Antidiabetic activity of *Schkuhria pinnata* – Biological screening, PK analysis and mode of action

by

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

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February 2016

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Prenitha Sewnarain

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Degree:	**Doctor of Philosophy**

ABSTRACT

The increasing reliance on drugs from natural sources has led to the development of several drugs from traditional plants which are present in abundance in Southern Africa. With the rapid increase of incidence of type 2 diabetes in South Africa with potentially devastating effects on healthcare, the need for alternative therapeutics is a priority. In this study, *Schkuhria pinnata* (Lam.) Kuntze was investigated for its antidiabetic potential.

Initial screening of two different solvent extracts of *S. pinnata* identified an aqueous extract that lowered blood glucose concentrations in a hyperglycaemic streptozotocin-induced diabetic rat. The classical bioassay approach was followed by using different solvents, drying processes and fractionation in order to produce the most active extract and attempt to isolate an active compound(s). An aqueous freeze dried extract was found to be the most active at stimulating glucose uptake activity in C2C12 and Chang cells. Fractionation of this extract in an attempt to identify the active compound yielded a novel crystalline compound 1 by NMR analysis. Screening for bioactivity of the extract and compound 1 using C2C12 muscle and Chang cells revealed that both extract and compound 1 were biologically active, however the activity of the aqueous extract was more significant overall. A butanone/pentane extract prepared for possible commercialization purposes was also shown to be active *in vitro*. To establish antidiabetic activity, the aqueous freeze dried extract, butanone/pentane extract and the enriched compound 1 fraction were tested in a streptozotocin (STZ) diabetic rat model showing hypoglycaemic effects for the aqueous freeze dried extract. Messenger RNA and protein studies on C2C12 muscle cells revealed that the aqueous freeze dried extract and compound 1 enhanced insulin receptor, GLUT-4, glycogen synthase, pyruvate kinase and pyruvate carboxylase expression, suggestive of an insulin mimetic mode of action, while the butanone/pentane extract enhanced adenosine monophosphate-activated kinase (AMPK) protein expression by a non-insulin dependent mechanism. A pharmacokinetic study (PK) established bioavailability of compound 1 following oral administration of the extracts, but not from the compound 1 enriched fraction.

From this study, the traditional use of *S. pinnata* has been scientifically validated as having antidiabetic properties. *In vitro* and *in vivo* bioassays, confirmed that an aqueous freeze dried extract which was prepared as per the traditional method had the most promising antidiabetic

activity. Compound 1 isolated from an active fraction was proven to be almost as effective as the parent extract in *in vitro* studies. This compound could therefore be the major active ingredient responsible for the uptake of glucose in cells and the hypoglycaemic activity *in vivo*.

In this study, the antidiabetic activities together with the mechanism of action of *S. pinnata* extracts and compound 1 were elucidated. The highlight of the study was the identification of a bioactive novel chemical entity (NCE) compound 1 (identified as 2-(2-{[(2E)-4-hydroxy-2-(hydroxymethyl)but-2-enoyl]oxy}-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)prop-2-enoic acid) isolated from an active fraction of *S. pinnata* that was proven to be almost as effective as the parent extract in *in vitro* studies. This compound could therefore be the major active ingredient responsible for the uptake of glucose in cells and the hypoglycaemic activity *in vivo*. The cellular mechanism of action of the *S. pinnata* extracts and compound 1 demonstrated both insulin mimetic and non-insulin dependent mechanisms (AMPK) in C2C12 muscle cells.

Further research in the form of preclinical and clinical trials need to be undertaken to make this extract or biologically active compound available as a herbal remedy or nutraceutical therapeutic for diabetes. To achieve this; safety, efficacy and mode of action studies will have to be established. The synthesis of compound 1 and/or analogues should also be investigated as an antidiabetic drug candidate.

Keywords: *S.pinnata*, streptozotocin-induced diabetic rat, hypoglycaemic, pharmacokinetic, novel chemical entity

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LIST OF ABBREVIATIONS

[α]	Specific optical rotation
μg/mL	Micrograms per millilitre
μL	Microlitre
μM	Micro molar
μm	Micrometre
^{0}C	Degrees Celsius
^{13}C	Carbon-13
^{1}H	Proton
2D	Two dimensional
ACN	Acetonitrile
ActB	Beta-actin
ADME	Absorption, distribution, metabolism, and excretion
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BW	Body weight
CA	California, USA
CD_3OD	Deuterated chloroform
cDNA	Complimentary DNA
cm	Centimetre
C_{max}	Maximum concentration

CO_2	Carbon dioxide
CoA	Coenzyme A
Conc	Concentration
COSY	Correlated spectroscopy
COX-1	Cyclooxygenase-1
CPM	Counts per minute
CSIR	Council for Scientific and Industrial Research
Ct	Threshold cycle
CYPs	Cytochrome P450s
d	Doublet
Da	Dalton
DAD	Diode array detector
DCM	Dichloromethane
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
ddH_2O	Double distilled water
ddt	Doublet of doublets of triplets
DEPT	Distortionless enhancement by polarisation transfer
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DMSO-D6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dictionary of Natural Products
DTT	1,4-Dithiothreitol
EC_{50}	Half maximal effective concentration

Eds.	Editors
ES	Electrospray ionisation
EtOAc	Ethyl acetate
EtOH	Ethanol
F	Pharmacokinetic parameter for bioavailability
FA	Formic acid
FCS	Foetal calf serum
FDA	Food and Drug Administration
g	Gram
g/L	Grams per litre
G6P	Glucose-6-phosphate
GLUTs	Glucose transporters
GMP	Good manufacturing practices
GSK3	Glycogen synthase kinase 3
H_2SO_4	Sulfuric acid
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HPLC/UV	High performance liquid chromatography with UV detector
hr	Hour
HRP	Horseradish peroxidise
HSQC	Heteronuclear single quantum coherence spectroscopy
Hz	Hertz
IDF	International Diabetes Federation
IR	Insulin receptor
IRS	Insulin receptor substrate

IUPAC	International Union of Pure and Applied Chemists
J	Spin-spin coupling constant
kg	Kilogram
kV	Kilovolt
L	Litre
L/hr	Litre per hour
LADME	Liberation, absorption, distribution, metabolism, and excretion
Lam.	Lamiaceae
LC-MS/MS	Liquid chromatography - mass spectrometry
LLOQ	Lower limit of quantitation
m	Multiplet (NMR)
MeOH	Methanol
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
mmol	Millimoles
MRC	Medical Research Council
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW	Molecular weight
N_2	Nitrogen
NCE	New chemical entity
ng/L	Nanograms per litre
NJ	New Jersey, USA
nM	Nano molar
nm	Nanometre
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear overhauser effect spectroscopy
NP40	nonyl phenoxypolyethoxylethanol
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Photo diode array
PDK1	Phosphosinsositide-dependent kinase 1
PFK	Phosphofructokinase
P-gp	P-glycoprotein
PI-4,5-P2	Phosphatidylinositol-(4,5)-biphosphate
PIP3	Phosphatidylinositol -3,4,5-triphosphate
PK	Pharmacokinetic
pKa	Ionizability
PKC–ζ	Protein kinase C–ζ
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
ppm	Parts per million
PVDF	Polyvinylidene difluoride
q	Quartet (NMR)

qRT-PCR	Quantitative Real Time – Polymerase Chain Reaction
RC DC	Reducing agent compatible and detergent compatible
RCF	Relative centrifugal force
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Reverse transcription
s	Singlet
SAR	Structure-activity relationship
SANBI	South African National Biodiversity Institute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
STZ	Streptozotocin
subsp.	Subspecies
t	Triplet
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TB	Tuberculosis
TBST	Tris-buffered saline and tween
TC	Tissue culture
TLC	Thin layer chromatography
T_{max}	Rate of absorption as measured over time
TOF	Time of flight
UCT	University of Cape Town
UK	United Kingdom

UPLC-DAD-MS	Ultra performance liquid chromatography-diode array detection with mass spectrometry
USA	United States of America
UV	Ultraviolet
V	Volt
Vd	Volume of distribution
WHO	World Health Organization

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1.1 Background

1.1.1 Burden of disease: Diabetes

The burden of diabetes is reflected not only in the increasing number of people with diabetes, but also in the growing number of premature deaths due to diabetes. In 2013, roughly half of all deaths due to diabetes in adults were in people under the age of 60, and in less-developed regions like sub-Saharan Africa, that proportion climbs to 75% (International Diabetes Federation, 2013). As the infectious disease burden (HIV and TB) decreases and life expectancy increases along with development that drives rapid changes in lifestyles, it is the developing regions that will succumb the most as the burden of diabetes increases.

Figure 1.1 Distribution of world diabetic population (20-79 years), 2013. (International Diabetes Federation, 2013)

Table 1.1 Top 10 countries with high diabetic population (20-79 years), 2013 (International Diabetes Federation, 2013)

Country	Number
China	98.4 million
India	65.1 million
United States of America	24.4 million
Brazil	11.9 million
Russian Federation	10.9 million
Mexico	8.7 million
Indonesia	8.5 million
Germany	7.6 million
Egypt	7.5 million
Japan	7.2 million

The above table shows the top 10 countries in the world with the highest diabetic population in 2013. However, according to the IDF statistics, they have predicted that by the year 2035, the highest percentage of people living with type 2 diabetes would be from the Middle East and North Africa (10.8%), followed closely by North America and the Caribbean (9.5%) and the with the lowest being Africa (6%).

Table 1.2 Prevalence (%) of diabetes (20 – 79 yrs) by IDF region, 2035

IDF Region	Percentage population by 2035
Middle East and North Africa (MENA)	10.8%
North America and Caribbean (NAC)	9.5%
South-East Asia (SEA)	9%
Western Pacific (WP)	8.1%
South and CentralAmerica (SACA)	8%
Europe (EUR)	6.9%
Africa (AFR)	6%

Diabetes currently affects 246 million people worldwide and is expected to affect 380 million by 2025 (Halban *et al*., 2010). Treatment of diabetes and its associated complications places an enormous burden on health costs especially in the developing world. Hence, there is a dire need for novel, non-cytotoxic, inexpensive and effective drugs to combat the predicted explosion in the prevalence of diabetes, especially Type 2 Diabetes (T2D), also known as a 'lifestyle disease' (http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Diabetes_Type_2).

It has been estimated by the International Diabetes Federation (IDF) that at least one in ten adults could have diabetes by 2030 (International Diabetes Federation, 2013), thus predicting the number of people living with diabetes is expected to increase from 366 million to 552 million by 2030 if no action is taken (http://www.globalbuzz-sa.com/forums/showthread.php?t=18423).

"There are 3.5 million South Africans (about 6% of the population) who suffer from diabetes, and many more who are undiagnosed. It is estimated that another 5 million South Africans have pre-diabetes, a condition where insulin resistance causes blood glucose levels to be higher than normal, but not high enough yet to be clinically classified as T2D. It has been found that the highest prevalence of diabetes is among the Indian population in South Africa (11 - 13%) as this group has a strong genetic predisposition for diabetes. This is followed by 8 - 10% in the coloured community, 5 - 8% among blacks and 4% among whites" (http://www.health24.com/Medical/Diabetes/About-diabetes/Diabetes-tsunami-hits-South-Africa-20130210).

Asian countries contribute to more than 60% of the world's diabetic population as the prevalence of diabetes is increasing in these countries (Ramachandran *et al.,* 2012). In recent decades, Asia has undergone rapid economic development, urbanization, and transitions in nutritional status that has led to an explosive increase in diabetes prevalence within a relatively short time (Hu, 2011). Asians have a strong ethnic and genetic predisposition for diabetes and have lower thresholds for the environmental risk factors (Ramachandran *et al.,* 2012). As a result, they develop diabetes at a younger age and at a lower body mass index and waist circumference when compared with the Western population. Other racial and ethnic groups that are more likely to develop type 2 diabetes, heart disease, and stroke are African Americans, Mexican Americans, American Indians, Native Hawaiians, Pacific Islanders and Asian Americans who have a higher risk for these deadly diseases (http://www.diabetes.org). In 2008, the prevalence of type 2 diabetes had reached nearly

10% of Chinese people suggesting that China had overtaken India as the global epicenter of the diabetes epidemic (Hu, 2011). However, in urban areas of south India, the prevalence of diabetes has reached nearly 20% (Hu, 2011).

1.1.2 Normal glucose metabolism

1.1.2.1 Regulation of glycaemia

Carbohydrates are the active fuel for the body and the main source of energy for cells. During the normal digestive process, starches (carbohydrates) are broken down into simple sugars (monosaccharides) mainly glucose before being absorbed into the bodies circulation. Glucose is stored as glycogen in the liver and muscles for later use as body fuel, at which time it is reconverted into glucose. Blood glucose rises after eating and in healthy individuals, returns to normal levels in about an hour or two. The amount of glucose in the blood is controlled mainly by the hormones insulin and glucagon (Maiti *et al*., 2004). Dysfunctional regulation i.e. too little or ineffectiveness of these hormones can cause blood sugar levels to fall too low (hypoglycaemia) or rise too high (hyperglycemia). Other hormones that influence blood sugar levels are somatostatin, cortisol, growth hormone and catecholamines (epinephrine and norepinephrine) (Bell *et al*., 1990).

The pancreas, a gland in the upper abdomen is responsible for producing insulin and glucagon. The pancreas is dotted with hormone-producing tissue called the islets of Langerhans, which contain amongst others, alpha and beta cells. Glucose is taken up by glucose transporter(s) (GLUT-2 exclusively in rodents but in humans GLUT-1, GLUT-2 and GLUT-3 are involved), into pancreatic beta cells where it is metabolized leading to increased levels of ATP (Thorens, 2015). This, in turn, increases the ATP/ADP ratio, which results in closing of potassium channels in the cell membrane and subsequent depolarization of the cell. As potassium channels close and cell plasma membrane depolarization increases, calcium channels in the cell membrane open to allow the flow of calcium into the cell. This accumulation of calcium causes the secretion of insulin into the blood by beta cells. Glucose is a hydrophilic compound which requires active transportation across the cell membrane by glucose transporters (GLUTs) (Mueckler and Hruz, 2001). Insulin facilitates the transport of glucose by these glucose transporters into insulin sensitive tissues such as muscle fat and the liver thereby maintaining blood glucose levels within the normal range (Gorovits and Charron, 2003). When blood sugar drops too low however, the alpha cells secrete glucagon.

Glucagon stimulates hepatic glycogenolysis and gluconeogenesis to release glucose into the circulation and raise blood sugar levels especially during periods of fasting (Meetoo *et al.*, 2007).

1.1.2.2 Insulin Stimulated Glucose Uptake

Insulin is an anabolic hormone that controls glucose homeostasis. To maintain stable plasma glucose (normoglycaemia), the β-cells of the pancreas secrete insulin in response to raised plasma glucose concentrations. The secretion of insulin stimulates glucose uptake in insulin sensitive tissues such as adipose and muscle tissue, while decreasing hepatic glucose output from gluconeogenesis and glycogenolysis (by inactivation of glycogen synthase kinase 3 (GSK3)) (Saltiel and Kahn, 2001), and by promoting glycogenesis (glucose storage as glycogen) (Czech and Corvera, 1999; Pessin and Saltiel, 2000). Insulin initiates peripheral glucose uptake in skeletal muscle and adipose tissue by binding to the insulin receptor (IR), a glycoprotein transmembrane, tetrameric enzyme complex consisting of two α- and two β-subunits. When insulin binds to the IR (Figure 1.2) on the extracellular α-subunit, the suppression of tyrosine kinase activity in the β-unit is released and phosphorylation of the cytoplasmic β-subunit is initiated. Once phosphorylated, the cytoplasmic and membrane portion of the subunit phosphorylates insulin receptor substrate (IRS) on its tyrosine residues thereby initiating the activation of a series of membrane-bound and cytoplasmic effector and adaptor molecules. These include a pivotal step involving the activation and membrane docking of phosphatidylinositol-3 kinase (PI3K) following the binding of phosphorylated IRS. Activation of PI3K initiates the phosphorylation of phosphatidylinositol-(4,5)-biphosphate (PI-4,5-P2) to form phosphatidylinositol -3,4,5-triphosphate (PIP3) (Gruzman *et al.*, 2009). Active PIP3 interacts with phosphosinsositide-dependent kinase 1 (PDK1) which recruits and phosphorylates protein kinase B (Akt) and protein kinase C-ζ (PKC-ζ). Activation of Akt and PKC-ζ induces cytoplasmic GLUT-4 translocation via exocytosis to the cell surface that facilitates glucose transport into the cell (Giri *et al.,* 2004).

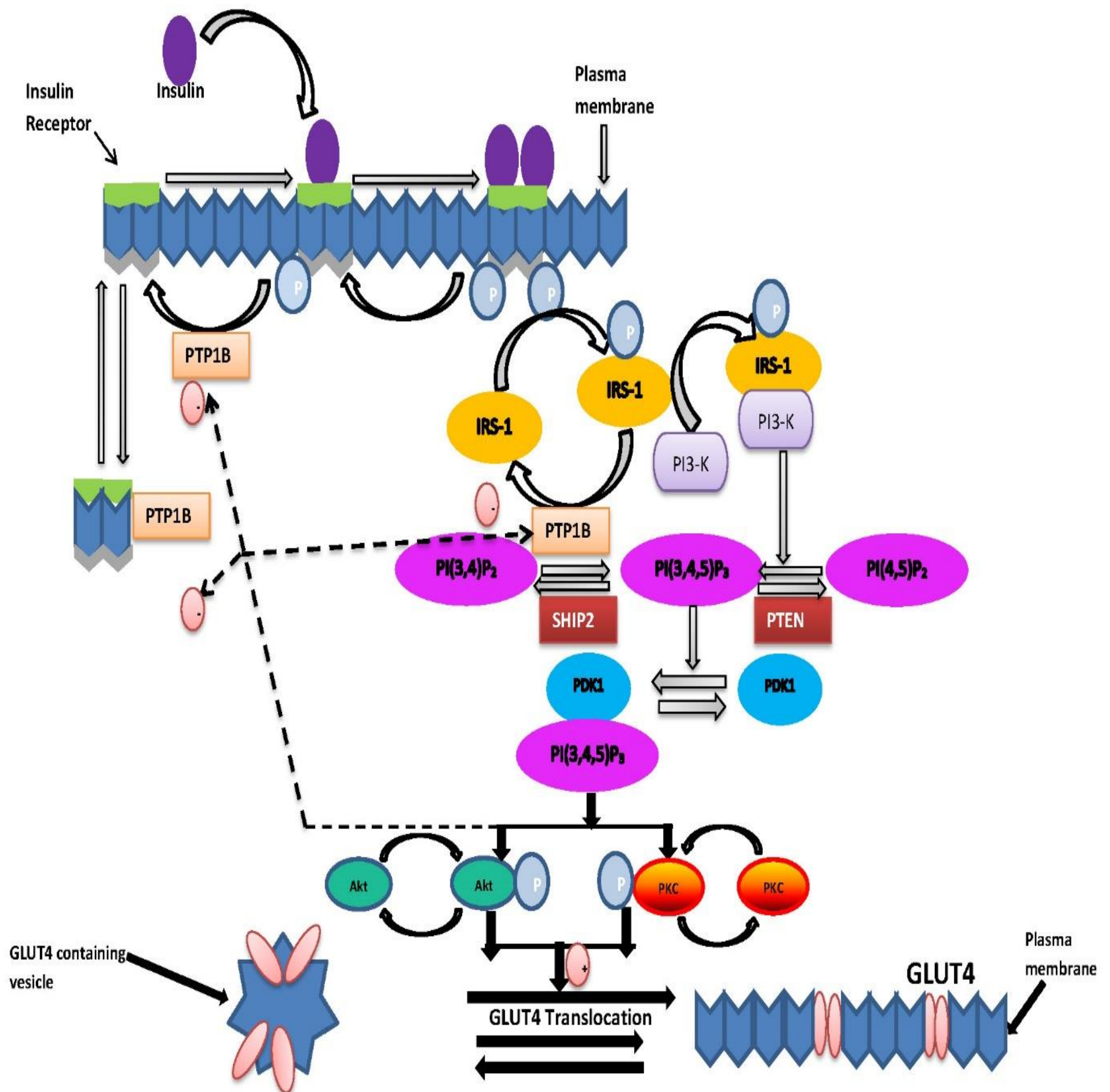

Figure 1.2 Illustrated view of insulin stimulated glucose transport.

1.1.2.3 Key Proteins and Receptors Involved in Glucose Uptake

The insulin receptor substrates-1 and -2 (IRS-1, IRS-2), and SHC (Src homology 2 domain containing) transforming protein 1 (SHC) are import initiators, potentiators and regulators of the insulin pathway. In particular IRS-1 is involved in accentuating the actions of insulin postprandially, while IRS-2 is thought to be more important during fasting (Long *et al*., 2011). Serine phosphorylation, in contrast to tyrosine phosphorylation of IRS proteins has

been shown to attenuate PI3K activation. Excessive serine phosphorylation of IRS-1 is linked to insulin resistance (Draznin, 2006).

Alternative non-insulin-dependent pathways linked to glucose uptake and metabolism include adenosine monophosphate-activated protein kinase (AMPK). This important regulatory protein is responsible for cellular energy balance and is considered a "master switch of glucose and lipid metabolism in various organs, especially in skeletal muscle and liver" (Gruzman *et al*., 2009). AMPK stimulates glucose transport in skeletal muscles and boosts fatty acid oxidation by decreasing glucose production, cholesterol and triglyceride synthesis in the liver (http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/pancreas/insulin_phys. html).

1.1.2.4 Glucose Transporters (GLUTs)

GLUTs are integral membrane proteins that transport glucose and related hexoses, either into or out of the cell. Binding of glucose to one site provokes a conformational change associated with transport, and releases glucose to the other side of the membrane. Each glucose transporter isoform plays a specific role in glucose metabolism determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression in different physiological conditions (Thorens *et al*., 1996).

Table 1.3 Glucose transporters GLUT-1 –GLUT-4 (Bell *et al.,* 1990)

Name	**Distribution**	**Notes**
GLUT-1	Is widely distributed in foetal tissues. In the adult, it is expressed at highest levels in erythrocytes and also in the endothelial cells of barrier tissues such as the blood-brain barrier. However, it is responsible for the low-level of basal glucose uptake required to sustain respiration in all cells.	Levels in cell membranes are increased by reduced glucose levels and decreased by increased glucose levels.
GLUT-2	Is a bidirectional transporter, allowing glucose to flow in 2 directions. Is expressed by renal tubular cells, small intestinal epithelial cells, liver cells and pancreatic β cells. Bidirectionality is required in liver cells to uptake glucose for glycolysis, and release of glucose during gluconeogenesis. In pancreatic β-cells, free flowing glucose is required so that the intracellular environment of these cells can accurately	Is a high-capacity and low-affinity isoform

	gauge the serum glucose levels. All three monosaccharides (glucose, galactose and fructose) are transported from the intestinal mucosal cell into the portal circulation by GLUT-2.	
GLUT-3	Expressed mostly in neurons (where it is believed to be the main glucose transporter isoform), and in the placenta.	Is a high-affinity isoform, allowing it to transport even in times of low glucose concentrations.
GLUT-4	Found in adipose tissues and striated muscle (skeletal muscle and cardiac muscle).	Is the insulin-regulated glucose transporter. Responsible for insulin-regulated glucose storage.

1.1.3 Diabetes

In diabetics, persistent hyperglycemia results from inefficient regulation of blood glucose by insulin, which is either absolute, as in insulin-dependent diabetes or type 1 diabetes (T1D), or relative, as in non-insulin-dependent diabetes or T2D (Meetoo *et al.*, 2007). Diabetes is the result of a disturbed glucose metabolism characterized by abnormal concentrations of glucose in the blood stream (hyperglycemia). To understand diabetes, it is imperative that one understands how blood glucose is regulated by the insulin pathway with the key proteins, receptors and glucose transporters involved in glucose uptake.

1.1.3.1 Type 2 Diabetes

In T2D relative insulin deficiency usually occurs because of resistance to the actions of insulin in muscle, fat, liver and an inadequate response by the pancreatic beta cells (Prashanth *et al.*, 2009). This pathophysiological abnormality results in decreased insulin stimulated glucose transport into responsive tissues, elevated hepatic glucose production, and increased breakdown of fat (Mueckler and Hruz, 2001). Initially, T2D is associated with increasing insulin resistance, and in response beta cells temporarily produce increased amounts of insulin resulting in hyperinsulinaemia (Alper *et al.*, 2006; Zimmet *et al.*, 2001). However as the disease progresses, beta cells fail and are increasingly lost by apoptosis (Cerasi *et al.*, 2000). If left untreated, diabetes can lead to various costly and dangerous neural and vascular

complications which can lead to damage of the eyes, kidneys, feet and heart, and in serious cases, result in early death (Tiwari *et al*., 2013).

T2D is one of the world's most common diseases, affecting more than 150 million people across the globe, and this number is predicted to double within the next 15 years (Rheeder, 2006). In Africa, the highest numbers of people with diabetes are from Nigeria, followed by South Africa (http://www.idf.org).

The epidemic increase in the prevalence of T2D is attributed to a synergism between genetic predisposition, environmental determinants, age and insulin resistance which is common in affluent western societies (Amos *et al*., 1997). Obesity is associated with a deterioration of glucose utilization and promotes the development of T2D, particularly in populations with a genetic predisposition (Bonetta, 2001).

No cure is available for T2D, and management of the disease typically involves maintaining a healthy weight and being physically active, home blood glucose testing, and treatment with oral pharmaceuticals or in advanced cases, medication with recombinant insulin. In response to the enormity of the growing problem, new efforts to identify and develop new pharmacological agents for T2D have increased dramatically (Nicholson and Hall, 2011). The application of genomics-based target discovery to T2D has long promised to provide new targets for medical intervention but, this has yet not materialized (Chen *et al*., 2008).

1.1.4 Medicinal Plants

1.1.4.1 Overview of traditional medicine as therapeutics

Plants have always been an exemplary source of drugs yielding directly or indirectly many important medicines in the past (Yin and Chen, 2000). During the last 20 years, there has been a resurgence of interest in herbals as remedies for self-medication in the Western world, which has been termed the 'green boom' (Mander *et al*., 1997). Although southern Africa contains approximately 10% of the world's plant diversity, little work has been done on medicinal plants from this region mainly due to erosion of indigenous knowledge passed down within the community and badly recorded literature. Therefore knowledge of indigenous plant use in the region needs urgent significant documentation before it is irretrievably lost to future generations (Van Wyk and Gericke, 2000).

Modern extraction and separation techniques have proved valuable in isolating pure compounds that can be applied against a myriad of biological activities. The reliance of the use of indigenous medicinal plants has a long history (Cunningham, 1988). Medicinal plants form a sizeable component of traditional medicine and are a mainstay for 80% of the people in developing nations. Estimates in 1996 showed that plant materials are present in, or have provided the models for 50% of Western pharmaceutical drugs (Robbers *et al*., 1996). In 1985, Farnsworth and Soejarto showed that 25% of the prescription drugs in the United States and Canada were modelled on plant-based products and 119 secondary plant metabolites were used globally as drugs, with hardly a dozen synthesized commercially or produced by simple chemical modification of active molecules. At a national level, it is estimated that 20,000 tonnes from over 700 medicinal plant species are traded in South Africa every year, with a market value of some $60 million (approximately R450 million) (Mander *et al*., 1997). Of approximately 3,000 species of higher plants used as medicine in South Africa, chemical investigations have mostly been on the 350 species most commonly used and traded as medicines (Van Wyk *et al*., 1997). Most of the medicines used in traditional medicine are prepared from medicinal plants, also called “muthi-plants”. As many of these plants are harvested in the wild, this has led to overexploitation that presents a severe threat to many medicinal plants.

In the last few decades there have been great changes to health care in the developing world, whereby traditional medicine can be viewed as a parallel system to conventional Western healthcare, as much as complementary medicine is in Europe and America. There is also a growing trend from conventional pharmaceutical drugs with associated side effects towards plant-derived drugs and natural products which are deemed to be as effective, but a healthier alternative by the population (Ventola, 2010). However, due to their proven efficacy for acute ailments, conventional pharmaceutical drugs are still regarded as the therapeutics of choice. There is a common misconception in the population that think because it is from a plant source, it is ‘natural’ hence ‘healthy’. Paracelsus (1538) has quoted “All substances are poisonous, there is none that is not a poison; the right dose differentiates a poison from a remedy” (Callahan, 2011). There are many chemical compounds found naturally in plants that are poisonous to humans in small doses; similarly, there are many man-made compounds which are perfectly harmless unless ingested at very high doses. All chemicals, if taken at a high enough doses are toxic, including water, which is considered essential to life. “The dose

makes the poison" is a rule that applies to all compounds, natural or man-made (Callahan, 2011).

Plant preparations are often considered useful for the treatment of less serious diseases, as supportive treatment of chronic diseases over long periods of time and possibly for prophylactic medication, with "no" harmful side-effects because they are more in tune with biological systems (Atal, 1983; Erasto, 2003). Thus, medicinal plants continue to receive attention from scientists for their chemical, pharmacological and clinical importance worldwide (Govil *et al*., 1993, Part 1). The plant kingdom has supplied some excellent drugs like morphine, codeine, thebaine, digitoxin, quinine and ergotamine (just to name a few). The ancient Ayurvedic, Siddha, Greek, Chinese and Arabian medical systems depend primarily on plant based material medica. The unaffordability of conventional Western medicine to most of Africa's population has necessitated a search for alternative ways of managing illnesses. Traditional medicine, which has always been practised in the indigenous cultures, is fast filling up this therapeutic gap (Sindiga *et al.,* 1995). Indigenous systems employ complex formulations with more than one plant ingredient and also prescribe a specific mode of application. These systems have stood the test of time for centuries and the natural plant medicines have served as useful prototypes for even better medicines (Govil *et al*., 1993, Part 2). This has led to renewed interest in biodiversity prospecting by both local and multinational companies. Phytochemical research has come a long way with several groups from both academia and industry being actively involved in screening programmes for wide-ranging plant activities.

1.1.4.2 Traditional medicines for Diabetes

Traditional medicines have great potential for scientists to find active compounds and develop new antidiabetic drugs. Despite their efficacy and better ADME (absorption, distribution, metabolism, and excretion)/Tox (toxicity) properties, only about 40% of the chemical scaffolds found in natural products are present in today's medicinal chemistry (Potterat and Hamburger, 2006).

Figure 1.3 Flowers of *Galega officinalis*. (http://www.womenfitness.net/blood_sugar.htm**)**

Galega officinalis, also known as goat's rue, French lilac, French honeysuckle, Italian fitch or professor-weed (Figure 1.3), was used in medieval times to treat high blood sugar levels in diabetics (Witters, 2001). The first half of the 20th century is considered an important milestone in the development of oral antidiabetic pharmacotherapy with the discovery of galegine (Figure 1.4), the glucose-lowering guanidine derivative (isoamylene guanidine) isolated from *G. officinalis* that formed the chemical basis of metformin (Bailey *et al.,* 2007). Metformin still remains the first line oral antidiabetic therapy for T2D today (Singh, 2014).

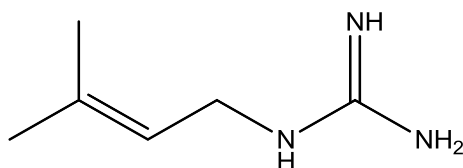

Figure 1.4 Structure of galegine ((3-Methylbut-2-enyl)guanidine;2-(3-methylbut-2-enyl)guanidine).

Many plants such as *Momordica charantia* L. (Cucurbitaceae), *Pterocarpus marsupium Roxb.* (Leguminoceae), *Asparagus racemosus, Butea monosperma, Catharanthus roseus,*

Coccinia indica, Gymnema sylvestre, Syzygium cumini, Aspalathus linearis and *Trigonella foenum graecum* L. (Leguminosae) have been shown to possess hypoglycemic properties to treat diabetic complications while Astragalus polysaccharides, found in the traditional Chinese anti-diabetic medicine Radix Astragali seu Hedysari possess activity to help regulate blood glucose two-dimensionally i.e. increase blood glucose during hypoglycemia and lower blood glucose during hyperglycemia (Li *et al*., 2004; Jung *et al*., 2006; Ayyanar *et al*., 2008). The active components commonly responsible for the hypoglycemic activities in natural products derived from plants include flavanoids, xanthones, triterpenoids, glycosides, alkylsulfides, aminobutyric acid derivatives, guanidine, polysaccharides and peptides (Wang and Ng, 1999). The mechanisms of action of these hypoglycaemic compounds are multimodal as they either enhance the release of insulin or enhance the peripheral utilization of glucose.

Many parts of the world's population are dependent on plants as their foundations for medicine (Dall'Agnol and von Poser, 2000). Natural products such as nopal (prickly pear cactus), fenu-greek, karela (bitter melon), gymnema, ginseng, tronadora, chromium, and alpha-lipoic acid have long been used in traditional medicine among people of different ethnicities for diabetes. People of Mexican descent commonly use nopal and *Tecoma stans* (commonly known as "tronadora") (Constantino *et al*., 2003) while those from Asia use karela for its hypoglycemic properties (Shapiro and Gong, 2002). Indians from Tamil Nadu South commonly use wild liquorice (*Abrus precatorius* L. (Fabaceae)) and the King of Bitters, *Andrographis paniculata* (Burm.f.) Wall. ex Nees (Acanthaceae) to treat their diabetic conditions (Rana *et al*., 1999). In Puerto Rico, the use of medicinal plants with antidiabetic activity has being reported in the ethnobotanical literature. Some of the plants reported include *Costus sp., Tapenoichilus ananassae, Lantana sp.* and *Rhoeo spathaceae* (Gurav *et al*., 2007).

Currently, there are no natural product medicines on the market that have been registered for the treatment of diabetes. However, there are a few in the market that are not FDA approved, e.g. Diabetes Daily Care (http://www.diabetes-daily-care.com) and Gluco Health (http://www.vedaliving.com). Thus, there is an increasing need to screen and obtain plant extracts and active compounds from traditional medicinal plants for the treatment of diabetes and related metabolic diseases. Of an estimated 250,000 higher plants, less than 1% has been

screened pharmacologically with approximately 1,200 reported to have anti-diabetic activity (Graf *et al*., 2010).

In the developing world the cost of acquiring modern antidiabetic drugs is beyond the reach of many people in the low income group and those living in rural areas. Therefore, because of the high morbidity, mortality and economic impact of diabetes in these regions, it is prudent to look for alternative but equally effective treatment options from herbal medicines (Barbosa-Filho *et al*., 2005). In the Eastern Cape, majority of the people are rural dwellers with no access to hospitals or medical care for diabetes. An ethnobotanical study of plants used by the traditional healers, herbalists and rural dwellers for the treatment of diabetes mellitus, conducted in the Eastern Cape revealed 14 plant species which are frequently used for the treatment of diabetes (Erasto *et al*., 2005). These people therefore still rely heavily upon traditional medicine for the treatment of diabetes (Erasto *et al*., 2005).

1.1.5 Pharmacokinetics

"Pharmacokinetics may be simply defined as what the body does to the drug, as opposed to pharmacodynamics which may be defined as what the drug does to the body" (Benet, 1984). Pharmacokinetics describes the time course of the drug concentration from a particular dosage treatment and also tells us how much of a drug and how often it must be given to attain the desired drug concentration. It also includes the study of the mechanisms of absorption, the chemical changes of the substance in the body and the effects and routes of excretion of the metabolites of the drug (Pharmacokinetics, 2006).

Pharmacokinetics can be divided into four basic categories known as the ADME or LADME scheme. ADME or LADME in pharmacokinetics commonly refers to liberation, absorption, distribution, metabolism and excretion of a drug in the body. ADME studies describe how a drug behaves in the human body and how it can be a major cause of drug failure or success if one can predict at an early stage which compounds not only possess good binding affinity for a specific target, but also pass the test for good bioavailability and toxicity. Liberation refers to the release of the drug from the formulation or it's dosage form and has been emphasized as playing an important role in pharmacokinetics (Koch and Ritschel, 1986; Ruiz-Garcia *et al.,* 2008). Once the drug is ingested, the body absorbs part of it and the substance enters the blood circulation. Bioavailability is often quantified by the pharmacokinetic parameter (F). The oral bioavailability is the percentage of the dose that actually reaches systemic

circulation after oral administration. Distribution is the dispersion of substances throughout the fluids and tissues of the body, which is usually expressed as the apparent volume of distribution (Vd).

A prodrug is a compound that has negligible or lower activity against a specified pharmacological target than one of its major metabolites. Prodrugs can be used to improve drug delivery or pharmacokinetics, to decrease toxicity, or to target the drug to specific cells or tissues (Montellano, 2013). Metabolism is the biotransformation of drugs to forms that can be more readily excreted from the body. Excretion refers to the elimination of the substances from the body and can be described by the pharmacokinetic parameter, clearance. The kidneys play a major role in the elimination of aqueous soluble drugs from the body while lipid soluble compounds are usually excreted via the bile system. As renal function degenerates, the dosage of medication needs to be adjusted to prevent build-up and the potential for toxic effects (Garbardi and Abramson, 2005).

