Identification of alpha-Glucosidase Inhibitors in Indigenous Plants used for Diabetes

Dissertation submitted in accordance with the requirements for the degree of

Master of Science

By

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Declaration

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 R. van Heerden and Co-supervision of Dr Muvhango Rasalanavho.

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 [redacted]

Nkosinathi Khulekani Ngcobo Date 19/08/2024

We hereby certify that this statement is correct:

Signed [redacted]

Professor Fanie R. van Heerden **(Supervisor)** Date 20/08/2024

Signed … [redacted] ……

Dr Muvhango Rasalanavho **(Co-supervisor)** Date 20 August 2024

Abstract

Diabetes, a global health concern, is a metabolic disorder with a broad range of micro and macro complications. There is still a need to develop new diabetic therapies. Hence, there is a resurging interest in isolating bioactive compounds with antidiabetic activity. One of the therapeutic therapies is the inhibition of α-glucosidase, an enzyme located in the digestive tract that facilitates the hydrolysation of carbohydrates into glucose. Plant-derived compounds and their derivatives have played a significant role in developing numerous drugs, such as the antidiabetic drug metformin.

With the intention to advance and explore new plant-derived compounds that can potentially be developed into new biologically active drugs that will be used to treat diabetes, several Indigenous South African species traditionally used to treat diabetes were selected for investigation. These plants were *Bulbine frutescens* (L.) Willd., *Carpobrotus dimidiatus* (Haw.) L.Bolus, *Cassia abbreviata* Oliv. subsp. *beareana* (Holmes) Brenan, *Gomphocarpus tomentosus* Burch. subsp. *tomentosus*, *Maerua angolensis* DC. subsp. *angolensis*, *Merwilla plumbea* (Lindl.) Speta, *Pappea capensis* Eckl. & Zeyh, *Sclerocarya birrea* (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro, *Senegalia mellifera* (Vahl) Seigel & Ebinger subsp. *detinens* (Burch.) Kyal. & Boatwr., *Senna italica* Mill. subsp. *arachoides* (Burch.) Lock, *Tabernaemontana elegans* Stapf, and *Ziziphus mucronata* Willd. subsp. *mucronata*.

Lead-like extracts were prepared for all the plant species, and the extracts were subjected to in vitro enzyme inhibition studies. The extract of *S. birrea* was selected for further investigation since it demonstrated the highest inhibitory activity against α-glucosidase. An active fraction of *S. birrea* stem bark was subjected to LC-MS analysis, and five compounds were identified in the extracts. Preparative chromatography was used to isolate four compounds: catechin, *β*-sitosterol, *β*-sitosterol glucopyranoside, and stigmasterol. Of the isolated compounds, only catechin showed reasonable inhibition of α-glucosidase.

Conference Contributions

The work from this study has been presented at the following conference:

- Nkosinathi Khulekani Ngcobo, Muvhango Rasalanavho, and Fanie R. Van Heerden, 44th South African Chemical Institute (SACI) National Convention, Stellenbosch (8–13 January 2023). Poster presentation, Identification of Active Compounds in Indigenous Plants Used for Diabetes.
- Nkosinathi Khulekani Ngcobo, Muvhango Rasalanavho, and Fanie R van Heerden, 16th Frank Warren Conference, Polokwane (3–7 December 2023). Poster presentation, "Identification of alpha-glucosidase inhibitors in indigenous plants used for diabetes".

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Abbreviations

°C	Degrees Celsius
DCM	Dichloromethane
DPP-4	Dipeptidyl peptidase 4
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
HPLC	High-performance liquid chromatography
HPLC-HRMS-SPE-NMR	High-performance liquid chromatography–high-resolution mass spectrometry–solid-phase extraction and nuclear magnetic resonance spectroscopy
HTS	High-throughput screening
MeOH	Methanol
mL	Millilitres
mg	Milligrams
mm	Millimetres
MW	Molecular weight
nm	nanometres
p-NPG	4-Nitrophenyl β-D-glucopyranoside
PDA	Photodiode-array detection
PMB	Pietermaritzburg
U/mL	Units per millilitre
UKZN	University of KwaZulu-Natal
UV	Ultraviolet

VLC	Vacuum liquid chromatography
WHO	World Health Organization

Chapter 1 Introduction

1.1 Traditional medicine

Traditional medicine (TM) is commonly known as ethnomedicine, native healing, or complementary and alternative medicine (CAM).[1] According to the World Health Organization (WHO), traditional medicine is "the knowledge, skills, and practices based on the theories, beliefs, and experience indigenous to different cultures used in the maintenance of health and prevention, diagnosis, improvement, or treatment of physical and mental illness".[2] Medicinal plants have played a key role in primary healthcare systems. People attribute this to several factors, such as easy accessibility of plants, affordability, and extensive knowledge. Traditional medicine dates back to primitive times when it was used to treat numerous diseases and served as the primary source of health care for a large percentage of the population in Africa's rural and urban areas.[3] In some nations, traditional medicine remains relevant and is still practised as the first line of defence against some diseases; for instance, in Ghana, Mali, Zambia, and Nigeria 60% of children suffering from malaria-related fever are treated with traditional medicine.[4,5]

There are different types of traditional medicine recognised around the world, namely traditional Chinese medicine (TCM), traditional Korean medicine (TKM), traditional Indian medicine (Ayurveda) (TIM), Kampo (Japanese), and African traditional medicine (ATM).[6] In developed countries, traditional medicine is referred to as alternative or complementary medicine, i.e., outside of modern/western medicine.[7]

The application of traditional, complementary, and alternative medicine (TCAM) is widespread globally and in Africa.[8] Traditional medicine knowledge is passed down from generation to generation through word of mouth. In the same way, as there are medical doctors, in traditional medicine, there are consultants available called traditional healers [Sangoma/ Nyanga (IsiZulu, South Africa), Babalawo (Yoruba-speaking people of Nigeria) or Shaman (the Americas)].[1,9,10] Treatment by traditional healers is regarded as a holistic approach (includes cultural and social practice) as opposed to modern medicine, which focuses mainly on the pertinent/sick area and aims to produce results instantly.

Approximately 80% of the people in Africa depend on the traditional health care system because Western medicine is considered expensive/unaffordable for a particular group of people (low/middle income). In some instances, Western medicine is not easily accessible as the health facilities are located in semi/urban areas where people have to travel long distances to get to facilities.[11,12]

1.2 Natural products

Humans used animals, plants, microorganisms, and marine organisms to treat different diseases since ancient times.[13,14] Natural products have increasingly played a significant role as a source of drugs and the discovery of novel compounds due to their structural and chemical diversity.[15,16] Out of 112 new drugs approved between 1999-2013 by the Food and Drug Administration (FDA), 28% were developed based on natural product pharmacophores.[17,18]

Apart from the unique chemical structures that natural products possess, there are downfalls in using natural products for the discovery and development of drugs, such as time-consuming isolation and identification of compounds, insolubility of isolated compounds, and unknown mechanisms of action.[18,19] Furthermore, the structural complexity and high molecular weights (MW ≥ 500) of several natural products do not obey Lipinski's "rule of five", which in turn affects the absorption of drugs into the blood.[19,20] Hence, developing natural products that obey Lipinski's rules is recommended to fast-track the isolation and discovery of novel bioactive compounds.

The development of natural products and derivatives has contributed to successfully treating human diseases in different therapeutic areas. This includes the development of drugs such as the vinca alkaloids and the terpenoid paclitaxel (anticancer drugs), penicillin (antibacterial), morphine (painkiller), and antimalarial drugs (quinine, artemisinin) (Figure 1.1).[14,21] Despite natural products' limitations, a few alterations in the structure, i.e., a change in the position of a substituent, may lead to the discovery of novel lead compounds that can be used to develop drugs that can cure diseases. For the last three decades (1981-2019), utilisation of natural products has been significant in developing novel biologically active drugs, especially

against cancer, where 64% of 185 small molecule drugs approved by the FDA were derived from natural products or natural product derivatives.[22]

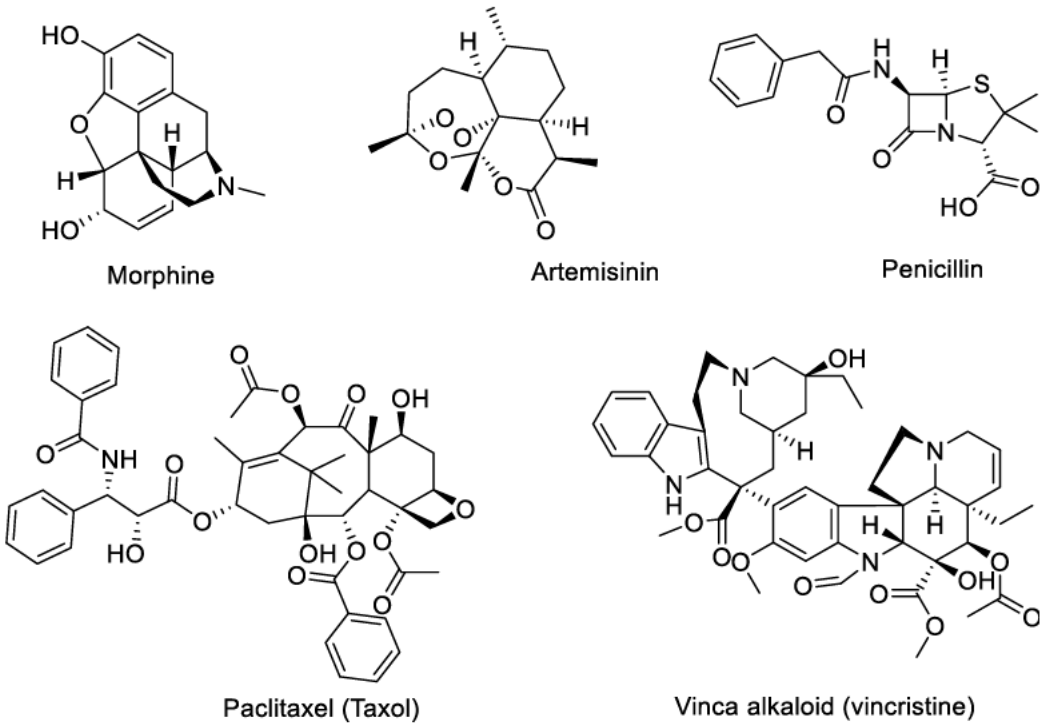

Figure 1.1. Structures of drugs developed from natural products.

1.3 Problem statement

Diabetes mellitus, commonly known as diabetes, is a metabolic disorder characterised by high glucose levels in the blood (hyperglycemia) due to the failure in insulin secretion from the pancreas (type 1 diabetes) or when the body is building up resistance toward insulin (type 2 diabetes).[23,24] Other less common types of diabetes are maturity-onset diabetes of the young (MODY), gestational diabetes, neonatal diabetes, and, lastly, secondary causes due to steroid use.[23,25,26]

Diabetes is the world's fastest-growing pandemic and is causing a threat to global health.[24] In 1980, the WHO estimated the number of diabetes cases to be 108 million; in 2017, 425 million cases were reported. This translates to a drastic increase of almost 400% in about 40 years.[27,28] By 2045, if sufficient and necessary actions are not implemented, the number is expected to

reach 625 million.[27,29,30] According to results published by Stats SA in 2021, for the period 2016-2018, diabetes was the second most underlying natural cause of death in South Africa among the general population. However, among females, diabetes was the leading cause of natural death.[31,32]

Failure to manage diabetes may lead to micro- and macrovascular complications such as blindness, renal failure, nervous system (Neuropathy, nerve damage), stroke, and coronary heart disease, leading to death.[33,34] Available treatments for diabetes, such as insulin, pharmaceutical drugs (acarbose, metformin), and diet modification,[27,35-37] are commonly used to control blood sugar levels. However, there are concerns about the side effects (nausea, skin reaction, liver disease, heart failure, diarrhoea, etc.) caused by the drugs.[38-40] The recommended drugs can keep sugar levels low but cannot cure diabetes. Therefore, safe and more potent antidiabetic drugs are still needed. Research has shown positive results in using and applying traditional medicine as an alternative to synthetic drugs for treating diabetes.[41] Medicinal plants are now commonly accepted worldwide as an alternative treatment for diabetes mellitus.[42-44] This is due to active compounds such as polyphenols, steroids, alkaloids, terpenoids, and glycosides in plant extracts.[45]

Traditional medicine has gained considerable attention due to its effectiveness in different types of diabetes.[46] This calls for further investigation of traditional medicinal plants used to treat diabetes.

The enzyme α-glucosidase is one of the targets for developing antidiabetic drugs. The current study intends to investigate the inhibitory effects of plant extracts and isolated compounds on α-glucosidase. The plants selected for the assays were *Bulbine frutescens* (L.) Willd., *Carpobrotus dimidiatus* (Haw.) L. Bolus*, Cassia abbreviata* Oliv. subsp. *beareana* (Holmes) Brenan*, Gomphocarpus tomentosus* Burch. subsp*. tomentosus, Maerua angolensis* DC. subsp. *angolensis, Merwilla plumbea* (Lindl.) Speta*, Pappea capensis* Eckl. & Zeyh., *Sclerocarya birrea* (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro*, Senegalia mellifera* (Vahl) Seigel & Ebinger subsp. *detinens* (Burch.) Kyal. & Boatwr*., Senna italica* Mill. subsp. *arachoides* (Burch.) Lock*, Tabernaemontana elegans* Stapf*,* and *Ziziphus mucronata* Willd. subsp*. mucronata.* Some of these plants could deliver a lead compound for developing new biologically active drugs for

treating diabetes. Limited research on the biological activities has been performed on the extracts of some of the selected species.

1.4 Aim and objectives

This project aims to identify extracts from indigenous plants used traditionally for treating diabetes that can inhibit the enzyme α-glucosidase and identify active compounds in one of these extracts.

Specific objectives of the study are to:

- Identify and collect plants used traditionally for diabetes
- Prepare lead-like extracts of the collected plants
- Perform α-glucosidase inhibitory assays on the crude extracts
- Select an active extract and develop an HPLC profile for this extract
- Fractionate the active extract into a 96-well plate by HPLC
- Perform an assay on the 96-well plate and identify wells that contain active compounds
- Isolate active compound(s) and determine the structure(s) of the compound(s)

In Chapter 2, a background to diabetes, drugs used to treat diabetes, and plant metabolites that inhibit α-glucosidase are given. Chapter 3 describes the preparation of lead-like extracts from a selection of plants and the results obtained in the inhibitory assay of the extracts on α-glucosidase. Chapter 4 discusses the large-scale extraction from an active plant, *Sclerocarya birrea*, and the identification of compounds in the extracts. Chapter 5 is the conclusion chapter.

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Chapter 2 A literature review on diabetes

2.1 Introduction

Diabetes mellitus, a chronic disease of global concern, is a metabolic disorder caused by insulin deficiency or hyperglycaemia. The two most common forms of diabetes are type 1 and type 2. With type 1 diabetes, there is inadequate insulin production due to the attack and destruction of β-cells in the pancreas. Type 1 diabetes accounts for 5-10% of all diabetic cases and is commonly called insulin-dependent or insulin-mediated diabetes. People with this type of diabetes require regular insulin injections. They must test several times per day for sugar levels in the blood (at least four times daily) if under intensive therapy.[1-4]

Type 2 diabetes is the most common form of diabetes and accounts for 90-95% of diabetes cases worldwide. In type 2 diabetes, the body becomes resistant to insulin, and sugar builds up in the blood. This is caused by an insufficient cellular response to insulin by vital organs, i.e., kidneys, heart, and nervous system. Type 2 diabetes is commonly called non-insulin-dependent diabetes mellitus (NIDDM) and significantly correlates with obesity, age, and genetics.[1,5-9] Insulin is a hormone produced by the β-cells in the pancreas and is responsible for controlling the glucose level in the blood.[1,6] Insulin resistance and pancreatic β-cells dysfunction are the two major defects that cause type 2 diabetes due to reduced glucose uptake in the blood.[5,10,11]

For the past few decades, the reported cases have increased exponentially.[12] Lack of physical exercise, unhealthy food habits, high calories, and excessive sugar consumption are significant contributors to the reported diabetes cases, especially for type 2 diabetes. For 2030, the prevalence of diabetes is predicted to be 578 million cases, with a further projection of 783.2 million cases for 2045.[13,14] Furthermore, young people are notably affected at a much younger age (≤ 5 years), and some are obese before puberty.[15] Obesity accounts for 100 million cases globally, and the numbers are still increasing. This figure comprises mostly nations affected by malnutrition.[16]

The imbalance of glucose levels in the bloodstream increases the risk for severe complications related to diabetes. Maintaining and managing glucose levels is crucial and must be monitored

continuously. Frequent blood glucose level check-ups and management assist with the following:

- Making one aware of the glucose level at a specific time
- Diagnosing factors influencing the fluctuation of blood glucose levels
- Identifying the influence of diet modifications and exercise on a diabetic patient.[1,2]

2.2 Medication currently available for diabetes mellitus

There is a variety of drugs available to treat different types of diabetes. However, concerns remain due to the unfavourable gastrointestinal side effects associated with the treatment. Medication is either administered orally or injected, depending on the type of diabetes. Different classes of antidiabetic drugs are described below.

2.2.1 Dipeptidyl peptidase 4 (DPP-4) inhibitors

This treatment is taken orally and prevents the hydrolysis of glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2), hormones responsible for lowering the blood glucose in the system.[17] Examples of this type of drug are saxagliptin and sitagliptin (Figure 2.1).

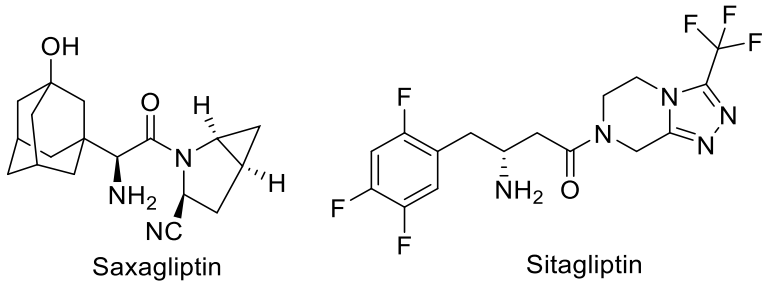


Figure 2.1. Structures of some DPP-4 inhibitors.

2.2.2 GLP-1 and dual glucose-dependent insulinotropic polypeptide (GIP)/ GLP-1 agonists

GLP-1 (glucagon-like peptide 1) and GIP (glucose-dependent insulinotropic polypeptide) (also known as incretins) are peptide hormones that have the ability to lower glucose levels in the blood by stimulating the secretion of insulin. Treatment with GLP-1 receptor agonists benefits patients with type 2 diabetes by lowering blood glucose and body weight.[18] An example of

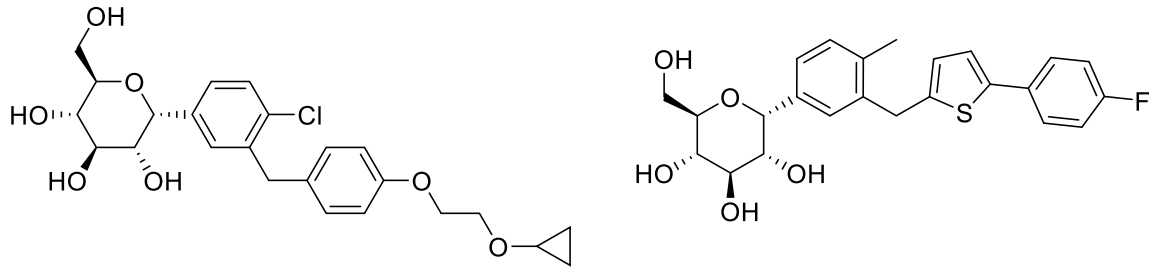
such a GLP-1 agonist is liraglutide (Figure 2.2). Liraglutide is present in products such as Ozempic®, which has recently become a popular weight-loss product.

H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(1)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH.palmitoyl-Glu(1)-OH

Figure 2.2. Structure of the injectable GLP-1 and dual GIP/GLP-1 agonist, liraglutide.

2.2.3 Sodium-glucose co-transporter-2 (SGLT2) inhibitors

The medication prevents the reabsorption of glucose into the blood in the kidneys. The side effects of this treatment are genital yeast infections, need for leg amputations, hypoglycaemia, and bladder cancer.[19] The SGLT2 inhibitor class of medications includes bexagliflozin and canagliflozin (Figure 2.3).



Bexagliflozin Canagliflozin

Figure 2.3. Commercially available SGLT2 inhibitors.

2.2.4 Sulfonylureas

This oral prescription medication is taken to stimulate the secretion of glucose in the pancreas.[20] Sulfonylurea drugs (Figure 2.4) are of the oldest antidiabetic agents, and when prescribed, a healthy lifestyle is recommended to minimise the development of chronic complications such as stroke and kidney problems.

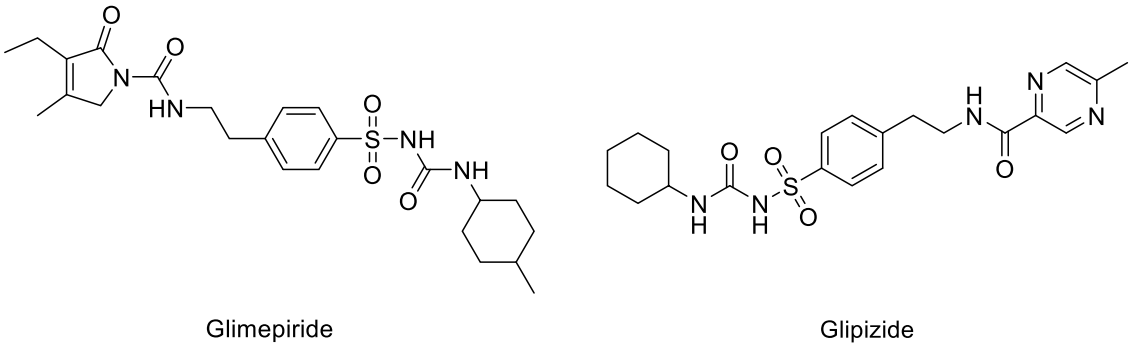


Figure 2.4. Structures of sulfonylurea drugs.

2.2.5 Thiazolidines (TZDs)

Thiazolidines (Figure 2.5) assist with the reduction of glucose produced in the liver and improve insulin sensitivity in the muscle and fat.[21,22] These drugs raise the risk of heart disease; hence, the medical staff monitors the heart's functioning when prescribed.

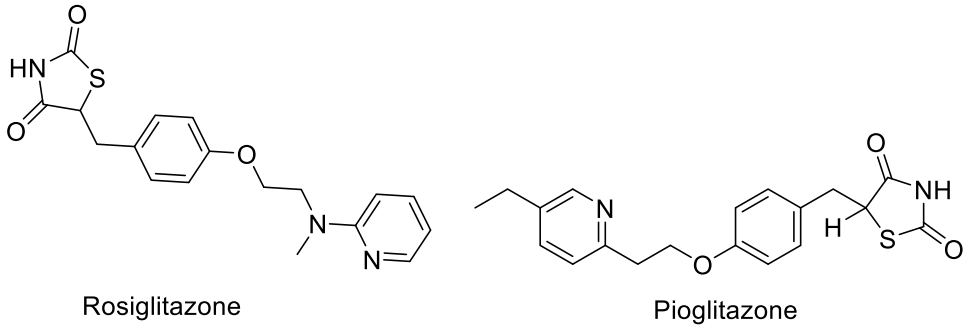


Figure 2.5. Structures of thiazolidine drugs**.**

2.3 α-Glucosidase inhibitors

Maintaining steady blood glucose levels between 5 and 10 mmol/L is significant for diabetic patients using hypoglycaemic medication. One of the standard therapeutic approaches for controlling high glucose levels (hyperglycaemia) involves the inhibition of α-glucosidase. This enzyme catalyses the hydrolysis of carbohydrate polymers (on the non-reducing end) into simple sugars, i.e., monosaccharides, leading to increased glucose levels in the

bloodstream.[23-25] An alternative approach to reducing postprandial and fluctuating blood glucose levels is slowing the glucose absorption from the gastrointestinal tract; hence, treatment targeting the enzyme α-glucosidase is significant for preventing and managing type 2 diabetes.

α-Glucosidase inhibitors are widely used for treating type 2 diabetes since they delay the absorption of glucose (monosaccharides) from the small intestine, thereby causing a decrease in postprandial blood glucose and insulin levels.[26] α-Glucosidase inhibitors are not invasive as the treatment causes mild and short-lived adverse effects such as diarrhoea, abdominal pain, and flatulence.[27] Until now, significant efforts have been made toward discovering new α-glucosidase inhibitors that are safe and have minimal side effects. This has led to the screening of natural products and synthetic organic compounds.[28] The currently available α-glucosidase inhibitors are miglitol, acarbose, and voglibose (Figure 2.6).[29]

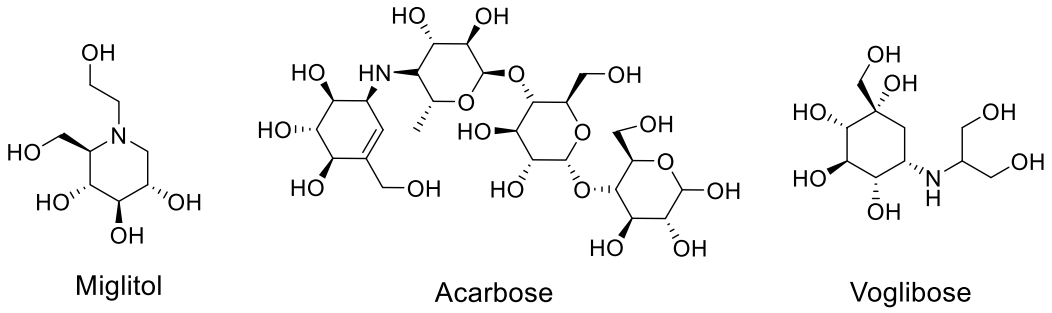
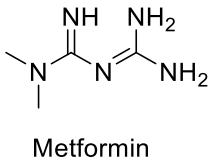


Figure 2.6. Structures of the clinical drugs used as inhibitors of α-glucosidase.

