# Phytochemical analysis and in vitro anti-diabetic activity of selected South African medicinal plants traditionally used to treat diabetes mellitus

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

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# DECLARATION OF RESEARCHER

I, Michelle Rose-Marie Stevens, hereby declare that this dissertation, entitled “Phytochemical analysis and *in vitro* anti-diabetic activity of selected South African medicinal plants traditionally used to treat diabetes mellitus”, is my own work submitted for the degree *Masters of Science in Pharmaceutical Chemistry* at the North-West University. This dissertation has not been submitted to any other institution of higher education before. Furthermore, I declare that the references used in this dissertation have been appropriately acknowledged and referenced.

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# PREFACE

This study aims to screen selected South African plants for antidiabetic activity, identify major compounds within the active plant's extract, test their antidiabetic activity and study their seasonal chemical variation.

This dissertation is prepared in article format, according to the guidelines stipulated by the North-West University of South Africa. The first chapter of the dissertation is an introduction, followed by Chapter 2 which is a review article that was submitted to the "*South African Journal of Botany*" and is currently under review. Chapter 3 consists of a short communication regarding the *in vitro* antidiabetic activity of a number of South African plants and it was prepared for submission to the journal "*Pharmacia*". This is followed by the final article in Chapter 4 detailing the chemical variation and bioactivity of the identified major compounds that was prepared for submission to the "*South African Journal of Botany*". Lastly, the final conclusions and future recommendations of this dissertation is presented in Chapter 5. Appendix A and B contains the Author Guidelines of each journal that was selected for submission. Supplementary data such as ethical approval, can be found in the Appendices as well.

**Author contributions**

I, Michelle Stevens, the study's main researcher, was responsible for the planning of the study, execution of experiments, data processing and analysis, as well as presenting the findings. In addition, I was responsible for the original planning, and writing and editing of each article, as well as that of the final dissertation.

Contributing co-authors are acknowledged and listed below:

**Prof. F. Van der Kooy**

Co-author and main supervisor responsible for the funding procurement, conceptual design, intellectual and expert inputs, and critical evaluation of research results and draft manuscripts for all three articles.

**Prof. J.H. (Sias) Hamman**

Co-author and co-supervisor responsible for reviewing all first drafts and providing guidance on research findings.

**Miss. S.E. van Niekerk**

Co-author in Chapter 4 for the collection of samples and for providing guidance with the LC-ESI-MS/MS method and analysis.

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# ABSTRACT

Diabetes Mellitus (DM) is a chronic disease that requires constant treatment and its prevalence in South Africa continues to increase. With many people not having access to proper health care or are unable to afford appropriate treatments, the need to find alternative, more affordable treatments are required. South Africa is well known for its diverse flora and for using herbal remedies to treat a wide variety of ailments, however, even though many of these medicinal plants used to treat DM are well documented, many still lack any form of scientific evidence to support its use.

In this study, South African medicinal plants were selected that are currently being used to treat DM, namely; *Artemisia afra, Bulbine natalensis, Cnicus benedictus, Elephantorrhiza elephantina, Heteromorpha arborescens and Tulbaghia violacea.* Extracts of these plants were screened for their *in vitro* antidiabetic activity using a bioassay that tested the extracts' ability to inhibit the α-glucosidase enzyme. For *B. natalensis*, this was the first report of its *in vitro* antidiabetic activity. A methanol extract made from the leaves of *E. elephantina* showed the most promise, obtaining a % inhibition higher than that of the positive control, with the second highest % inhibition shown by the *A. afra* tea infusion extracts.

Although herbal remedies are extensively used, they are also still widely critiqued. One aspect of contention is that plants are known to react to their environment, and as a result, chemical variation within and between plants can be expected, which will also likely lead to variation in biological activity. Therefore, the chemical variation of the major compounds within *A. afra* samples collected monthly over a 1- year period was investigated to better understand the expected chemical variation. Thirteen compounds were identified and comparatively quantified and were also individually tested for their antidiabetic bioactivity. The identified compounds showed substantial chemical variation over the course of the year, with the most notable observation that an interchangeable trend between the dicaffeoylquinic acids and monocaffeoylquinic acids concentrations were observed. The former being higher during summer whilst the latter was found to be higher during winter. It is believed that the dicaffeoylquinic acids, which have higher molar attenuation coefficients, than the monocaffeoylquinic acids (34.0 ϵ vs 18.3 ϵ at 330 nm), are therefore mainly biosynthesised during summer when UV radiation is most intense.

The α-glucosidase bioassay also revealed that two dicaffeoylquinic acids were far more active (85% and 89% inhibition) than the positive control, acarbose (48% inhibition), whilst the monocaffeoylquinic acids were far less active (ranging from inactive to 31% inhibition). These differences in bioactivity, in combination with the seasonal chemical variation, indicates that chemical variation can have a profound influence on bioactivity. This again highlights the

importance of quality control of herbal medicine, especially pertaining to the treatment of chronic diseases such as DM.

**Keywords:** *Artemisia afra,* Diabetes Mellitus, *Elephantorrhiza elephantina,* α-glucosidase, quality control.

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**CHAPTER 2**



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# LIST OF ABBREVIATIONS

**A**

*A. afra* - *Artemisia afra*

ACN - Acetonitrile

**B**

*B. natalensis* - *Bulbine natalensis*

**C**

*C. capensis* - *Cissampelos capensis*

*C. benedictus* - *Cnicus benedictus*

CGA - Chlorogenic acid

**D**

DCQA - Dicaffeoylquinic acid

DM - Diabetes Mellitus

DMSO - Dimethyl sulfoxide

DNS - Dinitrosalicylic acid

**E**

*E. elephantina* - *Elephantorrhiza elephantina*

*E. punctulatus* - *Eriocephalus punculatus*

**F**

FA - Formic acid

**H**

*H. arborescens* - *Heteromorpha arborescens*

HPTLC - High Performance Thin Layer Chromatography

**L**

*L. tetragona* - *Lauridia tetragona*

LC - Liquid chromatography

**M**

MeOH - Methanol

MS - Mass spectrometry

**N**

Neo-CGA - Neo-chlorogenic acid

NI - No inhibition

**P**

*P. prunelloides* - *Pentanisia prunelloides*

pNPG - p-nitrophenyl glucopyranoside

PPRF - polyphenol-rich fractions

PTFE - polytetrafluoroethylene

**S**

SD - Standard Deviation

**T**

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

*T. violacia* - *Tulbaghia violacia*

**U**

UV - Ultraviolet

UPLC - Ultra-High Performance Liquid Chromatography

**W**

WHO - World Health Organisation

# CHAPTER 1: INTRODUCTION

## 1.1 Dissertation layout

This dissertation consists of five chapters of which Chapter 1 serves as the introductory chapter, containing a summary of the background, research problem, aim and objectives and ethical aspects of this study.

Chapter 2 consists of a review article in which the botanical aspects and antidiabetic literature of the plants included in this study are summarised. This review article was submitted to the "*South African Journal of Botany*" and was prepared according to the guide to authors (Appendix A). The selected plants were prepared and underwent an initial screening for their antidiabetic potential and the results are presented in Chapter 3. This article was prepared for submission to the journal "*Pharmacia*", according to the guide to authors which can be found in Appendix B. Chapter 4 covers the chemical variation found in *Artemisia afra* and the impact this has on its antidiabetic activity. This chapter highlights the importance of proper quality control needed for herbal remedies. The reason for selecting *A. afra,* which showed the second-best activity of all the plants tested during screening, was due to the fact that sufficient plant material for *Elephantorrhiza elephantina,* which showed better activity*,* was not available. The final chapter, Chapter 5, gives the final conclusions and future recommendations from the study including the identification of the active compounds in *E. elephantina*. The guide to authors for each journal and supplementary data can be found in the appendices at the end of the document.

## 1.2 Diabetes mellitus

The World Health Organization (WHO) defines diabetes mellitus (DM) as a chronic illness characterised by high blood glucose levels, also known as hyperglycaemia, due to the body's inability to produce enough, or any, insulin or being unable to effectively utilise the insulin produced (WHO, 2021). Diabetes can be classified into two main types, namely, type 1 and type 2 DM.

When the body is unable to produce any insulin or produces too little insulin to control the blood glucose concentrations, the patient is said to have type 1 diabetes mellitus (T1DM) (Gupta et al*.*, 2017). With T1DM, the pancreatic beta cells that are responsible for secreting insulin, are either severely damaged or destroyed, along with an increase in lymphocytic infiltration. When these two factors are combined, the body's insulin production and secretion is decreased. Low levels of insulin then ultimately lead to the body's inability to maintain the blood glucose levels within the normal range (Khardori, 2021a).

Alternatively, if the body produces insulin but is unable to utilise it or has developed insulin resistance, it is characterised as type 2 diabetes mellitus (T2DM) (Gupta et al*.*, 2017). There are two main factors that contribute to T2DM, namely, resistance towards insulin seen in the peripheral tissue and inadequate

insulin secreted by pancreatic beta cells. There are many factors that lead to the body being unable to maintain a healthy blood glucose level with T2DM, namely, high levels of pro-inflammatory cytokines and free fatty acids which results in less glucose being transported and stored in the muscle and fat cells. In addition to this, the body's glucose production in the kidney increases, producing even more fatty acids and glucose (Khardori, 2021b).

According to the International Diabetes Federation (2019) there are approximately 19 million adults between the ages of 20-79 living with diabetes within the African region and this number is expected to grow to about 47 million by the year 2045. With the number of people affected by DM increasing and a lack of affordable and effective treatments available in developing countries, the search for alternative treatments continues. DM is also classified as a chronic illness which requires continuous treatment. The current oral glucose-lowering agent, metformin, was developed based on the use of *Galega officinalis L.* (Fabaceae) to treat diabetes (Modak et al., 2007). With plants proving to be a promising source of antidiabetic molecules in the past, this study will focus on selected South African plants with previous mention of antidiabetic potential or traditional use for DM in South Africa.

## 1.3 Plant species used by traditional healers to treat diabetes

### *1.3.1 Artemisia afra*

*Artemisia afra* Jacq. ex Willd. (Asteraceae) was mentioned by Afolayan and Sunmonu (2010) regarding its traditional use as an antidiabetic agent. A pre-clinical *in vivo* study was conducted as a follow up for this review to test the antidiabetic activity of an *A. afra* aqueous extract using diabetic induced mice. The study reported a significant reduction in blood glucose levels, as well as an increase in the insulin serum levels (Afolayan and Sunmonu, 2011). Another study was also performed by Afolayan and Sunmonu (2013) to test various concentrations of the *A. afra* extracts in diabetic rats and found that the extracts showed good hypoglycaemic activity. The *in vitro* antidiabetic activity of *A. afra* extracts were tested by Nkobole et al. (2011) by measuring the ability of the extracts to inhibit α-oxidase and α-amylase enzymes. Reviews by Mohammed et al. (2014) and Arulselvan et al. (2014) mention *A. afra* for its use as an antidiabetic agent and current treatment by traditional healers. Issa and Bule (2015) conducted i*n vivo* experiments where both methanolic and aqueous *A. afra* extracts were tested for antidiabetic activity using alloxan induced diabetic mice and found that both extracts showed a drastic reduction in the test animals blood glucose levels compared to that of the control. A review covering medicinal plants used in the Western Cape area for the treatment and management of hypertension and DM also included *A. afra* (Davids et al., 2016). This review mentioned that strong infusions are said to reduce sugar levels. However, no literature could be found where the molecule(s) in *A. afra* with hypoglycaemic activity was identified.

### *1.3.2 Bulbine natalensis*

Erasto et al. (2005) conducted an ethnobotanical survey and listed and discussed medicinal plants that are used in the Eastern Cape to treat DM. This included *Bulbine natalensis* Baker (Asphodelaceae). The ethnobotanical, chemical and biological properties of *B. natalensis* was reviewed by Musara and Aladejana (2020). In this review they refer to the ethnobotanical study by Erasto et al. (2005) and Oyedemi et al. (2009) again mentioning the use of *B. natalensis* roots in the management of a variety of illnesses, including DM. Besides these studies and surveys that report the traditional use of *B. natalensis* by healers to treat DM, there is no scientific data available to substantiate its antidiabetic use and claims.

### *1.3.3 Cissampelos capensis*

Van de Venter et al. (2008) studied 11 medicinal plants, of which *Cissampelos capensis* L.F. (Menispermaceae) was one that is currently being utilised to treat DM in South Africa. Existing literature of these plants was collected and reviewed, and a scoring system developed to rate the respective plants' antidiabetic potential. Three cell lines, namely, 3T3-L1 adipose cells, Charg liver cells and C2C12 muscles cells were used to measure the effect of the plant extract on glucose utilisation and uptake and found that the aqueous extract of *C. capensis* showed activity in the 3T3-L1 adipose cells, portraying the potential the plant processes as a possible antidiabetic agent. They also reported that the absence of *in vitro* toxicity for this plant was very encouraging. Philander (2011) conducted interviews in the Western Cape with traditional healers. From these interviews he noted that *C. capensis* is commonly used in this area to treat DM and other 'blood impurities'. The findings from Van de Venter et al. (2008) were mentioned in respective reviews compiled by Odeyemi and Bradley (2018) and Maroyi (2020) referring to the promising antidiabetic results obtained with the *C. capensis* extract. *C. capensis* was once again mentioned to be used by local communities in the Karoo region against DM (Hulley and Van Wyk, 2019).

### *1.3.4 Cnicus benedictus*

An inventory consisting of South African plants that are traditionally used to treat DM was compiled by Deutschländer et al. (2009) and included *Cnicus benedictus* L. (Asteraceae). A review by Hulley and Van Wyk (2019) also reported the traditional antidiabetic use of *C. benedictus.* Polyphenolic-rich extracts of *C. benedictus* were evaluated for their antidiabetic activity. These extracts obtained α-amylase and α-glucoside $IC_{50}$ values of 1.20 µg/mL and 48.54 µg/mL, respectively, showing that the plant is substantially more active than the positive control, acarbose, that had $IC_{50}$ values of 17.68 µg/mL and 272.58 µg/mL, respectively. These results support the traditional use of *C. benedictus* against DM and show the potential pertaining to the antidiabetic properties of the plant (Paun et al., 2019). An *in vivo* study used streptozotocin induced diabetic rats to measure the antidiabetic activity of *C. benedictus* and found that the extracts significantly reduce blood glucose levels by 44.81 to 66.04% (Bekale, 2016).

### *1.3.5 Elephantorrhiza elephantina*

*Elephantorrhiza elephantina* (Burch.) Skeels (Fabaceae) is reported to be one of the ten most frequently used medicinal plants in the Maseru district (Kose et al., 2015). A review by Balogun et al. (2016) mentioned the traditional use of *E. elephantina* by the Basotho people to treat DM, however, at the time the review was conducted, no scientific data was available to substantiate or support the plants antidiabetic properties. An *in vitro* study performed by Olaokun and King (2018) tested extracts of *E. elephantina* for their potential to stimulate glucose utilisation activity of C2C12 muscle cells. The most active extract was found to be a cold-water extract, which displayed 69.6 ± 0.04% glucose utilisation activity. The hot water, 95% ethanol and acetone extracts showed decreased activity of 57.5 ± 0.06%, 51.6 ± 0.03% and 46.0 ± 0.02% at a concentration of 0.5 mg/mL. In a follow up study, extracts made from the leaves of *E. elephantina* were evaluated by Olaokun et al. (2020) for their biological activity. The plant's antidiabetic, anti-inflammatory, anti-oxidant and cytotoxic activity was measured in this study. At a concentration of 500 µg/mL, the ethanol extract showed a 58.72% inhibition of α-glucoside, in comparison to the 78.48% inhibition shown by the positive control, acarbose. These results provide some credibility to the traditional use of the plant as was mentioned by Balogun et al. (2016) which was previously lacking, however, more studies will need to be conducted to confirm these findings as this was the first paper that focussed on the leaves and not the roots of the plant.

### *1.3.6 Eriocephalus punctulatus*

In a review of the medical ethnobotany of Lesotho, compiled by Moteetee and Van Wyk (2011), the use of *Eriocephalus punctulatus* DC (Asteraceae) for the treatment of DM and hypertension was reported. *E. punctulatus* is mentioned by Kose et al. (2015) as one of the plants used to treat DM in the Maseru district in Lesotho. Balogun et al. (2016) reviewed medicinal plants used by the Basotho people to treat DM and reported that *E. punctulatus* was one of the plants being used. They also stated that other bioactivities of the plant such as the anti-oxidant and anti-inflammatory properties have been tested *in vitro*, but *in vitro* or *in vivo* testing is yet to be done to study the antidiabetic properties of this plant.

### *1.3.7 Heteromorpha arborescens*

*Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. (Apiaceae) has been documented by Erasto et al. (2005) for being traditionally used to treat DM in the Eastern Cape. This review was then referred to by Afolayan and Sunmonu (2010) when they conducted a similar review about *in vivo* studies pertaining to potential antidiabetic plants. They reported the previous mentioned use of *H. arborescens* for diabetes treatment but provided no evidence of any *in vitro* or *in vivo* studies documenting the antidiabetic properties of this plant species. The antidiabetic, anti-oxidant and cytotoxicity properties of different *H. arborescens* leaf extracts were investigated by Abifarin et al. (2021). The results revealed that both the aqueous and

ethanol extracts obtained considerable inhibition against α-glucosidase enzyme with $IC_{50}$ values of 576.46 ± 3.21 µg/mL and 627.29 ± 4.62 µg/mL, respectively.

### *1.3.8 Lauridia tetragona*

Oyedemi et al. (2009) reported *Lauridia tetragona* (L.f.) R.H. Archer (Celastraceae) for its use to treat DM in the Nkonkobe municipality, Eastern Cape, South Africa. Odeyemi and Dewar (2019) identified polyphenolic-rich fractions (PPRF) found in *L*. *tetragona* extracts which they then tested for their *in vitro* glucose uptake and reported that some of the polyphenol-rich fractions (PPRF) showed good hypoglycaemic activity. Caffeine, coumarin and ferulic acid are some of the phytochemical compounds identified in the selected PPRF that have previous mention of having α-amylase and α-glucosidase inhibitory activity. In order to determine the exact pharmacological activity of these compounds, more testing will have to be done.

### *1.3.9 Pentanisia prunelloides*

Medicinal plants used by traditional healers as DM treatment in the Western Cape area, South Africa, mentioned *Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh.) Walp. (Rubiaceae) (Philander 2011). Kose et al. (2015) and Moteetee et al. (2019), in their respective surveys, reported that *Pentanisia prunelloides* was one of the most frequently mentioned medicinal plants used in Lesotho. Balogun et al. (2016) stated that the Basotho people also uses *P. prunelloides* to treat diabetes. The plants antibacterial, anti-inflammatory, antimycobacterial, anti-oxidant, and cytotoxic properties have been studied *in vitro*, however, the antidiabetic activity of the plant is yet to be tested to support its traditional use.

