an important role as a biological detergent emulsifying lipids for efficient hydrolysis by the digestive enzymes and subsequent absorption thereof.

2.4.1 Bile acids

Bile acids are derivatives of cholesterol that act as a physiological detergent that facilitate intestinal digestion and absorption of fats and fat-soluble molecules (Agellon, 2002). They are synthesised in the liver by the cytochrome P450-mediated oxidation of cholesterol. About half of daily human body cholesterol production is used to synthesise bile acids, thus the hepatic biosynthesis of bile acid is the major pathway for cholesterol metabolism (Sharma et al., 2012).

Prior to release into the small intestine, the bile acids are conjugated to taurine or glycine (primary bile acids). Conjugated bile acids readily ionise, increasing the amphiphatic nature of these compounds for efficient interaction with both hydrophobic and hydrophilic substances. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary bile acids common to mammalian species (Agellon, 2002). The primary bile acids are deconjugated and/or dehydroxylated by intestinal bacteria to produce secondary bile acids, predominantly deoxycholic acid (DCA) and lithocholic acid (LCA) (Styrellius et al., 1985). About 90% of the released bile acids, particularly primary bile acids, are actively reabsorbed by the terminal ileum and undergo an enterohepatic circulation (Hofmann and Hagey, 2008; Sharma et al., 2012). While only a small amount of LCA undergoes the enterohepatic circulation and sulfoconjugated before its secretion into bile, most of this bile acid is excreted in faeces (Chiang, 2009).

Accumulation of secondary bile acids has been associated with increased risk of developing colorectal cancer and some form of chronic diarrhoea (Peterlik, 2008; DiBaise and Islam, 2012). Bile acids sequestrants are important in preventing the enterohepatic circulation of bile acids, thus, favouring bile acids excretion and consequent low plasma cholesterol levels. Besides well-established roles of bile acids in dietary lipid absorption and cholesterol homeostasis, bile acids are also biological signalling molecules and inflammatory agents that rapidly activate nuclear receptors and cell signalling pathways that regulate drug, lipid, glucose, and energy metabolism (Lefebvre *et al.*, 2009; Chiang, 2012; Watanabe *et al.*, 2012).

2.5 Diabetes

Diabetes mellitus is a global health concern quickly reaching epidemic levels. Its continuously increasing prevalence is likely to reach 439 million people by 2030 (Shaw *et al.*, 2010). It is another serious chronic metabolic disorder characterised by chronic hyperglycaemia. The hyperglycaemia results from abnormal metabolism of carbohydrate, lipids and protein. The hyperglycaemia and hyperlipidemia, as the most common features of diabetes mellitus, result from defects in insulin secretion, insulin action, or both (Koti *et al.,* 2011).

Diabetes mellitus is commonly classified into type 1 and type 2. Type 1 diabetes mellitus is usually referred to as insulin-dependent since it results from failure of the pancreatic β-cells to secrete insulin and its complications are managed by injection of exogenous insulin. Type 2 (insulin-independent diabetes mellitus) results from cells' resistance to insulin or it can also be due to insulin deficiency. Diabetes is also commonly accompanied by obesity (Szkudelski, 2012). Among the different types of diabetes, type 2 diabetes is more frequent and accounts for 90-95% of all diabetic cases (Stolar *et al.*, 2008).

The hyperglycaemia and hyperlipidemia commonly observed in diabetes are considered the main contributors to the development of micro and macro vascular complications of diabetes (Ortiz-Andrade *et al.,* 2007; Gutierrez, 2013). These diabetic complications are likely to be mediated by oxidative stress. Experimental evidence supports the role of free radicals in the pathogenesis of diabetes and its

complications (Ramachandran *et al.,* 2012). Both increased oxidative stress and expression of inflammatory mediators are common in diabetes. Thus, control of postprandial hyperglycaemia, hyperlipidemia and reduction of oxidative stress is important in preventing diabetes-associated complications (Santos *et al.*, 2012).

Insulin stimulates cellular glucose transport via a family of facilitative membrane proteins referred to as glucose transporters (GLUT). Various isoforms of these proteins are expressed in different tissues. Insulin stimulates muscle glucose uptake by promoting translocation of GLUT4 via activation of phosphatidylinositol 3-kinase pathway (PI3-K) and Akt2 (Figure 2.4) (Czech and Corvera, 1999, Jiang *et al.*, 2003). The involvement of activated AMPK in the stimulation of muscle glucose uptake has been reported (Wright *et al.*, 2004). AMPK stimulate cellular glucose uptake independent of insulin. Its activation is dependent on the energy status of the cell. However, most of the currently available anti-diabetic agents require an insulin signalling pathway for their action.
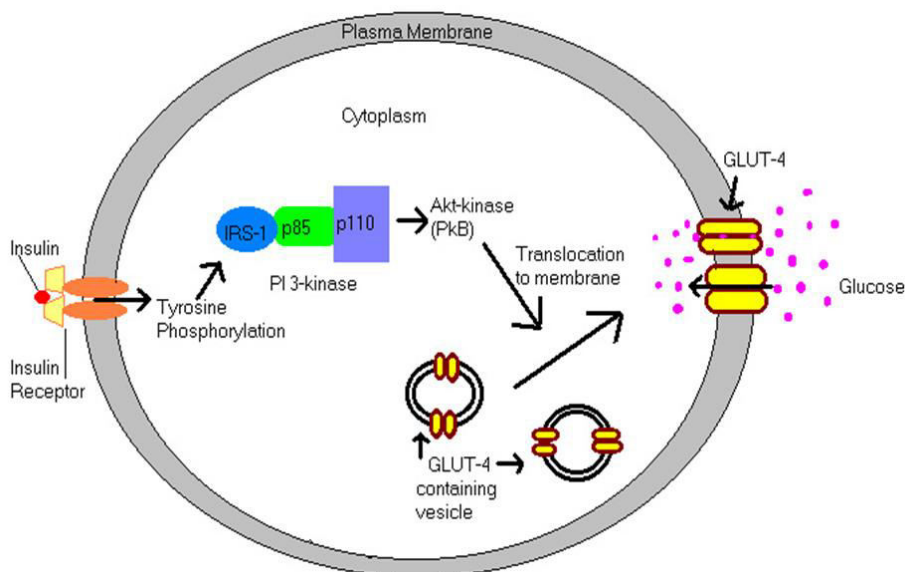
Figure 2.4: Insulin signalling pathway for glucose transport. Binding of insulin to the α-subunit of the insulin receptor causes conformational changes in its β-subunit causing activation of the cytoplasmic tyrosine kinase domain. This results in autophosphorylation of the receptor which in turn activates the downstream signalling pathways that stimulates muscle glucose uptake by promoting translocation of GLT4 via activation of PI3-K and Akt2.
(Picture from: http://student.biology.arizona.edu/honors2003/group05/bg.html, 05.12.2013)

In an effort to relief the burden of diabetes mellitus, new drugs are continually being tested and new strategies developed to prevent and treat diabetes. Current diabetic therapies focus on controlling postprandial hyperglycaemia by different approaches. These include enhancement of cellular glucose uptake through the use of insulin, insulin mimetics (Nankar and Doble, 2013), sensitizers (Zhou *et al.,* 2001, Hung *et al.*, 2012) and secretagogues (Hou *et al.*, 2009, Jackson and Robertson, 1989) or inhibitors of carbohydrates digestive enzymes (Hung *et al.*, 2012) thus limiting absorption of these nutrients (de Souza *et al.,* 2011). However, adverse effects (Hung *et al.,* 2012) and decreased efficacy over time, of the current antidiabetic drugs have stimulated a search for more new and effective agents.

Since plants are rich sources of bioactive compounds, the hypglycemic activity of some plants extracts has been reported to be through inhibition of some carbohydrates digestive enzymes (de Souza *et al.,* 2012; Salei *et al.*, 2013). Various multiple biological activities of plant-derived pentacyclic triterpenoids with apparent effects on glucose absorption, glucose uptake, insulin secretion and relief of diabetic complications have also been documented (Hou *et al.*, 2009; Lee and Thuong, 2010; Alqahtani *et al.*, 2013). Some other medicinal plants have shown the ability to enhance cellular glucose uptake without stimulating adipogenesis (Yang *et al.*, 2013).

In vivo models of antidiabetic activity of plant-derived compounds have also been used. Santos *et al.* (2012) investigated the antidiabetic and anti-hyperlipidemic of a triterpenoid mixture (α, β-amyrin) from *Protium heptaphyllum* in mouse models of STZ-induced diabetes and high-fat diet induced hyperlipidemia. The triterpenoid mixture did not only lower the blood glucose level, but also showed protective effects on the pancreatic β-cells, thus, increasing blood insulin levels. The pancreatic cells' protective effect of the compounds was linked to their anti-inflammatory and antioxidant properties (Santos *et al.*, 2012). Similar results were also obtained from the study carried out by Gutierrez (2013) on the triterpenoids from *Prosthechea michuacana.* Therefore, the hypoglycemic effect of the triterpenes was related to their ability to improve hyperlipidemia, insulin resistance and endogenous antioxidant system (Gutierrez, 2013).

Furthermore, the prevalence of type 2 diabetes due to cellular insulin resistance has currently shifted attention towards AMPK as an alternative therapeutic target (Park *et al.*, 2007, Krishnapuram *et al.,* 2012). Dimethylbiguanide (Metformin), a known AMPK activator (Fryer *et al.*, 2000, Park *et al.*, 2007), has been commonly used to lower blood glucose in type 2 diabetes. However, the use of this drug has also been associated with undesirable side effects (Hung *et al.,* 2012). This drawback also fuelled the search for new AMPK activators preferably of natural origin. Thus, a number of plant-derived antidiabetic agents such as resveratrol (Park *et al.,* 2007) berberine (Cheng *et al.,* 2006), ginsenoside Rc (Lee *et al.,* 2010) and chlorogenic acid (Ong *et al.*, 2012) have been tested and reported to regulate blood glucose level via AMPK activation. The antidiabetic activity of triterpenoids from *Momordica charantia* (Cheng *et al.,* 2008) and *Moutan cortex* (Ha *et al.*, 2009) has also been linked to activation of AMPK.

2.6 Oxidative stress

Oxidative stress is defined as an imbalance between production of oxidants (free radicals) and antioxidant such that the production of oxidants exceeds the antioxidant defense system. Free radicals are highly reactive molecules that can be classified as reactive oxygen species (ROS) or nitrogen reactive species (NOS). Reactive oxygen species (ROS) includes superoxide anion, hydrogen peroxide, hydroxyl radical, singlet molecular oxygen and organic peroxide radicals. Despite exogenous sources, free radicals are by-products of the body's normal energy producing biochemical reactions. Even though these molecules have some important physiological roles such as in phagocytosis, and intercellular signalling (Olorunnisola *et al.*, 2012); their production needs to be tightly controlled.

Accumulation of oxidants in cellular system results in impairment of normal biological functions. The cellular damaging effects of the oxidants are linked to increases in lipid peroxidation (Zhang *et al.*, 2013). Therefore, oxidative stress has been implicated in various pathophysiological disorders and diseases such as inflammation, hyperlipidemia, cancer, obesity, atherosclerosis, diabetes, and neurodegenerative diseases (Shibata and Kobayashi, 2008; Chang *et al.*, 2010; Shalaby and Hamouda, 2013).
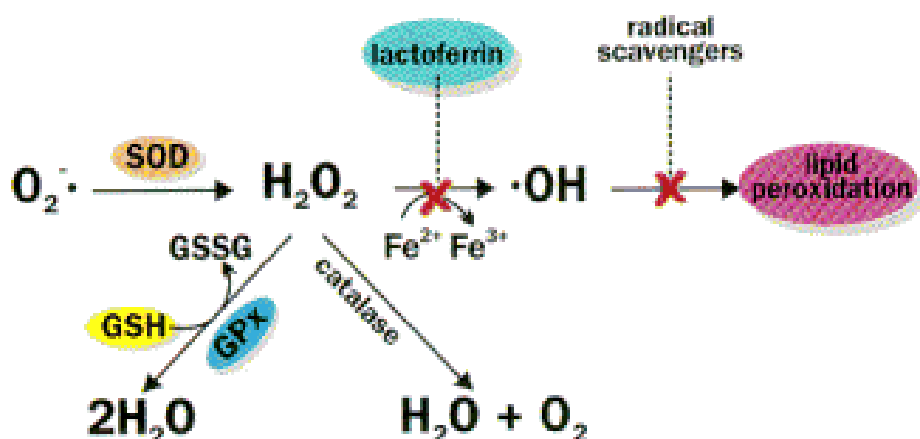
Figure 2.5: Schematic presentation showing activity of some antioxidant enzymes on some ROS. SOD- superoxide dismutase, GSH- reduced glutathione, GPx- glutathione peroxidase (http://www.rndsystems.com/mini_review_detail_objectname_MR97_ROS.aspx, 12.11.2013).

Under physiological conditions the destructive effects of free radicals are neutralised by endogenous antioxidant defense system (Figure 2.5), thus, producing harmless molecules. This antioxidant defense system comprises of enzyme (catalase, superoxide dismutase, glutathione peroxidases, and glutathione reductase) and non-enzymatic (i.e minerals, coenzymes, vitamins A, C, E) antioxidants. Under pathophysiological events, upregulation or increased activity of these enzymes is vital to prevent cellular damage as a result of oxidative stress. Interestingly a growing body of evidence reveals that plant extracts are capable of increasing activity of these antioxidant enzymes. A study conducted by Bouderbala *et al.* (2010) demonstrated the ability of iridoid extracts from *Ajuga iva* to suppress oxidative stress and significantly increased the antioxidant enzymes in red blood cells of hyperlipidemic rats. Similar results were also reported in kidney tissues of obese diabetic rats following oral administration of Genseng extract (Shalaby and Hamouda, 2013).

2.7 Thromboembolic Disorders

Under physiological conditions, blood coagulation, which is a process where liquid blood thickens and forms a clot (thrombus), is an essential component of haemostasis that needs to be tightly controlled. It is important to acknowledge the beneficial role of blood coagulation in maintaining haemostasis. This process is beneficial in stopping bleeding due to vascular injury, however inappropriate internal

formation of blood clots (thrombosis) could be fatal. Blood coagulation is a highly complex process that involves intertwined activation of enzymes, platelets and coagulation cascades. The involvement and importance of platelets in the blood coagulation process under both haemostatic and pathophysiological conditions is well documented (Xiang *et al.*, 2008; Jenning, 2009; Lee *et al.*, 2009; Fabre and Gurney, 2010). In addition to secreting some clotting factors, activated platelets also provide binding sites for factor XI and prothrombin, all important in the coagulation cascade (Jenning, 2009).

Thrombus, either arterial or venous, is composed of red blood cells, platelets, trapped neutrophils and polymerized fibrin (Sikka and Bindra, 2010). Thrombosis is one of the main causes of thromboembolic disorders such as pulmonary embolism, deep vein thrombosis, stroke and myocardial infarction. Interaction between atherosclerotic plaque and arterial thrombosis (atherothrombosis) underlies majority of cardiovascular diseases (Lee *et al.,* 2009). Prevalence of thromboembolic disorders has become a serious concern even in developing countries due to changing life styles, people living more sedentary lifestyle and consuming high fat diet. These disorders are therefore among the leading causes of morbidity and contribute to over 20% of annual deaths globally (Cohen *et al.,* 2011).

The health threat posed by thromboembolic disorders has led to discovery and development of antithrombotic drugs. Various *in vitro* and *in vivo* models that evaluate anti-platelet aggregation and anticoagulant properties are widely used in the screening and search for new antithrombotic agents. Bleeding time assay is a commonly used *in vivo* assay model indicative of platelet and coagulation function. The antithrombotic drugs are then classified as anti-platelet aggregation, thrombolytic (fibrinolytic) and anticoagulant drugs. Depending on the nature of thrombosis, anti-platelets are considered to be more important for arterial thrombosis while anticoagulants are basically more preferred in venous thrombosis (Kishore, 2013).

The currently used anti-platelet agents include commonly known cyclooxygenase antagonist acetylsalicylic acid (ASA) and $Y2Y_{12}$ ADP receptor antagonists (clopidogrel, prasugrel and ticlopidine). $Y2Y_{12}$ ADP receptor antagonists and aspirin are known to exert their therapeutic activity through irreversible inhibition of platelet

activation induced by ADP and thromboxane A2, respectively (Storey *et al.*, 2007), thus preventing initial clot formation. Fibrinolytic drugs include urokinase, streptokinase, and alteplase which exert their activity by hydrolysing fibrin polymers in the already formed thrombus (Sikka and Bindra, 2010). In addition to heparin and vitamin-K antagonists, there are also dabigatran etexilate, apixaban, edoxaban and rivaroxaban as new generation of oral anticoagulants reported effective against various thromboembolic disorders (Bauer, 2012). This class of drugs prevents thrombosis progression by directly inhibiting clotting mechanism (Figure 2.6). Apixaban, edoxaban and rivaroxaban are reversible inhibitors of factor Xa (a vitamin K-dependent glycoprotein that converts prothrombin to thrombin) while dabigatran is a reversible direct thrombin inhibitor, a serine protease responsible for catalytic conversion of soluble fibrinogen to insoluble fibrin polymers (Garcia *et al.*, 2010).
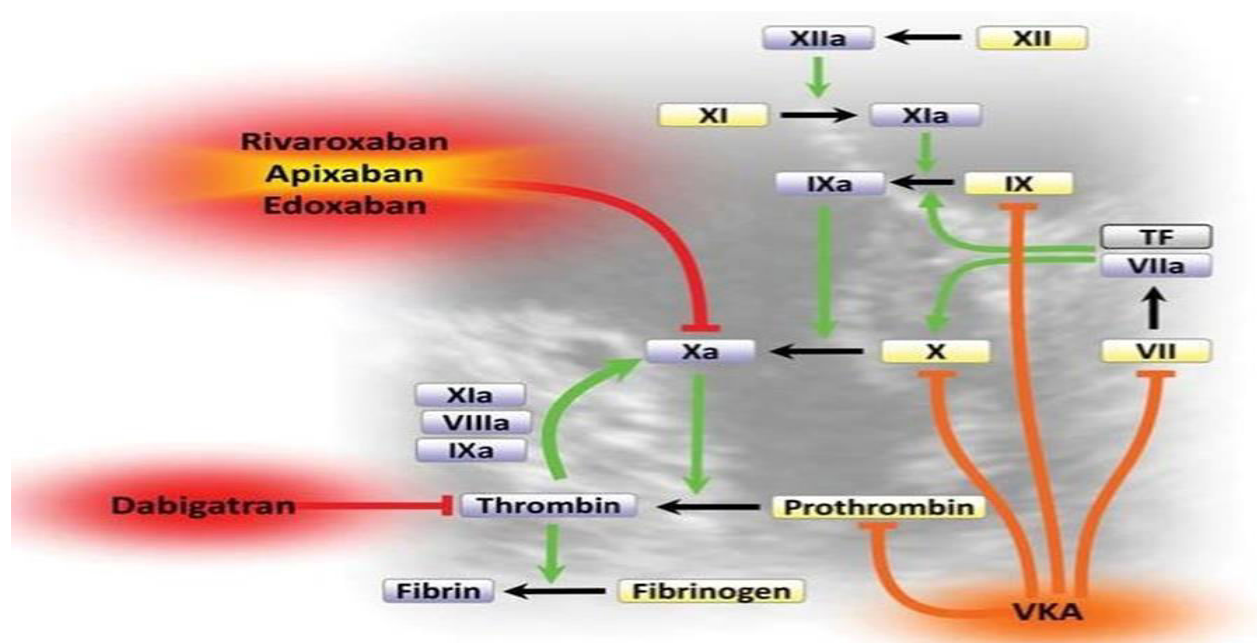
Figure 2.6: Site of action of the new oral anticoagulants in the coagulation cascade. Apixaban, edoxaban and rivaroxaban reversibly inhibit factor Xa while dabigatran is a reversible direct thrombin inhibitor. Source: Steffel and Hindricks (2012).

Despite availability and use of the current antithrombotic drugs, thromboembolic disorders continue to be among the leading causes of morbidity and mortality worldwide. The limited efficacy and safety of the currently used antiplatelet aggregation agents, mainly as a result of their single target and irreversible inhibition, are the major concerns. The new generation of anticoagulants, though deemed highly

effective, lack a specific antidote to reverse their anticoagulant activity should a need arise, which remains a concern (Fawole *et al.*, 2013). Therefore, in a continuous effort to discover more effective antithrombotic drugs with an improved safety profile, medicinal plants have become alternative sources of new effective and safe antithrombotic drugs.

Several traditionally used medicinal plants have been investigated for their antithrombotic activity. The antithrombotic activity of some Zulu medicinal plants has been reported to be partly mediated through inhibition of platelet aggregation (Mosa *et al.*, 2011a). Klafke *et al.* (2012) have also reported the anti-platelet, fibrinolytic and antithrombotic activities of *Campomanesia xanthocarpa* extract. Using rats, Zhang *et al.* (2013) demonstrated that total flavonoids from *Rosa laevigate* Michx fruit exert their antithrombotic activity in various ways. These included the inhibition of platelet aggregation, reduction of whole blood viscosity, and prolongation of thrombin and prothrombin time following a ten day oral administration of the extract. Several other plant-derived bioactive compounds including triterpenes have been reported to exhibit their antithrombotic activity through inhibition of platelet aggregation (Yang *et al.*, 2009; Mosa *et al.*, 2011b). Discovery of novel antithrombotic drugs with new mechanisms of action that would not increase risks of bleeding and interfere with haemostasis will be ideal. Therefore plant-derived compounds offer new exciting opportunities in novel therapeutic drug discovery and development.

2.8 Inflammation

Inflammation is a coordinated pathophysiological response of the body to harmful or injurious stimuli such as irritants, pathogens, allergens and toxic chemicals. The inflammatory process involves a complex biological cascade of molecular and cellular signals that alter physiological responses, ultimately resulting in the familiar clinical symptoms. Inflammation is characterised by dilation of local blood vessels, increased local blood flow, increased vascular permeability, accumulation of plasma fluid and blood cells at the injured tissue (Guyton and Hall, 2006; Lu *et al.*, 2009). These physiological reactions are triggered by chemicals released from tissues and migrating cells. The most strongly implicated chemicals include interleukin-1, platelet-activating factor, leukotrienes (LTs), serotonin, histamine, prostaglandins (PGs) and

bradykinin (Vane and Botting, 1987; Lu *et al.*, 2009). Though the inflammatory process is meant to remove harmful stimuli and initiate the healing process (Verhamme and Hoylaerts, 2009), its persistence may lead to chronic complications. Inflammation has also been linked with most chronic illnesses including diabetes, arthritis and cardiovascular diseases (Aggarwal *et al.*, 2006).

Inflammatory process induces oxidative stress (Chang *et al.*, 2010) and the produced free radicals intensify inflammation by stimulating secretion of inflammatory mediators from macrophages (Porfire *et al.*, 2009). Furthermore, these reactive species react with cell membranes and proteins, thus, impairing their function. Protein denaturation is a well documented factor that triggers inflammatory process (Chandra *et al.*, 2012). Other risk factors closely linked with inflammation include obesity-driven metabolic disorders and thromboembolic disorders. Shoelson *et al.* (2007) has indicated that accumulation of excess lipids in adipose tissue is commonly accompanied by inflammation. The enlarged adipocytes produce proinflammatory cytokines (tumour necrosis factor (TNF)-α, interleukin-1, interleukin-6) and chemokines. This role of adipose tissue is well supported by the work of Weisberg *et al.* (2003) where high levels of expression of the proinflammatory cytokines were observed in diet-induced obese mice model. An obesity-induced inflammation is known to promote insulin resistance, which in turn decreases Hsps expression, thus, permitting an accumulation of protein aggregates (Hooper and Hooper, 2008).

Furthermore, inflammation is also linked to blood coagulation under both physiological and pathophysiological conditions (Verhamme and Hoylaerts, 2009; Chu, 2011). Platelets play a central role in linking the two processes. In addition to their role in secreting prothrombogenic molecules, activated platelets also release cytokines and other proinflammatory mediators (Blair and Flaumenhaft, 2009). Jennings (2009) also indicated that the activated platelets interact with endothelial cells and leukocytes to promote inflammation. Thus, upon endothelial injury inflammation and blood coagulation become autocatalytic. Inflammation suppresses the anticoagulant system which favours high expression of prothrombogenic molecules (Verhamme and Hoylaerts, 2009).

Depending on duration and the type of immune cells involved, inflammation can be classified as either acute or chronic. Acute inflammation is a rapid short-term process that it is mediated by granulocytes. This is characterised by the usual cardinal signs clinical; redness, heat, swelling, pain and sometimes loss of function of the inflamed part (Underwood, 2000; Rote, 1994). In contrast to acute inflammation, which is self-limiting, chronic inflammation is prolonged (can take weeks to years) and it usually results from the host's failure to recover from acute inflammation. Chronic inflammation is mediated by monocytes and lymphocytes rather than granulocytes (Porth, 2005). It is also characterised by the infiltration of mononuclear cells, fibroblast proliferation and increased connective tissue formation (Beni *et al.*, 2011). Unlike in acute inflammation where inflammation is followed by repair, in chronic inflammation the two occur concurrently rather than in sequence. The repair is usually achieved by ingrowth of granulation tissue (Wakefield and Kumar, 2001).

2.8.1 Arachidonic metabolism in inflammation

Arachidonic acid (AA), a 20 carbon polyunsaturated fatty acid, is released from membrane phospholipids by phospholipase A2. Metabolism of AA is mediated by two major pathways namely cyclooxygenase (COX) and lipoxygenase (LOX) pathway. The COX pathway leads to biosynthesis of the PGs and thromboxanes (TXs) while the LOX pathway leads to the synthesis of leukotrienes (Figure 2.7). While both PGs and LTs are potent mediators of inflammation, TXs are prothrombogenic. It has been reported that dietary n-3 fatty acids (polyunsaturated fatty acids) exhibit anti-inflammatory properties (Poudyal *et al.*, 2013). These fatty acids are competitive inhibitors of AA metabolism in the COX pathway which leads to reduced production of prostanoids and thromboxane.
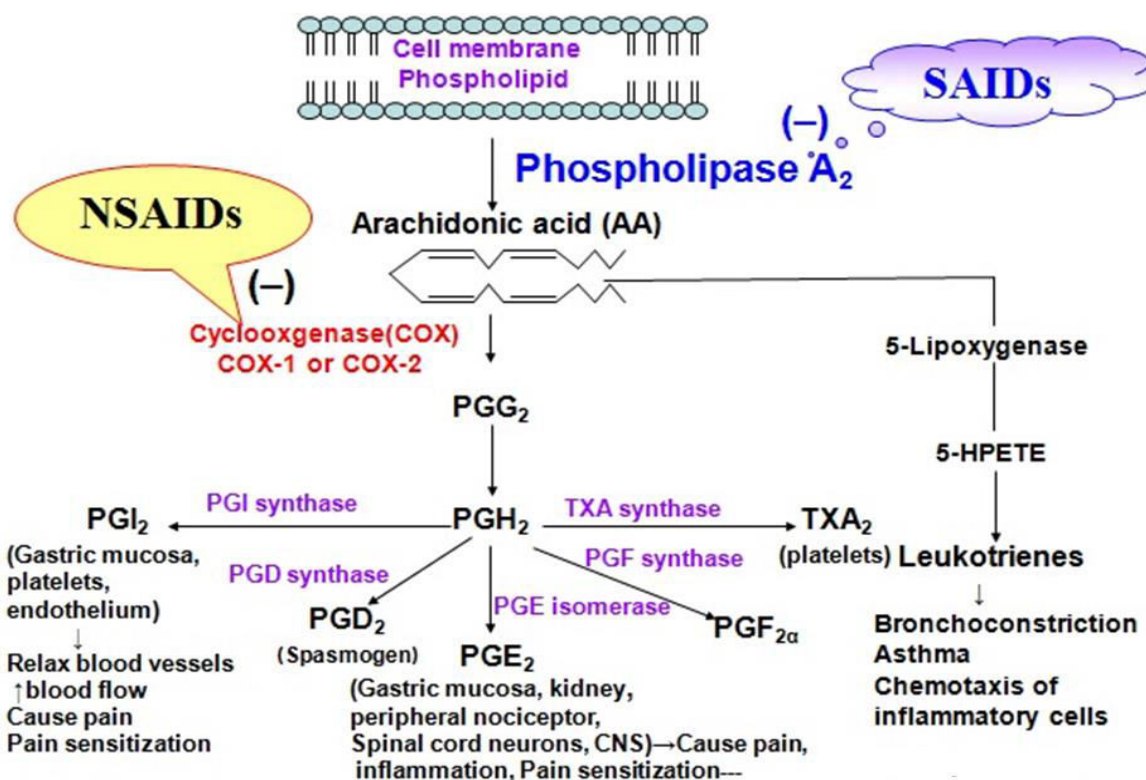
Figure 2.7: Pathways involved in AA metabolism and sites of action of anti-inflammatory drugs NSAIDs= non-steroidal anti-inflammatory drugs, PG = prostaglandins, TXA$_2$ = thromboxane A$_2$, 5-HPTETE = 5-hydroperoxyeicosatetraenoic acid (http://servingnature.blogspot.com/2013/04/what-are-nsaids-their-uses-and-side.html, 06.12.2013).

COX (Prostaglandin endoperoxide H synthase) exists in two isoforms namely COX-1 and COX-2. Several studies (Vane, 1996, Dubois *et al.*, 1998) have long revealed that COX-1 is constitutively expressed in most cells of the body and plays important roles in physiological processes. These include gastric epithelial cytoprotection, enhancing organ perfusion and regulating blood flow. COX-2 has been reported to be strongly induced by inflammatory stimuli (Roschek *et al.*, 2009). It has also been reported that during inflammation COX-2 is the main enzyme that is unregulated in macrophages and it is also expressed in some other non-inflammatory cells (Khansari *et al.*, 2009). Despite their differences, both enzymes are involved in the synthesis and release of homeostatic prostanoids during inflammation.

Inflammatory incidences are currently managed with two classes of anti-inflammatory drugs. These are steroidal anti-inflammatory drugs mainly corticosteroids (prednisone, betamethasone, dexamethasone) and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, aspirin, diclofenac, naproxen, ibuprofen,

rofecoxib, lumiracoxib and celecoxib. These two classes of drugs exert their therapeutic activity through different mechanisms of action (Figure 2.7). The steroidal anti-inflammatory drugs act by inhibiting phospholipase A2, an enzyme responsible for arachidonic acid synthesis. The NSAIDs, which are the most commonly used anti-inflammatory drugs, exert their anti-inflammatory activity through the inhibition of COX activity thereby preventing PG biosynthesis from AA. Traditional NSAIDs (indomethacin, aspirin, ibuprofen) have a non-selective inhibitory activity of the COX while coxib (celecoxib, rofecoxib, lumiracoxib) are COX-2 inhibitors.

Owing to the essential physiological functions of COX-1, prolonged use of non-selective NSAIDs is associated with adverse effects such as gastrointestinal complains and increased risks of bleeding (Fendrick *et al.*, 2008). The adverse effects of NSAIDs are associated with their inhibitory activity on COX-1 rather than COX-2. Thus, selective COX-2 inhibitors are considered much safer drugs. However, the use of the selective COX-2 inhibitors is also associated with some increased risk of cardiovascular events (Kearney *et al.*, 2006). This also indicates a need for a continuous search for more effective and safer therapeutic drugs, preferably from plants.

Protein denaturation assay is a commonly used *in vitro* assay to search for new anti-inflammatory drugs of natural origin. The carrageenan-induced paw edema model is widely used as a suitable *in vivo* model in the search for new anti-acute inflammatory agents from plant material. This model is considered to be biphasic in which the first phase (1 h) involves the release of inflammatory mediators; serotonin and histamine while the second phase (over 1 h) is mediated by prostaglandins (Vinegar *et al.*, 1969). Therefore, this model is considered suitable for the evaluation of acute anti-inflammatory agents exerting their therapeutic activity by inhibiting cyclooxygenases (COXs) which are involved in the synthesis of prostaglandins (Seibert *et al.*, 1994; Sawadogo *et al.*, 2006).

A large body of evidence from research using different *in vitro* and *in vivo* models has proven that plants possess anti-inflammatory properties (Nagar *et al.*, 2011; Chandra *et al.*, 2012). *Citrullus lanatus var. citroides,* traditionally used to treat rheumatism and swellings in Northern Sudan, has been reported to suppress swelling in carrageenan-

induced rat's paw edema. The anti-inflammatory activity of cucurbitacin E isolated from the plant extract was attributed to its COX inhibitory properties with more selectivity towards COX-2 (Abdelwahab *et al.*, 2011). Anti-inflammatory activity of several other plants have been reported to be through the inhibition of COX-2 (Kaur *et al.*, 2010; Chattopadhyay *et al.,* 2012), thus potential sources of safe anti-inflammatory drugs. Some triterpenes (Wang *et al.*, 2009; Araruna and Carlos, 2010) including a lanostane-type triterpenoid from the stem bark of *P. longifolia* (Mosa *et al.*, 2011b) have demonstrated their anti-inflammatory activity by significantly suppressing swelling in the carrageenan-induced paw edema model in rats. Natural anti-inflammatory drugs that could exert dual inhibition of COX and lipooxygenase activities could also be appreciated.

The cotton pellet-induced granuloma model is commonly used as an *in vivo* test to assess the transudative, exudative and proliferative components of chronic inflammation. Inflammation and granuloma often develops within several days. The granuloma is basically the product of macrophages, neutrophils and fibroblasts proliferation at the inflamed site (Ramakrishnan *et al.*, 2011). The weight of wet and dry cotton pellets correlates with the amount of formed transudate material and granuloma tissue, respectively. Thus, this model is suitable for the evaluation of the anti-proliferative activity of anti-inflammatory drugs. Efficacy of anti-inflammatory agents is then indicated by the inhibition of the fibroblast proliferation, collagen fibre synthesis and suppression of mucopolysaccharides during granuloma tissue formation (Verma *et al.*, 2010; Singh *et al.*, 2011). Some researchers have also proven using this *in vivo* model that medicinal plants have the ability to inhibit both the acute and proliferative phase of inflammation (Verma *et al.*, 2010; Nagar *et al.*, 2011).

A thorough knowledge of the inflammatory mechanism at molecular and cellular level is vital in the search for more effective drugs with novel mechanisms of action. This would include molecules or compounds with the ability to down regulate expression of some pro-inflammatory cytokines critical for the initiation and progression of inflammation. The anti-inflammatory activity of triterpenes from *Garnoderma lucidum* were reported to be through various mechanisms including suppression of cytokines release, inhibition of nitric oxide synthase and COX-2 in lipopolysaccharide-

stimulated macrophages (Dudhgaonkar *et al.*, 2009). Since protein denaturation causes tissue inflammation, the ability of anti-inflammatory agents to also prevent protein aggregation is important.

2.9 Heat shock proteins

Even though inappropriate protein aggregation is normally prevented by complex cellular mechanisms, under certain circumstances aggregation of proteins occurs. Misfolding and inappropriate aggregation of proteins leads to loss of biological function (Figure 2.8) and is commonly associated with a number of neurodegenerative diseases (Lendel *et al.*, 2009). Heat shock proteins (Hsps) are highly conserved ubiquitous proteins found in the cells of all living organisms. These proteins function as intra-cellular chaperones for other proteins, preventing protein misfolding and unwanted protein aggregation (Shonhai *et al.*, 2008). They are essential for normal cell function, viability and growth.
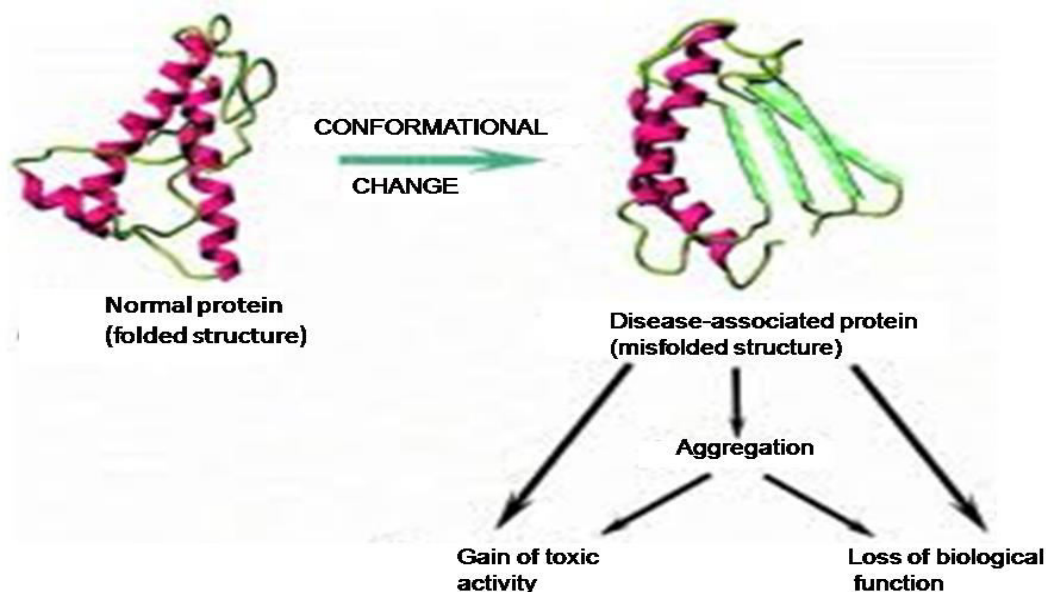
Figure 2.8: Misfolding and unwanted protein aggregation leads to loss of biological function and accumulation of toxic inclusion bodies (http://srxawordonhealth.com/tag/heat-shock-proteins/ 10.11.2013).

Different cellular compartments and/or organelles have specific Hsps adapted to meet their demands (Tsan and Gao, 2009). Hsps are classified according to their molecular weight (in kDa) as small Hsp_s (15 to 30 kDa), Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and Hsp110. Despite different molecular weights, these proteins

share common functional domains namely an adenine nucleotide-binding domain (binds and hydrolyses ATP) and a peptide-binding domain (binds exposed hydrophobic residues of substrate peptides) (Tsan and Gao, 2009). However, Barbatis and Tsopanomichalou (2009) have indicated that while high molecular weight Hsps are ATP-dependent chaperones, small Hsps are ATP-independent.

Hsps are not only confined within the cells, but are also released into the extracellular space. Their functions depend on their location. Intracellular Hsps have a protective function while the extracellular Hsps mediate immunological functions (Schmitt *et al.*, 2007). Their extracellular effects are mediated through cell surface receptors (See Figure 2.9). Due to their ability to function as signal molecules, extracellular Hsps are currently considered part of the "danger-associated molecular patterns" (Giuliano *et al.*, 2011) recognised by receptors of immune cells.
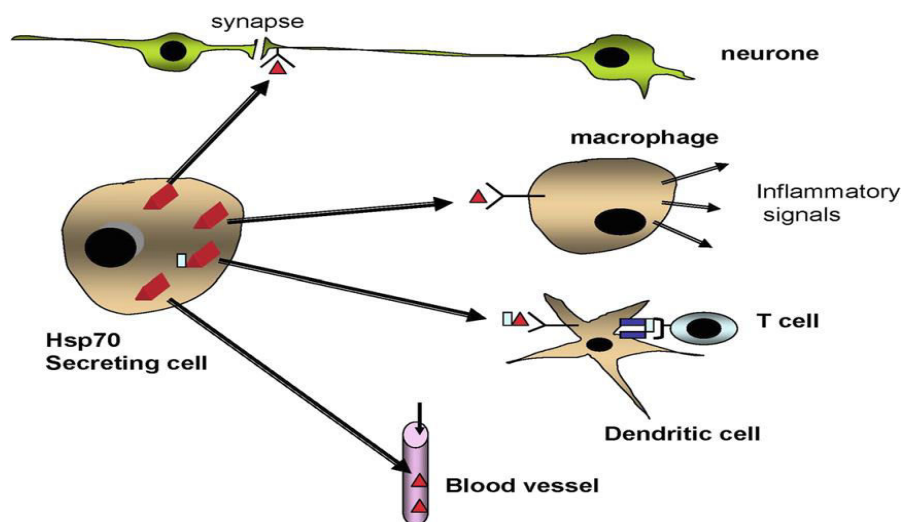
Figure 2.9: Hsp70 secreting cell releases Hsp70 and the released Hsp may interact with target cells to elicit various physiological responses. The released Hsp70 (red triangles) may interact with immune cells (monocytes or macrophages), neuronal cells or enter the circulation. Hsp70 may be taken up by antigen presenting cells to trigger the immune response (Adapted from Calderwood *et al.*, 2007).

2.9.1 Hsps expression in diseases

Hsps are involved in many disease processes including inflammation, cancer, cardiovascular and neurological diseases. Depending on the context or condition, Hsps are reported to have both anti-inflammatory as well as pro-inflammatory functions (Calderwood *et al.*, 2007). While intracellular Hsps are known to

predominantly down-regulate host inflammatory response, the extracellular proteins may down-regulate or upregulate the host's inflammatory response (Giuliano *et al.*, 2011). It has also been reported that the anti-inflammatory activity of Hsps is through suppression of inflammatory gene expression thus inhibiting synthesis of inflammatory cytokines. These proteins can also activate anti-inflammatory cytokines hence control the magnitude of the immune response (Barbatis and Tsopanomichalou, 2009).

Neurodegenerative diseases are characterised by the occurrence of extra- or intracellular fibrillar aggregates containing misfolded proteins (Arawaka *et al.*, 2010). The occurrence of these misfolded proteins and aggregates in the brain is regarded as the underlying cause of various neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease (AD), and the polyglutamine (polyQ) diseases (Nagai *et al.*, 2010, Tutar and Tutar, 2010). Hsps are not only capable of preventing unnecessary protein aggregation, but they can also reduce the formation of fibrils (Luo and Le, 2010). Therefore, Hsps with their chaperone activity in facilitating proper potein folding and preventing unwanted protein misfolding have now become new therapeutic targets (Sajjad *et al.*, 2010).

2.9.2 Hsp70

Hsp70 family is one of the most highly conserved chaperones and the major chaperones in all cellular compartments and organs (Wang *et al.*, 2003). These 70 kDa proteins are the most highly inducible Hsps and comprise both constitutive and inducible forms essential for cellular function and increase in response to environmental stress (Barbatis and Tsopanomichalou, 2009). These proteins do not only prevent protein misfoldings but they can also refold aggregated proteins. In preventing protein aggregation, Hsp70 dimerises with Hsp40 to facilitate protein folding using ATP/ADP exchange cycle. The Hsp40 provides ATPase activity that increases substrate binding to the Hsp70 (Fang *et al.*, 2003).

Hsp70 is currently a new drug target for treating neurodegenerative tauopathies, characterised by abnormal aggregation of tau protein (Miyata *et al.*, 2013). It has been found that this protein has a direct inhibitory effect on tau aggregation (Voss *et*

al., 2012). The study carried out by Hoshino *et al.* (2011) further showed the potential therapeutic benefit of Hsp70 in the prevention and treatment of neurodegenerative diseases such as AD. Amyloid-β peptide (Aβ) plays a key role in the pathogenesis of AD. Hoshino *et al.* (2011) demonstrated that the therapeutic activity of the Hsp70 is through upregulation of the expression of Aβ-degrading enzyme and cytokineTGF-β1, responsible for stimulating phagocytosis of Aβ by microglia and astrocytes.

Some common drugs such as aspirin have been found to promote the induction of Hsps (Jurivich *et al.*, 1992). While Celastrol, a plant-derived quinine methide triterpene has also been reported to induce expression of a wider range of Hsps in differentiated human neurons grown in tissue culture (Chow and Brown, 2007), Gaikwad *et al.* (2005) reported the suppressive effect of triterpenoid electrophiles (avicins) on the expression of Hsp70 in leukaemia cells. Furthermore, quercetin and several other flavonoids inhibited the synthesis of Hsp70 induced by heat shock in two human cell lines (Hosokawa *et al.*, 1990). There is currently a growing interest in discovery of pharmacologically active drugs that upregulate expression of Hsp70 as a potential therapeutic effect in some human diseases. However, drugs that can suppress expression of Hsps where cellular apoptosis is required could also be desirable.

2.10 Microbial Infections

Pathogenic microorganisms are the major cause of a wide range of infectious diseases. Microbial infections account for significant causes of morbidity and mortality worldwide (Tajbakhsh *et al.*, 2011). The devastating effects of microbial infections are more manifested in developing countries due to socio-economic problems, unavailability of medicine and proper treatment.

Despite great progress made in the discovery of antibiotics, the development of bacterial resistance to some of the current antibiotics is a serious global challenge. In addition to genetic modification, an indiscriminate use of the current antimicrobial agents has substantially contributed to the emergence and increase of antimicrobial resistance (Bansod and Rai, 2008). The biggest challenge currently facing drug discovery programs is to keep up with the rate at which antimicrobial resistance

develops. Therefore, there is urgent need to discover new antibiotic agents with novel mechanisms of action, improved efficacy and a better safety profile against new and re-emerging infections (Rojas *et al*., 2003, Zakaria *et al*., 2009).

Plants have always been an untapped source to provide bioactive compounds as potential therapeutic antimicrobial agents. Plant products, in various forms, provide a wide chemical diversity that is important in the development of new effective agents to combat infectious diseases resistant to conventional drugs (de León *et al*., 2005, Mahesh and Satish, 20008). Broth micro dilutions assays are widely employed in the search for new antimicrobials from natural sources such as plants. The antibacterial (Popova *et al*., 2009), antifungal (Han *et al*., 2009; Kanwal *et al*., 2010), antiviral (Zandi *et al*., 2011), and antiparasitic (Adams *et al*., 2010; Malebo *et al*., 2013; Simelane *et al*., 2013) activity of various plant-derived compounds including triterpenoids has been documented. Triterpenoids from *Momordica balsamina* (Ramalhete *et al*., 2011); *Alisma orientale* (Jin *et al*., 2012); and *Carpobrotus edulis* (Martins *et al*., 2011) exhibited antibacterial activity even on various resistant bacterial strains including the *Staphylococcus* spp. Some other triterpenes have also been reported to exhibit antimycobacterial activity against both antibiotic-sensitive and resistant *Mycobacterium tuberculosis* (Rojas *et al*., 2006; Jim´enez-Arellanes *et al*., 2007).

Antimicrobial drugs exert their therapeutic activity through various mechanisms which include inhibition of efflux pump, proteins, cell wall, cell membrane and nucleic acid synthesis (Riaz *et al*., 2011). Several plant extracts have been reported to exhibit efflux pump inhibitory activity (Chitemerere and Mukanganyama, 2011, Fiamegos *et al*., 2011) which indicated the potential ability of the plants to be used against resistant bacteria. Soyingbe *et al*. (2013) demonstrated that the essential oil from *Eucalyptus grandis* exerts their antimicrobial activity by inducing microbial membrane damage. Terpenoids have also been reported to exert their antimicrobial activity through disruption of bacterial cell membrane (Saleem *et al*., 2010) or possibly by inhibiting amongst others synthesis of cell membrane components, and prenylation of proteins (Nayak *et al.,* 2010). The DNA protective effect of some triterpenes has previously been reported (Ramos *et al*., 2010; Smina *et al*., 2011). Therefore, knowledge of the mechanism of action of various antimicrobial agents and their

various targets sites is very important in the development of new antibiotics with novel mechanisms of action.

Despite availability of the current clinical drugs, metabolic disorders and microbial infections continue to cause serious human health problems. The biggest challenge in therapeutic drug discovery is to keep up the pace with the ever evolving diseases. Thus, the need to search for more drugs with an improved efficacy and safety profiles is mandatory. Medicinal plants with their diverse chemical structures offer an opportunity to discover drugs with multiple beneficial effects.

2.11 Scope of the Work

2.11.1 Aim

This study aims to investigate the bioactivity of some triterpenes, from stem bark of *Protorhus longifolia,* and their derivatives.

2.11.2 Objectives

The objectives of the study were:

a) Collection and authentication of the plant
b) Extraction, isolation, purification and identification of triterpenes
c) Investigate some bioactivity of the triterpenes
    i. Determination of antioxidant activity
    ii. Investigation of anti-hyperlipidemic activity
    iii. Investigation of anti-diabetic properties
    iv. Evaluation of anticoagulant activity
    v. Evaluation of anti-inflammatory activity
    vi. Determining effects of the triterpenes on protein aggregation and expression of heat shock protein 70 (Hsp70)
    vii. Evaluation of antimicrobial activity
d) To carry-out structural modification (derivatisation) of the triterpenes
e) To investigate some bioactivity of the derivatives where the original compounds (triterpenes) showed significant activity.

References

Abdelwahab, S.I. Hassan, L.E. Sirat, H.M. Yagi, S.M. Koko, W.S. Mohan, S. (2011). Anti-inflammatory activities of cucurbitacin E isolated from *Citrullus lanatus var. citroides*: Role of reactive nitrogen species and cyclooxygenase enzyme inhibition *Fitoterapia* 82(8):1190-1197.

Adams, M. Christen, M. Plitzko, I. Zimmermann, S. Brun, R. Kaiser, M. Hamburger, M. (2010). Antiplasmodial lanostanes from the *Ganoderma lucidum* mushroom. *Journal of Natural Products* 73(5): 897-900.

Adisakwattana, S. Moonrat, J. Srichairat, S. Chanasit, C. Tirapongporn, H. Chanathong, B. Ngamukote, S. Mäkynen, K. Sapwarobol, S. (2010). Lipid-Lowering mechanisms of grape seed extract (*Vitisvinifera* L) and its antihyperlidemic activity. *Journal of Medicinal Plants Research* 4(20): 2113-2120.

Agellon, L.B. Metabolism and function of bile acids, In: Vance, D.E. Vance, J.E. (2002) (Eds). *Biochemistry of lipids, lipoproteins and membranes* (4th edition). New Comprehensive Biochemistry 36, pp 433-448.

Aggarwal, B.B. Shishodia, S. Sandur, S.K. Pandey, M.K. Sethi, G. (2006). Inflammation and cancer: How hot is the link? *Biochemical Pharmacology* 72: 1605-1621.

Ahmed, S.M. Clasen, M.D. Donnelly, M.D. (1998). Management of dyslipidemia in adults. *American Family Physician* 57: 2192-2204.

Ahn, J.H. Liu, Q. Lee, C. Ahn, M. Yoo, H. Hwang, B.Y. Lee, M.K. (2012). A new pancreatic lipase inhibitor from *Broussonetia kanzinoki*. *Bioorganic and Medicinal Chemistry Letters* 22: 2760-2763.

Ali, Y.B. Verger, R. Carrière, F. Petry, S. Muller, G. Abousalham, A. (2012). The molecular mechanism of human hormone-sensitive lipase inhibition by substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones. *Biochimie* 94: 137-145.

Alqahtani, A. Hamid, K. Kam, A. Wong, K.H. Abdelhak, Z. Razmovski-Naumovski, V. Chan, K. Li, K.M. Groundwater, P.M. Li, G.Q. (2013). The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Current Medicinal Chemistry* 20(7): 908-931.

Appendino, G. Pollastro, F. Plants: Revamping the oldest source of medicines with modern science. In: Buss, A.D. Burtler, M.S. (2010) (eds). *Natural Product Chemistry for Drug Discovery*. RCS publishing: Cambridge, UK, pp 164-165.

Araruna, K. Carlos, B. (2010). Anti-inflammatory activities of triterpene lactones from *Lactuca sativa*. *Phytopharmacology* 1(1): 1-6.

Arawaka, S. Machiya, Y. Kato, T. (2010). Heat shock proteins as suppressors of accumulation of toxic prefibrillar intermediates and misfolded proteins in neurodegenerative diseases. *Current Pharmaceutical Biotechnology* 11(2): 158-166.

Archer, R.H. (2000). Anacardiaceae, In: Leistner OA (Ed). Seed plants of Southern Africa. National Botanical Institute, Pretoria: *Strelitzia* 10: 56-59.

Bandeira, S.M. da Fonseca, L.J.S. Guedes, G.S. Rabelo, L.A. Goulart, M.O.F. Vasconcelos, S.M.L. (2013). Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. *International Journal of Molecular Sciences* 14: 3265-3284.

Bansod, S. and Rai, M. (2008). Antifungal activity of essential oils from Indian medicinal plants against human pathogenic *Aspergillus fumigatus* and *A. niger. World Journal of Medical Sciences* 3(2): 81-88.

Barbatis, C. and Tsopanomichalou, M. (2009). Heat shock proteins in inflammatory bowel disease. *Annals of Gastroenterology* 22(4):244-247.

Bauer, K.A. (2012). Reversal of antithrombotic agents. *American Journal of Hematology* 87: S119-S126.

Beni, J.M. Jayanthi, M.K. Suresha, R.N. (2011). Evaluation of the anti-inflammatory activity of *Aeglemarmelos* (Bilwa) root. *Indian Journal of Pharmacology* 43: 393-397.

Blair, P. and Flaumenhaft, R. (2009). Platelet alpha-granules: basic biology and clinical correlates. *Blood Reviews* 23: 177-189.

Bouderbala, S. Prost. J. Lacaille-Dubois, M.A. Bouchenak, M. (2010). Iridoid extracts from *Ajuga iva* increase the antioxidant enzyme activities in red blood cells of rats fed a cholesterol-rich diet. *Nutrition Research* 30(5): 358-365.

Bray, G.A. and Greenway, F.L. (2007). Pharmacological treatment of the overweight patient. *Pharmacology Reviews* 59(2):151-184.

Brown, A.W. Hang, J. Dussault, P.H. Carr, T.P. (2009). Plant sterol and stanol substrate specificity of pancreatic cholesterol esterase. *The Journal of Nutritional Biochemistry* 21(8): 736-740.

Bustanji, Y. Issa, A. Mohammad, M. Hudaib, M. Tawah, K. Alkhatib, H. Almasir, I. Al-Khalid, B. (2010). Inhibition of hormone sensitive lipase and pancreatic lipase by *Rosmarinus officinalis* extract and selected phenolic constituents. *Journal of Medicinal Plants Research* 4(21): 2235-2242.

Calderwood, S.K. Mambula, S.S. Gray, Jr. P.J. Theriault, J.R. (2007). Extracellular heat shock proteins in cell signaling. *FEBS Letters* 581: 3689-3694.

Chandra, S. Chatterjee, P. Dey, P. Bhattacharya, S. (2012). Evaluation of in vitro anti-inflammatory activity of coffee against the denaturation of protein. *Asian Pacific Journal of Tropical Biomedicine* 2012: S178-S180.

Chang, C. Zhang, L. Chen, R. Wu, C. Huang, H. Roy, M.C. Huang, J. Wu, Y. Kuo, Y. (2010). Quiquelignan A-H, eight new lignoids from the rattam palm *Calmus quiquesetinervious* and their antiradical, anti-inflammatory and antiplatelet aggregation activities. *Bioorganic & Medicinal Chemistry* 18: 518-525.

Chaput, J.P. St-Pierre, S. Tremblay, A. (2007). Currently available drugs for the treatment of

Chattopadhyay, P. Hazarika, S. Dhiman, S. Upadhyay, A. Pandey, A. Karmakar, S. Singh, L. (2012). *Vitex negundo* inhibits cyclooxygenase-2 inflammatory cytokine-mediated inflammation on carrageenan-induced rat hind paw edema. *Pharmacognosy Research* 4:134-137.

Cheng, H.L. Huang, H.K. Chang, C.C. Tsai, C.P. Chou, C.H. (2008). A cell-based screening identifies compounds from the stem of *Momordica charantia* that overcome insulin resistance and activate AMP-activated protein kinase. *Journal of Agricultural and Food Chemistry* 56 (16): 6835-6843.

Cheng, Z. Pang, T. Gu, M. Gao, A.H. Xie, C.M. Li, J.Y. Nan, F.J. Li, J. (2006). Berberine stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. *Biochimica et Biophysica Acta* 1760: 1682-1689

Chiang, J.Y.L. (2009). Bile acids: regulation of synthesis. *Journal of Lipid Research* 50: 1955-1966.

Chitemerere, T. and Mukankanyama, S. (2011). *In vitro* antibacterial activity of selected medicinal plants from Zimbabwe. *The African Journal of Plant Science and Biotechnology* 5(1): 1-7.

Chow, A.M and Brown, I.R. (2007). Induction of heat shock proteins in differentiated human and rodent neurons by celastrol. *Cell Stress Chaperones* 12(3): 237-244.