Bioavailability refers to the rate and extent to which the active ingredient or active moiety is absorbed from the pharmaceutical and becomes available at the site of action. Factors that can alter the bioavailability include the dosage form (e.g., capsule, tablet), the chemical form of the drug (i.e., salt or ester form of the drug), the extent of metabolism before reaching systemic circulation (e.g., first pass effect where drugs are metabolized in the gastrointestinal tract by microorganisms followed by absorption), and then absorption and dissolution characteristics of the drug itself. Dissolution is determined by the other effects such as aqueous solubility, ionizability (pKa), and lipophilicity. Log P is a crucial factor controlling passive membrane partitioning, as increasing log P enhances permeability while reducing solubility (Knights and Bronwen, 2002).

There is a medical need for the improvement of bioavailability of a large number of drugs. One of the most promising approaches is the co-administration of therapeutic agents with natural compounds promoting absorption and improving activity for oral drug delivery. Many natural compounds from medicinal plants have exhibited the ability to enhance the bioavailability of co-administered drugs by inhibiting efflux pumps or oxidative metabolism and perturbing the intestinal brush border membrane. These natural compounds include quercetin, genistein, naringin, sinomenine, piperine, glycyrrhizin and nitrile glycoside (Kang *et al.,* 2009). Quercetin and genistein, in particular show considerable P-gp inhibition activities (Kang *et al.,* 2009).

Although many molecules have exhibited potential therapeutic effects, they have low membrane permeability, which may be due to their physical characteristics like low lipophilicity, zwitterionic character at physiological pH and low water solubility (Raeissi *et al*., 1999) or the absorbed compounds may be effluxed by efflux proteins like P-glycoprotein (P-gp) of the enterocytes back into the intestinal lumen (Adachi *et al.,* 2003). Hence, improving oral drug absorption and bioavailability of drugs has become an important pharmaceutical industry issue. Several approaches to enhance intestinal absorption include the use of absorption enhancers, prodrugs and permeability-enhancing dosage forms such as liposomes and emulsions with minimal toxic effects. The co-administration of natural compounds is expected to be one of the promising approaches to enhance the absorption and bioavailability of drugs (Kang *et al*., 2009).

1.2 Study Outline

1.2.1 Objectives

- To prepare various forms of extracts from *S. pinnata* using different techniques and solvents.
- Isolate and identify the active ingredient using column chromatography and Nuclear Magnetic Resonance (NMR).
- Evaluate the biological efficacy for diabetes in a bioassay guided approach using *in vitro* and *in vivo* methods.
- To identify the possible mode of action of the active and extract/s using qRT-PCR (Quantitative Real Time – Polymerase Chain Reaction) and Western blot analysis.
- Analysis of a pharmacokinetic study to establish the bioavailability of the active ingredient versus the extract.

1.2.2 Hypothesis

S. pinnata has antidiabetic activity.

1.3 Structure of thesis

This study comprised of an investigation to identify and isolate the active compound responsible for lowering blood glucose levels. Screening of the plants' extracts and its isolated active fractions/compound for antidiabetic activity in *in vitro* and *in vivo* assays will validate its use for diabetes. The mode of action studies will help identify the key proteins and receptors responsible for stimulating the insulin glucose uptake pathway by examining the activity level of the genes. The pharmacokinetic (PK) study concludes the investigation by examining the fate of the active ingredient from the time that it is administered, to the point that it is utilized by the body. This study supports the potential use of *S. pinnata* as a traditional treatment for diabetes and also an unexplored source of new chemical entities (NCE) for the pharmaceutical industry.

Chapter 1: The introductory chapter consists of the general background on the incidence of diabetes. Normal glucose metabolism and insulin stimulated glucose uptake is explained as

well as the key proteins, receptors and glucose transporters involved in glucose uptake. Pharmacokinetics is defined, as well as an overview of all that it entails.

Chapter 2: This chapter involves a detailed discussion of the different chemistry techniques involved in isolating and identifying the active compound. It also covers various purification methods, a myriad of solvents to make several extracts and NMR experiments to elucidate the structure isolated.

Chapter 3: All the biological screening for diabetes by the various extracts and fractions are covered in this chapter. This includes *in vitro* screening showing glucose uptake in C2C12 muscle cells and Chang cells, as well as radio-labelled assays. *In vivo* studies involved the use of the STZ (Streptozotocin) rat model.

Chapter 4: This chapter on gene and protein expression in C2C12 muscle cells, analyzes the level of specific mRNAs involved in cell signaling after exposure to plant extracts and compounds. This may identify the possible mode of action of the active and extract/s using qRT-PCR– Poly and Western blot analysis.

Chapter 5: In this chapter, the PK study estimates the bioavailability of the extracts and active fraction over a 6 hr period. A standard curve involving a biomarker of the compound was used to attain a profile of the plasma samples containing the test extracts and active fraction.

Chapter 6: This is a summary of the whole study and gives a general discussion of the findings of each chapter, the limitations of the study and a conclusion.

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CHAPTER 2 - Chemical analysis of extracts of *Schkuhria pinnata* and isolation of biologically active compound

2.1 Introduction

2.2 Materials and Methods

2.2.1 Plant collection

2.2.2 Extract preparation

2.2.3 Extraction of plant material using aqueous and organic solvents

2.2.4 High performance liquid chromatography (HPLC/UV)

2.2.5 Concentration of the actives using liquid/liquid partitioning

2.2.6 Fractionation of ethyl acetate extract using column chromatography

2.2.7 Nuclear magnetic resonance (NMR) spectroscopy

2.2.8 UPLC-DAD-MS analysis

2.2.9 Targeted purification of compound 1 for in vivo studies

2.3 Results and Discussion

2.4 Conclusion

2.5 References

2.1 Introduction

Figure 2.1 Illustration of *S. pinnata.*

S. pinnata (Lam.) Kuntze ex Thell. belongs to the family Asteraceae. A few common names for this plant include canchalagua, canchalahua, dwarf marigold, dwarf Mexican marigold, kanchalawa, khakibush, pinnate false threadleaf and yellow tumbleweed (Taylor, 2006).

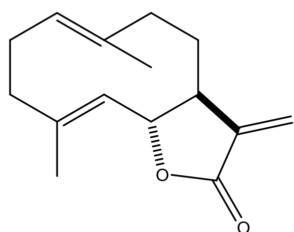
This plant is synonymous with a few other varieties such as *Amblyopappus mendocinus* Phil., *Hopkirkia anthemoides* DC., *Mieria virgata* La Llave, *Pectis pinnata* Lam., *Rothia pinnata* (Lam.) Kuntze., *Schkuhria wislizenia, Schkuhria wrightii, Schkuhria virgata* (La Llave) DC., *Schkuhria isopappa* Benth., *Schkuhria guatemalensis* (Rydb.) Standl. & Steyerm., *Schkuhria coquimbana* Phil., *Schkuhria anthemoides* (DC.) J.M. Coult., *Schkuhria advena* Thell., *Schkuhria abrotanoides* Roth. and *Tetracarpum guatemalense* Rydb.

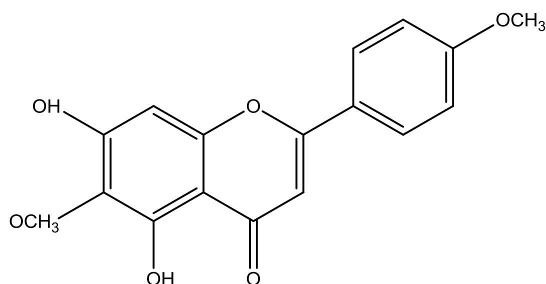
This weedy herb is indigenous to South America, but has been introduced to and cultivated in other parts of the world like Latin America, Mexico, Africa, and even in parts of southern Arizona and Texas. It is usually found in drier mountainous regions (Taylor, 2006). *S. pinnata* has escaped cultivation and flourishes as an annual weed growing up to 70 cm high. It has numerous small yellow flowers (Figure 2.1) on numerous airy erect stems which grow woodier as it matures.

The traditional preparation of *S. pinnata* involves drying, grinding and steeping of the aerial parts in boiling water (Watt and Breyer-Brandwijk, 1962). There are numerous ethnomedical uses of *S. pinnata* worldwide. In Africa and Mexico it is used for gastrointestinal problems, malaria, and stomach aches. The powdered leaf of *S. pinnata* is swallowed with water as a

remedy for malaria, colds, flu (Watt and Breyer-Brandwijk, 1962). Van Wyk and Gericke (2000) report its therapeutic use as an abortifacient and contraceptive. It is used by the Argentinians as an antibiotic and vulnerary; for diarrhoea, respiratory tract infections, urinary tract infections, and wounds (Taylor, 2006). The Peruvians use it as an anti-inflammatory, anti-tussive, capillary tonic, depurative, digestive, diuretic, hypoglycaemic, styptic and vulnerary; for acne, blackheads, dermatitis, detoxification, diabetes, digestive disorders, eczema, fleas, intestinal gas, kidney problems, kidney inflammation, lice, liver problems, liver stones, malaria, obesity, oily skin, pimples, prostate inflammation, renal problems, rheumatism, stomach problems, stomach aches, urinary tract problems, weight loss, wounds, and yeast infections (Taylor, 2006). In a study by Bussmann *et al* (2008), it was found that *S. pinnata* had promising antibacterial properties and effective against acne. Evaluation of the antimicrobial activity of *S. pinnata* revealed that it is active against several fungi, yeasts and certain mould strains. However, an extract made using the entire plant, rather than just the leaves, yielded much better activity (Quiroga *et al.,* 2001). Extracts made with only the leaves were found to only have antibacterial activity against *Bacillus.* Luseba *et al* (2007) showed that the dichloromethane extract of *S. pinnata* exhibited the highest antibacterial activity when screened against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* and showed selective inhibition of COX-1. It is also used as an insecticide in Paraguay and in Bolivia against fleas and malaria. Researchers in Bolivia reported that the ethanol extract made from the entire plant showed good antimalarial activity in rats (Munoz *et al.,* 2000). No contraindications or drug interactions with this plant have been reported (Taylor, 2006).

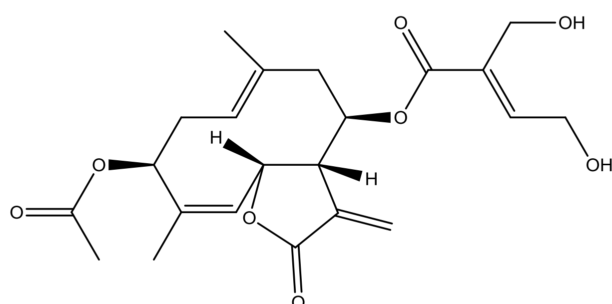
Germacranolides, heliangolides, sesquiterpene lactones, flavonoids, and sulphur compounds are some of the chemical compounds isolated from *S. pinnata*. Ganzer and Jakupovic (1990); Pacciaroni *et al*. (1995) and Bohlmann and Zdero (1977, 1981) have identified several compounds from *S. pinnata* to date. These include chromolaenide, chromolaenolide, costunolides **(i)**, dithiin, eucannabinolides, germacranolides, heliangolides, hiyodorilactones, loliolide, nerols, pectolinarigenin **(ii)**, santhemoidin A, schkuhrianol, schkuhrins **(iii)**, schkuhripinnatolides **(iv)**, schkurianol, thiarubrine A, thiophene (**v**), tridecapentayne and zaluzanin C (http://natural_herbal_remedies.my-online-store.net/list-of-medicinal-herbs-and-plants/ canchalagua.html). Costunolide, which is a sesquiterpene lactone, has been found to lower blood sugar and cholesterol levels in rat studies (Deutschländer *et al*., 2009) and to normalize the liver function enzymes levels of STZ-induced diabetic rats.

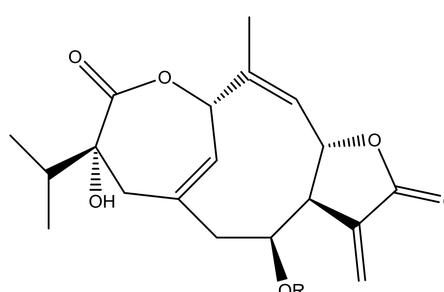




costunolide (i)

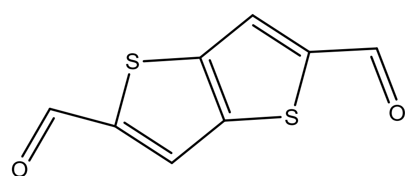
pectolinarigenin (ii)





schkuhrins (iii)

schkuhripinnatolide A (iv) R = H



thiophene (v)

Based on the historical success of natural products as antidiabetic agents and the ever increasing need for new antidiabetic agents, numerous South African medicinal plants have been investigated for their antidiabetic properties (Afolayan and Sunmonu, 2010). Although, the antidiabetic properties of *S. pinnata* have been investigated by Deutschländer *et al* (2009), its mode of action and active constituents have not been investigated. The findings from Deutschländer (2009) and van de Venter (2008) confirm *S. pinnata* to have *in vitro* hypoglycaemic activity in Chang cells but also indicate cytotoxicity on 3T3-L1

preadipocytes. However, the cytotoxicity results revealed that it was the acetone extract of *S. pinnata* which is toxic and raises concern for chronic use.

This chapter outlines the study aimed at the following:

- Preparation of suitable extracts from dried plant material of *S. pinnata* for biological assaying.
- Concentration and isolation of the active ingredient from the extract/s using column chromatography and liquid/liquid partitioning.
- Structure elucidation of the active ingredient using Mass spectrometry (MS) and NMR.

2.2 Materials and Methods

2.2.1 Plant collection

S. pinnata plant material was first collected in October 2001 from the Lebobombo mountains in KwaZulu-Natal (GPS co-ordinates: 32 2 4 E 27 22 24 S). The collection was undertaken by an independent plant collector, Hans Vahrmeijer, contracted to the CSIR. A voucher specimen (HV00248) with Genspec no. 92910002 of the aerial plant parts was identified as *S. pinnata* (Lam.) Cabrera and retained at the South African National Biodiversity Institute (SANBI) Herbarium in Pretoria. Permit applications were made to the Emakuleni Municipality for subsequent recollections of the aerial parts of *S. pinnata.*

A bulk recollection of 9 kg of plant material was collected by the same botanist just on the outskirts of Pretoria, South Africa in the Waterberg region, after some good rainfall and warm weather in mid-October. The yellow flowers, together with the leaves, were identified by the South African National Biodiversity Institute (SANBI) again who confirmed the identification of *S. pinnata*. This weed is an annual; hence it can only be collected once a year during October when flowers are abundant as it can easily be identified by the small yellow heads. *S.pinnata* grows abundantly along the roadside and favours sand-loamy soil and warm climate regions. This plant has strong erect woody stems which were cut using shears and allowed to dry on site for a few hours. The next day the plants were brought to the CSIR where it was immediately weighed and transferred to the ovens to dry.

2.2.2 *Extraction of plant material*

Aerial parts of *S. pinnata* (9 kg) were dried in an oven at 30–60°C for 3 days. Dried plant material (6.2 kg) was ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. The extraction procedure was based on the traditional method of preparation which was adding boiling water to the dried ground plant material (5 kg) and allowing to steep for 3 hrs. During the steeping process, there was intermittent stirring. The steeping procedure using boiling water was repeated 3 times on the same plant material and the extract was filtered to remove any sediment (for purification purposes in the laboratory). However, the aqueous extract was concentrated by freeze drying to keep it in a more stable form for research purposes. The freeze dried extract (340 g) was re-suspended in 2 L distilled water, mixed and centrifuged for 25 mins at 300 RPM. The supernatant was freeze dried and the sediment, which forms a compact pellet at the bottom, discarded. The freeze dried extract (200 g) was stored at 4°C. A summary of the extraction process is shown in Figure 2.2.

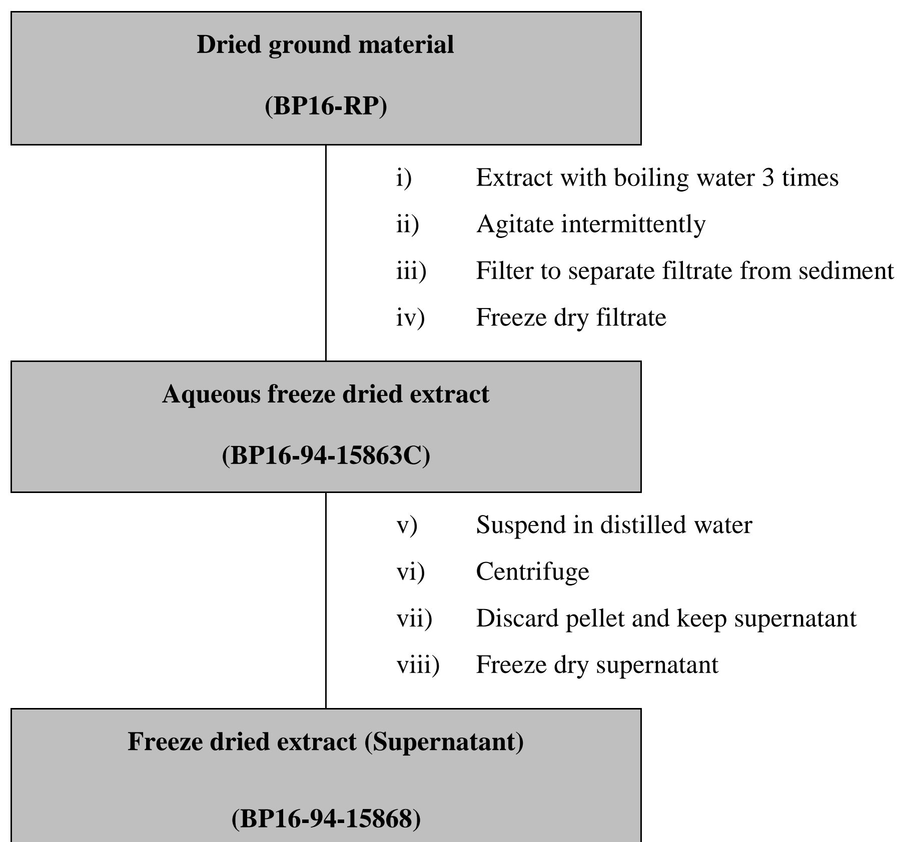


Figure 2.2 Summary of extraction process of the dried ground plant material.

2.2.3 Extraction of plant material using aqueous and organic solvents

Ground plant material (1.6 kg) was extracted using water and then separated into two equal portions; one portion was freeze dried (200 g) and the other portion spray dried (116 g). An ethanol extract (23 g) was also prepared by extracting the dried ground plant material (1.2 kg) with 96% ethanol for 24 hrs in large glass beakers. The glass beakers were covered with aluminium foil to prevent evaporation of the solvent overnight. Refer to Figure 2.3 for flowchart. All extracts were chemically profiled.

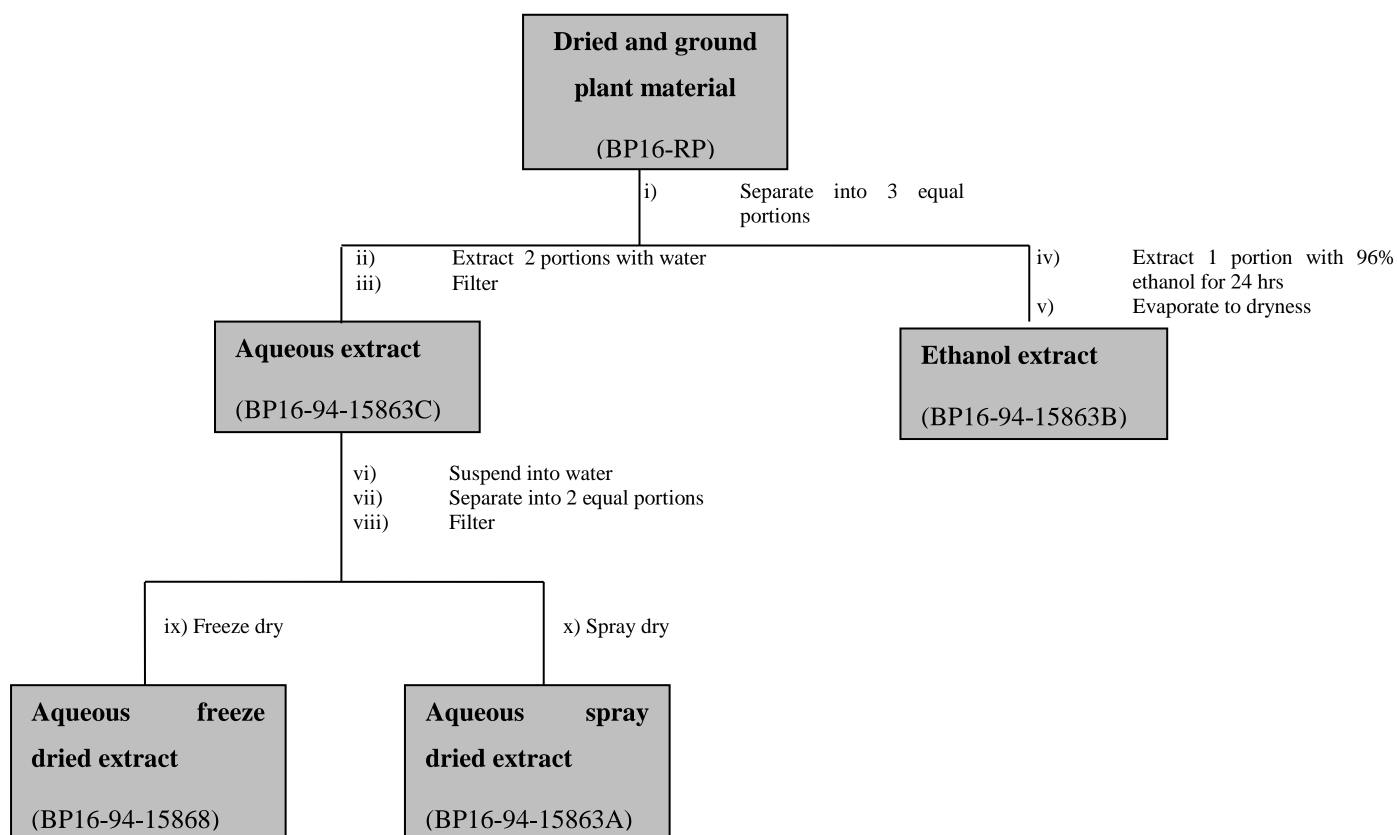


Figure 2.3 Flow diagram summarizing the different extracts produced.

2.2.4 High performance liquid chromatography (HPLC/UV)

The following extracts were prepared as follows:

Aqueous freeze dried extract (BP16-94-15868): 48.66 mg up to 4 mL in 50:50 MeOH: water

Aqueous spray dried extract (BP16-94-15863A): 43.21 mg up to 4 mL in 50:50 MeOH: water

Ethanol extract (BP16-94-15863B): 41.03 mg up to 4 mL in 50:50 MeOH: water

Method parameters:

Column: Phenomonex Luna C18 (2); 5µm; 250 x 4.6 mm

Column temperature: 45˚C

Flow rate: 1 mL/min

Injection volume: 10 µL

Detector: PDA (Photo diode array). Scanned wavelengths from 210 – 600 nm

Solvents: A = ddH_2O

B = MeOH

C = 0.1% Formic acid in ddH_2O

D = Acetonitrile

Table 2.1 Solvent gradient used for HPLC/UV

Time (min)	% Solvent A ddH_2O	% Solvent B MeOH	% Solvent C 0.1% FA in dH_2O	% Solvent D ACN	Flow rate (mL/min)
0	0	10	90	0	1
2	0	10	90	0	1
40	0	100	0	0	1
50	0	0	0	100	1
60	0	0	0	100	1
65	0	10	90	0	1
80	0	10	90	0	1

2.2.5 Concentration of the active extract using liquid/liquid partitioning

Liquid/liquid partitioning was performed on the aqueous freeze dried extract (73 g) by dissolving the extract in methanol/water (50:50) (100 mL) and adding ethyl acetate (4:1) (3 X 100 mL). To remove any traces of water present, 5 g of magnesium sulphate was added to the crude extract and then filtered using a funnel. The combined ethyl acetate layers were evaporated under pressure to yield the ethyl acetate-soluble extract (17 g). The ethyl acetate

extract was analysed using TLC with 5% methanol/dichloromethane as the solvent system. The plates were viewed under UV, developed using a vanillin: concentrated H_2SO_4 (1 g: 100 ml) spray reagent and then heated. The liquid/liquid partitioning process was also performed three times each on equal amounts (1 g each) of the freeze dried extract using 100 mL of butanone (1:1), butanol (1:1) and butanone/pentane (4:1). The extracts and the yields obtained were: butanol extract (220 mg), butanone extract (190 mg) and butanone/pentane extract (180 mg). Refer to Figure 2.4 for flow diagram.

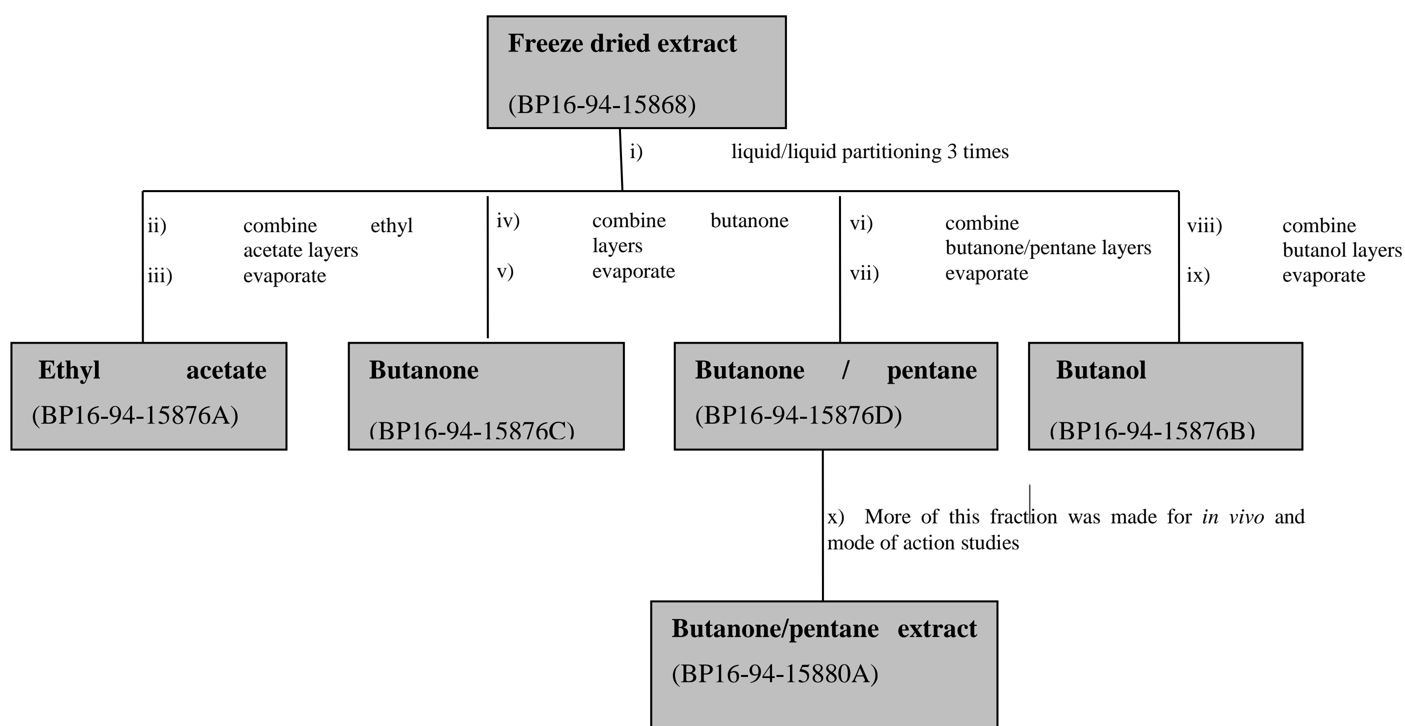


Figure 2.4 Flow diagram showing liquid/liquid partitioning using various solvents for extraction.

2.2.6 Fractionation of ethyl acetate extract using column chromatography

The ethyl acetate extract (4.8 g) was adsorbed onto silica gel by adding 5 g of silica gel to the extract. The mixture was gently dried using a rotary vacuum apparatus and added onto a silica gel column. The solvent system used initially was 2% methanol/dichloromethane and increased gradually to 5% methanol/dichloromethane. Time based fractions were collected. Fractions with similar TLC profiles were pooled together to yield 13 fractions. The last fraction 13, upon the solvent evaporating, yielded impure white crystals (32.8 mg) which was

later identified as compound 1. The impure crystals (7 mg) obtained were subjected to NMR experiments. Refer to figure 2.5 for flow diagram.

The filtrate from fraction 13 was subjected to prep TLC, yielding 3 bands. Each band was carefully scraped out, dissolved in methanol and evaporated. Only one of the bands crystallized resulting in further NMR experiments. This new band (BP16-94-15857) that crystallized turned out to be the same as BP16-94-15864H.

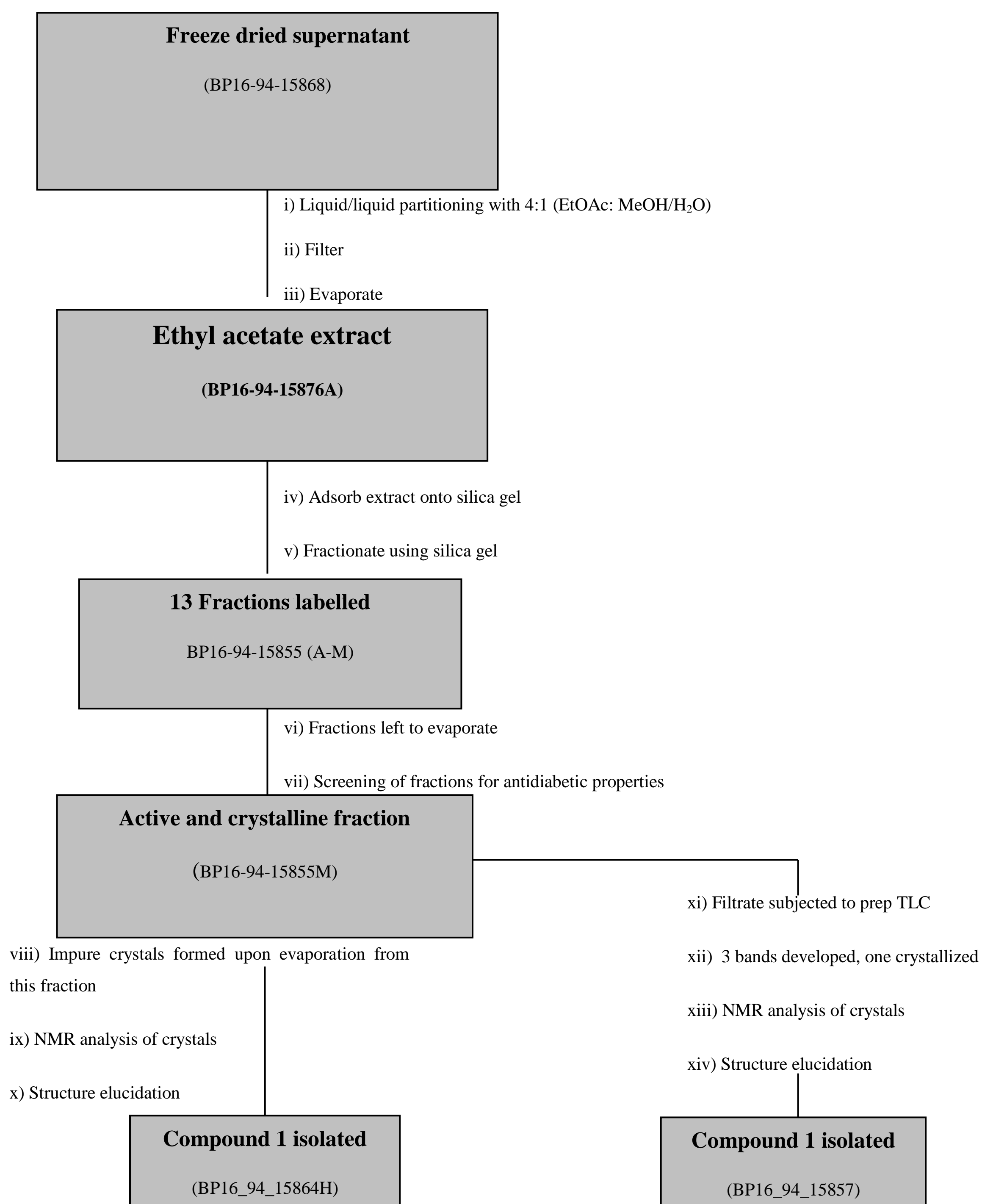


Figure 2.5 Flow diagram of isolation of compound 1.

2.2.7 Nuclear magnetic resonance (NMR) spectroscopy

Pure compounds are identified and characterized by mass spectrometry and NMR experiments. The pure compounds are based on detailed studies of the high field ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) and following two-dimensional (2D) NMR techniques (Croasmun and Carlson, 1987; Saunders and Hunter, 1987).

NMR data was acquired using a Varian VNMRS 600MHz NMR spectrometer using two different solvents. Compound 1 was acquired in CD_3OD (data set labelled BP16_94_15857) and DMSO-d6 (data set labelled BP16_94_15864H). The raw data files were processed using MNova 7.1.2.

2.2.8 UPLC-DAD-MS analysis

Compound 1 was analyzed using the UPLC-DAD-MS. UPLC-DAD-MS data were obtained with a Waters SYNAPT HDMS system consisting of an autosampler, ultra-pressure mixing pump, column oven and DAD connected to a TOF mass spectrometer. The system was controlled through MassLynx v 4.1 SCN639.

UPLC conditions: Acquity HSS T3 column (1.8 µm, 150 x 2.1 mm Waters), the solvent gradient used was as follows: with 5% (v/v) aqueous HPLC-gradient acetonitrile (A) in 0.1% formic acid and increased to 90% acetonitrile over 15 min in the following manner: 0 - 13 min, 90% A; 13 - 15 min 90% A with flowrates of : 0.35 mL/min (0 - 13 min) and 0.45 (13 - 15 min); Injection volume: 5 µL; Sample concentration: 16.6 mg/ml in water; DAD conditions: 200 - 500 nm.

ESI-MS conditions: Negative ion mode; Split ratio 1:4; Scan range: 100 - 1200 Da; Source temperature: 120°C; Analyzer: V-mode; Capillary voltage: 2 KV; Cone voltage: 30 V; Extraction cone: 5 V; Desolvation temperature: 400°C; Cone gas: 50 L/hr; Desolvation gas: 450 L/hr.

ESI-MS conditions: Positive ion mode; Split ratio 1:4; Scan range: 100 - 1200 Da; Source temperature: 120°C; Analyzer: V-mode; Capillary voltage: 2.5 KV; Cone voltage: 30 V; Extraction cone: 4 V; Desolvation temperature: 400°C; Cone gas: 50 L/hr; Desolvation gas: 500 L/hr. Harvard Model 11 syringe drive for lockmass infusion.

2.2.9 *Targeted purification of active for in vivo studies*

In order to isolate sufficient quantities of compound 1, a targeted purification of the active was pursued (refer to Figure 2.6). A total of 200 g of freeze dried supernatant was separated using ethyl acetate/methanol (4:1) and water by liquid/liquid partitioning using a separating funnel. The supernatant was extracted 3 times and the ethyl acetate layers were combined and evaporated to dryness. The ethyl acetate extract (90 g) was subjected to silica gel chromatography using a solvent system of 5% methanol/dichloromethane. Fractions were collected, spotted on TLC and similar fractions were pooled together to obtain a further 6 fractions. Using compound 1 as a marker, fraction 4 (30 g) was purified further using flash silica gel and the same solvent system. A further 4 fractions were obtained by pooling like fractions together based on TLC spotting. Fractions 2 and 3 (3 g) were combined and separated using flash silica gel again. This separation yielded 10 fractions. Compound 1 could not be isolated. However, fractions 5, 6, 7 and 8 were combined resulting in a compound 1 enriched fraction (BP16-94-15887) (1.138 g) as compound 1 was concentrated in this region, which was used for screening purposes and the mode of action studies.

