Collaborative research with traditional African health practitioners of the Nelson Mandela Metropole; antimicrobial, anticancer and anti-diabetic activities of five medicinal plants

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Collaborative research with traditional African health practitioners of the Nelson Mandela Metropole; antimicrobial, anticancer and anti-diabetic activities of five medicinal plants

By

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“…the Black man does not do anything unless there is a very good reason for it … Thus the Bantu will not participate enthusiastically in a … project unless they fully understand the reason for undertaking it.” (Mutwa, 1965)
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<table>
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<tr>
<td>5’AMP</td>
<td>5’adenosine monophosphate</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AICAR</td>
<td>5’-amino-4-imidazolecarboxamide-riboside</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immuno deficiency syndrome</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMP-kinase</td>
<td>5’adenosine monophosphate activated protein kinase</td>
</tr>
<tr>
<td>ANSA</td>
<td>1-amino-2-naphthal-4-sulfonic acid</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>ara</td>
<td>Adenine 9-β-D-arabinofuranoside</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CAP</td>
<td>Cbl associated protein</td>
</tr>
<tr>
<td>CBD</td>
<td>Convention on Biodiversity</td>
</tr>
<tr>
<td>Cbl</td>
<td>Casitas β-lineage lymphoma</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSIR</td>
<td>Council for Scientific and Industrial Research</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>DEET</td>
<td>N,N-diethyl-m-toluamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Median effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum beta-lactamase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>fbs</td>
<td>Fecal bovine serum</td>
</tr>
<tr>
<td>FCs</td>
<td>Furanocoumarins</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>G1P</td>
<td>Glucose-1-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GAR-HRP</td>
<td>Goat-anti-rabbit horseradish peroxidase</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanine triphosphatase</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoproteins</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papiloma virus</td>
</tr>
<tr>
<td>HTS</td>
<td>High through-put screening</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Inhibitory concentration₅₀</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>IDV</td>
<td>Integrated density value</td>
</tr>
<tr>
<td>IK</td>
<td>Indigenous knowledge</td>
</tr>
<tr>
<td>IKA</td>
<td>Indigenous knowledge agreement</td>
</tr>
<tr>
<td>IKS</td>
<td>Indigenous knowledge systems</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPR</td>
<td>Intellectual property rights</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>KRP-buffer</td>
<td>Krebs-Ringer phosphate buffer</td>
</tr>
<tr>
<td>LD</td>
<td>Lethal dose</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>MAP-kinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MCC</td>
<td>Medicines Control Council</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MgCl2</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>Sodium hydrogen carbonate</td>
</tr>
<tr>
<td>Na2HPO4·12H2O</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Na2SO4</td>
<td>Disodium sulphate</td>
</tr>
<tr>
<td>N/A</td>
<td>Not applicable</td>
</tr>
<tr>
<td>NC</td>
<td>Negative control</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NCR</td>
<td>National Cancer Registry</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NMM</td>
<td>Nelson Mandela Metropole</td>
</tr>
<tr>
<td>NMMU</td>
<td>Nelson Mandela Metropolitan University</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NRF</td>
<td>National Research Foundation</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBSA</td>
<td>Phosphate-buffered saline A</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PDK-2</td>
<td>Phosphoinositide-dependent kinase-2</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PI3,4,5-P3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PEPCk</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome-proliferator-activated receptor-gamma</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PTP-1</td>
<td>Phosphotyrosine phosphatase-1</td>
</tr>
<tr>
<td>PTPs</td>
<td>Phosphotyrosine phosphatases</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations / revolutions per minute</td>
</tr>
<tr>
<td>Sab</td>
<td>Sabouraud</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TDW</td>
<td>Triple distilled water</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor - α</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-hydroxymethyl-1,3-propanediol</td>
</tr>
<tr>
<td>UPE</td>
<td>University of Port Elizabeth</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UWC</td>
<td>University of the Western Cape</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Thesis layout

This section has been included to familiarise the reader with the layout of the thesis. The thesis contains nine chapters including the introduction and conclusion. Each chapter contains its own relevant literature thus no formal literature chapter has been included. Footnotes used throughout the chapters are marked with a superscripted number after the reference made to the relevant footnote. The complete list of footnotes is given after chapter nine. This was done because some of the same footnote references were used in different chapters. It thus prevented the duplication of footnote references. The reference list is given after the appendices.

Chapter one introduces the reader to the context of traditional healing in South Africa. It also outlines problems regarding research into indigenous plants and in particular collaborative research with traditional health practitioners. It then provides a short history of the initiation of the research collaboration. Chapter two continues with the collaboration development, describes the dynamics of the research project and the joint establishment of a medicinal garden. Chapter three includes the plant selection for this study, literature regarding the chosen plants and extraction processes employed.

Chapters four, five and six describe the antimicrobial, anticancer and anti-diabetic screening of the aqueous and ethanol extracts of the five selected plants, respectively. Apart from the short literature sections on the screenings, the complete methodology (including reagent information) is given as well as the results and discussion. Also included in each chapter is a short description of the feedback seminar at which the results were presented to collaborating health practitioners. Chapter seven describes different experiments done to explain the anti-diabetic mechanism of a jointly chosen extract. Literature in this chapter is limited, because most of the mechanisms have been covered in Chapter six. However, the discussion of the results focuses again on the pathophysiological and biochemical pathways of type 2 diabetes mellitus. Because of the many experimental methods that were used in this chapter, reagent information is listed in Appendix E and is thus not given in the text as in the previous three chapters.
Chapter eight ties together all collaborative experiences and lessons learnt with traditional health practitioners and gives the main outcomes of the collaborative project. Chapter nine concludes and summarises shortly all the most important findings and gives some recommendations and reflective comments gained from the research experience.
Summary

The promotion and development of indigenous knowledge pertaining to the traditional African healing system is one of the prime objectives set out by the South African government. Despite excellent research opportunities and funding, the biggest problem with ethnopharmacological research is a lack of interaction with indigenous communities, which tends to dilute the benefits this research has to offer these communities. The primary aim of this study was thus to promote the traditional African healing system through collaborative medicinal plant research with local traditional health practitioners. The research collaboration aimed to validate some biological activities of traditional remedies used by collaborating traditional health practitioners and ensured interactive sessions where scientific literature, research practices, findings and relevant legislation were discussed and debated. The joint development of a medicinal garden was a valuable tool in realising these goals.

Aqueous and ethanol extracts of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* were selected for antimicrobial, anticancer and antidiabetic screening, because of their sustainable utilisation potential. The ethanol extract of *T. violacea* produced the best antimicrobial activity on *Bacillus subtilis* (100% growth inhibition) and *Candida albicans* (89% growth inhibition) at 250 µg/ml. The EC$_{50}$ for the ethanol extract of *T. violacea* against HT29 colon cancer cells was 101 µg/ml. The aqueous extracts of *B. frutescens* and *T. camphoratus* (0.5 and 50 µg/ml) produced the highest overall glucose uptake activity in Chang liver and C2C12 muscle cells. *T. camphoratus* was unanimously chosen by participating practitioners as the plant to be investigated further. The aqueous extract of *T. camphoratus* increased glucose uptake in C2C12 muscle cells through increased translocation of GLUT4 to the plasma membrane and activation of the PI3-kinase and AMP-kinase pathways. It produced some alpha-glucosidase inhibitory activity at concentrations of ≥ 200 µg/ml.
Apart from interactive feedback seminars at which the findings were presented to participating practitioners, all scientific literature regarding the plants was summarised, translated, compiled and given to participating practitioners in written format. An indigenous knowledge agreement has been negotiated and will formalise the collaboration in future. It is recommended that future research focuses on plants with economic development potential that can be cultivated in the medicinal garden.
1 Introduction

1.1 Introduction

The trade in medicinal plants is a large and lucrative industry, with more than 700 mostly indigenous species traded for an estimated R 270 million per year in South Africa (Dold & Cocks, 2002). Historically, the use of these plants by traditional health practitioners was well controlled by their training to identify toxic plants and give appropriate concentrations of these plants in remedies (Tahraoui et al., 2007; Ziyyat et al., 1997). But the current size of this industry necessitates a degree of regulation (Springfield et al., 2005), which was previously unknown to indigenous knowledge holders. The Medicines Control Council (MCC) of South Africa has been attempting to develop and implement guidelines for the regulation of commercial herbal remedies as well as traditionally used medicinal plants, but so far not much progress has been made in this regard. The MCC’s primary concern is the lack of empirical data measures of safety, quality and efficacy of these plants and remedies to adequately protect the public who use it to self-medicate (Scott et al., 2004). There is thus a need for research on medicinal plants to make regulation possible, because it would be inappropriate to forbid this trade due to historical and cultural importance in South African society (National Department of Health et al., 2006). In addition, traditional health practitioners are valuable in assisting medicinal plant research because of their experience and expertise in the clinical use of these plants (Timmermans, 2003).

Another reason for accumulating scientific knowledge on indigenous medicinal plants is the actualisation of an African Renaissance (Soodyall, 2004), which carries much weight politically. To make these goals and ideals a reality, indigenous knowledge systems (IKSs) has become a focus area of all the major research funding organizations in South Africa, including the Innovation Fund, National Research Foundation (NRF), Medical Research
Council (MRC) and Council for Scientific and Industrial Research (CSIR) (Wynberg, 2001). The information gathered by indigenous knowledge (IK) research in South Africa will be compiled into the National Reference Centre for African Traditional Medicines, which was launched in August 2003 (National Department of Health et al., 2006). The launch of this centre set out a comprehensive theoretical plan to manage and develop traditionally used medicinal plants and the IK associated with it as well as

“fostering and identifying areas of collaboration in African traditional medicines research amongst research institutions, academics, … traditional practitioner associations, non-government organizations, and government departments” (National Department of Health et al., 2006).

The South African government has thus taken the route of protecting and developing IK by publicly displaying it. In public display lies a South African claim to this knowledge and thereby the hope of preventing its misappropriation. However, nothing can truly stop unauthorised use (Timmermans, 2003). It is thus imperative for research into IK and development thereof to focus on the promotion of indigenous knowledge systems and its practitioners, and not merely the documentation and dissemination thereof.

1.2 The context of traditional healing in South Africa

The colonial and post-colonial governments of South Africa have suppressed, denounced, excluded and prohibited the practice of traditional medicine for more than a century (Ehlers, 2000; Gordon, 2001; Pauskardt, 1990). This was done under the provisions of the Suppression of Witchcraft Act of 1957, which was first introduced in 1895 and last amended in 1970 (Ashforth, 2005). After this, the Health Act of 1974 prohibited traditional practitioners not registered with the South African Medical and Dental Council from practicing (Freeman & Motsei, 1992). This propaganda has caused an inherent distrust and lack of communication between the traditional and conventional medical communities in South Africa (Grierson & Afolayan, 1999). It also provided
traditional health practitioners the freedom to exist without any statutory control of their health system.

Even though South Africa has had a democratic government for more than 10 years, there still exists a lack of communication and mutual suspicion between traditional and conventional health practitioners (Peltzer, 2001). Referral between the two systems is one way, from traditional practitioners to western doctors. In the words of a Swazi healer:

“Medical people do not often refer patients to traditional healers because they do not know what the standard is, are not sure how their patients will be treated. The medical side needs more information on the healers.” (Campbell, 1998).

In an attempt to remedy this problem, the democratic government has developed legislation based on African and international policies to try and restore the dignity of traditional medicine in South Africa. These policies include among others, the Traditional Medicine Policy of the World Health Organization (WHO) (World Health Organization, 2002), the African Union’s Decade of Traditional Medicine (African Union, 2005) and the Convention on Biodiversity (CBD). They advocate the promotion of traditional medicine through conservation, sustainable utilisation and development of medicinal plants and IK, and the fair and equitable benefit-sharing arising from the development of these resources (Heinrich et al., 2004). Consequently:

- the Traditional Health Practitioners’ Act (No 35 of 2004) (South Africa, 2004b) gives traditional health practitioners legal status and uplifts their role in society,
- the National Environmental Management: Biodiversity Act (No 10 of 2004) (South Africa, 2004a) protects indigenous knowledge holders by regulating the exploitation of natural resources and providing for remuneration in these cases, and,
- the Patents Amendment Act (No 20 of 2005) (South Africa, 2005) acknowledges the claims of the indigenous knowledge holders on commercialisation of inventions developed from indigenous knowledge.
1.3 Problem statement

In the past, much research has been conducted in fields such as ethnonursing, ethnobotany, ethnopharmacology, ethnomedicine and the social sciences. The problem was that knowledge generated by this research had done very little to increase recognition of the indigenous medical system and much of the information had not been shared with indigenous communities or respondents (Mulaudzi, 2001). This is one of the reasons why the scientific community and its allies, while trying to assist traditional health practitioners through their research, continue to meet resistance from these practitioners (Etkin, 1993).

In addition to finding IK practitioners willing to participate in research projects, physically locating them may be just as challenging (UNAIDS, 2000). This is because the practice is unregulated. It has also been suggested that indigenous knowledge providers would be more willing to participate in IK research if they were involved in the decision-making processes of the research (Botes & van Rensburg, 2000). These aspects of research into indigenous knowledge make it very difficult for researchers to enter into any type of collaborative research, because minimal planning can be done before the IK practitioners are located. In addition, funding for this type of pre-endeavour is difficult to secure. Many academic researchers are thus discouraged by the time (UNAIDS, 2000) and resources involved in the preparative processes and choose not to pursue a collaborative approach.

Another fundamental problem in fostering collaborations is the fact that the African healing system is an alternative paradigm that is in many ways opposed to current concepts of modern science (Odora Hoppers, 2004). Some even postulate that issues between the systems exist on a much deeper level:

"the conflict that colonialism initiated with many indigenous cultures was ultimately corrosive, not only for indigenous cultures, but also western ability to accurately understand and appreciate indigenous technological achievements" (Cox, 1995).
It thus remains a challenge for scientists and traditional practitioners alike to co-exist with and acknowledge the value of the other paradigm (Swazo, 2005). However difficult, the aim of ethnoresearch is to promote traditional health practitioners and restore their dignity in the only way the westerners know how to, through western science.

“The hope is to propagate willingness to tolerate contradiction, and to act generously in situations of unresolved antagonisms (Odora Hoppers, 2004).”

1.4 Primary aim

The primary aim of this study was to build a mutually beneficial research collaboration between traditional African health practitioners of the Nelson Mandela Metropole (NMM) and scientists at the Nelson Mandela Metropolitan University (NMMU) through the conducting of collaborative medicinal plant research.

1.5 Background to this study

At the end of 2000, the Department of Biomedical Science at the Port Elizabeth Technikon was approached by the spokesperson of a group of three traditional African health practitioners. Initial meetings were held between traditional health practitioners and scientists of the Departments of Biomedical Science, Biochemistry & Microbiology and Pharmacy (latter two departments were from the University of Port Elizabeth, now NMMU). During these meetings, practitioners enquired about the possibility of having their remedies tested for biological activity. They wanted scientific proof for the potential biological effectiveness of their remedies. From interactions with the practitioners it seemed that they received a lot of criticism about their practices and remedies from both inside and outside their communities as
well as the media. Literature also supports the criticism from western trained practitioners (Hoppe et al., 1998). It appeared that this group wished to prove the effectiveness of their remedies in a way that would convince these critics. This might also explain partly why practitioners were willing to let the findings be published as long as they could not be identified by the audience. Practitioners were especially interested in biological activities of diseases such as diabetes, infantile diarrhoea, cancer, sexually transmitted diseases (including HIV/AIDS) and complications pertaining to circumcision, because they often had to treat these conditions.

The product of initial negotiations was a verbal agreement. The university would test ready-made remedies supplied by traditional health practitioners for *in vitro* anti-diabetic and antidiarrhoeal activities, without knowing the ingredients contained in the remedies. It was decided to share expenses for the laboratory testing of traditional remedies. The health practitioners would carry the cost of making the traditional remedies and the university would carry the costs for testing the remedies and providing feedback to the health practitioners on the results. The feedback was provided during private sessions with the practitioner who manufactured the remedy and included an explanation of methods used for testing and the interpretation of results with the assistance of a translator. The preliminary nature of the results was emphasised during the feedback discussions and that it did not conclusively prove the effectiveness of the remedy. This initial agreement was the prelude to a comprehensive collaborative research project between the NMMU and traditional health practitioners of the NMM.
2 Research setting

2.1 Introduction

The first chapter provided the context of where traditional African healing fits into the South African environment and a summary of the establishment of the initial collaboration. This chapter will introduce the reader to the paradigm of traditional African healing, the research setting and the collaboration development process.

The research was conducted in the Nelson Mandela Metropole (NMM), which is situated on the south coast of the Eastern Cape Province of South Africa. The NMM incorporates Port Elizabeth city and Uitenhage magistrate district (Figure 2.1) (Department of Social Development, 2006). It has one university, namely Nelson Mandela Metropolitan University (NMMU) which was established January 1st 2005 according to the government’s ‘Programme for the Transformation of Higher Education’ (National Department of Education, 2005). The NMM has a population of just more than one million people of which 59% are African (Department of Social Development, 2006) and approximately 57% speak isiXhosa (Eastern Cape Province, 2001). Furthermore, the people of the Eastern Cape tend to be more traditional and rural, and 41% of households still live in traditional dwellings (Dold & Cocks, 2002). To better understand the participating practitioners’ views and subsequent decisions in the collaboration process (Babbie, 2005), it will be important to firstly become familiar with the concepts of the traditional African paradigm.
Figure 2.1 The Nelson Mandela Metropole. The area incorporates the Port Elizabeth and Uitenhage magisterial district. The two black dots indicate the location of the medicinal garden in the KwaZakhele area and the Nelson Mandela Metropolitan University in the Summerstrand area.

2.2 The African paradigm

Traditional African healing is an ancient form of healing that has been developed for over 10 000 years (African Union, 2005). During this time traditional health practitioners acquired their knowledge and skills from observation, spiritual revelation, personal experience, training and from direct information supplied by their predecessors (African Union, 2005). It is a holistic system of healing which manages the unwell person on a physical, psychological, social and spiritual level (Onwuanibe, 1978). However, in the traditional paradigm no clear distinction is made between these levels (Ngubane, 1986).
2.2.1 Beliefs and theories

As with most traditional medical systems, the African system is based on certain beliefs and theories of the cosmos (Longmore, 1958). According to African lore the human being has two souls, the *ena* and the *moya* (Mutwa, 2003). The *moya* is the immortal soul, which passes through many evolutionary stages. However, for the purposes of this discussion the *ena* will be the focus. The *ena* develops as the person develops, from memories and experiences and has the form of the physical body, but is of a spiritual substance. After death the *ena* enters a higher state and is in close contact with the gods (Broster, 1981). These *ena*s are referred to as ancestral spirits and are greatly respected by the African community.

“This belief that a man lives solely to serve his ancestors is one of the most deep-rooted beliefs in the whole Africa, and tribal unity is based on this” (Mutwa, 1965).

For the *ena* to remain in a state of communication with the gods, it needs nourishment in the form of prayers and sacrifices of the living, otherwise it passes out of existence and a valuable communication with the gods is lost (Mutwa, 2003). Traditional health practitioners are the custodians of this culture of ancestor worship and serve as link between the African community and the spiritual world (Broster, 1981). The ancestors guide traditional health practitioners in the keeping of a peaceful and prosperous existence of their communities by the implementation of a strict code of conduct, named taboo’s (section 2.2.3).

2.2.2 Training

Training of traditional health practitioners is a complex process of attaining indigenous knowledge (IK) through different practices of purification and sacrifice. The process of becoming knowledgeable is a personal journey (Chikonzo, 2006). This is a major reason why IK is not shared with people who did not accomplish the specific requirements for its attainment (Odora Hoppers, 2004) and is kept secret to all who are not part of it (Kiteme, 1976). African IK is only transmitted orally (Ndeti, 1976) and / or through visions and dreams brought on by the ancestors. In addition, the ancestors prohibit the written documentation of IK and there is thus no requirement for apprentice
health practitioners to be literate. According to a Zulu healer (Campbell, 1998)

“… a child might be possessed or chosen. The ancestor wants that child to be out of school, to stop their formal education. You ask: “How can that be good” … But I say: “What is a schooling? Is this education?” This European education, it is only a small view.”

There are five main groups of traditional health practitioners which each have their own set of criteria for training (Abdool Karim et al., 1994). The five groups include diviners, herbalists, faith healers, traditional surgeons and traditional birth attendants.

Diviners, sangomas or amagqirha as they are called in isiXhosa do not choose to become health practitioners but are called by the ancestors into the profession (Broster, 1981). The calling is referred to as ukuthwasa and is manifested as an illness that can only be healed by a qualified traditional health practitioner by initiation into the traditional health system. Training is focused on interpretation of dreams and visions initiated by the ancestors and in this way training is unique for each individual.

“There is a training order that is followed. The ancestors guide each trainer; show them how to train and when to teach each important task. Each trainee is encouraged according to his or her talents; some may learn more or different types of healing practices than others. It all depends on the individuals, what they are ready to learn” (Campbell, 1998).

Training may thus last from one to ten years (Campbell, 1998). After graduation, diviners serve the community by acting as a medium to communicate between the spiritual and material worlds (Freeman & Motsei, 1992). They diagnose the cause of illness either by divining bones, through spirit possession or other supernatural methods (Chavunduka, 1994), depending on the training of the individual and his or her ancestors (Campbell, 1998). It is estimated that about 80% of diviners are women (Abdool Karim et al., 1994). The current collaboration is primarily with practitioners from this group.

Herbalists, inyange or ixhwele could be compared to pharmacists. They are experts of medicinal herbs but are not necessarily called into the profession.
Training may last a few years to ten years. They are mostly men (Abdool Karim et al., 1994). Faith healers or umthandazi are professed Christians and heal through prayer, lying on of hands and providing holy water or ash. They believe that their healing powers come directly from God (Ehlers, 2000). Traditional surgeons or iingcibi are trained men who have experience in conducting traditional circumcision (Ashforth, 2005). Traditional birth attendants are usually elderly women who have had at least two babies. Their training includes 15 to 20 years of apprenticeship and they handle all aspects of antenatal teaching, labour and delivery of babies (Abdool Karim et al., 1994).

2.2.3 Traditional African health and illness

According to the African belief system, diseases may be caused by natural or supernatural events (Abdool Karim et al., 1994). Diseases caused by natural agents are usually predictable in their duration and symptoms (Setswe, 1999). Medicinal plants or conventional medicine may be prescribed to resolve these diseases (Abdool Karim et al., 1994). However, when the duration and symptoms of a disease become unpredictable, it is ascribed to supernatural causes and it is important to resolve this type of illness with the aid of a traditional health practitioner (Chavunduka, 1994). When seeking the council of a traditional health practitioner, the patient and family of the patient would already have a suspicion as to the cause of the disease (Chipfakacha, 1994). Witchcraft or the breaking of a taboo which resulted in ancestral wrath and subsequent illness are the two most common causes of supernatural diseases (Onwuanibe, 1978).

In addition, it is commonly accepted that health is not only related to physical aspects, but also the mental, spiritual and social well-being of a person (Ndeti, 1976). The treatment of disease from whatever cause thus follows with a holistic approach to the person. Diseases are believed to manifest as a result of disharmony between any physical, mental, spiritual or social aspects affecting the sick person. These different concepts need to be brought into balance in order to cure the person (Kiteme, 1976). It is thus natural that treatment offered by traditional medicine generally includes diet, exercise and
Rituals. Traditional remedies and treatments include infusions or decoctions made of mixtures of herbs and other natural materials (Green & Makhubu, 1984). Plant material may also be dried and powdered to be used as snuff or it may be burned and the smoke inhaled (Abdool Karim et al., 1994). Traditional creams and ointments are provided for external application. Herbal enemas are extensively used. Plant material may be prescribed for bathing, inhalation steaming or full body steaming. Plants may also be given to the patient to wear as charms (Abdool Karim et al., 1994). Any traditional treatment prescribed is prepared by the traditional health practitioner on an individual basis for each patient.

Rituals are often prescribed when the cause of an illness is as a result of breaking a taboo. The Xhosa word for taboo is amaconini and it is basically a set of rules that covers all aspects of daily life and has to be adhered to in order to preserve the orderly and harmonious existence of the individual as well as the community at large. Each age group has its own set of rules regarding dress, beadwork, songs, dances, allocation of work, food and most other activities (Broster, 1981). In addition to appeasing the ancestors, rituals incorporate family, friends and the community as part of the cure (Setswe, 1999).

Before the ritual, the family is advised to retreat to a place close to nature for example the river and observe the essence thereof. When this is done, they invite family, friends and members of the community to a feast at the patient’s family home, which will involve slaughtering either a chicken, goat and/or cow depending on the severity of the punishment (Onwuanibe, 1978). This is excellent group therapy (Kiteme, 1976) for fixing relationships that may have caused the individual guilt, worries or heartache (Money, 2001; Okpako, 1999) in addition to other beliefs about the cause of the illness.

From this brief introduction, it can be seen that medicinal plants are but a small part of the whole African healing system. A long-term collaboration with traditional health practitioners may thus involve actions above and beyond simply the testing of traditional remedies.


2.3 Research setting

Negotiation is innate to the African culture. Accordingly, research policy suggests that indigenous people be involved in the planning, decision-making and execution of projects utilising IK (Moran et al., 2001; Pillsbury, 1982; Posey, 1996; Sen, 2005). This necessitated an inductive reasoning approach for the study and its subsequent development (Johnson, 1978). It also established the field research paradigm of participatory action research, which defines “the researchers’ function to serve as a resource to those being studied as an opportunity for them to act effectively in their own interest” (Babbie, 2005). Subsequently, the collaboration methodology was developed from suggestions and decisions made at meetings held with participating health practitioners. In this way, participating practitioners could; define their problems (lack of scientific proof of biological activity of their remedies), suggest possible solutions (biological activity testing of the remedies) and take the lead in designing the research that would help them realise their aims (section 1.5).

The nature of the study thus necessitated quantitative and qualitative components for the research design (Morgan, 2006). The quantitative component would include the testing of traditional remedies in the laboratory. The qualitative component would explore and describe the participation and views of traditional health practitioners before, during and after laboratory testing.

The qualitative component of the collaborative study was thus explorative, descriptive and interactive in design. Generally this type of research design will provide good validity for qualitative findings, but reliability may be questionable. However, the collaboration development process provided a longitudinal element to the study design (Chadwick et al., 1984). The longitudinal element made it possible to check decisions made in the development process with health practitioners and take appropriate action.
which might improve reliability of results (Bloor, 1997). However, the
generalisability of the results will be limited, due to the highly individualistic
nature of traditional health practitioners, but experiences will be linked to
literature sources where possible to improve it (Bloor, 1997). The following
sections will describe the collaborative research methodology in narrative
form, arranged to include methodological theory and practice.

2.3.1 Development of data generating and communication tools
The communication between scientists and traditional practitioners relied
greatly on two of the health practitioners who established first contact with the
university (section 1.5). They were the chairpersons of two traditional
practitioner organizations in the NMM area. The university would inform the
two health practitioners of discussions or meetings and they would then
phone members of their organizations who, in turn, would inform other
members that lived close to them.

The establishment of an effective communication system was probably the
most valuable asset of the collaboration. This was also reported by other
community participation projects (Botes & van Rensburg, 2000; UNAIDS,
2000). Many meetings and much discussion distinguished encounters
between the two collaborating parties. All communications, be it oral or
written, needed to be translated from English into isiXhosa. Interpreters with
a sound scientific background were used at meetings. However, the
language barrier was a constant problem and led to more than a few
miscommunications during the collaborative period.

The health practitioners suggested that formal records be held of meetings
and that annual reports be compiled to document progress of the
collaboration. Fieldnotes became the preferable method of documentation
(Emerson et al., 1995). In some instances tape recorders were used and
notes transcribed with the aid of a visiting anthropologist. However, recording
was not accepted by all health practitioners and permission was always asked
before recording.
Early on, it was decided not to approach practitioners individually but to negotiate everything with the group of healers that initially approached us as well as more healers that joined the collaboration later on. This led to the development of focus group discussions which became a characteristic means of communication with the participating traditional health practitioners. Focus group discussions simulated a traditional African environment (a close community of people) that provided participating health practitioners with the necessary peer support to make traditionally acceptable suggestions and decisions affecting the research (Odora Hoppers, 2004). Literature supports the use of focus groups in exploratory (Babbie, 2005) and applied research in which decision-making are required (Etkin, 1993; Fern, 2001). However, focus group discussions are limited to verbal behaviour (Morgan, 1997). In addition, the translation process stripped much of the richness of what had been said, such as disagreements encountered in the decision-making process among healers, jokes etc. Translation provided the bottom-line of a decision and not much else. However, it provided extra time for the taking of fieldnotes during conversations. The number of participants in the focus group discussions varied between three to 15 members per discussion.

Focus group discussions were also incorporated into interactive workshops in which the primary aim was to share scientific and traditional knowledge on a specific topic (Van Huyssteen et al., 2004). Health practitioners commented that interactive workshops should be aimed at making new participating health practitioners feel comfortable in the collaboration environment and empower them scientifically. The scientific information gained from the workshops could also help healers in their interactions with western health practitioners and understanding of these practitioners. Attending traditional practitioners also had the choice to use the scientific knowledge gained from these workshops to improve their practices if they wanted to. Health practitioners responded enthusiastically to interactive workshops. This enthusiasm was mirrored by the increase in the number of health practitioners on the collaboration list. Feedback on the workshops from the attending practitioners was overwhelmingly positive. Interactive workshops later provided the template for the development of group feedback seminars, which provided
participating practitioners with quantitative findings generated in the laboratory.

Because of the growing number of health practitioners participating in the collaboration it became difficult to keep everybody informed on developments in the research and accommodate them in a single workshop. It was proposed that a regular newsletter be distributed among the health practitioners. The Umongo Lwazi newsletters would keep participating health practitioners up to date with upcoming events as well as feedback on events that were finished. Still, network phone calls remained an important communication tool.

The most recently established communicative and collaborative tool was the medicinal garden (section 2.4.2). The garden provided a neutral setting for field research within which the participation process of the practitioners could be observed (Etkin, 1993). Observations made from field research complemented the focus group element of qualitative data gathering (Morgan, 1997) and provided a measure for triangulation of results. Generally, triangulation increases the validity of qualitative findings (Etkin, 1993; Fern, 2001). The medicinal garden also provided a practical solution to indigenous knowledge challenges faced by the collaboration (section 2.4.1). This research partnership was thus developed in two distinct settings. The settings included an academic environment of laboratories and lecture halls at the NMMU and the more traditional environment of a medicinal garden situated on the premises of an Old Age Home in the Zwide / KwaZakhele Township (refer to black dots on the map of Figure 2.1).

2.3.2 Traditional African health practitioners in the NMM

The network communication system that was developed between the university and participating practitioners mirrored the sampling process of the study. In this case a non-random sampling procedure, namely network or snowball sampling was used (Burns & Grove, 1987; Chadwick et al., 1984). This method is often used when a sample is difficult to obtain as is the case with traditional health practitioners (UNAIDS, 2000). At the moment, no
complete list of qualified traditional health practitioners is available for the Metropole. With this method, healers that belonged to the two organizations were informed of the collaboration and were invited to join. Because the participants were not independent of each other, a bias may have been introduced in the sampling method (Burns & Grove, 1987), which might influence reliability of results obtained from this sample.

Participation in the research collaboration was voluntary. Healers would come to a meeting or workshop, the collaboration process would be explained and they could decide if they wanted to be a part of it or not. Practitioners received transport money and some form of sustenance at meetings and workshops. This was done because many practitioners were poor and would not have been able to come if they did not receive transport money.

However, we soon found out that some traditional health practitioners did not agree with the idea of the collaboration. Participating health practitioners informed us that the “community and other health practitioners” were not happy with the proposed research collaboration - a sign of just how closely the African community functions. The opposed health practitioners were under the impression that participating health practitioners got paid for their participation and traditional remedies (which was not the case). It was later found that opposing health practitioners were also against the idea of sharing ancient knowledge with outsiders (section 2.2.2). The truth of the situation was that no information on the ingredients of the remedies has been required for participation in the collaboration. Participating practitioners also consulted their ancestors for permission before they prepared the remedies to be tested. For the university it would be a trial run to see if traditional remedies had any merit and further discussions such as revealing of the ingredients would depend on the results.
2.4 Collaboration development

At present there are details of about 150 practitioners of the NMM that have attended at least one focus group or interactive workshop during the duration of the collaboration period. During the three years of this study, there was weekly interactive contact with about four health practitioners through maintenance work in the medicinal garden. The practitioners attending to the garden differed from week to week and totalled about 12 different practitioners monthly. The newsletter was distributed to about 120 healers quarterly. Every semester either feedback seminars or IK workshops were held at the university with about 15 healers in addition to the healers reached monthly through the garden.

Some health practitioners came for a period of time and then lost interest. Some health practitioners moved away or died. The dynamic nature of the group of participating health practitioners was challenging at times, but also had its advantages. It ensured the discussion of difficult issues, to saturation, with traditional health practitioners joining for the first time or with those who were just curious; the discussions involved explanations of the aims of the research collaboration and methods used to reach these aims. These explanations sometimes wasted time that could have been spent on making new decisions, but it was important to get all the participants on the same page. Later on, it was common practice to start events with a short explanation of the founding of the collaboration, what had been decided so far and what was on the agenda for that meeting.

2.4.1 Indigenous knowledge

The issue of indigenous knowledge (IK) surfaced for the first time after the initial testing of traditional remedies, containing unknown ingredients (section 1.5). The results of the initial testing were indeed promising and the university was ready to move the collaboration to the next level. According to literature, our focus group discussions, interactive workshops and ongoing meetings were conducive to the development of trust between the collaborating
partners (Lipp, 1989; UNAIDS, 2000). We were thus confident that participating practitioners would be more open about the ingredients of the remedies that we tested. But when asked, the health practitioners assured us in no uncertain terms that the combination of plants used in traditional remedies was a secret. Literature confirmed that healers regard remedy combinations as trade secrets (Ashforth, 2005). Furthermore, participating practitioners explained their concerns that revelation of the ingredients might result in their exploitation. Literature confirmed exploitation of indigenous communities numerous times (Dutfield, 2000), and:

“The reason why indigenous people originally introduced secrecy and sacredness was to protect indigenous knowledge from misappropriation …” (Portfolio Committee on Arts Culture Science and Technology, 2000).

A healer explained that the Xhosa culture demanded great confidentiality in the ingredients of remedies and that this information was granted by the ancestors to a specific family. This knowledge is regarded as sacred and is for the benefit of that family. Literature confirmed that there were societies that recognised various forms of intellectual property rights and this knowledge was generally held by individuals, families or communities (Marshalkar, 2001). IK was indeed considered sacred (Struthers et al., 2004).

There was subsequent consensus among participating health practitioners that constituents of the remedies should not be revealed. They further explained that the ancestors may cause them to loose this knowledge once divulged to people who did not follow the proper processes of acquiring IK (refer to section 2.2.2). However, they conceded to the problem that herb sellers were giving out information on medicinal plants for a sum of money and in the process traditional health practitioners were being sold out.

At this stage, the establishment of a medicinal garden, which had long been one of the goals of the research collaboration², was brought to the table. According to participating practitioners, some plants were commonly used and known by most Xhosa people. The knowledge of these plants was subsequently not restricted. The garden would enable a shift in focus of
testing from combination remedies (which is secret) to individual plants (common knowledge).

2.4.2 The medicinal garden

Participating practitioners suggested that a list of common plants be compiled for the garden. However, this would have been of limited value to us, because we were not familiar with isiXhosa names for plants. Many isiXhosa names are not documented or do not correspond to documented plants with the same name or Zulu names (Dold & Cocks, 2002). It was thus always best to have a plant specimen when choosing plants. Plant sampling for the garden was thus done either informally, during tours of the university grounds at workshops, or formally at plant sampling meetings.

At plant sampling meetings, health practitioners were asked to bring specimens of plants that could be identified and planted. Or practitioners were shown colour photographs in books and chose plants according to the pictures and / or common names listed for the plants. Interestingly, common names of plants were helpful, for choosing plants practitioners bought from other parts of the country in dried form. Not all plants that were sampled were indigenous to South Africa or even the African continent. The plants were probably introduced into the traditional system when they were brought over by seafarers passing by the coast or settlers that relocated to Africa.

In addition to medicinal plants (Table 2.1), fruits and vegetables were also included in the practitioners’ choice. Fruits and vegetables included garlic, chillies, spinach, lettuce, beetroot and a peach tree. One of the reasons, for choosing the food plants was that healers believed that medicine should not be taken on an empty stomach and that many of their patients could not afford food. In addition, vegetables that needed to be planted for the ancestors included mealies, beans and pumpkins. It was also suggested that some of the harvest be sacrificed to the ancestors for thanks giving. Traditional health practitioners suggested distinctive sections for vegetables and medicinal plants in the garden.
Table 2.1 Partial list of plants growing in the medicinal garden.

<table>
<thead>
<tr>
<th>Latin name</th>
<th>Xhosa name (health practitioners)</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe ferox Mill.</td>
<td>Ikhala</td>
<td>Cape Aloe</td>
</tr>
<tr>
<td>*Aloe vera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artemisia afra Jacq. ex Willd.</td>
<td>Umhlonhyane</td>
<td>Wildeals</td>
</tr>
<tr>
<td>Atriplex semibracteata R. Br. var. appendiculata Aellen</td>
<td>Mabalabala</td>
<td>kruipsoutbos</td>
</tr>
<tr>
<td>Bulbine frutescens (L.) Willd.</td>
<td>Ficafican</td>
<td></td>
</tr>
<tr>
<td>Bulbine latifolia (L.f.) Schult. &amp; Schult. f.</td>
<td>Nxina</td>
<td>Rooiwortel</td>
</tr>
<tr>
<td>*Capsicum annuum L.</td>
<td></td>
<td>Chilli</td>
</tr>
<tr>
<td>Carissa macrocarpa (Eckl.) A. DC.</td>
<td></td>
<td>Noem-noem</td>
</tr>
<tr>
<td>Carpobrotus edulis (L.) L.Bolus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crassula spp.</td>
<td>Iphehula</td>
<td></td>
</tr>
<tr>
<td>Drimia spp.</td>
<td>Nonkozwana</td>
<td></td>
</tr>
<tr>
<td>Erythrina lysistemon Hutch.</td>
<td></td>
<td>Coral Tree</td>
</tr>
<tr>
<td>Euphorbia fimbriata Scop.</td>
<td>Umhlonlo</td>
<td>Speldekussing</td>
</tr>
<tr>
<td>Euphorbia ledienii A. Berger</td>
<td>Umhlonlo</td>
<td>Noors bush</td>
</tr>
<tr>
<td>Fockea edulis (Thunb.) K. Schum.</td>
<td>Inqatha</td>
<td></td>
</tr>
<tr>
<td>Gasteria bicolor Haw.</td>
<td>Intelezi</td>
<td></td>
</tr>
<tr>
<td>Harpephyllum caffrum Bernh.</td>
<td></td>
<td>Wild Plum</td>
</tr>
<tr>
<td>Helichrysum spp.</td>
<td>Imphepho</td>
<td>Everlasting</td>
</tr>
<tr>
<td>Hypoxis spp.</td>
<td>Nongwe</td>
<td>African Potato</td>
</tr>
<tr>
<td>Ledebouria ensifolia (Eckl.) S. Venter</td>
<td>Nongolwane</td>
<td></td>
</tr>
<tr>
<td>Olea europaea L. subsp. africana (Mill.) P.S. Green</td>
<td>Inqwebeba</td>
<td>Wild Olive</td>
</tr>
<tr>
<td>Ornithogalum longibracteatum Jacq.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pittosporum viridiflorum Sims.</td>
<td>Umkhwenkhwe</td>
<td>Cheesewood</td>
</tr>
<tr>
<td>Plumbago auriculata Lam.</td>
<td>Mvani</td>
<td>Syselbos</td>
</tr>
<tr>
<td>*Ricinus communis L. var. communis</td>
<td>Wyngreyti</td>
<td></td>
</tr>
<tr>
<td>*Ruta graveolens L.</td>
<td>Wynruit</td>
<td></td>
</tr>
<tr>
<td>Sanseveria hyacinthoides (L.) Druce</td>
<td>Isikolokotsho</td>
<td>Mother-in-law’s Tongue</td>
</tr>
<tr>
<td>Tarchonanthus camphoratus L.</td>
<td>Umqqemba</td>
<td>Wild Camphor Bush</td>
</tr>
<tr>
<td>Tetragonia calycina Fenzl</td>
<td>Inqatha</td>
<td></td>
</tr>
<tr>
<td>Tulbaghia violacea Harv.</td>
<td>Tswelelomlambo</td>
<td>Wild Garlic</td>
</tr>
</tbody>
</table>

* exotic

During the plant sampling process, two pieces of land were secured, by collaboration participants (although the municipality was asked for assistance\(^5\)). The one piece of land was already fenced off by the Local Department of Agriculture and it was decided to focus on this piece of land, because of the higher degree of security that it provided in the township. This piece of land was located on the premises of an Old Age Home in the Zwide Township (Figure 2.2).
Figure 2.2 The Umyezo Wamanyange Medicinal Garden during the initial phase of development. It is situated on the premises of an old age home (blue buildings in the background) in the Zwide / KwaZakhele area.

The Umyezo Wamanyange medicinal garden would be an asset for all stakeholders in the research collaboration. The vision for the garden is summarised in the following statements. The garden should;

- Provide health practitioners with medicinal plants of a high quality for use in traditional remedies,
- Provide the university with plant material for biological testing,
- Provide an educative facility where the different names as well as scientific information on the plants could be documented,
- Restore the community’s belief in traditional medicine as an effective treatment based on research findings,
- Provide the potential to be developed into a tourist attraction,
- Prompt government assistance and attract partners from the Departments of Forestry and Water Affairs,
- Show the community that the research collaboration was beneficial to participating practitioners, and
• Be a show case of the African culture.

The fact that plants grown under artificial conditions may not be suited for traditional medicine was argued away by traditional health practitioners. They said that having the plant was better than having no plants at all, because many medicinal plants have become scarce in the NMM area (Victor & Dold, 2003).

2.4.3 Medicinal plant research

Medicinal plants could now be selected from the garden for *in vitro* biological testing, as the healers had initially requested (section 1.5). An indication of sustainable utilisation could also be determined through cultivation of the plants (Van der Watt & Pretorius, 2001). According to the initial screening requests and the screens available at the university, selected plants were to be screened for antimicrobial, anticancer, and anti-diabetic activities. Depending on the results of the screening, the most promising plant and disease would be selected for more in-depth research.

Practitioners indicated that they needed complete information on the testing procedures followed in the laboratory. They explained that the information of the medicinal plants came from the ancestors and thus they needed to know what happened after we have harvested the plants and taken them to the laboratory. In addition to their own knowledge they had to keep the ancestors in the loop. The first step in the collaborative research would thus be to familiarise participating practitioners with extraction techniques and screening methodology. A decision was made to bring participating practitioners into the laboratory. Practitioners that would be selected for this purpose were required to speak and understand English and be able to write. A trial run would be conducted with one healer and recommendations made on how to proceed.

The following step would be to provide feedback on research results, which is said to be a very important aspect of collaborative research. Results were initially distributed among participating practitioners via the quarterly newsletter (Elisabetsky, 1991). Many practitioners did not understand the
feedback in the newsletter or even realise that that feedback was indeed
given in the newsletter upon questioning them about it. Group feedback
seminars were suggested by participating practitioners. In addition, the
feedback seminars ensured that health practitioners were provided with an
appropriate explanation of extraction, screening methodology and results of
the testing. Subsequent discussions allowed the participating practitioners to
give input on their view of the results as well as recommending what they
thought the next step of the research should be.

In addition to feedback given to participating practitioners, it was necessary to
present findings about the research at conferences, to ensure further funding
of the project. This was done according to the health practitioners’ request
that their names be withheld during research presentations for the time being
(section 1.5). The practitioners gave no reasons for this, but at one of the
conferences a group of attending practitioners from KwaZulu Natal
commented on the unusualness of practitioners approaching and
collaborating with scientists. Procedures regarding research outputs are
given in section 8.2.2.

2.4.4 Challenges
One of the main problems experienced in the collaboration was the constantly
changing dynamics of the participating healer organizations and relationships
between organizations and individual healers. The first obvious sign of a
division between the initial three practitioners was at the first formal plant
meeting when one organization chose to bring plants and the other
organization not. The chairperson of the latter organization informed the
university that they were not willing to work with the first organization anymore
and requested us to develop a separate garden for them. Resources and
sponsorship for the garden were scarce and the break away organization
could not be accommodated with their request. In an attempt not to alienate
them, the university kept contact and still invited them to research seminars
and workshops focused on collaboration related issues. Division in groups of
traditional health practitioners had been noted in literature (Sen, 2005;
Troskie, 1997). In addition to the split between the initial two organizations, a
new practitioner organization was formed to represent the NMM branch of the national Interim Traditional Health Practitioners Council as recommended in the Traditional Health Practitioners Act (no 35 of 2004) (South Africa, 2004b). One of the initial three members of the collaboration moved over to this organization. Currently, these three organizations participate in the partnership to different degrees. This may be ascribed to the different chairpersons, missions and activities of these organizations. Similar findings have been reported in literature (Botes & van Rensburg, 2000).

Still, the university was in a unique position to have health practitioners approach them for scientific assistance. However, the scientists themselves and the legal aid of the university were not prepared for the implications and rules pertaining to indigenous knowledge. The unpreparedness was further challenged by the initial scarcity and fast development of policies regarding traditional health practitioners and IK in South Africa during the research period (refer to Chapter 1).
3 The selected plants

3.1 Introduction

After the initial cultivation of plant specimens donated by participating practitioners (section 2.4.2), the plants for this study were selected. This chapter will explain the criteria used for the selection of the plants and summarise information that has been documented for each plant.

*Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) were selected for screening. Participating practitioners brought samples of these plants to meetings (Bf, Ol, Rg & Tv) or identified them from books during other meetings (Tv). Secondly, *B. frutescens* (Roberts, 1990), *O. longibracteatum* (Mulholland et al., 2004) and *R. graveolens* have been used as medicines by other cultural or geographic groups. Thirdly, to ensure sustainability, ease of cultivation was also included in the selection criteria (Cordell & Colvard, 2005). Some of the plants were easy to propagate (Bf), were fast growers (Rg & Tc), did not require much water (Bf & Ol), or were already being cultivated at the homes of participating practitioners (Bf, Rg & Tv). More specifically, two healers indicated that they used *T. camphoratus* leaves for diabetes. Using the leaves of *T. camphoratus* and its abundant occurrence in the Eastern Cape added to the sustainable context of its selection.
3.2 *Bulbine frutescens* (Asphodelaceae)

*Bulbine frutescens* (L.) Wild. (Figure 3.1) is a perennial herb and indigenous to southern Africa (Hey, 1994). Participating practitioners referred to it as *umficificane* and a list of common names are given in Table 3.1. It is also a popular plant, as ‘*Bulbine frutescens* folia’ has been included as one of the first 60 plants to be investigated by the University of the Western Cape (UWC) Pharmacopoeia Monograph Project (Scott & Springfield, 2006).

![Bulbine frutescens growing in the medicinal garden.](image-url)
Table 3.1 List of common names for *Bulbine frutescens*.

<table>
<thead>
<tr>
<th>Common names</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficaficane</td>
<td>Local /is/Xhosa name</td>
</tr>
<tr>
<td>cat’s tail, snake flower, burn jelly plant, grass aloe (Roberts, 1990)</td>
<td>English</td>
</tr>
<tr>
<td>balsem kopieva (Van Wyk &amp; Gericke, 2000), copaiba, geelkatstert (Roberts, 1990)</td>
<td>Afrikaans</td>
</tr>
<tr>
<td>Khomo-ya-Ntsukammele, sehlaere-sa-pekané, sehlaere-sa-mollo (Roberts, 1990)</td>
<td>Sotho</td>
</tr>
<tr>
<td>intelezi, ingelwane (Roberts, 1990) intelezi, ibhucu (Roberts, 1990) ibucu (Roberts, 1990)</td>
<td>isiXhosa isiZulu seTswana</td>
</tr>
</tbody>
</table>

3.2.1 Traditional uses

The fresh leaf produces a jelly-like juice that can be applied to burns, rashes, insect bites, cracked lips, prickly heat, acne, cold sores, mouth ulcers and areas of cracked skin (Hey, 1994; Roberts, 1990; Van Wyk & Gericke, 2000). The Rastafarians use it for the same purposes, but also use fresh leaf infusions for coughs, colds and arthritis. A warm poultice may be applied to treat eczema, arthritis, insect bites, cracked skin, sunburn, rashes and burns. A decoction of the roots is used to treat fever (Neuwinger, 2000). *Bulbine frutescens* has been used for various ailments, in particular wound healing, in other southern African countries as well (Mutanyatta et al., 2005).

3.2.2 Chemical constituents

*Bulbine* species have been investigated for 1, 8-dihydroxyanthraquinone derivatives and non-volatile organic acids in the leaves and roots. Anthraquinones were found, but no organic acids have been reported (Van Rheede van Oudtshoorn, 1964). 1,8-dihydroxyanthraquinones present in acetone extracts of dried *Bulbine frutescens* roots from the Olifantkop area included crysophanol, knipholone and isoknipholone (Van Wyk et al., 1995). However, asphodeline 10,7-bicrypsophanol, chryslandicin and knipholone anthrone were not present in the acetone extract (Van Wyk et al., 1995). Knipholone and knipholone anthrone (Figure 3.2) has also been identified.
from *B. frutescens* in other studies (Bringmann *et al.*, 1999; Van Staden & Drewes, 1994).

