

**ANTI-DIABETIC AND PHYTOCHEMICAL ANALYSIS OF
SUTHERLANDIA FRUTESCENS EXTRACTS**

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**Submitted in fulfillment of the requirements for the degree of
*Philosophiae Doctor***

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2016

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DECLARATION

I, Ogheneochuko Janet Adefuye, student number 212485520, hereby declare that except where due acknowledgements is made by reference, the thesis: Anti-diabetic and Phytochemical analysis of *Sutherlandia frutescens* extracts, for the degree of Philosophiae Doctor is my own work. No portion of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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Ogheneochuko J. Adefuye

January, 2016

ACKNOWLEDGEMENTS

I give all praise to Almighty God, the Creator of the heavens and the earth, the one who has every day of my life spread out before Him. I am forever grateful to God for the guidance, wisdom and the strength to complete this study successfully. You have exalted my horn like the horn of a unicorn and now my cup runs over with fresh oil. How excellent is Your name, Oh Lord my God, in all the earth. Thank you blessed redeemer.

I sincerely express my profound gratitude and appreciation to my supervisor, Professor Gill Dealtry, whom God has used to make this achievement a reality. I would like to thank you for your guidance, professional advice, support and encouragement throughout the duration of this project. You are everything a student could wish for in a supervisor.

I would also like to acknowledge Dr T Koekemoer, Steve Clarke for their expert advice in cell culture and all members of Prof Gill Dealtry group for their intellectual and moral support towards the successful completion of this work.

Thanks to all post-graduate students, as well as all members of staff of the Department of Biochemistry and Microbiology, NMMU for their assistance during the period of this study.

To the NMMU non-academic staff, Lawyer especially, thank you for your help and support.

Special thanks to Prof Graeme Bradley, Nhamo Mutingwende and Lara Kinghorn for providing Triple TOF LC-MS/MS services.

I would also like to thank National Research Foundation of South Africa (NRF) and the NMMU RCD for funding this project. I sincerely appreciate your support.

To my mother, sisters, brothers, uncles, aunts, friends, brother in-law, sisters in-law and mother-in-law, thank you so much for your prayers, love and support. May God bless you.

Thanks to Pastor Vic Klackers, Lathicia Klackers, Cynthia and Voke for the love, kindness, prayers and support.

To my darling husband and best friend, Dr Anthonio Oladele Adefuye, I say thank you for your prayers, unconditional love, endless support and encouragement during the period of this study. You have motivated and given me strength, and the rare qualities you possess inspire me every day. My lovely children, Mary Adeola and John Jesulayomi, thank you so much for your prayers, love, unending patience and understanding, indeed your presence in my life has been a great blessing.

DEDICATION

This thesis is

Dedicated to my beloved husband,

Dr Anthonio Oladele Adefuye

CONFERENCE PROCEEDINGS

O.J. Adefuye and G.B. Dealtry. Anti-diabetic and phytochemical analysis of extracts of *Sutherlandia frutescens*. **Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) Congress, 16-19 April 2015.**

O.J. Adefuye and G.B. Dealtry. Phytochemical analysis of the anti-diabetic activity of *Sutherlandia frutescens* extracts. **Physiology Society of Southern Africa (PSSA) Conference, 6-9 September 2015.**

PUBLICATIONS

O.J. Adefuye and G.B. Dealtry (2015). Anti-diabetic and phytochemical analysis of extracts of *Sutherlandia frutescens*. *Journal of Endocrinology, Metabolism and Diabetes of South Africa*. Volume 20 (1), p 44-45. (*Published abstract*)

O.J. Adefuye and G.B. Dealtry (2015). *Sutherlandia frutescens* prevents diabetic metabolic changes in insulin resistant hepatic cells. (*Manuscript ready for submission*)

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
μ	Mu
μL	Microlitre
$^{\circ}\text{C}$	Degrees Celsius
ACC	Acetyl-CoA Carboxylase
AMP	Adenosine 5'-Monophosphate
AMPK	AMP-activated Protein Kinase
aPKC	Atypical Protein Kinase C
ATP	Adenosine 5'-Triphosphate
ATP5B	Mitochondrial Adenosine 5'-Triphosphate synthase subunit β
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
ChREBP	Carbohydrate Response Element Binding Protein
C_q	Quantification Cycle
DAG	Diacylglycerol
ddH ₂ O	Deionised Distilled Water
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
dsDNA	Double Stranded DNA
EGP	Endogenous Glucose Production
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic Reticulum
F6P	Fructose 6-Phosphate
FAF-BSA	Fatty Acid-free Bovine Serum Albumin
FBS	Foetal Bovine Serum
FFA	Free Fatty Acid
g	Gram
G6P	Glucose 6-Phosphate
G6Pase	Glucose 6-Phosphatase
GLUT	Glucose Transporter
HBSS	Hank's Balanced Salts Solution
HEPES	N'-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
HGP	Hepatic Glucose Production
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome

HK	Hexokinase
IF	MCDB-201 medium supplemented with Insulin and Fructose
IFM	IF medium supplemented with metformin
IFSF	IF medium supplemented with <i>S. frutescens</i>
iNOS	Inducible Nitric Oxide Synthase
IR	Insulin Resistance
IRR	Insulin Receptor-related Receptor
IRS	Insulin Receptor Substrate
JIP1	JNK-interacting Protein 1
JNK	c-Jun N-terminal Kinase
kDa	Kilo Dalton
L	Litre
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
m	Milli
M	Molar
MAG	Monoacylglycerol
MAPK8	Mitogen Activated Protein Kinase 8
MIF	MCDB Supplemented with Insulin and Fructose
MPB	MCDB Supplemented with Palmitate-BSA
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
MTT	4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide
NAD ⁺	Nicotinamide Adenine Dinucleotide (Oxidised)
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NaOH	Sodium Hydroxide
NEAA	Non-essential Amino Acids
PB	MCDB-201 medium supplemented with Palmitate-BSA conjugate
PBS	Phosphate Buffered Saline
PDH	Pyruvate Dehydrogenase
PDK	Phosphoinositide-dependent Protein Kinase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate Carboxykinase
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C

PM	PB medium supplemented with metformin
PPAR α	Peroxisome Proliferator-activated Receptor α
PPAR γ	Peroxisome Proliferator-activated Receptor γ
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse Transcriptase
SH2	<i>Src</i> Homology 2
SIRT1	Sirtuin 1
SREBP-1c	Sterol Regulatory Element Binding Protein 1c
T2DM	Type 2 Diabetes Mellitus
T _a	Annealing Temperature
TAG	Triacylglycerol
TATA-BP	TATA Binding Protein
TLC	Thin-layer Chromatography
TNF- α	Tumour Necrosis Factor alpha

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SYNOPSIS

In Africa, the importance of medicinal plants in folklore medicine and their contribution to primary healthcare is well recognized. Across the continent, local herbal mixtures still provide the only therapeutic option for about 80% of the population. The vast floral diversity and the intrinsic ethnobotanical knowledge has been the backbone of localized traditional herbal medical practices. In Africa, an estimated 5400 of the 60000 described plant taxa possess over 16300 therapeutic uses. Similarly, with a therapeutic flora comprising of approximately 650 species, herbal medical practitioners in South Africa, make use of a plethora of plants to treat different human diseases and infections. Over the years, studies have identified numerous plant species with potential against chronic metabolic diseases including type 2 diabetes mellitus (T2DM).

Globally, the incidence and prevalence of T2DM have reached epidemic proportions affecting people of all ages, nationalities and ethnicity. Considered the fourth leading cause of deaths by disease, T2DM is a global health crisis with an estimated diagnosis and mortality frequency of 1 every 5 seconds and 1 every 7 seconds respectively. Though the exact pathophysiology of T2DM is not entirely understood, initial peripheral insulin resistance in adipose tissue, liver, and skeletal muscle with subsequent pancreatic β -cell dysfunction resulting from an attempt to compensate for insulin resistance is a common feature of the disease. The current approach to treating T2DM is the use of oral antidiabetic agents (OAs), insulin, and incretin-based drugs in an attempt to achieve glycaemic control and maintain glucose homeostasis. However, conventional anti-T2DM drugs have been shown to have limited efficacies and serious adverse effects. Hence, the need for newer, more efficacious and safer anti-T2DM agents.

Sutherlandia frutescens subsp. *microphylla* is a flowering shrub of the pea family (*Fabaceae/Leguminaceae*) found mainly in the Western Cape and Karoo regions of Southern

Africa. Concoctions of various parts of the plant are used in the management of different ailments including T2DM. However, despite extensive biological and pharmacological studies, few analyses exist of the chemical constituents of *S. frutescens* and no Triple Time of Flight Liquid Chromatography with Mass Spectrometry (Triple TOF LC/MS/MS) analysis has been performed.

The initial aim of this study was to investigate the phytochemical profile of hot aqueous, cold aqueous, 80% ethanolic, 100% ethanolic, 80% methanolic and 100% methanolic extracts of a single source *S. frutescens* plant material using colorimetric and spectrophotometric analysis. The hot aqueous extractant was found to be the best extractant for *S. frutescens*, yielding 1.99 g of crude extract from 16 g fresh powdered plant material. This data suggests that application of heat and water as the extractant (hot aqueous) could play a vital role in extraction of bioactive compounds from *S. frutescens* and also justifies the traditional use of a tea infusion of *S. frutescens*. Colorimetric analysis revealed the presence of flavonoids, flavonols, tannins, and phenols in all extracts with varying intensity. The organic extracts 100% methanol, 80% and 100% ethanol exhibited high color intensity (+++) for flavonoids and flavonols respectively, while all the extracts exhibited a moderate color intensity (++) for tannins and phenols. Spectrophotometric analysis of *S. frutescens* extracts revealed that all the organic extracts contained a significantly higher concentration (in mg/g of extract) of flavonols and tannins when compared to the aqueous extracts. All extracts contained approximately equal levels of phenols. These data confirm the presence of all four groups of bioactive phytochemicals in the *S. frutescens* extracts used in this study, and also confirm that different solvent extractants possess the capability to differentially extract specific groups of phytochemicals.

Analysis of the ion chromatogram generated from crude and solid phase extraction (SPE) fractions of *S. frutescens* extract, using the untargeted Triple TOF LC-MS/MS separation technique in positive and negative ionic mode, revealed the presence of multiple compounds

in individual extracts. Further comparison of these compounds with online databases of anti-diabetic phytocompounds led to the preliminary identification of 10 possible anti-diabetic compounds; α -Pinene, Limonene, Sabinene, Carvone, Myricetin, Rutin, Stigmasterol, Emodin, Sarpagine and Hypoglycin B in crude and solid phase extraction (SPE) fractions of *S. frutescens*.

Furthermore, using two hepatic cell lines (Chang and HepG2) as an *in-vitro* model system, the anti-T2DM properties of crude aqueous and organic extracts of *S. frutescens* was investigated and compared. Both aqueous and organic extracts of *S. frutescens* were found to decrease gluconeogenesis, increase glucose uptake and decrease lipid accumulation (Triacylglycerol, Diacylglycerol, and Monoacylglycerol) in Chang and HepG2 hepatic cell cultures made insulin resistant (IR) following exposure to high concentration of insulin and fructose.

Using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), the aqueous and organic extracts of *S. frutescens* were confirmed to regulate the expression of Vesicle-associated membrane protein 3 (VAMP3), Mitogen-activated protein kinase 8 (MAPK8), and Insulin receptor substrate 1 (IRS1) in insulin resistant hepatic cells. IR-mediated downregulation of VAMP3, MAPK8, and IRS1 mRNA in IR HepG2 hepatic cell cultures was reversed in the presence of aqueous and organic extracts of *S. frutescens*. The hot aqueous extract displayed the highest activity in all the assays, while all the organic extracts displayed similar potency.

In conclusion, this study reports that aqueous and organic extracts of *S. frutescens* possess numerous anti-diabetic compounds that can be further investigated for the development of new, more efficacious and less toxic anti-diabetic agents. The presence of multiple compounds in a single extract does suggest a synergistic or combinatorial therapeutic effect. These findings

support the burgeoning body of *in-vivo* and *in-vitro* literature evidence on the anti-diabetic properties of *S. frutescens* and its use in folklore medicine

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Medicinal plants represent the oldest source of pharmacotherapy used by mankind [1]. According to the World Health Organization (WHO), about 80% of the world's population now depend on traditional medicine for their primary healthcare needs [2] due to poverty and lack of access to modern medicine [3] (Figure 1.1) (Table 1). Hence, over the past decade the World Health Organization has undertaken various strategies to further identify and strategize on the future of traditional medicines (TM) around the world [4].

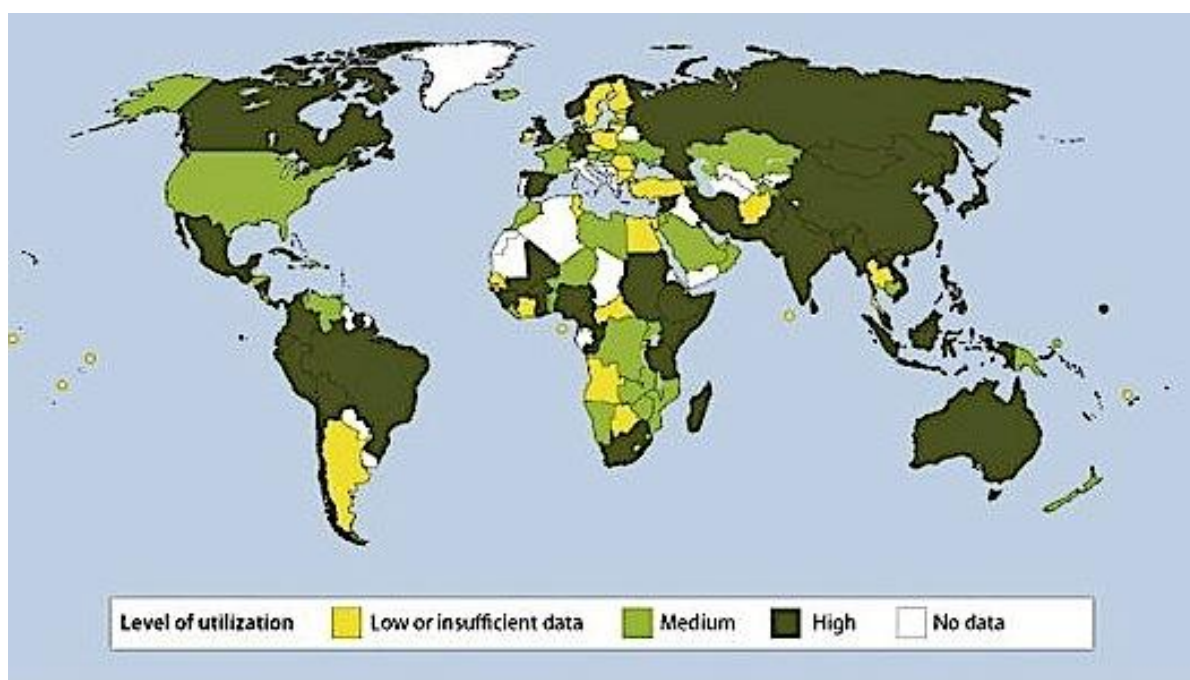


Figure 1.1: Global distribution of utilization of traditional medicine [5]

Table 1.1: Estimated global use of traditional medicine by patients and practitioners [6, 7]

Region or country	Extent of Use
Africa	<ul style="list-style-type: none">▪ Used by 80% of the population for primary health care.
China	<ul style="list-style-type: none">▪ Accounts for 30% - 50% of total health care.▪ Fully integrated into the health system, 95% of Chinese hospitals have TM units.
India	<ul style="list-style-type: none">▪ Widely used, about 2,860 hospitals provide TM.
Indonesia	<ul style="list-style-type: none">▪ Used by 40% of the entire population.▪ Used by 70% of the rural population.
Japan	<ul style="list-style-type: none">▪ 72% of physicians practice TM.
Thailand	<ul style="list-style-type: none">▪ TM integrated into 1,120 health centers.
Vietnam	<ul style="list-style-type: none">▪ Fully integrated into the health care system.▪ 30% of the population is treated with TM.
Western countries	<ul style="list-style-type: none">▪ TM not strongly integrated into the health care system.

In October, 2013, the World Health Organization launched the WHO Traditional Medicine Strategy 2014-2023 [8] with the view of developing TM based research innovation in line with the “Global Strategy and Plan of Action on Public Health, Innovation and Intellectual Property” adopted at the 61st World Health Assembly in 2008. It is now widely accepted that the traditional medical knowledge of medicinal plants and their use by indigenous cultures is not only useful for conservation of cultural traditions and biodiversity, but also for community

healthcare and drug development in the present and future [2]. Consistent with this, emerging evidence from pharmaceutical companies shows that natural products (including medicinal plants) represent a valuable source for the production of new chemical entities for the treatment of complex diseases [9, 10]. Based on recent technological advances in science, the options to produce high quality herbal medicinal products have been largely improved and the acceptance of phytotherapy is considered as an alternative or adjunct to conventional therapy synthetic drugs is presently on the rise within the general public [1]. However, in spite of this, the use of medicinal plants for disease management has been heavily criticized by physicians and their respective societies, owing to limited research and clinical trials that clearly demonstrate their efficacy and safety.

1.2 Phytotherapy in sub-Saharan Africa

In Africa, the importance of medicinal plants in folklore medicine and their contribution to primary healthcare across the continent is well recognized [11]. Across the continent, the use of medicinal plants has successfully complemented the orthodox medical system in the fight against various diseases and infections [12]. Local herbal mixtures still provide the only therapeutic option for about 80% of the population [13], a situation compounded by a persistently low medical doctor-to-patient ratio (1:40,000) [14] and long hospital waiting hours (6-12 hours) in some places [15]. Furthermore, a favorable ratio of one traditional medicine practitioner per 500 patients [16] has made traditional medicine the most accessible health care system for a large number of African populations resident in the rural and semi urban areas [17]. The therapeutic viewpoint of the use of medicinal plants is primarily based on their role in the prevention and promotion of good health, hence concoctions of plant extracts are regularly consumed as rejuvenators, tonics and/or nutritional supplements across

the continent [18]. This has led to a significant amount of biomedical and pharmacological research being done to validate the use of plant extracts as medicinal remedies in Africa [12].

1.2.1 Botanical diversity: backbone of traditional medical practices in Africa

The vast floral diversity in different parts of the African continent and the intrinsic ethnobotanical knowledge has been the backbone of localized traditional herbal medical practices for several years [19]. Most medicinal plants still remain locally derived, but are utilized worldwide for disease management. In Africa, an estimated 9% (5400) of the 60000 described plant taxa possess over 16300 therapeutic uses [20]. Over the years, scientists have identified numerous plant species with potential against common ailments including malaria [21], HIV/AIDS [22], Tuberculosis [23], microbial infection [24, 25], cancer [26], pain and inflammation [27], helminthiasis [28] and diabetes mellitus [29] (Table 2). Today, plants emanating from Africa constitute approximately 8% of the 1100 medicinal plants commercialized globally [30], for example, Niprisan®, is a phytomedicine used in Nigeria for the management of sickle cell disorder [31].

Table 1.2: Selected African plants of therapeutic importance [19, 30]

Family	Species	Common names	Biological activity
Aizoaceae	<i>Sceletium tortuosum</i>	Sceletium herb, Kanna, Channa, Kougoe	Sedative Anti-depressant
Annonaceae	<i>Xylopia aethiopica</i>	African grains of Selim, Ethiopian pepper, Senegal pepper	Anti-microbial Bronchitis Female infertility Anti-diuretic
Apocynaceae	<i>Cryptolepis sanguinolenta</i>	Ghanaian quinine, Yellow- dye root	Anti-cancer Anti-microbial Anti-malaria Anti-inflammatory Anti- hyperglycaemic
Burseraceae	<i>Boswellia sacra</i> Flueck.	Frankincense, olibanum-tree	Anti-microbial Anti-inflammatory Cytotoxic Rheumatoid arthritis
Canellaceae	<i>Warburgia salutaris</i>	Muranga, Pepper Bark Tree	Anti-microbial Molluscicidal Anti-diuretic Cytotoxic

Family	Species	Common names	Biological activity
Combretaceae	<i>Terminalia sericea</i>	Silver cluster-leaf, Silver terminalia	Anti-oxidant Anti-microbial Anti-inflammatory Anti-carcinogenic Anti-diabetic Adaptogen (stress and anxiety)
Convolvulaceae	<i>Ipomoea pes-caprae</i> subsp. <i>brasiliensis</i>	Beach morning glory, Goat's foot	Anti-microbial Anti-histaminic Anti-inflammatory Anti-spasmodic
Euphorbiaceae	<i>Euphorbia hirta</i>	Asthma plant, Red euphorbia	Anti-microbial Anti-spasmodic Anti-protozoal Anti-inflammatory
Geraniaceae	<i>Pelargonium sidoides</i>	Umckaloabo, South African Geranium	Acute bronchitis Anti-microbial Anti-virus
Leguminosae	<i>Sutherlandia frutescens</i>	Cancer bush, Balloon pea, Sutherlandia	Anti-oxidant Anti-inflammatory Anti-diabetic Anti-HIV Anxiety

Malvaceae	<i>Trichilia emetica</i> Vahl	Natal mahogany	Anti-microbial Anti-inflammatory Anti-trypanosomal Anti-plasmodial Anthelmintic Hepatoprotective
Pedaliaceae	<i>Harpagophytum procumbens</i>	Devil's claw	Anti-inflammatory Analgesic Antioxidant Anti-malaria
Rosaceae	<i>Prunus africana</i> (Hook.f.) Kalkman	Red Stinkwood	Anti-microbial Anti-malaria Anti-inflammatory Kidney disease
Strelitziaceae	<i>Ravenala madagascariensis</i> Sonn	Traveller's tree, Traveller's palm	Anti-microbial Anti-septic
Xanthorrhoeaceae	<i>Aloe ferox</i> Mill.	Cape aloe	Laxative Skin diseases
Zygophyllaceae	<i>Balanites aegyptiaca</i> (L.) Delil	Soapberry tree	Anti-microbial Anti-oxidant Anti-malaria Anti-diabetic Anthelmintic Molluscicidal

1.3 Medicinal plants and traditional medical practices in South Africa

Stretching between latitude 22°S to 35°S and longitude 17°E to 33°E, South Africa's surface area covers 1, 219 602 km² [32] inhabited by diverse ethnic groups/tribes including the Nguni's (Zulu, Xhosa, Ndebele and Swazi people); Tsonga and Venda; Sotho-Tswana; Afrikaners; colored (a group which include the descendants of unions between indigenous and European people and a Muslim minority known as the "Cape Malays"); English; Indians; and the Khoi and the San tribe [33], who depend on several species of medicinal plants for the treatment of diverse human ailments [34]. With a therapeutic flora comprising of approximately 650 species [35] of which 148 are identified as the most widely used [36, 37], herbal medical practitioners make use of a plethora of plants to treat different human diseases and infections.

In the Zululand, medicinal plants are used for the therapeutic management of neurological disorders (convulsion, epilepsy, Alzheimer's disease and Parkinson) [38], cutaneous bacterial infections (carbuncles) [39], gynaecological disorders, contraceptive and as an abortifacient [40]. Similarly, in the Limpopo Province, herbal concoctions are used for the management of gastrointestinal (GIT) disorders such as diarrhea [41], while in the Eastern and Western Cape Provinces herbal mixtures are used for the management of metabolic disorders such as diabetes mellitus [42] and cardiovascular conditions (hypertension) [43]. In addition, a number of these plants are also used as traditional veterinary medicine to treat common livestock diseases [44]. The dependence of a vast majority of the populace on medicinal plants as an alternative health care has prompted significant research investment in medicinal plants in the country, which can be regarded as the epicenter of innovation in African ethnopharmacology [12].

1.4 Bioactive phytochemicals: potent therapeutic principle in medicinal plants

The isolation of a pure pharmacological active compound “morphine” from seed pods of the poppy plant, *Papaver somniferum* by Friedrich Sertürner [45] initiated an era wherein phytochemicals from plants could be purified into drugs, to be studied and administered [46]. It is now widely accepted that the effectiveness of medicinal plants and their pharmacotherapeutic action is due to their complex diversity of chemical compounds [47]. Different plants may possess a wide spectrum of effects due to the presence of various groups of chemical compounds and various microelements [48]. Preparations obtained from a single plant can simultaneously be analgesic, sedative, cardiostimulant, anti-inflammatory, and expectorant [48].

1.5 Classification of bioactive phytochemicals

Phytochemicals are classified by their biogenetic origin. Based on their biosynthetic pathways, bioactive phytochemicals can be classified into the following groups: alkaloids, glycosides (saponins, phenols, cyanogenic glycoside and cardiac glycosides), lactones, and others (e.g. pigments and ecdysteroids) [48, 49].

1.5.1 Alkaloids

Alkaloids are large groups of naturally occurring nitrogen-containing organic bases found in plants and certain animal species [50, 51]. According to the dictionary of Natural Products more than 27,000 naturally occurring alkaloids have already been identified [52]. A single plant species usually contains few kinds of alkaloids, but certain plant families such as

Ranunculaceae (buttercups), *Solanaceae* (nightshades), *Amaryllidaceae* (amaryllis), and *Papaveraceae* (poppies family) are particularly rich in alkaloids [53]. Similarly, certain alkaloids such as latrunculin A and batrachotoxin can be found naturally occurring in animal species, such as *Negombata magnifica* (Red Sea sponge) and *Phyllobates* spp. (poison dart frogs) respectively [53].

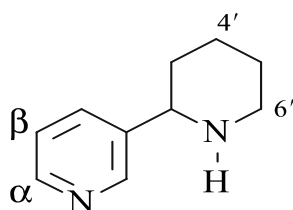
For over 300 years, alkaloid-containing extracts have been used as therapeutics [53]. Toxic alkaloids (from plant extracts) such as curare, aconitine, and tubocurarine are used to poison arrows in hunting, or as weapons of warfare [54]. Morphine became the first alkaloid to be isolated by modern chemistry in the 19th century [55], since then several other alkaloids such as caffeine, coniine, and nicotine have been discovered [53]. Because of their potent biological effects, alkaloids and their derivatives have an array of pharmacological applications in contemporary medicine. These include anti-hypertensive (e.g. reserpine), anti-pyretics (e.g. quinine), antibacterial (e.g. ciprofloxacin), anti-hyperglycemic (e.g. piperine) analgesic (e.g. morphine), anti-asthmatic (e.g. ephedrine), anti-arrhythmic (e.g. quinidine), and anti-cancer (e.g. eberberine) effects [53].

In contrast to other classes of natural compounds, alkaloids have practically unlimited structural frame works and contain an N atom in their molecules. For this reason, alkaloids are highly variable [56]. In plants, alkaloids are found mainly in leaves, seeds, or roots as salts of various organic and mineral acids. Their content varies widely depending on the species and its origin, the vegetation period of the plant, and the environmental conditions such as climate, soil, and humidity [56]. According to the distribution of their principal structure i.e. the principal C-N skeleton [56], alkaloids are classified into the following large groups: pyrrolidine, pyridine, quinoline, isoquinoline, indole, quinazoline, steroidal, diterpenoid, and other alkaloids, with each group subdivided into several subgroups depending on the structural

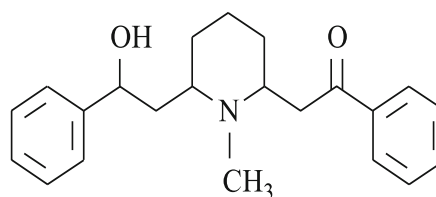
features of its representatives [57, 58].

1.5.1.1 Pyrrolidine, Pyridine, and Piperidine alkaloids

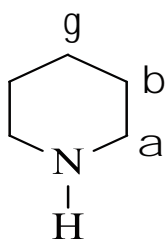
Pyrrolidine, pyridine and piperidine alkaloids are widely distributed in nature and have been found in about 27 plant species. These compounds are subdivided into simple derivatives of pyridine (3-methoxypyridine) and piperidine (coniine), and bi- and tricyclic uncondensed derivatives of pyridine (anabasine) and piperidine (lobeline). Codonopsine represent a new type of pyrrolidine base [56] (Figure 1.2).



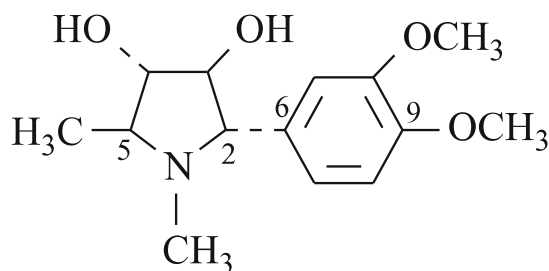
(+)-Anabasine [$C_{10}H_{14}N_2$]



(±)-Lobeline [$C_{22}H_{27}NO_2$]



Piperidine [$C_5H_{11}N$]

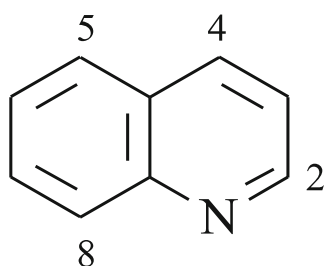


Codonopsine [$C_{14}H_{21}NO_4$]

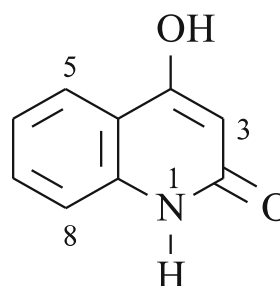
Figure 1.2: Chemical structure of Pyridine (Anabasine), Piperidine (lobeline and piperidine), and Pyrrolidine (Condonopsine) alkaloids [56].

1.5.1.2 Quinoline alkaloids

Quinoline alkaloids are found in about 13 plant families. However, most quinoline alkaloids have been isolated from plants of the genus *Haplophyllum* from the Central Asian region. 4-hydroxyquinolin-2-one is an example of a quinolone alkaloid [59] (Figure 1.3).



Quinoline [C₉H₇N]

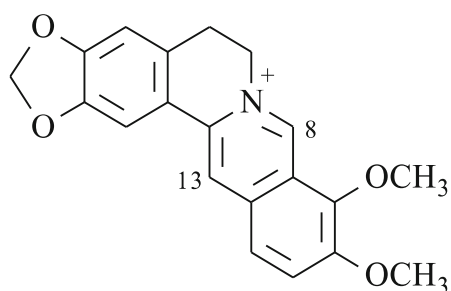


4-hydroxyquinolin-2-one [C₉H₇NO₂]

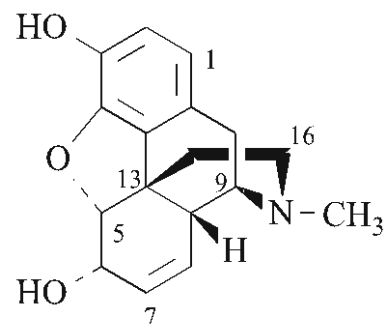
Figure 1.3: Chemical structure of quinoline (quinoline and 4-hydroxyquinolin-2-one) alkaloids [56]

1.5.1.3 Isoquinoline alkaloids (*Aporphine alkaloids*)

Isoquinoline alkaloids represent a large family (more than 700 compounds) [56, 60] of medicinal alkaloids found abundantly in plant families including Berberidaceae, Fumariaceae, Magnoliaceae, Papaveraceae, and Ranunculaceae [61]. Largely represented by aporphine alkaloids, isoquinoline alkaloids have a biphenyl system, which can be substituted (di-, tri-, tetra-, penta-, and hexa) with a hydroxyl, methoxyl, and methylenedioxy derivatives on all four rings [56]. Morphine and Berberine are examples of isoquinoline alkaloids (Figure 1.4)



Berberine [$C_{20}H_{18}N^+O_4$]



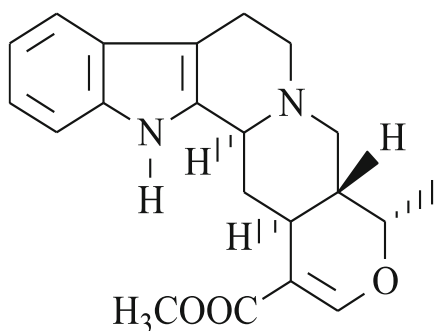
Morphine [$C_{17}H_{19}NO_3$]

Figure

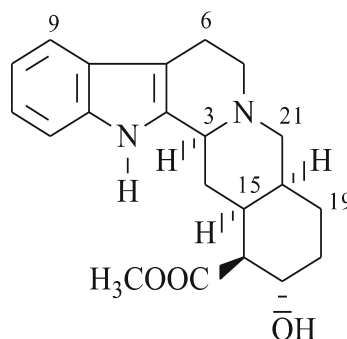
1.4:Chemical structure of isoquinoline (Berberine and Morphine) alkaloids [60, 61]

1.5.1.4 Indole alkaloids

Indole alkaloids consist of a large family of compounds (about 4100) [62] that contain an indole moiety (an aromatic heterocyclic organic compound) included in their structure [63]. In addition, many indole alkaloids also include isoprene groups and are thus called terpene indole alkaloids [64]. These complex organic compounds possess significant biological activities and some of them are used in medicine [64]. Examples are ajmalicine from *Catharanthus roseus* and yohimbine from *Rauwolfia serpentine* which are used as an anti-hypertensive and an adrenergic receptor blocker, respectively [64-66] (Figure 1.5).



Ajmalicine [$C_{12}H_{24}N_2O_3$]

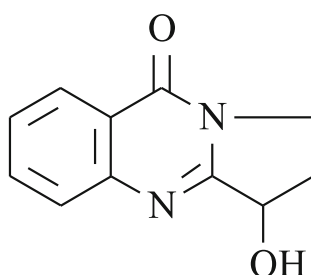


Yohimbine [$C_{21}H_{26}N_2O_3$]

Figure 1.5: Chemical structure of indole (Ajmalicine and yohimbine) alkaloids [56]

1.5.1.5 Quinazoline alkaloids

Quinazoline alkaloids form a small group of secondary natural occurring heterocyclic compounds [67]. Pharmacological studies of the properties of quinazoline alkaloids and their synthetic analogs revealed their anti-inflammatory [68], anti-HIV [69] and bacterial [70] properties. An example of quinazoline alkaloids is vasicinone, an anti-cancer compound obtained from methanolic extracts of *Peganum harmala* seed [71] (Figure 1.6).

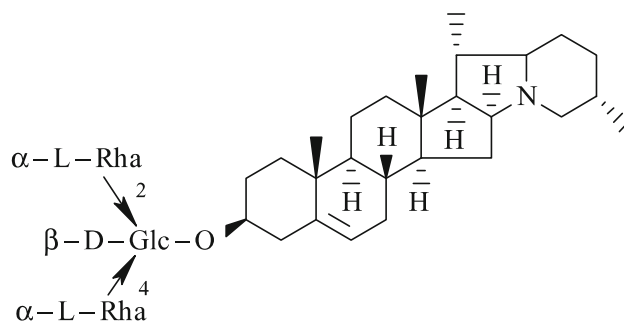


(±) Vasicinone [C₁₁H₁₀N₂O₂]

Figure 1.6: Chemical structure of quinazoline (Vasicinone) alkaloid [56]

1.5.1.6 Steroidal alkaloids

Steroidal alkaloids are an important class of alkaloids and secondary metabolites isolated from about 30 species of plant genera including *Veratrum*, *Petilium*, *Korolkowia*, *Rhinopetalum*, *Fritillaria*, *Zygadenus* (Liliaceae), *Solanum* (Solanaceae), and *Buxus* (Buxaceae) [72]. Similar to other alkaloids, steroidal alkaloids and their glycosides have been reported to have arrays of bioactivities, including anti-microbial [73], anti-inflammation [74] and anti-nociceptive [75] actions. An example of steroidal alkaloid is α -chaconine (Figure 1.7), which has been shown to exhibit anti-cancer activity *in-vitro* [76, 77].

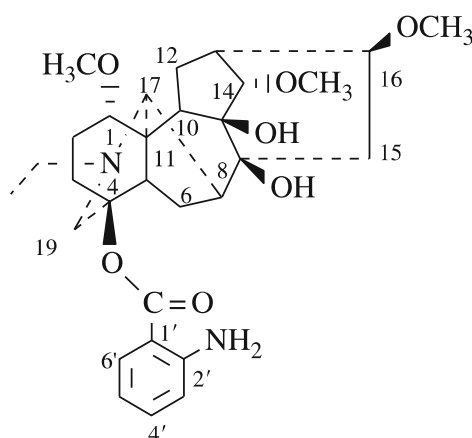


α-Chaconine [C₄₅H₇₃NO₁₄]

Figure 1.7: Chemical structure of steroidal (α-
chaconine) alkaloid [56, 77]

1.5.1.7 Diterpenoid alkaloids

Diterpenoid alkaloids were first isolated from roots of the plant genera *Aconitum* and *Delphinium* [78]. Based on extensive spectroscopic analyses diterpenoid alkaloids, have been structurally classified into three categories as C₁₈-, C₁₉-, and C₂₀- [79]. Diterpenoid alkaloids have been shown to exhibit anti-inflammatory (Lappaconitine) *in vitro* [80] (Figure 1.8), anti-cancer [81, 82] and anti-viral [79] activities.



Lappaconitine [C₃₂H₄₄N₂O₈]

Figure 1.8: Chemical structure of diterpenoid (Lappaconitine) alkaloid [56, 80]

1.5.1.8 General physical and chemical properties of alkaloids

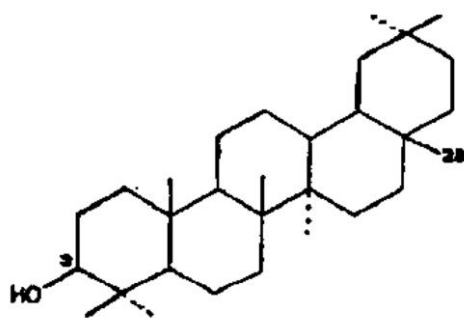
Alkaloids can exist in solid state (atropine) or liquid state. The solutions of alkaloids are known to be intensely bitter [83]. Alkaloids are basic in nature and the degree of basicity varies considerably, depending on the structure of the molecule and the presence and location of functional groups [84]. Alkaloids react with acids to form crystalline salts without the production of water [85]. Although alkaloids are sparingly soluble in water, most alkaloids and their salts are readily soluble in alcohol [86].

1.5.2 Glycosides

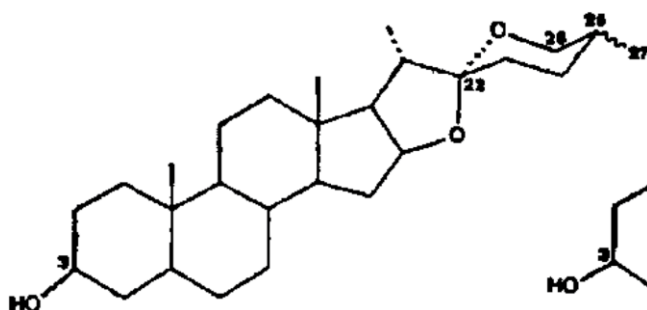
Plant glycosides are organic compounds composed of a sugar (glycoside, glycone) and a non-sugar (aglycone, genin) moiety [48, 85], with the aglycone forming the main physiologically active part [48]. The glycone (sugar moiety) can be attached to the aglycone in several ways. The most common bridging atom is oxygen (O-glycoside), however, it can also be sulphur (S-glycoside), nitrogen (N-glycoside) or carbon (C-glycoside). Based on the chemical nature of the aglycone, type of sugar constituent or pharmacological action, glycosides are categorized into saponins (the aglycones are triterpene and steroid compounds), phenolics (the aglycones are coumarins, flavonoids and others), cyanogenic glycosides (the aglycones contain prussic acid), cardiac glycosides (the aglycones are cardenolides and bufadienolides), anthraglycosides (the aglycones are derivatives of anthracene), and glycoalkaloids (the aglycones are nitrogen-containing steroid compounds) [48, 86]. Furthermore, based on the configuration of the hemiacetal hydroxyl group, glycosides can be classified as α -glycosides or β -glycosides. The majority of the naturally occurring glycosides are β -glycosides [87].

1.5.2.1 Saponins

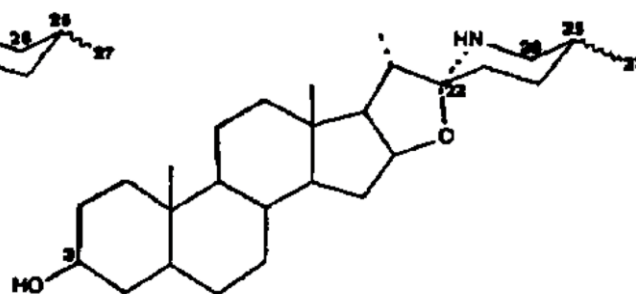
Saponins are naturally occurring high molecular weight glycosides originally isolated from the root of *Saponaria vaccaria* (soapwort plant) [88, 89]. Saponins are natural surfactants and detergents, giving stable foams in water [90]. The non-sugar (aglycone) portion of saponin is called sapogenin (genin) [90]. Depending on the type of genin present, saponin can be classified into three major classes: steroidal alkaloid saponin, steroidal saponin and triterpenoidal saponin [86, 90] (Figure 1.9). Furthermore, based on the numbers of sugars chains attached to the aglycone, saponins can be grouped into monodesmosidic (one sugar chain), bidesmosidic (two sugar chain) or tridesmosidic saponin (three sugar chain) (rarely found) [90] (Figure 1.10). Saponins are soluble in water and alcohol, but insoluble in non-polar organic solvents. Saponins are constituents of many plant drugs used in folk medicine. In contemporary medicine, the therapeutic uses of saponins include: expectorants, diuretics, hypocholesterolemics, and hypotensives [48]. However, in spite of their therapeutic usage, saponins are extremely toxic, causing irritation to the mucous membranes and hemolysis of the red blood cells when introduced intravenously [48, 91].



Triterpenoidal saponin



Steroidal saponin



Steroidal alkaloid saponin

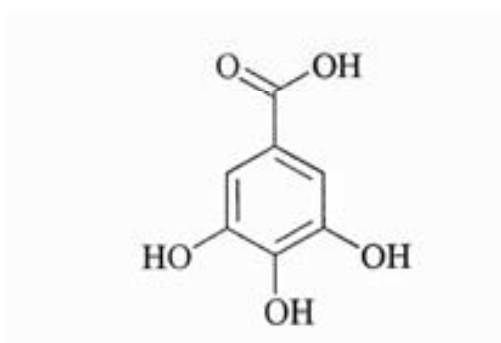
Figure 1.9: Chemical structure of Triterpenoidal saponin, Steroidal saponin and Steroidal alkaloid saponin [90]

1.5.2.2 Phenolics (*phenols*)

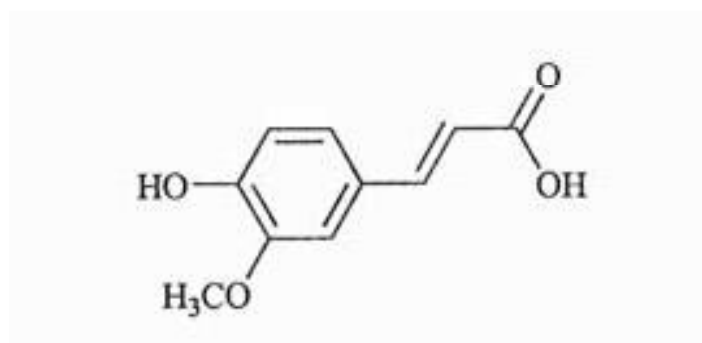
Phenolics are compounds that possess one or more aromatic ring with one or more hydroxyl groups [92]. Phenols are broadly distributed in plants, where they function in defense, as well as contributing to plants' colors [92]. More than 8,000 phenolic structures are currently known [92]. Phenols have the most diverse pharmacological activity. Amongst them are compounds with anti-hypoxic, anti-oxidant, choleretic, cardio-, angio-, and hepato- protecting and hemostatic actions [48]. Plant phenolics are classified into phenolic acids, flavonoids, tannins and the less common stilbenes and lignans [91, 92].

1.5.2.3 Phenolic acid

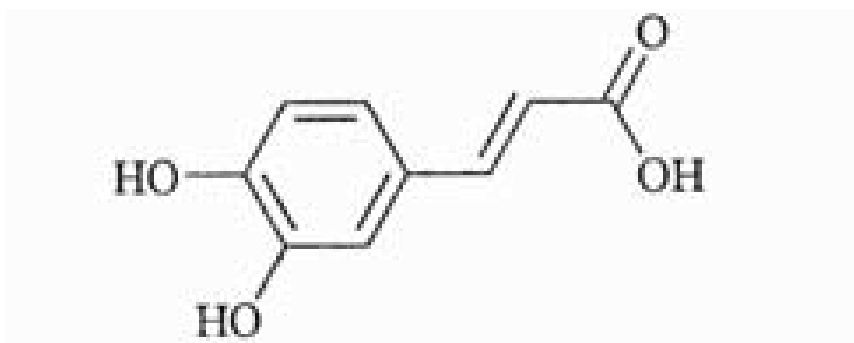
Phenolic acids can be divided into two groups: derivatives of benzoic acid (gallic acid) (Figure 1.11) and derivatives of cinnamic acid (coumaric, caffeic and ferulic acid). Caffeic acid is regarded as the most abundant phenolic acid, distributed in many fruits and vegetables [92].



Gallic acid



Ferulic acid

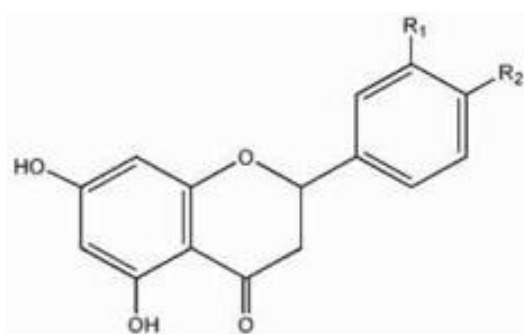


Caffeic acid

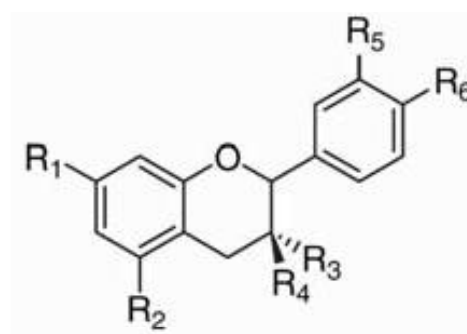
Figure 1.11: Chemical structures of phenolic (gallic, ferulic and caffeic) acids [92]

1.5.2.4 Flavonoids

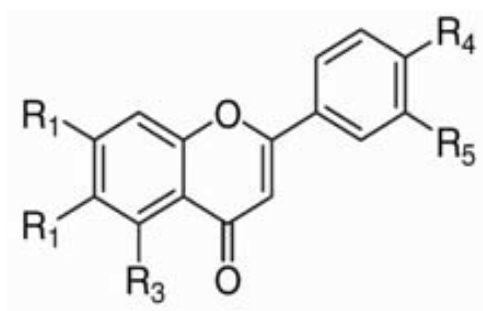
Flavonoids are a group of heterocyclic polyphenolic compounds widely distributed in plant flora [83]. The basic flavonoid structure is the flavan core, containing 15 carbon atoms arranged in three rings (C6-C3-C6), labeled A, B and C. Based on the oxidation state of the central C ring, flavonoids are divided into six subgroups namely: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins [86, 92] (Figure 1.12). Within each subgroup, structural variation is partly dependent on the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation.



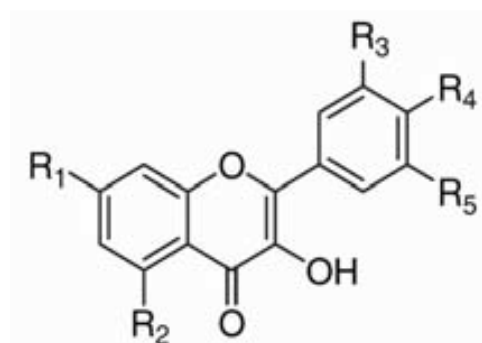
Flavanone



Flavanol



Flavone

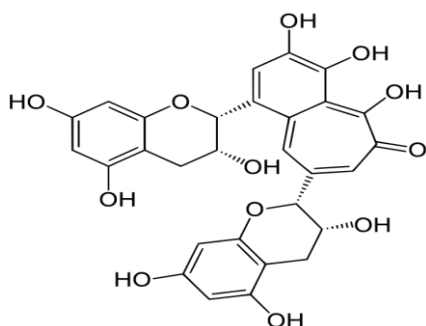


Flavonol

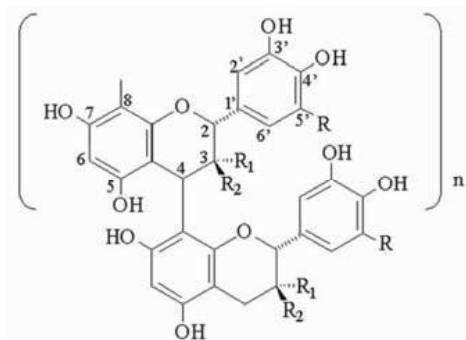
Figure 1.12: Chemical structures of flavonoids (flavanone, flavanol, flavone, flavonol) [92].

1.5.2.5 Tannins

Tannins are subdivided into two major groups: hydrolysable tannins and condensed tannins [83]. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, called gallotannins, or with hexahydroxydiphenic acid, called ellagitannins [93]. Common examples of hydrolysable tannins include theaflavins (from tea), daidzein, genistein and glycitein [93] (Figure 13). Condensed tannins also called proanthocyanidins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond (Figure 1.13). Tannins possess a wide variety of structures due to the many possibilities in forming oxidative linkage [93]. Tannin-rich medicinal plants are used as healing agents in a number of diseases including: diarrhea, leucorrhoea, and rhinorrhea [83].



Theaflavin



Proanthocyanidin

Figure 1.13: Chemical structure of Tannin (theaflavin and proanthocyanidin) [92]

1.5.2.6 Cyanogenic glycosides

Cyanogenic glycosides (CGs) are bioactive secondary metabolites of plants derived from amino acids with oximes and cyanohydrins (α -hydroxynitriles) as key intermediates [94]. The distribution of cyanogenic glycosides in the plant kingdom is relatively wide. About 2500 plant species belonging to families *Fabaceae*, *Rosaceae*, *Linaceae*, and *Compositae* are rich in CGs [95]. CGs are stable compounds, but when the β -glycosidic linkage is hydrolysed through the action of a β -glycosidase, the unstable cyanohydrin that is formed dissociates to release hydrogen cyanide in a process known as cyanogenesis [96]. Acute or chronic exposure to hydrogen cyanide can lead to severe morbidity or mortality in humans [94], and animals [97] due to its role in inhibiting the activity of cytochrome c oxidase, the final enzyme in the respiratory electron transport chain [98]. Prunasin and linamarin are examples of cyanogenic glycosides (Figure 1.14).

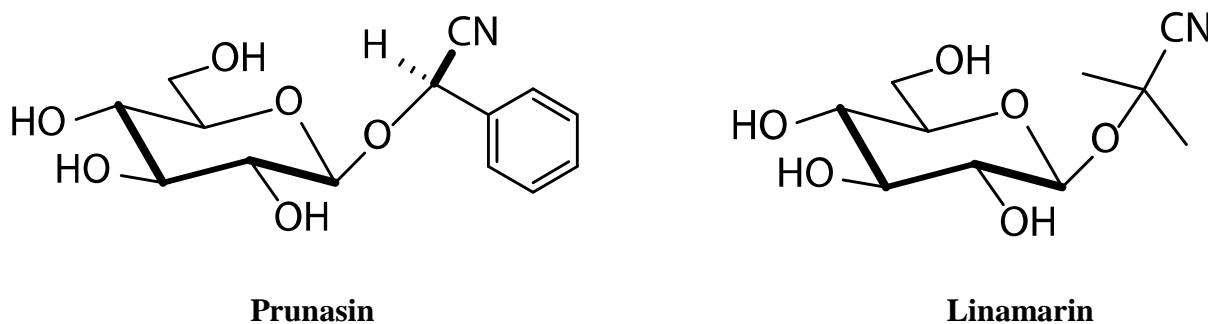
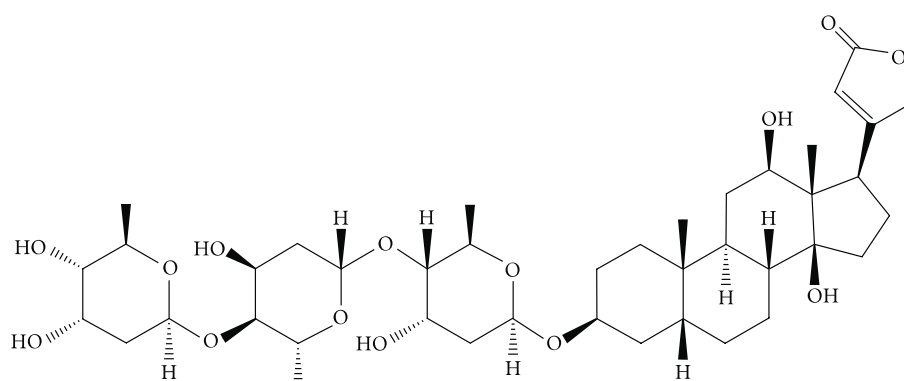


Figure 1.14: Chemical structures of prunasin and linamarin (cyanogenic glycosides) [94].

