Antidiabetic, antioxidant activities and metabolomics profile of *Amaranthus caudatus* and *Amaranthus hypochondriacus*

by

NIEL DE VILLIERS SNYMAN

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SUPERVISOR: DR. N. NKOBOLE

CO-SUPERVISOR: PROF. G. PRINSLOO

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Declaration

Name: Niel de villiers Snyman

Student number: 61990175

Degree: MSc in Agriculture

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I declare that the above dissertation is my own work and that all the sources that I have used have been indicated and acknowledged by means of complete references.

I further declare that I have not previously submitted this work, or part of it, for examination at UNISA for another qualification or at any other higher education institution.

12 May 2025

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LIST OF ACRONYMS

ABTS	2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AC	*Amaranthus caudatus*
AHG	*Amaranthus hypochondriacus* green
AHR	*Amaranthus hypochondriacus* red
ARC-VIMP	Agriculture Research Council Vegetable Industrial and Medicinal Plants
BCAA	Branched-chain amino acids
^{13}C-NMR	C-13 nuclear magnetic resonance spectroscopy
CE-EMS	Capillary electrophoresis-mass spectroscopy
CD_3OD	Deuterated methanol
D_2O	Deuterium oxide
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNS	3,5-dinitrosalicylic acid
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectroscopy
HCA	Hydroxycinnamic acid
HMDB	Human Metabolome Database
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
KH_2PO_4	Potassium dihydrogen phosphate
LAN	Limestone ammonium nitrate
LC-MS	Liquid chromatography-mass spectroscopy
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
MVDA	Multivariate data analysis
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
p-NPG	p-nitrophenyl α-D-glucopyranoside
PAA	Phenylacetic acid

PCA	Principal component analysis
PLA	Phenylalanine
T2DM	Type-2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TSP	Trimethylsilylpropionic acid sodium salt
VA	Vanillic acid

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GENERAL ABSTRACT

Diabetes mellitus is a chronic disorder caused by efficient insulin utilization by the body. Diabetes mellitus is one of the most common underlying causes of high mortality rates among adults and children in the world. Diabetes can be treated by using oral antidiabetic medicines. The main aim of this study was to to assess and compare the metabolomic profiles and antidiabetic activities of *Amaranthus caudatus* and *A. hypochondriacus*.

In this study, proton nuclear magnetic resonance (^{1}H-NMR) was used to chemically profile the untargeted metabolites of *Amaranthus caudatus* and *A. hypochondriacus* leaves. Thirty-one (31) compounds were reported, with most of the annotated compounds being similar across both *Amaranthus* species. Alanine, leucine, trehalose, trigonelline, and chlorogenic acid, to name just a few, were annotated in both *Amaranthus* species. Trigonelline has been found to be high in concentration in *A. caudatus* and low in concentration in *A. hypochondriacus*. Another compound that was annotated in this study, chlorogenic acid (CA), was higher in *A. caudatus* than in *A. hypochondriacus*.

The antidiabetic activity of this study demonstrated that methanol extracts of both *A. caudatus* and *A. hypochondriacus* leaves had moderate α-glucosidase inhibitory activity. According to the IC_{50} values of α-glucosidase, acarbose (positive control) had an IC_{50} value of 1.274 mg/mL, which was lower than any of the plant extract values. The IC_{50} value of the plant extracts against α-glucosidase ranged from 6.71 mg/mL *(A. hypochondriacus* green variety*)* to 8.39 mg/mL (*A. caudatus*), respectively. Only the *A. hypochondriacus* (green variety) extract showed activity against α-amylase with the IC_{50} value of 4.32 mg/mL, which was higher than acarbose (positive control), which demonstrated an IC_{50} value of 0.23 mg/mL.

This study demonstrated that *A. caudatus* and the two varieties of *A. hypochondriacus, A. hypochondriacus* red (AHR) and *A. hypochondriacus* green (AHG*),* respectively, have considerable antioxidant activity as evidenced by the ABTS radical scavenging assay. The methanol extracts showed dose-dependent inhibition of free radicals. The IC_{50} values of *A. caudatus, A. hypochondriacus* red variety and *A. hypochondriacus*

green variety were 0.06 mg/mL, 0.03 mg/mL and 0.04 mg/mL, respectively, when tested using the ABTS radical scavenging assay. Vitamin C (positive control) showed an IC_{50} value of 5.5 µg/mL.

Keywords: Amaranth, nuclear magnetic resonance spectroscopy, antidiabetic activity, α-amylase, α-glucosidase and antioxidant activity.

CHAPTER 1: INTRODUCTION

1.1. Background:

Diabetes mellitus (DM) is derived from the Greek word *diabetes*, meaning siphon—to pass through—and the Latin word *mellitus,* meaning sweet. DM is a chronic disorder caused by inefficient insulin utilization by the body (Global Burden of Disease Collaborative Network, 2020). Insulin is a hormone that modulates blood glucose concentrations. Hyperglycemia, also known as rising blood glucose or raised blood sugar, is a common outcome of untreated diabetes that, over time, leads to catastrophic damage to many of the body's systems, including neurons and blood vessels (Aamir *et al*., 2022). Diabetes is classified into two types: type 1 and type 2.

According to the Global Burden of Disease Collaborative Network (2020), between 2000 and 2019, there was a 3% increase in age-standardized mortality rates from diabetes. In lower-middle-income countries, the mortality rate due to diabetes increased by 13%. In 2021, diabetes affected an estimated 537 million people globally, or 10.5% of the adult population. Type 2 diabetes accounts for approximately 90% of all cases (Sun *et al*., 2022).

Type 1 diabetes:

Type 1 diabetes, also known as insulin-dependent diabetes or juvenile-onset diabetes, occurs when the body's immune system attacks the pancreas' insulin- producing cells, destroying more than 90% of them permanently. The pancreas, thus, produces little or no insulin (Attia *et al*., 2023). About 5 to 10% of all people with diabetes have type 1 disease (Brutsaert, 2022). Most people who have type 1 diabetes develop the disease before the age of 30, but it can develop later in life too (Brutsaert, 2022). Scientists believe that there is an environmental factor—possibly a viral infection or a nutritional factor during childhood or early adulthood that causes the immune system to destroy the insulin-producing cells of the pancreas (Brutsaert, 2022).

Type 2 diabetes:

In type 2 diabetes (also referred to as non-insulin-dependent diabetes or adult-onset diabetes), the pancreas often continues to produce insulin, sometimes even at higher-than-normal levels, especially if the disease is still in its earliest stages (Buzzetti *et al*., 2022). However, the body develops resistance to the effects of insulin, resulting in an insufficient supply of insulin to adequately fulfil the body's metabolic demands. As type 2 diabetes progresses, the insulin-producing ability of the pancreas decreases. Type 2 diabetes was once rare in children and adolescents but has recently become more common among them (Brutsaert, 2022). However, it usually begins in people older than 30 and becomes progressively more common with age (Brutsaert, 2022). About 26% of people older than 65 have type 2 diabetes (Brutsaert, 2022).

Less known types of diabetes mellitus are gestational diabetes, which develops during pregnancy and typically resolves after giving birth (Sweeting *et al*., 2022). Other variants are known as maturity-onset diabetes of the young (MODY) and neonatal diabetes (Deutschlander, 2010). For the sake of this study, the focus will only be on type 2 diabetes.

Currently, there is no cure for diabetes, and it has long-term complications like kidney disease, kidney failure, neuropathy, gangrene, cataracts, and retinopathy that may lead to blindness (Deutschlander, 2010). However, screening tests can be done to measure the glucose level in the blood. This will determine people who are at risk of diabetes but are showing no symptoms of it (Brutsaert, 2022).

Studies reveal that fruit and vegetables consist of nutraceuticals such as vitamins, minerals, and dietary fibres that can help combat diabetes (Kaur and Kapoor, 2001; Whitman, 2001; Asif, 2011; Ley *et al*., 2014). Nutraceuticals are a broad term used to describe any product derived from food sources with extra health benefits in addition to the basic nutritional value found in foods (Mestrovic, 2022). As depicted in figure 1.1 below, the term "nutraceutical" combines the two words "nutrient," which is a nourishing food component, and "pharmaceutical," which is a medical drug (Mestrovic, 2022).

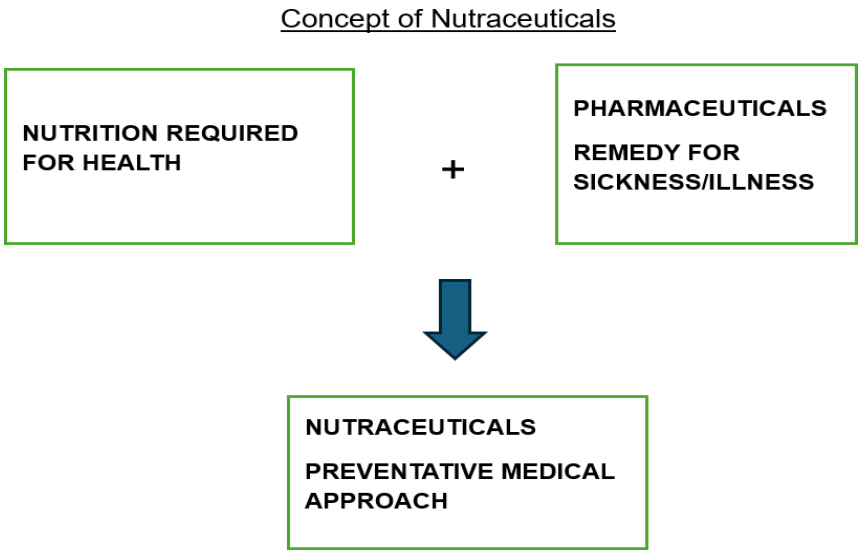

Figure 1.1: The concept of nutraceuticals (Nimesh and Ashwlayan, 2018).

Nutraceuticals promote wellness, prevent malignant processes, and control symptoms. Nutraceuticals can be categorized into three broad categories namely, nutrients, herbals, and dietary supplements. They are described as follows (Hathcock, 2001):

Nutrients: Nutrient-rich substances which include vitamins, minerals, amino acids, and fatty acids.

Herbals: Herbs or botanical products such as concentrates and extracts.

Dietary supplements: Reagents from different sources (e.g., pyruvate, chondroitin sulphate, steroid hormone precursors) are used for specific purposes, such as sports nutrition, meal replacements, and weight-loss supplements.

Amaranth is well recognised as a highly nutritious superfood with significant nutraceutical characteristics (Soriano-García and Aguirre-Díaz, 2019). It has been extensively utilised in various clinical and medical contexts due to its well-rounded nutritional composition and functional attributes, which have demonstrated notable therapeutic advantages (Soriano-García and Aguirre-Díaz, 2019).

The antidiabetic properties of plants can be attributed to secondary metabolites such as phenolic acids, glycosides, saponins, stilbenes, tannins, and others. Plant growth conditions, collection, drying, and storage techniques, as well as active compound extraction and purification techniques, all have an impact on the secondary

compounds. Therefore, a thorough analysis of how these factors affect the secondary compounds in plants is required (Sukhikh *et al*., 2023). Therefore, by studying the metabolome it allows scientists to see how the environment, genome, and metabolism all work together and find metabolites that are unique to a genus, species or family (Lankatillake *et al*., 2019). Analytical technologies including nuclear magnetic resonance (NMR) spectroscopy with multivariate data processing, and mass spectrometry (MS) can assess the impact of the environment on the plant's chemical profile of plants (Lankatillake *et al*., 2019). Metabolomics is a field of analytical biochemistry that uses traditional methods and multivariate statistical analyses to discover and quantify low-molecular-weight compounds in biological systems. Metabolomics can uncover biomarkers responsible for the plant's antidiabetic activities (Lankatillake *et al*., 2019).

1.2. Problem statement

Diabetes is one of the most common underlying causes of high mortality rates among adults and children in the world (Tao *et al*., 2015). Diabetes can be treated by using oral antidiabetic medicines; however, there are still a high number of limitations and adverse effects of these existing synthetic therapies that are available on the market (Shestra *et al*., 2017; Chaudury *et al*., 2017). For prevention and treatment of diabetes, diet in the form of nutraceuticals has been proposed (Nimesh and Ashwlayan, 2018). Nutraceuticals are medicinally or nutritionally functional foods or bioactive phytochemicals that have health promoting, disease preventing or medicinal properties (Zeisel, 1999).

South Africa has plenty of nutraceuticals, such as wild vegetables, that have not been scientifically explored yet. Wild vegetables contain nutrients like minerals and vitamins but also contain high amounts of phytochemicals (Sivakumar *et al*., 2018). Thus, if wild vegetables can be explored and promoted for their therapeutic purposes, they can, in the future, provide a solution. For example, *Amaranthus* has recently gained popularity because of the therapeutic properties that it contains. In a recent study, *Amaranthus cruentus* and *A. hybridus* showed potential in the treatment of diabetes *in vitro* (Nkobole *et al*., 2021). For the purpose of gaining more insight into the *Amaranthus* species, the proposed study focused on two other species of *Amaranthus*, namely

Amaranthus caudatus and *A. hypochondriacus.* Although *A. caudatus* has been explored for *in vitro* alpha-amylase antidiabetic activity before, to the best of the author's knowledge, its activity on alpha-glucosidase has never been reported.

1.3. Aim of the study:

The study aims to examine the metabolomic profiles and the antidiabetic effects of *Amaranthus caudatus* and *A. hypochondriacus*.

1.4. Objectives of the study:

1. To use a metabolomics approach to compare the metabolite differences in the leaves and young stems of *Amaranthus caudatus* and *A. hypochondriacus*.
2. To evaluate the effect of *Amaranthus caudatus* and *A. hypochondriacus* for their *in-vitro* α-amylase and α-glucosidase inhibitory properties.
3. To evaluate the antioxidant properties of *Amaranthus caudatus* and *A. hypochondriacus.*

1.5. Hypothesis:

Alternative hypothesis (H_1): *Amaranthus caudatus* and *A. hypochondriacus* will have a positive outcome in lowering blood glucose level *in-vitro.*

1.6. Overview of dissertation:

The dissertation consists of an introduction and literature overview, followed by three experimental chapters.

Chapter 1 focuses on the background of the study, the problem statement, aim of the study, objectives of study, hypothesis and the thesis layout.

Chapter 2 focuses on the literature review of the entire study. This chapter focuses on the *in vitro* antidiabetic activities of the *Amaranthus* species, an introduction to metabolomics, the different analytical tools used in metabolomics with some examples, and nutraceuticals used as antidiabetic treatments are discussed.

Chapter 3 focuses on ^{1}H-NMR based metabolomics profile of *Amaranthus caudatus* and *A. hypochondriacus.*

Chapter 4 presents *in vitro* α-amylase and α-glucosidase inhibitory activities of *Amaranthus caudatus* and *A. hypochondriacus* spp.

Chapter 5 explores the *in vitro* antioxidant activities of *Amaranthus caudatus* and *A. hypochondriacus.*

Chapter 6 comprises of the general conclusion and recommendations.

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CHAPTER 2: LITERATURE REVIEW

2.1. Introduction to Amaranth

2.1.1. *Amaranthus* species

Amaranthus means “immortal” in Greek. *Amaranthus* is derived from the Greek words, amárantos which means unfading and ánthos, which means flower in Greek. Thus, in Greek it means an unfading flower (Costea and Tardiff, 2003). *Amaranthus* species fall under the Caryophyllalles order, the Amaranthaceae family, the Amaranthoideae subfamily, and the *Amaranthus* genus (Peña *et al*., 2024). The *Amaranthus* genus consists of 60 species (Zhigila *et al*., 2014). DAAF, (2010), identifies *A. hybridus, A. spinosus, A. cruentus, A. caudatus,* and *A. thunbergii* as common species found in South Africa. Several amaranth species are known for their edible leaves and seeds, which are nutritious pseudo-cereals (similar to wheat) (Petruzzello, 2023).

2.1.2. Botanical description of *Amaranthus* plant species.

Amaranth plants have reddish stems or spines, as simple, alternatively arranged leaves, and pinkish taproot. Plants can be monoecious (producing both sexes flowers) or dioecious (producing only one sex’s flower per individual). The small flowers typically feature colourful spathe and are arranged in dense showy inflorescences. A single plant can produce many seeds, single in dry capsule fruits (Petruzzello, 2023).

Amaranth plant is typically an annual perennial plant that thrive in warm, humid environments (Muriuki, 2015). Amaranth plants use the C4 cycle for the photosynthetic system, making them highly efficient in utilizing sunlight and nutrients even at high temperatures (Lara and Andreo, 2011). Amaranth is drought-tolerant because of its ability to flourish in diverse climates.

Amaranth grows quickly and can be harvested after 4 to 6 weeks after planting (Shukla *et al*., 2006). Amaranth thrives in loamy soil with high water-holding capacity, but may grow in a variety of soil types, moisture levels and pH according to Nehuleni *et al*. (2007).

2.1.3. *Amaranthus* species:

For this study, the focus will only be on *Amaranthus caudatus* and *A. hypochondriacus*.

2.1.3.1. Amaranthus caudatus

Description

Amaranthus caudatus is well known for its spectacular flowering panicles, which can grow up to 90 cm long (Figure 2.1). The red variants of *A. caudatus* are attributable to a high betacyanin concentration (Li *et al*., 2015). Leaves and side branches emerge from the central stem and can begin as low as the plant's base (Fletcher, 2016). *A. caudatus* may readily grow to 1 to 2.5 meters and thrive in full sun for 4 to 6 months (summer months). Loam and loam-sandy soils with enough organic matter and sufficient drainage are ideal. Clay soils are unsuitable for *A. caudatus*. Furthermore, the pH must range from 6 to 7, yet the plant can grow at pH levels as high as 8.5 (Di Fabio and Parraga, 2017; Montserrat-de la Paz *et al*., 2021).

