

**Medicinal
Effects of
Agathosma
(Buchu) Extracts**



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Barbara Huisamen



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Research Justification

The research results presented in this book describe the quest of Cape Kingdom Nutraceuticals, Prof. Patrick Bouic and Prof. Barbara Huisamen to scientifically validate the health-promoting properties of an aqueous extract of the plant *Agathosma*. To accomplish this, they used both *in vitro* and *in vivo* models to understand and underscore the anecdotal information regarding the benefits of this product. Cell-based studies were utilised to highlight anti-inflammatory and anti-diabetic effects, whereas animal-based studies were utilised to confirm the anti-diabetic effects while further elaborating the anti-obesity properties. In addition, technology aimed at small animals was used to demonstrate, by means of blood pressure measurement, anti-hypertensive effects, while *ex vivo* perfused hearts were studied to show that this extract could also protect the heart against an ischaemic incident.

The research summarised in this book is novel, original and has not been published previously, and the results presented validate future use of this extract for the above-mentioned health-promoting properties. In view of the current pandemic of obesity and non-communicable diseases, the research results presented in this book will be of special interest to the scientific community and health practitioners interested in cardiometabolic diseases and nutraceuticals as an alternative treatment option. In light of the failure of many pharmaceuticals to curb non-communicable diseases, these results are deemed of high importance.

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Abbreviations, Figures and Tables appearing in the Text and Notes

List of Abbreviations

ACE	Angiotensin 1-Converting Enzyme
ActB	β -actin
ADH	Anti-diuretic Hormone
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BP	Blood Pressure
CAF	Central Analytical Facilities
CETP	Cholesteryl Ester Transfer Protein
CHO	Carbohydrate Content
CVD	Cardiovascular Diseases
DBP	Diastolic Blood Pressure
DG	Deoxy-glucose
DIO	Diet-induced Obesity
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	Food and Drugs Administration
FRET	Fluorescence Resonance Energy Transfer
GABA	γ -aminobutyric Acid (Gamma-aminobutyric Acid)
GRAS	Generally Recognized as Safe
HDL	High-density Lipoprotein
HFD	High-fat Diet
IAPP	Islet Amyloid Polypeptide
IPGTT	Intraperitoneal Glucose Tolerance Test
KH	Krebs Henseleit
LCAT	Lecithin-cholesterol Acyltransferase

LDL	Low-density Lipoproteins
LPS	Lipopolysaccharide
Maf A	Musculoaponeurotic Fibrosarcoma Homolog A
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOEL	No Observable Effect Levels
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator-activated Receptors
PVDF	Polyvinylidene Difluoride
RAAS	Renin-Angiotensin-Aldosterone System
RCT	Reverse Cholesterol Transport
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
SBP	Systolic Blood Pressure
SEM	Standard Error of the Mean
SG	Stafford GI
SOD	Superoxide Dismutase
SREBP	Sterol Regulatory Element-binding Proteins
TEAC	Trolox Equivalent Antioxidant Capacity
US	United States
USA	United States of America
VLDL	Very Low-density Lipoproteins
WHO	World Health Organization

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Barbara Huisamen obtained her PhD from the University of Stellenbosch in 1993, with a dissertation on the characterisation of the inositol trisphosphate receptor of the sarcoplasmic reticulum and its role in handling calcium levels. She continued her research in the Division of Medical Physiology of the Faculty of Medicine and Health Sciences at the Tygerberg Campus of the University of Stellenbosch, where she is currently an emeritus professor. She was also a Chief Specialist Scientist at the SA-MRC Biomedical, Research and Innovation Platform until 31 December 2018. Her preclinical research has 2 main focus areas, namely (1) myocardial insulin signalling in obesity, type 2 diabetes and hypertension and (2) pharmacological or nutraceutical interventions and treatment options to protect and improve pathology.

Prof. Huisamen has published 67 peer-reviewed articles on cardiometabolic diseases and cardioprotection, which drew about 2000 citations from peers. She has presented her work at more than 70 international conferences, also as invited speaker, and at more than 150 national meetings. She has also presented her work as a guest speaker. She holds various patents on the use of nutraceuticals as treatment options in disease states. Prof. Huisamen has a keen interest in training young scientists and has graduated 48 MSc and PhD students in her career to date.

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Patrick J.D. Bouic was trained as an immunopathologist in France and graduated with a PhD from the University of Claude Bernard, Lyon, in 1984. He has been a board-certified immunologist with the HPCSA since 1994 and has been with the University of Stellenbosch Faculty of Medicine and Health Sciences (in the Division of Pathology [Medical Microbiology]) since 1984. As an academic, he lectures to undergraduate students and oversees many postgraduate candidates registered with the university. He was the head of the Diagnostic Immunology Laboratory for 20 years before forming a private company, Synexa Life Sciences, in Cape Town in 2002, where he is one of the founding directors and serves as the Chief Scientific Officer. The company has its headquarters in Cape Town, but also has a laboratory in London and is currently opening a new facility in Berlin. These laboratories provide biomarker support services in Europe for larger pharma companies as well as smaller biotechnology groups.

Prof. Bouic was personally involved in the development of a natural immune supportive supplement (Moducare™), which was launched in the South African market in 2000 and is currently sold globally. He co-authored a book entitled *The Immune System Cure* with Lorna van den Haeghe, which received the gold medal for 2 years running as best seller by the Canadian Health Food Association in 2002 and 2003. This book outlines natural remedies that can be used to manage chronic diseases as well as those used to prevent immune conditions linked to the ageing process.

Patrick has a passion for knowledge sharing and to date he has done more than 150 conference presentations, both locally

and internationally. He has been widely published (with more than 150 peer-reviewed publications and several book chapters to his credit) and has numerous patents covering new formulations derived from natural sources. He serves on the SAB of Becton Dickinson, an international company that develops reagents and technologies for diagnostics and research purposes.

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Carmen Pheiffer began her career by studying antibiotic resistance in *Enterococcus faecium*, whereafter she obtained her MSc degree (with distinction) from the University of Cape Town, studying transcriptional regulation of the gonadotropin-releasing hormone receptor gene. She received a prestigious GSK PhD scholarship to study *Mycobacterium tuberculosis* protein expression and humoral immune responses of tuberculosis patients, under the supervision of Prof. Paul van Helden. Dr Pheiffer received postdoctoral training in the laboratory of Prof. Gennaro at the Public Health Research Institute, USA. In 2007, she joined the South African Medical Research Council to establish the molecular biology laboratory and coordinate gene and protein expression experiments at the Biomedical Research and Innovation Platform that primarily conducted contract research bound by confidentiality agreements. Her research involves using cell and animal models to investigate the anti-obesity and anti-diabetic potential of indigenous plant extracts. Recently, her research has expanded to include DNA methylation and microRNA regulation during obesity, type 2 diabetes and gestational diabetes in South African women. Dr Pheiffer supervises postgraduate students and has collaborations with scientists from several universities and the Agricultural Research Council.

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Mignon obtained her PhD in Medical Physiology from the University of Stellenbosch in 2018. Her research involved the link between natural substances and their effects on diabetes, hypertension and cardiovascular health. She was awarded several bursaries during her research career, namely University of Stellenbosch Merit Bursaries for 2014 to 2017, NRF Grant Holder-linked Bursaries respectively for 2015 and 2018, and Harry Crossley Foundation Bursaries for 2016 and 2017. She presented her research work at the Physiology Society of South Africa (PSSA) in 2014, at the University of Stellenbosch Academic Year Day in 2014 and 2016, and at the Biomedical Science Year Day in 2015 and 2017, where she respectively won the best poster presentation award, and the second price in the poster presentation section.

Foreword

Saartjie Roux

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I took great pleasure in reviewing this well-structured book on the medicinal use of *Agathosma* extracts. All the authors can be commended for their contribution towards novel scientific evidence on the anti-inflammatory-, anti-diabetic-, anti-obesity and anti-hypertensive properties of this plant extract. Novel information is also provided on protection shown by the plant extract against ischaemic incidences in *ex vivo* perfused hearts. In the light of the pandemic increase in obesity and related metabolic syndrome diseases, as well as the international interest in phytomedicine, this book is expected to be well received by scientists, health practitioners and people interested in phytomedicine.

Agathosma is one of South Africa's medicinal plants with claims of multiple healing properties. In the first chapter the editor provides a proper background on the plant, which is also known as buchu. The chapter continues with discussing other medicinal values of *Agathosma* that have been described before, but which were not part of the research described in this book. This chapter also acknowledges the hepatotoxicity that has been found before, but the toxicity is put into perspective, which explains why the extract has Food and Drug Administration (FDA) approval for use in the food industry.

With an international growing interest in ethnomedicine, the publication of this book is welcomed. The strength of this well-structured book is that it not only gives background on the plant, but also describes the research on the efficacy of the plant extract on diseases that are related to, and which form part of, the metabolic syndrome.

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A close-up photograph of a white Buchu flower (Lycium hirsutum) with green leaves. The flower is in focus, showing its five petals and prominent stamens. The background is blurred, showing more of the plant.

CHAPTER 1

Introduction and literature review

Description: An individual Buchu branch with flowers.

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Introduction and literature review

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■ Introduction

South Africa is rich in floral biodiversity as well as cultural diversity. The Cape region¹ of South Africa has veldt types, with one of the richest compositions of indigenous aromatic plant species in the country. One such aromatic plant is the genus *Agathosma*. There are about 150 different species within this genus, which derives from a greater family called Rutaceae, which also includes citrus plants.¹ These rutacious shrubs are part of the Cape Floral Kingdom (*fynbos*) vegetation found in the Western Cape Province of South Africa and are particularly abundant in the mountainous areas of the Cape.¹

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The term 'buchu' is primarily derived from the Khoisan (indigenous people of the western region of South Africa) and was used for any fragrant plant that could be dried and powdered.^{2,3} Therefore, it included a wide variety of aromatic plant species, not singling out a specific genus.² The Khoisan tribes relied on these plant materials for centuries to maintain and/or promote good health and longevity. For the Khoisan, dried buchu was the mainstay of the perfume repertoire of a woman and was also seen as an agent of physical and mental transformation, and was extensively used in medicine, perfume and certain rituals.³ In that era, herbal remedies formed the backbone of health care and were the main source of medication.³ Today, the term 'buchu' refers mostly to *Agathosma betulina* (round-leaf buchu, also known as *bergboegoe* or short buchu) and *Agathosma crenulata* (oval-leaf buchu, also known as *anysboegoe* or long-leaf buchu) which are the most common species.¹ *Agathosma betulina* is a small, multi-stemmed plant, which grows to a height of about 1m in the mountains of the Western Cape Province (from Clanwilliam in the north to Tulbagh in the south). *Agathosma crenulata* is a single-stemmed plant and grows to a height of approximately 2.5m, which grows between Gouda (north) and Stanford (south) and as far as the Outeniqua Mountains (east). The preferred soils of these plants are mostly nutrient-poor, sandy soils, with low pH levels varying between pH3.7 and pH5.3. Buchu plants grow naturally on steep slopes, with altitudes ranging between 737m and 2028m above sea level.³

Europeans were first introduced to buchu in the 1650s, upon their arrival in the Cape.¹ The knowledge and use of these plants then spread to Europe and later to America where it was extensively used for medicinal purposes.¹ In 1821, buchu was officially registered in the British Pharmacopoeia and described for its diuretic effects and use in the treatment of genito-urinary tract infections.³

In the meantime, more attention was given to the organoleptic properties of buchu rather than its impact on health, thus shifting the interest towards oil extraction. Oils are obtained

from these plants by means of hydrodiffusion, a method called hydrodistillation. Buchu oils are typically used in the perfume industry and as flavouring agents to enhance fruit flavours.¹ Currently, buchu products are found in virtually every store, ranging from tea bags to water, lotions, tonics, ointments and brandy, and buchu is probably the best-known herb for its medicinal properties, both locally and internationally.⁴ Buchu oil has been approved by the US FDA; (registration number 172.510 with a Generally Recognized as Safe [GRAS] status 2169) and the European Union for use in foodstuffs. It is the only plant in the world that genetically produces the chemical component diosphenol,³ a 2-hydroxypiperitone molecule, responsible for the signature blackcurrant scent and the flavour of the plant.³

Farming and cutting of buchu are usually performed during February and March, based on the seasonal variability of the polyphenolic and terpenoid contents of the plants and the fact that buchu is not harvested during the flowering season (i.e. October and November). This is to assure that the plants have ample time to regrow and propagate. A buchu plant, if properly handled, has a lifespan of up to 100 years. In the wild, the plant is only harvested every second or third year to ensure that there is enough time to regrow and seed. Currently, the Agricultural Research Council of South Africa, in collaboration with local farmers, has projects in place to monitor harvesting and ensure the protection of pure genetic strains of plants from which to propagate. This endeavour was strengthened by the study of Hüsselmann,⁵ who used amplified fragment length polymorphisms to genetically characterise *A. betulina* and *A. crenulata* and the possible hybrid plants that developed because different strains were transplanted out of their natural habitat. This is also important in view of the strictness with which the international markets are currently focussing on and buying organic produce. This latter movement of consumers to buy and use natural and organically produced products was a driving force for the current worldwide high demand of buchu.

In recent years, it has also been realised that good nutrition plays an important role in the prevention of chronic diseases, such as obesity, type 2 diabetes mellitus and hypertension. The term 'nutraceutical' was first introduced by Stephen DeFelice in 1979.⁶ He defined the term as 'a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease'⁶. To be classified as a nutraceutical, a food or an extract must have scientifically validated health benefits for both the treatment and the prevention of disease. The importance of this movement towards using nutraceuticals is underscored by numerous review papers listing, for example, plants with anti-diabetic activity⁷ as well as a list of plants with potential health benefits published by the World Health Organization (WHO) in its Essential Medicines and Health Products Information Portal: A World Health Organization resource.⁸ This list of plants with a full description of their constituents and uses is already in its fourth volume.

The medicinal properties of buchu that were exploited in the past include antimicrobial and anti-inflammatory activities of the buchu oil. More recently, the inherent antioxidant capacity of aromatic plants has been extended to the buchu plant, as well as several novel aspects of health benefits of different extracts prepared mostly from the buchu leaves. These include analgesic effects, wound healing and possible effects on the male reproductive system, which are discussed next.

■ Antimicrobial activity

A number of studies have evaluated the antimicrobial activity of buchu using the extracted hydrodistilled essential oil from both *Agathosma* species (*A. betulina* and *A. crenulata*) to perform minimum inhibitory concentration assays.⁴ Moolla and Viljoen¹ and Viljoen et al.¹⁰ showed that the oil was active against pathogens. The pathogens tested were *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Candida albicans*. These results indicated

that both extracts possess moderate antimicrobial activity⁴ and confirmed the study of Lis-Balchin et al.¹¹ who demonstrated low antimicrobial activity against *S. aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. The latter study could not detect antimicrobial activity against either *Enterococcus hirae* or *Pseudomonas aeruginosa*.

■ Anti-inflammatory activity

Inflammation is part of our innate immune response that is intended to protect and eliminate the cause of cell injuries.⁴ During the initial phases of acute inflammation, there is an increase in the production and release of vasoactive amines and eicosanoids that are responsible for initiating a process aimed at neutralising and removing harmful agents in order to allow the healing process to start. An integral part of the response of the host towards a site of inflammation is the attraction of leukocytes to and sticking onto the endothelium of the microvessels. The main eicosanoids are formed from arachidonic acid to render leukotrienes, prostaglandins and thromboxane.¹² Two main enzymes are involved in this process, lipoxygenase and cyclooxygenase, with the former leading to the formation of leukotrienes. Moolla and Viljoen¹ demonstrated that buchu oil has the ability to block the 5-lipoxygenase (5-LOX) enzyme, the key enzyme that produces leukotrienes, with IC_{50} values $50.37 \pm 1.87 \mu\text{g/mL}$ for *A. betulina* and $59.15 \pm 7.44 \mu\text{g/mL}$ for *A. crenulata*, respectively. Leukotrienes are known to be involved in a variety of inflammatory diseases. Because Viljoen et al.¹⁰ reported specifically that limonene could inhibit 5-LOX, the anti-inflammatory effects of the buchu oil were ascribed to this cyclic monoterpene that is also the major component in the oil of citrus fruit peel.

In 1989, Hansen published a review paper,¹³ highlighting that the formation of leukotrienes is associated with:

- contraction of the smooth muscle and thus also the smooth muscle of the airways leading to asthma

- reduced cardiac output because of vasoconstriction accompanied by reduced left ventricular contractility
- increase in pulmonary arterial pressure
- possible kidney disease
- atopic dermatitis and psoriasis
- ischaemia/reperfusion injury of the brain
- inflammatory bowel disease
- arthritis.

Hypothetically, the ability of buchu to inhibit 5-LOX should, therefore, be exploitable in all of these diseases; however, this needs to be investigated scientifically. Currently, leukotriene receptor inhibitors are available in the market as an adjunct treatment for asthma patients.

Moola and Viljoen¹ additionally reported on a double-blind placebo-controlled study undertaken by the Research Unit of Exercise and Sports Medicine, University of Cape Town, South Africa, where the efficacy of a topical gel containing buchu was tested on the inflammation caused by muscle damage. As reported by Lambert et al.,¹⁴ application of the gel was able to reduce swelling and pain in the damaged muscles. Furthermore, Cheraghali et al.¹⁵ showed that topical application of pulegone, a monoterpene found mainly in *A. crenulata*,¹⁶ in a vaseline and eucerin base, resulted in enhanced wound healing, with the significant restoration of stiffness and strength after both excisional and incisional wounds. This was accompanied by enhanced hydroxyproline content, implying enhanced collagen formation.

■ Antioxidant activity

Free radicals are highly reactive molecules containing an unpaired electron in an atomic orbital. Because of their nature, they can react with nearby stable molecules to gain an electron. In a cell, this could be lipids, DNA or proteins, thereby propagating free radical capability and damaging DNA, membrane systems and proteins. Within a cell, free radicals can be produced by normal enzymatic reactions, for example, by mitochondrial oxidative

phosphorylation reactions and the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase system, or can be supplied from external sources such as radiation and cigarette smoke (review by Lobo et al.).¹⁷ The human body has complex systems to counteract the damaging effects of free radicals and ensure homeostasis by means of antioxidant defence systems like superoxide dismutase (SOD), glutathione peroxidase and catalase. Some of the best-known exogenous antioxidants are vitamins A and E (fat-soluble) and Vitamin C (water-soluble). In addition, plant phenolics from our diets play a major role in our exogenous antioxidant defence system by protecting cells against oxidative damage. Their numerous effects are mainly attributed to their polyphenolic contents.¹⁸ These polyphenols are mostly found in teas, fruits, vegetables and spices.

Agathosma species are rich in flavonoids and various terpenes, one of them being the monoterpene limonene, which is thought to be involved in the anti-inflammatory effects of the buchu oil. As a component of citrus peel, limonene has been well studied and has a known antioxidant capacity. Despite this, studies by Moolla et al.^{1,2,10} reported on only weak antioxidant activity of the oil of both *A. betulina* and *A. crenulata*. Their studies corroborated the observations by O'Brien¹⁹ who also found a poor correlation between the total phenolic content of different extracts prepared from *Agathosma* and the radical scavenging activity of the extracts, despite the presence of quercetin, kaempferol and rutin, all of which have known radical scavenging properties.^{18,20} Most of the assays in the studies mentioned above were performed on the organic extractable fractions of the plant, but Thring et al.²¹ prepared aqueous extracts of a battery of plant material, including leaves from buchu, to test both the anti-ageing and antioxidant activities. Although this extract of buchu did not perform well against skin ageing, as tested by anti-collagenase and anti-elastase activities, the authors demonstrated a Trolox Equivalent Antioxidant Capacity (TEAC) of 11.8 μ M Trolox. In addition, they found SOD activity of 20.49% in comparison to 85.02% of the positive control (SOD, at 3.33 units final volume). At the time of

writing, this was the only study that used an aqueous extract to test for antioxidant activity, thereby demonstrating that this fraction of buchu indeed contains antioxidant potential.