Chu, A.J. (2011). Tissue factor, blood coagulation, and beyond: an overview. *International Journal of Inflammation* 2011: doi: 10.4061/2011/367284.

Cohen, A.T. Dobromirski, M. Lin. S.L. Wills, J.O. (2011). Venous thromboembolism: The magnitude of the problem in Europe and the world. *Advances in Venous Arterial Thrombosis* 1: 39-45.

Colombini, M.P. and Modugno, F. (Eds) (2009). Organic mass spectrometry in art and archeology. Wiley, Chichester, UK, pp 15-17

Czech, M.P. and Corvera, S. (1999). Signaling mechanisms that regulate glucose transport. *Journal of Biological Chemistry* 274: 1865-1068.

de León, L. Beltrán, B, Moujir, L. (2005). Antimicrobial activity of 6-Oxophenolic triterpenoids. Mode of action against *Bacillus subtilis*. *Planta Medica* 71: 313-319.

De Souza, P.M. de Sales, P.M. Simeoni, L.A. Silva, E.C. Silveira, D. Magalhães, P.O. (2012). Inhibitory activity of α-amylase and α-glucosidase by plant extracts from the Brazilian Cerrado. *Planta Medica* 78(4): 393-399.

Derosa, G. Salvadeo, S. Cicero, A.F. (2006). Prospects for the development of novel anti-hyperlipidemic drugs. *Current Opinion in Investigational Drugs* 7: 826-833.

Dewick, P.M. (2009). *Medicinal Natural Products: A biosynthetic approach*. 3rd edition. Wiley: Chichester, United Kingdom, pp 234-237.

Díaz, E.G. and Folgueras, T.M. (2011). Systematic review of the clinical efficacy of sibutramine and orlistat in weigth loss, quality of life and its adverse effects in obese adolescents. *Nutrición Hospitalaria* 26(3):451-457.

Díaz, E.G. Folgueras, T.M. (2011). Systematic review of the clinical efficacy of sibutramine and orlistat in weigth loss, quality of life and its adverse effects in obese adolescents. *Nutrición Hospitalaria* 26(3):451-457.

Dibaise, J.K. and Islam, R.S. (2012). In: Bile acids: An underrecognized and underappreciated cause of chronic diarrhea. Parrish, C.R. M.S .R.D. Series editor. Practical gastroenterology. *Nutrition Issues in Gastroenterology* series 110.

Dold, A.P. and Cocks, M.L. (2001). Traditional veterinary medicine in the Alice district of the Eastern Cape Province, South Africa. *South African Journal of Science* 97(9-10): 375-379.

Dold, A.P. and Cocks, M.L. (2002). The trade in medicinal plants in the Eastern Cape Province, South Africa. *South African Journal of Science* 98(11-12*): 594.

Dubois, R.N. Abramson, S.B. Crofford, L. Gupta, R.A. Simon, L.S. Van De Putte, L.B.A. Lipsky, P.E. (1998). Cyclooxygenase in biology and disease. *The Journal of the Federation of American Societies of Experimental Biology* 12: 1063-1073.

Dudhgaonkar, S. Thyagarjan, A. Sliva, D. (2009). Suppression of the inflammatory response by triterpenes isolated from the mushroom *Ganoderma lucidum*. *International Immunopharmacology* 9: 1272-1280.

Dzubak, P. Hajduch, M. Vydra, D. Hustova, A. Kvasnica, M. Biedermann, D. et al. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural Products Reports* 23:394-411.

Fabre, J. and Gurney, M.E. (2010). Limitations of current therapies to prevent thrombosis: A need for novel strategies. *Molecular BioSystems* 6(2): 305-315.

Fang, C. Lee, S. Cyr, D.M. (2003). Mechanisms for regulation of Hsp70 function by Hsp40. Cell stress chaperones 8(4): 309-316.

Fawole, A. Daw, H.A. Crowther, M.A. (2013). Practical management of bleeding due to the anticoagulants dabigatran, rivaroxaban, and apixaban. *Clevel and Clinic Journal of Medicine* 80(7), www.ccjm.org.

Fendrick, A.M. Pan, D.E. Johnson, G.E. (2008). OTC analgesics and drug interactions: Clinical implications (Review). *Osteopathic Medicine and Primary Care* 2(2): 2doi:10.1186/1750-4732-2-2.

Fiamegos, Y.C. Kastritis, P.L. Exarchou, V. Han, H. Bonvin, A.M.J.J. et al. (2011) Antimicrobial and efflux pump inhibitory activity of caffeoylquinic acids from *Artemisia absinthium* against Gram-positive pathogenic bacteria. *PLoS ONE* 6(4): e18127. doi:10.1371/journal.pone.0018127.

Fryer, L.G. Parbu-Patel, A. Carling, D. (2002). The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *Journal of Biological Chemistry* 277: 25226-25232.

Furukawa, S. Fujita, T. Shimabukuro, M. Iwaki, M. Yamada, T. Nakajima, Y. Nakayama, O. Makishima, M. Matsuda, M. Shimomura, L. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *Journal of Clinical Investigation* 114:1752-1761.

Gaikwad, A. Poblenz, A. Haridas, V. Zhang, C. Dvic, M. Gutterman, J. (2005). Triterpenoid electrophiles (avicins) suppress heat shock protein-70 and x-linked inhibitor of apoptosis proteins in malignant cells by activation of ubiquitin machinery: implications for proapoptotic activity. *Clinical Cancer Research* 11(5): 1953-62.

Garcia, D. Libby, E. Crowther, M.A. (2010).The new oral anticoagulants. *Blood* 115:15-20.

Ghosh, T. Maity, T.K. Singh, J. (2011). Antihyperglycemic activity of bacosine, a triterpene from *Bacopa monnieri*, in alloxan-induced diabetic rats. *Planta Medica* 77(8): 804-808.

Giuliano, Jr. J.S. Lahni, P.M. Wong, H.R. Wheeler, D.S. (2011). Extracellular Heat Shock Proteins: Alarmins for the host immune system. *The Open Inflammation Journal* 4: 49-60.

Gurevich-Panigrahi, T. Panigrahi, S. Wiechec, E. Los, M. (2009). Obesity: pathophysiology and clinical management. *Current Medicinal Chemistry* 16:506-521.

Gutierrez, R.M.P. (2013). Evaluation of the hypoglycemic and hypolipidemic effects of triterpenoids from *Prosthechea michuacana* in STZ-induced type 2 diabetes in mice. *Pharmacologia*, DOI: 10.5567/pharmacologia.2013.170.179.

Guyton, A.C. and Hall, J.E. (2006). *Textbook of Medical Physiology*, eleventh edition. Elsevier: Philadelphia, USA, pp 434.

Ha, D.T. Tuan, D.T. Thu, N.B. Nhiem, N.X. Ngoc, T.M. Yim, N.H. Bae, K.H. (2009). Palbinone and triterpenes from Moutan Cortex (*Paeonia suffruticosa*, Paeoniaceae) stimulate glucose uptake and glycogen synthesis via activation of AMPK in insulin-resistant human HepG2 Cells. *Bioorganic & Medicinal Chemistry Letters* 19: 5556-5559.

Hadváry, P. Sidler, W. Meister, W. Vetter, W. Wolfer, H. (1991). The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. *Journal of Biology and Chemistry* 266: 2021-2027.

Han, H. Yi, Y. Li, L. Liu, B. La, M. Zhang, H. (2009). Antifungal active triterpene glycosides from sea cucumber *Holothuria scabra. Acta Pharmaceutica Sinica* 44 (6): 620-624.

Heidrich, J.E. Contos, L.M. Hunsaker, L.A. Deck, L.M. Jagt, D.L.V. (2004). Inhibition of pancreatic cholesterol esterase reduces cholesterol absorption in the hamster. *BMC Pharmacology* 4:5.

Hofmann, A.F. Hagey, L.R. (2008). Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cellular and Molecular Life Sciences* 65: 2461-2483.

Hooper, P.L. and Hooper, P.L. (2008). Inflammation, heat shock proteins, and type 2 diabetes. *Cell Stress and Chaperones*. DOI 10.1007/s12192-008-0073-x.

Hoshino, T. Murao, N. Namba, T. Takehara, M. Adachi, H. Katsuno, M. Sobue, G., Matsushima, T. Suzuki, T. Mizushima, T. (2011). Suppression of Alzheimer's Disease-related phenotypes by expression of heat Shock protein70 in mice. *The Journal of Neuroscience* 31(14):5225-5234.

Hosokawa, N. Hirayoshi, K. Nakai, A. Hosokawa, Y. Marui, N. Yoshida, M. Sakai, T. Nishino, H. Aoike, A. Kawai, K. Nagata, K. (1990). Flavonoids inhibit the expression of heat shock proteins. *Cell Structure and Function* 15: 393-401.

Hou, J.C. Min, L. Pessin, J.E. (2009). Insulin granule biogenesis, trafficking and exocytosis. *Vitamins and Hormones* 80:473-506.

Hou, W. Li, Y. Zhang, Q. Wei, X. Peng, A. Chen, L. Wei, Y. (2009). Triterpene acids isolated from *Lagerstroemia speciosa* leaves as α-glucosidase inhibitors. *Phytotherapy Research* 23: 614-618.

Hung, H. Qian, K. Morris-Natschke, S.L. Hsu, C. Lee, K. (2012). Recent discovery of plant-derived anti-diabetic natural products. *Natural Product Reports* 29: 580-606.

Hutchings, A. Scott, A.H. Lewis, G. Cunningham, A. (1996). *Zulu medicinal plants: An inventory.* Pietermaritzburg: University of Natal Press.

Ibrahim, S.R.M. Mohamed, G.A. Banjar, Z.M. Hani K. M. Kamal, H.K.M. (2013). Natural antihyperlipidemic agents: Current status and future perspectives. *Phytopharmacology* 4(3): 492-531.

Islam, R.S. and Dibaise, J.K. In: Bile acids: An underrecognized and underappreciated cause of chronic diarrhea. Parrish, C.R. M.S. R.D. (series editor), (2012). *Nutrition Issues in Gastroenterology*, series #110.

Jackson, L. and Robertson, L. (1989). Sulphonylureas (specifically glibenclamide) and their correct dosage. *South African Medical Journal* 76(6): 286-289.

Jäger, S. Trojan, H. Kopp, T. Laszczyk, M.N. Scheffler, A. (2009). Pentacyclic triterpene distribution in various plants – rich sources for a new group of multi-potent plant extracts. *Molecules* 14: 2016-2031.

Jennings, L.K. (2009). Mechanisms of platelet activation: Need for new strategies to protect against platelet-mediated atherothrombosis. *Thrombosis and Haemostasis* 102: 248-257.

Jiang, Z.Y. Zhou, Q.L. Coleman, K.A. Chouinard, M. Boese, Q. Czech, M.P. (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA mediated gene silencing. *Proceedings of National Academic Science* (U.S.A) 100: 7569-7574.

Jim´enez-Arellanes, A. Meckes, M. Torres, J. Luna-Herrera, J. (2007). Antimycobacterial triterpenoids from *Lantana hispida* (Verbenaceae). *Journal of Ethnopharmacology* 111: 202-205.

Jin, H.G. Jin, Q. Kim, R.A. Choi, H. Lee, J.H. Kim, Y.S. Lee, D.G. Woo, E.R. (2012). A new triterpenoid from *Alisma orientale* and their antibacterial effect. *Archives of Pharmacal Research* 35: 1919-1926.

Jurivich, D.A., Sistonen, L., Kroes, R.A. Morimoto, R.I. (1992). Effect of sodium salicylate on the human heat shock response. *Science* 255: 1243–1245.

Kamesh, V. and Sumathi, T. (2012). Antihypercholesterolemic effect of *Bacopa monniera linn.* on high cholesterol diet induced hypercholesterolemia in rats. *Asian Pacific Journal of Tropical Medicine* 2012: 949-955.

Kanwal, Q. Hussaina, I. Siddiqui, H.L. Javaid, A. (2010). Antifungal activity of flavonoids isolated from mango (*Mangifera indica* L.) leaves. *Natural Products Research* 24(20): 1907-1914.

Karalis, D.G. Victor, B. Ahedor, L. Liu, L. (2012). Use of lipid-lowering medications and the likelihood of achieving optimal LDL-cholesterol goals in coronary artery disease patients. *Cholesterol* 2012: doi:10.1155/2012/861924.

Kaur, P. Kaur, S. Kumar, S. Singh, P. (2010). *Rubia cordifolia* L. and *Glycyrrhiza glabra* L. medicinal plants as potential source of COX-2 inhibitors. *American Journal of Biomedical Science* 2(2): 108-120.

Kearney, P.M. Baigent, C. Godwin, J. Halls, H. Emberson, J.R. Patrono, C. (2006). Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory

drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. bmj.com 332:1302.

Kee. N.L. Mnonopi, N. Davids, H. Naudé, R.J. Frost, C.L. (2008). Antithrombotic/anticoagulant and anticancer activities of selected medicinal plants of *South Africa. African Journal of Biotechnology* 7(3): 217-223.

Keirungi, J. and Fabricious, C. (2005). Selecting medicinal plants for cultivation at Ngabara on the Eastern Cape Wild Coast, South Africa. *South African Journal of Science* 101(11-12): 497-501.

Keller, A.C. Maillard, M.P. Hostettman, K. (1996). Antimicrobial steroids from the fungus *Fomitopsis pinicola. Phytochemistry* 41(4): 1041-1046.

Kelly, T. Yang, W. Chen, C. Reynolds, K. He, J. (2008). Global burden of obesity in 2005 and projections for 2030. *International Journal of Obesity* 32: 1431-37.

Khansari, N. Shakiba, Y. Mahmoudi, M. (2009). Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Patents on Inflammation & Allergy Drug Discovery* 3: 73-80.

Kishore, K. (2013). *In-vitro* and *in-vivo* screening methods for antithrombotic agents. *American Journal of Phytomedicine and Clinical Therapeutics* 1(5): 497-506.

Klafke, J.Z. da Silva, M.A. Rossato, M.F. Trevisan, G. Walker, C.I.B. Leal, C.A.M. et al. (2012). Antiplatelet, antithrombotic, and fibrinolytic activities of *Campomanesia xanthocarpa. Evidence-Based Complementary and Alternative Medicine* 2012, Article ID 954748, 8 pages doi:10.1155/2012/954748.

Ko, H.H. Hung, C.F. Wang, J.P. Lin, C.N. (2007). Anti-inflammatory triterpenoids and steroids from *Ganoderma luciduma* and *G. Tsugae. Phytochemistry* (2007): doi:10.1016/j.phytochem.2007.06.008.

Kobayashi, M. Ikegami, H. Fujisawa, T. Nojima, K. Kawabata, Y. Noso, S. et al. (2007). Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin. *Diabetes* 56(1): 239-47.

Koti, B.C. Gore, A. Thippeswamy, A.H.M. Swamy, A.H.M.V. Kulkami, R. (2011).). Alcoholic leaf extract of *Plectranthus amboinicus* regulates carbohydrate metabolism in alloxan-induced diabetic rats. *Indian Journal of Pharmacology* 43(3): 286-290.

Krishnapuram, R. Kirk-Ballard, H. Dhurandhar, E.J. Dubuisson, O. Messier, V. Rabasa-Lhoret, R. Hegde, V. Aggarwal, S. N.V. Dhurandhar, N.V. (2013). Insulin receptor-independent glucose uptake. *International Journal of Obesity* 37: 146-153.

Kumar, A.P and Sivashanmugam, T. (2012). Cholesterol esterase enzyme inhibitory and antioxidant activities of barks of *Terminalia arjuna roxb.* using *in vitro* models. *International Journal of Pharmaceutical and Allied Sciences Archive* 1(1): 09-16.

Lee, E.M. Lee, S.S. Chung, B.Y. Cho, J. Lee, I.C. Ahn, S.R. Jang, S.J. Kim, T.H. (2010). Pancreatic lipase inhibition by *c*-glycosidic flavones isolated from *Eremochloa ophiuroides. Molecules* 15: 8251-8259.

Lee, I. Kim, H. Youn, U. Kim, J. Min, B. Jung, H. Na, M. Hattori, M. Bae, K. (2010). Effect of lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* on adipocyte differentiation in 3T3-L1 cells. *Planta Medica* 76:1558-63.

Lee, J.J. Jin, Y.R. Yu, J.Y. Munkhtsetseg, T. Park, ES. Lim, Y. Kim, T.J. Pyo, M.Y. Hong, J.T. Yoo, H.S. Kim, Y. Yun, Y.P. (2009). Antithrombotic and antiplatelet activities of fenofibrate, a lipid-lowering drug. *Atherosclerosis* 206: 375-382.

Lee, M.S. Hwang, J.T. Kim, S.H. Yoon, S. Kim, M.S. Yang, H.J. Kwon, D.Y. (2010). Ginsenoside Rc, an active component of Panax ginseng, stimulates glucose uptake in C2C12 myotubes through an AMPK-dependent mechanism. *Journal of Ethnopharmacology* 127: 771-776.

Lefebvre, P. Cariou, B. Lien, F. Kuipers, F. Staels, B. (2009). Role of bile acids and bile acid receptors in metabolic regulation. *Physiological Reviews* 89: 147-191.

Lendel, C. Bertoncini, C.W. Cremades, N. Christopher, A. Waudby, C.A. Vendruscolo, M. Dobson, C.M. Schenk, D. Christodoulou, J. Toth, G. (2009). On the mechanism of nonspecific inhibitors of protein aggregation: Dissecting the interactions of R-synuclein with Congo red and Lacmoid. *Boichem*istry 48: 8322-8334.

Little, T. Horwitiz, M. Feinle-Bisset, C. (2007). Modulation by high-fat diets of gastrointestinal function and hormone associated with the regulation of energy intake: implications for the pathophysiology of obesity. *American Journal of Clinical Nutrition* 86: 531-541.

Liu, L. Sun, H. Wang, X. Mu, D. Liao, H. Zhang, L. (2007). Effects of oleanolic acid and maslinic acid on hyperlipidemia. *Drug Development Research* 68: 261-266.

Liu, Q. Ahn, J.H. Kim, S.B. Hwang, B.Y. Lee, M.K. (2012). New phenolic compounds with anti-adipogenic activity from the aerial parts of *Pulsatilla koreana*. *Planta Medica* 78: 1783-1786.

Lu, Y. Zhong, C.X. Wang, L. Lu, C. Li, X.L. Wang, P.J. (2009). Anti-inflammatory activity and chemical composition of flower essential oil from *Hedychium coronarium*. *African Journal of Biotechnology* 8(20): 5373-5377.

Luo, G.R. and Le, W.D. (2010). Collective roles of molecular chaperones in protein degradation pathways associated with neurodegenerative diseases. *Current Pharmaceutical Biotechnology* 11(2): 180-187.

Mahesh, B. and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World Journal of Agricultural Sciences* 4(S): 839-843.

Malebo, H.M. Wenzler, T. Cal, M. Swaleh, S.M. Hassanali, A. Machocho, A.K. et al. (2013). Anti-protozoal and structure-activity relationships of chemical constituents of *Acridocarpus chloropterus oliver* (Malpighiaceae) from Tanzania. *International Journal of Natural Products Research* 3(4): 74-81.

Martins, A. Vasas, A. Viveiros, M. Molnár, J. Hohmann, J. Amaral, L. (2011). Antibacterial properties of compounds isolated from *Carpobrotus edulis*. *International Journal of Antimicrobial Agents* 37: 438-444.

Miyata, Y. Lin, X. Lee, H. Jinwal, U.K. Srinivasan, S.R. Seguin, S.P. Young, Z.T. Brodsky, J.L. Dickey, C.A. Sun, D. Gestwicki, J.E. (2013). Synthesis and initial evaluation of YM-08, a blood-brain barrier permeable derivative of the heat shock protein 70 (Hsp70) inhibitor MKT-077, which reduces tau levels. *ACS Chemical Neuroscience 4*(6): 930-939.

Mosa, R.A. Lazarus, G.G. Gwala, P.E. Oyedeji, A.O. Opoku, A.R. (2011). *In vitro* antiplatelet aggregation, antioxidant and cytotoxic activity of extracts of some Zulu medicinal plants. *Journal of Natural Prod*ucts 4: 136-146.

Mosa, R.A. Oyedeji, O.A. Shode, F.O. Singh, M. Opoku, A.R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit antiplatelet aggregation activity. *African Journal of Pharmacy and Pharmacology* 5: 2698-2714.

Motlhanka, D. Houghton, P. Miljkovic-Brake, A. Habtemariam, S. (2010). A novel pentacyclic triterpene glycoside from a resin of *Commiphora glandulosa* from Botswana. *African Journal of Pharmacy and Pharmacology* 4(8): 549-554.

Mu, H. Høy, C.E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research* 43: 105-133.

Muffler, K. Leipold, D. Schellera, M. Haas, C. Steingroewer, J. Bley, T. Neuhaus, H.E. Mirata, M.A. Schrader, J. Ulber, R. (2011). Biotransformation of triterpenes. *Process Biochemistry* 46: 1-15.

Nagai, Y. Fujjkake, N. Popeil, H.A. Wada, K. (2010). Induction of molecular chaperones as a therapeutic strategy for the polyglutamine diseases. *Current Pharmaceutical Biotechnology* 11(2): 188-197.

Nagar, A. Shukla, A.K. Bigoniya, P. (2011). Anti-inflammatory potential of *Spinacia oleracea* leaf extract. *Journal of Natural Pharmaceuticals* 2: 80-87.

Nankar, R.P. and Doble, M. (2013). Non-peptidyl insulin mimetics as a potential antidiabetic agent. *Drug Discovery Today* 18: 15-16.

Nayak, B.S. Ramdath, D.D. Marshall, J.R. Isitor, G.N. Eversley, M. Xue, S. Shi, J. (2010). Wound healing activity of the skin of the common grape (*Vitis vinifera*) Variant, *Cabernet Sauvignon*. *Phytotherapy Research* 24(8):1151-1157.

Nielsen, T.R.H. Kuete, V. Jäger, A.K. Meyer, J.J.M. Lall, N. (2012). Antimicrobial activity of selected South African medicinal plants. *BMC Complementary and Alternative Medicine* 2012, 12:74, http://www.biomedcentral.com/1472-6882/12/74.

Ntuli, S.S.B.N. (2006). Ethnopharmacology and phytochemistry of some selected medicinal plants in KwaZulu Natal. MSc Theses, University of KwaZulu-Natal.

obesity: sibutramine and orlistat. *Mini Reviews in Medicinal Chemistry* 7: 3-10.

Olorunnisola, O.S. Bradley. Afolayan, A.J. (2012). Effect of methanolic extract of *Tulbaghia violacea* rhizomes on antioxidant enzymes and lipid profile in normal rats. *African Journal of Pharmacy and Pharmacology* 6(14): 1026-1030.

Ong, K.W. Hsu, A. Tan, B.K.H. (2012). Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK Activation: A contributor to the beneficial effects of coffee on diabetes. *PLoS ONE* 7(3): e32718. doi:10.1371/journal.pone.0032718.

Ortiz-Andrade, R.R, García-Jiménez, S. Castillo-España, P. Ramírez-Avilla, G. Villalobos-Milona, R. Estrada-Soto, S. (2007). Alpha glucosidase inhibitory activity of the methanolic extract from *Tournefortia hartwegiana*: an anti-hyperglycemic agent. *Journal of Ethnopharmacology* 109(1):48-53.

Panchal, S.K. Wong, W. Kauter, K. Ward, L.C. Brown, L. (2012). Caffeine attenuates metabolic syndrome in diet-induced obese rats. *Nutrition* 28: 1055-1062.

Park, C.E. Kim, M. Lee, J.H. Min, B. Bae, H. Choe, W. Kim, S. Ha, J. (2007). Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. *Experimental and Molecular Medicine* 39(2): 222-229.

Park, C.H. Chung, B.Y. Lee, S.S. Bai, H.W. Cho, J.Y. Jo, C. Kim, T.H. (2013). Radiolytic transformation of rotenone with potential anti-adipogenic activity. *Bioorganic and Medicinal Chemistry Letters* 23: 1099-1103.

Parmar, S.K. Sharma, T.P. Airao, V.B. Bhatt, R. Aghara, R. Chavda, S. Rabadiya, S.O. Gangwal, A.P. (2013). Neuropharmacological effects of triterpenoids. *Phytopharmacology* 4(2): 354-372.

Peterlik, M. (2008). Role of bile acid secretion in human colorectal cancer. *Wiener medizinische Wochenschrift* 158: 539-541.

Popova, M. Trusheva, B. Gyosheva, M. Tsvetkova, I. Bankov, V. (2009). Antibacterial triterpenes from the threatened wood-decay fungus *Fomitopsis rosea*. *Fitoterapia* 80: 263-266.

Porfire, A.S. Pârvu, A.E. Daicoviciu, D. Leucuta, S.E. (2009). Evaluation of anti-inflammatory activity of loposome encapsulated superoxide dismutase in rats. *Farmacia* 57: 412-423.

Porth, C.M. (2005). *Pathophysiology: Concept of altered health states*, 7th edition. Philadelphia: Lippincott Williams and Wilkins, pg 392-394.

Poudyal, H. Panchal, S.K. Ward, L.C. Brown, L. (2013). Effects of ALA, EPA and DHA in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. *Journal of Nutrition* 24(6): 1041-1052.

Prathapan, A. Krishna, M.S. Lekshmi, P.C. Raghu, K.G. Menon, A.N. (2012). Modulation of adipogenesis and glucose uptake by *Curcuma longa* extract in 3T3L1 and L6 cell lines - An *in vitro* study. *Asian Pacific Journal of Tropical Disease* 2012: S163-S165.

Rajadurai, M. Vidhya, V.G. Ramya, M. Bhaskar, A. (2009). Ethno-medicinal plants used by the Traditional Healers of Pachamalai hills, Tamilnadu, India. *Ethno-medicine* 3(1): 39-41.

Ramachandran, S. Rajasekaran, A. Manisenthilkumar, K.T. (2012). Investigation of hypoglycemic, hypolipidemic and antioxidant activities of aqueous extract of *Terminalia paniculata* bark in diabetic rats. *Asian Pacific Journal of Tropical Biomedicine* 2(4): 262-268.

Ramakrishnan, G. Allan, J.J. Goudar, K. Amit, A. (2011). Comparative evaluation of anti-inflammatory activity of different extracts of *Boswellia serrata* in wistar albino rats. *International Journal of PharmTech Research* 3: 261-267.

Ramalhete, C. Spengler, G. Martins, A. Martins, M. Viveiros, M. Mulhovo, S. Ferreira M.U. Amaral, L. (2011). Inhibition of efflux pumps in methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* resistant strains by triterpenoids from *Momordica balsamina*. *International Journal of Antimicrobial Agents* 37: 70-74.

Ramos, A.A. Pereira-Wilson, C. Collins, A.R. (2010). Protective effects of ursolic acid and luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells. *Mutation Research* 692: 6-11.

Riaz, S. Faisal, M. Hasnain, S. (2011). Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species in Pakistan. *African Journal of Biotechnology* 10: 6325-6331.

Roberts, D.L. Dive, C. Renehan, A.G. (2010). Biological mechanisms linking obesity and cancer risk: new perspective. *Annual Reviews of Medicine* 61: 301-316.

Rojas, R. Bustamante, B. Bauer, J. Fernandez, I. Alban, J. Lock, O. (2003). Antimicrobial activity of selected Peruvian medicinal plants. *Journal of Ethnopharmacology* 88: 199-204.

Rojas, R. Caviedes, L. Aponte, J.C. Vaisberg, A.J. Lewis, W.H. Lamas, G. Sarasara, C. Gilman, R.H. Hammond, G.B. (2006). Aegicerin, the first oleanane triterpene with wide-ranging antimycobacterial activity, isolated from *Clavija procera. Natural Products Research* 69(5): 845-846.

Roschek, B. Fink, R.C. Li, D. McMichael, M. Tower, C.M. Smith, R.D. Alberte, R.S (2009). Pro-Inflammatory enzymes, cyclooxygenase 1, cyclooxygenase 2, and 5-lipooxygenase, inhibited by stabilized rice bran extracts. *Journal of Medicinal Food* 12(3):615-623.

Rote, N.S. Inflammation, In: McCance, K.L. Heuther, S.E. (Eds). (1994). *Pathophysiology: The biological basis for disease in adults and children*, 2nd edition. St. Luois: Mosby, pp 234-235.

Sajjad, M.U. Samson, B. Wyttenbanch, A. (2010). Heat shock proteins: therapeutic drug targets for chronic neurodegeneration? *Current Pharmaceutical Biotechnology* 11(2): 198-215.

Saleem, M. Nazir, M. Ali, M.S. Hussain, H. Lee, Y.S. Raiz, N. Jabbar, A. (2010). Antimicrobial natural products: an update on future antibiotic drug candidates. *Natural Products Reports* 27: 238-254.

Salei, P. Asghari, B. Esmaeli, M.A. Dehghan, H. Ghazi, I. (2013). α-glucosidase and α-amylase inhibitory effect and antioxidant activity of ten plant extracts traditionally used in Iran for diabetes. *Journal of Medicinal Plant Research* 7(6): 257-266.

Sankaranarayanan, S. Bama, P. Ramachandran, J. Jayasimman, R. Kalaichelvan, P.T. Deccaraman, M. Vijayalakshimi, M. Visveswaran, M. Chitibabu, C.V. (2010). *In vitro* platelet aggregation inhibitory effect of triterpenoid compound from the leaf of *Elephantopus scaber* Linn. *International Journal of Pharmacy and Pharmaceutical Sciences* 2(2): 49-51.

Santos, F.A. Frota, J.T. Arruda, B.R. de Melo, T.S. da Silva, A.A. Brito, G.A. Chaves, M.H. Rao, V.S. (2012). Antihyperglycemic and hypolipidemic effects of α, β-amyrin, a triterpenoid mixture from *Protium heptaphyllum* in mice. *Lipids in Health and Disease* 11:98. doi: 10.1186/1476-511X-11-98.

Sawadogo, W.R. Boly, R. Lompo, M. Some, N. (2006). Anti-inflammatory, analgesic and antipyretic activities of *Dicliptera verticillata*. *International Journal of Pharmacology* 2(4): 435-438.

Schmitt, E. Gehrmann, M. Brunet, M. Multhoff, G. Garrido, C. (2007). Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *Journal of Leukocyte Biology* 81: 15-27.

Seibert, K. Zhang, Y. Leahy, K. Hauser, S. Masferrer, J. Perkins, W. Lee, L. Isakson, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase2 in inflammation and pain. *Proceedings of the National Academy of Sciences* (U.S.A*)* 91: 12013-12017.

Shalaby, M.A. and Hamouda, A.B. (2013). Antiobesity, antioxidant and antidiabetic activities of red Ginseng plant extract in obese diabetic rats. *Journal of Intercultural Ethnopharmacology* 2(3):165-172.

Sharma, K.R. (2012). Review on bile acid analysis. *International Journal of Pharmacy and Biomedical Science* 3(2): 28-34.

Shaw, J.E. Sicree, R.A. Zimmet, P.Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research Clinical Practice* 87: 4-14.

Shibata, N. and Kobayashi, M. (2008). The role for oxidative stress in neurodegenerative diseases. *Brain Nerve* 60(2):157-70.

Shoelson, S.E. Herrero, L. Naaz, A. (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology* 132: 2169-2180.

Shonhai, A. Botha, M. de Beer, T.A.P. Boshoff, A. Blatch, G.L. (2008). Structure- function study of a *Plasmodium falciparum* Hsp 70 using three dimensional modeling and *in vitro* analyses. *Protein Peptide Letters* 15:1117-1125.

Sikka, P. and Bindra, V.K. (2010). Newer antithrombotic drugs. *Indian Journal of Critical Care Medicine* 14(4):188-195.

Simelane, M.B.C. Shonhai, A. Shode, F.O. Smith, P. Singh, M. Opoku, A.R. (2013). Anti-plasmodial activity of some Zulu medicinal plants and of some triterpenes isolated from them. *Molecules* 18: 12313-12323.

Singh, S. Panchaksharimath, P. Devaru, S. (2011). Evaluation of anti-inflammatory activities of *Sida cordifolia* Linn in albino rats. *Journal of Chemical and Pharmaceutical Research* 3(6):136-142.

Smina, T.P. De, S. Devasagayam, T.P. Adhikari, S. Janardhanan, K.K. (2011). *Ganoderma lucidum* total triterpenes prevent radiation-induced DNA damage and apoptosis in splenic lymphocytes *in vitro*. *Mutation Research* 726: 188-94.

Soyingbe, O.S. Oyedeji, A. Basson, A.K. Opoku, A.R. (2013). The essential oil of *Eucalyptus grandis* W. Hill ex Maiden inhibits microbial growth by inducing membrane damage. *Chinese Medicine* 4: 7-14.

Staels, B. Vu-Dac, N. Kosykh, V.A. Saladin, R. Fruchart, J.C. Dallongeville, J. Auwerx, J. (1995). Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase. A potential mechanism for the hypolipidemic action of fibrates. *Journal of Clinical Investigation* 95(2): 705-712.

Steffel, J. and Hindricks, G. (2012). A changing landscape of anticoagulation. *European Heart Journal* 33(22): 2766-2768.

Stiti, N. and Hartmann, M. (2012). Nonsterol triterpenoids as major constituents of *Olea europaea*. *Journal of Lipids*, Article ID 476595, doi:10.1155/2012/476595.

Stolar, M.W. Hoogwerf, B.J. Gorshow, S.M. Boyle, P.J. Wales, D.O. (2008). Managing type 2 diabetes: going beyond glycemic control. *Managed Care Pharmacy* 14: s2-s19.

Storey, R.F. Husted, S. Harrington, R.A. Heptinstall, S. Wilcox, R.G. Peters, G. et al. (2007). Inhibition of platelet aggregation by AZD6140, a reversible oral P2Y12 receptor antagonist, compared with clopidogrel in patients with acute coronary syndromes. *Journal of the American College of Cardiology* 50:1852-1856.

Styrellius, I. Thore, A. Bjorkhem, I. (1985). Luminometric method. In: Methods of enzymatic analysis. (Ed. III). Bergmeyer, Hans Ulirch [Hrsg]. 8: 274-281.

Sudhahar, V. Kumar, S.A. Sudharsan, P.T. Varalakshmi, P. (2007). Protective effect of lupeol and its ester on cardiac abnormalities in experimental hypercholesterolemia. *Vascular Pharmacology* 46(6): 412-418.

Suleiman, M.M. McGaw, L.J. Naidoo, V. Eloff, J.N. (2010). Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. *African Journal of Traditional Complementary and Alternative Medicine* 7(1): 64-68.

Szkudelski, T. (2012). Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. *Experimental Biology and Medicine* 237(5): 481-490.

Tajbakhsh, S. Ilkhani, M. Rustaiyan, A. Larijan, K. Sartavi, K. Tahmasebi, R. Asayesh, G. (2011). Antibacterial effect of the brown alga *Cystoseiratrinodis. Journal of Medicinal Plants Research* 5: 4654-4657.

Tsan, M. and Gao, B. (2009). Heat shock proteins and immune system. *Journal of Leukocyte Biology* 85: 905-910.

Tucci, S.A. Boyland, E.J. Halford, J.S.G. (2010). The role of lipid and carbohydrate digestive enzyme inhibitors in the management of obesity: a review of current and emerging therapeutic agents. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 3: 125-143.

Tutar, I. and Tutar, Y. (2010). Heat shock proteins; an overview. *Current Pharmaceutical Biotechnology* 11(2): 216-22.

Underwood, J.C.E. (Ed). (2000). *General and systematic pathology,* 3rd edition.London: Churchill Livingstone, pp 202-203.

Van Wyk, B.E. Van Oudtshroorn, B. Gericke, N. (2009). *Medicinal Plants of South Africa*, 2nd edition, 1st impression. Pretoria: Briza, pp 7-8.

Vane, J. Botting, R. (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEBJ* 1: 89-96.

Vane, J.R. (1996). Introduction: mechanism of action of NSAIDS. *British Journal of Rheumatology* 35: I-3.

Vashka, H. (2013). Anti-HIV activity of selected South African medicinal plants. PhD Theses, Durban University of Technology.

Verhamme, P. and Hoylaerts, M.F. (2009). Haemostasis and inflammation: two of kind? *Thrombosis Journal* 7(15): http://www.thrombosisjournal.com/content/7/1/15.

Verma, S. Ojha, S. Raish, M. (2010). Anti-inflammatory activity of *Aconitum heterophyllum*on cotton pellet-induced granuloma in rats. *Journal of Medicinal Plant Research* 4: 1566-1569.

Vinegar, R. Schreiber, W. Hugo, R. (1969). Biphasic development of carrageenan oedema in rats. *Journal of Pharmacology and Experimental Therapeutics* 166: 96-103.

Voss, K. Combs, B. Patterson, K.R. Binder, LI. Gamblin, T.C. (2012). Hsp70 alters tau function and aggregation in an isoform specific manner. Biochemistry 51(4): 888-898.

W.H.O (2009). Obesity and overweight. <http://www.who.int/dietphysicalactivity/

Wakefield, D. and Kumar, R.K. (2001). Inflammation: Chronic. Encyclopedia of Life Sciences / & 2001 Nature Publishing Group / www.els.net

Wang, D. Chen, W. Xu, X. (2009). Anti-inflammatory effect of triterpene acids from *poria cocos. Anhui Medical and Pharmaceutical Journal* 2009-9.

Wang, S. Diller, K.R. Aggarwal, S.J. (2003). Kinetics study of endogenous heat shock protein 70 expression. *Journal of Biomechanical Engineering* 125: 794-797.

Watanabe, M. Morimoto, K. Houten, S.M. Kaneko-Iwasaki, N. Sugizaki, T. et al. (2012). Bile acid binding resin improves metabolic control through the induction of energy expenditure. *PLoS ONE* 7(8): e38286. doi:10.1371/journal.pone.0038286.

Weisberg, S.P. McCann, D. Desai, M. Rosenbaum, M. Leibel, R.L. et al. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigations* 112: 1796-1808.

Wright, D.C. Hucker, K.A. Holloszy, J.O. Han, D.H. (2004). Ca^{2+} and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* 53(2): 330-335.

Xiang, Y.Z. Kang, L.Y. Gao, X.M. Shang, H.C. Zhang, J.H. Zhang, B.L. (2008). Strategies for antiplatelet targets and agents (Review). *Thrombosis Research* 123: 35-49.

Xie, W. Wang, W. Su, H. Xing, D. Cai, G. Du, L. (2007). Hypolipidemic mechanisms of *Ananas comosus* L. leaves in mice: Different from fibrates but similar to statins. *Journal of Pharmacological Sciences* 103: 267-274.

Yadav, V.R. Prasad, S. Sung, B. Kannappan, R. Aggarwal, B.B. (2010). Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer . *Toxins 2*: 2428-2466.

Yang, C. An, Q. Xiong, Z. Song, Y. Yu, K. Li, F. (2009). Triterpenes from *Acanthopanax sessiliflorus* fruits and their antiplatelet aggregation activities. *Planta Medica* 75(6): 656-659.

Yang, M.H. Avula, B. Smillie, T. Khan, I. Khan, S.I. (2013). Screening of medicinal plants for PPARα and PPARγ activation and evaluation of their effects on glucose uptake and 3T3-L1 adipogenesis. *Planta Medica* 79: 1084-1095.

Yun JW (2010). Possible anti-obesity therapeutics from nature – A review. *Phytochemistry* 71: 1625-1641.

Zakaria, Z.A. Sufian, A.S. Ramasamy, K. Ahmat, N. Sulaiman, M.R. Arifah, A.K. Zuraini, A. Somchit, M.N. (2009). *In vitro* antimicrobial activity of *Muntingia calabura* extracts and fractions. *African Journal of Microbiological Research* 4: 304-308.

Zandi, K. Teoh, B. Sam, S. Wong, P. Mustafa, M.R. AbuBakar, S. (2011). Antiviral activity of four types of bioflavonoid against dengue virus type-2. *Virology Journal* 8:560, http://www.virologyj.com/content/8/1/560.

Zeng, X. Zhou, X. Xu, J. Chan, S.M.H. Xue, C.L. Molero, J.C. Ye, J.M. (2012). Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds. *Biochemistry and Pharmacology* 84: 830-837.

Zhang, S. Xu, Y. Jia, Y. Xu, L. Yin, L. Peng, J. (2013). Antithrombotic effects of the flavonoids from Rosa laevigata Michx fruit assessed by *in-vivo* models of thrombosis. *Phytopharmacology* 4(3): 481-491.

Zhang, X. Wang, Z. Li, J. Gu, D. Li, S. et al. (2013). Increased 4-hydroxynonenal formation contributes to obesity-related lipolytic activation in adipocytes. *PLoS ONE* 8(8): e70663. doi:10.1371/journal.pone.0070663.

Zhang, X. Wu, C. Wu, H. Sheng, L. Su, Y. et al. (2013). Anti-hyperlipidemic effects and potential mechanisms of action of the caffeoylquinic acid-rich *Pandanus tectorius* fruit extract in hamsters fed a high fat-diet. *PLoS ONE* 8(4): e61922. doi:10.1371/journal.pone.0061922.

Zhou, G. Myers, R. Li, Y. et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action," *Journal of Clinical Investigation* 108(8): 1167-1174.

Zhou, L. Zhang, Y. Gapter, L.A. Ling, H. Agarwal, R. Ng, K.Y. (2008). Cytotoxic and anti-oxidant activities of lanostane-type triterpenes isolated from *Poria cocos. Chemical and Pharmaceutical Bulletin* 56(10): 1459-1462.

CHAPTER 3

3.0 MATERIALS, METHODS, RESULTS AND DISCUSSION

The chapter consists of three parts: A, B and C.

Part A gives an account of the extraction, isolation, purification and identification of the pure compounds (triterpenes) from stem bark of *Protorhus longifolia*.

Part B describes the bioactivities (anti-hyperlipidemic, anti-hyperglycaemic, anticoagulant, anti-inflammatory and anti-protein aggregation activity) of the isolated triterpenes, and

Part C describes the antimicrobial and antiplasmodial activity of the isolated triterpenes.

Each section is presented in the general journals format of abstract, introduction, materials and methods, results, and discussion.

The details of the preparation of some reagents and of some methodologies are given in the Appendices A and B, respectively. Additional data is presented in Appendix D.

All the reagents and solvents used in the experiments were of analytical grade. Where animals were used, the guidelines for proper caring and conduct of animal experiments were followed. Approval (UZREC 171110-030 Dept 2012/23; see Appendix C) for use of animals and experimental procedures, was obtained from the University of Zululand Research Animal Ethics Committee.

3.1 Data Analysis

Unless stated otherwise, all experiments were repeated three times. Data generated were entered into Microsoft Excel® and analysed by calculating the means and standard deviation. Outliers were excluded using the Grubb's test. The results are reported as mean ± S.E.M. The statistical differences were determined using one way analysis of variance (ANOVA), followed by Tukey or Dunnett's *post hoc* test (GraphPad Prism version 5.03). The values were considered statistically significant where $p \leq 0.05$.

Unless stated otherwise, all the *in vitro* enzymatic inhibition experiments were repeated at least three times and data presented as mean ± SEM. Absorbance was read with BioTek[®] (ELx 808 UI) plate reader equipped with Gen5 software. Percentage inhibitions were calculated using the formula

$$\% \text{ Inhibition} = [(A_c - A_t)/A] \times 100$$

Where A_c = Absorbance of control sample, A_t = Absorbance of test sample. IC_{50} values were determined using GraphPad Prism version 5.03.

PART 3A: EXTRACTION, ISOLATION AND PURIFICATION, SYNTHESIS

Abstract

Protorhus longifolia (Benrh.) Engl. is commonly used by Zulu traditional healers to treat various diseases. Two triterpenes were isolated from the crude chloroform extract of the stem bark of the plant; their structures were established based on IR, NMR and MS spectroscopic data as 3β-hydroxylanosta-9,24-dien-21-oic acid (RA5) and methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA3). Structural modification of the isolated compounds was performed through acetylation and oxidation to yield two derivatives of each compound. Their structures were also confirmed based on the spectroscopic data analysis as 3β-acetyloxylanosta-9,24-dien-21-oic acid (R51), 3β-oxolanosta-9,24-dien-21-oic acid (R52), methyl-3β-acetyloxylanosta-9,24-dien-21-oate (R31) and methyl-3β-oxylanosta-9,24-dien-21-oate (R32).

3A.1 Introduction

Protorhus longifolia (Benrh.) Engl. of Anacardiaceae family is an evergreen tall tree (up to 15 m in height) indigenous to Southern Africa. The stem bark of the plant is commonly used by traditional healers to treat various ailments including blood-clotting related diseases. The leaf and stem bark extracts of the plant have been reported to possess antimicrobial (Suleiman *et al.*, 2010) and anti-platelet aggregation (Mosa *et al.* (2011a)) activity, respectively. Various triterpenes of the lupane type have been isolated from leaves of *P. longifolia* (Ntuli, 2006) while some lanostane type triterpenes have also been isolated from the stem bark of the plant (Mosa *et al.*, 2011b). Lanosteryl triterpenes have been commonly isolated from mushrooms (Ko *et al.*, 2007; Zhou *et al.*, 2008), thus this was the first time these compounds were isolated from a higher plant. Anti-platelet aggregation activity of the lanosteryl triterpenes from stem bark of *P. longifolia* has been reported (Mosa *et al.*, 2011b). Literature indicates diverse significant biological activities of plant-derived triterpenes (Zhou *et al.*, 2008; Lee *et al.*, 2010). Thus, these compounds have now become new targets for effective drug development against an array of diseases resistant to conventional medicines.

In this study, the extraction and isolation of two lanosteryl triterpenes (and their derivatives) from stem bark of *P. longifolia* have been reported.

3A.2 Materials

3A.2.1 Equipment

Silica gel 60 TLC aluminium sheets, Whatman No. 1 filter paper (Merck, Darmstadt, Germany), grinding mill (IKA)- Polychem supplies, rotary evaporator (Heidolph Instruments, Germany), glass columns (Sigma-Aldrich, St Louis, MO, U.S.A), Stuart SMP 11 melting point apparatus (Shalom Instruments supplies, Durban, R.S.A).

3A.2.2 Reagents/chemicals

Sigma-Aldrich, St Louis, MO, U.S.A: pyridine, acetic anhydride,

Merck, Darmstadt, Germany: chromium (VI) trioxide, sulphuric acid, silica gel 60, acid purified sea sand, methanol, acetone, ethyl acetate, chloroform and hexane.

3A.3 Methods

3A.3.1 Plant collection

Fresh plant material (stem barks) of *Protorhus longifolia* (Benrh.) Engl. was collected from Hlabisa, KwaZulu-Natal, South Africa. The plant (voucher specimen RA01UZ) was confirmed by Mrs. N.R. Ntuli, Department of Botany, University of Zululand. The plant material was cleaned with water, chopped into smaller pieces and air-dried. The dried plant material was ground into 2 mm mesh and stored in a sterile brown bottle at 4°C until required.

3A.3.2 Extraction and isolation

The method of extraction and isolation of the triterpenes from the stem bark of *P. longifolia* were followed as previously described by Mosa *et al.* (2011b). The powdered plant material was first defatted with *n*-hexane and then extracted (1:5 w/v) with chloroform (See Figure 3.1). The chloroform extract (13 g) was subjected to silica gel column chromatography (24 × 700 mm; Silica gel 60; 0.063 - 0.2 mm; 70-230 mesh ASTM), eluted with a gradient of hexane-ethyl acetate solvent system (9:1 to 3:7), collecting 20 ml fractions. Thin layer chromatography (TLC) (silica gel 60 TLC

aluminium sheets 20 cm × 20 cm, F_{254}, hexane-ethyl acetate solvent system 9:1- 7:3) was used to analyse the fractions. The TLC plates were first viewed under ultraviolet (UV) light, developed using a 10% H_2SO_4 spray reagent and then heated at 105-110°C. The collected fractions were combined based on their TLC profile to yield 18 combined fractions (Fr. A-R). The ninth and fourteenth combined fractions were further separately purified in *n*-hexane and ethyl acetate to afford the compounds RA5 (0.72 g) and RA3 (1.15 g), respectively. Stuart SMP 11 melting point apparatus was used to determine melting point of the compounds. Structures were established using infrared (IR) (Perkin-Elmer 100 FTIR), HRMS (in DCM, Waters Synapt G2),1D and 2D nuclear magnetic resonance (NMR) techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in CDCl$_3$ or DMSO-D6, Bruker 600 MHz). Chemical shifts were expressed in δ (ppm). The chemical structures also confirmed by comparing the spectra (See Appendix E) of the isolated compounds with reference compound in literature (Mosa *et al.*, 2011b).
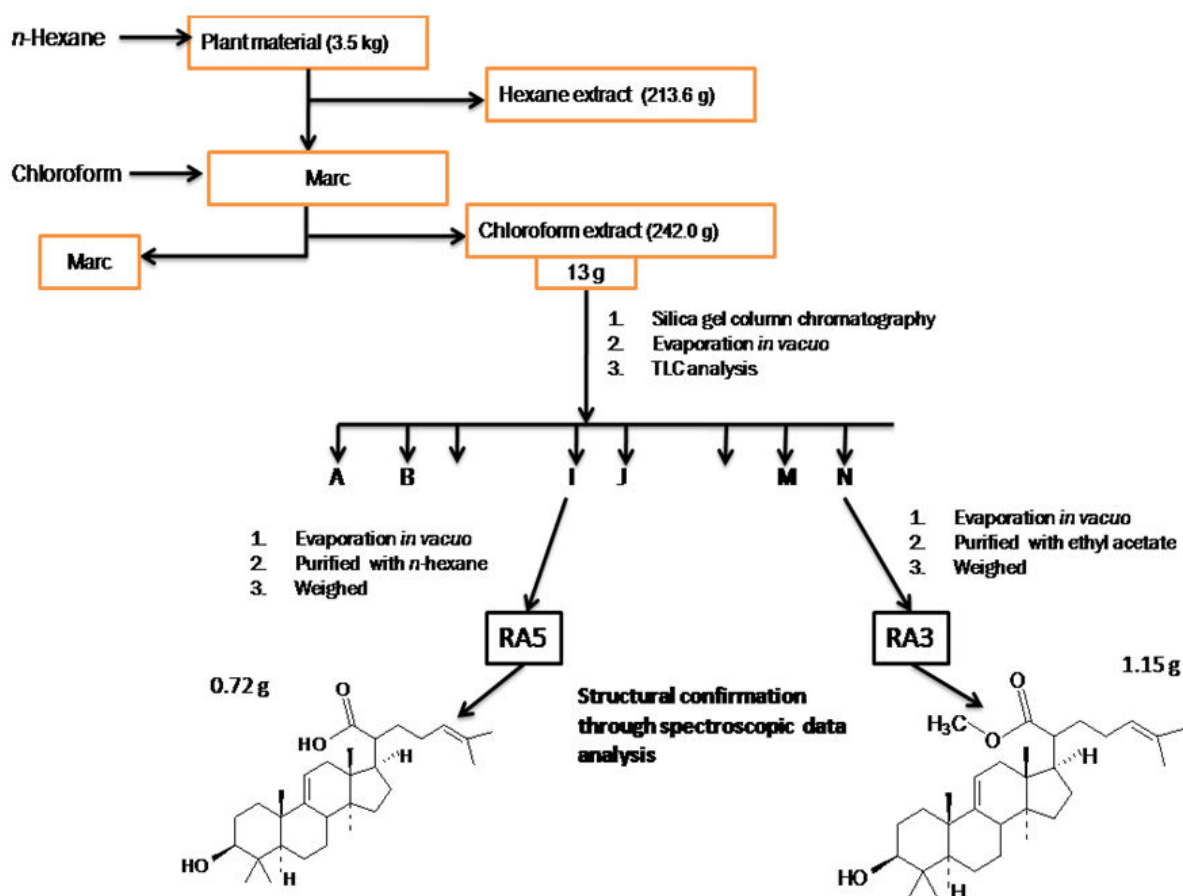
Figure 3.1: Schematic presentation of extraction and isolation of the two triterpenes from *P. longifolia*. Spectroscopic data analysis was used to establish and confirm the structures.

3A.3.3 Derivatisation reactions (synthesis)

3A.3.3.1 Acetylation

The compound (200 mg) was dissolved in a mixture (1:2 w/v) of acetic anhydride (10 ml) and pyridine (20 ml). See Figure 3.2 for the reaction scheme. The reaction mixture was refluxed for 3 h and was transferred into 250 ml of water in a beaker. This was allowed to cool and settle before the solid product was filtered out using a Whatman No.1 filter paper. The obtained solid product was dried and weighed. The acetylation of the compound was confirmed by the TLC and NMR spectral techniques, as above (3A.3.2). See Figure 3.2 for the reaction scheme.
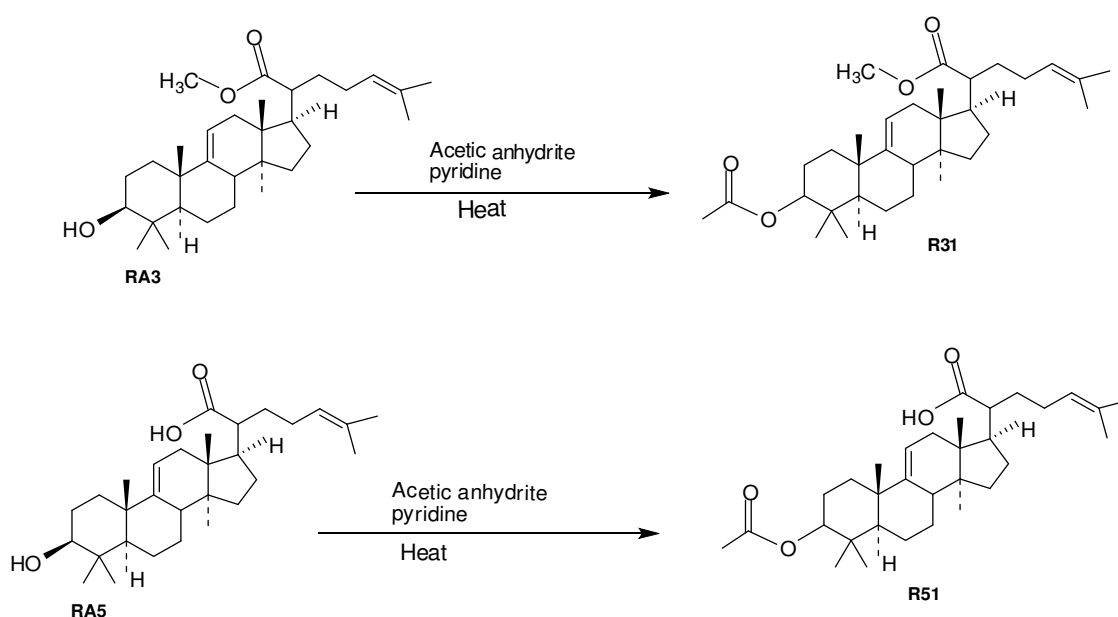
Figure 3.2: Reaction scheme for the acetylation of RA5 and RA3 to give R51 and 31, respectively.

3A.3.3.2 Oxidation (Jone's Oxidation)

The compound (200 mg) was dissolved in a small volume (2-4 ml) of acetone and cooled in ice. Jone's reagent ($Cr(VI)O_3$ in aqueous H_2SO_4, 2 M) was added drop wise to the solution until the colour of the solution (orange) turned green. This was followed by the addition of methanol to the solution (See Figure 3.3). The compound was precipitated by addition of 250 ml of water to the reaction mixture. The solid product was filtered out, dried and weighed. Oxidation of the compound was confirmed by the TLC and NMR spectral techniques (3A.3.2).
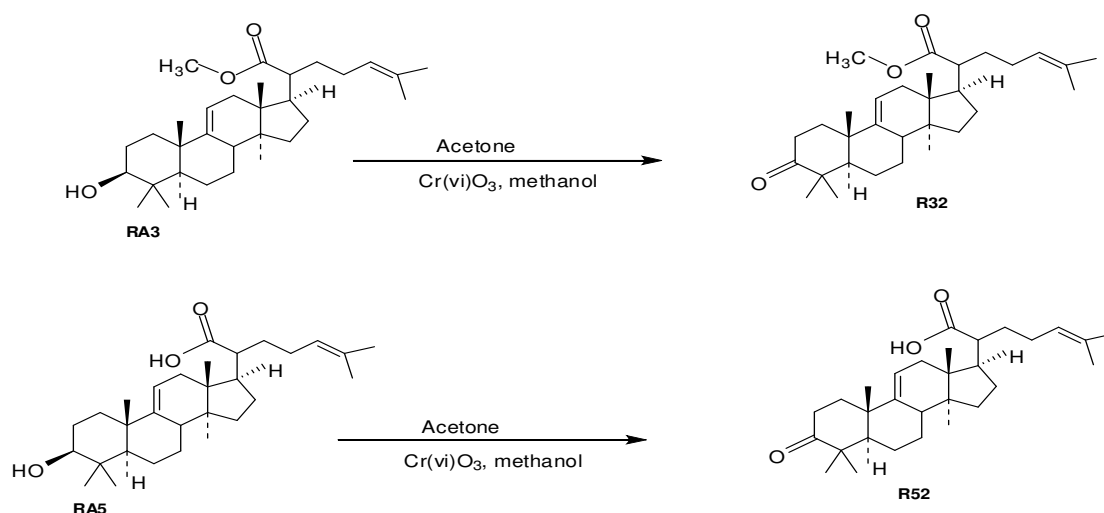
Figure 3.3: Reaction scheme for the oxidation of RA5 and RA3 to give R52 and R32, respectively.

