ISOLATION AND IDENTIFICATION OF ANTIDIABETIC COMPOUNDS FROM *BRACHYLAENA DISCOLOR* DC

Thesis submitted in fulfilment of the requirements for the degree

Master of Science

By

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ABSTRACT

Diabetes mellitus, which is a metabolic disease resulting from insulin deficiency or diminished effectiveness of the action of insulin or their combination, is recognized as a major threat to human life. Using drugs on a long term to control glucose can increase the hazards of cardiovascular disease and some cancers. Therefore, there is an urgent need to discover new, safe, and effective antidiabetic drugs. Traditionally, there are several plants that are used to treat/control diabetes by South African traditional healers such as *Brachylaena discolor*. This study aimed to isolate and identify antidiabetic compounds from *B. discolor*.

The plant materials of *B. discolor* was collected from University of KwaZulu-Natal botanical garden. Plant materials were dried under the fume hood for two weeks and ground to a fine powder. The powder was extracted with a mixture of dichloromethane and methanol (1:1). To investigate the antidiabetic activity, the prepared extract was tested *in vitro* for glucose utilization in a muscle cell line. The results revealed that blood glucose levels greater than 20 mmol/L, which measured after 24 and 48 hours of the experimental period, three fractions had positive (*$p<0.05$) antidiabetic activity compared to the control. The DCM:MeOH (1:1) extract of *B. discolor* leaves was subjected to column chromatography, yielding five fractions (A, B, C, D, and E). Fraction A yielded lupeol acetate and its Δ^{12}-isomer as a mixture of compounds. Two compounds identified as β-sitosteryl linelonate, and α-tocopherol were obtained from fraction B. Fraction E resulted in the isolation of genkwanin 5-*O*-β-D-glucopyranoside and a mixture of the α- and β-isomers of glucose.

The results of this study confirmed the antidiabetic properties of the leave extract *B. discolor,* which support the traditional use of the plants by healers. However, further research to investigate more compounds in this plant as well as testing the side effect of using these chemical compounds are proposed.

PREFACE

The experimental work described in this thesis was carried out in the School of Chemistry and Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Fanie van Heerden.

I hereby declare that these studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Signed: …………………………………

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Date:…………………………...

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Prof F R van Heerden (Supervisor)

Date:…………………………...

ACKNOWLEDGEMENT

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LIST OF ABBREVIATION

^{13}C Carbon-13

^{1}H Proton

AMU   Atomic mass unit

CC   Column chromatography

DCM   Dichloromethane

DEPT   Distortionless enhancement by polarization transfer

DM   Diabetes mellitus

DMEM   Dulbecco's modified Eagle's medium

DPP-4   Dipeptidyl peptidase 4

EtOAc   Ethyl acetate

FCS   Foetal calf serum

g   Gram

GC-MS   Gas chromatography - mass spectrometry

GLP-1   Glucagon-like peptide

h   Hour

Hex   Hexanes

HIV   Human immunodeficiency virus

HMBC   Heteronuclear multiple-bond correlation

HSQC   Heteronuclear single-quantum correlation

Hz   Hertz

IDF   International Diabetes Federation

LC-MS   Liquid chromatography - mass spectrometry

MeOH   Methanol

mg	Milligram
mL	Millilitre
mL/min	Millilitre/min
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
^{o}C	Degrees Celsius
ppm	Parts per million
R_f	Retention factor
SPE	Solid phase extraction
STZ	Streptozotocin
TLC	Thin-layer chromatography
TZD	Thiazolidinediones
USFDA	United State Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization
µL	Microlitre
µm	Micrometer

CHAPTER 1: INTRODUCTION

1.1   GENERAL INTRODUCTION

The World Health Organization (WHO) defines diabetes mellitus (DM) as "a chronic disease that occurs, either because the pancreas does not produce sufficient amounts of insulin, or the body cannot efficiently use the produced insulin".[1] Diabetes is considered as a controllable but not treatable disease. The WHO has reported a worldwide increase of diabetes in 2014, with an estimated 422 million cases in comparison to 108 million cases in 1980.[2] In addition, the prevalence of diabetes has almost doubled since 1980, increasing from 4.7% to 8.5% of the population with about 1.5 million deaths in 2012.[2] This disease can be controlled by direct therapies including insulin and other antidiabetic agents. Lately, people also turn to the use of herbal medicine and natural products because of their availability, low cost, and less side effects.[3]

The use of traditional medicine resulted from the combination of knowledge, skills, and practices which were established based on the observations, beliefs, and experiences in different cultures.[4] Major civilizations of the ancient world, e.g. Chinese, Indians, and Africans, provide strong evidence for the use of medicinal plants as therapy for a large diversity of illnesses.[5] Recently, people turned back to the use of medicinal plants due to adverse effects and cost of chemically synthesized drugs. Also, a large part of the population of the world consider traditional medicine as an essential element of their health care.[4] The basic components of herbal medicines are plants and plant products, minerals, and animals; generally, this medication is usually used on the body surface or inserted into the body.[6]

In organic chemistry, the term 'natural products' is used to describe purified organic components isolated from natural sources that are produced by secondary metabolic pathways.[7] Secondary metabolites often have the right chiral configuration to influence biological activity, without a "primary" function directly involved in the usual growth of an organism or its development.[8] It usually has a molecular weight of less than 1500 amu.[7] Several studies (e.g., Doughari,[9] Abo et al.,[10] and Nweze et al.[11]) reported that the secondary metabolites are responsible for plant protection from microbial infections. In

this context, Duraipandiyan et al.[12] evaluated 18 ethnomedicinal plant extracts for activity against nine bacterial and one fungal strain. They found that the plant extracts exhibited antimicrobial activity against the microorganisms at different concentrations.

Some of the old successes in the isolation of active compounds from plants are the isolation of the antimalarial agent quinine (**1.1**), which was isolated from the bark of *Cinchona* species and the development of the anti-inflammatory drug acetylsalicylic acid (**1.2**) (aspirin) from the natural products salicylic acid and salicin (**1.3**) isolated from the bark of the willow tree *Salix alba* L.[13] In addition, many alkaloid compounds such as morphine (**1.4**) isolated from *Papaver somniferum* L. (opium poppy) have been used as painkillers. Penicillin G (**1.5**), a well-known and one of the most famous natural products discovered in 1929, is derived from a fungus (microorganism) and have been used as an antibiotic.[14]
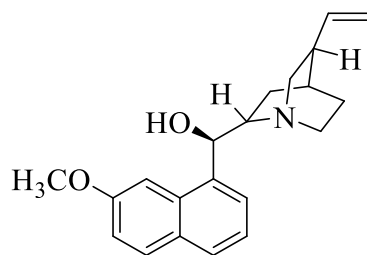
Quinine (**1.1**)
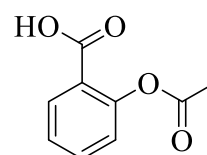
Acetylsalicylic acid (**1.2**)
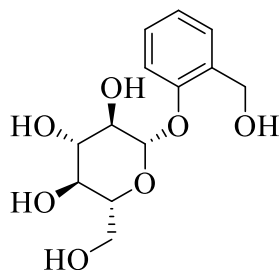
Salicin (**1.3**)
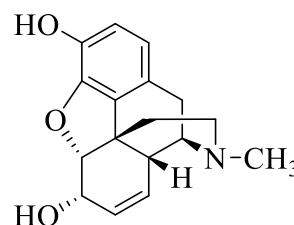
Morphine (**1.4**)
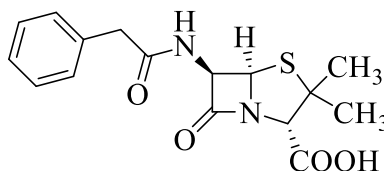
Penicillin (**1.5**)

Betulinic acid (**1.6**) is a natural product isolated from *Betula pubescens* Ehrh. bark and was recognized as an HIV inhibitor.[15] Betulinic acid also has anticancer properties, because it

has been found to induce apoptotic cell death in cancer cells.[16] Paclitaxel (Taxol®) (**1.7**), which was isolated from the bark of *Taxus brevifolia* Nutt. (Pacific yew), is the common drug for the treatment of breast cancer.[17] In addition, there are other antitumour compounds such as ingenol 3-*O*-angelate (**1.8**), a derivative of the polyhydroxy diterpenoid ingenol, which was isolated from *Euphorbia peplus* L. sap and is currently under clinical trials for the treatment of skin cancer.[18]

Digitalis purpurea L. (foxglove) contains four important glycosides and three of them are cardiac stimulants. The most powerful of the isolated compound is digitoxin (**1.9**), which has been used to treat heart cases through increasing the strength of the muscle tissue, especially heart and arterioles muscle.[19] Digitoxin and its analogues are also used in congestive heart failure control. However, their possible long-term negative effects lead to the replacement of **1.9** by other medications for the treatment of heart diseases.[18]

Betulinic acid (**1.6**)

Taxol® (**1.7**)

Ingenol 3-*O*-angelate (**1.8**)

Digitoxin (**1.9**)
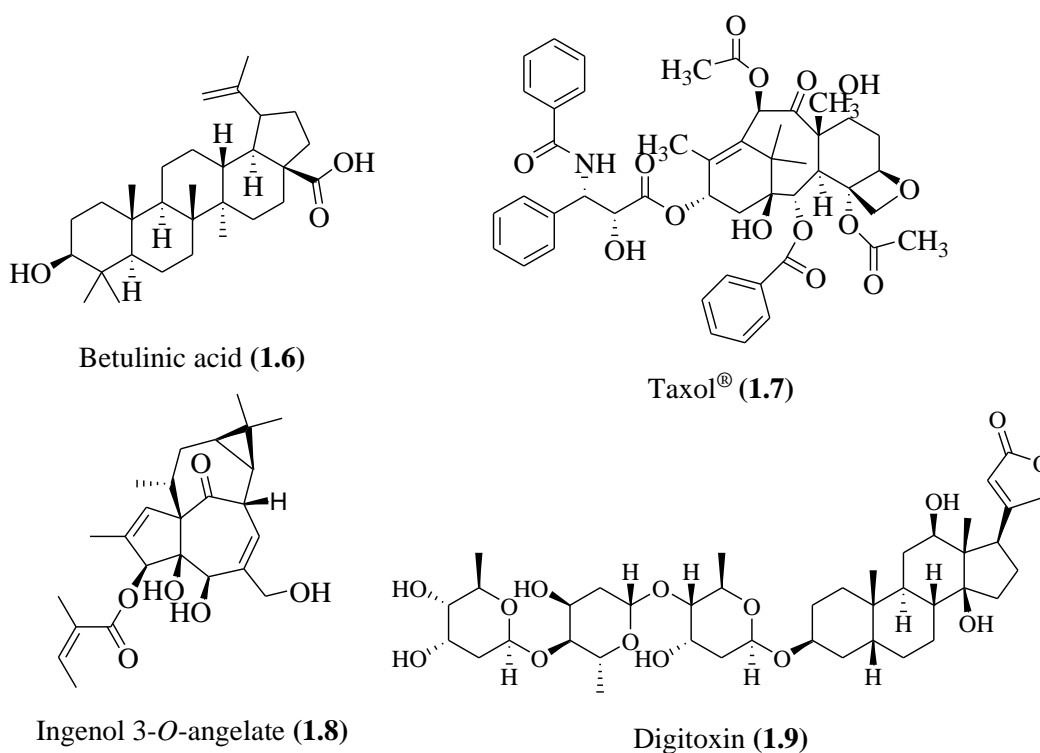
PG490-88 (**1.10**), which is also known as 14-succinyl triptolide sodium salt, was isolated from the vines and roots of *Tripterygium wilfordii* Hook.f., and is conventionally used by the Chinese for the treatment of inflammatory diseases affecting the immune system.[20] Other compounds, such as triptolide (**1.11**) obtained from the *Tripterygium* vine, are recognized as effective immunosuppressive compounds.[20]
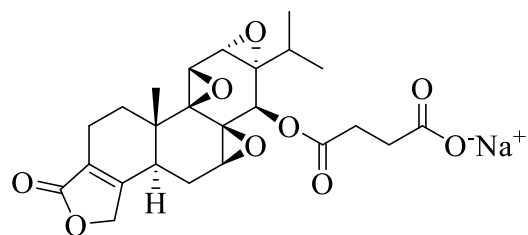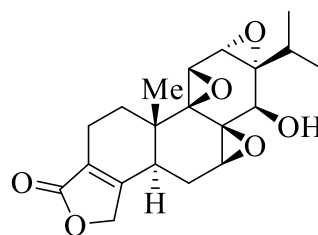
PG490-88 **(1.10)**   Triptolide **(1.11)**

One of the most common drugs used to treat diabetes, metformin, was first isolated from a plant, *Galega officinalis* L.[21]

1.2 AIMS AND OBJECTIVES OF THIS DISSERTATION

The aim of this thesis was to investigate *Brachylaena discolor* DC, a plant used traditionally for the treatment of diabetes.

The specific objectives were to:

- Confirm the antidiabetic activity of an extract of the leaves of *B. discolor*.
- Isolate the compounds in the extract.
- Determine the structure of the isolated compounds.

The rest of the thesis consists of three chapters as follows:

Chapter 2 provides an intensive literature review on diabetes and the medications used for the treatment thereof. In addition, the medicinal plants used for the treatment of diabetes in South Africa are discussed. Chapter 3 provides information about the medicinal plant (*B. discolor*) used in this study, including its taxonomy, description, distribution (in South Africa), and the phytochemistry of the species and *Brachylaena* genus. The procedures of isolating the components from *Brachylaena discolor* in this investigation are described. The presentation of the results and the associated discussions are also covered in this chapter. Finally, Chapter 4 provides a general conclusion and the implications of the finding of this study.

CHAPTER 2: DIABETES AND MEDICINAL PLANTS: A LITERATURE REVIEW

2.1   DEFINITION AND SYMPTOMS OF DIABETES

Diabetes mellitus is a group of symptoms related to the metabolic process, characterized by high blood glucose (hyperglycaemia) following a combined defect in insulin secretion and action. Long-term hyperglycaemia can cause eye damage, heart, and nerve dysfunction, and kidney failure.[22] The symptoms of diabetes mellitus can vary in individuals and sometimes there may be no signs. According to the International Diabetes Federation (IDF), the common symptoms of diabetes mellitus are a tendency to urinate more often, increased thirst, intense hunger, weight loss, tiredness, frequent infections, slow-healing wounds, and blurred vision.[23] Some of the specific diabetes symptoms are related to a certain type of diabetes.

2.2   CLASSIFICATION OF DIABETES

There are several types of diabetes, with the most common three types as follows:

- Type I: Insulin-dependent diabetes, which mostly results from the lack of insulin due to pancreatic beta cell destruction. This type of diabetes is usually diagnosed at a young age (children and young people) and can be treated using insulin injections.

- Type II: Non-insulin-dependent diabetes is a combination of insulin resistance and an insufficient insulin secretion defect. This type of diabetes is more common than type I representing about 90% of the diabetes cases worldwide. It mainly results as a consequence of sedentary behaviour, bad dietary habits, and genetic factors.[24]

- Gestational diabetes, commonly associated with pregnancy, is defined as a glucose intolerance that was not existing or known prior to pregnancy. In general, gestational diabetes mellitus affects about 2 to 5% of pregnant women and in most cases, may

increase the risk of developing type II (or, rarely, type 1) diabetes in the years following the delivery.[25]

Other types of diabetes include a wide variety of relatively uncommon conditions such as diabetes associated with defects in specific genes, diseases of the pancreas, special drugs that affect or damage the pancreas, infections, and other conditions.

When the blood glucose levels are higher than normal and at the same time not high enough to be diagnosed as diabetes, it is called prediabetes.[26] Prediabetes usually increases the risk factors for developing type 2 diabetes. The Centers for Disease Control and Prevention reported that about 86 million adults in the USA had prediabetes in 2012.[26]

Diabetes has become one of the greatest threats to human health in the 21st century and this is calling for increasing the efforts for its prevention and control.[27] A recent estimation showed that diabetes affects about 70 million people in Asia, 64 million in America and the Caribbean, 55 million in Europe, and about 15 million in Africa.[28] According to the most recent report of Stats South Africa, about 8% of the population in South Africa are affected by diabetes and it was recognized as the fifth most important disease that caused human death.[29]

2.3   MEDICATIONS USED TO TREAT DIABETES

Historically, symptoms of diabetic diseases were recorded 3,500 years ago, in Egypt, Arabia, and Asia. Diabetes has been diagnosed and medication for managing diabetes mellitus was discovered in the 20th century. The discovery of diabetic remedies such as insulin in 1922, significantly reduced death in the diabetic population.[30] Since that time, the diabetic remedies and medications were greatly improved. Nowadays, the pharmacological controls of diabetes are more effective. The drugs used to manage diabetes are discussed below.

2.3.1 Insulin

The discovery of insulin, a polypeptide, in 1922 was recognized as a major medicinal revolution in the treatment of diabetes.[30] Insulin is a hormone that is vital for metabolic processes, and is released in the body by pancreatic cells. Insulin works by stimulating glycogen formation and this function helps to maintain lower glucose levels in the blood. The subsequent development of insulin analogs was geared to improve diabetes control and reduce or delayed complications.[31] Although insulin is still considered as the basis for diabetes therapy, the discovery of new medications that can complement and enhance insulin action (e.g. sulfonylureas) were extremely important, because the pathophysiology of diabetes mellitus involves different mechanisms, which need to be considered.[31]

2.3.2 Sulfonylureas

Sulfonylureas are a class of oral antidiabetic compounds and are presently used as a second option to treat type 2 diabetes.[32] Table 2.1 provides a summary of the available sulfonylurea drugs, their classification, dosage, and side effects. The sulfonylurea medications can be divided into two groups: group one includes chlorpropamide (**2.1**), tolazamide (**2.2**), and tolbutamide (**2.3**), which are representing the first-generation agents. This group has longer half-lives, increased occurrence of hypoglycaemia, and more drug interactions. On the other hand, glimepiride (**2.4**), glipizide (**2.5**), and glyburide (**2.6**) are considered as second-generation agents (Fig. 2.1). The group of second-generation agents has a faster action, shorter half-lives, and a lower incidence of hypoglycaemia.[33]

There are some agents offered in a combination with other antidiabetic treatments. For example, glipizide (**2.5**) and glyburide (**2.6**) are both available in combination with metformin (**2.7**), while glimepiride (**2.4**) is available in combination with each of the thiazolidinediones (pioglitazone (**2.9**) and rosiglitazone (**2.10**)).[33] The sulfonylurea agents are not used in patients whose pancreas is not producing insulin (type 1 diabetes). Using sulfonylurea agents has many advantages (e.g. safe and cheap); however, the occurrence of hypoglycaemia is the major side effect.[33] In Table 2.1, a summary of the available sulfonylureas is given and the structures of these compounds are given in Fig. 2.1.

Table 2.1.   Available sulfonylurea agents, their classifications, dosage, uses, and side effects.

Drugs	**Dosage strength (mg)**	**Uses**	**Side effect**
Chlorpropamide (**2.1**)	100/200	Controlling blood sugar in type 2 DM.	Mild nausea, vomiting, diarrhoea, hunger, skin rash, and redness, itching
Tolazamide (**2.2**)	250/500	Control of mild to moderately severe, stable, type 2 DM	Feeling of stomach fullness, nausea, and heartburn.
Tolbutamide (**2.3**)	500 orally	Helping the body use insulin efficiently with diet and exercise.	Nausea, fullness, heartburn, headache, and changes in taste.
Glimepiride (**2.4**)	1/2/4 orally	Controlling of type 2 DM as an adjunct to diet and exercise.	Dizziness and nausea
Glipizide (**2.5**)	2.5/5/10	Reduce blood sugar levels which are caused by type 2 DM.	Low blood sugar and stomach problems.
Glyburide (**2.6**)	1.25/2.5/5	Adjunct to diet and exercise for the control of type 2 DM	Nausea, stomach fullness, heartburn and weight gain
Glipizide (**2.5**) & Metformin (**2.7**)	2.5/250 2.5/500 5/500	Adjunct to diet and exercise for improving glycaemic control associated with type 2 DM	Diarrhoea, headache, indigestion, stomach pain, and nausea
Pioglitazone (**2.9**) & Glimepiride (**2.4**)	4/1 4/2 4/4 8/2	Management of type 2 diabetes.	Headache, muscle pain, nausea, diarrhoea, and cold symptoms
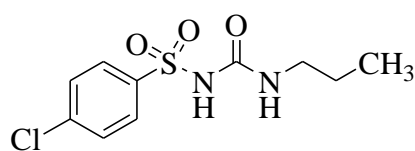
Chlorpropamide **2.1**
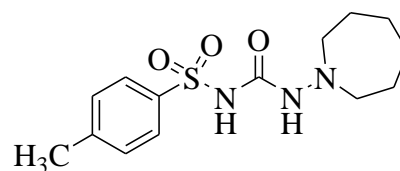
Tolazamide **2.2**
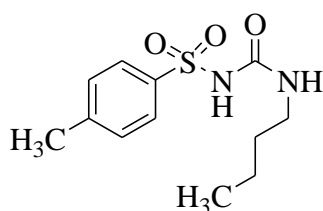
Tolbutamide **2.3**
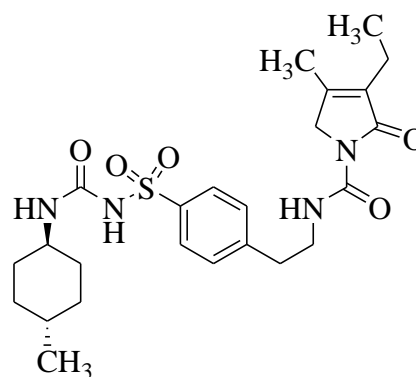
Glimepiride **2.4**
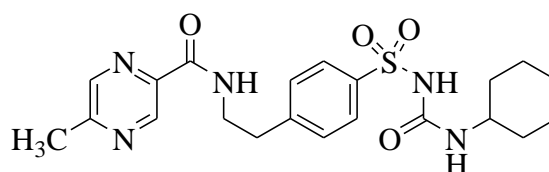
Glipizide **2.5**
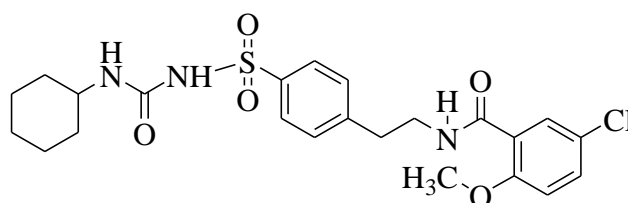
Glyburide **2.6**

Figure 2.1.   Chemical structures of sulfonylurea agents used for diabetes.

2.3.3   Metformin

Metformin (glucophage) (**2.7**), a biguanide, is the major medicine used to treat type 2 diabetes and is considered as significant for the control of the disease. Metformin was mainly developed and isolated from *Galega officinalis* L., (Fabaceae) a plant that was traditionally used in Europe as a diabetic drug.[21] This drug decreases the amount of glucose released by the liver, which in turn lowers the sugar levels in the blood.[34] It is used to treat insulin resistance associated with prediabetes, type 2 diabetes, and polycystic ovarian

syndrome. Metformin comes in tablet and in liquid form and the effective dose is 500 mg/day, with an optimum dose of 2 000 mg/day. However, as with all medicines, there are common side effects such as upset stomach, bloating, gas, diarrhoea, decreased appetite, lower back or side pain and heartburn.
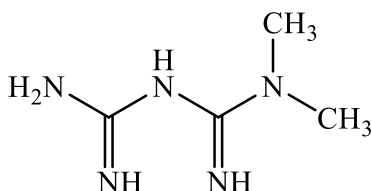
Metformin **2.7**

2.3.4   Thiazolidinediones (TZDs)

Thiazolidinediones (Fig. 2.2), also called glitazones, were first introduced in 1996 for the treatment of type 2 diabetes.[35] The thiazolidinediones are a class of oral antidiabetic drugs that enhance metabolic processes in type 2 diabetes through the enhancement of insulin sensitivity.[34] Although thiazolidinediones could be beneficial for the full insulin resistance syndrome, some of its members are known for their adverse side effects. For instance, troglitazone **(2.8)** was withdrawn from the market in 2000 due to idiosyncratic hepatoxicity, while pioglitazone **(2.9)** and rosiglitazone **(2.10)** continue to be widely used. These glitazones were found to be safe and is the most effective pharmacologic treatment for insulin resistance.[35]
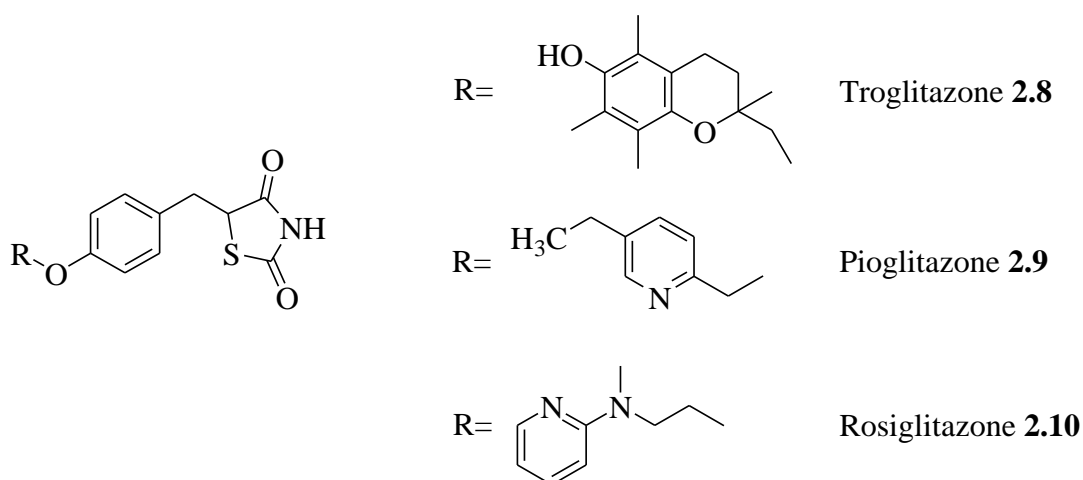
Figure 2.2.   Chemical structures of thiazolidinedione (TZDs) agents used for diabetes.

2.3.5 Meglitinides (glinides)

There is a similarity between meglitinides (Fig. 2.3) and sulfonylureas in terms of action but with a few major differences. Meglitinides are relatively new drugs compared to sulfonylureas. Repaglinide (**2.11**) and nateglinide (**2.12**) were the first and second agents approved by the USFDA in 1997 and 2000, respectively. Meglitinide medication works by stimulating insulin release from the pancreas, which in turn lowers blood glucose levels.[36] There are two meglitinide drugs in the United States; repaglinide (Prandin)® and nateglinide (Starlix)® can be used alone or in a combination with other medications for type 2 diabetes. The meglitinides can cause hypoglycaemia but at a rate lower than sulfonylureas.[36] Other reported side effects of meglitinides agents are feeling shaky, sweaty, hungry, and irritable.

Repaglinide **2.11**   Nateglinide **2.12**
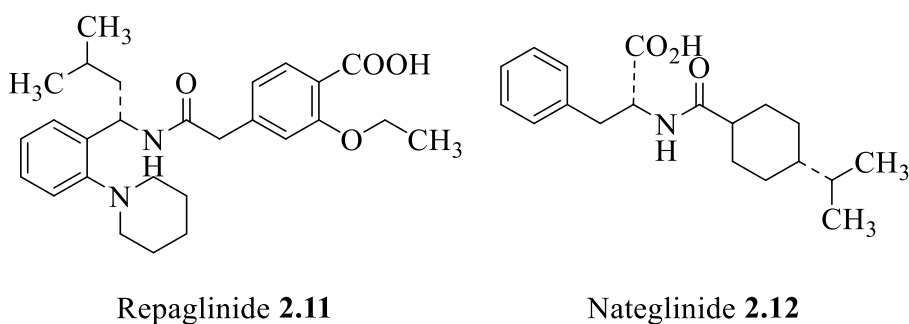
Figure 2.3.   Chemical structures of meglitinide agents used for type 2 diabetes.

2.3.6 α-Glucosidase Inhibitors

α-Glucosidase inhibitors (Fig. 2.4) are commonly used to treat patients with type 2 diabetes and have positive effects on glycaemic control but not on plasma lipids.[37] α-Glucosidase inhibiting agents slow the absorption of particular carbohydrates from the small intestine which in turn lowers the effect on postprandial blood glucose and consequently the insulin levels. Acarbose (**2.13**), voglibose (**2.14**), and miglitol (**2.15**) are α-glucosidase drugs available for therapy. Acarbose and voglibose are effective and safe drugs to be used in type 2 diabetes patients.[38]

Acarbose **2.13**   Voglibose **2.14**   Miglitol **2.15**
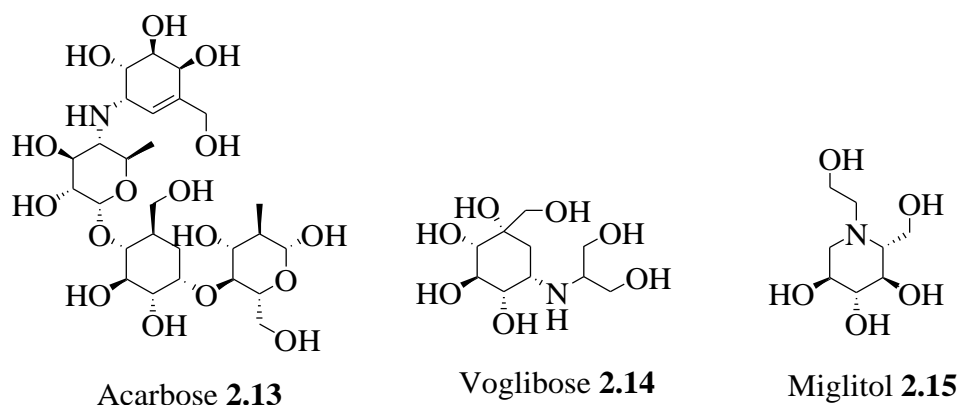
Figure 2.4.   Chemical structures of α-glucosidase inhibitors used for type 2 diabetes.

2.3.7 Glucagon-like Peptide (GLP)-1 and Dipeptidyl Peptidase 4 (DPP-4) Inhibitors

Incretin mimetics are a relatively new class of pharmacological agents for treatment of type 2 diabetes.[39] These drugs work by increasing the incretin levels, which is a group of intestinal hormones that are excreted from enteroendocrine cells into the blood within 10-15 minutes after eating.[40] These hormones increase the insulin production required by the body and lessen released liver glucose when it is not necessary.[41] In regards to incretin systems, two types of drugs have been modified: glucagon-like peptide (GLP)-1 receptor agonists (e.g. liraglutide (**2.16**) and exenatide (**2.17**)) and dipeptidyl peptidase 4 (DPP-4) inhibitors (e.g. sitagliptin (**2.18**), vildagliptin (**2.19**), saxagliptin (**2.20**)) for treating type 2 diabetes (Fig. 2.5). Long-term use of GLP-1 receptor agonists are increasing glycaemic control in patients with type 2 diabetes with a very low risk of hypoglycaemia. Nausea is a common side effect.[42]

H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-AlaLys(γ-Glu-palmitoyl)-Glu-Phe-Lle-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

Liraglutide **2.16**

H-His-Gly-Glu-Gly-xiThr-Phe-xiThr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Ala-Val-Arg-Leu-Phe-xille-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH$_2$

Exenatide **2.17**

Sitagliptin **2.18**   Vildagliptin **2.19**

Saxagliptin **2.20**
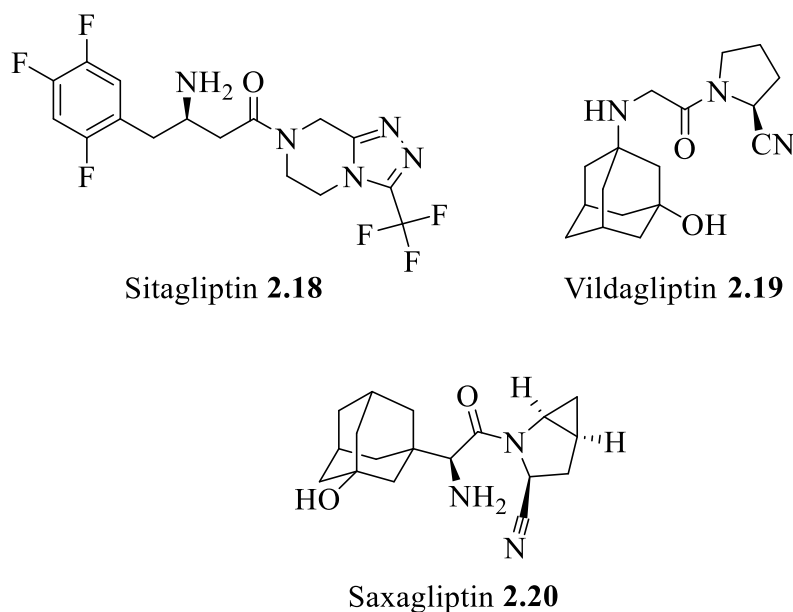
Figure 2.5.   Chemical structures of GLP-1 and DPP-4 agents used for type 2 diabetes.