■ Benefits for the reproductive system

The extracts of *Agathosma* species have a long-standing application as a diuretic or as a treatment modality for urinary tract infections. As previously mentioned, this was the first effect registered in the British Pharmacopoeia in 1821. In addition, it is listed as a possible treatment option for prostatitis.^{1,2,11,22} The unique 2-hydroxypiperitone diosphenol found in buchu oil is said to be responsible for the diuretic action, although the mechanism of this action is unknown. As summarised by Skosana et al.,²³ extracts of buchu may also be beneficial in the treatment of benign prostatic hyperplasia, inflammation of the prostate and various other urinary tract infections. By taking together the anti-inflammatory and antioxidant potential as discussed above, she concluded that buchu is a worthy candidate to regulate sperm function without having detrimental effects on the ability to reproduce. However, more scientific validation is required.

■ Analgesic properties

Scant information is available with regard to the analgesic properties of buchu extracts, although this should be expected in view of the anti-inflammatory properties of the extracts. However, in an elegant study bringing together physiology, chemistry, applied chemistry and nanotechnology, Chiguvare et al.²⁴ combined buchu leaf ethanolic extracts with silver nanoparticles to test possible analgesic properties. They used a scientifically validated mouse model and aspirin as a positive control in their study. In all aspects tested, the silver nanoparticle coupled with buchu extracts showed better analgesic properties than aspirin.

■ Anti-ageing properties

A Chronoscreen™ analysis of an aqueous extract of buchu performed by Sibelius in Oxford, UK, using both *S. cerevisiae* and *C. elegans* as model organisms, demonstrated a 62% increase in viability and a 21% improvement in median lifespan in yeast against an untreated control and a 33% increase in viability and an 8% increase in survival in the worm screen. In the yeast screen, this product performed better than Resveratrol. Even a 10 000 times dilution of the neat buchu extract was still able to improve the viability of *S. cerevisiae*.

The Chronoscreen™ platform has been developed specifically for the analysis of compounds and nutraceuticals for drug discovery and product development programmes. The nutraceutical Resveratrol is known to prolong lifespan and retard the onset of age-related markers.²⁵ It is used as a positive control in the Chronoscreen™ analysis.

■ Toxicity

The organic fractions (oil) of *Agathosma* contain a chemical that may be hepatotoxic when present in high concentrations. *Agathosma crenulata* is known to contain the monoterpene pulegone, while *A. betulina* does not.¹⁶ Pulegone is not water-soluble and has been approved by the US FDA for use in the food industry (with a Flavor and Extract Manufacturers Association [FEMA] GRAS status and listed among the authorised synthetic flavouring substances CFR 21-172.515). According to European standards,²⁶ pulegone has a No Observable Effect Levels (NOEL) of 100 mg/kg in beverages. An LC50 value of 25.91 mg/mL was recently reported by Raza et al.²⁷ Some of the earlier studies that showed hepatotoxicity (e.g. Moorthy et al.²⁸) used pulegone levels of 400 mg/kg to demonstrate significant decreases in the levels of liver microsomal cytochrome P-450 and haeme. Previously, Mizutani et al.²⁹ proposed that the hepatotoxicity of pulegone was because of a metabolite formed through the

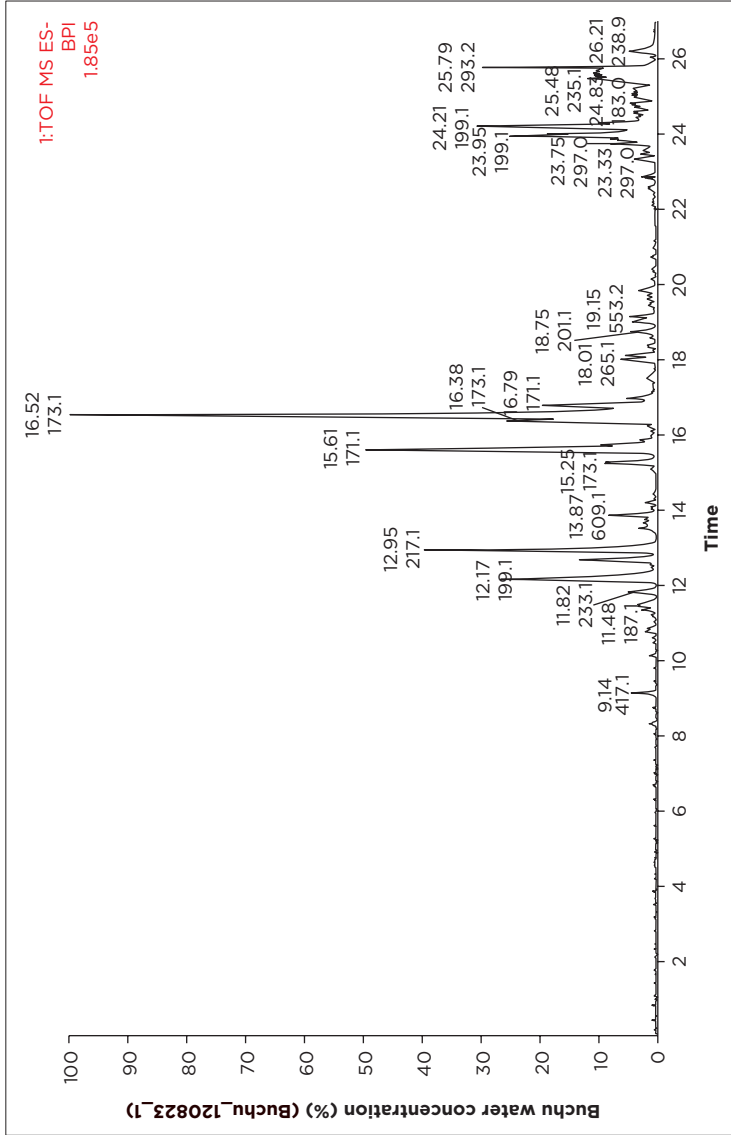
cytochrome P-450 system and not because of the parent compound. This was confirmed by Sztajnkrzyca et al.,³⁰ who suggested that CYP1A2 was the important isoform involved in pulegone metabolism. They furthermore demonstrated that inhibition of CYP P-450 mitigated the toxicity of pulegone in mice. In addition, the effects of pulegone were accompanied by depletion of the glutathione antioxidant system.³¹ The studies discussed above were all performed with pharmacologically high doses of pure pulegone.

■ Material used for *in vivo* model

The buchu oil required for the commercial market is extracted from the plant material and separated from the by-products using a steam distillation process. As the oil is distilled at low temperature but under high vacuum, the water used as part of the distillation process (cooling process) contains not only the more water-soluble molecules (hydrophilic compounds) but also some of the more volatile oil-soluble compounds usually found in the oil distillate. For the purposes of this research, it was decided to utilise the water distillate, knowing that it would contain the compounds of interest, albeit in a total mixture with some chemical identities only present in the oil.

The liquid chromatography-mass spectrometry (LC-MS) analysis of a representative sample of the water used, concentrated 180 times before analysis, shows a chromatogram of several molecular species as depicted in Figure 1.1. LC-MS is an analytical technique that combines the physical separation capabilities of liquid chromatography (or high-performance liquid chromatography) and the mass analysis capabilities of mass spectrometry (MS). The analysis was performed by the Central Analytical Facilities (CAF) of the University of Stellenbosch.

Targeted analysis using known standards revealed that such a sample of the water contained hesperidin, rutin, diosmin,



CAF, Central Analytical Facilities; LC-MS, liquid chromatography-mass spectrometry
 Note: This analysis was performed by the CAF of the University of Stellenbosch.

FIGURE 1.1: Analysis profile of the buchu aqueous extract utilised in these studies as generated by LC-MS.

quercetin and pulegone. Many other molecular species with unknown structures are also present in the water and could therefore account for the biological activity demonstrated both *in vitro* and *in vivo* as described in subsequent chapters.

The *in vitro* studies (Chapter 2) confirmed that the previously reported anti-inflammatory effects of *Agathosma* are fully retained in the aqueous extract utilised. In addition, cell-based studies indicated possible anti-glycaemic effects, paving the way for further preclinical studies in this regard. Chapter 3 summarises the data obtained in animal models on the effects of *Agathosma*, improving experimentally induced type 1 and type 2 diabetes. Furthermore, the aqueous extract showed both anti-obesity (Chapter 4) and anti-hypertensive effects (Chapter 5) in these animal models, thereby improving the full spectrum of the metabolic syndrome.

It is recognised that extrapolation of the effects observed in animal models to humans will need clinical trials for corroboration of the findings. This is especially true where pancreatic effects are involved, as it is known that rodent models do not have similar beta-cell biochemistry to that of humans.³² The results obtained from the current studies nevertheless substantiate the necessity of such clinical investigations.

The studies presented in this book were supported by Cape Kingdom Nutraceuticals (Pty) Ltd (contract numbers: S003211, S002594) who also supplied the aqueous extract of buchu. They were not involved in the studies or the interpretation of any of the results, and no conflict of interest is declared by the authors.

A photograph of a nursery for buchu seedlings. The plants are growing in a grid of white plastic trays within concrete raised beds. The entire nursery is covered by a green shade net supported by a metal frame. In the background, a rocky hillside is visible through the netting.

CHAPTER 2

Evaluation of buchu extracts *in vitro*: Evidence of its anti-glycaemic and anti- inflammatory properties

Description: Buchu seedlings in a nursery.

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Evaluation of buchu extracts *in vitro*: Evidence of its anti-glycaemic and anti-inflammatory properties

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■ Introduction

For many years, the water distillate resulting from the oil production phase of buchu was sold in the health market as herbal water. Many anecdotal testimonials were received from users of the buchu water, stating that the consumers with either insulin resistance or type 2 diabetes were managing their blood sugar levels (glycaemia) better since the intake of the product.

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It was therefore decided to investigate this *in vitro* by making use of cell lines that depend on an anti-glycaemic agent such as additional insulin or the drug metformin to survive in the presence of excess glucose during the culture phase. The cells normally grow in the presence of physiological concentrations of the glucose. However, if glucose is added to their culture medium in the presence of any agent/drug that can be actively transported across the cell membrane, then the uptake of the added glucose or its utilisation is vastly improved compared to that of the non-treated cells.

This *in vitro* model was used for testing the ability of both the buchu oil distillate and the water condensate to impact glucose utilisation of two specific cell lines as discussed here.

Furthermore, the historical use of the plant extracts related by the indigenous people of South Africa was for the management of conditions involving an inflammatory process, including cystitis and arthritis, among others. For this reason, we decided to use *in vitro* models of human inflammatory responses to investigate the effects of the various fractions obtained from the water condensate to evaluate this biological effect. We made use of human peripheral blood cells which, when triggered by biological response modifiers, upregulate (increase) the expression of membrane adhesion molecules (which aid during the chemotactic attraction of the innate cells of immunity), undergo an enzymatic response to produce the reactive and protective cellular response called the respiratory response (generation of O_2^-) or release potent inflammatory factors (monokines) implicated during the chronic inflammatory response.

■ **Materials and methods**

■ **Cell lines**

Two specific cell lines (Chang Liver cells and 3T3-L1 cells) were obtained from the American Type Culture Collection (ATCC) and

kept as long-term cultures in RPMI 1640 medium supplemented with 5% foetal bovine serum (FBS) and cultured at 37 °C and 5% CO₂. These cell lines have been reported to be useful *in vitro* tools when evaluating herbal extracts for their potential anti-diabetic properties of novel molecules with promise for *in vivo* evaluation.^{1,2}

The reported concentration of glucose in the RPMI medium, according to the manufacturer, is 2 mg/mL. The cells were cultured with the normal RPMI, FBS-supplemented medium, and on the day of experimentation, the cells were taken out of the culture flask, washed using sterile pure RPMI medium and placed back into the culture for 2 h to 'starve' the cells of essential nutrients. During this 'starvation period', various drugs or buchu extracts were co-incubated with the cells for 2 h. Then, the cells were seeded into 96-well microtiter plates at 100 000 cells/well; the medium containing increased glucose concentrations (additional 1 mg/mL, hence a total nominal concentration of 3 mg/mL) was added to all wells. Wells that contained cells having received added glucose-spiked medium but no added drug or extract were included as 'control wells'. The drugs or the extracts were therefore present throughout the total culture period.

Following a further overnight cell culture, the microtiter plate was centrifuged and the medium supernatant was removed for glucose measurement. Hence, the glucose measured in the 'control wells' served as 100%, and the drug-treated wells' concentrations were compared to those values. If the drug or the buchu preparations enhanced the glucose uptake and utilisation, then those wells would demonstrate a lower glucose concentration when compared to controls (results were expressed as percentage above control).

■ Peripheral blood cells from human blood

Blood was collected from volunteers by phlebotomy; anticoagulated samples (using Li-heparin tubes) were collected

and cells were prepared from the blood by density gradient centrifugation on Ficoll. The cells were recovered and washed several times using sterile RPMI medium before being placed into the culture in 96-well microtiter plates for 16 h. During this period, the cells were activated using bacterial endotoxin (Lipopolysaccharide [LPS], at 1 µg/mL) in the presence or absence of the buchu extracts. The plates were incubated for 16 h after which the culture supernatants were harvested by careful pipetting. Those wells that had not received any investigational compounds were regarded as maximal response wells, and all other wells were compared with those wells. The results were expressed as a percentage of maximal response. The release of the inflammatory cytokines tumour necrosis factor-alpha (TNF-α) or interleukin-6 (IL-6) into the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) techniques.

Further testing of the buchu extracts was conducted *in vitro* against the granulocytes (neutrophils and monocytes) by investigating their ability to mount the so-called respiratory burst (generation of the radical O₂⁻). This was measured using flow cytometry and fluorescently labelled substrates. Similarly, the expression of adhesion molecules at the surface of these cells was measured post-activation in the presence or absence of the extracts.

■ Drugs or buchu extracts used

Insulin was purchased as a ready-for-use formulation from our local medical supplier. This was in pre-filled graduated syringes of 1 mL volume equivalent to 80IU of the drug. The final concentration used was a 1 µM solution.

Metformin was purchased from Sigma-Aldrich (St Louis, Missouri) as a lyophilised powder. At the time of use, this was dissolved in 100% methanol as suggested by the distributor. The final concentration used was 1 µM.

The buchu extracts used were either the pure oil distillate (coded as CK_Oil) or the water condensate (coded as CK_H₂O); the water recovered during the distillation process to obtain the oil from the leaves was back-extracted using ethyl alcohol followed by drying under reduced pressure to finality and subsequently re-suspended into 100% methanol. The stock solution was made up in methanol at 600 µg/mL concentration. For the testing, this was diluted using distilled water before its addition to the wells containing the cells. Final dilutions used were 150X, 300X or 600X diluted from the 600 µg/mL stock solution.

■ Results

■ Effects on glucose metabolism

The commercial ELISA assay used yielded reproducible results, with a dynamic range of 0.031 µg/mL – 1.5 µg/mL and a correlation coefficient (R^2) of 0.993.

The culture supernatants recovered post 24-h incubation showed that the ‘control cells’ having received the increased (spiked) glucose (but no drug or extracts) still had 2.13 mg/mL of glucose present, implying that the cells were able to take up some of the extra added glucose and to metabolise this even in the absence of any added drug or buchu extract. However, those wells that had been treated with metformin had further decreased the glucose compared to the control wells, implying that these stimulated the uptake and metabolism of the added glucose.

Figure 2.1 shows a typical experiment using the Chang Liver cell line. The positive control (metformin at 1 µM) exhibited 23% – 25% increased glucose uptake. In parallel, the buchu oil showed a dose-dependent effect from 0.15 ppm to 0.6 ppm, with the wells having received 600 ppm of the oil showing the highest increase in glucose uptake and metabolism. However, it never reached the

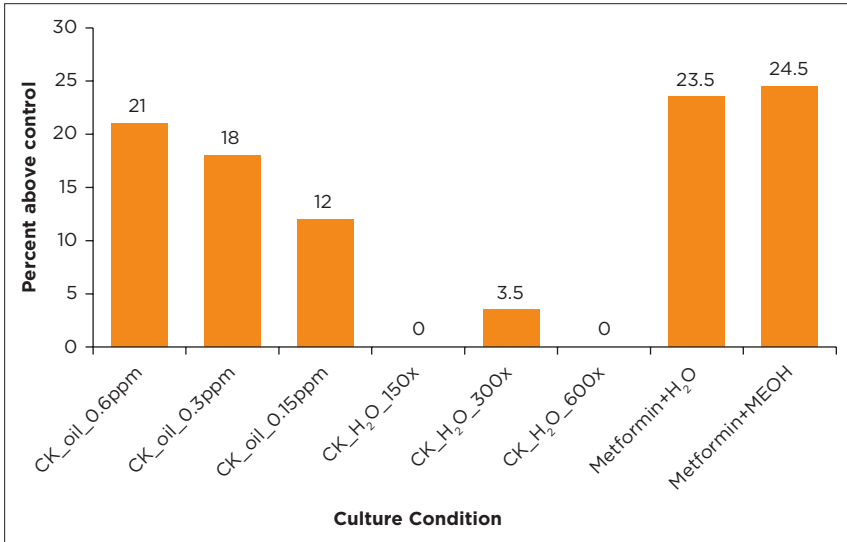


FIGURE 2.1: Glucose utilisation by Chang Liver cells.

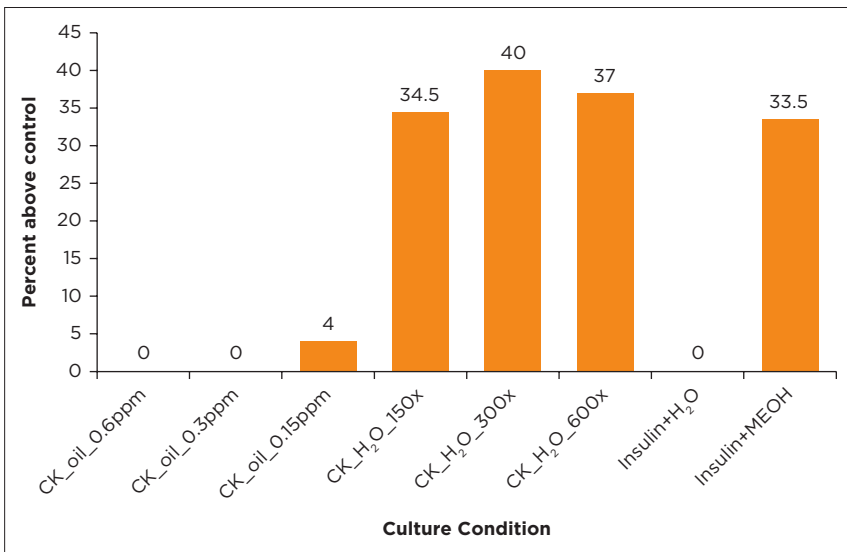


FIGURE 2.2: Glucose utilisation by 3T3-L1 cells.

same level of increased uptake or metabolism compared to the positive control metformin. It is interesting that when using this cell line, the CK-H₂O extract showed no significant effects on the glucose metabolism by these cells.

The results obtained using the 3T3-L1 cell line are shown in Figure 2.2. Once again, the addition of insulin at 1 μ M led to the increased uptake (by almost 35%) of the glucose added to the culture medium (positive control). In parallel, the addition of the CK-H₂O extract displayed an increase of 35% - 40% uptake of the glucose. No effects were measurable when the CK_Oil was used in these cells.

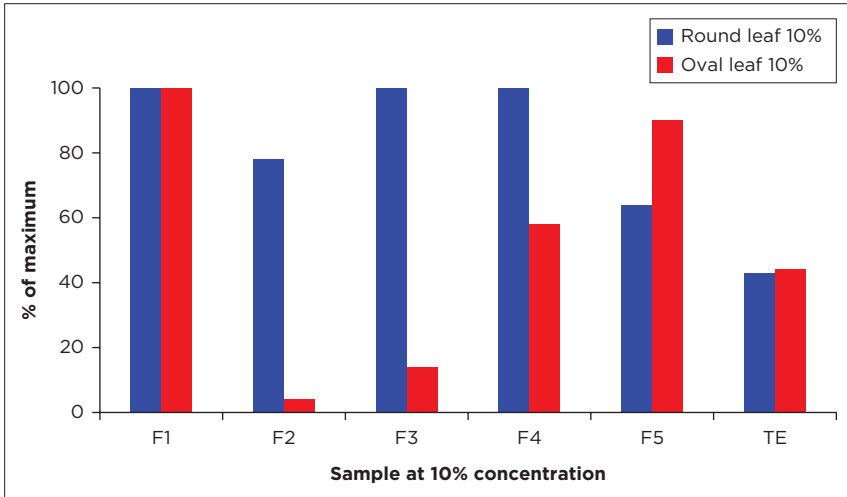
■ Anti-inflammatory activity

Firstly, when human peripheral blood cells were activated *in vitro* using LPS, the release of TNF- α and IL-6 was clearly evident in the culture supernatants as measured by ELISA (100% maximal response). Surprisingly, the addition of the buchu water condensate (which was back-extracted) to the cells led to significant inhibition of such cytokines, implying the ability of the fractions to inhibit the *in vitro* response (see Figure 2.3).