A B C

Figure 2.1 (A) plant without flowers; (B) flowering stage; and (C) the seeds of *Amaranthus caudatus* (Brun, 2025).

2.1.3.2. Amaranthus hypochondriacus

Description

Amaranthus hypochondriacus, sometimes known as Prince's Feather, is an herbaceous annual or short-lived perennial plant with magnificent feathery red flowers. It is commonly grown as an ornamental plant (Figure 2.2). *A. hypochondriacus* is a

lively, upright plant that can grow 40–200 cm tall (ANON, 2024). It is often grown for its flowers, which appear in dense, spike-like inflorescence in the summer and autumn. They are usually deep purplish red but may also be yellow green in colour (ANON, 2024).

A. hypochondriacus prefers well-drained, healthy soil in full sun. This plant is commonly grown in tropical places for its edible leaves and seeds. However, it is less common in northern climates due to its late maturing (ANON, 2024). Aphids can be a source of disease and pest issues. *Amaranthus* is not harmful, however when cultivated in nitrogen-rich soils, it can concentrate nitrates in its leaves (ANON, 2024). The “Green Thumb” cultivar of *A. hypochondriacus* is known for its bushy, upright growth, large, oblong, green leaves and erect, plume-like, bright yellow-green flowers (Figure 2.3).

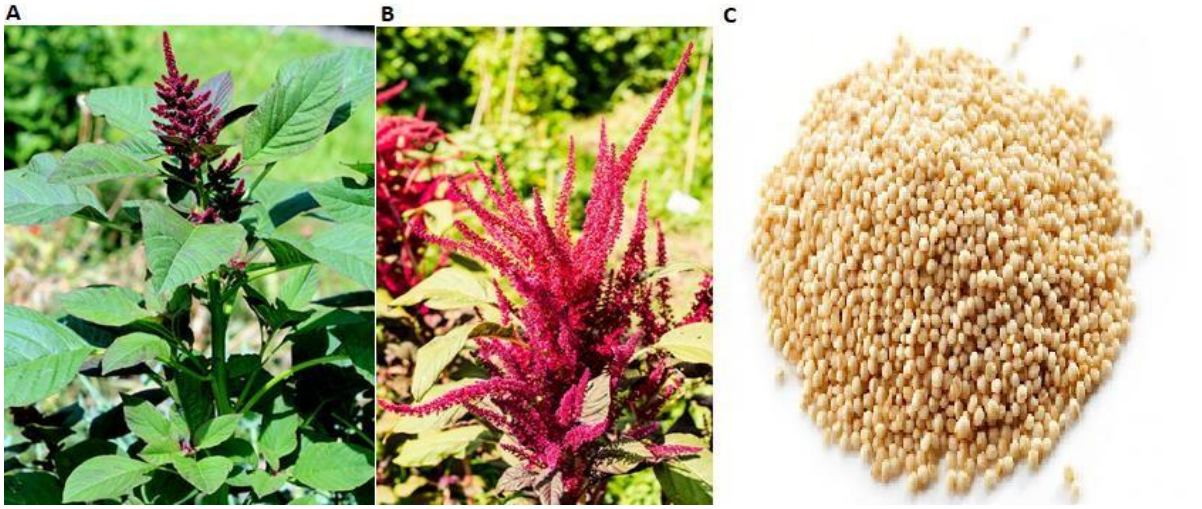

Figure 2.2 (A): plant without flowers; (B) flowering stage; and (C) the seeds of *Amaranthus hypochondriacus* red variety (BioExplorer.net, 2025).

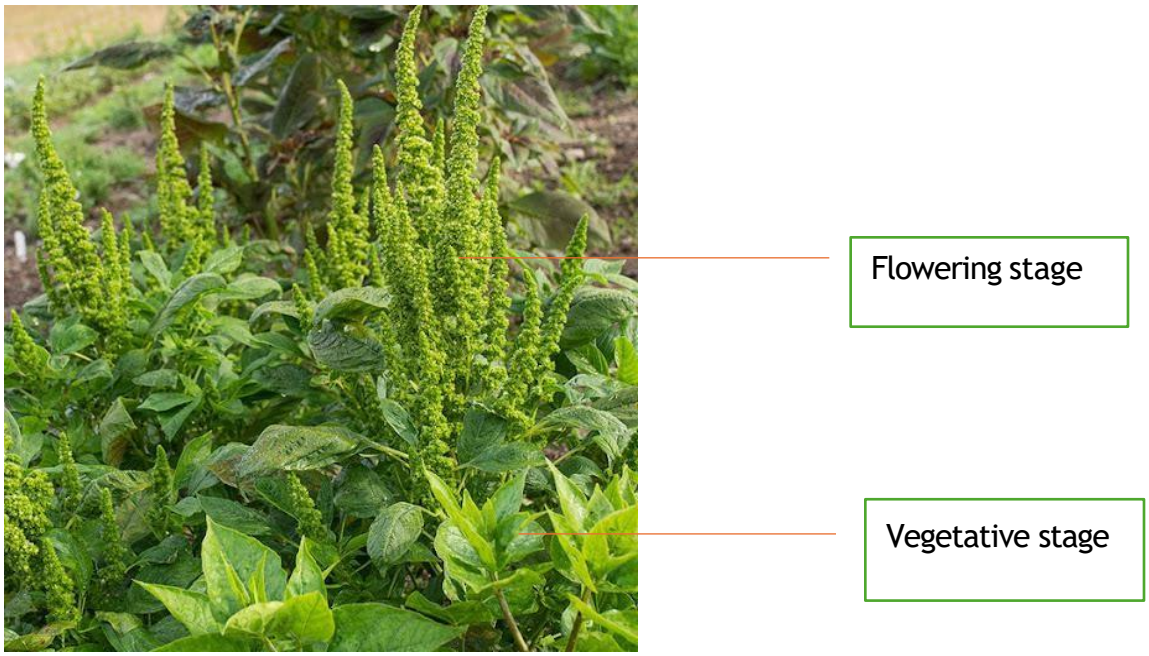

Figure 2.3: Vegetative stage and flowering stage of *Amaranthus hypochondriacus* green variety, also known as “green thumb” (Select seeds, 2025).

2.2. *In vitro* antidiabetic activities of *Amaranthus* species.

In reported studies, *in vitro* tests were described to assess the effects of plant extracts on the inhibition of two key enzymes that are involved in carbohydrate metabolism, namely α-glucosidase and α-amylase (Ali *et al*., 2006). Inhibiting these enzymes delays the breakdown of starch and oligosaccharides, lowering glucose absorption (Loizzo *et al*., 2008). Yang *et al*., (2020), describes α-amylase as a long-chain carbohydrate that is being catalyzed into smaller oligosaccharides. α-Glucosidase that resides in the small intestine catalyzes the conversion of oligosaccharides to glucose. The inhibition of these two enzymes may decelerate the production of postprandial blood glucose, which enhances hyperglycemia (Yang *et al*., 2020).

A total of 15 *in vitro* investigations were reported with α-amylase and α-glucosidase (n = 8) being the most commonly employed enzymes, followed by studies that explored only one of the enzymes, with α-amylase (n = 6) and α-glucosidase (n = 1). Concentrations of α-amylase and α-glucosidase ranged from 5.058 and 237 µg/ml up to as high as 46.73 mg/mL and 89 mg/mL for, respectively. The *in vitro* antidiabetic activities of *Amaranthus*, which have been investigated are summarized in table 1.

Table 1: Summary of *in vitro* antidiabetic activity of *Amaranthus* species.

Scientific name	Extract/Compound	Model	Dose 1 (α-amylase)	Dose 2 (α-glucosidase)	Results	Reference
A. caudatus	Methanol Acarbose	α-amylase	α-amylase 19.233 µg/mL 0.312 µg/mL		Inhibited α-amylase activity significantly	Kumar *et al*., 2011
A. caudatus	Oscar blanco seeds methanol extract Victor red seeds methanol extract	α-amylase	94.7±0.008 % 95.1 ±0.001%		Inhibited α-amylase activity	Conforti *et al*., 2005

A. caudatus	Acetone	α-glucosidase and α-amylase	α-amylase 46%	α-glucosidase 78%	Exhibited moderate inhibition of α-amylase and strong inhibition of α-glucosidase activity.	Kunyanga *et al*., 2011
A. caudatus	Methanol	α-amylase	IC_{50} = 19.233 µg/mL		Significant suppression of α-amylase enzyme in vitro, even at low doses.	Peter and Gandhi, 2017

A. spinosus	Methanol	α-amylase	IC_{50} =46.02 µg/mL		IC_{50} values of acarbose was lower than the extracts.	Kumar *et al*., 2010
A. cruentus	Methanol	α-amylase	IC_{50} value of 46.73 mg/mL			Ramalashmi. 2019
A. cruentus	Unprocessed leaf	α-amylase and α-glucosidase	α-amylase IC_{50} =0.19 mg/mL	α-glucosidase IC_{50} =0.32 mg/mL	Exhibited a dose-dependent reduction in α-amylase and α-glucosidase activities.	Oboh *et al*., 2013
A. cruentus (wild)	Methanol Palmitic acid	α-amylase and α-glucosidase	α-amylase (% inhibition) 23.47-39.63 mg/mL, acarbose= 71.37-89.00 mg/ mL 18.68-25.05, mg/mL, acarbose=	α-glucosidase (% inhibition) 41.85-87.13 mg/mL, acarbose= 66.31-80.20 mg/ mL 83.92-91.26 mg/mL, acarbose=	It inhibited α-glucosidase significantly and moderately inhibited α-amylase	Nkobole *et al*., 2021

			66.31-80.20 mg/mL	71.37-89.00 mg/mL		
	Pheophorbide A-methyl ester		7.23-49.84 mg/mL, acarbose= 66.31-80.20 mg/mL	53.16-75.41 mg/mL, acarbose= 71.37-89.00 mg/mL		
	α-Spinasterol		13.06-43.37 mg/mL, acarbose= 66.31-80.20	61.13-80.06 mg/mL, acarbose= 71.37-89.00		
A. cruentus (cultivated)	Methanol	α-glucosidase and α-amylase	α-amylase (% inhibition) 19.74-30.46 mg/mL, acarbose= 71.37-89.00 mg/mL	α-glucosidase (% inhibition) 61.61-85.46 mg/mL, acarbose= 66.31-80.20 mg/mL	It showed strong inhibition of α-glucosidase and moderate inhibition of α-amylase.	Nkobole *et al*., 2021

A. hybridus (wild) *A. hybridus* (cultivated)	Methanol Methanol	α- glucosidase and α-amylase α- glucosidase and α- amylase	α-amylase (% inhibition) 5.67-27.47 mg/mL, acarbose= 71.37-89.00 mg/mL α-amylase (% inhibition) 7.55-33.18 mg/mL, acarbose= 71.37-89.00 mg/mL	α-glucosidase (% inhibition) 89.92-97.10 mg/mL, acarbose= 66.31-80.20 mg/mL α-glucosidase (% inhibition) 63.85-79.19 mg/mL, acarbose= 66.31-80.20 mg/mL	It inhibited α-glucosidase well and moderately inhibited α-amylase	Nkobole *et al*., 2021
Red *Amaranthus* = *(Amaranthus gangeticus.Linn).*	Aqueous extracts	α-amylase and α-glucosidase	α-amylase (% inhibition): Leaves = 35-85 % inhibition from 0.25 to 1 mg.	α-glucosidase (% inhibition): Leaves = 30-80 % inhibition from 0.25 to 1 mg. Stems = 25-60 % inhibition	Aqueous extracts inhibited α-amylase and α-glucosidase activity, with red *Amaranthus* leaf extract having the strongest	Yang *et al*., 2020

			Stems = 30-70 % inhibition from 0.25 to 1 mg.	from 0.25 to 1 mg.	inhibitory impact. These studies suggest that these two vegetables may improve glycemic management by inhibiting the activity of α-amylase and α-glucosidase.	
White *Amaranthus* = *(Amaranthus inamoenus Willd.)*	Aqueous extracts	α-amylase and α-glucosidase	α-amylase (% inhibition): Leaves = 18-50 % inhibition from 0.25 to 1 mg. Stems = 20-70 % inhibition from 0.25 to 1 mg.	α-glucosidase (% inhibition): Leaves = 22-65% inhibition from 0.25 to 1 mg. Stems = 25-50% inhibition from 0.25 to 1 mg.		

A. spinosus	i). chloroform fraction of methanol extract; ii) – (14E, 18E, 22E, 26E) – methyl nonacosa – 14, 18, 22, 26 tetraenoate; iii) Acarbose	α-glucosidase		α-glucosidase IC_{50} i) 8.49 µM/mL ii) 6.52 µM/mL iii) 15.25 µM/mL	The ester of fatty acid exhibited a potent inhibition of enzyme α-glucosidase than the methanol extract and acarbose.	Mondal *et al*., 2015
A. spinosus	Ethanol	α-amylase and α-glucosidase	α-amylase IC_{50} = The values for acarbose inhibition on α-amylase were not shown. α-amylase	α-glucosidase IC_{50} = acarbose=36.98 µg/mL α-glucosidase: 237.06 µM/mL	The extract showed lower activity than acarbose in α-glucosidase.	Elya *et al*., 2015

			3.37±0.48 µM/mL			
A. viridis	Water	α-amylase	α-amylase IC_{50} = 5.058±0.41 µg/mL			Helen and Bency, 2019

2.3. *In vitro* antioxidant activities of *Amaranthus* species

Antioxidants are molecules that reduce the effects of free radicals, and they are essential for cancer defence and degenerative disorders (Peter and Gandhi, 2017). An example of *in vitro* antioxidant activities in *Amaranthus* species is the methanolic seed extract of *A. caudatus*, which was extremely effective in scavenging ABTS with an IC_{50} value of 114.25 µg/mL (Panday *et al.,* 2024).

Antioxidant activity, metabolomics analysis, and antidiabetic activity are all interconnected with each other. Metabolomics offers information about the specific metabolites that contribute to antioxidant and antidiabetic effects. Antioxidants, on the other hand, can protect against the damaging effects of hyperglycemia and improve glucose metabolism, which is crucial for glycemic control in diabetic patients, whereas metabolomics helps to identify and quantify these beneficial metabolites (Leonardo *et al*., 2024; Yu *et al*., 2024).

2.4. Introduction to metabolomics

Metabolomics is the study of the metabolome within cells, biofluids, tissues, or organisms to quantify low-molecular weight compounds and identify compounds in biological systems using high-throughput methods (Nalbantoglu, 2019). Metabolome is studied by researchers to identify relationships between the genome, the environment, and metabolism, as well as individual metabolites by genus, species or family (Lankatillake *et al*., 2019).

2.5. Analytical technologies used in metabolomics research

Metabolomics research typically utilizes a variety of analytical techniques. Common methods include gas chromatography mass spectrometry (GC-MS), capillary electrophoresis mass spectrometry (CE-MS), liquid chromatography mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR), and Fourier transform mass spectrometry (FT-MS) (Jiang *et al*., 2019). The following section focuses on the most widely used techniques in plant metabolomics: MS and NMR.

2.5.1. Mass Spectroscopy (MS)

Mass spectrometry measures the mass-to-charge ratio (*m/z*) of molecules in a sample. Mass spectrometers can discover unknown compounds, quantify known compounds, and analyze the structure and chemical properties of molecules (Broad Institute, 2025).

2.5.2. Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a widely utilized and powerful technique that leverages the magnetic properties of specific nuclei (Zia et al., 2019). NMR detects transitions between spin states unique to the nuclei of interest, as well as their chemical environments. These capabilities have made NMR valuable for determining chemical structures, monitoring reactions, studying cell metabolism, and for use in fields like medicine, biochemistry, and many other scientific disciplines (Raja and Barron, 2022).

NMR analysis can identify all metabolites in a sample, both primary and secondary, offering a more comprehensive overview (Kim *et al*., 2010). While NMR has lower sensitivity compared to Mass Spectrometry (MS), it is more reproducible and widely used, providing complete spectral data (Eghbalnia *et al*., 2017). Common NMR techniques in plant metabolomics include ^{1}H-NMR, P-NMR, and ^{13}C-NMR (Jiang *et al*., 2019).

2.6. Examples of metabolomics in plants/antidiabetic research.

Using H-NMR-based metabolomics, Mediani *et al*. (2016) identified glucose, choline, taurine, and creatine as the primary biomarkers in obese-diabetic rats compared to the normal group. The treatment with *Phyllanthus niruri* extract enhanced the level of pyruvate, which transforms to the acetyl-coenzyme A needed in the TCA cycle. Mediani *et al*. (2016) also found that the levels of TCA intermediates increased following the injection of *P. niruri* extract, resulting in an improvement of the TCA cycle. *P. niruri extract* significantly reduced the serum glucose levels and improved lipid profiles in obese diabetic rats. Another example of metabolomics applied in diabetes research was shown by Yang *et al*. (2022), which found that red ginseng extract may enhance islet tissue damage, regulate blood lipid levels, and perform a therapeutic role via amino acid metabolism. In their investigation, the authors discovered that phenylalanine levels in diabetic patients were dramatically lowered, which is comparable with the pattern of

phenylalanine levels in the rats' blood.

2.7. Nutraceuticals as antidiabetic treatments.

In recent years the use of nutraceuticals in medications has increased globally. Herbal medications offer better treatment choices for diabetes than synthetic drugs, with fewer diverse effects (Nimesh and Ashwlayan, 2018).

Few examples of nutraceuticals as antidiabetic treatments

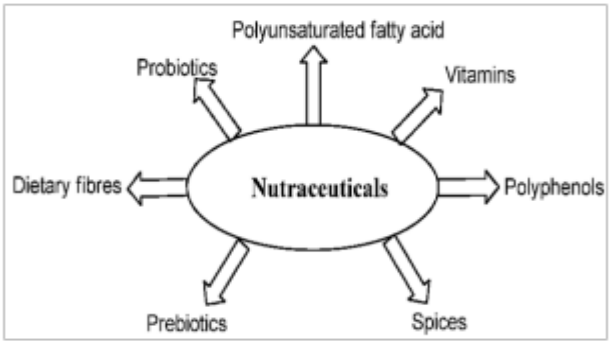

Figure 2.4: Functions of nutraceuticals (Nimesh and Ashwlayan, 2018).