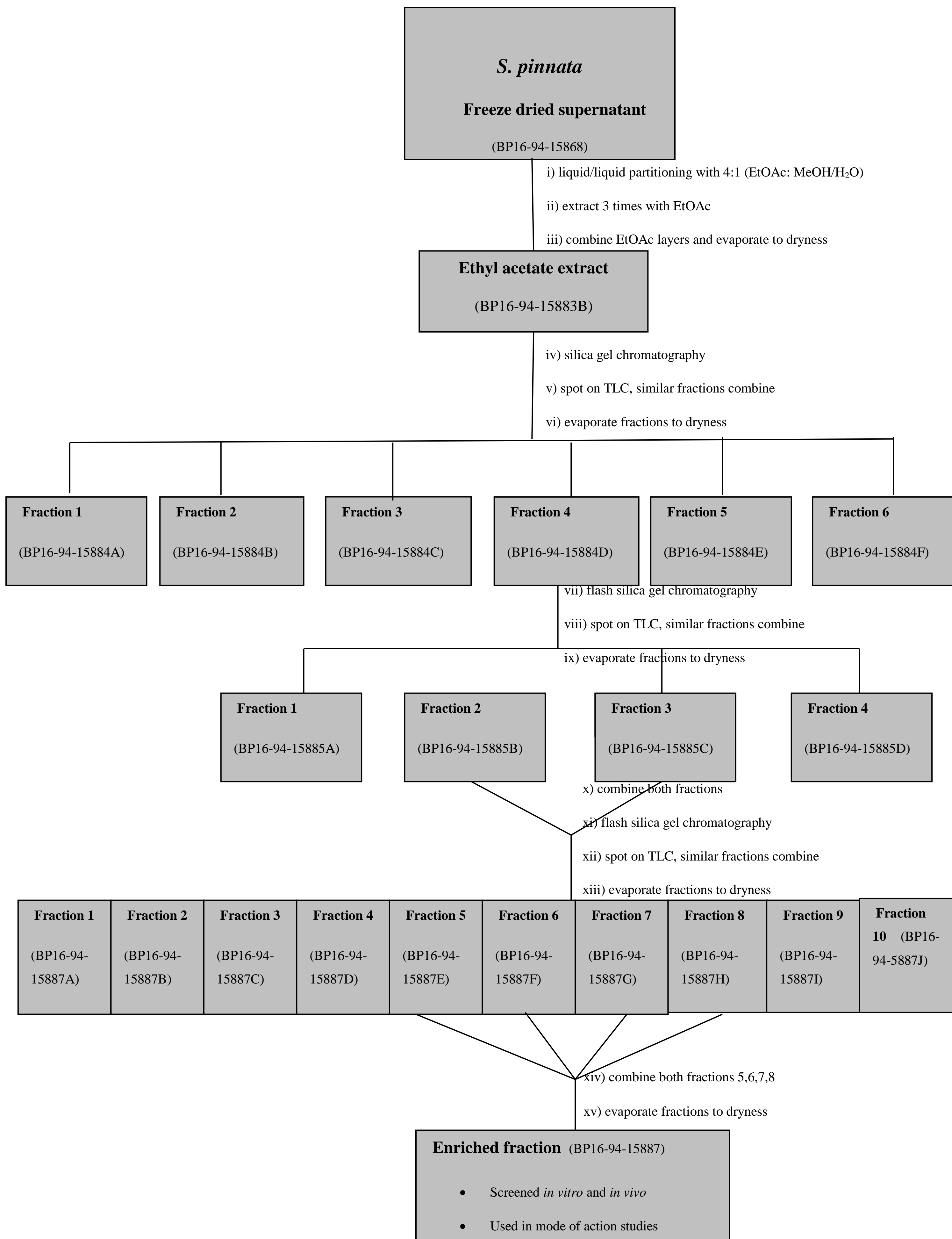


Figure 2.6 Summary of targeted purification of compound 1.

2.3 Results and Discussion

In order to identify the biologically active compound/s from *S. pinnata*, an aqueous bulk extraction of the plant material was performed and the aqueous extract was prepared as per the traditional use of the plant which was by making a tea of the dried ground plant material. However, for experimental studies, the aqueous extract was also centrifuged to get rid of fine sediment of insoluble plant material that appears in the filtrate. For research purposes, a wet extract is not feasible due to the sticky nature of most plant extracts and in that state and it also easily becomes contaminated with fungi. Therefore, freeze drying was introduced as an additional step to make the extract suitable for research purposes and to keep the extract in a stable form. Spray drying was also attempted to produce a dry free-flowing powder in a further attempt to keep the extract in a stable form to work with.

Liquid chromatography coupled to mass spectrometry is being adopted across all areas of drug discovery to provide early data that might help in understanding the chemistry of a drug candidate and, thus, saving time and reducing costs in the long-term. During the past 30 years, HPLC has become widely accepted and employed in laboratories worldwide (Croasman and Carlson, 1987). HPLC technology has become more reliable and easier to use. The overall result is faster analyses and better sensitivity.

A comparison of the freeze dried, spray dried and ethanol extracts were done to see which extract was most effective at promoting glucose uptake in C2C12 muscle and Chang cells (refer to Chapter 3 for screening results). Chemical profiling revealed the following chromatogram (Figure 2.7) obtained via HPLC/UV by overlaying and comparing the chemical profile of the three extracts. A long peak at a retention time of 26.097 was present in the freeze dried extract with a very much shorter (almost non-existent) peak in the spray dried extract at the same retention time. Speculation as to the absence of this peak in the spray dried extract could be that this compound may be volatile and with the heat involved in the spray drying process, it evaporated.

This peak was totally absent in the ethanol extract. There was another peak at retention time of 31.1 mins that was present in the freeze dried and spray dried extracts but absent in the ethanol extract.

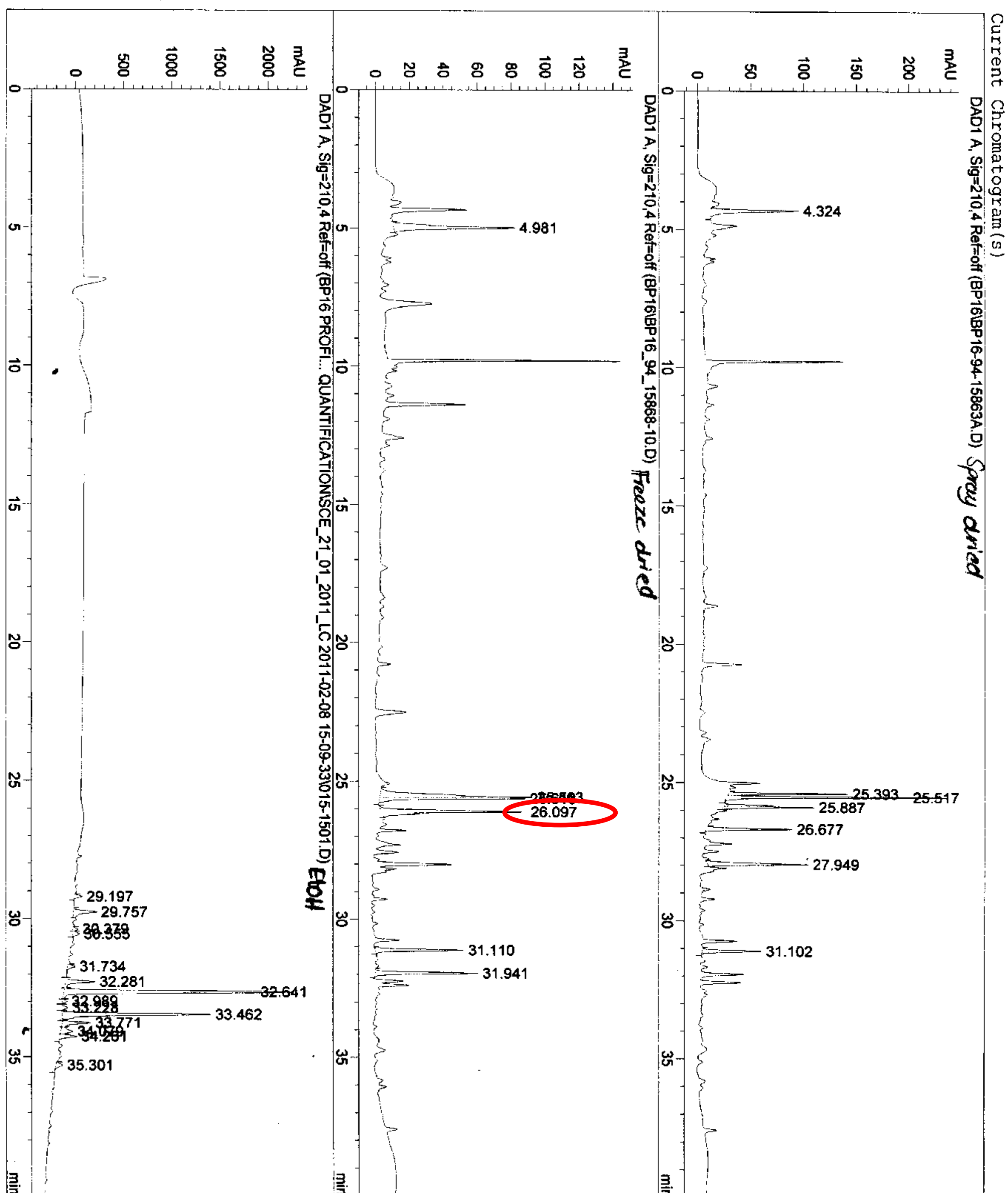


Figure 2.7 Chromatogram comparing chemical profiles of spray dried, ethanol and freeze dried extracts.

Pharmaceutically acceptable solvents are required by the FDA so that the end product would be less toxic and hence safe for all patients (http://www.pharmacopeia.cn/v29240/usp29nf24s0_c467.html). Butanone, butanol, pentane and ethanol are among a few of the pharmaceutically acceptable solvents allowed by the FDA and pharmacopeia for formulation activities. A standard liquid/liquid partitioning process was performed on the freeze dried aqueous extract with the above solvents and evaluated for antidiabetic activity. A qualitative analysis of the chemical profiles using HPLC of these different solvent extractions showed the butanone/pentane extract to be the least complex matrix. Hence it was decided to screen this extract *in vivo* as it contained compound 1 (albeit a very small concentration), appeared semi purified (did not contain many compounds) and most important, this decision was based on its *in vitro* biological efficacy (details in Chapter 3).

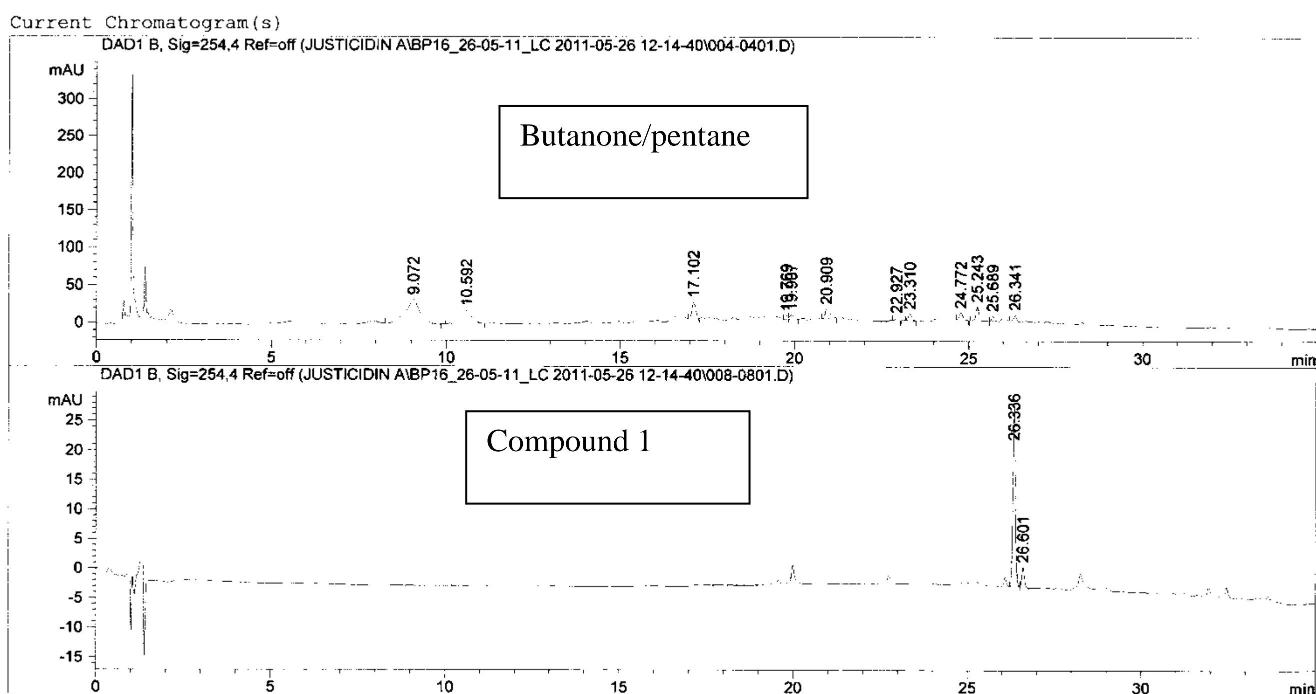


Figure 2.8 Chemical profile of butanone/pentane extract and compound 1.

The extracts prepared via liquid/liquid partitioning with the pharmaceutically acceptable solvents revealed that compound 1 was present in the butanone/pentane (Figure 2.8). However, judging by the size of peak, the butanone/pentane extract did not contain a very high percentage of compound 1. This extract was further investigated as the profile revealed

it did not contain too many other compounds, hence easier to work with and to isolate compounds.

The freeze dried extract was subjected to liquid/liquid partitioning with ethyl acetate and fractionated via silica gel column chromatography. Similar fractions were pooled together resulting in 13 fractions which were screened using a glucose uptake assay in C2C12 muscle and Chang cells.

During evaporation of the silica gel column fractions BP16-94-15855A to BP16-94-15855M, a crystalline compound precipitated out of the two last fractions, BP16-94-15855L and BP16-94-15855M. This was filtered to yield crystals of compound 1 (BP16-94-15864H). NMR studies were conducted on the isolated compound. The filtrate was subsequently subjected to preparative TLC to yield additional quantities of compound 1. Three bands were visible, cut out, washed, isolated and subjected to NMR. Just one band was the most similar to the compound already isolated. This compound (BP16-94-15857) was isolated and subjected to further 2D experiments, resulting in it being identified as the same as compound 1.

All structural characterizations were carried out using a combination of 1D(^{1}H,^{13}C) and various 2D Distortionless enhancement by polarisation transfer – DEPT, Correlation spectroscopy –COSY, Heteronuclear single quantum coherence – HSQC and Heteronuclear multiple bond correlation – HMBC) experiments.

Preliminary TLC, ^{1}H NMR and MS analysis revealed that compound 1 isolated from silica gel column chromatography was a mixture of 3–4 compounds; while the three compounds isolated via preparative TLC were the partially separated diastereomers of compound 1 with just one compound being similar to compound 1. An attempt using the semi-prep HPLC for the separation of the diastereomers was not successful as the NMR's of each showed traces of the other diastereomer. However, the NMR data of one diastereomer (compound 1 – isolated via silica gel chromatography) could be adequately determined. The structure of the pure compound could not be easily solved due to the nature of the diastereomers having the same R_f and molecular weight. However, with extensive 2D NMR experiments using two different solvents, conclusions could be drawn and the structure of compound 1 was concluded in combination with the results obtained with HPLC-UV, HPLC/MS and NMR. Compound 1

was identified as 2-(2-{[(2*E*)-4-hydroxy-2-(hydroxymethyl)but-2-enoyl]oxy}-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)prop-2-enoic acid, belonging to the class of compounds called terpenes. Terpenes are hydrocarbons that usually contain one or more C=C double bonds and consist of 5-carbon units, often called isoprene units. Overall, terpenes are used for practical applications especially in the fragrance and flavour industries, as well as in the pharmaceutical and chemical industries. However, in a review of phytomedicines for diabetes, Makheswari and Sudarsanam (2011) report that terpenes can be used for the prevention and/or treatment of T2D, obesity and neuropathy.

Table 2.2 ^{1}H and ^{13}C data for BP16-94-15857 in Methanol-d4

Atom Number	^{13}C (ppm)	^{1}H (ppm), J (Hz)	Type
1	139.8		=C
2	136.6		=C
3	131.5*	6.80, s	=C
4	136.9		=C
5	128.9	7.02, d, J=8.0	=CH
6	129.1	7.12, d, J=8.0	=CH
7	32.6	3.15, m	CH
8	32.1	1.66, dt, J=13.1, 3.3 2.26, m	CH_2
9	70.6*	5.52, ddd, J=11.1, 5.5, 2.8	CH
10	44.6*	4.62, d, J=5.6	CH
11	20.9	2.24, s	CH_3
12	24.6	1.33, d J=7.2	CH_3
14	145.5†		=C
15	128.7	4.88, s 6.30, s	$=CH_2$
16	172.0†		COO
19	167.6		COO

20	133.0		=C
21	145.9*	6.85, t, J=6.0	=CH
22	59.5	4.39, d, J=6.0	
25	56.9	4.30, m 4.28, m	CH_2

(* doubled up in ^{13}C spectrum, † broad in ^{13}C spectrum)

Atom numbering in Table 2.2 is generated by MNova and is non IUPAC. Coupling constants determined by MNova after spectral deconvolution.

Table 2.3 ^{1}H and ^{13}C data for BP16-94-15864H in DMSO-d6

Atom Number	^{13}C (ppm)	^{1}H (ppm), J (Hz)	Type
1	138.2		=C
2	135.3		=C
3	129.9	6.74, d, J=1.7	=C
4	135.0		=C
5	127.6	7.01, dd, J=8.0, 1.8	=CH
6	127.7	7.16, d, J=8.0	=CH
7	30.0	3.11, m	CH
8	31.5	1.60, ddd, J=13.0, 5.3, 2.6 2.13, ddd, 13.0, 10.0, 6.0	CH_2
9	68.2	5.37, ddd, J=10.0, 5.3, 2.6	CH
10	42.6	4.50, d, J=5.3	CH
11	20.5	2.20, s	CH_3
12	23.5	1.26, d J=7.1	CH_3
14	142.4		=C
15	128.4	4.97, s 6.28, s	=CH_2

16	168.2		COO
19	165.5		COO
20	130.9		=C
21	145.3	6.62, t, J=5.7	=CH
22	57.8	4.23, dd, J=16.0, 5.8 4.21, dd, 16.0, 5.8	CH_2
25	55.3	4.05, d, J=12.8 4.07, J=12.8	CH_2

Atom numbering for Table 2.3 is generated by MNova and is non IUPAC. Coupling constants are determined by MNova after spectral deconvolution.

Table 2.4 Summary of long-range ^{1}H-^{13}C correlations observed

Hydrogen(s)	**Long-range correlations**
3-H	C-11 (^3J), C-10 (^3J), C-5 (^3J), C-1 (^3J)
5-H	C-11 (^3J), C-6 (^2J), C-2 (^3J), C-1 (^3J)
6-H	C-7 (^3J), C-5 (^2J), C-2 (^3J), C-4 (^3J)
7-H	C-12 (^2J), C-8 (^2J), C-9 (^3J), C-1 (^2J)
8-H2	C-12 (^3J), C-7 (^2J), C-9 (^2J), C-10 (^3J), C-1 (^3J)
9-H	C-14 (^3J)*, C-19 (^3J)*
10-H	C-8 (^3J), C-9 (^2J), C-15 (^3J), C-3(^3J), C-2 (^2J), C-1(^3J), C-14 (^2J), C-18 (^3J)
11-H3	C-5 (^3J), C-2 (^3J), C-4 (^2J)
12-H3	C-7 (^2J), C-8 (^3J), C-1 (^3J)
15-CH2	C-10 (^3J), C-9 (^4J), C-14 (^2J), C-18 (^3J)
21-H	C-25 (^3J), C-19 (^3J), C-20 (^2J)
22-H2	C-20 (^3J), C-21 (^2J)
25-H2	C-20 (^2J), C-21 (^3J), C-19 (^3J)

* Correlations very weak

The stereochemistry was not determined.

A COSY spectrum acquired on BP16-94-15857 was used to identify ^{1}H-^{1}H coupling connectivities. Long-range ^{1}H-^{13}C (HMBC) spectra were acquired on both samples. From a combination of all the data sets and interpretation of ^{13}C chemical shifts, 4 isoprene units (**A** – **D**) can be identified as illustrated in Figure 2.9.

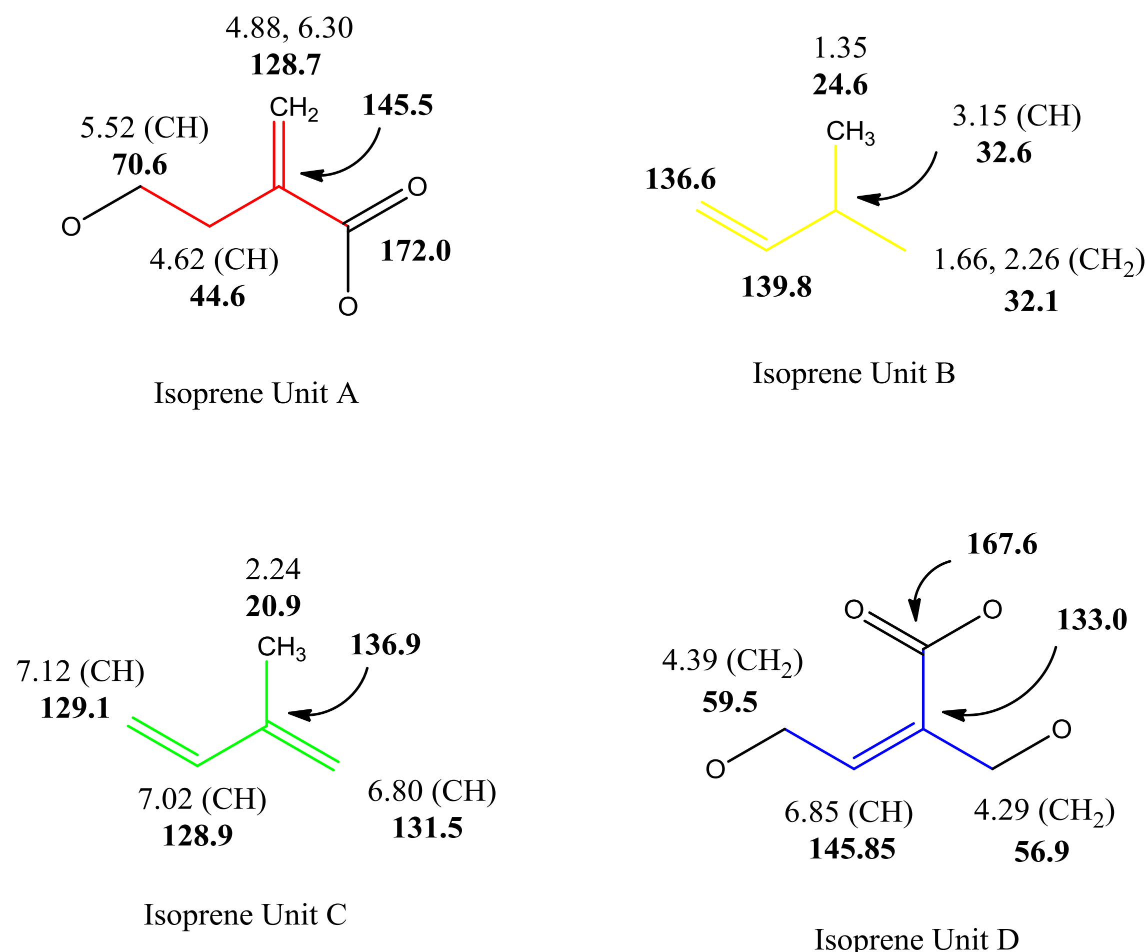


Figure 2.9 Structures of the 4 isoprene units (A-D) with their ^{1}H and ^{13}C (bolded) chemical shifts in CD_3OD.

From the COSY and HMBC data **A**, **B** and **C** can be linked together in a head to tail fashion followed by 2 cyclisation's to give partial structure (**E**) as shown in Figure 2.10 that is similar to a number of natural products (Figure 2.11).

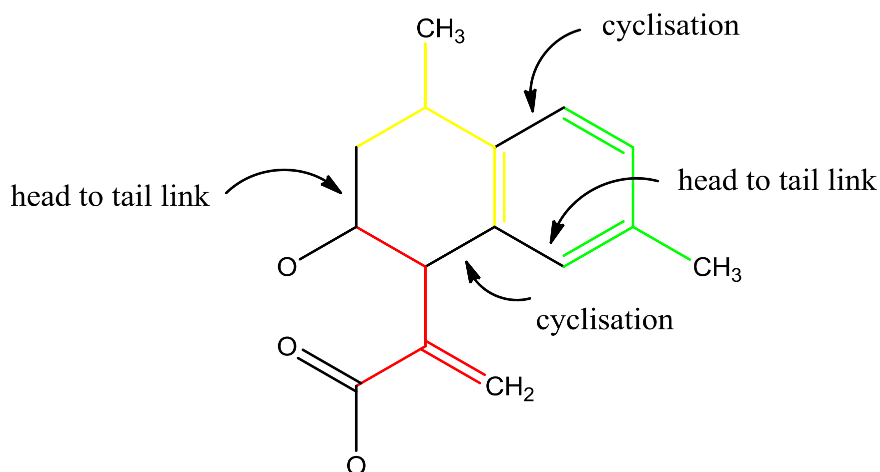
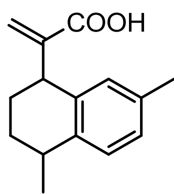
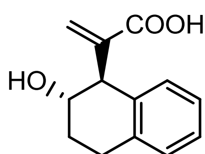
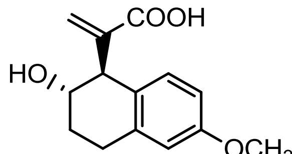
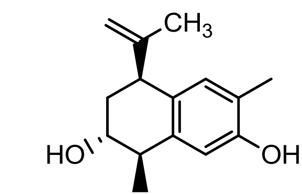


Figure 2.10 Partial structure (E) formed by head to tail linking of A, B and C together with 2 cyclisations.









J.Nat.Prod. 1993, 56(4), 552-563

J.Chem.Res. (S), 1995 (4), 120-121

Chem.Pharm.Bull. 2008, 56(8), 1143-1146

Figure 2.11 Examples of natural products with structures similar to partial structure E.

Some of the key ^{1}H-^{1}H (COSY) and long-range ^{1}H-^{13}C (HMBC) correlations for **E** are illustrated in Figure 2.12.

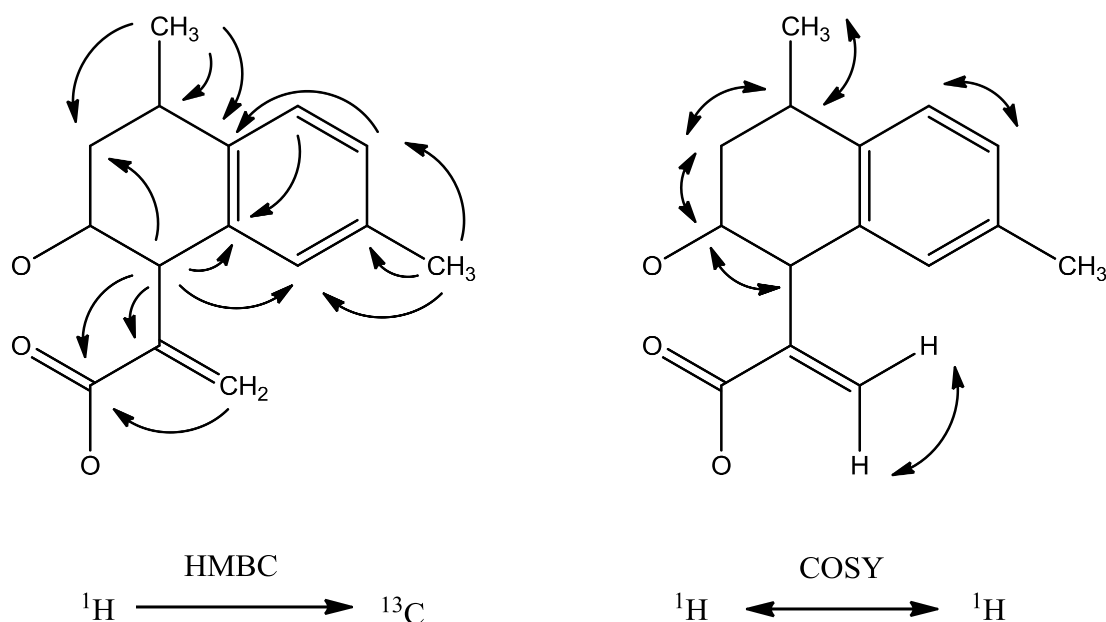


Figure 2.12 Key ^{1}H-^{1}H (COSY) and long-range ^{1}H-^{13}C (HMBC) correlations observed for partial structure E.

The location of isoprene unit **D** is not immediately clear from the data. Given the presence of 4 isoprene units, a formula of $C_{20}H_{24}O_6$ is consistent with the molecular weight of 360 that requires 9 double bond equivalents. Partial structure **E** accounts for 7 with the remaining 2 being accommodated within isoprene unit **D** itself. The ^{13}C chemical shifts of isoprene unit **D** are consistent with a COO and two OCH_2 groups. Either of these groups could be part of an ester functionality linking **E** to isoprene unit **D**. There are 3 possibilities (excluding stereochemistry) as summarised in Fig 2.13.

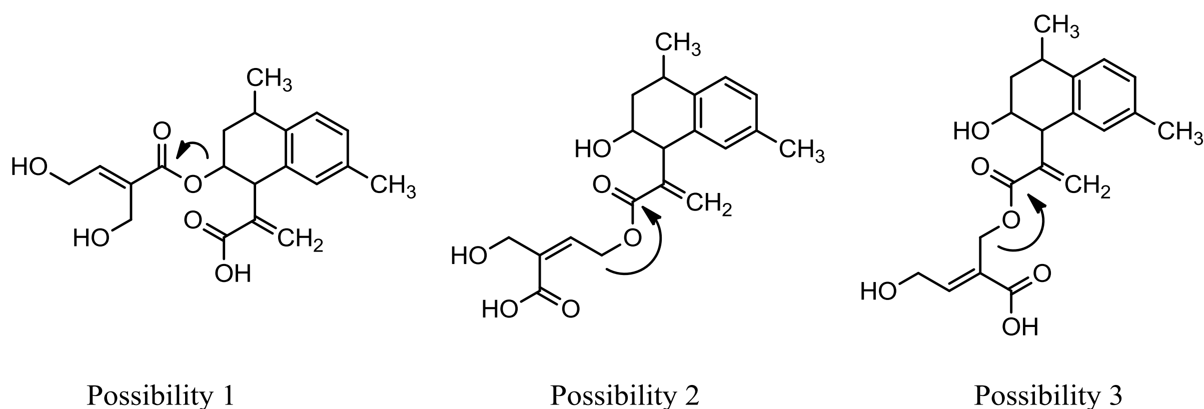


Figure 2.13 Possible structures for BP16-94-15857 and BP16-94-15864H (excluding stereochemistry). Possibility: 1 Ester formed from secondary alcohol of **E** with carboxylic acid of isoprene unit **D**. Possibilities 2 and 3: carboxylic acid of **E** with either

alcohol of isoprene unit **D**. Arrows indicate potential distinguishing long-range ^{1}H-^{13}C (HMBC) correlations.

Possibilities 2 and 3 would be expected to show a long range ^{1}H-^{13}C correlation from one of the CH_2 groups of isoprene unit **D** to the COO of **E** (Figure 2.13) but this is not observed. Since the CH_2 groups show several long-range ^{1}H-^{13}C correlations then the absence of this key correlation argues against these structures. In possibility 1, a long range ^{1}H-^{13}C correlation from a CH to the COO of isoprene unit **D** would be expected (Figure 2.13). This is not observed for BP16-94-15857 in CD_3OD although for BP16-94-15864H in DMSO-d6 there is an indication of a correlation, albeit barely above the noise level. Although one has to be careful with such weak correlations there are no clear long-range ^{1}H-^{13}C correlations from the CH to any other carbons. This could be a consequence of the width of this resonance as a result of its ddd splitting as well as the small number of scans (4) acquired per increment. Thus, possibility 1 appears to be the most likely structure for BP16-94-15857 and BP16-94-15864H (Figure 2.14).

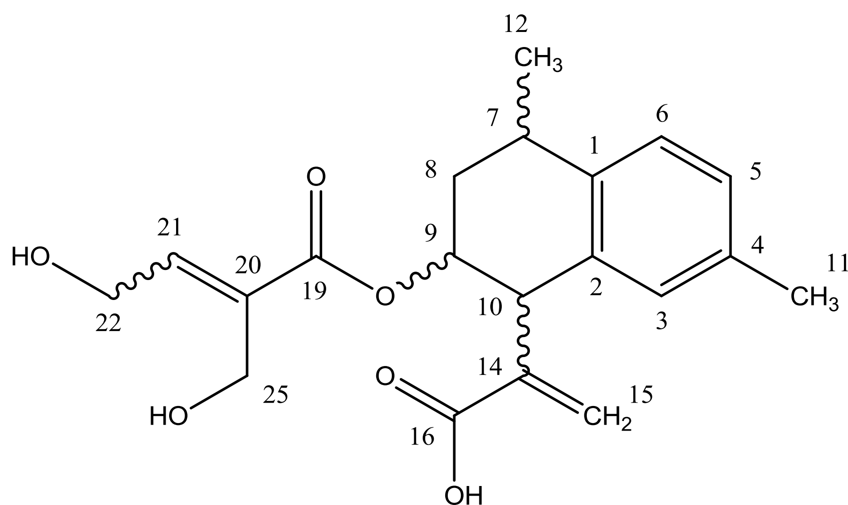
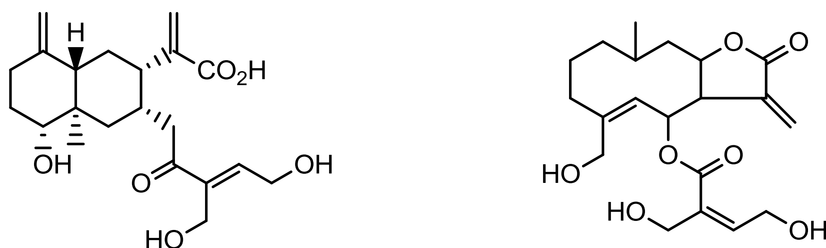


Figure 2.14 Compound 1 structure determined from BP16_94_15857 and BP16_94_15864H.

Atom numbering is that generated by MNova and is non IUPAC. Name generated by Chemdraw and DNP is 2-(2-{[(2*E*)-4-hydroxy-2-(hydroxymethyl)but-2-enoyl]oxy}-4,7-dimethyl-1,2,3,4-tetrahydronaphthalen-1-yl)prop-2-enoic acid. There is no published literature on this compound or biological activity.

A complete breakdown of the HMBC data is summarised in Table 2.4. A number of natural products have been reported that contain isoprene unit **D** ester linked to terpenes (Figure 2.15).



Biochemical Systematics and Ecology, 2001, 29(6), 633-647	Coll.Czech.Chem.Commun., 1960, 25, 2777-2782

Figure 2.15 Examples of natural products containing isoprene unit D ester linked to terpenes.

In conjunction with NMR, HPLC/MS was also performed to try to identify compound 1 from the Dictionary of Natural Products (DNP) based on its molecular weight. Figure 2.16 shows the intensity of the long molecular ion peak at *mz* 359 (ES-) to be compound 1 with a molecular weight of 360.169. Compound 1 ionizes in the ES- mode and the fragmentation pattern on several ion peaks losing/gaining either a H/H_2O/Na molecule fits that depicted in Table 2.5.

The UPLC-DAD-MS analysis revealed the chromatogram Figure 2.16.

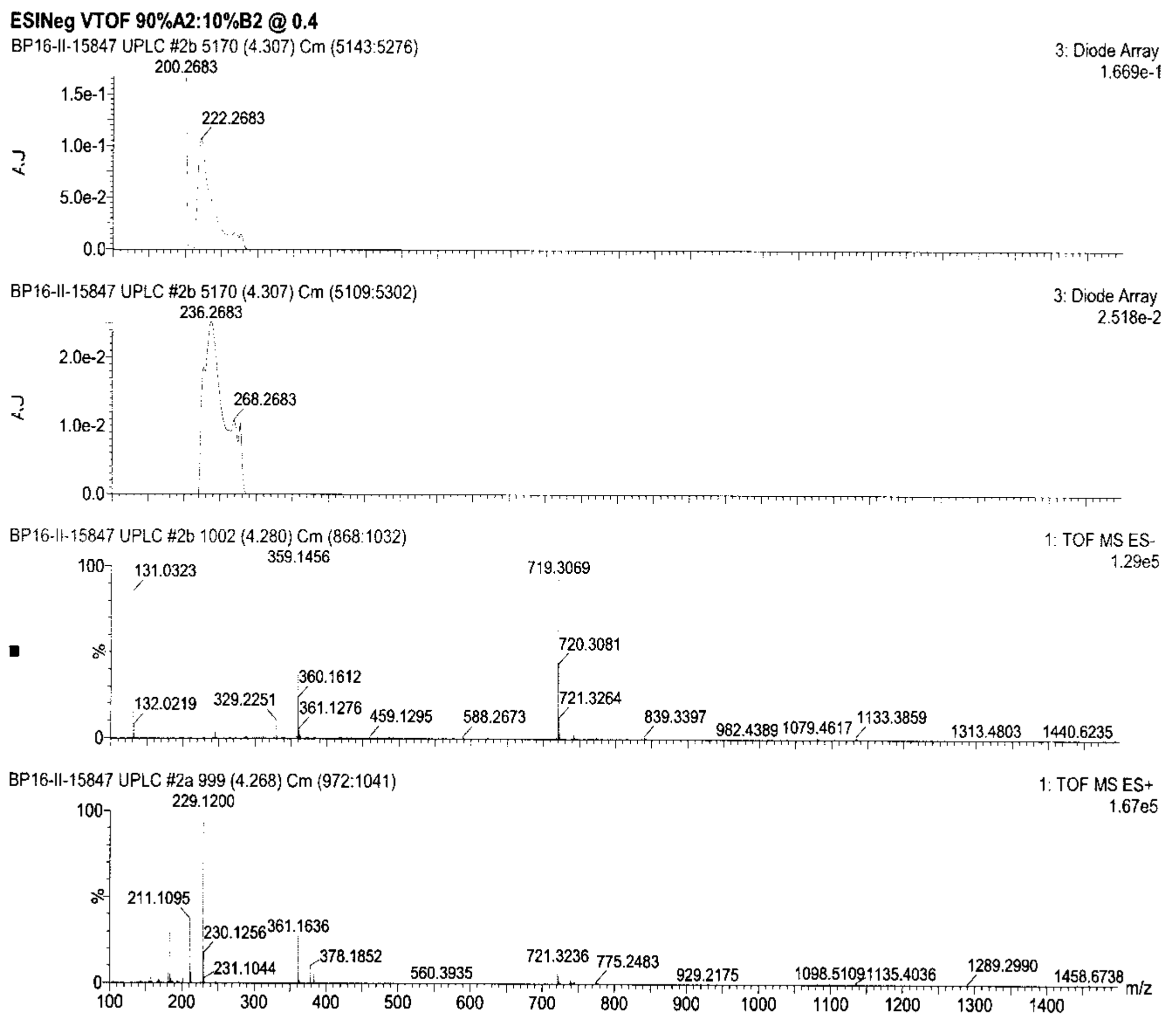


Figure 2.16 Chromatogram profile of active fraction showing ES+, ES- and diode array.