2.3.8   Amylin Agonists

Amylin, an islet amyloid polypeptide, or IAPP, is a 37-amino acid peptide hormone. It is co-stored and co-secreted with insulin and uses similar processing enzymes.[43] Adults with type 1 diabetes have no amylin, while those with type 2 diabetes have a relative deficiency in later stages of the disease.[44] Since the insoluble amylin is toxic to pancreatic beta cells, the discovery of a soluble amylin agonist was considered as a new diabetes medication. Pramlintide **(2.21)** (Fig. 2.6) is a synthetic analogue of amylin and it was approved by the USFDA in 2005 as an adjunct to preprandial insulin therapy. Nausea is the most common side effect.[31]

Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Pro-lle-Leu-Pro-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH_2

Pramlintide **2.21**

Figure 2.6.   Chemical structure of pramlintide.

2.3.9 Bromocriptine

Bromocriptine (Cycloset[®]) (**2.22**), a sympatholytic D2-dopamine receptor agonist, has been approved to treat patients with type 2 diabetes (Fig. 2.7).[45] Yet, its mechanism is not clear but based on some studies, it is believed that bromocriptine can increase dopamine activity in the brain. Since it is not possible to sample the human brain, all the hypotheses on the bromocriptine mechanism of action have been derived from animal experiments. Bromocriptine causes a reduction in glycated haemoglobin and lessens plasma triglyceride and the concentration of free fatty acids, whether it was used as monotherapy or in combination with other hypoglycaemic agents.[45] The benefits of Cycloset[®] include the absence of hypoglycaemia and weight neutrality.
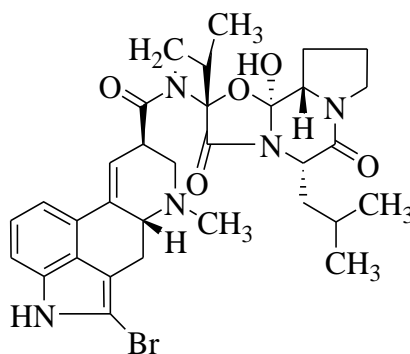
Bromocriptine **2.22**

Figure 2.7.　　Chemical structure of bromocriptine.

2.3.10 Colesevelam

Colesevelam hydrochloride (**2.23**) is a polymer formed from allylamine which was crosslinked with epichlorohydrin and alkylated with 1-bromodecane and (6-bromohexyl) trimethylammonium bromide and has an approximate molecular weight of 10^{14} Dalton (Fig. 2.8). It is a lipid-lowering agent and is used to reduce elevated low-density lipoprotein cholesterol in patients.[46] However, it was also approved in 2008 by the USFDA to treat type 2 diabetes.[31] Results from a clinical trial suggest that the antidiabetic mechanism of colesevelam involves an increase in incretin secretion and improved β-cell function, but gluconeogenesis or glucose absorption are not affected.[47]
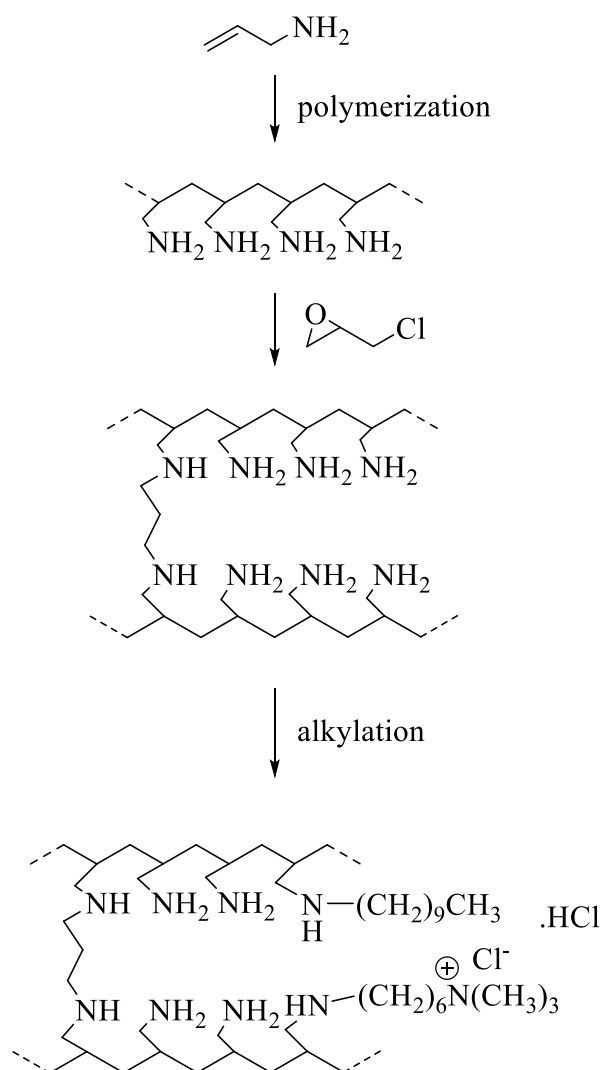
Colesevelam hydrochloride **2.23**

Figure 2.8.   Formation of colesvelam hydrochloride.

To conclude, there are many types and classes of available medication for the treatment of diabetes mellitus. The main goal of all these medications is to adjust blood glucose levels. These medications achieve this goal in different ways. In some cases, there is a need to combine more than one type of these drugs to target multiple processes that could possibly affect glucose levels in the blood. However, the major disadvantages of these drugs are the side effects, especially in the long term and the cost of the treatments. There is an opportunity to develop natural products in a scientific way to discover new active compounds that have less or no side effects and at the same time are affordable to people worldwide living under the poverty threshold.

2.4 MEDICINAL PLANTS USED FOR TREATMENT OF DIABETES

2.4.1 Introduction

Natural products from different sources, especially from plants, have long been used as remedies for human diseases and it will always remain a potential source of future drugs.[48] Many of the current drugs have been derived directly or indirectly from plant sources. In this context, out of 250 000 plants, approximately 1% have been tested pharmacologically and a much smaller number assayed against diabetes mellitus.[49] Reports on ethnobotany indicate that there are at least 800 plant species that could have antidiabetic potential. Amongst these plants, *Pterocarpus marsupium* (Fabaceae), *Momordica charantia* (Cucurbitaceae), and *Trigonella foenum greacum* (Fabaceae) are found to be beneficial for the treatment of type 2 diabetes.[50]

Recently, a review article considered all of the *in vivo* antidiabetic studies conducted between January 2000 to July 2013 on African plants and reported that the most investigated plant families are Asteraceae and Lamiaceae.[51] Moreover, while many studies reported promising findings, in only a few investigations the bioactive components have been identified and characterized and their mechanisms of action investigated.[51] In the next paragraph, the active principles of South African plants with antidiabetic properties are discussed.

2.4.2 Medicinal plants with antidiabetic properties used in South Africa

Plants with antidiabetic properties in South Africa have been extensively reviewed in the last decade.[51,52,53] Many plant species such as *Vernonia amygdalina* Del, *Leonotis leonurus* L. *(*Lamiaceae*)*, *Aloe ferox*, *Aloe greatheadii*, and *Brachylaena discolor* have been tested for antidiabetic properties by investigating the bioactivity in the extracts of the leaves, flowers, roots, and sometimes the whole plant.[54,55,56] Table 2.2 summarized the available literature of medicinal plants with antidiabetic properties used in South Africa.

Table 2.2. The *in vitro* and *in vivo* antidiabetic effects of some South African medicinal plants and the identification of the bioactive compounds.

Species	Plant part	Antidiabetic assay	Extracts and active compounds	Ref.
Adansonia digitata L. (Bombacaceae)	Stem bark, fruit pulp	STZ-induced diabetic rats	Methanol extract	[57]
Ajuga iva L. Schreberr (Medit) (Lamiaceae)	Whole plant	Normal and STZ-induced diabetic rats	Lyophilized aqueous extract	[54,58]
Aloe vera (L.) Burm.f. (Asphodelaceae)	leaves	STZ-induced diabetic rats	Chloroform and methanol extracts. Cycloartenol (**2.24**), 24-methylene-cycloartanol (**2.25**), lophenol (**2.26**), 24-methyllopenol (**2.27**), and 24-ethyllophenol (**2.28**)	[59]
Aloe ferox Mill. *Aloe greatheadii var. davyanan* (Asphodelaceae)	leaves	STZ-induced diabetic rats	Ethanol extract	[60]
Annona muricata L. (Annonaceae),	Leaves	STZ-induced diabetic rats	Aqueous extract	[61]
Artemisia afra Jacq.ex Willd. (Asteraceae)	Leaves roots	STZ-induced diabetic Wistar rats	Aqueous extract	[62]
Aspalathus linearis (Burm.f) R. Dahlgren	Shoots	insulin resistant cells line	Hot water extract	[63,64, 65,66]

(Fabaceae)		(C2C12 and KK-A^y)	β-Z-2-(-D-glucopyranosyloxy)-3-phenylpropenoic acid (**2.29**), 2-oxo-3-phenylpropanoic acid (**2.30**), aspalathin (**2.31**), nothofagin (**2.32**), rutin (**2.33**)	
Brachylaena discolor D.C. (Asteraceae)	Leaves	STZ-induced diabetic Wistar rats	Methanol extract.	56
Bulbine frutescens (L.) Willd. (Asphodelaceae)	Whole plant	Chang liver cells, C2C12 muscle cells	Aqueous and ethanol extracts	67
Catha edulis Forsk. (Celastraceae)	Leave, stems, roots	Patients with type 2 diabetic	Organic and aqueous extracts	52
Clausena anisata (Willd.) Hook. (Rutaceae)	Roots	Wistar rats (STZ)	Methanolic extract	68
Cyclopia intermedia (L) R.Br. (Fabaceae)	Shoots	STZ-induced diabetic rats	Hot water extract. Mangiferin (**2.34**), isomangiferin (**2.35**), eriodictyol glucoside (**2.36**), eriocitrin (**2.37**), hesperidin (**2.38**), luteolin (**2.39**), hesperetin (**2.40**)	69

Cyclopia maculata (Andrews) Kies *Cyclopia subternata* Vogel (Fabaceae)	The stems, leaves flowers	3T3-L1 cells	Hot water extracts vicenin-2 (**2.41**), scolymoside (**2.42**)	70,71
Elaeodendron transvaalense (Burtt Davy) R.H. Archer (Celastraceae)	Stem bark	Chang liver cells, 3T3-L1 preadipocytes, murine C2C12 myoblasts, α-glucosidase α-amylase assays	Acetone and ethanol extracts	72
Euclea undulata Thunb. (Ebenaceae)	Root bark	Chang liver cells, 3T3-L1 preadipocytes, murine C2C12 myoblasts, α-glucosidase α-amylase assays, Wistar rats	Acetone and ethanol extracts. α-amyrin3-*O*-ß-(5-hydroxyferulate) (**2.43**), betulin (**2.44**), lupeol (**2.45**), epicatechin (**2.46**)	72-74
Hypoxis hemerocallidea Fisch., C.A. Mey & Ave-Lall. (Hypoxidaceae)	Corms	STZ-induced diabetic rats	Aqueous extracts	75
Leonotis leonurus L. R. Br. (Lamiaceae)	Leave, flowers	STZ-induced diabetic rats	Aqueous, acetone and methanol extracts, premarrubiin (**2.47**), marrubiin (**2.48**)	76,77, 78

Momordica charantia Linn (Cucurbitaceae)	whole plants	STZ-induced diabetic Wistar rats	Aqueous extract sitosteryl glucoside (**2.49**), stigmasteryl glucoside (**2.50**), vicine (**2.51**) polypeptide-p	[79,80]
Musa sp. var. Nanjangud rasa bale (Musaceae)	Flowers	α-glucosidase and pancreatic amylase	Ethanol extract umbelliferone (**2.52**), lupeol (**2.45**)	[81]
Ornithogalum longibracteatum L. (Hyacinthaceae)	Bulb	C2C12 muscle cells	Aqueous and ethanol extracts	[67]
Pappea capensis L. (Sapindaceae)	Leaves, stem bark	Alloxan-induced diabetic	Aqueous and ethyl acetate extracts	[82]
Psidium guajava L. (Myrtaceae)	Leaves, toots	Alloxan and STZ-induced diabetic rats	Organic and aqueous extracts guiajaverin (**2.53**), quercetrin (**2.54**) ellagic acid (**2.55**)	[52,83]
Schkuhria pinnata (Lam.) Kuntze ex Thell. (Asteraceae)		Chang liver cells, 3T3-L1 preadipocytes, murine C2C12 myoblas, α-glucosidase α-amylase assays	Acetone and ethanol extracts	[72]

Sclerocarya birrea A. Rich. Hochst. (Anacardiaceae)	Stems, barks, roots	Normal and STZ-induced diabetic rats C2C12, 3T3-L1 and HepG2 cells	Aqueous and organic extracts	[52,84-86]
Strychnos henningsii (SH) (Loganiaceae)	Stem bark	Chang liver and 3T3-L1 cell lines. STZ-induced diabetic rats	Aqueous extracts	[87-89]
Sutherlandia frutescens (L.) R. Br. (Fabaceae)	Leaves, shoots	Human liver cells	Aqueous extract. Pinitol (**2.56**)	[90,91]
Tarchonanthus camphoratus L. (Asteraceae)	Leaves and soft twigs	C2C12 muscle cells	Aqueous and ethanol extracts	[67]
Terminalia sericea Burch. ex DC. (Combretaceae)	Stem bark	α-glucosidase and α-amylase enzymes	Acetone extract β-sitosterol (**2.57**), β-sitosterol-3-acetate (**2.58**), lupeol (**2.45**), and stigma-4-ene-3-one (**2.59**)	[92]
Tulbaghia violacea Harv. (Alliaceae)	whole plants	C2C12 muscle cells	Aqueous and ethanol extracts	[67]
Vernonia amygdalina Del. (Asteraceae)	Leaves	C2C12 muscle and Chang-liver cells	Ethanol, acetone, methanol, aqueous, and *n*-hexane/ isopropanol extracts	[55,93]

Ziziphus mucronata Willd. subsp.mucronata (Rhamnaceae)	Bark	C2C12, 3T3-L1 and HepG2 cells	Aqueous and methanol extracts	85

Aloe vera (L.) Burm.f. (Asphodelaceae) (synonym *A. barbadensis*) is an African plant that has been used traditionally to treat diabetes.[94] In order to confirm the antidiabetic properties of the plant, Tanaka et al.[59] evaluated its antihyperglycaemic effect and isolated five active compounds. These compounds were identified as cycloartanol **(2.24)**, 24-methylenecycloartanol **(2.25)**, lophenol **(2.26)**, 24-methyllopenol **(2.27)**, and 24-ethyllophenol **(2.28)** (Fig. 2.8). These compounds showed a significant reduction in blood glucose levels by as much as 64% in some cases confirming its potential in diabetes treatment.

Cycloartanol **(2.24)**: R = H

24-Methylenecycloartanol **(2.25)**: R+R =CH$_2$

Lophenol **(2.26)**: R = H

24-Methylenelophenol **(2.27)**: R+R=CH$_2$

24-Ethylenelophenol **(2.28):** R+R = CHCH$_3$
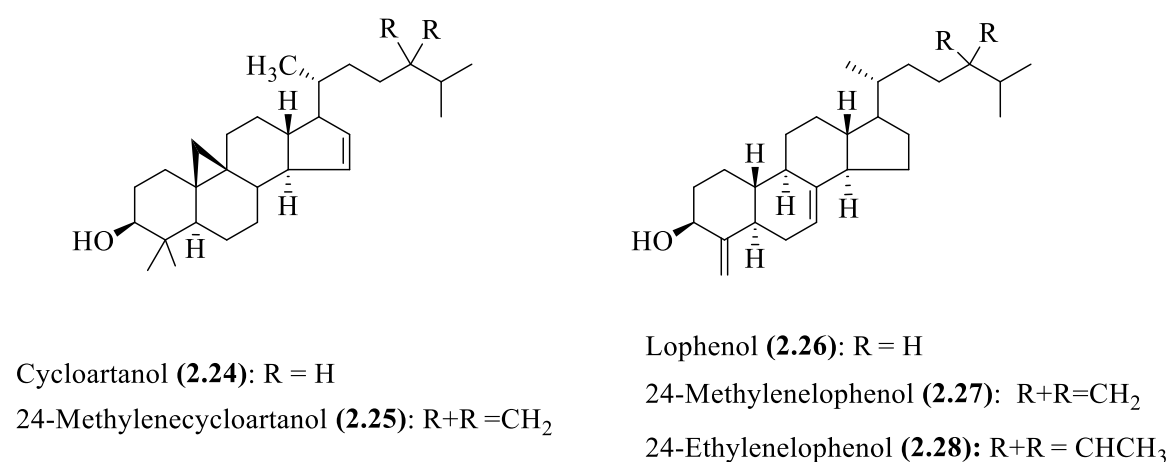
Figure 2.9   Chemical structures of antidiabetic compounds isolated from *Aloe barbadensis* Mill.

Aspalathus linearis (Fabaceae) is an indigenous South African plant, commonly known as rooibos. Rooibos herbal tea has become popular worldwide.[95] An investigation of rooibos shoots extract (rooibos tea) on streptozotocin-induced diabetic rats demonstrate a hypoglycaemic effect by reducing blood glucose in diabetic rats over a 6 hour period.[64] These findings led to other studies in which the active compounds of *A. linearis* were identified as *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid **(2.29)** and 2-oxo-3-phenylpropanoic acid **(2.30)** (Fig .2.10). These two compounds isolated from *A. linearis* increase the *in vitro* glucose uptake in Chang liver cells and improve glucose tolerance in

an insulin-resistant rat model.[63] Other active compounds, such as aspalathin (**2.31**), nothofagin (**2.32**), and rutin (**2.33**) (Fig. 2.10), which showed potential as new agents that can contribute to diabetes therapy, were also isolated from *A. linearis*.[64,65] Recently, it was reported that aspalathin (**2.31**) isolated from green rooibos extracts promoted the uptake of glucose in cells but that the effect was less than the effect of the crude extract.[66]

PPAG **(2.29)**

PPA **(2.30)**

Aspalathin **(2.31)**   R= OH

Nothofagin **(2.32)** R= H

Rutin **(2.33)**
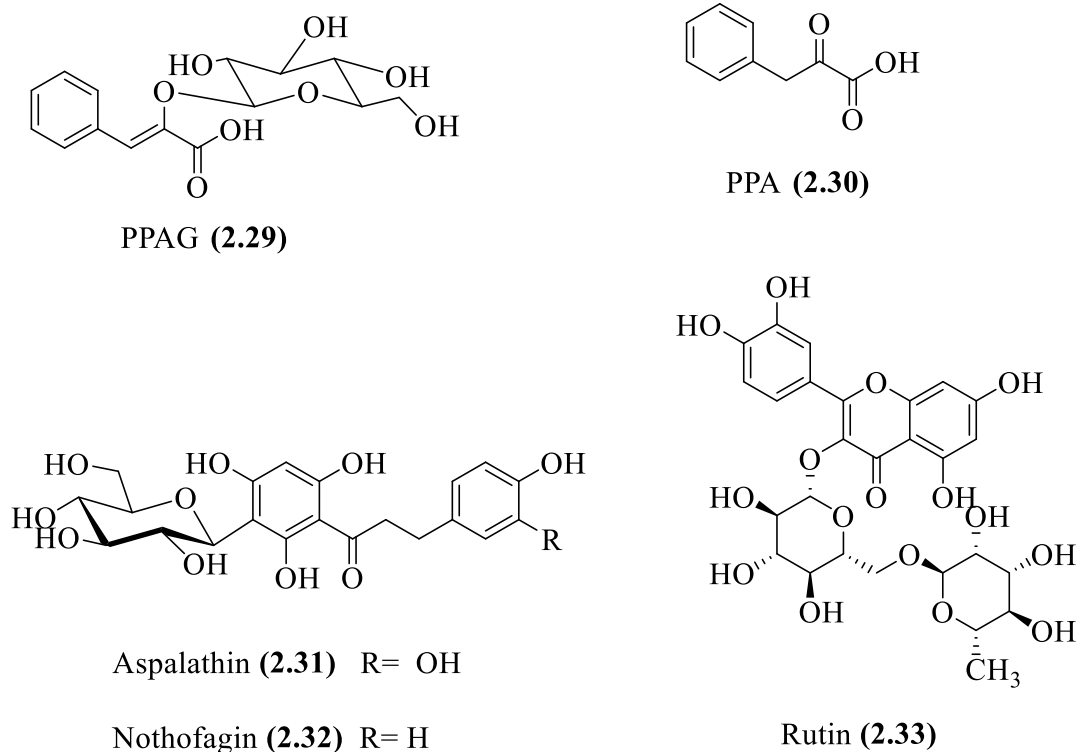
Figure 2.10   Chemical structures of antidiabetic compounds isolated from *Aspalathus linearis.*

Cyclopia species are native South African plants and *Cyclopia intermedia* (Fabaceae) is commonly known as honeybush tea. The honeybush tea prepared from *C. intermedia* has no caffeine, little tannin and other health benefits including antidiabetic properties.[96] The hot water extract of honeybush was found to be effective in reducing blood glucose levels in induced diabetic rat models. The antidiabetic properties were mainly attributed to the presence of phenolic compounds **2.34**-**2.40** (Fig. 2.11).[69]

Mangiferin **(2.34)**
(R^1= H, R^2 =C-β-D-glucoside)
Isomangiferin **(2.35)**
(R^1 =C-β-D-glucoside,R^2 =H)

Eriodictyol-glucoside **(2.36)**
(R^1, $R^{2,}$ R^3, R^4 = OH; O- or
C-β-D-glucosyl in unknown position)
Eriocitrin **(2.37)**
(R^1 = O-β-D-glucosyl; R^2=R^3=R^4= OH)
Hesperidin **(2.38)**
(R^1 = O-β-D-glucosyl; R^2=R^4 = OH;
R^3 = OCH$_3$)
Hesperetin **(2.40)**
(R^1=R^2=R^4 = OH; R^3 = OCH$_3$)

Luteolin **(2.39)**
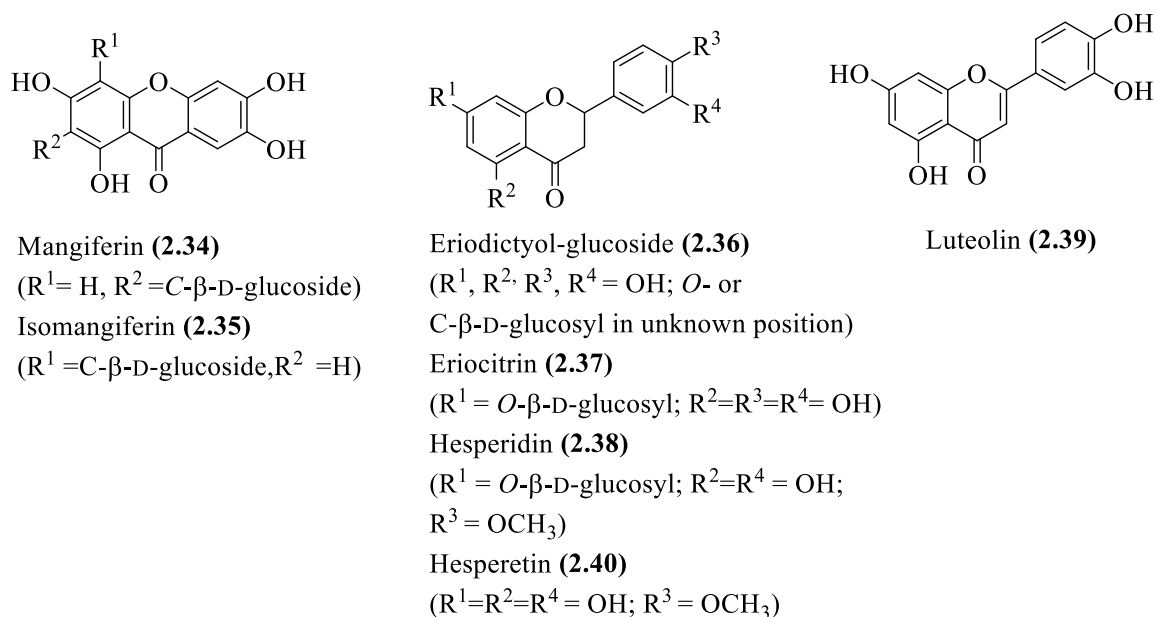
Figure 2.11 Chemical structures of phenolic compounds isolated from *Cyclopia intermedia*

Other *Cyclopia* species such as *C. maculata* and *C. subternata* were also investigated to evaluate their potential to treat different diseases such as anti-obesity and antidiabetic properties.[70,71] It is known that diabetes is associated with atherosclerotic and inflammatory diseases leading to cardiovascular complications.[97] Ku and Bae[70] showed that the flavonoids vicenin-2 **(2.41)** and scolymoside **(2.42)** (Fig. 2.12), reduced high-glucose-induced vascular inflammatory diseases in human endothelial cells and in mice. Therefore, honeybush may be able to diminish atherosclerosis as a complication of diabetes.

Vicenin-2 **(2.41)**

Scolymoside **(2.42)**
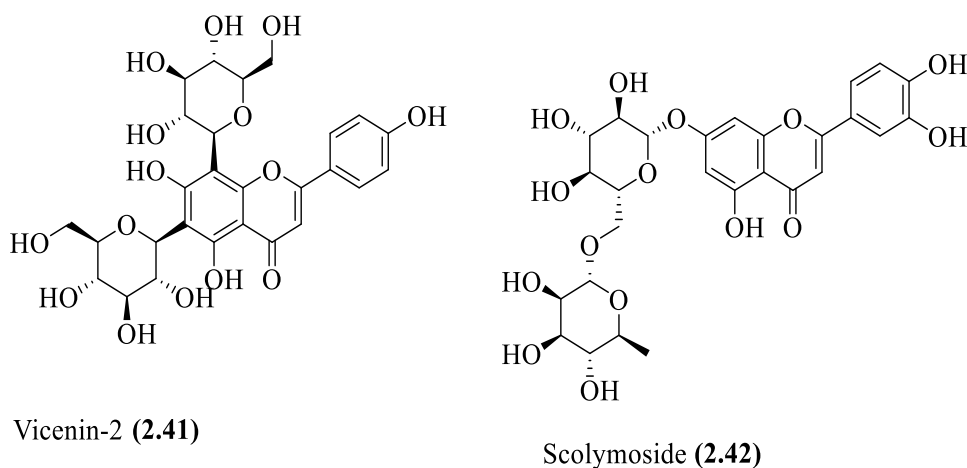
Figure 2.12.   Chemical structures of antidiabetic compounds isolated from *Cyclopia subternata*

Deutschländer et al.[72] investigated the hypoglycaemic activity of *Euclea undulata* Thunb. (Ebenaceae), a plant traditionally used to treat diabetes by South African healers. The screening of the acetone and ethanol extracts of the root and stem bark scientifically confirmed the traditional use of *E. undulata* for treatment of diabetes. Further investigations by Deutschländer et al.[74] yielded four antidiabetec compounds. Amyrin 3-*O*-β-(5-hydroxyferulate) (**2.43**), betulin (**2.44**), lupeol (**2.45**), and epicatechin (**2.46**) were isolated from a crude acetone extract of the root bark of *E. undulata* var. *myrtina* (Fig. 2.13).

α-Amyrin 3-*O*-ß-(5-hydroxyferulate) (**2.43**)   Betulin (**2.44**)

Lupeol (**2.45**)   Epicatechin (**2.46**)
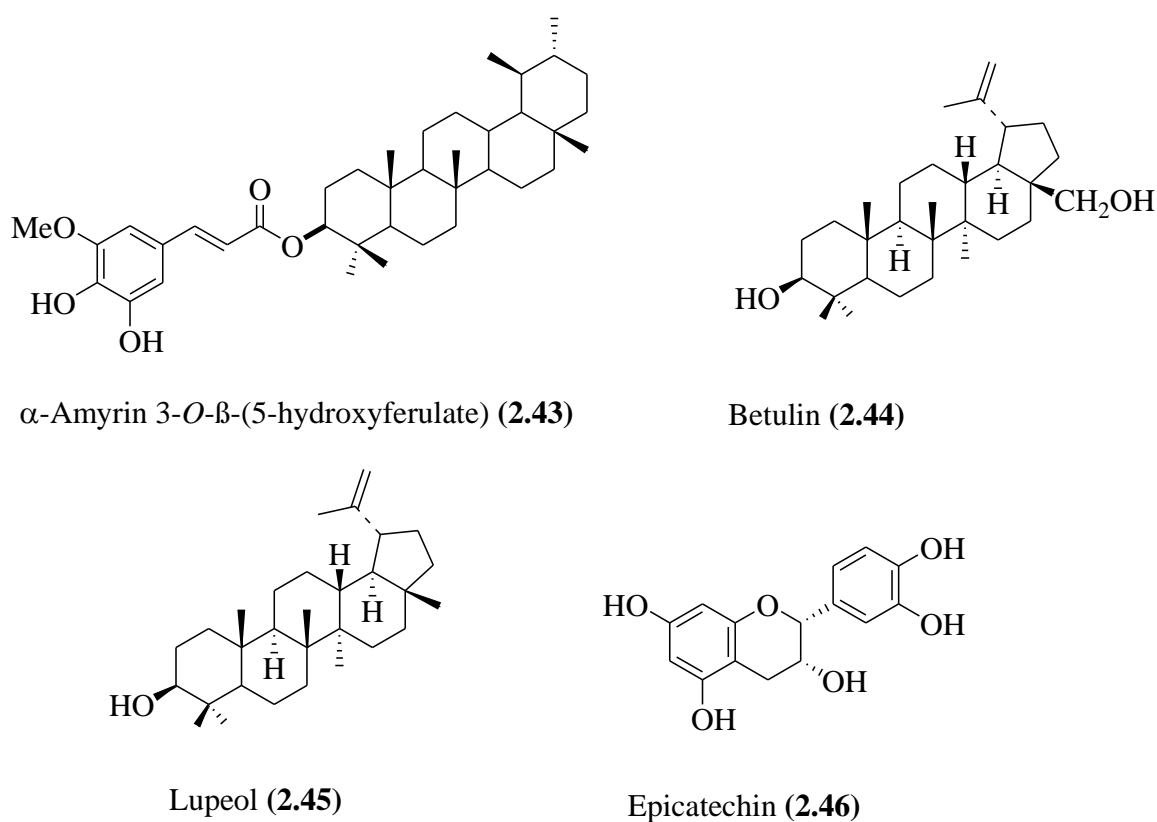
Figure 2.13.   Chemical structures of antidiabetic compounds isolated from *Euclea undulata*

Leaves and flowers of *Leonotis leonurus* L. (Lamiaceae) were also used traditionally for the treatment of diabetes and hypertension in South Africa.[105,106] The aqueous extract of the leaves showed hypoglycaemic effects in streptozotocin-induced diabetes in mice, by reducing the blood glucose.[106] Antidiabetic activities of *L. leonurus* were attributed to the different flavonoids, diterpenoids, and polyphenolics (Table 2.1).[107] Some of the isolated chemical components from *L. leonurus* are shown in Fig 2.14.
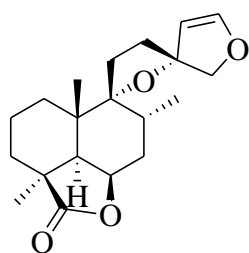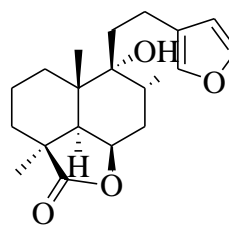
Premarrubiin **(2.47)**   Marrubiin **(2.48)**

Figure 2.14.   Chemical structures of antidiabetic compounds isolated from *Leonotis leonurus*.