2.7.1. Probiotics

Probiotics are the most often utilized nutritional supplements worldwide (Sanders *et al*., 2019). Probiotics can boost the production of glucagon-like peptide 1 (GLP-1), reduce lipogenesis and insulin resistance (IR), slow gastrointestinal motility, and suppress hunger by increasing short-chain fatty acids production. Secondly, they can improve IR by regulating intestinal permeability and bacterial lipopolysaccharide transport. Additionally, they can create bioactive peptides with antibacterial characteristics and inhibit amylases and glucosidases. Probiotics can reduce LPS -induced inflammation and oxidative stress by inhibiting the synthesis of NF-kB and ROS (Le *et al*., 2022).

2.7.2. n-3 long-chain polyunsaturated fatty acids (LCPUFAs)

n-3 long-chain polyunsaturated fatty acids (LCPUFA), often known as fish oils, is gaining attention for their potential role in preventing and managing Type 2 diabetes. Cione *et al*.,

2019) found that capsaicin analogues produced from n-3 polyunsaturated fatty acids diminish macrophages inflammation and promote insulin production by β cells *in vitro.* Dasilva *et al*., (2021) found that fish oil enhances pathway-oriented lipid mediators' profile in prediabetic rats' adipose tissue, promoting metabolic balance.

2.7.3. Plant-derived nutraceuticals

Plant phytochemicals exhibit antidiabetic properties by improving β cell function, insulin production, GLP-1 homeostasis, NF-kB signalling, inhibiting gluconeogenic enzymes, and protecting against ROS (Salehi *et al*., 2019; Unuofin and Lebelo, 2020). A systematic study found that blueberries which contain 9.1 to 9.8 mg of anthocyanins and cranberry consumption improved glucose control in T2DM patients (Rocha *et al*., 2019). *Ampelopsis grossedentata* (APL) supplementation for one month or ellagic acid (EA) administration (180 mg for 8 weeks) improved the glycemic control in adults with T2DM (Ran *et al*., 2019; Ghadimi *et al*., 2021).

2.8. Current treatments

2.8.1. Imeglimin (IMEG)

Imeglimin (IMEG) are hypoglycaemic medications used for the treatment of type 2 diabetes mellitus (T2DM). Imeglimin has been proven to improve 3 critical pathogenetic elements of T2DM. This includes increased gluconeogenesis, insufficient glucose-induced insulin production by β cells, and peripheral insulin resistance. After 16 weeks of treatment, IMEG had a peak effect on fasting plasma glucose (FPG) and glycated haemoglobin (HbA1c) levels. IMEG in humans increases insulin secretion and lowers fasting plasma glucose and glycated haemoglobin levels (Nowak and Grzeszczak, 2022).

2.8.2. Dapagliflozin

Dapagliflozin, is a selective SGLT2 (odium-glucose cotransporter 2) inhibitor, is approved as a complement to diet for improving glycaemic control in persons with type 2 diabetes. Dapagliflozin decreases blood glucose without insulin by blocking renal reabsorption, leading to increased excretion of glucose. Dapagliflozin can improve glycaemic indices in type 2 diabetes patients, whether treated alone or in conjunction used with metformin,

glimepiride, pioglitazone, sitagliptin, or insulin (Vivian, 2015).

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CHAPTER 3

^{1}H-NMR based metabolomics profile of *Amaranthus caudatus* and *A. hypochondriacus*

Abstract

Several studies that have been conducted so far on *Amaranthus* mainly focused on the physiological parameters, morphological properties, and nutritional composition, leaving a huge gap in the plant's metabolomics profile of its leaves. *Amaranthus* contains antidiabetic, antioxidant, antimicrobial, and anti-inflammatory properties. However, not much is known about its constituents that are linked to its health benefits. In this study, proton nuclear magnetic resonance (^{1}H-NMR) was used to chemically profile the untargeted metabolites of *Amaranthus caudatus* and *A. hypochondriacus* leaves by using multivariate statistical analysis. In this study, 31 compounds were reported, and these compounds were mostly similar in both *Amaranthus* species. Common metabolites that were reported in this study were amino acids (leucine, valine, threonine, GABA, alanine, tryptophan, etc.); sugars (glucose, sucrose, maltose, mannitol, and trehalose); organic acids (malic acid, mannonic acid and citric acid) and phenolic acids (gallic acid, vanillic acid, and ferulic acid). An important aromatic compound that was reported in both *A. caudatus* and *A. hypochondriacus* leaves was trigonelline, which acts as a stress protector against various abiotic stresses, such as heat, drought, high salinity, and ultraviolet rays. Chlorogenic acid, which is known for its antidiabetic and antioxidant properties was also annotated in both *A. caudatus* and *A. hypochondriacus* species. This study revealed that not only are amaranth leaves rich in minerals and proteins but that the plants contain important phytochemicals that have medicinal properties.

Keywords: NMR spectroscopy; *Amaranthus*; amino acids; phenolic acids; aromatic compounds; defence mechanism metabolites; growth promoting metabolites and stress regulating metabolites.

3.1. Introduction

Metabolomics is a field of analytical chemistry focused on measuring all metabolites within an organism, both qualitatively and quantitatively. This enables the creation of a comprehensive metabolic profile of a living organism under specific conditions (Kim *et al*., 2010). Metabolites are small molecules, compounds, and intermediates that are produced during metabolism. Metabolites are important in many ways, including fuel, signaling, enzyme stimulation and inhibition, defense, catalytic activity, structure and ingestion (Anand *et al*., 2022). In recent times, metabolomics gained attention, especially for analyzing secondary metabolites, due to their uncountable health benefits, which have accounted for an awakening interest in improving their production in various applications (Anand *et al*., 2022). These metabolites possess various therapeutic properties such as anti-inflammatory, anti-cancerous, anti-diabetic and antioxidant, which help in the prevention of various diseases such as cardiovascular, gastrointestinal, neurodegenerative and respiratory disorders caused by high oxidative stress (Anand *et al*., 2022). Secondary metabolites significantly improve plant defense enabling plants to survive under difficult environmental stresses (Zandalinas *et al*., 2018).

Advances in NMR spectroscopy, mass spectrometry, and chemical separation enable rapid identification and quantification of hundreds of metabolites (i.e., the metabolome) in various biological samples (German *et al*., 2005; Wishart, 2008). Metabolomics with the use of NMR spectroscopy and multivariate data analysis has the potential to serve as a valuable tool for rapid discovery of phytotherapeutics (Nkobole and Prinsloo, 2021).

Recently, *Amaranthus* spp. has become increasingly popular because of its nutritional benefits and medicinal properties found in both its seeds and leaves (Nkobole and Prinsloo, 2021). It offers high-quality protein, unsaturated fats, and is gluten-free (Nemadodzi and Managa, 2024). In rural South Africa, *Amaranthus* is grown organically in open fields for its leaves, with a harvesting season that peaks between five to six times from October to May. According to Nemadodzi and Managa, (2024), seeds are left to grow naturally and will reap the benefit the following season. Yet, in South Africa, both

smallholder and commercial farmers are skeptical about the growing of *Amaranthus* due to the lack of information available to these farmers.

Understanding the chemical profile of *Amaranthus* leaves depends on growing circumstance and environmental factors, as these compounds have been connected to the plants' health-promoting properties. Therefore, the main aim of this chapter is to use the metabolomics approach of ^{1}H-NMR spectroscopy to compare the metabolite differences in *A. caudatus* and *A. hypochondriacus* leaves.

3.2. Materials and Methods

3.2.1. Study site and planting of *Amaranthus* seeds

The seeds were sown in February 2024 at the Florida Science Campus, Roodepoort, South Africa (GPS coordinates: Latitude: 26°9′29.274″; Longitude: 27°55′17.663″). The seeds of *Amaranthus caudatus* and *A. hypochondriacus* were received from Agriculture Research Council Vegetable Industrial and Medicinal Plants (ARC-VIMP) gene bank in Roodeplaat. The seeds are stored to confirm their authenticity. Seeds were first placed in 200 cavity seedling trays with hygromix as a growing medium for germination. Twenty-one days (21 days) following emergence, seedlings were transplanted to the field. Two *A. hypochondriacus* varieties were noted in the seeding trays and they were labeled as *A. hypochondriacus* red (AHR) and *A. hypochondriacus* green (AHG).

The seedlings of both amaranth species were planted with a spacing of 10 cm x 20 cm (50 plants per square meter) in the soil under open-field conditions (Mulandana *et al*., 2009). Inorganic NPK fertilizer (2:3:4 (30)) was applied at a rate of 150 kg/ha prior to planting (Mhlontlo *et al*., 2018). Nitrogen fertilizer in the form of limestone ammonium nitrate (LAN), which contains 28% nitrogen, calcium, and magnesium, was subsequently applied at 100 kg/ha two weeks after planting (AdeOluwa *et al*., 2009). The plants were irrigated at least two times a week, but more often during hot periods to prevent stress and wilting.

3.2.2. Harvesting

The first harvest occurred in April 2024, two months after sowing, 10 a.m. or earlier. The second harvest occurred two weeks following the first harvest (end of April 2024). Plants are randomly selected from various locations in the field to ensure a representative sample batch for each species. To reduce the sweating of the leaves and increase airflow, each plant was sprayed with water, and each batch was loosely packed and tagged with essential details on the punctured black plastic bags. The bags were then taken to the laboratory where the plants were allowed to air-dry at room temperature protected from direct sunlight. When the leaves were dry, they were ground into a fine powder and kept at room temperature until they were analysed.

3.2.3. Reagents and buffer preparation.

Sigma supplied deuterium water (D_2O), deuterated methanol (CD_3OD), potassium dihydrogen phosphate (KH_2PO_4) and trimethylsilylpropionic acid sodium salt (TSP). To create a buffer, 1.232 g KH_2PO_4 was added to 100 mL of D_2O. A reference standard 10 mg TSP (0.01%) was also used.

3.2.4. Extraction of plant material based on NMR Spectroscopy method

The plant material extraction method was adapted from the works of Maree and Viljoen (2012) and Mediani *et al*. (2012). The extraction utilized the direct method. In brief, 50 mg of powdered leaf material was placed into 2 mL Eppendorf tubes. The samples were then mixed with 0.75 mL of deuterated methanol and 0.75 mL of buffered deuterium water (pH 6.0) containing 0.01% (w/w) TSP. The mixture was vortexed at room temperature and subjected to ultrasonic treatment for 20 minutes to break down cell walls and achieve homogenization (Kim *et al*., 2010). Afterward, the samples were centrifuged at 13,000 rpm for an additional 20 minutes to separate the supernatant from the pellet. The supernatant was transferred to a 5 mm NMR tube for analysis, where 32 scans were conducted on a 600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA).

3.2.5. Data mining and data pre-processing (MestReNova)

Pre-treatment of raw spectral data is crucial for creating trustworthy and interpretable models in multivariate analysis (Worley and Powers, 2013). Spectral data were pre-processed using MestReNova software (version 15.1, Mestrelab Research, Spain). All sample spectra underwent manual phase correction, baseline adjustment, normalization, and calibration against the internal standard TSP set at 0.0 ppm. The MestReNova software was employed to bucket the NMR spectra, dividing them into equal-sized sections (0.04 ppm each) ranging from 0.04 to 10.00 ppm. For multivariate analysis, the data was then loaded into SIMCA software (version 18.0, Umetrics, Umeå, Sweden) (Mediani *et al*., 2012).

3.2.6. Multivariate data analysis (SIMCA)

Mathematical modelling techniques known as multivariate data analysis (MVDA) were employed to extract relevant information from these large empirical data sets (Tugizimana *et al*., 2013). This work utilized principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) models, which are commonly used methodologies in metabolomics data analysis for data overview/descriptive exploration and explicative/predictive analysis, respectively (Tugizimana *et al*., 2016).

Principal Component Analysis (PCA) is a method employed to analyze large datasets with numerous dimensions or features per observation. It enhances data interpretability by retaining the most significant amount of information and enables visualization of multidimensional data (Jolliffe and Cadima, 2016). Hence, PCA is a statistical technique that reduces the dimensionality of a dataset. This is performed by linearly translating the data into a new coordinate system in which the variation may be expressed with fewer dimensions than the original data (Jolliffe and Cadima, 2016). The OPLS-DA approach is both explanatory and predictive, facilitating the identification of metabolites responsible for group differentiation (Tugizimana *et al*., 2013).

3.2.7. **Annotation of compounds**

Metabolites were identified and quantified with the Chenomx NMR program. The annotated compounds were verified using the Human Metabolome Database (HMDB) and previously published data (Wishart *et al*., 2008).

3.3. Results:

(i). Metabolite composition of *A. caudatus* and *A. hypochondriacus*

PCA and OPLS-DA were used to examine data from amaranth spp. extracts. Figure 3.1 depicts the PCA analysis of ^{1}H -NMR spectra from methanolic water extracts of *A. caudatus* and *A. hypochondriacus.* Each point on the PCA scatter plot represents an individual sample. The model demonstrated excellent fit ($R^2X_{(cum)}$= 0.95) and predictive performance ($Q^2_{(cum)}$ = 0.895). The majority of the *Amaranthus* samples showed positive loading along PC2, with the exception of, *Amaranthus caudatus* (AC3). The observed samples were evenly scattered around the plane, making it difficult to distinguish between them.

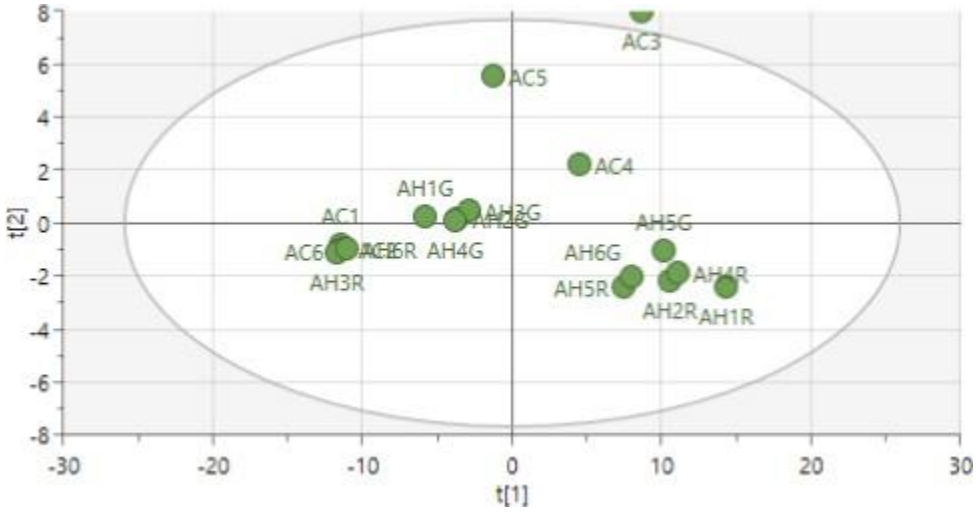
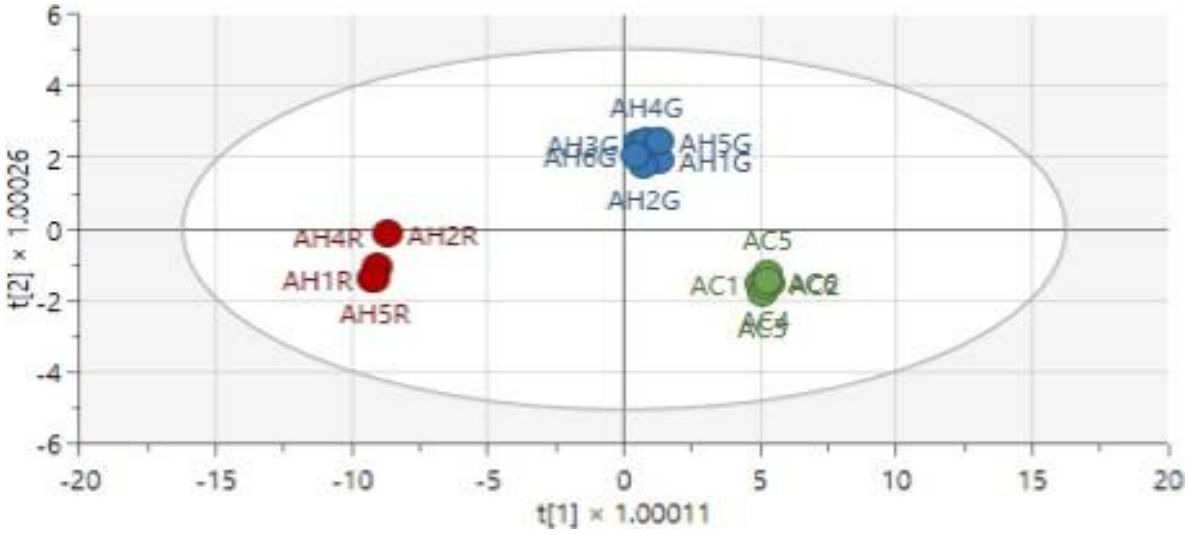


Figure 3.1: Score scatter plot of PCA of *A. caudatus* (AC); *A. hypochondriacus* red (AHR) and *A. hypochondriacus* green (AHG).

An OPLS-DA model was applied to identify compounds responsible for sample differences (Figure 3.2 A). The OPLS-DA statistical model distinguished between extracts of *Amaranthus* spp. The model had high goodness of fit (R^2X= 0.995) and predictability (R^2Y= 0.981) (Table 3.1). To validate the predictive capability of the OPLS-DA model, a response permutation test (with n = 100) was created. The test involves randomly assigning two groups, then fitting OPLS-DA models to each permuted class variable. The permutated models' values are compared to those of the actual models. The actual models had much higher R^2 and Q^2 (Figure 3.2 B) values, indicating the actual OPLS-DA models for each dataset outperforming the 100 permutated models.

