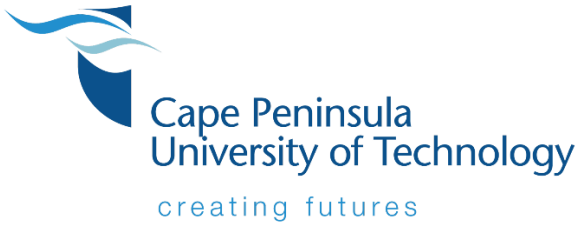


MODULATION OF HIGH GLUCOSE-INDUCED OXIDATIVE STRESS USING *CANNABIS SATIVA* PLANT EXTRACTS IN HEPG2 CELLS

By

ANEVAY ANBREN AFRICA

Thesis submitted in fulfilment of the requirements for the degree Master of Science: Biomedical Technology in the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology, Bellville

Supervisor: Prof. J.L. Marnewick

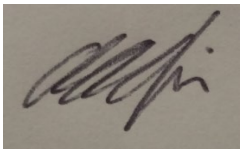
Co-supervisor: Dr. T. F. Docrat

External supervisor: Dr. N.S. Abdul

2025

DECLARATION

I, Anevay Anbren Africa, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



27 October 2025

Signed **Date**

ABSTRACT

Diabetes mellitus (DM) has become a major concern globally within the healthcare community, due to its prevalence and related complications. Type 2 diabetes mellitus (T2DM) is a metabolic disorder mainly characterised by hyperglycaemia. Hyperglycaemia can lead to oxidative stress (OS), caused by excessive generation of reactive oxygen species (ROS), which may result in the impairment of the antioxidant (AO) defense system. OS is known as one of the points of origin of diabetic complications. *Cannabis sativa* is a medicinal plant known to contain a variety of secondary metabolites such as phytocannabinoids and have become increasingly popular among patients who self-medicate. *C. sativa* is used traditionally in many parts of the world to alleviate the symptoms of various chronic illnesses. One of the cannabinoids, cannabidiol (CBD), has been known to exhibit antioxidant, anti-inflammatory and hepatoprotective properties. The number of patients using cannabis as a treatment for various health conditions is growing worldwide, it is imperative for further scientific research to be done which focuses on the selection, identification, and characterisation of the bioactive compounds. Current knowledge on extraction methods is insufficient and studies focusing on this field have been neglected. The hepatoprotective role of acetone, ethanolic and aqueous *C. sativa* extracts were evaluated in this study using an *in vitro* glucotoxic model, to address the limited knowledge on the effectiveness of different cannabinoid extraction methods against oxidative stress.

The phytochemical composition of the extracts used in this study were determined by the liquid chromatography/mass spectrometry (LC/MS) method. The resulting phytochemical fingerprint revealed compounds typical of cannabis, including well characterised phytocannabinoids: cannabidiol (CBD), and tetrahydrocannabinol (THC), which were identified as the primary constituents in the isolates. The total antioxidant (AO) content of *C. sativa* extracts showed an increase that was dependent on the dosage. The antioxidant capacity was evaluated in cell-free systems using three methods: total polyphenol content (TPC), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the ferric ion reducing antioxidant power (FRAP) assay. The biological effects of *Cannabis sativa* extracts on cell viability, metabolic activity, oxidative damage, and cellular death under hyperglycaemic (HG) conditions were assessed. After treating HepG2 cells for 24 hours, cell viability and metabolic activity were assessed using the MTT ([3-4,5-Dimethylthiazol-2-yl)-2.5-Diphenyltetrazolium Bromide]) and intracellular adenosine triphosphate (ATP) assays. Oxidative damage was measured through the thiobarbituric acid reactive substance (TBARS) assay, while redox status was evaluated using the reduced glutathione (GSH) assay. Cellular death was assessed via lactate dehydrogenase (LDH), caspase-8, and caspase-9 assays.

Results indicated that treatment with the *C. sativa* aqueous extract significantly elevated ATP levels ($p < 0.0001$) at a concentration of 200 µg/mL compared to the HG control, demonstrating its protective effect. While the aqueous extract preserved cell viability at lower concentrations (0-600 µg/mL), the acetone and ethanolic extracts demonstrated a dose-dependent decrease in cell viability.

Additionally, both the ethanol and aqueous extracts significantly ($p < 0.0001$) increased GSH concentrations compared to the HG control, with notable increase observed at 35 and 50 µg/mL for the ethanol extract and at 200 and 370 µg/mL for the aqueous extract. A significant decrease in caspase-8 activity was noted at 100 µg/mL of the acetone extract, while the highest concentration (600 µg/mL) of the aqueous extract significantly reduced caspase-8 activity. Moreover, both the acetone and ethanolic extracts showed a significant decrease in caspase-9 activity at their respective high concentrations (100 µg/mL and 50 µg/mL).

The current study demonstrates that *Cannabis sativa*, a well-known and widely used medicinal plant, at certain concentrations, may enhance the endogenous antioxidant defence system in The human liver cell line (HepG2) cells experiencing HG-induced oxidative stress. This enhancement is not true for all extracts and concentrations tested in this model, leading to the conclusion that different *C. sativa* extracts can potentially exhibit pro-oxidant (toxic) properties at certain concentrations. However, the findings of this study suggest the prospective use of *C. sativa* extracts in preventative and therapeutic strategies against metabolic disorders such as hyperglycaemia and DM. The results obtained in this study indicate that the aqueous *C. sativa* extract performed the best at the concentration of 200 µg/mL in the assays and showed to be less toxic to the HepG2 cells when compared to the acetone and ethanolic extracts. Further investigation into the mechanisms involved in the protective effects of the aqueous *C. sativa* extract need to be conducted.

Keywords: Diabetes mellitus; hyperglycaemia; oxidative stress; *Cannabis sativa*; hepatoprotective; antioxidant activity.

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OUTPUTS

- **Anevay Africa.** CPUT postgraduate conference 2023. SARETEC Auditorium in Bellville. 1 March 2023. Presenting author for oral presentation: Modulation of high glucose-induced oxidative stress “using *Cannabis sativa* plant extracts in HepG2 cells.

DEDICATION

To Yahweh (God Almighty), Jesus and the Holy Spirit, and finally, my parents. Thank you for all your love and support.

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LIST OF ABBREVIATIONS

Acronym/Abbreviation	Definition/Explanation
°C	Degree Celsius
µg/mL	Micrograms per millilitre
µL	Microlitre
AAE	Ascorbic acid equivalents
ADP	Adenosine diphosphate
$AlCl_3$	Aluminium chloride
ANOVA	One-way analysis of variance
ANT	Adenine nucleotide translocase
AO	Antioxidant capacity
ATP	Adenosine triphosphate
ARE	Antioxidant response element
AREC	Animal Research Ethics Committee
BCL-2	B-cell lymphoma 2
BHT	Butylated hydroxytoluene
CAT	Catalase
CB	Cannabinoid receptor
CBC	Cannabicrhomene
CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDA	Cannabidiolic aicd
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
CBNA	Cannabinolic acid
CBDV	Cannabidivarin
CBDVA	Cannabidivarinic acid
CDs	Conjugated dienes
CO_2	Carbon dioxide
CypD	Cyclophilin D
DCM	Dichloromethane
dH_2O	Distilled water
DM	Diabetes mellitus
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOH	Department of Health
DPPH	2,2-Diphenyl-beta-picrylhydrazyl
DR	Death receptor

ECS	Endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron chain transport chain
$FADH_2$	Flavin adenine dinucleotide
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
F-C	Folin-Ciocalteu
$FeSO_4$	Iron sulphate
$FeCl_3.6H_2O$	Iron (III) chloride hexahydrate
FFA	Free fatty acids
FHSO	Finola hempseed oil
FRAP	Ferric reducing antioxidant potential
GLUT	Glucose transporter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GA	Gallic acid
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
GCL	Glutamate cysteine ligase
G	Gram
HCl	Hydrochloric acid
HDL-C	High density lipoprotein cholesterol
HG	Hyperglycaemic
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HPLC-MS	High performance liquid chromatography mass spectrometry
H	Hours
IL	Interleukin
iNOS	Insoluble nitric oxide synthase
IR	Insulin resistance
LC-MS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
L-GCK	Liver glucokinase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MDA	Malondialdehyde
MetS	Metabolic syndrome
MPT	Mouse proximal tubular cells
MRC	Mitochondrial respiratory chain
mRNA	Messenger RNA
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

Min	Minutes
mL	Millilitres
M	Molar concentration
Mg	Milligram
mM	Millimolar concentration
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine
NAFLD	Nonalcoholic fatty liver diseases
NG	Normoglycaemic
NO	Nitric oxide
NOX	Nitrogen oxide
NRF2	Nuclear factor-erythroid 2-related factor
Nm	Nanometre
OS	Oxidative stress
OXPHOS	Oxidative phosphorylation
O_2	Oxygen gas
PEP	Phosphenolpyruvate
PC	Phenolic content
PDH	Pyruvate dehydrogenase
PFK-1	Phosphofructokinase
pH	Potential of hydrogen
PK	Pyruvate kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RIP1	Receptor interacting protein
Rpm	Revolutions per minute
RLU	Relative light units
RPMI	Roswell Park Memorial Culture Media
RT min	Retention time
SAHPRA	South African Health Products Regulatory Authority
SD	Standard deviation
TBA	2-Thiobarbituric acid
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
THCVA	Tetrahydrocannabivarinic acid
TNF	Tumour necrosis factor
TPC	Total phenolic content
TPTZ	2,4,6-tri[2-pyridyl]-s-triazine

T2D	Type 2 diabetes
T2DM	Type 2 diabetes mellitus
WHO	World Health Organisation
XO	Xanthine oxidase
Xg	Times gravity

GLOSSARY

Term	Definition/Explanation
Antioxidant capacity	A measure of an organism or food's ability to prevent free radicals from causing harm.
Diabetes mellitus	A group of diseases that lead result in an excess level of sugar in the blood (high blood glucose).
Free radical	An atom, molecule, or ion that contain at least one unpaired valence electron.
HepG2 cells	Human liver cancer cell line that are often used in research to study liver cancer, drug metabolism, and hepatotoxicity.
Hyperglycaemia	High blood sugar levels.

APPENDICES

CHAPTER ONE

1.1 Introduction

DM has become a major concern globally in the healthcare community due to its prevalence and related complications (Jubaidi et al., 2021). T2DM is a metabolic disorder mainly associated with hyperglycaemia, insulin resistance and hyperlipidemia (Arora et al., 2021). Hyperglycaemia can lead to OS, characterised by elevated production of ROS, which may result in the antioxidant level being depleted AO defense system (Scott and King, 2004). Superoxide, hydroxyl, peroxyl, hydroperoxyl and hydrogen peroxide radicals, are known to form part of ROS. (Johansen et al., 2005).

According to many hypotheses, OS is known as one of the points of origin of diabetic complications. A hypothesis that is currently widely accepted, was formulated at the turn of the century. This hypothesis emphasises ROS formation as the initial instigators of most of the major pathways that are linked to the onset and the worsening of diabetic complications, which may eventually lead to mortality (Iacobini et al., 2021).

Various metabolic processes under normal conditions produce ROS which function as signalling molecules, within the electron transport chain (ETC), which function as signalling molecules. OS may lead to the mitochondria producing superoxide, which drives the formation of intracellular ROS. Electron leakage is important in the physiological impact on mitochondrial coupling efficiency and generation of ROS (Misrani et al., 2021). When ROS are in excess, damage to major macromolecules such as carbohydrates, lipids, nucleic acid and proteins occur as well as compromised cell viability (Mullarky and Cantley, 2015). Excess cellular ROS production under pathological conditions may be caused by cell nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is cell membrane bound (Tabassum et al., 2020). Oxidative stress results due to the overproduction of ROS molecules (Oguntibeju, 2019).

Type 1 diabetes is a response to the pancreas being unable to regulate insulin, which helps regulate the production of glucose levels. Type 2 diabetes results due to high levels of glucose being present in the blood. Thus, both T1D and T2D results due to high glucose conditions, increasing ROS production and eventually leading to OS (Sempere-Bigorra, et al., 2021).

Due to the partial or ineffective results of diabetic treatments, there is a great need for the production and development of new therapeutic interventions (Nicholson and Hall, 2011).

Study-based evidence suggests that OS is significant in the pathogenesis of diabetes and the development of complications associated with diabetes (Deore and Sapakal, 2011). OS due to hyperglycaemia can also lead to impaired glycolysis. Glycolysis is a metabolic pathway involving the splitting of one glucose molecule into two pyruvate molecules, as the end products of this process. ATP is generated during glycolysis by pyruvate kinase (PK), which catalyses the final reaction of glycolysis (Teslaa and Teitell, 2015). Mazzio et al., (2004) showed that loss of glycolysis could involve hydrogen peroxide (H_2O_2) contributing to several factors. Mullarky and Cantley, (2015) concluded that ROS could inhibit glycolysis and that this inhibition can promote reduced nicotinamide adenine dinucleotide phosphate (NADPH) production and therefore protect cells against OS.

Type 2 diabetes in humans is characterised by decreased glucose disposal. The function of the insulin-dependent glucose transporter (GLUT4) receptor plays a central role in glucose transport and disposal (Huang and Czech, 2007). Hyperglycaemia develops when the rate at which glucose enters the blood plasma exceeds its rate of disposal in diabetic patients, hyperglycaemia is continuously present (Boden et al., 1996) and can damage tissues such as the kidneys, eyes, liver, nerves etc. Untreated high blood glucose levels could result in impaired glucose metabolism (Wu et al., 2016).

During glycolysis and other metabolic pathways, glucose metabolism supplies nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) with electrons, to produce ATP. When glucose overload occurs, NADH is in excess, this can lead to an imbalance between NADH and nicotinamide adenine dinucleotide (NAD^+), eventually leading to OS (Wu et al., 2016). The liver is the major organ of glucose utilisation and produces glucose to be utilised by other tissues in the body (Adeva-Andany et al., 2016). Liver damage can result from the underlying mechanisms of diabetes, that is, the combination of OS and inflammatory responses (Mohamed et al., 2016).

Antioxidants are compounds that can lower OS and its-induced damage to the mentioned cellular macromolecules including, deoxyribonucleic acid (DNA) mutations, malignant transformations, and other parameters of cell damage (Mazzio et al., 2004). Although the human body has several mechanisms to counteract OS, when this system fails, exogenous antioxidants may help in correcting the imbalance between ROS and endogenous antioxidants (Kurutas, 2016), thereby protecting an organism more efficiently from OS (Bouayed et al., 2010). Medicinal plants have been used for

generations by indigenous people globally and are known to be a good source of antioxidants (Tran et al., 2020). Medicinal plants with strong AO effects are becoming increasingly popular within the scientific community (Al Hroob et al., 2018).

Cannabis sativa is a medicinal plant known to contain a variety of secondary metabolites such as phytocannabinoids and is commonly used by patients who self-medicate (Romano and Hazekamp, 2013). Phytocannabinoids activate the cannabinoid receptors CB1 and CB2 (Brown et al., 2013). CBD has been reported to exhibit AO, anti-inflammatory, antipsychotic, anxiolytic and hepatoprotective properties (Erukainure et al., 2021). CBD oil significantly reduced the incidence of diabetes *in vivo* and its use as a strategy for treating diabetes, needs to be further investigated (Mattes et al., 2021).

Erukainure and colleagues showed that CBD exhibited antioxidant activity and improved glucose-lipid homeostasis (Erukainure et al., 2021). Since obesity is known as a comorbidity, beta-cell dysfunction in diabetes can be linked to adipocyte-derived factors (Zhao et al., 2006). Gallant and colleagues determined that the rate of adipogenesis decreased with increasing THC concentration (Gallant et al., 2009). Previous studies indicate that cannabinoids can induce apoptosis, however, the precise mechanisms through which they do so are not well understood (Brown et al., 2013). The endocannabinoid system (ECS) has diverse functions, including regulating OS in the body. The search for affordable and alternative treatments in combatting diabetes and its associated complications is a major health initiative globally (Erukainure et al., 2021).

The phytocannabinoids with CBD and THC, the major and well-known ones, can help regulate the ECS, and thus, aid in fighting against OS (Gojani et al., 2023). They can potentially prevent certain diseases (Brown et al., 2013) via their AO (Horváth et al., 2012 and Jitca et al., 2023) properties. Current scientific evidence suggests that CBD has beneficial effects on oxidative status and there is growing interest in CBD due to its AO effects, as it may prove to have a therapeutic use against conditions such as diabetes. Over the years, the use of cannabis for medicinal purposes has significantly increased and been used to mitigate physical distress (Wallis et al., 2022 and Asselin et al., 2022). The number of patients using cannabis for various conditions are growing worldwide (Bouso et al., 2020). Suitable cannabis extraction processes are imperative for further continued scientific research which focuses on the selection, identification, and characterisation of bioactive compounds.

Current knowledge on cannabis extraction methods and determining bio-activities are scarce as studies focusing on this aspect have been neglected. This leaves a significant gap in the literature that should be extensively explored (Dawidowicz et al., 2021 and Lazarjani et al., 2021). The CBD extracts prepared for medicinal use, often require increased purity to meet specific standards. Traditional methods for extracting compounds from cannabis include water and ethanol extraction and some of the best solvents are methanol and hexane. Acetone as a potential solvent for extracting compounds from the cannabis plant has not been well investigated to date. More studies are needed to further investigate the use of hot water, ethanolic and acetone extracts (Szalata et al., 2022).

1.2 Research aim

To investigate the protective role of *Cannabis sativa* solvent extracts against high glucose-induced oxidative stress in a liver-derived cell line, by assessing metabolic activity, programmed cell death, and the antioxidant response.

1.3 Research questions

- How do the chemical and antioxidant profiles of *C. sativa* differ between the various solvent extracts prepared?
- Do these extracts interfere with liver cell metabolism?
- Do the extracts exert cytoprotective properties in HepG2 cells under hyperglycaemic conditions?
- What is the possible mechanism/s involved in the hepatic cytoprotective effects in cells under hyperglycaemic conditions?

1.4 Research objectives

- To assess the antioxidant content and capacity of the different solvent extracts.
- To quantify the major chemical compounds in the various extracts.
- To determine an optimal *C. sativa* extract concentration in HepG2 cells that does not have a negative impact on energy output, viability, or metabolism via:
    - assessment of cell viability using the MTT assay.
    - assessment of the energy output using the ATP assay.
    - assessment of the apoptotic regulators such as caspases, using the Caspase 8, 9 activity assays.
    - assessment of cytotoxicity/necrosis (membrane leakage) via the LDH assay.
- To determine the oxidative lipid damage using malondialdehyde (MDA) quantification

- To determine the antioxidant response of *C. sativa* extracts in the HepG2 model by measuring reduced GSH levels

1.5 Hypothesis

It is hypothesised that cannabinoids as a source of antioxidants, will reduce oxidative stress caused by hyperglycaemia in a HepG2 derived cell line by mediating key cell stress responses.

1.6 Significance of research

The findings of this study may result in validation and the production of new and natural commercialised products that could be used as alternative treatment for T2DM in which oxidative stress and complications associated with it may be inhibited. This research may also aid in the development of home-based and laboratory methods of extraction within the Cannabis industry.

CHAPTER TWO

2.1. Diabetes

Metabolic syndrome (MetS) incorporates a group of disorders that may result in cardiovascular system defects, DM, obesity and other lifestyle disorders (Arora et al., 2021).Despite differences between the underlying mechanisms of both forms of diabetes, both type 1 and type 2 diabetes result due to high levels of glucose in the blood (Sempere-Bigorra, et al., 2021 and Ozougwu et al., 2013). The most common metabolic disorder characterised by hyperglycaemia is T2DM (Arora et al., 2021). Hyperglycaemia is caused by impaired blood glucose regulation, in which glucose levels are above the normal range (between 4.4 and 6.1 mmol/L) (Wensveen et al., 2021).

T2DM was reported to have a global prevalence of 9.3% in 2019 (You et al., 2021). It was reported by the International Diabetes Foundation that 463 million people worldwide were living with diabetes. It was projected that this number would increase by 51% to 700 million by 2045. An increase in the prevalence of diabetes increases the burden of other metabolic-related diseases. It has been estimated that diabetes accounts for 10% of global health care costs (Cefalu and Rodgers, 2024). In 2016 and 2017, diabetes was the second leading underlying cause of death in South Africa and has been rapidly increasing in its prevalence. An increase from 4.5% (2010) to 12.7% (2019) in the prevalence of diabetes in South Africa has been recorded (Grundlingh et al., 2022).

Medication used to treat T2DM targets the hallmarks of the disease, including insulin resistance, decreased insulin secretion, and inhibiting hepatic glucose output. Metformin (Glucophage) is commonly used to decrease mortality rates in patients suffering from T2DM and is known to be a first-line agent for its treatment. Metformin promotes glucose uptake and disposal (Ripsin et al., 2009).

Excessive free radical generation and OS results from diabetes (Yaribeygi et al., 2019). The progression of T2DM appears to be centred around excessive OS. At the cellular level, ROS/reactive nitrogen species (RNS) have major functional and dysfunctional roles, particularly in tissues that cause the progression of T2DM to be sped up. These tissues include pancreatic islets, muscle, adipose, and liver (Newsholme et al., 2016). Free radicals are chemicals that are toxic and when in excess, can damage cells and tissues in the human body. Free radicals are formed through hyperglycaemia, promoting the autoxidation of glucose. Antioxidants are substances that can neutralise free radicals, thus, removing them from the body. ROS are derived from the redox reactions involving free radicals and antioxidants (Chauhan et al., 2022). Focusing on the role of antioxidants and their sources will aid in the discovery and development of naturally derived diabetic treatments (Gautam et al., 2022).

2.2. Glucose transport

Homeostasis in the human body is maintained by glucose absorption and use. Glucose can be obtained via three main sources: intestinal absorption after the dietary carbohydrates have been digested, glycogenolysis and gluconeogenesis (Giugliano, et al., 2008; Gromova et al., 2021). Blood glucose levels that are dysregulated may lead to hyperglycaemia, which is a major issue leading to metabolically linked diseases such as, type 2 diabetes (T2D), metabolic syndrome, and obesity (Gromova et al., 2021).

Multiple metabolic pathways allow glucose to be transported into the cells in the body where it may be stored as glycogen, may undergo glycolysis, or may be released by the liver and kidneys into circulation (gluconeogenesis) (Giugliano et al., 2008). The liver and kidneys are the only organs containing glucose 6 phosphatase, the enzyme needed for glucose to be released into the circulation (Giugliano et al., 2008). In the human body, glucose is the main source of energy. Therefore, cells in the human body need a continuous supply of glucose (Głuchowska et al., 2021). This is achieved via the glucose transport system by the sodium-independent glucose transporters (GLUTs), sodium dependent glucose cotransporters (SGLTs), and uniporter, SWEET protein glucose transporters (Głuchowska et al., 2021). Glucose is a polar molecule which is hydrophilic in nature and needs specific proteins to undergo diffusion through the lipid bilayer (Głuchowska et al., 2021).

According to Gromova et al., (2021), the gut acts as a barrier and regulatory mechanism for maintaining blood glucose levels. It was reported that glucose absorption, glucose ingestion and metabolism are all interlinked (Gromova et al., 2021).

Glycolysis is a metabolic pathway needed for ATP synthesis. ATP is the source of energy that the body needs to function properly. Glycolysis plays a role in both aerobic and anaerobic conditions. Oxygen aids in the synthesis of ATP from the process of cellular respiration, beta-oxidation, lipid and protein catabolism. During anaerobic respiration, a buildup of NADH molecules occurs, which in turn limits the production of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose consumption (Seidler et al., 2013).

The liver is responsible for maintaining normal blood glucose levels. Glucose is produced during fasting and is stored in the liver. The liver utilises glucose and releases it to the systemic circulation (Adeva-Andany et al., 2016). The liver is important for generating glucose for other tissues (Han et al., 2016). The hepatocyte takes up blood glucose via the glucose transporter type 2 (GLUT2), which is independent of insulin signalling. GLUT2 is phosphorylated by glucokinase (LGCK) present in the liver (Bae et al., 2010). Glucose 6 phosphate is either further processed via glycolysis or utilised for the synthesis of glycogen (Bechmann et al.,

2012). Previous studies have indicated that glucose metabolism may be involved in diseases such as DM (Adeva-Andany et al., 2016).

2.3. Glycolysis and glucose disposal

Glycolysis is a metabolic pathway involving the formation of two pyruvate molecules from one glucose molecule (Chandel, 2023). In glycolysis, ATP is generated through substrate-level phosphorylation, specifically in two reactions. These reactions involve the transfer of a phosphate group from a high-energy substrate to ADP, forming ATP. Specifically, these are the reactions catalyzed by phosphoglycerate kinase and pyruvate kinase (Johnson et al., 2015). Substrates for energy production via the synthesis of ATP is provided by glycolysis (Guo et al., 2012).

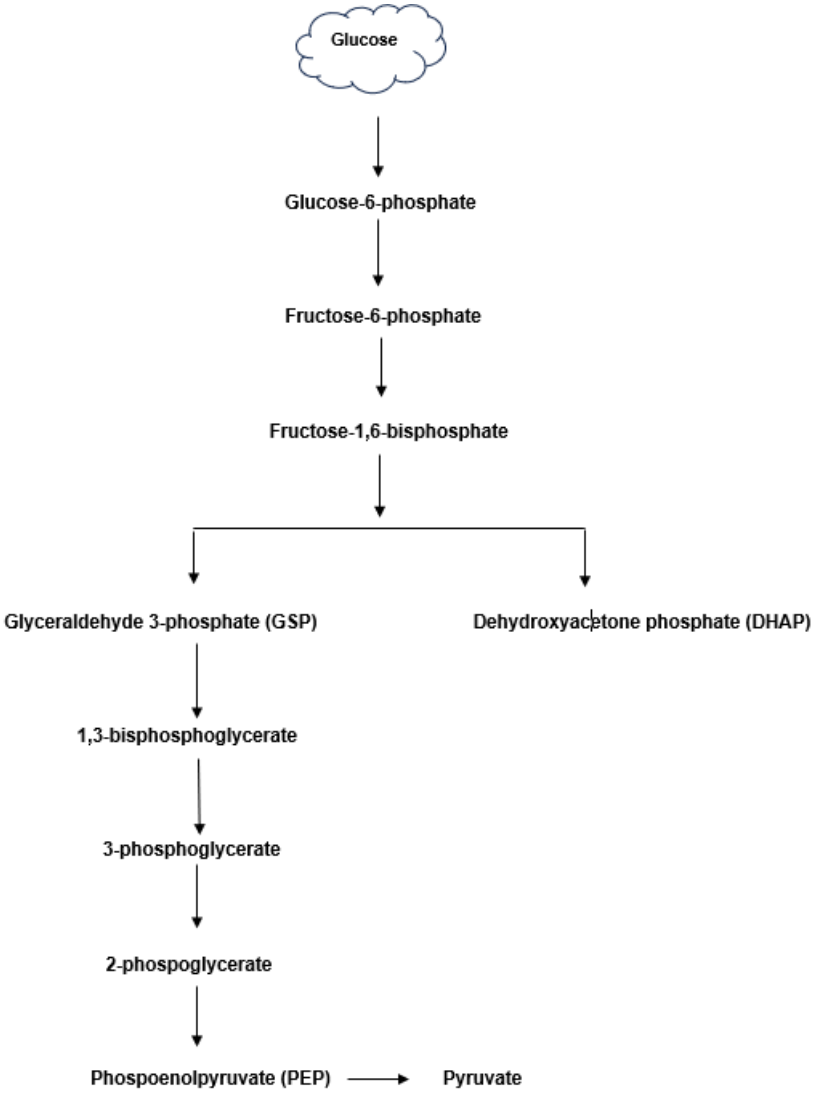


Figure 2.1:The major steps of glycolysis

Glycolysis involves ten major steps (Figure 2.1). Glycolysis can take place under aerobic and anaerobic conditions. Since the regulation of glucose uptake is essential for health, it is known that hyperglycaemia can trigger other pathways that may promote OS in human tissues. Aerobic glycolysis is increased by hyperglycaemia, producing superoxide molecules and causing mitochondrial dysfunction, via the mitochondrial ETC. A greater availability of glucose may lead to other pathways, for example, the polyol pathway, that could reduce the concentration of reduced GSH and impair the cellular antioxidant response.

Aerobic glycolysis results in pyruvate as an end product that enters the mitochondria and is oxidised to carbon dioxide (CO_2) and (H_2O) via the tricarboxylic acid (TCA) cycle and the ETC. In anaerobic glycolysis, pyruvate is reduced by LDH, which results in mitochondrial respiration being halted, Since the decarboxylation of pyruvate results in acetyl-CoA,this biochemical reaction links glycolysis to the TCA cycle (Steensma, 1997).This resulted in the assumption

that glycolysis reaches a dead-end point under anaerobic conditions (Schurr, 2017; Gonzalez et al., 2024).