### *1.3.10 Tulbaghia violacea*

In a study covering medicinal plants utilised by traditional healers in the Western cape, Philander (2011) recorded the traditional use of *Tulbaghia violacea* Harv. (Amaryllidaceaea) for a variety of ailments, of which DM was one of them. Five medicinal plants were selected by Van Huyssteen et al. (2011) and their effect on the glucose utilisation in muscle, liver and fat cells was studied. One of the selected plants included in this study was *T. violacea*. This study found that the greatest increase in glucose utilisation in the muscle cells was achieved by an ethanol extract of *T. violacea* obtaining a 140.5% increase compared the 117.9% of the positive control. Moodley et al. (2015) used diabetic induced rats to study the *in vivo* antidiabetic activity of *T. violacea* and found the plant to have hypoglycaemic effects. A follow up article of this study found that future studies are still required to identify potential active compounds responsible for the plant's hypoglycaemic activity (Moodley and Mackraj, 2016).

## 1.4 Role of alpha-amylase and alpha-glucosidase

Diabetes mellitus is a very complex disease and there are therefore many different *in vitro* and *in vivo* bioassays available to test for antidiabetic potential. An *in vitro* bioassay that measures an extract's ability to inhibit the digestive enzymes α-amylase or α-glucosidase, is one of the most frequently used *in vitro* test to determine antidiabetic potential. The first digestive enzyme, α-amylase, is responsible for breaking starch down to oligosaccharides whereafter the second enzyme, α-glucosidase breaks down these molecules even further to form monosaccharides that are then absorbed into the bloodstream (McIver and Tripp, 2020). If a plant extract or compound is found to inhibit either of these digestive enzymes, the body's ability to break down starches or sugars to the monosaccharide for absorption into the blood stream, is suppressed. Inhibition of these enzymes will therefore result in a more controlled glucose absorption, preventing any post-prandial blood glucose spikes. The body is therefore able to maintain its normal blood glucose levels.

## 1.5 Importance of quality control of herbal remedies

Although medicinal plants are very popular and extensively used, especially by traditional healers in South Africa, the use of these herbal remedies are still widely critiqued. Plants are subject to chemical variation due to them chemically reacting to their immediate environment (general climate, drought, waterlogging, pathogens, etc.). Chemical variation within and between different individual plants can and should be expected. Other aspects involved in the collecting and preparation of the extracts like post-harvest handling or storage conditions can also lead to chemical variation in extracts. To overcome this problem, various guidelines have been established to try and implement proper quality control in herbal medicine (WHO, 2011; Bensoussan et al*.,* 2016), however, in order to fully understand the impact and true extent of this barrier, more research will need to be done.

## 1.6 Research problem

Diabetes mellitus (DM) is a chronic illness that affects a large portion of South Africa's population, and its prevalence continues to increase. Due to high levels of poverty and the recent economic downturn, an increasing number of people cannot afford or do not have access to proper medical care. The chronic nature of DM means the disease will require continuous treatment. Many indigenous medicinal plants are currently being used to treat DM, but many also lack any form of scientific data to validate their use. There is a need for medicinal plants to be scientifically tested to validate their biological activities as well as to identify novel phytochemical compounds, which can potentially be developed into more effective treatments of DM. It is also well-known that the chemical profile of plants differ greatly which requires proper quality control.

## 1.7 Aims and objectives

The aim of this project was to screen extracts from selected medicinal plants, that have been reported as treatments of DM, for their antidiabetic activity by means of *in vitro* enzyme inhibition assays. The aim was then to further identify phytochemical compounds within these extracts that could have the potential to be developed into effective treatments of DM.

Objectives:

- Collection and sourcing of selected medicinal plant materials at different times during the course of the study.
- Optimisation of an extraction technique for each selected plant to provide maximum yield.
- Determination of the biological activities of the extracts from the selected plants using *in vitro* α-glucosidase.
- Development of an LC-ESI-MS/MS and UPLC chemical fingerprinting quality control method of the plant extracts that showed acceptable biological activity.
- Identification of major compounds in the active plant extract and determining their bioactivity.
- Study of chemical (seasonal) variation of the major compounds within the active plant.

## 1.8 Ethical aspects

Ethics approval for this study was acquired (NWU-00228-21-A1). The ethical approval certificate can be found in Appendix D. This study did not include any animal or human studies. Enzymes used to conduct the bioassay was purchased from Sigma-Merck. All plant material required for this study was either purchased from commercial sources (Mountain Herb Estate and Wildflower nursery) or collected from the North-West University Botanic Garden. All health and safety guidelines were adhered to, and Good Laboratory Practices were followed throughout performing and completion of this study.

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# CHAPTER 2: REVIEW ARTICLE: "ANTIDIABETIC ACTIVITY OF SOUTH AFRICAN MEDICINAL PLANTS TRADITIONALLY USED TO TREAT DIABETES MELLITUS – A MINI-REVIEW."

Chapter 2 is presented as a review article and was submitted on the 8th of September 2022 to the "*South African Journal of Botany*" (manuscript number: SAJB-D-22-01944). This manuscript was prepared according to the author guidelines of the "*South African Journal of Botany*" that can be found in Appendix A.

# Antidiabetic activity of South African medicinal plants traditionally used to treat diabetes mellitus – a mini-review

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**Abstract**

Diabetes mellitus (DM) is a chronic metabolic disease that affects a large portion of South Africa's population, and the prevalence continues to grow. The search for more affordable and more effective treatments therefore continues. The purpose of this mini-review is to collate published research data for selected South African medicinal plants (*Artemisia afra, Bulbine natalensis, Cissampelo capensis, Cnicus benedictus, Elephantorrhiza elephantina, Eriocephalus punctulatus, Heteromorpha arborescens, Lauridia tetragona, Pentanisia prunelloides, Tulbagia violacea*) with antidiabetic properties or with previous mention of traditional use for DM and to identify gaps where further research should be conducted. The first part of this mini-review discusses the different bioassays used, such as *in vitro* tests that measures the α-amylase and α-glucosidase inhibitory potential; *in vitro* tests that measure glucose uptake into cells; and *in vivo* tests that use alloxan or streptozotocin diabetic-induced rats. This is followed by a summary of the published research found on the antidiabetic effects of the selected medicinal plants. From the presented data, the traditional use of the plants seems to be well documented, but many studies lack scientific data to validate their purported antidiabetic properties, with some of the plants having no published *in vitro* or *in vivo* studies for their antidiabetic properties. However, the most promising and significant recent discovery is the ethanolic extract of *C. benedictus* which showed a 15-fold better activity than the positive control ($IC_{50}$ 1.2 µg/mL), followed by *A. afra* displaying reasonable inhibition at a concentration of 200 µg/mL.

**Keywords:** *Artemisia afra*, antidiabetic, Diabetes mellitus, *Elephantorrhiza elephantina*, *in vitro*

## 1. Introduction

Diabetes mellitus (DM) is defined as a chronic illness caused by the body's inability to either produce insulin or to use the insulin effectively, resulting in high blood glucose levels (hyperglycaemia). There are two main types of DM. Type 1 diabetes mellitus (T1DM) is when the body is unable to produce insulin or produces too little insulin (Gupta et al., 2017). In T1DM, the beta cells in the pancreas responsible for secreting insulin, are destroyed, along with an increase in lymphocytic infiltration. The combination of these two factors results in a decrease in insulin secretion, ultimately leading to the body being unable to maintain normal blood glucose levels (Khardori, 2021a).

Type 2 diabetes mellitus (T2DM) is when the body is unable to use the insulin that the body produces, resulting in an insulin resistance (Gupta et al., 2017). T2DM is characterised by a combination of two factors, namely, insulin resistance in the peripheral tissue and poor insulin secretion by the beta cells in the pancreas. As a result of high levels of free fatty acids and pro-inflammatory cytokines, less glucose is transported into the muscle and fat cells to be stored. In addition, the body produces more glucose and fatty acids by increasing glucose production in the kidney as well as increasing fat metabolism. All these factors lead to the body being unable to maintain the blood glucose levels, resulting in the onset of T2DM (Khardori, 2021b).

According to the International Diabetes Federation (International Diabetes Federation, 2019), there are approximately 19 million adults between the ages of 20–79 living with diabetes within the African region and this number is expected to grow to about 47 million by the year 2045.

With the number of people affected by DM increasing and a lack of affordable and effective treatments available in developing countries, the search for alternative treatments continues.

According to Newman and Cragg (2020), 63 new drugs for the treatment of diabetes was registered between 1981-2019 of which 9 were natural products or derivatives of natural products. With plants proving to be a promising source for antidiabetic molecules in the past and considering South Africa's diverse flora, this

mini-review will focus on the use of ten South African medicinal plants with previous mention of antidiabetic potential or traditional use for DM in South Africa (Figure 1).

## 2. Aim and structure of this mini-review

South Africa is known for its diverse range of medicinal plants used by traditional healers to treat a wide variety of illnesses and ailments. The traditional use of these plants is well documented, however, many are still lacking scientific data and testing regarding their claimed efficacy. The aim of this review is to collate published scientific data of ten selected South African medicinal plants commonly used by traditional healers, specifically to treat diabetes, and to determine what scientific data is available and identify which species might show potential for further investigation or testing.

A literature search was conducted using Scopus, Web of Science, Scifinder scholar, Google Scholar and Science Direct. Structure of review: The first part of the review will focus on the *in vitro* and *in vivo* bioassays that were used to determine the antidiabetic activity of plant-based extracts and compounds. This will be followed by a comprehensive description of all known scientific literature of each species that is traditionally used to treat DM. This will include plant parts used, type of extracts, bioassays used, phytochemicals identified and preclinical studies. Finally, a discussion on the most promising plant species for treatment of DM with future research recommendations will be provided.

## 3. *In vitro* and *in vivo* antidiabetic bioassays

Due to the complexity of diabetes and the many different metabolic pathways and processes that it affects, a wide variety of *in vitro* and *in vivo* methods are used in literature to test plant extracts for antidiabetic activity.

The most commonly used *in vitro* method measures the plant extract's ability to inhibit the α-amylase or α-glucosidase enzyme that is found in the digestive tract. α-Amylase is responsible for breaking down starch to oligosaccharides after which α-glucosidase breaks these oligosaccharides even further to monosaccharides (McIver and Tripp, 2020). When a plant extract shows promising enzyme inhibition, it

can be considered as having antidiabetic potential, as inhibiting these enzymes prevent post-prandial spikes in blood glucose levels.

In order to determine whether a plant extract has the ability to inhibit the α-amylase enzyme, the plant extract and enzyme are mixed together with starch and dinitrosalicylic acid (DNS) and the absorbance is measured at 540 nm. The absorbance is then compared to a negative control, which contains distilled water in the place of the plant extract, or a positive control such as acarbose (Kazeem et al., 2013). The α-glucosidase inhibition test uses p-nitrophenyl glucopyranoside (pNPG) and measures the yellow-coloured paranitrophenol released from the pNPG using spectrophotometry at 405 nm (Kazeem et al., 2013).

A variation on the above method uses High-Performance Thin Layer Chromatography (HPTLC) to compare the experiment samples with the control samples (Rocamora et al., 2020).

*In silico* studies using virtual docking software such as PyRx software (0.8 V), have also been used to screen plant extracts for α-amylase and α-glucosidase enzymatic inhibition potential (Akshatha et al., 2021).

Another popular *in vitro* method measures the glucose uptake in 3T3-L adipose and C2C12 muscle cells after being exposed to the relevant plant extract (Van de Venter et al., 2008). When the body produces insulin, it stimulates glucose uptake into muscle and fat cells in order to lower blood glucose levels, making this *in vitro* test an accurate test to determine a plant's antidiabetic activity (Satoh, 2014).

The antidiabetic potential of plant extracts can also be measured using *in vivo* tests. These involve alloxan (Issa and Bule, 2015) or streptozotocin (Afolayan and Sunmonu, 2011) diabetic-induced rat models. Blood glucose levels, body weight and glucose tolerance are some of the properties tested and monitored during these *in vivo* studies (Afolayan and Sunmonu, 2013).

## 4. Selected plant species used by traditional healers to treat diabetes

### 4.1. *Artemisia afra*

#### 4.1.1. Botanical aspects

*Artemisia afra* Jacq. Ex Willd (Asteraceae) (Fig. 1D) is a perennial shrub that can be found growing in the following provinces of South Africa; Limpopo, Gauteng, North-West, Free State, KwaZulu Natal, Northern

Cape, Western Cape and Eastern Cape. It has a thick woody stem at the base which thins and softens closer to the top. The leaves are small, divided, fern-like with a darker green top and grey/white under side (Van Wyk et al., 1997).

### 4.1.2. Antidiabetic literature

A review by Afolayan and Sunmonu (2010) regarding medicinal plants in South Africa, included *A. afra* as being traditionally used as an antidiabetic agent. This review was then followed up by a pre-clinical *in vivo* analysis. Aqueous extracts of *A. afra* were orally administered to streptozotocin-induced diabetic rats. The results showed that the extract significantly reduced blood glucose levels and increased the serum insulin levels of the rats (Afolayan and Sunmonu, 2011). A similar study was then performed where different dosages of an *A. afra* extract were tested in Wistar rats. They reported that the extracts did indeed show some hypoglycaemic activity, but that higher dosages could lead to impairment of certain kidney functions (Afolayan and Sunmonu, 2012).

Nkobole et al. (2011) included *A. afra* in a study in which the *in vitro* activity of numerous plants species was tested. This study measured the ability of the extracts to inhibit α-amylase and α-glucosidase. At a concentration of 0.2 mg/mL, the A. *afra* extract yielded an α-glucosidase inhibition of 47.2% and α-amylase inhibition of 74% compared to the positive control (acarbose) which yielded 80.6% and 73.4%, respectively. These results showed that this plant has antidiabetic potential.

The antidiabetic activity of *A. afra* discussed in the study by Afolayan and Sunmonu (2013) is later mentioned by Mohammed et al. (2014) in their review of African medicinal plants. Arulselvan et al. (2014) also mentions *A. afra* for its use in DM management.

*In vivo* experiments with alloxan induced diabetic Swiss albino mice conducted by Issa and Bule (2015) indicated that both methanolic and aqueous extracts of *A. afra* drastically reduced the blood glucose levels in the mice compared to that of the control group. They also found the $LD_{50}$ of the aqueous extract to be relatively high (9 833.4 mg/Kg) indicating its low toxicity.

An ethnobotanical survey of medicinal plants used in the Western Cape area for the management of hypertension and DM by Davids et al. (2016) included *A. afra*, stating that a strong infusion is said to reduce sugar levels. However, no literature could be found where the molecule(s) in *A. afra* with hypoglycaemic activity was identified.

### 4.2. *Bulbine natalensis*

#### 4.2.1. Botanical aspects

*Bulbine natalenis* Baker. (Asphodelaceae) (Fig. 1H) is a perennial succulent that is indigenous to South Africa. It is mainly found in KwaZulu Natal, Eastern Cape and Western Cape. The leaves are broad, fleshy and green to yellow and form a sharp point. When flowering it forms a long, thin flowering stem with small yellow clusters of flowers (Van Wyk et al., 1997).

#### 4.2.2. Antidiabetic literature

An ethnobotanical study by Erasto et al. (2005) which discussed medicinal plants being used for the treatment of DM in the Eastern Cape included *B. natalensis*. They stated that traditionally, the roots of the plant were boiled, and the infusion taken orally. Oyedemi et al. (2009) also mentioned the use of *B. natalensis* by traditional healers for DM treatment in the Nkonkobe municipality district, Eastern Cape in South Africa.

A review by Musara and Aladejana (2020) looked at the ethnobotanical use, biological and chemical properties of *B. natalensis*. In the review they refer to the studies of Erasto et al. (2005) and Oyedemi et al. (2009) and again mentioned the use of *B. natalensis* roots in the management of a variety of illnesses including DM. Besides these studies and surveys that report the use of *B. natalensis* by traditional healers for treatment of DM, there is no scientific data to substantiate its antidiabetic properties.

### 4.3. *Cissampelos capensis*

#### 4.3.1. Botanical aspects

*Cissampelos capensis* L.F. (Menispermaceae) (Fig. 1G) is a perennial climber mainly found in the Western parts of South Africa. It consists of twining stems that wraps around the stems of other plants to support

itself. The stems are covered with small, rounded leaves that are bright green in colour. The plant produces clusters of small, whitish-green flowers from which orange berries originate (Van Wyk et al., 1997).

### 4.3.2. Antidiabetic literature

In a study by Van de Venter et al. (2008) to screen and score 11 medicinal plants used in South Africa for DM treatment, *C. capensis* was included. The plants' effect on glucose utilisation was tested using three cell lines namely; Charg liver cells, 3T3-L1 adipose cells and C2C12 muscles cells. They reported that the aqueous extract of *C. capensis* showed better activity in the 3T3-L1 adipose cells than the positive control. They also reported that the absence of *in vitro* toxicity for this plant was very encouraging.

*Cissampelos capensis* was recorded for its use to treat DM and other 'blood impurities' in a study by Philander (2011) where traditional healers in the Western Cape were interviewed. Odeyemi and Bradley (2018) and Maroyi (2020) referred to the reports of Van de Venter et al. (2008) mentioning the promising antidiabetic results obtained by extracts of *C. capensis.* In a study by Hulley and Van Wyk (2019), *C. capensis* was once again mentioned to be used by local communities in the Karoo region against DM.

## 4.4. *Cnicus benedictus*

### 4.4.1. Botanical aspects

*Cnicus benedictus* L. (Asteraceae) (Fig. 1J) is an herb that grows to about 0.7 m high. It has become a naturalised plant in South Africa found in the Cape and Highveld areas, after being introduced to South African over 150 years ago. This plant's characteristic spiny leaves start by forming a basal rosette but disperse along the stem as the plant grows. The flowers are a bright yellow and are surrounded by spiny bracts (Van Wyk et al., 1997)

### 4.4.2. Antidiabetic literature

A review by Deutschländer et al. (2009) included *C. benedictus* in an inventory of plants traditionally used to treat diabetes in South Africa. Hulley and Van Wyk (2019) also reported the use of *C. benedictus* by traditional healers for the treatment of DM.

Paun et al. (2019) evaluated the antidiabetic activity of *C. benedictus* polyphenolic-rich ethanolic extracts. The $IC_{50}$ values obtained by the extract for the α-amylase and α-glucoside inhibition was 1.20 µg/mL and 48.54 µg/mL, respectively. This showed that the plant extract had a higher inhibitory potential that the control, acarbose, which had $IC_{50}$ values of 17.68 µg/mL and 272.58 µg/mL. This study reported findings that this plant can be utilised as a potential antidiabetic agent and therefore supported the traditional use of *C. benedictus* against DM.