Similar results were obtained when the release of IL-6 was evaluated, suggesting that chronic inflammatory responses could be inhibited when buchu is added to this *in vitro* model.

Secondly, further experiments were conducted in parallel whereby the ability of the buchu extracts to inhibit the upregulation of adhesion molecules (CD11b/CD18) on innate cells of immunity (namely granulocytes) was evaluated. For these experiments, the expression of these surface molecules was measured using fluorescent-labelled monoclonal antibodies and flow cytometry.

Once again, we demonstrated that when human cells are activated *in vitro* to express higher levels of the membrane



F1 - F5, correspond to the fractions; LPS, Lipopolysaccharide; TE, total extract prior to further fractionation. Note: Results are expressed as a percentage of maximum (cells in the absence of any added fractions).

FIGURE 2.3: The *in vitro* effects of various buchu water condensate fractions on the release of TNF- α in the culture supernatants of human peripheral blood cells activated by the bacterial endotoxin LPS.

adhesion molecules CD11b/CD18, the addition of the buchu extracts significantly inhibited this physiological response, implying that the buchu was able to inhibit the acute onset of inflammation (Figure 2.4). This representative experiment clearly shows that the buchu extract has more pronounced effects on the expression of the CD11b/CD18 membrane molecule of neutrophils compared to that expressed by monocytes; a clear dose-response effect is demonstrated by dilutions of 1:400 up to 1:3200 tested.

Thirdly, during the initial phase of neutrophil functions, the internalised organism or particle is destroyed by an oxygen-dependent process, which ultimately leads to the generation of oxygen radicals (O_2^-). These radicals are protective when they are generated in the cytoplasm of the cell in that it kills the bacterium. However, when these are released to the external

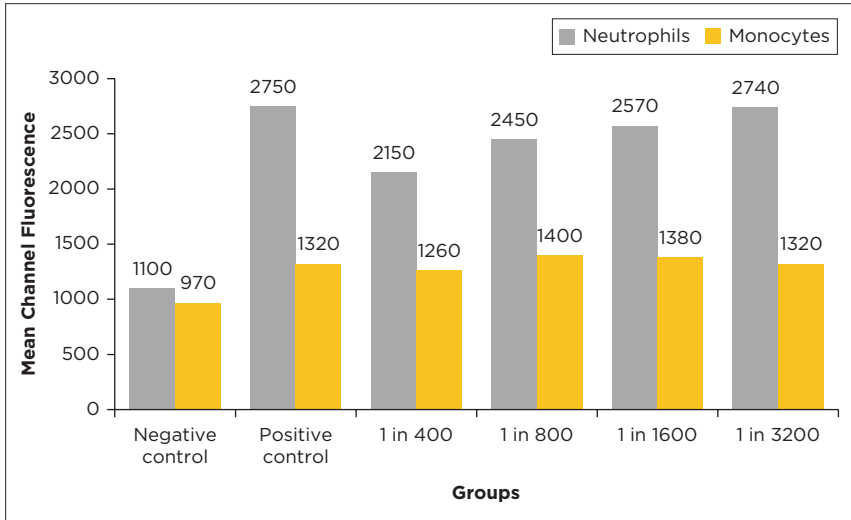


FIGURE 2.4: Expression of CD11b/CD18 on neutrophils and monocytes as measured by flow cytometry.

medium, it can damage healthy tissues in the vicinity of the phagocytic cell.

The ability of buchu extracts to influence the generation of oxygen radicals was measured by flow cytometry. As shown in Figure 2.5, it is evident that at different dilutions of the water extract, the oxidative burst (respiratory burst) of neutrophils was significantly inhibited by the watery distillate dilutions (from 1:400 to 1:3200 dilutions).

■ Discussion

The *in vitro* model used to investigate the possibility that buchu extracts could enhance the uptake and/or metabolism of glucose by two well-described cell lines proved to be easy to use and yielded results, which implied that the self-reported anecdotes from consumers that their blood glycaemia was better managed

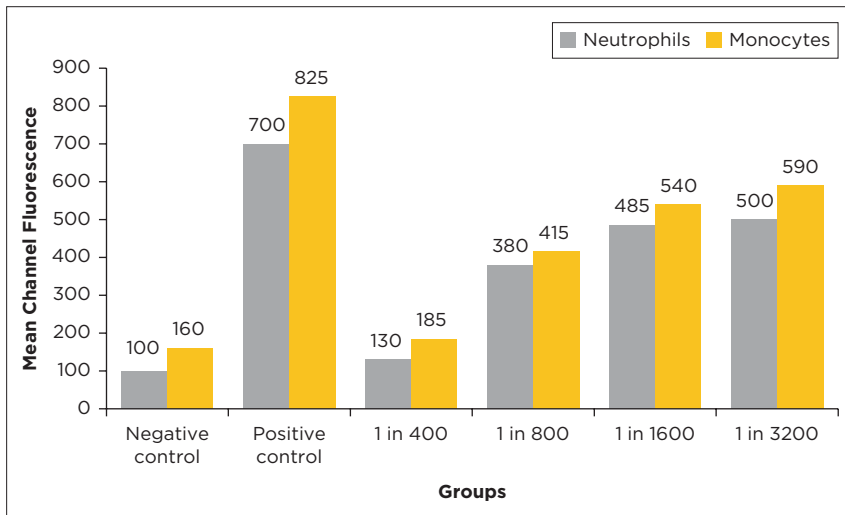


FIGURE 2.5: Respiratory burst by neutrophils and monocytes in the presence or absence of various dilutions of the buchu water condensate extracts (from 1:400 to 1:3200 dilutions) *in vitro*.

when using these products were correct. Although no mechanism of action of the extracts could be deduced from this model, it remains an interesting observation nonetheless. Furthermore, at this stage, we have no scientific proof of which compound(s) could be responsible for this observation, as the extracts used contain numerous molecules or chemical entities.

The model clearly showed that buchu oil extract (CK_Oil) had more pronounced effects on the Chang Liver cell line, with little or no effects seen when the CK_H₂O was tested in parallel. The converse was seen when the 3T3-L1 cell line was used as a model - the CK_H₂O had a significant effect on the 3T3-L1 cell line, whereas no effect was seen when the oil was tested. These cell lines have different origins - the 3T3-L1 cells are of adipose tissue origin, whereas the Chang cells are of hepatocyte origin. Even in this model, we were able to demonstrate differences in sensitivities to well-described anti-glycaemic agents, such as

insulin versus metformin. The Chang Liver cells were responsive to the added metformin, whereas the 3T3-L1 cells responded to the added insulin. It is therefore not surprising that the buchu oil showed differential effects on the cell lines (similar to the watery extract of the buchu).

The above results were sufficient evidence for us to take these into an *in vivo* model (as reported in subsequent chapters). To our knowledge, this is the first time that a natural herbal extract has been shown to control glycaemia *in vitro* as well as *in vivo* in an animal model. *Agathosma* remains a rich source of natural compounds with potent biological activities reported by the indigenous folk.

As far as the anti-inflammatory properties of the buchu are concerned, the *in vitro* model utilising human peripheral blood cells clearly demonstrated and supported the anecdotal use of this herbal extract by the indigenous peoples for the management of inflammatory conditions (such as cystitis and arthritis). Several assays were used to demonstrate this potent activity. The respiratory burst of neutrophils and monocytes was significantly inhibited and, simultaneously, the expression of adhesion membrane molecules was inhibited. Both of these physiological responses are implicated during the acute inflammatory phase. Furthermore, the extracts inhibited the release of potent cytokines such as IL-6 and TNF- α , both of which are directly implicated in the pathogenesis of chronic inflammatory conditions. It is also important to note that when tested *in vitro*, the two species of the buchu used (round and oval) yielded different results, with the oval leafed buchu species showing more potent inhibitory activity. The water condensate sold as a herbal remedy contains both species and therefore accounts for the effective anti-inflammatory properties reported by consumers. The anti-inflammatory property of *Agathosma* was previously reported by other groups, showing the ability of buchu to inhibit the 5-lipoxygenase enzyme that ultimately leads to leukotriene synthesis.³

Once again, we are unable to predict which bioactive molecules within these extracts are responsible for the anti-inflammatory potency. To isolate and identify individual chemical entities in these extracts presents challenges beyond the current infrastructure of the researchers.

■ Acknowledgements

The author would like to thank W. Kriek, Synexa Life Sciences (Pty) Ltd, Montague Gardens, Cape Town, for assisting in this study.



CHAPTER 3

Anti-diabetic properties of buchu

Description: Cultivated Buchu not flowering.

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Anti-diabetic properties of buchu

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■ Introduction

According to the WHO Factsheet, November 2017,¹ the current estimation of people with diabetes has risen to an astounding 422 million worldwide. This information was taken from projections of disease performed by Mathers and Loncar.²

Diabetes is a severe and chronic disease, which occurs when the pancreas does not produce enough insulin³ or when the body is unable to utilise the available insulin effectively. Both these conditions result in increased blood glucose levels that may over time lead to severe damage to the heart and vascular system, eyes, kidneys and the nerves.

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Three types of diabetes are recognised:

1. type 1 diabetes (T1D), formerly known as insulin-dependent or juvenile diabetes
2. type 2 diabetes (T2D), previously termed non-insulin-dependent or adult-onset diabetes
3. gestational diabetes.

The third is a condition where pregnancy results in increased blood glucose levels, but mostly this is resolved after delivery.

Type 1 diabetes is an autoimmune disease and has an active genetic component. It is characterised by the destruction of the pancreatic β -cells, leading to insulin deficiency.⁴ It accounts for 5%–10% of all diabetes; however, as reviewed recently,^{5,6} its prevalence is increasing globally, at a rate of 2%–3% per year. Although previously seen as a juvenile form of diabetes, it is currently estimated that more than 40% of all new cases of T1D occur in people over the age of 30 years.⁷

The aetiology of T1D informs that the pancreatic islet cells are progressively destroyed by autoantibodies. It is currently a disease without a cure, and although the understanding of the disease's progression has grown, there is no known preventative measure for disease development. All prevention trials to date have failed,⁶ and the only current treatment option is daily insulin injections. However, the interesting concept of pre-T1D with a staging of disease development has been suggested by the American Diabetes Association.⁸ This staging includes an important presymptomatic stage characterised by a steady decline in functional β -cell mass. Even at the beginning of the symptomatic phase, there is still a portion of functional β -cells available.

Type 2 diabetes describes the majority of people with diabetes and has a strong link with obesity and metabolic factors. However, the risk factors for the development of T2D also include genetic factors, ethnicity, physical inactivity, smoking and age, but is largely the result of excess bodyweight, especially abdominal fat

accumulation and physical inactivity.⁹ In addition, all these risk factors not only predispose to the development of gestational diabetes but also imprint a future risk of both obesity and T2D on the genome of the mother, which is carried into the next generation.

In the development of obesity-related T2D, the pre-diabetic state is characterised by peripheral insulin resistance (an inability to utilise available insulin in the body). Insulin is the primary stimulus for the muscle to utilise available blood glucose, the liver to stop producing glucose (*de novo* glucose synthesis) and the fat tissue to store any leftover glucose into fat. If this system malfunctions, the resultant increased blood glucose levels prompt a further increase in insulin secretion by the pancreas as glucose uptake and metabolism are the primary stimuli for insulin secretion. The pre-T2 diabetic state is therefore characterised by normal glucose levels but high insulin levels. The tipping point is when the pancreatic β -cells are not able to compensate further. The most recent paradigm for the progressive loss of function of the β -cell population lies in the concept of dedifferentiation of the β -cells caused by the post-prandial rise in blood glucose in the insulin-resistant state.¹⁰ Like all cells in the body, β -cells are derived from embryonic stem cells during the developmental stages.¹¹ During this process, certain transcription factors and their downstream gene clusters are activated or repressed.¹⁰ With dedifferentiation, this process is driven backwards, leaving the pancreas with a lower concentration of mature β -cells and more precursor cells. The differentiated state of the cell populations is recognised by the specific transcription factors expressed at a given time point.

At the tipping point, the blood glucose levels start to rise to pathologic levels, turning pre-diabetes and insulin resistance into T2D. It has been shown that this happens when 50% of the β -cell population is not functional anymore.¹² Because of this long, asymptomatic prelude to the development of obesity-related T2D, many of the detrimental consequences, for example,

cardiovascular complications, had a 5–10-year period to develop. Type 2 diabetes is recognised as a chronic disease state with no cure because of the destruction of mature, insulin-secreting pancreatic β -cells, although by a different mechanism. Prevention is therefore no longer an option at the time of diagnosis, but treatment is.

With these concepts of T1D and T2D in mind, we performed preclinical evaluations of the effectiveness of an aqueous extract of *Agathosma* (buchu) to act as a treatment option in both types of diabetes, or to act as a preventative option against the development of obesity-related T2D.

■ **Methods**

■ **Ethical considerations**

In all instances, only male, outbred Wistar rats, obtained from Charles Rivers Laboratories, Inc. (Wilmington, MA) and bred within the Central Research Facility of the University of Stellenbosch, were used. These rats were age- and weight-matched at the onset of experimentation. All animals were housed at the University of Stellenbosch Central Research Facility, which provided a simulated environment of the animals' natural habitat, exposing them to a temperature-controlled room (22 °C–24 °C) and a 12-h light–dark cycle.¹³ The animals had free access to water and food for the duration of the experiment. Control animals were fed normal rat chow, while the diet-induced obesity (DIO) or high-fat diet (HFD) animals were given an obesogenic diet as indicated. Specific groups in both diabetic models were treated with buchu water. The projects were approved by the Ethics Committee of the University of Stellenbosch (Faculty of Health Sciences – protocol number SU-ACUM11-00003) and conformed to the principles revised in the South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

■ Type 1 diabetes

The well-known streptozotocin (Stz) model to induce T1D by chemical destruction of the pancreatic β -cells was utilised.¹⁴ We have previously determined that a dose of 40 mg/kg of Stz injected in an adult rat will result in proportional destruction of β -cells, thereby representing the pre-T1D state.¹⁵ The Stz-induced model is deemed a suitable model for studying the complications of T1D because of the following:

1. continued β -cell derangement continues after Stz is eliminated from the body because of the established hyperglycaemia
2. Stz-induced diabetes can be reversed by insulin treatment, which indicates that the β -cell population can respond to treatment and has the ability to regenerate
3. Stz-induced diabetes can be ameliorated or alleviated by administration of phytochemicals.¹⁶

It must however be recognised that this is a chemically induced model, while the development of human T1D is an autoimmune disease, as previously discussed. It is therefore possible that effects of alleviation by the administration of any substance may always be overridden by the immune response.

Adult male Wistar rats (200 g – 250 g) were injected with a single intraperitoneal dose of Stz in citrate buffer (pH 4.5) at 40 mg/kg bodyweight. Vehicle-injected animals represented the controls. This amount of Stz results in a 50% ablation of β -cells.¹⁵ Blood glucose levels, measured via a drop of blood obtained from a tail prick using a handheld glucometer (GlucoPlus™, Montreal, Canada), were monitored daily for 3 weeks. Animals that spontaneously recovered normal blood glucose levels were removed from the groups. T1D animals were categorised into two groups, namely, those (1) with unfasted blood glucose levels below 20 mmol/L and (2) those with unfasted blood glucose levels between 20 mmol/L and 30 mmol/L. Stz-injected animals received the diluted buchu water from the 3-week time point.

After 14 weeks, the animals were fasted overnight to obtain fasting blood and perform an Intraperitoneal Glucose Tolerance Test (IPGTT).

■ Type 2 diabetes

A DIO model was used to render the animals insulin resistant. It is known that rats do not develop T2D as a result of obesogenic diets without a mechanism to also ablate some of the β -cell population. It is also postulated that rats do not form and deposit islet amyloid polypeptide in the pancreatic β -cells.¹⁷ The animals in our experiments therefore represent the pre-diabetic state. In the course of the studies described in this chapter and Chapters 4 and 5, two different variations of the diet were utilised. The composition of the DIO diet is described in this chapter and represents a diet high in sugar but not in fat (see Table 3.1).

Animals on this diet (DIO) do not develop hypertension; therefore, the diet was changed to also induce hypertension in the animals (described in Chapters 4 and 5). The main difference between these two diets lies in the elevated fat content as well as the higher fructose content available in the HFD. The composition of the HFD is provided in Chapter 4. The feeding regime of the animals was identical in all experimentation.

Young rats weighing 190 ± 10 g (6–7 weeks old) were divided into two groups, namely, a group receiving normal rat chow and a group receiving rat chow supplemented with sugar and

TABLE 3.1: Composition of diets for the experiment.

Specimen Group	Fat (g/100 g)	Cholesterol (mg/100 g)	Protein (%)	CHO (%)	Sugar (g/100 g)	Energy (kJ/100 g)
Control	4.8	3	17.1	34.6	6.6	1272
DIO	4.6	10	9.4	45.8	27.7	1173

Source: Analysis performed by Microchem Lab Services (Pty) Ltd. CHO, carbohydrate content; DIO, diet-induced obesity.

condensed milk^{18,19} (DIO) for a period of 16 weeks. Buchu water treatment was commenced after 8 weeks when the animals had gained substantial weight and already presented with insulin resistance.²⁰ Food intake and the weight of the animals were monitored throughout the experimentation.

To ensure that the buchu species were well blended over the 16-week period, multiple 1.5L sealed buchu water bottles were received from Cape Kingdom Nutraceuticals and diluted on a weekly basis.

The animals had free access to food, undiluted water or diluted buchu extract water, and the intake was monitored throughout the 16-week period. The extract dilution was calculated according to the equivalent surface area dosage conversion factors as described by Freireich et al.,²¹ assuming that an adult human (60 kg) would consume 250 mL of the water per day, while a rat weighing 250 g would consume a mean of 30 mL water per day.

Fifteen weeks after the onset of the diet period, the animals were fasted overnight, whereafter baseline glucose was determined and 1 mL of fasting blood was collected from the carotid artery. The blood was left on ice to clot, followed by serum collection and storage at -80°C . Fasting serum was used to determine insulin levels.

The animals were left to recuperate from the metabolic insult for 1 week before experimentation.²²

The animals were anaesthetised by intraperitoneal injection with 160 mg/kg bodyweight of sodium pentobarbital and monitored until deep anaesthesia, as determined by the lack of the pedal and eye reflexes.

Bodyweight was recorded, and death was induced by exsanguination. Unfasted blood was collected in SGVac gel serum collection tubes and left to clot on ice, followed by serum collection. Serum was stored at -80°C for biochemical analyses.

The pancreata were harvested, snap-frozen in liquid nitrogen and stored at -80°C for biochemical analyses. In addition, ventricular cardiomyocytes were prepared to determine the effects on myocardial insulin resistance.

■ Assays performed

□ On both type 1 diabetes and type 2 diabetes models

□ Intraperitoneal glucose tolerance test

An intraperitoneal glucose tolerance test was routinely performed to determine whole-body glucose tolerance.²³ The animals were fasted overnight, whereafter a blood sample was obtained from a tail prick to determine the blood glucose level. At the same time, 1 mL of fasting blood was collected from the carotid artery; serum was separated and stored at -80°C . Then, a dose of 1 g sucrose/kg bodyweight was injected intraperitoneally, and the disappearance of glucose from the blood was monitored over a 2-h period.

□ Only on type 1 diabetic animals

□ Daily monitoring of blood glucose levels

The blood glucose levels of these animals were followed daily for the full duration of 14 weeks by collecting blood samples from a tail prick in the morning and reading the value on a handheld glucometer.

□ Only on type 2 diabetic animals

□ Insulin radioimmunoassay

A commercially available Radioimmunoassay (RIA) kit (Coat-A-Count®; Siemens Medical Solutions Diagnostics, Los Angeles, CA) was used to determine the fasting insulin levels in the serum collected.

▣ C-peptide enzyme-linked immunosorbent assay

A commercial rat-sensitive ELISA kit was obtained from Abcam Ltd (Cambridge, UK) and used according to the manufacturer's instructions to determine the C-peptide levels in the stored serum. C-peptide levels are an indication of newly secreted insulin from the pancreatic β -cells.

