An investigation into the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *Tulbaghia violacea* collected from the Eastern Cape and Gauteng Province

A thesis submitted by

Tasmeera Kader

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Supervisor: Professor M.E. Mothibe

Co- supervisor: Dr N. Sibiya

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رَّبِّ زِدْنِى عِلْمًا

My Lord! Increase me in knowledge.

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ABBREVIATIONS

%	Percent
µg	Microgram
5-HT3	Serotonergic type 3
Abs	Absorbance
ABTS	2,2'-azino-bis-3-ethylbenthiazoline-6-sulphonic acid
Acetyl CoA	Acetyl coenzyme A
ACh	Acetylcholine
AChE	Acetylcholinesterase
AMPK	5' adenosine monophosphate- activated protein kinase
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BHT	Butylated hydroxytoluene
C	Carbon
C. albicans	*Candida albicans*
CAT	Choline acetyltransferase
CCK	Cholecystokinin
cm	Centimetre
CNS	Central nervous system
Cu	Copper
DMAPP	Dimethylallyl pyrophosphate

DME	Diabetic macular oedema
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-H	1-1,diphenyl-2-picryl-hydrazine
DPP-IV	Dipeptidyl peptidase IV
DTNB	5,5'- dithiobis- (2- nitrobenzoic acid)
E. coli	*Escherichia coli*
EC	Eastern Cape
ε	Epsilon
FC	Folin- Ciocalteu
FDA	Food and Drug Administration
g	Gram
GADA	Glutamic acid decarboxylase
GFR	Glomerular filtration rate
GIP	Glucose- dependent insulinotropic polypeptide
GIPR	Glucose- dependent insulinotropic polypeptide receptor
GLP-1	Glucagon- like peptide 1
GLUT	Glucose transporter
GP	Gauteng province
GPP	Geranyl diphosphate
H	Hydrogen
H_2O_2	Hydrogen peroxide
HbA1C	Glycated haemoglobin A1C

HCl	Hydrochloric acid
HGP	Hepatic glucose production
HLA	Human leukocyte antigen
IA2A	Insulinoma- associated autoantigen 2
IAA	Antibodies reactive to insulin
IPP	Isopentenyl diphosphate
IR	Insulin receptor
IRS	Insulin responsive substrate
IU	International units
K. pneumonia	*Klebsiella pneumonia*
K^+	Potassium
LDL	Low density lipoprotein
M	Molar
mAChR's	Metabotropic muscarinic receptors
mg	Milligram
MI	Myocardial infarction
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmol/l	Millimole per litre
N	Nitrogen
Na^+	Sodium
Na_2CO_3	Sodium carbonate
nAChR's	Iontropic nicotinic receptors

NaOH	Sodium hydroxide
NFT's	Neurofibillary tangles
nm	Nanometre
NMDA	N- methyl- D- aspartate
NO	Nitric oxide
NPDR	Non-proliferative diabetic retinopathy
O	Oxygen
^{o}C	Degrees Celsius
OH	Hydroxide
PDR	Proliferative diabetic retinopathy
pH	Potential of hydrogen
PI3K	Phosphatidylinositol- 3- kinase
PPAR-γ	Peroxisome proliferator- activated receptor- gamma
Rf	Retention factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
S. aureus	*Staphylococcus aureus*
SANBI	South African National Biodiversity Institute
SGLT	Sodium glucose cotransporter
SH2	Src- homology- 2- domain proteins
T. violacea	*Tulbaghia violacea*
TLC	Thin layer chromatography
TPC	Total phenolic content

UTI	Urinary tract infection
VAChT	Vesicular acetylcholine transporter
WHO	World Health Organization
ZnT8A	Zinc transporter 8
α	Alpha
β	Beta
γ	Gamma

PREAMBLE

Originally, the research study was going to compare the leaves of *T. violacea* collected from the Eastern Cape and Kwa-Zulu Natal. However, just before the collection of the plants, KZN was devastated by floods. This drastic change in environmental conditions made it difficult to collect sufficient quantities of plant material and could have affected the phytochemical composition of *T. violacea* collected from KZN at the time. This could have added an additional factor to consider and would not have made for a fair comparison study against the EC sample. For these reasons, it was decided that a sample of *T. violacea* from KZN would not be used in this study and that instead, the sample would be collected from the Gauteng Province in South Africa.

ABSTRACT

Introduction

The number of communicable and non-communicable diseases continues to rise and has become more prevalent. While drugs exist to manage and/ or treat majority of the communicable and non-communicable diseases, the rise in disease prevalence puts pressure on researchers to find new drug molecules to treat and manage these ailments. Traditional medicine refers to the knowledge, skills and practices which are based on the beliefs and experiences indigenous to cultures and is used to maintain health. Most of the research into traditional medicine focuses on the medicinal plants used. Medicinal plants are any plants in which one or more of its organs contain substances which are used for therapeutic purposes or for the synthesis of drugs. *Tulbaghia violacea* is a monocotyledonous genus of herbaceous perennial bulbs which is native to Africa and can be readily found throughout South Africa. It is popular for its antimicrobial, antifungal, anticoagulant, antioxidant and anticancer properties. It has been that ecological factors influence the composition and quantity of phytochemicals present in a plant.

Aim of the study

The aim of the study was to investigate the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *T. violacea* collected from the Eastern Cape and Gauteng Province.

Methods

The leaves of *T. violacea* were collected from the Eastern Cape and Gauteng Province. The leaves were dried and extracted using serial maceration with solvents hexane, acetone and methanol. The resulting extracts were subjected to qualitative preliminary phytochemical analysis and a quantitative total phenol content test was carried out using gallic acid as the standard. Thin layer chromatography (TLC) was performed to identify classes of compounds present in *T. violacea*.

Antioxidant activity of *T. violacea* was determined qualitatively using a dot-plot and quantitatively using a DPPH radical scavenging activity assay. Ascorbic acid was used as the standard.

Anti-diabetic properties of *T. violacea* were assessed using an α- amylase inhibition assay and an α- glucosidase inhibition assay. Acarbose was used as the standard for these assays.

The anti-Alzheimer properties of *T. violacea* leaf extracts was determined using and acetylcholinesterase (AChE) inhibition assay. Donepezil was used as the standard for this assay.

The DPPH radical scavenging activity, the α- amylase inhibition assay, the α- glucosidase inhibition assay and the AChE inhibition assay was combined with linear regression to determine the IC50 values of the *T. violacea* extracts and the standards. Statistical analysis was conducted to determine any differences between the plant samples and the standards as well as any differences between the EC and GP sample.

Results

The results of the qualitative phytochemical analysis revealed the presence of saponins, flavonoids, tannins, alkaloids, steroids, cardiac glycosides and phenolic compounds present in *T. violacea* collected from EC and GP. However, their presence in the samples were different based on where the plant was cultivated. The results of the total phenolic content test, revealed that the hexane, acetone and methanol extracts of *T. violacea* contained phenolic compounds with the highest quantity of phenolic compounds being present in the methanol extracts. Significant statistical difference in total phenolic content between the EC and GP samples were seen for the hexane and methanol extracts. The results of the TLC revealed the presence of multiple bands which confirmed the presence of multiple phytochemicals in *T. violacea*.

All of the extracts of *T. violacea* from EC and GP, showed antioxidant activity using both the dot-plot and the DPPH radical scavenging activity assay. The highest DPPH radical scavenging activity was seen by the hexane extract of the EC sample of *T. violacea*. The results showed significant statistical difference between the DPPH radical scavenging activity of the EC and GP samples.

The results of the α- amylase and α- glucosidase inhibition assays revealed that the extracts of *T. violacea* had inhibition activity comparable to or greater than that of the standard acarbose. With regard to the α- amylase inhibitory activity,, the hexane extract from the GP sample had an inhibitory activity of 85.667% at 50 µg/ml. With regard to the α- glucosidase inhibitory activity, the acetone extract from the EC sample had the highest inhibitory activity of 60.01% at 50 µg/ml. For both the α- amylase inhibition assay and the α- glucosidase inhibition assay, the inhibition by *T. violacea* leaf extracts and acarbose were concentration dependent. Significant statistical difference between the EC and GP samples were found for both the α- amylase and α- glucosidase inhibition assays.

The results of the AChE inhibition assay revealed a 60.123% inhibition by the methanol extract of the GP sample at 10 µg/ml. Donepezil showed a concentration dependent inhibitory activity against AChE. However, as concentration increased, the AChE inhibitory activity of the *T. violacea* plant extracts, decreased. It was also found that although the extracts from the EC and GP samples showed similar activity, significant statistical difference was seen between their results.

Conclusion

The leaves of *T. violacea* contain a variety of phytochemicals that have antioxidant, anti-diabetic and anti-Alzheimer properties. There is a significant statistical difference between *T. violacea* cultivated in the Eastern Cape and Gauteng Province, *in- vitro*. Further testing *in- vivo* should be conducted to assess the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *T. violacea*

CHAPTER 1

Introduction

1.1 Introduction

This dissertation is a report of a research study conducted at Rhodes University, Faculty of Pharmacy, Pharmacology Division, with the aim of investigating the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *Tulbaghia violacea* collected from the Eastern Cape and Gauteng Province, with a focus on its potential effects against diabetes and Alzheimer's disease, *in vitro*.

1.2 Background of the study

Over the past few decades, the number of communicable and non-communicable diseases have been increasing (1). There has also been an increase in the number of new emerging diseases and viruses such as the SARS-CoV-2 virus which was responsible for the COVID-19 pandemic. It was stated by the World Health Organisation (WHO), that chronic disease prevalence was expected to rise by 57% by the year 2020 and that they have become more prominent (2–4). While drugs exist to manage the majority of non-communicable/chronic diseases such as diabetes and hypertension, the rise in disease prevalence puts pressure on research and development to find new ways and new drugs to control and manage these ailments. This has led to scientists going back to nature to find natural products possessing compounds that could be possible drug candidates (5).

The use of natural products as medicine has been described as traditional medicine. According to the WHO, traditional medicine refers to the knowledge, skills and practices which are based on the beliefs and experiences indigenous to cultures and is used to maintain health (6). For centuries, people have and in many parts of the world, still do rely on traditional medicine for their health needs. When it is adopted outside of its traditional culture, it is often referred to as complementary or alternative medicine and the most widely used traditional medicine systems used today include those from China, India and Africa among others (7). The WHO states that trends in the use of traditional medicine and complementary medicines have been increasing (8). It has been stated that in Africa, Asia, Latin America and the Middle east, 75-90% of the

population still uses traditional medicine. It has also been purported that nearly one quarter of all modern medicine is derived from natural products of which many were originally used in traditional medicine (9).

Tulbaghia violacea is a monocotyledonous genus of herbaceous perennial bulbs which are native to Africa and can be readily found in the Eastern Cape of South Africa. It has been used traditionally for the treatment of a variety of conditions such as constipation, wounds, skin wrinkles, colds, fever, ulcers and tumours and its popularity may be due to its reported antimicrobial, antifungal, anticoagulant, antioxidant and anticancer properties (10, 11). The vast uses of *T. violacea* in traditional medicine makes it a plant of interest as the phytochemicals present in it could lead to new drugs which would be beneficial to millions of people globally.

1.3 Problem statement

The prevalence of chronic diseases is rising which obligates researchers to find new drugs to manage these conditions. *T. violacea* has been used in traditional medicine for decades, suggesting that it does indeed have pharmacological/ therapeutic properties. However, not all the mechanisms of its action have been explored. Many of the studies on *T. violacea* have investigated its antioxidant and antimicrobial properties. Interestingly, many studies have also found it to be effective against specific cancer cell lines, making this plant one of great importance (12, 13). Literature review on *T. violacea* revealed that only a few studies investigated its anti-diabetic activities and even fewer studies have investigated its anti-Alzheimer activities. Many authors have stated that environmental factors such as temperature and humidity may influence certain phytochemicals present in *T.* violacea, and that the class, content and quantity of the compounds may vary depending on the ecological factors in the area where the plant is cultivated (14). They have also stated that more research is needed in this area. For this reason, this study used samples of *T. violacea* grown in Gauteng and samples from the Eastern Cape, to assess the impact of ecological factors on the pharmacological activity of the plant. In doing so, the study sought to contribute to and narrow the gap in knowledge on the influence of ecological factors on pharmacological activity of *T. violacea*. All parts of *T. violacea* (leaves, bulbs and rhizomes) have been found to be pharmacologically active. However, extensive use of all parts of the plant could lead to destructive harvesting. For this reason, the study used only the leaves of *T. violacea* to reduce the impact on the environment and conduct a study in a sustainable manner.

1.4 Research question

What are the phytochemical compounds that influence the pharmacological and toxicological activities of *T. violacea* leaf extracts and how are these affected by ecological factors?

1.5 Aim of the study

The aim of the study was to investigate the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *T. violacea* collected from the Eastern Cape and Gauteng Province.

1.6 Objectives of the study

The objectives that addressed the aim and answered the research question are as follows:

- To determine and compare the phytochemical composition of *T. violacea* from the Eastern Cape and Gauteng Province
- To determine and compare the antioxidant activity of *T. violacea* from Eastern Cape and Gauteng Province
- To determine and compare the anti-diabetic activity of *T. violacea* from Eastern Cape and Gauteng Province
- To determine and compare anti-Alzheimer activity of *T. violacea* from Eastern Cape and Gauteng Province

1.7 Importance of the study

The literature review conducted on *T. violacea* suggested that only a small amount of work has been done to investigate the anti-diabetic properties of *T. violacea* and even less knowledge exists on its anti-Alzheimer properties. Researchers have stated that more information is required to determine the impact of geographical location on the phytochemical composition and pharmacological activity of plants. The literature review also showed that no study has been done comparing the antioxidant, anti-diabetic and anti-Alzheimer properties of *T.*

violacea from the Eastern Cape and Gauteng. Knowledge about the impact of geographical location will help both researchers and the community using *T. violacea* traditionally, to understand how the effects they see may vary depending on where the plant is cultivated. More research into the pharmacological properties of *T. violacea* may also potentially help researchers to find new natural sources of drug molecules.

1.8 Outline of the thesis

Preliminary pages

The preliminary section of the dissertation consists of the title page, declaration, acknowledgements, table of contents, list of figures, list of tables, list of abbreviations and the abstract.

Chapter 1

Chapter 1 consists of the background of the study, problem statement, research question, aim of the study, and objectives of the study, importance of the study and chapter references.

Chapter 2

Chapter 2 consists of literature review on traditional medicine, medicinal plants, *Tulbaghia violacea*, diabetes mellitus and Alzheimer's disease. This chapter is arranged in themes and subthemes.

Chapter 3

Chapter 3 consists of the materials and methods, results, discussion and summary of objective 1: Determine and compare the phytochemical composition of *T. violacea* from the Eastern Cape and Gauteng.

Chapter 4

Chapter 4 consists of the materials and methods, results, discussion and summary of objective 2: Determine and compare the antioxidant activity of *T. violacea* from Eastern Cape and Gauteng.

Chapter 5

Chapter 5 consists of the materials and methods, results, discussion and summary of objective 3: Determine and compare the anti-diabetic activity of *T. violacea* from Eastern Cape and Gauteng.

Chapter 6

Chapter 6 consists of the materials and method, results, discussion and summary of objective 4: Determine and compare anti-Alzheimer activity of *T. violacea* from Eastern Cape and Gauteng.

Chapter 7

Chapter 7 consists of an overall discussion and conclusion, limitations of the study and recommendations.

Reference list

This section consists of a list of all the references used in the dissertation, using Vancouver referencing style.

Supplementary pages

This section consists of the appendices and the abstracts from presentations done during the study, on the study.

CHAPTER 2

Literature review

2.1 introduction

This chapter focuses on the relevant literature available which relates to the study. The main topics discussed include traditional medicine, medicinal plants, *Tulbaghia violacea*, diabetes mellitus and Alzheimer's disease.

2.2 Traditional medicine

According to the World health organisation (WHO), traditional medicine refers to the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (6) . When it is adopted outside of its traditional culture, it is often referred to as complementary or alternative medicine and the most widely used traditional medicine systems used today include those from China, India and Africa (7).

The WHO has stated that trends in the use of traditional and complementary medicines have been increasing and that in Africa, Asia, Latin America and the Middle East, 75-90% of the population still uses traditional medicine (8) (figure 2.1). It has also been purported that nearly one quarter of all modern medicine is derived from natural products of which many were originally used in traditional medicine (9).

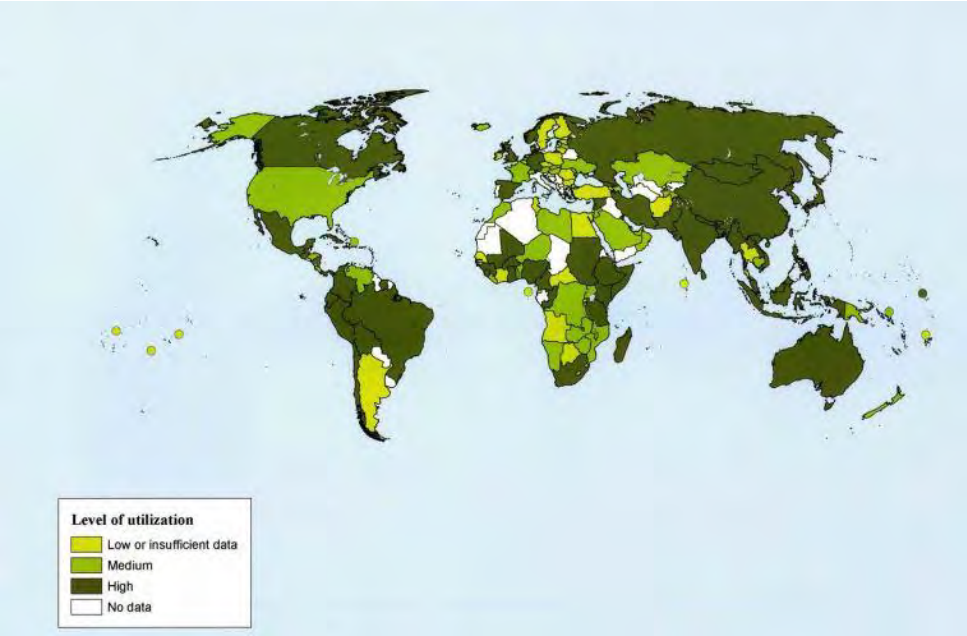


Figure 2.1: Utilisation of traditional medicine globally (15)

Conventional medicine is arguably based on the axiom of dualism, which separates the spirit from the body and focuses mainly on the latter whereas traditional medicine is based on both spiritual and physical components of personhood (16).

African traditional medicine is said to be one of the oldest and most diverse of all medicine systems even though they are not well documented (8). Even though African traditional medicine is one of the oldest medicine systems, it was rejected by European colonists and was described as being nugatory and carried many social stigmas (8, 16). During colonialism, many traditional healers were accused of practicing witchcraft by the colonists and an explanation for the rejection of traditional medicine is that they did not understand it and saw it as a threat to their authority. Today, South Africa has over 200 000 traditional healers who care for millions of people (16).

Over the decades, research into traditional medicine has increased and it is driven by the need to find new drugs due to the emergence of new diseases and drug resistance. There is also a need to validate traditional medicine for the claims made and to assess its efficacy and safety as well as to identify possible adverse events and drug interactions (8).

Most of the research into traditional medicine focuses on the medicinal plants used. The general approach in medicinal plant research starts with ethnobotanical studies followed by laboratory-based processes including solvent extraction, phytochemical screening, bioassay-guided fractionation, isolation of compounds and structural elucidation of the compounds. The medicinal plant extracts are then further investigated for pharmacological and toxicological activities (8).

2.3 Medicinal plants

Medicinal plants are any plants in which one or more of its organs contain substances which are used for therapeutic purposes, or are used for the synthesis of drugs. For centuries, medicinal plants have been used in the treatment of diseases. However, in recent times research shows that medicinal plants also play an important role in disease prevention (17). It has been reported that natural products and their derivatives represent more than 50% of all drugs use clinically and that higher plants (group of plants that have vascular tissue with veins to distribute resources through the plants) (18) contribute approximately 25% of the total (19). Well known medicines which have been derived from plants include morphine, colchicine, codeine, atropine, reserpine, digoxin as well as anticancer drugs, paclitaxel and vincristine (19).

When used in traditional cultures, plant products are often used in combination with psychological treatments to provide a holistic approach to health care. Modern formulations of medicinal plants can deviate from the traditional form. For example, the use of alcoholic extracts (tinctures) instead of water extracts (infusions) could result in better efficacy or may result in ineffective treatment or side effects. With all medicine, the correct dosage form and dose is essential in treating ailments and maintaining health (19).

2.3.1 Plant organs used in medicine

Usually, the entire plant is not used as the different parts of the plant contain different active ingredients where some parts may be harmless and others toxic (19). Parts of the plant that are used is represented in figure 2.2.

Root- the fleshy or woody roots of many plants are used medicinally. They may be solid, fleshy or even fibrous.

Bulb- is the fleshy structure made up of numerous layers of bulb scales, which are leaf bases.

Rhizome- is a woody or fleshy, elongated stem that generally grows horizontally below the ground and forms the leaves above the ground and roots on the ground.

Bark- is formed by layers of living cells above the ground and high concentrations of active ingredients can be found in bark.

Leaves- the leaves of the plant may be used alone or in combination with petioles (stalk that attaches the leaf to the plant stem).

Flowers- are popular in traditional medicine.

Fruit and seeds- the most commonly used fruit are the small, dry fruits. The seeds are contained within the fruit and are sometimes used on their own.

Gum and resins- gums are solid and consists of polysaccharides and are water soluble. Resins are a mixture of essential oils and polymerized terpenes and are usually insoluble in water.

Fatty oils and essential oils- fatty oils are non-volatile vegetable oils which are pressed from the seeds of fruits of the plant. Essential oils are volatile oils extracted from plants through steam distillation (19).

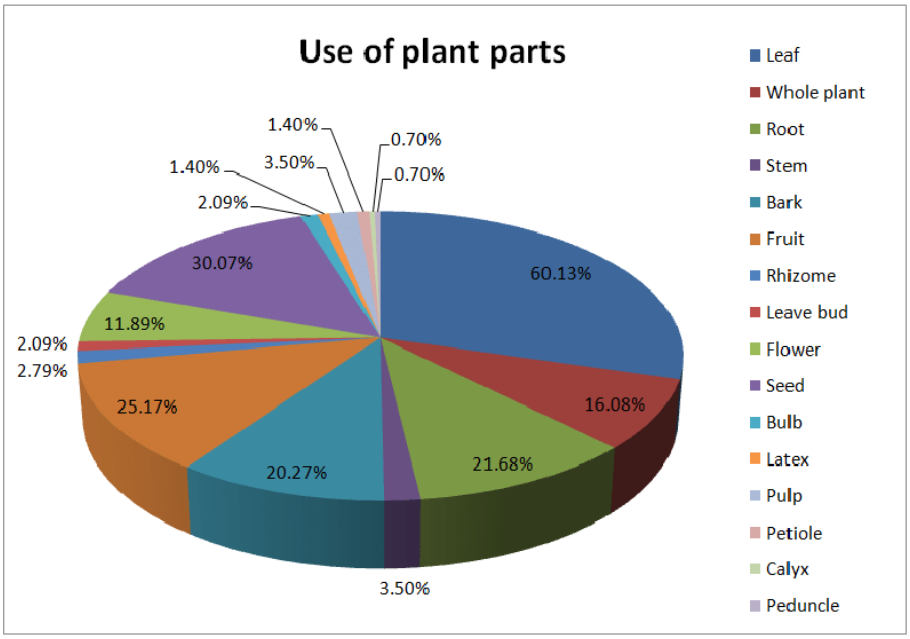


Figure 2.2: Plant parts used medicinally (20)

2.3.2 Dosage forms

Medicinal Plants can be made into a variety of dosage forms in order to exert their therapeutic effect. Some dosage forms of medicinal plants include (19):

Monopreparations- contain only a single plant or herb as the active ingredient.

Mixtures- contain two or more plants that act individually, additively or synergistically.

Extracts- are liquid, powder or viscous crude mixtures, which are extracted from plant material using water or organic solvents.

Tincture- is an alcoholic solution prepared from medicinal plant material.

Teas- are infusions prepared by steeping the plant or herb in boiling water.

Decoctions- are prepared by adding cold water to the plant. It is then heated and boiled and allow to simmer for a few minutes before being strained.

Ointments, gels and pastes- semi-solid preparations used for external application containing the medicinal plants in a suitable carrier.

The active ingredient from medicinal plants can also be made into tablets, capsules, suppositories, etc (19).

2.3.3 Phytochemicals found in medicinal plants

The chemistry of plants forms the basis for the therapeutic use of medicinal plants. Plants produce primary and secondary metabolites. Primary plant metabolites are involved in basic life functions, such as cell division and growth, respiration and reproduction. Primary metabolites include sugars, amino acids, Krebs cycle intermediates, proteins, nucleic acids and polysaccharides. Plants also produce secondary metabolites known as phytochemicals, which function to protect them against diseases, invasion and infection. Secondary plant metabolites possess biological effects which is the basis for their use in traditional medicine (21). The secondary plant metabolites are classified according to their chemical structures into various classes, including phenolics, alkaloids, glycosides and terpenes.

2.3.3.1 Phenolics

Phenolics constitute one of the largest group of plants secondary metabolites second only to carbohydrates (21, 22). They display a variety of structures from derivatives of simple phenols to complex polymeric materials. They share a common characteristic which is the presence of one or more phenol groups (figure 2.3). In plants they contribute to the colour, taste and flavour of many herbs (21). Phenolic compounds are known for their potential activity against viruses and they have immunomodulatory and anti-inflammatory activity (22). Phenolic molecules, especially flavonoids, are effective antioxidants and free radical scavengers. Phenolics can be classified into simple phenolics, tannins, coumarins, flavonoids, chromones and xanthones, stilbenes and lignans according to the different structures (21).

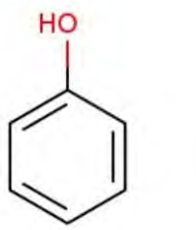


Figure 2.3: Basic structure of a phenol (23)

2.3.3.1 (a) Simple phenolics:

Phenolic acids are found among almost all plants. Free phenols are rare but gallic acid is widespread and is the parent compound of gallotannins. Gallic acid is also one of the most important phenolic compounds recognized from medicinal plants (21, 22). It is well known for its astringent properties but has also known antibacterial, antifungal, anti-inflammatory and antitumor properties *in-vitro*. The phenolic compounds in this group vary according to their functional group. The functional group may be hydroxyl, carboxylic or aldehydic. Another widely distributed simple phenol is hydroquinone. A shared property of all phenols is its antimicrobial activity and the pharmacological activities of many medicinal plants are attributed to its phenolic contents (21, 22).

2.3.3.1 (b) Tannins:

Tannins have the ability to precipitate proteins and are polyphenols. For decades, tannin molecules have been used to convert raw animal hide into leather as the tannin molecules crosslink the protein and make it more resistant to bacterial and fungal attacks. Tannins are classified into two groups: hydrolysable tannins and condensed tannins (figure 2.4). Several molecules of phenolic acid, example gallic acid and hexahydroxydiphenic acid are joined by ester linkages to a central glucose molecule to produce hydrolysable tannins (21, 22). Proanthocyanidins or condensed tannins are one of the oldest found secondary metabolites and are widespread in woody plants as well as can be found in fruit, seeds, nuts and bark (22). Their structures are based on oligomeric flavonoid precursors and vary based on the type of linkages between flavonoid units, hydroxylation patterns, stereochemistry of carbons 2,3 and 4 and the presence of additional substituents. Medicinal plants containing tannins are used as anti-diarrheal agents and have been used as antidotes in poisoning by heavy metals and alkaloids (21).

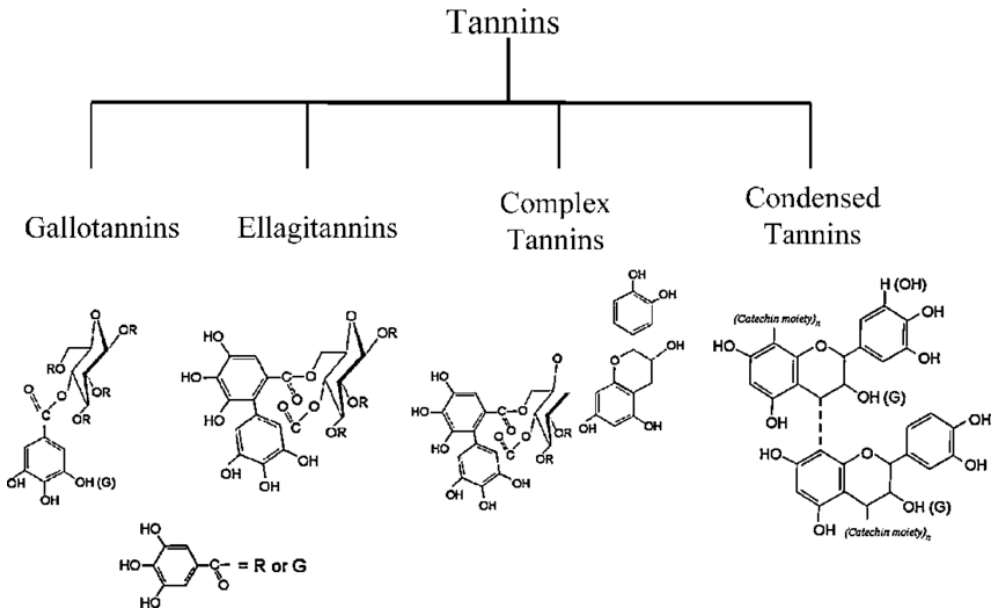


Figure 2.4: Main chemical structures of tannins (24)

2.3.3.1 (c) Coumarins:

Coumarins are derivatives of benzo-α-pyrone and have a molecular formula of $C_9H_6O_2$ (figure 2.5). They are used as additives in food and cosmetics. Coumarins have been reported to have antibacterial, anticancer, antioxidant, anti-inflammatory, anticoagulant and anti-Alzheimer

properties. Coumarins have been found in approximately 150 plant species belonging to 30 different families (21, 22).