*Cnicus benedictus* was administered at a dose of 100–400 mg/kg, i.p. in streptozotocin induced diabetic rats and was shown to significantly reduce blood glucose concentrations by 44.82 to 66.04% (Bekale, 2016)

### 4.5. *Elephantorrhiza elephantina*

#### 4.5.1. Botanical aspects

*Elephantorrhiza elephantina* Burch. Skeels (Fabaceae) (Fig. 1E) is a perennial subshrub which consists of aerial stems, which represent the canopy of the rest of the plant that is found growing underground. The branchlets are dark reddish to brown in colour and make up the much larger, underground tree. The leaves are a small, dull green and are present on the unbranched stems. This plant is mainly found Limpopo, Mpumalanga, Free State, Gauteng, North-West, KwaZulu Natal, Eastern Cape and Northern Cape provinces (Van Wyk et al., 1997).

#### 4.5.2. Antidiabetic literature

Kose et al. (2015) mentioned *E. elephantina* in their study as one of the ten most frequently used medicinal plants in the Maseru district. A review of the medicinal plants used by the Basotho people for their antidiabetic use mentioned *E. elephantina* as a medicinal plant species used by the people, but also stated that there had been no scientific proof that supported its antidiabetic properties at that time (Balogun et al., 2016).

A study by Olaokun et al. (2020) evaluated biological activities of the extracts from the leaves of *E. elephantina*. The anti-oxidant, anti-inflammatory, antidiabetic and cytotoxic activity was evaluated. An ethanol extract prepared from the leaves showed a 58.72% inhibition of α-glucoside at a concentration of

500 µg/mL, compared to acarbose which showed 78.48% inhibition. The findings of this may provide some credence for the traditional use of *E. elephantina* in the treatment of DM by the Basotho people as mentioned by Balogun et al. (2016). However, more studies will need to be conducted to confirm these findings as this was the first paper that focussed on the leaves and not the roots of the plant.

### 4.6. *Eriocephalus punctulatus*

#### 4.6.1. Botanical aspects

The genus *Eriocephalus* represents evergreen, woody, shrubs with small, simple leaves. The leaves are green and covered in pitted glands. The species, *Eriocephalus punctulatus* DC (Asteraceae) (Fig. 1F) is an erect shrub and produces many flowers, giving a bright white appearance. This plant is mainly found in the winter rainfall areas of South Africa and Namibia (Balogun et al., 2016).

#### 4.6.2. Antidiabetic literature

Moteetee and Van Wyk (2011), reviewed the medical ethnobotany of Lesotho, and recorded the use of *E. punctulatus* for the treatment of DM and high blood pressure. In a survey regarding medicinal plants used in the Maseru district in Lesotho, *E. punctulatus* was mentioned as one of the plants species used to treat DM (Kose et al., 2015).

In another review, Balogun et al. (2016) also mentioned the use of *E. punctulatus* by the Basotho people to treat DM. This review reported that the antioxidant and anti-inflammatory properties of this plant has been tested *in vitro,* but that there is a lack of data to support its antidiabetic properties.

### 4.7. *Heteromorpha arborescens*

#### 4.7.1. Botanical aspects

*Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. (Apiaceae) (Fig. 1A) is a small to medium size tree that can be found in all nine provinces in South Africa. One of the distinctive features of the tree is the dark, shiny bark that peels off the trunk in horizontal flakes. The leaves vary in shape and size and changes colour according to the seasons. The leaves start off grey/green before turning red during senescence and shedding

in Autumn. The common name of the plant is the parsley tree that is given due to the distinctive parsley smell given off when the leaves are crushed (Van Wyk et al., 1997).

### 4.7.2. Antidiabetic literature

Erasto et al. (2005) documented the use of *H. arborescens* for DM treatment in the Eastern Cape. An extract is made by boiling the roots and leaves of the plant and it is then taken orally. Afolayan and Sunmonu (2010) referred to the review by Erasto et al. (2005) in their review about *in vivo* studies on antidiabetic plants. They reported the aforementioned use of *H. arborescens* for diabetes treatment but provided no evidence of any *in vitro* or *in vivo* studies supporting the antidiabetic properties of this plant species.

## 4.8. *Lauridia tetragona*

### 4.8.1. Botanical aspects

*Lauridia tetragona (L.f.) R.H. Archer (Celastraceae) (*Fig. 1I), or better known as the climbing saffron, is a small shrub-like climber tree. The leaves range from dark green to blush red in spring and are glossy and serrated. The branchlets have small, reverse hooks that assist the plant in "climbing" and attaching to other surfaces, depending on the surrounding environment. The plant produces small reddish-purple and black fruit that are clustered together from December through to August. The distribution of *L. tetragona* include the following provinces: Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape (Archer and Van Wyk, 1997).

### 4.8.2. Antidiabetic literature

*Lauridia tetragona* is reported by Oyedemi et al. (2009) for its use to treat DM in the Nkonkobe municipality, Eastern Cape province, South Africa. In an article by Odeyemi and Dewar (2020), which studied the *in vitro* glucose uptake of polyphenolic compounds identified from *L. teragonia* extracts, they reported that some of the polyphenol-rich fractions (PPRF) showed good hypoglycaemic activity. Phytochemical compounds identified in the selected PPRF included diethyl phthalate ferulic acid, coumarin, 3-isopropylcatechol, herniarin, varencline, 3-tert-butyl-5-methycatechol, sterculic acid and caffeic acid. Of these identified structures, ferulic acid, coumarin and caffeine have been mentioned in

previous research regarding their alpha-amylase and alpha-glucosidase inhibitory activity. More research and testing is, however, still required to determine the exact pharmacological activity of these compounds.

### 4.9. *Pentanisia prunelloides*

#### 4.9.1. Botanical aspects

*Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh.) Walp. (Rubiaceae) (Fig. 1C) is a small, perennial herb found growing in the Eastern Cape and KwaZulu Natal. This herb consists of long, erect stems, covered by small, fine hairs, sprouting up from the rootstock. The leaves are small, green and ovate. A distinctive feature of the plant is the blue/lilac tubular flowers that grow in clusters at the ends of the stems (Van Wyk et al., 1997).

#### 4.9.2. Antidiabetic literature

Philander (2011) mentioned *P. prunelloides* among other plants, for its use by rural traditional healers in the Western Cape area, South Africa, to treat DM. Surveys conducted by Kose et al. (2015) in the Maseru district of Lesotho found *P. prunelloides* to be one of the ten most frequently mentioned medicinal plants used in this area. Data collected from interviews with the traditional healers reported the use of this plant to treat diabetes, however, no previous literature was found regarding its antidiabetic properties.

Balogun et al. (2016) also mentioned *P. prunelloides* for its use by the Basotho people to treat diabetes. The plants anti-inflammatory, antioxidant, antibacterial, cytotoxic and antimycobacterial properties have been studied *in vitro*, however, there are no scientific studies to support this plant's antidiabetic use.

*Pentanisia prunelloides* was reported to be one of the most frequently mentioned plants used by traditional healers in Lesotho. No specific reference of the plants' antidiabetic activity was mentioned in this study (Moteetee et al., 2019).

### 4.10. *Tulbaghia violacea*

#### 4.10.1. Botanical aspects

*Tulbaghia violacea* Harv. (Amaryllidaceae) (Fig. 1B) is a bulbous plant that naturally occurs in the Eastern Cape and KwaZulu Natal provinces. It is well known by its common name, wild garlic, due to the distinctive

garlic smell given off by the leaves and flowers when bruised or crushed. The leaves are thin, long and slightly fleshy and grow up out of the bulbous roots. The roots spread and clump together forming clusters of plants. The plants form tall flowering stalks that hold the pink-lilac tubular flowers that usually occur from January through to April (Van Wyk et al., 1997).

### 4.10.2. Antidiabetic literature

*Tulbaghia violacea* was recorded for its medicinal use, which include DM, by traditional healers in the Western Cape (Philander, 2011). Van Huyssteen et al. (2011) studied the effect of five medicinal plants on the glucose utilisation of cells, including *T. violacea*, in muscle, liver and fat cells. This study reported that an ethanol extract of *T. violacea* produced one of the highest increases in glucose utilisation of 140.5% in the muscle cells (positive control = 117.9%). These findings validate to some extent its traditional use for DM.

The *in vivo* antidiabetic activity of *T. violacea* was studied by Moodley et al. (2015) using a diabetic rat model. This study concluded that *T. violacea* extract has hypoglycaemic effects. This study was then followed up by another article, stating that more work and future studies are required in order to identify possible compounds responsible for the hypoglycaemic activity (Moodley and Mackraj, 2016).

## 5. Discussion and future perspectives

The conservation status of the selected plant species as described in this review was determined, and all are listed by the Red List of South African plants as being of least concern and are therefore not currently considered as being under threat.

The literature reviewed for the different plants indicates that although they are all mentioned for their medicinal use as antidiabetic treatments in South Africa, some plants show more promise than others. *Artemisia afra, C. benedictus* and *T. violacea* are mentioned in ethnobotanical surveys for their use against diabetes and have both *in vitro* and *in vivo* studies conducted. Compared to the positive controls used in these studies, acarbose and glibenclamide respectively, *A. afra* and *T. violacea* showed promising potential

by obtaining results similar to that obtained by the positive control, whilst *C. benedictus* showed substantially lower $IC_{50}$ values than the positive control.

The *in vitro* results obtained from a recent study on *C. benedictus* indicated that the plant extract had almost a 15-fold higher activity ($IC_{50}$ 1.2 µg/mL), than the control ($IC_{50}$ 17.68 µg/mL) and should therefore be considered a very promising plant for future studies (Paun et al., 2019). According to Bekale, (2016) several sesquiterpene lactones have been identified in *C. benedictus* such as cnicin, salonitenolide and artemisiifolin. These compounds are known for causing the distinctive bitter taste associated with the medicinal use of this plant, but no antidiabetic tests have yet been conducted with any of the main compounds found in *C. benedictus*. Although a significant discovery, no follow-up tests in order to confirm this finding or to identify the main active compounds have yet been conducted.

The information collected on *C. capensis, E. elephantina* and *L. tetragona* revealed both reports of their traditional medicinal use and *in vitro* studies. The results from these *in vitro* studies, although less active than the control, are still substantial enough to portray the plants antidiabetic potential, with *L. tertragona* the only species where a number of compounds were identified in the active extracts. The species *B. natalensis, E. punctulatus, H. arborenscens and P. prunelloides* were found to have no record of *in vitro* or *in vivo* studies regarding their antidiabetic activity, and the only literature on these plants is the mention of their medicinal use as a traditional antidiabetic treatment. Although this does not eliminate these plants from having any antidiabetic potential, the potential can only be determined once experiments are performed. No active compound(s) from any of these plants have yet been identified and tested for antidiabetic activity and this indicates the overall lack of necessary scientific scrutiny of the plants mentioned in this review.

The information collected during this review found that there is a wide variety of medicinal plants used to treat diabetes in South Africa. There are extensive records available for the plants that are used and by whom they are used. This being said, a large percentage of these plants have no scientific data to substantiate or validate their antidiabetic properties. The information collected for this review showed that

there is seemingly ample potential, but that more research and further testing should be done on the most promising plants, their extracts and eventually their most active compounds.

In conclusion, given the increase prevalence of DM, and the need to develop better treatments, promising medicinal plants should be investigated. *Cnicus benedictus* appears to be the most promising with a 15-fold better activity than the positive control and hence this significant find should first be replicated to confirm the reported activity, the actives should be identified, followed by *in vivo* studies and, if the data supports it, clinical trials.

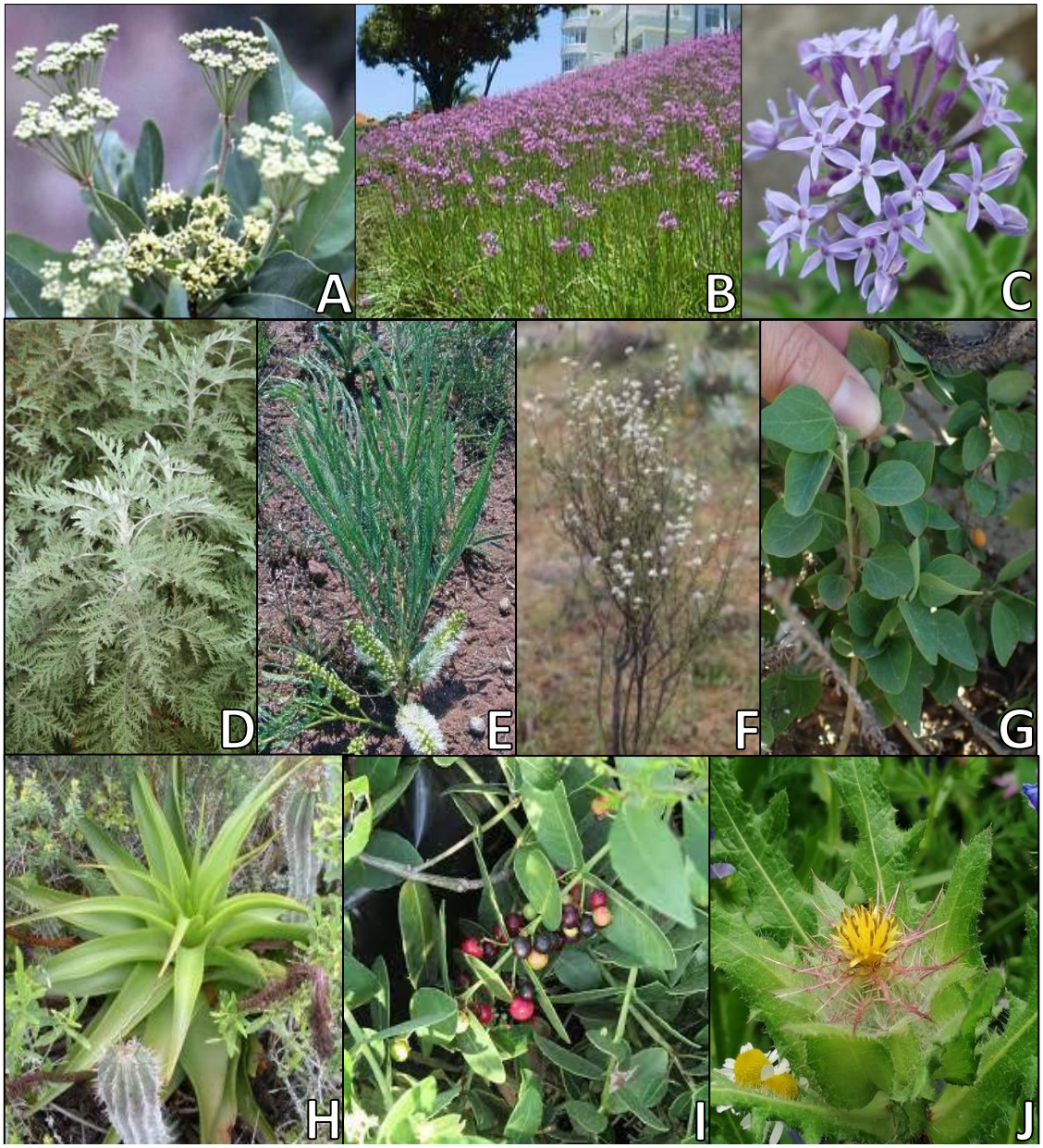


**Figure 1**: The plant species discussed in the review: *Heteromorpha arborescens* **(A)**, *Tulbagia violacea* **(B)**, *Pentanisia prunelloides* **(C)**, *Artemisia afra* **(D)**, *Elephantorrhiza elephantina* **(E)**, *Eriocephalus punctulatus* **(F)**, *Cissampelo capensis* **(G)**, *Bulbine natalensis* **(H)**, *Lauridia tetragona* **(I)**, *Cnicus benedictus* **(J)**. (Images from PlantZAfrica: http://pza.sanbi.org/)

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# CHAPTER 3: SHORT COMMUNICATION: "THE *IN VITRO* ANTIDIABETIC ACTIVITY OF PLANTS TRADITIONALLY USED FOR TREATMENT OF DIABETES IN SOUTH AFRICA"

Chapter 3 is presented as a short communication and was prepared for submission to the journal "*Pharmacia*". This manuscript was prepared as stated in the author guidelines of "*Pharmacia*" that can be found in Appendix B.

# The *in vitro* antidiabetic activity of plants traditionally used for treatment of diabetes in South Africa

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## Abstract

Many Southern African plants are traditionally used for the treatment of diabetes but lacks scientific evidence. In this study selected plants were screened, some for the first time, for bioactivity against diabetes. For each plant, tea and methanol extracts were prepared and tested which yielded varying results. All the plants tea extracts showed relativity weak inhibition with the highest being 7.83 ± 0.01% obtained for the *A. afra* sample. This was significantly lower than the positive control that yielded a 47.54 ± 0.07% inhibition. The results from the methanol extracts showed three samples inhibiting the enzyme, namely *A. afra*, the bulbs of *B. natalensis* and *E. elephantina* with percentage inhibitions of 1.14 ± 0.03, 1.10 ± 0.28 and 92.04 ± 0.01 respectively. *E. elephantina* extract was nearly double that of the positive control. This significant bioactivity, along with the plants low reported cytotoxicity shows potential for future studies.

**Keywords:** alpha-glucosidase, Diabetes Mellitus, *Elephantorrhiza elephantina, Artemisia afra*

## Introduction

The National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP) defines a chronic disease as a condition that lasts for at least 1 year or more and requires constant, ongoing medical treatment or the limiting of daily activities (CDC 2022). One such disease with a high prevalence in South Africa, which affects approximately 19 million adults between the ages of 20-79 in the African region alone, is Diabetes Mellitus (DM) (International Diabetes Federation 2019).

DM is a chronic disease defined by the body's inability to produce or use insulin effectively in order to control the blood glucose levels and is categorized in two main types namely Type 1 and Type 2 DM. Type 1 DM is when the body produces an insufficient quantity of insulin or is unable to produce insulin at all (Gupta et al. 2017). With no insulin, the body is unable to stimulate glucose uptake into cells for energy and results in a build-up of glucose in the bloodstream (hyperglycaemia). Type 2 DM is when the body is unable to use the insulin produced, which results in the patient developing insulin resistance and the body is unable to maintain healthy blood glucose levels. Diabetes is a complex, multifactorial disease involving many different genes, chemical steps, and physiological processes (Banday et al. 2020).

One of the current drugs on the market for treating type 2 DM is acarbose. Acarbose is a complex oligosaccharide that inhibits the alpha-glucosidase enzyme. This enzyme is responsible for breaking down oligosaccharides and disaccharides into monosaccharides that are then easily absorbed out of the digestive tract into the bloodstream (Martin and Montgomery 1996). By inhibiting this enzyme, the absorption of dietary carbohydrates is reduced, subsequently reducing the postprandial blood glucose and insulin levels.

Another antidiabetic drug used as a glucose-lowering agent in the treatment of type 2 DM, metformin, was discovered and developed based on the traditional use of *Galega officinalis* L. (Fabaceae) for the treatment of diabetes (Modak et al. 2007). Plants can be a promising source of new antidiabetic compounds, and South Africa's rich and diverse flora provide many possibilities. This being said, although South Africa has a large number of traditional medicinal plants that are mentioned in literature for their antidiabetic potential or that are currently used by traditional healers to treat diabetes, the majority of these plants have very limited or even no scientific data to support or substantiate their antidiabetic potential. There is a need for medicinal plants to be scientifically tested to validate their biological activities as well as to identify novel phytochemical compounds, which can potentially be developed into more effective treatments of DM. This study therefore focused on testing a variety of plant extracts for their antidiabetic potential. The plants selected were plants that occur in South Africa and have some previous mention of either having antidiabetic properties or are used by traditional healers for treating DM.

