ANTIDIABETIC ACTIVITY, METABOLOMICS AND NUTRITIONAL PROFILE OF WILD AND CULTIVATED *AMARANTHUS* SPP. AND ISOLATED COMPOUNDS

by

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I declare that the above dissertation is my own work and that all the sources that I have used or quoted 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.

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SIGNATURE **DATE**

PREFACE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

CH_3CN	Acetonitrile
ARC-VOP	Agriculture Research Council-Vegetable and Ornamental Plants
ALP	Alkaline phosphatase
ALVs	African Leafy Vegetables
^{13}C- NMR	C-13 nuclear magnetic resonance spectroscopy
CE-MS	Capillary electrophoresis-mass spectrometry
CAT	Catalase
$CHCl_3$	Chloroform
CC	Column chromatography
CD_3OD	Deuterated methanol
D_2O	Deuterium oxide
CH_2Cl_2	Dichloromethane
DNS	3, 5, di-nitro salicylic acid
d	Doublet
dd	Double doublet
EAC	Ehrlich's ascites carcinoma
EtOH	Ethanol
EtOAc	Ethyl acetate
HCOOH	Formic acid
GGT	Gamma-glutamyltransferase
GC-TOF-MS	Gas Chromatography Time-of-Flight Mass Spectrometry

GSH	Glutathione
GSSG	Glutathione disulphide
HMDB	Human metabolome database
LAN	Limestone ammonium nitrate
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoproteins
MDA	Malondialdehyde
MeOH	Methanol
MIC	Minimum inhibitory concentration
m	Multiplet
NPK	Nitrogen, phosphorous and potassium fertiliser
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
p-NPG	*p*-nitrophenyl-α-D-glucopyranoside
KH_2PO_4	Potassium dihydrogen phosphate
PCA	Principal component analysis
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
RE	Retinol equivalent
SGOT	Serum glutamic oxaloacetic transaminase
TG	Serum triglycerides
s	Singlet
Na_2CO_3	Sodium carbonate
SA	South Africa

SANHANES	South African National Health and Nutrition Examination Survey
TLC	Thin layer chromatography
TC	Total cholesterol
TT	Total thiols
TSP	Trimethylsilylpropionic acid sodium salt
t	Triplet
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VLDL	Very low-density lipoproteins
VAD	Vitamin A deficiency

GENERAL ABSTRACT

Diabetes mellitus is one of the major health problems in the world, with the incidence and associated mortality increasing. It is associated with high levels of blood sugar and inadequate regulation of blood sugar imposes serious consequences for health. Conventional antidiabetic drugs are effective, however, they have unavoidable side effects. Edible plants can act as an alternative source of antidiabetic agents. *Amaranthus cruentus* and *A. hybridus* (commonly known as Amaranth) belong to the Amaranthaceae family. Amaranth leaves are an inexpensive and excellent source of vitamins, including β-carotene, vitamin B6, vitamin C, riboflavin, folate, as well as dietary minerals like calcium, iron, zinc and potassium.

Previous studies focus mostly on the wild Amaranth variety and there is a dearth of information about the effect of cultivation on both primary and secondary metabolites which affect the activity of the plant. Thus, the study sought to assess the effect of wild and cultivated *A. cruentus* and *A. hybridus* leaf extracts on *in vitro* α-glucosidase and α-amylase enzymes. Furthermore, hypoglycaemic compounds from *A. cruentus* were isolated and elucidated and their antidiabetic activity determined. Vitamin A, zinc and iron content were also analysed.

The chemical profile of cultivated and wild *A. cruentus* and *A. hybridus* were determined by multivariate statistical analysis using Nuclear Magnetic Resonance (NMR) spectroscopy. Differences were observed in primary metabolites namely, sucrose and maltose. These metabolites were dominant in cultivated *Amaranthus* spp. when compared to their wild counterparts. In addition, a higher content of proline, an amino acid, was found in cultivated *A. cruentus* and *A. hybridus*, whilst leucine was only abundant in *A. hybridus*. Metabolites that were present in both wild and cultivated Amaranth in similar concentrations were: trehalose, *trans*-4-hydroxy-L-proline, trigonelline, betaine, valine, alanine, fumarate, formate and kynurenine. Trehalose and trigonelline are important contributors and regulators of water deficit stress responses in plants enabling *Amaranthus* to be resistant to water deficiency. Chlorogenic acid was annotated only on cultivated *A. hybridus*.

Findings on the antidiabetic activity of extracts demonstrate that methanol (MeOH) extracts of wild *A. hybridus* was a potent α-glucosidase inhibitor at the lowest concentration tested (0.125

mg/mL). Cultivated *A. cruentus* exhibited close to full inhibitory activity of the α-glucosidase enzyme. Notably, none of the extracts tested were able to inhibit the activity of α-amylase beyond 50%.

Wild *A. cruentus* showed good antidiabetic activity and a rich preliminary phytochemical profile. As a result it was selected for the isolation and identification of antidiabetic compounds. *Amaranthus cruentus* yielded three compounds; α-spinasterol, a plant sterol, and palmitic acid alongside pheophorbide A-methyl ester which was isolated from *A. cruentus* for the first time. The structures of isolated compounds were elucidated using proton (^{1}H) and carbon (^{13}C) NMR spectroscopy and comparison of spectral data with literature values. All compounds were potent α-glucosidase enzyme inhibitors. Palmitic acid in particular demonstrated the highest inhibition against α-glucosidase in all the concentration tested.

Finally, true to Amaranth's ability to store micronutrients, cultivated and wild Amaranth crops were rich in zinc. Cultivated *A. hybridus* and wild grown *A. cruentus* showed similar zinc concentration, 19.5 mg/100 g and 19.6 mg/100 g respectively. The highest iron amount was observed in cultivated *A. hybridus*. None of the samples accumulated vitamin A.

Keywords: *Amaranthus* spp., ^{1}H-NMR metabolomics, antidiabetic activity, cultivation, phytochemicals, α-glucosidase, α-amylase.

CHAPTER 1: GENERAL INTRODUCTION

1.1 BACKGROUND

Fruit and vegetables play an important role in human nutrition and health, particularly as sources of vitamin C, thiamine, niacin, pyridoxine, folic acid, minerals and dietary fibre (Asif, 2011). Other vital nutrients that fruit and vegetables provide include riboflavin, zinc, calcium, potassium, and phosphorus (Oguntibeju et al., 2013). The intake of vegetables has been strongly associated with improved gastrointestinal health, decreased risk of heart attack, certain forms of cancer and chronic conditions such as diabetes (da Silva Dias and Imai, 2017).

Diabetes mellitus (DM) is a serious, lifelong disease that has a significant impact on the lives and well-being of individuals, communities and societies around the world (Saeedi et al., 2019). DM is associated with high level of blood sugar, a situation where the body cannot effectively control the glucose metabolism, the primary energy source (Odeyemi and Bradley, 2018). It is characterized by hyperglycaemia due to a relative or absolute insulin deficiency or resistance to the cellular action of the hormone (Wadkar et al., 2008).

There are three major types of DM, Type 1 DM (T1DM), Type 2 DM (T2DM) and gestational diabetes (GDM which is prevalent in pregnant women) (Deutschlander, 2010). The focus of this thesis is on T2DM, thus the statistics presented below are concerned with T2DM.

DM is one of the top ten causes of adult mortality and was reported to have caused four million deaths worldwide in 2017 (International Diabetes Federation, 2017). Total diabetes health spending was projected at USD 727 billion in 2017 (IDF Diabetes Atlas, 2017). In 2009, 285 million people were estimated to have diabetes (T1DM and T2DM combined) (IDF Diabetes Atlas, 2009). This number had escalated to 425 million in 2017 (IDF Diabetes Atlas, 2017). According to the latest reports, the prevalence of global diabetes was estimated at 9.3% (463 million people) in 2019, rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saeedi et al., 2019). In 2017, there were 1,826,100 diabetes cases in South Africa with a prevalence of 5.4% (IDF Diabetes Atlas, 2017).

In order to understand diabetes aetiology, the normal physiological mechanism that occur during and after a meal must be understood. Food including proteins, fat and carbohydrate,

passes through the digestive system where nutrients are absorbed into the bloodstream (Steadman et al., 2019). The presence of glucose, a building block of carbohydrates, means that the hormone insulin is secreted by the endocrine pancreas (Nussey and Whitehead, 2013). Insulin causes almost all tissue types in the body to absorb and store sugar, particularly the liver, muscles and fat tissues (Röder et al., 2016). In T2DM, the body prevents the effects of insulin or does not produce enough insulin to maintain normal levels of glucose (Chatterjee et al., 2017). Long-term diabetes complications can ultimately become disabling or even life-threatening.

Currently, there is no cure for diabetes, but the likelihood of long-term complications with diabetes can be minimized through regulating blood sugar levels through a healthy diet, exercise and medication. According to Deutschlander, (2010); long-term complications that are experienced by diabetic patients are:

- kidneys – kidney disease and kidney failure
- feet – ulcers, infections, gangrene
- cardiovascular system – hardening of arteries, heart disease and stroke
- nerves – neuropathy (gradual damaging of nerves)
- eyes – cataracts and retinopathy (gradual damaging of the eye) that may lead to blindness

Remarkable progress has been made in development of synthetic drugs, however, investigations are being carried out to discover safe and cost-effective food sources for managing hyperglycaemia through diets rich in legumes (Gętek et al., 2014; Ademiluyi et al., 2015), fruits and vegetables (Asif, 2014), herbs and spices (Pereira et al., 2019). These plant foods consist of vital nutrients such as vitamins, minerals, dietary fibres, and important bioactive compounds such as polyphenols and carotenoids (Coman et al., 2020; Septembre-Malaterre et al., 2018; Pandey and Rizvi, 2009).

Amaranth has attracted attention in the last few decades due to the plant's genetic diversity, in addition to its extreme adaptability to adverse growing conditions (Achigan-Dako et al., 2014). Amaranth is not only an edible crop, but it is also reported to possess antidiabetic properties, among others (Kunyanga et al., 2012; Mondal et al., 2015). *Amaranthus hybridus* and *A. cruentus* were selected for the study on the basis of their availability and accessibility. Both of these species are grown and consumed in KwaZulu-Natal (KZN) Province. Due to limited

resources and time constraints, only KZN was selected as a collection site for wild Amaranth crops. In an effort to introduce *Amaranthus* in the urban areas particularly in the townships, a trial was established at the Mothong African Heritage Centre, Mamelodi, Pretoria. This site was chosen on the basis of its continued work of conserving and preserving indigenous plants of South Africa. The caretakers of the site are also at the forefront of teaching communities and surrounding schools about the importance of South African biodiversity.

The environment plays a vital role in the active substances responsible for plant's biological activity (Liu et al., 2016). Evaluating the effect of the environment on the plant's chemical profile can be achieved through the use of analytical tools such as NMR spectroscopy with multivariate data analysis (Lankatillake et al., 2019). This phenomenon is known as science of metabolomics. Metabolomics is a well-established multidisciplinary area of analytical biochemistry that combines classical analytical tools with multivariate statistical analyses to identify, semi- and/or quantify low molecular weight metabolites in biological systems (Lankatillake et al., 2019). In addition, through the use of metabolomics, it is possible to identify potential biomarkers that are responsible for the plant's known antidiabetic activities.

1.2 Aim of this study

The broad objective of the study is to apply NMR-based metabolomics in discriminating classes of primary and secondary metabolites / compounds in cultivated and wild Amaranth. In addition; the study seeks to evaluate antidiabetic properties of wild and cultivated *A. cruentus* and *A. hybridus*, as well as the isolated compounds. Finally; nutritional analysis of both cultivated and wild plant species was conducted.

Objectives of the study

- To determine the environment/genotype interaction of plants from different regions using NMR-based metabolomics.
- To evaluate the effect of wild and cultivated *A. cruentus* and *A. hybridus* for their *in vitro* α-glucosidase and α-amylase inhibitory properties
- To isolate hypoglycaemic compounds from *A. cruentus* and to determine their antidiabetic activity
- To evaluate iron and zinc content of *Amaranthus* spp. from different regions.

1.3 Scope of this thesis

The thesis consists of an introductory section and literature overview, followed by five experimental chapters.

Chapter 1 This chapter delineates between different types of diabetes mellitus, overall global diabetes mellitus statistics and the use of herbal medicine for the treatment of diabetes.

Chapter 2 This chapter discusses metabolomics in crop science research as well as the botanical and anatomical description of *Amaranthus* species. In addition; nutritional, pharmacological and phytochemical analysis of Amaranth plants are discussed.

Chapter 3 This chapter investigates the chemical variation of cultivated and wild *A. cruentus* and *A. hybridus* grown in different geographical areas using NMR based metabolomic analysis.

Chapter 4 The hypoglycaemic activity of wild and cultivated *A. cruentus* and *A. hybridus* is discussed.

Chapter 5 Deals with the isolation of the bioactive compounds from wild *A. cruentus* leaves as well as the evaluation of these isolated compounds for hypoglycaemic activity.

Chapter 6 Discusses the targeted analysis of 2-phenylethenamine in wild and cultivated *Amaranthus* spp.

Chapter 7 Discusses vitamin A, zinc and iron of cultivated and wild *Amaranthus* spp.

Chapter 8 Comprises a general discussion and conclusion.

Chapter 9 Appendices.

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CHAPTER 2: LITERATURE REVIEW

2. The role of plants in the treatment of diabetes mellitus

The treatment of diabetes mellitus (DM) in South Africa (SA) involves the use of oral drugs. The oral drugs that are currently used for the treatment of DM are delineated in Box 1. It is clear that DM is putting pressure on the already frail public health system in SA which caters for the majority of citizens (Delobelle, 2013). South Africa has a pluralistic health care system (Masola and Burman, 2018). Health pluralism is a term that refers to the coexistence of multiple health systems in a particular environment (Masola and Burman, 2018). About 80% of the population still relies on traditional medicines although the rate of use varies from province to province and from rural to urban areas (Nxumalo et al., 2011; Davids et al., 2014). Using plants for the treatment of diseases and ailments is not a new phenomenon in SA. This practice has been observed for many generations and is embedded in what is known as indigenous knowledge systems. Indigenous knowledge encompasses knowledge that is passed on from generations to generations by indigenous people in their communities (Dweba and Mearns, 2011). Such knowledge is central for the survival of the people it represents.

In the area of plant research; quite a number of authors have recorded the use of plants for the treatment of diseases or ailments in SA. For instance; Deutschländer et al. (2009), identified 32 species of plants, representing 20 families that are historically used to treat diabetes by healers. Plants belonging to 25 families that are traditionally used for the treatment of DM in the Eastern Cape were reported by Odeyemi and Bradley, (2018). Bapedi traditional healers in the Limpopo Province heavily rely on plants belonging to 20 families for the treatment of DM (Semenya et al., 2012).

There is a dearth of information with regard to the use of African leafy vegetables (ALVs) for the treatment of DM in SA. However, in the study conducted by Odhav et al. (2010), some local wild vegetables demonstrated the antidiabetic properties by inhibiting α-amylase enzyme, an enzyme responsible for increased blood glucose level. Even though the study was

not related to DM, Mokganya and Tshisikhawe, (2019), have reported 14 wild vegetables with medicinal value which are belong to 10 plant families. Wild vegetables have the potential to provide not only food for the SA population, but they are also a rich source of phytochemicals with medicinal properties. Since these wild vegetables are largely gathered from the wild, the demand for food and medicine can lead to the over exploitation of these vegetables.

The move towards promoting cultivation of wild species is a necessary step to cater for the growing demand for these species. The introduction of cultivation of wild vegetables open up avenues for new areas of research. As already alluded to in the previous chapter, metabolomics is one such research avenue that can be used to gain insight in the metabolite profile of wild and cultivated species. Although this technology is not new, its application in wild growing and cultivated ALVs in SA has never been done before. The first part of the sections that follows gives detailed information about metabolomics.

Dipeptidyl peptidase-4 inhibitors (DPP-4I)

These compounds are based on the incretin effect of a substance released from the intestine into the circulation in response to food ingestion modulating insulin secretion (Alhadramy, 2016). Two incretins are well characterized: the insulinotropic polypeptide (GIP) and the more strong GLP-1 (Kim and Egan, 2008). Both medications exhibit glucose-dependent insulin stimulation. This class can cause allergic reactions as extreme as angioedema and anaphylaxis (Karagiannis et al., 2014).

Alpha-glucosidase inhibitors

The two agents available in this class are acarbose and miglitol. Alpha-glucosidase inhibitors act by inhibiting pancreatic alpha-amylase and alpha-glucosidase enzymes found in the border cells that line the small intestine (Rosa and Dias, 2014). Acarbose and miglitol are active inhibitors of alpha glucosidase, modulating postprandial digestion and starch and disaccharides absorption (Rosa, and Dias, 2014). The two compounds' binding affinity differs, both acarbose and miglitol target alpha-glucosidases: sucrase, maltase, glycoamylase and dextranase. The clinical consequence of enzyme inhibition is to minimize the upper intestinal digestion and absorption of ingested starch and disaccharides in the distal small intestine, reduce glycemic post meal excursions and create an insulin-sparing effect (Deutschlander, 2010).

Insulin secretagogues: sulfonylureas

The major action of sulfonylureas is to increase insulin release from the pancreas by binding to 140kDa high-affinity sulfonylurea receptor (Proks et al., 2002). The binding of a sulfonylurea prevents the efflux of potassium ions through the

Sodium–glucose cotransporter-2 inhibitors (SGLT-2I)

The kidney plays a major role in homeostasis of glucose through the sodium-glucose cotransporter-2 (SGLT2) enzyme, which reabsorbs sodium and glucose in the proximal renal tubules. This enzyme can absorb about 90 percent of the glucose ingested daily (Gerich, 2010). There has been a development of potent inhibitors of this enzyme (SGLT-2I) (Karagiannis et al., 2014). Dapagliflozin was this group's first member, which was approved in 2012 in Europe. Dapagliflozin was not initially approved by the FDA because of concerns about breast and bladder cancer risk. However, after more safety data was available, the drug was approved in 2014 (Haas et al., 2014). In 2013, the FDA approved canagliflozin; whereas; empagliflozin was approved in 2014.

Biguanides

Biguanides are divided into three types: phenformin, buformin and metformin (Zhu et al., 2015). However, due to complications resulting to lactic acidosis, the US banned the use of phenformin and buformin (Gan et al., 1992). Metformin was derived from *Galega officinalis* L (Gunn et al., 2012). The active compound responsible for its antidiabetic property is known as galegine, a guanidine derivative (Rena et al., 2017). Although studies on the mechanism of action for metformin are inconclusive, it is believed to stimulate glycolysis in the tissue. Moreover, metformin increases the removal of glucose from the blood whilst reducing hepatic gluconeogenesis. As a result, glucose absorption from the gastrointestinal tract is slowed down with an increase in conversion of glucose to lactate by enterocytes and the reduction of plasmaglucagon levels (Deutschlander, 2010). Biguanides are most frequently prescribed for patients with refractory obesity whose hyperglycemia is due to insulin resistance (Olokoba

channel and results in depolarization of β-cell membrane. This, in effect, activates the opening of voltage-gated Ca^{2+} channels, resulting in Ca^{2+} inflow and an increase in intracellular Ca^{2+}, which stimulates the exocytosis of secretory granules containing insulin (Proks et al., 2002). The synthesis of insulin is not stimulated, and sulfonylureas may even decrease it. Some evidence suggests that serum insulin levels no longer increase but may even decrease after extended sulfonylurea therapy. Seven sulfonylurea drugs are available in the United States and are classified into agents of the first and second generations, which vary mainly in their potency. Tolbutamide, tolazamide, acetohexamide and chlorpropamide are included in the first generation, and glyburide, glipizide and glimepiride in the second generation (Alhadramy, 2016).

et al., 2012). Since metformin is an insulin-saving agent and does not increase weight or cause hypoglycaemia, it has an advantage over insulin and sulfonylureas in the treatment of hyperglycemia. The most common toxic effects of metformin are gastrointestinal and there is a risk of lactic acidosis (Blough et al., 2015).

Thiazolidinediones

Two types of thiazolidinediones are commercially available including rosiglitazone and pioglitazone (Ye, 2011). The exact mechanism of their action is not known, but their main action is to decrease insulin resistance in muscle and adipose tissue. Troglitazone was the first approved thiazolidinedione but was withdrawn due to its association with a low, but important incidence of idiosyncratic liver damage (Scheen, 2001). Two other thiazolidinediones, rosiglitazone and pioglitazone, showed similar efficacy to troglitazone, but with no evidence of hepatictoxoxicity (Scheen, 2001).

Box 1: Oral antidiabetic agents. Chemical structure of the compounds are found in the supplementary section.

2.1 Introduction of metabolomics applied to crop plants

Metabolomics is a well-established multidisciplinary field of analytical biochemistry combining conventional analytical tools with multivariate statistical analysis (Lankatillake et al., 2019). Its aim is the high-throughput identification and semi- and/or quantification of low molecular weight metabolites in biological systems (German et al., 2005; Roessner et al., 2011). The word "metabolome" refers to the complement of endogenous small molecules present in a cell or organism (primary or secondary metabolites) and is a direct representation of its physiological state and phenotype (Wishart, 2008). Studying the metabolome helps researchers to observe interactions between the environment, genome, and metabolism, and recognise particular family, genus, or species-specific metabolites (Lankatillake et al., 2019).

The application of metabolomics in food plants is diverse. This ranges from studies on the effect of water stress, salt stress, pest and disease infections and environmental interactions on crops to among others (Obata et al., 2015; Bowne et al., 2012).

For example, studies have shown that the expression of metabolites varied considerably between plants exposed to water stress and well-watered plants (Thomason et al., 2018). In a study performed by Obata et al. (2015), maize plants were grown under well-watered conditions (control) or exposed to drought, heat, and both stresses simultaneously. Findings reveal that drought stress caused many amino acids to accumulate, including isoleucine, valine, threonine, and 4-aminobutanoate. These amino acids have been reported in both field and greenhouse experiments in many plant species (Obata et al., 2015). In another study, levels of amino acids, most notably proline, tryptophan, and leucine, isoleucine, and valine branched chain amino acids, were increased in all cultivars under drought stress in bread wheat (Bowne et al., 2012). Metabolomics in crop research has also been applied to tomato to determine their metabolic profile in diseased and normal conditions (Galeano Garcia et al., 2018; Zeiss et al., 2019). The effect of salinity on crop plants has also been a subject of interest in metabolomics research. For an example; the gas chromatography-mass spectrometry (GC-MS) metabolic profiling of rice seedlings was performed under salt stress, which showed that important amino acids such as leucine, isoleucine, valine and proline were accumulated in higher levels (Gayen et al., 2019). Gupta et al. (2017), used the Gas Chromatography Time-Of-Flight Mass Spectrometry (GC-TOF-MS) technique to conduct comparative metabolic profiling of tolerant and sensitive rice where amino acid accumulation was observed in tolerant genotypes as compared to susceptible varieties. Finally, heat stress has been studied to determine its effect on crop plants such as soybean cultivars (Chebrolu et al., 2016), wheat (Qi, et al., 2017), maize (Sun et al., 2016) and tomato (Paupière et al., 2017). Results from Chebrolu et al. (2016) found that a vast array of antioxidant metabolites, including tocopherols, flavonoids, phenylpropanoids, and ascorbate precursors were found to be enriched in seed of the heat tolerant genotype. In a study by Qi et al. (2016), about 13 different metabolites including one organic acid, six amino acids, four sugars, and two polyols were identified under normal temperature in wheat. Further, 25 different metabolites including six organic acids, 12 amino acids, four sugars, and three polyols were identified under higher temperature. (Qi et al., 2016).

2.2 Analytical technologies commonly used in metabolomics research

For metabolomics studies, a variety of analytical techniques may be used, including GC-MS, capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-electrochemical spectrometry (LC-EC-MS), NMR spectroscopy, LC-NMR, direct infusion mass spectrometry (DIMS) and Fourier-Transform Mass Spectrometry (Tugizimana et al., 2013; Jiang et al., 2019). The section that follows delineates between MS and NMR, the two most popular techniques used in plant metabolomics analysis.

2.2.1 Mass Spectrometry (MS)

Chromatographic techniques distinguish components for the mobile and stationary phases, based on their affinity. The mobile and stationary phase can be used to classify chromatographic methods, for instance, into liquid chromatography (LC), gas chromatography (GC), and supercritical fluid chromatography (SFC) on the basis of the mobile phase (Fu et al., 2016). Mass spectrometry takes advantage of fragment ion mass-to-charge ratios derived from a molecule to analyse and classify structures (Mohimani et al., 2017). In addition, MS is preferred for its excellent sensitivity, high specificity, and simplicity without elaborate sample preparatory procedures (Liu et al., 2018). It can thus further enhance the resolution of unknown metabolites, particularly for isomer metabolites and poor chromatographic separation (Liu et al., 2014).

2.2.2 Nuclear Magnetic Resonance spectroscopy

The use of NMR-based fingerprints marked the start of metabolomics as a biochemical and phytochemical analysis tool (Dunn and Ellis, 2005). It is an unbiased, fast, non-destructive technique that involves little sample preparation and thus reduces the likelihood of sample failure or variation being introduced into the sample (Kim et al., 2010). When samples are put in a high magnetic field in a deuterated solvent and irradiated with a radio frequency, the energy absorption allows the nuclei to be transferred from low-energy to high-energy spin states. The resulting radiation emission during the relaxation process produces the resonances or signals reported as 'chemical changes' on an NMR scale, reflecting frequencies from all NMR visible nuclei in the sample relative to that of a reference proton in a reference compound (Tugizimana et al., 2013). Therefore, a NMR spectrum of an extract is the result of the superposition of the collective spectra of all NMR-visible individual compounds present in the sample under study. Moreover, an NMR analysis will typically provide a global view of all metabolites in a

sample (primary and secondary), given they are NMR detectable (Kim et al., 2010). Even though NMR is less sensitive, it provides comprehensive structural information due to its higher reproducibility and universality compared to MS (Eghbalnia et al., 2017). Popular NMR methods for plant metabolomics are ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR (Jiang et al., 2019).

2.3 Experimental Procedures/ workflow for NMR in metabolomics

Experimental procedures for NMR involves sample preparation, data processing and multivariate data analysis. These steps are discussed below.

2.3.1 Sample preparation for metabolomics research

The preparation of samples is one of the most important parts of metabolomics since it has a tremendous impact on the final results (Kim et al., 2010). Sample preparation is primarily aimed at separating metabolites from undesirable elements and enriching the desired metabolites. The best technique for sample preparation should be quick, economical, clear, convenient, and maintain the integrity of the sample (Causon and Hann, 2016). Several protocols have been developed in the last few years for metabolomics analysis and the choice depends on metabolites of interest (Vuckovic, 2012). In the extraction of metabolites, the option of extraction solvent is also very important. Aqueous MeOH, ethanol (EtOH), perchloric acid, acetonitrile (CH_3CN), and water are commonly used as solvents for extraction (Wu et al., 2014).

2.3.2 Data mining and processing

Metabolomics generates large data sets. To manage these large data sets and understand the metabolome data, automated software is required to identify peaks from raw data, compare peaks between different samples and replicates, and identify and measure each metabolite (Tugizimana et al., 2013). Data mining involves pre-processing information, pre-treatment data, and primary data statistical modelling. Since metabolomics analyses represent the cellular condition under specified conditions, metabolomics experiments are designed to measure the biological variation in the metabolome (Kim et al., 2010). However, the overall variation in metabolomics data is simply the amount of the biological variation predefined or induced and all other variations (non-induced biological, technological and analytical variation) (Tugizimana et al., 2013). Therefore, the data pre-processing and data pre-treatment procedures help to 'clean' the data to concentrate on the biologically relevant details in the data mining phase. Some of the methods of data pre-treatment are centering, scaling and transformation.

Through measuring the average of each variable and subtracting it from each measurement, the centering process allows the conversion of all concentrations to fluctuate around the zero value of coordinates. This method adjusts differences in the offset between high and low levels of compounds in samples (Tugizimana et al., 2013). Scaling, on the other hand, involves dividing each variable by a feature similar to its standard deviation (scaling factor) to change the variance between observed metabolites. The cleaned data are then analysed statistically, which provides model-based descriptions of the biological variation in the system being studied (Tugizimana et al., 2013).

2.3.3 Multivariate data analysis

Metabolomics studies generate highly complex data sets that are challenging to analyse and interpret by visual inspection or any conventional statistical univariate analysis. For the purposes of this study, only Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis OPLS-DA are discussed (refer to chapter three for more details of these models).

2.4. Botanical description of *Amaranthus* species

Amaranthus is an annual or short-lived perennial plant distributed worldwide in warm, humid regions (Muriuki, 2015). *Amaranthus* uses the photosynthetic pathway of the C_4 cycle which allows it to be uniquely efficient at high temperatures in the use of sunlight and nutrients (Lara and Andreo, 2011). Plants using the C_4 carbon fixing pathway tend to require less water than plants using the more traditional C_3 carbon fixing pathway (Lara and Andreo, 2011). Amaranth has often been called drought tolerant, probably because of the genus' ability to grow under a wide range of climatic conditions coupled with its competitive ability to grow with minimal management (Shukla et al., 2006).

Furthermore, Amaranth grows rapidly, and can be harvested in a short time (4-6 weeks after planting) (Shukla et al., 2010). *Amaranthus* grows best in loam or silty-loam soil with good water holding capacity, although it can also grow on a wide range of soil types, soil moisture and soil pH (Ebert et al., 2011). However, Beletse et al., (2012) reported that Amaranth could be produced in water-limited areas, although economic yield may be compromised as less irrigated treatments resulted in lower yields compared to the well irrigated treatment. The study further alluded to the fact that number of leaves, and leaf fresh and dry mass were highest in

the well irrigated treatment (Beletse et al., 2012). Another study reveal that water stress tolerance of Amaranth depends on the species; some species such as *A. graezizans* are more tolerant than *A. cruentus* (Nehuleni et al., 2007). Soil fertility is also an important consideration in growing *Amaranthus* spp. A recent study recorded that proportional increase in fertiliser levels from the control (0%) to 100% NPK fertilisation, Amaranth's above ground biomass increased significantly (Mndzebele et al., 2020). In another study, sheep kraal manure rates ranging from 0 to 10 t/ha was used to assess their effect of *A. cruentus* yield. NPK [2:3:4(30) + 0.5% Zn] fertiliser was used as a positive control at 150 kg/ha (Mhlontlo et al., 2007). Results indicated that low concentrations of manure (≤2.5 t/ha) resulted in plant heights and fresh material yields comparable to those under unfertilized control (Mhlontlo et al., 2007). Higher manure rates (5 and 10 t/ha) and NPK fertiliser used was responsible for increased plant heights and higher fresh plant material yields at both 30 and 60 days after transplant (Mhlontlo et al., 2007).

2.4.1 *Amaranthus* species

According to Montoya-Rodríguez et al., (2015) *Amaranthus* species belongs to Caryophyllales order, Amaranthaceae family, Amaranthoideae subfamily, and *Amaranthus* genus. Currently, the Caryophyllales contains 33 families, 692 genera, and 11 155 species (Wolosik and Markowska, 2019). Although the genus *Amaranthus* has received attention of many taxonomic studies, it is still classed as "difficult" due to numerous hybrid forms and a wide geographical distribution (Assad et al., 2017). There are about 60 species of the *Amaranthus* genus, of which 40 are native to America, the rest to Africa, Asia, and Europe (Zhigila et al., 2014). Out of these species, seventeen are eaten as leafy vegetables and three are categorised as grain Amaranth. Species commonly found in South Africa are, *A. hybridus, A. cruentus, A. spinosus, A. caudatus* and *A. thunbergii* (DAAF, 2010).

2.4.1.1 Amaranthus hybridus

Common names*:* **Utyuthu (IsiXhosa); Imbuya (Zulu); Thepe (Sotho); Pigweed (English); Misbredie (Afrikaans)**

The word *hybridus* is derived from the word hybrid which means sharing characteristics of two species. *Amaranthus hybridus* (Figure 2.1) is an erect, much branched, annual herb, which can grow up to 1 m high. Its stems are stout and grooved and the leaves are simple and alternate,

with narrow tips. The inflorescence is dense, 50-150 mm long during December to May. In South Africa, *A. hybridus* is distributed in KZN and the Limpopo Provinces.

