DEVELOPMENT OF A *BULBINE FRUTESCENS AND CARPOBROTUS EDULIS* CREAM IN COLLABORATION WITH AFRICAN TRADITIONAL HEALERS OF THE NELSON MANDELA METROPOLE

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DEVELOPMENT OF A *BULBINE FRUTESCENS AND CARPOBROTUS EDULIS* CREAM IN COLLABORATION WITH AFRICAN TRADITIONAL HEALERS OF THE NELSON MANDELA METROPOLE

By

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Submitted in fulfilment of the requirements for the degree of Philosophiae Doctor to be awarded at the Nelson Mandela Metropolitan University

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DECLARATION

I, Mbali Zamathiyane Keele, 199218692, hereby declare that the thesis for Philosophiae Doctor is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

Mbali Zamathiyane Keele
DEDICATION

This thesis is dedicated to my children Teboho and Bohlokoa, I can find no adequate expression of the love that I have for you two.
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LIST OF ABBREVIATIONS

ANC  African National Congress
ATH  African traditional healers
ATM  African traditional medicines
CFU  Colony forming units
CAM  Complementary and Alternative medicines
DOH  Department of Health
DMSO Dimethylsulfoxide
DPPH Diphenylpicrylhydrazyl free radical scavenging
DHS  District health systems
DMEM Dulbecco’s modified Eagles medium
EMA  European Medicines Agency
ET   Electron transfer
FRAP Ferric reducing antioxidant power
HSV  Herpes simplex virus
HPLC High performance liquid chromatography
HAT  Hydrogen atom transfer
ICH  International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MCC  Medicines Control Council

MIC  Minimum inhibitory concentration

MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMMU  Nelson Mandela Metropolitan University

ORAC  Oxygen radical absorbance capacity methods

PBS  Phosphate buffered saline

INT  $p$-iodonitrotetrazolium chloride

PHC  Primary health care

ROS  Reactive oxygen species

RO  Reverse osmosis

WHO  World Health Organisation
SUMMARY

Collaborations between researchers and African traditional healers on medicinal plants need to go beyond the flow of information from African traditional healers to researchers. Mutual beneficiation wherein African traditional healers reap rewards due to the information they possess is necessary and has been legislated in South Africa. The manner in which such beneficiation occurs and how it will be distributed should be subject to negotiation between (a) the researchers and holders of indigenous knowledge and (b) among holders of indigenous knowledge themselves. Beneficiation can be in the form of access to information, monetary or through shares in commercialised products, amongst others. African traditional healers involved in the collaboration with researchers of the Department of Biochemistry and Microbiology and the Department of Pharmacy at the Nelson Mandela Metropolitan University benefited by having access to knowledge and medicinal plants cultivated in the medicinal garden. Beneficiation was expected to extend to monetary gains from the sale of sewn garments and plants from a medicinal plants nursery which was to be developed and from a herbal topical product which was also to be developed.

The aim of this research project was to develop a wound healing herbal cream consisting of Bulbine frutescens and Carpobrotus edulis as active ingredients. B. frutescens demonstrated better wound closure properties when compared to C. edulis and the combined extracts. The scratch assay wound was completely closed after 24 hours at B. frutescens concentrations of 5 µg/ml and 10 µg/ml. B. frutescens was more toxic to 3t3-L1 cells than C. edulis, but it was less toxic than the combined extracts. None of the extracts were toxic to Vero cells and the combined extracts significantly promoted their proliferation. Antibacterial activity of all the extracts was low. C. edulis showed antiviral activity against human herpes simplex virus 1 at 62.5, 125 and 250 µg/ml, while the combined extracts were active at 250 µg/ml. The combined extracts exhibited synergistic antioxidant activity.
A reverse phase, gradient, high performance liquid chromatography method was developed and validated and used to conduct quality control tests on the extracts and the finished product. It was concluded that the use of whole chromatographic data instead of common peaks data is best for analysis of medicinal plants. The cream that was developed used buffered cream as the base and was stable at 25 °C/65% RH for one month with regard to organoleptic and rheological properties and microbial preservation.

**Keywords:** African traditional healers, *C. edulis*, wound healing, human herpes virus 1, 3t3-L1, Vero, *B. frutescens*, high performance liquid chromatography, herbal cream.
1. INTRODUCTION

1.1 Introduction

Scientific research intent on validating the traditional uses of medicinal plants has generated numerous articles which are available in databases and have been published in journals such as The Journal of Ethnopharmacology and the Pharmacognosy Journal amongst others. The majority of this research however is devoted to validating traditional uses by in vitro screening of single medicinal plants, e.g. antibacterial properties (Ningthoujam et al., 2012; Street, 2012). In most instances researchers used dried plant material in these assays for the following reasons: (a) there are fewer problems related to extracting dried versus fresh plant material, (b) the time between collecting and processing plant material can result in difficulties when using liquid-liquid extraction methods, (c) secondary metabolic components should be stable and (d) many plants are traditionally used in a dried state or as aqueous extracts (Eloff, 1998b).

The majority of research published on formulated products containing medicinal plants as active ingredients has mostly been undertaken in Asian countries e.g. China and India. Whilst having a rich plant biodiversity with over 3000 species being used medicinally, only a few South African products have been derived from medicinal plant species. Eighty three African medicinal plants have been successfully commercialised and only 14 Southern African species have been fully or partially commercialised (Coetzee et al., 1999; Van Wyk & Gericke, 2000; Van Wyk, 2011). The ultimate aim of current medicinal plant research is to develop monographs, assays and specifications for the testing of medicinal plant extracts and medicinal plant products. These assays would be used in animal studies and human trials, for quality control of medicinal plant products and also as part of dossiers which would need to be submitted for registration with regulatory authorities.
Whilst there are no guidelines and regulations published on the registration of African traditional medicines (ATM) in South Africa, regulations were published by the Department of Health (DOH) of South Africa for Complementary and Alternative medicines (CAM) in November 2013. In these, CAM are defined as “any substance or mixture of substances that –

a) originates from plants, minerals or animals;
b) is used or intended to be used for, or manufactured or sold for use in assisting the innate healing power of a human being or animal to mitigate, modify, alleviate or prevent illness or the symptoms thereof or abnormal physical or mental state; and
c) is used in accordance with the practice of the professions regulated under the Allied Health Professions Act, 1982 (Act No. 63 of 1982).”

Complementary and Alternative medicines will need to be registered with the Medicines Control Council (MCC) and assessed for safety; efficacy and quality; will have to comply with labelling requirements and contain patient information leaflets and package inserts (Department of Health, 2013).

The definition of medicine for CAM at face value differs from that of traditional medicine as defined by the Traditional Health Practitioners Act which states that “traditional medicine means an object or substance used in traditional health practice for:

- the diagnosis, treatment or prevention of a physical or mental illness; or
- any curative or therapeutic purpose, including the maintenance or restoration of physical or mental health or well-being in human beings but does not include a dependence-producing or dangerous substance or drug.” (Republic of South Africa, 2007).

When looking closely though, these two definitions are not as deviant as may be first thought, therefore, it is not farfetched to expect that requirements for ATM will be similar to those of CAM.

Historically, people requiring ATM obtained them (a) from African traditional healers (ATH), (b) they purchased or collected plant material to make such medicines at home.
or (c) they purchased finished products from informal medicinal plant street traders. Recently, African medicinal products of plant origin packaged in modern containers and labelled with printed labels bearing the name of the medicine, the indications, directions and medicinal plants contained in the medicine are being sold in supermarkets, muti-shops, street markets and pharmacies. It is these types of medicines that will require registration with the MCC as some of the claims made on them are questionable (Ndhlala et al., 2011).

According to the WHO (2000), “finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal”. Whether the MCC will adopt this approach for herbal ATM registered in South Africa is still unknown. What also needs to be cleared up is the interchangeability of the terms ATM, herbal medicine and medicinal plant products. It is feasible to think that they are not interchangeable taking into consideration definitions provided by the WHO and the fact that the active ingredients used in ATM are not restricted to medicinal plants. Deviation from definitions and groupings provided by the WHO has however been noted e.g. the WHO groups traditional and complementary medicines together whilst in South Africa they are not (WHO, 2000).

Active ingredients of medicinal plants are defined by the WHO (2000) as ingredients with therapeutic activity which may or may not have been identified. Where the active ingredient has not been identified, the whole herbal medicine may be considered as one active ingredient. Active constituents of most plants used in herbal medicines, including herbal ATM have not been identified, thus, for these medicines, whole plants are considered to be the active ingredients. This poses a challenge for traditional pharmaceutical assay methods which are used in the quality control of starting, intermediate and finished ATM products because of the numerous compounds that are present within the plant extracts. The reason for the challenge is due to the fact that the theory and development and validation of these assay methods has been optimised for pure, single pharmaceutical active ingredients. The determination of uniformity of content, purity and stability are thus straightforward. Alterations are needed where active ingredients comprising of multiple compounds which are not all
defined, identified or quantifiable are to be assessed and the use of multiple marker compounds or fingerprints at some point in the analysis is needed (Lazarowych & Pekos, 1998; WHO, 2012a).

1.2 Aim and objectives

The primary aim was to develop a pharmaceutically stable wound healing herbal cream and its associated quality control tests.

To achieve the aim, the following objectives were identified:

a) To screen the extract for wound healing and anti-herpes simplex virus 1 properties.

b) To develop a stability indicating high performance liquid chromatography (HPLC) assay to be used in the assessments of the extracts and proposed formulation.

c) To propose a formulation for a topical herbal preparation in which *Bulbine frutescens* and *Carpobrotus edulis* are the active ingredients.

d) To assess the formulation for stability using the developed assay.

1.3 Medicinal plants investigated

The plants selected for this study are individually known to be used traditionally for healing wounds and cold sores. Their use in combination to treat these conditions has not been reported or studied. The researchers did not enquire from the collaborating African traditional healers whether these plants are used in combination traditionally due to suspicion and secrecy of African traditional healers. Their individual, published uses were, however, confirmed by the African traditional healers.
The majority of medicinal plants documented for dermatological use are dedicated to wound healing and treatment of infective skin conditions at 41% and 32% respectively. These plants are applied topically as either pastes, plant juices or compresses. When formulated into pharmaceutical dosage forms, creams are better for the treatment of wet or acutely inflamed lesions than ointments which are more suitable for eczema and severe fungal infections (Mabona & Van Vuuren, 2013).

1.3.1 Bulbine frutescens

*Bulbine frutescens* belongs to the Asphodelaceae family, it has long, thin, juicy green leaves and yellow flowers and is found in the Cape provinces, KwaZulu-Natal, Swaziland, Free State and Lesotho. It is used traditionally to treat urticaria, wounds, cold sores and shingles. Root infusions are used to treat blood disorders, diabetes and diarrhoea (Felhaber, 1997; Van Wyk *et al.*, 1997).

The following compounds have been identified from the roots of *B. frutescens*: biaryl anthraquinones knipholone, isoknipholone, phenylanthaquinones (Gaboroquinones A and B), 4'-O-demethylknipholone-4'-O-β-D-glucopyranoside, sodium 4'-O-demethylknipholone 6'-O-sulfate and sodium isoknipholone 6'-O-sulfate (Van Staden & Drewes, 1994; Abegaz *et al.*, 2002; Mutanyatta *et al.*, 2005).

The gel of *Bulbine frutescens* has been commercially exploited with the production of Montagu Museum Bulbine Crème and the BulbAloe products. A tape impregnated with *B. frutescens* has been shown to improve wound healing and scar maturation (Widgerow & Chait, 1998). *In vivo* animal studies have validated the wound healing properties of the plant. Wound healing is hypothesised to be as a result of the glycoproteins and/or polysaccharides found in the gel of the leaves (Pather *et al.*, 2011; Pather & Kramer, 2012).

1.3.2 Carpobrotus edulis

*Carpobrotus edulis*, a member of the Aizoaceae family, is a perennial succulent plant, widely distributed from coastal towns to inland areas. Traditionally it is used to treat
sore throats, rashes, wounds, mouth ulcers, cold sores and thrush (Felhaber, 1997; Springfield et al., 2003; Thring & Weitz, 2006).

Flavonoids including rutin, neohesperidin, hyperoside, catechin and ferulic acid have been found in the leaves of *C. edulis* as well as catechol tannins (Van der Watt & Pretorius, 2001). Oleanolic acid, β-Amyrin, uvaol and monogalactosyldiacylglycerol have also been isolated from this medicinal plant (Martins et al., 2011).

### 1.3.3 Harvesting and storage

All the plants were harvested and the extracts obtained as explained below. Where there was deviation, the method used to obtain the extract will be described in the text. The plants were harvested from four areas of South Africa namely Port Elizabeth (Eastern Cape Province), Joubertina (Eastern Cape Province), Ladysmith (KwaZulu-Natal Province) and QwaQwa (Free State Province). The plants from Joubertina were collected in the wild. Those from Port Elizabeth were harvested from the gardens of the Nelson Mandela Metropolitan University and those from Ladysmith and Qwa-Qwa were harvested from private gardens.

*B. frutescens* and *C. edulis* leaves were washed twice with tap water and once with reverse osmosis (RO) water to remove the soil and then submerged for five minutes in a hypochlorite solution made by adding 100 ml of a 3.5% m/v hypochlorite solution to 4.9 L of RO water. The plants were rinsed with RO water three times and then submerged in 70% ethanol for five minutes. The juice of the leaves was then extracted (Liquafresh, Mellerware), freeze dried and stored at -20 °C.

The quantity of solid extract obtained per ml of liquid extract was greater for *C. edulis* where 200 ml of liquid extract resulted in 18.42 g of freeze dried extract. Two hundred millilitres of liquid extract yielded 12.4 g of freeze dried extract for *B. frutescens*. 
2. AFRICAN TRADITIONAL HEALERS: CURRENT STATUS AND RESEARCH COLLABORATION

2.1 Government interest in African traditional healing

Newly independent, resource constrained African countries recognised that African traditional healers (ATH) were a readily available, accepted and widely used source of primary health care practitioners. Steps to integrate African traditional healers within the state health systems began formally in the 1970s and the declaration of Alma-Ata was undertaken in 1978 (WHO, 1978; Homsy et al., 2004; Devenish, 2005). The integration of African traditional healers into the formal health care system was envisioned to be mutually beneficial. The states would benefit from primary healthcare services that African traditional healers would provide where there was a high disease burden and few resources (Gbodossou et al., 2005). African traditional healers would benefit by being included in the formal healthcare system of their respective countries and such inclusion could result in: (a) an increase in status, both in the community and amongst allopathic health workers (b) financial benefits from remuneration received either from the state and private institutions such as medical aids or from the increased number of patients who would consult them (Devenish, 2005).

The African National Congress (ANC) in its National Health Plan for South Africa, penned in 1994, also recognised the role played by African traditional healers in the health care of a large segment of the South African population. The ANC proposed that a coordinating body and mechanisms for integrating African traditional healers into the health system should be investigated, signalling that South Africa would also align itself with the declaration of Alma-Ata (African National Congress, 1994).
2.2 Prerequisites for inclusion into mainstream health care systems

Effective interaction amongst African traditional healers, the state and private health funders requires that the African traditional healers be organised into bodies which can communicate on behalf of the collective. In addition, African traditional healers have to professionalise and to achieve this objective effectively they need to:

- Create and register with a statutory professional body which ensures professional autonomy.
- Organise according to expertise thus ensuring monopoly on a knowledge base.
- Develop norms for practice and training.
- Have the respect and trust of the community (Bondi, 2004; Devenish, 2005; Starr, 2009).

2.2.1 Formation of and registration with a professional body

The Department of Health in South Africa was instrumental in driving this step of professionalisation. The Traditional Health Practitioners Act (Republic of South Africa, 2007) provides a statutory framework for professionalisation of African traditional healers in South Africa. The act requires the formation of a 22 member interim council which will have the following objectives:

- Ensure that appropriate practice standards and ethics are maintained.
- Set standards for training within African traditional health.
- Increase interest in traditional health practices through research, education and training.
- Compile a professional code for African traditional health practice (Republic of South Africa, 2007).

The act recognises and allows for registration of traditional health practitioners described below. Other forms of healing such as faith healers who may use traditional medicinal products and have been previously included when referring to African
traditional healers are excluded. They, along with African traditional health practitioners who do not register with the professional council will not be permitted by law to practice.

2.2.1.1 Herbalists

These are traditional healers who have extensive knowledge of medicinal herbs, animals and minerals; in general, herbalists combine a variety of herbs to treat an ailment and one herb may be used to treat multiple conditions (Kale, 1995). Some herbalists choose to follow this career without spiritual intervention, others are compelled by ancestors to become herbalists. In either case, the healer undergoes apprenticeship with a knowledgeable herbalist. The majority of herbalists are male (Ndenecho, 2009).

2.2.1.2 Diviners

Diviners are guided in their training and practice by ancestors. Guidance with regards to treatment of ailments, herbal therapy and sometimes the arrival of patients may be provided in the form of dreams (Hirst, 2005). The healers are able to interpret their dreams and sightings during divination and find the underlying or intended meaning. Diviners guide patients through rituals which may be necessary for healing in addition to providing herbal remedies (Kale, 1995). The majority of diviners are female.

2.2.1.3 Traditional birth attendants

Traditional birth attendants are mostly used in rural and peri-urban areas where homesteads are far from clinics and hospitals. Their knowledge of traditional medicine is mostly limited to herbs used to alleviate the complications and pain of labour. Traditional birth attendants are not called by spirits but rather apprentice for a long period of time. To become a traditional birth attendant, one has to have at least two biological children and have apprenticed for approximately twenty years (Kale, 1995).
2.2.1.4 Traditional surgeons

In the South African context, traditional surgeons are concerned mostly with the circumcision of young men. They may or may not have knowledge of medicinal herbs or guidance from ancestors (Peltzera et al., 2010).

2.2.2 Organising according to expertise in voluntary associations

The term African traditional healer encompasses a range of categories, as described above, each having its own set of expertise, practices and norms, which may be clan specific. Some African traditional healers simultaneously practice in more than one field and may require dual registration as proposed by the Traditional Health Practitioners Act e.g. an African traditional healer may be a diviner and traditional birth attendant simultaneously (Devenish, 2005). There are approximately 100 African traditional healers organisations in South Africa which range widely in the number of members registered. Whilst the condition of voluntary association is fulfilled, most of these organisations are small and many have internal strife, thus the objective of a unified, representative voice remains elusive (Kale, 1995; Devenish, 2005; Deutsche Gesellschaft fur Technische Zusammenarbeit, 2007). In order to pursue a common agenda effectively, the number of organisations will have to decrease and the numbers of African traditional healers registered within them increase.

2.2.3 Set norms for practice and training

Setting norms for practice and training for African traditional healers may prove challenging for the interim council. Although there are common practices and codes which permeate categories, some African traditional healers (especially diviners) are guided by their individual spirits or ancestors with regard to the treatment offered to patients as well as the manner in which the African traditional healer conducts him or herself. In addition, the training of African traditional healers varies amongst clans and tutors. To compound matters further, traditional healers are reluctant to share specialist information amongst themselves and are weary of being too close to other healers, especially those from a different clan (Hirst, 2005). They may as a result not be forthcoming with crucial information needed by the council to compile informed
guidelines. Notwithstanding the above, an African traditional healer’s organisation called the Traditional Healers Organisation, has drafted a code of ethics for their members. In this, the need to abide by the standards of the profession is stressed and a period of internship for initiates is suggested (Traditional Healers Organisation, 2010). The document however, does not articulate training standards.

2.2.4 Be respected by the population

A widely accepted statistic states that approximately 60% to 80% of the African population in South Africa consults African traditional healers for primary health care (Pretorius, 1999; Chatora, 2003). However, some studies have shown a drastic decrease in the number of people who reported consulting an African traditional healer (Grobler & Stuart, 2007; Nxumalo et al., 2011). The discrepancies in the statistics have also been in the public debate (Wilkinson, 2013).

Concerns expressed by allopathic practitioners regarding traditional medicines and messages from government warning of interactions between allopathic and traditional medicines, especially for patients being treated for chronic diseases may have caused the number of people consulting African traditional healers to decline (Meel, 2007). Another reason for the discrepancy could be that study participants were weary of admitting to consulting African traditional healers or it could be the manner in which the question was phrased as consultation in the preceding 30 days was questioned (Nxumalo et al., 2011). In addition, it is possible for people to self-medicate with traditional medicine without necessarily consulting a traditional healer, further skewing the results due to the formulated question (DeJong, 1991). The reported drop in consultation of African traditional healers cannot be discounted, whatever the reason for its cause. African traditional healers have to strive to regain or cement the trust of the community and counter the messages that may lead to a decrease in consultations if they are to remain relevant.
2.3 Progress in the professionalisation of South Africa’s African traditional healers

The South African government adopted the Primary Health Care strategy for improving health outcomes for its population in line with the agreements and declarations it had aligned its self with (WHO, 1978; Health Systems Trust, 2003). Formation, strengthening and decentralisation of primary health care (PHC) through district health systems (DHS) was envisioned (Department of Health, 2004). It can be envisaged that ATM would be institutionalised into the mainstream health system at the DHS level since ATM provides primary health care services. The World Health Organisation Africa region uses institutionalisation synonymously with integration (Samba, 2003) which is defined by the WHO as “a health system in which traditional medicine is officially recognised and incorporated into all aspects of health care provision” (WHO, 2002). The South African Department of Health in its draft policy for traditional medicine however, clearly states that it seeks institutionalisation and not integration of African traditional medicine with allopathic medicine. The department stated that it views the traditional and allopathic systems functioning “side by side within the health care system” (Department of Health, 2008). One wonders at this statement by the South African Government, is it that full integration as defined by the WHO is not sought or is it clarification that African traditional medicine will continue to function as it is currently and will not adopt practices of and ultimately disappear within the allopathic health system? Actions post release of the policy throw light on the matter.

