Investigating the antidiabetic potential of the combination of *Catharanthus roseus* and *Portulacaria afra* leaf extracts

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Dissertation accepted in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Sciences at the North-West University

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DECLARATION

I, Brunhildé De Vos, hereby declare that the dissertation "**Investigating the antidiabetic potential of the combination of *Catharanthus roseus* and *Portulacaria afra* leaf extracts**" is my own work and that all sources of information were acknowledged and referenced in accordance with the NWU Harvard referencing guideline. This research has not previously been submitted by myself at any university.

Signature:

Brunhildé De Vos
Date: 19/03/2021

PREFACE

The dissertation was written according to guidelines of the North-West University (NWU) for postgraduate studies.

All *in vitro* assays were carried out by myself after successful completion of short course on basic cell culturing techniques. Dr Wihan Pheiffer, co-supervisor, helped with the experimental design, research methodology, analysis of the data and interpretation of the results. The results were analysed and interpreted by myself and Dr Wihan Pheiffer.

The *in vivo* study was carried out by myself after successful completion of a short course in animal handling and the principles of research on animals. Dr Ashwell Ndhlala, assistant supervisor, conceptualised the project, provided the plants and assisted with the extraction methods of the aqueous leaf extracts. Prof Rose Hayeshi and Dr Trevor Nyakudya, supervisor and co-supervisor, assisted with the experimental design, analysis of data and the interpretation of results. The results were analysed and interpreted by myself, Prof Rose Hayeshi and Dr Trevor Nyakudya. animal study was carried out in fulfilment of the NWU code of conduct for researchers with ethics number NWU-00570-19-A5 in the AAALAC accredited animal facility (DSI/NWU Preclinical Drug Development Platform (PCDDP) Vivarium). The intravenous induction of hyperglycaemia (using streptozotocin) in rats was performed by the vivarium laboratory animal technologists, Mr Cornelius (Cor) Bester with help from Mr Jacobus (Kobus) Venter. Treatment of plant extracts via oral gavage and animal euthanasia were also carried out by Mr Venter and Mr Bester. I was involved in the oral gavage, body weight measurements, monitoring of animal well-being and collecting of organs upon euthanasia. Captured *in vivo* data were sent to Dr Erika Fourie for statistical analysis at the Statistical Consultation Services, NWU Potchefstroom Campus.

Chapter 2 is a literature review written in article format (unpublished) in accordance with the Authors guideline of Journal of Natural Medicines. Chapters 3 and 4 are also written in article format (unpublished). NWU Harvard referencing style is used throughout this dissertation and the references are provided at the end of each chapter.

DEDICATION

This study is dedicated to my iron-willed mother Reina De Vos, diagnosed with type 2 diabetes mellitus. Insulin and metformin managed your condition, but at a cost; you battled countless side effects. Being a strong/protective mother, you hushed a lot of suffering. One thing is for sure; no human being should ever have to choose between health and happiness. You can't go back and change the diagnoses, but we can start today and change the ending. It was your love for herbs that tickled my scientific curiosity. Together, we will find the remedy; one herb at a time.

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- I would also like to give special thanks to the financial support provided by the NRF and the DSI/NWU PCDDP.
- Lastly, a message to my Dad in heaven, I want to thank you so much for watching over me and guiding me through difficult times. Your love and grace have never failed me.

ABSTRACT

Background: Diabetes mellitus is a chronic disease that causes high blood sugar. The cost and side effects of modern antidiabetic drugs have become a major burden to patients suffering from diabetes, especially those from developing countries. As a result, medicinal plants are considered a favourable alternative for antidiabetic treatment in developing countries. In South Africa there is anecdotal evidence that the 1:1 combination of *Catharanthus roseus* (*C. roseus*) and *Portulacaria afra* (*P. afra*) are being used to treat symptoms of diabetes mellitus. There are multiple scientific reports on the *in vitro* and *in vivo* antidiabetic potential of *C. roseus*, whilst there is insufficient data on the antidiabetic effects of *P. afra* and its combination with *C. roseus*.

Aim: This present study investigated the antidiabetic potential of the aqueous leaf extract of *Catharanthus roseus* and *Portulacaria afra*, independently and in combination (1:1), in both *in vitro* and *in vivo* models.

Methods: In the *in vitro* study, the safety of the plants was determined by measuring the cytotoxicity against HepG2 cell line. In addition, the antidiabetic potentials of the plant extracts were evaluated by measuring the enzyme activity (α-amylase, α-glucosidase and hexokinase) — common markers of diabetes therapy —in the HepG2 cells. An *in vivo* study was conducted using a chemically induced diabetic rat model. Diabetes was induced in male Sprague Dawley rats (50) by a single intravenous injection of streptozotocin at 55 mg/kg body weight. Animals varying between seven to nine weeks of age were randomly assigned to six groups; non-diabetic (n = 10), diabetic non-treated (n = 8), metformin (500 mg/kg body weight, n = 8), *C. roseus* (CR, 100 mg/kg body weight, n = 8), *P. afra* (PA, 100 mg/kg body weight, n = 8) and 1:1 combination of *C. roseus* and *P. afra* (CR:PA, 100 mg/kg body weight, n = 8). The rats were treated by receiving an oral administration of the aqueous leaf extracts of CR, PA and CR:PA for 28 consecutive days.

Results: The 1:1 extract combination of *C. roseus* and *P. afra*, with a IC_{50} value of 4.10 µg/mL (i.e. NR assay), had the highest cytotoxicity on the HepG2 cell line, with statistical significance (p < 0.05), compared to *P. afra* (IC_{50} = 27.93 µg/mL). Extract of *C. roseus* (IC_{50} = 6.02 µg/mL i.e. NR assay) was also cytotoxic towards liver cells, whilst *P. afra* had the least cytotoxic effect on the liver cells (IC_{50} = 27.93 µg/mL i.e. NR assay). Further cytotoxic investigation (i.e. MTT assay) showed that the interference of copper compounds with MTT formazan, rendered the MTT assay to be less suitable for cell viability measurements when compared to the neutral red assay. The results of the *in vitro* assays showed that the plant extracts had no effects on the α-glucosidase activity in HepG2 cells. However, the inhibition activity of *P. afra* and CR:PA extracts on α-amylase and the activation of the liver hexokinase enzyme by the plant extracts, suggested potential antidiabetic effects that are commonly involved in the reduction of blood glucose levels. The *in vivo* study demonstrated that daily oral gavage with *C. roseus* effectively lowered plasma glucose in diabetic rats. In addition, administration of *C. roseus* showed improved reversal effects in the

relative weights of the heart, liver and kidneys, whilst also maintaining the body weights in diabetic rats. In contrast, oral treatment with *P. afra* resulted in elevated plasma glucose levels and caused a supplemental increase in the relative liver and kidney weights. The treatment with *P. afra* to diabetic rats resulted in a significant decline in body weights ($p < 0.05$). The administration of CR:PA to diabetic rats exhibited similar results in the liver and kidneys, thus displaying an antagonistic effect between *C. roseus* and *P. afra*. While *C. roseus* lowered the blood glucose levels in diabetic rats, *P. afra* raised it. Further investigation showed that the relative weights of the pancreas were unaffected for all treatment groups, except for CR:PA-treated rats, which displayed a significant decrease in comparison to non-diabetic rats ($p < 0.05$). The reason for this incident is still not well understood and would require further evaluation through organ histology. Understanding the major physiological function of these two plant extracts would be crucial for future studies.

Conclusion: The present *in vitro* data displayed potential antidiabetic effects of aqueous leaf extracts of *P. afra* and its 1:1 combination with *C. roseus,* whilst this was not the case for the *in vivo* study. The leaf extract of *P. afra* caused hyperglycaemic effects in diabetic rats, meaning that the alleged antidiabetic effects of *P. afra,* as presented by the inhibition activity of α-amylase and activation of hexokinase (as seen by the *in vitro* data), had no lowering effects on the blood glucose levels in animal models. The *in vivo* data demonstrated that the combination of CR:PA displayed antagonistic effects in glycaemic control, as an effect that was produced by the contrasting actions of *C. roseus* and *P. afra*. The current study evidently revealed the shortcomings associated with *in vitro* studies, one of which was the misleading antidiabetic results that was presented by the *in vitro* data. Our study has conclusively shown that the efficacy of drugs is better evaluated when using complex *in vivo* models such as animal. However, one of the disadvantages of STZ is that it specifically targets β-cells in the pancreas. Therefore, the STZ model could contain potential limitations to the proposed testing of the antidiabetic effects of the plants. In addition, one would need to perform a carbohydrate-loading test to see whether the increased postprandial blood glucose levels are decreased by the plant extracts. In conclusion, *P. afra* and its 1:1 combination with *C. roseus* (CR:PA) leaf extracts have shown no antidiabetic effects in Sprague Dawley rats, whereas leaf extracts of *C. roseus* did show antidiabetic activities.

Keywords: Diabetes mellitus, Traditional healers, Aqueous leaf extracts, Medicinal plants, *Catharanthus roseus, Portulacaria afra,* Cell viability, Enzyme biomarkers, Body weight, Streptozotocin, Hypoglycaemia

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARC-VOP	Agricultural Research Council-Vegetable and Ornamental Plant
ATP	Adenosine triphosphate
BC	Before Christ
BW	Body weight
CO_2	Carbon dioxide
CR	*Catharanthus roseus*
CR:PA	*Catharanthus roseus*: *Portulacaria afra* (1:1)
CVD	Cardiovascular diseases
DMEM	Dulbecco`s Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNC	Diabetic non-treated control
EDTA	Ethylenediamine tetra-acetic acid
FBG	Fasting blood glucose
FBS	Fetal bovine serum
GI	Gastrointestinal
G. Don	George Don (Binomial name)
G-6-P	Glucose-6-phosphatase
HepG2	Human hepatoma liver cell line
HK	Hexokinase
HPLC	High-performance liquid chromatography
IC_{50}	Concentration of plant extract that is needed to inhibit 50% cellular death, relative to an untreated control
IDF	International Diabetes Federation
ISO	International Standard Organisation
IVC	Individually ventilated caging
IV	Intravenous
Jacq.	Nikolaus Joseph von Jacquin (Binomial name)
L.	Carl Linnaeus (Binomial name)
IP	Intraperitonially
MET	Metformin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NDC	Non-diabetic control
NEAA	Non-essential amino acids (NEAA)
NR	Neutral red

NWU	North-West University
PA	*Portulacaria afra*
PBS	Phosphate buffered saline
pNPG	p-Nitrophenyl-α-D-glucopyranoside
RH	Relative humidity
ROS	Reactive oxygen species
SANS	South African National Standard
SANBI	South African National Botanical Institute
SD	Standard deviation
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
USA	United States of America
WHO	World Health Organisation
ZAR	South African Rand

LIST OF SYMBOLS/UNITS

®	Registered trademark
α	Alpha
β	Beta
Δ	Delta
°C	Degree Celsius
d	Effect size (practical significant difference)
g	Gram
g	Relative gravitational force
g/day	Grams per day
h	Hour(s)
kg	Kilogram(s)
mg	Milligram
mg/dL	Milligrams per decilitre
mg/mL	Milligram per millilitre
mg/min/mg protein	Milligram per minute per milligram protein
mg/kg	Milligram per kilogram
mg/kg body weight	Milligram per kilogram of body weight
mg/kg/day	Milligram per kilogram per day
mmol/L	Millimoles per litre
min	Minute(s)
mL	Millilitre
mL/g	Millilitre per gram
mL/kg	Millilitre per kilogram
nm	Nanometre
nmole/min/mL	Nanomole per minute per millilitre
%	Percentage
U/mg prot	Units per milligrams of protein
p	Statistically significant differences
v/v	Volume per volume
µg/mL	Microgram per millilitre
µL	Microliter

CHAPTER 1 RESEARCH SCOPE

1.1 Introduction

Diabetes mellitus is a common hazard to people in both poor and developed countries (Buowari, 2013). The lack of proper healthcare in low-income countries cause a higher percentage of deaths annually attributed to diabetes (WHO, 2020). Due to the lack of healthcare facilities, diabetic patients in low-income countries have limited access to synthetic antidiabetic medication (Grant, 2013). As an alternative, medicinal plants have been used for decades to manage diabetes in developing countries (Kasole *et al*., 2019). Research indicates that *Catharanthus roseus* (L.) G. Don, a flower plant in the Apocynaceae family, possess antidiabetic activities in diabetic-induced rats. On the contrary, *Portulacaria afra* Jacq., a flower plant in the Portulacaceae family, was reported to have positive antimicrobial effects in managing skin disorders (Nciki *et al*., 2016), whilst there is insufficient data on the antidiabetic effects of *P. afra* and its 1:1 combination with *C. roseus,* CR:PA. The antidiabetic potentials of *P. afra* and its 1:1 combination with *C. roseus* — CR:PA — were not previously reported by *in vitro* or *in vivo* studies.

The antidiabetic potentials of *P. afra* and its 1:1 combination with *C. roseus* (CR:PA) are mostly based on anecdotal evidence. Therefore, the treatment aim of these aqueous leaf extracts is to exhibit a blood glucose lowering effect in diabetic patients (Arumugam *et al*., 2013).

1.2 Research problem

In 2019, the number of diabetic patients worldwide was established at 463 million people with a projected increase to 700 million by 2045 (IDF Diabetes Atlas, 2019). The International Diabetes Federation (IDF, 2019) indicated that approximately 79.4% of all diabetic patients worldwide, aged between 20 to 79 years, live in low- and middle-income countries. For this reason, the increasing prevalence of diabetes is of global concern, especially in deprived countries where people are subjected to limited resources.

Synthetic antidiabetic treatments such as Symlin and metformin, exhibit adverse side effects and they are mostly unaffordable to the majority of diabetic patients in low-income countries (Marles & Farnsworth, 1995). According to Ekor (2014), the use of herbal medicinal supplements has increased significantly with approximately 80% of people worldwide relying on them as their primary health care. With the evolution of traditional drugs and technology, traditional medicinal plants are being used more readily in studies today. This provides researchers with the ability to investigate a more naturalised source of treatment for diseases such as diabetes mellitus (Ponnusamy *et al*., 2011), cancer and skin disorders (De Wet *et al*., 2013). The rich floral biodiversity of South Africa (Van de Venter *et al*., 2008) provides vast opportunities for antidiabetic research by investigating a more indigenous and naturalised source of diabetic treatment using

medicinal plants such as Madagascar periwinkle (*C. roseus*) and Elephant bush (*P. afra*). However, there is insufficient scientific evidence that supports the antidiabetic properties of *P. afra* and its 1:1 combination with *C. roseus*.

1.3 Aims and objectives

1.3.1 Aim

The aim of the current study is to investigate the antidiabetic activity of the 1:1 combination of aqueous leaf extracts of *C. roseus* and *P. afra* using *in vitro* and *in vivo* models *Catharanthus roseus* is a common antidiabetic drug and for this reason, this study would serve as a reiteration of its antidiabetic properties.

1.3.2 Objective

The aim of the study was achieved by the following objectives:

1. Evaluating the safety of aqueous *C. roseus* and *P. afra* leaf extracts individually and in 1:1 combination using *in vitro* assays

2. Determining the antidiabetic effect of *C. roseus* and *P. afra* leaf extracts *in vitro* by investigating their effects on enzymatic biomarkers relevant to diabetes therapy.

3. Evaluating the antidiabetic effect of *C. roseus* and *P. afra* leaf extracts, individually and in 1:1 combination, *in vivo* in streptozotocin-induced diabetic rats.

1.4 Study design

The antidiabetic effects of aqueous *C. roseus* and *P. afra* leaf extracts, independently and in combination, were evaluated using a combination of *in vitro* and *in vivo* studies.

1.4.1 *In vitro* (cell-based) study design

The safety of aqueous *C. roseus* and *P. afra* leaf extracts were evaluated *in vitro* using cytotoxicity and cell viability assays; MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and neutral red (NR) assays (Figure 1.1).

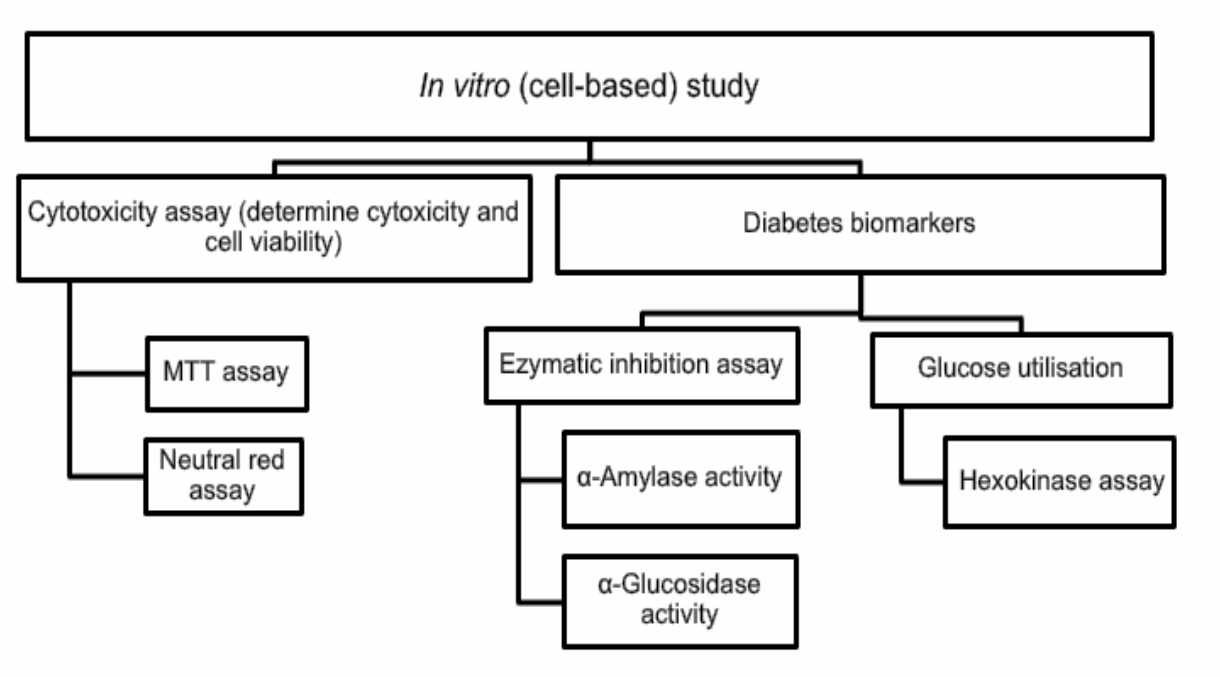

Figure 1.1: Study design of the *in vitro* assays used in the determination of antidiabetic activities.

The MTT assay is a colorimetric assay, of tetrazolium salt, that evaluates metabolic activities of cells as a measurement of cellular growth (Abhishek *et al*., 2018). The neutral red assay is used to measure the uptake of the neutral red dye by lysosomes (Repetto *et al*., 2008) which is a quantitative measurement of cell viability (Abhishek *et al*., 2018). The antidiabetic effect of *C. roseus* and *P. afra* leaf extracts were investigated *in vitro* by using diabetes biomarkers, i.e. the activity of enzymatic. The effects of *C. roseus* and *P. afra* extracts on α-amylase, α-glucosidase and hexokinase activity were determined according to the manufacturer's instructions in hepatocellular carcinoma (HepG2) cell line.

1.4.2 *In vivo* (animal) study design

Fifty Sprague Dawley male rats (N = 50) were used in this study with age varying between seven to nine weeks. Six groups comprising of eight to ten rats each (*n* = 8/10) were randomly divided into groups of two rats per cage (Figure 1.2). Type 1 diabetes mellitus was induced by a single intravenous injection of streptozotocin via tail vein at 55 mg/kg body weight to male Sprague Dawley rats. According to King (2012), the induction of type 1 diabetes is usually done by a single high dose of STZ. The effects of specific treatments, such as plant extracts, and their ability to enhance formulations of insulin are widely investigated using the high dose STZ model (King, 2012). In the current study, rats were chemically induced with a single high dose of STZ (55 mg/kg body weight) intravenously (IV). A high STZ induction volume is used to reduce the cost of the study, facilitating diabetes in rats in a shorter period of time. STZ has a rapid induction mechanism, making it a more favourable method, to ensure that the animals are not subjected to unnecessary long periods of STZ treatment. Long periods of STZ treatment give rise to multi-

organ streptozotocin toxicity that can adversely affect the wellbeing of rats and potentially cause death (ACUSC, 2016).

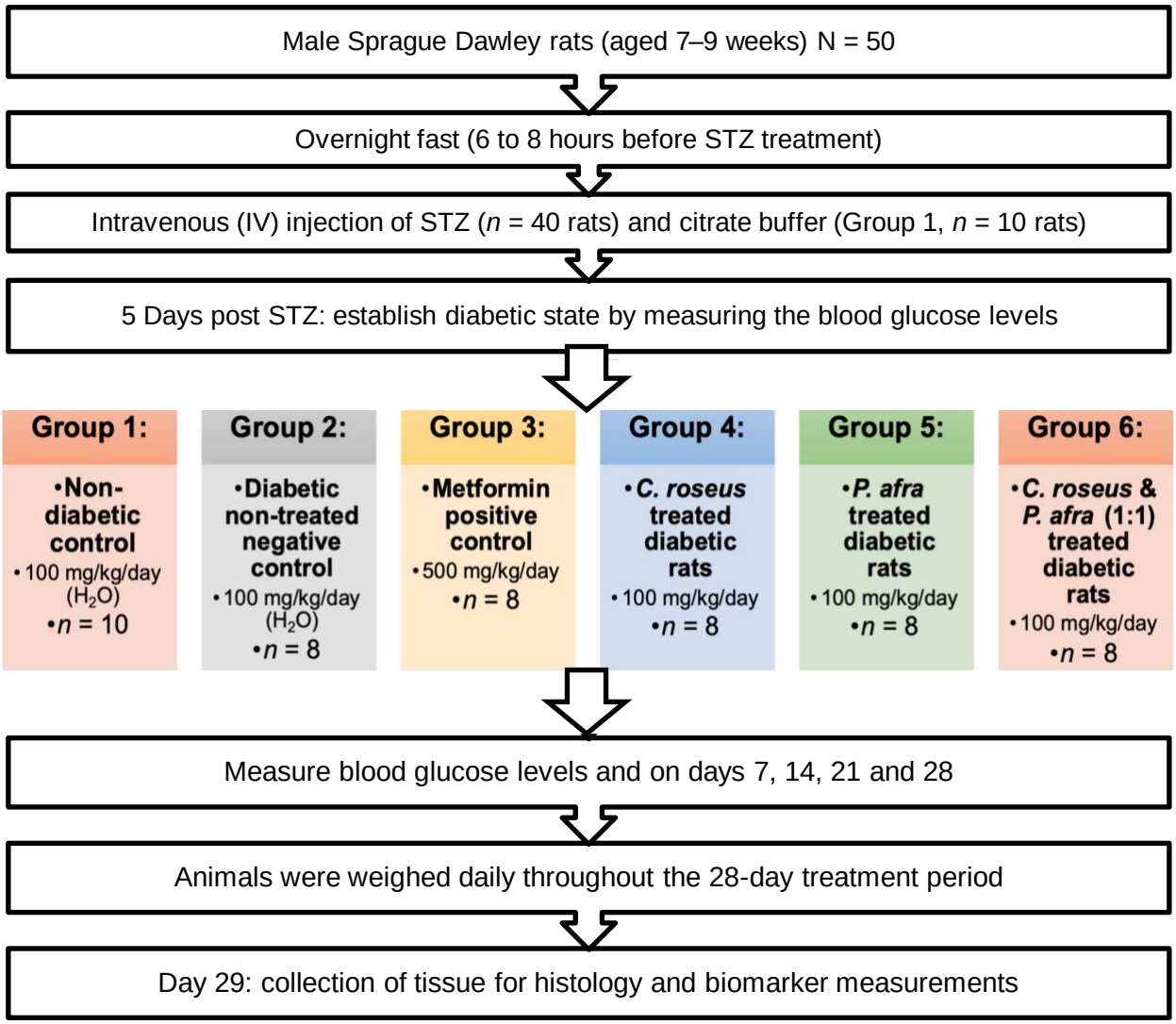

Figure 1.2: *In vivo* study design for evaluation of antidiabetic activities in male Sprague Dawley rats (*n* = 8 or 10).

1.5 Dissertation outline

Chapter 1 provides a brief description of the research background, research problem, aims, objectives, study design and dissertation outline.

Chapter 2 provides a comprehensive review of the literature on diabetes mellitus, with the prevalence of diabetes worldwide including Africa. In addition, this chapter will also describe the medicinal properties of *C. roseus* and *P. afra* together with literature background on their diverse applications. Finally, this chapter explores the antidiabetic activities of *C. roseus* and *P. afra*.

Chapter 3 investigates the cytotoxicity and enzymatic inhibition activities of the plant extracts by the *in vitro* screening of three biomarkers of diabetes; α-amylase, α-glucosidase and hexokinase activity.

Chapter 4 investigates the antidiabetic activities of aqueous leaf extracts of *C. roseus* and *P. afra* in animals.

Chapter 5 provides a brief summary of the study and gathers all the results to formulate a rational conclusion, with additional study limitations and future recommendations.

References presented at the end of each chapter.

Appendices added at the end of the dissertation.

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CHAPTER 2

Chapter 2 is a comprehensive review written according to the Authors guideline of Journal of Natural Medicines, an open access journal from Springer. For ease of reading, the figures and tables are inserted at their relevant positions. This manuscript will be submitted for publication upon confirmation of all contributing parties.

CHAPTER 2 LITERATURE REVIEW

A Review on the antidiabetic potential of *Catharanthus roseus* and *Portulacaria afra*

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Abstract: Diabetes mellitus is a common burden to patients in developed and developing countries. The lack of proper healthcare in low-income countries due to socio-economic challenges causes high mortality rates annually attributed to diabetes. Due to lack of healthcare facilities, diabetic patients in low-income countries have limited access to synthetic antidiabetic medication such as insulin. As an alternative, traditional medicinal plants have been used locally for decades to manage diabetes mellitus. These plants are commonly used as a health care option for the prevention of diseases and ensuring well-being. In numerous scientific literatures, *Catharanthus roseus* is reported to possess antidiabetic activities in diabetic-induced rats. On the contrary, *Portulacaria afra* is reported to have positive antimicrobial effects in managing skin disorders, whilst there is insufficient data on the antidiabetic effects of *P. afra* and its 1:1 combination with *C. roseus*. Traditional healers believe that the combination of *Catharanthus roseus* and *Portulacaria afra* (1:1) decreases blood glucose levels while also managing diabetes symptoms. The 1:1 mixture of these two plants, as an antidiabetic treatment are novel. In this literature review we gather information on the application and antidiabetic potentials of *Catharanthus roseus* and *Portulacaria afra,* which are believed to be attributed to their phytochemical constituents (flavonoids, alkaloids, tannins and saponins).

Keywords: Diabetes prevalence, Phytochemicals, *Catharanthus roseus, Portulacaria afra*, Traditional medicine, Antidiabetic effect.

2.1 Introduction

2.1.1 Diabetes mellitus

Diabetes mellitus is a well-known risk factor of metabolic disorders that are associated with the endocrine system (Li *et al*., 2004). It is characterised by persistent hyperglycaemia and disturbances of protein, carbohydrate and fat metabolism (Bhutkar, 2018). Diabetes mellitus either diminishes the production of insulin by the pancreas or the body does not appropriately respond to the insulin produced by the pancreas (Egan & Dinneen, 2019). Insulin is produced by the pancreatic beta (β)-cells and plays a crucial role in homeostasis of blood glucose levels (Gaikwad *et al.,* 2014). The pancreatic β-cells secrete insulin in the presence of high blood glucose levels, whereas the pancreatic α-cells release glucagon to raise the blood glucose levels when it drops too low. If there is insufficient insulin production by the β-cells, glucose cannot enter the cells for further metabolism and it builds up in the blood, leading to hyperglycaemia (Belinda, 2004). Hyperglycaemia is associated with severe complications, such as oxidative stress, abnormally high levels of lipids in the blood (hyperlipidaemia), cardiovascular diseases (CVDs) such as heart attacks (Firdous, 2014), and enzymatic protein glycation (Sagbo *et al.,* 2018).

Diabetes can be classified as either type 1 (insulin dependent), type 2 (non-insulin dependent) or gestational diabetes mellitus (Punthakee *et al.,* 2018). Type 1 diabetes mellitus (T1DM) occurs when the pancreas is unable to produce insulin, possibly due to dysfunction or damage to the pancreatic β-cells (Weinstock, 2018). In contrast, type 2 diabetes mellitus (T2DM) usually results from insufficient cellular response to adequate amounts of insulin possibly from defective receptors (Cantley & Ashcroft, 2015). However, both T1DM and T2DM are a result of multi-gene predisposition and environmental triggers (IDF, 2019). The International Diabetes Federation (IDF) reported in 2019 that 5–10% of all diabetic cases suffer from T1DM while 90–95% suffer from T2DM. Gestational diabetes emerges during pregnancy as a result of glucose intolerance (Punthakee *et al.,* 2018). Increased blood glucose levels during pregnancy are caused by the hormones released by placenta (Freemark, 2006). Other less common types of diabetes such as the monogenic diabetes, represents 1.5–2% of all cases globally. Monogenic diabetes is caused from a single gene mutation rather than the contribution of multiple genes, including environmental factors as seen in T1DM and T2DM (Murphy *et al*., 2008). This form of diabetes is very often misdiagnosed as either T1DM and T2DM.

2.1.2 Epidemiology of diabetes

Type II diabetes mellitus (T2DM) is a severe health issue worldwide and treatment with synthetic antidiabetic medication is expensive and shows adverse side effects (Gupta *et al*., 2016). According to the World Health Organisation (WHO, 2018), more than 400 million adults suffer

from T2DM with approximately 1.6 million deaths recorded in 2016 (WHO, 2016), owing to treatment failures, low patient compliance and dangerous side effects. However, a recent update from the International Diabetes Federation, IDF Diabetes Atlas in 2019, indicated an increase from 400 to 463 million people diagnosed with T2DM worldwide (Figure 2.1). Globally, the diagnosed diabetic cases are estimated to further increase to 578 million by 2030 and 700 million by 2045 (Figure 2.1). The prevalence of diabetes increases annually with the greatest increase being recorded in low- and middle-income countries (Koye *et al*., 2018).