Momordica charantia L., known as bitter gourd, was used traditionally by native people of Asia, America, India, the Caribbean islands, and Africa to treat diabetes. *M. charantia* was subjected to several animal model and biochemical studies to prove the antidiabetic effects that were known to the traditional healers. The isolated compounds such as sitosteryl glucoside **(2.49)**, stigmasteryl glucoside **(2.50)**, vicine **(2.51)** (Fig. 2.15), and polypeptide-p are known to decrease fasting blood glucose levels in both type 1 and 2 diabeties.[98,80]
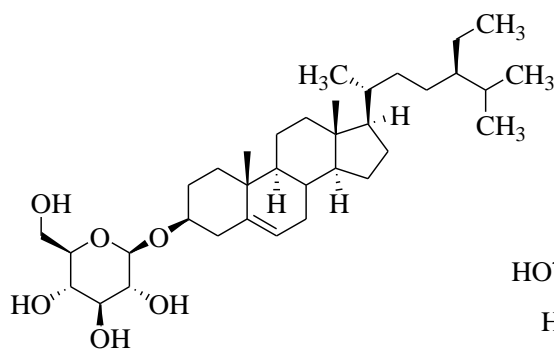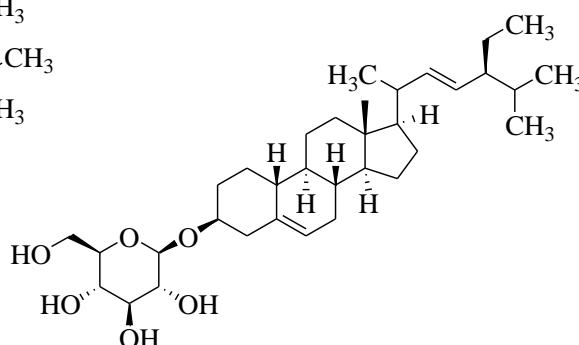
Sitosteryl glucoside **(2.49)**   Sigmasteryl glucoside **(2.50)**
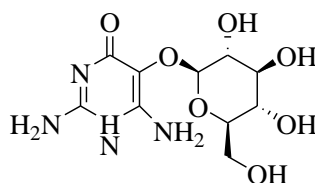
Vicine **(2.51)**

Figure 2.15.   Chemical structures of antidiabetic compounds isolated from *Momordica charantia*

Banana (*Musa sp*. var. Nanjangud rasa bale), is a common cultivated plant worldwide, mainly for the high nutritional value of its fruit. Banana flowers can be used as a vegetable

and it has been traditionally recommended by the Indian herbalists for diabetes.[99] An *in vivo* antidiabetic assessment of banana flowers in a hyperglycaemic rodent model by Ramu et al.[100] reported that banana flower extract and its constituents umbelliferone **(2.52)** and lupeol **(2.45)** (Fig. 2.16) enhanced the glycolysis reaction and significantly increased the utilization of glucose in diabetic rats.

Umbelliferone **(2.52)**   Lupeol **(2.45)**
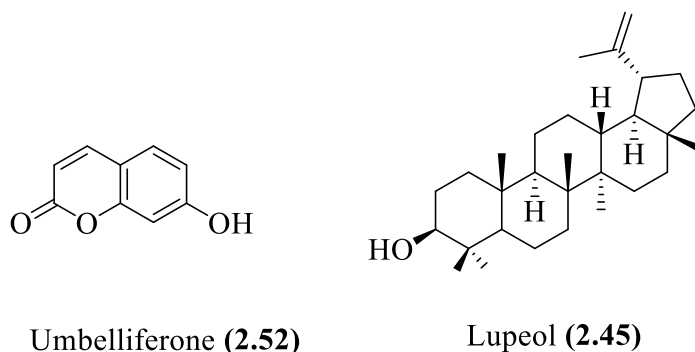
Figure 2.16.   Chemical structures of antidiabetic compounds isolated from *Musa* sp.

Psidium guajava L. *(Myrtaceae)* is not an indigenous South African plant, but it has been widely used, locally to treat diabetes. The hypoglycaemic activity of *P. guajava* organic extract of the leaves and the organic and aqueous extracts of the roots were found to be active in fat and muscle cells.[52] Another investigation of the hypoglycaemic and hypotensive effects of the aqueous extract of *P. guajava* leaves caused dose-related hypoglycaemia in normal and streptozotocin-induced diabetic rats. The hypoglycaemic effects of the aqueous extract of *P. guajava* leaves were speculated to be as a result of the presence of tannins, flavonoids, pentacyclic triterpenoids, guiajaverin **(2.53)**, quercetrin **(2.54)**, ellagic acid **(2.55)**, and other chemical compounds (Fig. 2.17).[83]

Guiajaverin **(2.53)**   R=L-arabinose
Quercetrin **(2.54)**   R=L-rhamnoside

Ellagic acid **(2.55**)
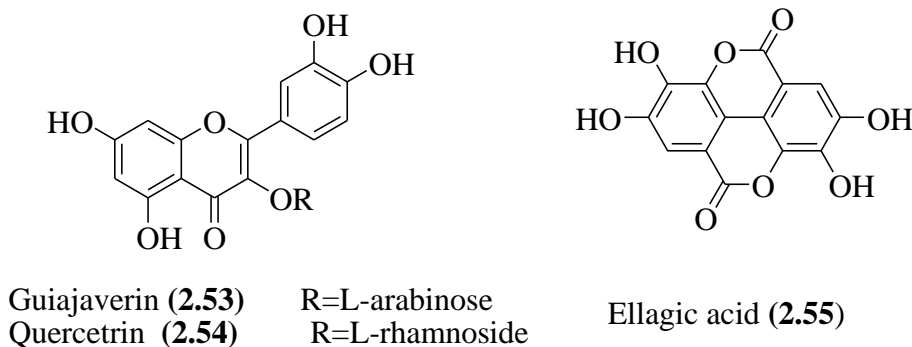
Figure 2.17.   Chemical structures of antidiabetic compounds isolated from

Psidium guajava

Sclerocarya birrea Rich. Hochst. (Anacardiaceae) is one of the common plants used traditionally to treat diabetes in South Africa. The stem-bark organic extract (dichloromethane:methanol, 1:1) of *S. birrea* was found to increase the glucose utilization in Chang liver and C2C12 muscle cells.[52] In support, Dimo et al.[101] reported that the organic extract of *S. birrea* bark decreased blood glucose and increased plasma insulin levels in streptozotocin rats. Different classes of chemical compounds have been isolated from *S. birrea* including flavonoids, alkaloids, triterpenoids, coumarins, ascorbic acid and amino acids.[84,102]

Sutherlandia frutescens (L.) R. Br. (Fabaceae) is commonly used by many cultural groups for different diseases such as peptic ulcer, cancer, heart failure, and diabetes.[90,91] *S. frutescens* aqueous extract was assayed in human liver cells and was found to prevent insulin resistance.[91] Chemical compounds isolated from *S. frutescens* include pinitol **(2.56)**, triterpenoid saponins, flavonoids, and amino acids.[90] Pinitol **(2.56)** is a well-known antidiabetic agent which has the same properties as insulin.[103] The chemical structure of pinitol **(2.56)** is shown in Fig. 2.18.
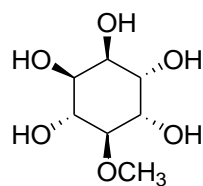
Pinitol **(2.56)**

Figure 2.18.   Chemical structure of an antidiabetic compound isolated from *Sutherlandia frutescens*

Terminalia sericea Burch.ex DC. (Combretaceae) is an indigenous southern African plant that has been widely used to treat different diseases and illness such as stomach problems, bilharzia, pneumonia, and diabetes.[104] The most common chemical constituents are stilbene glycosides and phenolic compounds. An acetone stem bark extract of *T. sericea* led to the isolation of four compounds, β-sitosterol **(2.57)**, β-sitosterol 3-acetate **(2.58)**, lupeol **(2.45)**, and stigma-4-en-3-one **(2.59)** (Fig. 2.19), which are known for their antidiabetic properties.[92]
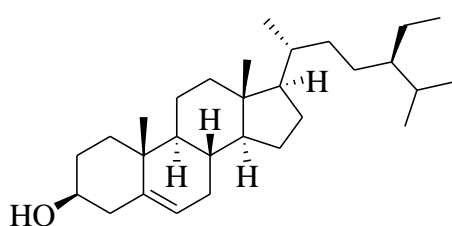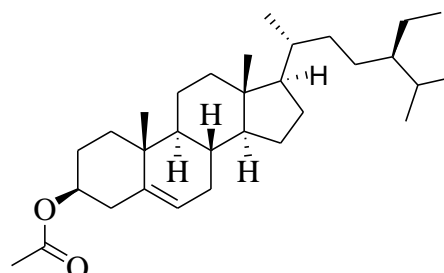
β-Sitosterol **(2.57)**

β-Sitosterol 3-acetate **(2.58)**
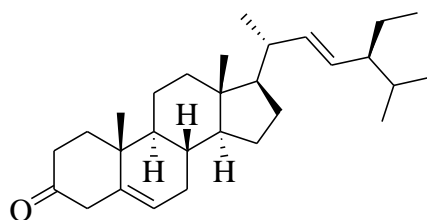
Stigma-4-en-3-one **(2.59**)

Figure 2.19.   Chemical structures of antidiabetic compounds isolated from *Terminalia sericea*.

A phytochemical analysis of the leave extract of *Vernonia amygdalina* Del. confirms the claimed ethnomedicinal uses of the leaves of *V. amygdalina* to treat different infectious diseases.[55] Erasto et al.[93] studied the effect of the leaf water extract of *V. amygdalina* on glucose utilization in C2C12 muscle, 3T3-L1 and Chang liver cells. Their results showed that the water extract significantly increased glucose utilization in Chang liver and C2C12 muscle cells, confirming the ethnomedical uses of *V. amygdalina* as an antidiabetic plant.

2.4   CONCLUSION

In conclusion, although large numbers of plants have been traditionally used to treat different diseases, only limited numbers of these plants have been scientifically investigated. In South Africa, while there are at least 73 plants traditionally used for diabetes treatments,[108] only a few plants were investigated for their antidiabetic activity (Table 2.2). The enhancement of the traditional experience to treat diabetes with original research could potentially discover a new safe and cheap drug.

CHAPTER 3: *BRACHYLAENA DISCOLOR:* TAXONOMY, PHYTOCHEMISTRY AND ANTIDIABETIC ACTIVITY

3.1   INTRODUCTION

For many centuries, several plant species from different families have been used traditionally for the management of the glucose level in human blood. Globally, about 800 plants are reported to have antidiabetic potential.[109] Out of these plants, only a few have been evaluated using sophisticated methods to investigate their efficacy in the treatment of diabetes. For example, some plants such as *Artemisia afra* Jacq. ex Willd., *Leonotis leonurus* (L.) R.Br. and *Catharanthus roseus* (L.) have been evaluated for their potential to treat diabetes in animals.[53] While research has been done on some plant species for their antidiabetic properties, little work was conducted on *Brachylaena discolor*. This study focuses on *B. discolor,* a member of the Asteraceae family.

3.2   AN OVERVIEW OF *BRACHYLAENA*

3.2.1   Asteraceae family

The Asteraceae is one of the major plant families, consisting of herbs, shrubs, and trees with more than 1620 genera and 23 600 species distributed worldwide.[110] The stems are usually vertical and in some species are prostrate to ascending. The leaves are generally alternate or opposite and sometimes in basal rosettes. The Asteraceae is commonly found in the arid and semiarid climate areas of the subtropics.[111] Asteraceae plants are a source of several active compounds such as essential oils, polyphenolic compounds, flavonoids, terpenoids, phenolic acids, alkaloids, lignans, saponins, stilbenes, sterols, and polysaccharides. Some species of this family are commonly used to treat different diseases like HIV and cancer.[112]

3.2.2   Phytochemistry of Asteraceae

Asteraceae is a plant family rich in chemicals that have biological activity and have potential for development of new drugs.[113] Sesquiterpene lactones are secondary

metabolites and a large class of natural compounds (over 5000 known compounds) that have been obtained from different plant families (e.g. Acanthaceae, Asteraceae, Anacardiaceae, Lauraceae, Apiaceae, Euphorbiaceae, Winteraceae, and Hepatideae).[114,115] According to Ohnishi et al.,[116] the highest number of sesquiterpene lactones were isolated from the Asteraceae, with about 3000 different structures.

3.2.3 *Brachylaena* genus

There are fifteen species/subspecies of *Brachylaena* R. Brown in eastern and southern Africa and Madagascar. In South Africa, there are nine species. The name *Brachylaena* is derived from the Greek words meaning shorter and cloak, which refers to the flowers being longer than the involucre.[117] Based on the pollen morphology, the southern African species could be categorized into two groups; group one includes *B. huillensis* O.Hoffm. and *B. ilicifolia* (Lam.) E.Phillips & Schweick., and group two includes *B. discolor* DC.*, B. elliptica* (Thunb.) DC.*, B. glabra* (L.f.) Druce*, B. neriifolia* (L.) R.Br., *B. rotundata* S.Moore, *B. transvaalensis* E.Phillips & Schweick., and *B. uniflora* Harv.[118,119]

Sesquiterpene lactones occur widely in the Asteraceae family and have also been isolated from *Brachylaena* species. Sesquiterpene lactones are mostly colourless lipophilic compounds.[120] Sesquiterpene lactones are terpenoids that naturally occur in plants and represent a large and unique chemical class of compounds which have a 15 carbon chain formed by three isoprene units with subsequent enzyme-mediated cyclization and oxidative transformation to produce *cis* or *trans*-fused lactones. The large number of sesquiterpenes can be attributed to the fact that one synthase may produce many sesquiterpene products.[121] Sesquiterpenes are further classified based on their carbocyclic skeletons into pseudoguaianolides (**3.1**), guaianolides (**3.2**), germanocranolides (**3.3**), eudesmanolides (**3.4**), heliangolides (**3.5**), and hyptocretenolides (**3.6**) (Fig. 3.1).[122]
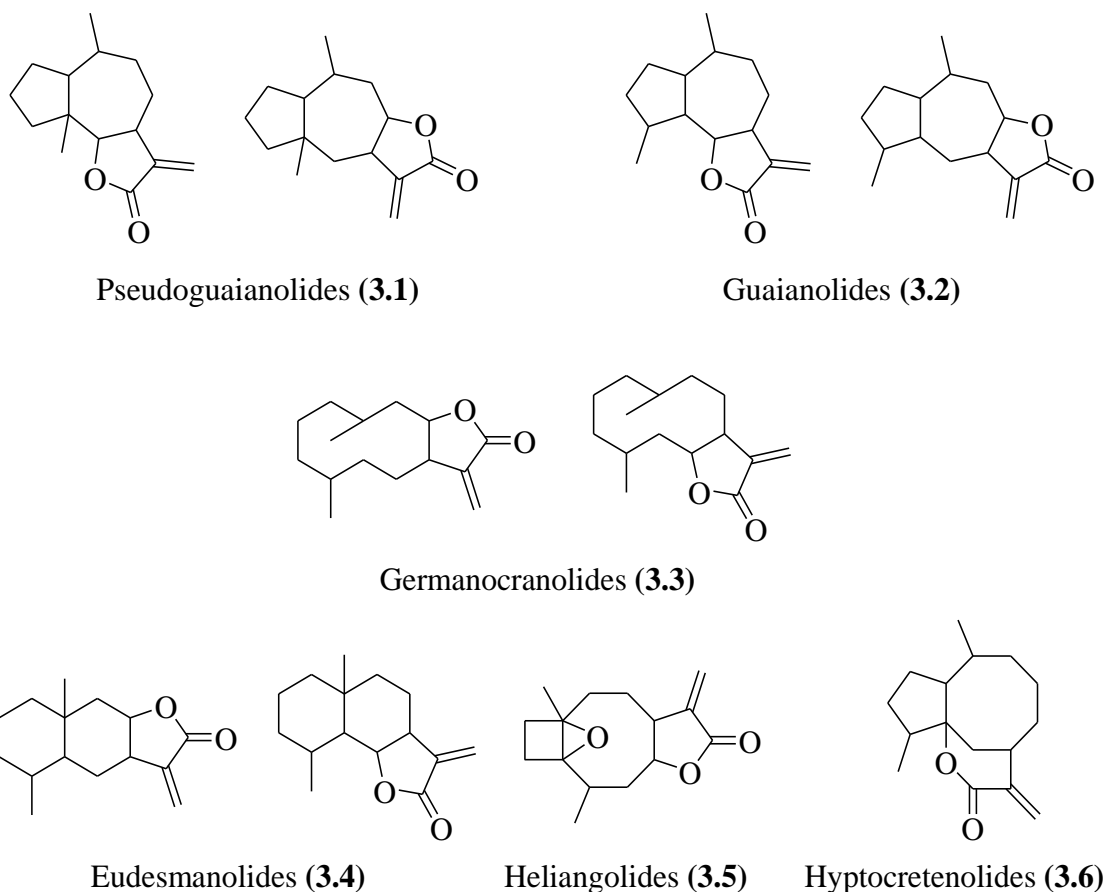
Figure 3.1.   The basic carbocyclic skeletons of different types of sesquiterpene lactones.

Health benefits of sesquiterpene lactones have been studied due to their various biological activities.[120,123] These studies focussed on the use of sesquiterpene lactones against a wide range of diseases. For example, some studies investigated their potential as anticancer, antitumour, anti-ulcer, anti-inflammatory, neurocytotoxic, and cardiotonic drugs.[124,120] Others studies focused on their uses as antimalarials, antimigraine activity and prevention of neurodegeneration, analgesic, and sedative activities and treating diarrhoea, flu, and burns.[125,120,126] The active sesquiterpene lactones isolated from different plants that showed potential to treat cancer, inflammation, and malaria will be briefly highlighted in the next section.

In general, 60% of all anticancer drugs were originally derived from natural products.[127] The structures of some sesquiterpenes lactones with *in* vitro cytotoxicity towards cancer cells are given in Fig. 3.2. Costunolide (3.7) and dehydrocostuslactone (3.8) are the most

well-known sesquiterpene lactones with anticancer properties. These compounds were isolated from plant species such as *Saussurea lappa* Decne and *Laurus nobilis* L.[128] The mechanisms of these compounds as anticancer agents were attributed to several pathways such as inhibition of growth of new cancer cells, angiogenesis, and reducing secondary malignancy. Their mechanisms also include differentional induction, regulation of new cancer cell cycle and reversal of the drug resistance of cancer cells.[128]

Arglabin **(3.9)**, another active compound that is classified as a guaianolide, was isolated from *Artemisia glabella* and was found to have potential for treatment of cancer-related malignancies such as breast, colon, ovarian, and lung cancers.[129] Micheliolide **(3.10)**, also a member of the guaianolides, was isolated from *Michelia compressa* and *Michelia champaca*.[130,131] Micheliolide **(3.10)** has shown high efficiency in treating leukaemia in mouse models. Other sesquiterpene lactones like parthenolide **(3.11)** and helenalin **(3.12)** isolated from different medicinal plants have anticancer and anti-inflammatory properties.

Costunolide **(3.7)**   Dehydrocostuslactone **(3.8)**

Arglabin **(3.9)**   Micheliolide **(3.10)**

Parthenolide **(3.11)**   Helenalin **(3.12)**
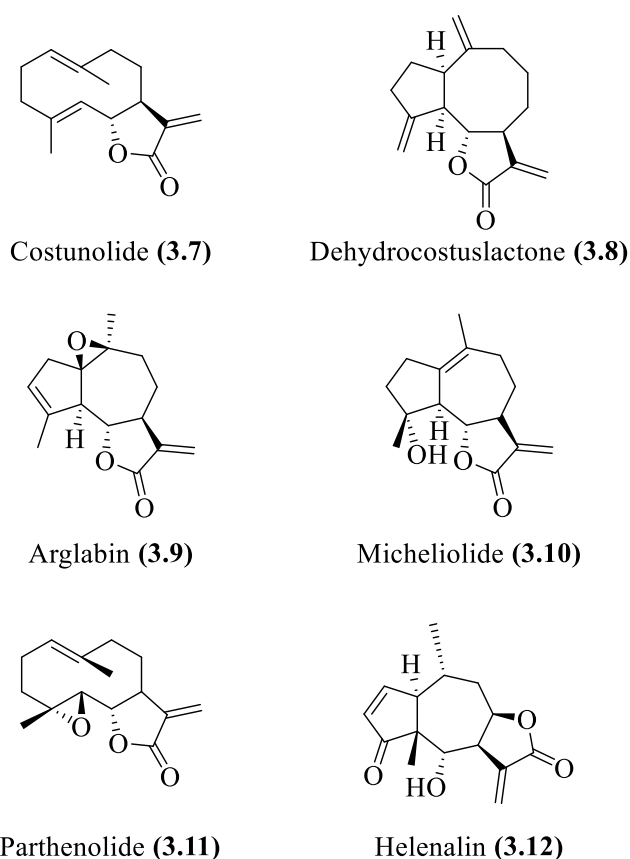
Figure 3.2.   Structures of sesquiterpene lactones with anticancer activity.

The structures of some sesquiterpene lactones with anti-inflammatory activity are given in Fig. 3.3. Parthenolide **(3.11)**, a sesquiterpene lactone isolated from *Tanacetum parthenium* (L.) Sch. Bip., a plant commonly known as "Feverfew", showed direct inhibition of pro-inflammatory enzymes such as phosphodiesterase-3, phosphodiesterase-4, and 5-lipoxygenase.[132] Helenalin **(3.12)** and dihydrohelenalin **(3.13)** were isolated from *A. montana* flower extracts and the proportions of each compound (helenalin **(3.12)** and dihydrohelenalin **(3.13)**) and their anti-inflammatory efficacy was reported to vary over the geographical range of *A. montana*.[133] Cynaropicrin **(3.14)** is a sesquiterpene lactone isolated from the roots of *Saussurea lappa*.[124] Cynaropicrin **(3.14)** was found to inhibit TNF-α and NO production, suggesting that the compound has anti-inflammatory properties. Finally, isoalantolactone **(3.15)** and alantolactone **(3.16)**, major bioactive ingredients found in many medicinal plants (e.g. *Inula racemo*se, *Inula helenium*, *Aucklandia lappa*, and *Inula japonica*), were found to have anti-inflammatory activity.[134,135]

Helenalin **(3.12)**

Dihydrohelenalin **(3.13)**   Cynaropicrin **(3.14)**

Isoalantolactone **(3.15)**   Alantolactone **(3.16)**
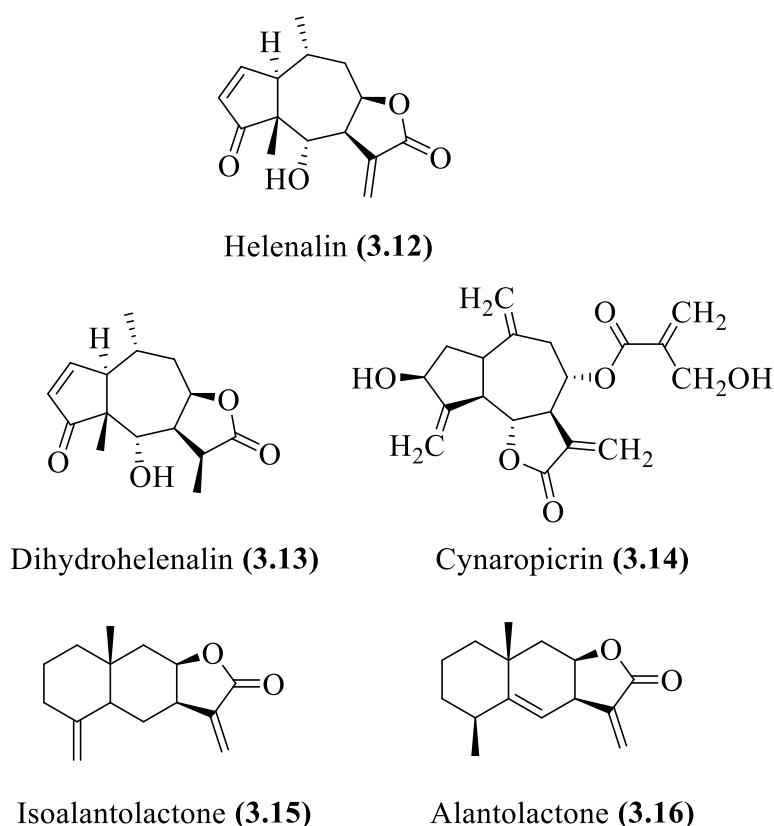
Figure 3.3.   Structures of sesquiterpene lactones with anti-inflammatory activity.

After the malaria parasite became resistant to the quinoline-based drugs (e.g. chloroquine and pyrimethamine), new antimalarial drugs needed to be developed. Artemisinin **(3.17)**, a natural product isolated from the leaves of *Artemisia annua* L. (Asteraceae), was found to be effective in killing malaria parasites.[136] The structures of some sesquiterpenoids with antimalarial activity are given in Fig. 3.4.

Three antimalarial active compounds, namely 8α-tigloyloxyhirsutinolide 13-*O*-acetate **(3.18)**, 8α-(4-hydroxymethacryloyloxy)hirsutinolide 13-*O*-acetate **(3.19)**, and vernolide D **(3.20)** were isolated from an extract of *Vernonia cinereal*.[125] Lactucin **(3.21)** and lactucopicrin **(3.22)** are also antimalarial compounds that were isolated from the roots of *Cichorium intybus* L.[137] Kraft et al.[138] isolated four sesquiterpenes (vernodalol **(3.23)**, dihydrovernodalin **(3.24)**, 11β,13-dihydrovernolide **(3.25)**, and 11β,13,17,18-tetrahydrovernolide **(3.26)**) from *Vernonia colorata.*
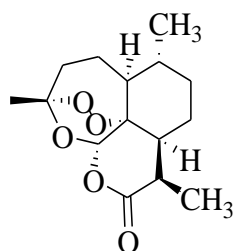
Artemisinin **(3.17)**
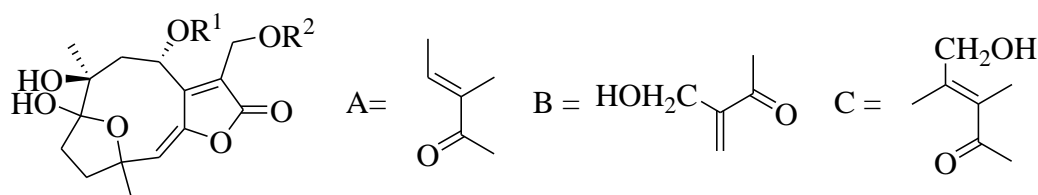
8α-Tigloyloxyhirsutinolide 13-*O*-acetate **(3.18)**: R^1=A, R^2=Ac
8α-(4-Hydroxymethacryloyloxy)hirsutinolide 13-*O*-acetate: **(3.19)** R=B, R^1=Ac
Vernolide D **(3.20)**: R^1=C, R^2=Ac

Lactucin (**3.21**)   Lactucopicrin (**3.22**)

Vernodalol (**3.23**)   Dihydrovernodalin (**3.24**)

11β,13-Dihydrovernolide (**3.25**) R=

11β,13,17,18-Tetrahydrovernolide (**3.26**) R=
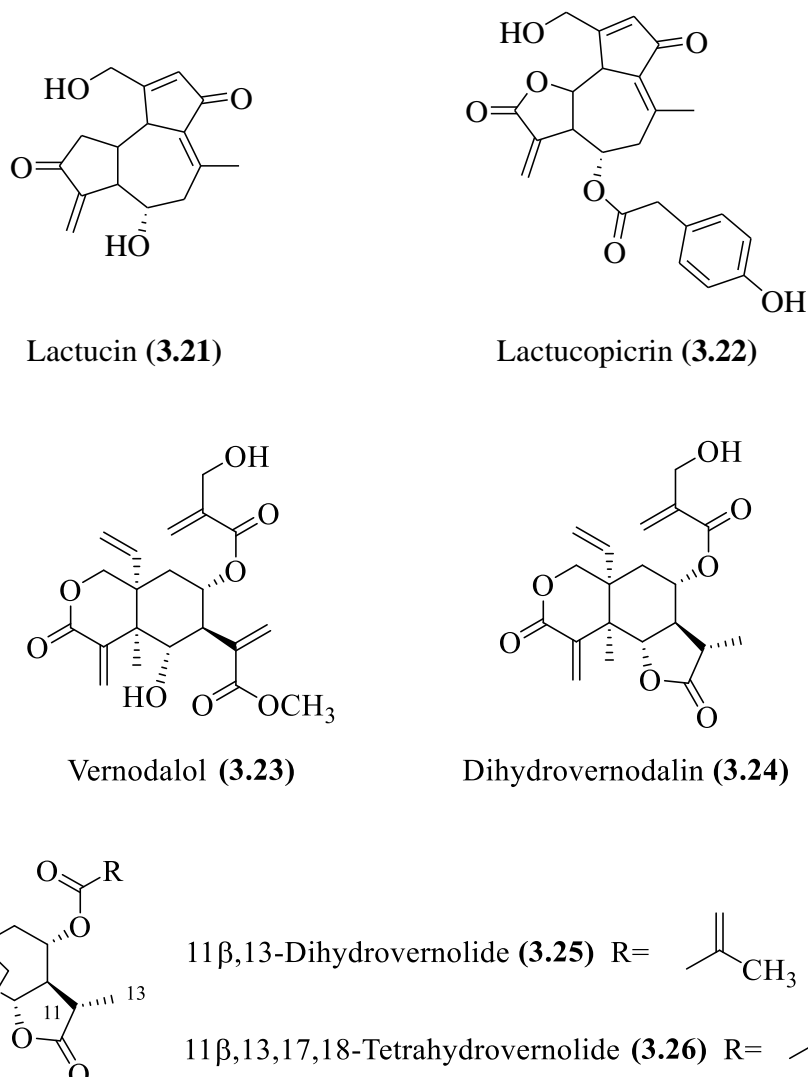
Figure 3.4.   Structures of sesquiterpene lactones with antimalarial activity.

Natural products provide a vital antiviral drug source and the mechanisms of these natural agents generally, is targeting the life cycle of the virus and the interactions between the virus and the specific host.[139,122] Some sesquiterpene lactones such as artemisinin (**3.17**) and its derivative artesunate (**3.27**), isolated from *Artemisia annua* L., showed good antiviral activity including the human cytomegalovirus, Herpesviridae family**,** hepatitis B & C virus and bovine diarrhoea virus.[139] Hwang et al.[140] tested several sesquiterpene lactones such as costunolide (**3.7**), parthenolide (**3.11**), helenalin (**3.12**), alantolactone (**3.16**), artemisinin (**3.17**), dehydrocostuslactone (**3.28**), epoxy-(4,5α)-4,5- dihydrosantonin (**3.29**), and 1,2-epoxy-1,2-dihydrosantonin (**3.30**) against hepatitis C virus. Their results showed that helenalin (**3.12**) was the best anti-hepatitis C viral compound. The structures of these antiviral compounds are given in Fig. 3.5.

Artesunate **(3.27)**　　　　　　　　Dehydrocostus lactone **(3.28)**

Epoxy-(4,5α)– 4,5-dihydrosantonin **(3.29)**　　1,2–epoxy-1,2-dihydrosantonin **(3.30)**
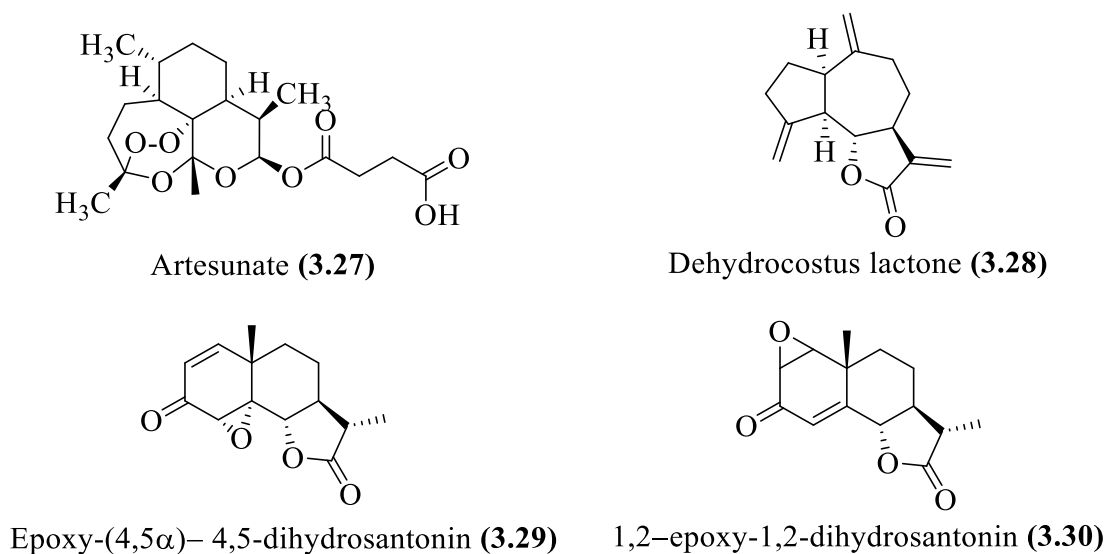
Figure 3.5.　　Structures of sesquiterpene lactones with antiviral activity.

