THE MEDICINAL PROPERTIES OF IPOMOEA OBLONGATA E.Mey. ex Choisy

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DECLARATION OF INDEPENDENT WORK

I, Ketlareng Liza Polori, identity number and student number , do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work, complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State, and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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SUMMARY

Traditional medicine has been known by mankind since ancient times as a healthcare system. All cultures have used herbs throughout history and it was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. Plants provided food, clothes, shelter and medicine and still play a vital role in rural villages of South Africa. The medicinal uses of plants and animals have been developed through trial and error. Herbal plants produce and contain a wide variety of chemical substances that act upon the body's immune system.

The medicinal properties of *Ipomoea oblongata* (E.Mey.ex Choisy) in this study was to determine the phytochemical content, antioxidant, anti-cancer and anti-diabetic properties of *I. oblongata* (Mothokho), a medicinal plant used by traditional medical practitioners of Thaba-Nchu in the Free State. The study was carried out in order to validate the ethnomedicinal claims made by the traditional medical practitioners and to evaluate the plant's potential as a novel therapeutic agent.

Twelve traditional medical practitioners of the Kopanang Dingaka Association were interviewed on the knowledge and use of *I. oblongata* (Mothokho). The phytochemical constituents of the plant were determined using standard screening methods. Testing for antioxidant properties (free radical scavenging activity) was carried out by means of the 2, 2- diphenylpicrylhydrazyl (DPPH) assays. The Sulforhodamine B assay was used to screen for anti-cancer activity in breast (MCF7), colon (HCT116) and prostate (PC3) cancer cell lines. Glucose uptake in C2C12 muscle cells was used to evaluate the anti-

diabetic potential of *I. oblongata*. Methanol extract was fractionated using Ultra-Pure Liquid Chromatography (UPLC) to identify active compounds.

The traditional medical practitioners cited the plant roots as the main ingredient in the treatment of respiratory infections, sexually transmitted infections, postnatal womb treatments, inflammation, arthritis, wounds and cancer. The methanol extract of *I. oblongata* showed remarkable (99.03%, 98.39, 71.31%) antioxidant potential in all triplicates tested which explains its use in oxidative stress-related diseases such as arthritis and cancer. Phytochemical tests showed the presence of carbohydrates, glycosides, steroids, terpenoids, alkaloids, flavonoids and tannins. However, the extracts were inactive against the cancer cell lines used. Glucose uptake by the C2C12 muscle cells was increased by over 150% and was comparable to that of insulin and metformin, suggesting good anti-diabetic activity. Predicted compounds found were dihydroquercetin pentaacetate, actinorhodin and actinorhodine, using Chemspider analysis. These are possible compounds that could be found in the *Ipomoea oblongata* extracts when using nuclear magnetic resonance (NMR) to determine the structure and names of the active compounds.

Ethnobotanical uses of *Ipomoea oblongata* were found to have a link with other ethnobotanical studies to capture the indigenous knowledge, culture and therapeutic uses. The phytochemical results of *Ipomoea oblongata* can contribute to the knowledge of new drug development as indicated by literature (Harbone; 1973, Soforowa, 1993; De *et al*., 2010) that plants contain phytochemical contents and phenolic compounds that are valuable as medicinal properties. Methanolic extract of *Ipomoea oblongata* presented high levels of antioxidant activity respectively, which can become a potential

antimicrobial agent and remedy oxidative stress related diseases. The anticancer properties were inactive and the cytotoxicity levels were found to below in *I. oblongata.* Low levels of cytotoxicity allow medicinal plants to be consumed by humans with fewer side effects however doses must be standardized. High levels of glucose uptake in muscle cells were indicated from the aqueous extract of *Ipomoea oblongata,* therefore showing great potential as an antidiabetic agent. Many medicinal plants have been validated as antidiabetic therapeutic agents in South Africa and the world. New treatment of cancer, diabetes, bacterial and viral infections is required from natural products that are cost effective with minimal side effects that can be used in health care systems.

There is a correlation between the medicinal properties found in *I. oblongata* and the ethnomedicinal uses cited by the traditional medical practitioners. The plant *I. oblongata* has six ethno-pharmaceutical uses*. I. oblongata* is a good source of anti-diabetic and antioxidant agents that can be developed further. However the plant is not a good source of anticancer properties. Future research will be into the isolation and identification of the active compounds.

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DEDICATION

This work is dedicated to the Father Almighty (man upstairs), my two children, Kabelo and Kagiso, and husband, Phegelelo, for their support and understanding through this, the most difficult journey of my entire career. I could not have completed this course if it had not been for their love and constant reminders of the bigger picture.

"Oh that You bless me indeed, and enlarge my territory, and let Your hand be with me and that You keep me away from evil."

1 Chronicles 4:9-10

Thank you.

Appendix A: Questionnaires

Interview guide

Interviewee's details

1. Age
2. Gender
3. Profession
4. Level of schooling

Use

5. Do you know this plant?
6. What are its names?
7. Do you use it?
8. What do you use it for?
    a. If also used for animals, which animals?
    b. Preparation for animal treatment
    c. Animal diseases it cures
9. Which part is used for medicine?

Preparation

1. How do you diagnose the disease? How do you know when to give this medicine?
2. How is it prepared?
3. Is the preparation the same for treating different diseases?
4. How is it taken by patients (e.g. orally) to treat different diseases?

5. Is it used fresh or dry?
6. Do you mix it or combine it with other things to work properly?
7. Dosages?
8. Is it used concentrated or diluted?
9. Does it have any side effects?
10. What are the side effects?
11. What causes the side effects?
12. Can it be prescribed to everyone?
    a. Recommended age for using the plant?
    b. Gender of patients

Finding and storing it

13. Are there any rituals that need to be observed when collecting/harvesting or giving the medicine?
14. Where do you find it?
15. When is it found (season)?
16. How is it harvested?
17. How are the harvested tubers stored?
18. How long can they be stored?

Propagation

19. Is finding it a problem?
20. Is it found in the wild only or are there any muthi gardens?
21. Do you cultivate it or would you like to cultivate it?
22. How is it propagated?

23. Is it easy or difficult to propagate?
24. How long does it take to germinate?
25. When does it germinate?
26. Which part is used to propagate the plant (seeds, vegetative)?
27. Conditions required for the plant to germinate and grow
28. What is production like (any diseases or temperature that lower the yield)?
29. Are there other plants that you can use in place of it or instead of it.

Trade

30. Do you sell the plant?
31. Where do you sell the plant?
32. How much do you collect for sale per day/week/month/year?

Appendix B: Abbreviations

1. µg: Microgram
2. µl: Mmicro litres
3. AP-1: Activator Protein-1
4. CSIR: Centre for Scientific and Industrial Research
5. CUT: Central University of Technology
6. DMSO: Dimethyl Sulfoxide
7. DNA: Deoxyribonucleic Acid
8. DPPH: 2,2-Diphenyl-1-picrylhydrazyl
9. DTP: Development Therapeutic Program
10. g: Grams
11. H2SO4: Sulfuric Acid
12. IDDM: Insulin Dependent Diabetes Mellitus
13. LC100: 100% Lethal Concentration
14. LC50: 50% Lethal Concentration
15. LCMS: Liquid chromatography Mass-Spectrometry
16. mg: Milligrams
17. ml: Millilitres
18. mM: milli-Molar
19. MRC: Medical Research Council
20. MRDM: Malnutrition-r Related Diabetes Mellitus
21. MTT: Mossman Tetrazole Test (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

22. NAOH: Sodium Hydroxide
23. NCI: National Cancer Institute
24. NF-K: Nuclear Factor-Kappa
25. NIDDM: Non-iInsulin Dependent Diabetes Mellitus
26. nm: nanometers
27. NMR: Nuclear Magnetic Resonance
28. O2: Oxygen
29. OH: Hydroxide
30. ROO: Peroxyl radical
31. ROS: Reactive Oxygen Species
32. SBR: Sulforhodamine B
33. SU: Sulphonylreas
34. T0: Drug Addition
35. TCA: Trichloroacetic Acid
36. TGI: Total Growth Inhibition
37. TZD: Thiazolidinedione
38. UK: United Kingdom
39. UN: United Nations
40. UPLC: Ultra-pure Liquid Chromatography
41. USA: United States of America
42. WHO: World Health Organization

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Chapter 1

General Introduction and Literature Review

1.1. Background

Alternative therapies are treatments that should solve problems in a way similar to conventional treatment methods. These therapies include compounds that are isolated and extracted from natural sources such as medicinal plants. Many cancer drugs originate from plants, soil and marine microorganisms and fungi (Koduru *et al*., 2007). Compounds that are derived from plants have contributed significantly in the development of some useful anti-cancer drugs such as *taxol, etoposide*, *vinblastine* and *vincristine*. Current cancer treatment poses numerous problems such as adverse side effects, drug resistance and toxicity to non-cancerous cells. Drug resistance and toxicity are not unique to cancer therapy but are widespread. This has led to the search for new antimicrobial, anticancer, antidiabetic and many more therapeutic agents from other sources like plants with minimal side effects (Hong-Fang *et al*., 2009).

Plants are natural sources that might have minimal or no side effects (Light *et al*., 2005). Medicinal plants in Southern Africa have been the point of interest for new drug development and the isolation of active compounds. Interest in this field has grown drastically in the last 10 years (Light *et al*., 2005). Medicinal plants can be seen as the way forward in future in terms of research and development on new therapeutic drugs (Makunga *et al*., 2008). According to Van Wyk (2011), more than 90 of the best known indigenous plants have shown commercial potential in a variety of applications as drug agents in South Africa. Traditional Medical practitioners, herbalists and rural dwellers have indigenous knowledge on using medicinal plants for treating ailments, but require support from researchers for scientific knowledge and validation of their work to be

recognized. These partnerships will lead to more plants being commercialized and documented to benefit all parties involved, including communities.

The South African population relies more on traditional herbal medicines for treatment than on Western medicines. This forms an important base of the primary healthcare system in the country, which is rich in biodiversity, cultural diversity and traditional knowledge (Nair *et al.,* 2011; Street *et al.,* 2008). According to Nair *et al*., (2011) and van Vuuren (2008), the Southern African region has a floral diversity of 30 000 species, which represents 10% of the world's flowering plant population. Indigenous plants have been trusted by traditional medical practitioners for the treatment of diseases such as respiratory infections, reproductive health, problems with pregnancy, cancer, diabetes, etc. (Louw *et al*., 2002).

1.2. Literature review

1.3 Cancer

Cancer is a malignant neoplasm (tumor), which involves unregulated cell growth that results in the formation of malignant tumors. The cancer cells are able to invade other parts of the body and may also spread to more distant parts of the body through the lymphatic system or bloodstream (Weinberg, 2007).

1.3.1. Factors increasing the risk of cancer

Tobacco use or tobacco smoke is a toxic mix of over 7 000 chemicals, which causes cancer of the mouth, nose, throat, kidney etc. (US Department of Health and Human

services, 2008). Continuous smoking damages deoxyribonucleic acid (DNA), tissue and cell structure and interferes with normal body processes (US Department of Health and Human Services, 2008). The transition from normal, healthy cells to cancer cells is a step-wise progression that requires genetic change in several different tumor suppressions. In order to generate a cancer cell, a series of mutations must occur in the same cell. Therefore, cancer is much more prevalent in elderly individuals (Hanna *et al.,* 2008). Cells in an elderly (70-year-old) person's body have had more time to accumulate the requisite genetic changes needed to develop cancer, unlike those in a child (Hanna *et al.,* 2008). Poor diets and obesity during the past several decades have enlarged the percentage of overweight and obese people. Obesity is associated with an increased risk of developing cancer of the esophagus, pancreas, colon and rectum, breast (after menopause), endometrium, kidney, thyroid and gall bladder (National Cancer Institute (NCI) Surveillance, 2007).

Epidemiological studies of environmental pollutants over the last 40 years have shown that ambient air pollution caused by the incomplete combustion of fossil fuels is associated with relatively small particles that increase the rate of lung cancer. The small particles from fossil fuel combustion are the most dangerous, being deeply inhaled into the lungs and resulting in acute changes in lung function and respiratory infection. These particles settle in the areas where the body's immune clearance mechanisms cannot eliminate them and become cancerous. Particulate matter is the source of the life- shortening effect of dry air, because polluted air contains small and large particles with chemicals. Chemicals found in the small particles tend to be active and become acidic, resulting in damage to lung cells (Cohen and Pope, 1995). Therefore an active

compound from plants is needed to assist in clearing inflammation of the lungs. The medicinal properties of *I. oblongata* will be investigated *in vitro* (artificial environment) for anti-cancer therapy to find a possible active compound(s) that could assist in eliminating chemicals causing cancerous cells.

1.3.2. Cancer treatment

The predictable methods of cancer treatment are radiation, surgery, chemotherapy, palliative care and some medicinal drugs (National Cancer Institute, 2010). Most of these treatments depend on the type of location and grade of cancer, as well as the person's health and wellness. For instance, surgery has good outcomes for some cancers (i.e. skin, breast, etc.), whereas radiation is normally applied in conjunction with surgery or chemotherapy. An overview of the common side effects of conventional treatments is listed in Table 1.1. It is rare for any treatment to be used independently to combat the illness on its own (National Cancer Institute, 2010).

Table 1.1 Overview of common side effects of conventional treatments

Conventional Treatment	**Side Effects**
1.Radiation therapy	Skin rashes (such as redness/rash), loss of appetite and decreased number of white blood cells.
2. Chemotherapy	Loss of appetite, nausea, vomiting, hair loss and mouth sores.
3.Hormonal	Nausea, vomiting, weight gain, interrupted menstrual

therapy	periods and vaginal dryness.
4.Surgery	Lymphedema (post-surgery), tingling or numbness.
General side effects	Constipation, sleep disorders, delirium, loss of fertility (from some anti-cancer drugs) and fatigue.

Source: National Cancer Institute, 2010

1.3.3. Cancer and inflammation

"Inflammation is a part of the host response to either internal or external environmental stimuli" (Aggarwal *et al.*, 2006). It has been described as a localized protective reaction of tissue to irritation, injury, or infection, characterized by pain, redness and swelling. Many studies have indicated that patients with chronic inflammatory diseases have a bigger risk of developing cancer, diabetes, etc. Chronic inflammation is the most common type of inflammation linked with cancer. Key factors associated with chronic inflammation are the roles played by pro-inflammatory cytokines, chemokines, adhesion molecules and inflammatory enzymes (Aggarwal *et al.*, 2006).

In a study by Bucala and Seamas (2007), macrophage migration inhibitory factor (MIF) was determined to have unique biological activities. The regulation of tumor-suppression genes, angiogenesis and cell senescence postulates MIF as playing an important role in linking chronic inflammation and cancer invasion. Long-term CD44 activation plays a vital role in malignancy by activating breast cancer cell invasion. In order to maintain the development of tumor cells, there is interaction between tumor cells and the host's microenvironment (Bucala *and* Seamas, 2007). The use of *I.*

oblongata as treatment might aid in preventing the development of the tumor cells if antioxidants and anti-inflammatory activities are found in the plant.