A



B

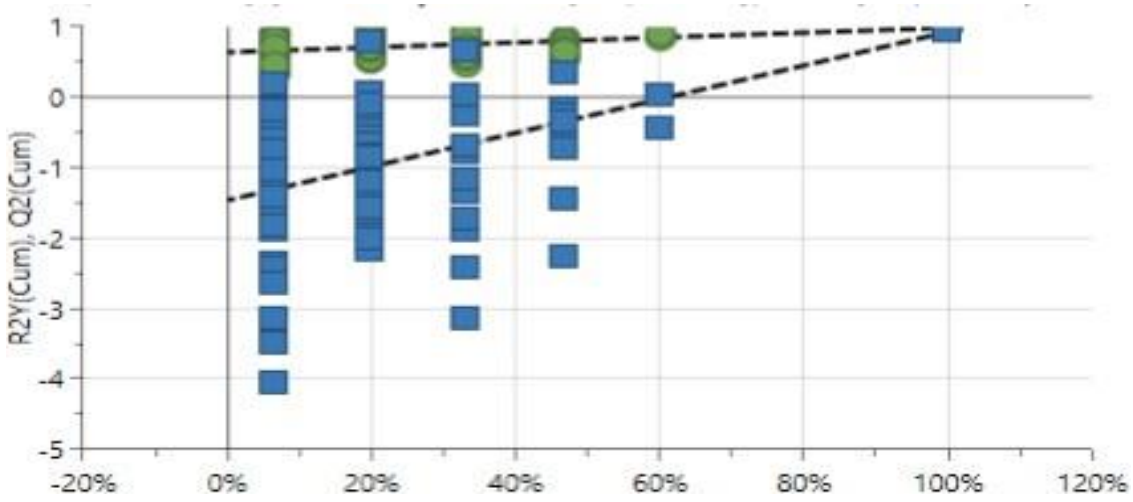


Figure 3.2 A: Score scatter plot of OPLS-DA of different *Amaranthus* spp. leaf extracts Green = *A. caudatus* (AC); red = *A. hypochondriacus* red variety (AHR) and blue = *A. hypochondriacus* green variety (AHG) cultivated in open field at UNISA Science Campus, Florida, South Africa. **Figure 3.2 B**: The response permutation test for the OPLS-DA model.

Table 3.1: Model Quality and description of OPLS-DA for *Amaranthus* spp.

OPLS-DA			Permutation (n = 100)	
R^2X	R^2Y	Q^2	R^2	Y^2
0.995	0.981	0.895	(0.0, 0.639)	(0.0, -1.46)

Contribution plots were generated to identify the key variables that influence the differentiation of samples between classes (Figure 3.3). These plots revealed that sugars and aliphatic compounds played a significant role in the variations observed among samples collected from the UNISA Science Campus in Florida, South Africa. The NMR regions corresponding to primary and secondary metabolites responsible for distinguishing *A. caudatus* from *A. hypochondriacus* were pinpointed using the contribution plot (Figure 3.3), with specific chemical shift regions identified.

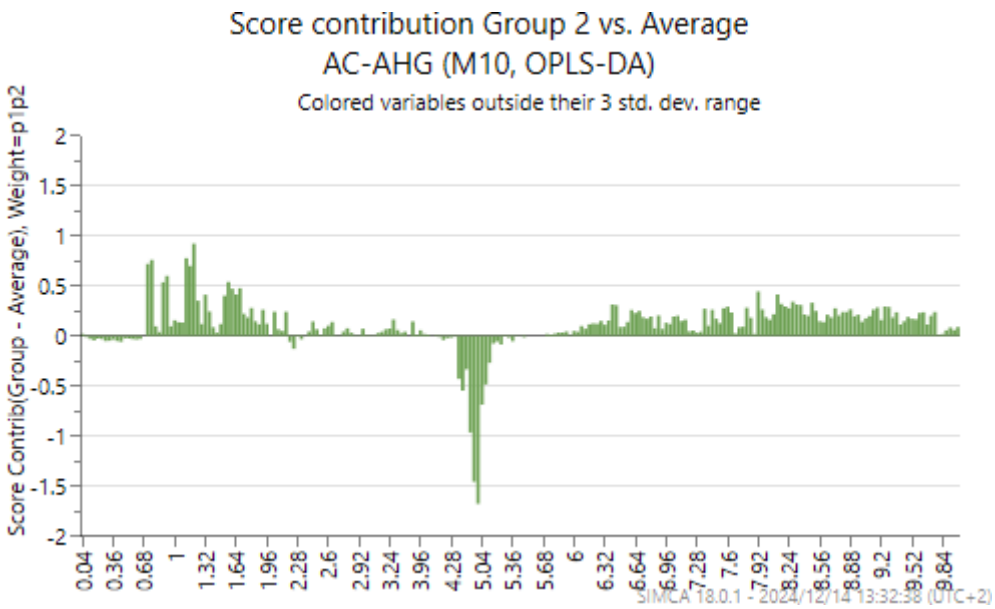


Figure 3.3: Contribution plot generated by comparing *A. caudatus* (AC) against *A. hypochondriacus* red variety (AHR). Both are red varieties. The positive bars above the line are positively associated with the *A. caudatus* samples.

The annotated metabolites of both *Amaranthus* species are represented in Table 3.2. In this study, Chenomx, Human Metabolome Database (HMDB), and published data of previous research articles were used to annotate metabolites that were found in both *Amaranthus* species. There were 31 compounds annotated in both *Amaranthus* species. Trigonelline and xylose were the identified chemicals that effectively separated *A. caudatus* from *A. hypochondriacus*, as they were present in higher concentrations in the *A. caudatus* samples.

Table 3.2: NMR peaks (ppm) of the compounds that were annotated in *A. caudatus* and *A. hypochondriacus* extracts.

Species name	Compound	NMR Region (ppm)	Chenomix 11.0 (ppm)	Human Metabolite Database	Reference (ppm)	Literature
Amaranthus spp.	Leucine (1)	0.92 0.95	0.9 1.0 1.7 3.7	0.94 1.70	0.94	Oh *et al*., 2018
	Isoleucine (2)	1.01 3.67	0.9 1.0 1.1 1.5 2.0 3.7	0.93 1.0 3.66	0.91 1.02 1.06	Oh *et al*., 2018; Lanzotti *et al*., 2022
	Valine (3)	0.98 1.03 3.62	1.0 1.0 2.3 3.6	1.02 1.02 2.26 3.60	1.0 1.05 2.32 3.6	Nkobole and Prinsloo, 2021; Lanzotti *et al*., 2022; Oh *et al*., 2018
	Threonine (4)	1.28 3.67	1.3 3.6 4.3	1.32 3.58 4.24	1.316 3.575 4.244	Maulidiani *et al*., 2018; Lanzotti *et al*., 2022

	Alanine (5)	1.47	1.5 3.8	1.47 3.8	1.49 3.8	Nemadodzi and Managa, 2024; Nkobole *et al*., 2021
	4-Aminobutyrate gamma-aminobutyric acid / (GABA) (6)	2.29	1.9 2.3 3.0	1.89 2.28 3.00	1.9 2.3 3.02	Oh *et al*., 2018; Managa *et al*., 2021; Lanzotti *et al*., 2022
	Chlorogenic acid (Chlorogenate) (7)	2.02 3.88 4.26 5.32 6.4 7.17 7.64	2.0 2.1 2.2 3.9 4.3 5.3 6.4 6.9 7.1 7.2 7.6	2.02 2.17 3.88 4.25 5.33 6.39 6.94 7.12 7.19 7.65	2.0 2.08 2.2 3.88 4.28 5.28 6.4 6.88 7.08 7.2 7.6	Nkobole and Prinsloo, 2021; Sabino *et al*., 2019; Lanzotti *et al*., 2022
	Glutamine (8)	2.14	2.1	2.11	2.10	Lanzotti *et al*., 2022; Nemadodzi

		2.46 3.76 6.86	2.4 2.5 3.8 6.9 7.6	2.42 2.46 3.76	2.43 3.86 6.89	and Managa, 2024
	Succinylacetone (9)	2.27 3.85	2.3 2.4 2.8 3.8	2.26 2.41 2.81 3.85	N/A	N/A
	Carnitine (10)	2.46 3.20	2.4 3.2 3.4 4.6	N/A	3.19	Nemadodzi and Managa, 2024
	Malate (Malic acid) (11)	 2.66 4.31	2.4 2.7 4.3	 2.73 4.44	2.36 2.67 4.30	Maulidiani *et al*., 2018; Villa-Ruano *et al*., 2019; Lanzotti *et al*., 2022
	Citric acid (Citrate) (12)	2.52 2.68	2.5 2.7	2.52 2.66	2.52 2.69	Oh *et al*., 2018; Maulidiani *et al*., 2018; Lanzotti *et al*., 2022

	Asparagine (13)	2.93 3.99	2.8 2.9 4.0 6.9 7.6	2.84 3.84	2.88 2.95 6.9	Lanzotti *et al*., 2022; Nemadodzi and Managa, 2024; Pei *et al*., 2022
	Phenyllactate (14)	3.5 7.31 7.4	2.9 3.1 4.3 7.3 7.4	2.87 3.09 4.26 7.33	N/A	N/A
	Phenylalanine (15)	3.9 7.31	3.1 3.3 4.0 7.3 7.4	3.27 3.98 7.29 7.42	3.07 7.28 7.42	Oh *et al*., 2018; Maulidiani *et al*., 2018; Lanzotti *et al*., 2022
	Xylose (16)*	3.42	3.2 3.3 3.4 3.5 3.7	3.52 3.52 3.68	3.23 3.31 3.43 3.52-3.70 “	Maulidiani *et al*., 2018

		3.94 4.59 5.19	3.9 4.0 4.6 5.2	3.87 4.0 4.63 5.18	 3.92 4.57 5.21	
	Betaine (17)	3.25 3.9	3.3 3.9	3.25 3.89	3.25 3.92	Nemadodzi and Managa, 2024; Nkobole and Prinsloo, 2021.
	Caffeine (Caffeic acid) (18)	3.34 3.52 3.95 7.88	3.3 3.5 3.9 7.9	3.34 4.04	3.29 3.5 3.9	Nemadodzi and Managa, 2024; Maulidiani *et al*. 2018.
	Maltose (19)	 3.41 4.66	3.3 3.4 3.6 3.7 3.8 3.9 4.0 4.6	3.27 3.41 3.66 3.70 3.84 3.9 3.96	3.24 3.4 3.6 3.72 3.8 3.92 4.0 4.56	Oh *et al*., 2018; Nkobole and Prinsloo *et al*., 2021; Mdlalose *et al*., 2022

		5.41	5.2 5.4	5.22 5.40	5.2 5.42	
	Tryptophan (20)	7.33 7.73	3.3 3.5 4.1 7.2 7.3 7.5 7.7 10.2	4.04 7.28	7.18 7.31 7.49 7.69	Lanzotti *et al*., 2022
	Trehalose (21)	3.4 3.81 3.86 5.16	3.4 3.6 3.8 3.9 5.2	3.42 3.81 3.85 5.18	3.41 5.18	Nemadodzi and Managa, 2024; Oh *et al*., 2018
	Sucrose (22)	3.56 3.8	3.5 3.6 3.7 3.8 3.9	3.46 3.55 3.82 3.89	3.67	Maulidiani *et al*., 2018; Nemadodzi and Managa, 2024; Lanzotti *et al*., 2022; Wishart *et al*., 2009

		4.03 5.38	4.0 4.2 5.4	4.04 4.21 5.4	3.87 5.39	
	Mannose (23)	 3.55 3.85 3.9 4.89	3.4 3.6 3.8 3.9 4.1 4.9 5.2	 3.66 3.81 3.95 4.05 4.92 5.21	3.37 3.57-3.93 “ “ 5.13	Maulidiani *et al*., 2018
	Arabinose (24)	 3.83 3.94 5.23 5.29	3.5 3.7 3.8 3.9 4.0 4.1 4.5 5.2 5.3	 3.68 3.81 3.87 4.0 4.12 5.21	 4.52 5.25 5.33	Maulidiani *et al*., 2018
	Galactose (25)		3.5	3.52		Villa-Ruano *et al*., 2019

			3.7 3.8 3.9 4.0 4.1 4.6 5.3	3.74 3.82 3.95 4.12 5.21	 4.73	
	Lactulose (26)	3.54 3.67 4.22 4.30 4.44 4.45 4.56	3.6 3.7 3.8 3.9 4.0 4.1 4.2 4.3 4.3 4.4 4.5 4.6	3.58 3.73 3.84 3.92 4.01 4.13 4.2 4.25 4.29 4.46 4.55	3.6 3.68 3.8 3.88 4.0 4.08 4.12 4.28 4.44 4.48 4.56	Nkobole and Prinsloo, 2021; Jayalakshmi *et al*., 2009

	Mannonic acid (Mannitol) (27)	3.68 3.79	3.7 3.8 3.9	3.65 3.77	3.79 3.90	Oh *et al*., 2018; Kim *et al*., 2011
	Ferulate (Ferulic acid) (28)	3.9 6.4 6.90	3.9 6.4 6.9 7.1 7.3	3.89 6.37 6.91 7.11 7.31	3.89 6.42 6.85 7.05	Managa *et al*., 2021
	Vanillic acid (Vanilate) (29)	3.91 6.93 7.53	3.9 6.9 7.5	3.90 6.93 7.52	6.94 7.55	Managa *et al*., 2021
	Syringic acid (Syringate) (30)	3.91 7.26	3.9 7.3	3.84 7.13	N/A	N/A
	Trigonelline (31)*	4.43 8.09 8.82 9.12	4.4 8.1 8.8 9.1	4.42 8.07 8.82 9.11	4.42 8.12 8.8 9.12	Maulidiani *et al*., 2018; Nkobole and Prinsloo, 2021; Lanzotti *et al*., 2022

*High in *Amaranthus caudatus* samples

3.4. Discussion

In this study, 31 compounds/metabolites were annotated in two *Amaranthus* species. These compounds are further divided into three regions, namely aliphatic (0.5 to 3.0 ppm), sugars (3.0 to 5.5 ppm), and aromatic (5.5 to 10.00 ppm).

Amaranth leaves are a valuable source of amino acids. There are 6 essential amino acids that are found in *A. caudatus* and *A. hypochondriacus* as shown in Table 3.2. The amino acids involved include isoleucine, leucine, phenylalanine, threonine, tryptophan, and valine. Additionally, the aspartate family of amino acids, which consists of isoleucine, aspartate, asparagine, and threonine, has also been identified in this study (Table 3.2). This group is characterized by a high degree of heterogeneity (Viola, 2001). According to Yang *et al*. (2020), amino acids are crucial products of primary metabolism, performing various functions in plants such as promoting growth, aiding in cell wall biosynthesis, serving as osmoregulatory agents, and acting as intermediates in secondary metabolite production. Furthermore, the amino acids annotated in this study are involved in plant defense mechanisms, responses to biotic and abiotic stresses, and are essential for plant growth (Grubb and Abel, 2006; Ziegler and Facchini, 2008).

On the other hand, alanine, leucine, and valine are synthesized from pyruvate, the end product of glycolysis. Along with isoleucine, leucine, and valine, they form a small group of branched-chain amino acids (BCAAs) (Ingrisano *et al*., 2023). BCAAs and their derivatives play a critical role in plant growth, stress responses, and the formation of food flavor components in plants (Xing and Last, 2017; Yang *et al*., 2020). In plants, the breakdown of BCAAs is particularly important during sugar deficiency, a condition that often arises during drought stress, as it provides the energy needed for survival (Izumi and Ishida, 2019). Bowne *et al*. (2012) found that the concentration of BCAAs increased in wheat cultivars under drought stress.

Two other important amino acids that were annotated in this study were tryptophan and phenylalanine. Both are described as primary metabolites. Tryptophan is an essential amino acid that plays a significant role in the production of precursors involved in plant growth, defense against biotic and abiotic stresses, and plant-insect interaction (Zemanová *et al*., 2014). Phenylalanine, on the other hand, is an essential amino acid

found in the aliphatic region in the plants. As noted by Pascual *et al*. (2016), phenylalanine is crucial for linking primary and secondary metabolism in plants. It serves as a precursor for a variety of compounds, including phenylpropanoids, flavonoids, anthocyanins, lignin, tannins, and salicylates. Additionally, it plays a significant role in plant growth, reproduction, and defense against both abiotic and biotic stresses (Pascual *et al*., 2016).

Other metabolites that were annotated in the aliphatic region include glutamate and arginine. These compounds belong to the glutamate family. According to Forde and Lea, (2007), glutamate plays a vital role in amino acid metabolism and is directly involved in both ammonia assimilation and the transferring of ammonia to other amino acids. Arginine was annotated in both *A. caudatus* and *A. hypochondriacus* species in this study. Arginine is a precursor of polyamine biosynthesis; hence, polyamines are accumulated under abiotic stress and play a vital role in protecting biomolecules and stimulating plant growth (Alcázar *et al*., 2020; Shao *et al*., 2022). Besides building blocks in protein biosynthesis, arginine contributes to stress tolerance in plants (Hayat *et al*., 2012; Winter *et al*., 2015).

Another two important amino acids that were annotated in *A. caudatus* and *A. hypochondriacus* were Gamma-aminobutyric acid (GABA) and valine. GABA is a non-protein amino acid, which acts as a signaling molecule in plants (Ramos-Ruiz *et al*., 2019). To support the findings, Shelp *et al*. (2021) reported the indirect role of GABA in plant growth, as well as the regulation of defence mechanisms against abiotic and biotic stresses. GABA also possesses important health-related properties, such as lowering the blood pressure of mildly hypertensive patients (Marseglia *et al*., 2014).