The roots of *B. frutescens* further yielded a few novel phenylanthraquinones and 6’-O-sulfated phenylanthraquinones. The novel phenylanthraquinones included 4’-O-demethylknipholone-4’-β-D-glucopyranoside and gaboroquinones A and B (Abegaz *et al.*, 2002). The novel 6’-O-sulfated phenylanthraquinones included sodium *ent*-knipholone 6’-O-sulfate, sodium 4’-demethylknipholone-4-β-D-glucopyranoside 6’-sulfate, sodium 4’-O demethylknipholone 6’-sulfate and sodium isoknipholone 6’-sulfate. The sulfates were prone to acid hydrolysis and yielded *ent*-knipholone, 4’-demethylknipholone-4-β-D-glucopyranoside, 4’-O demethylknipholone and isoknipholone (Mutanyatta *et al.*, 2005). In addition, the wound healing effects of *B. frutescens* may be attributed to the presence of glycoproteins, aloctin A and aloctin B, in the gel (Van Wyk & Gericke, 2000).

### 3.2.3 Previous biological activity investigations

Compounds isolated from *Bulbine frutescens* such as knipholone and 4’-O-demethylknipholone-4’-β-D-glucopyranoside have shown antiplasmodial activity in studies done on the individual components (Mutanyatta *et al.*, 2005). A study on isolated knipholone, showed significant inhibition of leukotrine synthesis by its inhibition of 5-lipoxygenase (LOX), less for 12-LOX and still less for cyclooxygenase (COX)-1 and COX-2 (Wube *et al.*, 2006). However, knipholone failed to produce anti-oxidant activity (Wube *et al.*, 2006).

Lectin-like proteins isolated from *B. frutescens* seeds produced weak inhibition of COX-1 and COX-2 (Gaidamashvili & van Staden, 2006). Dried
and ground plant material of *B. frutescens* was tested for antifungal activity against *Candida albicans* (strains taken from a 5-month old baby, an HIV positive adult and ATCC10231). Aqueous extracts had a minimum inhibitory concentration (MIC) of > 25 mg/ml and the organic extracts (ethanol, ethyl acetate and hexane) > 8.25 mg/ml (Motsei *et al.*, 2003).

### 3.3 *Ornithogalum longibracteatum* (Ornithogaloideae: Hyacinthaceae)

The hyacinthaceae family is divided into five subfamilies, of which one, the Ornithogaloideae occur in southern Africa (Mulholland *et al.*, 2004). Participating healers mostly referred to the plant as *inqwebeba* (also see common names in Table 3.2), but *buchu* and *eshwadi* were given as alternative names. Plant samples provided by participating practitioners were planted and were identified by a plant taxonomist from the Department of Botany at the NMMU. Participating practitioners used it to wash the skin, which improved the recollection of dreams and aided their communication with the ancestors. However, one practitioner warned to use only a small amount of the plant, because it was irritating to the skin. The plants are thriving in the medicinal garden (Figure 3.3) and are ready to be divided. Dividing of bulbs is recommended to be done every 2 to 3 years. Alternatively, the plant could be grown from seed (Pienaar, 1991).

<table>
<thead>
<tr>
<th>Common names</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inqwebeba</td>
<td>Local /isXhosa name</td>
</tr>
<tr>
<td>Buchu</td>
<td></td>
</tr>
<tr>
<td>Eshwadi</td>
<td></td>
</tr>
<tr>
<td>Wild onion (Watt &amp; Breyer-Brandwijk, 1962)</td>
<td>English (East Africa)</td>
</tr>
<tr>
<td>Kikwashi (Watt &amp; Breyer-Brandwijk, 1962)</td>
<td>Kikuyu</td>
</tr>
</tbody>
</table>
3.3.1 Traditional uses

The bulbs of *Ornithogalum longibracteatum* Jacq. have been used as a charm and irritant in South African traditional medicine (Verschaeve *et al.*, 2004). Its anti-inflammatory qualities are rated highly by the Zulu, who use it to reduce swelling (Mulholland *et al.*, 2004). The paste from crushed bulbs is also used as a dressing for wounds (Neuwinger, 2000).

3.3.2 Chemical constituents

Dichloromethane and methanol extracts of fresh bulbs of *Ornithogalum longibracteatum* yielded the homoisoflavanone, 7-O-methyleucomol (Figure 3.4) (Mulholland *et al.*, 2004). No cardiac glycosides were found in the dichloromethane crude extracts of *O. longibracteatum* (Mulholland *et al.*, 2004). Other southern African *Ornithogalum* species, namely *O. thyrsoides* and *O. saundersiae* have yielded a number of cholestane glycosides and their
derivatives. In contrast to their South African counterparts, several European *Ornithogalum* species contained cardenolide glycosides as their primary constituents (Mulholland *et al*., 2004).

![Figure 3.4 The structure of the homoisoflavanone 7-O-methyleucromol isolated by Mulholland *et al.* (2004).](image)

### 3.3.3 Biological activity and toxicity

In general, homoisoflavanone compounds have been shown to have anti-inflammatory properties (Della Logia *et al*., 1989; Du Toit *et al*., 2005) and extracted compounds produced topical anti-inflammatory activity comparable to that of indomethacin in dermatitis induced on mouse ear (Della Logia *et al*., 1989). Homoisoflavanones have also previously produced antibacterial, antihistamine, antimutagenic, angioprotective and potent phosphodiesterase inhibitory activities (Du Toit *et al*., 2005).

Bulbs of *Ornithogalum longibracteatum* were found not to be genotoxic (in the Ames and micronucleus tests) and did not influence the mutagenic effect of mytomycin C (MMC). However, the leaves were found to increase the mutagenic effect of MMC but were also not genotoxic (Verschaeve *et al*., 2004). The general toxicity of *O. longibracteatum* remains inconclusively demonstrated, possibly due to the occurrence of several geographic chemical races (Mulholland *et al*., 2004). However, *Ornithogalum* species is considered among the most poisonous plants in South Africa, because some of them contain different steroid or cardiac glycosides (Van Wyk *et al*., 2002). The whole plant has been reported to be toxic to animals, especially the fruit. The leaf and root are only toxic in large concentrations and pre-flowering young plants are more toxic than mature plants (Watt & Breyer-Brandwijk, 1962). In contrast to toxicity reports, an oral dose of 110 g of bulb showed no adverse effects on a rabbit and toxicity tests in Kenya have also given non-toxic results (Watt & Breyer-Brandwijk, 1962).
3.4 *Ruta graveolens* (Rutaceae)

*Ruta graveolens* L., or commonly known as rue, originates from south-eastern Europe (Heinrich *et al.*, 2004) and the Middle East (Hey, 1994). It a common herb grown in South African gardens and has become naturalised in some parts, including the southern part of the Eastern Cape Province of South Africa (Van Wyk & Gericke, 2000).

The participating practitioners referred to it as wyngreyti, which may have been derived from the Afrikaans common name, wynruit (Table 3.3). This might indicate that they learnt about the medicinal properties of this plant from European settlers. The crushed plant’s strong disagreeable smell (Watt & Breyer-Brandwijk, 1962) has contributed to its use to keep snakes and devils away. Participating practitioners also smear it on babies to calm restless and anxious ones. Practitioners had experience of *Ruta* cultivation, because it was growing at their homes. It has been easy to propagate by self seeding in the medicinal garden (Figure 3.5). However, due to it not being indigenous, it does need more water than the indigenous plants to flourish.

<table>
<thead>
<tr>
<th>Common names</th>
<th>Language</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wyngreyti</td>
<td>Local isiXhosa name</td>
<td>Afrikaans</td>
</tr>
<tr>
<td>Wynruit, binnewortel (Van Wyk <em>et al.</em>, 1997)</td>
<td></td>
<td>English</td>
</tr>
<tr>
<td>Rue, herb of grace (Van Wyk <em>et al.</em>, 1997)</td>
<td></td>
<td>German</td>
</tr>
<tr>
<td>Weinraute (Van Wyk &amp; Wink, 2004)</td>
<td></td>
<td>Italian</td>
</tr>
<tr>
<td>Ruta (Van Wyk &amp; Wink, 2004)</td>
<td></td>
<td>Spanish</td>
</tr>
<tr>
<td>Ruda común (Van Wyk &amp; Wink, 2004)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4.1 Traditional uses

In South Africa, *Ruta graveolens* leaf infusions have been used for the treatment of fits in children and hysteria (Van Wyk *et al*., 1997). Alternatively, the leaf juice may be administered to infants and children suffering from convulsions or fits (Watt & Breyer-Brandwijk, 1962). An infusion (Van Wyk *et al*., 1997) or decoction of *R. graveolens* leaves has traditionally been used for fevers, such as typhoid and scarlet fever (Watt & Breyer-Brandwijk, 1962). Bruised leaves may be put in the ear or tooth for related pain (Watt & Breyer-Brandwijk, 1962). Leaves may also be chewed to relieve stomach pain and headache (Neuwinger, 2000). Decoctions have been used to ease childbirth and alcoholic tinctures for respiratory and heart problems (Van Wyk *et al*., 1997). Rue has been used as an emmenagogue (agent that induces menstruation) and spasmolytic (Heinrich *et al*., 2004). A poultice made from the fruits of *R. graveolens* have been used to apply to swellings (Neuwinger, 2000). In a recent ethnobotanical survey in the Bredasdorp / Elim region in the Southern Cape, *R. graveolens* was one of two plants used by all the interviewed participants (the other plant was *Artemisia afra*). It was used for
bladder and kidney problems, convulsions, diabetes, fever, headache, stomach complaints, worms and sinus problems (Thring & Weitz, 2006).

*R. graveolens* has been used in Jordanian traditional medicine as an anti-inflammatory, spasmodic and analgesic as well as for sprains, bruises, wounds, colic and rheumatoid arthritis (Atta & Alkofahi, 1998). It has been used as emmenagogue and abortifacient in Brazil (De Freitas et al., 2005), Europe and America (Gutierrez-Pajares et al., 2003). In central Italy, the plant was reportedly used with sulfur on dogs afflicted with scabies. A decoction was given to pigs to deter flies and to calves and other livestock with helminthiasis. The fresh plant was believed to have repellent and insecticidal properties towards fleas, mosquitoes and other noxious insects (De Feo et al., 2002). People believed that the disagreeable smell would repel mice. An infusion or decoction of the aerial parts was used to eliminate head lice. A decoction of the leaves was also used for de-worming children (Guarrera, 1999).

3.4.2 Chemical constituents

Chemical compounds that have been identified from *Ruta graveolens* include coumarins such as herniarin, gravelliferon and rutaretin, furanocoumarins such as bergapten, psoralen and rutamarin, furanoquinolone alkaloids such as dictamnine, skimmianine and rutacridone with various derivatives as well as the flavonoids rutin (Van Wyk & Wink, 2004) and quercetin (Williamson, 2003). Methyl nonyl ketone is a major component of the volatile oil (Van Wyk & Wink, 2004) as well as 2-heptanol, 2-nonanone, limolene, pinene, anisic acid, phenol, guaiacol, linalyl acetate, menthol and others (Williamson, 2003).

*R. graveolens* roots were extracted in ethylacetate and yielded rutacridone epoxide which demonstrated potent selective algicidal activity. Gravacridondiol was also isolated, but was not as active (Meepagala et al., 2005).

*Ruta graveolens* has been identified as a possible source for the production of linear furanocoumarins (FCs) such as psoralen (10 to 15%), xanthotoxin (>25%), bergapten (>50%) and isopimpinellin (5 to 10%) (Milesi et al., 2001).
Fruits had the highest concentration (5 to 10 times) followed by stems and leaves and then roots. However, isopimpinellin was highly concentrated in the roots rather than other organs. Harvesting only fruits and leaves during the fructification stage of the plant in summer, will lead to the collection of 80% of the FCs produced by *R. graveolens*, thus leaving the stem and roots in place for further growth (Milesi *et al.*, 2001). No significant differences in furanocoumarin concentrations were observed between plants from 16 different origins in Europe.

### 3.4.3 Biological activity

Methanol extracts of *Ruta graveolens* (collected during Finland’s summer) showed slight inhibitory effects at a concentration of 500 μg/ml on the growth of *Bacillus subtilis* and *Staphylococcus aureus*, but not *Escherichia coli* or *Candida albicans* (Ojala *et al.*, 2000). Similarly, methanol extracts of *R. graveolens* (from Bulgaria) showed activity against *S. aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes* and *B. subtilis* (Ivanova *et al.*, 2005) in a disc diffusion assay. No activity was found against *E. coli*, *C. albicans* or *Corynebacterium diptheriae* (Ivanova *et al.*, 2005). Methanol and acetone extracts of *R. graveolens* (from Japan) produced positive results against Gram-positive bacteria, including *S. aureus* and *B. cereus*. The MIC for the methanol extracts were 2.64 mg/ml and 0.66 mg/ml respectively. Methanol extracts showed better results than acetone extracts (Alzoreky & Nakahara, 2003). Antimicrobial activity observed for *R. graveolens* has been ascribed to essential oils, flavonoids (Ojala *et al.*, 2000), furanocoumarins and / or furanoquinoline alkaloids (Van Wyk & Wink, 2004).

The aqueous extract of *R. graveolens* demonstrated a hypotensive effect in normotensive rats. *In vitro* investigations indicated that it had positive chronotropic and inotropic effects on isolated right atria (Chiu & Fung, 1997). It also relaxed pre-constricted (with potassium chloride) rat tail arteries, probably due to an effect on vascular smooth muscle (Chiu & Fung, 1997). Furthermore, a compound contained in *R. graveolens*, rutin, has been used as a capillary protectant (Williamson, 2003) and, supplementary to coumarins, for chronic venous diseases (Van Wyk & Wink, 2004). It also prolonged
survival of rats that received a diet filled with pro-blood clotting foodstuffs (Williamson, 2003).

The ethanol extract of *R. graveolens* showed concentration-dependent (100 and 200 mg/kg) central and peripheral analgesic effects on mice (Atta & Alkofahi, 1998). *R. graveolens* at a dose of 200 mg/kg showed an anti-inflammatory effect against chronic inflammation presented by cotton pellet granuloma in rats (Atta & Alkofahi, 1998). No acute anti-inflammatory effects were seen in xylene-induced ear swelling in mice. The *in vitro* anti-inflammatory effect of 50% methanol extract of the whole dried plant was demonstrated on murine macrophage cells challenged with lipopolysaccharides (Raghav *et al.*, 2006). Lipopolysaccharides induced an inflammatory response on macrophages by stimulating the release of nitric oxide (NO) and other inflammatory mediators. Upon treatment with the extract, significant inhibition of nitric oxide production was observed. The mechanism was found to be the inhibition of inducible nitric oxide synthase (iNOS) gene expression on the transcription level. Transcription of the COX-2 gene was also found to be inhibited (Raghav *et al.*, 2006).

Moderate acetylcholinesterase inhibitory activity (using an enzymatic assay) has been reported with methanol and water extracts. Higher inhibition was found with methanol than with water extracts (Adsersen *et al.*, 2006). Antiparasitic effects of *Ruta* spp. has been confirmed (Guarrera, 1999). Antispasmodic effects have been reported and have been attributed to the furanocoumarins and furanoquinolone alkaloids (Van Wyk & Wink, 2004). An extract of the dried herb has been shown to inhibit tumour formation induced by benzo(a)pyrene in mouse skin (Williamson, 2003).

The use of *Ruta* as an abortificant has given conflicting results. The aqueous extracts of powdered root and aerial parts were administered orally to female rats and hamsters (1-6 days post coition) and showed anti-conceptive activity in rats. Rutin a known chemical constituent was found to be inactive (Gandhi *et al.*, 1991). However, a study by De Freitas *et al.* (2005) in pregnant mice reported no significant embryonic loss or negative effect on implantation. Another study found a decrease in embryo quality and development when
water extracts were administered to mice during the pre-implantation phase (Gutierrez-Pajares et al., 2003).

3.4.4 Toxicity

*Ruta graveolens* has been used as an edible plant in China, Japan, Thailand and Yemen (Alzoreky & Nakahara, 2003). However, it should be used with caution, because it has caused strong phototoxic side effects (Heinrich et al., 2004) and may cause dermatitis in sensitive people. Adverse effects are caused by coumarins (Van Wyk et al., 2002). *R. graveolens* has also been labelled as mutagenic due to it containing furanocoumarins and related alkaloids (Van Wyk & Wink, 2004). In addition, the use of *R. graveolens* is contraindicated in pregnancy as it is a uterine stimulant (Williamson, 2003).

De Freitas et al. (2005) suggested that the 70% ethanol extract of dried *R. graveolens* (collected in Brazil) should not be used as a herbal remedy because it might cause multiple organ system failure and death. However, no acute toxicity was experienced in another study on mice with aqueous extracts of dried *R. graveolens* from Peru (Gutierrez-Pajares et al., 2003). Similarly, cytotoxicity assays on murine macrophage cells at concentrations of 100 to 500 µg/ml of 50% methanol extracts showed a cell viability of over 85% for all concentrations (Raghav et al., 2006). Methanol extracts of dried *R. graveolens* (grown in Bulgaria) was tested for brine shrimp toxicity and showed a strong cytotoxic activity at 10 µg/ml (Ivanova et al., 2005). In the same assay, toxicity of the aqueous extract was lower than that of organic extracts (Ivanova et al., 2005).

3.5 *Tarchonanthus camphoratus* (Asteraceae)

*Tarchonanthus camphoratus* L. is widely distributed on the African continent, from Somalia in the north to the Western Cape in the south (Venter & Venter, 2002). It occurs as an evergreen shrub or small to medium tree depending on the area where it grows. Leaves used in the study were harvested from a
positively identified medium-sized tree growing in a bushveld-type habitat on the NMMU campus (Figure 3.6).

Healers referred to *T. camphoratus* as *umgqeba* (Table 3.4). Small *T. camphoratus* trees have been planted in the garden to provide a wind-break for sensitive plants, because they grow moderately to fast, tolerate the sea breeze, severe draught, frost and strong winds (Venter & Venter, 2002). ‘*Tarchonanthus camphoratus herba*’, which refers to the leaves and twigs used in traditional medicine, has been included as one of 60 plants chosen by the UWC Pharmacopoeia Monograph Project (Scott & Springfield, 2006).

Figure 3.6 The *Tarchonanthus camphoratus* tree growing at Nelson Mandela Metropolitan University, which leaves were used in the screening assays.
Table 3.4 List of common names for *Tarchonanthus camphoratus*.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umqeba</td>
<td>Local /is/Xhosa name</td>
</tr>
<tr>
<td>Umnqebe</td>
<td>Nama</td>
</tr>
<tr>
<td>Mohata (Van Wyk et al., 1997)</td>
<td>seTswana</td>
</tr>
<tr>
<td>mofahlana (Van Wyk et al., 1997)</td>
<td>South Sotho</td>
</tr>
<tr>
<td>sefahla (Joffe, 1993; Venter &amp; Venter, 2002)</td>
<td>North Sotho</td>
</tr>
<tr>
<td>umgebe (Van Wyk et al., 1997)</td>
<td>isiXhosa</td>
</tr>
<tr>
<td>mathola (Van Wyk et al., 1997)</td>
<td>Shona</td>
</tr>
<tr>
<td>mkalambati</td>
<td>/isi/Xhosa</td>
</tr>
<tr>
<td>igqeba-elimhlope (Joffe, 1993; Venter &amp; Venter, 2002), siduli-sehlathi, (Van Wyk et al., 1997)</td>
<td>Swahili</td>
</tr>
<tr>
<td>amathola</td>
<td>isiZulu</td>
</tr>
<tr>
<td>wilde kanferbos, kanferboom, kapokbossie, vaalbos (Joffe, 1993)</td>
<td>Afrikaans</td>
</tr>
<tr>
<td>wild camphor bush (Van Wyk et al., 1997)</td>
<td>English</td>
</tr>
</tbody>
</table>

3.5.1 Traditional uses

The smoke from green branches of *Tarchonanthus camphoratus* has been inhaled for the treatment of blocked sinuses and headaches (Venter & Venter, 2002). Another report listed the use of the green branches as well as the seeds to be placed on a glowing coal and the smoke inhaled for the treatment of rheumatism, asthma (Van Wyk et al., 1997) and insomnia (Neuwinger, 2000). Leaves were dried and smoked by the Hottentot and Bushman to produce a slight narcosis (Watt & Breyer-Brandwijk, 1962).

A hot poultice of the leaves may also be applied to the chest to ease asthma and other chest conditions (Venter & Venter, 2002) as well as headache and inflammation (Van Wyk et al., 1997). An infusion of the leaves promotes perspiration, offers relief to spasmodic asthma, acts as a tonic (Watt & Breyer-Brandwijk, 1962), treats stomach complaints, fever, cough, bronchitis and influenza (Neuwinger, 2000). As an alternative to the leaf infusion, fresh leaves could also be chewed (Neuwinger, 2000) or a tincture of the leaves could be prepared (Van Wyk et al., 1997). The plant is also used as a toothache remedy (Watt & Breyer-Brandwijk, 1962).

3.5.2 Chemical constituents

According to chemical analysis done at the monograph research laboratories, the presence of tannins, saponins (2/3 collections), and reducing sugars (2/3
collections) has been detected in *Tarchonanthus camphoratus* (Scott & Springfield, 2005). *T. camphoratus* also contains pinocembrin, a flavanone (Van Wyk *et al.*, 1997). Various flavanones such as luteolin, apigenin, nepetin, and hispidulin have been identified from Egyptian collections as well as the sesquiterpene lactone, parthenolide and a quaternary alkaloid, tarchonanthine (Scott & Springfield, 2005).

The volatile oil of *T. camphoratus* has been shown to be very complex and variable at different locations (Omolo *et al.*, 2004; Van Wyk *et al.*, 1997; Watt & Breyer-Brandwijk, 1962). The combination of the essential oil of Kenyan *T. camphoratus* has been determined and composed of fenchol (15.9%), 1,8-cineole (14.3%), α-terpineol (13.2%), α-pinene (6.87%), *trans*-pinene hydrate (6.5%), terpinen-4-ol (4.74%), champhene (3.76%) and β-eudesmol (5.79%) among other minor constituents (Matasyoh *et al.*, 2007).

### 3.5.3 Pharmacological investigations and toxicity

The leaves do not appear to be toxic as it is eaten by domestic stock, such as cattle, sheep and goats (Watt & Breyer-Brandwijk, 1962), as well as game such as giraffe, gemsbok, eland, kudu, sable, black wildebeest, nyala, impala, springbok and grey duiker (Venter & Venter, 2002). In a recent study by Parker *et al.* (2003), a small group of giraffe fed on *Tarchonanthus camphoratus* only during summer.

There might be the slightest chance that animals prefer *T. camphoratus* in summer, because of its apparent ability to repel insects (Watt & Breyer-Brandwijk, 1962). The repellent effects of the essential oil of *T. camphoratus* from Kenya were investigated. It was found to be less potent than N,N diethyl-m-toluamide (DEET), a synthetic repellent (Omolo *et al.*, 2004). In another study the essential oil of *T. camphoratus* was found to be relatively toxic to *Anopheles gambiae* and had an LD$_{50}$ of $3.8 \times 10^{-3}$ mg/ml (Omolo *et al.*, 2005).

Pharmacological effects reported for *T. camphoratus* includes decongestant and antispasmodic effects (Van Wyk *et al.*, 1997). Water extracts of the leaves failed to produce antibacterial activity (Watt & Breyer-Brandwijk, 1962).
However, the essential oil from *T. camphoratus* (Matasyoh et al., 2007) produced antimicrobial activity using the disc diffusion method against *E. coli*, *S. aureus*, *Bacillus* spp. and *C. albicans* at concentrations $\leq 129$ mg/ml. *S. typhi*, *K. pneumonia* and *P. mirabilis* were inhibited at concentrations $\leq 900$ mg/ml. *P. aeruginosa* was resistant to the effects of the essential oil (Matasyoh et al., 2007).

Aqueous extracts of the leaves of *T. camphoratus* have demonstrated *in vivo* analgesic and antipyretic activity. Squirming of mice caused by contact with acetic acid and a hotplate were decreased. It was also effective in treating fever induced by lipopolysaccharides in rats (Amabeku et al., 2000).

### 3.6 *Tulbaghia violacea* (Alliaceae)

*Tulbaghia violacea* Harv. (Figure 3.7) is widely distributed in South Africa (Pienaar, 1985) and found along forest margins and stream banks, from Ladysmith to KwaZulu Natal (Manning, 2001). In addition, *T. violacea* has been widely cultivated (Marshalkar, 2003), also by participating practitioners in this study.

The species gets its name from the Latin word *violaceus*, which may be translated as violet coloured (Marshalkar, 2003). Participating practitioners refer to the plant as *tswelelo lambo* (common names listed in Table 3.5) and it is used for a range of different conditions. It also seems as if the species of *Tulbaghia* may be used interchangeably, because healers brought *T. capensis* and *T. violacea* for cultivation. In a similar trend, it has been postulated that *T. violacea* may have similar biological activities to garlic (*Allium sativum* L.) (Cox & Balick, 1994), because it smells of garlic when bruised (Manning, 2001) and belongs to the same family (Alliaceae). In addition, Motsei et al. (2003) found that the two plants had some similar active compounds, which support the potential for comparable biological activities (Motsei et al., 2003). ‘*Tulbaghia bulbus*’ has been included as one of 60
plants chosen by the UWC Pharmacopoeia Monograph Project (Scott & Springfield, 2006).

Figure 3.7 *Tulbaghia violacea* growing in the Umyezo Wamanyange medicinal garden.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tswelelomlambo</td>
<td>Local isiXhosa name</td>
</tr>
<tr>
<td>wild garlic</td>
<td>English</td>
</tr>
<tr>
<td>Wildeknoflok, wilde knoffel</td>
<td>Afrikaans</td>
</tr>
<tr>
<td>Incinsini (Van Wyk &amp; Gericke, 2000)</td>
<td>isiZulu</td>
</tr>
<tr>
<td>Mothebe</td>
<td>seSotho</td>
</tr>
</tbody>
</table>

**Table 3.5 Common names of *Tulbaghia violacea*.

### 3.6.1 Traditional uses

*Tulbaghia violacea* has been used as a traditional remedy for acute conditions such as earache, fever (Thring & Weitz, 2006), colds (Marshalkar, 2003) and stomach problems (Elgorashi *et al.*, 2003), such as constipation (Van Wyk &
Gericke, 2000). It may also be used for chronic conditions such as high blood pressure (Thring & Weitz, 2006), asthma, rheumatism and paralysis (Elgorashi et al., 2003). Decoctions may be administered as an enema (Manning, 2001). T. violacea leaves and bulbs (Elgorashi et al., 2003; Roberts, 1990) have also been used in the treatment of tuberculosis (TB) (Manning, 2001). The bulb alone has been recommended for pulmonary TB as well as an anthelmintic (Watt & Breyer-Brandwijk, 1962). The leaves are taken for oesophageal cancer (Van Wyk & Gericke, 2000).

The green parts and flowers may be eaten as a vegetable (Marshalkar, 2003; Watt & Breyer-Brandwijk, 1962) and it may also be combined with meat and potatoes (Roberts, 1990). The Zulu plant Tulbaghia around their houses as a snake repellent (Roberts, 1990; Van Wyk & Gericke, 2000).

3.6.2 Chemical constituents
A carbon-sulfur lyase was the first isolated compound from Tulbaghia violacea (Jacobsen et al., 1968). Subsequent chemical constituents isolated included sulfur compounds, 2,4,7-tetrathiaoctane-2 2-dioxide and 2,4,5,7-tetrathiaoctane, 15 flavones including flavonols such as kaempherol and quercetin and several sugars and steroidal saponins (Duncan et al., 1999). It was also found that T. violacea did not contain any tannins (Duncan et al., 1999).

Following on Jacobsen’s lead, Kubec isolated (R(S)R(C))-S-(methylthiomethyl)cysteine-4-oxide from the rhizomes of T. violacea. The content varied in different parts of the plant, but the quantities in the stems and rhizomes were almost equal (Kubec et al., 2002). S-methyl and S-ethylcystein derivatives were also detected in minute amounts as well as the major breakdown product 2,4,5,7-tetrathiaoctane-4-oxide to which the smell was attributed (Kubec et al., 2002).

3.6.3 Biological activity
The aqueous extract of the bulb of Tulbaghia violacea had an MIC of 3.25 mg/ml for the C. albicans strain isolated from a five month old baby; the
leaf extract had no activity. In addition, aqueous extract activity remained stable for two days when stored at 4°C (Motsei et al., 2003). Ethanol and ethyl acetate extracts produced MIC values of 0.26 and 0.13 mg/ml respectively against the C. albicans strain isolated from a five month old baby. Adult and ATCC strains had MIC values of 2.09 mg/ml for both ethanol and dichloromethane extracts. Lectin-like proteins that were extracted and purified from T. violacea produced agglutination of B. subtilis, but not S. aureus. Minimal agglutination occurred at a concentration of 45 µg/ml (Gaidamashvili & van Staden, 2002). In a follow-up study on lectin-like proteins, T. violacea produced very weak inhibition of COX-1 and COX-2 (Gaidamashvili & van Staden, 2006).

Ethanol and water extracts of the tuber of T. violacea has shown antinematodal activity at concentrations of 1 and 2 mg/ml against the nematode C. elegans. The water extracts were more effective than ethanol extracts and hexane extracts showed no activity (McGaw et al., 2000).

The water and ethanol extracts of the leaves and roots of T. violacea were tested for angiotensin-converting enzyme (ACE) inhibitory activity. The leaves had more inhibitory activity than the roots at a concentration of 25 µg of extract in 75 µl of incubation medium, while water extracts had better activity than the ethanol extracts (Duncan et al., 1999). In another study T. violacea showed β-adrenergic agonist activity in smooth muscle experiments (Duncan et al., 1999). The methanol extract of T. violacea produced anticoagulant / antithrombotic activities in vitro. It was found that this activity differed between collection sites and was lost during winter (Bungu, 2005).

Methanol extracts of the leaves and bulb of T. violacea showed antiproliferative and pro-apoptotic effects against breast (MCF7), cervical (HeLa), colon (HT29) and oesophageal (WHCO3) cancer cell lines (Bungu et al., 2006). The dichloromethane and 90% methanol extracts of T. violacea leaves were tested for genotoxicity using the bacterial Ames and VITOTOX® tests with and without metabolic activation (Elgorashi et al., 2003). It was not found to be genotoxic in any of the assays.
3.7 Extraction of medicinal plants

The selection of suitable extraction solvents for the study was guided by solvents already used by participating practitioners and solvents that would be easily obtainable by them. Participating practitioners mostly used water for remedy preparation. They either made infusions with hot or cold water, or decoctions. The use of water for extraction by traditional healers was confirmed by numerous sources (Eloff, 1998a; Grierson & Afolayan, 1999; Inngjerdingen et al., 2004; Kelmanson et al., 2000; Shale et al., 1999). In addition to water, alcohols such as ethanol and methanol have been used by traditional practitioners in South Africa. These solvents were reported to be relatively inexpensive and freely available (Louw et al., 2002). It was thus decided that cold water and ethanol extracts would be most appropriate to use in this study. Using the extraction solvents in this way, would also limit the destruction of any heat labile components. The use of water and ethanol thus ensured the accessibility and relevance of the results to participating practitioners. The concept of direct benefits has been said to be the most important challenge in ethnopharmacology (Cordell & Colvard, 2005). In addition, plant material collection and preparation was done as close as possible to the traditional way (Table 3.6).

The previously cut plant material was macerated at room temperature with just enough deionised water or 99% ethanol to cover plant material. Fresh solvent was added every 24 hours for three days. All extracts were vacuum-filtered through a Whatman No1 filter and stored in the dark until drying. Aqueous extracts were freeze-dried and the dried material was stored at 4°C in a desiccator, protected from light in capped 50 ml polypropylene tubes. Ethanol extracts were concentrated and dried in a rotary evaporator at a temperature of \( \leq 67°C \) for a maximum time of three hours. Concentrated ethanol extracts were transferred to 50 ml polypropylene tubes and were
freeze-dried. The dried ethanol extracts were transferred into light protected sterile polypropylene tubes and stored at 4°C, in a desiccator.

Table 3.6 Comparison of scientific and traditional plant collection and preparation methods.

<table>
<thead>
<tr>
<th>Scientific method</th>
<th>Traditional methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant material was collected in the morning (Abebe, 1984; Lipp, 1989).</td>
<td>Plant material was mostly collected in the early morning or sometimes in the afternoon. Practitioners rarely collected plants at night. Healers generally transported plants that they wanted to plant in the garden in plastic bags.</td>
</tr>
<tr>
<td>After collection, plant material was stored in a closed plastic bag during transportation which generally did not last more than an hour. Dust and soil were removed by rinsing the plant material under running water. The wet material was then patted dry with a paper towel and cut into 1-2 mm pieces. Plant material was extracted fresh.</td>
<td>Traditionally, plant material is washed under running water after collection to remove dust or residual soil. Traditional practitioners generally crush their plant material in a traditional ‘grinding machine’. This has been reported earlier (Abebe, 1984) Plant material was either used fresh or dried, depending on the time between collections from the bush. One of the practitioners remarked that she prefers working with fresh material.</td>
</tr>
</tbody>
</table>

3.7.1 Extraction of selected plants

The stems and leaves of flowering *Bulbine frutescens* plants were harvested in mid January from the medicinal garden at 10:00 in the morning. It was prepared and macerated according to the methods described above. Sixty-five grams of raw material yielded 1.34 g of dry powder for the water extract. That is a 2.05% yield. None of the ethanol extract yields were calculated for any of the plants, because the transfer from the round-bottom container used for rotary evaporation proved to be too challenging to get all the extract out. Accurate masses of the dried ethanol extract could thus not be measured.

The bulb of *Ornithogalum longibracteatum* was harvested in mid June from a non-flowering plant. It had been growing undisturbed in a black nursery bag for two years with minimal watering. The white bulb weighed 646.95 g. Both the ethanol and water extracts were very sticky and viscous, and could not be filtered through the normal Whatman No1 filter. It was thus filtered through cotton wool instead. The yield of dry water extract was 1.14%. The ethanol extract was very hygroscopic and had to be handled with care outside of the desiccator.
The aerial parts of a *Ruta graveolens* shrub were harvested in early December. The branches carried green and brown stems and green seedpods with black seeds. All were cut into 1 to 2 mm pieces. The water extract had a high viscosity and were filtered through a Whatman No1 filter with great difficulty. It also had the pronounced smell of crushed *Ruta*. The alcoholic extract had a viscosity close to that of pure ethanol and lacked the pronounced smell associated with the plant. The water extract produced a dry yield of 12.67%.

*Tarchonanthus camphoratus* leaves and twigs were cut off a tree early in the morning at the end of October. The water extract turned a red-brown colour and smelled of camphor. It filtered relatively easy through the Whatman No1 filter paper. Lots of bubbles were observed while filtering. The ethanol extract was green and filtered easily. The water extracts containing 14.79 g of fresh plant material yielded 0.95 g of dried extracted material, which indicated a yield of 6.45%.

The whole plant of *Tulbaghia violacea* was used for extraction. The water extract smelled very much of garlic and was filtered at a slow speed through the Whatman No.1 filter. The ethanol extracts only had hints of the garlic smell, much less pronounced than the water extract. The percentage yield of the water extract was 2.31%.

### 3.7.2 Collaborative extraction of medicinal plants

In an effort to realise the healers’ request for transparent research, it was decided to have a trial-run with one healer who could understand English, to demonstrate the actual extraction process (section 2.4.3). If this healer could see what the laboratory processes involved, she would be able to explain it to other participating practitioners much better than we could, because they shared the same frame of reference. During the exercise the practitioner assisted in the extraction process (Figure 3.8 and Figure 3.9) and even took over the procedure at times.
The collaborative extraction exercise taught us a lot about the practicality of trying to expose participating practitioners to real-time laboratory work. It was decided not to try and incorporate the participating healers in real-time procedures, because it took too long. Practitioners would not be able to afford being away from their surgeries for such an extended period of time. We also realised that to try and accommodate more than one practitioner at a time was not feasible. However, it was still an essential exercise for the collaborative...
research. Instead of real-time laboratory demonstrations, shortened demonstrations at feedback seminars and small group laboratory tours to introduce healers to the laboratory environment were pursued as an alternative.
4 Antimicrobial screening

Participating traditional health practitioners initially requested for biological evaluation of their remedies for diarrhoea and complications associated with circumcision, both of which have antimicrobial components. Antimicrobial screening was thus a suitable method to accommodate the practitioners’ request and facilitate the progress of the collaborative research study.

Screening of plant extracts for antimicrobial activity has indicated that higher plants may be a potential source for new anti-infective agents (Ojala et al., 2000). Bioprospecting programs directed towards infectious diseases, using ethnobotanical information have been found to be very useful in finding potential antimicrobial plants (Heinrich et al., 2004) and have been supported by a number of studies (Cardellina & Boyd, 1995; Khafagi & Dewedar, 2000). The increase in number of articles published on antimicrobial activity has demonstrated the increased global interest in medicinal plants (Rios & Recio, 2005) and their antimicrobial potential.

For centuries, indigenous people of South Africa have used a variety of plants to treat infections (Springfield et al., 2003) and literature for this study indicated that Ruta graveolens and Tulbaghia violacea probably had the best potential of producing some antimicrobial activity. The flavonoids (Ojala et al., 2000) as well as furanocoumarins or furanoquinoline alkaloids identified from R. graveolens have been suggested to be responsible for antimicrobial activity in some plants (Van Wyk & Wink, 2004). Allicin, which has been believed to provide the antimicrobial and antifungal properties associated with garlic, was also found in T. violacea which might indicate similar antimicrobial effects (Motsei et al., 2003). Bulbine frutescens has been used in many parts of Africa for the topical treatment of wounds, which might indicate some antimicrobial activity (Rabe & van Staden, 1997). Similarly, the paste from crushed bulbs of Ornithogalum longibracteatum has been used for the
dressing of wounds (Mulholland et al., 2004) and infusions of 
*Tarchonanthus camphoratus* has been used for the treatment of bronchitis (Neuwinger, 2000).

4.1 Infectious diseases

Statistics support the traditional health practitioners’ plea for the investigation of diarrhoea. A draft report by the Regional Office for Africa of the International Council of Science (International Council for Science Regional Office for Africa, 2006) conceded that the prevention of diarrhoeal diseases does not get the priority it deserves. In South Africa, diarrhoea was found to be the fifth most common cause of premature mortality in the general adult population and the third most prominent cause in children under the age of five years. Interestingly, diarrhoea was the second highest cause of premature mortality among adult females and only the sixth among adult males (Bradshaw & Nannan, 2003). According to the census data of 2001, the Eastern Cape was found to be particularly vulnerable to diarrhoea and other infectious diseases due to poor water provision and sanitation. It was later confirmed that diarrhoea was, in fact, the second largest cause (after HIV/AIDS) of premature mortality in the Eastern Cape (Bradshaw et al., 2006), which explained the practitioners’ concern for this disease.

In addition to diarrhoea, lower respiratory tract infections also had a mortality index of sixth overall for the adult population and fourth for children under the age of five years, nationally (Bradshaw & Nannan, 2003). In the Eastern Cape, the premature mortality rate for lower respiratory tract infections was rated fifth overall (Bradshaw et al., 2006). In addition, HIV infection and tuberculosis fuel diseases such as pneumonia and diarrhoea (Bradshaw & Nannan, 2003). It would thus be especially advantageous to screen local plants for antimicrobial activity, because of the high incidence and mortality of infectious diseases in the Eastern Cape Province.
4.1.1 Conventional antimicrobial agents and microbial resistance

Antimicrobial agents may be grouped according to their therapeutic targets. Targets may either be unique to the microorganism or be of more importance for the survival of the microorganism than for humans. Targets may be grouped according to the location in or on the microbe, for example the cell wall, the bacterial ribosome, or bacterial nucleic acids.

Antibacterials and antifungals that target the cell-wall, compromise its integrity by inhibiting critical processes in cell-wall synthesis (Chambers, 2004b). Examples of antibiotics that inhibit cell-wall synthesis include beta-lactam antibiotics, beta-lactamase inhibitors, vancomycin, teichoplanin, fosfomycin, bacitracin and cycloserine (Chambers, 2004b). Antifungal agents that inhibit cell-wall synthesis include amphotericin A and B and the azoles (Sheppard & Lampiris, 2004). Antibacterials binding to and interfering with bacterial ribosomes inhibit bacterial protein synthesis (Chambers, 2004c). Examples of bacterial ribosome targeting antibiotics include chloramphenicol, tetracyclins, macrolides, streptogrammins, iaxolidinones (Chambers, 2004c) and aminoglycosides (Chambers, 2004a). Bacterial nucleic acids are targeted by nucleoside synthesis and DNA replication inhibitors (Chambers, 2004d). These inhibitors include sulfonamides, quinolones and fluoroquinolones (Chambers, 2004d).

In spite of the many different types of antimicrobials available, microbial resistance is an alarming problem globally and has similarly, been reported to be one of the greatest threats to health care in the African region (International Council for Science Regional Office for Africa, 2006). Microbial resistance has also surfaced as a great problem in South African public hospitals. In addition, the current national antibiotic policy was found to be inadequate to prevent the spread and occurrence of resistance. It was suggested that specific resistance profiles should be monitored in areas in order to reduce further resistance development (Essack, 2006). However, backup strategies such as the development of new antibiotics must still be pursued. Accordingly, the national Department of Science and Technology
has funded a project for the bioprospecting of indigenous flora for novel antimicrobial compounds (Mossie, 2001).

4.1.2 Selection of pathogens for this study

Most antimicrobial studies utilise a combination of Gram-positive and Gram-negative bacteria as well as fungus to evaluate antimicrobial efficacy (Anani et al., 2000; Desta, 1993). This is due to selective toxicity that the plant may have for a certain pathogen or class of pathogen (Cos et al., 2006).

4.1.2.1 Bacillus subtilis

*Bacillus* species are endospore-forming Gram-positive, rod-shaped bacteria which live in the soil (Tortora et al., 1992). Due to their habitat, the chances of finding *B. subtilis* in medicinal plant remedies are high. In addition, some *Bacillus* may cause diarrhoea (McGaw et al., 2000), which makes it especially suitable for this study.

4.1.2.2 Staphylococcus aureus

*Staphylococcus* species are Gram-positive cocci and live in the soil and on the skin and mucous membranes of animals (Tortora et al., 1992). *S. aureus* may produce toxins that enhance its pathogenicity (Tortora et al., 1992). It is one of the most pathogenic staphylococci bacteria and is responsible for many infections such as toxic shock syndrome, scalded skin syndrome and post-operative infections (Nester et al., 2004). *S. aureus* was the first pathogen that became resistant to all known antibiotics (Ojala et al., 2000). As in other parts of the world (Machado et al., 2003), South Africa has experienced an emerging increased prevalence of methicillin-resistant *S. aureus* in the last few years (Brink et al., 2006). Because of the prevalence of *S. aureus* on the skin, it may be one of the agents causing complications during circumcision rituals, which makes it suitable for selection in this study.

4.1.2.3 Escherichia coli

*Escherichia* species are facultative anaerobic, Gram-negative, rod-shaped bacteria which grow in the soil and on plants (Tortora et al., 1992). *E. coli* together with other enterobacteria are the predominant organisms living in the
intestines of humans and animals (Nester et al., 2004). *E. coli* bacteria living in the large intestine are generally harmless. Infection is caused when it gains access to other sites (Tortora et al., 1992). Some strains of *E. coli* may cause diarrhoea and it is the most common cause of urinary tract infections (Nester et al., 2004). However, because of its constant presence in the body, the use of antibiotics for other conditions may cause inhabiting *E. coli* to develop resistance to these antibiotics (Nester et al., 2004). The incidence of multidrug-resistant *E. coli* has of late increased in South Africa (Brink et al., 2006). Enterohaemorragic *E. coli* have increasingly emerged as pathogens and are causing significant human diseases such as diarrhoea, haemorragic colitis and other complications (Vorav Ruthikunchai et al., 2004).

The poor sanitation in the Eastern Cape makes *E. coli* especially relevant to investigate in this study. Due to the nature of the demographics of patients visiting participating practitioners, they will probably encounter this type of infection regularly.

4.1.2.4 *Klebsiella pneumoniae*

Like *E. coli*, *Klebsiella* species are facultative anaerobic, Gram-negative, rod-shaped bacteria which live in the soil, on plants and in the gastrointestinal tract of animals (Tortora et al., 1992). Many important pathogens are found among this species (Tortora et al., 1992). *Klebsiella* species cause pneumonia in hosts whose defenses had been impaired and the best known of these is *Klebsiella pneumoniae* (Nester et al., 2004).

An analysis of a *Klebsiella* outbreak in 2005 at Mahatma Gandhi Hospital’s neonatal nursery revealed the presence of *K. pneumoniae* on the hands of 10% of the staff who worked in this unit. Resistance profiles of isolated *K. pneumoniae* revealed gentamycin and extended spectrum beta-lactamase (ESBL) resistance (Strum, 2005). In South Africa, *Klebsiella* spp. had a dramatic rise in ESBL production when resistance patterns were investigated (Brink et al., 2006). Some carbapenem resistant strains of *K. pneumoniae* have also been isolated (Brink et al., 2006). It is thus a suitable organism to
include in this study, because it is generally associated with the HIV/AIDS pandemic (Bradshaw et al., 2006).

### 4.1.2.5 Candida albicans

*Candida albicans* is a yeast which is part of the normal flora of the body, but may become opportunistically pathogenic if the host defenses are compromised or as a result of antibiotic therapy (Duarte et al., 2005; Nester et al., 2004). Infection in immunocompromised hosts may lead to oropharangeal thrush or vaginitis. It manifests as creamy-white patches in the oropharangeal area and results in swallowing and chewing difficulties. It is also associated with severe diarrhoea (Motsei et al., 2003). Factors that may lead to *Candida* overgrowth include disruption of normal flora due to hormonal imbalances or antibiotic treatment, AIDS, uncontrolled diabetes and severe burns (Nester et al., 2004). This yeast thus provides links to the HIV/AIDS pandemic and diabetes mellitus encountered by participating practitioners involved in this study (section 1.5).

### 4.2 Antimicrobial screening methodology

A number of antimicrobial evaluation methods exist, including agar diffusion, dilution and bio-autographic methods (Cos et al., 2006). For this study, an adaptation of the microdilution method was used (Eloff, 1998b; McCleland et al., 2004). The rationale for the method was to expose a standard concentration of microorganisms to a standard concentration of plant extract for a period of time. After the incubation period the viability of the microbial cultures was measured spectrophotometrically using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) as an indicator and percentage inhibition was calculated against the negative control. This method was also relatively easy to explain to the participating practitioners at the subsequent feedback seminar.
4.2.1 Routine maintenance of microbes

The microorganisms *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans* were obtained from the Department of Biochemistry and Microbiology (NMMU). These bacteria and fungi were basic wild-type clinical isolates. Pure cultures were kept on sloped nutrient agar in McCartney bottles and stored at 4°C for about two months. Prior to experiments, cultures were streaked out on either nutrient or sabouraud (for *C. albicans*) agar plates and stored at 4°C for about two weeks while experimentation proceeded.

4.2.2 Materials used for screening

4.2.2.1 Suspending and dissolution agents

Either nutrient (Biolab, Merck, Germany) or sabouraud (Sab) (Difco, Becton Dickinson and Co., USA) broth, were used as suspending agents for bacterial and fungal cultures, respectively. Both were sterilised at 121°C for 20 minutes with moist heat in an autoclave prior to use. The sterile broth was also used as vehicle for the dissolution of compounds used for screening.

4.2.2.2 Colourimetric substrate

MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was used as the colourimetric substrate in the assay (American Type Culture Collection, 2001). The assay measured cell proliferation and viability as a function of cell metabolism and growth (American Type Culture Collection, 2001; Roche Applied Science, 2006). Mitochondrial reductase of viable cells cause the reduction of soluble tetrazolium salts (yellow) to insoluble formazan salts (dark blue). The reduction is proportional to the amount of viable cells (Colombo *et al.*, 2001). The remaining solubilised tetrazolium salts are aspirated and the precipitated formazan salts left at the bottom are solubilised by the addition of dimethylsulfoxide (DMSO). The colour intensity of the solubilised salts is measured spectrophotometrically at 540 nm (Mosmann, 1983). These measurements are compared and percentage viability relative to untreated control cells determined.
MTT (Sigma Chemicals Co., USA) was made up with sterile phosphate-buffered saline (PBS; Formulae A.1) at a concentration of 0.5 mg/ml on the day of the experiment. The solution was filter sterilised through a sterile 0.22 µm syringe filter (Cameo 25AS acetate filter) and kept at 4ºC until needed.

4.2.2.3 Negative controls

The negative control represented the growth of untreated organisms during the test period. However, the addition of DMSO as co-solvent to aid dissolution of the positive control and test sample had to be accounted for, because DMSO could be toxic to microbes (Ojala et al., 2000). The negative control thus consisted of the bacterial suspension in the relevant broth as well as 1.25 or 2.5% DMSO (Associated Chemical Enterprises (Pty) Ltd, South Africa), to correspond with the concentration of DMSO used to dissolve the test samples. The negative controls thus represented 100% viability for the microbes taking in account the corresponding concentration of DMSO that was used.