▣ Cardiomyocyte glucose uptake

○ Preparation of cells

Calcium-tolerant adult ventricular myocytes in an unstimulated state were prepared as described previously.¹⁹ After isolation, myocytes were suspended in buffer A containing mmol/L HEPES 10, KCL 6, NaH_2PO_4 0.2, Na_2HPO_4 1, MgSO_4 1.4, NaCl 128, pyruvate 2, glucose 5.5, 2% BSA (fraction V, fatty acid-free) plus 1.25 mmol/L calcium, pH 7.4. The cells were left for 1h-2h under an oxygen atmosphere on a gently shaking platform to recover from the trauma of isolation. This procedure routinely rendered more than 80% of viable cells as measured by Trypan blue exclusion. After recovery, the cells were allowed to settle into a loose pellet, the supernatant was aspirated and the cells were washed twice with, and suspended in, a suitable volume of substrate-free buffer A (buffer B).

○ Determination of 2-deoxy-d-3[H] glucose (2DG) uptake

Cardiomyocytes (~0.5 mg protein) were assayed in a total volume of 750 μL of oxygenated buffer B (pH 7.4) as described previously.¹⁹ The cells were pre-incubated for 15 min in a shaking water bath (37°C) with or without phloretin (400 μM) for measurement of non-carrier-mediated glucose uptake. Each experimental series was incubated with or without 10 nM insulin for 30 min after which glucose uptake was initiated by addition of 2-deoxy-d-[3H] glucose (1.5 $\mu\text{Ci}/\text{mL}$; final concentration 1.8 μM). The glucose uptake was allowed to progress for a further 30 min before the reaction was stopped by adding 50 μL phloretin to give a final concentration

of 400 μ M. The cells were then microfuged for 1 min and the pellet was dissolved in NaOH (sodium hydroxide). An amount of 50 μ L of this solution was used to assay protein by the method of Lowry et al.,²⁴ while the rest was counted for radioactivity. The results were calculated as pmole 2DG/mg protein/30 min.

□ Pancreatic tissue western blotting of transcription factors

Frozen sections of pancreatic tissue were pulverised in a liquid nitrogen pre-cooled mortar and pestle and extracted in a standard Radioimmunoprecipitation Assay (RIPA) buffer containing in mM: Tris-HCl (pH 7.4) 20; EGTA 1; EDTA 1; NaCl 150; β -glycerophosphate 1; tetrasodium-pyrophosphate 2.5; sodium orthovanadate 1 with 1% Triton-X100, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin and 50 μ g/mL PMSF. The tissue was homogenised using a PolyTron PT10 homogeniser twice for 5 s at setting 4 and left on ice for 15 min to fully digest. The lysate was centrifuged at 15000rpm in a microfuge at 4 °C and the pellet was discarded. The protein content of the supernatant was determined according to the method of Bradford²⁵ and diluted to equal protein per sample. A three-times concentrated Laemmli sample buffer was added in a 1:2 v/v ratio and the sample was boiled for 5 min.

Sixty micrograms of protein were subjected to Polyacrylamide Gel Electrophoresis (PAGE) using a BioRad Mini Protean III system, and the resultant separated proteins were transferred to Polyvinylidene Difluoride (PVDF) membranes. Transfer and equal loading were confirmed by Ponceau Red reversible stain.²⁶ The non-specific binding sites on the membranes were blocked for 2h at room temperature with 5% fat-free milk dissolved in TBS-Tween. They were then incubated overnight at 4 °C with the suitable primary antibody, diluted according to the manufacturer's instructions. After rinsing thoroughly with TBS-Tween the next morning, the membranes were exposed for 1h at room temperature to the suitable secondary antibody coupled with horseradish peroxidase.

The signal of the specific antibodies bound to the target protein was captured by exposure to an enhanced chemiluminescent reagent reacting with the horseradish peroxidase to render a signal captured on film. The film was laser scanned and analysed using computer software (Un-Scan-It, SilkScientific, Orem, UT 84059, USA).

■ Statistical analyses

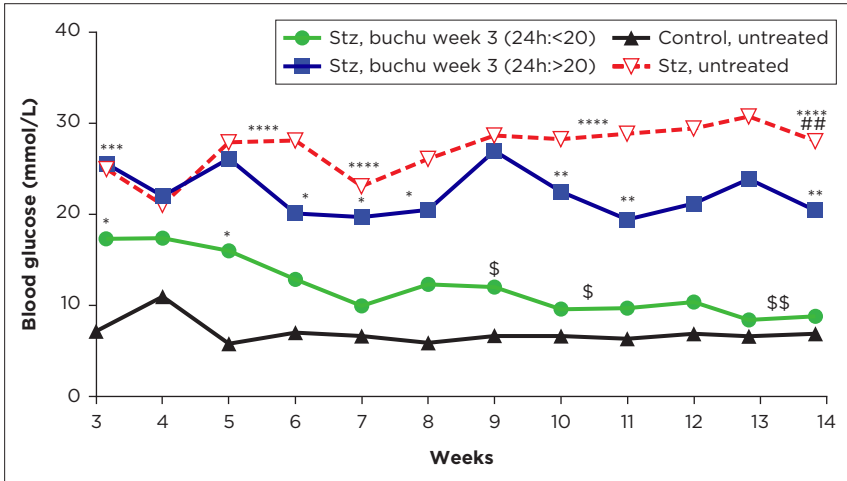
The statistical significance of results was analysed using GraphPad Prism 6. A one-way or two-way analysis of variance (ANOVA) was used as applicable, followed by a Bonferroni *post hoc* analysis to indicate how groups differed. Daily monitoring of blood glucose levels and the intraperitoneal glucose tolerance curves were analysed using a repeated measure two-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant.

■ Results: Type 1 diabetes

The ingestion of the aqueous extract of buchu, given as a treatment option to animals with partially ablated pancreatic β -cells and presenting with blood glucose levels of less than 20 mmol/L, completely normalised their random blood glucose levels, as shown in Figure 3.1 and Figure 3.2. The more robust ablation, leading to blood glucose levels of more than 20 mmol/L, was significantly lower than the untreated controls.

Results obtained from the IPGTT is shown in Figure 3.3 and demonstrate that whole-body glucose tolerance in animals that ingested buchu water after partial ablation of the β -cell population with Stz was normalised in both the groups with a low percentage of ablation of β -cells (24 h < 20 mmol/L) as well as a high percentage of ablation of β -cells (24 h > 20 mmol/L).

Figure 3.4 shows a compilation of the blood glucose values obtained after 120 min of monitoring from the time of onset of the IPGTT procedure. This shows that both groups of animals treated with buchu water were now able to effectively clear



Stz, streptozotocin.

Note: Treatment with buchu water commenced at week 3.

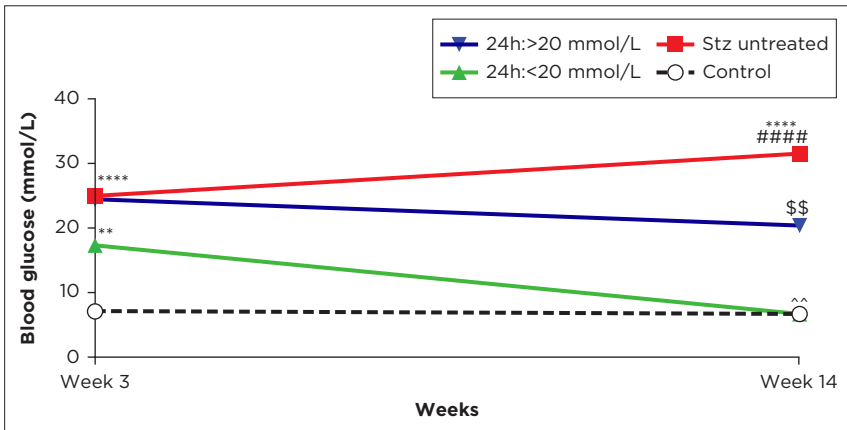
n = 8-10 per group

*, vs control; #, vs < 20; \$, vs Stz; ^, vs 3 weeks < 20

*, #, \$, p < 0.05; ##, \$\$, p < 0.01; ***, p < 0.001; ****, p < 0.0001

Effect of buchu, p < 0.0001

FIGURE 3.1: Blood glucose levels of the animals were measured on a weekly basis by collecting a drop of blood from a tail prick and reading the value using a handheld glucometer.



Stz, streptozotocin.

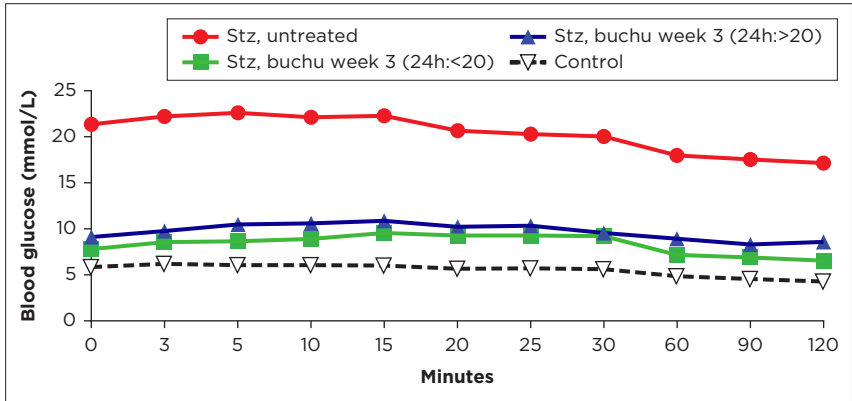
Note: The blood glucose levels of the different groups of animals at week 3 before the commencement of the buchu water treatment and at week 14 before termination of the animals.

n = 8-10 per group

*, vs. control; #, vs. < 20; \$, vs. Stz; ^, vs. 3 weeks < 20

** , ^, \$, p < 0.01; ****, #####, p < 0.0001

FIGURE 3.2: The change in blood glucose levels from weeks 3 to 14.



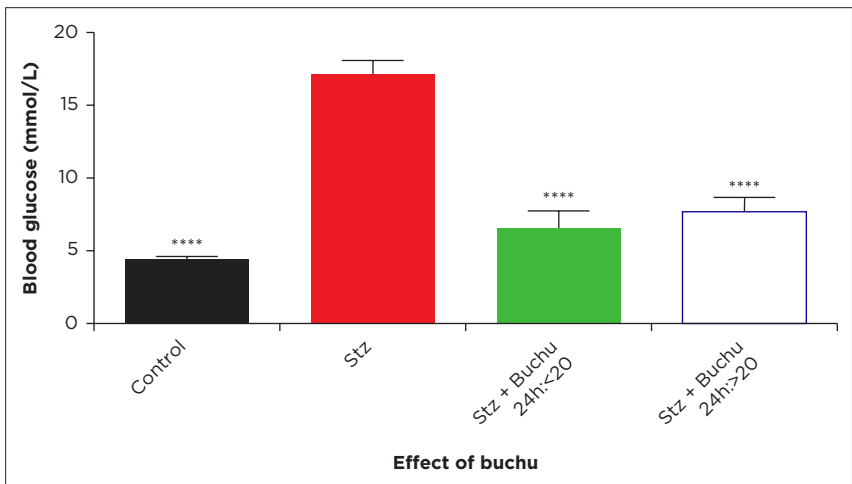
Stz, streptozotocin.

Note: Blood glucose levels were by means of blood samples from a tail prick over a 2-h period.

$n = 8-10$ per group

$p < 0.0001$

FIGURE 3.3: An IPGTT was performed after an overnight fast at week 14 of the treatment protocol, with point 0 on the graph representing the fasting value before administration of a 50% sucrose solution at 1g/kg bodyweight.



Stz, streptozotocin.

$n = 8-10$ per group

****, $p < 0.0001$ vs STZ

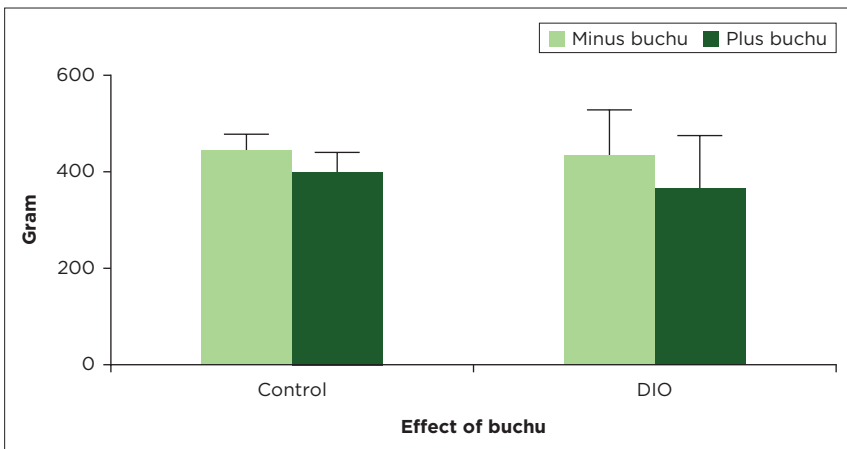
FIGURE 3.4: Blood glucose values obtained in the intraperitoneal glucose tolerance test 2h after ingestion of 1g sucrose per kg bodyweight.

glucose from the circulation within 2h in comparison to the untreated Stz group. The observed blood glucose values of the treated groups did not differ from those of the control animals that received no Stz and no buchu water.

■ Results: Type 2 diabetes

Figure 3.5 shows the bodyweight of the groups of animals that were fed either control rat chow (control) or a diet supplemented with sugar and condensed milk to induce hyperphagia (DIO) for a period of 16 weeks. There was no significant increase in bodyweight of the DIO animals over the course of the 16 weeks. However, ingestion of buchu water resulted in a significant effect of lowering bodyweight gain by animals in both the control and DIO groups.

Figure 3.6 shows that the DIO diet resulted in abdominal obesity in the animals as they presented with a significantly



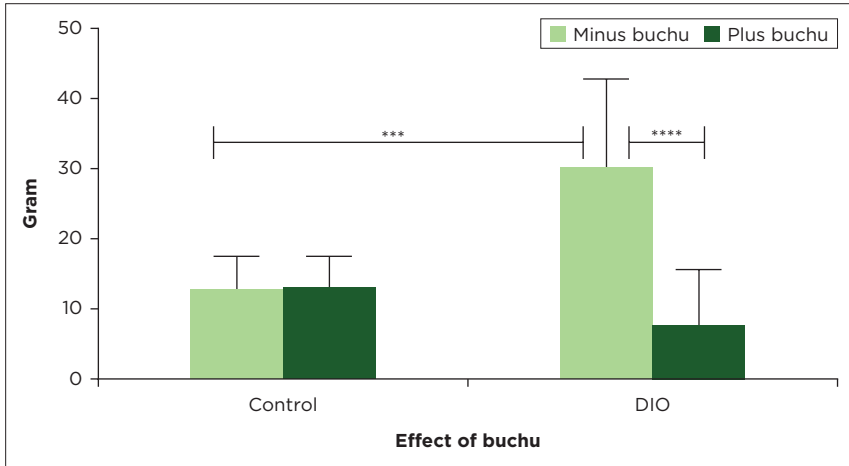
DIO, diet-induced obesity.

Note: A two-way ANOVA indicated a significant effect of buchu water.

$n = 10-16$ per group

$p = 0.018$

FIGURE 3.5: Bodyweight of animals from the four different groups indicating the mean bodyweight per group after 16 weeks of following the respective diets.



DIO, diet-induced obesity; IPGTT, Intraperitoneal Glucose Tolerance Test.

Note: After the euthanasia of the animals, the fat depots in the visceral cavity were dissected and weighed. This included the renal and epididymal fat pads.

$p = 10^{-16}$ per group

, $p < 0.001$; *, $p < 0.0001$

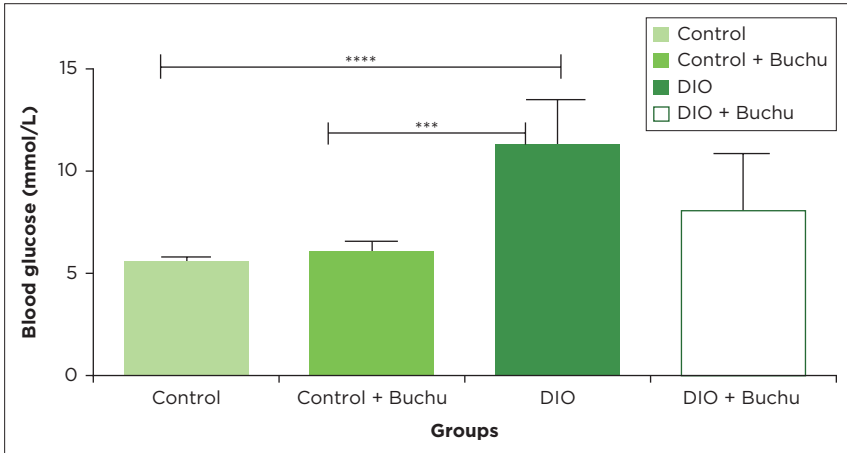
FIGURE 3.6: Intraperitoneal fat weight after 16 weeks of an obesogenic diet.

larger amount of accumulated visceral fat. In addition, the DIO animals ingesting buchu water did not accumulate this additional visceral fat.

Figure 3.7 depicts the fasting blood glucose levels of the groups of animals at 15 weeks after onset of the obesogenic diet. These are the values obtained at the start of the IPGTT. The animals on the DIO diet presented with significantly elevated fasting glucose levels. After the 8-week treatment period, the fasting blood glucose values of the DIO + buchu group did not differ significantly from values obtained from either control or control + buchu-treated animals.

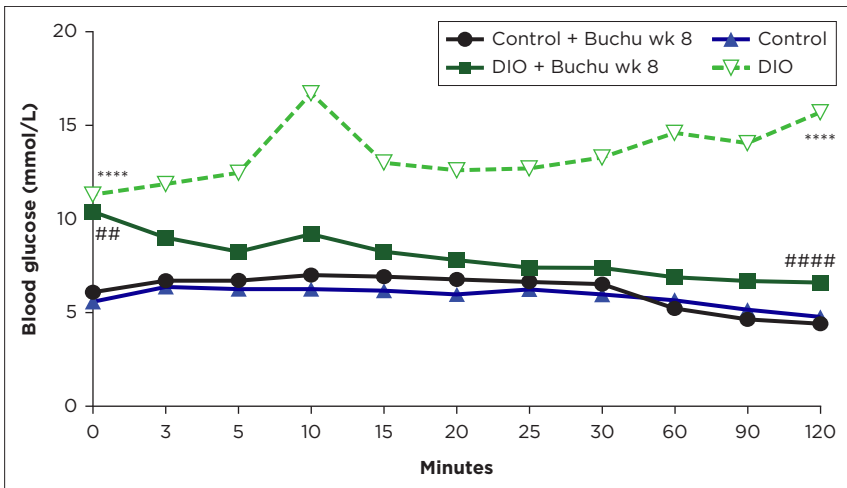
The results obtained by performing an IPGTT on control and DIO animals treated with buchu water from week 8 to week 16 of the respective diets are shown in Figure 3.8. This graph confirms the potential of the buchu water to normalise whole-body glucose tolerance, showing that after 8 weeks there were no significant

Anti-diabetic properties of buchu



DIO, diet-induced obesity; IPGTT, Intraperitoneal Glucose Tolerance Test.
 Note: Animals were fasted overnight to perform an IPGTT, and blood glucose levels were determined from a drop of blood collected from a tail prick and measured using a handheld glucometer.
 , $p < 0.001$; *, $p < 0.0001$

FIGURE 3.7: Effects on fasting blood glucose levels.