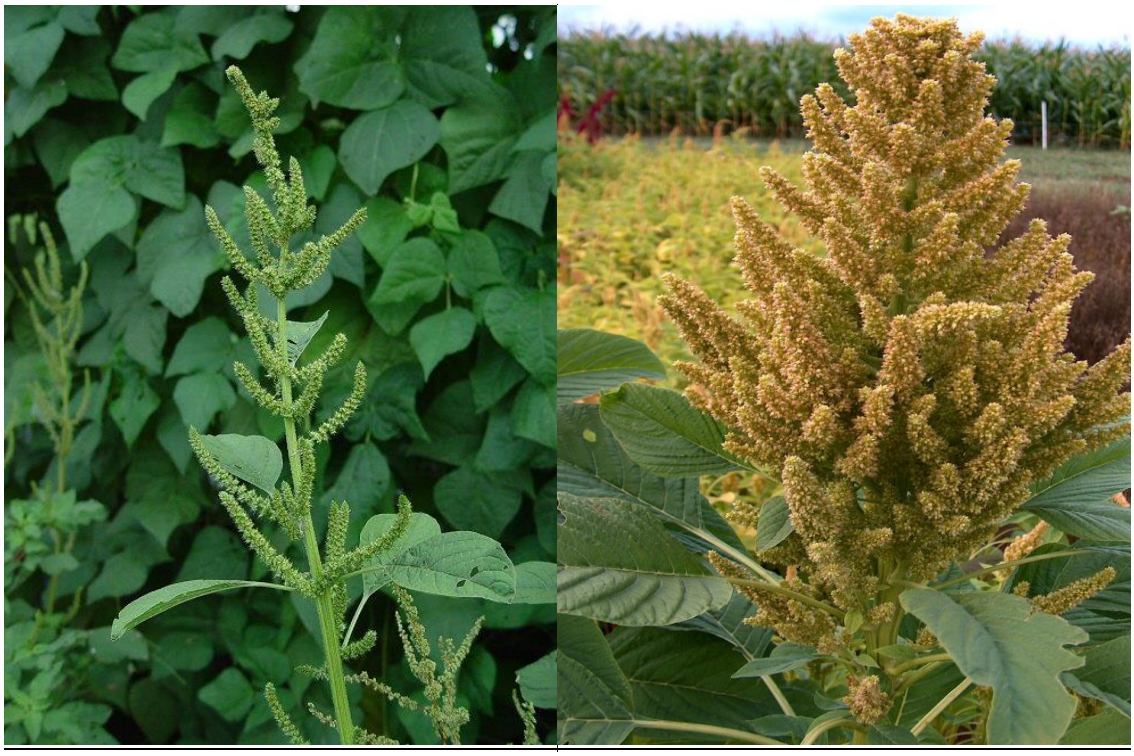


Figure 2.1: *Amaranthus hybridus* (source: Shutterstock. Available at: https://www.shutterstock.com/search/amaranthus+hybridus)

2.4.1.2 Amaranthus cruentus

Amaranthus cruentus (Figure 2.2) is an annual herbaceous plant which reproduces only by seeds and has a short growing period of four to six weeks (Makinde et al., 2010). The plant is characterised by one dominant, large tap root with thick stems that are often straight and branched (Wolosik and Markowska, 2019). The leaves are simple and their shape varies from ovate to rhombic-ovate. Numerous unisexual flowers are green and are arranged resembling finger-like spikes with a long and dense terminal panicle and axillary spikes below. The inflorescence which is characterized by high colour variability is about 50cm long and produces 50 000 seeds in round or lenticular shape, 1-1.5 mm in diameter, shiny, and dark brown (Robertson and Clemants, 2003). During maturity, the whole plant may be reddish (Grubben, 2004).

Figure 2.2: *Amaranthus cruentus* (source: Shutterstock. Available at: https://www.shutterstock.com/search/amaranthus+cruentus

2.5 Nutritional content of Amaranth

2.5.1 Macronutrient analysis

The macronutrients amounts in fresh leaves of nine species of *Amaranthus* species are shown in Table 2.1. The results showed that *A. viridis* had higher amount of protein (7.8 g/100g) followed by *A. tricolor* (Achigan-Dako et al., 2014; Srivastava, 2011). *Spinacia oleracea* (spinach) accumulated more proteins than Amaranth. With regard to carbohydrates, amounts in fresh leaves of Amaranth ranged between 4-11.2 g/100g. Among the species, *A. cruentus* contained more carbohydrates than the rest of the species (Table 2.1). The findings showed that in comparison with spinach, most of the *Amaranthus* species are rich sources of carbohydrates (Van Jaarsveld et al., 2014; Achigan-Dako et al., 2014; Kwenin et al., 2011; Muriuki et al., 2014; Ndlovu and Afolayan; 2008; Hanif et al., 2006).

Table 2.1: Macronutrient content (per 100g) of *Amaranthus* spp.

Botanical name	Common names	Moisture (g)	Carbs (g)	Prot (g)	Fibre (g)	Fat (g)	Ash (g)	Energy (kJ)	References
Amaranthus cruentus	Pigweed	79.2-82	10-11.2	3.2-4.4	3-6.7	0.3-1.6	1.5-2.38	272	Van Jaarsveld et al., 2014; Achigan-Dako et al., 2014; Kwenin et al., 2011; Muriuki et al., 2014
A. hybridus	Pigweed	83	6.09-7.5	3-6	2.81-3	0.5-2.5	2.2-4.91	222	Odhav et al., 2007; Muriuki et al., 2014
A. dubius	Pigweed	82.5-85	7.86	2.7-4	2.87	0.2-1.88	3.42	205	Odhav et al., 2007; Achigan-Dako et al., 2014; Muriuki et al., 2014
A. spinosus	Pigweed	91	4.30	4	2.48	0.6	2.76	113	Odhav et al., 2007
A. hypochondriacus	Pigweed	82.4	7.8	3.3	2.4	2.6	1.2		Muriuki et al., 2014
A. tricolor	Pigweed		9.7	3.9-6.1					Achigan-Dako et al., 2014; Srivastava, 2011
A. viridis	Pigweed		10.2	4.6-7.8	6.6		16.4	283	Achigan-Dako et al., 2014; Srivastava, 2011; Chauhan et al., 2016
A. albus	Pigweed	81.2	10	2.3	2.3	1.59	2.3		Muriuki et al., 2014

Spinacia oleracea	Spinach	91	4	*18.7	*4.13	*1.58	*17.41	*113	Ndlovu and Afolayan; 2008; Hanif et al., 2006

Carbs-carbohydrates; Prot-proteins; * values recalculated

2.5.2 Vitamin analysis

Table 2.2 shows the vitamin content of fresh Amaranth leaves. As a co-enzyme in carbohydrate metabolism, thiamine is an important dietary component (Miller and Peters, 2015). Thiamine is found in a variety of foods, but in small quantities. Thiamine levels of 160 µg/100g and 130 µg/100g have been reported for *A. gangeticus* and spinach respectively (Gupta et al., 2013; Steyn et al., 2001; Bhuvaneswari, 2015; Hanif et al., 2006; Tang, 2010). According to the South African National Health and Nutrition Examination Survey (SANHANES) of 2013; a persistently high prevalence of micronutrient deficiencies (vitamin A, iron and zinc) as well as anaemia (vitamin B12, iron and folate deficiency) among nearly one-third of women and children has been reported (Shisana et al., 2012). *Amaranthus cruentus* is remarkably rich in vitamin A (Table 2.2).

Table 1.2: Vitamin content of Amaranth species as compared to spinach

Botanical name	**Vit A (µg RE)**	**Tot β-Car**	**Thia**	**All *trans*-β-Car**	**Fol**	**Vit C**	**Ribov.**	**References**
		µg/100g				**mg/100g**		
A. cruentus	327-537	7138	0.04	5757	64 -75	2-36	0.1-0.4	Uusiku et al., 2010; Van Jaarsveld et al., 2014

A. hybridus	3290	-	2.75		75	25-126	4.24	Van Jaarsveld et al., 2014; Steyn et al., 2001; Akubugwo et al., 2007
A. gangeticus		*4670	*160			38.2		Gupta et al., 2013
A. tricolor		1000				62-100		Achigan-Dako et al., 2014; Sarker, and Oba, 2019
A. viridis			70			64	2.4	Achigan-Dako et al., 2014; Chauhan et al., 2016
Spinacia oleracea	469	4030-5626	78-130	9940	194	13.46-28	0.15-0.24	Steyn et al., 2001; Bhuvaneswari, 2015;Hanif et al., 2006 Tang, 2010

Vit A, vitamin A; Vit C-vitamin C; Ribov-riboflavin; Fol-folate; Thia –thiamine; Tot β-Car-total β-carotene; All *trans*- β-Car-all *trans* β-carotene

2.5.3 Mineral analysis

The mineral contents in fresh Amaranth leaves are presented in Table 2.3. When compared to spinach, the vegetable *A. cruentus* contained 18 times more iron (Uusiku et al., 2010; Van

Jaarsveld et al., 2014; Achigan-Dako et al., 2014; Kwenin et al., 2011; Muriuki et al., 2014). The human body requires iron for the synthesis of haemoglobin, the transport of oxygen, and the development of heme enzymes and other iron-containing enzymes that are particularly important for the production of energy, immune defence, and thyroid function (Food and Agriculture Organization) (FAO/WHO, 2001)). The daily iron requirements for children range between 7-15 mg/day and women of reproductive age require 15-18 mg/day while pregnant women require 27 mg/day (FAO/WHO, 2001), which could be met by various Amaranth species (Table 4.2). The levels of zinc ranged from 0.02-56 mg/100g (Table 2.3). *Amaranthus dubius* contains the highest zinc content (56 mg/100g). Amaranth species are also generally rich sources of calcium and magnesium.

Table 2.2: Mineral content of Amaranth as compared to spinach

	Mineral (mg/ 100g)									
Botanical name	**Fe**	**Ca**	**P**	**Na**	**Zn**	**Mg**	**K**	**Cu**	**Mn**	**References**
A. cruentus	0.3-40.5	222-443	78.9-81	10	0.02-8.4	242	459	0.17	2.34	Uusiku et al., 2010; Van Jaarsveld et al., 2014; Achigan-Dako et al., 2014; Kwenin et al., 2011; Muriuki et al., 2014
A. hybridus	10.5-21	198.4-2363	604	427	0.89-18	1317			24	Odhav et al., 2007; Muriuki et al., 2014
A. dubius	3.4-25	336.4-1686	487	347	0.6-56	806			82	Odhav et al., 2007; Achigan-Dako et al., 2014
A. hypochondriacus	9.5	131			1.3					Muriuki et al., 2014
A. spinosus	32	3931	629	393	1	1156			3	Odhav et al., 2007

A. tricolor	2.4-10	358-2000		34	0.8		3900			Achigan-Dako et al., 2014; Srivastava, 2011
A. viridis	8.9-15	410-1995		54			2230			Achigan-Dako et al., 2014; Srivastava, 2011
A. blitum	9	120		39						Srivastava, 2011
Spinacia oleracea	0.58-2.7	99	49	79	0.53	79	558	0.13	0.89-7	Steyn et al., 2001; Bhuvaneswari, 2015; Tang, 2010

Fe-Iron; Ca-calcium; P-phosphorous; Na-sodium; Zn-zinc; Mg-magnesium; K-potassium; Cu-copper; Mn-manganese

2.6 Pharmacological properties of *Amaranthus* species

There are several medicinal properties of *Amaranthus* spp. reported in the literature (Figure 2.3 below).

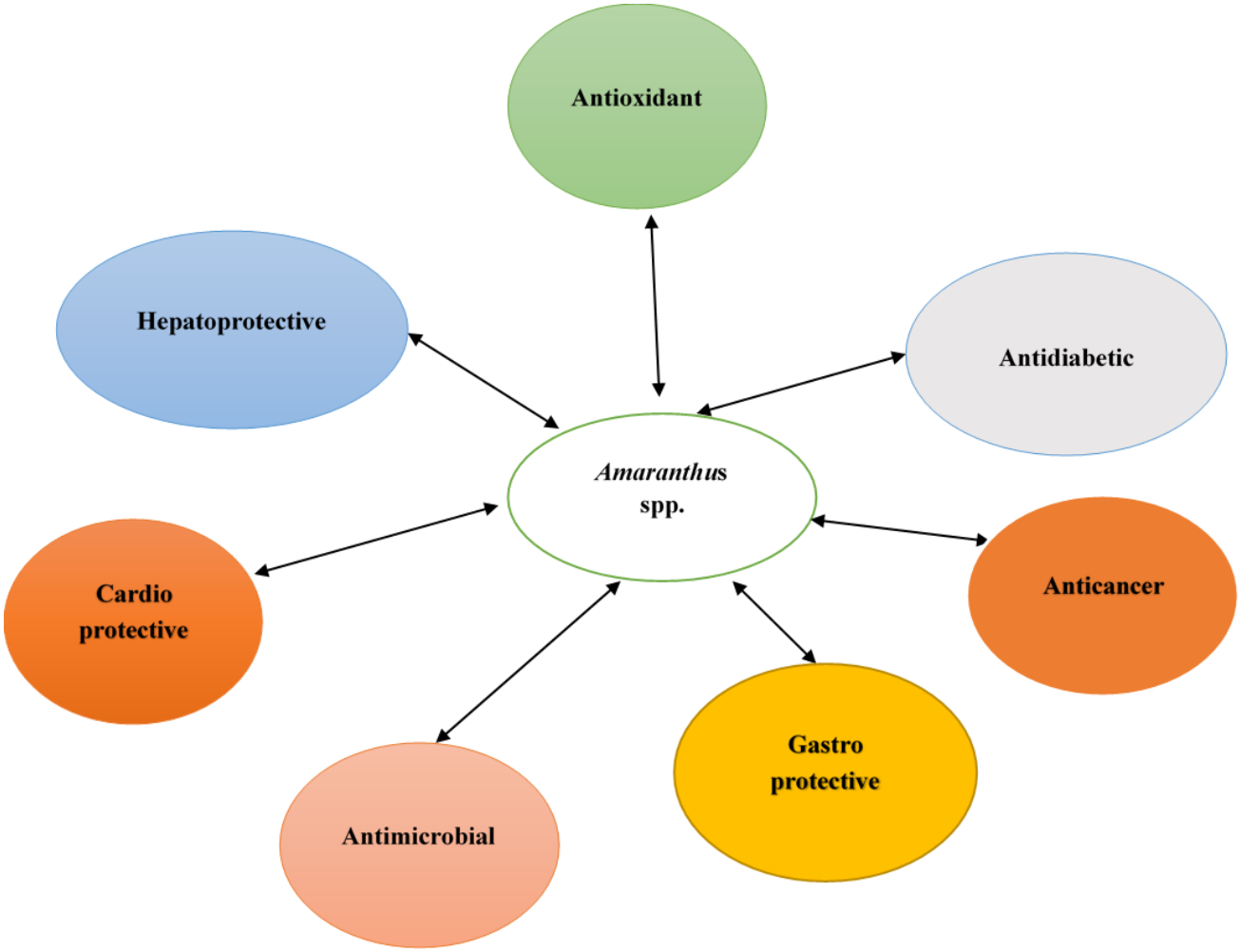


Figure 2.3: Diagrammatic representation of pharmacological properties of *Amaranthus* spp.

In the following sections, the pharmacological activities of Amaranth are addressed.

2.6.1 Antidiabetic properties

Diabetes mellitus is characterized by hyperglycaemia due to the impairment in glucose metabolism. Methanolic extracts of three species of *Amaranthus* spp, *viz A. caudatus*, *A. spinosus* and *A. viridis* demonstrated significant antidiabetic and anticholesterolemic activities (Girija et al., 2011). Clemente and Desai (2011) detected that the aqueous leaf extracts of *A.*

tricolor significantly reduced serum glucose, serum triglycerides (TG), total cholesterol, low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), and increased high density lipoproteins (HDL) ($p<0.05$) in alloxan induced diabetic rats compared to the diabetic control. Moreover, the extract prevented the decrease of body weight (BW) in treated diabetic rats and promoted haemoglobin levels. Extracts of *A. spinosus* from petroleum ether, chloroform ($CHCl_3$), MeOH and water (H_2O) extracts have been found to show a preventive effect on glycosylation of haemoglobin (Kumar et al., 2010). Hemoglobin is a useful model of non-enzymatic glycosylation of other proteins that may be involved in the long-term complications of DM. All the extracts however showed lower activity than the positive control, vitamin E. Methanolic leaves extracts of *A. caudatus* (Peter and Gandhi, 2017) and *A. spinosus* (Kumar et al., 2011) showed significant *in vitro* α-amylase enzyme inhibition even at very low concentrations (IC_{50} 19.233 µg/mL and IC_{50} 46.02 µg/mL, respectively). Acarbose had a lower IC_{50} values compared to the extracts. Orally administered methanolic leaf extract of *A. spinosus* and whole plant extract of *A. viridis* showed significant blood glucose reduction, malondialdehyde (MDA) and restored glutathione (GSH), catalase (CAT), total thiols (TT) levels in alloxan-induced oxidative stress in diabetic rats as compared to the diabetic control (Kumar et al., 2011; Kumar et al., 2012). In another study by Pandhare et al. (2012); aqueous stem extract of *A. viridis* significantly reduced blood glucose levels and modulated lipid profile in streptozotocin (STZ) induced diabetic rats. An ethanolic extract of leaves of *A. spinosus* was responsible for the significant reduction of plasma glucose and hepatic glucose-6-phophatase activity in type 1 and 2 diabetic rats (Bavarva and Narasimhacharya, 2013).

Results presented by Mishra et al. (2012) reported that 50% ethanolic leaf extract of *A. spinosus* caused a significant decrease in blood glucose in STZ induced diabetes in albino mice whilst the extract caused a significant increase in both enzymatic and non-enzymatic antioxidants (Mishra et al., 2012). A significant increase in oral hypoglycaemic activity of methanolic whole plant extract of *A. tricolor* at all doses tested was observed on glucose loaded Swiss albino mice (Rahmatullah et al., 2013). Betalains, which were identified from the leaves of *A. tricolor* inhibited the porcine pancreatic α-amylase activity by 22% compared to acarbose, a known enzyme inhibitor (Biswas et al., 2013).

2.6.2 Anticancer properties

The *n*-hexane, ethyl acetate (EtOAc) and MeOH extracts of *A. dubius* showed antiproliferative

properties with minimal side effects as determined in the COLO-320-DM human adenocarcinoma cell line (Baskar et al., 2012). The leaf extract of *A. spinosus*, given orally to Swiss albino mice with EAC (Ehrlich's ascites carcinoma), was evaluated for its antitumor potential in an *in vivo*. A decrease in tumour volume and viable cell count were observed, with parallel increase in mean survival time and non-viable tumour cell count along with restoration of normal haematological and biochemical parameters (Joshua et al., 2010). The cytotoxicity activities of the $CHCl_3$, *n*-hexane and EtOAc leaf extracts of *A. spinosus* were determined using brine shrimp lethality bioassay. The LC_{50} levels for vincristine sulphate (standard), $CHCl_3$, *n*-hexane and EtOAc extract were 7.55 µg/mL, 18.15 µg/mL, 29.51 µg/mL and 18.15 µg/mL respectively (Bulbul et al., 2011). Sreelatha et al. (2012) demonstrated that oral administration of *A. paniculatus* leaf extract induces a significant decrease in tumour size, viable cell count, tumour weight, and increased the lifespan of EAC-treated mice with an increase in the cellular antioxidant system. *Amaranthus caudatus* and *A. hybridus* feed have anticarcinogenic properties as is evident from reduced formation of micronuclei and also protect detoxifying enzymes such as gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP) in albino Wistar rats treated with sodium arsenite (Adewale and Olorunju, 2013). A protein isolate of *A. mantegazzianus* caused morphological changes and rearrangement of the cytoskeleton, inhibited cell adhesion and induced apoptosis and necrosis in the cell line UMR106, with potential antitumor properties (Barrio and Anon, 2010). The administration of *A. hybridus* seed and *A. lividus* stem extract resulted in a growth inhibition of 45 and 43% of EAC cells with up-regulation of p53, Bax and caspase-3 and down-regulation of Bcl-2 mRNA in *Amaranthus* treated mice (Al-Mamun et al., 2016). Another study showed that 50% of the hydroethanolic extracts of *A. viridis* Linn leaves had more antiproliferative activity against Jurkat, CEM and HL-603 (human leukemic cell lines) than the stem extract (Larbie et al., 2015).

2.6.3 Antioxidant potential of *Amaranthus* spp.

Antioxidants are molecules that reduce the effects of free radicals, and they are essential for cancer defence and degenerative disorders. The existence of significant levels of phenolics and flavonoids in Amaranth has been linked to the antioxidant potential (Peter and Gandhi, 2017). Pure and aqueous-methanolic leaf and seed extracts from *A. viridis* and EtOAc leaf extracts of *A. spinosus* showed superior antioxidant activity with IC_{50} values of 14.25–83.43 µg/mL and 53.68 µg/mL respectively (Iqbal et al., 2012; Bulbul et al., 2011). Research focused on hydroacetonic, methanolic and aqueous extracts from the aerial parts of *A. cruentus* and *A. hybridus*, showed that the extracts had an *in vitro* antioxidant and xanthine oxidase inhibitory

activities (Nana et al., 2012). Infusion of *A. gangeticus* leaves was investigated for its antioxidant potential on calf thymus genomic DNA and anti-lipid peroxidation and goat liver homogenates. Findings showed that the extract prevented free-radical associated oxidative damage and related degenerative diseases involving metabolic stress, genotoxicity and cytotoxicity (Dutta and Singh, 2011). SH-SY5Y cells pre-treated with the extracts of *A. lividus* and *A. tricolor* decreased cell toxicity and intracellular reactive oxygen species (ROS) production (Amornrit and Santiyanont, 2016). ROS production is associated with various pathologies including atherosclerosis and diabetes (Di Meo et al., 2016). In another study, partially purified alkaloids from *A. viridis* enhanced the antioxidant status on human erythrocytes and reduced levels of lipoproteins (LPO) by preventing the production of malondialdehyde (MDA). This confirms the ability of the partially purified alkaloids to act against free radical oxidative damage (Sasikumar et al., 2015). According to Kumar et al., (2011), it was found that the methanolic extract of *A. caudatus* was extremely effective in scavenging ABTS (2, 20-azino-bis (3-ethylbenzothiazo line-6-sulphonic acid-diammonium salt) (IC_{50} =48.75 ± 1.1 µg/mL). In another analysis, due to the presence of red anthocyanin pigment in *A. tricolor*, it has been found to contain more antioxidant capacity than *A. viridis* (Routray et al., 2012).

2.6.4 Gastroprotective effects

Plants contain compounds that have beneficial effects on the gastrointestinal tract mucosa. EtOH and EtOAc extracts of *A. tricolor* leaves demonstrated gastric-ulcer healing properties in chronic gastric ulcers caused by acetic acid. Moreover; an ethanolic extract showed gastric cytoprotective effects (Devaraj and Krishna, 2011). In another study, *A. roxburghianus* roots and piperine showed minimal ulceration, haemorrhage, necrosis and leucocyte infiltration in histopathological observation in rats with ulcerative colitis (Nirmal et al., 2013). Another study demonstrated that powdered leaves of *A. spinosus* may significantly protect Wistar albino rats from gastric ulcers induced by EtOH and duodenal ulcers caused by cysteamine (Ghosh, 2008).

2.6.5 Antimicrobial properties

Amaranthus viridis $CHCl_3$ leaf extracts showed antibacterial activity against *Bordetella bronchisseptica*, *Bacillus pumilus*, *Staphylococcus aureus* and *Proteus vulgaris* (Islam et al.,

2010). An EtOH extract of *A. viridis* inhibited the microbial activity of microorganisms namely; *Bacillus subtilis*, *B. bronchisseptica*, *B. cereus*, *B. pumilus*, *Micrococcus flavus*, *S. aureus*, *Sarcina lutea*, *Escherichia coli* and *P.* vulgaris by having values of growth inhibition zones in the ranges of 23.21 to 35.61 mm respectively (Islam et al., 2010). Plant extracts were tested at three concentrations (5, 50 and 100 mg/mL). Bacteria and fungi were inoculated in molten nutrient agar plates. Antimicrobial agents, ampicillin and streptomycin, were each tested at 1 mg/mL. The study did not provide details about the minimum inhibitory activity (MIC). In another study, *n*-hexane and EtOAc extracts of *A. spinosus* leaves showed medium to strong activity against a variety of Gram positive and Gram negative bacteria using diffusion technique (Bulbul et al., 2011). The extracts showed a zone of inhibition ranging from 14-15 mm against Gram positive bacteria and 14 mm against Gram negative bacteria. Cytotoxicity test was conducted using the brine shrimp lethality bioassay method and Vincristine Sulphate was used as positive control. The LC_{50} values of standard vincristine sulphate, $CHCl_3$, n-hexane and EtOAc extract were 7.55 μ g/mL, 18.15 μ g/mL, 29.51 μ g/mL and 18.15 μ g/mL respectively for the *Amaranthus spinosus* (Bulbul et al., 2011).

Root extracts prepared with petroleum ether, EtOAc, EtOH, and water were screened for antibacterial activity *in vitro* using an agar-well diffusion method. All the extracts were tested at a concentration of 2 mg/ mL. Although all extracts had an inhibitory effect on the tested bacterial strains; *A. hybridus* EtOAc root extract displayed the highest antibacterial activity against *B. subtilis* (24 mm) and *S. aureus* (22 mm) whereas *E. coli* was more inhibited by the alcoholic extract (21 mm) (Dahiya et al., 2010). Antibacterial drugs, streptomycin and oxytetracycline were used as positive controls and tested at a lower concentration (1 and 2.5 μ g/mL). These drugs were more potent than extracts. Ethanolic and aqueous *A. spinosus* root extracts tested at 100 mg/ mL showed comparative results with ciprofloxacin which was tested 50 mg/ml against Gram positive bacteria (Vardhana, 2011). In a study by Ahmed et al. (2013), the antibacterial activity of *A. viridis* was determined against various bacterial and fungal strains by detecting the MIC and zone inhibition. The MIC of the extracts varied between 178 and 645 µg/mL. There was no mention of the MIC values for the antimicrobial drugs used. (Ahmed et al., 2013). *n*-Hexane, EtOAc, dichloromethane (CH_2Cl_2) and MeOH extracts, prepared from *A. hybridus*, *A. spinosus* and *A. caudatus* leaves, EtOAc, CH_2Cl_2 and methanol displayed antibacterial activity against broad spectrum of bacteria. The MIC values for *A. spinosus* extracts against *S. typhi* was 129 mg/mL whereas, *A. hybridus* extracts against the tested organisms ranged from 200 mg/mL to 755 mg/mL. These MIC values were much higher

than MIC values for an antimicrobial, chloramphenicol, which ranged from 22.5-31.3 mg/mL. Finally, *A. caudatus* had MIC values between 162.2 mg/mL and 665 mg/mL on all the tested strains (Maiyo et al., 2010). Flower extracts of *A. spinosus* displayed good antibacterial activity against *Staphylococcus* spp., *E. coli*, *Pseudomonas* spp., *Klebsiell*a spp., *Paracoccus* spp. and three fungal strains of *Fusarium* spp., *Aspergillus* spp. and *Alternaria* spp.with maximum zones of inhibition ranging between 3.8-15 mm (Sheeba et al., 2012).

2.6.6 Cardioprotective properties

A number of studies demonstrate that consumption of a polyphenolic rich diet limits the incidence of coronary heart disease. According to Tiengo et al., (2011); Amaranth products, such as defatted Amaranth flour and protein concentrate, are capable of binding thus inhibiting the action of bile acids. These bile acids are associated with increased cholesterol levels in the body. Dried *A. viridis*, administered orally, altered the levels of C-reactive protein, total protein, albumin, globulin, ceruloplasm and glycoprotein in serum and in the heart of myocardial infarcted rats (Saravanan and Ponmurugan, 2012). The same plant demonstrated cardioprotective effect by lowering the levels of serum marker enzymes, cardiac troponin, glutathione disulphide (GSSG) and lipid peroxidation and elevated the levels of antioxidant enzymes and GSH (Saravanan et al., 2013). In another study, three novel peptides from grain of *A. cruentus* were evaluated for the hypocholesterolemic effect on HMG-CoA reductase, a key enzyme in cholesterol biosynthesis (Soares et al., 2015). The *in vitro* inhibition of HMG-CoA reductase by peptides, GGV, IVG, and VGVL from the grain Amaranth was different from that of the control (pravastatin). GGV and IVG presented 40% inhibition, but they were less effective than pravastatin (~90%). VGVL was the strongest HMG-CoA reductase inhibitor (~45%), as it was statistically similar to pravastatin when these data were analysed all together. All tests were performed at 4 μg/mL concentration (Soares et al., 2015).

2.6.7 Hepatoprotective properties

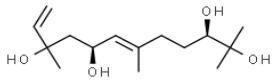
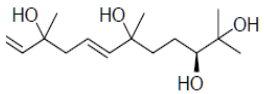
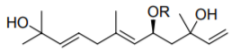
Research findings by Gul et al., (2011) indicated that the MeOH extract of *A. spinosus* administered orally significantly increased the protein and glycogen content in the liver of Sprague Dawley rats thus indicating that it to be applied for the treatment of liver problems. Flour and protein concentrate of *A. cruentus* seeds provoked antioxidant defences in Wistar

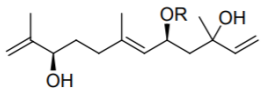
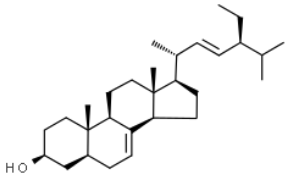
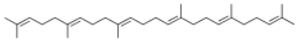
rats (Escudero et al., 2011). *Amarantus hypochondriacus* seed feed showed that it has protective effect in EtOH intoxicated rats, as it significantly increased the activity and gene expression of Cu, Zn-SOD and decreased activity of serum glutamic oxaloacetic transaminase (SGOT). In addition, the plants decreased the MDA ($p < 0.001$) content in serum; as well as NADPH oxidase transcript levels ($p < 0.05$) in the liver (López et al., 2011). Results of another study showed that 50% ethanolic whole plant extract of *A. spinosus* could provide significant protection against d-galactosamine/lipopolysaccharide-induced liver injury in rats (Zeashan et al., 2010). The whole plant methanolic extract of *A. spinosus* showed significant hepatoprotective activity in Wistar rats against paracetamol mediated hepatotoxicity (Kumar et al., 2010). Pretreatment with the aqueous extract of *A. tricolor* roots in Wistar albino rats significantly prevented physical, biochemical, histological and functional changes caused by paracetamol (Aneja et al., 2013).

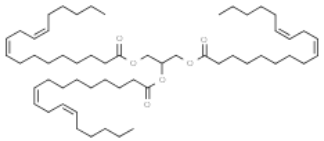
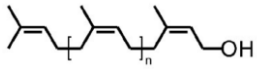
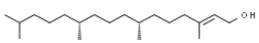
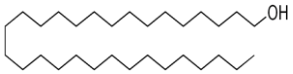
2.7 Compounds identified from *Amaranthus* species and their biological activities

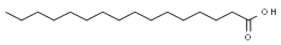
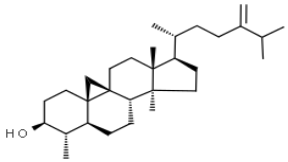
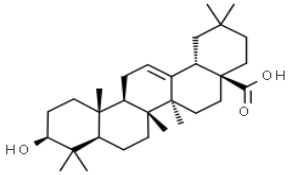
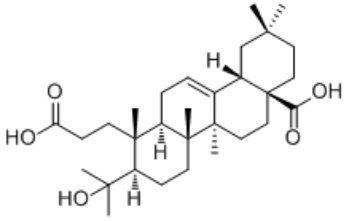
Several active constituents have been isolated from *Amaranthus* spp. (Table 2.4). Some of the compounds from *Amaranthus* spp. demonstrated biological properties. For example,α-spinosterol, isolated from *A. spinosus*, exhibited antiproliferative, analgesic and antidiabetic activities (Billah et al., 2013; Meneses-Sagrero et al., 2017; Brusco et al., 2017; Johann et al., 2011; Chukwujekwu et al., 2016).

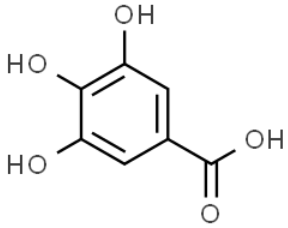
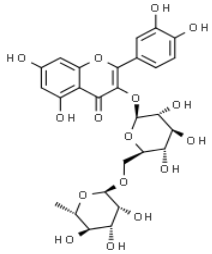
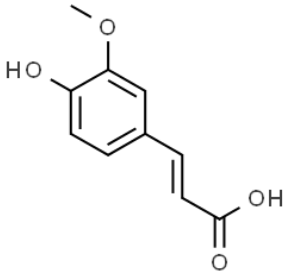
Table 2.3: Phytochemical and biological activities of compounds isolated from *Amaranthus* spp.