All the plants included in this study are mentioned in the literature for their medicinal use as antidiabetic treatments, however, some of the plants show more promise than others. *Artemisia afra* Jacq. *ex* Willd.(Asteraceae), *Cnicus benedictus* L. (Asteracea) and *Tulbaghia violacea* Harv. (Amaryllidaceae) are mentioned for their use against diabetes in ethnobotanical surveys and was studied in both *in vitro* and *in vivo* experiments.

*Elephantorrhiza elephantina* (Burch.) Skeels (Fabaceae) and *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. (Apiaceae) have been recorded for their traditional medicinal use, as well as *in vitro* studies, while the species *Bulbine natalensis* Baker. (Asphodelaceae) was found to have no record of any *in vitro* or *in vivo* studies regarding its antidiabetic activity with the only literature on this plant, the mention of the medicinal use as a traditional, antidiabetic treatment.

Although the lack of substantial scientific data does not eliminate these plants from having any antidiabetic potential, the potential can only be determined once experiments are performed. With South Africa being a developing country and the fact that DM requires constant, ongoing treatment, many people living in the

country cannot afford or do not have access to proper treatment. There is therefore a need for a more effective and affordable alternative treatment. This study aimed at screening extracts of the selected medicinal plants for α- glucosidase inhibition effects.

## Materials and method

### Chemical reagents

Ultrapure water for the preparation of the tea infusions was obtained from a Rephile Ultrapure water system (Boston, MA, USA) and methanol from Sigma-Merck. The enzyme (α- glucosidase), substrate (p-nitrophenyl glucopyranoside (pNPG)) and positive control (acarbose) used in the bioassay, was purchased from Sigma-Merck. DMSO used for reconstituting the samples for the bioassay was purchased from Sigma-Aldrich, Schaffhausen, Switzerland.

### Plant collection and authentication

An herbarium specimen for each plant species was deposited by the A.P Goossen herbarium, North-West University, Potchefstroom and accession numbers and barcodes were obtained. *Cnicus benedictus* was purchased as a dry powdered material from a commercial supplier (Mountain Herb Estate Nursery). The other plants purchased from commercial supplies was purchased as whole plants.

**Table 1**:Herbarium accession numbers and barcodes for each plant as well as where the plants were collected, and which parts were used.

| Plant name: | Where it was collected: | Plant part used: | Accession no: | Barcode: |
|---|---|---|---|---|
| ***Artemisia afra*** | (1) Collected from NWU Botanic Garden.<br>(2) Collected in Bronkhorstbaai | Leaves and twigs | 15474 | (1)PUC0015474<br>(2)PUC0015458 |
| ***Bulbine natalensis*** | Purchased from Mountain Herb Estate Nursery | (1)Leaves<br>(2)Roots/bulbs | 15476 | PUC0015476 |
| ***Cnicus benedictus*** | Purchased from Mountain Herb Estate Nursery | Whole plant | N/A | N/A |
| ***Elephantorrhiza elephantina*** | Purchased from Wildflower Nursery | Leaves and twigs | 15475 | PUC0015475 |
| ***Heteromorpha arborescens*** | Collected from NWU Botanic Garden | Leaves and twigs | 15478 | PUC0015478 |
| ***Tulbaghia violacea*** | Purchased from Mountain Herb Estate Nursery | Whole plant | 15477 | PUC0015477 |

All collected plant material were oven dried at 40°C for seven days to remove all moisture, except the leaves and bulbs of the *B. natalensis* which was freeze-dried for seven days due to the oven not being able to remove all the moisture from the succulent leaves. The dried plant material was then grounded into a fine powder using a food grade blender.

**Sample preparation and extraction**

Both methanol and aqueous tea extracts were prepared from the selected plant materials as listed in Table 1. The methanol extracts were made by adding dried plant material (2 g) to methanol (30 mL). The mixture was sonicated for 15 min and then left to infuse overnight, after which it was filtered. The filtrate (2 mL) was then again filtered through a polytetrafluoroethylene (PTFE) syringe filter (0.45 µm, 25 mm) into a Ultra-high performance liquid chromatography (UPLC) vial for chemical analysis. The remaining infusion was split into Eppendorf vials (1.5 mL in each vial) and dried in a speedvac to yield a primary dried infusion sample.

The tea extracts were made by adding dried plant material (2 g) to boiling deionized water (30 mL). The mixture was sonicated for 30 min and then left to infuse overnight, after which it was filtered. The filtrate (2 mL) was then again filtered through a polytetrafluoroethylene (PTFE) syringe filter (0.45 µm, 25 mm) into a UPLC vial for chemical analysis. The remaining water infusion was split into Eppendorf vials (1.5 ml in each vial) and frozen at -80°C, followed by freeze-drying to yield a primary dried infusion sample.

**Alpha-Glucosidase inhibitory assay**

All plant extract samples were first diluted in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Schaffhausen, Switzerland) to obtain stock solutions of 2000 µg/mL. All stock solutions were vortexed and sonicated to ensure the dried extract was fully dissolved. The stock solution was diluted further using a 20 mM phosphate buffer (pH 6.9) to a concentration of 1mg/mL which was used for the bioassay.

The method used was adapted and modified from Kazeem et al. (2013). $\alpha$-Glucosidase from *Saccharomyces cerevisiae* was used to determine the α-glucosidase inhibition. A p-nitrophenyl glucopyranoside (pNPG) solution was prepared in 20 mM phosphate buffer (pH 6.9). A volume of 100 µL α-glucosidase (1.0 U/mL) solution was preincubated with 50 µL of the different extracts for 10 min at 37°C in a transparent flat-bottom 96 well plate (Sarstedt AG, Sevelen, Switzerland). A volume of 50 µL of 3.0 mM pNPG dissolved in the phosphate buffer (pH 6.9) was added to the α-glucosidase/extract solution as the substrate to start the reaction. The reaction mixture was incubated at 37°C for 20 min. The activity was determined by measuring the yellow-coloured paranitrophenol released from the pNPG at 405 nm using a Spectramax™ paradime plate reader. All experiments were conducted in triplicate and averages obtained. Distilled water and acarbose was used in the place of the plant extract as negative and positive controls respectively. Once the absorbance was measured, the % inhibition of each sample was calculated using the following formula:

$$\% \text{ Inhibition} = \left(\frac{AbsNegative\ Control - AbsExtracts}{AbsNegative\ Control}\right) x100$$

**Results and discussion**

Table 2 shows the percentage enzyme inhibition obtained during the alpha-glucosidase bioassay for each of the selected plant extracts.

**Table 2**: Percentage alpha-glucosidase inhibition by the selected plant extracts at 1mg/mL.

| | Tea extract | MeOH extract |
|---|---|---|
| **Sample** | **% Inhibition ± SD*** | **% Inhibition ± SD*** |
| ***A. afra*- (1)** | 7.42 ± 0.04 | 1.14 ± 0.03 |
| ***A. afra*-(2)** | 7.83 ± 0.01 | NI |
| ***B. natalensis-leaves*** | 4.37 ± 0.10 | NI |
| ***B. natalensis-bulbs*** | 4.37 ± 0.02 | 1.10 ± 0.28 |
| ***C. benedictus*** | 0.15 ± 0.07 | NI |
| ***E. elephantina*** | 1.95 ± 0.05 | 92.04 ± 0.01 |
| ***H. arborescens*** | 2.68 ± 0.04 | NI |
| ***T. violacea*** | 2.52 ± 0.07 | NI |
| **Acarbose**** | | 47.54 ± 0.07 |

*All experiments were performed in triplicate (n = 3) and the results given as average ± standard deviation (SD)
** Positive control reconstituted in buffer at 1mg/mL.
NI = No Inhibition

All the tea extracts showed relatively weak inhibition of the alpha-glucosidase enzyme, with the highest percentage inhibition being obtained by the two *A. afra* samples. Although these two extracts showed some inhibition, it is substantially lower than that obtained by the positive control. For the methanol extracts, only three samples showed enzyme inhibition, namely: *A. afra*, *B. natalensis* bulbs and *E. elephantina*. The latter showed the highest percentage inhibition of 92.04 ± 0.01% at a concentration of 1 mg/mL, compared to the 47.54 ± 0.07% inhibition of the positive control.

Little information is available in literature regarding *E. elephantina*. Kose et al. (2015) mentioned *E. elephantina* in their study as one of the ten most frequently used medicinal plants in the Maseru district. A review of the medicinal plants used by the Basotho people as antidiabetics mentioned *E. elephantina* as one of the medicinal plant species, but also stated that there had been no scientific proof that supported its antidiabetic properties at that time (Balogun et al. 2016). Olaokun and King (2018) tested extracts of *E. elephantina* for their potential to stimulate glucose utilisation activity of C2C12 muscle cells. They found the most active extract to be a cold-water extract, which exhibited 69.6 ± 0.04% glucose utilisation activity. The hot water, 95% ethanol and acetone extracts showed decreased activity of 57.5 ± 0.06%, 51.6 ± 0.03% and 46.0 ± 0.02% at a concentration of 0.5 mg/mL. In a follow-up study, Olaokun et al. (2020), tested various extracts using the α-amylase and α-glucosidase bioassays and found the ethanol extract to show the most activity of 58.72% at 0.5 mg/mL, but again slightly less active than the positive control, acarbose which showed a 78.48% inhibition. This study confirmed this finding with the MeOH extract of the leaves showing better activity than acarbose. Olaokun et al. (2020), also tested cytotoxicity and showed that the extracts were only found to show cytotoxicity at high concentrations with the ethanol extract's $LC_{50}$ being greater that 1000 µg/mL when tested on H4IIE liver cells and C2C12 myotubules.

This was the first study to report *in vitro* results for the antidiabetic properties of *B. natalensis*, and although the percentage inhibition was not notably high, this study only tested the alpha-glucosidase enzyme, which is only one possible pathway to treating diabetes. In a study that looked at the cytotoxicity of selected indigenous South African medicinal plants, Vakele et al. (2022) reported that the aqueous extracts of *B. natalensis* roots and shoots showed 23.16% and 40.31% inhibition of HeLa and HepG2 cells, respectively. They, however, did not test the extract any further due to the percentage inhibition being below 50%. In this same study, *T. violacea* showed high cytotoxicity with a 90.2% at 100 µg/mL and a LC50 of 45.61 µg/mL (Vakele et al. 2022). In respective studies, the following plant extracts showed low to moderate cytotoxicity; *A, afra* (Mukinda and Syce 2007) *C. benedictus* (Paun et al. 2019) and *H. arborescens* (Abifarin et al. 2021)

## Conclusion

From the results obtained it can be concluded that all the selected plants' tea extracts showed some degree of inhibition, however, not as high as the *E. elephantina* MeOH extract, which showed the greatest percentage inhibition. Due to its low cytotoxicity and the fact that it showed better activity than the positive control, this plant species shows a lot of promise and hence the main active compound(s) needs to be isolated and identified and further evaluated in terms of antidiabetic activities.

## Conflicts of interest

The authors report no conflict of interest


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## Authors contributions

MR Stevens performed all experiments and wrote the article. F. van der Kooy and JH Hamman assisted with proofreading and editing of script.

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# CHAPTER 4: ARTICLE: "SEASONAL CHEMICAL VARIATION AND ANTIDIABETIC ACTIVITY OF MAJOR COMPOUNDS IN *ARTEMISIA AFRA* INFUSIONS."

Chapter 4 contains an article and was prepared for submission to the journal "*South African Journal of Botany*". This manuscript was prepared according to the author guidelines of "*South African Journal of Botany*" as presented in Appendix A.

Although, according to the initial screening process performed in the previous article, *A. afra* was only the second most biologically active plant, it was selected to continue with due to the lack and unavailability of sufficient plant material of *E. elephantina*, the most active plant.

# Seasonal chemical variation and antidiabetic activity of major compounds in *Artemisia afra* infusions

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**Highlights:**

- *Artemisia afra* is a popular herbal remedy used to treat diabetes
- Phytochemical variation complicates quality-control of herbals
- The seasonal chemical variation of *A. afra* samples were conducted
- Substantial phytochemical variation was observed over a 1-year period
- The major identified compounds were also tested for their antidiabetic activity

**Keywords:** *Artemisia afra*, chemical variation, diabetes, α-glucosidase, LC-ESI-MS/MS, quality control

**Abstract**

*Artemisia afra* is a very popular herbal remedy in Southern Africa. It is mainly used in the form of a tea infusion for the treatment of a wide variety of ailments, including diabetes. In this study the chemical variation of four *A. afra* samples collected monthly for a 1-year period was determined. Thirteen reference compounds were comparatively quantified in these samples to further illustrate the chemical variability. These compounds were also tested for their *in vitro* antidiabetic activity using the α-glucosidase bioassay. The results indicated that considerable chemical variation exist over a 1-year period within and between the plants tested. The main bioactive compounds, 1,5-dicaffeoylquinic acid and 3,5- dicaffeoylquinic acid, which showed better activity than the positive control, acarbose, revealed an interchangeable trend with its monomer, chlorogenic acid, with the former increasing during summer and the latter during the winter months. Being the most active compounds, this highlights the importance of quality control and the role that chemical variation can have on the bioactivity of herbal medicines.

## 1. Introduction

*Artemisia afra* Jacq. ex Willd. (Asteraceae) is a well-known herbal remedy in sub-Sahara Africa. It is used to treat a wide variety of ailments, in the form of an herbal tea, of which most commonly the treatment of flu like conditions. Upon review of available scientific literature, predominantly low-level evidence has been published on the activity of *A. afra* extracts and compounds used for the treatment of a wide range of medical conditions (Liu et al., 2009; Du Toit and Van der Kooy, 2019).

The hypoglycaemic activity of *A. afra* has been reported by Afolayan and Summonu (2011) who conducted a pre-clinical *in vivo* analysis of *A. afra* by orally administering an aqueous extract to diabetic rats. In a subsequent study, different dosages of an *A. afra* extract were tested in Wistar rats which revealed marked hypoglycaemic activity, however some toxicity was observed at higher dosages (Afolayan and Sunmonu, 2013).

*Artemisia afra* was also studied for *in vitro* activity using the α-amylase and α-glucosidase bioassays. At a concentration of 0.2 mg/mL the extract yielded an α-glucosidase inhibition of 47.2% and α-amylase inhibition of 74.0% compared to the positive control (acarbose) which produced 80.6% and 73.4% inhibition, respectively. This indicated that this plant had some potential as an antidiabetic agent. Issa and Bule (2015) conducted i*n vivo* experiments in diabetic Swiss albino mice and tested both methanolic and aqueous extracts of *A. afra* which drastically reduced the blood glucose levels in the mice compared to that of the control group. The $LD_{50}$ of the aqueous extract was also found to be relatively high (9 833.4 mg/Kg).

The use of herbal remedies is widely critiqued, for a number of reasons, including due to the naturally occurring chemical variation exhibited by medicinal plants. It is well known that plants chemically react to their immediate environment (soil type, drought, waterlogging, pathogens, general climatic anomalies etc.) and that chemical variation within and between individual plants can and should be expected. This chemical variation will also be exacerbated by post-harvest

handling and storage conditions. Various guidelines have been established to implement quality control in herbal medicines to overcome this problem, however, the effectiveness of these measures and the true extent and impact of this barrier is yet to be fully understood (WHO, 2011; Bensoussan et al., 2016).

Plants are chemically very complex, containing many different compounds, of which some are biologically active, and many may not be active at all. Taljaard et al. (2022) and Van Loggenberg et al. (2022) recently identified some of the compounds found in *A. afra* infusions but did not test their bioactivity. This study aimed to identify more of the compounds present in *A. afra* infusions, follow the variation in concentration of these compounds over a 1-year period and to test their antidiabetic activity.

## 2. Materials and Methods

### 2.1.Chemicals and reagents

Ultrapure water from a Rephile Ultrapure water system (Boston, MA, USA) was used to prepare the infusions, and as solvent for the LC-ESI-MS/MS analysis. The acetonitrile and formic acid used for LC-ESI-MS/MS analysis was obtained from Sigma-Aldrich (Johannesburg, RSA). The reference standards; caffeic acid, 4-chlorogenic acid (4-CGA), chlorogenic acid (CGA), 1,5-dicaffeoylquinic acid (1,5-DCQA), 3,5-DCQA, 4,5-DCQA, 3,4-DCQA, ferulic acid, luteolin, neo-chlorogenic acid (neo-CGA), quercetin, scopoline and scopoletin that were used for the comparative analysis was purchased from Chengdu Alfa Biotechnology (Chengdu, China). All reference standards had a purity grade of >98%.

### 2.2.Plant material and sample preparation

*Plant material:* The four *A. afra* plants used in this study is located in the Botanic Garden at North-West University (NWU), Potchefstroom, South Africa (GPS coordinates; -26.6823° S, 27.0950° E). Three of the plants are growing in a maintained section of the garden whilst the fourth plant

grows in an unmaintained section of the garden. Approximately 10 cm of the top part of a twig from all four plants was collected once a month. The material was immediately placed in an oven at 40°C for 6 days where after the dried leaves were stripped from the twigs and stored until analysis. Herbarium specimens have been deposited at the A.P. Goossens herbarium at NWU for positive identification (specimen numbers: PUC0015502, PUC0015503, PUC0015504, PUC0015505).

Table 1 provides the date, time of day and climatic conditions on each day of collection. More exact and expanded climatic data for collection days can be assessed from a weather archive (https://www.worldweatheronline.com/potchefstroom-weather-history/north-west/za.aspx).

*Tea infusion:* Samples were prepared by adding 1 g plant material to 5 mL boiled ultrapure water. Samples were left to infuse for 10 min. Samples were then vortexed for 30 sec to ensure proper mixing. Of each sample 1 mL was filtered through a 0.45 µm syringe filter into UPLC vials for analysis.

### 2.3.Reference standard preparation

The standard solutions for the thirteen marker molecules (caffeic acid, 4-CGA, CGA, 1,5-DCQA, 3,5-DCQA, 4,5-DCQA, 3,4-DCQA, ferulic acid, luteolin, neo-CGA, quercetin, scopolin and scopoletin) were prepared by accurately weighing 1 mg of each compound into individual 2 mL amber UPLC glass vials. The markers were then dissolved with methanol, to yield a 1000 µg/mL concentration. 100 µL of each reference standard was mixed together in another vial to yield a stock mixed solution with a final concentration of 76 µg/mL of each marker. The stock solution was stored at -20°C until use. A serial dilution in methanol was prepared from the stock solution to construct a standard curve for compound quantification.