1.3.4. Cancer and antioxidants

Atawodi, (2005) describes reactive oxygen species (ROS) or free radicals as a major cause of cellular damage. By using natural anti-oxidant compounds found in foods and medicinal plants, ROS can be scavenged for chemoprevention (Atawodi, 2005). Free radicals are generated as part of the body's normal metabolic processes. ROS can result in DNA damage that may alter signal transduction (transfer of genetic material) forces, including induction changes in transcription factors (i.e. NF-K and AP-1), which are intermediate cellular stress responses such as diabetes, cancer, aging and other degenerative diseases (Olayinka and Okoh, 2010; Nibha and Manjula, 2008). The increased level of radical activity (Atawodi, 2005), damage to the immune system and nervous system can be a result of chemical mobilization of fat stores under various conditions such as lactation, exercise, fever infections and even fasting (Atawodi, 2005). In cancer ROS are responsible for initiating the multistage carcinogenesis process, starting with DNA damage and accumulation of genetic events in one or a few cell lines. These cell lines lead to progressively dysplastic cellular appearance, deregulated cell growth and finally carcinoma. Therefore using free radical scavengers (antioxidants) has the potential to prevent and delay many of the known disorders (Atawodi, 2005).

1.4. Diabetes

The global prevalence of diabetes for 2000 and 2030 projections were conducted by age and sex from World Health Organization (WHO). The WHO state members and

United Nations (UN) population diabetes statistics was piloted for both rural and urban countries (Wild *et al*., 2004). The estimates for 2000 were 171 million people with diabetes at 2.8% and it was predicted that 366 million people would have diabetes by 2030 at 4.4% (Wild *et al*., 2004; WHO statistics). Leading countries in terms of the number of people with diabetes are India, China and the United States of America (USA); these are resourceful countries that can help develop technology for novel drugs from medicinal plants, which are natural sources. Factors contributing to the number of people with diabetes include population growth, aging, urbanization and others (Wild *et al*., 2004). In figure 1.1., the estimates of diabetes prevalence are shown in developed, developing countries and the world according to age. The age group of 64-65+ will have diabetes in the 2030 for the world and the developing countries. These prevalence plays as an indicator that age is a contributing factor in diabetes. The prevalence of diabetes is higher in men than in women, but more women are diagnosed with diabetes. There are more women who test for diabetes and are much more health conscious than men. Therefore there is a need to develop an anti-diabetic drug from natural sources to enable most people to benefit from low-cost medication (Wild *et al*., 2004).

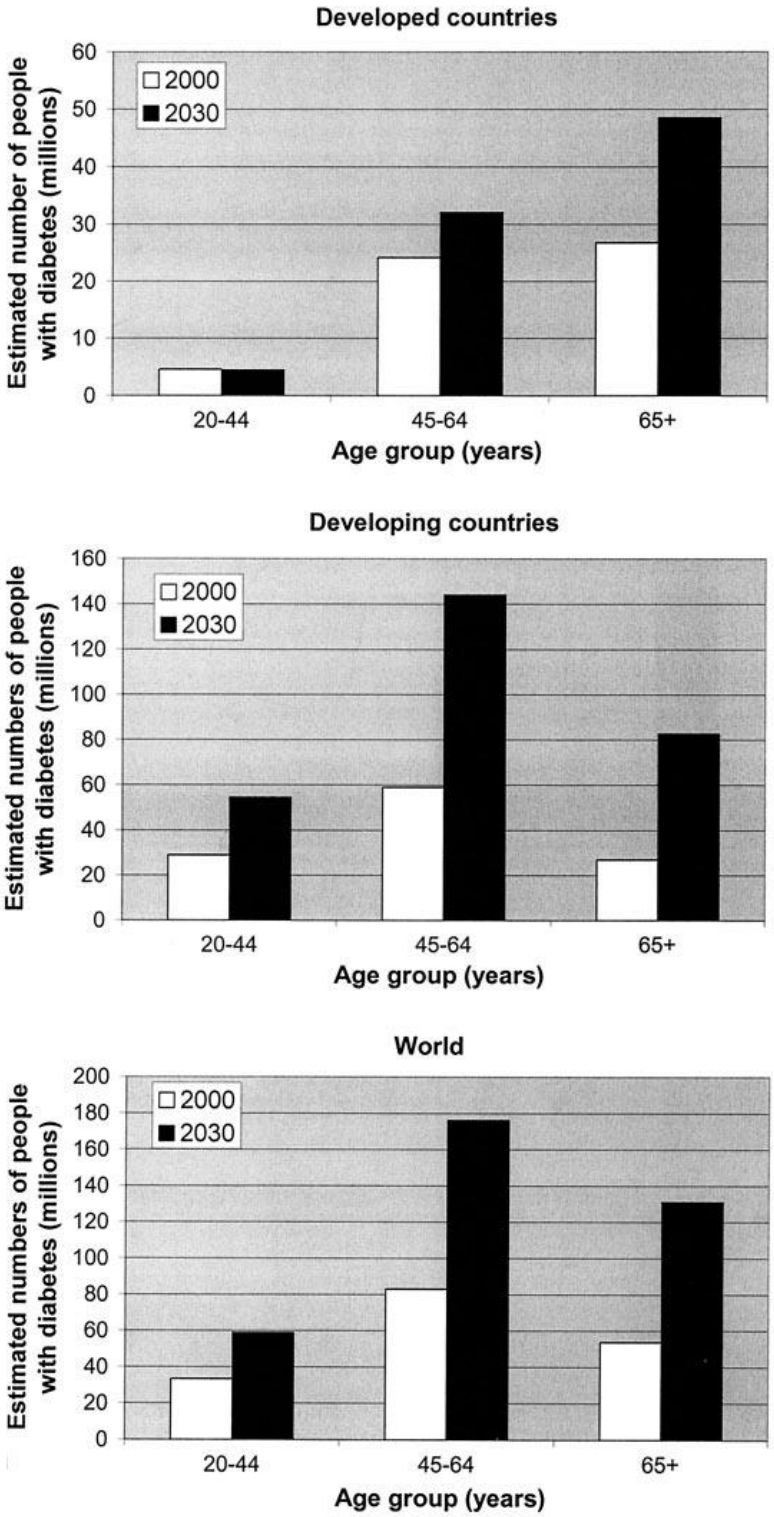

Figure 1. 1 Estimates of diabetes prevalence in developed and, developing countries and the world according to age (Source: Wild *et al*., 2004

The ethnobotany studies conducted on anti-diabetic activity show that about 800 plant species, such *as Momordica charantia, Pterocarpus marsupium and Trigonella foenum greacum,* have been reported to be potent for Type 2 diabetes (Patel *et al*., 2012a). Perez *et al*. (1998) indicated that plants demonstrating hypoglycemic effects have been used since ancient times to treat Type 2 diabetes. They also indicated the positive anti-diabetic effects caused by compounds isolated from medicinal plants. This justifies plants such as *I. oblongata* being tested for new leads as diabetes drugs. The plants cited most often as being used as anti-diabetic treatments belong to the following families: *Asteraceae, Fabaceae, Euphorbiaceae, Lamiaceae, Liliaceae and Poaceae* (Marles and Farnsworth 1995). The Eastern Cape Province used plants from the following families; *Asteraceae, Hypoxidaceae, Asphodelaceae, Apocynaceae, Apiaceae and Buddlejaceae* to treat diabetes. The plants parts commonly used were leaves, twigs, roots and corms as infusions taken orally as shown in Table 1.2. Mostly the infusions from fresh leaves were boiled or soaked in water and roots and corms were also boiled (Erasto *et al*., 2005). Medicinal plants have shown potential in treatment of diabetes in literature and the people utilizing plants. *Asteraceae* family indicates to be commonly used with antidiabetic activity.

Table 1.2 Plants used for the treatment of diabetes in the Eastern Cape Province, South Africa

Family & scientific name	Local name	Parts used	Preparation of medicine
Asteraceae *Herichrysum odoratissimum* L	Imphepho	Whole plant	The fresh plant is crushed and boiled and the infusion is taken orally.
Herichrysum nudifolium L.	Ichocholo	Leaves, roots	Fresh leaves or roots are boiled, then taken orally.
Herichrysum petiolare H & B.L.	Imphepho	Whole plant	A fresh plant is crushed, boiled and the concentrated solution is taken orally.
Artemisia afra Jacq.	Umhlonyane	Leaves, roots	Leaves or roots are boiled, then the infusion is mixed with sugar to mask the bitterness.
Vernonia oligocephala Sch. Bip.	Umhlunguhlungu	Leaves, twigs, roots	Fresh leaves, roots or twigs are pulverized, and the infusion is taken orally.
Vernonia amygdalina Del.	Umhlunguhlungu	Leaves	Pulverized fresh leaves are soaked in water and the solution is taken orally.
Brachylaena discolor DC.	UmPhahla	Leaves	Leaves are boiled and the infusion is taken orally
Hypoxidaceae *Hypoxis hemerocallidea Fisch. & C. A*	Inongwe	Corms	Fresh corms are crushed, boiled and taken orally
Hypoxis colchicifolia Bak. Asphodelaceae	Inongwe	Corms	Fresh corms are crushed, boiled and taken orally
Bulbine natalensis (Syn. *B. latifolia*) Mill.	Ibhucu	Roots	Fresh roots are boiled and the infusion is taken orally.
Bulbine frutescens L.	Ibhucu	Roots	An infusion is made from fresh boiled roots and is taken orally.
Apocynaceae *Catharanthus roseus*	Isisushlungu	Leaves	An infusion is made from boiled leaves and taken orally.
Apiaceae *Heteromorphica arborescens*. Hochst. Ex A. Rich.	Umbangandlala	Leaves and roots	An infusion is made from boiled leaves or roots and taken orally
Buddlejaceae *Chilianthus olearaceus Burch.*	Umgeba	Leaves and twigs	An infusion is made from leaves or twigs and taken orally.

Source: Erasto *et al*. (2005)

1.5. *Ipomoea oblongata* (E. Mey. ex Choisy)

I. oblongata (E. Mey. ex Choisy) with the synonym *Turbina oblongata (A. Meeus*) is a member of the *Convolvulaceae* family. Common vernacular names are wild morning glory (English), purperwinde (Afrikaans), Ubhoqo (Zulu), Mothokho (Sesotho) (Sobiecki 2008; Roux 2006). A image of *I. oblongata* is shown in Table 1.3*. Ipomoea oblongata* is widely distributed over Southern African countries such as Botswana, Malawi, South Africa (mainly in the Free State and KwaZulu-Natal), Swaziland, Zimbabwe and Tanzania (Sobiecki 2008; Roux 2006). The collection of *Ipomoea oblongata* was done at Selosesha location next to Serwalo in Thaba-Nchu, Free State. Figure 1.2. A shows *Ipomoea oblongata* taken in another province in Free State and figure 1.2.B shows the tuber of Ipomoea oblongata in a cross sectional view. This is how the traditional medical practitioners harvest the plant. The *Ipomoea oblongata* plant with its seeds taken in Thaba-Nchu is shown in figure 1.3.A and B. During harvest system the traditional medical practitioners are able to identify the plant as shown in the figures 1.2 A and B and figure 1.3 A and B. In literature there has not been any scientific work reported yet with the use of *Ipomoea oblongata.* This study is the first report to be presented with the medicinal properties of *Ipomoea oblongata* from *Convolvulaceae* family thus far.

Figure 1. 2 A: Ipomoea plant,

B: Tuber of I.oblongata; photograph taken at Bothaville

Table 1.3 Description of *Ipomoea oblongata*

Part	Description	Size	Shape
Tuber	Perennial large fusiform tuberous root	1 m long	
Stems	Annual, prostrate, petioles, leaves, penducles and calyx form a zigzag pattern; pubescent with yellow/brownish hairs	2 m long	Angular
Leaves	Vary in size and shape, either oblong or elliptic	20-150 x 4-8 mm	Ovate/ Linear
Base			Subcordate to rounded
Apex	Upper surface is yellowish strigose and lower surface is thin/more dense		Acute to emarginate, margin ciliate
Petiole	Shorter than leaves		
Penducles	One is severally flowered and shorter than leaves		
Bracteoles	Hairy, shorter than sepals		Lanceolate
Pedicles		≥ 6mm	
Sepals	Sub-equal, outer ones are hairy and inner ones are less hairy	12-25 mm long	Lanceolate to ovate, acute or acumianate
Corolla	Wide, magenta, mid-petaline areas usually thin and hairy	35-75 mm long	Funnel-shaped
Capsule	Style base persistent, glabrous, dark-brown and loosely enclosed by sepals	12-15mm diameter	Indehiscent subglobose, apiculate
Seed	1-4 seeds, punctate to smooth and grey. Flower in October- April and mostly in December-January	+7 mm long	Glabrous

Source: Roux (2006).

Figure 1. 3 A & B: *Ipomoea oblongata* with seeds taken at Thaba-Nchu

1.6. Purpose of the research

Traditional Medical Practitioners use *Ipomoea oblongata* for a variety of ailments. The aim of this study was to evaluate some medicinal properties of *Ipomoea oblongata.*

1.7. Objectives

In order to achieve the aim stated above, the study will focus on the following objectives:

- To document the medicinal use of *Ipomoea oblongata* in Thaba-Nchu, Free State.
- To determine the phytochemical contents of *Ipomoea oblongata* extracts and antioxidant properties.
- To evaluate the effects of the extracts in cancer cell lines *in vitro*.
- To determine the anti-diabetic properties of the plant extracts.
- To assess the cytotoxicity of extracts from *I. oblongata* on chang and muscle cells.

1.8. Overview of the dissertation

This study is presented in five sections (Chapters 2-6). Chapter 2 reports on he, Ethnobotanical uses of *Ipomoea oblongata* (Mothokgo). The plant part used and the preparation is documented. Chapter 3 discusses the phytochemical contents of the extracts. The standard screening methods were used to determine the contents from

methanol, water and dichloromethane. The next chapter (4) evaluates the antioxidant activity of *Ipomoea oblongata* using the free stable radical, 2, 2- diphenylpicrylhydrazyl (DPPH) method. Antioxidant activities played an important role in determining the other medicinal uses of *I.oblongata*. Chapter 5 discusses anti-cancer activity in medicinal plants and the cell lines used for this assay. The last chapter evaluates the effects of medicinal plants in treating diabetes and the cytotoxicity effects of *I. oblongata* as an anti-diabetic agent.

The plant material and extraction methods used in this study are the same for all experiments performed to determine the medicinal properties of *I. oblongata*.

This is the first scientific research done and reported on *I. oblongata* (E. Mey. ex Choisy).

