Pharmacological, Phytochemical and Safety Evaluation of Commercial Herbal Preparations Common in South Africa

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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STUDENT DECLARATION

Pharmacological, Phytochemical and Safety Evaluation of Commercial Herbal Preparations Common in South Africa

I, Ashwell Rungano Ndhlala, student number: 207526994 declare that:

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We hereby declare that we acted as Supervisors for this PhD student:

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1:

Ndhlala, A.R., Anthonissen, R., Stafford, G.I., Finnie, J.F., Verschaeve, L. and Van Staden, J. 2010. *In vitro* cytotoxic and mutagenic evaluation of thirteen commercial herbal mixtures sold in KwaZulu-Natal, South Africa. *South African Journal of Botany* 76: 132-138.

Contributions: Sample acquirement and laboratory analysis (mutagenic evaluation) was performed by the first author under the supervision of Dr J.F. Finnie and Prof. J. Van Staden. Dr R. Anthonissen and Prof L. Verschaeve performed the neutral red uptake assay for cytotoxicity as part of an ongoing collaborative link between the Research Centre for Plant Growth and Development and the Toxicology, Scientific Institute of Public Health, Brussels, Belgium. The second author, Dr G.I. Stafford helped in acquiring the commercial herbal preparations as well as in an informal survey to translate the Zulu words on the herbal preparations' packaging into English.

Publication 2:

Ndhlala, A.R., Stafford, G.I., Finnie, J.F. and Van Staden, J. 2009. *In vitro* pharmacological effects of manufactured herbal concoctions used in KwaZulu-Natal South Africa. *Journal of Ethnopharmacology* 122: 117-122.

Contributions: Sample acquirement and laboratory analysis was performed by the first author under the supervision of the last two authors. The second author, Dr G.I. Stafford helped in acquiring the commercial herbal preparations as well as in an informal survey to translate the Zulu words on the herbal preparations' packaging into English.

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1. Ndhlala, A.R., Stafford, G.I., Finnie, J.F. and Van Staden, J. Antimicrobial properties of selected plant constituents of a Zulu herbal mixture '*Imbiza ephuzwato*': 4th World Congress on Medicinal and Aromatic Plants (WOCMAP) – Using plants to benefit people, Cape Town, South Africa, 9-14 November, 2008.

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3. Ndhlala, A.R., Finnie, J.F. and Van Staden, J. Antibacterial, antifungal and anti-inflammatory activities of herbal concoctions used in South Africa. 34th Annual SAAB Conference and the 7th Southern African Society for Systematic Biology (SASSB). 14-17 January 2008. Drakensville resort, South Africa.

ABSTRACT

Herbal formulations claimed to cure several medical conditions including skin eruptions, chest pains, wounds, gout, menstrual pains, stress, nervous disorders, microbial and viral infections as well as stomach ailments have recently appeared as part of South African traditional medicine. The formulations consist of mixtures of extracts of different plant parts from several different plant species packaged in labelled bottles or boxes. The mixtures are available for sale in herbal shops and public places. While there has been widespread use of these herbal mixtures, there has been no scientific evidence to support their use. This project was aimed at documenting, validating claimed efficacy and testing the safety of fourteen unregistered commercial herbal preparations commonly sold in Pietermaritzburg. A detailed investigation of the pharmacological effects and safety of the plant components of one of the mixtures, *Imbiza ephuzwato* was also carried out.

Fourteen commercial herbal preparations, *Umzimba omubi, Umuthi wekukhwehlela ne zilonda, Mvusa ukunzi, Umpatisa inkosi, Imbiza ephuzwato, Vusa umzimba, Ingwe® traditional muthi mixture, Ibhubezi™, Supreme one hundred, Sejeso herbal mixture Ingwe®, Lion izifozonke Ingwe®, Stameta™ BODicare®, Ingwe® special muti and African potato extract™* were evaluated for activities against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria and the fungus *Candida albicans* using the microdilution assay to obtain minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC). *Imbiza ephuzwato*, *Ibhubezi™*, *Sejeso herbal mixture* and *Ingwe® special muthi*, which are multipurpose mixtures showed high antimicrobial activity ranging from 0.39 to 0.78 mg/ml. The fourteen herbal preparations were also evaluated for their ability to inhibit cyclooxygenase (COX-1 and COX-2), HIV-1 reverse transcriptase (RT) and acetylcholinesterase (AChE) enzymes. *Imbiza ephuzwato*, *Sejeso herbal mixture Ingwe®*, *Lion izifozonke Ingwe®*, *Ingwe® muthi mixture* and *African potato extract™* showed consistent potent activities against the four enzymes. *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Supreme one hundred*, *Sejeso herbal mixture Ingwe®* and *Ingwe® special muti* exhibited high antioxidant properties in the DPPH (2,2–diphenyl–1–picryl hydrazyl)