Side effects related to these treatments are dose-related; some nations, i.e., the US and the UK, still hesitate to use α-glucosidase inhibitors prescriptions due to the dosage (2/3 times a day) and its weak tolerance.[27] The International Diabetes Federation (IDF) and the American Association of Clinical Endocrinologists (AACE) have recognised natural α-glucosidase inhibitors as the first line of therapy. Hence, using natural extracts to discover highly effective novel compounds has attracted researchers' attention.[30]

Medicinal plants have led to the development of many commercial pharmaceutical drugs. Metformin, an FDA-approved antidiabetic agent, was first isolated from a herbaceous plant,

Galega officinalis L.[31] Metformin (Figure 2.7) is a biguanide-derivative, and it is administered orally to treat type 2 diabetes.[32,33] Metformin (1,1-dimethylbiguanide hydrochloride) was first synthesised in the early 1900s and has since been used as the first line of defence against type 2 diabetes. Recently, it has gained attention due to its unexpected effectiveness in treating other diseases such as cancer, cardiovascular disease, and liver disease.[34-36]



Metformin

Figure 2.7. Structure of metformin.

2.4 Extracts and plant metabolites as α-glucosidase inhibitors

Global plants of which the crude extracts inhibit α-glucosidase are listed in Table 2.1. However, active components in many of these extracts have not yet been identified.[37,38] The medicinal plants mentioned in Table 2.1 showed remarkable antidiabetic activity, either in vitro or in vivo studies. Although the phytochemicals responsible for the inhibition might not yet have been identified, the studies showed promising results that may lead to the discovery of new lead compounds.[39] Amongst the plants, *Chrysophyllum caimito* L. and *Ensete superbum* Roxb. Cheesman are known for the richness of valuable medicinal compounds such as gallic acid, lupeol, rutin, quercetin, proanthocyanidin, and *β*–amyrin.[40,41] However, more studies need to be conducted to determine these plants' efficacy, and toxicity.

Table 2.1. Some international plants that show α-glucosidase inhibition activity.

Medicinal plant	Plant part used	Type of extract
Adenosma bracteosum Bonati. (Scrophulariaceae)	Aerial parts	Ethanol and aqueous[42]
Annona senegalensis Pers (Annonaceae)	Leaves	Ethyl acetate and ethanol[43]
Chrysophyllum cainito L. (Sapotaceae)	Stem bark	Aqueous[44]
Ensete superbum (Roxb.) Cheesman (Musaceae)	Seeds	Methanol[45]
Hertia cheirifolia L. (Asteraceae)	Flower	Petroleum ether[46]
Homalium zeylanicum (Gardner) Benth. (Flacourtiaceae)	Bark	Ethanol[47]
Hypericum hircinum L. (Hypericaceae)	Aerial parts	Ethanol[48]
Hypericum scruglii Bacch., Brullo & Salmeri (Hypericaceae)	Aerial parts	Ethanol[48]
Liquidambar formosana Hance (Hamamelidaceae)	Leaves	Ethanol[39]
Quercus gilva Blume (Fagaceae)	Leaves	Methanol[49]
Quercus phillyreoides A. (Fagaceae)	Leaves	Methanol[49]
Mallotus japonicus (L.f.) Müll.Arg. (Euphorbiaceae)	Leaves	Methanol[49]

Dirir et al.[50] reviewed natural products (≥ 290) with inhibitory activity against α-glucosidase. The authors of this review identified the eight compounds (Figure 2.8) as promising α-glucosidase inhibitors for further development. The article discusses the plant extracts, their bioactive molecules, their pharmacokinetics uses, toxicity, and biological activity.

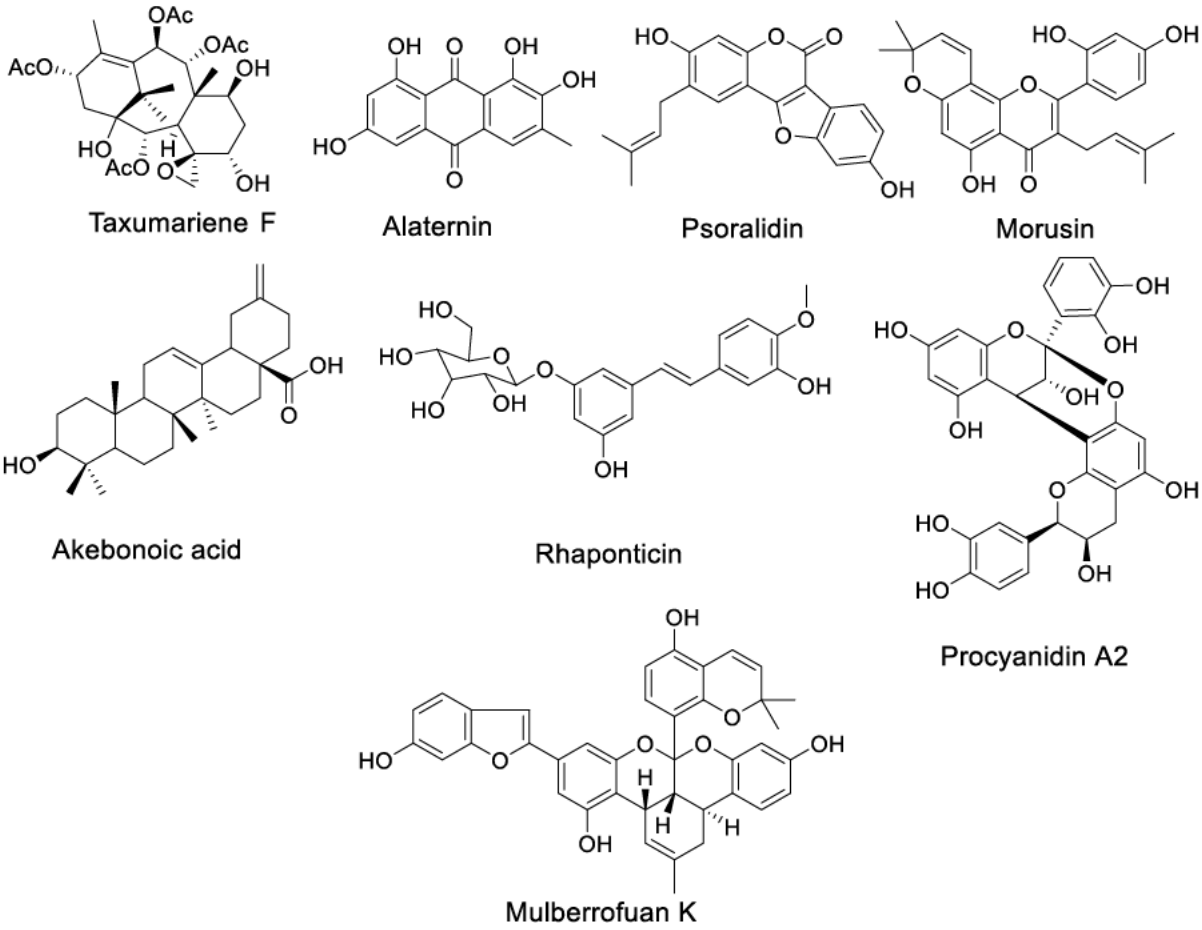


Figure 2.8. Structures of promising plant-derived α-glucosidase inhibitors.

2.4.1 South African plants with antidiabetic activity

There are several commercial treatments available to treat diabetes. However, for the past decades, there has been a rise in the use of medicinal plants to help manage and control diabetes. The estimated prevalence percentage of diabetes in South Africa ranges from 4-6%. South Africa has abundant biodiversity and cultural practices for using plants.[51-53] Thus, a high percentage of the people suffering from diabetes depend on traditional medicine.[51] Apart from this, there is still limited information on the South African flora used, and this is due to the little ethnobotanical information gathered. There is a variety of South African indigenous plants that are used to treat diabetes.

Among the plants listed in Table 2.2 with antidiabetic activity, some have not been extensively researched (biological activities), such as *Aloe arborescens* Mill, *Allium cepa* L., *Brachylaena*

elliptica (Thunb.) DC, *Bulbine latifolia* (L.f.) Spreng, *Coleonema album* (Thunb.) Bartl. & H.L. Wendl., and *Euclea natalensis* A.DC., to mention a few.

Table 2.2. South African plants used to treat diabetes.

Plant species	Part used
Acokanthera oblongifolia (Hochst.) Benth. & Hook.f. ex B.D.Jacks. (Apocynaceae)	Not specified[52]
Acokanthera oppositifolia (Lam.) (Apocynaceae)	Not specified[52]
Acorus calamus L. (Acoraceae)*	Not specified[52]
Adenia digitata (Harv.) Engl (Passifloraceae)	Not specified[52]
Albuca setosa Jacq (Hyacinthaceae)	Corms[53]
Allium cepa L. (Amaryllidaceae)*	Whole plant[54]
Aloe arborescens Mill. (Asphodelaceae)	Leaves[55]
Aloe ferox Mill.	Leaves, Roots[53]
Aloe greatheadii var. *davyana* (Schönland) Glen & D.S.Hardy (Asphodelaceae)	Leaves[56]
Aloe maculata All. (Asphodelaceae)	Leaves[57]
Aloe marlothii A. Berger (Asphodelaceae)	Leaves, Roots[58]
Aloe microstigma Salm-Dyck (Asphodelaceae)	Leaves[57]
Aloe vera (L.) Burm. f. (Asphodelaceae)	Leaves, gel[57]
Anacardium occidentale L. (Anacardiaceae)	Bark[56]
Anacampseros ustulata E.Mey. ex Fenzl	Corms[53]
Arctopus echinatus L. (Apiaceae)	Root[59]
Aristea africana (L.) Hoffmanns (Iridaceae)	Foliage[59]
Artemisia absinthium L. (Asteraceae)*	Leaves [60]
Artemisia afra Jacq. Ex Wild. (Asteraceae)	Leaves[61]
Asclepias crispa P.J. Bergius (Apocynaceae)	Not specified[60]
Brachylaena discolor DC. (Asteraceae)	Leaves[61]
Brachylaena elliptica (Thunb.) DC. (Asteraceae)	Leaves [62]
Bulbine latifolia (L.f.) Spreng. (Asphodelaceae)	Root[61]
Bulbine frutescens L. (Anacardiaceae)	Root [61]
*Bulbine natalensis (*Syn*. B. latifolia)* Mill. (Asphodelaceae)	Roots[61]
Cadaba aphylla (Thunb.) Wild. (Capparaceae)	Root[63]
Carpobrotus edulis (L.) N.E. Br. (Aizoaceae)	Leaves[58]
Cassia abbreviate Oliv (Leguminosae)	Stem bark[64]
Catharanthus roseus (L.) G. Don (Apocynaceae)*	Roots, Leaves[61]
Chamarea capensis (Thunb.) Eckl. & Zeyh. (Apiaceae)	Not specified[63]
Chironia baccifera L. (Gentianaceae)	Leaves, whole plant[65]
Chrysocoma ciliata L. (Asteraceae)	Leaves, roots[66]
Cissampelos capensis L.f. (Menispermaceae)	Leaves, root[53]
Coleonema album (Thunb.) Bartl. & H.L. Wendl (Rutaceae)	Foliage[59]
Dicerothamnus rhinocerotis (L.f.) Koekemoer (Asteraceae)	Leaves and stem[66]
Dicoma capensis Less. (Asteraceae)	Leaves[60]
Dittrichia graveolens (L.) Greuter (Asteraceae)*	Leaves, twigs[60]

Table 2.2 continued

Plant species	part used
Dodonaea viscosa (L.) Jacq. (Sapindaceae)	Leaves[63]
Elephantorrhiza elephantina (Burch.) Skeels (Leguminosae)	Rhizomes[67]
Eriocephalus punctulatus DC. (Asteraceae)	Leaves[63]
*Eucalyptus globulus** Labill. (Myrtaceae)	Not specified[63]
Euclea natalensis A.DC. (Ebenaceae)	Roots[64]
Euclea undulata Thunb (Ebenaceae)	Not specified[63]
Gazania krebsiana Less. (Asteraceae)	Leaves [67]
Lasiosiphon deserticola (Gilg) C.H.Wright (= *Gnidia deserticola* Gilg) (Thymelaeaceae)	Leaves, stems and roots[66]
Grewia flavescens Juss. (Malvaceae)	Roots[64]
Grewia villosa Willd. (Malvaceae)	Roots[64]
Gomphrena celosioides Mart. (Amaranthaceae)*	Roots[64]
Haplocarpha scaposa Harv. (Asteraceae)	Leaves, Roots[67]
Helichrysum caespititium (DC.) Sond. ex Harv. (Asteraceae)	Whole plant[58]
Helichrysum nudifolium L. (Asteraceae)	Leaves, roots[61]
Heichrysum petiolare Hilliard & B.L.Burtt (Asteraceae)	Whole plant[61]
Inula graveolens (L.)Desf. (Asteraceae)*	Foliage[59]
Jacobaea maritima (L.) Pelser & Meijden (Asteraceae)*	Not specified[63]
Kedrostis africana (L.) Cogn. (Cucurbitaceae)	Not specified[63]
Kedrostis nana Cogn. (Cucurbitaceae)	Root, tuber[63]
Lauridia tetragona (L.f.) R.H.Archer (Celastraceae)	Barks[53]
Leonotis leonurus (L.) R.Br. (Lamiaceae)	Leaves[53]
Lessertia frutescens (L.) Goldblatt & J.C. Manning (Leguminosae)	Leaves[63]
Merwilla plumbea (Lindl.) Speta. (Asparagaceae)	Leaves[68]
Momordica foetida Schumach. (Cucurbitaceae)	Leaves and stems[65]
Morella serrata (Lam.) Killick (Myricaceae)	Roots[67]
Nidorella ivifolia (L.) J.C.Manning & Goldblatt (= *Conyza scabrida* DC.) (Asteraceae)	Foliage[69]
Notobubon galbanum (L.) Magee (Apiaceae)	Foliage[59]
Opuntia ficus-indica (L.) Mill. (Cactaceae)*	Leaves[64]
Opuntia vulgaris Mill. (Cactaceae)*	Leaves[56]
Pelargonium antidysentericum (Eckl. & Zeyh.) Kostel. (Geraniaceae)	Roots[66]
Pentanisia prunelloides (Klotzch) Walp. (Rubiaceae)	Roots, leaves[67]
Pittosporum viridiflorum Sims (Pittosporaceae)	Bark[59]
Salix mucronata Thunb. (Salicaceae)	Foliage [63]
Sclerocarya birrea (A. Rich.) Hochst. subsp. *caffra* (Sond.) (Anacardiceae)	Stem bark and root[65]
Strychnos henningsii Gilg. (Loganiaceae)	Bark[53]
Solanum aculeastrum (Solanaceae)	Roots[53]
Tarchonanthus camphoratus L. (Asteraceae)	Leaves and soft twigs[58]
Terminalia sericea Burchell ex. DC. (Combretaceae)	Stem bark
Tulbaghia violacea Harv. (Amaryllidaceae)	Leaves, stem[70]
Viscum continuum E. Mey. (Santalaceae)	Not specified[63]
Ziziphus mucronata Willd. (Rhamnaceae)	Roots[64]

2.4.1 Isolated compounds from antidiabetic plants

Plants consist of chemicals with various activities and have been shown to be a significant source of new antidiabetic lead drugs.[71] The compounds often have numerous modes of action through interaction with different target enzymes related to diabetes. Given this and the urgent need for novel antidiabetic drugs, researchers continue to explore indigenous plants as possible candidates. Some plants with isolated antidiabetic compounds are briefly discussed in the following paragraphs.

Cassia abbreviata Oliv (Leguminosae) is widely distributed from East Africa (Somalia) to South Africa and is used to treat various diseases, including diabetes.[64] A study by Mongalo and Mafoko[72] reviewed the antidiabetic activity of the plant extract. The compounds (Figure 2.9) were isolated from this species, but the activity of the compounds was not been investigated.

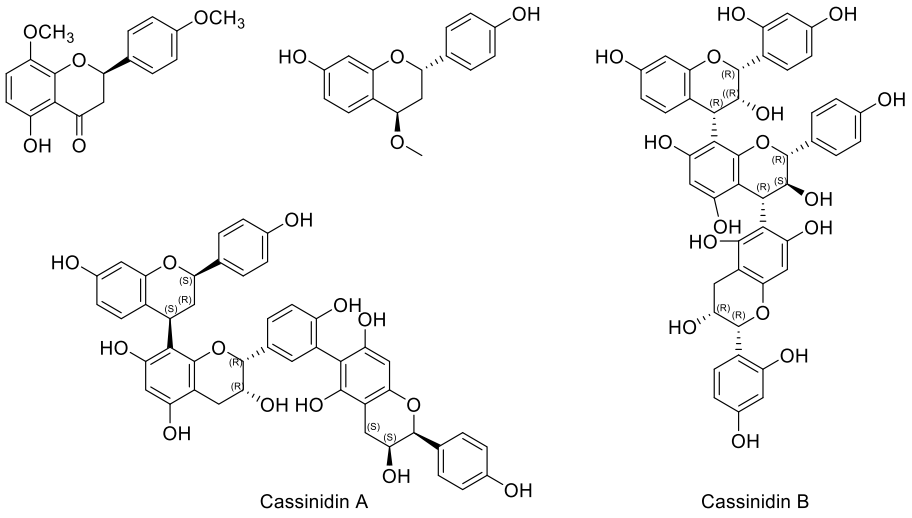


Figure 2.9. Isolated compounds from *Cassia abbreviata.*

Terminalia sericea Burchell ex. DC. (Combretaceae), also known as a multipurpose plant, is primarily used to treat diarrhoea, sexually transmitted infections, tuberculosis, and diabetes. Phytochemical investigation of the plant leads to the isolation of four known compounds

(Figure 2.10).[73,74] β-Sitosterol and lupeol had the highest inhibitory activity against α-glucosidase, IC_{50} 54.5 µM and 66.5 µM, respectively. This, in turn, substantiates the traditional use of this medicinal plant for managing and controlling diabetes.[74]

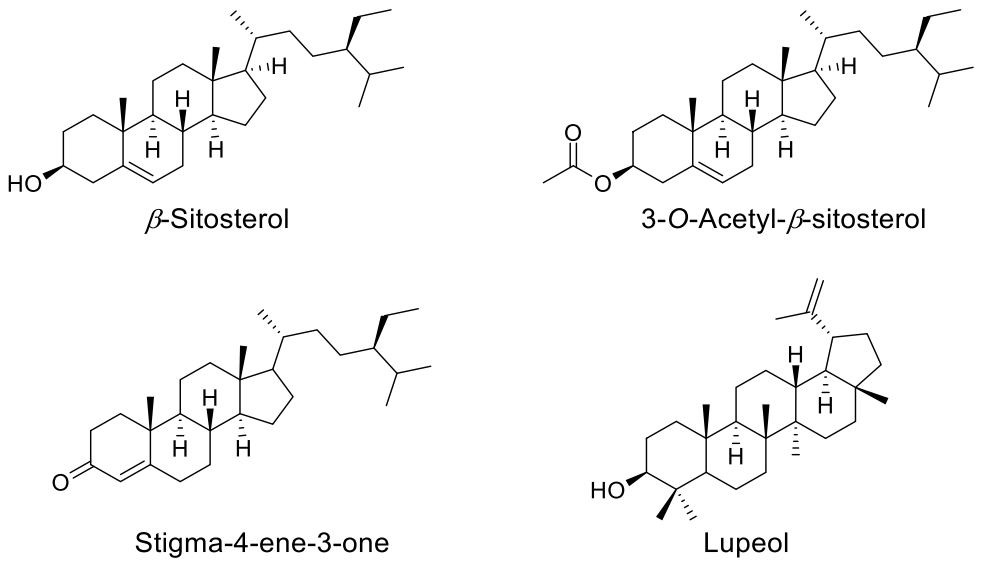


Figure 2.10. Active compounds isolated from *Terminalia sericea*.

Leonotis leonurus (L.) R.Br. (Lamiaceae) is a medicinal plant well-known as wild cannabis and mainly used by different South African tribes (Zulus, Xhosa, and Sotho) to treat numerous diseases, namely, epilepsy, chest infections, delayed menstruation, and diabetes mellitus.[75,76] In the literature, it has been reported that the plant can lower blood sugar (hypoglycaemic effect in vivo studies) due to the presence of the diterpenoids premarrubiin and marrubiin (Figure 2.11).[77,78]

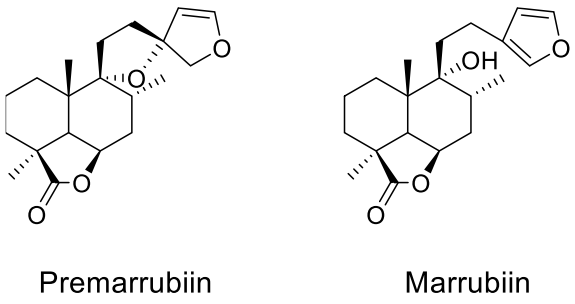
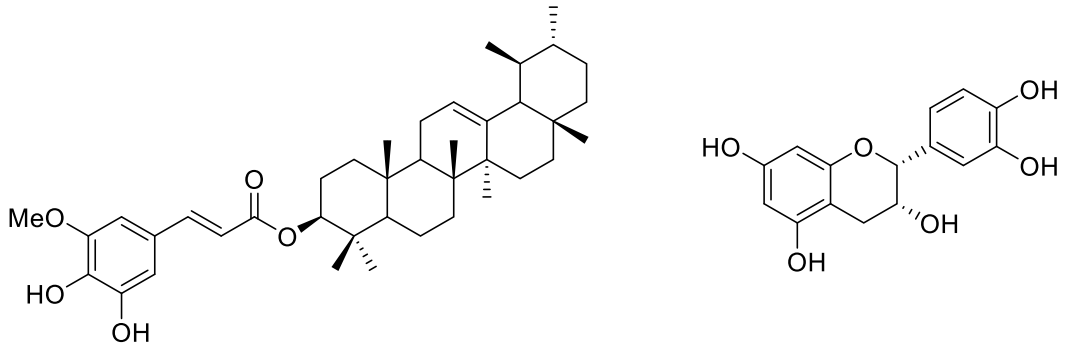


Figure 2.11. Active compounds isolated from *Leonotis leonurus* (L.) R.Br.

Euclea undulata Thunb. (Ebenaceae) is a dioecious shrub tree that is primarily used in tropical regions to treat diseases and ailments, including diabetes. The roots of the plants are sold in the "umuthi" shops/herbal shops or recommended by traditional healers as a treatment for diabetes.[79,80] Deutschländer et al.[81] conducted in vitro studies on C2C12 myocytes, which led to the isolation of four compounds, of which epicatechin (Figure 2.12) inhibited α-glucosidase (IC_{50} value of 4.79 µg/mL), with an activity comparable to the positive control, acarbose with IC_{50} value of 4.75 µg/mL).



α-Amyrin 3-*O*-(5-hydroxyferulate) Epicatechin

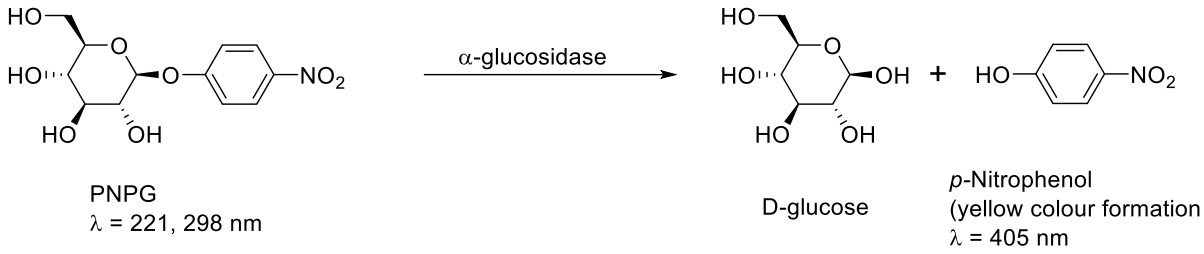
Figure 2.12. Compounds responsible for the biological activity of *Euclea undulata* Thunb.

Although many studies have investigated the antidiabetic activity of indigenous South African plants, there is still a sizable fraction (Table 2.2) of plants with unidentified active compounds responsible for the antidiabetic activity. This, in turn, shows the need for more investigations targeting the phytochemicals responsible for the biological activity in South African indigenous medicinal plants.

2.4.3 Identification of α-glucosidase inhibitors

A laboratory approach used for detecting active natural products is often to screen inhibitors of enzymes present in a crude extract based on spectroscopic methods. A general method for determining enzyme activity is to use a suitable substrate, which, after hydrolysis by the enzyme, produces a product that can be detected either by fluorescence or absorbance (colour) at a specific wavelength.

A substrate often used to determine the activity of α-glucosidase is *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG, a colourless compound) (Scheme 2.1).[82] Enzymatic hydrolysis of this compound yields *p*-nitrophenol (p-NP, yellow). The quantity of *p*-nitrophenol present is measured using spectrophotometry by setting the detection wavelength at 405 nm, the maximum absorbance for *p*-nitrophenol.[83,84] The calorimetric oxidase assay is another frequently used method to determine the α-glucosidase activity by estimating the amount of glucose present in a reaction.[50]



Scheme 2.1. Enzyme cleavage by α-glucosidase on a chromogenic substrate *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG).

2.4.4 High-throughput identification of active compounds in plant extracts

Although plants are an excellent source of bioactive molecules, there are some limitations in the processes involved in identifying these compounds, i.e., it is a time-consuming and cumbersome process and large amounts of solvents and chromatographic material are often needed to isolate pure compounds. Therefore, new analytical techniques are required to conduct faster and more accurate studies to identify active compounds.