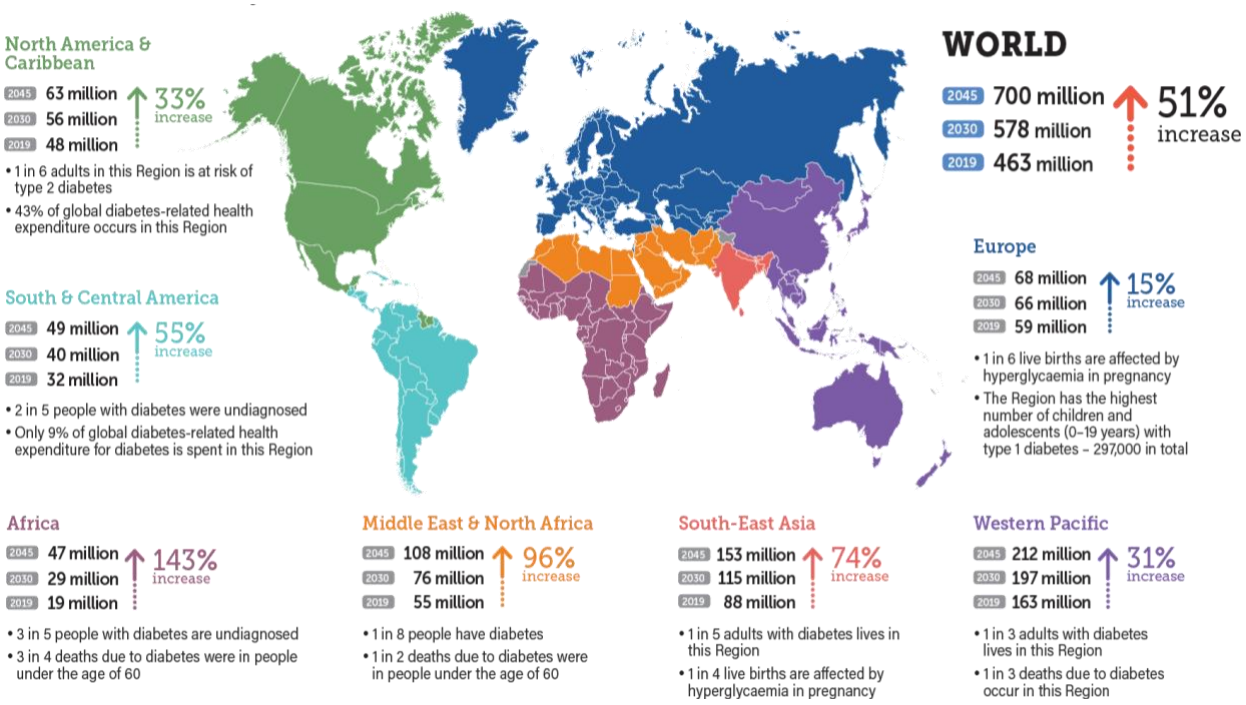

Figure 2.1: Number of adults, between the age of 20 and 79 years, diagnosed with diabetes worldwide and per region in 2019 with an estimate T2DM prevalence in 2030 and 2045 (with permission from International Diabetes Federation. IDF Diabetes Atlas, 9th ed. Brussels, Belgium: International Diabetes Federation, 2019. http://www.diabetesatlas.org)

Despite the alarming prevalence of T2DM, Glovaci *et al.* (2019) indicated that about 193 million people, globally, are unaware of their disease status due to the lack of healthcare facilities and minimal signs of symptoms. According to the IDF Diabetes Atlas (2019) an estimate of 73 countries worldwide do not have in-country data sources that satisfy the IDF Atlas inclusion criteria. The numbers displayed in Figure 2.2, show that the majority of countries without in-country data sources are from Africa. This makes it very difficult to accurately determine the global prevalence of T2DM. For example, in 2019, China was reported as the country with the highest number of people diagnosed with T2DM with an estimate of 116 million diagnoses (Saeedi *et al*., 2019). The elevated prevalence of diabetes in China is not because they are more prone to develop T2DM but due to their economic and technological advancement that allowed them to provide accurate data from in-country data sources (Figure 2.2). On the contrary, Africa has the lowest number (19 million) of people diagnosed with T2DM worldwide (Figure 2.1) not because

populations are less prone to develop diabetes but because Africa consists of numerous middle- to low-income countries whose ability to generate data from in-country sources are limited.

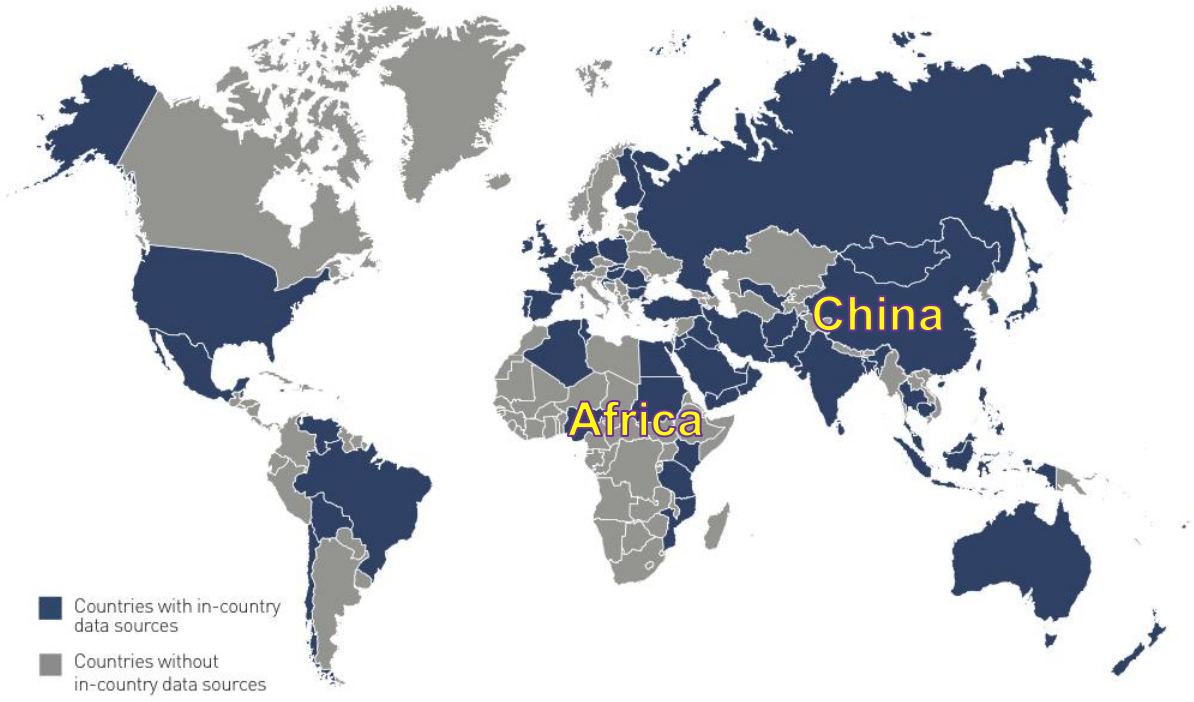

Figure 2.2: A visual representation of countries and regions with in-country data sources on the relative number of adults (20–79 years) with undiagnosed diabetes (with permission from International Diabetes Federation. IDF Diabetes Atlas, 9th ed. Brussels, Belgium: International Diabetes Federation, 2019. http://www.diabetesatlas.org)

Diabetes carries an enormous healthcare and financial burden worldwide (Htay *et al*., 2019). Management of T2DM contributes to a large percentage of the healthcare expenditures worldwide. In 2007, the health expenditure due to T2DM increased from USD 232 billion spent globally to USD 727 billion in 2017 (IDF Diabetes Atlas, 2019). The health expenditure for 2019 was at least USD 760 billion (IDF Diabetes Atlas, 2019). North America and the Caribbean region comprised the highest proportion (42.7%) of the total diabetes-related health expenditure in 2019, closely followed by the Western Pacific region (21.3%) and the European region (21.2%) (IDF Diabetes Atlas, 2019). According to Moucheraud *et al*. (2019) people in low- and middle-income countries tend to spend more for T2DM treatment due to their lack of health insurance. The cost of T2DM treatment depend greatly on the geographic location. In 2019, the highest percentage (19.4%) of the total health spending was allocated to diabetes in the South and Central America region, followed by 15.2% observed in the Middle East and Northern Africa region (IDF Diabetes Atlas, 2019).

2.1.3 Prevalence of type 2 diabetes mellitus in Africa

According to Cho *et al*. (2018), the highest percentage of undiagnosed diabetic patients (59.7%) are in Africa. Africa is one of the seven IDF continents that was subjected to diabetic research

and it was found that an astonishing 19 million adults (18–99 years) were living with T2DM in Africa, which represents a regional prevalence of 3.9% in 2019 (IDF, 2017). The number of T2DM cases is predicted to increase with 48% by 2030 and 143% by 2045, which is the highest growing increase of all the IDF regions (IDF Diabetes Atlas, 2019). It is also important to note that according to the IDF in 2019, the most densely inhabited countries in Africa (Table 2.1), had the highest number of people diagnosed with T2DM between the ages of 20 to 79 years (Cho *et al.,* 2018). In addition, an alarming 73.7% of all deaths (globally), attributed to diabetes before the age of 60, are found in the African region (IDF, 2017*;* Cho *et al*., 2018).

Table 2.1: Top Four African countries ranked from highest to lowest number of diabetic patients.

Rank	Country in Africa	Income group	Number of diabetic patients
#1	South Africa	Upper-middle	4.6 million
#2	Nigeria	Lower-middle	2.7 million
#3	Democratic Republic of Congo	Low	1.8 million
#4	Ethiopia	Low	1.7 million

According to IDF (2012), the number of diabetic patients changes according to the variation across regions and income groups. For analytical purposes, the World Bank (2018) categorises economies into four income groups; low, lower-middle, upper-middle, and high income (Appendix B: Figure B.1). The majority of African countries fall in the low and lower-middle income group. Furthermore, the IDF 2019 Diabetes Atlas indicated that 45.9% of the 19.4 million adults diagnosed with diabetes in the Africa region live in low-income countries while 54.1% live in middle-income countries. The socio-economic challenges in Africa correlate with the large and increasing burden for diabetic treatment. The high prevalence of T2DM in Africa is due to the lack of social, financial and economic development among other factors (Cho *et al.,* 2018). Poor dietary choices and lack of exercise have also been implicated in the development of metabolic dysfunction among Africans.

2.1.4 Prevalence of type 2 diabetes mellitus in South Africa

The IDF 2019 Diabetes Atlas shows that approximately 12.7% of adults (aged between 20 –79 years) in South Africa have diabetes. South Africa had the highest number of people diagnosed with diabetes (4.6 million) in 2019, which comprises of the largest proportion of all diabetic cases recorded in the Africa region (Table 2.1). The increase in urbanisation and population aging, pose an ever-growing challenge for T2DM (Basu *et al*., 2013; IDF, 2019). According to Erzse *et al*. (2019) T2DM requires continuous clinical care and management that consumes significant healthcare resources. They showed that the annual medical cost of diagnosed T2DM, attributed to 240,000 patients in South Africa, was recorded at roughly ZAR 2.7 billion in 2018 (Erzse *et al*.,

2019), which makes up nearly 12% of the national health budget. Treatment of diabetes focuses primarily on preventing hyperglycaemic complications. In order to manage T2DM, pharmacological therapy would be required.

2.1.5 Pharmacological treatment and management of diabetes

The three most common treatments include a healthy diet, oral hypoglycaemic medication (glipizide, metformin, repaglinide, etc) and insulin treatment (Ganesan & Sultan, 2019). The management of diabetes focuses primarily on glycaemic control (Imran *et al*., 2018). Diabetes complications have shown to increase the probability of microvascular and macrovascular complications (Harding *et al*., 2019). On the other hand, the strict and intensive management of blood glucose levels have proven to reduce these vascular complications (Cavaiola & Pettus, 2017). Overnutrition is one of the main factors that increases the risk of T2DM (Nathan *et al*., 2009).

2.1.6 Pharmacological treatment regimens used for T2DM

Various methods are used to manage type 2 diabetes mellitus (T2DM). The most common is through administration of oral pharmaceutical agents such as metformin or by adopting a healthy lifestyle. For instance, managing dietary intake and physical exercise are two of the basic elements used for the treatment of T2DM (Marin-Penalver *et al*., 2016). Having an unhealthy diet with high levels of calories, coupled with the adoption of sedentary lifestyles, contribute to an increase in body weight and obesity (Glovaci *et al*., 2019). Physical exercise benefits diabetic patients by improving glycaemic control, increasing insulin sensitivity (Phielix *et al*., 2010), and maintaining a healthy body weight. One of the most common pharmacological treatment of T2DM, still being used today, is metformin (Irons & Minze, 2014).

2.1.6.1 Antidiabetic treatment: metformin

Metformin is derived from the *Galega officinalis* plant and is well known for its hypoglycaemic effects (Goboza *et al*., 2016). According to Marin-Penalver *et al*. (2016) metformin decreases the fasting blood glucose by nearly 20% in T2DM patients. Metformin is physiologically responsible for decreasing hepatic production by increasing glucose utilisation in the gut. Although Rena *et al*. (2017) confirmed that metformin has multiple sites of action. It is thus understandable that studies such as Foretz *et al*. (2014) are also considering metformin as a cancer prevention drug since it is inexpensive and abide to common antineoplastic agents. As alluded to earlier, metformin acts physiologically on the liver by decreasing glucose production while also increasing glucose utilisation in the gut. According to Foretz *et al*. (2014), the hyperglycaemic effect of metformin is exhibited through inhibition of hepatic gluconeogenesis (the process whereby energy is converted into glucose). The primary site of action of metformin is the mitochondria. For this

reason, it is also responsible for enhancing insulin sensitivity, on a molecular level, by inhibiting the mitochondrial respiratory chain in the liver. Regardless of the mechanism of action, studies such as Rena *et al*. (2017), confirmed metformin's capability to manage diabetes-related complications with clear benefits associated with the glucose mechanism. Just like all synthetic medication, metformin has its own limitations which include side effects such as nausea, diarrhoea, abdominal discomfort and decreased intestinal absorption of Vitamin B12 (Marin-Penalver *et al*., 2016). Vitamin B12 keeps the body's nervous system and red blood cells healthy, and its deficiency may result in general tiredness and weakness among other symptoms (Chapman *et al*., 2016). For this reason, routine measurement of vitamin B12 will be required together with metformin treatment, which adds to the total cost of diabetes treatment. Not only is metformin treatment too expensive for people in developing countries (rural areas), but it is also not readily available to them (Chikowe *et al*., 2018). Due to the cost and availability of metformin, diabetic patients in developing countries rely on the use of traditional medicinal plants as a cheaper and safer alternative. There is an urgent need for people living with diabetes, especially in low- and middle-income countries (Table 2.1), to gain access to diabetic treatment and adjust their lifestyles as far as diet and exercise is concerned. As alluded to by Moucheraud *et al*. (2019), pharmacological antidiabetic treatment regimens are expensive for many people, in particular those from low-middle income countries. Moreover, low- and middle-income countries have limited access to proper diabetes management options. The average cost for healthcare in people diagnosed with diabetes in poor countries is twice as much in comparison to people without diabetes (Mbanya *et al*., 2010). According to Cho *et al*. (2018), the amount of money spent on global healthcare for diabetes, for people between the ages 20–79 years in 2017, was projected at USD 727 billion (ZAR 102 trillion) from which only 6% of the global healthcare budget originated from the Africa region. It is for this reason among others, that an increasing number of studies on alternative antidiabetic treatments have been initiated (Ipek *et al*., 2019; Sunny *et al*., 2019).

Given the high prevalence of T2DM and the adverse effects of pharmacological agents used in the treatment of T2DM, there is a need to investigate alternative and complementary approaches such as medicinal plants.

2.1.7 Therapeutic value of medicinal plants

Medicinal plants have been used as a source of medicine in the development of human cultures (Dar *et al*., 2017). Since time immemorial, humans have relied heavily on plants for food and their primary healthcare (Shava, 2000). According to Inoue *et al*. (2019), medicinal and aromatic plants were used for food flavouring, medicines, preservatives, beauty and decorations. Over time, our ancestors ate plants and likely discovered these medicines through trial and error. They managed to accumulate a lot of indigenous knowledge on medicinal plants, which was then passed down the generation over several years. The oldest record of medicinal herbs was found in the Alps,

5000–3000 BC, in a spectacularly preserved mummy known as Ötzi the Iceman (Dixon & Aldous, 2014). Medicinal plants have become an important part of human life. The time immemorial applications of medicinal plants are best represented by Chinese medicine, which dates back to more than 2000 years of history (Hao *et al*., 2017). The earliest inscription of Chinese medicine, found on tortoise shells and bones, were transcribed by the Chinese during the Shang Dynasty (11th to 15th century BC). Chinese were frontiers in traditional medicines in the first centuries. And because of that, numerous medicinal plants and herbal medicines are being used today as a more natural medication (Hao *et al*., 2017). Nowadays, scientific experimentation makes it possible to safely establish the curative qualities and therapeutic value of medicinal plants.

The therapeutic effects of medicinal plants have been assigned to the presence of biologically active compounds (phytochemicals) such as flavonoids, saponins, lignans and propenylphenols (Bacanl *et al.,* 2019). According to Koche *et al*. (2018), these phytochemicals can be classified as primary or secondary phytochemicals, depending on their role in plant metabolism. Thus, phytochemicals can be defined as constituents of herbal medicine (produced by plants) and medicinal plants with healing and therapeutic properties (Srivastava *et al*., 2019). According to Bacanli *et al*. (2019), certain phytochemicals can improve insulin sensitivity, which combats the complications associated with diabetes. Phenolic compounds, such as flavonoids, are considered good phytochemicals that have antioxidant, anti-inflammatory and antidiabetic potential (Olaokun *et al.,* 2017). Gaikwad *et al*. (2014) stated that phytomedicine is a progressing alternative therapy that can be used for diabetic treatment. The combination of phytochemicals are also known to have synergistic effects (Liu, 2003; Zhao *et al*., 2020).

A brief summary of the various biological activities represented by each phytochemical compound is given in Table 2.2. It is clear that phytochemical compound classes such as flavonoids, tend to have more than one biological activity (antioxidant, anti-inflammatory, Table 2.2). It is for this reason, that the phytochemical content of medicinal plants contributes greatly to their application in traditional treatments. The hereditary knowledge of medicinal plants is vital for the livelihoods of several African communities as it enhances traditional health deliverance (Mahomoodally, 2013; Mbuni *et al*., 2020).

Table 2.2: Phytochemicals and their role in health care

Phytochemical Classes	Phytochemical Sub-Classes	Role in healthcare (biological activity)	References
Phenolic compounds	Flavonoids	Antioxidant, anti-inflammatory, anticancer and antimicrobial activities	Koche *et al*., 2018 Panche *et al*., 2016 Singh and Kumar, 2019
Phenolic compounds	Tannins	Antimicrobial, anticancer, antiviral and anti-inflammatory	Sieniawska, 2015 Koche *et al*., 2018

Nitrogen compounds	Alkaloids	Antihyperglycaemic, anticancer, antimalarial and antimicrobial	Koche *et al*., 2018 Othman *et al*., 2019
	Saponins	Antioxidant, antimicrobial, antidiabetic and anti-inflammatory activity	Koche *et al*., 2018 Jin *et al*., 2017 Sparg *et al*., 2004
Phenolic compounds	Polyphenols	Antioxidant, anti-inflammatory, antimicrobial and metabolic regulation functions	Ly *et al*., 2014 Othman *et al*., 2019
Terpene compounds	Terpenoids	Antimicrobial, antimalarial, detoxifying agents and anticarcinogenic	Koche *et al*., 2018 Gutiérrez-del-Río *et al*., 2018

2.1.8 Medicinal plant-based antidiabetic treatment in South Africa

The floral biodiversity of South Africa expanded the cultural traditions of plant use (Van de Venter *et al.,* 2008). South Africa encompasses over 30,000 higher plant species from which 3,000 are being used in traditional medicines (Van Wyk *et al.,* 1997). It is also stated by Van Niekerk (2012), that in the year of 2012, there were an estimate of between 200,000 and 300,000 traditional healers in South Africa with a healer-patient ratio of 1:500–1200. These traditional healers are renowned for their extensive knowledge of herbs and plants (Van Niekerk, 2012).

Since time immemorial, there has been an exponential increase in investigations on traditional herbal remedies such as indigenous plants with medicinal properties, as affordable, natural and alternative remedies for diabetes. For example, the bitter gourd herb (*Momordica charantia*) is used as a native antidiabetic drug in Africa and Asia (Modak *et al*., 2007). This is because traditional plants are readily available in these countries and are considered as safe alternatives (Arumugam *et al.,* 2013; WHO, 2019). The evolution of traditional medicine provides numerous alternative and complementary treatment options to manage various diseases that pose as a threat to humanity. In fact, the World Health Organisation advocates for and support studies on the use of traditional plants for the treatment of various diseases such as T2DM, cancer and skin disorders (WHO, 2019).

There is an enormous amount of research opportunities in South Africa, especially on indigenous medicinal plants, to discover novel biological activities for medicinal plants (Gericke, 2011). These medicinal plants are used as remedies that were prepared by the folk, through the knowledge that was passed down from generation to generation and prepared using either the whole plant or different parts, like leaf, stem and flower, to name the few (Khan, 2011). Randrianarivony *et al*. (2017), reported that, of all the plant parts, the leaves were the most commonly used, which accounts for 54% of all herbal preparations, while the seeds and fruits were the least used (1%).

Traditional healers commonly prepare these herbs through a method called decoction, which is done by boiling the leaves in water and administering it orally for long periods of time (Erasto *et al.,* 2005). The exploitation of medicinal plants, by local people, is valuable in managing the community's health and biodiversity (Ajaib *et al.,* 2010).

According to Street and Prinsloo (2012), traditional plants such as the devil's claw, *Harpagophytum procumbens* and the African potato, *Hypoxis hemerocallidea*, are being used in South Africa as a cheaper alternative and more effective antidiabetic drug. Antidiabetic drugs are known for their hypoglycaemic effect that reduces blood glucose levels, enhances insulin activities and antioxidant levels (Duraisamy *et al*., 2012). A total of 54 medicinal plants have been reported to possess antidiabetic properties that have been identified in South Africa for the treatment of T2DM (Afolayan & Sunmonu, 2010; Davids *et al*., 2016). A compilation of these medicinal plants that are commonly used in the management of T2DM is shown in Table 2.3. However, Afolayan and Sunmonu (2010) found that previous literature only documented nine of these medicinal plants to possess *in vivo* antidiabetic activities. The remaining 23 species were evaluated for antidiabetic activities through various *in vitro* models. Unfortunately, studies that only focus on *in vitro* screening models are restricted to a single cell line, enzymes and metabolic pathways. The data in Table 2.3, emphasizes the use of multiple plant species for the management of diabetes mellitus. The health promotion and therapeutic effects of medicinal plants (Table 2.3) increases the search for new, naturalized sources of drugs.

Table 2.3: Documented medicinal plants used for the treatment of diabetes in South Africa.

Plant Family	Species	Common names	Part of plant used	References
Aizoaceae	*Carpobrotus edulis* (L.) N.E.Br.	Freeway iceplant	Leaves, juice, fruit	Davids *et al*., 2016
Aizoaceae	*Sceletium tortuosum* (L.) N.E. Br.	Kougoed[a]	Leaves, roots	Davids *et al*., 2016
Alliaceae	*Tulbaghia violacea Harv*.	Wild garlic, wildeknoffel[a]	Leaves, roots	Davids *et al*., 2016
Anacardiaceae	*Sclerocarya birrea Hochst*.*	Marula	Stem, bark, roots	Van de Venter *et al*., 2008
			Stem, bark	Van Wyk 2008b
Anacardiaceae	*Searsia burchellii* (Sond. Ex Engl.) Moffett.	Karroo Kuni-bush	Leaves, roots, stem	Davids *et al*., 2016
Apiaceae	*Heteromorphica arborescens* H.	Parsley tree, pietersieliebos[a]	Leaves, roots	Erasto *et al*., 2005
Apiaceae	*Lichtensteinia lacera Cham*. & Schltdl.		Leaves, stem	Davids *et al*., 2016
Apiaceae or Umbelliferae	*Petroselenium crispum* (*Mill*)	Parsley	Leaves	Thring and Weitz, 2006
Apocynaceae	*Catharanthus roseus* (L) G. Don*	Madagascar Periwinkle, Jasmine	Leaves	Erasto *et al*., 2005
			Leaves, twigs	Van de Venter *et al*., 2008

Apocynaceae	*Hoodia gordonii* (Masson) Sweet ex Decne*	Bitter ghaap[e]	Inner stem	Davids *et al*., 2016
Apocynaceae	*Vinca major L*.	Greater periwinkle or bigleaf periwinkle	Leaves, roots, stem	Van de Venter *et al*., 2008
Asphodelaceae	*Aloe ferox* Mill. *	Bitter aloe, cape aloe or red aloe	Leaves/juice	Davids *et al*., 2016
Asphodelaceae	*Bulbine natalensis Mill.*	Bulbine, Rooiwortel[a]	Roots	Erasto *et al*., 2005
Asphodelaceae	*Bulbine frutescens L.*	Stalked bulbine, snake flower, cat's tail or geelkatstert[a]	Roots	Erasto *et al*., 2005
Asteraceae	*Artemisia afra* Jacq. Ex Willd.*	Wormwood or Bitterals[a], Wildealsem	Leaves, roots Leaves Leaves Leaves Leaves	Erasto *et al*., 2005 Davids *et al*., 2016 Thring and Weitz, 2006 Van Wyk 2008b Erasto *et al*., 2005
Asteraceae	*Brachylaena discolor* DC.*	Coastal Silver-oak or Vaalbos[a]	Leaves, roots, stem	Van de Venter *et al*., 2008
Asteraceae	*Brachylaena elliptica* Thunb.	Pepperbark tree	Leaves	Van Wyk 2008a
Asteraceae	*Chrysocoma ciliate* L.	Bitter bush or bitterbos[a]	Leaves, roots	Davids *et al*., 2016
Asteraceae	*Conyza scabrida* DC.	Oven Bush	Leaves	Thring and Weitz, 2006
Asteraceae	*Elytropappus rhinocerotis* (L.f.)	Rhinoceros bush	Leaves	Thring and Weitz, 2006
Asteraceae	*Euryops abrotanifolius* (L.) DC.	Lace-leaf euryops, mountain resin bush	Leaves, stem	Davids *et al*., 2016
Asteraceae	*Helichrysum nudifolium* L.	Hottentot's tea	Leaves, roots	Erasto *et al*., 2005
Asteraceae	*Helichrysum odoratissimum* L.	Everlasting	Whole plant	Erasto *et al*., 2005
Asteraceae	*Helichrysum petiolare* H & B.L	Silver bush everlasting	Whole plant	Erasto *et al*., 2005
Asteraceae	*Tagetes minuta* L.	Khaki bush	Leaves	Davids *et al*., 2016
Asteraceae	*Vernonia oligocephala* Sch. Bip.	Bicoloured-leaved vernonia	Leaves, roots, stem Leaves	Erasto *et al*., 2005 Thring and Weitz, 2006
Asteraceae	*Vernonia amygdalina* Del.	English bitter leaf	Leaves	Erasto *et al*., 2005
Brassicaceae	*Cadaba aphylla* (Thunb.) Wild	Leafless wormbush, desert spray	Leaves, Stem	Davids *et al*., 2016
Buddlejaceae	*Chilianthus olearaceus* Burch.	Wild elder	Leaves, twigs	Erasto *et al*., 2005
Cannabaceae	*Cannabis sativa* L.	Cannabis	Leaves	Van de Venter *et al*., 2008
Celastraceae	*Catha edulis Forsk*. Ex Endl.	Somali tea	Leaves, roots, stem	Van de Venter *et al*., 2008
Compositae	*Dicerothamnus rhinocerotis* (L.f.) Koek.	Rhinoceros bush	Leaves, stem	Davids *et al*., 2016

Convolvulaceae	*Convolvulus capensis* Burm. f.	Cape Bindweed or Bobbejaantou[a]	Bulb	Davids *et al*., 2016
Crassulaceae	*Crassula muscosa* L.	Lizard's tail	Leaves, stem, roots, flower	Davids *et al*., 2016
Crassulaceae	*Tylecodon paniculatus* (L.f.) Toelken	Butter bush	Leaves, stem	Davids *et al*., 2016
Cucurbitaceae	*Momordica balsamina* L.*	African Cucumber, balsam Apple	Stem, flowers	Van de Venter *et al*., 2008
Cucurbitaceae	*Momordica foetida Schumach*.	Gifappel[a]	Whole plant	Van de Venter *et al*., 2008
Euphorbiaceae	*Ricinus communis* L	Castor bean	Leaves	Thring and Weitz, 2006
Fabaceae	Sutherlandia frutescens L. *	Cancer bush	Leaves	Van Wyk, 2008b
Fabaceae Lindl.	*Lessertia frutescens* (L.) Golblatt & J.C. Manning	Balloon Pea, Cancer bush	Leaves	Davids *et al*., 2016
Gentianaceae	*Chironia baccifera* L.	Christmas berry or bitterbossie[a]	Whole plant	Van de Venter *et al*., 2008
Geraniaceae	*Pelargonium antidysentericum* (Eckl. & Zeyh.) Kostel.	Rooistorm[a]	Roots	Davids *et al*., 2016
Hypoxidaceae	*Hypoxis colchicifolia Bak*.	Broad-leaved hypoxis	Corms	Erasto *et al*., 2005
Hypoxidaceae	*Hypoxis hemerocallidea Fisch*.*	Star flower, yellow star or sterblom[a]	Corms	Erasto *et al*., 2005
Lamiaceae	*Ballota africana (L.) Benth*	Cat herb	Leaves	Davids *et al*., 2016
Lamiaceae	*Leonotis leonurus* L.	Cape hemp	Leaves, flowers	Thring and Weitz, 2006
Lamiaceae	*Leonotis leonurus* (L.) R.Br.	Lion's tail, lion's ear, wild dagga or rooidagga[a]	Leaves, roots, flower	Davids *et al*., 2016
Lamiaceae	*Mentha longifolia* (L.) L.	Wild mint	Leaves, stem	Davids *et al*., 2016
Lamiaceae	*Salvia africana-caerulea* L.	Blue sage, wild sage, purple sage or blousalie[a]	Leaves	Davids *et al*., 2016
Menispermaceae	Cissampelos *capensis* L.f.	Davidjies[a]	Leaves	Van de Venter *et al*., 2008
Myrtaceae	*Psidium guajava* L.	Guava	Leaves	Van de Venter *et al*., 2008
Portulacaceae	*Portulacaria afra*	Spekboom[a], Elephant bush	Leaves	Hulley and Van Wyk, 2019
Rubiaceae	*Galium tomentosum* Thunb.	Old Man's Beard or Rooivergeet[a]	Roots	Van Wyk *et al*., 2008
Rutaceae	*Diosma oppositifolia* L.	Bitter buchu	Leaves, stem, flower	Davids *et al*., 2016
Rutaceae	*Ruta graveolens* L.	Rue, common rue or herb-of-grace	Leaves Leaves	Thring and Weitz, 2006 Van Wyk, 2008a
Thymelaeaceae	*Gnidia deserticola* Gilg	Night- or evening- scented bush	Leaves, stem, roots,	Davids *et al*., 2016

[*]Used *in vivo*
[a] *Afrikaans*
[e] *English*

2.2 Antidiabetic potential of selected medicinal plants: *Catharanthus roseus* and *Portulacaria afra*

In this review we have selected *Catharanthus roseus* and *Portulacaria afra* and we aim to provide the state of the current knowledge on the use of these medicinal plants and their therapeutically important phytochemicals in the management of diabetes.