2.4. Impaired glycolysis and glucose disposal

Impaired migration of glucose from the surrounding cell into the cell, reduced glycolysis, and the dysfunction of the mitochondria, are common features of both type 1 and 2 diabetes mellitus (Holecek, 2023). According to Lund et al., 2019, under hyperglycaemic conditions, increased lactate levels can occur because of impaired mitochondrial function, or simply due to a high concentration of glucose, resulting in increased glycolytic flux. This occurrence may cause pyruvate to be pulled toward lactate instead of entering the mitochondria. LDH functions as an enzyme that catalyses the conversion of pyruvate to lactate using NADH. When the production of ATP is disrupted, leading to the upregulation of LDH for energy production (Farhana and Lappin, 2023). Higher levels of lactate can be observed due to increased glycolysis (Haythorne et al., 2022).

2.5. Hyperglycaemia and oxidative stress

The production of free radicals, including ROS, can be increased by hyperglycaemia, leading to OS. The impact of these factors may be linked to diabetes being developed and the health complications associated with it, which can worsen over time (Johansen et al., 2005). Free radicals are by-products of metabolism, these molecules are continuously generated via enzymatic and nonenzymatic reactions. Free radicals can be generated by either external or internal processes. Free radicals can be generated from outside the body by sources such as chemmicals, ultra-violet (UV) rays, and environmental air pollution and they are converted to H2O2 by ionising radiation. The mitochondrial respiratory chain (MRC) is an internal process that produces the greatest number of free radicals, in which peroxide is involved in secondary oxidative activity (Nilsson and Liu, 2020). During chronic hyperglycaemia, the MRC is known to be a major potential source of free radical generation. Previous studies suggest that high glucose levels induce ATP synthase enzyme activity which leads to mitochondrial free radical generation. All of these processes ultimately lead to OS (Choi et al., 2009). The imbalance between free radicals (excess) and antioxidants can lead to multiple pathologies (Figure 2.2).

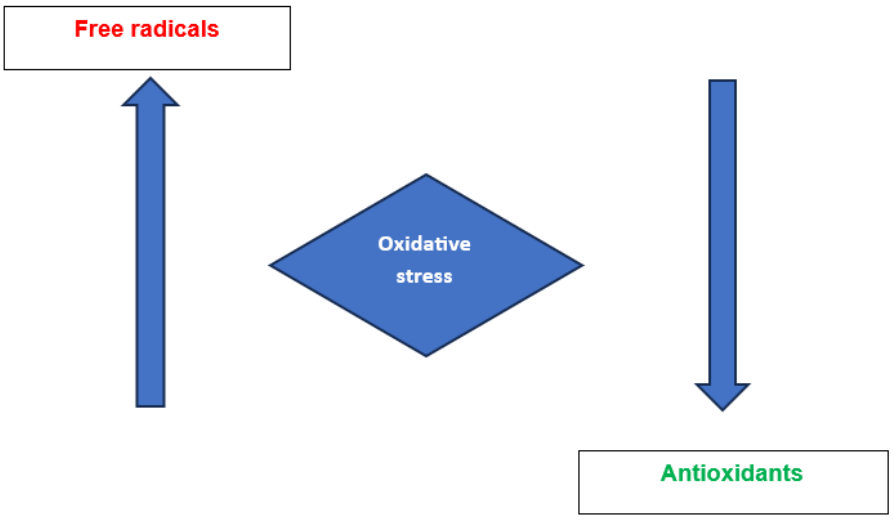


Figure 2.2: Oxidative stress: depicting the imbalance between free radicals and antioxidants

This interference in the free radicals and AO balance may lead to diabetic complications (Bonnefont-Rousselot et al., 2000). According to Lucchesi et al., (2013), it was concluded that OS in the liver is determined by diabetes, due to an increase in the levels of ROS and a significant reduction in their AO defences. It was stated that such an imbalance in liver cells may play a crucial role in the development of chronic liver disease in diabetes, including nonalcoholic fatty liver diseases (NAFLD) and the possibility of it leading to steatohepatitis and cirrhosis.

The amalgamation of increased OS and an aberrant inflammatory response is the underlying mechanism of diabetes. This combination contributes to liver damage. The process of transcription of pro-apoptotic genes is activated, thus, damaging hepatocytes (Mohamed et al., 2016). ROS and cytokines secreted from inflammatory cells are central to understanding hepatocellular injury as a result of toxins. It is now understood that parts of inflammatory cell products trigger liver injury. HepG2 cells can be used to study the effects of high glucose levels on the liver (Mohamed et al., 2016).

2.6. Reactive oxygen species and oxidative stress

Since hyperglycaemia results in increased levels of free radicals and ROS in diabetic patients, understanding how ROS form and function is important. In the human body, ROS is continuously being produced, simultaneously, antioxidants are formed and act as a mechanism of defense against ROS (Pisoschi et al., 2021). ROS accumulates when there is an imbalance in the production of ROS and antioxidants. This imbalance causes OS to occur (Charlton et al., 2020).

Table 2.1: Most abundant reactive oxygen species

Radicals	Non-radicals
Superoxide: $O_2^{\bullet-}$	**Hydrogen peroxide**: H_2O_2
Hydroxyl: $OH^{\bullet}$	**Hypochlorus acid**: HOCl
Alkperoxyl: $RO_2^{\bullet}$	**Alkyl hydroperoxide**: ROOH
Alkoxyl: $RO^{\bullet}$	**Ozone**: O_3
Hydroperoxyl: $HO_2^{\bullet}$	**Singlet oxygen**: O_2^{-}

Reactive oxygen species (Table 2.1) are a subset of free radicals that contain oxygen , and when they encounter another molecule, their unpaired valence electron can pair up with one of the other molecule's electrons (Southorn et al., 1988). Antioxidants can donate an electron to these free radicals to stabilise it (Pisoschi et al., 2021). However, these antioxidants become free radicals by donating their electrons (Forman et al., 2009). A chemical chain reaction then occurs and if the body does not have enough antioxidants to counterattack pro-oxidants, OS will be prolonged (Charlton et al., 2020). This process of OS being left unchecked, may cause a variety of chronic illnesses and diseases. High levels of ROS may lead to apoptosis (Patergnani et al., 2021).

2.7. Adverse effects of high glucose on antioxidant mechanisms

OS involves eliminating these free radicals and ROS via protective mechanisms, including antioxidants. This imbalance threatens an organism, as in the case of complications resulting from T2DM (Tu et al., 2019). Cells that are exposed to high concentrations of glucose and vascular tissues found in humans and animals with diabetes have high levels of OS markers. High levels of OS markers have been observed when cells and vascular tissues found in humans and animals with diabetes are exposed to excessive levels of glucose. These markers are namely gluco-oxidants, glycated compounds, oxidised low-density lipoprotein, superoxidants, and nitrotyrosine (Scott and King, 2004). Previous studies have shown that an increase in the levels of OS is due to AO defence mechanisms in the tissues being ineffective in neutralising increasing levels of oxidants produced by diabetes or insulin resistance (Scott and King, 2004).

It is known that protein carbonylation leads to lipid peroxidation, which occurs as a result of protein modification via carbonylation reactions, because of OS (Fritz and Petersen, 2011). Oxidative modifications such as lipid peroxidation products (MDA) and protein carbonylation result from accumulated ROS. The most useful current methods for quantifying ROS involve the quantification of peroxidation and protein carbonylation (Rodrigeuz-Garcia et al., 2020). In individuals with diabetes, ROS are formed by auto-oxidation reactions between sugars and

sugar adducts. Lipids are the main compounds targeted by ROS, this attack leads to lipid peroxidation (Blanco-Ayala et al., 2014; Dham et al., 2021).

The ECS is responsible for maintaining balance in the human body. The ECS maintains temperature, mood, and immune system homeostasis (Lowe et al., 2021). Pathophysiological diseases such as diabetes, cancer, cardiovascular diseases, and neurodegenerative diseases are associated with the ECS (Lowe et al., 2021).

2.8. Cell death

2.8.1. Metabolic changes associated with apoptosis

Hyperglycaemia can lead to apoptosis, also referred to as programmed cell death, and is a process of great importance in a wide range of biological systems. There are two phases in which apoptotic cell death occurs: a commitment to cell death, followed by an execution phase (Khoury et al., 2022). Apoptosis is a process in which biological functionality of the tissue maintains homeostasis and can be initiated via an extrinsic and intrinsic pathway. During this highly preserved and controlled mechanism, single cells are eliminated without causing disruptions to the normal functioning of the biological tissues. Recent studies of cholestatic liver injury have showcased the role of Kupffer cells (resident macrophages), which are involved in the pathogenesis of sustained inflammatory response following cell death (Schattenberg et al., 2006).

Caspases are activated during apoptosis, leading to all the morphological changes that are characteristic of this form of cell death (Docrat et al., 2018). Metabolic changes linked to hyperglycaemia associated with an increase in OS and inflammation, are the driving force behind diabetic complications (Jha et al., 2018). Several complications are associated with prolonged hyperglycaemia; retinopathy, peripheral neuropathy, peripheral arterial diseases, or macrovascular diseases (Liu et al., 2019). Apoptosis is essential for developing all multicellular organisms and maintaining homeostasis within these organisms. This process is central in diabetes and has links to pancreatic beta cell death and plaque progression. The extrinsic death receptor pathway can result in the activation of apoptosis or via the intrinsic mitochondrial pathway. This pathway could activate effector caspases, including Caspase-3 (Sun et al., 2021).

The intrinsic pathway is activated by various cellular stressors, which include deprivation of growth factors, disruption of the cytoskeleton, unfolded proteins accumulating, the occurrence of DNA damage, and insufficient cytokine support (Docrat et al., 2018). The activation of the extrinsic apoptotic pathway is caused by transmembrane death receptor-mediated reactions (Docrat et al., 2018). A HG environment promotes apoptosis and contributes to the gradual loss of renal function, which is another complication of diabetes (Sifuentes-Franco et al.,

2018). The increased oxidation of free fatty acids (FFA) by the liver, produces ROS. This can cause structural and functional changes in cells and cell death. The organism's antioxidant defences regarding physiological conditions, can neutralise the harmful effects of such substances. OS is initiated in diabetes and maintenance thereof to the hepatic level triggers the responses linked to adaptation in response to chronic stress by the liver. The changes in this biological cycle compromise the liver's replication and regeneration, which leads to apoptosis or cell death (Lucchesi et al., 2013). Since OS results from a hyperglycaemic environment, and hyperglycaemia promotes apoptosis, medicinal plants with strong AO effects have intrigued the scientific community. Medicinal plants have attracted much attention for the prevention of these complications (Al Hroob et al., 2018).

Significant hepatocellular damage can result from the overactivation of the apoptotic process. The proliferation and transformation of cells can be observed when the process of apoptosis is inhibited (Schattenberg et al., 2006).

2.8.2. Necrosis

Necrosis is morphologically different from "classical" apoptosis in which cells swell, leading to the collapse of the plasma membrane and eventually rapid cell lysis (Proskuryakov et al., 2003). A study showed that early swelling in ischemic liver cells in rats was due to ATP depletion. The lack of ATP can cause protein denaturation in living and dead cells (Ciechanover et al., 1994). ATP depletion is a biochemical hallmark of necrosis and is caused by mitochondrial oxidative phosphorylation (Kroemer et al., 2007). Necrosis can occur due to rapid ROS generation, a decrease in ATP and impairment of plasma membrane integrity (Samali et al., 1999). According to Samali et al., (1999), oxidant-induced caspase inactivation may lead to necrosis, concluding that strong oxidants inhibit caspases and can trigger necrosis in some cells. Late-stage apoptosis or autophagic cell death program can occur due to failed phagocytosis, which in turn, may lead to necrotic morphology being observed (Green and Llambi, 2015). The most studied and best characterised form of programmed necrosis is receptor interacting protein (RIP)-K-dependent necrosis (necroptosis) and requires RIP3 to be activated, potentially resulting in rapid cell death with features typical of necrosis (Cho et al., 2009; He et al., 2009). Previous studies have shown that RIP1 is linked to ROS production in the process of necrosis and the mitochondrial ROS accumulation is dependent on RIP1. RIP1 is localised to the mitochondria due to tumour necrosis factor (TNF) stimulation, which leads to the interaction between Adenine nucleotide translocase (ANT) and Cyclophilin D (CypD) being reduced and ATP being depleted, thus, leading to apoptosis or necrosis (Vanlangenakker et al., 2008; Li Y, et al., 2004). According to a study conducted by Lieberthal et al., (1998), ATP depletion either caused apoptosis or necrosis in mouse proximal tubular

cells (MPT) and it was determined that a narrow range of ATP depletion (~ 15 to 25% of control) determines whether cells die via necrosis or apoptosis (Lieberthal et al., 1998).

2.9. Reactive oxygen species detoxification

The mitochondria possess an AO defence system to help protect themselves against harmful effects produced by ROS (Snezhkina et al., 2019). Low levels of ROS are generated through the ETC in the mitochondria during oxidative phosphorylation (OXPHOS) (Gounden et al., 2015). However, under HG conditions, the TCA cycle generates more electron donors, resulting in an influx of electrons entering the ETC, leading to increased production of superoxide radicals (Gounden et al., 2015). Overproduction of ROS can lead to harmful diseases, and therefore, it is important to understand ROS generation and existing AO systems that help balance the redox state in mammalian cells (Snezhkina et al., 2019). Human cells are equipped with a variety of antioxidants, these antioxidants can counterbalance the effect of oxidants enzymatically and nonenzymatically. Enzymatic antioxidants include superoxide dismutases (SODs) (found in the mitochondria of cells), catalase (CAT), and glutathione peroxidase (GP*x*). CAT converts H_2O_2 into water (H_2O) and oxygen (O_2) (Stephenie et al., 2020). CAT and GPx are commonly present in the cytoplasm and mitochondrial matrix (Stephenie et al., 2020). Vitamins C, and E, beta-carotene, and GSH are all known as nonenzymatic antioxidants (Birben et al., 2012).

GPx utilise the reducing power of GSH. Vitamin E and glutathione are referred to as scavenging antioxidants and scavenging active radicals to prevent chain initiation, including the breaking of chain propagation reactions (Ighodaro and Akinloye, 2018).

2.9.1. Antioxidant mechanism: GSH free radical scavenger

Tripeptide glutathione (y-glutamyl-cysteinyl-glycine (GSH) plays a crucial role in intracellular defense against ROS-induced oxidative damage and is extensively used as a co-substrate by glutathione peroxidases (GP*x*) (Sharma, 2012; Lushchak, 2012). Both catalases and GP*x*s work together in the degradation of H_2O_2, these enzymes are found in different cellular compartments and GP*x* has a higher affinity for H_2O_2, and this is indicative of GP*x*s and catalases complementing each other (Lushchak, 2012).

Three forms of glutathione exist, namely, reduced glutathione (GSH), glutathione disulphide (GSSG), and GSH-conjugates, and can be excreted into extracellular regions (Lushchak, 2012). GSH acts as an AO and reacts chemically with $O_2^{\bullet-}$, $^{\bullet}OH$, H_2O_2 and functions as a free radical scavenger. This AO also has the ability to protect proteins, lipids, and DNA (Sharma et al., 2012) from OS. The molecule, glutathione and enzymes, glutathione reductase (GR), glutathione peroxidases and glutathione S-transferases all belong to the glutathione system.

(Kabel, 2014) Glutathione S-transferases are glutathione-dependent AO enzymes and are found at high levels in the liver, this is to aid in detoxification metabolism (Kabel, 2014).

Under sustained hyperglycaemic conditions, GSH is found at very low cellular levels, and can lead to tissue damage associated with OS (Sekhar et al., 2015). According to Sekhar et al., (2015), in type 2 diabetes, severely impaired production of glutathione is due to the availability of the GSH precursor being limited. Therefore, including GSH precursor amino acids into the diet could lead to the restoration of GSH synthesis, which can lead to lower OS damage whilst facing sustained hyperglycaemia (Sekhar et al., 2015).

2.10. Antioxidants as a source to combat diabetes-related complications

Current diabetic medications may show to be toxic to tissues and organs by causing OS. Thus, the need for new and improved medications that will reduce harmful side effects. Existing evidence depicts an increase in the formation of free radicals and a decrease in AO potential as being associated with DM. Numerous studies have shown OS markers being lowered by antioxidants (Rahimi et al., 2005). Medicinal plants are a good source of compounds/drugs for conditions associated with OS due to their AO activity (Rahimi-Madiseh et al., 2016). Many studies have shown that antioxidants sourced from various plants, such as, lycopene, retinol, and beta-cryptoxanthin to name a few, greatly reduced complications associated with diabetes (Ghasemi-Dehnoo et al., 2020). According to Kaneto et al., (1999), they found that treating diabetes with antioxidants is beneficial in the treatment thereof and provided protection for beta-cells against glucose toxicity in C57BL/KsJ-*db/db* mice (Kaneto et al.,1999).

2.11. The benefits of medicinal plants in the treatment of diabetes

The use of medicinal plants as a treatment for DM has been used traditionally for generations and has led to multiple studies being conducted. These studies have showcased the benefits of their use in both human and animal studies (Al Hroob et al., 2018). According to the World Health Organisation (WHO), an estimated 80% of the population of developing countries still depend on traditional medicines which are mostly derived from plants. These traditional medicines are often used to prevent or treat diseases (Tran et al., 2020). Parts of medicinal plants often used for therapeutic or medical effects include leaves, flowers, seeds, rhizomes, roots, stems, and barks (Škrovánková et al., 2012). Traditional medicine has been known to be more affordable and clinically effective and tends to have relatively fewer adverse effects than modern medicine (Tran et al, 2020).

Examples of plants that showcases antidiabetic activity include; Bitter Melon (*Momordica charantia*), Panax ginseng, Allium (*Allium cepa* Linn.), and Asphodelaceae (*Aloe vera* L.) to name a few. These plants contain bioactive compounds leading to hypoglycaemic activity and antidiabetic effects (Tran et al., 2020). Ginger (*Zingiber officinale*) has also exhibited potential

protective effects against diabetes-induced renal injury by ameliorating OS, inflammation and apoptosis (Al Hroob et al., 2018). A source of natural antioxidants are plants and their derivatives. Numerous epidemiological studies have shown that plant antioxidants are capable of removing/neutralising free radicals. Phenolic antioxidants such as phenolic acids can be found in many plants. The *Lamiaceae* family is a rich source of plants that possess AO activity. These include rosemary, oregano, sage, basil, marjoram, thyme, mints, and *Apiaceae* to name a few. Other examples of medicinal plants with AO activity belong to the *Asteraceae* family and one example is chamomile. Eucalyptus is another example of a plant that has antioxidant properties and belongs to the *Myrtaceae* family (Škrovánková et al., 2012).

2.12. *Cannabis sativa*

Cannabis sativa L. is a dioecious plant from the Cannabaceae family and is widely cultivated, and consumed across the world, despite it being known as a controversial plant (Stefkov et al., 2022). Most accept the viewpoint that cannabis is a monotypic genus and consists of a single species, *Cannabis sativa* L. The cultivation and utilisation of cannabis dates back to 3000 to 4000 years ago, according to ancient Chinese records and archaeological findings (Malik et al., 2021). *Cannabis* L. (Hemp) is known to contain over 150 active compounds called phytocannabinoids (Nigro et al., 2021). Cannabis has long been used as a natural medicinal remedy for the treatment of various ailments side effects caused by disease (Khoury et al., 2022). Recently, many countries have eased restrictions around research, commercial cultivation, and the selling of dried cannabis flowers and cannabis-based products. The easing of these restrictions may deepen our understanding of the *C. sativa* plant (Malik et al., 2021).

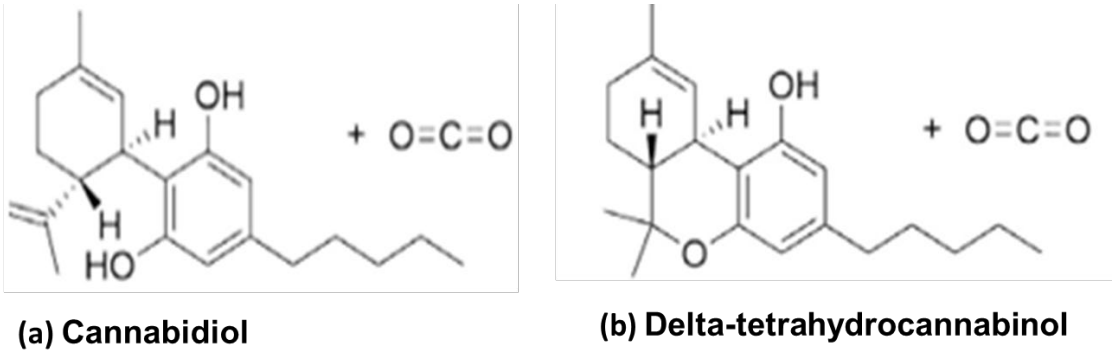
2.13. *Cannabis sativa* and its benefits

Cannabis sativa has been used to anecdotally treat anorexia, chronic pain, nausea, muscle spasticity and even issues relating to sleep. Previous studies have reported on the bioactivity of *C. sativa*, namely its AO and analgesic activities, its dietary significance, and its antimicrobial and anti-inflammatory activities (Datta et al., 2021). C. *sativa* has been reported to counter conditions such as diabetes, cancer, Parkinson's disease, nausea and vomiting. The cannabis plant has been used for its medicinal properties in indigenous communities worldwide (Rajput and Kumar, 2018).

2.14. Pharmacological benefits

The health benefits of *Cannabis sativa* are mainly due to its CBD and THC content and their ratios affects their pharmacological outcome when used in treating different diseases (Acquavia et al., 2023). THC is the main psychoactive compound in cannabis and It can induce sensations of euphoria (a feel-good state of mind and body), anxiety, and paranoia, but it also relieves nausea (used in anti-cancer treatments) and acts as an anti-inflammatory (Hurgobin et al., 2020). CBD (an isomer of THC) has an analgesic effect, and previous studies have

reported CBD as exhibiting neuroprotective, anti-cancer and anti-diabetic effects (Hurgobin et al., 2021). The THC and CBD levels are linked to their corresponding non-psychoactive carboxylated forms, tetrathydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), respectively (Acquavia et al., 2023). The figure below depicts the chemical structures of CBD and THC (Figure 2.3).



(a) Cannabidiol (b) Delta-tetrahydrocannabinol

Figure 2.3: Chemical structure of CBD and THC.

(Adapted from Malik et al., 2021).

As mentioned, cannabis extracts, also known as cannabis oils, have become increasingly popular among patients who self-medicate. Some patients have claimed cannabis oil as a cure for cancer (Romano and Hazekamp, 2013). In a previous study, CBD oil significantly reduced the incidence of diabetes in mice. This provides evidence that there is a great need for further studies that investigate the potential use of CBD to treat diabetes (Mattes et al., 2021).

As cannabis transitions from the black market to a legal market, there is an increased focus on product development and extraction methods. Traditional and modern methods for the processing of cannabis exist (Lazarjani et al., 2021). According to Lazarjani et al., (2021), the most convenient method for medicinal cannabis extraction uses organic solvents, although more research must be conducted on drying and other extraction methods. The use of home-based methods will allow larger populations of people to have access to cannabis extracts. However, developing green and sustainable cannabis extraction methods should be the focal point of future studies (Lazarjani et al., 2021).

2.15. The endocannabinoid system (ECS)

Growing evidence shows that the eECS) plays a pivotal role in various aspects of liver pathophysiology and controls glucose metabolism within the liver (Bazwinsky-Wutschke et al., 2019). Understanding how the ECS functions is important before understanding the effects of cannabinoids in the human body (Chye et al., 2021). The ECS consists of two main cannabinoid receptors which belong to the superfamily of G protein-coupled receptors,

cannabinoid receptor type 1 and 2 (CB1 and CB2) respectively (Chye et al., 2021). Endogenous cannabinoids function as naturally occurring ligands at the cannabinoid receptors. The participation of enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) facilitates their synthesis, uptake and metabolism, (Chye et al., 2021). It is widely accepted that both receptors can be found in the liver and that they play a role in a number of biological functions in different liver cell types including hepatocytes (Bazwinsky-Wutschke et al., 2019).

A study showed that daily expression of CB1 and CB2 messenger (mRNA) was observed in the liver tissue of young and middle-aged normoglycaemic Wistar rats and therefore demonstrated the potential link between food intake and the diurnal rhythm of cannabinoid receptors (Bazwinsky-Wutschke et al., 2019). In one study, the impact of cannabinoid receptors on insulin secretion and blood glucose recognition was investigated in knockout mice compared to wild-type mice. The study showed that glucose metabolism was impaired by the deletion of CB1 and CB2 in both knockout mouse lines (Zibolka et al., 2020). Bermudez-Silva et al., (2007), showed that CB2 receptor plays an important role in modulating glucose homeostasis, which suggests its therapeutic potential for the treatment of metabolic disorders such as diabetes (Bermudez-Silva et al., 2007).

2.15.1. Phytocannabinoids

Cannabis (genus: *Cannabaeae*) plants contain chemical compounds known as cannabinoids *(Cannabis sativa, Cannabis indica, and Cannabis ruderalis).* Some cannabis plants can contain more than 100 cannabinoids, however, all of these compounds lack full characterisation (Hossain et al., 2020). The first two cannabinoids characterised as being present in the flower of *Cannabis sativa* are Delta (Δ)-9-THC and cannabidiol (CBD) (Hossain et al., 2020). In a recent study, a novel phytocannabinoid, Δ9-tetrahydrocannabiphorol (Δ9-THCP), was isolated from *C. sativa.* The study showed a cannabimimetic activity that is seven times higher than its pentyl homolog THC. This finding opens a new perspective on the pharmacological effects that the cannabis plant exhibits, which are not limited to THC alone (Di Bartolomeo et al., 2021).

2.15.1.1. *Cannabis sativa* as an abundant source of phytocannabinoids

Phytocannabinoids are the best-known exogenous cannabinoids. Pharmacological effects have been observed for more than 180 phytocannabinoids isolated from *C. sativa* (Vacek et al., 2021). Analgesic, anti-inflammatory, and anti-cancer activities are a few of the pharmacological properties exhibited by THC and CBD (Isidore et al., 2021). Based on previous studies, CBD showcases no adverse effects associated with abuse, nor behavioural

changes due to prolonged use, memory loss or chronic intoxication in general (Vacek et al., 2021).

From the *C. sativa* plant, phytocannabinoids, which are also lipophilic phenolic terpenoids, have been isolated (Vacek et al., 2021). CBD is a non-psychoactive compound, but THC is known to have psychoactive effects (Hossain et al., 2020). Cannabis-plant-derived compounds may allow for new therapeutic approaches in the treatment of many diseases, including diabetes. In the future, cannabinoids may be used to target metabolic diseases (Charytoniuk et al., 2021). *Cannabis sativa* is used for the treatment of various medical conditions such as cancer-related symptoms, neuropathic, neurological, and HIV-related symptoms (Khoury et al., 2022). Cannabis has been known to act as an anecdote for reducing glucose levels and many reports on the medicinal use of cannabis by diabetic patients exist. Anecdotal reports on the stress and blood sugar levels in diabetic patients being reduced have been shared on the internet (Frisher et al., 2010). One study showed the therapeutic potential of CBD by reporting that CBD-treated adipocytes and preadipocytes promoted higher levels of glucose uptake, fat accumulation reduction and insulin resistance reversal (Zhang et al., 2023). A previous study showed that the effects of a cannabis extract to lean and obese rats displayed anticoagulant and antithrombotic activity. This is significant, due to the prothrombotic state of type 2 diabetes. Both the *in vitro* and *in vivo* studies showed that the cannabis extracts displayed anticoagulant activity via the inhibition of plasma clot formation (Coetzee et al., 2007).

There is a significant correlation between insulin resistance (IR) and T2DM, which are both metabolic disorders. According to (Charytoniuk et al., (2021), in both IR and T2DM, an increase in the concentration of sphingolipids is known to occur. Sphingolipids are also known to be a significant component in cell signalling pathways (Charytoniuk et al., 2021). Previous studies have reported that interferences in the sphingolipid pathway may have an impact on how IR develops (Charytoniuk et al., 2021). Charytoniuk et al., (2021) concluded that CBD influenced sphingolipid metabolism in insulin resistant brain tissue. In a previous study, the *in vitro* antioxidant activity of Finola hempseed oil (FHSO) was evaluated. The study concluded that the phenolic compounds might generally determine the significance of the antioxidant properties of FHSO (Smeriglio et al., 2016).

Most cannabinoids occur in nature as acidic precursors, of which Δ9-THCA, CBDA, cannabigerolic acid (CBGA) and cannabichromenic acid (CBCA) are the most abundant (Stefkov et al., 2022).

Cannabinoid acids are converted through the process of decarboxylation to generate their neutral counterparts; this process is induced by heat or age (Stefkov et al., 2022). Other

cannabinoids found in the cannabis plant include; cannabigerol (CBG, formed from CBGA), cannabichromene (CBC, formed from CBCA), cannabidivarin (CBDV, formed from cannabidivarin (CBDVA)), and tetrahydrocannabivarin (THCV, formed from tetrahydrocannabivarin (THCVA)) (Stefkov et al., 2022).

Phytocannabinoids have been linked to the ECS via receptor-dependent and receptor-independent mechanisms (Gojani et al., 2023). The management of diabetes can be improved by the secondary metabolites found in the cannabis plant (Suttithumsatid et al., 2022). Pre-clinical and clinical research provides evidence of CBD and THC being a potential alternative source for treating diabetes (Suttithumsatid et al., 2022).