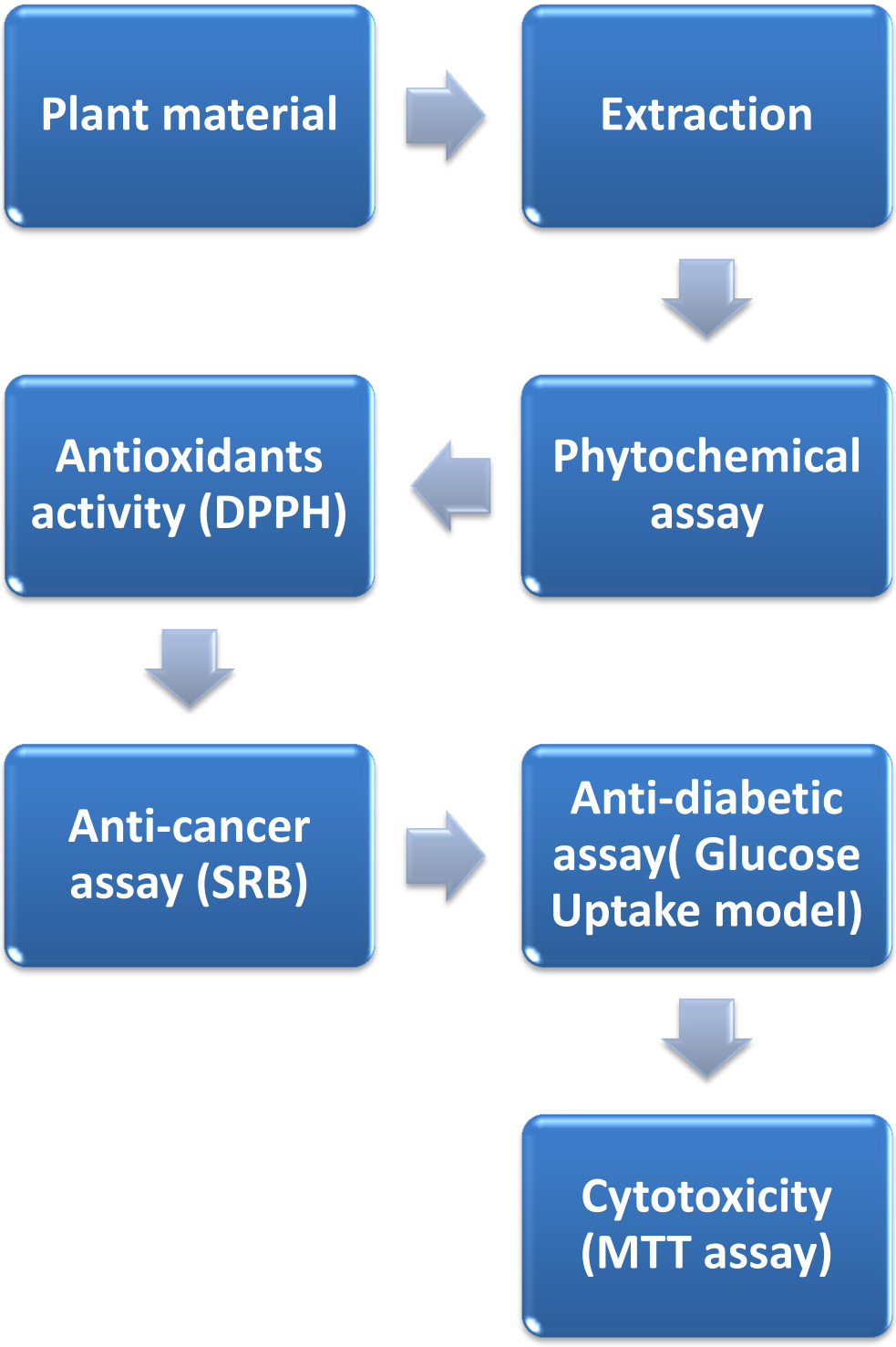

Figure 1. 4 Methodology summary

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Chapter 2

The use of *Ipomoea oblongata* in Thaba-Nchu, Free State

2.1. Introduction

Ethnobotany studies are common ways that are carried out by researchers to understand the use of traditional herbal medicines by traditional doctors, herbalists, rural dwellers and communities at large. This contributes to scientific literature, medicinal usage, conservation and biodiversity of plants and the knowledge of traditional medical practitioners. An ethnobotany study was conducted by Erasto *et al*. (2005) in the Eastern Cape to determine the use of medicinal plants to treat diabetes. The results of the survey showed that it is common practice to treat the ailment with traditional herbal medicines. Four plants (*Herichrysum odoratissimum*, *Herichrysum petiolare*, *Hypoxis hemerocallidea* and *Hypoxis colchicifolia)* are frequently used to treat both humans and livestock (Erasto *et al*., 2005).

The families *Asteraceae, Hypoxidaceae, Asphodelaceae, Apocynaceae, Apiaceae* and *Buddlejaceae* have proven to be the most effective treatment for diabetes in the Eastern Cape (Erasto *et al*., 2005). This validates the use of surveys and questionnaires among traditional medical practitioners as effective ways to determine how they use medicinal plants, which can play a partial role of novel drug discoveries. Researchers will investigate the claims on healing properties using appropriate scientific models in the laboratory to yield new active compounds that can lead into new therapeutic agents.

2.2. Aim of ethnobotany survey

The aim of this study was to determine the ethnobotanical uses of *I. oblongata* (Mothokgo) by traditional medical practitioners.

2.3. Methods

An interview guide about *I. oblongata* (Mothokho) that was prepared for the traditional medical practitioners of Kopanang Dingaka Association as shown in figure 2.2., from a small village called Serwalo (Koppie) in Thaba-Nchu, Free State Province, South Africa. Serwalo is located in Thaba-nchu, Free State, South Africa, with the following geographical coordinates: 29° 12' 0" South, 26° 47' 0" East. Each interview was conducted privately to eliminate repetition of what another candidate would mention refer to figure 2.2., as it is believed that each traditional healer uses medicinal plants differently, including *I. oblongata.*

Interview questionnaires were drafted and modified according to what the researcher wanted to understand about the traditional knowledge and uses of the plant by the traditional medical practitioners. The questionnaire was divided into six sub-headings: Interviewee's details, use, preparation, finding and storage, propagation and trade.

The following equation was used to determine the importance of the plant used:

UIV = ΣSs

Ss=F⁄(NmP) salience scores (Hoffman & Gallaher, 2007)

F= Frequency

mP= the mean position in which the species is mentioned

Figure 2. 1 Interviews at Serwalo Thaba-Nchu

Figure 2. 2 Kopanang Dingaka Association

2.4. Results and Discussion

The method of extraction used is boiling and mixing the plant with only water as a solvent. The traditional medical practitioners cited the plant roots as the main ingredient in the treatment of respiratory infections, STI's, postnatal womb treatments, inflammation, arthritis, wounds and cancer. The Use Importance Value formula is used to calculate how significant the common uses of *I. oblongata* are to the traditional medical practitioners and what the specific disease are that can or are being treated. In ethnobotany salience scores are utilized in order to determine the use importance value found in surveys about the plant use and its healing properties. Others use importance values may include everything significantly related to the plants properties, plant parts used, etc (Hoffman & Gallaher, 2007).

The illnesses were grouped into respiratory infections, pregnancy and postnatal, antimicrobial infections, cardiovascular diseases, and inflammatory diseases for the salience scores to determine their use importance and the salience were less than 1 as tabulated in table 2.1. The roots of *Ipomoea oblongata* are the most important plant part used to treat respiratory infections, inflammatory diseases, diarrhoea, and nausea, as shown in table 2.1., with reference to salience scores.

Other findings from the questionnaire showed that *Ipomoea oblongata* is found on mountains and river banks. The preferred method of storage is in bags, plastic jars with lids, newspapers and dry brown bags. In propagation, the harvest seasons are January to March, October to December are good seasons where *I. oblongata* has seeds, and flowers. In April to September the *I. oblongata* is still visible but very dry, therefore those

months are not suitable for harvesting. Kopanang Dingaka association utilizes digging to harvest the tuber. The association does not trade *Ipomoea oblongata* because they are in possession of recognized licenses to harvest indigenous plants.

Table 2.1. Uses of *I. oblongata* determined by salience scores

Ailments	Plant part used	Salience scores
Respiratory infections	Roots	0.5714
Inflammatory diseases	Roots	0.5436
Cardiovascular diseases	Roots	0.02787
Pregnancy and postnatal	Roots	0.3077
Diarrhoea and nausea	Roots	0.5000
Cancer	Roots and leaves	0.0833
Wounds	Roots	0.3000
STIs	Roots and leaves	0.2308

Preservation of the indigenous knowledge plays a vital role in the discipline of Ethnobotany science and conservation, (Kunwar and Bussmann, 2008). In this study twelve informants were interviewed with regard to indigenous use of *Ipomoea oblongata* (Mothokgo). The results of the surveys displayed ailments that are treated with *Ipomoea oblongata.* In this way plant-people relations are documented. The illnesses were

grouped into respiratory infections, pregnancy and postnatal complications, antimicrobial infections, cardiovascular diseases and inflammatory diseases for the salience scores to determine use, importance and salience. The salience score were found to be lower than 1, as presented in table 2.1. Salience score lower than 1, presented the use importance value in the medicinal properties claimed made by the informants. Data for Ethnobotanical surveys is collected through questionnaires and personal interviews to be explored for therapeutic uses of medicinal plant and safeguarding of indigenous knowledge concepts, (Mohammad[1] and Mohammed[2], 2013). After analyzing the survey and claims by traditional medical practitioners, the researcher valided some of the medicinal uses. The medicinal properties (anticancer, anti-inflammation, antidiabetic, and antioxidants) were major role players for validation. The plant was found to be the main ingredient (activity) that the traditional medical practitioners used to mix with other plant material to make concoctions. In a study conducted with the Taungya community, the outcomes reveled different application forms such as powder, juice, decoctions , infusions, poultice and oral administration of diverse medicinal plants, (Poonam and Singh, 2009). Some of the applications are the same as those that were indicated by the informants of *Ipomoea oblongata*. Table 2, is an indication of how the traditional medical practitioners are utilizing *Ipomoea oblongata* as the main source of treating different ailments. After analysing the survey and claims by traditional medical practitioners, we focused on cancer, diabetes and antioxidants (as the play major roles in healing and restoring above mentioned ailments). In a study conducted for the ethnobotanical uses of *Sansevieria Thunb (Asparaceae),* four parameters were identified (medicinal use, horticulture use, food administration and the

specific plant parts used) from interviewing the informants which were local communities (Nyenya *et al*., 2014). Traditional botanical knowledge of medicinal plants is retained in populations that utilized medicinal plants for therapeutic uses, (Cornara *et al*., 2014). The indigenous knowledge of medicinal plants was obtained from local communities, rural dwellers, and herbalist for documentation of ethnobotanical uses.

2.5. Conclusion

Ipomoea oblongata has a wide variety of medicinal applications. The plant is the main ingredient that the traditional medical practitioners use to make their herbal preparations. The root of *I. oblongata* was found to be the plant used most often to treat different ailments in the Thaba-Nchu community in the Free State.

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Chapter 3

Phytochemical Composition of *Ipomoea oblongata*

3.1. Introduction

Plants produce bioactive compounds known as phytochemicals, which are natural and non-nutritive. Phytochemical constituents of plants act as protective (nutritional and medicinal) agents against external stresses or attacks in the human body (Chew *et al*., 2011, Kolachi *et al*., 2010). The biosynthetic origin of phytochemicals allows them to be divided into the following categories; phenolics, alkaloids, steroids, terpenes, saponins and many more (Seeram *et al.*, 2004). Phytochemicals found in medicinal plants influence bioactivity, such as anti-mutagenic, anti-carcinogenic, antioxidant, antimicrobial and anti-inflammatory agents. Bioactive compounds that are found in *Ipomoea* species are flavonoids, glycosides, proanthrocyanidins (condensed tannins) and anthocyanins (Seeram *et al.*, 2004). Leaves, stems and tubers of other *Ipomoea* species have been validated for phytochemical compounds, antimicrobial, antibacterial and anti-inflammatory properties in literature. *I. oblongata* has not been scientifically validated yet for its activities and bioactive compounds.

Tannins are stable antioxidants that inhibit cell protein synthesis, aid in wound healing and inhibit the growth of microorganisms to make microbial proteins; they are also used for treatment of diarrhea and dysentery. Saponins and glycosides are responsible for upper respiratory tract inflammation properties, anti-diabetic, antitumor activity and antifungal properties (Singh *et al*., 2011). Alkaloids have analgesic, anti-inflammatory and adaptogenic properties to alleviate pain and develop resistance against diseases and stress. The last important phytochemical compound is flavonoids, which have antioxidant activities. All these compounds are naturally found in medicinal plants and play a significant role in primary health treatments. Natural phenolic compound

flavonoids form the largest part (group) and tannins form the most important group of the polymeric group of materials found in plants (Harbone, 1973). Phenolic compounds are also known for their wide range of plant substances, which are common in aromatic ring bearing. These bioactive compounds are water-soluble and they occur combined with glycosides to form the major group of phytochemical compounds (Harbone, 1973). Phenols are also known to be potent antioxidants and free radical scavengers because of their ability to act as hydrogen donors, metal chelators, singlet oxygen quenchers and reducing agents (Chew *et al*., 2011).

Preliminary phytochemical screening is essential for testing different extracts that were extracted using different solvents. In this case methanol, aqueous, and dichloromethane solvents were used in plant extraction and were used for the detection of different compounds by qualitative testing methods (De *et al.*, 2010). The aim of the study was to determine which phytochemicals are present in the root extract of *I. oblongata,* as this plant has not been studied thoroughly yet.

3.2. Methods

The procedure that was followed is described below and was used for all the experiments.

3.2.1. Plant material

I. oblongata was donated by one of the traditional medical practitioners of Kopanang Dingaka association. The plant was collected in Thaba-Nchu, identified by the traditional medical practitioners and a voucher specimen (KT001/2013) was kept at the Central University of Technology Free State and authenticated by scientists at the National

Botanical Gardens in Bloemfontein. Plant material (roots) were dried in the oven at 40^0C for 5 days, crushed into a fine powder and stored in a plastic jar. The plant was kept in a cool dry area in the laboratory (Mashele and Kolesnikova, 2010).

3.2.2. Preparation of extracts

For the preparation of aqueous (H_2O), methanol and dichloromethane extracts, *Ipomoea oblongata* roots were weighed to obtain 10 g. Plant roots were dried in the oven at 40^0C for 5 days, crushed into a fine powder material. The powdered material was soaked for 72 hours in 150 ml of the different solvents and stirred using a rotary shaker. The water, methanol and dichloromethane extracts were filtered through (Whatman No. 1) filter paper. The solvents were dried at 40ºC using a rotary evaporator to remove the solvents and make the final volumes of the original plant material.

3.2.3. Screening of *Ipomoea oblongata*

The *Ipomoea oblongata* extracts were used to detect saponins, carbohydrates, proteins, flavonoids, tannins, steroids, terpenoids, glycosides and alkaloids qualitatively (De *et al*., 2010). All the chemical tests were carried out on the methanolic and water extracts using standard procedure to identify the chemical constituents by colour changes (De *et al*., 2010; Trease *et al*., 1989; Harbone, 1973; Sofowara, 1993). The dichloromethane was excluded in the screening because the water and methanol extract displayed the type of phytochemical constituents present in the plant.