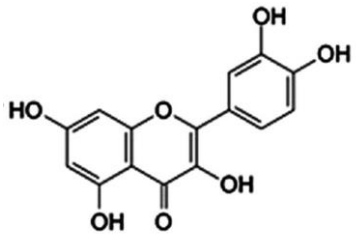
Plant species	Phytochemical		Chemical structure	Biological activity	References
A. retroflexus	Sesquiterpenes	Amarantholidol A		N/A	D'Abrosca et al., 2006
		Amarantholidol B		N/A	D'Abrosca et al., 2006
		Amarantholidol C	 **5** R=H	N/A	D'Abrosca et al., 2006

		Amarantholidol D	 **6** R=H	N/A	D'Abrosca et al., 2006
A.spinosus	Triterpene	α-spinasterol		Antiproliferative activity against cervical cancer cell line; analgesic effects; antifungal activity; antidiabetic activity	Billah et al., 2013; Meneses-Sagrero et al., 2017; Brusco et al., 2017; Johann et al., 2011; Chukwujekwu et al., 2016; Ragasa et al., 2015; Mozirandi et al., 2019
		(14E,18E,22E,26E) -methyl nonacosa-14,18,22,26 tetraenoate		Antibacterial activity, anticancer properties	Mondal et al., 2016; Xu, 2018
	Triterpene	Squalene		Emollient and antioxidant activities; antitumor activities	Huang et al., 2009

A.viridis		Trilinolein		Anticancer activity	Ragasa et al., 2015; Chou, 2011
	Diterpene	Polyprenol		Anti-tumour, anti-hepatitis C virus and anti-HIV effects	Ragasa et al., 2015; Zhang et al., 2015
		Phytol		Antioxidant, apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, and antimicrobial effects	Ragasa et al., 2015; Islam et al., 2018
	Fatty alchohol	Triacontanol		Anticancer properties	Hue et al., 2017

Fatty alchohol	Palmitic acid		Anticancer properties	Hue et al., 2017; Harada et al., 2002
Pentacyclic triterpene	Cycloeucalenol		Anti-leukemic cell proliferation activity	Hue et al., 2017; Suttiarporn et al., 2015
	Oleanolic acid		Antimicrobial activity	Hue et al., 2017; Ayeleso et al., 2017
Pentacyclic triterpene	3,4-seco-olean-12-en-4-ol-3,28-dicarboxylic acid		Antitumor effect	Hue et al., 2017; Tanaka et al., 2001

	Phenolic acid	Gallic acid		Antioxidant, anti-inflammatory, and antineoplastic properties	Paranthaman et al., 2012; Kahkeshani et al., 2019
A.caudatus	Phenolic acid	Rutin		Antifungal activity; antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities	Johann et al., 2011; Ganeshpurkar and Saluja, 2017
		Ferulic acid		Antioxidant, anti-inflammatory, antiviral, antiallergic, antimicrobial, antithrombotic, anticarcinogenic and hepatoprotective actions	Kim and Park, 2019

Quercetin		Anti-inflammatory, antihypertensive, vasodilator effects, antiobesity, antihypercholesterolemic and antiatherosclerotic activities	David et al., 2016

N/A: not available

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CHAPTER 3

^{1}H-NMR based metabolomics profile of wild and cultivated *Amaranthus* spp.

Abstract

Amaranthus has been rediscovered as a promising food crop mainly due to its ability to endure and thrive in diverse environments. Amaranth leaves are rich in proteins and micronutrients such as iron, calcium, zinc, vitamin C and vitamin A. In addition, the *Amaranthus* crop has been reported to possess antimicrobial, antidiabetic, and anticancer properties. However, little is known about the constituents of the crop that are directly linked to its health benefiting attributes. In South Africa, *Amaranthus* crops are mostly gathered from the wild, in some areas, women gather the vegetables from their gardens or nearby farms as they often grow as a weed alongside maize. The cultivation of *Amaranthus* species is still rare, and efforts are being made to market and commercialise this valuable crop. This study investigates the influence of cultivation and environment on the chemical profile of both cultivated and wild *A. cruentus* and *A. hybridus* by multivariate statistical analysis of spectral data deduced by NMR. In this study, it was found that maltose and sucrose increased in both cultivated *A. cruentus* and *A. hybridus*. Moreover, the amino acid, proline was present in cultivated *A. cruentus* in high amount whereas, proline and leucine were prominent in *A. hybridus*. Other compounds that were found in both wild and cultivated *A. cruentus* and *A. hybridus* were trehalose, *trans*-4-hydroxy-L-proline, trigonelline, lactulose, betaine, valine, alanine, fumarate, formate and kynurenine. Some of these compounds such as trehalose and trigonelline are stress protectors in plants enabling Amaranth to endure diverse climatic conditions such as drought. Chlorogenic acid was detected only in cultivated *A. hybridus*. This compound is reported to possess antioxidant and antidiabetic activities. In addition, trigonelline which is a known antidiabetic agent was detected in all Amaranth samples. The study has shown that not only is Amaranth rich in macro and micronutrients, the leaves also contain phytochemicals that have proven medicinal properties.

3.1 Introduction

Plant foods especially leafy vegetables contain phytochemicals which have potential to impact human health positively. Many studies have reported that Amaranth leaves contain diverse bioactive compounds which contribute to its health promoting attributes (Billah et al., 2013; Chukwujekwu et al., 2015; Ragasa et al., 2015). For an example, α-spinosterol and squalene isolated from *A. spinosus* demonstrated antidiabetic, antifungal and antitumor properties (Chukwujekwu et al., 2016; Huang et al., 2009). *Amarathus* spp. are largely collected from the wild, even though few selected cultivation incidences have been reported (Maseko et al. 2018), and most of the phytochemical studies were conducted on wild species. Since various compounds in *Amaranthus* leaves have been linked to the plant's health-promoting effects, it is important to better understand compound profiles depending on the conditions of cultivation and environmental factors.

To date, there has been no documentation of metabolomics profiles linked to geographical origin and the impact of cultivation on *Amaranthus* species. There is a growing interest in using metabolomics technology, because it does not require *a priori* knowledge of the species' chemical composition (Grebenstein et al., 2011). Metabolomics provides insights into the fundamental nature of plant phenotype in relation to development, physiology, and environment (Ward et al., 2007). Several analytical methods are applied in metabolomics, however, compared to other methods, proton nuclear magnetic resonance (^{1}H-NMR) is widely used (Kim et al., 2010[a, b]). This analytical method allows a wide range of metabolites to be identified simultaneously, offering an accurate and easily reproducible representation of the plant's metabolome (Verpoorte et al., 2008). This chapter explores the metabolome of wild and cultivated Amaranth leaves using ^{1}H-NMR to explain the general variations in chemical composition between wild and cultivated plants.

3.2 Materials and methods

3.2.1 Collection of wild vegetables from KwaZulu-Natal (KZN) Province, South Africa

Wild Amaranth plants collected from KwaZulu-Natal were confirmed by a botanist at the University of Zululand, Prof. Alfred Zobolo. *Amaranthus hybridus* leaves were bought from Esikhawini informal market during the month of April of 2018. Esikhawini is a peri-urban settlement situated in the district of Richards Bay in KwaZulu-Natal. The area is characterised by 1087 mm of rain per year, with most rainfall occurring mainly during summer. Esikhawini receives the lowest rainfall in June (42 mm) and the highest in March (133 mm) (Esikhawini climate, Map of South Africa, ND).

The leaves of *A. cruentus* were purchased from the informal market in Stanger which is situated in KwaDukuza in KwaZulu-Natal. Stanger normally receives about 866 mm of rain per year, most of which occurs during the summer (Stanger climate, Map of South Africa, ND). The leaves were sold in pierced plastic bags to avoid sweating of the leaves and to allow some airflow. Upon purchase, the leaves were transferred to brown paper bags to prepare for transportation to the lab. Upon arrival, the leaves were washed and left on the countertop to dry. After the leaves dried out, they were grounded to fine powder and stored at room temperature until analysis.

3.2.2 Planting of *A. cruentus* and *A. hybridus* at Mothong

Amaranthus cruentus and *A. hybridus* were grown in November of 2017 at Mothong African Heritage Centre garden in Mamelodi, Pretoria (GPS co-ordinates: $25^{0}41'49.7''S\ 28^{0}20'17.4''E$). The seeds of *A. cruentus* and *A. hybridus* were donated by the gene bank of the Agriculture Research Council-Vegetable and Ornamental Plants (ARC-VOP) where they are maintained to ensure that the material used in the study was true-to-type. Prior to planting, the seeds were sown in 98 cavity seedling trays using hygromix as a growth medium and kept under a 40% shade net. Seedlings were transplanted to the field 21 days after emergence. The plants were planted with a spacing of 10cm x 20cm (50 plants m^{-2}) (Mulandana et al., 2009). Limestone ammonium nitrate (LAN) in the form of 50 kg N ha^{-1} (containing 28% nitrogen, calcium and magnesium) fertilizer was applied on the freshly prepared soil on the day of planting the seedlings. Plants were irrigated once a week.

3.2.3 Harvesting

The first harvest was done two months after sowing early in the morning before 10:00 am. For each vegetable, three separate lots were randomly harvested from different positions in the field to obtain a representative sample batch for each species. Plants were dried at room temperature and ground to fine powder. Until investigation it was stored at room temperature.

3.2.4 Reagents and buffer preparation

Deuterated methanol (CD_3OD), potassium dihydrogen phosphate (KH_2PO_4), deuterium water (D_2O) and Trimethylsilylpropionic acid sodium salt (TSP), were purchased from Sigma. The buffer was prepared by adding 1.232 g KH_2PO_4 to 100 mL of D_2O with 10 mg TSP (0.01%) added as a reference standard.

3.2.5 Extraction of plant material

Metabolomics analysis followed the methods described by Maree and Viljoen (2012) and Mediani et al., (2012). Extracts were prepared using a direct extraction method for metabolomics. For extraction and analysis, pulverized leaf material of 50 mg per sample was weighed in 2 mL Eppendorf tubes. The samples were suspended in 0.75 mL CD_3OD and 0.75 mL KH_2PO_4, buffered in deuterium water (D_2O) (pH 6.0) containing 0.01% (w / w) TSP. For a homogenization, the mixture was vortexed for one minute at room temperature, then ultra-sonicated for 20 min to break down the cell walls. The sample was then centrifuged for an additional 20 min to remove the supernatant from the pellet. The supernatant was then transferred from each tube to a 5 mm NMR tube for analysis.

3.2.6 Data acquisition by NMR analysis

NMR spectral data were obtained with a 600 MHz NMR spectrometer (Varian Inc., California, USA) recording 32 scans.

3.2.7 Data mining and pre-processing

Pre-treatment of raw spectral data is essential in order to generate reliable and interpretable models using multivariate analysis techniques (Worley and Powers, 2013). Binning, which was

adapted in this study, takes care of chemical shifts which may occur as a result of changes in temperature, pH, ionic strength, and other factors that influence their electronic environment. For the pre-processing of spectral data, MestReNova from Mestrelab technology (9.0.1, Mestrelab Research Spain) was used. In all sample spectra, manual phase adjustment and baseline correction as well as calibration with an internal standard TSP to 0.0 ppm were performed. Excluded from further study were the chemical shifts of 4.6-5.0 and 3.28-3.36 ppm reflecting water and MeOH respectively (Mediani et al., 2012). In addition, MestReNova was used for bucketing NMR spectra. Consequently, the spectral intensities were reduced to combined regions of equal size (0.04 ppm each) corresponding to the area of 0.04-10.00 ppm, often referred to as buckets or bins. The generated ASCII files were then imported into Microsoft Excel 2013 for secondary variable labelling. For multivariate analysis, the data were imported into soft independent modelling by class analogy (Simca) software (15.0, Umetrics, Umeå, Sweden).

3.2.8 Multivariate data analysis

Metabolomics studies generate high-dimensional and complex data sets that are difficult to analyse and interpret by visual inspection or any conventional quantitative univariate analysis (Tugizimana et al., 2013). Thus, the techniques of multivariate data analysis (MVDA) – mathematical modelling approaches – are used to derive meaningful information from these broad empirical data sets (Tugizimana et al., 2013). This study made use of PCA and OPLS-DA models. The data was Pareto scaled to adjust for measurement errors to reach homogeneity of variance in the data (Tugizimana et al., 2016). PCA is a projection-based approach and a mathematically rigorous procedure that provides a global and qualitative visual representation of sample similarity or dissimilarity (without the use of category information; e.g. care vs. control) (Boccard et al., 2007). In the absence of a distinct discrimination pattern in the metabolic fingerprints, subsequent analysis was carried out using OPLS-DA, a supervised pattern recognition method. OPLS-DA method is an explanatory or predictive analysis which makes it easier to identify the metabolites responsible for group discrimination (Tugizimana et al., 2013).

3.2.9 Annotation of compounds

Detection and quantification of metabolites were performed using the Chenomx NMR suite (evaluation edition) with TSP used as a reference and the integrated metabolite spectral library. The annotated compounds were verified with the use of Human Metabolome Database (HMDB) (Wishart et al., 2013) and previously published data.

3.3 Results

A large amount of metabolic variance was observed in the experiment. For ease of discussion, the results will begin with a point by point analysis at two different levels (i) determining the effect of geographical location and cultivation by comparing the metabolite composition of the *A. cruentus* grown in Gauteng and the ones collected from KZN (ii) determining the effect of geographical location and cultivation by comparing the metabolite composition of the *A.hybridus* grown in Gauteng and the ones collected from KZN.

(i) Metabolite composition of the *A. cruentus* grown in Gauteng and KZN provinces

Figure 3.1 displays representative ^{1}H-NMR spectra showing profiles of cultivated and wild *A. cruentus* collected from different regions. The ^{1}H-NMR spectra revealed varying peak signals indicating differences in the chemical composition between the cultivated (shade net and open field) and wild *A. cruentus* leaf extracts. The strongest and highest intensity peak signals in all regions (aliphatic, sugar and aromatic) were consistently shown in cultivated samples grown in the open field. Compared to the samples collected from the wild, extracts of the cultivated (open field) *A. cruentus* showed higher intensities in aliphatic (0.5-3.00ppm), sugar (3.00-5.5ppm) and aromatic (5.5-10ppm) regions.

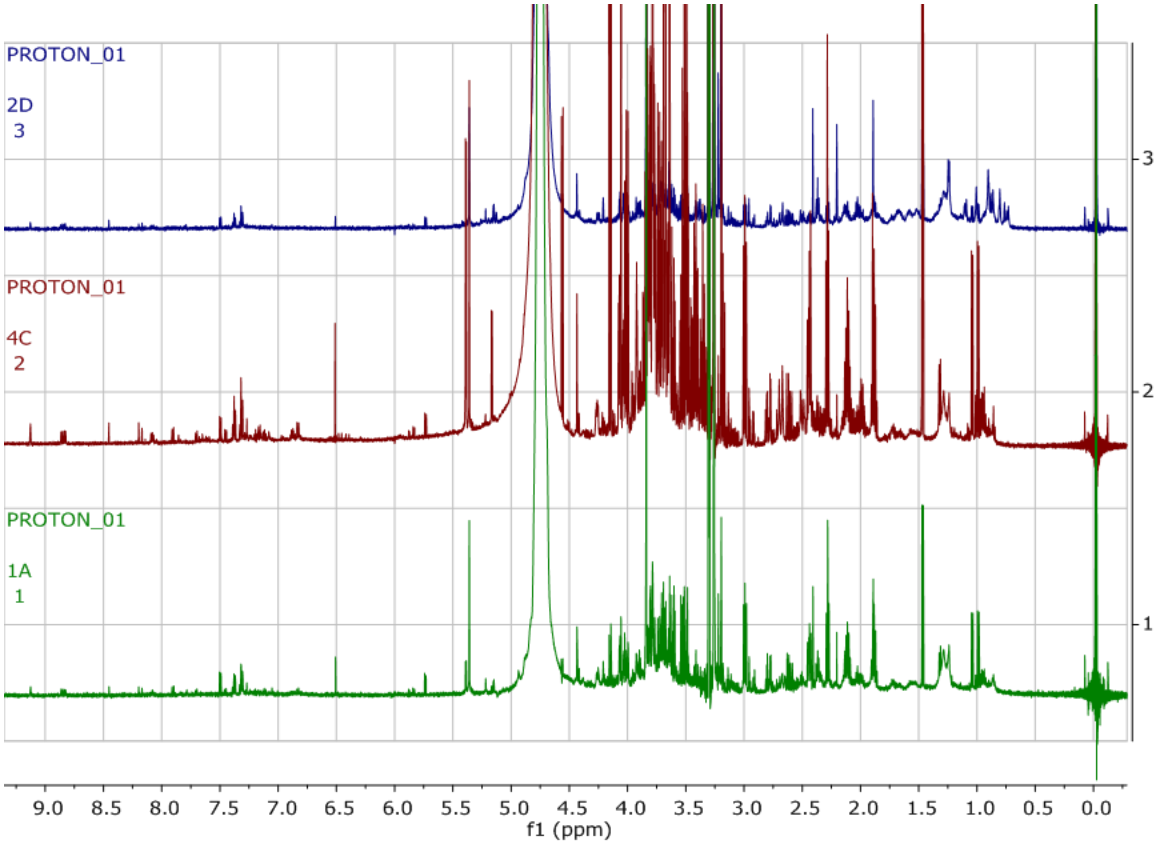


Figure 3.1: The 600 MHz ^{1}H-NMR spectra of *A. cruentus* leaf extracts (wild and cultivated). Red = cultivated at Mothong in the open field, blue = collected from the wild (KZN), and green = cultivated at Mothong in the shade net.

Multivariate data analysis of PCA and OPLS-DA were used to reduce, analyse and conceptualize the information contained in the *Amaranthus* spp. extracts collected from different geographical locations. The PCA analysis of ^{1}H-NMR spectra of MeOH extracts of wild and cultivated *A. cruentus* is shown in Figure 3.2. Each point in the PCA scatter plot is a representation of an individual sample. The two first axes Principal component (PC) PC1 and PC2 explained 70.6% of the variance. In addition, the model showed a great goodness of fit ($R^2X_{(cum)}$ = 0.95) and predictive ability ($Q^2_{(cum)}$ = 0.86). Most of the wild samples cluster together and showed positive loading along PC2 with an exception of one sample. There was no pattern observed for cultivated *A. cruentus* species (especially open field) as these samples were distributed throughout the plane, although the open field cultivated species are separated from the shade net samples to the right.

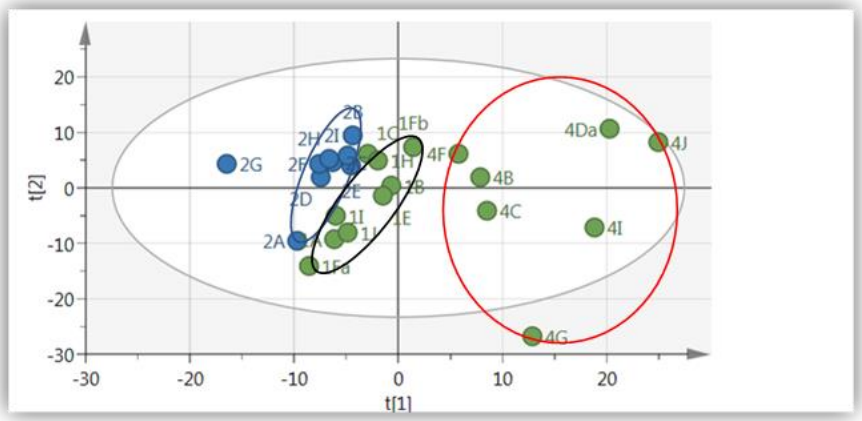
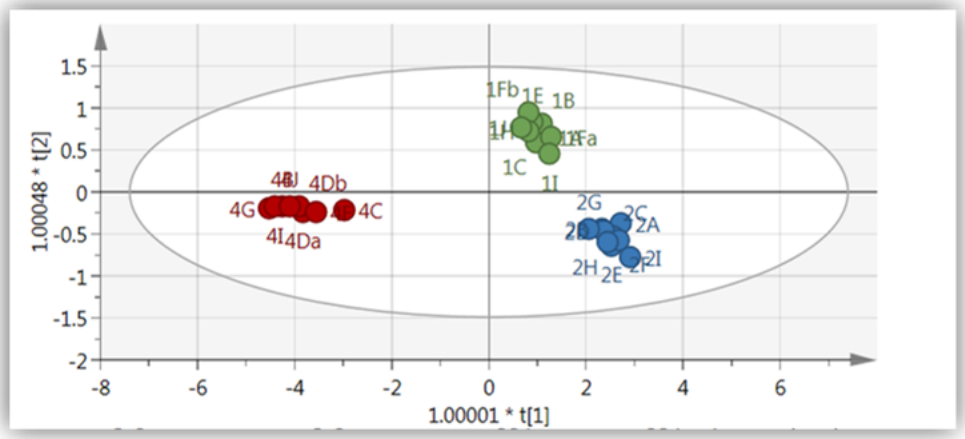


Figure 3.2: Score scatter plot of PCA of *A. cruentus* leaf extracts (wild and cultivated). Green = cultivated at Mothong in the open field (red circle) and shade net (black circle), blue = collected from the wild (KZN).

To improve the clustering and to identify the metabolites responsible for the differences between the samples collected from different areas, an OPLS-DA model (Figure 3.3A) was constructed. The OPLS-DA statistical model showed a clear distinction between extracts of the cultivated (shade net and open field) and wild *A. cruentus*. The model showed a goodness of fit and predictability as presented by R^2X of 0.95 and Q^2X of 0.89 (Table 3.1) respectively. Moreover, in order to validate the predictive capability of the computed OPLS-DA models, the response permutation test (with $n = 100$) was constructed. This statistical test compares the R^2 and Q^2 values of the true model to the permutated model. The test is conducted by assigning the two different groups at random, after which the OPLS-DA models are fitted to each permutated class variable. The values of R^2 and Q^2 for the permutated models are then determined and compared with the values of the true models. The results indicate that the measured models have much higher R^2 and Q^2 (Figure 3.3B) values and therefore the computed true OPLS-DA models for each dataset are statistically much better than the 100 permutated models.

A



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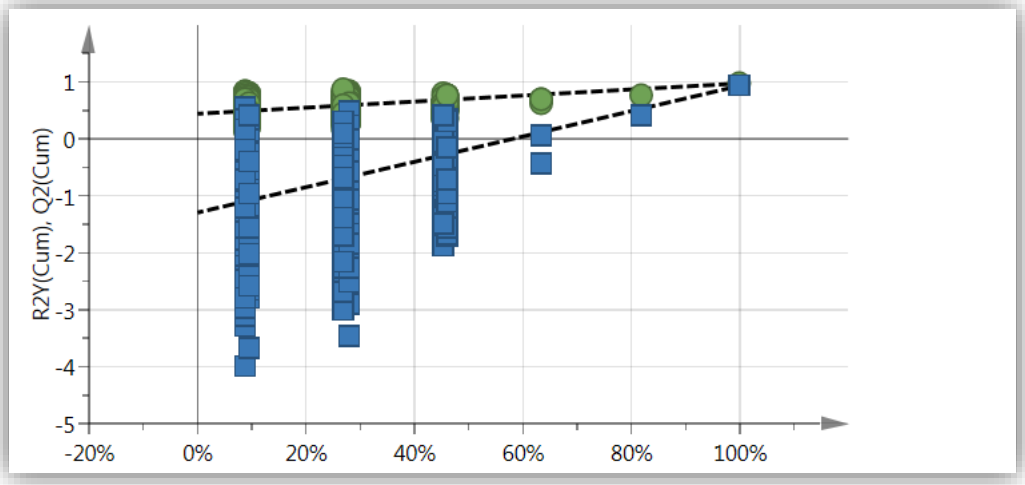


Figure 3.3: A: Score scatter plot of OPLS-DA of *A. cruentus* leaf extracts (wild and cultivated). Blue = Collected from the wild (KZN), Green = Cultivated at Mothong in the shade net, and Red = Cultivated at Mothong in the open field. B: The response permutation test (*n*=100) for the OPLS-DA model corresponding to y-axis intercepts (Table 3.1): R^2 = (0.0, 0.44) and Y^2 = (0.0,-1.29).

Table 3.1: Model Quality and Description of OPLS-DA for *A. cruentus*

	OPLS-DA		**Permutation (n=100)**	
R^2X	R^2Y	Q^2	R^2	Y^2
0.95	0.89	0.82	(0.0, 0.44)	(0.0-1.29)

To demonstrate the most relevant variables that affect the sample differentiation between the classes, corresponding contribution plots were produced (Figure 3.4). The contribution plots showed that the sugars and aliphatic compounds mostly contributed to the differences between the samples that were cultivated at Mothong (in the shade net) and the wild samples that were collected from KZN. NMR regions of primary and secondary metabolites responsible for separating wild and cultivated *A. cruentus* are observed in the contribution plot (Figure 3.4) by noting the regions of the chemical shifts. Annotated compounds that distinguished cultivated samples from wild samples were maltose, sucrose and proline (Table 3.3).

All the samples (cultivated and wild) showed the presence of trigonelline, lactulose, trehalose, betaine, leucine, valine, alanine, fumarate, formate, kynurenine and *trans*-4-hydroxy-L-proline.

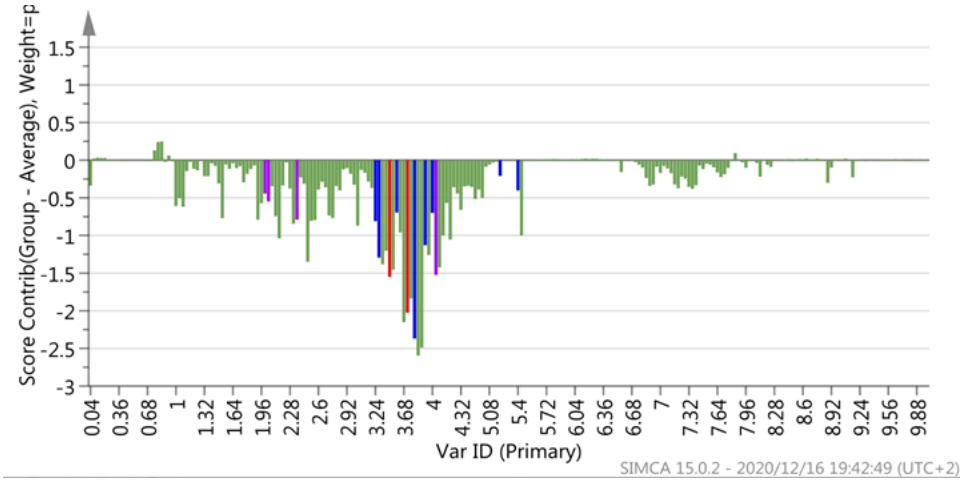


Figure 3.4: Contribution plot generated by comparing wild samples from KZN to cultivated samples (shade net). The blue, red and violet bars represent NMR regions that are associated with maltose, sucrose and proline, respectively. There may be overlaps between NMR regions of maltose, sucrose and maltose.

(ii) Metabolite composition of the *A. hybridus* grown in Gauteng and KZN provinces

Representative ^{1}H-NMR spectra demonstrating the chemical profile of wild and cultivated *A. hybridus* is shown in Figure 3.5. *Amaranthus hybridus* that was cultivated in the shade net showed strongest and high intensity peaks in aliphatic regions (0.5-3.0ppm). The visual inspection of the ^{1}H-NMR spectra shows that spectra are remarkably similar, but the peaks in the aromatic region (6.0-10ppm) of wild samples were less intense (as shown by the peak heights) compared to the cultivated samples grown under shade net, but not the open field (Figure 3.5).

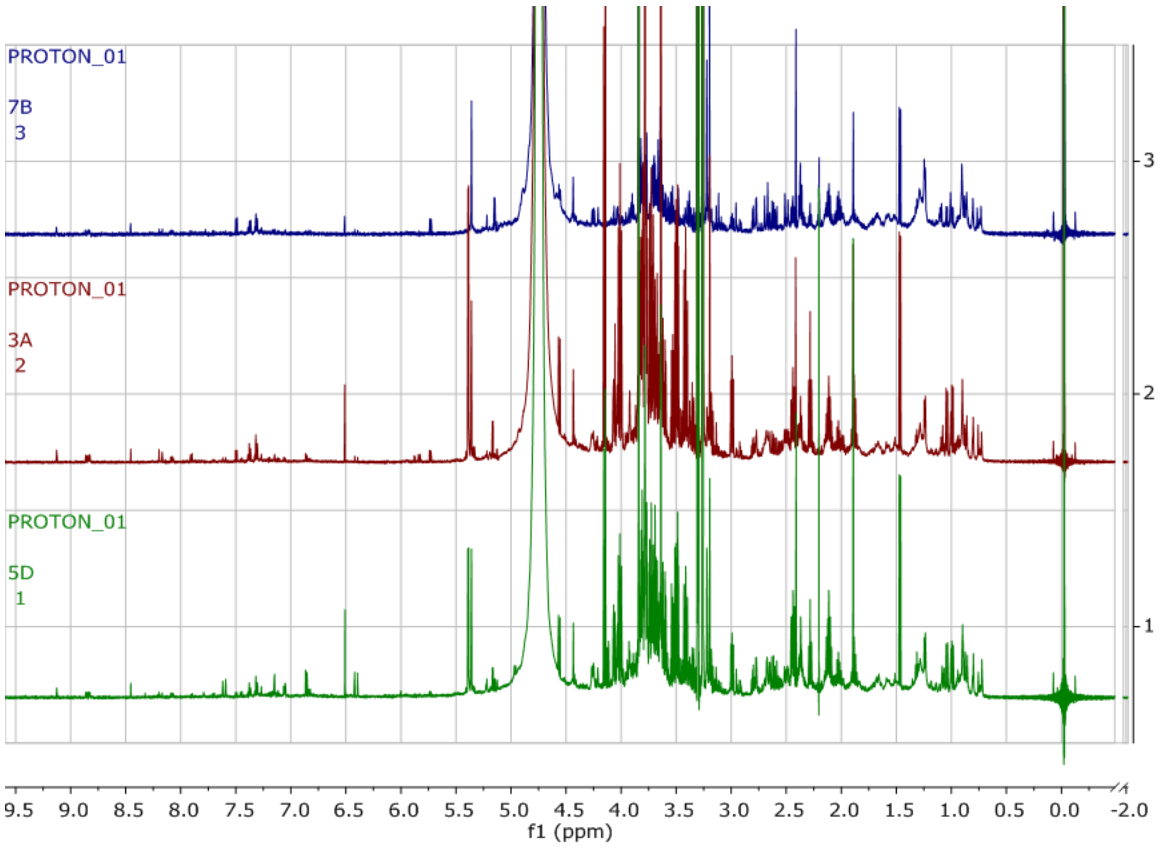


Figure 3.5: The 600 MHz ^{1}H-NMR spectra of *A. hybridus* leaf extracts (wild and cultivated). Red = cultivated at Mothong in the open field, blue = collected from the wild (KZN), and green = cultivated at Mothong in the shade net.

The PCA analysis of ^{1}H-NMR spectra of aqueous MeOH extracts of wild and cultivated *A. hybridus* is illustrated in Figure 3.6. Principal component (PC) 1 and PC2 explained 70% of the variance in the model. Moreover, the model showed a great goodness of fit ($R^2X_{(cum)}$ = 0.94) and predictive ability ($Q^2_{(cum)}$ = 0.81). With an exception of few samples, majority of the wild samples grouped together and showed positive loading along PC1 (Figure 3.6). Cultivated samples showed no particular order in terms of clustering together as these samples were dispersed throughout the plane.

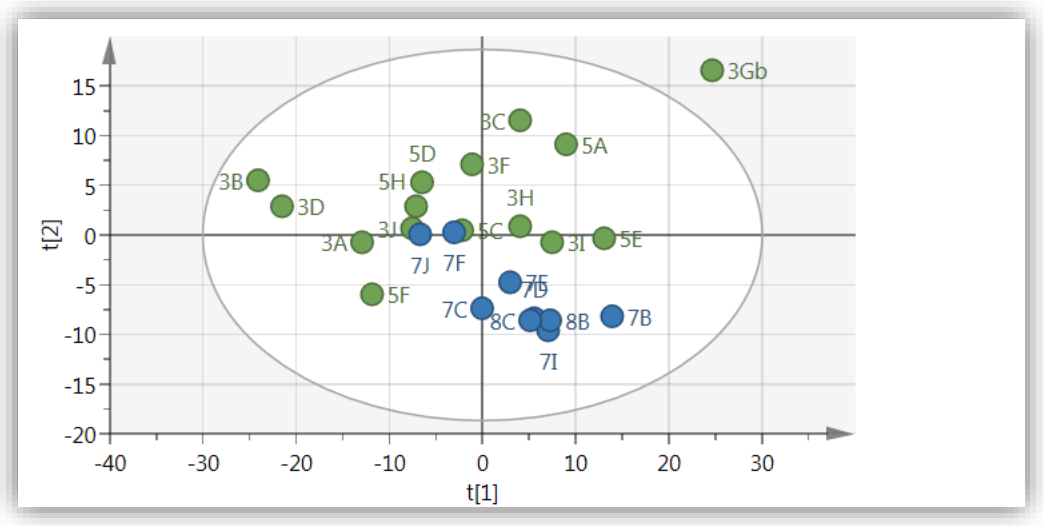
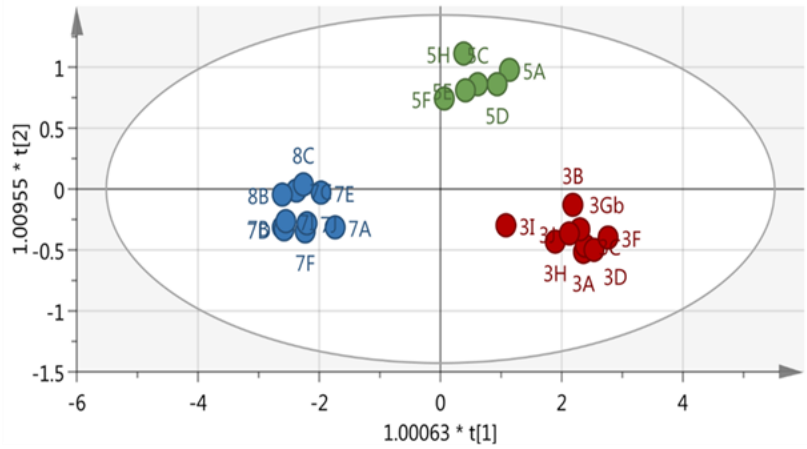


Figure 3.6: Score scatter plot of PCA of *Amaranthus hybridus* leaf extracts (wild and cultivated). Green = cultivated at Mothong in the shade net and open field, blue = collected from the wild (KZN).