CBD is not inclined to easily bind to CB1 and CB2 and is known to exhibit antioxidant and anti-inflammatory effects (Chan et al., 2021). CBD can be administered via oral ingestion, oromucosal spray, intravenous administration, and smoking. Previous studies investigated the pharmacologically relevant dosing in humans and concluded that effective dosage ranges from low nanomolar to, at most, a 2-3 μM range (Chan et al., 2021).

It is important to note that a synergistic relationship exists between cannabinoids, terpenes, and flavonoids. Terpenes modulate THC pharmacokinetics and ratios between terpenoids and phytocannabinoids significantly improve potential medical therapies (Nigro et al., 2021). THC partially initiates a response from CB1 and CB2, while CBD exhibits a higher affinity for CB2 than CB1 (Nigro et al., 2021). The THC/CBD ratio is related to the potency of the cannabis plant; in addition, pretreatment with CBD before treating with THC seems to have beneficial effects. According to one study, CBD prevented the transient psychotic symptoms of THC since it is known that CBD is non-psychoactive, whereas THC is psychoactive (Stasilowicz et al., 2021). Data that has been accumulated over the years, supports the therapeutic potential of cannabis integration and use in treating diseases. Cannabis-based products in clinical trials have shown the benefits and ease of symptoms when related to disease (Khoury et al., 2022).

2.16. Cannabis as a source of antioxidants

The scientific community has reported on the AO potential of ethanolic hemp root extracts. Kornpointner et al., 2021, reported on the antioxidant potential of *Cannabis sativa L.* roots using *in vitro* and *in vivo* methods. A total of 20 secondary metabolites were identified from ethanolic extracts and it was observed that the *in vitro* FRAP and TEAC assays of the ethanolic extracts showed a moderate AO activity. *Saccharomyces cerevisiae* was used as a model system for the determination of the cellular AO activity and the results were promising (Kornpointner et al., 2021). Vitorovic et al., (2021) reported that hemp seed oil had beneficial effects against H_2O_2-induced OS markers in *Drosophila melanogaster* larvae and contributed to the oil's antioxidant effects.

Rajesh et al., (2007), reported that CBD may attenuate HG-induced mitochondrial superoxide generation and increased NF-kB activation. Mitochondrial generation of superoxide may play the most vital role in complications associated with diabetes. HG-induced superoxide generation may lead to increased Insoluble nitric oxide synthase (iNOS) expression. This may be achieved through the activation of NF-kB, which in turn increases the production of nitric oxide (NO) (Rajesh et al., 2007). A clinical study showed that marijuana users had lower prevalence rates of obesity and DM in comparison to individuals who have never used marijuana. Lower levels of fasting insulin, a smaller waist size, glucose and high-density lipoprotein cholesterol (HDL-C) (Penner et al., 2013). It is suggested that the AO properties of cannabis are based on the molecular structure of cannabinoids such as CBD. The molecular structure of CBD can convert reactive species into weaker compounds. Thus, CBD may decrease the production of ROS and help regulate redox homeostasis (Jîtcă et al., 2023).

2.17. The liver

The liver plays an important role in maintaining energy homeostasis, by predominantly replenishing and storing energy reserves in glycogen, ensuring carbohydrate metabolism regulation, and neutralising and removing products from the body that are metabolic in nature (Arzumanian et al., 2021). Glucose metabolism and regulation are primarily carried out in the liver, and for this reason, the effects of hyperglycaemia can be studied in cultured liver cells (Chandrasekaran et al., 2010).

According to previous studies, exposing hepatocytes to high levels of glucose induces hepatocellular damage and therefore, results in excessive amounts of NADH via the glycolytic pathway (Bourebaba et al., 2021). Literature has shown that hepatic glucose uptake is impaired in patients with T2D, and contributes to glucose intolerance (Warner et al., 2020). Although the liver has a tremendous regenerative capacity to counter physical and chemical damage, adverse effects caused by drugs and chronic diseases such as diabetes, could impair physiological functions (Deng et al., 2019). Based on these facts, choosing a reliable liver model to investigate these adverse effects is important for developing new therapeutic drugs for liver diseases (Deng et al., 2019). Since hyperglycaemia is associated with most metabolic disorders such as type 2 diabetes, studying its effects is crucial. Exposure to high glucose levels for prolonged periods increases mitochondrial ROS production in cells, adversely affecting mitochondrial metabolism and cell signalling (Alnahdi et al., 2019). A major contributor to hyperglycaemia and organ damage associated with diabetes is increased gluconeogenesis. This increased gluconeogenesis takes place in the liver of patients with type 2 diabetes (Hatting et al., 2018).

The use of primary hepatocyte cells for *in vitro* studies is superior due to them resembling permanent cell lines. However, their availability is limited. Hepatic tumour cells are immortal unlike primary cell lines (Sefried et al., 2018).

2.17.1. Human hepatocarcinoma cells (HepG2)

There are currently 40 various hepatic tumour cell lines, however, the HepG2 cell line is the most widely studied and exhibits key characteristics of hepatocytes (Arzumanian et al., 2021). The physiological function of HepG2 cells is similar to that of lipid or glucose metabolism with normal functioning hepatic cells (Huang et al., 2007). Kamalian et al., (2015), found that the use of the HepG2 cell line to be valuable in the identification of mitochondrial toxins and in the assessment of ATP content and cytotoxicity, leading to a deeper understanding of the mechanisms involved in toxicity (Kamalian et al., 2015).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material and permit

Dried *Cannabis sativa* plant material was obtained from the South African Health Products Regulatory Authority (SAHPRA) licenced cannabis grower, Felbridge (https://felbridge.co.za/) in the Stellenbosch region of the Western Cape Province, South Africa. A permit was obtained by the supervisory team of this master's thesis from the Department of Health (DOH) to acquire, possess and use the dried cannabis plant material for analytical and research purposes. This permit expires on 30 July 2025 (permit number: POS 314/2024/2025, see Appendices 8.24 and 8.25 for the DOH permits for 2022-2023 and 2023-2024, respectively).

3.1.2. Chemicals

Folin Ciocalteu's phenol reagent, gallic acid (GA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), methanol, 2-thiobarbituric (TBA), glacial acetic acid, and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), sodium carbonate (Na_2CO_3), iron chloride hexahydrate ($FeCl_3$.6H2O). Sodium acetate, MDA, ethanol, hydrochloric acid (HCl), phosphoric acid (H_3PO_4), butylated hydroxytoluene (BHT), sodium hydroxide (NaOH), butanol, ethyl acetate, and dimethyl sulphoxide (DMSO), trypan blue dye, H_2O_2 were purchased from Merck (Johannesburg, South Africa). Chloroform (Kimix Chemical and Lab Supplies, South Africa).

Fetal bovine serum (FBS) and penicillin-streptomycin for the cell cultures were ordered from Thermo-Fisher (Johannesburg, ZA). Dulbecco's phosphate-buffered saline (PBS) was purchased from WhiteSci (Cape Town, ZA) and trypsin ethylenediaminetetraacetic acid (EDTA) from Lonza Group Ltd. (Verviers, Belgium). The Caspase -8 and -9 activity kits and the GSH-Glo™ Glutathione assay kit were obtained from Promega (Madison, USA). The LDH cytotoxicity detection kit was purchased from Roche (Germany), while the ATP CellTire Glo™ reagent was purchased from Sigma-Aldrich (South Africa).

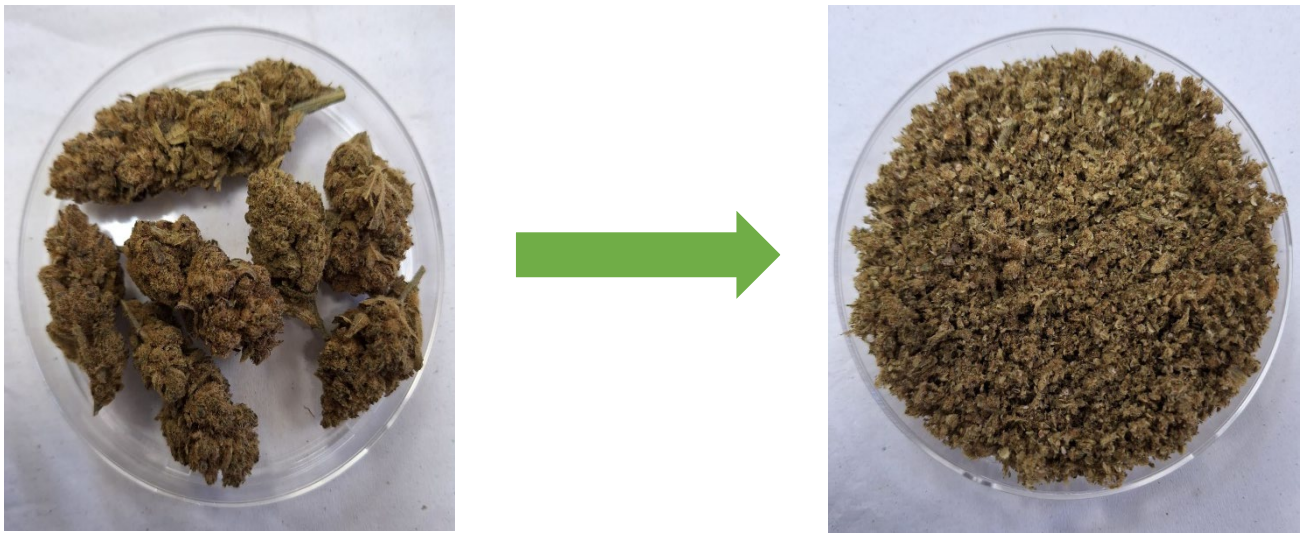
3.1.3. Consumables and cell line

Flasks, 96-well plates, 2 mL microcentrifuge tubes, 50 mL conical tubes, and 15 mL centrifuge tubes were all purchased from WhiteSci (Cape Town, South Africa). The HepG2 cell line was obtained from ATCC (American Type Culture Collection) while cell culture supplements were purchased from Thermo-Fisher. Lasec (Cape Town, ZA) supplied powder-free and nitrile-free examination gloves and reagent reservoirs (50 mL).

3.2. Methods

3.2.1. Plant material and extraction

Dried cannabis flower plant material (engineered to contain a low THC and a high CBD content) was ground into finer particles using a commercial blender before each extract preparation (Figure 3.1). Three different solvent extracts were prepared using the mentioned cannabis plant material. Factors such as solvent efficiency, cost, targeted compounds and potential future applications of the extracts were taken into consideration when comparing the different solvent extracts. Acetone and ethanol are known to be more potent than water, therefore a higher dilution of plant material to water was carried out to mimic the traditional preparation of this plant material, which is consumed by many people.



Unprocessed *Cannabis sativa* flower

Processed *Cannabis sativa* flower, prepared for extraction

Figure 3.1: Unprocessed and processed cannabis plant material

3.2.1.1. Aqueous extract

The method for the aqueous extract preparation was carried out according to Hazekamp et al., (2007). The use of a higher dilution of plant material to water was to intentionally mimic the traditional preparation of this plant material, consumed by many people. This allows us to understand the levels of compounds that individuals are typically exposed to (Chaachouaya et al., 2023 and Teske et al., 2002). One gram of dried plant material was extracted in 250 mL of freshly boiled distilled water, for 15 minutes, the extract was simmered with occasional stirring. The brew was then strained and filtered using Whatman 4 (185 mm) and Munktell Ahlstrom 3HW (240 mm) filter paper. The filtered extract was then placed in the freeze-dryer, (Virtis Genesis 25EL) resulting in a powdered extract and stored in the dark at -80°C until laboratory assays were done.

3.2.1.2. Acetone extract

The method for the acetone extract preparation was completed according to Teske et al., (2002) with slight modification. A higher plant material concentration was used for the acetone extract to potentially extract a broader range of less polar to moderately polar compounds, which may not be efficiently extracted with water alone. Five grams of dried plant material was extracted with 100 mL of acetone for 48 h in a dark environment, whereafter the mixture was strained and filtered using Watman 4 (185 mm) and Munktell Ahlstrom 3HW (240 mm) filter paper and placed in the rotary evaporator (Buch, Postfach, Switzerland) at 4°C to remove the acetone, yielding a tar-like liquid extract. The extract was then stored in the dark at -80°C until laboratory assays were done.

3.2.1.3. Ethanol extract

Extraction using ethanol was done according to the method published by Fischedick et al., (2010) with slight modification. Between the more concentrated acetone extract and the very dilute aqueous extract, ethanol, with its intermediate polarity, was tested at a ratio that aimed to strike a balance for the purposes of the study. One gram of dried plant material was added to 45 mL of 100% ethanol and shaken at 100 rpm for 60 minutes. The mixture was then strained and filtered using Whatman 4 (185 MM) and Munktell Ahlstrom (240 MM) 3HW filter paper and the supernatant collected. The same plant material was extracted twice with 25 mL of 100% (v/v) ethanol and the volume was adjusted to 100 mL. Thereafter, the filtered mixture was dried in a rotary evaporator (Buch, Postfach, Switzerland) at 4°C to remove the ethanol, yielding a sticky tar-like extract. The extract was then stored in the dark at -80°C until laboratory assays were done.

3.2.2. Quantification of the main cannabinoids

For new drug discovery, high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) is often used. This analytical technique is used for the analysis of newly synthesised compounds, that will become part of a compound library (Korfmacher, 2005). Liquid chromatography (LC) separates components of a sample which are analysed by the mass spectrometer MS. Charged ions are created and detected by the MS and the data generated may be used to provide information on the molecular weight, structure, identity. And quality of the specific components present in the sample. introduced to the MS, which creates and detects charged ions. The data generated may be used to provide information regarding the molecular weight, structure, identity, and quantity of specific components in the sample (Basics of LC/MS, A Primer) (NMISA, 2023). LC tandem MS (LC-MS) can analyse small volumes of sample, it is known to have fast analysis times, and improved specificity (Hawley and Keevil, 2016). The LC-MS method for identifying and analysing constituents in a sample is known to be highly sensitive (Rodrigues et al., 2013). LC-MS was used to analyse the major polyphenolic constituents of the acetone, ethanol, and aqueous extracts. LC-MS

was carried out on the extracts to extract, purify, and concentrate the analytes (cannabinoids) present in each extract.

The Shimadzu Nexera Series LC-40 coupled with the LCMS-9030 Q-TOF system was used to analyse the three extracts. The Shimadzu Nexera Series LC-40 couples the concepts of artificial intelligence (AI) and the internet of things (IoT). The Shimadzu LCMS-9030 Q-TOF system is a new high-resolution Q-TOF MS system and supports many ionisation sources (Jayanti et al., 2022). The Shimadzu Nexera Series LC-40 couples the concepts of artificial intelligence (AI) and the internet of things (IoT). The Shimadzu LCMS-9030 Q-TOF system is a new high-resolution Q-TOF MS system and supports many ionisation sources (Jayanti et al., 2022).

Quantification of the main cannabinoids present in the solvent extracts was done at the National Metrology Institute of South Africa (NMISA) in an accredited laboratory (See Appendix 8.1 to 8.20 for LC/MS cannabinoid chromatograms). The Shimadzu Nexera Series LC-40 coupled with the LCMS-9030 Q-TOF system with a Shim-pack Velox SP-C18 column (2.1 x 150 mm, 2.7 µm) was used to analyse the three solvent extracts. Using the identification of the cannabinoids isolated in the extracts was based on liquid chromatography retention time (RT min) and the analysis of high-resolution mass spectra (m/z) with standard compounds. The elements that make up an LC-MS system consists of an autosampler (loading the samples onto the HPLC), HPLC, ionisation (interface for LC to MS) and mass spectrometer (Figure 3.2).

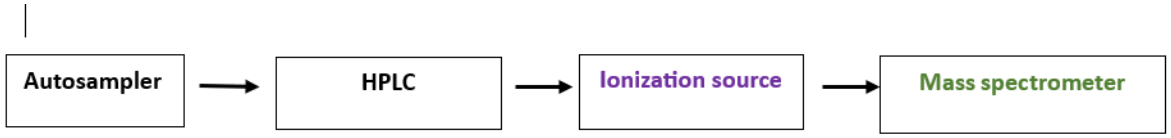


Figure 3.2: The elements of an LC-MS system

3.2.2.1. Assay

This method uses a Nexera LC-40 System coupled with a LCMS-9030 Q-TOF operated in positive ESI mode. Each sample (5 mg of each weighed out sample) was reconstituted in 12.5 mL of ethanol, the resultant concentration being 0.4 mg/mL, and thoroughly homogenised using a vortex. The reconstituted sample was filtered by passing a 1 mL aliquot through a 0.22 µm PFTE filter, followed by the addition of 9 mL of methanol to give a ten times dilution. The diluted sample was transferred into a 2 mL amber vial for liquid chromatography analysis. Prior to sample analysis, a calibration range containing 15 cannabinoids were prepared and run on the LC-qTOFMS system in MS/MS mode. The ions used for the quantification are noted in the results section. The calibration ranged from 0.025 to 5 µg/mL. It is to be noted that out of the 15 cannabinoids analysed, only the 12 that were positively identified were reported on. For

LC-qTOFMS injections, 5 µL of the samples and calibrations were injected on a Shim-pack Velox SP-C18 column (2.1 x 150 mm, 2.7 µm) at 40 °C. Chromatographic separation was performed using a gradient of mobile phase (A) water containing 0.1% (*v/v*) formic acid, and (B) acetonitrile containing 0.1% (*v/v*) formic acid. Internal mass calibration using clusters of NaI was performed. For data analysis, the LabSolutions software was used, and data processing was performed using LabSolutions Postrun and LabSolutions Browser (Shimadzu, Kyoto, Japan), and used in conjunction.

3.2.3. Extract screening for antioxidant content and capacity

3.2.3.1. Total phenolic content (TPC) assay

Phenolics are molecules that have a high potential to neutralise free radicals. The quantification of phenolic compounds (PC) is common practice when assessing its potential to protect against free radicals (Vuolo et al., 2019). The TPC of the three extracts were determined using the Folin-Ciocalteau (F-C) assay. This colourimetric method is based on electron transfer reactions between the F-C reagent and phenolics present in the extracts. The F-C reagent is reduced by the polyphenolic constituents in an alkaline medium. A blue-coloured complex is formed as a result (Figure 3.3), and it has a maximum absorbance of 765 nm. The absorbance is reported as GA equivalents and is directly proportional to the TPC (Malta and Liu, 2014).

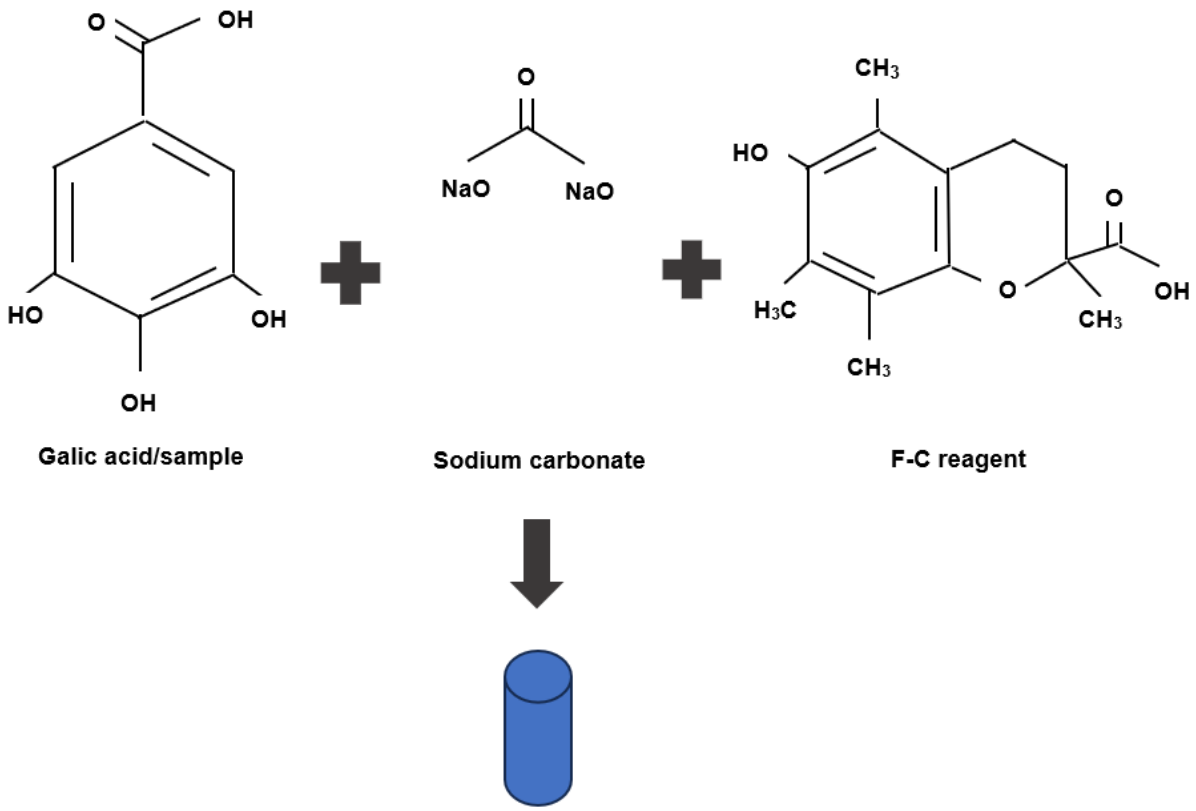


Figure 3.3: Principle of the total phenolic content assay

3.2.3.1.1. Assay

The TPC of the different *C. sativa* extracts was determined by the F-C spectrophotometric method of Singleton et al., (1999). Modifications as described by Ajuwon et al., (2013) were used, and all experiments were performed in triplicate. The TPC reagents were prepared (see Appendix 8.21 for the complete recipe for the TPC assay reagents). To a 96-well clear plate, 25 µL of *C. sativa* extract was added per well at concentrations of 0.250, 0.5, 0.750, 1, 1.5, and 2 mg/mL, followed by 125 µL of 0.2 M Folin-Ciocalteu reagent. The solutions were mixed with 100 µL of 7.5 % w/v Na_2CO_3 solution to create basic conditions (pH 10) for the redox reagent (F-C reagent), in order for the phenolic compounds present in the samples to be "oxidised". After a two-hour incubation period at room temperature (in a dark environment), absorbance was measured at 765 nm using a multiplate reader (SpectraMax i3X, San Jose, CA, USA). The GA standard curve involved a concentration range of 0 to 500 mg/L. The results were represented in GA equivalent (GAE) units per litre extract (mg/L).

3.2.3.2. 2, 2 – diphenyl – beta – picrylhydrazyl (DPPH) assay

The DPPH assay is used to measure the scavenging capacity of antioxidants, which can transfer electrons. DPPH is a chromophore, a stable radical cation with a purple colour (Figure 3.4). It shows the maximum absorbance at 517 nm. Antioxidant compounds with the capacity to donate an electron to the DPPH radical cation cause a decolouration of the solution when added to the DPPH radical. The spectrophotometer estimates the disappearance of colour during the reaction (Baliyan et al., 2022).

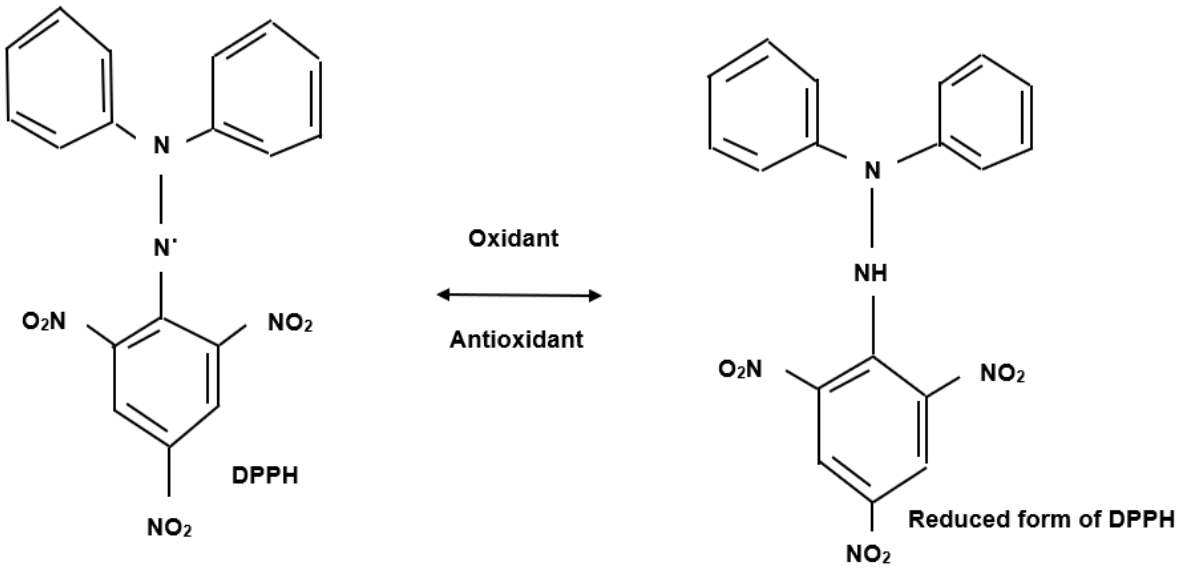


Figure 3.4: Principle of the DPPH assay

3.2.3.2.1. Assay

The assay was carried out according to the method of Xie and Schaich, (2014). A stock solution of DPPH radical cation was prepared: 0.0192g of DPPH (fridge) was weighed out in a 15 mL screw cap tube and 5 mL methanol (see Appendix 8.22 for the complete recipe for the DPPH assay reagents. This solution was mixed until dissolved (prepared fresh). A standard, trolox (6-hydrox-2,35,7,8-tetramethylchroman-2-carboxylic acid) was prepared: 0.0125g trolox was weighed in a 50 mL screw cap tube and 50 mL of ethanol was added. This solution was mixed (prepared fresh). The plate reader was set to read at 734 nm and the temperature set at 25°C. Trolox standards were prepared in six Eppendorf tubes and marked 1-6. The relevant amount of standard stock solution and diluents were added to each of these Eppendorf tubes. The trolox standard curve involved a concentration range of 0 to 500 mg/L.

Trolox standard wells: 25 µL of each standard was added per well in a clear 96-well plate. Sample wells: 25 µL of *C. sativa* extracts (0.250, 0.5, 0.750, 1, 1.5, and 2 mg/mL) were added in triplicate to the wells. The DPPH solution was diluted with ethanol to read absorbance of approximately 2 (±0.1), 275 µL of this DPPH solution was added to each well using a multichannel pipette. The plate was left for 30 minutes at room temperature before the reading was taken. The results were represented in Trolox equivalent (TEAC) units per litre extract (µmol TE/L).

3.2.3.3. Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay measures the antioxidants' reducing power to reduce ferric-tripyridyltriazine (Fe^{3+}) to ferrous tripyridyltriazine (Fe^{2+}) (Figure 3.5). Fe^{2+} can be observed as a blue product colour with an absorption maximum of 593 nm, and the change in absorbance is related to the antioxidant capacity (Njoya, 2021).

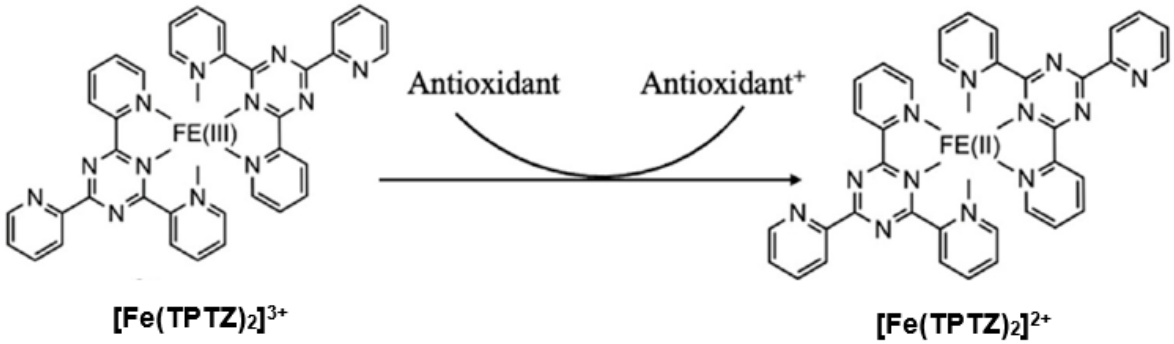


Figure 3.5: Principle of the FRAP assay

3.2.3.3.1. Assay

The FRAP assay was carried out according to the method described by Benzie and Strain, (1996) with slight modifications. To a 96-well plate, 10 µL of *C. sativa* extracts were added at concentrations 0.250, 0.5, 0.750, 1, 1.5, and 2 mg/mL. The ascorbic acid standard curve involved a concentration range of 0 to 500 mg/L. The ascorbic acid standard was mixed with 300 µL of FRAP reagent (10:1:1 v/v/v). The FRAP reagent consisted of acetate buffer at 300 mM, pH 3.6, TPTZ at 10 mM in 40 mM HCl, and $FeCl_3.6H_2O$ at 20 mM in a 96-well clear plate and incubated for 30 min at room temperature (see Appendix 8.22 for the complete recipe for the FRAP assay reagents). After incubation, the plate was read at 593 nm using a muliplate reader (SpectraMax i3X, San Jose, CA, USA). The results were expressed as micromole ascorbic acid equivalent per litre (µmol AAE/L).