2.2.1 Medicinal plant: Catharanthus roseus

Catharanthus roseus (L.) G. Don, also known as the Madagascar periwinkle (English), Kanniedood (Afrikaans) and *Isishushlungu* (isiZulu) belongs to the Apocynaceae family (Fapohunda, *et al.,* 2018). This plant grows 30–100 cm wide, the flower itself consists of five petals (Nisar *et al.,* 2016), forming a variety of pink-, white- or purple-coloured flowers (Fapohunda *et al.,* 2018). The leaves appear glossy green with an oval to oblong shape that can range between 2.5–9.5 cm in length and 1–3.5 cm in width (Nisar *et al.,* 2016). It is widely used as a garden ornament or traditional cure agent (Malathi *et al.,* 2010). This plant can be found near the coast and inland on riverbanks, in open forests or scrubs (Fern, 2019). It is a popular traditional medicine widely used in Africa and Asia (Fern, 2019). *Catharanthus roseus* is endemic to Madagascar and in South Africa, it can be found in the Limpopo, Gauteng, North West and KwaZulu-Natal provinces as an invasive species (Invasive Species South Africa, 2019). According to Barrales-Cureño *et al*. (2019), *C. roseus* has over 120 alkaloids of which 70 are pharmacologically active. This plant has therapeutic potential (Ponnusamy *et al.,* 2011) and according to Rasineni *et al*. (2010), the hot water decoction of *C. roseus* has been used as a chemotherapeutic agent because of their plant alkaloids: vincristine and vinblastine, which was also cited by Alexandrova *et al.* (2000) and Van der Heijden *et al.* (2004).

This plant has been used to treat various diseases in numerous countries. According to Tropical Plants Database (Fern, 2019), a decoction of leaf extracts of *C. roseus* are used to treat hypertension, asthma, menstrual irregularities, chronic constipation, diarrhoea, indigestion, malaria, dengue fever, diabetes, cancer and skin disease. *Catharanthus roseus* was first discovered by the Europeans (Afolayan & Sunmonu, 2010) and it was primarily used as a traditional cure for diabetes (Swanston-Flatt *et al.,* 1989). In countries such as Australia, Brazil, Thailand, England, South Africa, Pakistan and Jamaica, among others, *C. roseus* is consumed as a decoction for the treatment of diabetes, cancer and menorrhagia (Nisar *et al.,* 2016). In China, the whole plant is used as a menstrual regulator, cough medicine, diuretic and an astringent (Farnsworth, 1961; Virmani *et al.,* 1978). In India, the entire plant is boiled in water and

administered orally for cancer treatment (Virmani *et al.,* 1978). Evidence seems to suggest that *C. roseus* is mainly used for the treatment of T2DM.

2.2.2 Phytochemical constituents of *C. roseus*

Previous studies have shown possible antioxidant, antimicrobial, anti-inflammatory as well as antidiabetic potential in plant extracts of *Catharanthus roseus* (Barrales-Cureño *et al*., 2019; Kabesh *et al*. 2015: Oluwaseun & Saliu, 2018). A study conducted by Malathi *et al.* (2010), reported that alcoholic leaf and flower extracts of *C. roseus* possessed the following phytochemicals: flavones, phenol, tannins, glycosides, reducing sugars, and alkaloids. Vega-Avila *et al*. (2012), reported that aqueous leaf extract of *C. roseus* tested positive for polyphenols and glycosides. While aqueous root extract of *C. roseus* tested positive for alkaloids and terpenoids (Vega-Avila *et al*., 2012). All these phytochemical classes: alkaloids, flavonoids, glycosides and saponins are well-known for their antidiabetic effects (Gaikwad *et al*., 2014; Tiong *et al*., 2013). The most well-known alkaloids of *C. roseus* include vincristine and vinblastine, and according to Naeem and Khan (2017) these alkaloids are of medicinal and pharmaceutical importance.

2.2.3 Pharmacological actions of *C. roseus*

2.2.3.1 Antidiabetic activity

The antidiabetic potentials of *C. roseus* has been recorded in several studies. For example, the antidiabetic activity of *C. roseus* was confirmed in a study conducted by Vega-Avila *et al*. (2012), where they evaluated each part of the plant (flower, root, leaf and stem) in healthy and alloxan-induced diabetic mice. The study found that aqueous leaf extract of *C. roseus* (250 mg/kg) have shown blood glucose lowering effects (reduced plasma glucose by 42% after 6 h) in alloxan-induced diabetic mice (Table 2.4). Furthermore, aqueous extract of *C. roseus* decreased the plasma glucose by nearly half the initial fasting blood glucose levels, regardless of the plant part (flower, root, leaf and stem) that was used for the treatment (Table 2.4). In addition, they reported a maximum reduction in blood glucose levels of 50.61 ± 8.78% (6 h) in healthy mice treated with aqueous leaf extract of *C. roseus* (250 mg/kg). Not only does this indicate the hypoglycaemic effects of aqueous extract of *C. roseus*, but it also shows that *C. roseus* has the ability to lower blood glucose levels in non-diabetic cases as well, which ultimately makes it a good antidiabetic drug.

In addition, aqueous leaf extract of *C. roseus* used at a dose of 1 g/kg for 21 days was reported to reduce blood glucose levels by 20% (Table 2.5) in diabetic murine *in vivo* models in comparison to methanol and dichloromethane extracts, at a dose of 500 mg/kg for 7 to 15 days, which lowered the blood glucose levels by 49–58% (Singh *et al*., 2001). These results were also confirmed in

streptozotocin (STZ)-induced diabetic rats by Singh *et al*. (2001), using aqueous and dichlotomethane: methanol (1:1) extract at a dosage of 1g/kg and 500 mg/kg. It was found that the aqueous crude extract exhibited a 20% reduction in blood glucose within 21 days while dichlotomethane: methanol (1:1) exhibited a 58% hypoglycaemic activity within 15 days of treatment. A study conducted by Nammi *et al*. (2003), on alloxan-induced diabetic rabbits, reported a dose-dependent decrease in blood glucose levels within 20 hours of oral administration of the leaf juice of *C. roseus*. According to a study by Prasad *et al*. (2009), STZ-induced diabetic rats, receiving a daily dose of aqueous leaf extract of *C. roseus* (50 mg/day) for 15 days, displayed a significant decrease in blood glucose levels from 292.00 mg/dL to 156.33 mg/dL.

Table 2.4: Percentage blood glucose reduction displayed by aqueous extract of *Catharanthus roseus* in alloxan-induced diabetic mice (Vega-Avila *et al*., 2012)

Treatment (n = 6)	Dose (mg/kg)	Percent blood glucose reduction (6h)
Aqueous flower	250 mg/kg	51.68 ± 15.84%
Aqueous root	250 mg/kg	47.61 ± 5.34%
Aqueous leaf	250 mg/kg	41.25 ± 7.38%
Aqueous stem	250 mg/kg	52.94 ± 5.96%

For further elaboration, Table 2.5 provides a list of studies conducted on the antidiabetic effects of *C. roseus*. Chloroform leaf extracts of *C. roseus* (Table 2.5) was the only treatment that presented sudden deaths in animals. It was speculated that the deaths were due to the presence of some toxic alkaloids: tropane, piperidine, pyrrolizidine and indolizidine (Islam *et al*., 2009; Thawabteh *et al*., 2019). According to Rasineni *et al*. (2010), aqueous leaf extract of *C. roseus* at a dose of 100 mg/kg/60 days was the most effective antihyperglycaemic combination with a 78% reduction in blood glucose levels (Table 2.5). While methanolic whole plant extracts of *C. roseus* in male and female Wistar rats reported the lowest (13%) reduction in blood glucose levels (Mostofa *et al*., 2007).

An equal number of the studies in Table 2.5, used streptozotocin (STZ) or alloxan to induce diabetes. The differences between using STZ or alloxan-induced diabetic models are chosen based on the study's specific endpoint, limitations, financial reasons, product availability and/or the availability of animal strain/species at the facility. According to Kolb, 1987 (cited by Furman, 2015) some animal strains (Sprague Dawley and Wistar rats) are more sensitive to STZ in comparison to other strains. Furthermore, gender is also a factor when using STZ, since male rats are more sensitive. Male pancreatic islet β-cells are more prone to STZ-induced cytotoxicity than female rats (Furman, 2015). Furman, 2015 indicated that female rats show more resistance to STZ treatment than male rats, the reason for this aetiology is still not well understood. However, Deeds *et al.* (2011) and Yamabe *et al*., (2010) speculate that oestradiol's ability to protect the

pancreatic β-cells from apoptosis, could be the reason why female rats show such low sensitivity to STZ treatment. This statement could explain why Mostofa *et al*. (2007) reported such a low reduction in blood glucose levels (Table 2.5).

Table 2.5: Studies on the antidiabetic properties of *Catharanthus roseus*

Treatment of *C. roseus*	**Dose (mg/kg)**	**Period (days)**	**Animal**	**Diabetes induction method**	**Percentage reduction in blood glucose levels**	**Reference**
Methanolic leaf	250 mg/kg	7	Male Albino rats	Alloxan	69%	Aruljothi *et al*., 2016
Methanolic whole plant	300 and 500 mg/kg	14	Wistar rats (Male & Female)	Alloxan	25–50%	Ahmed *et al*., 2010
Dichloromethane: methanol (1:1) leaves and twigs	500 mg/kg	7 and 15	Male Sprague Dawley rats	STZ	49% (7 days) and 58% (15 days)	Singh *et al*., 2001
Aqueous	1000 mg/kg	21	Male Sprague Dawley rats	STZ	20.2%	Singh *et al*., 2001
Aqueous leaf	1000 mg/kg	14	Albino rats (Male & Female)	STZ	~13%	Mostofa *et al*., 2007
Ethanolic leaf (Petroleum ether, Chloroform & Ethyl acetate)	150 mg/kg	1	Long-Evans female Rats	STZ	52% petroleum ether 0% (chloroform) and 50% ethyl acetate	Islam *et al*., 2009
Ethanolic leaf	100 and 200 mg/kg	28	Male Wistar rats	STZ	46% (100 mg/kg) and 54%	Al-Shaqha *et al*., 2015
Methanolic leaf	200 and 400 mg/kg	13	Mice	STZ	58% (200 mg/kg) and 59%	Singh *et al*., 2014
Methanol leaf	250 mg/kg	7	Albino rats	Alloxan	37%	Ohadoma and Michael, 2011
Aqueous leaf	100 mg/kg	60	Male albino Wistar rats	STZ	78%	Rasineni *et al*., 2010
Dichloromethane: methanol (1:1) leaf	500 mg/kg	20	Male albino Wistar rats	Alloxan	54%	Jayanthi *et al*., 2010
Dry leaf powder	1.5 and 3.0 mg/kg	45	Male albino Wistar rats	STZ	59% and 61% (3.0 mg/kg)	Chauhan *et al*., 2012
Aqueous leaf	500 mg/kg	15	Albino rats	STZ	47%	Prasad *et al*., 2009
Ethanolic leaf	300 mg/kg	45	Male albino Wistar rats	Alloxan	53%	Manoharan *et al*., 2011
Ethanolic leaf	300 mg/kg	14	Albino rats	Alloxan	74%	Mohan *et al*., 2015
Aqueous leaf, flower and stems	25 mg/kg	7	Male albino Wistar rats	Alloxan	18%	Iweala and Okeke, 2005

Leaf juice	500, 750 and 1000 mg/kg	1	Rabbits	Alloxan	17%, 29% and 39% (1000 mg/kg)	Satyanarayana *et al*., 2008
Ethanolic leaf	150 mg/kg	14	Male and female albino rats	Alloxan	49%	Akhtar *et al*., 2007
Aqueous leaf	0.1 mg/kg (IP)	30	Albino Wistar rats	Alloxan	51%	Muralidharan, 2014

IP = Intraperitoneal administration

The antidiabetic effects of *C. roseus*, as indicated by the reduction in the blood glucose levels, was shown in numerous *in vivo* studies. Overall, it demonstrated that the effectiveness of *C. roseus* as an antidiabetic treatment, is not dependant on the animal species/strain (rats or mice), diabetic model (STZ or alloxan) or the plant extract (organic or aqueous). Thus, concluding that *C. roseus* is a good antihyperglycaemic agent for antidiabetic treatment.

2.2.4 Medicinal plant: *Portulacaria afra*

Portulacaria afra Jacq., also known as the Porkbush, Elephant bush/food (English), *Spekboom* (Afrikaans), *iNtelezi, isAmnilane, isiCococo* (isiZulu) and *iGqwanitsha* (isiXhosa). *Portulacaria afra* belongs to the Portulacaceae family (Hankey, 2009).

This plant can reach up to 2 meters in height, has red stems with round fleshy leaves and forms pink flowers. *Portulacaria afra* is found in warm conditions (Hankey, 2009), in the eastern parts of South Africa. It is widely used as a garden ornament (bonsai) or as a medicinal treatment for diseases such as diabetes mellitus (Hankey, 2009) and skin disorders, which include: sores, rashes, acne, boils, and warts (De Wet *et al.,* 2013). The leaves of *P. afra* can be eaten and are known to have a sour or bitter taste. It can either be chewed to treat sore throats and mouth infections or sucked to treat dehydration, exhaustion and heat strokes (Hankey, 2009). In rural Maputaland, people use the leaves as the preferable plant part (31%) to treat pimples, rash outbreaks, and insect bites (De Wet *et al.,* 2013). The leaves can be administered in various forms, as a liquid or a solid, resulting in different healing abilities. De Wet *et al*. (2013), reported that *P. afra* is one of nine plant species most frequently used as a treatment for skin disorders (dermatitis and chronic sores). This study was conducted in Northern Maputaland, which is one of the poorest regions in South Africa, with a very high HIV prevalence. Because a large part of the population has compromised immunity and burn wounds arising from cooking on open wood fires, it is expected that they are more prone to skin infections (De Wet *et al*., 2013). Consistent with Nciki *et al*. (2016), *P. afra* has positive antimicrobial effects, which validates its application to treat skin disorders. The leaves of the plant are the most frequent part used to treat skin disorders (Nciki *et al*., 2016) and diabetes (Olaokun *et al*., 2017).

The only study we could find that indicate the antidiabetic treatment of *P. afra* was reported by Hulley and Van Wyk (2019) whereby *P. afra* was used for the treatment of high blood pressure and diabetes. The majority of *P. afra* remedies are used for the treatment of skin disorders. Studies showed that *P. afra* contained phytochemical agents such as flavonoids (Olaokun *et al*., 2017), tannins and saponins (De Wet *et al*., 2013). Saponins have antimicrobial activities (Nciki *et al*., 2016), which explains why *P. afra* leaf extracts are used to treat skin ailments. We believe that the presence of flavonoids, which contain antioxidant and anti-inflammatory activities that cause a reduction in hyperglycaemia in diabetic patients. As chronic hyperglycaemia leads to the development of oxidative stress, which contribute to diabetic complications (Johansen *et al*., 2005).

2.2.4.1 Phytochemical constituents of *P. afra*

Olaokun *et al.* (2017), indicated that leaf acetone extracts of *P. afra* tested positive for flavonoid- and polyphenol content with antioxidant activities. Based on their findings they suggested that *P. afra* might have antidiabetic effects but would require further investigation.

2.2.5 Pharmacological actions of *P. afra*

2.2.5.1 Antidiabetic activity

There is limited published scientific literature to validate the antidiabetic properties of *P. afra*. The majority of published research papers such as De Wet *et al*. (2013), only report the antimicrobial effect of *P. afra* with little to no publications on the antidiabetic effect (Hankey, 2009; Hulley & Van Wyk, 2019; Parker, 2018). Olaokun *et al*. (2017) is one of few scientific publications, which purport that *P. afra* possesses some antidiabetic activities. Olaokun *et al*. (2017) indicated that *P. afra* enhanced glucose utilization of C2C12 muscle cells by 64% at 500 µg/mL. Showing that an increase in the activity of glucose utilization may attenuate hyperglycaemia.

Moreover, phytochemical analysis done by Olaokun *et al*. (2017), indicated that *P. afra* possessed flavonoids and polyphenols. These phytochemicals have been reported to hold antidiabetic effects (Wang *et al*., 2018). It is clear from Table 2.2, that flavonoids and polyphenols also possess antioxidant activities. Antioxidants are well known for their effectiveness in reducing diabetic complications (Bajaj & Khan, 2012). An increase in the antioxidant levels have also been proven to enhance the hypoglycaemic effect, which reduces the blood glucose levels and enhances insulin activities (Duraisamy *et al*., 2012). As such, the antidiabetic effects of *P. afra* seem promising enough to further investigate their novel antidiabetic potentials, individually and in combination with *C. roseus*.

Portulacaria afra is still a relatively novel medicinal plant used in antidiabetic research. However, indigenous community members have a rich body of knowledge about traditional ways of exploiting medicinal plants, such as *P. afra* for disease prevention such as diabetes. These traditional healers do not necessarily rely on scientific proof, but rather on their forefathers' traditional ways as well as their patient recoveries. It is for this reason, that the amount of information and knowledge provided by traditional healers, inspires further need for investigating the antidiabetic potentials of *P. afra*.

2.3 Conclusion

There has been an unprecedented increase in the global prevalence of diabetes. It poses a major health burden in low-income countries due to poor health facilities and a number of socio-economic challenges. Currently the management of diabetes in South African relies on pharmacological agents, which are often associated with adverse side effects and high costs. This calls for extensive research on alternative and complementary treatment in the management of T2DM. The rich floral biodiversity of Africa augmented the use of traditional plants as medicinal treatments for various diseases. Traditional healers developed therapeutic remedies from medicinal plants to treat metabolic diseases such as diabetes. These natural remedies are affordable, easily accessible and comparatively safer than synthetic medications. The only limitation of traditional medicinal plants is that they are mostly based on anecdotal evidence. The few studies that did evaluate the antidiabetic effects, were mainly *in vitro* studies. For this reason, there is need to study the antidiabetic potentials of plants *in vivo* is an imminent process necessary to fully clarify their antidiabetic activities.

From this literature review, it is reasonable to confirm the antidiabetic effect of *Catharanthus roseus.* Whilst *Portulacaria afra* presented some antihyperglycaemic effects, but the statement remains a hypothesis as it has not yet been fully confirmed. Apart from this, these plants grabbed the attention of traditional healers as potential antidiabetic drugs. The combination of *Catharanthus roseus* and *Portulacaria afra* might be a novel contribution to antidiabetic drugs.

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CHAPTER 3 *IN VITRO* STUDY

In vitro assessment of the antidiabetic potential of *Catharanthus roseus* and *Portulacaria afra* leaf extracts

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Abstract: The utilisation of medicinal plants for the treatment of diabetes has increased remarkably in developing countries. In South Africa there is anecdotal evidence that the combination of *Catharanthus roseus* and *Portulacaria afra* (1:1) is being used to treat symptoms of diabetes mellitus. There are multiple scientific reports on the antidiabetic potential of *C. roseus*, whilst there is insufficient data on the antidiabetic effects of *P. afra* and its combination with *C. roseus*. The major focus of this *in vitro* study was to investigate the antidiabetic activity of these medicinal plants by evaluating the activity of biomarker enzymes, specifically linked to diabetes therapy, against HepG2 cell line. This was done by investigating the safety (via cytotoxicity analysis using MTT and neutral red (NR) assays) and the activity of key digestive enzymes (via α-amylase, α-glucosidase and hexokinase assays). The 1:1 extract combination of *C. roseus* and *P. afra*, with a IC_{50} value of 4.10 µg/mL (i.e. NR assay), had the highest cytotoxicity ($p < 0.05$) on the HepG2 cell line compared to *P. afra* (IC_{50} = 27.93 µg/mL). Extract of *C. roseus* (IC_{50} = 6.02 µg/mL i.e. NR assay) was cytotoxic towards liver cells. Further cytotoxic investigation (i.e. MTT assay) showed that the copper presented within the plant extracts caused interactions with the MTT product which exhibited a two-fold increase in cytotoxicity, compared to the NR assay. The results of the *in vitro* assays showed that α-glucosidase presented no statistical significant changes after treatment with the plant extracts. However, the antidiabetic potentials of the plants were attributed to 1) the inhibition activity of α-amylase and 2) the activation of the liver hexokinase enzyme, which are known antidiabetic parameters involved in the reduction of blood sugar levels.

Keywords: Diabetes, medicinal plants, *Catharanthus roseus*, *Portulacaria afra*, HepG2 cell line, diabetic biomarker assays, viability assays.

3.1 Introduction

Diabetes mellitus is a well-known endocrine metabolic disease, epitomised by hyperglycaemia, caused by insulin deficiency or lack thereof (Shi *et al*., 2019). Diabetes prevalence over recent decades exhibited an increase in nearly all regions of the world, with 463 million people worldwide now living with diabetes (IDF Diabetes Atlas, 2019). An increase in the prevalence of diabetes has a profound effect on health services and economic costs, triggering an international burden (Hardin *et al*., 2019). The greatest problem associated with the management of diabetes, is the complications (Shi *et al*., 2018). Complications associated with diabetes include microvascular (neuropathy- nerve damage, nephropathy- kidney damage and retinopathy- eye damage) and macrovascular complications (cardiovascular disease, stroke and peripheral artery disease) (Papatheodorou *et al*., 2018). Diabetes-related complications are responsible for a great deal of the international burden associated with diabetes (Hardin *et al*., 2019).

The risk of diabetes-related complications can be lowered by maintaining glycaemic levels of diabetic patients with non-insulin treated drugs and Insulin (Corathers *et al*., 2013). According to Corathers *et al*. (2013), glycaemic levels can be controlled by different classes of synthetic antidiabetic medication (non-insulin treated drugs) such as: insulin sensitizers (metformin, pioglitazone and rosiglitazone), insulin secretors (glibenclamide, nateglinide and repaglinide), amylin analogues (Pramlintide) and specific digestive enzyme inhibition, such as α-glucosidase: acarbose, miglitol and voglibose (Corathers *et al*., 2013), and α-amylase inhibitors: buspirone, amlodipine and verapamil (Rodda *et al*., 2014). However, the use of these conventional antidiabetic treatments can lead to numerous adverse effects such as weight gain, cardiovascular risks, gastrointestinal effects, hypoglycaemia, diarrhoea and many more (Corathers *et al*., 2013; Osadebe *et al.,* 2014).

Synthetic antidiabetic drugs are not the only treatment used to manage high blood glucose levels. Medicinal plants (traditional medicine) are being used as an alternative for the treatment of diabetes, especially in developing countries such as Ghana, India and South Africa (Odeyemi & Bradley, 2018; Oyebode *et al*., 2016). Fokunang *et al*. (2011) best describe traditional medicine as a compilation of decade-old knowledge, beliefs and health practices to diagnose and prevent illnesses by using plants, animals and/or minerals for medicinal purposed. The diverse floral biodiversity of South Africa presents countless opportunities for the development of plant-based therapeutics (Moyo *et al*., 2011). According to Tuso *et al*. (2013), plant-based diets may present a more cost-effective solution for the management of chronic diseases such as diabetes mellitus.

In vitro studies are a cost-effective technique that allow a substance/drug to be studied safely in a controlled environment, before subjecting humans or animals to possible side effects or toxicity (Carvalho *et al*., 2019). *In vitro* models are mainly used to evaluate the efficacy of drugs to

determine the efficacy of the treatment to a particular disease (Pardons *et al*., 2019). In this regard, cytotoxicity assays are an important measurement of drug safety that evaluates the extent of cellular death in the presence of the test substance (Li *et al*., 2015; Taylor, 2019). Cell death is induced via activation of apoptosis, senescent death, necrosis and/or stress-induced cell death (Liu *et al.,* 2018). In the current study, the potential toxicity of the plants was assessed by using *in vitro* models; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) and neutral red assays for analysis of cytotoxicity to which cellular death was measured via cell parameters of cellular metabolic activity and lysosomal activity (Jain *et al.,* 2018).

The potency of therapeutic drugs is commonly associated with the metabolism rate (Singh *et al*., 2018). Determining the activity (inhibition) of digestive enzymes (e.g. α-amylase and α-glucosidase) provides valuable information about the potency of the drug, since digestion is an elementary process of drug metabolism in the gastrointestinal (GI) tract (Hani et al., 2018). Therefore, the GI tract is an important target site for evaluating antidiabetic treatments. The inhibition of key enzymes linked to antidiabetic treatment can further be evaluated in the liver, since this is the leading organ whereby substances would undergo the first pass metabolism (Singh *et al*., 2018). The effective control of hyperglycaemia in diabetes are associated with drugs that are insulin sensitizers, promotors of insulin secretion or inhibitors/regulators of carbohydrate enzymes (Kadan *et al*., 2013). Among these, α-amylase and α-glucosidase, are digestive enzymes that breaks down large dietary saccharides into smaller absorbable molecules such as glucose (He *et al.*, 2019). Treatment of diabetes can be achieved through the inhibition of α-amylase and α-glucosidase activities, known to significantly reduce post-prandial blood glucose, thus being a novel therapeutic target for diabetes (Agarwal, 2016; Tundis *et al*., 2010). Some of the most common antidiabetic drugs that act predominantly on inhibiting carbohydrate digestion are acarbose, miglitol and voglibose (Agarwal, 2016; DiNicolantonio, *et al*., 2015; Hirose *et al*., 2016). According to Osadebe *et al.* (2014) and Corathers *et al*. (2013), these antidiabetic drugs present side effects of gastrointestinal effects and hepatitis. A possible treatment of diabetes is to use natural sources as carbohydrate enzyme inhibitors as they present fewer side effects (Agarwal, 2016). Investigating the enzyme inhibition activity in medicinal plants and their phytochemical compounds may provide new therapeutic advances as antidiabetic medications (Agarwal, 2016).

Diabetes is one of many chronic diseases caused by oxidative stress (Zhang *et al*., 2015). Several phytochemicals such as flavonoids and phenolics are found to combat diabetes complications (Bacanli *et al*., 2019; Zhang *et al*., 2015). Phenolic compounds and flavonoids have been receiving much attention for their potential health benefits and for having antioxidant activity (Agati *et al*., 2020; Ali-Asgar, 2013). Potent antioxidant activities could be attributed to the synergistic effects of phytochemicals in the plant (Li, 2003). Recently, antioxidant phytochemicals have also

been found to have anti-inflammatory actions all suggesting antidiabetic activity (Zhang *et al*., 2015).

Over the years, increasing number of antidiabetic studies such as Rasineni *et al*. (2010) and Ponnusamy *et al*. (2011) focused on investigating alternative herbal remedies such as South African indigenous plants; *Catharanthus roseus* (Madagascar periwinkle) and *Portulacaria afra* (Elephant bush/ Spekboom) for the treatment and/or prevention of diseases. *Catharanthus roseus* is one of the most extensively investigated medicinal plants (Moreno *et al*., 1995). According to Tropical Plants Database (Fern, 2019), leaf extracts of *C. roseus* are used to treat hypertension, asthma, menstrual irregularities, chronic constipation, diarrhoea, indigestion, malaria, dengue fever, diabetes, cancer and skin disease. The leaves of *P. afra* are commonly chewed to treat sore throats and mouth infections or sucked to treat dehydration, exhaustion and heat strokes (Hankey, 2009). *Portulacaria afra* is most commonly used to treat skin disorders such as sores, rashes, acne, boils, and warts (De Wet *et al*., 2013). Although there is a paucity on the antidiabetic treatment of *P. afra,* limited studies such as Hulley and Van Wyk (2019) reported the use of *P. afra* for treatment of high blood pressure and diabetes. There is an urgent need for people living with diabetes, especially in low- and low-middle income countries, to be able to have access to affordable diabetic treatment (Oluwaseun *et al*., 2018). Approving the antidiabetic effects of traditional medicine with scientific evidence could potentially commercialise their use in drug development with the benefit of having fewer side effects.

The aim of this study was to investigate the antidiabetic potential of aqueous leaf extract of *C. roseus* (CR)*, P. afra* (PA) and CR:PA (1:1) against human hepatoma HepG2 liver cell line. This was achieved by investigating safety (by measuring the cytotoxicity), changes in the activities of enzyme relevant for diabetes (α-amylase, α-glucosidase and hexokinase).