4.2.2.4 Positive control

Chloramphenicol was used as the positive control for all the microbial cultures that were tested. Chloramphenicol is a bacteriostatic broad-spectrum antibiotic with activity against aerobic and anaerobic Gram-positive and Gram-negative microbes, as well as against rickettsia, but not chlamidiae (Chambers, 2004c). Gram-positive bacteria are generally inhibited at concentrations of one to 10 µg/ml and Gram-negative by concentrations of 0.2 to 5 µg/ml (Chambers, 2004c). Chloramphenicol may be bactericidal for highly susceptible *Haemophilus influenzae*, *Neisseria meningitides* and some strains of bacteroides (Chambers, 2004c).

Stock solutions of chloramphenicol (Sigma Chemicals Co., USA) were prepared on the day of the experiment with sterile nutrient or sabouraud (Sab) broth at concentrations of 10 and 20 µg/ml. The solutions were filter sterilised with 0.22 µm syringe filters and stored at 4ºC prior to experimentation.
4.2.2.5 Test samples

Dried plant extracts were weighed into sterile microtubes and dissolved in DMSO. Nutrient or sabouraud broth was added to make a final concentration of 1 mg/ml of test sample with a DMSO concentration of 5%. The mixtures were sterilised by passing the solution through a 0.22 µm syringe filter.

4.2.3 Antimicrobial assay

Each microbial culture was activated by transference of a loopful of single cultures from the spread plates (prepared in 4.2.1) into 10 ml of nutrient or Sab broth in the case of Candida albicans. Inoculation was followed by overnight incubation at 34°C or at 27°C for C. albicans in an orbital shaker (Labcon® orbital shaker) rotating at 100 rotations per minute (rpm). The next morning 100 µl of the inoculated broth were transferred into 10 ml sterile broth and incubated for a further two hours to ensure that the bacteria were in the log phase of growth during the experiment. The optical density of the fresh inoculant was then adjusted with sterile broth to 0.05 at 600 nm.

Experiments were performed in horizontal laminar flow units, using round bottom 96-well microtiter plates (Plastpro Scientific® Pty Ltd). The previously adjusted microbial suspension was diluted with equal parts of previously prepared stock solutions of either the positive or negative controls, or test samples to give a final volume of 100 µl in each well. The final concentrations of DMSO in the negative control wells were thus 1.25 and 2.5%. The dilutions of the positive control and test samples thus coincided with the DMSO concentrations in the negative controls. The final concentration of chloramphenicol was 5 and 10 µg/ml and the test samples were 250 and 500 µg/ml.

The cultures were exposed to the various compounds for 2 hours on the orbital shaker at 100 rpm at 34°C for bacteria and at 27°C for C. albicans. After incubation 40 µl of MTT solution was added to each well. The plates were again incubated at 34°C for 30 minutes to allow for the reduction of the soluble tetrazolium salts to insoluble formazan salts by viable cells. The plates were then centrifuged (Eppendorf Centrifuge 5804R) at 3000 rpm for
seven minutes to pellet the insoluble formazan salts. The supernatants were aspirated and 90 µl of DMSO added to each well to solubilise the pellet of formazan salts. The plates were shaken for one minute and read at 540 nm in a multiplate reader (Multiscan MS® version 4.0 Labsystem® type 352).

4.2.4 Data manipulation and statistical analysis

Absorbance readings obtained from the spectrophotometrical measurements were manipulated to give the percentage inhibition of cell growth for each sample compared to 100% viability represented by the measurement for the negative control with the corresponding DMSO concentration. Percentage growth inhibition was calculated according to the following equation:

\[
\% \text{ growth inhibition} = \left( \frac{\text{Avg: Negative control measurement} - \text{Sample measurement}}{\text{Avg: Negative control measurement}} \right) \times 100%
\]

Key: \(\text{Avg} = \text{average}\).

Statistical analysis of results was done using GraphPad Prism® 4 (GraphPad Software, 2003). The data that was analysed included four replicate readings for each sample measured during an individual experiment as well as the pooling of measurements for two or three individual experiments. For each sample the mean and standard error of the mean (SEM) were calculated. The SEM was used to compensate for variation between experiments. Significance determinations were obtained by applying a two-tailed unpaired t-test (\(p < 0.05\)).

4.3 Results

Individual measurements for each test were done in quadruplicate and experiments were performed three times for all microbes, with the exception of \(K.\ pneumoniae\) which were performed twice. Test samples were tested at concentrations of 250 and 500 µg/ml. Previous reports suggest that crude extracts with MICs above 1 mg/ml would not be clinically significant (Rios & Recio, 2005). For the purposes of this study, growth inhibitory activity was
considered to be clinically significant only above 50% of the control and growth stimulation activity at above 120%. However, growth stimulation activities would only be discussed and not indicated on graphs. The data in the figures are also represented in tables in Appendix B. These tables contain information such as the average % growth inhibition ± SEM, number of replicates (n) and p-values for comparison between sample and negative control and between different concentrations of the same sample. The findings are presented according to the organism tested.

4.3.1 Bacillus subtilis

The average percentage growth inhibition obtained for chloramphenicol was 98.35 ± 1.34% (n = 23) against *B. subtilis* for the aqueous and ethanol extract experiments. A summary of the percentage growth inhibition of aqueous extracts can be viewed in Figure 4.1 and Table B.1. *Bulbine frutescens* (500 µg/ml; 50.34 ± 16.97%, p = 0.0071) and *Tulbaghia violacea* (250 µg/ml; 76.34 ± 8.40%, p < 0.0001 and 500 µg/ml; 60.63 ± 8.82%, p = 0.0025) were the only aqueous extracts that produced any statistically significant growth inhibitory activity for *B. subtilis*. The findings and significance determinations for percentage growth inhibition of ethanol extracts on *B. subtilis* are summarised in Figure 4.2 and Table B.2. The two concentrations of the ethanol extract of *T. violacea* tested in the screening assay both showed 100% growth inhibition of *B. subtilis*. It can be deduced that the minimum inhibitory concentration of the ethanol extract of *T. violacea* is ≤ 250 µg/ml. The ethanol extract of *R. graveolens* also showed good growth inhibitory activity at 500 µg/ml (59.41 ± 21.53%, p = 0.0053).

It is noteworthy to explore the very large variation of the two concentrations of the ethanol extract of *Ruta graveolens*. There was a slight methodological difference between the three repeated experiments that seemed to significantly influence the performance of this extract. During the second experiment, the DMSO was added to solubilise the extracts, but the experiment could not be completed on that day, so approximately 2 mg of each sample was stored overnight at 4°C exposed to 50 µl of DMSO. If the results of the second experiment were omitted, the 500 µg/ml extract of *R.*
*graveolens* would have produced complete growth inhibition and the 250 µg/ml a 60.21 ± 15.19% growth inhibition of which both would have been statistically significant. The possibility exists that the *R. graveolens* extract might have lost some of its antimicrobial activity due to the prolonged exposure to DMSO.

The ethanol extract of *B. frutescens* indicated a concentration-dependent (p = 0.0162) growth stimulatory effect of this extract on *B. subtilis* (Figure 4.2). The ethanol extract of *Ornithogalum longibracteatum* (500 µg/ml) also stimulated growth of *B. subtilis* significantly (p = 0.0096).

![Figure 4.1 Percentage growth inhibition (± SEM) produced on *Bacillus subtilis* by aqueous extracts. Bacteria were exposed to either chloramphenicol (5 µg/ml) or aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for two hours at 34°C. Bars represent three individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).
4.3.2 Staphylococcus aureus

The average percentage growth inhibitory response obtained against *S. aureus* for chloramphenicol was 73.24 ± 5.06% (n = 22) for the aqueous and ethanol extract experiments. Influences on growth of *S. aureus* produced by aqueous extracts are summarised in Figure 4.3 and Table B.3. Aqueous extracts of *Ornithogalum longibracteatum* (250 and 500 µg/ml) and *Bulbine frutescens* (500 µg/ml) produced statistically significant growth inhibitory effects. However, none of the responses for aqueous extracts were clinically significant. Significant growth stimulation for *S. aureus* was produced by aqueous extracts of *Tulbaghia violacea* (500 µg/ml; which was also concentration-dependent; p = 0.0288) and *Tarchonanthus camphoratus* (250 µg/ml).
Figure 4.4 and Table B.4 summarise variation in the growth of *S. aureus* produced by ethanol extracts. The ethanol extract of *T. violacea* (500 µg/ml; 76.27 ± 10.27%, p < 0.0001) was the only extract that produced clinically significant growth inhibitory activity for *S. aureus*. The response for *T. violacea* was also concentration-dependent (p = 0.0073). The ethanol extract of *T. camphoratus* (250 and 500 µg/ml) produced significant growth stimulation for *S. aureus* in a concentration-independent manner (p = 0.0235).

![Figure 4.3 Percentage growth inhibition (± SEM) produced on *Staphylococcus aureus* by aqueous extracts. Bacteria were exposed to either chloramphenicol (5 µg/ml) or aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for two hours at 34ºC. Bars represent three individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).](image-url)
Figure 4.4 Percentage growth inhibition (± SEM) produced on *Staphylococcus aureus* by ethanol extracts. Bacteria were exposed to either chloramphenicol (5 µg/ml) or ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for two hours at 34ºC. Bars represent three individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005** and p < 0.0005***).

### 4.3.3 Escherichia coli

The average percentage growth inhibitory response obtained against *E. coli* for chloramphenicol (5 µg/ml) was 91.89 ± 4.862% (n = 19) for the aqueous and ethanol extract experiments. None of the aqueous (Table B. 5) or ethanol (Table B. 6) extracts had any growth inhibitory activity on *E. coli*. Growth stimulation of above 20% was produced by aqueous extracts of *Tulbaghia violacea* (250 µg/ml; -40.62 ± 16.37% and 500 µg/ml; -45.74 ± 15.56%) as well as ethanol extracts of *Ruta graveolens* (250 µg/ml; -52.70 ± 15.75% and 500 µg/ml; -35.67 ± 22.58%) and *T. violacea* (250 µg/ml; -52.42 ± 22.41% and 500 µg/ml; -64.57 ± 22.83%).
4.3.4 Klebsiella pneumoniae

The average percentage growth inhibitory response obtained against K. pneumoniae for chloramphenicol (5 µg/ml) was 92.48 ± 2.70% (n = 16) for the aqueous and ethanol extract experiments. The findings of the growth effects of aqueous extracts on K. pneumoniae are summarised in Figure 4.5 and Table B.7. Aqueous extracts of Ornithogalum longibracteatum (250 µg/ml; 57.94 ± 12.73%, p < 0.0001) and T. violacea (500 µg/ml; 61.75 ± 8.25%, p < 0.0001) inhibited growth of K. pneumoniae above 50% (Figure 4.5). None of the ethanol extracts promoted growth of K. pneumoniae (Table B.8). Ethanol extracts of Ruta graveolens (500 µg/ml; 54.98 ± 4.35%, p < 0.0001) and Tulbaghia violacea (500 µg/ml; 67.26 ± 5.71, p < 0.0001) inhibited growth of K. pneumoniae above 50% (Figure 4.6).

![Figure 4.5](image_url)

Figure 4.5 Percentage growth inhibition (± SEM) produced on Klebsiella pneumoniae by aqueous extracts. Bacteria were exposed to either chloramphenicol (5 µg/ml) or aqueous extracts (250 and 500 µg/ml) of Bulbine frutescens (Bf), Ornithogalum longibracteatum (Ol), Ruta graveolens (Rg), Tarchonanthus camphoratus (Tc) and Tulbaghia violacea (Tv) for two hours at 34ºC. Bars represent two individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005** and p < 0.0005***).
Figure 4.6 Percentage growth inhibition (± SEM) produced on *Klebsiella pneumoniae* by ethanol extracts. Bacteria were exposed to either chloramphenicol (5 µg/ml) or ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for two hours at 34ºC. Bars represent two individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005** and p < 0.0005***).

### 4.3.5 *Candida albicans*

The average percentage growth inhibitory response obtained against *C. albicans* for chloramphenicol (10 µg/ml) was 9.85 ± 4.04% (n = 23) for the aqueous and ethanol extract experiments. None of the aqueous extracts had any growth inhibitory response on *C. albicans* (Table B. 9). Aqueous extracts of *Bulbine frutescens* (250 µg/ml; -45.64 ± 6.38% and 500 µg/ml; -30.20 ± 6.11%), *Ruta graveolens* (500 µg/ml; -20.64 ± 7.31%) and *Tarchonanthus camphoratus* (250 µg/ml; -43.44 ± 5.71% and 500 µg/ml; -50.19 ± 3.25%) had growth promoting effects of above 20%. Generally, ethanol extracts had better growth inhibitory activity against *C. albicans* than aqueous extracts (Table B. 10). The ethanol extract of *Tulbaghia violacea* produced significant growth inhibitory activity above 50% at both concentrations tested (Figure 4.7).
Figure 4.7 Percentage growth inhibition (± SEM) produced on *Candida albicans* by ethanol extracts. Fungi were exposed to either chloramphenicol (10 μg/ml) or ethanol extracts (250 and 500 μg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for two hours at 27°C. Bars represent three individual experiments with four replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005** and p < 0.0005***).

### 4.4 Discussion

Generally, the aqueous extracts of *Bulbine frutescens* produced better growth inhibitory activity than the ethanol extracts. The most pronounced activity was produced by the aqueous extract (500 μg/ml) against *B. subtilis* which reached growth inhibition of 50.34 ± 16.97%. However, none of the other aqueous or ethanol extracts could reach an inhibitory value of over 50%. Generally, growth inhibition produced by aqueous extracts seem to be more selective towards Gram-positive than Gram-negative bacteria and absent for *C. albicans*. The ethanol extract of *B. frutescens* produced significant growth stimulatory activity for *B. subtilis* with a significant concentration-dependent
effect. It seems that the ethanol extract may be a source of nutrition for *B. subtilis*.

In previous studies, aqueous and various organic extracts of dried leaves of *B. frutescens* were inactive against *S. aureus* (1 mg/ml), *B. subtilis* (1 mg/ml) (Rabe & van Staden, 1997) and *C. albicans* (Motsei *et al.*, 2003). Both studies used leaves of *B. frutescens* dried at 50ºC and stored at room temperature prior to extraction (Motsei *et al.*, 2003; Rabe & van Staden, 1997) as opposed to fresh leaves used in this study. In addition, both studies also collected their plant material from different locations in KwaZulu Natal versus the Eastern Cape in this study. These results suggest that fresh leaves of *B. frutescens* have mild antiseptic activity which might support its use in wound healing.

Similar to *B. frutescens*, the aqueous extracts of *Ornithogalum longibracteatum* also produced slightly better growth inhibitory activity than the ethanol extracts. The aqueous extract (250 µg/ml) produced growth inhibition of 57.94 ± 12.73% against *K. pneumoniae*. However, the ethanol extract (500 µg/ml) stimulated growth of *B. subtilis* significantly. The reported use of crushed bulbs of *O. longibracteatum* for the dressing of wounds (Neuwinger, 2000) and its use for swelling (Mulholland *et al.*, 2004) may provide for a synergistic effect between an anti-inflammatory and mild aseptic action especially against *S. aureus* which resides on the skin. Evidence suggest that anti-inflammatory action may become more significant in future for the treatment of microbial infection as the infective process is better understood (Maeda *et al.*, 1996).

The ethanol extracts of *Ruta graveolens* produced higher growth inhibitory activity than the aqueous extracts. The ethanol extract (500 µg/ml) of *R. graveolens* produced growth inhibitory activities of above 50% for *B. subtilis* (59.41 ± 21.53%) and *K. pneumoniae* (54.98 ± 4.35%). Previous studies utilising disc-diffusion methods for *B. subtilis*, indicated antimicrobial activity of methanol extracts of *R. graveolens* at concentrations of 0.1 mg/disc (Ivanova *et al.*, 2005) and 126 µg/ml (Ojala *et al.*, 2000). The same studies indicated the following:
• A lack of activity against *E. coli* (Ivanova *et al.*, 2005; Ojala *et al.*, 2000), in this study growth was stimulated,

• A lack of activity against *C. albicans* (Ivanova *et al.*, 2005; Ojala *et al.*, 2000), in this study the ethanol extract (500 µg/ml; 40.47 ± 5.06%) produced weak but statistically significant growth inhibitory activity for *C. albicans*, and,

• A weak but significant activity against *S. aureus* (Ivanova *et al.*, 2005; Ojala *et al.*, 2000), similar to activities found in this study (500 µg/ml; 21.31 ± 6.44%).

Another study, which utilised the MIC micro-dilution method (Alzoreky & Nakahara, 2003) found the MIC for the methanol extract of *R. graveolens* to be 2.64 mg/ml for *S. aureus*. These results suggest that the ethanol extract of *R. graveolens* collected during summer in South Africa has better activity than the methanol extracts collected at the same time in Bulgaria (Ivanova *et al.*, 2005) and Finland (Ojala *et al.*, 2000). Conversely aqueous extracts of *R. graveolens* stimulated the growth of *B. subtilis* significantly in this study.

Extracts of *Tarchonanthus camphoratus* leaves could not inhibit the growth of any of the organisms tested, except weak growth inhibitory activity of the ethanol extract against *K. pneumoniae* (250 µg/ml; 42.40 ± 8.26% and 500 µg/ml; 30.57 ± 4.48%). The ethanol (250 µg/ml) and aqueous (500 µg/ml) extracts significantly stimulated the growth of *S. aureus* and *C. albicans*, respectively. The general absence of antimicrobial activity of the aqueous extracts of the leaves have been reported (Watt & Breyer-Brandwijk, 1962) and particularly the absence of activity against *K. pneumoniae* (McGaw *et al.*, 2000; Rabe & van Staden, 1997). The differences between this study and the two reporting no activity for *K. pneumoniae* included geographical (leaves harvested in the Eastern Cape versus KwaZulu Natal) and preparation differences (leaves used fresh versus dried at 50°C prior to extraction). Another interesting difference was that the leaves were harvested from May to July by McGaw *et al.* (2000) versus leaves harvested in October in the present study.
Interestingly *T. camphoratus* has been used in chest complaints such as asthma (Van Wyk *et al.*, 1997) and bronchitis (Neuwinger, 2000). This might indicate a synergistic effect between the weak antimicrobial effect against *K. pneumoniae* and anti-inflammatory action of the plant (Amabeku *et al.*, 2000) for these conditions. Inhalation as an alternative administration technique may also be advantageous as the essential oil from fresh *T. camphoratus* leaves (harvested in Kenya) showed inhibition of *E. coli, S. aureus, Bacillus* spp and *C. albicans* at concentrations ≤129 mg/ml and a less potent inhibition for *K. pneumoniae* (Matasyoh *et al.*, 2007).

Ethanol extracts of *Tulbaghia violacea* produced the highest growth inhibitory responses of all the extracts tested. The ethanol extracts showed significant inhibitory activity against all microbes tested, except *E. coli* for which both the ethanol and water extracts showed significant growth stimulatory activities at both concentrations. The aqueous extract of *T. violacea* produced growth inhibition of above 50% for *B. subtilis* (250 µg/ml; 76.34 ± 8.40% and 500 µg/ml; 60.63 ± 8.82%) and *K. pneumoniae* (500 µg/ml; 61.75 ± 8.25%). The activity against *B. subtilis* has been implicated previously when lectin-like proteins that were extracted and purified from *T. violacea* produced agglutination of *B. subtilis*, but not *S. aureus* (Gaidamashvili & van Staden, 2002). Aqueous extracts tended to follow the trend in activity of the Gaidamashvili study more closely, because the extraction solvent used in the Gaidamashvili study, PBS, resembled water more closely than ethanol (Gaidamashvili & van Staden, 2002).

The activity against *C. albicans* previously reported for ethanol and aqueous extracts of *T. violacea* was partially confirmed (Motsei *et al.*, 2003). The ethanol extract showed excellent activity in this study and correlated well with MIC values of 0.26 mg/ml and 2.09 mg/ml against a clinical isolate of *C. albicans* from an adult and ATCC strains, respectively, in the study by Motsei *et al.* (2003). The unexpected lack in activity in this study for the aqueous extract of *T. violacea* against *C. albicans*, could be explained by stability tests done by Motsei *et al.* (2003). According to these findings, a loss of antifungal activity was demonstrated after storage of the water extract for two days at
4°C (Motsei et al., 2003). The storage period of the aqueous extract in the present study exceeded this time period (section 3.7).

In contrast to this study, McGaw et al. (2000) found no activity against \textit{B. subtilis}, \textit{S. aureus}, \textit{K. pneumoniae} and \textit{E. coli} when the aqueous, ethanol and hexane extracts of \textit{T. violacea} were tested. The differences between the McGaw et al. (2003) study and the present one have been explained previously and included geographic, preparative and seasonal differences. \textit{T. violacea} was extracted during the summer months for the present study. Interestingly, a previous investigation has found that \textit{T. violacea} lost its blood clotting activity during the winter months (Bungu, 2005) and activity also depended on the geographic location where it grew, which might be true for the antimicrobial activity as well.

4.4.1 Feedback seminar discussions

The combined feedback seminar on the findings of antimicrobial and anticancer screening (chapter 5) of the plant extracts were presented to participating practitioners\textsuperscript{11}. The seminar also included some scientific information on the plants as well as the extraction procedures followed. There was a question about the type of \textit{Bulbine} used, because healers knew there were three types of this plant. They also mentioned that there was a bulb similar to \textit{Ornithogalum longibracteatum} which was red in colour. Practitioners were interested to hear that \textit{Ruta graveolens} was not an indigenous plant. They said that “as far as they could remember”\textsuperscript{12}, it has been used in traditional remedies. When asked, they confirmed that they have seen the plant grow in the wild (section 3.4). They were interested to know what the people in Asia used the plant for.

Extracts of \textit{Bulbine frutescens} did not produce the inhibitory activity that was expected by participating practitioners. This may be due to the methods of extraction, because the fresh leaf juice is used and not an extract. However, the application of the leaf juice on rashes caused by herpes zoster should be investigated for possible anti-viral activity.
The fact that ethanol extracts seemed to show higher growth inhibitory activity than the aqueous extracts were cumbersome to the participating practitioners, because generally traditional health practitioners used water as their most common extractant (Kelmanson et al., 2000; Lin et al., 1999). It has been proposed that water may well be an appropriate extraction solvent when considering the high doses that traditional medicine prescribe (four cups per day) (Shale et al., 1999). It should also be remembered that traditional health practitioners do not filter their remedies and some solid plant material is probably ingested which provides for a better in vivo dosage and a greater range of active components in ready-made traditional remedies. However, further investigation can be done into making extracts with relatively safe forms of ethanol such as cane spirit or brandy.

4.4.2 Summary of findings and limitations

This study again illustrated some common features which many previous antimicrobial screenings have suggested. The first is that Gram-positive organisms seemed to be more susceptible than Gram-negative organisms to the growth inhibitory activities of plants (Alzoreky & Nakahara, 2003; Anani et al., 2000; Kelmanson et al., 2000; McGaw et al., 2000; Meyer & Dilika, 1996; Rabe & van Staden, 1997; Shale et al., 1999). The resistance of Gram-negative bacteria towards antibacterial substances has been related to lipopolysaccharides in their outer membrane (Alzoreky & Nakahara, 2003).

The second common feature was that methanol and ethanol extracts seemed to be more active than aqueous extracts (Grierson & Afolayan, 1999; Kambizi & Afolayan, 2001; Kelmanson et al., 2000; McGaw et al., 2000; Rabe & van Staden, 1997; Shale et al., 1999). In a study which tested the extraction efficacy of different solvents for antimicrobial activity, it was found that no extractant did the best in all the parameters tested. Based on the rating scale employed during the study, acetone was found to be the best overall solvent (Eloff, 1998a). Another report suggested that methanol extracted more bacterial inhibitors than ethanol (Alzoreky & Nakahara, 2003). In this study, the best overall activity was shown by an ethanol extract (Tulbaghia violacea),
but some aqueous extracts did have better activity than their ethanol counterparts (*Bulbine frutescens* and *Ornithogalum longibracteatum*).

It has been suggested that screening should be followed by identification of active compounds by means of a bio-guided assay (Rios & Recio, 2005). However, fractionation of extracts often leads to a loss of biological activity through either compound break-down or the loss of synergistic effects between constituents (Cos *et al.*, 2006). The relatively high concentrations tested in the screening protocols (250 and 500 µg/ml) and the lack of adequate activity (Rios & Recio, 2005) of aqueous extracts, warranted no further investigations on antimicrobial activity of the selected plants. This is because the suggested use of ethanol extracts was not accepted very enthusiastically by participating practitioners, because of the high frequency of alcohol abuse encountered by them among their communities.

Limitations of the antimicrobial screening included the high screening concentrations, because generally concentrations above 150 µg/ml may lead to many false-positive results (Cos *et al.*, 2006). Also the lack of adequate standardisation of the microbial cultures before screening could have influenced the findings. However, the good activity of the positive control indicated that the number of cultures could not have been too high and the absorbance readings of the raw data were within the optimum range for the spectrophotometer. It is generally recommended that cytotoxicity evaluations should be done on antimicrobial drugs, to rule out non-selective toxicity (Cos *et al.*, 2006). This was done on Chang liver cells (section 5.3.3).
Reports on plants used for the treatment of cancer in South Africa are scarce (Steenkamp & Gouws, 2006). However, cancer was one of the initial illnesses requested by traditional health practitioners for investigation and it is well known that plant substances have been a great source of anti-neoplastic agents (Kamuhabwa et al., 2000). The most famous example is probably the isolation of the alkaloids, vincristine and vinblastine, from *Catharanthus roseus* (L.) G. Don, which is native to Madagascar (Chapuis et al., 1988; Charlson, 1980).

It has been recognised that plants used in traditional medicine are more likely to provide bioactive compounds than plants selected at random (Lee & Houghton, 2005). However, this assumption can be closely linked with the disease target (Cardellina & Boyd, 1995). In the case of cancer, there have been some conflicting reports on the advantage that ethnobotanically selected plants provide for lead identification. Some studies show no correlation (Kamuhabwa et al., 2000) and others a good correlation (Chapuis et al., 1988). This might be explained by the fact that ethnomedical data on cancer treatment may not be reliable. Cancer is a complicated and heterogeneous disease and this makes it difficult to diagnose accurately especially by traditional health practitioners (Chapuis et al., 1988; Kamuhabwa et al., 2000). Still, natural products, including plants, remain a focus area for the American National Cancer Institute (NCI) in their search for new anticancer drugs (Cardellina & Boyd, 1995).

In addition to direct use for cancer, ethnopharmacological uses that may indicate potential anti-neoplastic activity include immune and skin disorders, inflammatory, infectious, parasitic and viral diseases (Steenkamp & Gouws, 2006). *Bulbine frutescens* has been used as an anti-viral for cold sores and *Ornithogalum longibracteatum*, *Ruta graveolens* and *Tarchonanthus camphoratus* as anti-inflammatory agents. More on target, is the leaves of
Tulbaghia violacea that have been taken to treat oesophageal cancer (Van Wyk & Gericke, 2000) and this type of cancer may not be difficult to diagnose accurately.

5.1 Cancer

Cancer is a cellular disease which is characterised by the uncontrolled proliferation and differentiation of neoplastically transformed cells (Chu & Sartorelli, 2004). Some of the hallmarks of cancer include: acquisition of self-sufficient signals for growth, capacity for extended proliferation, resistance of growth inhibitory signals, ability to evade cell death signals, potential for tissue invasion and metastasis, and induction of blood-vessel formation (Albrecht, 2006). In almost all types of cancer, there have been associated activation of blood coagulation either directly, or indirectly by initiation of an inflammatory response (Lee, 2002).

Cancer may be related to a variety of factors such as gender, age, race, genetic predisposition and exposure to environmental carcinogens, the latter of which seem to be most important (Chu & Sartorelli, 2004). Excess weight has been associated with increased cancer mortality (Freeman, 2004). Some metabolic mechanisms associated with obesity such as increased plasma triglycerides, glucose and insulin along with insulin resistance are thought to play a role in the pathogenesis of cancer (Freeman, 2004).

5.1.1 Cancer chemotherapy

The main targets of cancer chemotherapy focus on inhibiting processes of cellular division. The basic groups of cancer chemotherapeutic drugs may be divided as follows: polyfunctional alkylating agents, antimetabolites, plant alkaloids, antitumour antibiotics, hormonal agents, miscellaneous anticancer drugs and investigational agents (Chu & Sartorelli, 2004). Only the first three groups of anticancer drugs will be discussed shortly, because of their relevance to this study.
Alkylating agents exert their cytotoxic effects by transfer of their alkylating groups to various cellular constituents, primarily the DNA within the cell nucleus (Chu & Sartorelli, 2004). Toxicity is dose-related and is worse in rapidly dividing tissues. Examples of alkylating agents include cyclophosphamide, melphalan, chlorambucil, nitrosoureas and cisplatin. Antimetabolites include methotrexate and purine and pyrimidine analogues. Generally these agents interfere with metabolic processes such as nucleotide and nucleic acid synthesis (Chu & Sartorelli, 2004). Drug resistance is a major problem for these two classes of drugs.

Plant alkaloids have substantially contributed in the development of cancer chemotherapeutics. Vincristine and vinblastine, isolated from *Catharanthus roseus*, cause mitotic arrest of the cell cycle. Podophyllotoxins from *Podophyllum peltatum* include the semi-synthetic etoposide and teniposide, which inhibit topoisomerase II resulting in blocking of cell division. Camptothecins from *Camptotheca acuminata* Decne. include topotecan and irinotecan. They inhibit topoisomerase I resulting in DNA damage. Taxanes from *Taxus brevifolia* Nutt. and *Taxus baccata* L. include paclitaxel (alkaloid ester) and docetaxel (semi-synthetic taxane), both of which are mitotic spindle poisons (Chu & Sartorelli, 2004).

### 5.1.2 Colon and colorectal cancer

Colorectal cancer is grouped with other cancers of the gastrointestinal tract such as gastric, oesophageal and pancreatic cancer (Chu & Sartorelli, 2004). Although not as aggressive as the others, colorectal adenocarcinoma is the most common type of gastrointestinal malignancy (Chu & Sartorelli, 2004). Colorectal cancer affects > 1 million people worldwide every year and causes approximately more than half a million deaths (Huerta et al., 2006). The incidence in men is about 50% greater than in women (Labianca et al., 2004) and also differs significantly between different parts of the world, with Africa given as a low-incidence area (Colorectal Cancer Progress Review Group, 2000). It was found that people migrating from a low to a high risk region experienced increases in the incidence of colorectal cancer within the same
generation (Colorectal Cancer Progress Review Group, 2000). This and other studies have proven the connection between colorectal cancer and lifestyle.

The incidence of colorectal cancer in Africa is characterised by an almost doubled incidence in females and more than doubled incidence for males in southern Africa as compared to the other regions of Africa (International Council for Science Regional Office for Africa, 2006). But the incidence in southern Africa is still lower than the incidence as compared to the rest of the world. Mortality data from the MRC indicate that colon cancer in men and women in South Africa are between 200 to 300% lower than in the USA (Albrecht, 2006). According to the 1998/1999 National Cancer Registry (NCR) of South Africa report, the new incidence of colorectal cancer was 4.1% in males and 3.05% in females over all age groups (Mqoqi et al., 2004). Colorectal cancer is one of the top five cancers in females and males and it occurs most commonly after the age of 50 years. Males have a 60% greater risk of developing colorectal cancer than females. It is most common among the white population in South Africa, with a nine-fold lower incidence in black men and a seven-fold lower incidence in black women (Mqoqi et al., 2004). Cancers of the digestive system ranked the 10th and 12th leading cause of death in males and females, respectively (Mqoqi et al., 2004).

Diet is the most important exogenous factor and it is estimated that 70% of colorectal cancers could be prevented by nutritional intervention (Labianca et al., 2004). There is evidence that a diet rich in vegetables helps to protect against colorectal cancer (Labianca et al., 2004). The most notable recent link between diet and colorectal cancer has been the consumption of red meat, which shows considerable evidence that the increased risk may be due to the presence of carcinogenic heterocyclic amines formed during high-temperature cooking of foods and burning of meat juices (Colorectal Cancer Progress Review Group, 2000). Evidence also suggest that low levels of physical activity increases the risk as well as cigarette smoking and alcohol consumption (Colorectal Cancer Progress Review Group, 2000). In addition, obesity and increased adiposity increased the risk of other gastrointestinal cancers, but with the strongest association with colorectal cancer (Freeman,
2004). Other findings suggest that high levels of c-peptide, caused by hyperinsulinaemia (Popovich et al., 2005), may also increase the risk of colorectal cancer (Colorectal Cancer Progress Review Group, 2000). Colorectal cancer has been linked to factors such as genetic polymorphisms as well as endogenous factors (Colorectal Cancer Progress Review Group, 2000). However, clinical data has shown that non-steroidal anti-inflammatory drugs ( NSAIDs) have anti-tumourogenic properties and reduce the risk of developing colorectal cancer (Chan, 2006).

Currently, the most effective treatment for colorectal cancer is surgery (Chell et al., 2006) with a 5-year survival rate of 70% (Beretta et al., 2004). Treatment regimens for colorectal cancer include primarily the antimetabolite agent from the pyrimidine antagonist group 5-fluorouracil (5-FU), which may be administered orally or intravenously (Beretta et al., 2004). 5-FU in combination with leucovorin is standard treatment where metastasis was present or 5-FU may be combined with irinotecan (Beretta et al., 2004). Alternatively, oxaliplatin may be added to the 5-FU / leucovorin regimen which has been shown to significantly prolong the disease-free survival of people diagnosed with stage II or III colon cancer (Andre et al., 2004). Recent implementation of targeted therapies against colon cancer using bevacizumab or cetuximab has increased the survival time (Huerta et al., 2006).

5.2 Cytotoxic screening methodology

Cell culture techniques play a key role in the development of new anticancer drugs by imposing certain biological constraints on the compounds being investigated. The advantages of cell-based assays include that successful compounds screened may have more than one target anticancer pathway, it ensures that the compounds have cell permeable properties and it allows for the identification of previously unknown drug targets (Aherne et al., 2002). Furthermore, cell culture techniques eliminate the exclusiveness of receptor interactions only, it allow for interactions with other cell receptors and for the
possibility of the compound being metabolised by the cells (Baguley et al., 2002). The microculture in combination with colourimetric assays for the measurement of cell proliferation has provided a basis for the large scale screening of cytotoxic and cytostatic drugs (Baguley et al., 2002) and was used in this study.

5.2.1 Routine maintenance of cell lines

HT29 cancer cells were isolated from human colon adenocarcinoma (Kamuhabwa et al., 2000) and have an epithelial-like morphology (HyperCLDB, 2006b). Human colorectal cancer is one of the most common malignancies (Chapuis et al., 1988) and is included in the 60 cell lines used by the NCI in their initial high through-put screening (HTS) assays.

Chang liver cells were isolated from non-cancer human liver cells, but display HeLa characteristics (HyperCLDB, 2006a). Cells have an epithelial-like morphology and contain human papilloma virus (HPV-18) (American Type Culture Collection, 2006). This cell line is often used as a model for normal liver cells.

Both cell lines were maintained in sterile RPMI-1640 (Biowhittaker, Walkersville, USA) growth medium supplemented with 10% fetal bovine serum (fbs) (Delta Bioproducts, Johannesburg, RSA) at 37°C in an incubator (Thermo Electron Corporation, Labotec) with a 5% CO₂ environment. Cells were routinely grown in 10 cm culture dishes with 10 ml culture medium. Cells were sub-cultured when 70% confluence in the dish was reached. During sub-culture, cells were detached from the dish by washing twice with 5 ml phosphate-buffered saline A (PBSA) (Formulae A.2) and incubated for 10 minutes at 37°C with 0.25% trypsin. Detached cells were resuspended in culture medium and the cell-suspension split at a ratio of one in five into new culture dishes. For the purpose of seeding cells, a viability count of the resuspended cell-suspension was done using trypan blue (Gurr, BDH Chemicals, Poole, England) on a haemocytometer.
5.2.2 Materials used for screening

5.2.2.1 Incubation buffers
The incubation buffer used to dissolve all materials was RPMI-1640 supplemented with 10% fetal bovine serum, with or without DMSO.

5.2.2.2 Colourimetric substrate
MTT was used as detecting agent for the assay (refer to section 4.2.2.2). A stock solution of MTT (Sigma Chemicals Co., USA) was made up at 5 mg/ml with PBS. It was filter sterilised with a 0.22 µm syringe filter and 1 ml aliquots were transferred to sterile 15 ml polypropylene tubes which were stored and protected from light at -20°C until it was needed. On the day of the assay, the aliquots were thawed and diluted 10 times with incubation medium, to reach a final concentration of 0.5 mg/ml. This was added to each well at a volume of 200 µl each.

5.2.2.3 Negative controls
Generally the negative control would comprise of the incubation buffer used for the preparation of the test samples. However, control cells were also treated with corresponding amounts of DMSO, depending on the amount used in either the samples or positive control. In this way, growth inhibition that may have been caused by DMSO was compensated for, by comparing the respective sample with the negative control containing the same amount of DMSO.

5.2.2.4 Positive control
Melphalan, a polyfunctional alkylating agent (Chu & Sartorelli, 2004), was used as the cytotoxic positive control for HT29 cancer cells. Melphalan (Sigma, Steinheim, Germany) was weighed prior to each experiment and dissolved in 50 µl DMSO in a microtube. After dissolution in the DMSO, it was made up to a concentration of 0.1 mM with incubation buffer and sterilised with a 0.22 µm syringe filter. The final concentration of DMSO in the solution
was 0.5% (v/v). The viability of the cells exposed to the melphalan solution was compared to the negative control which also contained 0.5% (v/v) DMSO. No positive control was used for Chang liver cell cytotoxicity determinations.

5.2.2.5 Test samples
Test samples for both the HT29 and Chang liver cells were prepared in the same way. Crude extracts were dissolved in incubation buffer and sterilised through a 0.22 µm syringe filter. Ethanol extracts were first dissolved in DMSO before dilution with incubation medium and filter sterilisation. Extracts were made up to 62.5 and 125 µg/ml before being added to the cancer cells. The final concentrations of DMSO for the ethanol samples were 0.31% for 62.5 µg/ml and 0.63% for the 125 µg/ml. Negative controls were prepared with the same concentration of DMSO for the two dilutions.

5.2.3 Cytotoxicity assay
Cells were seeded into flat-bottom 96-well microtiter plates, at a density of 6000 cells/well and incubated overnight at 37ºC. The incubation medium was replaced with 200 µl fresh incubation buffer containing different concentrations of crude extracts (125 and 62.5 µg/ml), melphalan (0.1 mM) or plain incubation buffer with (for ethanol extracts) or without DMSO (for aqueous extracts). The cells were exposed to the various buffers for 48 hours at 37ºC.

After incubation, the spent incubation buffer was aspirated and 200 µl of incubation buffer containing 0.5 mg/ml MTT was added to all the wells. Cells were incubated with MTT for 3 hours at 37ºC. The medium containing MTT was subsequently aspirated and 200 µl DMSO added to each well to solubilise the formazan crystals. The plate was shaken for about one minute and read the absorbance on a multiplate reader (Multiscan MS® version 4.0 Labsystem® type 352) at 540 nm.

5.2.4 Data manipulation and statistical analysis
Each sample had eight replicate wells in the microtiter plate and individual experiments were repeated a minimum of two times. Percentage growth
inhibition was calculated for each replicate using the 100% viability values provided by the relevant negative control (NC).

\[
\% \text{ growth inhibition} = \left( \frac{\text{Avg: NC readings} - \text{Sample reading}}{\text{Avg: NC readings}} \right) \times 100%
\]

Key: Avg = average; NC = negative control

Results were statistically analysed using GraphPad Prism® 4 (GraphPad Software, 2003). Means and standard error of the mean (SEM) values were calculated and used in statistical tests to determine significance. The unpaired t-test was used to compare the negative control values with sample values. Results were considered statistically significant if \( p < 0.05 \). Additionally, results were considered clinically significant when growth inhibition was \( \geq 50\% \) for HT29 cells and \( \geq 20\% \) for Chang liver cells.

5.3 Results

Crude extracts were tested for cytotoxicity against HT29 colon cancer cells and non-specific toxicity on Chang liver cells. All results in the figures are also presented in tables in Appendix C. Tables contain information on average percentage growth inhibition \( \pm \) SEM, number of replicates (n) and p-values for comparison between sample and negative control, and between different concentrations of the same extract.

5.3.1 HT29 cytotoxicity

Melphalan, which was used as the positive control in the anticancer screening of the HT29 cells, was used at a concentration of 0.1 mM and produced an average growth inhibitory effect of \( 24.11 \pm 1.82\% \) (n = 37; \( p < 0.0001 \)) throughout the screening experiments (Figure 5.1 and Figure 5.2). The water extract of *Tarchonanthus camphoratus* (125 \( \mu \text{g/ml} \); 40.17 \( \pm \) 5.32\%; n = 19; \( p < 0.0001 \)) showed the highest cytotoxic effect of all the aqueous extracts (Figure 5.1 and Table C. 1). Although the cytotoxic effect of *T. camphoratus* was also significantly concentration-dependent (\( p = 0.0121 \)), it remained under 50%
growth inhibition. The ethanol extract of *Tulbaghia violacea* (125 µg/ml; 59.75 ± 4.82%, n = 18, p < 0.0001) was the only ethanol extract that inhibited growth above 50% (Figure 5.2 and Table C. 2). In this case, the response was concentration-dependent (p = 0.0303).

Figure 5.1 Percentage growth inhibition (± SEM) produced on HT29 colon cancer cells by aqueous extracts. Cells were exposed to either melphalan (0.1 mM) or aqueous extracts (62.5 and 125 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37°C. Bars represent two to five individual experiments for each sample, with eight replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).
5.2 Percentage growth inhibition (± SEM) produced on HT29 colon cancer cells by ethanol extracts. Cells were exposed to either melphalan (0.1 mM) or aqueous extracts (62.5 and 125 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37°C. Bars represent three to five individual experiments for each sample, with eight replicates per sample per experiment. A solid gridline indicates 50% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.0005***).

5.3.2 Concentration-response curves for HT29 cytotoxicity

Concentration-response curves were generated for the aqueous extract of *Tarchonanthus camphoratus* and ethanol extract of *Tulbaghia violacea*. Concentration-response curves were calculated using non-linear regression for each experiment (Figure 5.3 and Figure 5.4). The equation used to calculate the curves was the “sigmoidal dose-response (variable slope)” or "four parameter logistic equation" (Motulsky & Christopoulos, 2003). Generally, there was a great deal of variation between the different experiments. The variation between experiments was greatest at lower concentrations of the extracts and seemed to stabilise at the higher concentrations. The variation for concentrations of *T. violacea* was also less than for *T. camphoratus*. It seems the more potent the extract the less variation there was at lower concentrations.
Figure 5.3 Concentration-response curves of percentage growth inhibition for *Tarchonanthus camphoratus* aqueous extract on HT29 colon cancer cells. Cells were exposed to different concentrations (15.6, 20.8, 31.3, 62.5, 125, 250, 500, 1000 and 2000 µg/ml) of aqueous extract of *T. camphoratus* for 48 hours at 37ºC. The graph show the curves of five experiments with four replicates per concentration tested.

Figure 5.4 Concentration-response curves of percentage growth inhibition for *Tulbaghia violacea* ethanol extract on HT29 colon cancer cells. Cells were exposed to different concentrations (15.6, 31.3, 62.5, 125, 250 and 500 µg/ml) of ethanol extract of *T. violacea* for 48 hours at 37ºC. The graph show the curves of five experiments with four replicates per concentration tested.

The concentration-response curves calculated for *T. camphoratus* were very scattered and were not very useful in calculating the EC$_{50}$ value (Figure 5.3). The EC$_{50}$s calculated for the five experiments were 114.4, 144.6, 45.74, 726.1 and 141.4 µg/ml. When comparing these calculated values with the screening results which averaged responses of 22.60 ± 4.00% for 62.5 µg/ml and 40.17 ± 5.32% for 125 µg/ml, it is theoretically not possible to get an EC$_{50}$ value of under 125 µg/ml. Thus experiments 2, 4 and 5 show the most plausible EC$_{50}$s, however, the variation is so great that one can not be sure which value is right. It can thus be concluded that the aqueous extract of *T. camphoratus*
showed such variable results that no EC\textsubscript{50} value could be extrapolated from the data. This might be because the aqueous extract of \textit{T. camphoratus} was not potent enough.

The concentration-response curves calculated for \textit{T. violacea} showed more constant results (Figure 5.4). With the exception of Experiment 1, the calculated EC\textsubscript{50} values were quite constant and substantiated. The calculated EC\textsubscript{50} values were 35.85, 108.7, 99.6, 102.1 and 95.42 µg/ml. The last four of these values also corresponded with the screening results which averaged 46.93 ± 3.28% for 62.5 µg/ml and 59.75 ± 4.82% for 125 µg/ml, which indicated that the EC\textsubscript{50} value should fall between 62.5 and 125 µg/ml. The average EC\textsubscript{50} value for the last four experiments was 101.46 µg/ml (± 5.56). It is quite possible that this EC\textsubscript{50} value may be correct for the ethanol extract of \textit{T. violacea} against HT29 cancer cells.

### 5.3.3 Cytotoxicity on Chang liver cells

No positive control was used to induce cytotoxicity on Chang liver cells. None of the aqueous extracts inhibited growth by more than 20% (Table C. 3). Growth of Chang liver cells was stimulated by more than 20% by aqueous extracts of \textit{Ruta graveolens} (62.5 µg/ml) (Figure 5.5). However, ethanol extracts of \textit{Bulbine frutescens} (62.5 µg/ml; 33.31 ± 5.94% and 125 µg/ml; 30.06 ± 3.72%), \textit{Tarchonanthus camphoratus} (125 µg/ml; 28.05 ± 3.94%, which also showed concentration-dependence with p < 0.0001) and \textit{Tulbaghia violacea} (125 µg/ml; 23.27 ± 4.76%) inhibited growth of Chang liver cells by more than 20% (Table C. 4 and Figure 5.6). The ethanol extract of \textit{Ornithogalum longibracteatum} stimulated growth of Chang liver cells by more than 20% (62.5 µg/ml).
Figure 5.5 Percentage growth inhibition (± SEM) produced on Chang liver cells by aqueous extracts. Cells were exposed to aqueous extracts (62.5 and 125 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37°C. Bars represent two to four individual experiments for each sample, with eight replicates per sample per experiment. A solid gridline indicates 20% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 **).

Figure 5.6 Percentage growth inhibition (± SEM) produced on Chang liver cells by ethanol extracts. Cells were exposed to ethanol extracts (62.5 and 125 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37°C. Bars represent two to four individual experiments for each sample, with eight replicates per sample per experiment. A solid gridline indicates 20% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).
5.4 Discussion

The ethanol extract of *Bulbine frutescens* was found to have potential hepatotoxic effects at high concentrations. The aqueous extract of *Ornithogalum longibracteatum* produced significant, but minor growth inhibitory effects on HT29 colon cancer cells (62.5 µg/ml; 26.83 ± 4.03%) and Chang liver cells (62.5 µg/ml; 18.14 ± 4.14%). The aqueous extract also produced more non-selective toxicity than the ethanol extract, which produced only slight growth stimulatory effects on Chang liver cells. The plant that was extracted for this study was approximately three years old and had flowered previous to extraction (section 3.3.3), which theoretically might have made it less toxic (Watt & Breyer-Brandwijk, 1962). In addition, participating practitioners used the bulb of *O. longibracteatum* and therefore the chances of encountering toxic side-effects were reduced because they did not use the fruits (Watt & Breyer-Brandwijk, 1962) or leaves (Verschaeve et al., 2004).

*Ruta graveolens* produced no toxicity in any of the screens, except growth stimulation by the aqueous extract on Chang liver cells. These findings are also supported by other studies that collectively indicate that methanol and other organic extracts are more toxic than aqueous or ethanol extracts (section 3.4.4) (De Freitas et al., 2005; Gutierrez-Pajares et al., 2003; Ivanova et al., 2005; Raghav et al., 2006).

*Tarchonanthus camphoratus* produced growth inhibition (± 40% for both aqueous and ethanol 125 µg/ml extracts) in HT29 cancer cells. Cytotoxicity of the aqueous extract may have been due to the presence of tannins (Scott et al., 2004). However, no EC$_{50}$ could be calculated for the aqueous extract, which was thought to be the result of poor potency (section 5.3.2). Selective toxicity was shown for *T. camphoratus* with a two-fold reduction in the growth inhibitory activity for the ethanol extract observed between cancer and liver cells and a loss of toxicity by the aqueous extract on Chang liver cells. Literature also reported regular feeding of animals on the leaves of
*T. camphoratus* (Venter & Venter, 2002; Watt & Breyer-Brandwijk, 1962) especially during summer (Parker *et al.*, 2003), which was when the leaves for this study were collected. The anti-inflammatory activity that has been reported for *T. camphoratus* (Amabeku *et al.*, 2000) may be advantageous in the treatment of colon cancer when taking into account the inverse correlation between colon cancer incidence and the use of non-steroidal anti-inflammatory drugs (risk reduction by ± 50%) (Bakhle, 2001; Chell *et al.*, 2006).