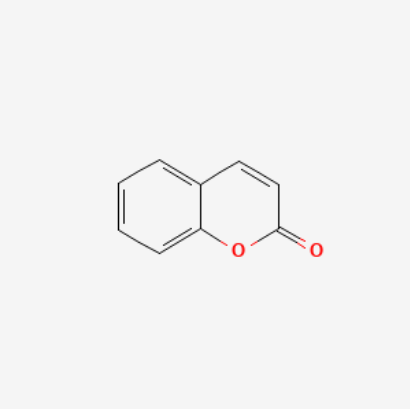


Figure 2.5: Structure of coumarin (25)

2.3.3.1 (d) Flavonoids:

More than 2000 flavonoids have been identified with approximately 500 occurring in the Free State of South Africa, making flavonoids the largest group of naturally occurring phenols. The chemical structure of flavonoids includes a chroman ring with an aromatic ring in position 2, 3 or 4. Flavonoids are divided into classes according to the oxidation level of the central ring and the most common are flavones, flavonols and anthocyanins (figure 2.6) (21). They are common in higher plants and in young tissue and are widely distributed in nature. Research has demonstrated the medicinal action of plants containing flavonoids and a number of these have been included in the British Pharmacopoeia. Medicinal plants used in Chinese traditional medicine contain flavonoids which have shown high antioxidant properties (22). Flavonoids are known for their anti-inflammatory, antithrombotic and vasoprotective properties as well as inhibition of tumour promotion (21, 22).

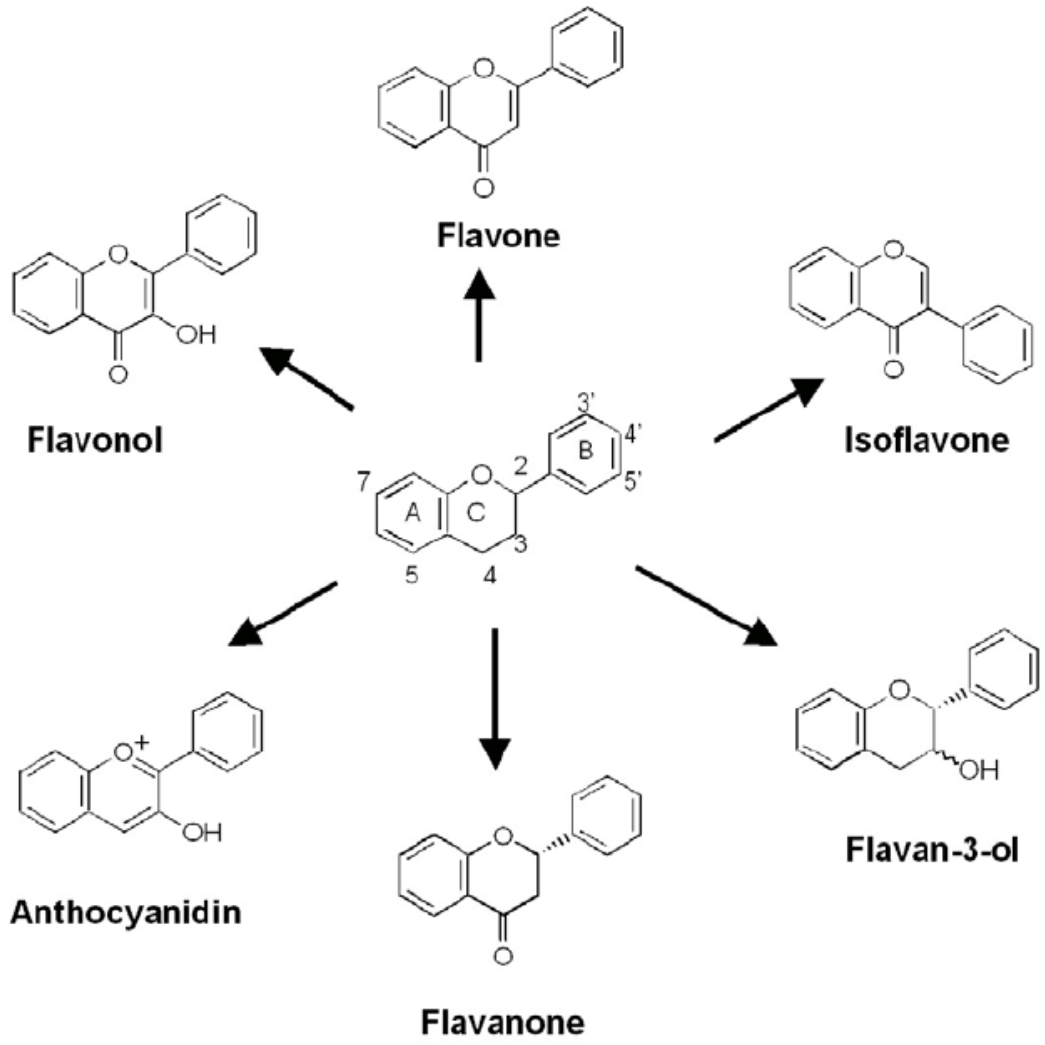


Figure 2.6: Basic structure of flavonoids (26)

2.3.3.1 (e) Lignans:

Lignans are dimeric compounds formed by two phenylpropene derivative molecules of which there are four subtypes: dibenzylbutane derivatives, dibenzylbutryolactones, monoepoxy lignans and derivatives of 3,7- dioxabicyclo (3.3.0)- octane. Lignans have shown antimicrobial, antifungal and cytotoxic activities (21).

2.3.3.2 Alkaloids

Alkaloids are small organic molecules which contain at least one nitrogen atom in a heterocyclic ring (figure 2.7) (21, 27). The nitrogen atom causes alkalinity of these compounds

(28). Alkaloids account for approximately 20% of secondary metabolites in plants (30) and pharmaceutical, traditional and modern uses of alkaloids are 25 to 75% in drugs (27). Based on its structure, alkaloids can be divided into classes such as indoles, quinolones, isoquinolines, pyrrolidines, pyridines, pyrrolizidines, tropenes, terpenoids and steroids. Another classification system connects the family of the plant species the alkaloid occurs in (21, 28). However, the two classification systems can cause confusion.

Pure alkaloids are generally odourless, colourless, crystalline solids but can be yellowish liquids. More than 3000 alkaloids are known in over 4000 different plant species (28) but they are not common in low plants.

Alkaloids are produced mainly by flowering plants and also by some animals. Groups of structurally related alkaloids are present in plants from few to 30 (28). In plants, alkaloids protect the plant from predators as well as regulates their growth (28, 29).

Therapeutically, alkaloids possess a variety of pharmacological actions including analgesia, local anaesthesia, vasoconstriction, muscle relaxation as well as antineoplastic, hypertensive and hypotensive properties. Examples of well-known alkaloids include nicotine, morphine, quinine, strychnine, ephedrine and vinblastine (21, 29).

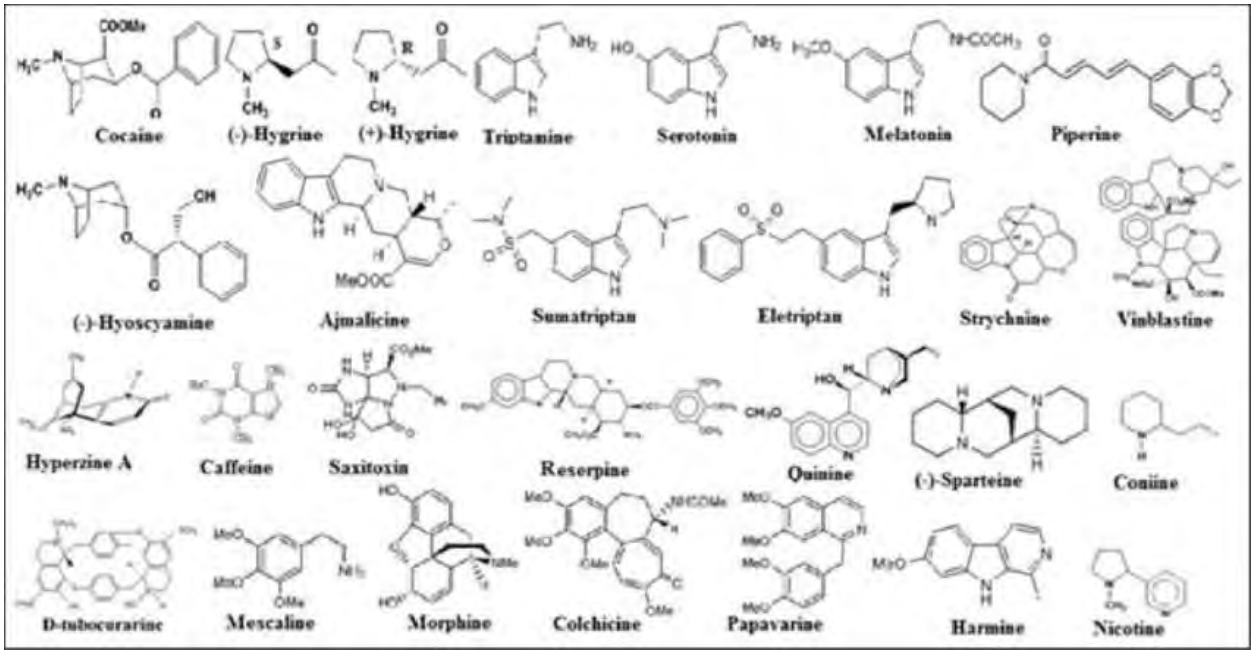


Figure 2.7: Structures of well-known alkaloids (30)

2.3.3.3 Glycosides

Glycosides are a large variety of secondary metabolic plant products which are widely distributed in plants (31, 33). Glycoside have many functions which include growth regulation, inhibition of other plant growth and defence mechanisms against herbivores and pathogens (31). Glycosides are organic compounds composed of two bound portions: a sugar moiety known as a glycone and a non-sugar moiety known as the aglycone or genin (31–33). Glycoside synthesis in plants is based on the modification of the secondary metabolites by glycosyltransferases. However, some glycosides require additional reactions such as acylation, oxidation and degradation (31). The bond between the sugar moiety and the aglycone is a hemiacetal linkage formed by the reducing group of the sugar moiety on an alcoholic or phenolic hydroxyl group of the aglycone (33) and the breakdown of glycosidic linkages are due to enzymes such as β-glucosidases or acids. The most common glycone is glucose and other glycones include one 1- rhamnose, 1-fructose, 1-arabinose and glucuronic acid (31).

Glycosides can be classified based on the number of saccharides. They can also be classified by the type of glucosidic linkage between the carbohydrates and the aglycone. For example, if the binding occurs via oxygen, then it is classified as O-glycosides and if binding occurs via nitrogen it is classified as N-glycosides. Another way glycosides are classified is based on the chemical group of the aglycone. Examples of these include anthraquinones, coumarin, cyanogen, terpenoids and saponins (31).

The pharmacological properties of glycosides are related to their structure and include analgesia, anti-inflammatory, antibacterial, antifungal, cardiotonic and anticancer properties. The most researched glycosides include anthraquinones, cardiac glycosides and saponins (31-33).

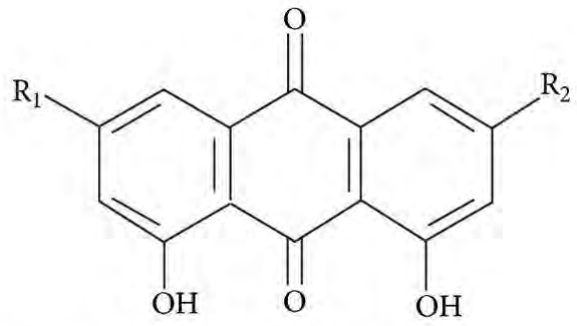
2.3.3.3 (a) Anthraquinones:

Anthraquinones also known as anthracenedione or dioxoanthracenes are members of the quinone family and are part of a large structural variety of compounds in the polyketide group (34). They are derived from plants from several families as well as some from fungal and lichen species (31) and are generally orange, red or red brown compounds (35). Not all anthraquinones are strictly quinones, for example sennosides are dianthrones consisting of two anthrone units (36). Anthraquinone glycosides have an aglycone group and usually occur as O-

glycosides or C-glycosides and the chemical structure of anthraquinones is based on anthracene (figure 2.8) (31).

Several studies have shown that anthraquinones possess pharmacological activities such as antiviral, antibacterial, antiparasitic, antioxidant and laxative activities. Anthraquinones include sennoside, emodin, rhein, chrysazon and anthrarufin (31).

It has been reported that an excessive dosage of anthraquinone laxatives causes dehydration, electrolyte imbalance, metabolic alkalosis and hypotension (31).



Aloe-emodin: $R_1 = CH_2OH; R_2 = H$

Chrysophanol: $R_1 = CH_3; R_2 = H$

Emodin: $R_1 = CH_3; R_2 = OH$

Rhein: $R_1 = COOH; R_2 = H$

Physcion: $R_1 = CH_3; R_2 = OCH_3$

Figure 2.8: Chemical structure of anthraquinone glycosides (37)

2.3.3.3 (b) Cardiac glycosides:

Cardiac glycosides are steroids which have the ability to exert action on cardiac muscles (38) and are primarily used in the treatment of congestive heart failure. Cardiac glycosides have two main classes of compounds which differ in the structure of their aglycone (figure 2.9). They are either C_{23} or C_{24} steroids with a basic nucleus of cyclopentanoperhydro phenanthrene at C_{17}. The cardenolides have a have a five membered lactone group at C_{17} with an α,β-unsaturated γ- lactone ring (butenolide) and bufadienolides have a doubly unsaturated six membered lactone ring at C_{17}. Commercially available cardiac glycosides include digoxin, gitoxigenin, ovabagenin, digoxigenin and strophanthidin (38).

Cardiac glycosides can be found in small quantities in seeds, leaves, stems, roots and bark of plants (38).

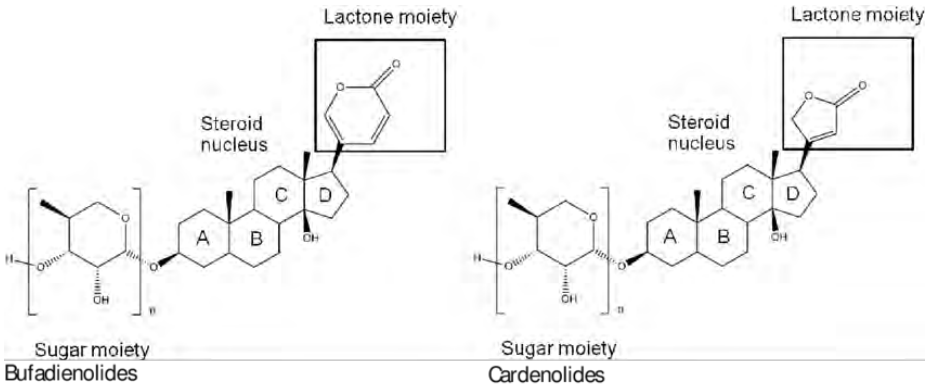


Figure 2.9: Structure of cardiac glycosides (39)

2.3.3.3 (c) Saponins:

Saponins possess a polycyclic aglycone moiety and either have a steroid or a triterpenoid attached to the carbohydrate unit. The sugar moieties are made up of pentoses, hexoses or uronic acids. Saponins form foam in aqueous solutions and cause haemolysis of blood erythrocytes. Saponins have been found in more than 500 plants from at least 90 Different families and can be found in leaves, stems, roots, bulbs, flowers and fruit of many plants but it is mainly concentrated in the roots of many species (21).

Saponins isolated from various species of plants have shown pharmacological properties such as anti-inflammatory, analgesic and antitussive properties (21).

2.3.3.4 Terpenes and terpenoids

Terpenes belong to one of the biggest classes of secondary metabolites. They are simple hydrocarbons which consists of five carbon isoprene units assembled in different ways (40). Terpenoids are a modified class of terpenes that have different functional groups as well as oxidized methyl groups added or removed at various positions (40), and can be found in all classes of living things (41).

The best known biosynthetic pathway of terpenoids is the mevalonate pathway with mevalonic acid as an intermediate (42). The primary metabolite for terpenoid synthesis is acetyl-CoA.

Mevalonic acid is the precursor for the five carbon building blocks known as isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The condensation of IPP and DMAPP provides a 10-carbon skeleton of geranyl diphosphate (GPP), which is the intermediate precursor of all the monoterpenes (42).

Terpenoids are classified according to the number of isoprene units and a prefix in the name indicates the number of isoprene units (21). They are divided into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and hemiterpenes/oids (figure 2.10). Due to their aromatic properties, terpenoids are widely used. Terpenoids contribute to the scent of cinnamon, clove, ginger and eucalyptus as well as the colour of sunflowers and the red colour seen in tomatoes (41). Most terpenoids are biologically active and have antibacterial, antitumour, anti-inflammatory and antifungal activity (21).

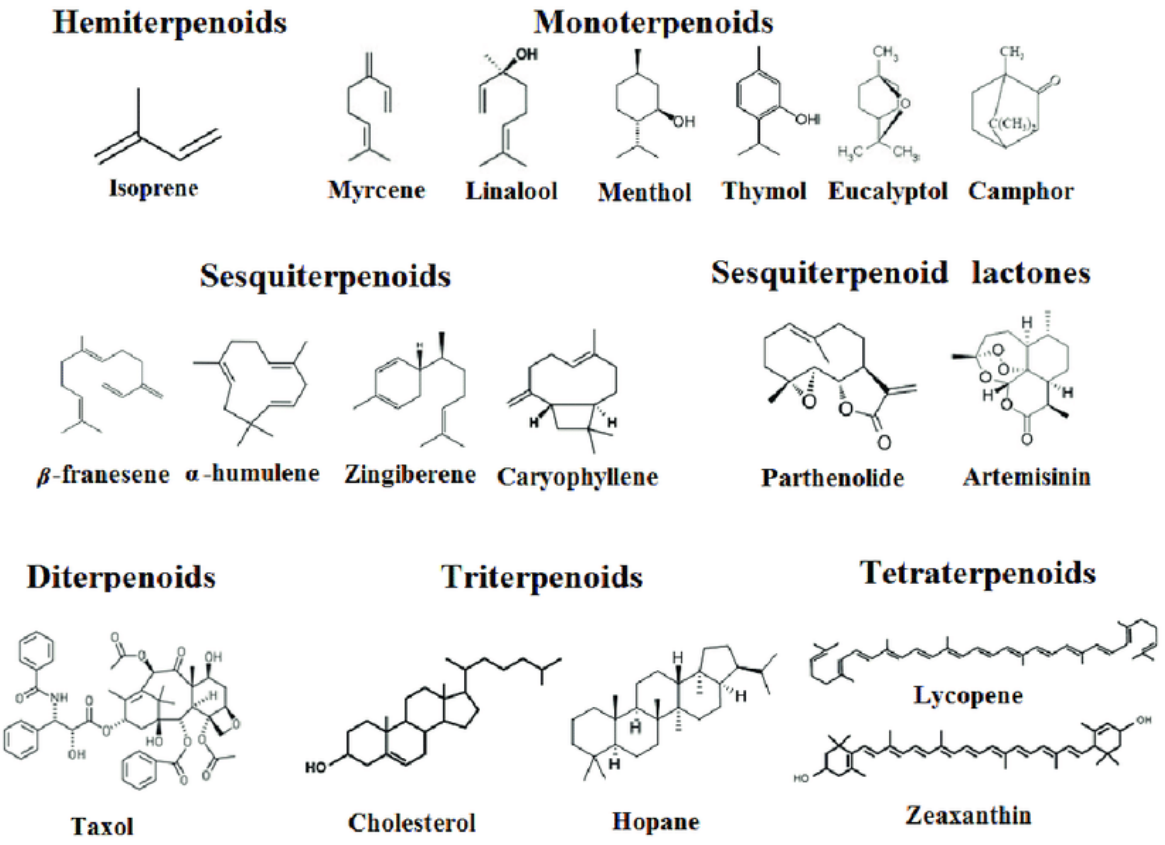


Figure 2.10: Classification of terpenoids (43)

2.3.4 Factors affecting the composition of secondary metabolites in plants

The medicinal properties of plants have been linked to the production of plant secondary metabolites such as phenols, tannins, alkaloids, terpenoids, among others. However, composition and quantity of the secondary metabolites are not always the same and is affected by factors such as seasonal variations, geographical location and other environmental factors including temperature, radiation, salinity, water among others (44) .

2.3.4.1 Seasonal variation or changes

Medicinal plants have shown distinct variations in the active ingredients during the different seasons. Variations have been attributed to changes in environmental factors such as temperature and rainfall in the different seasons (45). Many of the studies conducted on the seasonal effects on plants have shown that certain plants have better growth and active ingredients in certain seasons and slower growth in other seasons. There are many assumptions regarding the best season for the collection of various parts of medicinal plants. It has been shown that spring is the best time for the collection of bark, while during winter is the best time to collect essential oils (45). However, there are no generalities and medicinal plants should be evaluated for their optimum season of harvesting.

2.3.4.2 Geographical location

Geographical location is characterized by its soil type, soil pH, altitude, humidity and temperature. All which affect the composition of medicinal plants (46). Geographical locations also differ to each other as some are coastal regions, such as the Eastern Cape in South Africa, while some are inland such as Gauteng in South Africa.

A study conducted by Gololo *et al* (46). on the phytochemical constituents of leaf samples of *Senna italica* from four distinct locations in the Limpopo Province in South Africa found disparities in the phytochemicals. Some phytochemicals were found to be present in samples from certain locations while absent in samples from the other locations.

2.4 *Tulbaghia violacea*

Tulbaghia violacea is a fast-growing, bulbous plant with narrow, long, strap-like fleshy leaves (figures 2.11 and 2.12) which releases a strong garlic smell when bruised. It belongs to the *Alliaceae* family and is a geophyte. It is commonly known as *wild garlic* (English), *wildeknoffenel* or *wildeknoflok* (Afrikaans), *utswelane* (isiXhosa) and *isihaqa* (isiZulu) (10, 47).

Table 2.1: Scientific classification of Tulbaghia violacea (48)

Kingdom	Plantae
Phylum	Tracheophyta
Class	Liliopsida
Order	Asparagales
Family	Amaryllidaceae
Genus	*Tulbaghia* L.
Species	*Tulbaghia Violacea* Harv.

Figure 2.11: Tulbaghia violacea (49)

Figure 2.12: Flowers of Tulbaghia violacea (49)

It is a drought-resistant plant which can be found in the Eastern Cape, KwaZulu-Natal and Limpopo provinces in South Africa. Figure 2.13 depicts the worldwide distribution of the plant based on its use as traditional medicine. It does well in both dry and fertile areas. *T. violacea* is not a very tall plant and reaches a height of approximately 0.6 meters. It has mauve flowers which are clustered in groups of approximately twenty and are held on a stalk. The triangular capsules found on the plant are its fruit which groups together to form a head (10, 47).

Figure 2.13: Geographical distribution of Tulbaghia violacea (48)

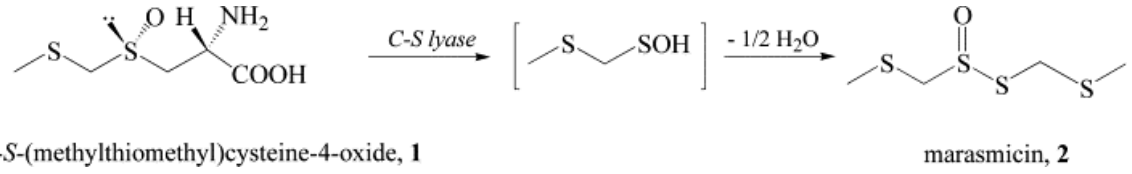
T. violacea has many uses both in traditional medicine as well as in food and for household purposes. Both the leaves and flowers are used in salads and some Zulu-speaking people use it in the same way as spinach due to its peppery-garlic taste. When crushed onto the skin, the strong smell is used to repel fleas, ticks and mosquitoes. In a medicated bath, *T. violacea* is

used to treat paralysis and rheumatism as well as to reduce the fever of a patient. Traditionally, the bulbs are boiled in water and the decoctions are taken orally to clear coughs and colds. It is also used as a remedy for pulmonary TB and intestinal worms. The leaves are used to treat oesophageal cancer and the rhizomes or bulbs are made into a tea for the treatment of a fever or high blood pressure (10, 47).

According to the Red List of South African Plants, *T. violacea* is classified as having a "least concern" status, thus a low risk of extinction (50). This is important to note as all parts of the plant can be used however, this could lead to over-harvesting or destructive-harvesting.

2.4.1 Compounds present in *T. violacea* and their biological activities

Studies on *T. violacea* have found the presence of a C-S lyase; 2,4,5,7-tetrathiaoctane-2,2-dioxide; 2,4,5,7-tetrathiaoctane and 3 S-substituted cysteine sulfoxide derivatives which were determined to be S-(methylthiomethyl) cysteine-4-oxide, also known as marasmin; S-methyl- and S-ethylcysteine derivatives (51, 53). Kubec *et al* (53). proposed that marasmicin decomposes to give various sulphur-containing degradation products which have been found to possess strong antimicrobial, antifungal and antithrombotic properties (figure 2.14).



(R_SR_C)-*S*-(methylthiomethyl)cysteine-4-oxide, **1** marasmicin, **2**

Figure 2.14: Formation of marasmicin in Tulbaghia violacea (53)

Phytochemicals are defined as bioactive nutrient plant chemicals found in fruit, vegetables, grains and other plant foods that may provide desirable health benefits that goes beyond basic nutrition to help in reducing the risk of major chronic diseases (53). They are derived from the secondary metabolism of plants and function to protect it from diseases, invasion and infection (11). Many chemical compounds have been identified in the family *Alliaceae*, to which *T. violacea* belongs. However, in comparison with the *Allium* plant species, only a few scientific articles on the chemical constituents of *T. violacea* have been published thus far (11, 51, 52).

Many qualitative and quantitative tests have been conducted on *T. violacea* in order to assess its phytochemicals and have found the presence of pharmacologically active compounds such as tannins, terpenoids, flavonoids, saponins, proteins, steroids, cardiac glycosides, phenols and coumarins in some organs of *T. violacea* (figure 2.15) (14, 54) . It is assumed that the scent of the plant is due to the odour-producing compounds from the decomposition of marasmicin (52). In 2017, Madike et al. found that the leaves of the plant contained more active compounds than the stems and roots when both water and 70% ethanol were used as extractants. They also pointed out that environmental extremes may influence certain compounds and that the class, content, and quantity of the compounds may be different depending on the ecological factors in the area where the plant is cultivated (14).

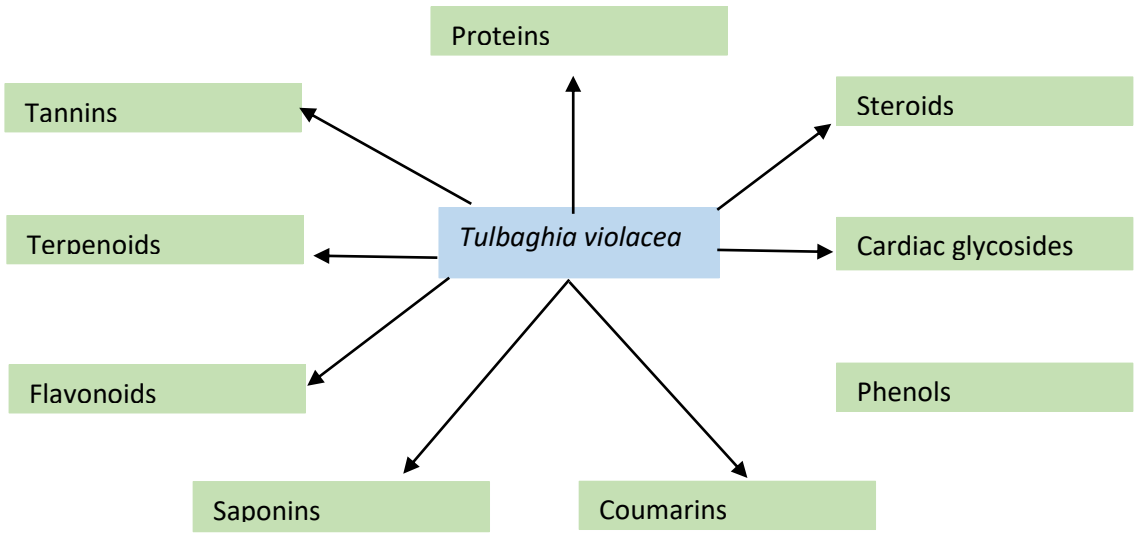


Figure 2.15: Phytochemicals found in Tulbaghia violacea

2.4.2 Antioxidant activity

Oxygen is an element crucial for life however; when cells use oxygen for energy free radicals are formed (55). A number of physiological and biochemical processes in the body produce free radicals and other reactive oxygen species as by-products. A free radical is defined as any molecule species capable of independent existence and contains an unpaired electron in an atomic orbital making it unstable and highly reactive. Free radicals can also come from external sources such as exposure to x-rays, cigarette smoke, air pollutants and industrial chemicals (57). An overproduction of free radicals in the human body can cause oxidative damage to

lipids, proteins and DNA among other biomolecules and can eventually lead to many chronic diseases and degenerative disorders such as arthritis, aging, cancer, autoimmune disorders, diabetes, cardiovascular and neurodegenerative disorders (54, 55).

Almost all organisms, including the human body, possess a complex antioxidant defence system which uses antioxidant enzymes such as glutathione peroxidase and superoxide dismutase as well as non-enzymatic antioxidants such as glutathione, thiol antioxidants, melatonin and carotenoids among others to protect itself against oxidative damage. While these systems do exist, they cannot completely prevent damage when there is an overproduction of free radicals (54).

Several studies on plants have reported radical scavenging activity due to the presence of phytochemicals. In particular, *T. violacea* has high antioxidant activity which has been proven through numerous studies (54, 57).