The second region (3.0 to 5.5 ppm) is highly dominated by soluble sugars and carbohydrates. Carbohydrates are major energy bearers in plants (Trouvelot *et al*., 2014). Carbohydrates such as sucrose, glucose, fructose, and raffinose are notable compatible solutes in plants (Krasavina *et al*., 2014). In this study, sucrose, xylose and maltose were annotated in both *A. caudatus* and *A. hypochondriacus*. Soluble sugars, which include sucrose, fructose, and glucose, play a vital role in the plant's physiology and preserve its overall structure and development (Rosa *et al*., 2009). In addition, sucrose is the most common fixed carbon form of carbohydrates for long-distance transport from mesophyll

leaves to non-photosynthetic sink organs during fruit and seed development (Aluko *et al*., 2021). In this study, xylose was present in higher concentrations in *A. caudatus* than *A. hypochondriacus* species, respectively. Xylose is a sugar component that is found in insoluble fiber alongside glucose, arabinose, galactose, and other sugars. It also appears in soluble fiber, which includes pectin, uronic acids, and other high-molecular-weight carbohydrates (Lamothe *et al*., 2015). To the best of the author's knowledge, there is no information available regarding the NMR profile of xylose in studies on *A. caudatus* species or any other related *Amaranthus* species. Thus, no comparison could be made with the NMR profile of xylose against other *Amaranthus* species.

In this study, adenosine, alanine and aspartate were found in both *A. caudatus* and *A. hypochondriacus*. Adenosine, alanine, and aspartate serve as stress-regulating metabolites. In the present study, it is considered that the green *Amaranthus* species (*A. hypochondriacus*) cultivated in the open field secrete aspartate, which allows for good tolerance to salinity in the soil compared to other vegetables, as indicated by Li *et al*., (2010).

Additionally, trehalose and glutamine were also detected in the current study, and previous studies suggest that they are secondary metabolites and responsible for the growth and development of *Amaranthus* species (Nemadodzi and Managa, 2024). Therefore, these compounds are referred to as growth-promoting metabolites, and this was confirmed by Nemadodzi and Managa, (2024). In plants, trehalose acts as a protectant against several abiotic stresses, such as drought, heat, ultraviolet rays, and high salinity (Garg *et al*., 2002). In addition, trehalose is responsible for seed maturation (Nadeem *et al*., 2013). Also of note is that trehalose found in the amaranth species of this study enables the plant to have the ability to endure harsh environmental and climatic conditions (Nkobole and Prinsloo, 2021). Arabinose was also reported in *A. caudatus* and *A. hypochondriacus*. Researchers have alluded to the fact that arabinose is a constituent of plant cell walls and plays a crucial role in the synthesis of cell wall intermediates, flavonoids, and signaling peptides (Rautengarten *et al*., 2017). Zayed, (2019), found that arabinose-based glycoproteins help plants tolerate salt stress better.

Betaine was also annotated in both green and red *Amaranthus* species in this study.

Betaines are secondary nitrogenous metabolites present in the seeds, fruits, flowers, leaves, stems, and roots of the Amaranthaceae family to which they confer their characteristic red-yellow pigmentation, as well as multiple properties, including antioxidant, anti-cancer, antilipidemic, and antimicrobial activity (Gengatharan *et al*., 2016; Hu *et al*., 2020; Otálora *et al*., 2019). Furthermore, betaine enhances the production of photosynthetic pigments, chlorophyll, and morphological features of plants (Schluepmann *et al*., 2009).

Organic acids that were annotated in *A. caudatus* and *A. hypochondriacus* species include fumaric acid, malic acid and succinic acid as illustrated in Table 3.2. These metabolites are intermediates of primary and secondary metabolism. Fumarate, malic acid, and succinate are intermediates of the tricarboxylic acid cycle (TCA) and are involved in regulating pH in plants (Araujo *et al*., 2011). In addition, succinic acid and malic acid have scavenging effects on reactive oxygen species (ROS), according to Guo *et al*. (2016). Malic acid is a predominantly organic acid associated with taste and flavouring. Malic acid also promotes plant growth by increasing the plant's chlorophyll content and mitigating stress damage to photosynthetic structures; therefore, it increases plant biomass (Chen *et al*., 2020).

Another organic metabolite that was annotated in this study was phenylacetic acid. Phenylacetic acid serves as a plant hormone (auxin). Auxins are a class of phytohormones that are essential for coordinating plant growth and development. Phenylacetic acid's main activity in plants appears to be in lateral root induction and root growth promotion (Cook, 2019; Perez *et al*., 2023).

The third region (5.5–10 ppm) is known as the aromatic region. One important metabolite that was annotated in this region was vanillic acid (VA). VA was annotated in both *Amaranthus* species of this study (Table 3.2). VA is a naturally occurring phenolic acid that can be found in many plants. VA helps plants tolerate environmental stresses by regulating biochemical and physiological processes (Matejczyk *et al*., 2024). Additionally, VA improves silage fermentation by decreasing the pH value, dry matter loss, and ammonia-N proportion. VA also acts as a signaling molecule or substrate for soil microorganisms, which can affect plant health (He *et al*., 2021). Another study described

VA as a flavouring agent and a benzoic acid derivative showing a broad range of biological activities, including antioxidants, anti-inflammatory, and neuroprotective effects (Ullah *et al*., 2021). Another important secondary metabolite detected in the present study is adenosine. Adenosine is reported to be induced as a signal and/or stimulus response to wounds and stress in plants, and this includes prolonged drought conditions (Prusinski, 2017).

Metabolites such as carnitine and caffeic acid were detected in both *A. caudatus* and *A. hypochondriacus* grown in the open field and serve as defence mechanism metabolites and were confirmed by Nemadodzi and Managa (2024). Lescano *et al*. (2016) reported that carnitine enhances the tolerance capacity of an organism against salinity stress and therefore plays an important role in the plant's defence. Jinal *et al*. (2020) reported that carnitine conjugates are derived from both the catabolism of branched-chain amino acids (BCAAs) and fatty acid oxidation. According to Oney-Birol (2019), carnitine is used to transport fatty across the membranes and serves as an alternative acyl-group receiver to buffer the BCAA pool (fatty acids), typically under stress conditions when BCAA becomes limited. Caffeic acid has been found to be slightly higher in concentration in *A. caudatus* samples than in *A. hypochondriacus* samples. Caffeic acid is a defence mechanism metabolite and has been reported to enable plants to exhibit a strong odour that repels herbivores (insects and pests), bacterial and viral infections, and inhibit pathogenic growth (Aneja and Gianfagna, 2001). Caffeic acid is a polyphenol and has been classified as a hydroxycinnamic acid (HCA) (Espíndola *et al*., 2019). Caffeic acid is a secondary metabolite in lignin biosynthesis and has been reported to have potent antioxidant properties (Espíndola *et al*., 2019).

Other notable metabolites that were annotated in the third region of both *Amaranthus* species in this current study include chlorogenate and trigonelline. In this study, chlorogenic acid was found to be higher in *A. caudatus* than in *A. hypochondriacus*. Chlorogenic acid (CA) is a phenolic compound commonly found in human plant-based diets. CA has been found to have hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant, and other pharmacological properties (Yan *et al*., 2020). In this study, trigonelline has been found to be high in concentration in *A. caudatus* and low

concentration in *A. hypochondriacus*. It has been shown in studies that trigonelline tends to be beneficial in diabetes cases by lowering blood glucose and lipid levels while boosting insulin sensitivity index and insulin content (Zhou *et al*., 2013). To the best of the author's knowledge, no comparison could be made between the NMR profile of trigonelline to other studies on *A. caudatus* species. However, another study reported that trigonelline was found in *A. cruentus* and *A. hybridus* species of their study but not in high concentration (Nkobole and Prinsloo, 2021).

Lastly, this study was done in the open field, and therefore environmental conditions played a vital role, and the plant's composition and reactions to these environmental conditions might have played a role in the release of pheromones, volatile organic compounds, and plant secondary metabolites to survive herbivore attacks (like insects and pests) and harsh environmental conditions (like weather, drought stress, etc.).

3.5. Conclusion and recommendations

NMR-based metabolomics was used to determine the compounds that play a vital role in *the A. caudatus* and *A. hypochondriacus* species. All the metabolites that were annotated in this study were present in all three *Amaranthus* extracts. The metabolomics approach using ^{1}H-NMR spectroscopy successfully annotated 31 metabolites, such as leucine, GABA, valine, alanine, arginine malic acid, betaine etc. as illustrated in Table 3.2. In this study, xylose and trigonelline were found to be high in *A. caudatus* samples. Trigonelline, which was present in both *Amaranthus* species, is known for its pronounced hypoglycemic effect. Caffeic acid, also present in both *Amaranthus* species of the current study, has been reported to possess potent antioxidant properties. Future studies should include growing these two species under greenhouse conditions to determine what the difference in compounds will be between open-field cultivation and greenhouse conditions. In addition, the use of liquid chromatography mass spectrometry (LC-MS) is advised to confirm the annotation of the metabolites that were found in this study.

3.6. References

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CHAPTER 4

In vitro α-amylase and α-glucosidase inhibitory activities of *Amaranthus caudatus* and *A. hypochondriacus* spp.

Abstract

Diabetes mellitus is a chronic metabolic disorder characterized by uncontrolled hyperglycemia that is usually due to lack of insulin secretion, its action or both. One of the therapeutic and prevention approaches of controlling postprandial hyperglycemia in Type 2 diabetes mellitus is inhibiting the digestion of dietary carbohydrates. Amaranth is well recognised as a highly nutritional superfood with significant nutraceutical characteristics and has demonstrated notable therapeutic advantages. Amaranth has extreme adaptability to adverse growing conditions, but it is also an edible crop and contains some important antidiabetic properties. The *in vitro* inhibitory activity of α-glucosidase and α-amylase was assessed in this study using *A. caudatus* and *A. hypochondriacus* leaf extracts. The results of this study demonstrated moderate α-glucosidase enzyme activity, with the highest inhibitory activity (52.67% inhibition) observed in *A. hypochondriacus* red variety, followed by *A. hypochondriacus* green variety (51.81% inhibition), and *A. caudatus* (50.10% inhibition) at the lowest concentration (3.125 mg/mL) tested. Acarbose (positive control) inhibited α-glucosidase at 53.16% at the lowest concentration (0.3125 mg/mL) tested. According to the IC_{50} values, acarbose had an IC_{50} value of 1.274 mg/mL which was lower than any of the plant extract values. The IC_{50} value of the plant extracts against α-glucosidase ranged from 6.71 mg/mL (*A. hypochondriacus* green) to 8.39 mg/mL (*A. caudatus*), respectively. Meanwhile, the extracts of *A. caudatus* showed low inhibitory activity (10.652 ± 0.034%) against α-amylase at the lowest concentration tested (1.56 mg/mL) whereas green *A. hypochondriacus* (AHG) variety showed highest enzyme inhibitory activity (63.169 ± 0.057%) at the same concentration tested. Acarbose inhibited α-amylase by 66.516 ± 0.026 at 0.125 µg/mL (the lowest concentration) tested. The green *A. hypochondriacus* plant extract (AHG) was a good inhibitor of α-amylase because it had potent inhibitory activity of 63.169 ± 0.057% at the lowest concentration (1.56 mg/mL).

The AHG extract had a higher IC_{50} value of 4.32 mg/mL against α-amylase inhibitory activity than acarbose which had an IC_{50} value of 0.23 mg/mL.

Keywords: diabetes mellitus, phytochemicals, blood glucose, *Amaranthus*, alpha-glucosidase, alpha-amylase.

4.1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by uncontrolled hyperglycemia that is usually caused by a lack of insulin secretion, its action or both (Moradi *et al*., 2018). In diabetes mellitus, elevated level of blood glucose over a long period can be associated with several acute or chronic complications (Löe, 1993). Inhibiting the digestion of dietary carbohydrates is a therapeutic and preventive method to manage postprandial hyperglycemia in Type 2 diabetes mellitus (T2DM). Pancreatic α-amylase is an enzyme that converts starch and other carbohydrates into monosaccharides during digestion. Furthermore, the monosaccharides are degraded by α-glucosidases to glucose which, on absorption, enters the bloodstream. Inhibiting α-amylase and α-glucosidase enzymes might delay glucose uptake and lower blood sugar levels, which, if uncontrolled, can lead to hyperglycemia (Kajaria *et al.*, 2013; Yang *et al*., 2020).

Miglitol, voglibose, and acarbose are the only α-glucosidase inhibitors that are currently being utilised in clinical practice for the treatment of patients with T2DM (Dirir *et al*., 2021). There is an urgent requirement to find more potent and efficient antidiabetic medications with minimal or no adverse effects. Throughout history, wild herbs have been utilized to naturally treat various illnesses, including diabetes mellitus (Showkat *et al*., 2020). Due to their cost-effectiveness and limited negative side effects, these plants are now seen as valuable medicinal herbs and carriers of potentially healing chemical components. Along with their nutritional value, these plants harbor a wide variety of bioactive compounds with beneficial nutraceutical qualities (Showkat *et al*., 2020).

The inhibition of α-amylase and α-glucosidase activity by plants is attributed to their phytochemical constituents. Natural α-glucosidase inhibitors derived from plants include alkaloids, flavonoids, anthocyanins, terpenoids, curcuminoids, and phenolic compounds (Kumar *et al*., 2011). Hussain *et al*., (2022) reported that the methanolic extract of *Momordica charantia* showed potent α-glucosidase inhibition activity and significantly improved blood glucose levels and insulin in diabetic rats. Acarbose showed higher α-glucosidase inhibition (79.91 ± 0.77%) *in vitro* than *M. charantia* methanolic extract (72.30 ± 0.30%). In another study done by Hbika *et al*. (2022), it was reported that ethyl acetate extracts of *Artemisia absinthium* (IC_{50} = 0.155 ± 0.0009 mg/mL) showed higher α-glucosidase inhibition activity *in vitro* than the aqueous extract, aqueous extract (IC_{50}= 0.170 ± 0.0002 mg/mL). Acarbose, which was used as a positive control had an IC_{50} value of 0.148 ± 0.002 mg/mL. Mahnashi *et al*., (2022) reported that β-caryophyllene epoxide isolated from *Persicaria hydropiper*, showed strong α-glucosidase and α-amylase inhibitory activities.

The wildly grown indigenous vegetable crops of South Africa can play a crucial role in the battle against diabetes mellitus through their wildly grown indigenous vegetable crops. For example, isolated compounds, namely rosmanol and 7-methoxyrosmanol which were isolated from *Salvia aurita* showed good α-glucosidase inhibitory activity while 12-methoxycarnosic acid and carnosol isolated from *Salvia aurita* showed strong α-amylase inhibitory activity (Etsassala *et al*., 2020). In another study, Jadalla *et al*., (2022) reported that the isolated compounds namely, 3-O-methylquercetin, allopatuletin, and 3-methylethergalangin which were isolated from *Helichrysum cymosum* possessed good α-glucosidase inhibitory activities while 3-O-methylquercetin isolated from *Helichrysum pandurifolium* showed poor α-amylase inhibitory activity.

In recent years, *Amaranthus* species started to gain more attention due to their pharmaceutical effect on T2DM and its nutraceutical profile (Kumar *et al*., 2011; Oboh *et al*., 2013; Nkobole *et al*., 2021). The present study aimed to investigate the *in vitro* inhibitory effects of *A. caudatus* and *A. hypochondriacus* leaf extracts on α-amylase and α-glucosidase, two diabetes-related carbohydrate metabolizing enzymes.

4.2. Materials and methods (α-glucosidase)

4.2.1. Components and chemicals

Sigma-Aldrich, South Africa supplied the α-glucosidase assay activity kit (MAK123), α-amylase enzyme (porcine pancreas), 3,5-dinitrosalicylic acid (DNS), sodium dihydrogen phosphate, potassium sodium tartrate tretahydrate, potato starch, acarbose and methanol.

4.2.2. Collection of plant material

Study site, planting and harvesting of plant materials are discussed in sections 3.1.1 to 3.1.3.

4.2.3. Extraction of plant material (sample preparation)

The leaves of *A. caudatus* (AC)*, A. hypochondriacus* red (AHR) and *A. hypochondriacus* green (AHG) species were pulverized into a homogenous powder using an electric blender. About a hundred grams (100 g) of the plant materials of each species were extracted in 2 mL of methanol (MeOH). The mixture was vortexed for 15 minutes and thereafter centrifuged for 15 minutes at room temperature for each plant sample. One sample per species was extracted and tested.

4.2.4. *In vitro* α-glucosidase inhibition assay

The α-glucosidase inhibition assay was done only one time. Using a commercially available kit (MAK123-1kt, Sigma-Aldrich, South Africa), the α-glucosidase activity was determined through a reaction in which α-glucosidase hydrolyses p-nitrophenyl-α-d-glucopyranoside. This reaction produces a colorimetric (405 nm) product that is proportional to the α-glucosidase activity. The concentrations of plant extracts ranged from 3.125 to 25 mg/mL. Acarbose was used as a positive control with concentration values of 0.3125 to 2.5 mg/mL. To summarise the procedure, 20 µL of water was added to two wells of a clear 96-well plate. In addition, 200 µL of water was placed into one well, and 200 µL of calibrator was added to the other. Approximately 20 µL of each sample

was transferred to different wells of the plate. Finally, each sample received 200 µL of the Master Reaction, which included 200 µL of phosphate buffer and 8 µL of α-NPG. The results were expressed as inhibition percentage, which were calculated using the following formula,

Inhibitory activity (%) = (Ac-As)/Ac ×100

where, Ac is the absorbance of control and As is the absorbance of the test substance.