Test for saponins

In this test 0.7 mg of the extract was dissolved in 3 ml of distilled water and shaken vigorously. The formation of foam was an expected outcome.

Test for carbohydrates

The Benedict's test was performed using 0.2 mg of the extract, which was dissolved in 2 ml of methanol. The sample of 1 ml (1000 ul) and 1 ml of Benedict's solution were put into a test tube. The expected color change is from green to bluish-green.

Test for protein

Biuret reagents were used to test for proteins. The test was carried out by measuring 0-2 mg of the extract, re-dissolved in 2 ml of methanol. A few drops of 1% copper sulfate ($CuSO_4$) and 4% sodium hydroxide (NaOH) was added to the extract. The expected result would be violet color change.

Test for flavonoids

To test for flavonoids 5 ml of dilute ammonia solution were added to the methanol and distilled water extracts before adding a few drops of concentrated sulfuric acid (H_2SO_4). A yellow coloration observed would indicate the presence of flavonoids.

Test for tannins

The methanol and water extracts were each re-dissolved in 2 ml methanol and 2 ml distilled water (solvents of extraction), then 3 drops of 0.1% ferric chloride solution were added. The formation of blue-black or brownish green precipitation in the solution indicated the presence of tannins.

Test for steroids and terpernoids (Salkowski test)

In this procedure 0.2 mg of extract was dissolved in 2 ml of solvents of extractions of methanol and distilled water, then the extracts were premixed with 1 ml of chloroform. Afterwards, concentrated sulfuric acid (H2SO4) was added to the sample. Formation of a layer would be visible. These experiments cause a reddish brown color precipitate at the border or interface of chloroform and H2SO4, to show the presence of steroids and terpenoids.

Test for cardiac glycosides (Keller-Killani test)

In this test 2 ml of methanol extracts and 2 ml of distilled water extracts were dissolved in the solvents of extraction. The extracts were both treated with 2 ml of glacial acetic acid and one drop of ferric chloride solution, before the addition of 1 ml concentrated sulfuric acid. A brown ring of the interface indicates the deoxysugar characteristic of cardenolides. A violet ring appears below the brown ring interface, while in the acetic acid layer, a greenish ring gradually forms throughout the thin layer.

Test for alkaloids (Meyer's test)

To test for alkaloids, 1 g of the methanolic and water extracts was re-dissolved in 3 ml methanol and 3 ml distilled water; 1 ml Meyer's solution (potassium mercuric iodide solution) was added to 200 ul of the samples. The expected color change was to cream and orange-brownish.

3.2.4. Determination of total phenolic content of extracts from *Ipomoea oblongata*

The principle behind this study was to evaluate the amount of total polyphenols found in the methanol extract, water extract and dichloromethane extract of *I. oblongata.* The Gallic acid standard curve was obtained by weighing 0.250 mg and dissolving it in 0.1 ml methanol to make a stock solution. The extracts in their solvents of extraction and Gallic acid were diluted to different concentrations (2.5 mg/ml and 5 mg/ml). Stock solutions were made from 0.010 mg of crude extracts (methanol, water and dichloromethane) and re-dissoved in 2 ml of each solvent of extraction.

A 50 ml volumetric flask was used for the experiment. In the flask 46 ml of distilled water were added, and then 5 ml of ethanol, 1 ml of Folin-Ciocalteu (FC) reagent from (Sigma) was also added. After 3 minutes the mixture was topped up to 50 ml by adding 2.9 ml of sodium bicarbonate and 0.01 mg of the Gallic acid (Waterhouse *et al*., 2003). This procedure was repeated for the methanol, water and dichloromethane extracts. The samples and the control were incubated in the dark for two hours. The absorbance was read at 760 nm using a spectrophotometer (Thermo Spectronic, Helious Epsilon model, USA)

The total phenolic content can be expressed as milligram (mg) Gallic acid equivalents (GAE) per milligram of the dry material (Gulluce *et al*., 2007; Waterhouse, 2003). This method was repeated for each extract.

3.2.5. Identification of active compounds by LCMS/UPLC

Ultra-pure Liquid Chromatography (UPLC) was conducted in order to establish which compounds were active in *I. oblongata.* The instrument used for UPLC was the Water Synapt G2 at a flow rate of 0.4 ml/min and 0.1% formic acid to acetonitrite gradient at Water UPLC BEN C18, 2.1 x 100 mm column. Electrospray negative was used as a source with capillary voltage 3 kV and cone voltage 15 V. This was done in collaboration with Stellenbosch University.

3.3. Results and Discussion

The phytochemical composition and primary metabolites of the water, methanol and dichloromethane extracts are shown in Table 3.1. The negative sign indicates absence of chemical composition. The presence of the constituents is indicated with the plus sign in the table. Total phenolic content of *Ipomoea oblongata* is represented in table 3.2 as gallic acid equivalence milligrams per liter. The gallic acid linear graph (fig.3.1) was used as standard to determine the phenolic contents of the *ipomoea* extracts. The active compounds that gives *I. oblongata* its medicinal properties is shown in table 3.3

Table 3.1. Phytochemical screening compound results

Phytochemicals	**Tests**	**Methanol extract**	**Water extract**
Carbohydrates	**Benedict's**	**+**	**+**
	Fehling's	**+**	**+**
Cardiac glycosides	**Keller-Killani test**	**+**	**+**
Saponins	**Sofowara**	**-**	**-**
Proteins	**Biuret's test**	**-**	**-**
Steroids	**Salkowski test**	**+**	**+**
Triterpernoids	**Salkowski test**	**+**	**+**
Alkaloids	**Sofowara**	**+**	**+**
Flavonoids	**Sofowara**	**+**	**+**
Tannins	**Sofowara**	**+**	**+**

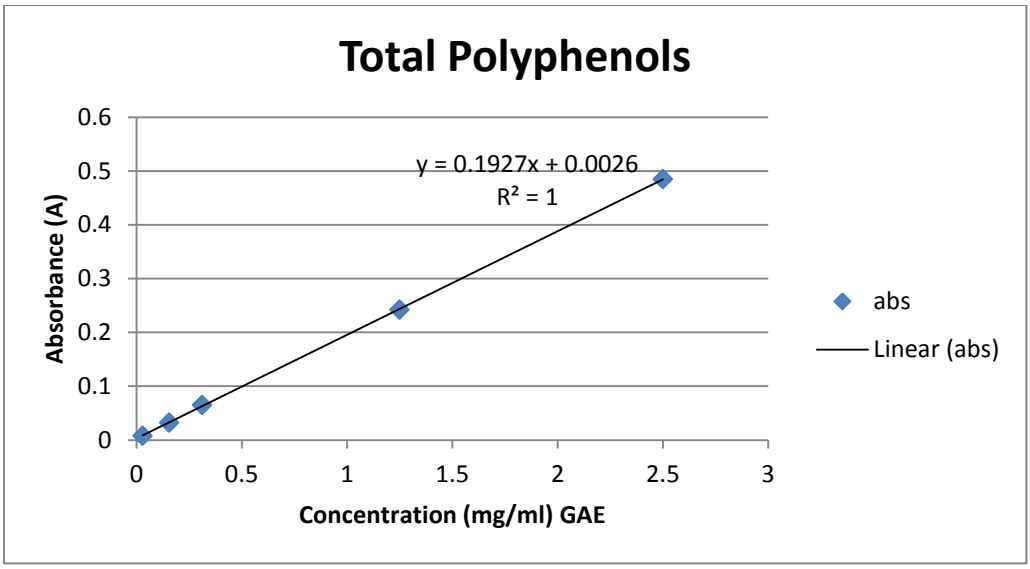

Figure 3. 1 Total phenol compounds using the Gallic acid equivalence (GAE) in mg/g

Table 3.2 Total phenolic compounds in GAE

Extracts	TPL (GAE)	Concentrations	% Content
Methanol	0.041109333	2.5	1.644373
Methanol	0.0801632	5	1.603264
Dichloromethane	0.041109269	2.5	1.644371
Dichloromethane	0.040595467	5	0.811909
Water	0.0319882	2.5	1.279528
Water	0.0608932	5	1.217864

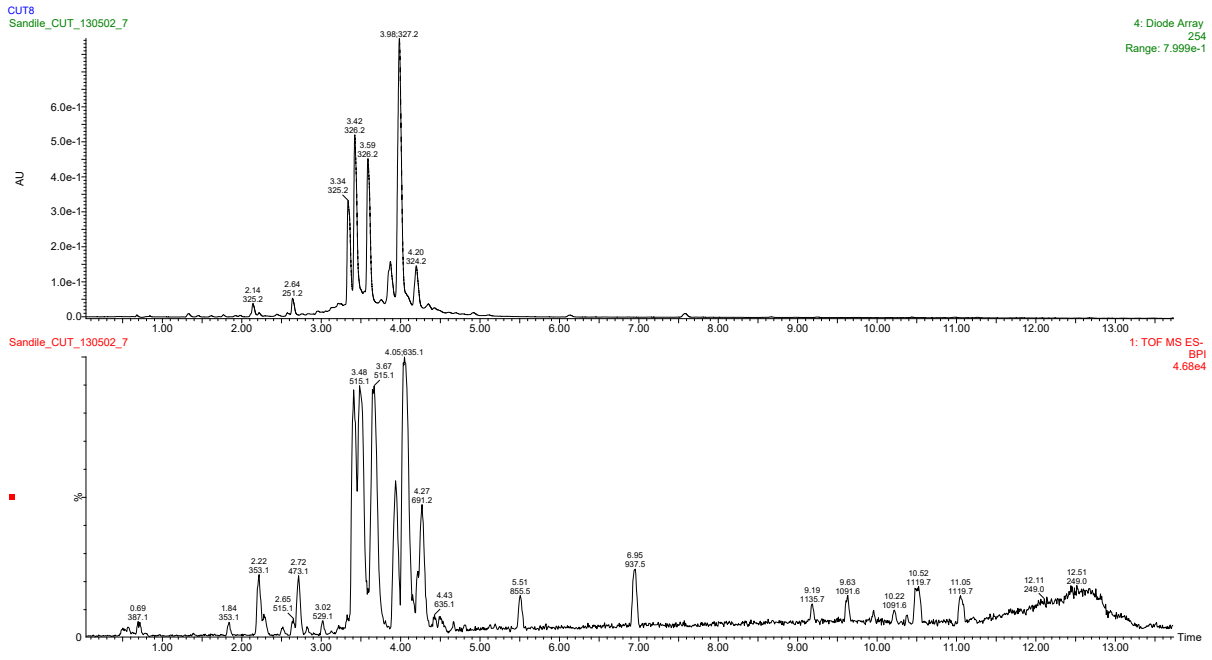

Figure 3. 2 UPLC detection peaks of I. oblongata methanol extracts

Table 3.3. Mass-spectrometry of *I. oblongata* methanol extracts (CUT8)

Peaks	Molecular weight	Chemical formula	Electrospray (ion)	Names of compounds
1	515.1182	C25H23O12	$M–H^+$	Dihydroquercetin pentaacetate
2	635.1384	C32H27O14	$M-H^+$	Actinorhdin Actinorhdine
3	937.5026	C63H69O7	$M-H^+$	

The plant *I. oblongata* was tested to see if it could form a database for the presence of phytochemical compounds and phenolic contents in the Convolvulaceae family. The aim was to determine the presence of phytochemical contents and phenolics. *I. oblongata* was found to have listed secondary metabolites as shown in Table 3.1, but the tests for saponins and proteins were negative. *Ipomoea* species *(Ipomoea batata L and Ipomoea carnea)* are known through literature to contain flavonoids, tannins, saponins, steroids, phenolic compounds and alkaloids (Khatiwora *et al*., 2010). Flavonoids are the most extensively studied phenolics and antioxidant compounds compared to alkaloids, saponins and many more bioactive compounds, which also play significant roles in pharmacology (Khatiwora *et al*., 2010). There is a gap to determine the mechanisms by which other compounds can be beneficial to drug development.

This shows that *I. oblongata* in this study has potential to be an antimicrobial agent because of the phenolics, flavonoids, glycosides and tannins that are present. This is the first time phytochemical compounds of *I. oblongata* have been studied or reported in literature.

The methanolic extracts and dichloromethane extracts as particularized in results had similarities at 2.5 mg/ml concentrations with a GAE of 0.04 and 1.64 % phenolic content found in each 2.5 mg/ml. The water extract was a little lower at the same concentration of 2.5 mg/ml with GAE of 0.03 and contained about 1.28% phenolic content in the same concentration as shown in Table 2. Phenolic contents of *I. oblongata* are influenced by the presence of flavonoids in the plant. The total phenolic contents are considered to be low, as the experiment was conducted at very low concentrations. The Gallic acid, flavonoids and other plant phenolics are reported by Khatiwora *et al*. (2010), as a

natural inhibitor of flowering in leaves and stems and are also found in bark and woody plants, so the results of this study correlate with the literature on the plants. Even at lower concentrations the phenolic compounds have substantial positive effects, such as antimicrobial, antioxidant, anti-inflammatory, antiviral, antimutagenic and chemo-preventative properties (Naidoo *et al.,* 2006; Okem *et al*., 2012). Figure 3.1 shows the standard curve obtained for the Gallic acid.

UPLC was able to detect peaks and indicate chemical formulas that could be active in the methanol extract (Table 3.3). This will lead to fractionation of the plant's active compound/s and structure using Nuclear Magnetic Resonance (NMR) spectroscopy of this compound.

3.4. Conclusion

This is an indication that *I. oblongata* can play an important role in treating diseases caused by oxidative stress, which releases ROS and free radicals. There is a correlation between antioxidant abilities and total phenolic content that is highly positive and indicates the major contribution of phenolic plants.

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Chapter 4

Antioxidant Activity of *Ipomoea oblongata*

4.1. Introduction

Antioxidants are described as a vital health-protecting factor, which can reduce the risk of ailments such as cancer and cardiological diseases. Antioxidants can be found naturally in plants, whole grains, fruit and vegetables. There are various known antioxidant compounds that can reduce the risk of ailments, such as vitamin C and E, carotenes, phenolic acids and phytoestrogens (Prakash *et al.*, 2001, Singh *et al*., 2011). The aim of antioxidant activity is to investigative its scavenging activity or potential against free radicals such as 2, 2- diphenylpicrylhydrazyl (DPPH) radical, superoxide anion radical (O2), the hydroxyl radical and the peroxyl radical.

It has been described that ROS or free radicals are a major cause of cellular damage, which occurs in biological systems as a result of different causes (Prakash *et al*., 2001; Seifried *et al.,* 2006, Singh *et al*., 2011). By using natural anti-oxidant compounds found in foods and medicinal plants, ROS can be scavenged for chemoprevention (Atawodi *et al*., 2005. ROS can result in DNA damage that may alter signal transduction (transfer of genetic material) forces, including induction changes in transcription factors (i.e. NF-K and AP-1), which are intermediate cellular stress responses such as diabetes, cancer, aging and other degenerative diseases (Olayinka and Okoh, 2010; Nibha and Manjula, 2008). Metabolism of stress hormones results in simpler, rather slow free radical molecules (Atawodi *et al*., 2005; Middleton *et al*., 2000).