The supervised multivariate analysis model (OPLS-DA) showed a clear discrimination between the sample groups within the 95% confidence interval (Figure 3.7A). The computed model showed a goodness of fit and predictability as presented by R^2X of 0.93 and Q^2X of 0.88 (Table 3.2) respectively. Furthermore, the predictive capability of the computed OPLS-DA model was validated using the response permutation test (with n = 100). This statistical test compares the R^2 and Q^2 values of the true model to the permutated model. Once again, it was observed that the true models have much higher R^2 and Q^2 (Figure 3.7B) values as compared to the permutated models.

A



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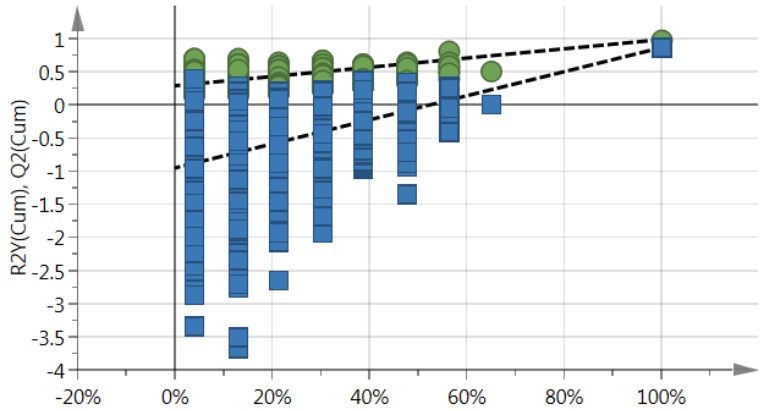

Figure 3.7: A Score scatter plot of OPLS-DA of *A. hybridus* leaf extracts (wild and cultivated). Green = cultivated at Mothong in the shade net, red= cultivated at Mothong on the open field, and blue = collected from the wild (KZN). B: The response permutation test (*n*=100) for the OPLS-DA model corresponding to y-axis intercepts (Table 3.2): R^2= (0.0, 0.28) and Y^2= (0.0-0.95).

Table 3.2: Model Quality and Description of OPLS-DA for *A. hybridus*

	OPLS-DA		**Permutation (n=100)**	
R^2X	R^2Y	Q^2	R^2	Y^2
0.93	0.88	0.82	(0.0, 0.44)	(0.0, -0.95)

The contribution plots (Figure 3.8) showed that the aliphatic, aromatic and sugar regions were strongly associated with samples that were cultivated at Mothong (shade net) and were negatively associated with the wild samples from KZN. Compounds that distinguished cultivated samples from wild samples were annotated as maltose, sucrose, leucine, proline and chlorogenic acid, the latter was only present in the samples grown under shade net (Table 3.3). Both cultivated and wild samples showed the presence of trigonelline, lactulose, trehalose, betaine, valine, alanine, fumarate, formate, kynurenine and *trans*-4-hydroxy-L-proline.

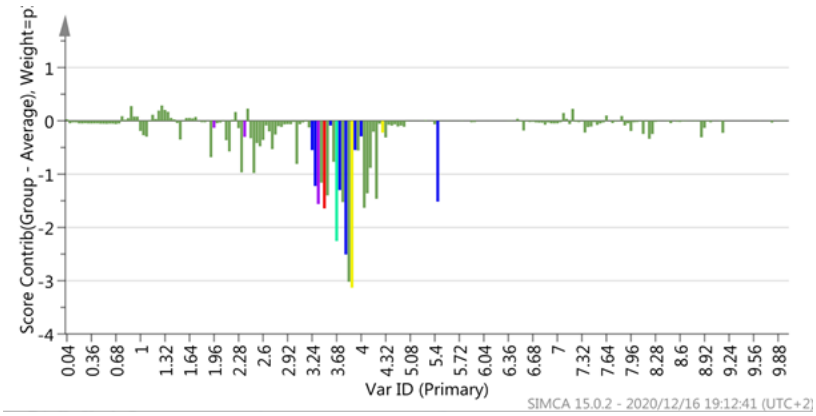

Figure 3.8: Contribution plot generated by comparing wild samples from KZN to cultivated samples (shade net). The blue, red and violet bars represent NMR regions that are associated with maltose, sucrose and proline, respectively. In addition, teal and yellow bars represent NMR regions associated with leucine and chlorogenic acid, respectively. There may be overlaps between NMR regions of identified metabolites.

The annotated metabolites are presented in table 3.3. Chenomx and HMDB were used to support the annotation and were compared to published data.

Table 3.3: NMR peaks (ppm) of the compounds that contributed to the separation of cultivated (shade net) and wild leaf extracts of *A. cruentus* and *A. hybridus.*

Group	Compound	H-NMR Chemical Shift (ppm)	Chenomx (ppm)	Human Metabolite Database	Reference (ppm)	Literature
Cultivated (Shade Net)	Maltose	3.24	3.3	3.27		Oh et al., 2018
		3.4	3.4	3.41		
		3.6	3.6	3.66		
		3.72	3.7	3.70		
		3.8	3.8	3.84		
		3.92	3.9	3.9		
		4.0	4.0	3.96		
		4.56	4.6			
		5.2	5.2	5.22	5.34	
		5.4	5.4	5.40	5.42	
	Sucrose	3.52	3.5	3.46	3.53	Maulidiani et al., 2018
		3.6	3.6	3.55	3.67	
		3.72	3.7	3.75		
		3.8	3.8	3.82	3.87	
		3.92	3.9	3.89		
		4	4.0	4.04	4.07	

		4.2	4.2	4.21	4.16	
		5.41	5.4	5.4	5.39	
	Proline	1.99	2.0	1.96	1.98	Oh et al., 2018
		2.06	2.0	2.04	2.02	
		2.34	2.3	2.32		
		3.33	3.3	3.24		
		3.41	3.4	3.4		
		4.1	4.1			
	* Leucine	0.88	0.9	0.94	0.94	Oh et al., 2018
		1.0	1.0			
		1.68	1.7	1.70	1.70	
		3.68	3.7			
	** Chlorogenic acid	2.0	2.0	2.02	2.03	Sabino et al., 2019
		2.08	2.1	2.17		
		2.2	2.2			
		3.88	3.9	3.88	3.80	
		4.28	4.3	4.25	4.23	
		5.28	5.3	5.33	5.33	
		6.4	6.4	6.39	6.39	
		6.88	6.9	6.94		
		7.08	7.1	7.12	7.05	
		7.2	7.2	7.19		
		7.6	7.6	7.65	7.59	
Wild and Cultivated	Trigonelline	4.4	4.4	4.42	4.41	Maulidiani et al., 2018; Conotte et al., 2018
		8.12	8.1	8.07	8.07	
		8.8	8.8	8.82	8.83	
		9.12	9.1	9.11	9.14	
	Trans-4-hydroxy-L-proline	2.4	2.4		2.6	Xavier et al., 2014
		3.4	3.4			
		3.48	3.5		3.63	
		4.28	4.3			
		4.56	4.7			
	Lactulose	3.6	3.6	3.582	3.5	Jayalakshmi et al., 2009
		3.68	3.7	3.732	3.6	
		3.8	3.8	3.836	3.8	
		3.88	3.9	3.919	3.9	
		4	4.0	4.012	4.0	
		4.08	4.1	4.125	4.1	
		4.12	4.2	4.2	4.2	
		4.28	4.3	4.249	4.3	
		4.4	4.4	4.286	4.4	
		4.48	4.5	4.46	4.5	
		4.56	4.6	4.553	4.6	
	Trehalose	3.41	3.4	3.44		Oh et al., 2018
		3.6	3.6	3.64		
		3.8	3.8	3.76		
		3.92	3.9	3.88		
		5.2	5.2		5.18	
	Betaine	3.24	3.3	3.25	3.22	Oh et al., 2018

	3.92	3.9	3.89	3.86	
Valine	1.0	1.0	1.02	1.05	Oh et al., 2018; Maulidiani et al., 2018
	2.32	2.3	2.26	2.22	
	3.6	3.6	3.60	3.60	
Alanine	1.44	1.5	1.47	1.47	Jung et al., 2011
	3.8	3.8	3.77		
Fumarate	6.52	6.5	6.51	6.5	Oh et al., 2018
Formate	8.4	8.4	8.44	8.46	Oh et al., 2018
Kynurenine	3.72	3.7	3.67	3.71	Jung et al., 2011
	4.12	4.1	4.10	4.15	
	6.8	6.8	6.75	6.80	
	6.92	6.9	6.83		
		7.4	7.37	7.44	
	7.8	7.8	7.79	7.84	

* High only in *A. hybridus*. ** detected only in *A. hybridus.*

3.4 Discussion

Carbohydrates are subdivided into several groups based on the number of sugar units and the way in which the sugar units are connected (Slavin and Carlson, 2014). Groups include sugars, starches, and fibres (Slavin and Carlson, 2014). In the present study, maltose and sucrose were annotated in both cultivated *A. cruentus* and *A. hybridus*. These sugars were present in wild species, but only in smaller quantities. Soluble sugars (sucrose, glucose, and fructose) play a significant role in the physiology of the plant while preserving the overall plant structure and development (Rosa et al., 2009). For an example; glucose causes stomatal closure and increases the adaptability of the plant to drought stress (Osakabe et al., 2013). Krasensky and Jonak, (2012) allude to the fact that during water stress, the role of sugars is membrane defense and radical scavenging (Krasensky and Jonak, 2012). Moreover, sugar accumulation prevents the oxidation of cell membrane under water deficiency (Arabzadeh, 2012). Finally, soluble sugars also preserve the turgidity of leaves and prevent membranes and proteins from being dehydrated (Sami et al., 2016). Xu et al. (2015) found that the condition of drought stress significantly increased the concentration of soluble sugar in roots and leaves of susceptible rice variety but not in resistant ones. In contrast, the concentration of soluble sugars in stems in both susceptible and resistant genotypes was significantly reduced under stress from drought (Xu et al., 2015). The low concentration of maltose in wild *A. cruentus* and *A. hybridus* suggests that the drought stress could potentially cause the concentration of soluble sugar to decrease in the wild plants compared to the cultivated samples. The more favourable conditions under

cultivation also support better growth and development in general, and consequently resulted in an increase of soluble sugars in the cultivated material.

In another study, the concentration of branched chain amino acids (BCAAs) leucine, isoleucine, and valine, was increased under stress from drought in wheat cultivars (Bowne et al., 2012). These results were comparable to the findings of Urano et al. (2009) which reported that accumulation of BCAAs, along with a number of other amino acids, increased under the stress of dehydration and was regulated at transcriptional level. In this study, leucine was annotated in cultivated *A. hybridus*, whereas, valine was annotated in cultivated *A. cruentus*. Leucine was established to play a key role in promoting acute secretion of insulin from pancreatic β cells (Nisoli et al. 2013) hence consumption of leucine rich foods may aid diabetic patients. In another study, it was established that leucine supplementation improves glucose homeostasis (Pedroso et al., 2015).

Other compounds were found in Amaranth e.g. trehalose, chlorogenic acid and trigonelline. Trehalose is a commonly occurring non-reducing sugar in bacteria, fungi, yeast, insects, and plants (Elbein et al., 2003). In plants, significant trehalose levels act as a protectant against various abiotic stresses, including heat, drought, high salinity and ultraviolet rays (Garg et al., 2002). The study proposes that the presence of trehalose in all of the samples investigated is indicative of the ability for Amaranth to endure harsh environmental and climatic conditions. Trigonelline is ubiquitous in herbaceous plants of saline dried habitats (Machado et al., 2013). Previously, trigonelline has been identified from young aerial parts of *A. hybridus* (Steffensen et al., 2011). In this study, trigonelline was found in both *A. hybridus* and *A. cruentus*. Zhou et al. (2013) found that trigonelline has beneficial effects for diabetes through lowering blood glucose and lipid levels, improved insulin sensitivity index and insulin content. In another study, trigonelline decreased the ratio of kidney weight/body weight and blood glucose levels, and lowered blood level of urea nitrogen, creatinine and albumin in type 2 induced diabetic rats (Li et al., 2019). Finally, chlorogenic acid was annotated only in cultivated *A. hybridus*. Previously, chlorogenic acid was detected from the leaves of *A. tricolor* through HPLC (Sarker and Oba, 2019; Khanam et al., 2013). Chlorogenic acid is the most abundant polyphenol in the human diet. It is an important component of coffee (Meng et al., 2013). Chlorogenic acid has many health benefits, including antioxidant, chemopreventive and other activities (Kabir et al., 2014). In addition, chlorogenic acid rich foods are associated with a lower risk of type 2 diabetes mellitus (Ong et al., 2013).

3.5 Conclusion

NMR-based metabolomics was used to determine the effect of cultivation and environmental changes in the leaf extracts of *A. cruentus* and *A. hybridus*. The chemical profiles of the samples of both wild and cultivated plants are comparable even though there are some differences, mostly in concentration of compounds. Remarkable similarity was found for all the *A. hybridus* samples, whereas this was not observed in case of *A. cruentus*, indicative of the differential responses of the two species to cultivation. Metabolomics approach using ^{1}H-NMR successfully determined proline, sucrose, maltose and leucine as the chemical constituents contributing to the variation of leaf extract metabolites and was positively associated with cultivated *A. cruentus* and *A. hybridus*. In addition, chlorogenic acid was positively associated with cultivated *A. hybridus*. This compound was not found in any of the other samples. Although chlorogenic acid was detected in *A. tricolor*, to the best of the author's knowledge, this phenolic acid is reported in *A. hybridus* for the first time. This study is also the first report of the presence of trehalose in the two species investigated and the first report of trigonelline in *A. cruentus*. Trigonelline, which was present in all the Amaranth samples is known for its pronounced hypoglycaemic effect. The presence of trigonelline in all the samples further warrants the next chapter which interrogates the antidiabetic potential of *Amaranthus* spp.

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CHAPTER 4

In vitro α-glucosidase and α-amylase inhibitory activities of wild and cultivated *Amaranthus* spp.

Abstract

Diabetes mellitus is a metabolic disorder involving the metabolism of glucose. Managing the level of blood glucose is the hallmark in treating this disorder thus preventing the complications that it presents. The goal of this study was to investigate the inhibitory effect of wild and cultivated *Amaranthus* spp. MeOH leaf extracts on the α-amylase and α-glucosidase activities. This was done by using *α*-amylase, isolated from porcine pancreas, and *α*-glucosidase yielded from *Saccharomyces cerevisiae.* Findings of the study demonstrate that wild *A. hybridus* showed potent α-glucosidase enzyme activity (89.92±0.04% inhibition) at the lowest concentration tested (0.125 mg/mL). Amongst the cultivated samples, *A. cruentus* extracts were responsible for inhibiting α-glucosidase enzyme activity at the lowest concentration by 84.95±0.04% compared to *A. hybridus* which exhibited inhibitory activities of 72.28±0.06%. None of the extracts altered the effectiveness of α-amylase beyond a 50% mark. Acarbose, a known antidiabetic drug, was used as a positive control which inhibited α-amylase by 71.37±0.01% at 0.062 mg/mL. In conclusion, the selective strong α-glucosidase inhibitory activity demonstrated by all plant extracts warrants a search for phytochemical constituents that are responsible for enzyme inhibition activity.

Keywords: diabetes mellitus, hyperglycaemia, phytochemicals, blood glucose

4.1 Introduction

Diabetes mellitus is a diagnostic term for a group of disorders characterized by abnormal homeostasis of glucose which results in high blood sugar level (American Diabetes Association, 2014). A sudden increase in blood glucose levels causing hyperglycaemia in patients with T2DM is due to pancreatic α-amylase starch hydrolysis and intestinal α-glucosidase uptake of glucose (Ahamad, and Naquvi, 2011). The inhibition of enzymes involved in starch breakdown (α-amylase) and glucose absorption (α-glucosidase) has been suggested as a useful approach to the treatment and prevention of T2DM (Oboh et al., 2012).

Current evidence supports the claim that known α-glucosidase inhibitors like acarbose and voglibose potentially reduce diabetes progression as well as micro- and macrovascular complications including; diabetic retinopathy, nephropathy and neuropathy (Sudhir and Mohan, 2002). However in diabetic patients, α-glucosidase and pancreatic α-amylase inhibitors have been documented to be associated with gastrointestinal side effects such as abdominal pain, flatulence and diarrhoea (Hanefeld, 1998).

Since ancient times, plants and herbal preparations have been used to treat diabetes mellitus, and are still used in traditional medicine (Rahmatullah et al., 2012). Several studies have indicated the capacity of various medicinal plants to inhibit α-glucosidase and α-amylase enzymes (Alqahtani et al., 2020; Sekhon-Loodu and Rupasinghe, 2019; Iheagwam et al., 2019; Kidane et al., 2018; Mourya, 2018; Mumtaz et al., 2018; Picot et al., 2014; Vadivelan et al., 2019). The ability of plants to inhibit the activity of α-glucosidase and α-amylase is linked to their phytoconstituents. Etsassala et al. (2019) reported that ursolic acid, β-amyrin and oleanolic acid isolated from *Salvia africana-lutea*, showed strong α-glucosidase inhibitory activities, whilst oleanolic acid also demonstrated high α-amylase inhibitory activity. EtOAc extract of *Rheum turkestanicum* and its isolated compounds emodin and daucosterol proved to be active against α-glucosidase and α-amylase enzymes. Moreover, rhododendron and emodin reported in the same study were highly active against α-glucosidase (Dehghan et al., 2018). Kaempferol, quercetin and myricetin, isolated from *Psidium guajava*, demonstrated high α-glucosidase and α-amylase activities (Wang et al., 2007). Other compounds that have been reported to suppress the activity of α-glucosidase enzyme include felamidin and suberosin which were isolated from *Ferulago bracteata* (Karakaya et al. 2018) while lupeol and β-sitosterol were isolated from *Terminalia sericea* (Nkobole et al., 2011).

Many edible plants are good sources of unique phytochemicals like polyphenols and flavonoids (Adisakwattana et al., 2012). Studies have demonstrated that food containing polyphenolic compounds and flavonoids have been linked to *in vitro* inhibitory activities of intestinal α-glucosidase and pancreatic α-amylase (Arise et al., 2016; Adisakwattana et al., 2012; Kor et al., 2010; Hargrove et al., 2011). Some of the consumed wildly occurring vegetables in South Africa have also played a vital role in the fight against diabetes mellitus. *Cleome gynandra* was found to be a potent intestinal α-glucosidase and pancreatic α-amylase inhibitor (Shaik et al.,

2013). Another commonly consumed wild vegetable in South Africa, *Corchorus olitorius*, showed high α-amylase inhibitory ability (Ademiluyi et al., 2015; Oboh et al., 2012). *Citrullus lanatus* was found to be a potent inhibitor of α-glucosidase and α-amylase (Sathya et al., 2015). *Amaranthus* spp. in particular has been subjects of pharmaceutical investigations and proved to be effective against T2DM (Kasozi et al., 2018; Balasubramanian et al., 2017; Ghosh et al., 2008; Sasikumar et al., 2015). These species are widely consumed in South Africa (Maseko et al. 2017) and have, in recent years, garnered interest in the academic community due to their nutritional profile. There is also overwhelming evidence supporting the medicinal claims associated with the use of Amaranth, thus there is a perceived increasing demand for the crop. Efforts should be made to promote the cultivation of Amaranth in order to meet the growing demand. Thus, the aim of the current study was to evaluate the *in vitro* inhibitory effects of wild and cultivated *Amaranthus* leaf extracts on the activities of selected diabetes-related carbohydrate metabolizing enzymes, α-amylase and α-glucosidase.

4.2 Materials and methods

4.2.1 Chemicals

α-Glucosidase (*Saccharomyces cerevisiae*), α-amylase (porcine pancreas), 3,5-dinitro salicylic acid (DNS), *p*-nitro-phenyl-α-D-glucopyranoside (*p*-NPG), sodium carbonate (Na_2CO_3), sodium dihydrogen phosphate, disodium hydrogen phosphate, acarbose and potato starch, and MeOH (HPLC grade) were purchased from Merck, South Africa.

4.2.2 Collection of plant Material

Collection, planting and harvesting of plant materials are described in sections 3.2.1-3.2.3.

4.2.3 Extraction of plant material

Plants are multicomponent systems hence, finding a suitable extractant is critical. Fifty grams (50 g) of dried plant materials were ground to a fine powder. Each plant sample was soaked overnight at room temperature in 50 mL of 100% MeOH. The solvent was then removed and replaced with the same solvent volume. This procedure was repeated two times. Extracts were filtered and evaporated to dryness under reduced pressure using a rotary evaporator (BUCHI-

Labotech, Switzeland). Dried extracts were stored in a closed cabinet at room temperature until analysis.

4.2.4 α-Glucosidase inhibitory activity

The inhibitory activity of plant extracts against intestinal α-glucosidase was determined following the method proposed by Telagari and Hullatti, (2015). The concentration of plant extracts ranged from 0.125-2 mg/mL. A negative control was prepared using the same procedure however, the plant extract was replaced with phosphate buffer (100 mM, pH = 6.8). A reaction mixture containing 50 µl phosphate buffer, 10 µl α-glucosidase (1 U/mL), and 20 µl of varying extract concentrations was pre-incubated at 37°C for 15 min in a 96-well plate. Acarbose (positive control) was tested at varying concentrations from 0.062–1 mg/mL (Ferron et al., 2020). In addition, 20 µl P-NPG (5 mM) was then added as a substrate and further incubated at 37°C for 20 min. The reaction was stopped with the addition of 50 µl Na_2CO_3 (0.1 M). The assay was performed in triplicate. The absorbance of the released p-nitrophenol was measured at 405 nm with a Thermo Scientific Varioskan Flash Spectrophotometer. The results were expressed as percentage inhibition, which was calculated using the formula,

Inhibitory activity (%) = $(1 - As/Ac) \times 100$

Where,

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

4.2.5 α-Amylase inhibitory activity

The assay was adapted from Telagari and Hullatti, (2015) wherein the concentration of plant extracts ranged from 0.125-2 mg/mL. The concentrations of the positive drug control acarbose ranged from 0.062-1mg/mL. A negative control was prepared using the same procedure however, the plant extract was replaced with phosphate buffer (100 mM, pH = 6.8). The reaction mixture containing 50 µl phosphate buffer, 10 µl α–amylase (2 U/mL), and 20 µl extract was pre-incubated for 20 min. at 37°C in a 96-well plate. One percent of a soluble potato starch (100 mM phosphate buffer pH 6.8) was then incorporated as a substrate and further incubated at 37°C for 30 min. A colour reagent, DNS (100 µl) was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using a Thermo

Scientific Varioskan Flash Spectrophotometer. The results were expressed as percentage inhibition, which was calculated using the formula,

Inhibitory activity (%) = (1 − As/Ac) ×100

4.3 Statistical analysis

All the analyses were carried out in triplicates and the results are expressed in mean ± SD. All of the data analyses were carried out using Microsoft Excel 2010.

4.4 Results

In vitro screening of *Amaranthus* spp. leaf extracts for enzyme inhibitory activities

Amaranthus spp. MeOH extracts were screened for their inhibitory activities against α-amylase and α-glucosidase.

4.4.1 α-Glucosidase inhibitory activity

Four plant extracts were tested for α-glucosidase and α-amylase enzyme inhibitory activities (Figures 4.1 and 4.2 and Tables 4.1 and 4.2). The inhibition percentage of extracts against α-glucosidase enzyme ranged from 61.61±0.05 - 97.10±0.05% at the highest concentration tested (2 mg/mL). The extracts of wild grown *A. cruentus* showed weakest activity (41.85±0.10%) against α-glucosidase at the lowest concentration tested (0.125 mg/mL) whereas wild grown *A. hybridus* showed highest enzyme inhibitory activity (89.92±0.04%) at the same concentration. Acarbose, which was used as a standard reference drug exhibited 80.20±0.13% and 68.18±0.11% inhibition against α-glucosidase at 0.125 mg/mL and 0.062 mg/mL respectively (Figure 4.1 and Table 4.1). It should be noted that the lowest concentration tested for acarbose was 0.062 mg/mL whereas that of extracts were 0.125 mg/mL. Among the cultivated samples, extracts of *A. cruentus* showed highest enzyme inhibitory activity (84.95±0.04%) at the lowest concentration (0.125 mg/mL) compared to *A. hybridus* which showed 72.28±0.06% inhibition.

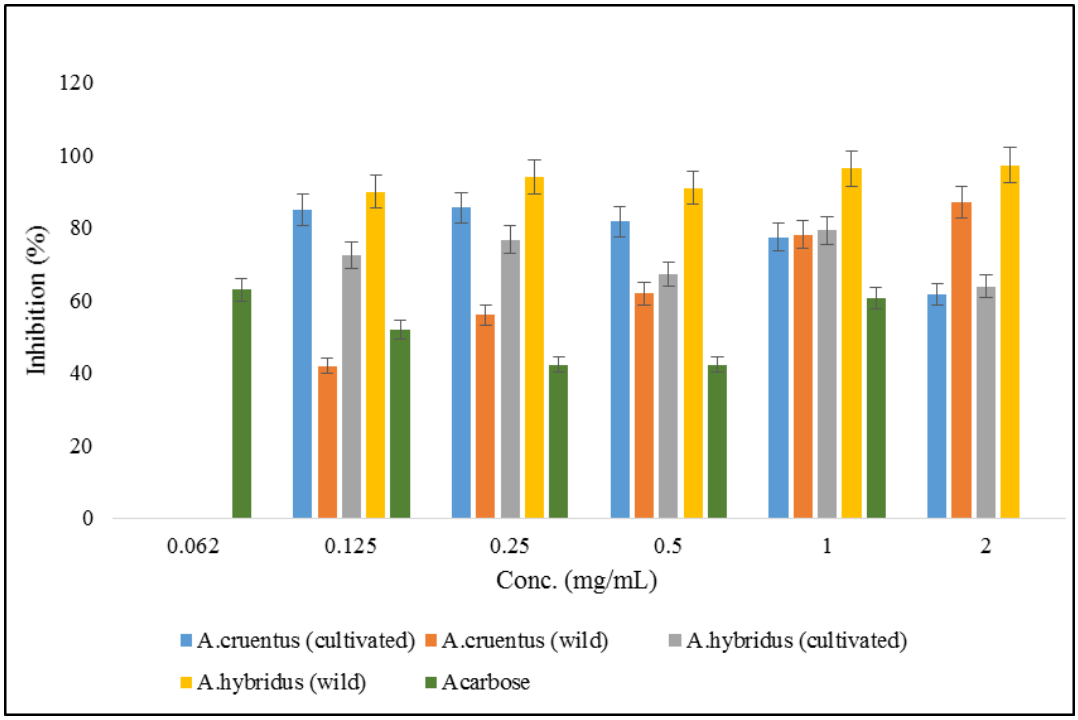

Figure 4.1: Inhibition of α-glucosidase using *p*-nitrophenyl α-D-glucopyranoside as a substrate, by the extracts and positive drug control; acarbose

Table 4.1: Effect of plant extracts on the inhibition of α-glucosidase enzyme

α-glucosidase (% inhibition)	**IC_{50} mg/mL**	**0.062 mg/mL**	**0.125 mg/mL**	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**	**2 mg/mL**
A.cruentus wild	0.144	N/T	41.85±0.10	55.96±0.13	61.85±0.13	78.12±0.34	87.13±0.18
A. cruentus, cultivated	ND	N/T	84.95±0.09	85.46±0.07	81.63±0.02	77.40±0.11	61.61±0.05
A. hybridus wild	ND	N/T	89.92±0.04	93.98±0.03	90.91±0.24	96.32±0.05	97.10±0.05
A. hybridus, cultivated	ND	N/T	72.28±0.06	76.59±0.23	67.11±0.25	79.19±0.13	63.85±0.16
acarbose	ND	72.72±0.24	80.20±0.13	73.41±0.16	66.31±022	68.18±011	N/T

N/T: not tested
ND: not determined

4.4.2 α-Amylase inhibitory activity

In vitro α-amylase inhibitory results demonstrated that none of the tested *Amaranthus* spp. leaf extracts screened inhibited pancreatic α-amylase beyond 50% (Figure 4.2, Table 4.2). The percent inhibition of all tested extracts ranged from -7.55±0.29 - 34.70±0.17%. Amongst the cultivated species, although not significant, *A. hybridus* had the highest α-amylase inhibitory activity (33.18±0.14%) at 0.125 mg/mL. On the other hand, wild grown *A. cruentus* had the highest α-amylase inhibitory activity (39.63±0.05%) at 0.25 mg/mL compared to wild *A. hybridus* which inhibited only the α-amylase by 20.56±0.06%. Acarbose showed enzyme inhibition ranging from 71.37±0.01-89.00±0.05% (Figure 4.2 and Table 4.2).

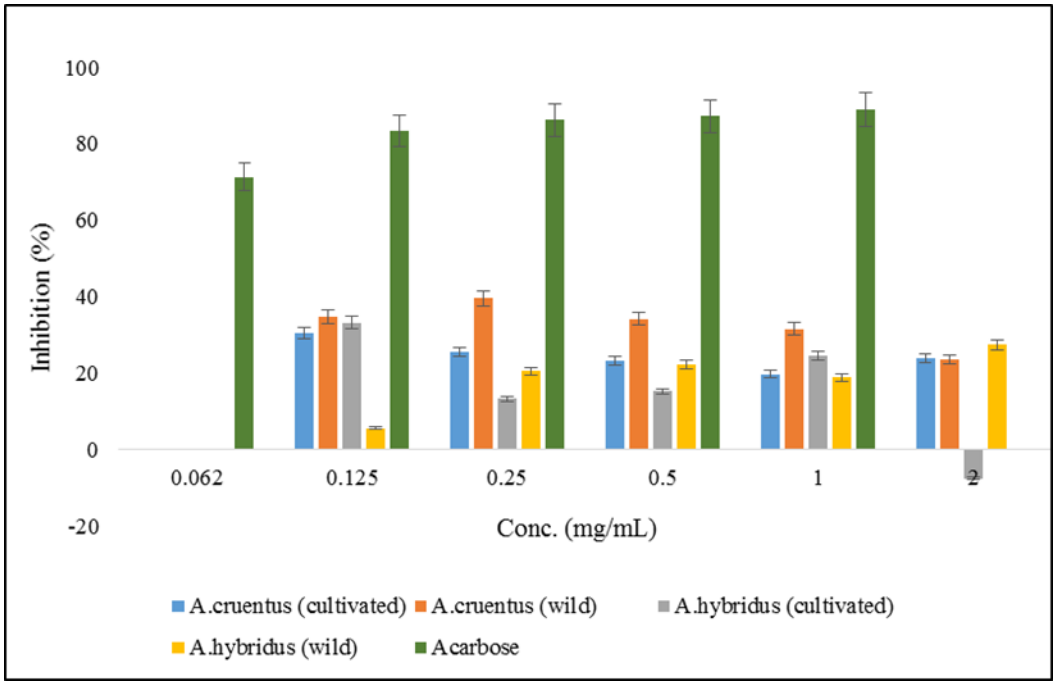

Figure 4.2: Inhibition of α-amylase using soluble potato starch as a substrate, by the extracts and positive drug-control; acarbose

Table 4.2: Effect of plant extracts on the inhibition of α-amylase enzyme

α-amylase (% inhibition)	**0.062 mg/mL**	**0.125 mg/mL**	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**	**2 mg/mL**
A.cruentus wild	N/T	34.70±0.17	39.63±0.05	34.25±0.05	31.62±0.01	23.47±0.02

A. cruentus, cultivated	N/T	30.46±0.07	25.57±0.14	23.39±0.12	19.74±0.14	24.03±0.12
A. hybridus wild	N/T	5.67±0.04	20.56±0.06	22.20±0.03	18.87±0.05	27.47±0.13
A. hybridus, cultivated	N/T	33.18±0.14	13.29±0.11	15.24±0.23	24.58±0.46	-7.55±0.29
Acarbose	71.37±0.01	83.41±0.01	86.29±0.02	87.22±0.04	89.00±0.05	N/T

N/T: not tested

4.5 Discussion

The MeOH extracts of wild *A. hybridus* and cultivated *A. cruentus* leaves demonstrated highest activities against α-glucosidase. Wild *A. hybridus*, in particular, showed potent *α*-glucosidase inhibitory effect which was higher than the positive control at all tested concentration. To the best of the author's knowledge, this is the first study reported on *A. hybridus*'s *α*-glucosidase inhibitory activity. In our investigation, the highest α-glucosidase inhibitory activity was observed in case of *A. cruentus*, in parallel with the work reported by Obah et al. (2013) where *A. cruentus* also inhibited α-glucosidase activity *in vitro*. Other *Amaranthus* species have also been evaluated for α-glucosidase inhibitory activity. MeOH extract of *A. spinosus* and its isolated compound (14*E*, 18*E*, 22*E*, 26*E*) –methyl nonacosa-14, 18, 22, 26 tetraenoate showed strong α-glucosidase inhibitory effect (Mondal et al. 2015) at 8.49 µM/L and 6.52 µM/mL. Kinetics analysis of the same study revealed uncompetitive type of inhibition for isolated compound and MeOH extract of *A. spinosus*. Although kinetics analysis was not conducted in our study, there is a possibility that an uncompetitive type of inhibition was observed between *A. cruentus* and the substrate. The ability of the plants to inhibit the effectiveness of α-glucosidase enzymes activity is attributed to phytochemicals such as luteolin (1.72×10^{-4} mol L^{-1}) (Yan et al. 2014), caffeic acid (27.4 µM) (Jabeen et al. 2013), vanillic acid (69.4 µM) (Mbaze et al. 2007), quercetin (15 µM) (Proença et al. 2017) and rutin (13.19 µM/mL) (Hong et al., 2013). It is therefore possible that the high inhibitory activity of *Amaranthus* spp. on α-glucosidase observed in the current study is due to the presence of these phytochemicals which have been isolated from *Amaranthus* spp. (Davids et al., 2016; Paranthaman et al., 2012).