Takaidza *et al* (2018). found that crude extracts of *T.violacea* had the highest scavenging activity for both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and therefore had the best antioxidant activities among the *Tulbaghia* species (54) . Assays carried out on an essential oil of *T. violacea* found that the DPPH radical scavenging activity of the oil had a concentration dependent activity close to that of synthetic antioxidants and vitamin C (11). Excess generation of nitric oxide (NO) has been implicated in various human diseases such as cancer and cardiovascular diseases (58). An extract of *T. violacea* showed strong concentration dependent inhibitory activities on NO generation when compared with butylated hydroxytoluene (BHT) and ascorbic acid (58, 59). The NO inhibiting activity of *T. violacea* could support its use in oxidative induced diseases such as cardiovascular conditions (58). Lipid peroxidation mediated by free radicals is a major mechanism of cell destruction and thus cell damage and has been implicated in the pathophysiology of many diseases (58, 59). An extract of the rhizome of *T. violacea* was shown to possess high anti-lipid peroxidative agents (59). The reducing power ability of a plant is a significant reflection of its antioxidant activity. The antioxidant potential of *T. violacea* was inferred by its ability to reduce Fe^{3+} to Fe^{2+} in a concentration dependent manner although its reducing power was lower than standard BHT (58, 59). Hydrogen peroxide (H_2O_2) can react with Fe^{2+} and Cu^{+} to produce toxic OH^{-} which can cause damage to biomolecules. *T. violacea* has shown significant H_2O_2 scavenging activity and it was postulated that this activity was due to phenols which could donate electrons to H_2O_2 and neutralise it into water (59).

2.4.3 Antimicrobial activity

Compounds shown to have antimicrobial properties *in-vitro* include flavonoids, alkaloids, tannins and terpenoids (60). The number of effective conventional antimicrobials is decreasing and this is rapidly becoming a global issue which has led to more research into plants as a potential source of new antimicrobials (61). The antimicrobial properties of the *Tulbaghia* species have been investigated against a wide variety of microorganisms. It has been shown that the crude extracts of *T. violacea* exhibit broad spectrum activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* (11). The essential oil of *T. violacea* revealed appreciable antimicrobial activity against *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *B. subtilis* and *Enterococcus faecalis* as well as against antibiotic resistant microorganisms (59). It inhibited the growth of antibiotic resistant strains of *S. aureus* and *Staphylococcus viridans* and was found to be more bacteriostatic than bactericidal. Ncube *et al.* (2011), compared the antimicrobial activities between outdoor grown and micropropagated *T. violacea*. It had good antimicrobial activity against *B. subtilis*, *K. pneumonia* and *S. aureus* but no significant activity against *E. coli* (62) . While many studies have been conducted on the antimicrobial properties of *T. violacea*, discrepancies were observed across the results. Some researchers have found significant antimicrobial activity while others have noted minimal antimicrobial activity (11). The differences in results may be due to many factors including the age of the plants and geographical location as the phytochemicals which are responsible for antimicrobial activity are produced in response to external stimuli such as moisture, light and temperature among others (62).

With regard to antifungal activity, the *Tulbaghia* species has been found to be fungicidal. *Candida albicans* is an opportunistic fungus that can cause skin infections, mucosal infections or systemic infections, mainly in immunocompromised individuals (11, 62). It is more resistant to plant extracts so the fungicidal activity demonstrated by *T. violacea* is promising for the treatment of candidiasis. A study found that the dichloromethane and water extracts of *T. violacea* rhizomes exhibited high activity against *C. albicans* (62).

2.4.4 Anticancer properties

Many current anticancer drugs have side effects and can also affect normal cells. For this reason, the number of medicinal plants being studied for their anticancer properties has increased. They are less toxic and cheaper than synthetic drugs (11, 63). Between 1980 and

2002, approximately 69% of anticancer drugs have been derived from or on the basis of knowledge of plants (63).

T. violacea crude plant extracts had a cytotoxic effect in cervical cancer cells but not in normal cells (12). It was found that *T. violacea* has an antiproliferative effect using HeLa cell lines with a concentration dependent activity and triggers DNA fragmentation and apoptosis in HeLa and ME-180 cervical cancer cell lines (12, 64). It also induces apoptosis in MCF7 cell lines but not in H157 and MG63 cell lines .Saibu *et al.* (2015), found that the growth inhibitory effects of *T. violacea* leaf water extracts were much higher than the extracts from the bulbs. However, these results were in contradiction to results of an earlier study which found the bulb extracts to have a higher growth inhibitory effect (12). It was shown that the growth inhibitory activity of *T. violacea* was due to induction of apoptosis and not necrosis as the treated cells stained with Annexin V but not with propidium iodide (13). Lyantagaye (2013) identified that *T. violacea* contained methyl-α-D-glucopyranoside, which selectively kills cancer cells through apoptotic mechanisms (65). From the research previously conducted on *T. violacea*, it is evident that it has potential as an anticancer agent.

2.4.5 The effect of storage and processing

The quantity of phytochemicals present in a plant is dependent on various factors including storage and processing of the plant material (66). Medicinal plant extracts are usually used over several days after preparation during which the bioactive compounds could undergo decomposition resulting in a decrease in their pharmacological activity. A study investigated the effect of storage and processing on the antimicrobial activity of *T. violacea* and concluded that the reaction that results in the formation of marasmicin is mediated by C-S lyase and that inactivation of this enzyme by sample drying or application of heat or organic solvents effectively reduces its formation. They also stated that fresh samples prepared by mild procedures are likely to exhibit a higher degree of antimicrobial activity and it is advisable to avoid using boiling water or organic solvents prior to/ during homogenisation of plant material (66).

2.5 Diabetes Mellitus

Diabetes mellitus is a chronic, metabolic disorder that occurs when the body does not produce enough insulin, or when the cells in the body stop responding to the insulin produced by the pancreas. Insulin is a hormone produced by the beta cells of the pancreas and its role is to regulate blood glucose levels. Hyperglycaemia commonly occurs due to uncontrolled diabetes and can cause serious health problems such as heart disease, vision loss, kidney problems and issues with the nervous system. It can cause long term damage to the eyes, kidneys, heart and blood vessels (67–70).

According to the International Diabetes Federation, 537 million people have diabetes in the world and in 2021, 4.2 million people in South Africa were found to have diabetes, which is approximately 11.3% prevalence of diabetes in adults in South Africa (70).

There are three main types of diabetes: type 1 diabetes mellitus, type 2 diabetes mellitus and gestational diabetes (diabetes occurring during pregnancy) (69).

2.5.1 Type 1 diabetes mellitus

Type 1 diabetes was previously known as insulin dependent diabetes mellitus (70). In type 1 diabetes there is an absolute deficiency in insulin secretion and is observed in about 10% of patients with diabetes mellitus (69). In the past, type 1 diabetes mellitus was considered a disorder found only in children and adolescents but this opinion has changed over the past decade. However, it is one of the most common chronic disorders that is diagnosed in children (70, 71). Most cases of type 1 diabetes mellitus are caused by an autoimmune mediated disorder however, not all patients diagnosed with type 1 diabetes mellitus have characteristics of an autoimmune mediated disorder which has led to the classification 1A (autoimmune) diabetes mellitus and 1B (idiopathic) diabetes mellitus (69- 71).

2.5.1.1 Pathophysiology of type 1 diabetes mellitus

Many research articles on the pathogenesis of type 1 diabetes mellitus state that type 1 diabetes mellitus results from an autoimmune destruction of the insulin secreting beta cells in the pancreas. The basis of this observation is the chronic inflammatory infiltrates that affects the pancreatic islets. It has also been shown that in patients who have had type 1 diabetes mellitus

for years, the pancreas lacks insulin producing cells and the beta cells that remain do not regenerate (70, 71) . Through the analysis of pancreas from individuals with type 1 diabetes mellitus, it has been suggested that around 70% of islets display complete insulin absence, 20% of insulin containing islets and 1% of insulin deficient islets are inflamed. Generally, symptoms of type 1 diabetes mellitus occur when 90 to 95% of beta cells are destroyed. Research has shown that among patients diagnosed with type 1 diabetes mellitus for more than five years, majority of the remaining islets are insulin deficient but contain a normal amount of the other hormone secreting cells such as alpha cells which secrete glucagon. This indicates that there is a selective loss of beta cells from the pancreas. CD8+ T cells were found to be the most predominant cells within the inflamed islets followed by macrophages, CD4+ T cells, B lymphocytes and plasma cells (70, 71).

2.5.1.2 Serological markers

One of the important distinguishing features of type 1 diabetes mellitus is the presence of autoantibodies against beta cell autoantigens. It has been found that more than 90% of people diagnosed with type 1 diabetes mellitus have one or more of the following autoantibodies: Antibodies reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A) and zinc transporter 8 (ZnT8A) (70, 71) .

Lipid and metabolite profiles have shown decreased phosphatidylcholine at birth, reduced triglycerides and antioxidant ether phospholipids which is then followed by increased pro-inflammatory lysophosphatidylcholine. Other studies have found higher incidence of odd chain triglycerides and poly-unsaturated fatty acid containing phospholipids as well as lower concentrations of methionine (70, 71).

2.5.1.3 Genetic and environmental factors

It has been shown that there can be a strong genetic predisposition to the development of type 1 diabetes mellitus. More than 85% of patients diagnosed with type 1 diabetes have no first degree relative with the disorder, suggesting that genetic and environmental factors play a role in the onset of type 1 diabetes mellitus (73).

Type 1 diabetes mellitus is a polygenic disorder (a condition which requires multiple factors to manifest) which currently has approximately 40 loci known to affect disease susceptibility (71). The human leukocyte antigen (HLA) locus found on chromosome 6 has been found to contribute to approximately 50% of the familial cases of type 1 diabetes mellitus (71, 72). The HLA genes DR4- DQ8 and DR3- DQ2 have been found to be present in 90% of children diagnosed with type 1 diabetes mellitus. The HLA gene DR15- DQ6 has been shown to have a protective role and is present in only less than 1% of children diagnosed with type 1 diabetes mellitus (72).

2.5.1.4 Idiopathic diabetes

Some patients are diagnosed with type 1 diabetes mellitus but its aetiology is unknown. This is referred to as idiopathic type 1 diabetes mellitus. Some of these patients present with permanent insulinopenia (insulin deficiency) and are prone to ketoacidosis but have no evidence of autoimmunity. This form of type 1 diabetes mellitus is strongly inherited, lacks evidence for beta cell autoimmunity and has no HLA association (70).

2.5.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus, previously known as non-insulin dependent diabetes or adult onset diabetes, is primarily caused by a combination of key factors, namely, defective insulin secretion by the beta cells of the pancreas, a defect in insulin mediated glucose uptake by the skeletal muscle cells (tissue insulin resistance) and an inadequate compensatory insulin secretory response (70, 73) .

Type 2 diabetes mellitus accounts for approximately 90 to 95% of cases in patients diagnosed with diabetes mellitus and it has been reported by the International Diabetes Federation that in 2019 diabetes mellitus was the cause of 4.2 million deaths (73). The risk factors for type 2 diabetes mellitus is a combination of metabolic, genetic and environmental factors and an individual's genetic predisposition to type 2 diabetes mellitus plays an important role in the risk of developing type 2 diabetes mellitus. Genome wide association studies, which help identify genes associated with a particular disease, has shown the complex polygenic nature of type 2 diabetes mellitus. It was found that most of the loci increase the risk of developing type 2 diabetes mellitus by having effects on insulin secretion and some act by reducing the action of

insulin (73). Diabetes mellitus is a disorder which is characterized by a failure in glucose homeostatic control (74).

2.5.2.1 Glucose homeostasis

Glucose is a simple sugar which is a vital energy source in living organs and tissues and is a component of carbohydrates. Glucose is essential to human health as it is the main source of energy and is not synthesized by brain tissue. Although blood glucose is essential for survival, its concentration in the blood has to be maintained adequately to prevent hypoglycaemia or hyperglycaemia. Glucose homeostasis is maintained by two main hormones, namely, insulin and glucagon (75). When an individual is in a fasting state, the plasma glucose concentration is determined by the amount and rate endogenous glucose is released. During this time, glucagon which is released from the alpha cells of the pancreas prevents a large decrease in plasma glucose levels by stimulating hepatic glucose production (HGP). At the same time, insulin secreted from the beta cells of the pancreas prevent drastic rise in plasma glucose levels. In a fed state there is a rapid increase in plasma glucose levels due to the consumption of carbohydrates. At this time, insulin levels spike and suppresses HGP and glucagon is also suppressed (76).

2.5.2.2 Digestion of carbohydrates

Digestion begins in the oral cavity by salivary amylases which is released during mastication. Salivary amylase is produced in the serous cells in the salivary gland and when a carbohydrate rich meal is consumed, it results in an increase in the secretion of salivary amylase. The role of amylase is to break down carbohydrates or starch into maltose and polysaccharides. However, amylase is sensitive to acidic environments hence very little carbohydrate digestion takes place in the stomach. One starch moves to the small intestine, pancreatic amylase is released from the acinar cells to continue the process of digestion. Amylase targets the α-1, 4 bonds of carbohydrates but it is unable to break α-1, 6 bonds or terminal bonds. The final step in carbohydrate digestion occurs by the brush border enzymes, which are synthesized in the endoplasmic reticulum. These enzymes include maltose (digests maltose to glucose), lactase (digests lactose to galactose and glucose), trehalase (digests trehalose to glucose), isomaltase

(which is a debranching enzyme responsible for digesting α-1, 6 bonds of limit dextrin to glucose) and sucrase (digests sucrose to fructose and glucose) (77, 79).

Once digestion of carbohydrates takes place, the resulting products need to be absorbed. The absorption of glucose occurs in the small intestine via the sodium glucose co-transporter one (SGLT-1) while fructose absorption occurs via the GLUT-5 transporter by facilitated diffusion. Glucose transport occurs via a sodium gradient across the apical cell membrane which is generated by the sodium (Na^+), potassium (K^+) ATPase pump (located in the basal lateral membrane of the enterocyte). The pump creates a low intracellular sodium concentration. It does this by transporting three sodium ions out of the cell and two potassium ions into the cell. The SGLT-1 transporter makes use of this gradient to transport glucose molecules. Two sodium ions bind to the outer surface of the transporter resulting in a conformational change, which allows glucose to bind. A second conformational change involves the rotation of the receptor which allows for the two sodium ions and the glucose molecule to be transferred to the cytoplasmic side of the membrane. The sodium ions are then expelled by the pump to maintain the gradient. The SGLT-1 transporter then undergoes another conformational change to allow for its binding site to be exposed at the apical surface. Data also suggests that passive glucose absorption does exist and that it is facilitated system mediated by glucose-dependent activation and that the GLUT-2 facilitative glucose transporter can be recruited to the brush border membrane in order to assist with the transport of glucose in the body (77, 79).

Postprandial hyperglycaemia refers to the increase in blood glucose concentration after a meal and this is more noticeable in a patient with diabetes mellitus. Therefore, inhibiting carbohydrate digestion can be beneficial in controlling postprandial hyperglycaemia in a patient with diabetes mellitus. This can be achieved by inhibiting alpha amylase and alpha glucosidase (77, 79).

2.5.2.3 The gastrointestinal tract

The influx of glucose after a meal result in the secretion of many glucoregulatory hormones such as amylin from the beta cells of the pancreas as well as glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide one (GLP-1) and cholecystokinin (CCK) from endocrine cells in the small intestine. These hormones play a role in glucose homeostasis via several mechanisms, including the regulation of insulin and glucagon. The incretin effect is a key contributor of glucose homeostasis by the gastrointestinal tract. The incretin effect refers

to the observation that an oral glucose load results in an augmented insulin response as a result of a signal passed from the gut. The hormones responsible for this effect are GIP and GLP-1 (incretin hormones). GLP-1 inhibits postprandial glucagon secretion, slows gastric emptying and reduces food intake, all which contribute to postprandial glucose regulation. Both hormones are rapidly inactivated by dipeptidyl peptidase IV (DPP-IV) (76).

GIP exerts its effect through the GIP receptor (GIPR) which is greatly expressed in the pancreas, stomach, small intestine, adrenal cortex, adipose tissue, testes, bone, brain, pituitary gland and lung. However, GIP is expressed mainly in the stomach and the K cells of the small intestine. It has been found that at position 2 there is an alanine present and this is a substrate for inactivation by DPP-IV. Therefore, GIP has a short half-life of approximately 7 minutes in healthy individuals. The stimulation of glucose- dependent insulin secretion is the main function of GIP and it also promotes insulin biosynthesis (80).

GLP-1 one can be found in two forms: GLP-1 $_{7\text{-}37}$ and GLP-1 $_{7\text{-}36}$ amide. The half-life of GLP-1 is approximately less than two minutes and it is quickly inactivated by DPP-IV and is cleaved by the kidneys. GLP-1 stimulates insulin secretion in a glucose-dependent manner, increases transcription of the gene encoding insulin, enhances the stability of the mRNA encoding insulin and enhances the stability of insulin biosynthesis (80). .

2.5.2.4 Insulin

Insulin is one of two main hormones involved in glucose homeostasis. It is secreted by the beta cells of the pancreas and is a 51 residue anabolic protein containing two chains (A and B) which are connected by disulfide bonds. The mature insulin hormone is a post-translational product of a single chain precursor (figure 2.16). The main functions of insulin are the stimulation of glucose uptake from the systemic circulation and suppression of hepatic gluconeogenesis (81).

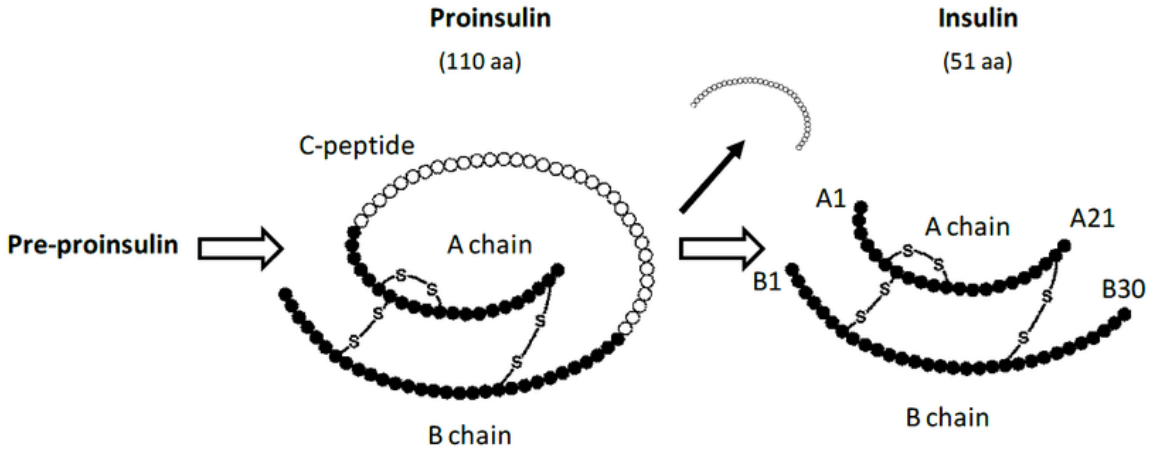


Figure 2.16: Structure of proinsulin and insulin. Proinsulin is formed from pre-proinsulin. Once the C chain is then removed, insulin is formed (82)

2.5.2.5 Insulin secretion by beta cells of the pancreas

An increase in postprandial blood glucose concentrations results in the secretion of insulin from the beta cells of the pancreas. When blood glucose concentration increases there are specific transporters which allow for the movement of glucose into cells (figure 2.17). The two main glucose transporters are sodium dependent and sodium independent. However, only sodium independent transporters (GLUT 1-7) result in an insulin response (81, 83).

Glucose enters beta cells through GLUT-2 transporters. Once inside the cell glucose is phosphorylated to glucose-6-phosphate by glucokinase, which is an enzyme related to other hexokinases but has one main distinguishing feature. It has a very low affinity for glucose and requires approximately 4-6 millimoles per liter (mmol/l) of glucose to be activated. An increase in glucose concentration results in an increase in oxidative metabolism, leading to an increase in adenosine triphosphate (ATP). Within the cell membrane there are ligand-gated potassium channels and the increased production of ATP causes these channels to close (depolarization). This results in the opening of voltage-gated calcium channels allowing calcium to flow or enter the beta cells. Within the cell, insulin is found in vesicles. Movement of calcium into the cell causes the vesicles to move to and fuse with the cell membrane. Insulin is then released from the beta cells into circulation (83).

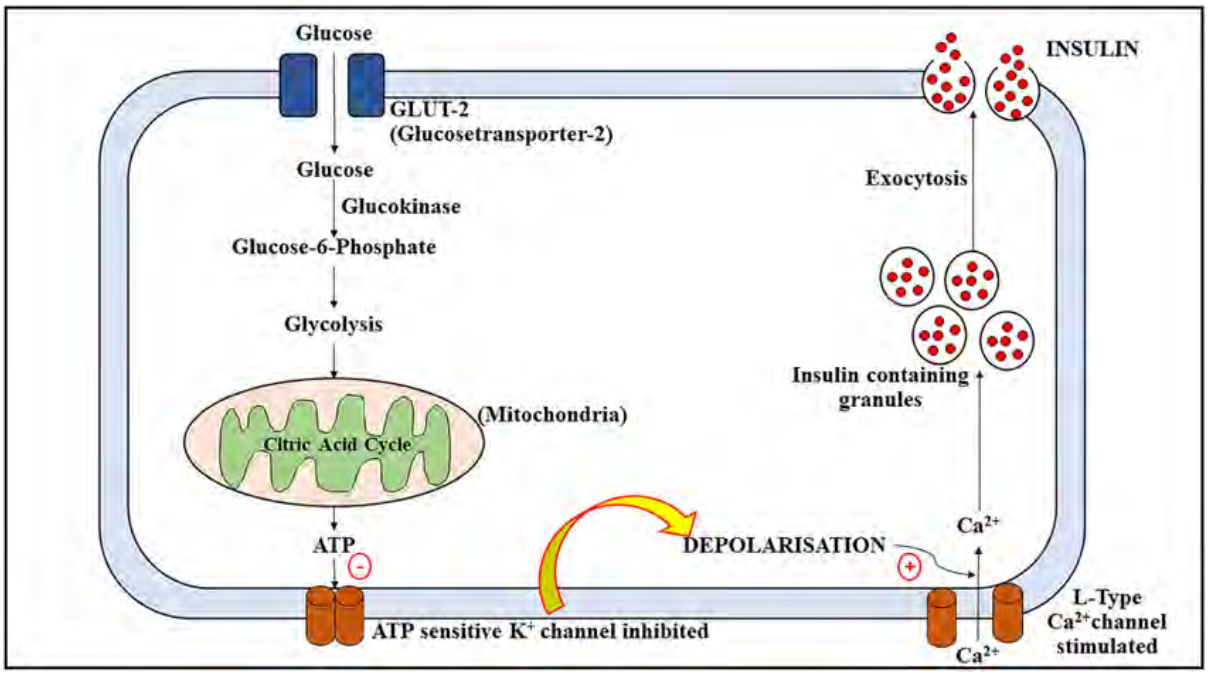


Figure 2.17: The release of insulin from beta cells of the pancreas. Glucose enters the cells via GLUT-2 transporters and undergoes phosphorylation. ATP is produced which causes the K+ channels to close and the Ca^{2+} channels to open. Influx of Ca^{2+} results in the movement of vesicles containing insulin to the cell membrane (84)

2.5.2.6 Insulin receptors and binding

The insulin that was released into circulation will carry out its effects by binding to insulin receptors (IR) (figure 2.18). The insulin receptor consists of 2α and 2β glycoprotein subunits and is found on the cell membrane. When insulin binds to the extracellular α subunit, it results in a conformational change which allows ATP to bind to the intracellular portion of the β subunit. The binding of ATP causes phosphorylation of the β subunit enhancing tyrosine kinase activity. This then allows for tyrosine phosphorylation of intracellular substrate proteins known as insulin responsive substrates (IRS), which can then bind to other signalling molecules which allow for more cellular actions of insulin (85). IRS proteins bind to specific src-homology-2 domain proteins (SH2) which include enzymes such as phosphatidylinositol 3- kinase (PI3K), an enzyme that facilitates glucose uptake through GLUT-4 translocation. The vesicles containing GLUT-4 move to the cell membrane and GLUT-4 allows glucose into the cells from the blood thus reducing blood glucose levels (85).

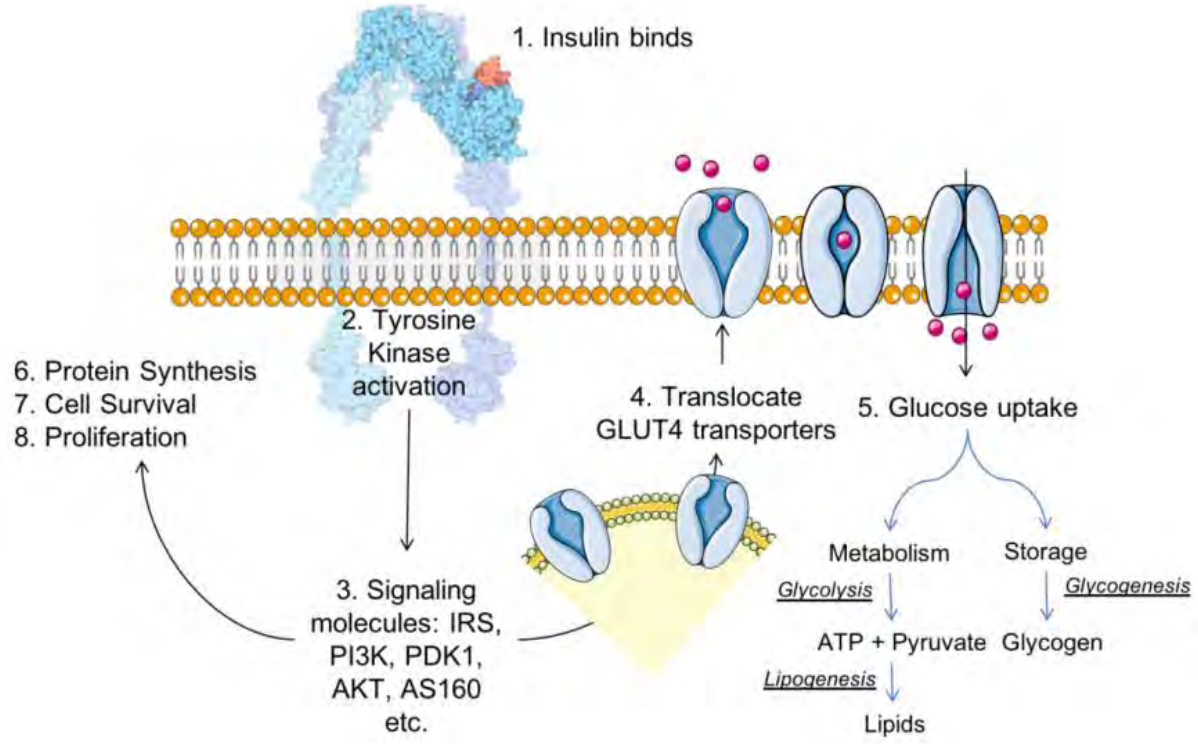


Figure 2.18: Insulin binding and signalling. Insulin binds to its receptor and activates tyrosine kinase. This results in a cascade of signalling molecules. Vesicles containing GLUT-4 move to and fuse with the cell membrane which allows glucose molecules to move into the cells from blood (86).

2.5.2.7 Pathophysiology of type 2 diabetes mellitus

Type 2 mellitus can be characterized by impaired insulin secretion, insulin resistance, excessive hepatic glucose production, abnormal fat metabolism and systemic low-grade inflammation (87). In the early stages, glucose tolerance appears to be near normal despite insulin resistance because the beta cells of the pancreas compensate for the insulin resistance by increasing production and release of insulin. As time progresses insulin resistance and the compensatory hyperinsulinemia progresses, the pancreatic cells are unable to sustain the hyperinsulinemic state. A decline in insulin secretion or an increase in glucagon secretion causes an increase in hepatic glucose production thus leading to fasting hyperglycaemia and eventually manifestation of type 2 diabetes mellitus (87).

The exact mechanism causing insulin resistance has not yet been elucidated. It has been found that insulin receptor levels and tyrosine kinase activity in skeletal muscle cells are reduced but this is not a primary defect. The defects in insulin regulated phosphorylation and

dephosphorylation play an important role in insulin resistance. The accumulation of lipid intermediates impairs mitochondrial oxidative phosphorylation and reduces insulin stimulated mitochondrial ATP production. The impaired fatty acid oxidation and accumulation of lipids may also generate reactive oxygen species such as lipid peroxidases. This can then generate low grade metabolic inflammation that worsens insulin resistance. The obesity that generally accompanies type 2 diabetes mellitus is thought to be part of the pathogenic process as the increased adipocyte mass leads to elevated levels of circulating free fatty acids and other adipocyte products. Adipose resident macrophages are an important source of metabolic inflammation in diabetes mellitus (87). Adipokines are an important modulator of appetite and satiety and they also modulate insulin sensitivity. The increase in free fatty acids and in adipokines may cause insulin resistance in skeletal and hepatic cells. Free fatty acids also impair glucose utilization in skeletal muscles, promote hepatic glucose production and impair beta cell function. In contrast, adiponectin (an insulin sensitizing peptide) is reduced in obesity and may enhance hepatic insulin resistance (88).

2.5.3 Complications of uncontrolled diabetes mellitus

In patients with diabetes mellitus, years of uncontrolled hyperglycaemia can lead to multiple vascular complications that affect the small vessels (microvascular) and large vessels (macrovascular). Vascular disease occurs via various mechanisms such as glycosylation of serum and tissue proteins, superoxide production, activation of protein kinase C (which increases vascular permeability and causes endothelial dysfunction), arterial microthrombosis, as well as pro-inflammatory and pro-thrombotic effects of hyperglycaemia that impair vascular autoregulation (89).

Microvascular disease is the cause of three common manifestations of diabetes mellitus, namely, retinopathy, nephropathy, and neuropathy (89).