3.2 Materials

3.2.1 Plant material

The plant material of *Catharanthus roseus* (*C. roseus*) (22°49’03’’S | 30°33’38’’E) and *Portulacaria afra* (*P. afra*) grown in a homestead (22°57’25’’S | 30°33’32’’E) was collected from Makonde, a small village located in Thohoyandou (Venda), South Africa. The pressed plant specimen of *C. roseus* (Voucher specimen #: KOT001) and *P. afra* (Voucher specimen #: KOT002) was identified and authenticated by the South African National Botanical Institute (SANBI). The leaves were oven dried and ground to fine powder to which the aqueous extracts were prepared using distilled water.

3.2.2 Cell Lines, media, reagents and assay kits

HepG2 cells were obtained from ATCC (ATCC® HB-8065™), LGC standards (Midrand, South Africa). The reagents and consumables for cell culturing was obtained from Gibco (Dun Laoghaire, Ireland), MERCK (St Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). The enzymatic activity assays (α-glucosidase and hexokinase) and viability assay kits were obtained from MERCK (St Louis, MO, USA). The α-amylase activity kit was purchased from Elabscience (Houston, Texas, USA). All reagents used in this study were of analytical grade.

3.3 Methods

3.3.1 Extraction of plant material

Leaves were harvested from the stalk and oven dried at 50°C. The dried leaves were ground into fine powder using an electronic blender. The aqueous extract was prepared by introducing 2 g of the powdered plant material into 20 mL distilled water. The mixture was extracted with distilled water at room temperature (20–25°C) by maceration overnight, using a magnetic stirrer. The extract of *C. roseus* was filtered using Whatman® filter paper (Grade 1 Qualitative) and *P. afra* through muslin cloth (owing to thick clay-like consistency) and stored at -80°C for 24 h. The frozen extracts were dried using a low temperature-low pressure freeze dryer (VirTis SP Scientific Sentry 2.0 Benchtop Scientific) for 48 h to obtain the final product which was used in this study. The dried leaf extracts were stored at 4°C and reconstituted in water for analysis.

3.3.2 Maintenance of HepG2 cell cultures

The human hepatocellular carcinoma cell line, HepG2 (ATCC® HB-8065™) were cultured at 37°C, under 5% CO_2 humidified atmosphere and 90% relative humidity, in complete growth medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 1% L-glutamine, 1% non-essential amino acids (NEAA) and 1% Penicillin/Streptomycin using T-75 cm^2 cell culture flasks. Once cells reached a confluency of 70–80% they were used in subsequent experiments.

3.3.3 Cell viability assay

The colorimetric MTT assay determines the safety of the plant extracts by measuring the cells ability to facilitate the metabolic conversion of tetrazolium salts into purple formazan in the mitochondria of viable cells (Bahuguna *et al*., 2017). The neutral red assay measures the capability of living cells to actively incorporate the neutral red dye, into the lysosomes of viable cells (Ates *et al*., 2017; Jain *et al*., 2018). Both the MTT and neutral red (NR) were used to determine the safety of *C. roseus* (CR), *P. afra* (PA) and CR:PA (1:1) by investigating the viability

of HepG2 cells exposed for 24 h to various concentrations of the leaf extracts; 0.66, 1.64, 4.096, 10.24, 25.6, 64, 160, 400 and 1000 µg/mL, prepared in plain growth media (DMEM).

The MTT assay was determined according to the method described by Mosmann (1983) and Vistica *et al*. (1991). In brief, 200 µL of HepG2 cells (1 x 10^6 cells/mL) were seeded into a 96-well plate and incubated for 24 h. After cell attachment, the old growth media was removed, and cells were exposed to treatment concentrations for 24 h. The spent growth media was aspirated and washed three times using 200 µL PBS per well. Next, 100 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (0.5 mg/mL in DMEM) was added per well and left to incubate for 1.5 h at 37°C (5% CO_2 and 90% relative humidity (RH)). After incubation, the cells were inspected under an inverted microscope for the development of formazan crystals. Once crystals were visible, the supernatant was removed, and cells were washed once with PBS. The formazan crystals were dissolved in 200 µL dimethyl sulfoxide (DMSO, Sigma, ≥99.5%) and left on plate shaker for 15–30 minutes to ensure complete dissolution of the crystals into the DMSO. The absorbance was measured at 560 nm and a background was measured at 630 nm. Absorbance was measured using SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer. The assay was done independently in quadruples. In this assay, metabolically viable cells were evaluated based on their ability to reduce MTT to purple formazan (Rai *et al*., 2018).

The cell viability was also determined using the neutral red (NR) assay according to the method described by Repetto *et al*. (2009). Cells were seeded and treated under the same conditions as described above. After 24 h exposure to treatment the spent media was aspirated, and cells were washed thrice with PBS (200 µL/well). The filtered NR dye (0.33% v/v in DMEM) of 200 µL was added to the cells and incubated for 2 h at 37°C (5% CO_2 and 90% RH). The neutral red solution was aspirated and washed once with 200 µL of PBS. NR solubilization solution (50% ethanol, 49% distilled water and 1% acetic acid) of 200 µL was added to all the wells. The plate was placed on the plate shaker for 15–30 minutes to ensure complete dissolution of the crystals into the neutral red solubilization solution. Absorbance was measure at 540 nm and 630 nm (Background) using SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer.

The percentage cell viability of both MTT and NR assay were calculated using the following equation:

$$\% \text{ Cell viability} = \left(\frac{\Delta \text{Sample}_{(630\text{-}560)}}{\Delta \text{Positive control}_{(630\text{-}560)}} \right) \text{ x } 100$$

Where; $\Delta \text{Sample}_{(630\text{-}560)}$ refers to the final absorbance value after the blank (630 nm) was subtracted from the absorbance of the treated cells (560 nm), $\Delta \text{Positive control}_{(630\text{-}560)}$ is the value after the blank (PBS) was subtracted from the absorbance of the untreated cells (positive control).

The positive control was treated with complete growth medium (DMEM), which acted as viability signal for healthy cells with an active mitochondrial activity. The cell viability is expressed in terms of the control. According to the International Organisation of Standardisation (ISO) and the South African National Standard (SANS), cell viabilities above 70 percent is non-cytotoxic; between 70 to 50 percent is considered slightly cytotoxic and below 50% strongly cytotoxic (ISO 10993-5: 2009; SANS 10993-5:2010). The IC_{50} values will be determined using the cell viability data of the neutral red assay, the reason for this will be addressed in the discussion.

3.3.4 Diabetes biomarkers

The antidiabetic effects of *C. roseus* and *P. afra* leaf extracts were investigated using enzymatic biomarker relevant to diabetes therapy. Enzymatic biomarkers are a common technique used to investigate diseases, by using common indicators of that specific disease, such as blood glucose in the case of diabetes. In this study, we measure the activity of three enzymes using colorimetric assays; α-amylase, α-glucosidase and hexokinase, frequently associated with antidiabetic agents. The α-amylase activity was measured using a coupled enzymatic assay, which results in the formation of a coloured product (540 nm), proportional to the amount of substrate (ethylidene-pNP-G7) cleaved by the amylase. The α-glucosidase activity of the plants was measured by a reaction in which α-glucosidase hydrolyses *p*-nitrophenyl-α-D-glucopyranoside (pNPG), producing a coloured product (405 nm), which is directly proportional to the α-glucosidase activity in HepG2 cells. The hexokinase assay is a quantitative, enzymatic determination of glucose in food and other material. Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalysed by hexokinase.

3.3.5 Extraction of crude enzyme from adherent cell samples:

3.3.5.1 Treatment and homogenate preparation

The assay cells were cultured and prepared according to the standard method as described by Repetto *et al*. (2008). HepG2 cells were seeded (1×10^6 cells/mL) into 6-well plate and incubated for 24 h. Each enzymatic assay had its own separate plate. The HepG2 cells for the α-amylase, α-glucosidase, and hexokinase assays were treated with the leaf extracts in plain DMEM (0.66, 1.64, 4.096 and 25.6 µg/mL) for 24 h. Treatment concentrations were based on the conservative IC_{50} values. After 24 h, cells were visually inspected (% cell confluency), followed by the removal of the old growth media. The wells were washed three times with 200 µL of phosphate buffer saline (PBS) and dislodged by brief exposure to 0.25% trypsin EDTA in PBS, for 4 minutes. After which it was centrifuged at 1734 × *g* for 4 minutes to remove trypsin. The cell pellet was resuspended in assay specific solvent (provided by the assay kit). For the α-amylase assay the cells were resuspended in 1 mL distilled water; for α-glucosidase in 100 µL PBS; and in 200 µL

ice-cold assay buffer for the hexokinase assay. The cells were lysed by sonicating using an ultrasonic water bath pre-set at 25°C for 2–4 minutes. After sonication, the homogenate was centrifuged again at 13 000 × *g* for 10 minutes to remove insoluble material. The supernatant was collected into tubes for further analysis.

3.3.5.2 Protein determination

The protein content of each assay (α-amylase, α-glucosidase and hexokinase) was determined according to Bradford (1976). In short, 5 µL of the supernatants, blanks (distilled water) and standards were added into a 96-well plate. Bradford's reagent (245 µL) was added to each well and allowed to incubate for 5 minutes. The absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as standard (0–2500 µg/mL), the protein content of each sample was determined using linear extrapolation.

3.3.6 Alpha (α)-amylase activity assay

The potential inhibition of α-amylase in HepG2 cells by the plant extracts was determine according to the manufacturer's instructions (Elabscience E-BC-K240). The collected supernatants (as prepared in Section 3.3.5.1) were further incubated at room temperature for 15 minutes whilst mixing every 5 minutes. The samples were centrifuged at 3000 × *g* for 10 minutes at 25°C to remove insoluble material. The supernatant was collected and diluted with distilled water to a total volume of 10 mL, in order to produce the amylase solution, which served as the enzyme stock solution. The protein activity of α-amylase was determined using the enzyme stock solution. For the α-amylase activity, a total volume of 75 µL amylase solution containing the samples and the untreated control were introduced into a 2 mL microcentrifuge tube. The tubes were incubated at 70°C for 15 minutes, after which it was left to cool down to room temperature. Once cooled, 75 µL of amylase substrate was added to the sample tubes, whereas 75 µL distilled water was added to the control group. Incubated at 40°C for 5 minutes, to which 150 µL of the colorphore was added to the samples and controls. Finally, all the samples were incubated at 95°C for 5 minutes then left to cool down and transferred to a 96-well microplate (200 µL/well) for analysis. Absorbance at 540 nm was measured using a Spectramax spectrophotometer.

Amylase activity was calculated using the regression equation under standard conditions; y = 2.481x - 0.1778. Where, x is the concentration of standard (mg/mL) and y is the absorbance (A).

α-Amylase activity calculated according to protein concentration of samples:

$$\text{α-Amylase activity (mg/min/mg protein)} = \left(\frac{[((A_{540})_{sample} - (A_{540})_{control} + 0.1778) \div 2.481 \times V_{total}]}{(V_{sample} - Cpr)}\right) \div T$$

Where; $(A_{540})_{sample}$ refers to the absorbance value of sample (α-amylase) and $(A_{540})_{control}$ is the absorbance value of control (α-amylase), V_{total} refers to the total volume of reaction system (0.15 mL), V_{sample} is the volume of sample added into the reaction system (0.075 mL), Cpr refers to the concentration of protein in sample (mg/mL) and T refers to the reaction time (5 minutes). The α-amylase activity was expressed as mg/min/mg protein where 1 mg reducing sugar catalysed by 1 mg of protein per minute is defined as an enzyme activity unit.

3.3.7 Alpha (α)-glucosidase activity assay

The α-glucosidase activity assay was carried out according to supplier's instructions (Sigma-Aldrich (MAK123)). After the exposure and homogenization of the cells (as described in Section 3.3.5.1), the supernatant (20 µL) was transferred into a clear flat-bottom 96-well plate. Only the control group contained ultrapure water (220 µL). The calibrator solution (represented the standard) of 200 µL was then added (equivalent to 250 U/L) to the wells and supplemented with 20 µL distilled water. All samples and controls were done in triplicate. The absorbance of the initial α-glucosidase activity was measured at 405 nm $(A_{405})_{initial}$, shortly after the α-NPG substrate reaction mix (200 µL) was added. The samples were incubated at room temperature for 20 minutes after which the final α-glucosidase activity was measured $(A_{405})_{final}$. The α-glucosidase activity was determined by measuring the yellow para-nitrophenol released from pNPG at 405 nm.

The α-glucosidase activity (units/L) was calculated as follows:

$$\text{α-Glucosidase activity (mg/min/mg protein)} = \frac{(A_{405})_{final} - (A_{405})_{initial}}{(A_{405})_{calibrator} - (A_{405})_{control}} \times 250 \text{ units/L}.$$

Where; $(A_{405})_{final}$ refers to the final absorbance value of α-glucosidase measurement and $(A_{405})_{initial}$ refers to the initial absorbance value of the α-glucosidase measurement, $(A_{405})_{calibrator}$ refers to the absorbance value of the calibrator, $(A_{405})_{calibrator}$ refers to the absorbance value of the control. The absorbance of the calibrator and the control were measured at 20 minutes.

3.3.8 Hexokinase assay

The hexokinase (HK) activity was determined according to the manufacturer instructions (Sigma-Aldrich, MAK091) protocol. In contrary to the previous two assays, this assay used a concentration range of 0.66, 1.640 and 4.096 µg/mL. After homogenisation of the cells (as described in Section 3.3.5.1), 40 µL, 30 µL and 20 µL of the supernatant was added to a 96-well plate, whereupon the volume was adjusted to 50 µL with hexokinase assay buffer. The reaction was initiated by introducing 50 µL of the reaction mix, which contains the enzymes, co-enzymes and developers. The plate was left to incubate for 5 minutes at room temperature to which the

initial absorbance was measurement at 450 nm. The absorbance was measured kinetically every 5 minutes for 15 minutes using a microtiter plate reader (SpectraMax® microplate reader). The plate was left to incubate at room temperature between measurements, while being protected from light.

Using the NADH standard curve a linear equation was calculated. Using the equation below, the x-value was extrapolated (ΔA_{450} as y-value) to give the amount of NADH generated during the reaction time.

$$\Delta A_{450} = (A_{450})_{final} - (A_{450})_{initial}$$

refers to the change in absorbance measurement between $T_{initial}$ (5 minutes) and T_{final} (15 minutes). The $(A_{450})_{final}$ refers to the absorbance value of the final hexokinase measurement and $(A_{450})_{initial}$ refers to the absorbance value of the initial hexokinase measurement. The amount of NADH generated between $T_{initial}$ and T_{final} is given as B in the equation below:

$$\text{HK activity} = \frac{\text{B} \times \text{Sample Dilution Factor}}{(\text{Reaction Time}) \times \text{V}}$$

Where, B refers to the amount (nmole) of NADH generated between $T_{initial}$ and T_{final}, reaction time refers to $T_{final} - T_{initial}$ (10 minutes) and V refers to the sample volume (mL) added per well.

3.4 Statistical analysis

Data were presented as mean ± standard deviation (SD). Distribution of data was determined using the Kolmogorov-Smirnov test. For non-parametric data, statistical significance between experimental groups and the control was determined by Kruskal-Wallis test and Dunn's multiple comparison test as *post-hoc* test. Statistical analysis on parametric data was done using a one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test. Statistical analysis was done using GraphPad Prism 9 statistical software. Statistical *p*-values smaller than 0.05 were considered significant. The 50% inhibition concentration of enzyme activity (IC_{50}) was determined using GraphPad Prism 9 software using non-linear regression (log(inhibitor) vs normalised response with variable slope).

3.5 Results

3.5.1 Effect of plant extracts on MTT and neutral red cell viability

The safety of aqueous leaves of *C. roseus* (CR), *P. afra* (PA) and 1:1 CR:PA were investigated by MTT and neutral red assays —in HepG2 cell line— after a 24 h treatment. A concentration dependent decrease in cell viability was noted for all three treatments (Figure 3.1 and 3.2). Cell viabilities greater than 70% were visualised for CR and PA extracts at the lower treatment

concentrations (0.66, 1.64,4.096 and 10.24 µg/mL), which was statistically significantly decreased ($p < 0.05$) compared to the control using the MTT assay (Figure 3.1). The cells treated with CR showed significantly decreased cell viability (less than 70% cell viability) from 25.6 µg/mL to the highest treatment concentration of 1000 µg/mL compared to the control (Figure 3.1). The cytotoxicity results revealed that PA extract displayed less cytotoxic effects to HepG2 cells at all doses — the only dosages that presented cytotoxic effects (cell viability lower than 50%) was concentrations of 400 µg/mL and 1000 µg/mL. The combination treatment of CR:PA leaf extracts showed no cytotoxic effects on HepG2 cells at the lowest two concentrations of 0.66 and 1.64 µg/mL (cell viability greater than 70%), whilst also presenting statistical significance ($p < 0.05$) to the control (Figure 3.1).

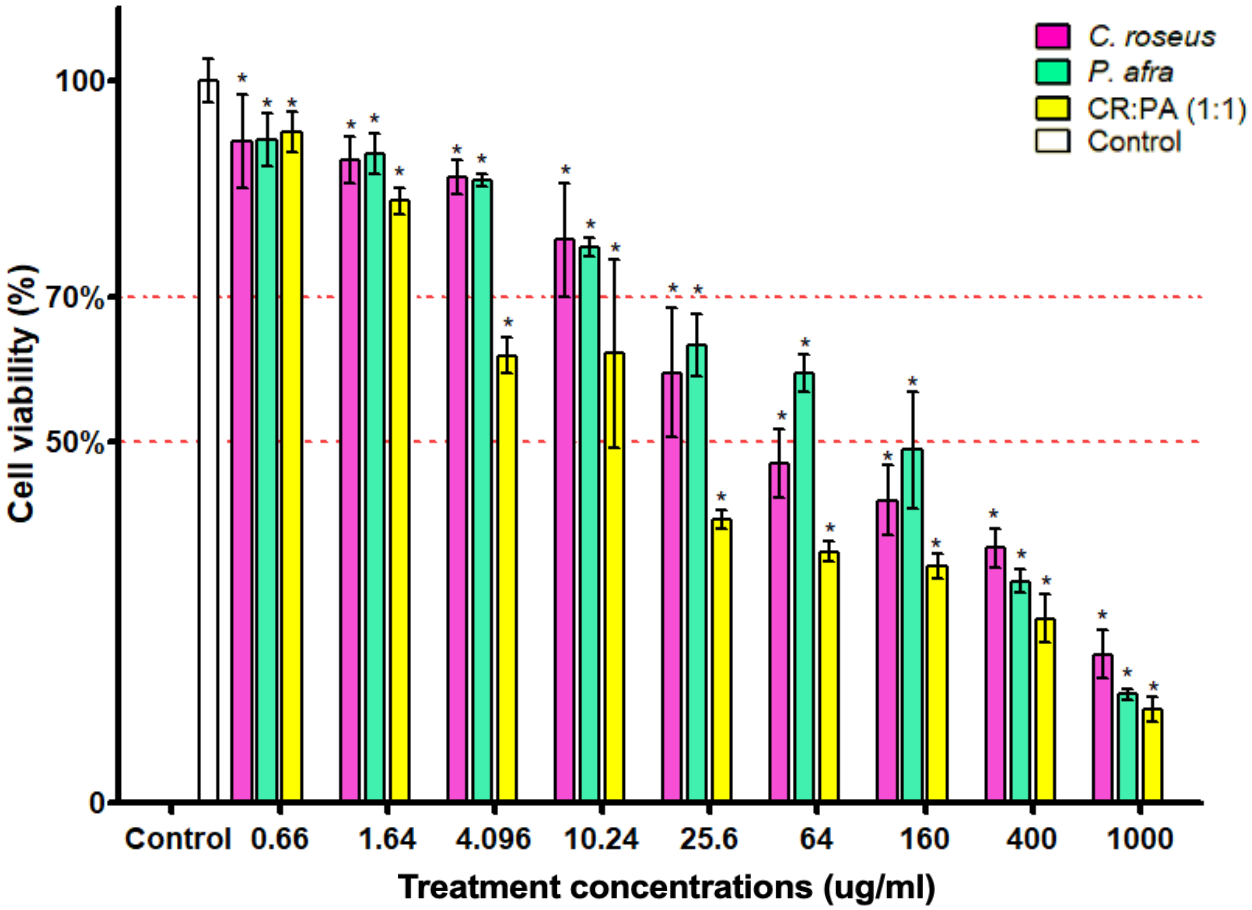

Figure 3.1: Viability of HepG2 cells exposed to aqueous leaf extracts of *C. roseus, P. afra* and CR:PA (1:1) between concentration range of 0.66–1000 µg/mL using MTT assay. * indicative of statistically significant difference in comparison to control ($p < 0.05$). All cell viability data of the MTT assay was presented as mean ± standard deviation.

For the NR assay, all extract treatments (CR, PA and CR:PA) resulted in significantly reduced cell viabilities in comparison to the control (Figure 3.2, $p < 0.05$). The cells treated with CR displayed significant increase in cytotoxicity (cell viability lower than 50%) in a dose-dependent from 25.6 µg/mL to 1000 µg/mL in comparison to the three lowest treatment concentrations (Figure 3.2, $p < 0.05$). The cells treated with CR:PA displayed similar cytotoxic results than that

of CR-treated cells by exhibiting increased cytotoxic effects (cell viability lower than 50%) from 25.6 µg/mL (Figure 3.2, $p < 0.05$). Only the cells that were treated with PA demonstrated less cytotoxic effects as seen for all concentrations (0.66–160 µg/mL) that had a percentage cell viability equal to or greater than 50%, with the only exception to 400 and 1000 µg/mL. These two concentrations of PA presented a significant reduction in cell viability (cell viabilities lower than 50%) when compared to the three lowest treatment dosages of PA (Figure 3.2, $p < 0.05$).

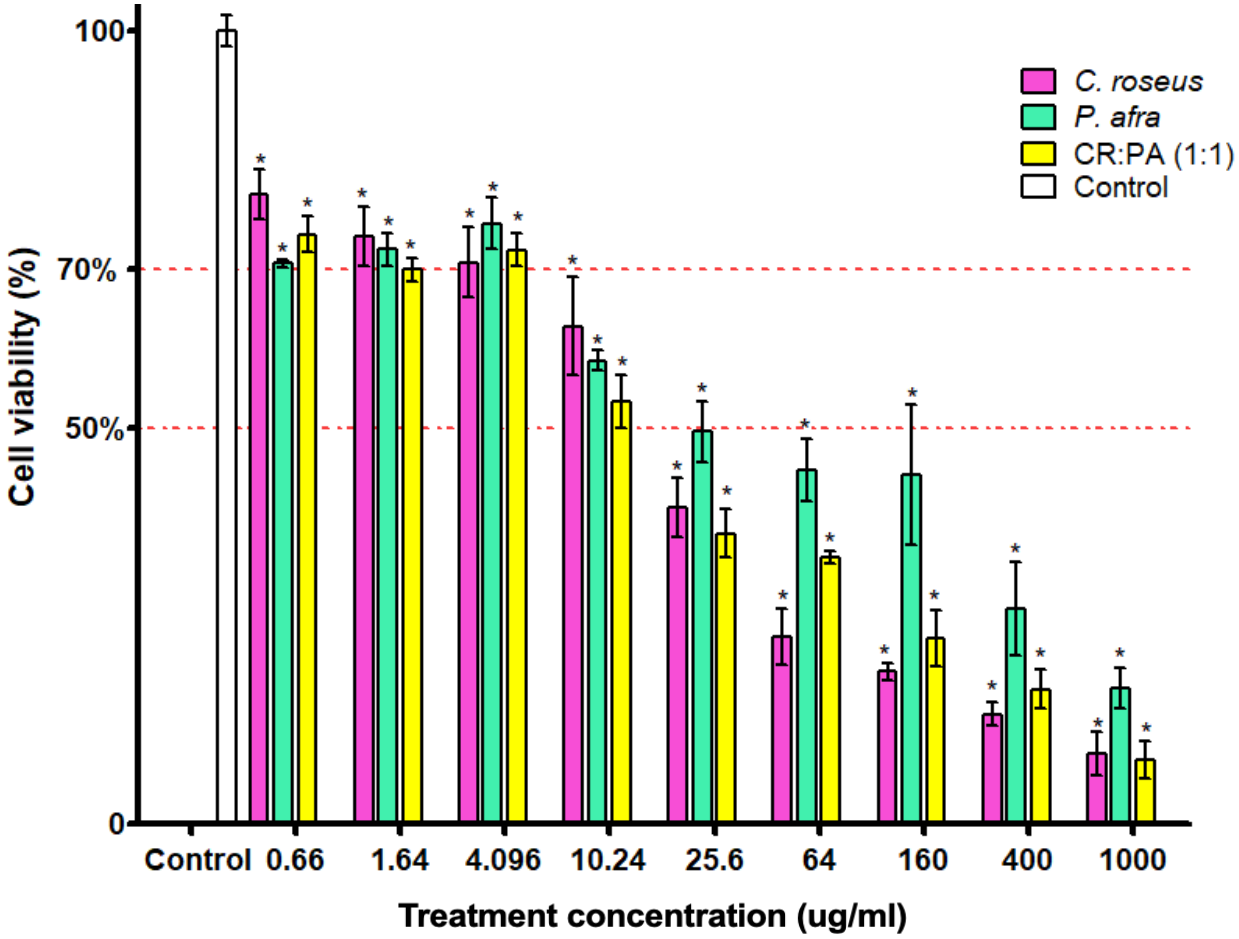

Figure 3.2: Viability of HepG2 cells exposed to aqueous leaf extracts of *C. roseus, P. afra* and CR:PA (1:1) between concentration range of 0.66–1000 µg/mL using neutral red assay. * indicative of statistically significant difference in comparison to control ($p < 0.05$). The data of the neutral red assay is presented as mean ± standard deviation.

3.5.2 IC_{50} determination of plant extracts

The IC_{50} values were used to establish the safety of the plant extracts by measuring the amount of extract that would be needed to inhibit 50% of the HepG2 cells activity (Aykul & Martinez-Hackert, 2016). The results of the MTT assay showed no statistically significant differences between the IC_{50} values of CR compared to CR:PA (Table 3.1). The cells treated with PA displayed increased IC_{50} values that were significantly higher compared to CR and CR:PA ($p < 0.05$), representing decreased toxicity to HepG2 cells. The results of the neutral red assay showed that PA had significantly higher IC_{50} values in comparison to treatment of CR and CR:PA ($p <$

0.05), with no statistical differences between CR and CR:PA (Table 3.1). Our study demonstrated that the IC_{50} values of the MTT were nearly two-fold higher than the IC_{50} values reported by the neutral red assays (Table 3.1). Consequently, the treatment concentrations of the enzymatic assays were based on the conservative IC_{50} values i.e. the NR IC_{50} values.

Table 3.1: The mean IC_{50} concentrations calculated from independent cytotoxicity analysis that were done in triplicate on both MTT and neutral red (NR) assay.

Aqueous leaf extract	**Cell viability assay**	
	MTT IC_{50} (µg/mL)	**NR IC_{50}** (µg/mL)
***Catharanthus roseus* (CR)**	18.83 ± 2.66	6.02 ± 3.0
***Portulacaria afra* (PA)**	57.32 ± 3.86*	27.93 ± 2.26*
CR:PA (1:1)	12.93 ±1.57	4.10 ± 0.65
Statistically significant differences are shown by asterisks (*) when comparing the IC_{50} values of PA to both CR and CR:PA, within each assay ($p < 0.05$).		

3.5.3 Effect of plant extracts on alpha (α)-amylase activity

The activity of α-amylase was investigated in HepG2 cells that were exposed to leaf extracts of *C. roseus* (CR), *P. afra* (PA) and CR:PA (1:1) for 24 h (Figure 3.3). The treatment range for this assay (0.66, 1.64, 4.096, 10.24 and 25.6 µg/mL) was determined using the conservative IC_{50} values obtained by the NR assay. We also validated our treatment range with the findings of a similar study conducted by Saliu *et al*. (2018), who treated aqueous extracts of *C. roseus* with α-amylase enzyme and reported an α-amylase IC_{50} concentration of 2.5 µg/mL. This approach ensured the cells remained viable for determination of enzyme activity. Cells were visually inspected for cytotoxicity, prior to assay examination, by means of percentage (%) cell confluency. A cell confluency lower than 75% indicated cytotoxic effects. None of the treatments showed a decrease in the confluency.

In this study, the results indicated that all three extracts exhibited a significant effect on α-amylase at all the tested concentrations (Figure 3.3). Compared to the CR control, the HepG2 cells treated with leaf extract of *C. roseus* showed significantly higher ($p < 0.05$) α-amylase activity at all tested concentrations, except for 4.069 µg/mL which exhibited significantly lower α-amylase activity than the control (Figure 3.3, $p < 0.05$). At the highest concentration (25.6 µg/mL) investigated, the α-amylase in the cells treated with aqueous CR displayed a significantly increased α-amylase activity by 25.8% relatively to the control (Figure 3.3, $p < 0.05$). On the contrary, the α-amylase activity was significantly decreased (by 14.4%) in the cells that were treated with CR at 4.096 µg/mL (Figure 3.3, $p < 0.05$). All treatment concentrations of PA and CR:PA displayed significantly lower α-amylase activity compared to their respective control groups (Figure 3.3, $p < 0.05$). The cells treated with PA displayed an α-amylase inhibition effect at all five treated concentrations

when compared to the control (Figure 3.3, $p < 0.05$). At the highest concentration (25.6 µg/mL), we discovered that the cells treated with CR:PA significantly decreased the α-amylase activity by 57.5% ($p < 0.05$). It appears as if the α-amylase activity of CR:PA-treated cells decreased in a dose-dependent manner from 1.64 to 25.6 µg/mL (Figure 3.3).