## 2.4.UPLC analysis

The samples were analysed on a Shimadzu i-Nexera UPLC system equipped with a quaternary pump, auto sampler, and a photodiode array detector. The system was fitted with an Agilent Poroshell 120 EC-C18, 3 X 150 mm, 2.7 µm column with the column oven set to 35°C. The solvent system consisted of water + 0.1% formic acid (A) and acetonitrile (ACN) + 0.1% formic acid (B) and a step-wise gradient system was employed: 0 min, 10% B; 5 min, 10% B; 6 min, 20% B; 10 min, 20% B; 11 min, 85% B; 16 min, 85% B; 16.10 min, 100% B; 17 min, 100% B, 17.10 min, 10% B, 20 min 10% B. UV detection was carried out at 254nm.

The flow rate was 0.49 mL/min and 10 µL of each sample was injected. After every 12 samples, a blank injection was conducted in order to test for and prevent any carry over between samples.

## 2.5.LC-ESI-MS/MS analysis

An Agilent 1100 Infinity binary pump and a 1260 Infinity auto sampler and column heater with an Agilent Poroshell 120 EC-C18, 3 X 150 mm, 2.7 µm column was used to separate the samples. The mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile (ACN) + 0.1% formic acid (B). The flow rate was 0.49 mL/min. The stepwise gradient system was set up as follows: 0 min, 10% B; 5 min, 10% B; 6 min, 20% B; 10 min, 20% B; 11 min, 30% B; 14 min, 30% B; 15.10 min, 40% B; 18 min, 60% B, 18.10 min, 100% B, 18.10 min 100% B; 19 min, 100%; 19.10 min, 10%; 22 min, 10%, and a post run time of 3 min in order to re-equilibrate the system. Total run time was 25 min. The optimization of the positive and negative ionization using an Agilent ESI probe and the Ultivo triple quadrupole detector for the 13 reference standards resulted in a highly sensitive multiple reaction monitoring (MRM) method for each marker. The retention times and MRM parameters for each marker is shown in Table 2. The optimised settings were as follow: gas temperature 350°C, $N_2$ gas flow 13 L/min, nebulizer pressure 60 psi, capillary voltage 4000V. The injection volume was 1 µL.

### 2.6.*In vitro* bioassay

The method used to determine the enzyme inhibition was adapted from Kazeem et al. (2013). $\alpha$-Glucosidase from *Saccharomyces cerevisiae* was used to determine the α-glucosidase inhibition. A p-nitrophenyl glucopyranoside (pNPG) solution was prepared in 20 mM phosphate buffer (pH 6.9). 100 µL α-glucosidase (1.0 U/mL) was preincubated with 50 µL of the reference standards (1 mg/mL) for 10 min at 37°C in a transparent flat-bottom 96 well plate (Sarstedt AG, Sevelen, Switzerland). 50 µL of 3.0 mM (pNPG) dissolved in the phosphate buffer (pH 6.9) was added to the α-glucosidase/reference standard solution as the substrate to start the reaction. The reaction mixture was incubated at 37°C for 20 min. The activity was determined by measuring the yellow-coloured paranitrophenol released from the pNPG at 405 nm using a Spectramax™ paradime plate reader. All experiments were conducted in triplicate and averages obtained. Distilled water and acarbose was used in the place of the reference standards as negative and positive controls respectively.

Once the absorbance was measured, the % inhibition of each sample was calculated using the following formula:

$$\%\text{Inhibition} = \left(\frac{AbsNegative\ Control - AbsExtracts}{AbsNegative\ Control}\right) x100$$

## 3. Results and discussion

### 3.1.UPLC

The overlayed chromatograms of the 12 samples collected monthly from each plant are presented in the supplementary data. It is clear that a large degree of chemical variation is present between individual plants but also over the course of the year. In order to better visualise these differences four seasonal samples were selected from plant 1 (growing in full shade) and plant 2 (semi-sun). The chromatograms from the eight selected samples as well as the reference sample mix is given in Fig 1. The most striking difference was the class of compounds eluting between 16.5-18.0 min

in samples prepared from plant 1. These compounds were completely absent in plants 2-4 with the main difference being that plant 1 grows in a shade environment whilst the other samples receive direct sunlight.

Moreover, not all our reference compounds were present in high concentrations with luteolin and quercetin being undetectable using UPLC. A number of unknown major compounds, notably at time 9 min in plant 1, and compounds eluting at 10 and 11 min in plant 2 remains unidentified.

### 3.2.LC-ESI-MS/MS

The seasonal comparative quantitative analysis confirms the large chemical variation within a plant and between the plants over the course of the year (Table 3). The flavonoids, quercetin and luteolin was detected but was present at very low levels. These flavonoids which are considered to play a significant role in the general bioactivity of *Artemisia* spp. are therefore either present in low concentrations in the plant or are not efficiently extracted with water due to their non-polar nature.

A recent in-house study analysing 6 marker molecules indicated an interchangeable nature in the seasonal quantity between CGA and 4,5-DCQA. The current study analysing 3 CGA derivatives and 4 DCQAs confirms this trend. During winter months the DCQAs are at their lowest whilst CGA and caffeic acid are at their highest concentration. In sample 1, growing in full shade, this trend was less pronounced (Table 3). This could possibly be ascribed to the molar absorptivity difference between CGA (18.3 $\epsilon$) and DCQA (34.0 $\epsilon$) at 329 and 330 nm, respectively, which implies that DCQA, with higher molar absorptivity, is biosynthesised during the summer months when UV radiation is generally higher (Kaeswurm et al. 2021). De Lazzari Almeidaa, et al. (2016) found a similar trend regarding the biosynthesis of CGA and DCQA while testing the seasonal variations in *Mikania laevigata* Schultz and *Mikania glomerata* Sprengel (Asteraceae).

Most of the other marker compounds showed a gradual decrease approaching winter and increase again approaching summer.

### 3.3.*In vitro* bioassay

Table 4 presents the results from the 13 identified reference compounds identified in the *A. afra* samples, showing which compounds inhibit the enzyme as well as the percentage inhibition obtained. Interestingly, two of the four DCQA derivatives (1,5-DCQA and 3,5-DCQA) showed activities above 80% compared to the positive control, acarbose, which inhibited only at 47.5% at a concentration of 1 mg/mL. CGA inhibited only at 31.05% at the same concentration. Etemadi-Tajbakhsh et al. (2020) reported a similar trend where 1,5-DCQA showed higher inhibition than CGA. El-Askary et al. (2022) tested 3,5-DCQA, 3,4-DCQA and 4,5-DCQA and reported that 3,5-DCQA had the most activity followed by 3,4-DCQA with 4,5-DCQA the least active. Our results confirm this with 3,5-DCQA showing better activity than the positive control with 1,5-DCQA being the most active. El-Askary et al. (2022) furthermore reported very weak toxicity for these compounds. The other compounds tested showed moderate (quercetin) to low inhibition with 4-CGA and scopoletin showing no inhibition at all.

## 4. Conclusions and final remarks

Our results confirm the well-known phenomenon of chemical geographical and/or seasonal variation as was also published by Sotenjwa et al., 2019. In our study, we indicate that this also occurs between plants growing in a very similar environment (soil type, climatic conditions). The main bioactive compounds were found to be 1,5-DCQA and 3,5-DCQA which showed better activity than the positive control acarbose. As DM is a chronic condition that needs treatment throughout the year and with our results indicating that the DCQA's significantly decrease during winter months it again highlights the difficulties in ensuring consistent quality in herbal medicine.

## 5. Acknowledgements

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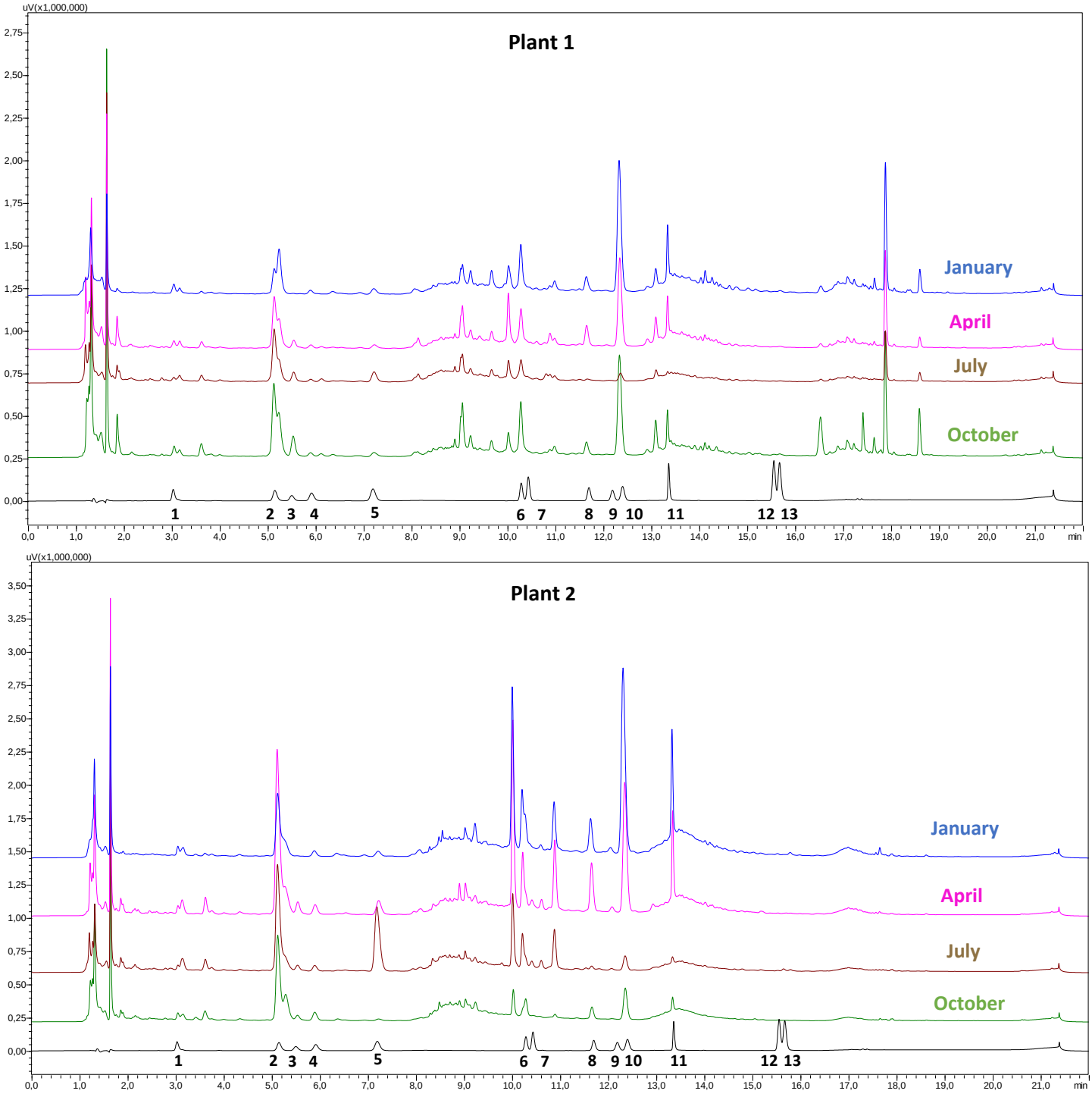


**Fig 1:** UPLC chromatograms of the selected *Artemisia afra* samples as well as the 13 reference standards; **(1)** Neochlorogenic acid **(2)** Chlorogenic acid **(3)** Scopolin **(4)** 4-CGA **(5)** Caffeic acid **(6)** Scopoletin **(7)** Ferulic acid **(8)** 3,4-DCQA **(9)** 1,5-DCQA **(10)** 3,5-DCQA **(11)** 4,5- DCQA **(12)** Luteolin **(13)** Quercetin

**Table 1**: Weather data in Potchefstroom for 2021 during plant collection

| Sample Month | Date | Time | Temp during collection (°C) | Min. temp preceding night (°C) | Average monthly temp (°C) | Average monthly rainfall (mm) | Weather conditions |
|---|---|---|---|---|---|---|---|
| 1 | 04/01/21 | 07:30 | 25 | 17 | 24 | 303.7 | Moderate to heavy rain showers |
| 2 | 04/02/21 | 07:50 | 18 | 16 | 23 | 232.6 | Cloudy with light rain showers |
| 3 | 05/03/21 | 08:10 | 25 | 21 | 23 | 63.0 | Partly cloudy |
| 4 | 06/04/21 | 08:30 | 23 | 22 | 23 | 38.4 | Cloudy with isolated showers |
| 5 | 04/05/21 | 08:20 | 13 | 16 | 17 | 1.3 | Partly cloudy |
| 6 | 04/06/21 | 08:30 | 9 | 8 | 15 | 0.6 | Partly cloudy |
| 7 | 07/07/21 | 08:30 | 10 | 7 | 13 | 0.0 | Partly cloudy |
| 8 | 11/08/21 | 08:45 | 10 | 5 | 18 | 1.2 | Partly cloudy |
| 9 | 10/09/21 | 08:15 | 19 | 14 | 24 | 2.2 | Partly cloudy |
| 10 | 04/10/21 | 08:10 | 20 | 14 | 24 | 22.2 | Partly cloudy |
| 11 | 04/11/21 | 07:30 | 27 | 19 | 16 | 32.3 | Cloudy with scattered thundershowers |
| 12 | 06/12/21 | 08:15 | 26 | 16 | 23 | 55.6 | Sunny |

**Table 2**: LC-ESI-MS/MS optimised MRM parameters and retention times for individual markers

| Marker molecule | Retention time (s) | Precursor (m/z) | Product ion (m/z) | Fragmentor voltage (V) | Collision energy (V) | Polarity |
|---|---|---|---|---|---|---|
| **4-CGA** | 3.26 | 353.1 | 135.2 | 86 | 37 | - |
| **Neo-CGA** | 3.26 | 353.1 | 134.7 | 81 | 37 | - |
| **CGA** | 5.46 | 353.1 | 190.9 | 71 | 17 | - |
| **Scopolin** | 5.77 | 355.1 | 192.9 | 66 | 13 | + |
| **Caffeic acid** | 7.59 | 181.0 | 163.0 | 56 | 5 | + |
| **Scopoletin** | 11.39 | 191.0 | 176.0 | 71 | 13 | + |
| **Ferulic acid** | 11.60 | 195.1 | 177.0 | 61 | 5 | + |
| **3,4-DCQA** | 13.31 | 515.1 | 191.1 | 131 | 37 | - |
| **1,5-DCQA** | 13.87 | 515.1 | 191.1 | 81 | 37 | - |
| **3,5-DCQA** | 14.11 | 515.1 | 191.1 | 141 | 37 | - |
| **4,5-DCQA** | 14.53 | 515.1 | 191.1 | 131 | 37 | - |
| **Luteolin** | 17.03 | 285.0 | 132.9 | 96 | 41 | - |
| **Quercetin** | 17.16 | 301.0 | 121.1 | 96 | 33 | - |

**Table 3**: LC-ESI-MS/MS comparative quantification results

| | | µg/mg plant material | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Month** | **Plant** | **Caffeic acid** | **4-CGA** | **CGA** | **1,5-DCQA** | **3,5-DCQA** | **4,5-DCQA** | **3,4-DCQA** | **Ferulic acid** | **Luteolin** | **Neo-CGA** | **Quercetin** | **Scopolin** | **Scopoletin** |
| **January** | 1 | 0.387 | 0.610 | 2.653 | 3.263 | 0.134 | 0.820 | 0.581 | 0.079 | 0.003 | 0.689 | 0.002 | 0.119 | 2.278 |
| | 2 | 0.541 | 0.784 | 7.769 | 8.249 | 0.252 | 2.342 | 1.793 | 0.023 | 0.006 | 0.897 | 0.005 | 0.183 | 1.668 |
| **April** | 1 | 0.239 | 0.495 | 5.890 | 2.661 | 0.355 | 0.649 | 0.809 | 0.032 | 0.003 | 0.565 | 0.001 | 1.703 | 1.575 |
| | 2 | 0.538 | 0.435 | 18.722 | 9.731 | 0.702 | 2.685 | 3.690 | 0.011 | 0.003 | 0.506 | 0.005 | 2.801 | 0.564 |
| **July** | 1 | 0.349 | 0.210 | 6.077 | 0.054 | 0.009 | 0.012 | 0.012 | 0.034 | 0.004 | 0.238 | 0.007 | 1.934 | 0.707 |
| | 2 | 2.050 | 0.272 | 18.081 | 0.816 | 0.091 | 0.146 | 0.257 | 0.024 | 0.006 | 0.314 | 0.015 | 1.579 | 0.355 |
| **October** | 1 | 0.328 | 0.852 | 8.773 | 4.875 | 0.362 | 1.011 | 0.840 | 0.057 | 0.002 | 0.969 | 0.001 | 4.321 | 2.599 |
| | 2 | 0.298 | 0.463 | 13.762 | 3.106 | 0.216 | 0.681 | 1.259 | 0.012 | 0.003 | 0.531 | - | 1.570 | 0.858 |

**Table 4**: *In vitro* bioassay results obtained by the 13 identified compounds at 1mg/mL.

| **Sample** | **% Inhibition ± SD*** |
|---|---|
| Caffeic acid | 4.05 ± 0.02 |
| 4-CGA | NI |
| CGA | 31.05 ± 0.06 |
| 1,5-DCQA | 88.96 ± 0.02 |
| 3,5-DCQA | 84.69 ± 0.03 |
| 4,5-DCQA | 25.55 ± 0.12 |
| 3,4-DCQA | 43.96 ± 0.58 |
| Ferulic acid | 11.74 ± 0.01 |
| Luteolin | 26.09 ± 0.04 |
| Neo-CGA | 18.52 ± 0.05 |
| Quercetin | 35.68 ± 0.02 |
| Scopolin | 2.43 ± 0.26 |
| Scopoletin | NI |
| Acarbose** | 47.54 ± 0.07 |

*All experiments were performed in triplicate (n = 3) and the results given as average ± standard deviation (SD)

** Positive control reconstituted in buffer at 1mg/mL.

NI= No inhibition

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# CHAPTER 5: FINAL CONCLUSION AND FUTURE RECOMMENDATIONS

## Final conclusion

From the literature review, substantial literature could be found stating the traditional use of all the plants selected for this study, however many of these plants lacked sufficient or any scientific evidence to support its use to treat DM. The results obtained from the initial screening process revealed some plants to have a higher degree of activity towards inhibiting the α-glucosidase enzyme than others. This was the first report of the *in vitro* antidiabetic activity of *B. natalensis* extracts, and although the extracts did not show a high level of inhibition, the bioassay focus only on one enzyme and therefore the extracts tested in this study should also be tested for inhibition potential using different bioassays. The most promising aqueous extracts was the *A*. *afra* extracts while for the MeOH extracts, *E. elephantina* leaves had the highest percentage enzyme inhibition, even higher than for the positive control.