3.2.4. Cell culture and exposure protocol

3.2.4.1. Cell culture

The HepG2 cell line is derived from a liver hepatocellular carcinoma of a 15-year-old Caucasian male and listed on the ATCC repository as a human cell line (HB 8065). These cells possess adherent properties, exhibit an epithelial-like morphology, are known to be non-tumorigenic and to have high proliferation rates. This cell line is used for investigating toxicity since the liver is the main detoxifying organ in the human body (Harjumäki et al., 2019). The cells were cultured in Roswell Park Memorial culture media (RPMI), consisting of reducing agent glutathione, Biotin, vitamin B12, vitamin PABA, high concentrations of inositol and

choline and supplemented with 10% FBS, 1% penstrepfungizone. Cultures were maintained at 37°C with 5% CO_2. Ethical clearance for the study was obtained from the Animal Research Ethics Committee (AREC) and the Faculty of Health and Wellness Sciences Research Ethics Committee (HWS-REC), with the reference number: 2022URI_NEG_001.

Cells were exposed to both a normoglycaemic (NG) and hyperglycaemic (HG) environment using media prepared as growth media for the HepG2 cells (Table 3.1). The cells were exposed to the NG and HG environment for 24 h.

Table 3.1: Recipe for the preparation of normoglycaemic (NG) and hyperglycaemic (HG) media

Reagents	Normoglycaemic media (5.5 mM)	Hyperglycaemic media (25 mM)
1 M Glucose	275 µL	1250 µL
5 mM HEPES	250 µL	250 µL
10% FBS	5000 µL	5000 µL
Sodium pyruvate	500 µL	None
1% Penstrep	2500 µL	2500 µL

3.2.5. Treatment of cells

Cells were allowed to reach a confluency of approximately 80-95% before exposing them to NG and HG conditions. The cells were treated with NG and HG media for 24 hours before the subsequent assays were performed on the cells.

3.2.6. Trypan blue method

The Trypan Blue (TB) method is often used to assess cell viability, and it is a dye exclusion method (Louis and Siegel, 2011). This method is based on the principle that the membranes of healthy cells remain intact, preventing the blue dye from entering the cell. TB will be allowed over the cell membrane in damaged or dead cells and cause those cells to be stained blue (Docrat, 2018). Numerous research articles have reported on the use of TB to quantify cell numbers and cell viability in culture (Piccini et al., 2017).

3.2.6.1. Assay

The HepG2 cells in culture were decanted from the flask and washed with PBS to rinse out old media and remove contaminants and dead cells. 1 mL of trypsin was added, and this cell and trypsin mixture was allowed to stand for 30 s to 1 min. 4 mL of RPMI media was added using a pipette and the cells were then centrifuged for 2 min at 1000 xg. The cells were then resuspended in 1 mL RPMI media. Into an Eppendorf tube 150 µL of RPMI media, 50 µL of TB dye, and 50 µL of cell suspension were back pipetted and 10 µL of this solution was added to a Neubauer haemocytometer consisting of a Neubauer grid with five quadrants. The

Neubauer haemocytometer was placed under the microscope and the stained viable cells in each quadrant were counted. The following formula (Equation 1) was used to calculate the volume of the cell solution needed to use in the subsequent assay:

$$Total\frac{cells}{mL} = \frac{Total\ cells\ counted\ \times Dilution\ factor\ \times 10\ 000\frac{cells}{mL}}{Number\ of\ squares\ counted}$$

Equation 1: Formula for haemocytometer cell counting

Thereafter, 200 µL of the final cell solution was added to each well for each treatment. Approximately 20 000 cells were added to each well and allowed to attach overnight.

3.2.7. Cell viability

3.2.7.1. The [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) assay

The MTT assay is based on the ability of the mitochondria of metabolically active cells to reduce the methylthiazol tetrazolium yellow water-soluble dye to its insoluble purple formazan (Figure 3.6). Colour change is only observed when the cell is viable and mitochondrial reductase enzymes are present (Abdul and Chuturgoon, 2016). The reaction that takes place in this assay only occurs in metabolically active cells. This assay measures the metabolic activity in the cell and the intensity of the formazan product and is directly proportional to the metabolic activity and cell viability (Abdul and Chuturgoon, 2016; Docrat et al., 2018).

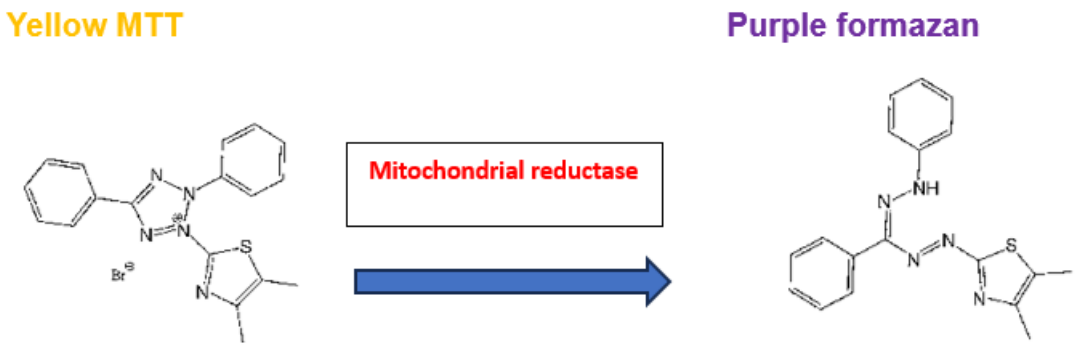


Figure 3.6: Principle of the MTT assay

3.2.7.1.1. Assay

The method was performed according to Docrat et al., (2018), with a few adjustments. Approximately 20 000 cells/well were seeded into 96 well microtitre plates. Cells were seeded in triplicate and allowed to attach overnight. Thereafter, cells were treated with varying concentrations of the different *Cannabis sativa* extracts (0 µg/mL – 1000 µg/mL) under normoglycaemic and (5.5 mM) and hyperglycaemic (25 mM) conditions at 37°C for 24 hrs in triplicate. After treatment, the cells were rinsed with 0.1M PBS and incubated (37°C, 2 hrs)

with 20 µL MTT salt solution (5 mg/mL in 0.1 PBS) and 100 µL RPMI (NG and HG). The supernatants were then removed and 100 µL of DMSO per well was added to solubilise the formazan product (15 mins; 37°C). The optional density of the formazan product was read using a spectrophotometer (SpectraMax 13X, San Jose, CA, USA) at 570 nm with a reference wavelength of 690 nm. The results were expressed as percentage cell viability (Equation 2), from which the IC_{50} (50% inhibitory concentration) was determined.

$$\%\ Cell\ viability = (Individual\ OD\ treated\ cells\ \div average\ OD\ control\ cells\ ) \times 100$$

Equation 2: Percentage cell viability determination equation

3.2.8. Intracellular adenosine triphosphate (ATP)

3.2.8.1. The ATP assay

The ATP assay is based on detecting illuminating light produced by the oxidative decarboxylation of luciferin in the presence of ATP, magnesium ion, and luciferase enzyme. Luciferase enzyme catalyses the change of luciferase to oxyluciferin, producing light (Figure 3.7). The emitted light has an intensity proportional to ATP present in the HepG2 cells and increased ATP levels degrade more luciferin. The CellTire Glo® kit (Promega) is based on bioluminescence was used to determine the ATP concentration (Docrat et al., 2018).

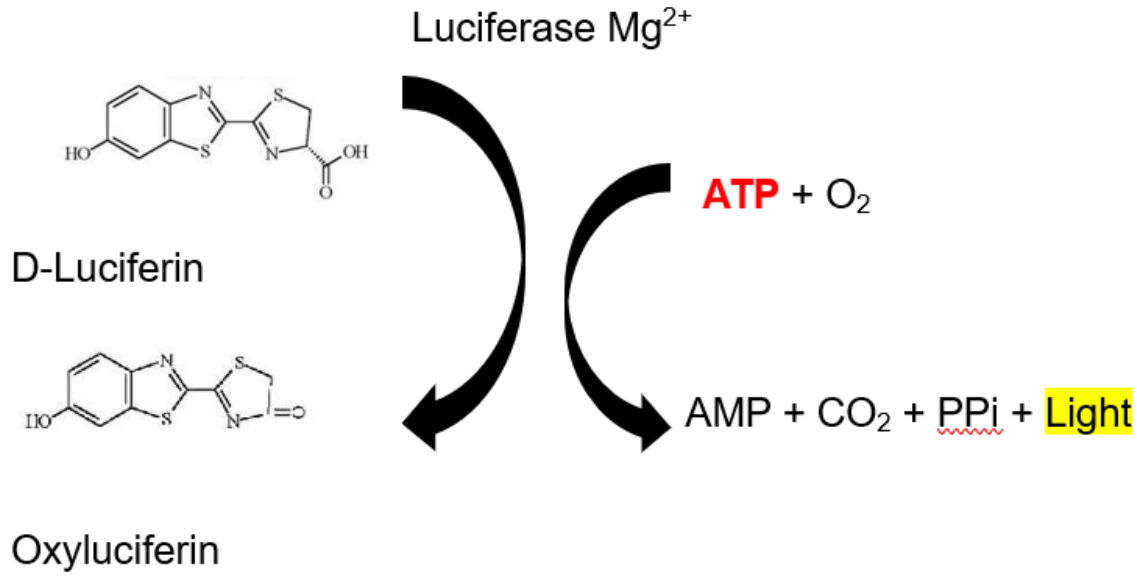


Figure 3.7: The luciferase reaction involving the mono-oxygenation of luciferase

3.2.8.1.1. Assay

ATP luminescence was detected using the Cell Titer-Glo® kit, with slight modifications, as previously described by Docrat et al., (2018). Approximately 20 000 cells/well were seeded using a white microtitre plate in triplicate. Thereafter, cells were treated with varying

concentrations of the three *Cannabis sativa* solvent extracts (µg/mL) under NG and HG conditions at 37°C for 24 h in triplicate. To each well, 50 µL PBS and ATP CellTire Glo™ reagent (Promega, Madison, USA) were added and incubated in the dark for 30 min at room temperature, for the reaction to occur. The luminescent signal was read using the SpectraMax i3X (Molecular Devices. Sunnyvale, USA). The signal is directly proportional to intracellular ATP concentration. The results obtained were expressed as mean relative light units (RLU).

3.2.9. Redox status

3.2.9.1. Reduced glutathione (GSH) assay

Glutathione is an important intracellular antioxidant, present in two forms: the reduced sulphydryl form GSH and the oxidised form, i.e. GSSG, (Dwivedi et al., 2020). Oxidative stress can decrease the body's GSH/GSSG ratio. Through a cascade of detoxification mechanisms, GSH can neutralise ROS (Lushchak, 2012; Sekhar et al., 2015).

The GSH-Glo™ test was used to determine GSH concentration and is dependent on a luciferin derivative being converted into luciferin in the presence of glutathione. Glutathione S-transferase (GST) acts as a catalyst and the signal produced in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample (Figure 3.8) (www.promega.com/protocols/).

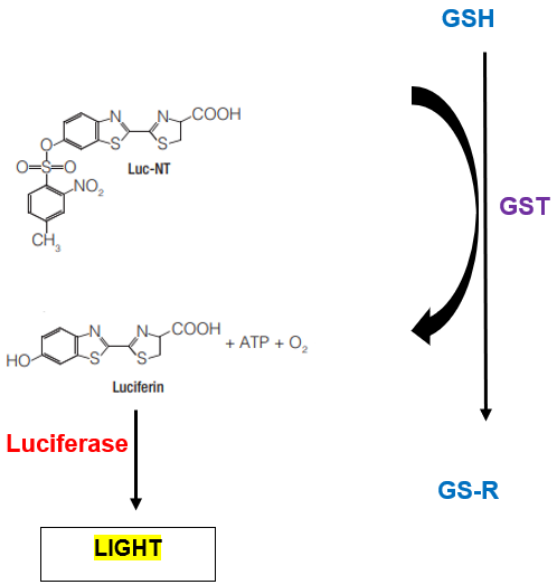


Figure 3.8: An overview of the GSH-Glo Glutathione principle

3.2.9.1.1. Assay

Into a white luminometric plate, 2.0×10^4 cells were seeded in triplicate. The cells were allowed to adhere to the bottom of each well overnight. The *C. sativa* extract concentrations (acetone: 20, 55, and 100 µg/mL ethanolic: 20, 35, and 50 µg/mL, and aqueous: 200, 370, and 600

µg/mL) were pipetted directly into the appropriate wells after the culture fluid was aspirated (24 h C. *sativa* treatment). Following the *C. sativa* and glucose treatment, the wells were then washed with 100 µL of 0.1 M PBS. The GSH-Glo™ assay reagents were prepared as directed by the manufacturer. Prepared GSH standard solution (glutathione, 5 mM) of known concentration (0-50 µM) was pipetted in triplicate (10 µL), which generated a standard curve. Into each well, 100 µL of the GSH-Glo™ test reagent was added and agitated for 30 seconds. The plate was incubated at room temperature in a dark environment for 30 minutes. The Luciferin Detection Reagent (100 µL) was then added, followed by a 15-minute incubation in a dark environment at room temperature. Luminometric emission was measured with a multiplate reader (SpectraMax i3X, San Jose, CA, USA). The data was presented as concentration (µM), which was determined using the standard curve.

3.2.10. Oxidative stress-status

3.2.10.1. Lipid peroxidation assay

Free radicals initiate lipid peroxidation and MDA is one of the end products of lipid peroxdiases and serve as a marker of lipid peroxidation. It is a reactive aldehyde that can form a complex with TBA and this complex, TBARS can be detected using spectrophotometry (Mas-Bargues et al., 2021).

The process is known as lipid peroxidation when lipids deteriorate due to oxidative stress. Lipids form part of the membranes of many cellular membranes and organelles, and therefore, damage caused by peroxidation can prove to be devastating to the function and survival of the cell. The TBARS assay is based on the quantification of MDA, which is a marker of oxidative stress. MDA reacts with TBA at a high temperature and low pH, this results in a pink chromagen being formed (Figure 3.9) (Ghani et al., 2017).

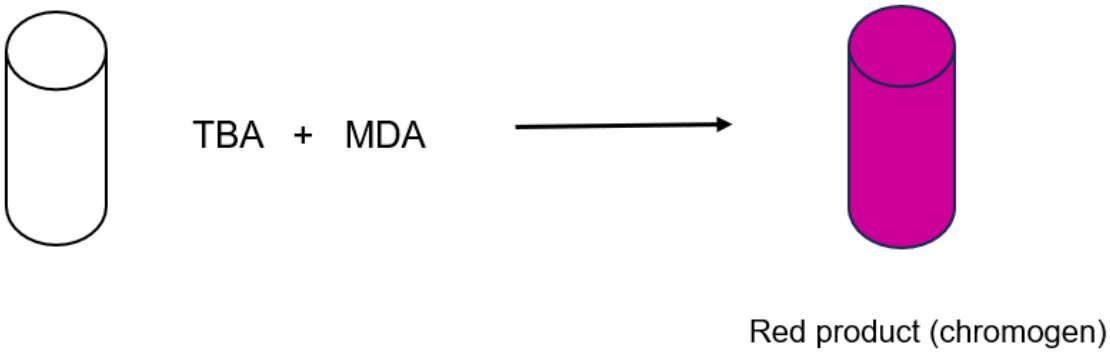


Figure 3.9: Principle of the TBARS assay

3.2.10.1.1. Assay

The TBARS assay was performed as described by Docrat et al., (2018). The reagents for the TBARS assay were prepared (see Appendix 8.23 for the complete recipe of the TBARS reagents. In a labelled glass test tube, 200 µL of 2% H_3PO_4 and supernatants (following treatment of cells with C. sativa extract and glucose treatment) were mixed. To each glass tube, 400 µL of TBA/BHT solution and 200 µL 7% H_3PO_4 was added. To remove background absorbance, 400 µL of 3 mM HCl was added to the blank sample tube, which acted as a negative control. MDA (1 µL) was added to the positive control test tube, thereafter, samples were vortexed and incubated at 100 °C for 15 minutes to facilitate MDA adduct hydrolysis before being cooled down at room temperature. The assay's sensitivity was enhanced by adding butanol (1500 µL), which precipitates the MDA-TBA adduct after cooling. After vortexing for 30 seconds, the samples were allowed to settle until two distinct phases were observed. The upper butanol layer (100 µL) was then aliquoted in triplicate into a 96-well clear microtitre plate and measured at 532 nm using a multiplate reader (SpectraMax i3X, San Jose, CA, USA) with a reference wavelength of 600 nm. The data (n=3) was expressed as MDA concentrations (mM), which were calculated using the equation below (Equation 3).

$$MDA\ concentration = [(individual\ OD\ of\ the\ sample - average\ OD\ blank) \div 156] \times 1000$$

Equation 3: Calculation to determine MDA concentration (in mM) and 156 is known as the extinction coefficient

3.2.11. Cytotoxicity

3.2.11.1. Lactate dehydrogenase (LDH) assay

The LDH assay (Roche CAT: 11644793001) was used to evaluate cytotoxicity. LDH is an enzyme that plays a role in most cells in the body. LDH is mainly found in the cytoplasm of the cells and becomes extracellular when the cell dies. Through oxidation, LDH is involved in catalysing pyruvate into lactate, and LDH catalyses pyruvate into lactic acid via the process of glycolysis (Figure 3.10) (Parhamifar et al., 2013).

The LDH assay is used to determine cellular toxicity. The LDH assay is based on the quantification of enzyme activity. Using the LDH assay, the LDH released from dead or severely damaged cells is assessed (Parhamifar et al., 2013).

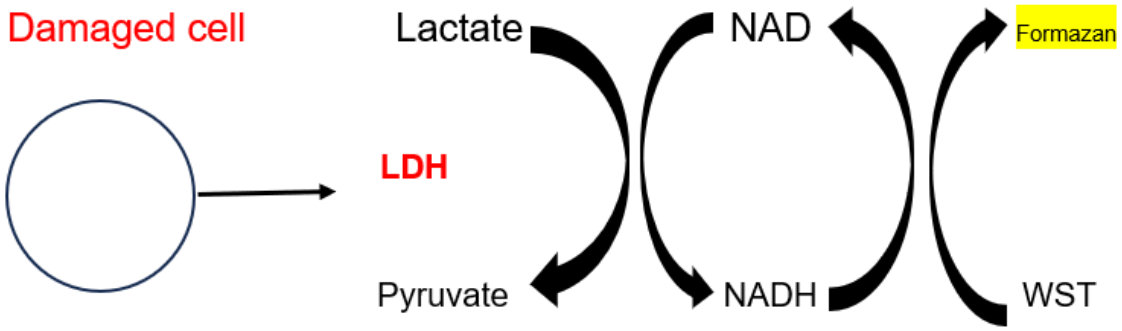


Figure 3.10: Principle of the LDH cytotoxicity assay

3.2.11.1.1. Assay

The protocol as described by Docrat et al., (2018) was followed with minor modifications. To a 96-well microtiter plate, 100 µL of supernatant (following the *C. sativa* extracts and glucose media) reaction mixture containing diaphorase/NAD^+ catalyst and a sodium lactate/tetrazolium salt was pipetted into each well and incubated at room temperature for 30 minutes and then read at 500 nm using a multiplate reader (SpectraMax i3X, San Jose, CA, USA). The results is expressed as the mean OD.

3.2.12. Apoptosis analysis

3.2.12.1. Caspase activity

The Caspase Glo -8, and -9 kits provided by Promega (Madison, USA) can be used to measure these different caspases. These assays are homogenous luminescent assays. Caspases -8 and -9 are initiator caspases and play a vital role in the intrinsic apoptotic pathway. The caspases provide a substrate and have been optimised for cell lysis, caspase and luciferase activity. The substrate is cleaved by the caspases and a luminescent signal is formed as a result of the luciferase reaction (Figure 3.11). Caspase activity and the luminescent signal are proportional to each other (Docrat et al., 2018).

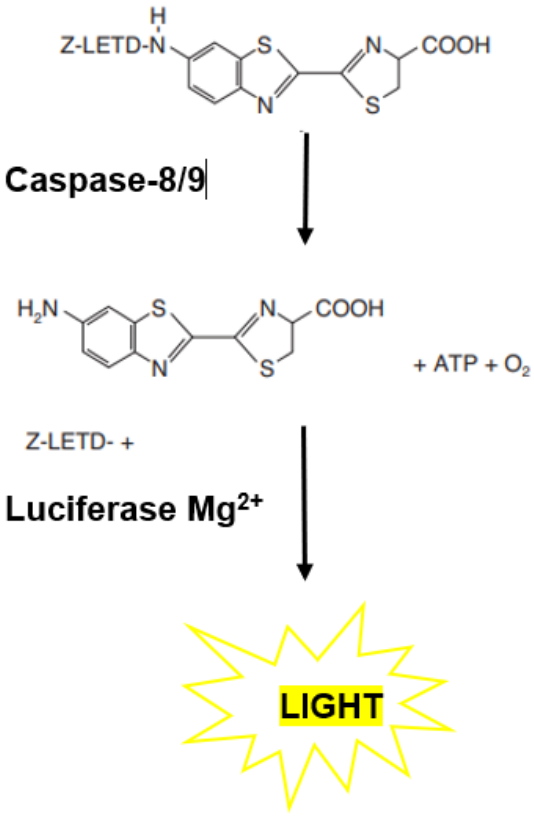


Figure 3.11: Caspase -8 and -9 cleavage of the luminogenic substrate containing the LEHD sequence

3.2.12.1.1. Assay

The Caspase Glo -8, and -9 kits provided by Promega (Madison, USA) was used to measure caspase activity. Cells were seeded into a 96-well white walled cell culture plate, at a density of 2.0 x 10^4 cells per well. The cells were pre-treated in triplicate with the different concentrations of *C. sativa* extracts and glucose media treatment for 24 h (acetone: 20, 55, and 100 µg/mL ethanolic: 20, 35, and 50 µg/mL, and aqueous: 200, 370, and 600 µg/mL). After incubation, the supernatants were aspirated and discarded. Caspase activities were determined with slight changes using the protocol given by Naidoo et al., (2017). The wells were rinsed with 100 µL PBS (0.1 M) to eliminate any residual media. Each well was treated with 25 µL of Caspase Glo® reagents (Caspases -8 and -9) and 25 µL of 0.1 M PBS before being incubated at room temperature for 30 minutes in a dark environment to allow cell lysis and the enzyme luciferase response. A multiwell plate reader (SpectraMax i3X, San Jose, CA. USA) was used to correlate the luminescent signal with caspase activity. Caspases -8 and -9 concentrations were expressed in RLU.

3.2.13. Statistical analyses

Each experiment was performed in triplicate (n=3, biological replicates). Results were presented as the mean ± standard deviation (SD). The differences among three or more

groups were analysed by one-way analysis of variance (ANOVA). Results were considered statistically significant at $p<0.05$. The statistic package GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California, U.S.A. www.graphpad.com) was used.

CHAPTER FOUR: RESULTS

4.1. Analysis of sample extracts by LC-qTOFMS

The LC-MS profile of the *C. sativa* extracts revealed the presence of 12 positively identified cannabinoids. The number of compounds isolated and identified from the aqueous was eight in total. CBDA was the most abundant cannabinoid detected in the aqueous extracts and CBD was the cannabinoid present in the second highest concentration. Cannabinol (CBN), CBC, THCA and CBCA were not detected in the aqueous extract. The acetone and ethanol extracts were shown to have the highest concentrations of cannabinoids (Table 4.1).

Table 4.1: A list of the cannabinoids quantified using LC-qTOFMS analysis.

Cannabinoid	Concentration (µg/g)		
	Acetone	**Ethanol**	**Aqueous**
CBDVA	1.0×10^3	8.0×10^2 ***	8.3×10^2 ***, ###
CBDV	3.4×10^2	3.6×10^2 ***	2.1×10^2 ***, ###
CBDA	**1.4×10^5**	**1.4×10^5 *** **	**8.9×10^4 ***, ###**
CBGA	3.6×10^3	2.8×10^3 ***	1.0×10^3 ***, ###
CBG	2.8×10^3	2.5×10^3 ***	1.3×10^2 ***, ###
CBD	**4.6×10^4**	**4.9×10^4 *** **	**2.6×10^3 ***, ###**
CBN	5.5×10^2	6.0×10^2 ***	n.d***, ###
THC	**5.8×10^3**	**4.8×10^3*** **	**2.0×10^2 ***, ###**
CBNA	7.0×10^2	4.6×10^2 ***	7.1×10^0 ***, ###
CBC	4.3×10^3	1.9×10^3***	n.d. ***, ###
THCA	2.1×10^3	1.0×10^3 ***	n.d. ***, ###
CBCA	4.6×10^3	4.9×10^3 ***	n.d. ***, ###

*The concentration of the cannabinoids isolated from the acetone, ethanol and aqueous extracts when compared to each other. Results are expressed as µg/g, mean and SD of triplicate evaluations (n=3). For significant difference acetone vs ethanol and aqueous ***p<0.0001. For significant difference ethanol vs aqueous ### p<0.0001, n.d=not detected. CBDVA: cannabidivarinic acid, CBDV: cannabidivarin, CBDA: cannabidiolic acid, CBGA: cannabigerol acid, CBG: cannabigerol, CBD: cannabidiol, CBN: cannabinol, THC: tetrahydrocannabinol, CBNA: cannabbinolic acid, CBC: cannabichromene, THCA: tetrahydrocannabinolic acid, CBCA: cannabichromenic acid.*

4.2. Antioxidant capacity of cannabis extracts

4.2.1. Total phenolic content

The total phenolic content of the cannabis extracts was measured using the F-C assay and expressed as gallic acid equivalents in acetone, ethanol and aqueous solvents. The results indicated a dose-dependent increase in total phenolic content for all three extracts (Table 4.2). At the concentrations of 250 - 750 µg/mL, the aqueous extract showed the highest ($p < 0.05$) total phenolic content in comparison to the acetone and ethanol extracts. The acetone extracts showed a lower total phenolic content at concentrations 500 and 750 µg/mL when compared to the ethanolic and aqueous extracts.

Table 4.2: Total phenolic content of the *Cannabis sativa* extracts in comparison to the extraction method.

Concentration (µg/mL)	Acetone (mg GAE/L) Mean (SD)	Ethanol (mg GAE/L) Mean (SD)	Aqueous (mg GAE/L) Mean (SD)
250	19.3 (0.3)	1.2 (0.1) ***	114.1 (0) ***, ###
500	54.2 (3.1)	126.4 (1.5) ***	147.5 (4.8) ***, ###
750	92.6 (4.5)	139.8 (1.0) ***	156.0 (1.2) ***, ns
1000	160.5 (9.9)	144.6 (5.4) ns	229.2 (7.3) ns, ns

*Total phenolic content of the acetone, ethanol, and aqueous Cannabis extracts compared to each other. Results are expressed as gallic acid equivalents (mg GAE/L), mean and SD of triplicate evaluations (n=3). Total phenolic content increased in a dose-dependent trend. For significant difference, acetone vs ethanol and aqueous is represented as ***p<0.0001 and ns p>0.05. For significant difference, ethanol vs aqueous is represented as ### p<0.0001 and ns p>0.05.*

At 250, 500, and 750 µg/mL, the aqueous extracts had a significantly ($p<0.05$) higher total phenolic content when compared to both the acetone and ethanolic extracts. However, when comparing the ethanol and aqueous extracts at 750 µg/mL, no statistical significance was observed in the total phenolic content. Similarly, the same trend was observed when comparing all three extracts at 1000 µg/mL.

4.2.2. FRAP activity

The ferric-reducing power of the acetone, ethanol and aqueous cannabis extracts was measured and expressed as ascorbic acid equivalents (AAE). All three extracts indicated a dose-dependent increase in antioxidant capacity measured as FRAP (Table 4.3). The aqueous extract had the lowest antioxidant capacity at concentrations of 250 - 1000 µg/mL in comparison to the acetone and ethanol extracts. The acetone extract was shown to have the lower antioxidant capacity at 500 and 750 µg/mL in comparison to the ethanol extract. At the concentration of 1000 µg/mL, the ethanol extract showed a higher antioxidant capacity in comparison to the acetone extract.

The ethanol extract showed to have the highest ferric reducing ability compared to the acetone and aqueous extracts ($p<0.05$). Whereas at 250 µg/mL, when compared to the acetone extract, the ethanol extract had a lower antioxidant capacity, ($p<0.05$). However, when comparing the acetone and aqueous extracts at 750 µg/mL, no statistical significance was observed in the antioxidant capacity. Similarly, this trend was observed when comparing all three extracts at 1000 µg/mL.

Table 4.3: Ferric reducing antioxidant power of the *Cannabis sativa* extracts in comparison to extraction methods.