Recently, the use of advanced bioanalytical techniques such as high-performance liquid chromatography–high-resolution mass spectrometry–solid-phase extraction, and nuclear magnetic resonance spectroscopy (HPLC-HRMS-SPE-NMR) to screen crude extract against a target enzyme has accelerated the identification of active compounds. This method has shown excellent results in identifying targeted active compounds in the extracts. The technique is used to isolate bioactive compounds from the crude extracts, such as α-glucosidase inhibitors, α-amylase inhibitors, aldose reductase inhibitors, antioxidants, and monoamine oxidase A inhibitors.[85-89]

Conclusion

The use of indigenous plants to treat diabetes mellitus is well-documented. However, subsequent validation studies on the efficacy and mechanism of action still need to be evaluated. Diabetes prevalence has become a world health crisis, and if no intervention is done, the number of people with diabetes will increase exponentially in the next decades. Besides people's lifestyle changes, new drugs are needed to combat this disease. Natural products will remain an essential contributor to the drug discovery process.

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Chapter 3 Preparation of lead-like extracts of medicinal plants and assay for α-glucosidase inhibition of these extracts

3.1 Introduction

Africa is well known for its rich plant biodiversity and traditional medicine used to treat various diseases, including diabetes.[1] The application of herbal medicines as alternative drugs for treating diabetes and diabetic side effects is expanding, and numerous plants are recognised globally to have antidiabetic properties.[2]

Southern Africa has about 23000 plant species, from which only about 3000 have been documented for therapeutic uses. Furthermore, fewer species have been assayed for antidiabetic properties. Species selection for biological studies can be challenging since literature reviews state the plant usage for a particular ailment rather than an alternative treatment for complex diseases such as diabetes, i.e., several plants are used for itchy skin, one of the symptoms of diabetes. However, excessive skin irritation can be misleading as it may be due to a chronic condition called pruritus.[3,4] Hence, there is uncertainty about whether a certain plant species can treat diabetes mellitus (DM) or associated ailments.

In the early 1970s, significant changes occurred in the drug discovery paradigm due to the need to improve the efficiency of pharmaceutical drugs and the growing knowledge regarding different diseases. Since then, screening methods used to identify active compounds have improved. This allowed scientists to question the selection methods of leads and possible candidates for clinical trials since it can affect the eventual outcome of launching a drug.[5,6] Lipinski's rules for "drug-like" concepts have significantly impacted drug discovery. It states that lead compounds outside the "rule of 5" are highly unlikely to be orally administered.[7] Subsequently, the "lead-likeness" concept was introduced in drug discovery. The lead-likeness is a tactical guide for selectivity since it offers a strong possibility to deliver a "drug-like" candidate that follows Lipinski's rule of five (i.e., MW < 500 Da, hydrogen bond donors ≤ 5, no more than ten hydrogen bond acceptors, and an octanol-water partition coefficient log P ≤ 5).[5,8]

High oral bioavailability is frequently a crucial factor to consider when developing new bioactive compounds that can be used to treat life-threatening diseases. Hence, highly flexible and polar compounds are not considered since they surpass Lipinski's rules and are, therefore, most likely less permeable in the cell membrane.[9,10]

Lipinski and other scientists' intensive research on the "drug-like" concept transformed drug discovery. Recently, the lead-likeness concept has been used as a key to defining a possible "drug-like" candidate.[11] Hann and Opera[11] define lead-likeness as "cut-off values in the physio-chemical profile of chemical libraries such that they have reduced complexity (MW < 400) and other restricted properties". Lead-likeness concepts are based on a smaller database than a "drug-like" score, which requires a wide range of chemical structures for statistical analysis.[12,13] Furthermore, studies by Hann et al. [13] showed that less complex compounds (one of the lead-likeness concepts) are more likely starting structures for drug discovery. This concept is one of the practical techniques used to pinpoint specific compounds with a particular activity. The concept of lead-like compounds has also been applied to plant extracts.[14]

Tannins consist of complex polyphenols that are widely distributed in various plants. They interact with several proteins by non-selectively binding enzymes, causing them to denature/ precipitate, thereby giving false readings/results.[15,16] A study by Wall et al.[16] showed that the activity of various plant extracts is due to the presence of tannins, which exhibit negative results after removing tannins. Furthermore, polyphenol-rich plant extracts make the isolation and characterisation of compounds in the extract very challenging. Hence, removing tannins from extracts before testing enzyme activity is preferred. One of many ways for tannin removal includes using polyamide, which is more effective than other methods. Treatment with polyamide is highly desirable for nearly completely removing tannins, and a small sample volume and low concentration are recommended. The method is rapid, reliable, robust, and cheap.[17]

The two main carbohydrate hydrolysing enzymes used as probes for discovering antidiabetic drugs are α-glucosidase and α-amylase. These enzymes are the leading cause of postprandial diabetes. α-Amylase is an enzyme that catalyses the hydrolysis of α-1,4 glycosidic bonds in starch/glycogen to form smaller fragments, usually di- or trisaccharides. α-Glucosidase

catalyses these oligosaccharides into monosaccharides.[18,19] Hence, inhibiting these enzymes is one of the approaches for managing and controlling diabetes.

This Chapter discusses the inhibition of α-glucosidase by several plant extracts. Plant species with evidence of potential antidiabetic properties were included in the research.

3.2 Selection of plants for this investigation

South Africa is known for its rich plant biodiversity; hence, traditional medicine practitioners use most of these plants to prepare remedies to cure numerous diseases. In the present study, some plants were selected based on indigenous knowledge of plants used to treat diabetes, listed in Table 2.2. Based on the ethnobotanical information, globally, 800 plant species show the potential to have antidiabetic properties. [20]

In this investigation, two approaches were followed for the selection of plants, namely, a targeted approach (plant species that was reported in the literature with known activity against α-glucosidase/used traditionally to treat diabetes) and an untargeted approach, where random plant species available were used to assay for enzyme inhibition. Table 3.1 shows the species that were collected for this study. These plants are briefly discussed in the paragraphs below.

Table 3.1. Plants selected to investigate for α-glucosidase inhibition.

Plant name	Family
Bulbine frutescens (L.) Willd.	Asphodelaceae
Carpobrotus dimidiatus (Haw.) L.Bolus	Aizoaceae
Cassia abbreviata Oliv. subsp. *beareana* (Holmes) Brenan	Fabaceae
Gomphocarpus tomentosus Burch. subsp. *tomentosus*	Apocynaceae
Maerua angolensis DC. subsp. *angolensis*	Capparaceae
Merwilla plumbea (Lindl.) Speta	Asparagaceae
Pappea capensis Eckl. & Zeyh	Sapindaceae
Sclerocarya birrea (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro	Anacardiaceae
Senegalia mellifera (Vahl) Seigel & Ebinger subsp. *detinens* (Burch.) Kyal. & Boatwr.	Fabaceae
Senna italica Mill. subsp. *arachoides* (Burch.) Lock	Fabaceae
Tabernaemontana elegans Stapf	Apocynaceae
Ziziphus mucronata Willd. subsp. *mucronata*	Rhamnaceae

Bulbine frutescens (L.) Wild. belongs to the Asphodelaceae and is a frequently used plant in South African medicine due to its effectiveness when applied to skin wounds and burns.[21,22]

The genus consists of 78 species, which are widely distributed in southern Africa, with some expanding into tropical Africa, and a few found in Australia.[23,24] Two species in the genus, namely *B. frutescens* and *B. natalensi*s (Oliv) Hutch, are traditionally used to treat diabetes.[25,26] Specific plant parts of these two species are used to prepare a concoction that is orally taken two/three times a day. In vitro studies done on *B. frutescens* showed some activity of the plant extract against enzymes and glucose uptake assays.[25,27] However, further studies need to be done to establish the biological activity of the plant.

Carpobrotus dimidiatus (Haw.) L. Bolus is a native plant distributed mainly in the coastal region of KwaZulu-Natal, South Africa.[21] The plant of interest is part of the Aizoaceae and one of the thirteen accepted species of *Carpobrotus*, of which seven are native to South Africa.[28] The South African species are widely used in traditional medicine to treat diabetes, wounds, inflammation, tuberculosis, high blood pressure, sore throat, dysentery, digestive ailments, and toothaches, and have bioactive properties such as antioxidant, antibacterial, and anticancer activities.[29-31] *C. edulis* (L.) N. E. Br. and *C. dimidiatus* (Haw.) are commonly used species in this genus and among the medicinal plants of commercial importance in South Africa due to their biological activities.[29,32] The phytochemistry of *C. dimidiatus* has yet to be described.

Ziziphus mucronata Wild., Buffalo thorn is classified under the Rhamnaceae.[33] A quick review of the family shows 11 tribes containing 60 genera and 900 species.[34] The *Ziziphus* genus has approximately 135 species, with 58 accepted species used for medicinal purposes to treat various diseases such as fever, diabetes, skin infection, and fungal infections.[33,35] The bioactivity of *Z. mucronata* has yet to be thoroughly investigated; hence, this study will evaluate the enzyme-inhibitory activity of the plant.

Sclerocarya birrea (A.Rich) Hochst (Anacardiaceae) is a deciduous tree indigenous to Africa.[36] Anacardiaceae, commonly known as the Cashew family of flowering plants, consists of about 80 genera and about 870 species, which occur mainly in the tropical and subtropical areas of the world, with a few found in the temperate regions.[37,38] *Sclerocarya birrea* (A.Rich) Hochst, also known as marula, is an essential tree to several African countries and serves different purposes in different countries. A bark concoction is used to treat diabetes, diarrhoea, and rheumatism. In Namibia, leaves are used to treat ear, nose, and throat infections and are

applied as a cream, ointment, and moisturiser. The oil obtained from marula seeds also serves a variety of cosmetic purposes.[39,40]

Cassia abbreviata Oliv. subsp. *beareana* (Holmes) Brenan belongs to the Fabaceae (subfamily-Caesalpinioideae) and is widely used to treat numerous diseases in Eastern and Southern African countries. The plant is a medium (6-7 m high) deciduous tree commonly spread in the tropical region of Africa. Decoctions of the plant are used as medicine for malaria and stomach ache. Furthermore, an infusion of different plant parts can be used against blood vomits, hernia, and skin disease.[41-43] The plant is used traditionally to manage diabetes and HIV, and a few reports have shown the extracts' activity against α-glucosidase.[44,45] Regardless of the plant's traditional uses to manage diabetes, safety and efficacy information is still missing.

Pappea capensis Eckl and Zegh belongs to the Sapindaceae (litchi family) and grows to 3.9 m tall. Different tribes in South Africa utilise the plant by taking a decoction/infusion from various plant parts for painful eyes, aphrodisiacs, and cattle (anaemia/ orally administered).[46,47] In vitro and in vivo studies have been conducted to test the antidiabetic activity of the plant extract, which exhibited activity.[48,49] However, further studies need to be undertaken.

Senna italica Mill. subsp. *arachoides* (Burch.) Lock (Fabaceae) is an indigenous plant in many African and some Asian countries. It is one of the 19 000 species in 730 genera of the Fabaceae.[50] *Senna* is a significant flowering plant genus that consists of 350 species and is unrestricted in tropical and subtropical regions.[51,52] *S. italica* is utilised for medicinal use against stomach aches, fever, skin problems, and ulcers.[53] Shai and colleagues[45] investigated the α-glucosidase inhibition of the plant extract, and the activity was noticed only at the highest concentration (1.8 mg/mL). Therefore, more studies must be conducted on the plant.

Maerua angolensis DC. (Capparaceae) is distributed in tropical Africa. The plant is taken as a decoction or inhaled steam of leaves soaked in hot/boiling water to treat stomach aches, headaches, skin cancer, and epilepsy.[54,55] However, the efficacy of these remedies is still uncertain. The pharmacological studies done on the plant show that the plant is used globally for its anti-inflammatory, anticarcinogenic, and antimicrobial activities.[56,57]

Merwilla plumbea (Lindl.) Speta belongs to the Hyacinthaceae. The *Merwilla* species is a well-known, traditionally used, yet endangered medicinal plant commercially available in South Africa. A phytochemical investigation shows that the plant contains compounds (homoisoflavanones) with anti-inflammatory and antibacterial activity.[58,59] However, there are still concerns due to overexploitation and anthropogenic factors.[60]

Apocynaceae consists of 3700 species and 424 genera. The genus *Tabernaemontana* Plum. ex L. is distributed worldwide in tropical and subtropical Africa, Oceania, America, and Asia,[61] and produces an abundance of indole and bisindole alkaloids that exhibit biological activity, some of which are potentially useful drugs.[62,63] *Tabernaemontana elegans* Stapf (Apocynaceae) is a species native to several African countries such as Kenya, Malawi, Mozambique Somalia, Tanzania, Zimbabwe, and South Africa. The species is commonly known as the toad tree because of the brown, wart-like skin of the fruits. *T. elegans* has several medicinal uses. It is used to treat pulmonary diseases, chest pains, cancer, heart disease, and as a wash for wounds.[61,64]

More information is needed on the pharmacological activities *of Gomphocarpus tomentosus* Burch. and *Senegalia mellifera* (Vahl) Seigel & Ebinger subsp. *detinens* (Burch.) Kyal. & Boatwr. making it an excellent candidate for exploring biological studies.

3.3 Results and discussion

The plants listed in Table 3.1 were dried, ground, and extracted by a method developed by Camp et al.[14] and optimised by Kratz et al.[65] Briefly, a fixed amount of plant material was extracted with hexane. The resulting extract was discarded because it consisted of fats and oils, which are too non-polar to be effective as drugs. A subsequent extraction with dichloromethane (DCM) and methanol (MeOH) afforded two extracts consisting of compounds with low to high polarity, respectively. The DCM and MeOH extracts were combined and dried to reduce the number of samples to assay. The dried extract was redissolved in MeOH and eluted through a polyamide-packed column to remove the tannins. Tannins are known to give false positive results in enzyme assays because of their non-selective binding ability. The yields of the extracts obtained are shown in Table 3.2.

Table 3.2. Lead-like small-scale extracts prepared from selected indigenous plants used to treat diabetes.

Name of plant	Extract code*	Mass (g) before SPE	Mass (g) after SPE
Bulbine frutescens	NKN-1-BF(l)-19a	0.0366	0.0317
	NKN-1-BF(l)-20b	0.0367	0.0310
	NKN-1-BF(r)-17a	0.0282	0.0214
	NKN-1-BF(r)-18b	0.0321	0.0198
Carpobrotus dimidiatus	NKN-1-CD(l)-23a	0.0778	0.0440
	NKN-1-CD(l)-24b	0.0616	0.0374
	NKN-1-CD(s)-21a	0.0468	0.0283
	NKN-1-CD(s)-22b	0.0681	0.0440
Cassie abbreviata	NKN-1-CA(sb)-36a	0.0358	0.0136
	NKN-1-CA(sb)-37b	0.0371	0.0144
Gomphocarpus tomentosus	NKN-1-GT(l)-38a	0.0239	0.0160
	NKN-1-GT(l)-39b	0.0313	0.0205
Maerua angolensis	NKN-1-MA(l)-52a	0.0285	0.0202
	NKN-1-MA(l)-53b	0.0232	0.0154
Merwilla plumbea	NKN-1-MP(b)-50a	0.0099	0.0063
	NKN-1-MP(b)-51b	0.0100	0.0063
Pappea capensis	NKN-1-PC(Ba)-40a	0.0281	0.0118
	NKN-1-PC(Ba)-41b	0.0127	0.0112
	NKN-1-PC(r)-42a	0.0145	0.0070
	NKN-1-PC(r)-43b	0.0138	0.0067
Sclerocarya birrea	NKN-1-SB(br)-25a	0.0206	0.0088
	NKN-1-SB(br)-26b	0.0160	0.0082
Senegalia mallifera	NKN-1-SM(l)-46a	0.0307	0.0091
	NKN-1-SM(l)-47b	0.0281	0.0057
Senna italica	NKN-1-SI(l)-44a	0.0112	0.0055
	NKN-1-SI(l)-45b	0.0113	0.0057

Table 3.2 continued

Name of plant	Extract code*	Mass (g) before SPE	Mass (g) after (high vacuum)
Tabernaemontana elagans	NKN-1-TE(l)-48a	0.0232	0.0152
	NKN-1-TE(l)-49b	0.0254	0.0163
Ziziphus mucronata	NKN-1-ZM(l)-27a	0.0388	0.0354
	NKN-1-ZM(l)-28b	0.0680	0.0348
	NKN-1-ZM(br)-29a	0.0099	0.0084
	NKN-1-ZM(br)-30b	0.0124	0.0114
Gnidia kraussiana	NKN-1-GK(r)-56a	0.1028	0.0705
	NKN-1-GK(r)-57b	0.1037	0.0714
	NKN-1-GK(s)-58a	0.0367	0.0168
	NKN-1-GK(s)-59b	0.0384	0.0171
	NKN-1-GK(fl)-60a	0.0173	0.0105
	NKN-1-GK(fl)-61a	0.0135	0.0081
	NKN-1-GK(lf)-62a	0.0438	0.0214
	NKN-1-GK(lf)-63b	0.0388	0.0194
	NKN-1-GK(rs)-64a	0.0246	0.0098
	NKN-1-GK(rs)-65a	0.0223	0.0073

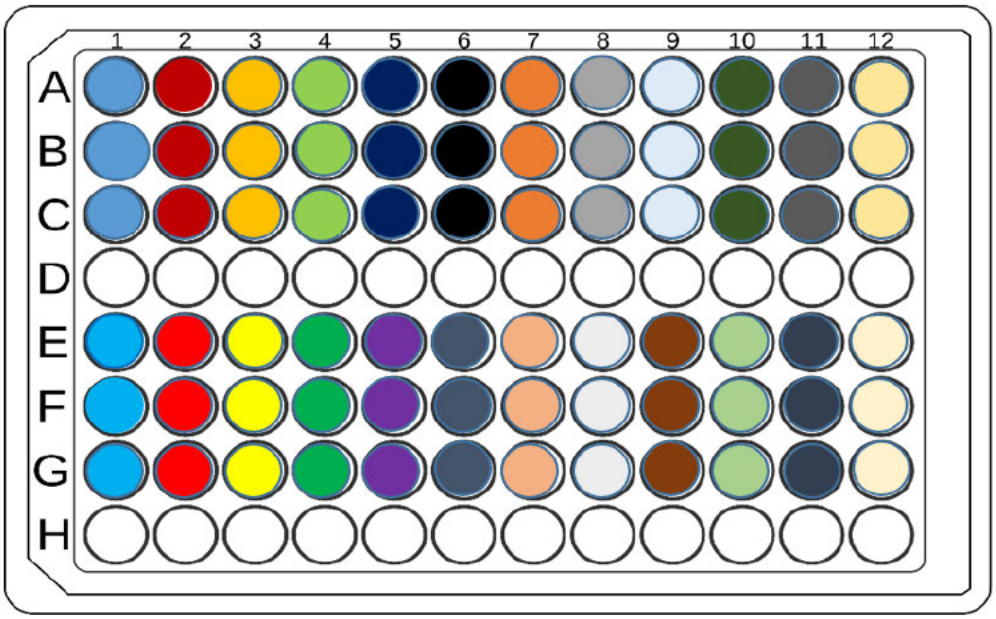
*l = leaves, r = roots, s = stem, br = branch, sb = stem bark, Ba= bark, b = bulb, fl= flower, rs=root skin

The assay used for identifying antidiabetic extracts was the inhibition of the enzyme α-glucosidase by the extracts. α-Glucosidase is an enzyme in the upper gastrointestinal tract that catalyses the hydrolysis of complex carbohydrates into glucose, resulting in hyperglycemia. When this enzyme is inhibited, the result is the delayed absorption of glucose and an improvement in glycemic management.[66] Three known α-glucosidase inhibitors, acarbose, miglitol, and voglibose (Figure 2.6), are currently on the market for the treatment of diabetes and are taken orally for the management of diabetes.[67] With only three α-glucosidase inhibitors on the market, there is room for further commercial α-glucosidase inhibitors.

The assay for inhibition of α-glucosidase is not a complex procedure and was done in-house by the author of this dissertation. Briefly, the assay is based on the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside, a colourless substrate, by the enzyme to produce *p*-nitrophenol, a yellow product with λ_{max} 405 nm, as described in Section 2.4.3. If the extract does not inhibit

the enzyme, the solution will turn yellow, whereas inhibition of the enzyme will result in a colourless solution.

The experiments were conducted in 96-well plates. The layout of the plate (Figure 3.1) was similar to that described by Lankatillake et al.[68] The assays were conducted in triplicate and included positive and negative controls.



- Negative control (50 µl Buffer + 30 µl enzyme + 20 µl substrate)
- Negative control blank (50 µl Buffer + 50 µl Buffer)
- Positive control (50 µl Acarbose + 30 µl enzyme + 20 µl substrate)
- Positive control blank (50 µl Buffer + 50 µl Acarbose)
- Test sample **1** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **1** blank (50 µl Buffer + 50 µl test sample)
- Test sample **2** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **2** blank (50 µl Buffer + 50 µl test sample)
- Test sample **3** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **3** blank (50 µl Buffer + 50 µl test sample)
- Test sample **4** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **4** blank (50 µl Buffer + 50 µl test sample)
- Test sample **5** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **5** blank (50 µl Buffer + 50 µl test sample)
- Test sample **6** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **6** blank (50 µl Buffer + 50 µl test sample)
- Test sample **7** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **7** blank (50 µl Buffer + 50 µl test sample)
- Test Sample **8** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **8** blank (50 µl Buffer + 50 µl test sample)
- Test sample **9** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **9** blank (50 µl Buffer + 50 µl test sample)
- Test sample **10** (50 µl extract + 30 µl enzyme + 20 µl substrate)
- Test sample **10** blank (50 µl Buffer + 50 µl test sample)

Figure 3.1. Plate layout for the α-glucosidase inhibition assay.

The plant extracts with a concentration of 0.5 mg/mL exhibited different α-glucosidase inhibition activity. Acarbose was used as a standard reference and showed activity ≥ 70% at a 10 mg/mL concentration. Two 96-well plates were used to assay the extracts collated in Table 3.1. The absorbance values and percentage inhibition of the extracts of NKN-1-GT(l)-38-56a, NKN-1-TE(l)-49a, NKN-1-GK(lf)-62a, NKN-1-PC(r)-43b, NKN-1-MP(b)-51b, and NKN-1-ZM(l) are shown in Figure 3.2 and Table 3.3. Likewise, the results of NKN-1-CD(s)-22b, NKN-1-SI(l)-45b, NKN-1-SM(l)-46a, NKN-1-PC(ba)-41b, NKN-1-CD(l)-24b, NKN-1-BF(r)-1a, NKN-1 MA(l)-52a, NKN-1-CA(sb)-36a, NKN-1-BF(l)-19a and NKN-1-SB(br)-25a are shown in Figure 3.3 and Table 3.4. Most plants showed poor inhibition activity. However, *S. birrea* showed remarkable inhibition, equivalent to the reference compound.

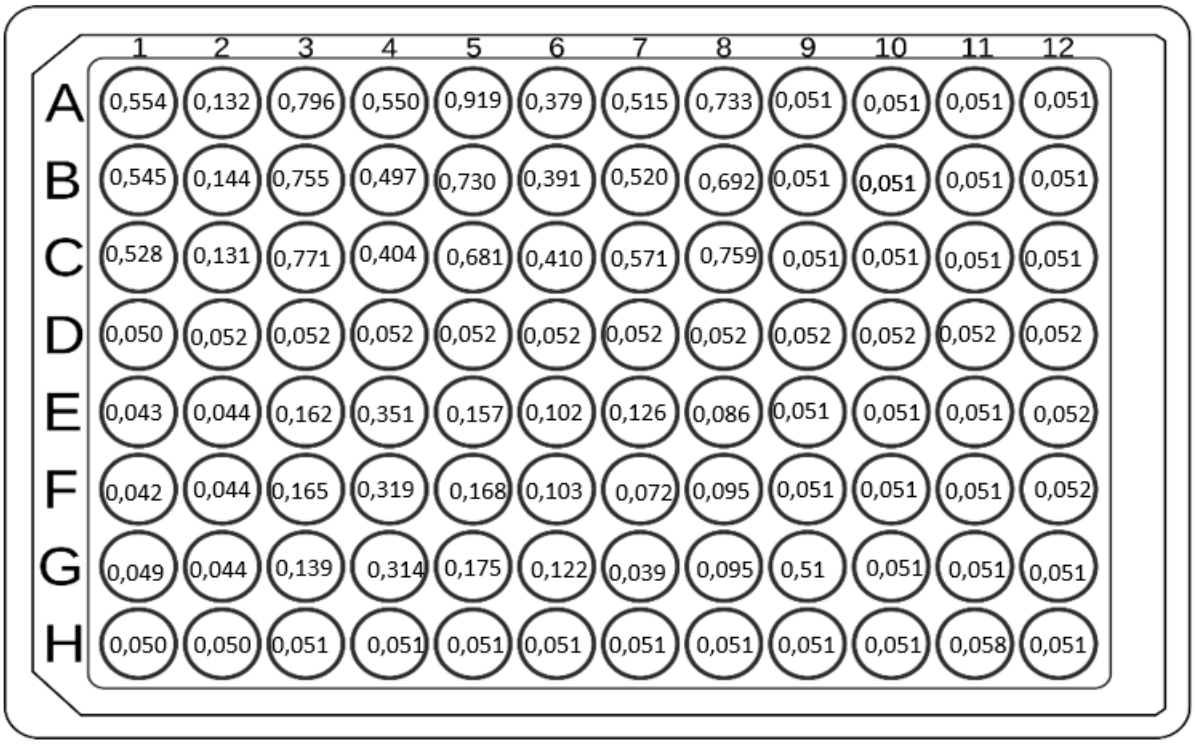


Figure 3.2. Absorbance reading of extracts on a 96-well plate. The negative control A1, B1, C1; positive control A2, B2, C2; NKN-1-GT(l)-38- 56a: A3, B3, C3; NKN-1-TE(l)-49a-64a: A4, B4, C4; NKN-1-GK(lf)-62a: A5, B5, C5; NKN-1-PC(r)-43b: A6, B6; NKN-1-MP(b)-51b: A7, B7, C7; NKN-1-ZM(l)-27b: A8, B8, C8, and blank wells are in rows E, F G.