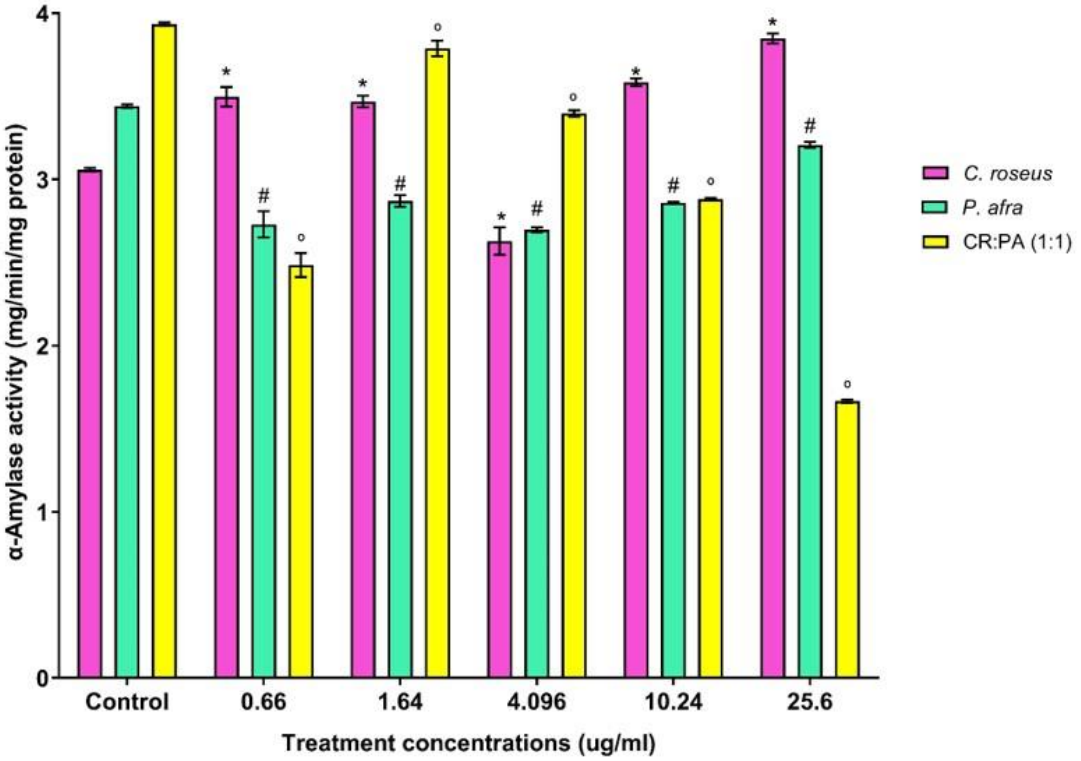

Figure 3.3: The effect of aqueous leaf extract of *C. roseus, P. afra* and CR:PA (1:1) were investigated on α-amylase activity in HepG2 liver cell line. Data expressed as mean ± standard deviation (SD) ($n = 6$). Statistically significant differences in comparison to the control are shown by common superscripts (*,#,°) ($p < 0.05$) * indicative of significant difference between untreated *C. roseus* control and *C. roseus* at 10.24 and 25.6 µg/mL ($p < 0.05$). # indicative of significant difference between untreated *P. afra* control and *P. afra* at all five concentrations ($p < 0.05$). º indicative of significant difference between untreated CR:PA control and CR:PA at all five concentrations ($p < 0.05$).

The percentage inhibition and activation effects of the three plant extracts on α-amylase (in HepG2 cells) were calculated and given in Figure 3.4. In this study, the results indicated that *C. roseus* exhibited activation of α-amylase at 0.66, 1.64, 10.24 and 25.6 µg/mL (13.42–17.22%). (Figure 3.4). The CR-treated cells demonstrated maximum α-amylase activation (%) of 25.83% at the highest treated concentration (25.6 µg/mL), whilst displaying a % inhibition of 14.05% at 4.096 µg/mL (Figure 3.4). The treatments of PA and CR:PA extracts displayed an inhibition effect on α-amylase at all five treated concentration (Figure 3.4). The cells treated with PA exhibited the greatest inhibition effect of 21.62% at 4.096 µg/mL (Figure 3.4). At the highest treated concentration, the PA cells displayed the lowest inhibition effect on α-amylase by 6.78%. The cells treated with CR:PA extract exhibited the highest inhibition effect of 57.66% on α-amylase at the highest treated concentration and the lowest inhibition effect was observed at 1.64 µg/mL presenting 3.71% (Figure 3.4).

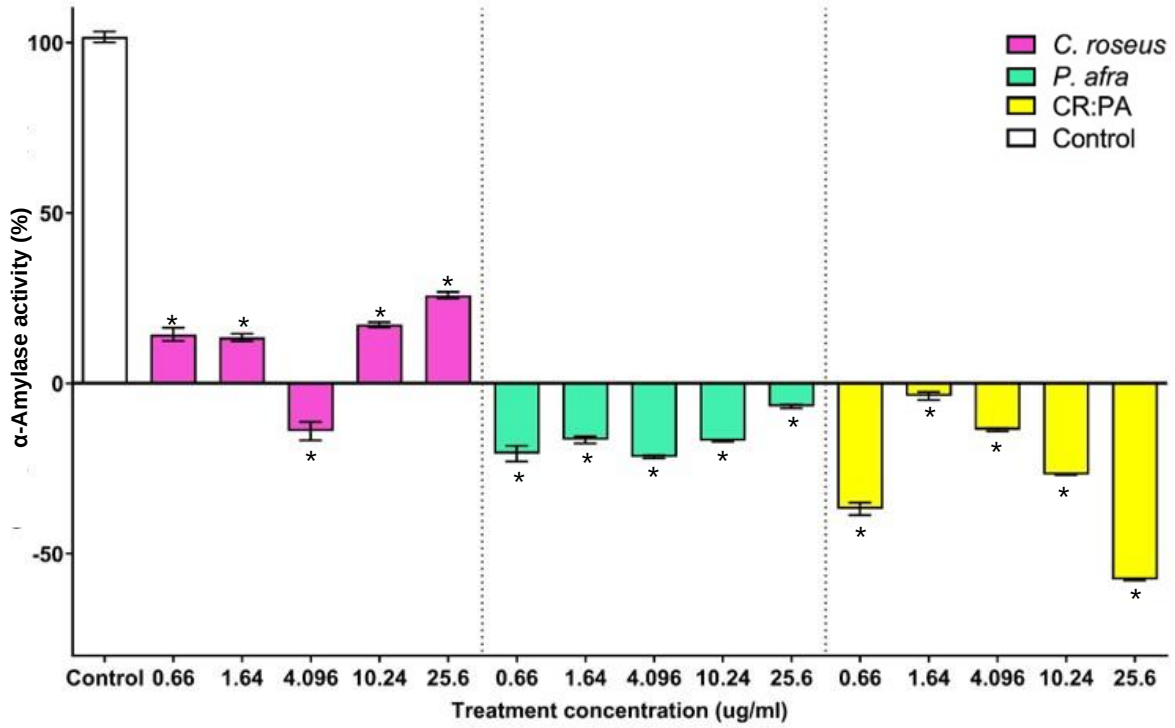

Figure 3.4: The effect of aqueous leaf extract of *C. roseus, P. afra* and CR:PA (1:1) on α-amylase activity screened against HepG2 cell line. Data expressed as percentage mean ± SD (n = 6). * indicative of statistically significant difference in comparison to control ($p < 0.05$).

3.5.4 Effect of plant extracts on alpha (α)-glucosidase activity

The effects of aqueous leaf extracts of *C. roseus*, *P. afra* and CR:PA (1:1) on α-glucosidase in HepG2 cells, are presented in Figure 3.5. No visual evidence of cytotoxicity was seen after treatment with plant extracts (i.e. between treatment concentrations 0.66–25.6 µg/mL). The α-glucosidase results showed that cells treated with aqueous leaf extract of *C. roseus,* exhibited the highest α-glucosidase activity at 25.6 µg/mL, which was also the only treatment concentration that showed a significant increase to the control (Figure 3.5, $p < 0.05$). The cells treated with PA and CR:PA extracts demonstrated no statistical significance to their respective control groups, showing no effect on the enzyme system owed to the large variations (SD) between data sets.

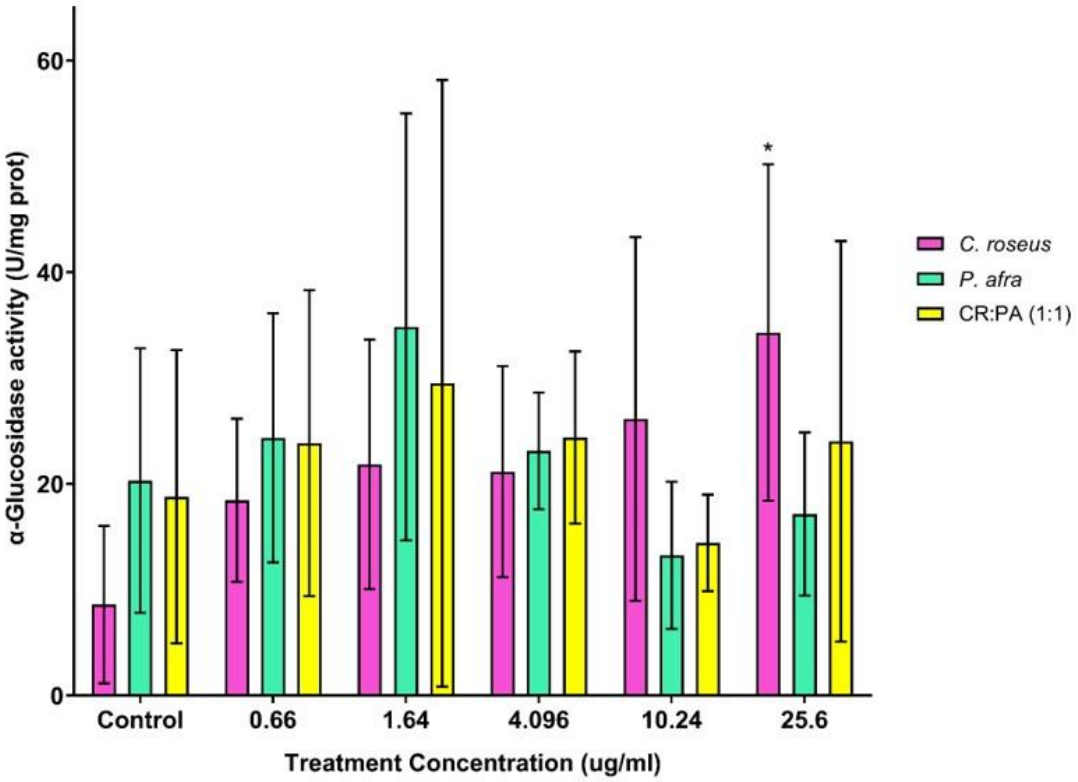

Figure 3.5: The effect of aqueous leaf extract of *C. roseus, P. afra* and CR:PA on α-glucosidase activity in HepG2 cells (1:1). Data expressed as mean ± SD (n = 6). Significant differences are shown by common superscripts in comparison to the control (*) ($p < 0.05$).

3.5.5 Effect of plant extracts on hexokinase activity

The *in vitro* hexokinase (HK) activity was evaluated in HepG2 cells treated for 24 h with aqueous leaf extracts of *C. roseus*, *P. afra* and CR:PA (1:1) and are presented in Figure 3.6. As previously mentioned, the confluency of cells was visually examined for toxicity before initiating assay. Upon inspection, we noted a significant reduction in cell confluency (< 30%) for all three plant extracts at the highest concentrations (10.24 and 25.6 µg/mL). As a result, concentrations of 10.24 and 25.6 µg/mL were omitted from this assay due to cytotoxic actions to cells. The reported concentration ranges for this assay were 0.66, 1.64 and 4.096 µg/mL. The HK assay was investigated using a single microplate (96-well), thus only one control was needed. The calculated equation of the linear standard curve; y = 0.043x - 0.002, was used to determine the hexokinase activity. In this study, the results indicated that all three plant extracts exhibited a significant increase in HK activity at the treatment dose of 1.64 µg/mL when compared to the untreated control (Figure 3.6, $p < 0.05$). The cells treated with leaf extract of CR displayed a maximum hexokinase activity at 1.64 µg/mL compared to the control. In addition, cells treated with 0.66 µg/mL of CR:PA, displayed elevated activity of hexokinase (increased by 39.8%) with statistical significance to the control (Figure 3.6, $p < 0.05$). At the highest concentration (4.096 µg/mL)

investigated, the treated cells of CR, PA and CR:PA displayed no statistically significant difference to the control group, which can be owed to the large variations within each sample.

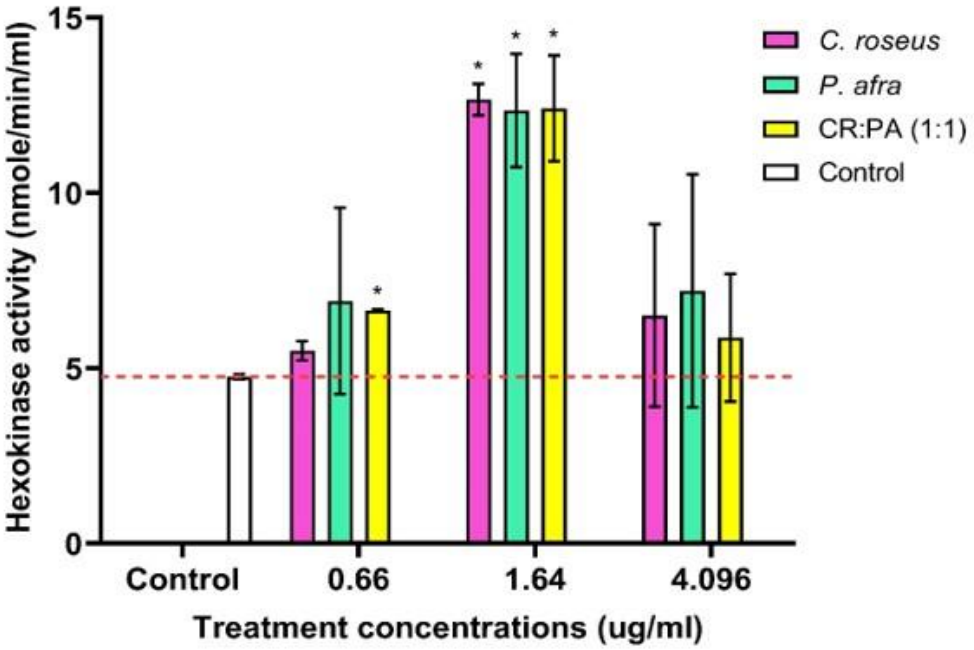

Figure 3.6: The hexokinase effect of aqueous leaf extract of *C. roseus, P. afra* and CR:PA (1:1) were investigated in HepG2 cell line. * indicative of statistically significant difference ($p < 0.05$) when compared to the untreated cells (control). Data expressed as mean ± SD ($n = 3$).

3.6 Discussion

Aqueous leaf extracts have been screened for antidiabetic properties since present-day traditional healers believe that the water decoction of *C. roseus* and *P. afra,* including the 1:1 mixture, has antihyperglycaemic effects. Diabetes causes hyperglycaemia because of insufficient insulin production (Arumugam *et al*., 2013). The use of these leaf extracts is believed to reduce insulin resistance and facilitate secretion of insulin in diabetic patients.

An analysis report was conducted on the phytochemical composition of *Catharanthus roseus* and *Portulacaria afra* (performed by the ARC-VOP under the guidance of Dr Ashwell Ndhlala (co-supervisor)), which revealed the presence of flavonoids and phenolics (Appendix C and D). These phytochemicals are believed to be promising antidiabetic agents (Al-Ishaq *et al*., 2019; Hajimehdipoor *et al*., 2014). In addition to being safe, flavonoids and phenolics demonstrated supplemental cytotoxic effects against cancer cells, by inducing cellular death in rapidly proliferating cells (Bharti *et al*., 2018). In the current study, the results of the MTT assay displayed a significant dose-dependent reduction in cell viability of HepG2 cells that were exposed to CR:PA leaf extracts —CR:PA-treated cells — when compared to HepG2 cells that were exposed to individual plant treatments of CR and PA (Figure 3.1). The elevated cytotoxicity that was observed for CR:PA-treated cells could be owed to the combination of flavonoids and phenolics (Appendix

E, Figure E.1 and E.2). These findings are in agreement with that of Sung *et al*. (2012), who reported that the combination of two or more phytochemicals, have shown increased cytotoxicity effects to cancer cells when compared to individual phytochemical treatment. This increase is often due to synergy of individual actives on cell death (Sung *et al*., 2012), which could be owed to the large phenolic content in CR:PA, presented in Figure E.1 (Appendix E). The MTT results (Figure 3.1) indicated that all plant treatments significantly reduced the mitochondrial activity in HepG2 cells, to which *P. afra* had the least cytotoxic effect when compared to CR and CR:PA (Figure 3.1).

To further investigate the cytotoxic effects of the plant extracts, the neutral red (NR) assay was also performed. This assay measures cell viability based on the cells ability to incorporate the NR dye into lysosomes (Aslantürk, 2018). The NR results indicated that all treatments exhibited significant growth inhibition in HepG2 cells in a dose-dependent manner (Figure 3.2). The combination of *C. roseus* and *P. afra* (1:1) significantly enhanced the growth inhibition of HepG2 cells when compared to individual plant treatments, which was also presented by the MTT assay (Figure 3.2). In addition, the NR assay indicated that PA extracts demonstrated significantly reduced cytotoxic effects than CR and CR:PA in HepG2 cells. The mitochondrial cell viability (MTT) and growth inhibition (NR) assays therefore demonstrated similar results in cell viability with the only variation owed to the intensity of dose-dependent decrease in HepG2 cells. As the gold standard, cell viability was measured as an endpoint of the MTT and NR assays and the reduction of viability was expressed as cytotoxic effects. However, the decrease in cell viability can also be attributed to apoptosis. For the current study, this difference is not distinguishable and requires further investigation.

The IC_{50} value refers to the cytotoxic concentration of the extracts responsible for causing death to 50% of the viable cells. Using the ISO and SANS cytotoxicity index from the cell viability measurements (specified in Section 3.3.3: ISO 10993-5: 2009; SANS 10993-5:2010), the IC_{50} values of the plant extracts, presented in Table 3.1, were considered cytotoxic for any treatment concentration that decreased the cell viability by 50% or more. The results obtained from the MTT assay, indicated that the IC_{50} values of the plant extracts, exhibited a two-fold increase in toxicity compared to the IC_{50} values presented by the NR assay (Table 3.1). A study conducted by Gomez-Perez *et al*. (2017), indicated decreased sensitivity to cytotoxicity studies performed by MTT assays when compared to the neutral red assay. Consequently, the MTT assay was regarded not suitable for cell viability measurement. Gomez-Perez *et al*. (2017) explain their hypothesis by indicating the interference of copper compounds ($Cu(II)Urea_2$, $Cu(II)Ser_2$ and $CuCl_2$) with MTT formazan, which attributed to inaccurate detection of the formazan product in MTT reduction. The sample analysis report revealed that the leaves of *C. roseus* contained 1.6 mg/kg of copper, whilst *P. afra* contained 0.9 mg/kg (Appendix C and D). The presence of copper

in all the treatments of the leaf extracts may support the two-fold increase that we see in the IC_{50} values of the MTT assay compared to NR assay (Table 3.1). Copper has many physiological functions such as anti-inflammatory and antioxidant effects, but it was also reported by Gomez-Perez *et al.* (2017), that copper may enhance cytotoxic effects when present in high concentrations. This may explain the two-fold increase in the IC_{50} values that was observed for the MTT results compared with the NR assay. Numerous studies reported decrease in cell viability after treatment with *C. roseus* against different cell lines, such as HepG2, Jurkat and HT29 cells (Hanan *et al*., 2018; Punnen, 2017; Vuanghao, 2015). In another study by Ahmad *et al*. (2010), it was reported that *C. roseus* showed a dose-dependent antiproliferative activity against HepG2 cell line due to the presence of vinblastine, a well-known alkaloid used for anticancer chemotherapy that attribute to apoptosis in proliferating cells (Ahmad *et al*., 2010). This apoptosis effect could be associated with the significant decreases in cell viability that was seen in the human hepatocarcinoma (HepG2) cells that were treated with CR (Figure 3.1 and 3.2).

Moreover, the pharmacological activity (antidiabetic, antimicrobial and anticancer effects) of plants has been attributed to their phytochemical constituents (Pandey *et al*., 2020; Pham *et al*., 2020). For example, the anticancer properties of *C. roseus* is owed to indole terpene alkaloids such as vinblastine and vincristine (Barrales-Cureño 2015; Bhagat & Singh, 2014). As confirmed by the analysis report (Appendix C and D), both *C. roseus* and *P. afra* contain phenolics and flavonoids (Appendix E, Figure E.1 and E.2). According to Lin *et al*. (2016), phenolic compounds are vital in defence responses, such as anti-proliferative, antioxidant and anti-inflammatory activities. The relatively low phenolic composition of oven dried PA (Appendix E, Figure E.1) may justify the decreased anti-proliferative activity presented by both viability assays (Figure 3.1 and 3.2), whilst the relatively large phenolic composition of CR:PA could have enhanced the anti-proliferative activity against liver cancer cells (Appendix E, Figure E.1). When comparing the IC_{50} values of *C. roseus* to CR:PA using NR results, we find that the combination was more toxic (Table 3.1). The combination of the leaf extracts induced reduction in proliferating cells, which demonstrated stronger cytotoxic effects against the cell lines compared to single-extract treatment of CR and PA. This effect may be due to the combination exhibiting an antagonistic effect of the mechanism of action between plant phytonutrients; Ca, Na, Mg, Mn, Al, B, N, S, P, K, Cl, Fe, Zn and Cu (Rietra *et al*., 2017; Yin *et al*., 2014), see analysis report of *C. roseus* and *P. afra* for micro-element composition (Appendix C and D), exhibiting negative interactions, decreasing the efficacy of the herbal remedy.

The results of our study confirmed inhibition of the α-amylase activity at all treatment concentrations of PA and CR:PA screened against HepG2 cell line. The inhibition of α-amylase activity by PA and CR:PA might delay the absorption of carbohydrates, offering an effective mechanism to lower post-prandial hyperglycaemia (Elya *et al*., 2015; Sudha *et al*., 2011).

Moreover, the biological activity of flavonoids is directly proportional to the potency of α-amylase inhibition (Witkowska-Banaszczak *et al*., 2020). The relatively high flavonoid content displayed in oven dried *P. afra* (Appendix E, Figure E.2) may be responsible for this inhibition effect (Figure 3.4) (Witkowska-Banaszczak *et al*., 2020). Cells treated with CR:PA showed significant inhibition ($p < 0.05$) of α-amylase activity at the highest investigated concentration (25.6 µg/mL). According to Singh *et al*. (2014), phenolic compounds (phytochemical) are known to inhibit α-amylase activity. The relatively high phenolic content of oven dried CR:PA (Appendix E, Figure E.1) may be responsible for the large inhibition effect displayed by the combination (Figure 3.4). Numerous studies reported a dose-dependent increase in the inhibition of α-amylase inhibition by *C. roseus* (Bhutkar & Bhise, 2012; Malathi *et al*., 2010; Saliu *et al*., 2018). In our study, aqueous leaf extract of *C. roseus* displayed an activation of α-amylase activity at 0.66, 1.64, 10.24 and 25.6 µg/mL (Figure 3.4). The reason why we do not see a dose-dependent increase in the inhibition of α-amylase activity, might be because both Saliu *et al*. (2018) and Bhutkar and Bhise (2012), tested the various extract concentration directly on the enzyme, whilst we examined the α-amylase activity through HepG2 cells.

Treatment of the HepG2 cell line with aqueous leaf extract of *C. roseus*, *P. afra* and CR:PA on α-glucosidase activity provided insight into the mechanism of action of HepG2 cells screened against different extract concentrations. In our study, the results of the α-glucosidase activity showed that CR-treated cells displayed increased activity of α-glucosidase at the highest treated concentration when compared to the control (Figure 3.5, $p < 0.05$). The data of the other two extracts displayed no significant effect on α-glucosidase activity owed to large variations between treatments (Figure 3.5). On another note, other studies have already reported inhibition of α-glucosidase activity of *C. roseus* leaf extracts, measured directly on the enzyme, without exposure to cell lines (Anand *et al*., 2019; Malathi *et al*., 2010). The assay results therefore highlight the variational effect between enzyme measurements conducted in cells compared to assaying the extracts directly on the enzyme.

What we can value from this assay is the potential phytochemical effects of the plants on the activity of α-glucosidase. Flavonoids are known to enhance inhibition of α-glucosidase to which α-glucosidase inhibitors are amongst the most commonly used classes of antidiabetic medications (Şöhretoğlu & Sari, 2019). The relatively high flavonoid content in oven dried PA (Appendix E, Figure E.2) may have contributed to the reduced α-glucosidase activity that was demonstrated in the cells at 10.24 and 25.6 µg/mL of treatment (Figure 3.5). This provides evidence to further investigate the synergistic effects of phytochemicals as potential antidiabetic agents.

According to Maideen and Balasubramaniam (2018) common antidiabetic herbs such as Fenugreek, Cinnamon, Ginseng, Aloe vera and Sesame exert hypoglycaemic effects by

increasing the activity of hepatic enzymes such as an increase in the hexokinase activity Hexokinase is known as the gateway enzyme of the glucose metabolism that phosphorylates glucose to glucose-6-phosphatase (G-6-P) by ATP (Calmettes *et al*., 2013). Glucose-6-phosphatase is the enzyme, mainly located in the liver and kidneys, that provides glucose to the body during starvation (Van Schaftingen & Gerin, 2002). The inhibition of G-6-P is believed to be involved in the action of insulin to control hepatic glucose production to which blood glucose levels decrease (Gardner, *et al*., 1993). The administration of *C. roseus* leaf powder to diabetic rats has been shown to stimulate the activity of hexokinase (Rasineni *et al*., 2010), in which a decrease in blood sugar levels was observed in diabetic animals. This decrease was a result of induced liver hexokinase activity therefore suppressing the G-6-P enzyme (Alam *et al*., 2019). These findings were in accordance with previous studies in which an increase in the activity of liver hexokinase, mediated by herbal medicines, have shown to decrease the blood sugar levels (Aba & Asuzu, 2018; Jayanthi *et al*., 2010; Pari & Srinivasan, 2010). According to Chude *et al*. (2001) the diabetic state of rats causes an increase in the G-6-P activity, to which treatment of *C. roseus* have shown to decrease the G-6-P activities to near normal (i.e. same as non-diabetic rats), therefore demonstrating hypoglycaemic effects in diabetic rats. The inhibition of G-6-P displayed by *C. roseus* (as reported by Chude *et al*., 2001) have been previously associated with an increase in the liver hexokinase activity (Jayanthi *et al*., 2010). In the current study, treatment of HepG2 liver cell line with *C. roseus* and *P. afra,* either individually or in combination, resulted in a significant increase ($p < 0.05$) in the liver hexokinase activity each at a concentration of 1.64 µg/mL when compared to hexokinase activity of the untreated (control) cells (Figure 3.6). This increase suggests that the leaf extracts of *C. roseus* and *P. afra* may stimulate hypoglycaemic effects in rats, based on the scientific discoveries that was made by Chude *et al*. (2001) and Jayanthi *et al*. (2010). Furthermore, the results demonstrated a non-significant increase in the hexokinase activity of all three plant extracts the highest treated concentration (4.096 µg/mL) against HepG2 cells. We found that the combination showed enhanced hexokinase activities therefore showing antidiabetic potential. On the basis of the current findings, the ability of PA and CR:PA extracts to be associated with increased hexokinase activity could suggest potential antidiabetic properties.

3.7 Conclusion

The aqueous leaf extract of *Catharanthus roseus* was toxic to HepG2 cells at the lowest treated concentrations (0.66–4.096 µg/mL), presenting IC_{50} value of 6.02 ± 3.0 µg/mL, whilst *Portulacaria afra* have shown to be less toxic to HepG2 cells over a larger concentration range with an IC_{50} value of 27.93 ± 2.26 µg/mL. The combination treatment of these plants was shown to be the most potent against the HepG2 cells with IC_{50} value of 4.10 ± 0.65 µg/mL. These plants are however regularly used by modern-day traditional healers, exercising treatment in patients with diabetes through prescribing a daily oral intake of the water decoctions. The results obtained in this study indicate that the combination of *C. roseus* and *P. afra* show potential antidiabetic activities. The antidiabetic activity can be attributed to the inhibition of α-amylase, as well as the induced liver hexokinase activities. Since the activation of hexokinase has known antidiabetic effects, the potential of these plants as natural diabetes treatments must further be investigated in complex and dynamic biological systems. Therefore, an *in vivo* study was conducted to confirm the potential antidiabetic or hypoglycaemic activities observed in the current *in vitro* study.

3.8 Reference list

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CHAPTER 4 *IN VIVO* STUDY

Antidiabetic potential of the combination of aqueous *Catharanthus roseus* and *Portulacaria afra* leaf extracts in streptozotocin-induced diabetic male Sprague Dawley rats

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Abstract: The treatment of diabetes mellitus using pharmacological agents have unavoidable side effects, additional expenses and is usually unattainable to less financially privileged patients. As an alternative, medicinal plants have been used for decades to manage diabetes mellitus, due to their affordability and availability. In this study, we evaluated the possible antidiabetic effects of aqueous leaf extracts of *Catharanthus roseus* and *Portulacaria afra* leaves in chemically induced diabetic rats. Diabetes was induced by a single intravenous injection of streptozotocin at 55 mg/kg body weight to male Sprague Dawley rats (N = 50). The animals were randomly divided into six experimental groups; non-diabetic (*n* = 10), diabetic non-treated (*n* = 8), metformin (500 mg/kg body weight, *n* = 8), *C. roseus* (CR, 100 mg/kg body weight, *n* = 8), *P. afra* (PA, 100 mg/kg body weight, *n* = 8) and 1:1 combination of *C. roseus* and *P. afra* (CR:PA, 100 mg/kg body weight, *n* = 8). The rats received an oral administration of the aqueous extracts of the leaves of CR, PA and CR:PA for 28 consecutive days. Results showed that administration of CR to diabetic rats lowered the plasma glucose and reversed the diabetic effects of organ enlargement. Whereas administration of PA to diabetic rats demonstrated elevated plasma glucose levels and significant weight loss. The liver and kidneys of PA-treated rats were significantly enlarged compared to diabetic non-treated rats. The administration of CR:PA to diabetic rats displayed antagonistic effects. While *C. roseus* lowered the blood glucose levels in diabetic rats, *P. afra* raised it. In conclusion, *P. afra* and CR:PA leaf extracts have shown no antidiabetic effects in Sprague Dawley rats, whereas leaf extracts of *C. roseus* did show antidiabetic activities in the management of diabetes mellitus.