Concentration (µg/mL)	**Acetone (µmol AAE/L) Mean (SD)**	**Ethanol (µmol AAE/L) Mean (SD)**	**Aqueous (µmol AAE/L) Mean (SD)**
250	50.1 (2.9)	40.6 (1.5) *	12.7 (3.9) ***, ###
500	69.4 (1.8)	101.1 (3.3) ***	45.3 (2.4) ***, ###
750	112.0 (2.2)	140.7 (3.4) ***	110.3 (3.1) ns, ###
1000	156.8 (3.1)	146.3 (0.9) ns	144.8 (7.3) ns, ns

*The ferric-reducing antioxidant power of the acetone, ethanol, and aqueous Cannabis extracts compared to each other. Results are expressed as ascorbic acid equivalents (µmol AAE/L), mean and SD of triplicate evaluations (n=3). Ferric-reducing antioxidant power increased in a dose-dependent trend. For significant difference acetone vs ethanol and aqueous *p<0.05, ***p<0.0001 and ns p>0.05. For significant difference ethanol vs aqueous ### p<0.0001 and ns p>0.05.*

4.2.3. DPPH capacity

The scavenging capacity of the cannabis extracts was measured and expressed as Trolox equivalents in the acetone, ethanol, and aqueous extracts. A dose-dependent increase in antioxidant capacity was observed for all three extracts (Table 4.4). Aqueous extract showed a higher antioxidant capacity when compared to the acetone and ethanol extracts at the concentration range 500 – 1000 µg/mL. The ethanol extract had a lower ($p<0.05$) antioxidant capacity when compared to both the acetone and aqueous extracts at the concentration range 250 – 1000 µg/mL.

Table 4.4: Radical scavenging activity of *Cannabis sativa* extracts in comparison to extraction methods.

Concentration (µg/mL)	**Acetone (µmol TE/L) Mean (SD)**	**Ethanol (µmol TE/L) Mean (SD)**	**Aqueous (µmol TE/L) Mean (SD)**
250	294.8 (15.4)	167.8 (7.0) ^{ns}	282.1 (50) $^{ns, ns}$
500	330.5 (4.9)	210.7 (5.2) ***	366.2 (11.8) $^{**, ###}$
750	374.4 (4.5)	301.1 (5.2) ***	428.3 (0.8) $^{***, ###}$
1000	384.7 (4.5)	340.7 (2.6) ***	454.3 (6.9) $^{***, ###}$

*The radical scavenging activity of the acetone, ethanol, and aqueous Cannabis extracts compared to each other. Results are expressed as Trolox equivalents (µmol TEAC/L), mean and SD of triplicate evaluations (n=3). Ferric-reducing antioxidant power increased in a dose-dependent trend. For significant difference acetone vs ethanol and aqueous **p<0.001, ***p<0.0001, and ns p>0.05. For significant difference ethanol vs aqueous $^{###}$ p<0.0001 and ns p>0.05.*

Based on the results indicated, the aqueous extract was shown to display the highest radical scavenging activity ($p<0.05$) in comparison to the acetone and ethanol extracts; at concentration of 250 µg/mL, no statistical significance was observed for all three extracts. Whereas at 250 µg/mL, when compared to the acetone extract, the ethanol extract had a lower antioxidant capacity ($p<0.05$).

4.3. Assessment of intracellular energy and metabolic status

The cell viability and intracellular ATP levels of the HepG2 cells treated with the various cannabis extracts were assessed via the MTT and ATP assays respectively.

4.3.1. Cell viability

The MTT assay is commonly used to evaluate cytotoxicity and cell viability as a measure of metabolic activity (Stockert et al., 2012). Figure 4.1 shows the results determined using the MTT assay after 24 hr treatment with increasing extract concentrations (µg/mL) in HepG2 cells cultured under NG (normoglycaemic) and HG (hyperglycaemic) conditions. The following dose range 10, 20, 50, 100, 150, 200, 400, 600, 800, and 1000 µg/mL was used for assessing the cell viability. Upon incubation of the cells treated with the extracts under NG and HG conditions, the results show an increase in cell viability at the lower tested concentrations when compared to the negative (NG) and positive (HG) controls respectively. At the higher tested concentrations, toxicity is observed, by a decrease in cell viability. Based on the IC_{50} (half maximal inhibitory concentration: in which 50% of the cells are killed upon treatment with extracts under HG conditions, after 24 hrs), the following concentration range was selected for the extracts: acetone - 20, 55, 100 µg/mL, ethanol – 20, 35, 50 µg/mL, and aqueous – 200, 370, 600 µg/mL for subsequent experimental evaluation.

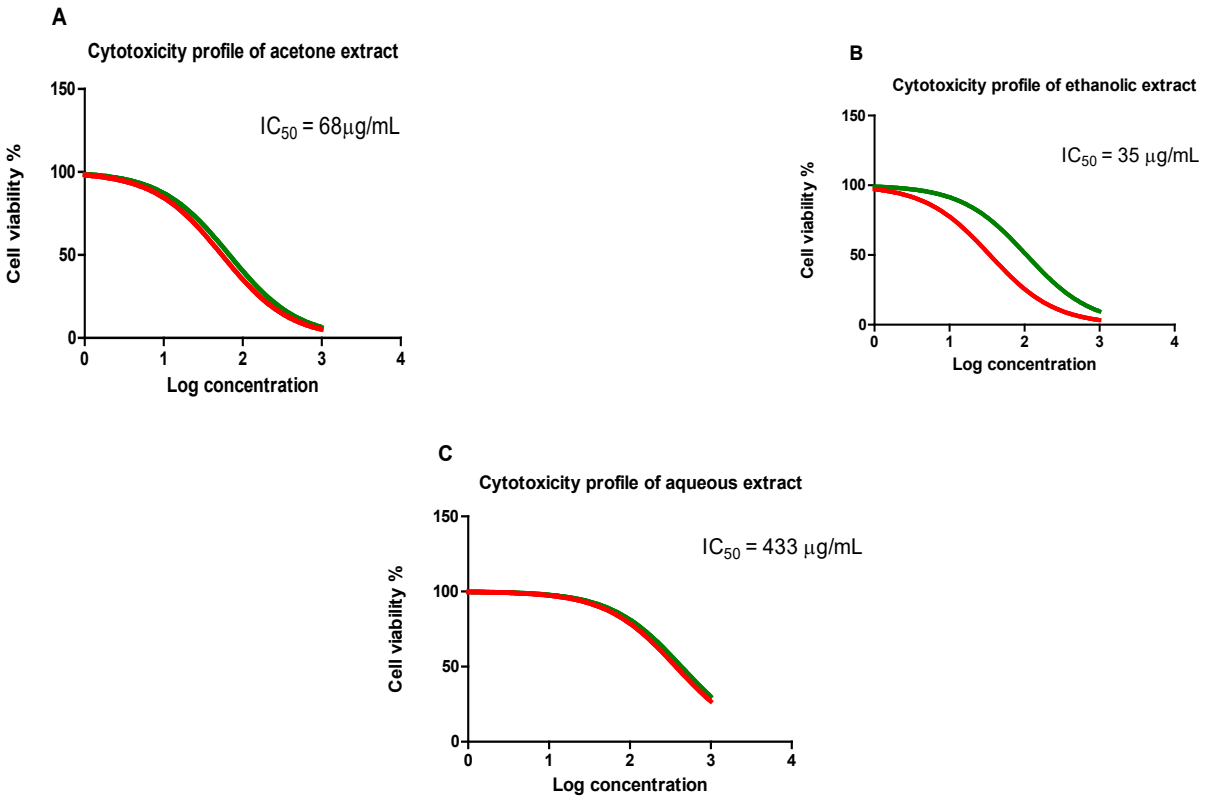


Figure 4.1: MTT assay demonstrating a dose-dependent decline in cell viability (red line: NG conditions, green line: HG conditions) following treatment with varying concentrations of *Cannabis sativa* extracts

Cannabis sativa extract concentration range used in the MTT assay: 0, 10, 20, 50, 150, 200, 400, 600, 800, and 1000 µg/mL.

4.3.2. Intracellular ATP levels

The ATP assay is one of the common methods for detecting cell viability (Adan et al., 2016). Upon exposure of the cells to NG and HG media only (negative and positive control respectively) for 24 hrs, ATP levels were significantly ($p<0.0001$) increased in cells treated only with HG media when compared to the NG media (Figure 4.6). Exposure of the cells to the extracts under HG conditions significantly decreased the ATP output with increasing concentration when compared to the positive control (HG). The higher concentrations of each extract were toxic to the cells after 24 hrs of treatment. For the cells treated with the aqueous extract under HG conditions, ATP levels were significantly elevated at 200 µg/mL ($p< 0.0001$) (HG control) (Figure 4.2 C), indicating the treatment with the extract was protective against the HG conditions. For both the acetone (Figure 4.2 A) and ethanol (Figure 4.2 B) extracts, cells treated with 20 µg/mL, showed results of no statistical significance, including cells treated with ethanolic extract at 35 µg/mL. However, for the acetone extract, at concentrations 55 µg/mL, ATP outputs significantly ($p<0.0001$) decreased when compared to the positive control (HG control). For the ethanol extract, at concentration 50 µg/mL, ATP levels significantly ($p<0.0001$) decreased when compared to the positive control. For the aqueous extract, at concentrations 370 µg/mL ($p<0.0001$), ATP levels were significantly decreased in comparison to the positive control.

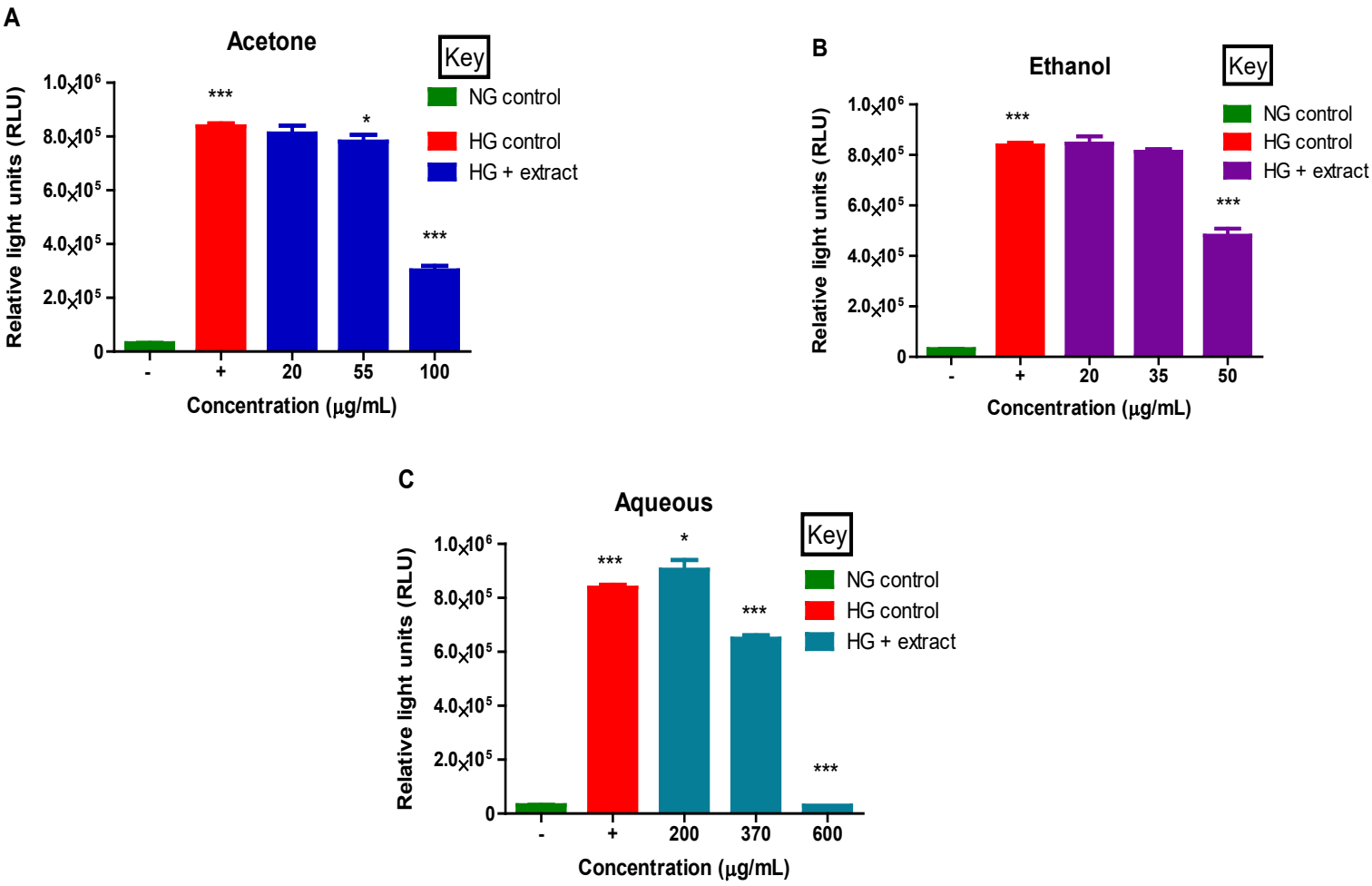


Figure 4.2: ATP assay demonstrating a decline at given concentrations under HG conditions

Compared to the HG control; acetone extract (A) and ethanol extracts (B) decrease ATP concentration. The aqueous extract (C) showed significant increase in ATP at 200 µg/mL when compared to the positive (HG) control. *$p < 0.05$; and ***$p < 0.0001$ relative to the negative (NG) and positive (HG) controls

4.4. Redox status

The effects of hyperglycaemia-induced oxidative stress in HepG2 cells were determined by measuring lipid peroxidation and GSH levels.

4.4.1. Glutathione level

The GSH-Glo™ Glutathione assay was used to assess the intracellular concentration of GSH, and results are represented in Figure 4.3. For the acetone extract, the results indicated no statistical significance in GSH levels. However, for cells treated with the ethanol and aqueous extracts, when comparing the positive control (HG) to the negative control (NG), the GSH

concentration increased significantly (p<0.0001). For cells treated with the ethanol extract at concentrations 35 and 50 µg/mL under HG conditions, respectively, GSH levels increased significantly (p<0.0001) when compared to the positive control. For the cells treated with the aqueous extract under HG conditions at 200 and 370 µg/mL respectively, the results indicated an increase (p<0.0001) in GSH levels when compared to the positive control.

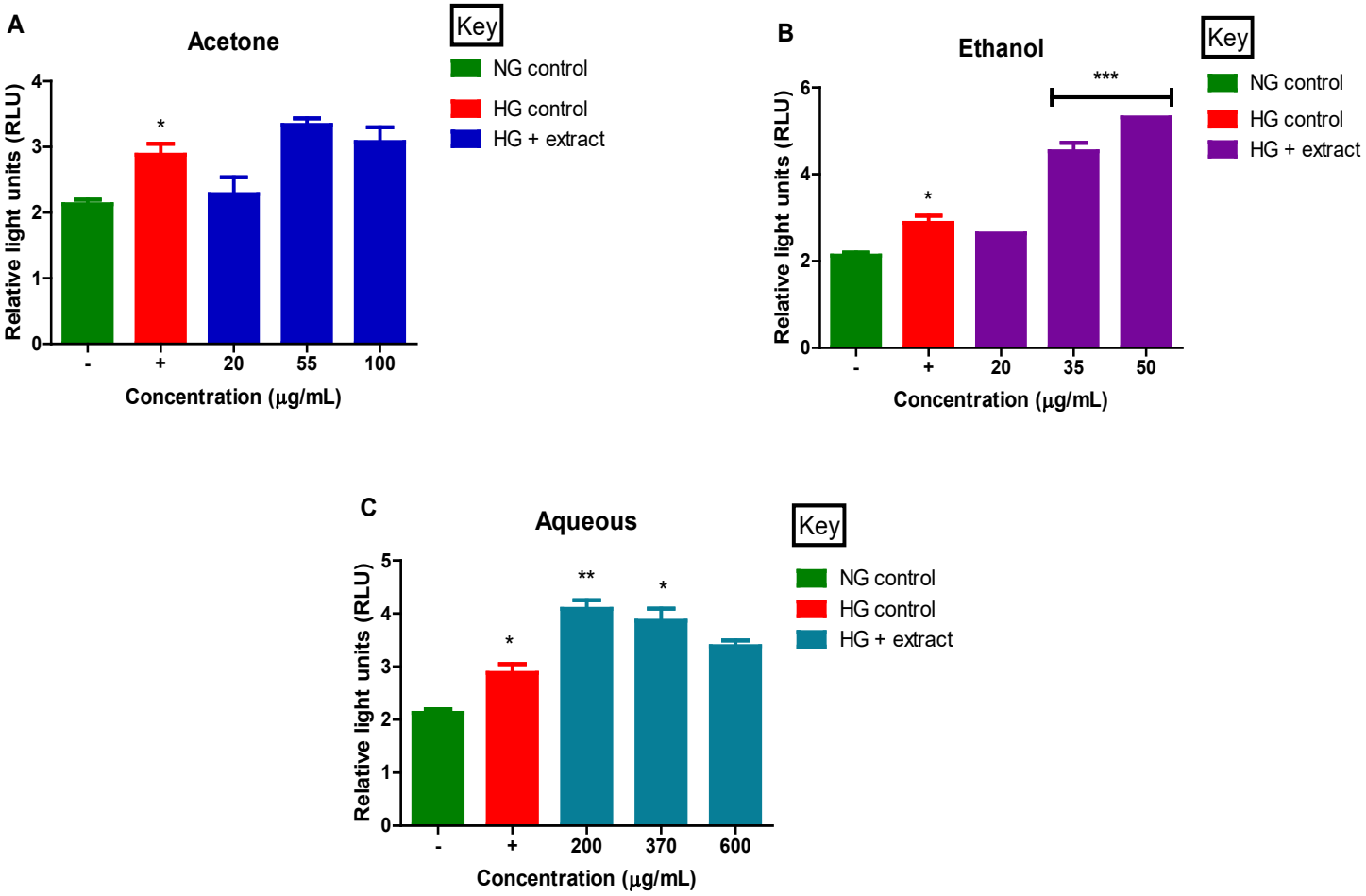


Figure 4.3: Glutathione concentration in HepG2 cells after treatment with *Cannabis sativa* extracts

Glutathione concentration increased in HepG2 cells after exposure to each extract. *p < 0.05; **p < 0.001; ***p < 0.0001 relative to the negative (NG) and positive (HG) controls.

4.5. Oxidative damage

4.5.1. Lipid peroxidation

The investigation into lipid peroxidation (MDA) levels, demonstrates its efficacy as a biomarker for assessing cellular oxidative stress (Lepara et al., 2020). Lipid peroxidation levels for the HG control increased significantly (p<0.0001) when compared to the negative control (NG) as shown in Figure 4.4 (a, b, and c), indicating an outcome of glucotoxicity in the cells treated with HG media. For the ethanol extract, no statistical significance in MDA levels was observed when compared to the positive control (HG). However, when cells were treated with the

acetone extract, at a concentration of 55 µg/mL, MDA levels were shown to increase significantly ($p<0.0001$) when compared to the positive control (HG). For the aqueous extract, at 200 µg/mL, MDA levels significantly ($p<0.0001$) increased at the lowest concentration in comparison to the positive control.

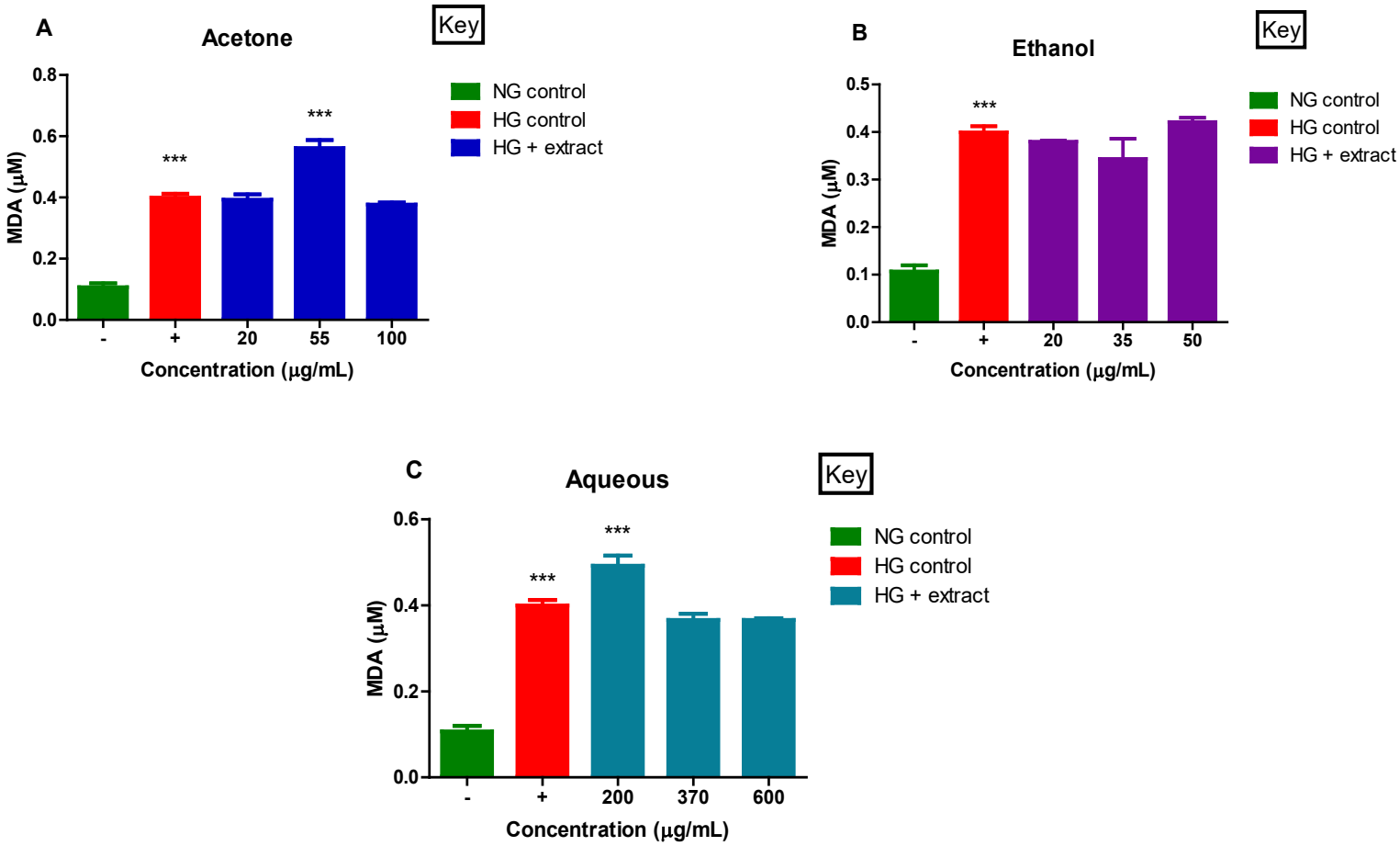


Figure 4.4: Levels of malondialdehyde (MDA) in HepG2 cells after treatment with the *Cannabis sativa* extracts

Levels of malondialdehyde (MDA) in the *cannabis sativa*-exposed cells. A) acetone extract showed decreased MDA levels at the lowest and highest concentrations, B) ethanol extract indicated decreased MDA levels at the lowest and IC_{50} concentrations, C) aqueous extract showed decreased MDA levels at the IC_{50} and highest concentrations when compared to the positive (HG) control. ***$p < 0.0001$ relative to the negative (NG) and positive (HG) controls.

4.6. Analysis of caspase activity

Initiator caspases such as caspases -8 and -9, activate executioner caspases and therefore facilitate apoptosis (programmed cell death). Caspase activity was quantified to assess apoptotic cell death using the Caspase-Glo® assays.

4.6.1. Caspase 8

Cells treated with the positive control (HG), significantly increased caspase-8 activity when compared to the negative control (NG) in Figure 4.5. At the IC_{50} concentrations for all extracts,

no statistical significance in caspase-8 activity was observed when compared to the positive control (HG). For cells treated with the acetone extract, at 100 µg/mL, a significant ($p<0.0001$) decrease in caspase-8 activity was indicated when compared to the positive control (HG). For cells treated with the ethanol extract, at concentration 20 µg/mL, a significant ($p<0.0001$) increase in caspase-8 activity was observed in comparison to the positive (HG) control in cells treated with the aqueous extract. However, a significant ($p<0.0001$) decrease in caspase-8 activity was observed at the highest concentration of 600 µg/mL in the cells treated with the aqueous extract in comparison to the negative control (NG).

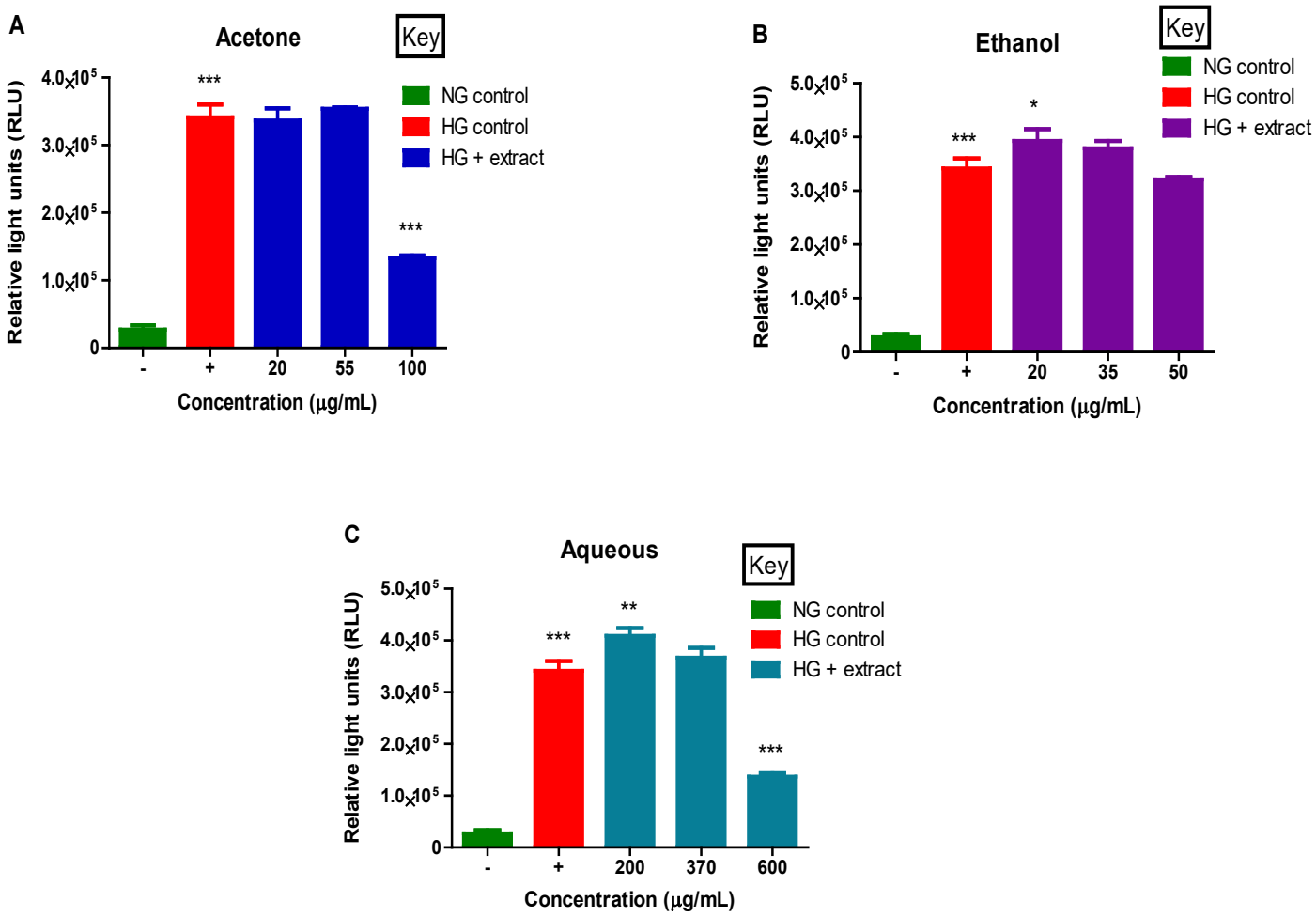


Figure 4.5: Caspase -8 activity

Caspase-8 activity for each treatment with *Cannabis sativa* extract. RLU: relative light units. A) acetone extract showed a significant decrease in activity at the highest concentration (100 µg/mL), B) ethanol extract significantly increased activity at 20 µg/mL and C) aqueous extract significantly increased and decreased activity at 200 and 600 µg/mL respectively when compared to the positive (HG) control. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ relative to the negative (NG) and positive (HG) controls.

4.6.2. Caspase 9

Cells treated with the positive control (HG), significantly increased caspase-9 activity when compared to the negative control (NG) as depicted in Figure 4.6. Cells treated with the highest concentration (100 µg/mL) of the acetone extract resulted in a significant ($p<0.0001$) decrease in caspase-9 activity when compared to the positive control (HG). For cells treated with the lowest concentration (20 µg/mL) of the ethanolic extract a significant ($p<0.0001$) increase in caspase-9 activity was indicated when compared to the positive control (HG). However, at the highest extract concentration (50 µg/mL), a significant ($p<0.0001$) decrease in caspase-9 activity was observed in comparison to the positive control (HG). For cells treated with the aqueous extract, a significant ($p<0.0001$) increase in caspase 9 activity was observed at 200 and 370 µg/mL respectively, when compared to the positive control.