The study also evaluated the effect of cultivation on α-glucosidase enzyme inhibition. Wild *A. cruentus* was less active than its cultivated counterpart. The effective dose of wild *A. cruentus*

was concentration dependent; the plant's effectiveness increased with an increase in dosage. As a result, it was possible to calculate the plant's IC_{50}=0.144 mg/mL. Unfortunately, in case of cultivated *A. cruentus* there was no relationship between the concentration and inhibition which means, as the concentrations of cultivated *A. cruentus* increased, the enzyme inhibition presented did not increase. Similar findings were detected in another study which examined α-glucosidase inhibitory effects of butanol fractions of *Salvia mirzayanii* and *Zataria multiflora* MeOH extracts and petroleum ether fraction of *Otostegia persica* EtOH extract. The study reported no relationship between the concentration level (in some fractions) and enzyme inhibition (Rouzbehan et al., 2017). *Amaranthus hybridus* showed opposite results whereby wild samples were more active than the cultivated samples at all concentrations tested. The results for *A. hybridus* correlate with findings from Chauhan et al. (2017) which illustrated that the wild variety of the plant showed more inhibition as compared to the cultivated *Rauwolfia serpentiana.*

With regard to α-amylase enzyme inhibition, all the extracts (wild and cultivated) of *A. hybridus* and *A. cruentus* were weak inhibitors. This is in line with earlier reports which showed that plant phytochemicals of *A. cruentus* and *Zea mays* are mild α-amylase inhibitors (Obah et al., 2013; Kwon et al., 2007). Contrary to this study, although the extracts were tested at higher concentrations (3 mg/mL), Odhav et al. (2010) reported that *A. hybridus* inhibited α-amylase enzyme by 52.76% (Odhav et al., 2010). Moreover, in another study, *A. cruentus* exhibited the highest inhibitory value of 55.40% at a concentration of 100 mg/mL (Ramalashmi, 2019). In a separate study, although the authors reported on different species than the ones utilised in this study, Conforti et al. (2005) demonstrated that MeOH, EtOAc and *n*-hexane extracts from two varieties of *A. caudatus* seeds showed α-amylase inhibitory activity (above 80% inhibition rate) at 0.25-1 mg/mL (Conforti et al., 2005). The mild α-amylase inhibitory activity observed in our study could be attributed, at least in part, to the lower concentrations of plant extracts.

4.6 Conclusion

Strong α-glucosidase and mild α-amylase inhibitory activity of *Amaranthu*s extracts could address the major disadvantages of commercially available α-glucosidase and α-amylase inhibitors. These drugs are associated with side effects such as abdominal discomfort, flatulence, and possibly diarrhoea. Noteworthy, and reported for the first time is the excellent α-glucosidase inhibitory activity of wild *A.hybridus* material (89.92±0.04 to 97.10±0.05%),

better than the positive control acarbose at all concentrations tested. Once again, in corroboration with the metabolomics analysis results in Chapter 3, the effect of cultivation and species are highlighted as cultivated material and material from different species showed significant differences in activity when compared to the two enzymes tested. The findings of this study showed that effective, cost-saving therapy using traditional vegetables could be a means of reducing untreated diabetes problems. Given the fact that diet is the cornerstone in the management of diabetes mellitus, a diet laden in fruits and vegetables is recommended. In line with an old adage by Hippocrates, "Let food be thy medicine, and medicine thy food", an inclusion of *Amaranthus* crop in people's plates not only provides minerals and micronutrients, but according to the findings of this study, the crop has medicinal properties. Furthermore, the findings of this study suggest that there is a need to isolate the active principle(s) that are responsible for antidiabetic properties associated with *Amaranthus* spp.

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CHAPTER 5

Isolation and antidiabetic activities of α-spinasterol, pheophorbide A-methyl ester and palmitic acid from *Amaranthus cruentus* L.

Abstract

A phytochemical investigation of *A. cruentus* led to the isolation of three known compounds; α-spinasterol, palmitic acid and pheophorbide A-methyl ester, which was isolated from *Amaranthus* spp. for the first time. The structures of isolated compounds were elucidated using proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy and comparison of spectral data with literature values. All compounds including acarbose, a commercially used antidiabetic drug, were tested for their antidiabetic activity against α-glucosidase and α-amylase enzymes. All compounds were potent α-glucosidase enzyme inhibitors. Palmitic acid in particular demonstrated the highest inhibition against α-glucosidase for all the concentrations tested. However, the isolated compounds were weaker α-amylase enzyme inhibitors. *Amaranthus cruentus* with associated compounds represents a new candidate for the management of diabetes mellitus.

Keywords: α-glucosidase, α-amylase, chromatography, spectroscopic techniques, phytochemicals

5.1 Introduction

Dietary starch hydrolysis is the main source of blood glucose, with α-amylase and α-glucosidase being the primary enzymes involved in starch breakdown and bowel absorption, respectively (Lordan et al., 2013). Inhibition of such enzymes is believed to substantially decrease the postprandial rise in blood glucose levels after a mixed carbohydrate diet, and may therefore be an effective tool in the management of type II diabetes-related hyperglycaemia (Kwon et al., 2008). Human α-amylase is one of the pancreas and salivary glands' main secretory products, which plays a role in the digestion of starch and glycogen (Lordan et al., 2013). α-Amylase catalyses the cleavage of starch, amylose, amylopectin, glycogen and various maltodextrins into shorter oligosaccharides (Etxeberria et al., 2012). The final stage of the digestive cycle is activated by a second enzyme, α-glucosidase, which is also present in the

brush-border surface membrane of intestinal cells. This exo-type carbohydrase enzyme facilitates the hydrolysis of complex carbohydrates and disaccharides to absorbable monosaccharides (Kim et al., 2010). Consequently, these hydrolytic enzyme inhibitors block the release of glucose from the intestinal tract into the blood vessels resulting in a drop in postprandial hyperglycaemia (Lordan et al., 2013).

As discussed in the previous chapter, current antidiabetic drugs, namely, acarbose, miglitol and voglibose are associated with adverse effects. It is for this reason that natural α-amylase and α-glucosidase inhibitors with less or no side effects are required in the management of diabetes.

In order to establish the best candidate for the isolation of active constituents, Thin Layer Chromatography (TLC) was done for wild *A. cruentus* and *A. hybridus*. Preliminary TLC results showed that *A. cruentus* contained a rich and diverse chemical profile when compared with *A. hybridus* (Figure 5.1). In addition, even though wild *A. hybridus* demonstrated higher α-glucosidase activity than all the samples, wild *A. cruentus* showed higher α-amylase activity (with an exception of 2mg/mL). As a result, *A. cruentus* was selected for further phytochemical work. The cultivated samples were excluded since there was not enough starting material (one kilogram) for phytochemical isolation.

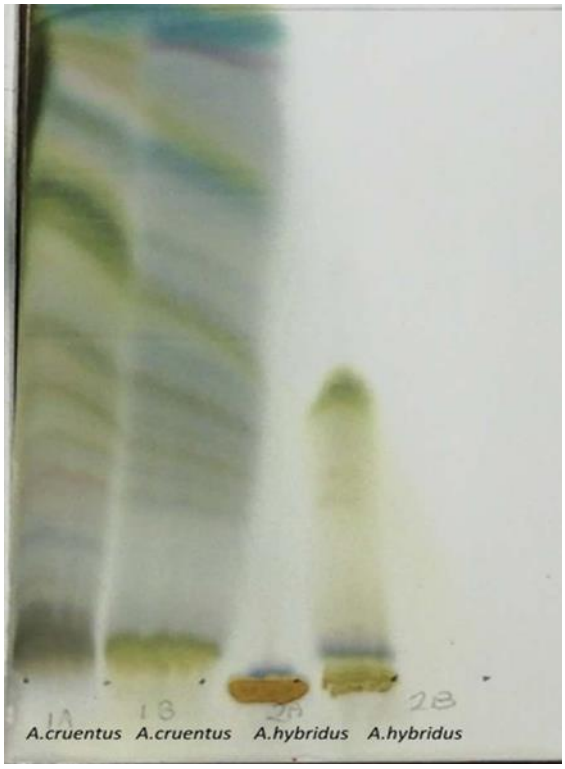

Figure 5.1: TLC chromatogram of crude MeOH leaf extracts of *A. cruentus* (1 A, B) and *A. hybridus* (2 A, B). Solvent system- CH_2Cl_2-MeOH (95:5). Detection: Vanillin in sulphuric acid

5.2 Materials and methods

5.2.1 Bulk extraction of active compounds from *A. cruentus*

A schematic diagram showing the isolation of *A. cruentus* active compounds is presented in Figure 5.2. Details about the transportation and storage of wild plant material are described in section 3.2.1. The air-dried powdered leaves of *A. cruentus* were ground into fine powder. The powdered leaf material (1 kg) was soaked in 5 L of MeOH-H_2O (8:2) overnight. The mixture (plant material and solvent) was filtered through Whatman no. four filter paper and stored at room temperature. This process was repeated twice. The filtrates were then combined and evaporated under reduced pressure using rotary evaporator (BUCHI-Labotech, Switzerland) to yield a semi solid extract. The semi solid material which amounted to 63.9 g was subjected to liquid-liquid partitioning using hexane, EtOAc and water. The EtOAc extract was subjected to column chromatography (CC, 10 x 20 cm) (Figure 5.3) with Merck silica gel 60 (0.040-0.063 mm) as stationary phase and eluted with hexane/EtOAc mixtures of increasing polarity (0-100%) followed by EtOAc/ MeOH mixtures of increasing polarity as mobile phases. A total of 36 sub-fractions (500 mL) were collected. The fractions were combined on the basis of TLC

profiles leading to thirteen main fractions (Figure 5.4). TLC of 13 pooled fractions were developed with hexane-EtOAc (8:2) and CH_2Cl_2- MeOH (95:5). TLC plates were then examined under 254 nm and thereafter dipped in vanillin solution (15 g vanillin, 250 mL EtOH and 2.5 mL concentrated sulphuric acid) and heated to detect compounds which do not absorb UV. Out of the 13 combined fractions, fraction one yielded two compounds, palmitic acid and α-spinosterol. In addition, fraction four yielded one compound, pheophorbide A-methyl ester, a derivative of chlorophyll which was isolated from *A. cruentus* for the first time.

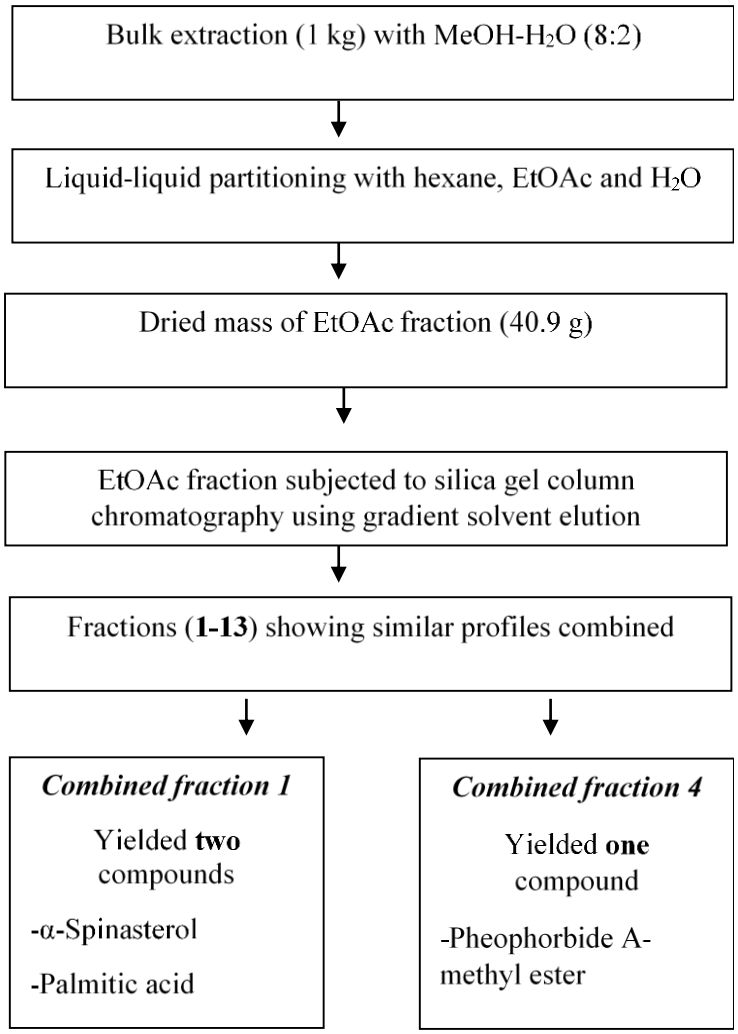

Figure 5.2: Schematic diagram of isolation of antidiabetic compounds from *A. cruentus*

Figure 5.3: Silica gel column chromatographic purification of EtOAc fraction of *A. cruentus.*

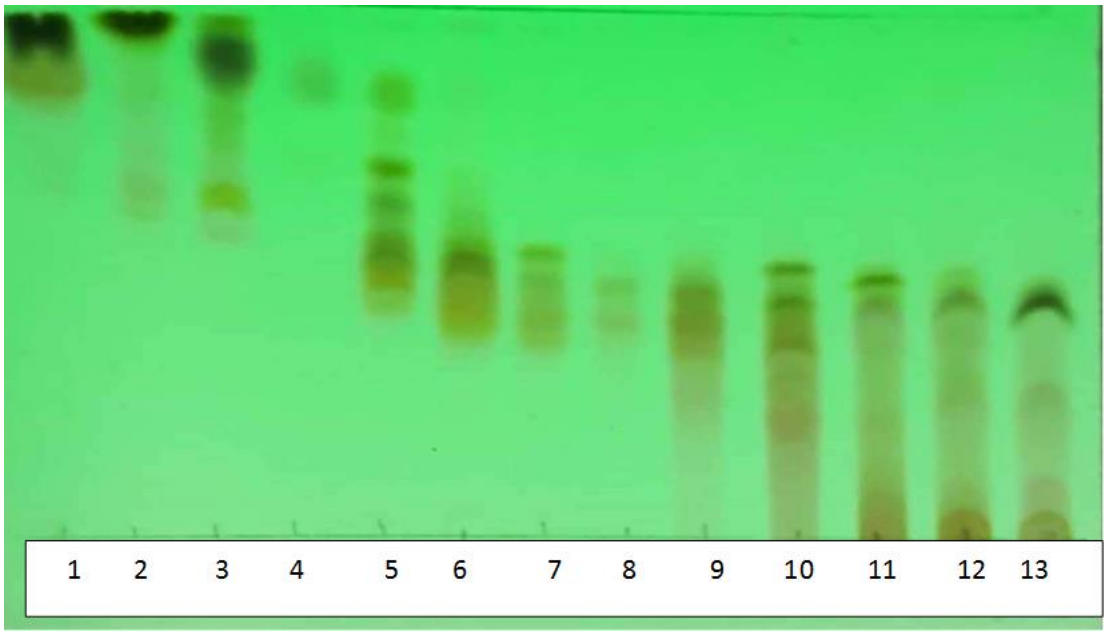

Figure 5.4: TLC chromatogram of combined fractions from chromatographic separation of *A. cruentus*. Solvent system- CH_2Cl_2-MeOH (95:5). **Detection**: Vanillin in sulphuric acid

5.2.2 α-Glucosidase and α-amylase inhibitory activity

α-Glucosidase and α-amylase inhibitory activities were performed as described in sections 4.2.4 and 4.2.5. Testing of the α-amylase activity followed a two way approach. i) Compounds were tested individually at concentrations ranging from 0.062-1 mg/mL. ii) The compounds were combined in a 1:1:1 ratio and tested at the same concentrations as before (0.062-1 mg/mL).

5.3 Results

5.3.1 Structure elucidation of compounds

Two compounds, α-spinasterol, 48.9 mg (**1**) and palmitic acid, 22.2 mg (**2**) were isolated from the combined fraction one. In addition, combined fraction four yielded one compound, pheophorbide A-methyl ester, 124.0 mg (**3**). The structures of isolated compounds are illustrated in Figure 5.5. Identification of the isolated compounds was achieved by comparing their spectroscopic data with those reported in literature. The NMR spectra of all isolated compounds are presented in Appendix A.

5.3.1.1 Characterization of α-spinasterol (1)

A colourless crystalline solid. ^{1}H-NMR ($CDCl_3$, 600 MHz): 0.53 (3H, s, H-18), 0.78-0.79 (6H, m, H-27, H-29), 0.83 (3H, s, H-19), 0.84 (3H, d, *J* = 6.0 Hz, H-26), 1.01 (3H, d, *J* = 6.5 Hz, H-21), 3.58 (1H, m, H-3), 5.01 (1H, dd, *J* = 15.0, 8.5 Hz, H-23), 5.12 (1H, m, H-7), 5.15 (1H, dd, *J* = 15.0, 8.5 Hz, H-22). ^{13}C-NMR ($CDCl_3$, 600 MHz): 12.05 (C-18), 12.22 (C-29), 13.02 (C-19), 19.00 (C-27), 21.06 (C-21), 21.37 (C-26), 21.56 (C-11), 23.02 (C-15), 25.38 (C-28), 28.48 (C-16), 29.69 (C-6), 31.48 (C-2), 31.87 (C-25), 34.23 (C-10), 37.16 (C-1), 38.00 (C-4), 39.48 (C-12), 40.29 (C-5), 40.78 (C-20), 43.30 (C-13), 49.48 (C-9), 51.26 (C-24), 55.13 (C-14), 55.94 (C-17), 71.06 (C-3), 117.45 (C-7), 129.47 (C-23), 138.14 (C-22), 139.57 (C-8).

5.3.1.2 Characterization of palmitic acid (2)

A slightly yellow coloured oily like semisolid compound. ^{1}H-NMR ($CDCl_3$, 600 MHz): 0.86 (3H, t, *J* = 6.7), 1.26 (28H, overlapping CH_2), 2.33 (2H, t, *J* = 6.7). ^{13}C-NMR ($CDCl_3$, 600

MHz): 14.67, 22.66; 24.68, 29.05, 29.22, 29.33, 29.41, 29.57, 29.57, 29.61, 29.63, 29.64, 29.66, 29.67, 31.90, 34.00, 179.69.

5.3.1.3 Characterization of pheophorbide A-methyl ester (3)

Pheophorbide A-methyl ester, a dark-green amorphous solid.^{1}H-NMR ($CDCl_3$, 600 MHz): 1.65 (3H, t, *J* = 7.8 Hz, H-31), 1.81(3H, d, *J* = 7.2 Hz, H-25), 2.31 (2H, m, H-2), 2.53 (2H, m, H-1), 3.15 (3H, s, H-29), 3.36 (3H, s, H-26), 3.57 (3H, s, H-36), 3.59 (2H, q, *J* = 7.6 Hz, H-30), 3.65 (3H, s, H-32), 3.87 (3H, s, H-34), 4.20 (1H, m, H-3), 4.45 (1H, dq, *J* = 7.2, 1.8 Hz, H-4), 6.12 (1H, dd, *J* = 11.4, 1.2 Hz, Ha-28), 6.14 (1H, dd, *J* = 17.9, 1.2 Hz, Hb-28), 6.24 (1H, s, H-21), 7.92 (1H, dd, *J* = 17.9, 11.4 Hz, H-27), 8.53 (1H, s, H-6), 9.28 (1H, s, H-11), 9.44 (1H, s, H-16). ^{13}C-NMR ($CDCI_3$, 600 MHz): 11.13 (C-29), 12.02 (C-26), 12.05 (C-32), 17.33 (C-31), 19.38 (C-30), 23.03 (C-25), 29.63 (C-2), 31.06 (C-1), 50.10 (C-4), 51.13 (C-3), 51.61 (C- 36), 52.79 (C-34), 64.70 (C-21), 93.08 (C-6), 97.48 (C-11), 104.36 (C-16), 105.26 (C-22), 122.69 (C- 28), 128.93 (C-19), 129.00 (C-27), 129.02 (C-18), 131.80 (C-8), 136.14 (C-9), 136.19 (C-10), 136.48 (C-13), 137.91 (C-17), 142.03 (C-7), 145.15 (C- 14), 149.64 (C-24), 150.91 (C-15), 155.58 (C-12), 161.18 (C-23), 169.54 (C-33), 172.14 (C-5), 173.30 (C-35), 189.56 (C-20).

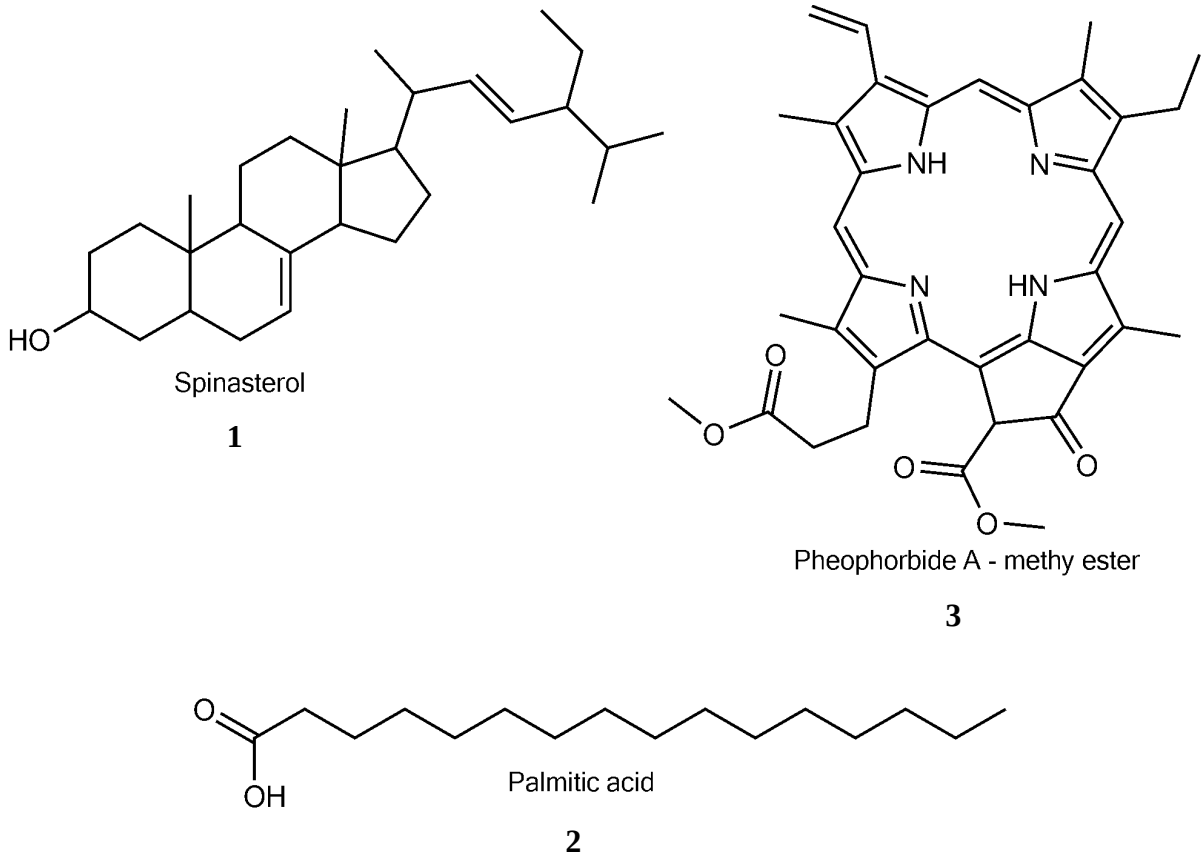

Figure 5.5: Chemical structures of isolated compounds

5.3.2 α-Glucosidase inhibitory activity

The percentage inhibition of the isolated compounds were compared to the percentage inhibition of the reference drug, acarbose. The inhibition values that were lower than 50% were considered to be insignificant. The isolated compounds demonstrated inhibition very similar to that of acarbose and in some cases the compounds were better inhibitors compared to acarbose. At both high and low concentrations tested, palmitic acid showed the highest inhibitory activity towards α-glucosidase enzyme, with inhibition of 83.26±0.03% and 91.26±0.01% respectively (Figure 5.6, Table 5.1).

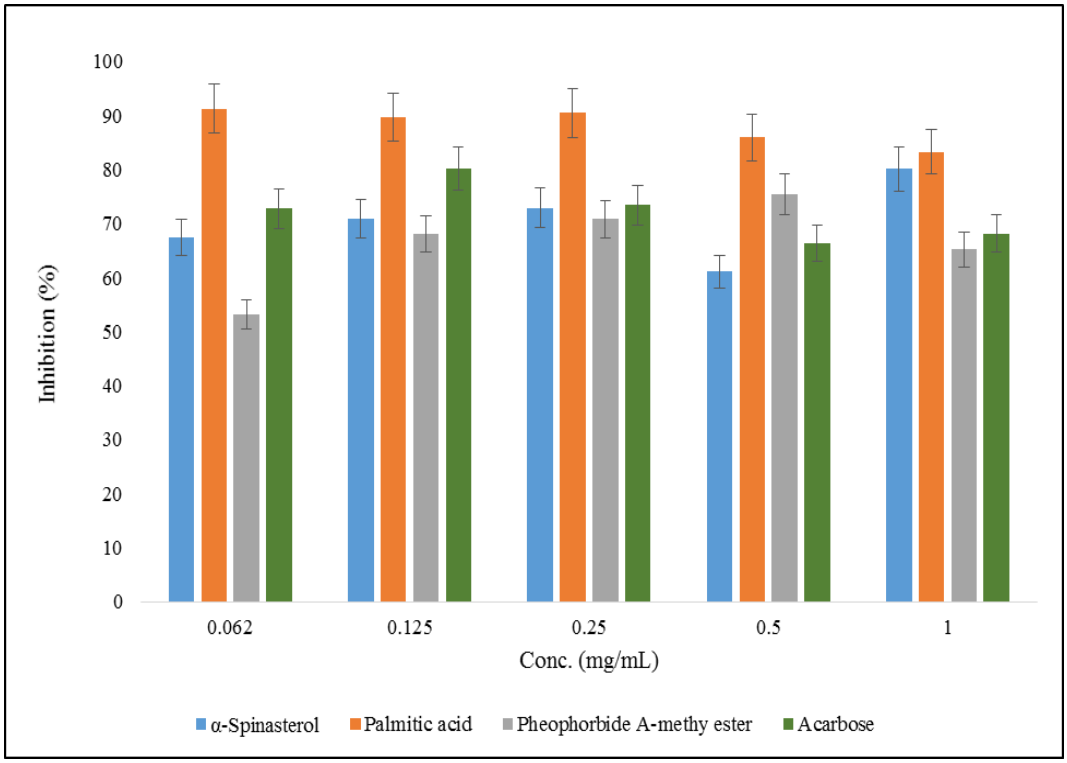

Figure 5.6: Inhibition of α-glucosidase using *p*-nitrophenyl α-D-glucopyranoside as a substrate, by the isolated compounds and positive drug-control; acarbose

Table 5.1: Effect of isolated compounds on the inhibition of α-glucosidase enzyme

α-glucosidase (% inhibition)	**0.062 mg/mL**	**0.125 mg/mL**	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**
α-Spinasterol	67.41±0.10	70.82±0.02	72.91±0.04	61.13±0.08	80.06±0.04
Palmitic acid	91.26±0.01	89.67±0.06	90.51±0.01	85.92±0.02	83.26±0.03
Pheophorbide A-methyl ester	53.16±0.43	68.08±0.30	70.78±0.11	75.41±0.03	65.22±0.06
Acarbose	72.72±0.24	80.20±0.13	73.41±0.16	66.31±022	68.18±011

5.3.3 α-Amylase inhibitory activity

Numerous chemical compounds have been isolated from a wide variety of plants which have demonstrated behaviour consistent with their potential use in diabetes treatment (Sales et al., 2012). In the present study, α-spinosterol, palmitic acid and pheophorbide A-methyl ester were tested and none of these isolated compounds inhibited α-amylase enzyme beyond 50% (Figure 5.7, Table 5.2). On the contrary, when the compounds were combined and tested collectively,

the compounds showed α-amylase inhibition of 58.49±002% at 0.5 mg/mL. At the same concentration (0.5 mg/mL), acarbose exerted high inhibitory activity of 87.22±004% (Figure 5.7, Table 5.2).

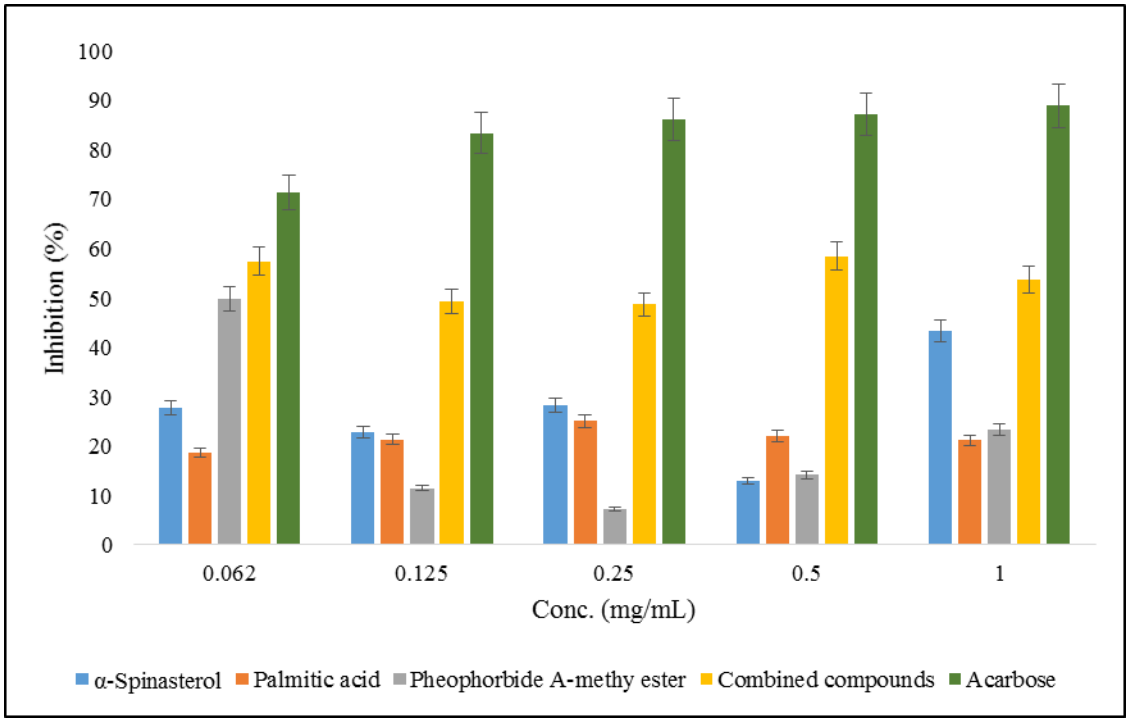

Figure 5.7: Inhibition of α-amylase using soluble potato starch as a substrate, by the isolated compounds, combined compounds and positive drug-control; acarbose

Table 5.2: Effect of isolated compounds on the inhibition of α-amylase enzyme

α-amylase (% inhibition)	**0.062 mg/mL**	**0.125 mg/mL**	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**
α-Spinasterol	27.73±0.17	22.86±0.10	28.22±0.12	13.06±0.23	43.37±0.15
Palmitic acid	18.68±0.07	21.340.08	25.05±0.02	21.99±0.03	21.16±0.07
Pheophorbide A-methyl ester	49.84±0.04	11.50±0.19	7.23±0.12	14.15±0.15	23.31±0.40
Combined compounds	57.45±0.04	49.32±0.04	48.69±0.05	58.49±0.02	53.65±0.04
Acarbose	71.37±0.01	83.41±0.01	86.29±0.02	87.22±0.04	89.00±0.05

5.4 Discussion

Purification of the EtOAc fraction of *A. cruentus* leaves resulted in the isolation of α-spinasterol (Kojima et al. 1990), palmitic acid (Bulama et al. 2014) and pheophorbide A-methyl ester (Rho et al., 2003). Pheophorbide A-methyl ester has been isolated from *A. cruentus* for the first time. Previously, pheophorbide A-methyl ester, was isolated from the leaves of *Combretum paniculatum* (Sowemimo et al. 2012), *Garuga pinnata* (Wongsinkongman et al. 2002) and *Neptunia oieracea* (Nakamura et al., 1996). α-Spinasterol is a plant-derived sterol present in a wide range of plants, including spinach leaves, alfalfa, cucumber, pumpkin seeds and other plants (Socała, and Wlaź, 2016). In *Amaranthus* spp., α-spinasterol was isolated from the stems of *A. spinosus* (Billah et al. 2013) and roots of *A. viridis* (Ragasa et al., 2015). Even though α-spinasterol was not isolated, researchers reported the presence of α-spinasterol in *A. cruentus* which was detected through gas chromatography coupled with mass spectrometry (Czaplicki et al., 2012). Palmitic acid is one of the most abundant saturated fatty acids in plants, humans, animals and microorganisms that make up approximately 16 to 45% of the lipid profile (Sidorov et al., 2014). Palmitic acid has been detected in *A. cruentus* in previous studies (Hlinková et al., 2013; He et al., 2002; Yánez et al., 1994).