2.5.3.1 Diabetic retinopathy

Diabetic retinopathy is a common cause of adult blindness and is a major complication of diabetes mellitus. Diabetic Retinopathy can be divided into two stages: Non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). The first stage of diabetic retinopathy is NPDR. During the stage there is increased vascular permeability and

capillary occlusion. Although the patient may be asymptomatic, retinal pathologies including micro-aneurysm, haemorrhages hard exudates may be detected. The later stage of diabetic retinopathy is PDR and it is characterized by neovascularization (formation of new blood vessels from existing vessels). Diabetic macular oedema (DME) is the most common cause of vision loss in patients with diabetic retinopathy. It is caused by the thickening or swelling of the macula due to sub and intra-retinal accumulation of fluid in the macula. Visual screening should be done regularly in both type 1 and type 2 diabetics (89,90).

2.5.3.2 Diabetic nephropathy

Diabetic nephropathy characterized by the thickening of the glomerular basement membrane, mesengial expansion and glomerular sclerosis is the leading cause of chronic kidney disease. The changes result in glomerular hypertension and a progressive decline in glomerular filtration rate (GFR). To detect nephropathy early, albumin levels should be monitored. Monitoring can also be conducted by measuring the albumin creatinine ratio (89).

2.5.3.3 Diabetic neuropathy

Diabetic neuropathy is a result of microvascular disease, effects of hyperglycaemia on neurons and intracellular metabolic changes that impair nerve function. There are various types of diabetic neuropathy including (89):

Symmetric polyneuropathy- most common, affecting both the hands and feet. It results in paraesthesia, dysthesia or a loss of sense of touch, vibration or temperature.

Autonomic neuropathy - can manifest as orthostatic hypotension, exercise intolerance, faecal and urinary retention and/or incontinence and constipation and/or diarrhoea among others.

Cranial neuropathy- when it affects the 3^{rd} cranial nerve can cause diplopia, ptosis and anisocoria. When it affects the 4^{th} or 6^{th} cranial nerve it causes motor palsies.

Mononeuropathies- can cause foot drop, weakness and numbness of the fingers and increases the risk of nerve compression disorders such as carpal tunnel syndrome.

Rodiculopathies- most commonly affect the proximal thoracic (T4- T12) nerves thus causing abdominal pain or affects the proximal lumber (L2- L4) nerves causing pain and weakness in the lower extremities (89).

2.5.3.4 Macrovascular disease

Hyperinsulinemia, dyslipidaemia and hyperglycaemia results in large vessel atherosclerosis and can manifest as angina pectoris and myocardial infarction (MI), transient ischemic attacks and strokes as well as peripheral arterial diseases (89). The diagnosis of macrovascular diseases is made through history and physical examination and management is via a multifactorial approach that includes the management of hypertension, dyslipidaemia and glycaemic control to reduce the risk of events.

2.5.3.5 Infection

Patients with uncontrolled diabetes mellitus have a higher risk of bacterial and fungal infections due to the effects of hyperglycaemia on the function of granulocytes and T cells. Vascular insufficiency in the lower extremities and diabetic neuropathy in patients with diabetes mellitus increases susceptibility to mucocutaneous fungal infections and bacterial foot infections including osteomyelitis. It was found that patients with diabetes mellitus that were infected with SARS-CoV-2, the higher blood glucose levels were associated with poorer outcomes including higher mortality (89).

2.5.4 The management of diabetes mellitus

Diabetes mellitus is a lifelong condition with no cure currently. However, hyperglycaemia associated with diabetes mellitus can be managed with the goal of therapy being to control blood glucose levels within the normal range and to prevent the complications associated with uncontrolled diabetes mellitus. Hyperglycaemia can be controlled with lifestyle modifications such as exercise, change in diet, cessation of smoking, etc (91). With lifestyle modifications, oral anti-diabetic drugs can be used to manage type 2 diabetes mellitus and improves quality of life, decreases mortality and decreases the risk of diabetes mellitus complications. Exogenous insulin is used in patients with type 1 diabetes mellitus and can also be used in

patients with type 2 diabetes mellitus when lifestyle modifications and oral anti-diabetic drugs are unable to adequately control glucose levels (91).

2.5.4.1 Insulin therapy

Insulin (exogenous) is required for all patients with type 1 diabetes mellitus as they do not produce insulin and will develop ketoacidosis without exogenous insulin. It can also be used in the management of type 2 diabetes mellitus. Insulin is administered either via daily subcutaneous injections or an insulin pump. Different types of insulin preparations exist and are differentiated by their onset of action and the duration of action (91).

Rapid acting insulin such as aspart and lispro are rapidly absorbed and have a time of onset of approximately 15 minutes and a short duration of action of less than four hours (91).

Intermediate acting insulin such as isophane has an onset of action of approximately two hours, reaches peak effects after 4 to 12 hours after administration a duration of action of 18 to 26 hours (91).

Long acting insulin such as insulin glargine and insulin determir provide a steady basal effect of approximately 24 hour (91).

The use of insulin pumps eliminates the need for multiple daily dosing, enhances patient adherence and reduces the variability in blood glucose levels. However, the disadvantages of the insulin pump include cost, mechanical failures and the constant use of an external device.

The most common side effect of insulin therapy is hypoglycaemia. Symptoms of hypoglycaemia include headaches, diaphoresis, palpitations, light-headedness, blurred vision, agitation and confusion. Other possible side effects of insulin therapy include local fat hypertrophy and lipoatrophy (91).

2.5.4.2 Sulfonylureas

Sulfonylureas such as glimepiride are known as insulin secretagogues. They decrease blood glucose levels by stimulating the release of insulin from pancreatic beta cells. Their mechanism of action involves the stimulation of insulin released from pancreatic beta cells by binding to blocking the ATP sensitive potassium channels in pancreatic beta cells (92). It has been shown

that glimepiride has an additional effect as it increases the sensitivity of peripheral tissues to insulin. Sulfonylureas are divided into two generations. First generation sulfonylureas include acetohexamide, chlorpropamide, tolazamide and tolbutamide. They are more likely to cause adverse effects and are not frequently used. Second generation sulfonylureas include glimepiride, glyburide, gliclazide and glipizide. Potential side effects of sulfonylureas include nausea and vomiting, hematologic reactions and weight gain (91, 92).

2.5.4.3 Biguanides

Biguanides (such as metformin) are considered to be peripheral insulin sensitizers and are the most commonly prescribed oral anti-diabetic. Biguanides suppress hepatic glucose production, increase peripheral glucose uptake and can moderately reduce low density lipoproteins (LDL) cholesterol and triglyceride levels. Biguanides activate 5' adenosine monophosphate- activated protein kinase (AMPK) thus improving insulin signalling and the metabolism of fat and glucose. Activation of AMPK by biguanides inhibits the expression of hepatic gluconeogenic genes and increases GLUT-4 positioning on skeletal muscle cells (93). The most common side effect of biguanides is gastrointestinal effects and a rare but serious side effect is lactic acidosis (91, 93).

2.5.4.4 Thiazolidinediones

Thiazolidinediones such as pioglitazone and rosiglitazone are insulin sensitizers as they reduce peripheral insulin resistance. They are currently approved for use by the FDA as either monotherapy or combined with metformin or sulfonylureas. They bind to peroxisome proliferator- activated receptor- gamma (PPAR-γ) which is a nuclear receptor present in fat cells and is involved in the transcription of genes regulating glucose and lipid metabolism. They also increase adiponectin levels which increases insulin sensitivity. Thiazolidinediones are administered orally once daily with or without food. Maximal glucose lowering effects are seen after 6 weeks to 6 months due to delayed onset of action via modification of gene expression (94). Potential side effects include weight gain, oedema and congestive heart failure, fractures and hepatic toxicity. Therefore, thiazolidinediones are contraindicated in patients with heart failure, pregnancy and moderate to severe hepatic impairment (91, 94).

2.5.4.5 Alpha glucosidase inhibitors

Alpha glucosidase inhibitors such as acarbose and miglitol are used alone or in combination in the management of diabetes mellitus but they can also be used in patients with impaired glucose tolerance to delay the occurrence of type 2 diabetes mellitus. They function by competitively inhibiting enzymes responsible for converting complex carbohydrates into simple sugars. These enzymes include glucoamylase, sucrase, maltase and isomaltase. By delaying the digestion of carbohydrates, they decrease or reduce the rise in postprandial blood glucose concentrations (95). The most common side effects of alpha glucosidase inhibitors include dyspepsia, flatulence and diarrhoea (91, 95).

2.5.4.6 DPP-IV inhibitors

Dipeptidyl peptidase IV inhibitors, known as gliptins, include sitagliptin, saxagliptin, linagliptin and alogliptin. Incretin hormones, GLP-1 and GIP maintain glucose homeostasis by increasing insulin secretion and decreasing glucagon secretion. However, these hormones are quickly degraded by dipeptidyl peptidase. DPP-IV inhibitors inhibit DPP-IV and thus prevent the degradation of the incretin hormones, resulting in an increase in insulin secretion by pancreatic beta cells and reduces postprandial hyperglycaemia. The gliptins are associated with a low incidence of side effects, but the most common side effect is hypoglycaemia. However, sitagliptin and saxagliptin are associated with side effects such as headaches, nasopharyngitis, urinary tract infections (UTI's) and arthralgia (96).

2.5.4.7 Sodium-glucose co-transporter 2 inhibitors

Sodium-glucose co-transporter 2 (SGLT2) inhibitors include canagliflozin, dapagliflozin, bexagliflozin, empagliflozin and ertugliflozin. SGLT2 proteins are expressed in the proximal convoluted tubules of the kidney and resorb filtered glucose. The SGLT2 inhibitors exert their effect by reducing the absorption of glucose, decreasing the renal threshold for glucose and promoting glycosuria which results in lowered blood glucose concentrations. SGLT2 inhibitors have been found to decrease mortality and the incidence of major cardiovascular events. The most common side effects include genitourinary infections and orthostatic symptoms (91).

2.5.4.8 Amylin analogues

Amylin is a hormone co-secreted with insulin from pancreatic beta cells and helps regulate postprandial glucose levels. An example of an amylin analogue is pramlintide which when administered with insulin results in a larger reduction in postprandial hyperglycaemia and reduction in glucagon levels. They have been shown to significantly reduce an individual's body weight, glycated haemoglobin A1c (HbA1c) and even the dosage of insulin that is required (97).

2.5.4.9 Glucagon like peptide-1 agonists

GLP-1 agonists include exenatide, lixisenatide, liraglutide, dulaglutide, albiglutide and semaglutide. They are administered subcutaneously and mimic the effects of GLP-1 thus enhancing glucose-dependent insulin secretion and slowing gastric emptying. They also reduce appetite and promote weight loss. The most common side effects are gastrointestinal (91).

2.6 Alzheimer's disease

Alzheimer's disease is a progressive form of dementia of unknown cause, and is characterized by progressive neurodegeneration of the CNS leading to a decline in cognitive function (98). According to the World Health Organization, Alzheimer's disease and dementia related deaths in South Africa were approximately 1.03% of all recorded deaths (99) and in 2011, there were approximately 2.2 million people in South Africa with some form of dementia (100), while Alzheimer's disease accounts for approximately 60 to 70% of all dementia cases (98). Alzheimer's disease can begin to manifest in the third decade of life but it is most common in elderly people. Patients with Alzheimer's disease often present with loss of episodic memory which is then followed by progressive dementia (101).

2.6.1 Epidemiology

The two most important risk factors for Alzheimer's disease are age and a positive family history. Genetic susceptibility to Alzheimer's disease is linked to the apolipoprotein E (APOE) genotype and a positive family history suggests a genetic contribution to Alzheimer's disease (98,101). It has been found that females who carry the APOE ε4 allele are more susceptible to developing Alzheimer's to males who carry the allele. Other risk factors for Alzheimer's disease include head injury, Down syndrome, depression and risk factors for vascular disease. Diabetes increases the risk of Alzheimer's disease by three-fold (101).

2.6.2 Clinical manifestation

The cognitive changes seen in Alzheimer's disease usually begins with memory impairment and progresses to deficits in language, executive and visuospatial function. In the early stages of Alzheimer's disease, it may go unrecognized or the memory loss may be attributed to old age. Eventually the cognitive issues start to interfere with daily activities and overtime, patients can become lost on walks or while driving. During the middle stages of Alzheimer's disease, the patient is unable to work, becomes easily confused or lost and language is affected. In the late stages of Alzheimer's disease there is loss of judgment and reasoning and delusions start to become prevalent. In end stage Alzheimer's disease patients may become rigid, mute and bedridden and hyperactive tendon reflexes occur spontaneously (101).

2.6.3 Pathophysiology of Alzheimer's disease

The pathological findings of Alzheimer's disease include intracellular neurofibrillary tangles (NFT's), degeneration of neurons, cortical atrophy and extracellular amyloid plaques in the cortex and medial temporal lobe (98).

2.6.3.1 β-amyloid protein aggregation

β-amyloid is a 4.3-kDa polypeptide, which is produced by proteolytic cleavage of larger proteins found in the brain known as amyloid precursor protein (APP) (102,103). Proteases, namely, alpha, beta and gamma secretase are responsible for cleaving β-amyloid from APP. APP is usually cleaved by alpha or beta-secretase and the resulting tiny fragments are not toxic to neurons. However, cleavage of β-amyloid by beta followed by gamma-secretase results in 42 amino acid peptides known as β-amyloid 42 and an elevation in these levels leads to aggregation of amyloid which causes neuronal toxicity (103). β-amyloid 42 favours the formation of aggregated fibrillary amyloid proteins. In Alzheimer's disease amyloid deposition occurs in the gray matter and around meningeal and cerebral vessels. The gray matter deposits join to form plaques. Plaques are round, microscopical lesions with a core of extracellular amyloid β-peptide surrounded by enlarged axonal endings. However, brain scans have found plaques in people without Alzheimer's disease and some people with Alzheimer's disease did not show evidence of having plaques (103).

2.6.3.2 Hyperphosphorylation of tau protein

The function of the tau protein is to stabilize axonal microtubules along neuronal axons and are vital for intracellular transport. In Alzheimer's disease, there is aggregation of β-amyloid which result in hyperphosphorylation of tau. This causes the formation of tau aggregates which form twisted paired helical filaments. These are known as neurofibrillary tangles and they are found first in the hippocampus and then throughout the cerebral cortex. The accumulation of hyperphosphorylated tau proteins and neurofibrillary tangles is associated with disruption of the microtubule network and axoplasmic flow. The number of neurofibrillary tangles is highly

linked to the degree of dementia indicating that they directly correlate with neuronal dysfunction in Alzheimer's disease (104).

2.6.3.3 The cholinergic hypothesis

Acetylcholine (ACh) is an ester of choline and acetic acid which serves as a neurotransmitter in the central and peripheral nervous systems. ACh can have excitatory or inhibitory effects as it can stimulate or block a response (105). The synthesis of ACh takes place in the terminal ends of axons where choline acetyltransferase (CAT) catalyses the reaction between choline and acetyl-CoA to create acetylcholine. In the axon terminal, the ACh molecules are stored in vesicles, which is acidified via an energy dependent pumped to create a gradient for ACh to enter via vesicular ACh transporter (VAChT) (106). Within the brain, ACh originates from the basal forebrain and the mesopontine tegmentum or the mesopontine tegmentum is found in the brainstem and mainly activates the M1 receptors in the brain stem. ACh in the brain alters neuronal excitibality, influences synaptic transmission, induces synaptic plasticity and coordinates the firing of neurons (107).

ACh appears to be a neuromodulator in the brain despite its role as a primary excitatory neurotransmitter in the periphery. A large number of models have been proposed to explain the actions of ACh in the CNS. ACh has been suggested to be critical for the response to uncertainty. It has also been suggested that ACh reinforces neuronal loops during learning and can also alter firing neurons on a rapid time scale (107).

The actions of ACh which are released from cholinergic cells are mediated through pre- and postsynaptic receptors throughout the brain. ACh signals through metabotropic muscarinic receptors (mAChRs) iontropic nicotinic receptors (nAChRs). ACh is linked to a variety of biochemical signaling cascades as muscarinic receptors are coupled either to Gq proteins that activate phospholipase C or to Gi/o proteins that negatively couple to adenylate cyclase. Also, mAChRs are located on both the presynaptic and postsynaptic neurons throughout the brain, which produces diverse consequences (107).

In the brain, ACh is involved in memory, motivation, arousal and attention (106) and thus has effects on a person's memory. Cholinergic signalling is essential for cognitive function (108). The termination of ACh activity in the synaptic junction occurs when ACh rapidly binds and unbind from its receptor and is cleaved by acetylcholinesterase into choline and acetate (106).

The cholinergic hypothesis was presented over 20 years ago, and it suggests that the dysfunction of ACh containing neurons in the brain contributes to the cognitive decline in those with Alzheimer's disease. It has been argued that ACh dysfunction is not the primary cause of Alzheimer's disease, but a consequence of Alzheimer's disease. However, attempts at correcting ACh deficiency in the brain of those with Alzheimer's disease is used for symptomatic treatment of Alzheimer's disease by means of acetylcholinesterase inhibitors (109).

2.6.4 Current treatments for Alzheimer's disease

Currently there is no cure for Alzheimer's disease, however by treating the symptoms of Alzheimer's disease, the goal is to retain quality of life and reduce long term clinical decline. Long term pharmacotherapy initially involves monotherapy with an AChE inhibitor followed by dual combination treatment with an AChE inhibitor and memantine (110,111).

2.6.4.1 Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors (AChEIs) prevent the breakdown of ACh by inhibiting the cholinesterase enzyme thus increasing the amount and duration of acetylcholine (112). Currently the acetylcholinesterase inhibitors which are approved for use in the management of Alzheimer's disease are donepezil, rivastigmine and galantamine.

Donepezil is a selective, reversible AChE inhibitor, which binds to the peripheral anionic site and exerts its effects by enhancing cholinergic function (112,113). Although the therapeutic use of donepezil is in palliative care of mild to moderate Alzheimer's disease. Some clinical studies have shown that donepezil result improves cognitive function in patients with severe Alzheimer's disease symptoms as well (112). Donepezil has a half-life of approximately 70 hours and easily crosses the blood brain barrier (BBB). The dosage for mild to moderate Alzheimer's disease is 5 or 10 milligrams (mg) daily and the dosage for moderate to severe Alzheimer's disease is 10 or 23 mg daily. It is available as tablets and orally disintegrating tablets. The most common side effects of donepezil are due to its cholinomimetic effects and include gastrointestinal side effects, such as nausea and vomiting and anorexia (113).

Rivastigmine is a slow, reversible carbamate inhibitor which blocks cholinesterase activity by binding to the esteratic part of the active site. In comparison to donepezil, rivastigmine inhibits AChE and BChE (112). For use in mild to moderate Alzheimer's disease the initial dose is 1.5 mg twice a day and after two weeks the dose is increased to 3mg twice a day and further to 4.5 mg twice a day and 6mg twice a day if tolerated with a minimum of two weeks at each dose (114).The main side effects include nausea, vomiting, diarrhoea, abdominal pain and dizziness and these side effects can be reduced by using the transdermal patch (112).

Galantamine is a selective, competitive, rapidly reversible AChE inhibitor which interacts with the anionic subsite and with the aromatic gorge. It is also an allosteric ligand at nicotinic receptors cholinergic receptors. It interacts with the nicotinic receptor at the binding site separate for acetylcholine and nicotinic agonists and enhances activity of nicotinic receptors in the presence of acetylcholine (112). Galantamine is an alkaloid which is isolated from the plant *Galanthus woronowii* and is used in the treatment of mild to moderate Alzheimer's disease. Treatment with a galantamine is initiated at 4 mg twice a day and is gradually increased to 12 mg twice a day (112,115).

2.6.4.2 N-methyl-D-aspartate receptor antagonist

N-methyl-D-aspartate (NMDA) receptor is a receptor of glutamate which is the primary excitatory neurotransmitter in the brain. It plays an important role in synaptic plasticity, which is a mechanism believed to be the basis of memory formation but NMDA receptors also appear to have involvement in a process called excitotoxicity which plays a role in the pathophysiology of many diseases including Alzheimer’s disease (116).

Memantine is an antagonist of the NMDA receptor subtype of the glutamate receptor. It is used to slow the neurotoxicity thought to be involved in Alzheimer's disease and exerts its effects by blocking the NMDA receptor subtype of glutamate preventing over activation while allowing for normal activity (117). Memantine also exhibits antagonist activity at the serotonergic type 3 (5-HT3) and nicotinic acetylcholine receptors. The starting dose of memantine is 5 mg daily and is titrated by 5 mg daily in weekly intervals to 20 mg daily. The most common side effects include dizziness, headaches, confusion, diarrhoea and constipation (117,118).

CHAPTER 3

Qualitative, quantitative and TLC analysis of various solvent extracts of *T. violacea* samples from the Eastern Cape and Gauteng

3.1 Introduction

For decades, people around the world have relied on plants as their source of medicine in the treatment of various ailments. As of recently, approximately 70 000 species of plants have been screened for their potential use as medicine and about 8 out of 10 drugs used to treat infection, cardiovascular disease, cancer or as immunosuppressants come from plants either directly or as their derivatives (119) . Plants produce both primary and secondary metabolites. Primary metabolites produced by plants are involved in the basic life functions of plants while secondary metabolites are produced by subsidiary pathways and function to protect the plant against pathogens, UV radiation and other environmental factors. These secondary plant metabolites have shown to possess various biological effects which provides a scientific backing for the use of medicinal plants to treat ailments (21). These secondary plant metabolites are classified according to their chemical structures and include phenolics, alkaloids, saponins, terpenes, lipids and carbohydrates (120).

The crude plant extracts possess a variety and complex mixture of secondary plant metabolites or compounds which make it difficult to assess their properties. Thin layer chromatography (TLC) is an affinity-based method which is used to separate compounds in a mixture (121). TLC is based on the chromatography principle in which components in a mixture are separated between a fixed stationary phase and a liquid mobile phase based on their affinities between the two phases. The stationary phase is a thin adsorbent material such as silica gel which is coated onto an inert plate surface such as aluminium. The plant sample is then spotted onto one end of the TLC plate and placed into a covered chamber containing the mobile phase. The mobile phase travels up the TLC plate and carries the sample components with it. When the mobile phase reaches the top of the TLC plate, the plate is removed, dried and read under UV light. The separated compounds appear as dots on the plate and from these dots the retention factor (Rf) of the compounds can be calculated. In this way, compounds are separated based on their polarity and affinity for either the stationary phase or the mobile phase (121).

T. violacea, also known as wild garlic, is a plant indigenous to South Africa and has been studied for its medicinal properties. Traditionally, *T. violacea* is used to treat a variety of ailments including fever, rheumatism, oesophageal cancer, pulmonary TB and coughs among others (10, 11).

This chapter reports on the phytochemicals present in *T. violacea* samples collected from the Eastern Cape and Gauteng provinces, using qualitative and quantitative phytochemical analysis as well as TLC fingerprinting.

3.2 Methods and materials

3.2.1 Sample collection, preparation and handling

Leaves of *T. violacea* were collected from Gauteng and Eastern Cape provinces of South Africa. Fresh samples were taken to the South African National Biodiversity Institute (SANBI) for botanical authentication. The leaves of *T. violacea* were separated from the flowers (appendix B) and stems and the roots were replanted and allowed to reshoot to prevent destructive harvesting. The leaves were cleaned to remove any sand or dust particles and allowed to dry completely at room temperature and pressure (appendix C). The leaves were kept out of direct sunlight during the drying process to avoid potential degradation of its phytochemicals. Once completely dry, the leaves were ground into a fine powder using a Powteq knife mill (Powteq Knife Mill, HM100, Huilongguan, Changping district, Beijing) and was then stored at room temperature for further use.

3.2.2 Extraction of plant material

The powdered plant samples were extracted using serial maceration extraction method with slight modifications and the solvents used were hexane, acetone and methanol. The extraction process was started with the solvents from lowest to highest polarity. The powdered plant samples were placed in beakers and covered with hexane solvent. They were then placed into a sonicator (Scientech, ultrasonic cleaner) at 37ºC for one hour (appendix D). After the hour, the supernatants were filtered using filter discs (Filter Discs Qual, 3 hw, 125 mm. BOECO, Germany). New hexane solvent was added and the process was repeated. The resulting supernatant was filtered and combined with the first one. The plant sample was once again

covered in hexane and was then placed on an orbital platform shaker (appendix D) (model 261, 8kg, 120w, Labotec, South Africa) overnight, set at 120 revolution per minute (rpm). The next morning, the supernatant was filtered and combined with the previous two batches. The filtrate was then concentrated using a Stuart rotary evaporator (RE 400, COLE-PARMER LTD. Stone, ST15 OSA, United Kingdom) at a temperature of 37ºC with a speed of 120 rpm (appendix E). The entire extraction process was repeated for both acetone and methanol. The resulting extracts were then stored at room temperature for further use.

3.2.3 Qualitative phytochemical analysis

All of the extracts were subjected to preliminary phytochemical analysis following standard methods as described by Madike et al. (14, 122- 123) with slight modifications, where the extracts were dissolved in the solvents used to extract them (ie. hexane, acetone and methanol). Phytochemical analysis detects the presence of various compounds based on their selective reaction with specific reagents and the identification is based on the development of a specific colour.

3.2.3.1 Saponins

Two millilitres (ml) of crude plant extract was added to two ml of distilled water and then shaken vigorously. The formation of foam and a persistent froth was an indication for the presence of saponins.

3.2.3.2 Anthraquinones

Two ml of plant extract was treated with approximately 20 drops of 10% ammonia solution. The appearance of a pink precipitate indicates the presence of anthraquinones in the plant extract.

3.2.3.3 Flavonoids

Three ml of plant extract was treated with one ml of dilute NaOH followed by the addition of one ml dilute HCL. A yellow colour that appeared after the addition of NaOH indicated the presence of flavonoids. A colourless solution that appeared after the addition of HCL, confirmed their presence.

3.2.3.4 Tannins

One ml of ferric chloride was added to two ml of the plant extract. The occurrence of black or blue-green colour indicated the presence of tannins.

3.2.3.5 Alkaloids

The Dragendorff reagent was used. Approximately two ml of extract was added to a test tube and 15 drops of Dragendorff's reagent was added. The formation of a reddish brown to orange precipitate indicated the presence of alkaloids.

3.2.3.6 Steroids

One ml of chloroform and one ml of concentrated sulfuric acid was added to two ml of plant extract. The appearance of a red colour in the lower chloroform layer indicated the presence of steroids.

3.2.3.7 Cardiac glycosides

Two ml of extract was treated with one ml glacial acetic acid, one ml of ferric chloride and one ml concentrated sulfuric acid. The presence of a green- blue colour of the solution indicated the presence of cardiac glycosides.

3.2.3.8 Phenols

Ferric chloride was added to one ml crude plant extract. A black or blue-green colour indicated the presence of phenols.

3.2.4 Quantitative phytochemical analysis

Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu (FC) reagent method with slight modifications (124, 125). For the reaction, 0.5 ml of plant extract was mixed with 0.5 ml of FC reagent (diluted 1:1 with distilled water). This was then incubated for 5 minutes at 22^{o}C. Thereafter, 2 ml of 20% Na_2CO_3 and 10 ml distilled water was added and the mixture was further incubated at 22^{o}C, in a dark space, for 90 minutes. Absorbance was then measured at 765 nm. A standard curve was created using different concentrations of gallic acid (10, 20, 30 40 and 50 µg/ml) (appendix F) and distilled water was used as a negative control. All determinations were performed in triplicate and the total phenol content was determined

from the regression equation Y = MX + C and expressed as mg/g gallic acid equivalents (GAE) using the formula:

C = XV/ M

where C is the concentration/ TPC in mg/g gallic acid equivalents;

X is the concentration from the standard curve;

V is the volume of the extract in ml;

M is the mass of the plant extract in grams.

3.2.5 Thin layer chromatography

The *T. violacea* plant extracts were redissolved in the solvents used to extract them before conducting TLC analysis. Pre-coated silica gel TLC plates were used and prepared. The size of the plates was prepared to 5 cm x 10 cm plates. Both provinces' extracts were spotted on the same plate, on a baseline drawn 1.5 cm from the bottom of the plate. Solvent systems consisting of different polarities were used as the mobile phases to develop the TLC chromatograms of the extracts. Hexane extracts were developed with hexane: ethyl acetate (8.5:1.5), acetone extracts were developed with hexane: ethyl acetate (7:3) and methanol extracts were developed with methanol: toluene (8:2). Once the solvent front travelled approximately 80% of the TLC plate, the plate was removed from the developing chamber and a line was drawn to indicate this point. The developed TLC plates were then observed under normal light, UV shortwave (254 nm) and UV longwave (365 nm).