Defense mechanism provided by the antioxidant systems is essential to the survival of human immunity. The mechanism can function at different levels within the cells through the following actions: prevention of the radical formation, stopping formed radicals,

repairing oxidative damage, increasing the elimination of damaged molecules and recognition of excessively damaged molecules (Middleton *et al*., 2000; Singh *et al*., 2011).

These entire preventative mechanisms are not repaired but rather eradicated to prevent mutations from occurring during cell replication. The anti-oxidant shield involves both enzymatic mechanisms (which utilize specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase), and non-enzymatic mechanisms, which utilize nutrients and minerals (Aggarwal and Shishodia, 2005; Middleton *et al*., 2000). The classification of non-enzymatic anti-oxidants is regarded as water-soluble or lipid-soluble depending on their action, primarily in the aqueous phase or in the lipophilic region of the cell membranes (Middleton *et al*., 2000). Other non-enzymatic anti-oxidants include antioxidant enzyme cofactors, oxidative enzyme inhibitors and transition metal chelators such as ethylene diamine tetra-acetic acid (Aggarwal and Shishodia, 2005; Middleton *et al*., 2000). There is a need to establish enzymatic or non-enzymatic mechanisms that are found in anti-oxidants from *Ipomoea oblongata*.

4.2. Methods

The plant material and extraction method used in this experiment is described in chapter 3, section 3.1.1, plant material and section 3.2.2, preparation of extracts above.

Principle

Free radical scavengers function by donating an electron to the free radical therefore pairs with the unpaired electron and thus stabilizes it.

4.2.1 (DPPH) (2.2-diphenyl-hydroxyl) Assay

The free radical scavenging activity screening was carried out using 0.004% of 2, 2-diphenylpicrylhydrazyl DPPH (Sigma) and Ascorbic acid as standard. DPPH solution without sample solution was used as negative control and ascorbic acid was used as a positive control. The blank used for both ascorbic acid and the sample was 1 ml of methanol. About 0.004 g of DPPH was weighed to make 1 mM and then added to 100 ml of methanol in a flask. For ascorbic acid 0.001 mg of ascorbic acid powder was weighed. It was then diluted with 1 ml of distilled water in the 50 ml tubes before 2 ml of DPPH was added to the tubes for serial dilutions using. The concentrations that were obtained for ascorbic acid were 1 mg/ml; 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; 0.06 mg/ml; 0.03 mg/ml and 0.015 mg/ml (Conforti *et al*., 2008; Brand-Williams *et al*., 1995). The ascorbic acid was incubated in the dark for an hour. The absorbance was read at 517 nm using a (Thermo Spectronic, Helious Epsilon model, USA) spectrophotometer (Gulluce *et al*., 2007). The test was done in triplicate.

The stock solution was 0.002 mg of the methanol root extract mixed into 1 ml of methanol solvent. The stock solution was made and dilutions were obtained using the same method as for the ascorbic acid. Afterwards 2 ml of the DPPH was added to the extract dilutions. The samples were incubated in the dark for an hour. The absorbance was read at 517 nm using a (Thermo Spectronic, Helious Epsilon model, USA) spectrophotometer (Gulluce *et al*., 2007). The following equation was used to calculate the scavenging percentage (antioxidant activity).

Equation: % scavenging=100-[(Abssample-Absblank/Abscontrol)x100

4.3. Results and Discussion

The antioxidant activity showed by the methanol extract of *I. oblongata,* was significantly higher (figure.4.1) in this experiment. The graph in figure 4.1., shows error bars with standard deviation. The other extracts did not show good antioxidant activity. Only methanol was reported because of its high antioxidant activity displayed. The scavenging activity was excellent when compared to the ascorbic acid which was used as a standard.

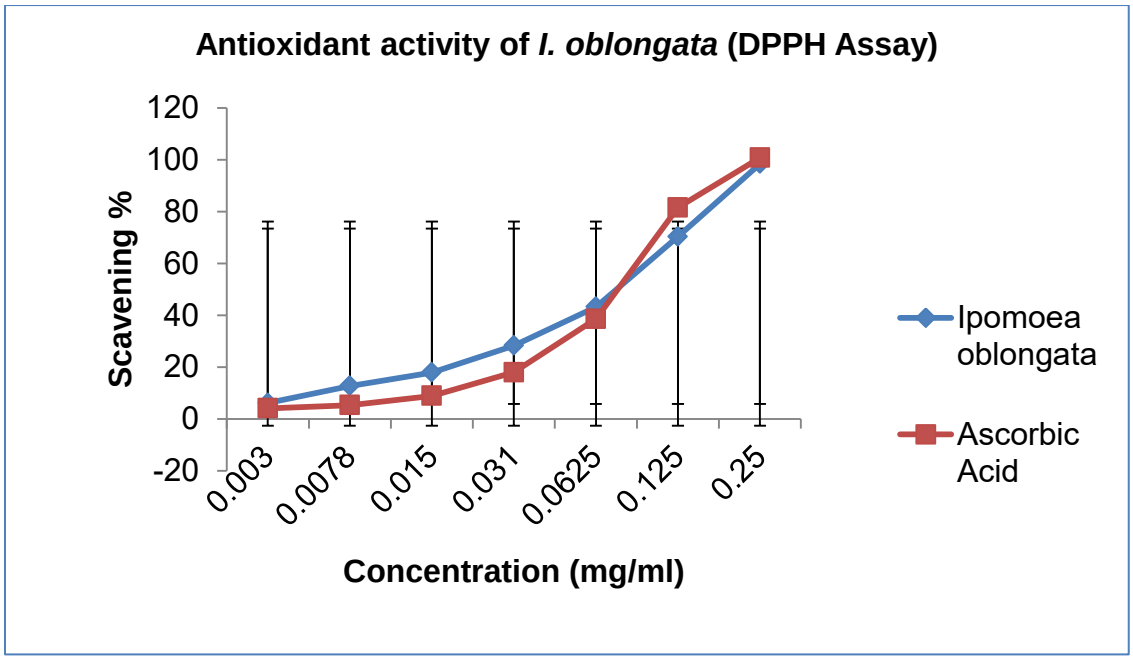

Figure 4. 1 DPPH, ascorbic acid and Ipomoea oblongata methanol extract scavenging activity comparisons.

Figure 4. 2 DPPH sScavenged free radical activity by the plant extract.

The methanolic extract of *I. oblongata* was found to have scavenging activity on the DPPH assay. At 0.25 mg/ml it was 98.39% and at 0.5mg/ml it was 99.03% scavenging ability, as shown in figure 4.1 and figure 4.2 respectively. *I. oblongata* was active as the ascorbic acid, (see figure 4.1), showed antioxidant activity. The hydrophilic antioxidants contain vitamin C (ascorbic acid) and other polyphenol flavonoid groups, while the lipophilic antioxidants include ubiquinols, retinoids, carotenoids, apocynin, procyanidins, certain polyphenol flavonoid groups and tocopherols (Aggarwal and Shishodia, 2006). Species from the Convolvulaceae family such as *I. batata L and I. carnea* possess antioxidant properties (antimicrobial properties and wound-healing activities from their leaf extracts) (Khatiwora *et al*., 2010). This correlates with the study indicating that *I. oblongata* presented with antioxidant activity.

Many activities such as anti-inflammatory, anti-atherosclerosis, antitumor, antimutagenic, anticarcinogenic, antinecrotic, hepatoprotecting drug, coronary heart

diseases, Alzheimer's, neurodegenerative diseases, antibacterial and antiviral effects are due to antioxidant compounds and their mechanism of action (Olayinka and Okoh, 2010; Ho *et al*., 2012). During microbial infections the hydrolysed phenolic substances (flavonoids) are synthesized by plants and they display antibacterial activity that forms complexes with extra-cellular and soluble proteins during disruption of microbial membranes (Mishra *et al*., 2013). Toxic phenolics that attack microbial pathogens are released by hydrolysed non-toxic glycoside found in many medicinal plants (Mishra *et al*., 2013). Literature, (Khatiwora *et al*., 2010; Mishra *et al*., 2013) states that medicinal plants such as *Ipomoea* have characteristics that can be used as therapeutic compounds that will aid in further research into drug development. These findings demonstrated that *I. oblongata* in the study has the potential to have antimicrobial properties.

The major bioactive phenolic compounds, such as tannins and flavonoids, act as primary antioxidants of free radical scavengers (Singh *et al*., 2011), which are present in *I. oblongata* and therefore contribute to the high antioxidant activity. These significant findings confirm that *I. oblongata* has potent antioxidant activity. The Convolvulaceae family (*Ipomoea* species) is naturally rich in total phenolics and phytochemical compounds that enhance their antioxidant activity.

4.4. Conclusion

The significant antioxidant activity found in methanolic extract of *I. oblongata* is a rich source of natural antioxidants that are able to play an important role in the prevention

mechanisms of oxidative stress-related diseases. It is concluded that the phytochemical compounds found in the plant enhances the antioxidant properties of *I. oblongata*.

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Chapter 5

Anti-cancer Activity of *Ipomoea oblongata*

5.1. Introduction

Conventional anti-cancer drugs show toxicity, resistance and side effects that interfere with normal cell functioning and cause DNA damage (Mashele and Fuku, 2011). This has prompted considerable scientific and commercial interest in the innovation and improvement of new therapeutic anti-cancer agents from natural sources such as plants. Herbal medicines have played a significant role in health systems worldwide. Investigation has pointed out and recognized plants as reliable sources since ancient times, as they have the potential to provide naturally active compounds that can be used as therapeutic agents (Mashele and Fuku, 2011). According to Webster *et al.* (2008), plants have provided analgesics, anti-inflammatories, antineoplastic drugs, anti-arrhythmic agents as well as medicines for asthma and hypertension. Plants are also natural sources that might have minimal or no side effects. A desirable anti-cancer drug must be selective, targeted to be cytotoxic only to cancerous cells and must not have the same destructive effects as conventional cytotoxic drugs (Mashele and Kolesnikova, 2010). However, research to test more medicinal plants for anti-cancer treatment is proceeding relatively slowly and more studies of plants with chemical diversity such as *I. oblongata* may aid in identifying and characterizing active anti-cancer components (Gautam *et al.,* 2007).

Research worldwide is currently engaged in the development of new cancer agents in clinical trials based on selective activity against cancer-related molecular targets. Flavopiridol and combretastinA4 phosphate showed some interesting activity as new drug leads in cancer clinical trials (Cragg and Newman, 2005). According to Brower (2008), some medicinal plants have hidden sets of genes that, when found and

expressed, yield more compounds with therapeutic properties. The plants that contain effective anti-cancer agents should be prioritized in preclinical development (Brower 2008).

Findings on the evaluation of plants as anti-cancer agents and for human health that were reported in literature are, “Hydroxynitrile lyase from the leaf of *Hevea brasiliensis* has the potential to be employed as a relatively low cost resource for various anti-microbial and anti-cancer activities due to the simplicity of latex preparation and the abundance of latex that can be obtained in the rubber producing regions” (Daruliza *et al.,* 2011). This could be vital for the environment, as rubber is a renewable source and decomposable. *Gynurapseudochina var. hispidia, Oroxylumindicum* and *Muehlenbeckia platycladai*n, Thailand, were found to be potential drug leads for anti-inflammatory discovery, whereas *Rhinacanthus nasutus* and *Pouzolzia indica* could possibly produce natural compounds as anti-cancer agents (Nisarat *et al.,* 2010).

These studies show more evidence that all around the world, by evaluating plants using diverse methods for new anti-cancer therapeutics, supplementary compounds can be made available for the market. Improved techniques or methods used for extraction should be made available to produce quality results that are not time-consuming and are less affected by human error. The development process should not take years to put drugs onto the market if there are structured relationships between plant-testing centers and drug developers to close gaps for natural products.

In South Africa Koduru *et al.* (2007) documented the use of 17 medicinal plants to treat cancer in the Eastern Cape Province. They provided information including the location,

plant names, plant parts used and different methods of preparation as revealed by traditional medical practitioners, herbalists and rural dwellers. However, work still needs to be done to validate the traditional use as well as to isolate, purify and identify the active compounds in the plants.

Steenkamp & Gouws (2006) worked on the cytotoxity of six South African medicinal plant extracts used in the treatment of cancer. *Bidens pilosa, Centella asiatica, Cnicus benedictus, Dicoma capensis, Hypoxis hemerocallidea and Sutherlandia frutescens* were used to test for cytotoxicity against three different human cancer cell lines. These cell lines were DU-145 prostate cancer cells, MDA-MB-231 and MCF-7 breast cancer cells and MCF-12A, a non-malignant breast cell line. This was done *in-vitro* and results showed that out of the six plants only *D. capensis* produced cytotoxic effects on the two breast cancer cell lines (Steenkamp & Gouws, 2006).The other plants showed no cytotoxicity at all. These are indications that if more testing is done on different medicinal plants, identification of a compound with activity can give better results than one particular plant.

Schrager (2006) refers to toxicology as a division of biochemistry and medicine associated with the study of adverse effects of chemicals on existing organisms and flora. Toxicology deals with indicators, mechanisms, usages and discovery of poisoning of medicinal plants in recent eras. Ernest (2010) describes a Greek physician who had made the first attempt to classify plants according to their toxic and therapeutic effect, so that indicators are known for conducting toxicology assessments. A lot of research has been carried out on the toxicology of plants and organisms.

This further indicates that the medicinal plants used traditionally have the properties they are presumed to cover. However, the anti-cancer agents derived from plant extracts proving to have anti-cancer, anti-inflammatory and antioxidant activities do not come from one specific plant. Testing several plants will give more options for the discovery of new compounds for modification of anti-cancer agents. Evaluating medicinal plants extensively in relation to others from the same botanical family could provide new drug leads in the development of anti-cancer therapeutics.

This is important, as it contributes to the database of anti-cancer treatment using medicinal plants in South Africa. Steenkamp & Gouws (2006) indicated that there is a lack of reports on plants used for cancer treatment in South Africa, hence the rationale for investigating *I. oblongata* as a locally collected plant that may contribute significantly to the country's anti-cancer database.

Anti-cancer assay

Cell lines derived from human tumors have been reported to be extensively used as investigational models of cancer disease. These cell lines are different from both normal and cancerous tissue. The model types of human tumors and normal tissue makes it promising that such common cell lines will stay in future as investigational models. "The National Cancer Institute's Developmental Therapeutics Program has carried out intensive studies of 60 cancer cell lines (the NCI60) derived from tumors from a variety of tissues and organs1–4" (Douglas *et al.,* 2000).

5.2. Methods

5.2.1 Plant material and extracts preparation

I. oblongata was donated by one of the traditional medical practitioners of Kopanang Dingaka association. The plant was collected in Thaba-Nchu, identified by the traditional medical practitioners and a voucher specimen (KT001/2013) was kept at the Central University of Technology Free State and authenticated by scientists at the National Botanical Gardens in Bloemfontein. Plant material (roots) were dried in the oven at 40^0C for 5 days, crushed into a fine powder and stored in a plastic jar. The plant was kept in a cool dry area in the laboratory (Mashele and Kolesnikova, 2010).