Table 3.3. Absorbance readings and % inhibition by the first batch of the extracts.*

Plant extracts/fraction	Average absorbance value (λ=405)	Average blank value	% inhibition of α-glucosidase
Negative control	0.542	0.045	NA
Acarbose	0.136	0.044	81
NKN-1-GT(l)-38a	0.775	0.155	24 (absolute)
NKN-1-TE(l)-49a	0.484	0.328	68
NKN-1-GK(lf)-62a	0.777	0.167	22 (absolute)
NKN-1-PC (r)- 43b	0.393	0.109	43
NKN-1-MP(b)-51b	0.535	0.0957	11.6
NKN-1-ZM(l)-27b	0.728	0.092	27 (absolute)

*Concentration of extracts 0.5 mg/mL

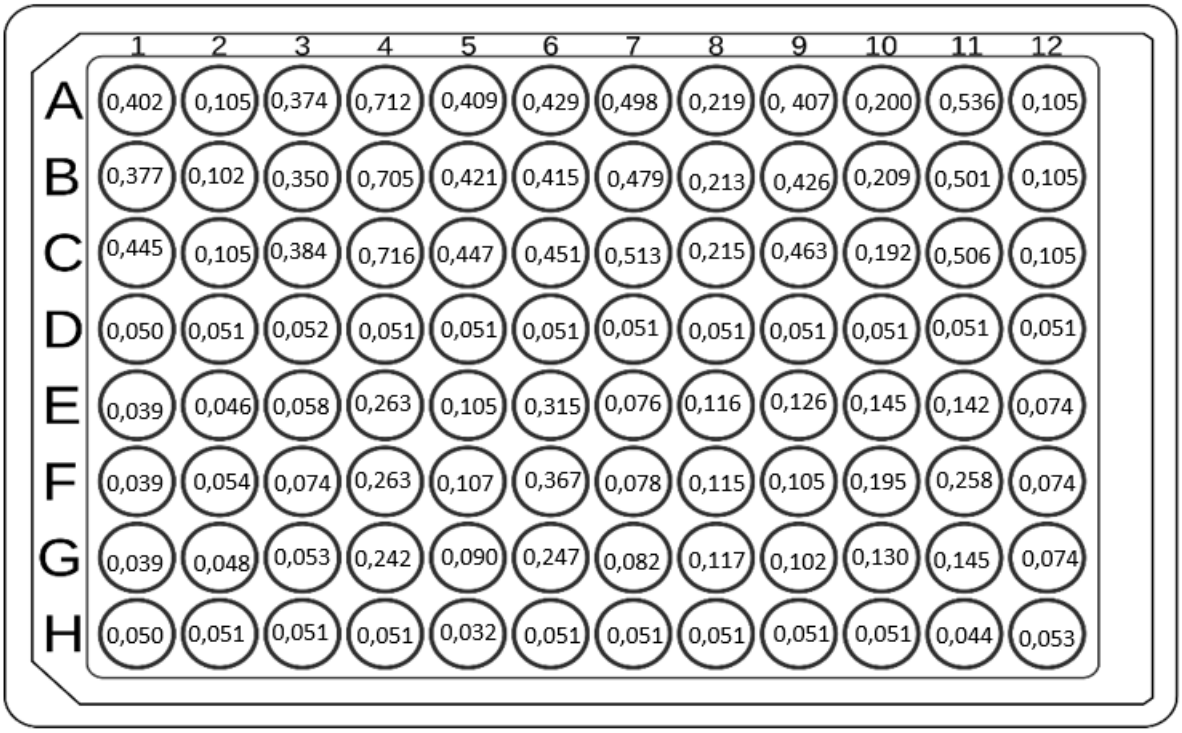


Figure 3.3. 96-Well plate showing absorbance reading of plant extracts. The negative control: A1, B1, C1; Positive control: A2, B2, C2; NKN-1-CD(s)-22b: A3, B3, C3; NKN-1-SI(l)-45b: A4, B4, C4; NKN-1-SM(l)-46a: A5, B5, C5; NKN-1-PC(ba)-41b: A6, B6, C6; NKN-1-CD(l)-24b:A7, B7, C7; NKN-1-BF(r)-1a : A8, B8, C8; NKN-1 MA(l)-52a -: A9, B9, C9; NKN-1-CA(sb)-36a A10, B10, C10; NKN-1-BF(l)-19a MeOH: A11, B11, C11; NKN-1-SB(br)-25a: A12, B12, C12; Blank wells are in rows E, F G.

Table 3.4. The % inhibition of the enzyme α-glucosidase by the second batch of extracts.*

Plant extracts/fraction	Average absorbance value (λ=405)	Average blank value	% inhibition of α-glucosidase
Negative control	0.408	0.039	N/A
Positive control (5mg/mL)	0.104	0.051	86
NKN-CD(s)-22b	0.369	0.061	16
NKN-SI(l)-45b	0.711	0.256	32 (absolute)
NKN-SM9l)-46b	0.426	0.101	12
NKN-PC(ba)-41b	0.431	0.309	67
NKN-CD(l)-24b	0.497	0.079	13 (absolute)
NKN-BF(r)- 18a	0.215	0.116	73
NKN-MA(l)-52a	0.432	0.111	13
NKN-CA(sb)-36a	0.200	0.157	86
NKN-BF (l)-19a	0.514	0.182	10
NKN-SB(br)-25a	0.104	0.074	91

*Concentration of extracts 0.5 mg/mL.

The results of the inhibition experiments are given in Table 3.3 and Table 3.4. The strongest inhibitions of an enzyme were the extracts of *S. birrea* branches [NKN-SB(br)-25a, 91%], *Cassia abbreviata* stem bark [NKN-CA(sb)-36a, 86%], *Bulbine frutescens* roots [NKN-BF(r)-18a, 73%], *Tabernaemontana elegans* leaves [NKN-1-TE(l)-49a, 68%], and *Pappea capensis* bark [NKN-PC(ba)-41b, 67% inhibition]. The extract of *S. birrea* was selected for further investigation since it demonstrated the highest activity.

Shai et al.[45] reported the inhibition of α-glucosidase of acetone extracts of the *Cassia abbreviata* stem bark and the roots of *Senna italica* at varying concentrations (0-3 mg/mL). The inhibition at the highest concentration tested was 88% for *C. abbreviata* and 60% for *S. italica*.[45] In the same study, it was found that the extract of *C. abbreviata* (IC_{50} 0.6 mg/mL) inhibited the enzyme at low concentrations (1 mg/mL, ≥ 70% inhibition). In contrast, the extract of *S. italica* (IC_{50} 1.8 mg/mL) did not demonstrate any inhibition with the same concentrations. Acarbose was used as a reference drug but did not show any inhibition at low concentrations, and IC_{50} 17 mg/mL was observed. The extract of *C. abbreviata* (86% inhibition, 0.5 mg/mL) was also active in the current study. Furthermore, Leteana et al.[46] showed that the extract of *C. abbreviata* is not cytotoxic active even in high concentrations (1000 µg/mL). These findings support the traditional use of the plant in controlling and managing diabetes. Utilisation of this species as a treatment for diabetic patients may yield desired outcomes.

A study conducted by Amoo and colleagues[69] on the inhibitory activity of acetone and petroleum ether plant extracts, including *Tabernaemontana elegans* (leaf) extract, *Cassia abbreviata* (leaves and stem bark) extract, and *S. birrea* (leaf, root bark, and stem bark) extract. From the stated species part, the leaf extract (acetone) of *C. abbreviata* could not be recorded (not determined) due to low activity at the highest concentration (1000 µg/mL). However, the acetone-prepared extracts of this species' other plant parts showed good activity (IC_{50} range of 34-606 µg/mL) compared to acarbose (IC_{50} 980 µg/mL). In contrast, all the petroleum ether extracts exhibited lower activity than acarbose. The results obtained by these authors are consistent with the ones reported herein, demonstrated by the potent enzyme inhibitions of *T. elegans* (68%), *C. abbreviata* (86 %), and *S. birrea* (91 %).

The activity of *C. abbreviata* and *S. birrea* extracts may be attributed to phenolics (specifically, proanthocyanidin), anthraquinones, and flavonoids. The positive results obtained from these species in biological activity suggest that these species can be considered potential candidates for new therapeutic approaches.

A study done on *Ziziphus muctonata* by Mousinho et al.[70] showed the activity of MeOH and aqueous crude extracts of the bark. The aqueous extract was more effective against α-glucosidase than the methanol extract.[70] The plant showed incredible activity on the antidiabetic parameters such as inhibition of α-amylase and α-glucosidase and glucose uptake. This supports the plant's historical use in the treatment of diabetes. Unfortunately, the current study observed low activity for the *Z. mucronata* (27%) extract. Various factors may influence such differences in results, i.e., the season the plant was harvested, the solvent used to prepare the extract, and the removal of tannins.[71,72]

The reference drug (acarbose) showed no inhibition at 1 mg/mL, the standard concentration used to prepare all the plant extract. Acarbose showed good inhibition at 10 mg/mL. it was observed in the literature that the IC_{50} value of acarbose differs substantially in different investigations.[45,73] A study by Oki et al.[73] showed that the IC_{50} values α-glucosidase inhibitors are affected by the enzyme origin, i.e., Baker's yeast, rat, rabbit and small intestine. Furthermore, substrate and enzyme concentration were reported to affect the IC_{50} of some inhibitors (competitive or noncompetitive).[74,75]

Various studies conducted on *S. birrea* showed exceptional inhibition activity for the stem bark and leaf extracts.[70,76-78] The consistent effectiveness of the plants in antidiabetic studies gives the green light for the plant to be tested in humans. *S. birrea* was selected for further investigation.

From literature reports,[69,70,76-79] it was noticed that the factor causing such a difference in these studies may be due to geographical area, season, incubation time during an assay, type of enzyme used, and mostly, the extraction solvent choice made for the study may influence the overall outcome. Numerous researchers have reported that solvent polarity may affect the type of compound extracts, which in turn influences the biological activity of the extracts.[80,81]

Conclusion

Five plant species, namely, *Pappea capensis*, *Tabernaemontana elegans*, *Bulbine frutescens*, *Cassia abbreviata*, and *Sclerocarya birrea*, showed good α-glucosidase inhibitory activity. In our study, *S. birrea* was selected for further investigation because the extract showed the best enzyme inhibition among the extracts investigated.

3.3 Experimental

3.3.1 General experimental procedures

Small-scale extractions of the plant material were performed in a Scientech ultrasonic bath**.** For the removal of tannins, empty 3 mL SPE tubes (Phenomenex) fitted with frits (pore size 20 µM, Phenomenex) and polyamide (particle size 0.05-0.16 mm, Carl Roth) were used. Assays were performed in 96-well microplates (Sigma-Aldrich), and the absorbance was measured at 405 nm using a multi-plate reader (Fisher Scientific, FB505) set at 37 °C.

Acarbose, α-glucosidase from *Saccharomyces cerevisiae*, 4-nitrophenyl β-D- glucopyranoside (*p*-NPG), dimethyl sulfoxide (DMSO), and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Sigma Aldrich. Hexane, DCM, and MeOH were bought from Honeywell.

3.3.2 Plant material

The aerial parts (leaves, stem, bark) of four species, namely, *Bulbine frutescens* (Asphodelaceae, accession number NU0092224), *Carpobrotus dimidiatus* (Aizoaceae, accession number NU0092223), *Sclerocarya birrea* (Anacardiceae, accession number NU0092226), and *Ziziphus mucronate* (Rhamnaceae, accession number NU0092227) were collected from the University of KwaZulu-Natal (UKZN) Botanical Garden in Pietermaritzburg. Plant material of *Cassia abbreviata* (Fabaceae, accession number SANBI 3536000), *Gomphocarpus tomentosus* (Apocynaceae, accession number SANBI 6787000), *Pappea capensis* (Sapindaceae, accession number SANBI 4784000), *Senna italica* (Fabaceae, accession number: SANBI 3536020), *Senegalia mallifera* (Fabaceae, accession number: SANBI 3446000), *Tabernaemontana elegans* (Apocynaceae, accession number: SANBI 6603000), *Merwilla plumbea* (Hyacinthaceae, accession number: SANBI 1093010), and *Maerua angolensis* (Capparaceae, accession number: SANBI 311200) were collected from two districts in Limpopo province, South Africa namely Vhembe (Xikundu and Phunda Maria villages) and Mopani district by Dr Candy Khosa (Agricultural Research Council). Lastly, *Gnidia kraussiana* (Thymelaceae) was collected in the Valley of a 1000 Hills, KwaZulu-Natal. Ms Alison Young, the curator of the UKZN botanical garden in Pietermaritzburg, identified plant species collected in the UKZN Botanical Gardens, and voucher specimens of these plants were deposited at the BEWS Herbarium, School of Life Science, UKZN (assisted by Dr Christina Curry). Plant species collected from Limpopo were collected by Dr M C Khoza and identified by a botanist from the South African National Biodiversity Institute (SANBI, Pretoria). These plant vouchers were deposited at the SANBI herbarium in Pretoria.

3.3.3 Preparation of lead-like small-scale extracts

The plant material was dried for two weeks at room temperature and ground to a fine powder using a coffee grinder. The ground material (300 mg) was defatted with 5 mL hexane by sonication of the sample for 15 minutes. After filtration, the plant material was extracted in a sonicating bath for 15 minutes at room temperature with DCM (7 mL) and then with methanol (13 mL). The extracts were filtered using filter paper fitted into a glass funnel in the same flask. The combined extract was dried under a vacuum using a rotary evaporator (65˚C). The masses

of the dried extracts were recorded before continuing with the next step (Table 3.2). Tannins were removed from the extracts using the following procedure: An empty 3 mL SPE tube was fitted with a frit and filled with polyamide gel (900 mg). The extract was dissolved by sonication in MeOH (4 mL) and filtered through the polyamide. The column was washed twice with methanol (4 mL). After that, the combined extract was dried first under a stream of nitrogen and then under a high vacuum. The dried lead-like-enriched extracts were weighed (Table 3.2) and stored in a refrigerator for further use.

3.3.4 α-Glucosidase assay

Dried extracts were assayed for α-glucosidase inhibition using a modification of a previously reported protocol.[68,82] Briefly, the procedure involved preparing a fresh buffer solution (buffer 1) consisting of a 100 mM sodium dihydrogen phosphate solution at pH 6.9 containing 2% DMSO. This solution was used to prepare extracts and acarbose (positive control) solutions. A working solution of the test samples (1 mg/mL) was prepared from the plant extracts. Buffer 2 was a sodium dihydrogen phosphate solution (100 mM, pH 6.9). This solution was used to prepare the enzyme, α-glucosidase (1 U/mL), and the substrate [4-nitrophenyl β-D-glucopyranoside (*p*-NPG)] (5 mM, final concentration 1 mM) solutions.

3.3.4.1 Protocol for the α-glucosidase assay

The assay was performed in a 96-well plate as described by Lankatillake et al.*,* [68] with minor modifications. The layout of the plate is illustrated in Figure 3.1. Buffer 1 (50 µL) was added to the negative control and blank wells. Acarbose and extract solutions (1 mg/mL working concentration) (50 µL) were added to the wells and the respective blank wells. After that, 30 µL of the enzyme [α-glucosidase (1 U/mL)] solution was added to the negative control and test wells. The contents of the wells were mixed gently using a multichannel pipette. The plate was then pre-incubated in the dark at 37 °C for 10 minutes. To initiate the reaction, 20 µL of the substrate (*p*-NPG) was added to all test wells (giving a final concentration of 0.5 mg/mL for the extracts), and well contents were again mixed using a multichannel pipette before incubating the plate in the dark at 37 °C for 20 minutes. 50 µL of Na_2CO_3 was added to all the wells to stop the reaction. All the assays were done in triplicate. The absorbances of the plate wells were read using the microplate reader set at 405 nm and a temperature of 37 °C.

The enzyme inhibition activity was calculated using the equation:

$$Enzyme\ inhibition = \left(1 - \frac{blank\ corrected\ absorbance\ of\ test\ well}{blank\ corrected\ absorbance\ of\ negative\ conrol}\right) \times 100$$

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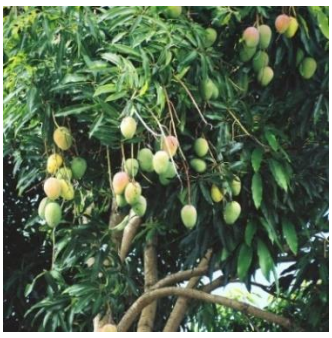
Chapter 4 Phytochemistry and α-glucosidase inhibition of *Sclerocarya birrea* extracts

4.1 Introduction to Anacardiaceae

In Chapter 3, the inhibition of α-glucosidase by twenty-one extracts prepared from thirteen plant species is reported. The most active extracts were prepared from branches of *Sclerocarya birrea* (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae).

Anacardiaceae, commonly known as the cashew family of flowering plants, consists of approximately 80 genera and 87 species in tropical, subtropical, and temperate areas.[1,2] This family includes plants of economic importance and some species that are only used in small communities. Anacardiaceae contains well-known plants with edible fruits, including mango (M*angifera indica* L.), cashew plant/nut (*Anacardium occidentale* L.), and pistachio nut (*Pistacia vera* L.) (Figure 4.1). Furthermore, this family includes species that are culturally significant in their respective countries and are also used as food, e.g., *Pistacia lentiscus* L. (fruit) and *S. birrea* (A.Rich.) Hochst. (marula fruit), *Schinus terebinthifolius* Raddi. (Brazilian pepper), *Schinus molle* L. (Peruvian pepper), and *Rhus coriaria* L. (sumac).[2,3]



Mangifera indica (Mango)[4]

Pistacia vera (Pistachio nut)[5]

Anacardium occidentale[6]

Figure 4.1. Well-known species from the Anacardiaceae.

4.2 *Sclerocarya birrea* (A.Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro

S. birrea occurs throughout the Savanah region and bushland, from West Africa (Nigeria and Cameroon) through central Africa (Ethiopia) to South Africa.[7-9] Three subspecies of *S. birrea* are recognised, i.e., *S. birrea* subsp. *birrea* that grows from tropical West Africa to Ethiopia and Tanzania, *S. birrea* subsp. *multifoliolata* (Engl.) Kokwaro occurs in Tanzania, and *S. birrea* supsp. *caffra* (Sond.) Kokwaro ranges from South Africa to Kenya, Mayotte, and Madagascar.[10] The shape and size of the leaves differentiate the three subspecies.

S. birrea, or marula, is an essential multi-purpose plant consumed by animals and used in rural communities as medicine, food, alcohol, and as a starting material for artistic work. As a result, the tree is often referred to as the 'Tree of Life'.[7] The tree's height ranges from 10-15 m with a cracked grey bark, green-white flowers, compound leaves, and mango-resembling fruit (yellow when ripe). It is more common in Limpopo province and is a protected species in South Africa.[11] Furthermore, locals act as vendors to harvest and sell the alcohol made from ripe fruits.

In Ghana, the aerial parts of *S. birrea* are used to treat snake bites, pruritus, pharyngitis, splenomegaly, and goitre problems. The Zulu people in South Africa prepare a decoction using stem bark to treat dysentery, fever, stomach ailments, ulcers, and diarrhoea.[12-14] Numerous researches have documented the bioactivities such as antibacterial, antimicrobial, analgesic, antioxidant, and antidiabetic properties.[8,13,15] Several surveys have been conducted in Limpopo province (South Africa) regarding the consumption or usage of the plant. This species provides numerous benefits for subsistence and the production of marketed and sold commodities. In Limpopo, About 30% of the people utilise the plant for beer and juice making.[16]

Numerous studies (phytochemistry and bioactivity) have been reported on *S. birrea*. Early studies by Braca et al.[17] on the leaves of the plant led to the isolation of nine phenolic compounds (**4.1**-**4.9**) from wild plant species and two additional epicatechin derivates from the cultivated species (**4**.**10**-**4.11**) with antioxidant activity (Figure 4.2). A comparison of the qualitative phenolic composition of both the wild and cultivated species was done, and the wild species was found to be richer in phenolic compounds.

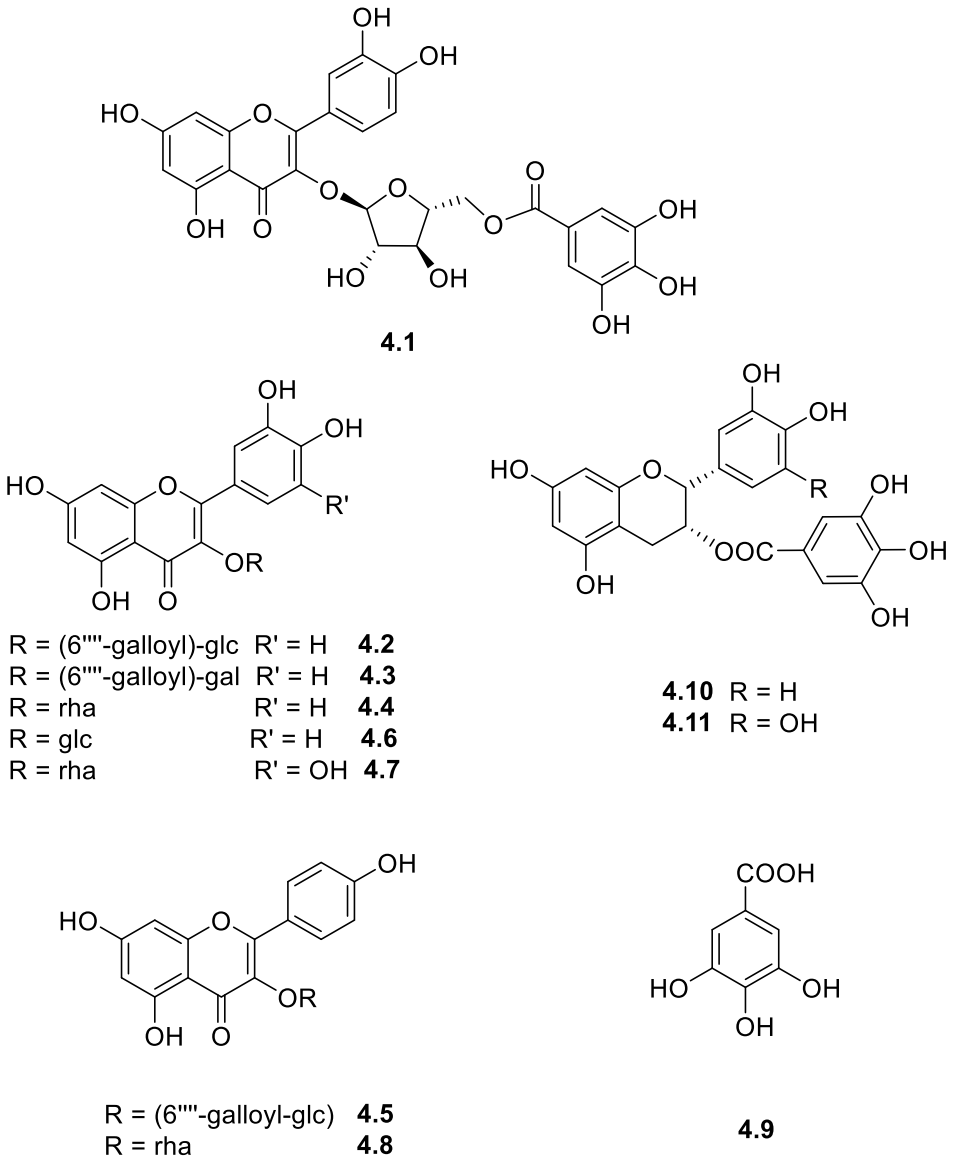
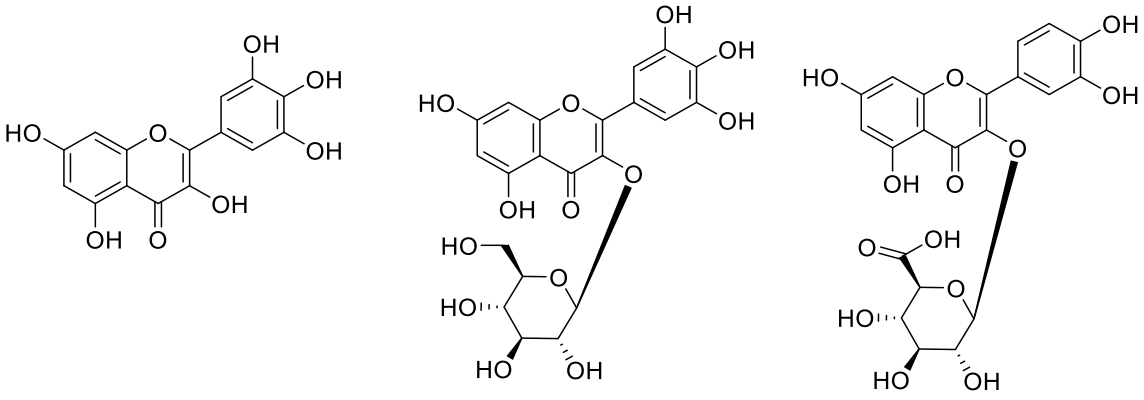


Figure 4.2. Chemical structures of compounds isolated from *Sclerocarya birrea* wild plant (**4.1-4 9**, MeOH extracts) and two purified (**4.10-4.11**) from cultivated species.

A recent paper reported the identification of eight compounds of which three (Figure 4.3) showed antidiabetic activity in a cell-based study. A 2-deoxy-glucose (2DG) technique was used to demonstrate the stimulatory effect on glucose uptake in differentiated C2C12 myocytes by spray-dried aqueous leaf extracts.[18]



4.12 **4.13** **4.14**

Figure 4.3. Structures of flavonol glycosides (**4.12-4.14**) isolated from *S. birrea* leaves.