The movement of the different compounds present in *T. violacea* plant extracts were expressed by their retention factor (Rf) values, calculated using the following equation:

$$Rf = \frac{Distance\ travelled\ by\ solute}{Distance\ travelled\ by\ solvent}$$

3.3 Results

3.3.1 Qualitative phytochemical analysis

Table 3.1: Qualitative phytochemical analysis

Phytochemical evaluated	**Extracts of different polarities**					
	Hexane (H)		**Acetone (A)**		**Methanol (M)**	
	EC	**GP**	**EC**	**GP**	**EC**	**GP**
Saponins	++	++	+	+/-	++	++
Anthraquinones	-	-	-	-	-	-
Flavonoids	+	+	-	-	-	-
Tannins	-	-	+	+	++	++
Alkaloids	+	+	++	+	+	+/-
Steroids	+++	+	++	+	+	-
Cardiac glycosides	++	+++	+	++	-	-
Phenols	+/-	+/-	+	+	+++	++

[Not present (-); trace presence (+/-); moderate presence (+); present in appreciable quantity (++); impressive presence (+++)]

EC- Eastern Cape Province; GP- Gauteng Province

3.3.2 Quantitative phytochemical analysis

Table 3.2: Total phenolic content of T. violacea plant extracts

Total phenolic content of *T. violacea* extracts in mg/g gallic acid equivalents						
	H (EC)	**H (GP)**	**A (EC)**	**A (GP)**	**M (EC)**	**M (GP)**
Average	2.463 ± 0.020	3.621 ± 0.062	9.958 ± 0.159	10.054 ± 0.165	16.905 ± 0.079	12.659 ± 0.183

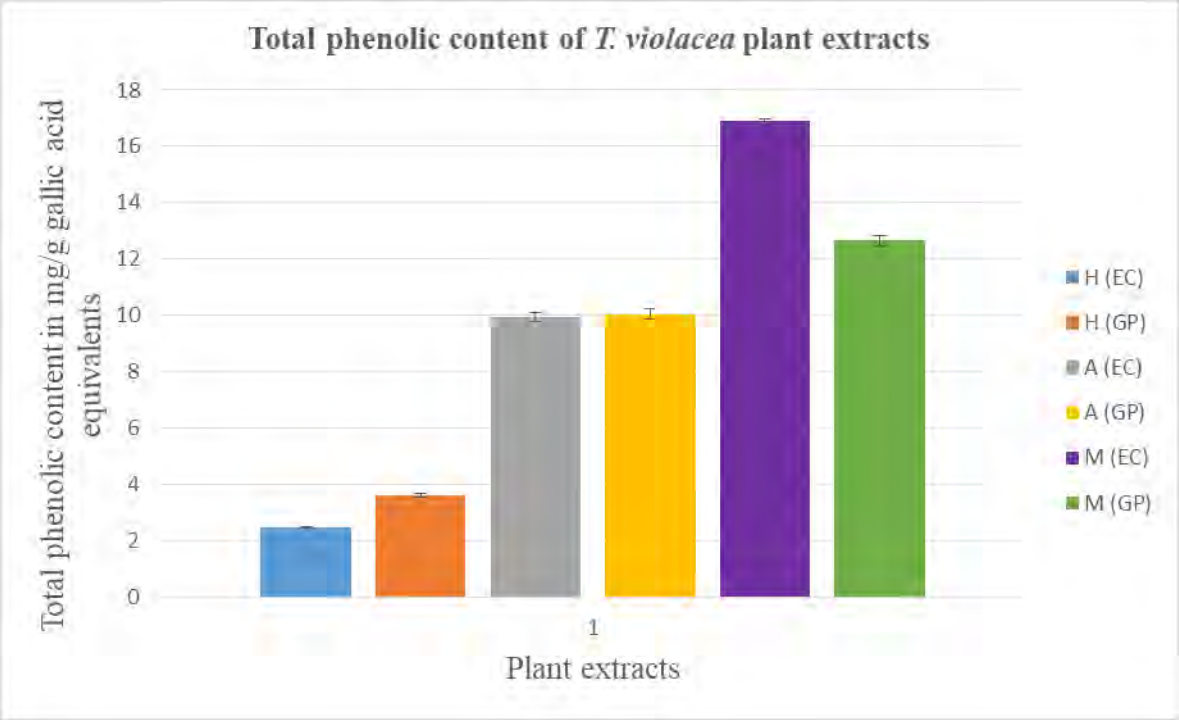


Figure 3.1: Total phenolic content of T. violacea plant extracts in mg/g gallic acid equivalents. The data is represented as columns representing the means ± standard deviation; H-hexane, A-acetone, M-methanol, EC-Eastern Cape, GP-Gauteng Province

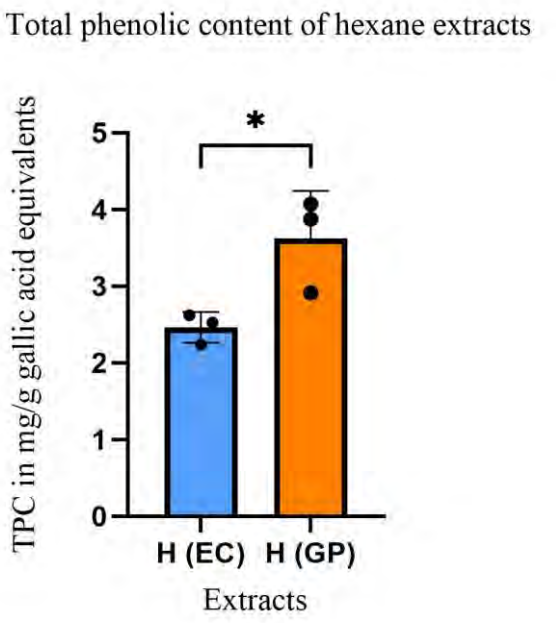


Figure 3.2: Total phenolic content of hexane extracts of T. violacea from EC and GP. The asterisk shows significant statistical difference between the two extracts with regard to total phenolic content; H-hexane, EC-Eastern Cape, GP-Gauteng Province

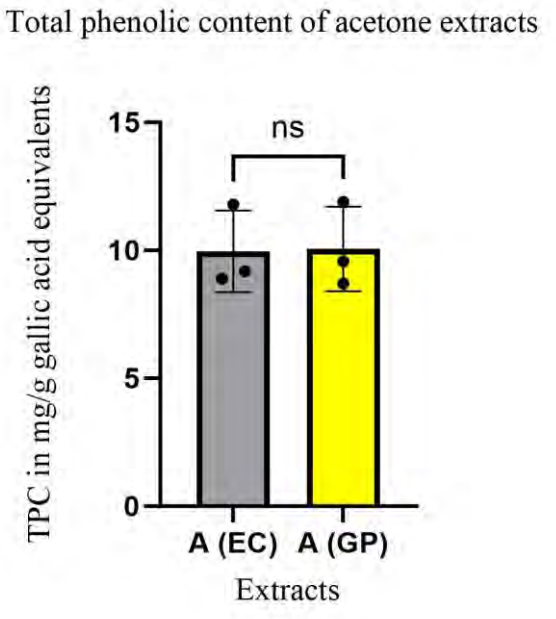


Figure 3.3: Total phenolic content of hexane extracts of T. violacea from EC and GP. There is no significant statistical difference between the two extracts with regard to total phenolic content; A-acetone, EC-Eastern Cape, GP-Gauteng Province

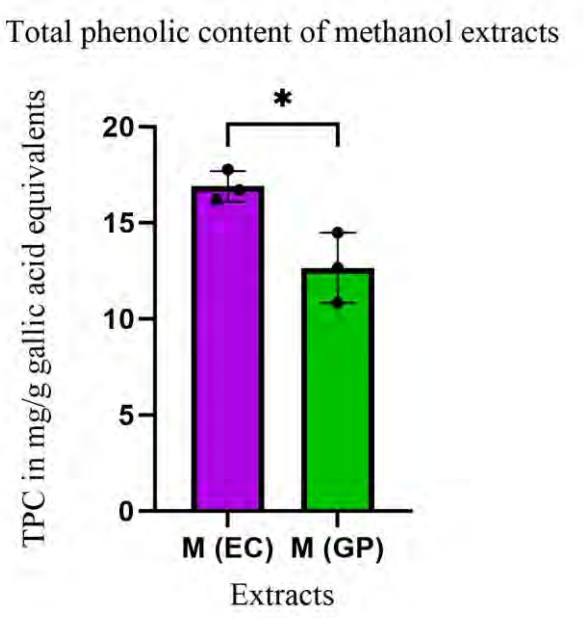


Figure 3.4: Total phenolic content of methanol extracts of T. violacea from EC and GP. The asterisk shows significant statistical difference between the two extracts with regard to total phenolic content; M-methanol, EC-Eastern Cape, GP-Gauteng Province

3.3.3 Thin layer chromatography

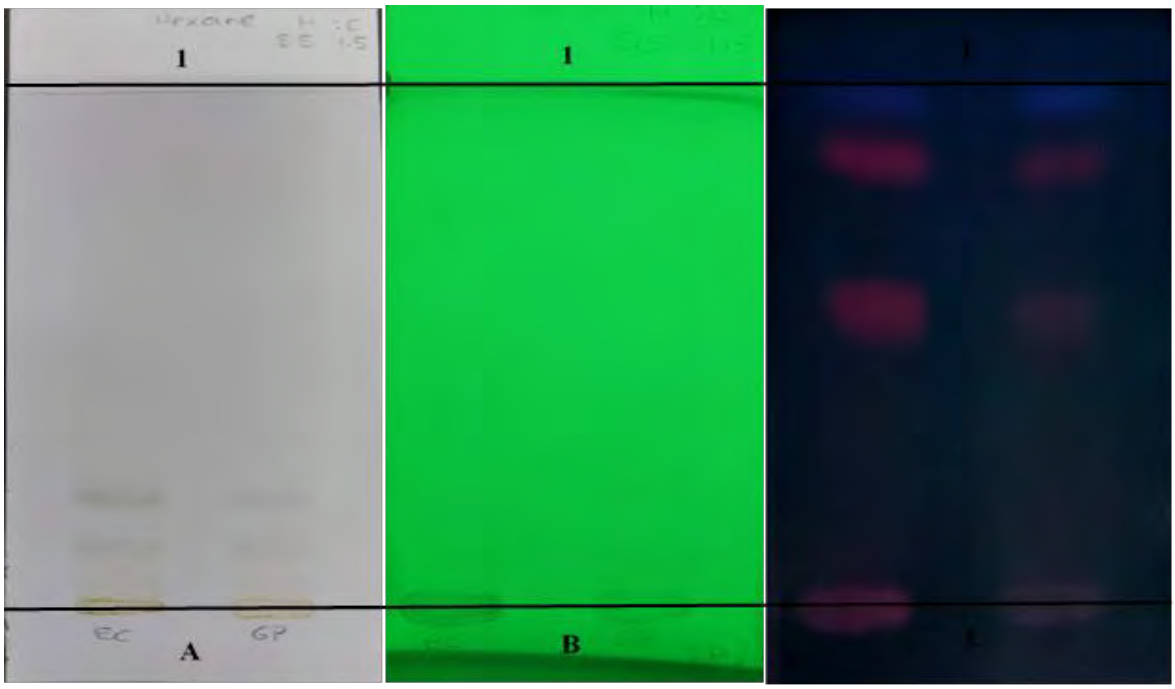


Figure 3.5: Chromatograms of the hexane extracts of T. violacea for EC (on the left) and GP (on the right). The chromatograms are visualised from left to right under A- visible eye, B- UV shortwave (254 nm) and C- UV longwave (365 nm); *EC-Eastern Cape, GP-Gauteng Province*

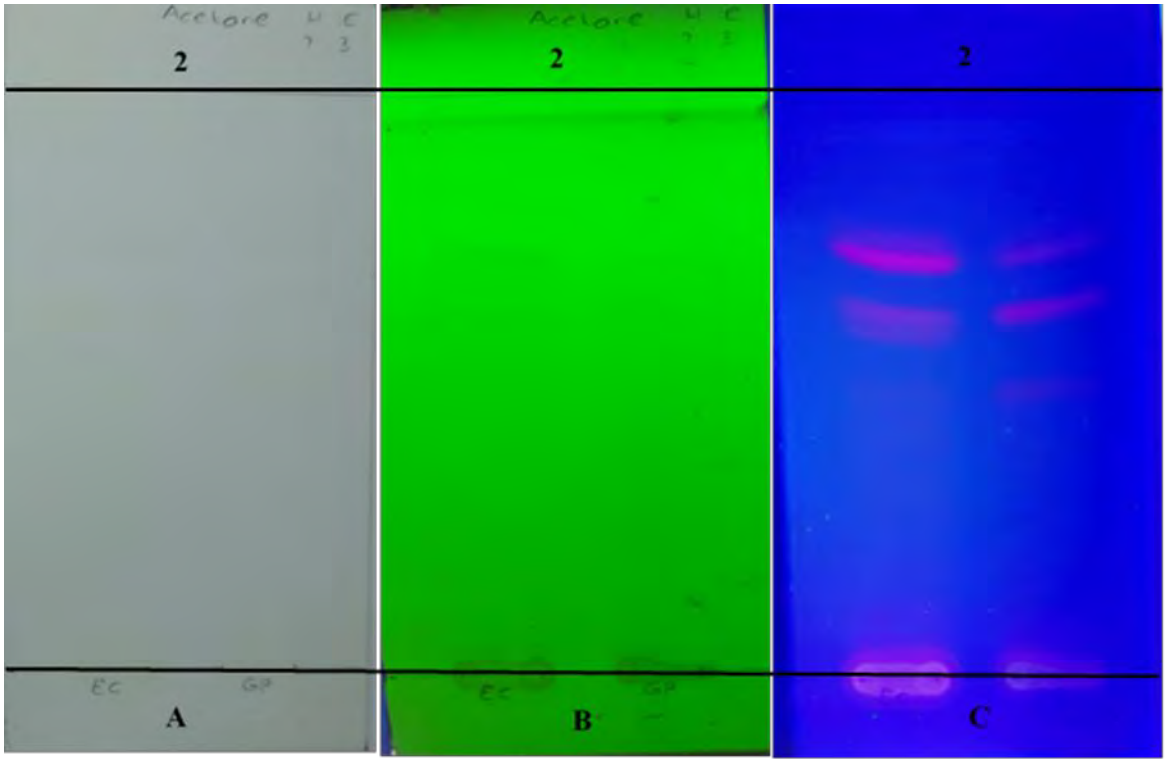


Figure 3.6: Chromatograms of the acetone extracts of T. violacea for EC (on the left) and GP (on the right). The chromatograms are visualised from left to right under A- visible eye, B- UV shortwave (254 nm) and C- UV longwave (365 nm); *EC-Eastern Cape, GP-Gauteng Province*

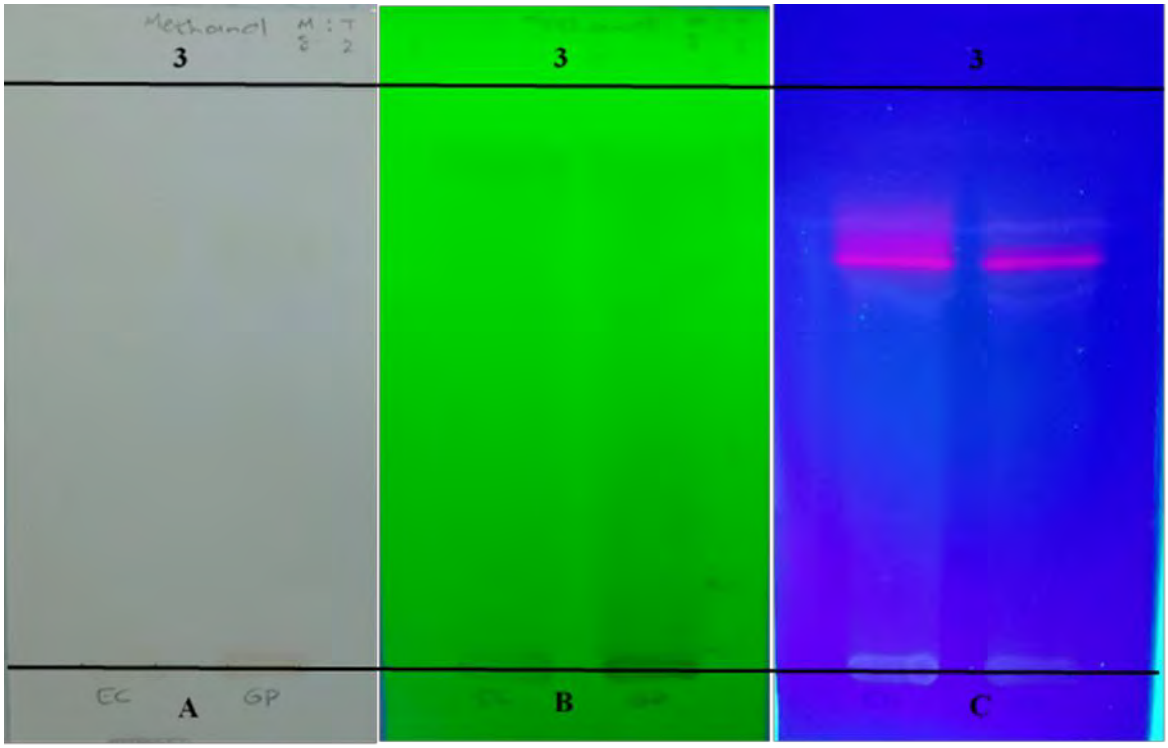


Figure 3.7: Chromatograms of the methanol extracts of T. violacea for EC (on the left) and GP (on the right). The chromatograms are visualised from left to right under A- visible eye, B- UV shortwave (254 nm) and C- UV longwave (365 nm); EC-Eastern Cape, GP-Gauteng Province

Table 3.3: Rf values for compounds extracted by hexane under visible light, UV shortwave (254 nm) and UV longwave (365 nm) for T. violacea from EC and GP

Hexane extracts					
Visible light		**UV shortwave (214 nm)**		**UV longwave (314 nm)**	
EC	**GP**	**EC**	**GP**	**EC**	**GP**
0.0625	**0.0625**	**0.9375**	**0.9375**	**0.5625**	**0.5625**
0.125	**0.125**			**0.9**	**0.9**
0.225	**0.225**			**0.9875**	**0.9875**
Total= 3	**Total= 3**	**Total= 1**	**Total= 1**	**Total= 3**	**Total= 3**

Table 3.4: Rf values for compounds extracted by acetone under visible light, UV shortwave (254 nm) and UV longwave (365 nm) for T. violacea from EC and GP

Acetone extracts					
Visible light		**UV shortwave (214 nm)**		**UV longwave (314 nm)**	
EC	**GP**	**EC**	**GP**	**EC**	**GP**
0.4875	**0.5**	**0.8375**	**0.8375**	**0.5**	**0.5**
0.6	**0.625**			**0.65**	**0.65**
0.7125				**0.725**	**0.725**
Total= 3	**Total= 2**	**Total= 1**	**Total= 1**	**Total= 3**	**Total= 3**

Table 3.5: Rf values for compounds extracted by methanol under visible light, UV shortwave (254 nm) and UV longwave (365 nm) for T. violacea from EC and GP

Methanol extracts					
Visible light		**UV shortwave (214 nm)**		**UV longwave (314 nm)**	
EC	**GP**	**EC**	**GP**	**EC**	**GP**
0.6875	**0.6875**	**0.875**	**0.875**	**0.65**	**0.65**
0.75	**0.75**			**0.7125**	**0.7125**
				0.7875	**0.7875**
Total= 2	**Total= 2**	**Total= 1**	**Total= 1**	**Total= 3**	**Total= 3**

3.4 Discussion

3.4.1 Qualitative phytochemical analysis

The preliminary phytochemical screening of *T. violacea* showed the presence of saponins, flavonoids, tannins, alkaloids, steroids, cardiac glycosides and phenolics. This is in correlation with the results published by Takaidza *et al*. (54) on the phytochemical contents of the *Tulbaghia* species, where it was found to contain flavonoids, glycosides, tannins, terpenoids, saponins and steroids. Table 3.1 shows the presence of alkaloids in all extracts from EC and GP but the study by Takaidza *et al.* found an absence of alkaloids from *T. violacea*. (54). Their study used the leaves of *T. violacea* collected from GP (54) and one of two samples in this study was also from GP. This difference in phytochemicals could be due to the different seasons the plants were harvested in, the soil type and pH and the specific locations within GP.

According to the results of the qualitative phytochemical analysis, saponins were found in the hexane, acetone and methanol extracts of *T. violacea* samples from EC and GP. However, the results revealed moderate presence of saponins from the acetone extracts from EC and trace presence from GP. Saponins are amphiphilic in nature, they contain an aglycone moiety which makes them soluble in non-polar solvents and a glycone moiety which makes them soluble in polar solvents (126). This chemical nature of saponins could be the reason they were found in

all of the extracts of *T. violacea*. Saponins being present in all the extracts of *T. violacea* is a good indication of its medicinal benefit as many studies have shown the pharmacological properties of saponins which includes anti-inflammatory, antitumour, analgesic and antitussive properties (21). One of the traditional uses of *T. violacea* is to clear coughs and the antitussive properties of saponins may be responsible for this effect/ use (127).

From the qualitative phytochemical analysis, anthraquinones were not found to be present in any of the *T. violacea* plant extracts.

Flavonoids were found to be present only in the hexane extracts of *T. violacea* in both the EC and GP samples. The chemical structure of flavonoids includes a chroman ring with an aromatic ring in position 2, 3 or 4 (21). This may be the reason why it was extracted by hexane (a non-polar solvent). The presence of flavonoids in *T. violacea* may be an indication that the plant has potential to have many biological activities such as anti-inflammatory, antithrombotic and vasoprotective properties as well as inhibition of tumour promotion (21, 22). Traditionally, *T. violacea* is used in the treatment of oesophageal cancer as well as hypertension (127). The presence of flavonoids in *T. violacea* may be responsible for these uses of the plant.

According to the results of the qualitative phytochemical analysis, tannins were found to be moderately present in the acetone extracts of *T. violacea* from EC and GP while being present in an appreciable quantity in the methanol extracts from EC and GP. These results make sense as tannins are polyphenols containing many hydroxyl groups indicating their solubility in polar solvents (21, 22).

From the qualitative phytochemical analysis, alkaloids were found in the hexane, acetone and methanol extracts of *T. violacea* from EC and GP. From the acetone extracts, alkaloids were present in appreciable quantity from the EC sample and in moderate presence from the GP sample. From the methanol extracts, alkaloids had moderate presence in the EC sample and trace presence in the GP sample. This indicates a difference in the phytochemical composition of alkaloids in *T. violacea* depending on the geographical location the plant is cultivated in, with *T. violacea* grown in the EC having a greater presence of alkaloids compared to *T. violacea* grown in GP. Alkaloids possess a variety of pharmacological activities including analgesia, vasoconstriction, muscle relaxation as well as antineoplastic, hypertensive and hypotensive properties (21). The vast activities of alkaloids may be one of the reasons for the popularity of *T. violacea* in traditional medicine.

Steroids were found to be present in the hexane, acetone and methanol samples of *T. violacea*. The hexane and acetone extracts of *T. violacea* from EC and GP contained steroids but only the methanol extract from EC contained steroids. The results showed that the hexane extracts of *T. violacea* from the EC sample showed impressive presence of steroids while the GP sample showed moderate presence. The acetone extracts from the EC sample showed steroids present in an appreciable quantity while the GP sample showed a moderate presence of steroids. This indicates that in all extracts, steroids had a greater presence in *T. violacea* collected from the EC compared to *T. violacea* collected from GP. Four interconnected rings of carbon atoms form the skeleton of steroids, making them more non-polar (128). This explains why hexane was able to extract steroids better than acetone and methanol. Plant steroids have a variety of medicinal properties including antitumour, immunosuppressive, hepatoprotective, antibacterial, antihelminthic, cytotoxic and cardiotonic properties (129, 130). The antibacterial and antihelminthic properties of steroids (127) could be the reason *T. violacea* is used in the treatment of pulmonary TB and intestinal worms, traditionally.

Cardiac glycosides were found in the hexane and acetone extracts but not in the methanol extracts. The structure of cardiac glycosides consists of an aglycone and a glycone so its presence in any solvent extract is expected (31, 32). In the hexane extracts of *T. violacea*, the sample from EC showed the presence of cardiac glycosides in an appreciable quantity and the GP sample showed an impressive presence of cardiac glycosides. In the acetone extracts, moderate presence of cardiac glycosides were found in the EC sample but an appreciable presence was found in the GP samples. This indicates that the *T. violacea* sample from GP contained a greater presence of cardiac glycosides compared to the sample from EC.

The results of the qualitative phytochemical analysis showed the presence of phenolics in the hexane, acetone and methanol extracts of *T. violacea*. It can be seen that the presence of the phenolic compounds increased with an increase in solvent polarity and this is due to the presence of hydroxyl groups making the compounds more polar (21). From the results of the methanol extracts, it can be seen that the sample from EC showed an impressive presence of phenolic compounds while the GP sample showed an appreciable quantity being present.

3.4.2 Quantitative phytochemical analysis

According to table 3.2, phenolic compounds were present in hexane, acetone and methanol extracts of *T. violacea* from EC and GP. This backs up the results of the qualitative

phytochemical analysis seen in table 1. According to table 3.1, table 3.2 and figure 3.1, the concentration of phenolic compounds increases with an increase in the polarity of the solvent used for extraction. This is due to the presence of hydroxyl groups found in phenolic compounds (16). The methanol extract of *T. violacea* from EC had the highest concentration of phenolic compounds with its value being 16.095mg/g GAE and the hexane extract of *T. violacea* from EC had the lowest total phenol content of 2.463 mg/g GAE.

Figures 3.2, 3.3 and 3.4 shows the results of the statistical analysis run on the total phenolic content of the hexane, acetone and methanol extracts of *T. violacea*. Statistical analysis was carried out using GraphPad Prism 10 and the test used was a t-test. Each analysis was run in triplicate and the analysis compared the results of the EC sample vs the GP sample for each of the solvent extracts. Significant statistical difference was seen when $p < 0.05$. The asterisks (*) seen on figures 3.2 and 3.4 indicate a significant statistical difference between the TPC of the EC and GP samples with regard to their hexane and methanol extracts. However, the acetone extracts of *T. violacea* from EC and GP showed no significant statistical difference with regard to TPC, as seen in figure 3.3.

3.4.3 Thin layer chromatography

It is known that certain metabolites or organic compounds such as aromatic compounds are not visible or are colourless under visible light but they can absorb UV light and become visible (131, 132) . Visualisation methods can either be non-destructive where the compound remains unchanged, or destructive where the compound is converted into something new (131, 132). The process of visualising a TLC plate under UV light is non-destructive (132) and is the method that was used in this study.

The fluorescent colour bands on the TLC plate under UV light allows for a preliminary detection of possible phytochemicals present in plant extracts. The blue/ blue violet bands seen on plates 1C, 2C and 3C (figures 3. 5- 3.7) could possibly indicate the presence of flavones, phenolic acids, terpenoids or saponins as these have been shown in previous studies (131, 133, 134). The pinkish violet bands seen on plates 1C, 2C and 3C could indicate the presence of glycosides (131, 133, and 134).

Majority of the bands on the TLC plate viewed under UV light have the same colour and Rf values for *T. violacea* samples from EC and GP. However, the intensity of the coloured bands

are greater in the EC samples for hexane and acetone as compared to the sample from GP. This may be due to the concentration of the phytochemicals present and this result correlates with the results of the qualitative and quantitative phytochemical analysis of *T. violacea*.

3.5 Chapter summary

The results of the phytochemical analysis revealed that the different extracts of *T. violacea* samples from EC and GP contain active phytochemical compounds including saponins, flavonoids, tannins, alkaloids, steroids, cardiac glycosides and phenolics. The results also revealed differences in the presence of phytochemicals between the EC and GP samples of *T. violacea* including differences in saponins, alkaloids, steroids, cardiac glycosides and phenolic compounds. Saponins, alkaloids, steroids and phenolic compounds had a greater presence in the EC sample of *T. violacea* while cardiac glycosides had a greater presence in the GP sample of *T. violacea*.

The results also revealed that the hexane, acetone and methanol extracts of *T. violacea* from EC and GP contained phenolic compounds with the methanol extract from the EC sample having the highest TPC. Significant statistical difference in TPC between EC and GP samples of *T. violacea* were seen for the hexane and methanol extracts.

These results indicate that there is a difference in the type and/or quantity of phytochemicals present in *T. violacea* based on the geographical location in which the plant is cultivated.

The TLC results confirmed the presence of a variety of phytochemicals present in *T. violacea*.

CHAPTER 4

Antioxidant capacity of various solvent extracts of *T. violacea* samples from the Eastern Cape and Gauteng

4.1 Introduction

A free radical is a molecule with one or more unpaired electrons in its outer shell (55, 57). Oxygen is an element which is essential for life and when cells use oxygen to generate energy, free radicals are produced. These by-products are generally reactive oxygen species (ROS) or reactive nitrogen species (RNS) and they have a dual role of being beneficial at low levels or toxic at higher levels. At higher concentrations, they result in oxidative stress which is a process that can result in damage to cell structures. The oxidative stress from high concentrations of free radicals plays a major role in the development of chronic conditions (55, 57) such as autoimmune disorders, cancer, arthritis, diabetes mellitus and Alzheimer's disease.

Antioxidants are molecules which are stable enough to donate an electron to a free radical and neutralise it. Some antioxidants, such as glutathione, ubiquinol and uric acid are produced via normal metabolism in the body, while other antioxidants are present in the food humans consume (55, 56). Research has shown that many medicinal plants possess antioxidant properties and research on the antioxidant properties of *T. violacea* have been conducted (54, 57-59). This chapter focuses on the antioxidant capacity of *T. violacea* samples, collected from the Eastern Cape and Gauteng provinces in South Africa, to determine if geographical location in which the plant is cultivated plays a role in its antioxidant capacity.

4.2 Methods and materials

4.2.1 Qualitative antioxidant activity

A method described by Gupta *et al.* (135) was followed with slight modifications. The solvent extracts of *T. violacea* were redissolved in the solvents that were used to extract them. A pre-coated silica 60 TLC plate was prepared in a way to accommodate all 6 extracts (hexane, acetone and methanol extracts from EC and GP samples).

Each extract was spotted in a dot form on the TLC plate and left to dry. Once dry, the TLC plate was sprayed with prepared 0.01 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. The white to pale yellow discoloration of the dots against the purple background of the sprayed TLC plate was taken as a positive indication of antioxidant activity.

4.2.2 Quantitative DPPH scavenging assay

The DPPH radical scavenging assay was determined according to a method described by Kwon *et al.* (136) with slight modifications. In a 96- well plate, 100 µl of freshly prepared DPPH (0.01 mM) was added to 200 µl of plant extracts of varying concentrations (10, 20, 30, 40 and 50 µg/ml). The reaction mixture was allowed to stand at room temperature in the dark for 30 minutes. Thereafter the absorbance was measured at 517 nm using a Spectramax M3-multimode microplate reader (Molecular devices, China). For the assay, ascorbic acid at varying concentrations (10, 20, 30, 40 and 50 µg/ml) was used as the standard compound and distilled water was used as a negative control. The assay was done in triplicate. The percentage of DPPH radical scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (%) = $[1- (Abs_{sample}/Abs_{control})] \times 100$,

Where: Abs_{sample} is the absorbance of the sample (plant extracts) measured at 517nm

$Abs_{control}$ is the absorbance of the control measured at 517nm

4.3 Results

The results for qualitative antioxidant analysis (dot-plot) are presented in figure 4.1 and those for quantitative antioxidant analysis (DPPH radical scavenging assay) are displayed in figures 4.2-4.5 below. The IC50 values of the various extracts are shown in Table 4.1.