1.5.2.7 Cardiac glycosides

Cardiac glycosides are secondary metabolites found in diverse groups of plants including

Digitalis purpurea and *Digitalis lanata* (foxgloves), *Nerium oleander* (common oleander), *Thevetia peruviana* (yellow oleander), *Convallaria majalis* (lily of the valley), *Urginea maritima* and *Urginea indica* (squill) [99]. In clinical practice, cardiac glycosides are used in the treatment of congestive heart failure and cardiac arrhythmia (Digoxin; derived from *Digitalis purpurea*) [100] (Figure 1.15).



Digoxin [C₄₁H₆₄O₁₄]

Figure 1.15: Chemical structure of cardiac glycoside (Digoxin) [100]

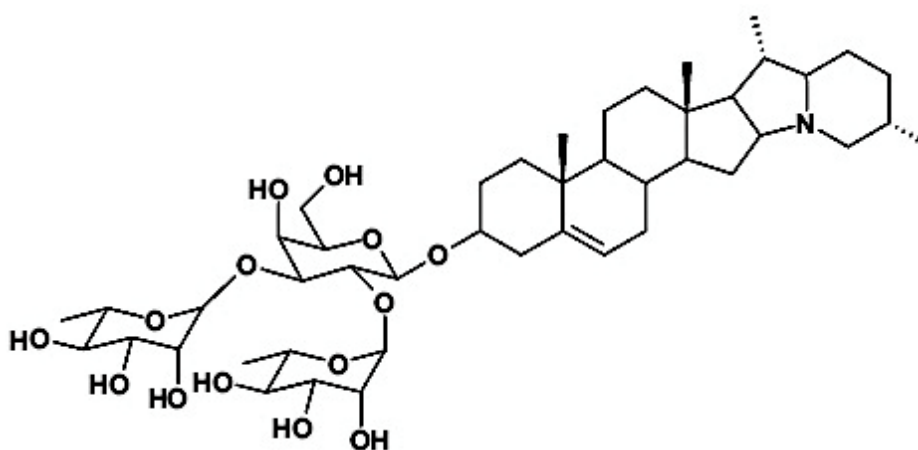
1.5.2.8 Anthraglycosides

Compounds belonging to the anthraglycosides are reddish in color. Plant extracts containing anthraglycosides exhibit purgative and choleretic actions [48].

1.5.2.9 Glycoalkaloids

Glycoalkaloids are secondary plant metabolites found in the family *Solanaceae* that include species such as *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato). They may be toxic to bacteria, fungi, viruses, insects, animals and humans at differing concentrations [101]. In plants, glycoalkaloids (e.g. solamargine) have been shown to exhibit phytoparasitic

nematocidal activity against root-node nematodes [102]. In humans, glycoalkaloids (solanine) (Figure 1.16) have been shown to exhibit anti-allergic properties in patients suffering from allergy to nightshade and cereals [101]. Similarly, in normal and adrenalectomized rats, intraperitoneal injection of solanine has been shown to induce a reversible decrease in blood sugar [101]. Furthermore, several *in-vitro* cell lines and animal studies have demonstrated the anti-pyretic, anti-inflammatory and anti-cancer properties of plant glycoalkaloids [101, 103, 104].



Solanine [C₄₅H₇₃NO₁₅]

Figure 1.16: Chemical structure of solanine (glycoalkaloid) [101]

1.5.2.10 General physical and chemical properties of glycosides

Glycosides are colorless, crystalline carbon, hydrogen and oxygen-, nitrogen-, or sulfur-containing water-soluble phytochemicals found in the cell sap. Glycosides are neutral in reaction and can be readily hydrolysed into their components with ferments or mineral acids [83].

1.6 Factors influencing levels of bioactive phytochemicals in medicinal plants

Variations in the level and type of phytochemicals found in plants largely depend on the active biosynthetic pathways during plant growth and development. Within the plant, biosynthetic pathways can be regulated by both intrinsic factors (genetic or species variation) and extrinsic factors (environmental conditions) [105].

1.6.1 *Species variation*

Over the years, several studies have shown that the phytochemical content found in plants exhibits high levels of interspecies and intraspecies variations [105]. However, while species variation invariably influences phytochemical content, interaction with environmental conditions can also influence the level of phytochemicals found in the same plant species.

1.6.2 *Environmental conditions*

Environmental factors such as soil composition, ultraviolet radiation, temperature variation and climatic conditions (rainfall and humidity) within a geographical location can affect the concentration of phytochemicals present in plants [105]. Wang et al. (2001) established a relationship between the level of phenolic acid, flavonols and anthocyanin content of plants with varying day/night temperature. In this study, a decrease in the level of phenolic acid was associated with day/night temperature of 18/22 °C, whereas an increase was observed with temperatures between 25/30 °C during the growing season [106]. In a similar study by Pék et al. (2013), levels of bioactive compounds in *Brassica oleracea* were found to vary significantly with environmental conditions and water status [107].

1.7 Processing of bioactive phytocompounds

1.7.1 Extraction of bioactive phytocompounds

Extraction of bioactive phytocompounds from plants materials is the principal step in the utilization of phytochemicals in therapeutic concoction or pharmaceutical products [92]. Phytocompounds can be extracted from fresh, frozen or dried plant samples. It has been shown that the extraction conditions can influence the level and bioavailability of phytochemicals in the therapeutic concoction. For example, freeze-drying has been shown to retain higher levels of phenolics content in plant samples than air-drying [108, 109]. Methods of phytochemical extraction include: solvent extraction, ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE), and microwave-assisted extraction (MAE) [110].

1.7.2 Solvent extraction

Solvent extraction is the most widely used procedure to prepare extracts from plant. A variety of chemical extractants are used, based on their ease of use, efficiency, and wide applicability [92]. Solvents of varying polarity (hexane, carbon tetrachloride, di-isopropyl ether, ethyl ether, methylene dichloride, tetrahydrofuran, chloroform, acetone, ethanol, ethyl acetate, methanol, water or mixtures of different solvents) are used on the plant material [111]. The yield and composition of extracted phytocompounds in chemical extraction is dependent on the solvent polarity, extraction time and temperature, sample-to-solvent ratio, as well as the chemical composition and physical characteristics of the samples [92]. Similarly, the type of solvent used may have an effect on the nature of the compounds extracted, and the resulting bioactivity of the extract [111]. To evaluate each extractant, parameters including the rate of extraction, the quantity extracted, the diversity of compounds extracted, the ease of subsequent handling

of the extracts, toxicity of the solvent in the bioassay process and the potential health hazard of the extractant must be considered [112].

1.7.3 *Ultrasound assisted extraction (UAE)*

Ultrasound-assisted extraction (UAE) is a potentially useful and cost effective technology that does not require complex instruments. UAE can be used for both small and large-scale phytopharmaceutical extraction [113]. The mechanism of action of UAE involves the shear force generated by implosion of cavitation bubbles upon the propagation of acoustic waves in the kHz range [114]. Collapse of bubbles can produce physical, chemical and mechanical effects, resulting in the disruption of biological membranes, that enhances penetration of solvent into cellular materials to augment the release of extractable compounds [115]. UAE has been widely used in the extraction of various phenolic compounds from different parts of plants, including leaves [116], stalks, fruits [117, 118], and plant seeds [119]. In addition to time conservation, UAE has been shown to cause less degradation of phytochemicals compared to other methods of extraction [120].

1.7.4 *Pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE)*

Pressurized liquid extraction (or ASE) is a relatively new method for phytochemical extraction that involves a high temperature and pressure. In PLE, applied pressure allows the use of extraction solvents at temperatures greater than their normal boiling point [92]. The combined use of high pressures (3.3-20.3 MPa) and temperatures (40-200°C) provides faster extraction processes that require small amounts of solvents (e.g. 20 min using 10–50 mL of solvent, compared to conventional extraction methods that require 10 – 48 hours and up to 200 mL of

solvent) [121]. High temperature and pressure improves analyte solubility and the desorption kinetics from the matrices [122]. Hence, extraction solvents including water which show low efficiency at low temperatures may be much more efficient at elevated PLE temperatures [92]. Another technique of compressed fluid extraction is the supercritical or subcritical fluid extraction (SFE) method that involves the use of compressed carbon dioxide as an extraction solvent. SFE is performed in the absence of both light and air; degradation and oxidation processes are significantly reduced in comparison with other extraction techniques [92].

Taken together, compressed fluid-base extraction techniques are more environmentally friendly procedures than other methods, in that they reduce use of organic solvents and allow extraction performed with nonpolluting, nontoxic solvents (water and CO₂). However, the complex instrumentation requirements and high cost often outweigh the technical benefits of these methods.

1.7.5 *Microwave-assisted extraction (MAE)*

Microwave-assisted extraction involves the use of microwave energy to facilitate partitioning of analytes from the sample matrix into the solvent. The main advantage of MAE is the reduced extraction time and solvent volume compared to conventional extraction methods [123]. MAE has been used for the extraction of small-molecule phytochemicals such as ellagic acid [124], isoflavone [125], and quercetin [126], which were shown to be stable under microwave-assisted heating conditions at temperature up to 100°C for 20 min [127].

1.8 Purification and fractionation of bioactive phytochemicals

Crude plant extracts usually contain a mixture of large amounts of phytochemicals present at various concentrations. Hence, there is a need to separate the bioactive components from other co-extractives, once extracted from the plant. Purification steps may involve the use of techniques such as sequential extraction, liquid-liquid extraction, solid phase extraction (SPE), and/or chromatographic (column chromatography) methods in order to fractionate the compounds based on their acidity, polarity or molecular size.

1.8.1 *Liquid-liquid extraction (LLE)*

Liquid-liquid extraction is one of the most common purification method used, particularly for the separation of organic compounds from aqueous matrices. The principle of LLE is centered on the relative solubility of an analyte in two immiscible phases (a liquid stationary phase and a liquid mobile phase). Analyte are separated according to their partition coefficients between the two solvent phases based on their solubilizing power (polarity) [128, 129].

1.8.2 *Solid phase extraction (SPE)*

Solid phase extraction is a method of sample purification that separates and concentrates analytes from a solution of crude extracts (liquid phase) by adsorption onto an adsorbent (solid-phase) cartridge. Post retention, analytes are subsequently recovered by elution with an appropriate solvent (mobile phase) [130]. SPE media include reverse phase, normal phase, and ion-exchange media. SPE is becoming popular since it is rapid, economical, and sensitive.

Different cartridges and discs with a great variety of sorbents can be used, making it possible

to simultaneously extract analytes with an extremely wide range of polarities [130].

1.8.3 Column chromatography (CC)

Column chromatography has also been employed for the fractionation of analytes from a crude plant extract. Although often labor-intensive and solvent consuming, CC provides greater amounts of fractions for subsequent isolation and identification of pure substances. Commonly used column sorbents are toyopearl [131], RP-C18 [132], LH-20 [133] and, to a lesser extent, polyamide resin [134]. While ethanol, methanol, acetone, and water at various volume combinations are frequently used as eluents.

1.9 Analysis and quantification of bioactive phytochemicals

The selection of the appropriate analytical strategy for studying phytochemical constituents of crude plant extracts depends on the purpose of the study, nature of the sample and the analyte [135]. Quantification of compounds in plant extract is influenced by the presence of interfering substances, the chemical nature of the analyte, assay method and selection of standards [136]. In the phytochemical evaluation of plant extracts, different colorimetric and spectrophotometric methods have been used. Spectrophotometric assays provide simple and fast screening methods to quantify classes of phytochemicals in crude plant extracts. However, modern high-performance chromatographic techniques (liquid chromatography (LC), high performance liquid chromatography (HPLC), and gas chromatography (GC)) combined with instrumental analysis are the “state of art” for analysis and quantification of bioactive compounds [92]. Furthermore, the recent approaches of applying chromatography

and mass spectrometry, such as liquid chromatography-mass spectrometry (LC-MS), high performance liquid chromatography-mass spectroscopy (HPLC-MS) and gas chromatography-mass spectroscopy (GC-MS) provide additional spectral information for qualitative analysis [137].

1.10 Mechanism of action of bioactive phytocompounds

Over the years, different mechanisms of action have been proposed for bioactive phytocompounds. Bioactive phytocompounds have been suggested to mediate their therapeutic effects by inhibiting microorganism growth (anti-microbial action), or modulating metabolic processes, gene expression and/or signal transduction pathways (anti-diabetic, anti-cancer action) [138-140]. In addition, in contrast to synthetic pharmaceuticals drugs, it has been suggested that many phytomedicines and herbal concoctions exert their therapeutic effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological processes [141]. Studies on how synergistic interactions of phytochemicals augment their therapeutic potency have been documented [141, 142]. In a study by Jamaluddin et al. (1994), the synergistic interaction between β -Sitosterol and Stigmasterol in mediating hypoglycemia in alloxan-induced diabetic rats was confirmed when individual compounds showed no hypoglycemic effects when tested separately [143]. This synergistic or additive pharmacological effect of a complex mixtures could also be more toxic since they possibly contain more xenobiotic compounds [141].

1.11 Anti-diabetic medicinal plants and anti-diabetic phytocompounds

Diabetes Mellitus (DM) is a chronic debilitating metabolic disorder, associated with severe complications, that affect millions of people globally, irrespective of their socio-economic

profile and geographical location [144]. Considered the fourth leading cause of death by diseases, DM is a global health crisis with an estimated diagnosis and mortality frequency of 5 seconds and 7 seconds, respectively [145, 146]. Management involves the use of conventional agents (oral hypoglycemic or insulin therapy) along with lifestyle modification [144]. However, despite the introduction and extensive utilization of hypoglycemic agents, diabetes and diabetes-related complications continue to increase globally, affecting nearly 10% of the world's population [147]. According to WHO, DM related deaths will increase by more than 50% in the next 10 years if the disease is not given urgent attention. Hence, research on DM treatment and discovery of new medications is gaining ground, as the world population of patients with diabetes increases from 382 million in 2013 to a projected 592 million by 2035 [148, 149].

Medicinal plants continue to be an important therapeutic aid for alleviating ailments of humankind, and because of their enormous therapeutic potential, medicinal plants are being extensively explored for their use in the management of DM [150]. This is due in part to the high economic cost [151, 152] and undesirable side-effects associated with conventional anti-diabetic drugs [153], leading WHO to support the utilization of herbal remedies for the management of diabetes [144]. Presently, numerous medicinal plants have been reported to be effective in diabetes therapy, however intensive research is still needed.

1.12 Incidence and prevalence of DM

According to the International Diabetes Federation (IDF) Atlas 2014, the global prevalence of diabetes is estimated to be 8.3%, representing 387 million people, and is expected to increase by an additional 205 million people by 2035 [146]. The occurrence of diabetes is higher in men than in women [144]. About 77-80% of people living with diabetes reside in the low and middle income countries [146, 154].

In Africa, an estimated 22 million cases, with a prevalence of 5.1%, has been reported [155]. In 2014, the Republic of South Africa was reported to have 2.713 million people within the age of 20-79 years living with DM [155].

1.13 Morbidity and mortality rate of DM

In 2012, an estimated 1.5 million deaths were directly caused by diabetes globally [154]. In Africa, an estimated 481,000 deaths was projected in 2014, 75% of which will be < 60 years old [155].

1.14 Classification

Based on etiology, pathophysiology and clinical presentation, diabetes mellitus is divided into three main types: Gestational diabetes (pregnancy related diabetes), type 1 (insulin-dependent diabetes mellitus or IDDM or juvenile-onset diabetes) and type 2 (non-insulin-dependent diabetes mellitus or NIDDM).

1.15 Pathogenesis of diabetes mellitus

1.15.1 Gestational diabetes

Gestational diabetes develops during pregnancy. Typically, it disappears after delivery, although the condition is associated with an increased risk of developing diabetes later in life [156].

1.15.2 Type 1 DM

1.15.2.1 *Autoimmune factor*

Type 1 diabetes mellitus (T1DM) is categorized as an autoimmune-mediated (type IA) or idiopathic (type IB) form of the disease. It is more pronounced in children and young adults, and is characterized by the destruction of pancreatic β -cell islets resulting in absolute insulin deficiency, hence patients depend entirely on an exogenous supply of insulin [157]. About 80% of patients with T1DM have islet cell autoantibodies [158]. Autoantibodies to a variety of β -cell constituents including insulin, isoforms of glutamic acid decarboxylase (GAD 65 and GAD 67), and the secretory granule protein islet cell antigen (ICA) 512 or IA-2, have been recognized [158]. The concept of T1DM being a chronic autoimmune disease is supported by the fact that islet antigen-directed antibodies may be present in asymptomatic first-degree relatives of patients [159]. The chance of developing T1DM is greater than 50% if autoantibodies are present to more than one β -cell antigen (i.e., insulin, GAD 65, ICA 512); T1DM rarely develops in antibody-negative relatives [159]. β -cell destruction is mediated by a variety of cytokines or by direct T-lymphocyte activity [159]. As the disease progresses, the islets become completely devoid of β -cells while the α , δ , and pancreatic polypeptide cells are left intact, thus illustrating the exquisite specificity of the autoimmune attack [159]. However, in addition to autoimmune factors, genetics and environmental factors have also been implicated in type1 pathophysiology.

1.15.2.2 *Genetic factors*

The role of genetic factors in T1DM is underlined by data in identical twins showing concordance rates of 30 to 40%. The HLA (human leukocyte antigen) genes located on short arm of chromosome 6 (p6) (i.e., the *IDDM1* locus) clearly play a dominant role in the genetic

susceptibility that leads to risk of T1DM [160]. Of the HLA types, HLA class II show the strongest association with T1DM, with haplotypes *DRB1*0401-DQB1*0302* and *DRB1*0301-DQB1*0201* conferring the greatest susceptibility, while *DRB1*1501* and *DQA1*0102-DQB1*0602* provide disease resistance [161]. Class I HLAs have also been implicated in the risk of T1DM, independent of class II molecules [160]. However, since concordance rates are not 100%, environmental factors must be important for disease expression.

1.15.2.3 Environmental factors

Although environmental factors such as diet and toxins have been proposed as triggers of T1DM, no environmental agent or agents have been proven to be a causative agent [158]. To date, scientific attention has focused on viral infections (e.g. coxsackievirus and cytomegalovirus) [162], early infant diet (e.g. breast feeding versus early introduction of cow's milk components) [163], and toxins (e.g., N-nitroso derivatives) [164].

1.15.3 Type 2 Diabetes Mellitus (T2DM)

Type 2 diabetes mellitus (T2DM or NIDDM) is the most common type of the disease characterized by a complex interaction between numerous genes, environmental and behavioural risk factors [165]. Onset of T2DM is generally in adulthood; largely occurring in people between the ages of 30-60 years. However early onset of the disease has recently been documented in children with an average age range of 10-20 years [165]. Prevalence is increasing rapidly throughout the world [166].

Although the pathophysiology of T2DM is not entirely understood, T2DM is a multifactorial disease characterized by an initial peripheral insulin resistance in adipose tissue, liver, and skeletal muscle with subsequent pancreatic β -cell dysfunction resulting from an attempt to compensate for insulin resistance [167, 168]. The development of insulin resistance and T2DM

progression are often associated with obesity [168, 169]. Insulin resistance in T2DM refers to the failure of cells to respond to normal levels of circulating insulin with respect to glucose uptake, metabolism or storage [170].

1.15.3.1 *Insulin biosynthesis and secretion*

Insulin is primarily an anabolic hormone that promotes the storage and synthesis of lipids, proteins and carbohydrates, and inhibits their breakdown and release into circulation [171]. Insulin is a peptide hormone comprising of 2 polypeptide chains; A (21 amino acid residues) and B (30 amino acid residues) with a molecular weight of 5808Da. Chains A and B are linked by disulphide bridges. In addition the A-chain contains an intra-chain disulphide bridge linking residue 6 and 11. The C chain, which connects the A and B chains, is liberated along with insulin after breakdown of proinsulin. Insulin monomers aggregate to form dimers and hexamers [172].

1.15.3.2 *Biosynthesis*

Insulin is synthesized in the beta (β) cells of the pancreas in the form of pre-proinsulin which is the ultimate precursor. Post synthesis pre-proinsulin is discharged into the cisternal space of the rough endoplasmic reticulum, where it is cleaved into proinsulin by proteolytic enzymes. Proinsulin with a C (connecting) chain linking the A and B chains is transported by microvesicles to the Golgi apparatus, with proinsulin then released in vesicles. Conversion of proinsulin to insulin then continues within maturing granules through the action of the prohormones convertase 2 and 3, and carboxypeptidase H [173]. Maturing granules are translocated to the β cell plasma membrane for secretion, with the help of microtubules and microfilaments.

1.15.3.3 Secretion

Insulin is secreted from the pancreatic β -cells in response to various stimuli such as glucose (major physiological determinant), arginine, and sulphonylureas [174]. Similarly, various neural, endocrine and pharmacological agents can also exert a stimulatory effect. Glucose is taken up by β cells via glucose transporter (GLUT)-2 receptors. Once in the β -cells, glucose is oxidized by glucokinase, which acts as a glucose sensor. Glucose concentrations below 90 mmol/L have been shown not to induce any insulin release [174]. At such sub-stimulatory glucose concentrations, K^+ efflux through open K_{ATP} channels keeps the β cell membrane at a negative potential, hence causing the closure of the voltage-gated Ca^{2+} channels. However, in the presence of increased plasma glucose concentration, glucose uptake and metabolism by the β cells act as insulin secretagogues by closing K_{ATP} channels to induce insulin secretion [174].

1.15.3.4 Insulin signaling

Insulin action is mediated via its binding to, and activation of its cell-surface receptor. The insulin receptor belongs to a subfamily of receptor tyrosine kinases (RTK) that includes the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (IRR) [175]. These receptors are tetrameric proteins consisting of two extracellular α subunits that bind insulin and two transmembrane β subunits with tyrosine kinase activity, linked by a disulphide bond into a $\alpha_2\beta_2$ heterotetrameric complex. Binding of insulin to the α -subunit on the IRR subsequently triggers the transphosphorylation of one β subunit, resulting in the increased catalytic activity of the kinase [176].

Once activated, the insulin receptor phosphorylates downstream signaling tyrosine molecules, including members of the insulin receptor substrate family (IRS1/2/3/4), the Shc adapter protein isoforms, SIRP family members, Gab-1, Cbl, and APS. Phosphorylation of the IRS

proteins create recognition sites for additional effector molecules containing Src homology 2 (SH2) domains. These include the small adapter proteins Grb2 and Nck, the SHP2 protein tyrosine phosphatase and, most importantly, the regulatory subunit of the class 1a phosphatidylinositol-3-kinase (PI3-kinase) [177]. Upon phosphorylation, activated PI3-kinase enzyme translocates to the plasma membrane where it utilizes the lipid phosphatidylinositol-4, 5-bisphosphate (PIP₂) as a substrate to generate phosphatidylinositol-3, 4,5-trisphosphate (PIP₃) [178]. PIP₃ then mediates the recruitment and/or activation of pleckstrin homology (PH) domain-containing proteins, including various enzymes, their substrates, adapter molecules, and cytoskeletal proteins. Among these is the Ser/Thr kinase PDK1, which phosphorylates and activates several downstream kinases, including Akt1, Akt2, Akt3, protein kinase C (PKC)ζ/λ, and glucocorticoid-inducible kinase (SGK) [179]. PIP₃ mediates the translocation of Akt to the plasma membrane, via its PH domain. Overexpression of a membrane-bound form of Akt (Akt1 and Akt2) in cells (adipocytes and myocytes) results in localization of glucose transporter (GLUT)-4 to the plasma membrane thus enhancing glucose transport into the cell [180] (Figure 1.17).

However, it has been shown that activation of the cytoplasmic PI3-kinase pathway alone is not sufficient for increased glucose transport observed in response to insulin. Separate insulin signaling pathways localized within the lipid raft microdomains (specialized regions of the plasma membrane) [181] have also been implicated in GLUT-4 translocation and glucose transport. Activation of the insulin receptor within the lipid raft microdomains causes the phosphorylation of the substrates Cbl and APS. Cbl interacts with CAP (Cbl associated protein), which can bind to the lipid raft protein flotillin. This interaction recruits phosphorylated Cbl into the lipid raft, resulting in the recruitment of the protein CrkII. CrkII binds constitutively to the exchange factor C3G, which can catalyze the exchange of GDP for GTP on the lipid-raft-associated protein TC10. Upon its activation, TC10 interacts with a

number of potential effector molecules, including CIP4, Exo70, and Par6/Par3/PKC λ , in a GTP dependent manner to mediate the docking of GLUT-4 vesicle to the plasma membrane [182] (Figure 1.17).

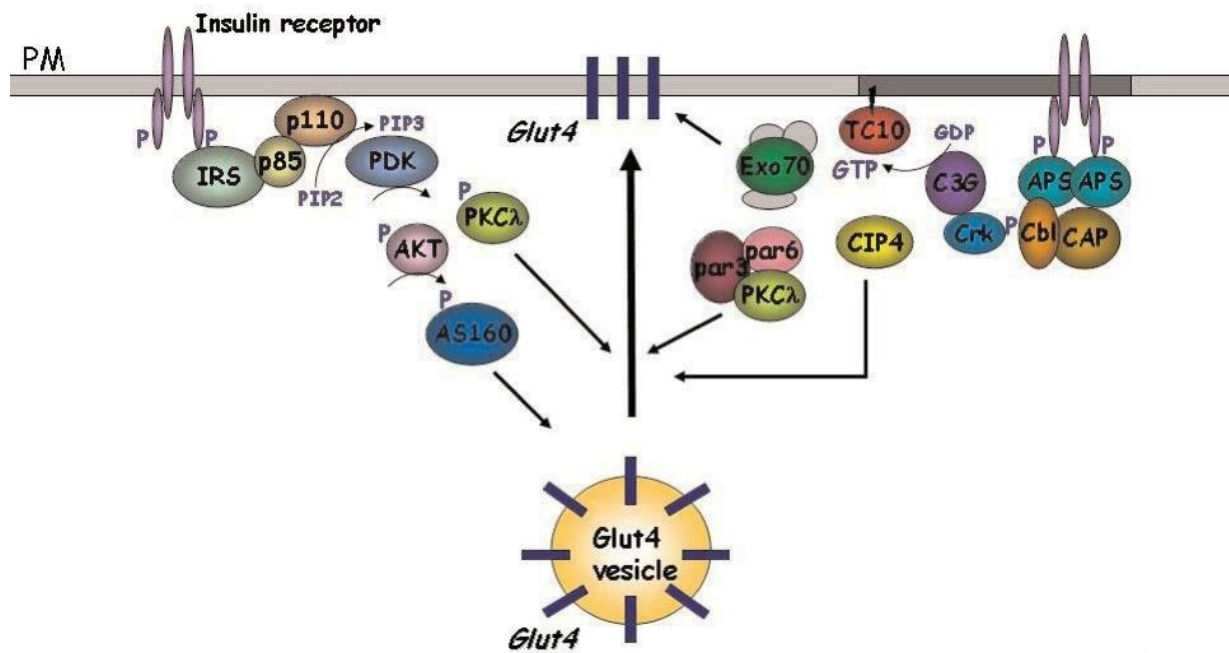


Figure 1.17: Signaling pathways of insulin in adipocytes and myocytes. Two signaling pathways are required for the translocation of the glucose transporter GLUT4 by insulin in fat and muscle cells. Tyrosine phosphorylation of the IRS proteins after insulin stimulation leads to activation of PI3-kinase to produce PIP₃, which in turn activates and localizes protein kinases such as PDK1. These kinases then initiate a cascade of phosphorylation events, resulting in the activation of Akt and/or atypical PKC. AS160, a substrate of Akt, plays an as yet undefined role in GLUT4 translocation through its Rab-GTPase activating domain. Similarly, a separate pool of the insulin receptor can also phosphorylate the substrates Cbl and APS. Cbl interacts with CAP, which can bind to the lipid raft protein flotillin. This interaction recruits phosphorylated Cbl into the lipid raft, resulting in the recruitment of CrkII. CrkII binds constitutively to the exchange factor C3G, which can catalyze the exchange of GDP for GTP

on the lipid-raft-associated protein TC10. Upon its activation, TC10 interacts with a number of potential effector molecules, including CIP4, Exo70, and Par6/Par3/PKC λ , in a GTP dependent manner to mediate the docking of GLUT4 vesicle to the plasma membrane [182].

1.15.3.5 Role of insulin signaling in physiology of glucose metabolism, transport and storage

In humans, glucose is necessary to ensure the proper function and survival of all organs. While hypoglycaemia (reduced blood glucose level) induces cellular death, chronic hyperglycaemia (persistent increased blood glucose level) also can result in organ damage. Therefore, the blood glucose level is maintained within a narrow range of about 5-7mmol/L, which is considered the physiological set point [167].

Glucose homeostasis is regulated primarily by the liver and skeletal muscle. The balance between glucose metabolism and production is maintained at equilibrium by two opposing hormones, insulin and glucagon which are secreted by the β and α -cells of the pancreas respectively. Both β and α -cells are extremely sensitive to glucose concentrations and can regulate hormone synthesis and release in response to small changes in plasma glucose levels [172]. In response to an elevation in plasma glucose and amino acids, as seen after consumption of a meal, GLUT-2 facilitates the transport of glucose into β -cells, via a gradient-dependent manner, such that the rate of glucose transport changes with fluctuations in blood glucose concentration [183].

Within the β -cells, phosphorylated glucose is oxidized and ATP is generated. The increased ATP/ADP ratio closes potassium channels, and the cell becomes depolarized [184] (Figure 18). This results in the opening of voltage-sensitive calcium channels, which increases intracellular calcium and thus stimulates the fusion of the insulin-containing secretory vesicles with the plasma membrane, resulting in the pulsatile release of insulin [184] (Figure 1.18). Insulin induces glucose uptake, utilization, and storage, while suppressing hepatic glucose production,

thus reducing plasma glucose levels. Conversely, in the presence of reduced plasma glucose level as seen during fasting or exercise, glucagon is secreted by the pancreatic- α cell. Glucagon then promotes the release of stored and newly synthesized glucose into the bloodstream (Figure 1.19). These two hormones act in concert to ensure the maintenance of glucose homeostasis in a wide variety of physiological conditions.

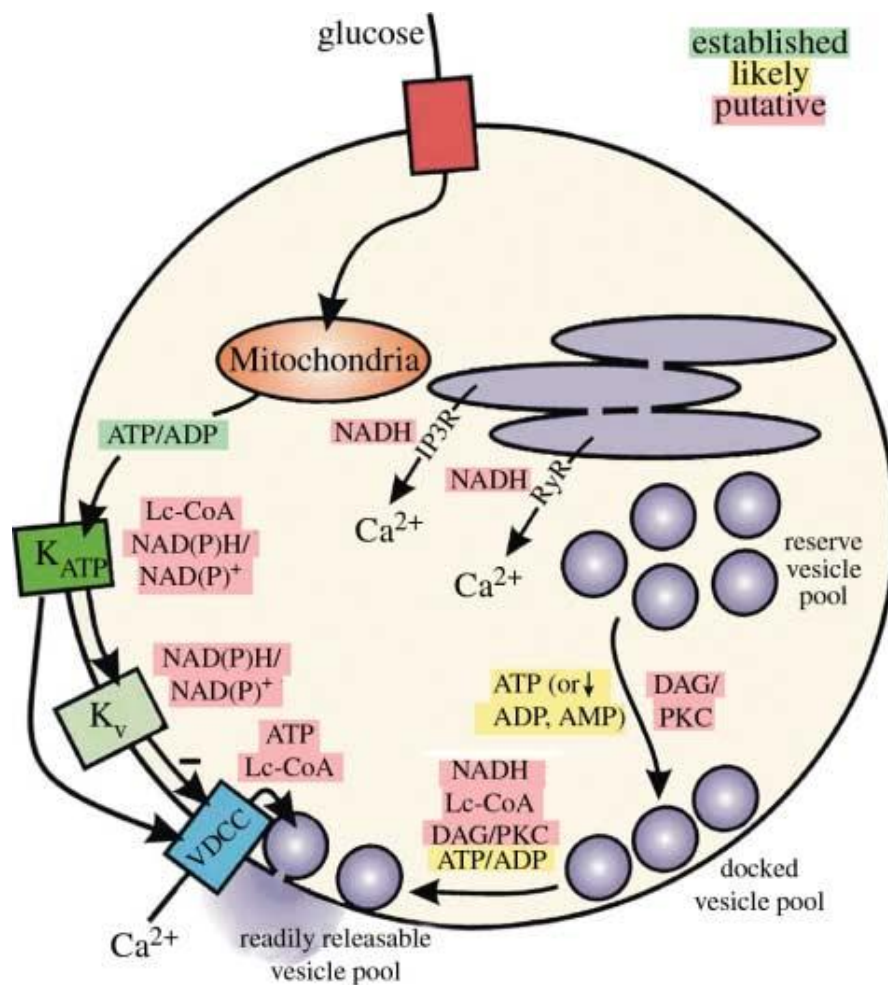


Figure 1.18: Glucose-mediated secretion of insulin from the pancreatic β -cell. Glucose entry and mitochondrial metabolism increases the intracellular ATP-to-ADP leading to closure of the K_{ATP} channels and membrane depolarization. This activates voltage-dependent calcium channels (VDCCs), allowing influx of Ca^{2+} , which triggers exocytosis of insulin granules. K_v channels also activate upon depolarization to mediate action potential repolarization, limiting

Ca²⁺ entry and insulin secretion [184].

The primary target organs/tissues for insulin are skeletal and cardiac muscle, adipose tissue and the liver. Glucose uptake is the rate-limiting step in glucose utilization and storage. Insulin binds to its receptor on the cell surface to stimulate the transport of glucose into muscle and fat cells via the translocation of the glucose transporter GLUT-4 from intracellular sites to the cell surface, thus increasing its concentration on the cell surface. About 75% of insulin-dependent glucose disposal occurs in skeletal muscle, while the adipocytes account for only a small fraction [185]. Once inside the muscle cell, glucose is rapidly phosphorylated by hexokinase and either subsequently stored as glycogen, due to the activation of glycogen synthase, or oxidized to generate ATP, via activation of enzymes such as pyruvate kinase (Figure 1.19).

In the adipocytes, glucose is stored primarily as lipids, due to increased uptake of glucose and activation of lipid synthetic enzymes. Insulin also profoundly inhibits lipolysis in adipocytes, primarily through inhibition of the hormone sensitive enzyme lipase. Most, if not all, of these insulin-dependent changes in enzyme activities are mediated by attenuation of the phosphorylation state of the relevant enzyme, due to a combination of protein kinase inhibition and phosphatase activation [185].

Glucose homeostasis in the liver is maintained through a delicate balance between hepatic glucose uptake and utilization and hepatic glucose production [185]. In hepatic cells insulin inhibits the production and release of glucose by the liver, by blocking the processes of gluconeogenesis and glycogenolysis thus regulating fasting glucose levels. Hepatic GLUT-2 facilitates the uptake of glucose into the hepatocytes when circulating levels of glucose are high, as well as the release of glucose out of hepatocytes into the plasma or bloodstream during the fasting state, via glycogenolysis and gluconeogenesis [185] (Figure 1.19). The direction is dictated by the glucose gradient across the plasma membrane.

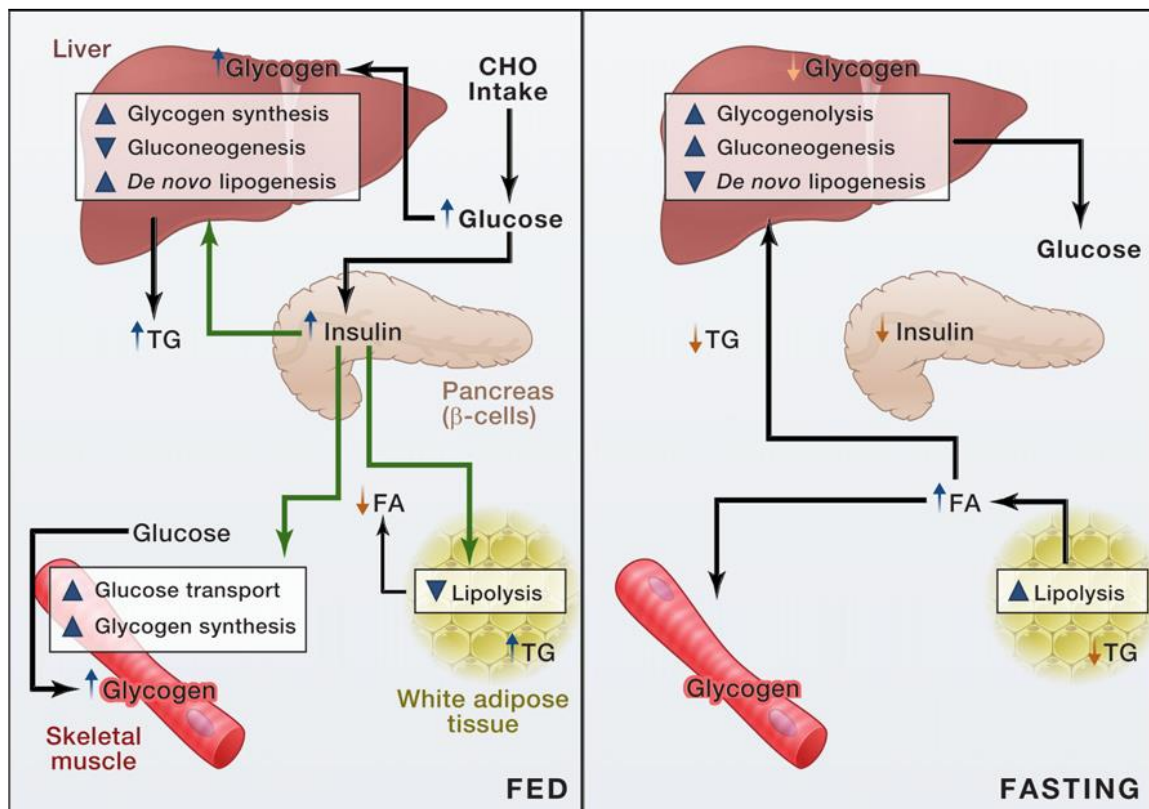


Figure 1.19: Role of insulin signaling in glucose metabolism, transport and storage. (Left) After consumption of a meal (fed state), dietary carbohydrate (CHO) increases plasma glucose level and promotes insulin secretion from the pancreatic β -cells. In the myocytes, insulin increases glucose transport, permitting glucose entry and glycogen synthesis. In the hepatic cells, insulin promotes glycogen synthesis and *de novo* lipogenesis while also inhibiting gluconeogenesis. In the adipocytes, insulin suppresses lipolysis and promotes lipogenesis. (Right) In the fasting state, insulin secretion is decreased. The drop in insulin increases hepatic gluconeogenesis and promotes glycogenolysis. Hepatic lipid production diminishes while adipose lipolysis increases [172]. TG; Triglycerol; FA; fatty acid.

1.15.3.6 Insulin signaling and T2DM

Insulin signaling is altered in diabetic states. Three major metabolic abnormalities have been identified to impair insulin signaling and contribute to hyperglycemia in T2DM [186]. These

abnormalities are classified into: (1) impaired insulin secretory responses to glucose, (2) elevated rates of hepatic glucose output (HGO), and (3) diminished ability of insulin to stimulate glucose uptake into peripheral tissues (insulin resistance). These functions involve cellular glucose transport in pancreatic β -cells, liver, adipose tissue, and skeletal muscle. In some instances, abnormalities in glucose transporter isoforms (GLUT) specifically expressed in these tissues may constitute key biochemical lesions underlying defective glucose homeostasis. Similarly, phosphorylation of the insulin receptor at the serine/threonine residue can inhibit the intrinsic tyrosine kinase activity of the receptor, thus altering the downstream signaling in a proportional manner [187]. It has been discovered that PKCs and MAPKs can phosphorylate the insulin receptor at this site, hence leading to decreased downstream signaling [188]. Furthermore, serine phosphorylation has also been found to be induced by the pro-inflammatory cytokine TNF- α [188] (Figure 1.18). SOCS (suppressor of cytokine signaling), activated during inflammatory conditions has been found to compete with IRS1/2 for IR binding, thus attenuating tyrosine phosphorylation and downstream signaling of IR1/2, and is also capable of inducing IRS degradation [189]. In addition, free fatty acids (FFA) and FFA derivatives are also capable of increasing serine phosphorylation of IRS through the activation of several serine/threonine protein kinases, such as PKCs, JNK, and inhibitor of nuclear factor- κ B kinase β (IKKB), to decrease the insulin-insulin receptor signaling cascade [190] (Figures 1.20). Furthermore, FFA also induces the stimulation of gluconeogenesis [172].

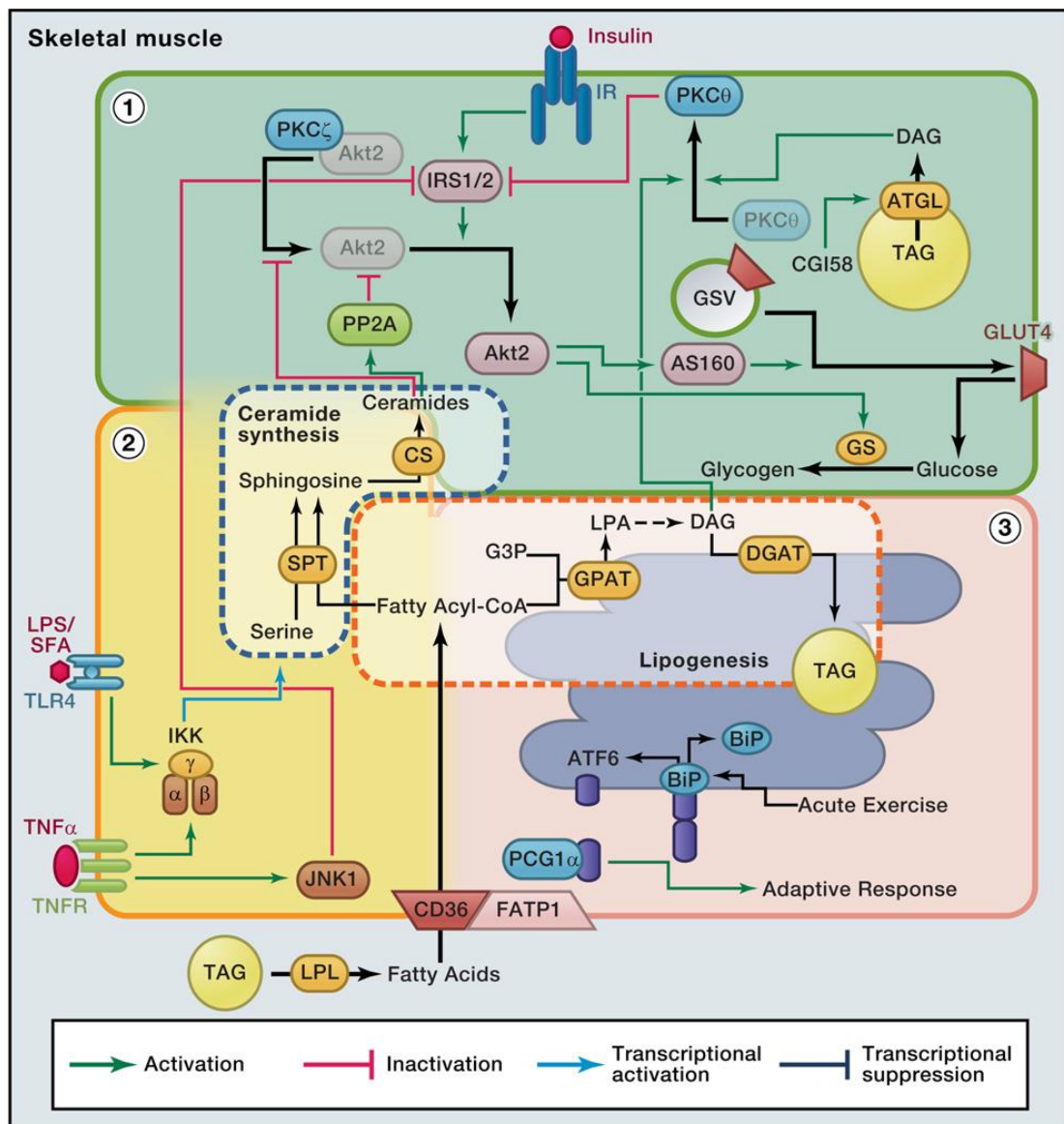


Figure 1.20: Pathways involved in insulin resistance in myocytes (muscle). Insulin activates the insulin receptor (IR) tyrosine kinase, which subsequently phosphorylates IRS1. This leads to activation of Akt2. Akt2 activation then promotes the translocation of GLUT4-containing storage vesicles (GSVs) to the plasma membrane, permitting the entry of glucose into the cell, and promotes glycogen synthesis via glycogen synthase (GS). (1) Represent mechanisms of lipid induced insulin resistance, notably diacylglycerol (DAG)-mediated activation of PKCq and subsequent impairment of insulin signaling, as well as ceramide-mediated increases in PP2A and increased sequestration of Akt2 by PKCz. Impaired Akt2 activation limits translocation of GSVs to the plasma membrane, resulting in impaired glucose uptake. (2) Depict several intracellular inflammatory pathways—notably, the activation of IKK, which

may impact ceramide synthesis, and the activation of JNK1, which may impair insulin signaling via serine phosphorylation of IRS1. (3) Depicts activation of the unfolded protein response (UPR), which may lead to activation of ATF6 and a PGC1a-mediated adaptive response. CS, ceramide synthase; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; SPT, serine palmitoyl transferase; TAG, triacylglycerol [172].

1.16 Clinical manifestation

Both T1DM and T2DM demonstrate common symptoms including hyperglycemia, polydipsia, polyphagia, polyuria, fatigue and blurred vision [191].

1.17 Criteria for clinical diagnosis of DM

To make a clinical diagnosis of DM one of the following criteria must be met: Glycosylated hemoglobin (HbA1c) $\geq 6.5\%$; or fasting plasma glucose (FPG) ≥ 126 mg/dL (7.0 mmol/L) (fasting is defined as no caloric intake for at least 8 hours); or Two-hour plasma glucose ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT) with 75g of anhydrous glucose dissolved in water; or patient with classic symptoms of hyperglycemia or hyperglycemic crisis; or a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L). Excluding a state of hyperglycemic crisis, all other criteria must be repeated to confirm diagnosis [191].

1.18 Conventional treatment of T2DM and their complications

T2DM is a multidimensional disorder characterized by a cluster of systemic pathologies, including obesity, hypertension, atherosclerosis, dyslipidaemia, hypercoagulation, impaired glucose tolerance and insulin resistance, collectively referred to as the “Metabolic syndrome” [192]. Hence, its management requires firm adherence to the prescribed treatment plan.

The existing treatment plan for T2DM is focused on suppressing and controlling blood glucose to a physiologic level, and lifestyle modification along with appropriate diet and weight control [193]. A number of medications are currently available, targeting numerous facets of the disease. Some of the first pharmacologic treatments for T2DM were aimed at increasing the insulin levels by using oral insulin secretagogues, such as sulfonylureas or meglitinides [194]. The insulin secretagogue binds to receptors on the pancreatic β cell to stimulate insulin secretion. In the event of failure of secretagogues, exogenous insulin can be administered. However both insulin secretagogues and exogenous insulin can induce hypoglycaemia [194]. Metformin (biguanide) is currently used as a first-line therapy in newly diagnosed T2DM patients [195]. Metformin acts by increasing the activation and phosphorylation 5'-AMP-activated kinase (AMPK). In the liver AMPK activation decreases lipid and glucose synthesis and increases fatty acid oxidation [196]. In muscle AMPK activation stimulates GLUT4 translocation [197]. However, the clinical limitation of metformin is that it is cleared through the kidneys, thus renal insufficiency/impairment could lead to toxic accumulation of the drug [194, 198].

Thiazolidinediones (TZDs) are relatively new class of drugs that were introduced in the late 1990s [199]. TZDs are peroxisome proliferator-activated receptor-gamma (PPAR γ) agonists that improve insulin sensitivity and glycemic control, with beneficial effects on lipid metabolism, blood pressure, vascular tone and endothelial function. However in clinical use TZDs have been shown to cause modest weight gain, increased risk of myocardial infarction, cardiovascular related death, and several cases of acute hepatitis and liver failure have also been reported [200, 201]. In addition to their individual debilitating side effects, anti-diabetic medication may sometimes involve polypharmacy (prescribing more than one drug at the same time), which can further augment the severity of these side effects. Hence, there is a need for

the development of novel anti-diabetic agents with more potent activity and lesser side effects than drugs currently in use in clinical practice.

1.19 Anti-diabetic medicinal plant and anti-diabetic phytocompound: Potential source of alternative therapy

Various herbal anti-diabetic remedies are used in traditional medical practices around the world. However, only a few of them have been scientifically assessed for their efficacy [144]. Anti-diabetic medicinal plants are widely distributed in six continental regions, and some specific regions around the world, such as in the Caribbean, Mediterranean and Middle East [150] (Figure 1.21). Asia (56%) and Africa (17%) dominate the global distribution of the anti-diabetic plants (Figure 1.21) [150]. This is not surprising as the two continents are located in the tropic and sub-tropical regions with large coverage of tropical rain forests, with diverse species of plants. Moreover, these regions have long established their traditional medicine systems [150].

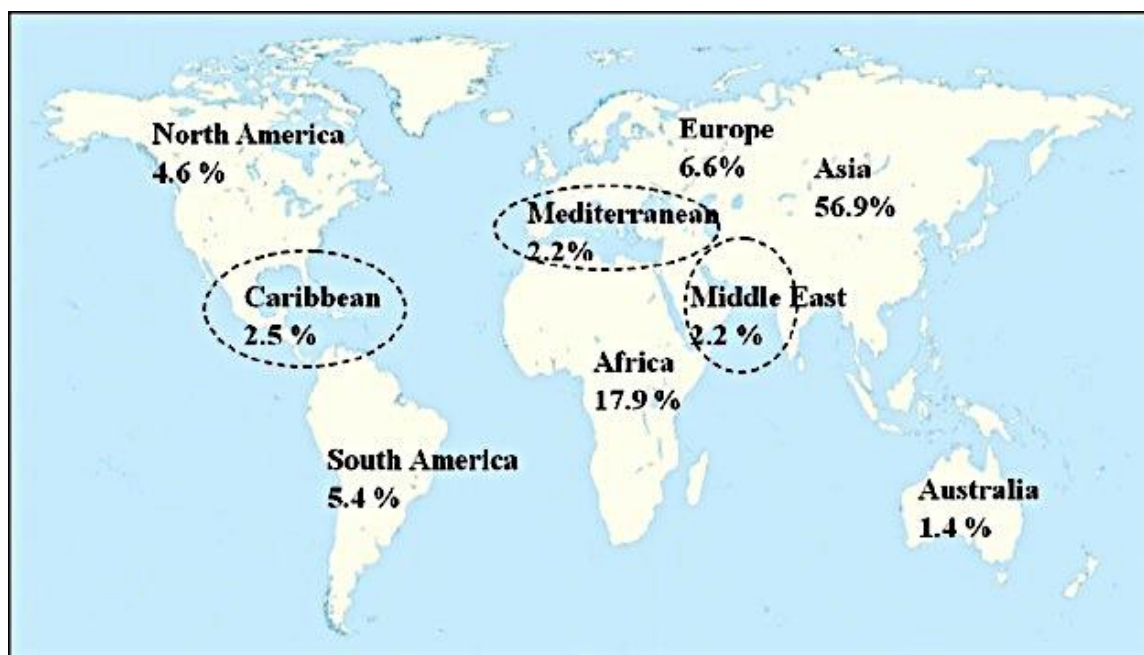


Figure 1.21: Global distribution of anti-diabetic medicinal plants [150]

In Africa, between 2000 and 2013, a total of 185 plant species from over 75 families were investigated for their anti-diabetic effects [29]. Of these, about 12.92% were reported from Southern Africa, with most (90%) of the studies originating from the Republic of South Africa [29]. Promising results were reported in several of these investigations, but only a few studies reported detailed characterization of bioactive principles [29]

1.20 *Sutherlandia frutescens*

1.20.1 *Ethnobotany*

Sutherlandia frutescens (L.) R. BR., Family *Fabaceae* subsp. *microphylla* is a flowering shrub of the pea family (*Fabaceae/Leguminaceae*) found mainly in the Western Cape and Karoo regions of Southern Africa (Figure 1.22) [202].