For the preparation of aqueous (H_2O), methanol and dichloromethane extracts, *Ipomoea oblongata* roots were weighed to obtain 10 g. Plant roots were dried in the oven at 40^0C for 5 days, crushed into a fine powder material. The powdered material was soaked for 72 hours in 150 ml of the different solvents and stirred using a rotary shaker. The water, methanol and dichloromethane extracts were filtered through (Whatman No. 1) filter paper. The solvents were dried at 40^0C using a rotary evaporator to remove the solvents and make the final volumes of the original plant material.

Three of the 60 human cell lines, namely MCF7 (breast), HCT116 (colon cancer) and PC3 (prostate cancer) were used in this study by sulforhodamine B (SRB) assay. SRB assay is a method developed by Skehan and collaborators to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein-basic amino acid residues of trichloroacetic acid-fixed cells. The protein dye also

binds to the fixed cellular protein under mild acidic and basic conditions. Therefore it can be extracted from cells and solubilized for measurement. The SRB assay was performed at the Council for Scientific and Industrial Research (CSIR) in accordance with the protocol of the Drug Evaluation Branch, NCI, and has been adopted for the specific screening of cancer. Maintenance of cell lines as monolayer cell cultures was routinely done according to Table 5.1:

Table 5. 1 Material used for the cancer assay

Temperature	Gas	Contents
37°C	5% CO2	5% fetal bovine serum
37°C	95% air	2 mM L-glutamine
	100% relative humidity in RPMI	50 µg/ml gentamicin

Blank = complete medium without cells, Control = cells without drug addition; Standard = Etoposide

The 96-well micro-titer plates were inoculated at a plating density of 7-10 000 cells/well (figure 5.1.). Inoculation time was 24 hours refer to figure 5.4. One plate was fixed with trichloroacetic acid (TCA), incubation time to present measurements of cell population for each cell line at the T0 (time of drug addition). Other plates with cells were treated with the experimental drug refer to figure 5.3., (*I. oblongata* extract). The extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in medium to produce concentrations of 6.25-100 µg/ml / 0.01-100 µM and the vortex process is shown in figure 5.2.. The second incubation time was 48 hours after addition of the test

compound (extracts) refer to figure 5.5.B. Viable cells that were fixed at the bottom of each well were washed with 50% TCA (figure 5.5.C), dried and the SRB dye was added as shown in figure 5.5 A. The unbound dye was removed. Protein-bound dye was extracted with 10 mM Trisbase to determine optical density as shown in figure 5.5.D. The optical density was measured at 540 nm using a multiwall spectrophotometer. Absorbance readings were used to calculate net percentage of cell growth. TGI means total growth inhibition. Ti means optical density of the test well after 48 hours of exposure. T(0) means optical density at time zero. C means control optical density.

[(Ti-T0)/(C-T0)] x 100 for concentrations at which Ti≥T0

[(Ti-T0)/T0] x 100 for concentrations at which Ti<T0.

The results of five dose screening were reported as TGI .The TGI is the concentration of the test drug where 100 x (T-T0)/(C-T0) = 0. The TGI signifies a cytostatic effect where cells are neither splitting nor growing. The assay conditions were as follows: one sample was screened with the test sample concentration of about 100- 6.25 µg/ml (5 x 2-fold serial dilutions). The standard (Etoposide) concentration was 100 - 6.25 µg/ml (5 x 2-fold serial dilutions) and the absorbance were read at 540 nm.

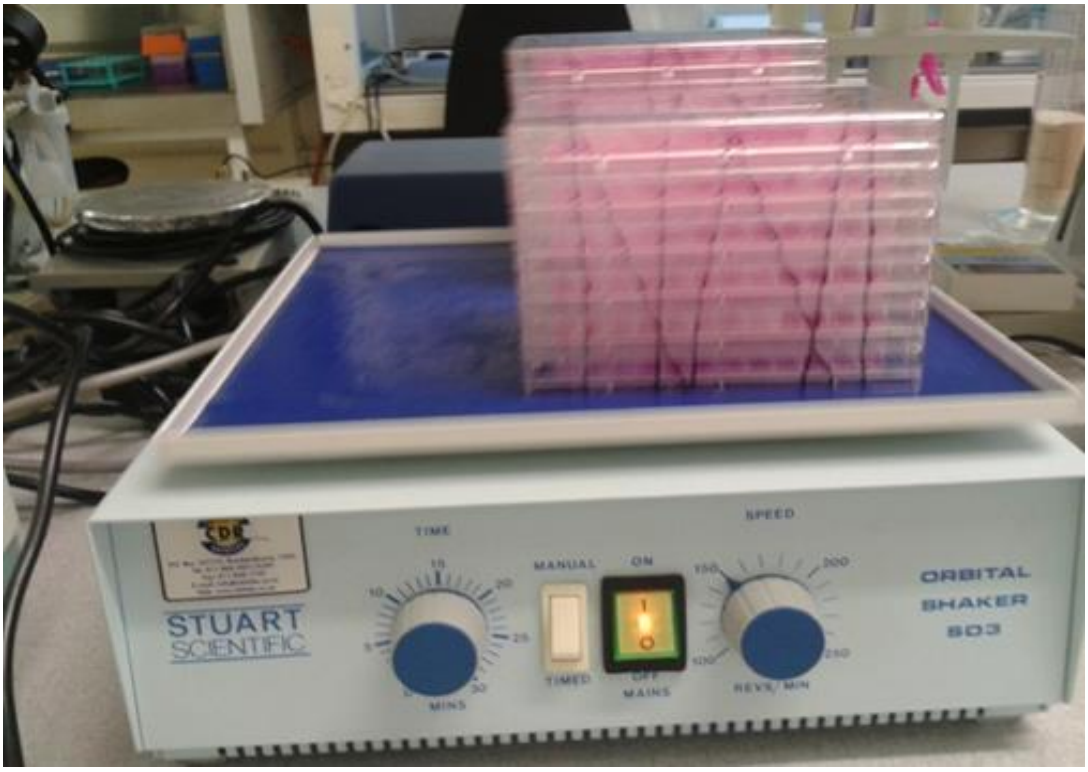

Figure 5. 1 Cell lines in the 96 microtiter plate placed on orbital shaker at 150rpm

Figure 5. 2 Vortex Process of I. oblongata extract

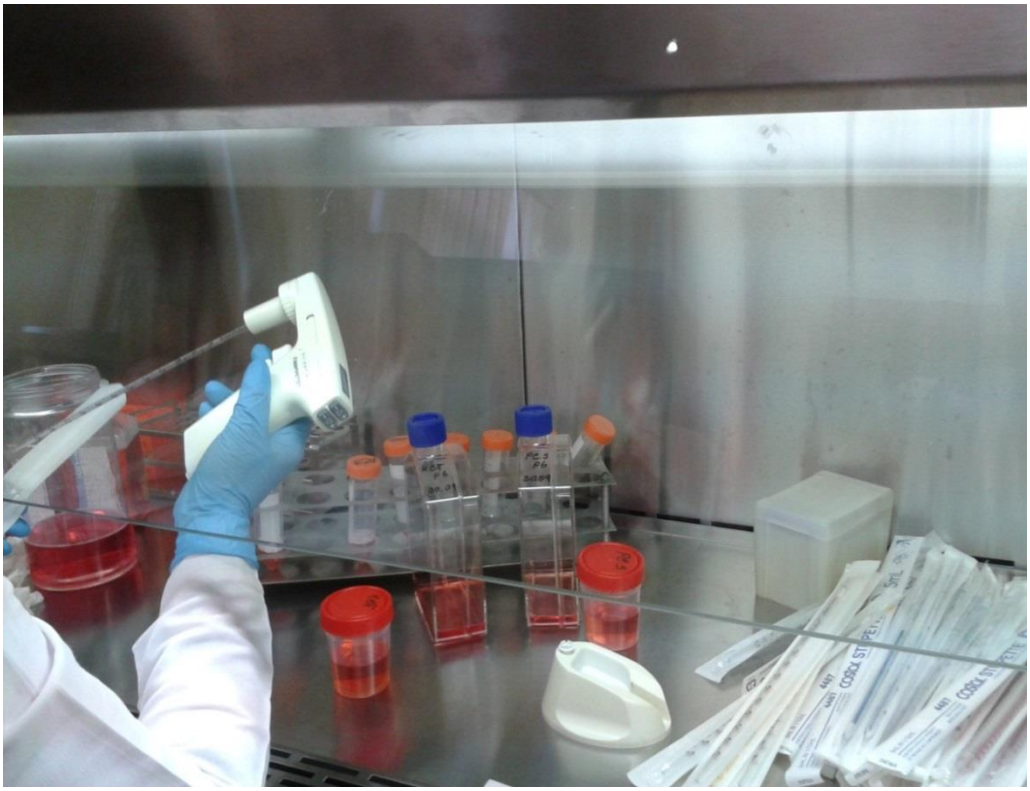

Figure 5. 3Inoculation of cell lines in different medium

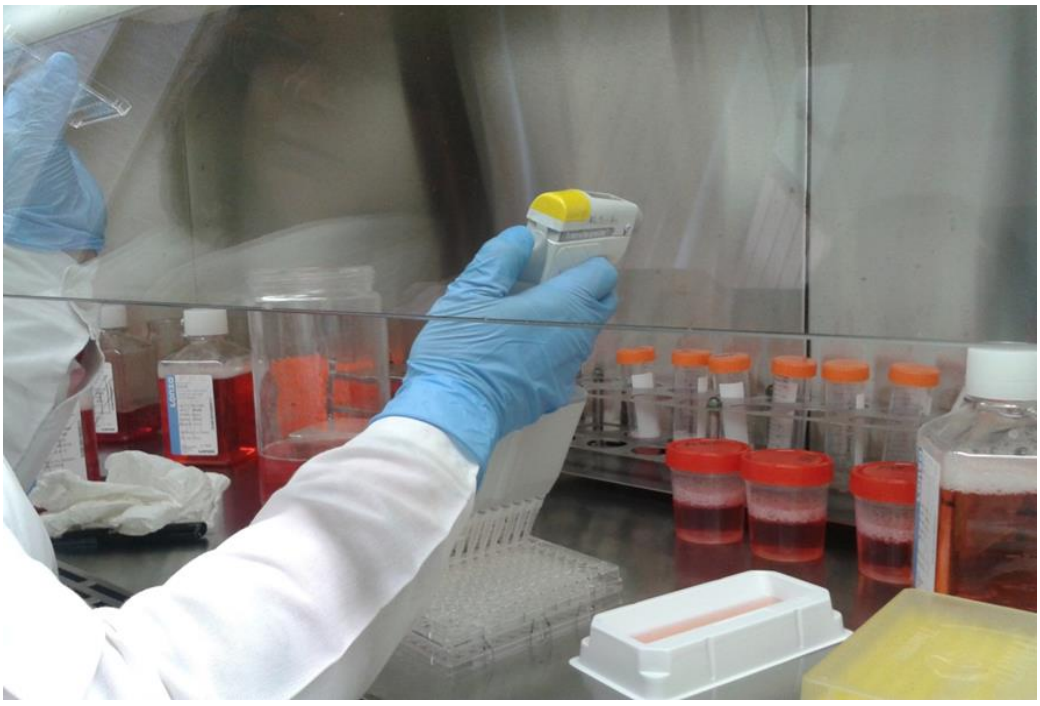

Figure 5. 4 Inoculation of cell lines in a microtiter plate using multi- pipette

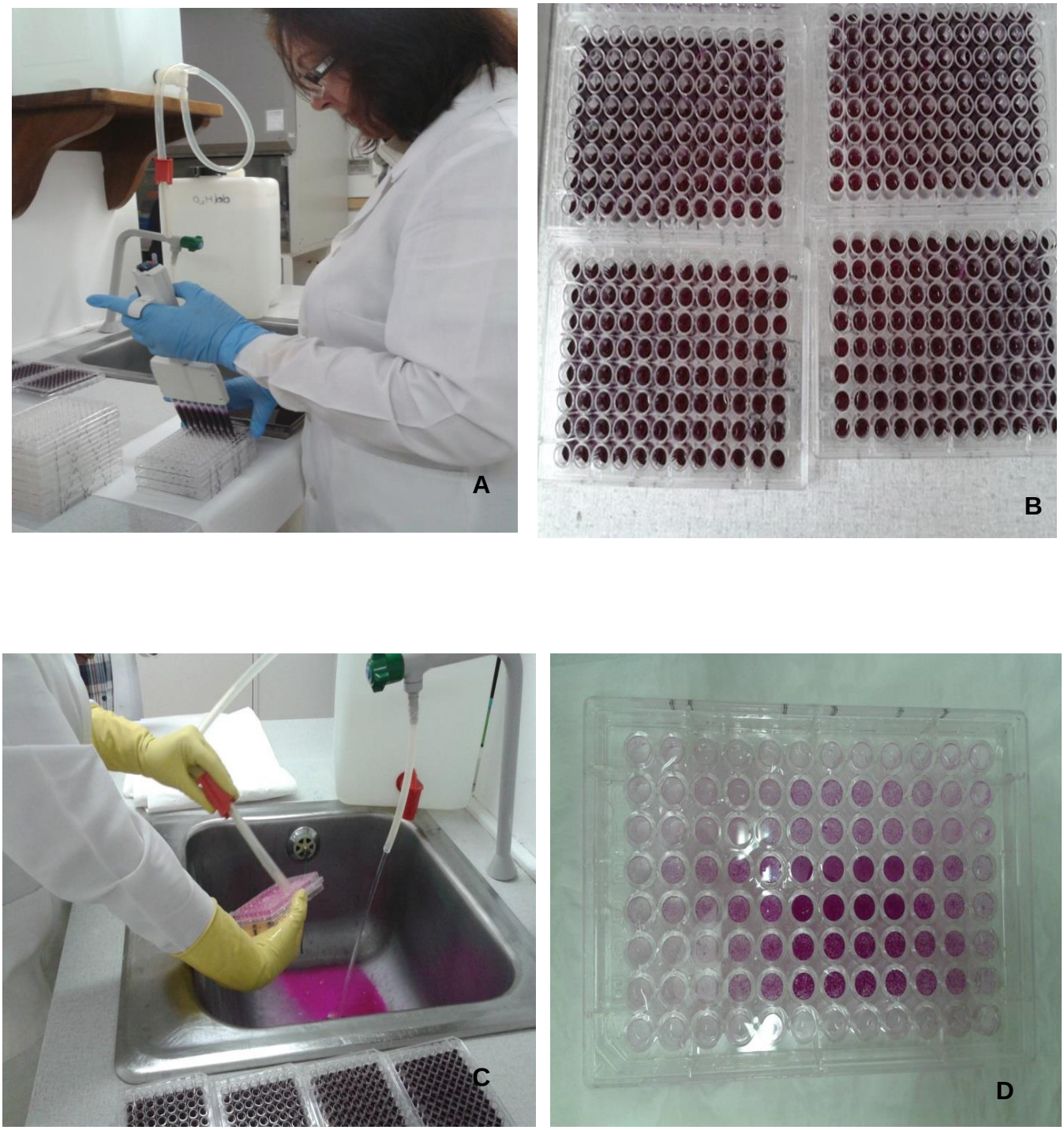

Figure 5. 5 A: Addition of Sulforhodamine B (SRB dye)

B: Unbound cell lines dyed and incubated at 48 hours

C: Rinsing of SBR dye from cell lines **D: Washed cell lines**

5.3. Results and Discussion

For each tested compound, four response parameters, GI50 (50% growth inhibition and signifying the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in TGI and signifies the cytostatic effect of the test agent), LC50 (50% lethal concentration, which signifies the cytotoxic effect of the test agent), LC100 (100% lethal concentration, signifying the cytotoxic effect of the test agent), were calculated for each cell line (Mashele and Fuku, 2011) with the Z' Factor equal to 0.8-0 for absorbance at 540 nm. According to the criterion of the CSIR, Pretoria, the extracts are considered inactive if the parameter TGI for two cell lines is higher than 50 µg/ml.