Among the methods used to identify and isolate bioactive compounds, analytical-based methods are gaining attention among researchers due to the time saved when using these techniques. Recently, Cádiz-Gurrea et al.[19] observed 71 compounds by HPLC–ESI–TOF–MS in aqueous and ethanol stem bark extracts of *S. birrea* as summarised in Table 4.1. Many flavonoid aglycones and galloyl esters were identified and may be responsible for the biological activity of the plant. Furthermore, Abdallah et al.[19] used LC-MS to determine the phenolic and flavonoid composition of the stem bark extract of *S. birrea*. He observed that gallic acid and catechins were the most abundant phenolic compounds in the extracts of *S. birrea*.

Table 4.1. Chemical composition of *Sclerocarya birrea* stem bark extract identified using LC–ESI–TOF–MS.[18,20,*]

Proposed Compound	RT	*m/z* calc.	*m/z* Meas	Mol. formula
Quinic acid	5.6	191.0561	191.0565	$C_7H_{12}O_6$
Sucrose	7.2	341.1089	341.1095	$C_{12}H_{22}O_{11}$
D-Raffinose	9.9	503.1618	503.1607	$C_{18}H_{32}O_{16}$
Galloyl glucose isomer 1	12.4	331.0671	331.0678	$C_{13}H_{16}O_{10}$
Galloyl glucose isomer 2	13.1	331.0671	331.0677	$C_{13}H_{16}O_{10}$
Gallo(epi)catechin dimer	13.4	609.1250	609.1228	$C_{30}H_{26}O_{14}$
Gallic acid	13.7	169.0142	169.0149	$C_7H_6O_5$
UK1[#]	14.4	411.0259	411.0239	$C_{17}H_8N_4O_9$
Bis(epi)gallocatechin monogallate 1	14.8	761.1359	761.1359	$C_{37}H_{30}O_{18}$
(Epi)gallocatechin isomer 1	14.8	305.0667	305.0670	$C_{15}H_{14}O_7$

Table 4.1 continued

Proposed Compound	RT	*m/z* calc.	*m/z* Meas	Mol. formula
Bis(epi)gallocatechin monogallate 2	15.2	761.1359	761.1365	$C_{37}H_{30}O_{18}$
(Epi)gallocatechin gallate (epi)catechin isomer 1	15.5	745.1410	745.1414	$C_{37}H_{30}O_{17}$
Protocatechuic acid	15.7	153.0193	153.0192	$C_7H_6O_4$
Procyanidin B dimer isomer 1	15.9	577.1351	577.1335	$C_{30}H_{26}O_{12}$
(Epi)catechin-(epi)gallocatechin	16.5	593.1301	593.1311	$C_{30}H_{26}O_{13}$
(Epi)gallocatechin gallate (epi)catechin isomer 2	16.7	745.1410	745.1402	$C_{37}H_{30}O_{17}$
Bis(epi)gallocatechin digallate	16.8	913.1469	913.1493	$C_{44}H_{34}O_{22}$
(Epi)gallocatechin gallate (epi)catechin isomer 3	17.4	745.1410	745.1402	$C_{37}H_{30}O_{17}$
(Epi)gallocatechin gallate isomer 1	17.8	457.0776	457.0769	$C_{22}H_{18}O_{11}$
(Epi)gallocatechin gallate (epi)catechin isomer 4	18.4	745.1410	745.1418	$C_{37}H_{30}O_{17}$
(Epi)gallocatechin gallate (epi)catechin isomer 5	18.6	745.1410	745.1414	$C_{37}H_{30}O_{17}$
(Epi)gallocatechin isomer 2	18.7	305.0667	305.0669	$C_{15}H_{14}O_7$
Catechin	19.4	289.0718	289.0725	$C_{15}H_{14}O_6$
(Epi)gallocatechin gallate (epi)catechin gallate isomer 1	19.5	897.1520	897.1543	$C_{44}H_{34}O_{21}$
(Epi)catechin gallate (epi)catechin isomer 1	20	729.1461	729.1471	$C_{37}H_{30}O_{16}$
(Epi)catechin gallate (epi)catechin isomer 2	20.5	729.1461	729.1476	$C_{37}H_{30}O_{16}$
Protocatechuic acid aldehyde	20.5	137.0244	137.0245	$C_7H_6O_3$
Dimethoxy-hydroxyphenyl-*O*-galloyl-glucopyranoside	20.8	483.1144	483.1134	$C_{21}H_{24}O_{13}$
(Epi)gallocatechin gallate isomer 2	21.9	457.0776	457.0783	$C_{22}H_{18}O_{11}$
(Epi)catechin gallate (epi)catechin gallate isomer 1	22.2	881.1571	881.1593	$C_{44}H_{33}O_{20}$
(Epi)catechin gallate (epi)catechin gallate isomer 2	23.1	881.15723.11	881.1586	$C_{44}H_{33}O_{20}$
Hydroxy-methoxyphenyl-*O*-galloyl-glucopyranoside	23.5	453.1038	453.1042	$C_{20}H_{22}O_{12}$
UK2 isomer 1[#]	23.6	439.0671	439.0668	$C_{20}H_{22}O_{10}$
Epicatechin	24.5	289.0718	289.0722	$C_{15}H_{14}O_6$
(Epi)gallocatechin gallate isomer 3	24.7	457.0776	457.0779	$C_{22}H_{18}O_{11}$
Eriodictyol-*O*-glucoside	24.9	499.1089	449.1069	$C_{21}H_{22}O_{11}$
(Epi)catechin gallate (epi)catechin gallate isomer 3	25.5	881.1571	881.1586	$C_{44}H_{34}O_{20}$

[*]The names of compounds in this table are the names given by the authors of the papers.[19,20] Not all the names are the correct systematic names.

[#]UK = unknown

Table 4.1 continued

Proposed Compound	RT	*m/z* calc.	*m/z* Meas	Mol. formula
(Epi)catechin gallate (epi)catechin gallate isomer 6	25.9	745.1410	745.1416	$C_{37}H_{30}O_{17}$
Galloyl glucosyl dihydroxy methoxy acetophenone	26.2	495.1144	495.1131	$C_{22}H_{24}O_{13}$
UK2 isomer 2[#]	26.5	439.0671	439.0664	$C_{22}H_{16}O_{10}$
(Epi)gallocatechin-(epi)catechin-gallate	26.6	743.1254	743.1282	$C_{37}H_{28}O_{17}$
(Epi)gallocatechin gallate (epi)catechin gallate isomer 2	26.7	897.1520	897.1516	$C_{44}H_{34}O_{21}$
Lyoniside	27.5	551.2134	551.2136	$C_{27}H_{36}O_{12}$
(Epi)catechin gallate isomer 1	27.9	441.0827	441.0836	$C_{22}H_{18}O_{10}$
(Epi)catechin gallate isomer 2	28.2	441.0827	441.0836	$C_{22}H_{18}O_{10}$
(Epi)catechin-3-*O*-glucoside-gallate	28.9	603.1355	603.1355	$C_{28}H_{28}O_{15}$
Procyanidin B dimer isomer 2	29.6	577.1351	577.1346	$C_{30}H_{26}O_{12}$
(Epi)catechin gallate isomer 3	30.1	441.0827	441.814	$C_{22}H_{18}O_{10}$
Dihydromyricetin isomer 1	31	319.0459	319.0465	$C_{15}H_{12}O_8$
(Epi)catechin gallate (epi)catechin gallate isomer 4	31.8	881.1571	881 .1602	$C_{44}H_{34}O_{20}$
(Epi)catechin gallate (epi)catechin isomer 3	32.1	729.1461	729.1488	$C_{37}H_{30}O_{16}$
(Epi)afzelechin gallate	33	425.0878	425.0892	$C_{22}H_{18}O_9$
Myricetin glucoside	33.5	479.0831	479.0849	$C_{21}H_{20}O_{13}$
Jaceidin triacetate	33.6	485.1089	485.1093	$C_{24}H_{22}O_{11}$
Phloretin-di-C-glucoside	34.6	597.1825	597.1807	$C_{27}H_{34}O_{15}$
Trihydroxystilbene glucosyl-*O*-gallate	34.7	541.1351	541.1362	$C_{27}H_{26}O_{12}$
Dihydromyricetin isomer 2	35.6	319.0459	319.0465	$C_{15}H_{12}O_8$
UK3[#]	35.7	439.1093	439.1073	$C_{16}H_{24}O_{14}$
Homaloside D	36.4	543.1508	543.1529	$C_{27}H_{28}O_{12}$
Phloretin-C-glucoside (nothofagin)	36.4	435.1297	435.1281	$C_{21}H_{24}O_{10}$
Rhamnetin	36.6	315.0510	315.0510	$C_{16}H_{12}O_7$
Dihydroquercetin	37.6	303.0510	303.0514	$C_{15}H_{12}O_7$
Pentamethoxystilbene isomer 1	37.7	329.1394	329.1402	$C_{19}H_{22}O_5$
Quercetin glucoside	37.9	463.0882	463.0883	$C_{21}H_{20}O_{12}$
Syringic aldehyde	38.7	181.0506	181.0503	$C_9H_{10}O_4$
Pentamethoxystilbene isomer 2	38.9	329.1394	329.1387	$C_{19}H_{22}O_5$
Ellagic acid	40.2	300.9990	301.004	$C_{14}H_6O_8$
Naringenin	40.8	271.0612	271.0609	$C_{15}H_{12}O_5$
Taxifolin	41.6	303.0510	303.0519	$C_{15}H_{12}O_7$
Nonanedioic acid (azelaic acid)	42.3	187.0981	187.0976	$C_9H_{16}O_5$
Flavanone	47.2	271.0612	271.0622	$C_{15}H_{12}O_5$
N,*N*-Dimethylaniline	0.043	121.0896		$C_8H_{11}N$

Table 4.1 continued				
Proposed Compound	**RT**	***m/z* calc.**	***m/z* Meas**	**Mol. formula**
Vidarabine	0.148	267.0971		$C_{10}H_{13}N_5O_4$
DL-Isoleucine	1.296	131.0949		$C_6H_{13}NO_2$
DL-Norleucine	1.601	131.0950		$C_6H_{13}NO_2$
L-Phenylalanine	1.937	165.0793		$C_9H_{11}NO_2$
Diisopropylethylamine	2.046	129.1521		$C_8H_{19}N$
2-Amino-1,3,4-octadecanetriol	2.305	317.2935		$C_{18}H_{39}NO_3$
N-Methyl-4-piperidone	2.371	113.0845		$C_6H_{11}NO$
1,8-Diazabicyclo[5.4.0]undec-7-ene	12.833	152.1312		$C_9H_{16}N_2$
Isophthalic acid	13.698	166.0256		$C_8H_6O_4$
Meprednisone acetate	15.444	414.2042		$C_{24}H_{30}O_6$
Gentisic acid	15.734	154.0260		$C_7H_6O_4$

[#]UK- unknown

Diabetes is the fourth leading cause of death in South Africa.[21] As such, a search for safe, efficient, and cheap DM drugs is of high importance. The current project aimed to isolate and characterise compounds from *Sclerocarya birrea* using analytical and chromatography techniques. The stem bark was selected for the study.

4.3 Results and discussion

4.3.1 Extraction of S. birrea bark and fractionation of the extracts

The ground stem bark of *S. birrea* was successively extracted with hexane, DCM-MeOH (1:1), and MeOH. The hexane extract contained mainly highly non-polar material, and was not investigated further. On the other hand, the methanol extract contained highly polar compounds (tannins) that might give false positive results in enzyme assays. Therefore, in the current study, the emphasis was on the DCM-MeOH extract of *S. birrea* bark. The DCM-MeOH extract was further fractioned into five fractions by vacuum liquid chromatography (VLC) according to polarity using the solvents hexane-DCM (9:1), DCM-EtOAc (20:1), EtOAc, EtOAc-MeOH (5:1), and MeOH.

4.3.2 Inhibition of α-glucosidase by plant extracts/fractions

The inhibitory effects of the VLC fractions [hexane-DCM (9:1); DCM-EtOAc (20:1); EtOAc; EtOAc-MeOH (5:1), and MeOH] on the enzyme α-glucosidase were investigated. Preliminary assay results showed that the plant extracts inhibited the activity of α-glucosidase by 95-99% at a concentration of 500 µg/mL (Table 4.2). Although the highest enzyme inhibition was

observed for the DCM-EtOAc fraction (99%), there was no substantial difference between the fractions, likely because of the high concentrations of the extracts used.

Table 4.2. % Inhibition of α-glucosidase by the *S. birrea* fractions.

Plant extract*	Average absorbance values (λ= 405 nm)	Average blank values	% inhibition of α-glucosidase
Negative control	1.758	0.045	N/A
Acarbose (5 mg/mL)	0.285	0.046	86
S. birrea (DCM/EtOAc, 20:1)	0.371	0.365	99
S. birrea (EtOAc)	0.328	0.213	93
S. birrea (EtOAc/MeOH, 5:1)	0.260	0.189	95
S. birrea (MeOH)	0.163	0.122	97

*Concentration 0.5 mg/mL

The IC_{50} values of the VLC fractions were also determined. The inhibition curves for the different fractions are given in Figure 4.4 and Figure 4.5. When considering the results (Table 4.3), it was observed that the EtOAc and the EtOAC-MeOH fractions had the lowest IC_{50} values and were the most active fractions.

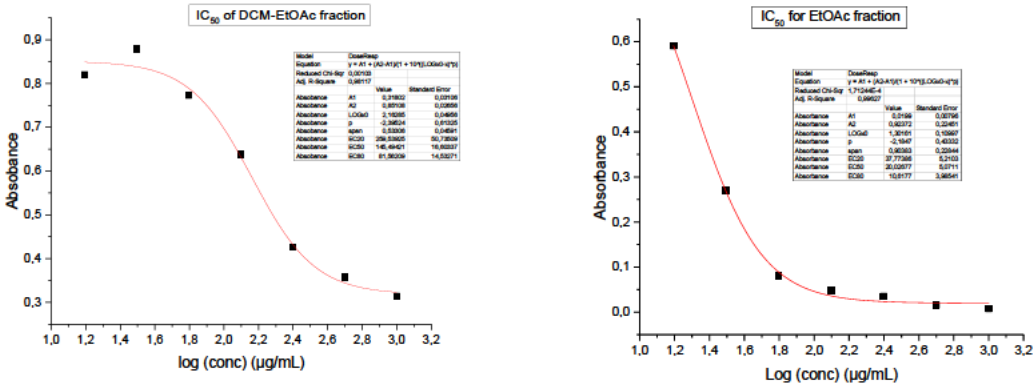


Figure 4.4. Inhibition curve of the DCM-EtOAc and EtOAc fractions.

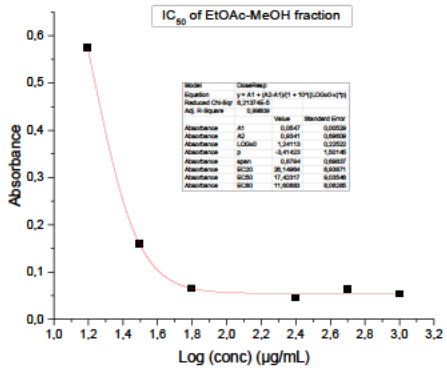


Figure 4.5. Inhibition curves of the VLC, EtOAc-MeOH fraction.

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Table 4.3. IC_{50} values of the VLC fractions for α-glucosidase inhibition.

Extract/ compound	IC_{50} (µg/mL) of α-glucosidase	Regression value
Acarbose	47.51 ± 6.02	0.9766
DCM-EtOAc (20:1)	145.49 ± 16.60	0.98117
EtOAc	20.03 ± 5.07	0.99637
EtOAc-MeOH (5:1)	17.42 ± 9.04	0.99809

4.3.3 UPLC-QTOF-MS-MS analysis of *S. birrea* extracts and fractions

The composition of the different fractions was investigated by UPLC-MS-MS. The UPLC (ultra-performance liquid chromatograph) separates the compounds according to polarity. Two detectors are attached in series to the UPLC: a diode array (UV) detector (DAD) and a mass detector. From the DAD, UV spectra of the compounds can be obtained. The different degrees of unsaturation of the various types of flavonoids influence the chromophores and the UV spectra of the compounds. Therefore, UV spectroscopy is particularly useful in identifying different classes of flavonoids. With a time-of-flight (TOF) mass spectrometer, high-resolution data of mass fragments allow the calculation of the molecular formula of a compound. With electrospray ionization (ESI) in mass spectrometry (MS), ionization can be performed in the positive mode, which results in positive ions, or in the negative mode, which results in negative ions. Ionization of polyphenols with acidic phenolic protons is generally more successful in the negative mode.

Figure 4.6 gives the LC-MS chromatograms of the crude DCM-MeOH extract and the VLC EtOAc fraction.

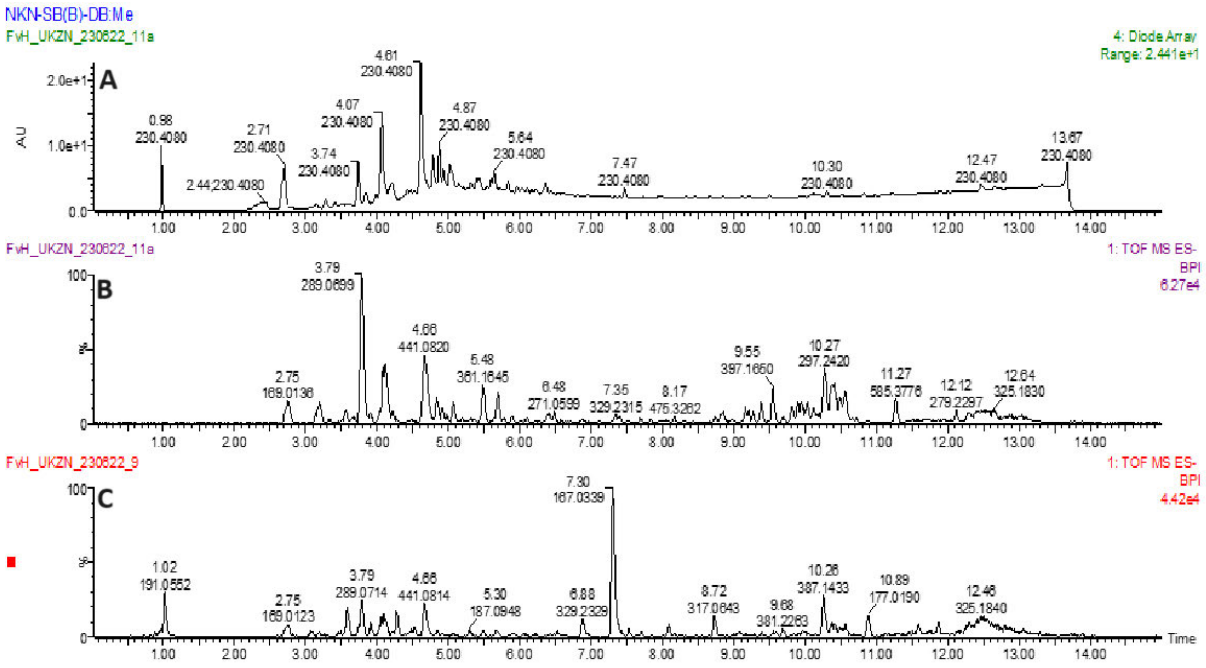


Figure 4.6. LC-MS chromatogram of (A) the VLC EtOAc fraction with PDA detection, (B) the VLC EtOAc fraction with ESI-(-)-MS detection, and (C) the DCM-MeOH crude extract with ESI-(-)-MS detection.

The chromatograms show that the VLC EtOAc fraction is a less complex mixture than the crude DCM-EtOAc extract and contains the compounds with R_t 2.50-6.00 minutes. Considering the activity of the VLC EtOAc fraction, it is likely that these compounds may play a role in the fraction's activity. Therefore, the structures of these compounds were investigated by UV spectroscopy and mass spectrometry. An expansion (R_t 2.20-7.00) of the VLC EtOAc fraction with the peaks of the identified compounds labelled is given in Figure 4.7.

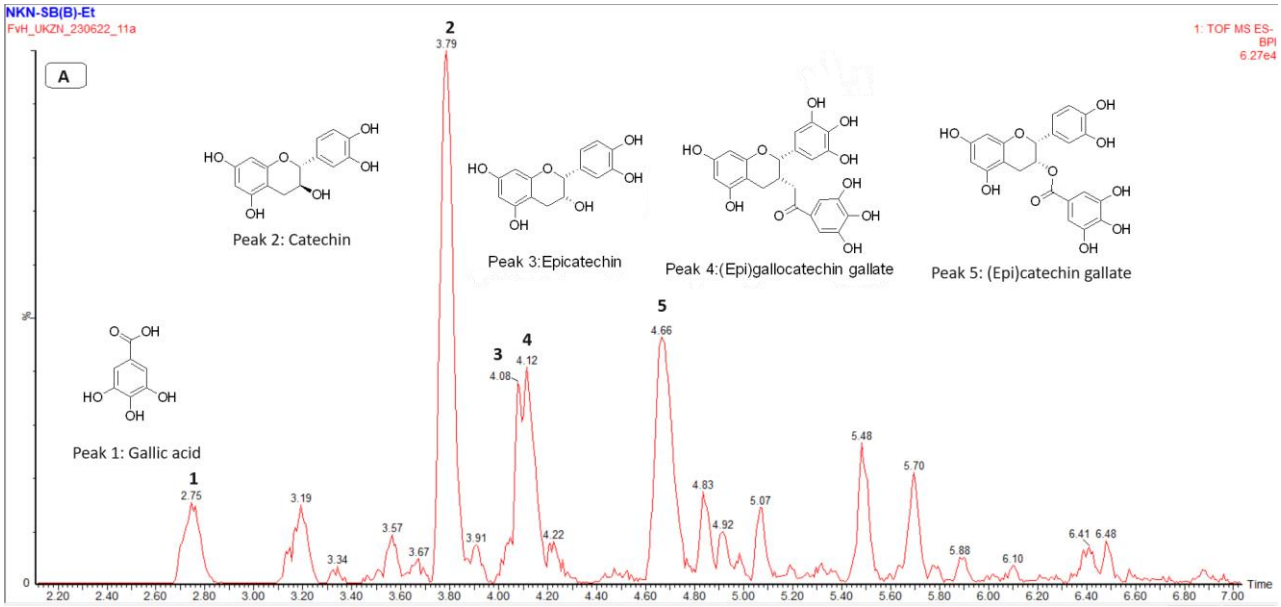
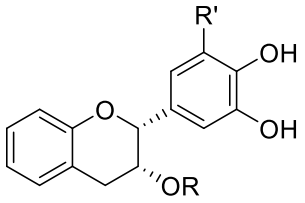
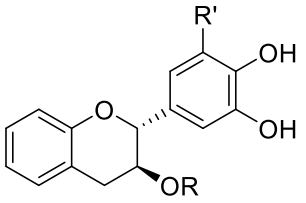


Figure 4.7. Compounds identified from the LC-TOF-MS chromatogram obtained from the EtOAc fraction of *S. birrea*.

In the HR-ESI-(-)-MS, the compound with R_t 2.75 displayed $[M-H]^-$ with *m/z* 169.0139 that corresponds to a molecular formula of $C_7H_8O_5$ (calc. for $C_7H_7O_5$, 169.0137) (Figure. S1). This compound was identified as gallic acid (**4.15**). A fragment ion was observed at *m/z* 125.0240, which is consistent with the loss of CO_2 (calc. for $C_6H_5O_3$, 125.0239), a common fragmentation for carboxylic acids. The UV spectrum of **4.15** (Figure. S2) agrees with the data for a benzoic acid.[22]

The compounds presented by peaks 2-5 all have a similar UV spectrum (Figure S3) and were assigned to catechin derivatives. The compounds with R_t 3.79 and R_t 4.08 both have $[M-H]^-$ ions at *m/z* 289.0699 and *m/z* 289.0696, respectively, in the mass spectra (Figure S4 and Figure S5), which correspond to a molecular formula of $C_{15}H_{14}O_6$ (calc. for $C_{15}H_{13}O_6$ 289.0712). These compounds were assigned as the isomeric catechin (**4.16**) and epicatechin (**4.17**). Based on literature reports, catechin has a shorter retention time than epicatechin.[23,24] Catechin and epicatechin often occur as gallate esters, e.g. compounds **4.10**, **4.11**, **4.18**, and **4.19** (Figure 4.8), in tannin-rich plant material such as black tea.[25]





R=H,	R'=H Epicatechin	**4.17**	R=H,	R'=H, Catechin	**4.16**
R=Gallate	R'=H Epicatechin gallate	**4.10**	R=gallate,	R'=H, Catechin gallate	**4.18**
R=Gallate	R'=OH Epigallocatechin gallate	**4.11**	R=gallate,	R'=OH, Gallocatechin gallate	**4.19**

Figure 4.8. Catechin, epicatechin, and gallate derivatives.

Two compounds with closely related retention times of 4.08 and 4.11 min. were assigned as epicatechin (**4.17)** and (epi)gallocatechin gallate (**4.11/4.19**), respectively. We could not assign this gallocatechin or the isomeric epigallocatechin and, therefore, use the term (epi)gallocatechin. With ESI (-) ionization, the deprotonated molecular ion of (epi)gallocatechin gallate was observed at *m/z* 457.0765, which corresponds to the molecular formula $C_{22}H_{18}O_{11}$ (calc. *m/z* 457.0768, $C_{22}H_{17}O_{11}$) and a dimeric adduct $[2M-H]^-$ was observed at *m/z* 915.1611. In the MS-MS spectrum, fragments observed at *m/z* 305.0626 correspond to the loss of gallate (calc. for $C_{15}H_{13}O_7$, 305.0661). The gallate ion was also observed at *m/z* 169.0127 (calc. for $C_7H_7O_5$, 169.0137). The presence of a fragment with *m/z* 169 in the MS-MS spectra of flavanols is a clear indication that a gallate ester is present. The epimer of this compound could not be observed in the chromatogram.