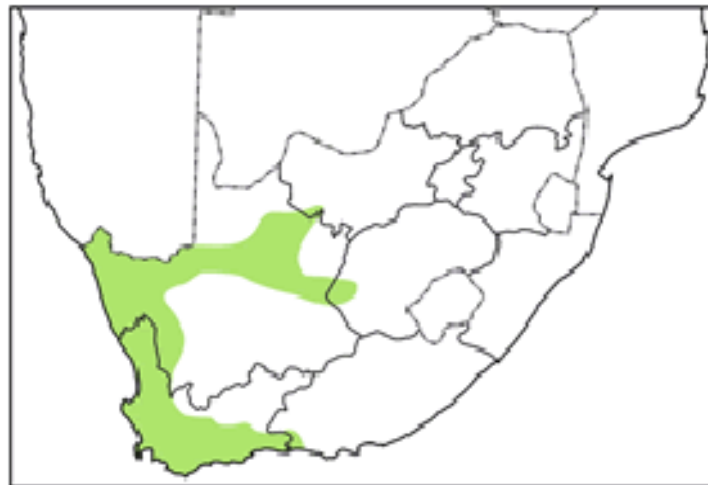


Figure 1.22: Geographical distributions of *S. frutescens* in Southern Africa[202]

S. frutescens grows to about 0.5-1 m in height and its leaves are pinnately compound. Each leaflet is about 4-10 mm long, greyish-green in color, giving the bush a silvery appearance (Figure 1.23) [203]. *S. frutescens* flowers are orange-red in color and can grow up to 35 mm long (Figure 1.23). The flowers appear in short clusters in the leaf axils at the tips of the branches in spring to mid-summer (September - December) [203]. Within the ethnic tribes of South Africa *S. frutescens* is known by different names including: cancer bush, balloon pea (English); umnwele (Xhosa); kankerbos, blaasbossie, blaas-ertjie, eendjies, gansiekeurtjie, klappers, hoenderbelletjie (Afrikaans); Unwele and Insiswa (Zulu); Musa-Pelo, Motlepelo an Phethola (Sotho) [204].



Figure 1.23: *Sutherlandia frutescens* growing in the wild.

1.20.2 Medicinal uses of *Sutherlandia frutescens*

S. frutescens is one of the best known multi-purpose medicinal plants in Southern Africa, and has diverse medical uses [205]. Medicinal preparations are generally produced from the leaves, but the stems are often also included. Concoctions of various parts of the plant are used in the treatment of ailments such as; stomach problems, cancer, influenza, chicken pox, diabetes, varicose veins, piles, inflammation, liver problems, backache, heart failure, urinary tract infections, rheumatism, stress and anxiety [205, 206].

In 2002, the South African Medical Research Council (MRC) launched a clinical research platform aimed at investigating the safety and efficacy of indigenous medicinal plants [207]. In the report of a test case, the first vervet monkey safety study of an indigenous plant, Matsabisa and colleagues reported that there was no indication of toxicity after feeding vervet monkeys with dried *S. frutescens* leaf powder for three months, at a dosage nine times the equivalent of the human dose [207]. Hence, the South African Ministry of Health concluded

that *S. frutescens* is safe for human consumption [208]. However, recent preclinical studies have indicated that concomitant administration of *S. frutescens* with prescription medicines (e.g. CYP3A4 substrates) could lead to therapeutic failure and adverse drug-herb interactions [209].

1.21 Pharmacological action of *S. frutescens* extracts

Various pharmacological actions have been attributed to *Sutherlandia frutescens* extracts as discussed below.

1.21.1 Immunomodulation

Several studies have established a role for *S. frutescens* extracts in immune modulation [210-212]. Using transcriptomic analyses, Lei et al. (2015) demonstrated the role of *S. frutescens* extract in regulating gene expression in an activated murine macrophage cell line [211]. Lei and colleagues observed that treatment of cells with *S. frutescens* resulted in the differential expression of 547 genes (DEGs) [211]. Pathway analysis identified that many of these DEGs participate in inflammatory signaling pathways, such as NF- κ B and MAPK [211]. Similarly, Kisten (2010), reported that *S. frutescens* extract regulated the expression of interleukin (IL) 6, IL-10 and IFN γ (interferon-gamma) in lipopolysaccharide (LPS) or Phytohaemagglutinin (PHA) stimulated whole blood [212]. However, these effects were found to be donor dependent [212].

1.21.2 Anti-oxidant

Natural anti-oxidants play an important role in health maintenance and the prevention of chronic and degenerative diseases [213]. By scavenging free-oxygen radicals (superoxide, peroxy radicals, hydroxyl radicals and singlet oxygen) anti-oxidants protect cells against the damaging effects of these reactive oxygen species. Studies by Tai et al. (2004) and Fernandes et al. (2004), showed that *S. frutescens* extracts possess superoxide as well as hydrogen peroxide-scavenging activities at low concentrations [214, 215]. Tai and colleagues reported that an ethanolic extract of *S. frutescens* reduced free radical cations production in cancer cell lines, at an estimated volume of 0.5 μ L of *S. frutescens* extract equivalent compared to 10 μ M of Trolox [214]. While Fernandes and colleagues showed that hot aqueous extract of *S. frutescens* reduced the rate of superoxide and hydrogen peroxide generation in L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) stimulated neutrophils, at a concentration of 10 μ g/mL [215].

1.21.3 Anti-cancer

Studies have shown that *S. frutescens* extracts have direct anti-cancer effects in some cancers. Chinkwo (2005) reported that crude aqueous extracts of *S. frutescens* induce apoptosis and cytotoxicity in cervical and ovarian neoplastic cells [216]. The pattern of *S. frutescens*-mediated DNA fragmentation seen in these neoplastic cells was similar to that mediated by other inducers of apoptosis such as staurosporine and ceramide [216].

1.21.4 Anti-HIV

S. frutescens has received international attention as a cheap herbal medicine that can improve the outcome of HIV infection. Clinical anecdotal evidence has shown that HIV-infected individuals on adjunct *S. frutescens* treatment have improved CD4 counts and decreased viral loads [217]. Similarly, Harnett et al. (2005) showed that both aqueous and organic extracts of *S. frutescens* contain inhibitory compounds active against HIV-1 reverse transcriptase (RT) enzyme [218]. In addition, Harnett and colleagues reported that a methylene dichloride extract of the *S. frutescens* leaves inhibited both α - and β -glucosidase enzymes, while no significant inhibitory activity was reported against HIV-II protease enzyme [218]. Consistent with this, the South African Ministry of Health recommended the use of *S. frutescens* as an adjunct therapy for HIV/AIDS treatment [219, 220].

1.21.5 Anti-diabetic (Hypoglycemic)

S. frutescens is among the most common and widely used herbal concoctions in Southern Africa for the treatment of T2DM. An aqueous extract of the shoots significantly prevented streptozotocin-induced hyperglycemia in mice when administered orally at various dosages (50–800 mg/kg) [221]. In a similar study by Chadwick et al. (2007) using rats fed a high fat diet to induce insulin resistance, a crude aqueous extract of *S. frutescens* leaves was shown to increase glucose uptake and utilization by peripheral tissues, with a decrease in intestinal glucose [222]. Furthermore, in a separate study, MacKenzie et al. (2009) showed that *S. frutescens* extracts reduced plasma free fatty acid levels in rats fed a high fat diet, subsequently preventing the development of insulin resistance in these rats [223]. In comparison to rats fed high fat diet only, levels of plasma free fatty acids (FFA) was significantly reduced after one week of *S. frutescens* treatment in the experimental group [223]. In addition, twelve weeks of

treatment with *S. frutescens* reduced the level of plasma free fatty acids below that of rats fed a normal diet [223].

1.22 Pre-isolated bioactive phytochemicals from *Sutherlandia frutescens*

Over the years, several bioactive phytochemicals including L-canavanine, D-pinitol, and γ -amino butyric acid (GABA) [35], triterpenoid glycoside (sutherlandioside B “SU1”) [206, 224, 225], flavonol glycosides [226], amino acids and small amounts of saponins [35] have been isolated from *S. frutescens* extracts. Specific bioactive principle constituents have been linked to the therapeutic application of *S. frutescens* in folklore medicine, however each isolated compound alone does not have the full activity of the complete extract. Three purified compounds have been analyzed in some detail:

1.22.1 Pinitol

Pinitol is a type of sugar found in many species of legumes and is classified as chiro-inositol [212]. Pinitol (Figure 1.24) has also been shown to exert an insulin-like effect, resulting in lower blood sugar levels and increased availability of glucose for cell metabolism [227]. Thus, pinitol has been proposed as a bioactive compound underlying the anti-diabetic effect of *S. frutescens*.

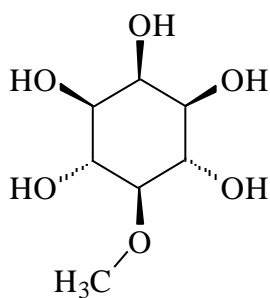


Figure 1.24: Chemical structure of pinitol.

1.22.2 Canavanine

Canavanine (Figure 1.25) is a potent L-arginine competitor and the L-2-amino-4-guanidino-oxy group, is a structural analogue of arginine [219]. It has been shown to possess both anti-cancer as well as anti-viral activities [219, 228], justifying the traditional use of *S. frutescens* as an anti-cancer agent [206].

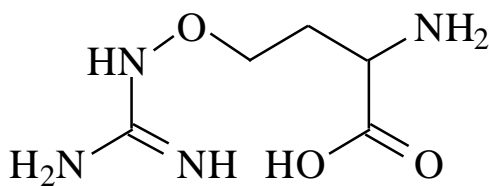


Figure 1.25: Chemical structure of canavanine

1.22.3 Gamma Amino Butyric Acid (GABA)

GABA (Figure 1.26) is a non-essential amino acid and the major inhibitory neurotransmitter

found in the mammalian nervous system [229]. The presence of GABA in the leaf extracts of *S. frutescens* justifies its use in treating anxiety and stress [35].

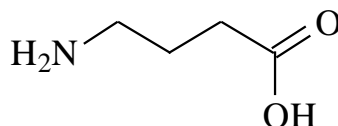


Figure 1.26: Chemical structure of GABA

Identification and characterization of these aforementioned phytochemicals suggests that crude extracts of *S. frutescens* contain these bioactive principles and potentially other compounds not identified by previous studies, which can be explored for their anti-diabetic activity. Analytical approaches using Triple Time-of-Flight Liquid Chromatography with mass spectrometry (Triple TOF LC-MS/MS) are a more effective tool for qualitative exploration, rapid profiling, and high-resolution quantification of medicinal plants or herb medicinal products.

1.23 Statement of the problem

In 2012, an estimated 1.5 million deaths were directly caused by diabetes globally [154]. Of these, T2DM accounted for about 90% of cases [230]. Presently, the global prevalence of diabetes is estimated to be 9% among adults aged 18+ years [231] with more than 80% occurring in low- and middle-income countries [154]. Although the pathophysiology of T2DM is not entirely understood, T2DM is a multifactorial disease characterized by initial peripheral insulin resistance in adipose tissue, liver, and skeletal muscle, and subsequent pancreatic β -cells dysfunction as a result of an attempt to compensate for insulin resistance [167, 168]. Presently, various pharmacological agents are available for the treatment of T2DM [232]. However, these conventional treatment modalities are expensive and could have short or long-term unpleasant side effects on the patients [233]. Hence there is a need to look for alternative and newer bioactive principles that will be cost effective and possess fewer side effects.

Africa has an enormous number of potential plant resources that may contain these potent anti-diabetic principles, thus *Sutherlandia frutescens* based on its folklore anti-diabetic usage can serve as a lead for the development of new treatments.

Prior studies in our laboratory have demonstrated the role of an aqueous extract of *S. frutescens* in the regulation of T2DM in a rat model system and in the regulation of key genes; including Vesicle-associated protein (VAMP)-3 and N-ethylmaleimide sensitive fusion proteins (NSF) in liver cells made insulin resistant [234]. However, the detailed comparison of the potent anti-diabetic phytochemicals present in the crude aqueous and organic extracts of the plant and the molecular mechanisms by which they mediate reversal of insulin resistance in hepatic cells *in-vitro* is yet to be elucidated.

In this study, the molecular role of crude organic and aqueous extracts of *S. frutescens* in the regulation of gene expression and reversal of insulin resistance in hepatic cell lines was investigated and compared *in-vitro*.

Furthermore, several bioactive phytochemicals [35, 206, 224-226] have been isolated from *S. frutescens* extracts. However, no conclusive study of the phytochemicals isolated and purified from the plant material, has rigorously connected these compounds to anti-diabetic activities. Hence, the exact compounds mediating the anti-diabetic effects of *S. frutescens* is still a subject of debate.

Using a more recent analytical approach (Triple TOF LC-MS/MS), qualitatively explore and profile the bioactive (anti-diabetic) principles present in crude and fractionated extracts of *S. frutescens* were qualitatively explored and profiled.

1.24 Hypothesis

I hypothesized that aqueous and organic extracts of *S. frutescens* contain anti-diabetic phytochemicals that can reverse insulin resistance in hepatic cells. Given the complexity of the extracts and their multiple activities, these extracts may display combinatorial or synergistic action, rather than containing a single active ingredient.

1.25 Overall aims/objectives

As a result of differences in activity reported between extracts from different plant samples and differing extraction procedures, the aim of this study was to investigate the phytochemical constituents and evaluate the relative anti-diabetic activities of aqueous and organic extracts of a single sample of plant material, as well as to identify potential active phytochemicals. These extracts will be used to analyze changes in expression of genes regulated by insulin resistance in hepatocyte cell lines *in-vitro*, following treatment with aqueous and organic extracts of *S. frutescens*.

1.26 Specific objectives.

1. To prepare crude extracts and fractions of *S. frutescens* extracts.
2. To elucidate the bioactive chemical compositions present in crude and fractionated extracts of *S. frutescens*.
3. To evaluate and confirm the efficacy of aqueous and organic extracts of *S. frutescens* in treating insulin resistant human cell cultures.
4. To quantify changes in expression of selected genes regulated by insulin resistance in hepatocyte cell lines with and without *S. frutescens* treatment.
5. To compare the molecular role and mechanism by which aqueous and organic extracts of *S. frutescens* mediate reversal of insulin resistance in hepatic cell *in-vitro*.

CHAPTER TWO

PHYTOCHEMICAL ANALYSIS OF *SUTHERLANDER FRUTESCENS* EXTRACTS

2.1 Introduction

The effectiveness of medicinal plants and their pharmacotherapeutic action has been attributed to their complex diversity of chemical compounds [47]. Hence, the use of analytical phytochemical investigations to identify compounds with therapeutic properties is of utmost importance in ethnopharmacological studies to justify the usage of traditional medicinal plants [235]. Similarly, identification, isolation and analysis of the chemical profiles of plant extracts could play an important role in drug discovery [236].

In traditional medical practises, aqueous and alcohol (ethanol) infusions are the most widely used procedures to prepare extracts from plants [237, 238]. These solvents have been shown to extract multiple chemically complex principles or compounds. Thus, analytical methods for plant extracts must be capable of concurrently identifying multiple compound entities sensitively and accurately [239].

In this study, using colorimetric and spectrophotometric analysis I investigated and quantified the phytochemical constituents of crude aqueous (hot and cold) and organic (ethanol and methanol) extracts of *S. frutescens*. Furthermore, using Triple Time-of-Flight Liquid Chromatography coupled with Mass Spectrometry (Triple TOF LC-MS), with superior sensitivity and selectivity, I identified specific phytochemicals present in crude and solid phase extractions of aqueous (hot and cold) and organic (ethanol and methanol) extracts of *S. frutescens*. Analysis of these compounds using online databases of anti-diabetic compounds and a bioinformatics tool revealed possible anti-diabetic compounds present within each extract, and highlights possible similarities and variations within each extract.

2.2 Materials and Methods

In traditional medicine, different parts (leaf, flower, stem and root) of medicinal plants have been utilized for the preparation of herbal mixtures [240]. *S. frutescens* leaves are the most widely used part of the plant in making infusions and decoctions [36]. In this study conventional methods of extraction and a range of solvents (hot aqueous, cold aqueous, 80% and 100% ethanol; 80% and 100% methanol) were used on air-dried *S. frutescens* leaves. These methods closely mimic the traditional medicine preparations.

2.2.1 Chemicals and Reagents

All chemicals used for this study were of high purity grade. Folin-Ciocalteu reagent, absolute (100%) ethanol and methanol, acetic acid, LC–MS grade methanol and trifluoroacetic acids (TFA) were purchased from Merck Chemicals (PTY) Limited (Gauteng, South Africa). Quercetins, tannic acid, sodium bicarbonate, LC-MS grade acetonitrile and aluminium chloride were purchased from Sigma Aldrich® (Cape Town, South Africa), while Whatman™ filter paper No 1 was purchased from GE Healthcare (UK). APCI negative calibration solutions, APCI positive calibration solutions and Phenomenex Strata™ X polymeric-based sorbent cartridges were purchased from Separations Scientific South Africa (PTY) Ltd. The LC–MS column and formic acid were purchased from Agilent (USA). Deionized water was used for all procedures.

2.2.2 Collection of Plant Material

Sutherlandia frutescens (L.) R. BR., Family *Fabaceae* subsp. *microphylla* was identified by Professor E Campbell, Botany Department, Nelson Mandela Metropolitan University. A voucher specimen has been deposited in the University herbarium.

Fresh *S. frutescens* plants were collected from a site in the Karoo region (Western Cape, South Africa) in August 2012 when the plants were in flower. The leaves of the plant material were removed from the stems and air dried at room temperature for two weeks away from direct sunlight. Then the dried leaves were ground to a fine powder using an electrical blender (Model BL-302, Pineware, South Africa), stored in clean labelled airtight bottles and extracted in six different solvents: hot water, cold water, 80% ethanol, 100% ethanol, 80% methanol and 100% methanol to extract potential bioactive constituents.

2.2.3 Preparation of plant extracts

2.2.3.1 Hot aqueous extracts

The hot water extract was prepared following the procedure of Chadwick et al. (2007) [222] which imitates the traditional preparation. Briefly, powdered plant material (2.5 g) was mixed with 100 mL boiling deionized water in a 1L flask and allowed to brew overnight. The extract was filtered using a Buchner funnel and Whatman No 1 filter paper. The filtrate obtained was frozen at -80 °C and then dried under vacuum over 2 days using the Savant Freeze Drying System (Kansas City, MO, USA). The dried material obtained was placed in eppendorf microcapped tubes as 30 mg samples, sealed and kept in the dark at 4 °C in a desiccator until use.

2.2.3.2 Cold aqueous extracts

The cold water extracts were prepared following the procedure of Harnett *et al.*, 2004 [218]. Powdered plant material (25 g) was added to 200 mL of deionized water in a 1L bottle and then extracted in the dark at 4 °C for four days. Each day the extract was filtered using Whatman No 1 filter paper, the filtrate retained and 200 mL of fresh deionized water was added to the plant material. This process was repeated thrice to produce a total of four extractions (exhaustive extraction). The combined filtrates were freeze-dried under vacuum over two days in the Savant freeze dry system. Dried extract obtained was placed in eppendorf microcapped tubes as 30 mg samples, sealed and kept in the dark at 4 °C in a desiccator until use.

2.2.3.3 Organic solvent extracts

Extracts from the organic solvents (ethanol and methanol) were prepared following the method described by Harnett *et al.* (2005) [218] with slight modification. Powdered plant material (25 g) was added to 200 mL of 80% or 100% ethanol, or 80% or 100% methanol. The material was allowed to soak for four days. Each day the extracts were filtered using Whatman No 1 paper and the appropriate 200 mL fresh solvent was added. This process was repeated thrice for a total of four extractions (exhaustive extraction) for each solvent system. The combined filtrates for each solvent were concentrated in a rotavapor (BUCHI R461, Switzerland) at a temperature not greater than 60°C. After rotary evaporation, a greasy residue was obtained for each of the organic extracts. The residues of the 80% organic extracts, were re-dissolved in 5 mL of relevant solvent and 45 mL of deionized water was added. For the rotary evaporated 100% organic extracts, the residue extracts were transferred to labelled vials and air dried to permit full evaporation of residual solvent. All extracts were freeze-dried and a powder subsequently obtained. Extracts were kept at 4 °C in the dark in a desiccator until use.

2.2.4 Determination of solvent extraction efficiency

To determine the extraction efficiency of individual solvent extractants, 16 g of dried powdered plant material was immersed in 192 mL of extracting solvent (hot and cold water, 80% ethanol, 100% ethanol, 80% methanol and 100% methanol) (single extraction). These were stirred, left on shaker overnight and then filtered. The aqueous extracts were frozen at -80°C and then freeze-dried, while the organic extracts were concentrated under vacuum at 60°C using the rotary evaporator. The concentrated residue was freeze-dried and a powder subsequently obtained. The extracted mass for each solvent was measured and is presented in a bar chart, as mass of extract against solvent [241, 242].

2.2.5 Phytochemical screening of crude *S. frutescens* extracts

The screening procedure involves preliminary qualitative chemical tests to characterize the classes of compounds present in each extract and quantitative spectrophotometric methods to determine the concentration of phytochemicals within the extracts.

2.2.5.1 Qualitative analysis

Aqueous (hot & cold) and organic (ethanol & methanol) extracts (sections 2.2.3.1-2.2.3.3) were subjected to preliminary screening to determine the presence or absence of the following bioactive phytochemicals; tannins, phenol, flavonoids and flavonols using a standard procedure as described by Wintola et al. (2011) [243]. The presence or absence of bioactive phytochemicals was determined by the presence or absence of observable visual color change after the addition of the phytochemical specific reagents. These color changes were estimated as low (+), moderate (++) and high (+++).

2.2.5.2 Quantitative analysis

2.2.5.2.1 Determination of total phenols

The concentration of total phenolic compounds in crude *S. frutescens* extracts was evaluated spectrophotometrically with Folin-Ciocalteu's phenol reagent as described by Otang et al. (2012) [244]. Individual solvent extracts (0.1 mg/mL) were mixed with 5 mL of Folin-Ciocalteu reagent, previously diluted with deionized water (1:9 v/v), and incubated for 5 minutes at room temperature. Post incubation, 4 mL of 7% sodium carbonate (Na_2CO_3) solution was added. The mixture was vortexed for 5 seconds and allowed to stand for 30 minutes at 40 °C for color development. Absorbance at 765 nm was measured using a Bio-Tek KC4 Power Wave XS micro plate reader (Analytical and Diagnostic Products, South Africa). The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg/g tannic acid equivalents. The experiment was conducted in triplicate and the results expressed as mean \pm SD.

2.2.5.2.2 Determination of tannin

Tannin content in crude *S. frutescens* extracts was determined by the modified AOAC (Association of Official Analytical Chemists) method as described by Wintola et al. (2011) [243]. To 0.20 g of crude plant extracts, 20 mL of 50% methanol in distilled water was added. This was shaken thoroughly to ensure uniform mixing and placed in a water bath at 80 °C for 1 hour. The mixture was filtered using Whatman filter paper No 1 and the filtrate collected into a 100 mL volumetric flask. To the filtrate, 20 mL of deionized water, 2.5 mL of Folin-Denis reagent and 10 mL of 17% Na_2CO_3 was added and then mixed thoroughly. The mixture was

made up to 100 mL with distilled water and allowed to stand for 20 minutes at room temperature. At the end of the reaction a bluish-green color developed of different concentrations ranging from 0 to 10 ppm. The absorbance at 760 nm was measured using a Bio-Tek KC4 Power Wave XS micro plate reader (Analytical and Diagnostic Products, South Africa). The tannic acid content in the standard solutions, as well as sample were expressed as mg/g of tannic acid equivalents using the calibration plot. The experiment was conducted in triplicate and the results expressed as the mean \pm SD.

2.2.5.2.3 Determination of total flavonoids

Total flavonoid content in crude *S. frutescens* extracts was determined using a flavonoid-aluminium chloride complex colorimetric method as, described by Ordonez et al. (2006) [245]. Briefly, 0.5 mL of individual solvent extract (0.1 mg/mL) was mixed with 0.5 mL of 2 % aluminium chloride (AlCl_3) prepared in ethanol and incubated for 1 hour at room temperature. The absorbance was measured at 420 nm using a Bio-Tek KC4 Power Wave XS micro plate reader (Analytical and Diagnostic Products, South Africa). A yellow colour change indicates the presence of flavonoids. Total flavonoid content in the test samples were calculated from the calibration plot and expressed as mg/g of quercetin equivalents. The experiment was conducted in triplicate and the results expressed as the mean \pm SD.

2.2.5.2.4 Estimation of total flavonols

Total flavonol content in crude *S. frutescens* extracts was determined using a modified Kumaran and Karunakaran method as described by Adedapo et al. (2008) [246]. The reaction

mixture consisted of 2.0 mL of the sample extract, 2.0 mL of 2% AlCl_3 prepared in ethanol and 3.0 mL of (50 g/L) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h of incubation at 20°C using a Bio-Tek KC4 Power Wave XS micro plate reader (Analytical and Diagnostic Products, South Africa). The content of flavonols was calculated as mg/g of quercetin equivalents using a standard curve obtained from various concentrations of quercetin. The experiment was conducted in triplicate and the results expressed as the mean \pm SD.

2.2.6 Fractionation/Purification of crude extracts by Solid Phase Extraction Method

Crude plant extracts contain multiple complex components. Hence, pre-concentration is needed to analyze targeted analytes by chromatographic methods. The use of SPE in the isolation and purification of bioactive compounds from crude plant extracts has been established by prior studies [247, 248]. SPE is a useful sample preparation technique that involves the use of a variety of media (sorbent) such as chemically bonded silica, usually with a C_8 or C_{18} organic group and a porous polystyrene polymeric sorbent to trap analytes over a wide range of polarities [249]. The choice of SPE media (sorbent) is determined by the sample matrix composition, analyte properties and interaction mechanism between the analyte and the media during the extraction process[250, 251].

For the purpose of this study, Strata - X polymeric reverse phase and ion exchange extraction methods were used to fractionate polar and non-polar groups of compounds from the crude plant extracts obtained in sections 2.2.3.1-2.2.3.3. Disposable Strata™ X polymer-based sorbent (200 mg/3 mL) mixed mode SPE syringe columns: Strata-X-C 33u polymeric strong cation, Strata-X-CW 33u polymeric weak cation, Strata-X-A 33u polymeric strong anion, Strata-X-AW 33u polymeric weak anion, and Strata-X 33u polymeric reversed phase

(Phenomenex, USA) were used to obtain a clean and concentrated target group of compounds prior to analysis by LC-MS.

2.2.6.1 Steps in solid phase extraction

The procedure of SPE generally consists of 5 main steps [252]. Step 1; conditioning (activation) of the sorbent with a suitable solvent to promote good surface contact between the sample and sorbent as well as to activate the functional groups on its surface to ensure a proper binding environment. Step 2; equilibration to remove the activation solvent. Step 3; passing of the sample through the sorbent bed by gravity, or application of vacuum such that the analyte will be retained by the sorbent. Step 4; washing away or removal of interfering compounds/impurities retained on the sorbent, while avoiding elution of analyte. Step 5; elution of the analyte retained on the sorbent using a suitable solvent or mixture of solvents, resulting in a highly purified extract (Figure 2.1).

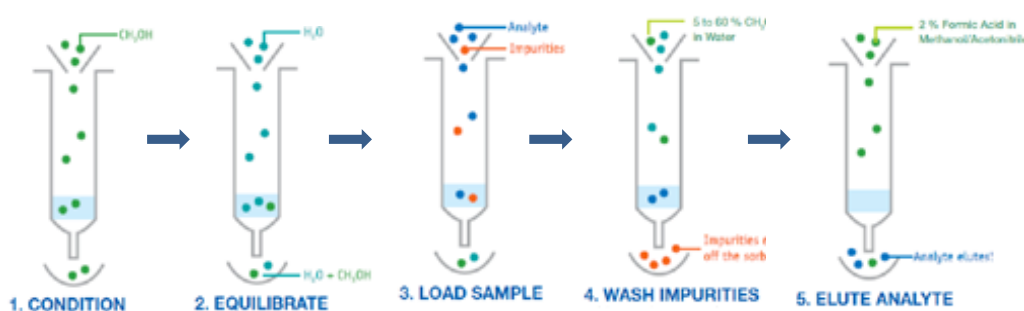


Figure 2.1: Steps in Solid Phase Extraction[252]

2.2.6.2 Extraction of basic compounds with $\text{pK}_a \leq 10.5$ by Strata™ X-C cation exchange SPE

A Strata-X-C 33u Polymeric Strong Cation cartridge (Phenomenex, USA) with a sorbent mass of 200 mg and sample volume of 3 mL was used to extract weak bases with $\text{pK}_a \leq 10.5$. The sorbent is composed of Strata X polymer with the surface modified with styrene divinylbenzene

and sulfonic acid functional group (highly selective for weakly basic compounds). This polymeric sorbent provides 3 mechanisms of retention; strong cation exchange (sulfonic acid ligand), π - π bonding, and hydrophobic interaction. This allows complete retention of basic compounds with $pK_a \leq 10.5$ and removal of matrix interferences such as phospholipids. The SPE phase selected for this extraction used a mixed-mode reversed phase and strong cation exchange mechanism. The solid phase extraction procedure was carried out as described by the manufacturer's instruction. The cartridge (sorbent) was conditioned with 1 mL methanol and equilibrated with 1 mL acidified water (1% trifluoroacetic acid in deionized H₂O). Post conditioning, 3 mL of dissolved acidified sample extract (0.06 g) was loaded onto the conditioned cartridge and then washed with 1 mL 0.1N HCl in water to remove all polar neutrals. This was then followed by a second wash step with 1 mL 0.1N HCl in methanol to remove all neutrals/acids. The samples were eluted from the cartridge with 1 mL (2x 500 μ l) of 5% NH₄OH in methanol to strip the column of retained bases (i.e. weak bases). This fraction was collected for LC-MS analysis.

2.2.6.3 Extraction of basic compounds with $pK_a > 8$ by Strata™ X-CW anion exchange SPE

A 200 mg/3 mL Strata-X-CW 33u Polymeric Weak Cation cartridge (Phenomenex, USA), was used to extract bases with $pK_a > 8$ (strongly basic compounds). The sorbent has extremely tight retention of basic compounds with a $pK_a > 8$ and is capable of removing matrix interferences. The sorbent is composed of Strata X polymer with the surface modified with styrene

divinylbenzene and carboxylic acid functional group (allows for reversible binding of strong bases and quaternary amines; highly selective for all basic compounds). This polymeric sorbent also provides 3 mechanisms of retention; weak cation exchange (Carboxylic acid ligand), π - π bonding, and hydrophobic interaction. The SPE phase selected for this extraction used a mixed-mode reversed phase and weak cation exchange mechanism. The solid phase extraction procedure was carried out as described by the manufacturer's instructions. The cartridge was conditioned with 1 mL methanol and equilibrated with 1 mL deionized water, pH 6.5. After the conditioning step, 3 mL of dissolved sample extract (0.06 g) pH 6.5, was loaded onto the conditioned cartridge and then washed with 1 mL water, pH 6.5. This was then followed by a second wash step with 1 mL methanol to remove all neutrals/acids. The samples were eluted from the cartridge with 1 mL (2x 500 μ L) of 5% formic acid in methanol to strip the column of retained strong bases elution, (E) 1, and eluted again with 1 mL (2x 500 μ L) of 5% NH_4OH in methanol to elute weak bases, E2. Fractions were collected for Triple TOF LC-MS analysis.

2.2.6.4 Extraction of Acidic Compounds with $\text{pK}_a > 2$ by Strata-X-A ion-exchange SPE

A Strata-X-A 33u Polymeric Strong Anion cartridge (Phenomenex, USA), 200 mg/3 mL was used to extract acids with $\text{pK}_a > 2$ (weakly acidic compounds). The sorbent is composed of Strata X polymer with the surface modified with styrene divinylbenzene and dimethyl butyl quaternary amine functional group (provides strong retention of weakly acidic analytes). Like the previous sorbents, this polymeric sorbent provides 3 mechanisms of retention; strong anion exchange (di-methylbutyl quaternary amine ligand), π - π bonding, and hydrophobic interaction. The sorbent allows for complete retention of weakly acidic compounds with $\text{pK}_a > 2$. The SPE phase selected for this extraction used a mixed-mode reversed phase and strong anion exchange mechanism. The solid phase extraction procedure was carried out as described by the

manufacturer's instructions. The cartridge was conditioned with 1 mL Methanol and equilibrated with 1 mL deionized water. Next, 3 mL of dissolved sample extract (0.06 g) pH 6.5 was loaded onto the conditioned cartridge and then washed with 1 mL 25 mM ammonium acetate buffered to pH 6.5. This was followed by a second wash step with 1 mL methanol to remove all neutrals/bases. The samples were eluted from the cartridge with 1 mL (2x 500 μ L) of 5% formic acid in methanol to strip the column of retained strong acids. The fraction was collected for subsequent Triple TOF LC-MS analysis.

2.2.6.5 Extraction of Acidic Compounds with $pK_a \leq 5$ by Strata X-AW ion exchange SPE

A Strata-X-AW 33u Polymeric Weak Anion cartridge (Phenomenex, USA) with a sorbent mass of 200 mg and sample volume of 3 mL was used to extract any acids with $pK_a \leq 5$ (strongly acidic compounds). The sorbent is composed of Strata X polymer with the surface modified with styrene divinylbenzene and di-amino functional groups (highly selective for charged acidic compounds). Again this polymeric sorbent provides 3 mechanisms of retention; weak anion exchange (di-amino ligand), π - π bonding, and hydrophobic interaction. This cartridge allows for complete retention of acidic compounds with $pK_a \leq 5$. The SPE phase selected for this extraction uses a mixed-mode reversed phase and weak anion exchange mechanism. The solid phase extraction procedure was carried out following the manufacturer's instructions. The cartridge was conditioned with 1 mL methanol and equilibrated with 1 mL deionized water (pH 6.5) after which 3 mL of dissolved sample extract (0.06 g), pH 6.5 was loaded onto the conditioned cartridge and then washed with 1 mL 25 mM ammonium acetate buffered to pH 6.5. This was followed by a second wash step with 1 mL methanol. The samples were eluted from the cartridge with 1 mL (2x 500 μ L) of 5% NH_4OH in methanol to strip the column of retained strong acids, elution (E) 1, and eluted again with 1 mL (2x 500 μ L) of 5% formic acid

in methanol to elute weak acids, E2. Fractions were collected for further Triple TOF LC-MS analysis.

2.2.6.6 Extraction of Neutral Compounds by Strata X Reverse phase SPE

A Strata-X 33u Polymeric reversed phase cartridge (Phenomenex, USA), 200 mg/3 mL was used to extract non-polar, hydrophobic and uncharged compounds (neutral and aromatic compounds). The cartridge is composed of Strata X polymer with a modified N-vinylpyrrolidone functional group surface. This polymeric sorbent provides 3 mechanisms of retention; π - π bonding, hydrogen bonding (dipole-dipole interactions) and hydrophobic interaction. This cartridge has high retention of neutral compounds. The SPE phase selected for this extraction uses a reversed phase mechanism. The solid phase extraction procedure was carried out following the manufacturer's instructions. The cartridge was conditioned with 1 mL methanol and equilibrated with 1 mL deionized water after which 3 mL of dissolved sample extract (0.06 g), was loaded onto the conditioned cartridge and then washed with 1 mL 60% methanol. The samples were eluted from the cartridge with 1 mL (2x 500 μ L) of 2% formic acid in methanol to strip the column of retained neutral compounds. The fraction obtained was collected for further Triple TOF LC-MS analysis.

2.2.7 Liquid Chromatography-Mass Spectrometry (LC-MS) analysis and identification of bioactive components

The above preliminary analysis revealed the presence of bioactive phytochemical groups (flavonoids, flavonols, tannin, phenols) within the aqueous and organic crude *S. frutescens* extracts. Prior studies have successfully used the LC-MS technique for the separation and

identification of targeted bioactive compounds from *S. frutescens* [253, 254]. Using the new AB SCIEX TripleTOF™5600 LC/MS/MS system (Applied Bio systems Sciex, USA), I further identified possible bioactive daughter phytochemicals present within crude *S. frutescens* extracts. The new AB SCIEX TripleTOF™5600 LC/MS/MS system is the next generation in quadrupole TOF technology. This instrument adds high resolution at the speed and sensitivity needed to give percentage of coefficient variations (%CVs) and limits of quantitation (LOQ) equivalent to those of high-end triple quadrupoles.

Mass spectrometry measures the mass-to-charge ratio of ions to identify unknown compounds and provide information about the structural and chemical properties of molecules. The TripleTOF®5600/5600+ system has a series of quadrupole filters that transmit ions according to their mass-to-charge (m/z) value. The QJet® ion guide is the first quadrupole in this series. The QJet ion guide does not filter ions, but focuses them before they enter the Q0 region. By prefocusing the larger ion flux created by the wider orifice, the QJet ion guide increases instrument sensitivity and improves the signal-to-noise ratio. The ions are again focused in the Q0 region before passing into the Q1 quadrupole.

In the Q1 quadrupole, the ions are sorted before they enter the Q2 collision cell. In the Q2 collision cell, the internal energy of the ions is increased through collisions with gas molecules to the point that molecular bonds break, creating product ions. This technique enables the design of experiments that measure the m/z of product ions to determine the composition of the parent ions.

After passing through the Q2 collision cell, the ions enter the TOF region for additional mass analysis, and then enter the detector. In the detector, the ions create a current that is converted into a voltage pulse. These voltage pulses are counted, and the number of pulses leaving the detector is directly proportional to the quantity of ions entering the detector. The instrument

monitors the voltage pulses and converts the information into a signal. The signal represents the ion intensity for a particular m/z value and the instrument displays this formation as a mass. For the purpose of this study, a non-targeted (without a preceding selection of compounds of interest) screening technique was employed to identify new or previously unrecognized bioactive compounds in the sample extracts.

2.2.7.1 Sample preparation

Both crude aqueous and organic extracts were weighed and reconstituted in deionized water/acetonitrile 50:50 (v/v) and methanol respectively to produce individual stock solutions (0.1 mg/mL). The mixture was then vortexed and centrifuged at 13000 rpm for 1 min, and the supernatant was filtered through a 0.2 μ m acrodisc filter (PALL, USA) and then placed into 2 mL LC-MS vials ready for analysis. Each SPE fraction was injected directly as an LC-MS sample.

2.2.7.2 Instrumentation and chromatographic conditions

LC-MS Triple-TOF qualitative analyses of extracts and fractions were performed with a high performance Agilent 1260 infinity liquid chromatography system coupled with the 5600 AB SCIEX Triple TOF hybrid mass spectrometer (Applied Bio systems Sciex, USA) and operated in both the positive and negative turbo ion spray (ESI) mode. The individual sample extracts (200 μ L) were separated/analyzed using a reverse phase Poroshell 120; EC-C18 column (7 μ m, 4.6 mm x 50 mm, Agilent Technologies) or ZORBAX SB-C18 column (5 μ m, 4.6 mm x 250 mm). The mobile phase used was 0.1% (v/v) formic acid in deionized water (Solvent A), and

0.1% (v/v) formic acid in acetonitrile (Solvent B). Extraction/elution of bound compounds was achieved using a flow rate of 0.5 mL/min with the following gradient: 95% A: 5% B to 5% A: 95% B for 10 min, kept constant for 1 minute. Finally, it was returned to the initial conditions in 0.5 min and kept constant for 5 minutes, giving a total run time of 17 min. The sample injection volume was 200 μ L. The column was equilibrated with deionized water/0.1% formic acid at a flow rate of 5 mL/min for 5 minutes. TOF MS parameter settings were as follows: the declustering potential (DP) was 60 V and Collision Energy (CE) was 35V with collision energy spread (CES) of \pm 35V. Product ion parameters were as follows: the IonSpray Voltage Floating parameter (ISVF) 5500, the Ion Source Gas 1 (GS1) parameter was 50 psi, the Ion Source Gas 2(GS2) parameter was 50 psi and Temperature (TEM) was 600⁰ C. An Agilent 1260 High Performance Auto sampler, with a 100 μ L syringe was used, with an injection volume of 200 μ L, to draw and inject the samples at a speed of 200 μ L/min. The Agilent column oven was set at 40°C for both right and left temperatures with a temperature tolerance of \pm 2°C.

2.2.7.3 LC-MS Data analysis

2.2.7.3.1 Generation of total ion chromatograms of untargeted analytes

Full scan acquisition to obtain total ion chromatograms (TICs) of untargeted analytes was performed with the aid of the PeakView Analyst software v1.1.1 (Absciex) (Figure 2.2)

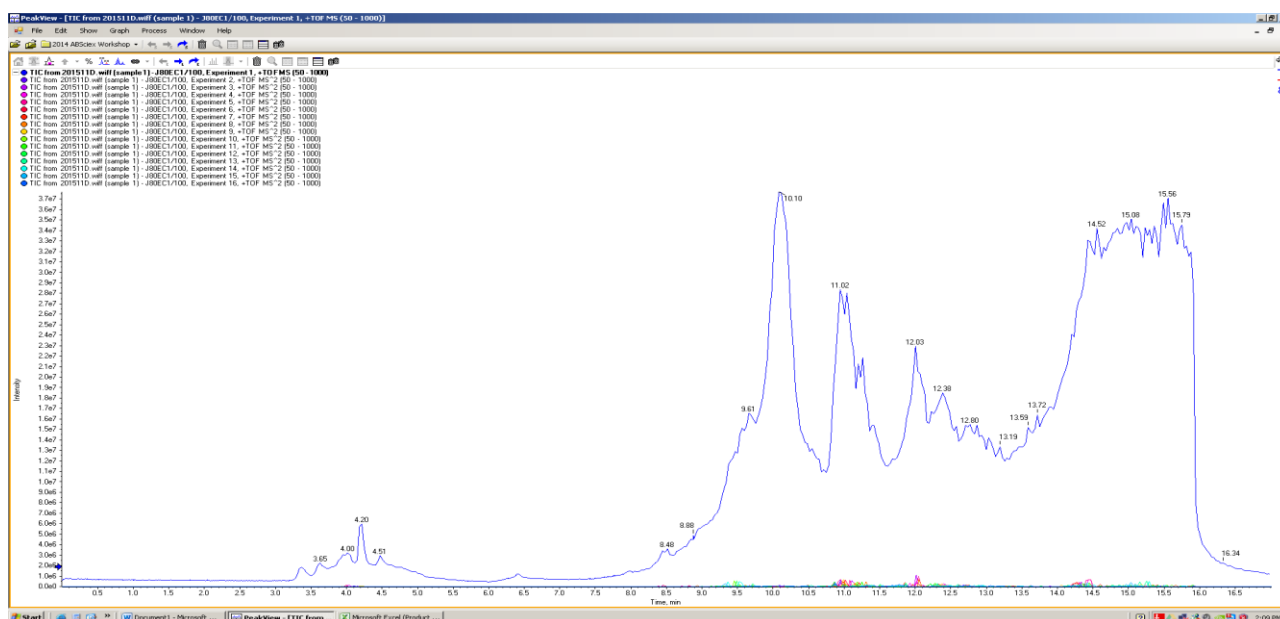


Figure 2.2: Total Ion Chromatograms obtained from crude extracts of *S. frutescens*

2.2.7.3.2 Identification of the elementary composition of untargeted analytes

The elementary composition of the untargeted compounds in crude *S. frutescens* extracts was obtained using the XIC manager/IDA (independent data acquisition) (Absciex). The XIC Manager is an add-in for the PeakView™ software. It allows the management of large lists of compounds and performs automatic extracted ion chromatogram (XIC) calculations from a table of defined list of masses or formulae. The XIC manager also has the ability to review the results of the XIC including any library search results.

2.2.7.3.2.1 Generation of XIC list

For the purpose of this study, the XIC list was generated from the Plant Metabolic Network (PMN) database (<http://pmn.plantcyc.org/PLANT/class-instances?object=Compounds>). PlantCyc database contains pathways and their catalytic enzymes and genes, as well as

compounds from over 350 plant species (2982 compounds) [255].



2.2.7.3.2.2 Defining an XIC list

The XIC table contains columns with values that can be edited, including Name, Formula, and Retention Time. To define an XIC, either a chemical formula or a mass must be defined. All other columns are either optional or contain default values [256].

2.2.7.3.2.3 XIC list column descriptions

The description of the columns in the XIC list is as seen in Table 2.1

Table 2.1: Descriptions of the columns in the XIC list [256]

Column Name	Description
#	The line number of the XIC when it was entered in the table. Used for reverting the list to its original order.
	Used to set which XICs will be calculated or have a specific action performed on them. See Exporting to MultiQuant™ Software for an example.
	<p>A visual representation of the quality of an XIC. This includes how well:</p> <ul style="list-style-type: none">the detected mass spectral peak matches the defined extraction mass.the detected retention time matches the expected retention time.the isotope pattern from the detected mass spectral peak matches the theoretical isotope pattern from the defined chemical formula.the purity score from the best library search hit. <p>The traffic lights for each of these values are displayed as red, yellow, or green. The criteria for this are set using the Options dialog.</p>

Column Name	Description
	If a value was not calculated then the traffic light is disabled.
Name	A name to represent the XIC. Used to label the XIC in the PeakView chromatogram panel.
Formula	Any valid chemical formula. The mass of this formula is calculated and then automatically entered in the Mass column. If the Name column is empty, then this formula is also used as the Name for the XIC.
Isotope	By default, the monoisotopic value for a formula is used to calculate the mass. For some formulae (for example, highly chlorinated and brominated compounds) it is preferable to use the second, third, or higher isotope. Use this column to define which isotope to use.
Mass (Da)	The neutral mass (not m/z) of the compound this XIC defines. This mass can be entered directly or it can be calculated automatically when a Formula is entered.
Adduct / Modification	<p>Use this column to enter any ionization, adduct, or modification chemical formula. The mass of this formula is added to the value in the Mass column to determine the actual Extract Mass used for generating the XIC. For most positive mode XIC, this column will be +H, to indicate protonation. A doubly-charged molecule is indicated using +2H.</p> <p>This value will default to the last Adduct / Modification value entered.</p>
Extraction Mass (Da)	This is a non-editable value that is calculated from the Mass (Da) and the Adduct / Modification values. This value is used for calculating the XIC.
Width (Da)	<p>The total width of the XIC window.</p> <p>This value will default to the last value entered.</p>
Found At Mass (Da)	The mass calculated from the mass spectral peak detected during the XIC. Spectral peak detection is performed at the apex of the XIC, at the rising portion of the XIC, and at the falling portion of the XIC. The spectral peak with an intensity greater than 300 and closest to the expected Extraction Mass is used. If no peak has an intensity greater than 300, then the peak detected at the apex of the XIC is used.
Error (ppm)	The mass error, in parts per million, of the Found At Mass compared with the Extraction Mass.

Column Name	Description
Error (mDa)	The mass error, in milliDaltons, of the Found At Mass compared with the Extraction Mass.
Expected RT (min)	If the retention time of the compound is known, enter the value here. This limits the XIC to only calculate the XIC in this region, greatly improving the calculation time.
RT Width (min)	The total width of the retention time window that is used to calculate the XIC. Only used if a non-zero Expected RT is specified.
Found At RT (min)	The largest peak in an XIC is automatically detected when an XIC is calculated. The actual retention time of the detected peak is displayed here.
Intensity	The intensity at the apex of the detected XIC peak.
Library Hit	The name of the compound with a matching library spectrum.
Purity Score	The purity score of the matching library spectrum compared with the acquired spectrum.

2.2.7.3.2.4 Confidence Settings

The thresholds for displaying green, yellow, or red for the traffic lights in the confidence column are defined in Figure 2.3.

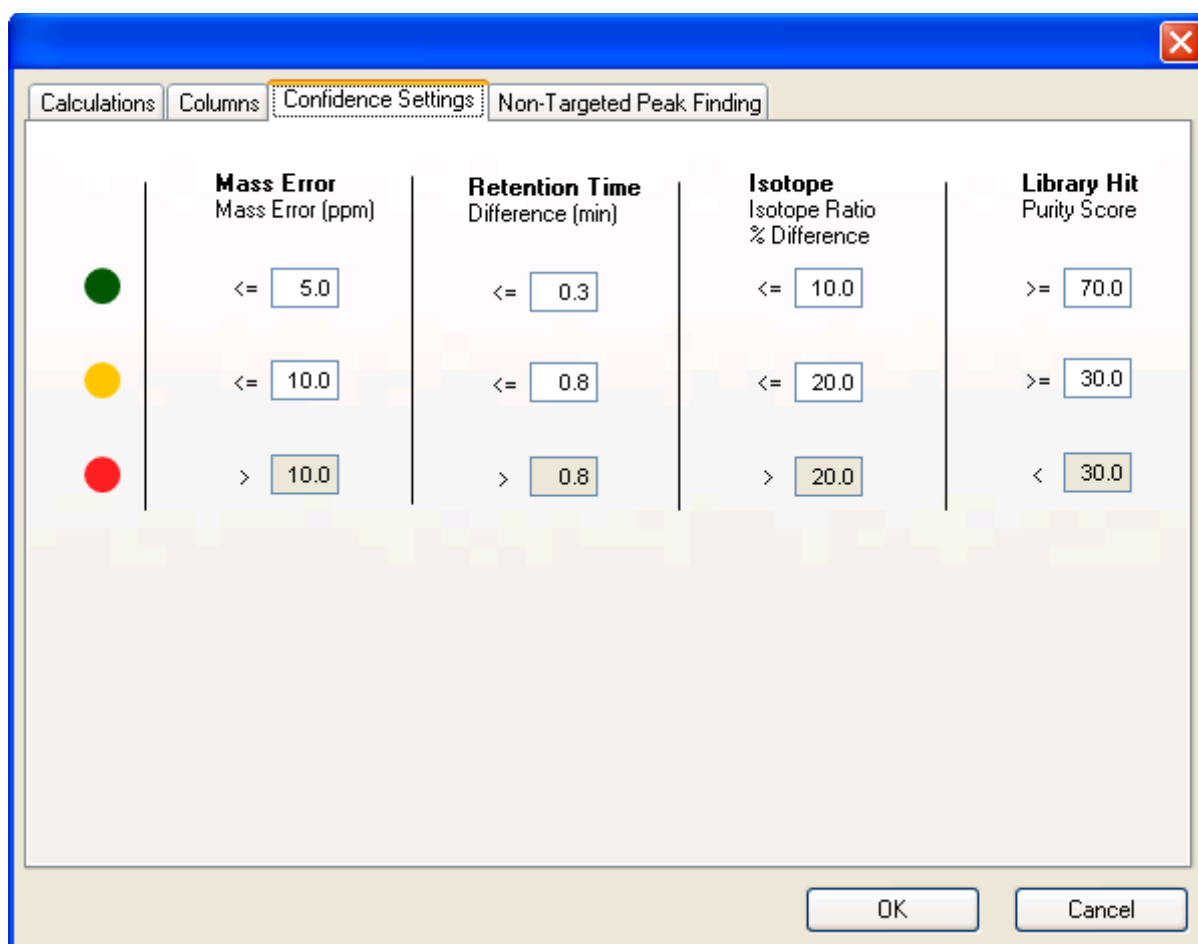


Figure 2.3: XIC manager confidence Settings

2.2.7.3.2.5 Displaying Results

The XIC is calculated from the mass spectra and the results are displayed in the XIC table, including found mass, mass error, found retention time, and library search results (Figure 2.4). The confidence in results is visualized using traffic lights. Each column of the result table can be used to sort generated results for each parameter. In addition, the filter XIC list function searches for specific information.