3A.4 Results and Discussion

Two compounds RA3 and RA5 (Figure 3.4a) were obtained from the column chromatography. Structures of the isolated compounds were established and confirmed through spectroscopic data analysis and by comparison with literature values (Keller *et al.*, 1996, Mosa *et al.*, 2011).
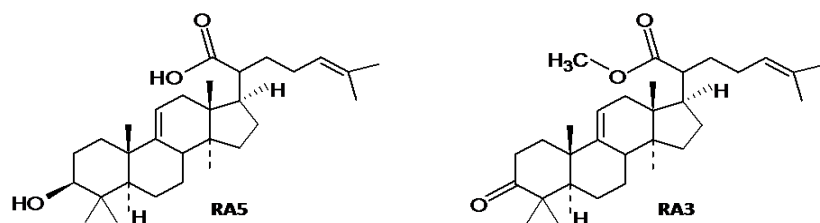
Figure 3.4a: Chemical structures of 3β-hydroxylanosta-9,24-dien-21-oic acid (RA5) and methyl**-**3β-hydroxylanosta-9,24-dien-21-oate (RA3).

Physical and spectral data of 3β-hydroxylanosta-9,24-dien-21-oic acid (**RA5**) has previously been given in Mosa *et al.* (2011b). The compound was obtained as white flakes (paper-like solids), mp 134-136°C, ESI-MS (positive mode) *m/z* % 455 [M+H]$^+$, protonated ion in HRMS at *m/z* 457.375 (calculated for $C_{30}H_{48}O_3$, 457.3682). The IR spectrum showed absorption bands for hydroxyl (3360, 2581cm^{-1}), and carbonyl (1702 cm^{-1}) functional groups which further confirmed the structure.

Methyl-3β-hydroxylanosta-9,24-dien-21-oate (**RA3**) was obtained as white crystals, mp 204-205°C, IR (KBr) v_{max}= 3469, 1683 cm^{-1}. ^{1}H and ^{13}C NMR (see Table 3.2b), the data suggested the molecular formula $C_{31}H_{50}O_3$, calculated 470.736.

The ^{1}H-NMR of the RA3 followed the same triterpenoid pattern with a large clusters of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8 observed in 3β-hydroxylanosta-9,24-dien-21-oic acid Mosa *et al.* (2011b). The ^{13}C-NMR of this compound was also similar to that of 3β-hydroxylanosta-9,24-dien-21-oic acid, with the presence of four olefinic carbon atoms between 145-118 ppm, and five quaternary carbon atoms confirming the lanosteryl skeletal structure. The presence of an ester carbon atom (δ 177.3 ppm) instead of a carboxylic carbon (δ 181.5 ppm) assisted in suggesting the methyl ester of 3β-hydroxylanosta-9,24-dien-21-oic acid. The absorption bands for hydroxyl (3469 cm^{-1}), and carbonyl (1683 cm^{-1}) functional groups observed on IR spectrum also further assisted in confirming the NMR structure.

In order to partially study structure-activity relationships, the structures of the isolated compounds were modified through acetylation and oxidation to afford two derivatives per compound (Figure 3.4b). Their structures were also confirmed through spectroscopic data analysis and comparison with the parent compounds. Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR is given in Table 3.1-3.2. See Appendix E for spectra of the compounds.
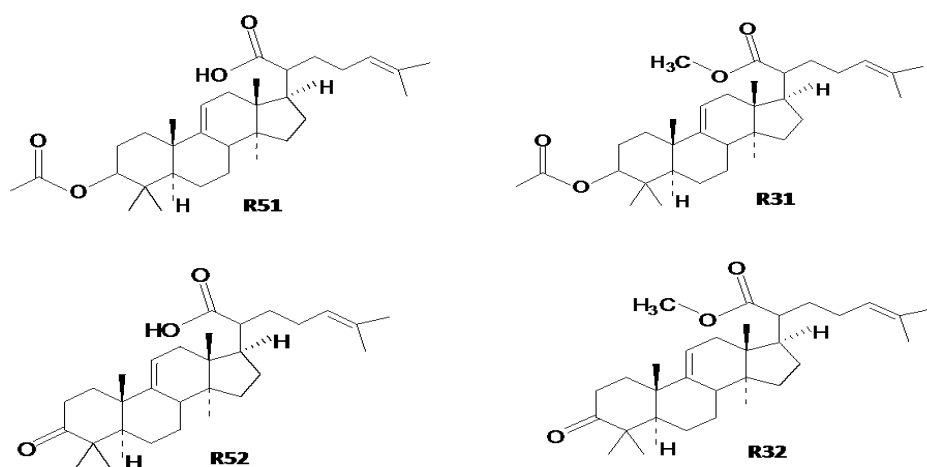
Figure 3.4b: Chemical structures of 3β-acetyloxylanosta-9,24-dien-21-oic acid (R51), 3β-oxolanosta-9,24-dien-21-oic acid (R52), methyl-3β-acetyloxylanosta-9,24-dien-21-oate (R31) and methyl-3β-oxylanosta-9,24-dien-21-oate (R32).

3β-acetyloxylanosta-9,24-dien-21-oic acid (**R51**): white powder (108 mg), mp 117-119°C, IR (KBr) v_{max}= 2935, 1715 cm^{-1}, calculated for $C_{32}H_{51}O_4$, 499.376.

3β-Oxolanosta-9,24-dien-21-oic acid (**R52**): white powder (150 mg) mp 158-162°C, IR (KBr) v_{max}= 2967, 1704 cm^{-1}, molecular formular $C_{30}H_{48}O_3$, 458.368.

Methyl-3β-acetyloxylanosta-9,24-dien-21-oate (**R31**): white powder (110 mg), mp 166-168°C, IR (KBr) v_{max}= 1736 cm^{-1}, molecular formula $C_{33}H_{52}O_4$, calculated 512.746.

Methyl-3β-oxylanosta-9,24-dien-21-oate (**R32**), white powder (123 mg), mp 181-183°C, IR (KBr) v_{max}= 1701 cm^{-1}, molecular formula $C_{31}H_{50}O_3$, calculated 469.735.

The ^{13}C-NMR and ^{1}H-NMR spectra of the derivatives of RA5 (R51, R52) and RA3 (R31, R32) exhibited closely related signals to those of their parent compounds. The differences were: the presence of carbonyl signals 170.8 ppm (R51) and 170.8 ppm (R31) was attributed to the acetyl group linked to C-3 in the structures of the compounds and this led to the compounds being assigned as acetyl derivatives of RA5 and RA3. The presence of carbonyl ketone at 216.9 ppm (R52) and 217.0 ppm (R32) confirmed oxidation of the compounds and were assigned as 3β-oxolanosta-9,24-dien-21-oic acid (R52) and methyl-3β-oxylanosta-9,24-dien-21-oate (R32), respectively.

Table 3.1: ^{13}C-NMR data of the compounds, chemical shifts are expressed in δ (ppm)

Position	Type	RA5 [a]	R51	R52	RA3	R31	R32
1	CH_2	30.3	32.0	30.0	30.4	29.9	30.0
2	CH_2	23.9	23.8	24.4	23.9	23.8	24.3
3	CH	77.2	78.3	216.9	74.3	78.3	217.0
4	C	37.3	37.1	38.6	37.4	36.6	38.6
5	CH	44.5	45.6	47.9	44.4	45.5	35.0
6	CH_2	17.7	17.6	17.8	17.3	20.9	17.8
7	CH_2	26.0	26.0	26.0	25.8	27.2	26.0
8	CH	49.7	49.8	49.8	49.6	49.8	49.7
9	C	145.7	145.6	145.5	145.9	145.6	145.2
10	C	34.9	34.7	34.9	34.7	34.7	34.8
11	CH	118.2	118.2	118.2	118.3	118.2	118.4
12	CH_2	29.3	30.0	35.5	28.6	29.4	30.0
13	C	43.3	43.3	43.3	43.3	43.3	43.4
14	C	51.0	51.1	51.0	51.0	51.0	50.9
15	CH_2	31.2	33.4	33.5	31.3	32.0	30.7
16	CH_2	27.3	27.2	27.2	27.0	27.2	27.4
17	CH	47.2	47.4	47.2	47.7	47.2	47.9
18	CH_3	16.5	12.9	12.7	13.3	12.8	12.7
19	CH_3	18.5	21.4	21.6	21.7	21.4	21.7
20	CH	48.2	48.3	48.2	48.4	48.3	48.2
21	C	181.5	181.9	181.4	177.3	178.5	177.7
22	CH_2	32.4	32.3	32.3	34.7	33.4	33.5
23	CH_2	25.3	27.2	24.5	25.5	23.4	24.5
24	CH	123.6	123.5	123.5	125.6	129.7	124.5
25	C	132.2	132.3	132.3	136.4	130.6	132.3
26	CH_3	17.6	17.6	17.6	17.4	17.6	16.4
27	CH_3	25.7	25.7	25.7	25.8	25.8	25.4
28	CH_3	21.8	21.6	21.7	21.9	21.6	21.5
29	CH_3	27.7	27.4	27.4	27.4	27.4	27.4
30	CH_3	22.7	22.9	21.6	22.1	22.9	24.3
-O$\underline{C}H_3$					59.8	63.1	52.4
-CO$\underline{C}H_3$			21.3			21.3	
-$\underline{C}OCH_3$			170.8			170.8	

[a] – Mosa *et al.* (2011b)

Table 3.2a: ^{1}H-NMR data of the compounds, chemical shifts are expressed in δ (ppm)

Position	RA5	R51	R52
1	1.76 (2H, t)		
2	1.28 (2H, qt)		
3	3.50 (1H, s,OH)	4.67 (1H, s)	
4			
5	1.41 (1H, t)		
6	1.45 (2H, qt)		
7	1.97 (2H, qt)		
8	1.90 (1H, t)		
9			
10			
11	5.20 (1H, t)	5.12 (1H, t)	5.12 (1H, t)
12	2.02 (2H, d)		
13			
14			
15	1.54 (2H, t)		
16	1.60 (2H, qt)		
17	1.50 (1H, s)		
18	1.20 (3H, s)		
19	1.32 (3H, s)		
20	2.28 (1H, qt)		
21			
22	1.45 (2H, qt)		
23	1.93 (2H, t)		
24	5.33 (1H, t)	5.30 (1H, t)	5.35 (1H, t)
25			
26	1.62 (3H, s)	1.60 (3H, s)	1.60 (3H, s)
27	1.65 (3H, s)	1.65 (3H, s)	1.69 (3H, s)
28	1.21 (3H, s)	1.27 (3H, s)	1.29 (3H, s)
29	0.92 (3H, s)	0.92 (3H, s)	0.91 (3H, s)
30	1.02 (3H, s)	1.02 (3H, s)	1.02 (3H, s)
-COC̲H$_3$		2.03 (3H, s)	
-C̲OCH$_3$			

Table 3.2b: ^{1}H-NMR data of the compounds, chemical shifts are expressed in δ (ppm)

Position	RA3	R31	R32
1	1.71 (2H, t)		
2	1.19 (2H, qt)		
3	4.24 (1H,s,OH)	4.64 (1H, s)	
4			
5	1.42 (1H, t)		
6	1.46 (2H, qt)		
7	1.92 (2H,qt)		
8	1.87 (1H, t)		
9			
10			
11	5.13 (1H, t)	5.20 (1H, t)	5.12 (1H, t)
12	1.26 (2H, d)		
13			
14			
15	1.46 (2H, t)		
16	1.69 (2H, qt)		
17	0.83 (1H, t)		
18	0.70 (3H, s)		
19	1.38 (3H, s)		
20	2.09 (1H, qt)		
21			
22	1.43 (2H, qt)		
23	1.21 (2H, t)		
24	5.22 (1H, t)	5.30 (1H, t)	5.33 (1H, t)
25			
26	1.65 (3H, s)	1.63 (3H, s)	1.54 (3H, s)
27	1.67 (3H, s)	1.66 (3H, s)	1.71 (3H, s)
28	1.37 (3H, s)	1.30 (3H, s)	1.36 (3H, s)
29	0.92 (3H, s)	0.95 (3H, s)	0.91 (3H, s)
30	1.04 (3H, s)	1.02 (3H, s)	1.03 (3H, s)
-OC̲H$_3$	3.86 (3H, s)	3.86 (3H, s)	3.79 (3H, s)
-COC̲H$_3$		2.02 (3H, s)	
-C̲OCH$_3$			

The spectroscopic data obtained from this study confirmed isolation of the two lanostane triterpenes (3β-hydroxylanosta-9,24-dien-21-oic acid, **RA5** and methyl-3β-hydroxylanosta-9,24-dien-21-oate, **RA3**) from stem bark of *P. longifolia* and their derivatives.

References

Keller, A.C. Maillard, M.P. Hostettman, K. (1996). Antimicrobial steroids from the fungus *Fomitopsis pinicola*. *Phytochemistry* 41(4): 1041-1046.

Ko, H.H. Hung, C.F. Wang, J.P. Lin, C.N. (2007). Anti-inflammatory triterpenoids and steroids from *Ganoderma luciduma* and *G. Tsugae*. *Phytochemistry* (2007): doi:10.1016/j.phytochem.2007.06.008.

Lee, I. Kim, H. Youn, U. Kim, J. Min, B. Jung, H. Na, M. Hattori, M. Bae, K. (2010). Effect of lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* on adipocyte differentiation in 3T3-L1 cells. *Planta Medica* 76:1558-63.

Mosa, R.A. Lazarus, G.G. Gwala, P.E. Oyedeji, A.O. Opoku, A.R. (2011). *In vitro* antiplatelet aggregation, antioxidant and cytotoxic activity of extracts of some Zulu medicinal plants. *Journal of Natural Prod*ucts 4: 136-146.

Mosa, R.A. Oyedeji, O.A. Shode, F.O. Singh, M. Opoku, A.R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit antiplatelet aggregation activity. *African Journal of Pharmacy and Pharmacology* 5: 2698-2714.

Ntuli, S.S.B.N. (2006). Ethnopharmacology and phytochemistry of some selected medicinal plants in KwaZulu Natal. MSc Thesis, University of KwaZulu-Natal.

Suleiman, M.M. McGaw, L.J. Naidoo, V. Eloff, J.N. (2010). Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. *African Journal of Traditional Complementary and Alternative Medicine* 7(1): 64-68.

Zhou, L. Zhang, Y. Gapter, L.A. Ling, H. Agarwal, R. Ng, K.Y. (2008). Cytotoxic and anti-oxidant activities of lanostane-type triterpenes isolated from *Poria cocos. Chemical and Pharmaceutical Bulletin* 56(10): 1459-1462.

PART 3B1: ANTI-HYPERLIPIDEMIA

Abstract

Hyperlipidemia, a metabolic disorder of lipids, is characterised by abnormally elevated plasma lipids levels. In an effort to discover new and possibly more effective drugs, this study aimed at investigating the *in vitro* anti-hyperlipidemic activity of the two triterpenes (and their derivatives) isolated from stem bark of *Protorhus longifolia*. Inhibitory activity of the triterpenes was evaluated on selected lipid (pancreatic lipase and cholesterol esterase) digestive enzymes and hormone sensitive lipase. Their ability to bind bile acids and inhibit intracellular lipid accumulation in 3T3-L1 adipocytes was also evaluated. The triterpenes effectively inhibited the activities of pancreatic lipase, cholesterol esterase, and hormone-sensitive lipase with IC_{50} values ranging from 60.1 to 677.8 µM. The compounds also showed a moderate bile acid binding ability. A significant reduction (34.8%) of intracellular lipid accumulation was also observed following a 48 h exposure of the 3T3-L1 adipocytes to RA5 at 25 µM. It is apparent that the triterpenoids possess hypolipidemic properties.

3B1.1 Introduction

Hyperlipidemia is a metabolic disorder of lipids that is characterised by abnormally elevated levels of plasma lipids. Hyperlipidemia has become a global concern and its prevalence is currently increasing at an alarming rate worldwide (Zhang *et al*., 2013). Plasma lipid levels are mainly influenced by exogenous lipid absorption, endogenous lipid synthesis and metabolism in the body (Xie *et al*., 2007). Chronic hyperlipidemia is strongly associated with the development of cardiovascular events and metabolic syndrome diseases (Adisakwattana et al., 2010). Thus, regulation of blood lipid levels is crucial in the management of hyperlipidemia (Derosa *et al*., 2006; Karalis *et al*., 2012).

In an effort to manage hyperlipidemia, inhibition of lipid digestive enzymes and limiting intestinal absorption of dietary lipids, as well as inhibition of adipogenesis are currently considered ideal therapeutic approaches to reduce incidences of hyperlipidemia and its related diseases (Ahn *et al*., 2012; Park *et al*., 2013). Despite

efficacy of the currently used hypolipidemic drugs, their adverse effects (Chaput *et al.*, 2007; Díaz and Folgueras, 2011) stimulate the search for new agents with improved efficacy and safety profile. Plants are rich sources of safe bioactive compounds with diverse therapeutic properties including hypolipidemic effects. Plant sterols and their esters have been reported to be effective not only in lowering intestinal cholesterol absorption but also in decreasing plasma levels of LDL. (Sudhahar *et al.*, 2007; Brown *et al.*, 2009). Adisakwattana *et al.* (2010) have reported that grape seed extract reduces dietary intestinal lipid absorption through the inhibition of pancreatic lipase and cholesterol esterase. A compound (2-phenylethyl 2,6-dihydroxybenzoate) isolated from the ethanolic extract of *Geophila herbacea*, exhibited *in vitro* lipid-lowering bioactivity (Luo *et al*, 2011). The 2, 4, 6-trihydroxyacetophenone isolated from *Myrcia multiflora* also showed anti-obesity and mixed hypolipidemic effects with the reduction of lipid intestinal absorption (Ferreira *et al.*, 2011).

In this study the *in vitro* hypolipidemic activity of the two triterpenes isolated from *P. longifolia* and their derivatives is reported.

3B1.2 Materials

3B1.2.1 Equipment

BioTek® plate reader (ELx 808 UI, BioTek Instrument Supplies), BioTek® 189 ELx800 plate reader (BioTek Instruments Inc., Winooski, VT, USA), 96-well microtitre plates, 24-well culture plates- Castor®, homogenizer (IKA® T10 basic), Platform shaker (Labcon)- Polychem supplies
Micropipettes (Eppendorf AG), Eppendorf centrifuge 5804 R- Merck.

3B1.2.2 Reagents/chemicals

Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A, Steinheim, Germany): porcine pancreatic lipase, porcine pancreatic cholesterol esterase, 4-nitrophenyl palmitate, *para*-nitrophenyl butyrate, trizma hydrochloride (Tris-HCl), orlistat, simvastatin, taurocholic acid, cholic acid, glycocholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, Hepes, bovine serum albumin (BSA), sucrose, collagenase,

protease inhibitor, bile acid binding kit, cellulose, cholestarymine, dimethyl sulfoxide (DMSO), dexamethasone, 3-isobutyl-1-methylxanthine (IBMX)

Glucose oxidase assay kit (Sigma, product code GAGO-20)

Bile acid kit (BQ Kits, San Diego, Cat no: BQ 042A-EALD)

Dulbecco's modified eagle medium (DMEM) (Lonza, MD, U.S.A), adipogenic differentiation medium (ADM), foetal calf serum (FCS) (GIBCO/Invitrogen Life Technologies, Paisley, U.K), Oil-Red-O stain, formalin, isopropanol, ethanol, crystal violet stain

3B1.3 Methods

3B1.3.1 *In vitro* study

3B1.3.1.1 Pancreatic lipase inhibition

Inhibitory activity of the triterpenes on pancreatic lipase was evaluated following the method described by Slanc *et al.* (2009). The reaction mixture consisted of 162 µl of Tris-HCl buffer (75 mM, pH 8.4), 12 µl of 5 mg/ml enzyme in Tris-HCl buffer, 16 µl of the triterpene (0-0.5 mg/ml in methanol), and 10 µl of 3,3 mM 4-nitrophenyl palmitate (4-PNP). The mixture was incubated at 37°C for 15 minutes before the addition of the substrate. In the controls, the test compound was replaced with a mixture (1:1) of methanol and water. Orlistat was used as a standard drug. The reaction mixture was further incubated for 10 min. The enzyme activity was determined by measuring the release of 4-nitrophenol at 405 nm. Percentage inhibition was calculated.

3B1.3.1.2 Hormone-sensitive lipase extraction

Hormone sensitive lipase (HSL) was extracted from the rat epididymal adipose tissue of *Sprague-Dawley* male rats as described by Morimoto *et al*. (1999) and Bustanji *et al*. (2010). Briefly, fat cells suspension in Krebs-Ringer bicarbonate buffer supplemented with 4% BSA (KRB-BSA) was diluted (1.0:1.125 ml) with homogenisation buffer (100 ml; 50 mM Tris-HCl, pH 7.0, 250 mM sucrose, and 1 crushed protease inhibitor tablet). HSL was then obtained following a series of centrifugations and diethyl ether (250 µl) was used to dissolve other fat contents. Following centrifugation of the homogenate at 1200 × g, 10 min, the diethyl ether

layer was aspirated and the subsequent supernatant was taken as HSL extract. The HSL extract was stored at -80°C until required.

3B1.3.1.3 Hormone-sensitive lipase inhibition

The inhibitory effect of the triterpenes on HSL activity was evaluated as described for the pancreatic lipase activity above (3B1.3.1.1).

3B1.3.1.4 Cholesterol esterase inhibition

The inhibitory activity of the triterpenes on cholesterol esterase was evaluated following a similar procedure to that described for pancreatic lipase (3B1.3.1.1). The composition of the reaction mixture was 50 µl of 100 mM sodium phosphate buffer (pH 7.0), 50 µl of 5.16 mM taurocholic acid, 50 µl of 100 µg/ml cholesterol esterase in the buffer, 50 µl of the compound (0-1.0 mg/ml in methanol), and 75 µl of 0.2 mM 4-nitrophenol-butyrate (4-NPB) in 6% acetonitrile. In the controls, the test compound was replaced with a mixture of methanol and water (1:1). The mixture was incubated at 25°C for 10 minutes before the addition of the substrate. The reaction mixture was further incubated for 5 min at 25°C. The absorbance was read at 405 nm. Simvastatin was used as a standard inhibitor of the enzyme. Percentage inhibition was calculated.

3B1.3.1.5 *In vitro* bile acid binding assay

In vitro bile acid binding activity of the triterpenes was evaluated following the method described by Matsumoto *et al.* (2011). Different concentrations (0.5-4.0 mM) of each bile acid (cholic acid, chenodeoxycholic acid, taurocholic acid, glycocholic acid, deoxycholic acid, dehyrocholic acid and lithocholic acid) were prepared in phosphate buffered saline (0.1 M, pH 7.4). The bile acid at different concentrations was mixed and incubated with 1% (w/v) of the compound. Cellulose and cholestyramine were used as negative and positive controls, respectively. The mixture was incubated at 37°C for 30 min with intermittent shaking. This was followed by centrifuging at 14000 rpm for 5 min. The supernatant was collected and used to determine concentration of residual bile acid. The concentration of the residual bile acid was determined using a bile acid kit (BQ Kits, San Diego, Catalogue No: BQ 042A-EALD) following manufacturer instructions. Concentration of the residual bile acid (total bile acid- TBA)

and percentage adsorption of the compounds were then calculated using the formulas

$$TBA \ (mM) = \frac{sample \ A_{405}/min}{std \ A_{405}/min} \times 100$$

$$\% \ Inhibition = \frac{(A_{std \ sample} - A_{test \ sample})}{A_{std \ sample}} \times 100$$

3B1.3.1.6 3T3-L1 adipocytes subculture and treatment

The triterpenes were stored desiccated in the dark and at constant room temperature. For the assay, the compounds were dissolved in 100 % DMSO to yield a stock solution of 87 mM and stored in 0.2 ml vials at -80°C. A fresh vial was used for each experiment. For the working concentration, the stock solution was dissolved in 10 % DMSO to yield the concentration of 1mM, which was further diluted in DMEM without phenol red to yield final concentrations of 1, 10, 25 and 100 µM. The final concentration of DMSO present in the working solution was less than 0.1%.

3B1.3.1.6.1 Sub-culturing

3T3-L1 preadipocytes (CL-173, American Type Culture Collection, VA, U.S.A) seeded at 20×10^4 cells per ml into 24-well culture plates were cultured in DMEM growth medium with 10% newborn calf serum at 37°C in humidified air with 5% CO_2 for 3 days. After 3 days, the media was replaced and cells were cultured for a further 2 days until confluent.

3B1.3.1.6.2 Differentiation

The confluent cells were differentiated in media comprising 10% foetal calf serum (FCS), 1 µM dexamethasone, 1 µg/ml insulin and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After 3 days of differentiation, the differentiation media was substituted with adipocyte maintenance medium (DMEM, 10% FCS, 1 µg/ml insulin) and cells were cultured for a further 2 days (day 5 post differentiation). On day five the media was changed to insulin-free DMEM containing 10% FCS and the cells were cultured for a further two days (day 7). On day seven, the fully differentiated pre-adipocytes to 3T3-L1 adipocytes, were treated with different concentrations (1,

10, 25 and 100 µM) of the triterpene, media control (adipogenic differentiation medium- ADM) and the vehicle control (< 0.1% DMSO) for 24 h and 48 h respectively. The 48 h plates were refreshed after the first 24 h. After the treatment, the intracellular lipid content was quantified.

3B1.3.1.6.3 Intracellular lipid content determination

The lipids accumulated in the adipocytes were quantified after staining the 3T3-L1 cells with Oil-Red-O staining. The culture medium was removed; the cells were washed with Dulbecco's phosphate buffered saline (DPBS) and fixed in neutral buffered formalin (10%) for 20 min. This was followed by washing with DPBS and staining with 0.7% (v/v) Oil-Red-O for 30 min. The Oil-Red-O stain was removed and the cells washed with DPBS, after which the Oil-Red-O was extracted from the cells using 100% isopropanol. The absorbance was measured at 510 nm using a BioTek® (189 ELx 800, BioTek Instruments Inc., U.S.A) plate reader equipped with Gen 5® 190 software for data acquisition.

To compensate for cell density, the remaining 3T3-L1 adipocytes in the wells were rinsed with 70% ethanol and stained with 0.01% (v/v) crystal violet for 5 min. Thereafter, the crystal violet stain was removed by aspiration, extracted using 70% ethanol and the absorbance was measured at 570 nm. Lipid content (Oil-Red-O measured at 510 nm) was normalised to cell density (crystal violet measured at 570 nm).

3B1.3.2 Animals

Adult rats (*Sprague-Dawley)* of either sex were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand. The animals were maintained under standard conditions (temperature 23 ± 2°C and 12 h light dark cycle); they had free access to standard pellet feed and enough drinking water.

3B1.3.3 *In vivo* bile acid binding activity

Adult rats (200 ± 20 g) of either sex were divided into three groups of four, housed individually. Normal control group was fed a normal basal diet while the experimental groups were fed the basal diet supplemented with triterpene or cholestyramine at 1%

(w/w). The animals were fed the diet accordingly for 14 consecutive days. This was followed by collection of faeces for 48 h after the 14 days of diet treatment. The method described by Matsumoto *et al.* (2011) was adapted to extract the faecal bile acids. Briefly, the collected faeces were freeze-dried and the faecal bile acids were extracted with 90% ethanol at 65°C for 4 h. Bile acid concentration was analysed using the bile acid kit (BQ Kits, San Diego).

3B1.4 Results

3B1.4.1 Effect on lipid digestive enzymes and HSL

It is apparent from the results in Table 3.3 that the compounds exhibited hypolipidemic properties as they strongly inhibited the activities of the enzymes with the IC_{50} values ranging from 60.1 to 677.8 µM (Table 3.3). Except RA3, all the compounds showed to varying degree more activity on HSL, a lipolytic enzyme. Apparently the structural modifications markedly decreased the activities of RA3 and RA5 on pancreatic lipase and cholesterol esterase, respectively.

Table 3.3: Inhibitory activity (IC_{50} µM) of the triterpenes on some lipid digestive enzymes

Sample	Pancreatic lipase	Hormone sensitive lipase	Cholesterol esterase
RA3	361.1	509.8[*]	233.7
R31	>1950.3[*]	195.0	370.6
R32	425.8[*]	149.0	255.5
RA5	328.0	87.5	677.8
R51	180.2	60.1	Not detected
R52	65.4	109.1	>2181.7[*]
Orlistat	20.2	20.2	-
Simvastatin	-	-	382.3

Data are expressed as mean ± SEM. * p < 0.05 vs positive control

3B1.4.2 Bile acid binding ability

The *in vitro* bile acid binding ability of the triterpenes from was determined on various primary and secondary bile acids. The results are presented in Figure 3.5 as percentage adsorption of the residual bile acid. Both isolated triterpenes exhibited a

concentration dependent high affinity for secondary bile acids (DCA and LCA), and only a weak affinity for some primary bile acids was recorded. Like cholestarymine, the adsorption rate of RA5 generally decreased as the concentration of the bile acids increased. The bile acids binding activity of RA3 was further evaluated *in vivo* using rats (RA5 was not tested due sample limitation). Although not as high as in the positive control group, the compound stimulated faecal bile acid excretion. A significantly ($p < 0.05$) higher total bile acid concentration in rats' faeces was recorded in the experimental group along with higher faecal content following the 14 days of consuming the triterpene-supplemented diet (Figure 3.6).
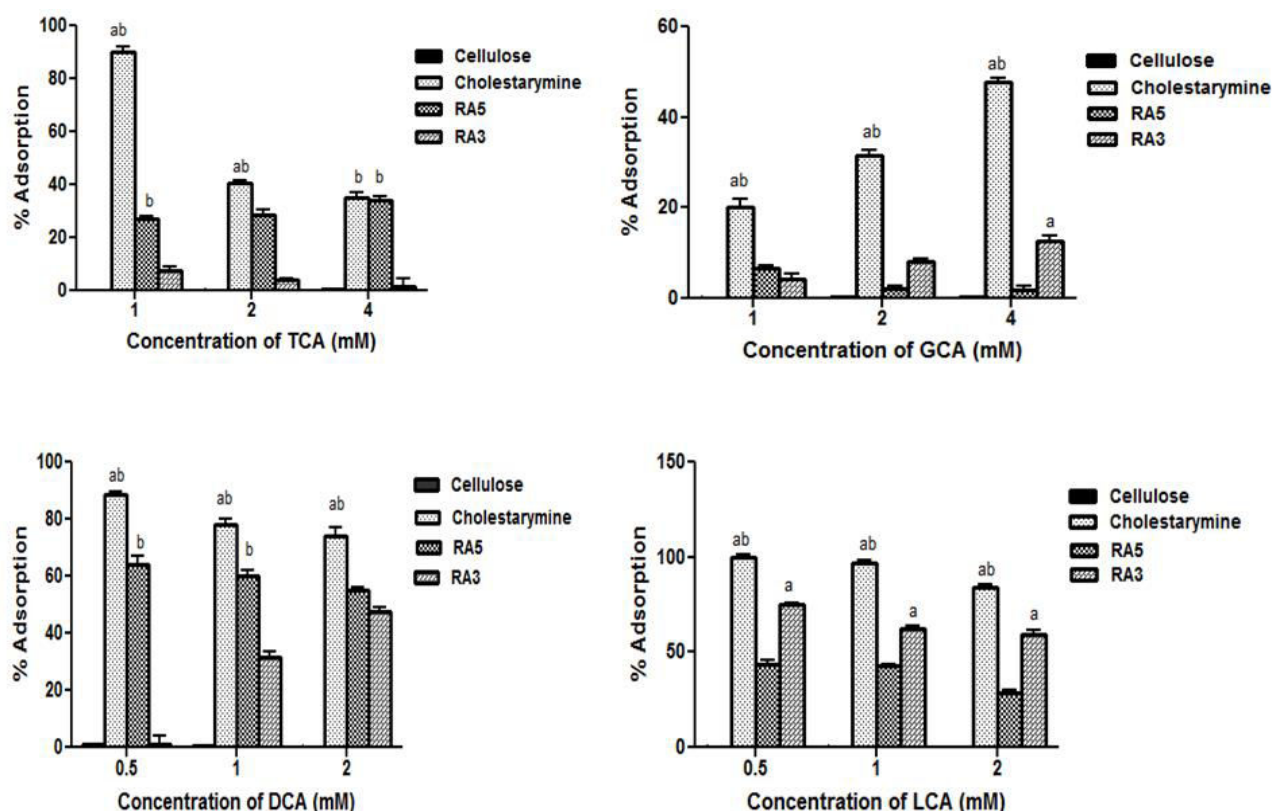
Figure 3.5: *In vitro* bile acid binding ability of the triterpenes. Various concentrations (0.5-4 mM) of the bile acids were incubated with the compound at 1% (w/v) for 30 min at 37°C. [a] $p < 0.05$ compared to RA5, [b] $p < 0.05$ compared to RA3
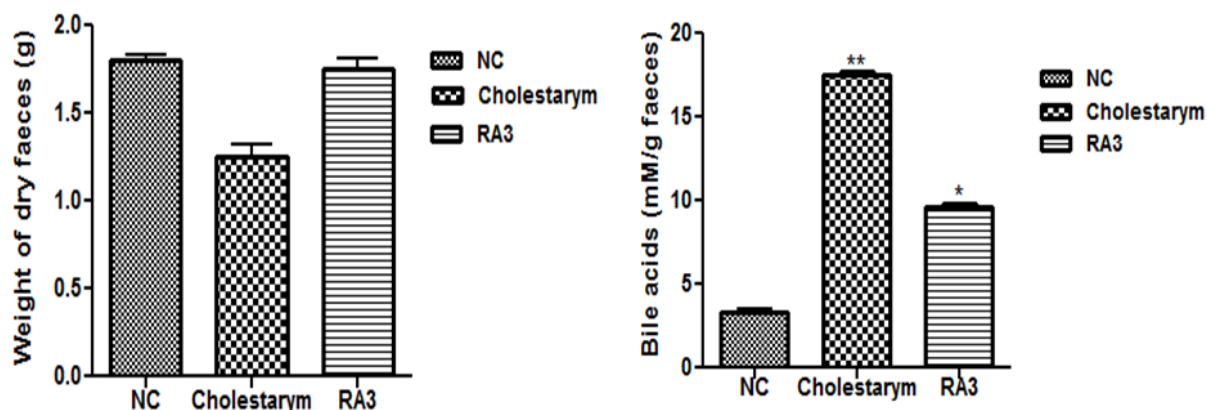
Figure 3.6: *In vivo* bile acid binding ability. The animals were fed a diet supplemented with 1% (w/v) of the triterpene (RA3). Cholestarymine (Cholestarym) was used as positive control. Faecal bile acids were extracted and measured from the 48 h faeces post diet treatment. NC- normal control, * $p < 0.05$, ** $p < 0.001$ vs NC

3B1.4.3 Intracellular lipid accumulation

The 3T3-L1 cell line is widely used to study adipogenesis and lipid metabolism, and has contributed to the current understanding of the biochemical mechanisms that anti-obesity agents have at a cellular level. Oil Red O staining assay was used to evaluate the effect of the compounds (RA5 and R52) on lipid (TG) accumulation in the 3T3-L1 adipocytes. Figure 3.7 indicates that only mature differentiated cells have increased lipid (TG) content and the controls (ADM and 0.1% DMSO) used did not have any effect on the TG accumulation. The compounds showed to varying degree a concentration dependent reduction of TG accumulation in the cells. While RA5 at the concentrations 1, 10 and 25 µM significantly ($p \leq 0.05$) reduced (34.8%) TG accumulation in mature differentiated 3T3-L1 adipocytes (Figure 3.8), R52 was significantly effective only at lower concentrations (1 and 10 µM) following a 48 h treatment (Figure 3.9). However, both compounds at the highest concentration of 100 µM showed no effect on the lipid content. The crystal violet stain used to normalise cell count, confirmed that the observed effect was not due to cytotoxicity.
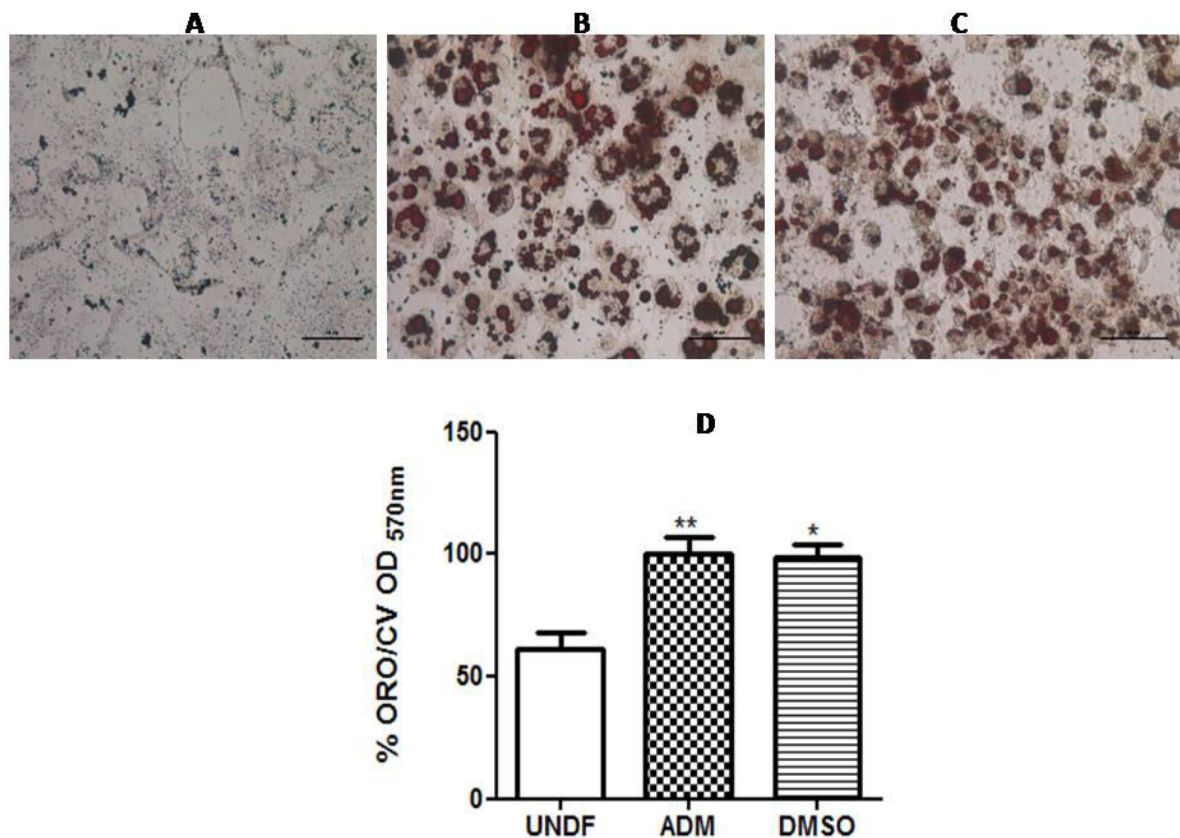
Figure 3.7: Lipid accumulation in 3T3-L1 adipocytes. Lipid accumulation following differentiation of 3T3-L1 cells were demonstrated by Oil Red O staining. Undifferentiated controls lacked staining (A), differentiated 3T3-L1 media and vehicle (< 0.1% DMSO) controls cells showed increased staining (B and C). The pictures are scaled at 60 μm. The intracellular TG content was quantified by measuring absorbance of the dye (D). The results are a mean of three independent experiments and data are expressed relative to the media control (ADM) set at 100%.*p ≤ 0.05 and ** p≤ 0.001
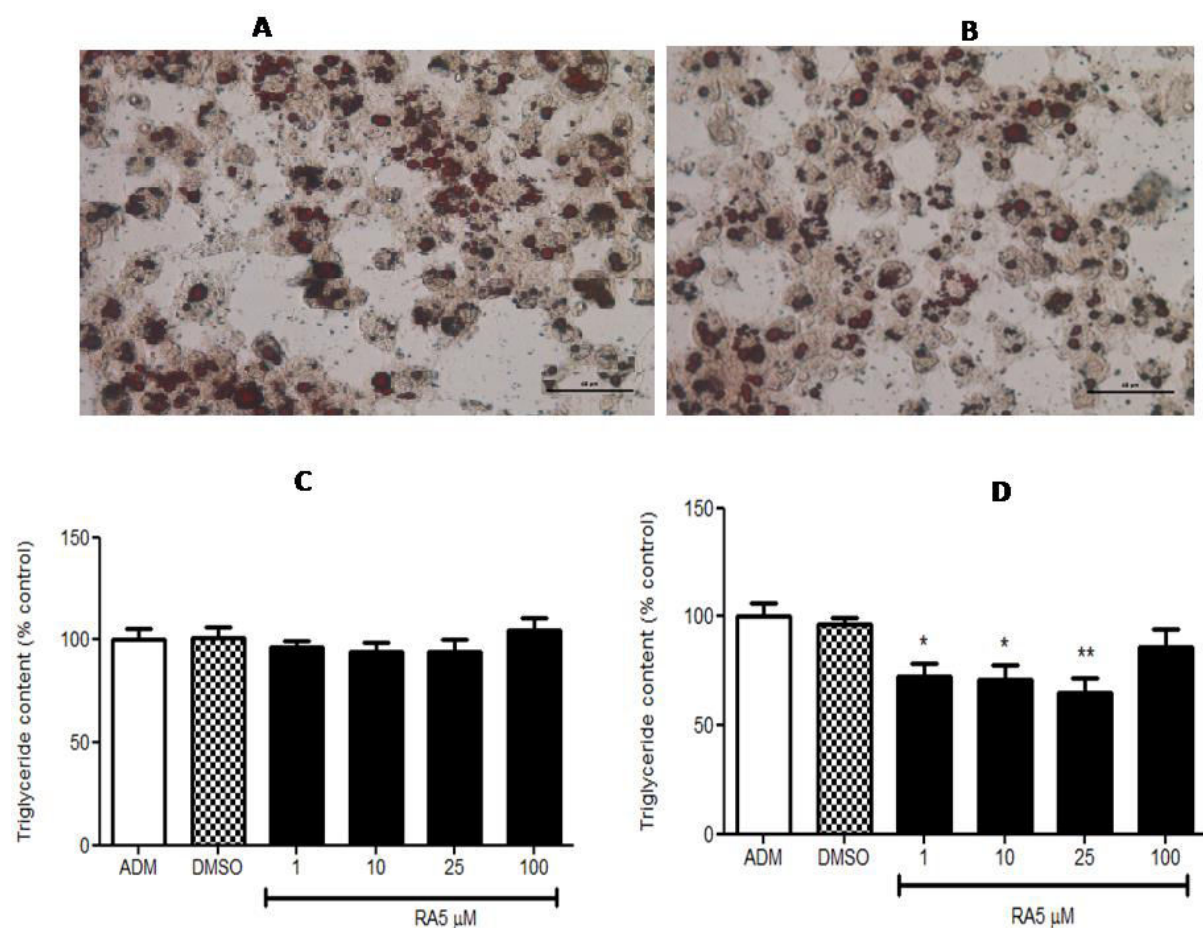
Figure 3.8: Effect of RA5 on lipid accumulation in 3T3-L1 adipocytes. The cells were exposed to the compound (RA5) at a range of concentrations (1, 10, 25 and 100 μM) for 24 h (A and C) and 48 h (B and D). The pictures are scaled at 60 μm. Data are expressed as mean of three independent experiments relative to the ADM control at 100%.* p ≤ 0.05 and ** p≤ 0.001.
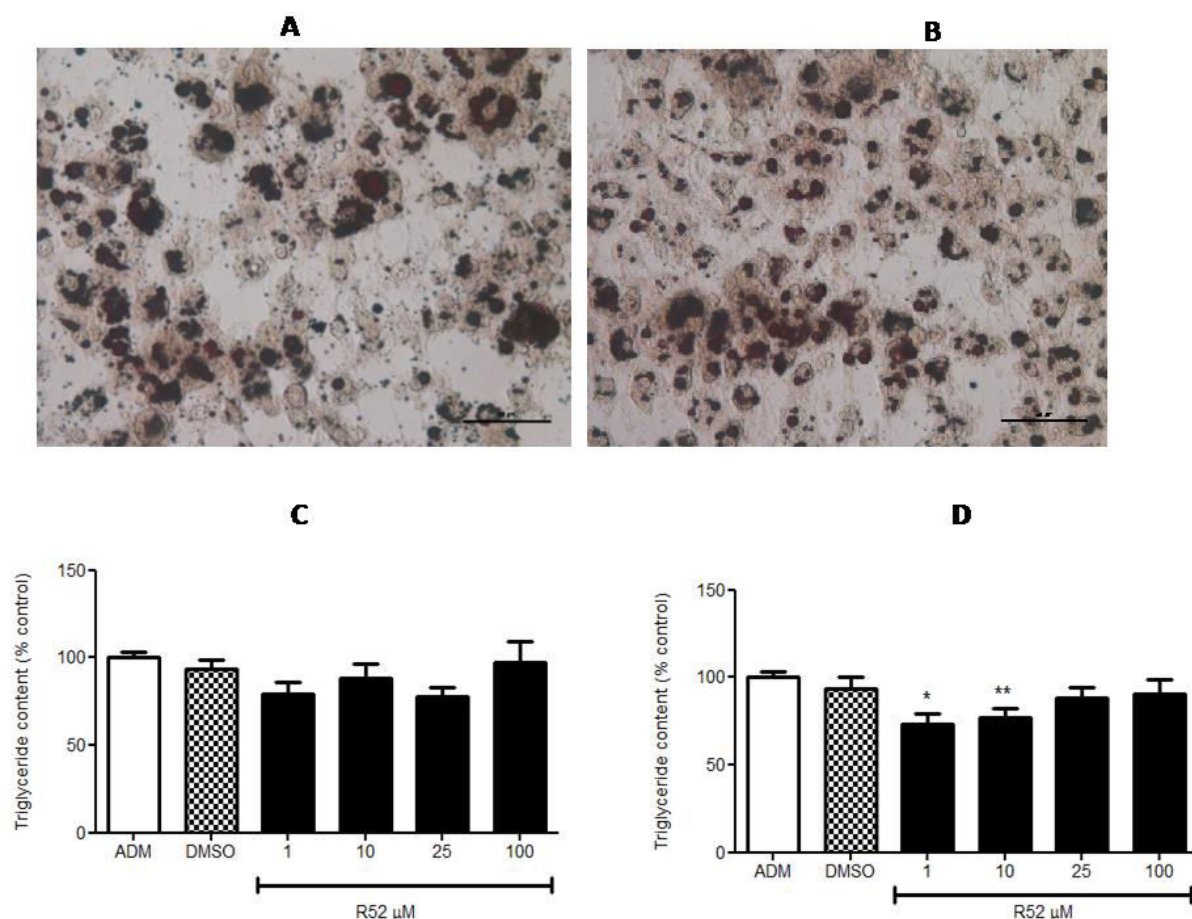
Figure 3.9: Effect of R52 on lipid accumulation in 3T3-L1 adipocytes. The adipocytes were exposed to R52 at different concentrations (1, 10, 25 and 100 µM) for 24 h (A and C) and 48 h (B and D). The pictures are scaled at 60 µm. Data are presented as mean of three independent experiments relative to the ADM control at 100%.* $p \leq 0.05$ and ** $p \leq 0.001$.

3B1.5 Discussion

Regulation and decreasing elevated blood lipids levels is crucial for the prevention and treatment of cardiovascular events (Derosa *et al.*, 2006; Karalis *et al.*, 2012). Inhibition of lipid digestive enzymes and limiting intestinal absorption of dietary lipids, as well as inhibition of adipogenesis are currently considered ideal therapeutic approaches to reduce incidences of hyperlipidemia and its related diseases (Ahn *et al.*, 2012; Park *et al.*, 2013).

The results obtained from this study indicate the hypolipidemic properties of the compounds from *P. longifolia* as they effectively inhibited the activities of pancreatic lipase, cholesterol esterase, and HSL (Table 3.3). The results suggest that the hypolipidemic activity of the compounds is partly mediated by inhibiting intestinal

absorption of dietary lipids. Evidence from research has shown that plant sterols lower intestinal cholesterol absorption (Sudhahar *et al.*, 2007). The poor activity exhibited by derivatives of RA3 and RA5 on pancreatic lipase and cholesterol esterase, respectively, indicates the essential role of the hydroxyl group at C-3 in the activity of the compounds. The strong inhibition of the triterpenes even on HSL is evidence that the compounds have potential to regulate dyslipidemia by inhibiting endogenous lypolysis as well. Inhibition of hormone sensitive lipase is also important for control of plasma glucose levels. Therefore, inhibitors of HSL are important drug targets in prevention of hyperlipidemia and consequent peripheral insulin resistance (Ali *et al.*, 2012).

The weak affinity of the triterpenes for the primary bile acids (Figure 3.5) indicates that their inhibitory effect on the lipid digestive enzymes is independent of the acids. However, their higher affinity for the secondary bile acids (Figure 3.5) suggests potential benefits of the compounds in maintaining cholesterol homeostasis and prevention of diseases associated with the bile acids accumulation (Peterlik, 2008; DiBaise and Islam, 2012). The ability of RA3 to increase faecal bile acid excretion in rats (Figure 3.6) further supports the bile acid binding activity of the compound, thus possibly interfering with the enterohepatic circulation of the bile acids. Similar results were also reported by Matsumoto *et al* (2011) where kaki-tannin from young persimmon fruit showed bile acid binding activity and increased faecal bile acid excretion in mice.

Fully differentiated 3T3-L1 adipocytes have most of the biochemical and morphological characteristics of adipocytes, and are used as an *in vitro* model to study lipogenesis and lipolysis (Morganstein *et al.,* 2008). These cells are representative of adipose tissue, one of the insulin sensitive tissue types involved in peripheral glucose clearance and maintenance of glucose homeostasis (Van de Venter *et al.*, 2008). Increase in adipocytes size due to excess intracellular lipid accumulation causes metabolic dysfunction of both lipids and glucose (Liu *et al.*, 2012). The ability of the triterpenoids (RA5 and R52) to significantly reduce intracellular lipid accumulation after only a 48 h treatment (Figures 3.8 and 3.9) strongly suggests their potent anti-hyperlipidemic effects. The reduction of lipid accumulation in adipose tissue is not only vital for the prevention and treatment of

hyperlipidemia, but also for obesity and insulin resistance in type 2 diabetes (Zeng *et al.*, 2012). Reduction of TG accumulation in 3T3-L1 cells as a measure of anti-adipogenic activity by a number of plant-derived compounds (Liu *et al.*, 2012) including lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* (Lee *et al.*, 2010) has also previously been reported. It is, thus, apparent that the hypolipidemic activity of these compounds could also be through inhibition of endogenous TG synthesis (lipogenesis).

This study demonstrated the potential hypolipidemic activity of the two triterpenes from *P. longifolia* and their derivatives. Apparently their hypolipidemic activity could be through inhibition of exogenous lipid absorption, endogenous lipid synthesis and lypolysis. The compounds have the potential to be developed in pharmacologically active hypolipidemic drugs.

References

Adisakwattana, S. Moonrat, J. Srichairat, S. Chanasit, C. Tirapongporn, H. Chanathong, B. Ngamukote, S. Mäkynen, K. Sapwarobol, S. (2010). Lipid-Lowering mechanisms of grape seed extract (*Vitisvinifera* L) and its antihyperlipidemic activity. *Journal of Medicinal Plants Research* 4(20): 2113-2120.

Ahn, J.H. Liu, Q. Lee, C. Ahn, M. Yoo, H. Hwang, B.Y. Lee, M.K. (2012). A new pancreatic lipase inhibitor from *Broussonetia kanzinoki. Bioorganic and Medicinal Chemistry Letters* 22: 2760-2763.

Ali, Y.B. Verger, R. Carrière, F. Petry, S. Muller, G. Abousalham, A. (2012). The molecular mechanism of human hormone-sensitive lipase inhibition by substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones. *Biochimie* 94: 137-145.

Brown, A.W. Hang, J. Dussault, P.H. Carr, T.P. (2009). Plant sterol and stanol substrate specificity of pancreatic cholesterol esterase. *The Journal of Nutritional Biochemistry* 21(8): 736-740.

Bustanji, Y. Issa, A. Mohammad, M. Hudaib, M. Tawah, K. Alkhatib, H. Almasir, I. Al-Khalid, B. (2010). Inhibition of hormone sensitive lipase and pancreatic lipase by *Rosmarinus officinalis* extract and selected phenolic constituents. *Journal of Medicinal Plants Research* 4(21): 2235-2242.

Chaput, J.P. St-Pierre, S. Tremblay, A. (2007). Currently available drugs for the treatment of obesity: sibutramine and orlistat. *Mini Reviews in Medicinal Chemistry* 7: 3-10.

Derosa, G. Salvadeo, S. Cicero, A.F. (2006). Prospects for the development of novel anti-hyperlipidemic drugs. *Current Opinion in Investigational Drugs* 7: 826-833.

Díaz, E.G. and Folgueras, T.M. (2011). Systematic review of the clinical efficacy of sibutramine and orlistat in weigth loss, quality of life and its adverse effects in obese adolescents. *Nutrición Hospitalaria* 26(3):451-457.

Dibaise, J.K. and Islam, R.S. (2012). In: Bile acids: An underrecognized and underappreciated cause of chronic diarrhea. Parrish, C.R. M.S .R.D. Series editor. Practical gastroenterology. *Nutrition Issues in Gastroenterology* series 110.

Ferreira, E.A. Gris, E.F. Rebello, J.M. Correia, J.F. de Oliveira, L.F. Filho, D.W. Pedrosa, R.C. (2011). The 2',4',6'-trihydroxyacetophenone isolated from *Myrcia multiflora* has antiobesity and mixed hypolipidemic effects with the reduction of lipid intestinal absorption. *Planta Medica* 77(14):1569-1574.

Karalis, D.G. Victor, B. Ahedor, L. Liu, L. (2012). Use of lipid-lowering medications and the likelihood of achieving optimal LDL-cholesterol goals in coronary artery disease patients. *Cholesterol* 2012: doi:10.1155/2012/861924.

Lee, I. Kim, H. Youn, U. Kim, J. Min, B. Jung, H. Na, M. Hattori, M. Bae, K. (2010). Effect of lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* on adipocyte differentiation in 3T3-L1 cells. *Planta Medica* 76:1558-1563.

Liu, Q. Ahn, J.H. Kim, S.B. Hwang, B.Y. Lee, M.K. (2012). New phenolic compounds with anti-adipogenic activity from the aerial parts of *Pulsatilla koreana*. *Planta Medica* 78: 1783-1786.

Luo, Y. Li, G, Li, G. Yan, J. Yi, J. Zhang, G. (2011). Discovery and identification of 2-phenylethyl 2,6-dihydroxybenzoate as a natural lipid-lowering lead. *Planta Medica* 77(18): 2047-2049.

Matsumoto, K. Kadowaki, A. Ozaki, N. Takenaka, M. Ono, H. Yokoyama, S. Gato, N. (2011). Bile acid-binding ability of kaki-tannin from young fruits of persimmon (*Diospyros kaki*) *in vitro* and *in vivo. Phytotherapy Research 25: 624-628.*

Morganstein, D.L. Christian, M. Turner, J.J.O. Parker, M.G. White, R. (2008). Conditionally immortalized white preadipocytes: a novel adipocyte model. *Journal of Lipid Research* 49: 679-685.