The ethanol extract of *Tulbaghia violacea* had significant growth inhibitory activity on both cell lines. Similar activity for *T. violacea* has been shown previously on HT29 cancer cells with methanol extracts (Bungu *et al.*, 2006), however, the present ethanol extracts had more potent activity. The previous study also established that growth inhibition of the methanol extract was produced by a caspase-3 dependent apoptotic pathway in HeLa cells (Bungu *et al.*, 2006). In addition, anti-thrombotic activity established for *T. violacea* (Bungu, 2005) might prove advantageous in cancer treatment (Lee, 2002). Furthermore, the growth inhibitory effect of the ethanol extract seemed to be more selective for the HT29 cancer cells because this effect was 2.5-fold more for HT29 cancer cells than for the Chang liver cells. Selective toxicity of *T. violacea* may be supported by the fact that the green parts and flowers of *T. violacea* had been consumed as vegetables (Marshalkar, 2003; Watt & Breyer-Brandwijk, 1962). In addition, it showed an absence of genotoxicity found with the Ames and VITOTOX® tests (Elgorashi *et al.*, 2003).

### 5.4.1 Feedback seminar discussions

These findings were shared with participating traditional health practitioners at a feedback seminar together with the antimicrobial results. Practitioners were concerned that alcoholic extracts seemed to have better anticancer potential than aqueous extracts, because they used water for the preparation of most, if not all of their remedies. According to other ethnobotanical reviews on medicinal plants (Itharat *et al.*, 2004; Kamuhabwa *et al.*, 2000), traditional healers used water-based decoctions to make most anticancer remedies. It is possible that decoctions may have different activity than cold water extracts.
Participating practitioners also remarked that they did not use any of the plants tested in this study for the treatment of cancer. However, ethnobotanical information reported the use of *Tulbaghia violacea* for oesophageal cancer (Van Wyk & Gericke, 2000). One practitioner specifically remarked that she did not expect positive results for *T. violacea* because she did not use it for cancer. It thus shows the individuality that is still observed in the use of medicinal plants especially among traditional health practitioners. This may also serve as evidence for the concept of ancestral impartation of knowledge, which will be more thoroughly discussed in section 8.2.1.

In contrast to the development of the traditional medical system via ancestral impartation of knowledge, it also seems that practitioners are willing to accept knowledge and medicine developed by science. This was evident when one of the practitioners asked during the feedback seminar, where she could buy the melphalan that was used as the positive control for the anticancer screening. This inquiry also illustrated the developing nature of traditional healing, which is not always acknowledged by scientists and is more often perceived as a stagnant and ancient system (Rao, 2006). Aspects of development in the traditional healthcare system are discussed further in section 8.2.1.

### 5.4.2 Summary of findings

Selected plants did not produce very promising cytotoxic activities. Especially when compared to the criteria of cytotoxic activity for crude extracts, as established by the American National Cancer Institute (NCI). The NCI advocates an IC$_{50}$ value of less than 30 µg/ml in the preliminary assay for the crude extract (Itharat *et al.*, 2004). Not even the ethanol extract of *Tulbaghia violacea*, which showed the highest growth inhibition of all extracts, came close to this value.

However, discovering one of these highly effective plants might complicate the collaboration because of toxicity and commercialisation concerns (Heinrich *et al.*, 2004). Historically, commercialisation has not benefited indigenous knowledge providers anyway (Kidal, 1993). The goal of the
current collaboration was to focus on development of formulations that could be easily used by participating practitioners.

It should also be noted that some plants used to treat cancer may exhibit their anti-tumour activity in vivo, but not in vitro. This phenomenon has been reported to be due to immunomodulation (Kamuhabwa et al., 2000; Steenkamp & Gouws, 2006), anti-inflammatory (Maeda et al., 1996) and anti-thrombotic effects (Lee, 2002). The use of medicinal plants in cancer treatment may thus represent a more holistic approach than using single compounds. In a similar trend, suggestions have been made to use a more inclusive stakeholder team in the treatment of cancer (Albrecht, 2006) and to include more unconventional agents and investigators in drug discovery programs to study the interface between complementary and alternative therapies and conventional care (Colorectal Cancer Progress Review Group, 2000). Traditional health practitioners may become one of these stakeholder groups to help in the research as well as the prevention and management of cancer.
The rise in occurrence of diabetes in developing countries has been linked to the changes in lifestyle associated with modernisation and urbanisation (Chacko, 2003; Hamdan & Afifi, 2004). The Nelson Mandela Metropole, being an urban location in a mostly rural province (Dold & Cocks, 2002), has all the factors for the potential development of diabetes mellitus in its population. The fact that traditional health practitioners requested investigation of diabetes mellitus may indicate some support for this assumption (section 1.5).

Diabetes mellitus is treated frequently with plants in many countries and cultures (Andrade-Cetto et al., 2006; Li et al., 2004; Mossa, 1985; Mukherjee et al., 2006; Singh et al., 2002; Tahraoui et al., 2007), including South Africa (Thring & Weitz, 2006; Van Wyk et al., 1997). Ethnobotanical information collected world-wide has documented between 800 (Alarcon-Aguilara et al., 1998) and 1 200 medicinal plants (Leduc et al., 2006) used for the treatment of diabetes mellitus. Due to the excessive number of plants used in diabetes, strategies have been developed to increase the likelihood of finding active plants by using tools such as disease-consensus in ethnobotanical surveys (Andrade-Cetto et al., 2006; Leduc et al., 2006). Diabetes mellitus is thus a common therapeutic area in natural product research. However, due to financial constraints in South Africa, the numbers of novel compounds and plants tested for diabetes are limited (Folb & Bhagwandin, 2005). Plants have been known to contain numerous hypoglycaemic compounds. The frequently used anti-diabetic drug, metformin was developed from the traditionally used plant, *Galega officinalis* L. (Li et al., 2004) and 4-hydroxyisoleucine from *Trigonella foenum-graecum* L. is currently undergoing clinical trials (Leduc et al., 2006).

Ethnobotanical information on the plants selected for this study includes the following: *Ruta graveolens* was reportedly used for the treatment of diabetes
according to an ethnobotanical survey in the Bredasdorp / Elim region in the Southern Cape, South Africa (Thring & Weitz, 2006). None of the other plants have been reported to be used for diabetes. However, *Allium sativum* L. has been used in Indian traditional medicine as a treatment for diabetes (Mukherjee *et al.*, 2006), which might indicate some potential for *Tulbaghia violacea* in the treatment of diabetes (refer to section 3.6). Interestingly, leaves of *Tarchonanthus camphoratus* have been used as an effective remedy for diabetes by participating traditional health practitioners as well as by indigenous people of the George / Knysna area in the southern Cape as reported by Yvette van Wijk¹³.

6.1 Overview and incidence of diabetes mellitus

Diabetes mellitus may be described as a group of metabolic disorders characterised by hyperglycaemia (Molleutze & Levitt, 2005). There are two primary types of diabetes, namely Type1 (insulin-dependent diabetes mellitus; IDDM) and Type 2 (non-insulin-dependent diabetes mellitus; NIDDM) (Frayn, 1996). Type 1 diabetes mellitus (T1DM) usually presents in slender children or adolescents and is thought to be caused by some kind of autoimmune response against the β-cells of the pancreas, causing β-cell destruction (Molleutze & Levitt, 2005). Type 2 diabetes mellitus (T2DM) generally develops from the mid-thirties onwards (Frayn, 1996). However, it is becoming more common in paediatric patients (Botero & Wolfsdorf, 2005; Gungor & Arslanian, 2004). Contributing factors to the occurrence of type 2 diabetes mellitus include genetics, foetal history, lifestyle factors and stress (Molleutze & Levitt, 2005). T2DM is the most common type of diabetes and accounts for 90% or more of diabetic cases (Giorgino *et al.*, 2005; Ye *et al.*, 2005).

Diabetes mellitus affects about 5 to 10% of the world population (Hamdan & Affi, 2004). The global burden of diabetes which used to be 124 million in 1997, is estimated to double by 2010 (Molleutze & Levitt, 2005). The number
of diabetic cases for southern Africa were estimated to be 0.82 million in 1997 and predictions indicate that the total number of diabetic cases (from 1995 to 2010) will increase with or without the expected impact of HIV/AIDS in South Africa (Molleutze & Levitt, 2005). It is thus important that diabetes remains a focus of the National Department of Health. The issue of diabetes mellitus has been highlighted under the ‘priority health programmes with specific reference to the promotion of healthy lifestyles’ in the health budget vote speech in the National Assembly (Parliamentary Monitoring Group, 2006).

Few epidemiological studies on the incidence of diabetes in South Africa have been done (Molleutze & Levitt, 2005). The largest study was done in 1998 and included interviews with 13 827 adults (≥15 years). The self-reported incidence of diabetes in this study was 2.4% in males and 3.7% in females. The incidence was highest in the Asian population followed by the coloured, white and black population groups. The difference in incidence between ethnic groups has been noted earlier (Botero & Wolfsdorf, 2005).

The total black population all over South Africa showed an incidence of 3% in females and 1.6% in males. The incidence of black females from rural areas was much less (2.2%) than of urban females (3.7%) (Molleutze & Levitt, 2005), which support the urbanisation link for the development of diabetes in developing countries (Chacko, 2003). In addition, diabetes mellitus was the 15th most prevalent cause of premature mortality for males and the 10th most prevalent cause of premature mortality in females. It scored 12th in the overall population (Bradshaw et al., 2003). The two studies correlate well with the higher incidence and mortality of diabetes found in the female population.

The Eastern Cape had a moderate diabetes incidence as compared to the other provinces with incidence rates of 3.5% for females and 2.7% for males (Molleutze & Levitt, 2005). Two interesting epidemiological facts were found which related to black people living in Umtata in the Eastern Cape Province; in a population of 374 factory workers, obesity was not found to be a risk factor for the development of diabetes and secondly, a strong genetic component for diabetes among Xhosa-speaking black people was found in a study among 1 111 diabetic patients (Molleutze & Levitt, 2005). From the
statistics regarding the Eastern Cape Province and the Xhosa people, research into diabetes is both important and necessary for traditional health practitioners of the NMM.

6.1.1 Biological processes important in glucose metabolism

As diabetes mellitus is primarily characterised by hyperglycaemia, it is important to understand the processes involved in glucose metabolism. Glucose homeostasis may be controlled in three ways; secretion of insulin by the β-cells of the pancreas, suppression of hepatic glucose production and stimulation of glucose uptake by the liver and muscles (Petersen & Shulman, 2002). The primary problem in both types of diabetes mellitus is the inadequate action of insulin due to the complete absence thereof (T1DM) or resistance of tissues to insulin’s effects (T2DM) resulting in hyperglycaemia.

Insulin is produced, stored and secreted by the β-cells located in the islets of Langerhans in the pancreas (Luzio & Thompson, 1990). It is a peptide hormone consisting of two peptide chains called the A and B chain, which are connected by disulphide bonds. It is synthesised as a continuous chain, called proinsulin. Proinsulin is stored in secretory granules and is proteolytically cleaved before secretion. The result of which releases free insulin and C-peptide into the bloodstream via exocytosis.

Insulin secretion is stimulated by elevated blood glucose (± 12 mM with a threshold of 5 mM) (Luzio & Thompson, 1990), mannose and leucine levels as well as vagal stimulation occurring after a meal (Frayn, 1996; Karam & Nolte, 2001). Stimulated insulin release results from accumulation of ATP in the β-cells following glucose metabolism as a result of hyperglycaemia. ATP accumulation leads to the closure of ATP-dependent potassium channels (Panunti et al., 2004). This results in the increase of intracellular potassium concentrations, (Mlinar et al., 2007) which opens voltage-gated calcium channels causing an influx of calcium. Subsequent depolarisation of the cell triggers secretion of insulin (Karam & Nolte, 2001) by modulating kinases and other signalling proteins (Mlinar et al., 2007).
Once secreted into the blood, insulin will bind to cell surface receptors called insulin receptors. The insulin receptor is a protein consisting of four subunits. Two extracellular α-subunits acting as the binding site for insulin and two β-subunits spanning the width of the cellular membrane (Karam & Nolte, 2001). The binding of insulin to the α-subunit causes phosphorylation of tyrosine residues on the β-subunit which activate second messenger tyrosine kinase or phosphatase signalling pathways (Granner, 2003).

There are three main biochemical pathways that may be activated when insulin binds to the insulin receptor, namely the phosphatidylinositol 3-kinase (PI3-kinase) pathway, the CAP/Cbl/TC10 pathway and the mitogen-activated protein kinase (MAP-kinase) dependent pathway (Mlinar et al., 2007). PI3-kinase interacts with phosphorylated tyrosine residues on insulin receptor substrate (IRS) molecules and is responsible for intermediate metabolic processes. Metabolic processes activated by PI-3 kinase occur primarily in skeletal and smooth muscle. Translocation of glucose transporter 4 (GLUT4) to the plasma membrane (Mlinar et al., 2007) in skeletal muscle cells increase glucose uptake and activation of the enzyme endothelial-nitric oxide synthase (eNOS) catalyses the synthesis of nitric oxide (NO) promoting the relaxation of smooth muscle (Lebovitz & Banerji, 2004). The second pathway activated by insulin-binding includes the adaptor protein CAP, which recruits proto-oncogene Cbl to the phosphorylated insulin receptor resulting in reinforced GLUT4 translocation (Mlinar et al., 2007). The third pathway requires IRS activation of a host of proteins which lead to MAP-kinase activation, cellular proliferation and differentiation via gene transcription regulation (Mlinar et al., 2007). There is also evidence, although still controversial, that MAP-kinase may play a role in the regulation of basal and insulin-dependent glucose transport activity in insulin-sensitive tissues (Kim et al., 2006).

After insulin has bound to the receptor the insulin-receptor complexes will gather in patches on the cell surface and be endocytosed. Inside the cells, receptor complexes can either be broken down by lysosomes or the receptors may be recycled back to the cell membrane. Insulin receptor number on the
cell surface and affinity for insulin may vary according to physiological conditions.

Insulin receptors are located on target tissues which mainly include liver, muscle and adipose tissue. Insulin facilitates the movement of glucose into muscle and fat cells by recruiting glucose transporters (GLUT4) from the intracellular reservoir (Mayes & Bender, 2003; Murray & Granner, 2003). In muscle cells insulin will cause an increase in glucose uptake, glycogen synthesis, amino acid uptake and protein synthesis (Binkley, 1995). Insulin also acts on adipose tissue by reducing the levels of free fatty acids in the blood and promoting triglyceride storage in adipocytes (Binkley, 1995). Insulin, which reaches the liver via the portal vein, stimulates the liver to take up more glucose. However, it is not directly involved in facilitation of glucose movement as in muscle and fat cells. Insulin acts on the liver by increasing glycogen synthesis, decreasing gluconeogenesis and decreasing ketogenesis (Binkley, 1995).

Insulin action ceases when glucose concentrations stabilise postprandially. This is termed the postabsorptive state and the body needs to keep blood glucose levels stable by mobilising stored intracellular glucose into the blood by the liver. The hormone responsible for this is glucagon, which is synthesised by the \(\alpha\)-cells of the islets of Langerhans as a single peptide chain consisting of 29 amino acids. The secretion of glucagon is stimulated by low blood glucose and high amino acid levels. Glucagon mainly acts on the liver by binding to glucagon receptors that stimulate adenylate cyclase via G-protein coupled receptors (Frayn, 1996). The action of glucagon on the liver triggers glycogenolysis (conversion of glycogen into glucose) and gluconeogenesis (production of glucose from non-glucose derived precursors such as glycerol, alanine, lactate and pyruvate). In this way the concentration of glucose in the blood is kept constant at times of fasting during sleep or starvation.
6.1.2 Pathophysiology of type 1 diabetes mellitus

IDDM develops because of a lack of insulin production. The lack of insulin results because of autoimmune destruction of β-cells in the islets of Langerhans, to a lesser extent because of genetic programming and more often as a result of a viral infection which confuses the immune system. However, in most cases the aetiology is unknown. The absence of insulin impairs postprandial insulin mediated glucose utilisation in target tissues, leading to hyperglycaemia (10 to 20 mmol/L). Glucagon action unopposed, leads to continuous glycogenolysis and gluconeogenesis (Frayn, 1996), which result in weakness and wasting due to the breakdown of glycogen supplies in muscle and fat tissue, respectively. Blood glucose concentrations of above 12 mmol/L overload glucose re-absorptive mechanisms in the kidneys, which causes glycosuria. Abnormal amounts of water are excreted along with the glucose, which leads to polyuria, dehydration and excessive thirst.

Lack of insulin also leads to continuous release of non-esterified fatty acids from adipose tissue. Adipocytes also fail to take up triacylglycerol from the blood because of a reduced activation of tissue lipoprotein lipase and thus cause its accumulation in the blood. The rise in fatty acid levels are accompanied by a rise in ketone bodies (acetone, acetoacetate and beta-hydroxybutyrate), which eventually lead to metabolic acidosis. The combination of hyperglycaemia, metabolic acidosis and high levels of circulating ketone bodies ultimately results in diabetic ketoacidosis. In addition to ketone body production, the liver may also produce very low density lipoprotein triacylglycerol from the fatty acids, which have undesirable effects on the patient’s cholesterol profile (Frayn, 1996).

6.1.3 Pathophysiology of type 2 diabetes mellitus

The progression from normal glucose metabolism to impaired glucose tolerance is associated with two core elements in the pathogenesis of T2DM which include impaired insulin action (insulin resistance) and dysfunctional insulin secretion (insulin deficiency) (Giorgino et al., 2005). The sequence of events leading to the development of T2DM is not yet fully understood and may differ between individuals (Giorgino et al., 2005). The initial dysfunctions
leading to the development of Type 2 diabetes mellitus has been associated
with the metabolic or x-syndrome which is a combination of metabolic
disturbances. The features of which include abdominal obesity, insulin
resistance, atherogenic dyslipidaemia, hypertension, pro-inflammatory and
prothrombotic states (Mlinar et al., 2007).

In many cases, events leading to the origin of T2DM are a tendency to insulin
resistance of peripheral tissues (Petersen & Shulman, 2002). The main
effects being uninhibited lipolysis in adipose tissue, decreased glucose uptake
in muscle tissue and uninhibited gluconeogenesis particularly in hepatic
tissue. It is possible that multiple inherited defects contribute to insulin
resistance which may be exacerbated by environmental factors (Ishida et al.,
1996). Unfortunately, the genetic component is not well understood, but it is
estimated that five to seven genes may contribute to this multigenetic disease
(Klover & Mooney, 2004). Partial mutations in IRS show more sequence
variation (Gillham et al., 1997) in people with type 2 diabetes and mutations in
the insulin receptor (Mlinar et al., 2007) have been observed. Whatever the
genetic component, evidence suggests that environmental factors such as
obesity, inactivity, diet (Panunti et al., 2004), aging, smoking and the use of
certain drugs (Mlinar et al., 2007) certainly aggravate the development of
diabetes. In addition, environmental changes such as diet and lifestyle
changes have been shown to relieve insulin resistance and prolong the period
towards β-cell failure.

The earliest defect that could so far be identified in insulin resistance is a
defect in the autophosphorylation of the insulin receptor and a decreased
amount of IRS-1 (Mlinar et al., 2007). Another significant contributing factor is
the observed abnormalities in adipose tissue metabolism (especially visceral
fat), which leads to the pro-inflammatory state. It has been shown that
visceral fat is morphologically different from subcutaneous fat. Visceral fat
tends to secrete more adiponectin, tumour necrosis factor-α (TNF-α) and
plasminogen activator inhibitor-1 (PAI-1) (Giorgino et al., 2005). Visceral fat is
also more sensitive to lipolytic hormones and less sensitive to the effects of
insulin as compared to subcutaneous fat (Lebovitz & Banerji, 2004). TNF-α
has been shown to activate the inflammatory cascade via activation of Nuclear Factor Kappa B (NFkB) (Klover & Mooney, 2004). Other inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 (IL-1) are also secreted in addition to TNF-α. It has been suggested that the inflammatory state may cause serine phosphorylation of IRS-1 and IRS-2 (Klover & Mooney, 2004), which would impede insulin signalling dependent on tyrosine phosphorylation.

In addition to the post-receptor signalling defects of the insulin receptor, the excessive release of free fatty acids from adipose tissue causes more problems (Mlinar et al., 2007). Insulin resistance in adipose tissue compromises the inhibition of lipolysis due to insufficient action of insulin and this causes the level of free fatty acids to rise in the blood (Panunti et al., 2004). There is also a decrease in GLUT4 mRNA production, translocation and activation in adipose tissue (Panunti et al., 2004).

In muscle cells, these altered processes cause serine / threonine phosphorylation of IRS-1 (Gillham et al., 1997) and PI3-kinase thereby inactivating them causing a decrease in GLUT4 translocation to the plasma membrane (Mlinar et al., 2007) and impaired glucose uptake (Figure 6.1). However, the MAP/Erk cascade remains unaffected by insulin resistance and there is an unopposed increase in secretion of PAI-1 and endothelin-1 (ET-1) (Giorgino et al., 2005), which increase the prothrombotic state and vascular resistance (Laight et al., 2000), respectively.

Hepatic insulin resistance results in uncontrolled glucose production because of unsuppressed glycogenolysis and gluconeogenesis. These processes are activated by a combination of a lack of insulin action, an increase in glucagon response, increased gluconeogenic precursors like lactate, alanine, glycerol and an increase in free fatty acid oxidation (Panunti et al., 2004). The free fatty acids provide an abundant substrate for the synthesis of triglycerides and very low density lipoprotein (VLDL) cholesterol (Mlinar et al., 2007), which aggravate the dyslipidaemic state.
The chronically elevated levels of insulin which compensates for insulin resistance also leads to a decrease in expression of insulin receptors on target cells, especially muscle tissue, which is the primary site of insulin resistance in T2DM (Panunti et al., 2004). The progressive worsening of the insulin resistance in peripheral tissues demands higher and higher amounts of insulin that needs to be produced by the β-cells of the pancreas. Because of the strain of producing such large amounts of insulin, progressive β-cell failure develops (Petersen & Shulman, 2002). In addition, people with T2DM have a genetic predisposition to abnormal β-cell function (Lebovitz & Banerji, 2004) and / or a genetically determined defect in β-cell GLUT 2, resulting in β-cells responding less well to the presence of glucose than normal (Gillham et al., 1997).

The high burden of the already genetically compromised β-cells causes an increased rate of apoptosis and loss of function of β-cells (Lebovitz & Banerji, 2004). In addition, prolonged exposure of β-cells to hyperglycaemia reduces
insulin gene transcription leading to a decrease in insulin synthesis (Lebovitz & Banerji, 2004; Panunti et al., 2004). Other contributing factors to β-cell failure include lipotoxicity (which also inhibits insulin synthesis and secretion, decreases the expression of glucose transporter-2 (GLUT2) and decreases the mitogenesis of β-cells via activation of various protein kinase C (PKC) isoforms (Mlinar et al., 2007)), and inflammation in the islets causing amyloid deposition (Lebovitz & Banerji, 2004). Another defect that has been observed with insulin production in T2DM is that there is a decreased conversion of proinsulin to insulin. A question mark remains if this may be due to an inflammatory response, because of the raised levels of plasma C reactive protein, an indicator of inflammation as well as a predictor of T2DM (Lebovitz & Banerji, 2004).

However, it should not be overlooked that genetic factors are aggravated by environmental factors. It has been shown that weight loss achieved by environmental changes increased insulin sensitivity and decreased triglyceride levels, the procoagulative and inflammatory states that are characteristic of the insulin resistant profile (Lebovitz & Banerji, 2004). A possible explanation is that with exercise induced weight loss, people loose visceral fat preferentially to subcutaneous fat (Lebovitz & Banerji, 2004). In addition, muscle contractions induced by exercise have powerful effects on the glucose transport process. Muscle contractions firstly activate GLUT4 translocation via insulin-dependent mechanisms (Henriksen, 2006) and increase the amount of GLUT4 mRNA in skeletal muscle (MacLean et al., 2002). Secondly, muscle contractions activate 5' adenosine-monophosphate activated protein kinase (AMP-kinase), which increases ATP generation through increased fatty acid oxidation, glucose transport into skeletal and cardiac muscle and glycolysis in cardiac muscle and white blood cells (Kelly et al., 2004; Ye et al., 2005). Thirdly, muscle contractions increase intracellular calcium levels which play an important role in the acute regulation of glucose transport (Henriksen, 2006). These findings might explain the successful management of mild T2DM with a strict diet and exercise program without drug therapy (Frayn, 1996).
6.1.4 Management of diabetes mellitus

Type 1 diabetes only responds to subcutaneous insulin injections as the major problem is a lack of insulin. However, in the case of type 1 insulin resistance, a biguanide may be added to the treatment regime and an alpha-glucosidase inhibitor may be added if the patient eats a diet high in starch (Karam & Nolte, 2001).

The treatment of type 2 diabetes depends on the severity of the hyperglycaemia as well as the lifestyle of the patient. Mild diabetes may be controlled by diet and exercise alone. If these changes are inadequate oral drug therapy is considered (Frayn, 1996). Proposed goals in treatment intervention include the improvement of β-cell function, decreasing hepatic glucose production and increasing glucose uptake peripherally (Kahn, 2000). However, new treatment strategies suggest prescribing according to the individual’s genetic pathogenic profile (Giorgino et al., 2005; Panunti et al., 2004). The classes of hypoglycaemics currently available include insulin secretagogues such as sulfonylureas and meglitinides, insulin sensitisers such as biguanides and thiazolidinediones, and glucose absorption modulators such as alpha-glucosidase inhibitors (Karam & Nolte, 2001).

The primary mechanism of action of sulfonylureas is to increase insulin release from the pancreas by binding to sulfonylurea receptors on the β-cell. Sulfonylureas also potentiate the exocytosis of insulin-containing granules by a direct action on the binding proteins, reduce glucagon serum concentrations and potentiate insulin action on target tissues (Karam & Nolte, 2001). However, sulfonylureas possess many undesirable side effects such as prolonged hypoglycaemia and have been shown to trigger apoptosis in β-cells (Giorgino et al., 2005), which may not be desirable when treating patients with identified β-cell pathogenesis. The meglitinides act by regulating potassium efflux through the potassium channels, but have no direct effect on insulin exocytosis. Meglitinides have a shorter half-life and therefore do not cause prolonged hypoglycaemia and have a more favourable effect on β-cell survival as compared to sulfonylureas (Giorgino et al., 2005).
Metformin, a biguanide, promotes insulin-stimulated glucose uptake in muscle, decreases hepatic glucose output and changes lipid metabolism by decreasing plasma triglycerides as well as free fatty acids (Hawley et al., 2002). However, the molecular mechanism of metformin remains partially explained. Metformin acts by phosphorylation and activation of AMP-kinase in hepatocytes (Fryer et al., 2002b) and skeletal muscle (Giorgino et al., 2005; Musi et al., 2002) which explains the decreased hepatic glucose production and increased glucose uptake in skeletal muscle (Fryer et al., 2002b). However, the mechanism of activation of AMP-kinase has not exactly been established (Fryer et al., 2002b; Hawley et al., 2002). Metformin’s effects on gene expression was investigated and it decreased glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) and increased glucokinase and L-pyruvate kinase gene expression (Fulgencio et al., 2001). The changes in gene expression explain the decrease in hepatic glucose output through decreasing gluconeogenesis. No effect on gene transcription for free fatty acid and VLDL synthesis was found (Fulgencio et al., 2001). Other mechanisms for increased glucose removal from the blood include enhancing insulin stimulatory activity in muscle cells through increased insulin receptor tyrosine kinase activity, increased GLUT4 transporter number and activity and enhanced glycogen synthesis (Panunti et al., 2004). However, data on the mechanism in skeletal muscle has been challenged and sources are divided as some believe that metformin has no direct effects on insulin-mediated muscle glucose uptake and any effects that have been observed are only as a result of an improvement in glucose toxicity and weight loss caused by metformin (Lebovitz & Banerji, 2004).

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a nuclear transcription factor, which is activated by the binding of thiazolidinediones. Once activated, it binds to certain regions of the DNA and activates the transcription of certain genes. The highest concentration of PPARγ receptors are found in adipocytes and genes that cause adipose cell differentiation are activated (Lebovitz & Banerji, 2004), especially subcutaneous adipocyte differentiation. There is a subsequent redistribution of fat from the visceral to the subcutaneous compartment (Giorgino et al., 2005). Furthermore, genes
that promote lipid uptake and storage are activated (Giorgino et al., 2005), which explain decreases of 25 to 35% in plasma fatty acids in treatment with thiazolidinediones (Lebovitz & Banerji, 2004). In addition, genes that modulate adipokine synthesis and secretion are activated, which result in lowering of circulating TNF-α and other cytokines and raising of plasma adiponectin levels (Lebovitz & Banerji, 2004). Apart from lipid metabolism, PPARγ also activates genes that regulate insulin action, (Lebovitz & Banerji, 2004), glucose uptake and energy expenditure. Insulin sensitivity in muscle cells and hepatocytes may also be enhanced by increased lipid uptake by adipocytes as well as decreased levels of circulating TNF-α (Lebovitz & Banerji, 2004; Petersen & Shulman, 2002; Wagstaff & Goa, 2002). It has also been found that rosiglitazone activates AMP-kinase by increasing levels of AMP within skeletal muscle cells, which is another way in which glucose uptake can be stimulated (Fryer et al., 2002b).

Alpha-glucosidase inhibitors such as acarbose and miglitol are competitive inhibitors of intestinal alpha-glucosidases. It thus slows the digestion of starch and disaccharides, which flattens the peak in glucose levels after meals (Karam & Nolte, 2001).

6.1.5 Diabetic complications

Diabetic complications include micro- and macrovascular atherosclerosis, nephropathy, neuropathy and eye complications including retinopathy (the most common cause of blindness) and the development of cataracts (Frayn, 1996). The relationship between glycaemic control and diabetic microvascular disease has been established in both type 1 and type 2 diabetes (Ratner, 2001). Although the underlying mechanisms for the development of diabetic complications may not be well understood, oxidative stress, induced by chronic hyperglycaemia (Kil et al., 2004) has been shown to be a major contributing pathway (Neri et al., 2005).

Increases in oxidative stress lead to increased production of reactive oxygen species (ROS) and potential enzymatic sources of ROS which lead to vasoconstriction (Kuyvenhoven & Meinders, 1999) due to the reduction of
nitric oxide (NO) bioavailability (Pratico, 2005). The resulting endothelial
dysfunction is a major contributing factor in the atherosclerosis of large and
medium-sized arteries (Laight et al., 2000). This may explain why additional
control of hypertension, which is the most common co-morbid state
associated with diabetes, appears to independently slow the progression of
both micro- and macrovascular complications of diabetes. Additionally,
proper glycaemic control, blood-pressure management and lipid modification
(lowering low density lipoproteins (LDL) and triglyceride levels and increasing
high density lipoproteins (HDL) levels) reduce the rates of diabetic
retinopathy, neuropathy, nephropathy and cardiovascular events (Ratner,
2001).

6.2 Anti-diabetic screening in liver cells

The liver plays an important role in the regulation of blood glucose control.
Hepatic glucose production is the most important determinant of fasting blood
glucose levels and uninhibited glucose production is a major problem in
diabetes (Panunti et al., 2004). Liver cells are freely permeable to glucose via
GLUT2 transporters in the cell membrane (Mayes & Bender, 2003). These
transporters make it possible for liver cells to take up as well as release
glucose (Mayes & Bender, 2003). If glucose uptake into liver cells could be
increased, it may indicate some potential use in treatment of diabetes.

6.2.1 Routine maintenance of Chang liver cells

Cell cultures were maintained in 10 cm culture dishes and incubated at 37°C
in a 5% carbon dioxide environment. Growth medium consisted of a mixture
of RPMI-1640 (BioWhittaker, Walkerville, USA) supplemented with 10% fetal
bovine serum (fbs) (Delta Bioproducts, Johannesburg, South Africa). Growth
medium was changed approximately every 48 to 72 hours. When about 70%
confluence was reached, cells were detached by washing with PBSA
(Formulae A.2) and incubation with 0.25% (v/v) trypsin in PBSA (Roche
Diagnostics, Manheim, Germany). Cells were routinely divided at a split ratio of one in six.

6.2.2 Materials used for screening

6.2.2.1 Incubation buffers
Two different buffers were used for the experiment. Incubation buffer A consisted of RPMI-1640 supplemented with 10% (v/v) fbs. Incubation buffer B was made up on the day of glucose uptake measurement and consisted of RPMI-1640 (glucose 11 mM) adjusted to 8 mM glucose by dilution with sterile phosphate-buffered saline (PBS; Appendix A.1) and 0.1% (m/v) bovine serum albumin (BSA; Roche Diagnostics, Germany).

6.2.2.2 Detection substrates
The amount of glucose left in incubation buffer B after exposure for three hours was determined with a glucose oxidase reagent kit (Sera-pak Plus, Hong Kong). Toxicity of chronic exposure of the extracts was determined with the MTT (Sigma, Germany) colourimetric assay (refer to section 4.2.2.2).

6.2.2.3 Negative control
The negative control reflected 100% glucose taken up by untreated cells. Only one negative control was prepared, because the concentration of DMSO used in the preparation of the ethanol extracts were so small, that the influence was negligible.

6.2.2.4 Positive control
Metformin hydrochloride was employed as the positive control because of its action on decreasing glucose output in hepatocytes (section 6.1.4). A 1 mM stock solution of metformin (Helm AG, Hamburg, Germany) was made up in RPMI-1640 supplemented with 10% (v/v) fbs. The solution was filtered through a 0.22 µm syringe filter. Aliquots of 1 ml were stored at -20°C. After thawing, sterile stock solutions could be kept up to 4 weeks at 4°C. The stock...
solution was diluted with either incubation buffer A or B to a concentration of 1 µM.

6.2.2.5 Test samples

Water extracts were weighed and dissolved directly in the relevant incubation buffer (1 mg/ml). Alcoholic extracts were weighed and dissolved in a maximum of 1.25% DMSO depending on the weight and dilution of the sample (1 mg/ml). Extracts were sterilised by filtration through 0.22 µm syringe filters. Screening involved chronic (48 hours) and acute (3 hours) exposure to the test samples. The chronic concentration was a quarter of the acute concentration. The screening concentrations were thus 0.125 or 12.5 µg/ml for chronic and 0.5 or 50 µg/ml for acute exposure, respectively. All concentration-response curves were calculated according to this ratio used during the screening.

6.2.3 Glucose uptake assay methodology

Chang liver cells were seeded into flat-bottom 96-well culture plates (NUNC, Roskilde, Denmark) at a density of 6 000 cells/well in a volume of 200 µl/well incubation buffer. After about 72 hours, 10 µl of fresh incubation buffer A, containing either no additive, metformin or test sample, was added to the 200 µl medium already in the well. The cells were chronically exposed to the test samples (0.125 or 12.5 µg/ml) or metformin (1 µM) for 48 hours before the glucose uptake assay was done.

On the day of the glucose uptake assay, incubation buffer B was prepared (section 6.2.2.1) containing either no additive, metformin (1 µM) or test sample (0.5 or 50 µg/ml). The spent medium was aspirated and 50 µl of each solution prepared from incubation buffer B, were added to each well. The cells were incubated at 37ºC for a further 3 hours for glucose uptake to occur.

After three hours of incubation at 37ºC with incubation buffer B, 10 µl aliquots were taken from each well and transferred to a second microtiter plate. Two hundred microliters of the glucose oxidase reagent was added to each 10 µl aliquot and developed for 15 minutes at 37ºC. The plate was read after
development at 492 nm in a multiplate reader (Multiscan MS® version 4.0 Labsystem® type 352).

Glucose standards were run with each experiment. Glucose standards were made up with the incubation buffer B at concentrations of 2, 4, 6, 7 and 8 mM diluted with PBS. PBS was also used as the blank. The exact concentration of glucose in incubation buffer B given to the cells at zero time, was also determined with the same method.

Concurrently with the glucose assay, a viability assay utilising MTT was also done in separate wells to detect any possible liver cell toxicity of the test samples during the 48 hour exposure period. One-hundred microliters of MTT made up in normal growth medium was added to each well and incubated at 37ºC for 3 hours. At the end of the incubation time, the solution was aspirated and 100 ul DMSO added to each well to dissolve the formazan crystals in the bottom. The plate was shaken for 60 seconds to dissolve the formazan salts and read at 540 nm with a multiplate reader (Multiscan MS® version 4.0 Labsystem® type 352).

6.2.4 Data manipulation and statistical analysis

Spectrophotometric readings of each sample were adjusted to represent the net amount of glucose taken up by the cells in each well by calculating the difference between the average of the readings at zero time and the absorbance in the test well after glucose uptake had occurred. The calculated differences were converted to express the readings as percentage glucose uptake for the sample as compared to the negative control which represented 100% glucose uptake. A toxicity correction was also made to accommodate for growth inhibition that might have occurred during the chronic exposure period (48 hours), to show the percentage glucose uptake as a measure of the viable cells in the well. The equation used was as follows:

\[
\% \text{ glucose uptake} = \left\{ \frac{(\text{Avg: } T_0) - \text{sample measurement}}{\text{Avg: (Avg: } T_0) - \text{NC measurements}} \right\} \times 100\% \times \left( \frac{\text{Sample MTT measurement}}{\text{Avg: NC MTT measurement}} \right)
\]

Key: Avg = average; NC = negative control; T0 = zero time.
Within each experiment, glucose uptake was measured in 10 wells per treatment and six wells per treatment were used for toxicity determinations. Individual experiments were repeated a minimum of three times, but not all experiments are presented in the results, because of variation between experiments. Theoretically, the effect of the positive control should be consistent between experiments. However, cell experiments have many variables that may significantly change the consistency of findings between experiments such as the transfer number, degree of differentiation and growth rate of the cells. These variables cause the response of the positive control to vary between experiments.

For the purpose of statistical analysis the data of the different experiments with comparable positive control values were pooled and analysed. Statistical analysis was done using GraphPad Prism® 4 (GraphPad Software, 2003). Outliers were calculated using the Z-test and accordingly excluded from significance calculations. The results were subjected to the two-tailed unpaired t-test and significance was taken at p < 0.05. Variation between individual experiments (as explained above) prevented the use of clinically significance parameters in the anti-diabetic screenings. For this reason, the response of the sample will rather be compared to the response achieved by the positive control to indicate potential effectiveness.

6.3 Anti-diabetic screening in muscle cells

Muscle cells are the major site of glucose utilisation and also for insulin resistance in type 2 diabetes mellitus (Panunti et al., 2004). Glucose transporter 4 (GLUT4) is responsible for glucose transport into muscle cells. The rate of glucose absorption is related to translocation of GLUT4 to the cell membrane and activation of the translocated GLUT4 to take up glucose at a maximal rate.
Cell cultures were routinely maintained as described in section 6.2.1. However, C2C12 cells were more sensitive to confluence conditions and had to be monitored closely to ensure the health of the cells.

6.3.1 Materials used for screening
The preparation of incubation buffer B (section 6.2.2.1), detection substrates (section 6.2.2.2), negative control (section 6.2.2.3) and test samples (section 6.2.2.5) were the same as for Chang liver cells. However, no MTT assay was done because exposure of the samples to the cells was acute only.

6.3.1.1 Positive control
Insulin was used as the positive control, because of the stimulatory effect it has on muscle cells for glucose absorption (Murray & Granner, 2003). A stock solution of crystalline insulin (Human, recombinant; Roche, Penzberg, Germany) was made with 4 mM hydrochloric acid at a concentration of 1 mg/ml. The solution was filtered through a syringe filter with 0.22 µm pores. Aliquots were transferred into sterile 1 ml microtubes and stored at -20ºC. Once thawed the undiluted stock solution could be stored at 4ºC for up to 4 weeks. Insulin was diluted with incubation buffer B and was added to the cells at a final concentration of 1 µM.

6.3.2 Glucose uptake assay
C2C12 muscle cells were seeded into flat-bottom 96-well culture plates (NUNC, Roskilde, Denmark) at a density of 5 000 cells/well in a volume of 200 µl/well incubation buffer. The plates were then incubated for three and a half days at 37ºC without changing the medium. On the day of the assay all reagents were made up with incubation buffer B. The spent growth medium was aspirated and 50 µl of either pure incubation buffer B, or incubation buffer B containing the insulin (1 µM) or test samples (0.5 and 50 µg/ml) were added to each well. The plates were incubated for one hour at 37ºC.

Afterwards, aliquots of 10 µl were removed from each well and placed in another 96-well microtiter plate. Two hundred microliters of glucose oxidase
reagent was added to each well and incubated again for 15 minutes at 37°C. After reagent development, the plate was read on a microtiter plate reader at 492 nm (Multiscan MS® version 4.0 Labsystem® type 352). Glucose standards and zero time readings were obtained as in section 6.2.3.

6.3.3 Data manipulation and statistical analysis

Spectrophotometric readings were manipulated as explained in section 6.2.4. However, in the case of C2C12 muscle cells no viability determination was done, because the cells were not exposed to the treatments prior to the glucose uptake assay. The following equation was used:

\[
\% \text{ glucose uptake} = \frac{[\text{Avg: } (\text{Avg: } T_0) - \text{ sample measurement}] \times 100\%}{\text{Avg: } (\text{Avg: } T_0) - \text{ NC measurements}}
\]

Key: Avg = average; NC = negative control; T₀ = zero time.

Data was recalculated in some cases to allow for the variation in insulin response between experiments, as the variation between experiments were more erratic for C2C12 cells than for Chang liver cells. The reasons being increased growth rate of C2C12 muscle cells as compared to Chang liver cells and the shorter period of the experiment. Slight variations in seeding densities, time of growth and differentiation could influence glucose uptake significantly. The data was expressed in these cases as a percentage of the insulin response (taken as 0%):

\[
\% \text{ GU as function of insulin response} = \frac{[\% \text{ GU of sample} – \text{ Avg: } (\% \text{ GU of insulin})] \times 100\%}{\text{Avg: } (\% \text{ GU of insulin})}
\]

Key: Avg = average; GU = glucose uptake; T₀ = zero time.

Within each experiment, measurements were done in 12 to 16 replicate wells for each sample and individual experiments were repeated at least three times, but not all experiments were shown in the results section due to variation of the response of the positive control. Data from experiments with relatively equal positive control responses were pooled for the statistical analysis.
Statistical analysis of results was performed utilising GraphPad Prism® 4. Outliers were calculated by using the Z-test (GraphPad Software, 2003). Identified outliers were excluded from statistical significance calculations. The two-tailed unpaired t-test was used for calculation of statistical significance with statistical parameters set for a two-tailed analysis at a 95% confidence interval. P-values of less than 0.05 were considered to be statistically significant and responses higher than the positive control were considered potentially effective.

6.4 Results

Data are presented in tables in Appendix D. Tables contain information on average glucose uptake response ± SEM, number of replicates (n) and p-values for comparison between sample and negative control, and between different concentrations of the same extract.

6.4.1 Glucose uptake in Chang liver cells (Chronic exposure)

The average percentage glucose uptake for metformin in the 10 experiments to test aqueous extracts was 115.4 ± 2.23% (n = 99; p < 0.001). Table D. 1 and Figure 6.2 provide a summary of the average percentage glucose uptake achieved by aqueous extracts tested at 0.5 and 50 µg/ml. Aqueous extracts that increased glucose uptake above that of metformin in Chang liver cells were Bulbine frutescens (0.5 µg/ml), Ornithogalum longibracteatum (0.5 µg/ml), Tarchonanthus camphoratus (50 µg/ml) and Tulbaghia violacea (50 µg/ml). Significant concentration-independent responses were observed for the aqueous extracts of B. frutescens (p < 0.0001) and O. longibracteatum (p = 0.0002) and significant concentration-dependent responses were recorded for the aqueous extracts of T. camphoratus (p = 0.0465) and T. violacea (p = 0.0015).
Figure 6.2 Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with aqueous extracts of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) at 37°C. Bars represent two or three individual experiments with 10 replicates per sample per experiment. The solid gridline indicates average percentage glucose uptake (115.5%) of metformin (1 µM). Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).

Growth inhibitory activities on Chang liver cells exerted by the aqueous extracts during chronic exposure are summarised in Table D. 2 and Figure 6.3. The average percentage growth inhibition produced by metformin over the 10 experiments was -0.06 ± 1.18% (n = 59; p = 0.9704). The only significant growth inhibitory activity for the aqueous extracts were found to be the 0.125 µg/ml concentration of *B. frutescens* and *O. longibracteatum* and the 12.5 µg/ml concentration of *T. violacea*. However, the average amount of growth inhibition produced by the three extracts was not higher than 10%. The 0.125 µg/ml aqueous extract of *T. violacea* was the only extract that produced statistically significant growth stimulatory activity (11.11 ± 3.07%).
Figure 6.3 Percentage growth inhibition (± SEM) produced on Chang liver cells. Cells were exposed to aqueous extracts (0.125 or 12.5 μg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37ºC. Bars represent two or three experiments for each sample with six replicates per sample per experiment. A dotted gridline indicates 10% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).

The average percentage glucose uptake produced by metformin in eight screening experiments performed for ethanol extracts was 110.1 ± 1.83% (n = 69; p < 0.001). The screening results for ethanol extracts are summarised in Table D. 3 and Figure 6.4. The ethanol extract of *T. camphoratus* (0.5 μg/ml) produced the greatest glucose uptake response. Other responses for ethanol extracts that were equal or greater than that of metformin included those of *B. frutescens* (0.5 μg/ml), *O. longibracteatum* (50 μg/ml) and *Ruta graveolens* (50 μg/ml). The average growth inhibitory response for metformin over the 10 experiments was 1.07 ± 1.10% (n = 42; p = 0.4789). A summary of the growth inhibitory responses for ethanol extracts can be viewed in Figure 6.5 and Table D. 4. The only two extracts that inhibited growth at a statistically significant level were the 12.5 μg/ml ethanol extracts of *R. graveolens* and *T. camphoratus*. However, the two extracts inhibited growth to only about 7% compared to the negative control.
Figure 6.4 Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with ethanol extracts of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) at 37°C. Bars represent one to three experiments for each sample with 10 replicates per sample for each experiment. The solid gridline indicates percentage glucose uptake (110.1%) of metformin (1 µM). Statistical significance is indicated with asterisks above the corresponding bar (p < 0.005 ** and p < 0.0005***).

Figure 6.5 Percentage growth inhibition (± SEM) produced on Chang liver cells by ethanol extracts. Cells were exposed to ethanol extracts (0.125 or 12.5 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) for 48 hours at 37°C. Bars represent one to three individual experiments per sample with six replicates per
sample per experiment. A dotted gridline indicates 10% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05).

6.4.2 Concentration-response curves on Chang liver cells

Concentration-response curves were performed on Chang liver cells with aqueous extracts of *Bulbine frutescens* and *Tarchonanthus camphoratus*, because it produced the largest glucose uptake response and the best concentration-dependent glucose uptake response, respectively.

Three independent concentration-response experiments were performed for the aqueous extract of *B. frutescens* (0.1, 0.5, 5, 10, 62.5, 125, 250 and 500 µg/ml). Figure 6.6 shows the trend of the concentration-response curve of the second experiment, which illustrates the basic trend followed by all the experiments. No 50% response calculation could be made, because the shape of the transformed curves did not follow a sigmoidal shape. From the combined data of all three experiments, a unique curve, which peaks at 0.5 and 62.5 µg/ml and troughs at 0.1 and 125 µg/ml, could be identified. The trend in the concentration-response curves also conformed to the screening averages obtained; 143.5 ± 5.69% for 0.5 µg/ml and 111.4 ± 2.40% for 50 µg/ml (Table D.1). The accompanying toxicity observed for each concentration is illustrated in Figure 6.7. The only statistically significant findings are those for 12.5 µg/ml which produced growth inhibition of 6.74 ± 2.26% (n = 6; p = 0.0380) and 0.025 µg/ml which produced significant growth stimulation -6.61 ± 3.17% (n = 6; p = 0.0487).
Figure 6.6 Concentration-response curve for glucose uptake by Chang liver cells. Cells were treated with the aqueous extract of *Bulbine frutescens* for 48 hours (1.25, 2.5, 15.6, 31.3, 62.5 or 125 µg/ml) and again for three hours (5, 10, 62.5, 125, 250 or 500 µg/ml) at 37ºC. Data points represent one experiment with 5 or 10 replicates per concentration. The solid gridline indicates percentage glucose uptake (110.6%) of metformin (1 µM).

Figure 6.7 Percentage growth inhibition (± SEM) produced on Chang liver cells by different concentrations of *Bulbine frutescens*. Cells were exposed to aqueous extracts at various concentrations (0, 0.025, 0.125, 1.25, 2.5, 15.6, 31.3, 62.5 or 125 µg/ml) of *B. frutescens* for 48 hours at 37ºC. Bars represent two or four experiments for each concentration, with six or three replicates per concentration. A dotted gridline indicates 10% growth inhibition. Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05).

Three independent concentration-response experiments were conducted for the aqueous extract of *T. camphoratus*. Figure 6.8 depicts the concentration-response curves of the third (concentrations; 5, 10, 62.5, 125, 250 and 500 µg/ml) and fourth (concentrations; 80, 100, 200 and 400 µg/ml) experiments. Again no 50% response could be calculated due to the non-sigmoidal shape.
of the transformed curve. For the concentrations tested, the curves showed a loss of activity at 5 µg/ml with increases in activity as the concentrations increase. The spike in activity at 500 µg/ml may not be a true representation of glucose uptake and the cells may have been in shock, due to the high concentration of the aqueous extract. When comparing the concentration-response curves to the screening averages for 0.5 µg/ml, 116.7 ± 5.83% and 50 µg/ml, 131.5 ± 4.20%, another peak at 0.5 µg/ml is evident (Table D.1). Toxicity evaluations for the concentration-response curves of aqueous extracts of T. camphoratus are represented in Figure 6.9. Only two concentrations were found to inhibit growth of Chang liver cells significantly, namely 62.5 µg/ml (8.23 ± 2.87%; n = 6; p = 0.0179) and 125 µg/ml (8.78 ± 2.45%; n = 6; p = 0.0107).