The compound with R_t 4.66 was identified as (epi)catechin gallate (**4.10**/**4.18**). In the HR-ESI-(-)-MS spectrum, the $[M-H]^-$ was observed at *m/z* 441.0820, corresponding to a molecular formula of $C_{22}H_{18}O_{10}$ (calc. for *m/z* 441.0822, $C_{22}H_{17}O_{10}$). The dimeric adduct $[2M-H]^-$ was also observed for this compound at *m/z* 883.1737. MS-MS fragmentation with a loss of a gallate was observed at *m/z* 271.6620. The gallate ion was also observed at *m/z* 169.0130. The epimer of this compound could not be observed.

The five phenolic compounds tentatively identified in this study, namely, gallic acid, catechin, (epi)catechin, (epi)gallocatechin gallate, and (epi)catechin gallate, were previously observed in LC-MS by other authors.[26,27]

Flavonoids are among plants' most important low-molecular-weight phenolic compounds and often occur as glycosides. These possess a wide range of medicinal benefits, including hypoglycemic, lipid-lowering, and anti-inflammatory properties.[28,29]

4.3.4 Isolation and structural elucidation of compounds

According to literature reports, numerous studies have been conducted on the stem and leaves of *S. birrea*.[17,18,20,30] Phytochemical screening, biological assays, and the LC-MS studies of *S. birrea* extracts have been reported. However, not many compounds have been isolated from the extracts. The isolation and structural elucidation of four compounds in the stem bark extract are described herewith.

The stem bark of *S. birrea* was extracted successively with hexane, DCM-MeOH (1:1, v/v), and MeOH. The crude DCM-MeOH extract was subjected to vacuum liquid chromatography. The fractions were further purified using column chromatography to afford compounds (**4.16**, **4.20**, and an inseparable mixture of **4.21** and **4.22**) (Figure 4.9). The structures were elucidated using NMR and TOF-MS.

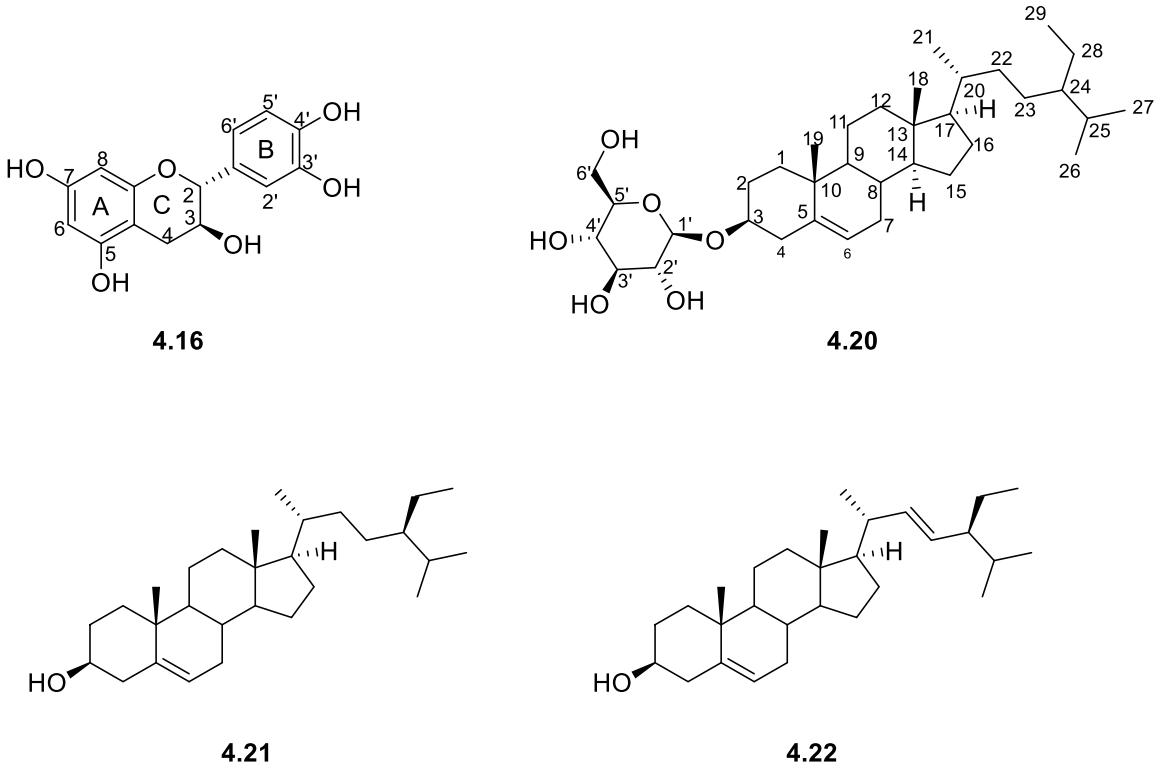


Figure 4.9. Structures of isolated compounds from the plant in the present study (**4.16-4.22**).

4.3.5 Structural elucidation of catechin (4.16)

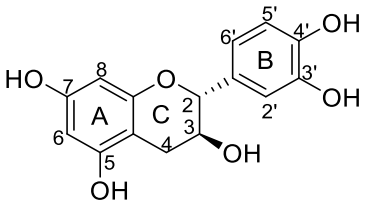


Figure 4.10. Structure of catechin (**4.16**).

Compound **4.16** was isolated as an orange powder. In the HR-ESI-(-)-MS spectrum (Figure 4.11), a $[M-H]^-$ deprotonated molecular ion was observed at *m/z* 289.0695 (calc. for $C_{15}H_{13}O_6$, 289.0712), which corresponds to a molecular formula of $C_{15}H_{14}O_6$.

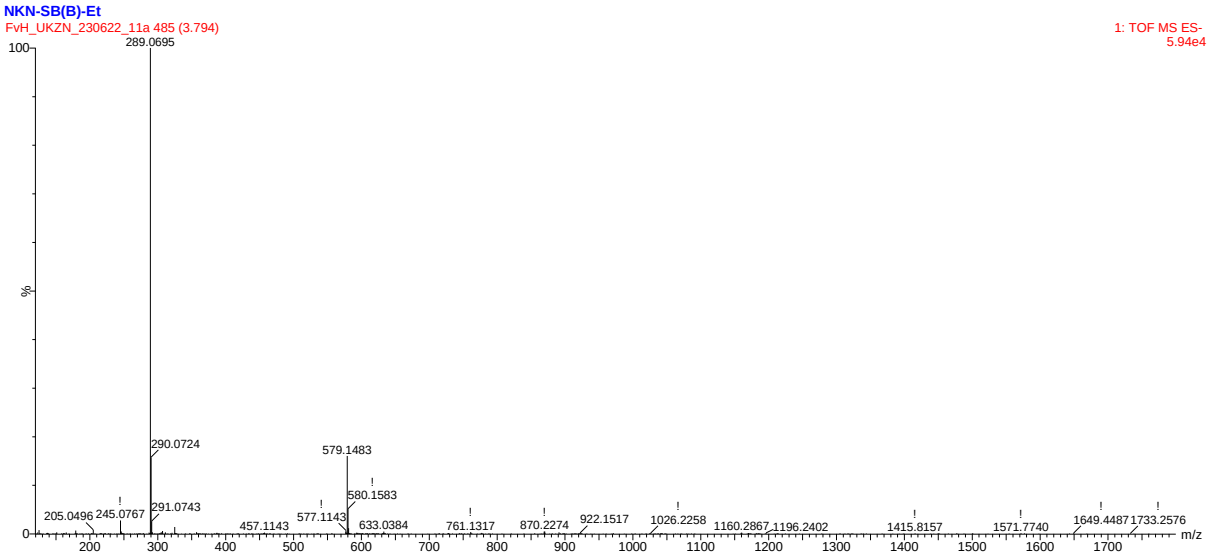


Figure 4.11. HR-ESI-(-)-MS Spectrum for compound **4.16**.

In the ^{1}H NMR spectrum of **4.16** (Figure 4.12), the two upfield signals were observed as doublets of doublets at δ_H 2.51 (dd, *J* = 16.1, 8.1 Hz) and δ_H 2.84 (dd, *J* = 16.2 Hz, 5.3 Hz), integrating for two protons, were assigned as diastereotopic methylene protons. A second signal resonating as a broad doublet of doublets at δ_H 3.98 was observed. The chemical shift of the proton indicated that it is bonded to carbon and also attached to an electronegative

atom (oxygen). A doublet at δ_H 4.57 (d, *J* = 7.6 Hz), integrating for one proton, was assigned to a methine proton. The coupling constant indicated a diaxial splitting of this proton with the previously assigned methine proton (δ_H 3.98). A COSY correlation was also observed between these protons. In the aromatic region, two one-proton resonances were observed as doublets at δ_H 5.84 and 5.94 (*J* = 1.9 Hz). The chemical shifts, coupling constant and the COSY correlation observed between the protons indicated that these are two *meta* protons on a phloroglucinol moiety. The remaining signals in the aromatic region were observed as a multiplet integrating for two protons at δ_H 6.74 and a doublet resonating at δ_H 6.84 (*J* = 1.8 Hz).

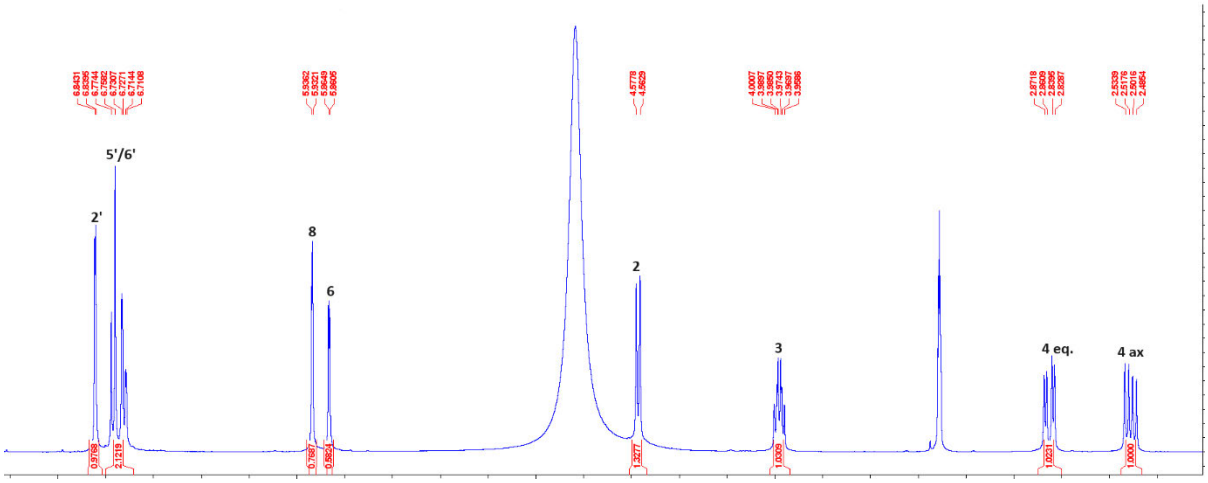


Figure 4.12. ^{1}H NMR spectrum of compound **4.16** in CD_3OD (500 MHz).

The methine proton (δ_H 3.98) was identified to be adjacent to the methylene protons (δ_H 2.51, 2.84) and the methine proton (δ_H 4.57), as was confirmed by the COSY correlations (Figure 4.13) of the proton to both the diastereotopic methylene protons and the methine proton. These correlations confirmed the presence of a pyran ring. Furthermore, a COSY correlation was also observed between the three aromatic protons of ring B. The COSY correlation between H-2' and H-6' was due to the *meta*-coupling in the benzene ring.

In the ^{13}C NMR spectrum (Figure 4.14), 15 carbons were observed. Using DEPT 135 NMR (Figure 4.15), the carbons were assigned as one methylene carbon (δ_C 28.6), two oxygen-bearing methine carbons (δ_C 69.1, 83.2), and five aromatic methine carbons (δ_C 95.8; 96.5;

115.5; 116.2; 120.2). Correlations in the HSQC (Figure 4.16) allowed for the assignments of directly bound protons and carbons.

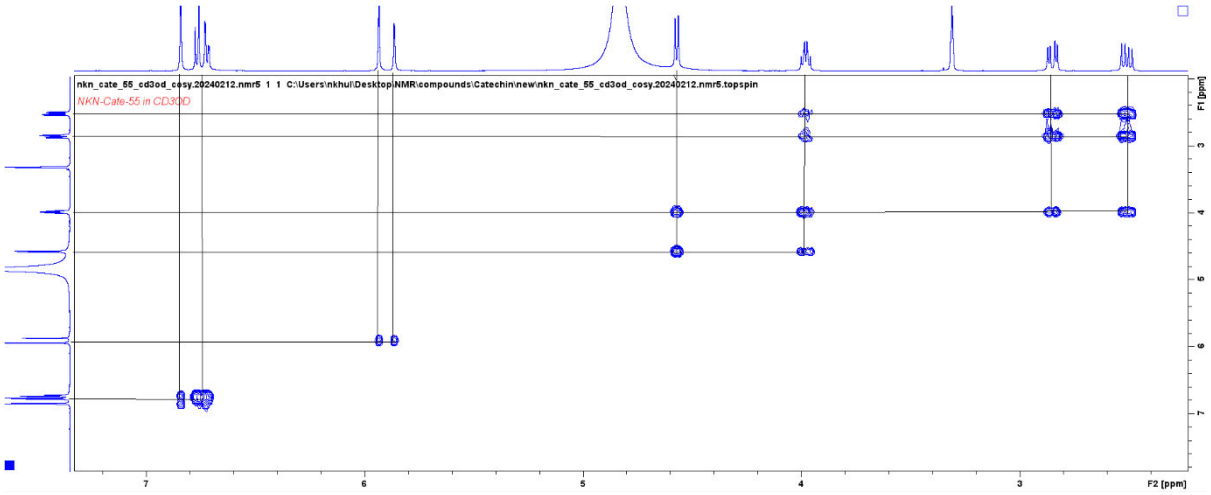


Figure 4.13. COSY correlation for compound **4.16**.

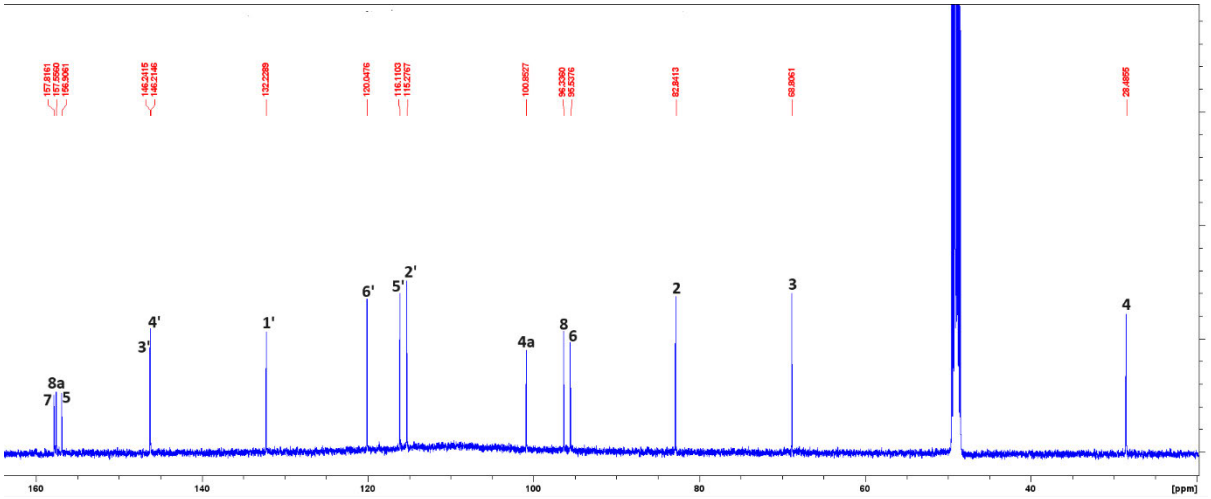


Figure 4.14. ^{13}C NMR spectrum for compound **4.16**.

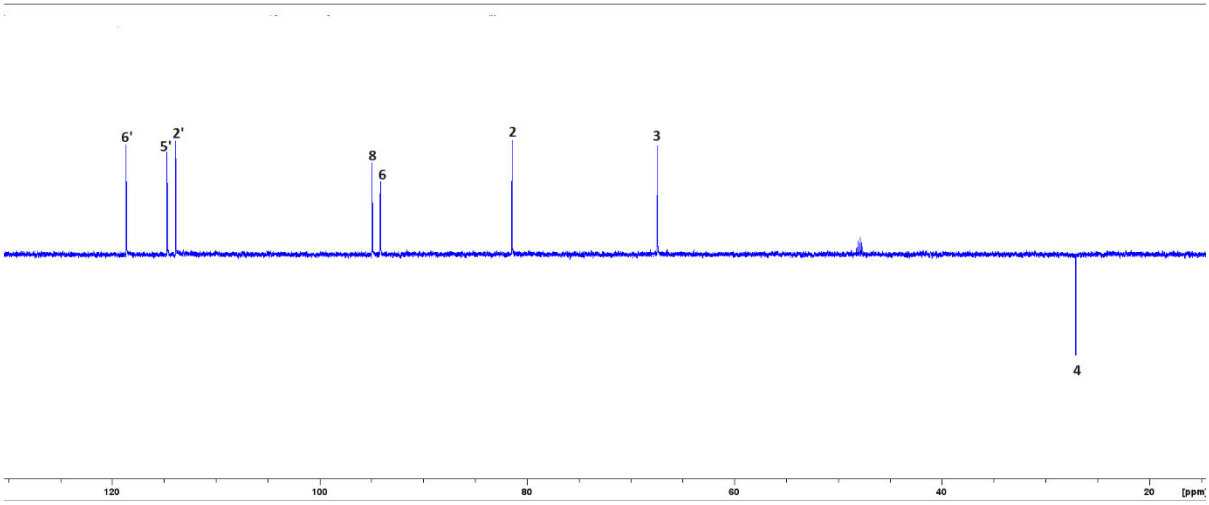


Figure 4.15. DEPT 135 NMR spectrum of compound **4.16** dissolved in CD_3OD.

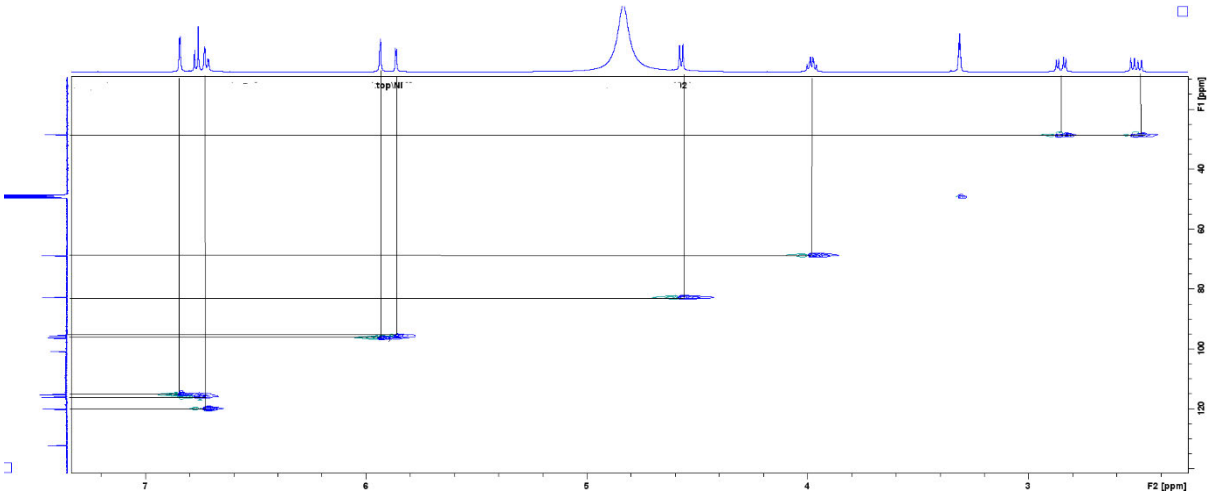


Figure 4.16. HSQC NMR spectrum of compound **4.16**.

In the HMBC spectrum (Figure 4.17), the diastereotopic protons (H-4) showed correlations to the carbons resonating at δ_C 68.8 (C-3), 83.2 (C-2), 101.1 (C-4a), and 157.7 (C-5). A ^2J correlation was observed from H-3 to δ_C 28.7 (C-4) and δ_C 83.2 (C-2), as well as a ^3J correlation from H-3 to δ_C 101.4 (C-4a) and 132.2 (C-1'). These correlations confirmed the connection between the pyran ring system and the aryl rings (A and B). Another HMBC correlation confirmed was the methine proton (H-2) to the carbons resonating at δ_C 28.7 (C-4); 68.8 (C-3); 116.1 (C-2'); 120.0 (C-6'); 132.3 (C-1'), and 156.8 (C-8a), confirming the presence of a benzopyran ring connected to the aryl ring. The HMBC correlations led to the identification of

compound **4.16** as catechin. The long-range couplings observed for compound **4.16** were displayed in Figure 4.18.

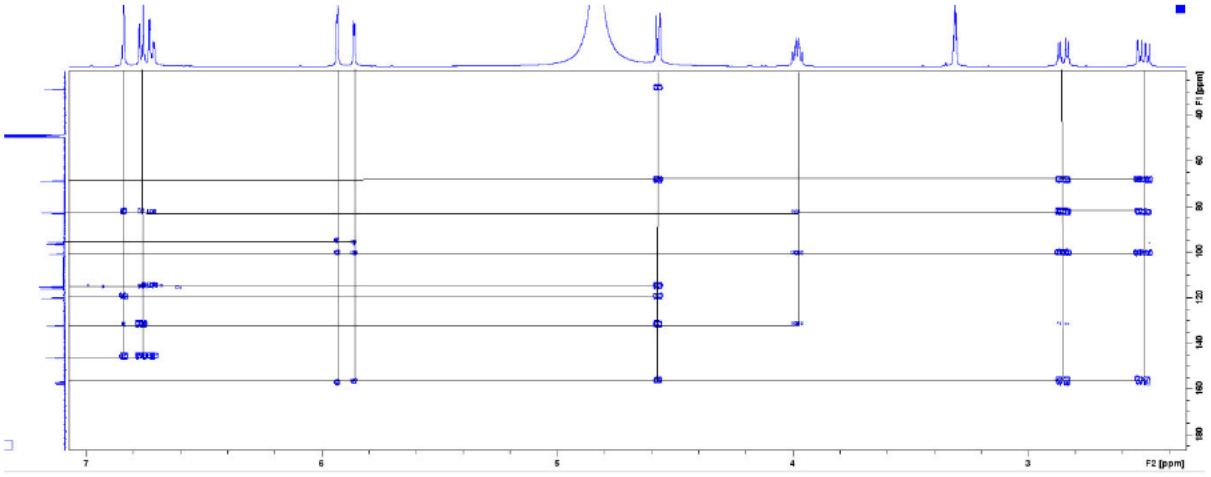


Figure 4.17. HMBC NMR spectrum for compound **4.16** in CD_3OD (500 MHz).

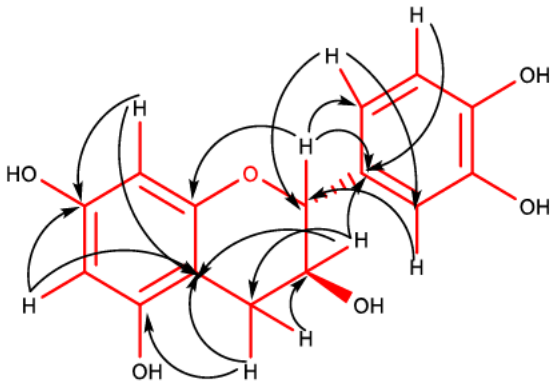


Figure 4.18. HMBC correlation observed for compound **4.16**.

Catechins, flavonoids from the flavanol subgroup, are naturally occurring polyphenol compounds in food and medicinal plants such as legumes, grapes, and tea.[31-33] Two compounds are generally referred to as catechins: catechin (**4.16**), with a *trans*-configuration in the C-ring and epimeric epicatechin (**4.17**), with a *cis*-configuration in the C-ring. The catechins are often present as gallate esters, e.g., catechin gallate, epicatechin gallate, and epigallocatechin gallate.[34] Several studies have shown the effectiveness of consuming foods high in catechins to prevent and treat chronic diseases such as inflammatory diseases.[33]

The IC_{50} value of catechin was determined and was observed to be 83.88 ± 19.82, which is higher than that of the VLC EtOAc and EtOAc-MeOH fractions. This suggests that the inhibitory effect of the fractions may be due to the combinational effects of all the compounds in the fractions. The inhibition curve of **4.16** is shown in Figure 4.19

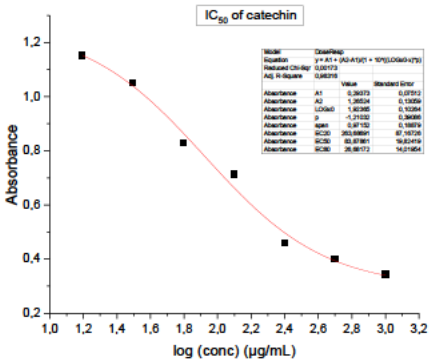
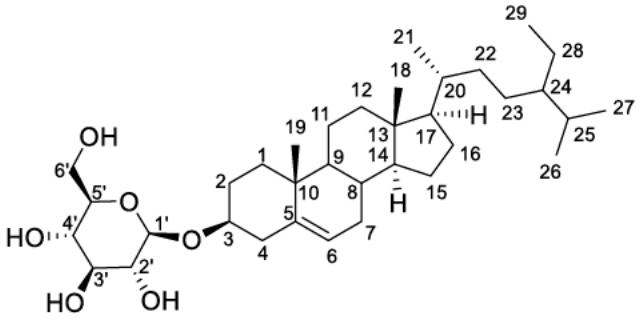


Figure 4.19. Inhibition curve for the isolated compound, catechin (**4.16**).