There has been strong evidence indicating that some of the sesquiterpene lactone compounds have antibacterial and antifungal activity.[122,141] Most of the sesquiterpene lactones biological activities were attributed to the presence of α-methylene-γ-lactone in their structure, which exerts its effect by the alkylation of thiol groups found in proteins.[141] The most common antibacterial sesquiterpene lactones are vernodalin **(3.34)** and vernolide **(3.35)**, which were isolated from different plants (e.g. *Vernonia colorata* and *Vernonia amygdalina*).[55,142] 6-*O*-Methylacrylylplenolin **(3.31)**, 6-*O*-angeloylplenolin **(3.32)**, 6-*O*-isobutyroylplenolin **(3.33)**, and other sesquiterpene lactones isolated from *Centipeda minima,* showed high antibacterial activity when they were screened against *Bacillus subtilis* and *Staphylococcus aureus.*[143]

The antifungal activity of sesquiterpene lactones were also confirmed in several studies worldwide.[122,144,120] There are many antifungal compounds isolated from different plant species. For example, isoalantolactone **(3.15)** and elema-1,3,11-trien-8,12-olide **(3.36)**, were isolated from *Ratibida mexicana,* while 8α-hydroxy-4-epi-sonchucarpolide **(3.37)** and 8α-(4-acetoxy-3-hydroxy-2-methylenebutanoyloxy) **(3.38)** were isolated from *Centaurea thessala* spp. *drakiensis* and *C. attica* spp. *attica.*[145] The structures of these compounds are given in Fig. 3.6.

6-*O*-Methylacrylylphenolin (**3.31**)   6-*O*-Angeloyphenolin (**3.32**)   6-*O*-Isobutyroylphenolin (**3.33**)

Vernodalin (**3.34**)   Vernolide (**3.35**)   Elema-1,3,11-trien-8,12-olide (**3.36**)

8α-Hydroxy-4-epi-sonchucarpolide (**3.37**)

8α-(4-Acetoxy-3-hydroxy-2-methylenebutanoyloxy-4-epi-sonchucarpolide) (**3.38**)
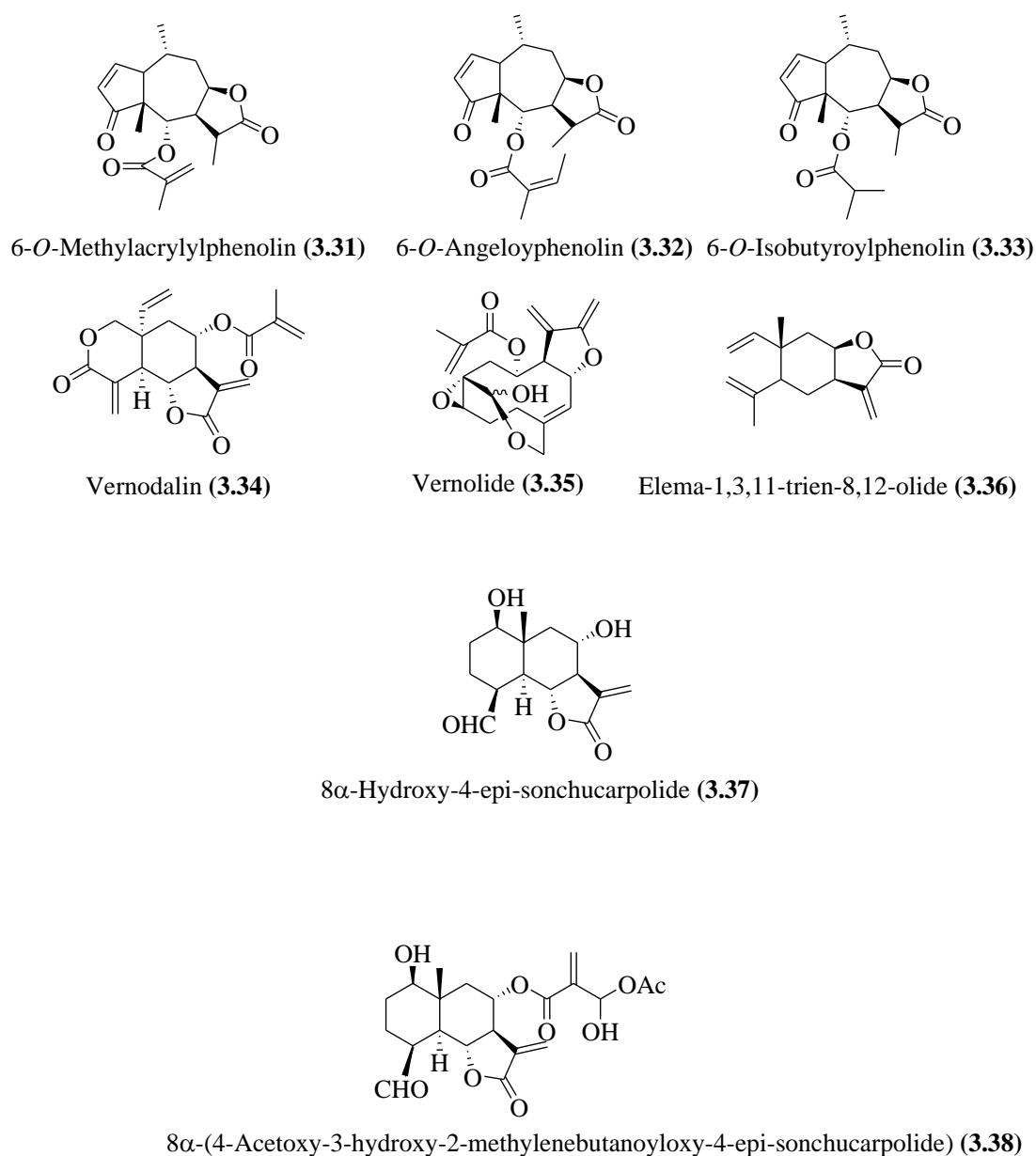
Figure 3.6.   Structures of antibacterial and antifungal sesquiterpene lactones.

3.2.4   Phytochemistry of *Brachylaena*

The investigation of the *Brachylaena* species started in 1982, when sesquiterpene lactones and other chemical ingredients such as a guaianolide (**3.2**), a germacranolide (**3.3**) and three eudesmanolides (**3.4**) were isolated from the aerial parts of *B. transvaalensis* and *B. rotundata*.[146] The study revealed that these two *Brachylaena* species have a number of chemical compounds such as acetylenic compounds, lupeyl acetate, linoleic and linolenic

acid, germacrene D **(3.39)**, and two germacranolide derivatives, onopordopicrin **(3.40)** and salonitenolide **(3.41)**.

The aerial parts of *B. rotundata* was also investigated together with three other *Brachylaena* species (*B. nereifolia, B. elliptica*, and *B. discolor*) by the same authors in 1987.[147] The second investigation of *B. rotundata* yielded onopordopicrin **(3.40)** and salonitenolide **(3.41)**. In addition, five guaianolide glucopyranosides and three germacranolides were isolated from the aerial part of *B. nereifolia*, while *B. elliptica* and *B. discolor* yielded a large amount of onopordopicrin **(3.40)**.[147,148]

Other guaianolides were isolated from the aerial parts of *B. huillensis* and *B. perrieri. B. huillensis* produced five compounds, cynaropicrin **(3.14)**, aguerin A **(3.42)**, aguerin B **(3.43)**, lupeol **(2.45)**, and its acetate, while *B. perrieri* yielded only lupeol **(2.45)** and its acetate (Fig. 3.7).[149]

Germacrene D **(3.39)**

Onopordopicrin **(3.40)**

Salonitenolide **(3.41)**

Cynaropicrin **(3.14)**

Aguerin A **(3.42)**

Aguerin B **(3.43)**

Lupeol **(2.45)**
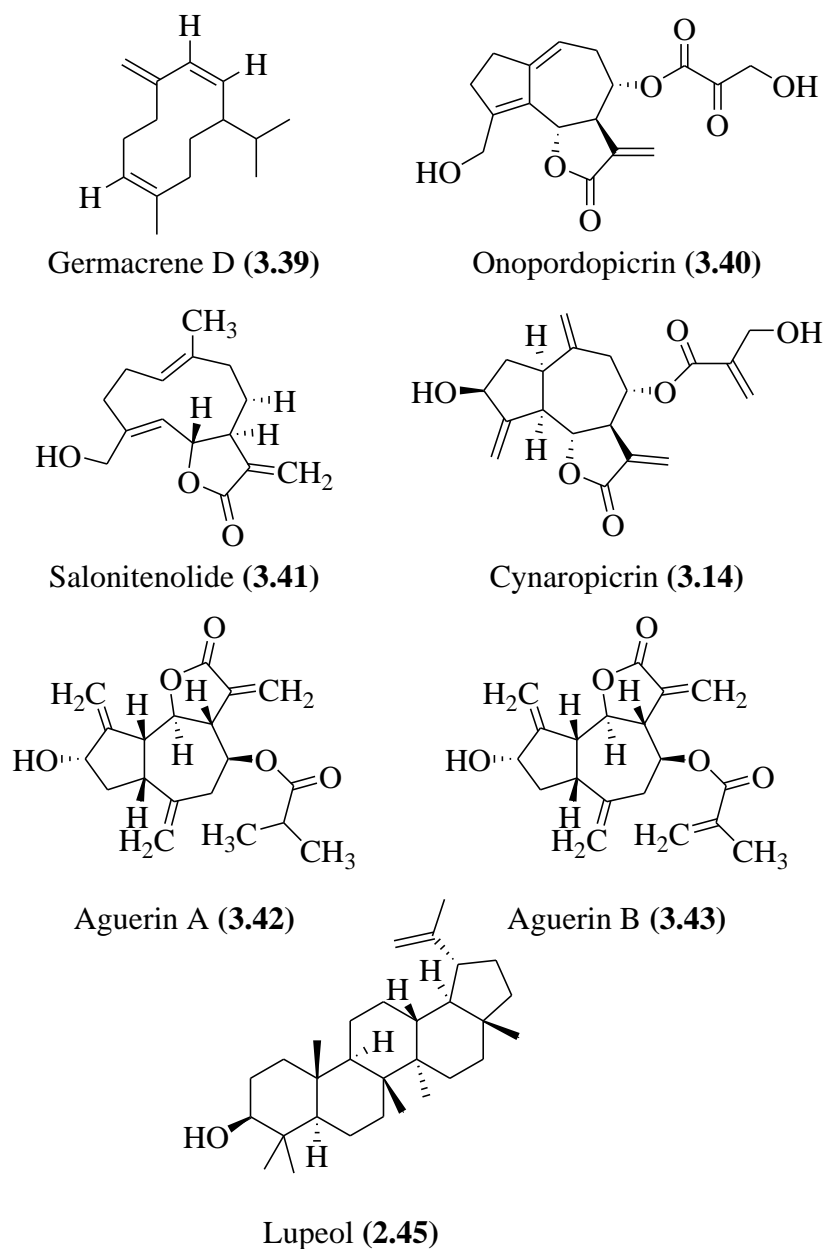
Figure 3.7.   Chemical structures of the components isolated from *Brachylaena* species

3.2.5   Medicinal uses of *Brachylaena* in South Africa

In traditional medicine in South Africa different plants are used to treat a wide range of diseases such as stomach illnesses and chronic diseases (heart failure, chronic respiratory, and diabetes).[52,150] Plants from the *Brachylaena* genus, which is indigenous to South Africa, have been used by the rural communities in South Africa to treat several diseases. In this

genus, the leaves of *B. transvaalensis*, *B. discolor, and B. ilicifolia* have been used for treatment of diarrhoea in humans and animals.[150,151,152] DCM-MeOH and aqueous extracts were used to evaluate the antimicrobial activity of *B. transvaalensis*.[153] Antimicrobial activity was observed for the combination of *B. transvaalensis* with other plants species such as *P. guajava.*

An *in vitro* anticancer screening of 7500 South African plants against three human cancer cell lines showed that DCM leaves extract of *Brachylaena rotundata* S. Moore has activity against cancer cell lines and the highest number of plants assayed were from the Asteraceae family.[154] The cytotoxic activity in *B. rotundata* could probably be attributed to the high concentration of sesquiterpene lactone compounds. Sesquiterpene lactones were found to increase cancer cell deaths by decreasing glutathione levels.[130]

In the *Brachylaena* genus, there are only three species that have been used traditionally for the treatments of diabetes, namely *B. elliptica, B. ilicifolia*, and *B. discolor*. The leaves of *B. elliptica* were traditionally used by Zulu and Xhosa tribes as an antidiabetic treatment.[155] The ethnomedicinal uses of *B. elliptica* and *B. ilicifolia* leaves to treat diabetes were attributed to the existence of antioxidant compounds (e.g. flavonoids, phenols, alkaloids, saponins, tannins, and other compounds).[156] Deutschländer et al.[155] reported that *B. discolor* is one of the plants that is used by indigenous people in South Africa to treat diabetes, renal problems and used as a tonic. Van de Venter et al.[52] conducted an antidiabetic screening against Chang liver, 3T3-L1 adipose, and C2C12 muscle cells of 11 South African plants. They found that *B. discolor* had the best results in term of activity scores and low toxicity. The compounds that have been previously isolated from the aerial parts of *B. discolor* were onopordopicrin (**3.40**) and lupeyl acetate (**3.44**)[157,147]

3.2.6 *Brachylaena discolor*

B. discolor is a shrub or small tree that grows naturally on the coastal dunes of the eastern part of South Africa and also occurs in Mozambique and Zimbabwe. It is commonly known as coast silver oak (English), kusvaalbos (Afrikaans), and iphahla, umpahla (isiZulu).[158]

It is an evergreen shrub or small tree, usually 4 to 10 m in height. Leaves are 5–11 cm long, dark green above, with distinctive silvery-grey undersides (Fig. 3.8). Flowers are creamy-white and arranged in dense terminal panicles. The tiny seeds are tipped with tufts of yellowish hairs. The plant is evergreen with a dark grey rough bark. The leaves are lanceolate with a dark green colour on the top and is pale whitish below, with both sides covered with dense hairs. *B. discolor* has small achene fruits covered with tufts of bristly hairs.[159,160] The distribution map of *B. discolor* is given in Fig. 3.8.
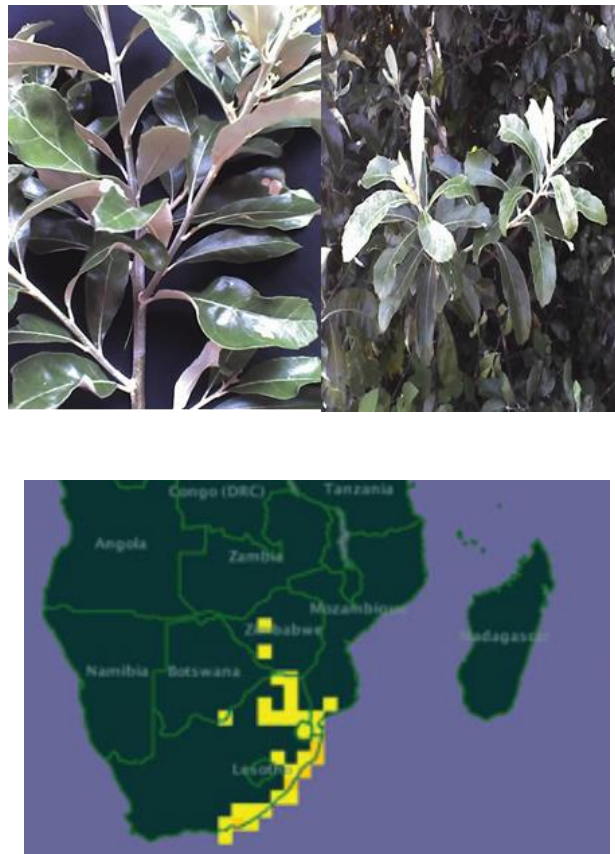

Figure 3.8.   Locations where *B. discolor* (both types) have been collected in Africa (Global Biodiversity Information Facility n.d.).

3.3 RESULTS AND DISCUSSIONS

3.3.1 Extraction and biological assay

In a previous investigation by Van de Venter et al.,[52] a number of plant extracts were assayed for antidiabetic activity and *B. discolor* was identified as one of the active plants.[52] These authors found that all plant parts of the plants were active. No cytotoxicity was observed for the extracts. Although the photochemistry of *B. discolor* has been investigated by Zdero and Bohlmann,[147] the active compound(s) present in *B. discolor* have not been identified.

In this investigation, we revisited the phytochemistry and activity of the leaves of *B. discolor*. Plant material of *B. discolor* was collected at the UKZN Botanical Garden in Pietermaritzburg. The leaves were dried, milled, and extracted with DCM-MeOH (1:1). Before analysing for activity, the crude extract was fractionated into five fractions by using a Diol SPE column (2 g/6 mL) (Table 3.4). This procedure was used to separate the polar fraction containing mostly tannins and carbohydrates from the medium polar compounds, which are often associated with biological activity. Diol is a stationary phase in which the silica is derivatised with 1,2-dihydroxypropane and can be used with a wide range of compounds. It is compatible with a wide range of the solvents, from hexane to water, and using the SPE columns is a reliable method to fractionate a crude extract into sub-fractions of different polarities which can be assayed for activity.[161]

The crude extract and the five fractions obtained from the Diol column were assayed in an in vitro assay where the effect of the different fractions on the glucose utilization on muscle cell line (C2C12) was investigated. The results of this experiment are illustrated in Fig 3.9.

In type 2 diabetes, cells are losing the ability to utilize glucose. Therefore, compounds which stimulate the uptake of glucose by the cells (observed by a lower glucose concentration in the solution) are considered as potential antidiabetic compounds. As can be seen from the results (Fig. 3.9), after 24 h the crude extract (2A) showed a moderate effect, but after 48 h a much higher activity was observed. Among the fractions, the highest activity was observed for Fractions A and E, both after 24 and 48 h. Both fractions B and C showed low antidiabetic activity after 24 h, but fraction C appeared to be more active after 48 h.

The results of this study confirm the finding by Van de Venter et al.[52] using the DCM-MeOH (1:1) extract of *B. discolor* against Chang liver, 3T3-L1 adipose, and C2C12 muscle cell. Mellem *et al.*[162] also reported that the methanolic leaves extract of *B. discolor* has high α-glucosidase inhibition with little toxicity. However, these two research groups only assayed the crude fractions and did not do any further fractionation. Unfortunately, our collaborator doing the antidiabetic assays, Prof C. T. Musabayane, passed away during this investigation and we were not able to conduct further bioassays.
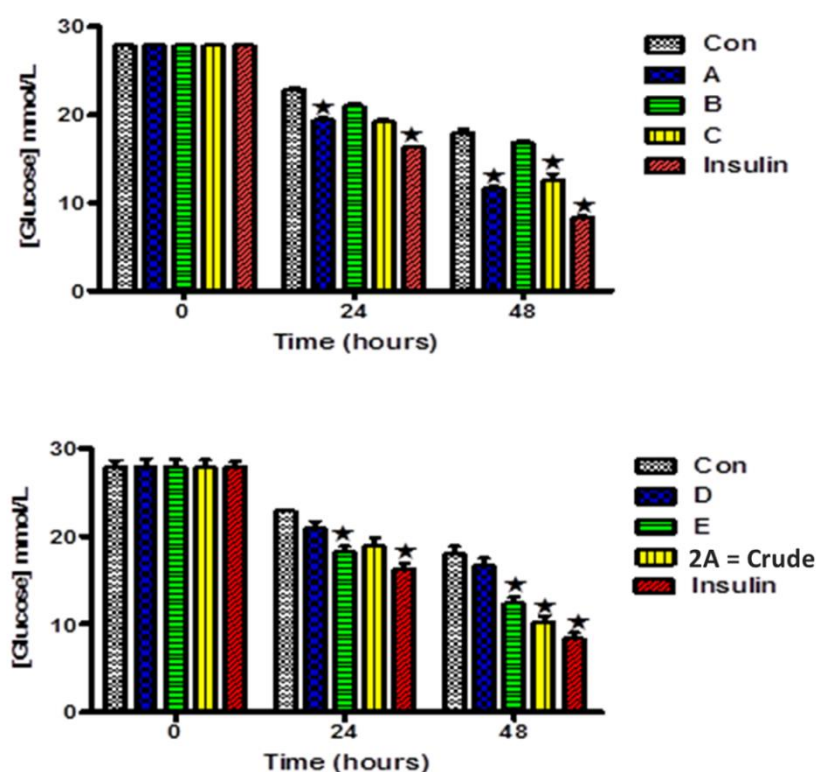

Figure 3.9.   The effects of plant extracts on glucose utilization in muscle cell line after 24 and 48 experimental periods. *p<0.05 in comparison to control.

3.3.2 Lupeol acetate and its Δ^{12} isomer (3.45 and 3.46)

Fractionation of the crude extract as described in the experimental section, led to the isolation of five compounds. Herewith, the structural elucidation of these compounds are described.

Fractionation with a DIOL SPE column is a convenient and reliable method to separate a crude extract into fractions of different polarities. However, the SPE columns can only be loaded with a small amount of extract (100 mg). To save on costs, we have adapted the method to a column chromatography procedure on silica gel for larger amounts of extract (see first step in isolation procedure, Fig. 3.14).

Fraction A (Fig. 3.15) yielded a white needle-like solid, which appeared to be one compound on TLC. However, NMR spectroscopy indicated that it consists of a pair of inseparable isomeric triterpenes, lupeol acetate [3β-acetoxy-20(29)-lupene] **(3.45)** and its Δ^{12} isomer, 3β-acetoxy-12-lupene **(3.46)**, also known as neolupenyl acetate.[163]

In the ^{1}H NMR spectrum, the following resonances were observed:
δ_H 5.26 (br d, H-12, neolupenyl acetate), δ_H 4.69 (br s, H-29a, lupeol acetate), δ_H 4.57 (br s, H-29b, lupeol acetate), δ_H 4.49 (m, H-3, lupeol acetate, neolupenyl acetate), δ_H 2.37 (dt, J 10.9 and 5.5 Hz, H-19, lupeol acetate), δ_H 2.043 (s, OAc (lupeol acetate), 2.037 (s, OAc, neolupenyl acetate) 0.79 (s, H-28, lupeol acetate, neolupenyl acetate), 0.73 (s, H-26, lupeol acetate, neolupenyl acetate). Unfortunately, when the other spectroscopic data were recorded, it was observed that the compounds had decomposed. The NMR data assigned to lupeol acetate **(3.45)** agrees with the shifts reported for the compound by Jamal et al.[164]

The high-resolution ESI-(+)-TOF mass spectrum, displayed a M+H ion at 469.4071, corresponding to a molecular formula of $C_{32}H_{52}O_2$ (calculated for $C_{32}H_{53}O_2$, 469.4046).

Lupeol acetate (**3.44**)   3β-Acetoxy-12-lupene (**3.45**)
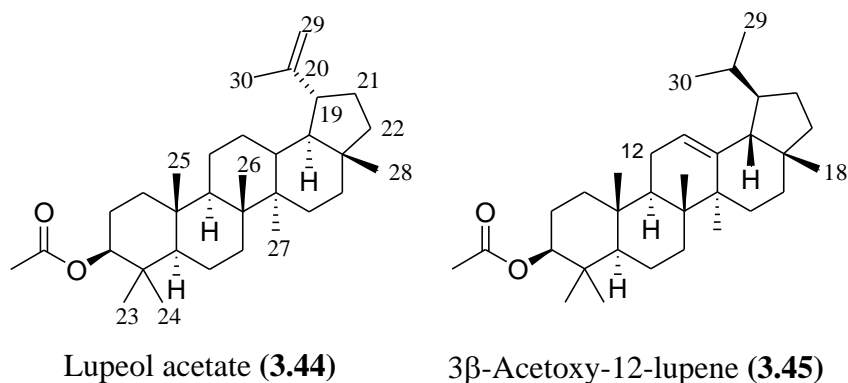
The polarity of these two isomeric compounds are similar and it came to no surprise that conventional column chromatography could not separate the two compounds. This chromatographic behaviour was also observed with two other isomeric triterpenoids, arjunolic acid and asiatic acid.[165,166]

The two compounds, lupeol acetate (**3.44**) and its Δ^{12} isomer (**3.45**), were also isolated from the aerial part of *B. discolor* by Zdero and Bohlmann.[147] However, these authors did not disclose any spectroscopic data for the two compounds. Recently, lupeol acetate (**3.44**) was isolated from *Pseudobrickellia brasiliensis* and its Δ^{12} isomer (**3.45**) from *Ficus sansibarica.*[167,168]

A number of triterpenes have been identified as promising agents to prevent diabetes complications.[169,170] Lakshmi et al.[171] reported that lupeol acetate (**3.44**) and its parent compound lupeol (**2.45**) showed *in vivo* antidiabetic activity comparable to metformin, a drug that is clinically used for the treatment of diabetes.[172] It was also reported that lupeol is a potent inhibitor of the enzyme protein tyrosine phosphatase (PTP1B).[172-174] PTB1B plays an important role in the inhibition of insulin action, development of Type 2 diabetes and obesity. Inhibitors of this enzyme are considered as potential leads for the development of new antidiabetic and antiobesity drugs. Lupeol and its derivatives are considered as promising candidates in this regard.[174,175]

A review on the health effects of lupeol also pointed out that lupeol has several other important pharmacological activities.[176] Neolupenyl acetate (**3.45**) is a rare compound in nature and there are no reports on its biological activity. Taking the evidence above into account, it is most likely that the antidiabetic activity of *B. discolour* can be attributed to the presence of lupeol acetate (**3.44**) and its Δ^{12} isomer (**3.45**).

3.3.3 β-Sitosteryl linolenate (3.46)

Fraction B yielded a fatty acid ester which was identified as β-sitosteryl linolenate (**3.46**).
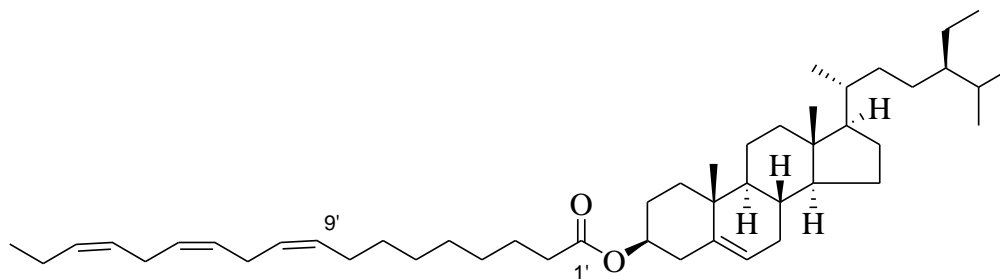
β-Sitosteryl linolenate **(3.46)**

The ^{1}H and ^{13}C NMR spectra of β-sitosteryl linolenate **(3.46)** are shown in Plate 2A and Plate 2B, respectively, and the data are collated in Table 3.1. The NMR spectra contained features of both a steroid and a fatty acid and initially the sample was considered as a mixture of two compounds. However, on careful inspection of the NMR spectra it was concluded that the sample consisted of a clean compound containing both fatty acid and steroid moieties.

The fatty acid moiety was identified by an ester carbon resonating at δ_C 173.3. The methylene group alpha to the ester carbon was identified by a long-range ^{1}H,^{13}C correlation in the HMBC (Plate 2G) between the signal at δ_C 173.3 and two protons resonating as a triplet at δ_H 2.26. In the COSY, the protons at C-2' further coupled with methylene protons resonating at δ_H 1.60. However, this region showed a large overlap of ^{1}H NMR resonances and it was concluded that an extended alkyl chain was attached to the ester. Another feature of the ^{1}H NMR spectrum was the presence of seven olefinic proton resonances as an overlapping multiplet at δ_H 5.42-5.48. One of these protons were assigned to the steroid moiety (see below), which led to the conclusion that the fatty acid moiety had three double bonds. This postulate was confirmed by the presence of seven olefinic methine resonances in the ^{13}C NMR spectrum at δ_C 132.0, 130.3, 128.30, 128.28, 127.7, 127.2, and 122.6.

One of the most common trienoic fatty acids is linolenic acid [(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid], an essential omega-3 fatty acid that is found widely in seeds, nuts, and vegetable oils. It was proposed that **3.46** might be an ester of linolenic acid. The terminal methyl protons (H-18') resonated as a triplet (*J* 7.4 Hz) at δ_H 0.98, which correlated

with a methyl carbon resonating at δ_C 14.3 in the HSQC spectrum (Plate 2E). In the COSY spectrum, this methyl signal (δ_H 0.98) correlated with a multiplet at δ_H 2.06, which showed further correlation with the olefinic proton multiplet at δ_H 5.42-5.48, thus confirming that the ester indeed contained an omega-3 fatty acid. In the HMBC spectrum of **3.46**(Plate 2G), no correlations were observed between the olefinic protons and olefinic carbons, which indicated that the three double bonds were not conjugated. Instead, the olefinic carbon resonances showed a correlation with a four-proton triplet resonating at δ_H 2.26, corresponding to two carbons resonating at δ_C 25.5 and 25.6. These signals were assigned to two methylene groups each located between two double bonds, as is present in the structure of linolenic acid. Comparison of the NMR data of **3.46** with those of methyl linolenate reported by Christie,[177] confirmed that **3.46** is an ester of linolenic acid (Table 3.1).

In the remaining parts of the NMR spectra there were evidence of six additional methyl groups: two methyl singlets at δ_H 0.68 (δ_C 11.8) and 1.02 (δ_C 11.8), three methyl doublets that appeared at δ_H 0.82 (δ_C 19.8), 0.84 (δ_C 19.1), and 0.92 (δ_C 18.8), and a methyl triplet at 0.85 (δ_C 12.0). The ¹H NMR spectrum also showed a proton resonance as a multiplet at δ_H 4.60 (δ_C 73.7) and an olefinic proton as part of the multiplet at δ_H 5.42-5.48. These spectroscopic features indicate that the remaining part of **3.46** is a steroid. The long-range ¹H,¹³C correlations observed in the HMBC spectrum confirmed that this steroid is β-sitosterol. Correlations were observed between the resonance at δ_H 1.02 (19-Me) and resonances at δ_C 37.0 (C-1), 139.8 (C-5), 50.1 (C-9), and 36.6 (C-10) and also between the methyl resonance at 0.68 and the carbon resonances at δ_C 39.8 (C-12), 42.3, (C-13) 56.7 (C-14), and 56.1 (C-17). The two *geminal* methyl groups, Me-26 and Me-27, resonated as doublets (*J* 6.8 Hz) at δ_H 0.84 and 0.82. The remaining methyl protons (Me-29) was observed as a triplet at δ_H 0.85, in agreement with an ethyl substituent on C-24.

The NMR data of **3.46** were in good agreement with those reported by Chaturvedula et al.[178] for β-sitosterol **(2.57)**. However, there was one major difference between the NMR data for β-sitosterol and those of **3.46** and that was the chemical shift of H-3. Instead of a chemical shift of δ_H 3.51 as reported for β-sitosterol,[178] H-3 of **3.46** had a chemical shift of δ_H 4.60. This was an indication that the oxygen on C-3 was part of an ester group. Therefore, the structure of **3.46** was assigned as β-sitosteryl linolenate.

Initial attempts to obtain a mass spectrum by electrospray ionization were not successful. By changing the ionisation method to API (atmospheric pressure ionisation), a technique suitable for extremely non-polar compounds, a mass spectrum could be obtained for **3.46**. In the API-(+)-TOF mass spectrum a peak of low intensity was observed at *m/z* 697, corresponding to the sodium adduct of the molecular ion. In a study of the mass spectrometry of sterol lipids by Wewer et al.[179] a fragment at *m/z* 397 resulting from the cleavage of the C-O bond between C-3 and the ester oxygen, was observed. A fragment with *m/z* 397 was also observed in the mass spectrum of **3.46.**

The isolation of **3.46** from the leaves of *B. discolor* has not been reported previously. β-Sitosterol **(2.57)** is a common plant metabolite and occurs in other plant species such as *Terminalia sericea* and *Solanum surattense*.[92,180] Both these studies linked antidiabetic activitiy to β-sitosterol. Although Scifinder listed only twenty-seven references for compound **3.46**, phytosterol esters of fatty acids occur in several plants.[180] As a result of the extremely non-polar nature of these compounds, they do not receive much attention from phytochemists.