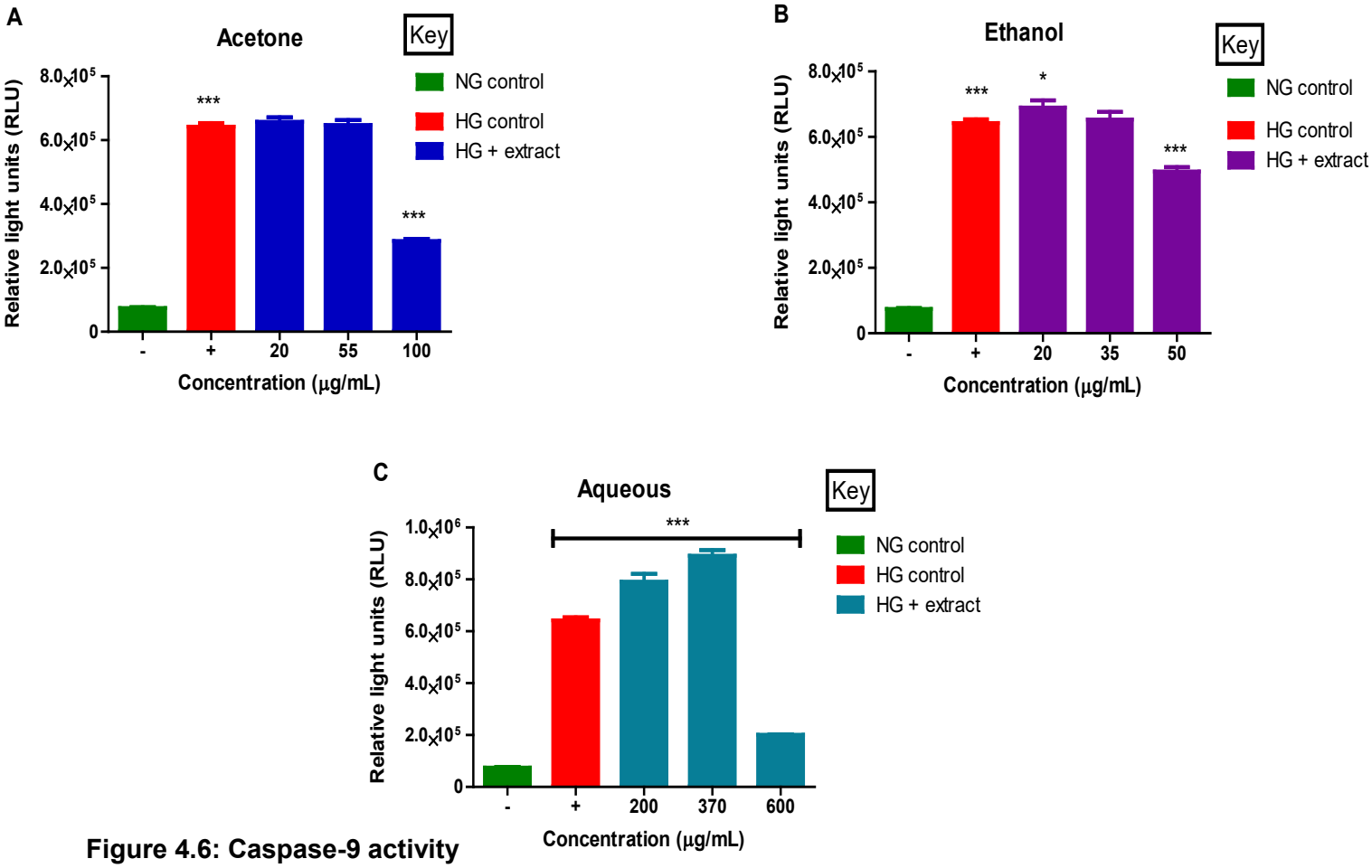


Figure 4.6: Caspase-9 activity

Acetone showed to significantly decrease activity at 100 µg/mL, (B) ethanol extract significantly increased and decreased activity at 20 and 50 µg/mL respectively and (C) aqueous extract significantly increased activity at 200 µg/mL and 370 µg/mL, including decreasing activity at 600 µg/mL when compared to the positive (HG) control. *$p < 0.05$; and ***$p < 0.0001$ relative to the negative (NG) control and positive (HG) controls. RLU: Relative light units.

4.7. Cellular death

4.7.1. Extracellular LDH concentration (Necrosis biomarker)

The integrity of the cell membrane was measured by determining the level of extracellular LDH, a marker used to assess tissue damage. The extracellular LDH measured for cells (Figure 4.7) treated with the positive control showed a significant increase compared to the negative control (NG). The results indicated an increase in extracellular LDH for the HepG2 cells treated with the acetone, ethanol and aqueous extracts, when compared to the positive control (HG). For the cells treated with the acetone extract, at 20, 55, and 100 µg/mL, the LDH activity increased significantly ($p<0.0001$) respectively, in comparison to the positive control. For cells treated with the ethanol extract, LDH activity increased significantly ($p<0.0001$) at 20, 35, and 50 µg/mL, in comparison to the positive control, respectively. For the cells treated with the aqueous extract at 370 and 600 µg/mL, the results indicated an increase ($p<0.0001$) in LDH activity when compared to the positive control, respectively.

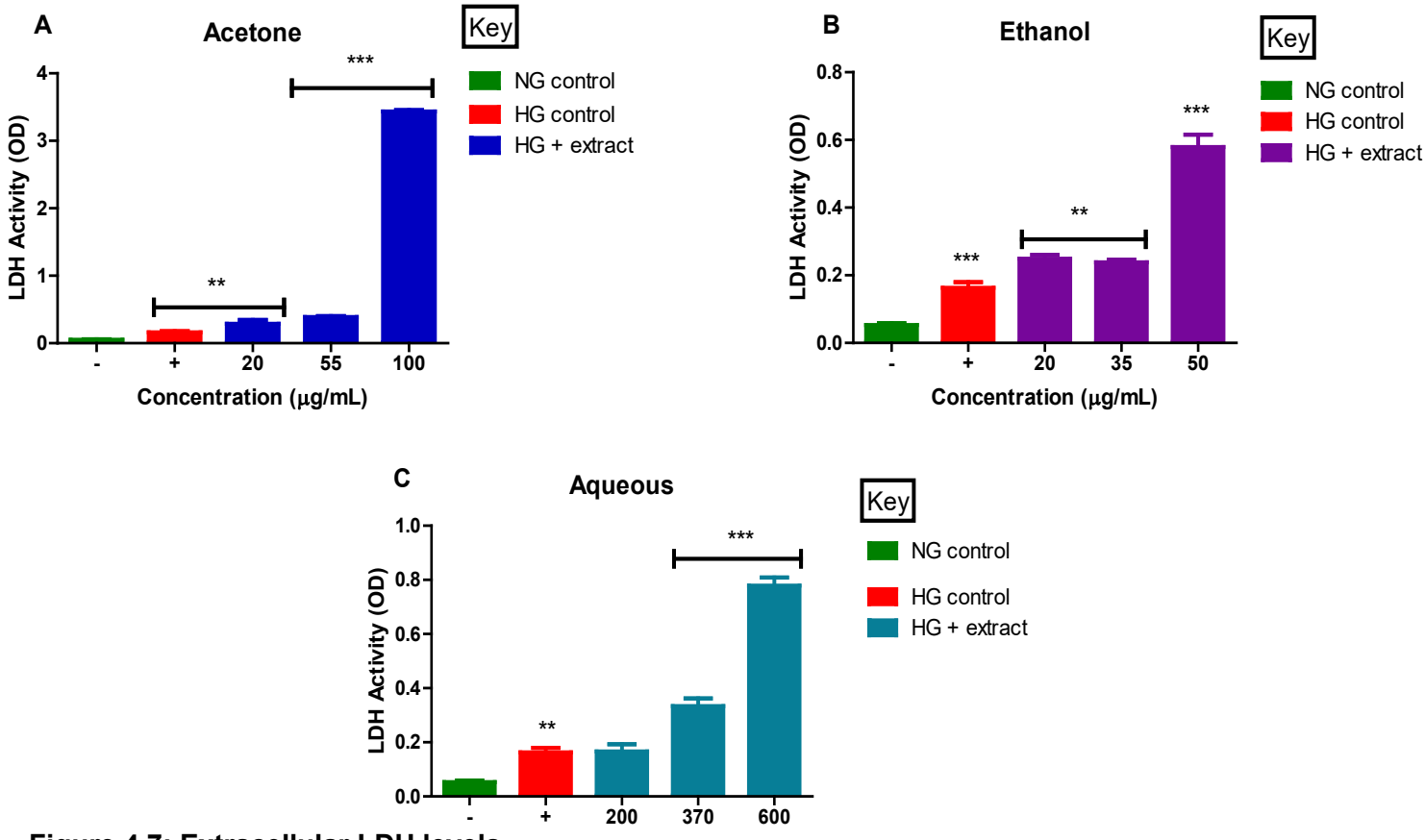


Figure 4.7: Extracellular LDH levels Extracellular LDH levels in cells treated with Cannabis sativa extract. A) acetone extract showed increased LDH activity at the highest concentration, ethanol (B) and aqueous (C) extracts indicated a dose-dependent increase in LDH levels, when compared to the positive (HG) control. **p < 0.001; ***p < 0.0001 relative to the negative (NG) and positive (HG) controls. OD: Optical density.

CHAPTER FIVE

5.1. Discussion

The focus of this study was to evaluate the AO properties of *Cannabis sativa* extracts against high glucose-induced OS in HepG2 cells. The high pharmaceutical potential of cannabis has made it crucial to develop analytical methods that are time and cost-efficient. Since new and updated legislation uses of cannabis is in effect in South Africa and globally, more opportunities exist for phytocannabinoid profiling via novel extraction techniques and analytical methods (Stefkov et al., 2022). Multidisciplinary research on cannabis has been

boosted by the discovery of the chemical structure of CBD and THC. THC and CBD are the best-known cannabinoids responsible for the pharmaceutical activity of cannabis (Stasilowicz et al., 2021).

The use of cannabis in medicine and research on its phytochemistry and pharmacology, has significantly increased toward the end of the nineteenth century (Stasilowicz et al., 2021). In a previous study, acetone, ethanol, and aqueous solvents were used to extract bioactive compounds in the *C. sativa* plant (Sasidharan et al., 2011). The phytochemical composition of the extracts used in this study was determined by LC-MS method and is presented in Table 4.1. The phytochemical fingerprint identified represents compounds typical of cannabis, including well characterised phytocannabinoids: CBD, THC, CBN, CBG, and CBC (Maayah et al., 2020). Liquid extraction with different solvents is the most commonly used method for isolating polyphenols. Acetone and ethanol are solvents best known to extract compounds with bioactivity (Borges et al., 2020). Water is non-toxic, has a low production cost and is safe for the environment, making it a desired solvent to use for extracting polyphenols (Castro-Puyana et al., 2017) and is highly used in Folk medicine (Erhabor et al., 2020).

The recovery of phenolic compounds depends on the polarity of the solvent used in the extraction. Cannabis components such as terpenes, terpenoids, flavonoids and polyphenols are extracted in different ratios. The different extraction ratios depend on the solvent that is used. Flavonoids and polyphenols are of more polar character, terpenes are non-polar (Predescu et al., 2016) and cannabinoids are soluble in both polar and non-polar solvents (Predescu et al., 2016). The TPC results indicate that the acetone, ethanolic and aqueous extracts extracted compounds in different quantities. The number of polyphenols found in the ethanol and aqueous extracts were higher than in the acetone extracts. Aqueous showed better characteristics as a solvent for polyphenols than ethanol, however, at 1000 µg/mL, both ethanol and aqueous extracts did not differ significantly. The two cannabinoids identified as having the highest concentration in the three extracts were found to be CBD and THC, CBD being in a higher concentration than THC. This correlates with the plant material used in this study being bred to have a higher CBD content than THC. CBD and THC are the most abundant phytocannabinoids found in cannabis and are commercially available in a variety of CBD to THC ratios. THC is psychoactive, while CBD does not display psychoactive effects (Meah et al., 2022).

The acetone, ethanol and aqueous cannabis extracts investigated in this study displayed high levels of polyphenols and were shown to have a high antioxidant capacity (Table 4.2). The ethanol and aqueous extracts had the highest antioxidant capacity using the FRAP and DPPH assays respectively. This agrees with several studies that provide evidence for a high total AO

capacity of cannabinoids and cannabis extracts. CBD, THC and other phytocannabinoids, exert beneficial AO properties that could contribute to the observed AO activity by synergistic effects. This could explain the different capabilities detected in the three extracts analysed. Indeed, differences were observed regarding phytocannabinoid content, with the increase in cannabinoid types justifying the pronounced and different AO activity. This suggests that there is a strong structure-activity relationship and that extracts retain a strong AO activity due to the massive presence of preserved bioactive compounds.

Well before the era of modern medicine, the cannabis plant was used for medicinal and therapeutic purposes (Malik et al., 2021). An analysis of the CANNUSE database highlighted ethnobotanical uses of cannabis from over 40 countries. In folk medicine the leaves of the versatile plant were shown to treat disorders of the skin, blood, subcutaneous tissue and circulatory system (Balant et al., 2021). *Cannabis sativa* is also used for its analgesic, antimicrobial, and anti-inflammatory properties (LaVigne et al., 2021). In recent years, the medical and pharmaceutical sciences have shown particular interest in the use of medical cannabis, therefore, more research into unlocking the full potential of the *C. sativa* plant is crucial (Malik et al., 2021). Studies on the bioactivity of CBD, demonstrate how this cannabinoid reduces inflammatory cytokine secretion (Gallant et al., 2009 and Huang et al., 2019). CBD can suppress oxidative and inflammatory responses, which leads to the protection of beta-cells against high glucose and high lipid content (Ehud et al., 2012). Phytocannabinoids found in cannabis may modulate the ECS resulting in anti-diabetic effects. Other phytocannabinoids can work synergistically with terpenes and flavonoids to control diabetes through anti-diabetic and anti-inflammatory effects against inflammation (Ghasemi-Gojani et al, 2022). Additionally, other biological activities associated with CBD include, anti-convulsant, anti-nausea, and analgesic effects (Li et al., 2020).

Several studies have demonstrated and highlighted the beneficial effects of cannabis extracts in treatment of diabetes and diabetic complications (Comelli et al., 2009). Therefore, it can be theorised that cannabis consumption is involved in the regulation of several metabolic pathways, including glucose regulation and insulin secretion (Horvath et al., 2012 and Mousavi et al., 2023). Ethnopharmacology may present new and underutilized strategies for preventing or delaying cell damage due to oxidative stress caused by glucotoxicity (Abdul and Marnewick, 2022). In the present study, the potential of *C. sativa* extracts to overcome OS induced by glucotoxicity in a liver-derived cell line was investigated. The study found that the cannabis extracts ameliorated oxidative damage in the HepG2 cells, by aiding in the improvement of the antioxidant status. The data presented in this study suggests that cannabis extracts can protect liver cells from oxidative damage caused by an increased glucose environment, such as in diabetic patients.

The biological effects of cannabinoids can play an important role in the health and disease of humans. There is accumulating evidence that as antioxidants, cannabinoids may protect cells against oxidative damage and, therefore, diminish the risk of diseases associated with OS. Recent studies have shown that DM, particularly T2DM, is associated with OS (Singh et al., 2022 and Brownlee, 2001) because of hyperglycaemia. A decrease in insulin secretion and an increase in insulin resistance is known to lead to glucotoxicitiy as a result of hyperglycaemia and is a prominent metabolic feature of T2D. Glucose metabolism disposal predomninantly takes place in the liver (Kawahito et al., 2009, Lima et al., 2022, Abdul and Marnewick, 2022).

Diabetes is associated with metabolic dysfunction and, therefore, measuring cell viability, as a measure of metabolic activity is important in understanding the effects of cannabis on cell metabolism in a glucotoxic environment. In the current study, the MTT and ATP assays were used to measure cell metabolic activity and output. Cell metabolic activity was preserved at the lower concentrations (0-600 µg/mL) of the aqueous *C. sativa* extracts in HG-supplemented HepG2 cells, however, a dose-dependent reduction in cell viability was observed with treatment after treatment with the acetone and ethanolic extracts. MTT reduction is considered an indicator of cellular metabolic activity (Takahashi et al., 2002), and may reflect enhanced mitochondrial oxidative activities related to increased glucose consumption (Abdul and Marnewick, 2022). CBD was shown to improve glucose uptake and utilization in adipocytes by increasing the activity of glucogenic enzymes. Our study suggests that the extracts could have a similar effect in liver cells as the endocannabinoid system is active in modulating hepatic glucose metabolism.

There was a marked decrease in cell viability with increasing extract concentration. According to Acquavia et al., (2023), hemp extracts with THC:CBD ratios of 1:9, displayed higher toxicity in HepG2 cells than pure cannabinoids. The cytotoxicity levels measured in the current study suggest that the extracts with a high CBD content may be toxic to liver cells with increasing concentration. The results from the MTT and ATP assays were cytotoxic and suppressed cell viability of HepG2 cells with increasing extract concentration after 24 h treatment. These results may be considered medically relevant as cannabis-based drugs to treat diabetic-related conditions lack sufficient investigation. Previous studies have shown THC to interfere with the action of insulin and its release. A study involving cultured adipocytes demonstrated how THC increased insulin and induced glucose uptake, since insulin resistance causes impairment of glucose uptake in T2D (Scherer et al., 2019). Studies have also shown the effects of TNF alpha on insulin and its effectiveness on glucose uptake. TNF alpha essentially interferes with insulin signalling and the expression of GLUT4 in adipocytes (Gallant et al., 2009, and Solomon et al., 2010). THC has been shown to decrease the level of TNF alpha in various experimental models (Misner and Sullivan, 1999, Bih et al., 2015, Seeman, 2016 and

Gomes et al., 2011). The THC present in the extracts used in the current study would likely enhance insulin action in diabetic patients. A study involving rat pancreatic islet cells showed that THC caused an increase in lipoxygenase activity which led to the cells releasing insulin and accelerating the metabolism of arachidonic acid (Nagarkatti et al., 2009). CBD has also been shown to improve disease manifestation in mice with latent diabetes or initial diabetes symptoms (Stella et al., 1997). Not many studies exist showing the direct effect of CBD on blood glucose levels, although, it has been shown to inhibit IL-12 production in splenocytes. IL-12 is a pro-inflammatory cytokine which plays a major role in autoimmune processes underlying diabetes (Stella et al., 1997). Modulating the immune system's response by decreased production of IL-12, CBD offers therapeutic benefits in alleviating the effects of diabetes. This could explain why the cannabis extracts used in the current study preserved cell viability at certain concentrations, as the extracts contained significant amounts of CBD and THC.

Clinical studies suggest that T2D is subjected to chronic OS, linked to glucose toxicity (Robertson et al., 2004). Hyperglycaemia is associated with the ROS formation from enzymes such as xanthine oxidase, nitrogen oxide (NOX) (NADPH oxidase) isoforms, which depletes the overall AO response. It has been reported that CBD prevents the formation of superoxide radicals. These molecules/compounds are mainly generated by xanthine oxidase (XO) and NADPH oxidase (NOX1 and NOX4), reduces ROS and can change the level and activity of antioxidants, thus, modifying the redox balance (Atalay et al., 2019). Other studies have shown both CBD and THC (partial agonists of CB2 receptor) as agonists of CB1 receptor and CB2 receptor, which are the most important receptors of the ECS (Gallant et al., 2009).

Strong evidence exists that links the excessive generation of ROS to glucotoxicity and the depletion of AO defense mechanisms (Abdul and Marnewick, 2022). The current study showed that the extracts had a protective effect on glucotoxicity, which is consistent with the elevated GSH levels in the high glucose supplemented HepG2 cells after treatment with the Cannabis extracts after 24 hours. According to Powell et al., 2001, high glucose decreased intracellular GSH levels in vascular smooth muscles cells and suggests that decreased levels of GSH within a HG environment are controlled by the availability of the rate-limiting substrate cysteine and the gene expression of the gamma-glutamylcysteine synthetase enzyme (Powell et al., 2001). Exhausted GSH levels were also present in diabetic rat models and patients (Aaseth et al., 2000; Tachi et al., 2001).

The intracellular antioxidant glutathione is important in the human defense system and is shown to be depleted in patients with diabetes. The low levels of GSH in diabetic patients may predispose cells to diabetic complications due to the oxidant/pro-oxidant imbalance generated

(Powell, et al., 2001). The synthesis of GSH involves the formation of glutamylcysteine from glutamate and cysteine, which is catalysed by glutamate cysteine ligase (GCL). Glycine is then added to glutathione synthetase to form GSH (Zhang and Forman, 2012). Most of the GSH in cells utilise GPx and this catalyses the reduction of H_2O_2 into water and GSSG (Zhang and Forman, 2012). According to Coskun, (2015), diabetes-induced oxidative damage was reduced by THC in the liver of T2D rats, showing that THC administration may serve as a protective measure to some extent for the liver in diabetes. This protective measure could block the OS state. (Karabulut et al., 2015). Several studies have shown the beneficial effects of polyphenols in a hyperglycaemic environment in the liver (Jasmine et al., 2021). Erukainure et al., 2021, reported that hexane, dichloromethane (DCM) and ethanol *Cannabis sativa* extracts as a treatment of isolated rat hepatic tissues incubated with iron sulphate ($FeSO_4$), significantly depleted GSH concentration, indicating the occurrence of OS and treatment with the cannabis extracts improved GSH levels. This leads to the observation that elevated GSH levels signify improved antioxidant defence in hepatic tissues (Erukainure et al., 2021). A separate study by Kubiliene et al., (2021), showed that *C. sativa* significantly reduced MDA concentrations after Aluminium chloride ($AlCl_3$)-induced OS and reached the same concentrations as the control group (Kubiliene et al., 2021). A similar trend was observed in the present study, for MDA levels after treatment with the acetone, ethanolic, and aqueous Cannabis extracts. The MDA levels reached concentrations similar to that of the HG-control, although not significantly, at certain concentrations of extract, suggesting that no further lipid peroxidation was induced. The extra ROS accumulated by glucotoxicity can be neutralised by AO systems consisting of enzymatic antioxidants such as glutathione peroxidases (GPxs) and ROS molecules can trigger OS in a feedback mechanism (He et al., 2017). Failure to scavenge free radicals via AO flux can initiate a dangerous cycle of dysfunction, ultimately leading to death (Dey et al., 2016). GSH is important in regulating cellular homeostasis and maintaining the redox cycle of cells. GSH peroxidases are involved in various forms of ROS and RNS (Georgiou-Siafis and Tsiftsoglou, 2023). Thus, the cannabis extracts used in the current study may reduce OS caused by glucotoxicity by improving the direct scavenging of ROS through increased concentrations or redox cycling of GSH.

High glucose levels in the blood may lead to excessive generation of ROS, disrupting oxidant and antioxidant enzyme balance. Thus, failure to inhibit excessive lipid peroxide generation, especially MDA, an imbalance may occur (Han et al., 2019). The underlying development of T2DM and the progression of its associated complications has been said to be due to OS, through the excessive production of ROS and may lead to liver cell damage (Pane et al., 2018). The observed increases in MDA levels at concentrations of 55 µg/mL for acetone extracts and 200 µg/mL for aqueous extracts, specifically under high-glucose treatments, are

noteworthy. Contrary to previous *Cannabis sativa* research, these results indicate that the extracts did not confer protection against lipid peroxidation at these concentrations. The increased MDA levels for the acetone and aqueous extracts may indicate that the extracts could not protect the cells against lipid peroxidation but prevented more damage. These results may suggest that the excessive OS may be a result of high glucose levels. According to Abdel-Salam et al., (2013), liver MDA was significantly increased after cannabis resin extract was given to mice subcutaneously after administering a single dose of lipopolysaccharide (LPS) to the mice. This study suggested that cannabis derived from the leaves and flowering tops, worsened the chemically induced liver injury and indicated that cannabis may enhance histological damage caused by administration of an endotoxin such as lipopolysaccharide (LPS) (Abdel-Salam, 2013). This highlights the complexity of biological responses to plant extracts at specific concentrations. The difference in composition and quantity of the various secondary metabolites found in the cannabis plant such as cannabinoids, terpenes, and flavonoids may contribute to the pharmacological effects of cannabis extracts (Stasiłowicz-Krzemień et al., 2023). Thus, the composition and quantity of the secondary metabolites found in the *C. sativa* extracts used in this study may be the cause of the inconsistent MDA levels measured. Previous studies indicate significant decreases in MDA levels post treatment with *C. sativa* extracts, which depict *C. sativa* extracts as having AO effects (Erhabor et al., 2020).

The current study shows that the administration of cannabis extracts caused a dose-dependent increase of LDH leakage in the HepG2 cell model, indicating cellular damage. Concurrently, depleted ATP levels with increasing extract concentration, suggests that reduced ATP may be a precursor for necrotic death. ATP depletion to hepatocytes can cause moderate cellular swelling, which is a prominent feature of necrosis. This swelling leads to plasma membrane damage known as blebbing, and eventually rapid cell lysis (Malhi et al., 2006). However, it is important to note that LDH release in cell cultures may not always be due to necrosis but could be attributed to late apoptotic cells (Benfalvi, 2017). Apoptotic cells *in vivo*, can undergo rapid phagocytosis. However, *in vitro*, conditions do not allow for this rapid process to occur, leading to possible discrepancies in cell death *in vivo* observations (Choi et al., 2009). A previous study demonstrated that rats treated with cannabis exhibited a significant increase in lactate dehydrogenase, which is said to play an important role generating NADPH. In turn, NADPH fuels ROS generation, this is achieved by NADPH oxidase catalysing electron transfer from NADPH to molecular oxygen, via the NOX catalytic subunit, thereby producing ROS (Oluwasola et al., 2023 and Sedeek et al., 2013). Impaired ATP regeneration may be caused by a variety of upstream mechanisms, and it is associated with increased mitochondrial ROS, primarily due to oxidative stress (Gollmer, et al., 2020). Particularly under hyperglycaemic conditions, mitochondrial dysfunction can cause ATP levels

to decrease. Hyperglycaemia is a known hallmark of T2DM, where insulin levels are markedly reduced. Thus, in the context of T2DM, reduced insulin may lead to mitochondrial dysfunction under hyperglycaemic conditions (Grubelnik, et al., 2020). The ATP depletion observed in the current study may be attributed to related HepG2 mitochondrial dysfunction due to high glucose conditions. Excessive generation of ROS induced by hyperglycaemia plays a crucial role in diabetes. High concentrations of glucose can serve as an important agent for initiating apoptosis in the liver. This proves detrimental since the liver is known to be the primary organ for metabolising and regulating glucose in the body (Chandrasekaran et al., 2010).

To discuss the findings of the current study, it is critical to emphasise the significant role of caspases, which are intracellular proteases that aid in the regulation of apoptosis (Docrat et al., 2018). The generation of mitochondrial ROS is enhanced by high glucose which results in the mitochondrial release of cytochrome-c, cleavage of procaspase-9 and caspase-9 enzyme activity (Abdul and Marnewick, 2022). The mitochondrial pathway of apoptosis is triggered by the weakening of the mitochondrial outer membrane. This loss of membrane integrity allows the release of proapoptotic factors (Green and Llambi, 2015). A previous study using HepG2 cells found that apoptosis was associated with cytochrome-c release, and caspase activation. The study involved the comparison of CD95-mediated apoptosis and menadione-induced necrosis (Samali et al., 1999). The initiator caspases -8 and -9 and are activated by dimerisation and not by cleavage (McIlwain et al., 2013). In the current study, the Cannabis extracts significantly reduced the activation of caspase-9 at the highest concentrations of extract and may be closely associated with the protective effects on the mitochondria. This indicates a close regulation of the intrinsic mitochondrial pathway of apoptosis and in vertebrates, is found to be the most common mechanism of apoptosis (Green and Llambi, 2015). Meanwhile, the caspase-8 activation pathway, or the extrinsic pathway, predominantly activated by the death receptor (DR) is a distinct mechanism of apoptosis (Green and Llambi, 2015). There are several studies suggesting that caspase-8 can be activated independently (Abdul and Marnewick, 2022), linking OS to reduced cell viability by inducing apoptosis (Marnewick et al., 2009).

The results of the current study indicate that hyperglycaemia-induced cytotoxicity *in vitro* is reduced by the antioxidant and cytoprotective properties of cannabis extracts, including acetone, ethanolic, and aqueous formulations, at higher concentrations. The role of phytocannabinoids in apoptosis is complex as studies show that both apoptotic and anti-apoptotic machinery is activated in different cells under different conditions. The current study supports the anti-apoptotic effects of cannabis as the extracts increased the concentration of GSH in hepatocytes. GSH is known to prevent cell death by interacting with proteins involved in apoptosis including the BCL-2 (B-cell lymphoma 2) family (Susnow et al., 2009). The

outcome of the study warrants further investigation of mechanistic profiles through *in vitro* and *in vivo* experiments to understand *C. sativa's* safety, efficacy and therapeutic value.

CHAPTER SIX

6.1. Conclusion

This is the first study to report on the quantification of the main cannabinoids present in the different solvent extracts used in this study. The present study showed that hyperglycaemic conditions impair the antioxidant response and that the *C. sativa* extracts can regulate induced glucotoxicity. The findings reveal that *C. sativa* exhibits hepatoprotective properties in the presence of glucotoxicity through the activation of antioxidant effects. These include improved cell viability at certain concentrations, increased glutathione levels, and reduced caspase activity across all extracts when used at their highest concentrations. The research

demonstrates the potential application of *C. sativa* extracts in treating and managing diabetes, and their therapeutic benefits should be further explored.