4.2.5. α-Amylase inhibitory activity protocol

This assay was adjusted from Telagari and Hullatti, (2015), where plant extract concentrations ranged from 1.56-25 mg/mL. The concentrations of acarbose, ranged from 0.125 to 2 µg/mL. Acarbose is the positive control of the study. A mixture of 50 µl phosphate buffer, 10 µl α-amylase (2 U/mL), and 20 µl extract was pre-incubated in a 96- well plate for 20 minutes at 37°C. As a substrate, one percent of a soluble potato starch (100 mM phosphate buffer pH 6.8) was added and incubated for 30 minutes at 37°C. The colour reagent, DNS (100 µl), was added and heated for 10 minutes. The absorbance of the resultant mixture was determined at 540 nm by using a Thermo 79 Scientific Varioskan Flash Spectrophotometer. The results were expressed as percentage inhibition, which were calculated by using the formula:

Inhibitory activity (%) = (Ac – As)/Ac ×100

where, Ac is the absorbance of control and As is the absorbance in the presence of test substance.

4.2.6. Statistical analysis

The results were expressed in mean ± SD. GraphPad (version 10.4) was used to calculate half-maximal inhibitory concentration (IC_{50}).

4.3. Results

4.3.1. α-Glucosidase inhibitory activity

Three plant extracts were evaluated for their α-glucosidase inhibitory activity (Figures 4.1–4.3; Tables 4.1 and 4.2). At the highest tested concentration (25 mg/mL), the extracts demonstrated inhibition ranging from 22.32% to 48.69%. Among them, *A. caudatus* (AC) showed the lowest inhibition (50.10%) at the lowest tested concentration (3.125 mg/mL), whereas the red variety of *A. hypochondriacus* (AHR) exhibited the highest inhibition (52.67%) at the same concentration (Figure 4.2 and Table 4.1). The extracts were associated with relatively high IC_{50} values: 8.39 mg/mL for AC, 8.16 mg/mL for AHR, and 6.71 mg/mL for AHG (Tables 4.1 and 4.2).

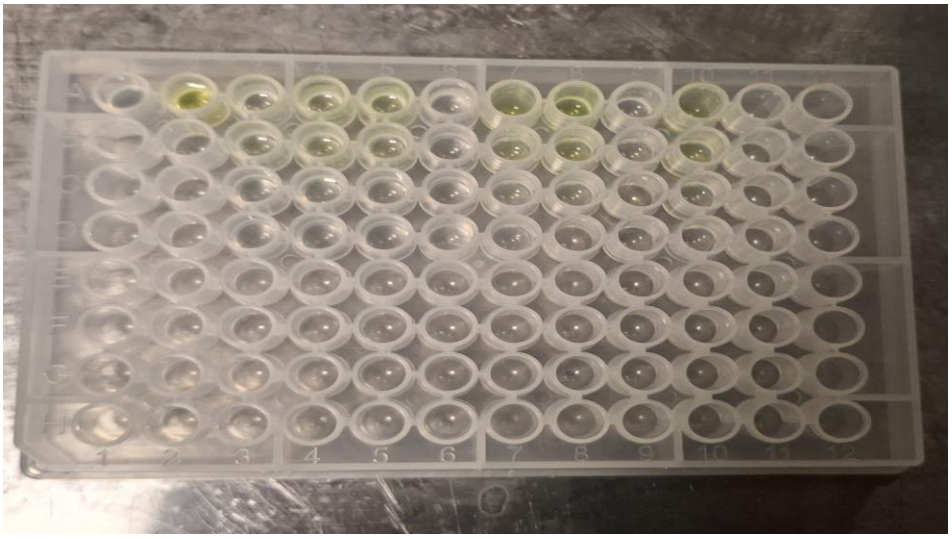

Figure 4.1: Inhibition of α-glucosidase enzyme by the plant extracts. Activation of the enzyme results in formation of yellow colour on 96-well plates, inhibition is indicated by light-yellow colour as compared to the wells where enzyme is activated.

Table 4.1: The inhibition of α-glucosidase enzyme and IC_{50} values.

α-glucosidase (% inhibition)	**3.125 mg/mL**	**6.25 mg/mL**	**12.5 mg/mL**	**25 mg/mL**	**IC_{50} mg/mL**
A. caudatus (AC)	50.10	51.82	39.76	41.74	8.39
A. hypochondriacus red (AHR)	52.67	50.00	38.61	36.38	8.16

A. hypochondriacus green (AHG)	51.81	52.46	41.99	22.32	6.71

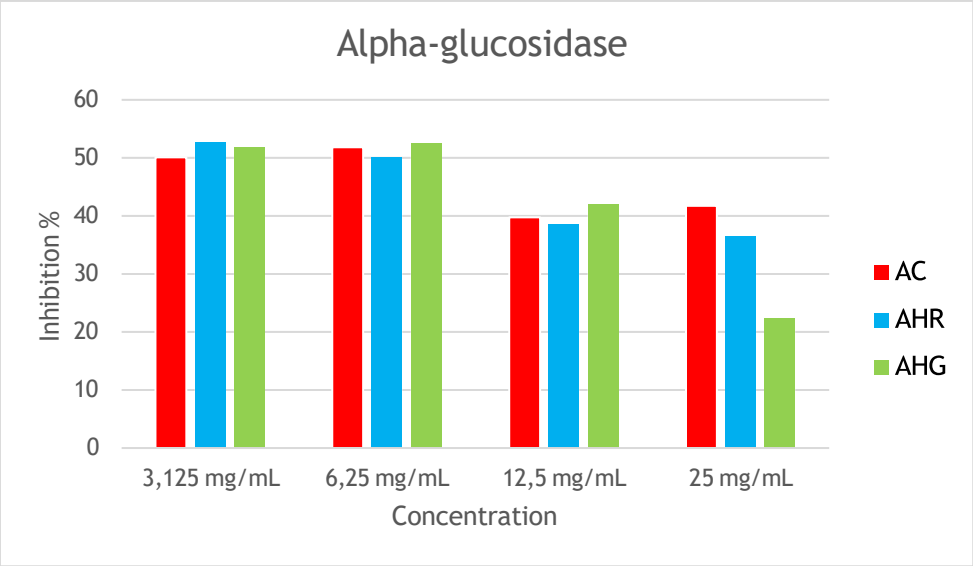

Figure 4.2: Inhibition of α-glucosidase by the plant extracts using p-nitrophenyl α-D-glucopyranoside as a substrate.

Acarbose was used as the standard reference drug (positive control), which exhibited 45.96% and 53.16% enzyme inhibition against α-glucosidase at 2.5 mg/mL and 0.125 mg/mL, respectively (Figure 4.3, and Table 4.2). The IC_{50} value for acarbose was 1.274 mg/mL, which is lower than the plant extracts values.

Table 4.2: Effect of acarbose (positive control) on the inhibition of α-glucosidase enzyme and IC_{50} values.

α-glucosidase (% inhibition)	**0.3125 mg/mL**	**0.625 mg/mL**	**1.25 mg/mL**	**2.5 mg/mL**	**IC_{50} mg/mL**
Acarbose (positive control)	53.16	53.17	53.21	45.96	1.274

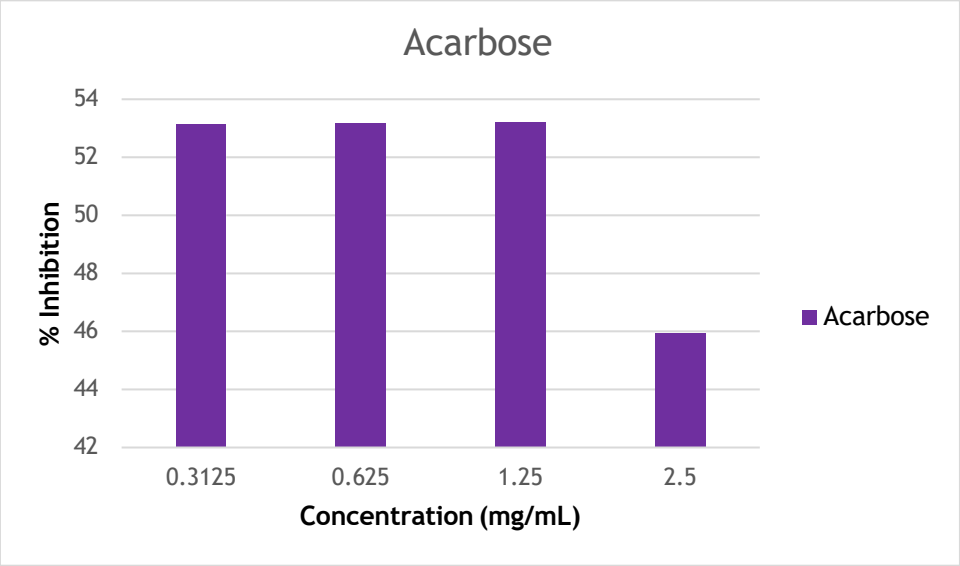

Figure 4.3: Inhibition of α-glucosidase by acarbose (positive control) using p-nitrophenyl α-D-glucopyranoside as a substrate.

4.3.2. α-Amylase inhibitory activity

Three plant extracts were tested for α-amylase enzyme inhibitory activities (Figures 4.4, 4.5 and 4.6; Tables 4.3 and 4.4 Both plant samples (*A. hypochondriacus* red and *A. caudatus*) showed a low inhibitory activity of α-amylase lower than 50%. Only *A. hypochondriacus* green (AHG) extract showed better activity at 63.169 ± 0.057% enzyme inhibition at the lowest concentration tested (1.56 mg/mL) (Figure 4.5 and Table 4.3). The extract of *A. caudatus* showed the weakest enzyme activity (10.652 ± 0.034%) tested against α-amylase at the lowest concentration (1.56 mg/mL). Only the green *A. hypochondriacus* (AHG) extract had an IC_{50} value of 4.32 mg/mL, as the other two plant extracts had no IC_{50} values, because their inhibition was lower than 50%.

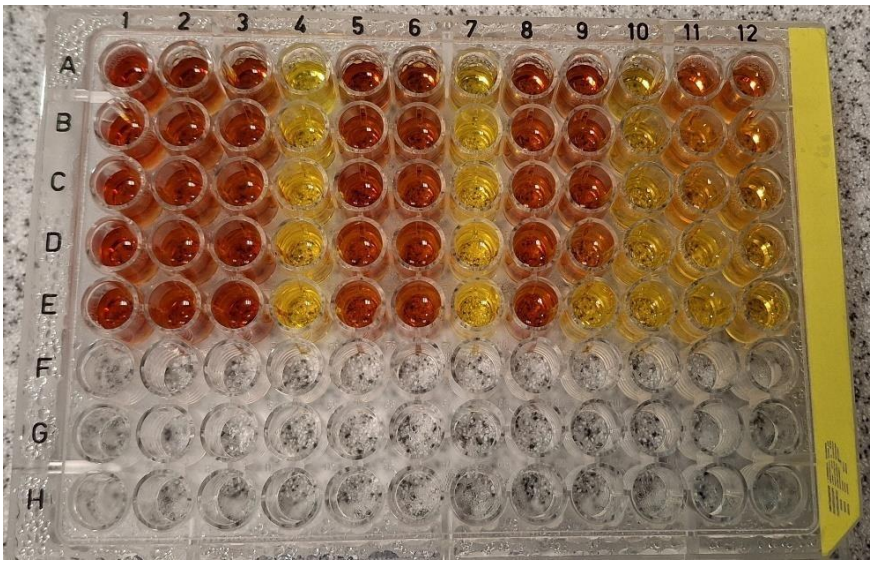

Figure 4.4: A 96-well plate based method for the estimation of α-amylase inhibition. Activation of the enzyme results in the formation of dark red colour on 96-well plates. Inhibition is indicated by light-yellow/ orange colour as compared to the wells where enzyme is activated.

Table 4.3: The inhibition of α-amylase enzyme and IC_{50} values.

α-amylase (% inhibition)	**1.56 mg/mL**	**3.125 mg/mL**	**6.25 mg/mL**	**12.5 mg/mL**	**25 mg/mL**	**IC_{50} mg/mL**
A. caudatus (AC)	10.652 ± 0.034	18.257 ± 0.047	32.122 ± 0.009	34.639 ± 0.216	34.195 ± 0.258	N/A
A. hypochondriacus red (AHR)	33.819 ± 0.223	32.574 ± 0.029	16.083 ± 0.214	24.550 ± 0.037	23.074 ± 0.008	N/A
A. hypochondriacus green (AHG)	63.169 ± 0.057	43.836 ± 0.013	28.823 ± 0.038	34.177 ± 0.414	31.60 ± 0.817	4.32

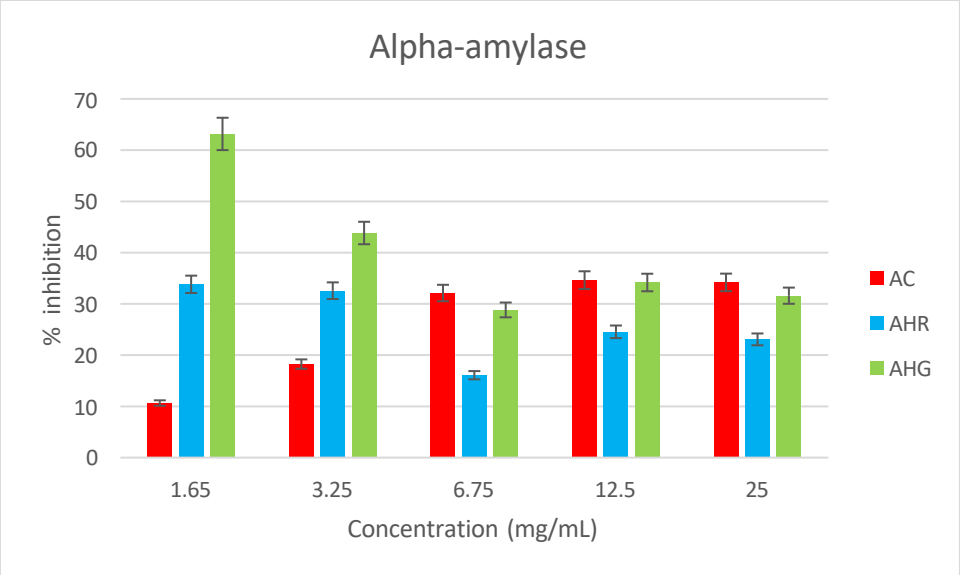

Figure 4.5: Inhibition of α-amylase using soluble potato starch as a substrate by the extracts.

Acarbose (positive control), had 66.516 ± 0.026% enzyme inhibition at the lowest concentration (0.125 mg/mL) and 29.614 ± 0.044% enzyme inhibition at the highest concentration (2 mg/mL) tested, respectively. In addition, the IC_{50} value of acarbose (positive control) was 0.23 mg/mL. When comparing the two IC_{50} values, *Amaranthus hypochondriacus* green (AHG) extract had a higher IC_{50} value than acarbose. AHG and acarbose showed potent enzyme inhibition and *A. hypochondriacus* red (AHR) and *A. caudatus* (AC) were weak inhibitors against α-amylase.

Table 4.4: Effect of acarbose (positive control) on the inhibition of α-amylase enzyme and IC_{50} values.

α-amylase (% inhibition)	**0.125 mg/mL**	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**	**2 mg/mL**	**IC_{50} mg/mL**
Acarbose (positive control)	66.516 ± 0.026	64.355 ± 0.004	54.578 ± 0.059	49.505 ± 0.032	29.614 ± 0.044	0.23

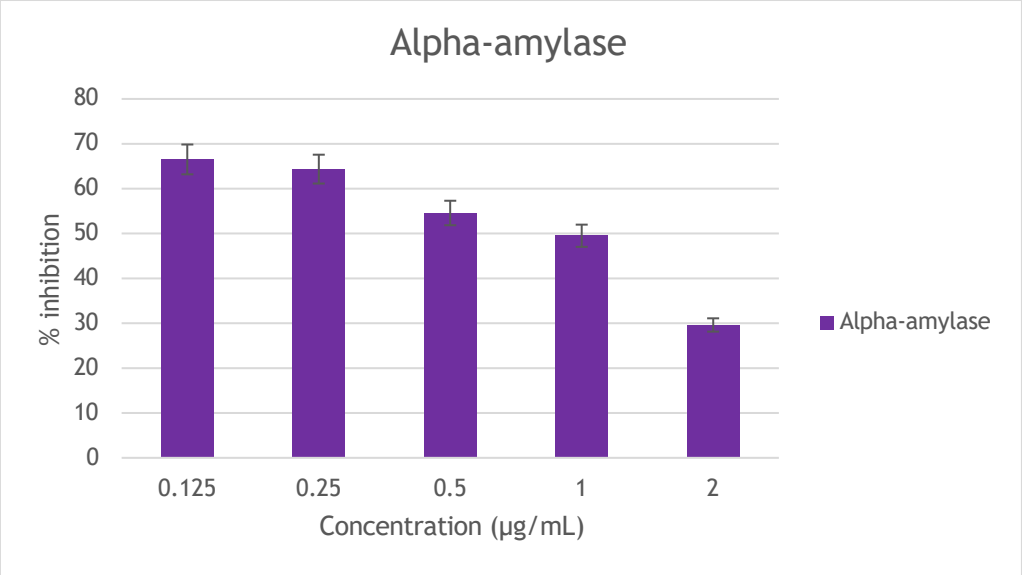

Figure 4.6: Inhibition of α-amylase using soluble potato starch as a substrate showing acarbose (positive control).