#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found at RT (min)	Intensity	Library Hit	Purity Score
2354	α-thiostine	C10H16	0	136.1252	+H	137.13248	0.02	137.13184	-4.6	0	2	10.61	1032		
26	(+)-pinene	C10H16	0	136.1252	+H	137.13248	0.02	137.13184	-4.6	0	2	10.61	1032		
84	(-)-6-phenylbornene	C10H16	0	136.1252	+H	137.13248	0.02	137.13184	-4.6	0	2	10.61	1032		
2272	OPC4-3-hydroxy-Cul	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
796	4a-formyl-5a-cholene	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
3881	α-terpinene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
1168	α-terpinene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
2911	α-terpinene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
122	(E)-6-caryophyllene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
121	(E)-α-bisabolene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
120	(E)-α-bisabolene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
421	2-methyl-3-hydroxy-6	C20H30O2	0	328.24023	+H	329.24751	0.02	329.24683	5.8	0	2	15.75	6434		
797	4a-hydroxy-5a-cholene	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
2273	OPC4-Cul	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
470	24-ethylsterol 2	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
109	(4a)-methyl-5a-ergo	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
2927	8-D-glucose-6-phosph	C19H34O11	0	340.1878	+H	341.19508	0.02	341.19434	-0.7	0	2	15.18	6910		
899	3-methylthiononylhyd	C20H30O1	0	296.22967	+H	297.23694	0.02	297.23619	-1.5	0	2	15.64	2813		
1849	isatin	C12H8O2	0	186.05551	+H	187.06279	0.02	187.06204	-1.5	0	2	15.64	2813		
243	10-oxygenal	C10H16O2	0	168.11503	+H	169.12231	0.02	169.12156	-2.5	0	2	13.89	2258		
39	(+)-7S-sabinene	C10H16	0	136.1252	+H	137.13248	0.02	137.13184	-4.6	0	2	10.61	1032		
3882	α-terpinene	C15H24	0	204.1878	+H	205.19508	0.02	205.19434	-0.7	0	2	15.18	6910		
2233	nitroperoxide ring	C42H64O14	0	732.42961	+H	733.43689	0.02	733.43614	3.5	0	2	15.86	1811		
2385	pelargonide-3,5-digly	C8H8O11	0	120.05751	+H	121.06479	0.02	121.06404	-1.2	0	2	5.02	1705		
1222	oceanophyllin II	C20H30O2	0	302.22458	+H	303.23186	0.02	303.23161	-0.8	0	2	13.74	27689		
1448	dihydromyricetin	C15H18O10	0	312.08307	+H	313.09035	0.02	313.08960	-0.4	0	2	9.48	1013		
2801	6-fenylacetylphorone	C18H20O3	0	276.27734	+H	277.28462	0.02	277.28387	0.1	0	2	14.28	1471		
2714	THF-L-glutamate	C10H16O5	0	210.10525	+H	211.11253	0.02	211.11178	-4.2	0	2	14.77	1240		
2866	jasconitrin	C6H8O10	0	210.06551	+H	211.07279	0.02	211.07204	-1.3	0	2	14.04	1165		
1258	CulG4	C10H16O5	0	222.09282	+H	223.09910	0.02	223.09835	1.1	0	2	12.36	1139		
2	(+)-5S-carvone	C10H16O2	0	150.10447	+H	151.11175	0.02	151.11100	2.1	0	2	13.87	1131		
1295	curcumin diglucoside	C30H40O11	0	426.38617	+H	427.39345	0.02	427.39270	-13.3	0	2	14.7	24259		
2853	oleone-ring	C30H40O1	0	426.38617	+H	427.39345	0.02	427.39270	-13.3	0	2	14.7	24259		

Figure 2.4: XIC manager showing automatically extracted ion chromatograms displayed in chromatographic pane obtained from crude extracts of *S. frutescens*.

The retention time chromatogram, TOF-MS and product ion of identified compounds from crude *S. frutescens* extracts were obtained by selecting the identified compound in the XIC manager and obtaining the associated MS spectra. The results are displayed as shown in figure 2.5. The TOF-MS pane displays the mother peak and its mass found at a specific retention

time, whilst the product ion pane displays broken down fragments of the mother peak.

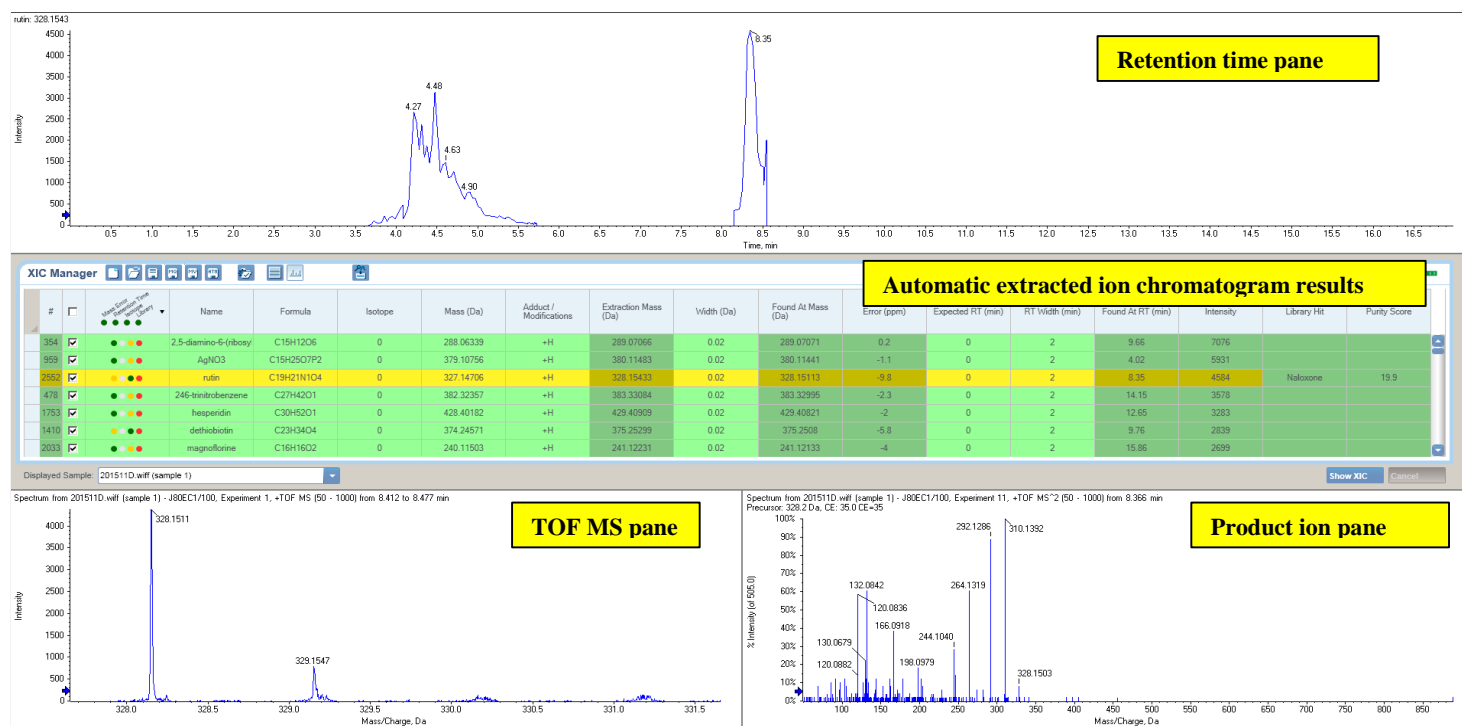


Figure 2.5: Result table obtained from non-targeted screening from organic crude extracts of

S. frutescens

2.2.8 Identification of bioactive (anti-diabetics) compounds from generated XIC results

Having identified the compounds present in crude *S. frutescens* extracts, the next procedure was to identify possible bioactive (anti-diabetic) compounds present in the XIC results generated for each solvent extract. In doing this, the XIC results of individual extracts with two online databases of anti-diabetic phytocompounds were compared: DIACAN: Integrated Database for Anti-diabetic and Anti-cancer Medicinal Plants (<http://www.kaubic.in/diacan>) [257] and Phyto diab care: Phyto remedial database for anti- diabetics

(<http://www.gbpuatcbsh.ac.in/departments/bi/database/phytodiabcare/HOME%20PAGE/Home%20page.html>) [258].

2.2.9 Chemical structures of identified possible anti-diabetic compounds

The chemical structures of identified anti-diabetic phytocompounds were obtained from ChEBI (Chemical Entities of Biological Interest) (<https://www.ebi.ac.uk/chebi/>), which is a freely available online dictionary of molecular entities, focused on chemical entities. Nomenclature, symbols and terminology used by ChEBI are endorsed by the International Union of Pure and Applied Chemistry (IUPAC) and the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) [259].

2.2.10 Comparison of anti-diabetic phytocompounds between extracts

An online bioinformatics tool (Bioinformatics & Evolutionary Genomics) (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to identify possible anti-diabetic phytocompounds common within groups of extracts.

2.2.11 Statistical analysis

The experimental results were expressed as the mean \pm standard deviation (SD) of three replicates. For the phytochemical analysis, One Way Analysis of Variance (ANOVA) was used to determine the significance of the difference in the extracts used.

2.3 Results

2.3.1 Extractant efficiency

Based on their extracting power and the technique of extraction (i.e. application of heat and/or mixing), different solvent extractants have been shown to exhibit remarkable differences in their extraction yield [242, 260]. In this study, using a single step extraction process, the extraction yield of hot aqueous, cold aqueous, 80% ethanol, 100% ethanol 80% methanol and 100% methanol from 16 g of fresh powdered *S. frutescens* plant material was evaluated. The results obtained, as shown in figure 2.6 revealed, that the hot aqueous extractant was quantitatively the best extractant, extracting 1.99 g of plant material compared to 80% ethanol (1.403 g), 100% methanol (1.37 g), cold aqueous (1.09 g) and 80% methanol (1.05 g), while 100% ethanol has the lowest mass extracted (0.52 g) (Figure 2.6). Thus suggesting that the application of heat (hot aqueous) could play a vital role in extraction of bioactive compounds from *S. frutescens*. Thus justifying the traditional use of a tea infusion of *S. frutescens* [261].

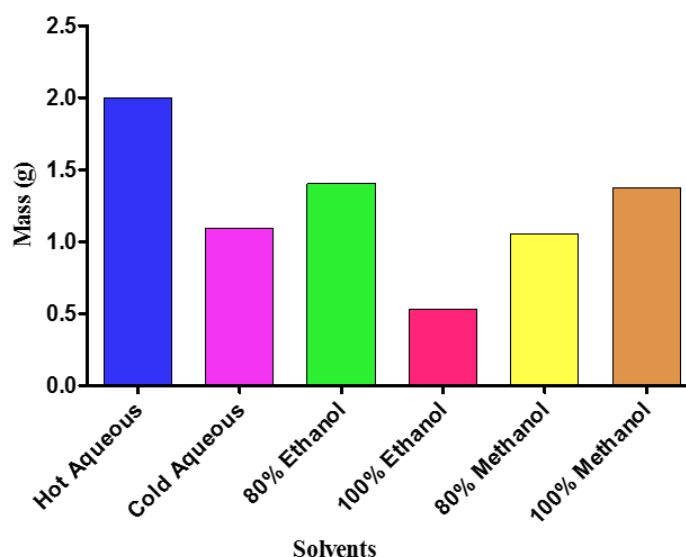


Figure 2.6: Mass of samples extracted by different solvents with varying polarity from 16 g of powdered *S. frutescens*.

2.3.2 Qualitative phytochemical analysis of *S. frutescens* extracts

Using colorimetric and spectrophotometric techniques, the levels of flavonoids, flavonols, tannins, and phenols present in aqueous and organic *S. frutescens* extracts were qualitatively and quantitatively investigated. Furthermore, using statistical tools (One-Way Analysis of Variance), the concentrations of these compounds in individual extracts were compared.

All extracts showed the presence of flavonoids (greenish yellow color), flavonols (pale yellow color), tannins (bluish green color), and phenols (bluish green color) with varying intensity. They all exhibited a moderate color intensity (++) for tannins and phenols (Table 2.2), while the organic extracts 100% methanol, 80% and 100% ethanol exhibited high color intensity (+++) for flavonoids and flavonols respectively (Table 2.2).

Table 2.2: Qualitative phytochemical analysis of flavonoids, flavonols, tannins and phenols in aqueous and organic extracts of *S. frutescens*.

Solvents	Flavonoids	Flavonols	Tannins	Phenols
Hot aqueous	++	++	++	++
Cold aqueous	++	++	++	++
80% Ethanol	++	+++	++	++
100% Ethanol	++	+++	++	++
80% Methanol	++	++	++	++
100% Methanol	+++	++	++	++

++; Low intensity

++; Moderate intensity

+++; High intensity

2.3.3 Quantitative phytochemical analysis of *S. frutescens* extracts

Spectrophotometric analysis of *S. frutescens* extracts revealed that all the organic extracts contained a statistically significant higher concentration (in mg/g of extract) of flavonols when compared to the aqueous extracts (0.79 mg/g, 0.62 mg/g, 0.495 mg/g and 0.42 mg/g for 100% ethanol, 80% ethanol, 100% methanol and 80% methanol, respectively vs 0.36 mg/g and 0.30 mg/g for hot and cold aqueous; $P < 0.001$ in all cases) (Figure 2.7 ii). Similarly, higher concentrations of tannins were found in the organic extracts, when compared to both aqueous

extracts (0.095 mg/g, 0.090 mg/g and 0.089 mg/g for 100% methanol, 80% methanol and 100% ethanol, respectively vs 0.074 mg/g and 0.073 mg/g for both hot and cold aqueous; $P < 0.001$ in all cases) (Figure 2.7 iii). With the exception of 100% methanol (0.028 mg/g), both aqueous extracts contained a slightly higher concentration of flavonoids compared to the remaining organic extracts. However this was not found to be statistically significant (0.027 mg/g and 0.028 mg/g for hot and cold aqueous vs 0.024 mg/g, 0.026 mg/g, 0.026 mg/g for 80% ethanol, 100% ethanol and 80% methanol, respectively) (Figure 2.7 i). All extracts contained approximately equal levels of phenols (Figure 2.7 iv). Taken together, these data confirm the presence of all four groups of bioactive phytochemicals in the *S. frutescens* extracts used in this study, and also confirm that different solvent extractants possess the capability to differentially extract specific groups of phytochemicals.

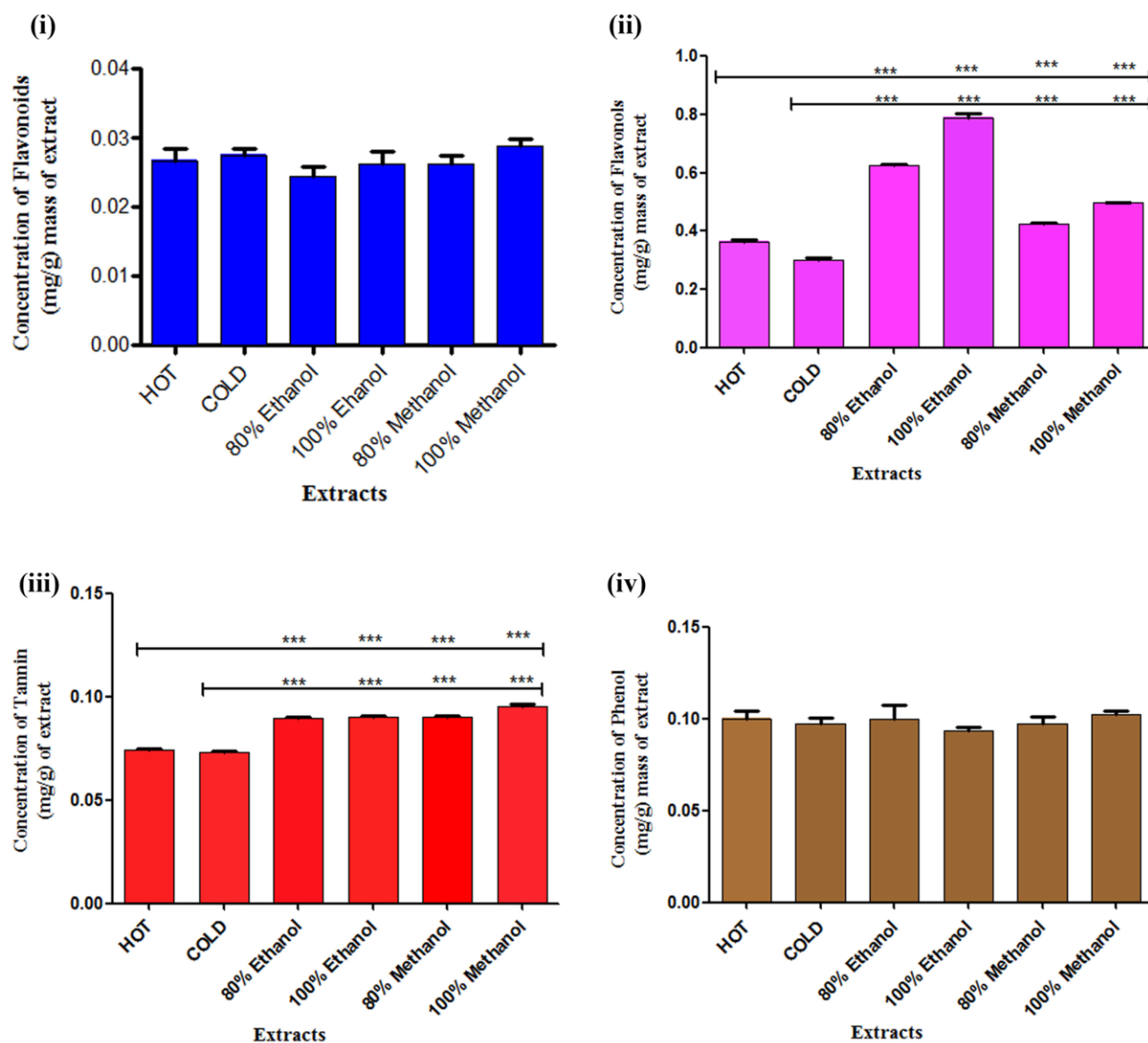


Figure 2.7: Concentrations of (i) flavonoids, (ii) flavonols, (iii) tannins and (iv) phenols found in aqueous and organic extracts of *S. frutescens*. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

2.3.4 LC-MS analysis of crude and SPE fractions of *S. frutescens* extracts

It is well established that plant mixtures contain hundreds of chemical constituents, but only a few components are bioactive [262]. Therefore, it is essential to identify and measure all of the bioactive constituents of medicinal plants [263]. However, the inherent complexity and variability of plant extracts has presented significant challenges for separation, detection and analysis of their chemical composition [264]. From the perspective of separation, modern chromatographic methods have been shown to be highly efficient in the analysis of natural products including plant extracts [265]. Hence, using the new AB SCIEX TripleTOF™5600 LC/MS/MS system, untargeted compound constituents present in crude and SPE fractionated *S. frutescens* extracts were identified.

Application of Triple TOF LC/MS/MS enables the separation of compounds in crude and SPE fractions of *S. frutescens* extracts by their ionic masses (ion chromatograms) and retention time. Further analysis with PeakView Analyst software v1.1.1 and XIC manager identified numerous compounds within each extract. List of some compounds identified are shown in Appendix I (Table I-XXII).

2.3.4.1 Identification of bioactive (anti-diabetics) compounds in crude aqueous and organic extracts of *S. frutescens*

Initially, the crude aqueous (hot and cold) and organic extracts of *S. frutescens* were screened for bioactive phytochemicals. To detect possible bioactive compounds in the identified list of compounds from crude extracts of *S. frutescens*, compounds identified by Triple TOF LC/MS/MS in each of the individual crude aqueous and organic extracts with a list of already identified anti-diabetic phytochemicals (327 compounds) obtained from the online databases DIACAN [257] and Phyto diab care [258] (Appendix II) were compared.

2.3.4.1.1 Anti-diabetic compounds in crude aqueous (Hot and Cold) extracts of *S. frutescens*

As shown in Figure 2.8 a and c, 3 anti-diabetic compounds common to the crude aqueous (hot and cold) extracts of *S. frutescens* were identified namely; α -pinene, Limonene and Sabinene. Stigmasterol and Myricetin, a hydroxyflavone, were found in the cold aqueous extracts alone (Figure 2.8 b and c). Compounds were found at retention times (RT) ranging from 4.90 minutes - 14.49 minutes (Table 2.3). Of these compounds, α -pinene had good confidence parameters (mass error (ppm) ≤ 5.0 and isotope ratio % difference ≤ 10.0) (Figure 2.9), while Sabinene and Limonene had fair confidence parameters (mass error (ppm) ≤ 5.0 and isotope ratio % difference ≤ 20.0) (Figure 2.10 – 2.11). Stigmasterol and Myricetin had poor confidence parameters (mass error (ppm) ≤ 10 and isotope ratio % difference ≤ 20.0), and therefore Myricetin (the poorest) is not shown. Further comparison between hot and cold aqueous extracts showed that both extracts had 21 other non-anti-diabetic compounds in common, 74 compounds were specific for the crude cold extract alone, whilst 20 compounds were specific for the crude hot extract (Figure 2.8 c).

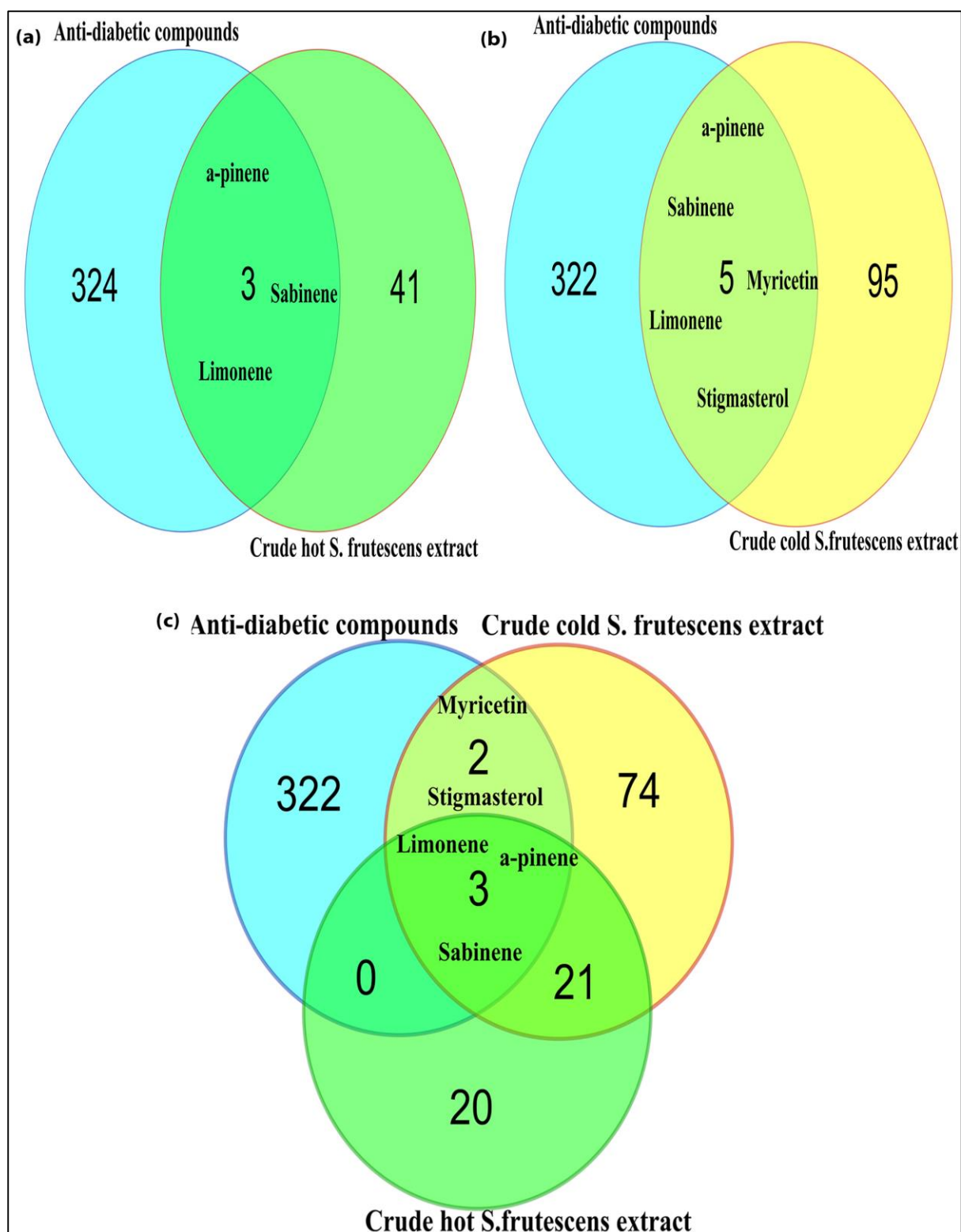


Figure 2.8: Anti-diabetic compounds identified in crude (a) Hot, (b) cold aqueous and (c) comparison of both aqueous extracts of *S. frutescens*

Table 2.3: Anti-diabetic compounds identified in crude aqueous (hot and cold) extracts of *S. frutescens*

Name	Formula	Mass (Da)	Found at mass (Da)	Found at RT (min)
α -Pinene	C ₁₀ H ₁₆	136.1252	137.13229	10.63
Limonene	C ₁₀ H ₁₆	136.1252	137.13192	13.04
Myricetin	C ₁₅ H ₁₀ O ₈	318.23510	318.23510	4.99
Sabinene	C ₁₀ H ₁₆	136.1252	137.13248	9.43
Stigmasterol	C ₂₉ H ₄₈ O	412.69082	412.69082	14.49

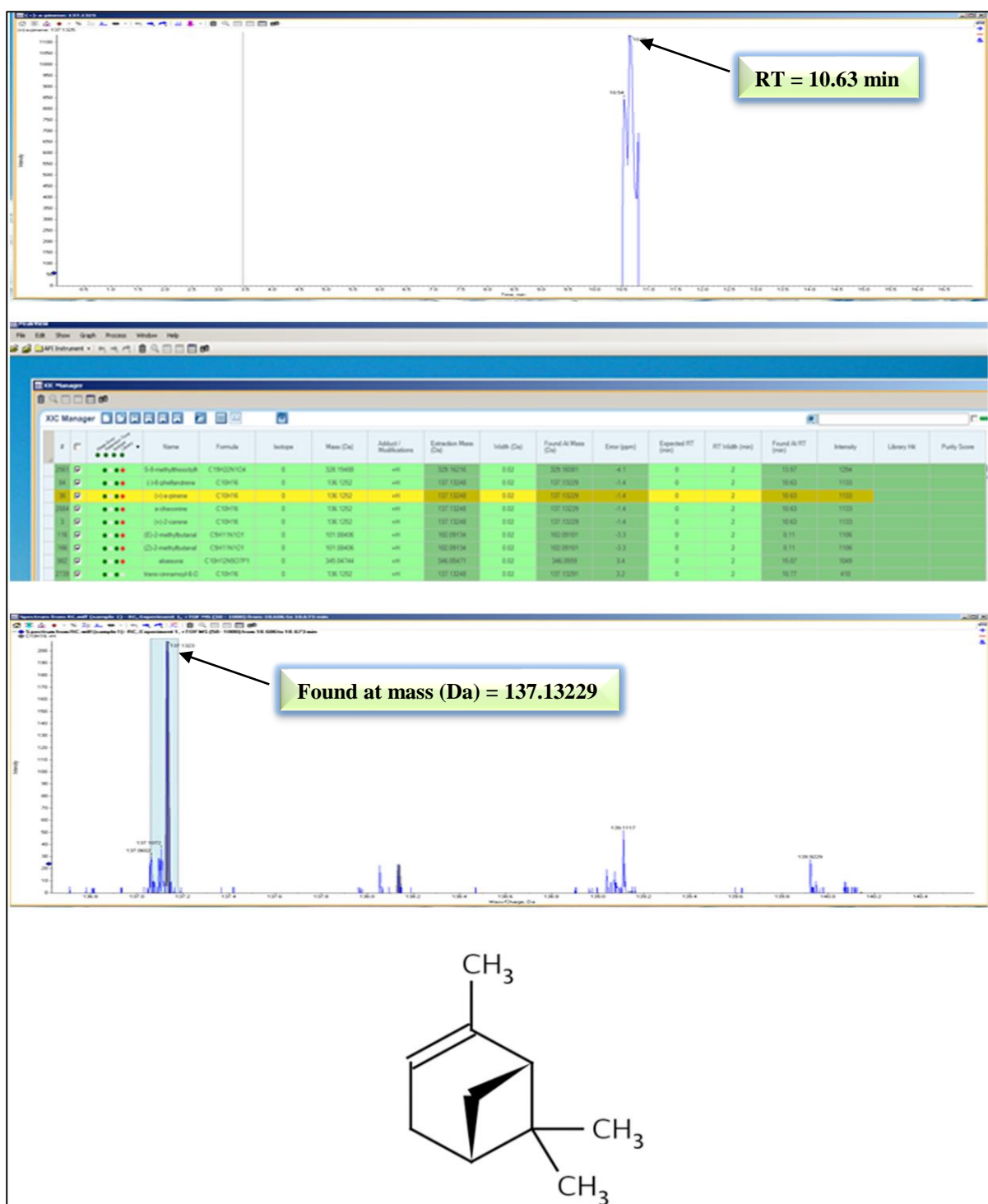


Figure 2.9: Chromatogram of α -pinene identified in crude aqueous (hot and cold) extracts of *S. frutescens*

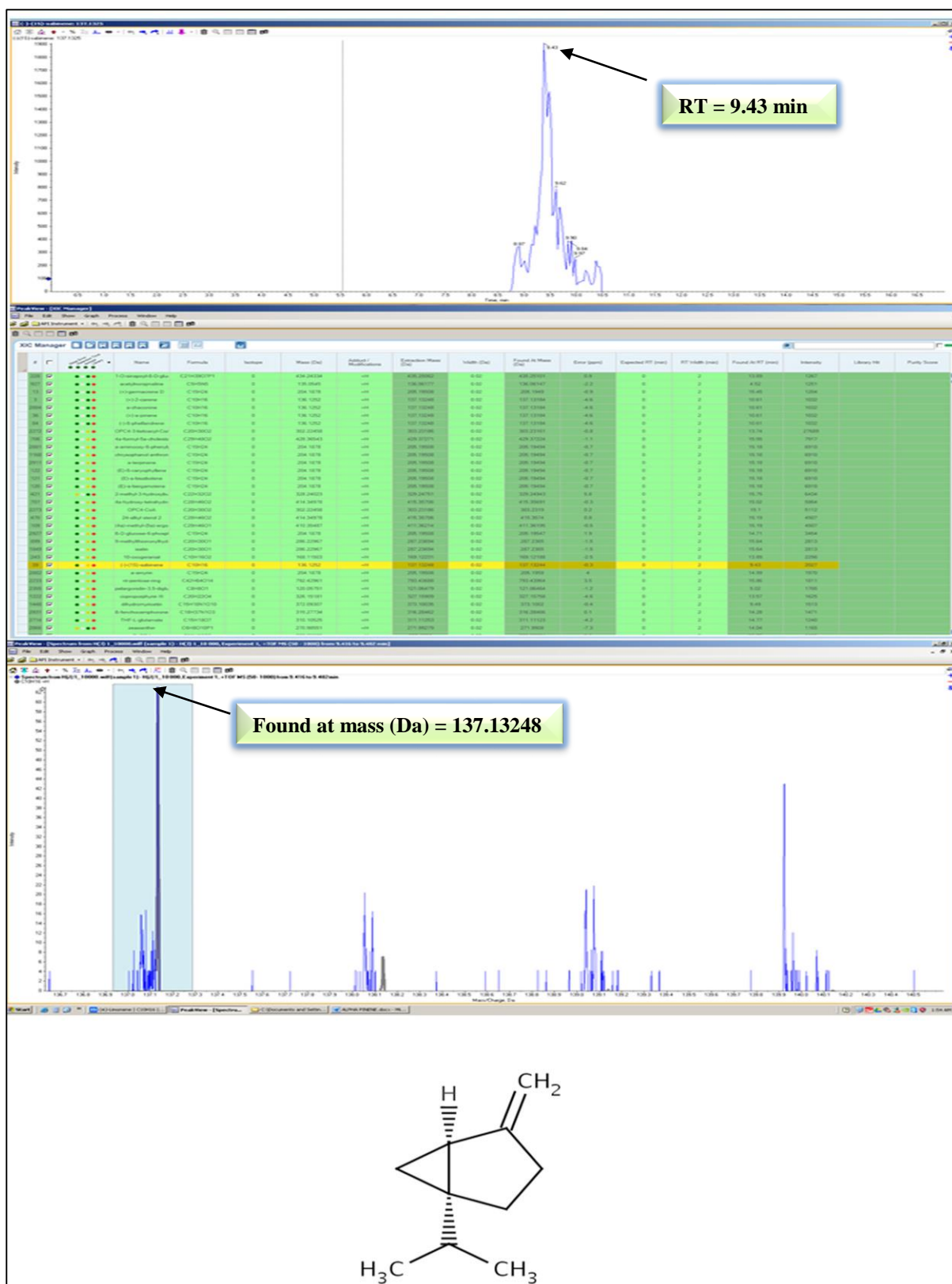


Figure 2.10: Chromatogram of Sabinene identified in crude aqueous (hot and cold) extracts of *S. frutescens*

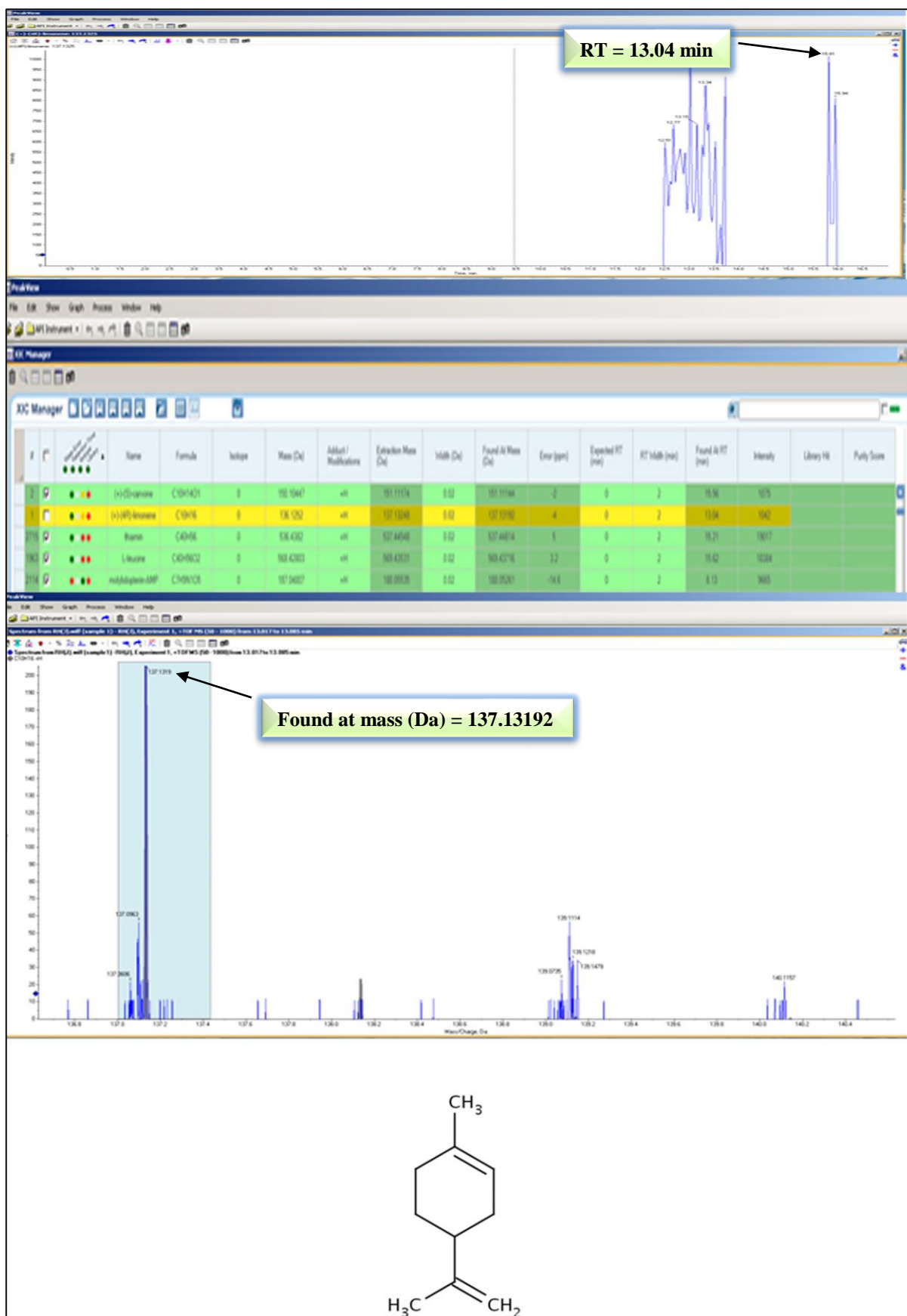


Figure 2.11: Chromatogram of Limonene identified in crude aqueous (hot and cold) extracts of *S. frutescens*

2.3.4.1.2 Anti-diabetic compounds in crude organic extracts of *S. frutescens*

Analysis of the crude organic (80% and 100% ethanol) extracts of *S. frutescens* identified 2 anti-diabetic compounds (Rutin and Carvone) common to both the 80% and 100% ethanolic extracts of *S. frutescens* (Figure 2.13 b and c) whilst Emodin and Limonene were found in the 80% ethanol extract alone (Figure 2.13a). Compounds were found at RT ranging between 8.35 minutes – 14.30 minutes (Table 2.4). Data from the XIC manger showed that Rutin and Emodin had fair confidence parameters (mass error (ppm) ≤ 10 and isotope ratio % difference ≤ 10) (Figure 2.14 and 2.15), whilst both Carvone and Limonene had poor confidence parameters (mass error (ppm) > 10 and isotope ratio % difference ≤ 20) (Figure 2.16 and 2.17). Further comparison between the two extracts (80% and 100% ethanol extracts) as seen in figure 2.13 c showed that 146 and 86 non-diabetic compounds were specific for 80% and 100% crude methanol extract while both extracts shared a total of 74 non-diabetic compounds.

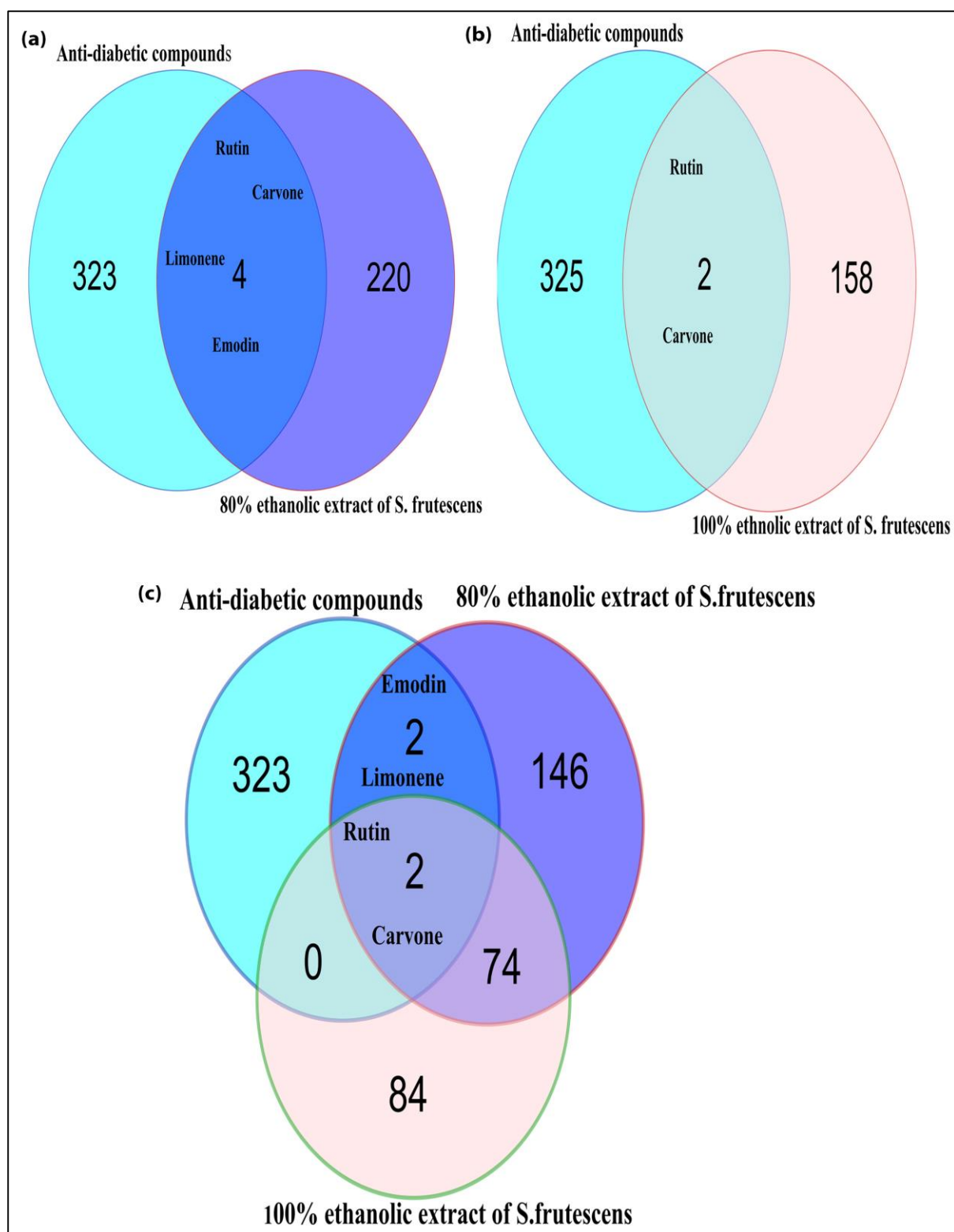


Figure 2.13: Anti-diabetic compounds identified in crude (a) 80% (b) 100% and (c) comparison of both ethanolic extracts of *S. frutescens*

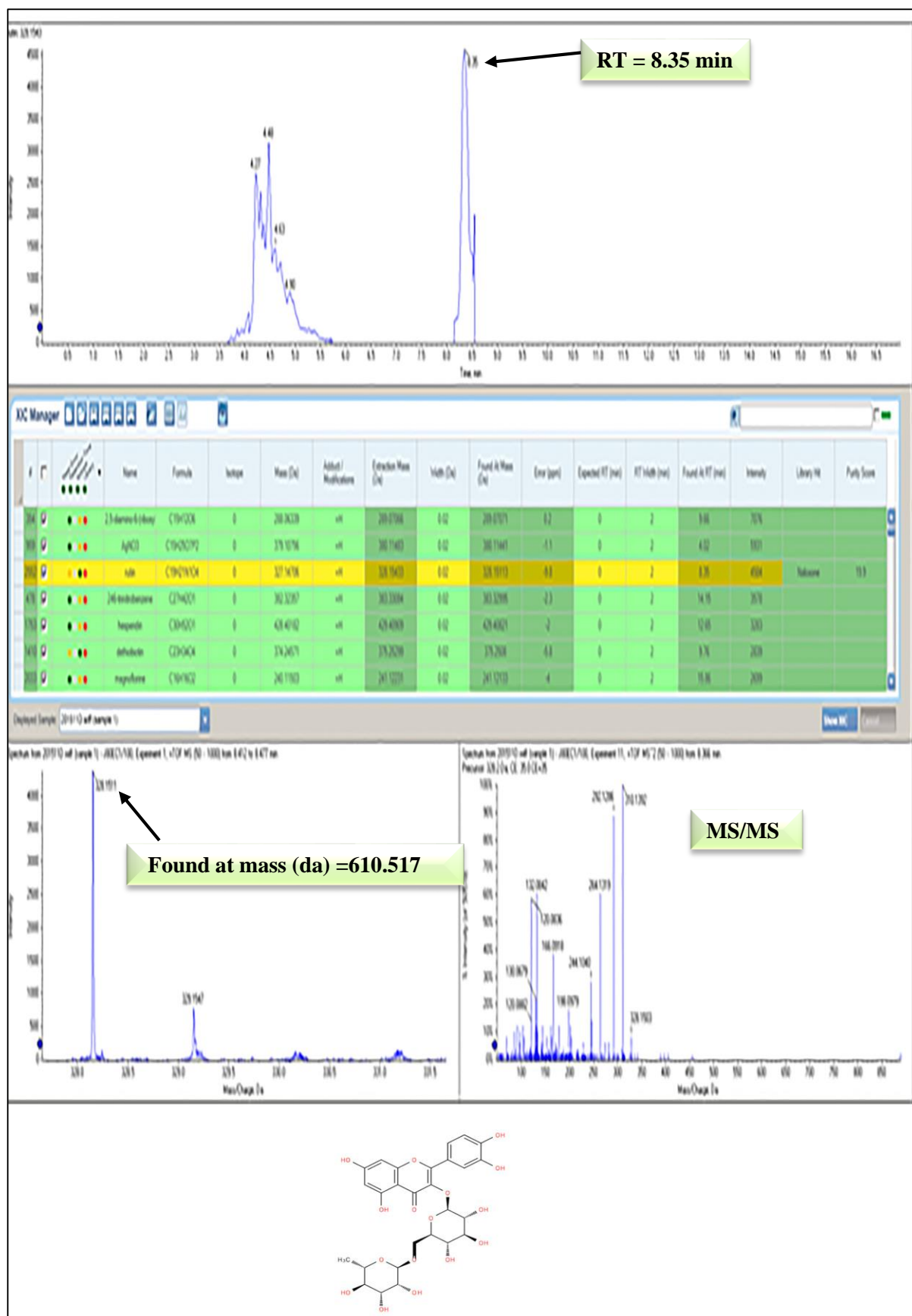


Figure 2.14: Chromatogram of Rutin identified in crude ethanolic extracts of *S. frutescens*

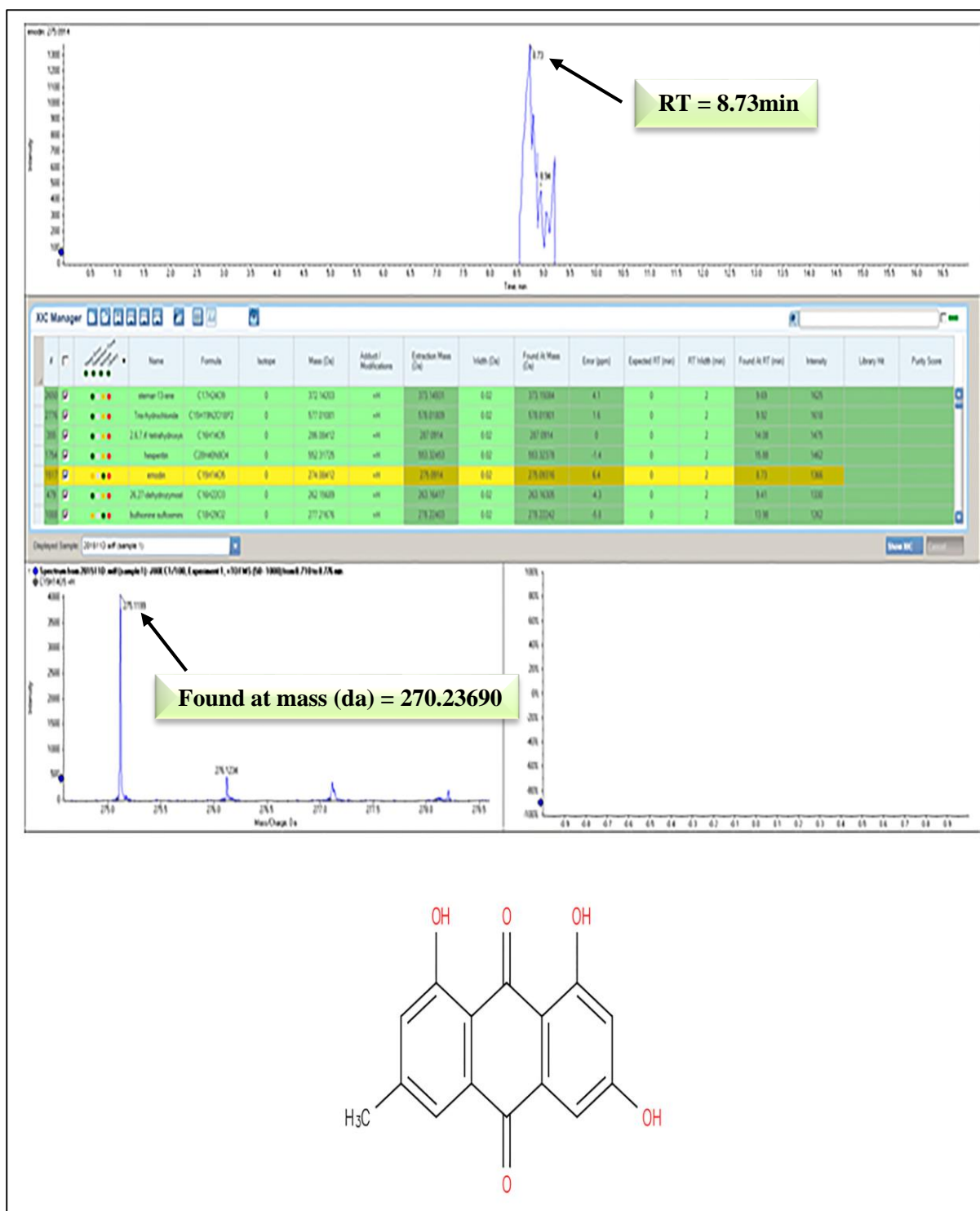


Figure 2.15: Chromatogram of Emodin identified in crude 80% ethanol extracts of *S. frutescens*

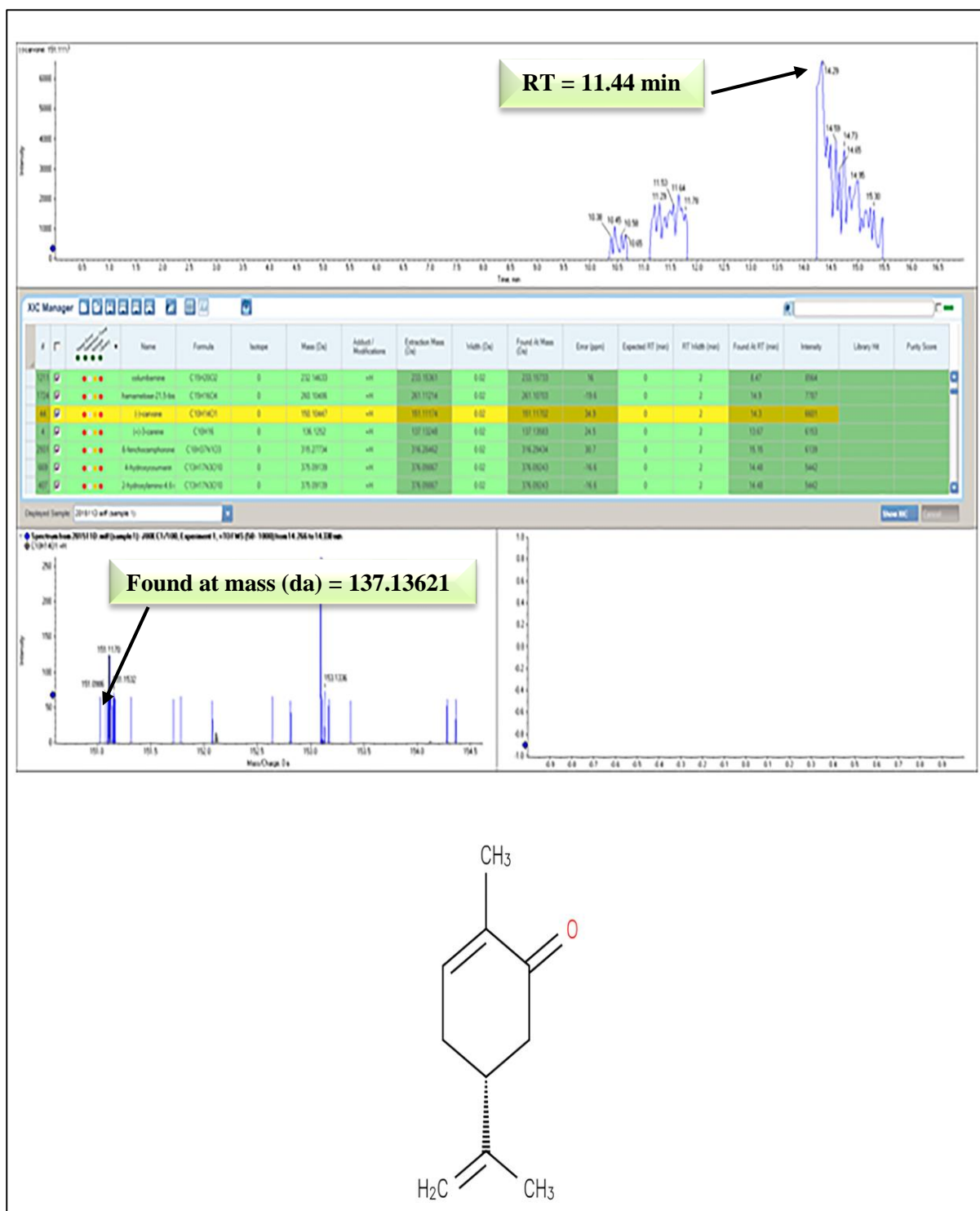


Figure 2.17: Chromatogram of Limonene identified in crude 80% ethanol extract of *S. frutescens*

Table 2.4: Anti-diabetics compounds identified in crude ethanolic (80% and 100%) extracts of *S. frutescens*

Name	Formula	Mass (Da)	Found at mass (Da)	Found at RT (min)
Rutin	C ₂₇ H ₃₀ O ₁₆	610.51750	610.51750	8.35
Emodin	C ₁₅ H ₁₀ O ₅	270.23690	270.23690	8.73
Carvone	C ₁₀ H ₁₄ O ₁	150.10447	151.11702	14.3
Limonene	C ₁₀ H ₁₆	136.1252	137.13621	11.44

Analysis of the *S. frutescens* methanolic extracts identified Hypoglycin B and Stigmasterol in both the 80% and 100% methanol extracts (2.18 c), whilst Sarpagine was identified in the 80% extract alone (Figure 2.18 a), and Limonene was found in the 100% (Figure 2.18 b) methanolic *S. frutescens* extract alone. The retention time (RT) for these compounds was within the range 9.69 min – 16.15 min (Table 2.5). As seen in figures 2.19-2.22, the confidence parameter of individual compounds was as follows; Hypoglycin B (Mass error (ppm) > 10 and isotope ratio % difference ≤ 10); Stigmasterol (Mass error (ppm) ≤ 5 and isotope ratio % difference >20); Sarpagine (Mass error (ppm) ≤ 5 and isotope ratio % difference ≤ 20) and Limonene (Mass error (ppm) ≤ 5 and isotope ratio % difference ≤ 20). Both methanolic extracts shared 76 non-diabetic compounds, whilst 120 non-diabetic compounds were specific for the 80% and 118 for the 100% methanol extract.