Table 3.1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for β-sitosteryl linelonate **(3.46)** in CDCl3.

C position			Chaturvedula et al.[178]	Christie[177]
	3.46		β-Sitosterol **(2.57)**	Methyl linelonate
C position	δ_C	δ_H	δ_C	δ_C
1	37.0		37.5	
2	29.7		31.9	
3	73.7	4.60 (1H, m)	72	
4	38.2	2.28 (1H, H-4eq)	42.5	
5	139.8		140.9	
6	122.6	5.42-5.48	121.9	
7	31.9		32.1	
8	31.9		32.1	
9	50.1		50.3	
10	36.6		36.7	
12	39.8	1.99, 1.16	39.9	
13	42.3		42.6	
14	56.7		56.9	
15	24.3		26.3	
16	27.8		28.5	
17	56.1		56.3	
18	11.8	0.68	12.0	
19	19.3	1.02 (3H, s)	19.0	
20	36.2		36.3	
21	18.8	0.92 (3H, d, *J* 6.6 Hz)	19.2	
22	34.0		34.2	
23	26.1		26.3	
24	45.9		46.1	
25	29.10*		29.4	
26	19.8	0.82 (3H, d, *J* 6.8 Hz)	20.1	
27	19.1	0.84 (3H, d, *J* 6.8 Hz)	19.6	
28	23.1		23.3	
29	12.0	0.85 (3H, t, *J* 7.5 Hz)	12.2	
1'	173.3			174.2
2'	34.7	2.26 (2H, t, *J* 7.8 Hz)		34.1
3'	25.1	1.60 (2H, m)		25.0
4'	29.21*	1.31		
5'	29.17*	1.31		
6'	29.11*	1.31		

7'	29.6	1.31		29.6
8'	27.2	2.06		27.3
9'	130.3	5.42-5.48		130.2
10'	127.7	5.42-5.48		127.8
11'	25.6[#]	2.81 (2H, t, J 6 Hz)		25.7
12'	128.30[†]	5.42-5.48		128.3
13'	128.28[†]	5.42-5.48		128.3
14'	25.5[#]	2.81 (2H, t, J 6 Hz)		25.6
15'	127.2	5.42-5.48		127.2
16'	132.0	5.42-5.48		131.9
17'	20.6	2.06 (2H, m)		20.6
18'	14.3	0.98, (3H, t, J 7.4 Hz)		14.3

[*],[#],[†]Assignments may be interchanged.

3.3.4 α-Tocopherol (3.47)

The NMR data of compound **3.47** are collated in Table 3.2. In the ^{1}H NMR spectrum of **3.47** (Plate 3A) there were only a few well-resolved signals. The ^{13}C NMR spectrum (Plate 3B) indicated the presence of 29 carbon atoms. A prominent feature in the ^{13}C NMR spectrum of **3.47** was the evidence of six aromatic carbon atoms without any attached protons. The chemical shifts of two of the carbons (δ_C 145.6 and 144.5) indicated that oxygens are attached to these carbon atoms. In the ^{1}H NMR spectrum, three methyl groups were observed at δ_H 2.16 (3H) and 2.11 (6H). The chemical shifts of the methyl protons indicated that these groups were attached to the aromatic ring. In the HMBC spectrum of **3.47** (Plate 3E), long-range ^{1}H,^{13}C-correlations were observed between the methyl protons and the aromatic carbons atoms, as indicted in Table 3.2. It was clear that compound **3.47** contains a fully-substituted benzene ring with three methyl groups, two oxygen substituents, and an alkyl substituent attached to it.

In the ^{1}H NMR spectrum, a two-proton triplet was observed at δ_H 2.60 that correlated with the carbon resonance at δ_C 20.8 in the HSQC spectrum (Plate 3G). In the COSY spectrum (Plate 3D), these protons are correlated with a pair of doublets of triplets with chemical shifts at δ_H 1.81 and 1.75. The HSQC spectrum (Plate 3G) showed that the last-mentioned pair of protons are attached to a methylene carbon resonating at δ_C 31.6. The coupling pattern of these protons indicated that they are in a ring where free rotation is not possible. In the HMBC spectrum, the methylene protons resonating at δ_H 2.60 (H-4) correlated to the carbon resonances at δ_C 145.6 (C-10), 117.4 (C-9), 74.5 (C-2) and 31.6 (C-3), whereas the two methylene protons resonances at δ_H 1.81 and 1.75 (H-3) showed long-rang correlations to an aromatic carbon resonating at δ_C 117.4 (C-9), an oxygen-containing carbon resonating at δ_C 74.5 (C-2), two methylene carbons at δ_C 39.8 (C-1') and 20.8 (C-4) and a methyl carbon resonating at δ_C 23.8 (2-CH$_3$). The NMR data discussed above enabled us to assign a chromane substructure for **3.47**. The long-range couplings observed in the HMBC spectrum agree with this substructure as shown in Fig. 3.10.
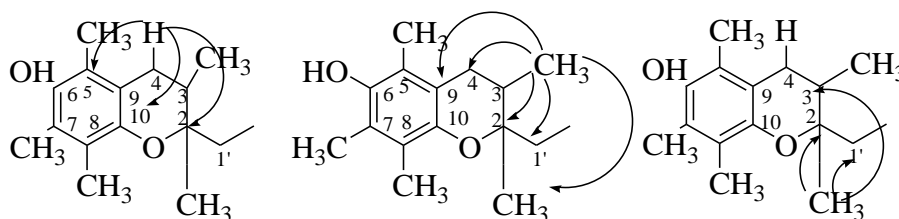
Figure 3.10.   HMBC correlations in compound (**3.47**).

In the ^{1}H NMR spectrum, the only other discernible feature was the presence of four methyl groups resonating as doublets at δ_H 0.87 (6H), 0.84 (3H), 0.86 (3H). Several methine and methylene protons were observed in the spectrum as broad, non-resolved resonances. In the HMBC spectrum, the two methyl group protons at δ_H 0.87 showed long-range coupling to the methyl carbons at δ_C 22.6 and 22.7, indicating the presence of an isopropyl group. These methyl protons (δ_H 0.87) also showed long-range coupling to the methylene carbon resonating at δ_C 24.4.

Each of the two remaining methyl group protons showed long-range ^{1}H,^{13}C couplings to two methylene carbons and one methine carbon. The only unaccounted carbons in the ^{13}C NMR spectrum are three methylene carbons. Based on biogenetic considerations, we then proposed a substructure containing a saturated linear sesquiterpene (Fig. 3.11).
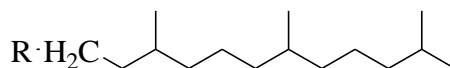

Figure 3.11.   Substructure based on a saturated linear sesquiterpene.

This substructure accommodated all the long-range correlations observed in the HMBC spectrum. The NMR data of this aliphatic chain is in close agreement with that assigned to phytol (Fig 3.12).[181]
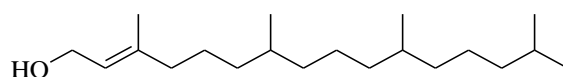

Figure 3.12.   The structure of phytol

Connecting the two sub fragments enabled us to assign the structure of compound **3.47** as α-tocopherol, commonly known as vitamin E. A paper describing the NMR assignment of

α-tocopherol was not available to us.[182] However, the NMR chemical shifts of the side chain is in good agreement with that of phytol, whereas the chemical shifts of the chromane moiety is in agreement with the values reported for α-tocopherol.[181]

The high-resolution ESI-(-)-TOF mass spectrum confirmed the molecular formula of α-tocopherol (**3.47**). A molecular ion was observed at 429.3737 [M-H]$^-$, which agreed with the calculated value for $C_{29}H_{49}O_2$ (429.3733).
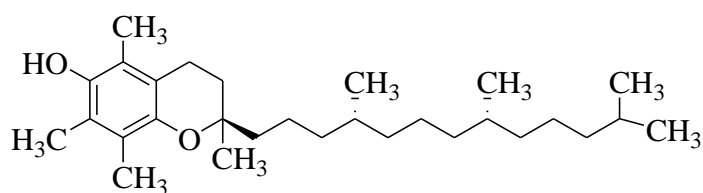
α-Tocopherol (**3.47**)

α-Tocopherol (**3.47**) or vitamin E was isolated (fraction B) for the first time from *B. discolor* leaves. Jamalan et al.[183] reported that α-tocopherol is a well-known antioxidant and that its consumption may decrease induced inflammatory response in patients with type 2 diabetes.

Table 3.2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for α-tocopherol (**3.47**) in CDCl$_3$.

C position	δ$_C$		δ$_H$	COSY	GHMBC
2	74.5	C			
2-CH$_3$	23.8	CH$_3$	1.23 (3H, s)		C-1',2,3
3	31.6	CH$_2$	1.81 (1H, dt, J 13.4 ,7.0 Hz)	H-4	C-1',2,2-CH$_3$,4,9
			1.75 (1H, dt, J 13.4 ,7.0 Hz)		C-1',2,2-CH$_3$,4,9
4	20.8	CH$_2$	2.60 (2H, t, J 7.0 Hz)	H-3	C-2,3, 9,10
5	118.5	C			
5-CH$_3$	11.3	CH$_3$	2.11 (3H, s)		C-6,9
6	144.5	C			
7	121.0	C			
8	122.6	C			
8-CH$_3$	11.8	CH$_3$	2.11 (3H, s)		C-7,10
9	117.4	C			C-10
10	145.6	C			
1'	39.8	CH$_2$	1.53 (2H, m)		
3'	37.5	CH$_2$	1.25 (2H, m)		
4'	32.7	CH	1.37 (2H, m)		
4'-CH$_3$	19.7	CH$_3$	0.85 (3H, d, J 6.7 Hz)		C-3',4',5'
5'	37.4	CH$_2$	1.25 (2H, m)		
6'	24.8	CH$_2$	1.27 (2H, m)		
7'	37.5	CH$_2$	1.25 (2H, m)		
7'-CH$_3$	12.2	CH$_3$	2.16 (3H, s)		C-6,8
8'	32.8	CH	1.37 (2H, m)		
8'-CH$_3$	19.6	CH$_3$	0.84 (3H, d, J 6.7 Hz)		C-7',8',9'
9'	37.3	CH$_2$	1.06 (2H, m)		
10'	24.4	CH$_2$	1.18 (2H, m)		
11'	39.4	CH$_2$	1.13 (2H, m)		
12'	28.0	CH	1.51 (1H, m)		
12'a-CH$_3$	22.7	CH$_3$	0.87 (3H, d)	H-12'	C-11',12',12'b-CH$_3$
12'b-CH$_3$	22.6	CH$_3$	0.87 (3H, d)	H-12'	C-11',12',12'a-CH$_3$

3.3.5   Genkwanin 5-*O*-β-primeveroside (3.48)

In the high-resolution mass spectrum of **3.48** (ESI-(-)-TOF), an ion was observed at 557.1554, corresponding to a formula of $C_{27}H_{29}O_{14}$ (calc. 577.1557), thereby confirming the molecular formula of $C_{27}H_{30}O_{14}$.

The elucidation of the structure of compound **3.48** was based on NMR spectroscopic data (Table 3.3). In the ^{1}H NMR spectrum (Plate 4A), several signals were observed in the aromatic region of the spectrum. Two two-proton doublets resonating at δ_H 7.88 (*J* 8.8 Hz) and 6.94 (*J* 8.8 Hz) were characteristic of a *para*-substituted benzene ring, with one substituent being an oxygen. Two one-proton doublets at δ_H 6.97 (*J* 2.4 Hz) and 6.95 (*J* 2.4 Hz) indicated the presence of two *meta*-coupled protons in a phloroglucinol derivative. Additionally, a one-proton singlet was observed at δ_H 6.63 and a three-proton singlet at δ_H 3.96. These data are consistent with a flavone that is substituted on C-5, 7, and 4'.

The ^{13}C NMR spectrum (Plate 4B) was in agreement with a flavone structure. A conjugated ketone carbon resonance was observed at δ_C 180.3 (C-4), in addition to five oxygen-containing sp^2-carbons at δ_C 166.1, 164.6, 162.9, 160.7, and 159.6, two two-carbon CH resonances at δ_C 129.4 (C-2',6') and 117.1 (C-3',5'), non-protonated carbon resonances at δ_C 122.9 (C-1') and 110.4 (C-4a), and three CH resonances at δ_C 106.8 (C-3), 104.4 (C-6), and 97.2 (C-8).

In the HMBC spectrum (Plate 4G), the singlet resonating at δ_H 6.63 (H-3) showed a correlation to the carbonyl resonance at δ_C 180.3 (C-4) and the resonance at δ_C 164.6, which could then be assigned to C-2. Both H-2' and H-3' correlated with the resonance at δ_C 162.9, which was assigned to C-4'. The methoxy protons (δ_H 3.97) showed a long-range correlation to the resonance at δ_C 166.1. Since H-2' and the methoxy protons are correlated to two different C-O sp^2 carbons, the B-ring must contain a hydroxy substituent and the methoxy substituent must be present on the A-ring. Both A-ring aromatic protons (H-6 and H-8) showed a correlation with the resonance at δ_C 166.1, which indicated that the methoxy substituent is present at C-7. If the methoxy substituent was at C-5, this correlation would have indicated a long-rang coupling from H-8 to C-5, which is less likely. The proposed flavone moiety of this compound is given in Fig. 3.13.
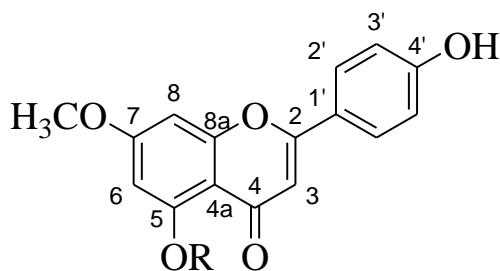
Figure 3.13.   Flavonoid moiety of **3.48.**

The remaining parts of both the ^{1}H and the ^{13}C spectra were in agreement with the presence of carbohydrate protons and carbons. The ^{1}H and the ^{13}C spectra indicated the presence of four anomeric hydrogens and four anomeric carbons. In the HSQC spectrum, correlations were observed between signals at δ_H 5.10 (d, J 3.8 Hz) and δ_C 94.0, δ_H 4.87 (d, J 7.7 Hz) and δ_C 104.7, δ_H 4.47 (d, J 7.8 Hz) and δ_C 98.2, and δ_H 4.33 (d, J 7.4 Hz) and δ_C 105.7. The deprotiated molecular ion [M-H]⁻ in the high-resolution mass spectrum (negative ionisation mode) suggested the presence of two carbohydrate moieties only. A careful inspection of the NMR spectra indicated that the major compound **3.48** was contaminated with a mixture of α- and β-D-glucopyranose, which will be discussed in the next paragraph. The carbohydrate moieties with anomeric protons resonating at δ_H 5.10 and δ_H 4.47, were assigned to this impurity.

Considering the evidence discussed in the previous paragraph, we proposed that compound **3.48** was a flavone containing a diglycoside substituent on C-5. In the ^{13}C NMR spectrum (Plate 4B), eleven carbohydrate carbons were observed for **3.48**, in agreement with the presence of a hexose and a pentose moiety. These resonances included two anomeric CH carbons resonating at δ_C 104.7 and 105.7, two methylene carbons resonating at δ_C 70.5 and 66.9 along with seven CH resonances in the region δ_C 71 – 78.

The coupling constants of the two anomeric protons resonating at δ_H 4.87 (d, J 7.7 Hz) and δ_H 4.33 (d, J 7.4 Hz) were characteristic of coupling between diaxial *vicinal* protons. This indicated that both these sugars were β-glycosides and also that the hydrogens on C-2 of both sugars were in the axial positions. The linkages of the glycosyl moieties were determined by observing the long-range ^{1}H,^{13}C correlations in the HMBC spectrum. The anomeric proton resonating at δ_H 4.87 (H-1″) showed a correlation with the carbon

resonance at δ_C 159.6, which was assigned to C-5. In the HMBC spectrum (Plate 4G), the second anomeric proton resonating at δ_H 4.33 correlated with a methylene carbon resonance at δ_C 70.5, which indicated that the second carbohydrate moiety was linked to C-6″ of the first carbohydrate moiety.

By comparison with literature data, the NMR resonances of the first carbohydrate moiety agreed with a glucose moiety.[184] For the second carbohydrate moiety, the coupling constants for H-2‴ (δ_H 3.23, dd, *J* 9.0 and 7.5 Hz) showed that the protons on C-1,2, and 3 of this moiety were all in axial positions. The resonances for the methylene group associated with the second carbohydrate moiety were observed at δ_H 3.86 (obscured multiplet) and 3.19 (dd, *J* 11.2 and 10.3 Hz) and δ_C 66.9. The large chemical shift difference between the two methylene protons provided evidence that the methylene group was located in a rigid ring and that the pentose moiety was in the pyranose form rather than the furanose form. Furthermore, the proton resonating at δ_H 3.19 (dd, *J* 11.2 and 10.3 Hz) had large *geminal* and *vicinal* coupling constants, which was only possible with an axial proton on C-4. Based on this evidence, the second carbohydrate moiety was identified as a β-xyloside.

The structure of **3.48** is indicted below. This compound was previously isolated from other plants such as *Aquilaria sinensis* and was given the name genkwanin 5-O-β-primeveroside.[184] Although using a different solvent (DMSO-d_6), NMR data reported for **3.48** in the literature were in good agreement with our results.[185] In Table 3.3, the experimental NMR data of **3.48** are compared with those of a derivative of genkwanin 5-*O*-β-primeveroside (B-ring is a 3,4,5-trimethoxyphenyl),[185] because these data were also acquired in CD$_3$OD.
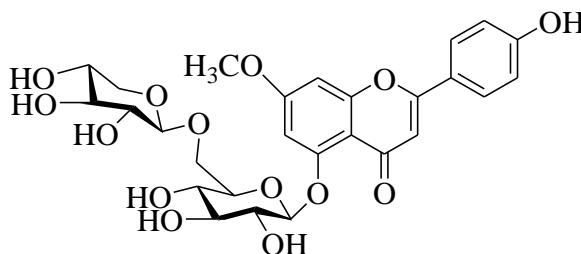
Genkwanin 5-O-β-primeveroside (**3.48**)

Table 3.3　　　¹H NMR (500 MHz) and ¹³C NMR (125 MHz) spectroscopic data for genkwanin 5-O-β-primeveroside **(3.48)** in CD₃OD.

|	**3.48**		**3.48** derivative*	
C position	Experiment		Zahir et al.[185]	
	δ_H	δ_C	δ_C	δ_H
2		164.6	163.1	
3	6.63 (1H, s)	106.8	108.5	6.95
4		180.3	179.8	
4a		110.4	110.3	
5		159.6	159.4	
6	6.97 (1H, d, *J* 2.4 Hz)	104.4	104.5	7.03
7		166.1	166.0	
8	6.95 (1H, d, *J* 2.4 Hz)	97.2	97.3	7.01
8a		160.7	61.4	
1'		122.9		
2',6'	7.88 (2H, *J* 8.8 Hz)	129 .4		
3',5'	6.94 (2H, *J* 8.8 Hz)	117.1		
4'		162.9		
7-OCH₃	3.96 (s)	56.8		
1"	4.87 (1H, d, *J* 7.8 Hz)	104.7	104.7	4.80
2"		75.0	74.7	
3"		77.3	77.2	
4"		71.5	71.5	
5"		77.5	77.4	
6"		70.5	70.4	
1'''	4.33 (1H, d, *J* 7.5 Hz)	105.7	105.6	4.32
2'''	3.23 (1H, dd, *J* 9.0, 7.5 Hz)	74.1	74.2	3.22
3'''		77.8	77.7	
4'''		71.1	71.1	
5'''	3.19 (dd, *J* 11.2, 10.3 Hz) 3.86	66.9	66.8	3.18;3.84

　　　*B-ring is 3,4,5-trimethoxyphenyl.

3.3.6   α- and β-D-Glucopyranose (3.49 and 3.50)

This material contained compound(s) that were highly polar. In the ^{13}C NMR spectrum of **3.49** and **3.50** (Plate 5B), 12 signals were observed. The DEPT-135 spectrum indicated that 10 of these resonances could be assigned to methine carbons and two of the resonances (δ_C 62.9 and 62.8) to methylene carbons. The chemical shifts of the two methylene carbons were consistent with two OCH_2 groups. Two of the methine carbon atoms resonated at lower field than the rest of the carbons (δ_C 98.2 and 94.0), indicating that these two carbons were attached to two oxygen atoms each. The other methine carbons resonated at δ_C 78.1, 78.0, 76.3, 74.9, 73.8, 73.0, 71.9, and 71.8. All the chemical shifts were consistent with aliphatic carbons attached to one oxygen substituent. These data suggested the presence of a carbohydrate. Three pairs of resonances had almost the same chemical shifts: δ_C 78.1 and 78.0, 71.9 and 71.8, and 62.9 and 62.8.

The ^{1}H NMR spectrum (Plate 5A) supported the assignment of the compound(s) as a carbohydrate. Two doublets are observed at δ_H 5.11 (*J* 4.0 Hz) and δ_H 4.48 (*J* 8.0 Hz), consistent with two anomeric protons (H-1), the first resonance characteristic of an equatorial proton, indicating an α-sugar, and the second of an axial proton, indicating a β-sugar. These two protons showed $^1J_{CH}$ coupling with the ^{13}C resonances at δ_C 94.0 and 98.2, respectively, in the HSQC spectrum. In the COSY spectrum a correlation can be observed between the proton resonating at δ_H 5.11 and a doublet of doublets at δ_H 3.36 (*J* 10.0, 4.0) and also between the proton resonating at δ_H 4.48 and a doublet of doublets resonating at δ_H 3.13 (*J* 9.0 and 7.7 Hz), indicating that the protons on both C-2 and C-3 were axial. In the HSQC spectrum, it was observed that the proton resonating at δ_H 3.36 was attached to the carbon resonating at δ_C 73.9, whereas the proton resonating at δ_H 3.13 was attached to the carbon resonating at δ_C 76.3. The other ^{1}H signals were not well resolved. The resonances of the protons attached to the carbons resonating at δ_C 78.1, 78.0, 71.9, and 71.8 were overlapping with the solvent signals at δ_H 3.27-3.34, whereas the resonances of the protons attached to δ_C 74.9 and 73.0 overlapped with the resonance of the four methylene proton resonances at δ_H 3.60 - 3.87.

From the data, it was clear that the material consisted of two glucose moieties. These two moieties could be attached to form a diglucoside or, alternatively, the material consisted of

an equimolar mixture of α- and β-glucose. If it was a diglucoside, long-range ^{1}H,^{13}C between H-1 of one unit and the ether carbon of the other carbohydrate unit should have been observed. In the HMBC spectrum, the axial anomeric proton (δ_H 4.48, J 8.0 Hz) did not correlate to any carbons, whereas the equatorial anomeric proton (at δ_H 5.11, J 4 Hz) only showed a correlation to the carbon resonating at δ_C 73.9, which was assigned to C-2 on the same unit. Therefore, it was clear that this material consisted of an equimolar mixture of α- and β-glucopyranose. These two compounds are in equilibrium with each other via the intermediate aldehyde, as illustrated in Scheme 3.1 and can normally not be obtained in pure form.

3.49 α-D-Glucopyranoside   **3.50** β-D-Glucopyranoside
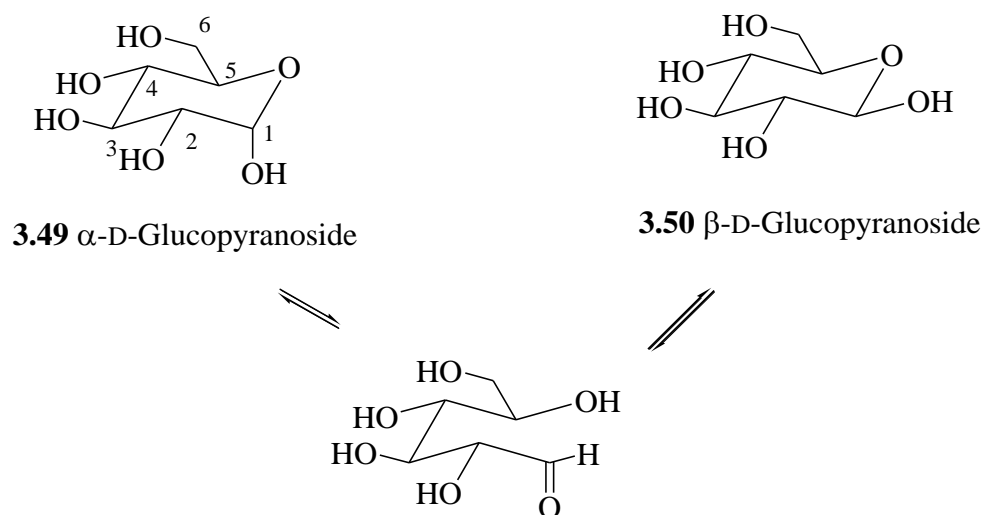
Scheme 3.1.   The equilibrium reaction between α-glucopyranose and β-glucopyranose.

In conclusion, an antidiabetic assay was conducted for the crude and subfractions of the leaves extract of *B. discolor,* confirmed the antidiabetic properties of the plant. Seven compounds were isolated from *B. discolor*, three of them were obtained as a single pure compound which are β-sitosteryl linolenate **(3.46)**, α-tocopherol **(3.47)**, and genkwanin 5-*O*-β-primeveroside **(3.48)**, while the others were isolated as two mixtures of two compounds, *i.e.* a inseparable mixture of lupeyl acetate **(3.44)** and its Δ^{12} isomer **(3.45)** as well as a mixture of α-glucopyranose **(3.49)** and β-glucopyranose **(3.50)**. Lupeol acetate **(3.44)**, its Δ^{12} isomer **(3.45), and** β-sitosterol **(2.57)** have been identified in the literature as antidiabetic compounds. Although it was envisaged that sesquiterpenes would be isolated as chemical constituents of this plant, in this study sesquiterpenes were not isolated.

3.4 EXPERIMENTAL

3.4.1 General experimental procedures

Analytical grade commercial solvents were used for extraction and chromatography. Ethyl acetate, dichloromethane (DCM), and methanol (MeOH) were used directly from the suppliers. Hexanes (a mixture of C_6 hydrocarbon isomers) was distilled before use.

Aluminium-backed thin-layer chromatography (TLC) plates (Kieselgel 60 F 254, 0.25 mm) were used to detect the different components in a mixture using non-destructive detection followed by dipping the plate in a reactive stain reagent and heating it with a heat gun. Non-destructive detection involves the examination of TLC plates containing a fluorescent indicator under a UV lamp at two different wavelengths, short wavelength (254 nm) and long wavelength (365 nm). An anisaldehyde/sulfuric acid stain reagent was used to detect compounds on TLC plates. This stain reagent was prepared by placing 845 mL of MeOH in a 1 L volumetric flask in an ice bath. While continuously shaking and maintaining a low temperature, the following reagents were added sequentially: acetic acid (100 mL), sulfuric acid (40 mL, dropwise), and anisaldehyde (5 mL, dropwise). This homogenous mixture was stored in a fridge.

Column chromatography was performed on glass columns packed with silica gel $60F_{254}$ (40-63 µm, Merck). The crude samples were applied to the column as dry material (adsorbed on silica gel) in case of the large amounts of crude plant extract or as a solution (dissolved in the solvent). Solvent systems with different polarities were used to elute the material, resulting in separation of compounds. Fig. 3.14 shows a summary of the general approach to column chromatography used in the isolation of the compounds.
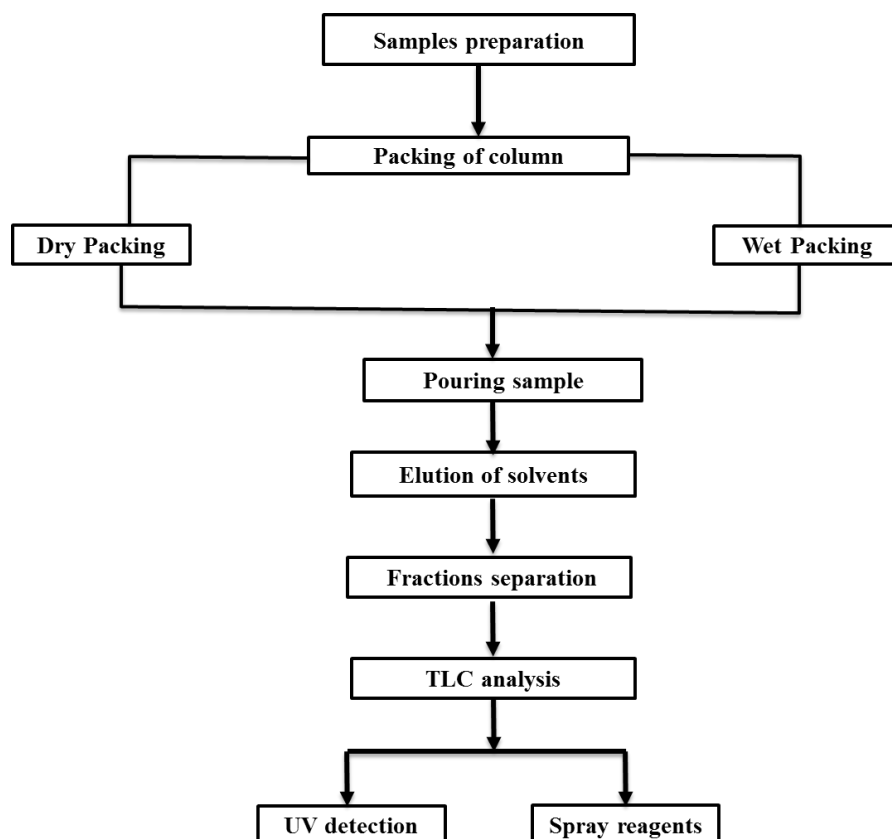
Figure 3.14.   A summary of column chromatography used for the isolation procedure.

Centrifugal chromatography, a modified *chromatographic technique* that combines the advantages of TLC and CC, was used to separate smaller amounts of compound mixtures. Centrifugal chromatography was performed on a Harrison Research Chromatotron®, model 7924. The circular plates used were coated with preparative silica gel (1, 2 or 4 mm thickness, Merck 7749 with binding agent) and dried in an oven at 50 °C before use. These plates were reused after a separation by washing it with MeOH and drying of the plates. The sample was dissolved in a small volume of eluting solvent and applied to the spinning adsorbent plate using the solvent system for development. Zones were detected by exposure of the plate to 254-265 nm UV light and fractions were collected in small vials.

For chromatography on Diol columns, Applied Separations SPE Diol columns (2 g/ 6 mL) were used. These columns were used to fractionate small fractions (100 mg) into 5 subfractions of different polarities. Diol is a stationary phase in which the silica is derivatised with 1,2-dihydroxypropane. This stationary phase is less polar than silica which allows it to be used for a wide range of compounds. It is also compatible with a wide range

of solvents from hexane through to water. The Diol [-OH] functional group is controlled and as such, provides more reproducible separation when compared to separation on silanols [-OH] from a bare silica surface.

NMR spectra were obtained on Bruker Avance III 500 (operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C) or Bruker Avance III 400 (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometers. The NMR experiments used for structural elucidation were ^{1}H, ^{13}C, COSY, NOESY, HSQC, HMBC, and DEPT-135. All spectra were recorded in deuterated chloroform and methanol at 30 ^{o}C using a 5 mm BBOZ probe or 5 mm TBIZ probe. Chemical shifts (δ) are given in ppm (part per million) relative to tetramethylsilane ($\delta = 0$) and are referenced to residual protonated solvent peaks, for CDCl$_3$: ^{1}H 7.26, ^{13}C, 77.0 and CD$_3$OD: ^{1}H 3.31, ^{13}C 49.1. Peak multiplicities were abbreviated as follows; s = singlet, d = doublet, dd = doublet of doublet, t = triplet, dt = doublet of triplet, and m = multiplet. Coupling constant (J) are given in Hz.

High-resolution mass spectra were collected on a Micromass LCT (Waters) spectrometer with electrospray ionization in either the positive or negative mode and with time-of-flight detection.

3.4.2 Collection of plant material

B. discolor plant material was collected on 8 April 2014 in the University of KwaZulu-Natal botanical garden and separated into leaves and branches. The leaves and branches were carefully examined for any damage. Healthy plant material was dried in a fume hood for two weeks. Once it was completely dry, the plant material was ground to a fine powder using a hammer mill and stored. *B. discolor* was identified by Ms Alison Young, curator of the botanical garden, University of KwaZulu-Natal, South Africa. A voucher specimen (Van Heerden 25) was deposited in the Bews Herbarium, Pietermaritzburg, UKZN.