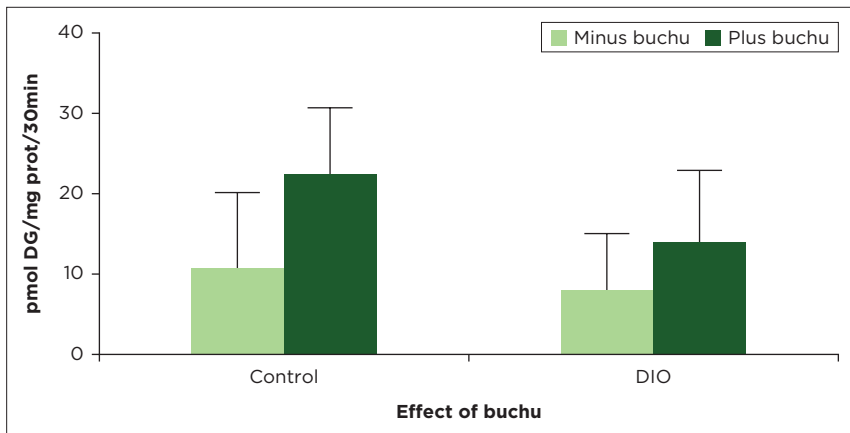


DIO, diet-induced obesity; IPGTT, Intraperitoneal Glucose Tolerance Test.
 $n = 3-8$ per group
 Effect of DIO, $p < 0.0001$; Effect of buchu on DOI, $p < 0.01$
 ##, $p < 0.01$, DIO + Buchu wk 8 vs Control and Control + Buchu wk 8; ****, $p < 0.0001$, DIO vs Control and control + Buchu wk 8; #####, $p < 0.0001$, DIO + Buchu Wk 8 vs DIO

FIGURE 3.8: IPGTT curves were generated as previously described and obtained from animals fed a control diet and those fed the obesogenic DIO diet, treated with buchu water or tap water as control.

differences between control animals and DIO animals that ingested buchu water from week 8 onwards. The 2-h values obtained through these graphs furthermore showed that the blood glucose values of the DIO + buchu animals were significantly lower than the values of the DIO animals and did not differ from that of control animals.

Figure 3.9 shows the results of the effects of ingestion of buchu water as a treatment option in pre-type 2 diabetic animals on insulin sensitivity at organ level using isolated ventricular cardiomyocytes. The ability of the cells to respond with glucose uptake to a concentration of 10 nM insulin was measured. In both control and DIO animals ingesting buchu water, insulin sensitivity at organ level was significantly enhanced. It can therefore be concluded that the ingestion of buchu water without a change in ingestion of an obesogenic diet was able to enhance the glucose utilisation at a given level of insulin in peripheral insulin-sensitive tissue.



DIO, diet-induced obesity.

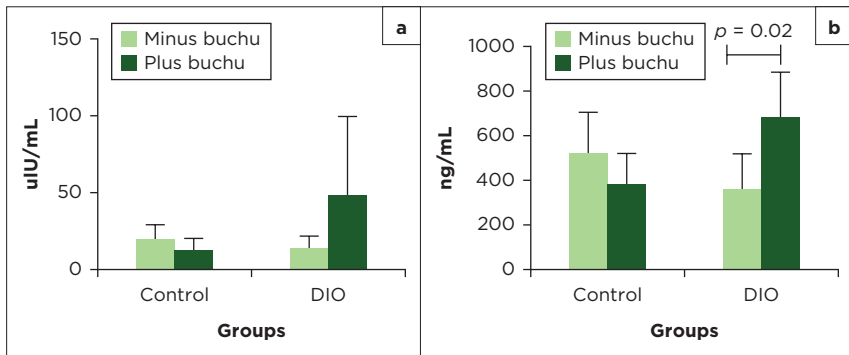
Note: Ventricular cardiomyocytes of hearts from control and DIO animals, with or without an 8-week treatment period, with buchu water were isolated by ex vivo collagenase perfusion. Cells were stimulated with 10 nM insulin as described in the 'Methods' section, and the accumulated DG was calculated per milligram protein over a 30-min period.

$n = 3-7$ per group

$p < 0.04$

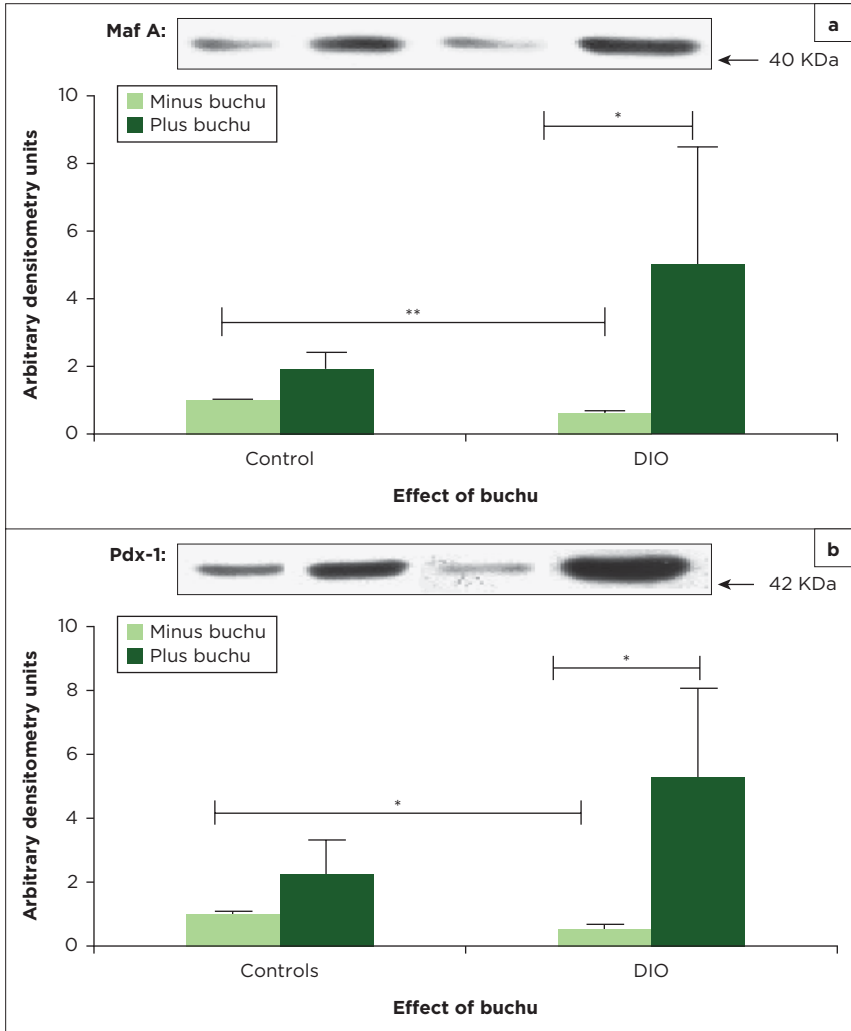
FIGURE 3.9: Cardiomyocyte accumulation of radio-labelled deoxy-glucose (DG).

Because of the observed effects of buchu water ingestion on glucose homeostasis in models of both type 1 and type 2 pre-diabetes, we investigated possible pancreatic effects. This was prompted by results showing enhanced insulin secretion in the DIO animals after ingestion of buchu water, as shown in Figure 3.10a. The secretion of insulin was further substantiated by an increase in the levels of C-peptide observed (Figure 3.10b). In addition, pancreatic transcription factors were analysed by western blotting. The two chosen transcription factors, Musculoaponeurotic Fibrosarcoma Homolog A (Maf A) and pancreatic duodenal homeobox 1 (Pdx-1), were significantly downregulated in the DIO animals (Figure 3.11). The ingestion of buchu water by DIO animals in combination with the obesogenic diet resulted in significant upregulation of both Maf A and Pdx-1. In addition, the overall effects of buchu ingestion, as indicated by a two-way ANOVA analysis, showed a highly significant effect. The enhanced expression of these transcription factors is an indication of β -cell neogenesis, probably by means of redifferentiation of pancreatic progenitor cells or acinar cells.²⁷



DIO, diet-induced obesity.

FIGURE 3.10: (a) Insulin levels of fasted serum obtained at the time of performance of the intraperitoneal glucose tolerance test were determined using a Coat-A-Count RIA (¹²⁵I radioimmunoassay) (*n* = 5-7). (b) The C-peptide levels were determined using a rat-sensitive ELISA from Abcam Ltd., Cambridge, UK (*n* = 5-7).



DIO, diet-induced obesity.

(a) $p = 0.01$; (b) $p = 0.007$

*, $p < 0.05$; **, $p < 0.01$

FIGURE 3.11: (a & b) Lysates were prepared from pancreata frozen at the time of euthanasia of the animals from the different experimental animal groups. They were analysed by western blotting and specific commercially available antibodies against musculoaponeurotic fibrosarcoma homolog A and pancreatic duodenal homeobox 1.

■ Discussion

The question that was posed was whether the aqueous extract of *Agathosma* as supplied by Cape Kingdom Nutraceuticals possessed any anti-diabetic properties as a treatment option. The research project tested this question by utilising two different animal models of diabetes simulating type 1 and type 2 diabetic populations. In both populations, it is recognised that there are stages of the disease that can be classified as pre-type 1 and pre-type 2 diabetes.⁸ The animal models used were manipulated to represent these so-called pre-stages. In T1D, this allows a remainder of pancreatic β -cells that has the potential to respond to treatment. If all β -cells were ablated, as is the scenario in end-stage T1D, manipulation would be impossible. Similarly, the DIO rat model, induced by feeding a diet rich in sugar and fatty acids, results in animals that are insulin resistant but, according to their fasting blood glucose levels, not type 2 diabetic.

From the results obtained, it is evident that buchu water was able to alleviate the pre-type 1 diabetes condition, in all instances, to very near normal levels when used as a treatment option. The animals presented with either >20 mmol/L or <20 mmol/L fasting blood glucose values and were grouped accordingly. This is also representative of the measure to which the pancreas was damaged. Highly significant statistical results were obtained showing normalisation of blood glucose levels in the <20 mmol/L group and a significant improvement in the >20 mmol/L group (Figure 3.1 and Figure 3.2). The IPGTT used to measure postprandial insulin responses to remove glucose from the circulation (Figure 3.3 and Figure 3.4), clearly demonstrated normalisation in both groups at the clinical relevant 2-h post-load values.²⁸ This is deemed a novel and important demonstration in that the aqueous extract of buchu has the ability to be used as a treatment option in newly diagnosed pre-type 1 diabetic patients. However, it must again be stressed that results obtained in an animal model must be re-tested by clinical trial to determine the efficacy in humans.

Anti-hyperglycaemic effects of buchu water were further substantiated in the diet-induced obese animals (Figure 3.7 and Figure 3.8). In addition, it was observed that the ingestion of buchu water resulted in a loss of intraperitoneal fat (IP fat). The effects of buchu water on fat tissue will be discussed in detail in Chapter 4.

To ascertain that the effects of buchu water on whole-body insulin sensitivity, as measured by the IPGTT assay, were also carried over to organ and cell level, we used myocytes isolated from the ventricles of the hearts of the animals after treatment. This is a sensitive measure to confirm the effects of buchu water on the glucose utilisation of peripheral insulin-sensitive tissue that is known to be responsible for the removal of post-prandial glucose from the circulation.²⁹ Figure 3.10 shows that the ingestion of buchu water as a treatment of pre-diabetes was able to significantly improve the ability of cardiomyocytes to accumulate glucose after stimulation with insulin. The exact cellular mechanism that was involved in this improvement was not elaborated.

As it was further noted in the DIO animals that the ingestion of buchu water stimulated insulin secretion (Figure 3.10a and b), we measured pancreatic transcription factors that are associated with the regeneration of pancreatic β -cells.

Musculoaponeurotic fibrosarcoma homolog A (Maf A) is a transcription factor of the basic leucine zipper family.³⁰ Maf A is a unique transcription factor expressed during late pancreatic development and is restricted to pancreatic β -cells.³¹ It is known to regulate insulin gene expression.^{32,33} In addition, it has been shown that the transcriptional activity of Maf A has a synergistic interaction with Pdx-1 that will enhance gene expression.³⁴

Pancreatic duodenal homeobox 1 protein is essential for early pancreatic development and β -cell survival.³⁵ Pdx-1 expression is restricted to pancreatic progenitor cells during early development, but later it becomes restricted to the β -cells. Low expression of Pdx-1 has been linked to a decline in β -cell population and

impaired glucose tolerance.³⁵ This decline in Pdx-1 is postulated to be because of enhanced DNA methylation induced by hyperglycaemia.³⁶ Eventually, this leads to β -cell death via apoptosis. When substantial β -cell loss occurs, it will result in permanent endocrine deficiency and irreversible diabetes.²⁷ It has been shown that patients suffering from T2D have a significantly lower β -cell population than healthy persons.²⁷ Current research to remedy this situation has a strong focus on different strategies to produce new endocrine islet cells. Preclinical studies have already shown Pdx-1 expression as a promising candidate in this arena as it was found that, when Pdx-1 progenitor cells were transplanted into mice, some of these cells differentiated into functional β -cells which could reverse diabetes.^{36,37} Furthermore, it was found that combination expression of Pdx-1 and Maf A, among others, could efficiently convert pancreatic acinar cells into β -like cells when these transcription factors were supplied to mouse pancreata through adenoviral vectors.^{38,39}

In light of the fact that both T1D and T2D are the result of substantial β -cell loss, and currently there is no treatment option to remedy or stop this loss, the results obtained in this study are of great importance. It is categorically stated by Zhou and Melton²⁷ that:

[/]f pharmacological means can be found to 'redifferentiate' the dedifferentiated β -cells, it could constitute a new therapeutic approach for diabetes and may be viewed as a distinct form of regenerative therapy. (p. 355)

The obesogenic diet fed to the rats in this study resulted in a significantly lower expression of both Maf A and Pdx-1, which is indicative of a loss of β -cell mass. The ingestion of buchu water as a treatment option significantly enhanced the expression of both these transcription factors, indicating an increase in functional β -cell mass. It must, however, be reiterated that human T1D is an autoimmune disease where the regeneration of pancreatic β -cells may be counteracted by the effects of the

autoantibodies and that this concept needs to be further explored through clinical trials.

It is recognised that different polyphenols in an extract, such as the one used in this study, have very low levels. In addition, the bio-availability of most of these polyphenols is also very low. In the past, this has repeatedly posed the question of how orally ingested polyphenols may change physiology. In this regard, a new paradigm has emerged, centring on the ability of nutrition to alter the inherent microbiome of the host ingesting any form of food.⁴⁰ This microbiome consists of various bacteria, viruses, protozoa and fungi⁴¹ and has an intricate relationship with the physiology of the host. It plays a major role in the way by which any substance ingested influences the physiology and pathophysiology of the host.^{41,42}

With regard to effects on pancreatic regeneration, γ -aminobutyric acid (GABA) has been recognised as a facilitator to reprogramme pancreatic β -cells. Treating mice with GABA resulted in a significant increase in pancreatic β -cell mass.⁴³ As reviewed by Foster et al.,⁴⁴ a definite link between the gut microbiome and the regulation of brain neurotransmitters has repeatedly been demonstrated. Bravo et al.,⁴⁵ for example, showed that probiotic modulation of the gut microbiota of mice could induce neurochemical changes that were relayed through the vagus nerve connections. It was also shown that the gut microbiota can act as an endocrine organ by directly producing metabolites that are transferred to the circulation. Among these neurotransmitters, both serotonin and GABA were identified, and it was demonstrated that oral ingestion of *Lactobacillus* increased GABA levels also in the brain.⁴⁶ If the aqueous extract of *Agathosma* utilised in this study could induce the gut microbiome to secrete substances such as GABA that have been shown to act on the most important transcription factors involved in pancreatic redifferentiation, it could explain our findings of enhanced β -cell neogenesis despite the low levels of polyphenols that can be expected to reach the circulation.

These findings are deemed novel and may indeed lead to the development of pharmacological means to redifferentiate β -cells and treat both pre-type 1 and pre-type 2 diabetes. Retrospectively, determination of serotonin and GABA levels in the circulation could have been performed to substantiate these arguments.

■ Acknowledgements

The author would like to thank V. Jansen and J.W. Lombard, Division of Medical Physiology, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, South Africa, for their assistance in the generation of data for this study.



CHAPTER 4

Anti-obesity effects of an aqueous extract of buchu

Description: Cultivated Buchu flowering.

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Anti-obesity effects of an aqueous extract of buchu

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■ Introduction

According to a WHO report, more than 1.9 billion adults worldwide are currently overweight and more than 650 million of these individuals are obese.¹ Obesity is associated with several health consequences, including cardiovascular diseases (CVDs) and T2D.² These conditions cause premature death or substantial disability, resulting in an economic burden.

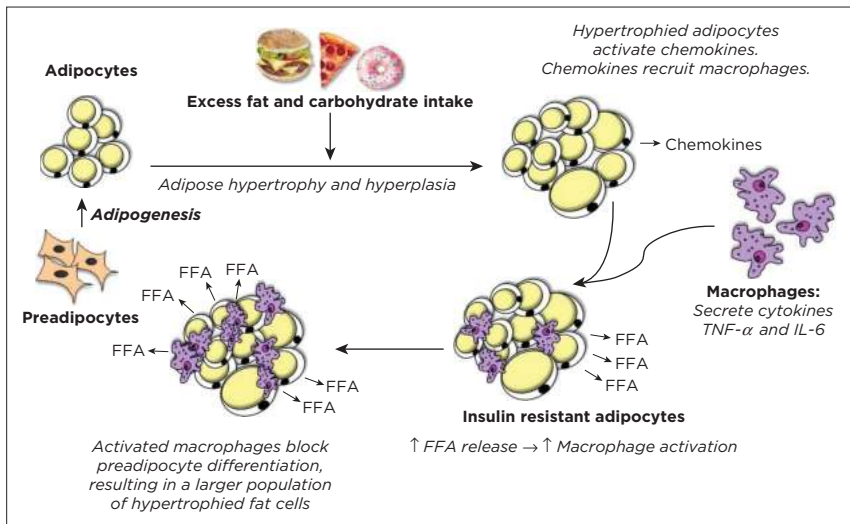
Obesity is defined as excessive fat accumulation, traditionally described as a result of an imbalance between energy intake versus energy expenditure. According to the first law of

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thermodynamics, energy in will be equal to energy out plus stored energy. This excess energy is stored in adipocytes, causing hyperplasia (increase in fat cell number) and hypertrophy (increase in fat cell size).³ However, the problem that isocaloric diets with different composition ratios of fat, carbohydrate and proteins do not always result in similar weight gain or weight loss was addressed in the carbohydrate-insulin model of obesity suggested by Ludwig and Ebbeling.⁴ The rationale of this model of obesity lies in the fact that a carbohydrate load is the strongest stimulus of insulin secretion, which, in turn, stimulates lipogenesis in fat cells. On the other hand, a low intake of carbohydrates will limit insulin secretion, thereby counteracting fat deposition and obesity. This concept can clinically explain how the composition of a diet, although isocaloric, by effects on the hormonal regulatory systems, can either cause obesity or have no effect on weight gain.

With diet management, however, adipocyte size can decrease, but their numbers are constant during adulthood.⁵ Hypertrophied adipocytes lead to the activation of several macrophage-attracting chemokines followed by macrophage recruitment.^{6,7,8} Macrophages secrete cytokines, including tumour necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), causing an inflammatory cascade and thereby inducing insulin resistance in the mature adipocytes.^{6,9,10,11} Activated macrophages block pre-adipocyte differentiation, resulting in a larger population of hypertrophied fat cells (Figure 4.1). According to the adipose tissue expandability hypothesis, this leads to the development of insulin resistance within the adipose mass, while adipose depots that consist of new or small adipocytes are associated with improved insulin sensitivity.^{6,12} Insulin-resistant adipocytes cause an elevation in Free Fatty Acids (FFAs) release and further macrophage activation, forming a vicious circle. It is postulated that insulin resistance in adipose tissue precedes the development of whole-body insulin resistance and lipid accumulation in other organs.^{6,13} It has also been shown in humans that even in non-obese individuals, adipocyte insulin resistance,

characterised by altered adipokine profiles, is associated with adipocyte hypertrophy and whole-body insulin insensitivity.⁹ In addition, Bremer et al. found that subcutaneous fat depots of patients suffering from metabolic syndrome were associated with increased biomarkers of both insulin resistance and low-grade inflammation.¹⁴ Peroxisome proliferator-activated receptors (PPARs) are important regulators of glucose and lipid storage and oxidation. In adipose tissue, PPAR γ is an important transcription factor controlling adipogenesis and adipocyte differentiation.^{6,15} Peroxisome proliferator-activated receptor α is associated with regulating the expression of genes controlling fatty acid oxidation.¹⁶ Sterol regulatory element-binding proteins (SREBPs) regulate lipid homeostasis by acting as transcription factors for lipid synthesis.^{15,17} Ferreira et al. demonstrated the upregulation of both PPAR γ and SREBP-1 (also termed Srebf1) in adipose tissue of high-fat-fed mice, while overexpression of Srebf1 in adipose tissue of mice resulted in adipocyte hypertrophy.^{18,19}



Source: Adapted from Virtue and Vidal-Puig⁶ and composed using Microsoft PowerPoint. FFA, free fatty acids; TNF- α , tumour necrosis factor-alpha; IL-6, interleukin-6

FIGURE 4.1: Adipose tissue expandability.