Keywords: Diabetes mellitus, Traditional healers, Medicinal plants, Rats, Streptozotocin

4.1 Introduction

Type 1 and type 2 diabetes mellitus are characterized by abnormally high blood glucose levels owing to failed insulin production or insulin deficiency (Tan *et al*., 2019). Both type 1 and type 2 diabetes mellitus are associated with complications such as cardiovascular disease, risk of heart attack, kidney failure, poor wound healing abilities and vision loss (Kuniss *et al*., 2019; Yaghoubi *et al*., 2020). Existing conventional treatment of type 1 and type 2 diabetes mellitus is largely comprised of synthetic drugs such as pramlintide acetate injection (Symlin) and metformin. Pramlintide acetate injection (Symlin) can be used to treat type 1 and type 2 diabetes (Laustsen, 2005), while metformin is mainly used to treat type 2 diabetes (Foretz *et al*., 2019). Despite the fact that these synthetic drugs can manage diabetes, they present various side effects such as diarrhoea, asthenia, nausea, myalgia, just to name a few (Hostalek *et al*., 2015).

Although synthetic drugs are affordable and available to the majority of middle-class people, they are too expensive and not easily accessible to people in countries with limited resources, especially in the underserved rural areas. In order to eliminate this problem, there is a need to investigate current scientific literature and traditional medicines to develop a beneficial ethnomedicinal plant-based antidiabetic treatment strategy that is available to people with socioeconomic challenges. Traditional medicine arose due to immemorial gathering of knowledge, skills, beliefs and cultural experiences that were conserved and passed down from generation to generation, with the sole purpose to sustain primary healthcare (Mahomoodally, 2013). Traditional medicinal plants are perceived to possess fewer side effects, readily available, easily accessible and overall inexpensive treatments (Kasole *et al*., 2019). However, there is a need to verify the efficacy of traditional medicinal plants in the management of diabetes through research. The current study was designed to investigate the antidiabetic effects of two traditional medicinal plants: *Catharanthus roseus* and *Portulacaria afra.* Numerous studies have reported the antidiabetic effects of *C. roseus* (Pham *et al*., 2020; Prasad *et al*., 2009; Singh *et al*., 2001; Tolambiya & Mathur, 2016).

According to Tolambiya and Mathur (2016), *C. roseus* is used for various traditional purposes including decoction of leaves to treat diabetes in the Philippines (Muralidharan, 2014), bitter leaves used as a vomitive in Madagascar (Muralidharan, 2014), leaf juices used for indigestion and dyspepsia in Mauritius (Muralidharan, 2014), flower decoction for asthma, tuberculosis and flatulence in the Bahamas (Muralidharan, 2014), gargling of leaves to ease sore throat in America and the plant is used for diabetes, hypertension, insomnia and cancer in Malaysia.

According to Hulley and Van Wyk (2019), *P. afra* is used for the treatment of pain, inflammation, diabetes, hypotension, kidney ailment, diarrhoea and wound healing as a poultice. The medicinal use of *Portulacaria afra* as a treatment for diabetes mellitus is also reported by Hankey (2009).

Traditional healers and nurses also claim that *P. afra* has hypoglycaemic and hypotensive effects (Parker, 2018). There is also anecdotal evidence which suggests the beneficial effects, such as hypoglycaemic and hypotensive activities, of *P. afra* and *C. roseus,* when used in combination and this prompted the need to verify the potential hypoglycaemic effects of the plants via *in vivo* experimental models. To the best of our knowledge, no *in vivo* antidiabetic studies have been conducted for *P. afra* (Spekboom).

Chemicals, such as Streptozotocin (STZ), are widely used for the induction of experimental diabetes in rodents for the understanding of the function of pancreatic β-cell glucotoxicity in diabetes (Wu & Yan, 2015). STZ has been used individually or in combination with other chemicals or with dietary supplements to induce type 1 or type 2 diabetes (Gajdosik *et al*., 1999). Type 1 diabetes can be induced in rodents by a single high-dose of STZ via intravenous injection in the tail vein (Furman, 2015). This model has been very useful in evaluating the efficacy of natural products that are potentially capable of lowering blood glucose levels (Kumar *et al*., 2012).

The aim of this study therefore was to investigate the potential of aqueous leaf extracts of *Catharanthus roseus* and *Portulacaria afra* individually and in combination to ameliorate chemically induced diabetes in male rats.

4.2 Materials and methods

4.2.1 Plant material

Plant material was collected and processed as described in Section 3.2.1 (*Plant material*) of this dissertation.

4.2.2 Preparation of aqueous leaf extracts

The aqueous leaf extracts were prepared as described in Section 3.3.1 (*Extraction of plant material*) of this dissertation.

4.2.3 Experimental animals and housing

Fifty (50) male Sprague Dawley rats, weighing between 200–400 g, were used in the study. The rats were bred and supplied by the Preclinical Drug Development Platform (PCDDP) Vivarium at the North-West University. The current study was carried out with permission by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC, ethics clearance no. NWU-00570-19-A5). Rats were acclimated for five days prior to the start of the experimental treatments. The rats were housed in pairs per rodent cage (Tecniplast Green Line IVC Sealsafe PLUS Rat), containing Vermiculite bedding, that were kept at standard

temperature of 24 ± 1°C and relative humidity of 55 ± 10%. A 12-hour light-dark cycle was maintained. Rats had *ad libitum* access to food (Nutrition Hub, Standard Rodent Feed) and water.

4.2.4 Experimental design

In this study, we adopted a diabetic model using single high dose of streptozotocin- as described by Furman (2015) and Rasineni *et al*. (2010). Type 1 diabetes mellitus was induced in male Sprague Dawley rats by a single intravenous injection of 55 mg/kg STZ (Furman, 2015). After the animals reached hyperglycaemic state of ≥ 11.1 mmol/L blood sugar levels, treatment was initiated for 28 consecutive days by oral administration of 100 mg/kg plant extract. The rats were weighed daily to adjust the treatment dosage and assess the health of the animals. The endpoint of the current study is reached by decapitation and collecting organs for histology.

Diabetic male rats (7–9 weeks old) were randomly assigned into six groups as follows:

Group 1: Non-diabetic control (**NDC**) group: rats in this group were non-diabetic and received distilled water for 28 consecutive days via oral administration.

Group 2: Diabetic negative control (**DNC**) group: rats in this group were diabetic and received distilled water for 28 consecutive days via oral administration.

Group 3: Metformin (**MET**) positive control group: treated orally with 500 mg/kg metformin for 28 consecutive days.

Group 4: *C. roseus* (**CR**) group: rats in this group were treated orally with aqueous leaf extract of *C. roseus* leaf (100 mg/kg) for 28 consecutive days.

Group 5: *P. afra* (**PA**) group: rats in this group were treated orally with aqueous leaf extract of *P. afra* (100 mg/kg) for 28 consecutive days.

Group 6**:** *C. roseus* and *P. afra* (**CR:PA**) group: rats in this group were treated orally with 1:1 combination of *C. roseus* (CR): *P. afra* (PA) at 100 mg/kg for 28 consecutive days.

4.2.5 Induction of type 1 diabetes using streptozotocin and treatment with plant extracts

Streptozotocin (STZ) (Sigma Aldrich SA (PTY) LTD, Kempton Park, Cat# S0130, ≥75% α-anomer basis, ≥98% (HPLC), powder) was freshly prepared at 68.75 mg/mL in 0.05 M ice-cold citrate buffer at pH 4.5 and stored at -20ºC while being protected from light (ensuring compound stability). The animals were fasted for 6 to 8 hours before STZ injection. Fasting blood glucose (FBG) was tested with a calibrated ACCU-CHEK® Instant glucometer (Roche Diabetes Care GmbH, Sandhofer Strasse 116, 68305 Mannheim, Germany, SN 95900683635) by collecting a small amount of blood via tail prick method using disposable lancet. The rats (groups 2–6) were induced with type 1 diabetes mellitus according to the method described by Furman (2015) by single

intravenous (IV) injection, in the tail vein, of the 55 mg/kg STZ solution (0.8 ml/kg). The rats in the control group (*n* = 10) received equal amounts of citrate buffer (0.8 ml/kg). Once the citrate buffer was introduced into the vial containing the pre-weighed STZ powder the injection needed to occur within 15 minutes after which the optimal effects of STZ diminishes. The decomposition rate of STZ rapidly increases after 15–20 minutes (Furman, 2015). Animals were provided with 10% sucrose water *ad libitum* for 48 h after STZ administration to prevent fatal hypoglycaemia from occurring (ACUSC, 2016; Furman, 2015), after 48 hours they received normal water *ad libitum*.

The fasting blood glucose (FBG) levels were measured five days after the injection of STZ, to establish hyperglycaemic state. Only rats with blood glucose levels ≥ 200–250 mg/dL (11.1–13.87 mmol/L) were included in the study and randomly divided into five groups (Singh *et al*., 2001).

Stock solutions of the treatments were prepared bi-weekly using distilled water and refrigerated at 4°C. Diabetic rats were treated daily, for a period of 28 days, receiving 100 mg/kg of plant extracts via oral gavage. The untreated diabetic rats (DNC) received an oral dosage of 0.2 mL water/0.25 kg rat. The stock solution of MET (group 3) was prepared at 300 mg/mL to give an oral dosage of 0.5 mL/0.30 kg rat. The plant extracts of CR (group 4), PA (group 5) and CR:PA (1:1, group 6) were prepared at 125 mg/mL with oral volume of 0.8 mL/kg rat.

Fasting blood glucose levels (mmol/L) were measured on days 7, 14 ,21, 28 and upon termination on day 29. On day 28, the rats were fasted overnight and euthanised the following day by decapitation. The brain, heart, liver, pancreas, kidneys and testes were collected, weighed and stored. Trunk blood was measured for plasma glucose upon decapitation, from rats fasted for 12 h.

The relative organ weight of the animals were calculated as:

$$\text{Relative organ weight (\%)} = \frac{\text{Total organ weight}}{\text{Terminal body weight}} \times 100$$

The well-being of the rats was monitored using a scoring system, generated by Wang-Fischer and Garyantes (2018), which contains a series of health measurements and diabetic severity assessments (Appendix F): a measure of fur cleanliness, faeces consistency and natural behaviour were used to measure distress. Owed to the characteristic symptoms of diabetes such as polyuria, vermiculite bedding was used instead of standard corncob bedding. Vermiculite is a softer, more lightweight material and better absorbent when compared to corncob bedding. With the vermiculite bedding, rodent cages could be changed every two days.

4.2.6 Statistical data analysis

Data was expressed as mean ± standard deviation (SD). Normality of data was determined using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) test was used to determine statistically significant differences (*p*) between the means of independent groups. The repeated measures ANOVA together with the Bonferroni *post-hoc* test was used to determine statistical significance ($p < 0.05$) between means from different experimental groups. The clinical relevance of the results was determined by calculating the effect sizes (*d*) between groups (Loots *et al*., 2011). The calculated effect sizes (*d*), also referred to as the practical significant differences, between the means of the groups was interpreted as follows: $d \leq 0.2$ small effect/no practically significant difference, $d \approx 0.5$ medium/practically visible difference, $d \approx 0.8$ large effect/practically significant difference for parametric data.

4.3 Results

4.3.1 The efficacy of STZ to induce hyperglycaemia

A total of 50 rats were used in this study but only 46 rats were considered for data analysis after the removal of 4 rats due to illness-related complications. Out of the 40 diabetic rats (Appendix G, Table G.1, $n = 40$), 36 managed to reach the study endpoint. All the rats exhibited normal glycaemic levels of 5.0 ± 0.21 mmol/L before injection of STZ (55 mg/kg body weight). Hyperglycaemic state (≥ 11.1 mmol/L) was confirmed within 5 to 21 days, following injection of STZ. Diabetic rats exhibited a mean FBG value of 22.02 ± 2.51 mmol/L ($n = 40$). The NDC rats ($n = 10$) exhibited a mean FBG value of 6.00 ± 0.71 mmol/L throughout the treatment period. Our study presented a STZ success rate of 66.7% (see Appendix G for details).

4.3.2 Effect of the plant extracts on blood glucose levels over the treatment period

The effect of aqueous leaf extracts of CR, PA and CR:PA on fasting blood glucose (FBG) are presented in Figure 4.1A. The blood glucose of the non-diabetic control group varied between 5.83–6.91 mmol/L, which is considered to be normal (Figure 4.1A). The blood glucose of the non-diabetic control was significantly lower when compared to that of the diabetic groups: DNC, MET, CR, PA and CR:PA, as was expected (Figure 4.1A, $p < 0.05$).

In the current study, results were described as statistically (*p* value) and/or practically (*d* value) significant. Statistical significance was used to describe an effect between two variables existing in the current study, whilst ‘practical significance’ was used to describe the difference between two variables as large enough to be considered meaningful in real life (demonstrated to be of high clinical relevance).

The FBG data showed that aqueous leaf extract of *C. roseus* maintained lower blood glucose levels compared to diabetic non-treated controls (DNC), although it was not statistically significant (Figure 4.1A, $p > 0.05$), effect size calculations showed that the blood glucose of CR was practically significantly lower compared to DNC rats from day 7 onwards ($d > 0.8$). The effect size calculations shows that CR maintained significantly lower levels of glucose when compared to other diabetic treated controls, from day 7 onwards ($d \geq 0.8$).

The rats that were treated with PA had elevated levels of blood glucose compared to the other groups throughout the treatment period (Figure 4.1A, $p > 0.05$, $d \geq 0.8$). When compared to DNC rats, PA showed no statistical significance ($p > 0.05$) although we did see a large effect size from day 7 onwards ($d > 0.8$), which is indicative of practical significance. The PA-treated rats displayed blood glucose levels that were significantly higher from day 14 when compared to CR-treated rats (Figure 4.1A, $p = 0.01$). Regardless, the effect size calculations demonstrated that the FBG levels of PA-treated rats were practically significantly ($d \geq 0.8$) higher, compared to all other experimental groups (Figure 4.1A).

The mean FBG between DNC- and CR:PA-treated rats showed no significant difference ($p > 0.05$). However, a medium effect size was calculated between FBG of DNC and CR:PA-treated rats from day 21 ($d = 0.55$) presenting a practically visible increase in FBG of clinical relevance for the rats treated with CR:PA ($p > 0.05$, $d \approx 0.5$). The FBG values of CR:PA-treated rats showed no clinical relevance in the reduction of blood glucose levels from day 1 to 14 when compared to DNC rats ($p > 0.05$, $d \approx 0.2$).

The rats that were treated with the 1:1 combination of *C. roseus* and *P. afra*, displayed blood glucose levels that were significantly higher than CR-treated rats ($d \geq 0.8$) but significantly lower ($d \geq 0.8$) compared to rats treated with PA extract (Figure 4.1A). Our results have shown that FBG levels were better controlled in CR:PA-treated rats when compared to PA although the combination did not exceed the hypoglycaemic effects of CR-treated rats (Figure 4.1A). The FBG levels of CR:PA-treated rats were maintained at lower levels for the first 14 days (Figure 4.1A). After day 14, the FBG levels of rats treated with CR:PA were significantly higher than that of DNC rats (Figure 4.1A, $d \geq 0.8$).

There were no statistically significant differences in glucose levels between the rats that were treated with metformin and that of diabetic non-treated (DNC) rats ($p > 0.05$). However, MET-treated rats maintained lower levels of glucose in the first week when compared with DNC ($d \geq 0.8$). After 14 days, a significant increase in FBG of MET rats were recorded ($d \geq 0.8$) compared to diabetic non-treated rats (Figure 4.1A). Although metformin did not show effective hypoglycaemia compared to DNC rats, it still displayed enhanced hypoglycaemic effects compared to PA-treated rats. This was confirmed by the effect size analysis that have shown that

MET rats had blood glucose levels that were significantly reduced compared to PA ($d \geq 0.8$), whilst still upholding weaker hypoglycaemic activities than CR-treated rats. Our results have shown no significant hypoglycaemic effects in the diabetic rats treated with metformin.

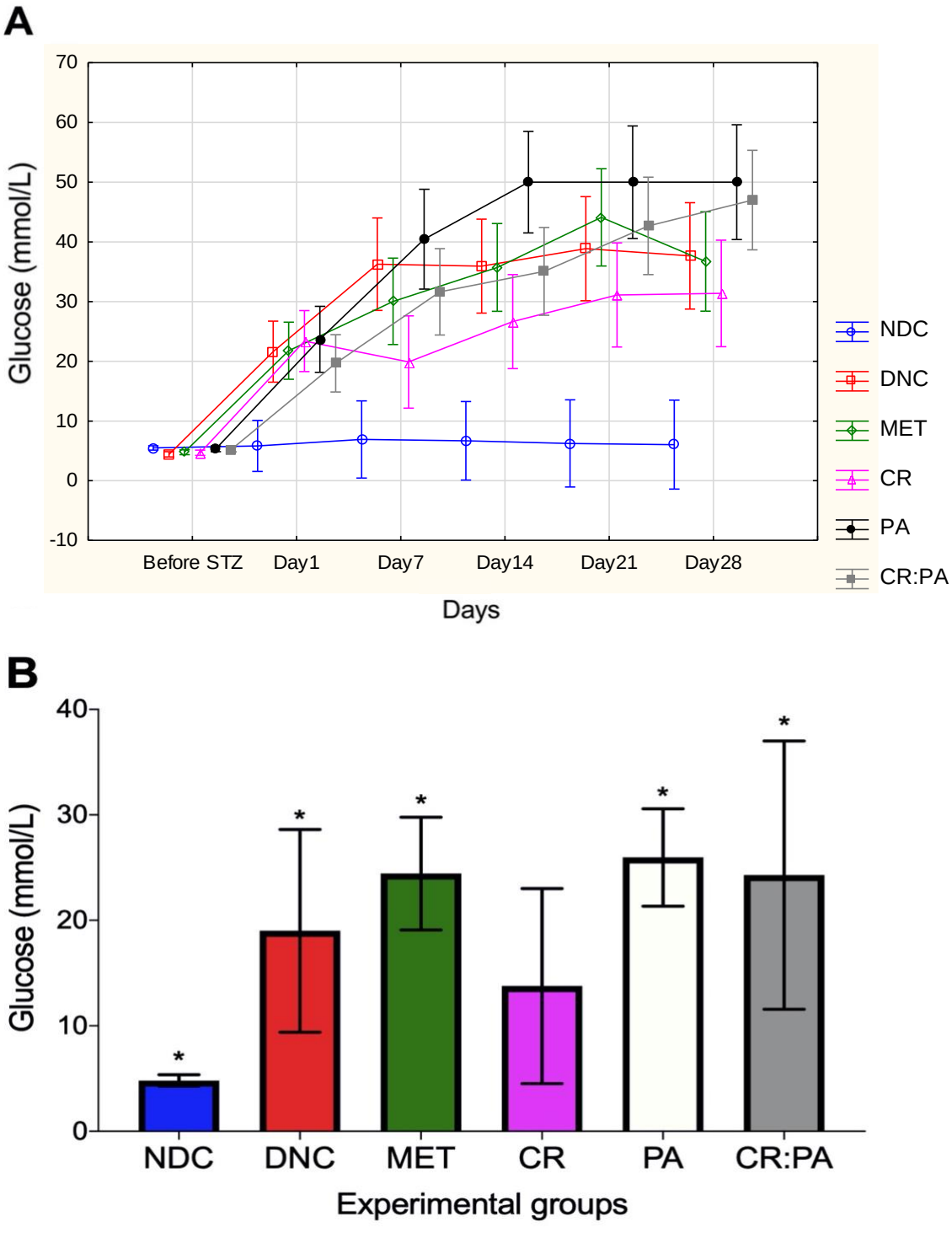

Figure 4.1: The effect of aqueous CR and PA leaf extracts on A) mean blood glucose levels of STZ-induced Sprague Dawley rats during treatment period, B) mean terminal blood glucose after euthanasia. Values are presented as mean ± 0.95 confidence interval. * indicative of significant difference of DNC, MET, PA and CR:PA in comparison to NDC ($p < 0.05$). NDC = Non-diabetic control ($n = 10$), DNC = Diabetic negative control ($n = 7$), MET = Metformin ($n = 8$), CR = *C. roseus* ($n = 7$), PA = *P. afra* ($n = 6$) and CR:PA = *C. roseus*: *P. afra* (1:1) ($n = 8$).

4.3.3 Effect of the plant extracts on terminal blood glucose

The results on the terminal blood glucose levels of the six experimental groups are presented in Figure 4.1B. Non-diabetic rats had significantly reduced terminal FBG (i.e. 24% lower) levels (4.82 mmol/L) compared to the FBG levels that were recorded during the 28-day treatment period. The terminal fasting blood glucose levels of all the diabetic groups, except for diabetic CR rats (Figure 4.1B, $p = 0.41$), were significantly elevated ($p < 0.05$) compared to NDC rats. The terminal blood glucose results of CR-treated rats show no statistically significant effect when compared to NDC rats, which could imply possible glycaemic control by *C. roseus* (Figure 4.1B, $p > 0.05$). There were no significant differences in the terminal glycaemic levels of diabetic rats treated with MET, CR, PA and CR:PA when compared to the non-treated diabetic rats ($p > 0.05$).

4.3.4 Effect of the plant extracts on body weight and growth performance

The normal non-diabetic rats gained, on average, 2.51 g/day during the 4-week treatment period demonstrating a 20.7% increase by the end of the study, which was expected (Figure 4.2, $p < 0.05$). The body weight of rats that were treated with DNC, metformin and *C. roseus* gained, on average, 0.37, 0.33 and 0.58 g/day (Figure 4.2). Whilst the rats that were treated with PA and CR:PA lost, on average, 0.39 and 0.22 g/day, respectively.

There were no significant differences between the body weights of MET and DNC rats ($p > 0.05$, $d < 0.2$), which means that metformin had no effect on the weight. The body weights of rats treated with CR were somewhat similar to both DNC- and MET-treated rats, but after 18 days a practically significantly increase ($d \geq 0.8$) was observed for CR. Whilst the body weight of PA-treated rats, from day 18, were practically significantly lower compared to both DNC and MET-treated rats ($d \geq 0.8$). Throughout the treatment period, the effect size calculations revealed that the body weights of CR-t rats were practically significantly higher compared to PA and CR:PA, respectively (Figure 4.2, $d \geq 0.8$). The statistical data indicated that treatment with aqueous leaf extracts of *P. afra* and CR:PA (100 mg/kg/day) showed an overall percentage body weight reduction of 4.17% (PA) and 3.85% (CR:PA).

The initial and terminal body weights (before STZ treatment) of the experimental groups were plotted in Figure 4.3. The terminal body weight of the diabetic rats treated with MET, CR, PA and CR:PA were significantly lower compared to that of normal non-diabetic rats. (Figure 4.3, $p < 0.0001$). The PA treatment resulted in 34.9% reduction in body weight at termination compared to normal NDC rats (Figure 4.3, $p < 0.0001$). The terminal body weight of rats treated with CR and CR:PA decreased by 23.8% and 29.3% when compared to NDC rats ($p < 0.0001$, $d > 0.8$), while both DNC and MET rats resulted in 27.7% reduction ($p < 0.0001$). The terminal body weights

of rats treated with CR was statistically significantly higher than those rats treated with PA (p = 0.04).

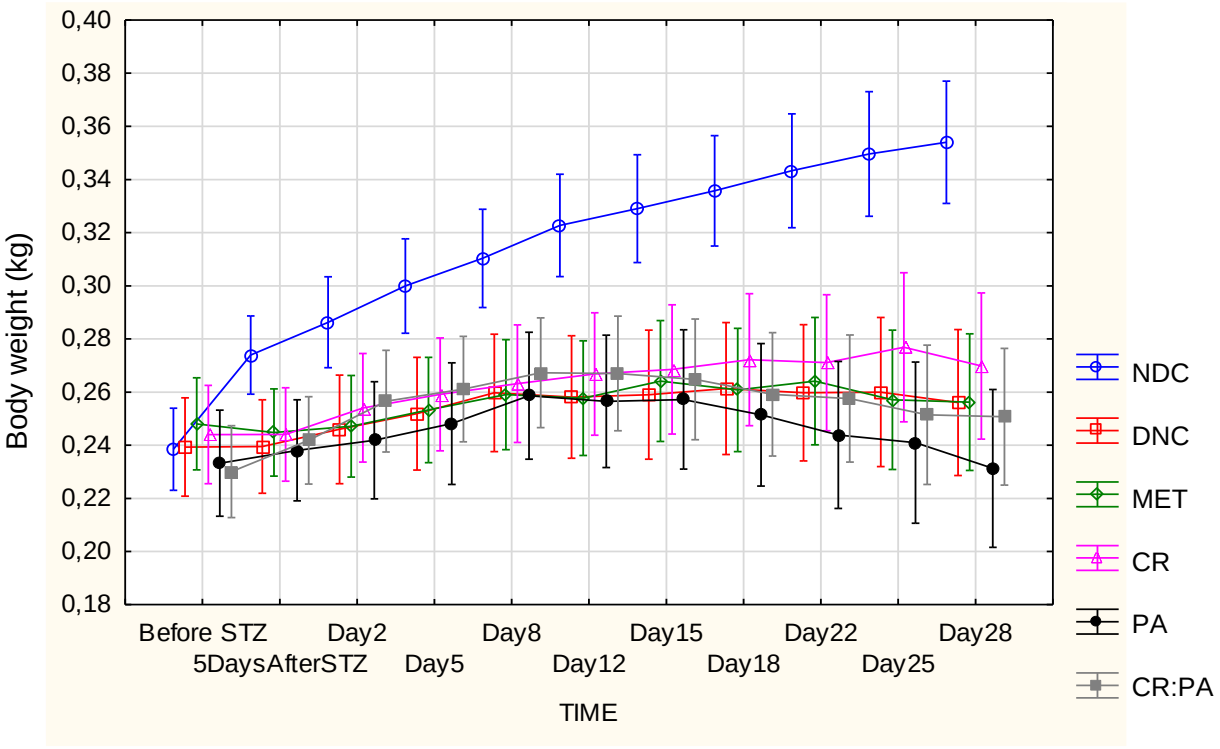

Figure 4.2: The effect of aqueous *C. roseus* and *P. afra* leaf extracts on the body weight of normal (NDC, n = 10) and STZ-induced Sprague Dawley rats over a time period of 28 days. All data is expressed as mean ± 0.95 confidence interval (CI). NDC = Non-diabetic control (n = 10), DNC = Diabetic negative control (n = 7), MET = metformin (n = 8), CR = *C. roseus* (n = 7), PA = *P. afra* (n = 6) and CR:PA = 1:1 mixture of *C. roseus: P. afra* (n = 8).

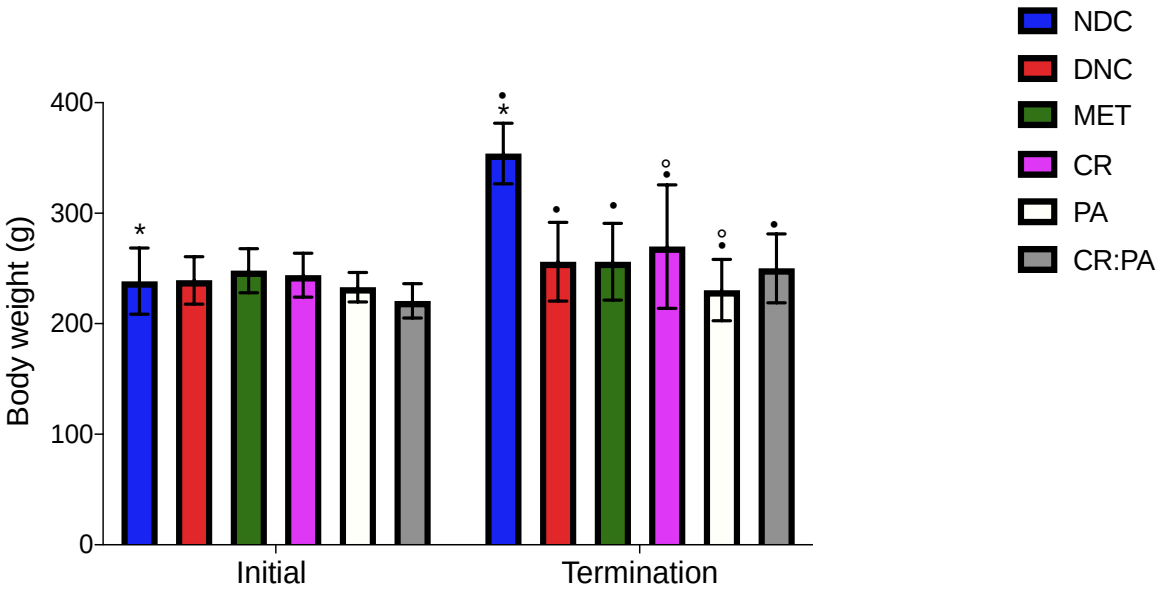

Figure 4.3*:* The effect of aqueous CR and PA leaf extracts on the initial and terminal body weight of the six experimental groups. *, •, ° indicative of significant difference compared to NDC rats (p < 0.05). 'Initial' = normal body weight before IV injection of STZ and 'Termination' = body weight of rats on day of decapitation (fasted for 12 h). NDC = Non-diabetic control (n = 10), DNC = Diabetic negative control (n = 7), MET = metformin (n = 8), CR = *C. roseus* (n = 7), PA = *P. afra* (n = 6) and CR:PA = 1:1 mixture of *C. roseus: P. afra* (n = 8).