The chemical variation within and between plants was studied using *A. afra* samples collected over a 12-month period and the results confirm the well-known phenomenon of seasonal and/or geographical, chemical variation found within and between plants. Compounds were identified in *A. afra* samples and tested for their antidiabetic activity. This indicated that some of the most active major compounds identified, such as 1,5-DCQA, showed substantial chemical variation within a period of a year, highlighting the importance of quality control of herbal remedies.

## Future recommendation

Due to the increasing prevalence of DM in South Africa and the chronic nature of the disease requiring constant treatment, plants showing bioactivity and possessing antidiabetic potential must be tested further.

- A wider variety of South African plants should be screened for bioactivity.
- Identification of main actives within the most bioactive plant extracts.
- Further studies of specifically *E. elephantina,* being the current most active extract, and identification of main active compounds should be conducted.
- Screening of plant extracts using different and more accurate bioassays.

# APPENDIX A: AUTHOR GUIDELINES "*SOUTH AFRICAN JOURNAL OF BOTANY*"

## Guide for Authors

### Introduction

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**Examples:** Type: South Africa. Western Cape, Beaufort West (3222): 'Renosterkopf' [Renosterkop] (-BB), 1850, *Zeyher 854* (TCD, lecto.- image!, here designated; SAM!, P - image! [2 sheets], isolecto.).

Type: South Africa. Western Cape, Caledon (3419): Ratelrivier flats (-DC), 12 Feb 1958, *Willems 19* (NBG, holo.!; NBG!, K!, iso.).

Type: South Africa. Precise locality unknown: 'Cap. Bonae Spae' [Cape of Good Hope], *Thunberg s.n.*THUNB-UPS 20137 (UPS, holo. - microfiche!).

Type: South Africa. KwaZulu Natal, Stanger (2931): Natal, near 'Port Natal' [Durban] (-CC), *Krauss 418* (MO, sheet with collection label in bottom left corner, lecto.!, here designated; BM!, K! [2 sheets], MO!, NBG!, S!, isolecto.).

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**EXAMPLES:**

**Additional specimens examined.**

Botswana. **2615 (Luderitz)**: Diamond Area No. 1, Sperrgebiet, south of Rotkuppe gate (-CD), 2 Aug 2001, *Mannheimer 1391* (WIND); Road to Grillental from Kaukausib, Blue ridge (-DC), 5 Sep 2002, *Mannheimer 2200* (WIND). **2715 (Bogenfels)**: Diamond Area No. 1, en route from Tsabiams to Grillenthal (-BA), 5 Sep 2002, *Bartsch, Loots and Mannheimer 1028* (WIND); Approach to Kaukausib Plain to south (-BA), 5 Sep 2002, *Mannheimer 2195* (WIND); Sandy-gravel plain east of Kaukausib Fountain (-BA), 12 Sep 2005, *Mannheimer 2769*(WIND, JRAU); Karas district, Sperrgebiet, Kaukausib Drainage (-BA), 3 Mar 2007, *Burke 7001* (PRE).

South Africa. WESTERN CAPE: **3218 (Clanwilliam)**: Near Eendekuil, western foot of Piekenierskloof Pass (-DB), 28 Aug 2009, *Magee, Boatwright, Manning and Goldblatt 161* (NBG, PRE, K, BOL). **3319 (Worcester)**: Tulbagh (-AC), Sep 1919, *Bolus 16734* (BOL); roadside near Gouda (-AC), 9 Sep 1951, *Esterhuysen 18840* (BOL [3 sheets], K, PRE).

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Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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***Submission checklist***
It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Please consult this Guide for Authors for further details of any item.
**Ensure that the following items are present:**
One Author designated as corresponding Author:
• E-mail address
• Full postal address
• Telephone and fax numbers
All necessary files have been uploaded
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Further considerations
• Manuscript has been "spellchecked" and "grammar-checked"
• Manuscript should have numbered pages, and line numbering throughout the text and preferably continuous not starting at 1 on each page.
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
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# APPENDIX B: AUTHOR GUIDELINES "*PHARMACIA*"

**Authors Guidelines**Due to alterations in the editorial policy of Journal **Pharmacia**, the following changes will be applied:

Since March 2019 the Journal will be published only in English, in printed and in electronic version. At the end of the year one printed copy will be submitted to libraries and other public scientific organizations. In order to provide for this, a publication fee will be imposed, since **Pharmacia** does not have any income source like subscription charges or as annual membership charges of author(s).

The published manuscripts will be available online in open access format at **https://pharmacia.pensoft.net/articles**.

## Content and structure of the manuscripts

**Body Text:** All papers should be in grammatically correct English. Authors are asked to certify that their manuscripts are written in fluent English or edited by a professional English language editor prior to submission. Use either British/Commonwealth or American English provided that the language is consistent within the paper. A manuscript must be written with precision, clarity, and economy. The voice - active or passive - and the tense used should be consistent throughout the manuscript. Avoid the use of parenthetical comments and italics or bold for emphasis.

**Spacing, Fonts, and Page Numbering:** Single-space all material (text, quotations, figure legends, tables, references, etc.). Separate paragraphs with a blank line. Use a 12-point font (preferably Times New Roman). All of the manuscripts should be presented in the native format of the word processor used.

The preferred text-processors are: Microsoft Word and Word Perfect. Please do **not** send ASCII files as relevant data may be lost. Include also the files containing computer generated graphics, artwork, bitmaps, and/or scanned images in one of the following formats: CDR, HPGL, WMF, EPS, TIF, PCX and JPG. For large image files, use one of the file compressing programs (ZIP, ARJ, RAR).

The manuscripts should not exceed the following limits:

***Original papers*** – the core text (without the abstract, bibliography, figures and tables) should fit 6-8 pages.

***Review articles*** (without the abstract, bibliography, figures and tables) should not exceed 12 pages.

***Preliminary communications*** – the core text (including the abstract, bibliography, figures and tables) should fit 1-2 pages.

***Short communications (notes)*** – Short communications are published as rapidly as possible. The length of a manuscript is limited to 3 pages (including short summary; subdivisions are not required; the "Experimental" - if there is one - should be marked), up to 15 citations of literature and a maximum of 2 supplementary materials (schemes, figures, tables) are allowed.

## Manuscript design

Due to the double-blind peer review system, you have to submit your manuscript (apart from the letter to the editor and supplementary material) as **two separate files** (for further details, see under Submission Guidelines):

**(i) Cover part (with personal information)**

**Authors and Affiliations:** Provide the complete names of all authors, and their addresses for correspondence, including e.g., institutional affiliation (e.g. university, institute), location (street, boulevard), city, state/province (if applicable), and country. One of the authors should be designated as the corresponding author. It is the corresponding author's responsibility to ensure that the author list and the individual contributions to the study are accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and their affiliations should be listed after the **Acknowledgements section**.

**(ii) Main part (anonymised)**

**The titles** of the submitted papers should be short and informative; double titles should be avoided. The title should be in a sentence case (only scientific, geographic or person names should be with a first capital letter, i.e. *Elater ferrugineus* L., Germany, etc.), and

should include an accurate, clear and concise description of the reported work, avoiding abbreviations.

**Headings and subheadings:** Main headings: The body text should be subdivided into different sections with appropriate headings. Where possible, the following standard headings should be used: **Abstract**, **Introduction**, **Materials and Methods (Experimental Part), Results, Discussion (or Results and Discussion), Conclusion, References.** These headings need to be in bold font on a separate line and start with a first capital letter. Please do not number headings or subheadings.

**Abstract and Keywords:**

**The abstract** of less than 150 words should contain solely the essential results and conclusions of the presented work. Textual formulations from the title should not be repeated and the findings rather than the aim of the work should be described. The abstract should be written in the third person and ready for input into the submission module.

The abstract should be accompanied by 3 to 5 **keywords**, given below the abstract to describe the content of the paper avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. Keywords should be in alphabetical order and ideally differ from the words used in the title.

- **Introduction** − The motivation or purpose of your research should appear in the Introduction, where you state the questions you sought to answer, and then provide some of the historical basis for those questions.
- **Methods** − Provide sufficient information to allow
- to allow someone to repeat your work. A clear description of your experimental design, sampling procedures, and statistical procedures is especially important in papers describing field studies, simulations, or experiments. If you list a product (e.g., animal food, analytical device), supply the name and location of the manufacturer. Give the model number for equipment used. Supply complete citations, including author (or editor), title, year, publisher, and version number, for computer software mentioned in your article. Explanation of the study design (randomization, group formation / stratification, crossover studies) and

experimental conditions; chemicals used; apparatuses and devices indicating the names and domiciles of the manufacturers/suppliers; detailed information about the experimental animals or cell lines along with keeping resp. culture conditions; information about the experiment and methods used (with literature references); detailed description of new methods; explanation of mathematical symbols and formulas; description of the statistical method used (referring to unpublished programs or computer models is not sufficient).

In the case of well known inorganic or organic compounds chemical formulae or common abbreviations may be used (e.g. NaCl, $H_2SO_4$, $CH_3OH$, $C_6H_6$: Ac, Eth, Me, Phe, DMSO) under "Experimental Part". In other parts of the paper this is not desirable.

For all newly synthesized compounds adequate evidence to establish identity and degree of purity must be provided. In general, this evidence should include elemental analyses for carbon, hydrogen and nitrogen (and/or halogen), if present. Supplying high-resolution mass spectral (HRMS) data in lieu of elemental analyses should be avoided whenever possible. HRMS data must always be accompanied with a proof of the degree of purity of the sample.

The following is the recommended style for analytical and spectral data presentation:

*Specific Rotation:*

$[a]^{23}_{D}$ –222 (*c* 0.35, MeOH).

Abbreviations: a = specific rotation; D = the sodium D line or wavelength of light used for determination; the superscript number, temperature (°C) at which the determination was made; In parentheses: *c* stands for concentration; the number following *c* is the concentration in grams per 100 mL; followed by the solvent name or formula.

*NMR Spectroscopy:*

$^1$H NMR (500 MHz, DMSO-$d_6$) d 0.85 (s, 3H, $CH_3$), 1.28–1.65 (m, 8H, 4´$CH_2$), 4.36–4.55 (m, 2H, H-1 and H-2), 7.41 (d, *J* 8.2 Hz, 1H, ArH), 7.76 (dd, *J* 6.0, 8.2 Hz, 1H, H-1'), 8.09 (br s, 1H, NH).

$^{13}C$ NMR (125 MHz, $CDCl_3$) d 12.0, 14.4, 23.7, 26.0, 30.2, 32.5, 40.6 (C-3), 47.4 (C-2'), 79.9, 82.1, 120.0 (C-7), 123.7 (C-5), 126.2 (C-4).

Abbreviations: d = chemical shift in parts per million (ppm) downfield from the standard; *J* = coupling constant in hertz; multiplicities s = singlet; d = doublet; t = triplet; q = quartet; and br = broadened. Detailed peak assignments should not be made unless these are supported by definitive experiments such as isotopic labelling, DEPT, or two-dimensional NMR experiments.

*IR Spectroscopy:*

IR (KBr) n 3236, 2957, 2924, 1666, 1528, 1348, 1097, 743 $cm^{-1}$.

Abbreviation: n = wavenumber of maximum absorption peaks in reciprocal centimetres.

*Mass Spectroscopy:*

MS *m/z* (relative intensity): 305 (M+H, 100), 128 (25).

HRMS–FAB (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{38}N_4O_6$, 442.2791; found, 442.2782.

Abbreviations: *m/z* = mass-to-charge ratio; M = molecular weight of the molecule itself; $M^+$ = molecular ion; HRMS = high-resolution mass spectrometry; FAB = fast atom bombardment.

*UV–Visible Spectroscopy:*

UV ($CH_3OH$) $l_{max}$ (log e) 220 (3.10), 425 nm (3.26).

Abbreviations: $l_{max}$ = wavelength of maximum absorption in nanometres; e = extinction coefficient.

*Quantitative analysis:*

Anal. Calcd for $C_{17}H_{24}N_2O_3$: C 67.08, H 7.95, N 9.20. Found: C 66.82, H 7.83, N 9.16.

All values are given in percentages.

*Melting and boiling points:*

mp 163–165 °C (lit. 166 °C)

mp 180 °C dec.

bp 98 °C

These specific abbreviations, listed above, should be used consequently as well as those described in "**Abbreviations**" part.

Studies in humans or animals must comply with the pertinent internationally valid legal provisions/guidelines. Refer to the obtaining of the approval of an ethics committee (humans/animals). Clinical studies must meet the requirements specified in the Declaration of Helsinki (Somerset West) ("informed consent").

- **Results, Discussion (or Results and Discussion).** Results must be presented precisely using tables and/or figures, if applicable. Avoid duplicate presentation of results in figures and tables.

In this part, no results must be repeated, but the importance of the study should be emphasized and conclusions drawn. The findings may be compared with results from other studies (referring to the respective literature).

- **Conclusion.** This should state clearly the main conclusions of the research and give a clear explanation of their importance and relevance. Summary illustrations may be included.
- **Acknowledgments:** An acknowledgment section (including financial support) may be included. It should be placed after the manuscript text and before the references.
- **References.** The list of References should be included after the final section of the main article body. A blank line should be inserted between single-spaced entries in the list. Authors are requested to include links to online sources of articles, whenever possible!

## Citations and References

**Citations within the text:** Before submitting the manuscript, please check each citation in the text against the References and vice-versa to ensure that they match exactly.

Citations in the text should be formatted as follows:

**One author:** Smith (1990) or (Smith 1990)

*Note:* The citations format depends on the way it is incorporated within the article's text:

*Example:*

1. According to Smith (1990), these findings...
2. These findings have been first reported in the beginning of the nineties (Smith 1990).

**Two authors:** Brock and Gunderson (2001) or (Brock and Gunderson 2001)

*Note:* When choosing between formats refer back to examples above.

**Three or more authors:** Smith et al. (1998) or (Smith et al. 1998)

*Note:* When choosing between formats refer back to examples above.

When **citing more than one source**, in-text citations should be ordered by the year of publication, starting with the earliest one:

(Smith et al. 1998, 2000, 2016; Brock and Gunderson 2001; Felt 2006).

*Note:* When you have a few citations from the same author but from different years (such as the case with Smith et al. above), the first year is taken into consideration when ordering the sources (in this case 1998, which is why Smith et al. come first in the list).

When having **two or more fully identical citations** (this can happen when you have more than one reference with exactly the same authors and years for one or two authors, or the same first author and year for author teams of three or more), the references are distinguished by adding the letters 'a', 'b', 'c', etc. after the years and this marking is followed in the in-text citations, respectively:

(Reyes-Velasco et al. 2018a, 2018b)

Authorship **references for species** should include a "," between author and year:

*Brianmyia stuckenbergi* Woodley, 2012.

**References:** It is important to format the references properly, because all references will be linked electronically as completely as possible to the papers cited. It is desirable to add a DOI (digital object identifier) number for either the full-text or title and abstract of the article as an addition to traditional volume and page numbers. If a DOI is lacking, it is recommended to add a link to any online source of an article.

List all authors cited in the References. For multiauthored papers, give all author names in full; the abbreviation "et al." is only allowed in the text. All journal titles should be spelled out completely and should not be italicized. Ensure that the References are complete and arranged according to name and year of publication. Personal communications and submitted manuscripts should be listed as unpublished results in the text and not listed in the References section.

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Polaszek A, Alonso-Zarazaga M, Bouchet P, Brothers DJ, Evenhuis NL, Krell FT, Lyal CHC, Minelli A, Pyle RL, Robinson N, Thompson FC, van Tol J (2005) ZooBank: The open-access register for zoological taxonomy: Technical Discussion Paper. Bulletin of Zoological Nomenclature 62: 210–220.

**Accepted Papers:**
Same as above, but "in press" appears instead of the year in parentheses.

**Electronic Journal Articles:**
Mallet J, Willmott K (2002) Taxonomy: Renaissance or Tower of Babel? Trends in Ecology and Evolution 18(2): 57–59. https://doi.org/10.1016/S0169-5347(02)00061-7

**Paper within conference proceedings:**

Orr AG (2006) Odonata in Bornean tropical rain forest formations: Diversity, endemicity and applications for conservation management. In: Cordero Rivera A (Ed.) Forest and Dragonflies. Fourth WDA International Symposium of Odonatology, Pontevedra (Spain), July 2005. Pensoft Publishers, Sofia-Moscow, 51–78.

**Book chapters:**

Mayr E (2000) The biological species concept. In: Wheeler QD, Meier R (Eds) Species Concepts and Phylogenetic Theory: A Debate. Columbia University Press, New York, 17–29.

**Books:**

Goix N, Klimaszewski J (2007) Catalogue of Aleocharine Rove Beetles of Canada and Alaska. Pensoft Publishers, Sofia-Moscow, 166 pp.

**Book with institutional author:**

International Commission on Zoological Nomenclature (1999) International code of zoological nomenclature. Fourth Edition. The International Trust for Zoological Nomenclature, London.

**PhD thesis:**

Dalebout ML (2002) Species identity, genetic diversity and molecular systematic relationships among the Ziphiidae (beaked whales). PhD Thesis, University of Auckland, Auckland, New Zealand.

**Link/URL:**

BBC News: Island leopard deemed new species http://news.bbc.co.uk/

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- Morphbank (www.morphbank.net)
- Genbank (www.ncbi.nlm.nih.gov/Genbank)

- BOLD (www.barcodinglife.org)

Providing accession numbers to data records stored in global data aggregators allows us to link your article to established databases, thus integrating it with a broader collection of scientific information. Please hyperlink all accession numbers through the text or list them directly after the References in the online submission manuscript.

All journal titles should be spelled out completely and should **NOT** be italicized.

Provide the publisher's name and location when you cite symposia or conference proceedings; distinguish between the conference date and the publication date if both are given. Do not list abstracts or unpublished material in the References. They should be quoted in the text as personal observations, personal communications, or unpublished data, specifying the exact source, with date if possible. When possible, include URLs for articles available online through library subscription or individual journal subscription, or through large international archives, indexes and aggregators, e.g., PubMedCentral, Scopus, CAB Abstracts, etc. URLs for pdf articles that are posted on personal websites only should be avoided.

Authors are encouraged to cite in the References list the publications of the original descriptions of the taxa treated in their manuscript.

**Ordering references:** All references should be ordered alphabetically by author name (but see below).

If the references have **the same first author and a varying number of co-authors,** the ordering should be based on the number of co-authors starting with the lowest; all articles with the same first author and two or more co-authors (thus cited as et al. in the text) should be listed chronologically, as follows:

Smith J (2018) Article Title. Journal Name 1: 1–10. https://doi.org/10.3897

Smith J, Gunderson A (2017) Article Title. Journal Name 1: 10–20. https://doi.org/10.3897

Smith J, Gunderson A, Brock B (2011) Article Title. Journal Name 1: 20–30. https://doi.org/10.3897

Smith J, Brock B, Gutierrez R, Gunderson A (2013) Article Title. Journal Name 1: 15–30. https://doi.org/10.3897

Smith J, Brock B, Gunderson A (2015) Article Title. Journal Name 1: 10–30. https://doi.org/10.3897

If both **the first author and year of publication match** within the categories above, the references are distinguished by adding the letters 'a', 'b', 'c', etc. after the year of publication and this marking is followed in the in-text citations, respectively.