Table 2.5 Fragmentation pattern of compound 1

Mass	UV data	M ions observed						
		M+H	M+Na	M-H	2M	2M+H	2M+Na	2M-H
360.161			383.151	361.169	720.322	721.33	743.312	719.314

The MS chromatogram in the ES- mode (Figure 2.16) shows the (M - 1) ion at *m/z* 361 which corresponds to the molecular formula of $C_{20}H_{20}O_4$.

In the ES+ mode, the addition of an H_2O molecule shows an ion peak at *m/z* 378.

A targeted extraction process and semi-purification of compound 1 was executed in order to obtain adequate quantities for *in vivo* studies. A chemical profile of the aqueous freeze dried extract (BP16-94-15868) (Figure 2.17) revealed that it contained at least twenty compounds.

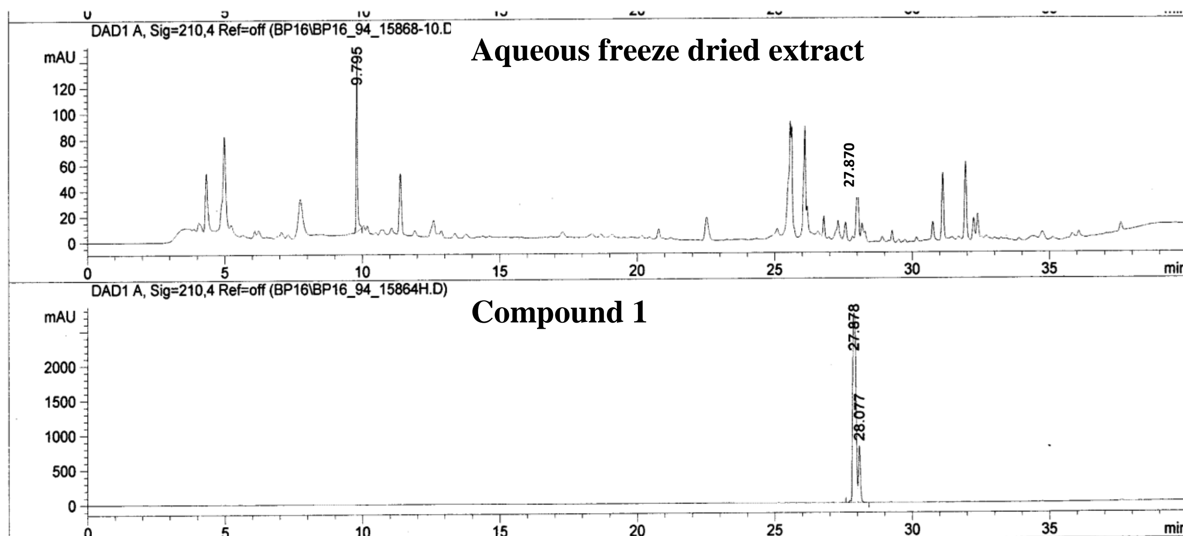


Figure 2.17 Chromatogram showing presence of compound 1 in freeze dried extract.

The aqueous extract was extracted again with ethyl acetate. Silica gel column chromatography was then conducted to yield three broad bands (BP16-94-15884A - C) and three smaller fractions (BP16-94-15884D - F) in the region where compound 1 was expected to elute. TLC analysis revealed that compound 1 was concentrated in fraction D (BP16-94-15884D). Repetitive silica gel column and flash silica gel chromatography was conducted in order to purify compound 1. This proved unsuccessful. Due to the scale of the purification, sufficient quantities of pure compound 1 could not be isolated. It was then decided to semi-purify the aqueous extract to concentrate compound 1 (active ingredient) within this fraction, resulting in a compound 1 enriched fraction (BP16-94-15887) and screen this together with compound 1 in an *in vivo* model.

2.4 Conclusion

Compound 1 was successfully isolated from *S. pinnata* and the structure elucidated. It is a novel compound as there are no published uses for it. The MS fragmentation has confirmed the compound for the molecular weight of 360. The structure is quite certain although future work would be necessary in order to have some harder evidence about the ester linkage. Some future work could look at the HMBC in DMSO-d6 which can be acquired with 8 (or 16) scans per increment to see the key correlation from 9-H to C-14 above the noise level. More NMR 2D studies with some nOe data (e.g. NOESY) would hopefully determine the relative stereochemistry of the 3 chiral centres and fix the double bond stereochemistry as *E* or *Z*. However, this was not considered relevant for the purposes of this study.

2.5 References

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CHAPTER 3 - Biological screening of *S. pinnata*

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3.1 Introduction

The importance of plant-derived medicinals in modern medicine is often undervalued. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethno-botanical information reports about 800 plants that may possess antidiabetic potential, with several medicinal plants showing antidiabetic activity when pharmacologically tested (Grover *et al*., 2002). Despite the obvious results that many plants and compounds do possess antidiabetic activity, there has been a dramatic decline for NCE's from natural sources (Newman and Cragg, 2007).

During the past decade, traditional systems of medicine have become a topic of global importance. Despite the fact that these plants are undeniably still being widely used by a large portion of the population of poorer countries, many developing countries in Western medical science still view such systems as lacking credibility and validity (Upton, 2015). In South Africa, a large section of the population relies heavily on traditional healers and medicinal plants to meet primary health care needs. Although modern medicine is available in this country to many, traditional medicines have often maintained popularity for historical and cultural reasons (Street and Prinsloo, 2013). Concurrently, many people in developed countries have turned to alternative or complementary medicines which include medicinal plants. Even with this vast array of data, few medicinal plant species have been scientifically evaluated for their possible medical application. Safety and efficacy data are available for only a few plants, their extracts and their active ingredients, and the preparations containing them. Furthermore, in most countries the herbal medicine market is poorly regulated and herbal products are often neither registered nor controlled (WHO, 2004; Ekor, 2013). Assurance of safety, quality and efficacy of medicinal plants and herbal products has now become a key issue in industrialised and in developing countries. Both the general consumer and health-care professionals need up-to-date information on the safety and efficacy of medicinal plants. Hence there is a need for validation of medicinal plants using various scientific techniques like the use of *in vitro* assays and *in vivo* models.

The use of *in vitro* studies to measure the glucose uptake in cell lines following stimulation with insulin and other active compounds, including indigenous plant extracts, is a direct and sensitive method of determining the antidiabetic effect of these agents (Van de Venter *et al*., 2008). *In vitro,* the cell physiology is mechanistically different for cells derived from different tissue types. Such cells retain, to a large extent, the characteristics of the original tissue type, also in terms of glucose uptake and metabolism. The C2C12 muscle and Chang cells utilized for this diabetic screening study were selected based on their different level of expression of the insulin sensitive glucose transporter GLUT-4, which is highly expressed in the C2C12 cells, (Brunetti *et al*., 1989; Nedachi and Kanzaki, 2006), but not in Chang cells (Rengarajan *et al*., 2007; Rodríguez-Enríquez *et al*., 2009). These cells therefore not only allow for rapid screening of compounds in terms of their efficacy and toxicity in an ethically acceptable manner, but also gives additional mechanistic information with regards to GLUT-4 induced glucose uptake.

Muscle derived cell lines such as C2C12 cells have been shown to be sensitive to insulin stimulation in culture, resulting in an increase of GLUT-4 activity and glucose uptake from the culture medium (Brunetti *et al.,* 1989; Nedachi and Kanzaki, 2006)**.** To maximize glucose receptor (GLUT-4) expression before glucose uptake experiments, the skeletal myoblast cells (C2C12 cells) need to be transformed into mature myocytic cells. This is achieved by the replacement of the growth factor rich foetal calf serum with horse serum in the medium once the cultures are near-confluent (80 – 90%). As the cells differentiate, they start to form visible myotubules, a syncytium of muscle cells with contractibility. The second cell line selected was Chang cells. They provide a well-established and validated cell line for *in vitro* glucose uptake screening (Van de Venter *et al.,* 2008, Deutschlander *et al*., 2009). Chang cells are derived from the HeLa cells that in contrast to the muscle cell line, express negligible levels of GLUT-4 and therefore is less sensitive to acute insulin stimulated glucose uptake (Rengarajan *et al.,* 2007; Rodríguez-Enríquez *et al*., 2009). Chang cells do not require media enhanced differentiation, but require a longer period of exposure (3 hrs) to the extract.

Assays performed for this project include glucose uptake from the media which was determined by measuring the glucose left in the media using a colourimetric method after 1 hr and 3 hrs exposure to the extracts or compounds for C2C12 and Chang cells respectively, and by measuring intracellular [^{3}H]-2-deoxy-D-glucose uptake. To further elucidate the effect(s) of the extracts on intracellular signalling and establish the mode of action, mRNA and protein expression was determined by quantitative real-time PCR and Western blot

analysis respectively (refer to Chapter 4).

In vitro models are usually simple, allows for high throughput bioactivity screening, uses little test substance and the results are obtained quickly with lower costs. There are fewer ethical problems in relation to studies conducted in animal or humans. The ethical aspect causes large public concern and animal and human studies should be used only when it is necessary. The disadvantage of *in vitro* screening is that it does not account for gastrointestinal bioavailability and metabolism of the substances.

In vivo studies using animal models are appropriate as they mostly involve similar molecular targets or pathways as humans, and hence such studies are valid for testing drugs. Mice are generally used and there are several genetic models available, including both natural and genetic generated mutations. The mouse also represents a model to identify gene regulation to determine the mechanism of action. *In vivo* tests are typically done only once and largely because of the expense, large quantities of the drug needed and time involved in conducting the studies, so reproducibility is unknown. "Nine out of ten experimental drugs fail in clinical studies because it cannot accurately predict how they will behave in people based on laboratory and animal studies" (Shanks *et al.*, 2009).

Traditionally, rats are another model of choice when performing scientific studies. However, the rat model has a number of limitations which makes extrapolation to humans really questionable, including a significantly different energy and food intake, a different lifespan and body proportion, differences in intestinal morphology and enteric microbiota, as well as other distinct physiological differences (Garcia and Diaz-Castro, 2013). Despite their shortcomings rodent studies illustrating safety and efficacy are needed for ethical approval of human trials which are extremely expensive and tightly regulated.

Although *in vitro* assessment of plant extracts and compounds provides much information regarding the effect(s) that these substances have at a cellular or molecular level, it is not fully representative of the *in vivo* situation. *In vitro*, digestion, absorption and the effect of metabolites derived from the substances tested is not represented and therefore *in vivo* confirmation of *in vitro* findings must be performed. Diabetic animal models commonly used for diabetes research include those induced by pharmacological agents, diet, surgical or genetic manipulations in several animal species. Most of the *in vivo* diabetes experiments are carried out on rodents, although some studies are still performed in larger animals (Frode and Medeiros, 2008; Meiton, 2006). Pancreatectomy in dogs is the most classical model employed by Banting and Best (Bliss, 2000).

In the field of ethnopharmacology, the majority of studies published between 1996 and 2006 employed the use of STZ (69%) and alloxan (31%) as diabetes inducing agents in rodent models (Frode and Medeiros, 2008). These models are by far the most frequently used for screening compounds, including natural products for their antihyperglycaemic activity and give additional information about *in vivo* bioactivity such as their insulinomimetic or insulinotropic activity (Srinivasan *et al.*, 2007). The dose of these agents required for inducing diabetes depends on the animal species, route of administration and nutritional status and can be administered intravenously, intraperitoneally or subcutaneously (Frode and Medeiros, 2008). Although both STZ and alloxan target and destroy the β-cells in a dose-related manner caused by the generation of intracellular reactive oxygen species (ROS), the mechanisms are different (Szkudelski, 2001). For this study, STZ was selected based on its better stability in solution and more predictable induction of hyperglycaemia. STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose), first demonstrated its use as a diabetogenic agent in dogs and rats in 1963, by specifically targeting pancreatic β-cells by entering the cells via GLUT-2 glucose transporters expressed on the surface of β-cells, but not by other islet cells (Srinivasan *et al.*, 2007). Inside the cell, STZ causes alkylation of deoxyribonucleic acid (DNA) and induces activation of poly adenosine diphosphate ribosylation, nitric oxide release, depletion of ATP and β-cell death by necrosis (Mythili *et al.*, 2004; Szkudelski, 2001). A possible problem encountered with using STZ is that its toxic effects are not restricted to the pancreas only, but leads to probable renal and hepatic injury, oxidative stress, inflammation and endothelial dysfunction (Lei *et al.*, 2005).

There are many effective classes of hypoglycaemic drugs currently in use to treat T2D. These include biguanides, sulphonylureas and thiozolidinediones. Unfortunately many of these drugs have potentially serious side effects and lose their effectiveness in the long run. In most cases, T2D patients on oral hypoglycaemic drugs, due to progressive β-cell failure, eventually revert to insulin injections to manage hyperglycaemia (UK Prospective Diabetes Study Group, 1995). Hence, there is an intensive search for new drugs without the side effects going on in several laboratories around the world that is concentrating on protecting against β-cell dysfunction or delaying the progressive loss of β-cell mass.

There are numerous uses of *S. pinnata* as an ethnobotanical agent that include both anecdotal and some scientific evidence of antidiabetic activity. Muthaura *et al.*, (2007) noted a low cytotoxicity of methanol and water extracts of *S. pinnata* to Vero E6 cells but no toxicity was observed in *in vivo* mice tests.

This chapter assesses the *in vitro* and *in vivo* antidiabetic activity of various extracts and semi purified fractions of *S. pinnata* in C2C12 muscle and Chang cells. Extracts/fractions exhibiting significant glucose uptake was further screened using radiolabelled [^{3}H]-2-deoxy-D-glucose experiments.

3.2 Materials & methods

3.2.1 *In vitro* Screening

3.2.1.1 Culturing and sub-culturing of cell lines

C2C12 (ATTC Cat. No. CRL 1722) and Chang cells (ATTC Cat. No. CCL-2) were seeded at 2,000 – 3,000 cells/cm^2 into 25 cm^2 tissue culture (TC) flasks with Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD, USA) supplemented with 10% FCS (Lonza, Walkersville, MD, USA), penicillin (100 IU) and streptomycin (100 µg/mL) (Sigma-Aldrich, St Louis, MO, USA), and incubated at 37°C in humidified air with 5% CO_2 for 3 to 4 days.

3.2.1.2 Seeding 96-well plates and cell transformation/differentiation

C2C12 cells were seeded into 96-well plates (5,000 cells/well) with DMEM supplemented with 10% FCS and penicillin (100 IU) and streptomycin (100 µg/mL) incubated at 37ºC with 5% CO_2 for 3 to 4 days. Once confluent, the 10% FCS in the DMEM was substituted by 2% horse serum (Sigma-Aldrich, St Louis, MO, USA) (differentiation/maturation media), and cultured further for 2 days at 37°C in humidified air with 5% CO_2 to induce myocytic differentiation and the formation of myotubules.

Chang cells were seeded into a 96-well plate (6,000 cells/well) and incubated at 37°C in humidified air with 5% CO_2 with DMEM media supplemented with 10% FCS, penicillin (100 IU) and streptomycin (100 µg/mL) for 2 days. On day 3, the cells were pre-sensitized with plant extract or metformin. The incubation was continued to day 5 when glucose uptake experiments were performed.

3.2.2 Radio-labelled glucose studies

Glucose uptake experiments are commonly used to measure cellular metabolic activity and glucose transport. A common technique of estimating glucose uptake is the use of radiolabelled [^{3}H]-2-deoxy-D-glucose, a glucose analogue. Once 2-deoxy-D-glucose has been taken up by cells, it is phosphorylated and cannot be metabolized further as it is trapped in the cell (Tanti *et al*., 2001).

For glucose uptake, differentiated C2C12 myoblasts were exposed to the plant extracts or fractions at a concentration of 0.05 µg/µL in fresh culture media containing 8 mM glucose. Modified DMEM with 8 mM glucose and 0.1% BSA (Sigma-Aldrich, St Louis, MO, USA) was added to each well and according to the protocol; either insulin (Sigma-Aldrich, St Louis, MO, USA) or extract was added. Plates were incubated for 1.5 hrs at 37°C in humidified air with 5% CO_2. Media was removed and the glucose concentration determined by a glucose-oxidase method (Biovision, Mountain View, CA, USA) and optical absorbance read at OD 570 nm using a 96-well plate reader.

Chang cells were pre-exposed to 0.0625 µg/µL of extract for 2 days after which cells were acutely exposed to the fractions at a concentration of 0.05 µg/µL in fresh culture DMEM containing 8 mM glucose. The glucose remaining in the media after 3 hrs incubation was determined using the Biovision colorimetric glucose oxidase assay.

Data was expressed as mmol/L glucose taken up by the cells with the DMSO vehicle values used as a comparative control.

3.2.2.1 Glucose Uptake -[^{3}H]-2-deoxy-D-glucose method

Serum starved cells were cultured for 20 mins at 37°C in humidified air with 5% CO_2 in modified DMEM with 8 mM glucose and 0.1% BSA. All extracts were dried under vacuum and the dried organic extracts re-dissolved in DMSO before being diluted into working concentrations. Insulin or plant extract was added to the incubation medium.

After the 1 hr incubation, glucose uptake in C2C12 cells was determined by adding 0.5 µCi/mL [^{3}H]-2-deoxy-D-glucose (American Radiolabelled Chemicals, St Louis, MO, USA) in glucose- and serum-free DMEM containing the extract/compound for 15 mins. Liquid scintillation counting was used to quantify radioactive 2-deoxy-D-glucose uptake in counts per minute (CPM). Insulin (1µM) was included as a positive control and metformin (1µM)

as a diabetic drug control. DMSO (0.02% v/v) was used as vehicle control. The experiment was done in triplicate with two replicates per concentration per plate (n = 6). Labelling was stopped by washing cells with ice-cold PBS. Cells were dissolved in 0.2 M NaOH for 1 hr at 60°C, the lysate was then transferred to scintillation vials with scintillant (Ready gel Ultima Gold™, Merck, Whitehouse Station, NJ, USA) before counting using a Scintillation counter.

3.2.2.2 Calculation of results

The amount of [^{3}H]-2-deoxy-D-glucose taken up by cells was recorded as CPM. Protein concentrations were determined using the Bradford (Bio-Rad, Hercules, CA, USA) protein determination method. The average CPM and protein concentrations were used to calculate the fmol/mg per sample with the GraphPad Radioactivity Calculator (http://www.graphpad.com/quickcalcs/radcalcform.cfm). The activity percentage was calculated as per the following formula:

$$\text{Activity (\%)} = \frac{(\text{Sample X}) - (\text{Vehicle control})}{(\text{Insulin control}) - (\text{Vehicle control})} \times 100,$$

where 'X' represents the fmol/mg values of the replicate samples tested.

Statistical differences were analysed using one-way ANOVA with a Dunnet's post hoc test using GraphPad 5 software (Graphpad Software Inc, CA, USA).

3.2.2.3 Data analysis

Data generated was entered into Microsoft Excel and analysed by calculating the means, standard deviation and by graphical representation. Results are expressed as mean and SD of a minimum of three replicates. Statistical significance was calculated using repeated-measures ANOVA with a Dunnett's multi-comparison *post hoc* test (Graphpad Prism version 5.0). Statistical significance of $p \leq 0.05$ is indicated by *, $p \leq 0.01$, is indicated by ** and $p \leq 0.001$ is indicated by ***.

3.2.3 *In vivo* Screening

3.2.3.1 Ethical approval

The ethical approval for this *in vivo* study was granted by the ethics committee at the Medical Research Council (MRC) of South Africa (ECRA 11/03/A). The study was performed in accordance with the principles and guidelines of the South African Medical Research Council as outlined in Guidelines on Ethics for Medical Research: Use of Animals in Research and Training, 2004 (http://www.mrc.ac.za/ethics/ethicsbook3.pdf).

3.2.3.2 Diabetic (Streptozotocin) Wistar rat model

Adult male Wistar rats (200 - 250 g) were used throughout for the *in vivo* studies. Rats were fasted for 3 hrs but given drinking water *ad libitum* before receiving an intramuscular (gluteal muscle) injection with streptozotocin (STZ; Sigma-Aldrich, St Louis, MO, USA)**,** at a dose of 33 mg/kg. This dose was sufficient to reduce their insulin producing cell numbers and induce hyperglycaemia at levels typical of late stage T2D. Animals were then rested for 3 days for the diabetes to stabilize (dose results in non-fasting glucose values of 15 – 22 mmol/L). During this period the animals received food and water *ad lib*. After 72 hrs, blood samples were taken from the tail vein. Plasma glucose concentrations were determined by using a glucometer (Ascensia, Bayer Healthcare). Rats with a blood glucose level of > 15 mmol/L were considered diabetic and selected for the studies.

3.2.3.3 Gavage procedure

Diabetic rats were lightly anaesthetized by halothane (2% halothane in 1% oxygen) inhalation to facilitate stress-free state for handling, but still retaining the swallow-reflex. After baseline plasma glucose levels were measured, a teflon gavage catheter was placed into the stomach via the mouth and oesophagus. One (1) mL of diluted extract/compound was injected directly into the stomach followed by an additional 1 mL water to flush any remaining extract from the gavage catheter.

3.2.3.4 Testing of extracts

1. Rats (after 3 hr fast) were randomized into treatment and control groups.

2. The rats were lightly anaesthetized with halothane (2% halothane in 1% oxygen) so that the gag reflex was retained but deeply enough to prevent stress due to vagal over-stimulation during gavaging.
3. Dosage of the extract was calculated so that each rat received 5 mL/kg BW of 100 mg/mL aqueous (GF-1-17A) or organic (GF-1-14A) extract dissolved in water which equates to dose of 500 mg/kg BW. Control animals received 5 mL water.
4. A plasma glucose baseline value was estimated directly before administration of the test substance (plant extract or compound enriched fraction) and then hourly thereafter for a 6 hr period.

3.2.3.5 Statistical analysis

Results were entered into an Excel spreadsheet and statistically analysed using Graphpad Prism® version 5 software.

3.3 Results and Discussion

When *S. pinnata* was brought to the CSIR for validation of its antidiabetic properties, a pilot *in vivo* screen revealed that the aqueous extract did indeed demonstrate hypoglycaemic properties (refer to Figure 3.1). A large collection of plant material of *S. pinnata* was made into extracts using various solvents and selected and fractionated further to identify the active compound. All tabulated data for the graphs can be viewed in the Appendix.

3.3.1 Pilot study *in vivo* screening of extracts of *S. pinnata* for antidiabetic activity

An initial pilot study was undertaken to investigate the efficacy of the aqueous (GF-1-17A) and organic extract (GF-1-14A) of *S. pinnata* in lowering blood glucose levels in an experimental diabetic STZ rat model.

For this pilot study, both the extracts were dissolved in water (heated slightly to solubilize it) just before administration via oral gavage and the whole dose of extract was administered. Both extracts were therefore evaluated against water as the vehicle control.

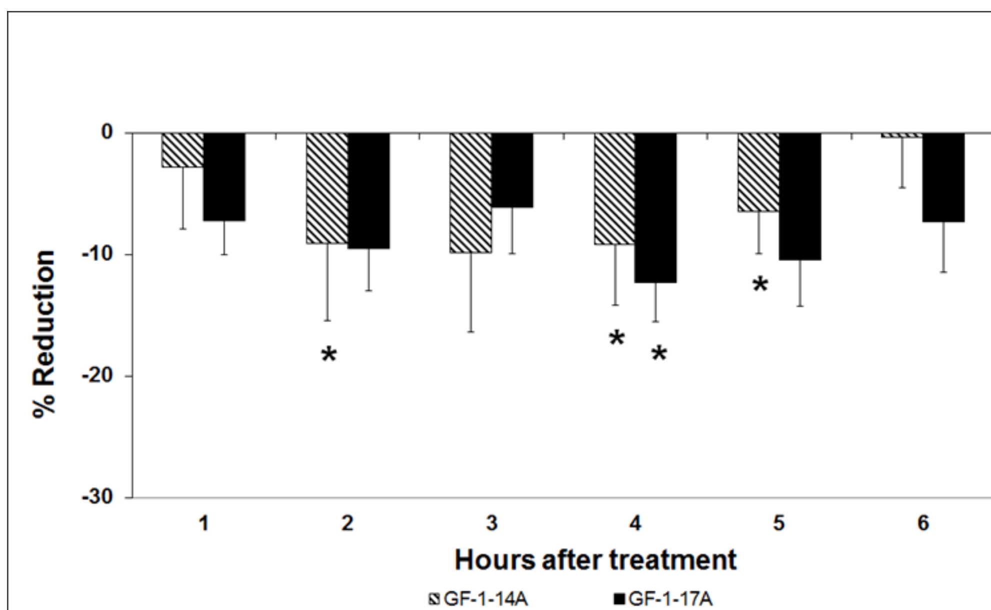

Figure 3.1 An initial pilot study showing a reduction in plasma glucose levels over a 6 hr period in STZ diabetic rats treated with aqueous (GF-1-17A) and organic (GF-1-14A) extracts of *S. pinnata* at a dose of 500 mg/kg. Results are expressed as the mean and SD of six rats (n = 6) per experimental group; * $p < 0.05$ ANOVA compared to placebo control (water).

The STZ rat model showed a significant reduction in plasma glucose in diabetic rats given 500 mg/kg of aqueous freeze dried extract (GF-1-17A) and the organic (GF-1-14A) extract. In comparison, the aqueous extract showed slightly better results than the organic extract. Hence, further work was carried out on the aqueous freeze dried extract to try and identify the active compound(s) responsible for lowering the glucose level. The extraction using water was also as per the traditional preparation of the tea given to patients by the traditional healer. However, seeing that aqueous extracts are not ideally the preferred extract of choice, a few other extracts were made to see which may be the most suitable to work with.

3.3.2 *In vitro* screening showing glucose uptake of aqueous and organic extracts of *S. pinnata*

Both the extracts were also screened *in vitro* using C2C12 muscle and Chang cells.

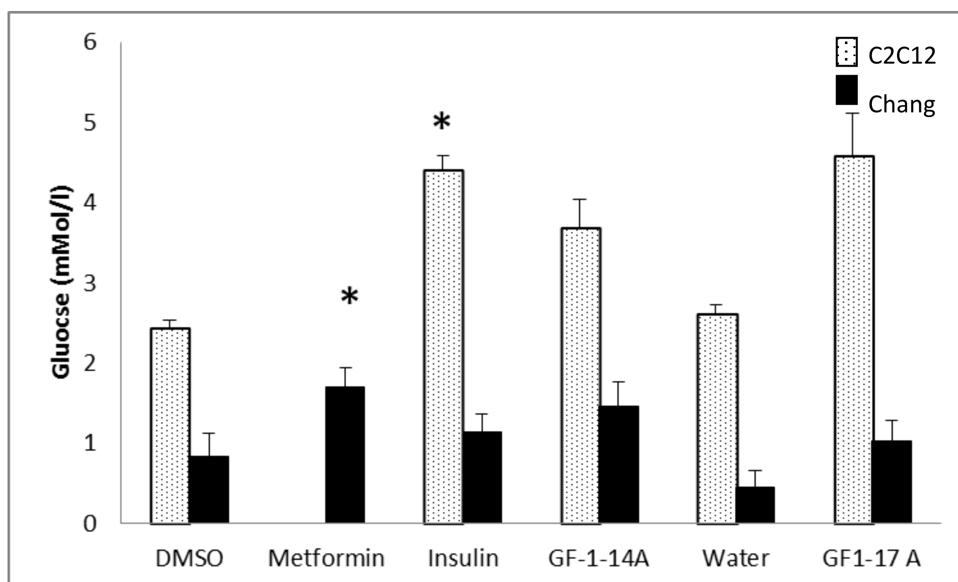

Figure 3.2 Glucose uptake activity of organic and aqueous extracts of *S. pinnata* in C2C12 cells and in Chang cells. Glucose uptake was calculated from the amount of glucose left in the media, determined by a glucose oxidase colourimetric assay. Results are expressed as the mean and SD of three independent experiments;* $p < 0.05$ compared to the vehicle control (water).

The results from Figure 3.2 indicate that both the aqueous and organic extracts increased glucose uptake from their respective vehicle (H_2O or DMSO) in C2C12 and Chang cells. However, the glucose lowering effect of the aqueous extract (GF1-17A) was more pronounced and comparable to that of the positive control (insulin) in C2C12 cells. In Chang cells, a similar trend was noted but the extracts were less effective than metformin which was the reference drug. The aqueous extract was slightly better at increasing glucose uptake from the media when screened on the C2C12 muscle cells. This was consistent with the initial *in vivo* pilot study.

The different extracts produced by freeze drying, spray drying and extraction using ethanol were screened *in vitro* using C2C12 muscle and Chang cells. In addition, the isolated compound 1 (BP16-94-15864H - refer to Chapter 2) was also screened. This was to see which extraction process produced the most effective extract in lowering glucose levels and if the isolated compound could compare to the activity of the extract. From the graph below

(Figure 3.3), the aqueous freeze dried extract (BP16-94-15868) and compound 1 (BP16-94-15864H) displayed the best antidiabetic activity.

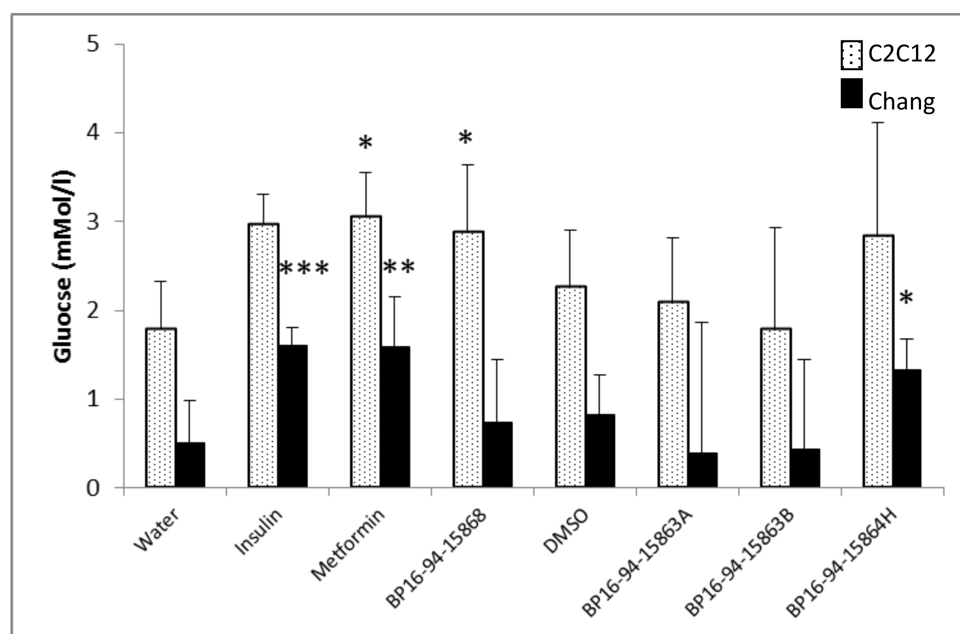

Figure 3.3 Glucose uptake of aqueous freeze dried extract (BP16-94-15868), spray dried extract (BP16-94-15863A), ethanol extract (BP16-94-15863B) and compound 1 (BP16-94-15864H) demonstrated in C2C12 muscle and Chang cells. Glucose uptake was calculated from the amount of glucose left in the media, determined by a glucose oxidase colourimetric assay. Results are expressed as the mean and SD of three independent experiments with two technical replicates per concentration ($n = 6$); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ ANOVA compared to vehicle control (water).

In C2C12 myocytes, the aqueous freeze dried extract significantly increased glucose uptake by 150%. Compound 1 significantly increased glucose uptake by almost 300% in Chang cells.

3.3.2.1 Bioassaying of fractions (BP16-94-15855 A-M)

The freeze dried extract was subjected to liquid/liquid partitioning with ethyl acetate. The ethyl acetate extract was fractionated using silica gel chromatography to yield 13 fractions. All fractions were screened in C2C12 muscle and Chang cells. Glucose uptake of C2C12 cells was measured following a 1 hr incubation with the relevant fractions, insulin and

metformin in serum and pyruvate free media containing 8 mM glucose. Glucose uptake was determined in Chang cells following a 3 hr acute glucose uptake assay with modified media containing 8 mM glucose. The glucose uptake was calculated by subtracting the amount of glucose left in the media of the wells containing the vehicle controls (water or DMSO), fractions, insulin and metformin minus the glucose concentration present in the media not exposed to cells (original glucose concentration).

The 13 fractions obtained after pooling similar fractions using silica gel column chromatography were screened *in vitro* using C2C12 muscle cells and Chang cells (Figure 3.4). The fractions that showed significant glucose lowering activity were BP16-94-15855A, BP16-94-15855B and BP16-94-15855G. These three fractions exhibited the same glucose lowering potential as the drug reference control metformin.

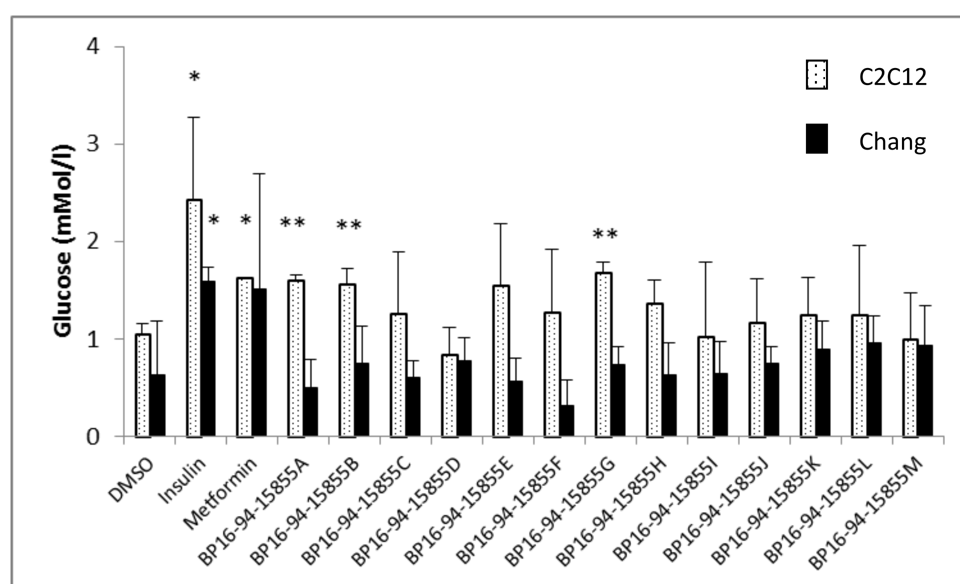

Figure 3.4 Effect of ethyl acetate extract fractions (BP16-94-15855A-M) on glucose uptake. Glucose uptake was calculated from the amount of glucose left in the media, determined by a glucose oxidase colourimetric assay. Results are expressed as the mean and SD of three independent experiments with two technical replicates per concentration (n = 6);* $p < 0.05$ and ** $p < 0.01$ ANOVA compared to vehicle control (water).

3.3.2.2 Dose concentration studies of aqueous freeze dried extract for antidiabetic activity

In addition to the above results, C2C12 cells were exposed to the freeze dried extract in serum-free media supplemented with 8 mM glucose for 1 hr at concentrations ranging from 50 µg/mL to 5 x 10^{-5} µg/mL. After the 1 hr incubation, glucose uptake in C2C12 cells was determined using pulse-labelling with [^{3}H]-2-deoxy-D-glucose in glucose- and serum-free media containing the aqueous freeze dried extract for 15 mins. The Chang cells were also exposed to the same concentration range between 50 µg/mL and 5 x 10^{-5} µg/mL of the aqueous freeze dried extract for 3 hrs in serum-free media supplemented with 8 mM glucose. Liquid scintillation counting was used to measure radioactive [^{3}H]-2-deoxy-D-glucose uptake in counts per minute (CPM). Insulin (1 µM) was included as a positive control and metformin (1 µM) as a diabetic drug control. The experiment was done in triplicate with two replicates per concentration per plate (n = 6).