4.4. Discussion

4.4.1. α-Glucosidase inhibitory activity:

α-glucosidase enzyme breaks down di- and oligosaccharides into glucose residues which are subsequently absorbed in the small intestines (Showkat *et al*., 2020). In this study, the methanol (MeOH) extracts of both *A. caudatus* (AC) and *A. hypochondriacus* (AHG and AHR) leaves showed moderate activities against α-glucosidase. A similar study done by Showkat *et al*. (2020) showed contrasting results than the present study, where *A. caudatus* enzyme inhibition was dose-dependent and had inhibition values ranging from 43.62% at the highest concentration (0.6 mg/mL) to 1.92% at the lowest concentration (0.01 mg/mL) tested, respectively with a recorded IC_{50} value of 0.68.74±0.13 mg/mL. The moderate α-glucosidase enzyme inhibitory activity of the three plant sample extracts tested in this study can be explained by α-glucosidase inhibitors present in extracts that decrease the rate of glucose absorption in the intestines through competitive and reversible inhibition of the intestinal α-glucosidase enzyme. This reduces the absorption of glucose after a meal and thus plays a beneficial effect in controlling the postprandial blood sugar levels (Chan *et al*., 2018). A similar study done by Oboh *et al*. (2013), showed

that ethanolic extracts of *A. cruentus* leaves were strong inhibitors of α-glucosidase but mild inhibitors of α-amylase, and this can be supported by the study of Nkobole *et al*., (2021).

Secondary plant metabolites with bioactive characteristics are responsible for the pharmaceutical properties of plants (Dirir *et al*., 2020). Phytochemicals such as terpenes and flavonoids that represent the largest chemical classes that exhibit inhibitory activities against this enzyme (Dirir *et al*., 2020). Higher α-glucosidase inhibitory activity was associated with an enrichment of terpenoids, fatty acids, and flavonoids (Daou *et al*., 2022). Amongst the identified molecules, active compounds with known α-glucosidase inhibitory activity were detected, including unsaturated fatty acids, triterpenoids, and flavonoid glycosides (Daou *et al*., 2022). Another example of phytochemicals are morusin and procyanidin A2 which were isolated from the shoots of *Wendlandia glabrata* showed good α-glucosidase inhibitory activity with IC_{50} values of 3.19 μM and 0.47 μM, respectively (Sheikh *et al*., 2019; Dirir *et al*., 2020). In another study, anthraquinones such as alaternin which inhibited the α-glucosidase enzyme had a good IC_{50} value of 3.45 μM (Jung *et al*., 2017).

4.4.2. α-Amylase inhibitory activity

This study showed very low α-amylase inhibitory activity (less than 50%) in two plant extracts of *A. caudatus* (AC) and *A. hypochondriacus* red (AHR) species. On the other hand, *A. hypochondriacus* green (AHG) species showed potent α-amylase inhibition activity (63.169 ± 0.057%) at the lowest concentration (1.56 mg/mL) tested with an IC_{50} value of 4.32 mg/mL. In a study done by Kazeem *et al*. (2015), a low percentage of α-amylase inhibition on *Senna alata* was reported, which can point to the fact that the plant is a mild inhibitor of the enzyme. In a similar study, Showkat *et al*. (2020) found that *A. caudatus* aqueous extract was shown to be dose dependent and had α-amylase inhibition of 1.51% at the lowest concentration (0.01 mg/mL) and 53.03% at the highest concentration (0.6 mg/mL) tested, respectively and had an IC_{50} value of 0.56 ± 0.047 mg/mL, which is contradicting the results found in the present study. In another study, the

methanolic extracts of *A. caudatus* against α-amylase enzyme inhibition was 44.01 ± 0.12%, 65.56 ± 0.18%, and 74.98 ± 0.11% at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL, respectively, and had an IC_{50} value of 19.233 µg/mL (Kumar *et al*., 2011). In another study, Nkobole *et al*. (2021) reported that *A. cruentus* and *A. hybridus* were weak inhibitors of α-amylase. In addition, *A. cruentus* enzyme inhibition against α-amylase showed 30.46% at the lowest concentration (0.125 mg/mL) and 24.03% at the highest concentration (2 mg/mL) tested. In the same study, *A. hybridus* showed 33.18% at the lowest concentration (0.125 mg/mL) and -7.55% at the highest concentration (2 mg/mL) tested, respectively. A similar study done by Conforti *et al*. (2005) demonstrated that two varieties of *A. caudatus* seeds in methanolic, ethyl acetate, and n-hexane extracts showed high α-amylase inhibitory activity (above 80%) at 0.25 -1 mg/mL concentrations tested. In contrast to this study, Das and Das, (2022) reported the methanolic extracts of *A. viridis* Linn. leaves had relatively high α-amylase inhibitory potential with values ranging from 57.12 ± 3.86 at 10 mg/mL; 63.37 ± 5.874 at 20 mg/mL; 75.17 ± 2.056 at 30 mg/mL; 75.89 ± 2.589 at 40 mg/mL; and 77.51 ± 3.053 at 50 mg/mL, respectively. A similar study done by Moein *et al*., (2017) reported that *Otostegia persica* had high α-amylase (99.4 ± 0.94%) activity tested at concentration of 7.4 mg/mL.

4.5. Conclusion

The two enzymes, α-glucosidase and α-amylase, are involved in carbohydrate metabolism and as a result, they can reduce blood glucose levels. Under *in vitro* conditions, *A. caudatus* and *A. hypochondriacus* varieties showed moderate α-glucosidase and low α-amylase inhibitory activities (less than 50%) except for the *A. hypochondriacus* green (AHG) variety, which showed good inhibitory activity against α-amylase. According to the IC_{50} values, *A. hypochondriacus* red (AHR) and *A. caudatus* varieties showed low α-amylase and moderate α-glucosidase inhibitory activity while *A. hypochondriacus* green (AHG) showed potent α-amylase and moderate α-glucosidase inhibitory activity. The IC_{50} value of acarbose was better than *A. hypochondriacus* green (AHG) enzyme extract against α-amylase. In conclusion, despite these results, more research should be done; for example, more types of enzymes against plant extracts can be tested to see the difference in inhibitory activity because not all inhibitory results will

give good results, and some will even show no enzyme activity. Moreover, research should be done to identify and isolate the compounds that might be responsible for the high activity in the plant extracts found in this study. In addition, mechanistic studies of methanolic *Amaranthus* extracts under clinical settings can be conducted before they can be recommended as an anti-diabetic agent.

4.6. References

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CHAPTER 5

In vitro antioxidant activities of *Amaranthus caudatus* and *A. hypochondriacus*

Abstract

Amaranth is well recognized as a highly nutritious superfood with significant nutraceutical characteristics and has demonstrated notable therapeutic advantages. Amaranth is considered a rich source of antioxidants, primarily due to the presence of phytochemicals such as flavonoids, phenolic acids, carotenoids, and vitamin C within the plants' leaves. Phytochemicals contribute to the plants significant antioxidant activity and this helps to counteract oxidative stress. The aim of the study was to evaluate the antioxidant activity of *Amaranthus caudatus* and *A. hypochondriacus* leaf extracts. The extract's ability to quench free radicals was investigated using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging method. Findings of this study showed that both *Amaranthus* spp. showed promising results with *A. caudatus,* showing an inhibitory percentage range of 63.92 to 11.51%. In addition, *A. hypochondriacus* red variety and *A. hypochondriacus* green variety showed a percent inhibition range of 68.55 to 29.18 % and 66.70 to 22.13% at 5 to 0.04 mg/mL concentration tested, respectively. The IC_{50} values of *A. caudatus, A. hypochondriacus* red variety and *A. hypochondriacus* green variety were 0.06 mg/mL, 0.03 mg/mL and 0.04 mg/mL, respectively, when tested using the ABTS radical scavenging assay. Vitamin C (positive control) showed an IC_{50} value of 5.5 µg/mL. In conclusion, this study demonstrated that *A. caudatus* and the two varieties of *A. hypochondriacus* have considerable antioxidant activity as evidenced by the ABTS radical scavenging assay.

Keywords: antioxidants; diabetes mellitus; hyperglycaemia; phytochemicals; *Amaranthus*

5.1. Introduction

Oxidative stress plays a significant role in the development of age-related diseases such as arthritis, diabetes, dementia, cancer, vascular diseases, obesity and metabolic syndromes (Tan *et al*., 2015; Liu *et al*., 2017). Oxidative stress occurs when the production of reactive oxygen species (ROS) surpasses the ability of a cell's antioxidant system to

neutralize them, and it can affect the photosynthesis, respiration, and nutrient uptake of plants and reducing growth and yield (Sun *et al*., 2024). ROS oprate as signaling molecules, regulating various cellular processes, such as gene expression, hormone synthesis, cell division and cell death, all of which are essential for plant growth, development, and stress responses (Sun *et al*., 2024). However, if ROS levels become too high, they can cause damage to plant cells if they accumulate in excess. ROS can disrupt the structure and function of lipids, nucleic acids, and proteins, causing oxidative stress. Plants must balance production and removal of ROS so that oxidative stress can be avoided (Sachdev *et al*., 2021).

Plants have developed a complex antioxidant system to remove excess reactive oxygen species (ROS) and protect cellular components from oxidative damage (Sun *et al*., 2024). The antioxidant system consists of both enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants consist of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), nonspecific peroxidase (POX), monodehydroascorbate reductase (MDHAR), monodehydroascorbate reductase (DHAR), glutathione *S*-transferase (GST), glutathione peroxidase (GPX), alternative oxidase (AOX), and peroxiredoxin (Prx) (Sachdev *et al*., 2021; Rajput *et al*., 2021). On the other hand, nonenzymatic antioxidants consist of ascorbic acid (AsA), glutathione (GSH), carotenoids, tocopherols, and flavonoids (Sachdev *et al*., 2021). Each of these antioxidants have a distinct mechanism of action and can target a variety of ROS.

There is a growing interest in medicinal plants as a potential source of antioxidants. Medicinal plants include significant antioxidant compounds with considerable promise in alleviating oxidative stress-related degenerative diseases while exhibiting low cytotoxicity (Arika *et al*., 2019). For example, the aqueous extracts of *Allium saralicum*, *Falcaria vulgaris*, and *Thymus kotschyanus* were all found to exhibit antioxidant effects through the degradation of free radicals (Hamelian *et al*., 2018; Mahdi *et al*., 2018; Goorani *et al*., 2019). They were found to increase the concentration levels of superoxide dismutase, catalse, glutathione peroxidase, and malondialdehyde while reducing levels of glutathione. Furthermore, *A. saralicum, F. vulgaris*, and *T. kotschyanus* were found to contain phytochemicals such as alkaloids, anthraquinones, flavonoids, phenolic acids,

saponins, steroids, and tannins, which have been reported to have antioxidant effects in cellular systems (Hamelian *et al*., 2018; Mahdi *et al*., 2018; Goorani *et al*., 2019). Many indigenous vegetables have been investigated in search of novel antioxidants in which *Amaranthus* species are included. For example, Amaranth leaf extracts were found to contain phenolics, to scavenge free radicals, and inhibit nitric oxide production (Conforti *et al*., 2011).

Various methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) assays, have been used to analyze the antioxidant activities of *Amaranthus* plant extracts. Therefore, the aim of the present study was to evaluate the *in vitro* antioxidant potential of *A. caudatus* and *A. hypochondriacus* leaf extracts and thus to confirm similar results as in previous studies.

5.2. Materials and methods

5.2.1. Chemicals

Sigma-Aldrich®, South Africa supplied 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), ascorbic acid and methanol.

5.2.2. Collection of plant Material

Collection, planting and harvesting of plant materials were discussed under sections 3.1.1 to 3.1.3.

5.2.3. Extraction of plant material (sample preparation)

One sample per species was extracted and tested. The leaves were pulverized into a homogenous powder using an electric blender. About a hundred grams (100 g) of the plant materials of each species were extracted in 2 mL of methanol (MeOH). The mixture was vortexed for 15 minutes and subsequently centrifuged for 15 minutes at room temperature.

5.2.4. ABTS scavenging assay protocol

The ABTS assay followed the method described by More and Makola, (2020). The free radical scavenging activity of plant extracts and ascorbic acid was determined using the ABTS radical cation decolorisation test. The ABTS cation radical production was generated by mixing 10 mg of ABTS and 2 mg potassium persulfate in water. The solution was stored in the dark at room temperature for approximately 12 to16 hours before use. The ABTS solution (1 mL) was then diluted with 60 mL of methanol. Measurements were then gradually carried out in duplicate. The percentage of inhibition of absorbance at 734nm which was measured in an ELISA microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Vantaa, Finland) was calculated by using the formula,

Inhibitory activity (%) = (Ac – As)/Ac ×100

where, Ac is the absorbance of control and As is the absorbance in the presence of test substance.

5.2.3. Statistical analysis

GraphPad was used to calculate half-maximal inhibitory concentration (IC_{50}).

5.3. Results

The ABTS assay was used to test the antioxidant activity of three *Amaranthus* plant extracts (AC- *A. caudatus*; AHR- *A. hypochondriacus* with red variety; AHG- *A. hypochondriacus* with green variety) with Vitamin C serving as the positive control (Figures 5.1, and Table 5.1).

The scavenging activity of the *Amaranthus* species leaf extracts tested at the concentration range of 0.04 to 5 mg/mL was assessed using the ABTS cation assay. The inhibited percentage of extracts on the antioxidant activity ranged from 59.48% to 63.92% at the highest concentration tested (5 mg/mL) and ranged from 11.51% to 29.28% at the lowest concentration (0.04 mg/mL) tested (Figure 5.1 and Table 5.1). The *A. hypochondriacus* red variety extract exhibited an IC_{50} value of 0.03 mg/mL, followed by

the green variety of the same species and *A. caudatus*, which had an IC_{50} value of 0.04 mg/mL and 0.06 mg/mL, respectively. Ascorbic acid/ vitamin C served as a positive control and was tested at 25 times lower concentrations (1.563- 200µg/mL) than those of the extracts. Vitamin C exhibited inhibition ranging from 98.789% and 15.721% against ABTS at 200 µg/mL and 1.563 µg/mL, respectively (Figure 5.2 and Table 5.2). The IC_{50} value for vitamin C was calculated to be 5.5 µg/mL (Table 5.2).

Table 5.1: Effect of plant extracts on the antioxidant activity (ABTS assay) and IC_{50}values.

ABTS (% inhibition) (mg/mL)	0.04	0.08	0.16	0.31	0.63	1.25	2.5	5	IC_{50}
A. caudatus (AC)	11.51	45.99	44.60	63.17	65.74	66.66	58.90	63.92	0.06
A. hypochondriacus red (AHR)	29.18	52.16	68.55	65.61	64.83	65.60	65.77	62.88	0.03
A. hypochondriacus green (AHG)	22.13	42.06	66.70	65.39	63.43	64.32	64.00	59.48	0.04

Table 5.2: Effect of Vitamin C (positive control standard) on the antioxidant activity (ABTS assay) and IC_{50}values.

ABTS (% inhibition) (µg/mL)	1.563	3.125	6.25	12.5	25	50	100	200	IC_{50}
Vitamin C	15.721	28.308	51.611	85.018	98.922	98.907	98.858	98.789	5.50

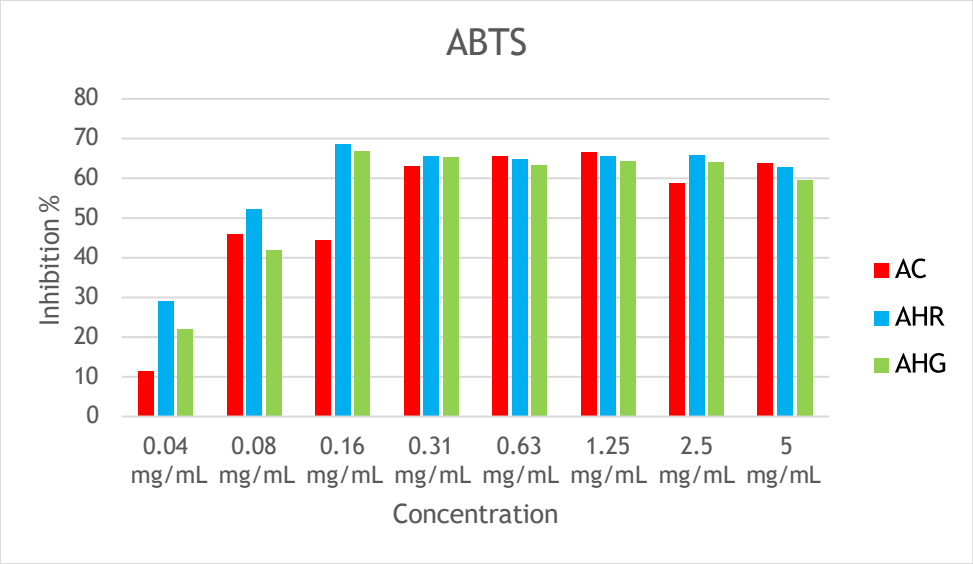

Figure 5.1: ABTS cation radical scavenging activities of various concentrations of *A. caudatus* (AC); *A. hypochrondriacus* red variety (AHR) and *A. hypochondriacus* green variety (AHG).

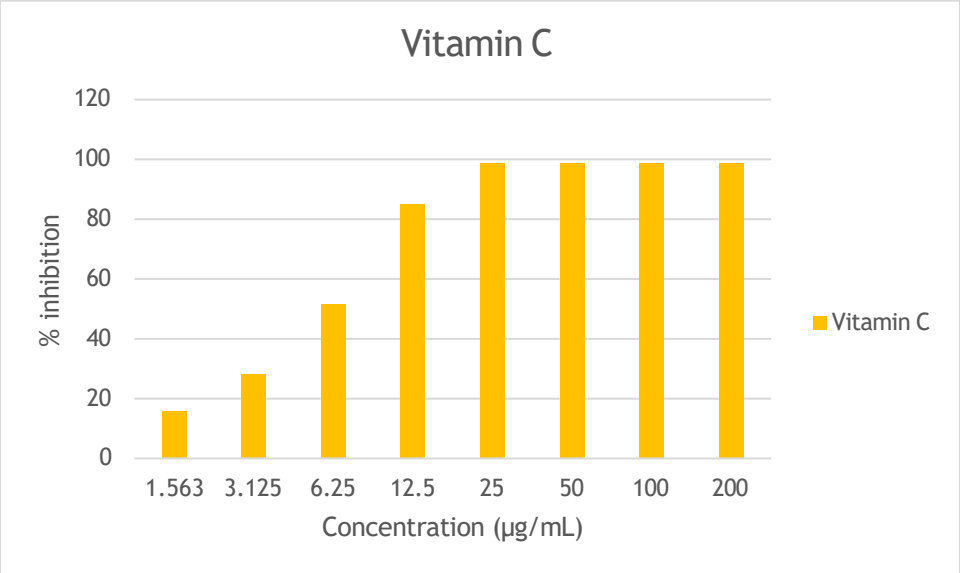

Figure 5.2: ABTS cation radical scavenging activities of various concentrations of Vitamin C (positive control).