Conditions necessary for the institutionalisation of ATM were identified and championed by the African Union, adopted by regional bodies and steps for implementation undertaken by individual countries. The South African government through the Department of Health proposed policies, plans and consultations focused on the legal framework needed for institutionalisation as described above. However, the vigour and enthusiasm with which these were pursued seems to have waned post 2008. The assented Traditional Health Practitioners Act which was published in the Government Gazette in January 2008 required that the registrar of the interim council
convene a meeting within three months of commencement of the act (Republic of South Africa, 2007). However, requests for nominations of members of the council only went out in 2011 with the closing date for nominations being January 2012 (Department of Health, 2011) and the council was inaugurated in February 2013. The green paper penned by the Department of Health stating plans for the overhaul of and improvements to the health system are silent on the role of ATH and ATM (Republic of South Africa, 2011). However, in her speech at the inauguration of the interim council, the deputy minister of health, Dr. G Ramokgopa, stated that the interim council’s work in the next three years would allow integration of ATH into the National Health Insurance system of South Africa (Medical Chronicle, 2013).

It will be mainly up to African traditional healers to keep themselves visible by garnering support from communities they serve and lobbying government. African traditional health practitioners seeking professionalisation through voluntary associations should be the driving force for this.

2.4 Validation of African traditional medicines

The WHO defines herbal medicines as “finished, labelled medicinal products that contain as active ingredients aerial or underground parts of plants, or other plant material, or combinations thereof, whether in the crude state or as plant preparations. Plant material includes juices, gums, fatty oils, essential oils, and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients” (WHO, 2000).

The safety and efficacy of African medicinal plants has, to a large extent, been demonstrated by a long history of use, however, some potential for toxicity still exist (Fennell et al., 2004). It is for this reason that the WHO (2000) advocates for well controlled double-blind clinical and toxicological studies of traditional medicine, these are, however, rare and most countries of the WHO Africa region have not established
safety monitoring systems for traditional medicines (Department of Health, 2004). Safety and efficacy concerns arise from the fact that active constituents of some plants are heat labile and others are destroyed by enzymatic processes that continue for a period of time after plant collection. In addition, the interaction between plant extracts and excipients in dosage forms may not be understood (Calixto, 2000).

Distinction needs to be made between products which have been in use traditionally for a long period of time and have been compounded by African traditional healers for their patients and new products which contain plants that have been in use for centuries. The former dosage forms have proven their safety and proof of concept over time and thus regulations regarding toxicity, safety and efficacy may be relaxed. The latter products have not been in use for a long period of time and require stringent safety, efficacy and toxicity data. Acquisition of such data should follow methodologies used to obtain safety, efficacy and toxicity data of conventional drugs. Both methodologies however, seek to determine the safety, efficacy, toxicity and pharmacological action of the herbal medicines, in animals and humans. The guidelines, recognising that a long history of use does somewhat prove safety and efficacy, state that animal studies may be omitted from the methodology provided that:

- a long history of use is documented; documentation could be oral
- there is literature published about the plant
- preparation is similar or identical to traditional methods

These requirements preclude many African traditional medicines currently being sold on the South African market from being tested using the abbreviated method because they do not meet all the requirements (WHO, 2000).

The South African government has through the Medicines and Related Substances Amendment Act of 2008 moved to regulate African Traditional Medicines (Republic of South Africa, 2008). This Act has allowed the Medicines Control Council (MCC) to form a ten member committee which will be tasked with registration and research of African traditional medicines. It is noteworthy that the African Traditional Medicines committee is separate from the Complementary and Alternative Medicines Committee.
Regulation by and registration of ATM by the MCC requires that standards against which these products are compared be developed. To this end the Association for African Medicinal Plants Standards has published the Africa herbal Pharmacopoeia (Brendler et al., 2010). The pharmacopoeia is by no means exhaustive as it contains only 50 African medicinal plants. The pharmacopoeia can be used as a quality control tool to identify whole plant extracts which have been extracted in the method specified in the document i.e. quality control of the raw materials. It does not address quality control of dosage forms containing plant extracts. The guidelines set by the WHO for the quality assessment of products containing plants and plant constituents along with the Africa herbal Pharmacopoeia can be used as the starting point for the development of quality assessment criteria for ATM products. Guidelines and specifications for analytical methods and stability studies laid out by the MCC and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) can be used to assess the quality of dosage forms containing plant extracts over a defined period of time in various environment conditions (ICH, 2003; Medicines Control Council, 2012).

2.5 Research collaboration with African traditional healers

Collaboration with African traditional healers at the Nelson Mandela Metropolitan University (NMMU) was initiated by African traditional healers of the Nelson Mandela Bay Municipality in the year 2000 who wanted their products validated. The growth and progress of the collaboration from 2000 to mid-2007 is documented in a thesis by the previous researcher (Van Huyssteen, 2007). In mid-2007, Van Huyssteen left the project and was replaced by the current researcher. To allay fears and suspicion, the researcher overlapped with Van Huyssteen for six months and rebuilt a relationship that had been established in 2003 when conducting research pertaining to the traditional healers’ attitudes and beliefs about hypertension (Luvuno, 2003). In 2005 a medicinal garden was established in which knowledge exchange, interaction and plant preservation could be conducted. Research seeking to validate the use of African
traditional medicinal plants documented in literature was ongoing with the participation of ATH. Workshops were being held on a regular basis where ATH were being informed of allopathic modes of treatment and there were discussions about the then new Traditional Health Practitioners Act (Republic of South Africa, 2007).

Requirements for initiating successful collaboration with traditional healers as listed below were present:

- Mutual respect between the collaborating parties had been built.
- It was stressed that both systems were complementary.
- There was transparency through dialogue.
- A system where genuine healers were selected for the collaboration was present (King, 2005; Kayombo et al., 2007).

In general an atmosphere of mutual benefit and reasonable trust is what the researcher inherited from Van Huyssteen. Existing points of contention were (a) a memorandum of understanding which had been drawn up and presented to the ATH which they were reluctant to sign and (b) tension existed between the collaborating ATH associations. It was the incoming researcher’s responsibility to get the memorandum of understanding signed and to take the research and collaboration one step further. The decision to expand the research collaboration was based on the positive groundwork that had been done which resulted in an efficiently conducted research collaboration.

2.5.1 Growing the research collaboration

The proposed project centred around two widely used and known medicinal plants. The efficacy of the medicinal plants on patients consulting the collaborating ATH was to be evaluated and the plant extracts were to be formulated into a topical dosage form. A meeting was called with the leaders of the three African traditional healers organisations which participated in the collaboration. They were asked to bring two other ATH who would form part of a nine member team. This was done following established etiquette in which the leaders were informed first and they distributed the information to members of their organisation.
The methodology proposed was that nine traditional healers would be involved in the project; they would recruit study participants and also collect some of the data for the research. The efficacy of the treatment would be evaluated using biomedical techniques which included assessment by professional nurses and laboratory testing of swabs taken from patients. The participating ATH would also provide commentary on the efficacy. The ATH were thus included as researchers in the validation of ATM as had been envisioned when the relationship was initiated in the year 2000.

Reasons for the research topic and the aims and objectives were provided, discussed and refined. The ATH present approved of the study and were willing to recruit patients if certain provisos were met which in the main were there to protect their reputation and the wellbeing of their patients. Final approval was however, needed from their mother bodies. To overcome a situation where researchers could be seen as “stealing” information, two plants were suggested as active ingredients for the topical preparation whose indications were sourced from literature.

Whilst the ATH were giving feedback to their colleagues and mother bodies about the proposed research, work to maintain the garden continued. Expenditure for the garden had been provided by funds received by the project leader from the National Research Foundation. Since this funding was not sustainable in the long term, income generating projects were pursued in earnest to keep the project viable. Some of these projects were put forward to the traditional healers whilst others were recommended by them. These projects included selling beaded wrist bracelets, ornamental grass baskets and decorative pots containing medicinal plants to people visiting the medicinal garden. The starting material for the aforementioned projects was provided by the researchers and visits were made to the local municipality to source funds for further materials and equipment.

During a scheduled visit to the garden the researcher discovered, along with some ATH, that the gate had been locked by one of the organisation’s leaders and access to it was forbidden. Attempts to get clarity on what caused the action proved fruitless; access to the garden was never granted again and all plans came to a halt.
What went wrong? It may be that a lack of trust by the healers in the researchers and rivalry over resources amongst the organisations were at the heart of the fallout.

2.5.1.1 Trust and Power

Traditional healers are weary of scientists who conduct research on what is essentially their livelihood (Kaboru et al., 2006). There is an ingrained conviction that scientists will take the information, extract the active constituents or synthesise them, protect the results through patents and keep the benefits from such outcomes for themselves (Scheinman, 2002; Kayombo et al., 2007). This fear is not unjustified as there have been reports of indigenous plants being commercialised while leaving the indigenous holders of that knowledge out e.g. hoodia (Vermeylen, 2007).

The collaborating ATH did not fully trust the researchers. After seven years of collaboration, none of their medicines recipes had been divulged. The researchers had to rely on the information and uses obtained from literature. Even this sparked questions and comments such as:

- Who are the people divulging this information for publication?
- If you cannot identify them by name or geographical location it means they were not properly identified and credited with the knowledge
- When was this information published and by whom?

It is possible that the re-introduction of a researcher and the leaving of another heightened fears which could have been exacerbated by the rapid pace at which the new researcher was introducing changes albeit known and previously discussed ones.

Traditional and biomedical healers differ in their understanding of causes and thus the treatments of diseases. Biomedicine focuses on physiological pathology and the treatment of diseases whilst traditional healers focus holistically on physical, spiritual and mental health. African traditional healers thus believe that even when a disease is being treated physically, full recovery may not occur unless the cause of such disease is identified and removed. The cause could be witchcraft or an imbalance within the community or with the ancestors (Kale, 1995). When validation of ATM includes trials
whose outcome will be assessed biomedically the exclusion of spirit and mind treatments may negatively skew the results to the detriment of ATM. Providing researchers with the full picture could have meant that the traditional healers needed to divulge more information around practice which they were not ready or willing to do. Although this was not communicated it is not unreasonable to think that concern around this matter might have been present. Kaboru and colleagues (2006) have suggested that time and patience around secrecy is needed, however, such secrecy may be a way of deflecting evidence based practice which could ultimately lead to the failing of collaborations.

Most of the training conducted during the collaboration was one sided, the ATH were informed of allopathic ways of treatment and the opposite was happening on an informal small scale. This was mainly due to the ATH reluctance to divulge information, it nevertheless created a relationship over time in which the majority of the power rested with the researchers. This was compounded by the fact that monetary support for the entire project was provided by the researchers. A power relationship favourably skewed towards allopathic practitioners usually exists when allopathic practitioners and/or scientists collaborate with traditional healers (Hillenbrand, 2006; Kaboru et al., 2006). The introduction of other allopathic health practitioners whose function was to assess the effectiveness of the ATH treatments could also have been a miscommunicated problem. African traditional healers have stated that allopathic health workers, including nurses, do not respect them and dismiss the work that they do (Mngqundaniso & Peltzera, 2008).

2.5.1.2 Organisations’ rivalry over resources

Most of the African traditional healers in the research collaboration lived amongst disadvantaged communities in which they are seen as leaders who should provide some form of relief to the everyday problems faced by the communities. As such the collaboration may have been viewed as more than just research but as a means of providing such relief. To illustrate this point, the ATH decided not to be provided with food when attending meetings but to rather to be given the money which would have been paid for catering purposes in order for them to buy food that could be shared by
many in their households. It also emerged that those ATH living closer to the meeting areas opted to walk even though they were provided with transport money.

The number of African traditional healer organisations who were attending to the established medicinal garden were greater than the number who were willing to work at establishing it. When they showed interest in the garden at a later stage they were not barred by the researchers. This caused discontentment amongst those who had worked at establishing the garden and reinforced power discrepancies. The returning healers were not barred because the researchers were wary of getting caught in rivalries and being seen as working with particular organisations and not others. A discussion ensued and resolution gained. It is however possible that resolution had not been achieved as initially thought and feelings of ownership and unfairness at having others reap rewards for which they did not work for were heightened when income generating and skills development projects were pursued.

2.6 Conclusion

The majority of conditions necessary for successful collaborations between allopathic and African traditional healers as set out by King (2005) existed. The tensions and mistrust present were thought to be well managed and a fairly smooth running project was in existence. Its collapse was thus unexpected and provided hard lessons which included the reformulation of this researcher’s methodology for the current study.

The researchers have not given up on collaborating with the African traditional healers and another medicinal garden is being created at Missionvale Campus of the NMMU. The lessons learnt regarding power dynamics and resource ownership are being put into practice and the collaboration is now being revived.
3. WOUND HEALING PROPERTIES OF MEDICINAL PLANTS

3.1 Medicinal plants and wound healing

A wound is defined as a disruption in the cellular, anatomical and functional continuity of the skin and underlying tissue (Thakur et al., 2011). Kumar and colleagues (2007) note that compared to Western medicines which have 1% - 3% of medicines in pharmacopoeia listed for wound healing, a third of traditional medicines are dedicated to wound healing. Such a high quantity of resources dedicated to one ailment implies that indigenous people were prone to wounds that needed some form of intervention. That the remedies used for wound healing were mastered is attested to by the fact that even to this day, treatment of wounds remains the most common reason why people consult Ayurvedic healers (Birhan et al., 2011).

Literature recording South African medicinal plants traditionally used to treat wounds is not specific in that the medicinal plant is indicated without giving the context under which it was used (Van Wyk, 2008; Ayyanar & Ignacimuthu, 2009; Aston Philander, 2011; Van Wyk, 2011). Answers to the questions listed below would give a greater indication of the type of properties to be expected from the medicinal plant. The questions are, was the plant used for:

- deep wounds that have been recently acquired?
- chronic or acute wounds that have become infected?
- wounds that are not healing as expected?

Mabona and Van Vuuren (2013) noted that when listing uses of plants, treatment of sores and ulcers were categorised separately from other skin ailments due to implications of severity. This difference in classification also exists in Ayurvedic medicine, see Table 3.1. Whilst not specifically answering the questions above, this form of classification can provide clues on the properties which could be expected from
the medicinal plant. As an example, if a plant or plant combination is administered for the first few days of treating a septic wound followed by a different plant combination for the duration of the healing period, it may be hypothesised that the first combination possesses greater antimicrobial properties and the second may have greater cell proliferation activity. When validating the use of these plants, it would not be surprising if the first combination did not demonstrate cell proliferation properties even though it is used for wound healing. It is this lack of specific knowledge which is most challenging when validating medicinal plants whose use is obtained from literature, however, as traditional healers fiercely guard specific information, literature proves to be the next best source of information.

Table 3.1: Ayurvedic causes and types of wounds

<table>
<thead>
<tr>
<th>Causes</th>
<th>Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wounds from working in the fields</td>
<td>Wounds/ulcers</td>
</tr>
<tr>
<td>Burns from cooking and sleeping near fires</td>
<td>Erysipelas</td>
</tr>
<tr>
<td>Fractures</td>
<td>Maggots in wounds</td>
</tr>
<tr>
<td>Motor vehicle accidents (modern cause)</td>
<td>Abscesses</td>
</tr>
<tr>
<td>/leg ulcers</td>
<td>Septic wounds</td>
</tr>
<tr>
<td>Injuries incurred in conflicts</td>
<td>Inflammatory changes of wounds</td>
</tr>
</tbody>
</table>

(Inngjerdingen et al., 2004; Kumar et al., 2007; Ayyanar & Ignacimuthu, 2009)

3.2 Validating wound healing properties

Notwithstanding the source of damage, wound healing follows overlapping processes that are illustrated in Figure 3.1. The goal of wound healing is to restore the anatomical continuity and functioning of the skin, to achieve this, allopathic wound care can involve dressings and the administration of antiinflammatories, antimicrobials and analgesics (Süntar et al., 2012).
In vitro assays have increasingly replaced whole animal and tissue experiments in ethnopharmacological studies due to high cost, ethical considerations and the unsuitability of animal experiments to bioassay-guided fractionation (Houghton et al., 2005). A single in vitro assay is however too reductionist to represent complex wound healing processes. Therefore, when validated with in vitro assays, medicinal plants should be proven to modulate at least two wound healing processes that are shown in Figure 3.1. Anti-oxidant properties of plants are also determined as the overproduction of free radicals can impede wound healing.

Wounds provide a moist, warm and nutritive environment which is optimal for microbial growth and the infection of wounds by microorganisms can hamper healing (Houghton et al., 2005). Antimicrobial properties of medicinal plants are thus also determined when validating wound healing properties. Microflora which have been isolated from wounds and used in in vitro assays include *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Escherichia coli*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus faecalis* and *Candida albicans* (Egbe et al., 2011).
3.2.1 Antioxidant and antiinflammatory activity

The involvement of low concentrations of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (10 nM – 1 µM) in cell proliferation in vitro has been demonstrated (Burdon, 1995). The ROS are thought to activate early growth genes such as c-fos and c-jun. Activation of c-fos expression is thought to be mediated through phospholipase A2-dependant release of arachidonic acid; this action activates the inflammation cascade which is essential for wound healing. It is due to this close action between oxidants and inflammation that the use of antioxidants during wound healing was thought to be deleterious to the processes of wound healing (Frum & Viljoen, 2006). That perception has changed and the delicate balance between anti and pro-oxidative species is recognised. It is now known that low concentrations of ROS promote wound healing and high concentrations may result in cell death (Burdon, 1995; Fitzmaurice et al., 2011). It is therefore, hypothesised that the direct reaction between ROS and plant extracts does not interfere with normal wound healing processes which is the reason why most plant extracts exhibiting wound healing properties also possess high radical scavenging capacities (Süntar et al., 2012).

A number of assays have been developed to determine and quantify antioxidant activity of compounds and extracts, some of which are indicated in Table 3.2. These have been classified into hydrogen atom transfer (HAT) based assays and single electron transfer (ET) based assays. Single electron transfer assays involve one redox reaction with the oxidant and HAT assays monitor competitive reaction kinetics (Huang et al., 2005).

In their study, Thaiponga and colleagues (2006) found that results from ferric reducing antioxidant power (FRAP), Diphenylpicrylhydrazyl free radical scavenging (DPPH), and oxygen radical absorbance capacity (ORAC) assays were comparable. The correlation between ET based assays is to be expected according to Huang and colleagues (2005) as the assays are based on similar redox reactions. The use of multiple ET based assays in the validation of wound healing properties is thus unnecessary.
Table 3.2: Antioxidant assays classified as either HAT or ET based assays.

<table>
<thead>
<tr>
<th>Electron transfer based assays</th>
<th>Hydrogen atom transfer based assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric reducing antioxidant power (FRAP)</td>
<td>Oxygen radical absorbance capacity methods (ORAC)</td>
</tr>
<tr>
<td>Diphenylpicrylhydrazyl free radical scavenging (DPPH)</td>
<td>Lipid peroxidation inhibition capacity</td>
</tr>
<tr>
<td>Copper (II) reduction capacity</td>
<td>Total radical trapping antioxidant parameter</td>
</tr>
<tr>
<td>Total phenols by Folin-Ciocalteu</td>
<td>Inhibited oxygen uptake</td>
</tr>
</tbody>
</table>

(Badarinath et al., 2012).

3.2.2 Cell proliferation activity

The study of cell proliferation is a basis for determining wound healing capabilities of medicinal plants because dermal fibroblast and keratinocyte proliferation and migration play an important role in wound healing processes. These processes include reepithelialisation, granulation tissue formation and remodelling of the extracellular matrix (Thakur et al., 2011). Three assays which detect cell proliferation activity are suggested, these are the fibroblast, keratinocytes and the chick chorioallantoic membrane assays. Fibroblast assays are most commonly used to determine wound healing properties. They include scratch, transmembrane, microfluidic chamber and cell exclusion zone assays. Good correlation of results has been observed between experiments conducted using fibroblast assays, however, results obtained from purchased test kits were found to be more reproducible and had lower coefficients of variance when compared to in-house scratch assays (Hulkower & Herber, 2011).

The scratch assay is based on the observation that upon creation of a wound in a monolayer of confluent, adherent cells, those at the edge of the “wound” will migrate and eventually close the gap (Rodriguez et al., 2005). It is possible to visually observe the morphology and movement of cells in real time allowing the measurement of velocity. The assay is also simple and uses equipment and chemicals commonly found in laboratories capable of culturing cells. This assay, to some extent, mimics in vivo cell migration which is an added advantage (Liang et al., 2007; Teferedegne et al., 2010).
Limitations of the scratch assay include the lack of reproducibility of the scratch intra- and inter-laboratories. This is because methods of creating the scratch may differ. Usually pipette tips or needles are used to create the scratch; these tools may differ from laboratory to laboratory. In addition, the size, spacing and shape of the scratch can also vary from well to well within the same experiment (Hulkower & Herber, 2011). Another disadvantage is the large quantity of cells and chemicals which are required to perform the assay.

Regardless of the type of assay chosen to identify wound healing properties, a cell viability assay, which measures the health of cells when exposed to the test compound, should be conducted. In addition to cell viability, cell proliferation is also determined when testing for wound healing properties. Cell proliferation measures actively dividing cells in the culture medium. It can be reported as the actual number of proliferating cells or as relative values to cell populations (Kresse, 2003).

There are a variety of methods which can be used to determine cell viability and proliferation. These include the measurement of (a) cell membrane integrity by measuring lactate dehydrogenase, (b) the metabolic activity of viable cells through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (c) adenosine triphosphate present in metabolically active cells and (d) the number of viable cells based on the uptake of the supravital dye, neutral red (Weyermann et al., 2005).