![Figure 6.8 Concentration-response curve for glucose uptake by Chang liver cells. Cells were treated with different concentrations of the aqueous extract of Tarchonanthus camphoratus for 48 hours (1.25, 2.5, 15.6, 20, 25, 31.3, 50, 62.5, 100 or 125 µg/ml) and again for three hours (5, 10, 62.5, 80, 100, 125, 200, 250, 400 or 500 µg/ml) at 37°C. Data points represent two individual experiments with 5 or 10 replicates per concentration per experiment. The solid gridline indicates percentage glucose uptake (109.1%) of metformin (1 µM).](image-url)
6.4.3 Glucose uptake by C2C12 muscle cells

The average response for insulin (1 µM) from 10 individual experiments was 119.7 ± 1.46% (n = 158). The percentage glucose uptake for different concentrations of aqueous extracts has been summarised in Table D. 5 and Figure 6.10. Aqueous extracts that increased glucose uptake more than insulin included both concentrations of *Bulbine frutescens* and the 0.5 µg/ml concentration of *Ornithogalum longibracteatum*, *Ruta graveolens* and *Tarchonanthus camphoratus* (Figure 6.10). Significant concentration-independent responses were observed for *B. frutescens* (p = 0.0241), *O. longibracteatum* (p = 0.0044) and *T. camphoratus* (p = 0.0201).
Aqueous extracts were also tested in combination with insulin to see whether the combination was more effective than when used alone. In this series of seven individual experiments, the average response for insulin (1 µM) was $126.1 \pm 1.97\%$ ($n = 111$). The percentage glucose uptake for different concentrations of aqueous extracts combined with insulin has been summarised in Table D.6. Generally, the responses of aqueous extracts increased when combined with insulin. The largest combined response ($148.3 \pm 5.40\%$) was produced by *Ornithogalum longibracteatum* (0.5 µg/ml).

Because of the variation of the insulin responses between individual experiments, it was decided to recalculate the activities of the aqueous extracts with and without insulin as a function of insulin response obtained in that specific experiment. All glucose uptake values were thus recalculated to obtain the percentage response compared to an insulin response of 0%. A summary of the percentage glucose uptake as compared to insulin for...
aqueous extracts with and without insulin can be viewed in Table D. 7 and Figure 6.11.

The 50 µg/ml aqueous extracts of *R. graveolens* and *T. camphoratus* produced statistically significant increased combined responses as compared to the extracts alone. This might indicate a possible additive effect with the addition of insulin to the 50 µg/ml aqueous extract of *R. graveolens* and *T. camphoratus*.

![Figure 6.11 Percentage glucose uptake (± SEM) produced on C2C12 muscle cells by aqueous extracts with and without insulin as compared to the insulin response (taken as 0%). Cells were treated for 1 hour with aqueous extracts (0.5 or 50 µg/ml) of *Bulbine frutescens* (Bf), *Ornithogalum longibracteatum* (Ol), *Ruta graveolens* (Rg), *Tarchonanthus camphoratus* (Tc) and *Tulbaghia violacea* (Tv) and these aqueous extracts combined with insulin (1 µM, represented with a “+” at 37ºC. Normal bars represent 10 experiments with 12 replicates per sample for the extract alone and checked bars represent seven individual experiments with the same amount of replicates per sample. The zero gridline indicates percentage glucose uptake of insulin (1 µM). Statistically significant changes in response to the extract with and without insulin are indicated with appropriately pointed arrows and asterisks above the corresponding arrow to indicate the degree of significance (p < 0.05* and p < 0.005**). Four individual experiments were performed for the screening of ethanol extracts. The average response for insulin (1 µM) during the four experiments was 117.9 ± 2.53% (n = 63; p < 0.0001). A summary of the percentage
glucose uptake achieved by ethanol extracts is shown in Table D. 8 and Figure 6.12. The largest increase in glucose uptake was produced by the 0.5 µg/ml ethanol extract of _T. violacea_ (140.5 ± 5.56%), which showed a significant concentration-independent response (p = 0.0109). The two extracts of _O. longibracteatum_ were the only ethanol extracts that produced a concentration-dependent increase in glucose uptake (p = 0.043).

![Figure 6.12](image_url)

Figure 6.12 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated for 1 hour with ethanol extracts (0.5 or 50 µg/ml) of _Bulbine frutescens_ (Bf), _Ornithogalum longibracteatum_ (Ol), _Ruta graveolens_ (Rg), _Tarchonanthus camphoratus_ (Tc) and _Tulbaghia violacea_ (Tv) at 37°C. Bars represent two experiments for each extract with 12 replicates per sample per experiment. The solid gridline indicates the percentage glucose uptake (117.9%) of insulin (1 µM). Statistical significance is indicated with asterisks above the corresponding bar (p < 0.005** and p < 0.0001***). The average response for insulin (1 µM) for the four experiments for ethanol extracts combined with insulin was 133.7 ± 4.60% (n = 64; p < 0.0001). Table D. 9 summarises the results of ethanol extracts combined with insulin. All the glucose uptake responses for the ethanol extracts combined with insulin were clinically and statistically significant. The largest response obtained for ethanol extracts combined with 1 µM insulin was 159.5 ± 9.68% from the 0.5 µg/ml concentration of _B. frutescens_.

As was done with the aqueous extracts, all glucose uptake values were recalculated to obtain the percentage response for the ethanol extract with
and without insulin as compared to a recalcualted insulin response of 0%. A summary of the percentage glucose uptake as compared to insulin for ethanol extracts with and without insulin can be viewed in Table D. 10 and Figure 6.13. The glucose uptake activity of the 50 µg/ml concentration of the ethanol extracts of B. frutescens (p = 0.0399), R. graveolens (p = 0.0066), T. camphoratus (p = 0.0105) and T. violacea (p = 0.0023) were significantly potentiated when it was combined with insulin, which may point to an additive or synergistic effect with insulin. However, glucose uptake activity significantly decreased when insulin was added to the 0.5 µg/ml ethanol extracts of T. camphoratus (p = 0.0089) and T. violacea (p = 0.0002) in comparison to the response with these extracts alone.

![Figure 6.13 Percentage glucose uptake (± SEM) produced on C2C12 muscle cells by ethanol extracts with and without 1 µM insulin as compared to the insulin response (taken as 0%). Cells were treated for 1 hour with ethanol extracts (0.5 and 50 µg/ml) of Bulbine frutescens (Bf), Ornithogalum longibracteatum (Ol), Ruta graveolens (Rg), Tarchonanthus camphoratus (Tc) and Tulbaghia violacea (Tv) and these extracts combined with insulin (represented with a “+” at 37ºC. Normal bars represent two experiments with 12 replicates per sample for the extract alone and checked bars represent two individual experiments per extract with the same amount of replicates per sample. The zero gridline indicates percentage glucose uptake of insulin (1 µM). Statistically significant changes in response to the extract with and without insulin are indicated with appropriately pointed arrows and asterisks above the corresponding arrow to indicate the degree of significance (p < 0.05*, p < 0.005** and p < 0.0005***).}
Concentration-response curves were done using the aqueous extracts of *Bulbine frutescens* and *Tarchonanthus camphoratus*.

Three independent concentration-response curves were done for *B. frutescens*. Two of the curves are represented in Figure 6.14. Concentrations for experiment 1 included 0.1, 0.5, 1, 2.5 and 5 µg/ml and for experiment 3 included 0.5, 5, 50 and 500 µg/ml. The concentration-response curve found for *B. frutescens* did not allow for the calculation of a 50% response because it was not sigmoidal in shape. The corresponding concentrations and variation of the three curves correlated with the screening averages which were 130.1 ± 2.43% for 0.5 µg/ml and 121.3 ± 3.05% for 50 µg/ml (Table D. 5).

![Figure 6.14 Concentration-response curve for glucose uptake in C2C12 muscle cells for the aqueous extract of *Bulbine frutescens*.](image)

Three independent concentration-response curves were done for *T. camphoratus*. Two of the curves are represented in Figure 6.15. Concentrations for experiment 1 included 0.1, 0.5, 1, 2.5 and 5 µg/ml and for experiment 2 included 0.5, 5, 50 and 500 µg/ml. If the insulin responses for the two experiments are taken into account, the two curves follow approximately the same trend in concentration activities. Once again no value could be calculated for the 50% response, due to the shape of the
curve. The average screening responses for *T. camphoratus* were 128.4 ± 3.27% for 0.5 µg/ml and 117.4 ± 2.75% for 50 µg/ml (Table D.5). The curves agree with the concentration-independent responses found in the screening.

![Concentration-response curves for glucose uptake in C2C12 muscle cells for the aqueous extract of *Tarchonanthus camphoratus*. Cells were treated for one hour with different concentrations (0.1, 0.5, 1, 2.5, 5, 50 and 500 µg/ml) of *T. camphoratus* aqueous extract at 37ºC. Data points represent two experiments with 12 or 16 replicates per concentration. The solid gridlines indicate colour coded average percentage glucose uptake (145.8 and 118.6%) of insulin (1 µM) for the two experiments.](image)

### 6.5 Discussion

Diabetes mellitus is associated with a number of pathophysiological mechanisms; the one most pronounced is hyperglycaemia. The current study thus focused on glucose uptake into Chang liver and C2C12 muscle cells.

*Bulbine frutescens* produced increased glucose uptake (except for the ethanol extracts in Chang liver cells) in both cell lines in a concentration-independent manner. Similar concentration-independent responses were observed for knipholone, one of the active ingredients contained in *B. frutescens*, in a leukotrine biosynthesis assay (Wube *et al.*, 2006). In addition, glucose uptake in C2C12 cells, initiated by the *B. frutescens* extracts was not adversely affected by the addition of insulin (1 µM). Indeed, glucose uptake was significantly enhanced for the 50 µg/ml ethanol extract when combined with
insulin as compared to the response of the extract alone. Growth inhibition of Chang liver cells that was induced by chronic exposure in the glucose uptake experiments concurred with the results found in section 5.3.3 for *B. frutescens* extracts.

The aqueous and ethanol extracts of *Ornithogalum longibracteatum* followed similar glucose uptake and concentration-independent trends in both cell lines as the trends previously discussed for the extracts of *B. frutescens*. Glucose uptake in C2C12 cells was not significantly different for the aqueous and ethanol extracts of *O. longibracteatum* with and without insulin. According to growth inhibitory findings in the current chapter and section 5.3.3 the possibility exists that the aqueous extract may be toxic at high concentrations. However, ethanol extracts of *O. longibracteatum* induced growth at concentrations of 62.5 and 125 µg/ml, which may indicate activation of mitogenic responses in Chang liver cells.

*Ruta graveolens* did not produce any significant effects related to glucose uptake in Chang liver cells. Similar to findings in Chapter 5 (Table C.4), the ethanol extract indicated toxicity in Chang liver cells at higher concentrations, but not the aqueous extracts (Table C.3). In contrast to the lack of glucose uptake activity found for *R. graveolens* in Chang liver cells, all but the 50 µg/ml aqueous extract, significantly increased glucose uptake in C2C12 muscle cells. The addition of insulin (1 µM) to the 50 µg/ml aqueous and ethanol extracts significantly increased glucose uptake activity by 4 and 14%, respectively as compared to the activity of the extracts alone. The presence of the hypoglycaemic flavonoid quercetin (Mukherjee *et al.*, 2006) in *R. graveolens* may have caused the enhancement of glucose uptake for the extracts. Quercetin’s antioxidant action (Wube *et al.*, 2006) may relieve oxidative stress and increase insulin sensitivity in muscle cells (Laight *et al.*, 2000). In addition, quercetin may alter Ca$^{2+}$ metabolism in cells causing muscle cell contraction and activation of AMP-kinase (Akerstrom *et al.*, 2006; Kelly *et al.*, 2004; Kim *et al.*, 2006). Activated AMP-kinase stimulates glucose transport via insulin-independent pathways (Henriksen, 2006). Furthermore, acetylcholinesterase inhibitory activity of *R. graveolens* (Adsersen *et al.*, 2006).
2006) may enhance muscle contraction in vivo by binding to nicotinic receptors activated by acetylcholine. The enhanced binding will cause increased Ca\(^{2+}\) levels in the cell and stimulate glucose transport (Henriksen, 2006).

_Tarchonanthus camphoratus_ showed definite increases in glucose uptake activity in both Chang liver and C2C12 muscle cells. The aqueous extracts were more effective in liver cells and the ethanol extracts in muscle cells. However, responses in both cell lines did not seem to follow any concentration-dependent trends, especially after concentration-response curves were compiled for the aqueous extracts. The extracts of _T. camphoratus_ had interesting glucose uptake responses in C2C12 cells when combined with insulin as compared to the response of the extract alone. The combined responses of the 50 µg/ml aqueous and ethanol extracts with insulin significantly increased glucose uptake (+ 9% and + 14%, respectively) as compared to the response of the extract alone. Conversely, the combined response of the 0.5 µg/ml ethanol extract was significantly decreased (-23%) as compared to the extract alone (p = 0.0089). Toxicity of especially the ethanol extract seemed suspect and should be monitored at higher concentrations as already determined in Chapter 5 (Table C. 4). _T. camphoratus_ contains many compounds that may be potentially hypoglycaemic, including saponins (Mukherjee et al., 2006), flavanones and tannins (Scott & Springfield, 2005).

The aqueous extract of _Tulbaghia violacea_ showed significant increased glucose uptake activity only at 50 µg/ml in Chang liver cells and no significant growth inhibition as found in Chapter 5 (Table C. 4). The ethanol extract of _T. violacea_ increased glucose uptake into C2C12 muscle cells following a concentration-independent trend. Similar to the ethanol extracts of _T. camphoratus_, the combination of _T. violacea_ extract with insulin decreased the response for 0.5 µg/ml and increased the response for 50 µg/ml, significantly as compared to the response of the extracts alone. The glucose lowering activity of _T. violacea_ may be attributed to sulfur-containing compounds, quercetin, kaempherol, sugars, and / or steroidal saponins.
(Duncan et al., 1999). The sulfur-containing compounds in garlic have been shown to be hypoglycaemic in diabetic animals and has been ascribed to antioxidant activity and the interaction of these compounds with thiol-containing proteins (Mukherjee et al., 2006).

6.5.1 Feedback seminar for participating practitioners

A diabetes feedback seminar, at which the results of the diabetic screening were explained, was held for participating practitioners. Previously, participating practitioners told us on different occasions that they used *Tarchonanthus camphoratus* for the treatment of diabetes. During the feedback seminar they also said that they used *Bulbine frutescens* and *Ornithogalum longibracteatum* for the treatment of diabetes.

It was explained that the 0.5 µg/ml concentration used during screening was very low. Participating practitioners confirmed that they used combinations of very small amounts of different plants in their diabetic remedies. Generally, ethnobotanical surveys confirmed the use of mixtures of plants for the treatment of diabetes (Alarcon-Aguilera et al., 1998; Li et al., 2004; Tahraoui et al., 2007; Ziyyat et al., 1997). It has also been reported that not all the plants in a diabetic remedy may necessarily have antihyperglycaemic activity (Alarcon-Aguilera et al., 1998). In accordance with this, practitioners attending the seminar explained that additional plants used in the remedies was to either enhance the activity or stop toxic side-effects of another plant. In accordance, ethnobotanical surveys have also reported the use of small amounts of toxic plants in diabetic remedies, to prevent toxicity in patients (Tahraoui et al., 2007; Ziyyat et al., 1997). Attending practitioners agreed that they did not use *T. camphoratus* alone, because it was too strong and that they mixed other plants with it “to stop the strongness”. Toxicity results from Chapter 5 and 6 confirm the possible toxicity of high concentrations of the ethanol extract of *T. camphoratus*.

When the practitioners were asked which plant should be investigated further, their answer was unanimous; *Umgqeba* (*T. camphoratus*). They said that “it is very strong and very good”. If they were not sure what to use, *umgqeba*
(T. camphoratus) or umhlonyane (Artemisia afra) were always a good choice, because “it is good for anything”. They said it worked on all systems of the body. Traditional Chinese medicine affirms the multiple workings of combinations of active ingredients found in plants, because it claims that it not only lowers blood glucose, but also prevents complications (Li et al., 2004). Recent evidence on the pathology of diabetes suggests an increasing amount of targets that might prolong the onset of T2DM i.e. treating causes of insulin resistance, inflammation, the procoagulative state, obesity etc. In light of this, natural products or crude extracts of natural products may exhibit more than one mechanism which may potentially (Raghav et al., 2006) prolong the development, improve treatment and/or prevent complications of T2DM.

The additional physiological effects of the selected plants support this view and their use in traditional medicine. B. frutescens and knipholone identified in B. frutescens has been found to have anti-inflammatory properties through the inhibition of COX-1 and COX-2 (Gaidamashvili & van Staden, 2006) and 5-LOX (Wube et al., 2006), respectively. Both of these enzymes are potential sources for the production of free radicals. There is thus the potential for endothelial protective properties and the associated prevention of diabetic complications (Pratico, 2005). Homoisoflavanones, as identified in O. longibracteatum have demonstrated anti-inflammatory, angioprotective and phosphodiesterase inhibitory activities (Du Toit et al., 2005). In addition, it has also been shown that related isoflavanones may increase glucose and lipid metabolism by PPARγ agonist activity (Mukherjee et al., 2006).

The antioxidant activity induced by quercetin has been discussed earlier as a possible mechanism of increased glucose uptake (Mukherjee et al., 2006) by Ruta graveolens. Antioxidant therapy has also shown to improve insulin resistance in skeletal muscle by activation of improved tyrosine phosphorylation of IR, IRS-1, PI3-kinase and serine phosphorylation of Akt (Henriksen, 2006; Sabu & Kuttan, 2002). Additionally, quercetin and rutin have shown inhibition of haemoglobin glycosylation via their anti-oxidant properties (Asgary et al., 1999). Furthermore, R. graveolens decreases protein expression of inducible nitric oxide synthase (iNOS) as well as COX-2
during inflammatory conditions (which are present during insulin resistance). This is thought to be as a result of the inhibition of nuclear factor Kappa B (Raghav et al., 2006), which might be due to the anti-oxidant properties of the extract (Stosic-Grujicic et al., 2004). *R. graveolens* may also be effective in the treatment of hypertension closely associated with diabetes mellitus. *R. graveolens* caused relaxation of pre-constricted smooth muscle in another experiment (Chiu & Fung, 1997), which may be related to the acetylcholinesterase inhibitory activity (Adsersen et al., 2006). This promotes smooth muscle relaxation through muscarinic receptor stimulation, which is an endothelial-independent vasodilator response (Kuyvenhoven & Meinders, 1999). Rutin, an alkaloid in *R. graveolens*, has also been used for its capillary protectant properties (Williamson, 2003). *R. graveolens* has also shown some properties of coagulation prevention in rats (Williamson, 2003).

Other effects that have been shown by *Tulbaghia violacea* include ACE-inhibitory properties (Duncan et al., 1999), which might be a welcome additional effect for its use in diabetes. Hypertension and diabetes mellitus have been known to be crucial risk factors in the onset of serious cardiovascular disease (Igarashi et al., 2007). It has been shown that angiotensin II inhibits insulin signalling at multiple levels thus interfering with glucose metabolism. This interaction is important in the progression of diabetic macroangiopathy (Igarashi et al., 2007), especially in the kidney (Laight et al., 2000). Another positive contribution that *T. violacea* may add to the insulin resistant syndrome is its anti-coagulative / anti-thrombotic effects that have been identified (Bungu, 2005). Additionally, the fact that *T. violacea* has anticancer properties (Bungu et al., 2006) may also be advantageous in the prevention of this associated, but sometimes controversial complication (Czyzyk & Szczepanik, 2000).

**6.5.2 Summary of findings**

It was found that four aqueous extracts compared to one ethanol extract had positive glucose uptake activity in Chang liver cells. However, for C2C12 muscle cells, all the ethanol extracts showed positive glucose uptake activity as compared to four aqueous extracts. The increased activity of the aqueous
extracts in the anti-diabetic screening as compared to the antimicrobial and anticancer screenings may be explained by water-soluble compounds being common anti-diabetic compounds (Gallaghar et al., 2003).

The fact that many anti-diabetic compounds are water extractable shows great promise for its use in traditional medicines. The extent of the mechanisms that may be beneficial in diabetes treatment may also provide for a potential complementary link between the conventional and traditional systems of medicine. We could probably learn from the Chinese in this respect. In Chinese medicine it is accepted that most western medicine would be more effective to lower blood glucose levels, but would have little effect on preventing diabetic complications (Li et al., 2004). Therefore Chinese doctors will often combine western medicine with traditional medicine to lower blood glucose and alter the course of diabetic complications, providing integrated care of the body (Li et al., 2004). The use of plant medicines in conjunction with conventional treatment has been noted in a survey done on diabetic patients in a rural teaching hospital in South Africa (Bopape & Peltzer, 2002). However, the use of traditional remedies in the South African setting was not very common (Bopape & Peltzer, 2002).

In addition to medication, control of hyperglycaemia is a multifactorial process, which includes diet, exercise and daily compliance to the medication regimen. Poor diabetic control has been linked to considerable psychological stress associated with the diagnosis of diabetes and psychosocial care has been recommended for both the patient and the family of the patient (Bopape & Peltzer, 2002). Traditional health practitioners would be well qualified to deal with this kind of counselling if given the chance to be integrated into the health care system in South Africa. The cost effectiveness of such an intervention might prove beneficial because of the positive link between increased glycaemic control and development of complications (Ratner, 2001).

Given the potential of traditional medicinal plants tested in this study to treat hyperglycaemia as well as potential to prevent diabetic complications, the incorporation of traditional medicinal plants could also be beneficial to the holistic care of diabetes in South Africa. More research on possible
hypoglycaemic mechanisms is necessary to identify possible side effects and drug interactions these plants may have. The next chapter will aim to shed some light on the hypoglycaemic mechanisms of the aqueous extract of *T. camphoratus*. 
7 Anti-diabetic activity of *Tarchonanthus camphoratus* aqueous extracts

7.1 Introduction

After feedback was given on the anti-diabetic screening results, participating practitioners suggested that we continue our investigations on *Tarchonanthus camphoratus*, as it was a frequently used plant for many different conditions and in particular diabetes mellitus. Knowledge generated from further testing would benefit participating practitioners, because it would pertain to the crude aqueous extract, which practitioners could easily produce themselves. This step took into account usefulness of knowledge to the indigenous community, which has been recommended to be an important consideration in ethnopharmacological research (Elisabetsky, 1991).

To ensure effective use of the *T. camphoratus* aqueous extract, seasonal variation of glucose uptake activity needed to be investigated as well as specific pathways by which the extract increased glucose uptake. The only seasonal information available on *T. camphoratus* was that a small group of giraffe consumed it during the summer months in the Shamwari Game Reserve in the Eastern Cape (Parker *et al.*, 2003) and that it flowered from April to June (Joffe, 1993). The screening results of this study showed that the aqueous extract of *T. camphoratus* harvested in November increased glucose uptake activity in both, Chang liver and C2C12 muscle cells as compared to the positive controls used in these experiments (section 6.4.1 and 6.4.3, respectively). The antimicrobial screening also indicated slight activity in leaves harvested in November as compared to leaves harvested in July (section 4.4).

With respect to the specific pathways that may be involved in glucose uptake, the primary mechanisms for glucose uptake in liver and skeletal muscle differ, because they contain different types of glucose transporters (Gould &
Holman, 1993). Subsequently, the two types of tissue are differently affected in T2DM. In skeletal muscle glucose uptake activity is impaired (Cheng & Fantus, 2005) and in liver glucose production is increased (Rutter, 2000), which both add to an increased blood glucose level. These differences between glucose transport in muscle and liver cells might suggest that the extract works differently in the two types of tissue.

In the case of skeletal muscle, glucose is taken up primarily through GLUT4 and is also the main site of glucose disposal in the body. GLUT4 are dynamic transporters moving between intracellular vesicles and the plasma membrane (Holman & Sandoval, 2001). Glucose uptake is one-way and once glucose is inside the cell, it can not be released. For glucose uptake to occur, GLUT4 need to be translocated from the intracellular vesicles and then fuse with the plasma membrane (Zorzano et al., 1998). GLUT4 translocation may occur as a result of various stimuli, of which, insulin-binding to the insulin receptor is a major activator (Mohammad et al., 2006). Insulin-binding activates a signalling cascade which involves the phosphorylation of tyrosine residues located primarily on IRS-2 and PI3-kinase (Konrad et al., 2001). The activation of these two proteins set the signalling cascade in motion thereby causing the translocation of GLUT4.

Apart from insulin-mediated GLUT4 translocation, other stimuli independent of insulin (Sakakibara et al., 2006) such as AMP-kinase activation increase glucose uptake (Abbud et al., 2000) and GLUT4 translocation. Although still controversial, the hypothesis of GLUT4 activation in addition to GLUT4 translocation is gaining much support (Somwar et al., 2001). This hypothesis advocates that the mere increase in translocation of GLUT4 to the plasma membrane is not the only mechanism by which glucose uptake may be increased. The hypothesis further suggests that some mechanisms activate the transporters already in the plasma membrane to take up more glucose. MAP-kinase, which is activated by insulin, seems to be one of the foremost activators of GLUT4, because it increases glucose uptake without increasing GLUT4 translocation to the plasma membrane (Somwar et al., 2001).
addition, MAP-kinase may also be activated by AMP-kinase (Imai et al., 2006).

In contrast to the one-way glucose uptake in muscle cells via GLUT4, the liver’s GLUT2 are permeable to glucose according to a concentration gradient, which allows for glucose to enter or leave the cell (Gould & Holman, 1993). The aqueous extract of *T. camphoratus* increased net glucose uptake into Chang liver cells, which implies that it either activated glucose utilisation pathways and / or inhibited glucose production pathways. Glucose utilisation inside the liver cell is only possible once it has been phosphorylated to glucose-6-phosphate (G6P) by glucokinase (Klover & Mooney, 2004). After the conversion, G6P may be utilised either for glycogen synthesis (by inactivation of glycogen synthase kinase-3 by insulin through PI3-kinase (Schenk & Snaar-Jagalska, 1999)), glycolysis (increased by the activation of AMP-kinase) or the pentose phosphate shunt (Roden & Bernroider, 2003). Alternatively, glucose production occurs through glycogenolysis (activated by glycogen phosphorylase) and / or gluconeogenesis. The final reaction in both of these pathways is catalysed by glucose-6-phosphatase (G6Pase) (Campbell, 2006). G6Pase produces glucose from G6P, thus opposing the action of glucokinase (Van Schaftingen & Gerin, 2002) and it has been established that gluconeogenesis is up-regulated in T2DM (Roden & Bernroider, 2003), which is not due to increased substrate availability (Jenssen et al., 1990). Furthermore, the action of insulin is impaired in insulin resistance by decreased tyrosine phosphorylation of the insulin receptor and IRS-2 (Wang et al., 2001). This is thought to be the result of up-regulation of phosphotyrosine phosphatases, which dephosphorylate phosphotyrosine residues (Gogg et al., 2001).

The final mechanism that will be investigated involves an *in vitro* enzymatic assay using α-glucosidase. This enzyme is situated in the brush-border of the intestine (Van de Laar et al., 2006). If it can be inhibited, digestion of carbohydrates would be delayed and postprandial hyperglycaemia prevented.
7.2 Methodology

The methods of all the assays will be described in the following section. Details of the reagents used in the assays, are listed in Appendix E.

7.2.1 Effect of seasonal variation and storage on glucose uptake

*Tarchonanthus camphoratus* leaves were collected at regular intervals throughout the year from the same tree on the NMMU campus. The tree was situated in a bushveld-type environment and received no irrigation water. Harvesting of the leaves was done at four month intervals to allow time for possible changes in chemical component levels (Table 7.1). Dried extracts were stored at 4°C in a moisture and light free environment (section 3.7).

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Weight of leaves (g)</th>
<th>Extraction method</th>
<th>Dry weight of extract (g)</th>
<th>% yield (m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 Oct 2004</td>
<td>8:30</td>
<td>14.79</td>
<td>Infusion</td>
<td>0.95</td>
<td>6.45</td>
</tr>
<tr>
<td>29 Nov 2005</td>
<td>7:45</td>
<td>32.2</td>
<td>Infusion</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>6 Mar 2006</td>
<td>9:00</td>
<td>26.71</td>
<td>Infusion</td>
<td>1.53</td>
<td>5.73</td>
</tr>
<tr>
<td>6 Mar 2006</td>
<td>9:00</td>
<td>23.83</td>
<td>Decoction</td>
<td>1.94</td>
<td>8.14</td>
</tr>
<tr>
<td>7 Jul 2006</td>
<td>± 8:00</td>
<td>NR</td>
<td>Infusion</td>
<td>0.67</td>
<td>NR</td>
</tr>
</tbody>
</table>

Key: NR = not recorded

The glucose uptake activities of the different extracts were tested to determine any variation between extracts made during different times of the year and storage stability of the dried extracts. Glucose uptake assays and statistical evaluations were performed on Chang liver and C2C12 muscle cells according to the methods described in section 6.2 and 6.3.
7.2.2 GLUT4 translocation

GLUT4 translocation was determined in C2C12 muscle cells by Western-blot analysis of the plasma membrane protein fraction (Gould & Holman, 1993). Quantification of GLUT4 was performed by densiometric scanning of the blots (Mohammad et al., 2006).

7.2.2.1 Materials

Prior to harvesting, cells were incubated in buffer consisting of RPMI-1640 supplemented with 1% (m/v) bovine serum albumin (BSA) with and without insulin (1 µM) or Tarchonanthus camphoratus aqueous extract (50 µg/ml).

After incubation, harvesting and membrane fractionation were done using different variations of the basic homogenising buffer. The basic homogenising buffer consisted of 10 mM sodiumhydrogen carbonate (NaHCO₃) and 0.25 M sucrose (pH 7.5). Homogenising buffer A contained protease inhibitor cocktail (10 µl/ml) and homogenising buffer B contained protease inhibitor cocktail (30 µl in 300 µl), nonidet P40 (0.04% v/v) and TWEEN 20 (0.02% v/v).

Protein determination was done according to the bicinchoninic acid (BCA) method (Caprette, 2005) and the protein determination reagent was made up of 50 parts BCA and 1 part copper sulfate (CuSO₄; 4% m/v) in water.

Proteins were prepared for SDS-polyacrylamide gel electrophoresis in reducing sample buffer (Formulae A.3). For the western blot, transfer buffer (Formulae A.4) was made up with 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS; 5.82 g), glycine (2.93 g), sodium dodecyl sulphate (SDS; 0.01% m/v), methanol (200 ml) and deionised water (800 ml). Tris-buffered saline (TBS; Formulae A.5), the wash buffer, was made up with sodium chloride (NaCl; 0.8% m/v), 1 M TRIS solution (2% v/v), TWEEN 20 (0.1% v/v) and nonidet P40 (0.04% v/v) (pH 7.4).

Blocking solution consisted of TBS with 5% (m/v) fat free milk powder. The primary (rabbit anti-GLUT4) and secondary (goat-anti-rabbit horseradish peroxidase GAR-HRP) antibodies were made up in blocking solution (1:20 000).
7.2.2.2 Methodology

C2C12 muscle cells were seeded into 10 cm culture dishes (NUNC) at 250 000 cells/plate. The plates were incubated at 37°C to reach confluence and to differentiate for three and a half days without changing the growth medium (section 6.3.2).

After the three and a half day incubation, each plate of C2C12 muscle cells was washed with 5 ml PBS supplemented with 0.1% (m/v) BSA. Then 10 ml PBS containing 1% (m/v) BSA was added to each plate and incubated for 10 minutes at 37°C. After this, the incubation medium was aspirated and 10 ml of incubation buffer with or without insulin (6 µg/ml or 1 µM) or T. camphoratus aqueous extract (50 µg/ml) added. The plates were incubated for a further 15 minutes at 37°C to allow for GLUT4 translocation to the cell membrane, after which the incubation buffer was aspirated and 1 ml ice cold homogenising buffer A added. The plates were subsequently frozen and stored at -80°C until the fractionation was performed.

Plasma membrane fractionation was initiated with three freeze-thaw cycles of the 10 cm plates to lyse the C2C12 muscle cells. After the last thawing, cell lysates were suspended, transferred to 15 ml sterile polypropylene tubes and syringed 10 times at 4°C to make sure lysis was complete. Tubes were centrifuged (Eppendorf centrifuge 5804 R) at 1 000 x g for 10 minutes at 4°C to remove nuclear material (Jung et al., 2006; Mohammad et al., 2006). The supernatant was then transferred to a centrifuge tube which was filled with about 4.5 ml basic homogenising buffer and spun (Beckman L8-80M ultracentrifuge) for another hour at 190 000 x g at 4°C (Mohammad et al., 2006). After this centrifugation, the supernatant was discarded and the pellet (containing the GLUT4 fraction (Jung et al., 2006)) suspended in 50 µl homogenising buffer B and transferred to a microtube. The centrifuge tubes were rinsed with another 30 µl of homogenising buffer B to ensure the complete removal of the pellet. A metal bead was added to each microtube and the pellet was homogenised (Retsch MM301) at 25 Hz for 2 minutes.

Protein determinations of the plasma membrane fractions were done using the BCA method (Caprette, 2005). Suspended and homogenised pellets
were centrifuged (Beckman Avanti™ J-25 centrifuge) at 10 000 x g for 10 minutes at 4ºC. Five microliters of supernatant protein was diluted with 20 µl of water and 10 µl of this solution was added to 200 µl of protein determination reagent, in a microtiter plate. The plate was incubated for 30 minutes at room temperature and absorbance read at 540 nm. Protein concentrations were determined from a BSA standard curve (31.25, 62.5, 125, 250 and 500 µg/ml).

Membrane protein fractions were prepared in reducing sample buffer at 4 µl of reducing sample buffer to 20 µl of protein, but application of the protein to the gel was done according to the calculated mass of protein (20 µg). Five microliters of protein standard solution (PageRuler, Fermentas) and 20 µg of prepared protein fractions were applied to a 7.5% SDS-polyacrylamide gel (Mini-PROTEAN® 3 Cell, BIO-RAD, USA) and separated by electrophoresis at 150 to 100 mV. After which, the SDS-polyacrylamide gel and nitrocellulose membrane (PVDF Western blotting membrane, Roche) were equilibrated in transfer buffer for 20 minutes. A semi-dry blot (Transfer-Blot® SD, Semi-dry Electrophoretic Transfer Cell, BIO-RAD, USA) was done for an hour to transfer proteins from the gel to the membrane. On completion of the transfer, the membrane was dipped in methanol and hung up to dry.

The membrane was incubated in blocking solution (5% fat free milk powder in TBS) containing the primary anti-GLUT4 antibodies (1:20 000) for an hour, after which it was washed with TBS. The washing steps included, rinsing the membrane twice in TBS, then washing in TBS for 15 minutes and washing another three times for 5 minutes. Washing incubation was done at room temperature on a rocker (Stuart scientific, 3D rocking platform STR9) at 30 rpm. After washing, the membrane was exposed to the secondary GAR-HRP antibodies (1:20 000) for 30 minutes and the previous wash steps repeated.

In a dark room, Amersham ECL™ substrate system (1 ml/membrane) was added to the membrane for a minute and bonded to the peroxidase enzyme on the membrane, which converted to luminescent peroxide on the immunoreactive bands of the GLUT4 protein fraction. After exposure to the substrate, the membrane was dried on a paper towel and put in a plastic
cover with the bubbles removed. X-ray film was positioned on top of the plastic-covered membrane and placed in a dark box for exposure to the luminescence from the membrane, which formed dark spots on the film. The X-ray film was subsequently developed for about 30 minutes and the spots fixed.

7.2.2.3 Data manipulation

Band quantification was done using an AlphalImage™ 3400 (Alpha Innotech). The GLUT4 blots on the X-ray film were scanned and rectangular boxes constructed around each GLUT4 band. An integrated density value (IDV) was calculated for each band, based on the sum of the pixel intensity in the rectangular box after a background correction. The average intensity of each band was calculated by dividing the IDV of each box by the area of the corresponding rectangular box.

7.2.3 Insulin-binding in C2C12 muscle cells

The amount of insulin-binding to the insulin receptors of semi-differentiated C2C12 muscle cells was determined. Relative competitive binding of the aqueous extract was calculated according to the ratio of insulin to $[^{125}\text{I}]$ insulin-binding.

7.2.3.1 Materials

C2C12 muscle cells were maintained as in section 6.3. $[^{125}\text{I}]$ insulin (rat) was obtained from Linco. It contained $[^{125}\text{I}]$ insulin at a concentration of 2.4 nM. The incubation buffer for the experiment was made by diluting radio-labelled insulin to 0.7 nM using RPMI 1640 containing 0.1% (m/v) BSA. Recombinant human insulin was used as the positive control at different concentrations to determine non-specific binding at very high concentrations and competitive insulin-binding at lower concentrations. $T.\ camphoratus$ aqueous extract was used at concentrations of 0.5 and 50 µg/ml. Sodium hydroxide (NaOH; 1 M) was used to lift the cells from the 24-well plate.
7.2.3.2 Methods

C2C12 muscle cells were seeded into 24-well plates (NUNC) at 37 500 cells/well in an incubation volume of 1 ml. The cells were left to reach confluence and partially differentiate for three and a half days (at 37°C and 5% CO₂) without changing the medium during this period.

Partially differentiated C2C12 muscle cells were exposed to 200 µl/well incubation buffer containing 0.7 nM [¹²⁵I] insulin with or without unlabelled insulin (0.001, 0.01 and 1 µM) or T. camphoratus aqueous extract (0.5 and 50 µg/ml). The exposed cells were then incubated at 20°C for 20 minutes in a waterbath. Afterwards the incubation medium was aspirated and cells were washed with 500 µl ice cold PBS containing 0.1% BSA to remove the unbound [¹²⁵I] insulin. After washing, sodium hydroxide (1 M) was added to each well at a volume of 100 µl to lift and lyse the cells, which were transferred to a 1 ml Eppendorf microtube. The wells were rinsed with 50 µl NaOH to ensure that all cells have been removed and added to the corresponding 1 ml microtube. One millilitre of scintillation cocktail (Ultima Gold) was added to each microtube and mixed. Each sample was counted in the scintillation counter (Liquid scintillation analyzer Tri-Carb 2300TR, Pacard) for 20 minutes.

7.2.3.3 Data manipulation

Counts per minute (CPM) data generated by the scintillation counter were converted to percentage [¹²⁵I] insulin bound to the insulin receptor. Untreated cells represented total binding (100%) of [¹²⁵I] insulin at a concentration of 0.7 nM. Cells treated with 1 µM insulin represented non-specific binding of [¹²⁵I] insulin to the plasma membrane of treated cells. Percentage binding was calculated according to the following formula:

\[
\% \text{ [¹²⁵I] insulin-binding} = \frac{(\text{CPM of sample} - \text{CPM avg: n-s binding}) \times 100\%}{(\text{CPM avg: t binding} - \text{CPM avg: n-s binding})}
\]

Key: avg = average; n-s = non-specific; t = total.

Quadruplicate results were obtained for each sample. Statistical analysis of results was performed utilising GraphPad Prism® 4 (GraphPad Software, 2003). The two-tailed unpaired t-test was used for calculation of statistical
significance with statistical parameters set at a 95% confidence interval. P-values of less than 0.05 were considered to be statistically significant.

### 7.2.4 Pathway inhibitors in C2C12 muscle cells

C2C12 muscle cells are commonly used to study aspects of skeletal muscle biology (Hill *et al.*, 2004; Jean-Baptiste *et al.*, 2005). The pathway through which the aqueous extract exerted its effect was investigated by the use of pathway inhibitors and activators for PI3-kinase, AMP-kinase and p38 MAP-kinase. The amount of glucose taken up in each instance was used as a measure of activity. The pathway activator would increase glucose uptake as compared to the untreated control and the pathway inhibitor would eliminate the increase in glucose uptake caused by that specific activator.

#### 7.2.4.1 Materials

The negative control contained incubation buffer made up of RPMI-1640 (of which the glucose concentration was adjusted with PBS to 5 mM) supplemented with 0.1% BSA, 0.2 µCi/ml [³H]-deoxyglucose and variable amounts of DMSO according to the concentration used for making up the pathway inhibitors and positive controls.

Wortmannin, a potent and selective inhibitor of PI3-kinase (Evans *et al.*, 1995), was used at 100 nM in the assay. A stock solution was made up at 1 mM in DMSO on the day of the experiment and was diluted with the different buffers. Insulin (1 µM) was used as the positive control for wortmannin, because it activates PI3-kinase. No DMSO was used for the preparation of the insulin.

Adenine 9-β-D-arabinofuranoside (ara) had been used by others to characterise the role of AMP-kinase (Derave *et al.*, 2000). However, it does not work directly on AMP-kinase and may not be the most adequate tool to study AMP-kinase action (Patel *et al.*, 2001), but no specific inhibitors for AMP-kinase have been found (Derave *et al.*, 2000; Fryer *et al.*, 2002a). Ara (1 mM) was used as the AMP-kinase inhibitor and made up at 1 M in DMSO.
The positive control for ara was dinitrophenol (DNP). DNP prevent ATP production in the cell by disrupting the mitochondrial proton gradient (Patel et al., 2001), thus decreasing ATP levels in the cell and activating AMP-kinase (Liu et al., 2006; Pelletier et al., 2005). DNP increases cell-surface GLUT4 and hexose uptake via a Ca\textsuperscript{2+} sensitive and conventional PKC-dependent mechanism (Patel et al., 2001). DNP was used at 0.5 mM in the assay (Patel et al., 2001) and a stock solution was made up at 0.5 M in DMSO, which was kept at -20ºC between experiments.

PD169316 is a selective inhibitor of p38 MAP-kinase and has been used at 10 to 20 µM to inhibit oxidant-stimulated glucose transport activity in epitrochlearis and soleus muscle (Kim et al., 2006). In this study it was used it at 5 µM in the assays. A stock solution that was kept at -20ºC was made up at 116 mM in DMSO. Insulin was used as positive control (1 µM).

Glucose uptake was quantified by the amount of radio-labelled [\textsuperscript{3}H] deoxyglucose taken up by C2C12 muscle cells. Krebs-Ringer phosphate buffer (KRP-buffer; Formulae A.5) was used to wash cells and NaOH (0.5 M) was used to lift the cells at the end of the experiment.

7.2.4.2 Methodology

C2C12 muscle cells were seeded into a 96-well microtiter plate at a density of 5 000 cells per well and left for three and a half days to reach confluence and induce partial differentiation. Spent medium was aspirated and the cells were washed twice with 100 µl of KRP-buffer. Each well was incubated with 50 µl of KRP-buffer supplemented with 0.1% (m/v) BSA either with or without inhibitor for 30 minutes at 37ºC. Afterwards, this incubation buffer was aspirated and a second incubation buffer added (50 µl/well). The second buffer consisted of KRP-buffer containing [\textsuperscript{3}H]-deoxyglucose (0.3 µCi/ml) and was used either pure (for the control) or with either the positive control or aqueous extract of T. camphoratus, with and with-out inhibitor (refer for Figure 7.1 for complete layout). The plate was incubated for 20 minutes at 37ºC. After incubation, wells were washed three times with 100 µl/well of ice cold KRP-buffer to remove any residual [\textsuperscript{3}H]-deoxyglucose. The cells were lifted
by addition of 50 µl of NaOH (0.5 M) to each well and left for 30 minutes. The lifted cell suspensions were transferred to microtubes. Wells were rinsed with another 50 µl of NaOH and transferred to the corresponding microtubes. One millilitre of scintillation cocktail (Ultima Gold) was added to each microtube, vortexed and read for 15 minutes in the scintillation counter (Liquid scintillation analyzer Tri-Carb 2300TR, Pacard) to detect the relative amount of [³H]-deoxyglucose taken up by the cells.

<table>
<thead>
<tr>
<th>Without inhibitor</th>
<th>Inhibitor 1</th>
<th>Inhibitor 2</th>
<th>Inhibitor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>With inhibitor</th>
<th>Inhibitor 1</th>
<th>Inhibitor 2</th>
<th>Inhibitor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>E1 E2 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>G1 G2 G3 G4 G5 G6 G7 G8 G9 G10 G11 G12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 7.1 Plate layout for pathway inhibitor experiment.

7.2.4.3 Data manipulation and statistical analysis
CPM data generated by the scintillation counter were converted to percentage [³H] deoxyglucose taken up into C2C12 muscle cells. The negative control without inhibitor represented 100% of glucose taken up. All other samples were calculated as a percentage of this value with the following equation:

\[
\% [³H] \text{ deoxyglucose uptake} = \frac{\text{CPM of sample}}{\text{CPM of NC}} \times 100\%
\]

Key: CPM counts per minute; NC negative control (without inhibitor)

Each experiment had four replicates per sample. Statistical analysis of results was performed utilising GraphPad Prism® 4 (GraphPad Software, 2003). The two-tailed unpaired t-test was used for calculation of statistical significance with statistical parameters set at a 95% confidence interval. P-values of less than 0.05 were considered to be statistically significant.
7.2.5 Enzymatic assays from crude rat liver homogenates

7.2.5.1 Materials and general method
The aqueous extract of *Tarchonanthus camphoratus* was made up in DMSO at a concentration of 1 mg/ml. Each enzymatic assay used 10 µg (10 µl) of extract.

Crude rat liver homogenates were prepared from liver harvested from Albino (Wistar-strain) male rats (Farhanullah *et al.*, 2004). After a 12 hour fast, they were sacrificed via cervical dislocation. The liver was removed and placed in isotonic potassium chloride (KCl) on ice. The liver was homogenised (Potter Elvejhem glass homogeniser and teflon pestle) and prepared as a 10% (w/v) homogenate in 150 mM KCl. The homogenate was centrifuged at 1 000 x g for 15 minutes at 4ºC (Farhanullah *et al.*, 2004). The supernatant was used as the enzyme source for all three enzymatic assays.

All experiments were performed on homogenate samples with and without *T. camphoratus* aqueous extract (10 µg/ml). Each of these samples also had its own sample control to compensate for interference of non-specific enzymatic activity.

7.2.5.2 Phosphotyrosine phosphatase-1B
Assay buffer for this experiment consisted of 10 mM p-nitrophenyl phosphate (PNPP) in 50 ml 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer (pH 7.0) with 1 mM dithiothreitol (DTT) and 2 mM ethylenediaminetetraacetic acid (EDTA), and was added at a final volume of 1 ml per sample. NaOH (0.1 N) was used as stop reagent for phosphotyrosine phosphatase-1B (PTP-1B) activity (Dixit *et al.*, 2007).

The assay buffer was pre-incubated for 10 minutes, which included 10 µl of sample, 100 µl of HEPES buffer, 100 µl of EDTA, 100 µl DTT, 500 µl of water and 100 µl of PNPP. After pre-incubation, 500 µl of NaOH were added to the sample controls and 20 µl of crude enzyme homogenate were added to the samples. This mixture was incubated at 37ºC for 30 minutes, after which 20 µl of enzyme homogenate was added to the sample controls and 500 µl of
NaOH added to the samples. The preparations were read at 410 nm (Dixit et al., 2007).

### 7.2.5.3 Glucose-6-phosphatase

Assay buffer consisted of sodium citrate buffer (0.3 M; pH 6.0), EDTA (28 mM) and sodium fluoride (NaF; 14 mM), made up with triple distilled water containing the appropriate amount of G6Pase crude enzyme homogenate. G6P (200 mM) was used as the substrate for G6Pase and trichloroacetic acid (TCA; 10% m/v) was used as the stop reagent for activity (Farhanullah et al., 2004).

In order to determine enzyme activity the inorganic phosphate (P_i) content of each sample had to be compared. The P_i-determination buffer was prepared with 30 g sodium metabisulfite in 195 ml triple distilled water (TDW) and 1 g sodium sulfite in 5 ml TDW, which was mixed. Five hundred milligram of 1-amino-2-naphthol-4-sulphonic acid (ANSA) was dissolved in 5 ml of this solution after which the rest of the solution was added and filtered. It was stored and protected from light.

For the experiment, assay buffer consisting of 100 µl citrate buffer, 100 µl EDTA, 100 µl NaF, 300 µl of triple distilled water and the appropriate amount of G6Pase crude enzyme homogenate, with and without 10 µl extract was mixed together. One millilitre TCA was added to the sample controls and 200 µl G6P (200 mM) to the samples. Sample controls and samples were incubated for 30 minutes at 37°C after which 200 µl G6P was added to the sample controls and 1 ml TCA to samples to stop the enzymatic reaction. The samples were left over night at 4°C, after which it was centrifuged at 3 000 rpm for 15 minutes in a microfuge.

The P_i content of the protein free supernatant was determined. After centrifugation, 50 µl of supernatant was mixed with 1 ml triple distilled water, 200 µl ammonium molybdate (2.5%, m/v), 100 µl sulfuric acid (H_2SO_4; 10 N) and 100 µl ANSA reagent. The mixture was vortexed, incubated at 37°C for 30 minutes and read at 625 nm.
7.2.5.4 Glycogen phosphorylase

The assay mixture consisted of 57 mg glycogen, 188 mg glucose-1-phosphate (G1P), 42 mg NaF, 4 mM 5' adenosine monophosphate (5'AMP) in 10 ml distilled water and 200 µl was used for each determination. TCA (10% m/v) was used as the stop reagent for enzymatic activity.