Morimoto, C. Sumiyoshi, M. Kameda, K. Tsujita, T. Okuda, H. (1999). Relationship between hormone-sensitive lipolysis and lipase activity in rat fat cells. *Journal of Biochemistry* 125: 976-981.

Park, C.H. Chung, B.Y. Lee, S.S. Bai, H.W. Cho, J.Y. Jo, C. Kim, T.H. (2013). Radiolytic transformation of rotenone with potential anti-adipogenic activity. *Bioorganic and Medicinal Chemistry Letters* 23: 1099-1103.

Peterlik, M. (2008). Role of bile acid secretion in human colorectal cancer. *Wiener medizinische Wochenschrift* 158: 539-541.

Slanc, P. Doljak, B. Kreft, S. Lunder, M. Janeš, D. Štrukelj, B. (2009). Screening of selected food and medicinal plant extracts for pancreatic lipase inhibition. *Phytotherapy Research* 23: 874-877.

Sudhahar, V. Kumar, S.A. Sudharsan, P.T. Varalakshmi, P. (2007). Protective effect of lupeol and its ester on cardiac abnormalities in experimental hypercholesterolemia. *Vascular Pharmacology* 46(6): 412-418.

Van de Venter, M. Roux, S. Bungua, L.C. Louw, J. Crouch, N.J. Grace, O.M. Maharaj, V. Pillay, P. Sewnarian, P. Bhagwandin, N. Folb, P. (2008). Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. *Journal of Ethnopharmacology* 119: 81-86.

Xie, W. Wang, W. Su, H. Xing, D. Cai, G. Du, L. (2007). Hypolipidemic mechanisms of *Ananas comosus* L. leaves in mice: Different from fibrates but similar to statins. *Journal of Pharmacological Sciences* 103: 267-274.

Zeng, X. Zhou, X. Xu, J. Chan, S.M.H. Xue, C.L. Molero, J.C. Ye, J.M. (2012). Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds. *Biochemistry and Pharmacology* 84: 830-837.

Zhang, X. Wu, C. Wu, H. Sheng, L. Su, Y. et al. (2013). Anti-hyperlipidemic effects and potential mechanisms of action of the caffeoylquinic acid-rich *Pandanus tectorius* fruit extract in hamsters fed a high fat-diet. *PLoS ONE* 8(4): e61922. doi:10.1371/journal.pone.0061922.

PART 3B2: ANTI-HYPERGLYCAEMIA (ANTIDIABETES)

Abstract

Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia. Control of postprandial hyperglycaemia is vital in the treatment of diabetes. Despite use of the current hypoglycaemic drugs, incidence of diabetes and related diseases continue to increase. The aim of this study was to evaluate the *in vitro* hypoglycaemic activity of the triterpenes from stem bark of *Protorhus longifolia*. The inhibitory activity of the triterpenes was evaluated on disaccharidases (maltase, sucrase, lactase) and glucosidases (α-, β-). The effect of the compounds on intestinal and cellular (C2C12 myotubules and 3T3-L1 adipocytes) glucose uptake was also evaluated. Cytotoxicity of the compounds was determined on the C2C12 and 3T3-L1 cells. The triterpenes showed to varying degree moderate inhibition on disaccharidases (maltase and sucrase) and α-glucosidase. No activity was observed on lactase and β-glucosidase. At 1% (w/v), RA5 effectively stimulated intestinal glucose uptake. Furthermore, the compounds at 50 µg/ml, also mimicked insulin character by effectively stimulating glucose uptake in both the C2C12 and 3T3-L1 cells. The compounds did not show any cytotoxic effects. The results indicate potential of the triterpenes from *P. longifolia* to be developed into active hypoglycaemic agents vital for treatment of diabetes.

3B2.1 Introduction

Diabetes mellitus is a global health concern quickly reaching epidemic levels. Its continuously increasing prevalence is likely to reach 439 million people by 2030 (Shaw *et al.*, 2010). Diabetes mellitus is one of the metabolic disorders characterised by chronic hyperglycaemia, with Type 2 diabetes responsible for over 90% of all cases of diabetes (Stolar *et al.*, 2008). Hyperglycaemia resulting from defects in insulin secretion, insulin action, or both, is considered the main cause of the debilitating effects of diabetes (Ortiz-Andrade *et al.*, 2007). Thus, control of postprandial hyperglycaemia is vital in the treatment of diabetes.

Current antidiabetic therapies focus on controlling postprandial hyperglycaemia by different approaches. These include enhancement of cellular glucose uptake or

inhibition of enzymes responsible for digestion of dietary carbohydrates therefore limiting their absorption (de Souza *et al.*, 2011). The currently used antidiabetic drugs are associated with some undesirable side effects and consequently limit their use (Hung *et al.*, 2012). There is a growing interest in medicinal plants and plant-derived compounds as alternative potentially safe antidiabetic drugs. Several studies (Santos *et al.*, 2012; Gutierrez, 2013) support the potential use of plant-derived triterpenes as hypoglycaemic agents.

This work was aimed at investigating the *in vitro* hypoglycaemic activity of the triterpenes from *P. longifolia*.

3B2.2 Materials

3B2.2.1 Equipment

See the list in section 3B1.2.1

3B2.2.2 Reagents/chemicals

Sigma-Aldrich: Glucosidases (α-, β-), acarbose, maltose, lactose, sucrose, 4-nitrophenol-α-D-glucose, sodium carbonate, pancreatin, glucose oxidase assay kit (product code GAGO-20) Dulbecco's Modified Eagle Medium (DMEM), D-glucose, NaHCO$_3$, BSA, 1,1-dimethylbiguanide hydrochloride (metformin), insulin, dexamethasone, isobutyl-methylxanthine, DMSO, ethylene glycol tetraacetic acid (EGTA), ATP, phosphoenolpyruvate, NADH, lactate dehydrogenase, ouabain, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)
 Horse serum (High veld Biological, Johannesburg, South Africa)

3B2.3 Methods

3B2.3.1 *In vitro* anti-hyperglycaemia

3B2.3.1.1 Alpha and beta-glucosidases inhibition

The method of Sancheti *et al.* (2011) was adapted with some modification to determine the inhibitory activity of the triterpenes on glucosidases. Components of

reaction mixture were: 50 µl of phosphate buffer (0.1 M, pH 7.0), 25 µl of 0.2 U/ml enzyme, 10 µl of compound (0 - 2.5 mg/ml), and 25 µl of 0.5 mM 4-nitrophenol-α-D-glucose (the β-isomer was used for the β-glucosidase activity, pH 7.0) as a substrate prepared in 0.1 M sodium phosphate buffer, pH 7.0. The reaction mixture was incubated at 37°C for 30 min. In the control sample, a carrier solvent was used to replace the tested compound. Acarbose was used as a standard drug. Enzyme activity was determined by measuring the release of 4-nitrophenol at 405 nm. Percentage inhibition was calculated. (See 3.1)

3B2.3.1.2 ATPase inhibition

Inhibitory activity of the triterpenes on Na^+/K^+-ATPase activity was determined by measuring the release of inorganic phosphate associated with hydrolysis of ATP (Vásárhlyi et al., 1997). The enzyme was obtained from rat's small intestines. Briefly, the rat's small intestines were homogenised in 0.1 M sodium phosphate buffer pH 7.0. The homogenate was centrifuged at 1200 rpm for 15 min. Supernatant was collected and kept at -20°C until use. Reaction mixture containing 250 µl of sample (0-2.5 mg/ml), 250 µl of homogenate was added to 4750 µl of reagent 1   [final concentration per litre: 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM tris-HCl (pH 7.4), 1 mM ATP, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 kU lactate dehydrogenase] and mixed well. The mixture was incubated at 37°C for 5 min after which 65 µl of 10 mM ouabain was added to inhibit ouabain-sensitive ATPase. Absorbance was read at 340 nm, Na^+/K^+ ATPase activity was calculated from the difference in absorbance (final – initial) at 340 nm.

3B2.3.1.3 Disaccharidases inhibition

Adult male rat (150-200 g) was sacrificed following a slight anesthetisation and upper part of the small intestines (duodenum and jejunum) was removed, and thoroughly cleaned with saline. The intestinal parts were then homogenised (1 g: 10 ml) in 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 10 000 × g for 10 min and supernatant was collected. The supernatant (enzymes extract) was kept at -20°C until use.

The method of Dahlqvist (1968) was followed with some modifications to evaluate inhibitory activity of the triterpenes on disaccharidases. The intestinal

disaccharidases (maltase, sucrase and lactase) activity was measured by determining the amount of glucose liberated from the corresponding disaccharides (maltose, sucrose and lactose). Reaction mixture consisted of 100 µl of enzymes extract, 100 µl of test compound (0.0 - 2.5 mg/ml) and 500 µl of 56 mM of appropriate substrate. The reaction mixture was incubated at 37°C for 60 min (15 min for maltase). The reaction was then terminated by a further incubation at 100°C for 5 min. The liberated glucose was measured with glucose oxidase assay kit (Sigma, product code GAGO-20) at 540 nm according to the manufacturer's instructions. Percentage inhibition was calculated. (See 3.1)

3B2.3.1.4 Intestinal glucose absorption

Effect of the triterpenes on intestinal glucose absorption was determined using inverted intestinal sac model (Said *et al.*, 2008; Wainstein *et al.*, 2012). The small intestines were obtained from male Sprague-Dawley rats (150-200 g), and thoroughly washed with physiologic buffer. The intestinal segment was inverted and the bottom part was tied before it was filled with Kreb's-Henselleit buffer. The sac was then placed in a flask containing 7 ml of 1% starch solution, 1% pancreatin and 1% (w/v) triterpene. In the control experiment, the compound was replaced with the buffer. The sac was incubated at 37°C for 120 min. The amount of glucose on both sides of the sac was measured with the glucose oxidase assay kit at 540 nm. The amount of glucose obtained on the outside and inside of the sac represented starch digestion and intestinal glucose absorption, respectively.

3B2.3.1.5 Cellular glucose uptake (*in vitro* cell culture models)

The compounds were separately dissolved in DMSO (1 mg/10 µl). Stock solutions were prepared by diluting the dissolved compounds (1:100) in Dulbecco's Modified Eagle Medium (DMEM) without phenol red, pyruvate and l-glutamine and supplemented with 8 mM D-glucose, 3.7 g/l $NaHCO_3$ and 0.1% bovine serum albumin (BSA). Subsequent working solutions were prepared from the stock solutions. The sample solutions were freshly prepared for each experiment.

3B2.3.1.5.1 Cell culture and treatment

The cell culture and cellular glucose uptake assays were conducted as described by Muller *et al.* (2012). Muscle cells (C2C12, CRL-1772) and adipocytes (3T3-L1) were seeded into 24-well plates at a density of 25 000 and 30 000 cells per ml respectively in DMEM foetal calf serum and incubated at 37°C in humidified air with 5% CO_2 for 3 days. To induce myotubule formation prior to performing glucose uptake assays, C2C12 cells were cultured for a further 2 days in DMEM containing 2% horse serum at 37°C in humidified air and 5% CO_2.

For 3T3-L1 preadipocytes differentiation, the preadipocytes were maintained in DMEM supplemented with 10% bovine calf serum at 37°C in a 5% CO_2 humidified incubator until they reach confluence. Two days after reaching confluence, cell differentiation was induced with a differentiation induction medium (DMI) mixture containing 1 mM dexamethasone, 1 µg/ml insulin, and 0.5 mM 3-isobutyl-methyl-xanthine in DMEM with 10% foetal bovine serum (FBS) for 2 days. The cells were then maintained in DMEM supplemented with FBS (10%) and insulin (1 mg/ml). The medium was being replaced every 2 days for 8 days.

3B2.3.1.5.2 Glucose uptake assay

C2C12 myotubules and 3T3-L1 were starved for 30 min using media (Kreb's media) containing 0.1% BSA, 3.7 g/l $NaHCO_3$ and no glucose prior to exposure to the compounds (50 µg/ml). The cells were then exposed to the compounds (50 µg/ml) in serum-free media supplemented with 8 mM glucose, 3.7 g/l $NaHCO_3$ and 0.1% BSA, incubated for 1 h at 37°C in humidified air with 5% CO_2. The media was collected and the glucose oxidase assay kit was used to determine the amount of glucose taken up by the cells. Glucose (8 mM) was used for normal control while 1 µM insulin and metformin (1 µM) were included as positive controls. Each experiment was done in triplicate. Three independent experiments were performed using C2C12 myotubules and 3T3-L1 adipocytes.

3B2.3.1.5.3 MTT cell proliferation assay (Cytotoxicity test)

After 1 h incubation for glucose uptake, the glucose media mixed with the compounds was removed from all the wells. This was transferred into a new plate for glucose

uptake detection or assay. The cells in the original plate were then refreshed with fresh glucose DMEM. The fresh glucose DMEM (200 µl) was added to each cell containing the cells. Then 50 µl of MTT (2 mg/ml PBS) was added to the cells. The cells were incubated at 37°C for 1 h. Thereafter the medium and MTT solution were removed from the wells and 200 µl of DMSO was added to each well (DMSO stops the reaction and dissolves insoluble formazan crystals). Sorenson's buffer (20 µl) was added to each well and absorbance was measured 570 nm. The experiment was replicated four times and 8 mM glucose and DMSO were used as controls. The percentage viability of the cells treated with the compounds was compared to those of the controls.

3B2.4 Results

3B2.4.1 Effect on carbohydrate digestive enzymes

The compounds exhibited a weak inhibition on the selected enzymes (Table 3.4). The compounds did not show any inhibitory activity on β-glucosidase and lactase. The effect of the triterpenes was also evaluated on the Na^+/K^+-ATPase activity, but no activity was observed.

Table 3.4: Inhibitory activity (%) of the triterpenes on some carbohydrate digestive enzymes

mg/ml	Acarbose	α-Glucosidase		Maltase		Sucrase	
		RA3	RA5	RA3	RA5	RA3	RA5
1.2	27.8 ± 0.21	14.0 ± 0.09	1.50 ± 0.02	18.3 ± 0.41	16.0 ± 0.03	1.54 ± 0.51	3.50 ± 0.11
1.4	38.0 ± 0.72	24.3 ± 1.17	1.90 ± 0.03	28.9 ± 1.22	24.0 ± 0.03	3.21 ± 0.27	3.80 ± 0.13
1.6	58.2 ± 2.44	29.1 ± 0.13	3.60 ± 0.35	27.5 ± 0.40	25.7 ± 0.06	4.76 ± 0.10	5.10 ± 0.12
1.8	67.8 ± 1.32	37.9 ± 0.48	8.00 ± 0.01	31.1 ± 0.72	27.6 ± 0.01	3.98 ± 0.04	4.80 ± 0.07
2.0	70.2 ± 1.64	49.7 ± 2.06	11.8 ± 0.04	41.5 ± 0.06	37.6 ± 0.10	5.88 ± 0.23	7.00 ± 0.07

Test compound (0 mg/ml) resulted in 100% activity. Data are expressed as mean ± SEM. * $p < 0.05$

3B2.4.2 Intestinal glucose absorption

The inverted intestinal sac model was used to also determine effect of the triterpene (RA5) on intestinal glucose absorption (RA3 was not tested due sample limitation). Figure 3.10 shows that RA5 significantly increased the intestinal glucose absorption.
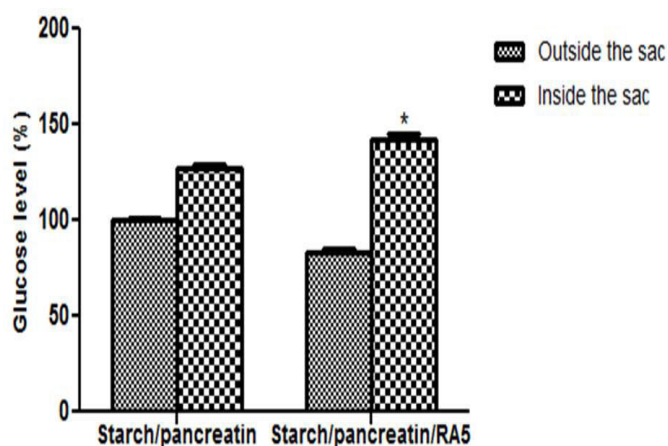
Figure 3.10: Effect of the triterpene on intestinal glucose uptake. The inverted intestinal sac was incubated for 120 min (at 37°C) in a solution containing 1% of starch, pancreatin and triterpene (RA5). Data are expressed as mean ± SD. * $p < 0.05$

3B2.4.3 Glucose uptake in C2C12 and 3T3-L1 cells

Several cell line culture models such as 3T3-L1 adipocytes, Chang (CCL-13) cells, C2C12 myotubules have been used in *in vitro* studies of cellular glucose transport and signalling mechanisms. In this study, the cellular glucose uptake stimulatory effect of the triterpenes and their derivatives was investigated in C2C12 and 3T3-L1 cells. The compounds mimicked insulin character by effectively stimulating cellular glucose uptake in both myotubules and adipocytes (Figure 3.11), an apparent hypoglycaemic property. The stimulatory effect exhibited by R31 on both cell types compared favourably even with the standard drugs (insulin and metformin) used. Furthermore, while R51 showed more activity on the C2C12 cells, R52 was active on the 3T3-L1 cells only.

The cytotoxicity of the compounds was also evaluated and the results are given in Figure 3.12. Interestingly, the tested compounds did not exhibit any potential cytotoxic effect.
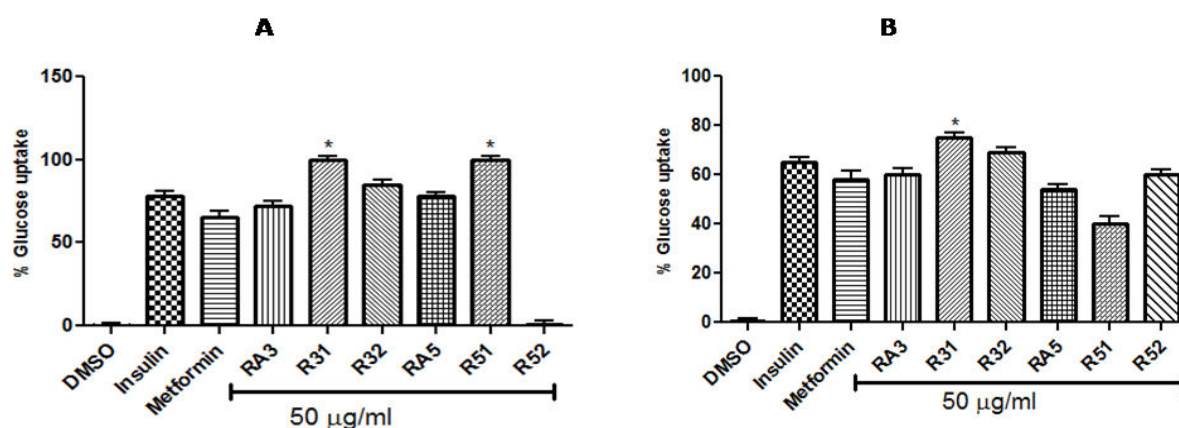
Figure 3.11: Effect of the compounds on glucose uptake in C2C12 myotubules (A) and 3T3-L1 adipocytes (B). The cells were exposed to the compounds (50 µg/ml) for 1 h at 37°C. Data are expressed as mean of three independent experiments relative to glucose (8 mM) control at 100%. * p ≤ 0.05 ***, p ≤ 0.0001
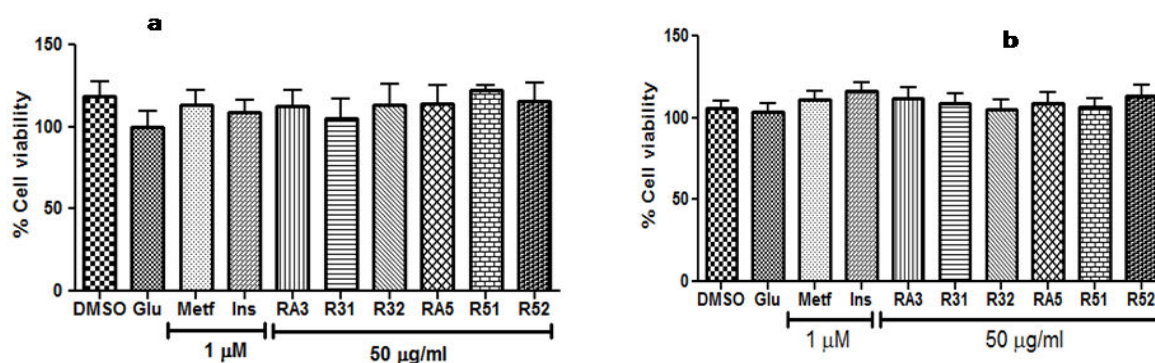
Figure 3.12: Effect of the compounds on the C2C12 (a) and 3T3-L1 (b) cells viability. The cells viability was determined following 1 h exposure (at 37°C) of the cells to the compounds at 50 µg/ml each. Data are expressed as mean of three independent experiments relative to glucose (8 mM) control at 100%. * p ≤ 0.05 ***, p ≤ 0.0001

3B2.5 Discussion

Chronic hyperglycaemia is considered the main cause of the debilitating effects of diabetes mellitus (Ortiz-Andrade *et al.*, 2007). Thus, control of postprandial hyperglycaemia is vital in the treatment of diabetes and its complications.

The results obtained in this work revealed that the triterpenes from *P. longifolia* possess anti-hyperglycaemic activity. The poor inhibitory activity on the carbohydrate digestive enzymes (Table 3.4) suggests that the hypoglycaemic activity of the compounds is mediated by mechanisms other than inhibition of carbohydrates digestion. Apparently the triterpenes exert their therapeutic effect through stimulation

of cellular glucose uptake as they effectively enhanced cellular glucose uptake in 3T3-L1 and C2C12 cells (Table 3.11). The significantly consistent higher activity of R31 in both 3T3-L1 and C2C12 cells could be attributed to the presence of acetyl group in the structure relative to the parent compound. Enhancement of cellular glucose uptake is currently considered one of the plausible approaches to control postprandial hyperglycaemia (de Souza *et al.*, 2011). Adipose and muscle tissues are the main sites for postprandial glucose clearance with about 75% of the blood glucose being cleared by skeletal muscles (Kim *et al.*, 2013). Thus, the obtained results strongly indicate potential of these compounds in controlling post prandial hyperglycaemia, thus preventing diabetes and its related diseases.

The results from this study are consistent with some other reports in literature. Several other studies (Mbaze *et al.*, 2007; Rahman *et al.*, 2008) have demonstrated that triterpenes are poor inhibitors of carbohydrate digestive enzymes, particularly alpha-glucosidase. It has also been reported that plant-derived triterpenes exert their hypoglycaemic activity through stimulation of cellular glucose uptake and insulin secretion (Lee and Thuong, 2010; Alqahtani *et al.*, 2013).

Though insulin signalling pathway and AMPK are considered as two distinct mechanisms responsible for the cellular glucose uptake (Kim e*t al.*, 2013), most of the currently available anti-diabetic agents require insulin signalling pathway for their action. However, the prevalence of type 2 diabetes has currently shifted attention towards AMP-activated protein kinase (AMPK) as an alternative therapeutic target (Park *et al.*, 2007, Krishnapuram *et al.,* 2012). AMPK has also been considered a major therapeutic target for management of obesity-linked disorders (Lage *et al.*, 2008). Several studies have linked antidiabetic activity of some triterpenoids to activation of AMPK (Cheng *et al.,* 2008; Ha *et al.*, 2009). The mechanism(s) through which the triterpenes from *P. longifolia* stimulate cellular glucose uptake needs to be explored.

It is noteworthy that despite some reports on the cytotoxicity of some other triterpenes (Lee *et al.*, 2007; Peteros and Uy, 2010), the isolated triterpenes and their derivatives did not exhibit any cellular cytotoxic effects (Figure 3.12). Lack of cytotoxicity of RA5 has previously been demonstrated in cancer cells (Mosa *et al.*,

2011b). The results encourage potential use of these compounds as new safe pharmacologically active antidiabetic drugs

Control of postprandial hyperglycaemia is vital in the treatment of diabetes and its complications. The results obtained indicate potential anti-hyperglycaemic activity of the triterpenes from *P. longifolia* and their derivatives. It is therefore apparent that the compounds exert their hypoglycaemic effect through stimulation of cellular glucose uptake.

References

Alqahtani, A. Hamid, K. Kam, A. Wong, K.H. Abdelhak, Z. Razmovski-Naumovski, V. Chan, K. Li, K.M. Groundwater, P.W. Li, G.Q. (2013). The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Current Medicinal Chemistry* 20(7): 908-931.

Cheng, Z. Pang, T. Gu, M. Gao, A.H. Xie, C.M. Li, J.Y. Nan, F.J. Li, J. (2006). Berberine stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. *Biochimica et Biophysica Acta* 1760: 1682-1689.

Dahlqvist, A. (1968). Assay of intestinal disaccharidases. *Analytical Biochemistry* 22(1):99-107.

De Souza, P.M. de Sales, P.M. Simeoni, L.A. Silva, E.C. Silveira, D. Magalhães, P.O. (2012). Inhibitory activity of α-amylase and α-glucosidase by plant extracts from the Brazilian Cerrado. *Planta Medica* 78(4): 393-399.

Gutierrez, R.M.P. (2013). Evaluation of the hypoglycemic and hypolipidemic effects of triterpenoids from *Prosthechea michuacana* in STZ-induced type 2 diabetes in mice. *Pharmacologia*, DOI: 10.5567/pharmacologia.2013.170.179.

Ha, D.T. Tuan, D.T. Thu, N.B. Nhiem, N.X. Ngoc, T.M. Yim, N.H. Bae, K.H. (2009). Palbinone and triterpenes from Moutan Cortex (*Paeonia suffruticosa*, Paeoniaceae) stimulate glucose uptake and glycogen synthesis via activation of AMPK in insulin-resistant human HepG2 Cells. *Bioorganic & Medicinal Chemistry Letters* 19: 5556-5559.

Hung, H. Qian, K. Morris-Natschke, S.L. Hsu, C. Lee, K. (2012). Recent discovery of plant-derived anti-diabetic natural products. *Natural Product Reports* 29: 580-606.

Kim, S. Hwang, J. Park, H.S. Kwon, D.Y. Kim, M. (2013). Capsaicin stimulates glucose uptake in C2C12 muscle cells via the reactive oxygen species (ROS)/AMPK/p38 MAPK pathway. *Biochemical and Biophysical Research Communications* 439: 66-70.

Kim, S.H. Sung, M.J. Park, J.H. Yang, H.J. Hwang, J.T. (2013). *Boehmeria nivea* stimulates glucose uptake by activating peroxisome proliferator-activated receptor gamma in C2C12 cells and improves glucose intolerance in mice fed a high-fat diet. *Evidence-Based Complementary and Alternative Medicine* 2013, Article ID 867893, 9 pages http://dx.doi.org/10.1155/2013/867893.

Krishnapuram, R. Kirk-Ballard, H. Dhurandhar, E.J. Dubuisson, O. Messier, V. Rabasa-Lhoret, R. Hegde, V. Aggarwal, S. N.V. Dhurandhar, N.V. (2013). Insulin receptor-independent glucose uptake. *International Journal of Obesity* 37: 146-153.

Lage, R. Dieguez, C. Vidal-Puig, A. Lopez, M. (2008). AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in Molecular Medicine* 14: 539-549.

Lee, I.S. Yoo, J.K. Na, M.K. Min, B.S. Lee, J.P. Yun, B.S. Jin, W.Y. Kim, H.J. Youn, U.J. Chen, Q.C. Song, K.S. Seong, Y.H. Bae, K.H. (2007). Cytotoxicity of triterpenes isolated from *Aceriphyllum rossii. Chemical and Pharmaceutical Bulletin* 55(9): 1376-1378.

Lee, M.S. and Phuong, T.T. (2010). Stimulation of glucose uptake by triterpenoids from *Weigela subsessilis.* Phytotherapy Research 24: 49-53.

Mbaze, L.M. Mbaze, H.M.P. Poumale, J.U. Wansi, J.A. Lado, S.N. Kahn, M.C. et al (2007). α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of *Fagara tessmannii* (Rutaceae). *Phytochemistry* 68: 591-595.

Muller, C.J.F. Joubert, E. de Beer, D. Sanderson, M. Malherbe, C.J. Fey, S.J. Louw, J. (2012). Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. *Phytomedicine* 20(1): 32-39.

Ortiz-Andrade, R.R, García-Jiménez, S. Castillo-España, P. Ramírez-Avila, G. Villalobos-Milona, R. Estrada-Soto, S. (2007). Alpha glucosidase inhibitory activity of the methanolic extract from *Tournefortia hartwegiana*: an anti-hyperglycemic agent. *Journal of Ethnopharmacology* 109(1): 48-53.

Park, C.E. Kim, M. Lee, J.H. Min, B. Bae, H. Choe, W. Kim, S. Ha, J. (2007). Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. *Experimental and Molecular Medicine* 39(2): 222-229.

Peteros, N.P. and Uy, M.M. (2010). Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants. *Journal of Medicinal Plants Research* 4(5): 407-414.

Rahman, A. Zareen, S. Choudhary, M.I. Akhatar, M.N. Khan, S.N. (2008). α-Glucosidase inhibitory activity of triterpenoids from *Cichorium intybus. Journal of Natural Products* 71: 910-913.

Said, O. Fulder, S. Khalil, K. Azaizeh, H. Kassis, E. Saad, B. (2008). Maintaining a physiological blood glucose level with 'glucolevel', a combination of four anti-diabetes plants used in the traditional Arab herbal medicine. *eCAM* 5(4): 421-428.

Sancheti, S. Sancheti, S. Lee S Lee, J. Seo, S. (2011). Screening of Korean medicinal plant extracts for α-glucosidase inhibitory activities. *Iranian Journal of Pharmaceutical Research* 10(2): 261-264.

Santos, F.A. Frota, J.T. Arruda, B.R. de Melo, T.S. da Silva, A.A. Brito, G.A. Chaves, M.H. Rao, V.S. (2012). Antihyperglycemic and hypolipidemic effects of α, β-amyrin, a

triterpenoid mixture from *Protium heptaphyllum* in mice. *Lipids in Health and Disease* 11:98. doi: 10.1186/1476-511X-11-98.

Shaw, J.E. Sicree, R.A. Zimmet, P.Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research Clinical Practice* 87: 4-14.

Stolar, M.W. Hoogwerf, B.J. Gorshow, S.M. Boyle, P.J. Wales, D.O. (2008). Managing type 2 diabetes: going beyond glycemic control. *Managed Care Pharmacy* 14: s2-s19.

Vásárhelyi, B. Szabó, T. Ágota Vér, Á. Tulassay, T. (1997). Measurement of Na^+/K^+-ATPase activity with an automated analyzer. *Clinical Chemistry* 43(10): 1986-1987.

Wainstein, J. Ganz, T. Boaz, M. Dayan, Y.B. Dolev, E. Kerem, Z. Madar, Z. (2012). Olive leaf extract as a hypoglycemic agent in both human diabetic subjects and in rats. *Journal of Medicinal Food* 15(7): 1-6.

PART 3B3: ANTICOAGULANT AND ANTI-INFLAMMATION (ANTI-PROTEIN AGGREGATION)

Abstract

Thromboembolic disorders are among the leading cause of morbidity and mortality worldwide. This work evaluated the anticoagulant, and anti-inflammatory activity of the triterpenes isolated from stem bark of *P. longifolia*. Tail bleeding time assay was used to evaluate the *ex vivo* anticoagulant activity. The effect of the triterpenes on the expression of heat shock protein70 (Hsp70) and their effect on thermally induced aggregation of malate dehydrogenase (MDH) and citrate synthase (CS) was studied. Cotton pellet-induced granuloma model in rats was used to evaluate the anti-inflammatory activity of the triterpenes. Granuloma formation was measured following 7 days of oral administration of the experimental rats with the triterpene at 50 and 250 mg/kg body weight. The activities of antioxidant enzymes (superoxide dismutase-SOD and catalase) and cyclooxygenases (COX-1 and COX-2) were also measured. The triterpenes significantly ($p < 0.05$) prolonged bleeding time up to 7.3 min as compared to 2.5 min in the normal control group. The triterpenes appreciably stimulated expression of Hsp70 *in E. coli* cells and in plasma of inflamed rats. They also improved the activity of Hsp70 on MDH and CS aggregation suppression. RA5 exhibited reduction of the granuloma formation by up to 40.3% along with increased SOD and catalase activity while COX activity was decreased. It is apparent that the triterpenes have potential to inhibit the aggregation of proteins.

3B3.1 Introduction

Thromboembolic disorders are the leading cause of stroke, myocardial infarction and pulmonary embolism. These disorders contribute to over 20% of annual deaths globally (Cohen *et al.*, 2011). Prevalence of thromboembolic disorders has become a serious concern even in developing countries due to changing lifestyles, people living more sedentary lifestyle and consuming a high fat diet. There is a direct link between inflammation and blood coagulation (Verhamme and Hoylaerts, 2009; Chu, 2011). This link is observed under both physiological and pathophysiological conditions. During endothelial injury, the two processes become autocatalytic. Inflammation

suppresses the natural anticoagulant system, which favours the expression and synthesis of prothrombogenic molecules (Verhamme and Hoylaerts, 2009).   In addition to their autocatalytic nature, both processes are mediated by the aggregation of proteins. Furthermore, both processes are mediated by activation and aggregation of proteins. Therefore, inappropriate protein aggregation if not checked could be responsible for development of various diseases and disorders.

Furthermore, inflammation has also been linked with most chronic illnesses including diabetes and cardiovascular diseases (Aggarwal *et al.*, 2006). Anticoagulants and anti-inflammatory compounds are thus important in the prevention and treatment of thromboembolic disorders (Hirsh *et al.*, 2007). The reported (Khanapure *et al.*, 2007; Mavrakanas *et al.*, 2011) undesirable side-effects of the current anticoagulant and anti-inflammatory agents have fuelled the search for a new generation of effective agents from natural sources. Anti-platelet (Sankaranarayanan *et al.*, 2010) and anti-inflammatory activity (Yadav *et al.*, 2010) of some plant-derived triterpenes have been reported. Thus, in this work, the anticoagulant and anti-inflammatory activity (anti-protein aggregation) of the lanosteryl triterpenes from *P. longifolia* is reported.

3B3.2 Materials

3B3.2.1 Equipment

See section 3B1.2.1 for the list, cotton (absorbent)

3B3.2.2 Reagents/chemicals

Sigma-Aldrich: porcine malate dehydrogenase, citrate synthase, tris-aminomethane, recombinant human heat shock protein70, BSA, acetylsalicylic acid, indomethacin, trisodium citrate, citric acid, thrombin, Tween 20, tris-HCl, tris-aminomethane, EDTA, Coomassie Brilliant Blue G-250, nitroblue tetrazolium (NBT), hydroxylamine hydrochloride, sodium nitroprusside, sulphanilic acid, 1-naphthylamine, ascorbic acid, xanthine, xanthine oxidase
Merck: isopropyl-1-thio-β-D-galactopyranoside (IPTG), sephadex beads, imidazole, sodium dodecyl sulphate (SDS), tryptone, nutrient agar, yeast, glycerol, phenylmethylsulfonyl fluoride, lysozyme

COX activity assay kit (Item No. 760151, Cayman Chemical Company, Michigan, U.S.A), Hsp 70 high sensitive ELISA kit (ab133060, Abcam®, Cambrige, U.K) Qiagen DNA Miniprep kit (Germany)

3B3.3 Methods

3B3.3.1 Malate dehydrogenase (MDH) and Citrate synthase (CS) Aggregation Suppression Assay

The ability of the triterpenes to prevent protein aggregation was evaluated on MDH and CS thermally induced aggregation. The heat stability of the protein and the triterpene was assessed following the method of Ramya *et al.* (2006) with some modifications. Hsp70 (Human recombinant or PfHsp70) and the triterpene (100µl) were separately prepared in the assay buffer (20 mM tris, pH 7.4; 100 mM NaCl) at a final concentration of 1.3 µM. Turbid metric changes at 48°C were then monitored at 340 nm for 45-65 min with BioTek® plate reader (ELx 808 UI, BioTek Instrument Supplies) using Gen5 software.

The effect of the triterpene to prevent thermally induced aggregation of MDH and CS at 48°C was conducted as described for heat stability assessment (Shonhai *et al.* 2008). MDH and CS (1.3 µM) were separately incubated with the Hsp70 (1.3 µM) in the absence and presence of the triterpene at different concentrations (1.3 and 5.2 µM). The thermally-induced aggregation of MDH and CS at 48°C was then separately followed by reading turbidity changes at 340 nm for 45-60 min. For control experiments, aggregation of MDH alone, aggregation of MDH in the presence of 1.3 µM BSA, and aggregation of MDH in the presence of Hsp70, was separately assessed. Similar controls were used for CS and the experiments were replicated three times.

3B3.3.2 Hsp70 expression in *E. coli*

3B3.3.2.1 Preparation of competent cells

Escherichia coli cells were grown on antibiotic-free agar plates (1.6% w/v tryptone, 0.5% w/v NaCl, 1.5% w/v nutrient agar and 1% w/v yeast) and incubated at 37°C, overnight. Positive colony was sub-cultured in 50 ml 2YT (yeast-tryptone broth) liquid broth (0.5% w/v sodium chloride, 1% w/v yeast and 1.6% w/v tryptone) at 37°C,

overnight with agitation at 162 rpm until OD_{600} of 03.6-0.6 was reached. The cells were then harvested following a series of centrifugation with intermittent incubation on ice. Finally, the obtained pellet was dissolved in 3 ml of 0.1 M $CaCl_2$ and 30% glycerol. The cells were aliquoted (100 µl) and stored at -80°C until required.

3B3.3.2.2 Transformation of competent cells

Plasmid DNA (pQE30/PfHsp70) was used to transform the *E. coli* competent XL1 blue cells. The cells were thawed on ice and 20 ng of plasmid DNA was added to 100 µl of the cells. The cells were then incubated on ice for 30 min. To induce the DNA uptake, the cells were heat shocked at 42°C for 45 sec in the heating block. This was followed by incubation on ice for 2 min. Antibiotic-free broth (2YT, 900 µl) was added to the cells and this was followed by incubation with shaking at 37°C for 1 h. The cells (50-100 µl) were then spread onto nutrient agar plates containing ampicillin (100 µg/ml). The plates were incubated at 37°C overnight in order for transformants to be selected.

3B3.3.2.3 Confirmation of the pQE30/PfHsp70 plasmid by restriction analysis

The integrity of the pQE30/PfHsp70 plasmid construct was confirmed through the use of restriction enzymes. The DNA was purified using Qiagen DNA Miniprep kit (Germany) following manufacturer's instructions. It was then digested with diagnostic restriction enzymes, Hind III and Bam HI. Agarose gel electrophoresis was used to analyse the resulting fragments and their sizes were estimated.

3B3.3.2.4 Protein Expression in *E. coli* XL1 blue cells

The effect of the triterpenes on the expression of *Plasmodium falciparum* heat shock protein 70 (*PfHsp70) in E. coli* XLI blue cells was investigated in the presence and absence of the triterpene*.* Overnight rich medium broth cultures of transformants of *E. coli* XL1 blue cells were allowed to grow until mid-log phase (OD_{600} 0.5–0.6) before the protein (PfHsp70) was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM as positive control. To evaluate the effect of the triterpenes on the protein expression, the IPTG was replaced with the triterpene at a final concentration of 0.05 and 0.5 mM. Assessment

of induction was continued at 37°C and 1 ml of each sample was collected at 1 h interval for 6 h. The collected samples were resuspended in ice cold phosphate buffered saline (PBS) and stored at -20°C for Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

3B3.3.2.5 Purification of PfHsp70 from *E. coli* XL1 blue cells

Following an overnight induction of the protein expression, the cells were harvested by centrifugation at 5000 × g for 20 min at 4°C. The pellets were resuspended in 10 ml of denaturing lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml lysozyme. The cells were stored overnight at −80 °C. The cells were then rapidly thawed and mildly sonicated. The cell debris was removed by centrifugation at 12000 × *g* for 30 min at 4°C. The lysates were left to bind to nickel-charged Sephadex beads in lysis buffer for 4 h at 4°C. In order to remove the unbound protein, the beads were washed twice with wash buffer (0.01 mM Tris, pH 7.5, 50 mM imidazole, 300 mM NaCl). The purified protein (PfHsp70) was eluted with the elution buffer (300 mM NaCl, 0.01 M Tris pH 7.5, 1 M imidazole). For each eluents collected, 50 µl were collected and put on the side. These were used for the protein analysis by (0.1%) SDS (12%) PAGE. The eluted protein was placed in pre-soaked Snakeskin™ pleated dialysis tubing, Mr 10 000 (Pierce, U.S.A). The protein was dialysed against the buffer (0.01 mM Tris, pH 7.5, 300 mM NaCl, 50 mM imidazole, 10% glycerol).

3B3.3.2.6 Western blotting

Following the SDS-PAGE, expression of pQE30/PfHsp70 was confirmed with Western blotting. Briefly, the protein was transferred to a nitrocellulose membrane for 1 h at 100v. The protein was visualised with ponceau stain. The membrane was washed two times with TBS (50 mM Tris pH 7.4, 150 mM NaCl) and then marked. This was followed by incubation in blocking buffer (5% w/v TBS) for 1 h. The membrane was then probed overnight 4°C with primary antibody for PfHsp70. After washing two times with TBS-T (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 20 min, the membrane was incubated with secondary antibody in 5% blocking buffer for 1 h on ice, with constant shaking. The membrane was again washed four

times with TBS-T (for 15 min per washing). Detection reagent was added to the blot and incubated for 60 sec in Chemidock (Bio-Rad) for the protein detection.

3B3.3.3 Animals

Adult rats (*Sprague-Dawley)* of either sex were used in the experiments and the guidelines for proper caring and conduct of animal experiments were followed.

3B3.3.4 Anticoagulant activity (*Ex vivo*)

3B3.3.4.1 Tail bleeding time assay

Tail bleeding time assay described by Gadi *et al.* (2009) was adapted with some modification to evaluate the anticoagulant activity of the triterpenes. The rats (220 ± 20 g) were randomly divided into four groups of five rats per group:

Group I: received carrier solvent 2% Tween 20
Group II: received acetylsalicylic acid (ASA) (30 mg/kg body weight- b.w)
Group III: received triterpene (50 mg/kg b.w)
Group IV: received triterpene (250 mg/kg b.w)

The rats were orally administered with the drugs 2 h before experiment. The animals were then slightly anaesthetised. Bleeding time was assessed by amputating the rat's tail tip (5 mm) and blood was blotted on a filter paper at 30 sec until the filter paper was no longer stained with blood. The period between amputation and the stop of bleeding was taken as the bleeding time (min).

3B3.3.4.2 Platelet preparation

Following the tail bleeding time assay, blood samples from the rats in the respective groups were collected to obtain platelets. A rat was killed by a blow to the head and the blood was immediately collected by cardiac puncture. The blood was then mixed with an anticoagulant (acid-dextrose-anticoagulant; 2% dextrose, 0.065 M citric acid, 0.085 M trisodium citrate). The platelets were obtained through a series of centrifugation and washing of the blood (Tomita *et al.,* 1983). The obtained platelets were suspended in a resuspending buffer (pH 7.4).

3B3.3.4.3 *Ex vivo* anti-platelet aggregation

The *ex vivo* anti-platelet aggregation activity of the triterpenes was tested on thrombin induced platelet aggregation (Mekhfi *et al.,* 2004). The platelets (200 µl), in a 96-well micro plate, were separately pre-incubated at 37°C for 5 min. The platelet aggregation was induced by addition of 20 µl thrombin (5 U/ml). The reaction was monitored by reading absorbance at 415 nm (at 30 sec interval for 20 min) with the Biotek plate reader. The experiment was replicated three times and the mean slope (A) ± SD was reported. The formula below was used to calculate percentage inhibition of platelet aggregation.

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where, A_0 is the mean slope of control and A_1 is the mean slope of the test drug.

3B3.3.5 Anti-inflammatory activity

3B3.3.5.1 Cotton pellet-Induced granuloma

Cotton pellet-induced granuloma model was used to investigate the anti-inflammatory activity of the triterpenes (Penn and Ashford, 1963). The rats (180-220 g) were randomly divided into four groups of five rats per group. The animals were orally pre-administered with the drugs thirty minutes before interscapular implantation (under slight anaesthetic) of pre-weighed sterile cotton pellets (20 mg).

Group I: received triterpene (50 mg/kg b.w)
Group II: received triterpene (250 mg/kg b.w)
Group III: received indomethacin (10 mg/kg b.w)
Group IV: received carrier solvent 2% Tween 20

The animals were orally administered with the drugs for seven consecutive days. On day 8, the implanted cotton pellets were carefully dissected out (under slight anaesthesia) and made free from the extraneous tissues. Weights of the wet pellets were determined and the pellets were dried at 37°C for 24 h. The pellets were separately re-weighed and increment in weight (relative to pre-weight cotton pellet) of the dry pellets was regarded equivalent to granuloma formation. The anti-proliferative

activity of the triterpenes was compared with the control. Percentage inhibition of granuloma formation was calculated using the formula

$$\% \text{ Inhibition} = (w_c - w_t / w_c) \times 100$$

where, w_c represents the pellet weight of the control group animals and w_t represents the pellet weight of the drug-treated group animals.

3B3.3.5.2 Biochemical Estimation

Tissue homogenate was prepared following the method described by Nagar *et al.* (2011), with some modifications. The granuloma tissue was homogenised (10 ml/g) in cold Tris-HCl buffer (0.1 M, pH 7.8, containing 1 mM EDTA). The homogenate was centrifuged at 10 000 × g for 15 min at 4°C. The supernatant was collected and kept at -80°C until use. The supernatant was used for biochemical estimation of protein content, catalase, superoxide dismutase (SOD) and COX activity.

3B3.3.5.2.1 Total protein

The Bradford assay was used to determine the protein content of the homogenate. Coomassie Brilliant Blue G-250 (5 ml) was added to 100 µl of the diluted (1:10) homogenate. The mixture was incubated for 5 min. BSA at different concentrations (5-100 µg/100 µl normal saline), was used as a standard. Absorbance was read at 595 nm. Protein concentration of the homogenate was determined from a standard curve.

3B3.3.5.2.2 Catalase activity

Catalase activity of the homogenate was estimated by measuring decomposition of hydrogen peroxide (H_2O_2) (Aebi, 1983). The reaction mixture contained 20 µl homogenate and 2 µl phosphate buffer (0.1 M, pH 7.2). The reaction was initiated by the addition of 250 µl H_2O_2 (30 mM). The enzyme activity was determined monitoring change in absorbance at 240 nm for 1 min at 15 sec intervals. The results were expressed as H_2O_2 decomposed/minute/mg protein, using 43.6 as the molar extinction coefficient of H_2O_2.

$$\text{Specific activity} = \frac{(\Delta A_{240}/min \times \text{volume of reaction mixture} / 43.6 \times \text{volume of enzyme})}{\text{concentration of protein}}$$

3B3.3.5.2.3 Superoxide dismutase activity

The nitroblue tetrazolium (NBT) reaction method was used to estimate SOD activity (Glannopolittis and Ries, 1977) in the tissue homogenate. The reaction mixture comprised 1 ml of 0.05 M sodium carbonate, 100 µl of homogenate, 200 µl of 1 mM EDTA and 400 µl of 0.01% NBT. Zero minute absorbance was read at 630 nm and the reaction was initiated by the addition of 400 µl of 2.4 mM hydroxylamine hydrochloride. This was followed by incubation at 25°C for 5 min. The enzymatic activity was determined by measuring reduction of NBT at 630 nm. One enzymatic unit was regarded as the amount of protein in the form of enzyme present in 100 µl of the homogenate, required to inhibit reduction of the NBT by 50%. The enzymatic activity of was expressed as a unit/mg protein.

3B3.3.5.2.4 Cyclooxygenase (COX) activity

COX activity of the tissue homogenate was determined using COX activity assay kit (Item No. 760151, Cayman). The preparation of the reagents and performance of the assay was according to the manufacturer's instructions. Briefly, the reaction mixture contained 120 µl of assay buffer, 10 µl of heme, 40 µl of homogenate (or COX standard). In the appropriate wells, inhibitor of COX-1 or COX-2 activity was also added. The mixture was incubated for 5 min at 25°C. Then 20 µl of calorimetric substrate (N,N,N',N'-tetramethyl-p-phenylenediamine-TMPD) was added and the reaction was initiated by adding 20 µl of arachidonic acid. This was mixed well and allowed to stand for 5 min at 25°C. Oxidation of TMPD was measured at 590 nm using BioTek® plate reader. One enzymatic activity was taken as the amount of enzyme required to oxidise 1.0 nmol of TMPD per min at 25°C. The experiment was done in triplicate. Percentage inhibition of COX activity was calculated using the formula

$$\% \text{ Inhibition} = \frac{\text{Total COX activity - COX activity}}{\text{Total COX activity}} \times 100$$

3B3.3.6 *In vivo* expression of Hsp70

B3.3.6.1 Plasma preparation

After the cotton pellets have been dissected out of the rats, whole blood (2 ml) was collected from the rats' tails into the separate EDTA (0.4 ml, 20 mM) containing tubes. The blood was centrifuged at 1000 × g for 15 min at 4°C. Supernatants were collected into the clean tubes and stored at -80°C until use.

3B3.3.6.2 Plasma content of Hsp70

Hsp70 high sensitivity ELISA kit (ab133060, Abcam$^®$) was used to measure content of Hsp70 in the plasma. The kit manufacturer's instructions were strictly followed in reagent preparation, handling and performing the assay. Briefly, 100 µl of samples and Hsp70 standard at different concentrations (0.20-125 ng/ml) were added to appropriate wells coated with antibody specific for Hsp70. The necessary incubations were all done at room temperature. Following a series of washing with assay buffer, addition of polyclonal antibody (100 µl) and horseradish peroxidase (100 µl) in all the wells except the blank, reaction was initiated by adding 100 µl of TBM substrate (3,3',5,5'-tetramethylbenzidine). Stop solution (100 µl) was added to all the wells and the resulting yellow colour was read at 450 nm. The experiment was replicated three times. Absorbance is directly proportional to the level of Hsp70 in the sample. The amount of the protein in the tested samples was determined from the standard curve and the results were compared to the control.

3B3.3.6 Antioxidant activity *in vitro*

3B3.3.6.1 Nitric oxide radical (NO$^.$) assay

Nitric oxide radical scavenging activity of the compounds was determined using Griess Illosvoy reaction (Garrat, 1964). Nitric oxide was generated from sodium nitroprusside. The reaction mixture comprising 0.5 ml of 0.1M phosphate buffer saline (pH 7.4), 2 ml of 10 mM sodium nitroprusside and 0.5 ml of the compound (0-50 µg/ml) were incubated at 25°C for 150 min. This was followed by the addition of 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid). The mixture was allowed to stand at room temperature for 5 min before adding 1 ml of 5% 1-naphthylamine, mixed well and allowed to stand for a further 30 min in diffused light.

Absorbance was measured at 540 nm. Ascorbic acid was used as a standard. The experiment was replicated three times. The nitric oxide radicals scavenging activity was calculated using the formula

$$\% \text{ scavenging activity} = ((A_c - A_t) / A_c \times 100)$$

where A_c is the absorbance of the control and A_t is the absorbance in the presence of the tested compound. The IC_{50} of the compounds was determined using GraphPad Prism version 5.03.

3B3.3.6.2 Superoxide radical assay

Superoxide radical scavenging activity of the compounds was determined following the method described by Martinez *et al.*, (2001) for determination of superoxide dismutase with some modifications. The assay is based on the capacity of the compounds to inhibit the photochemical reduction of NBT in the riboflavin–light–NBT system (Beauchamp & Fridovich, 1971). Reaction mixture containing 20 µl of each of the following: 3 mM xanthine, 0.15% BSA, 0.75 mM NBT, 3 mM EDTA, compound (0-50 µg/ml) and 480 µl sodium carbonate buffer (pH 10.5), was incubated at 25°C for 20 min. This was followed by addition of 20 µl of 6 µM xanthine oxidase and mixed well. The mixture was then incubated at 25°C for a further 20 min and this was followed by addition of 20 µl of 6 mM $CuCl_2$. The production of blue formazan was measured at 560 nm. The experiment was replicated three times and ascorbic acid was used as standard.

The inhibition of superoxide anion was calculated using the equation and the IC_{50} of the compounds was also determined.

$$\% \text{ Inhibition} = ((A_c - A_t) / A_c \times 100)$$

3B3.4 Results

3B3.4.1 Anti-protein aggregation

Figures 3.13 and 3.14 indicate that, like the Hsp70, the triterpenes clearly suppressed the thermally induced aggregation of MDH and CS (proteins), thus, exhibiting anti-protein aggregation activity. The triterpenes (1.3 and 5.2 µM) did not only show activity better than that of Hsp 70 (positive control), but they also improved its activity.
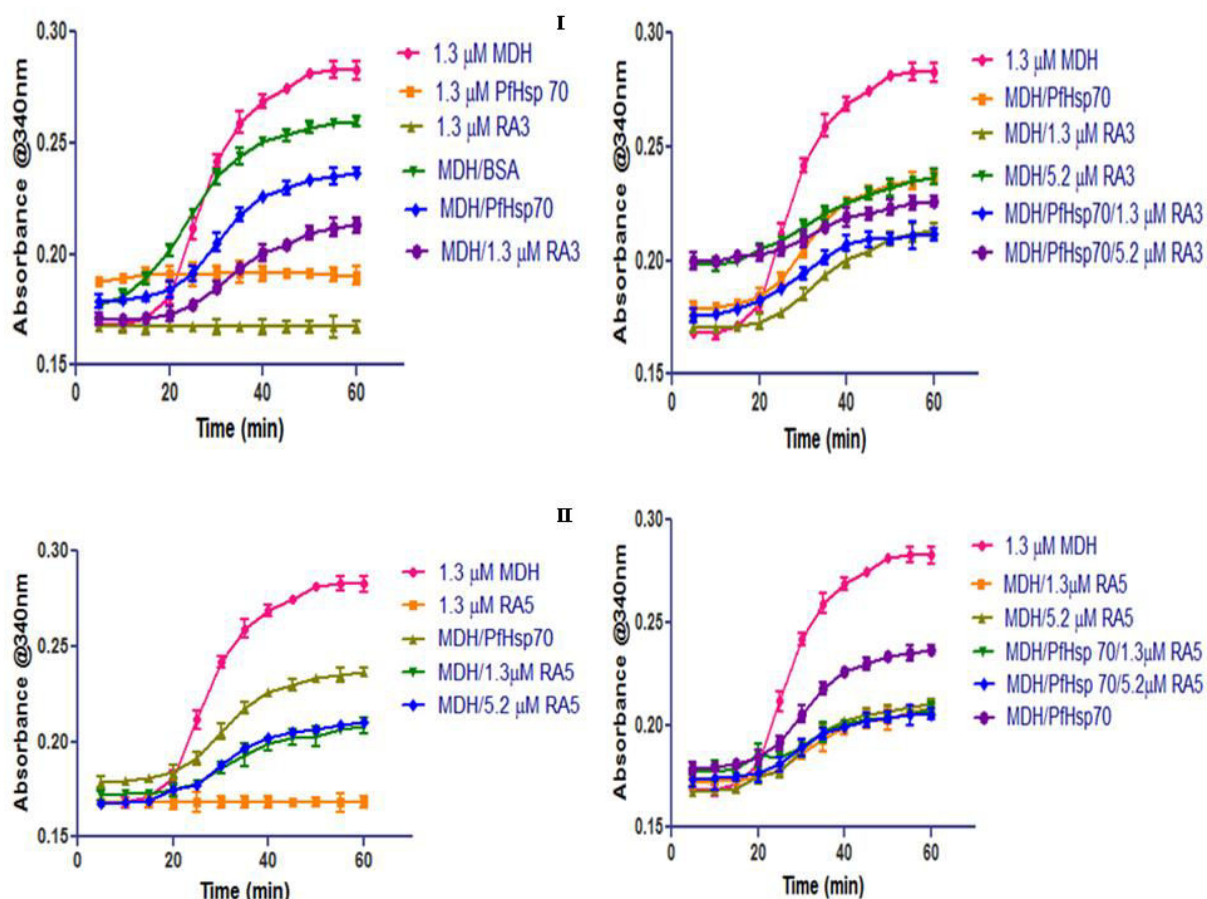
Figure 3.13a: Anti-protein aggregation activity of the triterpenes. Aggregation of MDH was thermally induced at 48°C in the presence and absence of the triterpene at different concentrations (1.3 and 5.2 µM). **I** and **II** refer to the effect of RA3 and RA5, respectively. For control experiments, aggregation of MDH alone, with BSA, and also in the presence of Hsp70 was separately assessed. Increase in absorbace indicates aggregation of the heat sensitive protein. Data were expressed as Mean ± SEM.
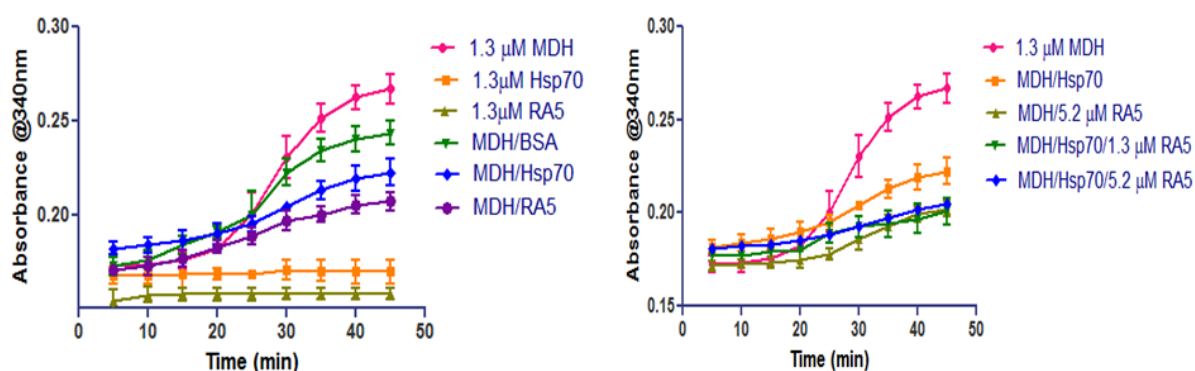

Figure 3.13b: Anti-protein aggregation activity of RA5. Similar controls were used as in Figure 3.13a except that recombinant human Hsp70 was used instead of PfHsp70.
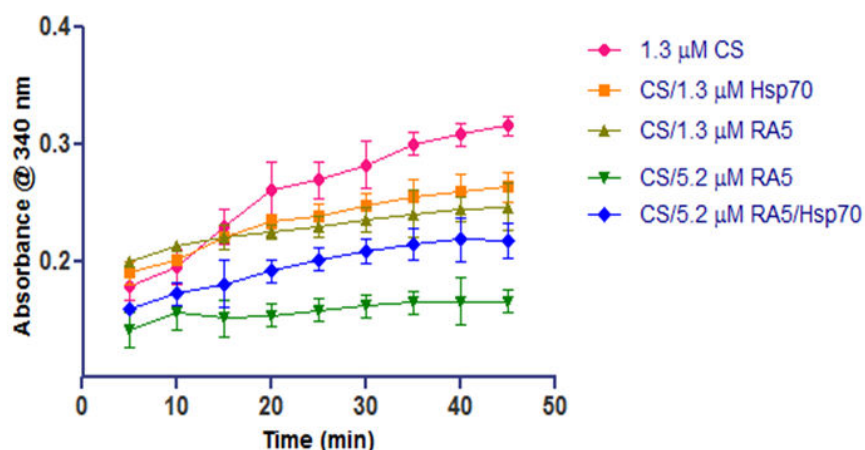
Figure 3.13c: Effect of RA5 on thermally induced aggregation of CS at 48°C. CS (1.3 µM) was exposed to heat stress in the presence and absence of the triterpene (1.3 and 5.2 µM). Aggregation of CS alone was separately assessed. Human Hsp70 was used instead of PfHsp70 Data were expressed as Mean ± SEM.