The ease of performing the MTT assay makes it widely used for determining cell viability and proliferation (Berridge et al., 1996). The principle of the assay is based on the assumption that mitochondrial activity is constant and therefore a change in the number of viable cells is linearly related to mitochondrial activity. Mitochondrial activity in living cells converts MTT into formazan crystals, these are then solubilised for homogenous measurement (Van Meerloo et al., 2011). The results from the MTT assays may be misleading if the compound in question only affects intracellular activities. In addition, plant extracts may possess an intrinsic reduction potential which can create inaccuracies in the assay. Removing the extracts and washing the cells
before addition of MTT can overcome this interference (Bruggisser et al., 2002; Wang et al., 2010).

3.3 Wound healing assays conducted

3.3.1 Cytotoxicity assays

3t3-L1 preadipocyte and Vero cells were obtained from the Department of Biochemistry and Microbiology of the Nelson Mandela Metropolitan University; Dulbecco’s modified Eagles medium (DMEM) from BioWhittaker, Walkersville, USA; foetal calf serum from Delta Bioproducts, Johannesburg, South Africa; trypan blue solution from Gurr, BDH Chemicals, Poole, England; dimethylsulfoxide (DMSO) from Associated Chemical Enterprises (Pty) Ltd, South Africa.

3.3.1.1 Maintenance of the cells

All manipulations were conducted using sterile reagents and equipment under aseptic conditions and all solutions were warmed to 37 °C before coming into contact with the cells. The cells were maintained in DMEM supplemented with 10% foetal calf serum. They were grown in 10 cm culture dishes in 10 ml of the supplemented DMEM and incubated at 37 °C in a humidified 5% CO₂ environment (Thermo Electron Corporation).

The medium was removed with the aid of a Pasteur pipette and the cells washed twice with 10 ml of calcium and magnesium free phosphate buffered saline (PBS) when the cells were 60% and 90% confluent for 3t3-L1 and Vero cells respectively. Two hundred microlitres of 0.25% trypsin in PBS was then added and the plate slightly agitated. Excess trypsin was removed to leave approximately 100 µl in the culture dish. The cells were incubated for 10 minutes to allow the cells to detach which was observed under a light microscope. One millilitre of supplemented DMEM was added to the detached cells to deactivate the trypsin. One hundred microlitres of the cell
suspension was transferred to a new culture dish and 10 ml of the supplemented DMEM added, this was incubated until 60% and 90% confluence was reached for 3t3-L1 and Vero cells respectively.

A viability count was done for seeding purposes using a 0.4% m/v trypan blue solution on a haemocytometer. The cells were seeded in a flat bottomed 96 well plate at a density of 6000 cells/well for cytotoxicity studies and at a density of 62 000 cells/well in a flat bottomed 24 well plate for the scratch assay. The plates were incubated at 37 °C in a humidified 5% CO₂ environment for 24 hours to allow the cells to attach to the bottom of the well.

3.3.1.2 Stock solutions of the extracts for cytotoxicity assays

*C. edulis* (0.01 g) was weighed and 50 µl of DMSO was added to solubilise and disinfect the extract followed by the addition of 4.95 ml of supplemented DMEM. The same was done for *B. frutescens*. To make the combined extract, 0.005 g of each extract was weighed, the two combined and the relevant solvents then added as mentioned above. Disinfection of the plant leaves with hypochlorite solution and ethanol and the addition of DMSO to the extracts nullified the need to sterilise the extracts further. No microbial contamination of the cells was experienced when the assays were being conducted. Fresh extract stock solutions were prepared on the day in which they would be used in the assays.

3.3.1.3 Conducting the assay

3t3-L1 cells were exposed to individual extracts at concentrations of 2000, 1500, 1000, 500, 300, 200, and 100 µg/ml. Vero cells were exposed to concentrations of 250, 125, 62, 31, 16, 8 and 4 µg/ml. Concentrations of individual extracts in the combined extract to which the cells were exposed were half of those listed above.

After 48 hours of exposure to the extracts, the incubation medium was removed and 100 µl of MTT (Sigma Chemicals Co., USA) at 0.5 mg/ml was added to the wells. The plates were then incubated for a further 4 hours. The MTT solution was removed and 200 µl of DMSO was added to the wells which were slightly agitated. The optical
density of the wells was determined at 560 nm using a multiplate spectrophotometer (Multiiscan MS® version 4.0 Labsystem® type 352). Cells not treated with the extract but whose incubation medium contained 1% DMSO (which is the highest vehicle concentration that the cells were exposed to) were used as the control.

3.3.2 Scratch assay

3t3-L1 cells were seeded in a 24 well plate at a density of 62 000 cells/well as described in section 3.3.1.1 above. A diagonal wound was created in the centre of the well using a pipette after 24 hours of incubation at 37 °C in a humidified 5% CO₂ environment. The incubation medium was then removed and the extracts were added in such a manner that the final concentration of individual extracts in the each well was either 5 µg/ml or 10 µg/ml. The individual extract concentrations in the combined extract were 2.5 µg/ml. Photographs of the wounds were taken at 24 and 48 hours. Wounds not treated with the extracts were used as the control. Visual inspection was used to determine the degree of wound closure.

3.3.3 DPPH Assay

To make a 2000 µg/ml stock solution of the individual plant extracts, 0.008 g of each extract was weighed and dissolved in 4 ml of methanol. The combined extract stock solution was made by weighing 0.004 g of each plant extract, combining them and dissolving them in 4 ml of methanol.

The stock solutions were diluted such that the following concentrations were achieved in the wells 1000, 500, 250, 125, 62.5, 31, and 16 µg/ml. The same was done with the B. frutescens and the combined extracts.

Fifty microlitres of 0.2 mg/ml DPPH solution in methanol which was freshly prepared on the day of the experiment was added to the wells containing plant extracts and the plates were then incubated in the dark for 30 minutes. Absorbance of the wells was determined at 540 nm using a multiplate spectrophotometer (Multiiscan MS® version 4.0 Labsystem® type 352). Ascorbic acid was used as the positive control and 50 µl of methanol combined with 50 µl of DPPH was used as the blank.
3.4 Statistical analysis of results

Cytotoxicity assays had eight replicates for each extract concentration and the average of the results is reported. Cell proliferation was calculated using the equation:

\[
\text{Cell proliferation (\%)} = \frac{\text{sample reading}}{\text{control}} \times 100
\]

Analysis of variance with Tukey as the post test and the two tailed, unpaired t-test were used to analyse the results using the GraphPad Prism 6® software program (GraphPad Software, San Diego, California). Results were considered statistically different when \( p < 0.05 \).

3.5 Results

3.5.1 Cytotoxicity assay

The manner in which the medicinal plants are used and the concentration of extract in the cream being produced determined the extract concentrations to which 3t3-L1 cells were exposed. Direct application of the leaf sap onto the wounds and the 10% w/w extract concentration in the cream could potentially expose individuals to large concentrations of the extracts.

In general, the extracts to varying degrees were toxic to 3t3-L1 cells as none of the experiments produced 100% viability as seen in Figure 3.2. \textit{B. frutescens} was less toxic to the cells when compared to \textit{C. edulis} at 100 µg/ml and 200 µg/ml, however, a reversal in toxicity was observed from 300 µg/ml. The viability of 3t3-L1 cells exposed to \textit{B. frutescens} at 100 and 200 µg/ml was significantly different to that of cells exposed to the combined extract at 100 and 200 µg/ml with \( p < 0.05 \).
There was a statistically significant difference between the viability of cells exposed to *C. edulis* when compared to those exposed to the combined extract at all concentrations with $p < 0.05$.

**Figure 3.2:** Cytotoxicity of *B. frutescens*, *C. edulis* and the combined extracts to 3t3-L1 cells. The degree of cell viability between *C. edulis* and the combined extracts was significantly different with $p < 0.05$ at all concentrations tested.

The combined extracts were more toxic to the cells than individual extracts with cell viability not exceeding 68%. An increase in the cytotoxic effects of medicinal plants used in combination has been documented by researchers, with some relating an increase in cytotoxicity to the increase in antimalarial and antibacterial properties of medicinal plant combinations (Briskin, 2000; Abd Razak *et al.*, 2007).

Whilst cytotoxicity was noted for 3t3-L1 cells, the fact that cell viability of more than 50% was attained at concentrations as high as 2000 µg/ml suggests that it may not be clinically relevant.

None of the plants were cytotoxic to Vero cells at the concentration range of 4 – 250 µg/ml. The combined extracts significantly increased cell viability when compared to *B. frutescens* and *C. edulis* alone as shown in Figure 3.3 with $p < 0.005$. 
Figure 3.3: Cytotoxicity of *B. frutescens*, *C. edulis* and the combined extracts to Vero cells. The degree of cell viability between the combined and individual extracts was significantly different with p < 0.005.

3.5.2. Scratch assay results

The process of wound healing in cell monolayers occurs through polarisation of the cells towards the wound, protrusion is then initiated followed by migration and finally closure of the wound (Yarrow *et al.*, 2004).

After 24 hours a larger degree of wound closure was obtained for cells exposed to 5 µg/ml for all extracts when compared to the control as seen in Figure 3.4.

There was greater wound healing for all extracts at 5 µg/ml and *B. frutescens* at 10 µg/ml when compared to the control wound. However, a reduction in the level of wound closure was observed when the concentration of *C. edulis* was increased to 10 µg/ml. This could not be explained by cytotoxicity as there was an increase in cell viability when *C. edulis* concentrations were increased.

The extent of wound healing obtained in the presence of individual extracts at 5 µg/ml was greater than that obtained when the extracts were used in combination. This correlates with cytotoxicity results of 3t3-L1 cells wherein lower cell viability was
obtained with the combined extracts when compared to *B. frutescens* and *C. edulis* alone.

Figure 3.4: Wound closure 24 hours after exposure to plant extracts. A larger degree of wound closure was obtained from *B. frutescens* at all concentrations followed by *C. edulis* at 5 µg/ml.

The constituents isolated by previous researchers from these plants may explain differences between the results. Tannins and flavonoids which have been isolated from *C. edulis* (Van der Watt & Pretorius, 2001) promote wound healing through antioxidant activity, promotion of wound contraction and increasing the formation of capillary vessels and fibroblasts (Nijveldt *et al.*, 2001; K. Li *et al.*, 2011; Pawar & Toppo, 2012). The wound healing properties of *B. frutescens* are thought to be due to the presence of polysaccharides and/or glycoproteins (Pather *et al.*, 2011). These promote wound healing through antioxidant activity and by increasing cell proliferation and migration of keratinocytes (Khamlue *et al.*, 2012; Krishnamoorthy *et al.*, 2012). Faster migration and proliferation thus occurred in the presence of *B. frutescens* resulting in faster wound closure in the scratch assay. The slower closure in the presence of *C. edulis* does not
indicate a lack of wound healing properties since at 5 µg/ml, wound closure was better than that of the control.

Figure 3.5: Wound closure 48 hours after exposure to plant extracts. The control wound and all wounds exposed to *B. frutescens* are closed. Wounds exposed to *C. edulis* and the combined extracts had not closed.

Wounds created at the start of the assay had closed after 48 hours in the control well and all wells containing *B. frutescens* alone. Wound closure in wells containing *C. edulis* and the combined extracts, shown in Figure 3.5, had also progressed but to a lesser extent. This can imply that whilst beneficial in the short term, *C. edulis* and combinations containing *C. edulis* are detrimental to wound healing when cells are exposed for an extended time. This however, does not correspond with cytotoxicity results and the clinical relevance needs to be determined.
3.5.3 Antioxidant activity based on the DPPH assay

The antioxidant activity of both *C. edulis* and the combined extract were significantly different from that of *B. frutescens* with \( p < 0.0005 \) and \( p < 0.005 \) respectively and the activity is depicted in Figure 3.6. The antioxidant activity of *C. edulis* was not significantly different from that of the combined extracts.

![Figure 3.6: Antioxidant activity of *B. frutescens* and *C. edulis* based on the DPPH assay.](image)

The antioxidant activity of *B. frutescens* cultivated under various conditions was investigated by Netshiluvhi (2012). The lowest IC\(_{50}\) obtained was 190 µg/ml using the DPPH assay. This is lower than what was obtained in this study, which was 660 µg/ml, however, an acetone extract obtained from leaves which had been air dried was used in Netshiluvhi’s study.

High antioxidant activity of *C. edulis* has been reported in the literature. In a study by Ibtissem and colleagues (2012), in which *C. edulis* extract had been obtained by boiling, the antioxidant activity determined by using the DPPH assay did not drop below 80% for concentrations in the 0.5 – 5 mg/ml range (Ibtissem et al., 2012). In another study, an aqueous extract of *C. edulis* was found to have higher antioxidant activity when compared to ethanol and acetone extracts with an IC\(_{50}\) of 15 µg/ml which
was comparable to that obtained in this study which was 11.32 µg/ml (Omoruyi et al., 2012).

The IC\textsubscript{50} values of the extracts which were 11.32 µg/ml, 660 µg/ml and 41.74 µg/ml for \textit{C. edulis}, \textit{B. frutescens} and the combined extracts respectively were plotted as an isobologram seen in Figure 3.7. Synergistic activity occurs when the dose of the combined extracts that is needed to provide a similar effect is less than the sum of the individual components. When using the isobologram method, synergism is indicated when the concentration of the combined extracts falls below the line of additivity (Hemaiswarya et al., 2008). The value for the combined extracts fell below this line indicating that \textit{B. frutescens} and \textit{C. edulis} had synergistic antioxidant activity when used in combination (Tallarida, 2006; Damaraju et al., 2007). The combination of these plants has not been investigated prior to this study, thus these results are not reported in the literature.

\textbf{Figure 3.7:} Isobologram analysis of the antioxidant activity of \textit{B. frutescens} and \textit{C. edulis}. The extracts demonstrated synergistic antioxidant activity when used in combination.
3.6 Conclusion

The extracts under study were not toxic to cells used to validate wound healing properties at the concentrations utilised in this study. To validate wound healing properties of plants with in vitro assays, two properties involved in the wound healing cascade should be modulated. *B. frutescens* and *C. edulis* did not individually meet this criterion with the tests utilised in this study. *B. frutescens* fared better in the scratch assay whereas *C. edulis* had better antioxidant properties. However, when used in combination, these plant extracts met the criteria needed to validate wound healing and produced synergistic antioxidant activity. Their use in combination to treat wounds was thus validated in vitro.
4. ANTIBACTERIAL AND ANTIVIRAL ACTIVITY OF C. EDULIS AND B. FRUTESCENS

4.1 The search for new antibiotics

Natural products such as medicinal plants and insects have been used for millennia by indigenous people throughout the globe for their curative properties. These medicines have been administered internally as teas, tinctures and powders or externally as poultices or by direct application of the sap onto the affected area. Many medicinal plants were used to treat bacterial infections, and as a direct result, clinically important antibiotics have been discovered (Balunas & Kinghorn, 2005). The use of quinine to treat malaria eventually led to the formation of drugs such as chloroquine and mefloquine. Artemisinin, a potent antimalarial and its derivatives were obtained from Artemisia annua leaves, this discovery proved useful since resistance to quinine and its derivatives had emerged (Gurib-Fakim, 2006). Arctostaphylos uva-ursi and Vaccinium macrocarpon are used to treat urinary tract infections (Ríos & Recio, 2005).

Natural products account for all but three classes of antibiotics, the majority of which have been sourced from natural products other than medicinal plants (Singh & Barrett, 2006). As examples, penicillin was obtained from the mould Penicillium notatum (Von Bubnoff, 2006) and streptomycin was obtained from the soil by Selman Waksman (Davies & Davies, 2010). The timeline for discovery and introduction of new antibiotics into clinical settings is shown in Figure 4.1 below.

In their review of new chemical entities introduced clinically, Newman and Cragg (2007) show that the number of antibiotics derived from natural sources decreased in the period 1994 – 2006 where one was introduced as compared to eight in the period 1981 – 1993 as depicted in Table 4.1.
**Figure 4.1:** Timeline for novel natural antibiotic drug class discovery. Adapted from (Bakshi & Singh, 2002; Davies & Davies, 2010).

**Table 4.1:** Number of antibacterial drugs introduced between 1981 and 2006. Adapted from (Newman & Cragg, 2007).

<table>
<thead>
<tr>
<th>Period</th>
<th>Total number</th>
<th>Natural product</th>
<th>Derived from a natural product</th>
<th>Synthetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981 - 1986</td>
<td>30</td>
<td>4</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>1987 – 1993</td>
<td>43</td>
<td>4</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>1994 – 1999</td>
<td>14</td>
<td>0</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>2000 – 2006</td>
<td>12</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>94</strong></td>
<td><strong>9</strong></td>
<td><strong>65</strong></td>
<td><strong>26</strong></td>
</tr>
</tbody>
</table>
The reasons for the decline, include amongst others, the shift in focus by large pharmaceutical companies from research and development pertaining to infectious diseases to that of lifestyle or chronic diseases (Von Bubnoff, 2006) and the move away from “functional cellular assay and animal models of disease” to “non-functional, non-biologic enzyme inhibition assays and receptor binding assays” as drug discovery tools (Rishton, 2008).

Along with the decline in new novel antibiotics, resistance of bacteria to the existing antibiotics was increasing after their widespread use. Such resistance was initially experienced in hospitals as nosocomial infections which developed soon after introduction of the antibiotic (Levy & Marshall, 2004). As examples, resistance to penicillin was observed within one year of its widespread use; within a few years 50% of bacteria were no longer susceptible (Alanis, 2005) and erythromycin was withdrawn from the Boston City Hospital in the 1950’s, less than a year after its introduction due to resistant bacteria (Davies & Davies, 2010). Resistance of bacteria to multiple agents emerged in the late 1950’s and was detected among enteric bacteria such as Salmonella.

Resistance has emerged among Group A Streptococcus to macrolides, Enterococci to vancomycin and multidrug resistant Mycobacterium tuberculosis is threatening to reverse strides made in the treatment of tuberculosis (Alanis, 2005). The general consensus is that resistance emerges largely due to inappropriate and excessive use of antibiotics. Loss of resistance can thus be attained through appropriate use of antibiotics, however, the process is slow and difficult when dealing with multidrug resistant strains (Levy & Marshall, 2004). The problem of resistance may however, never be fully overcome because it has been discovered that there is a large number of antibiotic resistance genes which are components of natural microbial populations (Davies & Davies, 2010).

The number of articles published in PubMed relating to research of medicinal plants as antimicrobial agents increased from 115 in the period 1966 – 1994 to 307 in the period 1995 – 2004. The critique of the methodologies was that the concentrations used in the tests were too high. It was suggested that concentrations above 1 mg/ml for extracts
and 0.1 mg/ml for isolated compounds should not be used, furthermore, antimicrobial activity below 100 µg/ml for extracts and 10 µg/ml for isolated compounds indicated good activity (Ríos & Recio, 2005).

Comparison of results relating to antimicrobial activity of medicinal plants can be difficult. Researchers determining the activity of the same medicinal plant may yield differing results. Variation can be due to:

- Environmental and climatic conditions during plant growth.
- Choice of extraction methods.
- Choice of plant extracts.
- Antimicrobial tests employed and
- Test organisms (Das et al., 2010).

Thus when comparing experimental results with published results a researcher should strive to compare with data which most closely resembles their methods.

4.1.1 Antimicrobial activity of South African Medicinal Plants

The antimicrobial activity of many South African medicinal plants has been identified indicating that with some persistence and correct methodologies, compounds may be isolated which can provide novel antibacterial agents (Grierson & Afolayan, 1999; Masika & Afolayan, 2002; Lewu et al., 2006; Mathabe et al., 2006; Thring et al., 2007). The antimicrobial activity of medicinal plants has been attributed to a variety of chemical compounds which include amongst others phenols, phenolics, flavones, tannins and coumarins (Das et al., 2010) with phenolics being the most active especially against Gram positive bacteria (Ríos & Recio, 2005).

The antimicrobial activity of *B. frutescens* has been investigated by researchers who utilised different extraction and testing methods and both articles report a lack of significant antimicrobial activity for this plant (Rabe & Van Staden, 1997; Coopoosamy, 2011). A negative feature of the Coopoosamy (2011) report was that plant extract activity against *Bacillus subtilis* and *Micrococcus kristinae* was obtained at high minimum inhibitory concentrations (MIC) which were 2.0 mg/ml and 3.0 mg/ml
respectively. The antibacterial activity of *C. edulis* and its fractions has been reported by researchers who used the disk diffusion method and thus did not report the MIC (Van der Watt & Pretorius, 2001).

Medicinal plants may be used in combination when treating diseases. The rationale is that use in combination produces synergistic activity. This has been validated by researchers who discovered that in the majority of instances, plant combinations produce better antimicrobial activity. Synergism is defined by Williamson (2001) as “an effect seen by a combination of substances being greater than would have been expected from a consideration of individual contributions”. There is a growing body of research which is proving antimicrobial synergistic interaction between medicinal plants (Williamson, 2001; Al-Bayati, 2008; Ncube et al., 2012). Hemaiswarya and colleagues (2008) demonstrated the synergistic activity of medicinal plants and synthetic antibacterials to which resistance had emerged. The synergism increased the susceptibility of the resistant microorganisms to the synthetic antibiotic, this gives hope that when formulated rationally these combinations may increase antibacterial activity of synthetic antibiotics rendered unusable by resistance. Synergism between different extracts of the same plant prove that the myriad of chemical constituents contained in medicinal plants work synergistically and isolating one fraction may not produce efficacy claimed by indigenous users or that obtained from whole plants (Hemaiswarya et al., 2008).