3.4.3 Extraction of plant material

The ground leaves (350 g) were extracted at room temperature for 24 h with a mixture of DCM-MeOH (1:1) (750 mL). The plant extract was filtered using Whatman no. 1 filter paper and the filtrate was taken to dryness on a rotovap with the water bath set at a temperature of 45 $^{\circ}$C. The concentrated extract was dried under high vacuum and 19.405 g

dry extract was obtained for the leaves. The residual plant material after filtration was then extracted with MeOH (750 mL) to yield 9.947 g of MeOH extract of the leaves. The dried extracts were stored in the fridge and used as a stock for appropriate applications.

3.4.4 Fractionation by SPE Diol column

To obtain fractions with different polarities, 100 mg of crude extract was dissolved in DCM-MeOH (1:1) (1 mL per 100 mg of extract per column) and the solution was applied to a Diol SPE column. To make sure there is no residual solvent present, the column was left to stand overnight and after that, a slight vacuum was applied for 15 min. A sequential vacuum elution of the column was performed with the 6 mL of each of the following solvent systems: hexanes-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1), and MeOH. Each fraction was transferred to a pre-weighed flask and the solvent was evaporated under vacuum. The dry fractions were weighed, transferred to a 2 mL capped vial and stored at 0 °C until further analysis. Table 3.4 shows the fractions obtained from the DCM-MeOH leave extract, their weight and colour description. These fractions were analysed by LC-MS and were used for the antidiabetic assay.

Table 3.4.   Fractions obtained from the crude DCM-MeOH extract of *B. discolor* after separation on a SPE Diol column.

Fractions	Mass of extract (mg)	Description
SA-1-5A	19.9	Dark green
SA-1-5B	9	Dark green
SA-1-5C	5.2	Light green
SA-1-5D	10.8	Yellow
SA-1-5E	46.9	Yellow
	Total mass recovered = 91.8 mg	

3.4.5   Isolation of pure compounds

The DCM-MeOH (1:1) extract of the leaves of *B. discolor* (3 g) was subjected to column chromatography over TLC grade silica gel. Five fractions (A, B, C, D, and E) were obtained after elution with solvents in order of increasing polarity, Hex-DCM (9:1), DCM-EtOAc (20:1), EtOAc (100%), EtOAc-MeOH (5:1), and MeOH (100%). All fractions were

collected and combined after TLC analysis and weighed to yield; A (164 mg), B (294 mg), C (656 mg), D (442 mg), and E (1150 mg). Fraction A was fractionated on the Chromatotron (2 mm plate) eluting with hexane-ethyl acetate (98:2) to yield a mixture of lupeol acetate (**3.44**) and its Δ^{12} isomer, neolupenyl acetate (**3.45**) as a mixture of compounds with the same R_f value (9 mg). The compounds were detected on TLC plates by spraying with anisaldehyde reagent and heating it. Fraction B was subjected to column chromatography (20 g of silica gel) with Hex-DCM (4:2) as the starting eluent and increasing the polarity to 1:1 ratio of the two solvents. Two compounds were obtained and they were identified as β-sitosterol (**3.46**) and α-tocopherol (**3.47**). Fractions C and D were extremely complex mixtures of compounds and were not investigated further. Fraction E resulted from MeOH (100%) as eluant. Column chromatography of 150 mg of this fraction using DCM-MeOH (9:1), (4:1), (2:1), and (1:1) as eluent resulted in the isolation of two fractions. One fraction contained a single compound, genkwanin-5-*O*-β-D-glucopyranoside (**3.48**), whereas the other fraction consisted of a mixture of two compounds, the α- and β-isomers of glucose (**3.49**) and (**3.50**), respectively.

The isolation of the compounds from the crude extract are summarised in Fig 3.15
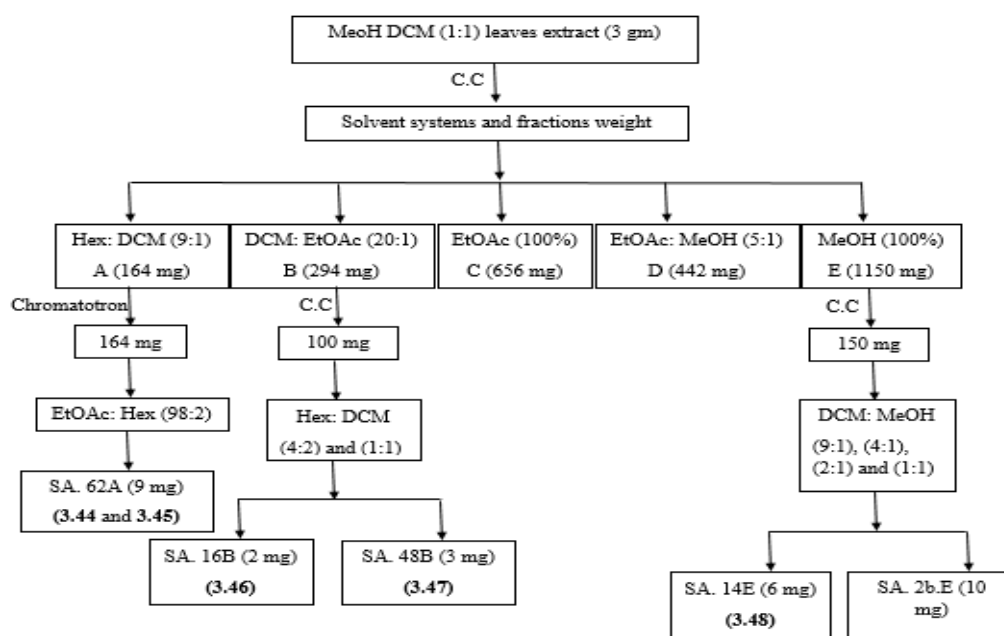
Figure 3.15.   A summary of the isolation procedure of the compounds.

The physical data for the compounds were as follows:

Physical data for lupeol acetate (3.44) and its Δ^{12} isomer (3.45)

Physical description: Needles

Melting Point: 211-218°C

Yield: 9 mg

Molecular formula: $C_{32}H_{52}O_2$

NMR data:

^{1}H NMR: Plate 1A

^{13}C NMR: Plate 1B

DEPT: Plate 1C

COSY: Plate 1D

HSQC: Plate 1E

HMBC: Plate 1G

NOESY: Plate 1H

Physical data β-sitosteryl linolenate (3.46)

Physical description: White

Molecular formula: $C_{47}H_{78}O_2$

Yield: 2 mg

^{1}H NMR: Plate 2A

^{13}C NMR: Plate 2B

D COSY: Plate 2D

DEPT: Plate 2C

HSQC: Plate 2E

HMBC: Plate 2G

NOESY: Plate 2H

Physical data α-tocopherol (3.47)

Physical description: Oil

Yield: 3 mg

Molecular formula: $C_{29}H_{50}O_2$

ESI-(-)-TOF MS: Observed 429.3737 [M-H]$^-$; calc. for $C_{29}H_{49}O_2$ 429.3733

^{1}H NMR: Plate 3A

^{13}C NMR: Plate 3B

COSY: Plate 3D

HSQC: Plate 3E

HMBC: Plate 3G

NOESY: Plate 3H

Physical data of genkwanin 5-*O*-β-primeveroside (3.48)

Physical description: Pale brown

Yield: 6 mg

Melting Point: 200 ˚C

Molecular formula: $C_{27}H_{30}O_{14}$

ESI-(-)-TOF MS: Observed: 557.1554 [M-H]⁻; calc. for $C_{27}H_{29}O_{14}$ 577.1557

^{1}H NMR: Plate 4A

^{13}C NMR: Plate 4B

DEPT: Plate 4C

COSY: Plate 4D

HSQC: Plate 4E

HMBC: Plate 4G

NOESY: Plate 4H

α- and β-D-Glucopyranose (3.49 and 3.50)

Physical description: white

Molecular formula: $C_6H_{12}O_6$

Yield: 10 mg

Melting Point: 146 ˚C

^{1}H NMR: Plate 5A

^{13}C NMR: Plate 5B

DEPT: Plate 5C

COSY: Plate 5D

HSQC: Plate 5E

HMBC: Plate 5G

NOESY: Plate 5H

3.4.5 Evaluation of antidiabetic effects of the crude extract and DIOL column fractions

The effect of extracts and fractions on the glucose utilization in muscle cell line (C2C12) was used as an indication of antidiabetic effect of an extract or fractions. These experiments were performed by Ntethelelo Sibiya under the supervision of the late Prof C T Musambayane in the Physiology Research Group in the School of Laboratory Medicine and Medical Science, Westville Campus, University of KwaZulu-Natal. The effects of the crude extract of the leaves *B. discolor* and the fractions obtained after separation on a diol column fractions (2A (crude), A, B, C, D, and E) were evaluated by the procedure described below and the results are graphically presented in Fig. 3.15

The muscle (C2C12) cell lines used for the study was donated by the Medical Research Council, Cape Town, South Africa. The cells were cultured in 25 mL flasks in DMEM (Dulbecco's modified Eagle's medium). Cells were used within 10 passage numbers to limit batch-to-batch variation. Culture media were supplemented with 10% foetal calf serum (FCS), 1% L-glutamate, and 1% penicillin/ streptomycin/ fungizone.

Cell culture was conducted using a well-established cell culture protocol. Briefly, muscle cells were cultured in 10 mL DMEM at 37 ºC, 5% CO_2, and 89% relative humidity. The cells could grow and become confluent; thereafter the confluent cells were trypsinised with trypsin (1 mL) after washing three times with PBS. The trypsinised cells were sub-cultured into new flasks and some were stored in 10% DMSO at -80 ºC for subsequent studies.

For the experiment, the muscle (C2C12) cells were seeded at a density of 1.5×10^5 cells/mL in a 24-well cell culture plate. The cells were allowed to grow and reach an 80% confluence monolayer. The cells were divided as follows: group 1 was treated with media containing glucose only, which served as the control, group 2 was treated with a standard drug (insulin at 0.04 mg/mL), and group 3 was treated with plant extract at a single dose of 50 µg/mL. This experimental protocol was followed for the crude plant extract and fractions obtained from the diol column. The glucose utilization experiment was performed as previously described by Van de Venter et al.[52] with slight modifications. The DMEM contained 28 mmol/L glucose. After seeded cells reached 80% confluence, the old media was replaced with fresh media. Plant extracts (100 µL) and plant extracts + insulin (100 µL) was added

into the media (1 mL) for treatment in each well. The culture plate was then incubated under the above-mentioned conditions. Glucose concentrations were measured at 0, 12, 24, and 48 hour intervals using a glucometer.

CHAPTER 4: CONCLUSION

Diabetes mellitus remains a major health problem and a leading cause of death worldwide. The number of people living with diabetes is increasing exponentially day after day. Many of the current antidiabetic drugs are based on synthetic compounds which are most likely to have side effects. Although a limited number of effective antidiabetic drugs from plant sources are currently in use, there is still a need for developing effective, safe, and cheap antidiabetic drugs. The aim of this thesis was to investigate *Brachylaena discolor* DC, a plant used traditionally for the treatment of diabetes with the specific objectives to confirm the antidiabetic activity of an extract of the leaves of *B. discolor*, isolate the compounds in the extract and determine the structures of the isolated compounds.

The *in vitro* assaying of the DCM-MeOH extracts of the leaves of *B. discolor*, showed that it had high antidiabetic activity against the C2C12 muscle cells line. The crude extract yielded five fractions (A, B, C, D, and E) which led to the isolation of two compounds characterised as lupeol acetate and its Δ^{12} isomer from fraction A. Fraction B also yielded two compounds identified as β-sitosterol and α-tocopherol. Fractions C and D showed a mixture of compounds which were not further investigated in this study. Finally, fraction E afford genkwanin 5-*O*-β-D-glucopyranoside and a mixture of two compounds, the α- and β-anomers of glucose. The antidiabetic activity observed in the DCM-MeOH extract can most likely be explained by the presence of these compounds. The available literature showed that lupeol acetate, β-sitosterol, and α-tocopherol have antidiabetic properties. The isolation of the two lupeol isomers is of particular interest since lupeol is considered as a possible drug for diabetes based on its inhibition of the enzyme protein tyrosine phosphatase (PTP1B).[175]

The results of this study confirm the traditional use of *B. discolor* to treat diabetes. While these results provide important findings, without doubt each research has some limitations which call for future studies. Therefore, the focus of future research could be on the investigation of other plant parts such as the branches and roots. The assays on both the extracts and pure compounds could be extended to the evaluation of the isolated compounds on C2C12 muscle cells line to determine which compounds has the best antidiabetic properties.

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Plate 1A. ¹H NMR spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45) in CDCl₃.
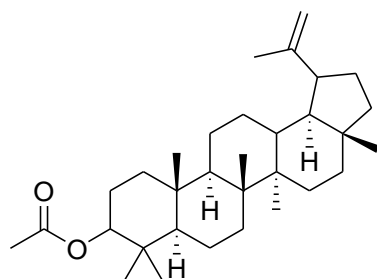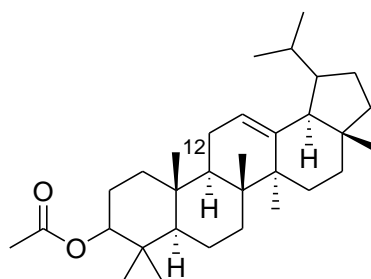
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
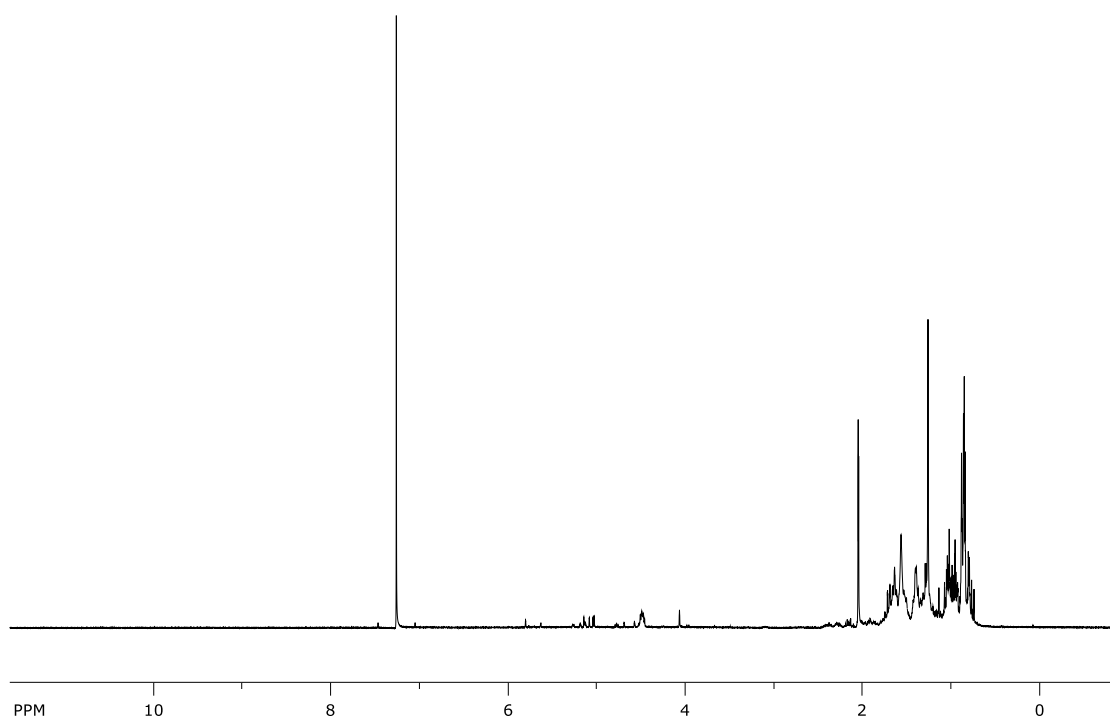
Plate 1B. ^{13}C NMR spectrum lupeol acetate (3.44) and its Δ^{12} isomer (3.45).
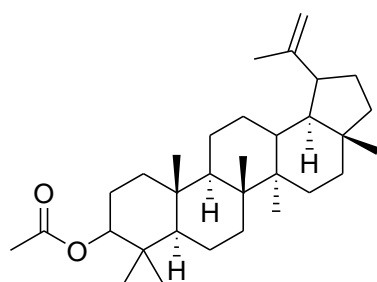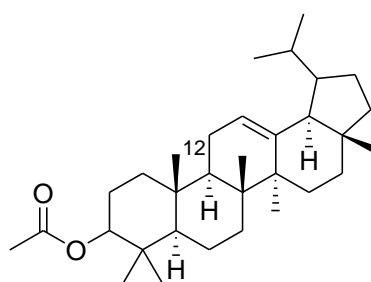
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
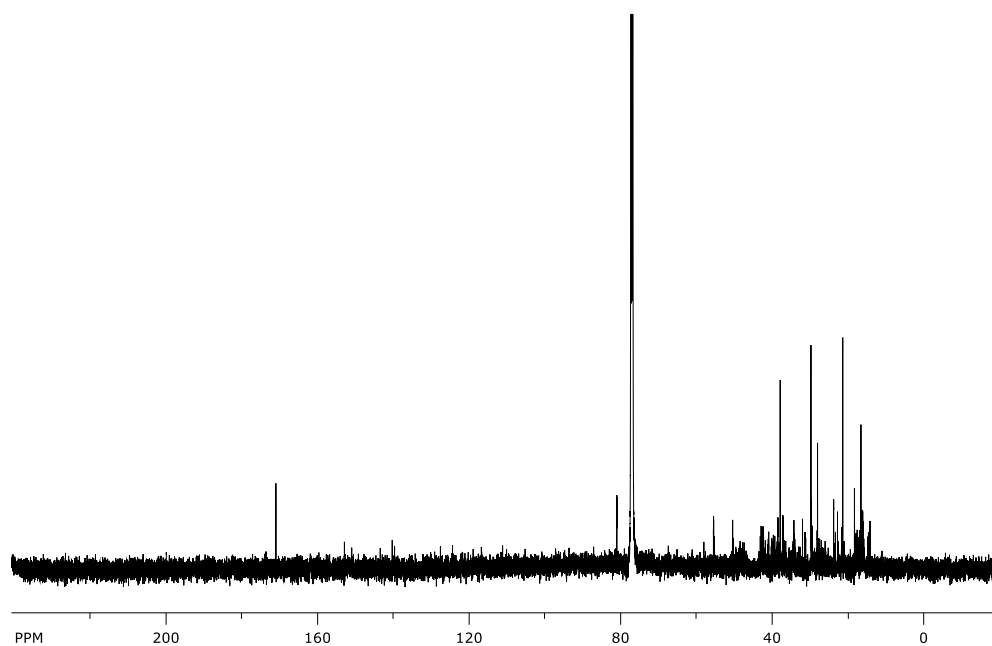
Plate 1C: DEPT-135 spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45).
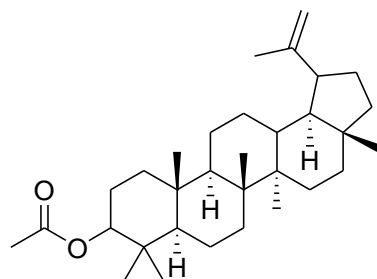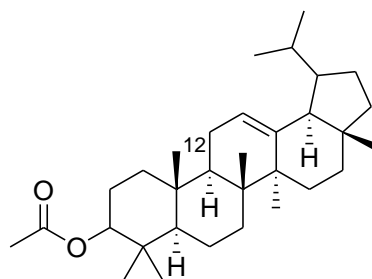
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
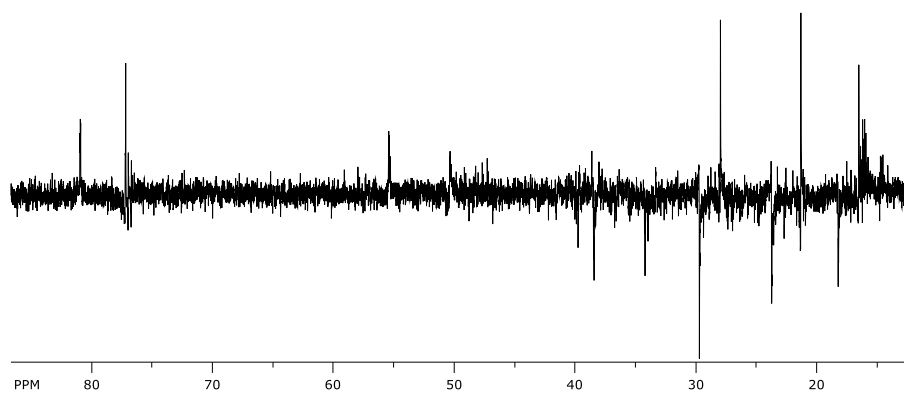
Plate 1D. COSY spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45).
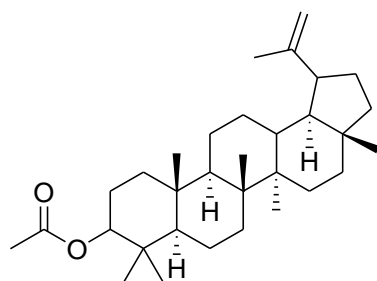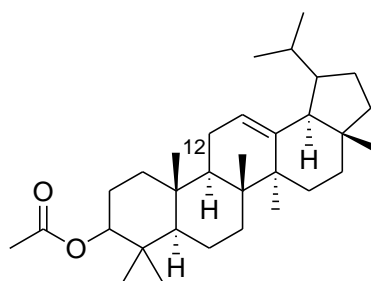
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
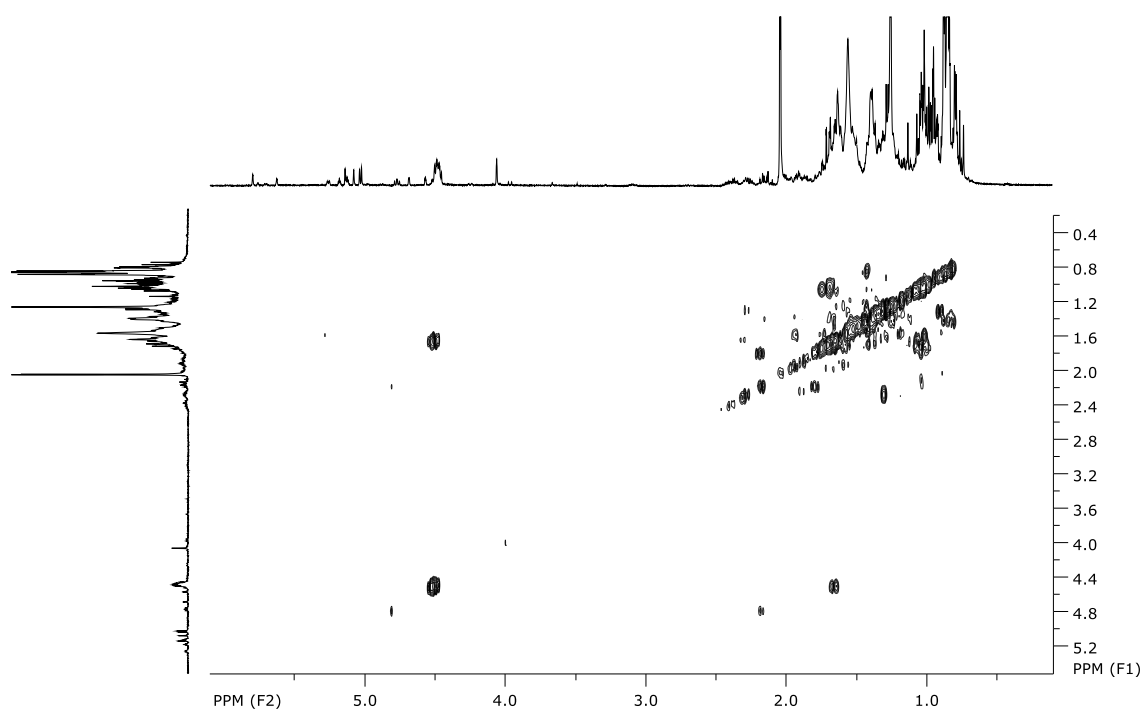
Plate 1E. HSQC spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45) in CDCl₃.
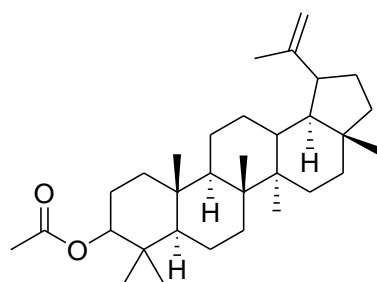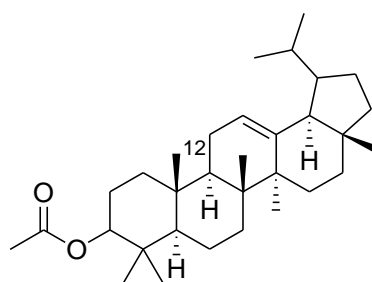
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
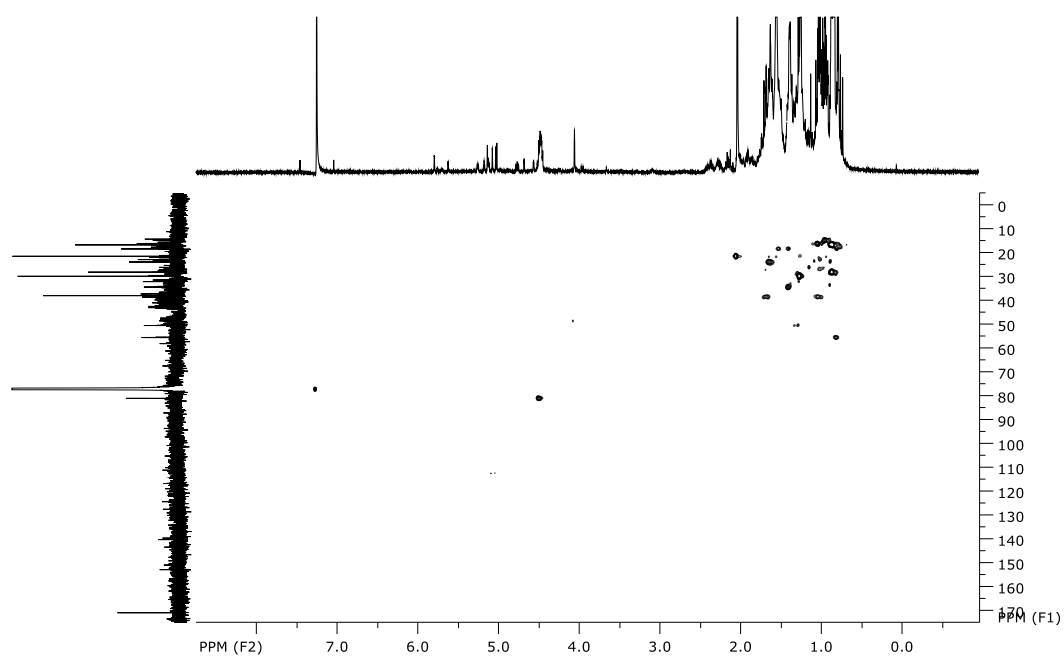
Plate 1F. HMBC spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45) in CDCl$_3$.
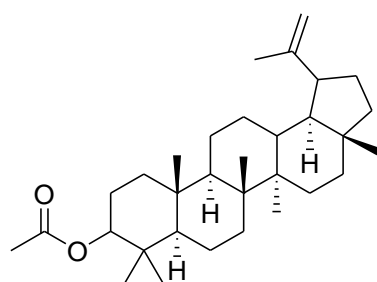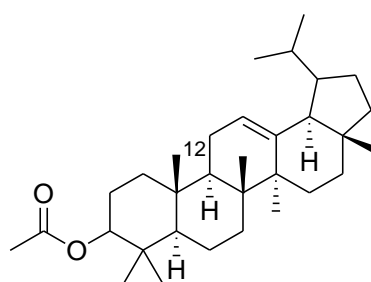
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
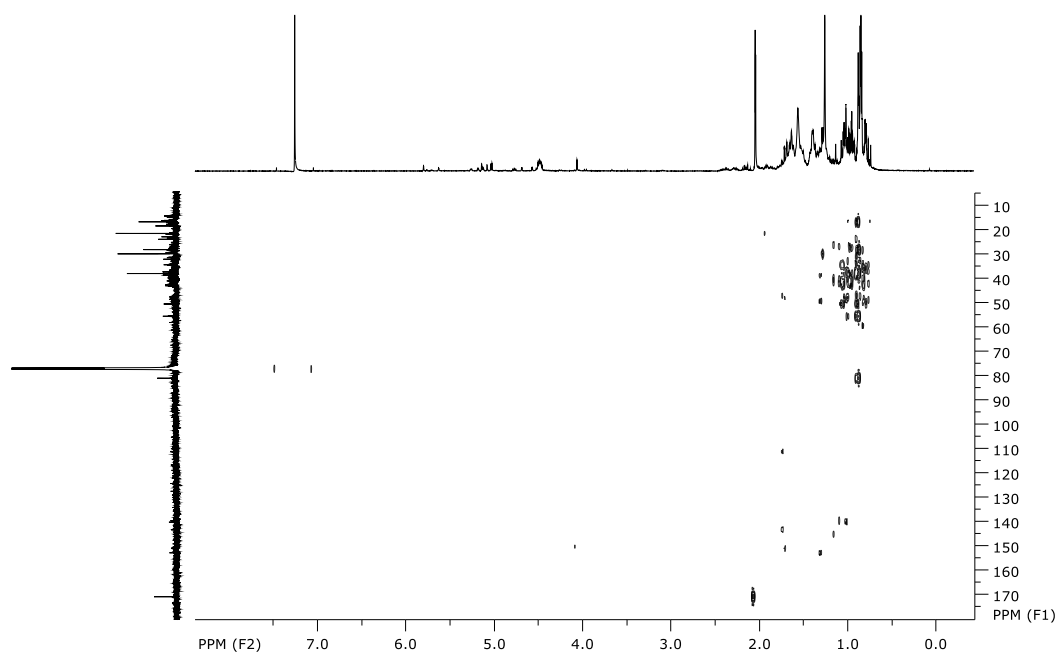
Plate 1G. NOESY spectrum of lupeol acetate (3.44) and its Δ^{12} isomer (3.45) in CDCl₃.
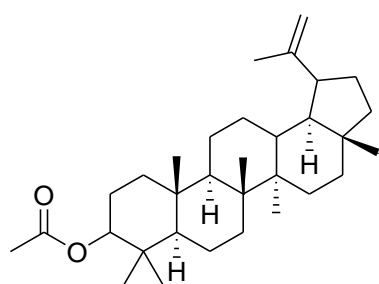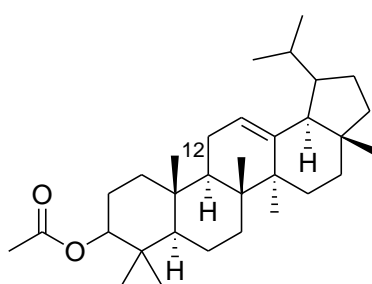
Lupeol acetate **(3.44)**   3β-Acetoxy-12-lupene **(3.45)**
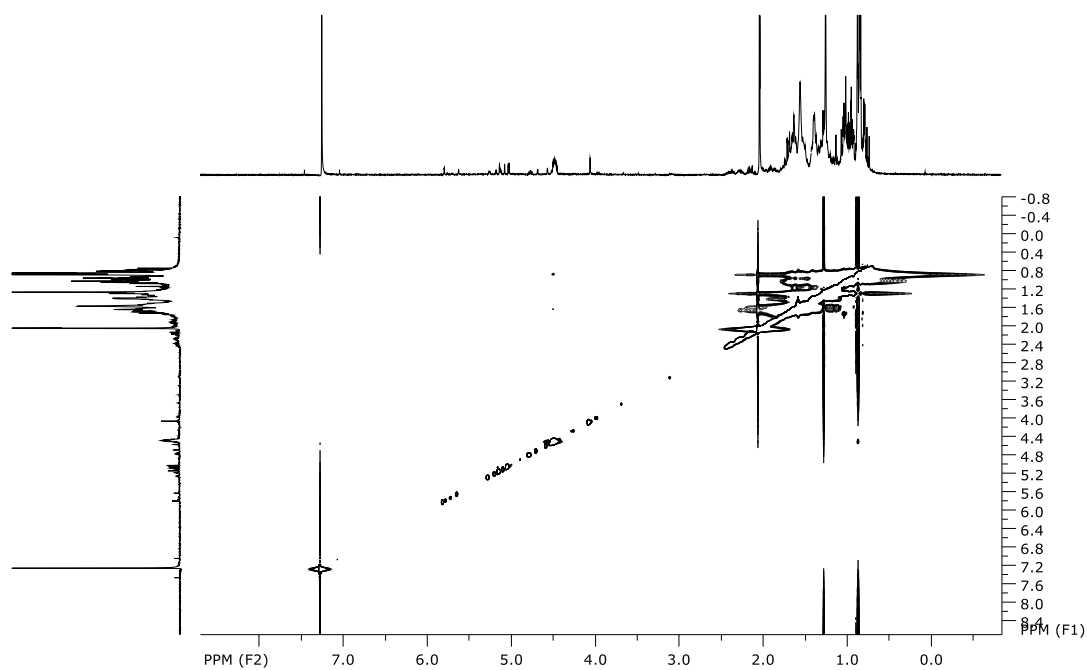
Plate 2A: ^{1}H NMR spectrum β-sitosteryl linolenate (3.46) in CDCl$_3$.
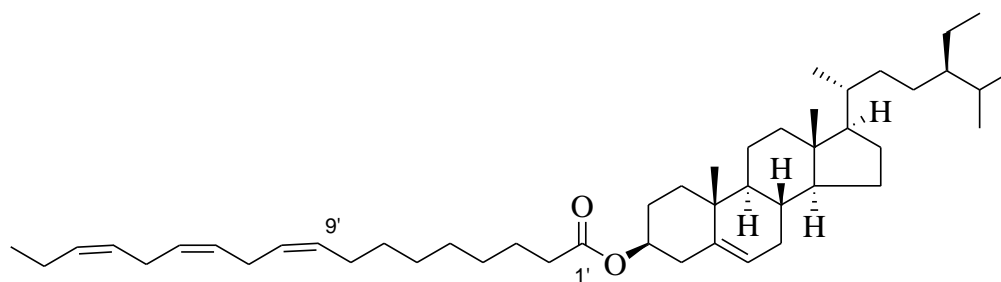
β-Sitosteryl linolenate **(3.46)**
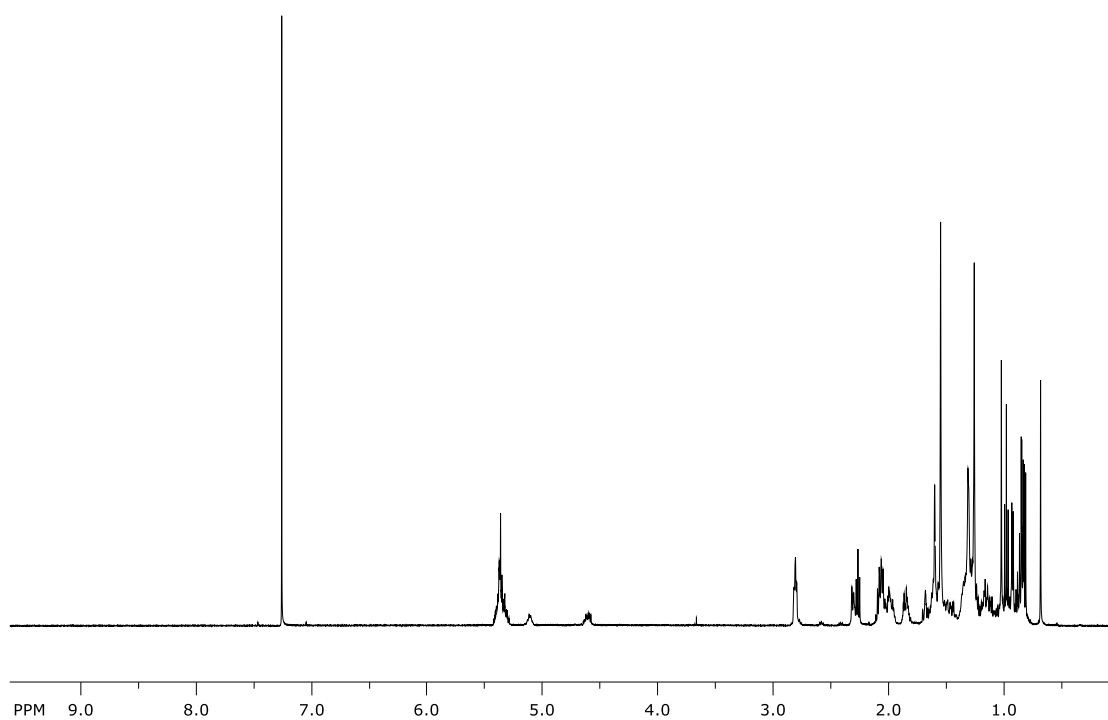
Plate 2B: ^{13}C NMR spectrum β-sitosteryl linolenate (3.46) in CDCl₃.