Assay mixture with and without 10 µl extract were prepared. The samples contained 100 µl of crude enzyme homogenate and the sample control 100 µl TCA, which was kept on ice. The assay mixtures were incubated for 30 minutes at 37°C and the reaction was stopped by adding 100 µl of 10% TCA to the sample and 100 µl crude enzyme homogenate to the sample control. Sodium acetate (100 mM) was added to all samples and sample controls at a volume of 400 µl to prevent spontaneous hydrolysis of G1P in the reaction mixture during the overnight incubation at 4°C. The samples were centrifuged at 3 000 rpm for 15 minutes. P_i was determined as for G6Pase (section 7.2.5.3).

7.2.5.5 Data manipulations

Each experiment had duplicate readings for samples and sample controls. Because of this no statistical tests were done on the data. Percentage enzyme inhibition was calculated as follows:

\[
\% \text{ inhibition} = 100 - \frac{\text{Sample reading}}{\text{Sample control reading}} \times 100\%
\]

7.2.6 Glycogen content determination

Glycogen content of fed and starved Chang liver cells was determined. Glycogen was first precipitated from cell lysates and hydrolysed to glucose, before the glucose concentration was determined with the use of glucose oxidase reagent.

7.2.6.1 Materials

Chang liver cells were maintained as in section 6.2.1 and the incubation medium was RPMI-1640 supplemented with 10% fbs, with or without
metformin (1 µM) or *T. camphoratus* aqueous extract (12.5 µg/ml). Cells were lysed using potassium hydroxide (30% m/v; KOH). Saturated disodium sulphate (Na₂SO₄) and 95% ethanol were used to precipitate the glycogen from the lysed cells. Hydrochloric acid (HCl; 1.2 M) was used to hydrolyse the glycogen pellet to glucose and sodium hydroxide pellets (0.5 M) to neutralise the HCl / glucose solution with the aid of phenol red indicator. A glucose standard curve was constructed (2 mM to 8 mM) to calculate the amount of glucose in the samples using glucose oxidase reagent.

### 7.2.6.2 Method

Chang liver cells were seeded in 10 cm culture dishes (NUNC) at a density of 300 000 cells/dish. The cells were left to grow for 72 hours before the cells were either (a) fed with 10 ml incubation medium with or without metformin (1 µM) or crude water extract of *T. camphoratus* (12.5 µg/ml), or (b) starved by adding 500 µl of incubation medium containing the same set of variables as for the fed cells. Cells were exposed to the various compounds in the different incubation mediums for a further 48 hours.

On the day of the assay, the cells were trypsinised, resuspended in growth medium in 15 ml tubes and counted using a haemocytometer. The cell suspensions were centrifuged (Eppendorf 5804R) at 1 500 rpm for three minutes and the growth medium aspirated. Cells were resuspended in 500 µl KOH to initiate cell lysis. The tubes were boiled in a water bath for 20 minutes to ensure that cell lysis was complete. After boiling, the tubes were transferred to an ice box for cooling. Next, 60 µl of Na₂SO₄ was added to each tube and vortexed to precipitate the glycogen contained inside the lysed cells. Ethanol (95%) was used as co-precipitant and was added to the tubes at a volume of 1.2 ml. The tubes were kept in the ice box for an additional 10 minutes to facilitate the precipitation process. The tubes were centrifuged (Eppendorf 5804R) at 1 900 x g for 25 minutes at 4°C. The supernatant fluid was aspirated and the precipitated glycogen suspended in 500 µl of water and transferred to a glass tube. One millilitre of HCl was added to each tube to hydrolyse the glycogen to glucose. Hydrolysis was facilitated by boiling the tubes for two hours in a water bath.
After boiling, the tubes were cooled and neutralised by the incremental addition of NaOH with phenol red as neutrality indicator. The neutralised samples were diluted with distilled water to ensure equal volumes and 50 µl of each tube was transferred to a 96-well plate. Glucose oxidase was added to give a final volume of 250 µl. The microplate was incubated at 37°C for 15 minutes and read at 492 nm in a multiwell plate reader (Multiscan MS® version 4.0 Labsystem® type 352).

7.2.6.3 Data manipulation and statistical analysis
Amount (mg) of glucose per replicate (10 cm culture plate) was calculated from the linear equation obtained from the glucose standard curve done during each experiment. In addition, the glucose mass was then divided by the number of cells (per 10 cm culture plate) and expressed as amount of glucose (mg; from glycogen) per million cells. Five replicates per sample were used during three individual experiments. Statistical analysis of results was performed utilising GraphPad Prism® 4. The two-tailed unpaired t-test was used for calculation of statistical significance with statistical parameters set at a 95% confidence interval. P-values of less than 0.05 were considered to be statistically significant.

7.2.7 Alpha-glucosidase assay

7.2.7.1 Materials
All reagents used for the assay were prepared in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (0.1 M; pH 6.5). The prepared buffer was stored at room temperature and protected from light. The substrate, ρ-nitrophenyl α-D-glucopyranoside, was made up with MES buffer at a concentration of 50 mM. It was aliquoted into microtubes and stored at -20°C until needed. On the day of the experiment it was thawed and diluted again with MES to 2.5 mM. The enzyme used was maltase from yeast (IUBMB Enzyme Nomenclature, 2006). A standard curve was done with different concentrations of the enzyme to determine the most accurate absorbance value read at 412 nm. A concentration of 10 µU/20 µl produced an
absorbance value closest to 1 and was chosen as the concentration for the experiment. The enzyme solution was also prepared with MES buffer. The crude \textit{T. camphoratus} aqueous extract was dissolved in MES buffer and tested at different concentrations.

7.2.7.2 Method
The experiment was performed in a 96-well microtiter plate (NUNC) with all tests repeated in triplicate as well as two extra wells without enzyme serving as blanks for the extracts to prevent the colour of the extracts interfering with absorbance values. Firstly, 20 µl of plain buffer or extract was added to the wells, then 20 µl of enzyme solution. The mixtures were left to equilibrate for 5 minutes before 160 µl of substrate was added to all the wells. The plate was incubated for 15 minutes at 37ºC, after which the enzyme reaction was terminated by the addition of 60 µl of glycine stop reagent. The microtiter plate was read on a multiwell plate reader at 412 nm (Multiscan MS\textsuperscript{®} version 4.0 Labsystem\textsuperscript{®} type 352).

7.2.7.3 Data manipulation and statistical analysis
Spectrophotometric readings were adjusted for the colour of the extracts interference and percentage enzyme inhibition was calculated as follows:

\[
\text{% enzyme inhibition} = \frac{(\text{Sample reading} - \text{sample blank reading}) \times 100}{\text{Average: control readings}}
\]

Readings were done in triplicate for each sample reading and in duplicate for each sample blank reading. Statistical analysis of results was performed utilising GraphPad Prism\textsuperscript{®} 4. The two-tailed unpaired t-test was used for calculation of statistical significance with statistical parameters set at a 95% confidence interval. P-values of less than 0.05 were considered to be statistically significant.
7.3 Results and discussion

All results, except those for section 7.3.1, have been summarised in tables (average ± SEM; n = n; p-value) in Appendix F.

7.3.1 Effect of extract storage on glucose uptake

The effect of storage on dried aqueous extracts was investigated as a function of glucose uptake. The dried extracts were stored in a moisture and light free environment at 4°C. As indicated by Figure 7.2, storage of the dried *Tarchonanthus camphoratus* aqueous extract decreased the glucose uptake ability at 0.5 µg/ml. However, the glucose uptake response for the 50 µg/ml concentration seemed erratic with an increase in storage period. This might be due to a corresponding increase in the concentration of degradation products in the higher concentration of stored extract, which produced a larger unpredictable response at this concentration.
**T. camphoratus** aqueous extract (Oct 04)

Figure 7.2 Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with *Tarchonanthus camphoratus* aqueous extracts prepared in October 2004, that were stored for different periods of time, thus showing storage stability of dried extracts (experiments done from June 2005 to January 2007). Bars represent one to two experiments with 10 replicates per sample per experiment. A solid gridline at 122.6% indicates the average response of metformin (1 µM). Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***).

Similar to the effect of *T. camphoratus* aqueous extract on Chang liver cells, the 0.5 µg/ml concentration also lost its activity in C1C12 muscle cells as the storage period progressed (Figure 7.3). Likewise, the 50 µg/ml concentration also lost its effect, but with more erratic fluctuations in glucose uptake over time than the lower concentration. This indicates selective degradation of active compounds that activate glucose uptake in both cell lines during storage of the dried extract.
T. camphoratus aqueous extract (Oct 04)

Figure 7.3 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated for one hour with Tarchonanthus camphoratus aqueous extracts (0.5 and 50 µg/ml, prepared in October 2004) that were stored for different periods of time to show storage stability of dried extracts (experiment performed from June 2005 to October 2006). Bars represent one experiment in a time period with 12 replicates per sample per experiment. A solid gridline at 120.7% indicates the average response of insulin (1 µM) obtained from the experiments. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 *, p < 0.005 ** and p < 0.0005 ***).

7.3.2 Effect of seasonal variation on glucose uptake

The findings relating to the storage stability of dried extracts should be kept in mind when interpreting the findings in the seasonal variation section. This is because some of the extracts have aged prior to the experiments. Three individual experiments (10 replicates per sample) were done for each extract on Chang liver cells, except for extracts made in July 2006 which were only tested twice. One experiment was done in March 2006 (Figure 7.4 and Table F. 1) and two experiments during January 2007 (Figure 7.5 and Table F. 2).

No significant seasonal differences in glucose uptake were observed for Tarchonanthus camphoratus aqueous extracts in Chang liver cells. Losses in glucose uptake activity for the 0.5 µg/ml concentration for the October 2004 and March 2006 infusion and decoction occurred during the January 2007 testing (Figure 7.5). This indicated that the extracts lost their activity during
storage (as was shown previously for the October 2004 extract in section 7.3.1). It also seemed that extracts made in March degraded more rapidly than those made in October. In addition, growth inhibitory responses were mostly not significant except for the ten month old March 2006 extract (50 µg/ml: 13.96 ± 2.70%; n = 12; p < 0.0001) and 26 month old October 2004 extract (50 µg/ml: 8.23 ± 2.65%; n = 12; p = 0.0115). This might indicate an increase in toxicity with a corresponding increase in storage period.

Figure 7.4 Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with Tarchonanthus camphoratus aqueous extracts that were prepared at different times of the year (October 2004, November 2005 and March 2006) thus showing seasonal variation and stability of dried extracts (experiment done in March 2006). Bars represent one experiment with 10 replicates per sample. A solid gridline at 121.1% indicates the response of metformin (1 µM). Statistical significance is indicated with asterisks above the corresponding bar (p < 0.05 *, p < 0.005 ** and p < 0.0005***). Key: (d) = decoction.
Figure 7.5 Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 μg/ml) and again for three hours (0.5 or 50 μg/ml) with *Tarchonanthus camphoratus* aqueous extracts that were prepared at different times of the year (October 2004, November 2005 and March 2006) thus showing seasonal variation and stability of dried extracts (experiment done in January 2007). Bars represent two independent experiments with 10 replicates per sample per experiment. A solid gridline at 126.6% indicates the average response of metformin (1 μM) for the two experiments. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 *, p < 0.005 ** and p < 0.0005***). Key: (d) = decoction.

Three individual glucose uptake experiments (12 replicates per sample) were performed on C2C12 muscle cells. Of these experiments, one was done in May 2006 (Figure 7.6) and another two in October 2006 (Figure 7.7). Glucose uptake for the November 2005 extract (Table F.5: 0.5 μg/ml: 123.1 ± 7.33%; n = 12; p = 0.017 and 50 μg/ml: 111.5 ± 9.24%; n = 12; p = 0.2592) correlated with the screening results of Chapter 6 (Table D.5: 0.5 μg/ml: 128.4 ± 3.27; n = 64; p < 0.0001 and 50 μg/ml: 117.4 ± 2.75%; n = 40; p < 0.0001). In contrast to the experiments on Chang liver cells, the time of collection does seem to play a role when looking at glucose uptake in C2C12 muscle cells. The glucose uptake ability of *T. camphoratus* aqueous extract was lost during March and July, but a decoction made during March retained its glucose uptake activity. The boiling process might have extracted a different range of compounds, thus the improved activity. These results further suggest that glucose uptake might be mediated by different compounds in the *T. camphoratus* aqueous extracts in liver and muscle cells.
Figure 7.6 Percentage glucose uptake (± SEM) by C2C12 muscle cells after one hour of exposure to test samples. Cells were treated with Tarchonanthus camphoratus aqueous extracts (0.5 or 50 µg/ml) that were prepared at different times of the year (October 2004, November 2005 and March 2006) to show seasonal variation and stability of dried extracts (experiment performed in May 2006). Bars represent one experiment with 12 replicates per sample. A solid gridline at 118.9% indicates the response of insulin (1 µM). Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 * and p < 0.005 **). Key: d = decoction.
Figure 7.7 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated for one hour with *Tarchonanthus camphoratus* aqueous extracts (0.5 or 50 µg/ml) that were prepared at different times of the year (October 2004, November 2005 and March 2006) thus showing seasonal variation and stability of dried extracts (experiment performed in October 2006). Bars represent two independent experiments with 12 replicates per sample per experiment. A solid gridline at 128.5% indicates the average response of insulin (1 µM) for the two experiments. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 *, p < 0.005 ** and p < 0.0005***). Key: (d) = decoction.

### 7.3.3 GLUT4 translocation

Altered glucose transport associated with defective GLUT4 translocation or the fusion of GLUT4-containing vesicles to the cell surface (Zorzano *et al.*, 1998) has been identified as major defects in T2DM (Anandharajan *et al.*, 2006). However, no mutations in the GLUT4 gene has been detected (Zorzano *et al.*, 1998). The effect of *Tarchonanthus camphoratus* aqueous extract on the amount of GLUT4 protein in the plasma membrane of C2C12 muscle cells may give insight into the mechanism through which the extract increased glucose uptake. Limited protein was available for the western blot and only duplicate values could be tested (Figure 7.8), thus no statistical analysis were performed on the data. However, the average blot intensities for the different samples did show observable differences when the X-ray film was analysed with Alphalmager™.

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Figure 7.8 GLUT4 in the plasma membrane fraction of C2C12 muscle cells after 15 minutes of exposure to insulin (1 µM) or *Tarchonanthus camphoratus* aqueous extract (50 µg/ml) at 37°C. The figure shows average blot intensity values (bars indicating the two duplicate values) calculated with Alphaimager™.

This experiment shows that the increase in glucose uptake found in C2C12 muscle cells caused by *T. camphoratus* aqueous extract may at least in part be due to an increase in GLUT4 translocation to the plasma membrane. Polyphenolic compound tannins have been shown to induce GLUT4 translocation through activation of an insulin-mediated signalling pathway in adipocytes (Jung *et al.*, 2006). Aqueous extracts of *T. camphoratus* have been shown to contain tannins (Scott & Springfield, 2005) and this effect could possibly be ascribed to their presence. Furthermore, insulin-mediated signalling which induce GLUT4 translocation may either be activated through the Rho-family GTPase TC10 pathway (Liu *et al.*, 2006; Watson & Pessin, 2006) and / or the IRS-1 / PI3-kinase pathway (Anandharajan *et al.*, 2006; Cheng *et al.*, 2006; Konrad *et al.*, 2001; Nishitani *et al.*, 2002; Somwar *et al.*, 2000). However, the TC10 pathway has been shown not to be functional in L6 myocytes, but functional in 3T3-L1 adipocytes (He *et al.*, 2007). If this applies to C2C12 muscle cells in particular, it probably is the PI3-kinase pathway that caused an increase in GLUT4 translocation. Insulin-mediated PI3-kinase activation of GLUT4 translocation would involve; increased production of phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P₃ or PI-3,4,5-P₃), which causes activation of phosphoinositide-dependent kinase-1
(PDK-1) and or PDK-2, which activates Akt / protein kinase B, (PKB) and atypical protein kinase C (aPKC) (Cheatham, 2000; Liu et al., 2006; Somwar et al., 2000; Tengholm & Meyer, 2002; Watson & Pessin, 2006) (Figure 7.9). No literature could, however, be found to verify the absence or presence of the TC10 pathway in C2C12 myocytes.

On the other hand, increased GLUT4 translocation may also have been induced by signalling pathways independent of insulin. Activation of AMP-kinase in skeletal muscle has been shown to increase the expression and translocation of GLUT4 to the plasma membrane (Hardie, 2003; Nakamaru et al., 2005). Other insulin-independent pathways that might have been activated by the extract include muscle contraction (activation of AMP-kinase) (Akerstrom et al., 2006; Cheng et al., 2006; MacLean et al., 2002), osmotic shock, and GTP-dependent pathways such as adrenergic receptor activation, which leads to an increase in GTP thus activating GLUT4 translocation (Cheatham, 2000).
7.3.4 Insulin-binding studies

The primary way in which insulin-mediated signalling is initiated, is by insulin receptor binding. Aqueous extracts may contain peptides or other compounds that could bind and activate the insulin receptor to increase glucose uptake. This was investigated in two individual experiments which had three to four replicates per sample. Data from one experiment is shown in Figure 7.10 (Table F.7) and indicate no statistically significant interference by *Tarchonanthus camphoratus* aqueous extract on insulin-binding to the insulin receptor on C2C12 muscle cells.

![Figure 7.10 Percentage [125I] insulin bound to insulin receptors on C2C12 muscle cells.](image)

The fact that the extract did not bind to the insulin receptor may indicate possible activation of insulin-independent pathways, activation of other membrane receptors causing GLUT4 translocation, or intracellular absorption of the extract, with subsequent activation of second messengers of either the insulin-mediated or insulin-independent signalling cascades. The absence of insulin receptor binding by the *T. camphoratus* aqueous extract might be welcome in the treatment of T2DM as defects in insulin receptor binding are not believed to play a significant role in T2DM (Rutter, 2000). Instead, defects
in insulin receptor signalling (Anandharajan et al., 2006) associated with reduced insulin receptor tyrosine kinase activity have been observed in T2DM (Panunti et al., 2004). Indeed, defects in IRS-1 tyrosine phosphorylation have been observed with subsequent decreased activation of PI3-kinase resulting in impairment of insulin-stimulated glucose disposal and glycogen synthase activity (Panunti et al., 2004). If the extract thus participates in tyrosine phosphorylation, it might be more useful by treating a pathological cause rather than a symptom.

7.3.5 Pathway inhibitors in C2C12 muscle cells

Inhibitors for PI3-kinase, MAP-kinase and AMP-kinase would provide valuable information regarding the glucose uptake pathways affected by the Tarchonanthus camphoratus aqueous extract. Two individual experiments that produced the same trends in $[^3H]$ deoxyglucose uptake were done with wortmannin as PI3-kinase inhibitor with four replicates per sample. The results of one of the experiments are illustrated in Figure 7.11 and Table F.8. These preliminary results showed that wortmannin decreased $T$. camphoratus induced glucose uptake significantly (50 µg/ml: $p = 0.0035$) in C2C12 muscle cells as compared to the extract without inhibitor. This indicated that an insulin-mediated pathway, namely PI3-kinase, was involved in the glucose uptake signalling of $T$. camphoratus aqueous extract. In addition, literature supports the fact that polyphenolic compound tannins induce GLUT4 translocation through activation of an insulin-mediated signalling pathway (Jung et al., 2006), in this case the signalling pathway has been shown to be the PI3-kinase pathway.

The extract may, however, work at any point upstream of PI3-kinase that causes its activation. PI3-kinase activation may be initiated upstream by insulin or other growth factors (Evans et al., 1995). We have eliminated the possibility of the aqueous extract activating PI3-kinase by binding to the insulin receptor (section 7.3.4). This still leaves possible binding to other membrane receptors or intracellular absorption and changing regulators of cell metabolism which may cause the activation of tyrosine kinases that phosphorylate either IRS-2 or PI3-kinase (Hill et al., 2004). Alternatively, the
extract may inhibit dephosphorylation of tyrosine residues on activated substrates (Wang et al., 2001).

Figure 7.11 Percentage [%] deoxyglucose uptake (±SEM) into C2C12 muscle cells. Cells were pre-incubated with and without wortmannin (100 nM) for 30 minutes at 37ºC. Then cells were either left untreated or were exposed to 1 µM insulin or Tarchonanthus camphoratus (Tc) aqueous extract (0.5 and 50 µg/ml) with and without wortmannin (100 nM) for 20 minutes at 37ºC. Averages and statistical evaluations were calculated from data of one individual experiment with four replicates per sample. P-values were calculated with the two-tailed unpaired t-test and considered significant if p < 0.05. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 *, p < 0.005 ** and p < 0.0005***). This experiment was repeated and similar results were obtained.

Preliminary results of four experiments done with the MAP-kinase inhibitor, PD 169316, and using insulin as the positive control produced no significant changes in glucose uptake between cells treated with and without inhibitor. In contrast, some literature indicate that MAP-kinase inhibitors inhibited insulin-stimulated glucose uptake in skeletal muscle (Lynge et al., 2003; Somwar et al., 2000; Somwar et al., 2001), and SB203580 in 3T3-L1 adipocytes and L6 muscle cells (Sweeney et al., 1999) without altering GLUT4 translocation (Geiger et al., 2005; Konrad et al., 2001). It has thus been suggested that MAP-kinase causes full activation of GLUT4 without affecting GLUT4 translocation (Cheng et al., 2006; Somwar et al., 2000; Somwar et al., 2002).
Despite the fact that, p38 MAP-kinase is present in undifferentiated, confluent C2C12 muscle cells (Yeow et al., 2002), the lack of response in our experiments may indicate that it has not been activated adequately. The expression of MAP-kinase may be induced by (among other signals) differentiation of C2C12 muscle cells (Keren et al., 2006). The starvation process used in the experiments might have induced differentiation, because serum levels in the growth medium would have become depleted (Cabane et al., 2004). Generally, differentiation of C2C12 cells has been characterised by the fusion of mononucleated myoblasts which align, elongate and fuse to form large multinucleated myotubes (Cabane et al., 2004). In our experiments, alignment and elongation of the cells were evident, but fusion may not have been complete. It may thus have been possible that MAP-kinase was not fully activated, because differentiation in C2C12 muscle cells was not complete. This may be the reason why no response was observed in these experiments.

Four individual experiments were done with ara as AMP-kinase pathway inhibitor. The results of only one experiment with four replicates per sample are shown in Figure 7.12 and Table F.9, because solubility problems regarding the inhibitor during the experiments prevented the response to the positive control from being properly inhibited in some cases. These results indicate that the glucose uptake activity induced by the aqueous extract of T. camphoratus is significantly inhibited (50 µg/ml: p = 0.0080; 0.5 µg/ml: p = 0.0352) in the presence of ara as compared to glucose uptake without the inhibitor. The extract may thus activate AMP-kinase as part of its glucose uptake activity in an ara-dependent way (section 7.2.4.1).

AMP-kinase may be activated in an acute fashion by AMP and phosphorylation of the catalytic α-subunit on 172-threonine residue by upstream kinases (Figure 7.13) (Hardie, 2003), such as LKB1 (Imai et al., 2006) and AMP-kinase kinase (Fryer et al., 2002b) 1 and 2 (Hawley et al., 2002). This might be one of the mechanisms involved, because exposure was for just 30 minutes.
Figure 7.12 Percentage [3H] deoxyglucose uptake (± SEM) into C2C12 muscle cells. Cells were pre-incubated with and without adenine 9-β-D-arabinofuranoside (ara; 1 mM) for 30 minutes at 37°C. Cells were then either left untreated or were exposed to dinitrophenol (DNP; 1 µM) or *Tarchonanthus camphoratus* (Tc) aqueous extract (0.5 and 50 µg/ml) with and without ara (1 mM) for 30 minutes at 37°C. Averages and statistical evaluations were calculated from data of one individual experiment with four replicates per sample. P-values were calculated with the two-tailed unpaired t-test and considered significant if p < 0.05. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 *, p < 0.005 ** and p < 0.0005***).

Alternatively, the extract may also have activated receptors that activate AMP-kinase such as the β2-adrenergic, G_q-coupled (Fryer *et al.*, 2002b) and adenosine A2 receptors, of which the first and last cause an increase in cAMP after stimulation (Campbell, 2006; Lynge *et al.*, 2003). Interestingly, receptors that activate G_q coupled proteins cause activation of phospholipase C and increase inositol 1,4,5-triphosphate and diacylglycerol inside the cell, leading to increased levels of PKC and cytoplasmic Ca^{2+} respectively. Furthermore, AMP-kinase is stimulated in skeletal muscle by leptin (Uotani *et al.*, 2006) and exercise and in liver by leptin and adiponectin (Sakakibara *et al.*, 2006) and generally catecholamines (Kelly *et al.*, 2004; Ye *et al.*, 2005). It is not stimulated by Ca^{2+} / calmodulin (Carling, 2005; Kim *et al.*, 2006). Glucose transport activated by AMP-kinase may have an additive effect with insulin because of the possible difference in cellular mechanism and / or differences in the cellular pools of GLUT4 being translocated (Abbud *et al.*, 2000).
might be an explanation for a significant additive effect that was observed when *T. camphoratus* aqueous extract (50 µg/ml) was combined with insulin in section 6.4.3 as compared to the response of the extract alone. In addition to glucose uptake AMP-kinase also activates fatty acid β-oxidation and glycolysis (Kurth-Kraczek *et al.*, 1999; Lee *et al.*, 2006; Pelletier *et al.*, 2005) and inhibits fatty acid, triglyceride and cholesterol synthesis (Polekhina *et al.*, 2005; Uotani *et al.*, 2006).

![Diagram of AMP-kinase activation and physiological effects](image)

**Figure 7.13** The activation and physiological effects of AMP-kinase (Carling, 2005).

### 7.3.6 Enzymatic assays with rat liver homogenates

Phosphotyrosine phosphatase-1B, glucose-6-phosphatase and glycogen phosphorylase are all linked to T2DM in their own specific ways. *Tarchonanthus camphoratus* aqueous extract did not show any inhibition of either of these hepatic enzymes. Experiments were done only once (with duplicate readings per sample), because no inhibition was found. Findings are summarised in Table 7.2.

**Table 7.2** Percentage enzyme inhibition produced in 30 minutes at 37°C by 10 µg/ml of aqueous extract of *Tarchonanthus camphoratus*. Percentages represent the mean of duplicate readings per sample of one experiment.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% inhibition by <em>T. camphoratus</em> aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphotyrosine phosphatase-1B</td>
<td>-2.40</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>-6.71</td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>-6.10</td>
</tr>
</tbody>
</table>
Protein tyrosine phosphatases (PTPs) dephosphorylate several phosphotyrosine residues (Gogg et al., 2001) including those on the insulin receptor and insulin receptor substrate. PTPs have been shown to be up-regulated in insulin resistance (Klover & Mooney, 2004) and are likely to down-regulate various growth factor and insulin functions (Umezawa et al., 2003). No direct inhibition of PTP-1B was found for the *T. camphoratus* aqueous extract. However, PI3-kinase-dependent glucose uptake activity found in C2C12 muscle cells in previous experiments suggests a phosphorylation effect rather than inhibition of dephosphorylation.

T2DM shows an increase in gluconeogenesis, which may be due to increased activity of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Panunti et al., 2004). No direct inhibition of G6Pase in the crude homogenate was found. However, PI3-kinase activation (which was found in C2C12 muscle cells) has been shown to inhibit the transcription factor, FOXO1, which is responsible for gluconeogenic enzyme gene expression (Klover & Mooney, 2004). It is therefore possible that the extract could decrease the amount of enzyme present in hepatocytes and thus reduce hepatic glucose output.

Finally, glycogen phosphorylase was not inhibited by the aqueous extract of *T. camphoratus*. This indicates that glycogen breakdown should remain normal in the presence of the aqueous extract of *T. camphoratus* and that the extract did not cause a net increase in glucose uptake into Chang liver cells through inhibition of glycogenolysis.

### 7.3.7 Glycogen assay
Glycogen content of Chang liver cells was determined in three individual experiments with four to five replicates for each sample (Figure 7.14). As expected, there were significant differences between the glycogen content of starved and fed cells for all the samples tested. The glycogen content per million Chang liver cells was not significantly different between untreated cells or cells treated with metformin or *Tarchonanthus camphoratus* aqueous extract in either the starved or fed cells.
Failure to increase glycogen synthesis and content in Chang liver cells may pose questions regarding the ability of *T. camphoratus* to activate PI3-kinase in these cells, because PI3-kinase activation was observed in C2C12 muscle cells. PI3-kinase should theoretically increase glycogen content because it inhibits glycogen synthase kinase-3 (GSK-3), which leaves glycogen synthase to work unopposed (Klover & Mooney, 2004; Liu *et al.*, 2006; Ueki *et al.*, 1998).

On a similar trend to *T. camphoratus*, 5’-amino-4-imidazolecarboxamide-riboside (AICAR), an AMP-kinase activator, did not affect glycogen levels in skeletal muscle and there was also no evidence of glycogen phosphorylase activation (Kurth-Kraczek *et al.*, 1999). AMP-kinase activation would inhibit glycogen synthesis through the inhibition of glycogen synthase (Carling, 2005; Hardie, 2003). The absence of any effect on glycogen content in liver cells by the aqueous extract of *T. camphoratus* might thus be explained by possible contradicting effects that the simultaneous activation of AMP-kinase and PI3-kinase may have on glycogen metabolism as shown in C2C12 muscle cells.

Alternatively, *T. camphoratus* may alter the activity of glucokinase (Ferrer *et al.*, 2003) or indirectly alter the activity of G6Pase via AMP-kinase activation, which would lead to increased G6P steered into the glycolysis pathway.
instead of glycogen synthesis. Still, the mechanism remains uncertain, because mechanisms activated for glucose uptake might differ between liver and muscle cell lines. A hint of this has been observed with the stability and seasonal variation testing, which suggested different compounds that might influence glucose uptake between the cell lines.

7.3.8 Alpha-glucosidase activity

Alpha-glucosidase inhibitors are used as adjunctive therapy in diabetes, because they prevent postprandial peaks in blood glucose. The aqueous extract of *Tarchonanthus camphoratus* showed weak α-glucosidase inhibitory activity (Figure 7.15 and Table F.10) in two (50 and 100 µg/ml) or three (200, 500 and 1000 µg/ml) independent experiments with triplicate values for each sample.

![Figure 7.15 Percentage α-glucosidase enzyme activity (±SEM) exposed to different concentrations of *Tarchonanthus camphoratus* aqueous extracts for 15 minutes at 37°C. Bars represent a minimum of two independent experiments with three replicates per sample. Statistical significance is indicated with asterisks above the corresponding bars (p < 0.05 * and p < 0.0005***)](image_url)

The concentrations used in the glucose uptake screening assays (50 µg/ml) did not show any significant inhibitory activity (Figure 7.15), but, enzyme activity did decrease significantly when the concentration of the extract was increased to ≥ 200 µg/ml (55.97 ± 2.39%; n = 9; p < 0.0001). The plateau in activity at the higher concentrations might indicate some non-specific effects if
the concentration is not well titrated. The much smaller concentration needed to elicit a response in the other assays might also be a realistic value which reaches the circulation after intestinal absorption of the extract.

7.4 Discussion

Possible active compounds that might be responsible for the anti-diabetic activity in the *T. camphoratus* aqueous extract include saponins and tannins. It has been shown in adipocytes, that polyphenolic compound tannins induced GLUT4 translocation through activation of an insulin-mediated signalling pathway (Jung *et al.*, 2006). In the case of *T. camphoratus* aqueous extract, GLUT4 translocation and glucose uptake into C2C12 muscle cells were increased through activation of PI3-kinase (insulin-dependent) and AMP-kinase (insulin-independent). Activation of PI3-kinase may also prevent the development of diabetes, because decreased ability to phosphorylate PI3-kinase has been linked to insulin resistance (Evans *et al.*, 1995). AMP-kinase activation may have additional favourable effects on the lipid profile as it decreases cholesterol and fatty acid synthesis (Abbud *et al.*, 2000; Cheng & Fantus, 2005; Lee *et al.*, 2006; Nakamaru *et al.*, 2005; Saha *et al.*, 2004; Sakakibara *et al.*, 2006).

A limitation of this study may be that AMP-kinase has been shown to increase glucose uptake in tissue expressing only GLUT1 (Abbud *et al.*, 2000). The presence of GLUT1 has been shown on muscle and fat tissue, and it is well-known that the transformation of cell culture lines produced a pronounced increase in GLUT1 protein and mRNA levels (Gould & Holman, 1993). GLUT1 may also be stimulated by mitogens as well as starvation (Gould & Holman, 1993). It is thus possible that some of the glucose uptake activity observed in the experiments was due to GLUT1.
8 Collaborative research: insights and comments

“…all foreigners try to evaluate what they learn about Africans in terms of their own preconceived ideas and against their own standards of civilisation and social and political thought. The African can only be studied against the strange workings of his own mind and those who do not appreciate this may as well refrain from trying to study the African.” (Mutwa, 1965)

8.1 Introduction

The following chapter will describe and discuss incidents observed and participated in during the collaborative process. One of the most important reminders involved in successful collaborative partnerships, is that one party can not possess unchallenged power (El Ansari et al., 2002). The current collaboration thus utilised various discussion and communication tools to ensure that decisions were made according to a compromise between the wishes of both collaborating parties (section 2.3.1). This was done, because the key to the distribution of power within a collaboration, lies in the decision-making processes (Warren, 1997). Negotiations were also important to aid the scientist's understanding of how indigenous knowledge (IK) functions as a basis for the indigenous community's decisions (Chikonzo, 2006). In addition, the understanding of IK was enhanced by experiencing and learning about the indigenous people’s culture, as indigenous knowledge is created by the individuality and culture of a community. The incidents described in this chapter will thus explore: the power dynamics within the collaboration, which was linked to the stakeholders' perceptions of knowledge; the perceived benefits arising from collaboration; perceptions of trust between the stakeholders; and, finally, how the experiencing of culture influenced the collaboration.
8.2 Power

Power was a dynamic entity in the collaborative research process and continuously shifted between researchers and practitioners. The researchers’ power was comprised of their scientific education, funds that would drive the project and technical know-how to execute the research. However, legislation and funding (Singer, 1990) issued by government had a tremendous impact on the way the researchers could exercise their power. The third power player in this study was thus the South African government. Two relevant laws were passed throughout the duration of this study, namely the Traditional Health Practitioners’ Act (South Africa, 2004b) and the Biodiversity Act (South Africa, 2004a). The passing of these laws necessitated the studying of the legislation, alignment of the research partnership with the recommended guidelines and the introduction of the legislative process and content of the laws to participating practitioners. In contrast to this scientific perception, practitioners found it difficult to relate to and understand the concept of legislation:

“She (a participating practitioner) has heard rumours about the Bill, but she don’t understand what is it, this Bill.”

“Because traditional healers are not used to that (legislation regarding themselves), it is new to us.”  

The group discussion introducing the Traditional Health Practitioners’ Bill / Act to practitioners, explained that it would regulate the practice and training of traditional African health practitioners in South Africa. Furthermore, regulations pertaining to the Act would be issued by the minister of Health after consultation with the Interim Traditional Health Practitioners’ Council. Practitioners’ responses regarding the Council were interesting:

“… What is going to be their (the Council members’) role, they (traditional practitioners) don’t want (Council members to be) puppets of the government.”

“… If the people of the Council will be traditional healers or people from the Minister’s (of Health) side.”  

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After clarifying that the bulk of Council would consist of traditional health practitioners, participating practitioners were still weary of the idea of this legislation. They had concerns about interference into their training practices and especially about inspections of their surgeries. They said that their surgeries were not for public display and if it were to be inspected, permission needed to be obtained from the ancestors before anyone would be allowed to enter. Practitioners were thus trying to integrate this legislation into the hierarchy of power of the traditional health care system (section 2.2). This power hierarchy was observed first hand during the collaboration period, when traditional health practitioners consulted their ancestors for permission to give their remedies to be tested (section 2.3.2). The practitioners concluded that the ancestors would have the final word, even in the case of legislation.

“Everything I do, I am controlled by my ancestors.” 15

Practitioners explained that generally, the ancestors would not be against something that is good for the traditional health care system and that the law would assist in the weeding out of charlatans among them. However, they had a problem with the exclusion of real healers just because their ancestors may not allow them to join the Council.

“There is nothing we can do. His ancestors don’t want him to (register with the Council), so he can not get arrested. I understand the Bill, it is protecting us but if his ancestors do not want him to, they don’t want him to. He is not sent by the Bill, he is sent by the ancestors, he must practice. There is no law that can forbid healing. Even (Thabo) Mbeki can not forbid it. They (the healers) can not be forced (to register).”

This difference in opinion among the ancestors has been observed previously when some practitioners’ ancestors were against the remedy exchange and testing (section 2.3.2). From these illustrations, one has to recognise the intangible power that the ancestors may have on this collaboration and that these types of problems are better left to be solved without outside interference. Scientists should rather keep their energy focussed on assisting practitioners to become aware and physically understand legislative and scientific concepts pertaining to collaborative research. However, in getting back to the more tangible power attributes of the collaboration, the power of
participating practitioners lay in their indigenous knowledge about medicinal plants, the preparation of traditional remedies and their inexplicable abilities to provide the research with an ever-present evaluative consciousness to truly promote the traditional health care system. In accordance with this process, the protection of the practitioners’ indigenous knowledge was a big priority.

8.2.1 Indigenous knowledge

The fact that knowledge was the power that lay in the hands of the participating practitioners within the partnership boundaries was not a new concept for practitioners. The distribution of knowledge in the traditional hierarchy has always been this way:

“Knowledge is controlled among the Bantu by the orders of the Chosen Ones. Only certain knowledge is passed on to the particular High Ones of the Tribes, such as they are required to know to execute their duties. Very little knowledge is passed on to the common people and nothing is ever disclosed to strangers” (Mutwa, 1965).

In addition, the nature of IK is spiritual and personal (Chikonzo, 2006) and this makes it most precious to the holder (Chavunduka, 1994) (refer to section 2.2.2).

“… indigenous knowledge systems can be described as ‘disciplines’ which embody ethical standards, standards of responsibility, standards of transmission and a system of rules and practices. They include different practices of earning and sacrificing to gain knowledge. You have to do different kinds of work to gain authorisation.” (Odora Hoppers, 2004).

In contrast to the acquisition and ownership of IK, the products of IK are shared freely with all who are in need of it, but the details are concealed in the mind of the practitioner. The benefit of which (be it monetary or other) is meant for that specific healer’s family. Similarly, knowledge of western medicine is protected through methods of registration and patenting (Teshome-Bahiru, 2002) and is for the benefit of the western practitioners owning the patent.

The following paragraph will discuss incidents relating to knowledge that one might perceive as different between the traditional and conventional systems. The first incident is related to the attainment process of indigenous knowledge
(section 2.2.2). This process provides an individual with a mostly unique collection of knowledge, although certain similarities may exist due to the training process and passing on of collective knowledge. Practitioners are thus aware that if they had a problem in treating a certain disease, he or she could consult another practitioner who has the knowledge to solve that problem\textsuperscript{16}, i.e. referral. Practitioners further elaborate that generally if a certain healer claims to heal anything and everything; it is possible that that practitioner is a fraud. Furthermore, traditional health practitioners are restricted to use only the knowledge gained personally from their ancestors. This concept may be illustrated by the following two examples. While working in a private garden, I asked a participating practitioner if we should consider planting \textit{Catharanthus roseus} in the medicinal garden, because it is such a well-known medicinal plant (World Health Organisation, 2004). The practitioner replied that she did not use it herself, because her ancestors did not tell her about it. However, she heard that another healer used it for diabetes. We asked her if she would try it. She replied that she was not going to use it, because she did not know how: “she never learnt it”\textsuperscript{10}. On another occasion, a different practitioner explained that she needed permission from her own ancestors if she wanted to use a plant recommended by another practitioner\textsuperscript{17}. This can probably be translated into conventional terms as quality control by the ancestors to keep practitioners to use plants that they are familiar with and know how to use. In accordance, the stealing of medicine from a practitioner is punished severely by the ancestors:

“He will not sleep”

Practitioners explained that the ancestors will not leave a culprit alone until he / she has given back the medicine that has been stolen. The medicine will also not be effective if it has been stolen, so there is no incentive to steal medicines in the traditional milieu. In addition to the knowledge gained in the training process, the attainment of indigenous knowledge continues throughout the healer’s journey with their ancestors. Drug discovery in the traditional system has been described on a few occasions by different practitioners. They explained that when they discover a new medicine
(usually shown to them by their ancestors in a dream or vision), they would collect it from its source, prepare it according to the instructions of their ancestors and test it on themselves, before giving it to a patient. Similar processes have been reported in literature (Abebe, 1984; Hillenbrand, 2006). Given the limited resources that traditional practitioners have to their disposal, this is probably the best way to ensure that a new medicine is safe. An interesting observation would be to see how traditional practitioners incorporate scientific knowledge gained from the collaboration into their practices. The Xhosa people have been known to show a willingness to incorporate new therapeutic technologies into their existing beliefs (Gordon, 2001) (also refer to section 5.4.1). Literature also supports the concept of IK development in a broader sense:

“each new generation adapt and add to this IK in a constant adjustment of changing circumstances and environmental conditions” (Odora Hoppers, 2004).

It is tempting to speculate on the assumption that if traditional health practitioners increase their scientific background, more diseases will start to fall under the heading of “natural causes” and be treated by traditional or conventional medicine (section 2.2.3). Be that as it may, the interpretation of supernatural causes deserves closer analysis too. The traditional system’s assumption that witchcraft is often the cause of a disease, receives much criticism from the conventional community, because it serves to shift the responsibility from the patient to another person. Indeed, jealousy is a word much heard in the traditional community. But, do genetic and environmental factors in the conventional sense not serve the same purpose as witchcraft in the traditional sense? Both shift the responsibility from the patient to something beyond his / her control. Similarly, the breaking of a taboo, has been equated to guilt in the modern sense (Okpako, 1999). The psychological (mind / body) connection between health and illness is being continuously proven by science on every level. It might be beneficial to reserve judgement on these issues until science have less paradoxical evidence to prove its point.
Bearing the previous comparisons in mind, the summoning of empathy for the traditional practitioners’ perspective might become easier. Literature agrees that traditional or indigenous knowledge (IK) is difficult to transmit to people who do not share the language, culture or traditions (Chisenga, 1991). In addition, scientists need to accept that the perceived benefits, logic and common sense connected to the modern scientific paradigm (Chikonzo, 2006) may not be so forthcoming to others. It is thus best to be prepared for some form of questioning and maybe even disregard for the value of science during the course of collaborative research. Still, scientists should rather remind themselves about the benefits that they receive from research collaborations, especially when the collaboration makes the research recent and relevant. This gives scientists the added opportunity to explore outside the limitations of the scientific box, as:

“… science is not a description of the natural world, it describes a paradigmatic world” (Swazo, 2005).

It is thus possible that the indigenous knowledge learnt through collaborative research might not make sense from the scientific point of view. However, the scientists’ ability to accept and incorporate the diversity of the paradigms into the research collaboration ensures that the participating practitioners keep interest in the research and gives the assurance to practitioners that the research is relevant to them (El Ansari, 2005). The win/win situation connected to the collaboration at the moment is that researchers have many medicinal plants to test for years to come and participating practitioners are free to keep their sacred indigenous knowledge a secret.

8.2.2 An indigenous knowledge agreement

In anticipation to the implementation of the Biodiversity Act, the negotiation of an indigenous knowledge agreement (IKA) (South Africa, 2004a), with knowledge of draft regulations was initiated. These negotiations were easy considering that the collaboration had been set-up to include and accommodate the opinions of participating practitioners in all research decisions (section 2.4.3).
The agreement aimed to define the roles and responsibilities of each stakeholder in all the activities of the research collaboration (see Appendix G for the last negotiated version). Firstly, the collaborative activities were defined and included meetings, interactive workshops, individual interviews, medicinal garden activities, medicinal plant research, research outputs and provisions in the case of commercialisation. Secondly, the roles, rights and responsibilities for each stakeholder were defined according to each collaborative activity. This was done during a number of interactive workshops dedicated to explaining and exploring relevant concepts contained in the IKA.

The concept of prior informed consent \(^{18}\) (Soejarto et al., 2005) and ethical research conduct during meetings, interactive workshops and individual interviews were explained during a workshop dedicated to these concepts (also refer to information and informed consent form: Appendix G.1). The joint development activities of the medicinal garden were stipulated. According to the activities, participating practitioners were only responsible to provide the collaboration with medicinal plants that they used frequently and the Xhosa names of these plants. It was also agreed that researchers could use medicinal plants from the garden to test for biological activity, providing they give feedback to the healers on the findings (also see vision for garden in section 2.4.2).

The procedures followed in medicinal plant research had to be thoroughly explained, because it would give practitioners an idea of where the current research fitted into the grand scheme of western drug discovery. A workshop was thus dedicated to the processes of drug discovery and registration\(^{16}\). It was important to explain the western drug discovery process in detail and how far off commercialisation (if any) was from where our research was at that stage (Elisabetsky, 1991). The actual medicinal plant testing was explained to the healers as best as possible, without having to show them the exact process in the laboratory (section 3.7.2). Simulations of how experiments were done in the laboratory and laboratory tours were given during feedback seminars to try and make the research as transparent as possible.
Research outputs have always been part of collaborative negotiations ever since the first verbal agreement (section 1.5). It was explained to the practitioners that funding for the research collaboration depended upon the production of research outputs. Publication of research results was thus included as one of the requirements for researchers’ participation in the collaboration. In time, as the collaboration and community knowledge of the collaboration had spread participating practitioners had given permission for their names and organizations to be acknowledged in research outputs (section 2.4.3). The procedure of producing research outputs was as follows:

- Participating practitioners were informed of the contents of presentations, posters or publications before it was presented or published\(^\text{19}\), in order for them to give their input if so required.
- The three organizations participating in the research collaboration would be acknowledged in research outputs for their participation in the research collaboration, according to the agreed format in Appendix G.3.
- Feedback on the comments and questions about presentations and posters were provided to practitioners at feedback seminars. Alternatively, copies of publications were given to practitioners at these seminars.

In spite of the fact that practitioners initially agreed to the dissemination of research findings, it was debated frequently by practitioners joining for the first time (section 2.4). During an interactive workshop dedicated to explaining research outputs and medicinal plant research\(^\text{19}\), the contents of a poster for an international conference was presented to two of the participating organizations of practitioners. The poster included information on the dynamic interactive research between healers and researchers and a brief summary of the screening results of Chapters 4, 5 and 6. Most of the practitioners were happy with the presentations and made some suggestions to improve it. However, a practitioner attending for the first time suggested that we keep the results a secret and only report on the collaboration between healers and the university. He was concerned that other people would steal the information at the congress.
Concerns regarding intellectual property and patenting (Raza, 2006) as well as the fear that other researchers may condemn and steal practitioners’ secrets are real (Hillenbrand, 2006). Our side of the argument for these concerns was that if we did not publish the information with acknowledgement of the participating practitioners’ associations for their input, another research group may publish similar data probably without acknowledgement or acknowledging other indigenous knowledge providers. Herb-sellers have often put practitioners in a precarious position, because they sell information on traditional plants (section 2.4.1). In this way, western medicine obtains indigenous knowledge and no recognition is given to traditional health practitioners (Teshome-Bahiru, 2002). Through publication of the results of collaborative research, participating practitioners are given protection by providing them with an indigenous knowledge claim if any patent should arise from the information (Rao, 2006) and recognition for their part (however small) in the research (Sen, 2005). The two initial practitioners’ organizations had no problem with publication of the experimental results, because it meant scientific evidence for the use of their traditionally used plants, the primary reason for them initiating the collaboration (section 1.5). In this case we could not accommodate the third group in their request to keep the results a secret. Fittingly, literature on collaborative projects reassures that different interest groups engaged in a project are often involved for different reasons and do not necessarily share common goals and objectives (Botes & van Rensburg, 2000).

The development of an indigenous knowledge agreement was probably advantageous for participating practitioners, because they seemed to be under constant scrutiny by their non-participating peers. According to a participating practitioner other practitioners asked her:

“How can you work with those people?”

The practitioner explained that the non-participating practitioners were under the impression that the healers participating in the partnership were telling us their “ancestors’ secrets”. With the indigenous knowledge agreement, the participating practitioners could prove to opposed practitioners that no unethical traditional practices were being followed. However, it is not clear at
this stage what the impact of an indigenous knowledge agreement would have on knowledge exchange or the convincing of opposed practitioners to join the collaboration.

8.2.3 Mutual benefits

The primary benefit that practitioners requested at the initiation of the research collaboration, was scientific proof for the efficacy of their traditional remedies (section 1.5). This was thus the initial objective of the research collaboration. In addition, the NRF IKS focus area, which funded the research, advocates empowerment as the primary interest that IKS practitioners have as stakeholders in collaborative programs. The NRF further stipulates that empowerment incorporates the protection, promotion and validation of IKSs, with a partnership in knowledge creation and equity. Participating practitioners should further establish forums to debate all these issues (Hechter, 1991), which is encouraged in this collaboration. However, it also became apparent in this collaboration that traditional health practitioners were in need of resources and money. IK has been said to be the social capital of the poor (Hillenbrand, 2006; Rao, 2006) and these practitioners were no exception. Indeed, a major reason why indigenous people may not be willing to share IK is, because IK is a livelihood and if it was exploited they could loose the benefits thereof (Chisenga, 1991). All the previous literature citings were true for practitioners participating in this collaboration24.