### 4.2 Antiviral drugs discovered from medicinal plants

There are approximately 50 antiviral agents that are in clinical use worldwide; about half of these are dedicated to treatment of the human immunodeficiency virus, the others are used to treat herpes simplex virus type 1 and 2, varicella-zoster virus, cytomegalovirus, hepatitis B and C virus, respiratory syncytial virus and influenza virus (De Clercq, 2005; Antonelli & Turriziani, 2012). The quest for new antiviral agents is
important and ongoing especially since resistance to some of the agents in use is being encountered in some patient populations.

Viruses are obligate intracellular parasites which pose a unique challenge to the development of antiviral agents. This is due to the nature of replication of viruses which relies on the hosts cellular mechanisms to propagate new viruses. Selectivity for the virus is thus challenging and the lack thereof is the cause of most toxic effects experienced with currently available antiviral agents (Antonelli & Turriziani, 2012). Numerous medicinal plants have been screened for antiviral activity using *in vitro* and *in vivo* assays with herpes simplex viruses being the most studied (Khan *et al.*, 2005; Kitazato *et al.*, 2007; Mukhtar *et al.*, 2008).

South African medicinal plants have demonstrated activity against a variety of viruses which include amongst others human immunodeficiency virus (HIV), feline herpesvirus-1 and polio virus (Louw *et al.*, 2002; Bagla *et al.*, 2012; Louvel *et al.*, 2013; Ndhlala *et al.*, 2013). No literature describing *in vitro* or *in vivo* antiviral properties of *B. frutescens* and *C. edulis* was obtained.

Direct and indirect methods may be utilised to determine antiviral activity of medicinal plants. Indirect methods include visual observation of the cytopathic effect, neutral red dye uptake assay and the MTT assay. Direct assays include amongst others the enzyme linked immunosorbent assay, hemagglutination assay, immunofluorescence assay and the plaque reduction assay.

The plaque reduction assay is the oldest and one of the most reliable methods for determining *in vitro* antiviral activity. It determines the effectiveness of the medicinal plant in reducing plaque forming units of the virus when compared to the controls. A general protocol is illustrated in Figure 4.2 (Khan *et al.*, 2005; Chattopadhyay *et al.*, 2009).
4.2.1 Herpes simplex virus infection

Herpes viruses represent a large group of clearly defined viruses that have the ability to persist in the nucleus of host cells causing latent infection. These viruses encode most of the enzymes they require for replicating viral DNA and this allows them to replicate in resting cells such as neurons. Reactivation triggers replication and recurrent or continuous infection (White & Fenner, 1994). The herpes simplex virus (HSV) has an affinity for mucous membranes, the skin and the nervous system. It may be spread by respiratory droplets, through direct contact with lesions or contact with virus containing fluids such as saliva or cervical secretions (Habif et al., 2001). There are three stages of infection; primary infection, maintenance and reactivation of the virus (Marques & Straus, 2000). Infection with HSV in immunocompromised patients

Figure 4.2: Schematic of a general plaque reduction assay. Sourced from Chattopadhyay et al., 2009.
causes serious concern due to a potential for resistance and lethality (Mukhtar et al., 2008).

4.3 Determining antimicrobial activity of B. frutescens and C. edulis

Extracts were dissolved in Mueller-Hinton broth and sonicated for 10 minutes followed by filtration sterilisation with a 0.22 µm syringe filter. Chloramphenicol palmitate 0.5 mg/ml (Sigma Chemicals Co., USA) and amoxicillin 1 mg/ml (Sigma Chemicals Co., USA), the positive controls, were dissolved in Mueller-Hinton broth (Sigma-Aldrich, St Louis, USA) and sterilised by filtration through a 0.22 µm syringe filter. Bacteria void of both the antibiotics and extracts were used as the negative controls.

All equipment, broth and agar were sterilised using moist heat at 121 °C for 21 minutes prior to use. Equipment which could not be sterilised such as syringes and filters were purchased sterile.

4.3.1 Methodology of the antibacterial assay

4.3.1.1 Microorganisms used in the assay

When evaluating antibacterial properties of agents, their activity against Gram negative and Gram positive microorganisms is determined. Bacillus subtilis, Pseudomonas aeruginosa (ATCC No. 27853), Staphylococcus aureus (ATCC No. 43300) and Escherichia coli (ATCC No. 38218) are the bacteria against which the plant extracts would be tested and were obtained from the Department of Biochemistry and Microbiology at NMMU.

Bacillus subtilis is a ubiquitous, rod shaped, Gram positive, spore forming bacterium that is often recovered from the soil, water and decomposing plant residue. It has low virulence in humans, however, a number of cases of infection with the bacterium have been reported (US Environmental Protection Agency., 1997). Most of the cases
reported are in patients who were immunocompromised or drug abusers (De Boer Sietske & Diderichsen, 1991).

*Pseudomonas aeruginosa* is an opportunistic Gram negative bacillus which has a ubiquitous occurrence in the environment with hospitals harbouring multidrug resistant strains. Nearly all reported cases of human infection with *P. aeruginosa* are in patients whose immune defences are compromised with the majority of infections occurring in patients with acquired immune deficiency syndrome, neutropenic patients undergoing chemotherapy, cystic fibrosis patients (causing chronic lung infection), severely burnt patients (causing bacteraemia) and users of soft contact lenses (resulting in acute ulcerative keratitis) (Lyczaka *et al.*, 2000). This microorganism is the third most prevalent microorganism that is associated with nosocomial urinary tract infections resulting from the use of catheters (Mittal *et al.*, 2009).

*Staphylococcus aureus* has been associated with surgical site infection, the infecting microorganism originating from either health care workers or from patients who are carriers of the microorganism (Crusz *et al.*, 2014). Other infections caused by the microorganism include skin and soft tissue infections, pneumonia, and bone and joint infections. Of great concern is infection with methicillin-resistant *S. aureus*, which whilst not adversely affecting the outcome of patients infected with *S. aureus* in the general population, resulted in higher mortality in patients with cancer, renal disease and those with *S. aureus* bacteraemia (Kang *et al.*, 2010; Ray *et al.*, 2013).

*Escherichia coli*, a Gram negative bacillus, is part of the normal intestinal flora of humans and animals where it causes no harm. In other parts of the body it can cause diseases such as urinary tract infections, bacteraemia and meningitis; enteropathogenic strains can cause acute diarrhoea (WHO, 2011; Fan *et al.*, 2013). It is also responsible for both community and nosocomial infections, especially those of the blood stream and urinary tract and the emergence of extended spectrum β-lactamase producing *E. coli* is of great concern (Rodriguez-Siek *et al.*, 2005; Wua *et al.*, 2010; Goulenok *et al.*, 2013).
4.3.1.2 Resuscitating the bacteria

A loop full of bacteria were transferred to 10 ml of sterile Mueller-Hinton broth and incubated overnight in an orbital shaker (Labcon Incubator Labotec (Pty) Ltd, SA) at 100 rpm at 37 °C. One hundred microlitres of the bacterial suspension was then transferred to 10 ml of Mueller-Hinton broth and further incubated in an orbital shaker at 37 °C at 100 rpm for 18 hours. The optical density of the bacterial suspension was read at 550 nm and Mueller-Hinton broth was used as the blank. The quantity required to make 10 ml of a suspension with an optical density of 0.2 was calculated using the equation below.

\[
\text{Suspension required} = 0.22 \times 10 \text{ ml} / \text{optical density of bacterial suspension}
\]

The volumes of each bacterial suspension used to make 10 ml of the adjusted suspension are indicated in Table 4.2.

**Table 4.2: Bacterial suspension volumes used to adjust turbidity to 0.2 at 550 nm**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Optical density obtained</th>
<th>Volume utilised (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.734</td>
<td>2.74</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1.382</td>
<td>1.52</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0.929</td>
<td>2.20</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1.061</td>
<td>1.95</td>
</tr>
</tbody>
</table>

4.3.1.3 *B. frutescens and C. edulis* extract concentrations used in the study

The assay used to determine antibacterial properties was a modified microplate method (Eloff, 1998a). The extracts and bacterial suspensions were pipetted into wells of a sterile 96 well plate such that the final concentrations listed in Table 4.3 were achieved. The plates were incubated for 18 hours in an orbital shaker at 100 rpm at 37 °C. The optical density of the wells was read using a multiwell plate reader (Multiscan MS® version 4.0 Labsystem® type 352) at 550 nm (Langfield et al., 2004; Mills-Robertson et al., 2012).
Table 4.3: Final extract concentrations used in the antimicrobial assay

<table>
<thead>
<tr>
<th></th>
<th>Individual extract concentration (mg/ml)</th>
<th>Concentration in the combined extract (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. edulis</em></td>
<td>0.225; 0.3; 0.375; 0.5 and 0.625</td>
<td>0.225; 0.3 and 0.375</td>
</tr>
<tr>
<td><em>B. frutescens</em></td>
<td>0.15; 0.2; 0.25; 0.375; 0.5 and 0.625</td>
<td>0.15; 0.2 and 0.25</td>
</tr>
</tbody>
</table>

Fifty microlitres of p-iodonitrotetrazolium chloride (INT) at 2 mg/ml was added to each well and the plates were again incubated in an orbital shaker at 100 rpm at 37 °C for two hours. The final optical density of the wells was again measured at 550 nm. The use of INT salts is based on the reduction of INT, a colourless solution by biologically active cells to INT formazan, a red, water insoluble deposit (Jeffrey & Paul, 1986). No solubilisation is necessary when INT is used as the colorimetric indicator (Grare *et al.*, 2008). All experiments were conducted in triplicate. (Felhaber, 1997)

4.3.2 Methodology of the antiviral assay

Facilities at the NMMU did not allow for the safe and reproducible testing of the extracts antiviral properties. The antiviral assay was therefore outsourced to VIRAPUR laboratories in the United States of America; cytotoxicity studies were however, conducted at NMMU laboratories.

The Vero cell line which is derived from the African green monkey was used as the host cell, it is extensively used for plaque reduction assays (Sheets, 2000). When conducting antiviral assays, extract concentrations which are not toxic to the host cells should be used (Gebre-Mariam *et al.*, 2006). The cytotoxicity of *B. frutescens*, *C. edulis* and the combined extracts to Vero cells at the concentrations used in the antiviral assay was tested and reported in section 3.5.1.

Antiviral properties of *B. frutescens*, *C. edulis* and a 1:1 combination of *B. frutescens* and *C. edulis* were determined against Human Herpes Simplex Virus Type 1 (HSV-1) Strain KOS using the plaque reduction assay. The extracts were initially solubilised in 50 µl of DMSO and then made to a stock concentration of 100 mg/ml with tissue culture media consisting of Dulbecco’s Modified Eagles medium (HyClone) with 2%
fetal bovine serum (HyClone, Central American Sourced) and antibiotic (Mediatech Antibiotic Antimycotic Solution). The assay was conducted at the following concentrations: 250, 125, 62.5, 31.25, 15.6 and 7.8 μg/ml. Cidofovir at 0.32, 3.2 and 32 μg/ml was used as the positive control. Cidofovir is an acyclic monophosphate nucleotide analogue; it has a broad spectrum antiviral activity against cytomegalovirus, herpes viruses, pox viruses and papilloma viruses. It acts by competitively inhibiting the incorporation of deoxycytidine triphosphate into viral DNA by viral DNA polymerase (Cundy, 1999). Concentrations were maintained throughout the virus absorption and the two day infection period.

Six well plates were seeded with Vero cells, the extracts and cidofovir were then added the next day resulting in the concentrations given above. Approximately 50 – 100 HSV-1 plaque forming units (VIRAPUR Lot # A1212A1) were added to each well after one hour of incubation. The virus was allowed to adsorb onto the cells for 2 hours and then removed, agarose solution at the required concentrations was then added to appropriately infected cells. The plates were then incubated at 37 °C for 48 hours. Cultures were fixed and stained with methanol and crystal violet after incubation to visualise plaques which were counted and whose numbers were recorded. Experiments were conducted in duplicate.

The HSV-1 KOS stock contains two plaque morphologies which do not clone out from each other on dilution cloning. Large plaques which are syncytial, some of which have clear centres and some centres are dark can be observed. Smaller non-syncytial plaques with clear centres are also observable.

4.3.3 Statistical analysis of the results

Results of replicate experiments are reported, antibacterial experiments were conducted in triplicate and antiviral experiments in duplicate. Inhibition of bacterial and viral proliferation was calculated as:

\[
\% \text{ inhibition} = \left\{ \frac{(\text{control} - \text{sample reading})}{\text{control}} \right\} \times 100
\]
A one way analysis of variance (ANOVA) using the Bonferroni post-test and the two-tailed, unpaired t-test were used to analyse the results using the GraphPad Prism 6® software program (GraphPad Software, San Diego, California). The purpose was to determine if statistically significant differences in antimicrobial activity existed; statistical difference was defined as p < 0.05.

4.4 Results and discussion

4.4.1 Antibacterial activity of *B. frutescens* and *C. edulis*

The results for the antimicrobial activity of the extracts are presented from Table 4.4 to Table 4.7. No appreciable antibacterial activity was obtained for any of the extracts as none of them produced more than 60% inhibition of all microorganisms used in the assays alone or in combination.

*B. frutescens* had greater antibacterial activity against Gram positive vs Gram negative bacteria. The greater sensitivity of Gram positive bacteria correlates with the findings of Coopoosamy (2011). In that study, aqueous leaf extracts were found to have activity against *B. subtilis* with a minimum inhibitory concentration of 2 mg/ml, no activity was noted against *S. aureus* and *E. coli*. Minimum inhibitory concentrations of *B. frutescens* were not determined in this study, however, based on the results obtained from the highest concentration studied, 0.625 mg/ml, from which a 40.14% inhibition was obtained, it should be reasonable to expect that MIC would also be above 1 mg/ml. Rabe and van Staden (1997) failed to find antibacterial activity of aqueous and methanol extracts of *B. frutescens* against *S. aureus*, *E. coli* and *B. subtilis*. 
Table 4.4: Antibacterial activity of *B. frutescens*. The best activity was obtained against *S. aureus* at 0.625 mg/ml.

<table>
<thead>
<tr>
<th></th>
<th>0.15 mg/ml</th>
<th>0.2 mg/ml</th>
<th>0.25 mg/ml</th>
<th>0.375 mg/ml</th>
<th>0.5 mg/ml</th>
<th>0.625 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>29.25</td>
<td>31.88</td>
<td>32.50</td>
<td>34.01</td>
<td>35.91</td>
<td>37.25</td>
</tr>
<tr>
<td>Std. Error</td>
<td>11.29</td>
<td>1.08</td>
<td>7.31</td>
<td>0.66</td>
<td>1.94</td>
<td>7.80</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>26.70</td>
<td>28.95</td>
<td>29.89</td>
<td>31.93</td>
<td>32.37</td>
<td>34.32</td>
</tr>
<tr>
<td>Std. Error</td>
<td>8.43</td>
<td>4.09</td>
<td>5.94</td>
<td>2.03</td>
<td>1.28</td>
<td>6.10</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>31.02</td>
<td>30.22</td>
<td>34.95</td>
<td>34.11</td>
<td>35.77</td>
<td>40.78</td>
</tr>
<tr>
<td>Std. Error</td>
<td>13.20</td>
<td>7.95</td>
<td>13.44</td>
<td>0.94</td>
<td>1.39</td>
<td>8.80</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>32.81</td>
<td>32.42</td>
<td>32.60</td>
<td>35.78</td>
<td>37.48</td>
<td>40.14</td>
</tr>
<tr>
<td>Std. Error</td>
<td>9.39</td>
<td>7.38</td>
<td>13.46</td>
<td>1.20</td>
<td>2.84</td>
<td>11.11</td>
</tr>
</tbody>
</table>

The differences in antibacterial activity of aqueous leaf extracts of *B. frutescens* could be due to differences in sample preparation. In the other studies, the plants were dried either in the sun or in an oven before solvent extraction whereas the juice of the fresh plant was freeze dried in this study. Sample preparation also differed amongst the three studies (Rabe & Van Staden, 1997; Coopoosamy, 2011). It is also possible that the locations from where these plants were harvested and the seasons in which they were harvested contributed to differences in plant constituents and thus antibacterial activity.

The antibacterial activity of *C. edulis* has been investigated by a number of researchers. Van der Watt and Pretorius (2001) demonstrated that fractions of the plant had activity against amongst others *B. subtilis*, *S. aureus*, and *E. coli* using the disk diffusion method which does not provide for quantitative analysis. Chokoe and colleagues (2008) quantified the antibacterial activity and determined minimum inhibitory concentrations (MIC) of the plant against *Enterococcus faecalis*, *S. aureus*, *P. aeruginosa*, and *E. coli*. Minimum inhibitory concentrations ranged from 0.16 mg/ml to 2.5 mg/ml depending on the season in which the plant was harvested and the solvent used for extraction (Chokoe *et al.*, 2008).

The good antibacterial activity against *S. aureus*, *P aeruginosa* and *E. coli* were confirmed by others (Ibtissem *et al.*, 2012). In addition, Martins and colleagues (2011)
found that compounds extracted and purified from *C. edulis* were effective against methicillin resistant *S. aureus*.

**Table 4.5:** Antibacterial activity of *C. edulis*. highest inhibition was against *S. aureus* 0.625 mg/ml yielding 48.49% proliferation inhibition.

<table>
<thead>
<tr>
<th></th>
<th>0.225 mg/ml</th>
<th>0.3 mg/ml</th>
<th>0.375 mg/ml</th>
<th>0.5 mg/ml</th>
<th>0.625 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>27.88</td>
<td>28.77</td>
<td>28.98</td>
<td>30.01</td>
<td>32.11</td>
</tr>
<tr>
<td>Std. Error</td>
<td>3.74</td>
<td>1.46</td>
<td>4.57</td>
<td>1.11</td>
<td>1.29</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>29.58</td>
<td>29.75</td>
<td>31.30</td>
<td>33.86</td>
<td>35.04</td>
</tr>
<tr>
<td>Std. Error</td>
<td>5.40</td>
<td>4.29</td>
<td>3.63</td>
<td>9.62</td>
<td>3.26</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>38.24</td>
<td>44.20</td>
<td>45.74</td>
<td>46.95</td>
<td>48.49</td>
</tr>
<tr>
<td>Std. Error</td>
<td>6.57</td>
<td>6.47</td>
<td>7.51</td>
<td>4.08</td>
<td>16.33</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>26.99</td>
<td>28.86</td>
<td>32.50</td>
<td>34.76</td>
<td>37.02</td>
</tr>
<tr>
<td>Std. Error</td>
<td>7.88</td>
<td>8.34</td>
<td>8.01</td>
<td>7.04</td>
<td>10.85</td>
</tr>
</tbody>
</table>

The MIC values were not determined in this study, however, the concentrations studied are within the MIC range obtained by Chokoe and colleagues (2008). No complete inhibition of microbial proliferation was obtained, the highest inhibition was obtained against *S. aureus* at a concentration of 0.625 mg/ml which yielded a 48.49% inhibition of microbial proliferation. It should be noted however, that all three studies differed in their methods of sample preparation and extraction. The similarities and differences in antibacterial activity of *C. edulis* may also be influenced by growing, harvesting and extraction methods.

Using the extracts in combination did not improve the degree of antibacterial activity, the highest inhibition obtained was at 0.625 mg/ml against B. subtilis which yielded a 52.28% inhibition of proliferation.

Possible synergistic activity of the extracts was not determined as 50% inhibition of antimicrobial growth was only obtained with the combined extracts at 0.625 mg/ml against *B. subtilis*, thus IC₅₀ could not be calculated. No statistical difference in the antimicrobial activity of all extracts whether used as single agents or when used in combination was obtained.
Table 4.6: Antibacterial activity of the combined *B. frutescens* and *C. edulis*. *B. subtilis* was most sensitive at 0.625 mg/ml of extract yielding 52.28% growth inhibition.

<table>
<thead>
<tr>
<th></th>
<th>0.375 mg/ml</th>
<th>0.5 mg/ml</th>
<th>0.625 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>24.51</td>
<td>28.87</td>
<td>33.27</td>
</tr>
<tr>
<td>Std. Error</td>
<td>11.23</td>
<td>9.14</td>
<td>5.78</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>23.77</td>
<td>28.13</td>
<td>31.70</td>
</tr>
<tr>
<td>Std. Error</td>
<td>5.46</td>
<td>1.17</td>
<td>5.55</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>37.03</td>
<td>39.65</td>
<td>40.39</td>
</tr>
<tr>
<td>Std. Error</td>
<td>4.93</td>
<td>7.27</td>
<td>3.75</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>43.10</td>
<td>48.49</td>
<td>52.28</td>
</tr>
<tr>
<td>Std. Error</td>
<td>9.65</td>
<td>6.36</td>
<td>8.66</td>
</tr>
</tbody>
</table>

4.4.2 Antiviral activity of *B. frutescens* and *C. edulis*

When using direct assays to determine antiviral activity of medicinal plants, a 50% or more reduction in viral titre of treated cells when compared to untreated cells indicates antiviral activity. In addition to this, antiviral activity at two subsequent dilutions is required for antiviral activity to be considered relevant (Chattopadhyay et al., 2009). *C. edulis* was inhibitory against HSV-1 at concentrations of 250, 125 and 62.5 μg/ml. *B. frutescens* exhibited no activity against the virus as indicated in Figure 4.3. The combined extracts were active at 250 μg/ml. The antiviral activity of the combined extracts can be attributed to that of *C. edulis* which was inhibitory against the virus at 250 μg/ml and 125 μg/ml, and is present at 125 μg/ml in the 250 μg/ml combined extract. The loss of antiviral activity in the combined extracts at concentrations less than 250 μg/ml coincides with a reduction in the activity of *C. edulis* at 62.5 μg/ml and below.