β-Sitosteryl linolenate **(3.46)**
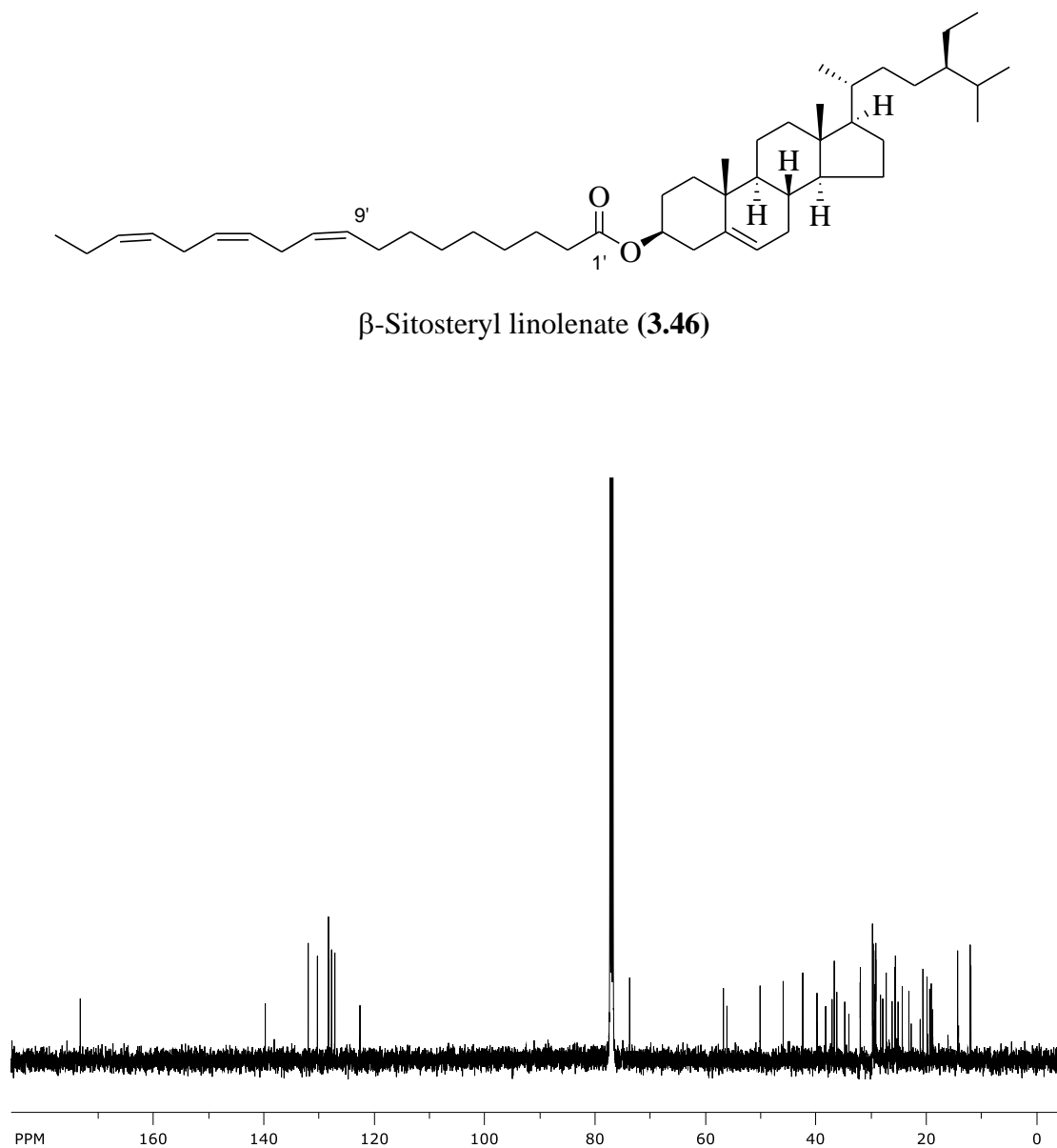
Plate 2D: COSY spectrum of β-sitosteryl linolenate (3.46) in CDCl₃.
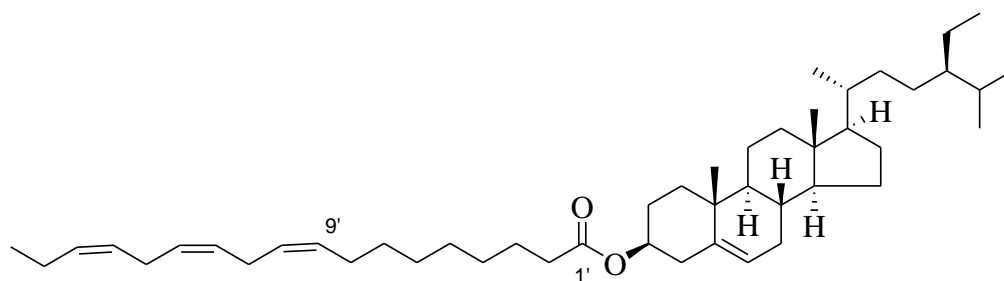
β-Sitosteryl linolenate **(3.46)**
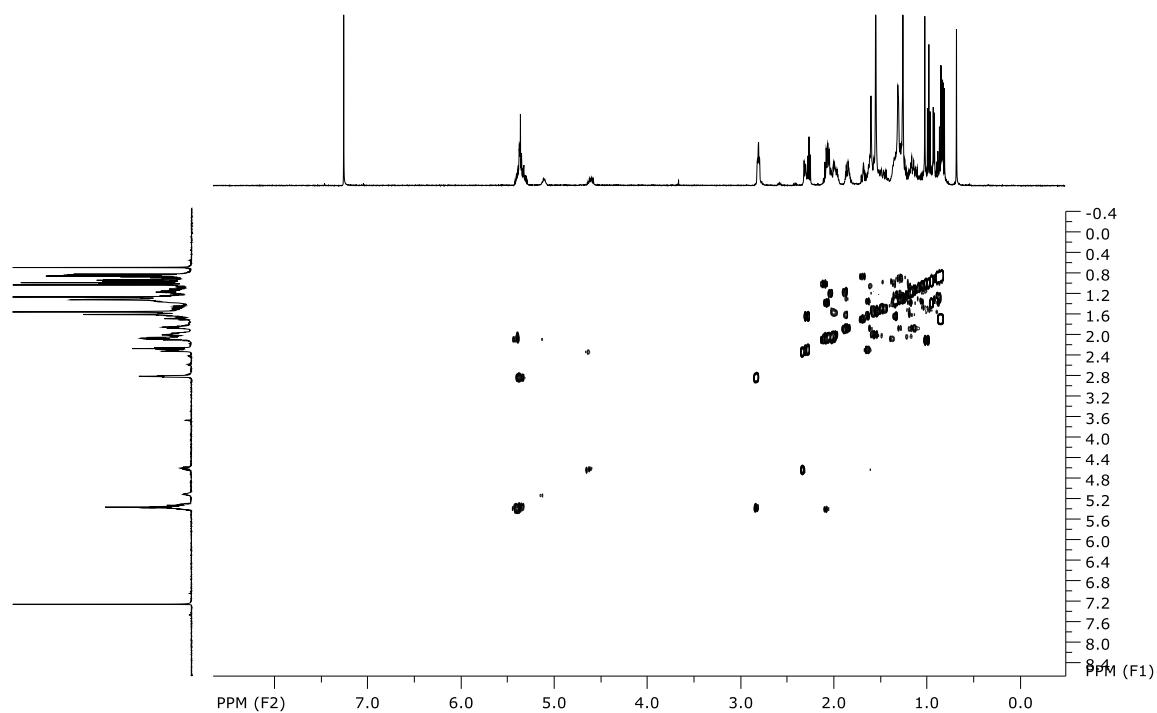
Plate 2E: HSQC spectrum of β-sitosteryl linolenate (3.46) in CDCl₃.
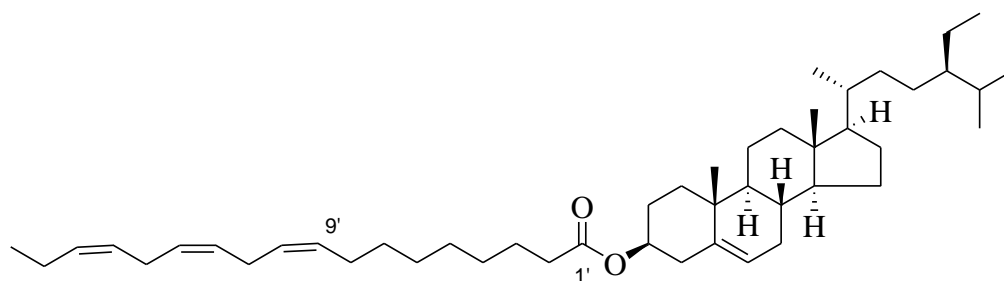
β-Sitosteryl linolenate **(3.46)**
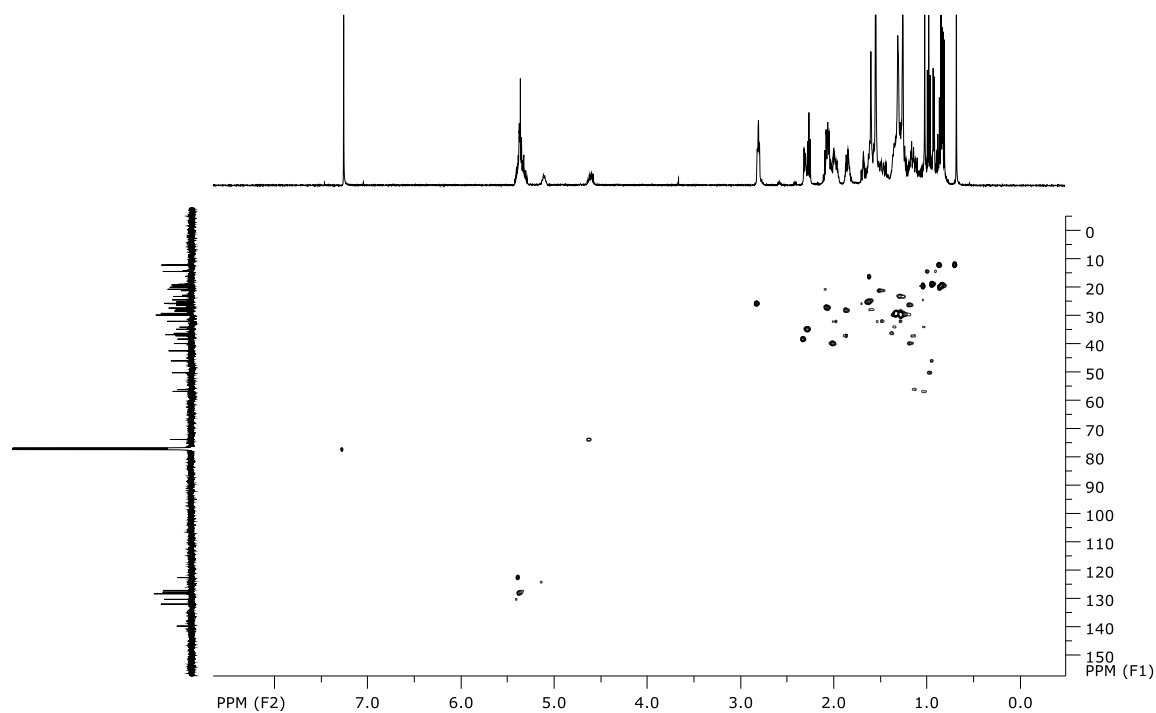
Plate 2G: HMBC spectrum of β-sitosteryl linolenate (3.46) in CDCl₃.
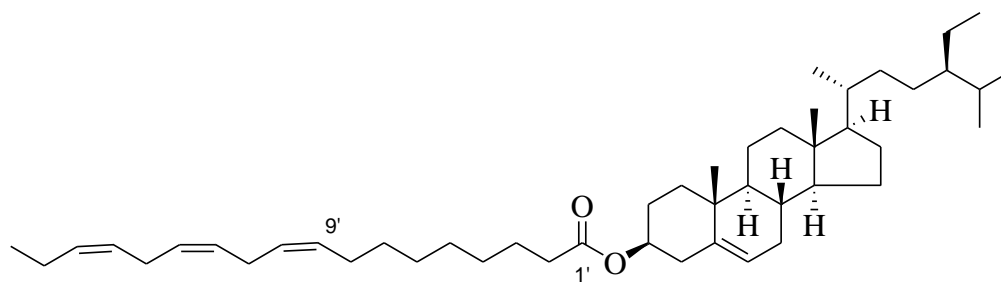
β-Sitosteryl linolenate **(3.46)**
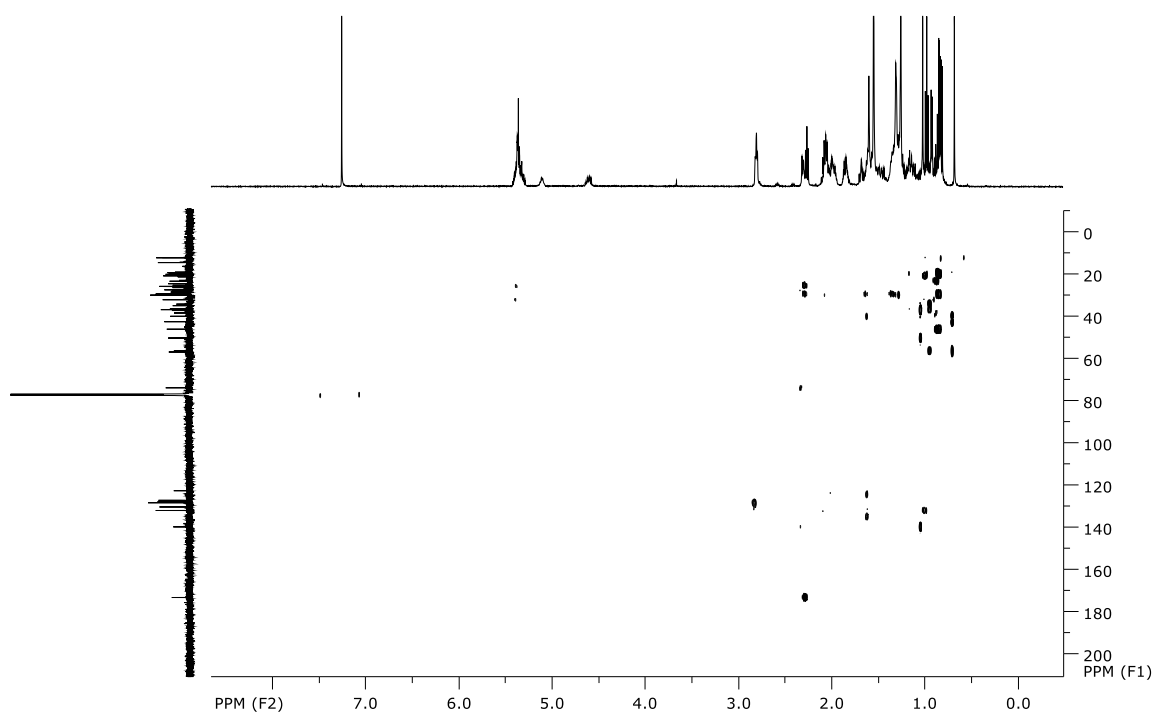
Plate 3A: ¹H NMR spectrum of α-tocopherol (3.47) in CDCl₃.
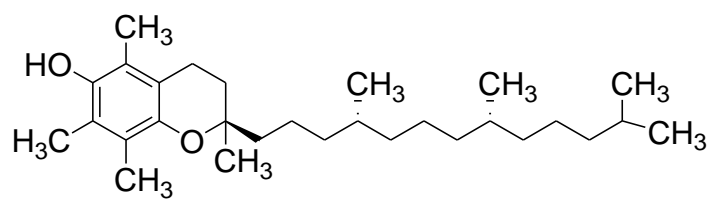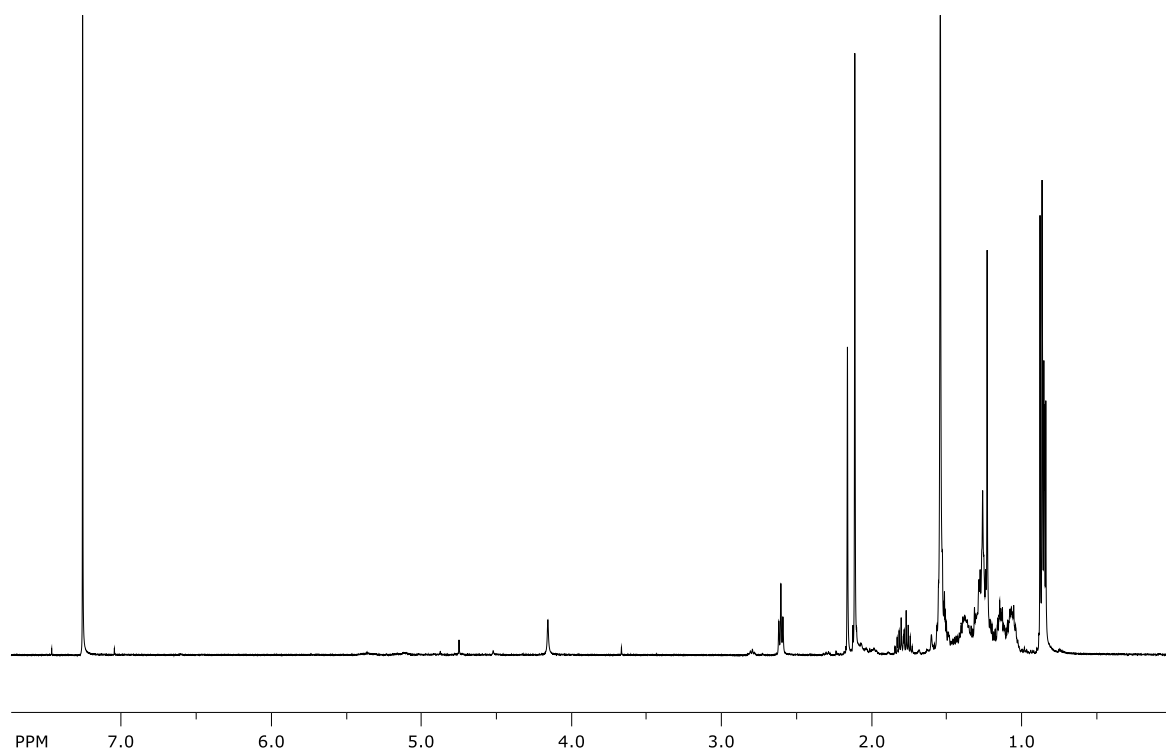
α-tocopherol **(3.47)**

Plate 3B: ^{13}C NMR spectrum of α-tocopherol (3.47) in CDCl₃.
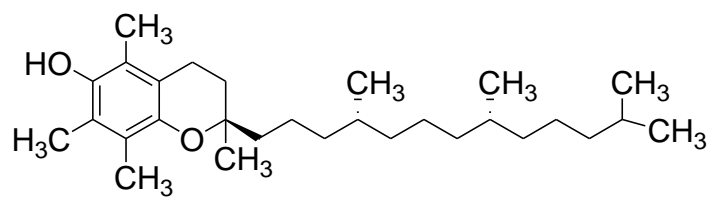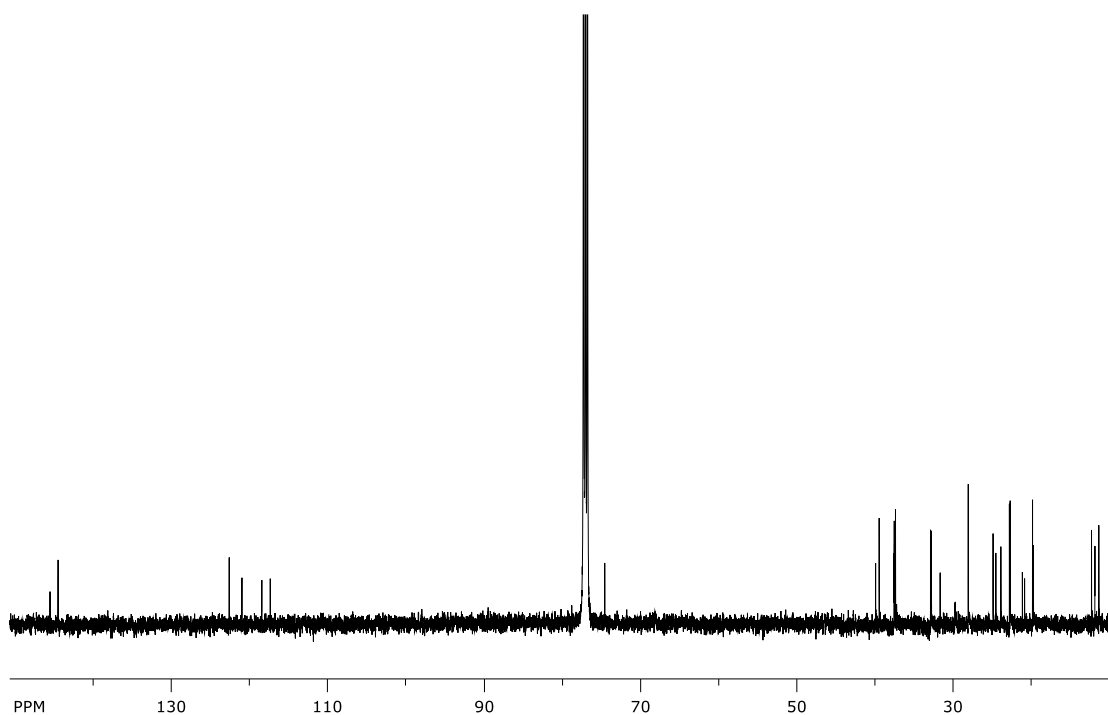
α-tocopherol **(3.47)**

Plate 3D: COSY spectrum of α-tocopherol (3.47) in CDCl₃.

α-tocopherol **(3.47)**

Plate 3E: HSQC spectrum of α-tocopherol (3.47) in CDCl₃.

α-tocopherol **(3.47)**

Plate 3G: HMBC spectrum of α-tocopherol (3.47) in CDCl₃.
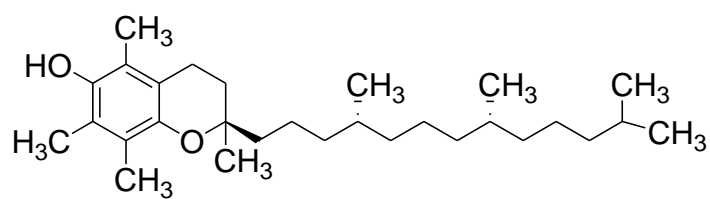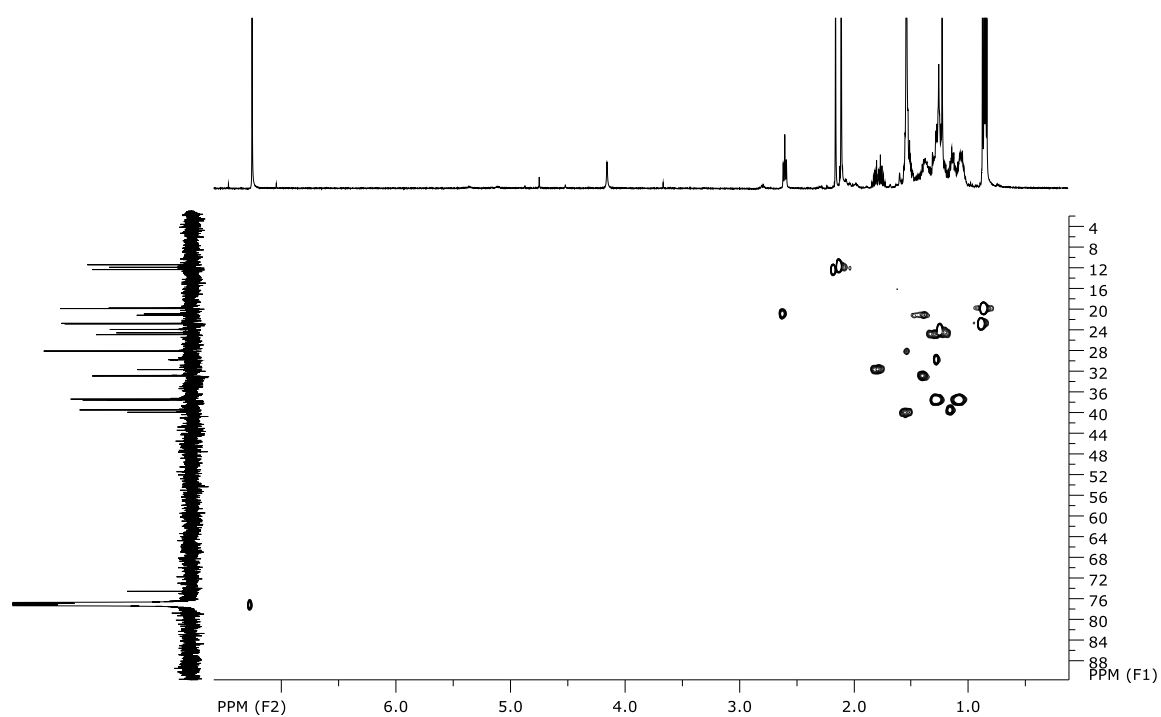
α-tocopherol **(3.47)**

Plate 3H: NOESY spectrum of α-tocopherol (3.47) in CDCl₃.
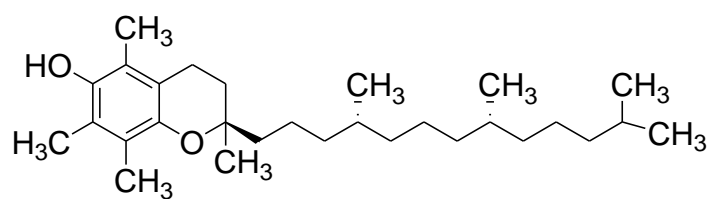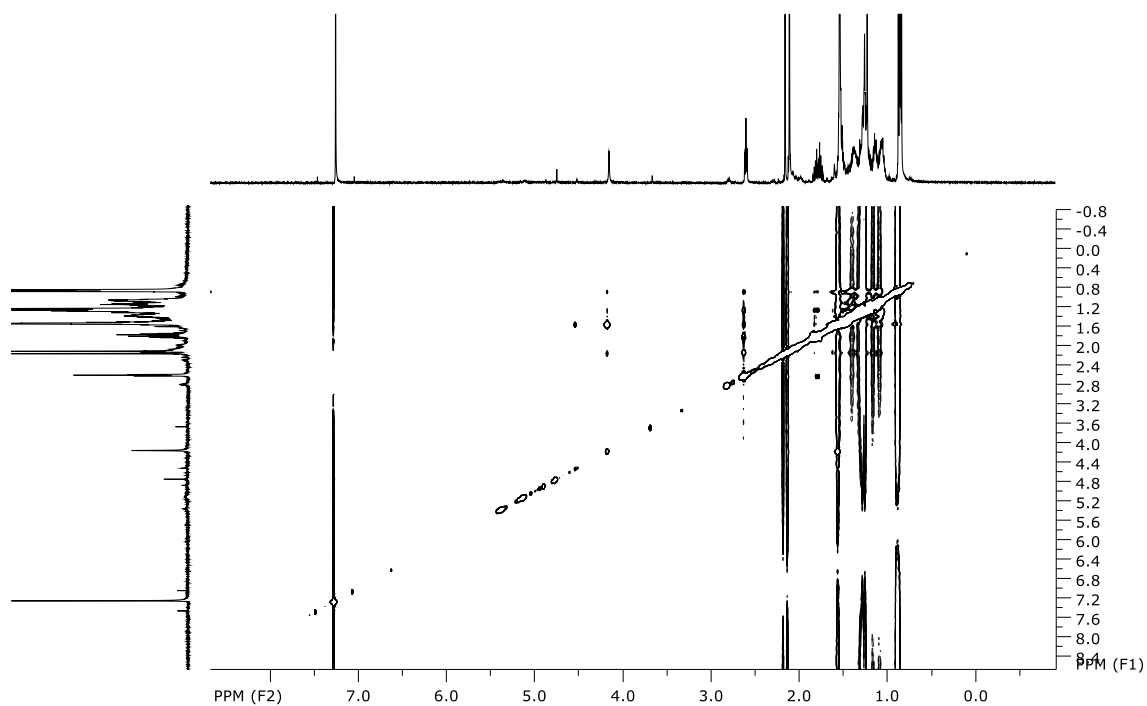
α-tocopherol (**3.47**)

Plate 3I: High-resolution mass spectrum (ESI-) of α-tocopherol (3.47).