The methanolic extract of *I. oblongata* was considered inactive for all three cell lines (MCF7, HCT116 & PC3) in all four parameters used in determining the cell viabilities and cytotoxicity to the cancer lines by using the lethal concentration (LC) parameter at 50% and 100%. The positive control (Etoposide) was also inactive for all cell lines within the four parameters, especially TGI, LC50 and LC100, which are significantly higher (>100). The growth inhibition at 50% (GI50) for the cell lines MCF7, HCT116 and PC3 for the drug Etoposide was less inactive compared to that of the crude extract, which was highly inactive. The TGI value was significantly higher than the required TGI to be regarded as potent (refer to figure 5.1 and figure 5.2 as indicated above).

Table 5. 2. CSIR criteria for anticancer activity

TGI	Status	TGI	Status
> 50 µg/ml	Inactive	< 15 > 6.25 µg/ml	Moderate activity
< 50 >15 µg/ml	Weak activity	< 6.25 µg/ml	Potent activity

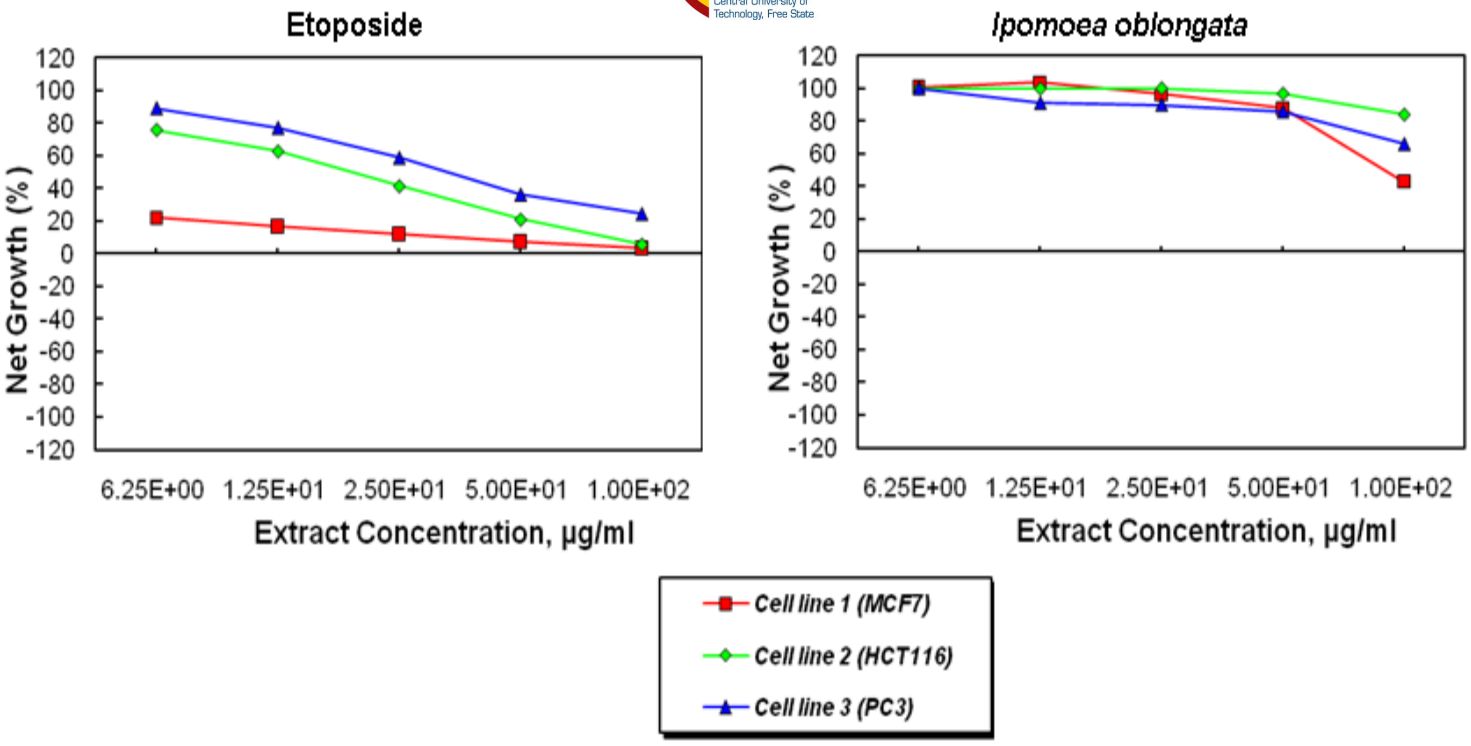

Figure 5. 6 Anti-cancer activity of Etoposide and the methanolic extract of Ipomoea oblongata

Table 5.3. Anti-cancer activity of *Ipomoea oblongata*

Extract	Activities	GI50	TGI	LC50	LC100	Status
Etoposide	**MCF7**	<6.25	>100	>100	>100	**Inactive**
	HCT116	19.96	>100	>100	>100	**Inactive**
	PC3	34.64	>100	>100	>100	**Inactive**
Ipomoea oblongata	**MCF7**	92	>100	>100	>100	**Inactive**
	HCT116	>100	>100	>100	>100	**Inactive**
	PC3	>100	>100	>100	>100	**Inactive**

Key notes; (GI50 = 50% growth inhibition), (TGI = total growth inhibition; drug concentration resulting in total growth inhibition), (LC50 = 50% lethal concentration), (LC100 = 100% lethal concentration)

South Africa has contributed significantly to research for potential therapeutic drugs using medicinal plants, which were used and are still used by traditional medical practitioners to treat a list of diseases including cancer. Traditional Medical Practitioners used infusions without any scientific research into their efficacy and safety but still showed remarkable results after use for a long time. For example, in KwaZulu-Natal, *Cyphostemma natalitium* (Vitaceae), a Zulu traditional medicinal plant, was evaluated for anti-inflammatory and anti-microbial activity and showed potential in treating cancer patients. Other potential cancer chemopreventive agents such as resveratrol (with anti-inflammatory and anti-mutagenic properties) from *Vitis vinifera* have been derived from Vitaceae (Lin *et al.,* 1999).

The plant *I. oblongata* was inactive for anticancer activity, but its traditional uses by traditional medical practitioners were reported to have anti-inflammatory activities. Anticancer activity of *Ipomoea oblongata* and the etoposide are represented in table 5.3, showing TGI, LC50 and LC100 of more 100% inhibition of the breast, colon and prostate (MCF7, HCT116 and PC3). The GI50 of the etoposide in all 3 cancer cell lines showed cell inhibition of less than 100% as wells as the breast cancer cell line of *Ipomoea oblongata*. Cell viability of the test extract and the standard are presented in figure 5.7., in all the 3 cancer cell lines. The results were anticipated to have some anticancer activity since *I. oblongata* had claims of anti-inflammatory properties and its methanolic extract of showed antioxidant activities in chapter 4. As a result the anti-cancer screening will be repeated using different cell lines and control to eliminate any errors that might have occurred during the experiment.

The breast cancer cell line (MCF7) growth was inhibited (inactive) by the extracts of *Hypoxis hemerocallidea* (Steenkamp & Gouws, 2006). There is still a need to develop drug agents from medicinal plants. In a study conducted on the anti-cancer activity and cytotoxicity of *Bauhinia variegata* extracts, the ethyl acetate extracts showed cytotoxicity to leukemia (THP-1) and breast cancer (MCF-7) cell lines, whereas the aqueous fraction was the most potent extract with anti-cancer activity and considerable cytotoxic effect on all cell lines used (Mishra *et al*., 2013). The cellular composition of the cell lines used in this study could have contributed to the inactivity of the *I. oblongata* and the Etoposide.

Extensive research was done using plant extracts and cell lines in an attempt to identify the chemotherapeutic and chemoprevention agents that can offer potent anti-cancer therapy with fewer or no toxic effects compared to the current chemotherapy (Mishra *et al*., 2013). The anti-cancer effect produced by plant extracts stems from bioactive compounds such flavonoids, saponins and anthraquinones that function like antioxidants. *I. oblongata* has the potential for anti-cancer activities owing to the presence of flavonoids and high levels of antioxidants, as shown in chapter 4, figure. 4.1.

5.4. Conclusion

The extracts of *Ipomoea oblongata* and the standard Etoposide were inactive for the cell lines MCF7, HCT116 and PC3, but further anti-cancer screening should be done using different standards and cell lines.

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Chapter 6

Antidiabetic and Cytotoxicity Activities

6.1. Introduction

Diabetes mellitus is an endocrine disorder characterized by abnormal protein, carbohydrates and fat metabolism. This can result from lack of normal insulin production and release by the islet cells of the pancreas, causing degenerative diseases (Marles and Farnsworth, 1995; Patel *et al*., 2012a). Complications associated with diabetes mellitus are cardiovascular ailments, stroke, blindness, neuropathy, gangrene, nephropathy and kidney failure (Marles and Farnsworth, 1995). According to WHO there are three types of diabetes. Type 1-insulin-dependent diabetes mellitus is characterized by lack of or insufficient insulin production. It is treated by injecting the patient with insulin to prevent catabolic flow resulting in diabetes ketoacidosis, coma and even death and β-cells causing further insufficient insulin secretions in the pancreas (Marles and Farnsworth, 1995).

Type 2-non-insulin-dependent diabetes mellitus (NIDDM) is regarded as a decrease in β-cells compared to α-cells. There is enough insulin to oppose the ketogenic processes of glucagon but it is unable to prevent hyperglycaemia. NIDDM mostly occurs in elderly (above 65 years) and obese people and it can be corrected with diet and drugs from natural sources with minimal side effects (Marles and Farnsworth, 1995). It is also a progressive ailment identified by higher levels of glucose caused by the inability of the pancreas to secrete insulin, hyperglucagoneamia and decreased insulin sensitivity (Marles and Farnsworth, 1995; Vilsboll *et al*., 2009). Type 3 is malnutrition–related diabetes mellitus (MRDM), which occurs in young people with a history of malnutrition such as kwashiorkor and other diseases from tropical areas in developing countries (Marles and Farnsworth, 1995).

Type 2 is the form of diabetes mellitus studied most intensively because of its micro- and macrovascular complications resulting in damage to organs such as the heart, eyes, blood vessels, nerves and kidneys (Vilsboll *et al*., 2009). It has become the most important research aspect in seeking drug derivatives from medicinal plants to assist in regulation of glucose homeostasis (Vilsboll, *et al*., 2009). Any plant that will provide this mechanism will prevent adverse events such as death, weight gain, hypoglycaemia and the risks associated with macro- and micro-vascular complications (Vilsboll, *et al*., 2009).

Conventional treatment methods for Type 2 diabetes do not address the serious progression fairly. The low levels of β-cells mass are washed out. There is a need to supplement treatment with insulin injections or equivalents that do not result in weight gain and hypoglycaemia (Vilsboll *et al*., 2009). Sulphonylreas (SU) has been shown to intensify apoptosis in normal human beta cells. There are complications with the use of SU such as increase in body weight and hypoglycaemia (Vilsboll, *et al*., 2009). Vilsboll *et al*. (2009) reported findings about anti-diabetic drugs from traditional plants such as thiazolidinedione (TZDs), repaglinides and metformin with alpha glycosidase inhibitors. These drugs have adverse effects such as weight gain, hypoglycaemia and gastrointestinal effects (Vilsboll, *et al*., 2009).

Researchers aim to find and isolate other compounds from plants that lower the blood glucose with as few side effects as possible, preferably none. Many more plants such as *I. oblongata* in Southern Africa can be explored as new leads in diabetes mellitus treatment and in view of its antioxidant activity discussed in chapter 5, it could possibly be a potent anti-diabetic agent. Valuable drug leads for diabetes can be derived from

herbal remedies to help in the pathology of diabetes mellitus. The cost and availability of medication will be reduced for many developing countries, unlike the current treatment (Marles and Farnsworth, 1995). A review was conducted on 1200 plant species and reported ethnobotanical uses in treating diabetes (Marles *et al*., 1995). The data reported on the mechanisms of action, hypoglycaemic activity bioassays of hypoglycaemic agents and cytotoxicity problems associated with active constituents of medicinal plants used in diabetes treatment (Marles and Farnsworth 1995). A study was conducted on 65 medicinal plant species with hypoglycaemic properties, which included part of the plants used, mode of reduction in blood glucose and active phytochemical contents having insulin properties (Patel *et al*., 2012b).

6.2. Materials and Methods

6.2.1. *In vitro* glucose uptake model in C2C12 muscle cells

The cells (C2C12) were cultured in DMEM medium containing 10% fetal bovine serum at 37ºC in humidified air with 5% CO_2. Muscle C2C12 cells were seeded at a density of 4 000 cells per well into a 96-well culture plate and cultured for three days. C2C12 cells were differentiated for another two days in DMEM supplemented with 2%. After differentiation, cells were acutely exposed for three hours to the methanol, water. The extracts were diluted at concentrations of 50 µg/mL, metformin (1 µM), insulin (1 µM). The solvent control (DMSO/water) was added to the modified DMEM media supplemented with 8 mM of glucose. The glucose concentrations remaining in the wells were determined using a commercial fluorimetric kit. After three hours of exposure to the media containing glucose, glucose uptake was calculated by subtracting the glucose

concentration remaining in the test wells from the glucose concentration of media measured in wells not containing cells. The cell passage number was <10. All samples were tested in triplicate (Medical research council SA).

6.2.2. *In vitro* glucose uptake model Chang liver cells

The Chang cells were cultured in EMEM growth medium containing 10% fetal bovine serum at 37ºC in humidified air with 5% CO_2. Cells were planted at 6 000 cells per well into a 96-well culture plate and cultured for up to five days. Thereafter cells were acutely exposed for three hours to methanol, water and decoction extracts at different concentrations of 50 µg/mL, metformin (1 µM), insulin (1 µM). The solvent control (DMSO/water) was added to the modified EMEM media supplemented with 8 mM of glucose. The glucose uptake was determined after three hours using a glucose oxidase fluorimetric assay. The glucose uptake from the media was calculated as described for C2C12 muscle cells. The cell passage number was <10, (Medical research council SA).