The antiviral activity is not due to cytotoxicity of the extracts towards Vero cells as no cytotoxicity was observed during cytotoxicity studies and when the cells were visually inspected during the antiviral assay. Catechin, which is present in *C. edulis* leaves has been demonstrated to be active against HSV-1 (Kaul et al., 1985). It alone or in combination with other compounds within the extract possesses antiviral activity. Antiviral activity against HSV-1 was thus obtained only for *C. edulis*, subsequent
concentrations of the combined extracts did not demonstrate antiviral activity and thus failed to meet the required criteria.

**Figure 4.3:** Antiviral activity of *B. frutescens*, *C. edulis* and the combined extracts.

The IC$_{50}$ value for *C. edulis* was 44.67 µg/ml, this extract is thus a strong candidate for further evaluation of antiviral activity and the development of antiviral products as an IC$_{50}$ value below 100 µg/ml is considered a stringent endpoint for extracts (Cos *et al.*, 2006).

### 4.5 Conclusion

Aqueous extracts of *B. frutescens* and *C. edulis* did not demonstrate significant antimicrobial activity. These findings do not differ greatly from those obtained by other researchers who studied the antibacterial properties of the two plants. Some of the differences noted in results could be as a result of growing, harvesting, extraction and testing methods. Combining *B. frutescens* and *C. edulis* aqueous extracts did not increase or decrease antibacterial activity against the organisms used in this study.

The use of *C. edulis* aqueous extracts in the treatment of HSV-1 infections is thus validated.
5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.1 HPLC method development and validation

Medicinal products produced for human consumption should adhere to quality attributes that are laid out by regulatory authorities with which they are registered. African traditional medicines sold in South Africa are not currently registered with the MCC and are thus not required to meet the stringent safety, efficacy and reliability requirements that are laid out for registered medicines. A consequence of this is that proof of efficacy of ATMs is not scientifically demonstrated leading to the challenges discussed in Chapter 2. Another consequence is that the quality of manufacture of ATMs cannot be assured as adherence to validations and current Good Manufacturing Practices (cGMP) are not a requirement for marketing and sale of such medicines.

There is a concerted effort by organisations such as the WHO and the South African Government through the MCC to correct this phenomenon. Therefore, along with professionalisation of the providers of ATM i.e. African Traditional Healers there is a drive to register ATM with the MCC thus improving conditions under which they are manufactured and marketed. In order to meet conditions required for the production of quality medicines as laid out for other registered medicines, efficacy, toxicity and clinical studies need to be conducted, the methodology depending on the route of administration and the time over which these traditional medicines have been used. Standards and specifications need to be set; along with development of testing methods. Among these are tests for starting materials, especially the plant and plant extracts which are the active constituents of the products. The WHO has set guidelines for the testing and method development for herbal products which are intended to be used by regulatory authorities for traditional medicines they intend to register (WHO, 2000).
The reproducible production of reliable, safe and effective medicines is based on pillars which include amongst others current good manufacturing practices and quality assurance of which quality control is an integral part. Chromatography is one of the most widely used quality control methods for plant derived raw materials. This is because it can provide information on the quality of the extracts in question along with the quantity of selected chemical constituents within the extract (Fennell et al., 2004).

The stability, quality and identity of pharmaceutical products is ascertained through stability indicating assays which may be spectroscopic or chromatographic. Chromatography and specifically high performance liquid chromatography is extensively used due to its simplicity, specificity, low cost and abundance of equipment (Giri et al., 2010). When stability indicating assays are developed, it is a requirement that the quantity and quality of the API is specified that and its degradation products are identified and quantified even in the presence of excipients (Bakshi & Singh, 2002). Accuracy, precision, robustness, range, linearity and specificity are determined when such assays are being validated. Guidelines for determining these properties are provided by the ICH, FDA and MCC in various guidance documents, these are however not detailed to an extent that provides specific methodologies. It is through the perusal and interrogation of the guidelines, together with literature published on the development of stability indicating assays that information on where to start and how to conduct the necessary tests is obtained. During the pre-clinical stages of development, emphasis is given to specificity, linearity, accuracy and precision studies. The other validation stages are usually conducted along with phase III clinical studies for novel products (Putheti et al., 2008). The ensuing discussion will focus on high performance liquid chromatography as it is the method used for stability determination and quality control in this study.
5.2 HPLC and medicinal plants

The stability and quality control of pharmaceutical APIs using HPLC is based on the chromatogram that is produced by each API; quantitative and qualitative determinations can then be made from it (Lazarowych & Pekos, 1998). The fingerprint or chromatogram produced by single chemical entities and that produced in the presence of degradation products and excipients is used to assess stability over a period of time and for quality control during manufacture of the products (Bakshi & Singh, 2002). Stability studies and quality control have assured the quality of pharmaceutical products over an extended period of time and as such, similar quality control measures should ideally be used when other medicinal products, specifically plant derived medicinal products are produced.

Developing and validating stability indicating HPLC assays for herbal products however presents challenges that are not encountered when assaying for a single chemical pharmaceutical API which tend to be pure.

Firstly, the active constituents for most herbal products are unknown; they have thus not been isolated and purified in order to obtain simple to use single entity chemical compounds. It should also be remembered that the multiple constituents of plants from which extracts are obtained are believed to function synergistically, thus if active constituents were indeed identified, they too could be complex if activity similar to that of the whole plant extract is to be preserved. Secondly, there are multiple constituents that will be eluted when a chromatogram of the plant extract is produced. Selecting which of these will be used for quality control, identification and stability assessment can be challenging (Lazarowych & Pekos, 1998; Giri et al., 2010). Finally, the quantity of chemical constituents within plant extracts varies depending on the environmental conditions encountered during growth, harvesting and post harvesting handling. The chromatogram produced may also vary depending on the extraction method and solvents used during assay (Therapeutic Goods Administration, 2011).
HPLC method development and validation for plant derived products needs to be adapted to the abovementioned challenges while it endeavours to adhere to the principles and guidelines set out for single entity pharmaceutical products. Compounds which are known to be present in the plant extract and which are available as standards are used as marker compounds, these should preferably be unique to the plant and the least stable marker should be used. Markers are defined by the European Medicines Agency (EMA) (2009) as “chemically defined constituents or groups of constituents of a herbal substance, a herbal preparation or a herbal medicinal product which are of interest for control purposes independent of whether they have any therapeutic activity. Markers serve to calculate the quantity of herbal substance(s) or herbal preparation(s) in the Herbal Medicinal product if that marker has been quantitatively determined in the herbal substance(s) or herbal preparation(s) themselves” (European Medicines Agency, 2009). Presence of the marker compound in the plant can be confirmed by literature or be shown when the marker co-elutes with a compound within the plant extract. Identification of the co-eluting constituents can be done through ultraviolet (UV) analysis or mass spectrometry. The limitation of using marker compounds as sole determinants of quality is that the developer is not certain that the marker is specific only to that plant extract. Selecting a few peaks for quality control may fail to detect changes to constituents in the chromatogram which have not been selected as markers for quality control (Lazarowych & Pekos, 1998; S. P. Li et al., 2011).

5.3 Data analysis for selectivity and specificity

Principal component analysis (PCA), an unstructured, multivariate, dimension reducing method is used in the grouping of HPLC fingerprints obtained from medicinal plant extracts. It is popular because it is a simple, non-parametric method which can reveal characteristic data structures and provide reduced dimension representation of the original data and does this without losing qualitative and quantitative information of the
original data set (Nyamundanda et al., 2010). Data manipulation prior to analysis with PCA is paramount; pre-treatment through normalisation is advantageous in that it does not create negative baseline shifts and noise enhancement when analysing chromatographic data (Mecozzi et al., 2012). Although advantageous for the analysis of chromatographic data, PCA has limitations, these include the fact that it does not have an associated probabilistic model, thus assessing the fit of PCA to the data is difficult. The methods ability to deal with missing data is also problematic necessitating manipulation of data produced by chromatograms.

Once extracts have been grouped using PCA, Hierarchical Cluster Analysis (HCA) can be further used to analyse and group the data. Hierarchical Cluster Analysis is also a multivariate analysis technique which is used to classify samples according to groups (Wu et al., 2013). The number of groups need not be known prior to the analysis and clusters can have subgroups within them which in turn can also have subgroups, that is useful in:

- Developing typology or classification
- Investigating conceptual schemes for grouping entities
- Generation of hypothesis through data exploration and
- Hypothesis testing i.e. attempting to determine whether classifications defined using other methods are in fact present (Aldenderfer & Blashfield, 1984; Beckstead, 2002).

The method is extensively used in the analysis of chromatographic fingerprint data which provides visual representation of the data. HCA however does not allow for the determination of statistical weighing of variables and does not identify variables which account for similarity or dissimilarity among samples (Wu et al., 2013).
5.4 Method development for *B. frutescens* and *C. edulis*

One of the aims of this project was to produce a herbal cream. A method for quality control of the medicinal plant extracts for the herbal cream needed to be developed in order to meet quality requirements as discussed in section 5.1. In developing and validating the HPLC method for the two plant extracts, both qualitative and quantitative methods were used. Qualitative methods were used to establish specificity and selectivity of the method for the plant extracts. Standards and markers were utilised when quantitative methods were used during the validation. These methods are used in conjunction to offer greater accuracy.

5.4.1 HPLC method development

One of the aims of this project was to formulate a topical herbal cream. In order to meet quality requirements as stated above, one of the tests that would have to be developed is one for quality control of the active ingredients i.e. the plant extracts. This method would also be used for the quality control of the formulated product.

The initial conditions for the analysis of the plant extracts were obtained from the research done by Springfield and Weitz (2006) where in *Carpobrotus mellei* was analysed using a gradient method with methanol and 5% acetic acid (SMM instruments, Vorna Valley) as the mobile phases.

*C. edulis* and *B. frutescens* were prepared for analysis by weighing 0.01 g of each extract and dissolving them separately in 4 ml methanol:5% acetic acid (20:80). The sample solvent was matched with the mobile phase at the start of the gradient run to ensure that peaks which were not abnormally wide or misshapen were obtained and to eliminate the occurrence of ghost peaks especially since the organic phase was methanol (Egi & Euyanagi, 1998; Bruno, 1999). After sonicating for 15 minutes they were allowed to return to room temperature, made up to 5 ml with the sample solvent and then filtered through a 0.45 µm membrane filter. After this, they were analysed using a Luna C$_{18}$ 250 x 4.6 mm column with a 5 µm internal diameter (Phenomenex,
Torrance, USA) and a guard column (Phenomenex, Torrance, USA) on a LC2020 HPLC system including a binary pump and a photodiode array detector and fitted with LC Solutions software (Shimadzu, Tokyo, Japan). The column temperature was 35 °C, the injection volume was 10 μl and the flow rate was 0.6 ml/min. The mobile phases were 5% acetic acid (A) and methanol (B) and the detection wavelengths were 254, 270, 300 and 360 nm. The gradient method was conducted over 52 minutes with the organic solvent changing from 20% to 90% over 52 minutes as indicated in Table 5.1 below.

Table 5.1: HPLC gradient method 1.

<table>
<thead>
<tr>
<th>Change in methanol concentration (%)</th>
<th>Change in time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0 - 1</td>
</tr>
<tr>
<td>20 – 40</td>
<td>1 - 10</td>
</tr>
<tr>
<td>40</td>
<td>10 - 20</td>
</tr>
<tr>
<td>40 – 45</td>
<td>20 - 31</td>
</tr>
<tr>
<td>45 – 50</td>
<td>31 - 34</td>
</tr>
<tr>
<td>50</td>
<td>34 - 36</td>
</tr>
<tr>
<td>50 – 90</td>
<td>36 - 39</td>
</tr>
<tr>
<td>90</td>
<td>39 - 52</td>
</tr>
</tbody>
</table>

The wavelength that produced the best result was 270 nm. Chromatograms were viewed at the other wavelengths throughout method development, however, 270 nm consistently produced the best chromatograms, and thus all chromatograms shown in this document were read at 270 nm.

The method did not produce good results which are labelled Figures 5.1 and 5.2. It also became evident on successive runs that there was retention of compounds on the column and these were interfering with subsequent analyses.
Figure 5.1: Chromatogram of *B. frutescens* produced by HPLC gradient method 1.

In attempting to improve the results and remove adhering compounds the solvent used for sample preparation was changed to methanol:5% acetic acid (40:60). In addition, methanol alone was used as another sample solvent to see if this would improve the quality of chromatograms obtained. A period was introduced after the analysis wherein 90% methanol was run through the column for 30 minutes to remove bound constituents. Changes made to the gradient run are shown in Table 5.2. The time allocated to lower methanol concentrations was increased to improve separation of the
compounds because of the congested peaks that were being produced in the first 10 minutes of the analysis. Fewer peaks were present between 22 minutes and 36 minutes which coincided with 40% - 45% of the organic phase thus the time spent at these concentrations was shortened resulting in a reduction of the gradient method from 52 minutes to 30 minutes.

**Table 5.2:** HPLC gradient method 2.

<table>
<thead>
<tr>
<th>Change in methanol concentration (%)</th>
<th>Change in time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 – 20</td>
<td>0 - 5</td>
</tr>
<tr>
<td>20</td>
<td>5 - 15</td>
</tr>
<tr>
<td>20 – 45</td>
<td>15 - 18</td>
</tr>
<tr>
<td>45 – 50</td>
<td>18 - 22</td>
</tr>
<tr>
<td>50 – 90</td>
<td>22 - 29</td>
</tr>
<tr>
<td>90</td>
<td>29 - 30</td>
</tr>
</tbody>
</table>

Figures 5.3 to 5.6 below show that the changes made produced chromatograms which were an improvement on those obtained earlier, they were however, also not acceptable. The problem of sample retention on the column and elution with later runs continued to be experienced.

**Figure 5.3:** Chromatogram of *B. frutescens* dissolved in methanol:5% acetic acid (40:60), produced by HPLC gradient method 2.
**Figure 5.4:** Chromatogram of *B. frutescens* prepared with methanol, produced by HPLC gradient method 2.

**Figure 5.5:** Chromatogram of *C. edulis* dissolved in methanol:5% acetic acid (40:60), produced by HPLC gradient method 2.
Figure 5.6: Chromatogram of *C. edulis* prepared with methanol, produced by HPLC gradient method 2.

Since the sample preparation method and organic solvent used were not producing the required results, acetonitrile was used instead of methanol; the ratio of organic to aqueous solvent remained at 40:60. The gradient method was again changed to increase the time in which the organic solvent was being passed through the column shown in Table 5.3. Other changes included increasing the flow rate to 1 ml/min and the column to temperature 40 °C. The column was flushed with 100% acetonitrile for 60 minutes in-between analyses.

Table 5.3: HPLC gradient method 3.

<table>
<thead>
<tr>
<th>Change in acetonitrile concentration (%)</th>
<th>Change in time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0 - 4</td>
</tr>
<tr>
<td>10 - 20</td>
<td>4 - 13</td>
</tr>
<tr>
<td>20</td>
<td>13 - 23</td>
</tr>
<tr>
<td>20 – 45</td>
<td>23 - 28</td>
</tr>
<tr>
<td>45 – 55</td>
<td>28 - 33</td>
</tr>
<tr>
<td>55</td>
<td>33 - 36</td>
</tr>
<tr>
<td>55 - 70</td>
<td>36 - 40</td>
</tr>
<tr>
<td>70 – 90</td>
<td>40 - 44</td>
</tr>
</tbody>
</table>
The chromatograms obtained, shown in Figures 5.7 and 5.8, were greatly improved along with the compound retention problem. The majority of the compounds were however, still eluting within the first 10 minutes producing congested chromatograms.

**Figure 5.7**: Chromatogram of *B. frutescens* obtained using HPLC gradient method 3.

In order to resolve this, HPLC gradient method 3 was inverted as shown by Table 5.4. The sample solvent remained acetonitrile:5% acetic acid (40:60) and the period of flushing with 100% acetonitrile was retained.

**Figure 5.8**: Chromatogram of *C. edulis* obtained using HPLC gradient method 3.
Table 5.4: HPLC gradient method 4.

<table>
<thead>
<tr>
<th>Change in acetonitrile concentration (%)</th>
<th>Change in time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>0 - 5</td>
</tr>
<tr>
<td>90 - 70</td>
<td>5 - 9</td>
</tr>
<tr>
<td>70 - 55</td>
<td>9 - 14</td>
</tr>
<tr>
<td>55 – 45</td>
<td>14 - 18</td>
</tr>
<tr>
<td>45 – 30</td>
<td>18 - 22</td>
</tr>
<tr>
<td>30 – 20</td>
<td>22 - 27</td>
</tr>
<tr>
<td>20 -10</td>
<td>27 - 32</td>
</tr>
</tbody>
</table>

A satisfactory fingerprint was obtained with the *B. frutescens* extract indicated in Figure 5.9 below.

![Chromatogram](image)

**Figure 5.9:** Chromatogram of *B. frutescens* obtained using HPLC gradient method 4.

Obtaining a suitable result with *C. edulis* was however, difficult. When the extract was prepared for analysis using different solvents the chromatograms shown in Figure 5.10 were obtained. Chromatogram A was obtained by dissolving the extract in acetonitrile:5% acetic acid (40:60), sonicating for 15 minutes, filtering and analysing using HPLC gradient method 4. Chromatograms B and C were obtained by dissolving the extracts in ethyl acetate and chloroform respectively, sonicating for 15 minutes, filtering and analysing using HPLC gradient method 4.
When none of these produced a suitable chromatogram a new method of preparing *C. edulis* was undertaken. The extract was weighed and 1 ml of water added to it, this was then thoroughly shaken. Five ml of ethyl acetate was added to the slurry and mixed, it was then centrifuged (Eppendorf Centrifuge 5804R) at 3000 rpm for 10 minutes. The ethyl acetate layer was removed and evaporated using compressed air in a fume cupboard. The resultant dried substance was dissolved in 2 ml of acetonitrile:5% acetic acid (40:60) solvent, filtered through a 0.45 µm membrane filter and analysed using HPLC gradient method 4. The chromatogram obtained, shown in Figure 5.11, was satisfactory.

**5.4.1.1 Final conditions**

HPLC gradient method 4 was adopted as the method for analysis of the extracts in this study. *B. frutescens* was prepared for analysis by dissolving it in acetonitrile:5% acetic acid (40:60). Sample preparation for *C. edulis* included obtaining an ethyl acetate fraction which was then dried and dissolved in acetonitrile:5% acetic acid (40:60) prior to analysis. The amount of extract used in all analyses was standardised at 0.02 g.
5.5 Method validation

5.5.1 Standards

Rutin and catechin were used as standards for method validation as both have been identified compounds present in *C. edulis* (Springfield *et al.*, 2005). Sulfated phenyl anthraquinones and their analogues isoknipholone and sodium 4'-O-demethylknipholone 6'-O-sulfate and joziknipholones A and B have been isolated from the roots of *B. frutescens*, these were not available to the researcher as standards and as such their presence and use as suitable markers for *B. frutescens* leaf extracts could not be determined. The scarcity of suitable markers and standards for quality control of plant extracts is challenging, as an illustration, Li and colleagues (2011) report that a single marker is used to evaluate the quality of 154 herbs included in the Chinese Pharmacopoeia. Due to a lack of suitable slavianolic acid B reference substance, methylparaben was used in the quality evaluation of Radix Salviae Miltiorrhizae and Compound Danshen tablets (S. P. Li *et al.*, 2011). Although they have

![Figure 5.11: An acceptable chromatogram of *C. edulis* obtained using HPLC gradient method 4.](image-url)
not been identified in the leaves of *B. frutescens*, rutin and catechin were used as substitute markers.

Individual standards were prepared by dissolving 50 mg of each standard in 30 ml acetonitrile:5% acetic acid (40:60) and sonicating for 15 minutes. It was then allowed to return to room temperature and then made to 50 ml with acetonitrile:5% acetic acid (40:60). Five millilitres of the above was then made to 50 ml to make a 0.1 mg/ml stock solution.

5.5.2 Limit of detection and quantification

The limit of detection (LOD) and quantification (LOQ) were initially calculated from the calibration curve using Equation 5.1 and 5.2 for LOD and LOQ respectively (ICH, 2005).

\[
\text{Limit of detection} = \frac{3.3\delta}{S}
\]

**Equation 5.1:** Limit of detection

\[
\text{Limit of quantification} = \frac{10\delta}{S}
\]

**Equation 5.2:** Limit of quantification

where: \(\delta\) = the standard deviation of the slope

\(S\) = the slope of the calibration curve

The LOQ was then determined experimentally by analysing a concentration range in the vicinity of the theoretically determined LOQ. Concentrations of 0.25, 0.2, 0.100, 0.05, 0.025 and 0.0125 µg/ml were analysed in triplicate. The lowest quantifiable concentration would be the lowest concentration whose response has a relative standard deviation of less than 5%. The LOD was calculated as a third of the result of the LOQ.
5.5.3 Precision and Accuracy

Precision of the method was determined by analysing injections of five samples and determining the relative standard deviation of the responses which should be less than 2%. Accuracy was determined by performing triplicate injections of three standard concentrations and determining the concentration recovered by the assay. The relative deviation from the known concentration should be less than 2% for the method to be deemed accurate.

5.5.4 Linearity

Linearity was determined by constructing a calibration curve of the standards and determining the correlation coefficient of the regression line and the deviation of the y-intercept from zero. The concentrations used to generate the calibration curve were prepared from the 0.1 mg/ml stock solution. Concentrations of 1.0, 1.5, 2, 2.5, 3 and 3.5 µg/ml were prepared by taking 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 ml of the stock solution and making to 10 ml with acetonitrile:5% acetic acid (40:60). The standards were prepared in triplicate, filtered through a 0.45 µm filter and analysed using the HPLC gradient method.