Because of the effects of ingestion of buchu water on blood glucose handling, bodyweight and, especially, the intraperitoneal (IP) fat weight, this study was conducted to document changes in the fat tissue harvested from the animals with regard to the aspects described above.

Materials and methods

Animal model

In this study, the animals were randomly divided into a control group and a HFD group and kept on the respective diets for 16 weeks (Table 4.1). This specific diet renders the animals both insulin resistant and hypertensive, which is further elaborated on in Chapter 5. All other aspects of handling the animals were similar, as described in Chapter 3.

In this study, both renal and gonadal fat pads were removed to determine the weight. A specific portion of fat with a piece of muscle attached was dissected from the renal pads for histological examination of fat cell size. About 50 mg of fat was collected in Ribonucleic Acid (RNA) later® (Thermo Fischer Scientific, Waltham, MA USA 02451) for real-time polymerase chain reaction (PCR).

Histology

Fat was cryo-fixed and eosin-stained for the histological determination of fat cell size. Three areas (1mm × 1mm) per sample were selected using low magnification, a Zeiss Axioskop 2

TABLE 4.1: Composition of diets.

Specimen Group	Fat (g/100 g)	Cholesterol (mg/100 g)	Protein (%)	Carbohydrates (%)	Sugar (g/100 g)	Fructose (g/100 g)	Energy (kJ/100 g)
Control	4.8	3	17.1	34.6	6.6	0.5	1272
HFD	27.9	6.4	14.6	29.5	13.3	11	1829

Source: Analysis performed by Microchem Lab Services (Pty) Ltd. HFD, high-fat diet.

microscope with AxioVision v4.7 software and an Axiocamera. Each area was enlarged 10 times and then the diameter of every cell in the specific area was measured. Data from the different groups of animals were compiled and statistically analysed.

■ Biochemical analyses

ELISA kits (Abcam[®], Cambridge, UK) were used to determine unfasted serum leptin, adiponectin, TNF- α and IL-6 levels. Insulin levels were determined using fasting serum and a RIA coat-a-count assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA).

■ Polymerase chain reaction

Total RNA was extracted from 50 mg of fat in RNA later[®] using Qiazol reagent and purified using the RNeasy Mini Kit, according to the manufacturer's instructions (Qiagen). The RNA concentration and purity were determined spectrophotometrically using the Nanodrop, ND-1000 (Nanodrop Technologies, Thermo Fischer Scientific, Waltham, MA 02451, USA) and integrity was determined using the Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA 95051, USA).

DNase-treated RNA (Turbo DNA-free DNase, Life Technologies, Thermo Fischer Scientific, Waltham, MA 02451, USA) was reverse-transcribed to cDNA using the high-capacity reverse transcription kit (Life Technologies). Real-time quantitative PCR was performed in a total volume of 10 μ L using cDNA templates and pre-formulated Taqman gene expression assays (Table 4.2) according to the manufacturer's protocol (Life Technologies). The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification was carried out on the 7500 real-time PCR system (Life Technologies). The default settings for the threshold cycle (Ct) and baseline were used. The mRNA levels of each gene were normalised to those of the housekeeping genes β -actin (ActB) and hypoxanthine phosphoribosyltransferase 1 (Hprt).

TABLE 4.2: Taqman gene expression assay.

Taqman gene expression assay	Assay number
Srebf1	Rn01495769_m1
Fatty acid synthase (Fasn)	Rn01463550_m1
PPAR α	Rn00566193_m1
PPAR γ	Rn00440940_m1
ActB	4326315E
Hprt1	Rn01527840_m1

PPAR, Peroxisome proliferator-activated receptors.

Note: Taqman gene expression assays were performed according to the manufacturer's protocol (Life Technologies). The mRNA levels of each gene were normalised to those of the housekeeping genes β -actin (ActB) and hypoxanthine phosphoribosyltransferase 1 (Hprt).

Total liver cholesterol, liver triglycerides, plasma phospholipids and plasma triglycerides analysis

Plasma and liver samples were analysed for concentrations of lipids, including total cholesterol, triglycerides and phospholipids.

Total cholesterol, triglycerides and phospholipids concentrations were determined using enzymatic colorimetric kits (LabAssay™ Cholesterol [catalogue number 294-65801], LabAssay™ Triglyceride [catalogue number 290-63701] and LabAssay™ Phospholipid [catalogue number 296-63801]; Wako Chemicals, Neuss, Germany) and using a SPECTRA-max Plus 384 spectrophotometer with SoftMax Pro 4.8 microplate data acquisition and analysis software (Molecular Devices [US/Canada]; Labotec Industrial Technologies [Pinelands, South Africa]). The inter-assay coefficients of variation for these analyses were all less than 3%.

Livers were stored at -80°C for lipid analysis. Liver tissue was homogenised on ice, using a Vir Tis hand shear (The VirTis Company, Gardiner, NY, USA), in 10 volumes of 75mM phosphate buffer containing 0.5M EDTA (Sigma-Aldrich, Johannesburg, South Africa), pH 7.4. Extraction of lipids was carried out by the Bligh and Dyer method,²⁰ a modified method of Folch et al.²¹ Briefly, lipids were extracted into a monophasic mixture of chloroform and

methanol (Merck [Pty] Ltd South Africa, Cape Town). This mixture was finally separated into two phases, one of which was the organic phase containing the purified lipids. This layer was dried under nitrogen gas, after which it was dissolved in chloroform and divided into aliquots, dried once again under nitrogen headspace and stored at -80°C . For lipid analyses, the extracts were solubilised in ethanol (Merck [Pty] Ltd South Africa, Cape Town) and Triton X-100 (Sigma-Aldrich [Pty] Ltd South Africa, Cape Town), and this was followed by the addition of physiological saline. This was used for the determination of total cholesterol, triglyceride and phospholipid concentrations in the liver samples. Liver cholesterol, triglyceride and phospholipid concentrations were determined using the enzymatic colorimetric kits as described for plasma above.

■ Statistical analysis

All data were statistically analysed using GraphPad Prism 5. All values are given as mean \pm standard error of the mean (SEM). A two-way ANOVA, followed by a Bonferroni *post hoc* test, was used to determine the significance and a p -value of <0.05 was considered as significant.

■ Results

■ Biometric data

Table 4.3 summarises the biometric parameters and daily food and water consumption of the animals. The HFD significantly increased the animals' bodyweight from $381.3\text{g} \pm 9.5\text{g}$ to $451.8\text{g} \pm 15.1\text{g}$ ($n = 10/\text{group}$; $p < 0.001$) and IP fat from $8.6\text{g} \pm 0.4\text{g}$ to $24.5\text{g} \pm 2.7\text{g}$ ($n = 10/\text{group}$; $p < 0.0001$) when compared to the controls. However, buchu water consumption resulted in less weight gain (from $451.8\text{g} \pm 15.1\text{g}$ to $394.1\text{g} \pm 14.0\text{g}$; $n = 10/\text{group}$; $p < 0.01$) and less IP fat gain (from $24.5\text{g} \pm 2.7\text{g}$ to $15.9\text{g} \pm 1.3\text{g}$; $n = 10/\text{group}$; $p < 0.01$) in the HFD animals. The effect of buchu consumption on lowering bodyweight gain was further indicated as highly significant ($p = 0.003$).

TABLE 4.3: Biometric parameters of different animal groups.

Biometric parameter	Control	Control + buchu	HFD	HFD + buchu
Bodyweight (g) n = 10/group	381.3 ± 9.5	363.2 ± 5.5	451.8 ± 15.1***	394.1 ± 14.0##
IP fat weight (g) n = 10/group	8.6 ± 0.4	8.7 ± 0.4	24.5 ± 2.7****	15.9 ± 1.3##
Blood glucose (mmol/L) n = 5/group	6.2 ± 0.1	6.0 ± 0.1	7.3 ± 0.2**	6.2 ± 0.2#
Insulin (mU/mL) n = 5/group	16.6 ± 3.3	11.8 ± 4.7	21.8 ± 3.8	12.2 ± 0.5
Food intake (g/rat/day) n = 20/group	21.4 ± 0.5	20.4 ± 0.4	21.7 ± 1.0	19.3 ± 0.7
Water intake (mL/rat/day) n = 20/group	33.8 ± 0.9	28.7 ± 0.9*	29.7 ± 1.7	30.4 ± 1.0

HFD, High-fat Diet; SEM, Standard Error of the Mean; IP, Intraperitoneal.

Note: Data are expressed as mean ± SEM using two-way ANOVA ($n = 5-20$). The effect of buchu on bodyweight: $p = 0.003$; IP fat: $p = 0.007$; and blood glucose levels: $p < 0.01$. The effect of HFD on IP fat: $p < 0.0001$.

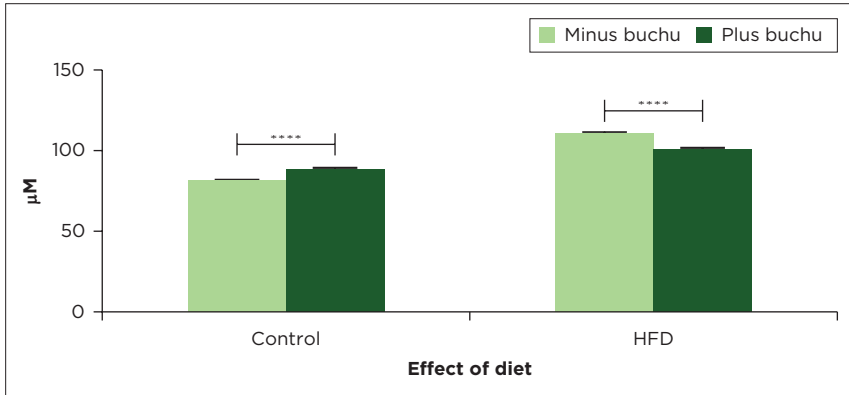
* $p < 0.05$ versus control; ** $p < 0.01$ versus control; *** $p < 0.001$ versus control; **** $p < 0.0001$ versus control; # $p < 0.05$ versus HFD; ## $p < 0.01$ versus HFD.

Furthermore, similar analyses of IP fat accumulation showed that the HFD had a significant effect ($p < 0.0001$), while the effect of buchu consumption on lowering IP fat content was significant with $p = 0.007$. These changes were not associated with differences in the amount of food consumed by the animals; however, the HFD has a higher caloric density compared to the control diet (Table 4.1). A slightly diminished buchu extract intake was noted in the control animals, but not in the HFD animals.

The HFD animals presented with increased fasting blood glucose levels ($p < 0.01$), while buchu consumption had a significant overall effect on blood glucose levels ($p < 0.01$) and prevented the rise in blood glucose in the HFD animals ($p < 0.05$).

■ Adipocyte hypertrophy

In Figure 4.2, data are summarised to show that the HFD animals presented with enlarged fat cells ($p < 0.0001$), while the ingestion of buchu in the HFD animals decreased the fat cell size ($p < 0.0001$). Then again, fat cells in the control animals were overall significantly larger after buchu ingestion ($p < 0.001$).



HFD, high-fat diet.

Note: Histological examination of fat cell size with $n = 3-5$ biological replicates analysed with an n -value of 650-1050 individual cells per group was performed and the data were compiled.

****, $p < 0.0001$

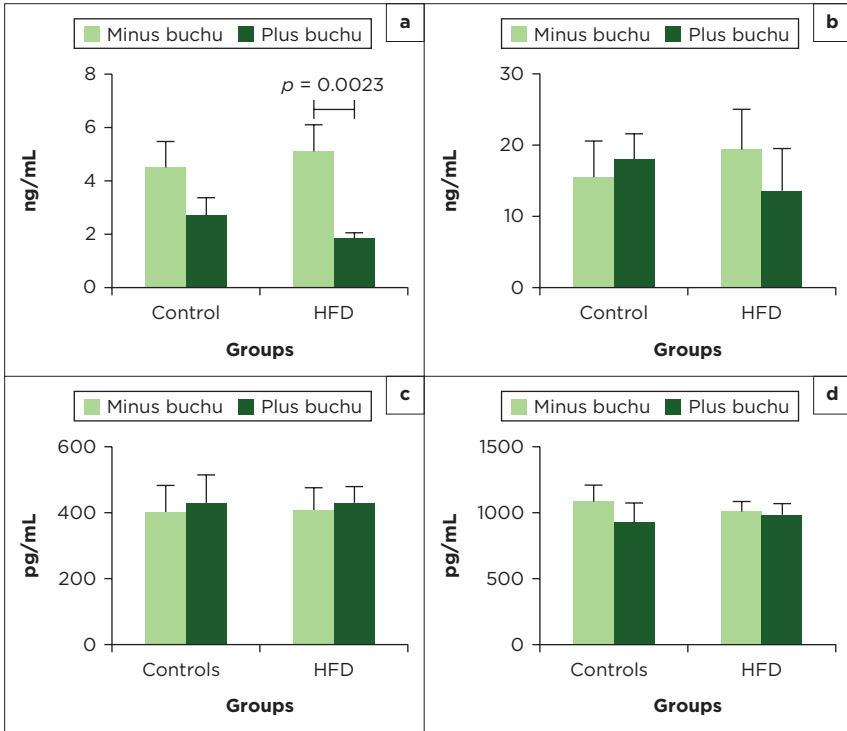
FIGURE 4.2: Adipocyte hypertrophy.

Biochemical analysis

Blood serum indicated that buchu ingestion had an overall significant effect on lowering leptin levels ($p = 0.0003$, Figure 4.3a). This was accentuated by significantly lower leptin levels in the HFD animals ($p = 0.0023$). No significant differences were observed in the adiponectin (Figure 4.3b), TNF- α (Figure 4.3c) and IL-6 (Figure 4.3d) levels.

Transcription factors

According to PCR analyses of the fat tissue, the HFD elevated the mRNA levels of PPAR γ ($p < 0.05$, Figure 4.4a), while ingestion of buchu lowered these levels to control values ($p < 0.05$). The overall effect of the HFD on the mRNA of Srebf1 was also indicated as significant ($p = 0.05$, Figure 4.4c). These levels were not affected by the ingestion of buchu. Peroxisome proliferator-activated receptor α showed no significant changes (Figure 4.4b).



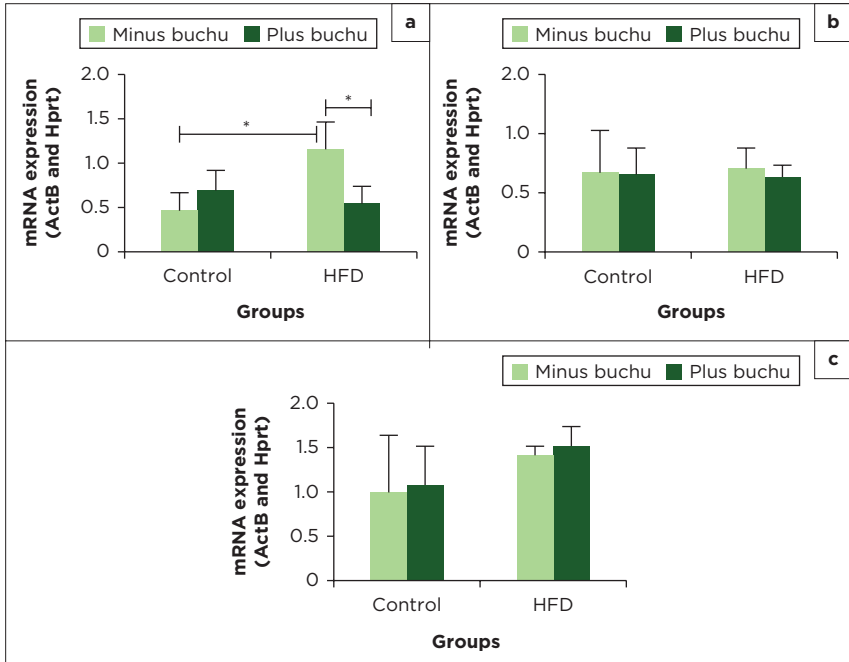
HFD, high-fat diet.

Note: Values are given as mean \pm SEM ($n = 5-8$ biological replicates per group). Analyses were performed in triplicate. The overall effect of buchu on leptin levels was significant ($p = 0.0003$).

FIGURE 4.3: Biochemical markers leptin, adiponectin, TNF- α and IL-6. (a) Plasma leptin, (b) adiponectin, (c) TNF- α and (d) IL-6 levels from fasted buchu-treated and untreated animals determined by enzyme-linked immunosorbent (ELISA) assay kits (Abcam).

■ Total liver cholesterol, liver triglycerides, plasma phospholipids and plasma triglycerides

Liver analysis indicated that treatment with buchu significantly decreased total cholesterol levels in both the control and HFD animals ($p = 0.03$). Furthermore, buchu treatment decreased plasma phospholipids with borderline significance ($p = 0.07$) in both animal groups, and plasma triglycerides in the HFD animals ($p = 0.03$) (see Figure 4.5).



HFD, high-fat diet.

Note: mRNA levels were normalised to the housekeeping genes β -actin (ActB) and hypoxanthine phosphoribosyltransferase 1 (Hprt). Additionally, a two-way ANOVA indicated the effect of the HFD on Srebf1 levels to be significant, with $p = 0.05$.

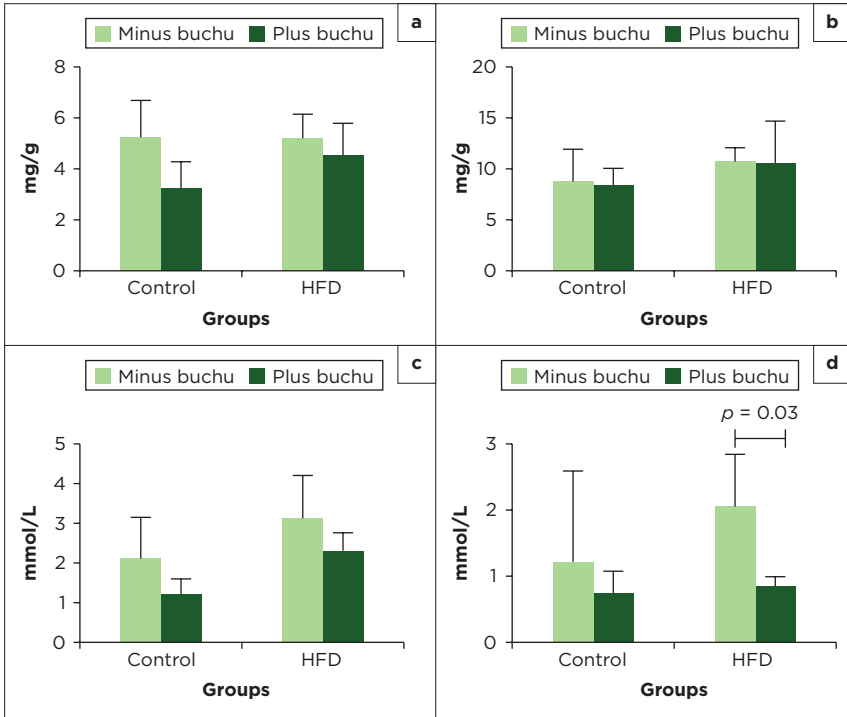
$n = 4$ biological replicates per group

*, $p < 0.05$

FIGURE 4.4: Transcription factors PPAR γ , PPAR α and Srebf1. For (a) PPAR γ , (b) PPAR α and (c) Srebf1 determination, total RNA was extracted from 50 mg of fat in RNAlater® for quantitative real-time PCR analysis.

Discussion

The current pandemic of obesity is a known risk factor for several non-communicable diseases, including cardiovascular disease, T2D and various cancers. Treatment strategies include lifestyle changes, drugs and bariatric surgery. Lifestyle changes have proved to be difficult to adhere to; drugs have at best modest benefits, while bariatric surgery, although successful in affecting weight loss, has serious long-term consequences.^{22,23} Nutraceutical therapies have been explored with various degrees of success in



HFD, high-fat diet.

Note: These were determined using commercially available assays ($n = 3-5$ biological replicates per group). A two-way ANOVA indicated that buchu had an overall positive effect on total cholesterol levels ($n = 0.03$) and plasma phospholipids ($p = 0.07$, borderline significance). Buchu also significantly decreased plasma triglyceride levels in the HFD animals ($p = 0.03$).