4.3.1 Qualitative antioxidant screening (dot-plot method)

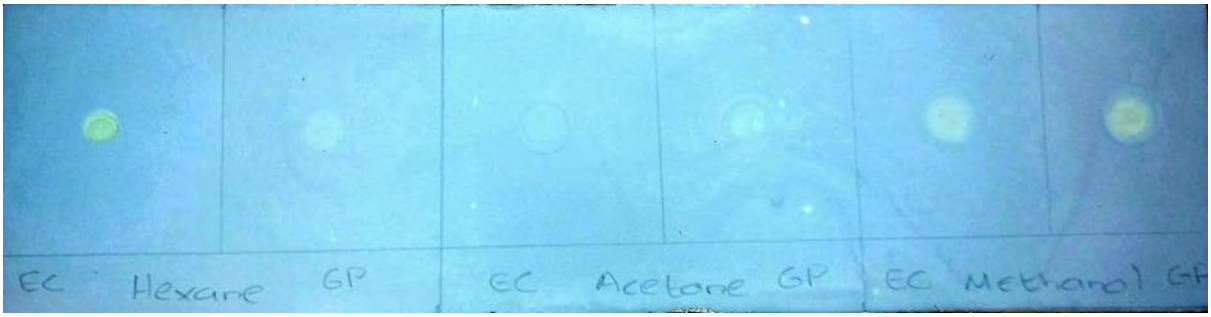


Figure 4.1: Dot-plot of T. violacea extracts from EC and GP after spraying with DPPH. The hexane extract from the EC sample showed the brightest yellow spot while the acetone extracts showed the smallest colour change; , EC-Eastern Cape, GP-Gauteng Province

4.3.2 DPPH radical scavenging assay

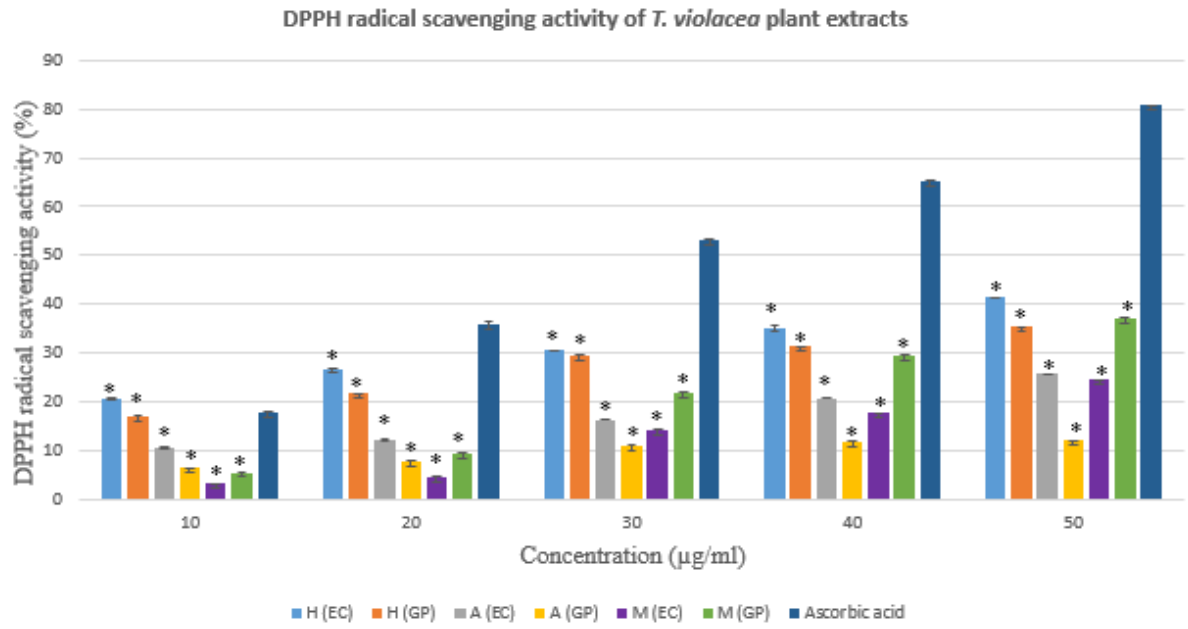


Figure 4.2: DPPH radical scavenging activity (%) of T. violacea extracts (EC and GP) and ascorbic acid. The asterisks (*) above the bars represent significant statistical difference between the test compound and ascorbic acid; H-hexane, A-acetone, M-methanol, EC-Eastern Cape, GP-Gauteng Province

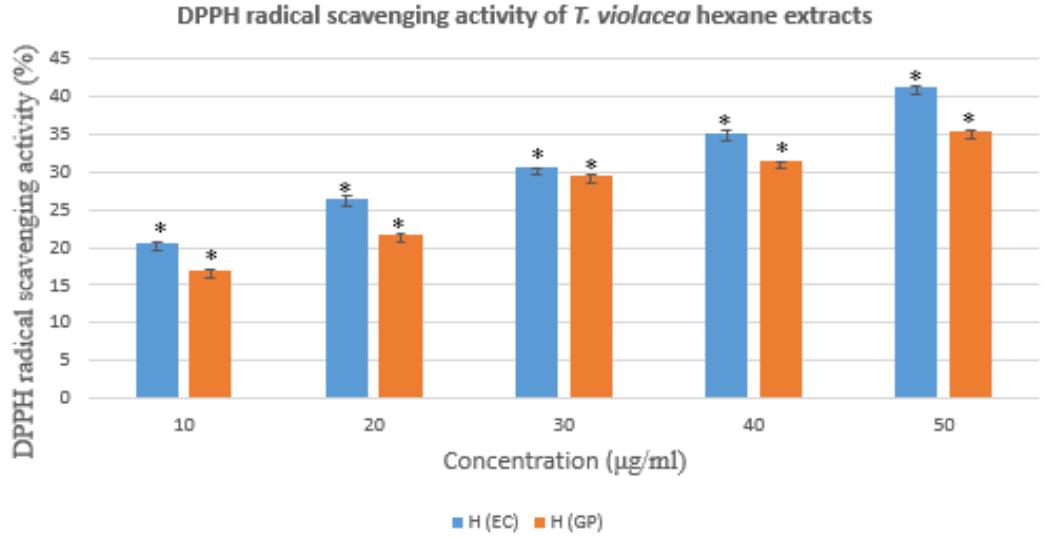


Figure 4.3: DPPH radical scavenging activity (%) of T. violacea hexane extracts. The asterisks (*) above the bars represents significant statistical difference; H-hexane, EC-Eastern Cape, GP-Gauteng Province

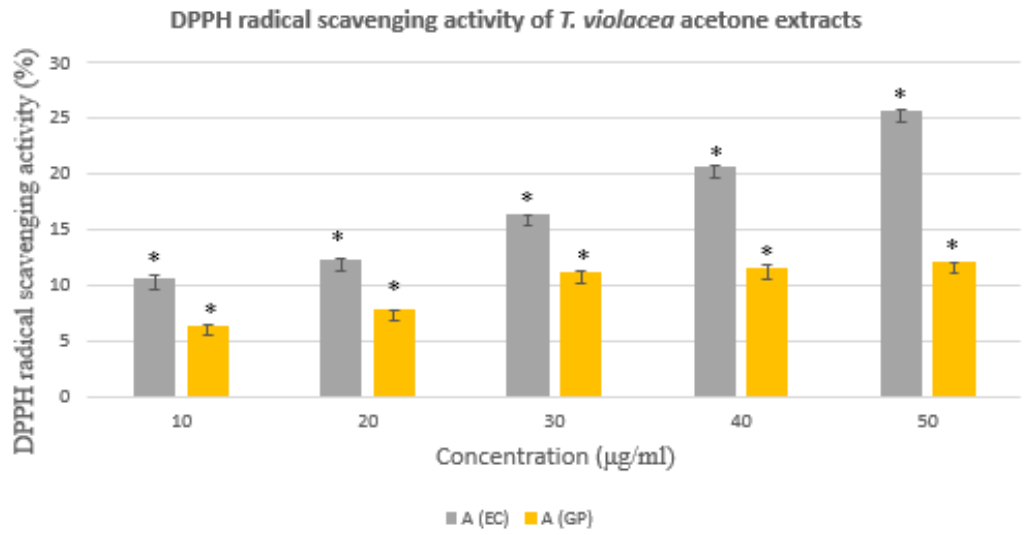


Figure 4.4: DPPH radical scavenging activity (%) of T. violacea acetone extracts. The asterisks (*) above the bars represents significant statistical difference; A-acetone, EC-Eastern Cape, GP-Gauteng Province

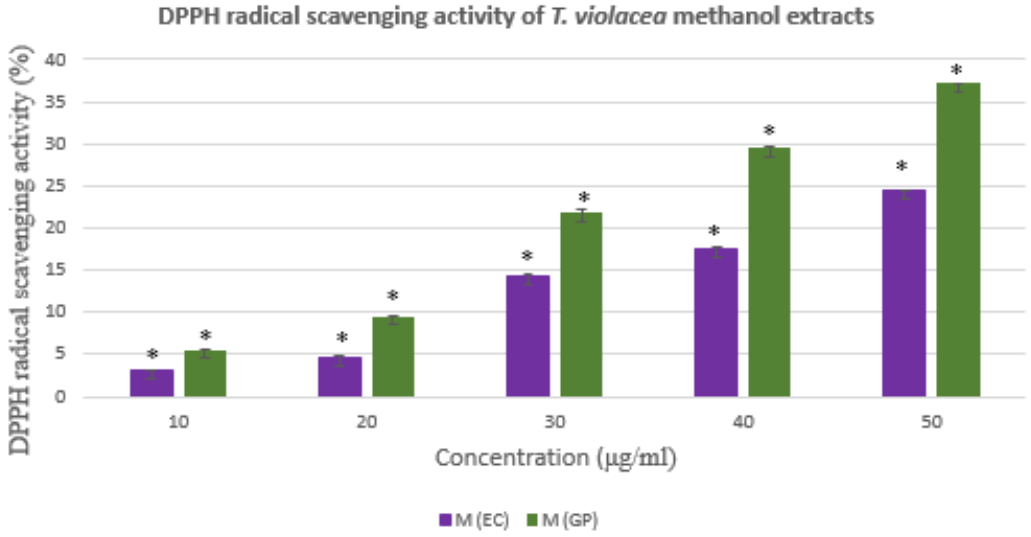


Figure 4.5: DPPH radical scavenging activity (%) of T. violacea methanol extracts. The asterisks (*) above the bars represents significant statistical difference; M-methanol, EC-Eastern Cape, GP-Gauteng Province

Table 4.1: IC_{50} values of various extracts of T. violacea (EC and GP) and the standard ascorbic acid

Extracts and standard	**IC_{50} value (µg/ml)**		
	T. violacea		**Ascorbic acid**
	EC	**GP**	
Hexane	68.259	79.565	
Acetone	115.11	295.568	
Methanol	97.105	65.138	
Ascorbic acid			29.595

4.4 Discussion

Both the qualitative antioxidant analysis (dot-plot) and quantitative antioxidant analysis (DPPH radical scavenging assay) used DPPH as the free radical, to evaluate the antioxidant potential of *T. violacea* plant extracts.

DPPH is a free radical that has a deep violet/ purple colour to it. It is a molecule which is soluble in methanol and has a maximum absorption at 517 nm (137, 138). In the presence of a hydrogen donor or a free radical scavenger (antioxidant), the odd electron is paired and forms 1-1, diphenyl-2, picryl-hydrazine (DPPH-H) which is a pale yellow in colour. The change in colour reduces the absorbance at 517 nm and the antioxidants reduce the DPPH free radical colour in a concentration dependent manner (137, 138). Therefore, the decrease in absorbance is proportional to the antioxidant capacity of the inhibitor (*T. violacea* plant extracts).

The results of the dot-plot, shown in figure 4.1, showed that the hexane, acetone and methanol crude extracts of *T. violacea* from EC and GP have antioxidant activity as all of the extracts changed the purple DPPH free radial to yellow. From figure 4.1, it can be seen that the hexane extract of *T. violacea* could have the strongest antioxidant capability as it discoloured the DPPH the most, followed by the methanol extract of *T. violacea* from GP. However, a conclusion cannot be made from only the qualitative analysis hence the need for a quantitative DPPH radical scavenging assay.

The results of the radical scavenging assay, as depicted in figure 4.2, correlates with the results of the dot-plot, indicating that all extracts (hexane, acetone and methanol) of *T. violacea* from EC and GP have antioxidant activity. It can also be seen that the activity of the plant extracts is concentration dependent. As the concentration of the extracts increased, its % DPPH radical scavenging increased. Of the *T. violacea* plant extracts, the hexane extract from EC had the highest radical scavenging activity with a concentration of 50 µg/ml having a radical scavenging activity of 41.314%. This was followed by the methanol extract of *T. violacea* from GP, with a radical scavenging activity of 37.202% at a concentration of 50 µg/ml. These results confirm the assumptions drawn from the dot-plot. The acetone extract of *T. violacea* from the GP sample had the lowest radical scavenging activity of 12.069% at a concentration of 50µg/ml.

The values from the graph depicted in figure 4.2 were used to determine the IC_{50} values of *T. violacea* plant extracts. Microsoft Excel and GraphPad Prism 10 were used to plot the graphs and calculate the IC_{50} values. Calculation of the IC_{50} values was done using the linear regression method where the linear equation was drawn to best represent the data points obtained from each of the concentrations for each extract. The IC_{50} values for each extract are depicted in table 4.1. Although all the *T. violacea* extracts from EC and GP showed antioxidant activity, none of the activity or IC_{50} values were close to that of the standard used (ascorbic acid). The hexane sample from EC had an IC_{50} value of 68.259 µg/ml, making it the closest to the IC_{50} value of ascorbic acid which was found to be 29.595 µg/ml.

Statistical analysis was carried out on the results to check if there was a significant difference between the radical scavenging activity of *T. violacea* plant extracts and the standard ascorbic acid. The experiment was carried out in triplicates and statistical analysis was done using GraphPad Prism 10. Statistical analysis was conducted using one-way ANOVA trailed by Dunnett's post-hoc test. Significant statistical difference was accepted when $p < 0.05$. The asterisks (*) seen above the bars in figure 4.2 represent significant statistical difference between *T. violacea* plant extracts and ascorbic acid. The figure shows that all extracts of *T. violacea*, at all concentrations, had a radical scavenging activity that was statistically different to the radical scavenging activity of ascorbic acid at the same concentrations.

Figures 4.3, 4.4 and 4.5 compares the results of the EC samples vs the GP samples for hexane, acetone and methanol extracts of *T. violacea*. A t-test was used as the statistical test to compare the radical scavenging activity of the EC sample to the radical scavenging activity of the GP sample of *T. violacea*. Significant statistical difference was accepted when $p < 0.05$ and the

asterisks (*) above the bars represent this difference. From figures 4.3, 4.4 and 4.5 it can be seen that the hexane, acetone and methanol extracts of *T. violacea* from the EC sample had a radical scavenging activity that was significantly different to the radical scavenging activity of the GP sample.

With regard to the hexane extracts, the EC sample had a higher radical scavenging activity (41.314%) than the GP sample (35.362%).

With regard to the acetone extracts, the EC sample had a higher radical scavenging activity (25.762%) compared to the GP sample (12.069%).

With regard to the methanol extracts, the EC sample had a lower radical scavenging activity (24.443%) compared to the GP sample (37.202%).

The differences in the radical scavenging activities and antioxidant capacity of *T. violacea* samples from EC and GP could be due to the differences in the phytochemical composition/ concentrations as seen in chapter 3.

Antioxidants protect the body from oxidative stress caused by free radicals and reduce the risk of chronic diseases such as diabetes mellitus and Alzheimer's disease.

A study conducted on the phytochemical contents and antioxidant activities of crude extracts of the *Tulbaghia* species, found that *T. violacea* had the highest scavenging activity for DPPH among the *Tulbaghia* species. The results also showed that it had a low IC50 value and had a concentration dependent activity (54). This was in correlation with the results presented in this chapter.

Olorunnisola *et al.* (57) reported that *T. violacea* showed DPPH radical scavenging activity at all concentrations, in a concentration dependent manner. They also found that the DPPH scavenging activity of *T. violacea* reached 89.2%. This is in comparison to the results of this study which found the hexane extract of *T. violacea* from EC to have a DPPH radical scavenging activity of 41.314%. However, the findings of the study by Olorunnisola *et al.* (57) were for *T. violacea* at 0.5 mg/ml, while the results of this study were for *T. violacea* at 50 µg/ml. Both studies confirm the antioxidant activity of the plant however, further studies at other concentrations need to be done.

Another study, conducted on the essential oils of *T. violacea* found it to be a weak scavenger of DPPH, with a DPPH scavenging activity of 49.25% at 100 mg/ml, although it exhibited a

concentration dependent activity (58). This is in comparison with the results of this study, done on the crude leaf extracts of *T. violacea*, which found it to have a DPPH scavenging activity of 41.314% at 50 µg/ml. The difference in results could be due to different extraction methods or testing at different concentrations.

Since it has been found that extracts of *T. violacea* do have antioxidant activity, it supports their use in traditional medicine and may have potential in managing chronic conditions such as diabetes mellitus and Alzheimer's disease.

4.5 Chapter summary

This chapter has revealed the presence of antioxidants, which are able to scavenge DPPH free radicals, in all extracts of *T. violacea* from EC and GP samples. The results of the quantitative analysis showed that the radical scavenging activity of *T. violacea* is concentration dependent. It also showed that there is significant statistical difference between the radical scavenging activity of *T. violacea* collected from EC and *T. violacea* collected from GP. These results are important as it shows that an individual using *T. violacea* collected from different geographical locations would be provided with varying amounts of antioxidant activity.

CHAPTER 5

Determination of anti-diabetic activity of various solvent extracts of *T. violacea* samples from the Eastern Cape and Gauteng

5.1 Introduction

According to the International Diabetes Federation, 4.2 million people were found to have diabetes in South Africa (139). Diabetes mellitus is a chronic, metabolic disorder resulting in hyperglycaemia. It occurs when the body does not produce enough insulin or when cells in the body stop responding to the insulin that is produced (insulin resistance) by the beta cells of the pancreas. Uncontrolled diabetes mellitus can result in serious complications and health problems including vision loss, kidney problems, heart disease and issues with the nervous system (67-70).

There are 2 main types of diabetes mellitus: Type 1 diabetes mellitus and type 2 diabetes mellitus. In type 1 diabetes mellitus, there is an absolute deficiency in insulin secretion and this form of diabetes is observed in approximately 10% of patients diagnosed with diabetes (68).

Type 2 diabetes mellitus is caused a combination of factors such as defective insulin secretion by the beta cells of the pancreas or when skeletal muscle cells develop insulin resistance. Type 2 diabetes mellitus accounts for approximately 90-95 % of all diabetes mellitus cases (69, 73).

Currently, no cure for diabetes mellitus exists however, there are drugs available to help control the hyperglycaemia associated with diabetes with the goal of therapy being to control blood glucose levels within the normal range and to prevent the long term complications associated with uncontrolled diabetes.

For the management of type 1 diabetes, exogenous insulin is available for use with multiple dosage regimens. For the management of type 2 diabetes, lifestyle modifications are combined with oral anti-diabetic drugs. Classes of oral anti-diabetics include sulfonylureas, biguanides, thiazolidinediones, alpha- glucosidase inhibitors, DPP-IV inhibitors and sodium- glucose co-transporter 2 inhibitors (91).

Although many classes of drugs exist for the management of type 2 diabetes, each class is associated with multiple side effects. For this reason, medicinal plants are screened for their

anti-diabetic activity as they are more cost efficient, widely available and generally associated with a lower incidence of side effects.

5.2 Method and materials

5.2.1 The α- amylase inhibition assay

The α-amylase inhibitory activity was determined by the method described in the Worthington Manual with slight modifications (140). For the reaction, 500 µl plant extract (10, 20, 30, 40 and 50 µg/ml) was added to 500 µl of a 1% α-amylase solution (1 U/ml). This was incubated at 37^{o}C for 15 minutes. Then 500µl of a 1% starch solution in 0.02 M phosphate buffer (pH= 6.9) was added and the mixture was incubated at 37^{o}C for a further 10 minutes. This was followed by addition of 1 ml of 3,5- Dinitrosalicylic acid (DNSA) reagent was added to each test tube and the mixture was boiled for 10 minutes. The reaction mixture was then diluted by adding 2 ml distilled water. Absorbance was read at 540 nm using a Spectramax M3-multimode microplate reader (Molecular devices, China). Acarbose (10, 20, 30, 40 and 50 µg/ml) was used as the standard. The experiment was carried out in triplicate and the α-amylase inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(Abs\ control - Abs\ sample)}{Abs\ control}\ X\ 100\%$$

Where: Abs_{sample} is the absorbance of the sample (plant extracts) measured at 540 nm

$Abs_{control}$ is the absorbance of the control measured at 540 nm

Microsoft Excel and GraphPad Prism 10 were used to plot the graphs and calculate the IC50 values. Calculation of the IC50 values was done using the linear regression method where the linear equation was drawn to best represent the data points obtained from each of the concentrations for each extract. Statistical analysis was conducted using one-way ANOVA trailed by Dunnett's post-hoc test. Significant statistical difference was accepted when $p < 0.05$.

5.2.2 The α-glucosidase inhibition assay

The α-glucosidase inhibitory activity was determined by the method described in the Worthington Manual with slight modifications (141). In a 96- well plate, the reaction mixture containing 100 µl phosphate buffer (0.1 M, pH= 6.9), 40 µl α- glucosidase (1 U/ml) and 80 µl

plant extracts (10, 20, 30, 40 and 50 µg/ml) were incubated at 37ºC for 10 minutes. Then 20 µl para-nitrophenyl-α-D-glucopyranoside (5 mM) was added and the reaction mixture was incubated for 10 minutes at 37ºC. The reaction was stopped by the addition of 100 µl of 0.1 M Na_2CO_3. Absorbance was read at 405 nm using a Spectramax M3- multimode microplate reader (Molecular devices, China). Acarbose (10, 20, 30, 40 and 50 µg/ml) was used as the standard. The experiment was carried out in triplicate and the α- glucosidase inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(Abs\ control - Abs\ sample)}{Abs\ control}\ X\ 100\%$$

Where: Abs_{sample} is the absorbance of the sample (plant extracts) measured at 405 nm

$Abs_{control}$ is the absorbance of the control measured at 405 nm

Microsoft Excel and GraphPad Prism 10 were used to plot the graphs and calculate the IC50 values. Calculation of the IC50 values was done using the linear regression method where the linear equation was drawn to best represent the data points obtained from each of the concentrations for each extract. Statistical analysis was conducted using one-way ANOVA trailed by Dunnett's post-hoc test. Significant statistical difference was accepted when $p < 0.05$.

5.3 Results

5.3.1 The α- amylase inhibition assay

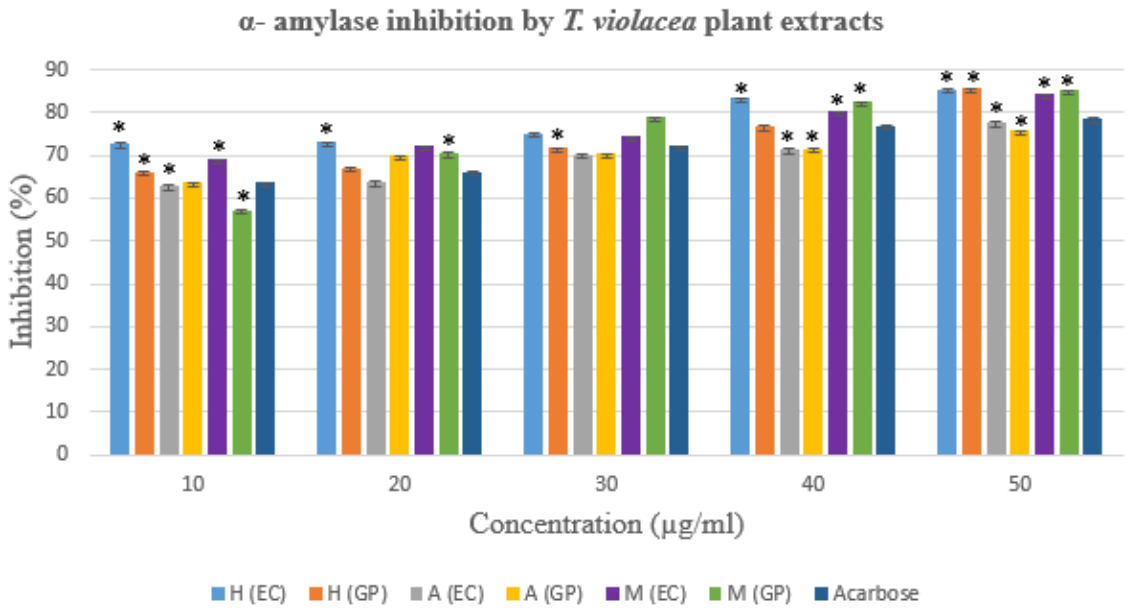


Figure 5.1:The α- amylase inhibition (%) of T. violacea extracts (EC and GP) and acarbose. The asterisks (*) above the bars represent significant statistical difference between the test compound and acarbose; H-hexane, A-acetone, M-methanol, EC-Eastern Cape, GP-Gauteng Province

Table 5.1: IC_{50} values of various extracts of T. violacea (EC and GP) and the standard acarbose

Extracts and standard	**IC_{50} value (µg/ml)**		
	T. violacea		**Acarbose**
	EC	**GP**	
Hexane	9.022	15.083	
Acetone	16.189	16.504	
Methanol	16.146	16.201	
Acarbose			16.366

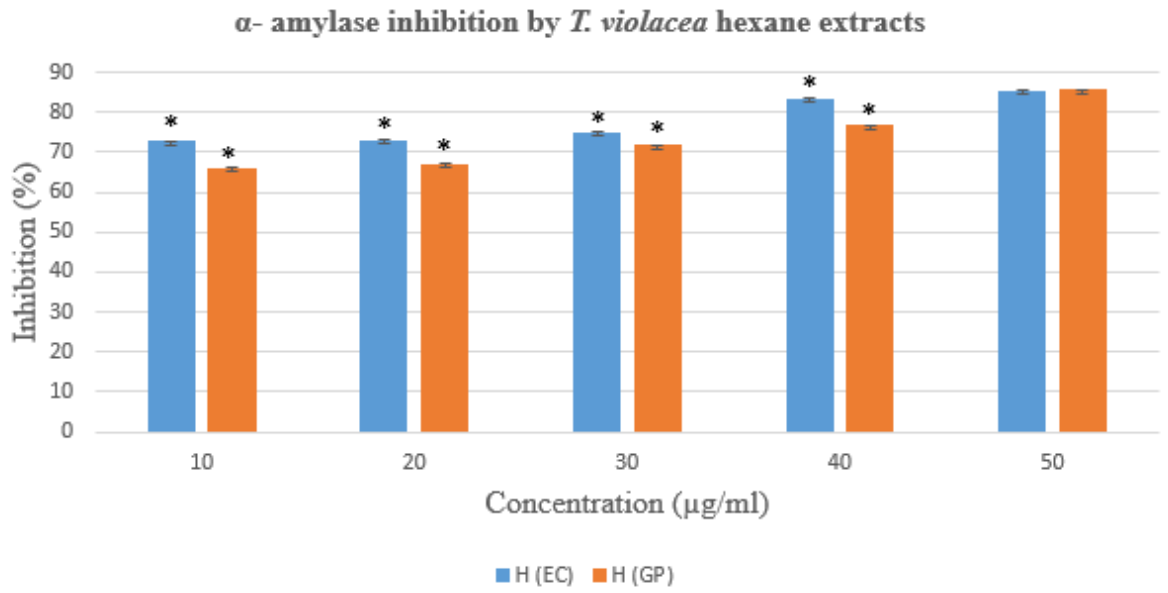


Figure 5.2:The α- amylase inhibition (%) of T. violacea hexane extracts. The asterisks (*) above the bars represents significant statistical difference; H-hexane, EC-Eastern Cape, GP-Gauteng Province

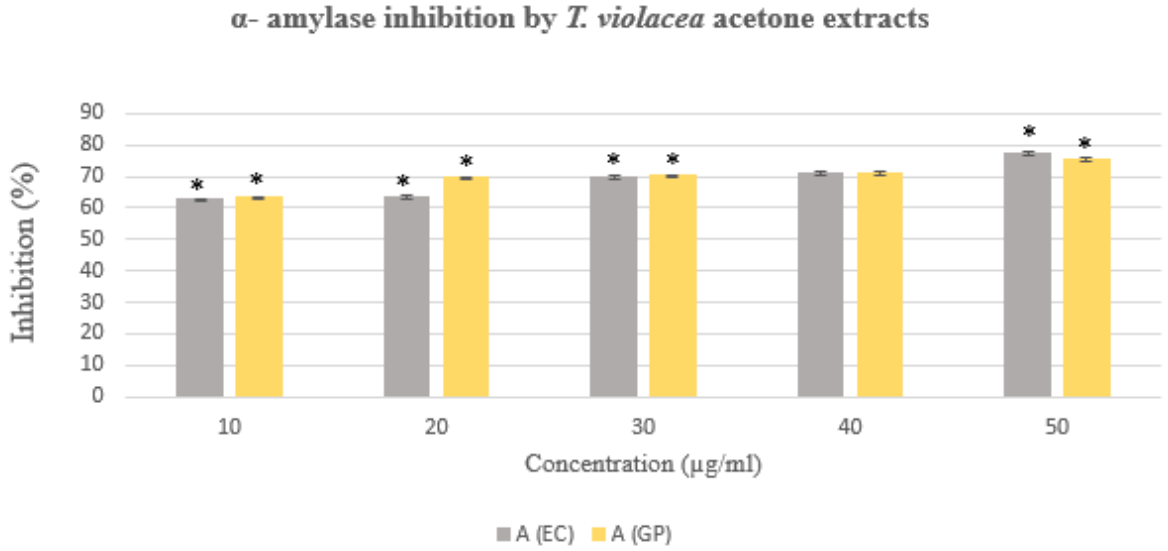


Figure 5.3:The α- amylase inhibition (%) of T. violacea acetone extracts. The asterisks (*) above the bars represents significant statistical difference; A-acetone, EC-Eastern Cape, GP-Gauteng Province