### Illustrations, Figures and Tables

**Figures and illustrations** are accepted in the following image file formats:

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- **PNG** (preferred format for photos or images)
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- **GIF**
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Should you have any problems in providing the figures in one of the above formats, or in reducing the **file below 20 MB**, please contact the Editorial Office at journals@pensoft.net.

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Illustrations of measurable morphological traits should bear mute scale bars, whose real size is to be given in the figure captions.

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For nomenclature of peptides, see *Neuropeptides*, Vol. 1, 1981, p. 231.

The nomenclature of receptors and their subtypes should conform to the *TIPS 1995 Receptor & Ion Channel Nomenclature Supplement (Trends Pharmacol. Sci.* Receptor Nomenclature Supplement 1995).

The trivial name of the enzyme may be used in the text, but the systematic name and classification number according to *Enzyme Nomenclature*, rev. edn. (Academic Press, New York, NY, 1984) should be quoted the first time the enzyme is mentioned.

**Units of measurement.** Units of measurement are determined by the directions of the International Units System SI as symbols; M instead of mol/l or mol * l-1 is allowed.

**Botanical names.** Botanical names (species, genus) should be marked in italics.

**Abbreviations.** The following abbreviations should be used consequently (except in the title and all subtitles). All other abbreviations have to be explained in the manuscript at first usage, if aforementioned directions are not applicable. Abs. = absolute: anh. = anhydrous; b.p.; b.r. = boiling point, -range; calcd. = calculated; CC = column chromatography; conc. = concentrated; dec. = decomposition , eq. = equation; Fig. = figure; GC = gas chromatography, - chromatogram, HPLC = high performance liquid chromatography, - chromatogram; i.m. = intramuscular; i.p. = intraperitoneal; IR = infrared; i.v. = intravenous; lit. = literature value; m.p.; m.r. = melting point, -range; MS = mass spectometry, mass spectrum; NMR = nuclear magnetic resonance spectrum; PC = paper chromatography, - chromatogram, % = per cent, percentage, p.o. = peroral; s.c. = subcutaneous; TLC = thin

layer chromatography, - chromatogram; UV = ultraviolet; ADP, CDP, GDP, IDP, UDP = 5'-pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine; AMP etc. = adenosine 5'-monophosphate etc.; ADP etc. = adenosine 5'-diphosphate etc.; ATP etc. = adenosine 5'-triphosphate etc.; CM-cellulose = carboxymethylcellulose; CoA and acetyl-CoA = coenzyme A and its acyl derivatives; DNA = deoxyribonucleic acid; EGTA = ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; FAD = flavin-adenine dinucleotide; FMN = flavin mononucleotide; NAD = nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate; NMN = nicotinamide mononucleotide; RNA = ribonucleic acid; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol.

**Correspondence.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

**Tables:** Each table should be numbered in sequence using Arabic numerals (i.e. Table 1, 2, 3 etc.). Tables should also have a title that summarizes the whole table, maximum 15 words. Detailed legends may then follow, but should be concise.

Small tables can be embedded within the text, in portrait format (note that tables on a landscape page must be reformatted onto a portrait page or submitted as additional files). These will be typeset and displayed in the final published form of the article. Such tables should be formatted using the 'Table object' in a word processing program to ensure that columns of data are kept aligned when the file is sent electronically for review. Do not use tabs to format tables or separate text. All columns and rows should be visible, please make sure that borders of each cell display as black lines. Colour and shading should not be used; neither should commas be used to indicate decimal values. Please use a full stop to denote decimal values (i.e., 0.007 cm, 0.7 mm).

Larger datasets can be uploaded separately as Supplementary Files. Tabular data provided as supplementary files can be uploaded as an Excel spreadsheet (.xls), as an OpenOffice spreadsheets (.ods) or comma separated values file (.csv). As with all uploaded files, please use the standard file extensions.

Tables and diagrams should be designed in a fashion that enables their understanding without referring to the text. Presentation of the same results in figures and tables will not be accepted.

## Supplementary Files

Online publishing allows an author to provide datasets, tables, video files, or other information as supplementary information, greatly increasing the impact of the submission. Uploading of such files is possible in Step 6 of the submission process.

The maximum file size for each Supplementary File is 20 MB.

The Supplementary Files will not be displayed in the printed version of the article but will exist as linkable supplementary downloadable files in the online version.

While submitting a supplementary file the following information should be completed:

- File format (including name and a URL of an appropriate viewer if format is unusual)
- Title of data
- Description of data

All supplementary files should be referenced explicitly by file name within the body of the article, e.g. 'See supplementary file 1: Movie 1" for the original data used to perform this analysis.

Ideally, the supplementary files should not be platform-specific, and should be viewable using free or widely available tools. Suitable file formats are:

For supplementary documentation:

- **PDF** (Adobe Acrobat)

For animations:

- **SWF** (Shockwave Flash)

For movies:

- **MOV** (QuickTime)
- **MPG** (MPEG)

For datasets:

- **XLS** (Excel spreadsheet)
- **CSV** (Comma separated values)
- **ODS** (OpenOffice spreadsheets)

As for images, file names should be given in the standard file extensions. This is especially important for Macintosh users, since the Mac OS does not enforce the use of standard file extensions. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet).

## Revising Your Article

Authors **must** submit the revised version of the manuscript using Track Changes/Comments tools of Word so that the Subject Editor can see the corrections and additions.

Authors **must** address all critiques of the referees in a response letter to the editor and submit it along with the revised manuscript through the online editorial system. In case a response letter is not submitted by the authors, the editor has the right to reject the manuscript without further evaluation. When resubmitting a manuscript that has been previously rejected with resubmission encouraged, authors **must** include the response letter to the article text file, and the pdf review version, so that it gets to the Subject Editor and the reviewers during the peer review.

When submitting corrections to proofs (during the layout stage), authors **must** upload the latest proof (in PDF format) containing their revisions as track changes.

## Concise Copyediting Instructions

The copyediting instructions below represent a concise summary of the journal's formatting requirements. The instructions are intended for use by the authors during preparation of the final revised versions of their manuscripts, technical editors, copy editors and typesetters.

## Author names

- Omit titles, degrees, etc.
- Provide ORCID if available

## Affiliation

(Department,) Institution, City, Country

## Article title

Title of article: Subtitle of article

- Title: Sentence case
- Colon between title and subtitle (if any)
- No footnotes
- No bold (use when needed sub-/superscript, and/or italics only for the terms in Latin)
- Higher taxa within the title should be separated with commas and **not** with a semicolon

## Running head

- A short version of title up to 50 characters (including spaces); normally the short title should have been suggested by the authors and checked for clarity by the copy editor

## Abstract

- No references to tables, figures, etc., no footnotes
- No citations (preferably)
    - If citations unavoidable: Complete citations, allowing unambiguous identification of cited publication!
- Should be written consistently in either third or first person
- Note: The abstract has to be a stand-alone entity, to present a really well written and concise summary of the article! A special care for copy editors to check!

- Designations of nomenclatural novelties should be in bold and spelled in the way suggested (**sp. nov., gen. nov., comb. nov.**)

### Keywords (up to 8 words)

keyword a, keyword b, keyword n

- Do not repeat words from the title
- Listed in *alphabetical order* and separated by commas
- Lowercase letters, except proper names
- No bold font
- Without any punctuation marks after last keyword

### Tables

- Table caption: Start with label "Table N." in bold. Sentence case, i.e.:
    - **Table 2.** Table caption text.
- Numbered consecutively with Arabic numerals
- Heading for every column (including the leftmost!)
- No shading of cells, rows, columns; no colored fonts
- No horizontal or vertical lines in table body
- Same number of decimal places for same statistics (usually within same column)
- Text formatting in the cell without paragraph and line break
- Table must be in an editable format (.docx, .xlsx, etc., not as images)
- Caption and footnotes as texts (not as part of a table)

### Figures

- Figure caption: Start with label "Figure N." in bold. Sentence case, i.e.:
    - **Figure 6.** Figure caption text.
- Numbered consecutively with Arabic numerals
- Figure parts: Use capital letters in bold. No punctuation separator, i.e.:
    - **Figure 1.** Figure general caption text. **A** part caption text **B** part caption text **N** part caption text.
- If abbreviations are used, these are placed after the parts with a colon, i.e.: Abbreviations: xxxx

- If there are scale bars on the figure parts, reference to them is last and in the format: Scale bars: 20 µm (**D, N, O, Q**); 50 µm (**F, K**); 10 µm (**G, P**); 5 µm (**H**); 100 µm (**M**).
- High quality (at least 300 dpi)
- Text sharp and readable (e.g., no overlap of text and graphical elements like lines)
- White or transparent background
- No image border
- Caption as text (not as part of the image)

## Capitalization

- Article title: Sentence case
- Running head: Sentence case
- Section and subsection titles:
    - For separated titles (usually H1-H3): Sentence case
    - For paragraph titles (usually H4): Sentence case
- Table captions: Sentence case
- Headings of table rows and columns:
    - Sentence case or lower case (check for consistency only!)
- Figure captions: Sentence case
- In text body: Nouns followed by numerals/letters (citations of figures, tables, appendices and supplementary files) e.g.:
    - Fig. 4; Figs 1, 2; Table 2; Appendix 1
- In text body: Titles of articles, book chapters, books, tests
- In references: Sentence case

## Equations and statistical symbols

- Typeface
    - standard typeface for Greek letters, sub-/superscripts, and abbreviations that are not variables
    - italic typeface for all other statistical symbols
- Space before and after equal/inequality signs
- Same number of decimal places for decimal values

- Omit the zero before a decimal fraction, when the statistic cannot exceed 1, e.g., $p = .34$
    - Alternative A: Omit the zero before a decimal fraction only for the following statistics: $p$, $r$, $R$ (and $R^2$), α (Cronbach's α), $\eta^2$ (Eta-Square, also $\eta_p^2$) .
    - Alternative B: If zero is omitted before a decimal fraction, this should be done consistently for the respective statistic.
- Standard formats for common statistics, e.g., $t(23) = 3.51$, $p = .002$
    - commas (not semicolons!) between test statistics and *p* values
    - exact *p* values, if *p* not less than .001

## Text body

- **Regular font usage:**
    - Main text
    - Abbreviations e.g., i.e., et al., etc., cf., vs.
    - Greek letter e.g., α, β, γ, δ, ε, σ, φ, χ, ω
- **Italic font usage:**
    - Scientific names of taxa of species and genera (authorities in regular font, not in italics)
    - Long direct quotations
    - Symbols for variables and constants, such as *p*, *F*, *U*, *T*, *N*, *r*, but not for SD (standard deviation), SE (standard error), DF (degrees of freedom), and NS (non significant). These symbols in illustrations and equations should be in italics to match the text.
    - Do **not** use italics for emphasis
- **No underlining**
- **Bold font usage:**
    - Subheadings, sections and subsections
    - Figure captions – For the label and designation of figure's parts:
        - **Figure 1.** Figure general caption text. **A** part caption text **B** part caption text **N** part caption text.
    - Table captions – For the label:
        - **Table 1.** Table caption text.
    - In systematic sections for specimen designation such us: **holotype**, **paratype, syntype**, **lectotype**, **isotype**, etc.

- Abbreviations of institutions or morphological characters or indices listed alphabetically in the section Materials and methods, i.e.:
  - **NHML** Natural History Museum, London
  - **MW** Naturhistorisches Museum, Vienna
  - **EL** length of elytra
  - **EW** maximum width of elytra
  - **TL** total length (PL+EL)
- In species descriptions – designation of main anatomical structures followed by a colon mark, i.e. **Head:**..., **Thorax:**..., **Legs:**..., **Abdomen:**..., etc., in this case these should be followed by a section describing other anatomical organs and structures attached to these.
- Subsection "Specimens examined" - the preferred order is as follows, HOWEVER THESE FINE-GRAINED FORMATTING GUIDELINES ARE NOT COMPULSORY. Authors who follow the guidelines will benefit from the submission of their specimen records to GBIF after publication. The records on GBIF will bear the article citation details contributiing to a wider dissemination and re-use of the published data.
  - COUNTRY • specimens [e.g. 1 ♂, size ]; geographic/locality data [from largest to smallest]; coordinates; altitude/elevation/depth [using alt./m a.s.l. etc.]; date [format: 16 Jan. 1998]; collector [followed by "leg."]; other collecting data [e.g. micro habitat/host/method of collecting]; barcodes/identifiers [e.g. GenBank: MG779236]; institution code and specimen code [e.g. CBF 06023].
    For Example: **Holotype:** CHINA • ♀; Sichuan, Kangding; 30.04°N, 101.57°E; 15.VI.2017; Yanzhou Zhang leg.; Hyp-2018-06, original number ZYZ-2017-28. **Paratypes:** CHINA • 1♀1♂; Sichuan, Kangding; 29.VI.2017; Yanzhou Zhang leg.; Hyp-2018-01, Hyp-2018-02, original number ZYZ-2017-08 • 1♀; Sichuan: Kangding; 2.VIII.2017; Yanzhou Zhang leg.; Hyp-2018-03, original number ZYZ-2017-20 • 1♂, Sichuan: Kangding; 29.VI.2017; Yanzhou Zhang leg.; Hyp-2018-08, original number ZYZ-2017-029.
  - Punctuation:
    A bullet point "•" (unicode: 2022) is used to signify the beginning of a material citation. Within each citation, the different fields are

delimited by a semicolon. A single field can be composed of several elements, which are separated by commas (e.g. the details region, area, town and street for the 'locality' field). Semicolons should not be used elsewhere in a material citation.

        - Repetitive data: Authors can indicate repetitive data with indications such as "same data as for holotype", "same data as for preceding", "same locality", "ibid", etc. as long as the same method and wording are used consistently throughout the paper.
        - 'Missing' elements: It is not necessary to include information such as "no date" or "no locality data"; just list the elements that are available.
        - see more details here

- **Quotation marks**
    - Avoid quotation marks except for direct quotations, words defined by the author, and words used in unusual contexts.
    - Short quotations should be embedded in the text and enclosed in double quotation marks ("). Long quotations should be on a separate line, italicized, but without quotation marks.
    - Single quotation marks are to be used only for a quotation that occurs within another quotation.
- **Hyphen and dash characters**
    - Consistent use of (-, –, —).
    - In contrast to parentheses an em-dash can be used alone.
    - En-dashes and em-dashes should not be spaced.
        - Hyphens (-) are used to:
            - link words such as personal names, some prefixes and compound adjectives (the last of which vary depending on the style manual in use)
        - En-dash (–) or en-rule (the length of an 'n') is used to:
            - link spans.
            - link numerals, sizes, dates and page numbers (e.g., 1977–1981; figs 5–7; pp. 237–258)
            - geographic or name associations (e.g., Murray–Darling River; a Federal–State agreement)

        - character states combinations (e.g., long–pubescent or red–purple).
    - Em-dash (—) or em-rule (the length of an 'm') should be used rarely:
        - only for introducing a subordinate clause in the text that is often used much as we use parentheses.

### Section hierarchy

- No more than 4 levels, from hierarchical level 1 (H1) to hierarchical level 4 (H4)
- Unambiguous hierarchy levels
- No numbering of hierarchical levels

### Section titles

- Capitalization:
    - For separated titles (usually H1-H3): Sentence case
    - For paragraph titles (usually H4): Sentence case

### Mandatory statements

- Funding
    - If missing, add the following statement (depending on the number of authors):
        - The author has no funding to report.
        - The authors have no funding to report.
- Competing interests
    - If missing, add the following statement (depending on the number of authors):
        - The author has declared that no competing interests exist.
        - The authors have declared that no competing interests exist.
- Acknowledgments (= non-financial support)
    - If missing, add the following statement (depending on the number of authors):
        - The author has no support to report.
        - The authors have no support to report.
- Data Resources (mandatory for empirical articles)

## Geographical coordinates

One of the following formats should be used:

- Degrees, Minutes and Seconds (DMS), i.e.:
  - 36°31'21"N; 114°09'50"W
- Degrees and Decimal Minutes (DDM), i.e.:
  - 36°31.46'N; 114°09.84'W
- Decimal Degrees (DD), i.e.:
  - 36.5243°S; 114.1641°W
  - −36.5243; −114.1641 (using minus to indicate southern and western hemispheres)

## In-Text Citations

- **References**
  - 1-2 authors
    - Jackson and Miller (2012) found out that...
    - A recent study (Jackson and Miller 2012) confirmed that...
  - 3 or more authors
    - Jackson et al. (2012) found out that...
    - A recent study (Jackson et al. 2012) confirmed that...
  - Multiple sources in chronological order:
    - same authors different years - separated by a comma:
      - Jackson and Miller (2012, 2015) found out that...
      - Recent studies (Jackson et al. 2012, 2015) confirmed that...
    - different authors - separated by a semicolon:
      - (Smith et al. 1998, 2000, 2016; Brock and Gunderson 2001; Felt 2006)
    - two or more fully identical citations (the same authors and years) are distinguished by adding the letters 'a', 'b', 'c', etc. after the year:
      - Jackson 2008a, 2008b
      - Jackson and Miller 2014a, 2014b
      - Reyes-Velasco et al. 2018a, 2018b
  - Sources with page numbers

        - Jackson and Miller (2012: 120–121) found out that
        - A recent study (Jackson and Miller 2012: 120) confirmed that
- **Figures:**
    - Fig. 1
    - Fig. 1A, B
    - Fig. 1A–D
    - Figs 1, 2
    - Figs 1–3
    - Figs 1A, B, 3F, G, 7A
- **Tables:**
    - Table 1
    - Tables 1, 2
    - Tables 1–3
- **Appendixes:**
    - Appendix 1
    - Appendices 1, 2
    - Appendices 1–4
- **Referenced materials from other sources:**
    - All figures, tables, etc., from other sources should be written with small letters i.e.: see fig. 2 in Author (Year) …

## References

- Author names: surname first; all given names abbreviated, no full stops, commas or spaces, i.e.:
    - Lyal CHC
    - van Tol J
    - de Albuquerque PRA
- Different authors separated by comma
- Year in brackets; no comma or full stop after it
- No italics (except for Latin terms)

**Published papers:**

Polaszek A, Alonso-Zarazaga M, Bouchet P, Brothers DJ, Evenhuis NL, Krell FT, Lyal CHC, Minelli A, Pyle RL, Robinson N, Thompson FC, van Tol J (2005) ZooBank: The open-access register for zoological taxonomy: Technical Discussion Paper. Bulletin of Zoological Nomenclature 62: 210–220.

**Accepted papers:**

Same as above, but "in press" appears instead of the year in parentheses.