6.2.3. Glucose detection method

The measurement of glucose concentration in the media was determined using a glucose oxidase fluorimetric assay. Fluorescence was measured at Ex/Em = 535/587 nm using a BioTek FX800 fluorimeter. According to the protocol of Biovision, glucose oxidase plays an important role in oxidation of beta-D-glucose into hydrogen peroxide and D-glucacon-1-5-lactone and is finally hydrolyzed into gluconic acid. Glucose uptake fluorimetric assay is a non-radioactive, sensitive reaction used to determine glucose uptake at low concentrations and can be applied in various cell lines, as shown in figure 6.3, b, c and d with the protocol standards (Biovision, USA). Glucose uptake is an

important biological process for studying cell signaling and glucose metabolism. The glucose transporters metabolize glucose into 2-Deoxyglucose-6-phosphate (2-DG6P) and during this stage excess glucose accumulates in the cells and cannot be metabolized further (Biovision, USA) as shown in figure 6.1. The accumulated 2-DG6P is oxidized by enzyme reaction and later coupled with Pico Probe (Biovision, USA), which produces fluorescence in the presence of NADPH.

6.2.4. Cytotoxicity assay

The cells' viability in the study was 100% that was represented in wells and assessed using the Mossman tetrazole test (MTT) assay. The cell passage number was below 10. The samples were tested in triplicate in a single experiment. MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium-bromide is a colorimetric assay that measures the reduction of the yellow MTT color by mitochondrial dehydrogenase to dark purple formazan crystals in living cells (Mosmann, 1983; Ulukaya *et al*., 2008). The solubilized formazan precipitate was measured by spectrophotometer at 570 nm. The method used in this study was described by Mosmann (1983), and carried out in collaboration with the Medical Research Council.

6.2.5. Data analysis

The data generated was analyzed using Microsoft Excel by calculating the mean and standard deviation and by graphical representation. Results are expressed as mean and standard error mean (SEM) of a single experiment with triplicates per sample.

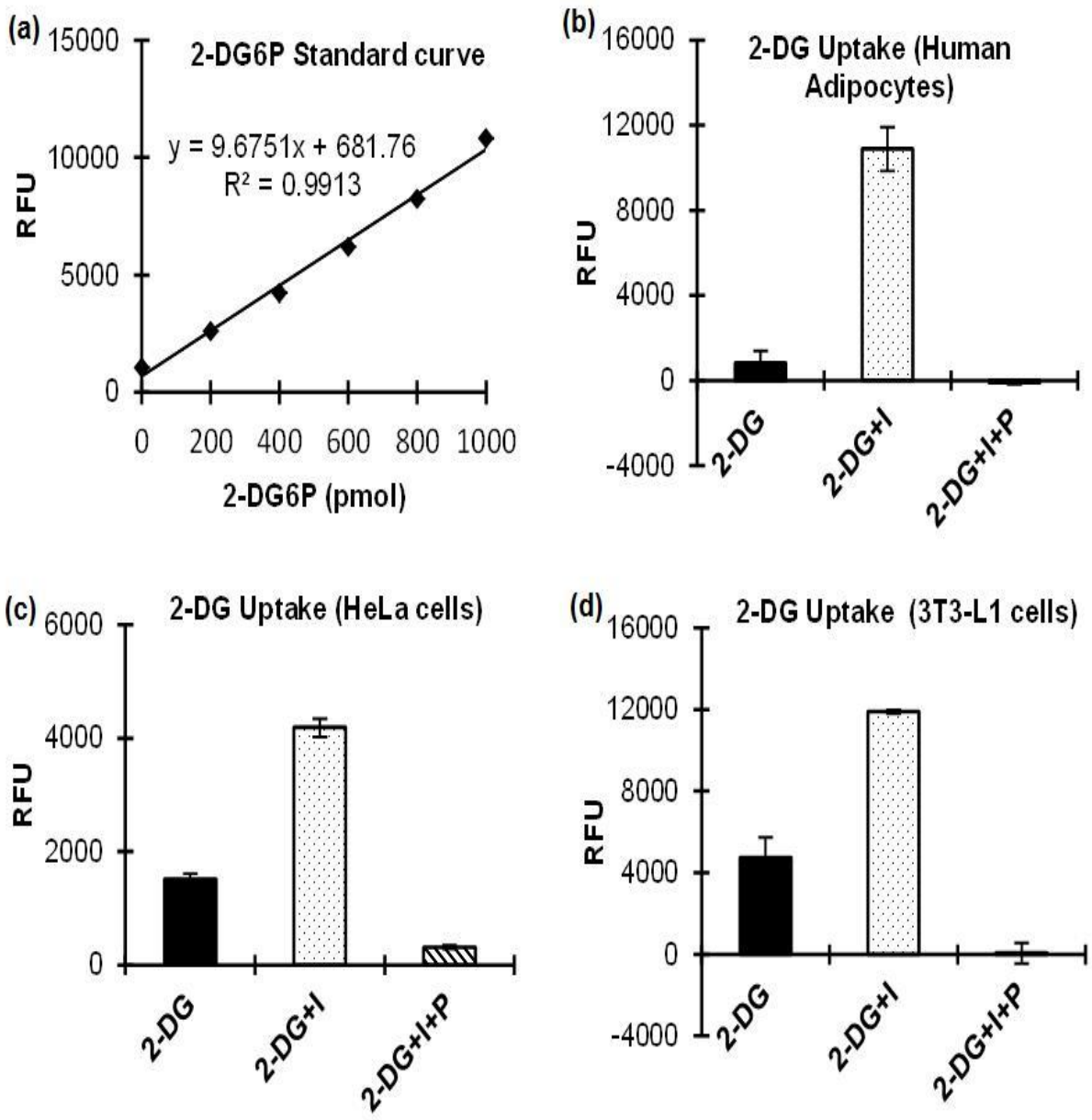

Figure 6. 1 Glucose Uptake Fluorimetric Assay kit (Biovision, USA)

6.3. Results and Discussion

The percentage glucose uptake for antidiabetic activity of *Ipomoea oblongata* methanol extract was 66.3% lower than the vehicle control (20% DMSO) 100% and metformin with 157% in the muscle cell line. The insulin was 193% glucose uptake as compared to

the water extract that was 182% higher than vehicle control and metformin when compared to insulin (see table 6.1). Therefore the water extract showed antidiabetic activity in muscle cell lines similar to that of insulin. In table 6.2., the antidiabetic percentage glucose uptake in the chang liver cancer cell lines displayed activity in both water (88.40%) and methanol (99.81%) extracts which are comparable to the vehicle control (100.9%). Metformin (116.79%) and insulin (114.41) illustrated higher levels of antidiabetic properties in the glucose uptake of chang cells. Cytotoxicity is represented in figure 6.1., with methanol and water extracts having cell viability below 100%. The plant is regarded as a low hazard plant.

Table 6. 1 Anti-diabetic percentage gGlucose uptake in C2C12 cell line

	20% DMSO	Metformin	Insulin (1)	Aqueous extract	Methanol extract
Ave	100	**157.02**	**192.23**	**182.35**	66.3
SD	1.88	34.13	54.59	47.05	3.31
SEM	0.94	19.7	19.3	27.16	1.65

Table 6. 2 Anti-diabetic percentage glucose uptake in Chang cell lines

	20% DMSO	Metformin	Insulin (1)	Aqueous extract	Methanol extract
Ave	100.9	**116.79**	**114.41**	**88.40**	99.81
SD	12.60	1.28	3.02	24.09	4.16
SEM	4.20	0.57	1.35	9.84	1.70

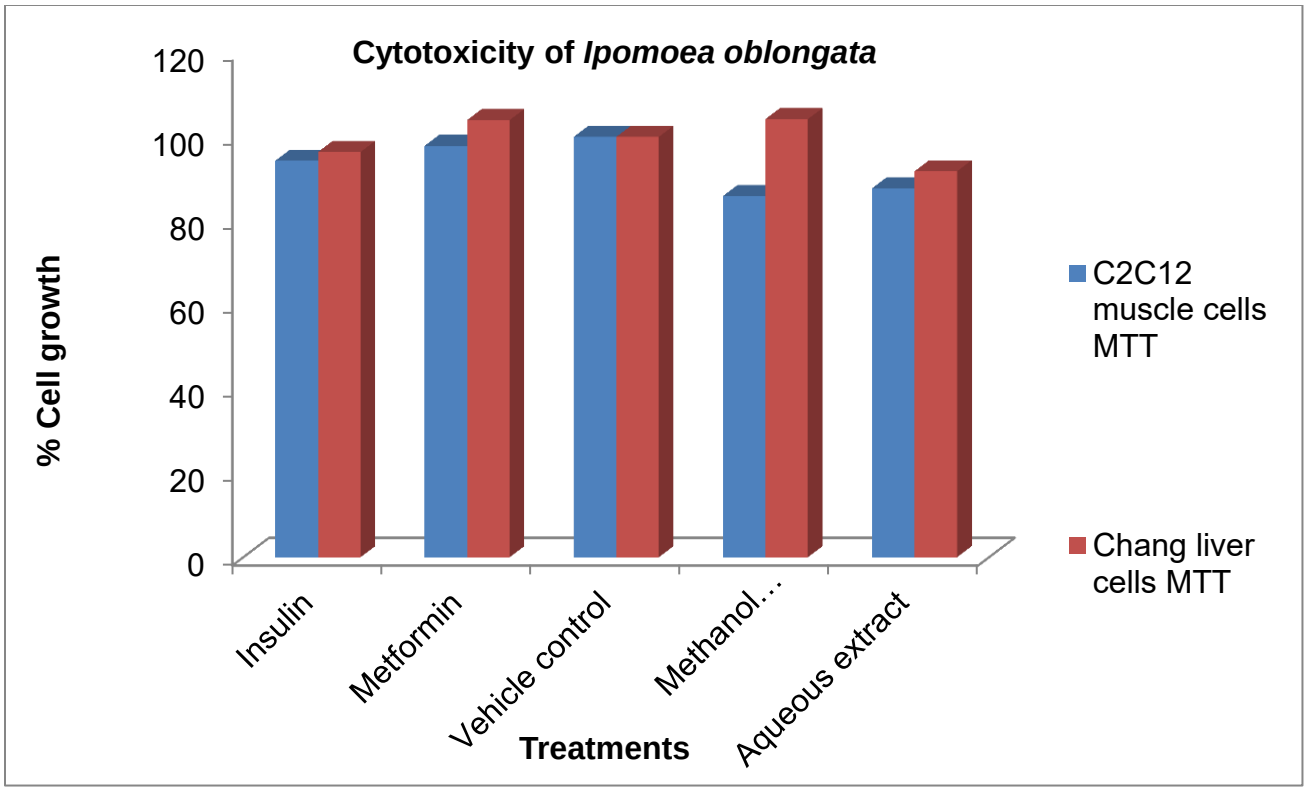

Figure 6. 2 Cytotoxicity using MTT assay in C2C12 cell lines and Chang cell lines

The treatment of diabetes is mainly focused on controlling plasma glucose levels and achieving insulin derivative effects (Lopez-Viseras *et al*., 2013). Glucose uptake by the aqueous extract in the C2C12 muscle cells was increased by over 182.35%, which is comparable to that of insulin (positive control) and metformin (reference drug control), suggesting good anti-diabetic activity (Table 6.1). The leaves of *Ipomoea aquatica* are reported to have anti-diabetic activity (Patel *et al*., 2012c).

However, in the Chang liver cells the water extract reduced the glucose uptake by 88.4% (refer to Table 6.2). Glucose uptake and insulin release are suggestive of hypoglycemic effects. Hypoglycemic effects are due to the action on hepatic gluconeogenesis or glycogenesis (Patel *et al*., 2012b). When there is glucose uptake

efficiency by peripheral tissue, impairment occurs as a result of decreased insulin secretion and defective cellular insulin action (Patel *et al*., 2012a). Insulin as a protein structure requires compounds such as vanadium, which has shown hypoglycaemic action used in diabetes therapy (Lopez-Viseras *et al*., 2013). The enzymatic and non-enzymatic antioxidative mechanism can reduce the generation of reactive oxygen species liable for degenerative diseases (Vilsboll *et al*., 2009). Anti-diabetic plants with different activities, such as *Cuscuta reflexa* (Convolvulaceae), containing high phenolic constituents, were described by Patel *et al*. (2012c). *Ipomoea* species have proven to be anti-diabetic plants from which compounds can be isolated for use as new diabetic drugs agents.

The methanol extract of *I. oblongata* reduced glucose activity and it is not regarded as a good anti-diabetic extract. Methanolic extract of *I. oblongata* reduced MTT activity by >15%, thus was suggestive of cytotoxicity, as shown in figure 6.5. The toxicity as indicated by the SBR assay on the Vero cells showed that the methanol extract had low hazard activity. Therefore it means that the cytotoxicity is low and can be tested further with ATP assay to confirm cytotoxicity levels. The mechanisms of action found in the tuber of *Ipomoea batata* was in the reduction of insulin resistance and blood glucose level (Patel *et al*., 2012c). This is an indication that plants from the Convolvulaceae family are strong anti-diabetic agents because of their ability to imitate insulin activities. The water extract had no adverse effect on C2C12 muscle cell viability and Chang cell viability as estimated by MTT assay at the concentration tested (50 µg/mL). It is evident that it is possible for medicinal plants to have no cytotoxicity when tested. Literature states that toxicological effects of many crude extracts have been overlooked because

of the belief that medicinal plants have better compatibility with the human body owing to fewer side effects. Cytotoxicity testing plays an important role as a standard procedure of the biological evaluation of pharmacology (Ashafa *et al*., 2013). An important discovery has been the mononuclear zinc compound's anti-diabetic activity (Lopez-Viseras *et al*., 2013). Deficiency in zinc occurs in diabetic patients and this activity thus reverses the diabetes-induced zinc status (Lopez-Viseras *et al*., 2013). Zinc affects the structural position of proteins, including insulin-enhancing activities (Lopez-Viseras *et al*., 2013). There is a need to find structural protein from medicinal plants that will not affected by the zinc.

Bioactive compounds such as polyphenols, alkaloids, flavonoids, glycosides, carotenoids, terpenoids, coumarins and other phytochemical constituents greatly contribute to the anti-diabetic activity of medicinal plants, causing reduction in blood glucose levels (Patel *et al*., 2012b). The presence of the phytochemical compounds present in *I. oblongata,* as indicated in Chapter 4 (Table 4.1), contributed to the ability of this plant to have anti-diabetic activity. The anti-hyperglycemic effect of the plants is to restore the function of pancreatic tissues. There should be an increase in insulin output and/or inhibition of intestinal absorption of glucose. It should also facilitate the metabolites available in insulin-dependent processes (Patel *et al*., 2012a).

6.4. Conclusion

Aqueous extract of the *I. oblongata* is a good anti-diabetic source, whereas the methanol extract is not a good source. Cytotoxicity is regarded to be a low hazard in

Ipomoea *oblongata* for human cell lines. The active compounds for anti-diabetic activity will be isolated in later studies.

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