4.3.5 The effect of plant extracts on the terminal organ morphometry

The relative organ weights, expressed as a percentage of body weight, are presented in Table 4.1. The relative weight of the pancreas was the same in all the rats, except in those treated with CR:PA. Analysis of the effect sizes showed that the relative weight of the pancreas of CR:PA rats were practically significantly decreased by 25.6% in comparison to NDC group (d = 0.9) while also showing a practical significant decrease of 32.6% when compared to DNC group (Table 4.1, $^{\#}d$ > 0.8). Moreover, the relative pancreatic weight of CR:PA-treated rats are 29.3% smaller than CR rats (d = 1.15) and 27.5% smaller than PA-treated rats (d = 1.07), which are both of high practical relevance.

In diabetic rats, the weight of the brain was increased by 46.2%, which means the diabetic state induced some changes in the organ (Table 4.1). In comparison to diabetic non-treated rats, treatment with MET, CR and PA showed no decrease in the weight back to baseline level (i.e. same as NDC), meaning that the CR, PA and MET could not reverse the effect of diabetes in the brain ($p > 0.05$, $d < 0.2$). Upon treatment with CR:PA, the weight of the brain decreased by 15.8% ($^{\#}d$ = 0.65) compared to DNC, which means that the CR:PA reversed the effects of diabetes (Table 4.1). This therefore would suggest that CR:PA has antidiabetic effects.

The relative heart weights of diabetic rats were significantly increased ($p < 0.05$) by up to 20% compared to NDC, indicating that the diabetic state caused heart enlargement (Table 4.1). The diabetic rats that were treated with MET and CR showed a practically (d > 0.5) visible decrease in the weight by 4.17%, meaning that treatment with MET and CR reversed the effects of diabetes by inhibiting the enlargement of the heart, therefore suggesting antidiabetic effects.

When we look at the relative liver weights of diabetic rats, we find that the liver was significantly ($p < 0.05$) enlarged (by up to 46.3%) compared to NDC rats (Table 4.1). Upon treatment with PA, the weight was further increased by 12.6% (i.e. greater than DNC), meaning that the PA could not reverse the effects of diabetes but rather enhanced liver enlargement (Table 4.1, $^{\#}d$ = 1.35). Comparing the relative liver weights of PA-treated rats with treatment of CR and CR:PA, showed that the weight of the liver was 25.7% larger than CR-treated rats (d = 1.36) and 11.6% larger than CR:PA-treated rats, *(*d = 1.17), meaning that PA caused additional effects in the organ. Furthermore, diabetic rats that were treated with CR, demonstrated a significant (d = 0.62) decrease (by up to10.4%) in the liver (i.e. compared to DNC) back to baseline level, meaning that CR reversed the effects of diabetes in the organs. This therefore suggests that CR has antidiabetic effects. The weight of the liver in diabetic rats treated with CR:PA, were further increased (i.e. compared to DNC, d > 0.8), meaning that CR:PA could not reverse the effects of diabetes on organ enlargement (Table 4.1).

In diabetic rats, the state of hyperglycaemia induced a 46% increase in the weight of the kidney, relatively to the body weight (i.e. compared to NDC), meaning that the diabetic state induced organ enlargement (Table 4.1). Upon treatment with CR, the weight significantly ($d \geq 0.5$) decreased by 7.1% in comparison to DNC, meaning that CR reversed the effect of diabetes in the organ. This therefore would suggest that CR has antidiabetic effects. According to the effect size calculations, treatment with PA, demonstrated a practically significant increase (by up to 6.3%) in the relative kidney weights i.e. compared to DNC (Table 4.1, $^{\#}d \geq 0.8$). This therefore implies that treatment with PA caused additional effects in organ enlargement. The diabetic rats treated with CR:PA caused kidney enlargement (by up to 3.9%), meaning that the CR:PA could not reverse the effects of diabetes in the kidney ($d \geq 0.5$),

The elevated blood glucose levels in diabetic rats caused a significant enlargement (36.3% larger i.e. compared to NDC) in the relative testis weights (Table 4.1, $p < 0.05$, $d > 0.8$). The ability of the plants to exhibit antidiabetic effects in diabetic rats would be represented by a decrease in the relative weights of the testis back to the baseline value (i.e. same as NDC). In diabetic rats, treatment with PA caused significant enlargement in the weight of the testis (enlarged by 20.1%), meaning that PA could not reverse the antidiabetic effects in the testis (Table 4.1, $^{\#}d = 1.51$). In addition to that, the relative testis weights of PA-treated rats were significantly ($d > 0.8$) larger than that of metformin and CR groups (Table 4.1). Upon treatment with CR:PA, the weight increased by 10.1% i.e. compared to DNC rats ($d \geq 0.5$), whilst also being 8.38% smaller than that of PA-treated rats ($d = 0.51$). This, therefore, reveal that the combination presented better-quality antidiabetic effects in the testis than PA, whilst the other two plant treatments caused an increase in the relative testis weights. The treatment with CR displayed no significant difference ($d = 0.13$) to DNC rats, suggesting that CR inhibited the testis from enlarging therefore, suggesting antidiabetic effects.

Table 4.1*:* The effect of aqueous CR and PA leaf extracts on the relative organ weights to body weight percentage of non-diabetic and STZ-induced diabetic rats after a treatment period of 28 days.

Relative Organ Weight	**Treatment group**					
	NDC ($n = 10$)	**DNC** ($n = 7$)	**MET** ($n = 8$)	**CR** ($n = 7$)	**PA** ($n = 6$)	**CR:PA** ($n = 8$)
Brain (%)	0.52 ± 0.06	0.76± 0.11$^{*\bullet}$	0.75± 0.09$^{*\bullet}$	0.73± 0.17$^{*\bullet}$	0.76± 0.13$^{*\bullet}$	0.64± 0.20$^{\#}$
Heart (%)	0.40± 0.04	0.48± 0.04$^{*\bullet}$	0.46± 0.04$^{\bullet}$	0.46± 0.05$^{\bullet}$	0.47± 0.03$^{\bullet}$	0.47± 0.09$^{\bullet}$
Liver (%)	3.09± 0.19	4.52± 0.42$^{*\bullet}$	4.76± 0.33$^{*\bullet}$	4.05± 0.77$^{*\bullet}$	5.09± 0.31$^{*\bullet\#}$	4.56± 0.46$^{*\bullet}$
Kidney (%)	0.87± 0.05	1.27± 0.08$^{*\bullet}$	1.26± 0.07$^{*\bullet}$	1.18± 0.21$^{*\bullet}$	1.35± 0.11$^{*\bullet\#}$	1.32± 0.11$^{*\bullet}$
Pancreas (%)	0.39± 0.10	0.43± 0.11	0.40± 0.06	0.41± 0.08	0.40± 0.10	0.29± 0.11$^{\bullet\#}$

Testis (%)	1.02± 0.06	1.39± 0.18*•	1.47± 0.19*•	1.37± 0.20*•	1.67± 0.18*•#	1.53± 0.26*•

All weights are presented as a percent of total body weight. All data are presented as mean ± standard deviation. * indicate statistically significant increase when compared to NDC ($p < 0.05$). • indicative of practically significant difference compared to NDC ($d > 0.8$). # indicative of practically significant difference compared to DNC ($d > 0.8$).

NDC = Non-diabetic control, **DNC** = Diabetic negative control, **MET** = Metformin, **CR** = aqueous *C. roseus* leaves, **PA** = aqueous *P. afra* leaves and **CR:PA** = 1:1 mixture of *C. roseus* and *P. afra*.

4.4 Discussion

In the current study, the effects of aqueous leaf extracts of *Catharanthus roseus* and *Portulacaria afra* on the lowering of plasma glucose levels were investigated in male rats induced with insulin-dependent (type 1) diabetes mellitus.

Diabetes mellitus was successfully induced in rats (66.7% success rate, see Appendix G for detailed description) via a single high dose of streptozotocin (STZ) at 55 mg/kg. The characteristic diabetogenic property of STZ to human type 1 diabetes mellitus includes the following symptoms: hyperglycaemia, polydipsia and polyuria (Furman, 2015). This is in agreement with the findings of Wang-Fischer and Garyantes, (2018), depicting identical symptoms in type 1 diabetic rats: increased thirst (polydipsia), excessive urination (polyuria), increased hunger (polyphagia), gastric dysfunction, unexplained weight loss and fatigue. Our study demonstrated that normal (non-diabetic) rats appeared healthy, active and gained weight, whilst the chemically induced diabetic rats appeared ill, inactive, presented polyuria (observed through frequent cage changes) and weight loss, which mimic the signs of potential type 1 diabetes. We presuppose that our animals had type 1 diabetes mellitus based on their symptoms and the protocol specific guidelines we followed for inducing type 1 diabetes as described by Furman (2015).

Polydipsia is a common sign of type 1 diabetes mellitus (Dheir *et al*., 2019). Owed to the diabetic state of the rats, in our study, polyuria occurred, which was clearly visible in the wetness of the bedding. Some concern was raised by previous studies that the frequency at which rat cages were changed could negatively affect the well-being of the rats (Rasmussen *et al*., 2011). These studies suggested that it causes some disturbance to which elevated stress levels could increase the blood glucose levels (Duke *et al*., 2001; Rosenbaum *et al*., 2009; Yitshak-Sade *et al*., 2020). In our study, the statistical analysis of the FBG values in NDC rats, demonstrated no statistical significance between rats ($p > 0.05$). For this reason, contributors of stress such as bedding changes did not result in major interferences with the FBG values of rats and were considered negligible (Figure 4.1A).

In addition, we assessed the consistency of rat faeces as a hyperglycaemia marker (see Appendix F for severity score). According to Wang-Fischer and Garyantes (2018), diabetic rats are

presented with a soft or pasty stool, which was in accordance with the findings of our experimental observations.

The blood glucose levels of fasted STZ-induced diabetic rats were significantly elevated when compared to non-diabetic rats. STZ is a diabetogenic agent well-known for its destructive effects to pancreatic β-cells, resulting in hyperinsulinaemia and hyperglycaemia as we have recorded in the current study (Konda *et al*., 2020). The FBG levels of MET treated rats were significantly higher than NDC rats, as would be expected for diabetic animals. Treatment with metformin, in the first 2 weeks, maintained lower blood glucose levels than DNC rats but after 14 days were significantly increased. This increase implies that metformin (500 mg/kg/day) had no significant plasma glucose lowering effects in STZ-induced type 1 diabetic rats. This is in contrast to Baloyi *et al*. (2019) who reported effective results when metformin was used together with STZ-induced diabetic research.

The diabetic model (type 1 or type 2 diabetes mellitus) could further be elucidated by evaluating the efficacy of metformin towards lowering plasma glucose levels in rats as either a treatment for type 1 or type 2 diabetes. According to Vella *et al*. (2010), metformin will have no plasma glucose lowering effects in type 1 diabetic rats (type 1; present little or no insulin production in the body), because metformin requires the presence of insulin to take action. The effect of metformin in type 1 diabetes was also confirmed by Meng *et al*. (2018), which indicated that it had no effect on lowering the blood glucose levels of type 1 diabetic patients. However, metformin will show anti-hyperglycaemic effects in rats with type 2 diabetes owed to the presence of insulin (Foretz *et al*., 2014). In our study, the results have shown that metformin had no plasma glucose lowering effects in diabetic rats and for this reason we can logically conclude that our animals had insulin-dependent type 1 diabetes. Our findings showed that metformin treated rats exhibited a minor blood glucose reduction of 33.54% while the terminal blood glucose of CR, PA and CR:PA-treated rats were reduced by up to 56.1%, 48.06% and 48.3%. The terminal blood glucose levels of metformin treated rats were still elevated enough to exhibit only a minor FBG decrease even after 12 h fast. Treatment with metformin did not have significant effects on hypoglycaemic activity.

The leaf water decoction of *C. roseus* is a common hypoglycaemic agent used in folk medicine (Tolambiya & Mathur, 2016). In the current study, the effect size calculations demonstrated a significant decrease in the blood glucose of CR-treated rats, from day 7 onwards, when compared to the other 4 diabetic groups ($d > 0.8$). Therefore, suggesting that treatment with *C. roseus*, managed to exhibit antihyperglycaemic effects despite the fact that the rats experienced a state of severe hyperglycaemia. Our findings are in agreement with numerous studies, which reported hypoglycaemic effects in rodents that were treated with *C. roseus* (Mostofa *et al*., 2007; Rasineni *et al*., 2010; Singh *et al*., 2001; Vega-Avila *et al*., 2012). It should be mentioned that the state of severe hyperglycaemia, observed by the elevated FBG levels (Figure 4.1A), could be associated

with the β-cell toxicity effects of STZ causing an ongoing destruction of pancreatic β-cells (Figure 4.1A). According to Shpilberg *et al*. (2012), severe hyperglycaemia occurs when FBG levels of rats are increased by 60% when compared to the baseline values, which is what was seen from our results (FBG was increased by 77%). Eleazu *et al*. (2013b), Adeghate and Ponery (2002) indicated that the destruction of insulin secreting β-cells in rats usually starts 3 days after STZ injection and can reach its peak hyperglycaemic effects between 2 to 4 weeks. These findings substantiate the increase in plasma glucose that was observed in the diabetic rats when compared to the non-diabetic rats, because our treatment took place during the time that STZ was still facilitating severe destruction of β-cells. Regardless of the severity of the diabetic state, CR still managed to display hypoglycaemic effects.

The potent antihyperglycaemic effect exhibited by CR was not particularly observable in the rats that were treated with PA. However, treatment with CR:PA demonstrated improved antihyperglycaemic effects when compared to PA but still not as potent as seen with CR treatment. We showed that the blood glucose levels of rats treated with 100 mg/kg/day of CR:PA, were significantly increased following 2 weeks of treatment compared to DNC rats. The administration of CR:PA to diabetic rats displayed an antagonistic effect between *C. roseus* and *P. afra*. While *C. roseus* lowered the blood glucose levels in diabetic rats, *P. afra* raised it. Further investigation showed that the relative weights of the pancreas were unaffected for all treatment groups, except for CR:PA-treated rats, which displayed a significant decrease in comparison to non-diabetic control. The reason for this incident is still not well understood and would require further evaluation through organ histology. Understanding the major physiological function of these two plant extracts would be crucial for future studies.

In the current study, we showed that the terminal plasma glucose of all the diabetic groups were significantly decreased, between 33–50%, when compared to their respective FBG values on day 28 (Figure 4.1B). Studies such as Heikkinen *et al*. (2007) suggested that for publication purposes overnight fasted rats would be considered ideal for it has the advantage of producing low and stable blood glucose levels. The typical fasting periods of rats during treatment are either a fast of 6 h or an overnight fast of 16 h to ensure a stable baseline measurement and to obtain consistent data (Bowe *et al*., 2014). Because the diabetic rats in our study were already experiencing involuntary weight loss, owed to the chemical induction of type 1 diabetes, a once weekly overnight fast would express a major concern as the animals would be placed under extreme risk of mortality as a result of extreme weight loss. Consequently, we fasted our animals for 6 h during the treatment period and 12 h prior to terminal procedures. Comparing the terminal FBG to that measured on day 28 revealed an important connection between fasting time and blood glucose levels (Figure 4.1B). Thus indicating that prolonged fasting times (12 h vs 6 h) in both diabetic and non-diabetic rats had supplemental hypoglycaemic effects as was seen in the

terminal FBG results (Figure 4.1B). These findings are in agreement with a similar study conducted by Bowe *et al*. (2014), purporting increased hypoglycaemia in rats that were exposed to prolonged food restrictions. On the other hand, our results demonstrated no significant differences between the terminal glycaemic levels of diabetic rats (treated with MET, CR, PA and CR:PA) and that of non-treated diabetic rats ($p > 0.05$). This implies that the treatment of the plant extracts, both independently and in combination, showed no supplemental plasma glucose lowering effects upon termination, meaning that the detected decrease was a result of prolonged fasting times.

Analysis of the phytochemical composition of the oven dried leaves of CR and PA, as well as CR:PA indicated that CR:PA contained considerable amounts of phenolics (Appendix E, Figure E.1) but fewer amounts of flavonoids (Appendix E, Figure E.2). The dried leaf of PA indicated that it contained considerable amounts of flavonoids but lower amounts of phenolics. Oven dried leaf of CR contained moderate amount of phenolics and flavonoids. Flavonoid and phenolics have been associated with antihyperglycaemic activities (Islam *et al*., 2009; Middleton *et al*., 2000; Singh *et al*., 2014). This statement was justified by numerous other studies: Brahmachari, (2009), Qi *et al*. (2010) and Gaikwad *et al*. (2014), purporting hypoglycaemic effects of flavonoids as antioxidant agents. Phenolics have shown to inhibit digestive enzymes such as α-amylase, which in turn delay the carbohydrate breakdown and ultimately control the glycaemic index (as seen in the *in vitro* study for PA and CR:PA, Figure 3.4– Section 3.5.3) (Suryanarayana *et al*., 2004). Flavonoids, as antioxidants, have been associated with the inhibition of progressive pancreatic β-cell destruction (caused by oxidative stress), therefore reversing the effects of diabetes (Eleazu *et al*., 2013a). However, in our study, treatment with PA (100 mg/kg/day) showed blood glucose levels that were significantly increased compared to all diabetic groups, meaning that PA could not reduce the effects of diabetes, regardless of its high flavonoid composition ($p < 0.05$, $d \geq 0.8$). The hyperglycaemic activity that was observed in PA-treated rats was contradicting to the findings of previous studies purporting hypoglycaemic effects with flavonoids. However, this phenomenon is not well understood and would require further investigation.

Weight loss in type 1 diabetic rats is a common sign of hyperglycaemia (Akbarzadeh *et al*., 2007; Tamborlane, 2008). The body weight of non-diabetic rats showed a percentage increase of 20.7% (gained 2.51 g/day), whilst the body weight of all 5 diabetic groups were significantly reduced (i.e. compare to NDC rats). On average, the body weight of CR-treated rats displayed a significant ($p < 0.05$) increase of 8% (gained 0.58 g/day) during the 4-week treatment period. Thus, CR showed successful inhibitory effects on the reduction of body weights in type 1 diabetic rats. These findings are in accordance with Prasad *et al*. (2009) and Rasineni *et al*. (2010), who reported an increase in body weight of diabetic rats treated with *C. roseus*. On the contrary, treatment with PA showed additional decline in body weight. This decline may be owed to the fact that PA was

exacerbating the hyperglycaemic state of the animals. A similar weight loss effect was observed in diabetic rats treated with CR:PA, suggesting that PA displayed single agent dominance (antagonism) in weight loss when used in combination with CR. The extract of *C. roseus* seemed to be the most promising in maintaining growth performance as measured by the body weight. Our study also revealed that metformin had no effect on increasing the body weight of diabetic rats. This reduction was also reported by another study that showed declined body weight of diabetic rats treated with metformin as a result of metformin causing loss of adipose tissue (Pournaghi *et al*., 2012; Yanardag *et al.,* 2005).

The diabetic state of rats caused significant enlargement in the relative weights of the brain, heart, liver, kidney and testis in proportion to the body weight. This is consistent with what has been reported by similar studies using STZ-induced diabetic models (Cosyns *et al*., 2007; Khaneshi *et al*., 2013; Shahreari *et al.*, 2010; Zafar & Naqvi, 2010).

Pancreas: According to Akbarzadeh *et al*. (2007) and Mythili *et al*. (2004), β-cell necrosis is an apparent effect of STZ, which either causes morphology changes to the pancreatic islets or completely destroys it. The results of the current study revealed that the relative weight of the pancreas in all treated groups were unaffected, except for CR:PA-treated rats, when compared to non-diabetic rats (Table 4.1). Although the diabetic rats had elevated blood glucose levels, the results revealed that the weight of the pancreas in diabetic rats were non-significant to non-diabetic rats, meaning that it was normal. On the contrary, we noted a significant reduction in the relative weight of the pancreas in CR:PA-treated rats when compared to diabetic control ($d > 0.8$). This decrease suggests potential synergistic effects when plants were used in combination, which could suggest anti-inflammatory effects by reversing the symptoms of diabetes (Akbarzadeh *et al*., 2007). Our study has indicated that a single IV injection of 55 mg/kg STZ, did not cause swelling of the pancreas when compared to the pancreas of non-diabetic rats. Further histology of the pancreas will be required to evaluate the morphological changes in the islets as a connotation to β-cell toxicity and organ weight.

Brain: The diabetic state caused a practically significant increase in the relative brain weights in all diabetic rats ($d > 0.6$) in comparison to normal NDC control. A study conducted by Jing *et al*. (2013) indicated that hyperglycaemia can cause inflammation and oxidative stress in the brain, which causes neurodegeneration. Moreover, Yang *et al*. (2013) associates oxidative stress in the brain tissue to STZ treatment, causing pathological changes to the cortex and vessel cell structures. Another study confirmed this statement where diabetic rats treated with STZ-induced type 1 diabetes, without glycaemic control, exhibited brain injuries (Huang *et al*., 2012). The relative brain weight of CR:PA-treated rats were significantly lower compared to DNC rats, indicating that treatment of CR:PA may have promoted antioxidant and anti-inflammatory effects thus causing neuroprotection in the brain. Whilst treatment with CR, PA and MET showed no

significant difference in the relative brain weights compared to DNC rats, which means that the weight of the brain was only affected by the diabetic state of the rats and not the plant extracts. It is important to note that further clarification of brain damage will rely on organ histology.

Heart: Heart disease and multiple organ failure is one of the most common diabetic complications (Liu *et al*., 2018). According to Cosyns *et al*. (2007), enlargement in the heart can be owed to the diabetic state (i.e. seen in DNC rats). This enlargement may be attributed to cardiac hypertrophy (Howarth *et al*., 2017). The relative heart weights in MET and CR-treated rats were practically visibly lower in comparison to DNC rats, indicating potential inhibition of cardiac hypertrophy. Treatment with MET, CR, PA and CR:PA reversed the effects of diabetes in the heart of STZ-induced diabetic rats.

Liver: The increase in the liver weight in proportion to the body weights of diabetic rats is attributed to increased triglyceride accumulation or the influx of fatty acids (Eleazu *et al*., 2013a; Zafar & Naqvi, 2010). Increased influx of fatty acids leads to enlarged liver (Eleazu *et al*., 2013b). The diabetic rats treated with aqueous *P. afra* leaf extracts, revealed a practical significant increase of 12.61% in relative liver weights when compared to DNC rats ($d > 0.8$). This increase may be due to the elevated FBG levels causing increased accumulation of triglyceride. The relative liver weights of MET treated rats were 5.31% larger than that of DNC rats ($p = 1.0$, $d = 0.62$). This is important to note because the size of the liver in MET treated rats were still 6.93% smaller compared to PA-treated rats ($p = 1.0$, $d = 0.99$). The higher liver weights displayed in MET and PA-treated rats may have been the result of increased influx of fatty acids into the liver due to hyperinsulinemia. The relative liver weights of the diabetic rats that administered *C. roseus* were practically visibly lower than that of diabetic non-treated control ($d = 0.62$) indicating the potential of *C. roseus* to ameliorate the build-up of fat in the liver in diabetes. Interestingly, the relative liver weights of CR:PA-treated rats were practically non-significant in comparison to DNC rats, which means that the combination of CR:PA decreased the build-up of fat in the liver thus reversing the effects of diabetes in the liver. This suggests that CR displayed single agent dominance (antagonism) in maintaining the weight of the liver when used in combination with PA. The extent to which this is plausible will require further histology of the liver tissue.

Kidney: Kidney enlargements occur due to the development of renal hypertrophy as a direct effect of insulin-like growth factor 1 also known as somatomedin C (Zafar & Naqvi, 2010). The diabetic state of the rats significantly increased the relative kidney weights compared to NDC control ($p < 0.05$, $d \geq 1.5$). The relative kidney weights of CR-treated rats were significantly decreased by up to 7.1% in comparison to DNC rats ($d < 0.5$), indicating that treatment with *C. roseus* may have improved renal efficiency than blocked renal hypertrophy in untreated diabetic rats. This is in agreement with the findings of Eleazu *et al*. (2013b) where treatment with ginger showed ameliorative potential by rejuvenating the renal structure of biological tissue. However, it

seems like the rats that were treated with PA and CR:PA, respectively, have shown contributing effects to kidney enlargement when compared to untreated diabetic rats ($p = 1.00$, $d \geq 0.5$). This therefore suggest that treatment of *P. afra* in the 1:1 combination of CR:PA may have caused renal deterioration as a result of increased tubular basement membrane thickening (Habib, 2018). The enlarged kidneys of diabetic rats that were treated with CR:PA showed antagonistic effects. The leaf extract of CR decreased the relative weights of the kidneys in diabetic rats and PA increased it, therefore PA presented single agent dominance (antagonism) when used in combination with CR.

Testis: Two testes of each rat were measured and regarded as one observation. The enlarged testis of the diabetic rats is attributed to hyperglycaemia. Hyperglycaemia in type 1 diabetes is a result of insufficient insulin production that causes oxidative stress thus increasing production of reactive oxygen species (ROS), which is responsible for changes in the seminiferous epithelium of diabetic rats (Shahreari *et al.*, 2010). The size of testis is a reflection of the germinal cell numbers which is related to sperm production (seminiferous epithelium) and Sertoli cell quantity (Slegtenhorst-Eegdeman *et al*., 1998). It is for this reason that ROS in STZ-induced diabetes reduce the level of testosterone and Sertoli cell function in which the size of testis is reduced (Khaneshi *et al*., 2013). The relative testis weights (1.37 ± 0.20%) in CR-treated rats were non-significant ($d < 0.2$) to DNC rats, indicating that treatment with CR did not cause supplemental enlargement of the testis. The increase in the testis weight in proportion to the body weights of the PA and CR:PA-treated rats, compared to DNC, MET and CR-treated rats ($d > 0.8$), may be attributed to increased production of testosterone, reducing oxidative stress, to which the size of the testis was enlarged. We believe that the increase in the relative testis weights of CR:PA-treated rats can be owed to single agent dominance (antagonism) exerted by PA.

From the above results, we can conclusively state that organ enlargement occurred as a result of hyperglycaemia. In general, the treatment with CR displayed antidiabetic effects in diabetic rats by restraining organ enlargement. Diabetic rats treated with *P. afra* exhibited potential damage to the liver and kidneys as a result of significant enlargement. The treatment with CR:PA displayed antagonistic effects, produced by the contrasting actions of CR and PA leaf extracts. The leaf extracts of *C. roseus* restrained organ enlargement while leaf extracts of *P. afra* caused significant organ enlargement. The results suggested that PA had single agent dominant (antagonism) effects in organ enlargement when used in combination with CR. Histology of organs will be needed for further investigation towards the antidiabetic effects of *P. afra* and CR:PA (1:1).

4.5 Conclusion

STZ-induced diabetic rats exhibited a significant increase in blood glucose levels and relative organ weights with declined body weight. The fundamental approach to control diabetes mellitus

is lowering plasma glucose. Here, the antidiabetic potentials of *C. roseus* and *P. afra* plant extracts were assessed in male Sprague Dawley rats through their reversal effects on diabetes. The study showed that *C. roseus*, alone, presented a pronounced hypoglycaemic effect in male rats, compared to untreated diabetic rats. As confirmed by the effect size calculations of the plasma glucose, body weight and the relative organ weight findings, aqueous leaf extract of *P. afra* revealed no glycaemic control in male diabetic rats, meaning that it could not reverse the effects of diabetes but amplified it. The lack of glycaemic control exerted by *P. afra* further appears to adversely impact the size of the liver and the kidneys by causing significant organ enlargement. The administration of CR:PA to diabetic rats displayed antagonistic effects. Whilst the aqueous leaf extracts of *C. roseus* lowered the blood glucose levels in diabetic rats, *P. afra* raised it, therefore suggesting that *P. afra* displaying single agent dominance. In conclusion, *P. afra* and CR:PA leaf extracts have shown no antidiabetic effects in Sprague Dawley rats, whereas aqueous leaf extracts of *C. roseus* showed antidiabetic activities in the management of diabetes mellitus in rats.

4.6 Reference list

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CHAPTER 5 STUDY CONCLUSION AND FUTURE RECOMMENDATIONS

5.1 Conclusion

The present study aimed to investigate the antidiabetic activity of aqueous leaf extracts of *Catharanthus roseus* and *Portulacaria afra*, independently and in combination (1:1) in both *in vitro* and *in vivo* models. This was done by evaluating the safety of the plants by measuring the cytotoxicity against HepG2 cell line. In addition, the antidiabetic potentials of the plant extracts were evaluated by measuring the enzyme activity (α-amylase, α-glucosidase and hexokinase) — common markers of diabetes therapy —in the HepG2 cells. The *in vivo* hypoglycaemic activity was determined using a streptozotocin (STZ)-induced diabetic rat model.

The *in vitro* study involved the use of HepG2 cell cultures to evaluate the potential of the plant extracts as antidiabetic drugs. Firstly, the safety of the leaf extracts was screened using cytotoxicity assays. After which the potency was gauged from the IC_{50} values. Low IC_{50} values suggest that the plant is potent at low concentrations (Berrouet *et al*., 2020). Therefore, the 1:1 mixture of *C. roseus* and *P. afra* displayed the most potent effects against HepG2 cell viability, exhibiting a low IC_{50} value of 4.10 µg/mL. A similar effect was observed for *C. roseus* (IC_{50} value of 6.02 µg/mL). In contrast, the extract of *P. afra* showed less potent effects on the cell viability with a high IC_{50} value of 27.39 µg/mL. Furthermore, the potential *in vitro* antidiabetic effects of the plants were confirmed by the inhibition activity of α-amylase by the *P. afra* and combination treatments, and the activation of the liver hexokinase activity by all treatment groups. The inhibition of α-amylase, an enzyme involved in carbohydrate digestion, has been shown to reduce post-prandial increase of plasma glucose in diabetes mellitus (Tundis *et al*., 2010). The potential antidiabetic properties of *P. afra* could be attributed to the presence of flavonoids, as antioxidant agents, whilst the presence of higher phenolic content (found within CR:PA) could be linked to inhibition of α-amylase enzyme. The ability of these traditional plants to increase hepatic enzymes such as hexokinase, suggested effective hypoglycaemic activities (Maideen & Balasubramaniam, 2018). The cells that were treated with all three leaf extracts exhibited activation of hexokinase. Thus, from the *in vitro* study it can be concluded that the aqueous leaf extract of *C. roseus* and *P. afra*, independently and in combination, showed antidiabetic potential.