**Electronic journal articles:**

Mallet J, Willmott K (2002) Taxonomy: Renaissance or Tower of Babel? Trends in Ecology and Evolution 18(2): 57–59. https://doi.org/10.1016/S0169-5347(02)00061-7

**Paper within conference proceedings:**

Orr AG (2006) Odonata in Bornean tropical rain forest formations: Diversity, endemicity and applications for conservation management. In: Cordero Rivera A (Ed.) Forest and Dragonflies. Fourth WDA International Symposium of Odonatology, Pontevedra (Spain), July 2005. Pensoft Publishers, Sofia-Moscow, 51–78.

**Book chapters:**

Mayr E (2000) The biological species concept. In: Wheeler QD, Meier R (Eds) Species concepts and phylogenetic theory: A debate. Columbia University Press, New York, 17–29.

**Books:**

Goix N, Klimaszewski J (2007) Catalogue of Aleocharine Rove Beetles of Canada and Alaska. Pensoft Publishers, Sofia-Moscow, 166 pp.

**Book with institutional author:**

ICZN [International Commission on Zoological Nomenclature] (1999) International code of zoological nomenclature. Fourth Edition. The International Trust for Zoological Nomenclature, London.

**PhD thesis:**

Dalebout ML (2002) Species identity, genetic diversity and molecular systematic relationships among the Ziphiidae (beaked whales). PhD Thesis, University of Auckland, Auckland, ## pp.

**Link/URL:**

BBC News (2012) Island leopard deemed new

# APPENDIX C: SUPPLEMENTARY DATA FOR CHAPTER 4

Supplementary Data

## Seasonal chemical variation and antidiabetic activity of major compounds in *Artemisia afra* infusions

Michelle Rose-Marie Stevens, Suzanne Elaine van Niekerk, Josias Hendrik Hamman, Frank van der Kooy*

Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa

*Corresponding author.

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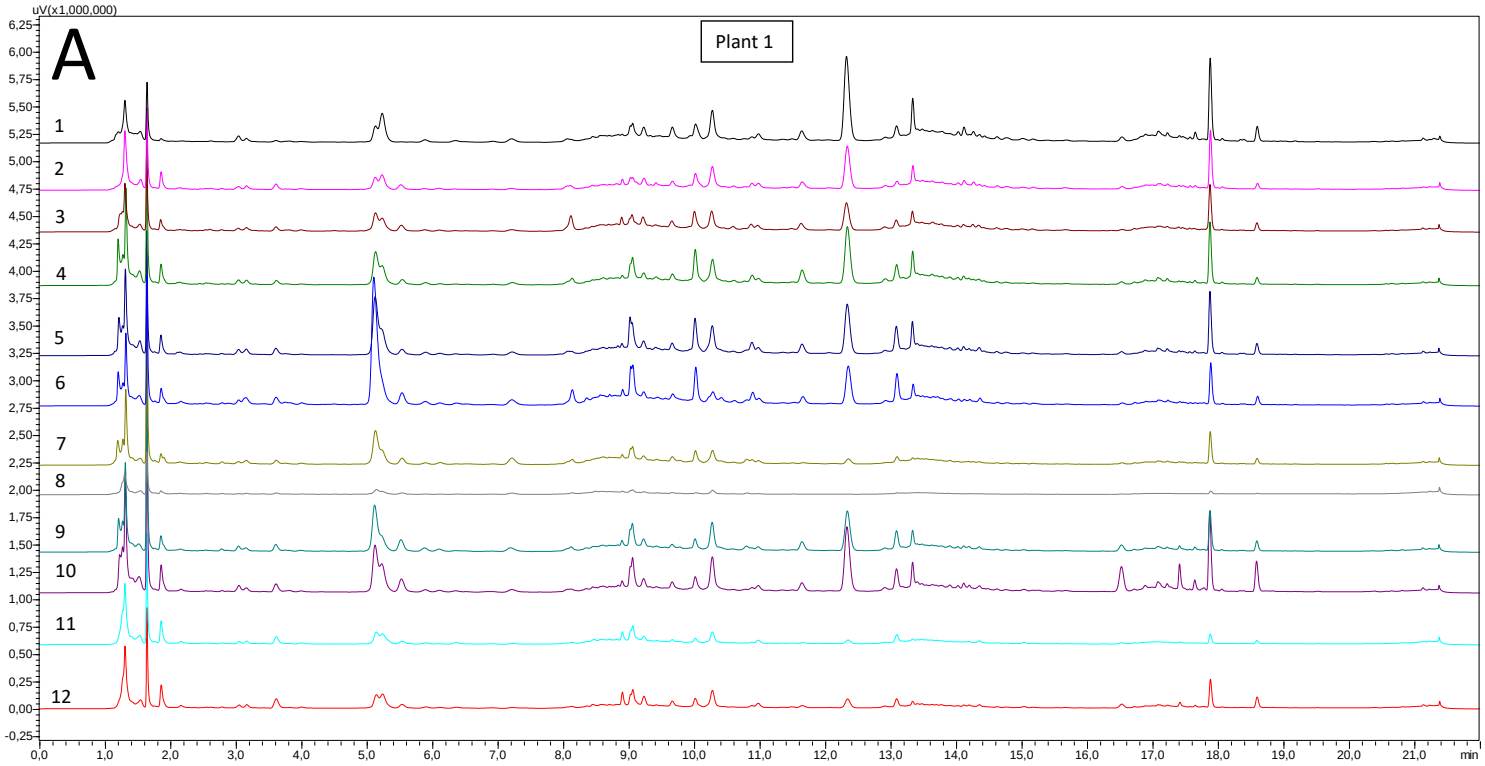
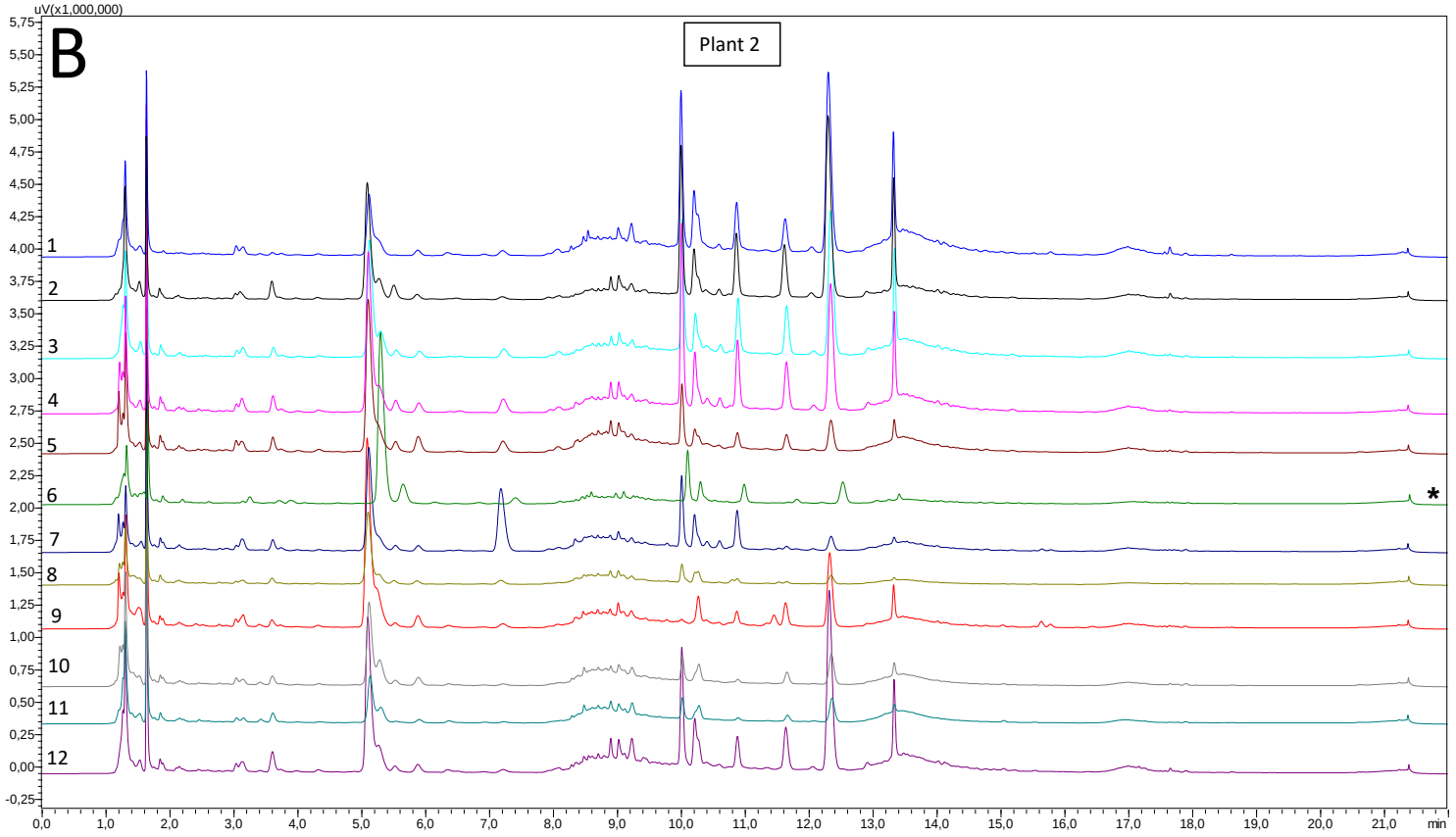
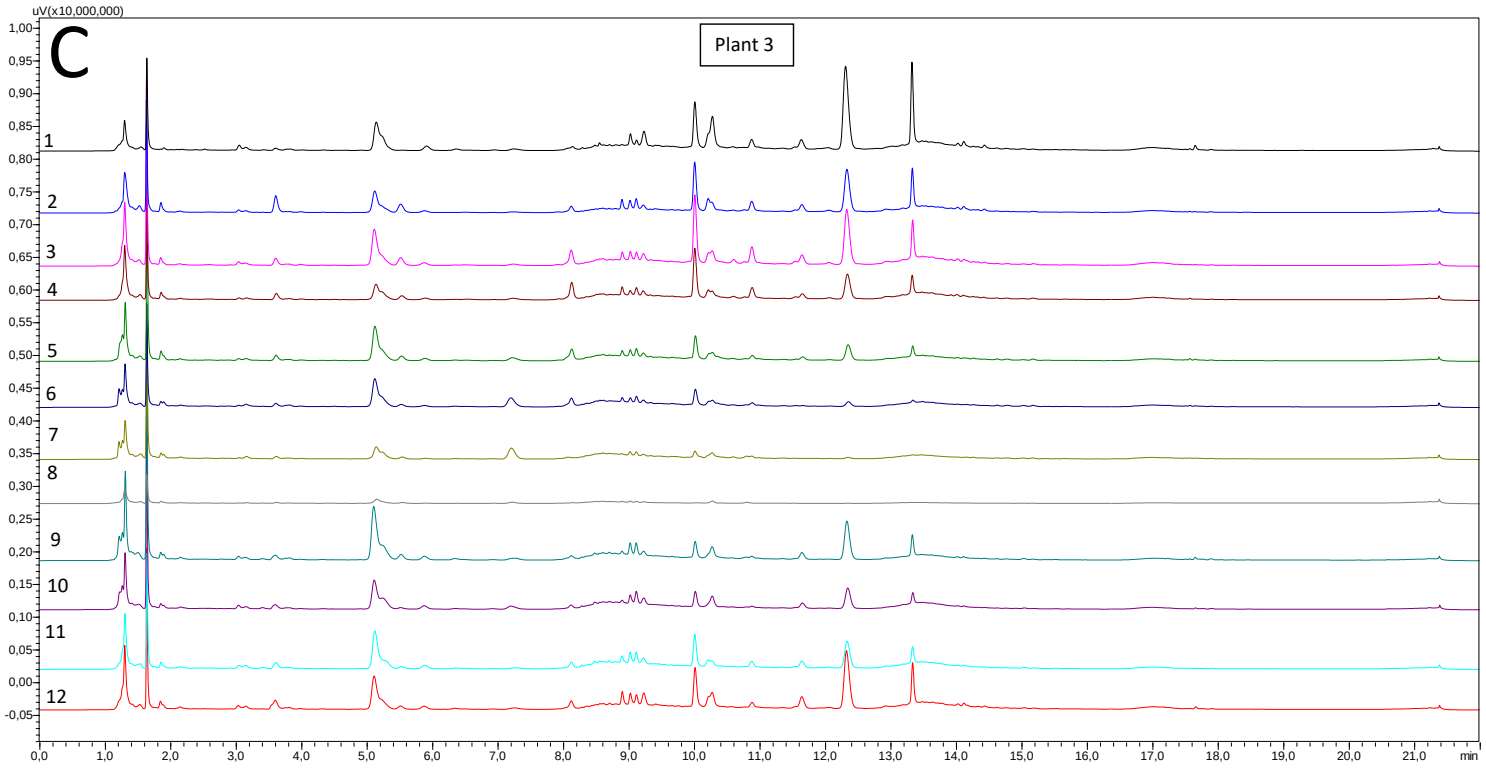
## Contents

**Fig 1S:** Overlayed UPLC Chromatographs of the 4 *A. Afra* aqueous extracts, measured at 254nm, and ranging over a 12-month period starting from January **(1)** down to December **(12)**. Each chromatogram represents the following: Plant 1 **(A)** Plant 2 **(B)** Plant 3 **(C)** Plant 4 **(D)**.




*Slight shift in retention time due to the sample being run on a later date.



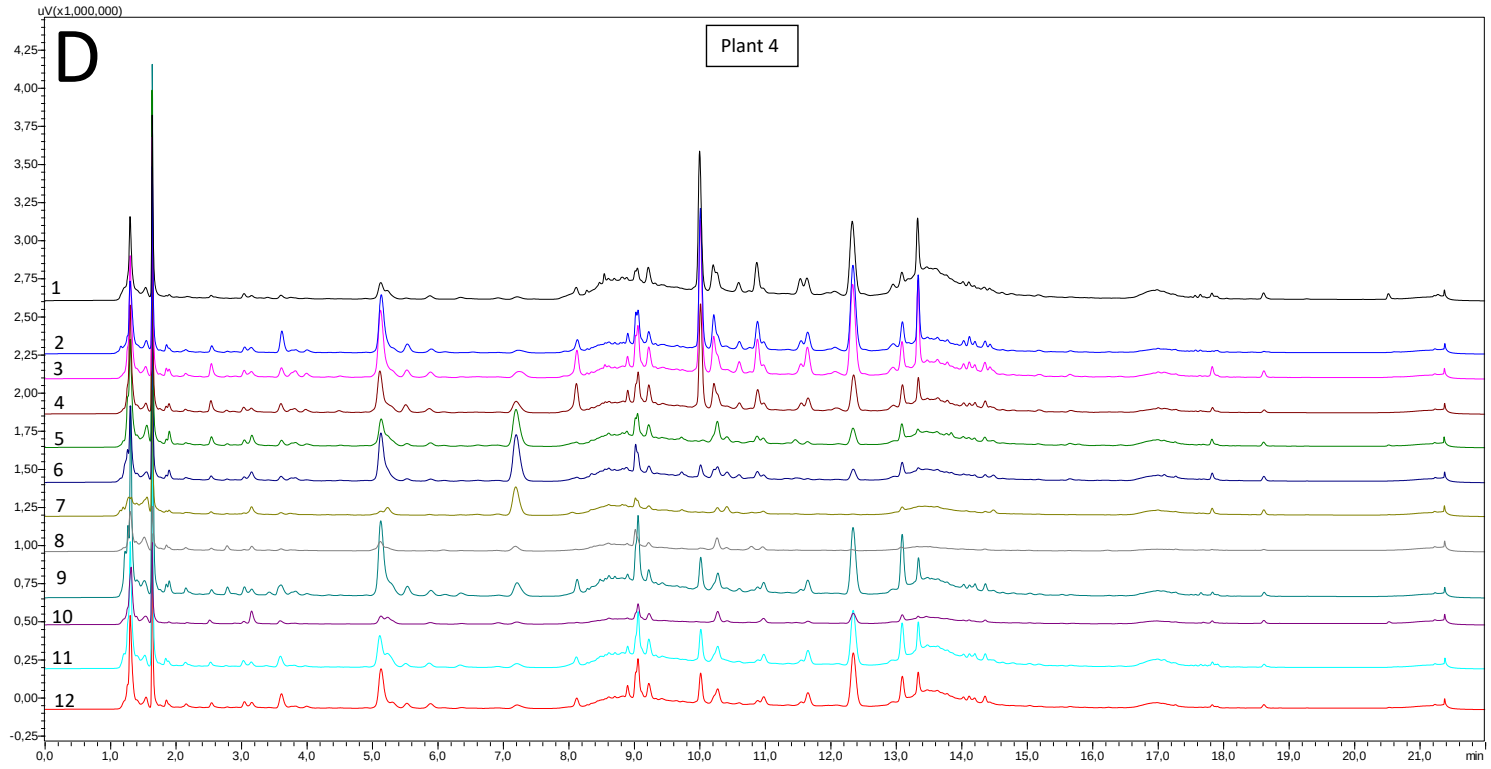




**Fig 1S:** Overlayed UPLC Chromatographs of the 4 *A. Afra* aqueous extracts, measured at 254nm, and ranging over a 12-month period starting from January **(1)** down to December **(12)**. Each chromatogram represents the following: Plant 1 **(A)** Plant 2 **(B)** Plant 3 **(C)** Plant 4 **(D)**

# APPENDIX D: ETHICAL APPROVAL CERTIFICATE

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za

**North-West University Health Research Ethics Committee (NWU-HREC)**

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

26 July 2021

## RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 26/07/2021, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

**Study title: Phytochemical analysis and *in vitro* anti-diabetic activity of selected South African medicinal plants traditionally used to treat diabetes mellitus**
**Principal Investigator/Study Supervisor/Researcher: Prof F van der Kooy**
**Student: M Stevens - 28463269**

**Ethics number**:

| N | W | U | - | 0 | 0 | 2 | 2 | 8 | - | 2 | 1 | - | A | 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Institution | | | | Study Number | | | | | | Year | | | Status | |

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

**Application Type: Single study**
**Commencement date: 26/07/2021**

**Risk:** No Risk

**General conditions:**

*The following general terms and conditions will apply:*

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC 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;*
  - *withdraw or postpone clearance 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-HREC or that information has been false or misrepresented;*
    - *submission of 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-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

**Special conditions of the research approval due to the COVID-19 pandemic:**

**Please note:** Due to the nature of the study i.e. (laboratory work involving the *in vitro* analysis of the antidiabetic activity of certain medicinal plants), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

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

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2021.07.26 20:58:14 +02'00'

NWU-HREC Chairperson

Digitally signed by Gordon Wayne Towers
Date: 2021.07.26 16:12:59 +02'00'

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

Current details: (13210572) G:\My Drive\My Documents 20190227\NWU-HREC\NWU-HREC_Applications\NWU-HREC_Applications-2021\NWU-HREC_App06-20210730\NWU-00228-21-S1(F van der Kooy-M Stevens)-NR\NWU-00228-21-S1(F van der Kooy-M Stevens)-LoD\9.1.5.4.3_LOD_NWU-00228-21-A1_20210708.docm
7 July 2021

File reference: 9.1.5.4.3