5.4. Discussion

In this study, the methanol extracts of *A. caudatus* and *A. hypochondriacus* leaves exhibited good antioxidant activity where especially *A. caudatus* showed promising antioxidant activity from 69.32% to 11.51% at concentration of 5 to 0.04 mg/mL, respectively. A similar study demonstrated that the methanolic extract of *A. caudatus* was also found to be extremely effective in scavenging ABTS radical activity (IC_{50} value of 48.75 ± 1.1 µg/mL) (Kumar *et al*., 2011). In another study, *A. tricolor* had higher phenolic content and antioxidant capacity compared to *A. viridus*, due to the presence of a red anthocyanin pigment (Routrey *et al*., 2013). In a study by Bang *et al*. (2021), it was reported that the antioxidant activities of the grain species (*A. caudatus, A. cruentus*, and *A. hypochondriacus*) were lower than those of the vegetable species of *Amaranthus* (*A. tricolour L., A. blitum L., A. dubious*, and *A. viridis*) and the three weed species (*A. hybridus, A. spinosus, and A. crispus*). Consequently, the authors alluded to the fact that the vegetable species and weed species had higher amounts of phytochemicals compared to the grain species types.

Antioxidants play a vital role in preventing and managing diabetes by counteracting the excessive production of free radicals (oxidative stress), which is a significant contributor to diabetic complications, including damage to blood vessels, nerves, and organs (Zhu *et al*., 2023). In diabetes mellitus, oxidative stress is perpetuated by the production of free radicals and the suppression of the existing antioxidant system (Caturano *et al*., 2023). Oxidative stress, which is mainly mediated by hyperglycemia-induced generation of free radicals, contributes to the development and progression of diabetes, thus alleviating oxidative stress through treatment with antioxidants is an effective strategy for reducing diabetic complications (Johansen *et al*., 2005).

The leaves of the *Amaranthus* species such as *A. caudatus* and *A. hypochondriacus* are rich in phytochemicals such as lysine-rich protein, β-carotene, various vitamins, minerals and dietary fiber (Das, 2016). The phytochemical compounds that are present in the *Amaranthus* species, such as phenolic acids and flavonoids, contribute to its antioxidant

potential (Sarian *et al*., 2017). Phenolics and flavonoids from plants are thought to be potential classes of nutraceuticals with strong antioxidant activities (Fatima *et al*., 2020). Antioxidant agents, like polyphenols and flavonoids, are direct scavengers of free radicals, resulting in more stable, less-reactive radical species (Nijveldt *et al*., 2001; De Mello Andrade and Fasolo, 2014). Polyphenols dramatically reduce ROS/reactive nitrogen species, including hydroxide, oxygen, nitric oxide, and peroxynitrite, preventing biomolecule damage or the creation of additional ROS (Bang *et al*., 2021).

Furthermore, the antioxidant activity in the *Amaranthus* spp. is largely attributed to its diverse array of phytochemicals, including betacyanins (red pigments), betaxanthins (yellow pigments), and tocopherols (vitamin E) (Sarker *et al*., 2024). Given its antioxidant potential, neuroprotective, and anti-inflammatory properties, *Amaranthus* spp. shows promise as a natural therapeutic agent for managing oxidative stress-related diseases, such as diabetes mellitus.

5.5. Conclusion:

This study demonstrated that *A. caudatus* and the two varieties of *A. hypochondriacus* have considerable antioxidant activity as evidenced by the ABTS radical scavenging assay. The methanolic extracts showed dose-dependent inhibition of free radicals. The phytochemical compounds that are present in the *Amaranthus* species, such as phenolic acids and flavonoids, are likely to contribute to its antioxidant potential. *Amaranthus* spp. shows promise as a natural therapeutic agent for managing oxidative stress-related diseases because it possesses antioxidant potential and anti-inflammatory properties. Future research should focus on isolating specific compounds responsible for these effects and testing their efficacy in clinical settings.

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CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1. General Conclusion

In recent times, the amaranth plant gained popularity due to its high nutritional and medicinal properties. It has been extensively utilised in various clinical and medicinal contexts because of its therapeutic advantages (Soriano-García and Aguirre-Díaz, 2019). Additionally, it is quite adaptable to harsh growing conditions like drought, amaranth is also an edible crop that possesses antidiabetic and antioxidant properties. This study therefore investigated the chemical profile of two Amaranth species, namely *A. caudatus* and *A. hypochondriacus.* Furthermore, the antidiabetic and antioxidant activities of *A. caudatus* and *A. hypochondriacus* were also determined in this study.

Plant material utilised in the study were established at the University of South Africa's Science campus in Florida, which is situated in Johannesburg, Gauteng. Objective one of this study was to use a metabolomics approach (in this study NMR) to compare the metabolite differences in the leaves of *A. caudatus* and *A. hypochondriacus*. NMR technology with multivariate analysis was successful in analyzing the chemical profile differences, as well as annotating metabolites that contributed to groupings between *A. caudatus* and *A. hypochondriacus* samples. The experimental protocol for NMR in metabolomics was meticulously followed to obtain a trustworthy outcome.

By combining ^{1}H-NMR analysis and OPLS-DA chemometrics, variations leading to samples grouping of the two *Amaranthus* species were done in this study. The OPLS-DA model of this study showed high goodness of fit (R^2X= 0.995) and predictability (R^2Y= 0.981). Subsequently, chemical annotation was performed to determine which compounds were predominant in the *A. caudatus* and *A. hypochondriacus* samples. Contribution plots were performed to detect metabolites that might be accountable for the clustering found in the OPLS-DA score plots between samples. Compounds such as trigonelline and xylose were more pronounced in the samples of *A. caudatus* compared to *A. hypocondiacus*. Chenomx, the Human Metabolome Database, and published literature were used to successfully annotate the metabolites that were found in the two samples by means of correlating the chemical shifts to classes of metabolites (Table 3.2).

This study found that the compounds annotated are mostly similar in both species. Noticeably, there were 31 compounds annotated in this study. Some compounds that were reported in *A. caudatus* and *A. hypochondriacus* of this study were amino acids (leucine, valine, threonine, GABA, alanine, tryptophan, etc.); sugars (glucose, sucrose, maltose, and trehalose); organic acids (malic acid, mannonic acid and citric acid); and phenolic acids (chlorogenic acid, caffeic acid, vanillic acid, and ferulic acid). An important aromatic compound that was reported in both *A. caudatus* and *A. hypochondriacus* leaves was trigonelline, acts as a stress protector against various abiotic stresses, such as heat, drought, high salinity, and ultraviolet rays. Chlorogenic acid, which is known for its antidiabetic and antioxidant properties, was also annotated in both *A. caudatus* and *A. hypochondriacus* species. Trigonelline was reported to be higher in *A. caudatus* according to Table 3.2. Trigonelline and chlorogenic acid have been reported to contain antidiabetic properties and were annotated in both *A. caudatus* and *A. hypochondriacus* of this study.

From the metabolomics chapter of this study, the two compounds that contain antidiabetic properties, trigonelline and chlorogenic acid, have been annotated in both *A. caudatus* and *A. hypochondriacus* although trigonelline was higher in *A. caudatus*. All the samples underwent *in vitro* tests to determine their α-amylase and α-glucosidase inhibitory activity to hypothesize whether the presence of trigonelline and chlorogenic acid could have any impact on biological activity. To manage type 2 diabetes, blocking α-amylase and α-glucosidase enzymes can decrease the conversion of starch to glucose (Oboh et al., 2012). It should be noted that all extracts showed moderate α-glucosidase inhibitory activity. According to the IC_{50} values, acarbose had an IC_{50} value of 1.274 mg/mL which was lower than any of the plant extract values. The IC_{50} value of the plant extracts against α-glucosidase ranged from 6.71 mg/mL (*A. hypochondriacus* green) to 8.39 mg/mL (*A. caudatus*), respectively. On the other hand, *A. caudatus* and *A. hypochondriacus* red variety extracts did not inhibit α-amylase beyond 50%. The *A. hypochondriacus* green variety extract had a higher IC_{50} value of 4.32 mg/mL against α-amylase inhibitory activity than acarbose which had an IC_{50} value of 0.23 mg/mL. Thus, *A. caudatus* and *A.*

hypochondriacus leaves possess noticeable *in vitro* α-glucosidase inhibitory activities and mild α-amylase inhibitory activities.

From the metabolomics chapter (Chapter 3), trigonelline has been found to be high in concentration in *A. caudatus* (0.1 mM) and low concentration in *A. hypochondriacus* (0.03 mM). Comparing this to the antidiabetic activity (Table 4.1 and 4.3) which showed that the inhibition % of α-glucosidase and α-amylase were similar in both *Amaranthus* species, probably indicating that trigonelline is not the major antidiabetic compound in these *Amaranthus* species. Synergism between trigonelline and other compounds, however, could be a possibility, resulting in higher antidiabetic activity in *A. caudatus*. Trigonelline achieves amelioration of diabetes, the mechanisms of which include the modulation of insulin secretion, a reduction in oxidative stress, and the improvement of glucose tolerance and insulin resistance (Liang *et al*., 2023).

Another compound that was annotated in this study, chlorogenic acid (CA), which is known for its antidiabetic and antioxidant activities, was found to be higher in *A. caudatus* (0.2 mM) than in *A. hypochondriacus* (0.1 mM). CA reduces the production of reactive oxygen species (ROS) and the morphological changes of cells caused by STZ, thereby protecting *β* cells (Yan *et al*., 2020) and might therefore be one of the compounds contributing to the antidiabetic activity. Synergism with other compounds might again be resulting in higher antidiabetic activity in *A. hypochondriacus*.

The contribution plot (Figure 3.3) and NMR peaks (Table 3.2) showed that *A. hypochondriacus* had a higher concentration of the sugars (region 4-5). For example, sucrose in *A. hypochondriacus* showed a concentration of 1.04 mM whereas *A. caudatus* extract, the concentration was 0.38 mM using the anomeric proton peaks at 5.4 ppm for qualitative analysis. The higher sugar (sucrose) concentrations in *A. hypochondriacus* cannot be linked with any antidiabetic activity studies. In contrast, only xylose was different and higher in *A. caudatus* (0.64 mM at 5.2 ppm) than *A. hypochondriacus* (0.46 mM), respectively. Even though there was slightly higher xylose concentration in *A. caudatus*, the antidiabetic results (Table 4.1 and 4.3) showed that the inhibition % of α-glucosidase was higher in *A. hypocondriacus* and similar for α-amylase for both

Amaranthus species. Researchers alluded to the fact that D-xylose as a sugar complement regulates blood glucose levels by suppressing phosphoenolpyruvate carboxylase (PEPCK) in streptozotocin-nicotinamide-induced diabetic rats and by enhancing glucose uptake *in vitro* (Kim *et al*., 2016). Kim *et al*., (2016) also reported that *in vitro,* 2-[N-(7-notrobenz 2-oxa-1,3-daizol-4-yl)amino]-2- deoxy-d-glucose (2-NBDG) uptake by C2C12 cells and insulin secretion by INS-1 cells were increased with D-xylose supplementation in a dose-dependent manner compared to treatment with glucose alone.

The last objective focused on the antioxidant activity of the two *Amaranthus* species. Antioxidants play a vital role in preventing and managing diabetes by counteracting the excessive production of free radicals (oxidative stress), which is a significant contributor to diabetic complications, including damage to blood vessels, nerves, and organs (Zhu *et al*., 2023). In diabetes mellitus, oxidative stress is perpetuated by the production of free radicals and the suppression of the existing antioxidant system (Caturano *et al*., 2023). Oxidative stress, which is mainly mediated by hyperglycemia-induced generation of free radicals, contributes to the development and progression of diabetes, and alleviating oxidative stress through treatment with antioxidants is an effective strategy for reducing diabetic complications (Johansen *et al*., 2005). This study demonstrated that *A. caudatus* and the two varieties of *A. hypochondriacus (*AHR and AHG*)* have considerable antioxidant activity as evidenced by the ABTS radical scavenging assay. The methanolic extracts showed dose-dependent inhibition of free radicals. Findings of this study showed that both *Amaranthus* spp. showed promising results. The IC_{50} values of *A. caudatus* was 0.06 mg/mL; *A. hypochondriacus* red variety, 0.03 mg/mL and *A. hypochondriacus* green variety, 0.04 mg/mL, respectively, in the ABTS radical scavenging assay. Vitamin C (positive control) had an IC_{50} value of 5.5 µg/mL. Given its antioxidant potential, neuroprotective, and anti-inflammatory properties, *Amaranthus* spp. shows promise as a natural therapeutic agent for managing oxidative stress-related diseases.

By integrating metabolomics (chapter 3) with the antioxidant activity results (Chapter 5) the following results were noted. Caffeic acid has been found to be slightly higher in concentration in *A. caudatus* samples (0.06 mM using 7.9 ppm as a reference peak) than in *A. hypochondriacus* samples (0.02 mM at 7.9 ppm). Linking the antioxidant activity with

the slightly higher caffeic acid concentration found in *the A. caudatus* species do not support the lower antioxidant activity when compared to the better activity in *A. hypochondriacus* species. Caffeic acid scavenges reactive oxygen species (ROS) generated under abiotic stress conditions, thereby protecting plant cells from oxidative damage. Caffeic acid can upregulate the expression of genes encoding antioxidant enzymes, and stress-related proteins, enhancing the plant's ability to cope with abiotic stresses (Mughal *et al*., 2024). The slight differences in the concentration of the compound can therefore not explain the difference in antioxidant activity.

To conclude, the aim of this study was achieved, as the metabolites found in the two *Amaranthus* species through the NMR-based metabolomics approach are reported. In addition, antidiabetic and antioxidant properties were successfully demonstrated, although the link between the metabolites, antidiabetic and antioxidant activity should be investigated in more detail.

6.2. Recommendation

Recommendations that can be made for future studies include the following:

- In metabolomics, analytical tools such as LC-MS should be incorporated to identify compounds that cannot be detected by NMR, such as secondary metabolites.
- Molecular docking can establish the ligand binding of the metabolites that are annotated in the study and further investigate the compound’s mode of action against α-glucosidase and α-amylase enzymes.
- *In vitro* cell culture models can further be used to investigate the antidiabetic potential of the two *Amaranthus* species used in this study to validate the *in vitro* results. In addition, cytotoxicity studies should be carried out to assess the potential toxicity of the samples before considering preclinical studies that involve animals.
- It is also recommended that prospective studies should focus on using grains instead of leaves of *Amaranthus* (*A. caudatus* and *A. hypochondriacus*) employing the same experimental methods as this study to determine if the results yield comparable outcomes.

6.3. Referencing

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7. APPENDIXES

APPENDIX I: COLLEGE ETHICS APPROVAL

UNISA university of south africa

College of Agriculture and Environmental Sciences_Health REC

NHREC Registration # : REC-170616-051
Ref #: 2024/CAES_HREC/2686
Name: Mr Niel Snyman
Student #: 61990175

Date: 23/01/2024

Dear: Mr Niel Snyman

Decision: Ethics Approval from 18/01/2024 to 31/12/2026

Researcher: Mr Niel Snyman
61990175@mylife.unisa.ac.za 0662061248

Supervisor: Dr Nolitha Nkobole nkobon@unisa.ac.za

Antidiabetic, antioxidant activities and metabolomics of *Amaranthus graecizans* and *A. hypochondriacus*

Qualification: MSc Agriculture

Thank you for the application for research ethics clearance by theCollege of Agriculture and Environmental Sciences_Health RECfor the above mentioned research study. Ethics approval is granted for three years, **subject to submission of yearly progress reports. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report is submitted.**

Due date for progress report: 18 January 2025

The **low risk application** was **reviewed** by College of Agriculture and Environmental Sciences_Health REC on 18 January 2024 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the College of Agriculture and Environmental Sciences_Health REC .
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.

5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.

6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data requires additional ethics clearance.

7. No field work activities may continue after the expiry date (18 January 2025). Submission of a completed research ethics progress report will constitute an application for renewal, for Ethics Research Committee approval.

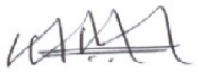
Additional Conditions

1. Disclosure of data to third parties is prohibited without explicit consent from Unisa.
2. De-identified data must be safely stored on password protected PCs.
3. Care should be taken by the researcher when publishing the results to protect the confidentiality and privacy of the university.
4. Adherence to the National Statement on Ethical Research and Publication practices, principle 7 referring to Social awareness, must be ensured: "Researchers and institutions must be sensitive to the potential impact of their research on society, marginal groups or individuals, and must consider these when weighing the benefits of the research against any harmful effects, with a view to minimising or avoiding the latter where possible." Unisa will not be liable for any failure to comply with this principle.

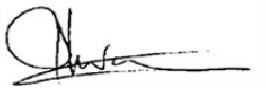
Note

The reference number 2024/CAES_HREC/2686 should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.

Kind regards,



Prof MA Antwi
Chair of College of Agriculture and Environmental Sciences_Health REC
E-mail: antwima@unisa.ac.za



Executive Dean / By delegation from the Executive Dean of College of Agriculture and Environmental Sciences_Health REC
E-mail: ntwasmm@unisa.ac.za