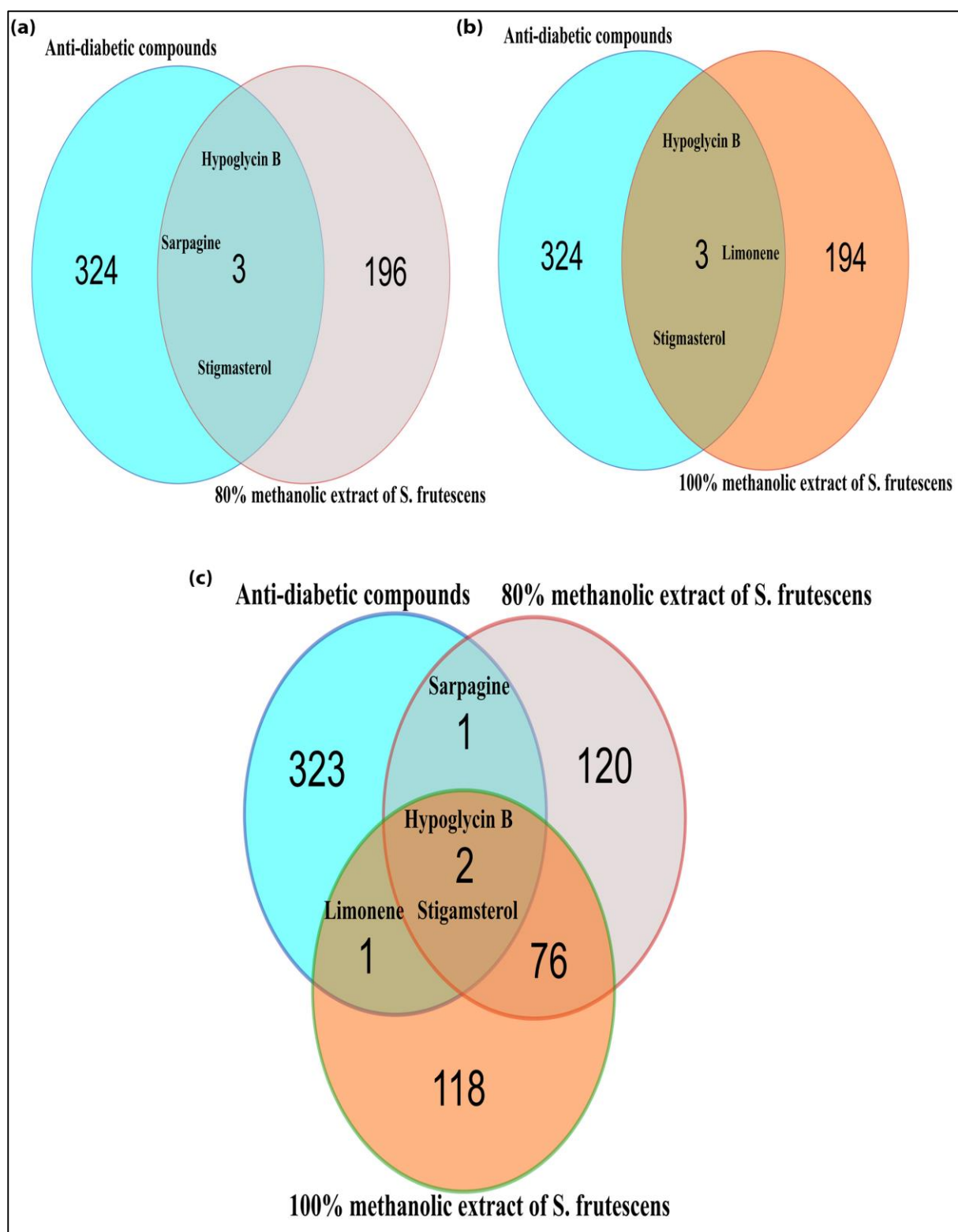


Figure 2.18: Anti-diabetic compounds identified in crude (a) 80% (b) 100% and (c) comparison of both methanolic extracts of *S. frutescens*

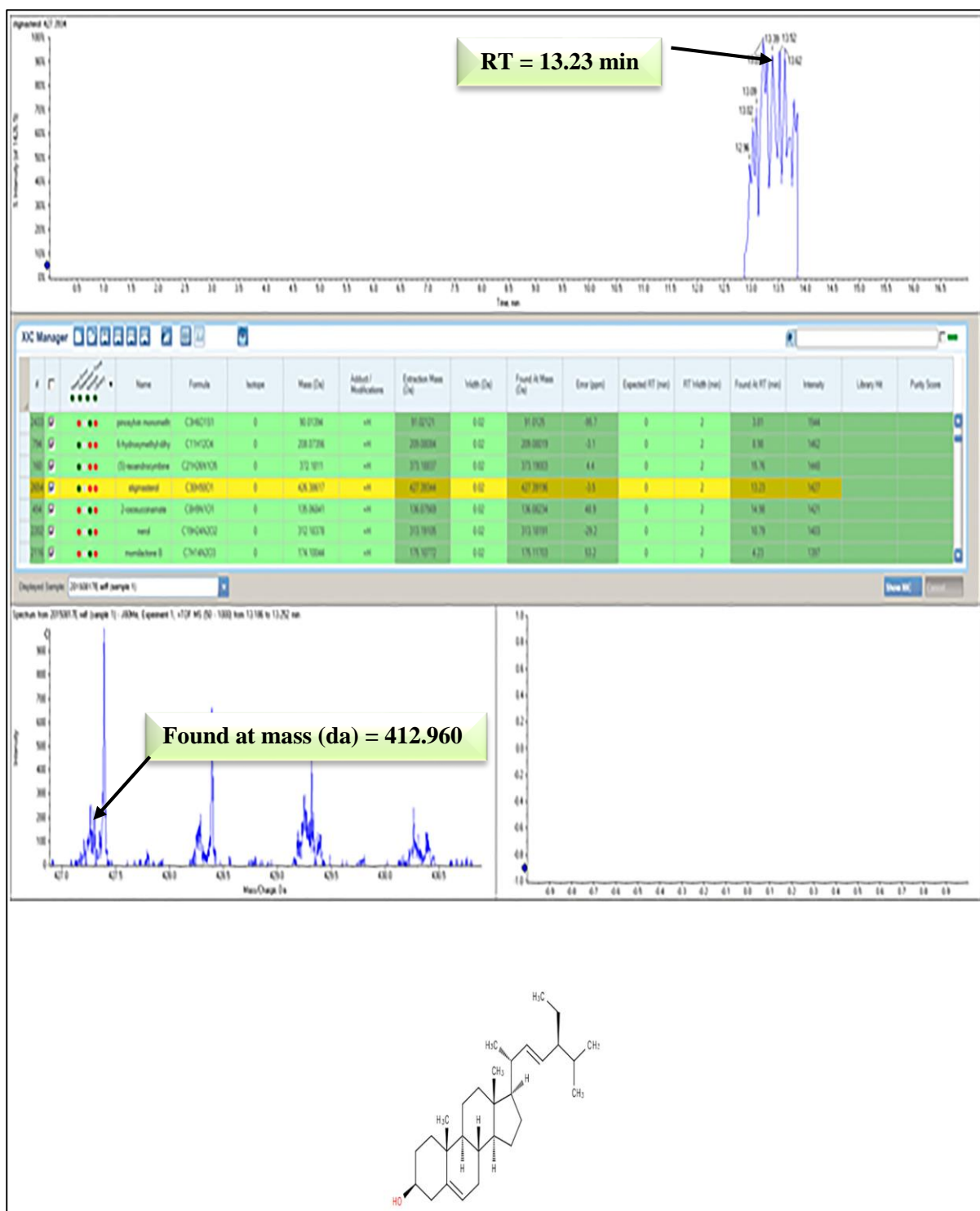


Figure 2.20: Chromatogram of Stigmasterol identified in crude methanolic extracts of *S. frutescens*

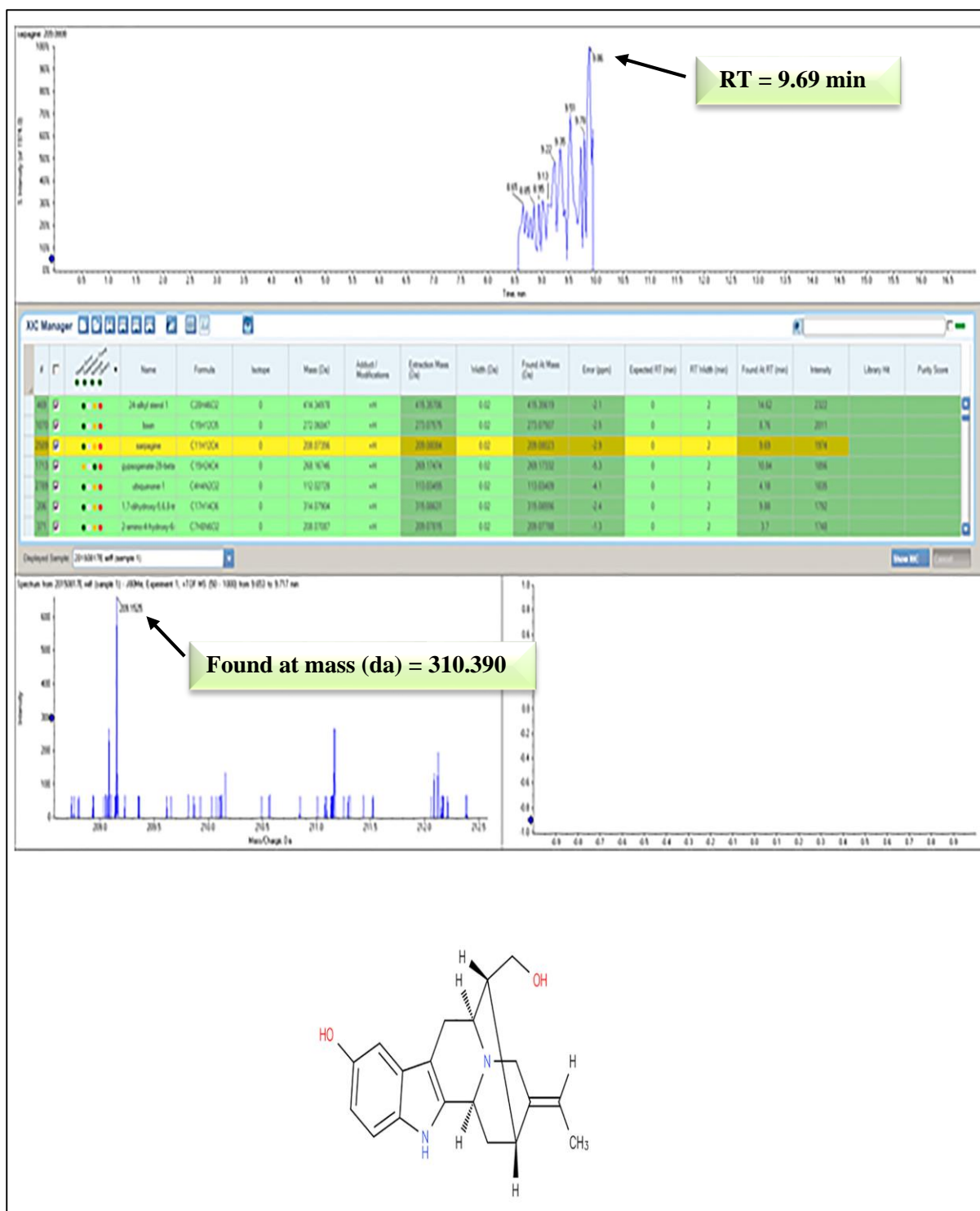


Figure 2.21: Chromatogram of Sarpagine identified in crude 80% methanol extract of *S. frutescens*

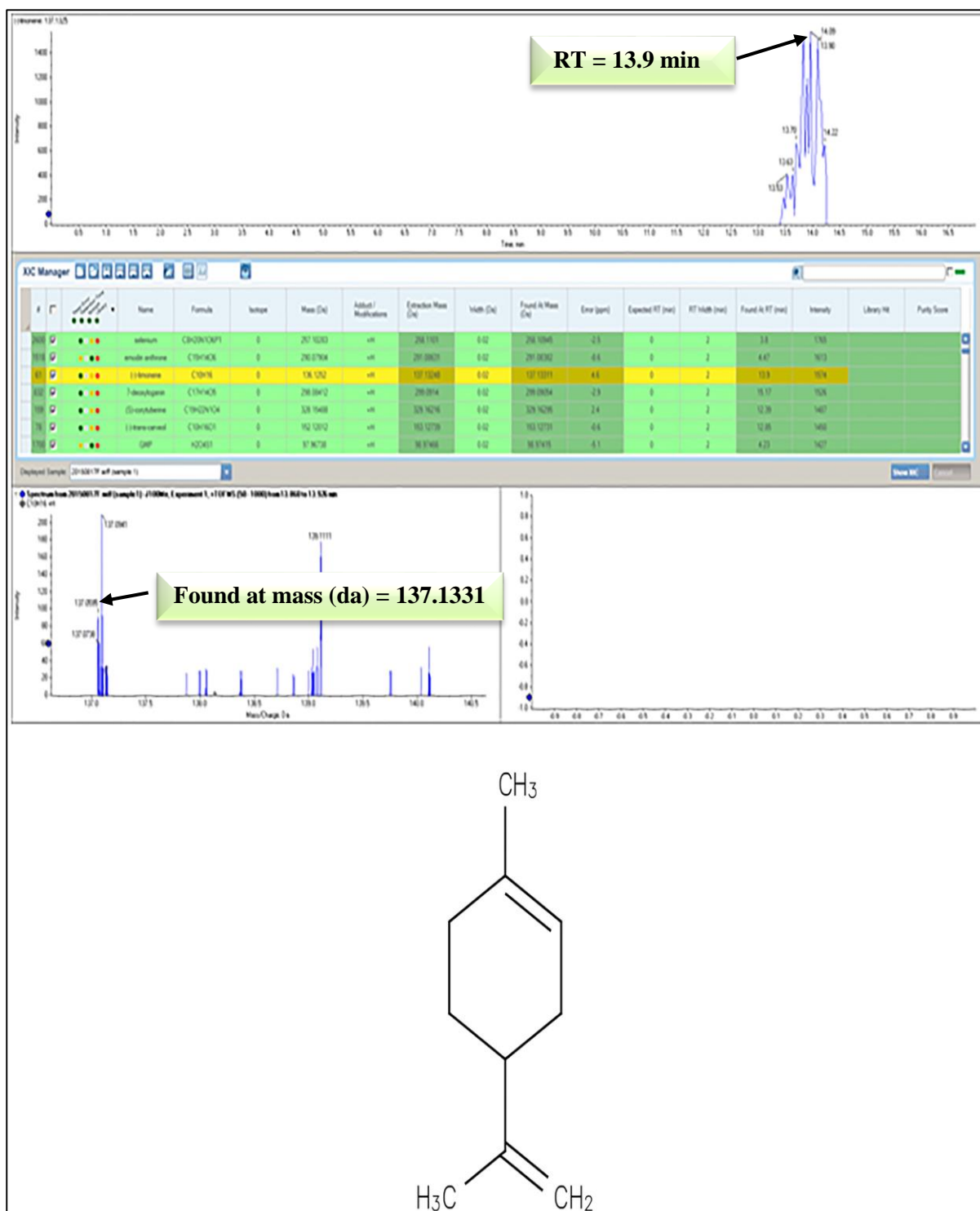


Figure 2.22: Chromatogram of Limonene identified in crude 100% methanol extract of *S. frutescens*

Table 2.5: Anti-diabetic compounds identified in crude methanolic (80% and 100%) extracts of *S. frutescens*

Name	Formula	Mass (Da)	Found at mass (Da)	Found at RT (min)
Hypoglycin B	C ₁₂ H ₁₈ N ₂ O ₅	270.28172	270.28172	16.15
Stigmasterol	C ₂₉ H ₄₈ O	412.69082	412.69082	13.23
Sarpagine	C ₁₉ H ₂₂ N ₂ O ₂	310.390	310.390	9.69
Limonene	C ₁₀ H ₁₆	136.1252	137.1331	13.9

2.3.4.1.3 Anti-diabetic compounds in both crude aqueous and organic extracts of *S. frutescens*

Comparison analysis between individual extracts revealed anti-diabetic compounds identified in each individual extract. Results obtained showed that no single compound was found in all the extracts. However, Limonene was found in all the extracts except 100% ethanol and 80% methanol extracts (Table 2.6).

Table 2.6: Comparison of anti-diabetic compounds identified in individual aqueous and organic extracts of *S. frutescens*

Cold aqueous	Hot aqueous	80% Ethanol	100% Ethanol	80% Methanol	100% Methanol
α -Pinene	α -Pinene	Rutin	Rutin	Hypoglycin B	Hypoglycin B

Limonene	Limonene	Limonene	Carvone	Stigmasterol	Limonene
Myricetin	Sabinene	Carvone		Sarpagine	Stigmasterol
Sabinene		Emodin			
Stigmasterol					

Taken together, the data obtained from this preliminary untargeted LCM-MS screening of crude aqueous and organic extracts of *S. frutescens* suggest that *S. frutescens* extracts contain several anti-diabetic compounds that may work in synergy, or alone at a single specific site, or multiple sites in a cell to mediate their biological effects.

2.3.4.1.4 Anti-diabetic compounds in SPE fractions of *S. frutescens*

Analysis of ion chromatograms obtained from SPE fractions of *S. frutescens* aqueous, 100% ethanol and 100% methanol extracts, acquired in positive [H⁺] ion spray (ESI) mode revealed similar anti-diabetic compounds as seen in the individual crude extracts. However, compounds were found separated by their individual ionic charges as seen in table 2.7. Other non anti-diabetic compounds identified in individual SPE fractions are listed in Appendix I.

2.3.4.1.5 Comparison of compounds in crude and SPE fractions of *S. frutescens* acquired in both the positive and negative ion spray (ESI) mode.

Further comparison of ion chromatograms obtained from crude and SPE fractions of *S. frutescens* acquired in both the positive [H⁺] and negative [H⁻] ion spray showed the presence of similar compounds in each extract, found at the specific ionic spray i.e. [H⁺] or [H⁻] (data not shown).

Table 2.7: Anti-diabetic compounds identified in SPE fractions of crude aqueous and organic extracts of *S. frutescens*

Extracts	SPE fractions				
	Strong anions	Weak anions	Strong cations	Weak cations	Reverse Phase
Cold aqueous	-	-	Limonene		Limonene
			α -pinene		α -pinene
			Sabinene	Stigmasterol	
			Stigmasterol		
Hot aqueous	-	-	α -pinene		
			Limonene		
			Sabinene	-	-
100% Methanol	-	-	Limonene		
			Stigmasterol	-	Hypoglycin B
100% Ethanol	-	Rutin	Carvone	-	Emodin

-; no anti-diabetic compound found

2.4 Discussion

For many decades, the use of synthetic chemicals as drugs has been effective in the treatment of diseases. However, the increased rates of resistance, rising costs and the possible toxicity of some synthetic drugs has led to the search for new therapeutics particularly from ethnopharmacology and traditional herbal medicine. Hence, over the past 20 years interest in traditional medicines has increased considerably in many parts of the world [266]. Many plant based (herbal) medicines have been shown to play an important role in health care [267]. Medicinal plant species are reservoirs of natural products with remarkably high biodiversity, which can serve as a source of novel chemical entities with potential as drug treatments [236]. Phytochemicals are natural bioactive compounds found in medicinal plants, usually in leaves, flowers and roots. They act as plant defense system to combat against diseases. More than 1000 different phytochemicals have already been shown to possess interesting therapeutic activities [268]. Phytochemicals comprises of a diverse range of chemical entities, such as polyphenols (tannins), flavonoids, flavonols, phenols, steroidal saponins, organosulphur compounds and vitamins [267].

Sutherlandia frutescens (L.) R. BR., Family *Fabaceae* subsp. *microphylla*, is a multipurpose medicinal plant widely used in the Western Cape province of South Africa [269, 270]. Crude extracts of the plant have been shown to possesses anticancer [271], antidiabetic [222], antibacterial and antioxidant properties, as well as inhibitory activity against HIV target enzymes [272]. However, despite extensive biological and pharmacological studies, few analyses exist of the chemical constituents of this medicinally important herbal plant [273], and no Triple TOF LC/MS/MS analysis has been performed.

Based on their extracting power, different solvents exhibit remarkable differences in their extraction yield [242, 260]. However, in traditional medical practices, water is the chief solvent

and major extractant used in preparing herbal mixtures and extracting bioactive compounds from medicinal plants [242]. Moreover, water is not harmful to humans and is generally cheap and easily acquired. In South Africa, the use of *S. frutescens* tea infusion as a herbal mixture is a common practice [274].

In this study, crude aqueous (hot and cold) and organic (80% and 100% methanol and 80% and 100% ethanol) extracts of *S. frutescens* were qualitatively and quantitatively investigated for the presence of phytochemicals tannins, flavonoids, flavonols, and phenols. Furthermore, using the Triple TOF LC-MS/MS, a preliminary phytochemical screening was performed to identify possible bioactive (anti-diabetic) chemical constituents of these extracts.

The hot aqueous extractant was quantitatively the best extractant, extracting 1.99 g of extract from 16 g starting plant material, while the organic solvent (100% ethanol) was a poor extractant yielding 0.52 g of extract from 16 g starting plant material. It is therefore very plausible that the biological effects mediated by hot aqueous extract of *S. frutescens* are a consequence of its greater extracting power leading to extraction of more bioactive compounds from a given amount of plant material.

Plant phenols range from simple low-molecular-weight phenolic glycosides to polymeric compounds [275]. Phenols and their metabolites are believed to play important roles in plant defense mechanisms [276]. However, in human medicinal therapy, phenols and phenolic compounds have been explored for their anti-cancer [277], anti-oxidant, anti-atherosclerotic, anti-bacterial, anti-inflammatory, and anti-viral properties [278, 279]. In this present study, aqueous and organic extracts of *S. frutescens* were investigated for their total phenolic content. All extracts contained approximately equal concentration of phenol. However, further analysis showed differences in the concentration of polyphenolic compounds: flavonoids, flavonol and tannins in each individual extract.

Flavonoids are polyphenolic compounds distributed widely in the plant kingdom and include several subclasses, such as flavones, flavanols, flavanones, isoflavones and anthocyanins [280]. Flavonoids are found in several medicinal plants and plant mixtures containing flavonoids have been used in folk medicine around the world [281]. Flavonoids display a number of biological activities including antiallergic, anti-inflammatory, antioxidant, anticarcinogenic, and modulation of enzymatic activities [282-285]. The enzyme modulatory action of flavonoids was demonstrated in a study by Zhang et al. (2013). Zhang and colleagues showed that total flavonoids obtained from *Rosa laevigata* fruit significantly decreased serum liver enzymes: Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activities in non-alcoholic fatty liver diseased rats [286]. In addition, emerging studies have established a neuroprotective and neuromodulatory role for plant-derived flavonoids. By interacting within ERK and PI3-kinase/Akt signaling pathways, medicinal plant flavonoids was shown to increase the expression of neuroprotective and neuromodulatory proteins and increase the number and strength of connections between neurons [287]. Furthermore, Zayachkivska et al. (2005) demonstrated the gastroprotective properties of plant-derived flavonoids on ethanol-induced gut damage in male Wistar rats [288]. Data presented herein revealed that both aqueous and organic extracts of *S. frutescens* contained flavonoids and in particular flavanols. The presence of flavonoids and flavanols in these extracts thus support the folklore use of herbal mixture of *S. frutescens* as an anxiolytics, anti-inflammatory and in the treatment of stomach problems [206]

Spectrophometric quantitative analysis showed that all extracts had approximately equal concentration of flavonoids while the organic extracts had a significantly higher concentration of flavanols than both aqueous extracts, with the 80% and 100% ethanolic extracts having the highest concentration (0.62 mg/g and 0.79 mg/g). This is in agreement with similar findings

by Brandao et al. (1997) [289] and Hufford et al. (1993) [290] reviewed in Cowan et al. (1999) [291] where it was shown that ethanol is a major extractant of flavonol compounds. Similarly, all *S. frutescens* extracts in this study contained tannins. Tannins are polymeric phenolic compounds with molecular weights ranging from 500 - 3,000 Da [292] found in almost every plant part: bark, wood, leaves, fruits, and roots [293]. Tannins are divided into two groups, hydrolysable and condensed tannins [293]. Over the years, tannins have received a great deal of attention owing to their health benefits. The consumption of tannin-rich beverages, particularly green teas and red wines have been suggested to cure or prevent human ailments including, cancer and cardiovascular diseases [294].

The anti-cancer properties of tannins have been heavily linked to their beneficial physiological role as a scavenger of reactive oxygen intermediates (ROI) [295]. Labieniec and colleagues demonstrated this in a study where tannins was shown to significantly decrease H₂O₂ mediated DNA damage at low concentration in fresh water bivalve mollusc (mussel *Unio tumidus*) [296]. In addition, using disc diffusion method Langer et al. (2013), demonstrated the anti-bacterial (gram positive and gram negative) properties of tannins [297]. Furthermore, in an experiment to elucidate the anti-inflammatory properties of tannins, Yoshimura and colleagues discovered that oenothien B a unique macrocyclic ellagitannin found in several medicinal plants inhibited dendritic cell (DC) differentiation. In addition, this compound also inhibited the production of inflammatory cytokines such as IL-1 β and IL-6 in a dose-dependent manner [298]. Taken together, the identification of tannins in *S. frutescens* extracts as seen herein may, in part, explain the traditional use of *S. frutescens* herbal mixture in the treatment of a variety of inflammatory diseases including inflammatory bowel disease, celiac disease, and rheumatoid arthritis [206] and as an anti-cancer agent [216].

Analysis of the ion chromatograms generated from crude aqueous and organic *S. frutescens* extracts, using untargeted Triple TOF LC-MS/MS separation technique in positive ionic mode,

revealed the presence of multiple compounds in individual extract. Further comparison of these compounds with online databases of anti-diabetic phytochemicals led to the preliminary identification of 10 possible anti-diabetic compounds in crude aqueous and organic extracts of *S. frutescens*. These compounds include: α -Pinene, Limonene, Sabinene, Carvone, Myricetin, Rutin, Stigmasterol, Emodin, Sarpagine and Hypoglycin B.

Pinenes and bicyclic terpenes are the major chemical constituents found in the essential oils of coniferous trees (pine) [299]. Pinenes have two active constitutional isomers: α - and β -pinene both having enantiomers known in nature as $(-)$ - α -pinene, $(+)$ - α -pinene, $(-)$ - β -pinene and $(+)$ - β -pinene [299]. Alpha-pinene (α -pinene) is a monoterpene (Figure 2.23) [300] that has been suggested to possess a variety of interesting pharmacological properties including anti-inflammatory [301], anti-bacterial [299, 302], and anti-fungal [303] properties. Diaz et al. (2008) demonstrated the cytotoxic effect of α -pinene a major constituent (97.2%) of *Schinus molle* essential oil on breast cancer and leukemic cell lines [304]. In addition, Pinheiro et al. (2015) demonstrated the gastroprotective and anti-ulcerogenic effect of α -pinene. In their study, Pinheiro and colleagues showed that α -pinene pre-treatment inhibited ethanol-induced gastric lesions, reduced volume and acidity of the gastric juice and increased gastric wall mucus in male Swiss mice [300]. The anti-diabetic effect of α -pinene has been demonstrated by various *in vitro* and *in vivo* studies showing the hypoglycaemic and insulin-like effects of α -pinene-rich extracts or oil of medicinal plants [305, 306]. In an *in-vivo* study, Bakirel et al. (2007) showed that ethanol extracts (100 and 200 mg/kg) of *Rosmarinus officinalis*, a plant highly rich in α -pinene [307, 308], significantly lowered blood glucose level and increased serum insulin concentration in alloxan-diabetic rabbits [305]. In an *in-vitro* study sought to investigate the inhibitory potential of rosemary extracts on key enzymes related to diabetes such as α -amylase and pancreatic lipase activities, Ben Ali et al. (2014) demonstrated that α -pinene-rich essential oil of *Rosmarinus officinalis* inhibited α -amylase and pancreatic lipase

activities with an IC_{50} ranging from 28.36 - 34.07 $\mu\text{g/mL}$, suggesting strong anti-diabetic and anti-obesity effects of *Rosmarinus officinalis* [309]. Furthermore, ethanol extracts of *Nigella sativa* L. seed, a plant containing α -pinene [310] was shown to significantly reduce the elevated levels of blood glucose, lipids and improved altered levels of lipid peroxidation products in streptozotocin (STZ) induced diabetic rats [309].

In this present study, (+)- α -pinene was identified in crude aqueous (hot and cold) extracts of *S. frutescens*. It is therefore very plausible that aqueous extracts of *S. frutescens* can act in similar ways to mediate anti-diabetic effects.

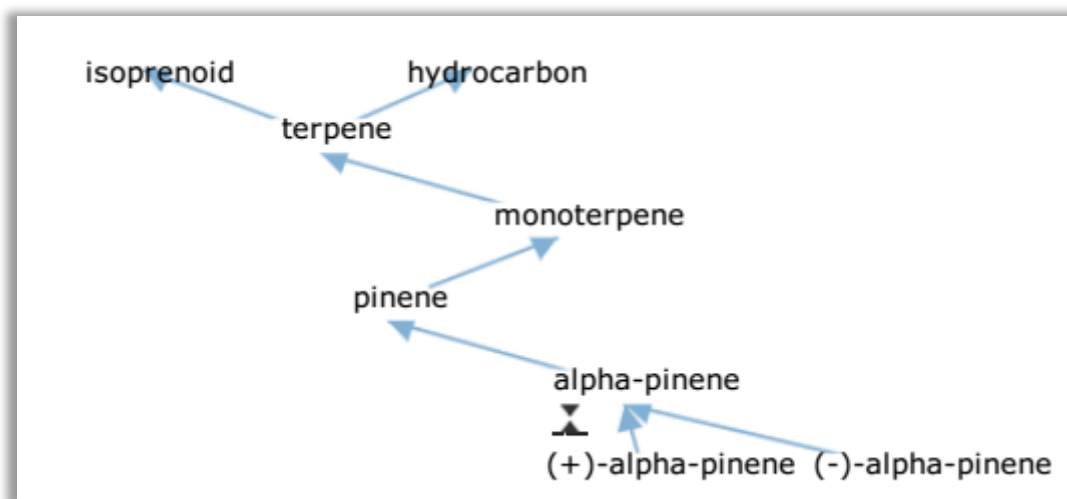


Figure 2.23: Chemical ontology of α -pinene [311].

Limonene is also a monocyclic monoterpene (Figure 2.24), that has been isolated from a number of medicinal plants such as *Citrullus colocynthis*, *Adansonia digitata* and Finger citron fruits [312-314]. Limonene, has been shown to have both chemopreventive and therapeutic effect against several human cancers [315]. Haag et al. (1992), showed that Limonene mediated regression of mammary gland cancer in Wistar-Furth female rats [316]. Similarly, recent clinical trial data have shown a promising therapeutic effect of Limonene in human breast carcinoma [317, 318]. To demonstrate the anti-inflammatory properties of

Limonene, d'Alessio and colleagues showed that Limonene significantly reduced intestinal inflammation in TNBS-colitis rats. This was associated with lowered serum concentrations of TNF- α compared to the untreated TNBS-colitis rats [319]. Furthermore, the anti-diabetic properties of Limonene has been documented in a study by Murali et al. (2012) in which orally administered Limonene (100 mg/kg) was found to decrease plasma glucose and glycosylated haemoglobin, down regulate activities of gluconeogenic enzymes such as, glucose 6-phosphatase and fructose 1, 6-bisphosphatase and up-regulate the activity of glycolytic enzyme, glucokinase in streptozotocin-induced diabetic rats [320]. Identification of Limonene in aqueous (cold and hot) and organic (80% ethanol and 100% methanol) extracts in this present study suggests that cold aqueous, hot aqueous, 80% ethanol and 100% methanol extracts of *S. frutescens* could serve as a potent anti-cancer and anti-diabetic agents.

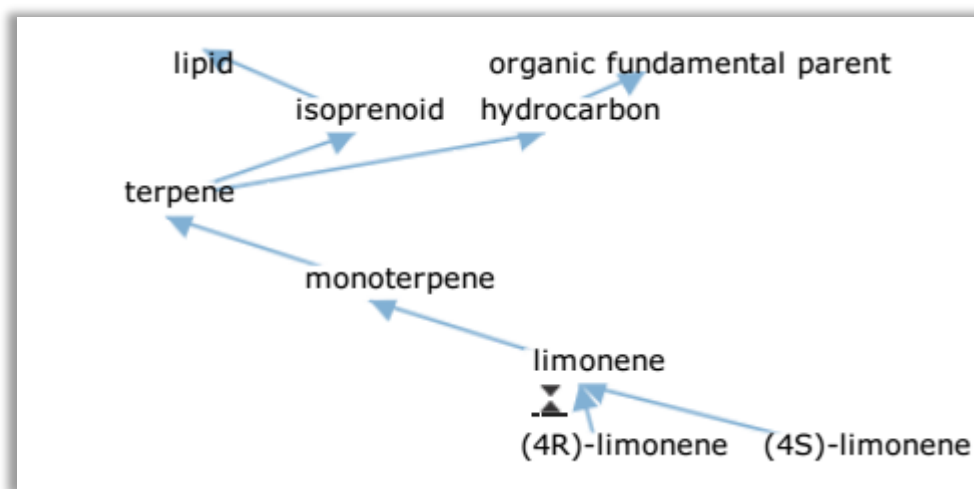


Figure 2.24: Chemical ontology of Limonene [311]

Sabinene is a natural bicyclic monoterpene (Figure 2.25), isolated from essential oils of plants including *Quercus ilex* and *Picea abies* [321, 322]. To date, several medicinal plant extracts

rich in Sabinene have been used as anti-pyretic [323], anti-microbial [324], anti-hypertensive, anti-convulsant and anti-asthma [323] therapeutic agent. The anti-inflammatory effect of Sabinene was demonstrated in a study by Min-Jin et al. (2013) where it was shown that hydro-distilled essential oils (HEOs) of *Citrus unshiu Marcov*, rich in Sabinene (34.7%), suppressed lipopolysaccharide (LPS)-mediated cyclooxygenase-2 (COX-2) protein synthesis via the inhibition of nitric oxide (NO) and prostaglandin E₂ (PGE₂) secretion in LPS-stimulated macrophage cells [325]. In addition, HEOs of *Citrus unshiu Marcov* downregulated the production of inflammatory cytokines, tumour necrosis factor (TNF)- α , Interleukin (IL)-6, and IL-1 β in these cells [325]. Similarly, Sabinene has been suggested to be a potent anti-diabetic compound in a study by Saini et al. (2013). Saini and colleagues showed that oral administration of ethanolic extract (250-500 mg/kg) of Sabinene-rich seed of *Helianthus annuus L.* reduced blood glucose level, restored lipid profile, showed improvement in body weight, liver glycogen content, glycosylated haemoglobin, plasma malondialdehyde, glutathione level and serum insulin levels in streptozotocin-nicotinamide induced diabetic rats [326]. Hence, it is likely that Sabinene present in aqueous extracts of *S. frutescens* as seen in this study, can as an anti-inflammatory and anti-diabetic therapeutic agent.

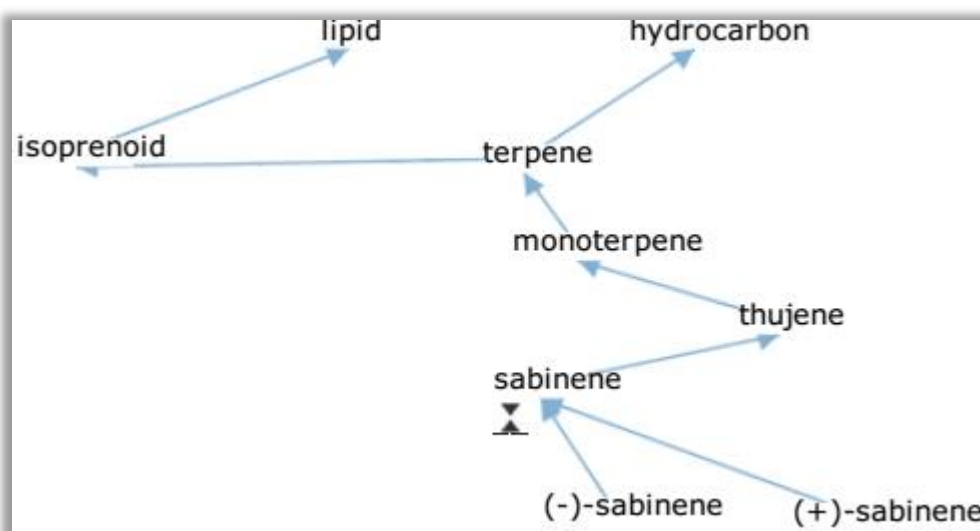


Figure 2.25: Chemical ontology of Sabinene [311]

Carvone is a monoterpene ketone (Figure 2.26) found as the active component of several essential oils [327]. Well-known sources of Carvone include spearmint oil and *Carum carvi* (caraway) oils [328]. Carvone has been proposed to have depressant and consequently anticonvulsant-like activity in the central nervous system (CNS) [328]. The anti-diabetic properties of Carvone was suggested in a study by Eddouks et al. (2004) where oral administration of aqueous of *Carum carvi* (20 mg/kg) decreased blood glucose levels in STZ-diabetic rats [329]. Similarly, Sadiq et al. (2010) showed that aqueous extracts of *Carum carvi* (60 mg/kg) decreased diabetic-mediated systemic and renal changes (increase in the serum levels of glucose, urea, creatinine, total urinary protein and microalbuminuric levels) in male Wistar rats [330]. Thus, confirming the reno-protective properties of Carvone in diabetic nephropathy state. It can then be suggested that Carvone found in ethanolic extracts of *S. frutescens* can reduce elevated glucose level and confer reno-protective function in diabetic patients.

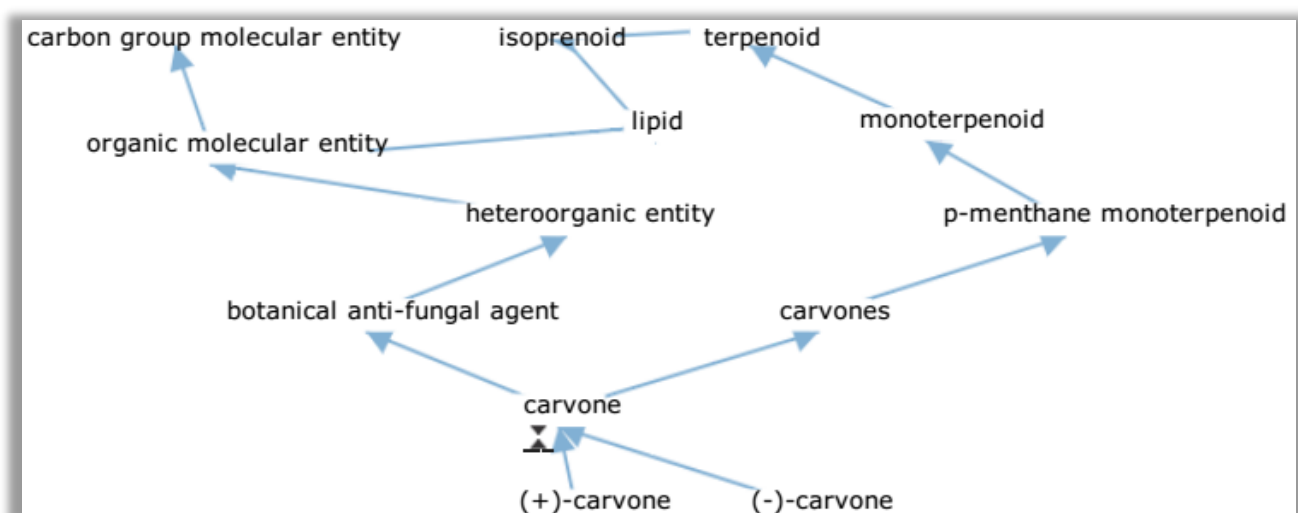


Figure 2.26: Chemical ontology of Carvone [311]

Myricetin is a member of the flavonoid class of polyphenolic compounds (Figure 2.27). Myricetin is present in several medicinal plants and edible fruits [331, 332]. The therapeutic potentials of Myricetin include, as an anti-inflammatory [333], anti-carcinogenic [334], anti-microbial [335] and anti-viral agent [336].

Phillips et al. (2011) demonstrated the anti-carcinogenic effect of Myricetin in a study, in which Myricetin was shown to mediate pancreatic cancer cell death *in vitro*. Furthermore, Phillips and colleagues demonstrated that *in vivo* treatment of orthotopic pancreatic tumors with Myricetin resulted in tumor regression and decreased metastatic spread in female nude mice [337]. In a similar study, Iyer et al. (2105), showed that Myricetin inhibited PAK1/Ras/Wnt/Erk/Akt/Bcl-2 signaling to mediate intrinsic caspase-mediated mitochondrial apoptosis in hepatocellular carcinoma cells [338]. In addition to its anti-carcinogenic effect, Myricetin has also been shown to be a potent anti-diabetic agent. In a study by Ong et al. (2000), 12 hourly intraperitoneal injection of Myricetin (3 mg) reduced hyperglycaemia by 50% and normalized associated hypertriglyceridemia in diabetic male Wistar Rats. These was

associated with increased hepatic glycogen and glucose-6-phosphate content and increased hepatic glycogen synthase I activity [339]. Ong and colleagues reported no indication of serious hepatotoxicity with Myricetin treatment in the experimental group of rat [339]. Similar findings was reported by IM et al. (2005) where bolus intravenous injection (1.0 mg/kg) of Myricetin decreased plasma glucose concentrations in a dose-dependent manner in STZ-diabetic rats [340]. In this present study, Myricetin was found in cold aqueous extracts of *S. frutescens* with poor confidence parameter, thus suggesting that it could be present in minimal quantity in this extract. However, it is probable that nominal Myricetin found in the cold aqueous extract of *S. frutescens* can mediate anti-cancer and anti-diabetic activity.

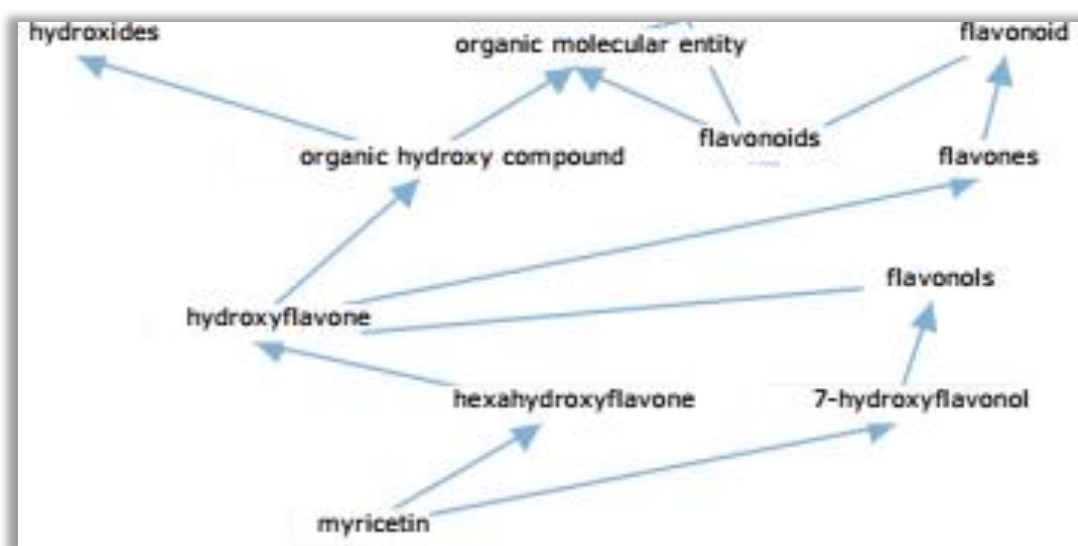


Figure 2.27: Chemical ontology of Myricetin [311]

Rutin is a flavonoid (Figure 2.28) found in many plants, fruits, and vegetables [341, 342], and has been suggested to possess a wide range of biological activities. Rutin exhibit broad biochemical and pharmacological activities including anti-carcinogenic, vasoprotective, anti-thrombic, and cardioprotective activities [343]. In addition, Rutin possess anti-oxidant, anti-inflammatory, anti-allergic, and anti-viral activities [344, 345]. In a study aimed at investigating the oral anti-diabetic activity and anti-hyperlipidemic activity of four flavonoids

including Rutin in STZ-nicotinamide induced diabetic rats, Jadhav et al. (2012) showed that Rutin significantly diminished total cholesterol, triglyceride compared with the control group [346]. This was associated with significant increase in glucose uptake and decrease glucose transport activity observed in the rat hemi-diaphragm [346]. In addition, Jadhav and colleagues reported that Rutin was the most active compound of all four flavonoids in both experiments [346]. This in agreement by prior study by Prince et al. (2006) where it was reported that Rutin improves glucose homeostasis in STZ-diabetic tissues by altering glycolytic and gluconeogenic enzymes [347]. Hence, it is likely that Rutin found in ethanolic (80% and 100%) extracts of *S. frutescens* as seen in this present study, can act in similar manner to mediate glucose homeostasis and therapeutic anti-diabetic function.

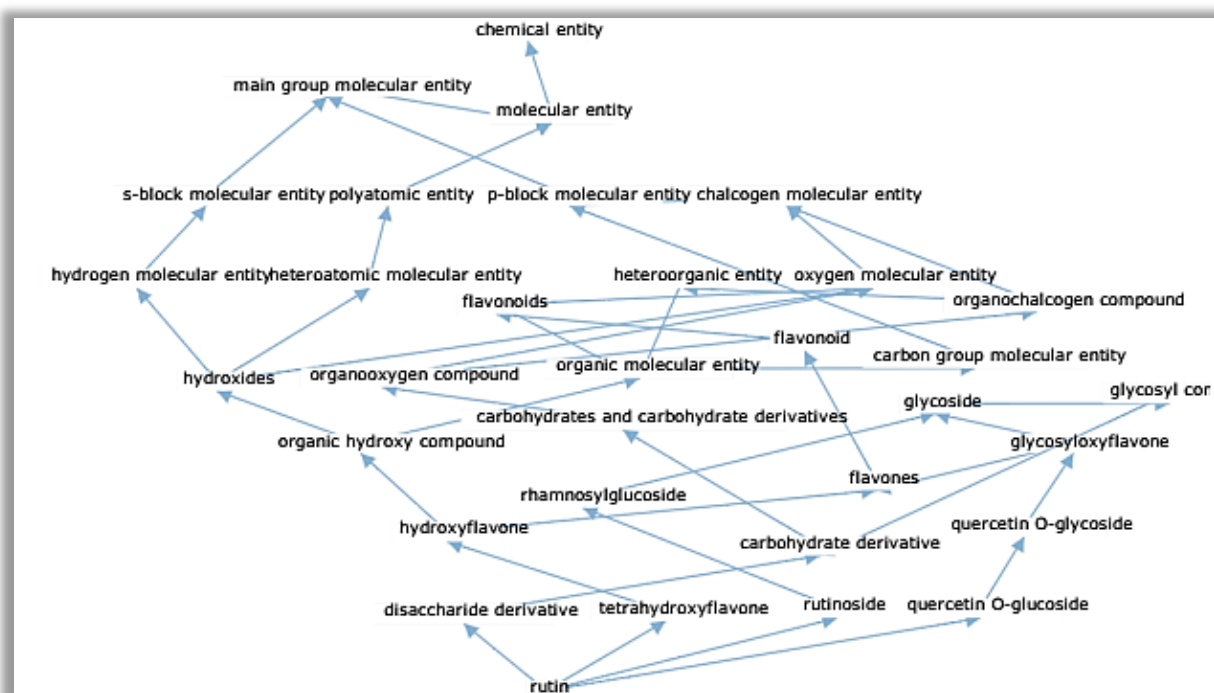


Figure 2.28: Chemical ontology of Rutin [311]

Stigmasterol belongs to the family of phytosterols (Figure 2.29) that include β sitosterol, campesterol, brassicasterol, delta-7 Stigmasterol, and delta-7-avenasterol, that are chemically similar to animal cholesterol [348]. Phytosterols are insoluble in water but soluble in most organic solvents. Thus agreeing with findings in this present study, as Stigmasterol was found only in organic (80 and 100% methanol) extracts of *S. frutescens*. Thiers (1953) proposed the first therapeutic role of Stigmasterol as an anti-stiffness factor in the therapy of rheumatic disease, as shown in an *in vivo* experiment using guinea pigs [349]. Hence, Stigmasterol is also known as Wulzen anti-stiffness factor [349]. Similarly, using new born mouse chondrocytes and human osteoarthritis (OA) chondrocytes in primary culture, Gabay and colleagues demonstrated that Stigmasterol (20 μ g/mL) downregulated the expression of pro-inflammatory gene IL-6 and genes involved in cartilage turn-over: MMP-3, MMP-13, ADAMTS-4,-5, type II collagen, and aggrecan in IL-1 β -stimulated cells [348]. Thus, confirming the anti-inflammatory and anti-osteoarthritic properties of Stigmasterol. Stigmasterol has been reported to reduce plasma cholesterol levels and inhibit hepatic synthesis and intestinal absorption of cholesterol in the rat [350]. Furthermore, oral administration of chloroform extracts of *Parkia speciosa* seeds, containing Stigmasterol (34%), significantly reduce blood glucose levels in alloxan-induced diabetic rats [143]. Data presented herein showed the presence of Stigmasterol in methanolic extracts of *S. frutescens* thus suggesting a therapeutic role for *S. frutescens* methanol extracts in the treatment of inflammatory conditions and diabetes.