The *in vitro* studies are usually used for screening and do not provide comprehensive information of a given treatment. To investigate the *in vivo* hypoglycaemic activity of *C. roseus*, *P. afra* and CR:PA, male Sprague Dawley rats were induced with diabetes through chemical injection by streptozotocin (STZ). The volumes for oral administration (100 mg/kg body weight) were selected from literature of animal studies of *C. roseus* used as an antihyperglycaemic agent for diabetic treatment. The oral gavage treatment of diabetic rats with the aqueous leaf extract of *C. roseus*

demonstrated a significant lowering of plasma glucose compared to the diabetic non-treated control over a 28-day period. Whilst treatment of the rats with *P. afra* demonstrated an opposite effect as seen by the elevated hyperglycaemic activity, thus implying no glycaemic control in diabetes. The 1:1 mixture of CR:PA demonstrated increased plasma glucose levels in diabetic rats when compared to *C. roseus*-treated rats, which is most probably owed to the exacerbating effect (antagonism) of *P. afra*. Although treatment with CR:PA did not display an effective decrease in plasma glucose as seen for *C. roseus*, it still accomplished improved glycaemic control compared to *P. afra*.

The leaf extract of *C. roseus* appeared to be the most promising in maintaining growth performance in diabetic rats, as measured by body weight. A significant decline in body weight was observed in all the diabetic groups that were treated with STZ. The mean body weight of *P. afra* treated rats demonstrated an additional decline, which could be harmful when used for type 1 diabetic treatment, already having the characteristic symptom of unintended weight loss. Further evaluation on the relative liver and kidney weights of diabetic rats demonstrated adverse effects in rats receiving oral treatment of *P. afra*. These adverse effects were warranted by the enlargement of the liver and kidneys. The treatment of *C. roseus* substantiated antidiabetic properties by reversing the effects of organ enlargement in diabetic rats (heart, liver, kidney and testis) by decreasing the weights of the organs back to baseline levels (i.e. same as non-diabetic control).

The present study confirmed that aqueous leaf extracts of *C. roseus* demonstrated hypoglycaemic potential in diabetic rats and thus support the therapeutic usage of this plant. On the other hand, the leaf extract of *P. afra* had the exact opposite effect by elevating the blood glucose levels in diabetic rats. This suggests that the alleged antidiabetic effects of *P. afra,* as presented by the inhibition activity of α-amylase and activation of hexokinase (as seen by the *in vitro* data), had no blood glucose lowering effects. The use of the 1:1 mixture of *C. roseus* and *P. afra,* in combination, demonstrated antagonistic effects. The leaf extracts of *C. roseus* displayed hypoglycaemic effects in diabetic rats whilst *P. afra* exacerbated the effects of hyperglycaemia, thus indicating that PA exerted single agent dominance (antagonism) when used in combination with CR.

The current study evidently exposes the shortcomings associated with *in vitro* studies, one of which was the misleading antidiabetic activities that was presented by the *in vitro* data. Our study has conclusively shown that the efficacy of drugs is better evaluated when using complex *in vivo* models such as animal. In conclusion, the aqueous leaf extracts of *P. afra* and its 1:1 combination with *C. roseus* have shown no antidiabetic effects in Sprague Dawley rats.

5.2 Limitations and future recommendations

- Instead of evaluating the enzyme activity (α-amylase and α-glucosidase) via the process of exposing the cells to the plant extracts, measuring the effects of the enzymes directly on the organ tissue (collected upon termination), might provide better justification towards the potential antidiabetic inhibition effects of the plants.
- Evaluating the quantitative enzyme inhibition activity of the plant extracts directly on the isolated enzymes. This will eliminate supplemental interference of the glucose metabolism of HepG2 cells with the mechanism of action of the digestive enzymes.
- Before commencing with the treatment of medicinal plants, it would be recommended to postpone treatment by 3 to 4 weeks, post-injection of STZ, since hyperglycaemia will reach its peak effect between 2 to 4 weeks (Adeghate & Ponery, 2002). This approach will eliminate the interference of ongoing hyperglycaemia with the treatment of natural products.
- The need to undertake a carbohydrate loading test to measure the effects of enzyme inhibition in the gastrointestinal tract could be beneficial in future studies since enzyme inhibition occurs at the level of the gastrointestinal tract. Therefore, the STZ model limits the proposed testing of antidiabetic effects of the plants. In addition, one would need to perform a carbohydrate-loading test to see whether the postprandial blood glucose increase is decreased by the plant extracts.
- In addition, the study could have been improved by measuring the digestive enzymes and isoenzymes in the gastrointestinal (GI) tract, seeing that it is the target area of antidiabetic treatments. This can be done by 1) conducting an *in vivo* fluorescence imaging of orally administered peptide drugs, isolated from natural sources such as insulin, to monitor the degradation of these drugs by GI enzymes as described by Fuhrmann & Leroux (2011) or by 2) measuring the enzyme activity of the plant extracts directly on the organ tissue using *in vitro* assays.
- Finally, the study could have been improved by measurement of insulin as the positive control in type 1 induced diabetic rats since metformin showed poor glycaemic control in type 1 diabetic rats. Although studies such as Baloyi *et al*. (2019), suggested using metformin with STZ-induced models, our results clearly revealed non-significant plasma glucose lowering effects of metformin in STZ-induced diabetic rats.
- The addition of organ histology will provide further confirmation towards the biological effects of the plant extracts on the selected organs such as the liver, heart, pancreas, testis and kidneys. This analysis will specify whether the plant extracts caused tissue damage or cell rejuvenation.

- The use of alternative toxicology analysis (absolute organ weights) could also be implemented to measure the function of the liver and kidneys as well as the reproductive capacity and testosterone function, in treated rats compared to the control. The addition of surrogate markers of brain injury could also be measured in future studies.

5.3 Reference list

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APPENDIX A

Ethics approval and certificates of research ethics training

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North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

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24 November 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 24/11/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Investigating the antidiabetic potential of the combination of *Catharanthus roseus* and *Portulacaria afra* leaf extracts
Principal Investigator/Study Supervisor/Researcher: Prof RK Hayeshi
Student: B de Vos - 26098466

Ethics number:

N	W	U	-	0	0	5	7	0	-	1	9	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single
Commencement date: 24/11/2019
Expiry date: 30/11/2020

Risk: Category 3

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of November annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Chairperson: NWU-AnimCareREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

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Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Faculty of Health Sciences Ethics Office for Research, Training and Support

Tel: 018 299 2092
Email:minrie.greeff@nwu.ac.za

8 March 2019

Ms Brunhilde de Vos

Dear Ms de Vos

PROOF OF ATTENDANCE AND ASSESSMENT

This letter certifies that you have attended the 2-day ethics training entitled:

The Basics of Health Research Ethics

(Accreditation number: PSB002/110/01/2019 from University of Free-State CPD accreditation department accredited by the HPCSA)

Presenter: Prof M Greeff (Head of the Health Sciences Ethics Office for Research, Training and Support) on the **22nd – 23rd January 2019.**

This letter of attendance, serves as proof of ethics training and assessment and is valid for three (3) years and expires on 31 January 2022. (Where applicable, Ethics CEUs awarded: 14 CEUs)

Yours sincerely

Digitally signed by Prof Minrie Greeff
Date: 2019.03.15 15:44:41 +02'00'

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Prof Jeanetta du Plessis
Deputy Dean: Research and Innovation
Faculty of Health Sciences

Current details: (20536690) G:\My Drive\9. Research and Postgraduate Education\9.1.5.7 Training\ 9.1.5.7.6_Letter of Attendance_Template_BCHIRE.docm
8 March 2019

File reference: 9.1.5.7.6

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Faculty of Health Sciences Ethics Office for Research, Training and Support

Tel: 018 299 2092
Email: minrie.greeff@nwu.ac.za

Ms Brunhildé de Vos

8 March 2019

Dear Ms de Vos

PROOF OF ATTENDANCE

This letter certifies that you have attended the half-day ethics training entitled:

The SANS document: As regulation for research with animals

Presenter: Prof CB Brink (Chairperson: NWU-AnimCareREC) on the **24 January 2019.**

This letter of attendance, is recognised by the NWU-AnimCareREC and the Health Science Ethics Office for Research, Training and Support and is valid for three (3) years and expires on 31 January 2022.

Yours sincerely

Digitally signed by Prof Minrie Greeff
Date: 2019.03.20 16:16:44 +02'00'

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Prof Jeanetta du Plessis
Deputy Dean: Research and Innovation
Faculty of Health Sciences

Current details: (20536690) G:\My Drive\8. Research and Postgraduate Education\6.1.5.7 Training\6.1.5.7.6_Letter of Attendance_ SANS.docm
7 March 2019

File reference: 6.1.5.7.6

APPENDIX B

Map of the world by Income

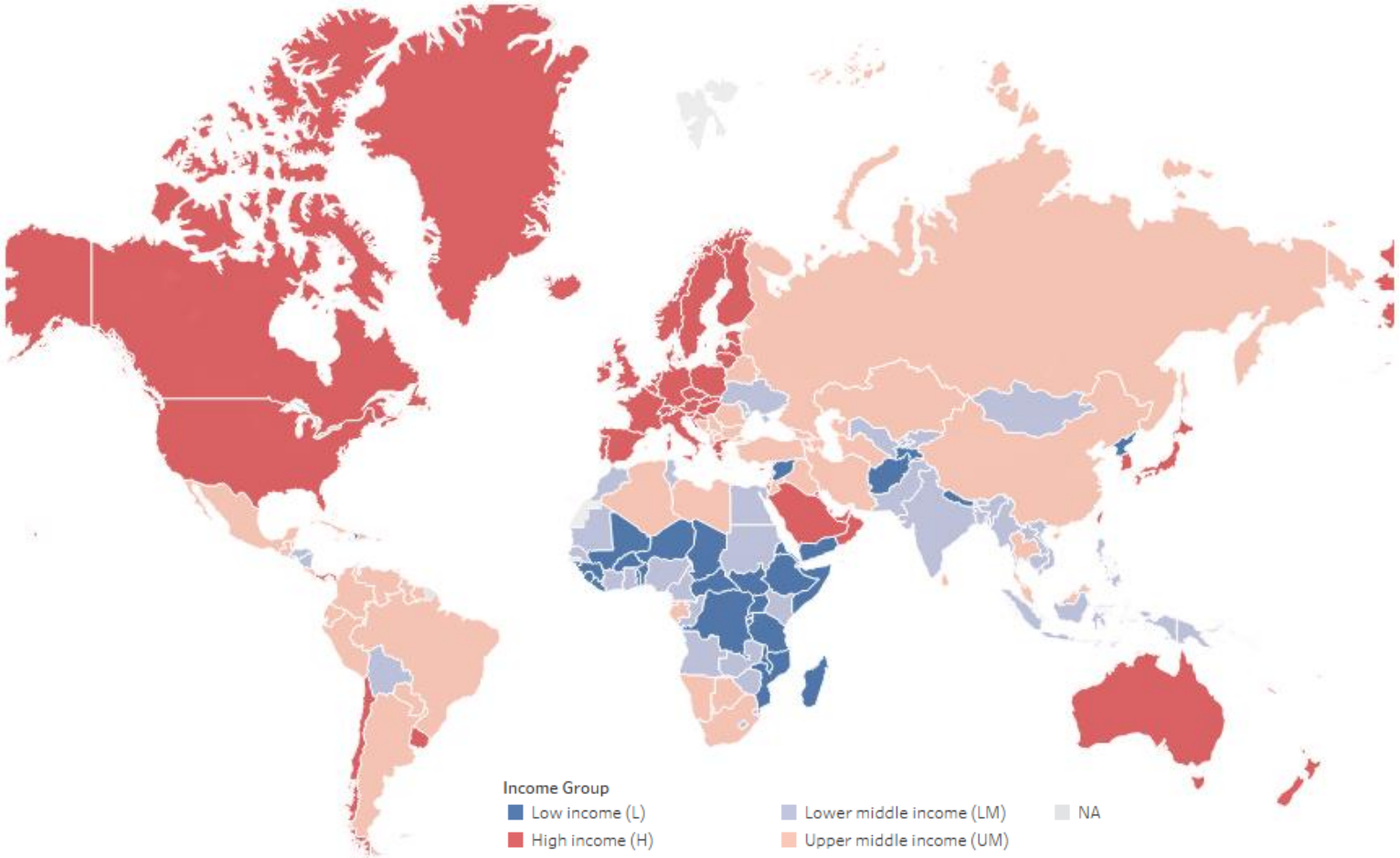

Figure B.1: The world by Income and region where countries are being classified based on the Gross National Income per capita data in U.S. dollar (World Bank, 2018).

APPENDIX C

Certificate of analysis for *Catharanthus roseus*

ARC LNR

AGRICULTURAL RESEARCH COUNCIL ROODEPLAAT:
Vegetable and Onamental Plants
Agro-Processing of Medicinal and Food Crops Lab

Private Bag X293
Pretoria
0001
SOUTH AFRICA
Tel: +27 12 808 8000
Fax: +27 12 808 0844
E-mail: NdhlalaA@arc.agric.za

REPORT OF ANALYSIS

Product: *Catharanthus roseus*
(Madagascar periwinkle)

DETAILS OF MANUFACTURER
Agricultural Research Council
Gauteng
South Africa

Batch No./Bar Code number	None
Production date	Sep-19
Product shelf life	6 months

IDENTITY

Medicinal/herbal specimen	USE: Ethnopharmacology/IKS utilisation
Botanic name	*Catharanthus roseus* (L.) G. Don
Preferred Common Name	Madagascar periwinkle
Plant part	Leaf powder (aqueous extract)
Place where plant is grown	Throughout South Africa

ORGANOLEPTIC	SPECIFICATION	RESULTS
Apperance	Brown powder	Complies
Colour	Brown powder	Complies
Oudor	Characteristic	Complies

ANALYTICAL		RESULTS	
Thermocycles	pH	Stable	4-42 °C
	Colour	Stable	4-42 °C NB. May get darker with
			time (1 week) when exposed to high temps
	Aroma	Stable	4-42 °C
Light exposure		Turns brown	Midday light intensity of 800 µmol m^{-2} s^{-1}
Shelf life		1 month opened	Temps 30/15 °C (16-h day/8-h night)
Shelf life		6 months	
pH		Balanced	6.45 - 6.49 (Liquid state)
Freeze drying			13,49 %

Fibre (crude)		1,56	%
Neutral detergent fibre		5,15	%
Acid detergent fibre		2,66	%
Acid detergent lignin		1,6	%
Tot. C		11,4	%
Tot. N		0,723	%
K		3584	mg/kg
Ca		3992	mg/kg
Mg		790	mg/kg
S		402	mg/kg
P		374	mg/kg
Na		146	mg/kg
Mn		24,4	mg/kg
Al		55,7	mg/kg
Fe		75,5	mg/kg
Zn		9,4	mg/kg
B		6,7	mg/kg
Cu		1,6	mg/kg
Phytochemical composition			
protocatechuic acid		Low	
p-hydroxybenzoic acid		Low	
Total phenolics (TP)		High (2,25%)	
Flavanoids (TF)		High (12%)	
Alkaloids		Not detected	
Teroids or triterpenoids		Not detected	
Oleic acid		Low (Undetectable)	
Llinoleic acid		Low (Undetectable)	
Palmitic acid		Low (Undetectable)	
Efficacy			
Antimicrobial activities			
Antibacterial		Low activity >12.50 mg/ml	
Antifungal activity		Low activity >12.50 mg/ml	
Antioxidant activities			
DPPH scavenging		Moderate activity	
Protection against LDL oxidation		Low activity	

MICROBIAL SPECIFICATIONS **RESULTS**

Total plate count	3 x 10^3 CFU/100ml	Complies
E. coli	0 CFU/100ml	Complies
Salmonella sp	0 CFU/100ml	Complies
Listeria sp	0 CFU/100ml	Complies

ALLEGENS & ANIMAL INGREDIENTS

None

No pollen

Does not contain any viable plant materials such as seeds
Does not contain any animal derived ingredients

HUMAN HEALTH SAFETY ASSESSMENT

Genotoxicity: Not Determined
Repeated Dose Toxicity: Contain a group of toxic alkaloids including vinchristine and vinblastine
Developmental and Reproductive Toxicity: Contain a group of alkaloids including vinchristine and vinblastine
Phototoxicity/Photoallergenicity: Not phototoxic/photoallergenic (UV Spectra)
Local Respiratory Toxicity: No NOAEC available. Exposure is below the TTC.

CULTIVATION CONDITIONS

Cultivated in gardens and parks worldwide

STORAGE AND STABILITY

Keep in a cool dry place. See stability results above. Refrigerate after opening

NB: The information contained in this specification is to our knowledge true and correct and presented in good faith. The information is offered soley for your consideration and verification.

Issued by: Ashwell R. Ndhlala (PhD, DPhil)
NdhlalaA@arc.agric.za
0762059531
2019-10-23

Photo provided by Dr Ashwell R. Ndhlala

APPENDIX D

Certificate of analysis for Portulacaria afra

AGRICULTURAL RESEARCH COUNCIL ROODEPLAAT:
Vegetable and Onamental Plants
Agro-Processing of Medicinal and Food Crops Lab
ARC LNR

Private Bag X293
Pretoria
0001
SOUTH AFRICA
Tel: +27 12 808 8000
Fax: +27 12 808 0844
E-mail: NdhlalaA@arc.agric.za

REPORT OF ANALYSIS

Product: *Portulacaria afta*
(Porkbush)

DETAILS OF MANUFACTURER
Agricultural Research Council
Gauteng
South Africa

Batch No./Bar Code number	None
Production date	Sep-19
Product shelf life	6 months

IDENTITY

Medicinal/herbal specimen	USE: Ethnopharmacology/IKS utilisation
Botanic name	*Portulacaria afra* Jacq.
Preferred Common Name	Porkbush
Plant part	Leaf (aqueos extract)
Place where plant is grown	Throughout South Africa

ORGANOLEPTIC	SPECIFICATION	RESULTS
Apperance	Brown powder	Complies
Colour	Brown powder	Complies
Oudor	Characteristic	Complies

ANALYTICAL		RESULTS	
Thermocycles	pH	Stable	4-42 °C
	Colour	Stable	4-42 °C NB. May get darker with
			time (1 week) when exposed to high temps
	Aroma	Stable	4-42 °C
Light exposure		Turns brown	Midday light intensity of 800 µmol m^{-2} s^{-1}
Shelf life		1 month opened	Temps 30/15 °C (16-h day/8-h night)
Shelf life		6 months	
pH		Balanced	6.23 - 6.49 (liquid state)
Freeze drying			30,24 %
Fibre (crude)			2,13 %

Neutral detergent fibre		5,15	%
Acid detergent fibre		2,83	%
Acid detergent lignin		2,1	%
Tot. C		4,6	%
Tot. N		0,238	%
K		3385	mg/kg
Ca		1387	mg/kg
Mg		1535	mg/kg
S		181	mg/kg
P		138	mg/kg
Na		966	mg/kg
Mn		13,2	mg/kg
Al		44,4	mg/kg
Fe		50,2	mg/kg
Zn		5,5	mg/kg
B		4	mg/kg
Cu		0,9	mg/kg
Phytochemical composition			
protocatechuic acid		Low	
p-hydroxybenzoic acid		Low	
Total phenolics (TP)		High (2,5%)	
Flavanoids (TF)		High (0,45%)	
Alkaloids		Not detected	
Teroids or triterpenoids		Not detected	
Oleic acid		Low (Undetectable)	
Llinoleic acid		Low (Undetectable)	
Palmitic acid		Low (Undetectable)	
Efficacy			
Antimicrobial activities			
Antibacterial		Low activity >12.50 mg/ml	
Antifungal activity		Low activity >12.50 mg/ml	
Antioxidant activities			
DPPH scavenging		Low activity	
Protection against LDL oxidation		Low activity	

MICROBIAL SPECIFICATIONS **RESULTS**

Total plate count	2.1 x 10^3 CFU/100ml	Complies
E. coli	0 CFU/100ml	Complies
Salmonella sp	0 CFU/100ml	Complies
Listeria sp	0 CFU/100ml	Complies

ALLEGENS & ANIMAL INGREDIENTS

None
No pollen
Does not contain any viable plant materials such as seeds
Does not contain any animal derived ingredients

HUMAN HEALTH SAFETY ASSESSMENT

Genotoxicity: Not genotoxic
Repeated Dose Toxicity: Not cytotoxic
Developmental and Reproductive Toxicity: Not toxic
Phototoxicity/Photoallergenicity: Not phototoxic/photoallergenic (UV Spectra)
Local Respiratory Toxicity: No NOAEC available. Exposure is below the TTC.

CULTIVATION CONDITIONS

Cultivated in gardens and parks worldwide

STORAGE AND STABILITY

Keep in powder in a cool dry place.

NB: The information contained in this specification is to our knowledge true and correct and presented in good faith. The information is offered soley for your consideration and verification.

Issued by: Ashwell R. Ndhlala (PhD, DPhil)
NdhlalaA@arc.agric.za
0762059531
2019-10-23

Photo provided by Dr Ashwell R. Ndhlala

APPENDIX E

Analytical report on the phytochemical constituents of C. roseus, P. afra and CR:PA (1:1)

E.1

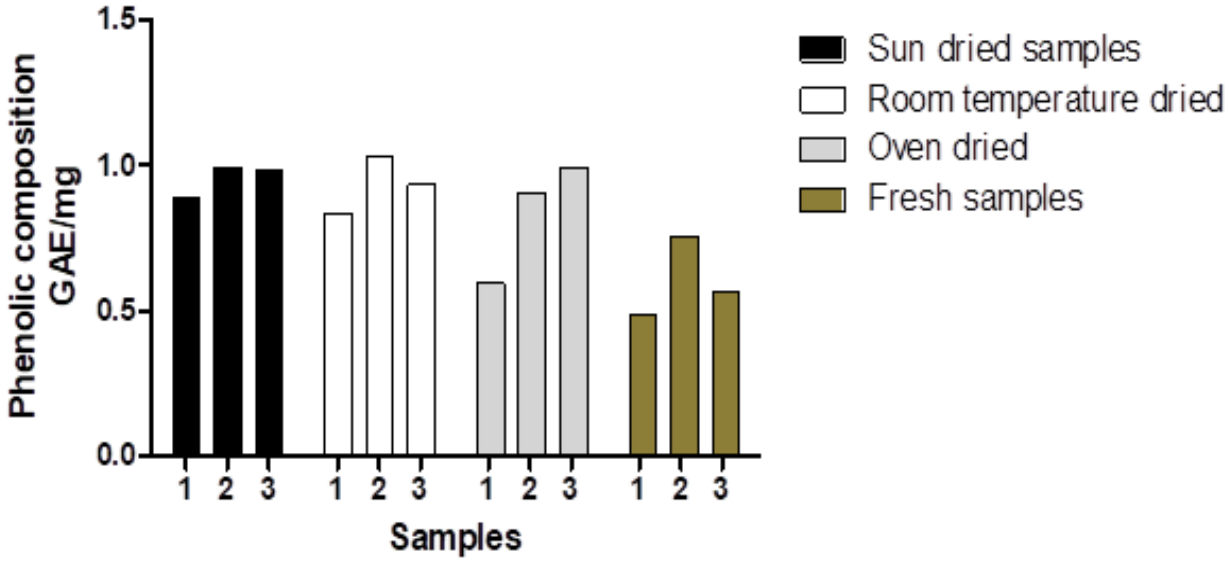

Figure E.2: Relative phenolic composition in 1) *Portulacaria afra*, 2) *Catharanthus roseus* and 3) 1:1 mixture of *C. roseus* and *P. afra* dried under different conditions and fresh samples.

E.2

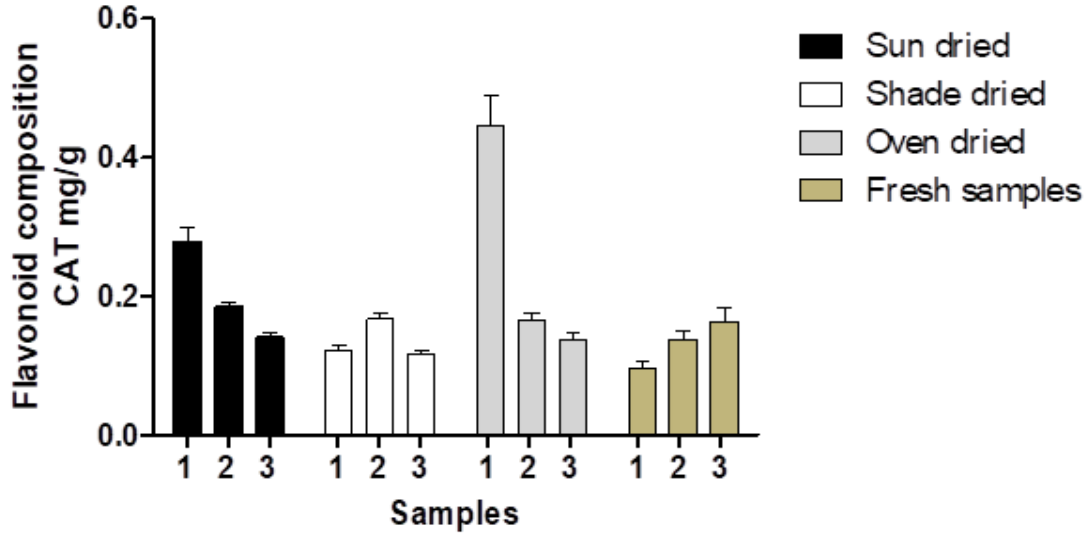

Figure E.3: Relative flavonoid composition in 1) *Portulacaria afra*, 2) *Catharanthus roseus* and 3) 1:1 mixture of *C. roseus* and *P. afra* dried under different conditions and fresh samples.

APPENDIX F

Animal well-being and health check scores

#	HEALTH INDICATOR	SCORE	
		VALUE	**EXPLANATION**
1.	**Loss in Body Weight (BW)**	0	Gain/ No Loss in BW
		1	Loss in BW ≤ 5%
		2	5% < Loss in BW ≤ 20%
		3	Loss in BW > 20%
2.	**Appearance**	0	Normal and clean
		1	Dirty Tail but otherwise clean fur
		2	Dirty tail, some brown colour/ faeces on rodent's back fur
		3	Dirty tail, wet dirty belly and some faeces on rodents back fur
3.	**Clinical Signs**	0	Normal
		1	Rapid breathing
		2	Polydipsia (thirst) and Polyuria (urination)
		3	Unexplained weight loss and fatigue
4.	**Natural Behaviour**	0	Normal
		1	Minor changes
		2	Abnormal, reduced mobility, decreased alertness, inactive
		3	Unsolicited vocalisations, self-mutilation, either very restless or immobile
5.	**Faecal Consistency (AKA Provoked Behaviour)**	0	Full solid
		1	Loose faeces
		2	Pasty diarrhoea
		3	Watery diarrhoea
Total Possible Score		**15**	**Consider euthanasia whenever a score of ~15 is assigned for 3 consecutive days**
The designated Veterinarian (Dr Nico Minnaar) conduct final assessment for euthanasia			

APPENDIX G

Success rate of streptozotocin and related complications

All the groups received a single dose of STZ (55 mg/kg) except Group 1 (non-diabetic control, *n* = 10), which received equal amounts of citrate buffer (*n* = 10, non-diabetic control). None of the rats died in the week following STZ injection. However, STZ presented an overall mortality rate of 6.7%, which is considered adequate (< 20%), owing to the toxicity of STZ (Furman, 2015). The animals were given a window period of 5 to 21 days, post STZ injection, to develop hyperglycaemia. (Furman, 2015). After 21 days, the remaining animals, that did not develop diabetes, were euthanised. A total of 70 rats were involved in this study; 40 rats developed diabetes via intravenous injection of STZ (55 mg/kg), 10 rats served as the non-diabetic control and a total of 20 rats failed to develop type 1 diabetes.

Animals were obtained in staggered formation throughout the study period of four months as presented in Table G.1 (Group A, B and C). Due to the unexplained failure of STZ injection exhibited by group B (Table G.1), 10 out of the 11 rats did not develop diabetes. The reason for this incident is still unknown. However, it was revealed by scientists in Research Gate that the potency of STZ rapidly decreases when opened, whilst also displaying discrepancy within animal groups injected days apart. Based on our findings, STZ (batch 1) presented poor reproducibility with various inconsistencies between group A and B (Table G.1). While a second unopened batch of STZ displayed similar complications in group C. Out of the 29 rats that were IV injected with STZ (group C), 31% did not develop diabetes. Taking all the animal numbers into consideration, this study exhibited a moderate STZ success rate of 66.7%.

Table G.1*:* Staggered formation of animals throughout study period of four months. Also used to assess the success rate of intravenous injection of STZ in Sprague Dawley male rats.

Staggered groups	**Group A** (BW 210–290g)	**Group B** (BW 170–280g)	**Group C** (BW 200–340g)	**Total**
Age (weeks)	**7–8**	**8–9**	**8–9**	
Number of male Sprague Dawley rats	25	13	32	70
Number of rats receiving single STZ 55 mg/kg IV	20	11	29	60
Number of rats receiving no STZ (non-diabetic)	5	2	3	10
Number of rats with hyperglycaemia (5 to 21 days post STZ injection)	19	1	20	40
Number of rats that did not developed diabetes (post STZ injection)	1	10	9	20
Total rats used for treatment	24	3	23	50

Animals from group A and B were IV injected with the same batch of STZ (#1). Animals from group C were IV injected with STZ from a new unopened batch (#2).

G. Reference List

Furman, B.L. 2015. Streptozotocin-induced diabetic models in mice and rats. *Current Protocol in Pharmacology*, 70(1):5.47.1–5.47.50. https://doi.org/10.1002/0471141755.ph0547s70