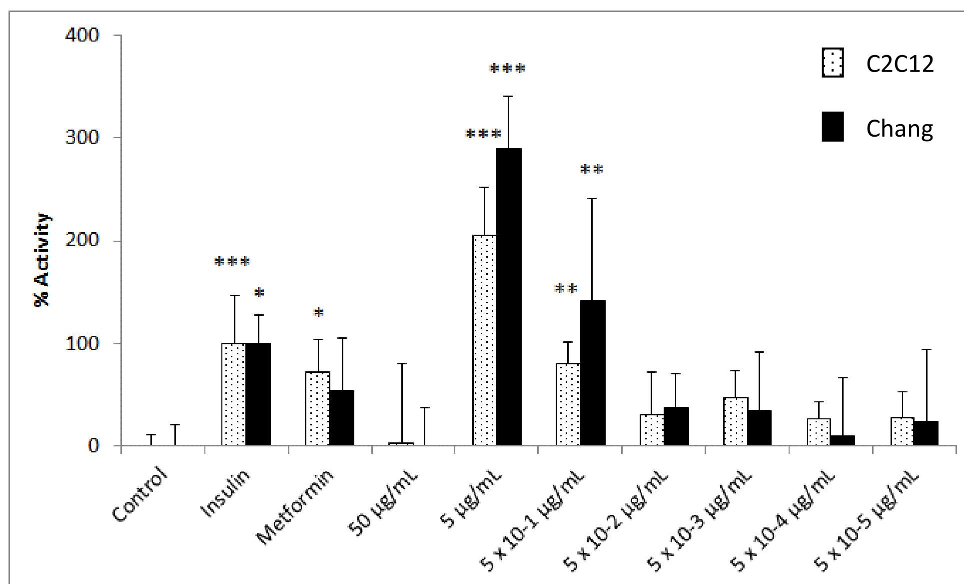

Figure 3.5 [^{3}H]-2-deoxy-D-glucose uptake in C2C12 myocytes and Chang cells of varying concentrations of the aqueous freeze dried extract (BP16-94-15868). Results are expressed as the mean and SD of three independent experiments with two technical replicates per concentration (n = 6) relative to the control set at 0% and insulin (positive control) at 100%; * $p < 0.05$, * $p < 0.01$ and *** $p < 0.001$ ANOVA compared to vehicle control (DMSO).

Figure 3.5 shows the relative glucose uptake activity in C2C12 and Chang cells of the aqueous freeze dried extract at different log doses over 1 and 3 hrs, respectively. Of the concentrations tested, 5 and 0.5 µg/mL showed a significant increase in glucose uptake when compared to the control (0% = basal glucose uptake). Maximal glucose stimulation was achieved at a concentration of 5 µg/mL in both cell lines, which was superior to both insulin (positive control set at 100%) and metformin, the reference control drug. At 50 µg/ml, the stimulatory effect was lost but as this effect was not as statistically different to the control, it was not regarded as having a cytotoxic effect.

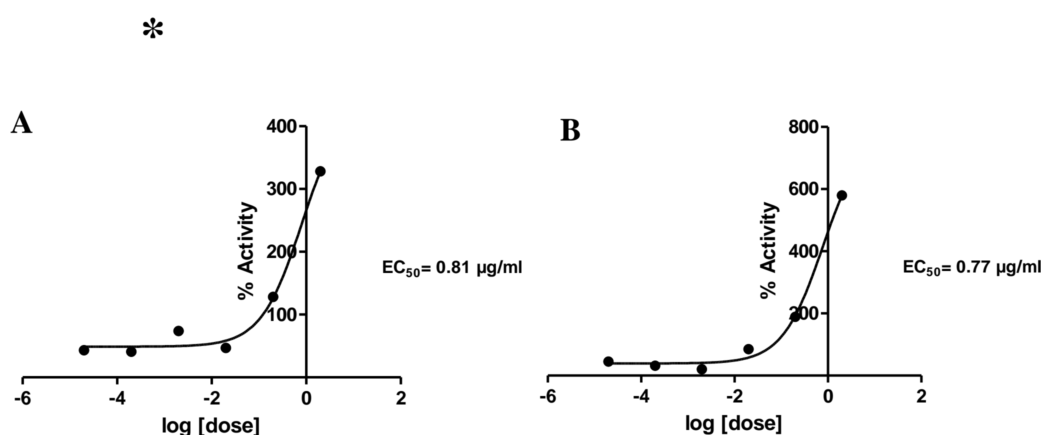

Figure 3.6 Estimated EC_{50} in (A) C2C12 myocytes; and (B) Chang cells of aqueous freeze dried extract (BP16-94-15868). EC_{50} values for glucose stimulated uptake in C2C12 and Chang cells were calculated from the aqueous freeze dried extract concentration study (Figure 3.5) using GraphPad vers. 5 Software.

The aqueous freeze dried extract (BP16-94-15868) effectively stimulated glucose uptake in a dose related manner in both C2C12 myocytes and Chang cells. Maximal glucose uptake stimulation was achieved at a concentration of 5 µg/mL. The EC_{50} was estimated from the peak value of 5 µg/mL to 0.00005 (5 x 10^{-5}) µg/mL. The EC_{50} was calculated at 0.81 µg/mL for C2C12 cells and 0.77 µg/mL for Chang cells.

3.3.2.3 Dose concentration studies of butanone/pentane extract for antidiabetic studies

C2C12 and Chang cells were exposed to the butanone/pentane extract in serum-free DMEM supplemented with 8 mM glucose for 1 hr at concentrations ranging from 50 µg/mL to 5 X

10^{-5} µg/mL. After 1 and 3 hrs, respectively, glucose uptake in C2C12 and Chang cells was determined by [^{3}H]-2-deoxy-D-glucose uptake. Liquid scintillation counting was used to measure radioactive [^{3}H]-2-deoxy-D-glucose uptake in counts per minute (CPM). Insulin (1 µM) was included as a positive control and metformin (1 µM) as a diabetic drug control. The experiment was done in triplicate with two replicates per concentration per plate (n = 6).

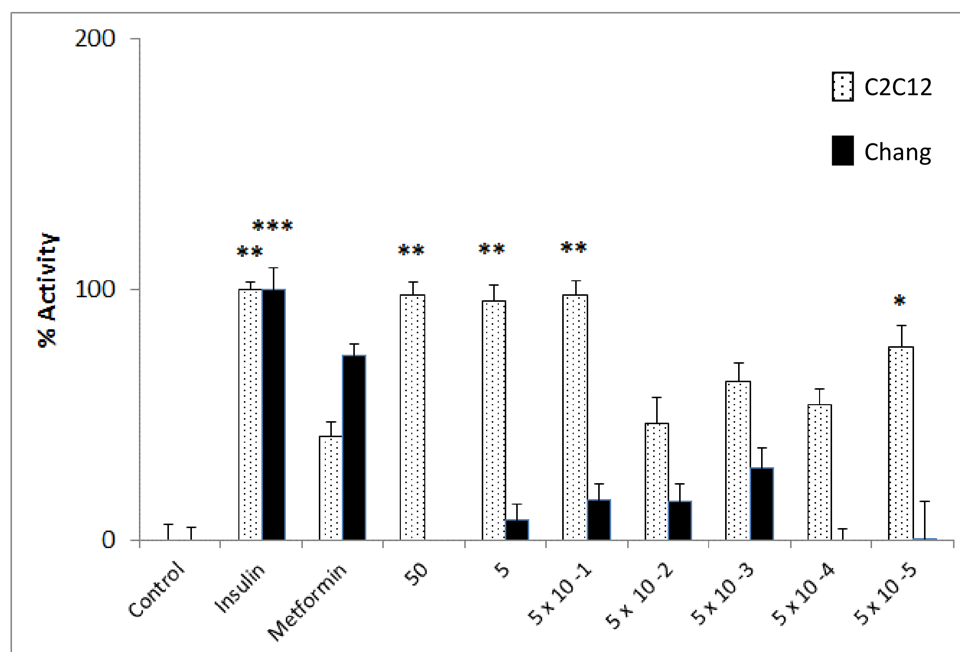

Figure 3.7 [^{3}H]-2-deoxy-D-glucose uptake by C2C12 myocytes and Chang cells of the butanone/pentane extract. Results are expressed as the mean and SD of three independent experiments with two technical replicates per concentration (n = 6) relative to the control set at 0% and insulin (positive control) at 100%;* $p < 0.05$, * $p < 0.01$ and *** $p < 0.001$ ANOVA compared to vehicle control (DMSO).

The dose concentration study investigating the efficacy of the butanone/pentane extract (Figure 3.7) showed a significant increase in the glucose uptake activity of the C2C12 cells at 50 µg/mL, 5 µg/mL and 5 X 10^{-1} µg/mL concentration points, respectively. The EC_{50} concentration was calculated to be 13.6 µg/mL. This activity was comparable to the action displayed by insulin and metformin. The extract did not significantly improve the glucose uptake activity of the Chang cells.

3.3.2.4 Dose concentration studies of compound 1

Compound **1** (BP16-94-15864H) was investigated for glucose uptake by C2C12 myocytes and Chang cells following exposure to decreasing log doses (20 μg/mL – 2 X 10^{-5} μg/mL) of the compound.

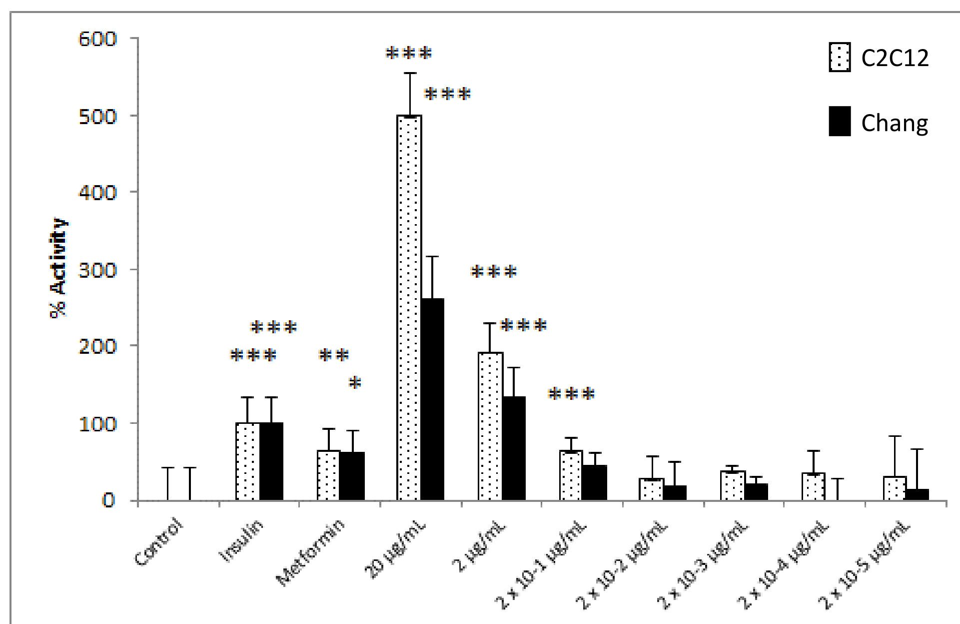

Figure 3.8 [^{3}H]-2-deoxy-D-glucose uptake in C2C12 myocytes and Chang cells of varying concentrations of compound 1. Results are expressed as the mean and SD of three independent experiments with two technical replicates per concentration (n = 6) relative to the control set at 0% and insulin (positive control) at 100%; * $p < 0.05$, * $p < 0.01$ and *** $p < 0.001$ ANOVA compared to vehicle control (DMSO).

The isolated compound 1 (BP16-94-15864H) exhibited high relative activity in both C2C12 myocytes and Chang cells, with concentrations of 20 μg/mL and 2 μg/mL, significantly stimulating glucose uptake in excess of 150% (Figure 3.8). As maximal glucose uptake stimulation was seen at the highest concentration of 20 μg/mL, an EC_{50} value for the active compound could not be calculated from these results.

3.3.3 *In vivo* screening of aqueous and organic extracts of *S. pinnata*

The STZ diabetic rat model was used for *in vivo* studies. The aim was to chemically (streptozotocin) induce an insulin deficient, mildly hyperglycaemic rat capable of maintaining glucose levels of between 15 – 22 mmol/L without insulin support. The rats maintained stable hyperglycaemia for several weeks without becoming ketoacidotic.

The best extracts that induced the greatest glucose lowering potential when screened *in vitro* were subjected to an *in vivo* study. This *in vivo* study was undertaken involving 33 rats to assess the effects of plasma glucose levels on various extracts/compound. There were four groups consisting of 10 rats each, except one group (group 3) that had only 3 rats. This was due to the limited quantity of the compound 1 enriched fraction available.

The four groups where the rats were given:

- GROUP 1: Rats (n = 10) received 5 mL/kg BW of 100 mg/mL aqueous freeze dried extract (BP16-94-15868) which equates to dose of 500 mg/kg.
- GROUP 2: Rats (n = 10) received 5 mL/kg BW of 100 mg/mL butanone/pentane extract (BP16-94-15875C and BP16=94-15880A) which equates to a dose of 500 mg/kg.
- GROUP 3: Rats (n = 3) received 1.67 mL/kg BW of 33.33 mg/mL compound 1 enriched fraction (BP-16-compound) which equates to a dose of 55.7 mg/kg.
- GROUP 4: the control group rats (n = 10) received the solvent vehicle i.e. water 5 mL/kg BW.

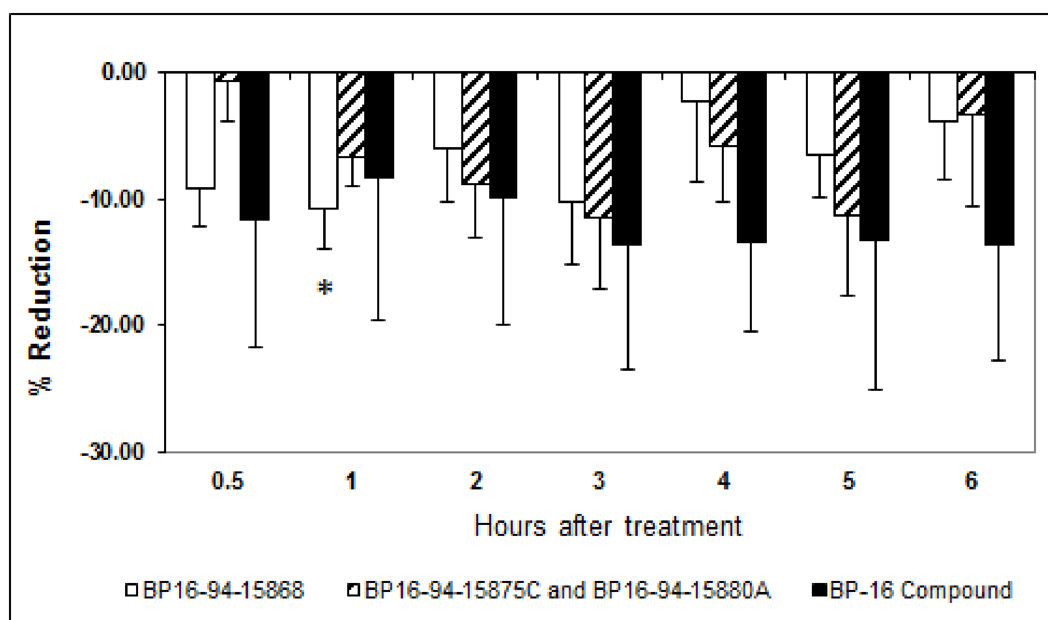

Figure 3.9 Reduction of blood glucose levels in STZ diabetic rats treated with aqueous freeze dried extract (BP16-94-15868; 500 mg/kg BW) was 15%, butanone/pentane extract (BP16-94-15875C and BP16=94-15880A; 500 mg/kg BW) and compound 1 enriched fraction (BP-16-compound; 55.7 mg/kg BW) was 15 – 20%, respectively. Results are normalised to the control (reduction in controls subtracted) and expressed as the mean and SD of six rats (n = 10) per experimental group for the extracts and three rats (n = 3) for the compound, respectively; * $p < 0.05$ ANOVA compared to placebo control (water).

Plasma glucose levels were measured in control and experimental rats over a 6 hr period. STZ treated diabetic rats showed a reduction in levels of blood glucose when treated with the extracts and the compound enriched fraction as compared to the control rats (Figure 3.9). Gavage administration of the aqueous freeze dried extract at 5 mL/kg showed a significant effect by reducing the plasma glucose level by 15%. The compound enriched fraction (BP-16-compound) was most effective over the 2 - 6 hr period at keeping the glucose levels reduced at around 15 – 20%. Statistically the STZ data was disappointing with only the 1 hr time point for the aqueous freeze dried extract being significantly reduced. Trends towards significance ($p < 0.1$) were demonstrated for the aqueous freeze dried extract at 30 mins and the butanone/pentane extract at 1 and 2 hrs using the t-test.

3.4 Conclusion

The isolated compound 1 (BP16-94-15864H) appears to be one of the major compounds or is the active compound in the extract responsible for lowering glucose activity. This can be concluded from the *in vivo* activity and the radio-labelled *in vitro* studies that show equal, if not better antidiabetic activity when the compound is screened together with the aqueous freeze dried extract and the compound enriched fraction. *In vitro,* the compound at the highest concentration tested induced greater glucose uptake than the positive controls, insulin and metformin.

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CHAPTER 4 - Gene and protein expression analysis in C2C12 muscle cells

4.1 Introduction

4.2 Materials & Methods

4.2.1 Culturing of C2C12 cells

4.2.2 Harvesting of cells for mRNA and protein extraction

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4.2.4 Reverse transcription of mRNA

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4.2.6 Protein extraction

4.2.7 Western blot analysis

4.2.8 Statistical analysis

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4.3.2 Western Blot

4.4 Conclusion

4.5 References

4.1 Introduction

Gene and protein expression studies are commonly employed to elucidate the mechanism of action that compounds such as phytochemicals have on cell signalling pathways, biological processes and function, is also referred to as expression profiling (Ulrich-Merzenich *et al.*, 2007). In expression profiling, the expression level of specific mRNAs involved with cell signalling or function in a given cell population are examined after exposure to test substances including plant extracts and compounds.

As described in Chapter 1, T2D results from a defective response to insulin by insulin responsive tissues such as muscle, adipose tissue and liver. This could be due to genetic and/or acquired factors. Insulin unresponsiveness causes dysfunctional regulation of glucose and lipid metabolism which, depending on the severity, can present itself as glucose intolerance, hyperglycaemia, dyslipidaemia and T2D. Apart from the portal system, skeletal muscle accounts for ~80% of the glucose removal from the peripheral circulation (Abdul-Ghani and Defronzo, 2010). Metabolically, muscle contraction consumes large amounts of energy which is mostly derived from glucose and therefore, apart from movement, it plays an essential part in ensuring glucose homeostasis (Hardie *et al.,* 2003). In this context, muscle cell lines such as C2C12 are often used to screen phytochemicals and extracts for antidiabetic activity. At a subcellular level, insulin controls a whole myriad of essential cellular functions including anabolic and catabolic metabolism of glucose. Activation of the insulin pathway is initiated the moment insulin binds to the extracellular α-subunit of an insulin receptor. The signal is perpetuated into the cytoplasm by autophosphorylation of the intracellular β-subunit of the receptor. Downstream of the receptor the PI3K/AKT pathway cascade is activated via IRS1/2 which culminates in the translocation of GLUT-4 to the cell surface and subsequent influx of glucose into the cell (Kahn *et al.,* 2005). In addition, insulin signalling via PI3K/AKT/mTOR pathway also affects the synthesis of various important proteins, including GLUT-1 and GLUT-4, thereby regulating glucose transport in response to insulin stimulation. The activation of the insulin pathway is reliant on the sequential phosphorylation of various signalling proteins which propagate the activation signal downstream in the insulin signalling pathway. Insulin resistance, a characteristic manifestation of T2D, is the result of dysfunctional insulin signalling at various levels. Decreases in the concentrations of receptor and/or effector signalling molecules, ineffective or altered kinase activity contributes either collectively or singularly to insulin resistance and T2D. Often these defects involve an attenuation of early signalling events from either the IR

itself or from IRS1/2 affecting the efficacy of downstream signalling, and in terms of glucose uptake in muscle, ultimately GLUT-4 translocation (Holmes *et al*., 2004). Therefore, the identification of phytochemicals that enhance insulin signalling or alternative non-insulin dependent signalling pathways involved in glucose utilization are regarded as potentially hypoglycaemic and possibly antidiabetic (Kahn *et al.,* 2005). Non-insulin dependent pathways such as the AMP-activated protein kinase (AMPK) pathway have been regarded as an important drug target, particularly for the alleviation of insulin resistance. Despite low concentrations in skeletal muscle, peroxisome proliferator-activated receptor-gamma (PPAR-gamma) PPAR-γ, a nuclear receptor, known to increase insulin sensitization is a further target for phytochemicals with hypoglycaemic activity (Verma *et al*., 2004). AMPK and PPAR-γ are pharmaceutical targets for the biguanides (metformin) and the thiazolidinediones (glitazones), respectively.

Further, apart from glucose transport across the plasma membrane, the rate whereby glucose is taken up into muscle and adipose cells is rate-limited by hexokinase, the enzyme responsible for the conversion of glucose to glucose-6-phosphate (G6P) in the first irreversible step of glycolysis (except in the liver where glucose-6-phosphatase releases the glucose) (Rothman *et al.,* 1992). As this step uses cellular ATP, hexokinase activity is auto-regulated by increased levels of G6P. Phosphofructokinase (PFK), the rate limiting enzyme for glycolysis, is allosterically activated by increased levels of AMP and inhibited by ATP. Together, these enzymes along with glycogen synthase which is allosterically activated by the substrate G6P, regulate glucose metabolism (glycolysis and glycogenesis), cellular energy consumption and thereby have a rate-limiting effect on glucose uptake. The amounts of these important enzymes are varied by the regulation of transcription in response to metabolic needs (Berg *et al.,* 2002).

Therefore, in terms of elucidating possible mechanism(s) whereby the extracts and compound could elicit their hypoglycaemic effects, quantitative real time polymerase chain reaction (qRT PCR) using Taqman® probes relevant to genes involved with glucose metabolism, have been applied in this chapter. In addition, Western blot analysis was used to study the protein expression of AMPK and GLUT-4 which are key proteins involved with glucose uptake.

4.2 Materials & methods

4.2.1 Culturing of C2C12 cells

C2C12 cells were cultured at 37ºC in humidified air with 5% CO_2 in DMEM growth medium (Lonza, Walkersville, MD, USA) with 10% (v/v) foetal bovine serum (Lonza, Walkersville, MD, USA). C2C12 murine skeletal muscle cells (cat no. CRL 87 1722, American Type Culture Collection, Manassas, VA, USA) were seeded at a density of 75,000 cells per well into 6-well CellBIND® culture plates (Corning Inc., Lowell, MA, USA) and cultured for 3 days until 80 - 90% confluent. The C2C12 cells were allowed to differentiate into myotubules for a further 2 days in DMEM supplemented with 2% (v/v) horse serum (Sigma-Aldrich, St Louis, MO, USA). Differentiated C2C12 myotubules were exposed to different treatments (as listed below) in bicarbonate-buffered DMEM (without pyruvate) and phenol red containing 4.5 g/L glucose for 3 hrs, at the respective optimal concentrations for glucose uptake (Chapter 3).

Treatment 1: 0.1% (v/v) DMSO (vehicle control)

Treatment 2: 1 µM Insulin

Treatment 3: aqueous freeze dried extract (BP16-94-15868) (5 µg/mL)

Treatment 4: butanone/pentane extract (BP16-94-15880A) (20 µg/mL)

Treatment 5: butanone/pentane extract (BP16-94-15880A) (2 µg/mL)

Treatment 6: compound 1 enriched fraction (BP16-94-15887) (20 µg/mL)

Treatment 7: compound 1 enriched fraction (BP16-94-15887) (2 µg/mL)

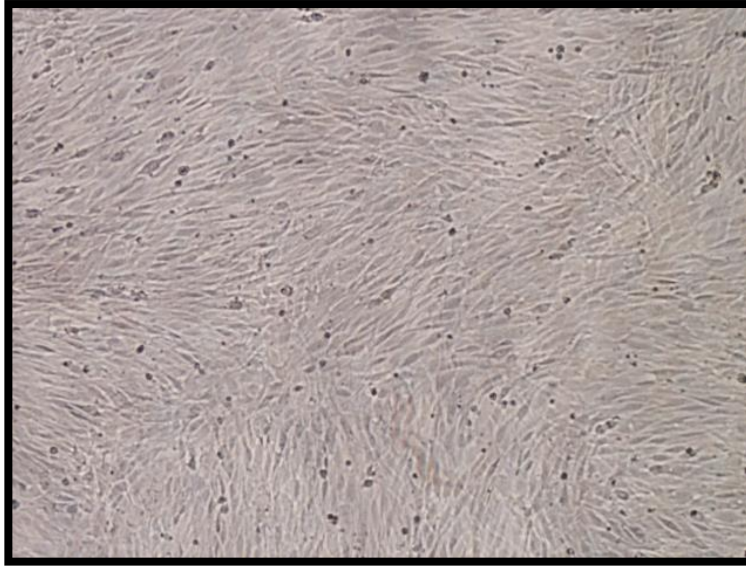

Figure 4.1 C2C12 myotubules in culture. A photomicrograph demonstrating early stages of myotubule formation in a confluent C2C12 cell culture (X100 original magnification).

4.2.2 Harvesting of cells for mRNA and protein extraction

C2C12 skeletal muscle cells for mRNA and protein extraction were prepared by seeding 6-well plates (CellBIND®) and exposing 3 adjacent wells per treatment for mRNA and the whole plate (6 wells) for protein extraction. For mRNA extraction, the media was aspirated and the cells washed by adding 3 mL PBS (Phosphate buffered saline) to each well. After aspiration of the PBS, 300 µL QIAzol® lysis reagent (Qiagen®, Hilden, Germany) was added per well and the adherent cells scraped from the well surface using a plastic cell scraper (Techno Plastic Products AG, Trasadingen, Switzerland). The pooled lysates from 3 wells per treatment were transferred to respective 2 mL Eppendorf microfuge tubes and stored at -20°C until further analysis for mRNA.

For protein extraction, the wells were rinsed with pre-warmed PBS, before adding 300 µL of lysis buffer (50 mM Tris pH 7.5, 1 mM DTT, 50 mM NaF, 100 µM Na_3VO4, 1% NP40, 1% Triton X114, 10 U DNase, 25 µg/mL RNase, 1 mM PMSF) and protease and phosphatase inhibitor tablets (all from Roche Applied Science, Basel, Switzerland) were added and cells were dislodged using a cell scraper. The pooled lysates of one 6-well plate per treatment were transferred to respective 2 mL Eppendorf microfuge tubes and stored at -20°C for further protein analysis.

4.2.3 Total RNA extraction

Cell lysates were homogenised in a TissueLyser (Qiagen®) at 25 Hz for 2 mins, centrifuged at 4°C and relative centrifugal force (RCF) of 12,000 g for 15 mins. Supernatant fractions were transferred to clean 2 mL microfuge tubes. Thereafter, 200 µL chloroform (Sigma-Aldrich) was added, samples were shaken vigorously for 15 sec, and then incubated at room temperature for 3 mins with occasional mixing. Samples were centrifuged at 12,000 g for 15 mins at 4°C and the upper aqueous phase was transferred to clean microfuge tubes. RNA was precipitated by addition of 500 µL of isopropanol and mixed well for 30 secs before storage at -20°C overnight. The following day, samples were subjected to centrifugation at 12,000 g for 20 mins at 4°C. Pellets were washed by adding 1 mL of 75 % (v/v) ethanol and centrifugation at 12,000 g for 15 mins at 4°C. The wash step was repeated. After the second wash, pellets were air dried for 30 mins. Excess liquid was removed by blotting tubes on paper towel occasionally during incubation. Pellets were resuspended in 100 µL of RNase-free water and incubated at 55°C for 10 mins. Thereafter, RNA was purified with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen®) and concentrations were determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Residual genomic DNA was removed from the total RNA with Turbo DNA-free DNase (Ambion® supplied by Life Technologies Corp. Carlsbad, CA, USA) by incubation at 37°C for 90 mins according to the manufacturer's instructions. Briefly, 20 µg of total RNA was incubated with 1.5 µL (3 units) of DNase, 5 µL of DNase buffer, and nuclease-free water in a final reaction volume of 50 µL for 45 mins at 37°C. Thereafter, another 3 units of DNase was added and incubated for a further 45 mins. DNase was inactivated with the addition of 10 µL of the DNase inactivation reagent. Reactions were incubated at room temperature for 2 mins, and spun at RCF 10,000 g for 90 secs. The supernatant was removed and RNA concentrations were again measured using the Nanodrop ND-1000 spectrophotometer. RNA samples with absorbance A_{260}:A_{280} ratio of more than 1.9 was used for further analysis. The quality of the DNase-treated RNA was determined with the RNA 6000 Nano kit using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

4.2.4 Reverse transcription of RNA

Total RNA was converted to complimentary DNA (cDNA) using the High Capacity Reverse Transcription kit (Applied Biosystems, California, USA) as recommended by the manufacturers. In brief, 1 μg of DNase-treated RNA was added to nuclease-free water in a volume of 10 μL. Thereafter, 2 μL reaction buffer, 0.8 μL dNTPs, 2 μL random primers, 1 μL RNase-inhibitor, 1 μL reverse transcriptase, and 3.2 μL nuclease-free water were added. The same reaction without the reverse transcription enzyme (minus RT reaction) was set-up to detect the possible presence of residual genomic DNA. For cDNA synthesis, reactions were incubated at 25°C for 10 mins and 37°C for 120 mins. In order to inactivate the reverse transcriptase, samples were incubated at 85°C for 5 secs. cDNA samples were stored at -20°C for subsequent use.

4.2.5 Quantitative Real-Time PCR, data collection and evaluation

The presence of genomic DNA was investigated by performing qRT-PCR with exon spanning β-actin (ActB) primers: forward primer, 5' CGTGCGTGACATCAAAGAGAA 3'; reverse primer, 5' GGCCATCTCCTGCTCGAA 3'. Briefly, undiluted cDNA (plus and minus RT reactions) was mixed with 12.5 μL of SYBR Green mix (Applied Biosystems), 1 μL of 10 μM ActB Forward Primer (400 nM), 1 μL of 10 μM ActB Reverse Primer (400 nM), and nuclease-free water to a final volume of 25 μL. After all the reagents had been added, the PCR tubes were briefly spun to ensure that all solutions were at the bottom of the tubes. The PCR reactions were conducted on a ABI 7500 Real Time PCR System (Applied Biosystems) using the following universal cycling conditions: 50°C for 2 mins and 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Data was collated. After the run, default settings for the threshold cycle (Ct) and baseline were used and Ct values were exported to Excel for analysis.

For gene analysis using expression probe assays, cDNA was diluted 1:5 times and mixed with 5 μL Taqman universal PCR master mix (Applied Biosystems), 1 μL gene-specific primer and probe mixtures (Taqman gene expression assays, Applied Biosystems), and nuclease-free water in a final volume of 10 μL. The Taqman assays that were used are listed in Table 4.1. PCR reactions were conducted on the ABI 7500 Real Time PCR system

(Applied Biosystems) using the universal cycling conditions as described above. All samples were run in duplicate and normalised to the endogenous control ActB (Table 4.1). Data generated by the ABI 7500 v1.4 software was imported into Microsoft Excel for analysis.

Table 4.1 Taqman gene expression assays used in the study

Gene Name	**Assay ID**
ActB	4352341E
Gapdh	4352339E
Glut -4	Mm01245502_m1
IRS1	Mm01278237_m1
IRS2	Mm03038438_m1
InsR	Mm01211881_m1
PI3K	Mm00803160_m1*
Pyruvate Kinase (PK)	Mm01176125_g1
Pyruvate Carboxylase (PC)	Mm00500992_m1*
Glycogen Synthase (GS)	Mm00472712_m1
Glucose-6-Phosphatase	Mm00839363_m1

[†]Validated gene expression probe assays were purchased from Applied Biosystems.

[‡]Assay number indicates the product number of the probe assay.

[*]Accession number indicates the Genbank number.

4.2.6 Protein extraction

For protein extraction, the pooled lysates were homogenised in the TissueLyser (Qiagen) at 25 Hz for 1 min, followed by cooling on ice for another 1 min. This step was repeated 3 times. The homogenate was centrifuged at 12,000 g for 10 mins at 4°C, the supernatant removed and the lysates precipitated with acetone. Briefly, a 3X volume of acetone was added to the protein lysate, mixed well and incubated at -20°C for 1 hr. Thereafter, proteins were recovered by centrifugation at 12,000 g for 10 mins at 4°C, and resuspended in 300 µL lysis buffer (extraction buffer (FNN011, Invitrogen) containing protease Inhibitor Cocktail

Tablet (Roche diagnostic)) and phenylmethyl sulfonyl fluoride according to manufacturer's instruction. The addition of PMSF and protease inhibiting cocktail prevent protein modification or proteolysis of the dissolved proteins.. Protein concentrations were determined using the RC DC assay (Biorad Laboratories, Hercules, CA, USA) after which proteins were stored at -20°C.

4.2.7 Western blot analysis

Prior to Western blot analysis proteins were denatured by heating the lysates to 95°C for 5 min. For Western blot analysis, 60 µg of heat denatured proteins were separated by SDS-PAGE using a 4% stacking gel over a 10% resolving gel and proteins were transferred to a onto a pre-wetted (methanol) PVDF-P membrane (Amersham Pharmacia) for 75 min at 160 V at -4°C by tank (wet) electrotransfer. Transfer efficiency was assessed by staining the membrane with Ponceau S (Sigma-Aldrich). Non-specific protein binding sites were blocked by incubating the membrane in 5% (w/v) low-fat milk powder in TBST (TBS, pH 7.2; 0.1% (w/v) Tween 20) at 4°C overnight. The following day the membranes were incubated with anti-rabbit monoclonal phospho-AMPKα (Thr172) (Cell Signalling, diluted 1:500), AMPKα (Cell Signalling, diluted 1:1000), GLUT-2 (Calbiochem, diluted 1:1000), GLUT-4 (Calbiochem, diluted 1:000) and Malonyl-CoA (Abcam, diluted 1:1000) at 4°C overnight. After washing in TBST, the membranes were incubated with horseradish peroxidise (HRP) labelled anti-rabbit IgG (Santa Cruz) diluted 1:4000, at room temperature for 1 hr. Membranes were washed and proteins were detected using chemiluminescent detection reagents (KPL laboratories, Gaithersburg, MD) as recommended by the manufacturers. Proteins were visualised using the Chemidoc-XRS (Biorad) and quantified using the Quantity One software (Biorad). To correct for protein loading and transfer efficiency, antibodies were stripped from membranes using a commercial kit (Pierce) and membranes reprobed with anti-rabbit polyclonal β-Tubulin (Cell Signalling, diluted 1:1000) and detected as described previously. The Cruz Marker (Santa Cruz) was used to determine protein sizes since the Cruz Marker has epitopes that will bind to the HRP-labelled secondary antibody.

Table 4.2 List of antibodies used in the study

Primary Antibody	Dilution	Size (kDa)	Company	Secondary Antibody
GLUT-4 mAb	1:1000	45	Cell Signalling	Anti-mouse
AKT (pan) (11E7) mAb	1:1000	60	Cell Signalling	Anti-rabbit
Phospho-AMPKα (Thr172 (40H9) Rabbit mAb	1:500	62	Cell Signalling	Anti-rabbit
AMPKα	1:500	62	Cell Signalling	Anti-rabbit
β -tubulin (c4) mAb	1:200	55	Cell Signalling	Anti-mouse

mAb, monoclonal antibody

pAb, polyclonal antibody

Table 4.3 Extracts and enriched fraction used for mRNA and protein studies

Extract no.	Extract type
BP16-94-15868	Freeze dried aqueous extract
BP16-94-15880A	Butanone/pentane extract
BP16-94-15887	Compound 1 enriched fraction

4.2.8 Statistical analysis

Statistical analysis of normalised gene and protein expression data was performed using the unpaired t-tests (GraphPad Prism version 5.02 Software, San Diego, California, USA). Statistical significance was indicated by a p value ≤ 0.05. Additionally, to compensate for the small number of experimental repeats ($n = 3$), a fold change cut-off of ≥ 1.5 was deemed to indicate relevance.

4.3 Results and Discussion

In an attempt to understand the mechanism(s) whereby the selected extracts elicit their glucose uptake enhancing activities at an *in vitro* level, mRNA and protein analysis were performed on C2C12 cells exposed to the extracts during culture. mRNA expression reflects

gene activation levels that precede protein synthesis. Western blot analysis was used to estimate the actual levels of specific proteins present in the cytoplasm of the C2C12 muscle cells. The Taqman® gene expression probes selected were for specific genes related to insulin signalling, the insulin sensitive glucose transporter GLUT-4, and key enzymes involved with intracellular glucose metabolism.