Table 4.4. ^{1}H (500 MHz), ^{13}C (125 MHz), and DEPT 135 NMR data of compound **4.16** in CD_3OD.

Position	δ_H(ppm)	δ_C(ppm)	DEPT 135
2	4.57(1H, d, *J* = 7.5 Hz)	83.2	CH
3	3.97 (m, 1H)	68.8	CH
4_{ax}	2.51 (dd, 2H)	28.7	CH_2
4_{eq}	2.849 (dd, 2H)	28.7	CH_2
4a	-	101.1	
5	-	156.9	
6	5.93, (1H, d, *J*= 2.2 Hz)	95.5	CH
7	-	157.8	
8	5.86 (1H, d, *J*= 2.1 Hz)	96.7	CH
8a	-	157.6	
1'	-	132.3	
2'	6.84 (1H, d, *J*=1.8 Hz)	116.1	CH
3'	-	146.2	
4'	-	146.2	
5'	6.74 (1H, m)	96.3	CH
6’	6.74 (1H, m)	120.0	CH

4.3.6 Structural elucidation of β-sitosterol 3-*O*-D-glucopyranoside (4.20)



4.20

Figure 4.20. Structure of β-sitosterol 3-*O*-D-glucopyranoside (**4.20**).

Compound **4.20** was isolated as a white solid from the EtOAc-MeOH fraction of *S. birrea*. In the ^{1}H NMR (Figure 4.21), two upfield singlet signals, integrating for three protons each, resonating at δ_H 0.57 and 0.89, were assigned for two methyl groups (H–18 and H–19, respectively). A vinyl proton was observed at δ_H 5.25 (H–6), confirming the presence of the double bond. An anomeric proton resonating at δ_H 4.29 (d, J = 7.9 Hz, H–1') was observed. A signal at δ_H 0.81 (d, J = 6.3 Hz, H–21) integrating for three protons was assigned to one methyl group. In addition, a significant overlap of signals between δ_H 0.69–0.79 was integrated for nine protons assigned to three methyl groups (CH_3–26, 27, and 29). The HSQC correlation assisted with assigning the protons to their respective carbons.

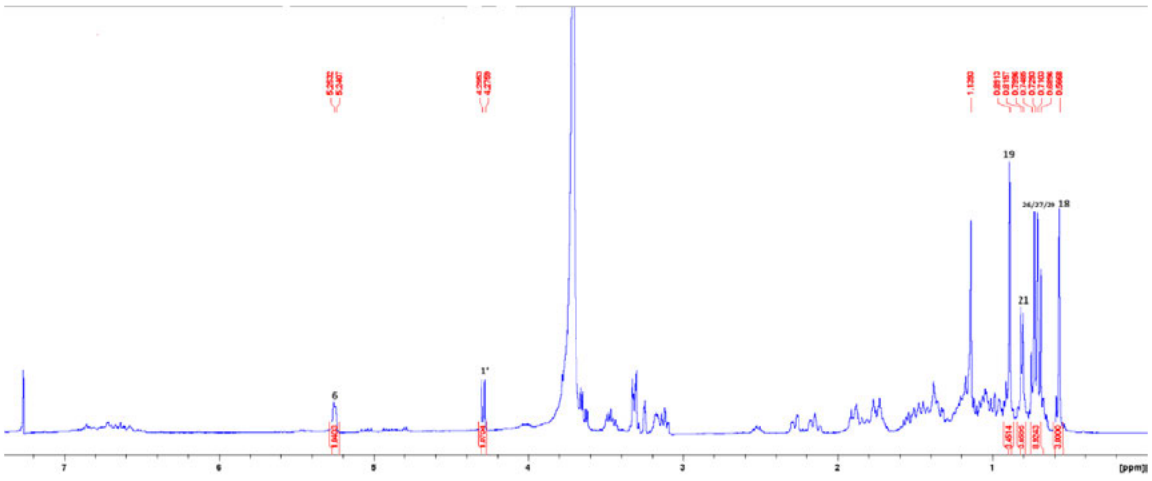


Figure 4.21. ^{1}H NMR spectrum for compound **4.20** in CD_3OD-$CDCl_3$ (1:1, v/v).

The ^{13}C and DEPT–135 NMR spectra (Figure 4.22) indicated 35 carbon resonances. Seven C–O carbons resonated at δ_C 61.81–79.59 ppm. The spectrum also showed three quaternary carbons resonating at δ_C 42.2, 37.4, and 141.2 ppm. The remaining steroid methine and methylene carbons resonated at δ_C 12.02-57.36 ppm. The HSQC spectrum (Figure 4.23) confirmed the presence of a glucose moiety (six carbons).

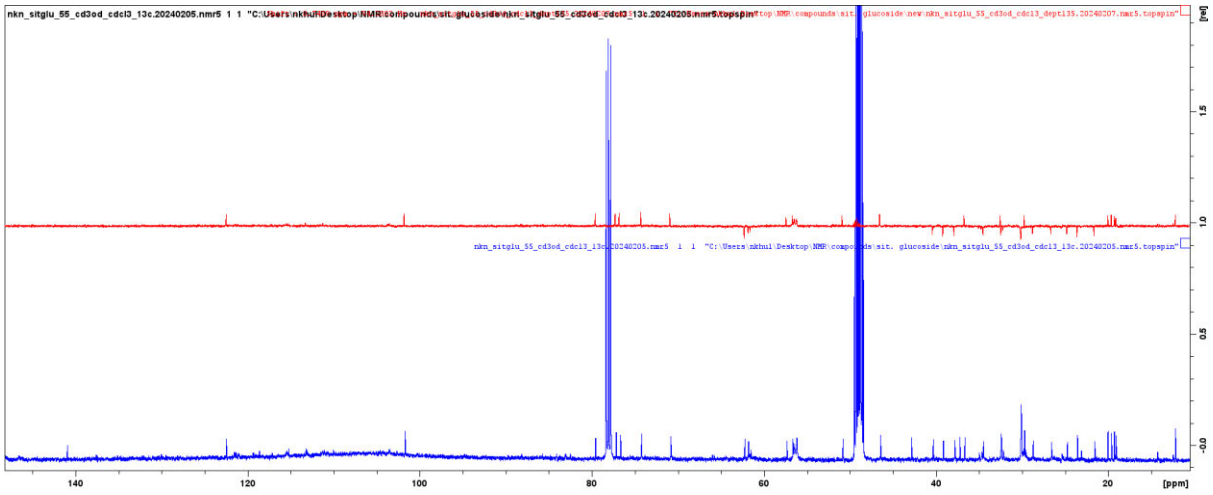


Figure 4.22. ^{13}C and DEPT 135 NMR spectra of the compound **4.20** in CD_3OD-$CDCl_3$ (1:1, v/v).

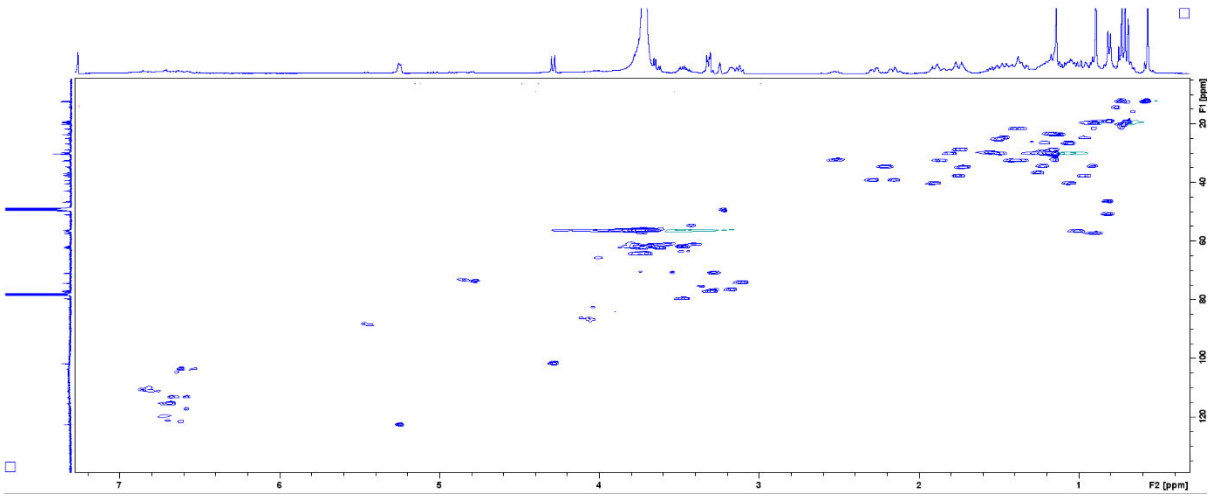


Figure 4.23. HSQC NMR spectrum for compound **4.20**.

In the COSY correlation spectrum (Figure 4.24), the anomeric proton (H-1', δ_H 4.29) correlated to H-2' (δ_H 3.15) of the glucose moiety, which further correlated with a proton at δ_H 3.61 (H-3'). The vinyl proton observed at δ_H 5.24 (H-6) correlated with diastereotopic protons at δ_H 1.46 and 1.91 (H-7).

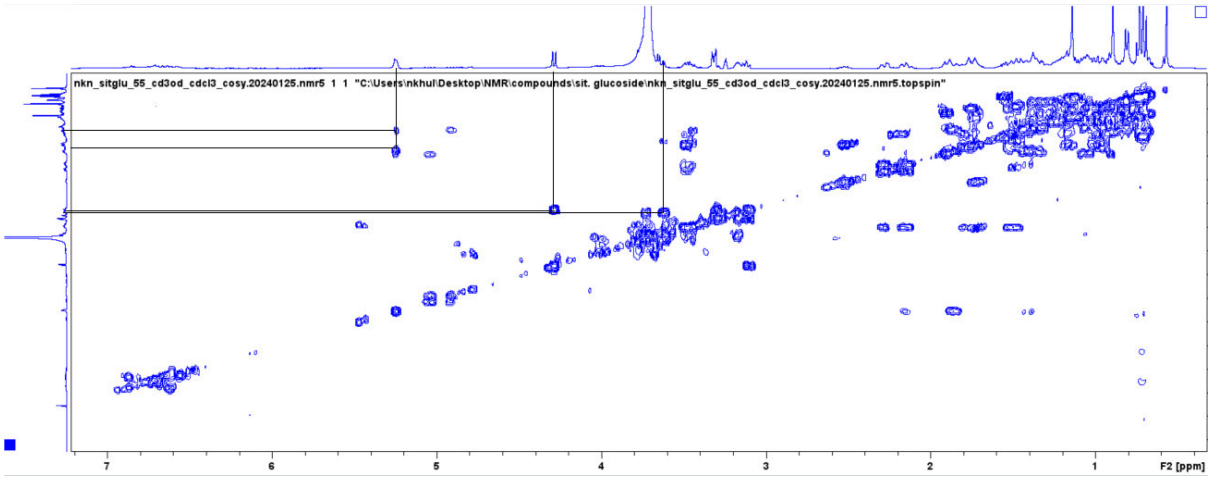


Figure 4.24. COSY correlations observed for compound **4.20**.

The HMBC spectrum (Figure 4.25) was used to analyse the long-range ^{1}H and ^{13}C correlations. An *O*-glycosidic bond was assigned based on a ^{3}J correlation observed between the anomeric proton [δ_H 4.29 (H-1')] of the sugar moiety and the carbon (steroid moiety) resonating at δ_C 80.0 ppm (C-3). This proton's chemical shift and coupling constant (*J*=7.9 Hz) confirmed the presence of an *O*-glycosidic bond and β-anomeric proton. The methyl protons (H-19) were observed to correlate by a ^{2}J with δ_C 37.1 (C-10) and as well as $^{3}J_{CH}$ correlation to δ_C 141.2 (C–5) and δ_C 51.5 (C–9) (yellow circles in HMBC spectrum). The long-range correlation of the steroid's remaining methyl protons (red circles in HMBC spectrum) and the NMR spectra suggested that the steroid is a *β*-sitosterol derivative. The *O*-glycosidic bond assigned using HMBC indicates that compound **4.20** is *β*-sitosterol 3-*O*-*β*-D-glucoside. The long-range couplings observed for compound **4.20** are displayed in Figure 4.26. The NMR data for compound **4.20** are collated in Table 4.5.

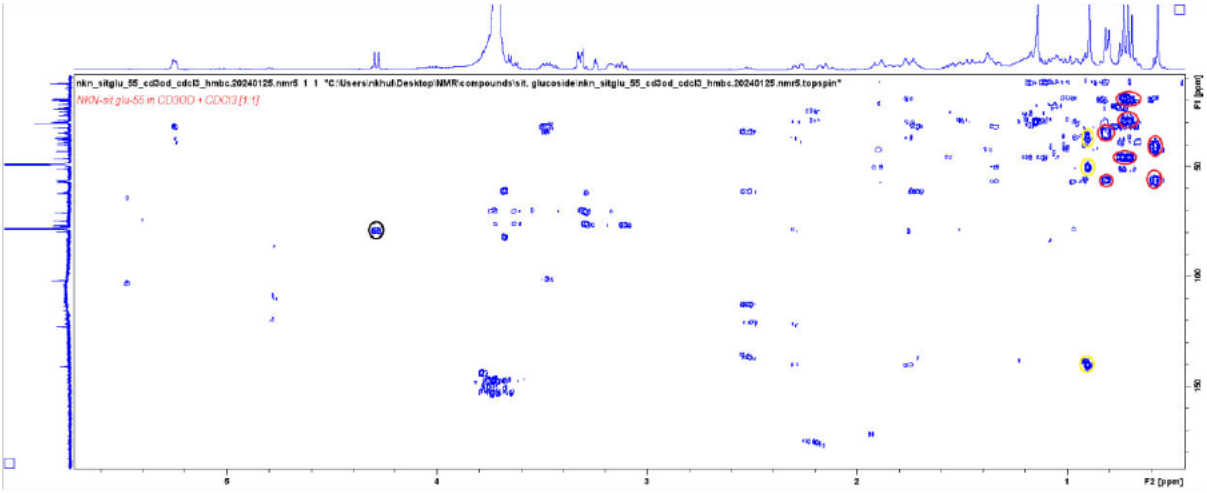


Figure 4.25. HMBC NMR spectrum observed for compound **4.20** at 500 MHz.

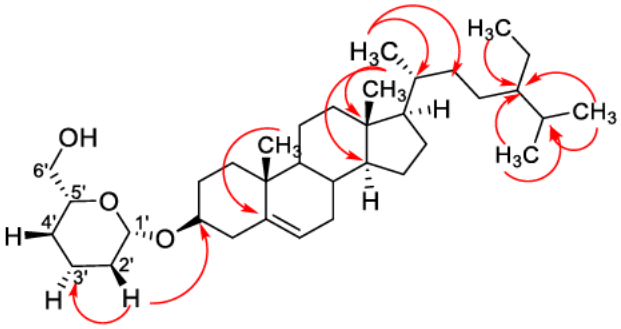


Figure 4.26. HMBC correlation of compound **4.20**.

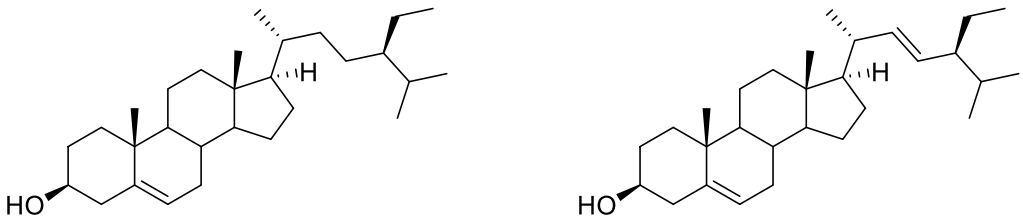
β-Sitosterol 3-*O*-β-D-glucopyranoside (**4.20**) is a plant-derived phytosterol glucoside reported as a bioactive molecule with therapeutic characteristics such as anti-parasitic, anti-inflammatory, anticancer, and immunomodulatory activity.[35-37]

The IC_{50} value for the inhibition of α-glucosidase by compound **4.20** was very high to the point that it was undetectable.

Table 4.5. NMR data for *β*-Sitosterol 3-*O*-*β*-D-glucopyranoside (**4.20**) (^{1}H (500 MHz), ^{13}C (125 MHz).

Position	δ_H(ppm)	δ_C(ppm)
1	0.97, m	38.1
2	1.48, m	25.1
3	3.48, m	79.7
4	2.16, brd	39.5
5	-	141.4
6	5.25 (1H, brd)	122.7
7	, m	32.8
8	1.38, m	32.9
9	0.82	51.3
10	-	37.6
11	1.06	27.1
12	2.15, m	39.5
13	-	43.2
14	0.89	57.9
15	1.13	23.8
16	1.06,	27.1
17	1.01, m	57.0
18	0.57 (3H, s)	12.3
19	0.89 (3H, s)	19.8
20	1.26, m	37.0
21	0.81 (3H, d, *J* = 6.3 Hz)	19.4
22	1.24, m	34.8
23	-	26.9
24	1.56, m	46.8
25	0.82, m	30.1
26	0.91	20.2
27	0.80, d	19.3
28	1.41, m	21.9
29	0.72, t	12.3
1’	4.29 (1H, d, *J*=7.9 Hz)	102.0
2’	3.08, m	74.6
3’	3.25, m	77.8
4’	3.20, m	71.2
5’	3.16, m	77.3
6’	3.64	61.8

4.3.7 Structural elucidation of a mixture of β-sitosterol (**4.21**) and stigmasterol (**4.22**)



4.21 **4.22**

Figure 4.27. Structures of compounds found in the inseparable mixture (**4.21** and **4.22**).

From the MeOH extract, one fraction was obtained as a yellow-white solid. Although a single spot was observed for the fraction on TLC, NMR spectroscopy indicated that the fraction was a mixture of two closely related compounds. In the ^{1}H NMR spectrum (Figure 4.28), the signals of the two compounds that overlap were two singlets at δ_H 0.68 (3H, H-18), 1.01 (3H, H-19) and a doublet at δ_H 0.92 (3H, H-21, *J*= 6.7). The difference between the signals of the ^{1}H NMR spectra of the **4.21**, and **4.22** was the presence of an alkene moiety as a doublet of doublets at δ_H 5.15 and 5.02 (both, dd; *J*=15.1 Hz, 8.7 Hz) in the ^{1}H NMR spectrum. The coupling constant (*J*=15.1 Hz*)* indicated a *trans*-alkene system.

The resonances in the ^{1}H NMR spectrum (Figure 4.28) resembles those of a ubiquitous plant steroid, *β*-sitosterol (**4.21**). The only difference between the two compounds was the presence of the second double bond with *trans* configuration (*J*=15.1 Hz) in **4.22**. The only way to accommodate a *trans* alkene in a steroid is if the double bond is in the chain (alkyl group). Therefore, compound **4.22** was identified as ergosterol. Because of the limited amount of material available and the complexity of the spectra, the ^{13}C NMR of the mixture was not investigated.

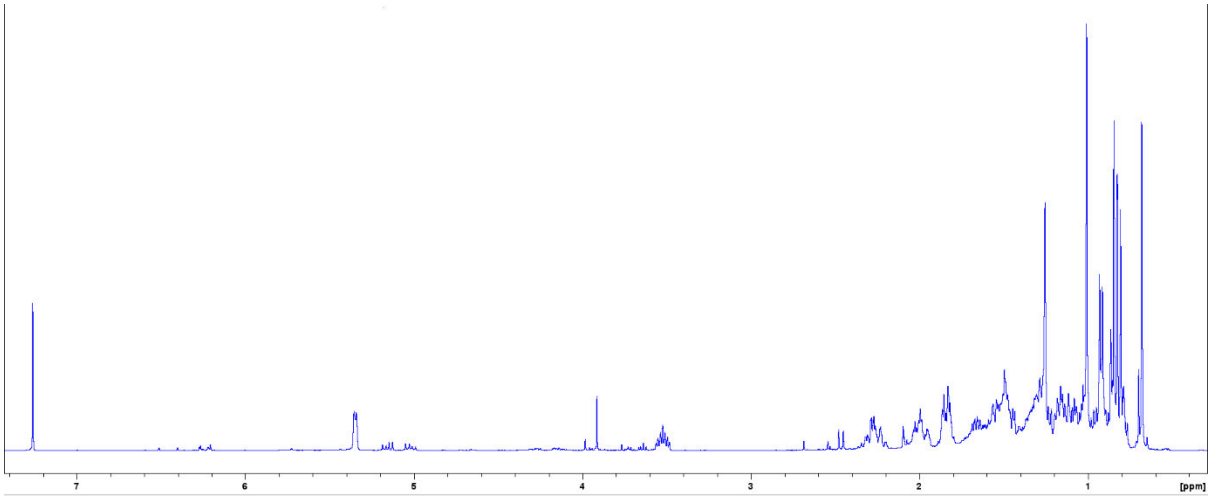


Figure 4.28. ^{1}H NMR spectrum of the mixture compound **4.21** and **4.22**.

The phytosterols **4.21** and **4.22** are present in many plants. These phytosterols play a significant role in treating and maintaining various diseases. Several studies have been conducted about assaying these phytochemicals for antibacterial, antimicrobial, control cholesterol levels, anticancer, and anti-inflammatory activities.[38-42] These plant sterols are among the most abundant in lipid-rich plants and play a crucial role in the functioning and maintenance of the structure of the cell membranes.[43]

Table 4.6. ^{1}H NMR (400 MHz) data for the inseparable mixture of β-sitosterol (**4.21**) and stigmasterol (**4.22**).

Position	**δ_H(ppm)**
6	5.35 (1H, brd)
18	0.68 (3H, s),
19	1.01 (3H, s)
21	0.92 (3H, d, *J*= 6.7)
23a	5.02 (dd; *J*=15.1 Hz, 8.7 Hz)
23b	5.15 (dd; *J*=15.1 Hz, 8.7 Hz)

4.3.8 α-Glucosidase inhibition studies

The initial aim of this project was to separate the crude plant extracts in 96-well plates using HPLC. A plate would then be subjected to an enzyme assay to determine which wells the active compounds were, and a separate 96-well plate with fractionated extract would be subjected

to LC-MS to determine the structure(s) of the active compounds. Due to the low concentration of the compounds in the 96-well plates, we could not get usable results. This approach needed further optimization and was abandoned.

The enzyme inhibitory activity of the isolated compounds of *S. birrea* was investigated as described in Chapter 3, and the results are shown in Table 4.7. The compounds' α-glucosidase inhibition percentage (%) was compared with that of acarbose. The results indicate that out of the three compounds, **4.16** and **4.20** exhibited good anti-glucosidase activity, and the mixture (**4.21** and **4.22**) showed the lowest inhibitory activity. However, when determining the IC_{50} for **4.16** and **4.20**, the IC_{50} was very high to the point that it could not be determined.

Several studies (*in vivo* and in vitro) have been conducted to investigate the antidiabetic activity of the isolated compounds. **4.16** and **4.20** have shown positive results in these studies.[44-47] The present study indicated that **4.16** could be useful in managing postprandial diabetes due to the inhibition of α-glucosidase, the key enzyme responsible for the hydrolysis of carbohydrates.

Table 4.7. The α-glucosidase inhibition activity of the isolated compounds of *S. birrea* at specific concentrations.

Name of compound	**Average absorbance (405 nm)**	**Average blank absorbance (405 nm)**	**% inhibition activity**
Negative control	3.159	0.053	-
Acarbose	0.074	0.043	99
Catechin (**4.16**)*	0.398	0.217	94
β-sitosterol 3-*O*-β-D-glucoside (**4.20**)*	0.753	0.255	83
β-sitosterol (**4.21**) and stigmasterol (**4.22**)*	1.843	0.153	46

*concentration 0.5 mg/mL

4.4 Conclusion

In the current study, it was observed that fractions obtained from the DCM-MeOH and MeOH extracts demonstrated a significant α-glucosidase inhibitory activity. LC-MS proved to be an excellent method to investigate complex fractions. Fractionation using HPLC was shown to be time efficient, and solvent consumption was decreased excessively. However, fractionated fractions showed low % enzyme inhibition. HPLC method development for some fractions

(EtOAc, EtOAc-MeOH, and MeOH) was very challenging, which led to the isolation of compounds using classical column chromatography. *S. birrea* contains bioactive compounds that have shown several biological activities such as antidiabetic and anti-inflammatory activity. The EtOAc and EtOAc-MeOH fractions displayed the lowest IC_{50} values, 20.03 ± 5.07 and 17.42 ± 9.04, respectively, compared to that of the standard control, acarbose with IC_{50} 47.51 ± 6.02. The isolated compound (catechin) showed a higher IC_{50} 83.88 ± 19.82 compared to the fraction, suggesting that the fraction's inhibitory effect may be due to the combinational effects of the compounds it contains.

4.5 Experimental

4.5.1 General experiment

NMR spectra (^{1}H and ^{13}C) were recorded on either a Bruker Avance III 500 or Bruker Avance III 400 spectrometer at frequencies of 400 MHz/500 MHz for ^{1}H and 100 MHz/125 MHz for ^{13}C, using either a 5 mm BBOZ probe [^{1}H], a BBIZ probe, or a TBIZ probe. The experiments were conducted at 30 °C. Residual protonated solvent signals were used as references: 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C) for $CDCl_3$, 3.31 ppm (^{1}H) and 49.0 ppm (^{13}C) for CD_3OD. Coupling constants are reported in Hertz.