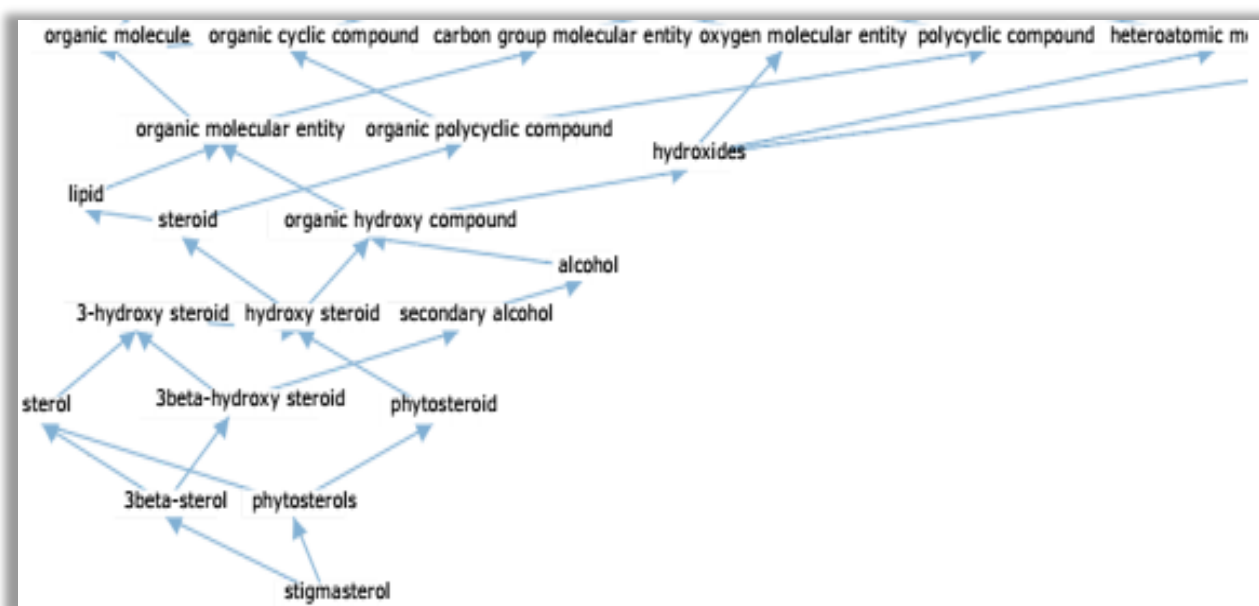


Figure 2.29: Chemical ontology of Stigmasterol [311]

Emodin is a natural occurring phytochemical that belongs to the class of hydroxyanthraquinones (Figure 2.30) [351]. Emodin has been reported to exhibit anti-bacterial [352], anti-inflammatory [353], immunosuppressive [354], anti-ulcerogenic [355], and anti-cancer [356, 357] activities. In an *in vivo* experiment aimed at investigating the anti-diabetic properties of Emodin, Xue et al. (2010) demonstrated that three weeks intraperitoneal injection of Emodin into STZ-induced diabetic mice resulted in significant decrease in the level of blood glucose, triglyceride and total serum cholesterol [358]. These were also associated with improved glucose tolerance and insulin sensitivity in Emodin-treated mice. Furthermore, Xue and colleagues suggested that Emodin mediates its anti-diabetic action via the activation of Peroxisome proliferator-activated receptor- γ (PPAR γ) and the modulation of systemic metabolism-related genes [358]. It is therefore very likely that the ethanolic (80%) extracts of *S. frutescens* can mediate anti-diabetic function consequent to the presence of Emodin.

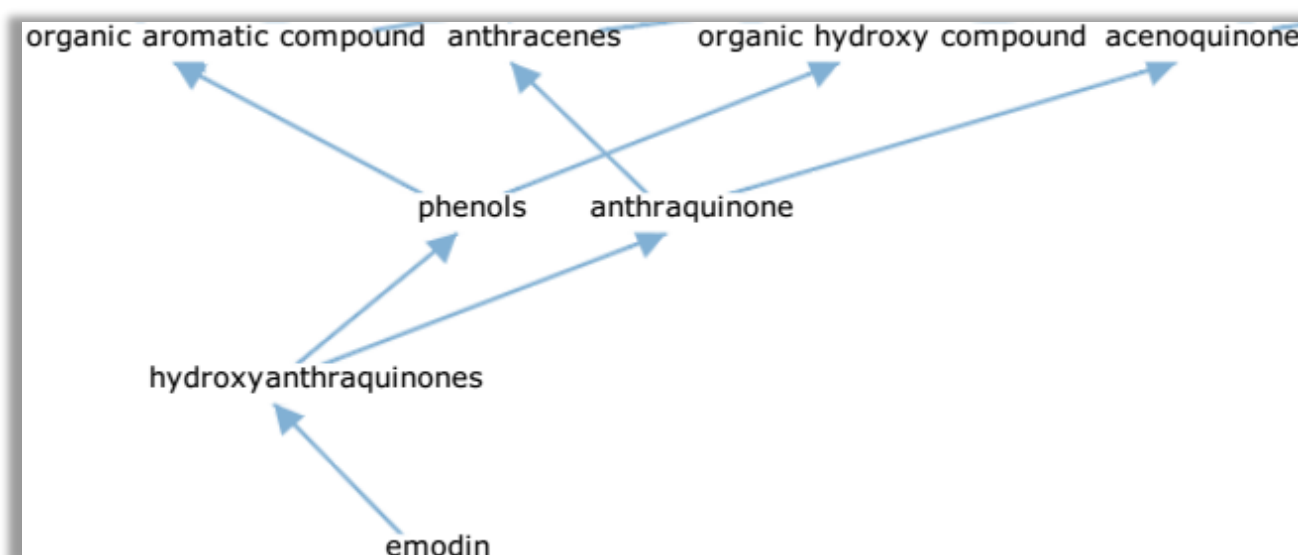


Figure 2.30: Chemical ontology of Emodin [311]

Sarpagine is an indole alkaloid (Figure 2.31), that have structural similarity to the essential amino acid tryptophan and related metabolites, such as the neurotransmitter serotonin [359]. Sarpagine has been isolated from various plant species most importantly genus *Rauwolfia*, *Corynanthe*, *Alstonia*, and *Strychnos* [359]. Extracts of *Rauwolfia* has been shown to possess potent anti-cancer [360] and anti-hypertensive [361] properties. Similarly, the anti-bacterial and anti-oxidant properties of *Rauwolfia* ethanolic extract has been reported [362]. In 2009, Qureshi and colleagues demonstrated that methanolic root extract of *Rauwolfia* can mediate hypoglycaemic, hypolipidemic and hepato-protective function in alloxan-induced diabetic rats [363]. In addition to this, Ganugapati et al. (2012) discovered that alkaloids present in *Rauwolfia serpentine* act as potential activators of insulin receptor [364]. The presence of Sarpagine alkaloid in 80% methanolic extract of *S. frutescens* as presented herein thus suggests a similar anti-diabetic role for *S. frutescens*.

CHAPTER THREE

***SUTHERLANDIA FRUTESCENS* PREVENTS DIABETIC METABOLIC CHANGES IN INSULIN RESISTANT HEPATIC CELLS.**

3.1 Introduction

Globally, the incidence and prevalence of chronic non-communicable diseases including chronic respiratory diseases, cardiovascular diseases, neoplasia and T2DM have reached epidemic proportions affecting people of all ages, nationalities and ethnicity [368-370].

Though the exact pathophysiology of T2DM is not entirely understood, initial peripheral insulin resistance in adipose tissue, liver, and skeletal muscle with subsequent pancreatic β -cell dysfunction resulting from an attempt to compensate for insulin resistance is a common feature of the disease [167, 168]. Thus, the classical clinical features of T2DM include peripheral hyperglycemia with underlying dyslipidaemia, including increased free fatty acid flux, elevated triglyceride levels and reciprocally low levels of high-density lipoprotein (HDL) [371, 372]. Altered liver metabolism disrupts normal maintenance of glucose homeostasis [373], through impaired suppression of hepatic glucose production [374]. In addition, accumulation of lipids within the liver can interfere with insulin signalling and cause insulin resistance [375]. Dyslipidaemia is a major risk factors for premature cardiovascular morbidity and mortality in T2DM patients [376, 377]. Hence, multiple anti-hyperglycaemic and anti-hyperlipidaemia drugs are often needed for effective management of T2DM [378, 379]. Over the years, conventional anti-T2DM drugs have been shown to have limited efficacies and serious adverse effects [371]. Hence, the need for newer, more efficacious and safer anti-T2DM agents [380, 381]. Medicinal plants offer great potential for the development of natural effective anti-T2DM agents, often with minimal or no side effects [29].

In my previous chapter, preliminary Triple TOF LC-MS/MS analysis revealed the presence of several potential anti-diabetic phytochemicals in crude aqueous and organic extracts of *S. frutescens*. Hence, the aim of this part of my present study was to investigate and compare the anti-T2DM properties of crude aqueous and organic extracts of *S. frutescens* using two cell lines (Chang and HepG2) as an *in-vitro* model system, in which glucose uptake, gluconeogenesis and lipid accumulation were analyzed.

Furthermore, using a multiplex gene array, prior research from our laboratory has shown that an aqueous extract of *S. frutescens* can regulate diabetes related genes in IR hepatic cells [382]. Hence, using quantitative real-time RT-PCR, the inter-extract variability in the regulation of expression in IR hepatic cells of three diabetes-associated genes previously shown to be regulated by *S. frutescens*: Vesicle-associated membrane protein 3 (VAMP3), a transporter gene; Mitogen-activated protein kinase 8 (MAPK8), a signal transducer; and Insulin receptor substrate 1 (IRS1) were investigated.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

All chemicals used for this study were of molecular biology grade. Cell culture media (EMEM) was purchased from Lonza, South Africa. Fetal bovine serum (FBS) and non-essential amino acids (NEAA) were purchased from HyClone, South Africa. DMSO, absolute ethanol and methanol were purchased from Merck Chemicals (PTY) Limited (Gauteng, South Africa). Tissue culture plates and flasks (TTP) were purchased from Separations, South Africa. Fatty acid-free BSA (FAF-BSA), MCDB-201 medium, Palmitate, D-fructose, MTT, Sodium lactate, Sodium pyruvate, Oil-Red-O, Nile Red, 2-deoxy glucose, Silica gel TLC plates, HEPES, L-carnithine transferase, L-carnithine, Aldrithiol and Acetyl CoA were purchased from Sigma

Aldrich, Cape Town, South Africa. Insulin was obtained from Roche, South Africa. Glucinet reagent, 2-DOG (0.2 μ Ci/ml 2-[3H]-DOG was purchased from New England Chemicals, UK. Optiphas Supermix scintillation cocktail was purchased from PerkinElmer, USA. AurumTM RNA extraction kit, iScript cDNA synthesis kit, SoFastTM Evergreen Supermix were purchased from Bio-Rad and PCR primers synthesised by Inqaba Biotech, Johannesburg, South Africa. All reagents were prepared according to manufacturers' instructions.

3.2.2 Collection of Plant Material

Collection of *S. frutescens* was as described in section 2.2.2

3.2.3 Preparation of plant extracts

3.2.3.1 Hot aqueous extract

A hot aqueous extract of *S. frutescens* was prepared as described previously in section 2.2.3.1

3.2.3.2 Cold aqueous extract

A cold aqueous extract of *S. frutescens* was prepared as described previously in section 2.2.3.2

3.2.3.3 Organic solvent extracts

Organic solvent extracts were prepared as described previously in section 2.2.3.3.

3.2.3.4 Preparation of extracts for cell culture

Each dry *S. frutescens* extract was dissolved in 1 mL 50% (v/v) dimethyl sulfoxide (DMSO) in deionized water, vortexed for 5 min and further diluted in MCDB-201 medium (Sigma). The extract suspension was filter sterilised using 0.2 µm acrodiscs (Pall), in a laminar flow hood using aseptic techniques. All extracts were used at a final concentration of 12.5 µg/mL. The concentration of *S. frutescens* used in this study was based on previous dose response studies on C2C12, 3T3-L1, and Chang cells [383-385]. The DMSO serves to sterilize the extract and once diluted (0.25%) has no effect on the biological assays or cell viability [386].

3. 2.4 Cell lines

3.2.4.1 Chang liver cell

The human hepatoma Chang cells were purchased from Highveld Biological, South Africa. These are adherent epithelial-like cells that grow as monolayers of small aggregates. Chang cells were originally derived from a normal human liver tissue, but were subsequently found to have been contaminated by HeLa cell based on isoenzyme analysis, HeLa marker chromosomes and DNA fingerprinting [387-390]. Although HeLa markers have been identified in this cell line, published work done in our laboratory [384] and studies from other laboratories [391, 392] have provided evidence of hepatocyte functions of these cells.

3.2.4.2 HepG2 cell

The HepG2 cell was purchased from Highveld Biological, South Africa. HepG2 cells are adherent, epithelial-like cells similar to liver parenchymal cells, which are mostly responsible

for the synthesis and secretion of plasma proteins characteristic of normal human liver cells [393].

These two human liver cell cultures were used to strengthen the significance of the results obtained in the experiments and determine whether the results were cell line-related or hepatocyte-related.

3.2.5 Cell culture.

Cells were routinely maintained in Eagle's minimum essential medium (EMEM) cell culture medium, containing 25 mM HEPES and 2 mM L-glutamine (Fisher, Philadelphia, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Cape Town, South Africa) and 1% non-essential amino acids (NEAA) (HyClone, Cape Town, South Africa) at 37 °C and 5% CO₂. The medium was stored at 4 °C, and was warmed to 37 °C before use. Monolayer cultures of hepatic cells were grown in 10 cm diameter tissue culture (TC) dishes (TPP, UK) and kept in a humidified incubator (37 °C, 95% relative humidity and 5% CO₂). Cellular growth was monitored every 48 hours using an inverted microscope and cells were passaged when 70-80% confluent. To passage, cell cultures were placed in a laminar flow cabinet and the culture medium aspirated using a sterilized Pasteur pipette attached to a pump. The cells were then washed twice in pre heated (37 °C) sterile phosphate buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KH₂PO₄, 2.9 g/L Na₂HPO₄, and 0.2 g/L KCl, pH 7.4) and detached from the TC dish with 1 mL sterile 1x trypsin (HyClone, South Africa) and incubated for 5 min at 37°C. The culture was viewed under an inverted microscope to confirm cell detachment. The cells were then transferred into a new culture dish with complete medium at a ratio of 1 in 3. Alternatively cells were seeded onto culture dishes/plates for experimentation, or frozen in liquid nitrogen for later use. Early passage cells were frozen by re-suspending the trypsinized cells (1×10⁶ cells/mL) in

FBS containing 10% DMSO and transferred into sterile cryogenic vials. The vials containing cell aliquots (1mL each) were placed upright in a -80 °C freezer for 24hrs and later transferred to liquid nitrogen for long term storage.

3.2.6 Preparation of Metformin

Metformin (Sigma Aldrich, South Africa), an established first line medication for the treatment of insulin-resistance (IR) and T2DM, was used as a positive control for the treatment of IR. Metformin was prepared as a 1 mM stock solution in 90% DMSO (Merck, South Africa) and filter sterilized with 0.2 µm acrodisc filters. Aliquots of 100 µL were stored at -20°C. Before use, the stock solution was diluted to 20 µM/mL with MCDB-201 medium and used at a final concentration of 1 µM/mL. Diluted stock solutions were stored at 4°C and discarded after one week.

3.2.7 Conjugation of Palmitate to Fatty Acid Free-BSA

Palmitate-BSA complex was prepared following the method described by Pappas and Ruddock [394, 395]. Palmitate is a saturated fatty acid with low solubility in aqueous solutions. This becomes a challenge in cell-based assays since it is not readily available to the cells. In order to create an aqueous-soluble reagent, palmitate was conjugated to a stabilizing agent for insoluble fatty acids (e.g. fatty acid free-BSA). Briefly, sodium palmitate (100 mM) solution was added to 0.1 M sodium hydroxide and heated to 65 °C with stirring until dissolved. A 0.1 mL aliquot of the resulting fatty acid solution was added, with stirring, into 1.3 mL 10% (w/v) fatty acid free-BSA (FAF-BSA) solution (Sigma Aldrich, South Africa) at 50 °C. After 15 min of slow stirring to allow clarification of the solution, 0.6 mL sterile deionized water (dH₂O)

was added to bring the final concentration to 5 mM fatty acid. The solution was filter sterilised using an acrodisc (0.2 µm pore size) and stored in aliquots at -20 °C for up to 6 months.

3.2.8 Cellular investigations

3.2.8.1 Generation of Insulin-Resistance in Hepatic cells

3.2.8.1.1 Cell treatment with Insulin and Fructose

Separate Chang and HepG2 cell cultures were each seeded at a density of 2.5×10^4 cells/mL and grown for 48 hours in EMEM containing 10% FBS and 1% non-essential amino acids in a humidified incubator at 37 °C and 5% CO₂. After 48 hours, the culture medium was aspirated and replaced with either serum-free MCDB-201 (Sigma Aldrich, South Africa) medium (control) or serum free MCDB-201 supplemented with 0.1 µM insulin (Roche, South Africa) and 1 mM fructose (Sigma Aldrich, South Africa) (MIF) to induce insulin-resistance, as described by Williams (2010) [384]. In addition, cells were exposed to MIF induction medium supplemented with either a 12 µg/mL (final concentration) aqueous or organic extract of *S. frutescens* (MIF+SF) or 1 µM metformin (MIF+MET) (positive control for treatment) for 24 hours [384].

3.2.8.1.2 Treatment with Palmitate-BSA

Separate Chang and HepG2 cell cultures were each grown in complete culture medium at a density of 2.5×10^4 cells/mL for 48 hours, after which the culture medium was aspirated and replaced with either serum-free MCDB-201 medium (control) or serum-free MCDB-201 supplemented with palmitate-BSA (0.25 mM) (PMB) to induce insulin-resistance. Parallel cultures were exposed to PMB-induction medium supplemented with either 12 µg/mL (final

concentration) aqueous or organic extract of *S. frutescens* (PMB+SF) or 1 μ M metformin (PMB+MET) (positive control for treatment) for 24 hours [395].

3.2.9 Cell Viability Testing

The viable cell number was determined in parallel experimental plates using the 4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide MTT assay (Sigma, South Africa) [396]. A stock solution of MTT (5 mg/mL) was made in PBS and filter sterilized with a 0.2 μ m filter. A working solution (10%) was made from the stock solution in EMEM containing 10% FBS. An aliquot of 200 μ L of the working solution was added to three parallel wells for each medium condition tested. The MTT assay was based on the metabolic reduction of the yellow tetrazolium salt (4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT), by dehydrogenase enzymes present in viable cells, to a water-insoluble, purple formazan product. The MTT was removed after a 4hr incubation at 37°C and the formazan product (purple crystals) extracted from the cells by the addition of DMSO. Absorbance was measured spectrophotometrically against a DMSO blank at 540 nm. The amount of purple formazan extracted from the cells is proportional to the number of viable cells in the culture [397].

3.2.10 Verification of insulin resistance

3.2.10.1 Glucose Production Assay

Chang and HepG2 cell cultures were each grown in complete medium. After 48 hours of growth, the medium was aspirated and replaced with the various treatments as described in section 3.2.8.1.2 and the cultures incubated for a further 24 hours. The glucose content of each culture medium after treatment with the insulin resistance inducing agents was analysed using

a glucose oxidase colorimetric method, as described by Gao et al. (2010) [398]. Briefly, the medium was aspirated and cells washed twice with PBS to remove any residual glucose contained in the culture media. The cells were then incubated for 16 hours in glucose production medium (glucose- and phenol red-free EMEM containing gluconeogenic substrates, 20 mM sodium lactate and 2 mM sodium pyruvate) (Sigma) with the addition of 0.1 μ M insulin during the last 3 hours. The glucose content of medium from each sample was analysed by glucose oxidase colorimetric determination using a Gluci-net reagent kit (Bayer, France). Glucose oxidase in the Gluci-net reagent, oxidises the D-glucose to gluconic acid producing hydrogen peroxide. This causes the oxidation of sodium hydroxyl-benzoate-aminophenazone to form stable quinine complexes varying in color according to the concentration of glucose in the medium from each sample. To each aliquot in the 96-well plates, 200 μ L of Gluci-net reagent was added and incubated for 15 minutes in a humidified 5% CO₂ incubator at 37°C. The optical density was read at 510 nm using a Bio-Tek KC4 Power Wave XS micro plate reader (Analytical and Diagnostic Products, South Africa) The viable cell population was measured with the MTT method described in section 3.2.9 and used to normalize assay results.

3.2.10.2 Deoxy-glucose uptake assay

Glucose uptake by Chang and HepG2 cell cultures was measured using tritium labelled 2-Deoxy-D-glucose ([2-³H]-DOG), adopting the method of Ralser et al. (2008) [399] as described by Williams et al. (2013) [384] with slight modification. 2-Deoxy-D-glucose (2-DOG) is a stable analogue of glucose that can be taken up and phosphorylated, but cannot be fully metabolized by the cell; consequently 2-DOG-6-phosphate accumulates in the cell [399]. Briefly, Chang and HepG2 liver cell cultures were seeded into 96-well cell culture plates at 5 x 10³ cells per well in 200 μ L of growth medium. After 48 hours, growth medium was removed

from the cells and replaced with plant extract or metformin treated media, or control media described in section 3.2.8.1.2. The cells were chronically exposed to these conditions for 24 hours. The following day, cells were washed twice with 200 μ L Krebs-Ringer phosphate (KRP) buffer (10mM phosphate (pH 7.2), 136mM NaCl, 4.7mM KCl, 1.25mM CaCl₂, 1.25mM MgSO₄). After washing with KRP-buffer, cells were stimulated with 1 μ M insulin in KRP-buffer without glucose (starvation buffer) for 15 min. After stimulation, starvation buffer was removed and 200 μ L 2-DOG (0.2 μ Ci/ml 2-[³H]-DOG (New England Chemicals) in 1 μ M unlabelled 2-deoxyglucose) was added and cells were incubated for 15 minutes. Cells were then washed thrice with ice cold PBS and solubilized in 100 μ L 0.1N NaOH. A PerkinElmer optiphase supermix scintillation cocktail (100 μ L) was added to each cell suspension, the solution was vortexed until it was clear, and thereafter each of the samples was counted for 15 minutes in a PerkinElmer MicroBeta2 2450 microplate counter (PerkinElmer, USA).

3.2.11 Lipid Accumulation Assays

Two lipid stains, Oil-Red-O and Nile Red, were used to determine lipid accumulation in the Chang and HepG2 cell cultures after exposure to the treatment conditions. The Oil-Red-O assay detects cellular neutral lipids, while Nile Red staining is used for the detection of neutral lipids (yielding a yellow-gold fluorescent colour) and phospholipids (yielding an orange-red fluorescence).

3.2.11.1 Oil-Red-O Assay

Chang and HepG2 liver cell cultures were seeded into 96-well cell culture plates at 5x10³ cells per well in 200 μ l of growth medium. After 48 hours, growth medium was removed from the cells and replaced with the same media described in section 3.2.5-2. The cells were chronically exposed to these conditions for 24 hours. The next day, the medium was removed and cells

fixed in 10% (v/v) formaldehyde in PBS (pH 7.4) for 10 min. Cells were rinsed with deionized H₂O followed by 70% (v/v) ethanol, and stained with 200 µL of 3% (v/v) Oil-red-O solution (Sigma, South Africa) (6 parts Oil-Red-O stock in isopropyl alcohol: 4 parts water) for 15 min. The stain was removed and cells washed with 70% (v/v) ethanol and finally deionized water. The stain was then extracted by addition of isopropyl alcohol and the absorbance measured at 520 nm [398]. After the Oil-Red-O assay was completed, cell viability was determined with the MTT assay, as described in section 3.2.9.

3.2.11.2 Nile Red Assay

Chang and HepG2 liver cell cultures were seeded into 96-well cell culture plates at 5×10^3 cells per well in 200 µL of growth medium. After 48 hours growth medium was removed from the cells and replaced with the same media described in section 3.2.5. The cells were chronically exposed to these conditions for 24 h, after which, the medium was removed and cells were washed with Hanks Balanced Salts Solution (HBSS; pH 7.4), and background fluorescence determined (535nm excitation, 580nm emission) in a Fluoroskan Ascent FL (Thermo Labsystems) plate reader. A 1mM stock solution of Nile red dye (Sigma, South Africa) was prepared in DMSO and stored in the dark at -20°C. The 1mM Nile red stock solution was freshly diluted in DMSO with 1% Pluronic F127 (Sigma, South Africa) in HBSS to form a 1 µM working solution, 100 µL of the working solution was then added to each culture well. After 4 hour incubation at room temperature in the dark, Nile Red was removed and cells were washed once with HBSS. After a further incubation for sixteen hours in HBSS at room temperature in the dark, fluorescence was again determined as above and background subtracted to obtain bound Nile Red fluorescence [384]. After the Nile red assay was completed, cell viability was determined with the MTT assay as described in section 3.2.9.

3.2.12 Thin Layer Chromatography Analysis for Lipids Classes

Thin Layer Chromatography (TLC) on silica gel was used to separate and determine lipid components in the Chang and HepG2 cell cultures after exposure to the treatment conditions. After 24 hour exposure to the conditions indicated in section 3.2.5, the culture medium was aspirated and cells gently scraped into 1 mL growth medium. Cells were then transferred to 2 mL microcentrifuge tubes and centrifuged for 5 min at 400 \times g. The supernatant was removed and the cell pellet re-suspended in 0.75 mL chloroform-methanol (1:2, v/v) to extract total lipids. Lipids and polar contaminant phases were separated by the addition of 0.25 mL deionized H₂O and 0.25 mL chloroform, followed by centrifugation at 1000 \times g for 5 min. The lower phase containing neutral lipids (e.g., triacylglycerides, free fatty acids, diacylglycerides, monoacylglycerides, waxes and sterol esters) and polar lipids (mainly phospholipid) was dried and re-dissolved in 50 μ L chloroform and spotted on silica gel TLC plates (Sigma, South Africa). The total lipid extract in the lower phase was separated using diethylether-heptane-acetic acid (75:25:1, v/v/v). The solvent used to resolve the TLC plates causes the separation of the neutral lipid class. Thus, waxes and sterol esters migrate most quickly, followed by triacylglycerides (TAGs), free fatty acids (FFAs), diacylglycerides (DAG), and monoacylglycerides MAG, while polar lipids remain at the sample origin. Once the solvent had run to approximately $\frac{3}{4}$ of the length of the TLC plate, the TLC plates were stained using 0.003% (w/v) Coomassie blue in 100 mM NaCl in 30% (v/v) methanol for 30 min. The plates were de-stained in the dye-free 100mM NaCl in 30% (v/v) methanol solution for 5 min [400]. Developed TLC plates were imaged using an AlphaImagerTM 3400 (Alpha Innotech). Densitometry on visualized lipid bands was done using ImageJ version IJ 1.46r (imagej.nih.gov/ij/) [401].

3.2.13 Quantification of changes in gene expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyse levels of mRNA expression of VAMP3, MAPK8 and IRS1 in Chang and HepG2 cell cultures after exposure to various induction media or treatments as indicated in section 3.2.8.1.1. qRT-PCR allows for accurate and reproducible measurement of mRNA present within the tissue sample [402]. In the qRT-PCR technique data are collected throughout the PCR process as it occurs, therefore combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity [403]. The qPCR technique combines PCR amplification of the gene sequence of interest, with fluorescent probe detection of the amplified product. Fluorescence intensity is recorded during every cycle. The cycle in which target amplification is first detected as significantly above a defined background threshold is referred to as the threshold quantification cycle (C_q). The greater the quantity of target cDNA in the starting material, the smaller the number of PCR amplification cycles required to increase the fluorescent signal above the background threshold, yielding a low C_q value [404].

In order to evaluate the level of gene expression, mRNA must first be extracted from the samples and reverse transcribed to cDNA, which is quantified in the qPCR experiment. Reverse transcription and PCR may be performed in a one-step reaction where both occur in the same tube or in a two-step process, where these occur in two separate tubes. This study employed the two-step procedure.

The PCR process involves 3 steps: (i) denaturation at 94-96°C: where double stranded DNA (dsDNA) is denatured into single stranded DNA (ssDNA); (ii) annealing: where the forward and reverse primers bind with their complimentary ssDNA sequences on either side of the sequence to be amplified at lower temperatures, generally of 50-65°C; (iii) elongation or

extension: where the Taq polymerase extends the DNA sequence from the primers in the 5' to 3' direction at 72°C. This process is repeated for 40 cycles. Amplification curves are generated from the PCR reactions to determine the C_q value, which is inversely proportional to the amount of specific nucleic acid sequence in the original sample [405]. Comparison of PCR template quantity can be performed in two ways: (1) Relative quantification: where changes in the steady-state mRNA levels of a gene of interest are measured following normalization with one or more reference genes, and (2) Absolute quantification: which requires a sample of known quantity (copy number) of the gene of interest diluted to generate a standard curve. The unknown samples are compared to this standard curve for absolute quantification [405].

In this study, the relative quantification procedure was used. This procedure relies on the use of control genes or sequences (referred to as reference or housekeeping genes) and a normalization procedure. During the analysis of gene expression, several variables need to be controlled, such as the amount of starting material, enzymatic activity, and differences between the overall transcription activities of the experimental cells [406]. One strategy to normalize for these variations is the use of reference or housekeeping genes which should not vary in any of the experimental and control cells.

3.2.13.1 Quantitative real-time PCR detection Chemistries

During qPCR, the accumulation of amplified DNA product is detected and measured as the reaction progresses. Two types of detection methods can be employed. The first is a non-specific detection method using an intercalating dye such as SYBR green that binds to any dsDNA. The second method consists of specific fluorogenic probes which are activated during the amplification process [40]. The signal from a single fluorophore is observed for each amplified molecule synthesized. It has high specificity, but probe production is expensive. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor

the fluorescence in each cycle as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle (Bio-Rad laboratories Inc, USA).

In this study, SYBR Green dye was used to detect PCR products. SYBR Green dye chemistry uses the SYBR Green dye to detect polymerase chain reaction (PCR) products by binding to double-stranded DNA formed during PCR. As the double-stranded PCR products accumulate during cycling, more dye can bind and emit fluorescence. Thus, the fluorescence intensity is directly proportional to synthesized dsDNA concentration [407]. Advantages of this method include: (a) it can be incorporated into optimized and previously established PCR protocols simply by adding the dye as a reagent to the PCR reaction cocktail of standard reactions and (b) it is significantly cheaper than using specific probes. Disadvantages of the non-specific SYBR Green dye technique include: indiscriminate binding to any dsDNA, which can result in fluorescence readings in the “no template controls” (NTC) due to dye molecules binding to primer dimers. This problem can be addressed by using software capable of melt curve analysis. This method makes use of plotting fluorescence as a function of temperature to generate a melting curve. This is done by slowly increasing the temperature above the melting temperature (T_m) of the amplicon and measuring fluorescence. A single characteristic melting peak at the T_m of the amplicon distinguishes it from the shorter primer-dimers that melt at lower temperatures with broader peaks [404]. A qPCR plot shows two phases: an exponential phase and a plateau phase. DNA replication in the exponential phase doubles with each cycle. As the reaction progresses, one or more reagents are used up and become limiting. At this point the reaction will slow and enter the plateau phase where no more amplification will take place. Therefore, for quantification purposes, the C_q value, representing the cycle in which the target amplification is first detected as significantly above a background threshold and the reaction is in the early exponential phase, is used.

3.2.13.2 RNA Extraction

After exposure to the various induction media or treatments as described in section 3.2.8.1.1 the cells were exposed to either an equal volume of 1× PBS (pH 7.4) or 0.1 μM insulin for 30 minutes. Thereafter, the medium was removed and cells were washed twice with PBS before RNA was extracted using Bio-Rad Aurum™ Total RNA extraction kit (Bio-Rad Laboratories Inc, USA) following the manufacturer's instructions. Throughout the RNA extraction, a ribonuclease (RNase) free environment was maintained by wiping all the surfaces with RNase-ZAP® (Sigma). Briefly, lysis buffer contained in the extraction kit, was added directly to the culture wells at 350 μL per 2×10^6 cells. A cell scraper was used to loosen the cells off the plate, and the scraped cells were then transferred to a sterile 2 mL Eppendorf capped microcentrifuge tube. The lysed cells were homogenized with a 2 mL syringe and 21 gauge needle for one minute. 350 μL volume of 70% ethanol was added to the sample and mixed thoroughly by pipetting up and down. 700 μL of the RNA lysate was transferred onto a RNA binding column and centrifuged for 30 seconds at 13400 xg in an Eppendorf minispin microfuge. The filtrate was discarded leaving the RNA bound to the column. 700 μL low-stringency wash solution was added to the RNA binding column, centrifuged for 30 seconds at 13400 xg and the filtrate discarded. Any contaminating genomic DNA was removed from the RNA by the addition of 70 μL chilled diluted DNase 1 (Bio-Rad Laboratories Inc, USA) to each column. The DNase I digest was incubated for 15 minutes at room temperature. Once digestion was complete, the columns were centrifuged for 30 seconds at 13400 xg and the digestion buffer discarded from the wash tube. 700 μL high stringency wash solution was added to the RNA binding column to wash off the DNase 1, centrifuged for 30 seconds at 13400 xg and then discarded. 700 μL low-stringency wash solution was added to the RNA binding column, centrifuged for 1 minute at 13400 xg and discarded. The sample was centrifuged for an additional 2 minutes at 13400 xg to remove any residual wash solution. The RNA binding

column was transferred to a new sterile 1.5 ml capped microcentrifuge tube. 30 μ L of elution solution was pipetted onto the RNA binding column. The sample was incubated for 1 minute to allow complete soaking and saturation of the membrane. The sample was then centrifuged for 2 minutes at 13400 xg to elute the total RNA, which was stored at -80 °C for later use.

The yield and quality of the extracted RNA was assessed with RNA 6000 Nano chips in the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). The Agilent 2100 Bioanalyzer uses a fluorescent assay involving electrophoretic separation to evaluate RNA samples qualitatively and quantitatively. The amount of fluorescence is measured as the RNA sample is pulsed through a microchannel and the Agilent Bioanalyzer software creates an electropherogram. The electropherogram is a diagram showing the fluorescence changes over time. For each sample the software creates a gel image to accompany the graph. It also displays the sample concentration and the ratios of the 18S and 28S ribosomal subunits. By determining the quantity of RNA in the sample, the volume needed to reverse transcribe the RNA to cDNA can be calculated.

In order to standardize the process of RNA integrity interpretation, Agilent Technologies has developed the RNA Integrity Number (RIN) to remove individual interpretation in RNA quality control. RIN is based on a software algorithm that works with the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip kit. The algorithm was developed from a large database of eukaryote total RNA samples (mainly human, mouse and rat) to enable the classification of eukaryotic total RNA, based on a numbering system from one to ten, with one being the most degraded profile and ten being the most intact. In the present study only samples with a RIN value > 8 were used.

A RNA ladder (Agilent technologies, Germany) was used for the quantitation and quality testing. The ladder was reconstituted according to the manufacturer's instructions. The Agilent RNA 6000 Nano Gel Matrix was prepared according to manufacturer's instructions. Prior to

analysis, the electrodes of the Agilent RNA 6000 BioAnalyzer were cleaned according to the manufacturer's instructions. The gel, dye concentrate and marker were allowed to equilibrate at room temperature for 30 minutes before use. The RNA 6000 Nano dye concentrate was protected against light, vortexed for 10 seconds and pulse spun down in an Eppendorf minispin microfuge to allow proper mixing. 1 μ L of dye concentrate was pipetted into a 65 μ L aliquot of the gel, vortexed thoroughly and visibly inspected to ensure proper mixing. Immediately before use, the tube was spun for 10 minutes at room temperature at 13400 xg in an Eppendorf minispin microfuge (Eppendorf, Germany). A new RNA Nano chip was placed on the chip priming station with 9. of the gel-dye mix. Two further 9.0 μ L aliquots of the gel-dye mix were pipetted into the chip. The fluorescent marker was vortexed and 5 μ L pipetted into each of the 12 sample wells and the RNA ladder well. 2 μ L of each RNA sample was pipetted into a microcentrifuge tube and heated at 70 °C for 2 minutes and placed on ice. 2 μ L RNA ladder was also heated at 70°C for 2 minutes and placed on ice. The mixtures were pulsed down for 15 seconds before use. 1 μ L of the ladder was pipetted into the ladder well and 1 μ L of each sample was pipetted into each of the 12 sample wells. The Nano chip was vortexed for 60 seconds at 2000 xg using the IKA vortex mixer (IKA Works, Inc, USA). The Nano chip was inserted into the Agilent BioAnalyzer and run according to the manufacturer's instructions. Extracted RNA was stored at -80 °C until reverse transcribed.

3.2.13.3 Reverse transcription (cDNA synthesis)

RNA samples were thawed on ice and reverse transcribed to cDNA using the reverse transcription BioRad iScript® cDNA synthesis kit (Bio-Rad Laboratories Inc, USA.) according to the manufacturer's instructions. The volume of RNA template added was dependent on the concentration of RNA obtained from the Agilent BioAnalyzer results. The volume of RNA used was calculated to provide 1 μ g of RNA. The total reaction volume was 20 μ L. Each reverse

transcriptase reaction was set up as indicated in Table 3.1. The reaction mixture was incubated for 5 minutes at 25 °C to allow RNA unfolding, 30 minutes at 42 °C for the reverse transcription to occur and finally incubated for 5 minutes at 85 °C to degrade the enzyme and stop the reaction. The samples were then frozen at -80 °C until the qPCR experiment.

Table 3.1: Reverse Transcription Reaction Mix. Relative amounts of each component from the iScript cDNA synthesis kit added per reaction.

Components	Volume per Reaction
5× iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Nuclease-free water	x µl
RNA template (1µg of total RNA)	x µl
Total Volume	20 µl

3.2.13.4 Quantitative real-time PCR reaction analysis

Quantitative real-time PCR was performed on the samples using a SoFast™ Evergreen Supermix ((Bio-Rad Laboratories Inc, USA) in a 20 µL reaction under sterile conditions according to the manufacturer’s instructions. Briefly, a master mix (Table 3.2) containing a final concentration of 1× SoFast™ Evergreen Supermix, 500 mM final concentration forward and reverse primers, and nuclease-free PCR-grade water (Ambion), was prepared. The mixture was vortexed, pulse spun and then placed on ice until needed. Prior to performing qPCR, the total number of reactions in each experiment was calculated (plus one extra reaction per 10

reactions to accommodate pipetting error and residual reagents left in the tube) in order to accurately prepare a qPCR master mix. Each cDNA sample was diluted 1 in 10 in nuclease-free PCR grade water (Ambion, USA) and placed on ice. The mixtures were vortexed and pulse spun prior to use. 10 μ L of the above diluted cDNA was mixed together with 30 μ L of the master mix. 10 μ L of nuclease-free PCR grade water (Ambion, USA) was used in place of the cDNA sample in no template controls (NTC). All tubes were vortexed, pulse spun and placed on ice. A 96 well PCR plate was placed on a chilled IsoFreeze plate holder to ensure all reactions remained cold while transferring the reactions to the plate and 20 μ L of the appropriate master mix-cDNA sample was added to each of the wells, with NTC included in 2 wells. Each sample and NTC was run in duplicate. The plate was sealed with optical tape, (BioRad Laboratories Inc, USA), centrifuged at 1500 xg for 2 minutes at 4 °C on an Eppendorf centrifuge 5804 R (Eppendorf, Germany), and run in a CFX96™ qRT-PCR detection system (BioRad, South Africa). A reference positive control was also added in duplicate. To normalize the gene of interest, a reaction mix containing ALUSx primers and corresponding cDNA samples was included in the experiment to serve as an internal reference control. ALUSx sequence was provided by Jan Hellemans. Ferdricks et al. 2013 [408] showed that ALUSx was one of the most stable reference genes analyzed for human colon tissue when the average expression stability value M was calculated using geNorm in qbase+ 2.5.1 software (Biogazelle, Belgium).

The protocol for all qPCR runs comprised of 3 minute Taq polymerase activation at 95 °C and 40 cycles of denaturing at 95 °C for 30 seconds, 30 seconds at appropriate annealing temperature (see Table 3.2) and extension at 72 °C for 30 seconds. The quantification cycle (Cq) defined as the number of qPCR cycles to reach a fluorescence threshold was determined for each sample. Cq was measured at the start of the exponential phase of PCR amplification where the reagents are not limited; hence qPCR can be reliably and accurately used to calculate

the initial amount of template present in the reaction (Bio-Rad Laboratories Inc, USA). Once the 40 cycle amplification reaction was completed, a melt curve was generated to verify the specificity of the reaction. The plate was heated to 95 °C, and then cooled to 2°C below the annealing temperature for each primer pair to ensure that the entire DNA was double stranded. The temperature was increased in increments of 0.5 °C for 30 seconds up to 95 °C to melt the double stranded DNA. The C_q values for the reference genes were transformed into relative quantification data using the $2^{-\Delta\Delta C_t}$ method [409]. All the relevant genes used in the analyses in this study are listed in Table 3.2, with their respective forward and reverse sequences as well as melting or annealing temperature.

The T_a for each primer pair was determined by performing a temperature gradient experiment. This involves the preparation of a qPCR reaction as described above, but using a cocktail of the cDNA samples. This cocktail was set up by mixing equal volumes of each cDNA sample in a single tube from which 2 µL was used per qPCR reaction. For each primer pair, a theoretical T_a was reported by the supplier (Inqaba Biotech, South Africa) which was used as a guideline for the temperature gradient experiment. The CFX was programmed to run a temperature gradient ranging from ~2 °C below the theoretical T_a to ~65 °C, providing 8 temperatures for testing. Once the PCR reaction was completed, the highest T_a at which the amplification efficiency was still as high as the most-efficient lower T_a was chosen as the experimental T_a to be used. The melt curve was checked to ensure that no non-specific reaction occurred. The qPCR data was recorded on the CFX software, and the C_q values were exported for analysis. Relative changes in gene expression were compared.

Table 3.2: qPCR conditions used for each of the reference and target genes. The annealing temperature for each primer pair varied between genes (designated T_m) as listed in Table 3.3.

Step	Number of Cycles	Temperature (°C)	Time
Denaturation	1	95	3 minutes
Denaturation	40	95	30 seconds
Annealing		T _a	30 seconds
Extension		72	30 seconds
Denaturation	1	95	30 seconds
Melt Curve	1	Variable	30 seconds
Hold	1	18	∞

Table 3.3: Primers used for the reference genes or sequences and genes of interest. Primer sequences and respective annealing temperatures of each primer pair were determined using a temperature gradient of which the highest, most efficient temperature was chosen to be used.

Target	Primer Sequences		T _a (°)
	Sense	Anti-sense	
IRS1	5'-TCTGTAAGTCTGTCTCCTA-3'	5'-CCTAATGTGATGCTCTGT-3'	59
MAPK8	5'-ATGTCCTACCTTCTCTATCA-3'	5'-TTACTACTATATTACTGGGCTTTA-3'	56
VAMP3	5'-TCCAACCTTATGCCTTCCA -3'	5'-CGAGAGTCAGTAGTGCTTA -3'	59
ALU _{sx}	5'-TGGTGAAACCCCGTCTCTACTAA-3'	5'CCTCAGCCTCCCGAGTAGCT-3'	60

3.2.14 Calculation of relative mRNA expression using the comparative C_q method

The comparative C_q method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample [402]

In this study, the $\Delta\Delta Cq$ method, also known as the Livak method [409] was used to determine relative mRNA expression of genes assayed. This method normalizes the expression of the target genes relative to a single reference gene and expressed relative to the reference sample [409]. Calculations adapted from Livak et al. (2001) are summarized below;

$$\Delta Cq = \text{Average } Cq \text{ (target assay)} - \text{Average } Cq \text{ (Reference assay)}$$

$$\Delta\Delta Cq = \Delta Cq \text{ (Test sample)} - \Delta Cq \text{ (Reference sample)}$$

$$\text{Relative quantification (RQ)} = 2^{-\Delta\Delta Cq}$$

3.2.15 Data Analysis

All cell assay data in this study were normalised to cell number as determined by the MTT assay and expressed as a percentage of the control (MCDB-201). All experiments were performed in triplicate and each experiment contained three replicate cultures for each treatment. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA) to determine the significant difference between treatments and control IR cultures, followed by Newman-Keuls post-tests with $p < 0.05$ considered significant.

3.3 Results

3.3.1 Cell Viability

Data obtained revealed that *S. frutescens* extracts were not toxic to the cells at the concentration used in this study.

3.3.2 Crude aqueous and organic extracts of *S. frutescens* decrease gluconeogenesis production in IR Chang and HepG2 liver cells

In the physiologic state, insulin inhibits gluconeogenesis and glycogenolysis, thus blocking the production and release of glucose by the liver [185]. This occurs via the direct effect of insulin on the liver cells [410]. However, in IR hepatic cells, there is an increase in gluconeogenesis and consequently an increase in glucose production, in an attempt to compensate for IR. In this present study, the glucose content of the culture medium of IR Chang and HepG2 cell cultures was measured using the oxidase assay. Result as presented in Figures 3.1a and b show that there was significantly more glucose in the medium after culture in MIF than in the control MCDB culture medium (Figures 3.1a and b; ***, $P < 0.001$). Thus indicating that these cells were IR. Further analysis using One-way ANOVA followed by Newman-Keuls post-test indicated that simultaneous treatment of IR Chang and HepG2 cells with crude aqueous or organic extracts significantly decreased glucose production by these cells (Figures 3.1a and b; $P < 0.001$ in all cases) and maintained glucose at the same level as the control (MCBD) (no significant difference was observed between control and *S. frutescens* extract treated MIF cultures) (Figures 3.1a and b). Data obtained from the IR Chang cell culture showed that the hot aqueous extract gave a better reduction in glucose production when compared to the other extracts, although this was not found to be statistically significant (108.53% for hot vs 138.78%, 154.53%, 134.07%, 159.75%, and 136.83% for cold aqueous,

80% ethanol, 100% ethanol, 80% methanol and 100% methanolic extracts, respectively) (Figure 3.1a). Similarly, treatment with metformin also maintained the glucose content of the culture medium at the level of the control culture (Figures 3.1a and b).

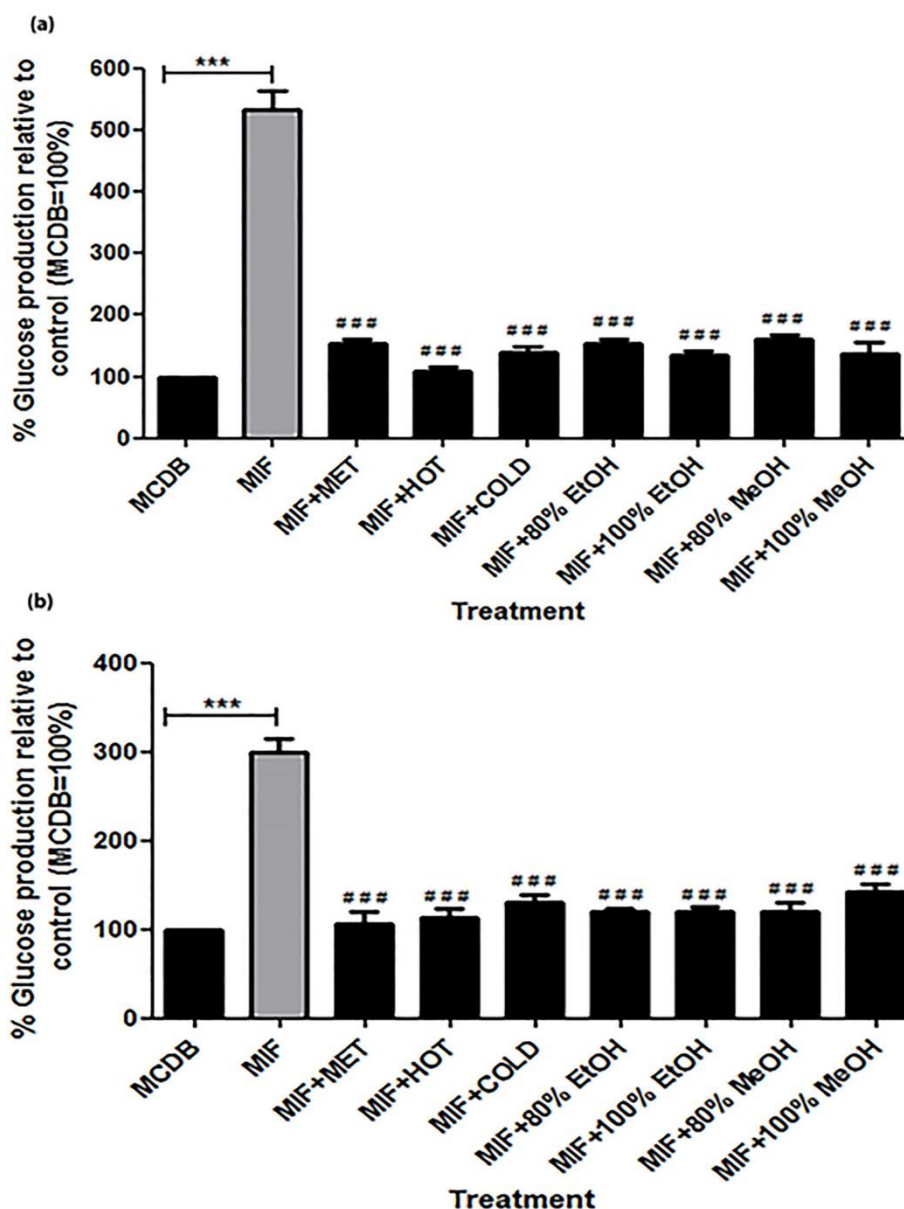


Figure 3.1: Hepatic glucose production in IR (a) Chang and (b) HepG2 liver cells as measured by the glucose oxidase assay, is expressed as a % of the control MCDB culture. Chang and HepG2 cells were cultured in MCDB-201 medium containing insulin and fructose (MIF) for 24 hrs, with *S. frutescens* extracts or metformin as positive control. The effect of *S. frutescens* extracts or metformin on net glucose concentration in the

culture medium is shown. Data are represented as mean \pm SEM from 3 independent experiments of 3 wells per experiment for all analysed conditions. One-way ANOVA was conducted on the means of the replicates between MIF and MCDB (control) and, MIF and cells treated with *Sutherlandia frutescens* extracts or metformin.* or #, ** or ## and *** or ### indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.3.3 Crude *S. frutescens* extracts increased glucose uptake in IR Chang and HepG2 liver cells

The 2-[3H]-DOG taken up by the experimental or control Chang and HepG2 liver cells, was calculated relative to the untreated control culture of Chang and HepG2 liver cells in MCDB-201 medium (MCDB). Compared to the control culture (MCDB), Chang and HepG2 liver cells made IR, by treatment with 0.1 mM insulin and 1 mM fructose in MCDB-201 medium (MIF) for 24 h, took up significantly less 2-[3H]-DOG (Figures 3.2a and b; $P < 0.001$), thus indicating the IR state in these cells. Treatment of IR Chang and HepG2 liver cells with metformin significantly reversed the effect of IR-mediated changes on glucose uptake in these cells, returning 2-[3H]-DOG uptake to that of the control culture (Figures 3.2a and b; $P < 0.001$ and $P < 0.05$, respectively). In the IR Chang cell culture, simultaneous treatment of cells with aqueous or organic extracts of *S. frutescens* significantly increased 2-[3H]-DOG uptake, returning it to the control level, with the hot aqueous extract displaying the highest activity (100% for hot aqueous vs 80%, 84%, 77%, 78.33% and 84.33% for 80% ethanol, 100% ethanol, 80% methanol and 100% methanolic extracts, respectively; $P < 0.001$ for hot aqueous, 80% and 100% ethanolic extracts and $P < 0.01$ for cold aqueous 80% and 100% methanolic extracts, respectively) (Figure 3.2a). However, in the IR HepG2 culture, hot aqueous, cold aqueous and 80% ethanolic extracts were the only extracts found to significantly return 2-[3H]-DOG uptake to that of the control culture (Figure 3.2b; $P < 0.001$ for hot aqueous and $P < 0.05$ for cold aqueous and 80% ethanolic extracts, respectively).