4.3.1 qRT PCR

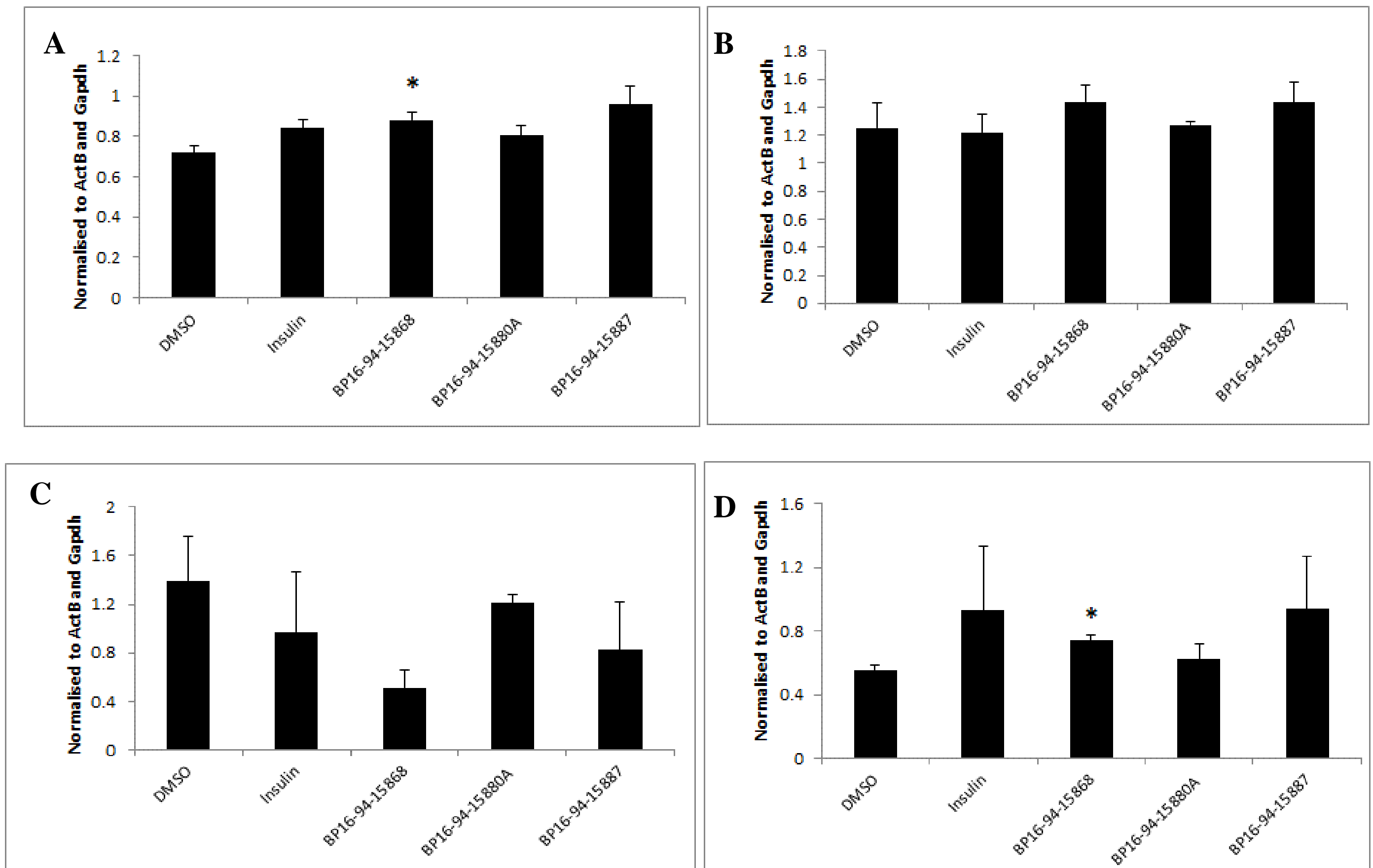

Figure 4.2 mRNA expression of insulin receptor (A), insulin receptor substrate 1 (B), PI3-kinase (C) and GLUT-4 (D) key effector proteins involved in insulin signalling and glucose uptake in C2C12 myocytes. mRNA expression has been normalised to the reference housekeeping genes beta actin (ActB) and glyceraldehyde 3-phosphate dehydrogenase (Graphpad). Results are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$ compared to DMSO vehicle control (unpaired t-test).

Relative to the vehicle control, (A) mRNA expression of the insulin receptor was increased by the aqueous freeze dried extract (BP16-94-15868) and albeit not significantly by the compound 1 enriched fraction (BP16-94-15887) (1.3-fold); (B) extracts had no effected on IRS1 mRNA expression, (C) PI3K expression appeared to be suppressed by the aqueous freeze dried extract ($p = 0.09$) and by a lesser extent the compound 1 enriched fraction and (D) the aqueous freeze dried extract significantly increased GLUT-4 expression while the compound 1 enriched fraction induced a 1.7-fold increase which was similar to that demonstrated for insulin, which is expected to increase GLUT-4 expression. Note: mRNA expression levels of IRS2 were below the detection levels of the method and are therefore not illustrated. Results are expressed as the mean ± SEM of three independent experiments.

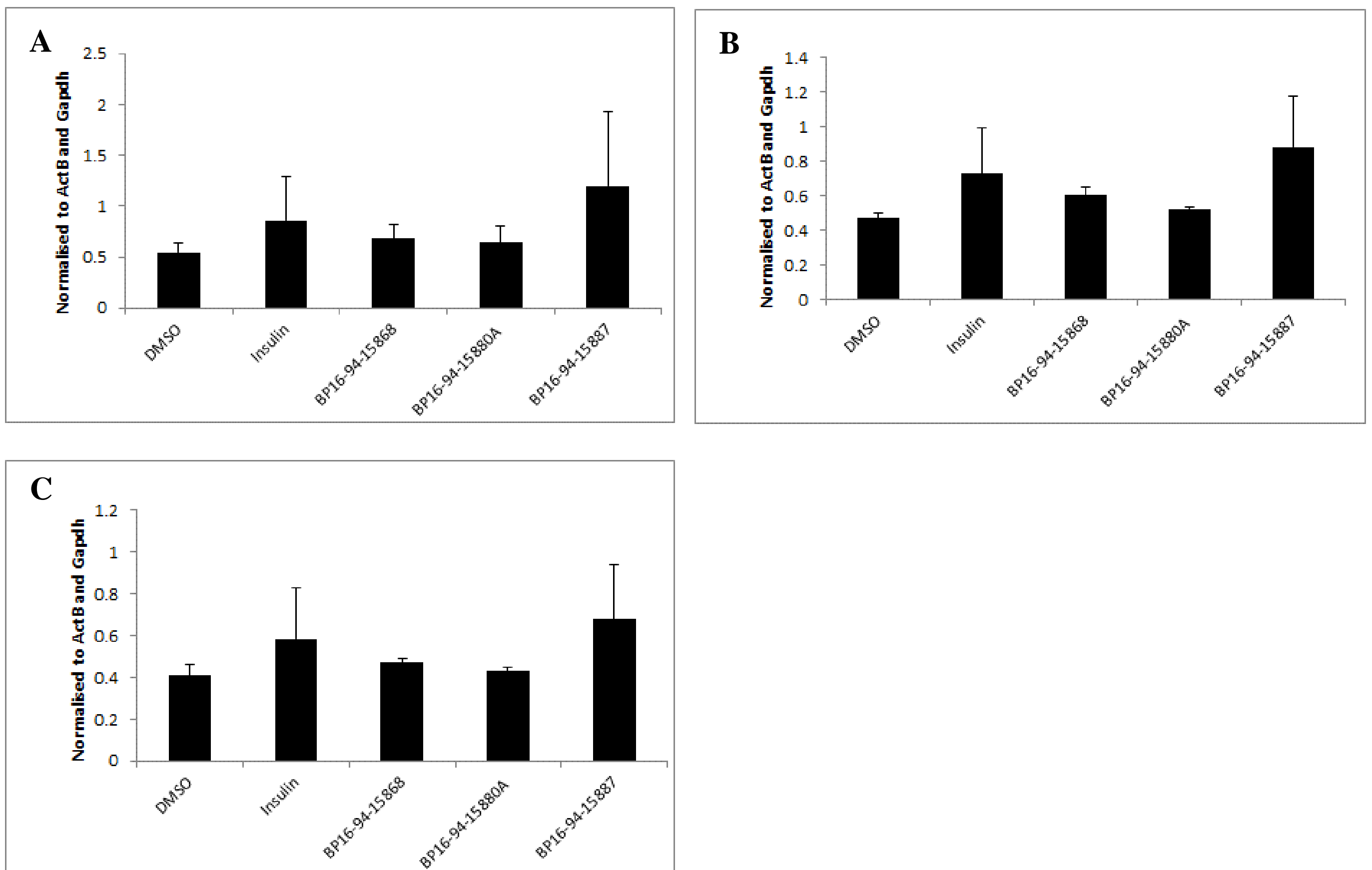

Figure 4.3 mRNA expression of enzymes glycogen synthase (A), pyruvate kinase (B) and pyruvate carboxylase (C) related to intracellular glucose metabolism in C2C12 myocytes. mRNA expression has been normalised to the reference housekeeping genes beta actin (ActB) and glyceraldehyde 3-phosphate dehydrogenase (Graphpad). Results are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$ compared to DMSO vehicle control (unpaired t-test).

Relative to the vehicle control, glycogen synthase expression (A) was substantially upregulated 2.2-fold by the compound 1 enriched fraction; the compound 1 enriched fraction also increased the expression of pyruvate kinase (B) 1.9-fold as well as pyruvate carboxylase expression (C) 1.7-fold which was similar to that by insulin. Note: mRNA expression levels of hexokinase were below the detection levels of the method and are therefore not illustrated.

4.3.2 Western Blot

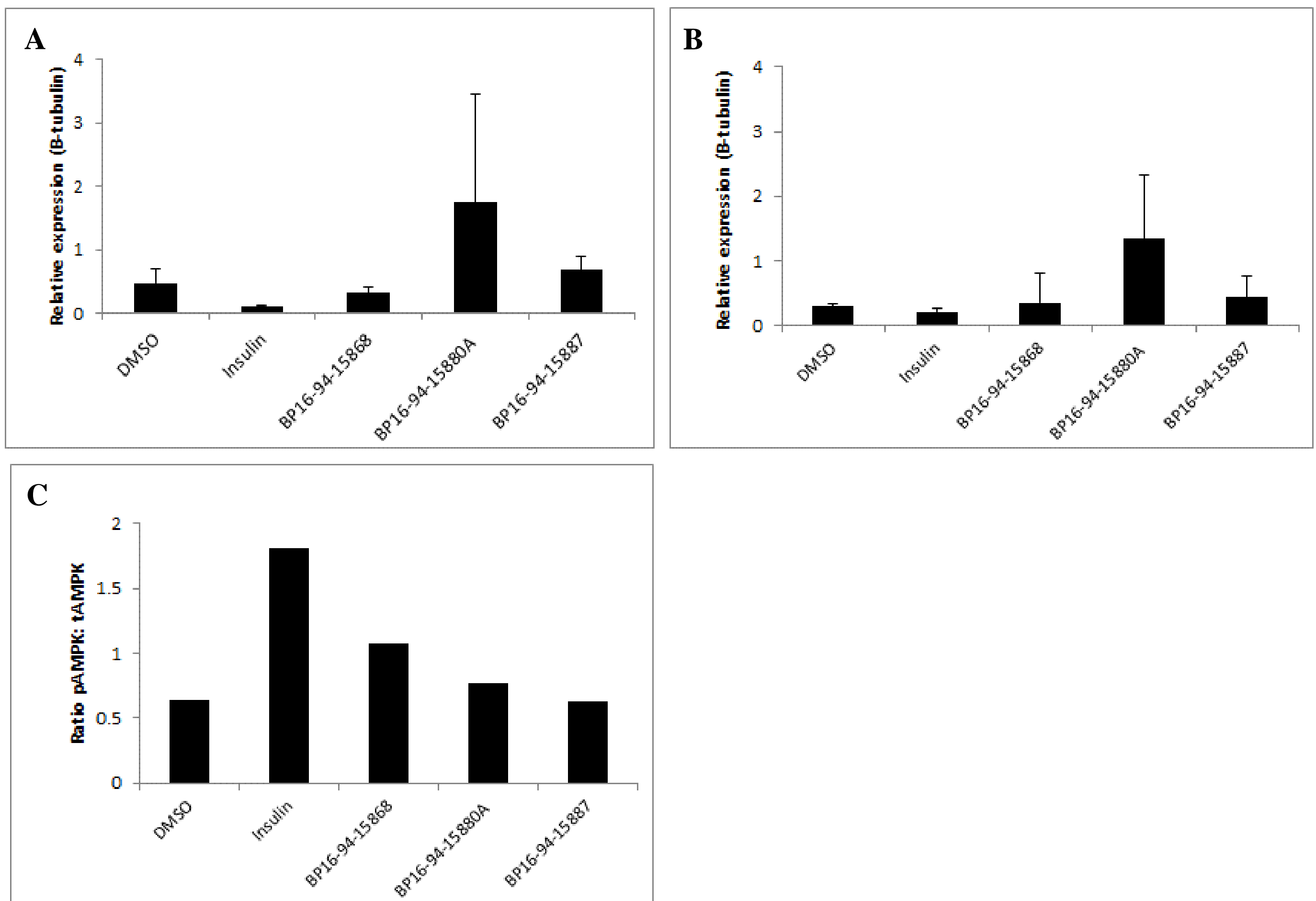

Figure 4.4 Protein content of total AMP-activated protein kinase (AMPK) (A), phosphorylated-AMPKα (Thr172) (B) and phospho-AMPK α: total AMPK ratio (C). Protein expression has been normalised to beta tubulin (B-tubulin). Results are expressed as the mean ± SEM of three independent experiments. The ratio phospho-AMPK α: total AMPK was calculated from their respective mean values.

Compared to the vehicle control, the butanone/pentane extract (BP16-94-15880A) increased both (A) tAMPK and (B) pAMPK protein content, relative to the β-tubulin protein content, by 6.4 and 4.4-fold respectively. Protein levels of GLUT-4 and AKT (pan) were too low to detect and quantify using standard Western blots.

An important alternative non-insulin dependent mechanism for regulating energy homeostasis exists which involves the activation of AMP-activated protein kinase (AMPK). AMPK is a major cellular regulator of lipid and glucose metabolism. AMPK increases the translocation of GLUT-4 to the plasma membrane during periods of fasting, hypoxia or contraction (exercise). In isolated rat skeletal muscles, metformin stimulates glucose uptake corresponding with AMPK activation (Zhou *et al*., 2001).

The butanone/pentane extract (BP16-94-15880A) increased the protein expression of both total AMPK and the activated phosphorylated form. Essentially, insulin signalling and AMPK activation are antagonistic. Insulin stimulation occurs at times of excess energy consuming anabolic processes, while AMPK which is activated in times of need, suppresses these processes and activates energy generating catabolic processes (Hutber *et al*., 1997). However, these opposing pathways do overlap and in the case of skeletal muscle culminate in increased glucose uptake via GLUT-4. Metabolically, the fate of glucose is different. Insulin stimulates glycogen synthesis via glycogen synthase while AMPK increases glucose oxidation (Towler and Hardie, 2007; Jorgenson and Rose, 2008).

In terms of the aqueous freeze dried extract (BP16-94-15868) and the compound 1 enriched fraction (BP16-94-15887), their glucose uptake enhancing effects are related to increased levels of GLUT-4 mRNA expression, independent of insulin. However, in contrast to the butanone/pentane extract (BP16-94-15880A), both total and activated AMPK protein levels were not affected by the aqueous freeze dried extract and the compound 1 enriched fraction. Further evidence upstream of involvement with the insulin signalling pathway is lacking. PI3K, a pivotal enzyme in insulin signalling, which together with AKT/PKB is responsible for the majority of insulin's metabolic actions including regulation of glucose catabolism (Yang *et al*., 2009) was either not affected or suppressed by the extracts. Contrary to expectations, PI3K mRNA expression was also suppressed by insulin. Insulin signalling is

however extremely complex. Prolonged or chronic exposure of muscle cells to insulin induces insulin resistance with reduced PI3K activation and signalling (Yang *et al*., 2012). In a similar way, chronic insulin exposure rapidly (within 24 hrs) reduces insulin receptor expression by up to 60% in C2C12 myotubules (Yang *et al*., 2012). In terms of our findings, a 3 hr exposure to insulin and the extracts only marginally increased insulin receptor mRNA expression and had no effect on IRS1 expression. This suggests that it is unlikely that the extracts are direct transcriptional agents for these targets. However, this does not provide us with an insight into the activation status of the IR, IRS1/PI3K pathway. For this, further protein studies are required.

At an intracellular metabolic level, the aqueous freeze dried extract and the compound 1 enriched fraction had similar effects to that of insulin on mRNA expression of key enzymes involved with glucose catabolism and anabolism. As glucose is the principle source of energy for skeletal muscle cells, its usage or storage based on energy demand is tightly controlled by several enzymes. Of these, the mRNA expression of three key enzymes which are pyruvate kinase (glycolytic enzyme that catalyses phosphoenolpyruvate to pyruvate), pyruvate carboxylase (carboxylates pyruvate to oxaloacetate) and glycogen synthase provides evidence of enhanced intracellular glucose utilization. In muscle, these enzymes regulate the rate of glycolysis, supplements intermediates to the citric acid cycle and synthesizes glycogen, a stored form of glucose, respectively. Collectively, these processes contribute to the clearance of glucose and its glycolytic pathway intermediates from the cytoplasm thereby increasing the uptake of glucose from the extracellular space via the glucose transporters including GLUT-4. Expression for mRNA of glycogen synthase, pyruvate kinase and pyruvate carboxylase were increased by ~1.5-fold by insulin. The compound 1 enriched fraction had similar but greater mRNA fold-increase effects than insulin.

4.4 Conclusion

Treated C2C12 cells with the aqueous freeze dried extract (BP16-94-15868) and the compound 1 enriched fraction (BP16-94-15887) showed a tendency that enhanced GLUT-4, glycogen synthase, pyruvate kinase and pyruvate carboxylase mRNA expression. Insulin had similar effects. In contrast, the butanone/pentane extract (BP16-94-15880A) greatly enhanced the protein expression of both total and activated AMPK. This implies that the

aqueous freeze dried extract (BP16-94-15868) and the compound 1 enriched fraction (BP16-94-15887) mechanistically have insulin mimetic effects while the butanone/pentane extract (BP16-94-15880A) affects AMPK protein expression which could affect cellular energy and metabolism, independently of insulin.

4.5 References

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CHAPTER 5 - Pharmacokinetic study

5.1 Introduction

5.2 Materials and Methods

5.2.1 *In vivo* PK evaluation

5.2.2 PK parameters and analytical method

5.3 Results and Discussion

5.4 Conclusion

5.5 References

5.1 Introduction

The use of *in vitro* studies to evaluate the effects on cell function and cell signalling is an important first phase to identify plant products such as extracts, extract fractions or isolated compounds that show significant activity. However, pharmacokinetics is essential as it relates to the determination of the bioavailability for administered therapeutic substances (Midha *et al.,* 2005). For any substance to be effective, it has to reach its target tissues or organs in sufficient quantities to elicit a therapeutic effect. *In vitro* experiments do not account for *in vivo* factors such as digestion, absorption, metabolism and excretion (ADME). A pharmacokinetic study (PK) is therefore essential to establish if the active compounds are absorbed into the circulation and establish parameters such as the maximum concentration of the substance in the serum (C_{max}) and the rate of absorption as measured over time (T_{max}) (Midha *et al.,* 2005). From this, other calculations can be made that include excretion or clearance rates (half-life) and thereby determine an effective dose.

A lack of PK studies seems to be the biggest hindrance towards the transformation of traditional/herbal products as there is no way to establish the bioequivalence between products prepared by modified method as compared to the traditional method (Hussain *et al*., 2011). PK of natural products is challenging due to the complexity of extracts with known and unknown compounds and the unavailability or inadequacy of standards and methods. PK studies can assist physicians in prescribing drugs safely and effectively to those patients who are consuming herbal products, because these plant extracts may work in a synergistic or antagonistic manner with other drugs, herb-drug interactions (Hussain *et al*., 2011).

Natural products such as herbal remedies are generally considered safe and advantageous to the human body. Certain natural products interact with drugs by upsetting the biological processes that regulate metabolism and elimination. A family of enzymes known as the cytochrome P450s (CYPs) are involved in drug metabolism (Dresser *et al*., 2000). One of the most important subtypes of CYPs is CYP3A4 that has been predicted to oxidize at least 60% of all drugs to metabolites (Bailey and Dresser, 2004). It is expressed in the small intestine and liver, hence, it is able to inactivate orally administered drugs before they even enter the systemic circulation, thereby, reducing the drug's bioavailability (Bailey and Dresser, 2004).

By changing the activity of CYP3A4, the bioavailability of drugs that are oxidized by this enzyme is also affected (Figure 5.1).

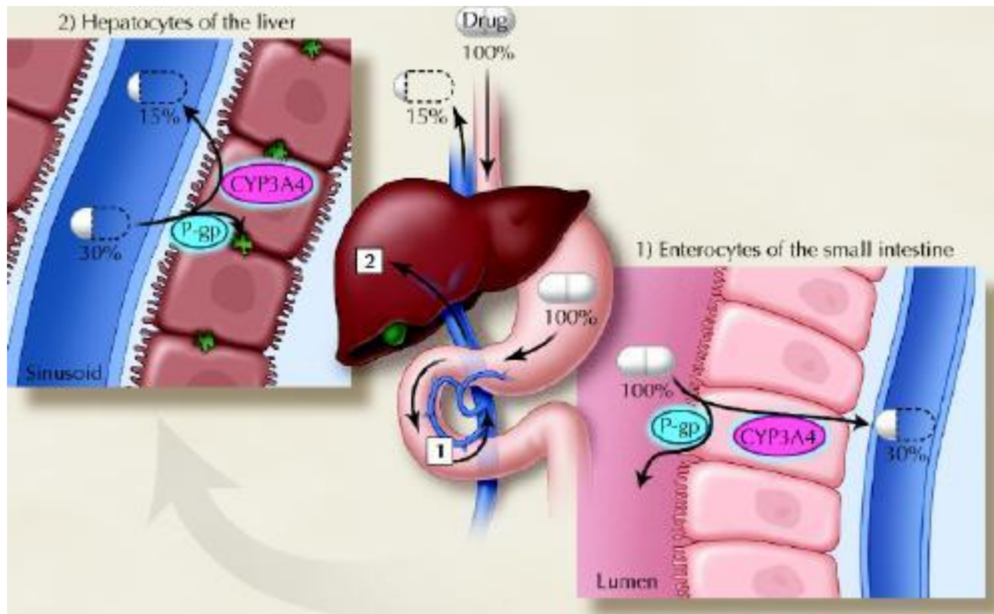

Figure 5.1 Chronological transport of a drug through metabolism by CYP3A4 and/or efflux by P-glycoprotein (P-gp) in enterocytes of the small intestine and then hepatocytes of the liver. The percentage of the initial drug dose that is available before and after passage through the gut wall and liver is presented. Although the drug is 100% absorbed from the gastrointestinal tract, its bioavailability is only 15% after oral administration (Bailey and Dresser, 2004).

One of the ways that a natural product can change the action of an enzyme is to inhibit its action. Maskalyk (2002) found that grapefruit can significantly reduce CYP3A4 activity in the small intestine, thus elevating the bioavailability of drugs metabolized by enteric CYP3A4 to potentially toxic levels. Another example is the use of St. John's wort which is a herb that is commonly used for the treatment of depression. However, this herb has the opposite effect whereby it increases the activity of CYP3A4, resulting in more oxidation of the drug causing a cascade reaction of a higher metabolism meaning lower bioavailability and hence a weakened clinical response. St. John's wort also increases the activity of the drug transporter P-glycoprotein (P-gp). P-glycoprotein, an efflux protein acts to push drugs back

into the intestinal lumen directly from the gut wall and indirectly from the liver via bile, thus reducing the bioavailability of orally administered drugs (Lin and Yamazaki, 2003). P-gp is also gaining significance in absorption enhancement due to its substrate selectivity and distribution at the site of drug absorption. The combined action of CYP3A4 and P-glycoprotein in the gut and liver can significantly reduce bioavailability and diminish effectiveness.

For this PK study, the focus was specifically directed at establishing the antidiabetic potential of *S. pinnata*.

5.2 Materials and Methods

5.2.1 In vivo PK evaluation

To estimate the bioavailability of the extracts and compound, plasma samples were obtained at various time intervals over a 6 hr period by bleeding the tail vein of the rat. The rats were subjected to two extracts and the compound enriched fraction which was screened for *in vitro* and *in vivo* antidiabetic efficacy.

- BP16-94-15868 – aqueous freeze dried extract of *S. pinnata* was administered orally at a dose of 500 mg/kg BW.
- BP16-94-15880A – butanone extract derived from liquid-liquid partitioning of the aqueous *S. pinnata* extract using butanone and pentane, administered orally at a dose of 500 mg/kg BW.
- BP16-94-15887 – compound 1 enriched fraction that was obtained from silica gel chromatography by pooling similar fractions that contained compound 1 as the major component when spotted using TLC. The fraction was administered orally at a dose of 55.7 mg/kg BW.
- BP16-94-15864H – The isolated compound 1 (identified in Chapter 2) which was used as a biomarker for the PK profiles.

The *in vivo* rat study and collecting of blood samples was conducted at MRC, Diabetes Platform. The extracts and compound were administered by gavage at a dose of 500 mg/kg and 55.7 mg/kg respectively to adult Wistar rats. Blood samples were collected at 1 hr intervals over a 6 hr period. The samples were centrifuged for 15 min to separate the serum from the blood cells and platelets. The serum was transferred into sterile Eppendorf tubes for

analysis. PK profiling of the serum was conducted at UCT, Pharmacology Department. Test compounds were subsequently extracted and levels were determined by LC-MS/MS.

5.2.2 PK parameters and analytical method

An AB Sciex AB 3200 Q trap LC mass spectrometer was used for the analysis. The mass spectrometer was coupled in tandem with a Shimdzu Prominence HPLC system. A Gemini C_{18} (50 mm x 2.1 mm, 5 µm) column was used. The column was kept at 40 °C in a column oven. Mobile Phase A comprised of 0.1% formic acid and 4 mM ammonium acetate in water. Mobile Phase B was acetonitrile. Gradient elution was from 5% B to 95% B over 4 min at which point the column was equilibrated to starting conditions for 7 mins prior to the next injection. Quantification was performed using multiple reaction monitoring (MRM) of the transitions *m/z* 361.0 --> 229.2, *m/z* 361.0 --> 183.2.

Standards of the biomarker which was a mixture of the two diasteromers were prepared in the range 5 – 10 000 ng/mL. Diastereomerism occurs when two or more compounds having the same molecular weight have different configurations at one or more of the equivalent (related) stereocenters, but are not mirror images of each other. 50 µL of standard and test samples were prepared and protein precipitated by addition of 150 µL of acetonitrile. Samples were vortexed, sonicated and centrifuged for 5 min at 13,000 RPM to remove the protein. 10 µL of the supernatant was injected onto the column. LLOQ (lower limit of quantitation) was 5 ng/mL. Standard curve was made up ranging from 0.005 ug/ml to 10 ug/ml.

5.3 Results and Discussion

Under the chromatographic conditions described above, compound 1 was eluted at 2.31 and its diastereomer at 2.35 min (Figure 5.2). No obvious interfering endogenous substances were observed at the retention time of compound 1 in the blank plasma sample.

Figure 5.2 Chromatogram of blank plasma – precipitated.

Preparation of a standard curve (Figure 5.3) involved dissolving 5 mg of compound 1 (BP16-94-15864H) to make up a standard solution and then plasma was spiked in the range of 5 – 10,000 ng/mL.

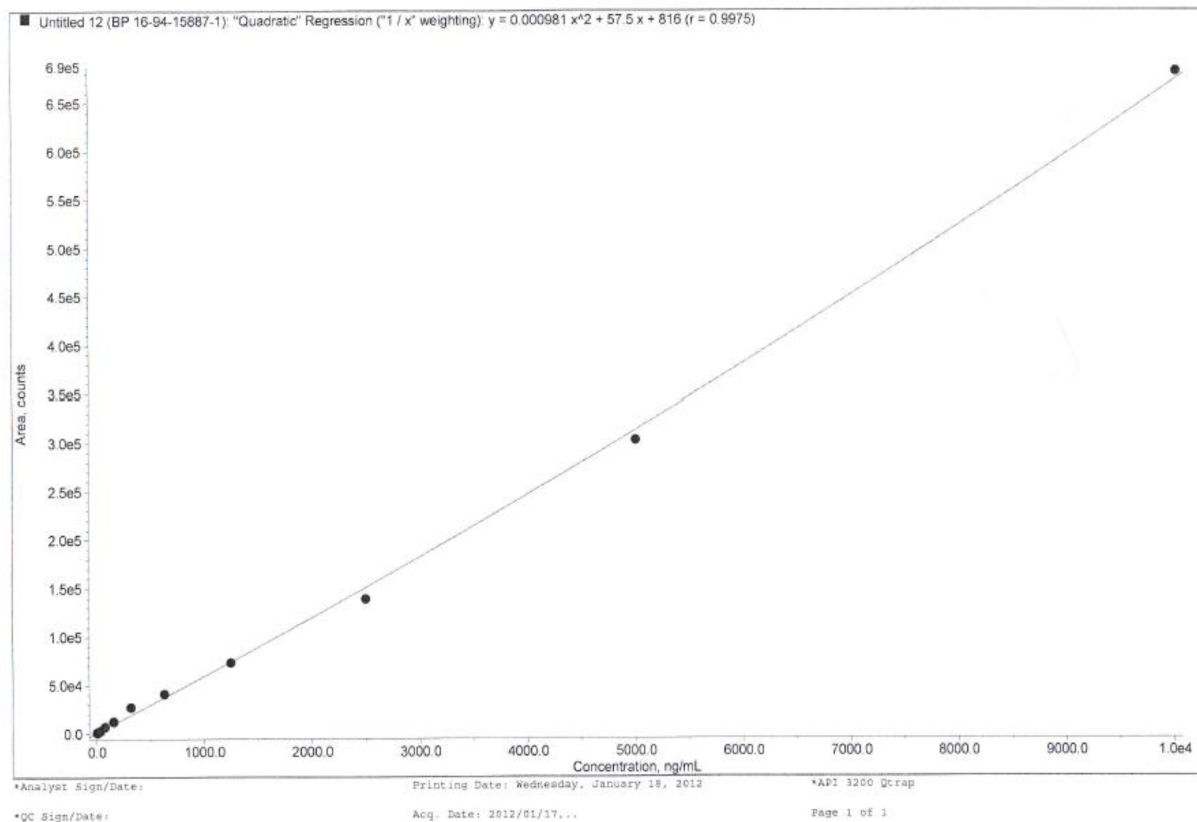

Figure 5.3 Standard curve of biomarker (compound 1 – BP16-94-15864H).

It could be clearly viewed from the chromatogram showing the 10 μg/ml standard in the plasma that compound 1 is a mix of two diastereomers (Figure 5.4) with the same molecular weight and being eluted at the same time.

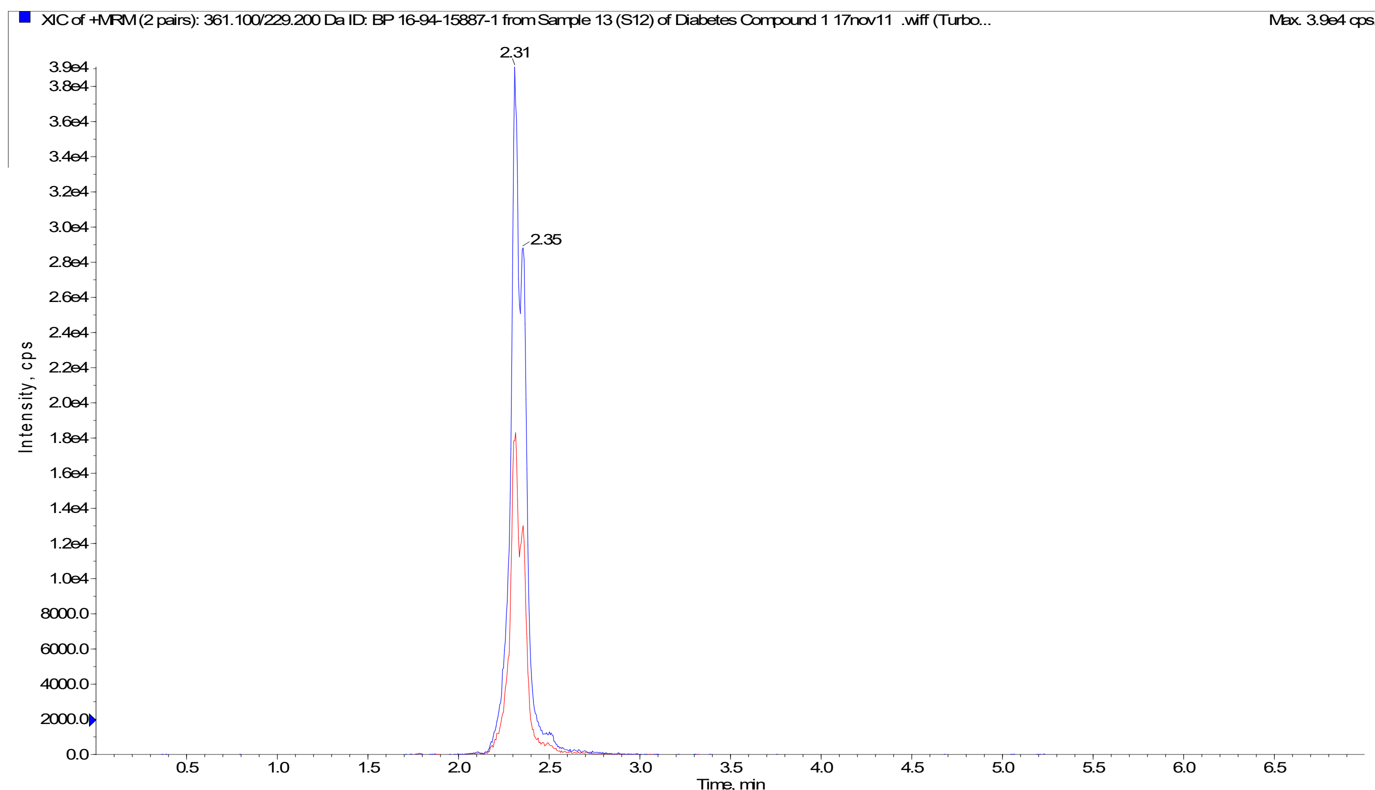

Figure 5.4 Chromatogram of standard 10 µg/ml in plasma – precipitated.

The MS fragmentation pattern (Figure 5.5) also confirmed the molecular weight of 361 with the following transitions:

fragment 1: 361 - 229.2

fragment 2: 361 - 183.2

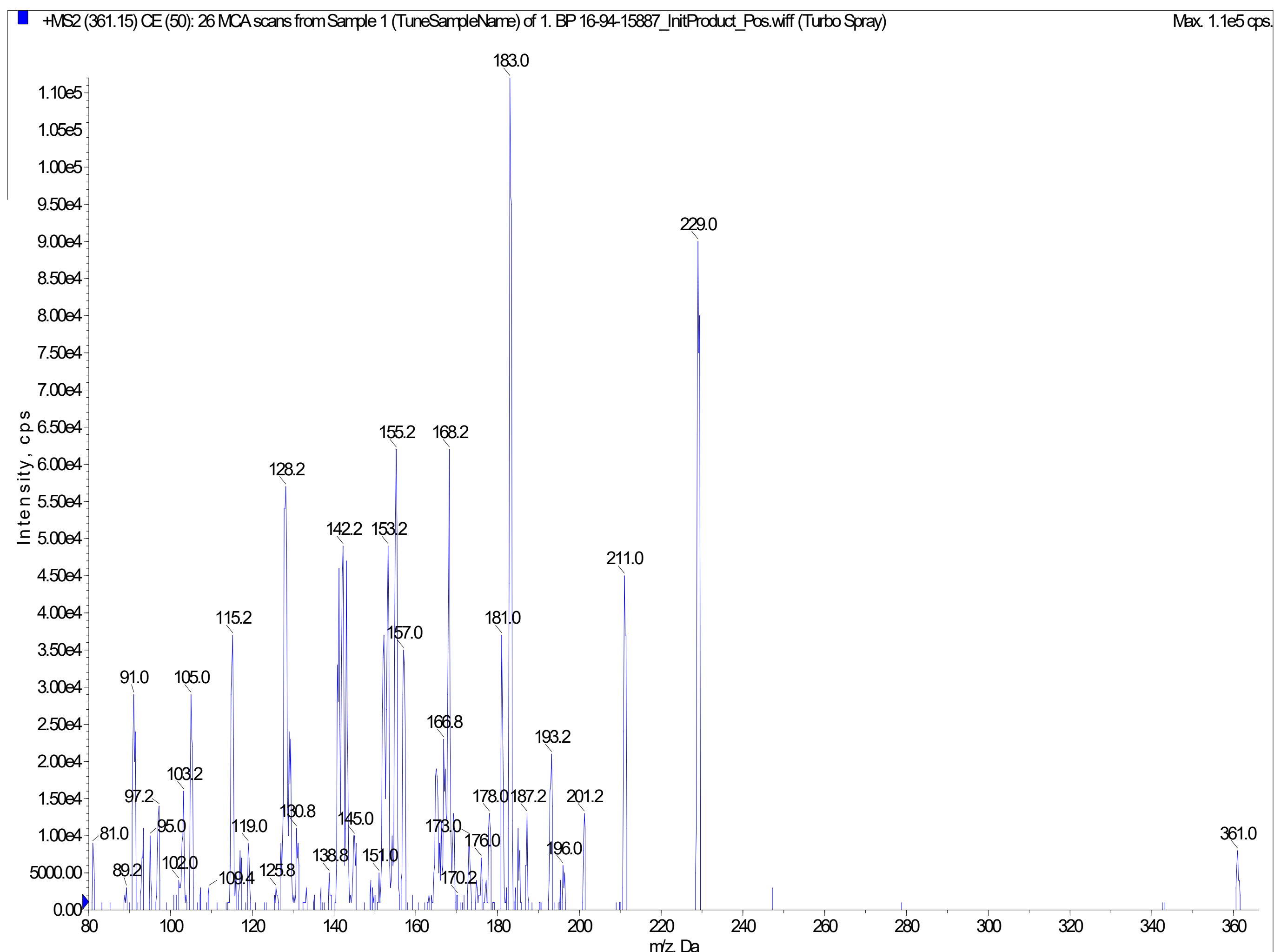

Figure 5.5 Fragmentation pattern of mass 361.