LC-MS-MS experiments were performed on a Waters Acquity UHPLC system coupled to a Synapt G2 quadrupole-time-of-flight (q-TOF) instrument. For MS detection, ESI− (ESI negative) or ESI+ (ESI positive) mode was used. The UHPLC-MS instrument was equipped with an Acquity HSS T3 C18 column (2.1 mm × 50 mm, 1.8 µm, Waters). The mobile phase for the separations consisted of H_2O and acetonitrile at a flow rate of 0.4 mL/min and a total run time of 15 minutes. The program was as follows: (1) initial 100% H_2O; (2) 0.5 min 100% H_2O (3) 12 min 100% acetonitrile (4) 12.5 min 100% acetonitrile (5) 13 min 100% H_2O (6) 15 min 100% H_2O.

HPLC experiments were performed on a Shimadzu HPLC with an autosampler (SiL-20A), a binary pump and a PDA (SPD-M20A) detector. The PDA spectral range was configured to a 210-600 nm wavelength range. A Gilson FC 203B fraction collector was used to fractionate extracts into 96-well plates. Analytical HPLC experiments were performed on a Phenomenex

Luna® C18 (5 µm) column (250 × 4.6 mm). The sample solutions were filtered through CHROMAFIL PTFE (pore size 0.45 µm, membrane diameter 25 mm) filters (Machinery-Nagel).

Silica gel 60 (0.040–0.063 mm, 230-400 mesh ASTM) was used for fractionation by vacuum liquid chromatography. Assays were performed in 96-well microplates, and the absorbance was measured at 405 nm using a multi-plate reader (Fisher Scientific FB 505).

Large-scale extraction of plant material was performed using an Infors AG CH-4103 Bottmingeni orbital shaker.

4.5.2 Large-scale extraction

The bark of the active plant part, *S. birrea*, was harvested in large quantities from the botanical gardens (UKZN, PMB) and was dried at room temperature. The dried plant material was pulverized using a hammer mill. The powdered plant material was extracted successively with hexane (1500 mL), DCM-MeOH (1500 mL, 1:1), and methanol (1200 mL) for 16 h. The plant extracts were filtered using a funnel fitted with cotton wool and concentrated under a vacuum using a rotatory evaporator (65 °C). The hexane extract was discarded. The mass of the ground material used was 600 g, and the extracts obtained were 24 g (DCM-MeOH) and 10 g MeOH extract. The extracts were transferred to pre-weighed flasks and stored in a fridge at 7 °C until required.

4.5.3 Fractionation of the DCM-MeOH extracts using vacuum liquid chromatography (VLC) and isolation of compounds

The DCM-MeOH (1:1) extract (10 g) was dissolved in a 35 mL DCM-MeOH (1:1) solvent mixture, mixed with 40 g of silica, and the material was then left to dry for a few minutes in the fume hood. The impregnated silica was crushed into a fine powder using a glass rod and loaded on a VLC column packed with 150 g of silica. The column was eluted with the solvent mixtures given in Table 4.7. Each solvent eluate was collected as one fraction, concentrated on a rotary evaporator, and dried. The masses obtained are shown in Table 4.8.

Table 4.8. VLC fractions collected from the DCM-MeOH crude extract of *S. birrea*.

Solvent	Volume (mL)	Fraction	Dry fraction obtained (g)
Hexane-DCM (9:1)	600	NKN-2-H-DCM-46	1.22084
DCM-EtOAc (20:1)	600	NKN-2-DCM-EtOAC-46	1.3953
EtOAc	600	NKN-2-EtOAc-46	1.0856
EtOAc-MeOH (5:1)	600	NKN-2-EtOAc-MeOH-46	3.8906
MeOH	600	NKN-2-MeOH-46	2.5001

The EtOAc fraction (800 mg) was subjected to column chromatography (29 x 4 cm) on silica gel. The column was eluted using different solvent mixtures, namely, EtOAc-chloroform (8:2), EtOAc-chloroform (9.5:5), and lastly, EtOAc-MeOH (9:1). The subfractions obtained (4-8 mL) were analysed by TLC and were combined based on the TLC profiles observed to afford four new subfractions. The EtOAc fraction consisted of a mixture of flavonoids identified using the UPLC-MS. Using column chromatography, attempts to isolate and purify the flavonoid in the subfractions were unsuccessful. However, compound **4.16** (5 mg, catechin) was obtained from the purification of subfraction A using the chloroform-MeOH (8:2) solvent mixture and, finally, flashing of the column using chloroform-MeOH (1:1) as the solvent to afford three subfractions.

Purification of the EtOAc-MeOH fraction (1.5 g) was conducted in the same manner as stated above. However, the column was eluted using DCM-MeOH (8:2) as the solvent system to afford seven subfractions (Et-Me, A-F), and one subfraction (D) was identified to be compound **4.20** (β-sitosterol 3-*O*-D-glucoside).

The MeOH crude extract (7 g) was dissolved in 25 mL MeOH and mixed with 30 g silica, and the mixture was left to dry in the fume hood. The silica mixture was then wet-packed on the column with silica gel (100 g). The column was eluted with a gradient method as follows: DCM (600 mL), DCM-MeOH (8:2, 300 mL), DCM-MeOH (7:3, 400 mL), DCM-MeOH (1:1, 400 mL), and lastly with MeOH (500 mL). The 50 fractions (25 mL each) were combined into seven subfractions (A–G) (Table 4.9).

Table 4.9. Combined fraction obtained by column chromatography of MeOH crude extract**.**

Fractions	Mass (g)
A	1.7
B	0.3
C	0.2
D	1.5
E	0.2
F	0.7
G	1.0

Fraction A was subjected to isocratic column chromatography (chloroform-MeOH, 8:2, 500 mL) to afford five subfractions. One of the subfractions was a mixture of β-sitosterol (**4.21**) and stigmasterol (**4.22**), which could not be separated.

Experimental data of compounds isolated

Catechin (**4.16**): Orange powder (5 mg), specific rotation $[\alpha]_D^{22.5}$ -19.7, HR-ESI-(-)-MS m/z 289.0699 (calc. for $C_{15}H_{13}O_6$ 289.0712) see figure 4.8, ^{1}H: see Figure 4.9 and Table 4.5 and ^{13}C: see Figure 4.11 and Table 4.5.

β-Sitosterol glucopyranoside (**4.20**): White powder (5 mg), ^{1}H see Figure 4.17 and Table4.5 and ^{13}C: see Figure 4.18 and Table 4.5.

Inseparable mixture of *β*-sitosterol (**4.21**) and stigmasterol (**4.22**): Yellow-white solid (4 mg), ^{1}H: see Figure 4.24 and Table 4.6.

4.5.4 Analysis of the extracts

Method development for high-performance liquid chromatography (HPLC)

The HPLC-UV/PDA experiments were performed at room temperature on a Phenomenex Luna® C18 (5 µm) column (250 × 4.6 mm). For HPLC analyses, 3 mg of the extract fractions dissolved in 10 mL MeOH or a mixture of MeOH and acetonitrile, depending on the polarity of the fraction.

For the DCM-EtOAc fraction (20:1), the binary mobile phase consists of solvent A: H_2O and solvent B: acetonitrile, both containing 0.1 % formic acid, at a flow rate of 0.70 mL and injection volume of 20 µL for 35 minutes. The method was isocratic and was run at 70%

acetonitrile. The PDA coupled to the HPLC system was set in a spectrum range starting at 210 nm and ending at 500 nm.

After confirmation of the Method, the HPLC was connected to the fraction collector. The injection volume was increased to 50–100 µL, and the extract was fractionated into a 96-well plate. The plate was dried in the fume hood and was subjected to α-glucosidase inhibition studies.

4.5.5 α-Glucosidase assay

Dried 96-well plates were assayed for α-glucosidase inhibition using the adjusted previously reported protocol.[48,49] Briefly, the procedure involved preparing a fresh buffer solution of 100 mM sodium dihydrogen phosphate at pH 6.9. This solution was used to prepare solutions of an enzyme, α-glucosidase (1 U/mL), substrate (p-NPG) (5 mM, final concentration 1.25 mM) solutions, and acarbose (positive control). Dimethyl sulfoxide (DMSO) solution was prepared at 25 % (final well concentration 6.25%)

4.5.6 Protocol for the α-glucosidase assay in the 96-well plate

The assay was performed at 37 °C in 100 mM phosphate buffer at pH 6.9. In a 96-well plate, the contents in each well were dissolved in 50 µL DMSO (25%) before the addition of 100 µL α-glucosidase (1U/ mL) solution, and the plate was pre-incubated for 10 minutes in the dark. To start the reaction, 50 µL of p-NPG was added to all the wells, including the controls, and contents were mixed well using a multichannel pipette before incubation for 20 minutes. The measurement of *p*-nitrophenol quantity was determined at 405 nm using a microplate reader set at 37 °C, and the absorbance was recorded for 10 minutes in 30-second intervals.

4.5.7 Determination of α-glucosidase inhibition activity of compounds

The protocol and preparation followed are similar to that done in Chapter 3. In a 96-well plate, the assay mixture containing 50 µL phosphate buffer (for the negative control and blanks), 30 µL enzyme and 50 µL of varying concentrations of fractions and compounds (15.625-1000 µg/mL) was incubated for 10 minutes at 37 °C in the dark. This was followed by the addition of 20 µL of substrate in all the test wells, and the mixture was incubated for 20 minutes at 37 °C in the dark. The reaction was terminated by the addition of 5 mM of sodium carbonate.

The absorbance was measured at 405 nm. Acarbose was the standard used and was prepared at varying concentrations of (15.625-1000 µg/mL). The percentage inhibition was calculated using the equation:

$$Enzyme\ inhibition = \left(1 - \frac{blank\ corrected\ absorbance\ of\ test\ well}{blank\ corrected\ absorbance\ of\ negative\ conrol}\right) \times 100$$

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Chapter 5 Conclusion and Future Work

Plant extracts continue to play a significant role in drug discovery, especially new lead-like for new therapies. The ethnobotanical records for utilising South African indigenous plants to treat diabetes are reported, and the activity of crude extracts was investigated. However, subsequent isolation of compounds and validation studies have been neglected. Numerous species of the Anacardiaceae are of economic importance, and compounds isolated have proven to be pharmacologically active towards several diseases.

Lead-likeness concepts have gained considerable attention due to their chemical properties and adherence to the 'rule of five'. This is considered one of the techniques most likely to deliver "drug-like" candidates/ leads. Lead-like small-scale extracts were prepared for all the species investigated in this study.

Lead-like extracts were prepared from some South African indigenous plants, namely, *Bulbine frutescens*, *Carpobrotus dimidiatus*, *Sclerocarya birrea*, *Ziziphus mucronata, Gomphocarpus tomentosus, Pappea capensis, Senna italica, Senegalia mellifera, Tabernaemontana elegans, Merwilla plumbea, Maerua angolensis,* and *Gnidia kraussiana*. The lead-like extracts were assayed for α-glucosidase inhibition studies, and *S. birrea* was selected for further studies.

Analysis by LC-TOF-MS of the fractions of *Sclerocarya birrea* led to the identification of five compounds in the extract. The compounds were identified based on high-resolution data of the molecular ions and fragments in the MS spectra. Only one of the detected compounds by LC-MS was isolated and characterized.

Three well-known compounds were isolated from *Sclerocarya birrea*, namely, catechin (**4.16**), β-sitosterol 3-*O*-β-D-glucoside (**4.20**), and a mixture of β-sitosterol (**4.21**) and stigmasterol (**4.22**). Catechin is well known for its antioxidant effect, and studies have shown its effects in other diseases, including diabetes. The *β*-sitosterol and its glycoside have been studied for the effectiveness in treating hyperglycemia and other diabetic-related diseases.

The crude extracts of the plant exhibited poor to good inhibition of α-glucosidase, a target for developing antidiabetic drugs. The isolated compound (catechin) inhibited of α-glucosidase.

Advanced bioactivity assays were not conducted and will be addressed in future work to validate these findings.

In future work, in vivo studies must be conducted on the isolated compounds. Considering the fraction's activity, more compounds need to be isolated for biological assays. Many South African plants are used for the treatment of diabetes, and active extracts should be subjected to LC-MS analysis. Isolated compounds from active extracts need to be assayed against the relevant enzymes.

Supplementary Information

Index

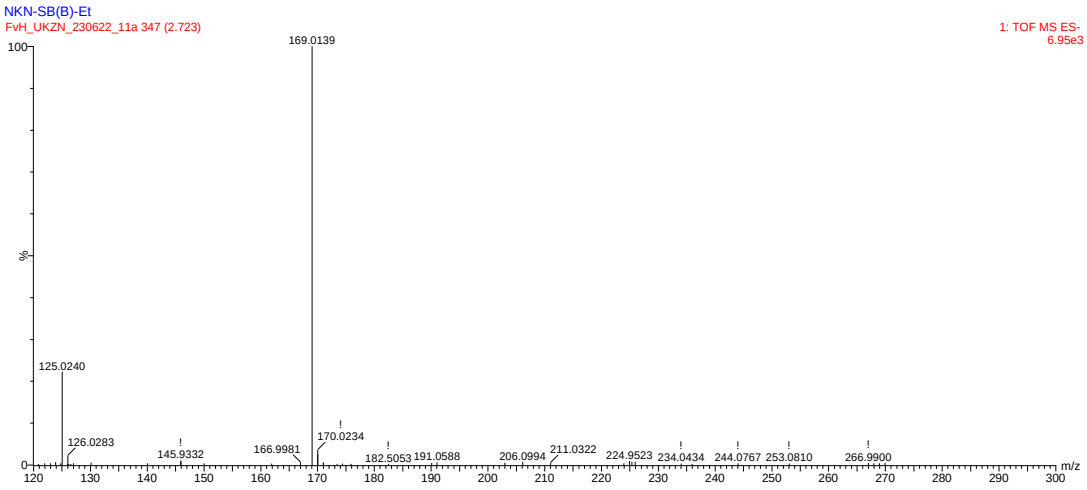

Figure S1. HR-ESI-(-)-MS of gallic acid (**4.15**).

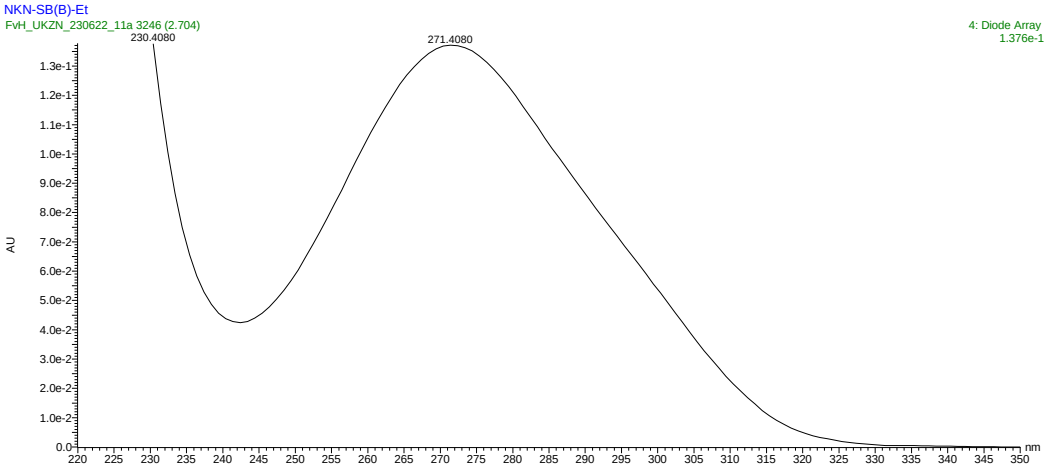

Figure S2. UV spectrum of gallic acid (**4.15**).

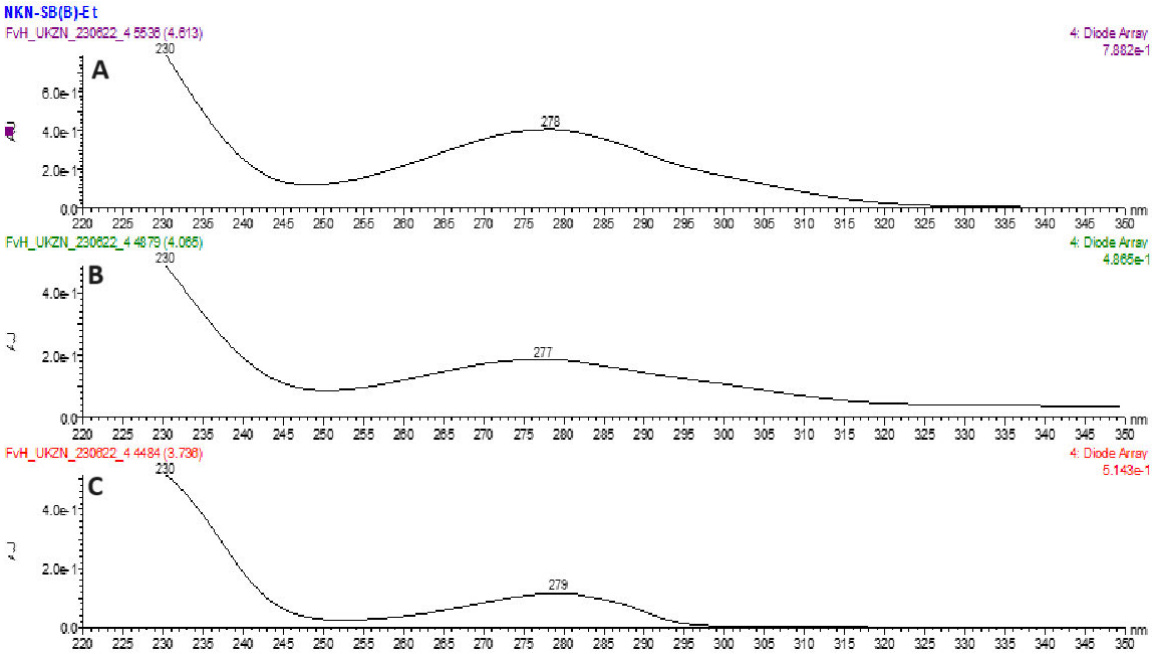

Figure S3. The UV spectrum of the gallate derivative (A), epicatechin (B) and catechin(C) identified in the EtOAc fraction of *S. birrea*.

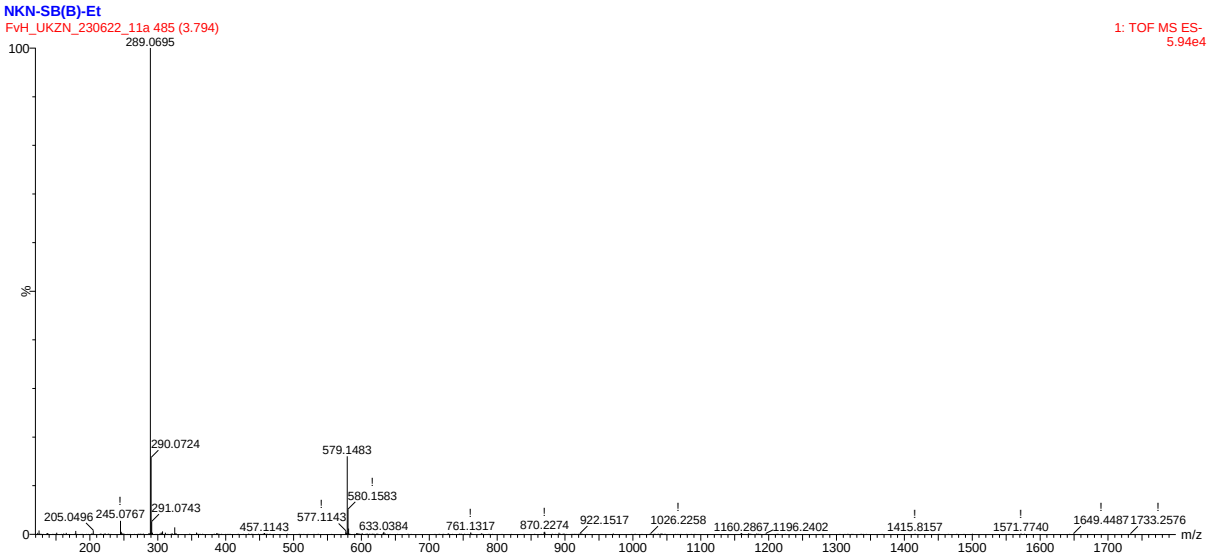

Figure S4. HR-ESI-(-)-MS of catechin (**4.16**).

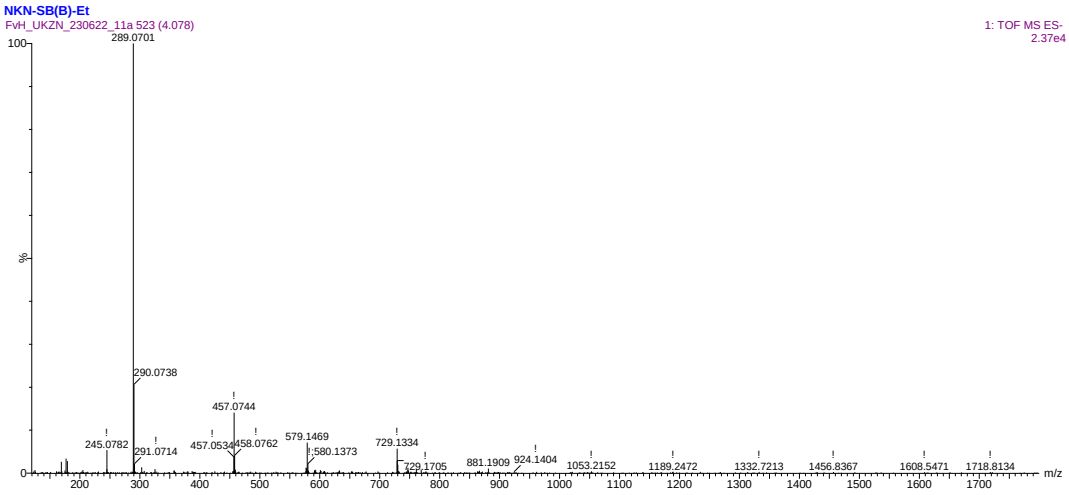

Figure S5. HR-ESI-(-)-MS of epicatechin (**4.17**).

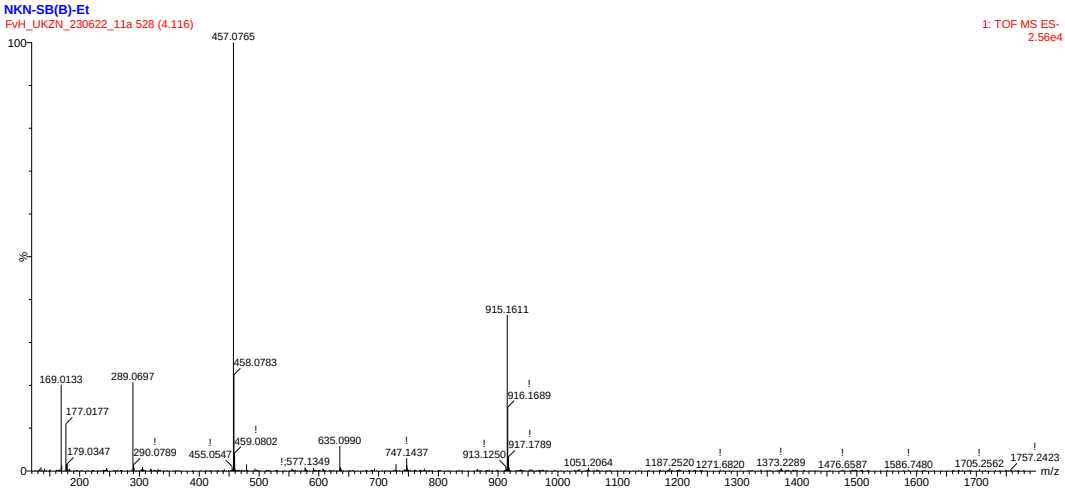

Figure S6. HR-ESI-(-)-MS of (epi)gallocatechin gallate (**4.11**).

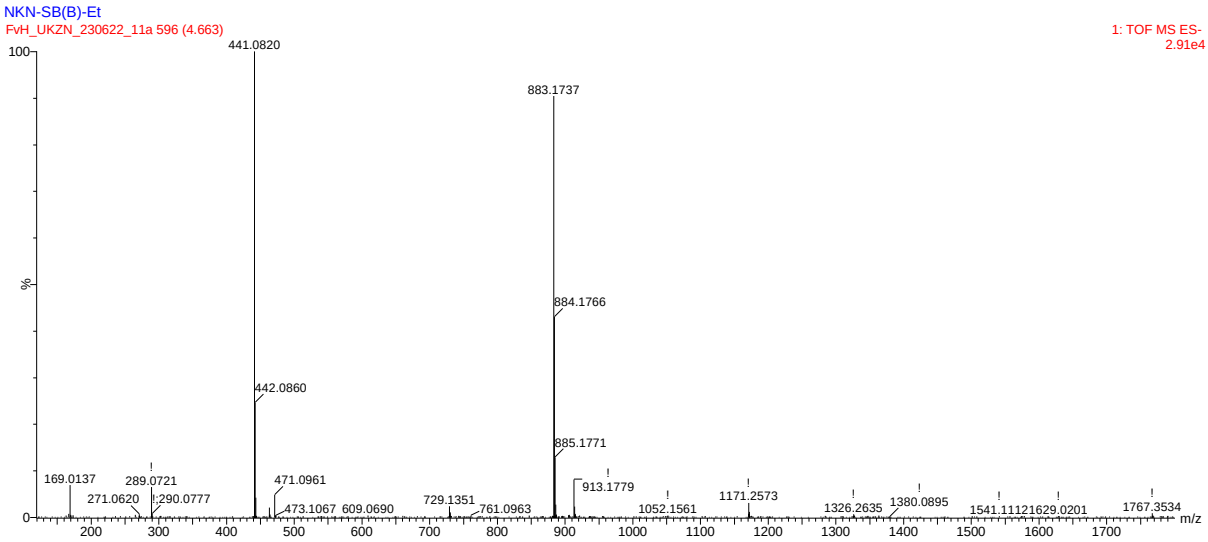

Figure S7. HR-ESI-(-)-MS of the (epi) catechin gallate (**4.10**).