All the compounds tested in this study showed good activity against α-glucosidase enzyme. In a study by Chukwujekwu et al. (2016), α-spinasterol isolated from the leaves of *Buddleja saligna* showed α-glucosidase enzyme inhibition with IC_{50} value of 10.7 µg/mL. In another study, α-spinasterol moderately inhibited the α-glucosidase (IC_{50}= 200 µM) (Jeong et al., 2015). In a different antidiabetic assay model, α-spinasterol was found to ameliorate the development and progression of diabetic nephropathy in streptozotocin-induced diabetic mice (in the same manner as the insulin did) (Jeong et al., 2004). Song et al. (2013) established that α-spinasterol, which was isolated from roots of *Phytolacca americana*, was able to ameliorate the development and progression of diabetic nephropathy by inhibiting the transforming growth factor-β (TGF-β) production). TGF-β is the central cytokine that plays a role in the development of diabetic nephropathy (Song et al., 2013).

Pheophorbide A-methyl ester is a chlorophyll A-derivative (Rho et al., 2003). There are no previous reports, at least to the best of the author's knowledge, about the inhibitory activity of pheophorbide A-methyl ester on *in vitro* α-glucosidase and α-amylase. However, Kim et al. (2019) reported that pheophorbide A, an unesterified analog isolated from *Gelidium amansii* caused significant decrease in the activity of α-glucosidase and α-amylase when compared to acarbose (Kim et al., 2019). The authors of the study purported that inhibitory effects of pheophorbide A on α-glucosidase and α-amylase were partly due to the hydroxyl group within chemical structure. In the same study, pheophorbide A suppressed blood glucose levels in STZ-induced diabetic mice than in the control group. Even though pheophorbide A-methyl ester was not active against α-amylase enzyme in the current study, its ability to effectively inhibit α-glucosidase may partly be due to an existence of the hydroxyl group as suggested by Kim et al., (2019). Moreover, pheophorbide A-methyl ester has a potential to be used as a nutraceutical agent for the alleviation of postprandial hyperglycemia (Kim et al., 2019).

Palmitic acid showed the highest α-glucosidase activity in all the concentrations tested ranging from 324.69-355.89 µM (83.26±0.03-91.26±0.01 mg/mL). These results are comparable with findings reported by Cherigo and Martínez-Luis (2018) in which palmitic acid showed α-glucosidase inhibitory activity of 237.5 µM. The authors reported that palmitic acid inhibited the activity of α-glucosidase enzyme and its activity was similar to acarbose (241.6 µM). In the same study, the authors established that palmitic acid acted as a competitive inhibitor and that the compound binds to the same site as acarbose in the human intestine (Cherigo and Martínez-Luis 2018). In another study, it was found that palmitic acid showed a moderate inhibitory effect on both α-amylase and α-glucosidase activities at concentrations of 3000 µM and 750 µM respectively (Su et al., 2013). Pheophorbide A-methyl ester; palmitic acid and α-spinasterol were weak inhibitors of the α-amylase enzyme. However, when the compounds were tested as a mixture, their activity was enhanced, at least in the case of α-amylase. Thus, the study proposes that the synergistic effects as well as individual activity of phytochemicals in vegetables are responsible for their potent antidiabetic activities, and that the benefit of a diet rich in vegetables is attributed to the complex mixture of phytochemicals present in whole foods. Although the mechanism of action for enzyme inhibition was not determined in this study, it is possible that compounds isolated from *A. cruentus* can either bind to the enzyme at a catalytic site (Escandón-Rivera et al. 2012); or can act as a non-competitive inhibitor (Kim

et al., 2013; Wang et al., 2013). In other cases, compounds can act as competitive inhibitors (Kang et al., 2009).

5.5 Conclusion

This study was undertaken to investigate the chemical components underlying the beneficial antidiabetic effect of *A. cruentus* extract. Three compounds were isolated and identified from the EtOAc extract, namely α-spinasterol, palmitic acid and pheophorbide A-methyl ester. This is the first report of the isolation of pheophorbide A-methyl ester from *Amaranthus* spp. All the compounds were effective against α-glucosidase enzyme, with palmitic acid resulting in the highest activity (91.26±0.01%), better than the positive control acarbose, at 72.72±0.24% inhibition at the lowest concentration tested (0.062 mg/mL). The study highlights the importance of inclusion of different assays or targets. As it was demonstrated in this study, very good activity can be achieved in one assay with no or low activity in another. Moreover, the capacity of pheophorbide A-methyl ester to ameliorate diabetes mellitus has never been reported before, although very good activity was reported for pheophorbide A, supporting the activity reported in this study.

5.6 References

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CHAPTER 6

Targeted metabolomic analysis of the crude extracts of wild and cultivated *Amaranthus* spp.

Abstract

Amaranthus in particular, wild *A. hybridus* and cultivated *A. cruentus* showed α-glucosidase activity. Targeted analysis for isolated compounds, revealed no link to clustering of these compounds. Comparison of the cultivated vs the wild samples of the two *Amaranthus* spp., as well as the active samples of the two species, led to the identification of common NMR regions in samples with better α-glucosidase activity. The focus was specifically on the comparison of wild *A. hybridus* with the cultivated samples, as wild material exhibited the highest α-glucosidase activity. Similar for *A. cruentus*, the cultivated material was compared with the wild samples, as the cultivated material showed higher α-glucosidase activity. NMR regions, common between the samples with higher α-glucosidase inhibitory activity (wild *A. hybridus* and cultivated *A. cruentus*) was therefore targeted. Upon the inspection of proton NMR spectra, peaks at δ 5.75, 7.07, 7.29, 7.34 and 7.51 were pronounced and distinctly higher in all the samples. None of the compounds isolated in Chapter 5, matched these peaks, and literature and database searches for matches to these peaks were undertaken. The presence of these peaks fits well to the NMR profile of an alkaloid 2-phenylethenamine. A number of alkaloids have been isolated from *Amaranthus* species before and some of these alkaloids have been proven to be potent α-glucosidase inhibitors. The compound 2-phenylethenamine is therefore proposed as a possible determinant for clustering of the samples and possibly contributing to the activity of *Amaranthus* species and cultivation practises. Furthermore, the presence of 2-phenylethenamine in *Amaranthus* spp. was confirmed by LC-MS. The compound however has not been isolated and activity of the compound should be confirmed although this is the first report of 2-phenylethenamine in *Amaranthus*.

6.1 Introduction

2-Phenylethenamine is an alkaloid derived biosynthetically from the aromatic amino acid, phenylalanine (Irsfeld et al., 2013). It is not a well-known compound, although 2-

phenylethylamine (PEA) is a more common relative, differing with only one double bond from 2-phenylethenamine. PEA is present in many algae (Guven et al., 2010), fungi and bacteria (Kim et al., 2012) and various plant species (Le Thi et al., 2014). In plants, PEA is found in members of the family Fabaceae, which is the second-largest family of seed plants and is comprised of trees, shrubs, vines, herbs (such as clover), and vegetables (such as beans and peas) (Irsfeld et al., 2013). In addition, PEA has been isolated and identified from *Prosopis chilensis* pods (Astudillo et al., 2000). PEA and 2-phenylethenamine however have not been identified in *Amaranthus* before.

The presence of PEA in plants is associated with allelopathy. In a study by Bunse et al., (2020) N-*trans*-cinnamoyltyramine, a β-phenylethylamine, showed allelophatic effect in Vietnamese rice cultivar. This research postulates that the presence of 2-phenylethenamine in the crude extracts of *Amaranthus* can be linked to the plant's allelopathic activity. In a study by Prinsloo and Du Plooy (2018), the extracts of the whole *Amaranth* plant significantly inhibited the seed germination of vegetables as well as weed seeds of *Conyza bonariensis* under experimental conditions (Prinsloo and Du Plooy, 2018). The study did not identify the compounds responsible for *Amaranthus's* allelopathic effect.

6.2 Materials and methods

6.2.1 Metabolomics analysis

The metabolomics analysis as described in 3.2.6 was used in the targeted analysis of the data in this Chapter. A two phased approach was followed.

Firstly, the cultivated vs the wild samples of each of the two *Amaranthus* spp. were compared, to identify NMR regions discriminating between the samples, focusing on the samples with better activity. Wild *A. hybridus* exhibited the highest α-glucosidase activity, and the profile was therefore compared with the cultivated samples of *A. hybridus*. Similar for *A. cruentus*, the cultivated material which showed higher α-glucosidase activity was compared with the wild samples of *A. cruentus*. NMR regions, common between the samples with higher α-glucosidase inhibitory activity (wild *A. hybridus* and cultivated *A. cruentus*) were therefore identified.

Secondly, the regions identified in the first phase were compared for the wild *A. hybridus* and cultivated *A. cruentus* samples. The common NMR regions present in both were recorded and used for targeted analysis.

6.2.2 Liquid chromatography-mass spectroscopy (LC-MS)

Triple quad based LC-MS (LC-QqQ-MS) was used in order to confirm the presence of 2-phenylethenamine in *Amaranthus* spp.

6.2.2.1 Sample preparation

Five milligram of dried *Amaranthus* leaf materials was extracted with MeOH-H_2O (75:25). Samples were sonicated for 5 min and thereafter centrifuged for 15 min at 15 000 rpm. To extract and remove cell debris, the supernatant was filtered through Sartorius Minisart RC 4 0.2 µM syringe filters with 1 mL plastic pipette.

UPLC analysis

A Waters UPLC coupled in tandem to a Waters SYNAPT G1 HDMS MS was used to generate accurate mass data. Optimisation of the chromatographic separation was done utilising a Waters HSS T3 C18 column (150 mm x 2.1 mm, 1.8 µm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (HCOOH) (natural pH of 2.3) and CH_3CN (Eluent B) containing 10 mM HCOOH. The initial conditions were 98% A at a flow rate of 0.4 mL/min and were maintained for 1 minute, followed by a linear gradient to 5%A at 25 min. The conditions were kept constant for 2 min. and then changed to the initial conditions. The runtime was 30 min. and the injection volume was 2 µL. Samples were kept cool at 6 °C in the Sample Manager during the analysis.

Liquid Chromatography TOF Mass Spec analysis

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to enable detection of phenolic and other ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in both ESI positive and negative modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 4.0 V.

The scan time was 0.1 sec. covering the 50 to 1200 Dalton mass range. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. Nitrogen gas was used as the nebulisation gas at a flow rate of 550 L/h and cone gas was added at 50 L/h. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 872).

The raw data was processed and extracted ion chromatograms (XICs) obtained based on the known compounds found in published literature. The accurate mass data of each detected compound was submitted for elemental composition, double bond equivalence (DBE) as well as isotopic fit calculations. Compounds tentatively identified using the abovementioned criteria are listed in Table A1 (Appendix). Compound identification was further enhanced by analysing all samples with low and high collision energy settings of the collision cell. To minimise compound fragmentation a low energy setting of 3 eV was used, but to enhance fragmentation of molecules various collision energies between 10 and 40 eV were used (MS^E). Fragmentation spectra were submitted to the NIST mass spectral library (NIST 2014, Version 2.2 build Jun 2014) as well as the mass spectral libraries developed on the Synapt G1 system.

6.2.2.2 Annotation and identification of compounds

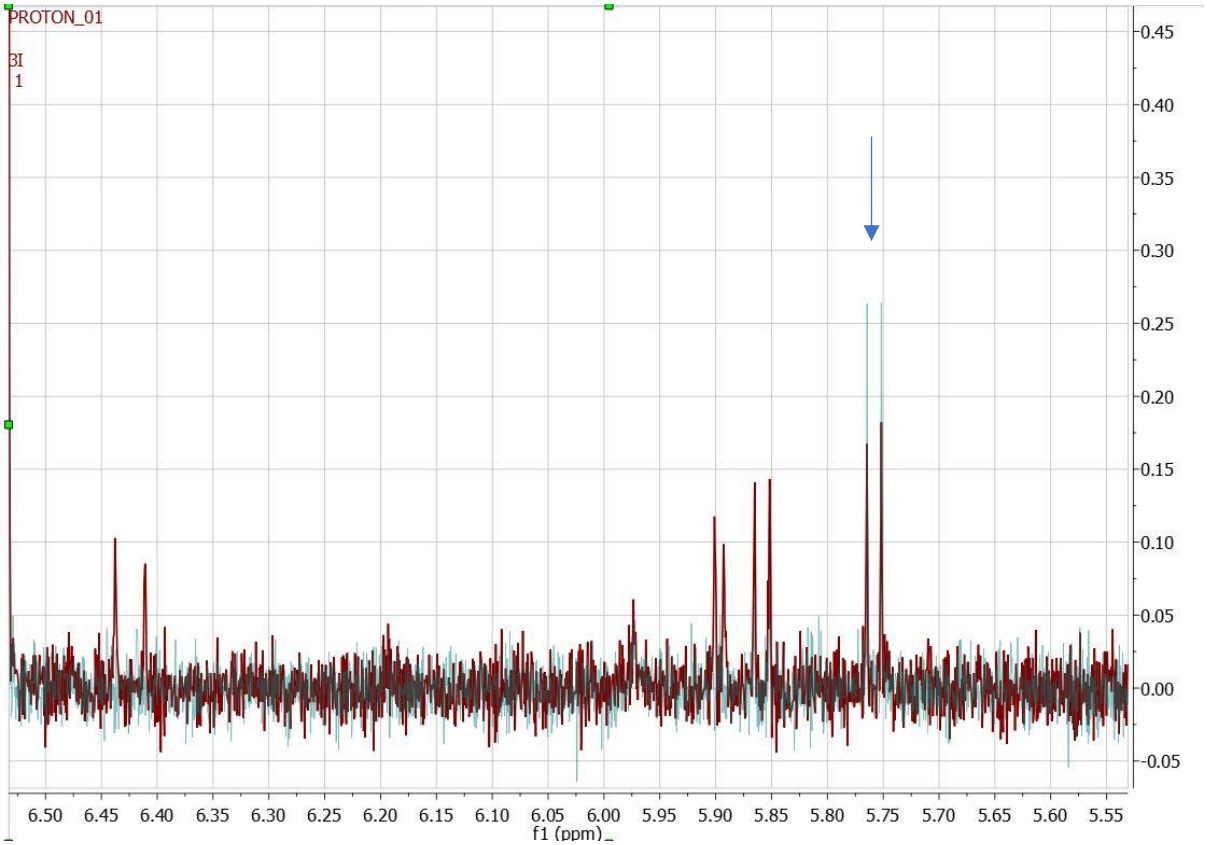
An automated chemical structure annotation and identification of 2-phenylethenamine in Amaranth extracts was carried out using MAGMa and CSIFingerID respectively. The compound was detected in positive ionisation mode producing a precursor ion $[M+H]^+$ of 120.0825 (theoretical accurate mass of 119.0735). The double bond equivalence (DBE) calculation was 5 which is in accordance with published data. Further MS^e fragmentation produced a product ion at 103.0587 which corresponds to the loss of NH_2 and a DBE count of 6.

6.3 Results and discussion

The presence of 2-phenylethenamine in wild and cultivated Amaranth species was detected by using proton NMR spectra of samples acquired for metabolomics analysis and comparing their spectroscopic data with those reported in literature. Chemical shifts for proton NMR for 2-phenylethenamine are delineated as follow: 5.75 (1H, d, *J*=16.1Hz), 7.07 (1H, tt, *J*=7.7, 1.3 Hz), 7.29 (2H, tdd, *J*=7.6, 1.3, 0.5 Hz), 7.34 (2H, dddd, *J*=7.6, 1.8, 1.3, 0.5 Hz), 7.51 (d, *J*=16.1 Hz). ^{1}H-NMR spectra of *Amaranthus* spp. aqueous MeOH crude extracts are illustrated in

Figure 6.1 and Figure 6.2. The doublet of one proton at δ 5.75 was observed in all the samples although higher in wild *A. hydridus* and cultivated *A. cruentus*. The signal at δ 7.07 is also higher in wild *A. hybridus* (Figure 6.1) and cultivated *A. cruentus* (Figure 6.2) and is characteristic of 2-phenylethenamine (Figure 6.3A). Moreover, signals at δ 7.29, 7.34 and 7.51 were present in all the samples, although higher in wild *A. hybridus* and cultivated *A. cruentus*. Additionally it was confirmed with LC-MS (Figure 6.4).

Although there is no available studies reporting the direct link of 2-phenylethenamine or PEA on DM, some publications reported that alkaloids had to α-glucosidase and α-amylase inhibitory activities. For an example, betalains with antidiabetic inhibitory properties have been isolated from *A. tricolor* (Biswas et al., 2013). Betalains are not strictly alkaloids, because they are acidic in nature due to the presence of several carboxyl groups, however, similarly to alkaloids, betalains possesses at least one heterocyclic nitrogen atom (Schliemann et al., 2001). Yin et al., (2014) reported two alkaloids (vasicine and vasicinol) which were isolated from the MeOH extract of the leaves of *Adhatoda vasica* Nees. These two compounds are potent inhibitors of α-glucosidase (IC_{50} = 125 and 250 μM respectively), both being competitive enzyme inhibitors. In another study, three alkaloids, miliusacunine E, consanguine B, and polyalthiacinnamine B exhibited α-glucosidase inhibitory activity varying from IC_{50}= 11.3-43.2 μM, better than acarbose (IC_{50} =83.5 μM). Harmanyl β-d-glucopyranoside, a potent α-glucosidase inhibitor isolated from *Buthus martensii* Karsch showed non-competitive glucosidase inhibition, with an IC_{50} value of 24 μM (Kim, 2013). The molecular docking study of alkaloids conducted by Zafar et al., (2016) indicates good α-glucosidase inhibition exerted by the compounds, attributed to their good docking score and binding mode. Good α-glucosidase inhibition activity of crude extracts of *Amaranthus* is therefore proposed to be linked to the presence of 2-phenylethenamine.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-0.05
6.50 6.45 6.40 6.35 6.30 6.25 6.20 6.15 6.10 6.05 6.00 5.95 5.90 5.85 5.80 5.75 5.70 5.65 5.60 5.55
f1 (ppm)

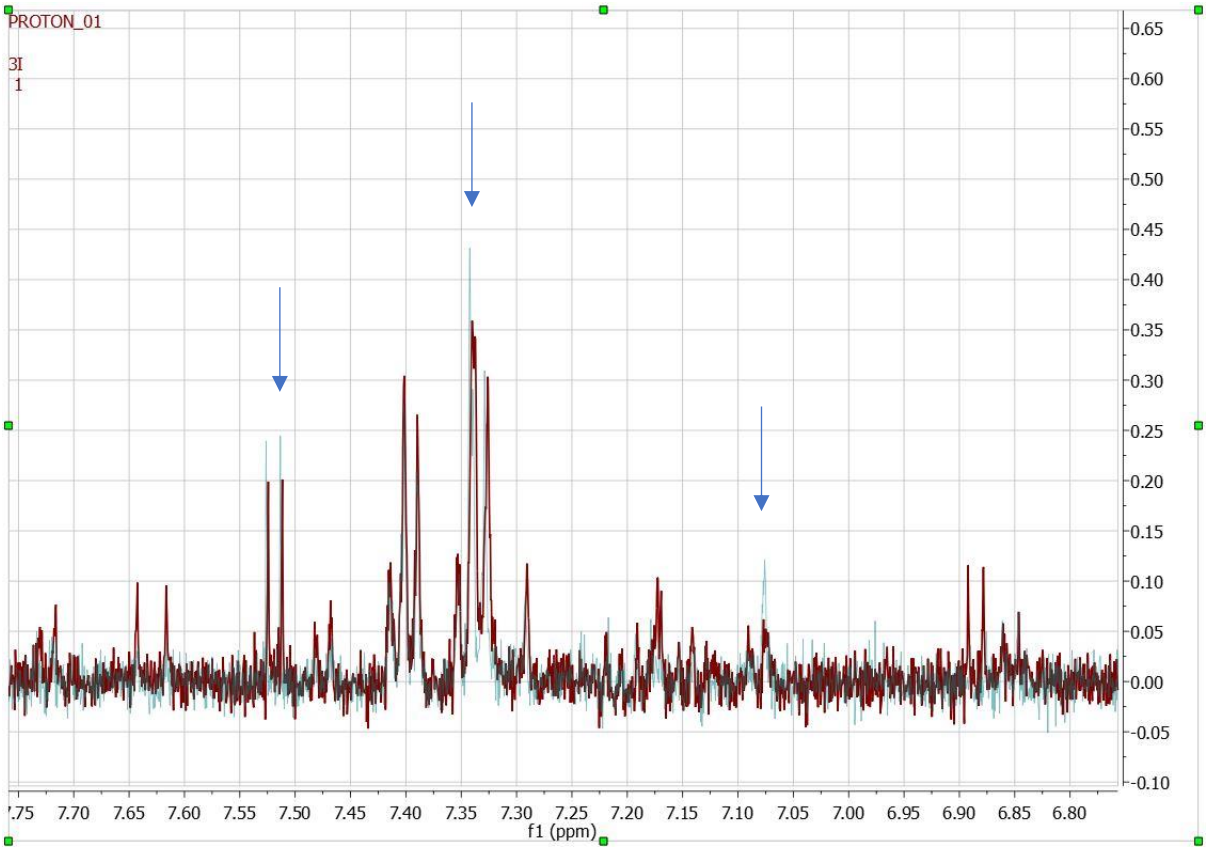
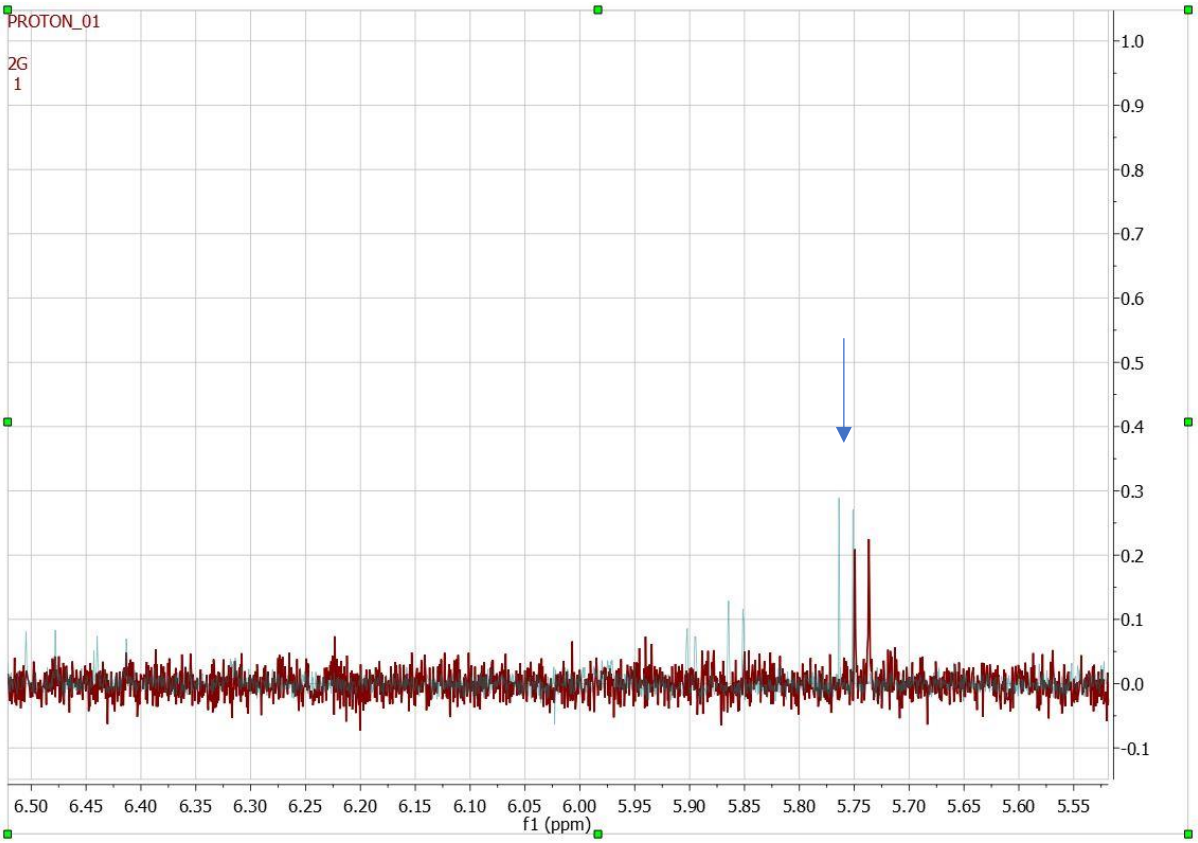


Figure 6.1: Comparison of wild (blue spectrum) and cultivated *A. hybridus* (red spectrum). Peaks at δ 5.75, 7.07, 7.29, 7.34 and 7.52 are indicated by arrows. Height of the peaks are indicative of the concentration of the compound.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1.0
0.9
0.8
0.7
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0.5
0.4
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0.2
0.1
0.0
-0.1
6.50 6.45 6.40 6.35 6.30 6.25 6.20 6.15 6.10 6.05 6.00 5.95 5.90 5.85 5.80 5.75 5.70 5.65 5.60 5.55
f1 (ppm)

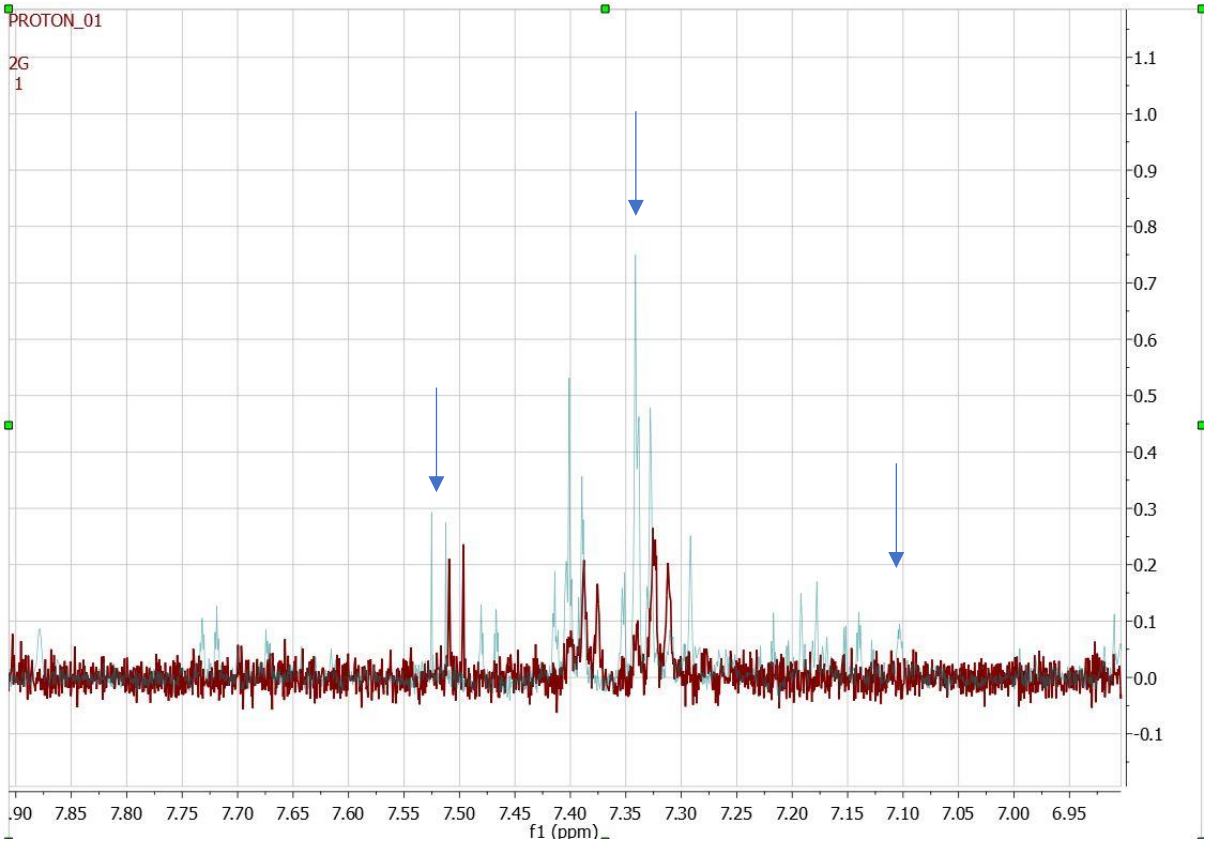
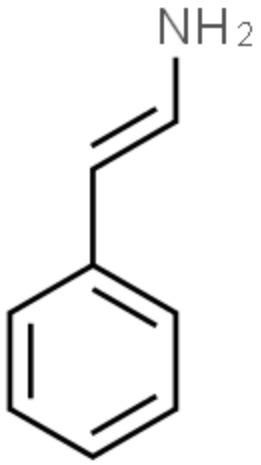


Figure 6.2: Comparison of wild (red spectrum) and cultivated *A. cruentus* (blue spectrum). Peaks at δ 5.75, 7.10, 7.29, 7.39 and 7.50 are indicated by arrows. Height of the peaks are indicative of the concentration of the compound

A



B

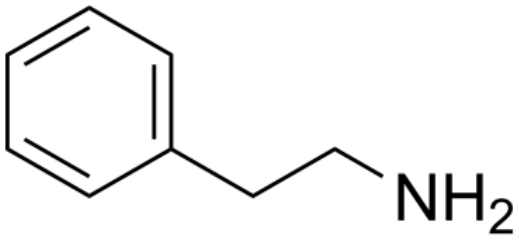


Figure 6.3: Structures of A: 2-phenylethenamine; B: 2-phenylethylamine

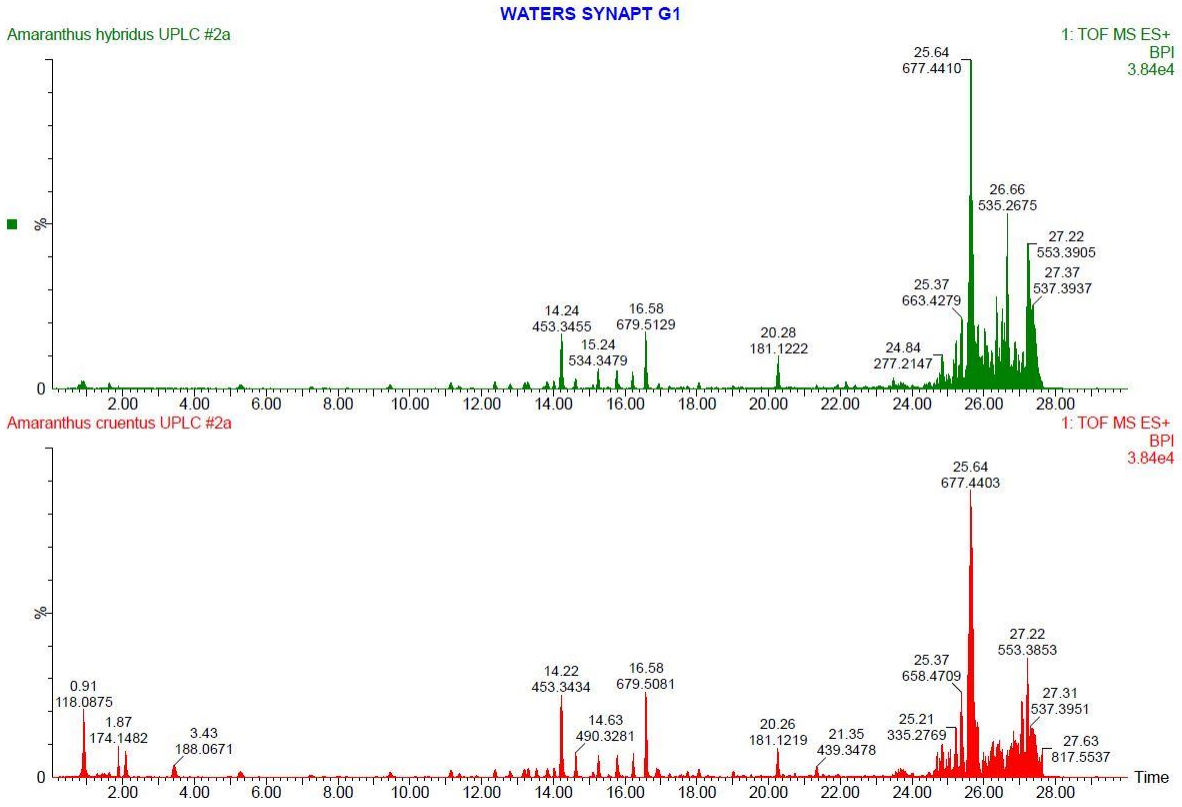


Figure 6.4: Chromatograms of the wild *Amaranthus hybridus* and *A. cruentus* extracts analysed by LC-QqQ-MS (positive ionisation mode)

Considering Figures 6.1 and 6.2 peaks observed at 5.75, 7.29, 7.39 and 7.50 were higher in wild *A. hybridus* and cultivated *A. cruentus*. These peaks align to an alkaloid, 2

Phenylethenamine. Additionally the presence of 2-phenylethenamine was confirmed by LC-MS (Figure 6.4). Even though this compound was not isolated from *Amaranthus* spp., the study proposes that the high α-glucosidase activity observed in wild *A. hybridus* and cultivated *A. cruentus* is linked to the presence and concentration levels of 2-phenylethenamine.