3B3.4.2 Effect of the triterpenes on the expression Hsp70

The effect of the triterpenes on the expression of Hsp70 was first evaluated in *E. coli* cells. Plasmid DNA (pQE30/PfHsp70) was used to transform the *E. coli* competent XL1 blue cells and the integrity of the pQE30/PfHsp70 plasmid construct was confirmed through the use of restriction enzymes (BamHI and HindIII). Agarose gel electrophoresis was used to analyse the resulting fragments and their sizes were estimated (Figure 3.14). Then expression of PfHsp70 was induced with the triterpene at 0.05 and 0.5 mM. The results are presented in Figure 3.15a-c. The triterpenes, at the different concentrations mimicked the IPTG character by effectively inducing expression of the protein (PfHsp70). It is worth noting that the compounds were able to induce expression of the protein at the concentrations lower than that of IPTG (positive control). The expressed protein was confirmed with Western blotting (Figure 3.15c)
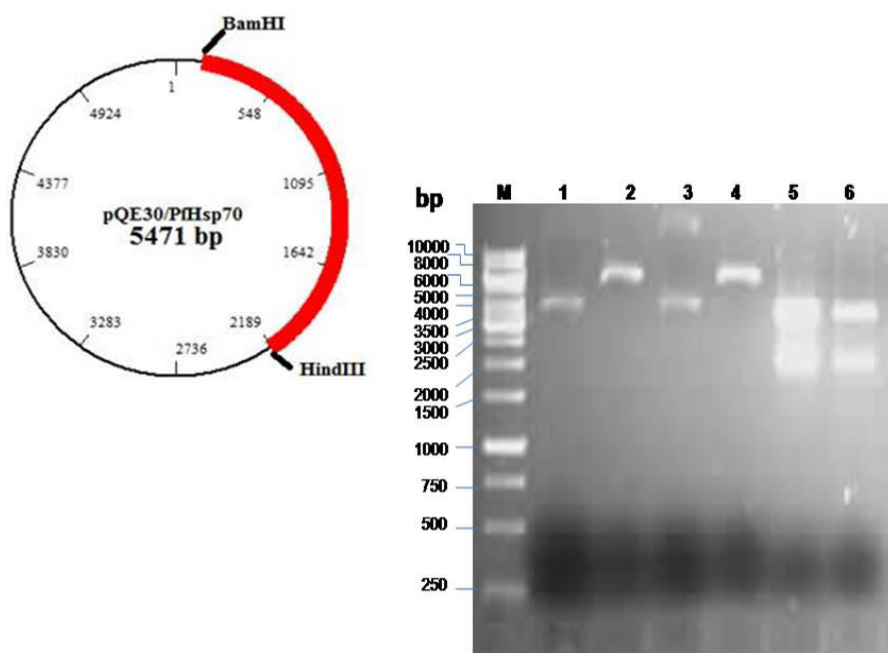
Figure 3.14: DNA restriction by *HindIII* and *BamHI*. M-marker, lane 1- uncut DNA, lane 2- cut with *BamHI*, lane 3- cut with *HindIII*, lane 4- cut with *BamHI*, lane 5 and 6 - cut with both *BamHI* and *HindIII*.
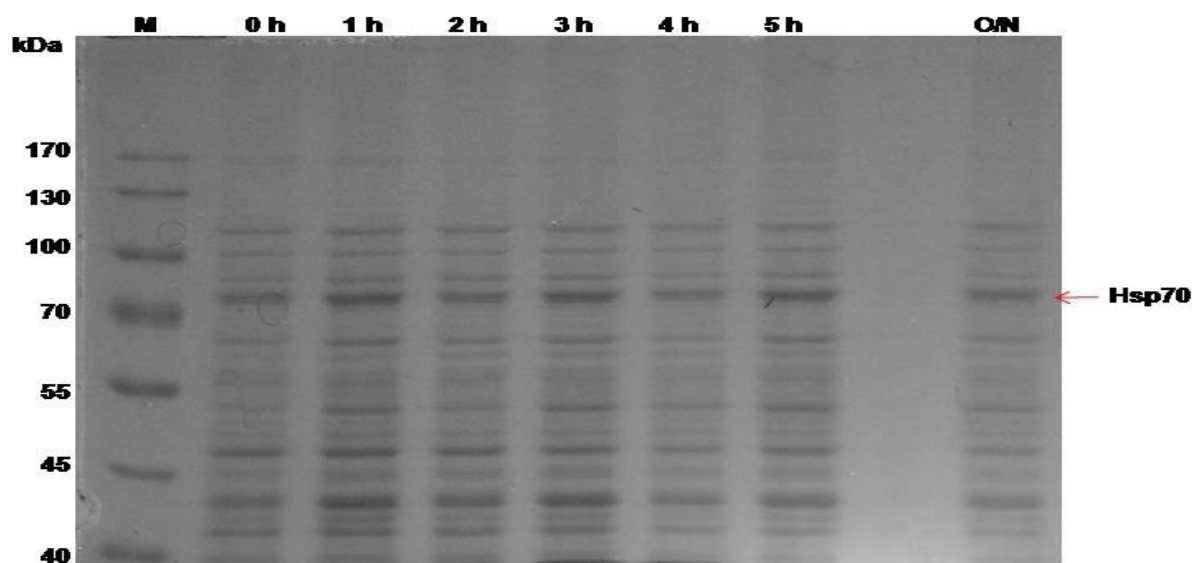

Figure 3.15a: Expression of PfHsp70 in *E. coli* cells. Expression of the protein was induced with IPTG. O/N- over night
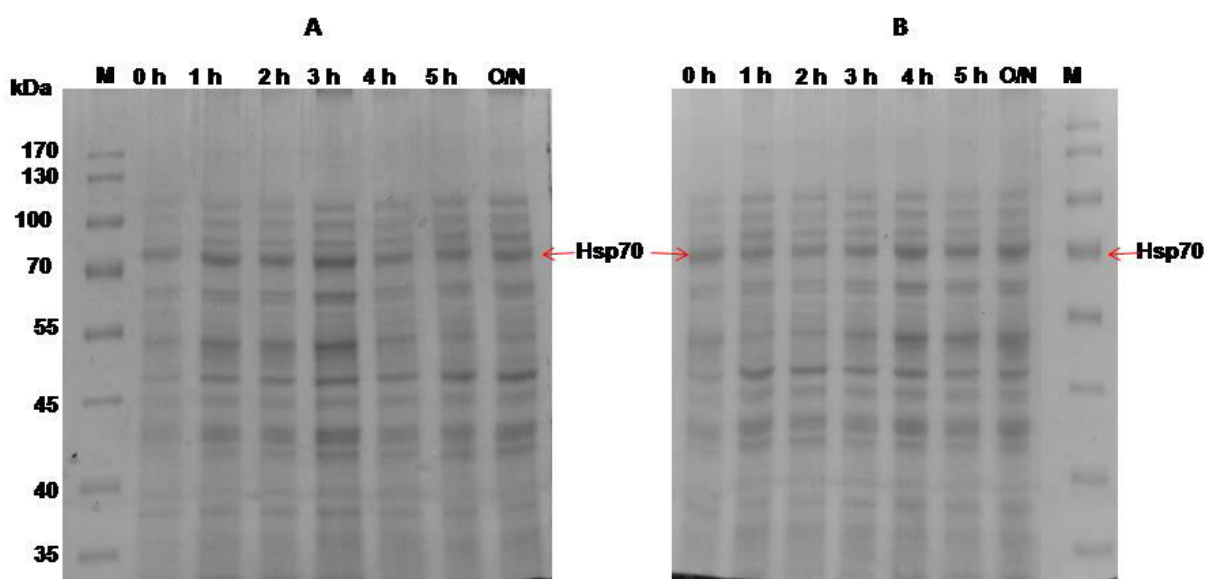
Figure 3.15b: Effect of RA5 at 0.05 mM (A) and 0.5 mM (B) on the expression of PfHsp70 in *E. coli* cells

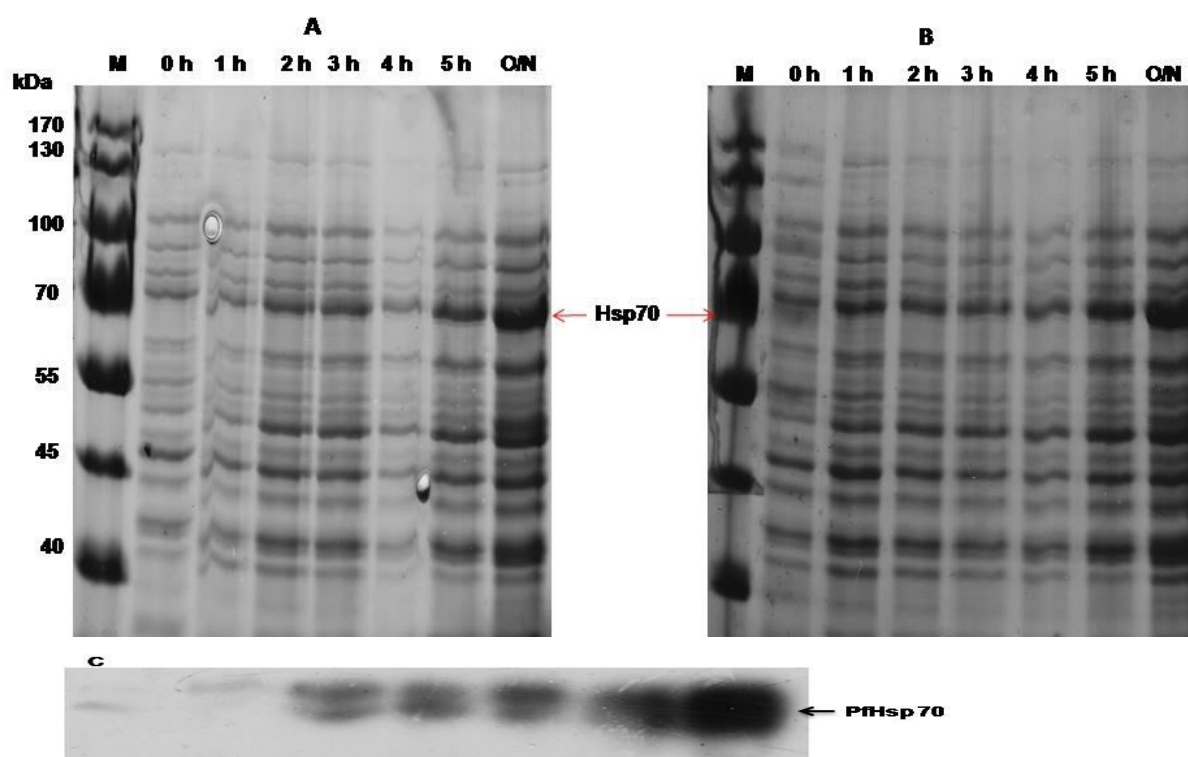**Figure 3.15c:** Effect of RA3 at 0.05 mM (A) and 0.5 mM (B) on the expression of PfHsp 70 in *E. coli* cells. The protein expression was confirmed with Western blot (C)

3B3.4.3 *Ex vivo* anticoagulant activity

The *ex vivo* anticoagulant activity of the triterpenes was evaluated using tail bleeding time assay. It is apparent that the triterpenes significantly ($p < 0.05$) prolonged

bleeding time up to 7.3 min (by RA3 at 250 mg/kg) as compared to 2.5 min in the normal control group (Table 3.5) and thus exhibit anticoagulation properties. The *ex vivo* anti-platelet aggregation activity of the triterpenes was, however, relatively not as high as that of ASA - a standard anti-platelet agent.

Table 3.5: Effect of the triterpenes on tail bleeding time and inhibition of platelet aggregation

Group	Dose (mg/kg)	Bleeding time (min)	Anti-platelet aggregation (%)
Control	2% Tween 20	2.50 ± 0.20	
ASA	30	4.50 ± 0.46[*]	19.3
RA3:	50	5.60 ± 0.96[*]	12.0
	250	7.30 ± 1.44[*]	15.8
RA5:	50	7.00 ± 1.04[*]	4.53
	250	6.00 ± 0.79[*]	9.32

Values are expressed as mean ± SEM, n = 5. [*] $p < 0.05$ compared to control

3B3.4.4 Anti-inflammatory activity

The cotton pellet-induced granuloma model was used to investigate the anti-proliferative activity of the triterpenes. This model is commonly used as an *in vivo* test to assess the transudative, exudative and proliferative components of chronic inflammation. The weight of wet and dry cotton pellets correlates with the amount of formed transudate material and granuloma tissue, respectively. Both triterpenes at 50 and 250 mg/kg significantly ($p < 0.05$) decreased transudates, and granuloma formation in the rats (Table 3.6). RA5 (250 mg/kg) effectively inhibited the granuloma formation by up to 40.3%. The inhibitory effect of the compounds (at 250 mg/kg) compared favourably with that of indomethacin (10 mg/kg), a standard non-steroidal anti-inflammatory drug (NSAID).

Table 3.6: Effect of the triterpenes on transudative and granuloma formation

Group	Dose mg/kg	Granuloma wet weight (mg)	Granuloma dry weight (mg)	Transudative weight (mg)	Granuloma inhibition (%)
Control		338 ± 4.05	96.6 ± 0.51	241.4 ± 4.33	
Indomethacin	10	168 ± 2.43^{**}	64.8 ± 2.43[*]	103.2 ± 2.33^{**}	32.9
RA3:	50	228 ± 1.02[*]	70.2 ± 0.11[*]	157.8 ± 0.05[*]	27
	250	174 ± 0.93^{**}	64.7 ± 1.01[*]	109.3 ± 1.35[*]	33
RA5:	50	222 ± 2.90[*]	67.9 ± 0.76[*]	154.1 ± 1.42[*]	29.7
	250	152 ± 2.15^{**}	57.7 ± 0.81[*]	94.3 ± 3.18^{**}	40.3

Values are expressed as mean ± SEM, n = 5. * $p < 0.05$, ** $p < 0.001$ compared to control

3B3.4.5 Biochemical estimation of protein content, catalase, SOD and COX activities

The protein content, catalase, SOD and COX activities were estimated. Table 3.7a shows that the triterpenes did not only reduce the protein content in the granulation tissue, but also significantly increased the activity of catalase. Though RA3 could not increase SOD activity, an appreciable increase (up to 1.08 units/mg protein) in the activity of SOD was observed following rats treatment with RA5 compared to the control group (0.75 units/mg protein). Compared to the control group, both compounds at 250 mg/kg, further showed a significant inhibition of COX activity (Table 3.7b). Activity of the compounds was in a manner similar to that of indomethacin. Even though the compounds showed more inhibition on COX-1 than on COX-2, the difference was basically not significant. The highest inhibitory activity (26.5%) by RA3 was observed on COX-1.

Table 3.7a: Effect of the compounds on protein content, catalase and SOD activity on the cotton pellet-induced inflammation in rats

Group	Dose (mg/kg)	Protein content (mg/ml)	Catalase activity (µmol H_2O_2 decomposed/min/mg protein)	SOD activity (units/mg protein)
Control		0.68 ± 0.11	96.7 ± 2.88	0.75 ± 0.42
Indometh	10	0.56 ± 0.19	$119.1 \pm 1.01^{*}$	1.26 ± 0.75
RA3:	50	0.60 ± 0.23	98.0 ± 0.08	0.25 ± 0.09
	250	0.45 ± 0.10	$121 \pm 0.15^{*}$	0.27 ± 0.26
RA5:	50	0.48 ± 0.94	$242.5 \pm 0.83^{**}$	1.04 ± 0.09
	250	0.52 ± 0.07	$256.4 \pm 1.22^{**}$	1.08 ± 0.26

Values are expressed as mean $\pm$ SD, n = 5. *p < 0.05, **p < 0.001 compared to control

Table 3.7b Effect of the compounds on COX activity

Sample	Dose (mg/kg)	COX Activity (U/ml)			% Inhibition	
		Total COX	COX-1	COX-2	COX-1	COX-2
COX Std		29.4 ± 0.04				
Control		27.7 ± 0.03	24.1 ± 0.04	23.9 ± 0.01	13.1	13.7
Indomethacin	10	29.7 ± 0.03	23.9 ± 0.012	25.2 ± 0.13	19.3^{*}	15.0
RA3	50	30.8 ± 0.07	27.1 ± 0.10	27.2 ± 0.16	12.2	11.8
	250	32.9 ± 0.05	24.3 ± 0.02	25.5 ± 0.01	26.3^{*}	25.5^{*}
RA5	50	27.5 ± 0.01	23.3 ± 0.01	24.0 ± 0.00	15.2	12.5
	250	17.9 ± 0.03	14.4 ± 0.01	14.8 ± 0.01	19.5^{*}	17.2

Data are expressed as mean $\pm$ SEM, n = 5. *p < 0.05 compared to control

3B3.4.6 Plasma content of Hsp70

The effect of the triterpenes on expression of Hsp70 was also determined by measuring plasma content of Hsp70 in the triterpene-treated inflamed rats. The results are shown in Table 3.8 as plasma concentration (ng/ml). The results indicate concentration dependent plasma content of the protein and at 250 mg/kg, both compounds significantly (p < 0.05) stimulated more expression of the protein even

better than indomethacin (10 mg/kg). Thus, it is evident that the triterpenes have the ability to stimulate Hsp70 expression *in vivo*.

Table 3.8: Plasma content of Hsp70 in inflamed rats

	Control	Indometh		RA3		RA5	
Dose	*2% Tween 20*	*10 mg/kg*	*50 mg/kg*	*250 mg/kg*	*50 mg/kg*	*250 mg/kg*	
Hsp70 (ng/ml)	1.20 ± 0.02	$6.80 \pm 0.03^*$	3.24 ± 0.44	$8.80 \pm 0.03^*$	1.68 ± 0.01	$7.12 \pm 0.04^*$	

Values are expressed as mean ± SEM, n = 5. $^*p < 0.05$ compared to control, indometh- indomethacin

3B3.4.7 *In vitro* antioxidant activity

Superoxide dismutase and nitric oxide radical assays were used to determine the antioxidant activity of the two triterpenes. Both compounds at the various concentrations (0-50 µg/ml) did not exhibit any radical scavenging activity.

3B3.5 Discussion

Inflammation is associated with excess production of superoxide radicals (Chang *et al*., 2010) which intensify inflammation by stimulating secretion of inflammatory mediators from macrophages (Porfire *et al*., 2009). Endogenous antioxidant enzymes such as catalase and SOD help to destroy the free radicals. Though the triterpenes did not show any direct *in vitro* antioxidant activity, their ability to significantly increase activity of the antioxidant enzymes (Table 3) partly explains a relatively higher inhibitory activity of the triterpenes (250 mg/kg) observed on the granuloma formation. While some studies have reported antioxidant activity of triterpenes (Russel and Paterson, 2006), lanostane triterpenes are known to be poor antioxidants (Zhou *et al*., 2008). The poor antioxidant activity of RA5 has previously been demonstrated using DPPH and ABTS radicals (Mosa *et al*., 2011b). It is apparent that the ability of the compounds to increase activity of the antioxidant enzymes (SOD and catalase) rather than direct antioxidant effect is vital in the compound's anti-inflammatory activity.

Furthermore the significant inhibitory activity shown by the compounds at 250 mg/kg on the COX activity (Table 3.7b) suggests that the anti-inflammatory activity of the compounds could also be mediated by inhibition of the COX pathway, thus reducing production of prostanoids and thromboxane. A slightly higher inhibitory activity on COX-1 than COX-2 further indicated the NSAIDs character of the compounds. Owing to the essential physiological functions of the constitutive COX-1 (Vane, 1996, Dubois *et al.*, 1998), selective inhibitors of the inducible COX-2 are highly desired and considered much safer drugs. However, COX-2 inhibitors have also been associated with some risk of cardiovascular events (Kearney *et al.*, 2006). It has also previously been suggested that selective COX-2 inhibitors prevent synthesis of prostacyclin (the anti-thrombotic prostaglandin) by endothelial cells, thus promoting thromboxane-induced platelet aggregation (McAdam *et al.*, 1999). Like aspirin, the slightly higher inhibitory activity on the COX-1 than COX-2 may partly explain the anticoagulant or anti-platelet aggregation activity of the compounds. Anti-inflammatory agents with anticoagulant activity are therapeutically important in the prevention of thromboembolic disorders.

In contrast to the results obtained in this study, several other triterpenes from different plants have shown to be potent selective COX-2 inhibitors (Kaur et al., 2010). The anti-inflammatory activity of triterpenes from *Garnoderma lucidum* were reported to be through various mechanisms including suppression of cytokines release, inhibition of nitric oxide synthase and COX-2 in lipopolysaccharide-stimulated macrophages (Dudhgaonkar *et al.*, 2009).

Even though inappropriate protein aggregation is normally prevented by complex cellular mechanisms, however under certain circumstances aggregation of proteins occurs. Misfolding and inappropriate aggregation of proteins is commonly associated with a number of neurodegenerative diseases (Lendel *et al.*, 2009). Hsps are ubiquitous proteins found in the cells of all living organisms. These proteins function as intra-cellular chaperons for other proteins, preventing protein misfolding and unwanted protein aggregation (Shonhai *et al.*, 2008). Hsps are involved in many disease processes, including inflammation, cardiovascular and neurological diseases (Whitley *et al.*, 1999, Luo and Le, 2010). Therefore, there is a growing interest in the

discovery of pharmacologically active drugs that up regulate expression of Hsps as a potential therapeutic effect in human disease.

The ability of the compounds to improve the activity of Hsp70 (Figure 3.13-14) suggests that the triterpenes could partly be exerting their therapeutic properties through inhibition of protein aggregation. The ability of the compounds to effectively induce expression of Hsp70 both in *E. coli* cells and plasma of inflamed rats (Table 3.8) further strongly supports the anti-protein aggregation activity of the compounds. The induction of Hsp70 and subsequent inhibition of protein aggregation are vital in the prevention of inflammation and blood coagulation. While several flavonoinds (Hosokawa *et al.*, 1990) have been reported to inhibit synthesis of Hsp 70, Celastrol (a plant-derived triterpene) and aspirin (a famous anti-platelet drug) have been reported to promote induction of Hsps (Jurivich *et al.*, 1992; Chow and Brown, 2007).

The *in vitro* anticoagulant and/or anti-platelet aggregation activity of a number of triterpenoids from various plants have been reported (Habila *et al.*, 2011; Lee *et al.*, 2012). The ability of RA5 to inhibit platelets aggregation induced by thrombin and other platelet agonists has also been reported (Mosa *et al.*, 2011b). The results obtained demonstrate that the triterpenes possess anticoagulant properties as they significantly prolonged the tail bleeding time in rats (Table 3.5). The lower anti-platelet aggregation activity activity relative to the standard drug could be due to the bioavailability of the compound. Furthermore, previous studies (Mekhfi *et al.*, 2008; Gadi *et al.*, 2009) have shown that there is not always a direct correlation between inhibition of platelet aggregation and the bleeding time. Also the possibility that ASA could have permanently inhibited activation and aggregation of the platelets could not be ruled out in contributing to the higher anti-platelet aggregation activity in the positive control.

The anti-inflammatory activity of RA5 using the carrageenan-induced paw edema model (suitable for acute inflammation) has previously been reported (Mosa *et al.*, 2011b). In this study, the anti-proliferative activity of the two triterpenes was investigated using the cotton pellet-induced granuloma model. Chronic inflammation is characterised by infiltration of mononuclear cells, fibroblast proliferation and increased connective tissue formation (Beni *et al.*, 2011). Cotton pellet-induced

granuloma model is commonly used as an *in vivo* test to assess the transudative, exudative and proliferative components of chronic inflammation.

The anti-proliferative activity of NSAIDs is characterised by a decrease in granuloma tissue formation, collagen fibre generation, and suppression of muccopolysaccharides (Verma *et al*., 2010). The results from this study (Table 3.6) suggest the NSAIDs character of the triterpenes from *P. longifolia* which indicates their potential to prevent chronic inflammation. The anti-inflammatory activity of other triterpenes has been previously reported (Yadav *et al*., 2010).

In conclusion this study demonstrated that the triterpenes from stem bark of *P. longifolia* possesses anticoagulant and anti-inflammatory activity. The observed activities of the triterpenes could be attributed to their ability to inhibit protein aggregation. Compounds with anti-inflammatory activity may significantly prevent inappropriate blood coagulation and, therefore, alleviate thromboembolic disorders.

References

Aebi, H. (1983). Catalase, In: Bergmeyer HU, Ed. *Methods of Enzymatic Analysis* 2. New York: Academic Press, 673-684.

Aggarwal, B.B. Shishodia, S. Sandur, S.K. Pandey, M.K. Sethi, G. (2006). Inflammation and cancer: How hot is the link? *Biochemical Pharmacology* 72: 1605-1621.

Beni, J.M. Jayanthi, M.K. Suresha, R.N. (2011). Evaluation of the anti-inflammatory activity of *Aeglemarmelos* (Bilwa) root. *Indian Journal of Pharmacology* 43: 393-397.

Blair, P. and Flaumenhaft, R. (2009). Platelet alpha-granules: Basic biology and clinical correlates. *Blood Reviews* 23: 177-189.

Chang, C. Zhang, L. Chen, R. Wu, C. Huang, H. Roy, M.C. Huang, J. Wu, Y. Kuo, Y. (2010). Quiquelignan A-H, eight new lignoids from the rattam palm *Calmus quiquesetinervious* and their antiradical, anti-inflammatory and antiplatelet aggregation activities. *Bioorganic & Medicinal Chemistry* 18: 518-525.

Chow, A.M. and Brown, I.R. (2007). Induction of heat shock proteins in differentiated human and rodent neurons by celastrol. Cell Stress Chaperones 12(3): 237-244

Chu, A.J. (2011). Tissue factor, blood coagulation, and beyond: an overview. *International Journal of Inflammation* 2011: doi: 10.4061/2011/367284.

Cohen, A.T. Dobromirski, M. Lin, S.L. Wills, J.O. (2011). Venous thromboembolism: The magnitude of the problem in Europe and the world. *Advances in Venous Arterial Thrombosis* 1: 39-45.

Dubois, R.N. Abramson, S.B. Crofford, L. Gupta, R.A. Simon, L.S. Van De Putte, L.B.A. Lipsky, P.E. (1998). Cyclooxygenase in biology and disease. *The Journal of the Federation of American Societies of Experimental Biology* 12: 1063-1073.

Gadi, D. Bnouham, M. Aziza, M. Ziyyat, A. Legssyera, A. Legrand, C. Lafeveb, F.F. Mekhfi, H. (2009). Parsley extract inhibits *in vitro* and *ex vivo* platelet aggregation and prolongs bleeding time in rats. *Journal of Ethnopharmacol*ogy 125: 170-174.

Ghosh, T. Maity, T.K. Singh, J. (2011). Antihyperglycemic activity of bacosine, a triterpene from *Bacopa monnieri*, in alloxan-induced diabetic rats. *Planta Medica* 77: 804-8.

Glannopolittis, C.N. and Ries, S.K. (1977). Superoxide dismutase occurrence in higher plants. *Plant Physiol*ogy 59: 309-314.

Habila, J.D. Shode, F.O. Opoku, A.R. (2011). Triterpenoids from *Eucalyptus grandis* Hill ex Maiden inhibits platelet aggregation. *African Journal of Microbiology Res*earch 5: 4646-4651.

Hirsh, J. O'Donnell, M. Eikelboom, J.W. (2007). Beyond unfractionated heparin and warfarin: current and future advances. *Circulation* 116: 552-60.

Jäger, S. Trojan, H. Kopp, T. Melanie, N. Laszczyk, M.N. Scheffler, A. (2009). Pentacyclic triterpene distribution in various plants – rich sources for a new group of multi-potent plant extracts. *Molecules* 14: 2016-2031.

Kaur, P. Kaur, S. Kumar, S. Singh, P. (2010). *Rubia cordifolia* L. and *Glycyrrhiza glabra* L. Medicinal plants as potential source of COX-2 inhibitors. *American Journal of Biomedical Science* 2(2): 108-120.

Kearney, P.M. Baigent, C. Godwin, J. Halls, H. Emberson, J.R. Patrono, C. (2006). Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. bmj.com 332:1302.

Khanapure SP, Garvey DS, Janero DR, Letts LG (2007). Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. *Current Topics in Medicinal Chemistry* 7: 311-340.

Lee, W. Yang, E.J. Ku, S.K. Song, K.K. Bae, J.S. (2012). Anticoagulant activities of oleanolic acid via inhibition of tissue factor expressions. *BMB Reports* 45: 390-395.

Lendel, C. Bertoncini, C.W. Cremades, N. Christopher, A. Waudby, C.A. Vendruscolo, M. Dobson, C.M. Schenk, D. Christodoulou, J. Toth, G. (2009). On the mechanism of nonspecific inhibitors of protein aggregation: dissecting the interactions of R-synuclein with Congo red and Lacmoid. *Boichem*istry 48: 8322-8334.

Liu, J. Sun, H. Wang, X. Mu, D. Liao, H. Zhang, L. (2007). Effects of oleanolic acid and maslinic acid on hyperlipidemia. *Drug Development Research* 68: 261-266.

Luo, G.R. and Le, W.D. (2010). Collective roles of molecular chaperones in protein degradation pathways associated with neurodegenerative diseases. *Current Pharmaceutical Biotechnology* 11(2): 180-187.

Mavrakanas, T. and Bounameaux, H. (2011). The potential role of new oral anticoagulants in the prevention and treatment of thromboembolism. *Pharmacology &Therapeutics* 130: 46-58.

McAdam, B.F. Catella-Lawson, F. Mardini, I.A. Kapoor, S. Lawson, J.A. FitzGerald, G.A. (1999). Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. *Proceeding of National Academic Sciences (U.S.A)* 96: 272-277.

Mekhfi, H. Gadi, D. Bnouham, M. Ziyyat, A. Legssyer, A. Aziz, M. (2008). Effect of argan oil on platelet aggregation and bleeding time: a beneficial nutritional property. *Journal Complementary and Integrative Medicine* 5: 1553-3840.

Mekhfi, H. Haouari, M.E. Legssyer, A. Bnouham, M. Aziz, M. Atmani, F. Remmal, A. Ziyyat, A. (2004). Platelet anti-aggregant property of some Moroccan medicinal plants. *Journal of Ethonpharmacol*ogy 94: 317-322.

Mosa, R.A. Oyedeji, O.A. Shode, F.O. Singh, M. Opoku, A.R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit antiplatelet aggregation activity. *African Journal of Pharmacy and Pharmacology* 5: 2698-2714.

Nagar, A. Shukla, A.K. Bigoniya, P. (2011). Anti-inflammatory potential of *Spinacia oleracea* leaf extract. *Journal of Natural Pharmaceuticals* 2: 80-87.

Penn, G.B. and Ashford, A. (1963). The inflammatory response to implantation of cotton pellets in the rat. *Journal of Pharmacy and Pharmacology* 15: 798-803.

Porfire, A.S. Pârvu, A.E. Daicoviciu, D. Leucuta, S.E. (2009). Evaluation of anti-inflammatory activity of loposome encapsulated superoxide dismutase in rats. *Farmacia* 57: 412-423.

Ramya, T.N.C. Surolia, N.N. Surolia, A. (2006). 15-Deoxyspergualin modulates *Plasmodium falciparum* heat shock protein function. *Biochemical and Biophysical Research Communications* 348: 585-592.

Russell, R. and Paterson, M. (2006). *Ganoderma*- A therapeutic fungal biofactory. *Phytochemistry* 67(18): 1985-2001.

Shonhai, A. Botha, M. de Beer, T.A.P. Boshoff, A. Blatch, G.L. (2008). Structure- function study of a *Plasmodium falciparum* Hsp 70 using three dimensional modeling and in vitro analyses. *Protein and Peptide Letters* 15: 1117-1125.

Tomita, T. Umegaki, K. Hayashi, E. (1983). Basic aggregation properties of washed rat platelets: Correlation between aggregation, phospholipid degradation, malondialdehyde, and thromboxane formation. *Journal of Pharmacological Methods:* 10: 31-44.

Vane, J.R. (1996). Introduction: mechanism of action of NSAIDS. *British Journal of Rheumatology* 35: l-3.

Verhamme, P. and Hoylaerts, M.F. (2009). Haemostasis and inflammation: two of kind? *Thrombosis Journal* 7: http://www.thrombosisjournal.com/content/7/1/15.

Verma, S. Ojha, S. Raish, M. (2010). Anti-inflammatory activity of *Aconitum heterophyllum*on cotton pellet-induced granuloma in rats. *Journal of Medicinal Plant Research* 4: 1566-1569.

Whitley, D. Goldberg, S.P. Jordan, W.D. (1999). Heat shock proteins: A review of the molecular chaperons. *Journal of Vascular Surgery* 29(4): 748-751.

Yadav, V.R. Prasad, S. Sung, B. Kannappan, R. Aggarwal, B.B. (2010). Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer. *Toxins 2*: 2428-2466.

Zhou, L. Zhang, Y. Gapter, L.A. Ling, H. Agarwal, R. Ng, K.Y. (2008). Cytotoxic and anti-oxidant activities of lanostane-type triterpenes isolated from *Poria cocos. Chemical and Pharmaceutical Bulletin* 56(10): 1459-1462.

PART 3C: ANTIMICROBIAL and ANTIPLASMODIAL STUDY

Abstract

Antimicrobial resistance of pathogenic strains has hugely contributed to the wide spread of new and re-emerging infectious diseases. There is, thus, a need for the discovery of new antimicrobials with an improved efficacy and a better safety profile. The antimicrobial activity of the triterpenes from *P. longifolia* against fungi, a panel of selected general and antibiotic resistant Gram positive and Gram negative bacteria was evaluated. The antiplasmodial activity of the triterpenes was also investigated against chloroquine sensitive (CQS) strain of *P. falciparum* (D10). The broth microdilution assay was used to determine the antimicrobial activity of the isolated compounds. The *in vitro* antiplasmodial activity of the compounds was quantitatively assessed using parasite lactate dehydrogenase assay. The compounds exhibited antibacterial activity against most of the tested bacteria with MIC and MBC values ranging from 0.16 to 5.00 mg/mL and 0.31 to 5.00 mg/mL, respectively. RA3 exhibited MIC_{99} of 37.6 µg/ml on *Mycobacterium tuberculosis* (H37Rv). The triterpenes did not exhibit any bacterial DNA damaging effects, but apparently RA3 affected the microbial cell membrane integrity. The compounds did not exhibit any antiplasmodial activity and only showed antifungal activity when combined, indicating synergism. The triterpenes could be potentially effective antibacterial agents to combat infectious diseases.

3C.1 Introduction

Pathogenic microorganisms are the major cause of a wide range of infectious diseases. Microbial infections account for significant causes of morbidity and mortality worldwide (Tajbakhsh *et al.*, 2011). Despite the availability of the current antimicrobial drugs, the development of antimicrobial resistance to some of the current antibiotics is a serious global challenge (Hoffmann *et al.*, 2011; Chung *et al.*, 2013). The indiscriminate use of antimicrobial drugs has hugely contributed to this natural phenomenon. The incidence of antimicrobial drug resistance has been increasing at an alarming rate. However, only a few new, effective antimicrobial drugs have been developed. Discovery and development of new effective antimicrobial agents with

novel mechanisms of action (Rojas *et al.*, 2003, Zakaria *et al.*, 2009) is currently a public health priority.

Tuberculosis, a chronic infectious disease caused by *Mycobacterium tuberculosis*, continues to be a major common health problem. Evidence from research studies has revealed plants as potential sources of new active antimycobacterial compounds (Robles-Zepeda *et al.*, 2013). Similarly malaria, a parasitic disease caused by the drug resistant *Plasmodium falciparum*, has a huge health impact, infecting and killing millions of people each year (Alonso *et al.*, 2011). Thus, the continuous search for new generation of drugs is necessary.

A growing body of evidence supports medicinal plants as rich sources of bioactive compounds with significant potential antimicrobial and/or antiplasmodial activity (Mahesh and Satish, 2008; Nethengwe et al., 2012; Malebo *et al.*, 2013). Numerous reports have indicated triterpenes as one of the most active class of plant-derived compounds with potent antimicrobial (Jim´enez-Arellanes *et al.*, 2007; Martins *et al.*, 2011) and antiplasmodial (Simelane *et al.*, 2013) activity.

Thus, the antimicrobial and *in vitro* antiplasmodial activity of the two lanostane-type triterpenes isolated from stem bark of *P. longifolia* was evaluated.

3C.2 Materials

3C.2.1 Equipment

See the list in 3B1.2.2

3C.2.2 Reagents

Sigma-Aldrich: Ampicillin, neomycin, amphotericin B, iodonitrotetrazolium chloride (INT), phenazinemethosulfate, nicotinamide adenine dinucleotide (NAD$^+$), lactic acid, triton X-100, DMSO, Rhodamine 6G, erythromycin, berberine, sodium azide
Merck: nutrient agar, nutrient broth, Mueller-Hinton broth, Mueller-Hinton agar, Sabourad broth, Sabouraud dextrose agar (Oxoid), tris aminomethane, boric acid, ethidium bromide, EDTA

ZR Fungal/bacterial DNA MiniPrep™ kit (Zymo Research, U.S.A, Catalogue No. D6005)

Glycerol-alanine-salts-Tween (GAST/Fe) medium, rifampicin, kanamycin, alamar blue, chloroquine (CQ), artesunate, MMV390048

3C.2.3 Microorganisms

The Gram positive and Gram negative bacteria (Table 3.9) were obtained from the Department of Microbiology, University of Zululand while fungal strains (*Aspergillus terreus, Rhizomucor pusillus, Absidia corymbifera, Candida rugosa, Cryptococcus neoformans, Cryptococcus albidus, Candida albicans, Asperigillus niger, Penicillium notatum)* were obtained from Univeristy of Fort Hare. *Mycobacterium tuberculosis* (H37RvMa) *and Plasmodium falciparum* (D10, NF54) were obtained from University of Cape Town. Antibiotic resistant strains (Table 3.10) of clinical isolates were obtained from the Lancet Pathology Laboratory (Durban, South Africa). Mueller-Hinton agar and Sabouraud dextrose agar (Oxoid) were used to maintain bacterial and fungal stock cultures, respectively, and these were kept at 4°C.

Table 3.9: List of general Gram negative and Gram positive bacteria

Gram negative bacteria	Gram positive bacteria
Salmonella spp., KZN	*Bacillus pumilus,* ATCC 14884
Enterobacter cloacae, ATCC 13047	*Staphylococus aureus,* ATCC 6538
Escherichia coli, ATCC 8739	*Staphylococus aureus,* KZN
Escherichia coli, KZN	*Bacillus subtilis,* KZN
Klebsiella pneumoniae ATCC, 10031	*Staphylococcus faecalis, KZN*
Pseudomonas aeruginosa, ATCC 19582	
Proteus mirabilis, KZN	
Shigella flexneri, KZN	
Serratia marscens, ATCC 9986	
Klebsiella pneumoniae, ATCC 10031	

Table 3.10: List of antibiotic resistant bacterial strains and antibiotics they are resistant to.

Bacterial strain		Resistant to
Pseudomonas aeruginosa	T3374	Cotrimoxazole
Staphylococcus aureus	P12702	Cipro: Levo, Clindamycin
Staphylococcus aureus	P12724	Cipro: Levo, Clindamycin
Staphylococcus aureus	B10808	Oxa: Clox, Oxa: Meth, Gentamicin, Penicillin
Streptococcus viridans	S17141	Oxa: Meth, Oxa: Clox

Clox- Cloxacillin, Cipro- Ciprofloxacin, Levo- Levofloxacin, Meth- Methicillin, Oxa- Oxacillin

3C.3 Methods

3C.3.1 Disk diffusion method

Antimicrobial activity of the triterpenes was investigated by the agar disc diffusion method (Vlientik *et al.*, 1995). Overnight bacterial cultures were diluted to a final cell density (1.0 × 10^8 CFU/ml) equivalent to 0.5 McFarland standard, and 1.0 x 10^4 spore/ml of fungi. Sterile paper discs (6 mm diameter), impregnated with the triterpene (20 mg/ml 10% DMSO) were placed on nutrient agar (Sabouraud agar for fungi) which was inoculated with the bacterial (1.0 × 10^8 CFU/ml) or fungal (1.0 × 10^4 spore/ml) suspension. DMSO (10%) was used as a negative control while ampicillin and neomycin both at the concentration of were used as positive controls for bacteria, and amphotericin B was used as a positive control for fungi. The inoculated plates were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the zone of inhibition (mm) against the tested organism.

3C.3.2 Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

The MIC of the triterpenes on the tested organisms was determined by the broth microdilution method (Eloff, 1998). Overnight bacterial cultures in Muller-Hinton broth were standardized to 0.5 McFarland. The MIC and MBC of the compounds were quantitatively determined using 96-well plates. The tests were replicated three times and the mean values reported.

Nutrients broth (50 μl) was put into all wells of the 96-well plate and 50 μl of the test compound (20 mg/ml) was added to the wells in the first row (A). This was mixed well and a 2-fold serial dilution was performed down the rows by initially removing 50 μl of the sample mixture from all the wells in the row A into the wells in row B. This was continued until the last row and the last 50 μl of the sample mixture was discarded. Subsequently, the respective bacterial cultures (50 μl) were added into the appropriate wells. For controls, 10% DMSO instead of the test compound was used (negative control); ampicillin and neomycin were used as standard antibiotics (positive controls). The plates were well covered and then incubated at 37°C for 24 h. Iodonitrotetrazolium chloride (INT) (20 μL, 0.2 mg/ml) was introduced to all of the wells and the plates were covered, then incubated for a further 30 min at 37°C. The MIC was recorded as the lowest concentration of the tested compounds that inhibited bacterial growth.

The MBC of the triterpenes along with the positive controls (ampicillin and neomycin) was determined by transferring 10 μl of each culture medium from the wells with no observable bacterial growth onto sterile nutrient agar plates. The plates were well covered and then incubated at 37°C for 24 h. Following the incubation, the plates were observed for presence or absence of bacterial growth. The MBC was recorded as the lowest concentration at which no bacterial growth recurred.

3C.3.3 Lactate dehydrogenase (LDH) release assay

The cytosolic LDH release assay was used to evaluate the effect of the triterpenes on the bacterial membrane integrity (Tadić *et al.*, 2012). Fresh cultures of the most susceptible bacteria were prepared. The cultures were then exposed to the MBC of the compounds at 37°C for 24 h. This was followed by centrifugation (5000 × g for 5 min) of the bacterial suspension. The supernatant was collected and used for the assay. The supernatant (100 μl) was mixed with 100 μl of reaction mixture (54 mM lactic acid, 1.3 mM NAD$^+$, 0.28 mM of phenazinemethosulfate, 0.66 mM INT). The mixture was incubated at 37°C for 10 min. The pyruvate-mediated reduction of INT into highly-coloured formazan (red) was measured at 490 nm. For controls, 10% DMSO and 3% triton X-100 were used as negative control and positive control, respectively. The experiment was repeated three times and the mean values were

reported. Percentage (%) LDH released upon the cellular membrane damage was calculated using the formula:

% LDH released = [(A_E-A_C)/(A_T-A_C)] ×100,

where A_E- absorbance of test compound-treated cell culture, A_C- absorbance of control (cell medium alone), and A_T-absorbance of Triton X-100 lysed cells [representing maximal (100%) LDH released].

3C.3.4 Bacterial DNA damage

The effect of the triterpenes on bacterial DNA was evaluated following the method described by Liu *et al.* (2011). The bacteria that were most susceptible to the compounds were chosen and used for the assay. Fresh bacterial cultures were prepared and treated with the MBC of the compound. For control, 10% DMSO was added instead of the test compounds. Both untreated and triterpene-treated bacteria were incubated at 37°C for 24 h. Bacterial DNA from the respective cultures was extracted and purified using ZR Fungal/bacterial DNA MiniPrep™ kit (Zymo Research, U.S.A) according to the manufacturer's instructions. Briefly, bacterial samples were put into ZR lysis tubes and centrifuged at 10 000 × g for 10 min. Supernatant was discarded while the remaining pellet was resuspended in the lysis solution for 5 min. DNA (Fungal/Bacterial) Binding Buffer was then introduced to the suspension and mixed well. The suspension was centrifuged at 10 000 × g for 10 min and the resulted supernatant was collected. The DNA was precipitated and purified following a series of centrifugation and washing with buffers. Elution buffer was used to eventually elute the pure DNA. For analysis and confirmation of the DNA damage, agarose gel electrophoresis (150v for 30 min) was used to analyse the DNA extracted from both the untreated and triterpene-treated bacteria. DNA maker was run along the tested bacterial DNA. Vilberlourmate Gel documentation system was used to visualize the DNA.

3C.3.5 Antimycobacterium activity

3C.3.5.1 Broth micro dilution method

Broth micro dilution method (Collins *et al.,* 1997; Collins *et al.,* 1998) was used to evaluate the antimycobacterium activity of the two triterpenes from *P. longifolia.*

Briefly, a 10 ml of *Mycobacterium tuberculosis* (H37Rv) culture (Loerger *et al.*, 2010) was grown to an OD_{600} of 0.6 - 0.7. The culture was then diluted (1:100) in GAST/Fe medium. Stock solutions of the test compounds were made up to 12.8 mM in DMSO, and diluted to 640 µM in GAST/Fe medium.

GAST/Fe medium (50 µl) was added to all wells of a 96-well microtitre plate from Rows 2-12. The compounds to be tested were added to Row 1 in duplicate, at the final concentration of 640 µM. This was followed by a two-fold serial dilution, transferring 50 µl down the rows until the last row where the last 50 µl of the sample mixture was discarded so as to bring the final volume in all the wells to 50 µl. The diluted (1:100) *M. tuberculosis* culture (50 µl) was added to all wells in Rows 2-12. Cells were not added in Row 1 which served as a contamination control. Media only, 5% DMSO, Rifampicin and Kanamycin were used as controls. The 96-well plate was stored in a secondary container and incubated at 37°C for 7-14 days with humidifier (to prevent evaporation of liquid). The lowest concentration of drug that inhibits growth of more than 99% of the bacterial population was considered to be the MIC_{99}. The MIC_{99} values were scored visually by observing the presence (white pellet at bottom of a well) or absence (no pellet) of bacterial growth at 7-days and 14-days after inoculation. Alamar blue is a colorimetric growth indicator that turns pink in the presence of bacterial growth or remains blue in the absence of growth. At day 14 alamar blue was added to the wells to confirm the results.

3C.3.7 *In vitro* antiplasmodial activity

The test compounds were tested in triplicate on one occasion against chloroquine sensitive (CQS) strain of *P. falciparum* (D10) or (NF54). A method of Trager and Jensen (1976) with modification was used to continuously maintain *in vitro* cultures of asexual erythrocytes stages of *P. falciparum*. A modified method of Makler (1993) was followed to quantitatively assess *in vitro* antiplasmodial activity of the compounds using the parasite lactate dehydrogenase assay.

Stock solutions (20 mg/ml) of the samples were prepared in 100% DMSO and stored at -20°C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ), artesunate and MMV390048 (in-house control) were used as the reference drugs in all the experiments. Test samples were tested at 20 µg/ml, 10 µg/ml and 5 µg/ml, while the reference drugs were tested at 30 ng/ml, 15 ng/ml and 7.5 ng/ml.

The concentration inhibiting 50% of parasite growth (IC_{50}-values) was determined by performing a full dose-response on active samples. Test samples were tested at initial concentration of 100 µg/ml (1000 ng/ml for reference drugs), followed by a 2-fold serial dilution in complete medium to give 10 concentrations; with the lowest concentration being 0.2 µg/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). GraphPad Prism v.4.0 software was used to obtain the IC_{50}-values from a non-linear dose-response curve fitting analysis.

3C.4 Results

The results of the antimicrobial activity of the triterpenes from *P. longifolia* are given in Tables 3.11-5. The compounds exhibited antibacterial activity against most of the tested bacteria with zones of inhibition greater than 7 mm (Table 3.11). The compounds exhibited the MIC and MBC values ranging from 0.16 to 5.00 mg/ml and 0.31 to 5.00 mg/ml, respectively (Table 3.12-3.13). Table 3.14 further indicates that RA3 (MIC_{99} = 37.6 µg/ml) even effectively inhibited growth of *Mycobacterium tuberculosis* H37Rv.

Table 3.11: Zones of inhibition (mm) of the triterpenes on some sensitive and antibiotic resistant bacteria

Bacteria	Resistant to	RA5	RA3	Ampicillin	Neomycin
E. coli (ATCC 8739)	-	10 ± 0.01	13 ± 0.12	14 ± 0.07	12 ± 0.05
Salmonela spp. (KZN)	-	12 ± 0.00	15 ± 0.10	11 ± 0.01	10 ± 0.09
P. aeruginosa ATCC 19582	-	13 ± 0.20	15 ± 0.45 :	14 ± 0.15	13 ± 1.00
S. aureus (KZN)	-	13 ± 0.03	12 ± 0.01	14 ± 0.01	13 ± 0.01
P. mirabilis (KZN)	-	11 ± 0.05	14 ± 0.03	12 ± 0.00	11 ± 0.10
P. aeruginosa T3374	Cotrimoxazole	8.0 ± 1.00	13 ± 0.90	11 ± 0.01	14 ± 1.20
S. aureus P12724	Cipro: Levo, Clindamycin	11 ± 1.01	13 ± 0.60	10 ± 0.24	11 ± 0.50
S. aureus B10808	Oxa: Meth, Penicillin	11 ± 0.00	16 ± 1.14	13 ± 0.10	9.0 ± 0.10
S. aureus P12702	Cipro: Levo, Clindamycin	12 ± 0.15	15 ± 0.10	13 ± 1.50	12 ± 0.08
S. viridans S17141	Oxa: Meth, Oxa: Clox	10 ± 0.50	16 ± 1.24	8.0 ± 0.00	11 ± 0.25

The results are expressed as mean ± SD. Clox- Cloxacillin, Cipro- Ciprofloxacin, Levo- Levofloxacin, Meth- Methicillin, Oxa- Oxacillin

Table 3.12: MIC and MBC in mg/ml of the triterpenes on the general bacteria

Bacteria	RA5		RA3		Ampicillin		Neomycin	
	MIC	MBC	**MIC**	MBC	**MIC**	MBC	**MIC**	MBC
E. coli (ATCC 8739)	**1.25**	1.25	**1.25**	1.25	**0.16**	1.25	**0.63**	2.50
Salmonela spp. (KZN)	**0.16**	1.25	**1.25**	2.50	**0.63**	0.63	**0.31**	1.25
P. aeruginosa ATCC	**0.63**	5.00	**0.63**	5.00	**0.16**	0.63	**1.25**	1.25
S. aureus (KZN)	**0.31**	2.50	**0.16**	1.25	**0.31**	1.25	**0.16**	0.31
P. mirabilis (KZN)	**1.25**	5.00	**0.31**	1.25	**0.16**	0.31	**0.31**	2.50

Table 3.13: MIC and MBC in mg/ml of the triterpenes on the resistant bacteria

Bacteria	1		2		Ampicillin		Neomycin	
	MIC	MBC	**MIC**	MBC	**MIC**	MBC	**MIC**	**MBC**
P. aeruginosa T3374	**0.63**	1.25	**0.63**	1.25	**0.31**	2.50	**2.50**	2.50
S. aureus P12702	**2.50**	5.00	**1.25**	2.50	**1.25**	1.25	**0.63**	5.00
S. aureus P12724	**0.16**	0.63	**1.25**	2.50	**0.63**	2.50	**1.25**	1.25
S. aureus B10808	**1.25**	1.25	**0.63**	1.25	**1.25**	1.25	**0.63**	2.50
S. viridans S17141	**5.00**	5.00	**5.00**	5.00	**2.50**	5.00	**1.25**	2.50

Table 3.14: *In vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv

Sample	**Rifampicin**	**Kanamycin**	**RA3**	**RA5**
MIC$_{99}$ (µg/ml)	0.01	3.125	37.6	> 73

RA3 showed some membrane damaging effect (Table 3.15) with high LDH release observed in antibiotic sensitive Gram negative bacteria (*P. mirabilis*, 89.6%; *E. coli*, 76.3%). Both compounds did not show any DNA damaging effect (Figure 3.16a-b). Table 3.16 shows that the compounds individually lack antifungal activity, but the activity was only observed when mixed.