FIGURE 4.5: Total liver cholesterol (a), liver triglyceride (b), plasma phospholipids (c) and plasma triglycerides (d).

this field and, if scientifically validated, can supply a readily available and safe alternative to pharmacotherapy. Therefore, in this study, we tested the anti-obesity potential of an aqueous extract of buchu, a product currently commercially available.

The *Agathosma* plant, especially *A. crenulata*, contains one possible hepatotoxic substance, pulegone.²⁴ Pulegone, however, is not water soluble and has been approved by the US FDA for use

in the food industry (with a FEMA GRAS status and listed among the authorised synthetic flavouring substances CFR 21-172.515). The no-effect level of pulegone in beverages is 100mg/kg.²⁵ The buchu extract contained 74.22 mg/L \pm 1.1mg/L pulegone, which is well below the acceptable level indicated by the FDA and the LC50 value of 25.91mg/mL.²⁶ Buchu-treated animals in our study would have ingested a maximum of 1.7 mg/kg/day of pulegone and showed no observable signs of hepatotoxicity according to liver cholesterol and triglyceride analyses (Figure 4.5).

The water of buchu-treated animals was replaced with a four times dilution of the aqueous buchu extract. This rendered a concentration in mg/L of diosmin (0.0005), quercetin (0.007), hesperidin (0.001) and rutin (0.0035), of which the animals consumed a mean of 30mL per day. The ingestion of these bioflavonoids was therefore extremely low. Despite this, the HFD buchu-treated animals presented with significantly less IP fat weight gain and lower blood glucose values when compared to HFD-untreated animals (Table 4.3). In view of the adipose expandability hypothesis⁶ and the known effects of bioflavonoids on obesity,^{27,28,29} we examined changes brought about in the fat depots of the animals to understand the lesser weight gain.

As endocrine organ, fat secretes certain adipokines, such as leptin and adiponectin. In obese individuals, increased leptin levels increase the macrophage phagocytic activity, along with their production of pro-inflammatory cytokines, resulting in decreased small adipocyte formation.^{5,8} As shown in Figure 4.2, the HFD resulted in increased adipocyte size, while buchu ingestion resulted in significantly smaller adipocytes, accompanied by significantly lower leptin secretion.

Leptin acts as an appetite suppressant by inhibiting neuropeptide Y via negative feedback. However, our study documented no difference in the amount of food consumed by the animals (Table 4.3), therefore indicating that, in the buchu-treated HFD animals, the lower leptin secretion was because of smaller adipocytes, as larger cells secrete more adipokines.¹¹

Buchu ingestion significantly attenuated blood glucose levels in both animal groups, indicating the improvement of insulin sensitivity (Table 4.3).

Thus, buchu ingestion in the HFD animals decreased adipocyte size and leptin levels, thereby increasing insulin sensitivity and pre-adipocyte formation¹² (Figure 4.2 and Figure 4.3a).

Adipogenesis and lipogenesis are regulated by multiple transcription factors coding for different enzymes modulating fat deposition and lipolysis. To further elucidate the anti-obesity effects of buchu, we determined the mRNA levels of three regulators of adipogenesis and fatty acid oxidation: PPAR γ , PPAR α and Srebf1 (Figure 4.4a–c). No changes were observed in PPAR α levels. Peroxisome proliferator-activated receptor γ has long-chain fatty acids as endogenous ligands and, as also found in this study, is known to be increased in adipose tissue following a HFD.¹⁸ In accordance with the findings in mice fed with HFD,^{17,19} the transcription factor Srebf1, which is known to be involved in lipid synthesis, was also elevated in this study and associated with adipocyte hypertrophy.

Peroxisome proliferator-activated receptor γ (PPAR γ) expression is regulated, among others, by the β -catenin pathway, which, in turn, is regulated by Wnt-GSK-3 β signalling.³⁰ As GSK-3 is overexpressed in adipose tissue of obese mice,³¹ it can be expected that β -catenin levels are low, leading to increased PPAR γ levels. The increased insulin sensitivity of the adipose tissue after buchu ingestion may therefore lead to the decrease in PPAR γ levels via normalisation of inhibition of GSK-3. It is also known that Sirt1 promotes fat loss by repressing PPAR γ through acetylation³²; however, Sirt1 expression was not determined in this study. In contrast to the decrease in PPAR γ levels induced by buchu ingestion in the HFD animals, we observed no significance in the control animals' PPAR γ levels, but found an increase in adipocyte hypertrophy. This may be explained by an observation by Ferreira et al.¹⁸ that diet composition differentially affects adipocyte hypertrophy. In addition, despite this small increase in adipocyte size, no increase in the IP fat deposition was detected.

Reverse cholesterol transport (RCT) refers to the process where cholesterol from the peripheral tissues are returned to the liver via the plasma.³³

The first step entails the movement of peripheral cholesterol and phospholipids to the pre-high-density lipoprotein (HDL) via the ATP-binding cassette transporter (ABCA1). Following this, the cholesterol present on the pre-HDL is converted by the Lecithin-cholesterol Acyltransferase (LCAT) enzyme to cholesteryl esters, which are stored in the core of the HDL. Consequently, the cholesteryl esters will be exchanged for triglycerides from other lipoproteins, for example, Very Low-density Lipoproteins (VLDL) and Low-density Lipoproteins (LDL) through the cholesteryl ester transfer protein (CETP). The mature HDL will bind to the scavenger Receptor class B type 1 (SRB1) present on the liver surface, followed by the release of cholesteryl esters. The cholesterol will be converted into bile and transferred to the intestine for excretion.^{34,35}

Interestingly, in rats and mice, RCT is altered because of a deficiency in the sequences coding for functional CETP activity.³⁶ Thus, because of this deficiency, the plasma cholesterol, phospholipid and triglyceride levels of rats will be more accurate when measured in the plasma, as compared to the liver.

In this study, buchu decreased (with borderline significance) plasma phospholipids in both the animal groups and plasma triglyceride levels in the HFD animals (Figure 4.5c and d). Both these results are indicative of decreasing the risks associated with the metabolic syndrome.

Although it is recognised that the known bioflavonoids in the buchu extract, especially hesperidin and quercetin, have the ability to regulate the levels of PPAR γ and Srebf1,^{27,29,36} the concentrations of these substances in even the original, undiluted buchu extract are so low in comparison to the concentrations used in these studies that, in isolation, they cannot be deemed to elicit the physiological effects observed.

■ Conclusion

We conclude that the buchu extract tested in this study has beneficial effects as nutraceutical to counteract obesity, with all the accompanying health benefits of less weight gain. The exact mechanisms of how this is accomplished are possibly multifactorial, but include insulin sensitisation of fat cells. The extract may contain a hitherto unrecognised substance that may have elicited the observed changes, or the combination of bioflavonoids may be responsible.

■ Limitations of the study

Limitations of this study include:

- inflammatory mediators (TNF- α and IL-6) were only determined in circulation and not in the adipose tissue itself
- Sirt1 expression levels were not determined, which may have shed more light on the mechanisms involved
- as the study was conducted on the prevention of weight gain, a further study should address the buchu extract as a treatment option.

■ Acknowledgements

The authors acknowledge the assistance of Mr Reggie Williams, Department of Anatomy, Faculty of Health Sciences, University of Stellenbosch, for technical assistance with histological analysis of the fat tissue and Dr Dee Blackhurst, Division of Chemical Pathology, University of Cape Town, for the liver analyses.

A person wearing a cap and work clothes is harvesting green bushes in a field. They are using a curved harvesting tool to cut the plants, which are being collected into a large, light-colored sack. The background shows a vast, open landscape with green fields and a reddish-brown hillside under a clear sky.

CHAPTER 5

Anti-hypertensive and cardioprotective effects of buchu

Description: Manual harvesting of Buchu.

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Anti-hypertensive and cardioprotective effects of buchu

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■ Introduction

Cardiovascular disease (CVD) is currently the leading cause of mortality in the world, resulting in approximately 17.3 million deaths annually, with 80% of these deaths occurring in low- and middle-income countries.^{1,2} Both obesity and elevated blood pressure (BP) increase the risk of developing CVD, renal failure and stroke, thereby further increasing the risk of mortality from CVD.² Hypertension is classified as the leading preventable cause of death worldwide³ and is causally linked to stroke, myocardial infarction, end-stage renal disease, congestive heart failure, peripheral vascular disease and blindness. In South Africa, current

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statistics show an overall 55% incidence of hypertension and 25% of pre-hypertension. Hypertension is defined as Systolic Blood Pressure (SBP) ≥ 140 mmHg and/or Diastolic Blood Pressure (DBP) ≥ 90 mmHg; pre-hypertension is defined as SBP between 120 mm Hg and 139 mm Hg and/or DBP between 80 mm Hg and 89 mm Hg in the absence of a diagnosis of hypertension or treatment with medication for high BP.⁴

Despite global efforts to combat hypertension, rates of control of the disease remain very low. Given the continued significant health and economic burden on low- and middle-income populations, there is an urgent need to address the problem by way of novel approaches.²

One such approach utilises the anti-hypertensive effects of polyphenols found in plant extracts.^{5,6,7} We tested the efficacy of the aqueous extract of *Agathosma* to lower BP and to protect the heart using obese, hypertensive Wistar rats. In previous chapters, we have already discussed the significant effects of obesity and hyperglycaemia, both risk factors for the development of CVD and hypertension.

■ **Methods**

■ **Animal model**

Hypertension was induced by feeding randomly selected animals (6–7 weeks old) a diet consisting of normal rat chow supplemented to contain 40% saturated fat, 10% fructose, 10% casein and 1% cholesterol for 14 weeks (HFD).⁸ These were the same animals from which the fat depots were harvested, analysed and described in Chapter 4.

■ **Diuretic activity**

At the commencement of treatment with the buchu water and again at the end of the treatment, in this specific set of experiments, groups of animals were placed individually in

metabolic cages to collect 24-h urine production as a measurement of diuretic activity.

■ Blood pressure measurement

A non-invasive computerised tail-cuff system (Kent Scientific Corporation, CT, USA) was used to measure BP. Rats were trained for 2 weeks before the commencement of data collection. The animals were placed on a warming platform (35°C) within a restrainer fitted with a black nose cone. It was previously shown that temperature fluctuation of the tail may affect the measurement of BP.⁹ Each session included two sets of five measurements so that a total of 10 measurements were used to determine the BP of each rat on a single day. Data were collected at weekly intervals over the 14-week period.

At the end of 14 weeks, the animals were anaesthetised using sodium pentobarbital (160 mg/kg bodyweight) until deep anaesthesia indicated by lack of the pedal reflex or blinking when touching the eye. The animals were weighed and euthanised by exsanguination, and the blood sample was collected in serum collection tubes for biochemical analysis. Intraperitoneal fat was dissected out and weighed.

■ Determination of myocardial infarct size

After removal, the rat hearts were arrested in ice-cold Krebs Henseleit (KH) medium (in mM: NaCl 119, NaHCO₃ 25, KCl 4.75, KH₂PO₄ 1.2, MgSO₄·7H₂O 0.6, Na₂SO₄ 0.6, CaCl₂·2H₂O 1.25, glucose 10) and immediately (within 30 s) mounted onto the aortic cannula of a perfusion rig. The pulmonary vein was connected to a second cannula in order to perform perfusions in the working-heart mode, with a preload of 15 cm H₂O and an afterload of 100 cm H₂O, as described previously.^{8,10} The perfusion medium was continuously gassed with 95% O₂/5% CO₂. The hearts were fitted with a temperature probe and the temperature was kept constant at 36.5°C – 37°C. After a stabilisation period of 30 min, the rat hearts

were subjected to 35min regional ischaemia by coronary artery ligation, followed by reperfusion for 1h, as described previously.^{8,10} Infarct size was determined according to a well-established protocol, followed by planimetry, and expressed as the percentage of the area at risk.^{8,10} Planimetry was performed blindly by a third party.

■ Biochemical analyses

Serum insulin and leptin levels were determined using enzyme-linked immunosorbent (ELISA) assays (Abcam Ltd, Cambridge, UK) according to the manufacturer's instructions, while aldosterone was determined using a Coat-a-Count RIA kit (Diagnostics Corporation Ltd, Los Angeles, CA, USA). Both the aqueous extract and the serum collected from the animals were assayed for Angiotensin 1-Converting Enzyme (ACE) inhibitor activity using fluorescence resonance energy transfer (FRET) as described by Carmona et al.¹¹ The ACE fluorescent peptide substrates specific for the N (Abz-SDK(Dnp)P-OH) and C (Abz-LFK(Dnp)-OH) domains of ACE were obtained from Sigma-Aldrich. Angiotensin 1-converting enzyme activity of the water was tested on aortic endothelial cells in culture.¹² Captopril was used as a positive control.

■ Statistical analyses

The changes in BP were analysed by a biostatistician from the University of Stellenbosch and are presented with 95% confidence intervals. All other data are expressed as mean \pm SEM and were analysed using GraphPad Prism 6 software by either one-way or two-way ANOVA, with $p < 0.05$ considered as significant.

■ Results

■ Biometric measurements

As summarised in Table 5.1, the HFD diet caused significant bodyweight gain ($449\text{g} \pm 13.3\text{g}$ vs. $377\text{g} \pm 8.0\text{g}$, $p < 0.001$ and

TABLE 5.1: Biometric parameters of four animal groups.

Biometric parameters	Control	Control + buchu	HFD	HFD + buchu
Bodyweight (g), <i>n</i> = 15/group	377.5 ± 8.0	363.4 ± 8.1	449.5 ± 13.3 ^c	395.8 ± 13.4 ^e
IP fat (g), <i>n</i> = 15/group	8.8 ± 0.3	8.6 ± 0.6	24.3 ± 2.4 ^d	15.4 ± 1.0 ^f
Food intake (g/rat/day), <i>n</i> = 20/group	21.4 ± 0.5	20.4 ± 0.4	21.7 ± 1.0	19.3 ± 0.7
Water intake (mL/rat/day), <i>n</i> = 20/group	33.8 ± 0.9	28.7 ± 0.9 ^a	29.7 ± 1.7	30.4 ± 1
Insulin (mIU/mL), <i>n</i> = 5/group	16.7 ± 2.7	12.4 ± 3.7	19.6 ± 4.4	16.5 ± 8.4
Urine output 8 wks (mL/24 h), <i>n</i> = 10/group	11.3 ± 0.1	17.5 ± 1.6 ^b	9.5 ± 1	7.5 ± 1
Urine output 14 wks (mL/24 h), <i>n</i> = 10/group	11.0 ± 0.8	16.8 ± 1.2 ^b	4.1 ± 0.9 ^a	5.6 ± 1 ^g

HFD, high-fat diet; IP, intraperitoneal.

Note: In addition, by two-way ANOVA – the effect of buchu on weight loss, $p = 0.0009$; on IP fat, $p = 0.004$.

^a $p < 0.05$ versus control; ^b $p < 0.01$ versus control; ^c $p < 0.001$ versus control; ^d $p < 0.0001$ versus control;

^e $p < 0.05$ versus HFD; ^f $p < 0.01$ versus HFD; ^g $p < 0.001$ versus control + buchu.

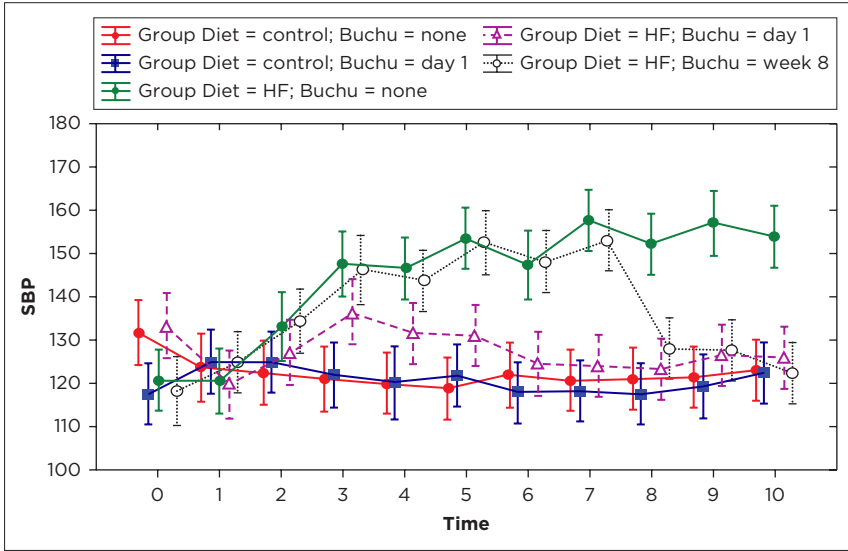
intraperitoneal (IP) fat ($24.3 \text{ g} \pm 2.4 \text{ g}$ vs. $8.8 \text{ g} \pm 0.3 \text{ g}$, $p < 0.0001$) gain, while ingestion of buchu resulted in less weight gain in the HFD animals – bodyweight $395.8 \text{ g} \pm 13.4 \text{ g}$, $p < 0.05$ and IP fat $15.4 \text{ g} \pm 1 \text{ g}$, $p < 0.01$, having no weight loss effect in control animals. This was not accompanied by a difference in food intake but only by a slightly reduced buchu water intake in control rats. Furthermore, the HFD diet resulted in water retention by the animals as evidenced by a significantly smaller 24h urinary output ($4.1 \text{ mL} \pm 0.9 \text{ mL}$ vs. $11.0 \text{ mL} \pm 0.8 \text{ mL}$). This was not prevented by the buchu water ingestion ($5.6 \text{ mL} \pm 1 \text{ mL}$). In addition, the well-known diuretic effect of buchu was only observed in control animals ($17.5 \text{ mL} \pm 1.6 \text{ mL}$ and $16.8 \text{ mL} \pm 1.2 \text{ mL}$, respectively).

Table 5.1 presents a summary of the biometric parameters measured in the four groups of animals involved in this study.

■ Blood pressure measurements

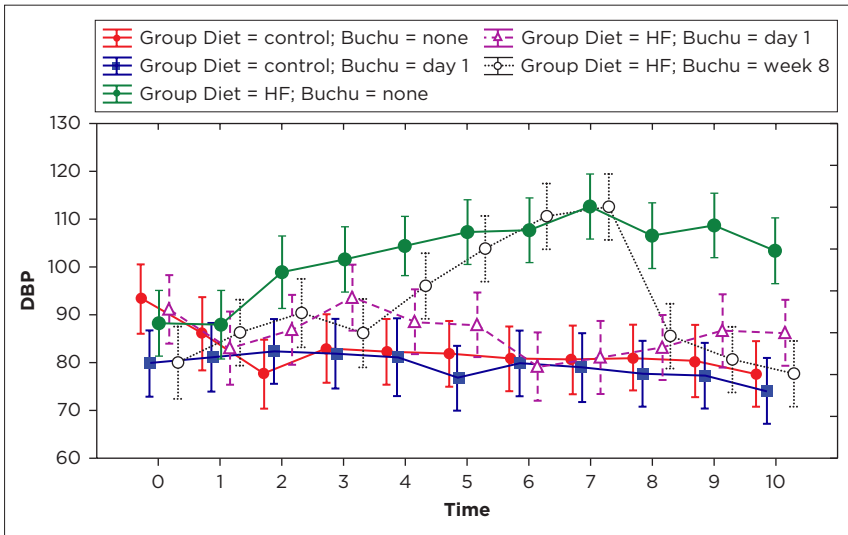
The changes in systolic and diastolic BP observed over the course of the experimental protocol are shown in Figure 5.1 and Figure 5.2. BP was measured at the same time each

Anti-hypertensive and cardioprotective effects of buchu



BP, blood pressure; SBP, systolic blood pressure; HF, high-fat. Type III decomposition Vertical bars denote 0.95 confidence intervals. Current effect: $F(40, 419) = 4.4808, p = 0.0000$

FIGURE 5.1: Systolic BP.



BP, blood pressure; DBP, diastolic blood pressure; HF, high-fat. Type III decomposition Vertical bars denote 0.95 confidence intervals. Current effect: $F(40, 419) = 4.4808, p = 0.0000$

FIGURE 5.2: Diastolic BP.

morning by the same person. The animals were trained until a stable baseline BP was obtained in all the groups. Data are presented showing 95% confidence intervals and were analysed and interpreted by a biostatistician from the University of Stellenbosch.