"(Healer x) said that it was part of the business also, they (western practitioners) don’t tell and why should we (traditional practitioners)? It would be normal that nobody said what he or she uses."

The guarding of indigenous knowledge for business reasons have been reported earlier (Teshome-Bahiru, 2002). There is thus a need to link innovation, enterprise and investment when working with local communities (Marshalkar, 2003), because, generally, the only monetary benefits participants may expect from a research collaboration is long-term and requires a waiting period of 8 to 10 years, if commercialisation is ever achieved (King et al., 1996). To sustain the research partnership, it is recommended that incentives are generated which aim to provide immediate,
short and medium-term benefits for research participants (King et al., 1996). Benefits that have been stipulated by the regulations (Department of Environmental Affairs and Tourism, 2007) of the Biodiversity Act are listed in Table 8.1.

**Table 8.1 Benefits as listed in the regulations of the Biodiversity Act (Department of Environmental Affairs and Tourism, 2007). Benefits that participating practitioners may expect from the collaboration are ticked.**

<table>
<thead>
<tr>
<th>To be completed if stakeholder is an indigenous community</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ongoing communication of research objectives, methods and findings, translated into local languages.</td>
<td>√</td>
</tr>
<tr>
<td>Simplified and popularised posters, manuals, pamphlets and other documents translated into local languages.</td>
<td>√</td>
</tr>
<tr>
<td>Co-authorship of publications</td>
<td></td>
</tr>
<tr>
<td>Access to research data</td>
<td>√</td>
</tr>
<tr>
<td>Copies of photographs and slides</td>
<td>√</td>
</tr>
<tr>
<td>Inclusion in the research of local collaborators, assistants, guides and informants</td>
<td></td>
</tr>
<tr>
<td>Training of local people as appropriate in relevant scientific, legal and management issues</td>
<td>√</td>
</tr>
<tr>
<td>Equipment and infrastructure support</td>
<td>√</td>
</tr>
<tr>
<td>Co-ownership of any intellectual property rights</td>
<td></td>
</tr>
<tr>
<td>Other (specify) Financing of traditional ceremonies held for the research collaboration</td>
<td>√</td>
</tr>
<tr>
<td>Other (specify)</td>
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In the case of our research partnership, the benefiting indigenous community at the moment includes three traditional practitioner organizations. Because of the differences between the organizations regarding their participatory collaborative activities, the benefits offered by the collaboration differed accordingly. All participating organizations were always informed of collaborative research activities. At these activities, attending practitioners were informed of research progress, relevant scientific literature, legal developments or management issues. Attending practitioners were also provided with translated notes of the proceedings and they could enquire for more details on the research, copies of presentation slides etc. Practitioners that attended research activities were also reimbursed for their transport
expenses and given a light meal at such events. All that were required from practitioners was the time to attend the interactive sessions.

In our partnership, money was not available to provide healers with compensation for the time away from their surgeries during workshops and the principle was voluntary participation. However, transport money was an essential part of the participation process, because many of the participating practitioners would not have been able to voluntarily participate, if not for the transport reimbursement (section 2.3.2).

“(Certain practitioners) could not come (to the garden), because they did not have money to get there.”

The viewpoints of the research partners regarding their needs were taken into consideration (King et al., 1996) and the medicinal garden was established after their suggestions. The medicinal garden now acts as one of the collaborative tools that may be developed into a profitable resource (Etkin, 1993), if practitioners are willing to invest time and effort into it. A part of the research budget is spent on the development of the garden, providing equipment and infrastructure support, financing for traditional ceremonies and transport money, as suggested by ethno-botanical collaboration programs (Gollin, 1999; King et al., 1996). However, some of the groups are reluctant to embrace the garden. Luckily, the requirement for the review of the agreement every 12 months makes it flexible enough to include more individuals or organizations as the collaboration becomes more wide-spread and acceptable to non-participating practitioners.

Benefits that are being sought, but have not been realised include grants for development and environmental education projects. Practitioners have brought application forms from various funding organizations and were assisted in completing them. However, to date, no funding has been secured. Co-ownership of intellectual property rights are being negotiated with the university before we take it to the practitioners, as the legal department of the university has been slow in responding to our requests. Because of the lack of commercialisation none of the benefits such as upfront payments, milestone payments or royalties have been put forward.
8.3 Trust

The issue of trust was more difficult to define, because of its intangible nature. Trust can not be negotiated, it is governed by perceptions and it reaches to the essence of a paradigm. Due to the far reaching differences between the two paradigms attempting to collaborate, much could be said among the two parties about the perceived motivations and intent of the other towards the collaboration. In this case, scientists were just as much victims of their paradigm as traditional practitioners were.

“(Healer x) replied (sort of in triumph): This is it. There is no trust…”

If we look at some motivations for western society in South Africa to support collaboration programs with traditional health practitioners, the issues become clearer. In the case of government, traditional health practitioners have been identified as valuable resources to advocate the primary health care approach, which was adopted by the public health care system. The focus of primary health care is disease prevention and for the concept to succeed, more people are needed on the ground to enforce it. Traditional health practitioners are ideally distributed among the population to reach all communities. In addition, community health education programs are central to illness prevention, the success of which is dependent on the beliefs and attitudes of that community (Govere et al., 2000), in which health practitioners would play a leading role. The HIV/AIDS pandemic has hastened integration programs of which the aims are to increase psychological support to families and referral to western practitioners (Giarelli & Jacobs, 2001). The intended roles of traditional health practitioners in these contexts are supportive at best. Therefore it is essential that community health programs be transparent about objectives, inherent benefits and limitations of the program (Jinabhai et al., 1997). Because of certain shortcomings in the aims and implementation of these programs, practitioners have become disillusioned with the whole scheme.

“They (government) never wanted to cooperate with us, only because of the epidemic (AIDS)”
Practitioners realise that they still play an inferior role to that of western health practitioners and the programs, if any, are not promoting their own needs. Government’s solution was to fast track recognition of traditional health practitioners. The Traditional Health Practitioners’ Act was thus hastily tabled and accepted (South Africa, 2004b). The problem with the whole process was that traditional health practitioners were still being promoted in accordance with the western paradigm, which will always leave them inferior to western methods. The specific provisions that made the Act questionable for traditional health practitioners was that they were not authorised under the Act to treat or claim to treat terminal diseases such as HIV/AIDS or cancer.

“We heal cancer! We heal cancer!”

The decrease in the number of diseases that may be treated by traditional health practitioners once incorporated into the health care system has long been one of the threats to traditional practitioners’ status and remuneration (Freeman & Motsei, 1992). The challenge for government to incorporate traditional health practitioners into the health care system as fair and equitable as possible, remains. Unfortunately, at the moment, government is not doing so well according to the participating practitioners:

“... the Department of Health only invited the healers to some occasions and do not really work with them all the time. …when the premier … came to visit … the healers are invited, but after that they (Department of Health) don’t keep on the collaboration.”

“Even now the government doesn’t ask for their (the healers) needs. They (the government) just want the information. The medicine (traditional remedies) comes from the ancestors and they just can’t tell anyone. Why can’t the medicine just be tested to see if it works? Why do they (the government) want to know the ingredients?”

To make the situation more interesting, the Traditional Health Practitioners’ Act was declared invalid by the Constitutional Court (SAMA Legal Unit, 2006). The petitioners being a western practitioner organization that previously claimed that by regulating traditional practitioners, recognition would be given to the occult. They further said that traditional healers’ medicine was not tested scientifically (Pienaar & De Villiers, 2004). In their appeal to the Constitutional court, they argued lack of public input. In a much earlier and more inclusive report, it was also found that physicians were generally
reluctant to share power with traditional practitioners, because they perceived traditional practitioners as opposition (Green, 1988). Furthermore, western practitioners often criticise traditional healing for their perceived physiologically harmful medicines, while traditional practitioners accuse western practitioners of having a superiority complex (Hopa et al., 1998). These and other encounters with western medicine and practitioners of western medicine have made it difficult for participating practitioners to believe in the integrity of western health practitioners:

“The western doctors have the tendency to take things for their selves and then tell that they have found it on their own. They don’t want to share. …the healers have never been recognised.” 24

Western practitioners have thus been referred to as ignorant in the respect of traditional medicine (Ngubane, 1986). In spite of government’s attempts to remedy a solution, generally, the gap between western and traditional medical systems remains to be very big16.

“Western doctors and traditional healers will never work in the same place … it will take a very long time …” 25

Not escaping this debate, western researchers take a lot of sometimes self-inflicted criticism from traditional practitioners. Our concept of validating the use and safety of traditional medicine (Lin et al., 1999; Ngom, 2004) and training of practitioners in biomedicine, with the belief that it will benefit them (Neumann & Lauro, 1982) is not shared by them. We have to admit that these beliefs may bring us in disdain with practitioners who are not of our thinking. Be that as it may, as scientists, we are interested to do research into traditional medicine, because we suspect that it might have scientific merit (Zerbe, 2005). Evidence from ethnopharmacological exploration (with field inquiry) has yielded various drug developments (Raza, 2006). The continued use of traditional medicines by the majority of third-world countries affirms the potential effectiveness of traditional medicine further (Light et al., 2005). However, these suspicions can not be taken as fact by scientists before it is proven. Only when a remedy’s safety or efficacy has been proven, can it be believed and trusted by the western community (Light et al., 2005). This very concept of belief and trust only after scientific validation has proven to be
puzzling to participating practitioners. They perceived this half-belief as a lack of trust from the scientist’s side towards them.

With history (and the present) providing scientists with, at best, questionable credibility to traditional practitioners, the process of building trust through long and consistent communication remains. In this instance scientific literature suggest that discussion and feedback are very important ways to build trust (Elisabetsky, 1991). However, change only happens slowly (El Ansari, 2005; UNAIDS, 2000).

“She (a healer) said to the researcher (without being nasty or mean) that she would know she (the researcher) is a bit too fast and not patient enough.”

Encouragingly, the future success of medicinal plant research has been linked to a high level of collaboration and mutually beneficial partnerships (Cordell, 2000). Previous collaboration programs with traditional health practitioners support the recognition of IK as a means to earn the trust of practitioners, for example the right of practitioners to keep their remedies a secret (UNAIDS, 2000). However, the understanding of IK may sometimes be confusing to westerners, because rational concepts are not separated from non-rational ones (Rao, 2006). It has thus been recommended that the study of cultural background may facilitate understanding of IK, because IK is embedded in broader cultural traditions (Ngom, 2004).

8.3.1 Culture

“Seeing the mosquito under the microscope is not enough. He wants to know who sent the mosquito to bite him and why” (Elliot, 1984).

Culture provides the meaning for traditional practitioners to participate in collaborative processes (Odora Hoppers, 2004). A sensitive approach and full respect for the spiritual and cultural aspects of traditional medicine has been recommended, because this is the only way in which practitioners will fully collaborate in any program (Hillenbrand, 2006). This is probably the reason why it has been reported that “dealing with traditional medicine” incorporates issues relating to “promotion of cultural heritage” (Timmermans, 2003). One of the problems with the incorporation of traditional healing into
the modern health care sector is that the cultural aspect of traditional healing is likely to be undermined (Freeman & Motsei, 1992). This is a major problem for health practitioners, because:

“Indigenous knowledge system stresses interrelatedness and interdependence of all phenomena – biological, physical, psychological, social and cultural” (Odora Hoppers, 2004).

One of the ways that culture can be learnt and understood is by experiencing it in action (Wendler & Struthers, 2002). The launch of the co-developed medicinal garden provided a great opportunity to observe the culture of the participating practitioners. A ritual ceremony involving the slaughtering of a goat and cow was proposed;

“… because it was the proper way to do as people of the ancestors.” 26

The ceremony would symbolise the asking of the blessing of the ancestors upon the garden (Broster, 1981), without which the healers believed that the garden would not succeed. However, funding was limited and only a goat could be afforded. Healers consulted the ancestors and they agreed that a goat would be fine if it was promised that a cow would be bought when the finances were right.

Preparations for the ceremony started a few days before to allow for the brewing of the traditional beer (umqhombhoti). The day previous to the tree planting ceremony involved the slaughtering of the goat and eating of the intestines. It is customary for the goat to cry before it dies. The day of the ceremony started with traditional song and dance. Traditional prayer (ukuphahla) under the sounds of drumming and singing commenced. The holy smoke of the imphepho and the foam of the izilawo were prepared to inform the ancestors of the proceedings27. Part of most traditional ceremonies is to drink or sprinkle on the ground a tot of gin and / or brandy in honour of your ancestors. After the proceedings were over, everybody was invited to lunch. The researchers were requested to be present and partake in the eating of the right front leg of the goat, which was slaughtered to symbolise the partnership. It is important to serve this meat on Olea twigs and leaves.
Participating practitioners felt that the way the ceremony was conducted and the attendance by a number of healers and members of the local community made it a success. Indeed, stakeholders from the old age home were also pleased to see the community joining in the festivities\(^{28}\). An after blessing ceremony was held about ten days afterwards to thank the ancestors for sending so many of the local community to join in the festivities.

“A person is a person through other people. … I am human, because I belong” (Odora Hoppers, 2004).

At minor ceremonies, where no slaughtering takes place, it is customary to serve meat (not fish) as part of lunch, because it is associated with the ancestors. It has been shown that if a project respects the traditional customs, it would be successful (UNAIDS, 2000), because it conveys respect and caring (Wendler & Struthers, 2002). The experience of the cultural side of traditional medicine, gave me a better understanding of the perspectives of participating practitioners. It also sensitised me to ways of preparing and approaching different aspects of the research. I have learnt that the correct approach to an issue may ease tensions and provides for a smoother discussion process, thus saving much of the time that would have been spent on talking in circles.

8.4 Challenges to collaboration

In general, the main stumbling block which prevented traditional health practitioners from achieving their true potential in the collaboration was the lack of unity among them (section 2.4.4). This has also been noted in previous reports regarding government, which documented that the many organizations representing traditional practitioners decentralise control of traditional healing and weakens their bargaining power (Oyebola, 1981). The pockets of power among traditional health practitioners may have been a contributing factor in the invalidation by the Constitutional Court of the Traditional Health Practitioners’ Act (SAMA Legal Unit, 2006). According to
the preamble to the Bill, in the legislative development process, cooperation was found with a few healers that were prominent in certain organizations and these healers were consulted rather than going through the grass roots process, which can be very time-consuming and repetitive. Although healers from our collaboration were familiar with two of the healers who were consulted in the legislative process\textsuperscript{29}, they still felt that they were left out:

“No one came here…” \textsuperscript{15}

It was thus difficult for them to accept the legislation, because the African culture welcomes and indeed demands negotiation with all involved. Although not the proper way, with limited resources this was the most inclusive way for government that legislation regarding traditional practitioners could be developed, especially with the prominent divisions in the ranks of traditional practitioners. However, it has been recommended that when devising policies, it is important to involve all stakeholders – “not just those with whom policymakers have pre-established relations” (Timmermans, 2003). The invalidation of the Act was thus a paradoxical blessing for traditional practitioners, because now the legislative process would be forced to be done in a more inclusive way. More discussion may also alleviate much of the problems traditional practitioners have with certain aspects of the Act (section 8.2).

In our experience with participating practitioners of the three collaborating organizations, the reason for the fragmentation in the ranks of traditional health practitioners were many times severe differences in opinion between the organizations, especially pertaining to cultural differences between tribes. Two of the most prominent complaints concerned differences in training of practitioners and the conducting of ceremonies. Because training is so individualised and varied among traditional practitioners even in the same tribe, practitioners of different tribes often question the training of the other. For example, one group of practitioners would train for years and much emphasis would be put on the proper conducting of rituals, while another group would train for six months and focus more on the knowledge gained from the ancestors. The differences in training might be the reason why ceremonies and rituals are conducted differently. The reason why this may
become a problem is that when a ceremony does not ‘work’, it is mostly because of the reason that it was conducted ‘improperly’. The first group rationalise that they know the proper way of conducting a ceremony or ritual, because they had the longer training. This collaborative partnership might provide an environment for practitioners from different backgrounds to discuss and debate together about issues such as these.

In addition, internal politics between organizations is another problem that fragments traditional health practitioners. Literature suggests that internal politics of healer organizations should be avoided, because it distracts healers from the issues at hand (UNAIDS, 2000). Due to the longitudinal nature of this collaboration, such an issue was encountered when the preamble to the indigenous knowledge agreement was discussed. It required information pertaining to the practitioners and organizations who initiated the collaboration. The discussion of which distracted practitioners so severely that they asked for it not to be discussed. Although it was mostly not difficult to maintain neutrality in immediate arguments, the establishment of the medicinal garden (section 2.4.2) made it almost impossible not to ‘take sides’. With the establishment, the second organization would not work in a garden on a site secured by the first organization. It was well beyond our resources to establish a second garden for the break away group and we kept on developing the first garden without them (section 2.4.4). In addition, the third organization accused the first of “taking over” the medicinal garden. These allegations came after they were continuously invited to garden meetings, after we found out of their existence. The garden problem can be summarised as follows:

“In the beginning the garden was for all healers, but some did not come.” 30

Luckily, with perseverance and many meetings, the second group agreed to join the garden. However, the third group still need some convincing. Not participating in the garden is a problem, because most of the benefits from the partnership go into the garden or are made available at the garden. This may be another reason why the third group was so reluctant to participate; they received the minimum benefits from the collaboration (section 8.2.3). The
continued running of the collaboration was thus a big challenge. However, the voice of reason did appear every now and then:

“… the healers was confused and this is the reason that there is so much fighting. … just because you belong to an organization does not mean that that organization takes over your being as a traditional healer. Yes, that organization has its own constitution and you abide by that constitution, but in the end you are still a traditional healer, and the person that trained you is still your trainer. You were called to heal, you were born like this. In the end your calling is much more important than the organization.” 31

8.5 Collaboration outcomes

The completion of this research project provided the first literature for the garden information centre. The documents that were presented to participating practitioners included translated summaries of the literature reviews done on the plants in Chapter 3 and of the screening results32. Healers would also be able to enquire for more detailed explanations of the results if they so wish. A copy of this thesis would be available at the garden. With this information, practitioners can choose to adjust their training to incorporate more scientific literature for students or qualified practitioners can choose to expand their knowledge base. Practitioners that received copies of the above mentioned documents were happy, because the documents were evidence that they could use to prove to opposed practitioners that they were getting information from the collaboration and not merely (as the opposed practitioners perceived it) giving it out.

The negotiated indigenous knowledge agreement was provisionally accepted by the initial two groups, but it was not formally signed. One group that provisionally accepted it, sent it to their legal advisors and are awaiting their response. Although being reassured by many different practitioners that the agreement will be signed as it stands, the major factor stifling this action was the lack of adequate communication between practitioners and organizations. The signing would have taken place at the end of 2006 at the medicinal garden, but on that day, two of the three organizations did not show up,
although being invited and assuring us of their attendance. The attending organization would not sign without the others being present, because some of the people in the other organizations also belonged to the attending organization and they wanted them to know about the signing. This ties-up with the reason the practitioners would not accept the Bill:

“The government must more try to reach those who are not affiliated to an organization in the rural areas so that people can’t turn around saying: ‘you sold us out, we did not know about it’; everybody must have a copy.” 15

Frustrating as it was, the indecision that the fragmentation of practitioners brought about, can not be remedied by anyone but themselves. Again, collaborative initiatives cannot force practitioners to agree, but can only facilitate discussion of the issues and attempt to guide the debate in a logical and legally acceptable direction. In addition to the non-unity of the practitioners, the university’s legal department was not equipped to guide our indigenous knowledge agreement negotiations, because at that stage the regulations to the Biodiversity Act had not been printed. It seems that this is a common problem in the ethnopharmacological field as the statement has been made that “agreements made the one year may not meet the ethical standards of a subsequent year” (Cordell, 2000). Still, much have been learnt through the process of collaborative medicinal plant research and it is important to document the learning process even if very little concrete conclusions can be made (Botes & van Rensburg, 2000).

The realisation and accepting of the different paradigms in the collaboration and the potential barrier that it was and still is should be kept in mind and challenged, because:

“Participation is about moving away from a ‘them and us’ mentality towards a partnership of mutual benefit” (El Ansari et al., 2002).

One recommendation to improve community participatory projects is the acknowledgement of skills of the participants (El Ansari et al., 2002). This aspect was difficult, because of the limited IK that practitioners were willing to share about medicinal plants. However, if this collaboration has amounted to anything, it was to stress the importance for indigenous knowledge providers
to be a part of the evaluation process for their medicinal plants. Their involvement ensures better understanding, true recognition and promotion of traditional medicine. It also provides ethnopharmacological science with a holistic purpose and consciousness:

“Ask: Whose interest does our work further. Who is empowered by our efforts; on whose standards does our work confer social legitimacy; what is the source of our ideas and taken-for-granted understandings of social reality; and, what is the structure of social relationships that our endeavours reinforce or reproduce?” (Singer, 1990).
9 Conclusion

The founding of this research collaboration was born from the action of three traditional health practitioners who wanted to promote their health care system through the evidence produced by science (section 1.5). Essentially, this collaboration thus focused on promotion of the traditional health care system through the conducting of medicinal plant research (section 1.4). The willingness of both parties to work together was the first step in fostering a mutual positive regard and initiated a quest for understanding the view of the other (section 1.3). The first three practitioners helped further to expand the awareness of the collaboration in their communities and among other practitioners in these communities (section 2.3.2). Through this action, other research projects have been accommodated in locating practitioners to participate in other studies locally and even in other parts of the country. The most important characteristic of this research collaboration was an interactive communication system, which aspired to following a transparent research path, which was maintained during the establishment of goals, conducting of experiments and dissemination of findings (section 1.3).

In addition to the interactive communication system, the nature of the collaboration required time, effort and knowledge from both sides to succeed. The most beneficial joint action was the establishment of a medicinal garden. The Umyezo Wamanyange medicinal garden became our meeting place. Notwithstanding all the important field observations that were made regarding interactions with traditional practitioners, it also imparted on the collaboration a unified purpose which strengthened mutual respect and understanding for each other. More even, this garden provided solutions to IK differences (section 2.4.1), the identification of individual plants (section 2.4.2) and cultivation data which guided the selection of the medicinal plants that were tested in this study (section 3). Aqueous and ethanol extracts of Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus and Tulbaghia violacea were tested for in vitro antimicrobial,
anticancer and anti-diabetic activities. Participating practitioners already have access to these plant materials and may reproduce these extracts if they were found to be effective.

9.1 Summary of laboratory findings and collaborative outcomes

Bulbine frutescens aqueous extract produced mild growth inhibition on Bacillus subtilis and increased glucose uptake into Chang liver and C2C12 muscle cells in a concentration-independent manner. Caution is advised for the ethanol extract, which showed toxicity towards Chang liver cells at high concentrations.

Ornithogalum longibracteatum aqueous extract produced mild growth inhibition on Klebsiella pneumoniae and mildly increased glucose uptake activity in especially Chang liver cells. However, the aqueous extract showed toxicity towards Chang liver cells in a concentration-independent manner.

Ruta graveolens ethanol extract produced mild growth inhibitory activity on B. subtilis and K. pneumoniae and increased glucose uptake into C2C12 muscle cells with an additive effect when combined with insulin. In both cases the ethanol extract produced better results than the aqueous extract, but also produced more toxicity on Chang liver cells.

Tarchonanthus camphoratus ethanol extract produced weak growth inhibitory activity on K. pneumoniae and both the ethanol and aqueous extracts produced mild growth inhibition on HT29 cancer cells. Extracts were more toxic to HT29 cancer cells than to Chang liver cells, with the ethanol extract being more toxic than the aqueous extract. The aqueous extract produced better increased glucose uptake in Chang liver cells and the ethanol extract in C2C12 muscle cells. Both the effects were concentration-independent. The higher concentrations produced an additive glucose uptake effect in combination with insulin in C2C12 muscle cells, but the lower concentration’s effect decreased with insulin. During the anti-diabetic feedback seminar,
practitioners suggested the continued investigation of the aqueous extract of *T. camphoratus*. The aqueous extract of *T. camphoratus* showed:

- Increased GLUT4 translocation, no binding to the insulin receptor, PI3-kinase involvement in glucose uptake, no effect on MAP-kinase, and, a small chance of activation of AMP-kinase in C2C12 muscle cells,
- No direct inhibition of PTP-1B, G6Pase or glycogen phosphorylase, and no effect on glycogen content in Chang liver cells, and,
- Alpha-glucosidase inhibitory activity at concentrations of ≥ 200 µg/ml.

*Tulbaghia violacea* ethanol extract produced significant growth inhibitory activity on *B. subtilis* (100%), *Candida albicans* (89%) and *Staphylococcus aureus* (76%) and the aqueous extract mild growth inhibitory activity on *B. subtilis* and *K. pneumoniae*. The ethanol extract produced growth inhibitory effects on HT29 colon cancer cells with an EC$_{50}$ of 101.46 µg/ml. The aqueous extract of *T. violacea* increased glucose uptake in Chang liver cells and the ethanol extract in C2C12 muscle cells. The higher concentrations produced an additive glucose uptake effect in combination with insulin in C2C12 muscle cells, but the lower concentration’s effect decreased with insulin.

The information of these laboratory findings and corresponding literature surveys have been summarised, translated and made available to participating traditional health practitioners as the first documents of the information database planned for the medicinal garden (section 2.4.2). This formal exchange of knowledge as compared to the continuous feedback seminars was important, because practitioners referred to the exchange as the first “fruits” of the collaboration (section 8.5). In addition to the continuous communication with the scientific community, different practitioners’ organizations were put in contact with each other, which may contribute to the unifying of practitioners in this community. Scientists’ understanding of traditional practitioners and the health care system is expanding as they learn more about African culture and ways of doing transparent research. Although no IKA has been formally accepted (section 8.2.2), the partnership thus far has mutually benefited both practitioners and scientists.
9.2 Recommendations

Objectives should be recalibrated according to the needs of both collaborative sides. Preparation of plants for experiments should reflect traditional methods more accurately, such as using the leaf juice (in the case of *B. frutescens*), crushed bulb or decoctions prepared by participating practitioners in the laboratory. Also, greater emphasis should be placed on economic development of the medicinal plants showing promising laboratory results. It is thus recommended that *B. frutescens* be investigated further for its topical anti-viral properties. If proven, this plant could be actively marketed by practitioners for topical use. Although systemic effectiveness will be more difficult to prove, the anti-diabetic testing on *B. frutescens*, *O. longibracteatum* and *T. camphoratus* should continue, because it is used by practitioners for diabetes (section 6.5.1) and more scientific data will be required before it is deemed safe. Interesting hypotheses for further research regarding the glucose uptake mechanisms of *R. graveolens* can be made, because of the host of literature available on other biological mechanisms and chemical components (section 6.5.1). *T. violacea* is also an exciting candidate for glucose uptake mechanisms, because of its ACE inhibitory activity, which would be a welcome additional benefit in the treatment of diabetes. An interesting angle would be to start testing combinations of plants effective for the same diseases to ascertain synergistic effects (Gilani & Atta-ur-Rahman, 2005), also because healers used the plants in this way (section 6.4.1).

Practitioners and the legal department of the university should come to an agreement as soon as possible regarding the IKA and intellectual property rights (IPR) of the research. It has been postulated that IPR law, biodiversity prospecting and indigenous people’s rights in research may become one of the most important debates of the twenty-first century (Posey, 1996). Collaborative researchers should have a certain authority to lead this debate.
as it was suggested that universities be one of the stakeholders in this process (Posey, 1996).

9.3 Reflective comments

Apart from the undisputable fact that collaborative research looks good on paper, the unwritten realities of this research were less pretty and yet possessed true beauty. Many times the research process was difficult, time-consuming, messy and depressing. In the same breath, it built unexpected friendships, compelled me to think out of the box and bestowed a sense of purpose to my research.

I will briefly present two illustrations that mark the most defining moments in this research project for me. In a small way, these two moments illustrated my own ignorance of how caught up I was in my own paradigm. The following remark was made by one of the initiators of the collaboration after five years of working together. This specific practitioner had attended 10 workshops, 21 meetings, 5 group discussions, had participated in 2 slaughtering ceremonies in the garden and is an active member of the medicinal garden (it was her first feedback seminar).

"that she (a participating practitioner) was not sure that it was a good thing to collaborate with western people … Now she does not have any doubts and that she is happy about the collaboration, we all learned from each other."12"

On another occasion healers exclaimed that they did not want to be westernised, because they did not want to:

"loose the essence of their calling".16

Despite the fact that the research collaboration was flawed and produced minor successes in scientific terms, I think I can present to you one of the keys to collaborative research. Mike Pantsi used to encourage me by saying:

"As long as we are talking, we are going forward."
List of footnotes

1 On January 1st 2005 the University of Port Elizabeth and Port Elizabeth Technikon merged to form the Nelson Mandela Metropolitan University according to the government’s restructuring policy of Higher Education Institutions in South Africa.


3 Meeting: 4 September 2002, Plant meeting.

4 Meeting: January 2004, Meeting for garden culture sub-committee.


6 Meeting: 15 October 2003, Medicinal garden planning meeting.

7 Fieldnotes: 8 June 2005, in the garden.

8 Fieldnotes: 22 March 2006, in the garden.

9 Fieldnotes: 23 June 2004 after working in the medicinal garden.

10 Fieldnotes: 16 June 2004; Garden preparations.

11 Feedback seminar: 30 March 2005; Antimicrobial and anticancer screening activities.

12 Feedback seminar: 9 November 2005; Anti-diabetic screening activities.

13 Research visit: 30 July 2001

14 Focus group discussion: 18 August 2004, Traditional Health Practitioners’ Bill.

15 Fieldnotes: 27 August 2004, Informal discussion of the Traditional Health Practitioners Bill, additional transcripts provided by Julia Heckl, medical anthropologist.

16 Interactive workshop: 10 May 2006; Drug discovery and registration of traditional medicine.

17 Fieldnotes: 28 June 2006 at the medicinal garden in Zwide.


20 Fieldnotes: 15 September 2006 at the medicinal garden.

21 Fieldnotes: 22 March 2006 at the medicinal garden.

22 Fieldnotes: 10 November 2004 meeting at medicinal garden.

23 Fieldnotes: 22 February 2006 at the medicinal garden.

24 Focus group discussion: 7 April 2004, HIV/AIDS, additional fieldnotes provided by Julia Heckl, medical anthropologist.

25 Fieldnotes: 12 November 2005 at the house of a participating practitioner.

26 Fieldnotes: 4 August 2004, meeting at medicinal garden.

27 Fieldnotes: 3 August 2005, student ceremony at medicinal garden.

28 Meeting: 1 September 2005, with stakeholders at the medicinal garden.

29 Fieldnotes: 8 November 2004, at the house of a healer.

30 Meeting: 7 December 2006, initial meeting of indigenous knowledge agreement.

31 Fieldnotes: 11 November 2005, meeting with a healer at the university.

A. Formulae

A.1 Phosphate-buffered saline (PBS)

Sodium chloride (NaCl), Associated Chemical Enterprises; Glenvista, South Africa

Potassium dihydrogen phosphate (KH₂PO₄), Saarchem, Krugersdorp, South Africa

Disodium hydrogen phosphate (Na₂HPO₄·12H₂O), Merck, South Africa

Potassium chloride (KCl), BDH AnalaR, Midrand, South Africa

Distilled H₂O

pH 7.4

A.2 Phosphate-buffered saline A (PBSA)

Sodium chloride (NaCl), Associated Chemical Enterprises; Glenvista, South Africa

Potassium dihydrogen phosphate (KH₂PO₄), Saarchem, Krugersdorp, South Africa

Disodium hydrogen phosphate (Na₂HPO₄·12H₂O), Merck, South Africa

Potassium chloride (KCl), BDH AnalaR, Midrand, South Africa

Ethylenediaminetetra-acetic acid (EDTA), BDH AnalaR, Gauteng, South Africa

Distilled H₂O

pH 7.4
A.3 Reducing sample buffer

Solution C

Glycerol, Merck, Gauteng, South Africa
2 ml

Solution D

β-mercaptoethanol, Merck, Germany
1.6 ml

BPB
0.8 ml

A.4 Transfer buffer

2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), Merck, Gauteng, South Africa
3.2 ml

Glycine, Merck, Gauteng, South Africa
5.82 g

Sodium dodecyl sulfate (SDS), BDH Laboratory Supplies, England
2.93 g

Methanol (MeOH), Associated Chemical Enterprises, Glenvista, South Africa
0.1 g

Deionised water
800 ml

A.5 TRIS-buffered saline (TBS)

Sodium chloride (NaCl), Associated Chemical Enterprises; Glenvista, South Africa
0.8%

1 M TRIS, Merck, Gauteng, South Africa
2%

TWEEN 20, Sigma, St Louis, USA
0.1%

Nonidet P 40, Fluka, Steinheim, Germany
0.04%

pH 7.6
A.6 Krebs-Ringer phosphate buffer (KRP-buffer)

Potassium chloride (KCl), BDH AnalR, Midrand, South Africa 4.7 mM

Calcium chloride (CaCl₂), NT Laboratory Supplies, Johannesburg, South Africa 1.25 mM

Magnesium chloride (MgCl₂), Associated Chemical Enterprises, Southdale, South Africa 1.2 mM

Potassium dihydrogen phosphate (KH₂PO₄), Saarchem, Krugersdorp, South Africa 1.2 mM

Sodium chloride (NaCl), Associated Chemical Enterprises; Glenvista, South Africa 119 mM

Dissolve compounds in the order it is given in deionised water.

pH 7.4
B. Result tables: Chapter 4

Table B. 1 Percentage growth inhibition (± SEM) produced on *Bacillus subtilis* after exposure to aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values in the table represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>41.64 ± 11.66</td>
<td>0.0002</td>
<td>0.6767</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>50.34 ± 16.97</td>
<td>0.0071</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>-9.55 ± 16.78</td>
<td>0.4453</td>
<td>0.9252</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-7.19 ± 17.83</td>
<td>0.8931</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>-19.76 ± 18.30</td>
<td>0.1553</td>
<td>0.3891</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-44.35 ± 20.85</td>
<td>0.0675</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>-15.42 ± 8.78</td>
<td>0.0759</td>
<td>0.4599</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-31.70 ± 19.75</td>
<td>0.1947</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>76.34 ± 8.40</td>
<td>&lt;0.0001</td>
<td>0.2117</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>60.63 ± 8.82</td>
<td>0.0025</td>
<td></td>
</tr>
</tbody>
</table>

Table B. 2 Percentage growth inhibition (± SEM) produced on *Bacillus subtilis* after exposure to ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

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<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>-28.02 ± 11.43</td>
<td>0.0075</td>
<td>0.0162</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-91.09 ± 21.35</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>-1.34 ± 8.66</td>
<td>0.8735</td>
<td>0.0171</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-67.25 ± 24.04</td>
<td>0.0096</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>14.23 ± 28.47</td>
<td>0.4956</td>
<td>0.2258</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>59.41 ± 21.53</td>
<td>0.0053</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>-11.33 ± 8.28</td>
<td>0.1748</td>
<td>0.7813</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-15.45 ± 12.08</td>
<td>0.5258</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>100.0 ± 0.0</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100.0 ± 0.0</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

Key: NC Not calculated, because there was no growth in the wells, the percentage inhibition calculated from the spectrophotometrical measurements was negative which is practically not possible, thus a value of 100% inhibition was assigned to these measurements with no standard deviation. Because of the lack of variance the program could not calculate p-values for the data set.
Table B. 3 Percentage growth inhibition (± SEM) produced on Staphylococcus aureus after exposure to aqueous extracts (250 and 500 µg/ml) of Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus and Tulbaghia violacea for two hours at 34ºC. Values in the table represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
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<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. frutescens</td>
<td>250</td>
<td>8.37 ± 17.98</td>
<td>0.6380</td>
<td>0.4028</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>26.61 ± 11.57</td>
<td><strong>0.0193</strong></td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum</td>
<td>250</td>
<td>38.38 ± 12.82</td>
<td><strong>0.0101</strong></td>
<td>0.8178</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>34.92 ± 7.48</td>
<td><strong>0.0004</strong></td>
<td></td>
</tr>
<tr>
<td>R. graveolens</td>
<td>250</td>
<td>13.49 ± 15.57</td>
<td>0.4006</td>
<td>0.6113</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3.15 ± 12.65</td>
<td>0.7839</td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>250</td>
<td>-25.98 ± 7.08</td>
<td><strong>0.0186</strong></td>
<td>0.3644</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-12.79 ± 12.36</td>
<td>0.2638</td>
<td></td>
</tr>
<tr>
<td>T. violacea</td>
<td>250</td>
<td>-3.48 ± 10.88</td>
<td>0.7404</td>
<td><strong>0.0288</strong></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-33.59 ± 6.88</td>
<td><strong>0.0022</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table B. 4 Percentage growth inhibition (± SEM) produced on Staphylococcus aureus after exposure to ethanol extracts (250 and 500 µg/ml) of Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus and Tulbaghia violacea for two hours at 34ºC. Values represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. frutescens</td>
<td>250</td>
<td>-13.59 ± 15.57</td>
<td>0.3973</td>
<td>0.4764</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3.85 ± 18.33</td>
<td>0.7955</td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum</td>
<td>250</td>
<td>19.85 ± 11.42</td>
<td>0.1360</td>
<td>0.6137</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>13.03 ± 6.85</td>
<td>0.1423</td>
<td></td>
</tr>
<tr>
<td>R. graveolens</td>
<td>250</td>
<td>-10.88 ± 22.29</td>
<td>0.6075</td>
<td>0.1792</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>21.31 ± 6.44</td>
<td><strong>0.0174</strong></td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>250</td>
<td>-36.14 ± 5.44</td>
<td><strong>0.0008</strong></td>
<td><strong>0.0235</strong></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-18.30 ± 4.91</td>
<td><strong>0.0288</strong></td>
<td></td>
</tr>
<tr>
<td>T. violacea</td>
<td>250</td>
<td>27.02 ± 13.10</td>
<td><strong>0.0257</strong></td>
<td><strong>0.0073</strong></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>76.27 ± 10.27</td>
<td>&lt;<strong>0.0001</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table B. 5 Percentage growth inhibition (± SEM) produced on *Escherichia coli* after exposure to aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values in the table represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250</td>
<td>-14.70 ± 3.95</td>
<td>0.1632</td>
<td>0.3671</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>500</td>
<td>-7.43 ± 6.89</td>
<td>0.3622</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>1.02 ± 10.06</td>
<td>0.9345</td>
<td>0.2366</td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>500</td>
<td>-17.83 ± 11.78</td>
<td>0.0969</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-0.88 ± 8.95</td>
<td>0.9414</td>
<td>0.3032</td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>500</td>
<td>12.06 ± 8.40</td>
<td>0.1754</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-16.00 ± 6.94</td>
<td>0.1605</td>
<td>0.1800</td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>500</td>
<td>-2.26 ± 7.10</td>
<td>0.7831</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>-40.62 ± 16.37</td>
<td>0.0247</td>
<td>0.8226</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-45.74 ± 15.56</td>
<td>0.0053</td>
<td></td>
</tr>
</tbody>
</table>

Table B. 6 Percentage growth inhibition (± SEM) produced on *Escherichia coli* after exposure to ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250</td>
<td>2.31 ± 8.82</td>
<td>0.8463</td>
<td>0.1470</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>500</td>
<td>-18.55 ± 10.71</td>
<td>0.0687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-18.83 ± 4.69</td>
<td>0.0822</td>
<td>0.6289</td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>500</td>
<td>-15.54 ± 4.81</td>
<td>0.0399</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-52.70 ± 15.75</td>
<td>0.0015</td>
<td>0.5425</td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>500</td>
<td>-35.67 ± 22.58</td>
<td>0.0434</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-16.97 ± 7.85</td>
<td>0.1482</td>
<td>0.9692</td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>500</td>
<td>-17.49 ± 10.72</td>
<td>0.0854</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>-52.42 ± 22.41</td>
<td>0.0164</td>
<td>0.7076</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-64.57 ± 22.83</td>
<td>0.0046</td>
<td></td>
</tr>
</tbody>
</table>
Table B. 7 Percentage growth inhibition (± SEM) produced on *Klebsiella pneumoniae* after exposure to aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values in the table represent quadruplicate measurements of samples tested in two independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 8)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>41.67 ± 13.92</td>
<td>0.0044</td>
<td>0.2973</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>24.54 ± 7.52</td>
<td>0.0122</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>57.94 ± 12.73</td>
<td>0.0001</td>
<td>0.1713</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>34.15 ± 10.50</td>
<td>0.0034</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>31.20 ± 9.13</td>
<td>0.0093</td>
<td>0.2517</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>19.33 ± 3.91</td>
<td>0.0199</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>22.15 ± 9.86</td>
<td>0.0616</td>
<td>0.1124</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-0.60 ± 9.12</td>
<td>0.9509</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>45.89 ± 7.44</td>
<td>&lt;0.0001</td>
<td>0.1756</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>61.75 ± 8.25</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table B. 8 Percentage growth inhibition (± SEM) produced on *Klebsiella pneumoniae* after exposure to ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 34°C. Values in the table represent quadruplicate measurements of samples tested in two independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 8)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>29.69 ± 15.96</td>
<td>0.0484</td>
<td>0.5244</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>45.55 ± 18.31</td>
<td>0.0060</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>28.88 ± 12.72</td>
<td>0.0311</td>
<td>0.2364</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.295 ± 11.95</td>
<td>0.5194</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>49.91 ± 6.36</td>
<td>&lt;0.0001</td>
<td>0.5216</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>54.98 ± 4.35</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>42.40 ± 8.26</td>
<td>0.0006</td>
<td>0.2291</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>30.57 ± 4.48</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>30.36 ± 16.87</td>
<td>0.0230</td>
<td>0.0572</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>67.26 ± 5.71</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>
Table B. 9 Percentage growth inhibition (± SEM) produced on *Candida albicans* after exposure to aqueous extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 27°C. Values in the table represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>-45.64 ± 6.38</td>
<td>&lt;0.0001</td>
<td>0.0944</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-30.20 ± 6.11</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>1.83 ± 7.54</td>
<td>0.7740</td>
<td>0.3636</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>10.75 ± 5.96</td>
<td>0.0899</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>3.95 ± 5.66</td>
<td>0.4608</td>
<td>0.0143</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-20.64 ± 7.31</td>
<td>0.0047</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>-43.44 ± 5.71</td>
<td>&lt;0.0001</td>
<td>0.3157</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-50.19 ± 3.25</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>-3.69 ± 8.06</td>
<td>0.5889</td>
<td>0.4287</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>6.58 ± 9.85</td>
<td>0.4331</td>
<td></td>
</tr>
</tbody>
</table>

Table B. 10 Percentage growth inhibition (± SEM) produced on *Candida albicans* after exposure to ethanol extracts (250 and 500 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for two hours at 27°C. Values represent quadruplicate measurements of samples tested in three independent experiments compared to the corresponding negative control values. P-values were calculated with the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (n = 12)</th>
<th>P-value for difference between sample and negative control</th>
<th>P-value for difference between two concentrations tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>250</td>
<td>1.99 ± 8.95</td>
<td>0.7831</td>
<td>0.5206</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>10.21 ± 8.86</td>
<td>0.1919</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>250</td>
<td>6.36 ± 5.82</td>
<td>0.2449</td>
<td>0.9902</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>6.46 ± 5.36</td>
<td>0.2798</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>250</td>
<td>19.55 ± 5.25</td>
<td>0.0005</td>
<td>0.0089</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>40.47 ± 5.06</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>250</td>
<td>-3.74 ± 4.53</td>
<td>0.4358</td>
<td>0.0123</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>15.81 ± 5.55</td>
<td>0.0122</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>250</td>
<td>89.36 ± 0.51</td>
<td>&lt;0.0001</td>
<td>0.7961</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>89.52 ± 0.33</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>
C. Result tables: Chapter 5

Table C. 1 Percentage growth inhibition (± SEM) produced on HT29 colon cancer cells. Cells were exposed to aqueous extracts (62.5 and 125 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for 48 hours at 37°C. Averages and p-values were calculated from two to five independent experiments for each sample, with eight replicates per sample per experiment. P-values were calculated using the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant (number of experiments)</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM (number of replicates)</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0.00000012 ± 3.04 (n = 39)</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.1 mM</td>
<td>24.11 ± 1.82 (n = 37)</td>
<td>&lt;0.0001</td>
<td>Not applicable</td>
</tr>
<tr>
<td><em>B. frutescens</em> (n = 4)</td>
<td>62.5</td>
<td>5.98 ± 3.23 (n = 24)</td>
<td>0.1405</td>
<td>0.0100</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-11.54 ± 5.67 (n = 24)</td>
<td>0.0276</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em> (n = 2)</td>
<td>62.5</td>
<td>26.83 ± 4.03 (n = 13)</td>
<td>&lt;0.0001</td>
<td>0.0526</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>16.80 ± 2.81 (n = 13)</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em> (n = 5)</td>
<td>62.5</td>
<td>4.04 ± 5.55 (n = 30)</td>
<td>0.4414</td>
<td>0.6616</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>7.80 ± 6.50 (n = 30)</td>
<td>0.1853</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em> (n = 3)</td>
<td>62.5</td>
<td>22.60 ± 4.00 (n = 19)</td>
<td>&lt;0.0001</td>
<td>0.0121</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>40.17 ± 5.32 (n = 19)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em> (n = 2)</td>
<td>62.5</td>
<td>-1.91 ± 4.29 (n = 13)</td>
<td>0.7060</td>
<td>0.5197</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-6.66 ± 5.98 (n = 12)</td>
<td>0.2331</td>
<td></td>
</tr>
</tbody>
</table>

Table C. 2 Percentage growth inhibition (± SEM) produced on HT29 colon cancer cells. Cells were exposed to ethanol extracts (62.5 and 125 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for 48 hours at 37°C. Averages and p-values were calculated from three to five independent experiments for each sample, with eight replicates per sample per experiment. P-values were calculated using the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant (number of experiments)</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0.00000012 ± 3.04 (n = 39)</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.1 mM</td>
<td>24.11 ± 1.82 (n = 37)</td>
<td>&lt;0.0001</td>
<td>Not applicable</td>
</tr>
<tr>
<td><em>B. frutescens</em> (n = 4)</td>
<td>62.5</td>
<td>6.62 ± 5.32 (n = 25)</td>
<td>0.1991</td>
<td>0.6733</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>2.74 ± 7.43 (n = 25)</td>
<td>0.6982</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em> (n = 5)</td>
<td>62.5</td>
<td>9.90 ± 5.60 (n = 31)</td>
<td>0.0814</td>
<td>0.2462</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>0.80 ± 5.39 (n = 31)</td>
<td>0.8922</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em> (n = 5)</td>
<td>62.5</td>
<td>8.19 ± 5.85 (n = 31)</td>
<td>0.1621</td>
<td>0.4912</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>2.01 ± 6.75 (n = 30)</td>
<td>0.7697</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em> (n = 3)</td>
<td>62.5</td>
<td>27.61 ± 2.97 (n = 17)</td>
<td>&lt;0.0001</td>
<td>0.0033</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>40.84 ± 2.91 (n = 17)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em> (n = 4)</td>
<td>62.5</td>
<td>46.93 ± 3.28 (n = 21)</td>
<td>&lt;0.0001</td>
<td>0.0303</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>59.75 ± 4.82 (n = 18)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>
Table C. 3 Percentage growth inhibition (± SEM) produced on Chang liver cells. Cells were exposed to aqueous extracts (62.5 and 125 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for 48 hours at 37°C. Averages and p-values were calculated from two to four independent experiments for each sample, with eight replicates per sample per experiment. P-values were calculated using the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant (number of experiments)</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0.51 ± 2.78 (n = 48)</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td><em>B. frutescens</em> (n = 3)</td>
<td>62.5</td>
<td>-9.67 ± 6.44 (n = 21)</td>
<td>0.0926</td>
<td>0.3081</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-18.15 ± 5.10 (n = 21)</td>
<td><strong>0.0009</strong></td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em> (n = 2)</td>
<td>62.5</td>
<td>18.14 ± 4.14 (n = 15)</td>
<td><strong>0.0021</strong></td>
<td>0.9318</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>17.53 ± 5.80 (n = 15)</td>
<td><strong>0.0056</strong></td>
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<tr>
<td><em>R. graveolens</em> (n = 4)</td>
<td>62.5</td>
<td>-22.96 ± 7.30 (n = 29)</td>
<td><strong>0.0008</strong></td>
<td>0.6798</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-18.71 ± 7.18 (n = 29)</td>
<td><strong>0.0048</strong></td>
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<tr>
<td><em>T. camphoratus</em> (n = 3)</td>
<td>62.5</td>
<td>-3.55 ± 4.51 (n = 24)</td>
<td>0.4255</td>
<td>0.1472</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>5.56 ± 4.11 (n = 21)</td>
<td>0.3169</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em> (n = 4)</td>
<td>62.5</td>
<td>4.65 ± 2.99 (n = 12)</td>
<td>0.4773</td>
<td>0.6594</td>
</tr>
</tbody>
</table>