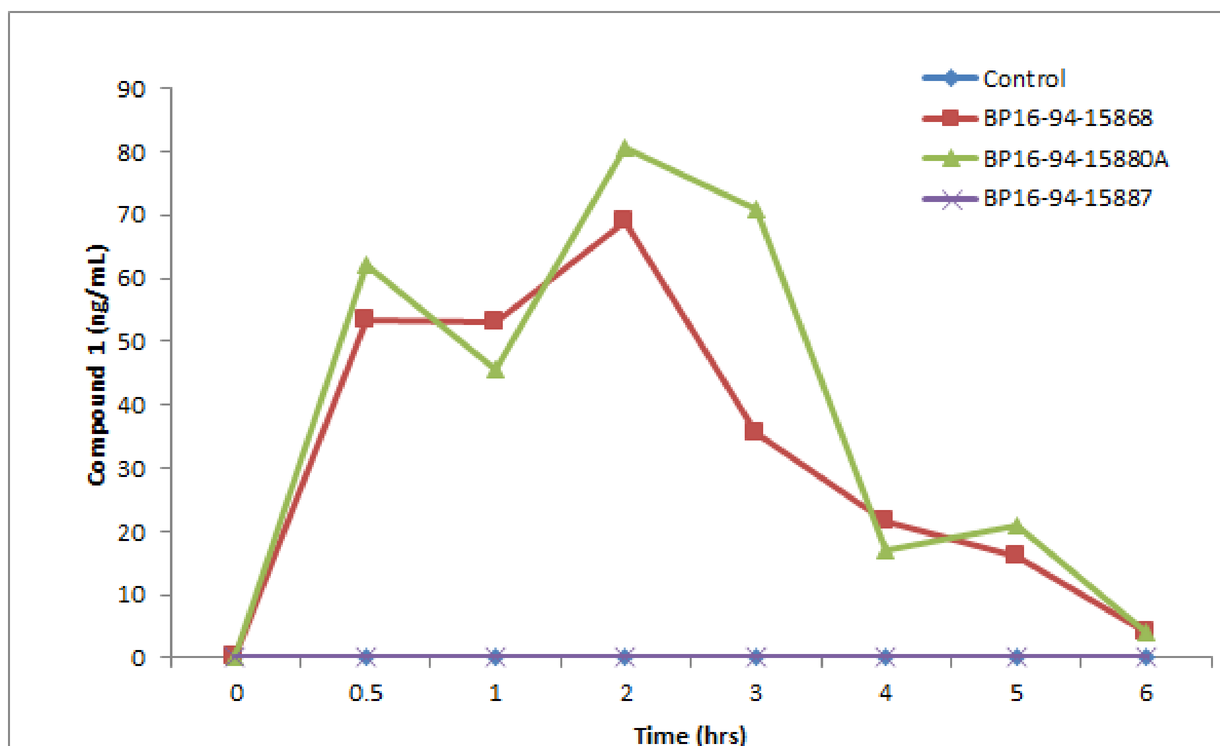

Figure 5.6 PK profile of compound 1 in the plasma samples of adult Wistar rats following the oral administration of 500 mg/kg BW aqueous freeze dried extract (BP16-94-15868), 500 mg/kg BW butanone/pentane extract (BP16-94-15880A) and 55.7 mg/kg BW compound enriched fraction (BP16-94-15887), respectively over 6 hr period. The results are the mean of two analysis batches (LC-MS).

The molecular mass of *m/z* 361.169 was confirmed for the biomarker (standard) using the Q-trap LC-MS. This was also found in the compound 1 enriched fraction which was run as two isomers. It was very difficult to separate; hence it was quantitated together (fragment 1: 361 - 229.2 and fragment 2: 361 - 183.2). The plasma was spiked with the pure compound and a standard curve was set up ranging from 10 – 10,000 ng/mL. Analysis of the samples confirmed the presence of the compound in the control samples. The compound was detectable in samples from rats that received both the freeze dried extract (BP16-94-15868) and butanone/pentane extract (BP16-94-15880A) (Figure 5.6). However, it was not detectable in any of the rats which received the enriched fraction.

In the rats receiving the aqueous freeze dried and butanone/pentane extracts, the 0.5 hr samples had similar serum concentrations (53.5 ng/mL and 62 ng/mL) confirming its rapid

absorption from the gastrointestinal tract. In both of these extracts the Cmax serum concentrations of compound 1 were achieved at 2 hrs with 69 ng/mL and 80.5 ng/mL, respectively. Thereafter the enriched fraction serum concentrations decreased steadily to below 10 ng/mL at 6 hrs. The PK AUC values of 210.5 and 256.6 were similar for the butanone/pentane extracts, respectively. This suggests that in terms of bioavailability, these two extracts behaved as insulin would which usually reaches the bloodstream within 30 mins after injection and peaks anywhere from 2 to 3 hrs after injection (Frode and Medeiros, 2008).

The biomarker (compound 1) was not detected in any of the serum samples of the rats that received the compound 1 enriched fraction. Reasons for low bioavailability could include the physical characteristics such as solubility, stability, pH and low membrane permeability of molecules due to low lipophilicity and zwitterionic character at physiological pH, or efflux by P-gp (Kang *et al*., 2009). As is the case for resveratrol, a rapid rate of metabolism in the intestine/liver may also be a rate-limiting step in the compounds bioavailability (Walle *et al*., 2004). Alternatively the matrix and other chemical entities present in the extracts could have enhanced the bioavailability of compound 1. Further PK studies will be required to elucidate this interaction.

5.4 Conclusion

In vitro assays are cost-effective and easy to perform, but to establish the relevance of those results to real clinical outcomes in animals or human, pharmacokinetics is the key essential. Following oral administration of the *S. pinnata* aqueous freeze dried and butanone/pentane extracts, the presence of compound 1 (marker compound) was detectable from 30 min up to 6 hrs, demonstrating that compound 1 is readily bioavailable if administered orally in the form of an extract. Compound 1 could not be detected in any of the blood samples where the rats were given the compound enriched fraction.

5.5 References

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CHAPTER 6 – General Discussion

6.1 General Discussion

6.2 Conclusion

6.3 Limitations of the study

6.4 Future studies

6.5 References

6.1 General Discussion

Diabetes presents the biggest health challenge globally since the advent of HIV, and is labelled as a societal disaster. By 2030 it is expected that 80% of the estimated 552 million diabetics worldwide will live in low and middle-income countries. This will have devastating consequences to the health-care system and impact directly on the sustainable development in these regions (IDF, 2015). In these regions, conventional oral hypoglycaemic drugs and insulin are often either unobtainable or unaffordable (Beran and Yudkin, 2006). In most of the low–income countries, even the cheapest available generic combination treatments for diabetes often equates to two days of salary and up to eight days in Ghana (MDG Gap Task Force report, 2009). Apart from their greater availability and affordability, the use of herbal medicine for the treatment of chronic diseases, including type 2 diabetes has grown dramatically, not only in the low-income developing countries, but also in the developing countries where there is a growing demand for effective natural products with less side effects (Modak *et al*., 2007). The WHO has also supported the use of herbal medicine as a treatment strategy for diabetes in the developing world (WHO, 2013).

Historically, medicinal plants have been used for diabetes throughout the world. Currently more than 1,200 plants are claimed to have antidiabetic activity (Habeck, 2003). In South Africa, ethnobotanical surveys found that at least 32 plants are commonly used to treat diabetes. Of these, *in vivo* antidiabetic activity has only been demonstrated in nine plants (Afolayan and Sunmonu, 2010). More effort is therefore urgently needed from the scientific community to investigate the safety and efficacy these traditional remedies from in our occidental societies (Bailey and Day, 1989). The opportunity and need exists in South Africa to develop new and effective antidiabetic therapeutics and to identify active NCE's from indigenous plants that can potentially develop into new oral hypoglycemic drugs (Rout *et al.,* 2009).

In this study, *S. pinnata* was subjected to detailed scientific investigation and a mechanism-based approach using *in vitro* assays so that this plant may provide antidiabetic treatment and justify the role of such novel traditional medicinal plants as antidiabetic therapeutics. The plant was initially brought by a traditional healer to the CSIR claiming its use for inflammation and swollen legs (CSIR, *pers.comm.,* Book 2). The traditional preparation of *S.*

pinnata is by adding boiling water to the dried ground plant material, allowing it to steep for 1 – 2 hrs, filter, and then it's drunk as a tea. Two extracts of *S. pinnata*, GF-1-14A (organic) and GF-1-17A (aqueous) were screened from the CSIR repository for glucose uptake using C2C12 muscle and Chang cells. Activity was compared with respect to metformin (reference oral antidiabetic drug), insulin (positive control) and the solvent vehicle (DMSO/water) resulting in the aqueous extract proving to effectively increase glucose uptake *in vitro*, being as effective as insulin. Following on this promising data, a pilot *in vivo* study using a STZ diabetic rat model involving the same two extracts, confirmed significant glucose lowering effects of the aqueous extract. It was then decided to investigate *S. pinnata* further for its antidiabetic properties, thus forming the basis of this study.

The study included isolation and characterization of the active compound, chemical profiling of several extracts of *S. pinnata*, evaluating the biological efficacy of the extracts/fractions, mode of action studies and pharmacokinetic analysis of the blood samples of the rats that were given the various treatments.

The dried ground plant material of *S. pinnata* was extracted with water and the aqueous extract was divided into two batches; one to be spray dried and the other freeze dried, in order to obtain a suitable working extract for laboratory use that is free from contamination. Using the bioassay guided fractionation approach, the aqueous freeze dried extract (BP16-94-15868) exhibited the most significant glucose lowering potential as compared to the spray dried (BP16-94-15863A) and ethanol (BP16-94-15863B) extracts. A [^{3}H]-2-deoxy-D-glucose assay was then used to estimate an effective concentration range of the aqueous freeze dried extract. In the C2C12 and Chang cells, the aqueous freeze dried extract significantly increased glucose uptake activity in the concentration range of 5 μg/mL to 5 X 10^{-5} μg/mL, yielding an EC_{50} concentration of 0.81 μg/mL and 0.77 respectively μg/mL.

The aqueous freeze dried extract was subjected to liquid/liquid partitioning using ethyl acetate for laboratory purposes and fractionation via silica gel column chromatography. Similar fractions were pooled together and screened using the glucose uptake assay in C2C12 muscle cells and Chang cells. Several fractions, BP16-94-15855A, BP16-94-15855B and BP16-94-15855G, induced an increase in glucose uptake by >150% in C2C12 cells.

However, in the Chang cells, the same fractions did not significantly increase glucose uptake. The highest glucose uptake in the Chang cells were noted in fractions BP16-94-15855K, BP16-94-15855L and BP16-94-15855M. Further fractionation of these latter three fractions resulted in a crystalline compound (compound 1) being isolated. With much hard work and success of analyzing the NMR spectral data, compound 1 (Figure 2.14) was elucidated and identified to be 2-(2-{[(2*E*)-4-hydroxy-2-(hydroxymethyl)but-2-enoyl]oxy}-4,7-dimethyl-1,2,3,4 tetrahydronaphthalen-1-yl)prop-2-enoic acid using The Dictionary of Natural Products. This novel compound has been confirmed with MS fragmentation with the compound having a molecular weight of 360.

The chemical profile of the spray dried, freeze dried and ethanol extracts were over laid to view the differences in compounds using HPLC. The aqueous freeze dried extract had a major peak with a retention time of 26.097 and the same but much shorter peak in the spray dried extract. It is possible that the compound was lost during the spray drying process. This compound was absent in the ethanol extract. These extracts were screened for antidiabetic activity resulting in the aqueous freeze dried extract being the most active at reducing glucose levels, followed by the spray dried extract being less active and the ethanol extract being inactive. It was therefore deduced that the compound with retention time 26.097 could be the active compound responsible for increasing glucose uptake.

The aqueous freeze dried extract was also subjected to extraction using pharmaceutically acceptable solvents such as butanone, butanol and pentane (http://www.pharmacopeia.cn/v29240/usp29nf24s0_c467.html) to determine which would be the most effective extract thereof for commercialization purposes should the extract prove to have antidiabetic activity. These solvents chosen are few of the pharmaceutically acceptable solvents allowed by the FDA and pharmacopeia for formulation activities. HPLC analysis revealed the butanone/pentane extract to have the least complex matrix of compounds (Figure 2.8). When this extract was over laid with that of compound 1, it was found that the major peak (compound 1) at retention time 26.6 was present in the butanone/pentane extract but in a very small concentration judging by the shortness of the peak. A [^{3}H]-2-deoxy-D-glucose assay was subsequently used to estimate the maximal effective glucose uptake concentration of the butanone/pentane extract. Compared to the aqueous extract, the C2C12 cells in the

butanone/pentane extract was effective over a wider concentration range from 50 μg/mL to 5 X 10^{-5} μg/mL. However, the EC_{50} concentration was higher at 13.6 μg/mL. At this concentration range, glucose uptake was ca. double that of metformin, the reference drug. In the Chang cells, the efficacy of the extract was lost. The fact that the butanone/pentane extract was more effective in C2C12 cells at the higher concentration of 50 μg/mL and the loss of activity in the Chang cells could be related to the loss of chemical constituents and possibly a lower concentration of compound 1, compared to the aqueous freeze dried extract.

Compound 1 was also screened using [^{3}H]-2-deoxy-D-glucose to establish a concentration response curve. The starting concentration was at 20 μg/mL as this was a pure compound. Glucose uptake was maximally increased at this concentration in both the C2C12 and Chang cells, thereafter tapering down drastically to the following lower concentrations. An EC_{50} value could therefore not be determined for compound 1 as the highest concentration of 20 μg/mL elicited the highest response.

The *in vitro* results paved the way as to which extracts and fractions to take forward for the *in vivo* study involving a large group of 33 rats using the STZ diabetic rat model. The aqueous freeze dried extract and the butanone/pentane extract was given orally at a dose of 500 mg/kg BW, and the compound 1 enriched fraction received a dose of 56 mg/kg BW. In the rats given the freeze dried extract, blood glucose levels were reduced significantly 1 hr after administration but the activity declined thereafter (Figure 3.9). The butanone extract only showed a trend ($p < 0.1$) towards significantly lowering plasma glucose at 3 hrs. These findings are in keeping with the *in vitro* glucose uptake experiments where the freeze dried aqueous extract was also more effective than the butanone/pentane extract. The compound 1 enriched fraction showed the highest glucose reduction at 3 hrs and was able to maintain this reduced glucose concentration for the remainder of the monitoring period. Although the compound 1 enriched fraction appeared to be the most effective, the glucose lowering effect was not significant due to the small number of rats ($n = 3$) that were treated with the limited quantity of the enriched fraction available. On the whole, glucose reduction achieved was between 10 - 15% by the extracts and the compound enriched fraction after the controls were subtracted.

Gene and protein analysis in C2C12 muscle cells revealed that the aqueous freeze dried extract (BP16-94-15868) and the compound 1 enriched fraction (BP16-94-15887) enhanced GLUT-4, glycogen synthase, pyruvate kinase and pyruvate carboxylase mRNA expression, which demonstrates that the extract has insulin mimetic effects. This could have important implications for insulin resistance in which the efficacy of insulin is reduced. However, for this study it was required to establish if this mode of action would be additive or competitive to that of insulin *in vivo*. The butanone/pentane extract (BP16-94-15880A) greatly enhanced protein expression of both total and activated AMPK, unlike the aqueous extract and compound 1 enriched fraction. Activation of AMPK is a well-known target for hypoglycaemic drugs. Metformin, the first line of oral hypoglycaemic drug prescribed for T2D, activates AMPK. This mode of action is however not novel as several other phytochemicals (berberine, genistein, resveratrol and aspalathin to name a few) elicit their antidiabetic effects by the activation of AMPK (Chang *et al.,* 2012). Although these extracts show different modes of action and the effects of insulin and AMPK are often physiologically antagonistic, there is substantial overlap between these divergent pathways specifically related to increasing skeletal muscle glucose uptake via AKT/GLUT-4. These mechanistic findings suggest that these extracts could potentially have insulin sensitising effects as is the case of metformin.

Rapid advances in drug design technologies have introduced many drugs, but many of these compounds exhibit poor bioavailability. The results of the PK study were based on a standard curve and the molecular mass of 360 which also corresponds to the data generated via LC-MS. Compound 1 was chromatographed as a doublet and both peaks were quantified. In the control samples, no compound was detected. LC-MS analysis of the blood serum samples of the rats treated with the aqueous freeze dried and butanone/pentane extracts at a dose of 500 mg/kg BW showed that compound 1 was rapidly absorbed i.e. levels of compound 1 quickly increased from 30 mins (53.5 and 62 ng/mL, respectively) and peaking at 2 hrs (69 and 80.5 ng/mL), followed by a steady decline to below 10 ng/mL at 6 hrs (Figure 5.3). In contrast to the extracts, compound 1 was not detected in the blood serum from rats given the compound enriched fraction at a dose of 56 mg/kg BW. Reasons for this is not easily explained. However, apart from its physical characteristics (solubility and stability), the pharmacokinetics and pharmacodynamics of phytochemicals such as compound 1 after ingestion is greatly affected by absorption, distribution, metabolism and

elimination from the body (Rein *et al.,* 2012). In general, compounds in extract preparations are often more readily absorbed than the pure compounds itself. This could be attributed to the presence of other phytoconstituents and their interactions with absorption and metabolism (Manach *et al*., 2004). Reasons for this are unknown for the increased presence of compound 1 in the plasma from the aqueous freeze dried and butanone/pentane extracts, but not from the compound 1 enriched fraction.

6.2 Conclusion

As is evident in this study, the biological data (*in vitro* and *in vivo*) clearly provides evidence which substantiates the traditional use of *S. pinnata* for diabetes by increasing glucose utilization. The aqueous freeze dried extract that mimics the traditional way of preparing *S. pinnata* proved to be the most effective method of extraction at increasing glucose uptake *in vitro* and lowering plasma glucose in the diabetic rats, thus supporting the traditional method of preparation. The highlight of this study was the identification and isolation of a NCE, compound 1; (2-(2-{[(2E)-4-hydroxy-2-(hydroxymethyl)but-2-enoyl]oxy}-4,7-dimethyl-1,2,3,4 tetrahydronaphthalen-1-yl)prop-2-enoic acid) which was demonstrated to be at least partially responsible for the antidiabetic activity observed in *S. pinnata.* This finding warrants further investigation into the development and synthesis of compound 1 as a possible drug candidate.

6.3 Limitations of the study

Time and budget constraints limited the further elucidation and antidiabetic efficacy *in vivo* of the isolated compound 1. It was not possible to isolate sufficient quantities of the active compound for the *in vivo* study. A limited amount of an enriched fraction of compound 1 was produced for the *in vivo* assessment, but this was only sufficient to treat three rats, thus subsequently compromising the statistical power of the treatment group.

Further *in vivo* studies using a different diabetic model such as the *db/db* mouse could provide additional information regarding the hypoglycaemic potential of the extract and compound 1 specifically related to its possible insulin sensitising effect. Functional studies

such as oral glucose tolerance and glucose stimulated insulin secretion rates would have been of great value at estimating such effects.

Funding limited the extent to which the mechanism of action could be further pursued using multi array technologies that offer pathway-focused gene expression RT^2 Profiler PCR arrays and protein multiplex systems. For this study with a limited budget, only the most likely genes and proteins were selected.

6.4 Future Studies

Provided that sufficient funding can be obtained, the following future studies are proposed: Investigate the potential for commercialising a standardised pharmaceutical grade GMP *S. pinnata* extract as a natural hypoglycaemic product. To achieve this, further supporting studies including toxicity, efficacy and mode of action studies will be needed. The health risk benefits of consuming such an extract either as a monotherapy or in combination with other chronic medications will also have to be established.

Based on the evidence for compound 1 as an antidiabetic agent, the synthesis of compound 1 and/or analogues should be investigated as antidiabetic drug candidates. To achieve this objective, a multi-disciplinary approach will be required. The feasibility of applying Lipinski's rule of 5 and structure-activity relationship (SAR) studies aimed at establishing the structural requirement(s) for pharmacological activity will have to be undertaken. Toxicity and efficacy testing of the synthesised compound(s) will have to be repeated in *in vitro* and *in vivo* models.

6.5 References

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CHAPTER 7 - Appendix

7.1 Compound 1 (BP16_94_15857) in methanol

7.1.1 H^1 spectrum

7.1.2 C^{13} spectrum

7.1.3 COSY

7.1.4 HSQC

7.1.5 HMBC

7.2 Compound 1 (BP16_94_15864H) in DMSO

7.2.1 H^1 spectrum

7.2.2 C^{13} spectrum

7.2.3 HSQC

7.2.4 HMBC

7.3 Biological screening of *S. pinnata*

7.3.1 *In vivo* STZ-induced diabetic rat pilot study data (see Figure 3.1) to assess the glucose lowering efficacy of an aqueous (GF-1-17A) and organic (GF-1-14A) extracts of *S. pinnata*.

7.3.2 *In vitro* glucose uptake data for aqueous (GF-1-14A) and organic (GF-1-17A) extracts in C2C12 and Chang cells (see Figure 3.2).

7.3.3 *In vitro* glucose uptake data for aqueous freeze dried extract (BP16-94-15868), spray dried extract (BP16-94-15863A), ethanol extract (BP16-94-15863B) and compound 1 (BP16-94-15864H) in C2C12 muscle and Chang cells (see Figure 3.3).

7.3.4 *In vitro* glucose uptake data of the aqueous extract fractions BP16-94-15855A-M in C2C12 and Chang cells (see Figure 3.4).

7.3.5 ***In vitro* [3H]-2-deoxy-D-glucose uptake data of varying concentrations of the aqueous freeze dried extract in C2C12 myocytes and Chang cells (see Figure 3.5).**

7.3.6 ***In vitro* [3H]-2-deoxy-D-glucose uptake data of the butanone/pentane extract by C2C12 myocytes and Chang cells (see Figure 3.7).**

7.3.7 ***In vitro* [3H]-2-deoxy-D-glucose uptake data of compound 1 by C2C12 myocytes and Chang cells (see Figure 3.8).**

7.3.8 Blood glucose concentrations and glucose reduction over 6 hrs in the STZ diabetic rat model treated with aqueous freeze dried extract (BP16-94-15868; 500 mg/kg BW), butanone/pentane extract (BP16-94-15875C and BP16=94-15880A; 500 mg/kg BW) and compound 1 enriched fraction (BP-16 – compound; 55.7 mg/kg BW), respectively (see Figure 3.9).

7.4 Gene and protein expression analysis in *S. pinnata* treated C2C12 muscle cells

7.4.1 mRNA expression data of insulin receptor (A), insulin receptor substrate 1 (B), PI3-kinase (C) and GLUT-4 (D) in C2C12 myocytes (see Figure 4.2).

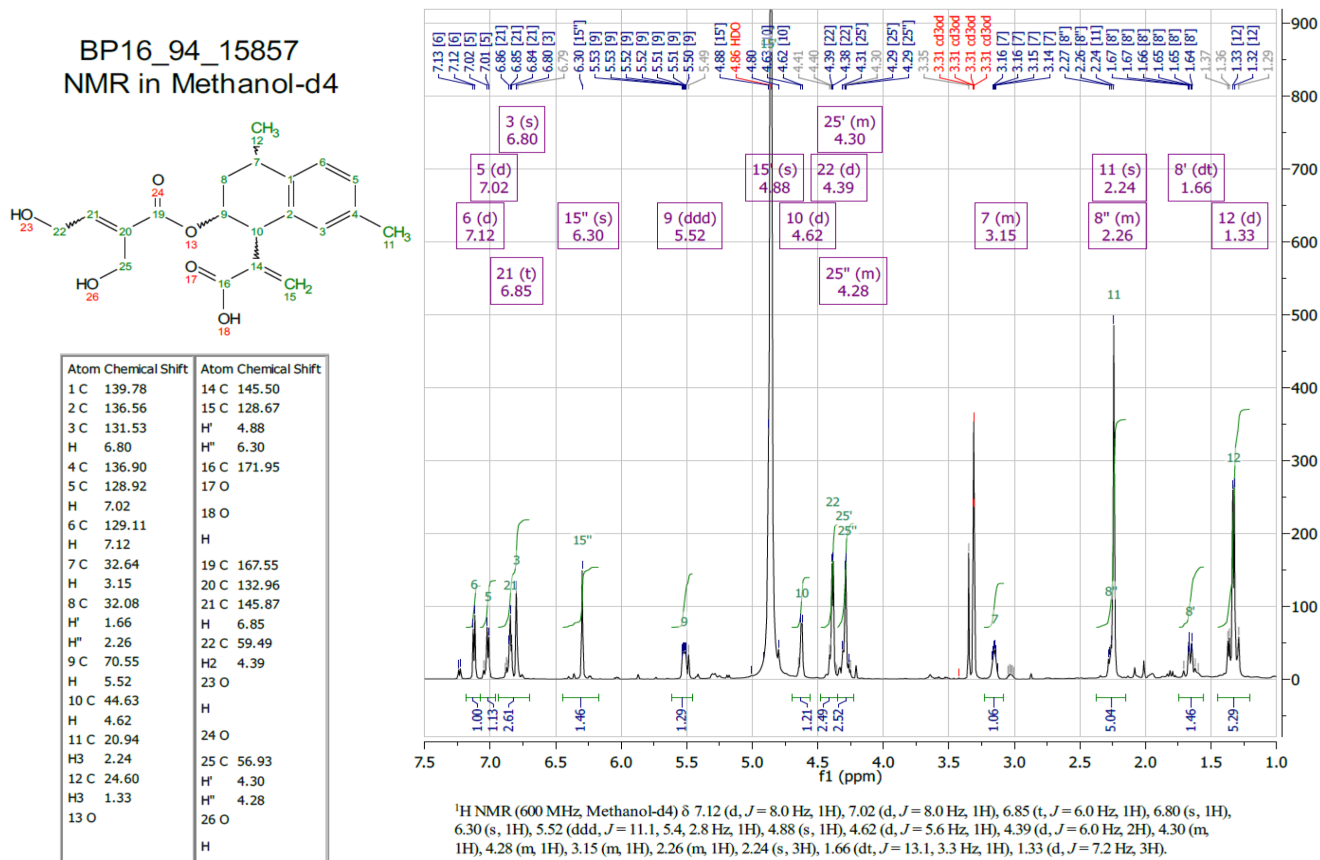
7.4.2 mRNA expression of enzymes glycogen synthase (A), pyruvate kinase (B) and pyruvate carboxylase (C) in C2C12 myocytes (see Figure 4.3).

7.4.3 Protein content of total AMP-activated protein kinase (AMPK) (A) and phosphorylated-AMPKα (Thr172) (B) (see Figure 4.4).

7.5 Ethical approval of the study

7.1 Compound 1 (BP16_94_15857) in methanol

7.1.1 H¹ spectrum



Atom	Chemical Shift	Atom	Chemical Shift
1 C	139.78	14 C	145.50
2 C	136.56	15 C	128.67
3 C	131.53	H'	4.88
H	6.80	H"	6.30
4 C	136.90	16 C	171.95
5 C	128.92	17 O	
H	7.02	18 O	
6 C	129.11	H	
H	7.12	19 C	167.55
7 C	32.64	20 C	132.96
H	3.15	21 C	145.87
8 C	32.08	H	6.85
H'	1.66	22 C	59.49
H"	2.26	H2	4.39
9 C	70.55	23 O	
H	5.52	H	
10 C	44.63	24 O	
H	4.62	25 C	56.93
11 C	20.94	H'	4.30
H3	2.24	H"	4.28
12 C	24.60	26 O	
H3	1.33	H	
13 O			

Figure 7.1.1 H¹ spectrum of compound 1 (BP16_94_15857) in methanol.

7.1.2 C^{13} spectrum

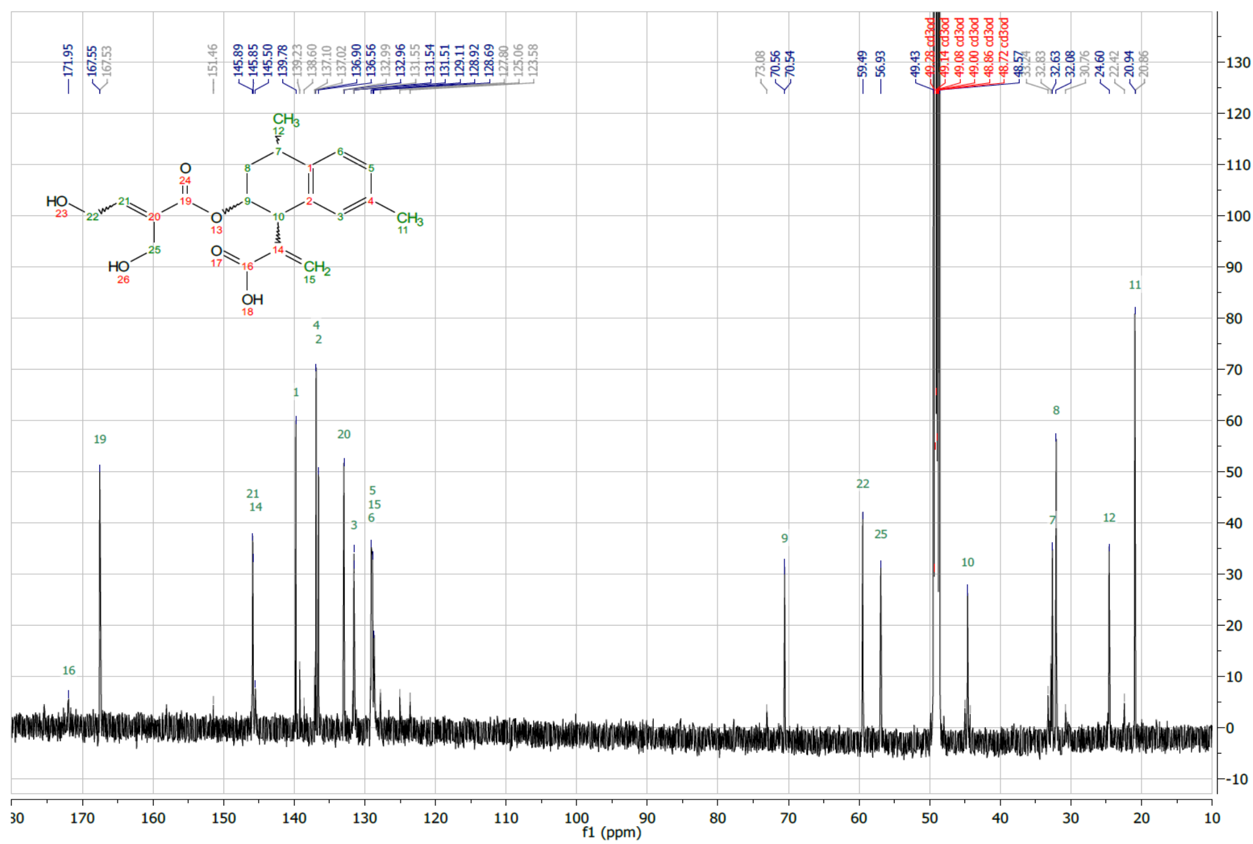


Figure 7.1.2 C^{13} spectrum of compound 1 (BP16_94_15857) in methanol.

7.1.3 COSY

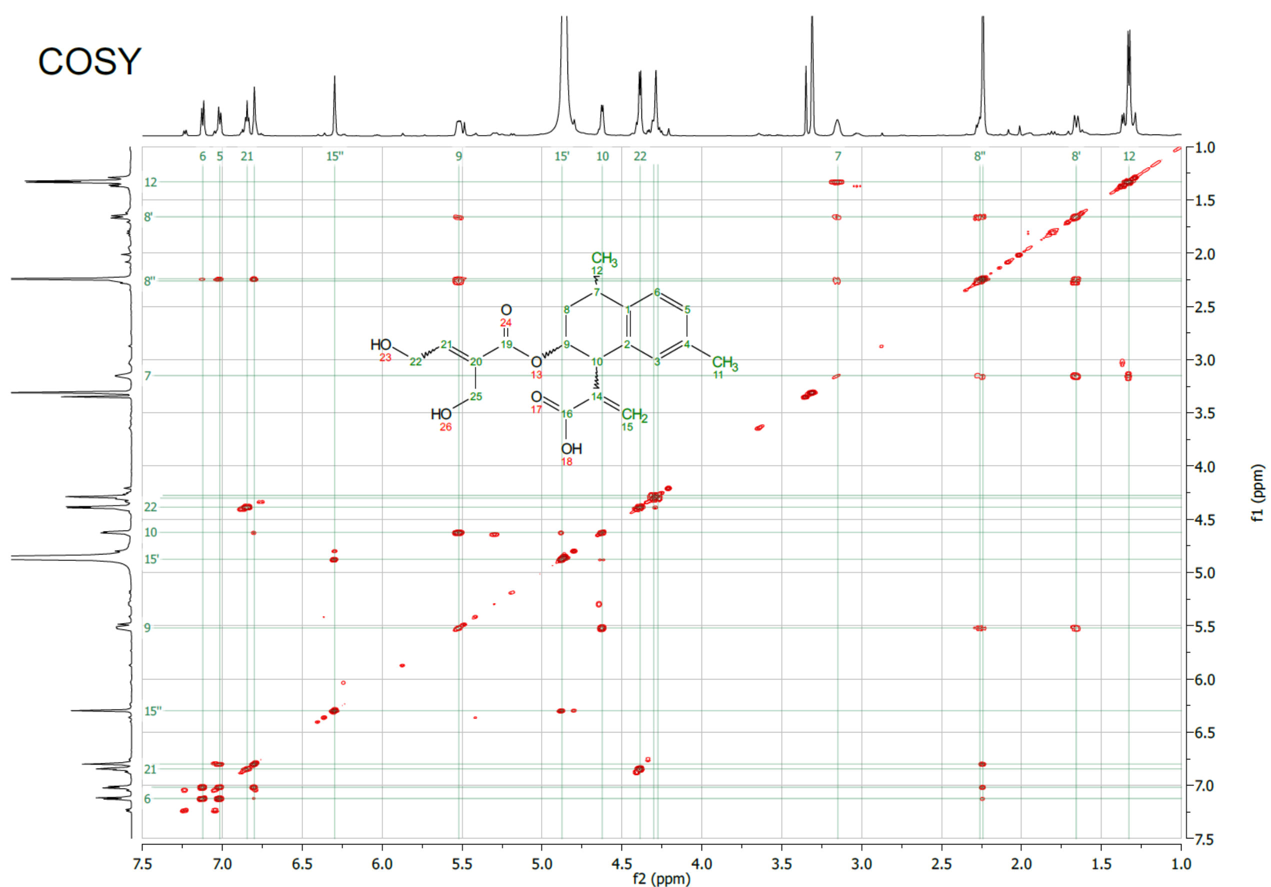


Figure 7.1.3 COSY spectrum of compound 1 (BP16_94_15857) in methanol.

7.1.4 HSQC

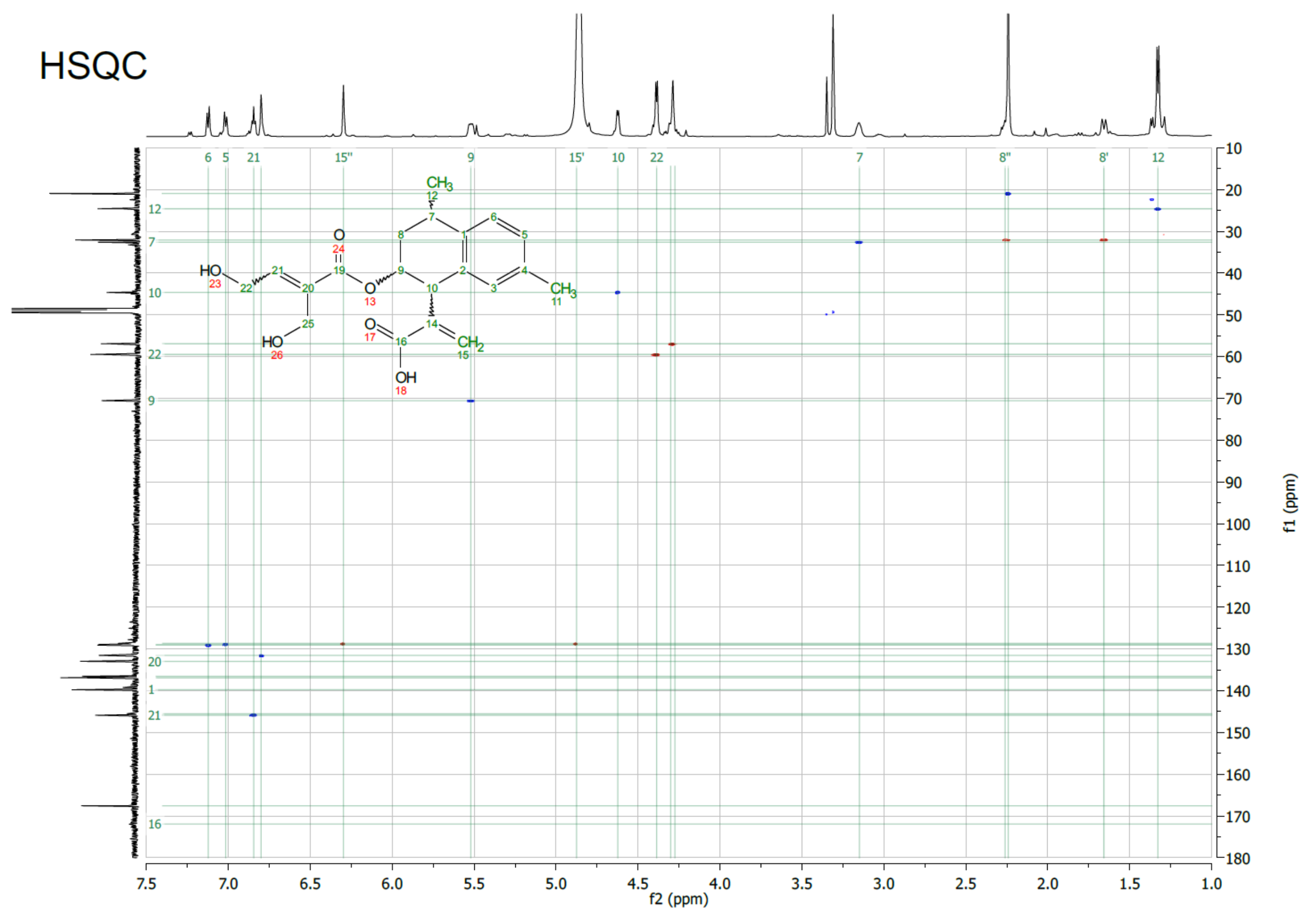


Figure 7.1.4 HSQC spectrum of compound 1 (BP16_94_15857) in methanol.