5.5.5 Selectivity and specificity

Plants were harvested from different geographical areas in South Africa and analysed to determine selectivity and specificity. Both plants *C. edulis* and *B. frutescens* were harvested from Port Elizabeth (Eastern Cape), Joubertina (Eastern Cape), Ladysmith (KwaZulu-Natal) and QwaQwa (Free State). Sample preparation and analysis was carried out as described in section 5.4.1.

Plants from Joubertina were furthermore extracted differently to determine whether changes, if present, would be identified by the method developed. Leaves of *C. edulis* and *B. frutescens* were dried in an oven at 50 °C and ground to a powder using a mortar and pestle. The plants were extracted overnight by adding 10 ml of either methanol or acetone:water (50:50) to 10 g of plant material. The supernatant was evaporated using compressed air in a fume cupboard and the resulting extract was
prepared as described in section 5.4.1 and analysed using the HPLC gradient method 4.

To further test for selectivity and specificity, the Port Elizabeth extract was stressed by:

- exposing it to 115 °C for 20 minutes in an oven
- keeping it at 40 °C / 75% RH for 3 months in a stability chamber
- refluxing at 80 °C with 1N NaOH for 2 hours
- heating with 1M HCl at 80 °C for 2 hours

5.5.6 Analysis

Statistical analysis was conducted using Statistica 11.0 software (StatSoft Inc., Tulsa, OK, USA) and the mean chromatogram was generated using the program SpecAlign 2.4 (Jason Wong, Oxford, U.K.). Relative retention areas were used in all analyses. PCA along with HCA using Ward’s method as the amalgamation rule and Euclidean distances as the distances measured were used to analyse the data. Ward’s method groups clusters by minimising functions based on the sum of squared deviations between entities and the centres of each cluster and produces homogenous clusters with maximum distances (Beckstead, 2002; Ferreira & Hitchcock, 2009). Similarity among the extracts was also determined using correlation coefficients.

5.6 Results

5.6.1 Precision, accuracy and linearity

Figures 5.12 and 5.13 below compare B. frutescens and C. edulis chromatograms when 2.5 µg/ml of rutin and catechin standards were added to the plant extracts and when they were excluded. The standards co-eluted with compounds present in the plant extracts and proof of their presence was thus only demonstrated by an increase
in the area of peaks coinciding with the retention time of the standards, such retention
times being shown in Figure 5.12.

![Rutin and Catechin Peaks](image)

**Figure 5.12:** Rutin and catechin peaks.

That the standards co-elute with compounds present in *C. edulis* is expected since
both standards are known to be present in the plant. It is unknown which compounds
they are co-eluting with in *B. frutescens*.

![Chromatograms](image)

**Figure 5.13:** Chromatograms of the Port Elizabeth extract of *B. frutescens*. The extract
resulting in the pink chromatogram was spiked with the standards and the black one
was void of standards.

Port Elizabeth extracts were spiked with 1 ml of rutin and catechin at a concentration of
1.5 µg/ml and the quantity recovered determined. 3.4 µg/ml and 2.70 µg/ml of rutin
were recovered for *B. frutescens* and *C. edulis* respectively implying that 1.9 and 1.2
µg/ml was already present at that specific retention time prior to addition of the
standard. When unspiked Port Elizabeth extracts were analysed it was found that 2.31
74 µg/ml and 0.458 µg/ml were present in *B. frutescens* and *C. edulis* respectively. The discrepancies in the quantities added and recovered from the extracts could be caused by interactions between the numerous compounds in the extracts and the added standards. Whilst it is desirable to accurately recover added concentrations, the fact that they are not accurately recovered is not detrimental to the validation of this method as these will be used as external standards to quantitate constituents within the extracts. They will thus not interact with compounds distorting the contents.

![Figure 5.14](image-url): Chromatograms of the Port Elizabeth extract of *C. edulis*. The extract resulting in the pink chromatogram was spiked with the standards and the black one was void of standards.

The retention areas of catechin recovered for both *B. frutescens* and *C. edulis* were large and fell significantly beyond that which could be extrapolated to the calibration curve. The quantity recovered could thus not be calculated, similarly the retention areas of the unspiked extracts were so large preventing quantification using the constructed calibration curve.

The calibration curves for catechin and rutin are shown in the Figures 5.15 and 5.16 below. They both have a high linearity indicated by a correlation coefficient ($r^2$) ≥ 0.999. Deviation of the y-intercept from zero was further used to determine the linearity. The y-intercept needs to deviate by less than 2% from the 50% response to be acceptable. A 50% response was calculated based on a 0.3 µg/ml maximum concentration as
there was no 0.175 µg/ml concentration studied. It was calculated to deviate by 1.92% and 1.93% for catechin and rutin respectively.

**Figure 5.15**: Calibration curve for catechin (n = 3 for each point, \( y = 9350.571x + 11.380, r^2 = 0.9995 \)).

**Figure 5.16**: Calibration curve for rutin (n = 3 for each point, \( y = 286999.048x + 485.49, r^2 = 0.9990 \)).

The method was demonstrated to be accurate as the actual and measured concentrations did not deviate from each other by more than 2% for any of the concentrations as shown in Table 5.5.

The limit of detection is the lowest concentration within a sample that can be detected by a specific instrument but may not necessarily be quantitated under specific experimental conditions. The limit of quantification refers to the lowest concentration of the sample that can be determined with acceptable precision and accuracy (Van Iterson, 2005).
Table 5.5: Accuracy results for rutin and catechin.

<table>
<thead>
<tr>
<th>Actual concentration (µg/ml)</th>
<th>Rutin</th>
<th>Catechin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration (µg/ml)</td>
<td>Deviation (%)</td>
</tr>
<tr>
<td>1.000</td>
<td>1.016</td>
<td>1.610</td>
</tr>
<tr>
<td></td>
<td>1.009</td>
<td>0.869</td>
</tr>
<tr>
<td></td>
<td>1.012</td>
<td>1.232</td>
</tr>
<tr>
<td>1.500</td>
<td>1.481</td>
<td>1.912</td>
</tr>
<tr>
<td></td>
<td>1.512</td>
<td>1.211</td>
</tr>
<tr>
<td></td>
<td>1.506</td>
<td>0.629</td>
</tr>
<tr>
<td>3.500</td>
<td>3.520</td>
<td>2.000</td>
</tr>
<tr>
<td></td>
<td>3.517</td>
<td>1.747</td>
</tr>
<tr>
<td></td>
<td>3.520</td>
<td>1.972</td>
</tr>
</tbody>
</table>

The limit of quantification for catechin and rutin was 0.05 µg/ml (RSD = 2.57%) and 0.25 µg/ml (RSD = 4.81%) respectively. The theoretically determined LOQ’s were 0.0273 µg/ml and 0.206 µg/ml for catechin and rutin respectively which did not deviate greatly from those obtained experimentally. The limits of detection were calculated to be 0.017 µg/ml and 0.083 µg/ml respectively.

Precision was established since the relative standard deviation of the responses for both standards was less than 2% as shown in Table 5.6.

Table 5.6: Precision for rutin and catechin.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Response</th>
<th>Average response</th>
<th>Standard deviation</th>
<th>Relative standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>58923</td>
<td>58380</td>
<td>58160</td>
<td>58375</td>
</tr>
<tr>
<td>Catechin</td>
<td>23945</td>
<td>24449</td>
<td>23330</td>
<td>23815</td>
</tr>
</tbody>
</table>
5.6.2 Specificity and selectivity

Research methodology pertaining to specificity and selectivity of a HPLC assay for analysing plant extracts is not standardised, some researchers have opted to use standards to demonstrate these traits whilst others have used plant extracts. The same data analysis methods are used to group and determine similarity of extracts obtained from various geographic regions (Itharat & Sakpakdeejaroen, 2010; Zheng, 2011; Maji et al., 2012; Landim et al., 2013). Selectivity and specificity for this project were determined using plant extracts instead of standards because it is believed that the method should be selective and specific to the active ingredient which in this case is the plant extract.

To generate data for selectivity and specificity using plant extracts, data of chromatograms from plants harvested from different regions was obtained. A simulated chromatogram was then produced, either as a mean or median of the multiple extracts. There is again no guideline as whether one should compare against a median or a mean of the chromatograms, however, there was no difference in the simulated chromatogram generated by either the mean or median that was identified by Xie and colleagues (2006). A simulated chromatogram based on the mean of the results was used for this study.

Similarity of the plant extracts to each other is determined through a combination of analyses. One such determination is through the use of correlation coefficients. There is again great variation in the type of data used for comparison, some researchers use the whole chromatogram for comparison, whilst others select common peaks and use these for comparison. Whilst the use of common peaks is an improvement in the use of single standards for quality control, this method of analysis still has shortcomings in that changes to peaks not selected for analysis can be overlooked. There is also no standardised or accepted minimum or maximum number of peaks which should be common, as a result, the number of common peaks vary widely among researchers (Sun & Liu, 2007; Zhou et al., 2008; Xu et al., 2009; Li et al., 2010; Wu et al., 2011; Wu et al., 2013). Analysis were conducted using both whole chromatographic and common peaks in order to determine the accuracy of both methods.
The mean chromatogram was generated using data from the Ladysmith, QwaQwa, Joubertina aqueous and Port Elizabeth extracts. These extracts were created from leaves which were extracted, stored and generally manipulated in a similar manner. A chromatogram obtained from a 1:1 combination of *B. frutescens* and *C. edulis* extracts was included in the analysis to ascertain whether such combination would be deemed as different to the individual extracts by the method developed. All the chromatographic data was converted to relative retention data before analysis.

5.6.2.1 *B. frutescens* results

Similarity based purely on visual examination divided the extracts into two groups, one formed by the Joubertina acetone, Ladysmith and Port Elizabeth extracts and the other comprising of Joubertina methanol, Joubertina aqueous and QwaQwa extracts as seen in Figure 5.17. This classification of the extracts could also be made based on the PDA spectra shown in Figure 5.17 below.

![Comparison of chromatograms of B. frutescens: (A) Joubertina methanol, (B) Joubertina acetone, (C) Ladysmith, (D) Joubertina aqueous, (E) Port Elizabeth, (F) QwaQwa.](image)

**Figure 5.17:** Comparisons of chromatograms of *B. frutescens*: (A) Joubertina methanol, (B) Joubertina acetone, (C) Ladysmith, (D) Joubertina aqueous, (E) Port Elizabeth, (F) QwaQwa.
Figure 5.18: PDA spectra of *B. frutescens*.

Nine common peaks whose relative retention data is presented in Table 5.7 were identified.

Table 5.7: Relative retention areas of *B. frutescens* common peaks.

<table>
<thead>
<tr>
<th>Joubertina Methanol</th>
<th>Joubertina Acetone</th>
<th>Joubertina aqueous</th>
<th>QwaQwa</th>
<th>Ladysmith</th>
<th>Port Elizabeth</th>
<th>Combined extracts</th>
<th>Mean Chromatogram</th>
<th>Std deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.117</td>
<td>0.158</td>
<td>0.176</td>
<td>0.141</td>
<td>1.179</td>
<td>0.296</td>
<td>5.441</td>
<td>0.448</td>
<td>0.492</td>
</tr>
<tr>
<td>0.408</td>
<td>0.558</td>
<td>0.531</td>
<td>0.555</td>
<td>8.806</td>
<td>5.412</td>
<td>17.557</td>
<td>3.826</td>
<td>4.036</td>
</tr>
<tr>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>0.324</td>
<td>1.215</td>
<td>1.521</td>
<td>1.368</td>
<td>1.489</td>
<td>1.784</td>
<td>3.953</td>
<td>1.540</td>
<td>0.175</td>
</tr>
<tr>
<td>0.697</td>
<td>1.348</td>
<td>1.294</td>
<td>1.330</td>
<td>1.492</td>
<td>1.087</td>
<td>6.145</td>
<td>1.301</td>
<td>0.167</td>
</tr>
<tr>
<td>0.292</td>
<td>0.710</td>
<td>0.380</td>
<td>0.521</td>
<td>2.429</td>
<td>1.087</td>
<td>3.998</td>
<td>1.104</td>
<td>0.935</td>
</tr>
<tr>
<td>0.550</td>
<td>0.664</td>
<td>0.366</td>
<td>0.596</td>
<td>1.269</td>
<td>0.759</td>
<td>2.022</td>
<td>0.747</td>
<td>0.384</td>
</tr>
<tr>
<td>0.112</td>
<td>0.777</td>
<td>1.299</td>
<td>0.527</td>
<td>0.918</td>
<td>0.645</td>
<td>0.488</td>
<td>0.847</td>
<td>0.343</td>
</tr>
<tr>
<td>0.095</td>
<td>0.093</td>
<td>0.551</td>
<td>0.104</td>
<td>0.561</td>
<td>0.189</td>
<td>0.753</td>
<td>0.351</td>
<td>0.239</td>
</tr>
</tbody>
</table>

All extracts with the exception of the Ladysmith extract had low similarity to the mean chromatogram when analysed using the whole chromatogram as indicated by the low correlation coefficients shown in Table 5.8. There was good correlation between the
Ladysmith, Port Elizabeth and Combined extracts when compared to the mean chromatogram if whole chromatographic data was used.

**Table 5.8:** Correlation coefficients of *B. frutescens* extracts vs. the mean chromatogram.

<table>
<thead>
<tr>
<th></th>
<th>Joubertina Methanol</th>
<th>Joubertina Acetone</th>
<th>Joubertina Aqueous</th>
<th>QwaQwa</th>
<th>Ladysmith</th>
<th>Port Elizabeth</th>
<th>Combined Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole chromatographic</strong></td>
<td>0.13</td>
<td>0.13</td>
<td>0.15</td>
<td>0.16</td>
<td>0.98</td>
<td>0.54</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Common peaks data</strong></td>
<td>0.17</td>
<td>0.20</td>
<td>0.07</td>
<td>0.24</td>
<td>0.95</td>
<td>0.99</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Figures 5.19 and 5.20 below show PCA results. No clear clusters were identified by PCA when whole chromatographic data was analysed, this differed from correlations results which divided the extracts into three groups. Two groups were revealed by principal component analysis and correlation results when common peaks were used in the analysis. As with correlation results, PCA of the common peaks failed to show that the combined extracts should be different.

**Figure 5.19:** Whole chromatogram principle component analysis of *B. frutescens* extracts.
Figure 5.20: Common peaks principle component analysis of *B. frutescens* extracts.

Four clusters which are indicated in Figure 5.21 were constructed from HCA of whole chromatographic data. This method grouped the extracts in a manner corresponding with correlation results. Results from HCA based on common peaks were congruent with those of PCA and similarity based on correlation. The exception was that with HCA, the combined extract was identified as being different (Figure 5.22). Linkage distances shown in Figures 5.22 and 5.24 confirm the number of clusters obtained for each type of data.

Figure 5.21: Whole chromatogram HCA tree of *B. frutescens* extracts.
Figure 5.22: HCA linkage distance of *B. frutescens* extracts based on whole chromatographic data.

Figure 5.23: Common peaks HCA tree diagram of *B. frutescens* extracts.

Whole chromatogram results of HCA and similarity based on correlation grouped the QwaQwa extract together with all Joubertina extracts. The rest of the extracts differed from each other to an extent that they did not form any groups. PCA, correlation and HCA results using common peaks were consistent in grouping the QwaQwa extract with all Joubertina extracts, corresponding to results based on whole chromatographic data. The difference in the results of the two data sets was with the grouping of the remaining extracts. These formed a second distinctive group according to correlation results and PCA. HCA was the only analysis method based on common peaks that established that there was a difference between the combined extract and all the others.
Figure 5.24: HCA linkage distance of *B. frutescens* extracts based on common peaks data.

5.6.2.1.1 Stressed *B. frutescens* extracts

Heat at 110 °C and NaOH produced the greatest degradation of *B. frutescens* which is depicted in Figure 5.25. There was a low correlation between stressed and unstressed extracts; extracts stresses with NaOH, HCl, storage at 45 °C/75% RH for 3 months and storage at 115 °C for 15 minutes had correlation coefficients of 0.21, 0.33, 0.84 and 0.47 when compared to the unstressed extract respectively. Storage at 45 °C/75% RH produced the least degradation.

Figure 5.25: Stress degraded *B. frutescens*, Port Elizabeth extract (black – heat at 115 °C for 15 minutes; pink – storage at 40 °C/75% relative humidity for 3 months; blue – 2 M HCl for 2 hours; brown – reflux with 1 N NaOH for 2 hours; green – unstressed).
5.6.2.2 C. edulis results

Similarly to B. frutescens, whole chromatographic and common peaks data were used to analyse C. edulis extracts. The mean chromatogram was generated from the Ladysmith, QwaQwa, Joubertina aqueous and Port Elizabeth extracts. Visually, the chromatograms appeared to be similar in relation to the peaks obtained as indicated in Figure 5.26. The difference amongst them was mostly due to their peak areas and differences were as a result of the content present in the extracts as the quantity used for analysis was standardised at 0.02 g.

Visual inspection of the chromatograms showed that the Port Elizabeth extract was the most different when compared to the other extracts as seen in Figure 5.26 below whilst the other extracts produced similar chromatograms.

![Figure 5.26: Chromatograms of C. edulis: (A) Port Elizabeth, (B) Joubertina aqueous, (C) Joubertina methanol, (D) QwaQwa, (E) Ladysmith, (F) Joubertina acetone.](image-url)

If groupings were to be made simply by viewing DAAD spectra then Joubertina aqueous and Port Elizabeth extracts would form one group, Joubertina methanol, QwaQwa and Joubertina acetone extracts another, and the Ladysmith extract would
not belong to any of the groups. Nine common peaks were identified, their relative retention areas are given in Table 5.9.

![Figure 5.27: PDA spectra of C. edulis.](image)

**Table 5.9:** Relative retention areas of *C. edulis* common peaks.

<table>
<thead>
<tr>
<th>Joubertina Methanol</th>
<th>Joubertina Acetone</th>
<th>Joubertina aqueous</th>
<th>QwaQwa</th>
<th>Ladysmith</th>
<th>Port Elizabeth</th>
<th>Combined extracts</th>
<th>Mean Chromatogram</th>
<th>Std deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.656</td>
<td>0.175</td>
<td>0.163</td>
<td>0.006</td>
<td>0.004</td>
<td>0.034</td>
<td>2.600</td>
<td>0.052</td>
<td>0.076</td>
</tr>
<tr>
<td>0.267</td>
<td>0.156</td>
<td>0.035</td>
<td>0.008</td>
<td>0.006</td>
<td>0.033</td>
<td>0.099</td>
<td>0.020</td>
<td>0.016</td>
</tr>
<tr>
<td>0.154</td>
<td>0.175</td>
<td>0.089</td>
<td>0.037</td>
<td>0.008</td>
<td>0.021</td>
<td>0.211</td>
<td>0.039</td>
<td>0.036</td>
</tr>
<tr>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>0.171</td>
<td>0.122</td>
<td>0.073</td>
<td>0.188</td>
<td>0.431</td>
<td>0.233</td>
<td>0.550</td>
<td>0.231</td>
<td>0.149</td>
</tr>
<tr>
<td>0.222</td>
<td>0.132</td>
<td>0.048</td>
<td>0.047</td>
<td>0.022</td>
<td>0.090</td>
<td>0.358</td>
<td>0.052</td>
<td>0.028</td>
</tr>
<tr>
<td>0.078</td>
<td>0.128</td>
<td>0.100</td>
<td>0.063</td>
<td>0.073</td>
<td>0.121</td>
<td>0.158</td>
<td>0.089</td>
<td>0.026</td>
</tr>
<tr>
<td>0.210</td>
<td>0.874</td>
<td>0.897</td>
<td>0.218</td>
<td>0.519</td>
<td>0.605</td>
<td>0.432</td>
<td>0.560</td>
<td>0.280</td>
</tr>
<tr>
<td>0.070</td>
<td>0.033</td>
<td>0.116</td>
<td>0.015</td>
<td>0.035</td>
<td>0.0100</td>
<td>0.050</td>
<td>0.045</td>
<td></td>
</tr>
</tbody>
</table>

That the extracts were similar was largely confirmed by results of correlation coefficients obtained from both whole chromatogram and common peaks analysis. Differences between the two data sets was with the grouping of the combined extract. As shown in Table 5.10 whole chromatogram analysis identified that the combined extracts were different and assigned a correlation of 0.290 whilst common peaks not
only failed to distinguish that the sample was different, it assigned a correlation coefficient of 1. In addition, the dissimilarity of Joubertina methanol to the Mean chromatograph was larger with whole chromatographic data at 0.100 against 0.68 assigned using common peaks data.

**Table 5.10**: Correlation coefficients of *C. edulis* extracts vs. the mean chromatogram when whole chromatographic data and common peaks data were used.

<table>
<thead>
<tr>
<th></th>
<th>Joubertina Acetone</th>
<th>Joubertina Methanol</th>
<th>Joubertina Aqueous</th>
<th>Ladysmith</th>
<th>QwaQwa</th>
<th>Port Elizabeth</th>
<th>Combined Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole Chromatographic data</strong></td>
<td>0.860</td>
<td>0.100</td>
<td>0.910</td>
<td>0.910</td>
<td>0.910</td>
<td>0.930</td>
<td>0.290</td>
</tr>
<tr>
<td><strong>Common peaks data</strong></td>
<td>0.92</td>
<td>0.68</td>
<td>0.940</td>
<td>0.980</td>
<td>0.940</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

When extracting principle components using both whole chromatographic and common peaks data one distinct component which contained the majority of the extracts under review was obtained. The Joubertina methanol and combined extracts were sufficiently removed from this to indicate that they did not belong to the said groupings.