Table C. 4 Percentage growth inhibition (± SEM) produced on Chang liver cells. Cells were exposed to ethanol extracts (62.5 and 125 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* for 48 hours at 37°C. Averages and p-values were calculated from two to four independent experiments for each sample, with eight replicates per sample per experiment. P-values were calculated using the two-tailed unpaired t-test. Statistical significance (p <0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Plant (number of experiments)</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>-0.18 ± 3.50 (n = 44)</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td><em>B. frutescens</em> (n = 3)</td>
<td>62.5</td>
<td>33.31 ± 5.94 (n = 19)</td>
<td><strong>&lt;0.0001</strong></td>
<td>0.6424</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>30.06 ± 3.72 (n = 20)</td>
<td><strong>&lt;0.0001</strong></td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em> (n = 4)</td>
<td>62.5</td>
<td>-23.66 ± 9.09 (n = 29)</td>
<td><strong>0.0401</strong></td>
<td>0.4229</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-15.30 ± 5.29 (n = 31)</td>
<td><strong>0.0152</strong></td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em> (n = 4)</td>
<td>62.5</td>
<td>-7.51 ± 7.63 (n = 31)</td>
<td>0.4301</td>
<td>0.3560</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>1.65 ± 6.24 (n = 31)</td>
<td>0.7856</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em> (n = 2)</td>
<td>62.5</td>
<td>-9.31 ± 3.69 (n = 19)</td>
<td>0.1247</td>
<td><strong>&lt;0.0001</strong></td>
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<tr>
<td></td>
<td>125</td>
<td>28.05 ± 3.94 (n = 20)</td>
<td><strong>&lt;0.0001</strong></td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em> (n = 4)</td>
<td>62.5</td>
<td>19.38 ± 5.19 (n = 26)</td>
<td><strong>0.0019</strong></td>
<td>0.5833</td>
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<tr>
<td></td>
<td>125</td>
<td>23.27 ± 4.76 (n = 26)</td>
<td><strong>0.0002</strong></td>
<td></td>
</tr>
</tbody>
</table>
D. Result tables: Chapter 6

Table D. 1 Percentage glucose taken up (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with aqueous extracts of *Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus* and *Tulbaghia violacea* at 37°C. Averages and p-values were calculated with the two-tailed unpaired t-test from two to three experiments with 10 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 10)</td>
<td>100.0 ± 1.98 (n = 99)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM (n = 10)</td>
<td>115.4 ± 2.23 (n = 99)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 3)</td>
<td>143.5 ± 5.69 (n = 28)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>111.4 ± 2.40 (n = 20)</td>
<td>0.0134</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 3)</td>
<td>131.9 ± 4.25 (n = 29)</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>109.5 ± 2.26 (n = 20)</td>
<td>0.0389</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 3)</td>
<td>114.2 ± 5.37 (n = 28)</td>
<td>0.0029</td>
<td>0.9517</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>114.7 ± 3.45 (n = 20)</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 2)</td>
<td>116.7 ± 5.83 (n = 20)</td>
<td>0.0015</td>
<td>0.0465</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>131.5 ± 4.20 (n = 20)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 2)</td>
<td>98.44 ± 3.46 (n = 30)</td>
<td>0.7011</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>50 (n = 3)</td>
<td>124.5 ± 7.01 (n = 30)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table D. 2 Percentage growth inhibition (± SEM) produced on Chang liver cells by aqueous extracts. Cells were treated for 48 hours with aqueous extracts (0.125 or 12.5 µg/ml) of *Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus* and *Tulbaghia violacea* at 37°C. Averages and p-values were calculated with the two-tailed unpaired t-test from two to three experiments with six replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% growth inhibition ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 10)</td>
<td>0.00 ± 0.99 (n = 60)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM (n = 10)</td>
<td>-0.06 ± 1.18 (n = 59)</td>
<td>0.9704</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.125 (n = 3)</td>
<td>8.08 ± 2.03 (n = 18)</td>
<td>0.0003</td>
<td>0.0144</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>-0.58 ± 2.70 (n = 12)</td>
<td>0.8169</td>
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</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.125 (n = 3)</td>
<td>4.66 ± 2.08 (n = 18)</td>
<td>0.0320</td>
<td>0.2231</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>-0.35 ± 3.81 (n = 12)</td>
<td>0.9007</td>
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</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>0.125 (n = 3)</td>
<td>3.29 ± 1.92 (n = 18)</td>
<td>0.1196</td>
<td>0.1594</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>-1.78 ± 3.19 (n = 12)</td>
<td>0.4992</td>
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</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.125 (n = 2)</td>
<td>-1.07 ± 3.12 (n = 12)</td>
<td>0.6826</td>
<td>0.1997</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>4.54 ± 2.87 (n = 12)</td>
<td>0.0790</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.125 (n = 2)</td>
<td>-11.11 ± 3.07 (n = 18)</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 3)</td>
<td>7.35 ± 3.07 (n = 18)</td>
<td>0.0037</td>
<td></td>
</tr>
</tbody>
</table>
Table D. 3 Percentage glucose taken up (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with ethanol extracts of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* at 37ºC. Averages and p-values were calculated with the two-tailed unpaired t-test from two to three experiments with 10 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 7)</td>
<td>100.0 ± 1.71 (n = 70)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM (n = 7)</td>
<td>110.1 ± 1.83 (n = 69)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 3)</td>
<td>110.8 ± 2.60 (n = 30)</td>
<td>0.0008</td>
<td>0.2660</td>
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<tr>
<td></td>
<td>50 (n = 2)</td>
<td>104.1 ± 6.17 (n = 20)</td>
<td>0.3768</td>
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</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 3)</td>
<td>106.7 ± 4.41 (n = 30)</td>
<td>0.0889</td>
<td>0.4814</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>110.7 ± 2.08 (n = 20)</td>
<td>0.0022</td>
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</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 3)</td>
<td>101.3 ± 2.05 (n = 30)</td>
<td>0.6607</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>50 (n = 1)</td>
<td>117.7 ± 3.07 (n = 10)</td>
<td>0.0003</td>
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</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 3)</td>
<td>126.9 ± 3.84 (n = 29)</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>50 (n = 1)</td>
<td>101.4 ± 3.87 (n = 10)</td>
<td>0.7699</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 2)</td>
<td>100.1 ± 2.69 (n = 20)</td>
<td>0.9830</td>
<td>0.9406</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>100.6 ± 5.73 (n = 20)</td>
<td>0.2250</td>
<td></td>
</tr>
</tbody>
</table>

Table D. 4 Percentage growth inhibition (± SEM) of Chang liver cells by ethanol extracts. Cells were treated for 48 hours with ethanol extracts (0.125 or 12.5 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* at 37ºC. Averages and p-values were calculated with the two-tailed unpaired t-test from one to three experiments with six replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% growth inhibition ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 8)</td>
<td>0.00 ± 1.02 (n = 42)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM (n = 8)</td>
<td>1.07 ± 1.10 (n = 47)</td>
<td>0.4789</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.125 (n = 3)</td>
<td>0.18 ± 2.57 (n = 18)</td>
<td>0.9370</td>
<td>0.9567</td>
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<td></td>
<td>12.5 (n = 2)</td>
<td>0.38 ± 2.14 (n = 12)</td>
<td>0.8652</td>
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<tr>
<td><em>O. longibracteatum</em></td>
<td>0.125 (n = 3)</td>
<td>-3.35 ± 2.86 (n = 18)</td>
<td>0.1725</td>
<td>0.1722</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>2.56 ± 2.86 (n = 12)</td>
<td>0.2985</td>
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<tr>
<td><em>R. graveolens</em></td>
<td>0.125 (n = 3)</td>
<td>-1.89 ± 2.60 (n = 18)</td>
<td>0.4146</td>
<td>0.0679</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 1)</td>
<td>7.53 ± 3.22 (n = 6)</td>
<td>0.0142</td>
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<tr>
<td><em>T. camphoratus</em></td>
<td>0.125 (n = 3)</td>
<td>2.73 ± 2.73 (n = 17)</td>
<td>0.2519</td>
<td>0.3537</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 1)</td>
<td>7.43 ± 2.92 (n = 6)</td>
<td>0.0143</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.125 (n = 2)</td>
<td>0.55 ± 3.10 (n = 12)</td>
<td>0.8268</td>
<td>0.7080</td>
</tr>
<tr>
<td></td>
<td>12.5 (n = 2)</td>
<td>2.19 ± 2.96 (n = 11)</td>
<td>0.3845</td>
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</tr>
</tbody>
</table>
Table D. 5 Percentage glucose taken up (± SEM) by C2C12 muscle cells. Cells were treated for one hour with aqueous extracts (0.5 or 50 µg/ml) of Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus and Tulbaghia violacea at 37°C. Averages and p-values were calculated with the two-tailed unpaired t-test from three to six experiments per sample and with 12 replicates per sample for each experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 13)</td>
<td>100.0 ± 1.53 (n = 159)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin 1 µM (n = 13)</td>
<td>119.7 ± 1.46 (n = 158)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>B. frutescens 0.5 (n = 6)</td>
<td>130.1 ± 2.43 (n = 79)</td>
<td>&lt;0.0001</td>
<td>0.0241</td>
<td></td>
</tr>
<tr>
<td>B. frutescens 50 (n = 4)</td>
<td>121.3 ± 3.05 (n = 56)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum 0.5 (n = 4)</td>
<td>129.2 ± 4.19 (n = 48)</td>
<td>&lt;0.0001</td>
<td>0.0044</td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum 50 (n = 3)</td>
<td>113.9 ± 2.72 (n = 40)</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>R. graveolens 0.5 (n = 4)</td>
<td>124.7 ± 3.30 (n = 48)</td>
<td>&lt;0.0001</td>
<td></td>
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</tr>
<tr>
<td>R. graveolens 50 (n = 3)</td>
<td>118.1 ± 1.68 (n = 40)</td>
<td>&lt;0.0001</td>
<td>0.0974</td>
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</tr>
<tr>
<td>T. camphoratus 0.5 (n = 5)</td>
<td>128.4 ± 3.27 (n = 64)</td>
<td>&lt;0.0001</td>
<td>0.0201</td>
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<tr>
<td>T. camphoratus 50 (n = 3)</td>
<td>117.4 ± 2.75 (n = 40)</td>
<td>&lt;0.0001</td>
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<td></td>
</tr>
<tr>
<td>T. violacea 0.5 (n = 4)</td>
<td>119.0 ± 2.57 (n = 63)</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td>T. violacea 50 (n = 3)</td>
<td>116.8 ± 2.20 (n = 48)</td>
<td>&lt;0.0001</td>
<td>0.5172</td>
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</tr>
</tbody>
</table>

Table D. 6 Percentage glucose taken up (± SEM) by C2C12 muscle cells. Cells were treated for one hour with aqueous extracts (0.5 or 50 µg/ml) of Bulbine frutescens, Ornithogalum longibracteatum, Ruta graveolens, Tarchonanthus camphoratus and Tulbaghia violacea combined with insulin (1 µm) at 37°C. Averages and p-values were calculated with the two-tailed unpaired t-test from three individual experiments with 12 replicates per sample for each experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 10)</td>
<td>100.0 ± 1.86 (n = 111)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin 1 µM (n = 10)</td>
<td>126.1 ± 1.97 (n = 111)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>B. frutescens 0.5 (n = 3)</td>
<td>146.5 ± 5.01 (n = 36)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>B. frutescens 50 (n = 3)</td>
<td>118.8 ± 2.58 (n = 40)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum 0.5 (n = 3)</td>
<td>148.3 ± 5.40 (n = 36)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>O. longibracteatum 50 (n = 3)</td>
<td>122.7 ± 2.63 (n = 40)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. graveolens 0.5 (n = 3)</td>
<td>138.1 ± 4.05 (n = 36)</td>
<td>&lt;0.0001</td>
<td>0.0058</td>
<td></td>
</tr>
<tr>
<td>R. graveolens 50 (n = 3)</td>
<td>125.9 ± 1.86 (n = 40)</td>
<td>&lt;0.0001</td>
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<td></td>
</tr>
<tr>
<td>T. camphoratus 0.5 (n = 3)</td>
<td>144.3 ± 6.60 (n = 35)</td>
<td>&lt;0.0001</td>
<td>0.1050</td>
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</tr>
<tr>
<td>T. camphoratus 50 (n = 3)</td>
<td>133.4 ± 2.32 (n = 40)</td>
<td>&lt;0.0001</td>
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<td></td>
</tr>
<tr>
<td>T. violacea 0.5 (n = 3)</td>
<td>137.2 ± 5.68 (n = 48)</td>
<td>&lt;0.0001</td>
<td>0.0179</td>
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</tr>
<tr>
<td>T. violacea 50 (n = 3)</td>
<td>122.0 ± 2.73 (n = 48)</td>
<td>&lt;0.0001</td>
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</tr>
</tbody>
</table>
Table D. 7 Percentage glucose uptake (± SEM) produced on C2C12 muscle cells by aqueous extracts with and without 1 µM insulin as compared to the insulin response (taken as 0%). Cells were exposed at 37ºC for one hour to aqueous extracts of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* alone (0.5 or 50 µg/ml) or these aqueous extracts (0.5 or 50 µg/ml) combined with 1 µM insulin (shown in red). P-values were calculated with the two-tailed unpaired t-test from three to six individual experiments, which had 12 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM compared to insulin</th>
<th>P-value (difference between sample and insulin)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 6) 7.71 ± 2.07 (n = 80)</td>
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<td>0.0001</td>
<td>0.2790</td>
</tr>
<tr>
<td></td>
<td>0.5 (n = 3) 11.98 ± 3.63 (n = 36)</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 (n = 4) 3.69 ± 2.26 (n = 56)</td>
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<td>0.0982</td>
<td>0.0679</td>
</tr>
<tr>
<td></td>
<td>50 (n = 3) -2.30 ± 2.16 (n = 40)</td>
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<td>0.3496</td>
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<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 4) 5.80 ± 3.08 (n = 48)</td>
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<td>0.0208</td>
<td>0.1265</td>
</tr>
<tr>
<td></td>
<td>0.5 (n = 3) 13.35 ± 3.90 (n = 36)</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td></td>
<td>50 (n = 3) -4.70 ± 2.13 (n = 40)</td>
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<td>0.0566</td>
<td>0.0956</td>
</tr>
<tr>
<td></td>
<td>50 (n = 3) 0.37 ± 2.12 (n = 40)</td>
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<td>0.8788</td>
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<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 4) 2.66 ± 2.79 (n = 48)</td>
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<td>0.2734</td>
<td>0.4613</td>
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<tr>
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<td>0.5 (n = 3) 5.76 ± 3.08 (n = 36)</td>
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<tr>
<td></td>
<td>50 (n = 3) -1.17 ± 1.12 (n = 40)</td>
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<td>0.6194</td>
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<tr>
<td></td>
<td>50 (n = 3) 2.88 ± 1.31 (n = 40)</td>
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<td>0.2245</td>
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<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 5) 6.10 ± 2.52 (n = 64)</td>
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<td>0.0063</td>
<td>0.3944</td>
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<tr>
<td></td>
<td>0.5 (n = 3) 10.49 ± 5.21 (n = 35)</td>
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<tr>
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<td>50 (n = 3) 0.49 ± 2.25 (n = 40)</td>
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<td></td>
<td>50 (n = 3) 9.01 ± 1.73 (n = 40)</td>
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<td>0.0002</td>
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<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 4) -3.83 ± 2.48 (n = 64)</td>
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<td>0.0609</td>
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<tr>
<td></td>
<td>0.5 (n = 3) 5.55 ± 4.66 (n = 48)</td>
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<td>0.0581</td>
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<tr>
<td></td>
<td>50 (n = 3) -1.80 ± 1.66 (n = 48)</td>
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<td>0.4195</td>
<td>0.4791</td>
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<tr>
<td></td>
<td>50 (n = 3) 0.04 ± 1.99 (n = 48)</td>
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<td>0.9857</td>
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</tbody>
</table>

Key: + 1 µM insulin
Table D. 8 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated at 37ºC for one hour with ethanol extracts (0.5 or 50 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea*. Averages and p-values were calculated with the two-tailed unpaired t-test from two experiments for each sample and 12 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 4)</td>
<td>100.0 ± 2.78 (n = 64)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 µM (n = 4)</td>
<td>117.9 ± 2.53 (n = 63)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 2)</td>
<td>128.3 ± 6.65 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.5217</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>123.4 ± 3.41 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 2)</td>
<td>118.3 ± 5.26 (n = 22)</td>
<td>0.0017</td>
<td>0.0436</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>133.0 ± 4.74 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 2)</td>
<td>136.9 ± 10.85 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.2698</td>
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<tr>
<td></td>
<td>50 (n = 2)</td>
<td>124.1 ± 3.74 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 2)</td>
<td>131.6 ± 6.54 (n = 23)</td>
<td>&lt;0.0001</td>
<td>0.8023</td>
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<tr>
<td></td>
<td>50 (n = 2)</td>
<td>129.7 ± 4.31 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 2)</td>
<td>140.5 ± 5.56 (n = 31)</td>
<td>&lt;0.0001</td>
<td>0.0109</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>117.7 ± 6.66 (n = 32)</td>
<td>0.0049</td>
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</tr>
</tbody>
</table>

Table D. 9 Percentage glucose taken up (± SEM) by C2C12 muscle cells. Cells were treated at 37ºC for one hour with ethanol extracts (0.5 or 50 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* combined with insulin (1 µM). Averages and p-values were calculated with the two-tailed unpaired t-test from two individual experiments per extract with 12 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake ± SEM</th>
<th>P-value (difference between sample and negative control)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 (n = 4)</td>
<td>100.0 ± 5.06 (n = 64)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 µM (n = 4)</td>
<td>133.7 ± 4.60 (n = 64)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 2)</td>
<td>159.5 ± 9.68 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.1664</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>144.0 ± 5.37 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 2)</td>
<td>150.8 ± 8.32 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.3806</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>142.5 ± 4.40 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.0065</td>
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<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 2)</td>
<td>159.3 ± 8.50 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.3343</td>
</tr>
<tr>
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<td>50 (n = 2)</td>
<td>149.5 ± 5.26 (n = 24)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 2)</td>
<td>126.5 ± 8.99 (n = 24)</td>
<td>&lt;0.0001</td>
<td>0.0048</td>
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<tr>
<td></td>
<td>50 (n = 2)</td>
<td>158.6 ± 6.09 (n = 24)</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 2)</td>
<td>133.3 ± 7.58 (n = 32)</td>
<td>0.0003</td>
<td>0.4382</td>
</tr>
<tr>
<td></td>
<td>50 (n = 2)</td>
<td>140.2 ± 4.53 (n = 32)</td>
<td>&lt;0.0001</td>
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</tr>
</tbody>
</table>
Table D. 10 Percentage glucose uptake (± SEM) produced on C2C12 muscle cells by ethanol extracts with and without 1 µM insulin as compared to the insulin response (taken as 0%). Cells were exposed at 37°C for one hour to ethanol extracts (0.5 or 50 µg/ml) of *Bulbine frutescens*, *Ornithogalum longibracteatum*, *Ruta graveolens*, *Tarchonanthus camphoratus* and *Tulbaghia violacea* alone or these ethanol extracts (0.5 or 50 µg/ml) combined with 1 µM insulin (shown in red). P-values were calculated with the two-tailed unpaired t-test from two individual experiments with 12 replicates per sample per experiment. Statistical significance (p < 0.05) is indicated in bold.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration (µg/ml) (number of experiments)</th>
<th>% glucose uptake ± SEM compared to insulin</th>
<th>P-value (difference between sample and insulin)</th>
<th>P-value (difference between two concentrations tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. frutescens</em></td>
<td>0.5 (n = 2) 14.18 ± 5.91 (n = 24)</td>
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<td>0.0047</td>
<td>0.8444</td>
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<tr>
<td></td>
<td>0.5 (n = 2) 15.88 ± 6.25 (n = 24)</td>
<td></td>
<td>0.0019</td>
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</tr>
<tr>
<td></td>
<td>50 (n = 2) 0.20 ± 2.83 (n = 24)</td>
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<td>0.9636</td>
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</tr>
<tr>
<td></td>
<td>50 (n = 2) 10.86 ± 4.17 (n = 24)</td>
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<td>0.0197</td>
<td></td>
</tr>
<tr>
<td><em>O. longibracteatum</em></td>
<td>0.5 (n = 2) 5.30 ± 4.59 (n = 22)</td>
<td></td>
<td>0.2726</td>
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</tr>
<tr>
<td></td>
<td>0.5 (n = 2) 9.46 ± 5.21 (n = 24)</td>
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<td>0.0505</td>
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<tr>
<td></td>
<td>50 (n = 2) 7.89 ± 3.83 (n = 24)</td>
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<td>50 (n = 2) 9.68 ± 3.40 (n = 24)</td>
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<td>0.0326</td>
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<tr>
<td><em>R. graveolens</em></td>
<td>0.5 (n = 2) 21.77 ± 9.58 (n = 24)</td>
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<td>0.0003</td>
<td>0.6144</td>
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<tr>
<td></td>
<td>0.5 (n = 2) 16.12 ± 5.71 (n = 24)</td>
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<td>0.0013</td>
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<td>50 (n = 2) 0.70 ± 3.03 (n = 24)</td>
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<td>50 (n = 2) 15.09 ± 4.05 (n = 24)</td>
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<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 (n = 2) 17.10 ± 5.74 (n = 23)</td>
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<td>0.0007</td>
<td>0.0089</td>
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<td>0.5 (n = 2) -6.86 ± 6.57 (n = 24)</td>
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<td>50 (n = 2) 5.24 ± 3.50 (n = 24)</td>
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<tr>
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<td>50 (n = 2) 19.59 ± 4.10 (n = 23)</td>
<td>&lt;0.0001</td>
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</tr>
<tr>
<td><em>T. violacea</em></td>
<td>0.5 (n = 2) 25.07 ± 4.90 (n = 31)</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
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</tr>
<tr>
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<td>0.5 (n = 2) -2.72 ± 4.95 (n = 32)</td>
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<tr>
<td></td>
<td>50 (n = 2) -8.45 ± 3.79 (n = 31)</td>
<td>0.0443</td>
<td>0.0023</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 (n = 2) 7.86 ± 3.47 (n = 32)</td>
<td>0.0545</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: + 1 µM insulin
E. List of reagents

1-amino-2-naphthol-4-sulfonic acid (ANSA), Local supplier (analytical grade), India
2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), Merck, Gauteng, South Africa
2-(N-morpholino)ethanesulfonic acid (MES), Sigma, Steinheim, Germany
4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), Sigma-Aldrich Co, USA
5’adenosine monophosphate (5’AMP), Sigma-Aldrich Co, USA
[^125I] insulin, Linco Research Inc., St Charles, USA
[^3H] deoxyglucose (deoxy-D-glucose, 2[1,2-3H(N)]), Perkin Elmer life and analytical sciences, Boston, USA
Adenine 9-β-D-arabinofuranoside (ara), Sigma, Steinheim, Germany
Ammonium molybdate, Sigma-Aldrich Co, USA
Bicinchoninic acid (BCA) (disodium salt), Sigma, Steinheim, Germany
Bovine serum albumin (BSA), Roche Diagnostics, Mannheim, Germany
Carbon dioxide (CO₂), Afrox, South Africa
Copper sulphate (CuSO₄), Saarchem, Krugersdorp, South Africa
D- (+)-glucose, Associated Chemical Enterprises, Glenvista, South Africa
Dimethylsulfoxide (DMSO), Associated Chemical Enterprises, Southdale, South Africa
Dimethylsulfoxide (DMSO) (section 7.2.5.1), Sigma-Aldrich Co, USA
Dinitrophenol (DNP), Merck, Germany
Disodium sulphate (Na₂SO₄), Merck, Germany
Dithiothreitol (DTT), Sigma-Aldrich Co, USA
Ethylendiaminetetra-acetic acid (EDTA), BDH AnalR, Gauteng, South Africa
Ethanol 95% (ethyl alcohol 4111), Hazchem, South Africa
Fetal bovine serum (fbs), Delta Bioproducts, Johannesburg, South Africa
Goat-anti-rabbit horseradish peroxidase antibodies(GAR-HRP), Bio-Rad
Glucose-1-phosphate (G1P), Sigma-Aldrich Co, USA
Glucose-6-phosphate (G6P), Sigma-Aldrich Co, USA
Glucose oxidase, Sera-pak Plus, Hong Kong
GLUT4 antibodies (Rabbit anti-GLUT4 43 kD, polyclonal antibody, AB1346), Chemicon International
GLUT4 substrate (Amersham ECL™ western blotting analysis system), Amersham Biosciences, Buckinghamshire, England
Glycogen, Sigma-Aldrich Co, USA
Glycine, Merck, Gauteng, South Africa
Hydrochloric acid (HCl), Ibhayi Laboratory Supplies, Port Elizabeth, South Africa
Insulin (human, recombinant), Roche Diagnostics, Mannheim, Germany
Maltase (from yeast), Sigma, Steinheim, Germany
Metformin, Helm AG, Hamburg, Germany
Methanol (MeOH), Associated Chemical Enterprises, Glenvista, South Africa
Milk powder, Elite (fat-free), Rhoodepoort, South Africa
Nonidet P40, Fluka, Steinheim, Germany
PD169316, Sigma-Aldrich Co, USA
Phenol red, May & Backer, USA
p-nitrophenyl α-D-glucopyranoside, Sigma-Aldrich Co, USA
p-nitrophenyl phosphate (PNPP), Sigma-Aldrich Co, USA
Potassium chloride (KCl) analytical grade (section 7.2.5.1), local suppliers, India
Potassium hydroxide (KOH), SMM Chemicals (Pty) Limited, Booyens, South Africa
Protease inhibitor cocktail (mammalian), Sigma-Aldrich Co, USA
RPMI-1640 with 25mM HEPES and L-glutamine, BioWhittaker, Walkersville, USA
Scintillation cocktail (Ultima Gold), Perkin Elmer Life and Analytical sciences, Boston, USA
Sodium acetate, Sigma-Aldrich Co, USA
Sodium chloride (NaCl), Associated Chemical Enterprises; Glenvista, South Africa
Sodium citrate (section7.2.5.3; analytical grade), Local suppliers, India
Sodium dodecyl sulphate (SDS), BDH Laboratory Supplies, England
Sodium fluoride (NaF), Sigma-Aldrich Co, USA
Sodiumhydrogen carbonate (NaHCO3), Associated Chemical Enterprises, Southdale, South Africa
Sodium hydroxide (NaOH), Analysed Analytical Reagents, Vorna Valley, South Africa
Sodium hydroxide (NaOH) analytical grade (section 7.2.5.2), Sigma-Aldrich Co, USA
Sodium metabisulfate (analytical grade), Local suppliers, India
Sodium sulfite (analytical grade), Local suppliers, India
Sucrose, BDH AnalR, Gauteng, South Africa
Sulfuric acid (H2SO4) analytical grade, local suppliers, India
Trichloroacetic acid (TCA) (section 7.2.5.3), Sigma-Aldrich Co, USA
TWEEN 20, Sigma, St Louis, USA
Wortmannin (from *Penicilium funiculosum*), Sigma, Steinheim, Germany
Table F. 1  Percentage glucose taken up (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with *Tarchonanthus camphoratus* aqueous extracts. Extracts were prepared in October 2004, November 2005 and March 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiment done in March 2006). Averages and p-values were calculated from one experiment with 10 replicates per sample. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake (± SEM) March 2006</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 2.81 (n = 20)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM</td>
<td>121.1 ± 4.29 (n = 20)</td>
<td>0.0002</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>106.3 ± 3.98 (n = 10)</td>
<td>0.2088</td>
<td>0.1963</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>114.2 ± 4.36 (n = 10)</td>
<td>0.0086</td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>126.0 ± 3.97 (n = 10)</td>
<td>&lt;0.0001</td>
<td>0.8695</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>125.2 ± 3.39 (n = 10)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>133.9 ± 6.06 (n = 10)</td>
<td>&lt;0.0001</td>
<td>0.0657</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>120.2 ± 3.50 (n = 10)</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>126.3 ± 5.00 (n = 10)</td>
<td>&lt;0.0001</td>
<td>0.0909</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>140.0 ± 5.78 (n = 10)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table F. 2  Percentage glucose uptake (± SEM) by Chang liver cells. Cells were treated for 48 hours (0.125 or 12.5 µg/ml) and again for three hours (0.5 or 50 µg/ml) with *Tarchonanthus camphoratus* aqueous extracts. Extracts were prepared in October 2004, November 2005, March 2006 and July 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiments done in January 2007). Averages and p-values were calculated from two independent experiments with 10 replicates per sample per experiment. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake (± SEM) January 2007</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 3.34 (n = 60)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM</td>
<td>126.6 ± 4.78 (n = 57)</td>
<td>&lt;0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>99.82 ± 5.31 (n = 20)</td>
<td>0.9777</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>145.7 ± 5.42 (n = 20)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>132.2 ± 5.69 (n = 20)</td>
<td>&lt;0.0001</td>
<td>0.7544</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>135.0 ± 6.73 (n = 20)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>89.86 ± 10.59 (n = 20)</td>
<td>0.2294</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>186.7 ± 12.46 (n = 20)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>96.16 ± 6.85 (n = 18)</td>
<td>0.5934</td>
<td>0.0269</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>130.2 ± 13.03 (n = 18)</td>
<td>0.0018</td>
<td></td>
</tr>
<tr>
<td>July 2006</td>
<td>0.5</td>
<td>116.3 ± 5.46 (n = 19)</td>
<td>0.0171</td>
<td>0.2480</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>127.7 ± 8.08 (n = 18)</td>
<td>0.0004</td>
<td></td>
</tr>
</tbody>
</table>
Table F. 3 Percentage growth inhibition (± SEM) produced on Chang liver cells by *Tarchonanthus camphoratus* aqueous extracts. Cells were treated for 48 hours with (0.125 or 12.5 µg/ml) extracts that were prepared in October 2004, November 2005 and March 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiment done in March 2006). Averages and p-values were calculated from one experiment with 10 replicates per sample. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% growth inhibition (± SEM) March 2006</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0.00 ± 1.74 (n = 12)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM</td>
<td>2.02 ± 2.42 (n = 12)</td>
<td>0.5037</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>-2.26 ± 2.66 (n = 6)</td>
<td>0.4753</td>
<td>0.1459</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.14 ± 2.15 (n = 6)</td>
<td>0.2945</td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>7.21 ± 4.50 (n = 6)</td>
<td>0.0875</td>
<td>0.4563</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.02 ± 3.00 (n = 6)</td>
<td>0.3630</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>5.04 ± 2.61 (n = 6)</td>
<td>0.1199</td>
<td>0.4862</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.66 ± 2.00 (n = 6)</td>
<td>0.3629</td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>-0.28 ± 1.09 (n = 6)</td>
<td>0.9162</td>
<td>0.0278</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.68 ± 1.59 (n = 6)</td>
<td>0.1047</td>
<td></td>
</tr>
</tbody>
</table>

Table F. 4 Percentage growth inhibition (± SEM) produced on Chang liver cells by *Tarchonanthus camphoratus* aqueous extracts. Cells were treated for 48 hours with (0.125 or 12.5 µg/ml) extracts that were prepared in October 2004, November 2005, March 2006 and July 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiments done in January 2007). Averages and p-values were calculated from two independent experiments with 10 replicates per sample per experiment. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% growth inhibition (± SEM) January 2007</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0.00 ± 1.57 (n = 36)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 µM</td>
<td>0.83 ± 1.28 (n = 36)</td>
<td>0.6852</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>-7.75 ± 3.27 (n = 12)</td>
<td><strong>0.0235</strong></td>
<td><strong>0.0010</strong></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.23 ± 2.65 (n = 12)</td>
<td><strong>0.0115</strong></td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>1.22 ± 2.04 (n = 12)</td>
<td>0.6841</td>
<td>0.1576</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-3.87 ± 2.82 (n = 12)</td>
<td>0.2286</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>-1.74 ± 3.13 (n = 12)</td>
<td>0.5974</td>
<td><strong>0.0010</strong></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>13.96 ± 2.70 (n = 12)</td>
<td>&lt;<strong>0.0001</strong></td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>4.12 ± 2.74 (n = 12)</td>
<td>0.1977</td>
<td>0.0916</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-3.11 ± 3.05 (n = 12)</td>
<td>0.3419</td>
<td></td>
</tr>
<tr>
<td>July 2006</td>
<td>0.5</td>
<td>-4.46 ± 2.34 (n = 11)</td>
<td>0.1619</td>
<td>0.1519</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.56 ± 2.42 (n = 11)</td>
<td>0.8599</td>
<td></td>
</tr>
</tbody>
</table>
Table F. 5 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated with *Tarchonanthus camphoratus* aqueous extracts (0.5 or 50 µg/ml) that were prepared in October 2004, November 2005 and March 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiment performed in May 2006). Averages and p-values were calculated from one experiment with 12 replicates per sample. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake (± SEM) May 2006</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 5.47 (n = 22)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 µM</td>
<td>118.9 ± 6.30 (n = 24)</td>
<td>0.0294</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>96.04 ± 9.55 (n = 12)</td>
<td>0.7003</td>
<td>0.1447</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>115.1 ± 8.19 (n = 12)</td>
<td>0.1247</td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>123.1 ± 7.33 (n = 12)</td>
<td>0.0170</td>
<td>0.3382</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>111.5 ± 9.24 (n = 12)</td>
<td>0.2592</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>95.43 ± 6.60 (n = 12)</td>
<td>0.6101</td>
<td>0.5591</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>89.95 ± 6.45 (n = 12)</td>
<td>0.2629</td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>114.0 ± 8.71 (n = 12)</td>
<td>0.1634</td>
<td>0.2522</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>127.3 ± 7.26 (n = 12)</td>
<td>0.0054</td>
<td></td>
</tr>
</tbody>
</table>

Table F. 6 Percentage glucose uptake (± SEM) by C2C12 muscle cells. Cells were treated for one hour with *Tarchonanthus camphoratus* aqueous extracts (0.5 or 50 µg/ml). Extracts were prepared in October 2004, November 2005, March 2006 and July 2006, thus showing seasonal variation and storage stability on glucose uptake of dried extracts (experiment performed in October 2006). Averages and p-values were calculated from two independent experiments with 12 replicates per sample per experiment. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Time of extraction</th>
<th>Concentration (µg/ml)</th>
<th>% glucose uptake (± SEM) October 2006</th>
<th>P-value (compared to the control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 3.96 (n = 41)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 µM</td>
<td>128.5 ± 5.37 (n = 41)</td>
<td>&lt; 0.0001</td>
<td>N/A</td>
</tr>
<tr>
<td>October 2004</td>
<td>0.5</td>
<td>94.42 ± 14.24 (n = 11)</td>
<td>0.5982</td>
<td>0.9469</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>95.60 ± 10.03 (n = 11)</td>
<td>0.6342</td>
<td></td>
</tr>
<tr>
<td>November 2005</td>
<td>0.5</td>
<td>138.8 ± 12.92 (n = 12)</td>
<td>0.0003</td>
<td>0.0253</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>88.75 ± 16.53 (n = 11)</td>
<td>0.3257</td>
<td></td>
</tr>
<tr>
<td>March 2006</td>
<td>0.5</td>
<td>93.05 ± 6.91 (n = 24)</td>
<td>0.3500</td>
<td>0.3613</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>85.31 ± 4.76 (n = 24)</td>
<td>0.0235</td>
<td></td>
</tr>
<tr>
<td>March 2006 (Decoction)</td>
<td>0.5</td>
<td>123.3 ± 10.46 (n = 21)</td>
<td>0.0145</td>
<td>0.4923</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>132.5 ± 8.01 (n = 21)</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>July 2006</td>
<td>0.5</td>
<td>107.2 ± 5.98 (n = 24)</td>
<td>0.3032</td>
<td>0.5271</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>101.9 ± 5.71 (n = 24)</td>
<td>0.7808</td>
<td></td>
</tr>
</tbody>
</table>
Table F. 7 Percentage $^{125}$I insulin bound to insulin receptors on C2C12 muscle cells. Cells were exposed to insulin or Tarchonanthus camphoratus aqueous extract for 20 minutes at 20ºC. Averages and p-values were calculated from quadruplicate values of one experiment. Statistical significance is indicated in bold (p < 0.05). This experiment was repeated and similar results were obtained.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% $^{125}$I insulin bound (± SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100.0 ± 20.29 (n = 4)</td>
<td>NA</td>
</tr>
<tr>
<td>Insulin (1 µm)</td>
<td></td>
<td>-0.000000005961 ± 2.77 (n = 4)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Insulin (0.01 µm)</td>
<td></td>
<td>10.84 ± 3.76 (n = 3)</td>
<td>0.0143</td>
</tr>
<tr>
<td>Insulin (0.001 µm)</td>
<td></td>
<td>46.82 ± 22.63 (n = 3)</td>
<td>0.1425</td>
</tr>
<tr>
<td>T. camphoratus (50 µg/ml)</td>
<td></td>
<td>65.95 ± 12.52 (n = 4)</td>
<td>0.2032</td>
</tr>
<tr>
<td>T. camphoratus (0.5 µg/ml)</td>
<td></td>
<td>79.63 ± 11.78 (n = 4)</td>
<td>0.4186</td>
</tr>
</tbody>
</table>

Table F. 8 Percentage $^{3}$H deoxyglucose uptake (± SEM) into C2C12 muscle cells. Cells were pre-incubated with and without wortmannin (100 nM) for 30 minutes at 37ºC. Then cells were either left untreated or were exposed to insulin 1 µM or Tarchonanthus camphoratus aqueous extract (0.5 and 50 µg/ml) with (W) and without wortmannin for 20 minutes at 37ºC. Averages and statistical evaluations were calculated from data of one individual experiment with four replicates per sample. P-values were calculated with the two-tailed unpaired t-test and considered significant if p < 0.05.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% $^{3}$H deoxyglucose uptake (± SEM; n = 4)</th>
<th>P-value (compared to negative control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 2.71</td>
<td>N/A</td>
<td>0.3896</td>
</tr>
<tr>
<td>Negative control W</td>
<td>0</td>
<td>104.2 ± 3.66</td>
<td>0.3896</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>1 µM</td>
<td>133.3 ± 3.73</td>
<td>0.0004</td>
<td>0.0015</td>
</tr>
<tr>
<td>Insulin W</td>
<td>1 µM</td>
<td>107.4 ± 2.83</td>
<td>0.1088</td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>0.5 µg/ml</td>
<td>109.6 ± 1.77</td>
<td>0.0281</td>
<td>0.0524</td>
</tr>
<tr>
<td></td>
<td>0.5W µg/ml</td>
<td>102.4 ± 3.08</td>
<td>0.5160</td>
<td></td>
</tr>
<tr>
<td>T. camphoratus</td>
<td>50 µg/ml</td>
<td>118.4 ± 1.97</td>
<td>0.0013</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>50W µg/ml</td>
<td>101.9 ± 2.25</td>
<td>0.6596</td>
<td></td>
</tr>
</tbody>
</table>
Table F. 9  Percentage $[^{3}H]$ deoxyglucose uptake (± SEM) into C2C12 muscle cells. Cells were pre-incubated with and without adenine 9-β-D-arabinofuranoside (ara; 1 mM) for 30 minutes at 37ºC. Then cells were either left untreated or were exposed to dinitrophenol (DNP; 1 µM) or *Tarchonanthus camphoratus* aqueous extract (0.5 and 50 µg/ml) with (A) and without ara for 30 minutes at 37ºC. Averages and statistical evaluations were calculated from data of one individual experiment with four replicates per sample. P-values were calculated with the two-tailed unpaired t-test and considered significant if p < 0.05.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% $[^{3}H]$ deoxyglucose uptake (± SEM; n = 4)</th>
<th>P-value (compared to negative control)</th>
<th>P-value (compared between concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0</td>
<td>100.0 ± 1.86</td>
<td>N/A</td>
<td>0.1937</td>
</tr>
<tr>
<td>Negative control A</td>
<td>0</td>
<td>103.0 ± 1.25</td>
<td>0.1937</td>
<td></td>
</tr>
<tr>
<td><strong>DNP</strong></td>
<td>0.5 mM</td>
<td>163.9 ± 3.23</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>DNP A</strong></td>
<td>0.5 mM</td>
<td>112.9 ± 3.22</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 µg/ml</td>
<td>109.3 ± 3.83</td>
<td>0.0194</td>
<td>0.0352</td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>0.5 µg/ml</td>
<td>98.37 ± 2.23</td>
<td>0.6523</td>
<td></td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>50 µg/ml</td>
<td>112.3 ± 3.39</td>
<td>0.0057</td>
<td>0.0080</td>
</tr>
<tr>
<td><em>T. camphoratus</em></td>
<td>50 µg/ml</td>
<td>98.85 ± 3.31</td>
<td>0.6941</td>
<td></td>
</tr>
</tbody>
</table>

Table F. 10  Percentage α-glucosidase activity (± SEM) for different concentrations of *Tarchonanthus camphoratus* aqueous extract. The enzyme was exposed to different concentrations of the extract for 15 minutes at 37ºC. Averages and p-values were calculated from a minimum of two independent experiments with three replicates per sample per experiment. Statistical significance is indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th><em>T. camphoratus</em> concentration</th>
<th>Percentage α-glucosidase enzyme activity (± SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg/ml</td>
<td>100.0 ± 1.40 (n = 8)</td>
<td>NA</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>87.13 ± 10.04 (n = 6)</td>
<td>0.1661</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>79.72 ± 10.38 (n = 6)</td>
<td>0.0441</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>55.97 ± 2.39 (n = 9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>56.99 ± 4.47 (n = 9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1000 µg/ml</td>
<td>55.73 ± 4.75 (n = 9)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
G. Indigenous knowledge agreement between traditional African health practitioners of the NMM and Department of Biochemistry and Microbiology, Department of Pharmacy, Department of Biomedical Science of the Nelson Mandela Metropolitan University

The purpose of a written agreement between the Nelson Mandela Metropolitan University and traditional healers in the Nelson Mandela Metropole is to define the research collaboration formally. Many misconceptions exist among some traditional healers about the role of the university and the traditional healers participating in the collaboration. The university does not want to exclude any healers from the collaboration or force any healer to participate. Participation is exclusively voluntary and all participating healers are under no obligation to participate beyond any level that is comfortable for them.

Primary aim of the research collaboration

The primary aim of the collaboration is to promote the traditional African healing system through the sharing of traditional as well as scientific knowledge.

Collaboration activities

Activities of the collaboration include:
1. Interactive workshops, group discussions and meetings,
2. Individual interviews,
3. Medicinal garden activities,
4. Medicinal plant research,
5. Research outputs, and
6. Commercialisation.

Healers are free to choose in which activities they would like to participate or not.

1. Interactive workshops, group discussions and meetings

An interactive workshop may be defined as a workshop that aims to provide healers with an understanding of scientific information on a specific topic (e.g., diabetes mellitus). During the workshop healers are encouraged to share their traditional understanding of the same topic.

Group discussions and meetings are held in order to generate input from healers on the development and ways to continue the collaboration. All suggestions and comments are noted and incorporated into the partnership if practically possible.
2. Individual interviews

Individual interviews will be conducted with healers if more detailed information on a specific research topic is required after an initial group discussion session. The healers will always be informed of the topic and purpose of the interview beforehand and will be reminded that they are under no obligation to answer any questions if they do not want to. The interviews will be held with healers willing to participate, who have given written informed consent prior to the interview (refer to Appendix G.1: NMMU Information and Informed Consent Form).

3. Medicinal garden

The medicinal garden will be established with the help of the university and any interested sponsors (refer to Appendix G.2 for business plan). The purpose of the garden is to aid communication between healers and scientists.

Participating healers will be expected to;
- Attend cultivation workshops,
- Help with the maintenance of the garden,
- Provide Xhosa names of the plants growing in the garden, and
- Take over the managing role once the garden has been developed.

The university will be expected to;
- Host cultivation workshops,
- Provide Latin names of the plants growing in the garden,
- Provide existing monographs on plants growing in the garden,
- Test plants growing in the garden for biological activity, and
- Give over the managing role to the healers once the garden has been developed.

The ultimate aim of the garden is to provide:
- Healers with medicinal plants,
- The university with medicinal plants for research,
- Healers with scientific information on the plants growing in the garden by means of the development of a database available in the garden,
- A place to do cultural rituals and ceremonies, and
- Healers with an economic development possibility in the form of a tourist destination in future.

4. Medicinal plant research

- Biological activity screening will be done on medicinal plants growing in the garden.
- Healers may suggest plant candidates that they want investigated.
- Results of these experiments will be distributed among participating healers via the newsletter and/or via research seminars held at the university.
• Healers will be able to comment on findings and recommend on further research at the seminars or via the newsletter.
• Healers’ suggestions and recommendation will be noted and followed as far as practically possible.

5. Research outputs

It has been established that the production of research outputs is necessary in order to ensure the continuation of funding for the research collaboration. Research outputs may be defined as posters, podium presentations or publications resulting from research done on medicinal plants selected from the garden.

In the case of producing a research output:
• Chairpersons and members of participating organisations will be consulted about poster or podium presentations for scientific conferences as well as articles written for scientific journals.
• They will be asked to sign an informed consent form to acknowledge that they have indeed been informed of any research outputs.
• The chairpersons of the participating organisations will afterwards be provided with feedback on the conferences as well as any publications of the research.
• All research outputs will acknowledge the healers for their part in the research collaboration (refer to Appendix G.3).

6. Commercialisation

At present, research is in the discovery phase and it is unknown if any part of the research will be of use in the development of commercial products. If commercialisation is a relevant possibility, a benefit-sharing agreement will be negotiated with all participating healers according to the stipulations of the National Environmental Management: Biodiversity Act (No 10 of 2004), Patents Amendment Act (No 20 of 2005) and any acts that may make reference to indigenous knowledge in the future. The three organisations who have participated in the project will be the primary beneficiaries of any commercialisation and benefit sharing will be determined as set out in the legislation.

Revision of the Agreement

The contents of this agreement will be reviewed by all parties every year from the date of signing of the agreement (any party that would like to join at the annual revision meeting will be welcomed). If funding for the research is terminated the provisions of the agreement will stand until that date and any continuous benefit-sharing agreements produced by the collaboration will continue.
Signing of the agreement:

We, the undersigned agree that the contents of this agreement are true and will uphold the agreement.

Organization:
Chairperson:
Vice-chairperson:
Contact details:

Nelson Mandela Metropolitan University
Department of:
Coordinator:
Contact details:
## Title of the research project

### Reference number

### Principal investigator

### Address

### Postal Code

### Contact telephone number

*(private numbers not advisable)*

## DECLARATION BY OR ON BEHALF OF PATIENT/PARTICIPANT

I, the undersigned

I.D. number

Address

## HEREBY CONFIRM AS follows:

I / the participating traditional healer was invited to participate in the above-mentioned research in the capacity of a knowledgeable person in a project that is being undertaken by ……………………………………………………………

Of the Department of ……………………………………………………………

In the Faculty of ………………………………………………………………………

Of the Nelson Mandela Metropolitan University.

The following aspects have been explained to me/the patient/participant:

### Aim:

The investigators are studying ………………………………………

The information will be used to/for ………………………………………

### Procedures:

I understand that ………………………………………

### Risks:

………………………………………………………………………….

### Possible benefits:

As a result of my participation in this study

### Confidentiality:

My identity will not be revealed in any discussion, description or scientific publications by the investigators, **unless I request my identity to be disclosed.**
**Access to findings:** Feedback will be given ………………………………

**Voluntary participation/refusal/discontinuation:** My participation is voluntary. My decision whether or not to participate will in no way affect my present or future lifestyle.

The information above was explained to me / the participant by

<table>
<thead>
<tr>
<th></th>
<th>Afrikaans</th>
<th>English</th>
<th>Xhosa</th>
<th>Other</th>
</tr>
</thead>
</table>

and I am in command of this language / it was satisfactorily translated to me by

I was given the opportunity to ask questions and all these questions were answered satisfactorily.

No pressure was exerted on me to consent to participation and I understand that I may withdraw at any stage without penalisation.

Participation in this study will not result in any additional cost to myself.

**I HEREBY CONSENT VOLUNTARILY TO PARTICIPATE IN THE ABOVE-MENTIONED PROJECT**

Signed/confirmed at…………………………………………….  …………………………………

Signature or right thumb print of participant  Signature of witness 20..
I, ………………………, declare that

- I have explained the information given in this document to ……………………………………………………………………….. and/or his/her representative ………………………………………………………………………..

- he/she was encouraged and given ample time to ask me any questions;

- this conversation was conducted in [ ] Afrikaans [ ] English [ ] Xhosa [ ] Other

and this conversation was translated into

by

Signed/confirmed on 20…

at ………………………………………………………………………………………………………

……………………………………………………………………………………………………

Signature or right thumb print of participant Signature of witness
Appendix G.2 Business Plan for Umyezo Wamanyange Medicinal Garden

(Please contact author for information)

Appendix G.3 Acknowledgement of healers on Poster, Papers and Publications

We would like to acknowledge the following healers and organisations for their contribution to the research; Fezeka Kosi and members of Nyangazezizwe Traditional Healers Association, Mr PJ Rala and members of Traditional Health Practitioners: Nelson Mandela Bay Metro and Tandiwe Mazamane and members from the Eastern Cape Task Team.

Appendix G.4 Preamble

(Please contact author for this information, the nature of which is sensitive)
### References


Andre, T., Boni, C. & Mounedji-Boufiaf, L., 2004. "Adjuvant chemotherapy with oxaliplatin, in combination with fluorouracil plus leucovorin prolongs disease-free survival, but causes more adverse events in people with stage II or III colon cancer,". *Cancer Treatment Reviews*, vol. 30, no. 8, pp. 711-713.


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