6.4 Conclusion

In this chapter, targeted analysis was conducted to determine if the compounds isolated in the previous chapter distinguished the more active samples from the samples with lower activity. None of the compounds isolated could be linked to the differences in the samples with improved activity. By comparing the samples with higher and lower activity, NMR regions could be identified which were consistently associated with samples with higher activity. These peaks were linked to an alkaloid 2-phenylethenamine. In addition, LC-MS which is more sensitive than NMR further confirmed the presence of 2-phenylethenamine in both *Amaranthus* spp. This is the first report of 2-phenylethenamine in *Amaranthus*. It is therefore proposed that 2-phenylethenamine, might be a major component, resulting the higher activity found in some samples. The compound should however be isolated, and tested for activity on α-glucosidase enzyme, to confirm the role of 2-phenylethenamine in the antidiabetic activity of the *Amaranthus*.

6.5 References

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CHAPTER 7

Zinc, iron and vitamin A analysis of wild and cultivated *Amaranthus* spp.

Abstract

Amaranthus has been rediscovered as a promising food crop mainly due to its ability to endure and thrive in diverse environments and its potential to store diverse micronutrients. The concentrations of two minerals (zinc and iron) and vitamin A were determined in samples of naturally growing and cultivated *A. hybridus* and *A. cruentus*. The total metal contents were analysed by inductively coupled plasma optical emission spectrometry. Vitamin A was detected using High Performance Liquid Chromatography (HPLC). Cultivated *A. hybridus* and wild grown *A. cruentus* showed similar zinc concentration, 19.5 mg/100 g and 19.6 mg/100 g respectively. The highest iron amount was observed in cultivated *A. hybridus*. None of the samples accumulated vitamin A. Consumption of *Amaranthus* can aid in supplementing iron and zinc especially in households that have limited access to nutrient rich food.

Keywords: micronutrient deficiencies, dietary diversification, food fortification, leafy vegetables

7.1 Introduction

Mineral and vitamin deficiency has become a threat to the health of the world's more than two billion population (Bailey et al., 2015). It is well established that women and children are affected the most with regard to micronutrient deficiencies (Harika et al., 2017). Due to its degrading effects on health, learning ability and productivity, particularly on the already disadvantaged population, dietary deficiency contributes largely to the vicious circle of underdevelopment (WHO, 2000). The dietary requirement refers to lowest continuing intake of a nutrient that will maintain a defined level of nutritive need in an individual for a specified indicator of adequacy (Jimoh et al., 2018). Iron, vitamin A, and zinc are amongst the most common micronutrient deficiencies (Muthayya et al., 2013). In South Africa, vitamin A deficiency (VAD) is estimated at 22% in women of reproductive age (Harika et al., 2017). Vitamin A is an important element for proper functioning of the visual system, immune system,

cell maintenance, reproduction and epithelial cell integrity (Ahmad and Ahmed, 2019). Deficiency of vitamin A in children is associated with measles and diarrhoea whereas the deficiency manifests as night blindness in adults (Akhtar et al., 2013). Vitamin A rich food such as foods of animal origin for example dairy products, liver and egg yolks are not always readily available (Akhtar et al., 2013).

Iron is very important during the first five years of life; as a result, children are the most vulnerable group to iron deficiency anaemia because of their increased iron requirements in the periods of rapid growth (Hlatswayo et al., 2016). Children that are iron deficient suffer from increased childhood morbidity and impaired cognitive development and school performance (Hlatswayo et al., 2016). The daily iron requirements for children range between 7-15 mg/day and women of reproductive age require 15-18 mg/day while pregnant women require 27 mg/day (FAO/WHO, 2001). Zinc deficiency, on the other hand, is associated with impaired development, depressed immune function, increased susceptibility and severity of infections, and neurobehavioral abnormalities (Motadi et al., 2015). Currently, there are no recent estimates on zinc deficiency at national level (Hess, 2017). The 2005 National Food Fortification Baseline Survey showed that 44% of children between the ages of one and nine had deficient zinc status and were thus at risk of zinc deficiency (Labadarios et al., 2008).

Lack of dietary diversity is linked to micronutrient deficiencies mentioned above in South Africa (Faber et al., 2007). Many countries including South Africa have adopted a number of strategic approaches such as food fortification, supplementation and dietary diversification (Govender et al., 2017). With respect to dietary diversity, the promotion of underexploited traditional foods offers a great potential in South African households. *Amaranthus* spp. in particular, are endowed with minerals and vitamins that can contribute towards food and nutrition security (Jimoh et al., 2018). Thus, the aim of this study was to assess the vitamin A, zinc, and iron content of fresh *Amaranthus* spp. leaves grown in different geographic areas in South Africa.

7.2 Materials and methods

7.2.1 Collection of plant material

Collection, planting and harvesting of plant materials are described in sections 3.2.1-3.2.3.

7.2.2 Processing of leaves for analysis

The leaves were soaked and washed vigorously with tap water (three to four times) and then rinsed with distilled water to remove the soil debris. The leaves were dried at room temperature. Dried leaves were crushed into fine powder. Measured portions (150 g) were transferred to marked centrifuge tubes with screw caps. The containers were kept at room temperature in a cabinet until required for analysis.

7.2.3 Chemical analyses of selected nutrients

7.2.3.1 Vitamin A analysis

The method used for the determination of vitamin A is adopted from American Association for Clinical Chemistry (AACC) 86-06. The samples of dried leaves (1.0 g) were macerated for an hour in 20 mL of petroleum ether. The mixture was filtered and evaporated to dryness. A solution (0.2 mL) of chloroform-acetic anhydride (1:1, v/v) was added to the residue, followed by 2 mL of trichloroacetic acid (TCA) dissolved in $CHCl_3$ (30%, w/v). The purity of vitamin A standard (purchased from Sigma, South Africa) was tested on HPLC and the concentration of standard was adjusted accordingly. Chromatography was performed with reverse phase C18 column, 10-μm (4.6 × 250 mm) (Agilent Poroshell EC-C18 column, Agilent Technologies), capable of separating *cis* and *trans* isomers of retinol with a resolution of 1.0 or greater. The mobile phase consisted of MeOH: deionized water (80:20), with a flow rate of 1 mL/min. The detector wavelength was set at 328 nm for the detection of vitamin A.

7.2.3.4 Mineral analysis

Iron and zinc content were determined by means of Optical Emission Spectrometry Inductively Coupled Plasma (ICP-OES) using AOAC Method 984.27. A 100 g portion of the dried leaf samples were digested with nitric-perchloric acid at temperatures up to a maximum of 200°C. The Varian Liberty 200 sequential ICP-OES with a Varian SPS-5 autosampler (Agilent Technologies, Inc., Santa Clara, CA, USA) measures each wavelength immediately after each other, using a single photomultiplier tube detector with a diffraction grating monochromator. The instrument uses a V-groove nebuliser with a radial orientated Sturman-Masters spray chamber with the torch. The instrument was calibrated against iron and zinc standard solutions.

Each element was measured at an appropriate emission wavelength (iron, 259.940 nm and zinc, 213.856 nm) chosen for high sensitivity and lack of spectral interferences.

7.3 Results

The zinc and iron content of wild and cultivated *Amaranthus* spp. are presented in Figure 7.1 and Table 7.1. These values are compared to the widely consumed vegetables such as cabbage and spinach. Values for iron and zinc are given in mg per 100 g of fresh edible leaf mass.

The zinc content of Amaranth in the study ranged from 8.4-16.6 mg/100g (Table 7.1, Figure 7.1) while that of spinach and cabbage was 0.53 and 0.18 mg/100 g respectively. Wild grown *A. cruentus* collected from KZN demonstrated the highest content (19.6 mg/100g) of zinc, whereas cultivated *A. cruentus* concentrated the least amount of zinc compared to all other samples. According to the findings in this study, wild *A. cruentus* collected from KZN provided the most abundant zinc out of all the samples tested.

There is a 7-fold variation in the content of iron in cultivated and wild grown *A. hybridus.* The latter showed iron content of 15.8 mg/100 g and cultivated *A. hybridus* concentrated 124.0 mg/100 g iron (Table 7.1, Figure 7.1). Wild grown *A. cruentus* accumulated more iron (117.0 mg/100g) than its cultivated counterpart (81.9 mg/100 g). With an exception of wild grown *A. hybridus*, all the plants provided exceptionally high amount of iron beyond the recommended 29.4 mg daily intake for adult females (Tables 7.1 and 7.2). Vitamin A was not detected in any of the samples that were tested. Contrary to this, spinach accumulated high amount of vitamin A (672 μg RE) making it a good candidate for vitamin A supply.

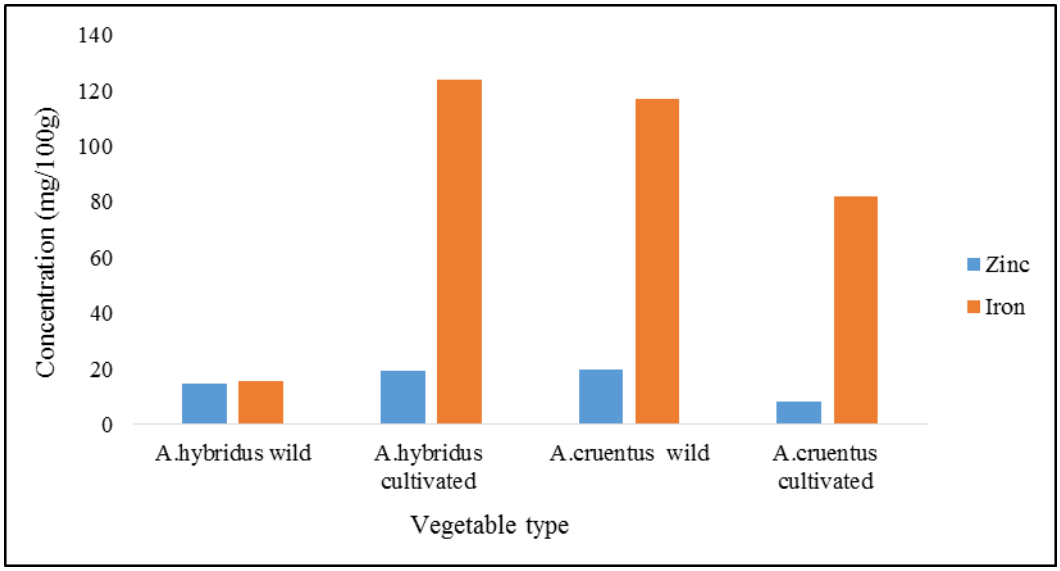

Figure 7.1: Zinc and iron content of *Amarantus* spp. Vitamin A was not detected

Table 7.1: Zinc, iron and vitamin A content of *Amarantus* spp. and widely consumed vegetables namely [1]*Spinacia oleracea* (spinach) and [2]*Brassica oleracea* var. *capitata* (cabbage)

Plant	Zinc mg/100 g	Iron	Vitamin A (µg RE)
A. hybridus wild	14.5	15.8	*ND
A.hybridus cultivated	19.5	124.0	*ND
A.cruentus wild	19.6	117.0	*ND
A.cruentus cultivated	8.4	81.9	*ND
[1]*Spinacia oleracea*	0.53	2.7	672
[2]*Brassica oleracea* var. *capitata*	0.18	0.47	5

*ND: not detectable, [1]Hadley and Fordham, 2003, [2]Gebhardt et al., 2008

Table 7.2: Recommended daily nutrient intakes (RNI) of selected micronutrients for different age groups (FAO, 2001)

Age (years)	Sex	Vitamin A (µg RE)	Iron[a] (mg)	Zinc[b] (mg)
1-3		400	5.8	8.3

4-6		450	6.3	9.6
7-9		500	8.9	11.2
10-18	Male	600	14.6 (10-14 yrs.)	17.1
			18.8 (15-18 yrs.)	
	Female	600	32.7 (10-14 yrs.)	14.4
			31.0 (15-18 yrs.)	
19-65	Male	600	13.7	14.0
	Female	500	29.4	9.8

[a] Based on a diet with 10% iron bioavailability
[b] Based on a diet with low zinc bioavailability

7.4 Discussion

Zinc is a micronutrient of biological importance since most biochemical processes and physiological functions cannot occur without it (Parveen et al., 2017). Zinc is required for more than 300 enzyme activities, it is vital for DNA stabilisation and for gene expression (Frassinetti et al., 2006). The study demonstrated that both cultivated and wild grown fresh leaves of *Amaranthus* spp. were rich sources of zinc.

The zinc content of wild and cultivated *A. hybridus* in the present study were substantially higher than values reported by Akubugwo et al. (2007) which was reported to be 3.8 mg/100 g for the same species. However, findings from Odhav et al. (2007) (18 mg/100 g) were comparable with the results from the present study. In another study (Ntuli, 2019), zinc levels of semi cultivated *A. hybridus* grown in KZN were recorded to be 93.0 mg/100 g, which far exceeded the amount of zinc in *A. hybridus* (or *A. cruentus*) reported in this study. The amount of zinc, 19.6 mg/100 g, for wild grown and 8.4 mg/100 g for cultivated *A. cruentus* reported in this study is higher than published data by Van Jaarsveld et al. (2014) and Chege et al. (2014) on the same species who reported values of 0.70 mg/100 g and 3.18 mg/100 g respectively. The amount of zinc offered by widely consumed vegetables, spinach (0.53 mg/100 g) and cabbage (0.18 mg/100 g) were significantly lower compared to Amaranth (Hadley and Fordham, 2003; Gebhardt et al., 2008).

Plant sources contain vitamin A in the form of carotenoids which must be converted into retinol during digestion before it can be used by the body (Gilbert, 2013). β-Carotene is the most abundant member of the group of carotenoids (Grune et al., 2010). Vitamin A was not detected in any of the plants tested in this study. This is contrary to the findings by Uusiku et al. (2010) with the amount of vitamin A recorded to be 327 µg RE for *Amaranthus* spp. Moreover, *A. cruentus* reported by Van Jaarsveld et al. (2014) accumulated 537 µg RE vitamin A, which is comparable to that of spinach (672 µg RE) (Hadley and Fordham, 2003).

Iron is an important mineral nutrient that is involved in multiple biological processes for all living species (Ancuceanu et al., 2015). Its deficit is the cause of the world's most common type of anaemia: iron deficiency anaemia (IDA) (Ancuceanu et al., 2015). Anaemia is characterized as concentration of haemoglobins below the cut-off levels (Ancuceanu et al., 2015). In the current study, it was shown that the amount of iron in the leaves of cultivated *A. hybridus* (124 mg/100 g) was found to be significantly higher than reported by Oyelola and Banjoko (2014) and Muchuweti et al. (2008) at 12.5 mg/100 g and 11.4 mg/100 g respectively. The wild material showed only a slightly higher value at 15.5 mg/100 g. Contrary to the results of this study, findings by Ntuli (2019) depicts that the leaves of *A. hybridus* had significantly higher iron amount (518 mg/100 g). The difference in iron content in leaves between the two studies is unclear, however, it is postulated that these differences could be ascribed to the differences in analytical tools or the solvents used for the extraction of minerals. In Ntuli's paper, the minerals were analysed using atomic absorption spectrophotometer as opposed to Optical Emission Spectrometry Inductively Coupled Plasma used in this study. The latter is more sensitive, thus is able to measure elements that are difficult to analyse in atomic absorption spectrometry (He et al., 2017).

The variation in iron content of the same species (*A. hybridus*) which was collected from two geographical areas is also supported by Jinazali et al. (2017), reporting major variations in the iron content (22.9-44.7 mg/100 g) of *Cleome gynandra* (another widely consumed ALVs) leaves collected from different agro-ecological zones in Malawi. The variations in iron contents in the study were attributed to the differences in soil types from the different districts (Jinazali et al., 2017). The observed intra-species variation in iron amount could be attributed, at least in part, to soil conditions (Dróżdż et al. 2018), which support the previous statements. Jumberi et al. (2001) found that soils with high pH were associated with a decrease in iron uptake by

barley and rye plants. It is possible that the soil from which wild *A. hybridus* leaves were collected had limited availability of iron as well as other factors such as pH that may have potentially limited the ability of the crop to accumulate iron. This is supported by the high accumulation by the cultivated material at 124 mg/ 100 g for *A. hybridus* and 81.9 mg/100 g for *A. cuentus*, both produced in the same soil under the same cultivation conditions. Under these conditions, it was even found that *A. hybridus* accumulated more iron than *A. cruentus*.

The amount of iron measured in *A. cruentus* in this study (117 mg/100 g for wild and 81.9 mg/ 100 g for cultivated) far exceeds that of *A. cruentus* that was reported in previous studies as stated by Chege et al. (2014) and Van Jaarsveld et al. (2014) at 8.47 mg/100 g and 5.1 mg/100 g respectively. Once again, soil type and conditions could be the reason for the difference in the reported values, as was also alluded for *A. hybridus*.

Iron bioavailability, defined as the degree to which iron is absorbed from the diet and used for normal body functions, should be considered in plant-based diets (Hurrell and Egli, 2010). Phytate is the primary inhibitor of absorption of iron in many plant-based diets (Hurrell and Egli, 2010). The negative effect of phytate on iron absorption has been shown to be dose-dependent (Abbaspour et al., 2014). Phytate has been detected in *Amaranth* species (Essack et al., 2017). However, methods of food processing and preparation including milling, heat treatment, soaking, and fermentation have been used to degrade phytate to varying degrees (Suri and Tanumihardjo, 2016). Vitamin C/ ascorbic acid on the other hand, has been shown to enhance iron absorption in human studies (Hurrell and Egli, 2010). *Amaranthus* plants contain an appreciable amount of vitamin C, which have the propensity to enhance the absorption of iron. The vitamin C level reported in *Amaranthus* spp. range from 2-126 mg/100 g (Uusiku et al., 2010; Van Jaarsveld et al., 2014, Steyn et al., 2001; Akubugwo et al., 2007). Efforts should be made to create awareness on the effect of preparation on the nutrient content of these vegetables to avoid overcooking them, which may denature some heat sensitive vitamins. Moreover, consumers and specific communities should be sensitised on the high nutritional profile of *Amaranthus* spp. as these crops have proportionally higher mineral profiles than widely conventional vegetables.

7.5 Conclusion

The current study revealed that the Amaranth vegetables are a good source of zinc and iron. It is also clearly shown that the ability of the plants to store nutrients is not only a function of environmental conditions, but also influenced by the plant genotype which has an impact on the accumulation of minerals. While there is no one-size-fits-all solution; it is clear that Amaranth is able to provide some of the essential required nutrients for the South African population regardless of species and where the plants are grown. Undesirable anti-nutrients such as phytate in Amaranth which may hinder zinc and iron absorption can be reduced through cooking and implementing other existing food processing and preparation methods. Cultivated and wild *Amaranthus* spp. have the potential to supplement a starch rich diet in poor rural communities in view of the fact that iron and zinc are one of the most prevalent forms of micronutrient malnutrition in the world.

7.6 References

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CHAPTER 8

8.1 GENERAL CONCLUSION AND RECOMMENDATIONS

Amaranthus has attracted attention worldwide due to its nutritional and medicinal attributes. Cultivation of this crop in South Africa is negligible despite its potential to contribute towards food and nutrition security. The leaves of *Amaranthus* are highly nutritious and in some cases, their mineral and vitamins levels surpass those of commonly cultivated leafy vegetables. Amaranth's pharmacological activities, such as antidiabetic, anti-tumour, anti-ulcer, hepatoprotective etc. are well documented (Nahar et al., 2018). The study therefore investigated the effect of cultivation and geographical location on the chemical profile of two widely consumed Amaranth species, *A. cruentus* and *A. hybridus*. Moreover, antidiabetic and nutritional profile of wild and cultivated *A. cruentus* and *A. hybridus* were determined.

As a bid to heed the call to introduce cultivation of Amaranth crop in urban areas, the trial of two species, *A. cruentus* and *A. hybridus*, was successfully established in the township of Mamelodi, Pretoria in Gauteng Province. In this regard, the first objective of the study was to conduct a comparative untargeted metabolomics analysis and identify differences and subsequently unique metabolites in leaves of cultivated *A. cruentus* and *A. hybridus* versus leaves of wild *A. cruentus* and *A. hybridus* growing in two geographically separated areas, namely KZN and Gauteng Provinces. The objective was to determine if *Amaranthus* can be cultivated without affecting the chemical profile and ultimately its pharmacological activity. ^{1}H-NMR metabolomics coupled with multivariate data analysis was used. If no chemical differences were observed, the NMR spectrums of wild and cultivated *A. cruentus* collected from different regions would look identical. Similarly, NMR spectrums of wild and cultivated *A. hybridus* would look similar. In addition, no clustering would be present therefore, hypothetically speaking, geographical location would have no impact on the chemical profile of the plants. NMR technology equipped with multivariate analysis proved successful in evaluating and clearly showing the chemical profile differences and annotating metabolites that contributed to groupings between wild and cultivated samples. In essence, experimental procedure or workflow for NMR in metabolomics was followed with precision to ensure a desired outcome. Deuterated methanol water was a solvent of choice due to its ability to extract

diverse metabolites. The incorporation of potassium dihydrogen phosphate buffer and controlling the pH in extraction solvents prevent the fluctuations in chemical shifts of signals in the NMR spectrum. Ensuring that reliable and accurate data was obtained, all the metabolomic analysis workflow starting from sample preparation, data processing and multivariate data analysis were carefully observed.

By combining ^{1}H-NMR analysis and OPLS-DA chemometrics, variations leading to samples grouping by site, suggested strong plant-environment interactions as factors influencing metabolite composition. The clustering observed indicates that the OPLS-DA model was a valid model for the current study as it showed high goodness of fit (R^2 and R^2Y) as well as good predictability values (Q^2 and Q^2Y). It was therefore determined that cultivation and geographical location significantly affect the chemical profile of *A. cruentus* and *A. hybridus*. Subsequently annotation of compounds was done to determine the compounds that were prominent in wild and cultivated *A. cruentus* and those that were discriminating wild and cultivated *A. hybridus*. Contribution plots were used to detect metabolites possibly responsible for the clustering observed in OPLS-DA score plots between samples that were collected in different locations. Correlating these chemical shifts to classes of metabolites found within the plant kingdom by means of Human metabolome database, Chenomx and literature search led to successful annotation of metabolites distinguishing the extracts of different localities. Table 3.3 lists constituents that distinguishes wild from cultivated *A. cruentus* and *A. hybridus* samples. Cultivated samples of *A. cruentus* had higher quantities of maltose, sucrose, leucine, lactulose, trehalose and valine than the wild samples. With regard to *A. hybridus*, cultivated samples accumulated more maltose, sucrose, proline, leucine and chlorogenic acid, compared to the quantities which were much lower in wild species. Chlorogenic acid was only found in cultivated samples of *A. hybridus*. It should be mentioned that chlorogenic acid annotated from cultivated *A. hybridus* has been reported to have antidiabetic properties.

Comparison of the plants on the species level reveal that cultivated *A. cruentus* and *A. hybridus* had a similar profile with regard to the annotated compounds. In both of these cultivated species, maltose, sucrose, and proline were quantitatively higher than in wild *A. cruentus* and *A. hybridus*. The difference observed between the two species was related to the presence of amino acids, leucine and valine. Valine was higher in cultivated *A. cruentus* but lower in cultivated *A. hybridus*, whereas, leucine was higher in cultivated *A. hybridus* and lower in

cultivated *A. cruentus*. Leucine plays an important role in promoting the secretion of insulin from pancreatic β-cells (Nisoli et al. 2013) thus its presence in cultivated *A. hybridus* could be linked to the plant's antidiabetic activity. The accumulation of high maltose, sucrose, trehalose, trigonelline and proline observed in cultivated species may have been influenced by the environment since both of these species were cultivated in the same place. The high accumulation of valine in cultivated *A. cruentus* samples and leucine in cultivated *A. hybridus* seem to suggest that even though these two species were grown in the same environment, their ability to accumulate amino acids, leucine and valine is related to species rather than environmental factors.

Since metabolomics analysis revealed clustering and therefore chemical profile differences between samples that were collected from geographically separate areas, the next step of the study was to determine the effects of environment and cultivation on diabetic activity. From the metabolomics chapter, two compounds that have antidiabetic activity were annotated: trigonelline, was annotated in all samples whereas chlorogenic acid was present only in cultivated *A. hybridus*. In order to determine whether or not, the presence of trigonelline have bearing on biological activity, all the samples were subjected to *in vitro* experiments to determine their α-glucosidase and α-amylase inhibition activity. One of the effective ways to manage T2DM is to slow down the conversion of starch to glucose by inhibiting α-amylase and α-glucosidase enzymes. α-Amylase and α-glucosidase enzyme assays are rapid, reliable and cost effective methods. α-Glucosidase enzyme used in this assay was isolated from *Saccharomyces cerevisiae*. All extracts showed very good α-glucosidase inhibitory activity especially wild *A. hybridus* and cultivated *A. cruentus* with inhibitory activity of 89.92±0.04 and 84.95±0.09 mg/mL respectively. Cultivated *A. hybridus* which contained chlorogenic acid showed remarkable α-glucosidase inhibitory activity (84.95±0.09) at the lowest concentration tested (0.125 mg/mL). The activity of wild *A. hybridus* was comparable to that of acarbose. The good inhibitory activity shows a higher binding affinity of the extracts to α-glucosidase enzyme. The extracts are predicted to either be non-competitive or competitive inhibitors of the enzyme. Wild *A. hybridus* was a more potent inhibitor of α-glucosidase (89.92±0.04) compared to wild *A. cruentus* (41.85±0.10) at lower concentrations (0.125 mg/mL). Since both of these wild species were collected from the same geographical area, the study showed that on the species level, *A. hybridus* is a better α-glucosidase inhibitor than *A. cruentus*. The study showed that cultivation, species and geographical location affected the plant's bioactivity.

As a result of the good antidiabetic activity obtained, it was pertinent to determine the active ingredients that were responsible for the plant's bioactivity. The therapeutic ability of plant extracts depends on the pharmacological effect produced individually or synergistically by the active compounds with other compounds within the extracts. To further explore Amaranth chemical diversity, chemical constituents that contributed toward the plant's antidiabetic activity were isolated. The techniques used to isolate and purify plant-derived compounds are mainly based on chromatography (Sasidharan et al., 2011). The samples that were cultivated at Mothong in Pretoria were inadequate in yield to carry out isolation of compounds, therefore, these samples were excluded for further analysis. Isolation and identification of compounds is both a time consuming and expensive exercise, a decision to select a viable candidate for the work was carefully considered. The first step involved preliminary Thin layer chromatography analysis which showed a rich profile for wild *A. cruentus* compared to *A. hybridus*. Secondly, even though wild *A. hybridus* showed exceptional α-glucosidase activity, wild *A. cruentus* showed higher α-amylase activity (with an exception of 2 mg/mL). Based on these observations, wild *A. cruentus* was selected for isolation and identification of compounds. Three compounds namely, α-spinasterol, palmitic acid alongside pheophorbide A-methyl ester were isolated. In the present study, pheophorbide A-methyl ester is reported in *Amaranthus* spp. for the first time.

The compounds that were successfully isolated were also subjected to *in vitro* evaluation against α-glucosidase and α-amylase enzymes. All the compounds showed strong α-glucosidase activity with palmitic acid showing highest activity exceeding positive control, acarbose, in all the concentrations tested. For an example, at the low concentration tested (0.062 mg/mL), α-glucosidase inhibitory activity demonstrated by palmitic acid was 91.26±0.01 compared to acarbose (72.72±0.24). Pheophorbide A-methyl ester, which was tested on α-glucosidase for the first time, also showed good activity (75.41±0.03-53.16±0.43). Mild α-amylase inhibition by extracts was observed. This shows the extracts have less affinity to bind α-amylase enzyme. However, the compounds showed good inhibitory effect on α-amylase that ranged from 48.69±0.05-58.49±0.02 when tested together. A synergistic effect between the compounds may have contributed to the compounds' ability to inhibit α-amylase activity. This study therefore contributes to a better understanding of the efficacy of the compounds in treatment and management of the diabetes challenge by inhibiting α-glucosidase. Additionally the difference in activity on two assays also shows the importance of using a variety of assays since compounds may have high affinity and specificity for a particular protein (enzyme) but

demonstrate low affinity for another. In the case of this study, crude extracts were potent α-glucosidase enzyme inhibitors but mild α-amylase inhibitors. This is especially desirable since excessive α-amylase inhibition is associated with abdominal discomfort as a result of undigested starch.

The good activity obtained and the annotation of various compounds in the extracts, such as chlorogenic acid, necessitated more in depth analysis of the samples to link compounds to the clustering and activity. Cultivated *A. hybridus* which contained chlorogenic acid demonstrated strong α-glucosidase inhibition at low concentration, however, compared to its wild counterpart, which was void of the compound, the values were slightly lower. Moreover, the presence of chlorogenic acid did not contribute to the plant's activity to inhibit α-amylase. It is therefore evident that other metabolites contribute to the high activity observed in wild species, particularly for *A. hybridus*, as these were more active on α-glucosidase (at low and high concentrations) than the cultivated samples.

Additionally, compounds with significant antidiabetic activity were isolated, and targeted analysis could be performed to determine if these compounds were responsible for the clustering of the samples in the metabolomics analysis. If these compounds could be linked to the clustering of the samples, cultivation practises, regions of planting and species selection could be managed. Targeted analysis is performed by matching characteristic NMR peaks of the compound to the profiles. Additionally, NMR is quantitative, and higher peaks of compounds therefore indicate an increase in concentration of the compounds which translates to better biological activity. Unfortunately none of the isolated compounds' peaks could be linked to the clustering of the samples according to geographical region and species selection. Literature alludes to the fact that although NMR spectroscopy is reproducible and reliable, it is deemed less sensitive than other available analytical tools such as liquid chromatography mass spectroscopy (LC-MS) and gas chromatography-mass spectrometry (GC-MS) (Emwas et al., 2019). It is possible that there may be other undetectable compounds through NMR that were responsible for clustering/ grouping of samples. Moreover, the polar solvent used, could contribute to low extraction of the apolar compounds, therefore not clearly present in the extracts.

A two phased approach was followed whereby the first step involved comparing cultivated vs the wild samples of each of the two *Amaranthus* spp. to identify NMR regions discriminating between the samples, focusing on the samples with better activity. Wild *A. hybridus* exhibited the highest activity against α-glucosidase and thus the profile was compared with the cultivated *A. hybridus* samples. Likewise for *A. cruentus*, the cultivated material which showed higher α-glucosidase activity was compared with the wild samples of *A. cruentus*. NMR regions, common between the wild *A. hybridus* and cultivated *A. cruentus* which showed higher α-glucosidase inhibitory activity were identified. The second step of the targeted analysis involved comparing NMR regions identified in the first phase for the wild *A. hybridus* and cultivated *A. cruentus* samples. This led to identification of specific peaks, consistently higher in samples with better activity. The aromatic region in the proton NMR of extracts showed consistently higher peaks at δ 7.07, 7.29, 7.39 and 7.50 ppm. By comparing the NMR peaks of known structures in literature, it was deduced that the peaks could be linked to an alkaloid compound 2-phenylethenamine. In addition, the samples were analysed and further detected in LC-MS which is a more sensitive and robust tool compared to NMR. This compound belongs to a class of secondary compounds, alkaloids, which have been reported with potent α–glucosidase activity. It is possible that the presence of 2- Phenylethenamine could have been responsible for better activity of wild *A. hybridus* and cultivated *A. cruentus* activity on α-glucosidase, although this compounds has never been reported in *Amaranthus* species. Since this compound has not been isolated, this proposed link to activity should be investigated further by isolating the compound and testing it for α-glucosidase activity. Additionally synergistic effects of the isolated compounds with the alkaloid is also possible, and will also yield valuable information especially α-amylase enzyme which was not inhibited by compounds when tested individually.

Finally, regarding nutritional analysis, although no vitamin A was detected in all the samples, inductively coupled plasma optical emission spectrometry was successfully used to identify the amount of zinc and iron levels in wild and cultivated *Amaranthus* spp. Compared to conventional vegetables, *Amaranthu*s spp. are rich sources of zinc and iron in quantities more than the widely consumed spinach and cabbage.