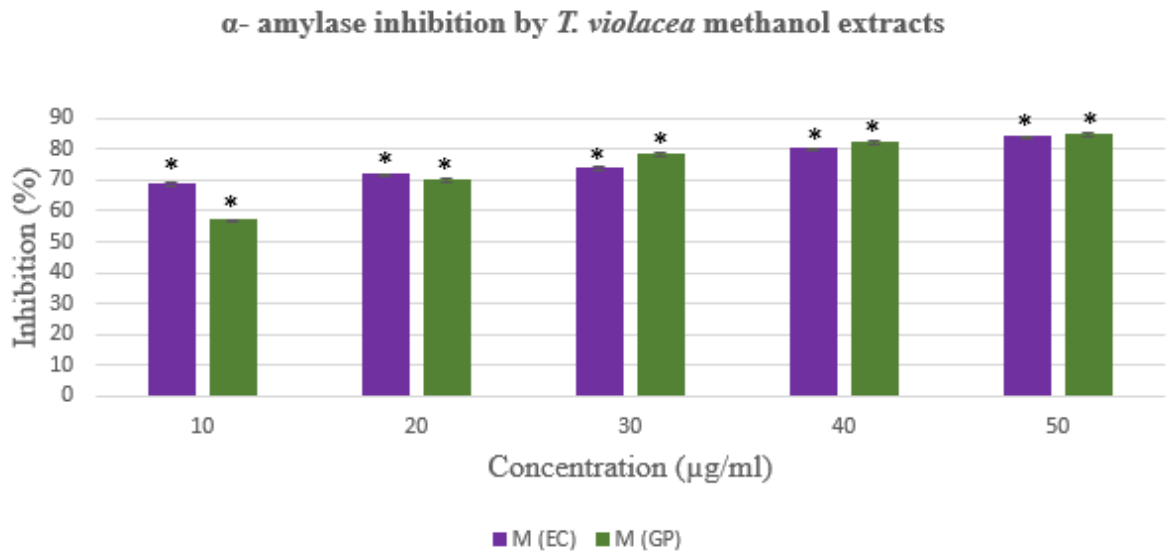


Figure 5.4:The α- amylase inhibition (%) of T. violacea methanol extracts. The asterisks (*) above the bars represents significant statistical difference; M-methanol, EC-Eastern Cape, GP-Gauteng Province

5.3.2 The α- glucosidase inhibition assay

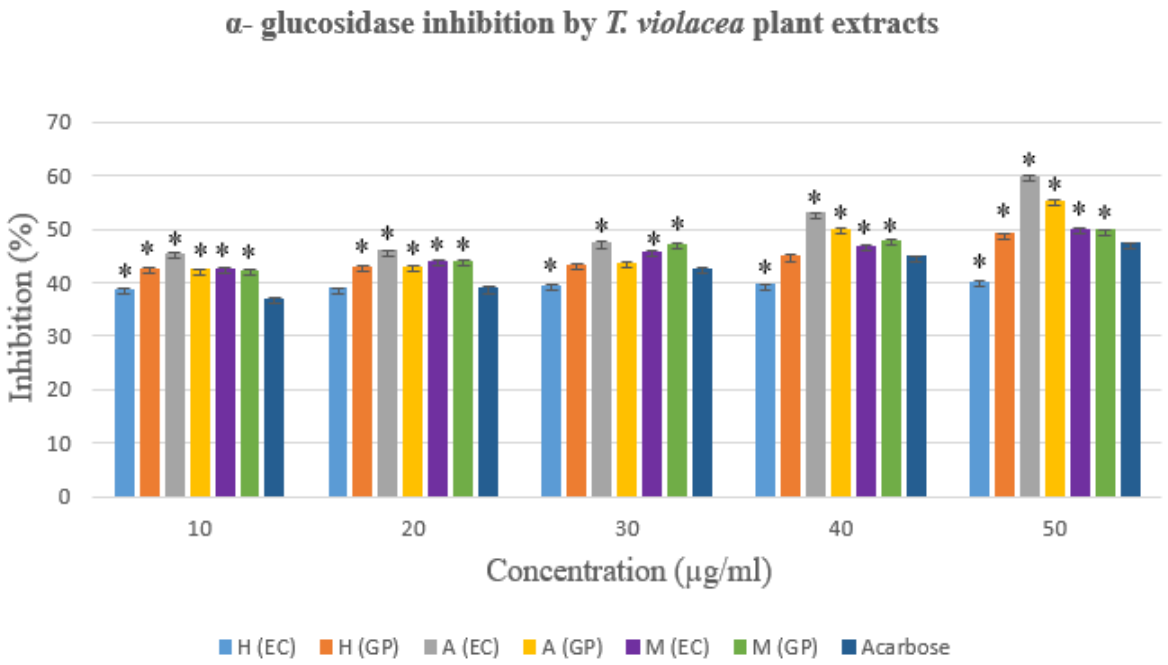


Figure 5.5:The α- glucosidase inhibition (%) of T. violacea extracts (EC and GP) and acarbose. The asterisks (*) above the bars represent significant statistical difference between the test compound and acarbose; H-hexane, A-acetone, M-methanol, EC-Eastern Cape, GP-Gauteng Province

Table 5.2: IC_{50} values of various extracts of T. violacea (EC and GP) and the standard acarbose

Extracts and standard	**IC_{50} value (µg/ml)**		
	T. violacea		**Acarbose**
	EC	**GP**	
Hexane	42.2	43.332	
Acetone	34.570	38.878	
Methanol	41.607	41.148	
Acarbose			45.609

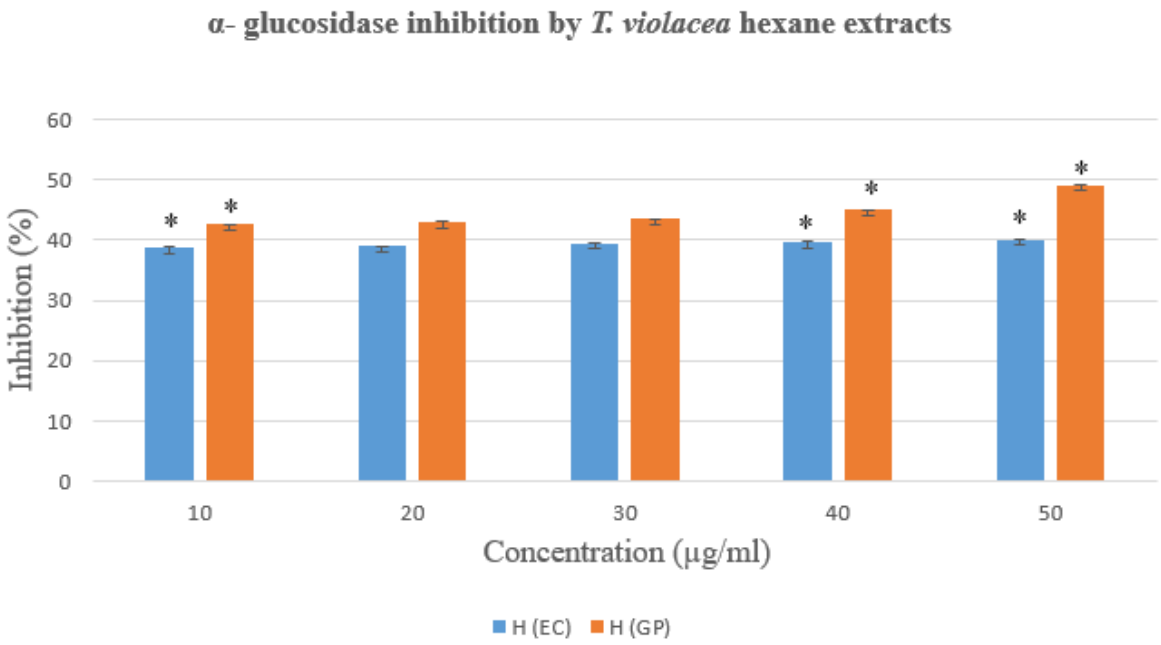


Figure 5.6: The α- glucosidase inhibition (%) of T. violacea hexane extracts. The asterisks (*) above the bars represents significant statistical difference; H-hexane, EC-Eastern Cape, GP-Gauteng Province

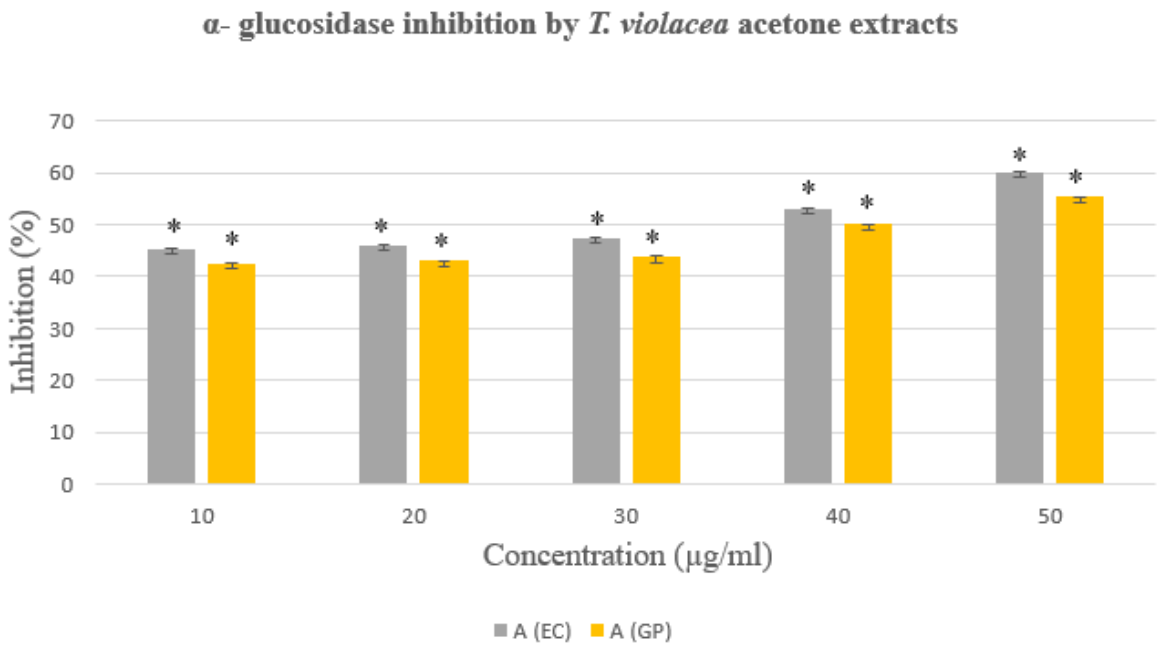


Figure 5.7: The α- glucosidase inhibition (%) of T. violacea acetone extracts. The asterisks (*) above the bars represents significant statistical difference; A-acetone, EC-Eastern Cape, GP-Gauteng Province

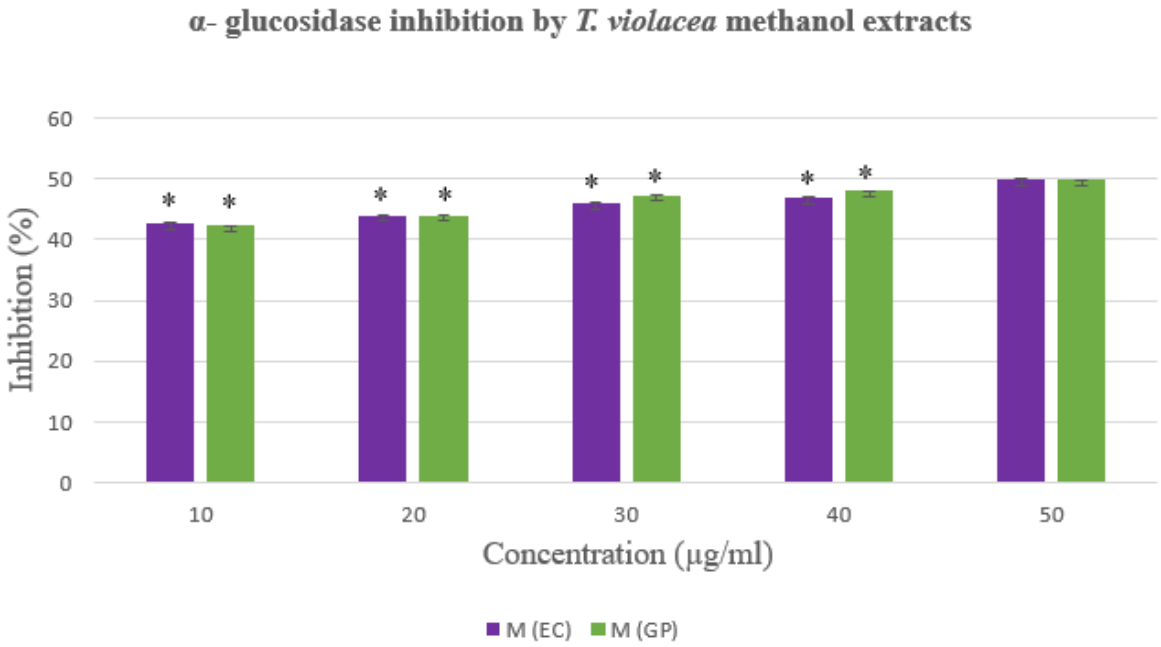


Figure 5.8: The α- glucosidase inhibition (%) of T. violacea methanol extracts. The asterisks (*) above the bars represents significant statistical difference; M-methanol, EC-Eastern Cape, GP-Gauteng Province

5.4 Discussion

5.4.1 The α- amylase inhibition assay

The role of amylase in the body is to break down carbohydrates or starch into maltose and polysaccharides. After carbohydrates are broken down they are absorbed and cause an increase in blood glucose levels (77, 78). Inhibiting carbohydrate digestion can be beneficial in controlling postprandial hyperglycaemia in diabetic individuals and this can be achieved by inhibiting α- amylase and α- glucosidase.

The results of the α- amylase inhibition assay, as depicted in figure 5.1, indicates that all extracts (hexane, acetone and methanol) of *T. violacea* from EC and GP have α- amylase inhibitory activity. The inhibition of α- amylase by *T. violacea* extracts and acarbose (standard) is concentration dependent. As the concentration of the extracts increased, its α- amylase inhibitory activity increased. All the extracts of *T. violacea* had good inhibition of α- amylase with the hexane extract from GP having the highest inhibitory activity of 85.667% at 50µg/ml, followed closely by the hexane extract from EC with 85.500% inhibition. This was then followed by the methanol extracts and the lowest inhibition was seen by the acetone extracts of *T. violacea* from EC and GP.

Interestingly, the only extracts which showed % inhibition of α- amylase lower than the standard acarbose was the acetone extracts of *T. violacea*. Both the hexane and methanol extracts of *T. violacea* from EC and GP showed greater α- amylase inhibition than the standard which showed 78.611% inhibition at 50 µg/ml. At the time of this study, no other studies on the α- amylase inhibitory effects of *T. violacea* had been conducted.

The graph depicted in figure 5.1 was used to determine the IC50 values of *T. violacea* plant extracts. The IC_{50} values for each extract and acarbose are depicted in table 5.1. Acarbose was found to have an IC_{50} value of 16.366 µg/ml. the IC_{50} values of all the *T. violacea* extracts from EC and GP were comparable to or better than the IC_{50} value of acarbose.

Statistical analysis was carried out on the results to check if there was a significant difference between the % α- amylase inhibition of *T. violacea* plant extracts and the standard acarbose.

It was observed that the majority of the *T. violacea* extracts from EC and GP at all concentrations, had an α- amylase inhibitory activity that was found to be statistically different to that of acarbose.

Figures 5.2, 5.3 and 5.4 compares the results of the EC samples vs the GP samples for hexane, acetone and methanol extracts of *T. violacea*. A t-test was used as the statistical test to compare the α- amylase inhibitory activity of the EC sample to the α- amylase inhibitory activity of the GP sample of *T. violacea*. With regard to the hexane extracts, the EC sample had a higher inhibitory effect than the GP sample at concentrations 10, 20, 30 and 40 µg/ml and at these concentrations their activity was significantly different. At concentration 50 µg/ml, their activity was comparable and was not found to be statistically different.

With regard to the acetone extracts, the EC sample had a higher inhibitory activity than the GP sample at concentrations 10, 20, 30 and 40µg/ml while at 50µg/ml, the GP sample had a greater inhibitory effect. Only at concentration 40µg/ml, were the results of the acetone extracts from EC and GP found not to be statistically significant.

With regard to the methanol extracts, at all concentrations, the results of the inhibition assay by the EC and GP samples of *T. violacea* were found to be statistically different. At concentrations 10 and 20µg/ ml, the EC sample showed a greater inhibitory effect while at concentrations 30, 40 and 50µg/ml, the GP sample showed a greater inhibitory effect.

The results of the α- amylase inhibition assay revealed that all extracts of *T. violacea* from EC and GP have an inhibitory effect that is comparable to or greater than that of acarbose. This shows that *T. violacea* does have anti-diabetic properties. This suggests that a person who uses *T. violacea* traditionally to treat diabetes mellitus may get a similar result compared to a person using acarbose. However, this would have to be confirmed through *in vivo* studies.

Although the results of the assay show good inhibitory activity by both the EC and GP samples, at most concentrations their inhibition was found to have significant statistical difference. This correlates with the results seen in chapters 3 and 4, in which differences in the phytochemical composition and antioxidant capacity of *T. violacea* extracts collected from EC and GP were noted.

5.4.2 The α- glucosidase inhibition assay

The results of the α- glucosidase inhibition assay, as depicted in figure 5.5, indicates that all extracts (hexane, acetone and methanol) of *T. violacea* from EC and GP have α- glucosidase inhibitory activity. It can also be seen that inhibition of α- glucosidase by *T. violacea* extracts and acarbose (standard) is concentration dependent. As the concentration of the extracts

increased, its α- glucosidase inhibitory activity increased. All the extracts of *T. violacea* had good inhibition of α- glucosidase with the acetone extract from the EC having the greatest inhibitory activity of 60.01% at 50 µg/ml, followed by the acetone extract from GP with 55.273% inhibition at the same concentration. This was then followed by the methanol extracts and the lowest % inhibition was seen by the hexane extracts. All of the extracts of *T. violacea* except the hexane extract from EC, showed a higher % inhibition compared to acarbose, which showed 47.434% inhibition at 50 µg/ml.

The graph depicted in figure 5.5 was used to determine the IC50 values of *T. violacea* plant extracts. The IC_{50} values for each extract and acarbose are depicted in table 5.2. Acarbose was found to have an IC_{50} value of 45.609 µg/ml. The IC_{50} values of all the *T. violacea* extracts from EC and GP were better than the IC_{50} value of acarbose, as seen in table 5.2.

Statistical analysis was carried out on the results to check if there was a significant difference between the α- glucosidase inhibitory activity of *T. violacea* plant extracts and the standard acarbose. It was observed that the majority of the *T. violacea* extracts from EC and GP at all concentrations, had an α- glucosidase inhibitory activity that was found to be statistically different to that of acarbose.

Figures 5.6, 5.7 and 5.8 compares the results of the EC samples vs the GP samples for hexane, acetone and methanol extracts of *T. violacea*. A t-test was used as the statistical test to compare the α- glucosidase inhibitory activity of the EC sample to the α- glucosidase inhibitory activity of the GP sample of *T. violacea*. Significant statistical difference was accepted when $p < 0.05$ and the asterisks (*) above the bars represent this difference.

With regard to the hexane extracts, the GP sample had a higher α- glucosidase inhibitory than that of the EC sample. However, significant statistical difference was only found for the results at concentrations 10, 40 and 50 µg/ml.

With regard to the acetone extracts, the EC sample had a higher inhibitory effect than the GP sample at all concentrations and significant statistical difference in the results were seen at all concentrations.

With regard to the methanol extracts, the EC sample had a higher inhibitory effect overall, compared to the GP sample. Significant statistical difference was seen for concentrations 10-40 µg/ml but no statistical difference was noted for the results at 50 µg/ml.

The results of the α- glucosidase inhibition assay revealed that all extracts of *T. violacea* from EC and GP have an inhibitory effect that is comparable to or greater than that of acarbose. This shows that *T. violacea* does have anti-diabetic properties. A person who uses *T. violacea* traditionally to treat diabetes mellitus may get a similar result compared to a person using acarbose. However, this would have to be confirmed through in vivo studies.

Medicinal plants have the ability to reduce blood glucose levels due to the presence of phytochemicals such as flavonoids, saponins, alkaloids, tannins, glycosides and terpenes (142). Chapter 3 showed that *T. violacea* possesses saponins, tannins, alkaloids, flavonoids and phenolic compounds, all which are associated with the anti-diabetic properties of medicinal plants and may the reason for the α-amylase and α-glucosidase inhibitory effects of *T. violacea*.

At the time of this study, no research on the α- amylase and α- glucosidase inhibitory activities of *T. violacea* had been reported. However, a study on streptozotocin diabetes- induced rat models, found that *T. violacea* rhizome methanolic extracts improved body weights, significantly reduced fasting blood glucose levels, improved glucose tolerance and significantly increased plasma insulin and liver glycogen content (143). These results of the study were verified by another study in which *T. violacea* extracts reduced blood glucose and increased plasma insulin. However, the study also stated that further work is needed to identify the mechanism of *T. violacea* anti-diabetic effects (144).

In another study, *T. violacea* was reported to improve glucose- stimulated insulin secretion in INS-1 pancreatic beta cells and glucose uptake in Chang liver cells (145).

The results of the previous studies on *T. violacea* and of this study, shows potential for follow up testing towards new drugs for the management of diabetes, derived from *T. violacea*.

5.5 Chapter summary

The results of this chapter revealed that all extracts (hexane, acetone and methanol) of *T. violacea* from EC and GP have potential anti-diabetic properties. All extracts were able to inhibit α- amylase and α- glucosidase in a concentration dependent manner and showed inhibitory activity comparable to or better than the standard acarbose. This justifies the use of *T. violacea* in the management of diabetes mellitus.

Although the samples from EC and GP showed similar activity, significant statistical difference was seen in their results.

CHAPTER 6

Determination of anti-Alzheimer activity of various solvent extracts of *T. violacea* samples from the Eastern Cape and Gauteng

6.1 Introduction

According to the World Health Organisation, there are currently more than 55 million people who have dementia worldwide, of which 60% live in low to middle income countries. Alzheimer's disease is the most common cause of dementia worldwide and accounts for 60-70% of all dementia cases (146). Alzheimer's disease is a progressive form of dementia of unknown cause and it is characterised by progressive neurodegeneration of the central nervous system (CNS) leading to a decline in cognitive function (98). The changes in cognitive function as seen in Alzheimer's disease usually begins with memory impairment and progresses to defects in language and executive function (101) . In the early stages of Alzheimer's disease, the symptoms may go unnoticed or are attributed to old age however, eventually they begin to interfere with daily activities.

The pathological findings of Alzheimer's disease include intracellular neurofibrillary tangles, degeneration of neurons, cortical atrophy and extracellular amyloid plaques (98).

Currently there is no cure for Alzheimer's disease but drugs such as acetylycholinesterase (AChE) inhibitors and memantine exist to manage symptoms of Alzheimer's disease (111, 147). By managing the symptoms of Alzheimer's disease, the goal of therapy is to retain patient quality of life and reduce long term clinical decline.

Medicinal plants contain a large variety of phytochemicals which have proven biological effects. The screening of medicinal plants for anti-Alzheimer activity has increased over the past few years in an attempt to find new drug molecules for the management of Alzheimer's disease.

6.2 Method and materials

Acetylcholinesterase inhibition assay

The AChE inhibition assay was carried out using the Ellman's method with slight modifications (148, 149). The acetylcholinesterase enzyme hydrolyses the substrate acetylcholine to thiocholine. Thiocholine reacts with the Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid, DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm (150).

In a 96- well plate, 150 µl of Tris-HCL (50 mM, pH= 8), 20 µl DTNB (10 mM) and 80 µl *T. violacea* plant extracts (10, 20, 30, 40 and 50 µg/ml) were added. Then 10 µl acetylcholinesterase enzyme (6.67 IU/ml) was added and the reaction mixture was incubated at 37^{o}C for 15 minutes. After incubation, 10 µl acetylthiocholine iodide was added to each well and absorbance was measured at 412 nm using a SpectraMax M3 multi-mode plate reader (California, USA). Donepezil (10, 20, 30, 40 and 50 µg/ml) was used as the control standard. The experiment was carried out in triplicate and the AChE inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(Abs\ control - Abs\ sample)}{Abs\ control}\ X\ 100\%$$

Where: Abs_{sample} is the absorbance of the sample (plant extracts) measured at 412 nm

$Abs_{control}$ is the absorbance of the control measured at 412 nm

Microsoft Excel and GraphPad Prism 10 were used to plot the graphs and calculate the IC50 values. Calculation of the IC50 values was done using the linear regression method where the linear equation was drawn to best represent the data points obtained from each of the concentrations for each extract. A t-test was used as the statistical test to compare the AChE inhibitory activity of the EC sample to the AChE inhibitory activity of the GP sample of *T. violacea*. Significant statistical difference was accepted when $p < 0.05$.