Table 3.15: Effect of the triterpene (**RA3**) on bacterial cell membrane

Bacteria	LDH released (%)
E. coli ATCC 8739*	76.3
P. mirabilis*	89.6
S. aureus P12702	12.8
S. aureus P12724	62.2
S. aureus B10808	4.36
P. aeruginosa T3374	25.4

*- Non resistant strain
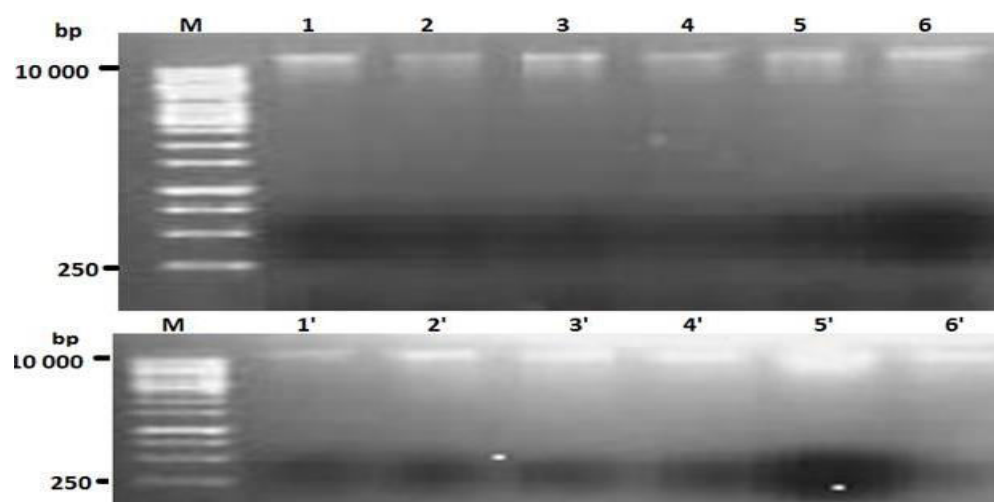
Figure 3.16a: Effect of triterpene (**RA3**) on bacterial (*E. coli* ATCC 8739, *S. aureus* P 12702, *S. aureus* P12724*, P. aeruginosa* T 3374, *S. aureus B 10808*, *P. mirabilis) DNA.* M- DNA marker, lane 1-6- untreated DNA, lane 1'-6'- treated DNA.
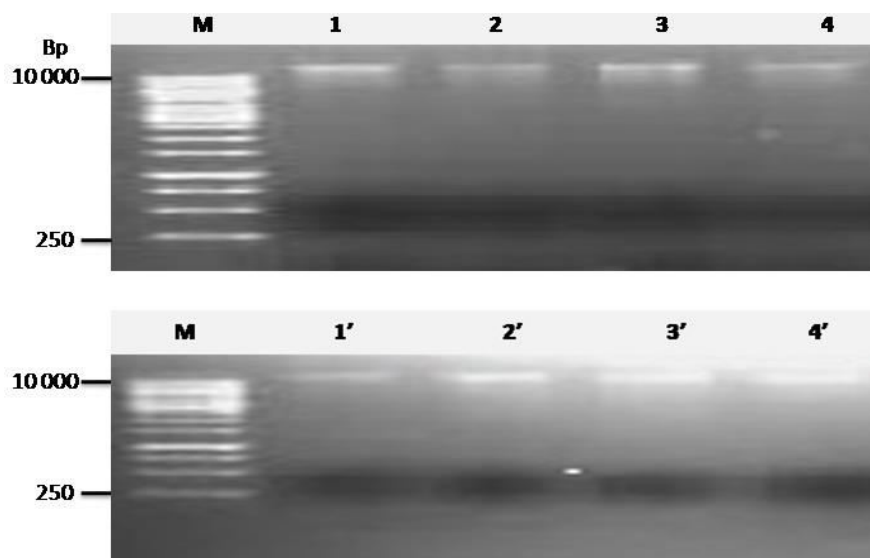
Figure 3.16b: Effect of triterpene (**RA5**) on bacterial (*E. coli* ATCC 8739, *S. aureus* P 12702, *S. aureus* P12724, *P. aeruginosa* T 3374) *DNA.* M- DNA marker, lane 1-4- untreated, DNA, lane 1'-4'-treated DNA.

Table 3.16: Antifungal activity of the triterpenes presented with zones of inhibition (mm)

Fungal strain				RA3:RA5		Amphotericin B
	RA3	RA5	1:1	2:1	1:2	
A. terreus	NA	NA	20	25	19	24
R. pusillus	NA	NA	20	21	15	20
A. corymbifera	NA	NA	20	19	16	24
C. rugosa	NA	NA	25	26	19	22
C. neoformans	NA	NA	26	26	18	24
C. albidus	NA	NA	19	21	17	16
C. albicans	NA	NA	19	26	19	30
A. niger	NA	NA	21	22	16	24
P. notatum	NA	NA	18	25	17	13

NA- Not active

Both compounds did not show any antiplasmodial activity (Table 3.17).

Table 3.17: *In vitro* antiplasmodial activity against *P. falciparum* (CQS) D10 strain.

Sample	Parasite survival (%)			IC_{50} (µg/ml)
	20 µg/ml	*10 µg/ml*	*5 µg/ml*	
RA3	> 80	> 80	> 90	> 20
RA5	78.1	80	89.9	> 20
	30 ng/ml	*15 ng/ml*	*7.5 ng/ml*	IC_{50} *(ng/ml)*
CQ	< 20	< 40	< 50	5.2
MMV390048	< 30	< 50	< 60	8.9
Artesunate	< 20	< 40	< 50	2.1

3C.5 Discussion

Plants have always been an untapped source to provide structurally diverse bioactive compounds as potential therapeutic agents, including antimicrobials. Triterpenoids from *Momordica balsamina (*Ramalhete *et al*., 2011*)*; *Alisma orientale* (Jin *et al*., 2012); and *Carpobrotus edulis* (Martins *et al*., 2011) also exhibited antibacterial activity on various resistant bacterial strains including the *Staphylococcus* spp.

Popova *et al.* (2009) also reported the antibacterial activity of lanostane triterpenes from wood-decay fungus *Fomitopsis rosea* against *S. aureus*. The ability of the two triterpenes from *P longifolia* to exhibit strong activity even on the resistant bacterial strains indicates their potential to be developed into effective antimicrobial agents.

According to Robles-Zepeda *et al.* (2013) an extract with MIC of ≤ 200 µg/ml against *Mycobacterium tuberculosis* is considered active. The appreciable activity demonstrated by the triterpenes from *P. longifolia* with RA3 exhibiting MIC_{99} of 37 µg/ml (Table 3.14) against *Mycobacterium tuberculosis* H37Rv indicates potent antimycobacterial activity of the compounds. Antimycobacterial activity of several other plant triterpenoids (Ge *et al.*, 2010, Jadulco *et al.*, 2011) including lanostane triterpenes from *Ganoderma orbiforme* BCC 22324 (Isaka *et al.*, 2013) has recently been reported against *Mycobacterium tuberculosis* H37Rv and/or H37Ra.

Antimicrobial drugs exert their therapeutic activity through various mechanisms which include inhibition of proteins, cell wall, cell membrane and nucleic acid synthesis (Riaz *et al.*, 2011). The antimicrobial activity of terpenoids has been associated with among others, bacterial cell membrane disruption by the lipophilic compounds (Saleem *et al.*, 2010). The evaluation of the bacterial DNA damaging effect of the triterpenes indicated that the investigated triterpenes did not exhibit any DNA damaging effect. DNA protective effect of some triterpenes has previously been reported (Ramos *et al.*, 2010; Smina *et al.*, 2011). However, the release of the cytosolic LDH into the extracellular medium is indicative of the cell membrane damaging effect of the triterpene (RA5 was not enough to carry out the assay). It is noteworthy that despite the complex Gram negative bacteria cell wall, the highest percentage LDH release was observed in *P. mirabilis* (89.6%) and *E. coli* (76.3%) (Table 3.15). While some compounds act by damaging both membrane and DNA (Liu *et al.*, 2011), it is apparent that the triterpenes exert their antibacterial activity by affecting the microbial cell membrane integrity rather than damaging DNA.

While some lanosteryl triterpenes from *Ganoderma annulare* inhibited growth of some fungal strains (Smania *et al.*, 2003), the two lanosteryl triterpenes from *P. longifolia* only showed antifungal activity when combined (Table 3.16), indicative of synergism. The observed inactivity of the individual triterpenes against the fungal

strains may not be surprising since lanostane-type triterpenes have mostly been isolated from fungi (Popova *et al.*, 2009; She *et al.*, 2012; Yang *et al.*, 2012). Thus their role in fungi still needs investigation. It is apparent that the antimicrobial activity of the triterpenes from *P. longifolia* is through their antibacterial effect.

Several studies have demonstrated potency of some plant-derived triterpenes as potential antiplasmodial agents (Adams *et al.*, 2010; Simelane *et al.*, 2013). Also the lanostane triterpenes from *Ganoderma orbiforme* BCC 22324 have recently been reported to exhibit both antimycobacterial and antiplasmodial activity against *Plasmodium falciparum* K1 (Isaka *et al.*, 2013). However the triterpenes from *P. longifolia* did not exhibit any appreciable antiplasmodial activity at the concentrations tested (Table 3.17). The observed inactivity of the triterpenes against the malarial parasite (*P. falciparum*) could partly be linked to their ability to induce expression of PfHsp70- the protein is vital for survival of the parasite (Shonhai *et al.*, 2007).

In conclusion even though the triterpenes did not show any antiplasmodial activity, the bactericidal activity of the compounds even on the antibiotic resistant strains indicates their potential to be used as templates to develop effective antimicrobial agents to combat infectious diseases resistant to conventional drugs.

References

Adams, A. Christen, M. Plitzko, I. Zimmermann, S. Brun, R. Kaiser, M. Hamburger, M. (2010). Antiplasmodial lanostanes from the *Ganoderma lucidum* mushroom. *Journal of Natural Products* 73(5): 897-900.

Alonso, P.L. Brown, G. Arevalo-Herrera, M. Binka, F. Chitnis, C. *et al.* (2010). A research agenda to underpin malaria eradication. *PLoS Medicine* 8(1): e1000406.

Chung, P.Y. Chung, L.Y. Navaratnam, P. (2013). Transcriptional profiles of the response of methicillin-resistant *Staphylococcus aureus* to pentacyclic triterpenoids. *PLoS ONE* 8: e56687. doi:10.1371/journal.pone.0056687.

Collins, L. and Franzblau, S.G. (1997). Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrobial Agents and Chemotherapy* 41: 1004-9.

Collins, L.A. Torrero, M.N. Franzblau, S.G. (1998). Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 42: 344-7.

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: 711-713

Ge, F. Zeng, F. Liu, S. Guo, Na. Ye, H. Song, Y. et al. (2010). *In vitro* synergistic interactions of oleanolic acid in combination with isoniazid, rifampicin or ethambutol against *Mycobacterium tuberculosis*. *Journal of Medical Microbiology* 59: 567-572.

Gonzalez, A.G. Leon, F. Rivera, A. Padron, J.I. Gonzalez-Plata, J. Zuluaga, J.C. Quintana, J. Estevez, F. Bermejo, J. (2002). New lanostanoids from the fungus *Ganoderma concinna*. *Journal of Natural Products* 65: 417-421.

Hoffmann, K. Wagner, G. Apfalter, P. Maier, M. (2011). Antibiotic resistance in primary care in Austria- a systematic review of scientific and grey literature. *BMC Infectious Diseases.* 11:330 doi:10.1186/1471-2334-11-330.

Ioerger, T.R. Feng, Y. Ganesula, K. Chen, X. Dobos, K.M. Fortune, S. Jacobs, W.R. Mizrahi, Jr. V. Parish, T. Rubin, E. Sassetti, C. Sacchettini, J.C. (2010). Variation among genome sequences of H37Rv strains of *Mycobacterium tuberculosis* from multiple laboratories. *Journal of Bacteriology* 192: 3645-3653.

Isaka, M. Chinthanom, P. Kongthong, S. Srichomthong, K. Choeyklin, R. (2013). Lanostane triterpenes from cultures of the *Basidiomycete Ganoderma orbiforme* BCC 22324. *Phytochemistry* 87: 133-139.

Jadulco, R.C. Koch, M. Van Wagoner, R. Pond, C. Gideon, O.G. Matainaho, T Pius, P. Barrows, L.R. (2011). New antimycobacterial triterpenoids from *Rhus taitensis*. *Planta Medica* 77(14): 1651-1654.

Jin, H.G., Jin, Q. Kim, R.A. Choi, H. Lee, J.H. Kim, Y.S. Lee, D.G. Woo, E.R. (2012). A new triterpenoid from *Alisma orientale* and their antibacterial effect. *Archives of Pharmacal Research* 35: 1919-1926.

Liu, X. Zhang, X.J. Fu, Y.J. Zu, Y.G. Wu, N. Liang, L. Efferth, T. (2011). Cajanol inhibits the growth of *Escherichia coli* and *Staphylococcus aureus* by ccting on membrane and DNA damage. *Planta Medica* 77: 158-163.

Mahesh, B. and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World Journal of Agricultural Sciences* 4(S): 839-843.

Makler, M.T. Ries, J.M. Williams, J.A. Bancroft, J.E. Piper, R.C. Gibbins, B.L. Hinrichs, D.J. (1993). Parasite lactate dehydrogenase as an assay for *Plasmodial falciparum* drug sensitivity. *The American Society of Tropical Medicine and Hygiene* 48: 739-741.

Malebo, H.M. Wenzler, T. Cal, M. Swaleh, S.M. Hassanali, A. Machocho, A.K. et al. (2013). Anti-protozoal and structure-activity relationships of chemical constituents of *Acridocarpus chloropterus* Oliver (Malpighiaceae) from Tanzania. *International Journal of Natural Products Research* 3(4): 74-81.

Martins, A. Vasas, A. Viveiros, M. Molnár, J. Hohmann, J. Amaral, L. (2011). Antibacterial properties of compounds isolated from Carpobrotus edulis. *International Journal of Antimicrobial Agents* 37: 438-444.

Nethengwe, M.F. Opoku, A.R. Dludla, P.V. Madida, K.T. Shonhai, A. Smith, P. Singh, M. (2012). Larvicidal, antipyretic and antiplasmodial activity of some Zulu medicinal plants. *Journal of Medicinal Plants Research* 6(7): 1255-1262.

Popova, M. Trusheva, B. Gyosheva, M. Tsvetkova, I. Bankov, V. (2009). Antibacterial triterpenes from the threatened wood-decay fungus *Fomitopsis rosea*. *Fitoterapia* 80: 263-266.

Ramalhete, C. Spengler, G. Martins, A. Martins, M. Viveiros, M. Mulhovo, S. Ferreira, M.U. Amaral, L. (2011). Inhibition of efflux pumps in methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis* resistant strains by triterpenoids from *Momordica balsamina*. *International Journal of Antimicrobial Agents* 37: 70-74.

Ramos, A.A. Pereira-Wilson, C. Collins, A.R. (2010). Protective effects of ursolic acid and luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells. *Mutation Research* 692: 6-11.

Riaz, S. Faisal, M. Hasnain, S. (2011). Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species in Pakistan. *African Journal of Biotechnology* 10: 6325-6331.

Rojas, R. Bustamante, B. Bauer, J. Fernandez, I. Alban, J. Lock, O. (2003). Antimicrobial activity of selected Peruvian medicinal plants. *Journal of Ethnopharmacology* 88: 199-204.

Saleem, M. Nazir, M. Ali, M.S. Hussain, H. Lee, Y.S. Raiz, N. Jabbar, A. (2010). Antimicrobial natural products: an update on future antibiotic drug candidates. *Natural Products Reports* 27: 238-254.

She, G. Zhu, N. Wang, S. Liu, Y. Ba, Y. Sun, C. Shi, R. (2012). New lanostane-type triterpene acids from *Wolfiporia extensa. Chemistry Central Journal* 6:39: http://journal.chemistrycentral.com/content/6/1/39.

Shonhai, A, Boshoff, A, Blatch, G.L. (2007). The structural and functional diversity of Hsp70 proteins from *Plasmodium falciparum. Protein Science* 16(9): 1803-1818.

Simelane, M.B.C. Shonhai, A. Shode, F.O. Smith, P. Singh, M. Opoku, A.R. (2013). Anti-plasmodial activity of some Zulu medicinal plants and of some triterpenes isolated from them. *Molecules* 18: 12313-12323.

Smania, E.F.A. Monache, F.D. Smania, Jr. A. Yunes, R.A. Cuneo, R.S. (2003). Antifungal activity of sterols and triterpenes isolated from *Ganoderma annulare. Fitoterapia* 74: 375-377.

Smina, T.P. De, S. Devasagayam, T.P. Adhikari, S. Janardhanan, K.K. (2011). *Ganoderma lucidum* total triterpenes prevent radiation-induced DNA damage and apoptosis in splenic lymphocytes *in vitro. Mutation Research* 726: 188-94.

Tadić, V.M. Jeremic, I. Dobric, S. Isakovic, A. Markovic, I. Trajkovic, V. Bojovic, D. Arsic, I. (2012). Anti-inflammatory, gastroprotective, and cytotoxic effect of *Sideritis scardica* extracts. *Planta Medica* 78: 415-427.

Tajbakhsh, S. Ilkhani, M. Rustaiyan, A. Larijan, K. Sartavi, K. Tahmasebi, R. Asayesh, G. (2011). Antibacterial effect of the brown alga *Cystoseiratrinodis. Journal of Medicinal Plants Research* 5: 4654-4657.

Trager, W. and Jensen, J.B. (1976). Human malaria parasite in continuous culture. *Science* 193 (4254): 673-675.

Vlientink, J. Van Hoof, L. Lasure, A. VandenBerghe, D. Rwangabo, P.C. Mwakiyumwani, J. (1995). Screening of a hundred Rwandese medicinal plants for antibacterial and antiviral properties. *Journal Ethnopharmacology* 46: 31-47.

Yang, S.X. Yu, Z.C. Lu, Q.Q. Shi, W.Q. Laatsch, H. Gao, J.M. (2012). Toxic lanostane triterpenes from the basidiomycete *Ganoderma amboinense*. *Phytochemistry Letters* 5: 576-580.

Zakaria, Z.A. Sufian, A.S. Ramasamy, K. Ahmat, N. Sulaiman, M.R. Arifah, A.K. Zuraini, A. Somchit, M.N. (2009). *In vitro* antimicrobial activity of *Muntingia calabura* extracts and fractions. *African Journal Microbiology Research* 4: 304-308.

CHAPTER FOUR

4.0 GENERAL DISCUSSION

Triterpenes have become new targets for drug development against an array of diseases. This study investigated some bioactivity of the two lanosteryl triterpenes [3β-hydroxylanosta-9,24-dien-21-oic acid (RA5) and methyl-3β-hydroxylanosta-9,24-dienoate (RA3)] from *P. longifolia* and their derivatives.

Obesity is the major risk factor of hyperlipidemia and hyperglycaemia. The abnormally elevated plasma lipids, particularly free fatty acids, observed in obesity (Zhang *et al.*, 2013) inhibit glucose uptake and utilisation by peripheral tissues. This leads to the development of insulin resistance and type 2 diabetes.

The results obtained in this study revealed that the compounds possess hypolipidemic properties. Apparently their hypolipidemic effect is partly mediated by the inhibition of lipid digestive enzymes and lipolysis. Though the triterpenes are poor inhibitors of carbohydrate digestives enzymes, the significant effect of the compounds in stimulating cellular glucose uptake in both C2C12 and 3T3-L1 cells, is indicative of their hypoglycaemic effect. Other reports have further shown that while some plant extracts effectively inhibit adipocytes differentiation with only minimal effect on cellular glucose uptake (Prathapan *et al.*, 2012), others enhance cellular glucose uptake without stimulating adipogenesis (Yang *et al.*, 2013). Thus the ability of the compounds (RA5 and R52) to significantly reduce intracellular lipid accumulation and also stimulate cellular glucose uptake is vital to the prevention of hyperlipidermia, obesity and insulin resistance in type 2 diabetes (Zeng *et al.*, 2012).

Enlarged adipocytes have been reported to produce proinflammatory molecules (Weisberg *et al.*, 2003; Shoelson *et al.*, 2007). An obesity-induced inflammation is known to promote insulin resistance, which in turn decreases Hsps expression, thus permitting the accumulation of protein aggregates (Hooper and Hooper, 2008). The ability of the triterpenes to stimulate Hsp70 expression in inflamed rats together with their direct anti-protein aggregation activity could play an important role in the prevention of obesity-induced inflammation and its consequent effects.

Inflammation is also strongly linked with blood coagulation (Verhamme & Hoylaerts, 2009; Chu, 2011). Platelets play an important role in linking the two processes of inflammation and blood coagulation. Activated platelets release cytokines and pro-inflammatory mediators (Blair & Flaumenhaft, 2009). Consequently, Inflammation suppresses the natural anticoagulant system, which favours synthesis and expression of prothrombogenic molecules (Verhamme & Hoylaerts, 2009). It is apparent that the anti-inflammatory property of the triterpenes plays an important role in their anticoagulant activity. The anticoagulant and anti-inflammatory activity of the triterpenes is linked to their ability to inhibit platelets and protein aggregation. The anti-inflammatory and significant anticoagulant activity of the compounds is therapeutically important in the prevention of thromboembolic disorders.

Despite the inactivity against fungi, the antibacterial activity exhibited by the triterpenes even on the resistant strains is important in the fight against infectious diseases. Reports have also linked some intestinal bacteria to the development of obesity, systemic inflammation and insulin resistance (Mohammed, 2012). Thus the antibacterial activity of the triterpenes could also partly contribute to the prevention of diet-induced obesity and its related diseases. However, ability of the isolated triterpenes to induce expression of PfHsp70 (vital for survival of malarial parasite) could also partly be responsible for the lack of antiplasmodial activity of the compounds.

The biggest challenge in new therapeutic drug discovery is to keep up the pace with the ever evolving diseases resistant to conventional medicines. Medicinal plants with their diverse chemical structures offer an opportunity to discover drugs with multi-beneficial effects. Integration of a multi-target action approach in the treatment of diseases is considered highly possible with the use of plant-derived bioactive compounds (Appendino and Pollastro, 2010). Thus, the overall results reported in this thesis encourage the development of the isolated triterpenes into potential pharmacologically active drugs with beneficial multiple effects. The low cytotoxicity of the tritepenes on HEK293 and HepG2 cell lines (Mosa *et al*, 2011b) suggests their safe use in the management of metabolically related diseases.

References

Appendino, G. Pollastro, F. Plants: Revamping the oldest source of medicines with modern science. In: Buss, A.D. Burtler, M.S. (2010) (eds). *Natural Product Chemistry for Drug Discovery*. RCS publishing: Cambridge, UK, pp 164-165.

Blair, P. and Flaumenhaft, R. (2009). Platelet alpha-granules: Basic biology and clinical correlates. *Blood Reviews* 23: 177-189.

Chu, A.J. (2011). Tissue factor, blood coagulation, and beyond: an overview. *International Journal of Inflammation* 2011: doi: 10.4061/2011/367284.

Hooper, P.L. and Hooper, P.L. (2008). Inflammation, heat shock proteins, and type 2 diabetes. *Cell Stress and Chaperones*. DOI 10.1007/s12192-008-0073-x.

Mohammed, N.K. (2012). Diet, bacteria and inflammation: the intestinal mucosa and metabolic syndrome. *Theses and Dissertations--Nutritional Sciences.* Paper 4. http://uknowledge.uky.edu/nutrisci_etds/4

Mosa, R.A. Oyedeji, O.A. Shode, F.O. Singh, M. Opoku, A.R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit antiplatelet aggregation activity. *African Journal of Pharmacy and Pharmacology* 5: 2698-2714.

Prathapan, A. Krishna, M.S. Lekshmi, P.C. Raghu, K.G. Menon, A.N. (2012). Modulation of adipogenesis and glucose uptake by *Curcuma longa* extract in 3T3L1 and L6 cell lines - An *in vitro* study. *Asian Pacific Journal of Tropical Disease* 2012: S163-S165.

Shoelson, S.E. Herrero, L. Naaz, A. (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology* 132: 2169-2180.

Verhamme, P. and Hoylaerts, M.F. (2009). Haemostasis and inflammation: two of kind? *Thrombosis Journal* 7: http://www.thrombosisjournal.com/content/7/1/15.

Weisberg, S.P. McCann, D. Desai, M. Rosenbaum, M. Leibel, R.L. et al. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigations* 112: 1796-1808.

Yang, M.H. Avula, B. Smillie, T. Khan, I. Khan, S.I. (2013). Screening of medicinal plants for PPARα and PPARγ activation and evaluation of their effects on glucose uptake and 3T3-L1 adipogenesis. *Planta Medica* 79: 1084-1095.

Zeng, X. Zhou, X. Xu, J. Chan, S.M.H. Xue, C.L. Molero, J.C. Ye, J.M. (2012). Screening for the efficacy on lipid accumulation in 3T3-L1 cells is an effective tool for the identification of new anti-diabetic compounds. *Biochemistry and Pharmacology* 84: 830-837.

Zhang, X. Wang, Z. Li, J. Gu, D. Li, S. et al. (2013). Increased 4-hydroxynonenal formation contributes to obesity-related lipolytic activation in adipocytes. *PLoS ONE* 8(8): e70663. doi:10.1371/journal.pone.0070663.

CHAPTER FIVE

5.0 CONCLUSION

Hyperlipidemia and obesity often coexist and these metabolic disorders favour the expression of prothrombogenic and proinflammatory molecules (Grundy, 2004). Obesity-induced inflammation promotes cellular insulin resistance, which in turn downregulates the expression of Hsps, thus causing accumulation of harmful protein aggregates (Hooper and Hooper, 2008). Hypolipidemic drugs which can also prevent protein aggregation are vital in the management of obesity and its co-morbidities.

This study demonstrated that the two lanosteryl triterpenes isolated from stem bark of *P. longifolia* and their derivatives possess hypolipidemic and hypoglycaemic properties. Apparently the hypolipidemic and hypoglycaemic activity of RA5 and R52 are mediated through the reduction of intracellular lipid accumulation and the stimulation of cellular glucose uptake. The anti-protein aggregation activity of the triterpenes and their ability to upregulate the expression of Hsp70 could very well be linked to their anticoagulant and anti-inflammatory activity. The triterpenes did not show any antioxidant activity which clearly suggests that the various bioactivity of the compounds are mediated by mechanisms other than antioxidant activity. Despite the lack of antifungal and antiplasmodial activity, the antibacterial activity of the two triterpenes even on the antibiotic resistant strains is important in the fight against infectious diseases.

The diverse bioactivities of the compounds are vital to the management of cardiovascular disease. This indicates the potential of the compounds to be used directly or as templates to develop pharmacologically active drugs. Overall results demonstrate potential new approaches towards development of pharmacologically active agents with beneficial multiple effects.

5.1 Limitations

The availability of isolated triterpenes due to among other factors seasonal variation, has been the major limitation. Owing to the smaller yields of the compounds, it was difficult to perform all the set experiments as anticipated.

5.2 Suggestions for Further Studies

- o Since the triterpenes exhibited inhibitory activity on the selected lipid digestive enzymes and HSL, evaluation of mechanism(s) of action involved on the enzymes kinetics is recommended for future work.
- o *In vivo* activities on hyperlipidemia, diabetes, hypertension, and cardiovascular protective effects of the compounds need to be evaluated.
- o More animal studies are necessary to show whether the anti-inflammatory responses are useful. These would include tissue histology and measurement of inflammatory cytokines
- o Bioavailability studies of the compounds are recommended.
- o Mechanism(s) of action on the anti-adipogenic activity require evaluation.
- o It is also important to investigate effect of the compounds on expression of genes involved in hyperlipidemia and obesity.

The targeted studies in animal models of human disease, and then clinical trials, will be necessary to bring these compounds closer to therapeutic use.

References

Hooper, P.L. and Hooper, P.L. (2008). Inflammation, heat shock proteins, and type 2 diabetes. *Cell Stress and Chaperones*. DOI 10.1007/s12192-008-0073-x.

Grundy, S.M. (2004). Obesity, metabolic syndrome, and cardiovascular disease. The Journal of Clinical Endocrinology & Metabolism 89(6): 2595-2600.

APPENDIX A

A. Details of preparation of some reagents

A1. PNP (3.33 mM)

A 10 mM *p*-nitrophenol palmitate was prepared in acetonitrile and the solution was further diluted with ethanol to reach the final concentration of 3,33 mM.

A2. KRB-BSA solution pH 7.4

Solution was prepared by mixing 120 mM NaCl, 3 mM KCl, 12.0 mM $MgSO_4$, 12.0 mM $CaCl_2$, 21.9 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 9.99 mM Hepes in 1000 ml of distilled water and 4% BSA was added to the solution and the pH was adjusted to 7.4.

A3. Homogenisation buffer pH 7.0

Tris-HCl (50 mM), 250 mM of sucrose and 1 crushed protease inhibitor tablet were mixed together and the volume was made up to 100 ml with distilled water.

A4. Coomassie Brilliant Blue G-250

Coomassie blue G250 (0.1 g) was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (w/v) phosphoric acid. The mixture was made up to 1000 ml with distilled water. After the dye has completely dissolved, the solution was filtered through Whatman filter paper several times (×6) until the colour was brown.

A5. Resuspending buffer (pH 7.4)

NaCl (140 mM), 15 mM Tris-HCl and 5.0 mM glucose were prepared and made up to 100 ml with distilled water. The pH was adjusted to 7.4.

A6. Washing buffer (pH 6.5)

NaCl (113 mM), 4.3 mM Na_2HPO_4, 4.3 mM of K_2HPO_4, 24.4 mM of NaH_2PO_4, 5.5 mM glucose and 1 mM EDTA were prepared and made up to 1000 ml with distilled water. The pH was adjusted to 6.5.

A7. Tris buffer (pH 7.4) containing EDTA and NaCl

Tris-HCl (50 mM), 7.5 mM EDTA, and 175 mM NaCl were prepared and made up to 1000 ml with distilled water. The pH was adjusted to 7.4.

A8. ADA (acid-dextrose-anticoagulant)

Dextrose (0.555M), 0.065 M citric acid and 0.085 M trisodium citrate were prepared in water and volume was made up to 100 ml with distilled water.

A9. GAST/Fe Media

0.3 g of Bacto Casitone (Difco)

4.0 g of dibasic potassium phosphate

2.0 g of citric acid

1.0 g of L-alanine

1.2 g of magnesium chloride hexahydrate

0.6 g of potassium sulphate

2.0 g of ammonium chloride

1.80 ml of 10 M sodium hydroxide

10.0 ml of glycerol

0.05% Tween 80

0.05 g of ferric ammonium citrate

The above components were dissolved in distilled water and the volume was made up to 1000 ml with water. pH was adjusted to 6.6. The medium was then filter sterilised (using 0.2 µM filter) and stored at 37°C.

A10. Wash buffer pH 7.5

0.01 mM Tris-HCl, pH 7.5, 50 mM imidazole, 300 mM NaCl.

A11. Elution buffer pH 7.5

300 mM NaCl, 0.01 M Tris-HCl pH 7.5, 1 M imidazole

A12. Tris buffered saline (TBS) pH 7.4

50 mM Tris-HCl, 150 mM NaCl

A13. Tris buffered saline with Tween 20 (TBS-T) pH 7.4

50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20

APPENDIX B

B. Details of some methodologies

B1. Extraction of hormone sensitive lipase

Hormone sensitive lipase (HSL) was extracted from adult male *Sprague-Dawley* rat epididymal adipose tissue. The animal was killed by a blow to the head, dissected and epididymal fat pads and rinsed in ice-cold normal saline. Weight of the tissue was determined and the tissue was minced into smaller pieces. The minced tissue was then treated (1 g: 3 ml) with KRB-BSA (pH 7.4), followed by 10 mg of collagenase. The mixture was incubated at 37°C for 2 h with constant shaking at 120 rpm. Fat cells were then liberated from the tissue fragments by gentle stirring with a rod. This was followed by centrifugation at 400 × g for 1 min at 20°C. Supernatant (fat cells) was collected while sediment (stromal-vascular cells) was discarded. The fat cells were then washed by being suspended in 10 ml of warm (37°C) KRB-BSA solution. The suspension was again centrifuged at 400 × g for 1 min at 20°C, a second round of removing stromal-vascular cells. The cells washing procedure was repeated three times.

In order to extract HSL, the fat cells suspension (in KRB-BSA solution) was further diluted (1:250) with KRB-BSA solution and incubated at 37°C for 30 min. the cells suspension was further centrifuged at 100 × g for 1 min and the supernatant was collected (fat cells suspension). The cells suspension was mixed (1:1.25) with homogenization buffer (each 100 ml prepared from 50 mM Tris-HCl, pH 7.0, 250 mM sucrose, and 1 crushed protease inhibitor tablet) and the mixture was manually agitated 20 times. The homogenate was centrifuged at 4540 × g for 10 min at 4°C and the supernatant was collected. Afterward, 250 μl of diethyl ether was added to the supernatant, abruptly shaken and centrifuged at 1200 × g for 10 min at 4°C. The upper layer (ether layer) was aspirated. The subsequent supernatant was taken as HSL extract and was stored at -80°C for later use.

B2. Preparation of competent cells

Escherichia coli cells were grown on antibiotic-free agar plates (1.6% w/v tryptone, 0.5% w/v NaCl, 1.5% w/v nutrient agar and 1% w/v yeast) and incubated at 37°C,

overnight. Positive colony was sub-cultured in 5 ml 2YT liquid broth (0.5% w/v sodium chloride, 1% w/v yeast and 1.6% w/v tryptone) at 37°C, overnight with agitation at 162 rpm. Following the incubation, 250 µl of the culture was diluted with 50 ml of fresh 2YT liquid broth, mixed well and this was incubated at 37°C for 1 h with constant shaking at 162 rpm until an OD600 of 03.6-0.6 was reached. Then cells were centrifuged for 10 min at 5000 x g at 4°C. Supernatant was discarded and pellet (cells) was re-suspended in 10 ml of 0.1 M $MgCl_2$. The suspension was allowed to stand for 20 min on ice. The suspension was then centrifuged at 4000 × g for 10 min and again supernatant was discarded. The remaining pellet was re-suspended in 10 ml of 0.1 M $CaCl_2$, mixed well and incubated on ice for 2-4 h. The suspension was again centrifuged at 4000 × g for 10 min, supernatant discarded and the remaining pellet was finally dissolved in 3 ml of After centrifuge (4000 x g for 10 min) the pellet was dissolved in 3 ml of 0.1 M $CaCl_2$ and 30% (v/v) glycerol. Finally, the obtained pellet was dissolved in 3 ml of 0.1 M $CaCl_2$ and 30% (v/v) glycerol. The cells were aliquoted (100 µl) and stored at -80°C until required.

B2.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of proteins

The plates (short glass plate and spacer plate) were put on flat surface in a green casting frame and the frame was then clamped. Separating gel (7.5%) (1.5 M Tris pH 8.8, 10% SDS 30% bis-acrylamide, 10% ammonium persulfate- APS and N,N,N´,N´-tetramethylethylenediamine-TEMED) was loaded between the glasses and this was levelled up with distilled water. The gel was allowed 30 min to solidify and the distilled was removed. Subsequently, 4% of stacking gel (0.5 M Tris pH 6.8, 30% bis-acrylamide, 10% SDS, 10% APS and TEMED) was loaded on to the gel and wells were created with a comb. This was allowed to stand for 30 min and the comb was then removed. The gel was placed into the electrophoresis tank and SDS running buffer was added into the tank. Diluted (4:1) protein sample was loaded on the second wells following addition 2 µl of protein ladder. The gel was allowed to run for 1 h at 100v after which it was put transferred into Coomassie blue solution and left shaking for 3 h. The Coomassie blue was then washed off and distaining solution was added to the gel, left shaking until the gel was clear. The Page Silver TM staining kit (Fermentas Life Sciences) was also used following the supplier's instructions, due to the poor visualization of the protein.

APPENDIX C

C. Ethic Clearance

UNIVERSITY RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-30)

UNIVERSITY OF ZULULAND
Website: http://www.unizulu.ac.za

Private Bag X1001
KwaDlangezwa 3886

Tel: 035 902 6645
Fax: 035 902 6222
Email: ViljoenD@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 Dept. 2013/23					
Project Title	The biological activity of the triterpenes from some Zulu medicinal plants					
Principal Researcher/ Investigator	Prof. A. Opoku					
Supervisor and Co-supervisor						
Department	Biochemistry					
Nature of Project	Honours/4th Year		Master's	Doctoral	Departmental	x

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate. Special conditions, if any, are also listed on page 2.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

The Principal Researcher must report to the UZREC in the prescribe format, where applicable, annually and at the end of the project, in respect of ethical compliance.

Page 1 of 2

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these may also require approval.)
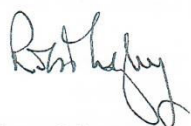
Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			
Health Research Ethics Committee recommendation			X
Ethical clearance application form			X
Project registration proposal	X		
Informed consent from participants	X		
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language			X
Other data collection instruments		Only if necessary	
		Only if used	

Special conditions: Documents marked "To be submitted" must be presented for ethical clearance before any data collection can commence.

The UZREC retains the right to

- Withdraw or amend this Certificate if

  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.

Professor Rob Midgley
Deputy Vice-Chancellor, Research and Innovation
Chairperson: University Research Ethics Committee
01 March 2013

PROF. JR MIDGLEY
DEPUTY VICE-CHANCELLOR
RESEARCH & INNOVATION

1   MAR 2013

UNIVERSITY OF ZULULAND
PRIVATE BAG X1001
KWADLANGEZWA, 3886

Page 2 of 2

150

D. ADDTIONAL DATA

D1. Effect of the compounds on lipids digestive enzymes and HSL
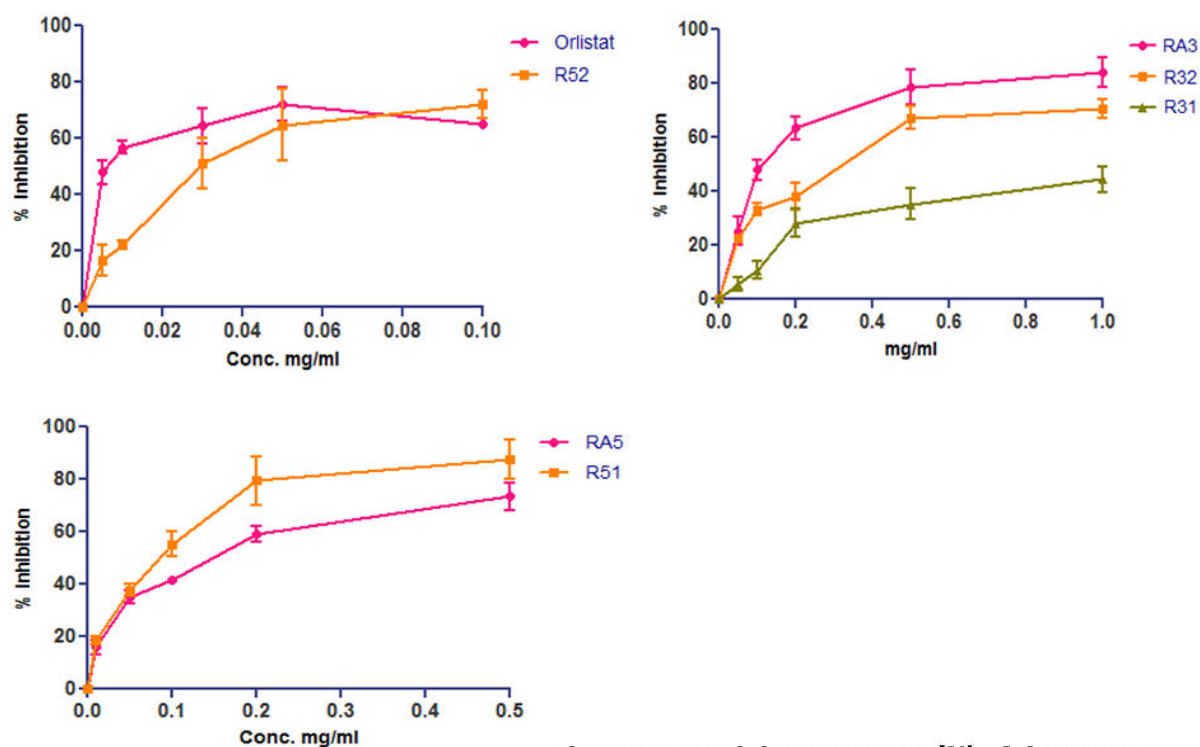
Figure D1.1: Inhibitory activity (%) of the triterpenes and their derivatives on pancreatic lipase
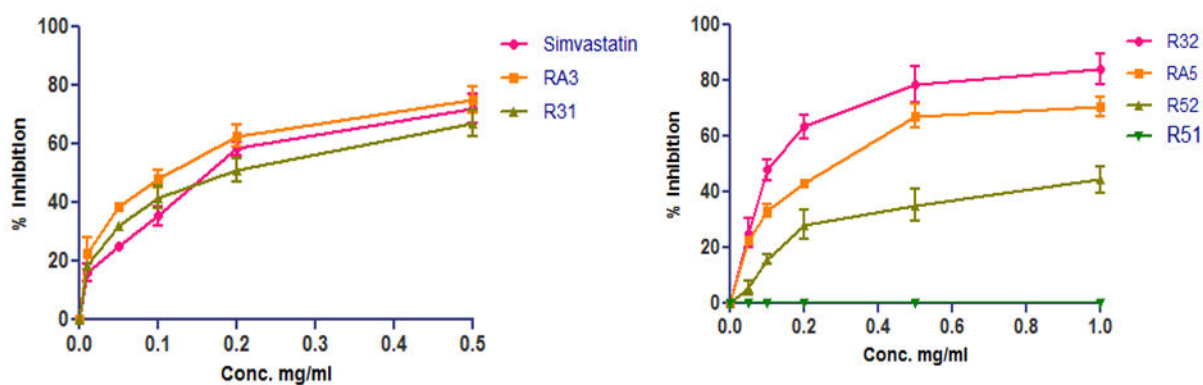
Figure D1.2: Inhibitory activity (%) of the triterpenes and their derivatives on cholestreol esterase
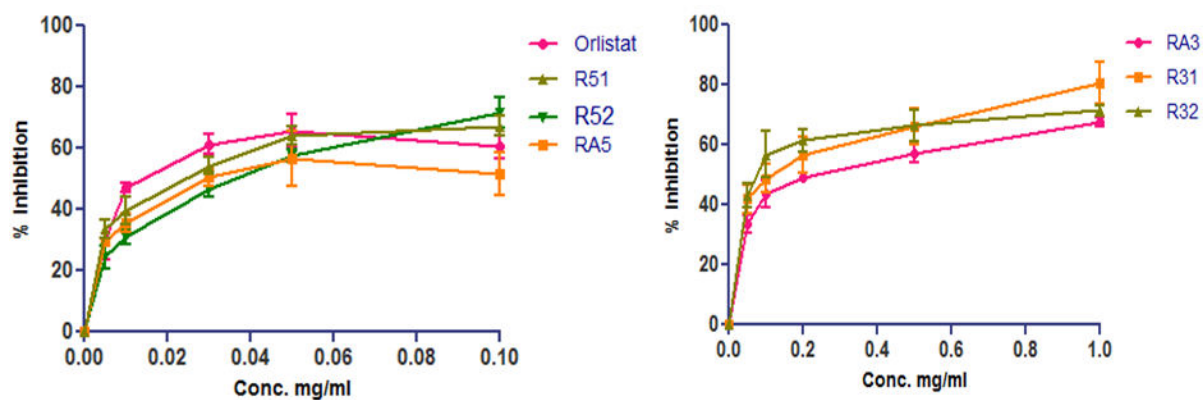
Figure D1.3: Inhibitory activity (%) of the triterpenes and their derivatives on hormone sensitive lipase

D2. Effect of the compounds on lipid accumulation in 3T3-L1 cells
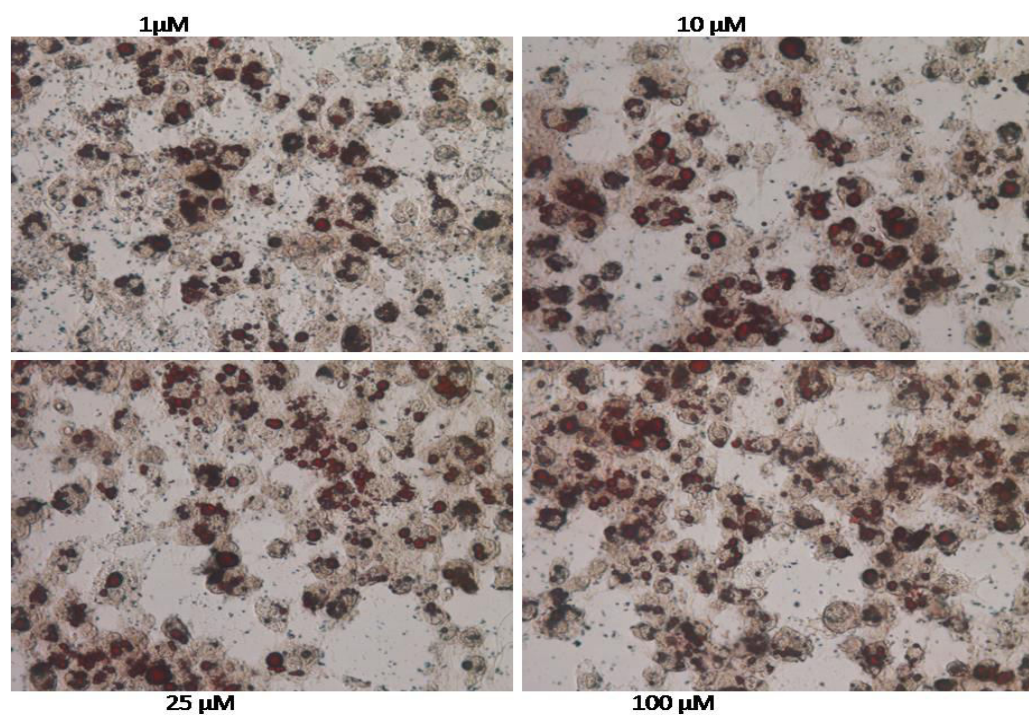
Figure D2.1a: Effect of RA5 at different concentrations (1, 10, 25 and 100 μM) on lipid accumulation in 3T3-L1 cells after 24 h exposure
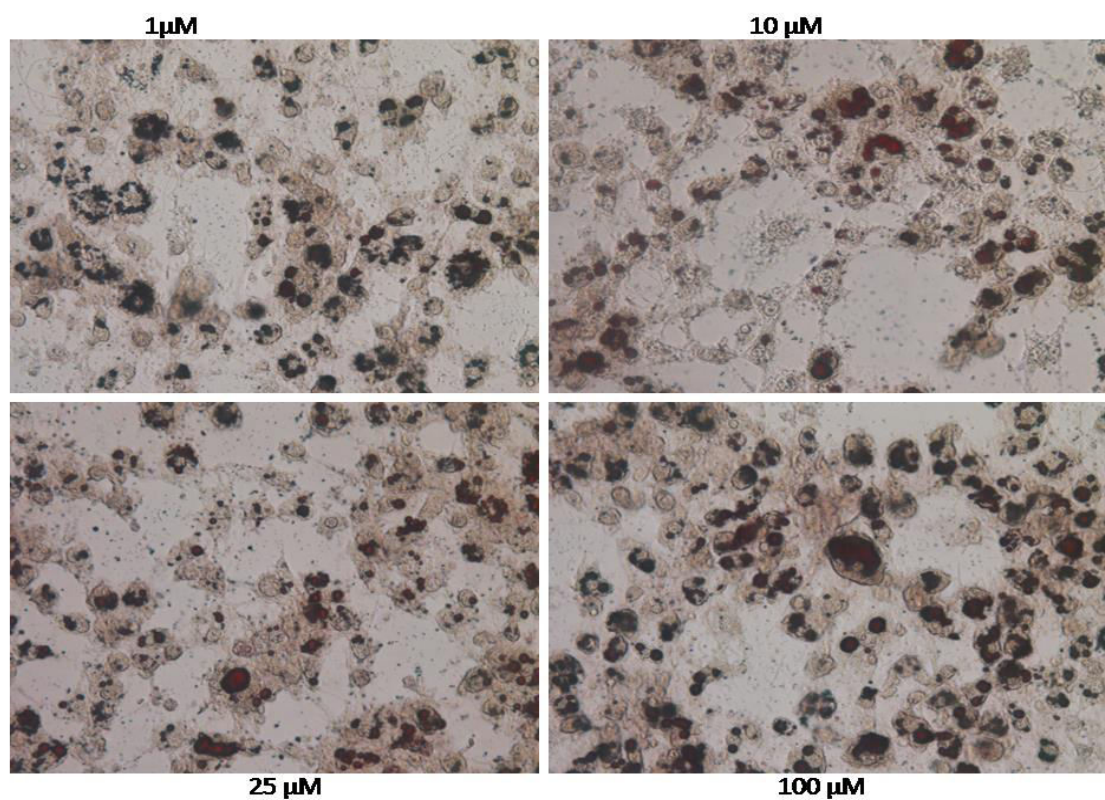
Figure D2.1b: Effect of RA5 at different concentrations on lipid accumulation in mature 3T3-L1 cells after 48 h exposure
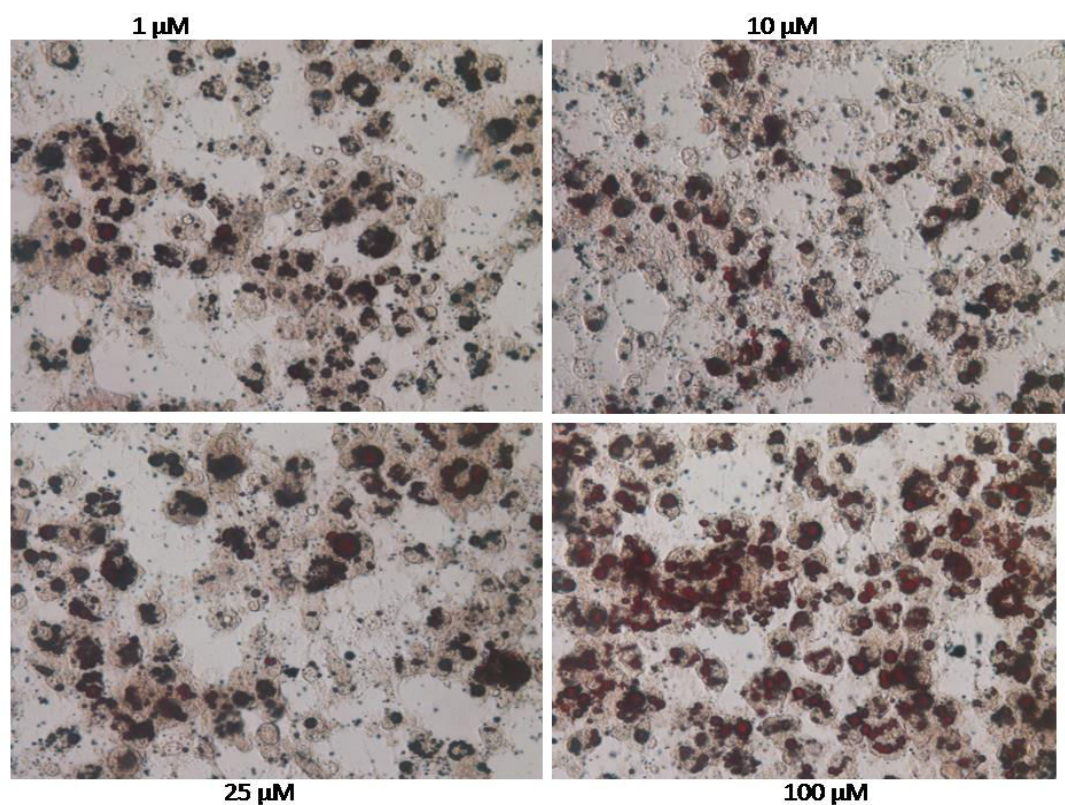
Figure D2.2a: Effect of R52 at different concentrations on lipid accumulation in mature 3T3-L1 adipocytes following a 24 h exposure
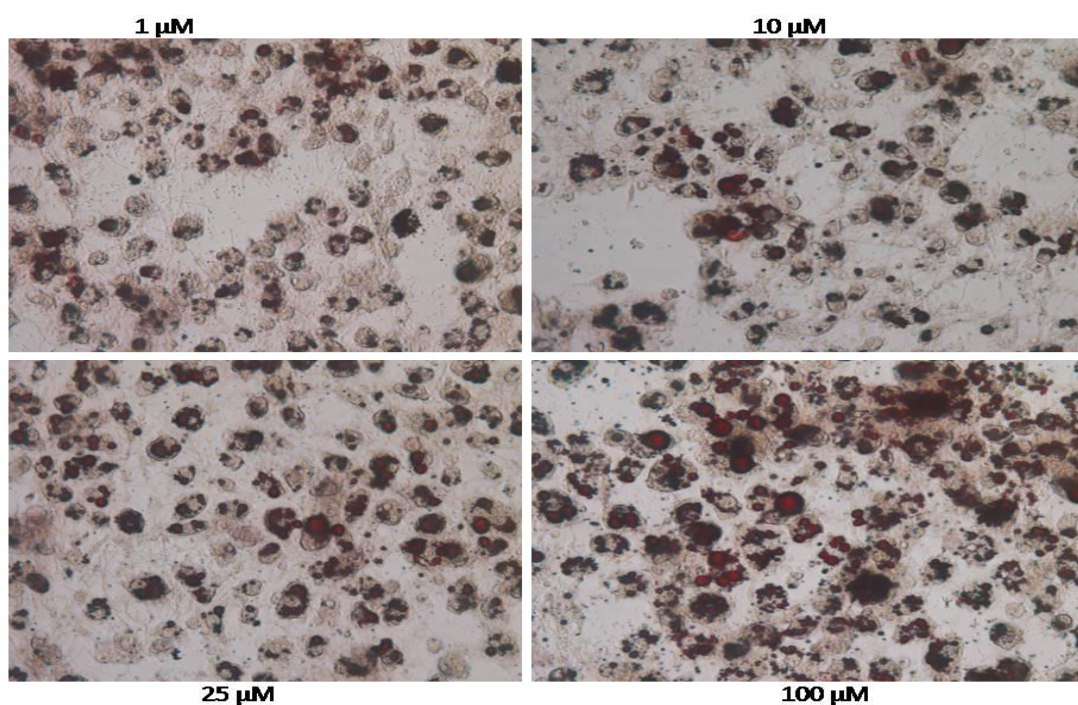
Figure D2.2b: Effect of R52 on lipid accumulation in 3T3-L1 following a 48 h exposure

D3. Bile acid binding ability of the triterpene
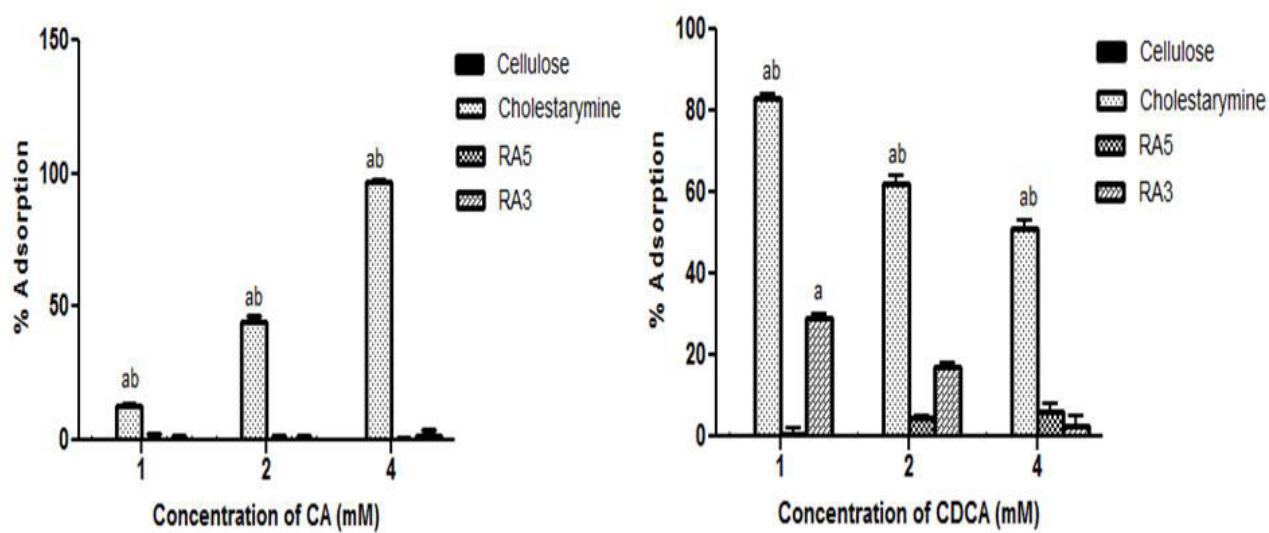
Figure D3.1: *In vitro* bile acid binding ability of the triterpenes. [a] $p < 0.05$ compared to RA5, [b] $p < 0.05$ compared to RA3