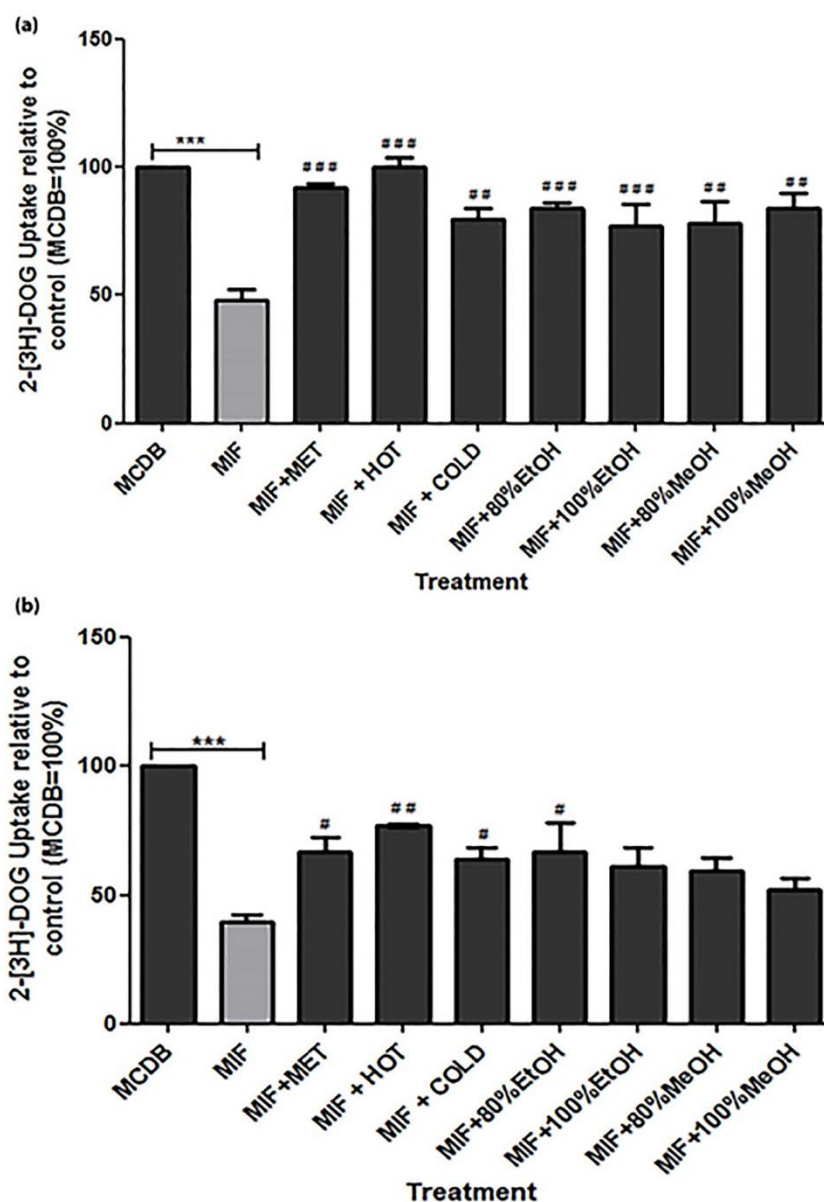
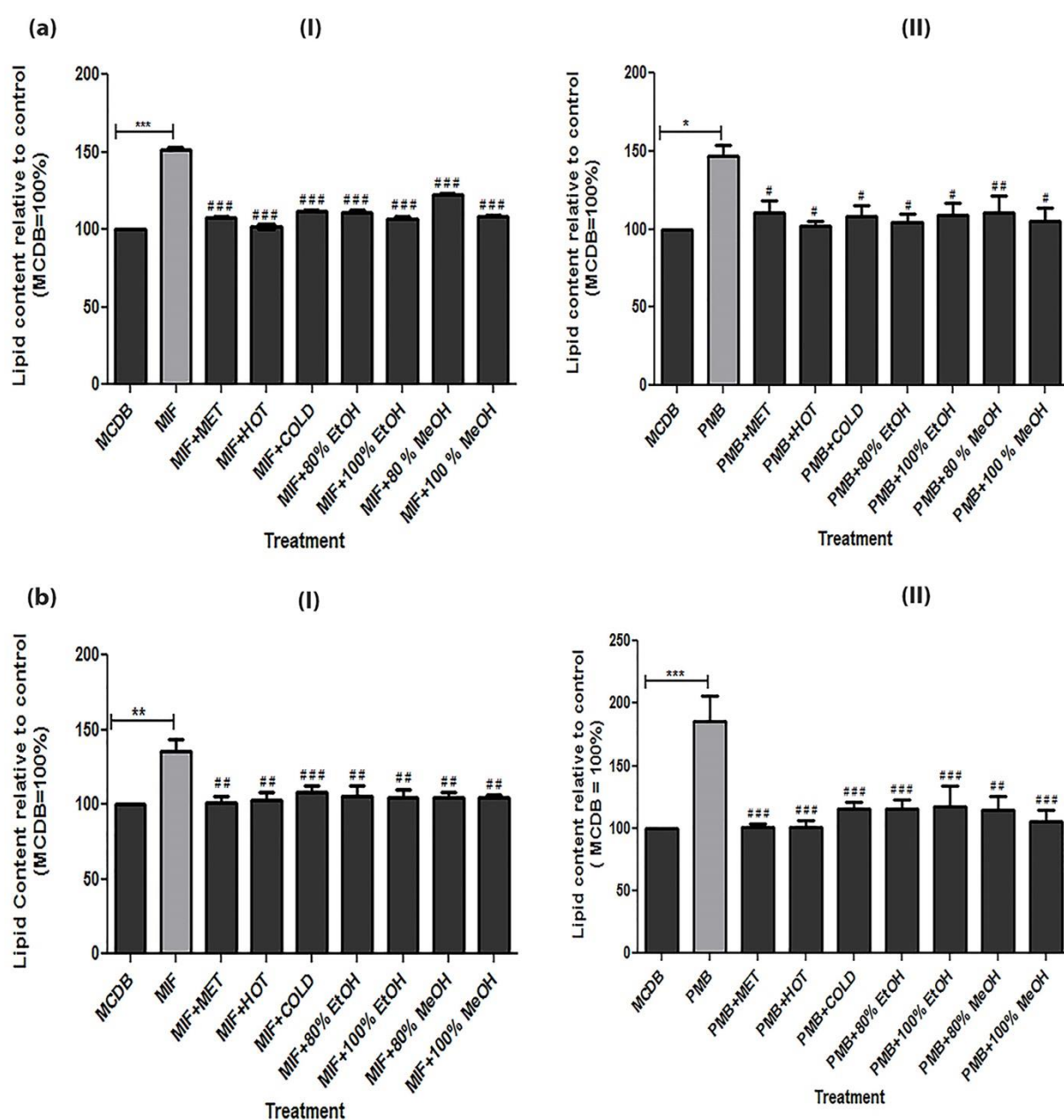


Figure 3.2: Glucose uptake (2-[3H]-deoxyglucose) in (a) IR Chang, (b) HepG2 liver cells. Chang and HepG2 cells were cultured in MCDB-201 medium containing insulin and fructose (MIF) for 24 hrs, with *S. frutescens* extracts, or metformin as positive control. 2-[3H]-DOG uptake measured relative to the control MCDB culture. Data are represented as mean \pm SEM from 3 independent experiments of 3 wells per experiment for all analysed conditions. One way analysis of variance (ANOVA) was conducted on the means of the replicates between MIF and MCDB (control) (*) and, MIF and cells treated with *Sutherlandia frutescens* or metformin (#). * or #, ** or ## and *** or ### indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.3.4 Aqueous and organic extracts of *S. frutescens* regulates lipid accumulation in IR Chang and HepG2 liver cells

In addition to changes in glucose metabolism, altered lipid metabolism has been observed in IR state [382]. In this present study, lipid accumulation following insulin and fructose (MIF) or palmitate (PMB) treatment of Chang and HepG2 liver cells was determined using the Oil-red-O stain to detect neutral triacylglycerols and cholesteryl esters [411, 412], and the fluorescent Nile Red stain to detect cytoplasmic phospholipids and neutral lipid droplets [413]. One-way ANOVA indicated that there was significant variation between the groups. Newman-Keuls post-test showed that Chang and HepG2 liver cells treated with MIF or PMB accumulated significantly higher levels of neutral triacylglycerols and cholesteryl esters than the control cell culture (MCDB) as determined by the Oil-red-O stain (Figures 3.3a I-II and b I-II; $P < 0.001$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively). Similarly, data obtained from the fluorescent Nile Red stain showed that both cells accumulated higher levels of cytoplasmic phospholipids and neutral lipid droplets than the control cell culture, though this was not significant in the HepG2 cell cultures (Figures 3.3c I-II; $P < 0.001$). The presence of *S. frutescens* extracts or metformin significantly reduced the amount of lipid accumulation in cells cultured in MIF or PMB, compared to the MIF or PMB treatment alone, returning lipid accumulation to a similar level to that observed in cells cultured in MCDB alone (Figures 3.3a I-II and b I-II; $P < 0.05$ in all cases). In the Chang cells cultured in MIF or PMB and HepG2 cells cultured in PMB, the hot aqueous extract of *S. frutescens* displayed a greater activity when compared to the other extracts (101.66% for hot aqueous extract vs 111.33%, 111%, 107%, 122.33% and 108.33% for cold aqueous, 80% ethanol, 100% ethanol, 80% methanol and 100% methanolic extracts, respectively) (Figure 3.3a I), (102 % for hot aqueous extract vs 108.5%, 104.5%, 109%, 110.5%, 105.5% for cold aqueous, 80% ethanol, 100% ethanol, 80% methanol

and 100% methanolic extracts, respectively) (Figure 3.3a II) and (100.33 for hot aqueous extract vs 115.33%, 115.66%, 117.66%, 114.33%, 105.33% for cold aqueous, 80% ethanol, 100% ethanol, 80% methanol and 100% methanolic extracts, respectively) (Figure 3.3b II). Furthermore, aqueous and organic extracts of *S. frutescens* were also found to significantly decrease the levels of cytoplasmic phospholipids and neutral lipids droplets in MIF treated Chang liver cells, as determined by the Nile Red stain (Figures 3.3c I-II; $P < 0.001$ in all cases).



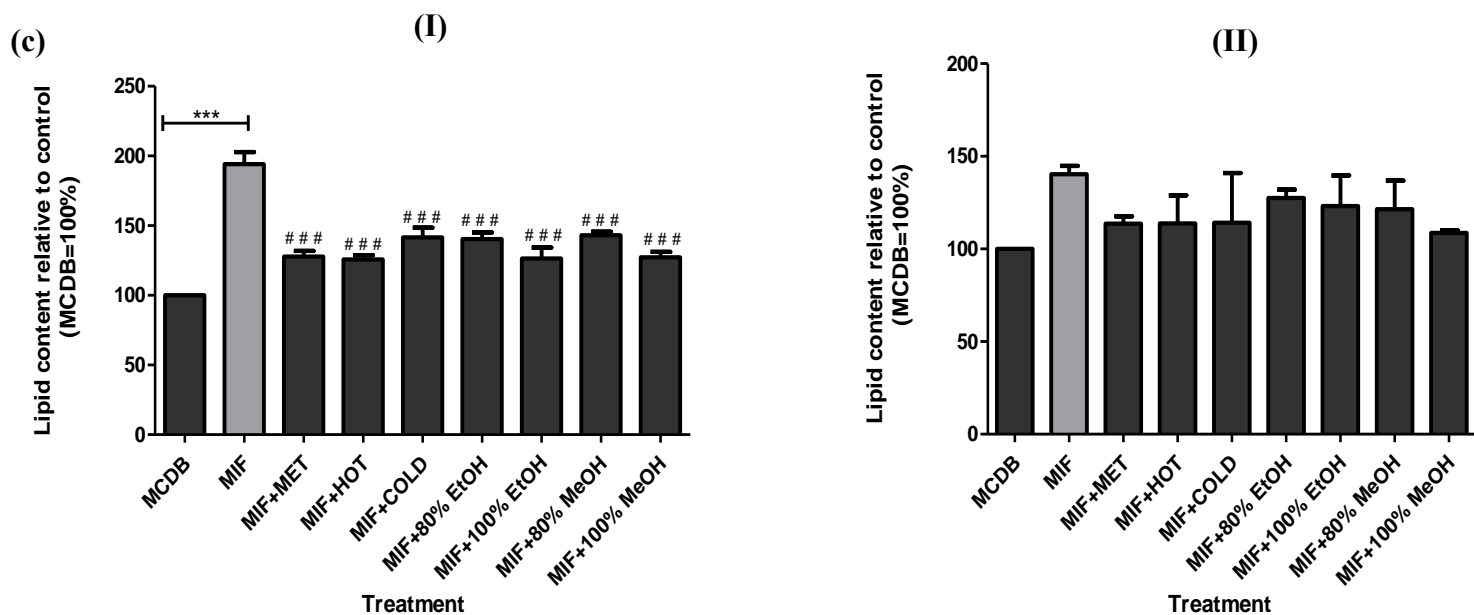
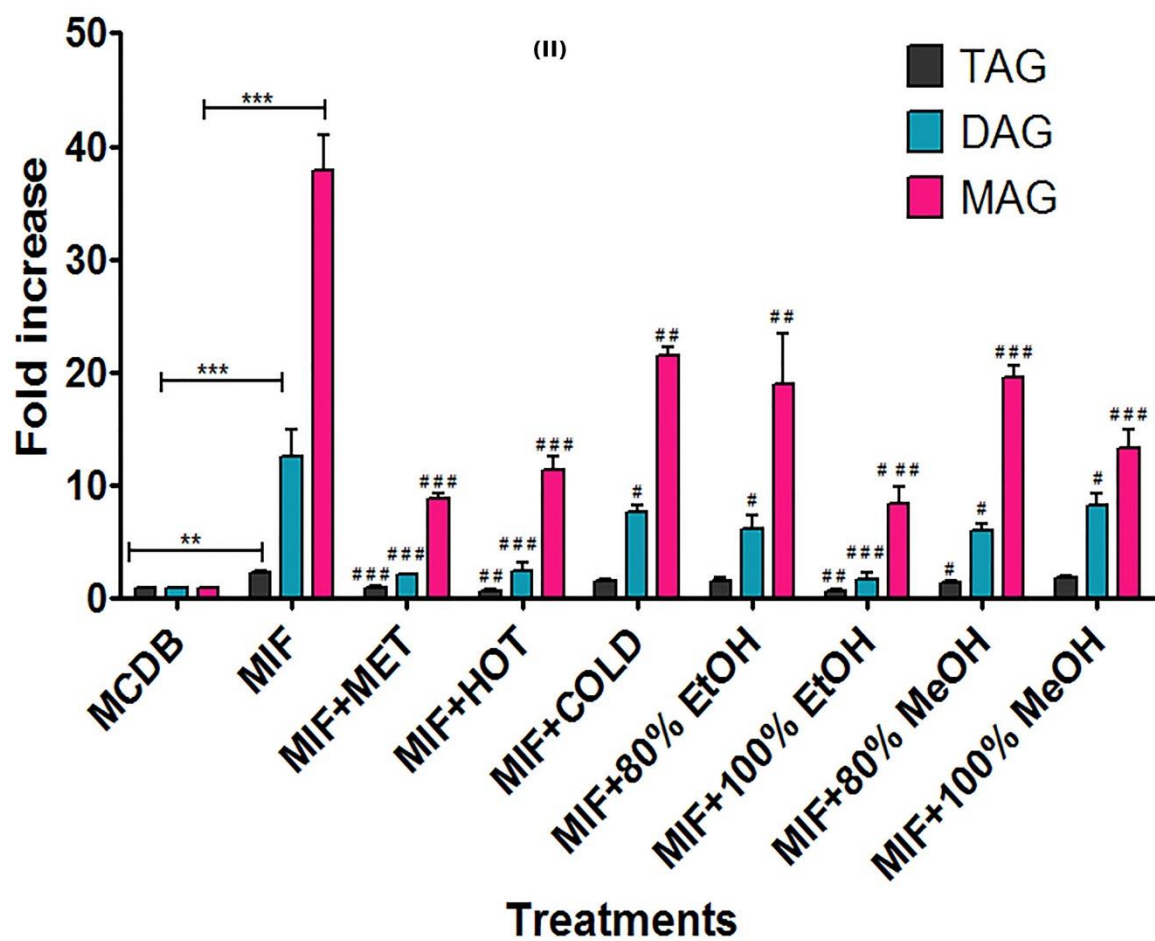
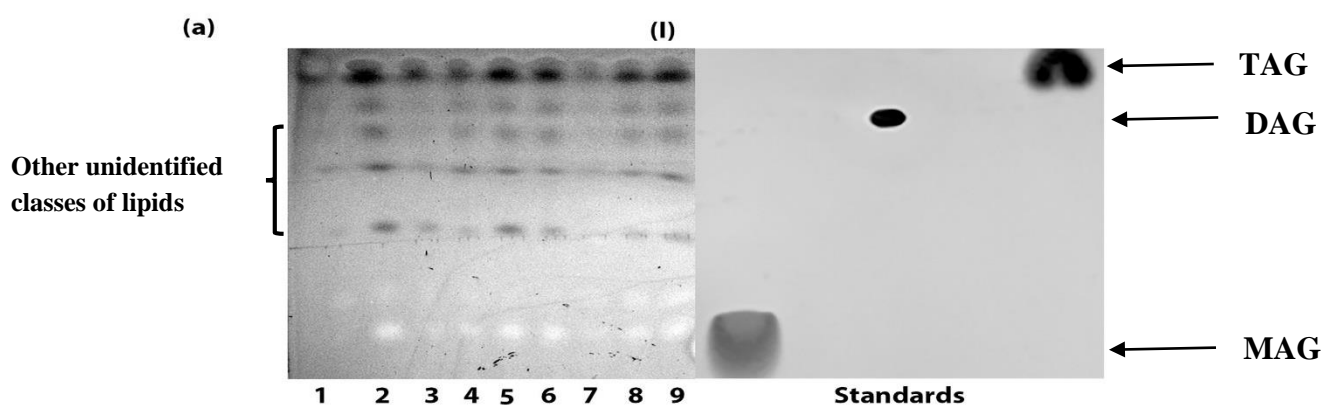


Figure 3.3: Lipid content in (a) Chang, (b) HepG2 liver cells determined using Oil-red-O staining and (c) Chang and HepG2 liver cells determined by Nile Red stain. Chang and HepG2 cells cultured in MCDB-201 medium containing MIF (aI, bI and cI-II) or palmitate (PMB) (aII and bII) are compared to the control MCDB culture, *S. frutescens* or metformin. Data are represented as mean \pm SEM from 3 independent experiments of 3 wells per experiment for all analysed conditions. One way analysis of variance (ANOVA) was conducted on the means of the replicates between MIF and MCDB (control) (*) and, MIF and cells treated with *S. frutescens* or metformin (#). * or #, ** or ## and *** or ### indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.3.5 Thin Layer Chromatography Analysis for Lipids Classes

Thin Layer Chromatography was used to separate and determine lipid components in the Chang and HepG2 cell cultures after exposure to MIF treatment in the presence or absence of metformin or extracts of *S. frutescens*. In the Chang cell culture, treatment with MIF increased the levels of triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) by 2.25, 14.47 and 37.81 fold above the control (MCDB), respectively ($P < 0.001$) (Figures 3.4a

I and II). Subsequent treatment with metformin or extracts of *S. frutescens* significantly decreased the accumulation of these classes of lipid in comparison to the MIF-treated Chang cells (Figures 3.4a I and II) ($P < 0.05$), although MAG and DAG levels remained significantly higher than those of the control (MCDB) ($p < 0.05$) (Figure 3.4a II). Similarly, treatment of HepG2 cells with MIF increased the levels of TAG, DAG and MAG by 1.47, 1.74 and 2.48 fold above the control (Figure 3.4b I and II; $P < 0.01$). The presence of *S. frutescens* extracts or metformin was found to significantly reduce the levels of these classes of lipids compared to the MIF-treated HepG2 cells alone (Figure 3.4b I and II; $P < 0.01$ in all cases for DAG and MAG).



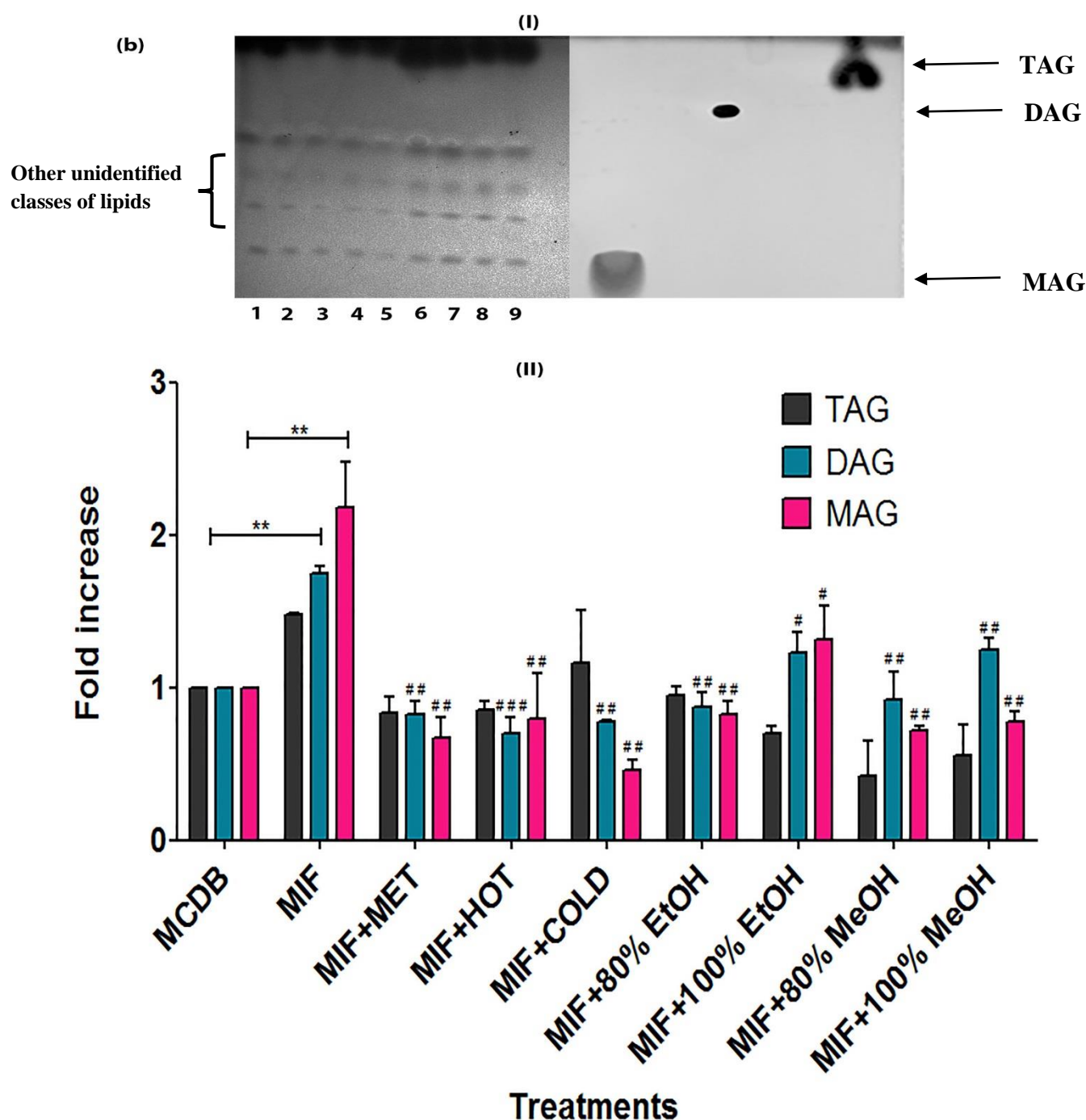


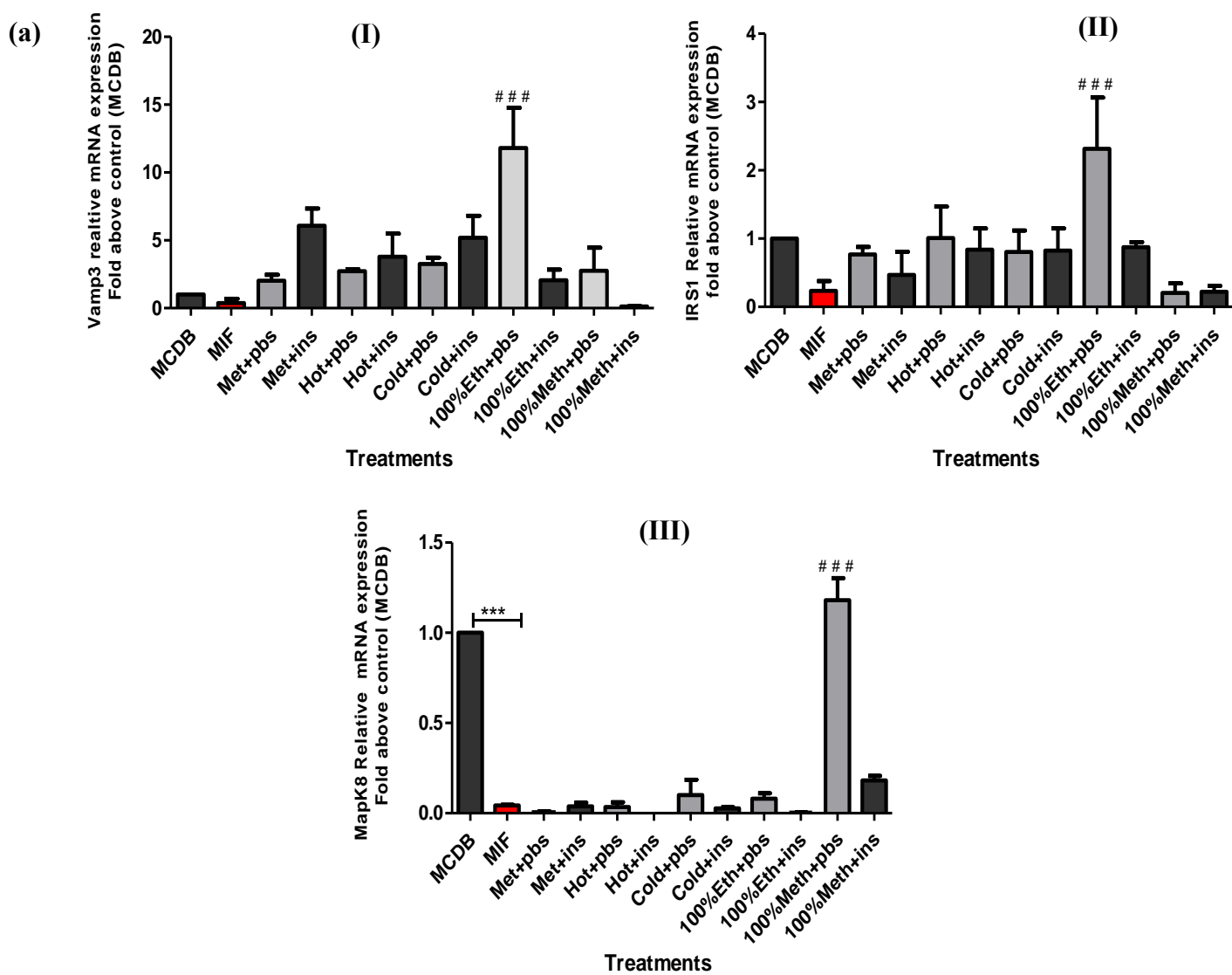
Figure 3.4: Thin layer chromatography of lipid fractions in (a) Chang and (b) HepG2 liver cells. The cellular lipid profile of Chang and HepG2 cells grown for 24 hours in MCDB or MIF or MIF together with metformin or extracts of *S. frutescens* extract was analysed by (I) representative thin layer chromatograph and (II) subsequent densitometry. In the chromatograph, lanes represent the samples as 1) MCDB, 2) MIF, 3) MIF+Metformin, 4) MIF+hot aqueous extract, 5) MIF+cold aqueous extract, 6) MIF+80% Ethanolic extract, 7) MIF+100% Ethanolic extract, 8) MIF+80% Methanolic extract, and 9) MIF+100% Methanolic extract of *S. frutescens*. Data are represented as fold increase compared to the control (MCDB)

± S.D. Statistical analysis was performed using two-way ANOVA (n = 2). * or #, ** or ## and *** or ### indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. MAG = monoacylglycerol, DAG = diacylglycerol, TAG = triacylglycerol.

3.3.6 *S. frutescens* regulates the expression of VAMP3, MAPK8, and IRS1 in IR hepatic cells.

Data obtained from the quantitative real-time RT-PCR analysis showed that IR state induced by treatment with insulin and fructose (MIF) downregulated the expression of VAMP3, IRS1 and MAPK8 in the HepG2 cultures when compared to the control MCDB culture (Figures 3.5a I-III; $P < 0.001$ for MAPK8 expression). However, this downward regulation was reversed in the presence of metformin and *S. frutescens* extracts. For VAMP3 expression, the 100% ethanolic extract of *S. frutescens* had the highest activity, significantly increasing the expression of VAMP3 by 11.81 fold above the control (Figures 3.5a I; $P < 0.001$). In addition, it was observed that the presence of insulin augmented the activity of the aqueous extracts and metformin while a reduction in the level of gene expression was observed in the organic extracts in the presence of insulin (3.79 fold increase compared to control vs 2.73 for hot+insulin vs hot+pbs, 5.182 fold increase vs 3.25 for cold+insulin vs cold+pbs, 2.06 fold increase vs 11.81 for 100% ethanol+pbs vs 100% ethanol+insulin, and 0.17 fold expression (decrease) vs 2.76 for 100% methanol+insulin vs 100% methanol+pbs). Similarly, for IRS1 expression, the 100% ethanolic extract significantly up-regulated the expression of IRS1 gene by a 2.31 fold increase above the control (Figure 3.5a II; $P < 0.001$). This was also associated with a reduction in gene expression in the presence of insulin (Figure 3.5a II). With the exception of 100% methanolic extracts, all other extracts were not able to reverse IR-mediated downregulation of MAPK8 in IR HepG2 hepatic cells (Figure 3.5a III; $P < 0.001$).

Interestingly, in the Chang cell cultures, the IR state had no downregulatory effect on the expression of all three genes when compared to the control MCDB (Figures 3.5b I-III). Nonetheless, in the presence of insulin, metformin and the cold aqueous extract of *S. frutescens* were observed to significantly up-regulate the expression of VAMP3 above the MIF insulin resistant culture or MCDB control (Figure 3.5b I; $P < 0.05$). While the hot aqueous extract in absence of insulin and the methanolic extracts with or without insulin augmentation, maintained VAMP3 gene expression at the level of the MCDB control in IR Chang hepatic cells (Figure 3.5b I). As seen in Figure 3.5b II, metformin with insulin and 100% methanolic extracts without insulin up-regulated IRS1 expression above the MCDB control, but this was significantly less than the MIF-mediated IRS1 expression in these cells. In addition, a combination of metformin and insulin was found to significantly up-regulate MAPK8 expression above the MIF or MCDB control cultures (Figure 3.5b III; $P < 0.01$). Although, the hot aqueous extract without insulin and cold aqueous extract with insulin up-regulated MAPK8 expression, this was not found to be significant (Figure 3.5b III).



(b)

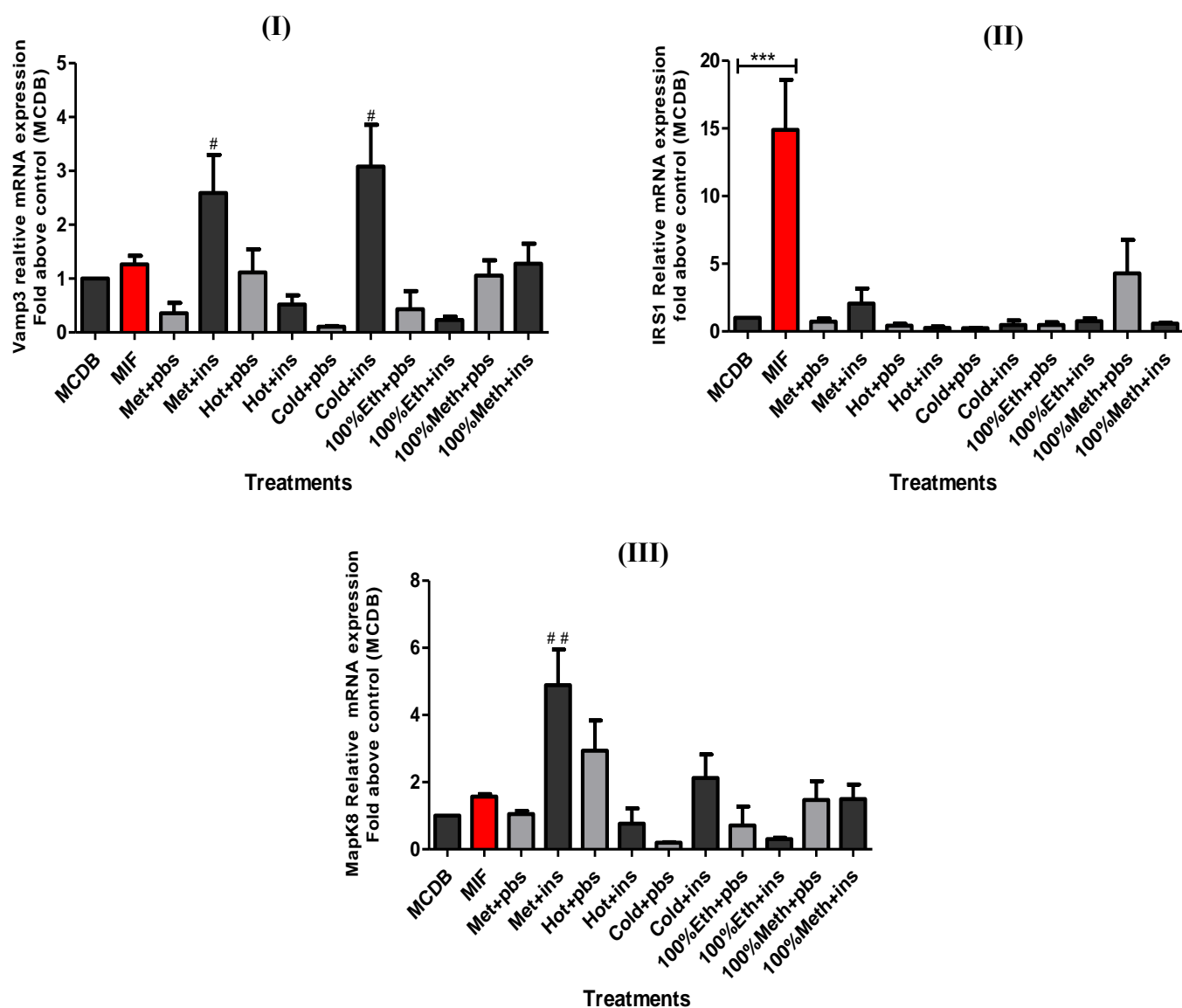


Figure 3.5: Relative mRNA expression of VAMP3, IRS1, and MAPK8 in (a) IR HepG2 cells and (b) IR Chang cells. Chang and HepG2 cells were cultured in MIF for 24 hr in the presence or absence of metformin or extracts of *S. frutescens* with or without insulin augmentation. The relative mRNA expression of VAMP3, IRS1, and MAPK8 was determined by quantitative real-time RT-PCR, with fold changes in expression calculated by the $\Delta\Delta C_q$ Livak method [409]. Data are represented as fold increase above control (MCDB) \pm S.D. Statistical analysis was performed using One-Way ANOVA (n = 3). * or #, ** or ## and *** or ### indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3.4 Discussion

It is estimated that by the year 2020, approximately 250 million people will be affected with T2DM [414]. Though the primary etiological factor for this disease remain unknown, it is accepted that IR plays a key role in the development of T2DM [415]. This is supported by findings from longitudinal and cross sectional studies that show the presence of IR 10–20 years before the onset of T2DM [416, 417] and prospective studies demonstrating that IR is the best predictor of whether or not an individual will become diabetic later in life [416, 417]. The current approach to treating T2DM is the use of oral anti-diabetic agents (OAAs), insulin, and incretin-based drugs in an attempt to achieve glycemic control and maintain glucose homeostasis [418]. But current anti-diabetic agents lack efficacy and also possess undesirable side effects [419].

Extracts of *S. frutescens* have been shown to contain a number of potential anti-diabetic phytochemicals [420, 421]. Prior studies by Chadwick et al. (2007) and Mackenzie et al. (2009 and 2012) have demonstrated the anti-diabetic potential of both a laboratory prepared aqueous extract and a commercial aqueous preparation of *S. frutescens* in the treatment and prevention of IR in rats fed with high fat diet [261, 422, 423].

Solvent extraction is the most widely used procedure to prepare extracts from plants. The type of solvent used may have an effect on the nature and the groups of compounds extracted, affecting the resulting bioactivity of the extract [111]. It is therefore very plausible that different solvent extracts of *S. frutescens* may possess varying biological activity consequent on the groups of compounds extracted. In this present study, six different solvent extracts; hot aqueous, cold aqueous, 80% ethanol, 100% ethanol, 80% methanol and 100% methanol extracts of *S. frutescens* were investigated for their anti-diabetic activity and the ability to prevent IR-mediated metabolic changes in IR Chang and HePG2 liver cell lines. Furthermore, the activity of individual extracts was compared with each other, MIF treated cells and the

MCDB control. *In-vitro* insulin resistance was established in Chang and HepG2 cells by treating cultures with 0.1 mM insulin and 1 mM fructose (MIF), or 0.25 mM palmitate (PMB) for 24 hours. Characteristic metabolic changes found in the IR state include reduced glucose uptake, increased hepatic glucose production and the accumulation of circulating lipids and ectopic fat deposits [382].

De novo synthesis of glucose in the liver is a central mechanism to provide the organism with glucose in times of starvation [424, 425]. However, when glucose is directly available from external sources, hepatic gluconeogenesis becomes dispensable and consequently needs to be shut off [426]. Insulin is the most important hormone that inhibits gluconeogenesis [426]. It acts predominantly by suppressing key gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) [425, 426]. However, in the diabetic state, the occurrence of IR, leads to the inability of insulin to regulate the activity of glucogenic enzymes, thereby causing an increase in hepatic glucose output and elevated blood glucose levels [426]. In patients with T2DM, the rate of hepatic gluconeogenesis has been found to be considerably elevated compared to healthy subject [425]. Similarly, an increased hepatic glucose production with elevated levels of G-6-Pase and PEPCK was reported in mice with an organ-specific insulin receptor knockout in the liver [410].

In this study, treatment of Chang and HepG2 cells with MIF resulted in a significant increase in glucose production and accumulation within the culture medium (Figures 3.1a and b) compared to the control. Simultaneous treatment of these cells with *S. frutescens* extracts or metformin, prevented IR-mediated gluconeogenesis by significantly decreasing the percentage glucose production in IR Chang and HepG2 cells (Figure 3.1a and b; $P < 0.001$) and maintaining it at the same level as the control (MCBD). Furthermore, in the Chang cell culture, the hot aqueous extract was found to be most active, decreasing the percentage glucose

production levels by 79.7% when compared to metformin (71.2%) and all the other solvent extracts, Figure 3.1a although this was not statistically significant. Several studies on anti-diabetic medicinal plants have suggested that extracts of these plants mimic insulin in downregulating hepatic gluconeogenesis. In a study by Pari et al. (2004), 4 weeks oral administration of 200 mg/kg aqueous extract of *Boerhaavia diffusa* L. leaf to diabetic albino rats was found to significantly increase the activity of hepatic hexokinase while downregulating the activity of hepatic G-6-Pase glucose and fructose-1,6-bisphosphatase in normal and diabetic rats [427]. G-6-Pase catalyzes the final step of gluconeogenesis, the production of free glucose from glucose 6-phosphate [426]. It is therefore very likely that extracts of *S. frutescens* act in a similar manner to prevent IR-mediated gluconeogenesis in Chang and HepG2 liver cells.

Futhermore, *S. frutescens*-mediated prevention of glucose production in IR-hepatic cells seen herein is in agreement with findings by Williams et al. (2013), where an aqueous extract of *S. frutescens* was found to prevent the development of IR-mediated glucose production in Chang liver cells [382]. This was associated with up-regulation of the phosphatidylinositol 3-kinase (PI3-kinase)-protein kinase B (PKB/Akt) and the mitogen-activated protein kinase (MAP kinase) signaling pathways [382], required for insulin-mediated regulation of glucogenic enzymes [428]. Likewise, in this present study, both aqueous and methanolic extracts of *S. frutescens* up-regulated MAPK8 expression in IR Chang hepatic cells, while the methanolic extract alone regulated MAPK8 expression in the HepG2 cell cultures. Futhermore, the inability of metformin alone (Met+pbs) to significantly up-regulate MAPK8 expression does partially support the theory that metformin inhibits hepatic gluconeogenesis via other pathways, in particular the AMPK-activated protein kinase [429]. However, a recent finding has suggested metformin can act via other non-AMPK dependent pathways [430].

Insulin receptor substrate (IRS) 1 and 2 are abundantly expressed in the liver and are thought to be responsible for transmitting insulin signaling from the insulin receptor to the intracellular effectors in the regulation of glucose and lipid homeostasis [431]. The binding of insulin to its receptor on the hepato-cellular membrane, promotes the autophosphorylation of regulatory loop tyrosine residues, removing the inhibition of the insulin receptor tyrosine kinase activity toward IRS [432]. Tyrosine phosphorylation of IRS1 by the insulin receptor generates binding sites for Src homology 2 (SH2) domain proteins, including the regulatory subunits of class 1A phosphatidylinositol 3-kinase (PI3K) [433]. Up-regulation of IRS1 expression by aqueous and organic extracts of *S. frutescens* in IR HepG2 seen in this present study suggests that use of *S. frutescens* herbal mixture can reverse the IR state, possibly as a result of increased IRS1 expression. This is in agreement with similar findings by Williams et al. (2013) where extracts of *S. frutescens* up-regulated IRS1 expression in IR Chang cells [382]. However, in contrast to their findings, the *S. frutescens* extracts used in this present study did not induce IRS1 expression in IR Chang cells. Thus suggesting that further work is needed to determine the roles *S. frutescens* in the regulation of IRS1 expression.

Activation of PI3-kinase leads to downstream phosphorylation and activation of protein kinase B (PKB/Akt) [434, 435]. PKB has shown to be partially responsible for the regulation of G-6-Pase gene transcription via the insulin-response unit (IRU) within the G-6-Pase promoter [436]. In addition, overt expression of PKB in hepatoma cells and primary hepatocyte cultures has been shown to downregulate PEPCK and G-6-Pase gene transcription [437]. Similarly, activation of mitogen-activated protein kinase by phorbol ester has been shown to mediate the suppression of PEPCK and G-6-Pase gene transcription [437, 438]. Hence, it is plausible that extracts of *S. frutescens* used in this present study act via the PI3-kinase-Akt and MAP kinase signaling pathways to inhibit PEPCK and G-6-Pase gene transcription and prevent gluconeogenesis. However, reduced extract-mediated expression of MAPK8 in the presence of

significant biological activity seen herein suggests that these extracts might be acting on multiple intracellular target pathways to significantly decrease glucose production in IR hepatic cells. Thus corroborating the hypothesis that a herbal mixture has a significant advantage over conventional pharmaceuticals synthesised from a single chemical [439].

In the post-prandial state, peripheral blood glucose is taken up by the hepatocyte via the hepatic glucose transporter type 2 (GLUT2), a membrane bound transporter with high capacity and low affinity for glucose [440]. The expression and activity of GLUT2 is independent of insulin signaling [440], but dependent on the concentration gradient of glucose across the hepatocyte plasma membrane [441]. GLUT2 maintains intracellular glucose in equilibrium with extracellular glucose [441]. Thus, the generally accepted role of GLUT2 is to take up glucose during the absorptive phase and to release it in the blood during fasting [442]. Hepatic insulin resistance, created by exposing hepatic cells Chang [382] and HepG2 [443] to high doses of fructose or glucose and insulin has been shown to down-regulate the expression of GLUT2 and reduce glucose uptake [443]. The significant reduction in uptake of 2-[3H]-DOG by Chang and HepG2 cells treated with 0.1 mM insulin and 1 mM fructose seen herein thus suggest that IR inhibits hepatic GLUT2 in these IR hepatic cells. This reduction in glucose uptake was prevented by simultaneous treatment with 12.5 µg/mL *S. frutescens* extracts or 1 µM metformin (Figure 3.2a and b; $P < 0.05$). In the Chang cell culture, both aqueous and organic extracts of *S. frutescens* were found to significantly induce 2-[3H]-DOG uptake above the MIF treated cells while the hot aqueous, cold aqueous and 80% ethanolic extracts of *S. frutescens* were found to significantly induce 2-[3H]-DOG uptake in the HepG2 culture. Furthermore, in both cell cultures, the hot aqueous extract was found to be the most active extract increasing 2-[3H]-DOG by 100% in the Chang cell culture and 76.6% in the HepG2 culture, respectively when compared to metformin and other extracts. Induction of 2-[3H]-DOG uptake in IR Chang cells by *S. frutescens* observed in this present study is consistent with

similar findings by Williams et al. (2013) [382]. Similarly, Cordero-Herrera et al. (2014) showed that 1 µg/mL of phenolic extracts of *Theobroma cacao* prevented high-glucose induced downregulation on GLUT2 levels and glucose uptake in HepG2 cells [443]. Hence it is possible that extracts of *S. frutescens* act in a similar manner to mediate glucose uptake in these cells. Moreover, we have previously shown that hot aqueous *S. frutescens* can upregulate the expression of transporter genes including Vesicle-associated membrane protein 3 (VAMP3), N-ethylmaleimide-sensitive factor (NSF), Synaptosomal-associated protein 25 (SNAP25), VAMP-associated protein A (VAPA), and Syntaxin binding protein 2 (STXB2) in IR hepatic cells [382]. Likewise, I found that the aqueous and organic extracts of *S. frutescens* were found to up-regulate the expression of VAMP3 in IR HepG2 and Chang hepatic cells with varying intensity (Figures 3.5a I and bI). The combinatorial effect of insulin and the aqueous extract of *S. frutescens* on VAMP3 expression, partly supports the adjunct use of *S. frutescens* by T2DM patients.

In the adipocytes and myocytes, insulin mediated exocytosis of GLUT4 bears a resemblance to the regulated exocytosis of synaptic vesicles found in the axon end of motor neurons [444, 445]. In particular, GLUT4 vesicles contains vesicle-soluble NSF attachment protein receptor (v-SNARE) proteins VAMP2 and VAMP3, which physically interact with their target (t)-SNARE counterparts (syntaxin 4 and SNAP family) in the plasma membrane during GLUT4 vesicle translocation [446]. Although, GLUT4 is not expressed in the hepatocytes, most of the GLUT4 trafficking machinery has been found in the liver [447], making it very likely that *S. frutescens* enables glucose uptake in IR hepatocytes by increasing GLUT2 translocation.

Furthermore, in their study, Cordero-Herrera and colleagues showed that treatment with 10 µM (–)-epicatechin, a natural occurring plant flavonoid [448], also upregulated GLUT2 levels and induced glucose uptake in HepG2 cells made IR by exposure to a high concentration of glucose and insulin [443]. Prior studies by Shaik et al. (2011) and Tobwala et al. (2014) have shown

that aqueous and organic extracts of *S. frutescens* leaf contain substantial levels of flavonoids [449, 450]. It is therefore plausible that flavonoids present in the *S. frutescens* extracts used in this study are responsible for the induction of 2-[3H]-DOG uptake in these cells. In addition, Tobwala and colleagues reported that the hot aqueous extract contained the highest concentration of total flavonoids (28.7 µg/mg) when compared to cold aqueous, acetone, acetonitrile, methanol and ethanol extracts respectively [450]. Hence, it is very likely that the high concentration of flavonoids present in the hot aqueous extract might be responsible for its higher activity, as observed in this study.

The association between lipids and insulin resistance is widely accepted [451]. A variety of abnormalities in lipid metabolism have been described in insulin-resistant state [452]. In the liver, IR has been identified as a crucial pathophysiological factor in the accumulation of various classes of lipids [453], leading to hepatic steatosis [375], and subsequent development of non-alcoholic fatty liver disease (NAFLD) [454, 455]. In this present study, the effect of the IR state, created by exposure to combined insulin and fructose or to the saturated fatty acid palmitate, on lipid accumulation in Chang and HepG2 cells was assessed using the Oil-red-O assay and the fluorescent Nile Red stain, in the presence or absence of metformin or extracts of *S. frutescens* (Figures 3.3a, b and c). Data obtained indicate that treatment with insulin and fructose or palmitate significantly increased lipid accumulation in both Chang and HepG2 liver cells ($P < 0.001$). However, treatment with extracts of *S. frutescens* or metformin significantly reversed the effects of fructose and insulin or palmitate on lipid accumulation ($P < 0.01$). These *in-vitro* findings agree with prior observations by Mackenzie et al. (2009, 2012) who showed that ingestion of a high fat diet by Wistar rats led to the development of the IR state, with subsequent free fatty acid (FFA) accumulation. These metabolic changes were prevented and completely reversed by *S. frutescens*[422, 423]

Fructose has been shown to enter hepatocytes in an unregulated manner [456, 457] and fructolysis lacks the tight metabolic regulation present in glycolysis [375]. In the liver, fructose is rapidly phosphorylated by fructokinase to fructose-1-phosphate, a precursor for the triacylglycerols (TAG) backbone, which is subsequently converted to glycerol and acetyl-CoA for fatty acid synthesis [458]. Hence, excess fructose can lead to increased intra-cellular accumulation of TAG [458, 459]. This is corroborated by findings from my thin layer chromatography analysis of the classes of lipid components within Chang and HepG2 cells treated with a high concentration of fructose and insulin (MIF). I found that exposure to a high concentration of fructose and insulin significantly increased the levels of triacylglycerols (TAG) (2.25 fold increase), diacylglycerol (DAG) (14.47 fold increase) and monoacylglycerol (MAG) (37.81 fold increase) in Chang cells (Figures 3.4a I and II; $P < 0.001$) and diacylglycerol (DAG) (1.74 fold increase) and monoacylglycerol (2.48 fold increase) in HepG2 cells (Figures 3.4b I and II). In addition, to TAG, DAG and MAG, I observed that MIF also increases the levels of other unidentified classes of lipids (e.g. sterols). This suggests that these groups of lipids are responsible for the total lipid accumulation observed in MIF treated Chang and HepG2 cells, as discussed earlier. This is in agreement with similar *in vitro* findings by Huang et al. (2011), where exposure to a combination of glucose and fructose increased intra-cellular TAG (palmitate and oleate) in HepG2 cells [460]. Furthermore, I found that the increase in intra-cellular accumulation of these classes of lipids was significantly reversed by extracts of *S. frutescens* or metformin ($P < 0.05$) (Figures 3.4a I, 4b I and 4a II, 4b II). This suggests that *S. frutescens* mediates a reduction in IR-mediated intra-cellular lipid accumulation by inhibiting the synthesis of TAG, DAG, and MAG classes of lipids. As discussed earlier, we have previously shown that *S. frutescens* induces the expression of NSF and VAPA genes in IR Chang cells [382]. NSF has been shown to be involved in lipid droplet formation, movement and fusion; while VAPA has been suggested to regulate lipid metabolism

by binding to lipoproteins and targeting them to the endoplasmic reticulum for processing [461]. This suggests that the effects of *S. frutescens* extracts on IR mediated intra-cellular lipid (TAG, DAG and MAG) accumulation could be as a result of up-regulation of NSF and VAPA gene expression. Furthermore, by inducing Peroxisome proliferator-activated receptor alpha (PPAR α) expression [382], *S. frutescens* can induce mitochondrial and peroxisomal fatty acid β -oxidation in the hepatic cells [462] to provide energy for the cells and at the same time lower intra-cellular fatty acids, subsequently reducing lipid accumulation.

Taken together, using a novel two hepatic cell line (Chang and HepG2) *in-vitro* model system, I have shown that aqueous and organic extracts of *S. frutescens* can prevent the development of hepatic IR, a vital step in the development of T2DM [463]. Here I show that aqueous and organic extracts of *S. frutescens* prevented and reversed insulin and fructose (MIF) mediated IR metabolic changes: increased accumulation of glucose in the culture medium, reduction in glucose uptake and increased intra-cellular accumulation of lipids in Chang and HepG2 liver cells. The expression of biological activities by all the extracts used in this study suggests that a common active principle might be responsible, though at a different concentration, as all the extracts displayed varying levels of activity. In conclusion findings presented herein suggest that aside from the frequently investigated aqueous extract, organic extracts of *S. frutescens* can also be explored for the development of a newer and efficacious anti-diabetic principle. A schematic diagram showing the proposed role of *S. frutescens* in IR mediated metabolic changes in IR hepatocyte is shown in Figure 3.6.