6.3 Results

Acetylcholinesterase inhibition assay

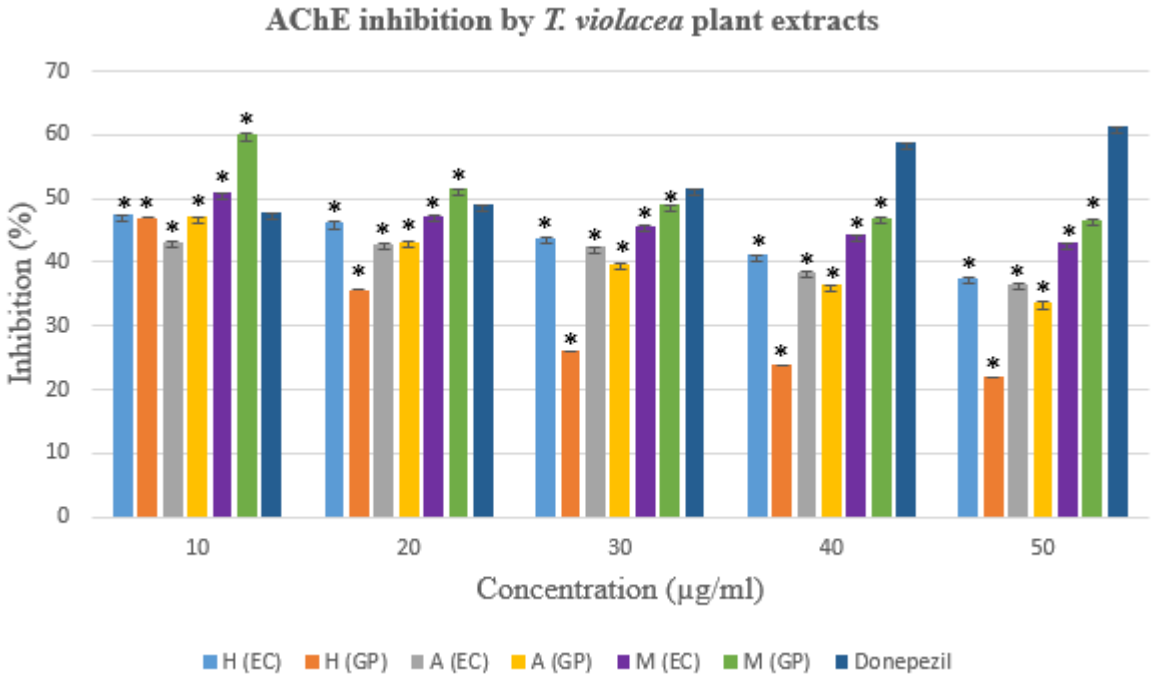


Figure 6.1: AChE inhibition (%) of T. violacea extracts (EC and GP) and donepezil. The asterisks (*) above the bars represent significant statistical difference between the test compound and donepezil; H-hexane, A-acetone, M-methanol, EC-Eastern Cape, GP-Gauteng Province

Table 6.1: IC_{50} values of various extracts of T. violacea (EC and GP) and the standard donepezil

Extracts and standard	**IC_{50} value (µg/ml)**		
	T.violacea		**Donepezil**
	EC	**GP**	
Hexane	54.410	92.736	
Acetone	76.842	74.657	
Methanol	66.540	60.560	
Donepezil			52.177

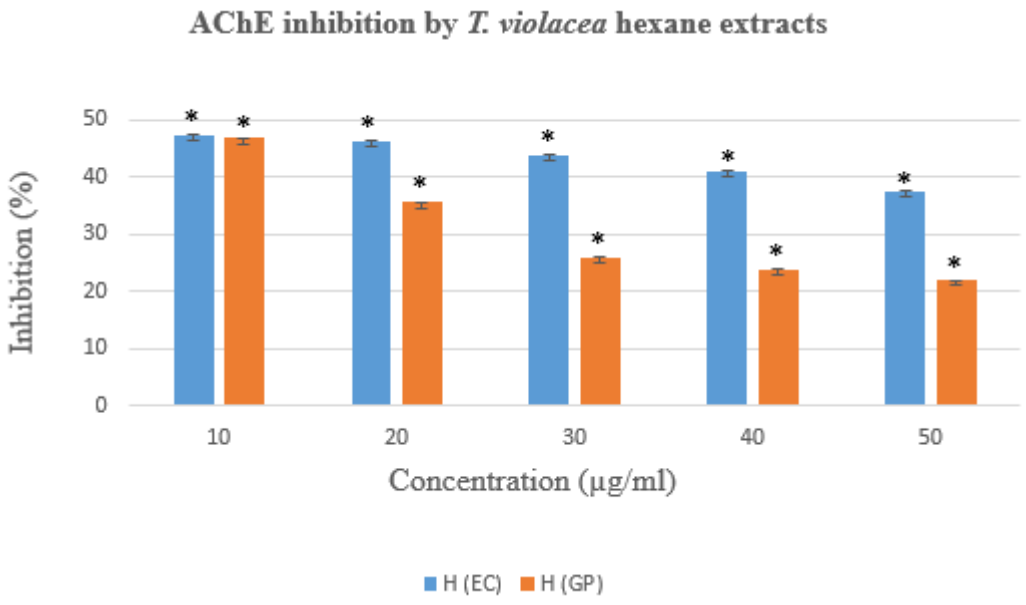


Figure 6.2: AChE inhibition (%) of T. violacea hexane extracts. The asterisks (*) above the bars represents significant statistical difference; H-hexane, EC-Eastern Cape, GP-Gauteng Province

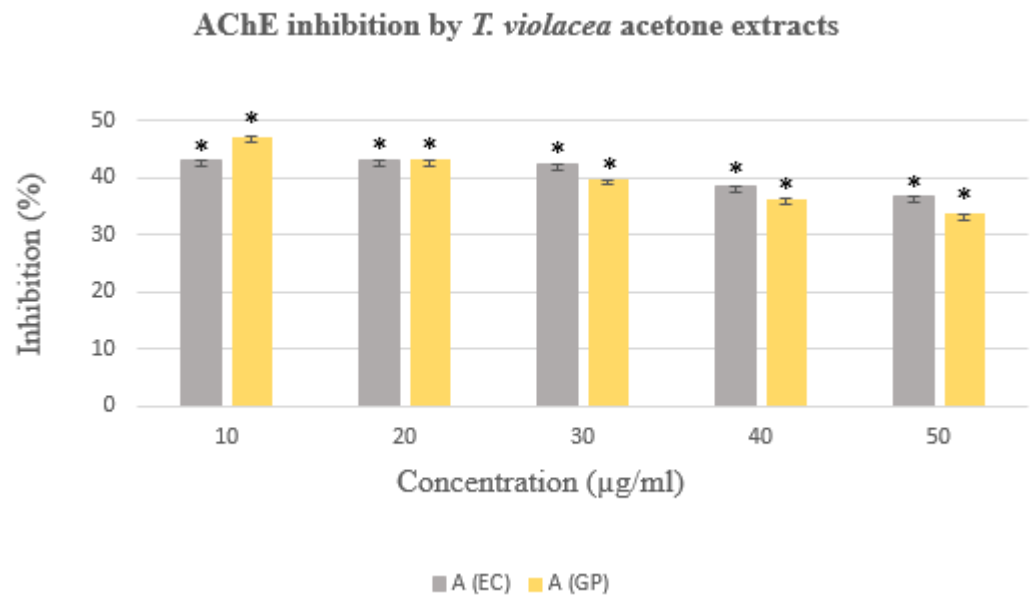


Figure 6.3: AChE inhibition (%) of T. violacea acetone extracts. The asterisks (*) above the bars represents significant statistical difference; A-acetone, EC-Eastern Cape, GP-Gauteng Province

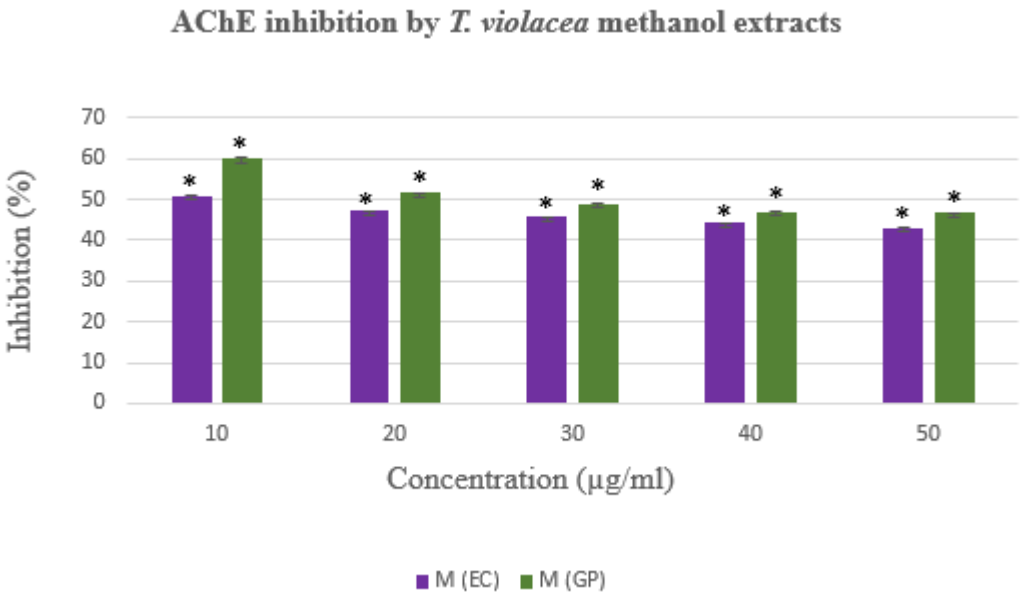


Figure 6.4: AChE inhibition (%) of T. violacea methanol extracts. The asterisks (*) above the bars represents significant statistical difference; M-methanol, EC-Eastern Cape, GP-Gauteng Province

6.4 Discussion

The cholinergic hypothesis is one of the most common hypotheses in the pathologic processes involved in Alzheimer's disease (109). It has been suggested that acetylcholine (ACh) is critical for the response to uncertainty and reinforces neuronal loops during learning. In the brain, ACh is involved in memory, motivation, arousal and attention. The cholinergic hypothesis suggests that a dysfunction in ACh containing neurons in the brain contributes to the cognitive decline seen in Alzheimer's disease (109). Attempts at correcting ACh deficiency in the brain is used for symptomatic treatment of Alzheimer's disease by using acetylcholinesterase (AChE) inhibitors.

The results of the AChE inhibition assay, depicted in figure 6.1, indicated that all extracts (hexane, acetone and methanol) of *T. violacea* from EC and GP had AChE inhibitory activity. The inhibition of AChE by donepezil (figure 6.1), was concentration dependent. As the concentration of donepezil increased, its inhibitory effect increased. However, with all extracts of *T. violacea* from EC and GP, as concentration increased, the inhibitory effect decreased. This means that the lower the concentration of *T. violacea* extracts, the greater its inhibitory effect, and an increase in its concentration results in a lower inhibition of AChE.

The highest inhibitory effect of AChE by *T. violacea* was observed from the methanol extracts. At a concentration of 10 µg/ml, the GP sample inhibited 60.123% of the AChE enzyme and the EC sample inhibited 50.864%. This was in comparison to donepezil which had an inhibition of 47.778% at 10 µg/ml.

With the 10 µg/ml concentration, the lowest inhibitory effects were observed from the acetone extracts of *T. violacea* from both samples of the EC and GP. However, as concentration of the extracts increased, the biggest drop in inhibitory effect was exerted by the hexane extract of *T. violacea* from GP with 22.025% inhibition of AChE at 50 µg/ml.

The graph depicted in figure 6.1 was used to determine the IC50 values of *T. violacea* plant extracts. The IC_{50} values for each extract are depicted in table 6.1. Donepezil was found to have an IC_{50} value of 52.177 µg/ml and from all of the *T. violacea* plant extracts, the hexane extract from the EC sample had the closest IC_{50} value to donepezil of 54.41 µg/ml.

Statistical analysis was carried out on the results to check if there was a significant difference between the AChE inhibitory activity of *T. violacea* plant extracts and the standard donepezil. All extracts of *T. violacea* from EC and GP, at all concentrations, had an AChE inhibitory

activity that was statistically different to the AChE inhibitory activity of donepezil at the same concentrations.

Figures 6.2, 6.3 and 6.4 compares the results of the EC samples vs the GP samples for hexane, methanol and acetone extracts of *T. violacea*, respectively. From figures 6.2, 6.3 and 6.4 it can be seen that the hexane, acetone and methanol extracts of *T. violacea* from the EC sample had an AChE inhibitory activity that was significantly different to the AChE inhibitory activity of the GP sample.

With regard to the hexane extracts, the EC sample had a greater AChE inhibitory effect compared to the GP sample at all concentrations. The highest % inhibition was noted for the EC sample at 10 µg/ml with a % inhibition of 47.407%. At concentration 50 µg/ml, the EC sample had 37.531% inhibition while the GP sample had 22.025% inhibition of AChE.

With regard to the acetone extracts, the GP sample had a higher % inhibition at 10 and 20 µg/ml but at concentrations 30-50 µg/ml, the EC sample showed greater inhibition. The highest AChE inhibitory activity for the acetone samples was by the GP sample at 10 µg/ml, with 47.160% inhibition while the EC sample had 43.210% inhibition at the same concentration.

With regard to the methanol extracts, the GP sample showed a higher % inhibition of AChE than the EC sample, at all concentrations. At 10 µg/ml, the GP sample resulted in 60.123% AChE inhibition while the EC sample had 50.864% inhibition.

At all concentrations, significant statistical difference was noted for the hexane, acetone and methanol extracts of *T. violacea* with regard to its AChE inhibitory effects. The differences in its activity could be due to the differences that were found in chapter 3 with regard to its phytochemical content as the phytochemicals present in a plant are responsible for its pharmacological effects.

At 50 µg/ml, the standard donepezil had an AChE inhibition of 61.235%. At this concentration, none of the *T. violacea* plant extracts from EC or GP had a % inhibition that was comparable. However, at 10 µg/ml, the methanol extract of *T. violacea* collected from GP, had a % inhibition of 60.123%, indicating that *T. violacea* methanolic extract has potential to have similar AChE inhibitory activity comparable to donepezil, at a different concentration. Further research would be needed in this area to assess why a lower concentration of *T. violacea* plant extracts were required to produce a similar activity to donepezil and why the AChE inhibition activity of *T. violacea* decreased with an increase in concentration.

At the time of this study, no data on the AChE inhibition potential of *T. violacea* extracts could be found and very few studies on the anti-Alzheimer properties of *T. violacea* extracts had been published.

An *in-vivo* Alzheimer's disease assay was conducted in *Caenorhabditis elegans* by Rivas-Garcia et al. The results of this study found that *T. violacea* extracts led to a reduction in the 1-42 beta amyloid peptide formation and prevented oxidative stress (151).

The results of the study by Rivas-Garcia and the results of this study indicates that *T. violacea* plant extracts have anti-Alzheimer properties and there is potential towards the discovery of new drugs in the management of Alzheimer's disease, from *T. violacea*. More research is needed to determine the mechanisms by which *T. violacea* works to manage Alzheimer's disease.

6.5 Chapter summary

This chapter revealed that the leaf extracts of *T. violacea* from EC and GP have anti-Alzheimer properties by inhibiting AChE. Donepezil was found to inhibit AChE in a concentration dependent manner (as concentration increased, inhibition of AChE increased). However, as concentration of the *T. violacea* plant extracts increased, its inhibition of AChE decreased. This means that a very small quantity of *T. violacea* is required to inhibit AChE.

Traditionally, most medicinal plants are used in water (a polar solvent) and the methanol (polar) extracts of *T. violacea* showed the highest AChE inhibition. This means that when used in a traditional manner, *T. violacea* plant extracts could have good AChE inhibition.

Although the samples from EC and GP showed similar activity, significant statistical difference was seen in their results.

CHAPTER 7

Overall summary of the study, conclusions, limitations and recommendations

7.1 Overall summary

The qualitative phytochemical screening of *T. violacea* leaf extracts revealed that the extracts of *T. violacea* samples from both EC and GP contain phytochemical compounds including saponins, flavonoids, tannins, alkaloids, steroids, cardiac glycosides and phenolics. The results also showed that while the phytochemicals were present in both the EC and GP samples, the strength of their presence varied. Saponins, alkaloids, steroids and phenolics had a greater presence in the EC sample while cardiac glycosides had a greater presence in the GP sample of *T. violacea*. However, an exact difference could not be determined from a qualitative analysis.

A quantitative analysis on the phytochemicals present in *T. violacea* was carried out by means of a total phenolic content test. The results of this test revealed that the hexane, acetone and methanol extracts of *T. violacea* from EC and GP contained phenolic compounds. It was found that the methanol extract of *T. violacea* from EC had the highest TPC and significant statistical difference in TPC was seen between the EC and GP samples for their hexane, acetone and methanol extracts.

The results of the TLC confirmed the presence of a variety of phytochemicals in *T. violacea* samples from EC and GP, which correlate with the results of the qualitative phytochemical analysis.

The dot-plot and DPPH radical scavenging assay revealed that *T. violacea* leaf extracts have radical scavenging activity. The results of the dot-plot showed that the hexane, acetone and methanol extracts from the EC and GP samples have radical scavenging activity as they discoloured the purple DPPH to yellow. The hexane extracts of *T. violacea* discoloured the purple DPPH the most, followed by the methanol extract of *T. violacea* from EC. However, a conclusion could not be made only from a qualitative test hence the need for the quantitative DPPH radical scavenging assay.

The results of the DPPH radical scavenging assay revealed that all extracts of *T. violacea* from EC and GP had antioxidant capacity, correlating with the results of the dot-plot. It also showed that the radical scavenging activity *T. violacea* leaf extracts was concentration dependent and that at each concentration its radical scavenging activity was statistically different to the DPPH radical scavenging activity of ascorbic acid. The results of the assay showed that the EC sample had a DPPH radical scavenging activity that was significantly different to the DPPH radical scavenging activity of the GP sample. The hexane and acetone extracts of the EC sample had a higher DPPH radical scavenging activity compared to the GP sample, while the methanol extract of the GP sample had a higher DPPH radical scavenging activity compared to the EC sample.

The results of the anti-diabetic assays proved that *T. violacea* leaf extracts have anti-diabetic properties. The hexane, acetone and methanol extracts of *T. violacea* from EC and GP were able to inhibit α-amylase and α-glucosidase and its inhibition was in a concentration dependent manner. The α-amylase and α-glucosidase inhibitory effects of *T. violacea* were comparable to or greater than the inhibitory effects of acarbose. Although the plant samples from EC and GP had similar activity, there was a significant statistical difference between their results.

With regard to the α-amylase inhibition assay, *T. violacea* collected from EC had better inhibitory activity than *T. violacea* collected from GP, at most concentrations.

With regard to the α-glucosidase inhibition assay, at most concentrations, the sample of *T. violacea* from EC showed better inhibitory activity than the GP sample.

Since all the extracts exhibited inhibition on amylase and glucosidase, indicates the great potential of this plant towards the search for anti-diabetic compounds, and is a strong motivation for future research.

The results of the acetylcholinesterase inhibition assay proved that the leaf extracts of *T. violacea* have anti-Alzheimer properties, by inhibiting AChE. Donepezil was found to inhibit AChE in a concentration dependent manner. However, as the concentration of the T. violacea leaf extracts increased, its % AChE inhibition decreased. This was found to be true for the samples from both EC and GP.

Interestingly, the results of the AChE inhibition assay showed that the acetone and methanol extracts of *T. violacea* from GP had a higher % inhibition compared to the EC sample. It was

also found that there was significant statistical difference between the EC and GP samples of *T. violacea*, for all the plant extracts (hexane, acetone and methanol).

Since all the extracts exhibited inhibition on AChE, indicates the potential of this plant towards the search for anti-Alzheimer compounds, and is a motivation for future research.

7.2 Conclusion

The use of medicinal plants for the treatment of diseases (communicable and non-communicable) has been practised for decades and research into medicinal plants is becoming more popular as scientists try to find new drug candidates for the treatment of various diseases.

T. violacea is a plant native to Africa which has many uses in traditional medicine. It has also been stated that the type and/or quantity of phytochemicals present in a plant may be affected by geographical location, seasonal variations and other environmental factors. This study investigated the impact of geographical location on the phytochemical composition and pharmacological activities of *T. violacea* leaf extracts collected from EC and GP.

The results of the study found that *T. violacea* from EC and GP contain a variety of phytochemicals and has antioxidant, anti-diabetic and anti-Alzheimer properties.

The results of the preliminary phytochemical analysis found the presence of saponins, flavonoids, tannins, alkaloids, steroids, cardiac glycosides and phenolic compounds present in *T. violacea* collected from EC and GP. The results of the TLC confirmed these results by the appearance of multiple bands on each TLC plate.

The total phenolic content test revealed that all extracts (hexane, acetone and methanol) of *T. violacea* contained phenolic compounds with the methanol extracts having the greatest quantity of phenolic compounds from all of the extracts.

The dot-plot and DPPH radical scavenging activity assay confirmed the antioxidant potential of *T. violacea* that were mentioned in previous studies. All of the extracts of *T. violacea* had a DPPH radical scavenging activity that was concentration dependent.

This study found that *T. violacea* inhibited α- amylase and α- glucosidase in a concentration dependent manner and that its inhibition was comparable to or greater than that of the standard acarbose. This indicates great potential of *T. violacea* towards the search for new anti-diabetic compounds and is motivation for future research.

With regard to anti-Alzheimer properties, *T. violacea* leaf extracts inhibited AChE. Interestingly, as the concentration of the extracts increased, the AChE inhibitory activity decreased. Further research would need to be conducted to determine the reason for this as well as determining other pathways in which *T. violacea* may inhibit the pathological processes involved in Alzheimer's disease.

Although the results of the study found that *T. violacea* from EC and GP contain a variety of phytochemicals and has antioxidant, anti-diabetic and anti-Alzheimer properties, significant statistical differences in the results between the EC sample and the GP sample of *T. violacea* were observed across all of the tests. This proved that geographical location does play a role in the type (quality) and/or quantity of phytochemicals present in a plant, which affects its pharmacological activities. This is important as it suggests that people using *T.violacea* as traditional or herbal medicine in one province may experience different pharmacological effects compared to a person using the same plant collected from a different province. More research is needed in this area to assess the impact of seasonal variations and other environmental factors on the phytochemical composition of plants.

7.3 Limitations of the study

- In this study, only the leaves of *T. violacea* collected from EC and GP were used. The impact of geographical location on the phytochemical composition and pharmacological activities of the other plant organs (roots, stems and flowers) could not be determined.
- The impact of seasonal variations on the phytochemical composition and pharmacological activities of *T. violacea* was not assessed as the leaves of *T. violacea* from EC and GP were harvested in the same season, as to not have multiple variables affecting the results.

7.4 Recommendations

- Studies on the impact of geographical location on the phytochemical composition and pharmacological activities of *T. violacea* roots, stems and flowers can be carried out.

- Studies on *T. violacea* leaves collected in different seasons could be carried out to determine the impact of seasonal variations on the phytochemical composition and pharmacological properties of *T. violacea*.
- Further studies including isolation of the compounds present in *T. violacea* could be carried out.
- At the time of this study, very few studies on the anti-diabetic properties of *T. violacea* had been conducted, hence more research is needed in this area to determine other pathways in which *T. violacea* may act to control blood glucose levels.
- At the time of this study, there were no reports on the AChE inhibitory potential of *T. violacea*. Therefore the current study is the first to report on this activity. Further anti-Alzheimer assays on *T. violacea* can be carried out to determine whether other pathways in the pathophysiology of Alzheimer's disease may be affected by *T. violacea*.

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APPENDIX A

Rhodes University Approval Letter

RHODES UNIVERSITY
Where leaders learn

Faculty of Pharmacy
Artillery Road, Makhanda, 6139
PO Box 94 Makhanda 6140
South Africa
Tel: +27 (0) 46 603 8589
Fax: +27 (0) 46 603 7566
E-mail: dean.pharmacy@ru.ac.za

www.ru.ac.za

08/12/2021

RESEARCH PROPOSAL REVIEW OUTCOME

Title: Investigation of the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *Tulbaghia violacea* collected from the Eastern Cape and Kwazulu-Natal
Researcher: Tasmeera Kader (17K5953)
Supervisor: Prof M Mothibe
Co-supervisor: Dr N Sibiya
Degree: MSc Pharmacy

Dear Researcher

Your proposal was tabled at the meeting of the Division of Pharmacology for HDC.

The Committee had made specific comments to be considered and recommended that minor corrections be addressed to the satisfaction of the supervisor.

This letter serves to inform you that after review, the committee has APPROVED your revised proposal.

Sincerely

Chairperson of committee

Prof ME Mothibe
Associate Professor and HOD: Pharmacology
Rhodes University

APPENDIX B

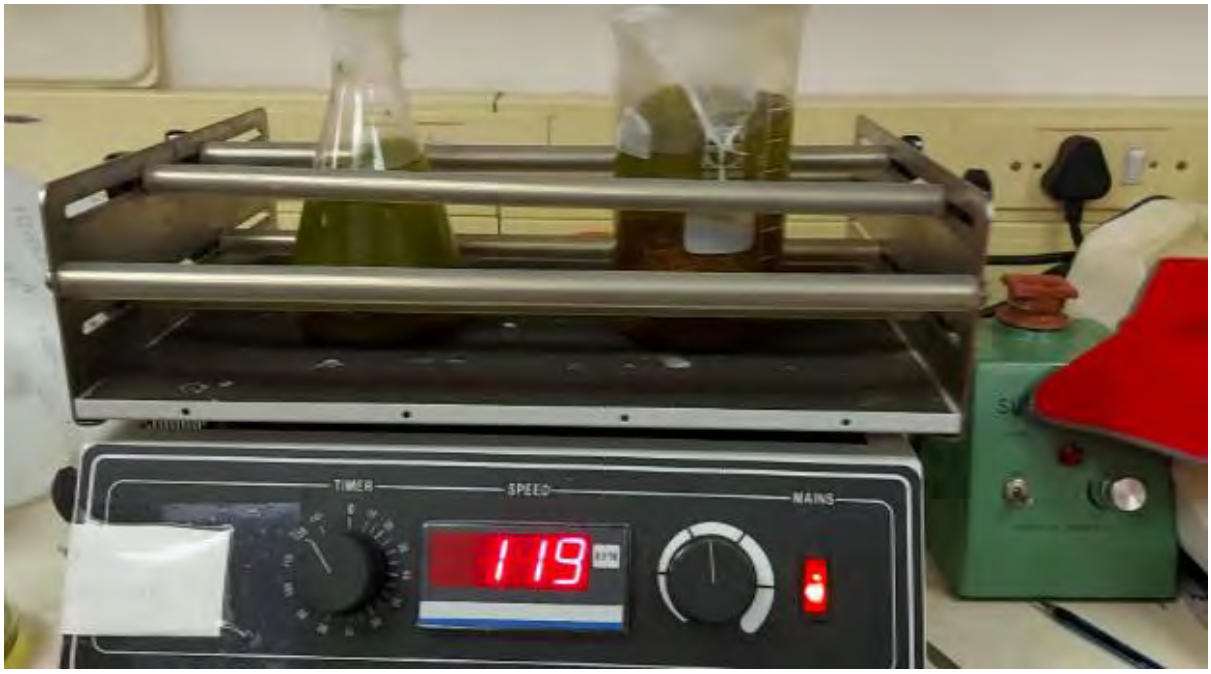
Plant preparation and processing

APPENDIX C

Plant preparation (drying process)

APPENDIX D

Extraction process

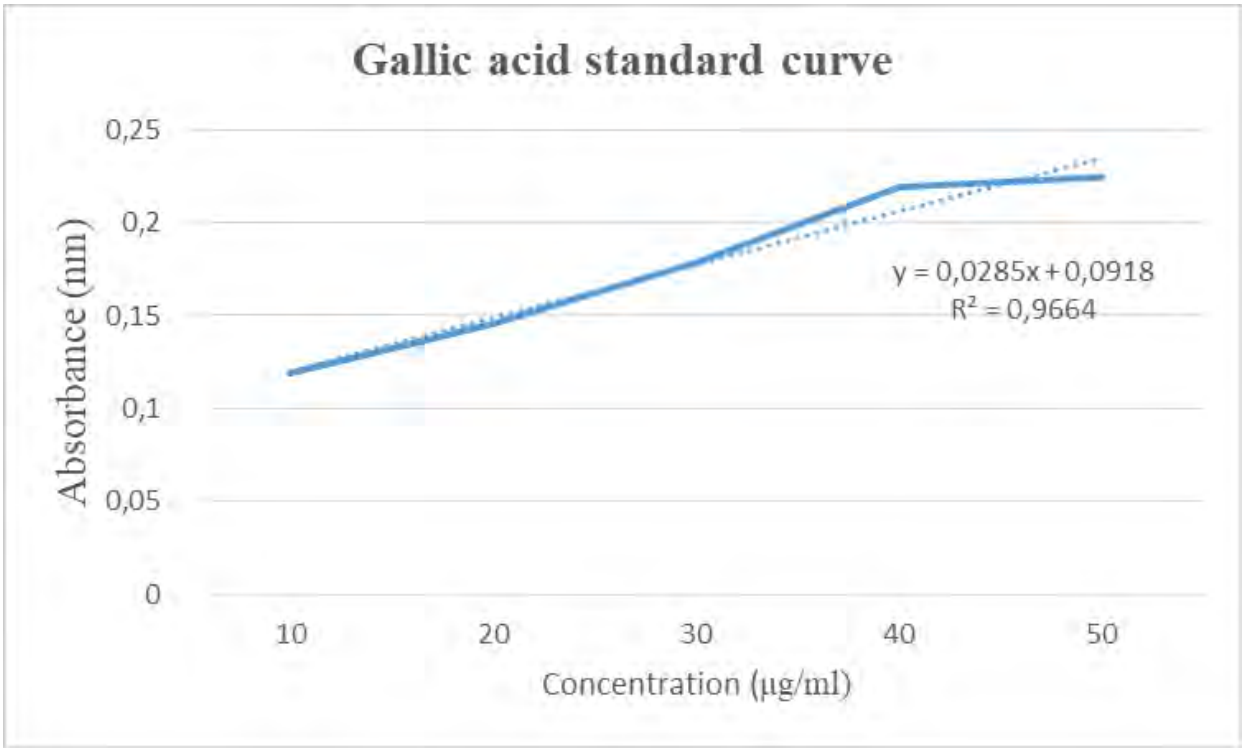


APPENDIX E

Extraction process continued

APPENDIX F

Gallic acid standard curve used to calculate Total phenol content of *T. violacea* plant extracts



APPENDIX G

Abstract from the 2021 virtual Postgraduate conference at Rhodes University

Miss Tasmeera Kader

- M.Sc. Candidate

An investigation into the impact of geographical location on the phytochemical composition, pharmacological and toxicological activities of *Tulboghia violacea* collected from the Eastern Cape and Kwazulu-Natal

Tasmeera Kader, Professor ME. Mothibe, Dr. N. Sibiya

Pharmacology

Traditional medicine refers to the knowledge, skills and practices which are based on the beliefs and experiences indigenous to cultures and is used to maintain health [1]. Over the years, disease rates have been increasing which has resulted in scientists looking at natural products and traditional medicine to find new drug molecules [2]. *Tulbaghia violacea* is a plant native to Africa and is readily available in the Eastern Cape of South Africa [3-5]. It is popular for its antimicrobial, antifungal, anticoagulant, anti-oxidant and anticancer properties [3-5]. Traditionally, it is used for the treatment of various conditions such as constipation, wounds, colds, fever, ulcers and tumours [3-5]. Ecological factors have been said to influence the composition and amount of phytochemicals present in a plant [6]. This study aims to identify phytochemicals present in *Tulbaghia violacea* and investigate if these phytochemicals possess antioxidant, anti-diabetic and anti-Alzheimer activities *in-vitro*. It also aims to identify potential differences in pharmacological activity depending on the growing environment of the plant. This will be done by comparing samples obtained from two provinces in South Africa, viz. KwaZulu- Natal and the Eastern Cape.

Keywords: *Tulbaghia violacea*, phytochemicals, assay, anti-diabetic, anti-Alzheimer

Figure 1: Tulbaghia violacea

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[illegible]

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APPENDIX H

Abstract from the 2022 Postgraudate Research Day, hosted by the Faculty of Pharmacy at Rhodes University

Tasmeera Kader

- *MSP candidate*

THE HISTORY OF TRADITIONAL MEDICINE

Tasmeera Kader, ME. Mothibe, Dr. N. Sibiya

Pharmacology Division

According to the World health organisation (WHO), traditional medicine refers to the knowledge, skills and practices which are based on the beliefs and experiences indigenous to cultures and is used to maintain health. For centuries, people have and in many parts of the world, still do rely on traditional medicine for their health needs. When it is adopted outside of its traditional culture, it is often referred to as complementary or alternative medicine and the most widely used traditional medicine systems used today include those from China, India and Africa among others. The WHO states that trends in the use of traditional medicine and complementary medicines have been increasing. It has been stated that in Africa, Asia, Latin America and the Middle east, 75-90% of the population still uses traditional medicine. It has also been purported that nearly one quarter of all modern medicine is derived from natural products of which many were originally used in traditional medicine.

APPENDIX I

Certificate of participation at the 2023 conference of The South African Society for Basic and Clinical Pharmacology at Rhodes University

Certificate of Participation

This is to confirm that

Ms Jasmeera Kader

Has attended the 2023 Conference
of
The South African Society for Basic and Clinical Pharmacology
at Rhodes University
on the 24th - 27th August 2023
(accredited for 18 CPD points)

ANNUAL CONFERENCE 56th

"Pharmacology celebrating resilience: Current and Future strategies"

Prof Mamza Mothibe
Chairperson: 2023 Organising Committee

Ms Tamusha Dukhan
Secretary: 2023 Organising Committee