D3. Effect of the compounds on carbohydrate digestive enzymes
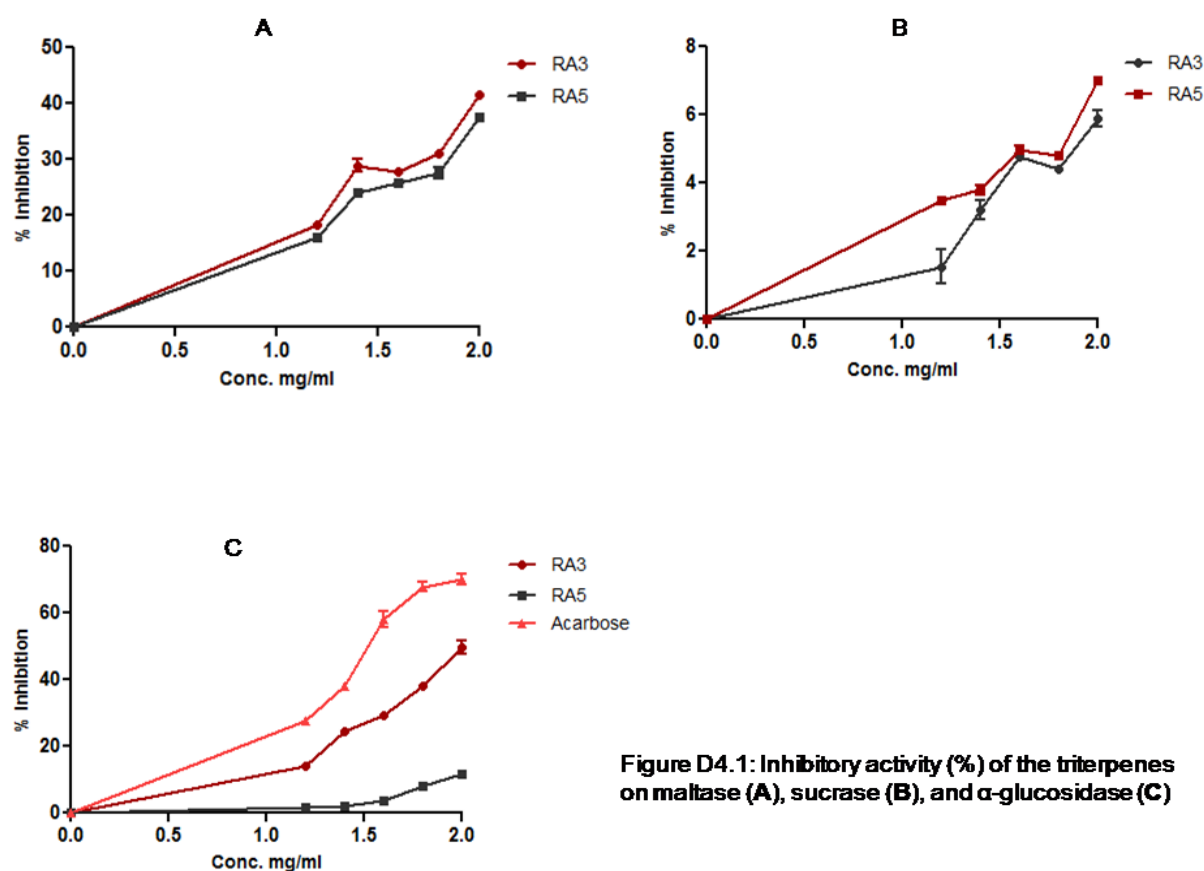
Figure D4.1: Inhibitory activity (%) of the triterpenes on maltase (A), sucrase (B), and α-glucosidase (C)

D5. Antimicrobial Activity

Table D5.1: Zones of inhibition (mm) of the triterpene (RA5) on some antibiotic sensitive bacteria

Microorganism	Strain	RA5	Penicillin	Cloxacillin
E. cloacae	ATCC 13047	11	11	11
K. pneumonia	ATCC 10031	10	8	9
S. flexneri	KZN	10	9	10
S. marscens	ATCC 9986	10	10	11
B. cereus	ATCC 1072	0	0	0
B. pumilus	ATCC 14884	11	9	9
B. subtils	KZN	8	7	8
E. faecalis	KZN	9	0	0
S. aureus	ATCC 6538	10	10	8
S.faecalis	ATCC 29212	10	10	7

Table D5.2: MIC and MBC in mg/ml of the triterpene (**RA5**) on some antibiotic sensitive bacteria

Microorganism	RA5		Penicillin		Cloxacillin	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. cloacae ATCC 13047	1.25	2.50	5.00	5.00	ND	ND
K. pneumonia ATCC10031	5.00	5.00	0.16	1.25	ND	ND
S. flexneri KZN	5.00	5.00	ND	ND	ND	ND
S. marscens ATCC 9986	1.25	1.25	ND	ND	ND	ND
B. cereus ATCC 1072	-	-	-	-	-	-
B. pumilus ATCC 14884	2.50	2.50	0.16	ND	ND	5.00
B. subtils KZN	5.00	5.00	0.16	0.16	0.16	5.00
E. faecalis KZN	1.25	2.50	5.00	ND	ND	ND
S. aureus ATCC 6538	2.50	2.50	0.63	ND	0.31	2.50
S. faecalis ATCC 29212	5.00	5.00	ND	ND	ND	ND

ND- Not detected

APPENDIX E

E. SPECTRA

E1. Spectra of RA5
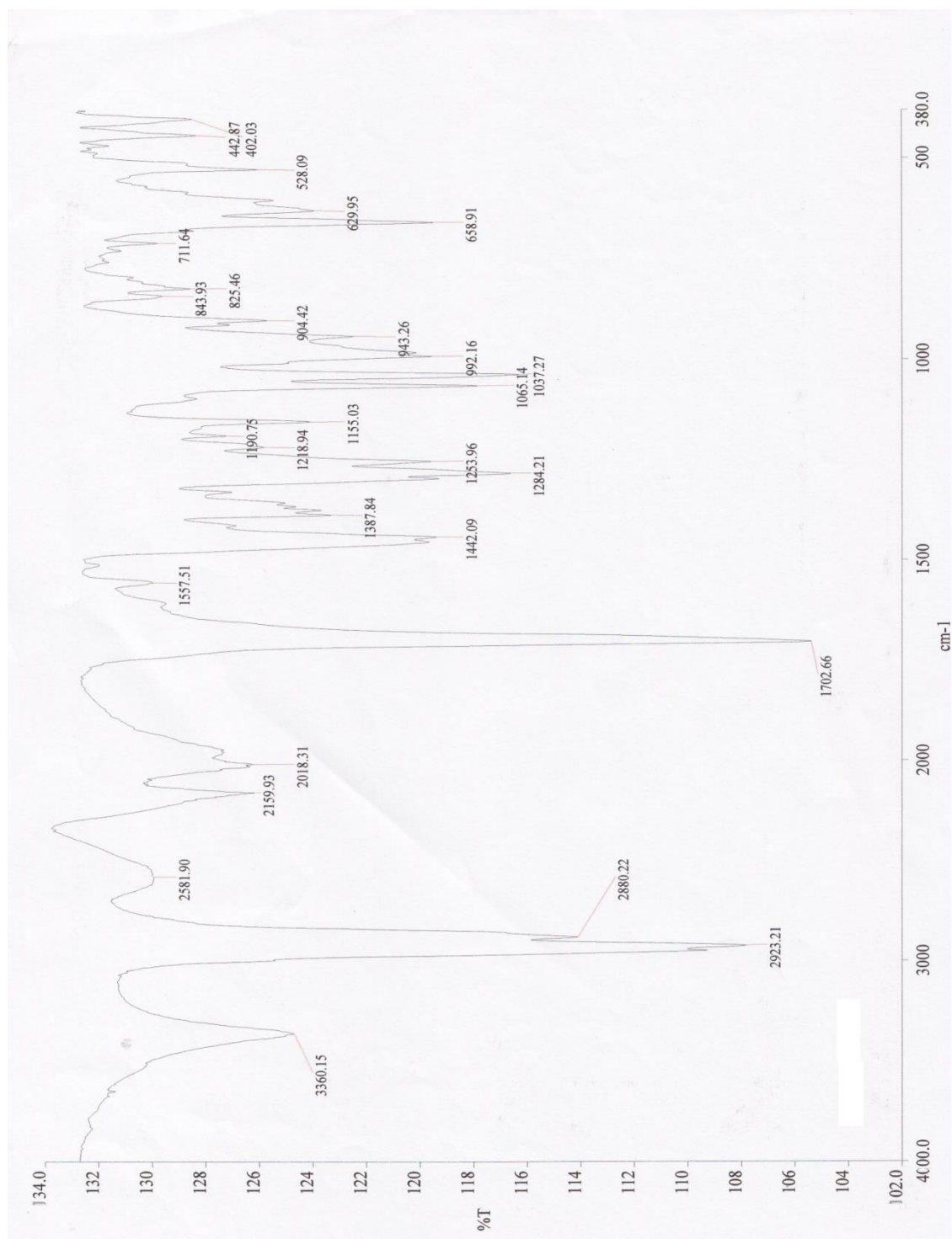
Figure E1.1: IR spectrum of compound RA5
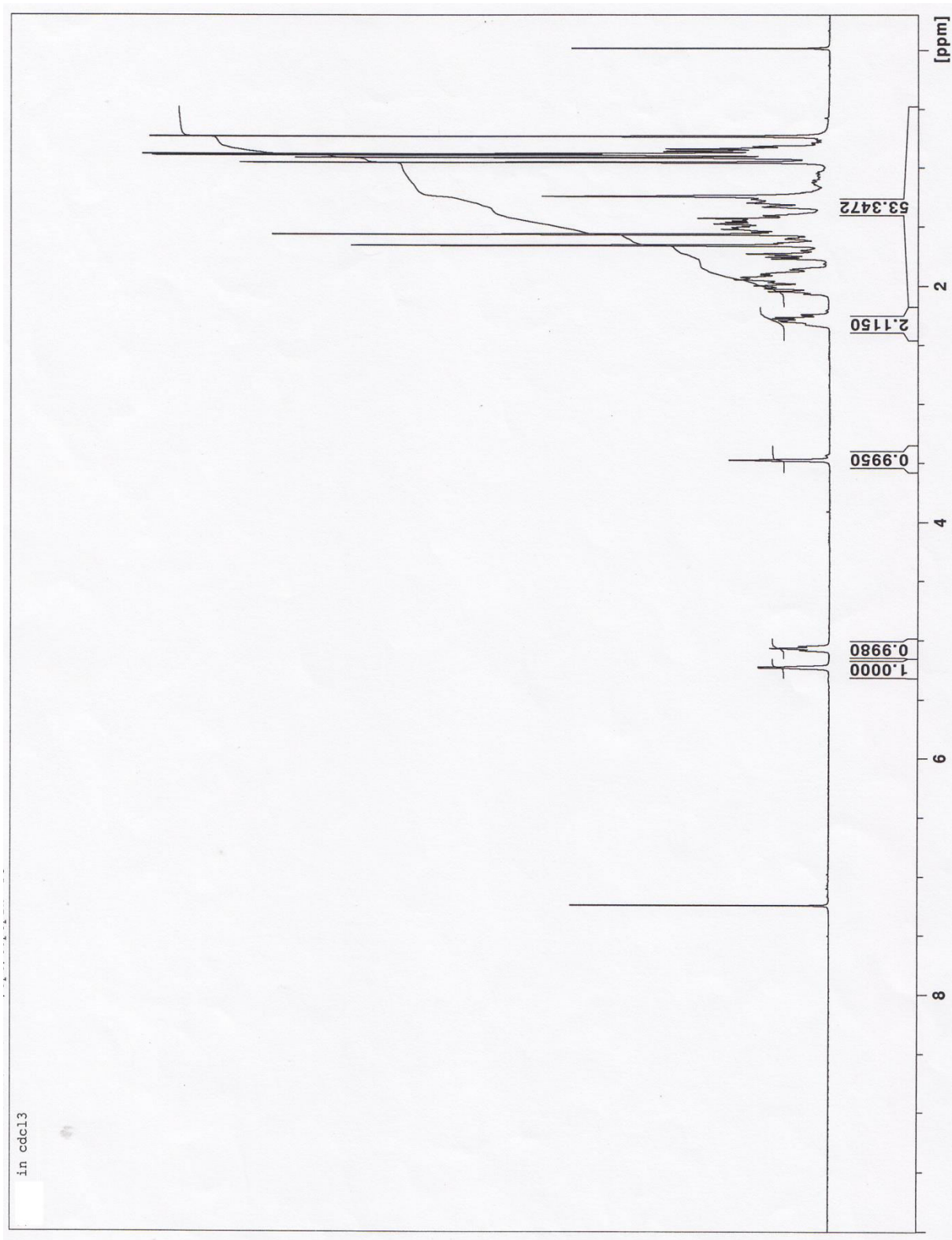
Figure E1.2: ^{1}H-NMR spectrum of compound RA5
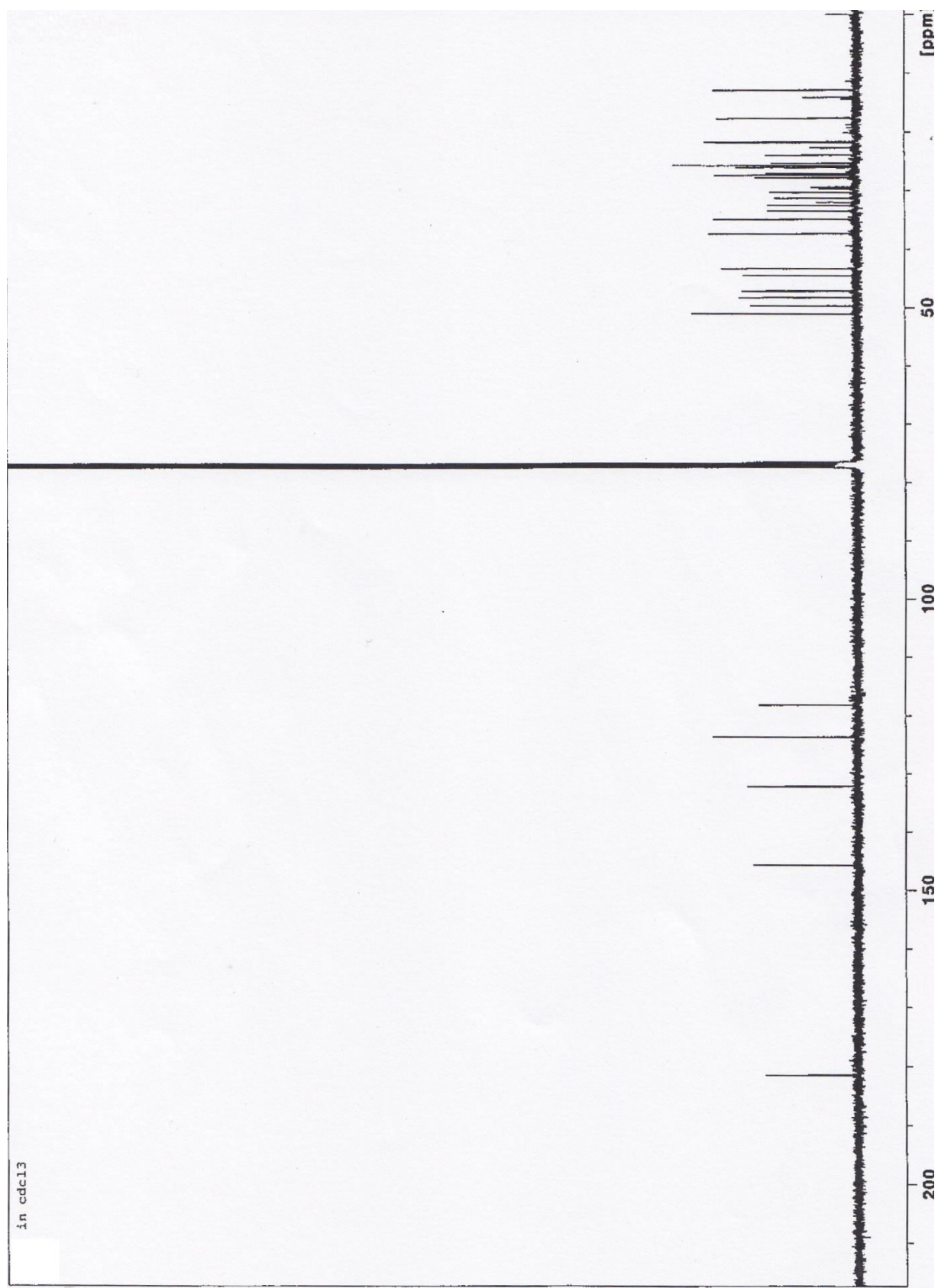
Figure E1.3: ^{13}C-NMR spectrum of compound RA5
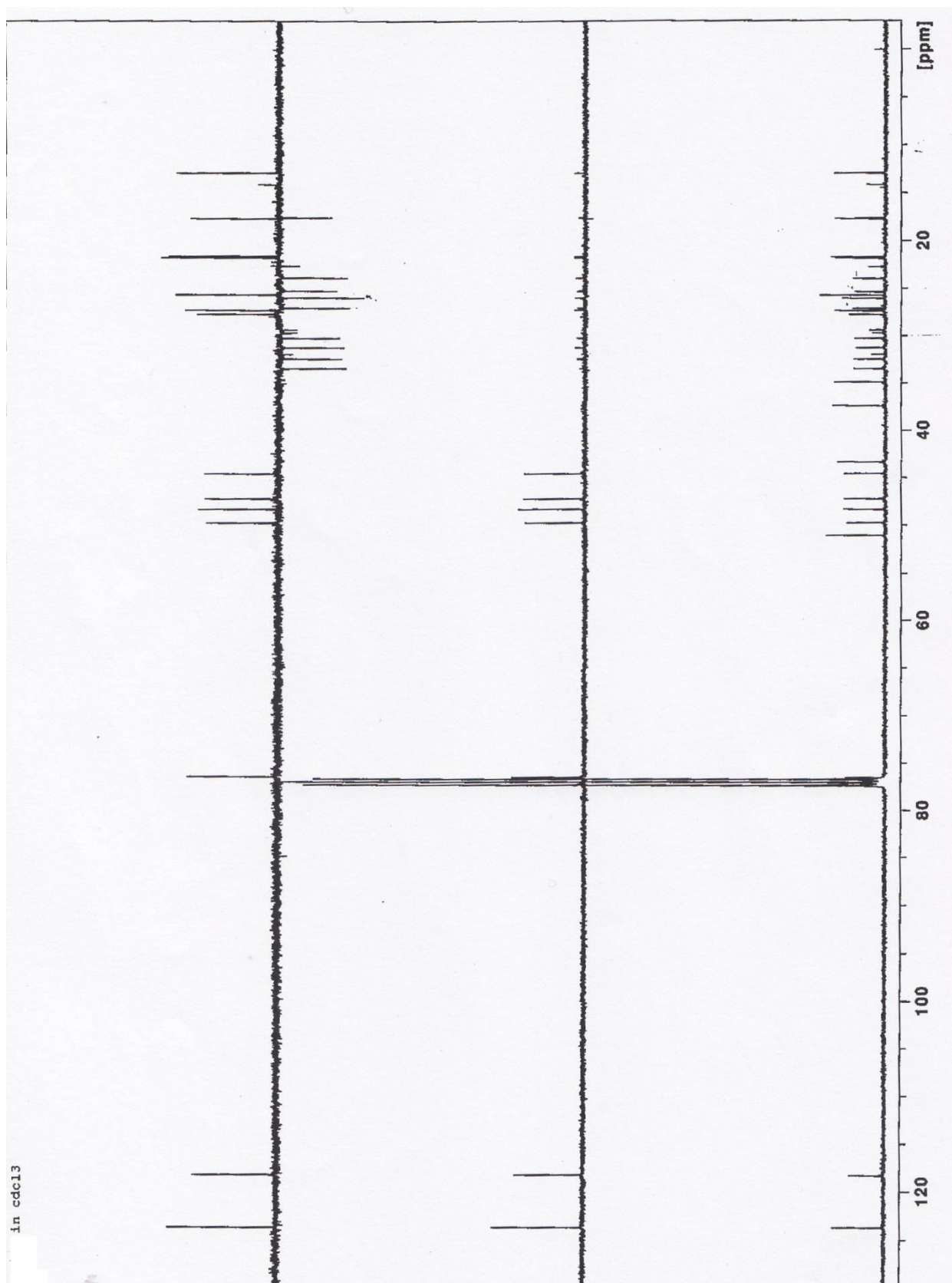
Figure E1.4: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound RA5
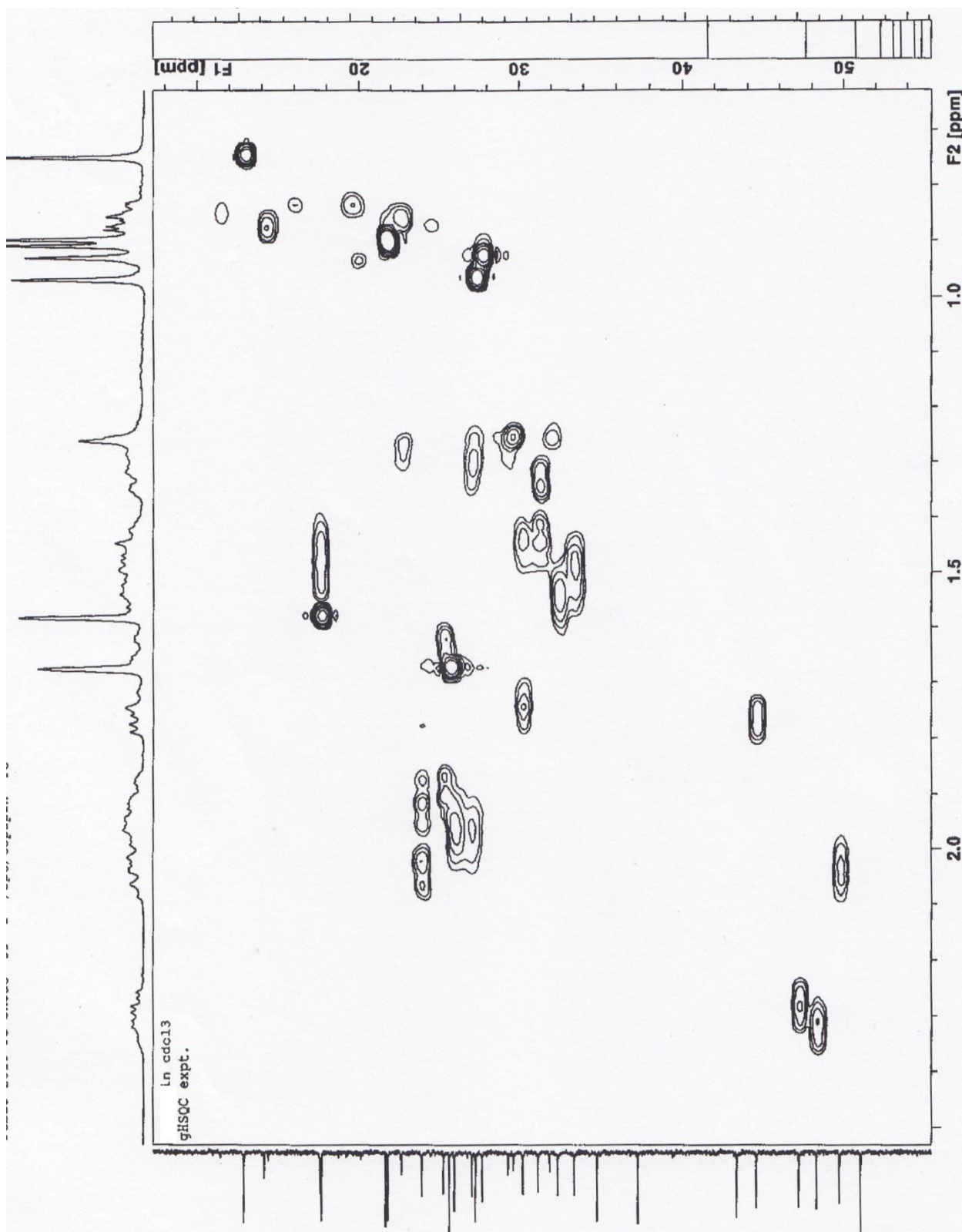
Figure E1.5: HSQC NMR spectrum of RA5
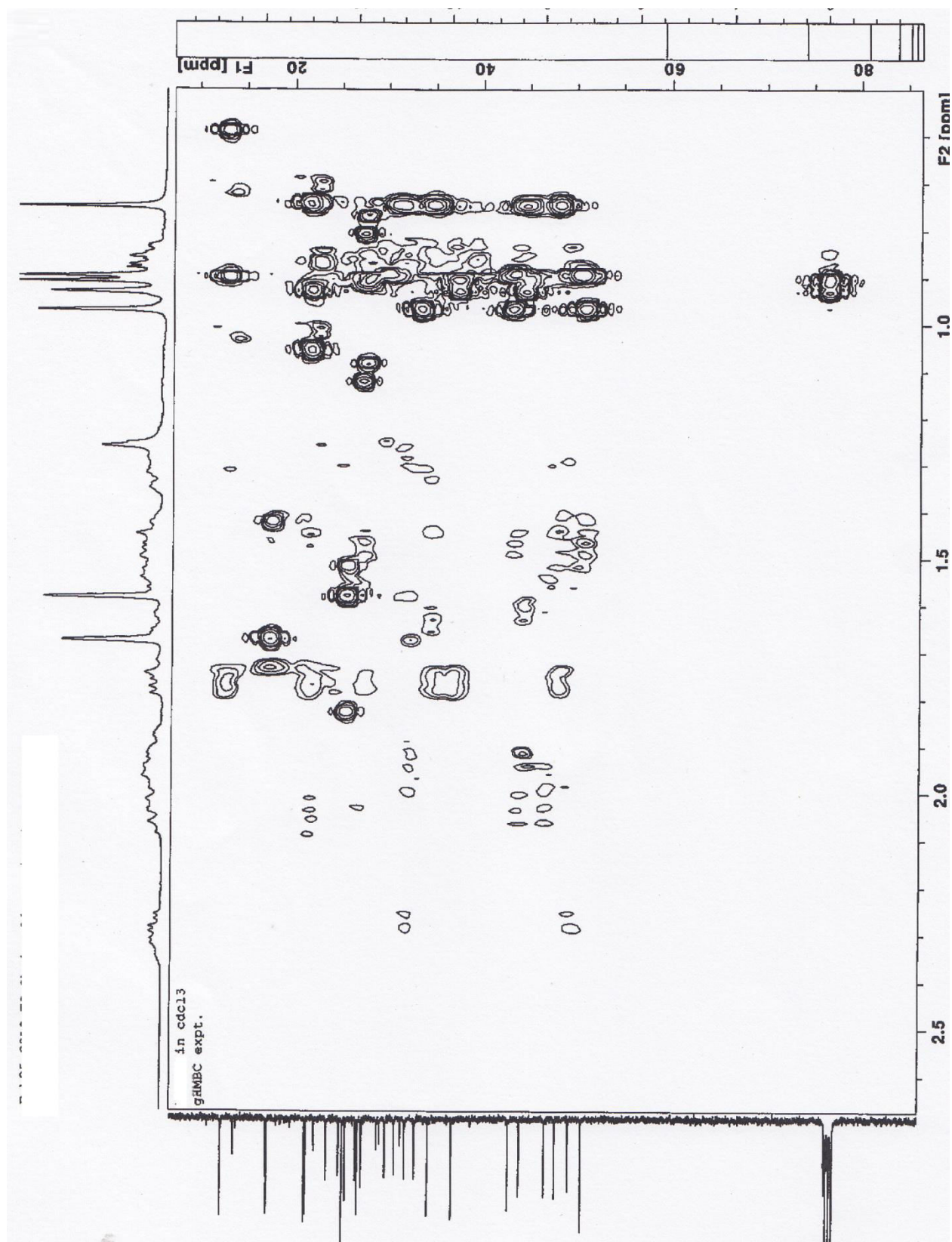
Figure E1.6: HMBC NMR spectrum of RA5
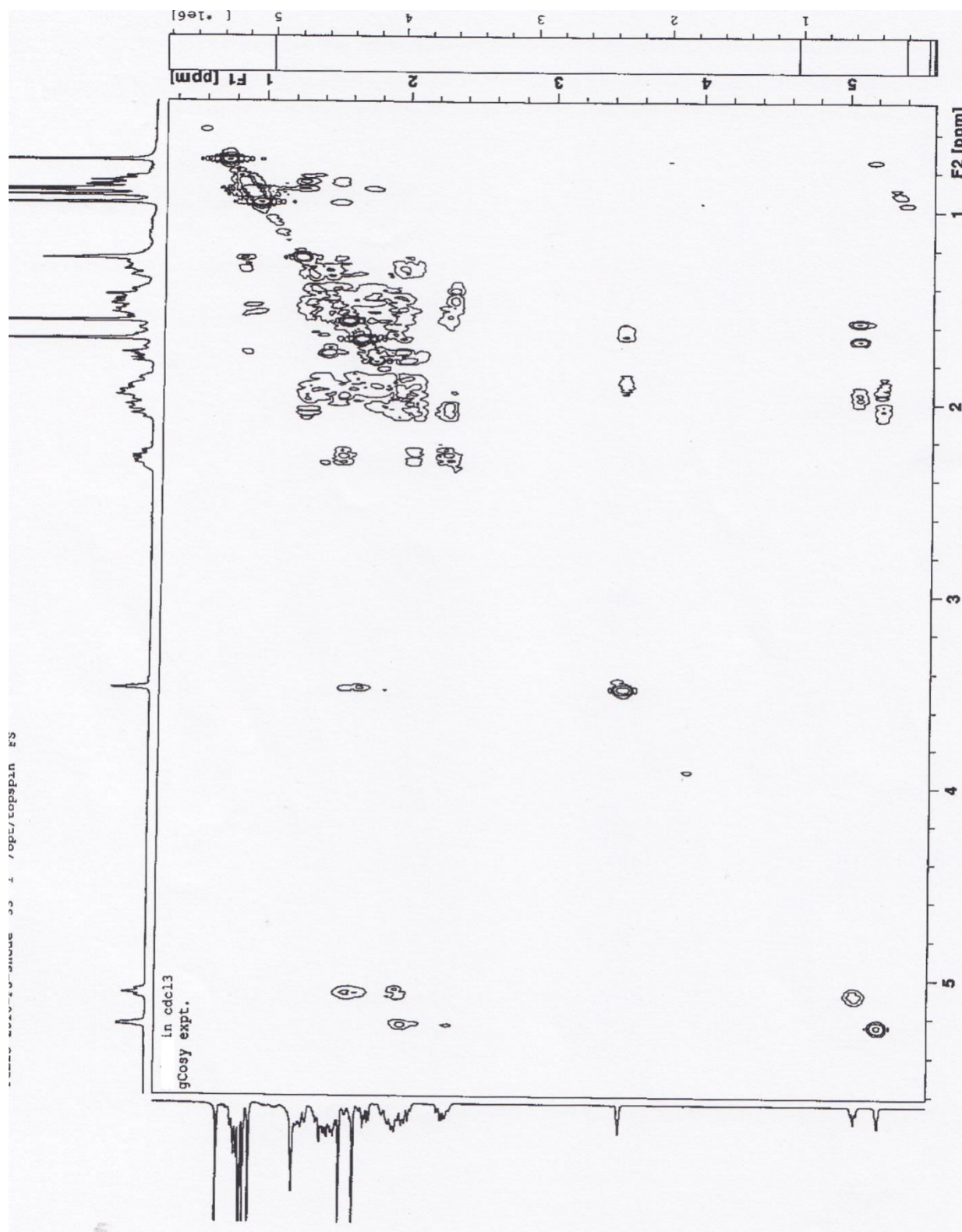
Figure E1.7: COSY NMR spectrum of RA5
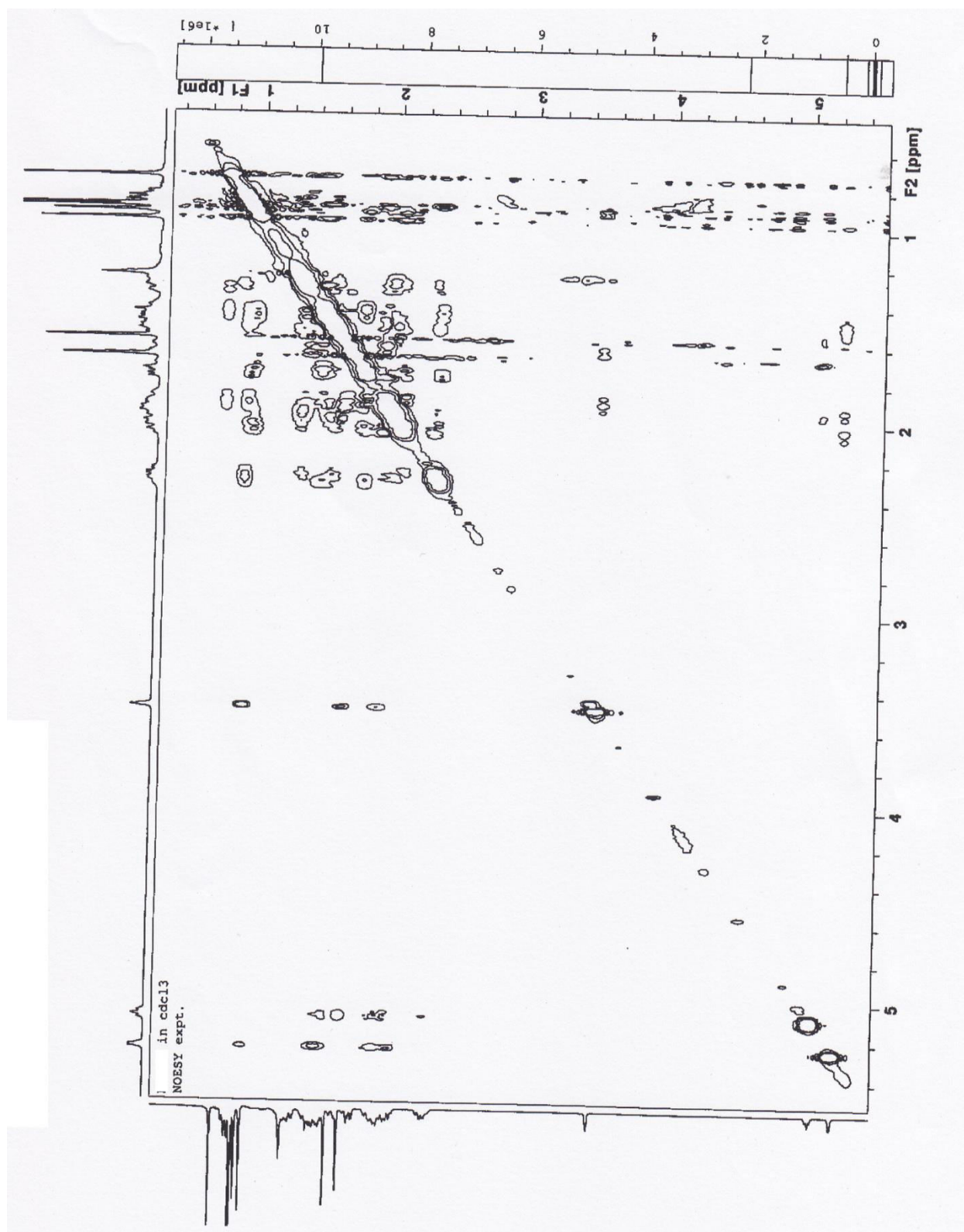
Figure E2.8: NOESY NMR spectrum of RA5
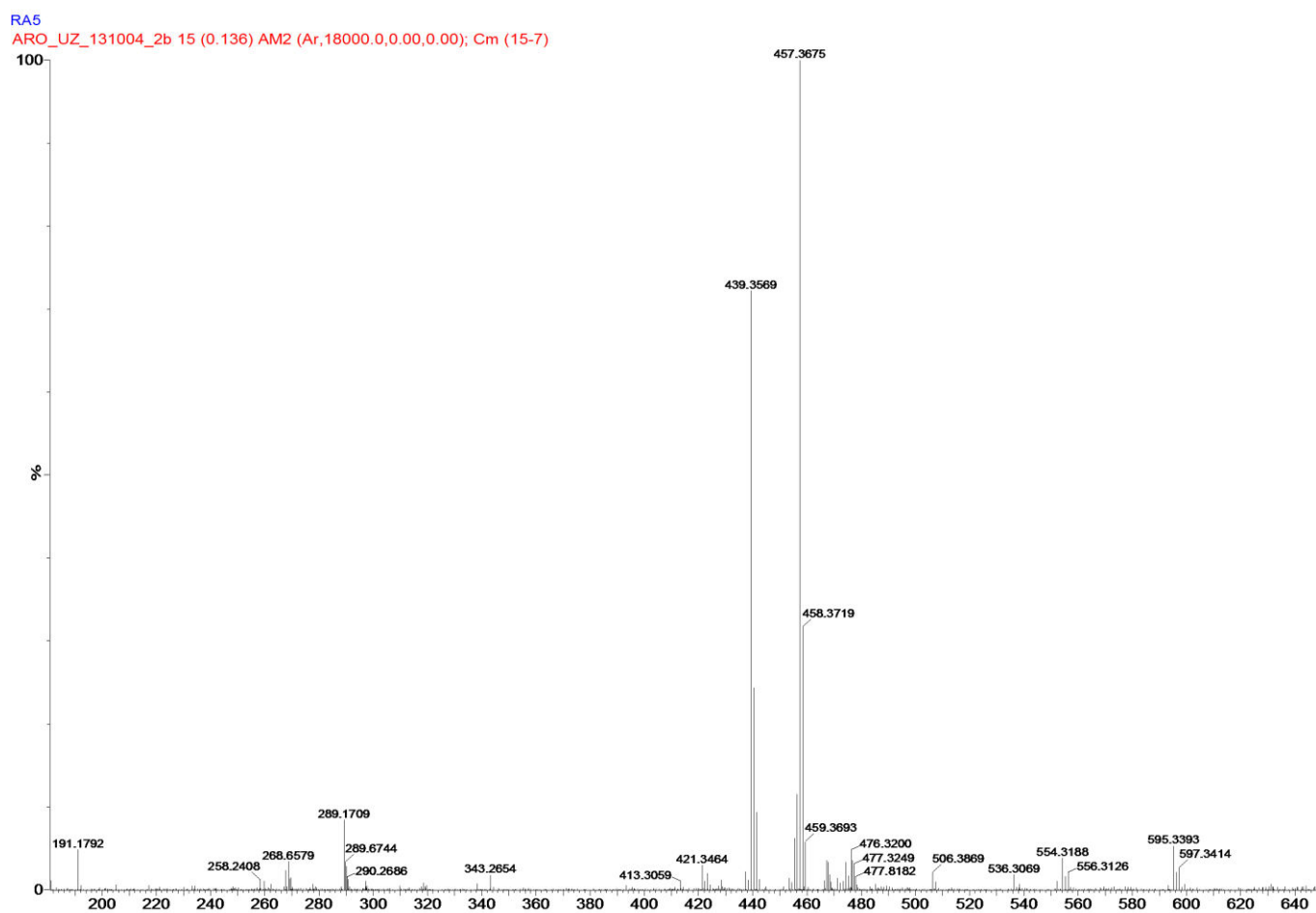
Figure E1.9a: MS spectrum of RA5
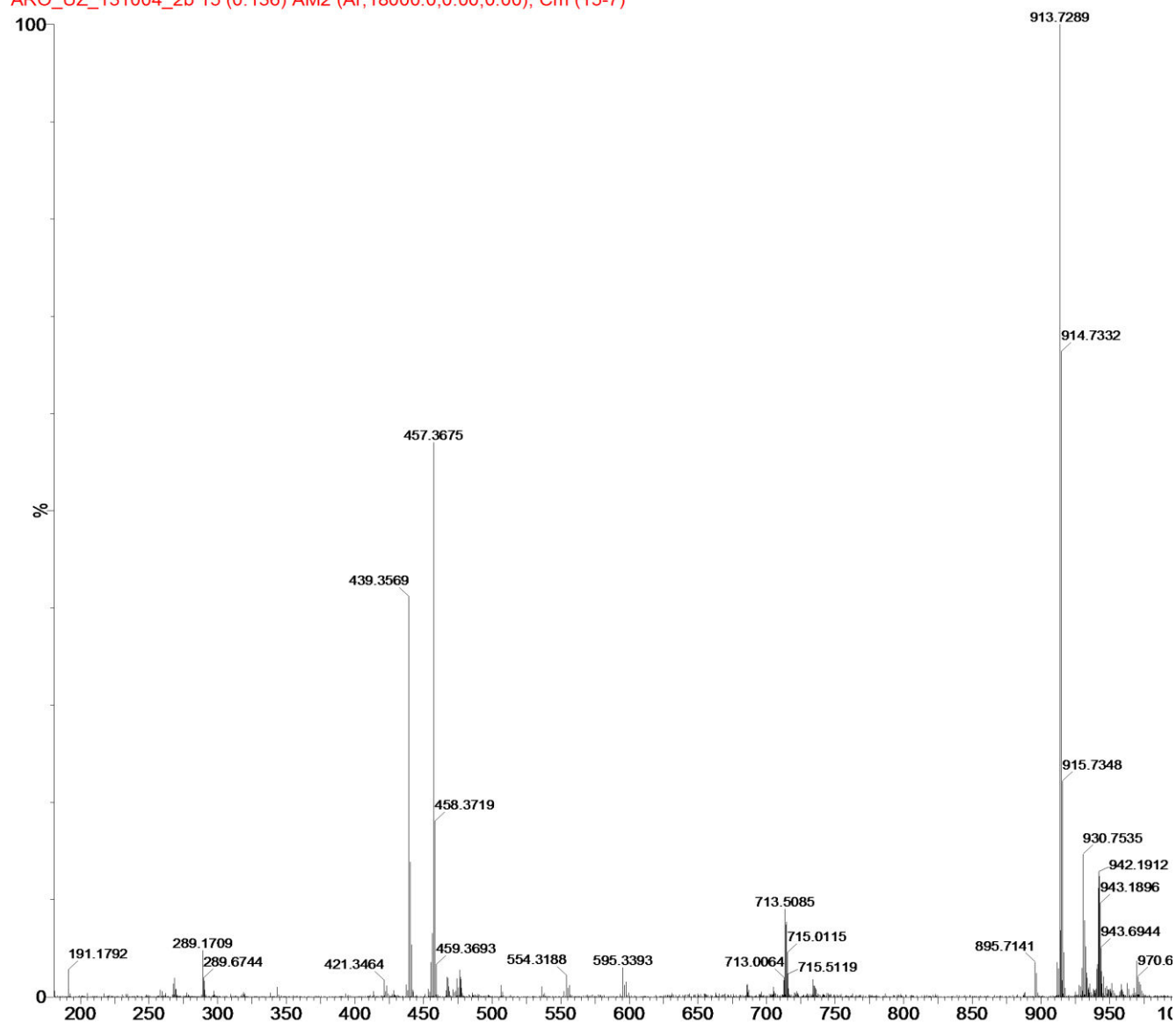
Figure E1.9b: MS spectrum of RA5

E2. Spectra of RA3
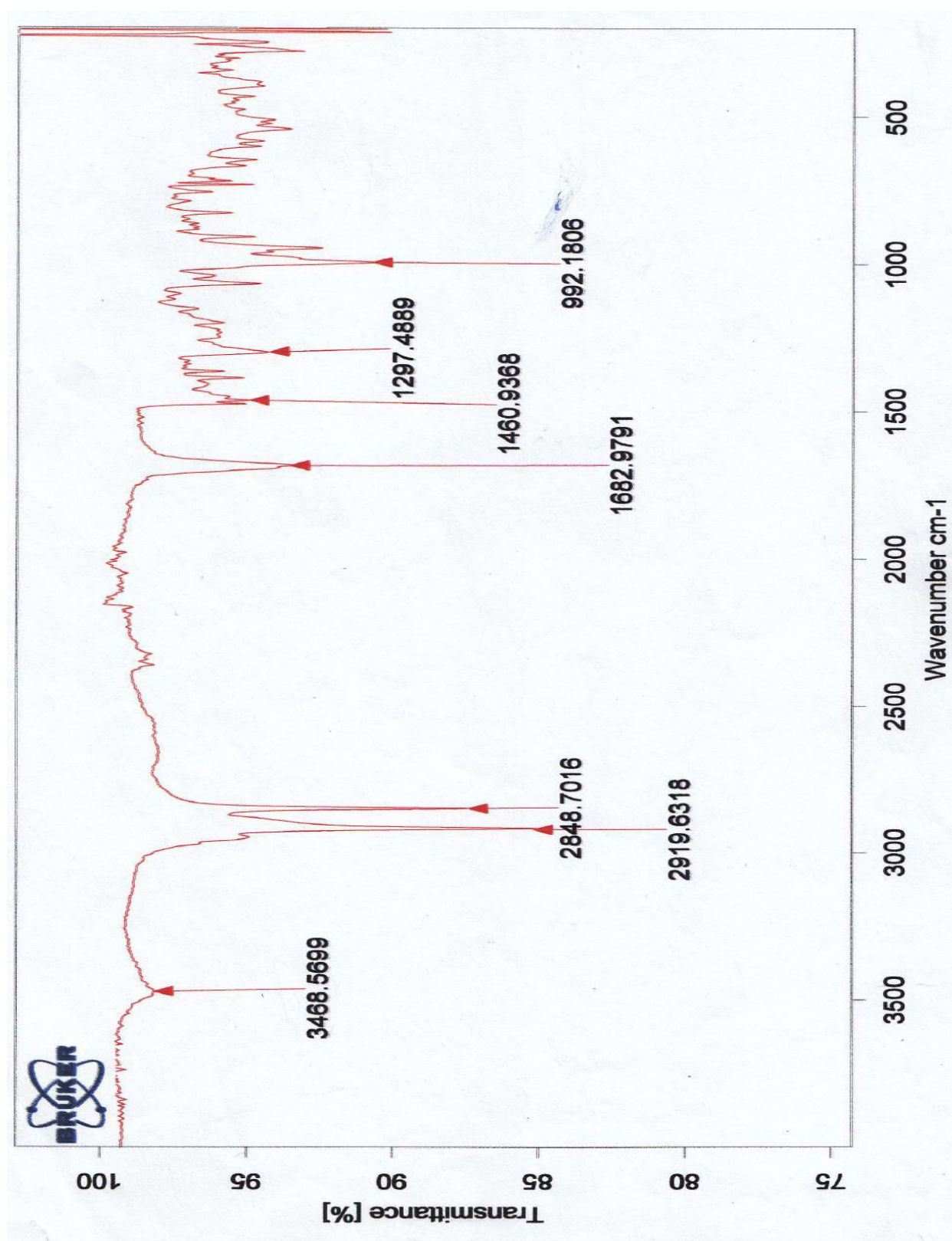
Figure E2.1: IR spectrum of RA3
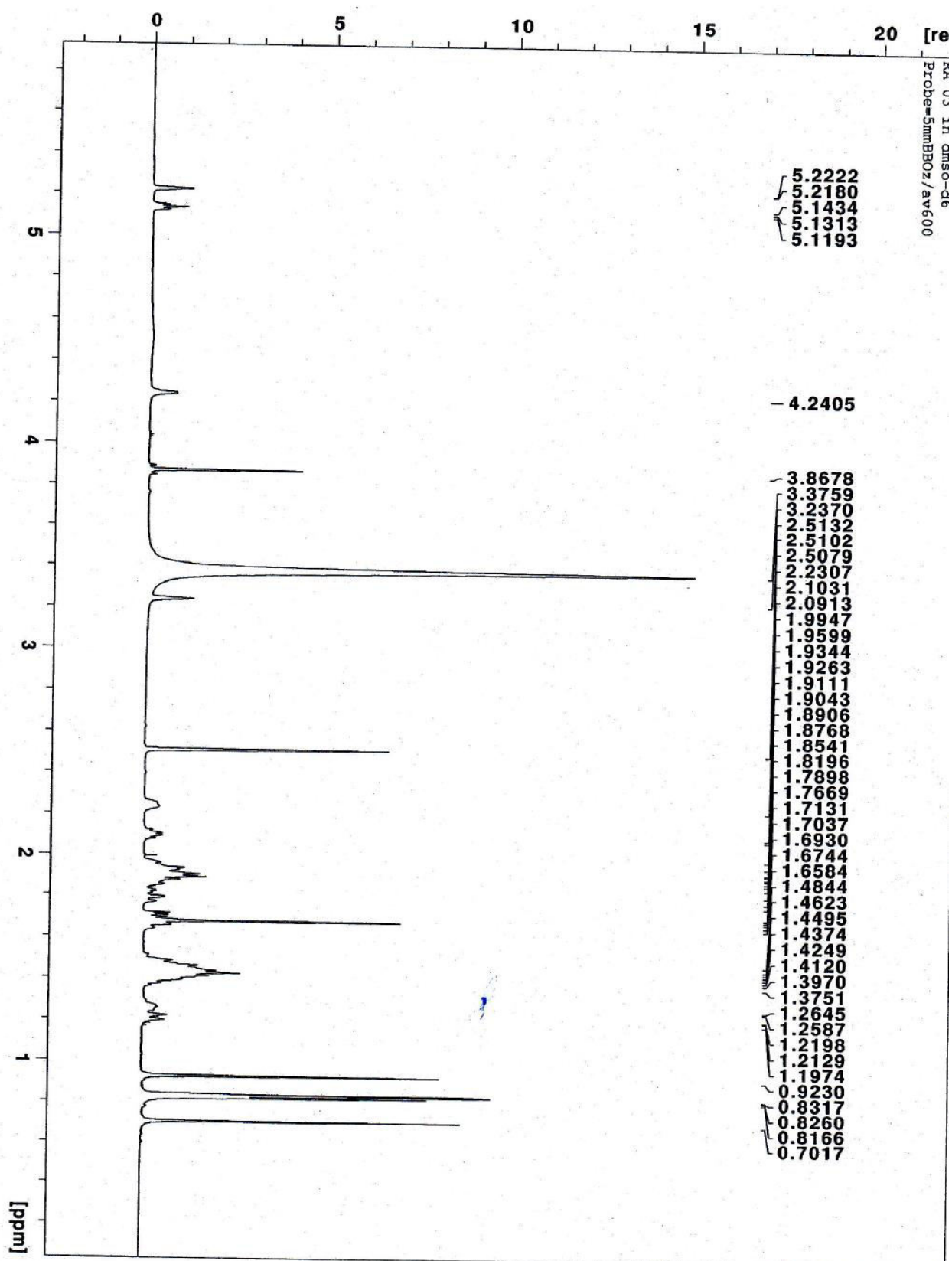
Figure E2.2: ^{1}H-NMR spectrum of RA3
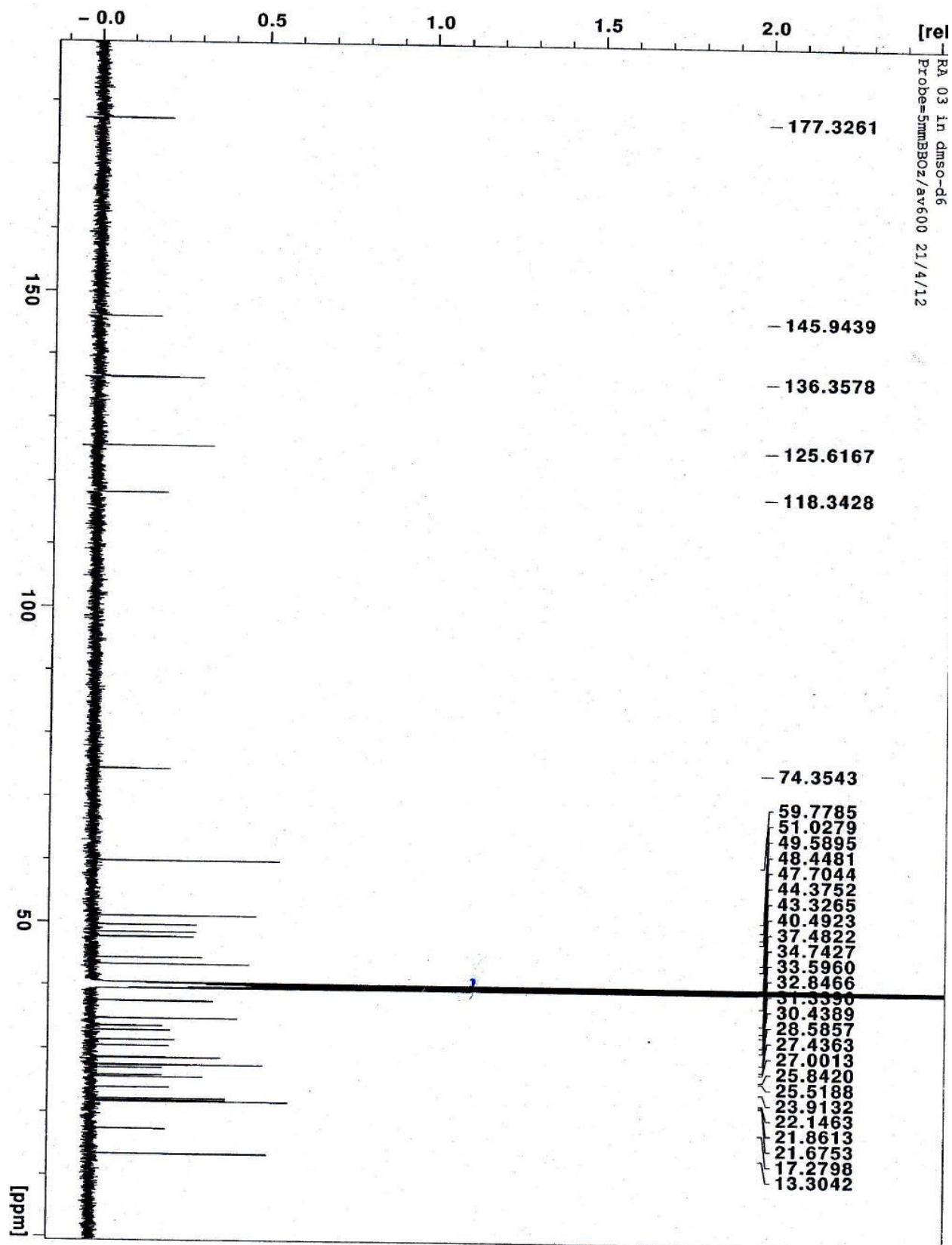
Figure E2.3: ^{13}C-NMR spectrum of RA3