![Figure 5.28: Whole chromatogram PCA of *C. edulis* extracts.](image-url)
Hierarchical cluster analysis of both whole chromatographic data and common peaks data also yielded one main cluster in which most of the extracts shown in Figures 5.30 and 5.32 were included. Linkage distances shown in Figures 5.31 and 5.33 confirm the number of clusters obtained. Most of the extracts except for the Joubertina methanol and combined extracts were placed in one grouping.

**Figure 5.30:** Whole chromatographic data HCA tree diagram of *C. edulis* extracts.
Figure 5.31: HCA linkage distance of *C. edulis* extracts based on whole chromatographic data.

Figure 5.32: Common HCA peaks tree diagram of *C. edulis* extracts.

Figure 5.33: HCA linkage distance of *C. edulis* extracts based on common peaks data.
5.6.2.2.1 Stressed C. edulis extracts

The method adequately identified changes to the stressed extracts caused by adverse conditions. Those extracts challenged with a temperature of 115 °C for 15 minutes and 1N NaOH for 2 hours exhibited the greatest change as depicted in Figure 5.34 and supported by correlation coefficients. These were 0.10, 0.08, 0.50 and 0.3 for extracts stressed using NaOH, HCl, storage at 40 °C/75% RH for 3 months and storage at 115 °C for 15 minutes respectively. The developed method is able to distinguish between extracts altered by storage conditions which may be missed by visual inspection of chromatograms alone.

**Figure 5.34:** Stress degraded C. edulis Port Elizabeth extracts (black – heat at 115 °C for 15 minutes; pink – storage at 40 °C/75% RH for 3 months; blue – 2M HCl for 2 hours; brown – reflux with 1N NaOH for 2 hours; green – unchallenged C. edulis).
5.6.3 General Discussion

Results obtained from whole chromatographic and common peaks data were, in the majority, similar. The greatest difference between the two lay with the grouping of the combined extracts. Correlation and PCA analysis using common peaks did not identify that it was different; this was true for both extracts. HCA did however consistently link the combined extract to the other clusters at such a distance that the difference was evident. Results obtained using whole chromatographic data was similar to common peaks results, however, it consistently showed that the combined extracts should not be grouped with any of the other extracts for both B. frutescens and C. edulis. In addition, the level of dissimilarity amongst the extracts was consistently less when common peaks data was analysed. The failure by two of the three analysis methods to identify plant adulteration when using common peaks is worrying. It shows that adulteration can potentially escape detection should the added components have peaks which are common with the desired extract.

There is an appreciation of the complexity of analysing numerous and complex chromatographic data and the benefits afforded by data reduction. Data reduction can however, reduce the sensitivity of data analysis method. It is granted that at least one of the three analysis methods was able to detect the change, however, with the lack of standardisation of analysis methods, it is possible that HCA may not be conducted and thus adulteration not identified.

Changes resulting from stressing of the extracts were identified by this method. The method developed can thus be said to be specific and selective when whole chromatographic data is used.

B. frutescens could not be clearly grouped implying that it is more sensitive to environmental growth conditions when compared to C. edulis in which the extracts were more similar regardless of the area in which they were harvested. Differences among extracts of the same plant are being attributed to environmental conditions encountered during growth as the plants were harvested, handled and extracted in a similar manner.
5.7 Conclusion

Appropriate tests used to validate developed HPLC methods were conducted and these confirmed that the method is sound. Accuracy, precision and linearity were tested using standards as the use of complex chromatographic data obtained from plant extracts was unsuitable for this type of analysis. The selected standards were also used as markers for quantification of plant constituents.

Selectivity and specificity were determined using plant extracts since these were the active ingredients. It has been shown that the method is suitably specific and selective and that the use of correlation coefficients, PCA and HCA in conjunction with whole chromatographic data enables quality control of *B. frutescens* and *C. edulis* (Xie *et al.*, 2008; Hua-Bin *et al.*, 2012; Arceusz & Wesolowski, 2013). Whilst HCA and PCA are good analysis methods, they should be combined with other methods, which in conjunction, would indicate whether the extract under study is indeed a genuine one. Such methods could amongst others include microscopic examination of whole plants and determination of total ash. These tests were not included in this study as it limited itself to the use of HPLC for quality control (Xie *et al.*, 2006).

Based on the results especially those obtained for *B. frutescens* in which the variation of the plant extracts precluded adequate grouping, it is suggested that extracts intended for use in medicinal products be standardised. This can be done through control of growing conditions along with the seeds, seedlings, cuttings etc. used to propagate the plants. Soil treatment and composition should also be standardised across suppliers. Differences of plant extracts harvested from different areas have been noted by researchers establishing chromatographic fingerprints of plants (Dong *et al.*, 2003; Zhao *et al.*, 2005; Randriamampionona *et al.*, 2007). These differences are mostly in the quantity of chemicals present in the plant rather than the number of chemicals present in the plant (Hua-Bin *et al.*, 2012). Authentication and raw material testing should depend on whether the manufacturer will provide fresh plant material or extracts. If the whole plant or part of the plant is provided then the manufacturer should conduct other physical identification tests such as microscopic examination and species identification amongst others. If an extract is provided then a certificate of
analysis should accompany the extract wherein the quantity of peaks and plant identity are provided. Some of these can be verified by the manufacturer on receipt of raw material prior to release for use in manufacture of herbal products.

For analysis of the data using correlation coefficients, HCA and PCA are adequate, however, it is my assertion that the whole chromatographic data should be used instead of common peaks as is currently the case with research being published. Using common peaks for analysis defeats the purpose and intention behind using fingerprint analysis for plants in the first place.
6. B. FRUTESCENS AND C. EDULIS CREAM

6.1 Determining stability of products

One of the fundamental goals of pharmaceutical formulation is the development of products which are fit for their intended purpose; these products should be stable throughout their shelf-life (Allen Jr, 2008). The objective of pharmaceutical development is to design a quality product and manufacturing process to consistently deliver the intended performance of the product. The information obtained during dosage form development can be used for quality risk assessment including quality control. A good formulation must be manufacturable, chemically and physically stable throughout manufacture and shelf life, and bioavailable at administration of the drug. It must also meet quality standards and special requirements to ensure efficacy and safety of the final manufactured product (Zheng, 2009).

The purpose of stability testing according to the ICH is “to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and to establish a re-test period for the drug substance or a shelf life for the drug product and recommended storage conditions” (ICH, 2003). Thakur and colleagues (2011) emphasise the point, stating that “the stability is aimed at assuring that the drug/drug product remains within the specifications established to ensure its identity, strength, quality and purity. It can be interpreted as the length of time under specific conditions and storage that a product will remain within the pre-defined limits for all its important characteristics.”

The MCC requires that stability testing of pharmaceutical products should cover chemical and biological stability, physical and organoleptic properties and where relevant preservative activity. Assays and preservative efficacy tests should also be carried out. Importantly it further states that “stability should be established for the
whole period of intended use under the intended storage conditions”. In South Africa, long term testing should be conducted at 25 °C/60% RH, 30 °C/65% RH or 30 °C/75% RH and accelerated studies at 40 °C ± 2 °C/75 ± 5% RH (Medicines Control Council, 2012).

Physical instability of medicinal products containing herbs may arise from product-container incompatibility or the presence of microorganisms or infestation by insects. Chemical instability as a result of growth conditions, post-harvest storage and processing, enzymatic degradation and herb excipient incompatibility can also be encountered (Thakur et al., 2011).

Semisolid oil in water emulsions (creams) are widely used in pharmaceutical product development as vehicles for topical drug substances (Eccleston, 1997). Such formulations need to overcome the barrier mechanism of the stratum corneum to pass into the skin (Marks, 2004). The molecular weight, solubility and molecular configuration greatly influence the rate of penetration, in addition, components of the vehicle may interact with the skin and drug substance altering the expected bioavailability; this effect may or may not be intentional (Cevc, 1997). Physical and chemical stability of the formulation and partition across the stratum corneum should be considered when formulating creams. In addition, the formulations should be non-toxic to the skin, be easy to apply and remove and should allow the incorporation of additives such as antioxidants and preservatives (Soriano, 2001).

Creams, which are semisolid oil in water emulsions, need to satisfy criteria relating to long term stability, consistency and safety. Long term stability refers to a shelf-life of 2 to 3 years and physical stability would have to be maintained under variable conditions of temperature and stress. Consistency, which is the feel of the cream during application, spreading and good delivery of the active ingredients also need to be maintained (Tadros, 2004). Rheology provides a simple technique for comparison of structural properties of the creams and consistency, it also provides information about the creams resistance to external forces indicating the stability of the cream over a period of time (Thorgeirsdóttir et al., 2006).
6.1.1 Registration of formulated ATM with regulatory authorities

African Traditional medicines are generally prescribed, compounded and dispensed by ATH only for the period that they will be used which varies from a few days to a few weeks depending on the condition being treated and the length of time for which the patient has been ill (Igoli et al., 2005; Togola et al., 2005). The current debate and action around ATM by regulatory bodies is centred on the validation of these medicines as currently dispensed by ATH along with those that may be used to self-medicate and available for purchase at shops and pharmacies. The challenge and scale of work needed to adequately validate ATM can be appreciated when one thinks that whilst the indications of some medicinal plants are generally known, the quantity used and other medicinal plants included in the final preparation may differ vastly. In addition, some ATM are known only to a small number of ATH because they have been dreamt or have been passed down only in that clan. If changes to plants caused by environmental conditions experienced during growth and differing extraction methods are included then the number of medicines to be validated becomes enormous. Based on the above, it is feasible to deduce that the validation and registration of ATM will be, at least initially, for those plants and products that are:

- At present being sold on the market as ATM
- Widely known to have medicinal properties and functions and are used by a large number of people when self-medicating i.e. in the public domain
- Those which are being and will be formulated for sale on a large scale in some type of market.

Part of this study was intended to initiate the process of standardising manufacture by producing a cream from widely known medicinal plants. As has been mentioned in Chapter 2, when this study was initiated, a meeting with ATH was held in which the use of the medicinal plants for wounds and herpes was confirmed. The amount to be used, the frequency of use and the duration of use were however not disclosed. ATH envisioned a state in which a company and associated manufacturing plant would be created in which ATM would be manufactured as long term goal. The short term goal was to create products and intermediate products which the ATH could use to make

95
final formulations in their surgeries. Intermediate products would be especially useful in ensuring some measure of standardisation and quality control whilst affording the ATH the opportunity to keep critical parts of ATM a secret.

To initiate this, a decision was made to produce a herbal cream using simple standard pharmacopoeial recipes. A preliminary study was conducted by Chuang (2008) under this researcher’s guidance in which the following was concluded:

- A cream base is better than an ointment base for organoleptic appeal.
- The product needed to be preserved as colonisation by microorganisms was experienced. The best results were produced by a combination of methyl hydroxybenzoate and propyl hydroxybenzoate.

The use of bases containing synthetic constituents for the production of herbal medicines has been criticised by Kapoor and Saraf (2010), who pointed out that these pharmaceutical excipients are toxic. They do however, concede to the fact that formulating a cream base only from excipients of natural origin is cumbersome. There is also the added danger of microbial contamination. The majority of researchers formulating herbal creams have used bases with synthetic excipients in their formulations (Anchisi et al., 2001; Saleem et al., 2008; Aswal et al., 2013).

6.2 Methods

Unless specified to the contrary, tests described below were conducted in triplicate when the creams were initially produced (T0), after one month (T1), two months (T2) and three months (T3) of storage at 25 °C/60% RH and 40 °C ± 2 °C/75% RH in a Binder KBF240 climatic chamber (Binder GmbH, Tuttlingen, Germany).
6.2.1 Compounding the cream

The buffered cream which was used as the base was prepared by dissolving 20 g disodium hydrogen phosphate dodecahydrate, 4 g citric acid monohydrate, 1.6 g methylhydroxybenzoate and 0.24 g propylhydroxybenzoate in 300 g of purified water heated to 60 °C. It was added to 240 g of emulsifying ointment, made to 800 g with RO water (all components were at 60 °C when they were combined) and then cooled with continuous stirring. The emulsifying ointment had been prepared by melting 120 g emulsifying wax, 200 g white soft paraffin and 80 g liquid paraffin in a beaker and then stirred until cool.

As there was no indication as to the dose of plant extract that is used to treat wounds and herpes, the total concentration of extract added to the cream was based on concentrations used in the production of other herbal creams which in total did not exceed 10% w/w (Gebre-Mariam et al., 2005; Saleem et al., 2008; Aswal et al., 2013). To make the final product, 5 g of *B. frutescens* and 5 g of *C. edulis* leaf extract were weighed and ground to a fine powder using a mortar and pestle. The powdered material was then incorporated into 180 g of buffered cream. The cream was packed in weights of 10 g in glass containers with high density polyethylene closures. Some of the containers were stored at 40 °C/75% RH and others at 25 °C/60% RH in a stability chamber. An equal quantity of cream base without extract was stored at these conditions in similar containers. All equipment used was previously sprayed with 70% ethanol and air dried.

6.2.2 pH and separation

To measure the pH, 5 g of each cream was weighed and mixed with 10 ml water. This was heated to 45 °C for 3 minutes and filtered through a Whatman No 1 filter after which the pH was measured at 22 °C with a pH meter (Metrohm) (Verma et al., 2011). Separation was tested for by centrifuging (Eppendorf Centrifuge 5804R) 15 g of the cream at 3500 rpm for 15 minutes at 25 °C (Realdon et al., 2002).
6.2.3 Permeation

The quantity of extract released from the creams was determined using 5 ml Franz diffusion cells with a cellulose nitrate membrane (0.45 µm pore size) as the permeation barrier. The membranes were prepared for the test by immersion in the receptor medium for 16 hours prior to use. The receptor medium was acetonitrile:5% acetic acid (40:60) which is the sample preparation medium used to dissolve extracts prior to HPLC analysis. Five millilitres of the receptor medium was placed in a Franz diffusion cell ensuring that there were no bubbles beneath the membrane. Ideally the receptor medium should mimic physiological conditions as closely as possible, however, preparation of the extracts prior to analysis was best when acetonitrile:5% acetic acid (40:60) was used.

A synthetic membrane was used during the analysis since the purpose was for quality control and not determination of bioequivalence in which case human skin equivalents are desirable (Permegear Inc.; Thakker & Chern, 2003). The medium was kept at 32 °C ± 2 °C and a magnetic stirrer was used to keep it in motion. Five grams of the cream was placed in the donor cell ensuring that the membrane was completely covered. Sampling was done at 0.5, 1, 2, 4, 6 and 24 hours; 2 ml was removed from the diffusion cell at each sampling time and replaced with an equal volume of the receptor medium which had been warmed to 32 °C before addition. The samples were filtered through a hydrophilic PVDF 0.45 µm filter (Millipore® Inc., Massachusetts, USA) and analysed using the method developed in Chapter 5. The sampling port and donor chamber were covered to prevent evaporation of the receptor medium and drying of the cream during the experiment.

The cumulative quantity of extract released (µg/m²) per sampling time was calculated as follows:

\[ Q = \frac{(C_nV + \sum_{i=1}^{n-1} C_iS_i)}{A} \]
Where:

- **Q** = Cumulative amount of extract released per surface area of membrane (μg/cm²)
- **Cₙ** = Concentration of extract (μg/ml) determined at nᵗʰ sampling interval.
- **V** = Volume of individual Franz diffusion cell
- \( \sum_{i=1}^{n-1} C_i S \) = Sum of concentrations of extract (μg/ml) determined at sampling intervals 1 through n-1
- **S** = Volume of sampling aliquot
- **A** = Surface area of sample well (Thakker & Chern, 2003).

Catechin and rutin which had been used to validate the HPLC method were used as markers for quantification of permeating compounds. The retention areas of compounds eluting at the retention times coinciding with those of catechin and rutin were used in the calculation.

The concentration of the marker constituents in the creams was determined by mixing 0.5 g of the cream with 15 ml of RO water and heating the contents to 50 °C for 5 minutes. It was allowed to cool and then centrifuged at 3000 rpm for 10 minutes. The supernatant was placed in a beaker and evaporated with compressed air. 5 ml of the HPLC dissolution medium was added to the beaker, mixed, filtered and analysed using the developed HPLC method.

### 6.2.4 Microbial tests

#### 6.2.4.1 Microbial quality of the extracts

The total aerobic count of the extracts was determined by dissolving 0.5 g of the extracts in 25 ml of Fluid Soybean-Casein Digest broth for bacteria and Sabouraud broth for fungi. One millilitre of this was transferred to a petri dish and 15 ml Soybean-Casein Digest or Sabouraud agar (cooled to 45 °C) added. The contents were thoroughly mixed using a figure of 8 motion. The petri dishes were allowed to cool, inverted and incubated at 37 °C for 48 hours for bacteria and 25 °C for 5 days for fungi after which the colony forming units (CFU) were counted (WHO, 2012a).
Microorganisms used in the preservative challenge test were *Pseudomonas aeruginosa* (ATCC No. 27853), *Staphylococcus aureus* (ATCC No. 43300), *Escherichia coli* (ATCC No. 38218) and *Candida albicans* (ATCC No.10231). The microorganisms used in the test are not those prescribed by the harmonised ICH methodology, however, researchers have found that using reference and non-reference microorganisms in preservative challenge tests led to similar conclusions (Abu Shaqra & Al-Shawagfeh, 2012).

Microorganisms were resusciitated by placing a loop full in 10 ml Soybean-Casein Digest broth and Sabouraud Dextrose broth for the bacteria and *C. albicans* respectively. They were incubated at 32 °C and 25 °C overnight for bacteria and fungi respectively in a shaking water bath. One hundred microlitres of the microbial suspension was transferred to McCartney bottles containing 10 ml of Soybean-Casein Digest [Sigma-Aldrich (Pty) Ltd, (Kempton Park, SA)] or Sabouraud Dextrose broth [Sigma-Aldrich (Pty) Ltd,( Kempton Park, SA)] and incubated for 18 hours. Ten millilitres of a $10^5$ serial dilution was made with normal saline of which 100 µl was plated with Soybean-Casein digest agar for bacteria and Sabouraud Dextrose for *C. albicans*, the remainder was kept in a refrigerator. The petri dishes were incubated for 24 hours at 32 °C and 25 °C for the bacteria and *C. albicans* respectively. The number of colonies was then counted and the number of cfu/ml in the refrigerated microbial sample calculated. The quantity used to make 5 ml of a standardised $1 \times 10^8$ cfu/ml concentration of bacteria or fungi was calculated using the formula $C_1 V_1 = C_2 V_2$.

Five grams of the cream that was to be used in the test was transferred from the original container into sterile polypropylene bottles. 0.05 ml of the standardised $1 \times 10^8$ cfu/ml suspension was transferred to the container and the contents thoroughly mixed using a vortex with a resultant microbial concentration of $1 \times 10^6$ cfu/g. The containers with inoculated product were incubated at 32 °C and 28 °C for the bacteria and *C. albicans* respectively. Sampling and testing was conducted at 14 and 28 days of incubation. To conduct the test, 0.1 g of the cream was sampled from the bulk and placed in a sterile polyethylene container. It was then thoroughly mixed with 15 ml
Soybean-Casein Digest agar at 40 °C and plated on petri dishes. The petri dishes were incubated for 48 hours at 35 °C and 25 °C for the bacteria and *C. albicans* respectively and the number of colonies counted (USP, 2007). Preservative efficacy was challenged at $T_0$ immediately after the cream had been compounded and after three months of storage had passed, the cream was again sampled and the efficacy of the preservative determined.

6.2.4.3 Rheology

Rheological tests were conducted on an Anton Paar RheolabQC rheometer fitted with *RheoPlus™* software with CC27 cylindrical sensor. The temperature was controlled at 25 °C ± 1 °C using a water bath (Anton Paar ViscoTherm VT2). The tests were conducted in phases. The first consisted of a preshear phase conducted over 30 seconds where the shear rate was 5 s$^{-1}$ to homogenise the sample. The sample was then allowed to rest for 30 seconds. The next interval consisted of a shear stress ramp wherein the shear rate increased from 0.5 s$^{-1}$ – 120 s$^{-1}$ followed by a reduction from 120 s$^{-1}$ to 0.5 s$^{-1}$.

6.3 Results

6.3.1 Organoleptic properties

The cream base without extract was smooth and white with a mild pleasant odour. It changed to a light green colour when the extracts were added and the odour remained mild and pleasant. After one month of storage at 40 °C/75% RH, a change in colour was noted. The light green colour had a tinge of brown; although not unpleasant, the odour of the cream had changed. Creams stored at 25 °C/60% RH were not exhibiting this change and there was no change in the appearance or odour of the base devoid of extract.
After 2 months of storage at 40 °C/75% RH creams containing the extract had become brown and foul smelling; those stored at 25 °C/60% RH had also changed colour and had an unpleasant odour but to a lesser extent when compared to those stored at 40 °C/75% RH. Creams without the extract remained white and smooth with a mild pleasant odour.

There was no change in the organoleptic properties between creams at 2 and 3 months of storage at 40 °C/75% RH. They continued to look brown and still had the unpleasant odour. There was also no change in those stored at 25 °C/65% RH from what was noted after 2 months. The base without extracts stored at both storage conditions remained white, smooth and had a mild pleasant odour.

6.3.2 pH and separation

Ideally, the pH of the skin should be between 4 and 7 to maintain an adequate antimicrobial barrier and facilitate healing (Duncan et al., 2013). The base of the cream without extract remained constantly at pH 6 as shown in Table 6.1. Addition of the extract at T₀ reduced the pH from 6.12 to 4.95 which was still within the range required for adequate skin function. However, with time, the pH increased, and the creams stored at accelerated storage conditions had the highest increase, breaching the upper limit of 7 after three months of storage. There was however, no statistical difference in the pH of the creams with p < 0.05. Addition of the extracts did not adversely change the pH of the cream, however, caution should be exercised as the pH rises with time (Hachem et al., 2003). No phase separation was observed for any of the creams that were centrifuged.