6.2. Limitations of study

Addressing the limitations associated with the *in vitro* investigation is vital to ensure the study's comprehensiveness and applicability. Studies conducted solely on cells removed from their natural environment neglect the interaction and protective mechanisms intact within whole systems. Therefore, the results obtained from this *in vitro* study may differ from that derived from an *in vivo* study. This study may be improved by studying a primary hepatocyte cell model and the use of an *in vivo* mouse or rat model, as this will allow for further investigation of the effects of hyperglycaemia-induced oxidative stress.

Additionally, this study focused on a 24-hour exposure period to protect HepG2 cells under hyperglycaemic conditions and may not capture the complete range of cellular responses. Extending the hyperglycaemic conditions (4,6 hours) to longer durations (48 hours) may provide a better understanding of the cells' response to hyperglycaemia.

6.3. Future studies

Further studies using other cell lines are required as well, to corroborate the effects of *C. sativa* on diabetes. It is important to conduct *in vivo* studies, that allow for the investigation of *C. sativa* extracts as potential therapeutic drugs in treating diabetes, as well as the conducting of randomised clinical trials. Further analysis of the molecular pathways involved can give a better understanding of the extracts' potential to enhance therapeutic applications in a diabetic model. Investigating *C. sativa* combined with other potentially therapeutic medicinal plants may also enhance its effects. It can be concluded that *C. sativa* is potentially a therapeutic intervention in diabetes.

The cost and availability of diabetes medication for impoverished communities in South Africa and globally have contributed to the importance for new drug discovery, specifically drugs derived from medicinal plants. Medicinal plants can be grown and harvested for their therapeutic benefits in these impoverished communities, making it possible for diabetic patients within these communities to have access to life-changing medication. The side effects caused by diabetic medicines found in the market today, are another reason to develop new therapeutic interventions, such as cannabis-based drugs, that could minimise or eliminate these potentially harmful effects.

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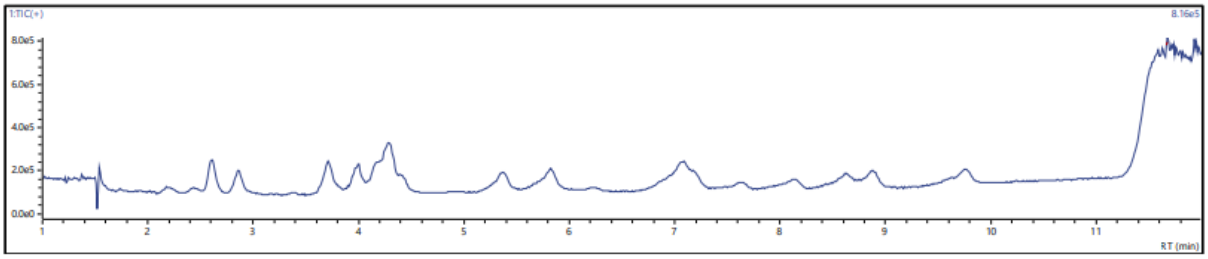
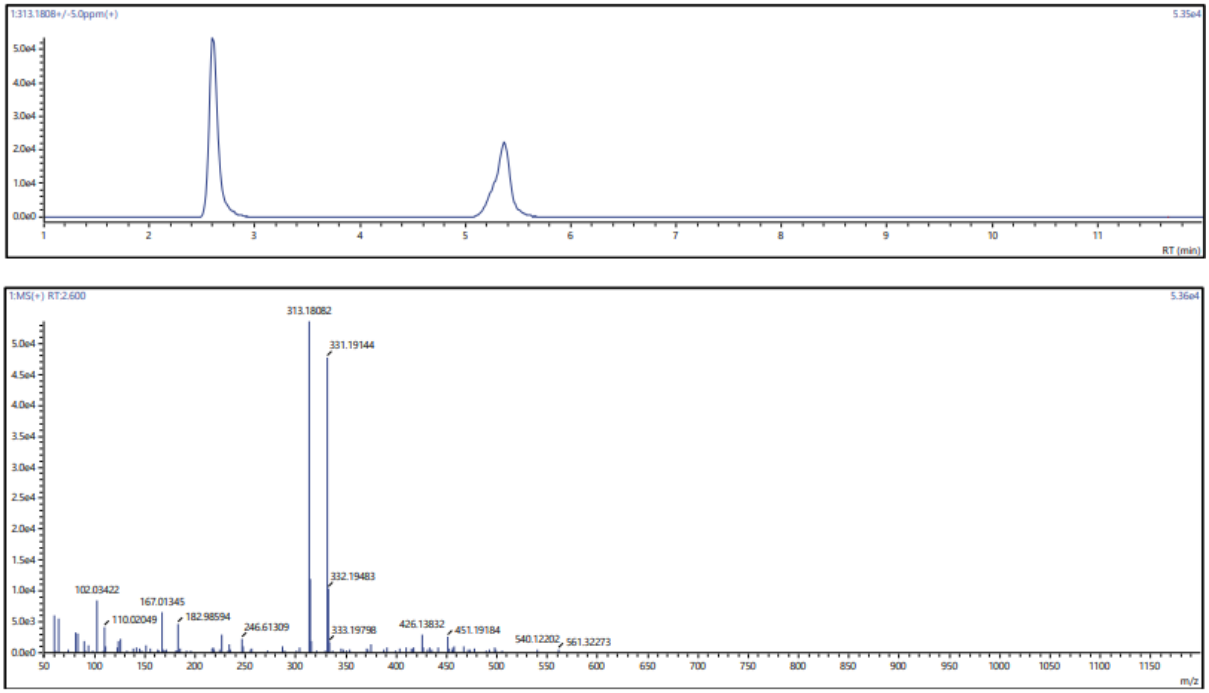
APPENDICES

Below are the LC-MS chromatograms for the cannabinoid standards used in the LC-MS analysis, the total ion chromatograms for the acetone, ethanolic and aqueous extracts. The recipes for the TPC, FRAP, DPPH and TBARS assays as well as the DOH permits for 2022-2023 and 2023-2024 respectively, can also be found below.



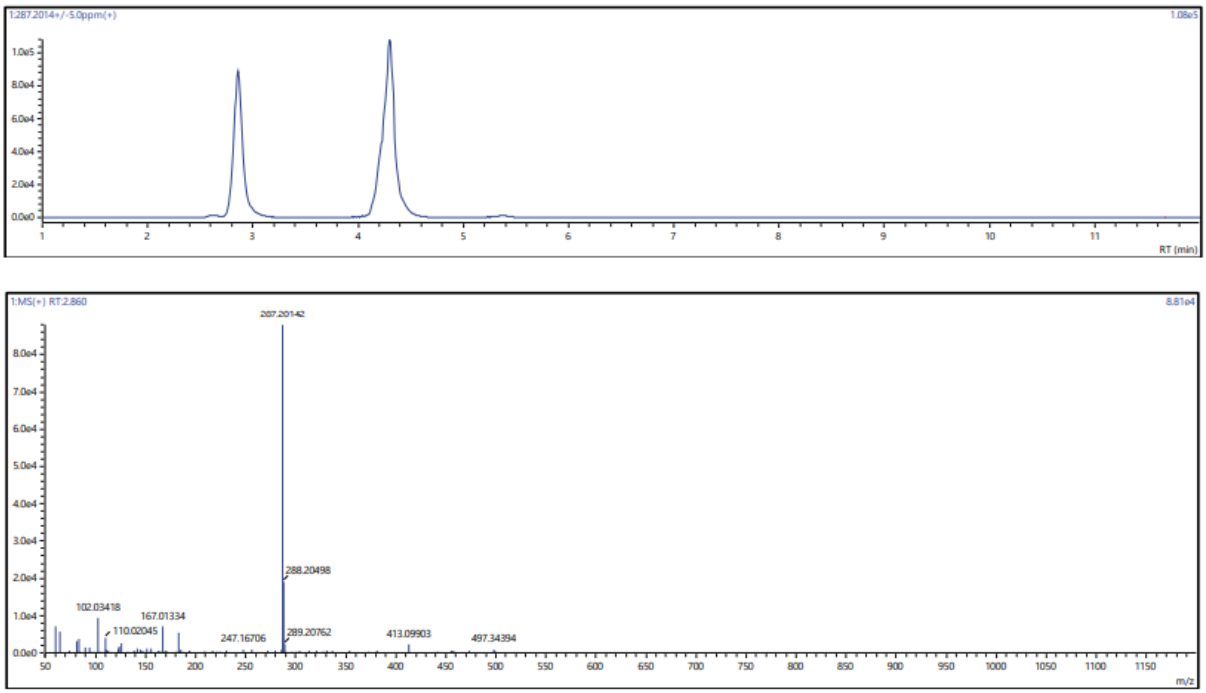
Appendix 8.1: Total ion chromatogram of the 15 cannabinoid standards

Chromatogram was formed using the Shim-pack Velox SP-C18 column (2.1 x 150 mm, 2.7 µm)



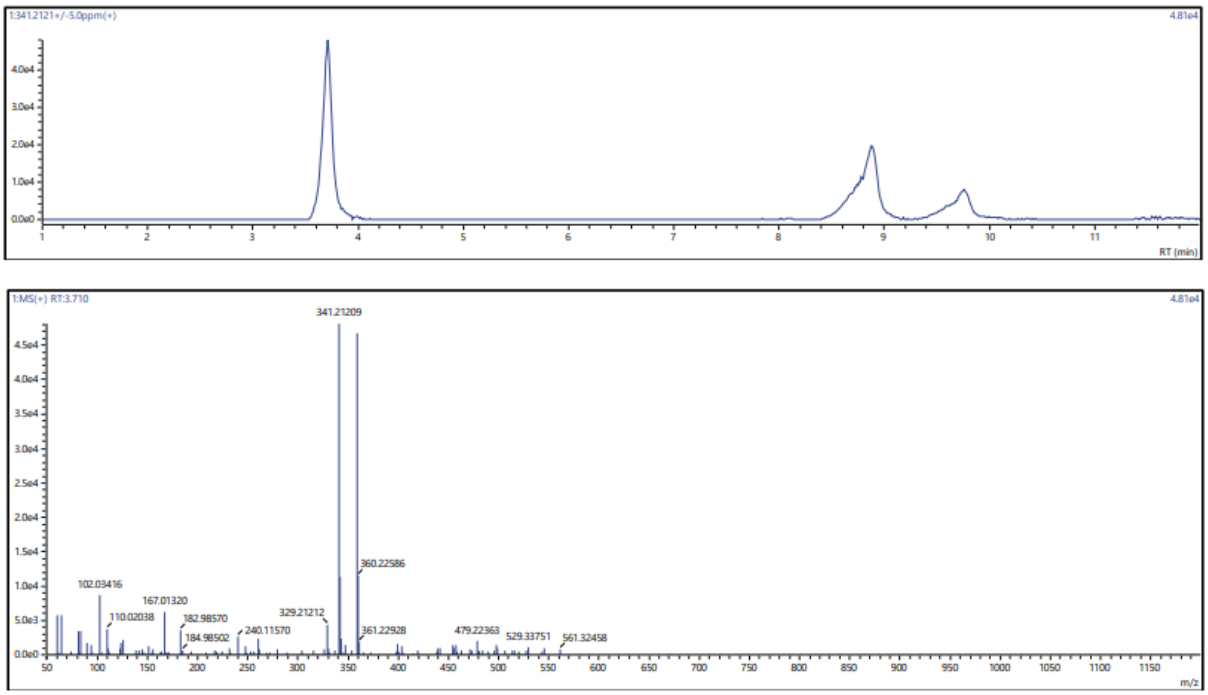
Appendix 8.2: LC-MS chromatogram of cannabidivarinic acid (CBDVA)

Liquid chromatography – mass spectrometry (LC-MS) chromatogram of cannabidivarinic acid (CBDVA) for m/z 313. 1808, at retention time 2.600 min



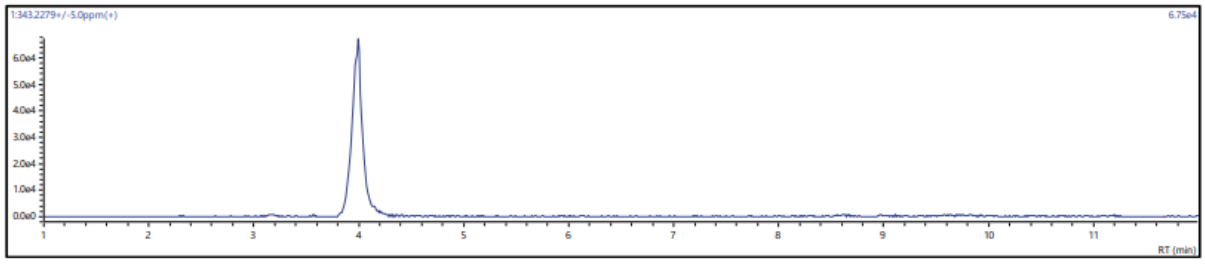
Appendix 8.3: LC-MS chromatogram of cannabidivarin (CBDV)

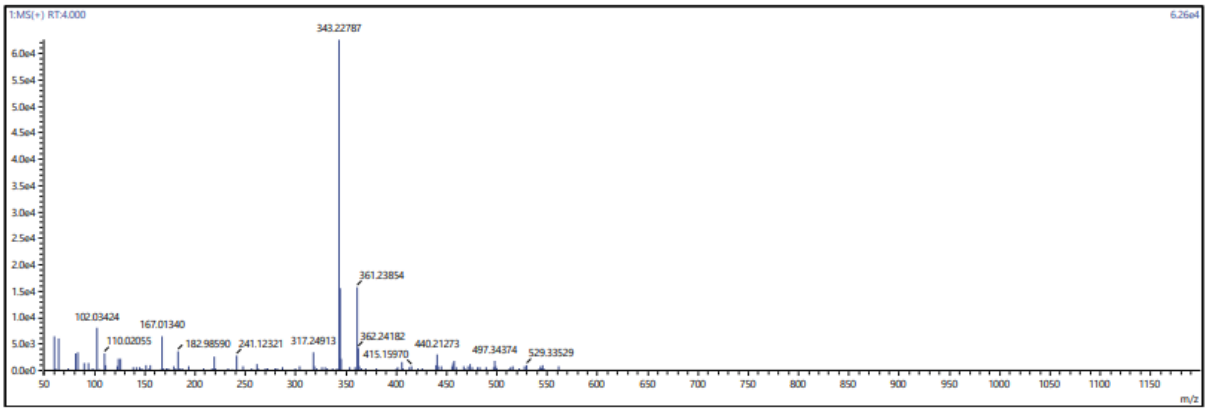
Liquid chromatography – mass spectrometry (LC - MS) chromatogram of cannabidivarin (CBDV) m/z 287.20142, at retention time 2.860 min.



Appendix 8.4: LC-MS chromatogram of cannabidiolic acid (CBDA)

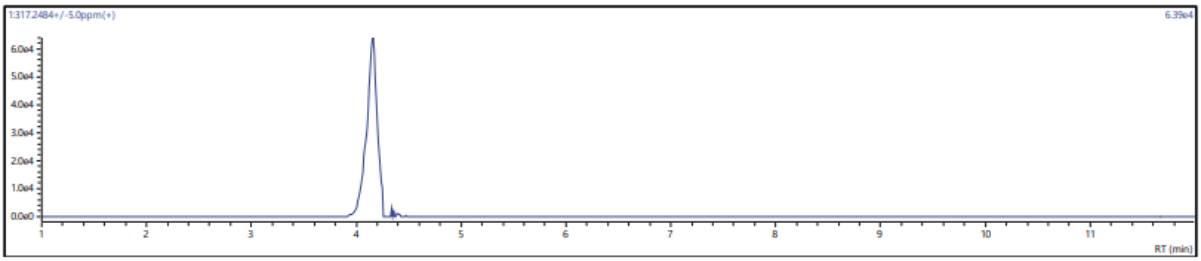
Liquid chromatography – mass spectrometry (LC-MS) chromatogram of cannabidiolic acid (CBDA) for m/z 341.21209, at retention time 3.710 min

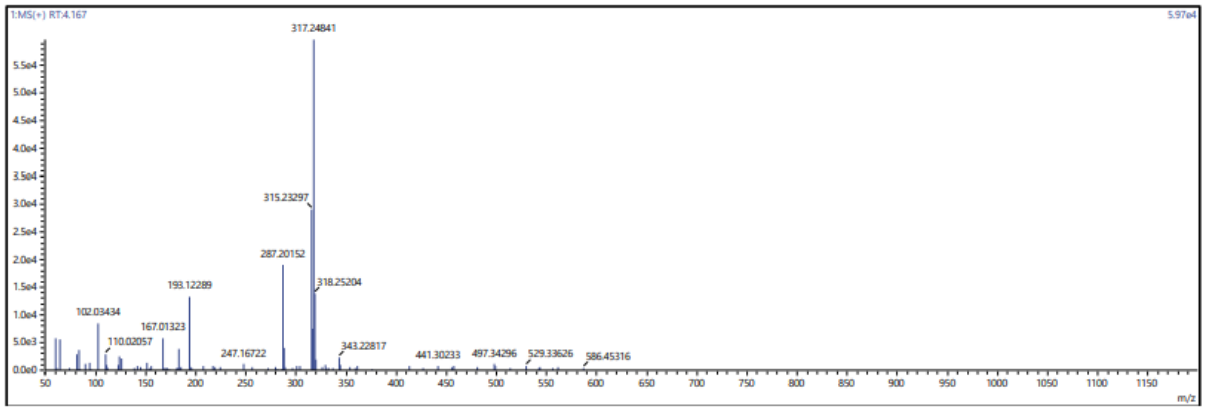




Appendix 8.5: LC-MS chromatogram for cannabigerol acid (CBGA)

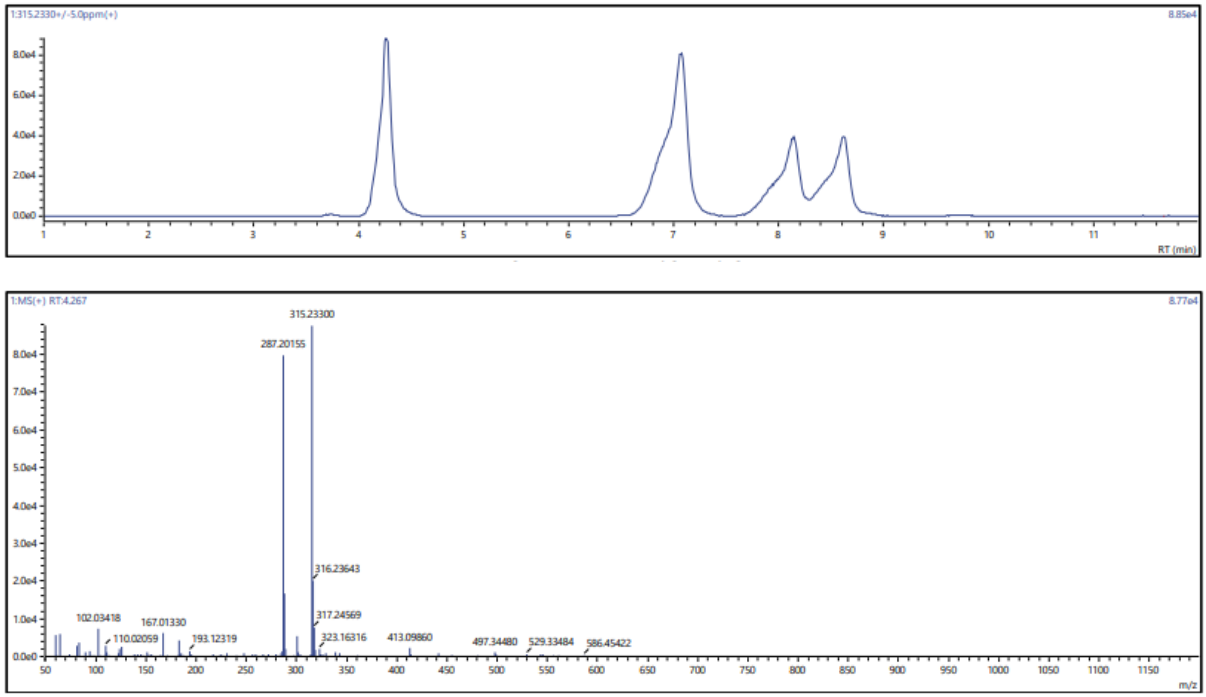
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for cannabigerol acid (CBGA) for m/z 343.22787, at retention time 4.000 min





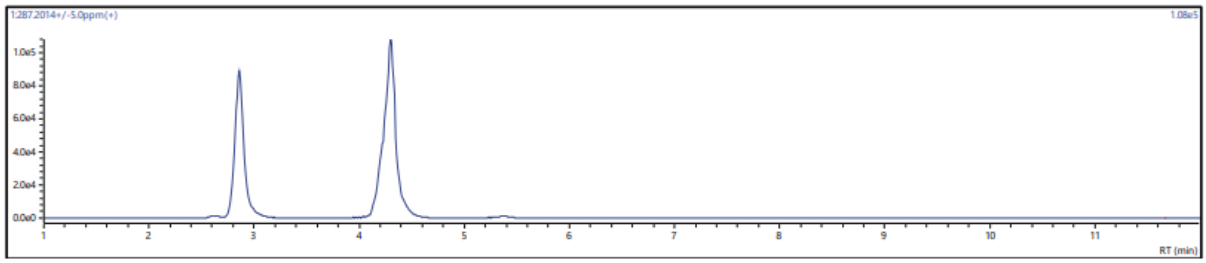
Appendix 8.6: LC-MS chromatogram for cannabigerol (CBG)

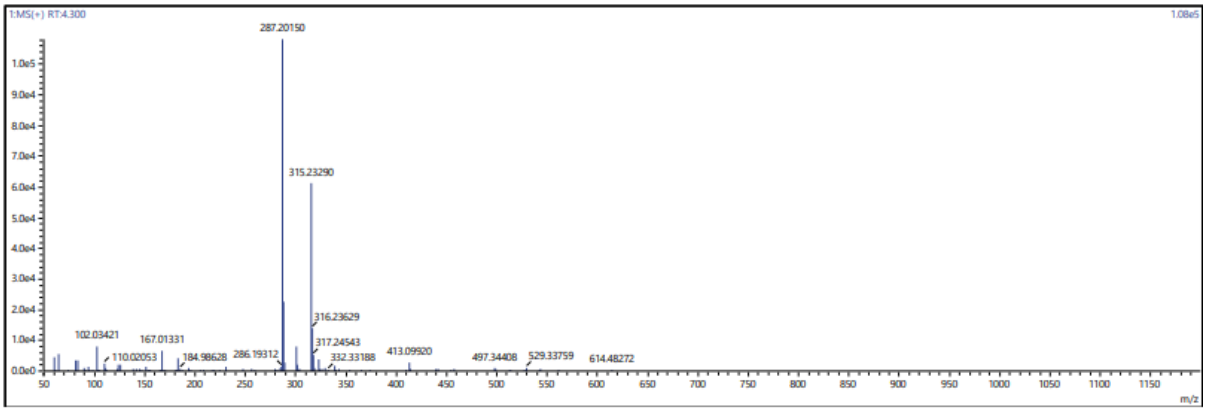
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for cannabigerol (CBG) for m/z 317.24841, at retention time



Appendix 8.7: LC-MS chromatogram for cannabidiol (CBD)

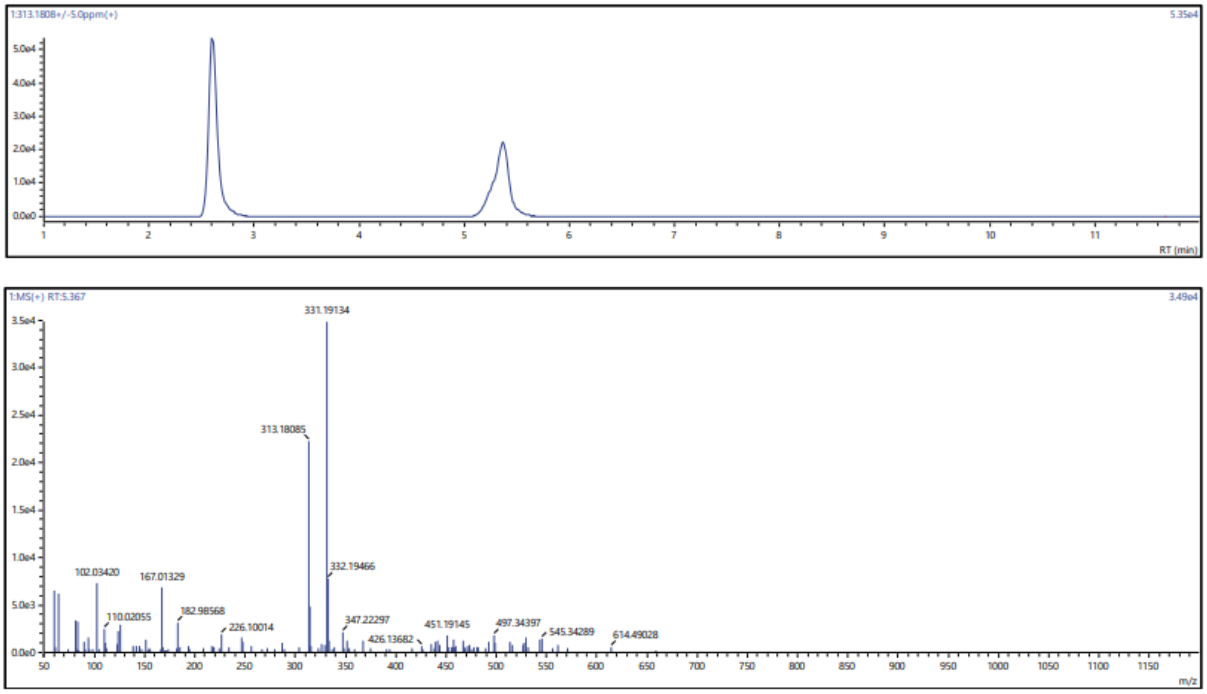
Liquid chromatograph - mass spectrometry (LC-MS) chromatogram for cannabidiol (CBD) for m/z 315.23300, at retention time 4.267 min





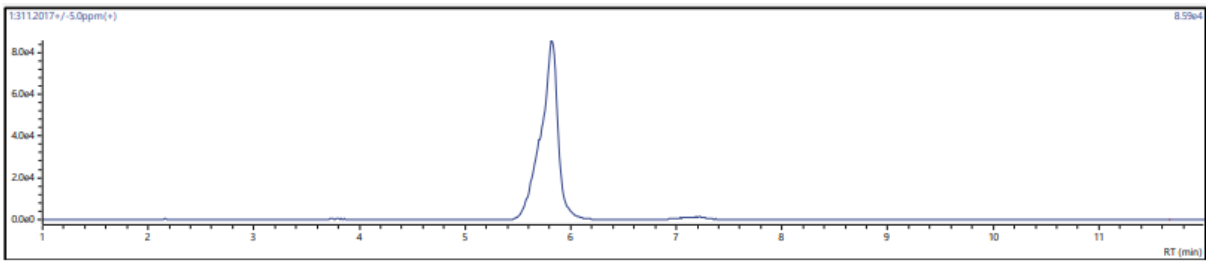
Appendix 8.8: LC-MS chromatogram for tetrahydrocannabivarin (THCV)

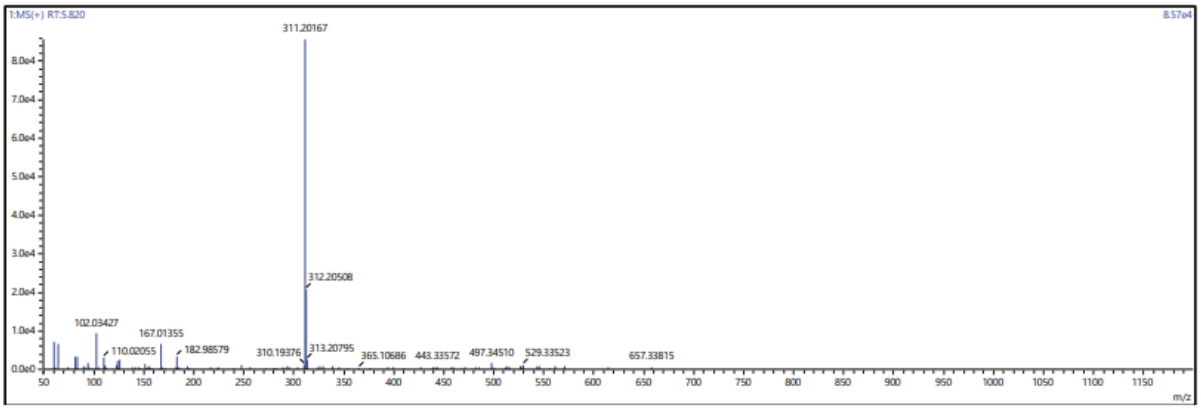
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for tetrahydrocannabivarin (THCV) for m/z 287.20150, at retention time 4.300 min



Appendix 8.9: LC-MS chromatogram for tetrahydrocannabivarin acid (THCVA)