E3. Spectra of R51
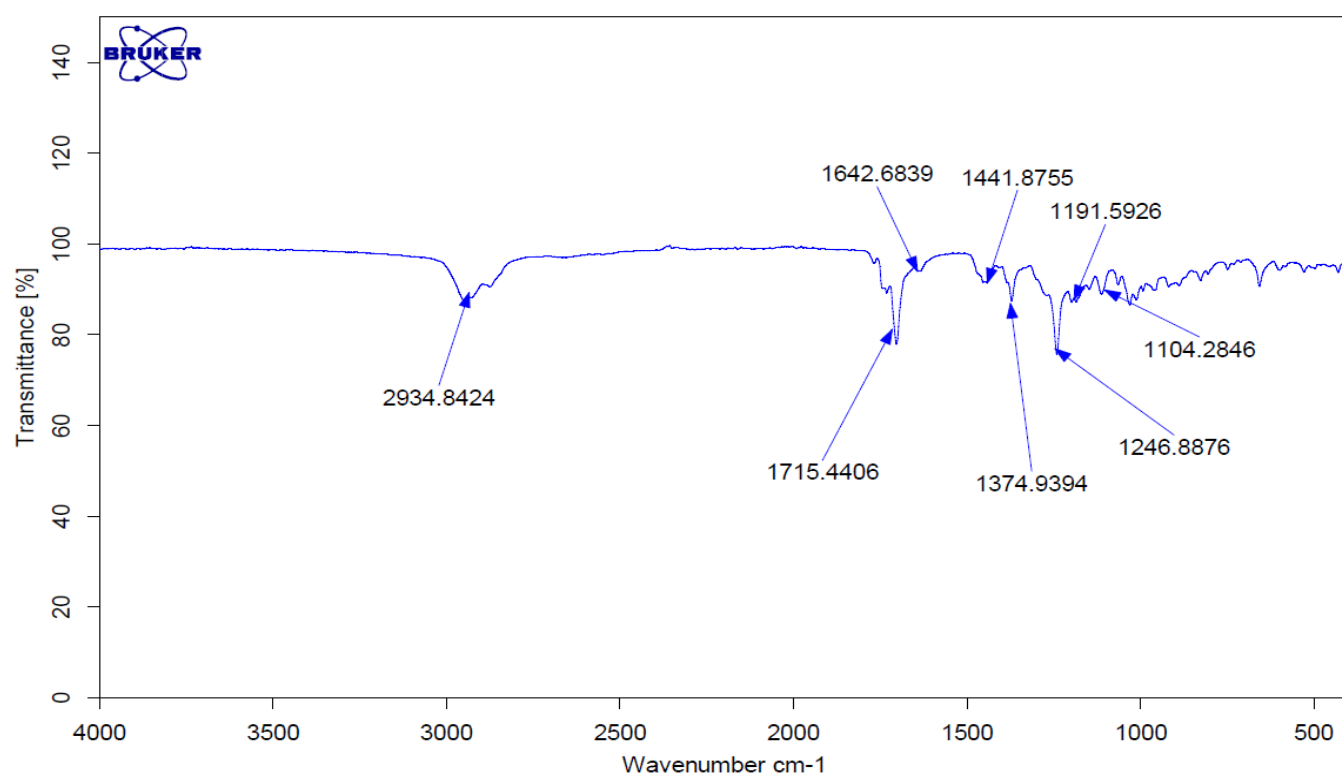
Figure E3.1: IR spectrum of R51
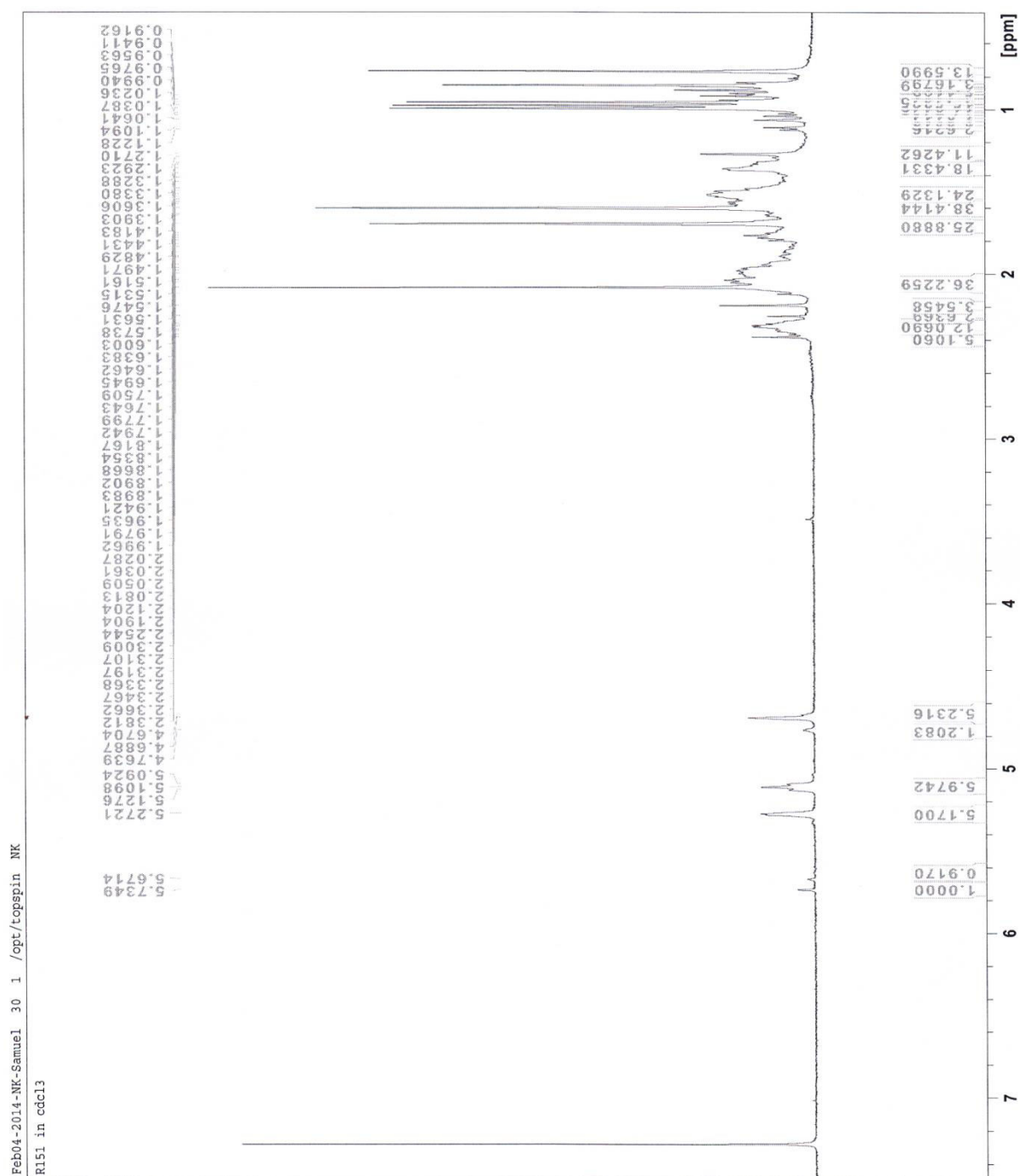
Figure E3.2: ^{1}H-NMR spectrum of R51
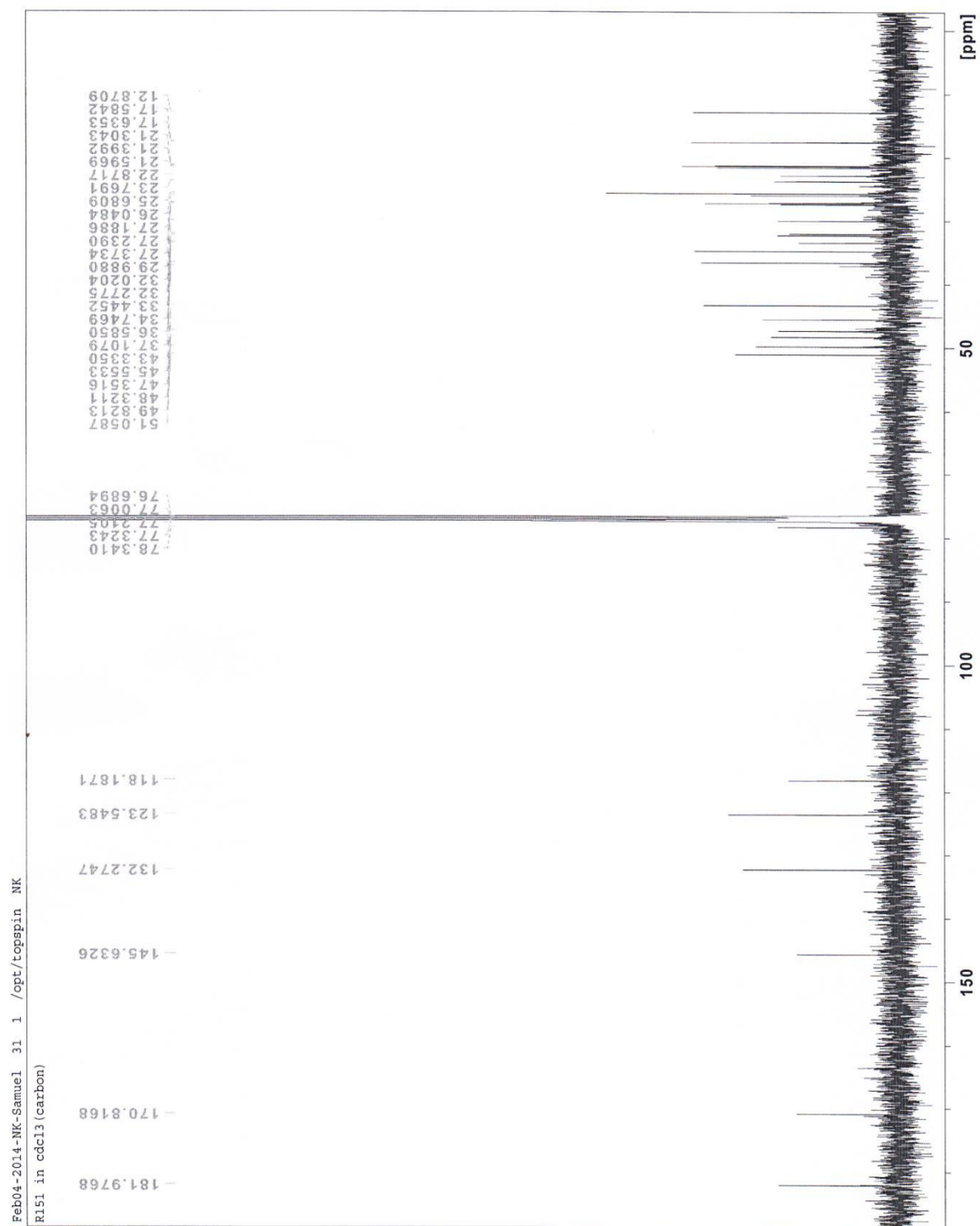
Figure E3.3: ^{13}C-NMR spectrum of R51

E4. Spectra of R52
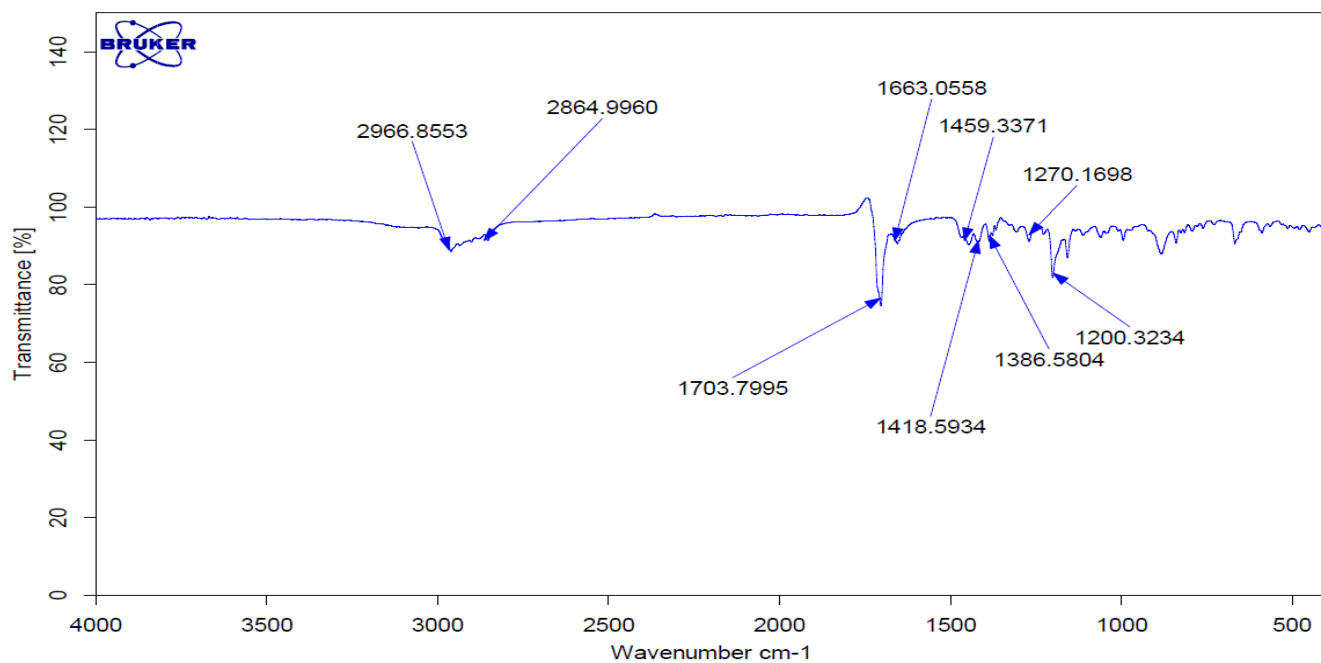
Figure E4.1: IR spectrum of R52
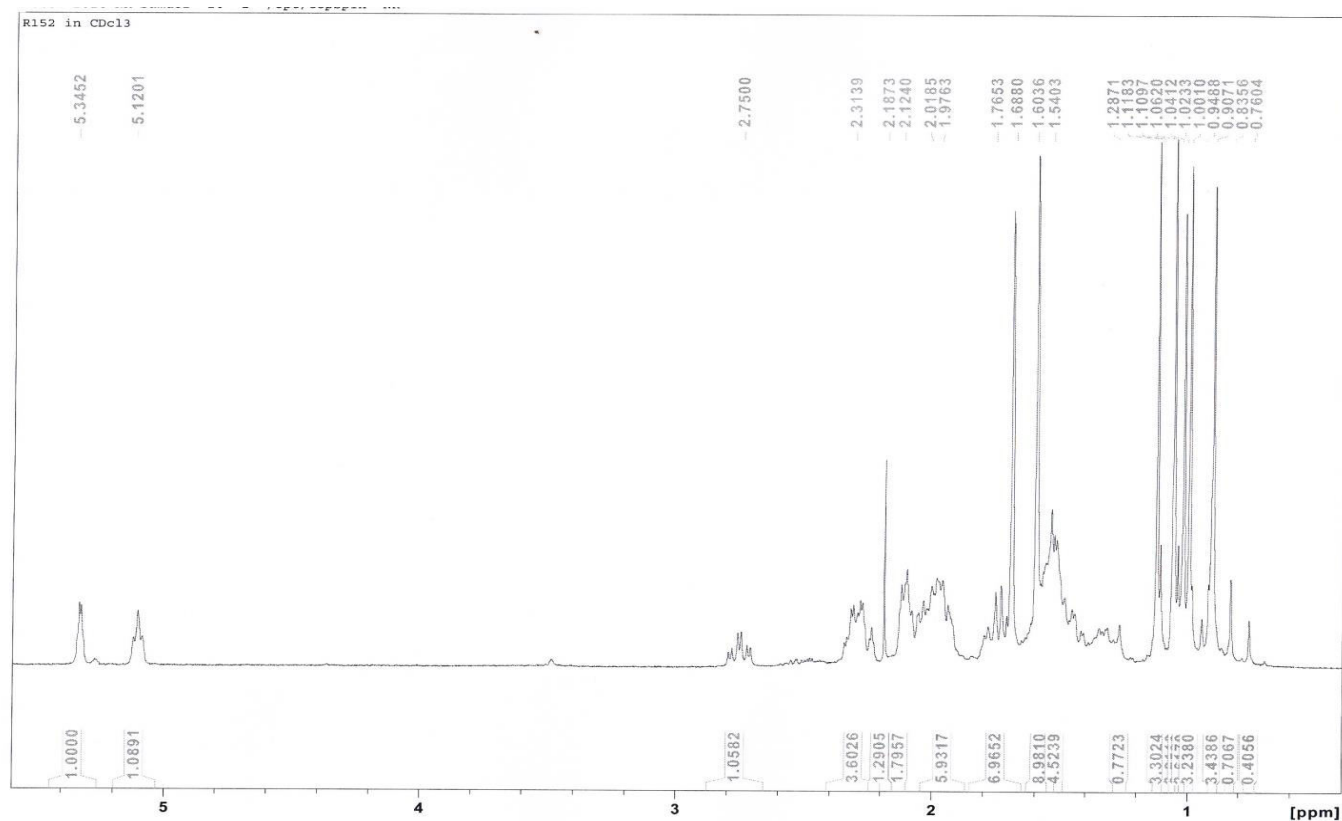
Figure E4.2: ^{1}H-NMR spectrum of R52

Figure E4.3: ^{13}C-NMR spectrum of R52

E5. Spectra of R31
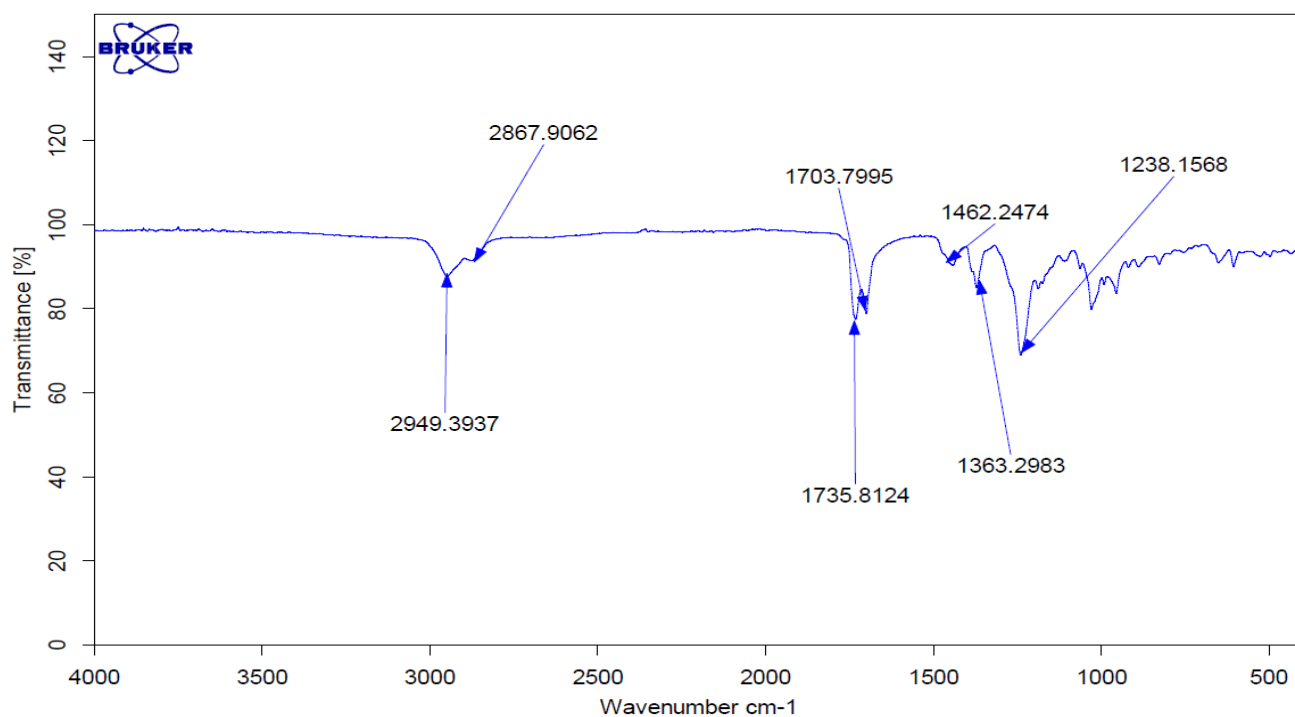
Figure E5.1: IR spectrum of R31
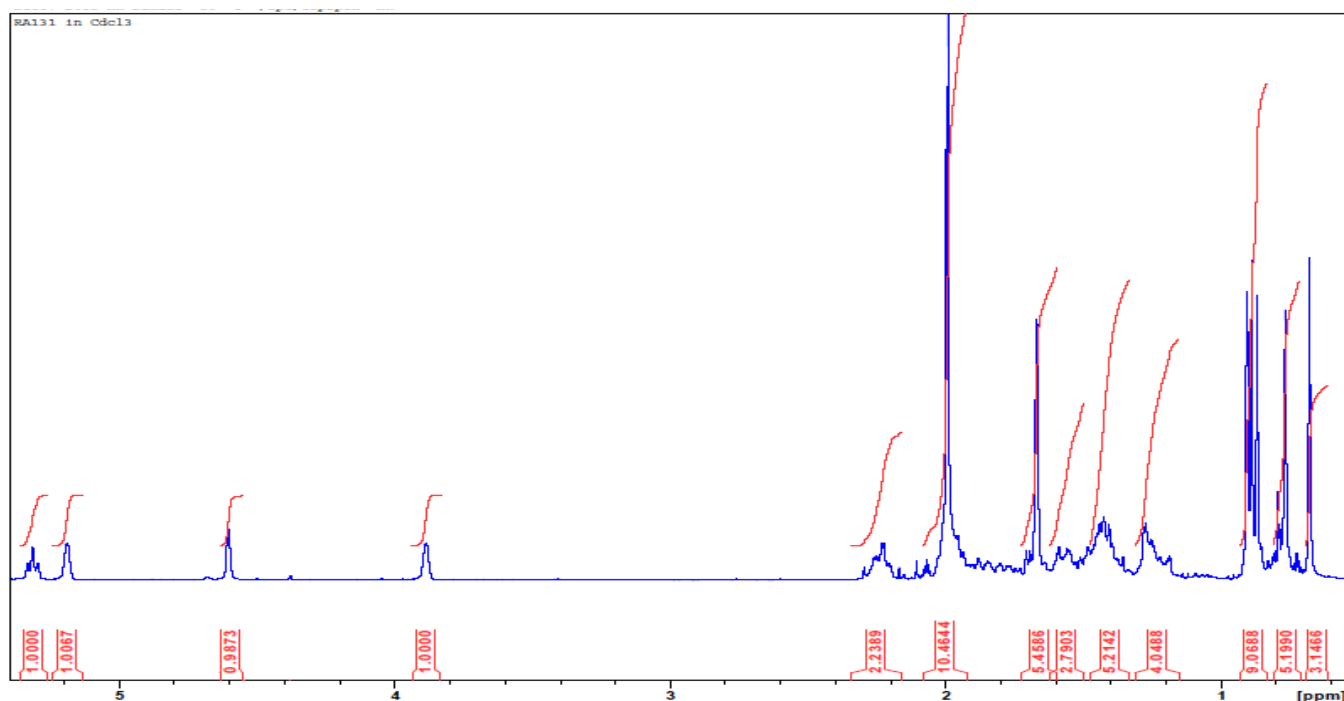
Figure E5.2: ^{1}H-NMR spectrum of R31
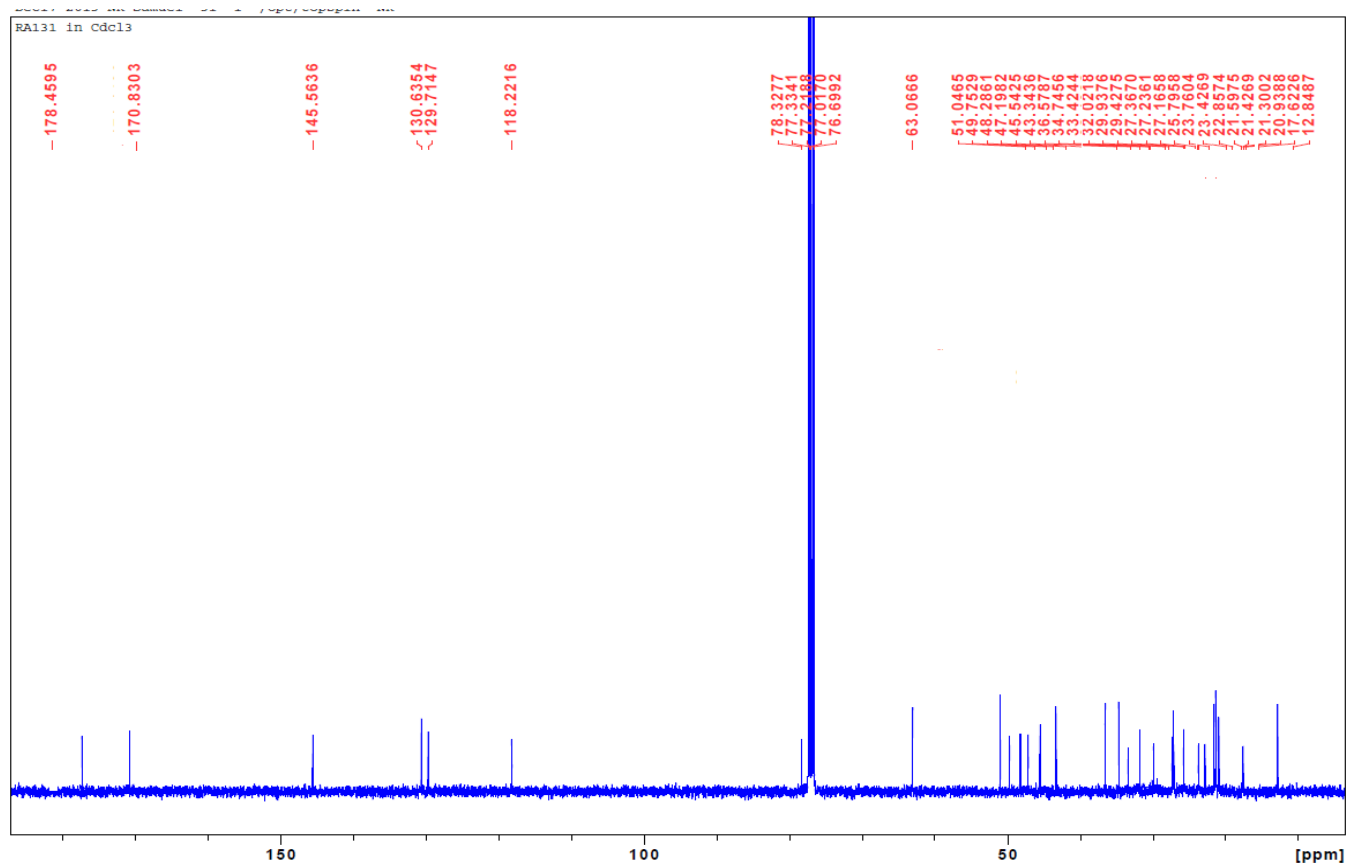
Figure E5.3: ^{13}C-NMR spectrum of R31

E6. Spectra of R32
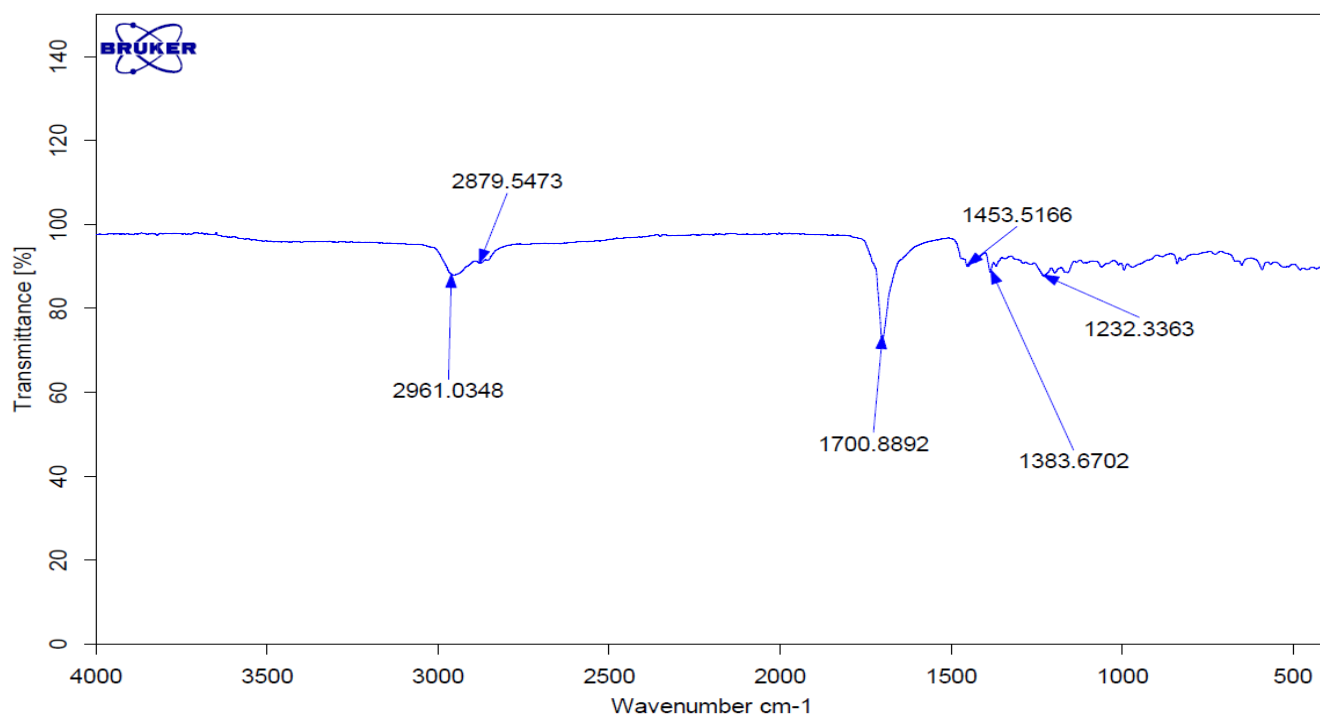
Figure E6.1: IR spectrum of R32
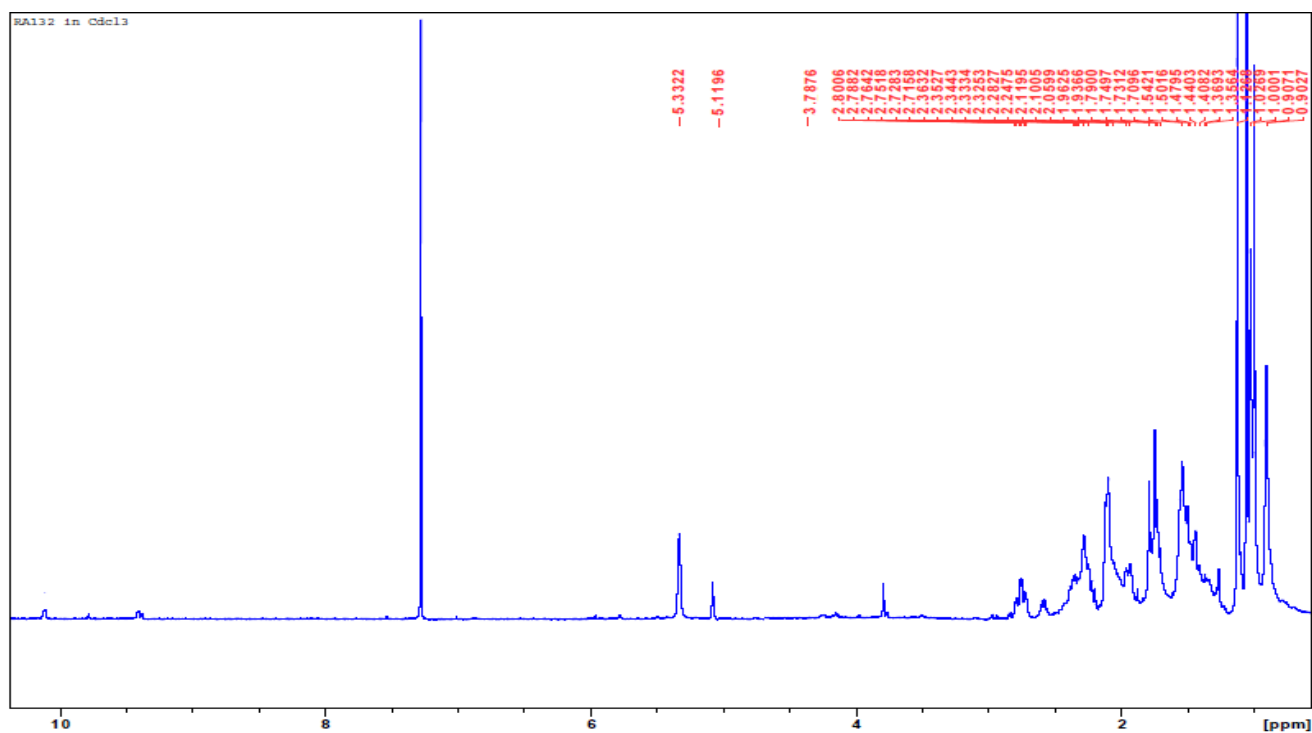
Figure E6.2: ^{1}H-NMR spectrum of R32
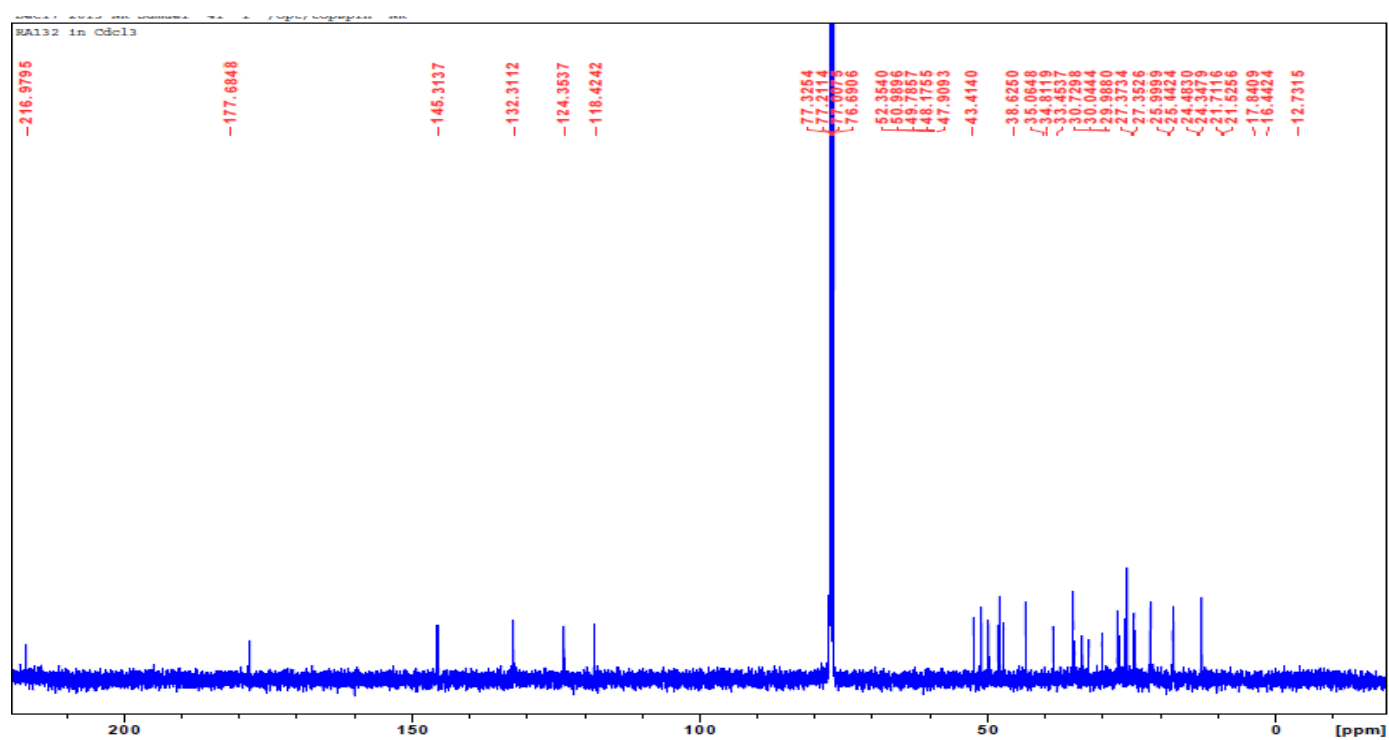
Figure E6.3: ^{13}C-NMR spectrum of R32

APPENDIX F

F. RESEARCH OUTPUT

In Vitro Antihyperlipidemic Activity of Triterpenes from Stem Bark of *Protorhus longifolia* (Benrh.) Engl.

Mosa RA[1], Naidoo JJ[1], Nkomo FS[1], Opoku AR[1], Muller CJF[2]

[1]Department of Biochemistry & Microbiology, University of Zululand, P. Bag X1001, KwaDlangezwa, 3886, Republic of South Africa
[2]Diabetes Discovery Platform, Medical Research Council (MRC), P.O. Box 19070, Tygerberg 7505, South Africa
E-mail: rebamang@gmail.com

Introduction

Hyperlipidemia, a metabolic disorder characterized by abnormally elevated blood lipids levels, is crucial to the pathogenesis of cardiovascular disease[1]. Undesirable side effects of the current hypolipidemic drugs[2,3] has triggered a search for new effective drugs with improved safety profile. Current literature supports plant derived triterpenes as new targets for drug development. Thus this work investigated the antihyperlipidemic activity of two triterpenes from stem bark of *Protorhus longifolia*.

Methodology

o Chromatographic techniques were used to isolate the compounds from the chloroform extract of the plant. Structures of the isolated compounds were established and confirmed based on the Spectroscopic data and literature[4] (Fig. 1).

o Inhibitory activity of the triterpenes on selected lipid and carbohydrate digestive enzymes was evaluated by colorimetric assays following hydrolysis of the respective *pnp*-substrate[3].

o Bile acid kit was used to determine bile acid binding ability of the compounds[5].
o Effect on cellular glucose uptake was evaluated using C2C12 and 3T3-L1 cell lines[6]
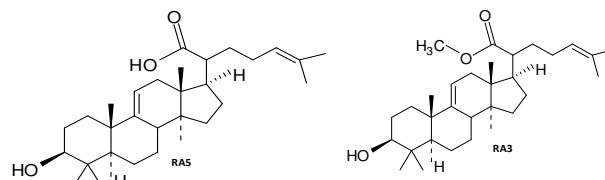
Fig.1: Chemical structures of 3β-hydroxylanosta-9,24-dien-21-oic acid (**RA5**) and **m**ethyl-3β-hydroxylanosta-9,24-dienoate (**RA3**).
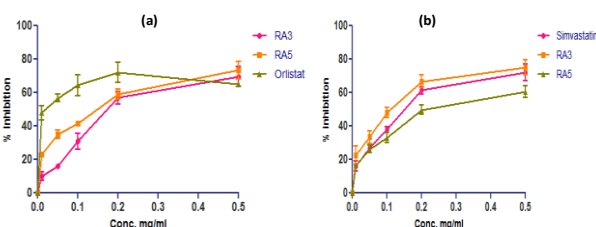
Results

Fig.2: Inhibitory effect of the triterpenes on (**a**) pancreatic lipase- IC$_{50}$ (mg/ml): orlistat (**0.01**); RA3 (**0.17**); RA5 (**0.15**) and (**b**) cholesterol esterase- IC$_{50}$ (mg/ml): simvastatin (**0.16**); RA3 (**0.11**); RA5 (**0.31**).
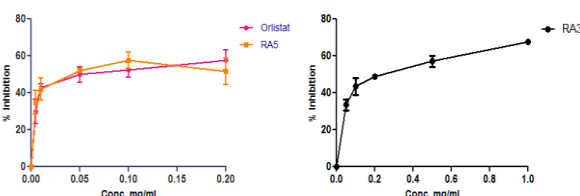
Fig.3: Inhibitory effect of the triterpenes on hormone-sensitive lipase- IC$_{50}$ (mg/ml): orlistat (**0.01**); RA3 (**0.24**); RA5 (**0.04**).
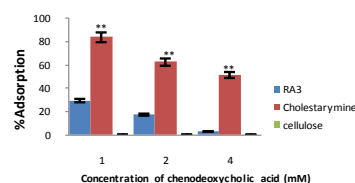
Fig. 4: Bile acid binding ability of the compounds. * P < 0.05; ** p < 0.001; *** p < 0.0001;

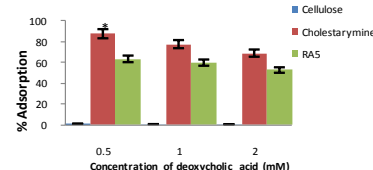**Table 1:** Inhibitory activity (%) of the triterpenes on some carbohydrate digestive enzymes

mg/ml	α-Glucosidase		Maltase		Sucrase	
	RA3	RA5	RA3	RA5	RA3	RA5
1.2	14.0 ± 0.09	1.50 ± 0.02	18.3 ± 0.41	16.0 ± 0.03	1.54 ± 0 .51	3.50 ± 0.11
1.4	24.3 ± 1.17	1.90 ± 0.03	28.9 ± 1.22	24.0 ± 0.03	3.21 ± 0.27	3.80 ± 0.13
1.6	29.1 ± 0.13	3.60 ± 0.35	25.7 ± 0.40	27.5 ± 0.06	4.76 ± 0.10	5.10 ± 0.12
1.8	37.9 ± 0.48	8.00 ± 0.01	31.1 ± 0.72	27.6 ± 0.01	3.98 ± 0.04	4.80 ± 0.07
2.0	49.7 ± 2.06	11.8 ± 0.04	41.5 ± 0.06	37.6 ± 0.10	5.88 ± 0.23	7.00 ± 0.07

Fig. 5: Effect of the compounds on C2C12 and 3T3-L1 Glucose uptake. * P < 0.05; ** p < 0.001; *** p < 0.0001.

References

1. Nelson, R.H 2013. Hyperlipidemia as a risk factor for cardiovascular disease. *Primary Care: Clinics in Office Practice* 40(1): 195-211.
2. Zhang. X., Wu, C., Wu, H., Sheng, L., Su, Y., et al. 2013. Anti-hyperlipidemic effects and potential mechanisms of action of the caffeoylquinic acid-rich *Pandanus tectorius* fruit extract in hamsters fed a high fat-diet. PLoS ONE 8(4): e61922. doi:10.1371/journal.pone.0061922
3. Bustanji, Y., Issa, A., Mohammad, M. ,Hudaib, M. ,Tawah, K. ,Alkhatib, H., Almasir, I. , Al-Khalid, B. 2010. Inhibition of hormone sensitive lipase and pancreatic lipase by *Rosmarinus officinalis* extract and selected phenolic constituents. *Journal of Medicinal Plants Research* 4(21): 2235-2242
4. Mosa, R. A., Oyedeji, O.A., Shode, F.O., Singh, M., Opoku, A.R., 2011. Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity. *African Journal of Pharmacy and Pharmacology*, **5**: 2698-2714.
5. Matsumoto, K. ,Yokoyama, S. ,Gato, N. 2010. Bile acid-binding activity of young Persimmon (*Diospyros kaki*) fruit and its hypolipidemic effect in mice. *Phytotherapy Research* 24: 205–210.
6. Muller, C.J.F., Joubert, E., de Beer, D., Sanderson, M., Malherbe, C.J., Fey, S.J., Louw, J . 2012. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. *Phytomedicine*
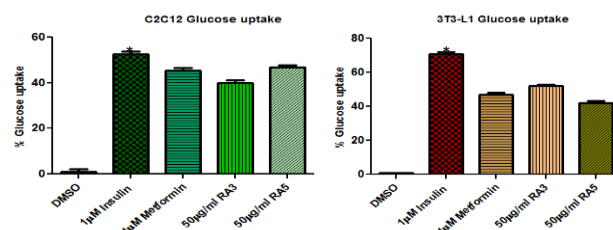
Conclusion

The two triterpenes from *P. longifolia* effectively exhibited inhibitory activity on the tested enzymes. It is apparent that the compounds possess hypolipidemic properties.

In Vivo Antihyperlipidemic Activity of a Triterpene from Stem Bark of *Protorhus longifolia* (Benrh.) Engl.

Opoku AR, Mosa RA, Cobongela SZZ, Machaba KE

Department of Biochemistry & Microbiology, University of Zululand, P. Bag X1001, KwaDlangezwa, 3886, Republic of South Africa
E-mail: OpokuA@unizulu.ac.za

Background

Hyperlipidemia, characterized by abnormal high levels of blood cholesterol and triglycerides, is the major risk factor of coronary heart diseases[1]. Adverse side effects of the currently available antihyperlipidemic drugs[2,3] indicate the need for the search of alternative medicine, preferably of natural origin. Plant-derived compounds have become very important in the development of effective therapeutics.

Objective

This study investigated the antihyperlipidemic activity of a triterpene (Methyl-3β-hydroxylanosta-9,24-dienoate) from stem bark of *Protorhus longifolia*

Methodology

o Chromatographic techniques were used to isolate and purify the compound from the chloroform extract of the plant[4]. Spectral techniques [NMR, IR] and literature[4,5] were used to establish and confirm structure of the isolated compound (Fig. 1).

o The *in vitro* antihyperlipidemic activity of the compound has been reported[6]. In this study the *in vivo* activity was investigated on high-fat-diet induced hyperlipidemia in rats[3] (Fig. 2). Liver was used for histology while serum was used to determine lipids components.
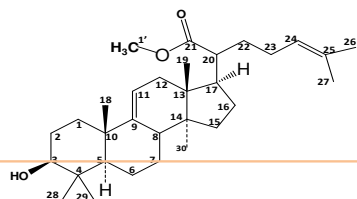

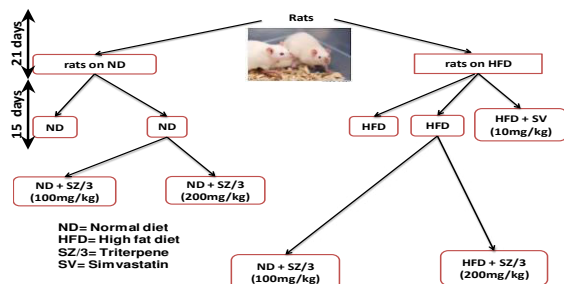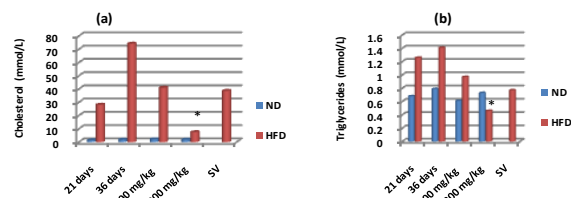**Fig.1: Chemical structure of methyl-3β-hydroxylanosta-9,24-dienoate (SZ/3)**

Fig.2: Schematic presentation of the animal experiment. Rats (≈200g) were randomly divided into 2 groups (normal and high-fat-diet).

ND= Normal diet
HFD= High fat diet
SZ/3= Triterpene
SV= Simvastatin

Results

*p < 0.05, compared to 36 days HFD untreated group

Fig.3: Effect of SZ/3 at different concentrations on serum **cholesterol (a)** and **triglycerides (b)** levels after 15 days treatment
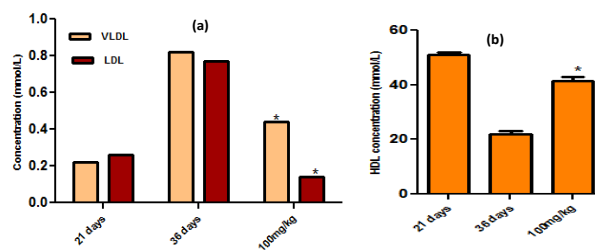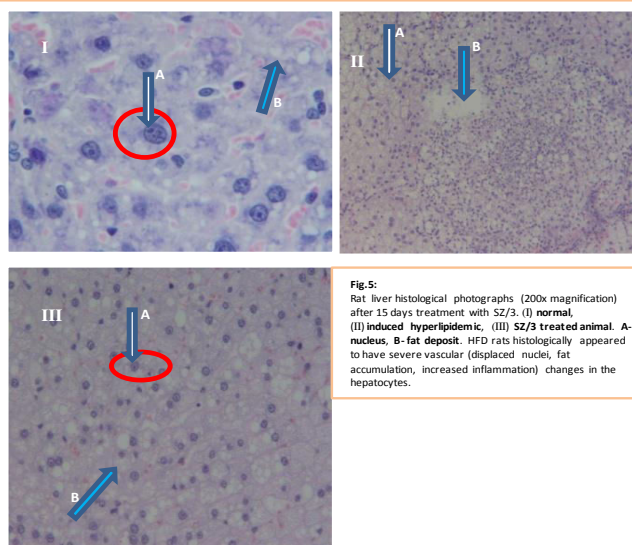

Fig.4: Effect of SZ/3 on serum **VLDL and LDL (a)**, and **HDL (b)** levels after 15 days treatment

Fig.5:
Rat liver histological photographs (200x magnification) after 15 days treatment with SZ/3. (I) **normal**, (II) **induced hyperlipidemic**, (III) **SZ/3 treated animal. A-nucleus, B - fat deposit**. HFD rats histologically appeared to have severe vascular (displaced nuclei, fat accumulation, increased inflammation) changes in the hepatocytes.

Conclusion

It is apparent that the triterpene from *P. longifolia* has hypolipidemic effect. The is a potential use of plant derived triterpenes as antihyperlipidemic agents.

References

1. Pande, V. V., Dubey, S., 2009. Antihyperlipidemic activity of *Sphaeranthus indicus* on atherogenic diet induced hyperlipidemia in rats. International Journal of Green Pharmacy, **3**:159-6
2. Wang, T., Choi, R.C., Li, J., Bi ,W.C., Zang , L., Liu, Z., Dong , T.T.X., Bi , K .,Tsim , K.W.K., 2010. Antihyperlipidemic Effect of Protodioscin, an Active Ingredient Isolated from the Rhizomes of *Dioscorea nipponica*. *Planta Medicine*, **76**:1642-1646.
3. Hor SY, Farsi E, Yam FM, Nuysh NM and Asmawi MZ, 2011. Lipid-lowering effects of *Coriolus versicolor* extract in poloxamer 407-induced hypercholesterolaemic rats and high cholesterol-fed rats. *Journal of Medicinal Plants Research*, **5**:2261.
4. Mosa, R. A., Oyedeji, O.A., Shode, F.O., Singh, M., Opoku, A.R., 2011. Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity. African Journal of Pharmacy and Pharmacology, **5**: 2698-2714.
5. Keller, A.C., Maillard, M.P., Hostettman, K., 1996. Antimicrobial steroids from the fungus *Fomitopsis pinicola*. *Phytochemistry*, **41**: 1041-1046.
6. Opoku, A., Mosa, R., Naidoo, J., Nkomo, F. The in vitro effects of a triterpene from the stem bark of Protorhus longifolia (Benrh) Engl. on some lipid and carbohydrate disgestive enzymes. III International Symposium on Medicinal and Nutraceutical Plants. 14-19 October 2012. Aracaju, Brazil.

181

The *In Vitro* Effect of a Triterpene from the Stem Bark of *Protorhus longifolia* (Benrh) Engl. on some Lipid and Carbohydrate Digestive Enzymes

Andy Opoku, Rebamang Mosa, Javan Naidoo, Fezile Nkomo

Department of Biochemistry & Microbiology, University of Zululand, P. Bag X1001, KwaDlangezwa, 3886, Republic of South Africa
E-mail: aropoku@pan.uzulu.ac.za

INTRODUCTION

Obesity is a common disorder of carbohydrate and fat metabolism. Lowering blood lipid levels has been shown to decrease the risk of coronary artery disease[1]. Natural products prepared from traditional medicinal plants have always presented an exciting opportunity for the development of new therapeutic agents.

Stem-bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae) is used by Zulu traditional healers to treat various diseases. The anti-platelet and anti-inflammatory activity of a lanosteryl triterpene from stem bark of the plant has been reported[2]. This study evaluated the *in vitro* inhibitory effect of the triterpene from the stem bark of *Protorhus longifolia* on lipid and carbohydrate digestion.

MATERIALS AND METHODS

❖ The triterpene (Fig. 1) was extracted, isolated and characterized from the chloroform extract[2]; the structure was analyzed and confirmed through IR, NMR, LC-MS spectral data[2].

❖ *The in vitro* effect of the triterpene on the selected lipid [hormone-sensitive lipase (HSL), pancreatic lipase and cholesterol esterase] and carbohydrate (dissacharidases, α- and β-glucosidases) digestive enzymes[3,4,5] was investigated by the hydrolysis of the respective *pnp*-substrate.

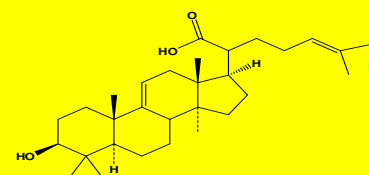❖ Bile acid binding ability of the compound was determined using bile acid kit[6].

Fig. 1: 3β-Hydroxylanosta-9,24-dien-24-oic acid (RA05)

Fig. 5: % adsorption of bile acids (taurocholic, cholic and deoxycholic acid) by the triterpene
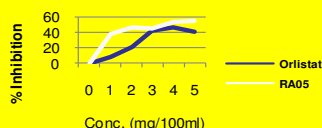
RESULTS

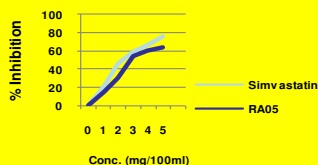Fig. 2: inhibitory activity of RA05 on pancreatic lipase

Fig. 3: inhibitory activity of RA05 on cholesterol esterase

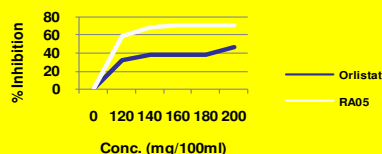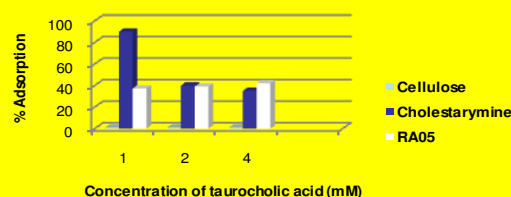Fig. 4: inhibitory activity of RA05 on hormone sensitive lipase

Concentration of taurocholic acid (mM)

Concentration of Cholic acid (mM)
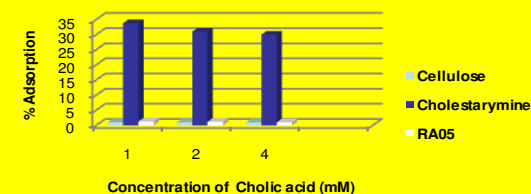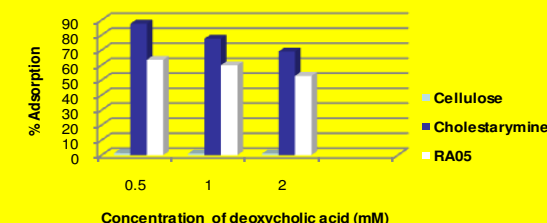
Concentration of deoxycholic acid (mM)

Table 1: % inhibitory activity of the triterpene on some carbohydrate digesting enzymes

Conc. of (mg/100ml)	α-Glucosidase	Maltase	Sucrase	Lactase
120	1.50 ± 0.019	16.0 ± 0.025	3.50 ± 0.110	0.00 ± 0.005
140	1.90 ± 0.028	24.0 ± 0.026	3.80 ± 0.126	0.00 ± 0.075
160	3.60 ± 0.350	27.5 ± 0.064	5.10 ± 0.115	0.00 ± 0.057
180	8.00 ± 0.009	27.6 ± 0.011	4.80 ± 0.072	0.00 ± 0.045
200	11.8 ± 0.039	37.6 ± 0.095	7.00 ± 0.067	0.00 ± 0.006

DISCUSSION AND CONCLUSION

The triterpene effectively inhibited the *in vitro* activities of pancreatic lipase, hormone-sensitive lipase, cholesterol esterase, and exhibited bile acid binding ability. It also showed effective inhibition of maltase and other disaccharidases. It is apparent that the compound has a potential antilipidemic property.

REFERENCES

1. Karalis, D.G., Victor, B., Ahedor, L., Liu, L. (2012). Use of Lipid-Lowering Medications and the Likelihood of Achieving Optimal LDL-Cholesterol Goals in Coronary Artery Disease Patients. Cholesterol 2012: doi:10.1155/2012/861924
2. Mosa, R. A., Oyedeji, O.A., Shode, F.O., Singh, M., Opoku, A.R. (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity. African Journal of Pharmacy and Pharmacology Vol. 5(24), pp. 2698-2714.
3. Bustanji, Y. Issa, A. Mohammad, M. Hudaib, M. Tawah, K. Alkhatib, H. Almasir, I. Al-Khalid, B. (2010). Inhibition of hormone sensitive lipase and pancreatic lipase by *Rosmarinus officinalis* extract and selected phenolic constituents. Journal of Medicinal Plants Research **4**(21): 2235-2242
4. Hou, W., Li, Y., Zhang, Q., Wei, X., Peng, A., Chen, L., Wei, Y. (2009). Triterpene acids isolated from *Lagerstroemia speciosa* leaves as α-glucosidase inhibitors. *Phytotherapy Research* 23: 614–618
5. Dahlqvist, A. (1968). Assay of intestinal disaccharidases. *Analytical Biochemistry* 22: 99-107.
6. Matsumoto, K. Yokoyama, S. Gato, N. (2010). Bile acid-binding activity of young Persimmon (*Diospyros kaki*) fruit and its hypolipidemic effect in mice. *Phytotherapy Research* **24**: 205–210.