Table 6.1: pH of the creams as measured at measured at 20 °C.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cream with extract (stored at 25 °C/60% RH)</th>
<th>Cream with extract (stored at 40 °C/75% RH)</th>
<th>Cream without extract (stored at 25 °C/60% RH)</th>
<th>Cream without extract (stored at 40 °C/75% RH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Std deviation</td>
<td>pH</td>
<td>Std deviation</td>
</tr>
<tr>
<td>T₀</td>
<td>4.95</td>
<td>0.25</td>
<td>4.95</td>
<td>0.25</td>
</tr>
<tr>
<td>T₁</td>
<td>5.00</td>
<td>0.84</td>
<td>5.76</td>
<td>0.10</td>
</tr>
<tr>
<td>T₂</td>
<td>5.07</td>
<td>0.35</td>
<td>5.13</td>
<td>0.86</td>
</tr>
<tr>
<td>T₃</td>
<td>6.11</td>
<td>0.69</td>
<td>7.63</td>
<td>0.57</td>
</tr>
</tbody>
</table>
6.3.3 Permeation

Figure 6.1 below shows that the cream base did not interfere with quantification of the marker compounds for the extracts as none of the cream base compounds eluted at retention times coinciding with catechin and rutin which are 13 minutes and 16 minutes respectively.

Determining the amount of active ingredient released by a cream during stability studies is important since variation in the levels released gives an indication of the stability of the product (Food and Drug Administration, 1997). The quantity released by the cream over a period of time is indicated in Figures 6.2, 6.3 and Table 6.2.

![Chromatogram of buffered cream base without extracts.](image)

**Figure 6.1:** Chromatogram of buffered cream base without extracts.

The quantity of catechin released at T₀ was higher than at any other time. Additionally, consistently higher concentrations were obtained from creams stored at long term conditions when compared to accelerated conditions. This implies that catechin and constituents represented by catechin are affected by heat. The quantity released after storage at accelerated conditions at T₂ was less than that released at T₃ under the same storage conditions. It may be that interactions with in the product caused reduced release of the compounds after three months of storage. Analysis using ANOVA found that there was a statistical difference in the concentration released at T₀ vs. T₂ and T₃ at accelerated and long term storage conditions; significant difference being p < 0.05.
The quantity and pattern of release for rutin was consistent throughout the storage conditions with no significant difference in the release of extract from the various creams. This implies that the constituents represented by rutin were less affected by storage conditions compared to those represented by catechin.

**Figure 6.2:** Permeation of extract from the cream using catechin as a marker (n = 3 for each point).

**Figure 6.3:** Permeation of extract from the cream using rutin as a marker (n = 3 for each point).
The methodology used during the permeation studies does not allow for determination of bioavailability as stated in Section 6.2.3, an attempt at estimating it will thus not be made. However, when contrasting the largest cumulative concentration of catechin and rutin combined which is 2.39 µg/ml (T₀ concentrations) with concentrations of extract at which other in vitro studies were conducted then one is encouraged. For the combined extracts, the lowest active antiviral concentration was 250 µg/ml, the IC₅₀ for the DPPH assay was 41 µg/ml and wound closure was complete after 24 hours when a concentration of 10 µg/ml was used during the scratch assay. Considering that rutin and catechin represent two of a wide range of compounds that are present in the extracts it is reasonable to deduce that the final concentration permeating through the membrane may be higher and could be in the range demonstrating in vitro activity as mentioned above.

Table 6.2: Permeation results for the creams.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sampling interval (hr.)</th>
<th>Catechin (µg/ml)</th>
<th>Rutin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>0.5</td>
<td>0.141</td>
<td>0.307</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.405</td>
<td>0.470</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.673</td>
<td>0.609</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.876</td>
<td>0.702</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.019</td>
<td>0.858</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.109</td>
<td>1.220</td>
</tr>
<tr>
<td>T₁ (40 °C/75% RH)</td>
<td>0.5</td>
<td>0.072</td>
<td>0.299</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.112</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.239</td>
<td>0.561</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.319</td>
<td>0.684</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.528</td>
<td>0.800</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.682</td>
<td>1.173</td>
</tr>
<tr>
<td>T₁ (25 °C/60% RH)</td>
<td>0.5</td>
<td>0.092</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.174</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.329</td>
<td>0.583</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.487</td>
<td>0.688</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.663</td>
<td>0.799</td>
</tr>
</tbody>
</table>
The level of reduction in the concentration of the extracts should not be greater than 5% from the initial assay value at the end of the proposed shelf-life for herb containing medicinal products. Deviation from the 5% reduction can be allowed in exceptional circumstances if justified and allowed by regulatory bodies. The level of deviation depends on the herbs in question (European Medicines Agency, 2013). The lowest reduction for this product was obtained with cream stored at 25 °C for one month resulting in a 17.07% reduction (Table 6.3). Catechin could not be used in this

<table>
<thead>
<tr>
<th></th>
<th>24</th>
<th>0.821</th>
<th>1.182</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T₂ (40 °C/75% RH)</strong></td>
<td>0.5</td>
<td>0.064</td>
<td>0.237</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.074</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.091</td>
<td>0.401</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.113</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.111</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.385</td>
<td>0.727</td>
</tr>
<tr>
<td><strong>T₂ (25 °C/60% RH)</strong></td>
<td>0.5</td>
<td>0.068</td>
<td>0.361</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.106</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.146</td>
<td>0.510</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.156</td>
<td>0.641</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.228</td>
<td>0.766</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.700</td>
<td>1.129</td>
</tr>
<tr>
<td><strong>T₃ (40 °C/75% RH)</strong></td>
<td>0.5</td>
<td>0.081</td>
<td>0.222</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.080</td>
<td>0.291</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.101</td>
<td>0.358</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.157</td>
<td>0.457</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.254</td>
<td>0.538</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.527</td>
<td>0.770</td>
</tr>
<tr>
<td><strong>T₃ (25 °C/60% RH)</strong></td>
<td>0.5</td>
<td>0.058</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.066</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.116</td>
<td>0.410</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.116</td>
<td>0.541</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.128</td>
<td>0.666</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.500</td>
<td>1.029</td>
</tr>
</tbody>
</table>
quantification as the size of the retention areas did not allow for extrapolation to the calibration curve that was constructed. When permeation concentrations (which include catechin and rutin) were used to calculate the deviation from the initial assay value, the 5% specification was also not met, the lowest reduction obtained was 14.00% from the cream stored at 25 °C/60% RH after one month of storage.

Table 6.3: Assay results of the cream using rutin as a marker.

<table>
<thead>
<tr>
<th></th>
<th>Rutin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>3.34</td>
</tr>
<tr>
<td>T₁ (40 °C/75% RH)</td>
<td>2.16</td>
</tr>
<tr>
<td>T₁ (25 °C/60% RH)</td>
<td>2.77</td>
</tr>
<tr>
<td>T₂ (40 °C/75% RH)</td>
<td>1.70</td>
</tr>
<tr>
<td>T₂ (25 °C/60% RH)</td>
<td>2.45</td>
</tr>
<tr>
<td>T₃ (40 °C/75% RH)</td>
<td>1.59</td>
</tr>
<tr>
<td>T₃ (25 °C/60% RH)</td>
<td>2.45</td>
</tr>
</tbody>
</table>

6.3.4 Microbial studies

6.3.4.1 Microbial quality of the extracts

Microorganisms were present in the extracts despite treatment of the whole plants with ethanol and hypochlorite prior to extraction and the concentration of microorganisms is shown in Table 6.4. A limit of 10² aerobic microbial count and 10¹ combined yeast/moulds count has been set by WHO for cutaneous pharmaceutical products. This limit, however, does not apply to herbal medicinal products and none have been set for these types of products (WHO, 2012a, 2012b).

Table 6.4: Total aerobic counts of the extracts.

<table>
<thead>
<tr>
<th></th>
<th>Aerobic microbial count (cfu/ml)</th>
<th>Combined yeast/moulds count (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. frutescens</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>C. edulis</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>
6.3.4.2 Preservative challenge

A bacterial log reduction of 2 or more from the initial count at 14 days without an increase in subsequent days along with no increase in the amount of *C. albicans* is required for the preservative function to be acceptable (United States Pharmacopoeia, 2004). The microbial log concentration in the cream on the day of inoculation was 5.0. All the creams exhibited a 2.0 of more log reduction in the concentration of bacteria shown in Table 6.5 and no increase in the number of microorganisms was evident on day 14 and 28 for *C. albicans*.

Table 6.5: Preservative challenge results.

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Test day</th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cfu/ml</td>
<td>log [ ]</td>
<td>cfu/ml</td>
<td>log [ ]</td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;0&lt;/sub&gt;</strong></td>
<td>14</td>
<td>930</td>
<td>2.97</td>
<td>850</td>
<td>2.92</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>890</td>
<td>2.95</td>
<td>840</td>
<td>2.92</td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;3&lt;/sub&gt; at 25 °C</strong></td>
<td>14</td>
<td>10</td>
<td>1.00</td>
<td>50</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>10</td>
<td>1.00</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;3&lt;/sub&gt; at 40 °C/75% RH</strong></td>
<td>14</td>
<td>20</td>
<td>1.30</td>
<td>10</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>20</td>
<td>1.30</td>
<td>10</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*[] = concentration

The number of surviving microorganisms in T<sub>3</sub> creams was less than those surviving in T<sub>0</sub> creams. This reduction in numbers could possibly be explained by hostile environmental conditions within the cream. The change in organoleptic properties of the cream may have been accompanied by toxic constituents which could have been unfavourable for proliferation of microorganisms.
6.4 Rheology

Rheological properties of creams can influence their performance, specifically, viscosity affects a cream’s application to treatment sites and the diffusion of active constituents from the cream and thus the delivered dose (Ueda et al., 2009). The yield value is the quantity of shear stress which needs to be overcome in order for the cream to flow. The yield stress of the creams increased from 8.309 Pa to 39.52 Pa when extracts were added to the cream base. The yield stress required for emulsions to have a good feeling on the skin is 6.5 Pa – 13 Pa (Brummer & Godersky, 1999). The high apparent viscosity and yield values shown in Tables 6.6 and 6.7 thus result in a cream that has an unappealing “feel” on the skin. However, when considering that this is a cream to be applied on wounds and herpes lesions and thus needs to be in contact with the skin for an extended period of time, such combination becomes beneficial (Colo et al., 2004).

Table 6.6: Yield values of the creams.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cream with extract (stored at 25 °C/60% RH)</th>
<th>Cream with extract (stored at 40 °C/75% RH)</th>
<th>Cream without extract (stored at 25 °C/60% RH)</th>
<th>Cream without extract (stored at 40 °C/75% RH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield Value (Pa)</td>
<td>Yield Value (Pa)</td>
<td>Yield Value (Pa)</td>
<td>Yield Value (Pa)</td>
</tr>
<tr>
<td></td>
<td>T₀</td>
<td>39.52</td>
<td>39.52</td>
<td>8.31</td>
</tr>
<tr>
<td></td>
<td>T₁</td>
<td>36.61</td>
<td>28.54</td>
<td>9.49</td>
</tr>
<tr>
<td></td>
<td>T₂</td>
<td>18.59</td>
<td>9.18</td>
<td>9.50</td>
</tr>
<tr>
<td></td>
<td>T₃</td>
<td>16.51</td>
<td>6.04</td>
<td>12.35</td>
</tr>
</tbody>
</table>

On storage the yield values of creams with extract decreased from 39.52 Pa at T₀ to 16.51 Pa and 6.04 Pa at T₃ for creams stored at long term and accelerated storage conditions respectively.
Table 6.7: Apparent viscosity of creams containing extracts.

<table>
<thead>
<tr>
<th>Shear rate (1/s)</th>
<th>$T_0$</th>
<th>$T_1$ (stored at 40 °C/75% RH)</th>
<th>$T_1$ (stored at 25 °C/60% RH)</th>
<th>$T_2$ (stored at 40 °C/75% RH)</th>
<th>$T_2$ (stored at 25 °C/75% RH)</th>
<th>$T_3$ (stored at 40 °C/75% RH)</th>
<th>$T_3$ (stored at 25 °C/75% RH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.20</td>
<td>7.08</td>
<td>11.60</td>
<td>14.00</td>
<td>5.17</td>
<td>4.95</td>
<td>5.57</td>
<td>6.56</td>
</tr>
<tr>
<td>50.10</td>
<td>2.70</td>
<td>4.60</td>
<td>5.95</td>
<td>2.06</td>
<td>1.92</td>
<td>2.26</td>
<td>1.51</td>
</tr>
<tr>
<td>102.00</td>
<td>1.74</td>
<td>3.06</td>
<td>3.74</td>
<td>1.43</td>
<td>1.36</td>
<td>1.55</td>
<td>1.34</td>
</tr>
</tbody>
</table>

This was not the case with the extract free cream base whose yield value remained relatively constant. The decrease in yield value, accompanied by a reduction in the apparent viscosity points to the loss of “stickiness” of the product i.e. the property of extended contact with the skin is lost and this may be related to a reduction in the stability of the product over time. A reduction in viscosity on addition of active ingredients of vegetative origin was experienced by Anchisi and colleagues who calculated a 20% reduction in apparent viscosity on addition of extracts (Anchisi et al., 2001).

The loss of cream stability can be appreciated when the results of viscosity vs. shear rate are observed in Figures 6.4 and 6.5. Thixotropy refers to an isothermal system in which the apparent viscosity of a material decreases under shear stress followed by a steady recovery when the stress is removed and the size of the area being indicative of the magnitude of the disturbance. The hysteresis loop method is the most popular method of characterising thixotropic behaviour of non-Newtonian systems. It involves measurement of the area enclosed by the up and down curves (Lee et al., 2009).
Figure 6.4: Rheograms of creams stored at 40 °C/75% RH (A) with extract, (B) without extract [red = T₀, yellow = T₁, blue = T₂, green = T₃].

The hysteresis area of the creams reduced with increased storage time of the creams. Whilst the pattern of reduction of the hysteresis area was the same for all creams, it was visually determined to be greater for creams with extract stored at accelerated storage conditions for two and three months indicating a greater change in the internal structure of these creams and thus instability.

Figure 6.5: Rheograms of creams stored at 25 °C/60% RH (A) with extract, (B) without extract [red = T₀, yellow = T₁, blue = T₂, green = T₃].
6.5 Conclusion

It was evident from organoleptic properties of the creams that storage beyond one month whether at long term or accelerated storage conditions reduced the stability of the creams. They became brown and developed an offensive odour. Stability related to permeation and pH were favourable up until two months of storage after which the pH increased beyond 7 at accelerated storage conditions and the concentration of compounds released during permeation studies reduced. The preservative was still effective after three months of storage at both storage conditions. The best indicator of the loss of stability was obtained from rheological studies. From Figure 6.4 and 6.5 the changes were contrasted against the two storage conditions for creams with and without extract and whilst instability over time is noted for both, it is marked for creams with extract at T₂ and T₃.

Based on the results obtained, storage and use of the cream beyond one month is not advocated. The proposed shelf-life is adequate since this particular product would be compounded and dispensed to patients of ATH on diagnosis and for use over a short time and not packaged for mass sale.

The cream base void of extract is largely stable and instability is created by the addition of the extracts. Thus for production of a product with long term stability, active (in this case extracts) excipient studies will have to be conducted to establish which excipients are adversely interacting and could be substituted with ones serving a similar function but which are stable in the presence of the extracts. Supercritical carbon dioxide extraction can be used to produce extracts with increased stability (Thakur et al., 2011). Whilst this is not a sterile product, the presence of materials of natural origin make it much more susceptible to microbial contamination and the instability associated with it. Preservative efficacy studies were positive, however, a suggestion that the extracts be sterilised prior to large scale manufacture is recommended. Gamma sterilisation can also be investigated since microorganisms were still present after disinfection with ethanol and hypochlorite while heat degrades the extracts.
7. CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

This research was influenced by African traditional healers who had from 2000 – 2008 collaborated with researchers from NMMU on a number of projects. Interactions with them had highlighted the need to produce a herbal topical product comprising of widely known medicinal plants. *B. frutescens* and *C. edulis* were selected due to their wide distribution and for the fact that their use is publicly known and documented. Wound healing and antiviral activity against HSV-1 were tested.

The individual and combined wound healing properties of the two plants were tested using a number of *in vitro* assays. *B. frutescens* proved to have good wound closure properties, was not cytotoxic to Vero cells and was minimally cytotoxic to 3t3-L1 cells. It did not have appreciable antibacterial and no antiviral activity against HSV-1. *C. edulis* was also mildly cytotoxic to 3t3-L1 cells but to a lesser degree than *B. frutescens*, the response of Vero cells to this plant extract was similar to that of *B. frutescens*. Wound closure was slower than that observed for *B. frutescens*. Antibacterial activity of *C. edulis* was also not notable, however, antiviral activity against HSV-1 was obtained at 250, 125 and 62.5 μg/ml. The combined, cumulative dose of compounds represented by rutin and catechin that permeated from the cream was 2.33 and 2.00 μg/ml for the T₀ and T₁ cream stored at 25 °C/65% RH. This value was obtained from two peak areas out of a total of 21 peaks. The total cumulative dose of permeating constituents could be closer to the concentrations exhibiting antiviral activity if all permeated constituents were quantified.

*C. edulis* showed good antioxidant activity using the DPPH assay, which was better than that of *B. frutescens*. The combined extracts were more cytotoxic to 3t3-L1 cells than the individual extracts but proliferation of Vero cells was greater with the combined extracts. This phenomenon could be due to differing sensitivities of the cell
lines. No increase in antibacterial activity was obtained with the combined extracts and they were active against HSV-1 at 250 μg/ml. Wound closure was faster than that of *C. edulis* but slower than *B. frutescens*. Antioxidant activity of the combined extracts showed synergistic activity. The use of these plants either individually or in combination for wound healing was validated. *C. edulis* was further validated for antiviral activity against HSV-1.

A gradient HPLC method for analysis of *B. frutescens* and *C. edulis* aqueous extracts was developed. Its ability to identify extracts obtained using different extraction methods and those altered by adverse conditions was demonstrated, it was also validated using routine pharmaceutical methods for validation of HPLC methods. Suitable markers and standards could not be obtained for *B. frutescens*, thus, surrogate markers were used.

The developed herbal cream which has a buffered cream as the base was stable at 25 °C/65% RH for one month. After this time the organoleptic properties began to deteriorate. Storage at accelerated conditions produced instability within one month. The preservatives are however, efficacious throughout the time that the cream was stored at all storage conditions.

**7.2 Limitations and recommendations**

It is not enough to prove wound healing and antiviral activity *in vitro, in vivo* studies should be conducted for full validation to be obtained. Thus the next step is to determine wound healing in animal studies. The combined extracts and the cream do not qualify for abbreviated toxicity studies as described by the WHO since their long term use has not been recorded.

Active-excipient studies should be conducted for the cream in order to identify and rectify areas where instability could be emanating to increase the products’ shelf-life.
Sterilisation methods besides heat sterilisation and chemical disinfection with hypochlorite and ethanol should be investigated.

7.3 The future of the collaboration

There is a great need for continued collaboration between scientists and African traditional healers. Such collaborations, if structured well, can be mutually beneficial to both parties leading to validation of current remedies and plants and the development and discovery of drugs from medicinal plants. Such collaborations though can be frustrating if there is no mutual trust and harmony between researchers and ATH and among ATH organisations within the collaboration.

As far as the collaboration between the Departments of Biochemistry and Microbiology and Pharmacy with ATH of the Nelson Mandela Bay Municipality is concerned, the collaboration is being resurrected and construction of the medicinal garden at the Missionvale campus of NMMU is progressing. Clinical studies of a *B. frutescens* and *C. edulis* cream with a shelf-life of more than one year will have to be undertaken in collaboration with the African traditional healers i.e. the clinical methodology that had to be abandoned by this researcher has be revived if the dream of a registered cream is to be realised.
REFERENCES


Homsy, J., King, R., Balaba, D., & Kabatesi, D. (2004). Traditional Health Practitioners Are Key to Scaling up Comprehensive Care for Hiv/Aids in Sub-Saharan Africa. AIDS, 18(12), 1723-1725. doi: 10.1097/01.aids.0000131380.30479.16


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APPENDICES

Appendix A

A1. Phosphate-buffered saline (PBS)

NaCl, Associated Chemical Enterprises; Glenvista, South Africa  
8 g

KH$_2$PO$_4$, Saarchem, Krugersdorp, South Africa  
0.2 g

Na$_2$HPO$_4$·12H$_2$O, Merck, South Africa  
2.9 g

KCI, Saarchem, Krugersdorp, RSA,  
0.2 g

RO water to  
1000 ml

Adjust to pH 7.4

A2. Buffered cream

Disodium hydrogen phosphate dodecahydrate; Merck, South Africa  
20 g

Citric acid monohydrate; Associated Chemical Enterprises (Pty) Ltd  
4 g

Methyl hydroxybenzoate; Minema, Johannesburg  
1.6 g

Propyl hydroxybenzoate; Minema, Johannesburg  
0.24 g

Emulsifying ointment  
240 g

RO water heated to 60 °C to  
800 g
A3. Emulsifying ointment

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Emulsifying wax</td>
<td>120 g</td>
</tr>
<tr>
<td>White soft paraffin</td>
<td>200 g</td>
</tr>
<tr>
<td>Liquid paraffin</td>
<td>80 g</td>
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