The use of *Amaranthus* spp. as food and medicine, especially for the treatment of diabetes is validated, although only tested *in vitro*. The identification of known antidiabetic compounds

however, supports the use of the plants for antidiabetic properties. Promotion of these species to urban areas is encouraged as data in this study shows that these plants still retain their nutritional status even under cultivation. It is also important to note that different species of Amaranth behave differently. For an example, *A. cruentus* behaved differently than *A. hybridus* with regard to nutrients accumulation. Wild *A. cruentus* accumulated more zinc and iron than cultivated *A. cruentus*. Contrary to this, cultivated *A. hybridus* accumulated more nutrients than its wild counterpart. Also observed in metabolomic analysis, wild samples of *A. cruentus* clustered together and were distinguished from those of cultivated *A. cruentus*. The same is true for *A. hybridus* wherein wild samples clustered together and cultivated samples grouped together. Regarding biological activity, for *A. cruentus*, cultivated samples were more effective than the material that was collected in the wild. However, wild samples of *A. hybridus* were effective in inhibiting enzyme activity linked to diabetes. There are approximately 60 species of the *Amaranthus* genus that have been recorded. This study has proven that two selected species out of the 60 Amaranth species showed unique characteristics. For an example, when considering nutrients (zinc and iron in this case), wild *A. cruentus* is a better candidate, however, its cultivated counterpart gave better antidiabetic results. The trends are reversed for *A. hybridus* in that wild samples were potent α-glucosidase inhibitors whereas, cultivated *A. hybridus* accumulated more nutrients than its wild counterpart.

The aim of the study was therefore achieved as the study successfully demonstrated that through NMR-based metabolomics, metabolites that discriminate between cultivated and wild Amaranth were annotated. In addition; antidiabetic properties of wild and cultivated *A. cruentus* and *A. hybridus*, as well as the isolated compounds were reported. Lastly, this is the first study to highlight the nutritional analysis of both cultivated and wild *Amaranthus* spp.

8.2 RECOMMENDATION AND FUTURE WORK

One study cannot answer all the existing questions. Based on this premise, the study recommends the following:

- In the present study, all the compounds that showed anti α-glucosidase activity were isolated from the non-polar extracts. It is therefore recommended that future studies should also include non-polar solvents for metabolomic analysis to determine the contribution of the isolated compounds to the pharmacological effect.

- Based on the results obtained in this study, promising species should be promoted and their sites of cultivation expanded to other areas. It is recommended that future studies should use plant metabolomics studies to investigate other widely consumed *Amaranthus* spp. in South Africa such as *A. greazicans*, *A. spinosus*, *A. deflexus*, *A. hypochondriacus* and *A. viridis*.
- Exploring *in vivo* models to further ascertain diabetic activities of wild and cultivated Amaranth to validate the *in vitro* results
- Incorporate other analytical tools such as GC-MS and/or LC-MS to identify compounds that cannot be detected by NMR.
- Molecular docking studies to compare the binding affinity of known drugs with compounds isolated in this study.
- More extensive phytochemical analysis of the extracts of other solvents to provide a more comprehensive chemical profile.
- Isolation of 2-phenylethenamine to confirm its contribution to the biological activity as this compounds has not been identified in any *Amaranthus* species nor linked to antidiabetic activity of *Amaranthus*.

A step towards developing commercially viable antidiabetic compounds from *Amaranthus* spp. can be achieved through the implementation of the recommendations given above. The direction towards commercialising outputs from universities bridges the gap between publically funded universities and the private sector and creates long-term partnership between the two. In addition, small scale farmers stand to benefit financially by cultivating Amaranth for profit. By introducing Amaranth in their diets, communities located in urban areas can also diversify their diets thus improving their overall wellbeing.

8.3 References

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Nahar, K., Kabir, F., Islam, P., Rahman, M.M., Al Mamun, M.A., Faruk, M., Subhan, N., Rahman, G.S., Reza, H.M. and Alam, M.A., 2018. Cardioprotective effect of *Amaranthus tricolor* extract in isoprenaline induced myocardial damage in ovariectomized rats. *Biomedicine & Pharmacotherapy*, *103*, pp.1154-1162.

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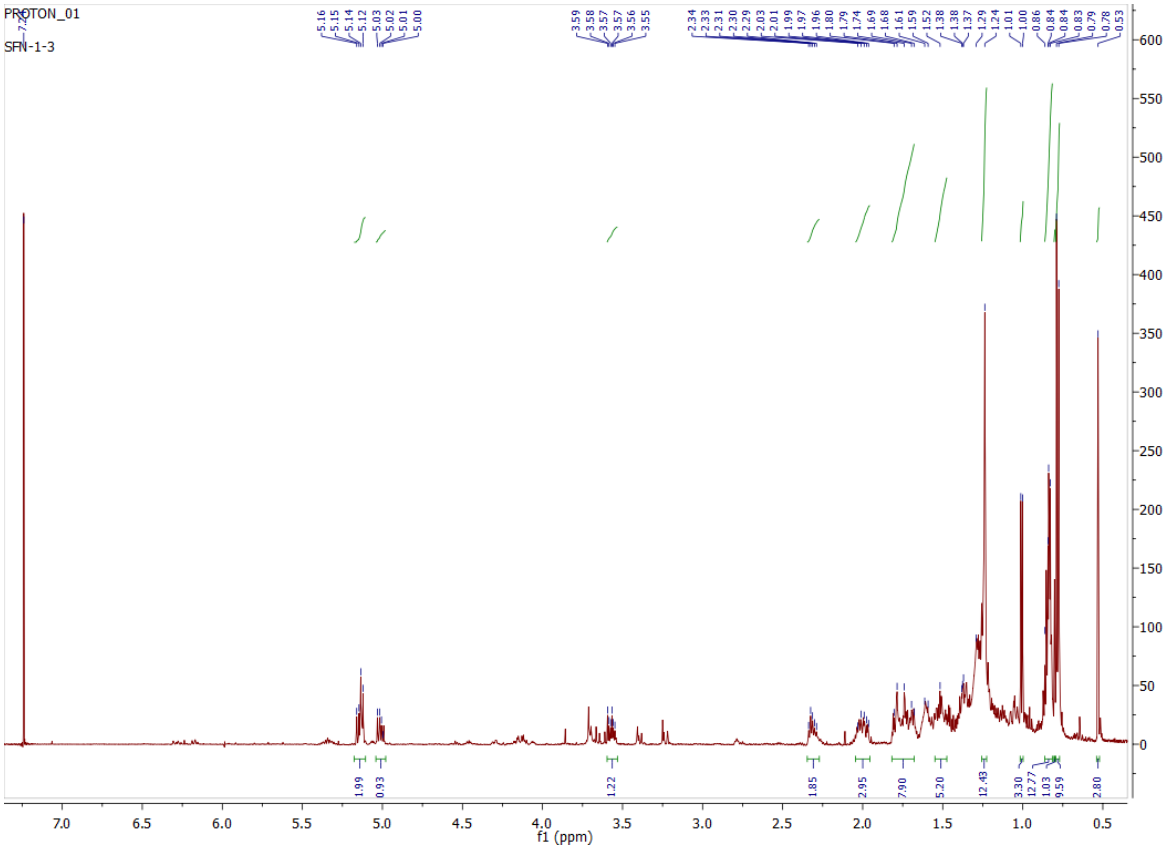
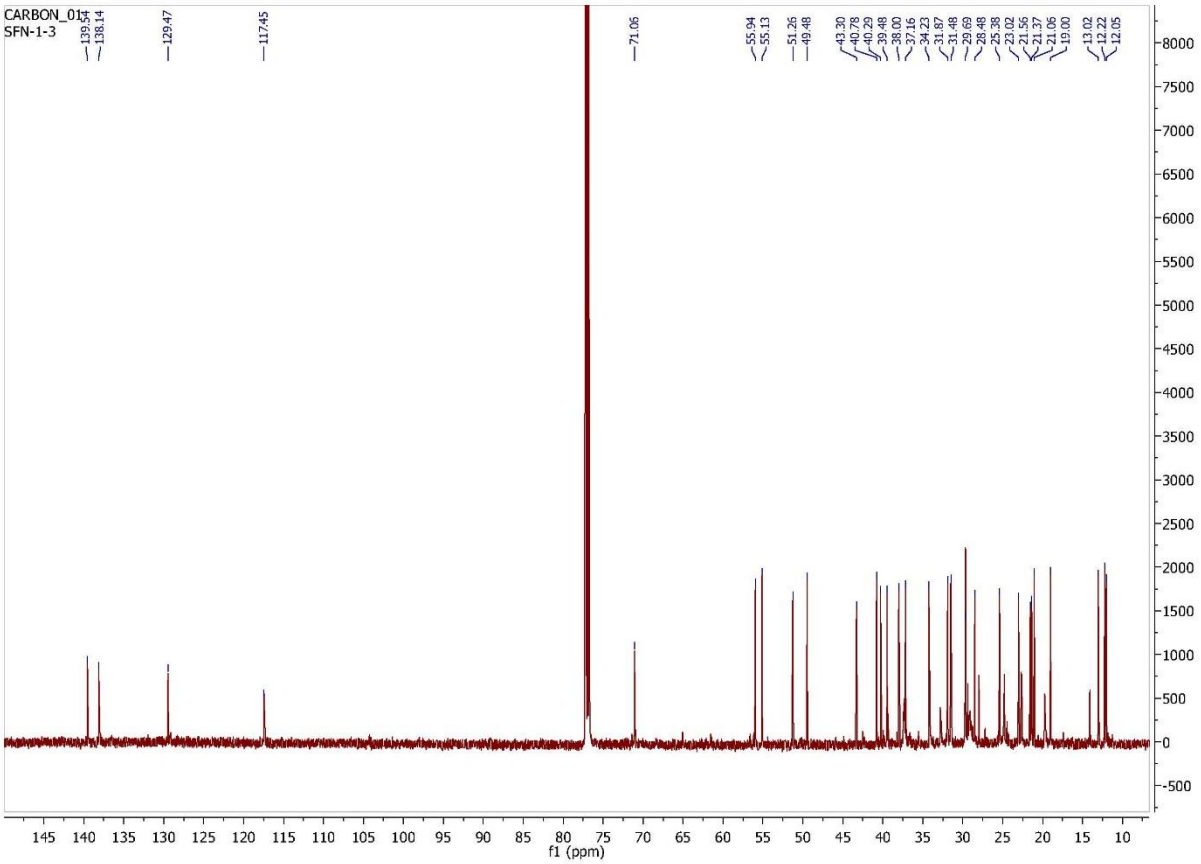
CHAPTER 9

9.1 APPENDICES

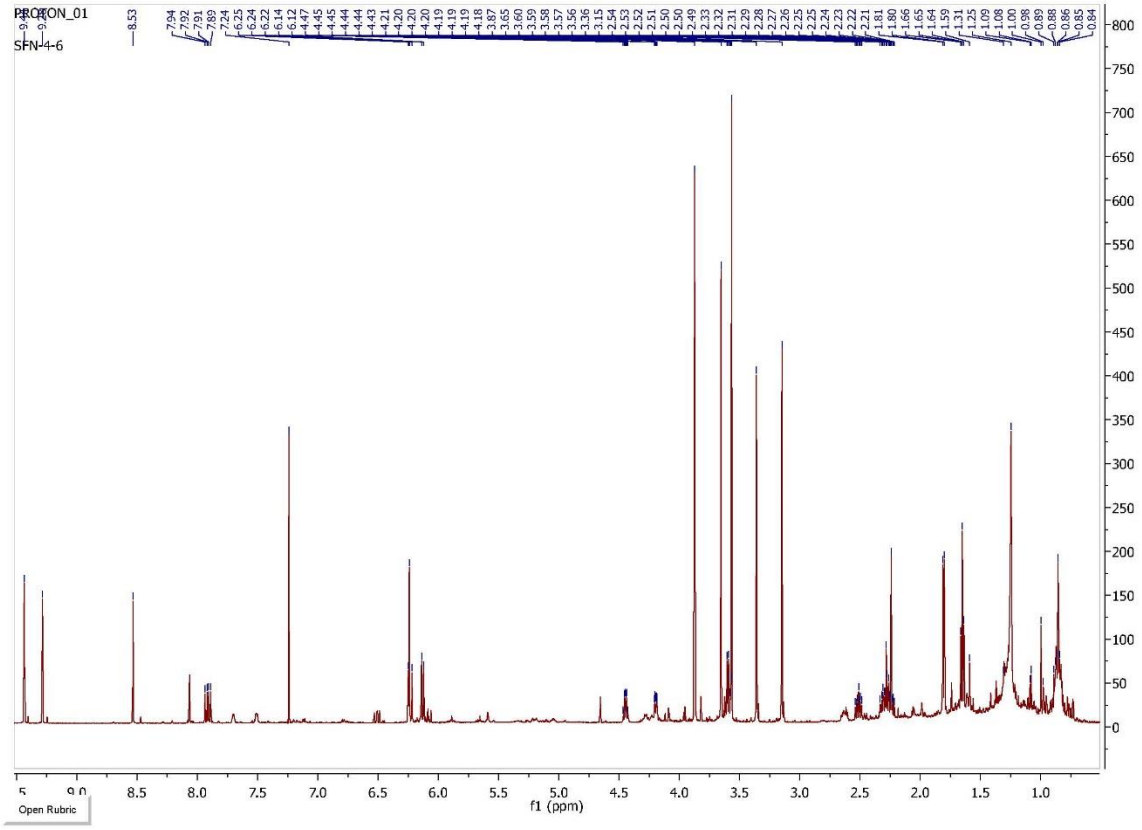
Supporting information consisting the NMR spectras of the isolated compounds and other supplementary data.



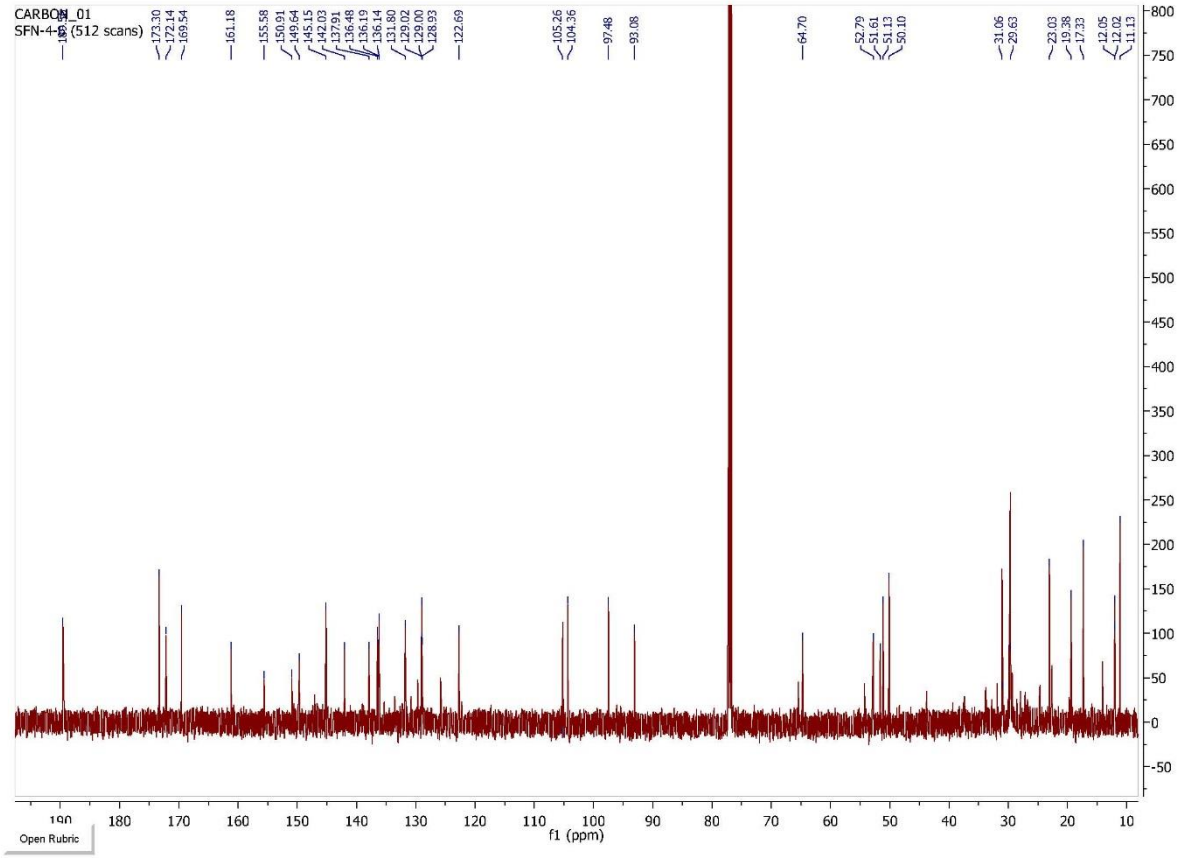
Appendix A1: ^{1}H-NMR of isolated α-spinosterol isolated from *A. cruentus*



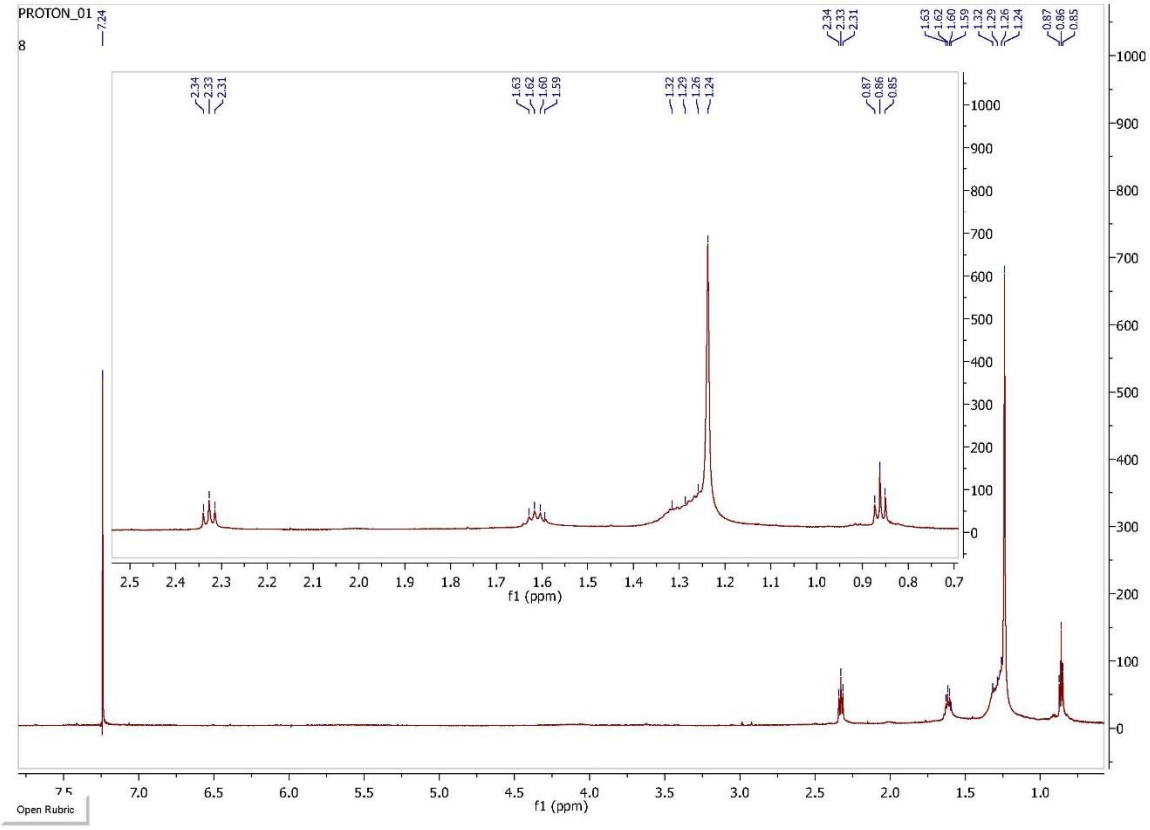
Appendix A2: ^{13}C-NMR of α-spinosterol isolated from *A. cruentus*



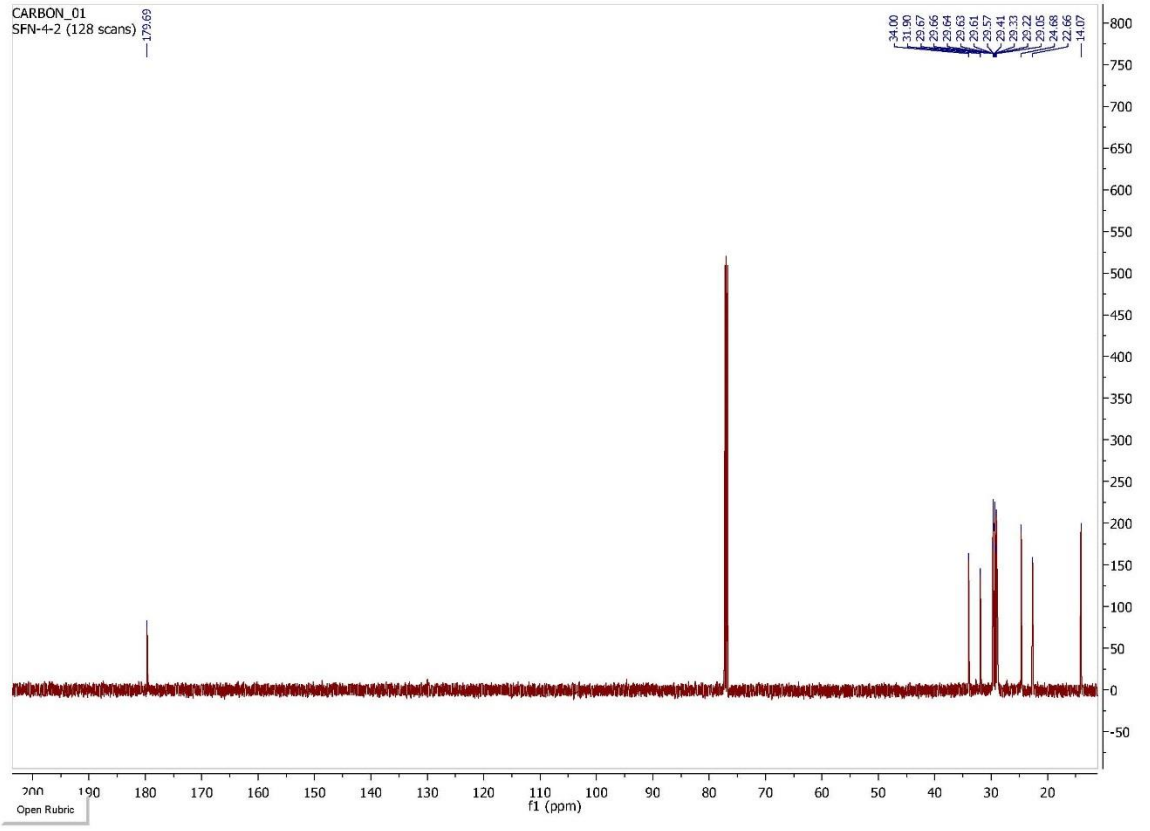
Appendix A3: ^{1}H-NMR of pheophorbide A-methyl ester isolated from *A. cruentus*



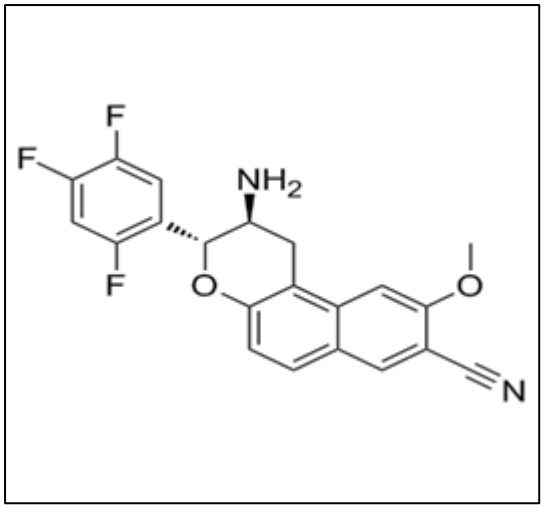
Appendix A4: ^{13}C-NMR of pheophorbide A-methyl ester isolated from *A. cruentus*



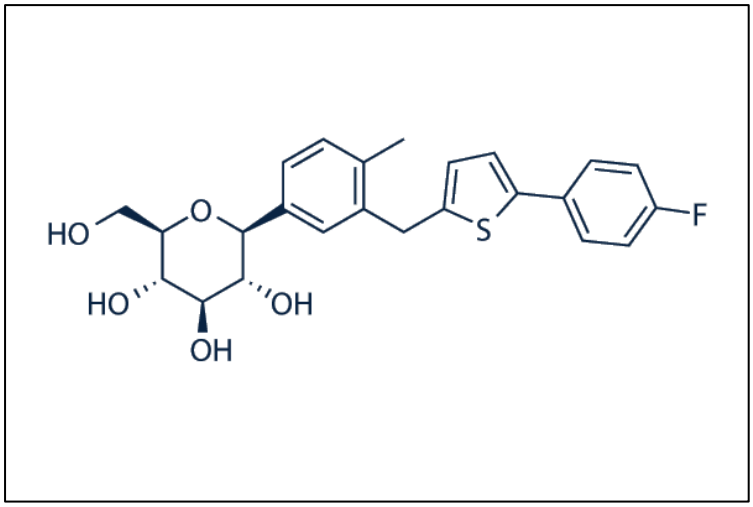
Appendix A5: ^{1}H-NMR of palmitic acid isolated from *A. cruentus*



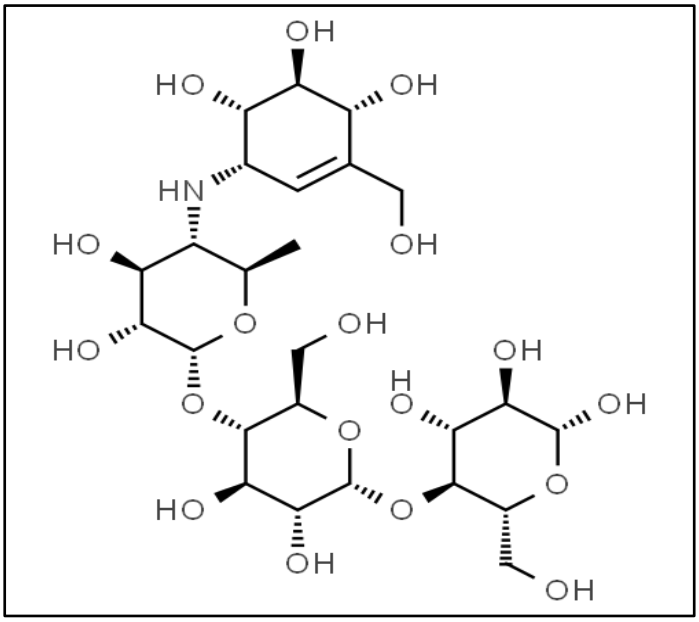
Appendix A6: ^{13}C-NMR of palmitic acid isolated from *A. cruentus*



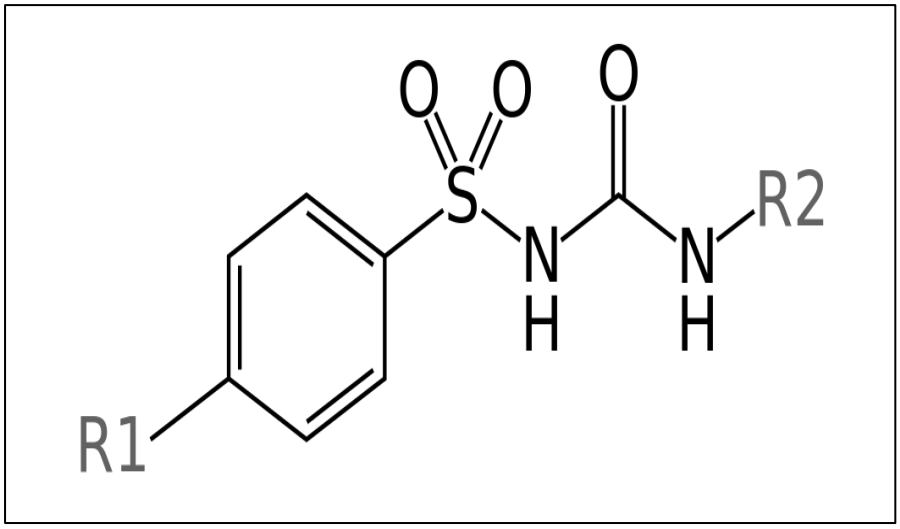
Appendix A7: Chemical structure of dipeptidyl peptidase-4 inhibitors (DPP-4I)



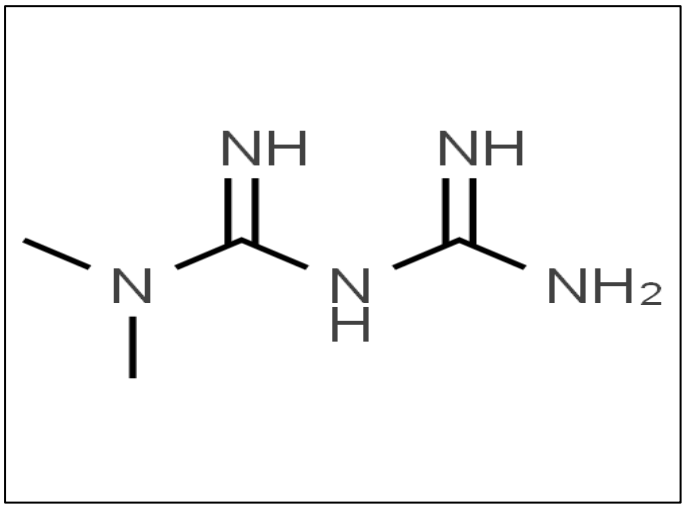
Appendix A8: Chemical structure of sodium–glucose cotransporter-2 inhibitors (SGLT-2I)



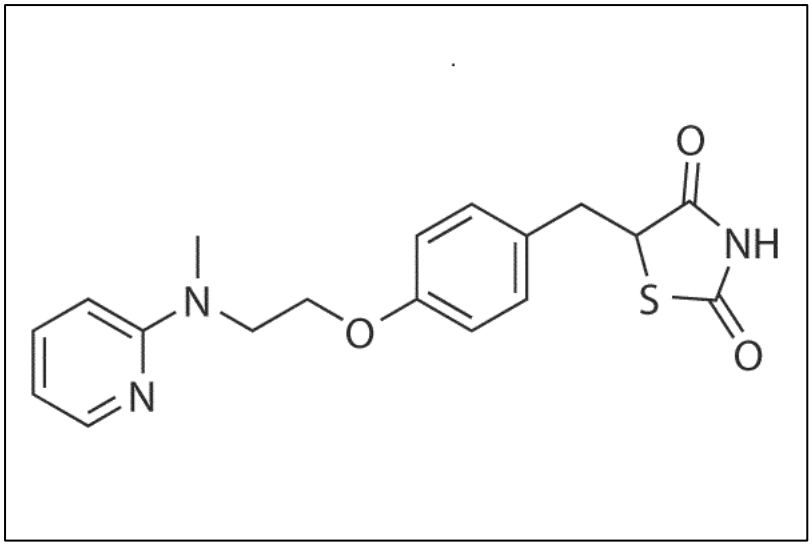
Appendix A9: Chemical structure of alpha-glucosidase inhibitors **(**acarbose)



Appendix A10: Chemical structure of sulfonylureas



Appendix A11: Chemical structure of biguanides (metformin)



Appendix A12: Chemical structure of thiazolidinediones (rosiglitazone)

Table A1: Compounds identified from the wild *Amaranthus hybridus* and *A. cruentus* extracts analysed by LC-QqQ-MS

Compound	Empirical Formula	Ret time (Min)	Measured Mass	Theoretical Mass	Mass Error (mDa)	DBE	Mode (+/−)	*Amaranthus Cruentus*	*Amaranthus Hybridus*
2-Phenylethenamine	C_8H_9N	1.72	120,0809	119,0735	0.4	5	+	✓	Trace
2-Phenylethylamine	$C_8H_{11}N$	-	-	121,0891	-	-	ND	×	×
L-Tryptophan	$C_{11}H_{12}N_2O_2$	2.62	205,0977	204,0899	1.4	7	+	✓	×
Phenylalanine	$C_9H_{11}NO_2$	1.85	166,0851	165,07890	1.7	5	+	✓	×
Kaempferol	$C_{15}H_{10}O_6$	7.22	287,0559	286,0477	0.3	11	+	✓	×
Ferulic acid	$C_{10}H_{10}O_4$	-	-	194,0579	-	-	+	×	×
Epicatechin	$C_{15}H_{14}O_6$	-	-	290,0790	-	-	ND	×	×
Chlorogenic acid	$C_{16}H_{18}O_9$	-	-	354,0951	-	-	ND	×	×
Vanillic acid	$C_8H_8O_4$	-	-	168,0422	-	-	ND	×	×
Quercetin	$C_{15}H_{10}O_7$	6.66	303,0477	302,2357	0.2	-	+	✓	×
Rutin	$C_{27}H_{30}O_{16}$	6.41	609,1456	610,1534	1.9	13	+/−	✓	✓
Trigonelline	$C_7H_7NO_2$	0.93	138,0587	137,0477	3.2	5	+	✓	✓
Amaranthussaponin I	$C_{48}H_{76}O_{19}$	15.51	955,4903	956,4981	2.6	11	-	✓	✓