TOF MS ES-   2.89e+005

Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
429.3737	429.3733	0.4	0.9	5.5	560.0	0.0	C29 H49 O2
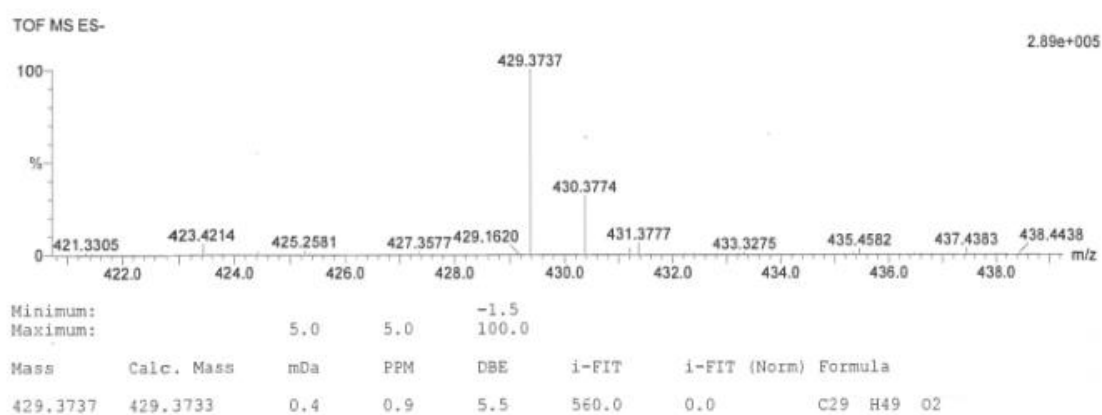
Plate 4A: ^{1}H NMR spectrum of genkwanin 5-*O*-β-primeveroside (3.48) in CD₃OD.
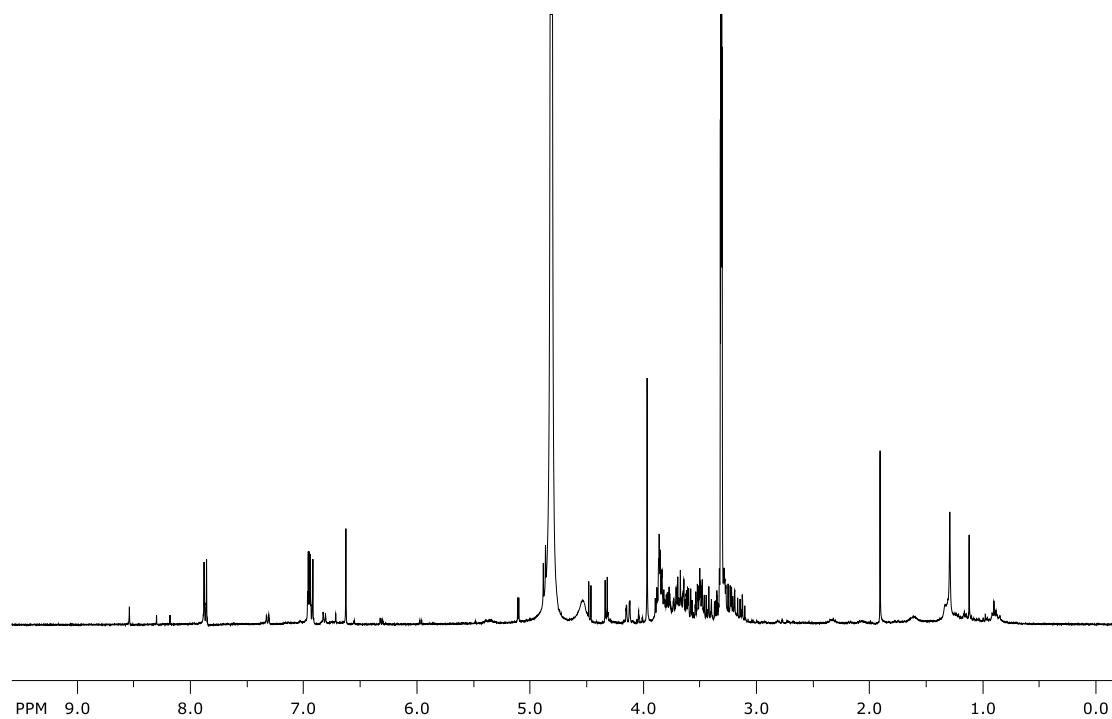
Plate 4B: ^{13}C NMR spectrum of genkwanin 5-*O*-β-primeveroside (3.48) in CD₃OD.
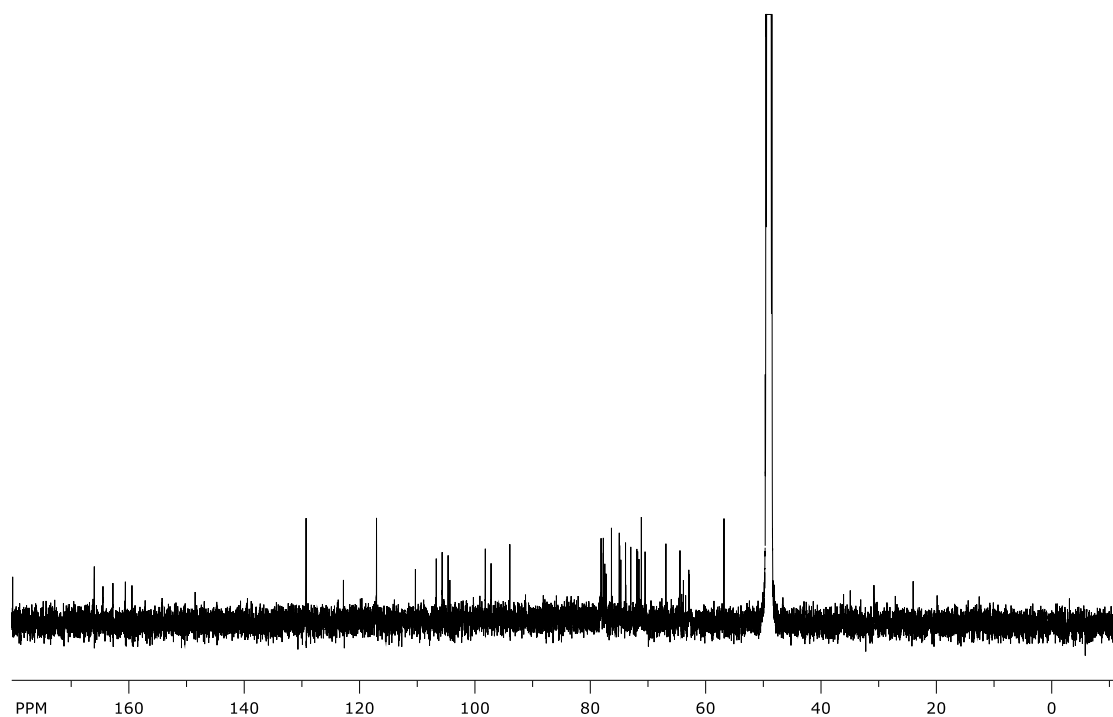
Plate 4C: DEPT spectrum of genkwanin 5-*O*-β-primeveroside (3.48) in CD₃OD.
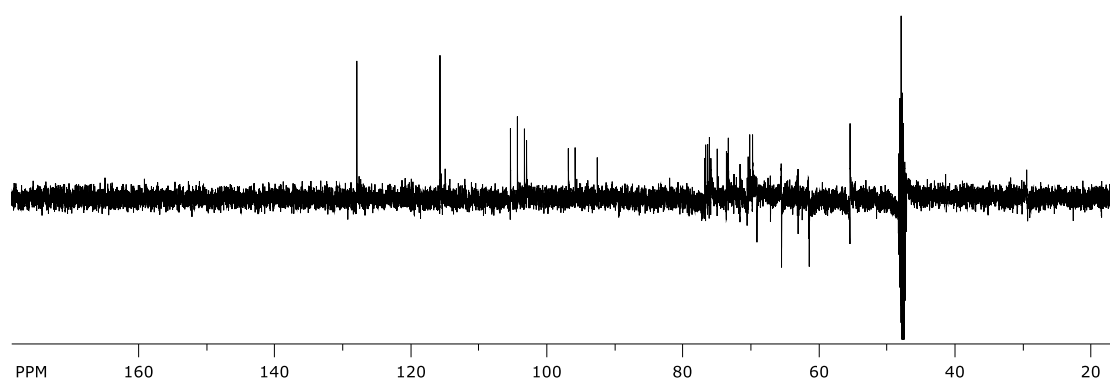
Plate 4D: COSY spectrum of genkwanin 5-*O*-β-primeveroside (3.48) in CD₃OD.
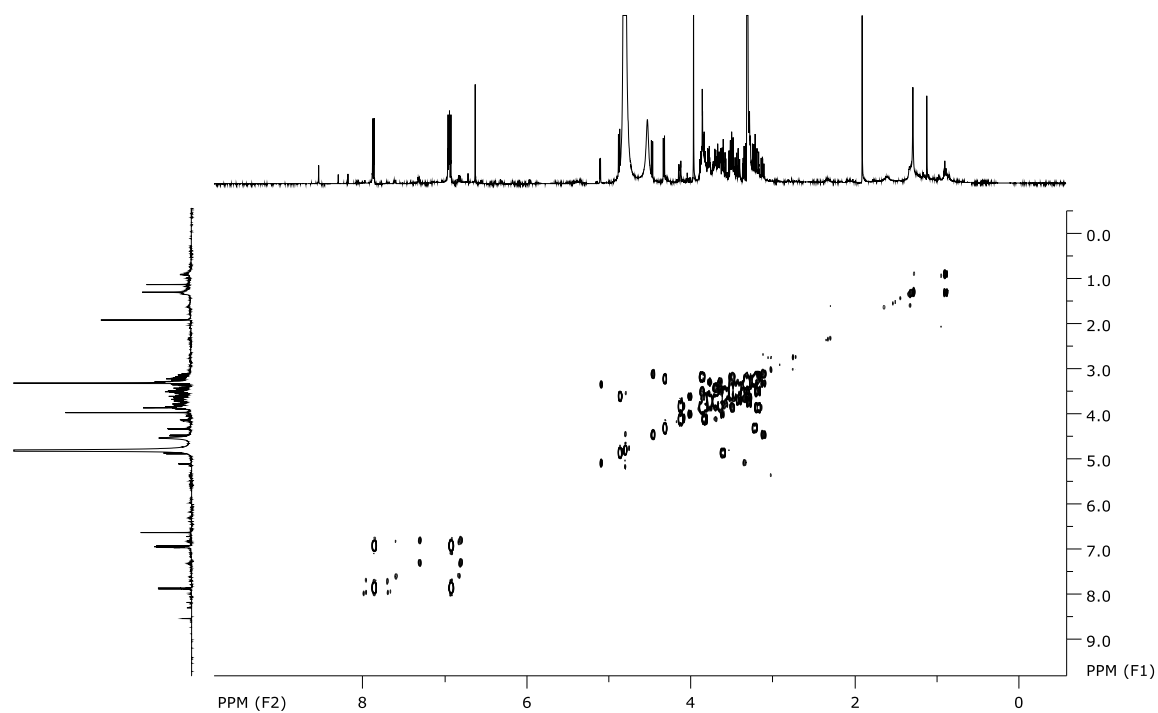
Plate 4F: HMBC spectrum of genkwanin 5-*O*-β-primeveroside (3.48) in CD₃OD.
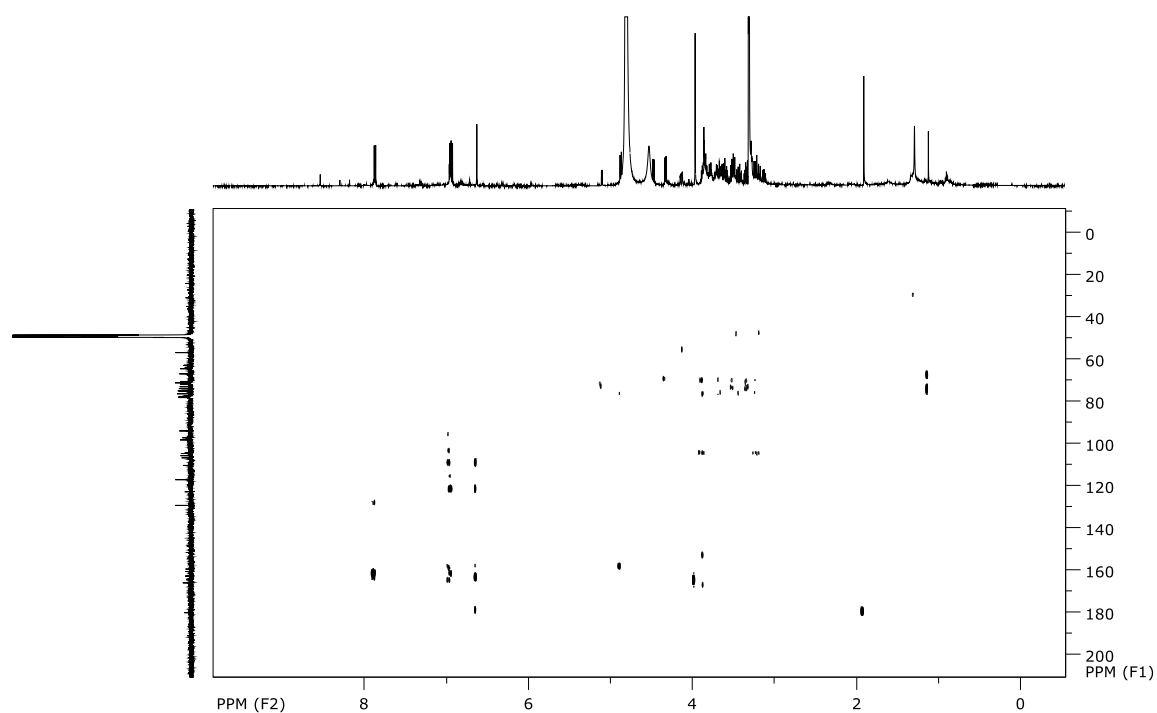
Plate 4I: High-resolution mass spectrum (ESI-) of genkwanin 5-*O*-β-primeveroside (3.48).

TOF MS ES-
3.08e+005

Minimum:			-1.5
Maximum:	5.0	5.0	100.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
577.1554	577.1557	-0.3	-0.5	13.5	522.8	0.0	C27 H29 O14
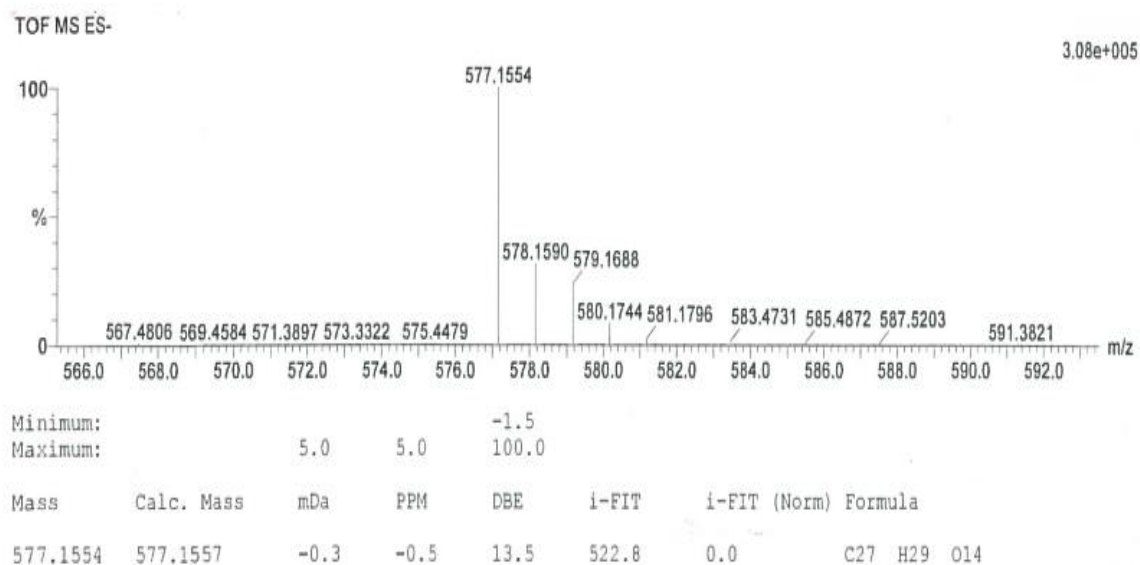
Plate 5A: ^{1}H NMR spectrum of α- and β-D-glucopyranose (3.49) and (3.50) in CD₃OD.
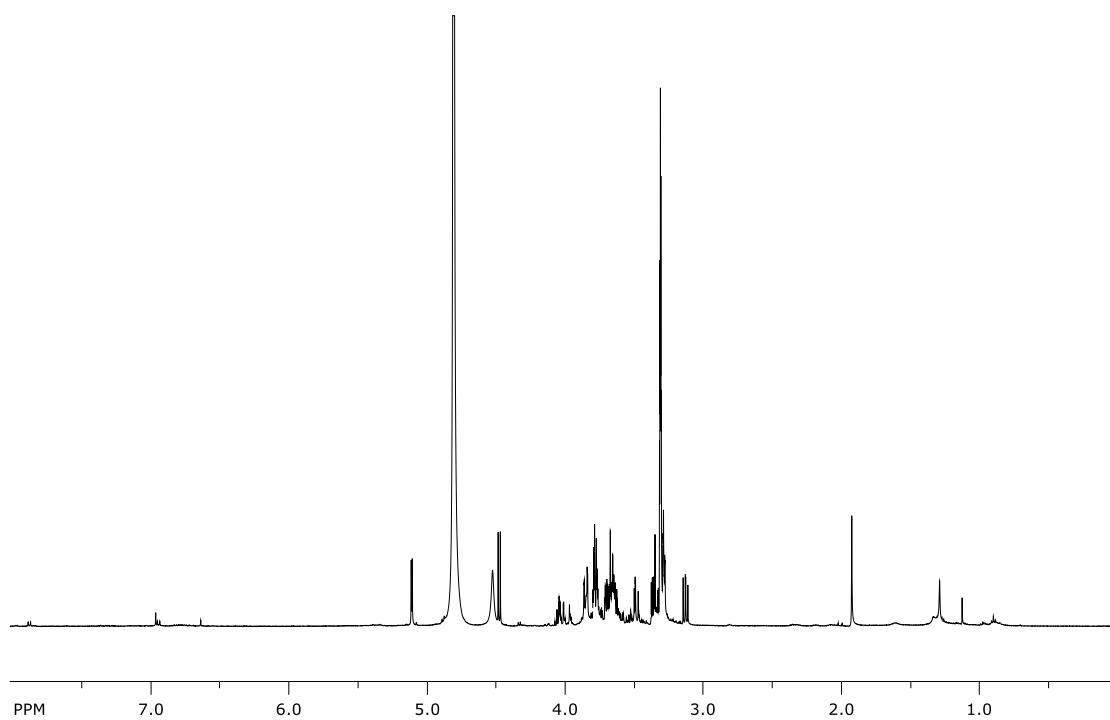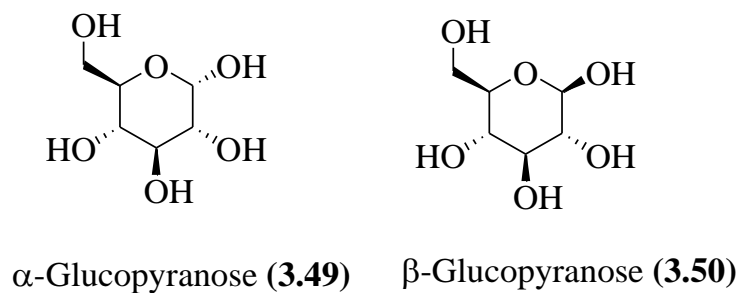
α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**

Plate 5B: ^{13}C NMR α- and β-D-glucopyranose (3.49) and (3.50) in CD₃OD.

α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**
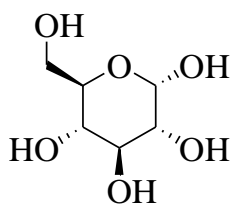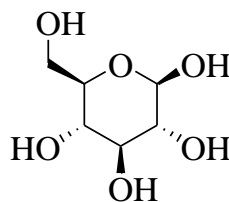
PPM   110   100   90   80   70   60   50   40   30
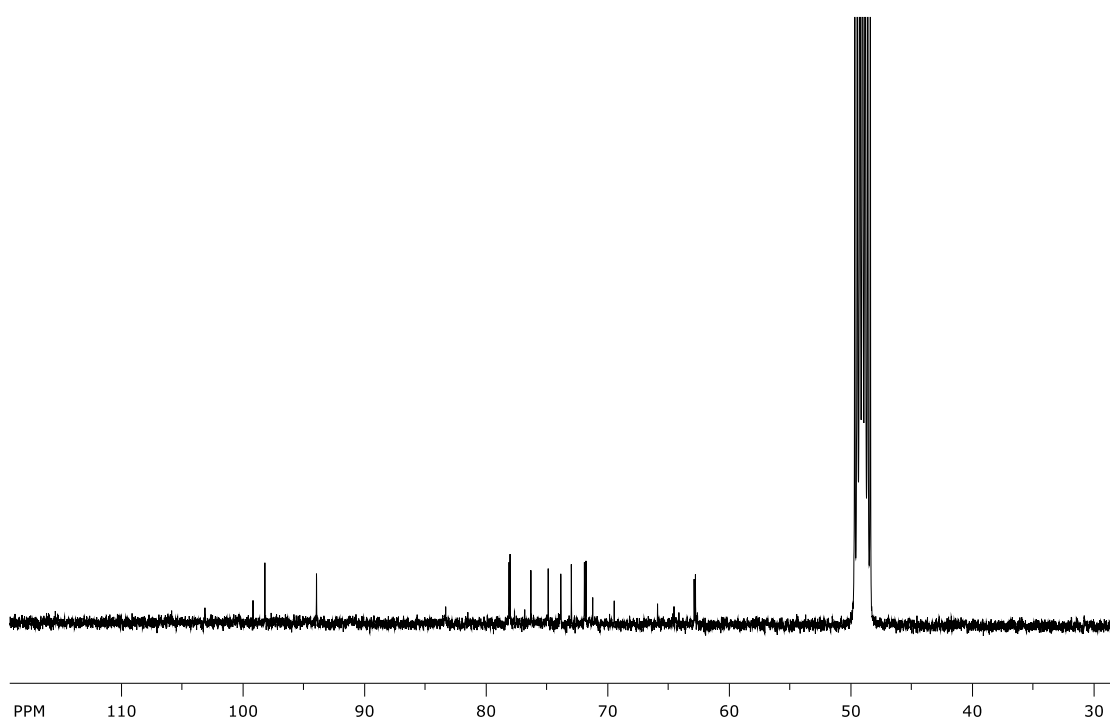
Plate 5C: DEPT spectrum of α- and β-D-glucopyranose (3.49) and (3.50) in CD₃OD.
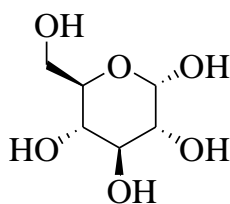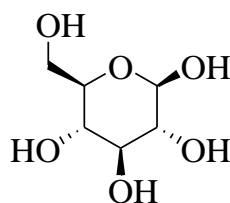
α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**
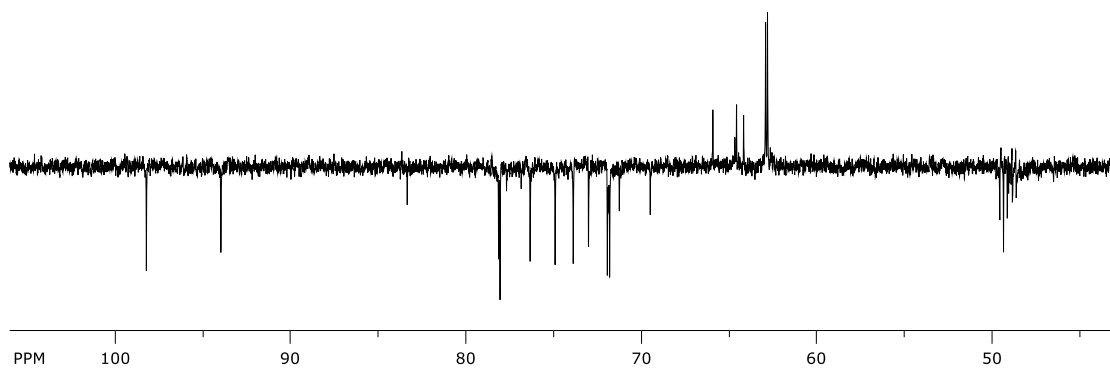
Plate 5D: COSY spectrum of α- and β-D-glucopyranose (3.49) and (3.50) in CD₃OD.
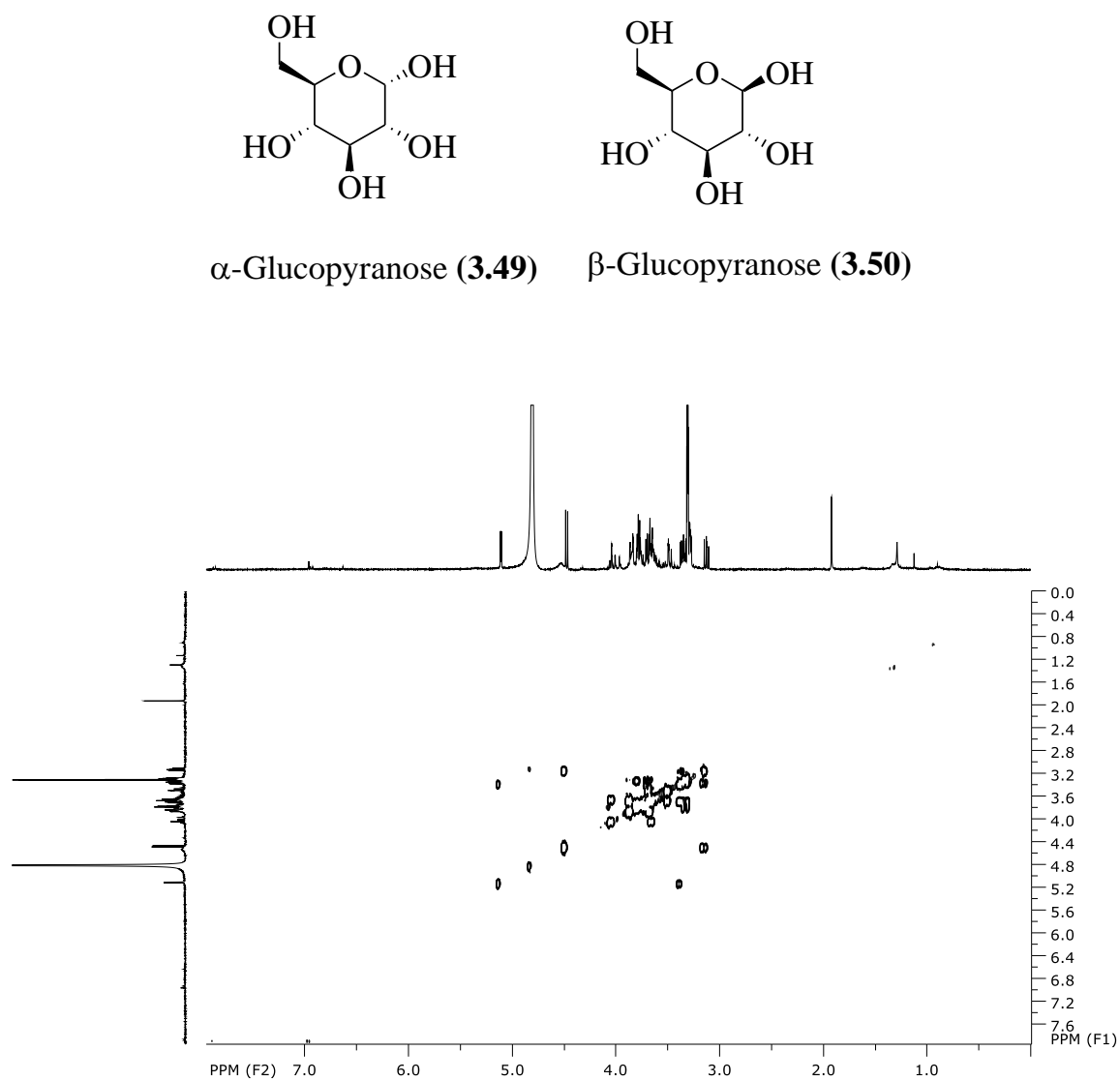
α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**
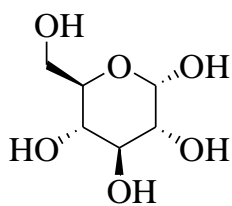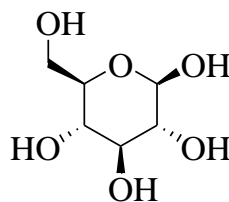
α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**
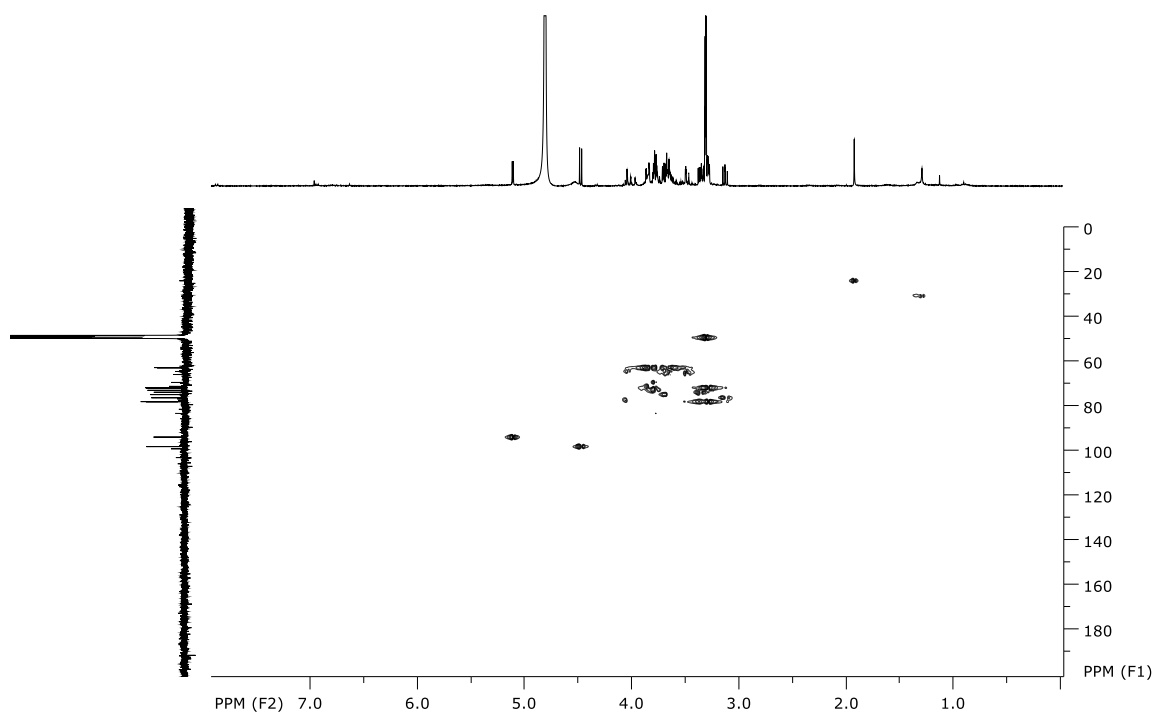
Plate 5G: HMBC spectrum of α- and β-D-glucopyranose (3.49) and (3.50) in CD₃OD.
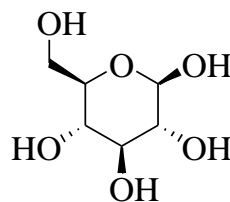

α-Glucopyranose (**3.49**)   β-Glucopyranose (**3.50**)

α-Glucopyranose **(3.49)**   β-Glucopyranose **(3.50)**