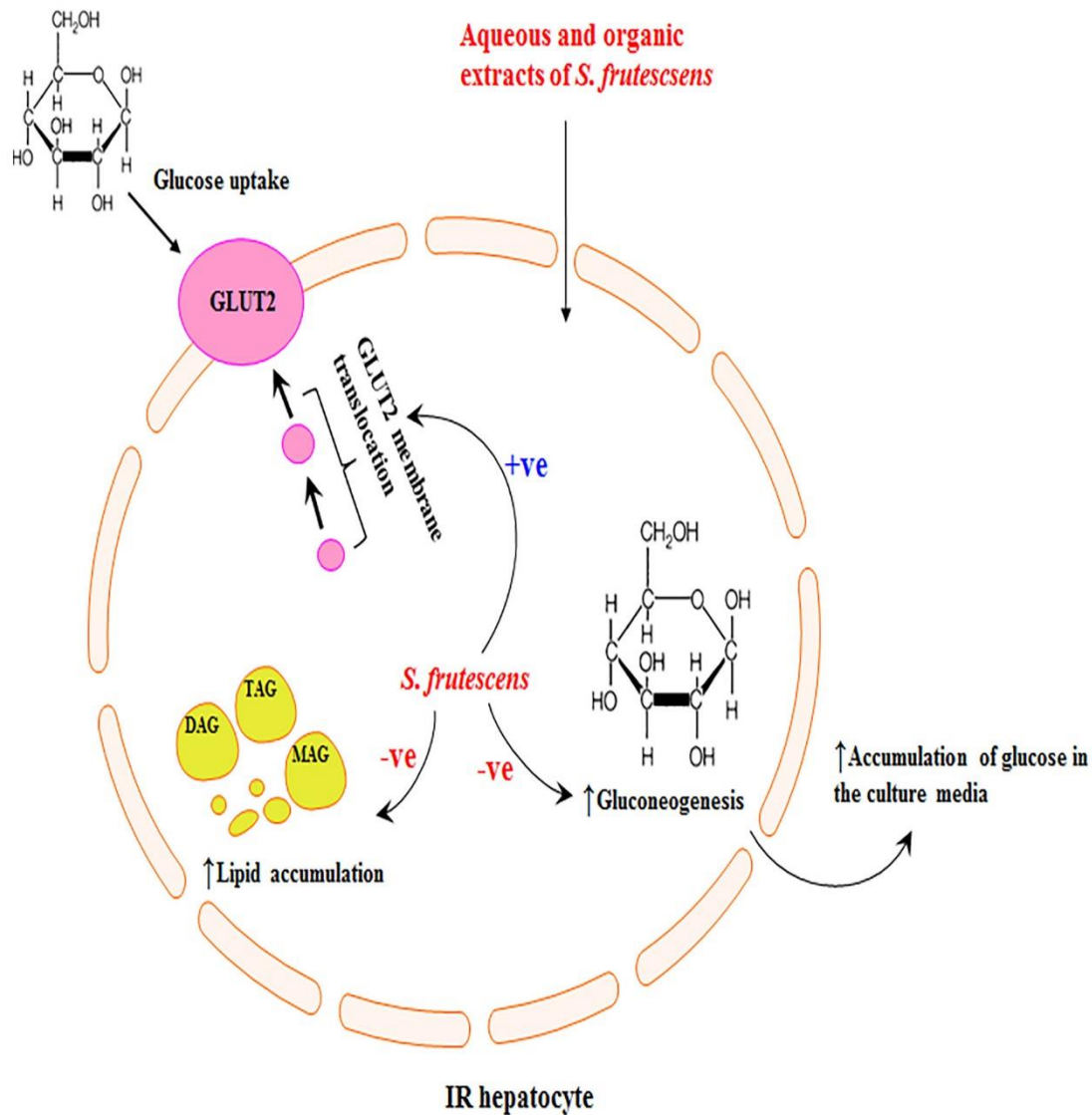


Figure 3.6: Schematic diagram showing the role of *S. frutescens* in reversing IR mediated metabolic changes in IR hepatocyte. In the IR hepatocyte, metabolic changes such as increased gluconeogenesis, increased lipid accumulation and decreased GLUT2 membrane translocation and subsequent decrease in hepatic glucose uptake are a common phenomenon. Exposure of this cell to *S. frutescens* extracts prevents and reverse this IR mediated metabolic changes via its positive effect on GLUT2 membrane translocation to increase hepatic glucose uptake and its inhibitory (negative) effects on hepatic lipid accumulation and hepatic gluconeogenesis to prevent increase in hepatic glucose output.

CHAPTER FOUR

GENERAL DISCUSSION

4.1 General discussion

Presently, glycaemic control with the use of oral anti-diabetic agents (OAAs) is the main stay of clinical management of T2DM [418]. But due to the lack of efficacy and undesirable side effects of current OAAs [419], there is a need for the development of newer more efficacious and less toxic drugs. Medicinal plants have long played important roles in the treatment and management of varying forms of human disease including T2DM [464]. To date, about 7000 natural compounds are in use in modern medicine [465, 466]. In contrast to conventional pharmaceuticals which are based upon a single synthetic chemical, many herbal mixtures exert their biological effects through the additive, or synergistic action of several chemical compounds acting at single site or multiple target sites associated with a physiological process [439]. Various herbal anti-diabetic remedies are used in traditional medical practices around the world. However, only a few of them have been scientifically assessed for their efficacy [144]. Hence, in this present study using an *in-vitro* cell line model system, the anti-diabetic properties of different aqueous and organic extracts of *S. frutescens* was investigated. A single source of plant material was used to make all the extracts, to ensure that the starting plant content was identical and so control for any differences resulting from growth conditions or other variation in the plant material.

The effectiveness of medicinal plant extracts and their pharmacotherapeutic action is due to their complex diversity of chemical compounds [47]. Different plants may possess a wide spectrum of effects due to the presence of various groups of chemical compounds and various microelements [48]. However, successful extraction of biologically active compounds from plant material is largely dependent on the extraction procedure and type of solvent used [467].

Hence, using the conventional hot aqueous solvent extraction method and five other solvent methods: cold aqueous, 80% ethanol, 100% ethanol, 80% methanol and 100% methanol, I have extracted bioactive phytochemicals from *S. frutescens*. Of these solvents, the hot aqueous extractant was quantitatively the best extractant, extracting 1.99 g of plant material, compared to the other solvent methods, and while the 100% ethanol had the lowest mass extracted (0.52 g). This suggests that the application of heat and water (hot aqueous) could play a vital role in extraction of bioactive compounds from *S. frutescens* and also justifies the traditional use of a tea infusion of *S. frutescens* [261].

Based on their chemical structure, bioactive phytochemicals can be classified into alkaloids, glycosides (saponins, phenols, cyanogenic glycoside and cardiac glycosides), lactones, and others (e.g. pigments and ecdysones) [48, 49]. Using colorimetric analysis, the presence of flavonoids, flavonol, phenol and tannins were confirmed in aqueous and organic extracts of *S. frutescens*. This agrees with findings by Shaik et al. (2011) and Tobwala et al. (2014) where aqueous (hot and cold) and organic (ethanol and methanol) extracts of *S. frutescens* were found to contain phytochemicals including flavonoids, phenol and tannins [449, 450]. Furthermore, quantification of these compounds using a spectrophotometric method showed higher concentration of flavonols and tannin in the organic extracts. Similarly, higher concentration of flavonoids was found in both the aqueous (hot and cold) and 100% methanolic extracts, while all the extracts contained approximately equal levels of phenols. This is consistent with findings by Tobwala et al. (2014) where hot aqueous, cold aqueous and methanolic extracts of *S. frutescens* contained higher concentrations of flavonoids when compared to acetone, acetonitrile, and ethanolic extracts [450]. Flavonoids and flavonols are polyphenolic compounds distributed widely in the plant kingdom [280]. Plant mixtures containing flavonoids have been used in folk medicine around the world [281]. Flavonoids display a number of biological activities, including anti-allergic, anti-inflammatory, anti-oxidant, anti-

carcinogenic activity, and modulation of enzymatic activities [282-285]. In humans, phenols and phenolic compounds have been explored for their anti-cancer [277], anti-oxidant, anti-atherosclerotic, anti-bacterial, anti-inflammatory, and anti-viral properties [278, 279]. Similarly, tannins have been suggested to be potent anti-cancer agents due to their beneficial physiological role as scavengers of reactive oxygen intermediates (ROI) [295]. The presence of these compounds in *S. frutescens* gives credence to the folklore use of herbal mixtures of *S. frutescens* as an anti-inflammatory and anti-cancer agents.

Several bioactive phytochemicals have been isolated from *S. frutescens* extracts [35, 206, 224-226]. Some of these bioactive principle constituents have been linked to the therapeutic application of *S. frutescens* in folklore medicine, however each isolated compound alone does not have the full activity of the complete extract. Despite extensive biological and pharmacological studies, few analyses exist of the chemical constituents of *S. frutescens* [273], and no Triple TOF LC/MS/MS analysis has been performed. In this study, untargeted Triple TOF LC-MS/MS analysis in positive and negative ionic mode, revealed the presence of multiple compounds in individual crude and SPE fractions of *S. frutescens* extracts. Comparison of these compounds with online databases of anti-diabetic phytochemicals led to the preliminary identification of 10 possible anti-diabetic compounds: α -Pinene, Limonene, Sabinene, Carvone, Myricetin, Rutin, Stigmasterol, Emodin, Sarpagine and Hypoglycin B in extracts of *S. frutescens*. These are all glycosidic compounds: α -Pinene, Limonene, Sabinene and Carvone are monoterpenes; Myricetin and Rutin are flavonoids; Stigmasterol is a steroid; Emodin is a phenol; Sarpagine is an alkaloid, and Hypoglycin B is an amino acid. The identification of these groups of anti-diabetic compounds in part supports the folklore use of *S. frutescens* herbal mixture for the management of T2DM. In addition, many other phytochemicals were detected by the Triple TOF LC-MS analysis that has not previously been recognised as anti-diabetic compounds and it is possible that these contribute to *S.*

frutescens activity. The identification of these multiple bioactive compounds in a single extract does suggest a combinatorial or synergistic interaction to effect a biological function.

Furthermore, using an *in-vitro* two hepatic cell line model system, made IR by exposure to high concentration of fructose and insulin, the anti-diabetic properties of *S. frutescens* extracts was confirmed. Initial cell viability assays showed that *S. frutescens*, at the concentration used in this present study was not toxic to the cells. Aqueous and organic extracts of *S. frutescens* were found to significantly prevent and reverse IR-mediated metabolic changes in IR Chang and HepG2 cells. *S. frutescens*-mediated prevention and reversal of increased glucose production in IR hepatic cells seen herein agrees with prior findings in our laboratory [382]. This suggests that *S. frutescens* mimics insulin activity by inhibiting the activities of gluconeogenic enzymes G-6-pase and PEPCCK. More so, I showed that extracts of *S. frutescens* were shown to regulate the mRNA expression of MAPK8 and IRS1 in IR hepatic cells. This corroborates prior findings in our laboratory that show *S. frutescens* can mediate the up-regulation of PI3-kinase-Akt, MAP-kinase and IRS1 genes [382], to activate their respective signaling pathways and prevent hepatic gluconeogenesis in IR hepatic cells. In both the metabolic assay of glucose production and the molecular assays of MAPK8 and IRS1 mRNA expression, all extracts acted to prevent or reverse the changes caused by IR, but the hot aqueous extract showed the greatest activity. This may indicate that *S. frutescens* acts via the IRS1 intra-cellular signalling pathway to regulate cellular responses to metabolic changes, with the compounds in the hot aqueous extract providing the best response. In addition, all of the *S. frutescens* extracts were found to prevent and reverse IR-mediated decrease in hepatic glucose uptake. This was also associated with up-regulation of VAMP3 expression, a transporter gene required for successful membrane translocation of members of the GLUT family. This suggests that the *S. frutescens* extracts investigated in this present study can act via VAMP3-mediated translocation of GLUT2 to the hepatic cell membrane to mediate an increase in hepatic glucose uptake. These findings are in

accordance with similar *in vitro* findings by Williams et al. (2013), where *S. frutescens* was shown to increase glucose uptake in IR Chang cell cultures [382].

Furthermore, *S. frutescens* was shown to prevent hepatic lipid accumulation, in particular TAG, DAG and MAG in IR Chang and HepG2 cells. This corroborates with prior findings by Mackenzie et al. (2009, 2012) who showed that IR-mediated free fatty acid (FFA) accumulation in Wistar rats fed on a high fat diet was prevented and completely reversed by *S. frutescens* [422, 423]. The hot aqueous extract is the most potent of all the extracts investigated in this study, though this was not statistically significant, while all the organic extracts displayed similar potency.

Taken together, the findings presented in this present study support the burgeoning body of *in vitro* literature evidence on the anti-diabetic properties of *S. frutescens* and its use in folklore medicine. Overall, the hot aqueous extract was the optimal anti-diabetic extract. Previous studies have also indicated the activity of a hot aqueous extract and generally the organic extracts are reported to be less active. This is the first study to directly compare different extracts prepared from the same single source of plant material, thus I have shown that the combination of phytochemicals within the hot aqueous extract forms the most effective anti-diabetic concoction.

CHAPTER FIVE

CONCLUSION AND FUTURE STUDIES

5.1 Conclusion

Taken together, this study showed that extracts of *S. frutescens* contain potential anti-diabetic compounds that can be explored for the production of newer more efficacious and less toxic anti-diabetic agents. Ten anti-diabetic principles were identified from the DIACAN and Phyto diab care databases following Triple TOF LC-MS analysis, it is likely that some of these compounds combine or interact synergistically to confer the medicinal/biological activities.

Furthermore, between 44 (hot aqueous extract) and 224 (80% ethanol extract) known phytocompounds were identified using the Plant Metabolic Network (PMN). Although most of these compounds have not previously been identified as anti-diabetic, it is possible that they have hitherto unknown activity, or that they synergise with the known anti-diabetic compounds identified in an extract to enhance the overall activity of that extract.

The above biochemical analysis is strengthened by the findings from the *in-vitro* cell line assays in which extracts of *S. frutescens* were shown to prevent and reverse IR-mediated changes in hepatic cells. The hot aqueous extraction method extracted more plant material and was subsequently the most active of all the extracts investigated, while all the organic extracts displayed similar potency. Therefore the potency of the hot aqueous extract may be a result of a higher concentration of a key compound(s), and/or the presence of a specific anti-diabetic compound(s), or finally it may be the result of the unique combination of compounds within that extract.

Furthermore, findings from the quantitative real-time RT-PCR showed that *S. frutescens* can act at multiple target sites to regulate the expression of diabetic related genes and to mediate

anti-diabetic activities, again indicating the potential action of multiple compounds within the extracts.

This study provides scientific evidence for the folklore use of *S. frutescens* as an adjunct therapy in the management of T2DM. However, more explorative study is needed to determine, isolate, purify and characterize the anti-diabetic chemical principles in crude and partially purified (SPE) extracts of *S. frutescens*. More so, since previous work by other research groups has shown that none of the purified compounds from *S. frutescens* tested to date demonstrated anti-diabetic effects equal to a complete crude *S. frutescens* extract. It is possible that previously unidentified compounds also confer activity. Therefore the chemical analysis presented herein has revealed 10 potential anti-diabetic compounds, which can be tested for activity alone and in combination.

5.2 Future studies

In view of the above, future studies will be to;

- ❖ Confirm the anti-diabetic compounds in the crude and SPE fractions of *S. frutescens* extracts by using specific standards on the TOF LC-MS/MS, since an untargeted analysis was done in this present studies.
- ❖ Investigate further the expression of signaling proteins involved in normal and impaired insulin signaling to determine how *S. frutescens* regulates functional protein expression using qRT-PCR to quantify mRNA expression of selected genes, flow cytometry to quantify the protein products and Western blot analysis to investigate their post-translational modification and/or activation (eg phosphorylation state).
- ❖ Intracellular localization of proteins regulated by *S. frutescens* during the IR state, using immunocytochemistry and confocal analysis.

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APPENDIX I

List of compounds found in untargeted LC-MS/MS analysis of crude and SPE fractions of *S. frutescens* extracts

Table I: List of compounds in crude cold aqueous extract of *S. frutescens*

Name	Found at RT (min)
(+)-(4R)-limonene	10.63
(+)-2-carene	10.63
(+)-3-carene	10.63
(+)-sesamolinal	15.26
(+)- β -pinene	10.63
(-)-camphene	10.63
(-)- β -phellandrene	10.63
(-)- β -pinene	10.63
(22a)-hydroxy-campest-4-en-3-one	15.7
(E)-2-methylbutanal oxime	10.28
(E)- β -ocimene	10.63
(S)-2,3-epoxysqualene	15.35
(Z)-2-methylbutanal oxime	10.28
stigmasterol	4.99
myricetin	4.99
(Z)- β -ocimene	10.63
1,3,7-trihydroxyxanthone	9.72
acetylnorajmaline	4.54
acidomycin	4.54
amorpha-4,11-diene	5.08
curcumin diglucoside	15.35
curcumin monoglucoside	15.35

Table II: List of compounds in crude cold aqueous strong anionic extracts of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	13.31
(+)-isopimara-7,15-diene	10.61
(+)-maackiain	11.67
(+)-pisatin	11.78
(+)-sesamin	10.39
(-)-9 β -pimara-7,15-diene	10.61
(-)-abietadiene	10.61
(-)-maackiain	11.67
(-)-maackiain-3-O-glucoside	9.87
(-)-sophorol	13.31
(4a)-methyl-(5a)-ergosta-8,14,24(28)-trien-3 β -ol	15.33
(E)-2-methylbutanal oxime	9.27
(E)-2-methylpropanal-oxime	0.29
(Z)-2-methylbutanal oxime	9.27
(Z)-2-methylpropanal-oxime	0.29
1,3,7-trihydroxyxanthone	9.86
1,4-dihydroxy-2-naphthoyl-CoA	13.35
1,5,7-trihydroxy-6,8-dimethoxyanthraquinone	13.31
1,6,7-trihydroxy-2-methylanthraquinone	13.31
1,6-kestotetraose	13.31
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.78
1-dodecanol	3.95
1D-chiro-inositol	11.78
2-amino-4,6-dinitrotoluene glucoside	9.44
2-hydroxyferulate	11.67
2-methyl-6-farnesyl-1,4-benzoquinol	11.21
2-methylbutyrate	3.52
serinol	15.87

Table III: List of compounds in crude cold aqueous weak anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6 <i>a</i> -hydroxymaackiain	11.26
(+)-pisatin	11.78
(-)-sophorol	11.26
1,6-kestotetraose	11.26
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.78
1D-chiro-inositol	11.78
2-fluorobenzoate	9.24
2-isopropyl-3-oxosuccinate	10.49
2-methyl-6-geranylgeranyl-1,4-benzoquinol	15.77
2-oxindole-3-acetate	8.49
3,7-di-methylquercetagenin	9.99
3-deaza-GMP	15.66
3-hydroxy-15-dihydrolubimin	13.43
3-hydroxypropionyl-CoA	10.49
3-rha-gal-glc soyaapogenol B	15.62
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.87
6,7-dimethyl-8-(1-D-ribityl)lumazine	15.6
6-hydroxymellein	11.26
6-hydroxymethyl-dihydropterin	10.49
7-hydroxy-2-oxindole-3-aceate glucoside	11.26
conhydrine	9.19
curcumin diglucoside	15.69
curcumin monoglucoside	15.69
cysteamine	6.48

Table IV: List of compounds in crude aqueous strong cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-(4R)-limonene	2.36
(+)-2-carene	2.36
(+)-3-carene	2.36
(+)-pisatin	11.78
(+)- β -pinene	2.36
(-)-camphene	2.36
(-)-maackiain	11.68
(-)- β -phellandrene	2.36
(-)- β -pinene	2.36
(22a)-hydroxy-campest-4-en-3-one	15.66
(E)- β -ocimene	2.36
1D-chiro-inositol	11.78
7-O- β -D-glucosyl-apigenin	12.71
curcumin diglucoside	15.78
curcumin monoglucoside	15.78
cyanoborohydride	4.17
epicatechin	5.51
esculetin	10.29
estolide	11.77
levopimaradiene	15.78
linamarin	10.01
luteolin 7-O-[β -D-glucuronosyl-(1,2)- β - D-glucuronide]-4'-O- β -D-glucuronide	15.78
quercetin 3, 7, 4'-trisulphate	10.29
α -curcumene	2.36

Table V: List of compounds in crude cold aqueous weak cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	13.29
(+)-maackiain	11.67
(+)-pisatin	11.78
(-)-maackiain	11.67
(-)-sophorol	13.29
(4-hydroxyphenyl)acetaldehyde	10.41
(R)-pantolactone	14.12
(S)-2,3-epoxysqualene	15.67
1,3,7-trihydroxyxanthone	9.9
1,5,7-trihydroxy-6,8- dimethoxyanthraquinone	13.29
1,6,7-trihydroxy-2- methylantraquinone	13.29
1,6-kestotetraose	13.29
1,7-dihydroxy-5,6,8- trimethoxyanthraquinone	11.78
1-O-methyl-scylo-inositol	8.91
1D-chiro-inositol	11.78
2-butyl-4-hydroxy-5-methyl-3(2H)- furanone	5.59
1,2-diaminopropionate	10.5
1,3,7-trihydroxyxanthone	9.86
1,5,7-trihydroxy-6,8- dimethoxyanthraquinone	13.66
1,6,7-trihydroxy-2- methylantraquinone	13.66
1,6-kestotetraose	13.66
1,7-dihydroxy-5,6,8- trimethoxyanthraquinone	11.77
1-hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate	10.23

Table VI: List of compounds in crude hot aqueous extract of *S. frutescens*

Name	Found at RT (min)
(+)-2-carene	12.69
(+)-3-carene	12.69
(+)- β -pinene	12.69
(-)-camphene	12.69
(-)- β -phellandrene	12.69
(-)- β -pinene	12.69
(E)- β -ocimene	12.69
(Z)- β -ocimene	12.69
4a-methylfecosterol	3.89
Acidomycin	4.58
adenosine 2'-monophosphate	15.64
aureusidin 6-O-glucoside	12.23
Bisdemethoxycurcumin	13.29
D-arabinose 5-phosphate	4.67
D-myo-inositol (1,2,3,4) tetrakisphosphate	4.67
Deguelin	11.16
Dihydrosanguinarine	3.29
heliocide B3	11.46
Norfuraneol	15.61
peonidin-3-(p-coumaroyl)-rutinoside-5-glucoside	5.04
serinol phosphate	15.61
Sulfuretin	12.69
Tetranitromethane	11.46
a-chaconine	12.69
a-curcumene	12.69
a-fenchene	4.67
a-phellandrene	12.69

Table VII: List of compounds in crude hot aqueous strong anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	13.31
(+)-maackiain	11.68
(+)-pisatin	11.79
(-)-maackiain	11.68
(-)-sophorol	13.31
(E)-2-methylpropanal-oxime	1.15
(S)-2,3-epoxysqualene	15.83
(Z)-2-methylpropanal-oxime	1.15
1,3,7-trihydroxyxanthone	9.83
1,5,7-trihydroxy-6,8-dimethoxyanthraquinone	13.31
1,6,7-trihydroxy-2-methylanthraquinone	13.31
1,6-kestotetraose	13.31
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.79
1-deoxy-D-xylulose 5-phosphate	8.04
1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one	8.48
1D-chiro-inositol	11.79
2-hydroxyferulate	11.68
2-mercaptobenzothiazole	6.15
2-oxo-3-carboxy-4,5-cyclopropylhex-5-enoate	3.42
24-epi-campesterol	15.83
246-trinitrobenzene	15.61
3-hydroxy-15-dihydrolubimin	13.41
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.83
4-methylpentanal	14.91
5-hydroxyisourate	11.27
5-methylthiopentylsulfoglucosinolate	10.46
5-phosphoribosyl-N-formylglycineamidine	15.61
6-hydroxymellein	13.31
7-deoxyloganin	13.41
7-hydroxy-2-oxindole-3-aceate glucoside	13.31

Table VIII: List of compounds in crude hot aqueous weak anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	10.54
(+)-maackiain	11.64
(+)-pisatin	11.76
(-)-maackiain	11.64
(-)-sophorol	10.54
1,3,7-trihydroxyxanthone	9.8
1,5,7-trihydroxy-6,8-dimethoxyanthraquinone	10.54
1,6,7-trihydroxy-2-methylanthraquinone	10.54
1,6-kestotetraose	10.54
1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one	8.45
1D-chiro-inositol	11.76
2-hydroxyferulate	11.64
2-mercaptobenzothiazole	6.17
2-methyl-6-geranylgeranyl-1,4-benzoquinol	15.86
(+)-6a-hydroxymaackiain	10.54
3,4,6-trihydroxy-cis-cinnamate	4.19
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.88
6-hydroxyflavone	10.23
6-hydroxymellein	11.25
6-O-galloylglucose	10.23
7-hydroxy-2-oxindole-3-aceate glucoside	11.25
Acetylnorajmaline	6.12
Acidomycin	6.12
Benzoate	15.64
Cinchonine	8.42
curcumin diglucoside	15.64
curcumin monoglucoside	15.64

Table IX: List of compounds in crude hot aqueous strong cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-maackiain	11.67
(+)-pisatin	11.79
(-)-maackiain	11.67
(-)-maackiain-3-O-glucoside-6"-malonate	4.24
1,3,7-trihydroxyxanthone	9.96
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.79
1-(3-aminopropyl)-4-aminobutanal	3.15
1D-chiro-inositol	11.79
2'-hydroxygenistein	8.93
2-hydroxyferulate	11.67
2-methyl-6-geranylgeranyl-1,4-benzoquinol	15.92
246-trinitrobenzene	15.63
3,4-dihydroxypyridin	10.68
3-dehydroquinat	11.17
3-methoxybenzaldehyde	15.61
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.96
4,4'-diisothiocyanostilbene-2,2'-disulfonate	14.92
5-phosphoribosyl-N-formylglycineamidine	15.63
Avenacin	8.44
Berbamunine	4.24
Butylamine	11.67
Cyanoborohydride	4.15
D-arabinose 5-phosphate	3.66
D-myo-inositol (1,2,3,4) tetrakisphosphate	3.66
D-myo-inositol (1,2,3,4,6)-pentakisphosphate	3.66

Table X: List of compounds in crude hot aqueous weak cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	13.32
(+)-maackiain	11.67
(+)-pisatin	11.79
(-)-maackiain	11.67
(-)-sophorol	13.32
(E)-2-methylbutanal oxime	2.44
(Z)-2-methylbutanal oxime	2.44
1,3,7-trihydroxyxanthone	9.84
1,5,7-trihydroxy-6,8- dimethoxyanthraquinone	13.32
1,7-dihydroxy-5,6,8- trimethoxyanthraquinone	11.79
1-amino-propan-2-one-3-phosphate	3.83
10-hydroxygeranial	12.62
1D-chiro-inositol	11.79
4-hydroxybutylglucosinolate	8.98
6-hydroxymellein	13.31
7-deoxyloganin	13.41
7-hydroxy-2-oxindole-3-aceate glucoside	13.31
7-O-acetylsalutaridinol	9.32
Acetylnorajmaline	5.98
Acidomycin	5.98
adenylo-succinate	13.41
aureusidin 6-O-glucoside	12.23
D-octopine	6.5
dephospho-CoA	8.77
geranylgeranyl-chlorophyll a	4.21
indole-3-acetate	10.5

Table XI: List of compounds in crude 100% methanolic extract of *S. frutescens*

Name	found at RT (min)
Acetylnorajmaline	4.2
L-aspartate-semialdehyde	3.6
Shikonin	14.24
Shisonin	14.24
glucono-d-lactone	4.2
L-histidine	4.2
D-myo-inositol (1,2,6) trisphosphate	3.95
Shikimate	12.86
Acidomycin	4.2
L-glutamate	4.5
(Z)-2-methylpropanal-oxime	4.2
Anol	14.06
7-methylthioheptylhydroximoyl- glutathione	3.94
Vanillylamine	14.96
glyceollidin I	4.22
Butylamine	14.1
Serinol	15.72
D-myo-inositol (1,3,4)-trisphosphate	3.35
Vitexin	15.66
Isoalliin	15.52
9-methylthiononylhydroximoyl- glutathione	15.52
Strictosidine	15.52
n-propanol	15.52
indole-3-acetate	8.47
L-histidinol-phosphate	4.2
Linamarin	9.68
emodin anthrone	4.47

Table XII: List of compounds in crude 80% methanolic extract of *S. frutescens*

Name	Found at RT (min)
acetylnorajmaline	4.18
artemisinic aldehyde	3.79
L-aspartate-semialdehyde	3.63
shikonin	14.24
shisonin	14.24
L-gulose	8.93
shikimate	12.86
linamarin	9.69
L-glutamate	4.48
vanillylamine	14.85
dihydrohomopteroate	4.08
another strange group	15.52
L-histidinol-phosphate	4.18
glyceollidin I	4.21
N-prenylagmatine	10.33
P(1),P(5)-di(adenosine-5'-)pentaphosphate	10.33
glyceraldehyde	15.86
serinol	14.87
S-8-methylthiooctylhydroximoyl-L- cysteine	13.26
sn-glycerol-3-phosphate	10.35
sodium azide	10.35
(-)-9 β -pimara-7,15-diene	10.35
heliocide H4	11.03
echinenone	10.6
L-tyrosine	10.6
15-cis-phytoene	11.71
acidomycin	4.12
10-hydroxydihydrosanguinarine	14.03
24-alkyl sterol 2	14.67

Table XIII: List of compounds present in crude 100% methanolic strong anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-bornane-2,5-dione	9.21
(+)-maackiain	11.66
(+)-pisatin	11.78
(-)-maackiain	11.66
(-)-medicarpin	12.32
(E)-2-methylpropanal-oxime	0.54
(S)-2,3-epoxysqualene	15.64
(Z)-2-methylpropanal-oxime	0.54
1,3,7-trihydroxyxanthone	9.8
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.78
1-O-galloyl- β -D-glucose	3.91
10-methyl-5,6,7,8-tetrahydropteroylglutamate	9.21
1D-chiro-inositol	11.78
2'-hydroxypseudobaptigenin	12.32
2-fluorobenzoate	9.17
2-hydroxyferulate	11.66
2-oxosuccinamate	4.2
24-epi-campesterol	15.64
3-glc-glc-medicagenic acid	15.03
3-hydroxy-15-dihydrolubimin	13.41
3- β -hydroxy-5- α -pregnane-20-one	16.16
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.8
4',2,6,7-tetrahydroxyisoflavone	3.91
4-methoxy-3-indolylmethylamine	10.66
4a-methylfecosterol	3.91
6-methoxy, 4',5,6,7-tetrahydroxyisoflavone	12.61
7-deoxyloganin	13.42
8-aminoethyl-2,6-anhydro-3,8-dideoxy-D-glycero-D-talo-octonate	8.96
9-oxo-nonanoate	14.39

Table XIV: List of compounds in crude 100% methanolic weak anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-heliannuol K	16.06
(+)-maackiain	11.67
(+)-pisatin	11.76
(-)-heliannuol E	16.06
(-)-maackiain	11.67
(-)-maackiain-3-O-glucoside	9.73
(-)-medicarpin	12.5
(E)-2-methylpropanal-oxime	4.21
(R)-amygdalin	4.22
(S)-2,3-epoxysqualene	15.57
(Z)-2-methylpropanal-oxime	4.21
1,3,7-trihydroxyxanthone	9.65
1,3-diphosphateglycerate	3.73
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.76
1-O,6-O-digalloyl- β -D-glucose	8.72
16a, 17-epoxy gibberellin A4	8.99
1D-chiro-inositol	11.76
(+)-(4R)-limonene	3.59
(+)-2-carene	3.59
(+)-3-carene	3.59
(+)-maackiain	14.32
(+)-pisatin	11.79
(+)-sesamolinol	15.26
(+)- β -pinene	3.59
(-)-camphene	3.59

Table XV: List of compounds in crude 100% methanolic strong cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-2-carene	3.67
(+)-3-carene	3.67
(+)-isopimara-7,15-diene	10.52
(+)-maackiain	11.64
(+)-pisatin	11.74
(+)- β -pinene	3.67
(-)-9 β -pimara-7,15-diene	10.52
(-)-abietadiene	10.52
(-)-camphene	3.67
(-)-maackiain	11.64
(-)- β -phellandrene	3.67
(-)- β -pinene	3.67
(4a)-methyl-(5a)-ergosta-8,14,24(28)-trien-3 β -ol	14.58
(E)-2-methylbutanal oxime	9.39
(E)- β -ocimene	3.67
(Z)-2-methylbutanal oxime	9.39
(Z)- β -ocimene	3.67
1,3,7-trihydroxyxanthone	9.79
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.74
1D-chiro-inositol	11.74
2-hydroxyferulate	11.64
246-trinitrobenzene	15.72
3-(phosphonoacetylamido)-L-alanine	3.64
3 β -Hydroxy-9 β -pimara-7,15-diene-19,6 β -olide	9.79
4,4-dimethyl-14a-formyl-5a-cholesta-8,24-dien-3 β -ol	14.58

Table XVI: List of compounds in crude 100% methanolic weak cationic extract of *S. frutescens*

Name	Found at RT (min)
(+)-6a-hydroxymaackiain	13.31
(+)-isopimara-7,15-diene	10.47
(+)-pisatin	11.78
(-)-9 β -pimara-7,15-diene	10.47
(-)-abietadiene	10.47
(-)-sophorol	13.31
(E)-2-methylbutanal oxime	9.29
(R)-Prunasin	8.65
(S)-2,3-epoxysqualene	15.73
(Z)-2-methylbutanal oxime	9.29
1,3,7-trihydroxyxanthone	9.68
1,4-dihydroxy-2-naphthoyl-CoA	13.81
1,5,7-trihydroxy-6,8-dimethoxyanthraquinone	13.31
1,6,7-trihydroxy-2-methylanthraquinone	13.31
(+)-(4R)-limonene	3.64
(+)-2-carene	3.64
(+)-3-carene	3.64
(+)-6a-hydroxymaackiain	13.31
(+)-isopimara-7,15-diene	10.53
(+)-maackiain	11.67
(+)-pisatin	11.76
(+)- β -pinene	3.64
(-)-(1S)-sabinene	3.64
(-)-9 β -pimara-7,15-diene	10.53
(-)-abietadiene	10.53
(-)-camphene	3.64
(-)-maackiain	11.67

Table XVII: List of compounds in crude 100% ethanolic extract of *S. frutescens*

Name	Found at RT (min)
24-alkyl sterol 3	15.18
acetylnorajmaline	6.39
purine	10.2
decaprenyl diphosphate	10.2
dUTP	10.41
butylamine	14.05
hesperitin	15.9
246-trinitrobenzene	14.53
2-keto-isovalerate	3.83
coniine	15.04
L-glyceraldehyde	4.18
D-erythro-imidazole-glycerol- phosphate	12
campest-4-en-3 β -ol	10.71
beta-methylenecyclopropyl pyruvate	9.95
β -D-apiofuranosyl-(1->6)-D-glucose	14.21
precorrin-2	8.61
3S,3'S-astaxanthin	9.59
N-methylputrescine	9.57
shikimate-3-phosphate	11.02
2,5-diamino-6-(ribosylamino)-4- (3H)-pyrimidinone 5'-phosphate	9.59
(+)-7-iso-jasmonate	15.92
punicate	10.2
gibberellin A4 methyl ester	11.36
gibberellin A9 methyl ester	11.36
gibberellin A44 diacid	11.36
(S)-coclaurine	8.4
5-pentadecatrienyl resorcinol-3- methyl ether	10.63

Table XVIII: List of compounds present in crude 80% ethanolic extract of *S. frutescens*

Name	Found at RT (min)
acetylnorajmaline	6.37
purine	10.21
decaprenyl diphosphate	10.21
pelargonidin	4.02
ribavirin-5'-monophosphate	4
4-methoxy-3-indolylmethyl	3.94
glucosinolate aglycone	
6-hydroxyprotopine	9.44
3S,3'S-astaxanthin	9.67
N,N-dihydroxyvaline	3.81
24-alkyl sterol 3	14.88
chelidamate	9.91
2,5-diamino-6-(ribosylamino)-4-(3H)- pyrimidinone 5'-phosphate	9.66
246-trinitrobenzene	14.15
Hesperidin	12.65
Dethiobiotin	9.76
magnoflorine	15.86
phosphoryl-choline	4.26
Adenosine	11.5
kaempferol-3-rhamnoside-7- rhamnoside	3.97
Harmol	3.76
stemar-13-ene	9.69
Tris-hydrochloride	9.92
2,6,7,4'-tetrahydroxyisoflavanone	14.08
Hesperitin	15.88
26,27-dehydrozymosterol	9.41
buthionine sulfoximine	13.98

Table XIX: List of compounds in crude 100% ethanolic strong anionic extract of *S. frutescens*

Name	Found at RT (min)
OPC4-trans-2-enoyl-CoA	11.32
OPC6-3-hydroxyacyl-CoA	11.32
OPC4-trans-2-enoyl-CoA	11.32
acetylnorajmaline	4.18
2-hydroxyformononetin	12.07
5a-cholesta-7,24-dien-3 β -ol	12.04
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	11.55
acidomycin	4.2
(+)-maackiain	11.04
shikonin	14.17
shisonin	14.17
CTAB	9.56
shikimate-3-phosphate	14.18
isatin	11.32
9-methylthiononylhydroximoyl-cysteinyglycine	11.32
shikimate	14.06
serinol	14.88
anol	14.75
uracil	13.38
3-cyanopyridine	4.21
4,4'-diisothiocyanostilbene-2,2'-disulfonate	14.6
1D-chiro-inositol	13.14
15-cis-phytoene	11.63
vanillylamine	14.91
dihydromyricetin	14.06
D-sorbitol	14.91
OPC4-3-ketoacyl-CoA	12.15
(6aR,11aR)-3,9-dihydroxypterocarpan	11.45
umbelliferone	11.55
(-)-curcuhydroquinone	11.46

Table XX: List of compounds in crude 100% ethanolic weak anionic extract of *S. frutescens*

Name	Found at RT (min)
(+)-maackiain	11.66
shikonin	14.19
shisonin	14.19
campestanol	13.52
acetylnorajmaline	6.32
linamarin	10.33
pyridoxamine	9.16
6-hydroxymellein	12.51
E-pyridine-3-aldoxime	10.11
dTDP-a-L-rhamnose	12.73
decaprenyl diphosphate	8.62
purine	8.62
adenylo-succinate	10.49
sodium azide	10.29
(-)-9 β -pimara-7,15-diene	10.29
sn-glycerol-3-phosphate	10.29
(4-hydroxyphenyl)acetaldehyde	8.38
hydroxypyruvate	9.06
hydroxymethylpyrimidine phosphate	9.06
OPC6-3-hydroxyacyl-CoA	13.29
OPC4-trans-2-enoyl-CoA	13.29
OPC4-trans-2-enoyl-CoA	13.29
7-O-methylvitexin 2"-O- β -L-rhamnoside	10.08
D-myo-inositol (1,2,6) trisphosphate	3.94
9-methylthiononanaldoxime	10.3
aminoxyacetate	12.49
isorhamnetin 3-sulphate	8.41

Table XXI: List of compounds in 100% ethanolic strong cationic extract of *S. frutescens*

Name	Found at RT (min)
acetylnorajmaline	4.19
acidomycin	4.24
kaempferol-3-O-gentiobioside-7-O-rhamnoside	3.96
glucosyl-(mannosyl)9-(N-acetylglucosaminy)2-diphosphodolichol	4.08
2-hydroxyferulate	10.8
norfuraneol	15.19
(+)-piperitol	11.55
(Z)-2-methylpropanal-oxime	4.21
pelargonidin-3,5-diglucoside-5-O-p-coumaroylglucoside	6.75
indole-3-acetate	8.73
P(1),P(5)-di(adenosine-5'-)pentaphosphate	12.98
N-prenylagmatine	12.98
3-methoxytyramine	4.22
columbamine	8.44
L-histidine	6.32
L-tyrosine	10.38
echinenone	10.38
sn-glycerol-3-phosphate	11.52
(-)-9 β -pimara-7,15-diene	11.52
1D-chiro-inositol	9.77
linamarin	9.49
10-oxogeranial	15.69
(+)-pinoresinol	10.59
D-myo-inositol (1,2,6) trisphosphate	3.95
leucopelargonidin	15.85

Table XXII: List of compounds in 100% ethanolic weak cationic extract of *S. frutescens*

Name	Found at RT (min)
conhydrine	9.08
(-)-maackiain	11.16
shisonin	14.17
shikonin	14.17
norfuraneol	12.55
linamarin	9.73
serinol	14.91
acetyl norajmaline	6.24
purine	8.64
decaprenyl diphosphate	8.64
dTDP-a-L-rhamnose	11.21
methylsuccinate	15.89
(+)-pulegone	15.32
acetylenedicarboxylate	4.19
7-hydroxy-2-oxindole-3-aceate glucoside	13.1
aminooxyacetate	12.43
1D-chiro-inositol	13.14
5-O-(indol-3-ylacetyl-myo-inositol) D- galactoside	8.28
levopimaradiene	13.98
(E)-2-methylbutanal oxime	4.02
β -fenchocamphorone	15.55
heliocide H4	12.36
2,5-diamino-6-(ribosylamino)-4-(3H)- pyrimidinone 5'-phosphate	11.57
quinate	12.29
7-O- β -D-glucosyl-apigenin	6.73
uroporphyrin I	11.63
dalcochinin-8'-O- β -glucoside	11.52
abscisic acid glucose ester	16.8

APPENDIX II

Names of Phytocompounds with Anti-diabetic properties compiled from online Databases DIACAN and Phytoremedial Database for Anti-diabetic.

a-pinene

allicin

aminoguanidine

ampelopsin

andrographolide

arbutin

arecolin

a-tocopherol

acetophenone

achyrofuran

acylglucosyl Sterols

ajmalicine

ajmaline

alpha-Homonojirimycin

amarogentin

amaroswerin

andrographolide

apigenin

arecoline

bassic acid

berberine

bergenin

beta Amyrin

beta-sitosterol

biguanidine

bakuchiol

bellidifolin

bengalenoside

berberin

bergenin

beta Sitosterol

beta-Sitosterol 3-D-Glucoside

beta-Sitosterol-D-Glycoside

betavulgarosides 2

breviscapine

chlorogenic Acid

corosolic acid

chrysophanol

cryptolepine

crispatine

curcumin

caffeic Acid

capsaicin

carvone

castanospermine

catechin

chamaemeloside

charantin

christinin-A

cinchonain Ib

cinnamaldehyde

coixan A

coixan B

coixan C

coumarin

coutareagenin

cryptolepine

cycloartanol

dianex

dioscoretine

D-chiro-inositol

desmanthin-1

dibenzocyclooctadiene

dioscoran A

dioscoran B

dioscoran C

dioscoran D

dioscoran E

dioscoran F

D-pinitol (3-O-Methyl-Chiroinositol)

emodin

ellagic acid

eleutheran-A

eleutheran-B

eleutheran-C

eleutheran-D

eleutheran-F

eleutheran-G

emblicanin-A

emblicanin-B

enhydrin

ephedran A

ephedran B

ephedran C

ephedran D

ephedran E

epicatechin

epicatechin-3-O-Gallate

epigallocatechin

epigallocatechin Gallate

eremanthin

eugenol

ferulic acid

fagomine

galegine

gallotannins

geraniol

ginkgolides

glibenclamide

glycyrrhizic acid

galactomannan

galegine

gallic acid

gamma-conglutin

geraniin

ginsenosides

girinimbine

girinimbiol

glycyrin

glycyrrhizin

gossypol

guaijaverin

gymnemic Acid

harpagoside

harpagoside-B

hederagenin

hydnocarpin

hydnowightin

hydroxytyrosol

hydroxyvernolide

hypoglycin A

hypoglycin B

hypophyllanthin

isopimpinellin

isorhamnetin

indoleacetic Acid

inositol

isoorientin

jatrorrhizine

jiangtangsu

kaempferitrin

kalopanaxsaponin A

kaempferol

kaempferol-3-O-Beta-Glucopyranoside

kaempferol-3-O-Sophoroside-4'-O-'Beta-Glucoside

kalopanaxsaponin A

kaurenoic Acid

kolaviron

kotalagenin 16-Acetate

Kotalanol

luteolin

lagerstroemin

lathyrine

lectin

L-Ephedrine

lepidine

leucodelphinidin

leucopelargonidin

leucopelargonidin-3-O-Alpha-L-Rhamnoside

leucosceptoside A

lilium-A-Glucomannan

lilium-J-Glucomannan

lilium-S-Glucomannan

lilium-S-Glucomannan

limonene

lithosperman A

lithosperman B

lithosperman C

lophenol

luffin-A

lupanine

lycoris-R-Glucomannan

lycoris-S-Glucomannan

maltodextrin

masoprocol

myrcene

myricetin

mangiferin

mangiferin-7-O-Betaglucoside

masoprocol

MDG-1

mearnsitrin

methylbellidifolin (Swerchirin)

methylenecyclopropyl Glycine

methylswertianin

mimosine

momordicosides A

momordicosides B

momordin-A

moracin M

moracin M-3-O-Beta-D-Glucopyranoside

moran A

mucuadinine

mucunadine

mucunine

mulberrofuran U

mycaminose

myrciacitrin III

myrciacitrin IV

myrciacitrin V

myrciacitrins I

myrciacitrins II

myrciaphenone A

myrciaphenone B

myricitrin

myristicin

myrtillin

neohydnocarpin

neriin

N-Hydroxyethyl-1-Deoxynojirimycin (Miglitol)

N-Hydroxyphenyl-1,4-Dideoxy-1,4-Imino-D-Arabinitol

nigellone

nuciferine

nymphayol

oleanolic acid

oleandrin

oleanoic Acid-28-O-Beta-D-Glucopyranoside

oleo-Gum-Resin

Oleuropein

paeoniflorin

proanthocyanidins

paeoniflorin

palmatine

pandanus Odorus (Toei-Hom) A 4- Hydroxybenzoic Acid

paniculatan

pectin

pedunculagin

peptidoglycan MVS-1

peptidoglycan MVS-IIA

perlargonidin 3-O-Alpha-L Rhamnoside

phanoside

phloridzin

phyllanthin

pinitol

p-Insulin

proanthocyanidin

procyanidin B-2

punigluconin

purpureaside C

quercetin

quercetin-3-O-Beta-Glucopyranoside

quercetrin

quercitrin

quinoline-2-Methanol

rutin

rebaudioside A

regeol A

rehmannioside A

rehmannioside B

rehmannioside C

rehmannioside D

roseoside

rosmarinic Acid

sabinene

salacinol

sarsasapogenin

scopoletin

swerchirin

saccharan A

saccharan B

saccharan C

saccharan D

saccharan E

saccharan F

salacinol

salacinol

salacinol

salaquinone A

salasol A

salasone A

salasone B

salasone C

salidroside

S-Allyl Cysteine Sulphoxide (SACS)

sanguinarine

sarpagine

scropolioside-D

serpentine

sesquiterpene

sesquiterpene

skimmianine

skimmin

S-Methyl Cysteine Sulfoxide (SMCS)

solasonine

sonchifolin

sparteine

sparteine

stevioside

stigmasterol

sweroside

swertiamarin

tecomine

trans β - Ocimene

tannic Acid

thymoquinone

tingenine B

tingenone

tormentic Acid

trans-Dehydrocrotonin

trichosan A

trichosan B

trichosan C

trichosan D

trichosan E

trigonelline

triptocalline A
 umbelliferone
 ursolic acid
 uniflorine A
 uniflorine B
 urs-12-En-3beta-Ol-28-Oic Acid 3beta-D-Glucopyranosyl-4'-Octadecanoate
 uvedalin
 vinblastine
 vincamine
 vernodalin
 vernolide
 vernonioside A1-A4
 v-Insulin
 wedelolactone
 yohimbine
 zingiberene
 1,4-Dideoxy-1,4-Imino-D-Ribitol
 18beta-Glycyrrhetic Acid
 1-Deoxymannojirimycin
 1-Deoxynojirimycin
 2-(2,3,6-Trihydroxy-4-Carboxyphenyl)Ellagic Acid
 2,5-Dideoxy-2,5-Imino-D-Mannitol
 2,5-Dihydroxy-4,3'-Di(Beta-D-Glucopyranosyloxy)-Trans-Stilbene

2,5-Dihydroxymethyl-3,4-Dihydroxypyrrolidine
 2,5-Imino-1,2,5-Trideoxy-D-Mannitol
 24-Ethyl-Lophenol
 24-Methylene-Cycloartanol
 24-Methyl-Lophenol
 28Nor-22(R)Witha 2,6,23-Trienolide
 2-Heptyl Acetate
 2-Hydroxy 4-Methoxy Benzoic Acid
 2-Methylbutyl Acetate
 3,22 -Dihydroxyolean-12-En-29-Oic Acid
 3-Caffeoylquinic Acid/Chlorogenic Acid
 3-Epifagomine
 4-Hydroxy-Alpha-Tetralone
 4-Hydroxy-Alpha-Tetralone-4-O-Beta-D-[6'-O-(3'4'5'-Trihydroxybenzoyl)
 Glucopyranoside
 4-Hydroxybenzoic Acid
 4-O-Beta-D-Glucopyranosylfagomine
 6-Gingerol
 6-Methyl-4-Chromanone
 6"-O-Acetyl-5-O-Beta-D-Galactopyranosyl-7,3',4'-Trihydroxy-4-Phenylcoumarin
 6"-O-Acetyl-5-O-Beta-D-Galactopyranosyl-7,4'-Dihydroxy-4-Phenylcoumarin
 7-O-Beta-D-Glucopyranosyl Alpha-Homonojirimycin
 8-Debenzoylpaeoniflorin

APPENDIX III

MTT Cell Viability Assay of Chang and HepG2 cells

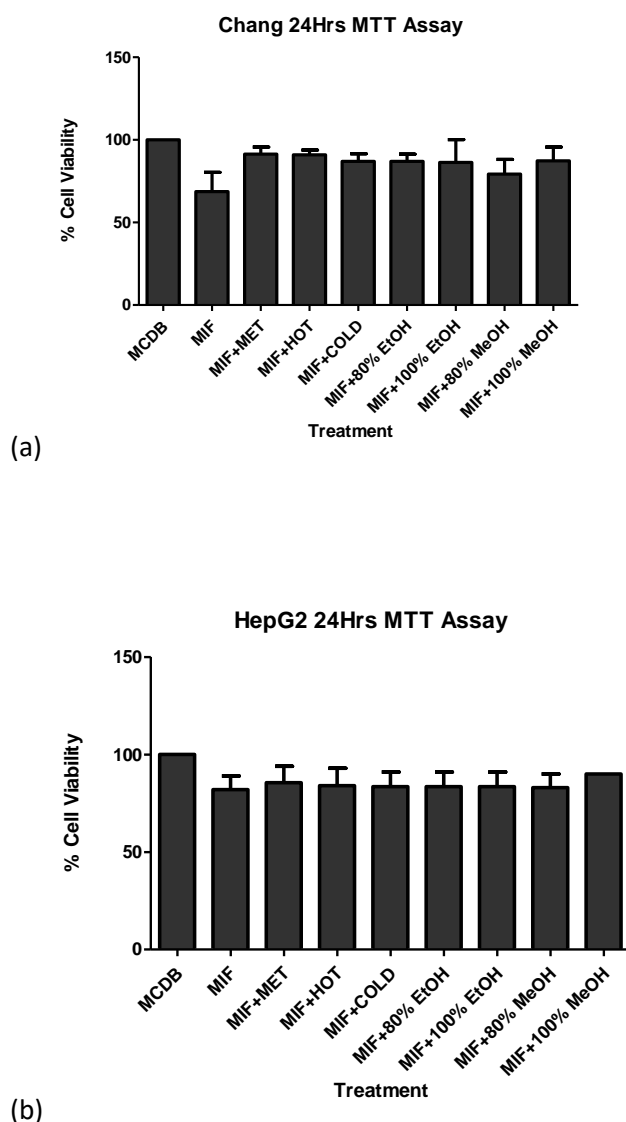


Figure 3.6: Cell viability of (a) Chang cells (b) HepG2 cells as measured by MTT assay, is expressed as a % of the control MCDB culture. Chang and HepG2 cells were cultured in MCDB-201 medium containing insulin and fructose (MIF) for 24 hrs, with *S. frutescens* extracts or metformin as positive control. The effect of *S. frutescens* extracts or metformin on cell viability in the culture medium is shown. Data are represented as mean \pm SEM from 3 independent experiments of 3 wells per experiment for all analysed conditions.