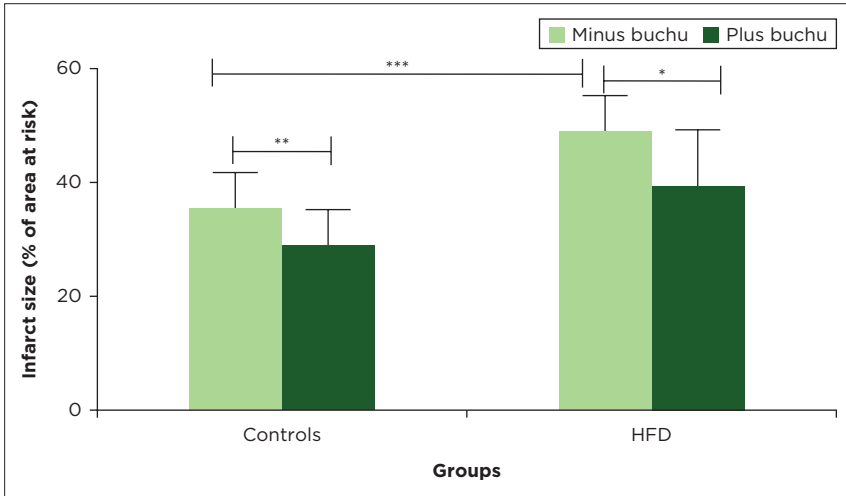
Through the monitoring of BP of the animals over the 14-week period (intervals 1-10 in the figures), the following was observed:

1. The HFD diet elevated SBP ($157.1\text{mmHg} \pm 8.7\text{mmHg}$ vs. $122.4\text{mmHg} \pm 8.2\text{mmHg}$, $p < 0.001$), reaching significance after 4 weeks. Changes in DBP ($102.5\text{mmHg} \pm 1.3\text{mmHg}$ vs. $82.2\text{mmHg} \pm 1.4\text{mmHg}$, $p < 0.0001$) showed similar reactions.
2. In untreated HFD animals, BP increased steadily over the 14 weeks.
3. The aqueous extract of buchu prevented the rise in BP (SBP $125.9\text{mmHg} \pm 12.0\text{mmHg}$; DBP $86.2\text{mmHg} \pm 13\text{mmHg}$ at week 14).
4. Treating animals from week 8 normalised their BP within 2 weeks, from $153.1\text{mmHg} \pm 8\text{mmHg}$ to $123.1\text{mmHg} \pm 10.7\text{mmHg}$, $p < 0.001$ ($N = 10$ in all groups).

The initial slight rise in BP in HFD animals treated with buchu from day 1 did not become significant, and declined to control values at week 14. Buchu ingestion had no hypotensive effects in control animals.

■ Infarct size measurement

The cardioprotective effects of treatment with an aqueous extract of buchu are demonstrated in Figure 5.3. The diet resulted in the development of larger infarcts in the hearts of these animals, with a p -value of <0.0001 . Furthermore, in both chow-fed animals and HFD animals, infarct development was significantly smaller after ingestion of the buchu water as compared to their respective controls. In addition, a two-way ANOVA indicated this effect of the buchu water ingestion as highly significant, with a p -value of 0.0002 .



HFD, high-fat diet.

n = 8–18 group

$p < 0.0002$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

FIGURE 5.3: Hearts of the animals on the control and the HFD were harvested and perfused *ex vivo*. They were perfused in the working mode and subjected to 35-min regional ischaemia, followed by 1h reperfusion.

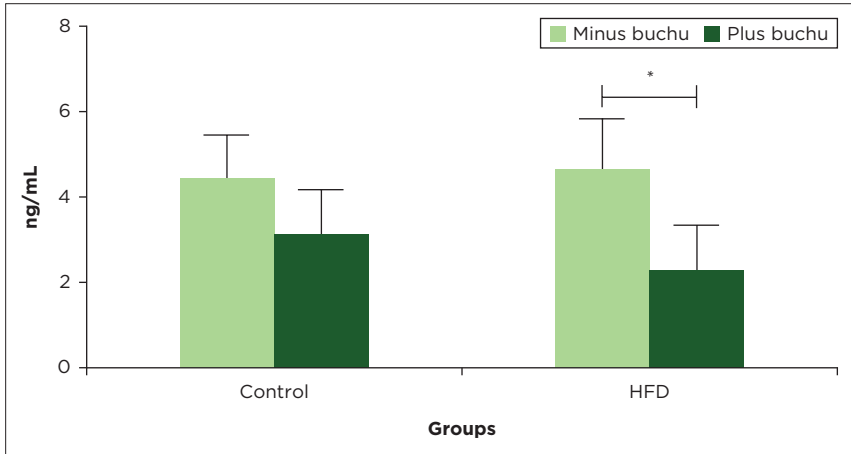
■ Adipokine determination

□ Leptin and aldosterone

Serum leptin levels (Figure 5.4) were not significantly elevated in the HFD animals. However, ingestion of buchu resulted in significantly lower leptin levels in HFD animals. Serum aldosterone levels (Figure 5.5) as an indicator of Renin–Angiotensin–Aldosterone System (RAAS) activity were significantly elevated in HFD animals ($619.9 \text{ pg/mL} \pm 136.1 \text{ pg/mL}$ vs. $119.24 \text{ pg/mL} \pm 19.1 \text{ pg/mL}$, $p < 0.05$, $n = 6$), while the ingestion of buchu lowered aldosterone levels in the HFD animals to $259.7 \text{ pg/mL} \pm 65.5 \text{ pg/mL}$, $p < 0.05$, $n = 6$.

■ Angiotensin 1-converting enzyme inhibitor activity

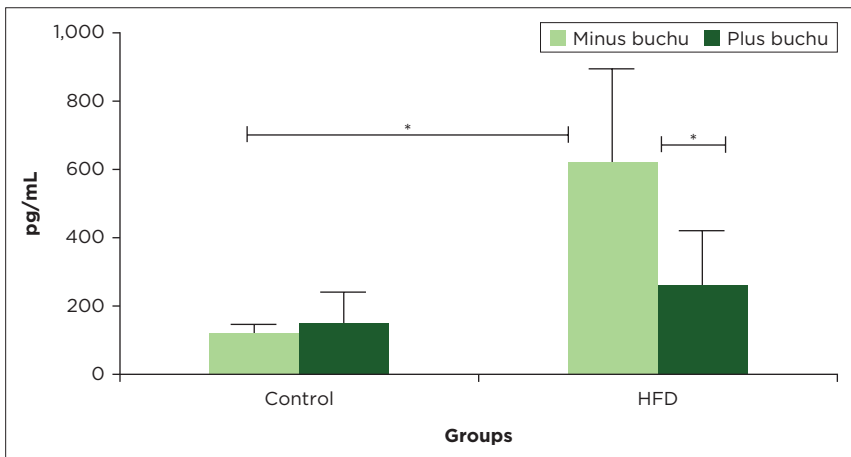
Using a FRET assay as previously published,¹² and commercially available fluorescent peptides, we were not able to detect any



HFD, high-fat diet.

Note: Serum was collected upon the euthanising of the animals and analysed as described in the 'Methods' section. Ingestion of buchu water lowered the serum leptin levels of the HFD animals significantly, while a two-way ANOVA indicated the effect of buchu water on serum leptin as significant, with a p -value of 0.006, $n = 5$. * $p = 0.0158$

FIGURE 5.4: Leptin levels.



HFD, high-fat diet.

Note: Serum collected upon the euthanising of the animals was analysed for aldosterone levels as described in the 'Methods' section.

$n = 5$

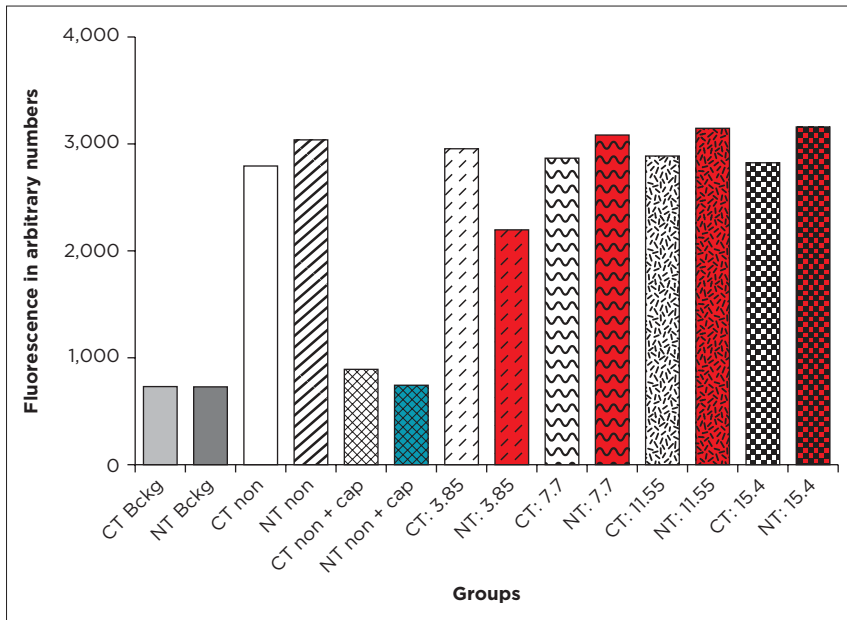
* $p < 0.05$

FIGURE 5.5: Aldosterone levels.

ACE inhibitor activity in a serial dilution of the buchu water (Figure 5.6) nor any elevated ACE inhibitor activity in serum collected from the control and HFD animals (Figure 5.7).

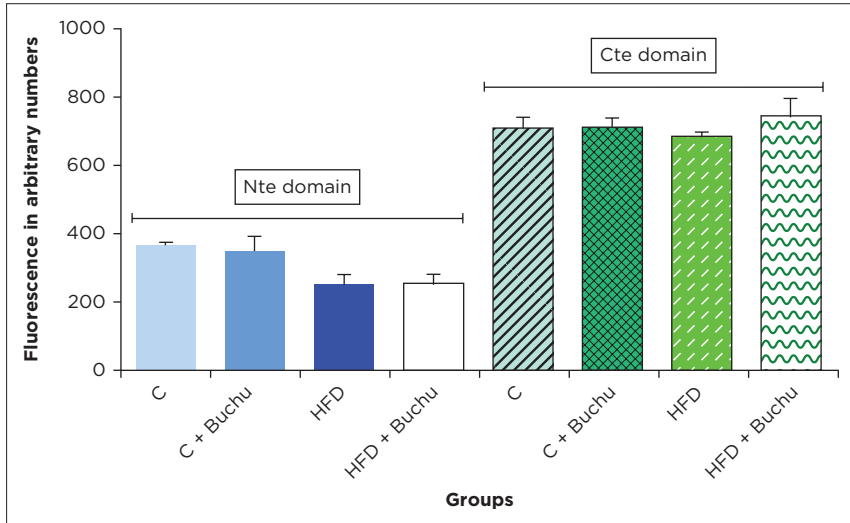
Discussion and conclusion

The main findings of this study were that ingestion of the aqueous extract of *Agathosma*, commonly known as buchu, at an equivalent human dose of 250 mL/day, was able to both prevent and successfully treat hypertension. In addition, the same extract demonstrated cardioprotective properties, as shown by a significant reduction in infarct size in both HFD and control animals. Hypertension, coupled with obesity, was elicited by diet



CT, C-terminal probe activity; NT, N-terminal probe activity; Bckg, background fluorescence; Non, negative control; Non + Cap, positive control; 3.85-15.4 = serial dilutions of the buchu water with distilled water.

FIGURE 5.6: Angiotensin 1-converting enzyme inhibitor activity of a dose-response with buchu water.



HFD, high-fat diet; C, control.

Note: After euthanasia of the control (C) or obese (HFD) animals with or without buchu treatment, blood was collected and serum was stored. The serum was tested for possible enhanced ACE inhibitor activity using endothelial cells in culture and probes against both the N (Nte domain) and the C (Cte domain) terminal of the ACE enzyme.

FIGURE 5.7: Angiotensin 1-converting enzyme inhibitor activity in serum of control (C) and hypertensive (HFD) rats.

manipulation in male Wistar rats. To date, as reviewed by Rahimi et al. and Forouzanfar et al., hypertension is still the leading risk factor for mortality and morbidity worldwide.^{13,14}

As already indicated by data from the NHANES III¹⁵ report, there is a positive relationship between abdominal obesity and development of hypertension and, as summarised by Poirier in 2006 on behalf of the American Heart Association, this further increases the risk of mortality from CVD as well as renal disease.¹⁶

The profile of changes observed in this study included elevated aldosterone levels (Figure 5.5). In the literature, activation of the RAAS has been linked with obesity-related hypertension both in animal models and humans. As reviewed by Stiefel et al., this is a multifactorial relationship, including effects on insulin sensitivity, oxidative stress, vascular reactivity and water and salt retention or excretion.¹⁷ The adipokine leptin is also described as a possible

link between obesity and hypertension.^{18,19,20} In addition, it was shown both *in vitro* and *in vivo* that leptin, besides its central actions, directly affects the production of aldosterone by acting on leptin receptors in the rat adrenal glomerulosa.^{20,21} We acknowledge that this effect was more pronounced in female mice than in male mice. In the latter, leptin seemed to exert anti-hypertensive effects via a central mechanism.²² However, Huby et al. also demonstrated that adrenocortical cells in culture dose-dependently increase aldosterone production when stimulated with leptin.²³

In view of the significantly lower weight and IP fat gain that we documented with ingestion of buchu, we argue that this resulted in significantly lower leptin levels observed (Figure 5.4), which, in turn, resulted in lower aldosterone production. The observation that food intake was not affected corroborates the observation that leptin resistance in obesity desensitises this central effect of the hormone while not affecting its effects on aldosterone secretion.²⁰

A contrasting observation was that ingestion of buchu water, despite significantly lowering aldosterone levels, did not lower water retention in the HFD rats. As we did not measure anti-diuretic hormone (ADH) levels, the counter-regulatory hormone on water retention, it is possible that this is still a mechanism that may be explored. Furthermore, the expression or activity of the SGLT2 glucose transporter was not measured. The SGLT2 glucose transporter is responsible for the reabsorption of ~90% of the glucose in the plasma, while SGLT1 is responsible for the remaining 10% glucose being excreted in the urine. SGLT2 has a low Michaelis-Menten constant (K_m) for glucose, therefore a high affinity, and is a sodium co-transporter transporting glucose into the cells from the tubular lumen. Gluts then transport the glucose back into the blood. It is known that the SGLT2 transporter is upregulated in obesity and type 2 diabetes mellitus.²⁴ It is therefore possible that this was also the case in our model of obesity-hypertension. If such a high expression is not rectified by

the treatment, water retention would still be observed despite the lower aldosterone levels. The levels of vasoconstrictive molecules, for example, endothelin-1, were also not measured, although the contractile responses of aortic tissue to phenylephrine contraction followed by acetylcholine relaxation *ex vivo* were studied, which showed no significant differences between control and HFD animals.

Many phytochemicals have anti-hypertensive properties, for example, extra virgin olive oil was shown to affect kidney angiotensinase, thereby attenuating the diet-induced rise in BP in spontaneously hypertensive rats.^{6,25} Phytochemicals may also act as diuretics or ACE inhibitors.²⁶ As three of the main polyphenols found in the aqueous extract of buchu may have ACE inhibitor activity, we tested whether such activity may be involved in the lower aldosterone levels. However, as shown in Figure 5.6 and Figure 5.7, we could not detect this as an anti-hypertensive mechanism.

At least two of the polyphenols in the buchu water may have assisted in the lower weight gain observed, namely the citrus flavonoid hesperidin and the flavonoid quercetin. Hesperidin has been identified as a treatment option for dyslipidemia,²⁷ while quercetin is well known as an anti-obesity treatment.^{28, 29} In addition, quercetin was recently the focus of a meta-analysis of clinical trials, where this systematic review highlighted a significant effect of quercetin supplementation on lowering BP.³⁰

As buchu has the FDA approval for use in the food industry, we conclude from this study that the ingestion of this aqueous extract may act as a readily accessible anti-hypertensive treatment that should be further evaluated. However, the complexity of plant extracts in the treatment of a multifactorial disease, as eloquently addressed by Fernandez-Arroyo,⁵ should always be kept in mind. In addition, the possible effects of buchu treatment on the intestinal microbiome, as discussed in Chapter 3, should also be considered as a mediator of observed actions.

■ Acknowledgements

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CHAPTER 6

Summary

Description: Manual harvesting of Buchu.

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Summary

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The chapters presented in this book summarise both the *in vitro* and *in vivo* health-promoting effects of a specific aqueous extract obtained from *Agathosma*. These effects were demonstrated either by exposing different cells to the extract or by animals ingesting the extract. The dose used in the treatment of the animals was equivalent to a human weighing approximately 70 kg drinking 250 mL/day of the extract.

Cell-based studies corroborated previous findings of anti-inflammatory effects but, most importantly, pointed to possible metabolic effects that may lead to improved glucose homeostasis. These effects were observed both in liver cells and in adipocytes. This observation was further elaborated in the animal-based studies which showed that this aqueous extract of buchu had profound effects not only on the fat depots of obese, insulin-resistant animals, causing weight loss, but also

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anti-hyperglycaemic effects. These effects were demonstrated by different research teams from different laboratories over the course of more than 5 years. Different diet regimes were used to induce obesity and a pre-diabetic state, but the main findings of metabolic change held true. The ingestion of the buchu water furthermore caused changes in pancreatic transcription factors of the diabetic animals, indicating possible redifferentiation of the cells from a diabetic profile to a healthy profile. This is one of the novel findings of these studies. However, as stated previously, it must be cautioned that the pancreatic biochemistry of a rat differs from that of a human and that these results need to be confirmed in clinical trials.

In addition to the above effects, animal-based studies showed that the buchu water has anti-hypertensive properties as it effectively lowered the BP of hypertensive animals. In conjunction with this, it was also cardioprotective in different models of obesity and insulin resistance, where the hearts of the animals were compromised because of their obesogenic diet. The buchu water ingestion protected the hearts of the animals against an ischaemic incident (a simulated heart attack), with resultant less tissue damage afterwards.

An important observation of these studies is that no detrimental effects whatsoever were observed. Normal animals did not become hypoglycaemic on ingestion of the buchu water, nor did their BP fall. They, however, also lost some intraperitoneal fat and presented with lower levels of liver cholesterol and triglycerides – all health-promoting effects.

At the end of this journey with the product, we can therefore conclude that the ingestion of buchu water is safe and promotes an improved health status on all accounts tested. Limitations of the presented results lie in the shortfall of data obtained in human trials, although this is a product that is widely consumed by humans in different forms.

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The aim of this book was to provide scientific evidence for the many anecdotal reports on the beneficial health effects of an aqueous extract from leaves of the *Agathosma* (buchu) plant. The medicinal properties of buchu that were focused on in the past include the antimicrobial and anti-inflammatory activities of buchu oil in particular. Recently, a byproduct of the oil extraction process, namely buchu water, which contains the more water-soluble molecules, was investigated by two well-established research groups at the University of Stellenbosch to scientifically validate its suggested health promoting properties. LC-MS analysis of buchu water showed that it contained compounds such as hesperidin, rutin, diosmin, quercetin, pulegone and a number of unknown structures which could all contribute to its biological activity. Using in vivo and in vitro experimental approaches, professors Huisamen, Bouic and co-workers demonstrated the anti-diabetic, anti-obesity and anti-hypertensive properties of an aqueous buchu extract. These studies form the foundation for further studies in the identification of the active compound(s), which, in turn, may have significant clinical implications.

**Prof. Amanda Lochner, Department of Biomedical Sciences,
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Tygerberg, South Africa.**

This work is novel, innovative, and makes use of a natural and local plant which is relevant to the South African context. The promising findings of this work contribute to the growing body of knowledge of this herb and its efficacy in the animal model, primarily, with some preliminary work in human cell lines. This work is important and needs to be progressed into human randomised controlled trials in order to establish its efficacy in the human population group. Hopefully this founding work will be used as a strong platform for future rigorous trials. It is pleasing to see that herbs and natural remedies that have been in existence for a long time are being studied under rigorous research conditions. The science of buchu needs to be progressed rather than dismissed as fringe medicine supported only by anecdotal evidence. This work makes an important contribution to the field of herb and plant-based science and its efficacy in managing health and disease.

**Dr Caryn Zinn, School of Sport and Recreation, Faculty of Health and
Environmental Sciences, Auckland University of Technology,
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