7.1.5 HMBC

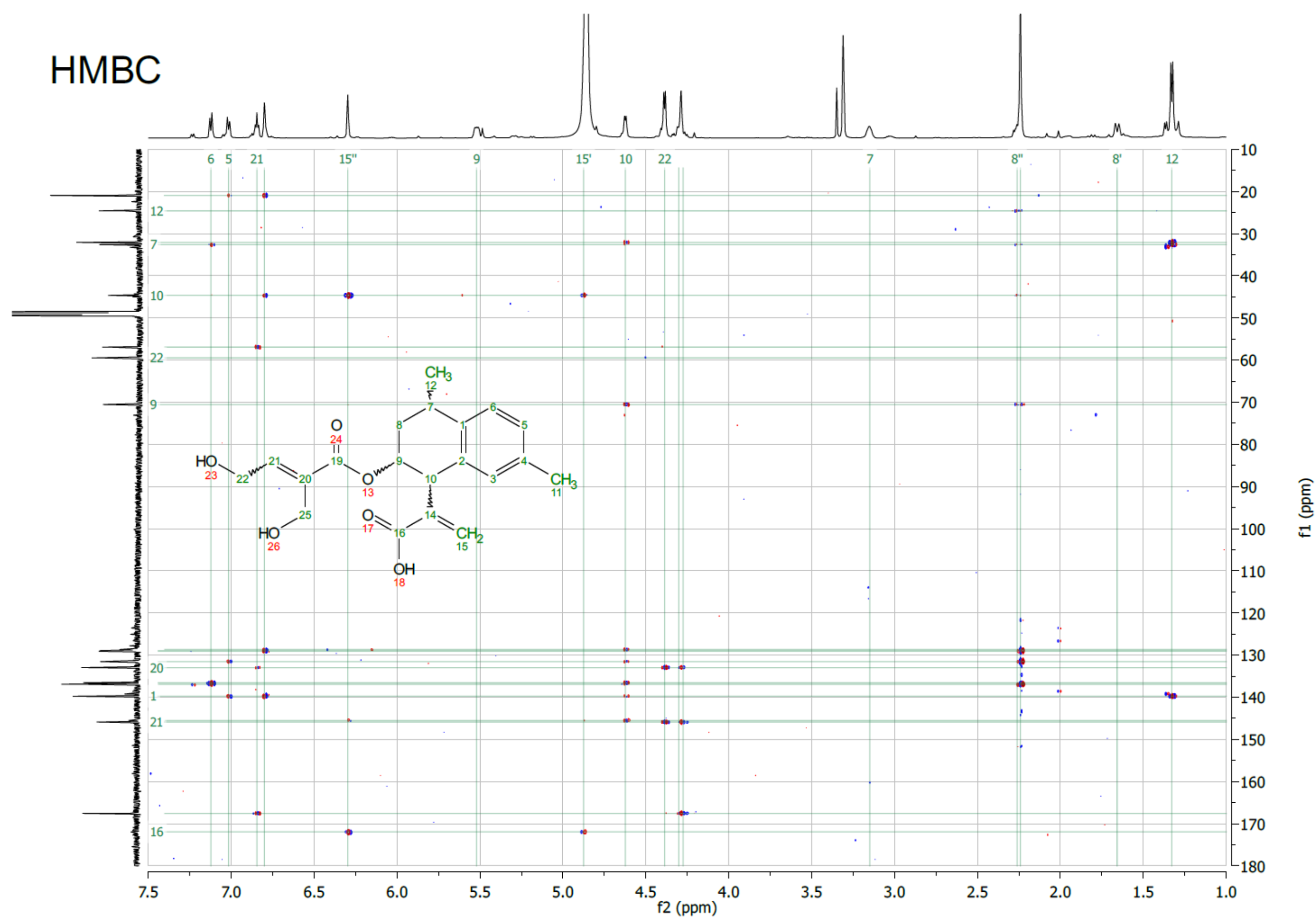


Figure 7.1.5 HMBC spectrum of compound 1 (BP16_94_15857) in methanol.

7.2 Compound 1 (BP16_94_15864H) in DMSO

7.2.1 H^1 spectrum

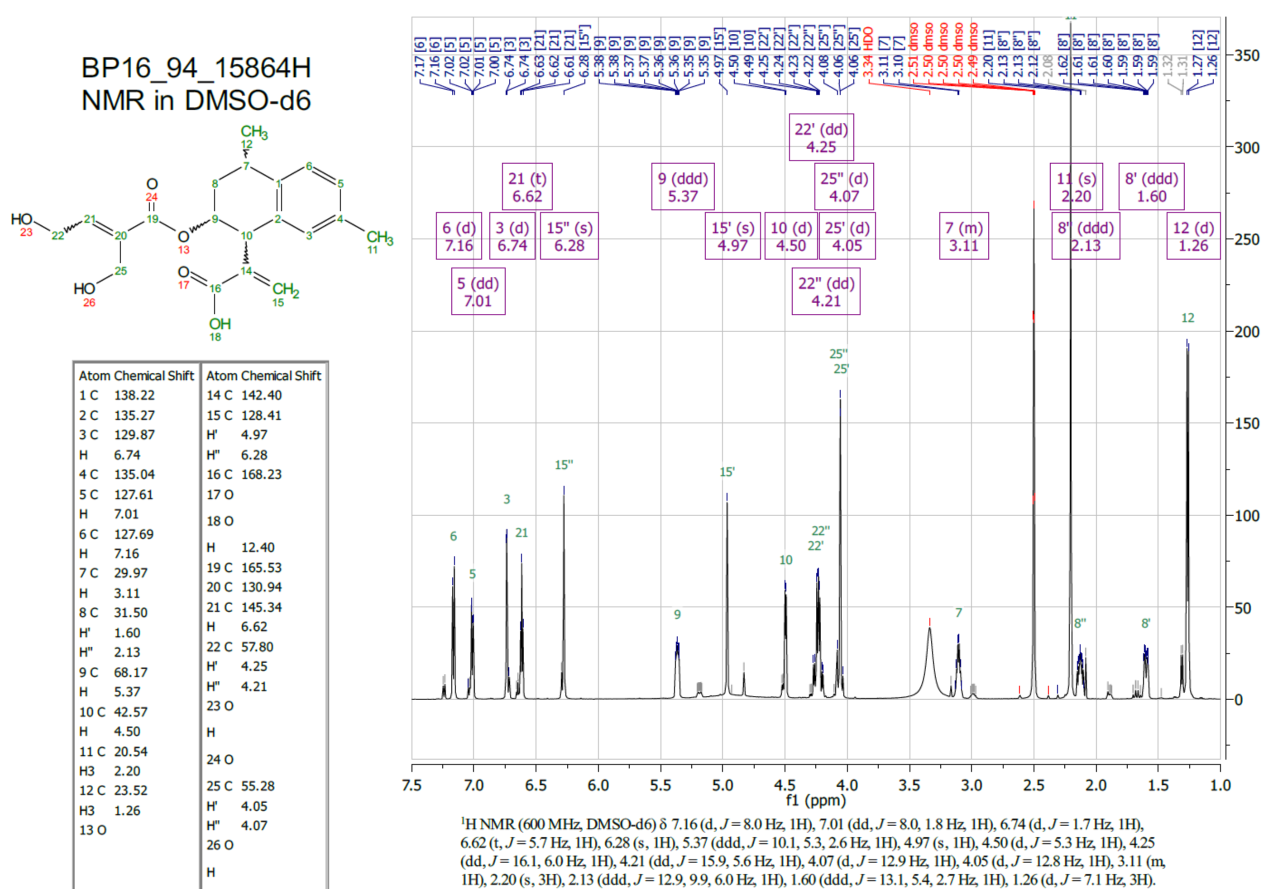


Figure 7.2.1 H^1 spectrum of compound 1 (BP16_94_15864H) in DMSO.

7.2.2 *C^{13} spectrum*

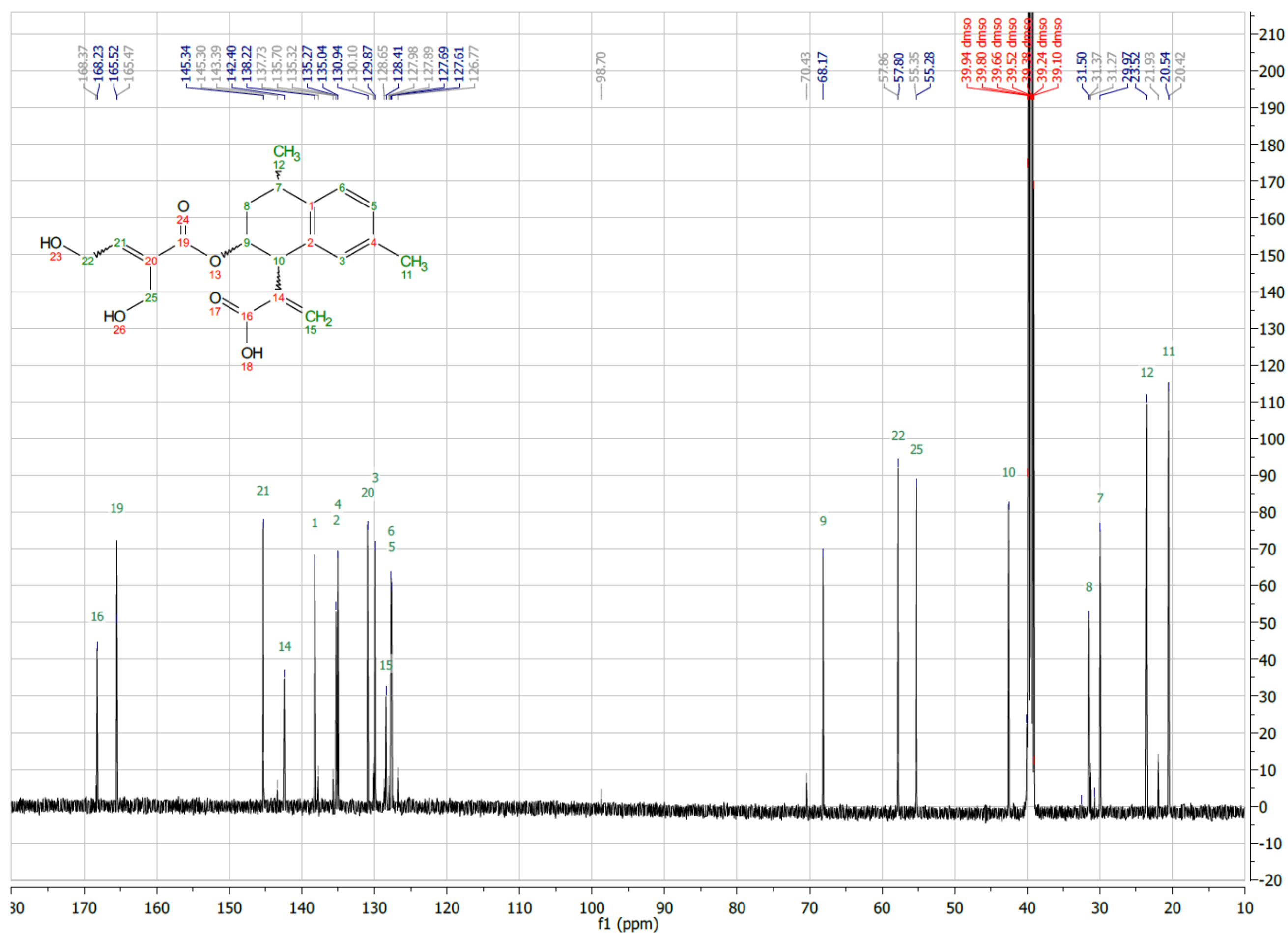


Figure 7.2.2 C^{13} spectrum of compound 1 (BP16_94_15864H) in DMSO.

7.2.3 HSQC

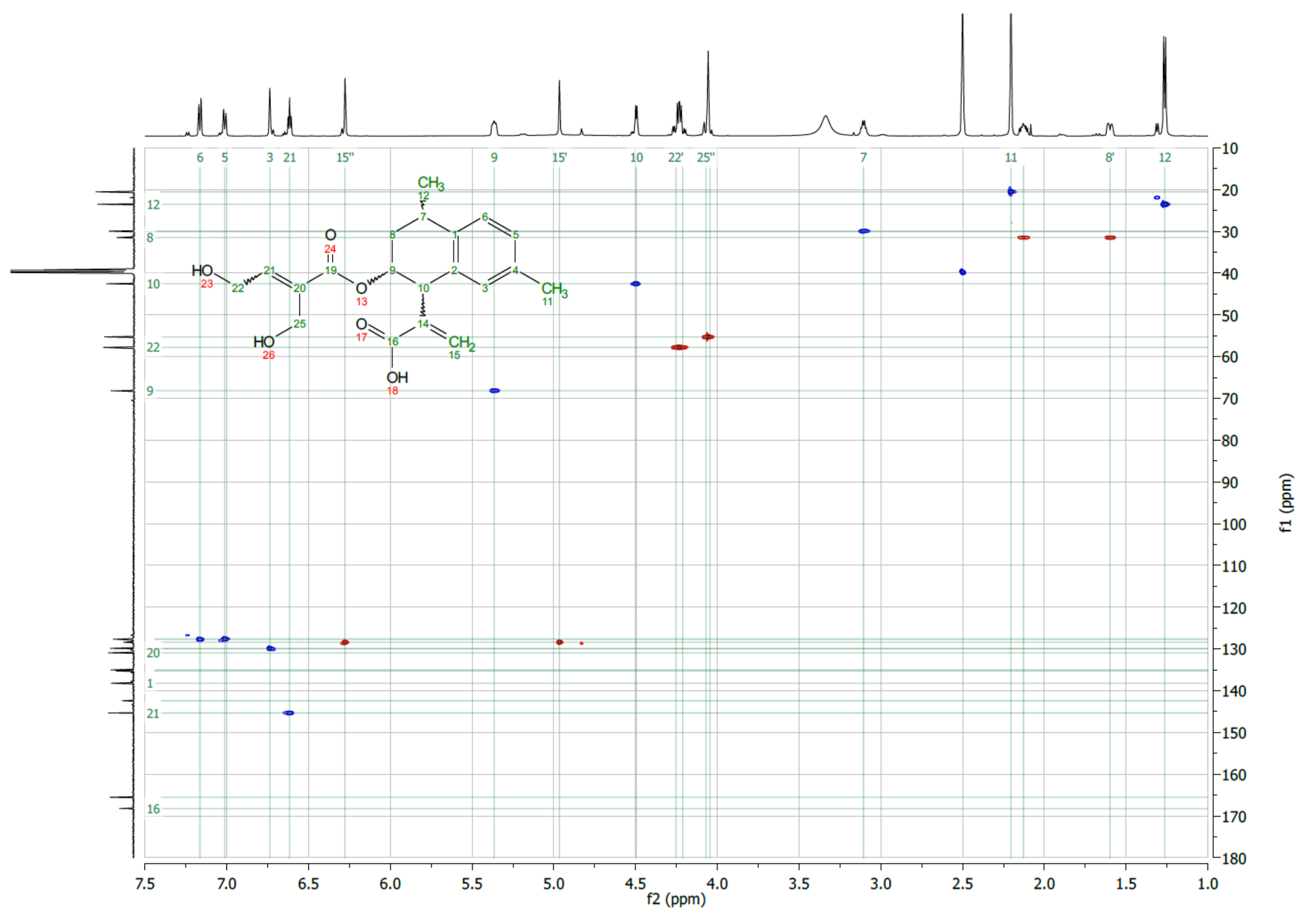


Figure 7.2.3 HSQC spectrum of compound 1 (BP16_94_15864H) in DMSO.

7.2.4 HMBC

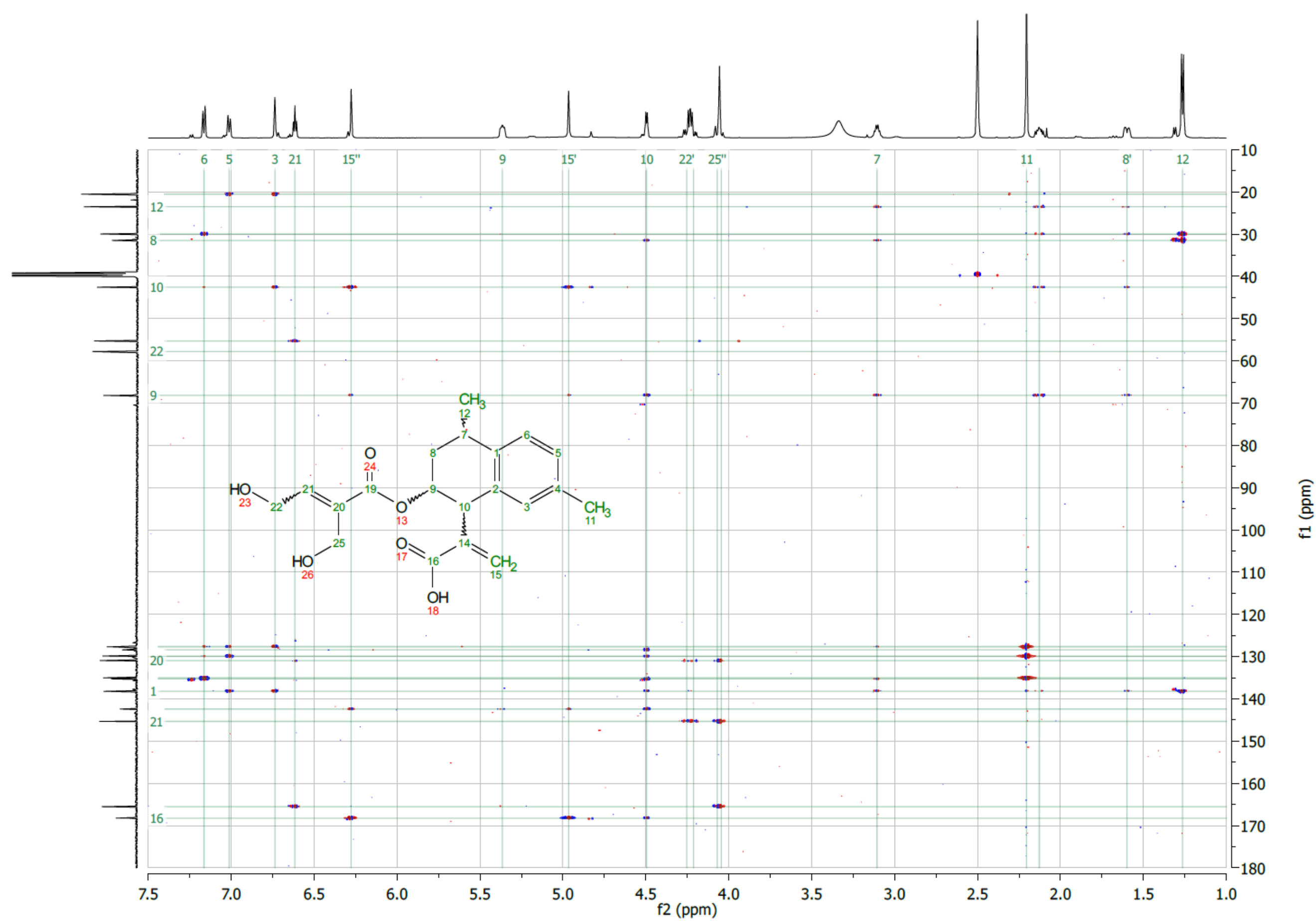


Figure 7.2.4 HMBC spectrum of compound 1 (BP16_94_15864H) in DMSO.

7.3 Biological screening of *S. pinnata*

Table 7.3.1 *In vivo* STZ-induced diabetic rat pilot study data (see Figure 3.1) to assess the glucose lowering efficacy of the aqueous (GF-1-17A) and organic (GF-1-14A) extracts of *S. pinnata*

		Glucose (mmol/l)							% Reduction with time					
		0	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	Mean	19.60	19.02	18.98	17.66	16.98	16.72	15.94	-3.07	-3.34	-9.88	-13.38	-14.69	-18.54
	SD	0.509	0.746	0.933	0.552	0.664	0.528	0.494	1.564	2.723	1.828	2.514	1.684	2.783
GF-1-14A	Mean	19.52	18.28	16.97	15.54	15.04	15.32	15.74	-5.87	-12.48	-19.72	-22.55	-21.20	-18.96
	SD	0.551	0.698	0.985	0.983	0.771	0.440	0.526	5.157	6.329	6.554	5.006	3.420	4.141
	Control subtracted								-2.80	-9.15	-9.83	-9.17	-6.52	-0.42
	P value								NS	<0.05	NS	<0.05	<0.05	NS
GF-1-17A	Mean	21.02	18.76	18.18	17.50	15.52	15.60	15.42	-10.30	-12.86	-16.06	-25.72	-25.17	-25.87
	SD	0.999	0.544	0.240	0.285	0.525	0.432	0.244	2.822	3.528	3.787	3.200	3.834	4.131
	Control subtracted								-7.24	-9.52	-6.18	-12.34	-10.49	-7.33
	P value								NS	NS	NS	<0.05	NS	NS

Table 7.3.2 *In vitro* glucose uptake data for aqueous (GF-1-14A) and organic (GF-1-17A) extracts in C2C12 and Chang cells (see Figure 3.2)

		DMSO	Metformin	Insulin	GF-1-14A	Water	GF-1-17A
C2C12 cells	Mean	2.44		4.40	3.68	2.61	4.58
	SD	0.10		0.19	0.36	0.12	0.53
	P value			<0.05	NS		NS
Chang cells	Mean	0.84	1.70	1.15	1.46	0.45	1.03
	SD	0.29	0.25	0.22	0.31	0.21	0.26
	P value		<0.05	NS	NS		NS

Table 7.3.3 *In vitro* glucose uptake data for aqueous freeze dried extract (BP16-94-15868), spray dried extract (BP16-94-15863A), ethanol extract (BP16-94-15863B) and compound 1 (BP16-94-15864H) in C2C12 muscle and Chang cells (see Figure 3.3)

		Water	Insulin	Metformin	BP16-94-15868	DMSO	BP16-94-15863A	BP16-94-15863B	BP16-94-15864H
C2C12 cells	Mean	1.79	2.97	3.05	2.88	2.26	2.08	1.78	2.84
	SD	0.53	0.33	0.5	0.76	0.64	0.73	1.15	1.28
			NS	<0.05	<0.05		NS	NS	NS
Chang cells	Mean	0.51	1.6	1.59	0.74	0.82	0.4	0.44	1.33
	SD	0.48	0.2	0.57	0.71	0.45	1.46	1.01	0.35
			<0.001	<0.01	NS	NS	NS	NS	<0.05

Table 7.3.4 *In vitro* **glucose uptake data of the ethyl acetate extract fractions BP16-94-15855A-M in C2C12 and Chang cells (see Figure 3.4)**

		DMSO	Insulin	Metformin	BP16-94-15855A	BP16-94-15855B	BP16-94-15855C	BP16-94-15855D	BP16-94-15855E
C2C12 cells	Mean	1.05	2.42	1.62	1.6	1.56	1.26	0.84	1.55
	SD	0.11	0.85	0.01	0.06	0.17	0.63	0.28	0.63
	P value		<0.05	<0.05	<0.01	<0.01	NS	NS	NS
Chang cells	Mean	0.64	1.6	1.51	0.51	0.76	0.61	0.78	0.57
	SD	0.55	0.14	1.18	0.28	0.38	0.17	0.23	0.23
	P value		<0.05	NS	NS	NS	NS	NS	NS

		BP16-94-15855F	BP16-94-15855G	BP16-94-15855H	BP16-94-15855I	BP16-94-15855J	BP16-94-15855K	BP16-94-15855L	BP16-94-15855M
C2C12 cells	Mean	1.27	1.68	1.36	1.02	1.16	1.25	1.25	0.99
	SD	0.65	0.11	0.25	0.77	0.46	0.38	0.71	0.49
	P value	NS	<0.01	NS	NS	NS	NS	NS	NS
Chang cells	Mean	0.32	0.74	0.63	0.65	0.76	0.9	0.96	0.94
	SD	0.26	0.19	0.34	0.33	0.16	0.29	0.28	0.41
	P value	NS	NS	NS	NS	NS	NS	NS	NS

Table 7.3.5 *In vitro* **[^{3}H]-2-deoxy-D-glucose uptake data of varying concentrations of the aqueous freeze dried extract in C2C12 myocytes and Chang cells (see Figure 3.5)**

		Control	Insulin	Metformin	50 µg/mL	5 µg/mL	5×10^{-1} µg/mL	5×10^{-2} µg/mL	5×10^{-3} µg/mL	5×10^{-4} µg/mL	5×10^{-5} µg/mL
C2C12 cells	**Mean**	0.00	100.00	71.97	3.54	204.39	81.03	30.99	47.66	27.19	28.60
	SD	11.26	47.33	32.69	76.94	48.06	19.53	41.09	25.78	16.35	24.12
	P value		<0.001	<0.05	NS	<0.001	<0.01	NS	NS	NS	NS
Chang cells	**Mean**	0.00	100.00	53.61	-0.34	289.96	141.80	38.21	35.50	9.78	24.11
	SD	20.53	26.81	51.77	38.25	50.83	98.46	32.41	56.67	56.17	69.82
	P value		<0.05	NS	NS	<0.001	<0.01	NS	NS	NS	NS

Table 7.3.6 *In vitro* **[^{3}H]-2-deoxy-D-glucose uptake data of the butanone/pentane extract by C2C12 myocytes and Chang cells (see Figure 3.7)**

		Control	Insulin	Metformin	50 µg/mL	5 µg/mL	5×10^{-1} µg/mL	5×10^{-2} µg/mL	5×10^{-3} µg/mL	5×10^{-4} µg/mL	5×10^{-5} µg/mL
C2C12 cells	**Mean**	0.00	100.00	41.32	97.77	95.74	97.76	46.62	63.25	54.32	77.02
	SD	6.36	2.96	5.78	5.17	6.02	5.70	10.32	7.41	6.39	9.06
	P value		<0.01	NS	<0.01	<0.01	<0.01	NS	NS	NS	<0.05
Chang cells	**Mean**	0.00	100.00	73.72	-16.81	8.00	16.37	15.79	28.95	-4.99	0.00
	SD	5.30	8.99	4.67	5.78	6.27	6.49	6.70	8.24	9.56	15.99
	P value		<0.001	NS	NS	NS	NS	NS	NS	NS	NS

Table 7.3.7 *In vitro* **[^{3}H]-2-deoxy-D-glucose uptake data of compound 1 by C2C12 myocytes and Chang cells (see Figure 3.8)**

		Control	Insulin	Metformin	20 µg/mL	2 µg/mL	2×10^{-1} µg/mL	2×10^{-2} µg/mL	2×10^{-3} µg/mL	2×10^{-4} µg/mL	2×10^{-5} µg/mL
C2C12 cells	**Mean**	0.00	100.00	65.02	499.17	191.45	63.38	26.73	36.34	34.36	30.97
	SD	43.02	34.56	27.34	55.94	38.64	17.80	31.15	9.52	28.91	53.37
	P		p<0.001	p<0.01	p<0.001	p<0.001	p<0.001	NS	NS	NS	NS
Chang cells	**Mean**	0.00	100.00	62.27	261.35	132.78	43.93	19.13	21.06	-0.41	12.53
	SD	43.02	34.56	27.34	55.94	38.64	17.80	31.15	9.52	28.91	53.37
	P=		p<0.001	p<0.05	p<0.001	p<0.001	NS	NS	NS	NS	NS

Table 7.3.8 Blood glucose concentrations and glucose reduction over 6 hrs in the STZ diabetic rat model treated with aqueous freeze dried extract (BP16-94-15868; 500 mg/kg BW), butanone/pentane extract (BP16-94-15875C and BP16=94-15880A; 500 mg/kg BW) and compound 1 enriched fraction (BP-16-compound; 55.7 mg/kg BW), respectively (see Figure 3.9)

		Glucose (mmol/l)							% Reduction with time					
		0	1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	Mean	21.00	21.32	20.00	20.24	20.62	20.74	20.02	1.31	-4.75	-3.65	-1.69	-1.25	-4.76
	SD	0.680	1.095	0.719	1.005	1.061	1.164	1.313	2.505	1.676	3.263	4.554	4.459	4.868
BP16-94-15868	Mean	20.95	18.98	18.68	17.98	19.95	19.30	19.07	-9.45	-10.72	-13.97	-3.97	-7.75	-8.56
	SD	0.666	0.976	1.036	0.994	0.898	0.831	0.754	3.176	4.330	4.954	6.335	3.396	4.607
	Control subtracted								-10.76	-5.97	-10.32	-2.28	-6.49	-3.80
	P value								NS	<0.05	NS	NS	NS	NS
BP16-94-15875C	Mean	19.45	18.23	17.17	16.95	18.25	17.57	18.55	-5.39	-13.55	-15.20	-7.45	-12.51	-8.16
and	SD	2.886	2.584	2.698	2.761	2.889	2.939	3.271	2.402	4.267	5.662	4.538	6.366	7.291
BP16-94-15880A	Control subtracted								-6.70	-8.80	-11.55	-5.76	-11.26	-3.40
	P value								NS	NS	NS	NS	NS	NS
BP-16 Compound	Mean	20.98	18.75	17.75	16.43	17.33	17.18	16.38	-7.07	-14.68	-17.32	-15.10	-14.51	-18.45
	SD	4.987	3.622	3.836	2.994	3.388	3.452	3.061	11.192	10.145	9.777	7.150	11.854	9.146
	Control subtracted								-8.37	-9.93	-13.67	-13.41	-13.26	-13.69
	P value								NS	NS	NS	NS	NS	NS

7.4 Gene and protein expression analysis in *S. pinnata* treated C2C12 muscle cells

Table 7.4.1 mRNA expression data of insulin receptor (A), insulin receptor substrate 1 (B), PI3K (C) and GLUT-4 (D) in C2C12 myocytes (See Figure 4.2)

	IR				IRS1			
	Mean	SD	SEM	Fold	Mean	SD	SEM	Fold
DMSO	0.72	0.06	0.04	1.00	1.25	0.30	0.17	1.00
Insulin	0.84	0.07	0.04	1.17	1.22	0.21	0.12	0.97
BP16-94-15868	0.88	0.06	0.04	1.23	1.44	0.21	0.12	1.15
BP16-94-15880A	0.81	0.09	0.05	1.13	1.27	0.04	0.02	1.01
BP16-94-15887	0.95	0.16	0.09	1.33	1.43	0.25	0.14	1.14
	Pi3k				GLUT4			
	Mean	SD	SEM	Fold	Mean	SD	SEM	Fold
DMSO	1.39	0.63	0.36	1.00	0.61	0.04	0.02	1.00
Insulin	0.97	0.86	0.50	0.70	0.97	0.68	0.39	1.59
BP16-94-15868	0.51	0.26	0.15	0.37	0.76	0.07	0.04	1.25
BP16-94-15880A	1.22	0.10	0.06	0.88	0.66	0.18	0.10	1.07
BP16-94-15887	0.82	0.69	0.40	0.59	1.02	0.72	0.41	1.66

Table 7.4.2 mRNA expression of enzymes glycogen synthase (A), pyruvate kinase (B) and pyruvate carboxylase (C) in C2C12 myocytes (See Figure 4.3)

	Glycogen Synthase				Pkm (Pyruvate kinase)			
	Mean	SD	SEM	Fold	Mean	SD	SEM	Fold
DMSO	0.55	0.17	0.10	1.00	0.47	0.06	0.03	1.00
Insulin	0.86	0.73	0.42	1.58	0.73	0.46	0.27	1.55
BP16-94-15868	0.68	0.23	0.13	1.25	0.60	0.08	0.05	1.28
BP16-94-15880A	0.64	0.28	0.16	1.17	0.52	0.02	0.01	1.12
BP16-94-15887	1.20	1.25	0.72	2.20	0.88	0.51	0.30	1.88

	Pcx (Pyruvate carboxylase)			
	Mean	SD	SEM	Fold
DMSO	0.41	0.10	0.05	1.00
Insulin	0.58	0.43	0.25	1.42
BP16-94-15868	0.47	0.03	0.01	1.15
BP16-94-15880A	0.43	0.03	0.02	1.06
BP16-94-15887	0.68	0.45	0.26	1.65

Table 7.4.3 Protein content of total AMP-activated protein kinase (AMPK) (A) and phosphorylated-AMPKα (Thr172) (B) (see Figure 4.4)

	pAMPK				tAMPK				% pAMPK/tAMPK
	Mean	**SD**	**SEM**	**Fold**	**Mean**	**SD**	**SEM**	**Fold**	**Ratio**
DMSO	0.40	0.19	0.10	1.00	0.48	0.38	0.22	1.00	83.82
Insulin	0.31	0.18	0.10	0.78	0.12	0.03	0.01	0.07	264.03
BP16-94-15868	0.50	0.53	0.30	1.26	0.33	0.14	0.08	0.36	154.04
BP16-94-15880A	1.94	1.43	0.82	4.87	1.74	2.43	1.72	6.35	111.68
BP16-94-15887	0.56	0.52	0.30	1.39	0.68	0.39	0.22	1.01	81.21

7.5 Ethical approval of the study

GRANTS INNOVATION & PRODUCT DEVELOPMENT
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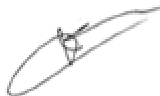
08 February 2016

To whom it may concern

This letter certifies that Ethical approval for Ms. Prenitha Sewnarain's PhD study was granted by the ethics committee at the South African Medical Research Council of South Africa (ECRA 11/03/A). The study was performed in accordance with the principles and guidelines of the South African Medical Research Council as outlined in Guidelines on Ethics for Medical Research: Use of Animals in Research and Training, 2004 (http//www.mrc.ac.za/ethics/ethicsbook3.pdf).

Yours sincerely



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CHAPTER 8 - Acknowledgements

8.1 Acknowledgements

8.1 Acknowledgements

My sincere thanks and appreciation goes out to my co-supervisor, Dr Christo Muller for his motivation and help, because without you this would probably not have been possible. Thank you for your priceless knowledge, technical contribution, support, time and guidance during the supervision of this work. My supervisor Prof Pete Smith, thank you for leading the way forward and your help and assistance when needed.

Particular attention also must be made to my research group leader (RGL), Dr Gerda Fouche for her technical advice, input, encouragement and support. To all my fellow colleagues, it has been quite a ride, but thank you for your part in it.

A special and huge thank you goes to my MRC colleagues; Dr Carmen Pheiffer, Dr Rabia Johnson, Dr Micheline Sanderson and Dr Sithandiwe Mazibuko. Another distinctive acknowledgment is to Torren Peakman for his invaluable help with a very difficult part of this project. I would like to also acknowledge my UCT colleagues; Prof Pete Smith and Lubbe Wiesner for their contribution to the pharmacokinetic analysis.

I dedicate this thesis to two important sets of people in my life; the memory of my late dad Satish, and my mum Monica, for their unwavering love and support because without them this would not be possible. My inspiration, my mom, I want to say thank you for getting me started and the part you played in my life. A heartfelt appreciation and genuine thanks for my two very good friends for life, Adhika Singh and Mohini Moodley for their heartfelt friendship and keeping me sane through this insane process. A special thank you to Niron Singh for going out of his way and helping me whenever things were not going right. Love and recognition must be mentioned to my husband Ravin, brother Dinesh, sister Manisha, bother in law Rishen, sister in law Risha and other friends that have played an instrumental part of my life. And last but not least, my two beautiful children Keshav and Kitania, I hope you both grow up and value the education given to you.

~~Jai Sri Krishna~~