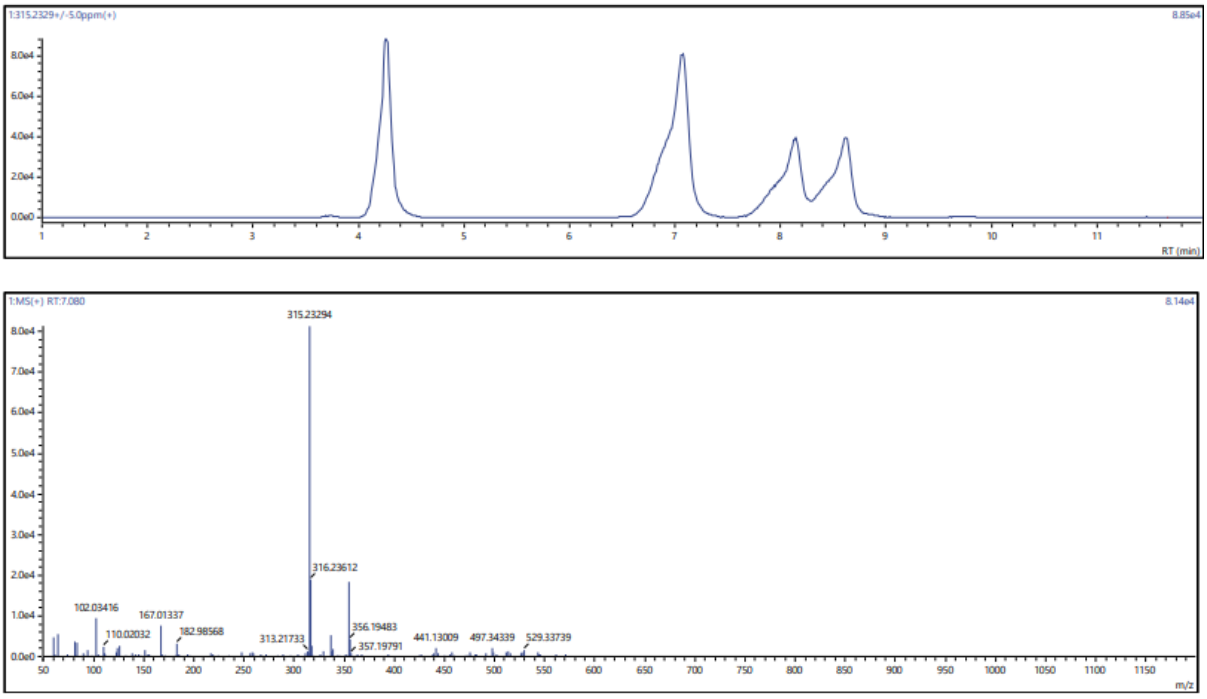
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for tetrahydrocannabivarin acid (THCVA) for m/z 313.18085, at retention time 5.367 min





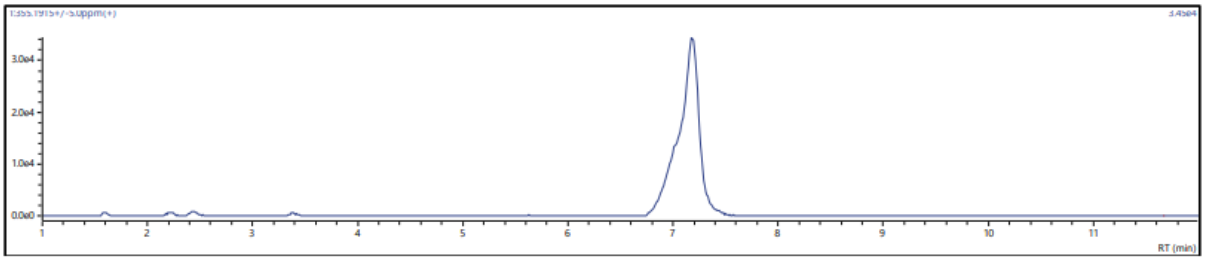
Appendix 8.10: LC-MS chromatogram for cannabinol (CBN)

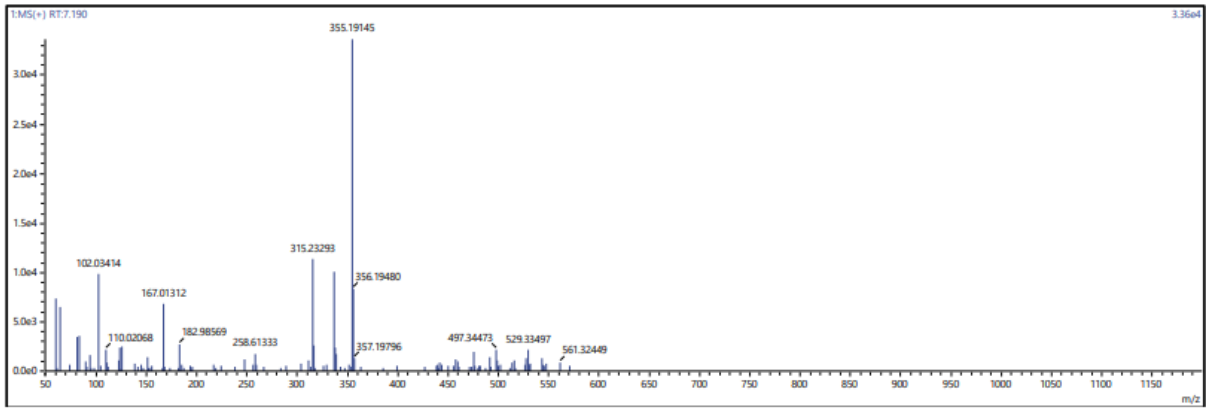
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for cannabinol (CBN) for m/z 311.20167, at retention time 5.820 min



Appendix 8.11: LC-MS chromatogram for tetrahydrocannabinol (THC)

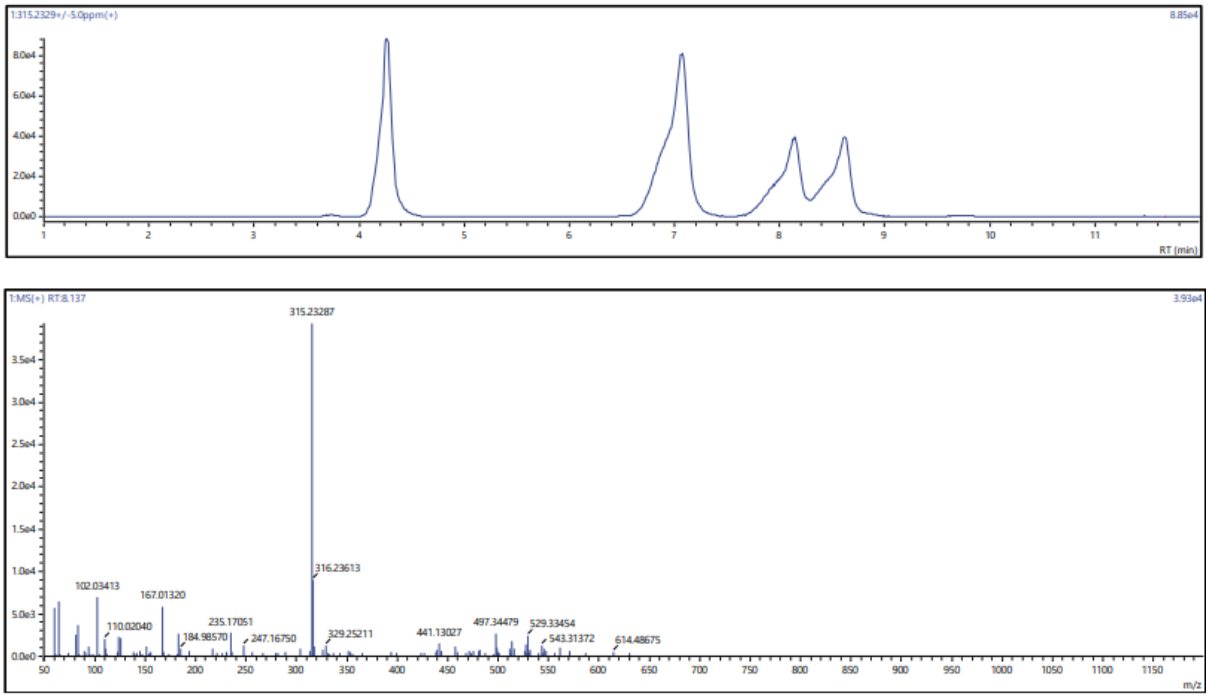
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for tetrahydrocannbinol (THC) for m/z 315.23294, at retention time 7.080 min





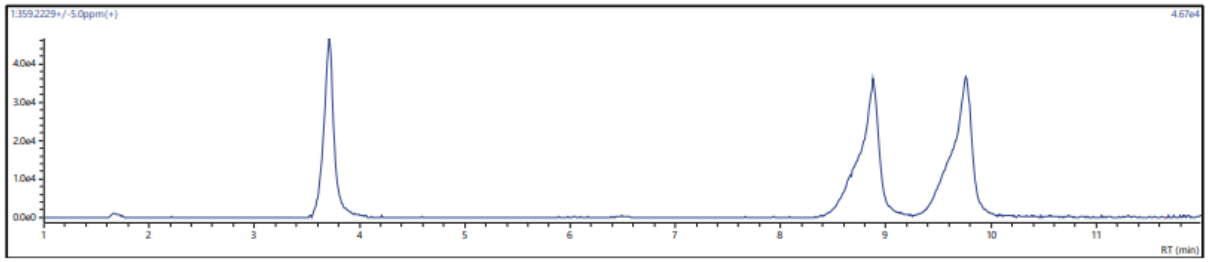
Appendix 8.12: LC-MS chromatogram for cannabinolic (CBNA)

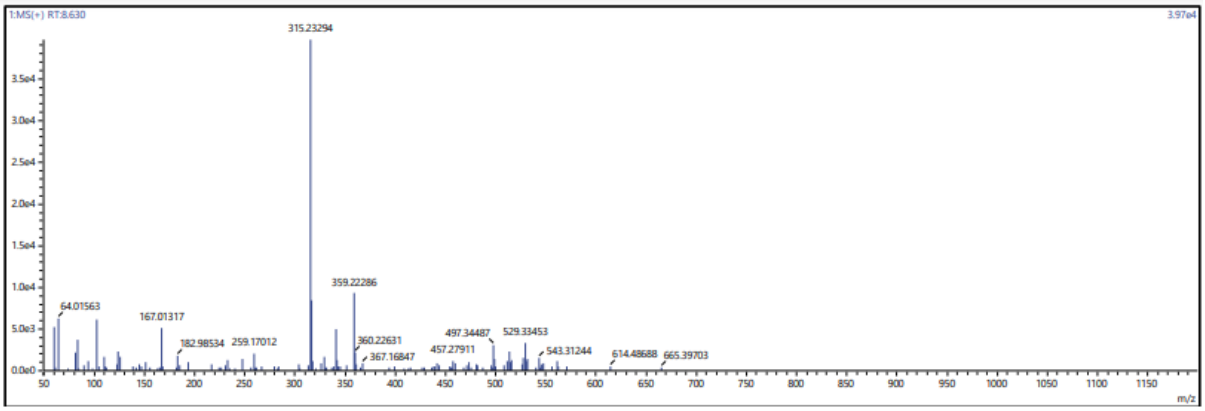
Liquid chromatorgraphy - mass spectrometry (LC-MS) chromatogram for cannabinolic acid (CBNA) for m/z 355.19145, at retention time 7.190 min



Appendix 8.13: LC-MS chromatogram for cannabicyclol (CBL)

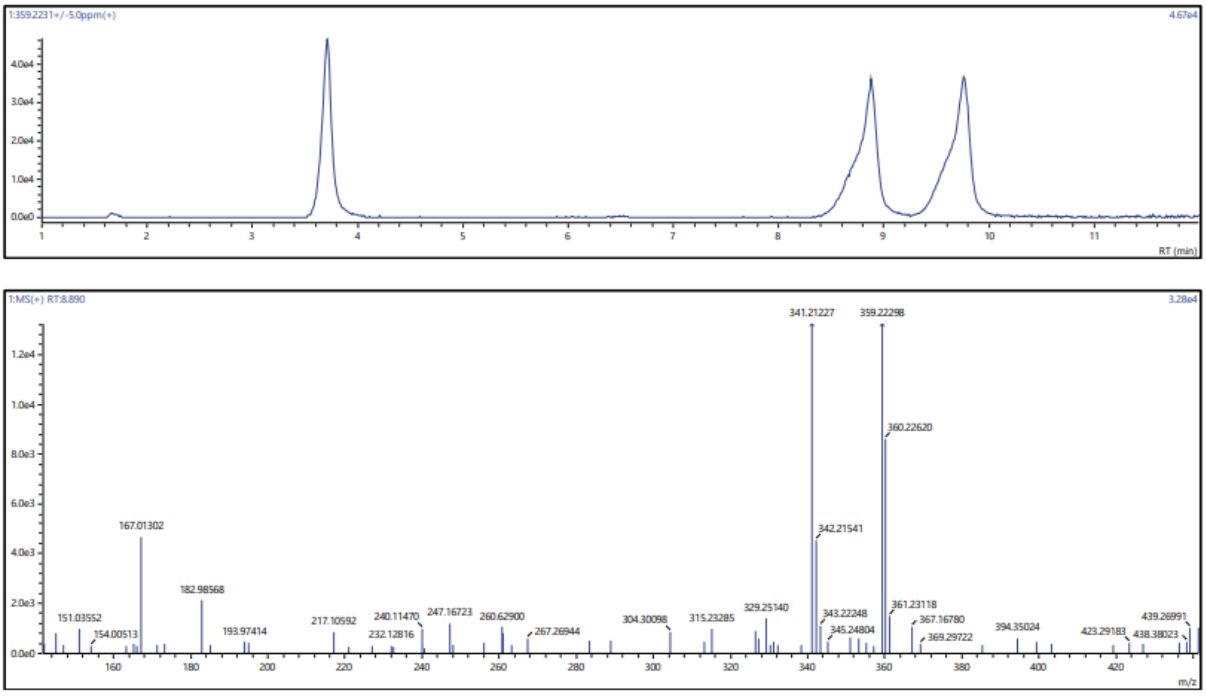
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for cannabicyclol acid (CBL) for m/z 315.23287, at retention time 8.137 min





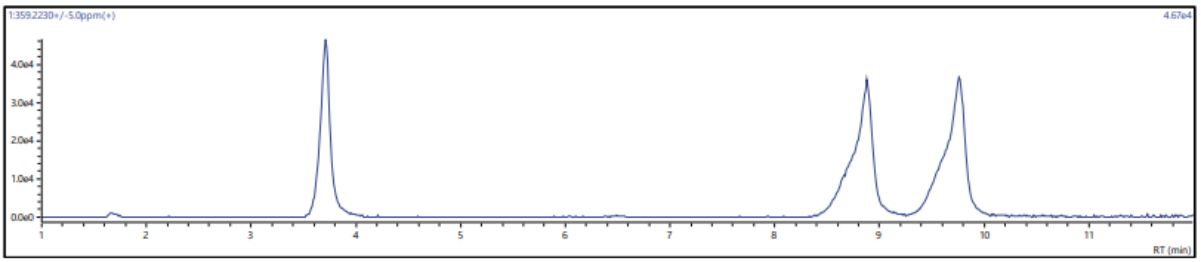
Appendix 8.14: LC-MS chromatogram for cannabichromene (CBC)

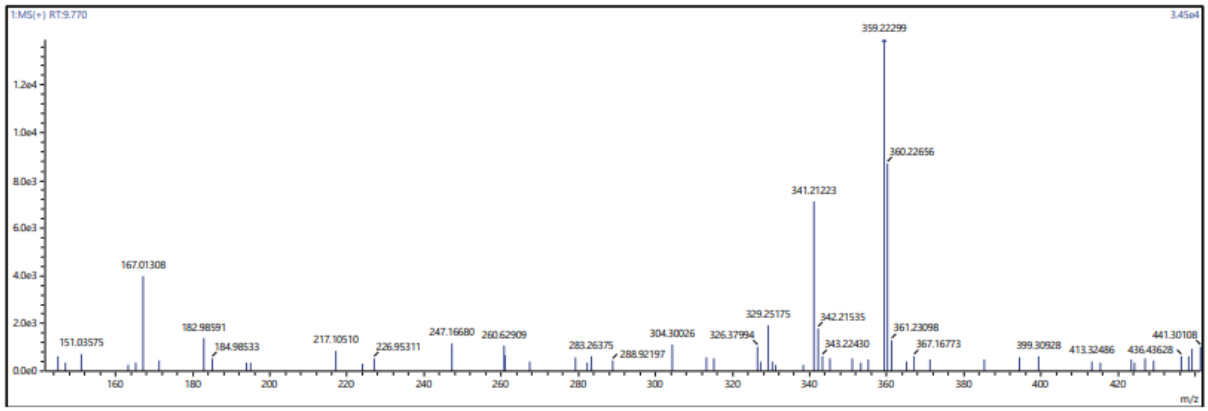
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for cannabichromene (CBC) for m/z 359.22286, at retention time 8.630 min



Appendix 8.15: LC-MS chromatogram for tetrahydrocannabinol acid (THCA)

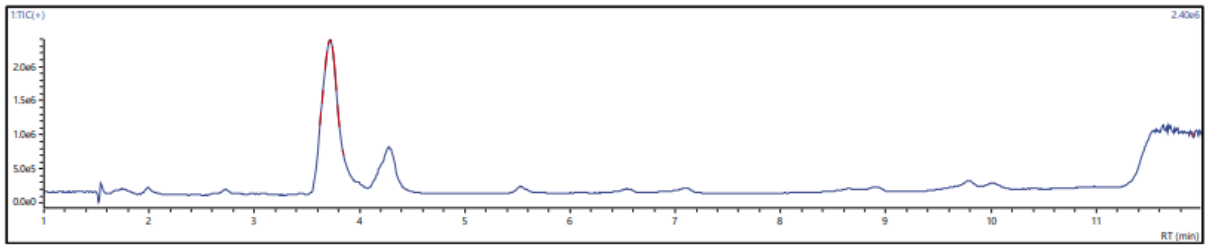
Liquid chromatography - mass spectrometry (LC-MS) chromatogram for tetrahydrocannabinolic acid (THCA) for m/z 359.22298, at retention time 8.890 min



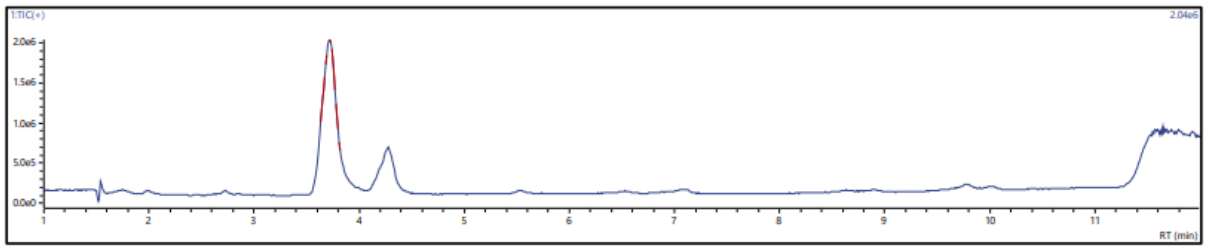


Appendix 8.16: LC-MS chromatogram for cannabichromenic acid (CBCA)

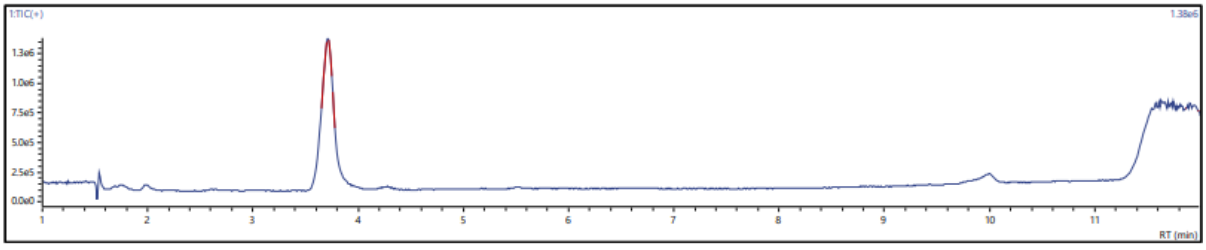
Liquid chromatography - mass spectrometry (LC-MS) for cannabichromenic acid (CBCA) for m/z 359.22299, at retention time 9.770 min



Appendix 8.17: LC-MS total ion chromatogram for the acetone extract



Appendix 8.18: LC-MS total ion chromatogram for the ethanolic extract



Appendix 8.19: LC-MS total ion chromatogram for the aqueous extract

Appendix 8.20: Recipe for the preparation of the TPC reagents

Ethanol:	10%: in a 1L media bottle, add 100 mL of ethanol (Saarchem Cat Nr: 2233540LP) to 900 mL H_2O.

Folin Reagent:	In a 15 mL screw cap tube, add 1 mL Folin-Ciocalteu phenol reagent (Merck Cat Nr: 109001) (Reagent rack) to 9 mL H_2O and mix well. Prepare fresh on day of analysis. This mix should have a bright yellow colour, if otherwise, discard.
Sodium Reagent:	7.5%: In a 100 mL media bottle, weigh 7.50 g Na_2CO_3 (Aldrich Cat Nr: 223530) (Reagent Rack) and add 100 mL H_2O. Mix until dissolved. Store at room temperature for up to a month.
Gallic acid standard:	In a 50 mL screw cap tube, dissolve 40 mg (0.40 g) gallic acid (Sigma Cat Nr: G7384) (Reagent rack) in 50 mL 10% ethanol to give a stock concentration of 800 mg/L. Prepare fresh. Use this solution as the stock standard. Check: When diluted 50 x with 10% ethanol, this solution should give an absorbance of 0.509 ± 0.010 at 280 nm.
Control:	In a 50 mL screw cap tube, dissolve 10 mg (0.0010 g) gallic acid (Sigma Cat Nr: G7384) (Reagent Rack) in 50 mL 10% ethanol (200 mg/L). Prepare fresh. Use this solution as the control. Check: When diluted, 12.5 x with 10% ethanol solution should give an absorbance of 0.509 ± 0.010 at 280 nm.

Appendix 8.21: Recipe for the preparation of the FRAP reagents

Acetate Buffer:	300 mM, pH 3.6 In a 1 L media bottle, add the following: 1.627 g sodium carbonate (Reagent rack). 16 mL glacial acetic acid (SAARCHEM Cat Nr: 1021000) (Reagent rack). Make up with dH_2O to 1 L.

	Check pH, store at room temperature for up to 1 month.
Dilute HCl:	40 mM (SAARCHEM Cat Nr: 100319 LP) In a 1 L media bottle, add the following: 1.46 mL concentrated HCl (32% HCl) (Reagent rack). Make up with dH_2O to 1 L. Store at room temperature for up to 6 months.
TPTZ (2,4,6-tri[2-pyridyl]-s-triazine):	10 mM (Sigma Cat Nr: T1253). In a 15 mL conical tube, add the following: 0.0093 g TPTZ (Fridge). 3 mL of 40 mM HCl. Make fresh on the day of assay.
Iron (III) chloride hexahydrate:	20 mM (F2877) In a 50 mL conical tube, add the following: 0.054 g $FeCl_3.6H_2O$ (Reagent rack). 10 mL dH_2O Make fresh on the day of assay.
L-Ascorbic acid standard:	L-Ascorbic acid (Sigma Cat Nr: A5960)

	Prepare 1 mM solution: Weigh 0.0088 g Ascorbic acid (Reagent rack) in a 50 mL centrifuge tube and add 50 mL dH_2O. Mix until dissolved. Prepare fresh. Use this solution as the stock solution.
Control:	L-Ascorbic acid 400 µM (Sigma Cat Nr: A5960) Weigh 0.00352 g ascorbic acid (Reagent rack) in a 50 mL centrifuge tube and add 50 mL dH_2O. Mix until dissolved. Use this solution as the control. Check: In an Eppendorf tube, dilute 311 µL of this solution with 1189 µL H_2O to obtain a concentration of 83 µM. This solution should give an absorbance of 0.830 ± at 265 nm.

Appendix 8.22: Recipe for the preparation of the DPPH reagents

DPPH:	7 mM (Sigma Cat Nr: A1888) Weigh out 0.0192 g of DPPH (Fridge) in a 15 mL screw cap tube and add 5 mL MeOH. Mix until dissolved. Prepare fresh.
Standard (Trolox, also known as 6-hydrox-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid):	1 mM (Aldrich Cat Nr: 238831) Weigh 0.0125 g Trolox in a 50 mL screw cap tube and add 50 mL of ethanol (SAARCHEM Cat Nr: 2233540LP) (Reagent rack). Mix until dissolved. Prepare fresh. Use this solution as the stock standard. Check: When diluted 5 x with ethanol, this solution should give an absorbance of 0.650 ± 0.015 at 289 nm.
Control (Trolox, also known as 6-hydrox-2, 5, 7, 8-	200 µM (Aldrich Cat Nr: 238831):

tetramethylchroman-2-carboxylic acid):	Dissolve 0.0025 g Trolox (Reagent rack) in a 50 mL of ethanol (Saarchem Cat Nr: 2233540LP). Prepare fresh, use this solution as the stock control. Check: When used as is, this solution should give an absorbance of 0.650 ± 0.015 at 289 nm.
	3 mL of 40 mM HCl.

Appendix 8.23: Recipe for the preparation of TBARS reagents

Phosphoric acid (H_3PO_4) stocks can be stored at room temperature:	Prepare 2% H_3PO_4 by pipetting 1177 µL H_3PO_4 in 48.823 mL dH_2O in pre-labelled glass test tubes. Prepare 7% H_3PO_4 by mixing (4.1 mL H_3PO_4 in 45.9 mL dH_2O). Prepare 1 M HCl, 4.92 mL from 32% HCl topped up to 50 mL. 3 mM HCl (30 µL from 1 M stock in 9.97 mL dH_2O). 20 mM Butylated hydroxytoluene (BHT) stock by [440.8 mg BHT in 100 mL ethanol]. TBA/Butylated hydroxytoluene (BHT) solution 0.1 g sodium hydroxide [NaOH]; 0.5 g TBA; 250 µL BHT from 20 mM stock, all dissolved and made up to 50 mL using dH_2O.

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health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE ANALYTICAL & RESEARCH PURPOSES.

Date of Issue: 04 May 2021	Expiry Date: 05 May 2022	Permit No: POS 019/2021/2022

Authority is hereby granted in terms of Section 22A (9)(a)(i) of the above-mentioned Act to **Dr Naeem Sheik Abdul** of to **Cape Peninsula University of Technology,Applied Microbial and Health Biotechnology Institute, Symphony Way (off Robert Sobukwe drive), Bellville, Cape Town,7530** acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **04 May 2021** to **05 May 2022.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
± 5kg Cannabis plant to be cultivated	Schedule-6 Schedule-4	± 35 [grams] Tetrahydrocannabinol (THC) ± 79 [grams] Cannabidiol (CBD)

Total Items: 2

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical & Research Purposes** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Dr Naeem Sheik Abdul**

   ID Number: **9212095116085**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Dr Naeem Sheik Abdul**

   ID Number: **9212095116085**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **Cape Peninsula University of Technology,Applied Microbial and Health Biotechnology Institute, Symphony Way (off Robert Sobukwe drive), Bellville, Cape Town,7530** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **05 May 2022** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

DocuSigned by:
Dr Sandile Buthelezi
BCE9DAE489C04EE...
DIRECTOR-GENERAL:
DR SANDILE BUTHELEZI

Appendix 8.24: Department of Health (DOH) permit for 2022 – 2023 for *Cannabis sativa* plant material

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE ANALYTICAL & RESEARCH PURPOSES.

Date of Issue: 08 June 2023	Expiry Date: 07 June 2024	Permit No: POS 202/2023/2024

Authority is hereby granted in terms of Section 22A (9)(a)(i) of the above-mentioned Act to **Dr Naeem Sheik Abdul** of to **Cape Peninsula University of Technology, Applied Microbial and Health Biotechnology Institute, Symphony Way (off Robert Sobukwe drive), Bellville, Cape Town,7530** acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **08 June 2023** to **07 June 2024.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
± 5kg Cannabis plant to be cultivated	Schedule-6	± 35 [grams] Tetrahydrocannabinol (THC)
	Schedule-4	± 79 [grams] Cannabidiol (CBD)

Total Items: **2**

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical & Research Purposes** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Dr Naeem Sheik Abdul**

   ID Number: **921209 5116 085**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Dr Naeem Sheik Abdul**

   ID Number: **921209 5116 085**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit.**
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **Cape Peninsula University of Technology, Applied Microbial and Health Biotechnology Institute, Symphony Way (off Robert Sobukwe drive), Bellville, Cape Town,7530** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **07 June 2024** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

Digitally Signed by:
Sandile Buthelezi

DIRECTOR-GENERAL OF HEALTH:
DR SANDILE BUTHELEZI
DATE:
23/06/2023 08:36:55 AM

Appendix 8.25: Department of Health (DOH) permit 2023 – 2024 for *Cannabis sativa* plant material