radical scavenging assay, ferric-reducing power assay and the ability to delay or halt the bleaching of *β*-carotene-linoleic acid in a model system. Phytochemical analysis revealed high total phenolic compounds, gallotannins and condensed tannins and lower levels of flavonoids in *Ingwe*® *special muti*, *Ibhubezi*™ and *Stameta*™ BODicare®. *Imbiza ephuzwato* exhibited high flavonoid concentrations and less total phenolic compounds, condensed tannin and gallotannin. Thin layer chromatography (TLC) was used to investigate the chemical profiles of each of the fourteen herbal preparations. It was not surprising to note that *Ingwe*® *muthi mixture*, *Sejeso herbal mixture Ingwe*®, *Lion izifozonke* and *Ingwe*® *special muti* showed similar chemical profiles as shown by their TLC profiles. The neutral red uptake inhibition bioassay in human liver (HepG2) cells was used to assess potential toxicity of the commercial herbal preparations. The results revealed that the most toxic herbal mixture was *Umpatisa inkosi*, with a 50% inhibition of neutral red uptake (NI_{50}) value of 0.016 mg/ml. The least toxic herbal preparation was *Stameta*™ *BODicare*® with an NI_{50} value of 28.00 mg/ml. The Ames test results revealed that all fourteen herbal preparations were non-mutagenic towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 without metabolic activation. However, five of the fourteen herbal mixtures (*Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Stameta*™ *BODicare*® and *African potato extract*™) showed indirect mutagenic effect toward the tester strain TA98 after metabolic activation.

A detailed study was done on one of the herbal mixtures, *Imbiza ephuzwato* which had shown consistent activities in the bioassays performed. The petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water extracts of the 21 plants used to make *Imbiza ephuzwato* herbal mixture were evaluated against two Gram-positive and two Gram-negative bacteria, *Candida albicans*, inhibitory effects against COX-1 and -2 as well as AChE enzymes. *Gunnera perpensa* rhizomes and *Rubia cordifolia* roots were the only plant species used to manufacture *Imbiza ephuzwato* that had water extracts which showed good antimicrobial activity.

The water extracts of *Hypericum aethiopicum* leaves, *Gunnera perpensa* rhizomes, *Drimia robusta* bulbs, *Vitellariopsis marginate* roots, *Scadoxus puniceus* bulbs and *Momordica balsamina* leaves at a final concentration of 2 mg/ml, had over 70% inhibition of COX-1. For COX-2 enzyme, the water extracts of *Gunnera perpensa*

rhizomes, *Cyrtanthus obliquus* bulbs, *Momordica balsamina* leaves and *Tetradenia riparia* leaves at a final concentration of 2 mg/ml exhibited inhibitory activity above 70%. Water extracts of *Gunnera perpensa* rhizomes, *Cyrtanthus obliquus* bulbs, *Vitellariopsis marginate* roots, *Asclepias fruticosa* roots and *Watsonia densiflora* corms showed good AChE inhibitory activity (> 80%).

The Ames test results revealed that all the water extracts of the 21 plant species used to make *Imbiza ephuzwato* were non-mutagenic towards the *Salmonella typhimurium* strain TA98 for the assay with and without S9 metabolic activation. The results obtained in this study reveal high levels of pharmacological activities by most of the herbal mixtures, providing scientific validation for their use.

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LIST OF ABRREVIATIONS

AChE.....	Acetlycholinestrerase
AD……	Alzheimer's Disease
AIDS.....	Acquired Immune Deficiency Syndrome
APL.....	Acute promyelocytic leukaemia
ATCC.....	American Type Culture Collection
ATM.....	African Traditional Medicine
ATMSA…	African Traditional Medicine for South Africa
ARV.....	Antiretroviral
BHT.....	Butylated hydroxytoulene
CNS.....	Central Nervous System
COX.....	Cyclooxygenase
CTE.....	Catechin equivalents
CTM.....	Chinese Traditional Medicine
CV.....	Crystal Violet
CYP450...	Cytochrome P450
DCM…….	Dichloromethane
DIG-POD.	Digoxigenin-peroxidase
DMEM….	Dulbecco's modified Eagle's culture medium
DNA…….	Deoxyribonucleic acid
DNDi……	Drugs for Neglected Disease initiative
DPM…….	Disintegrations per minute
DPPH…...	Diphenyl–1–picryl hydrazyl
EC.....	Effective concentration
ELISA…..	Enzyme-linked immunoassay
EtOH……	80% Ethanol
EU……	European Union
GAE…….	Gallic Acid Equivalents
GMP.....	Good Manufacturing Practice
HIV……..	Human immunodeficiency virus
HPLC…...	High Performance Liquid Chromatography
IC…..……	Inhibitory concentration
IKS……...	Indigenous Knowledge System
INT……...	Iodonitrotetrazolium chloride
IOCD….....	International Organization for Chemical Sciences
ISDNP…..	International Society for the Development of Natural Products
IUCN….....	International Union for the Conservation of Nature and Natural Resources
LC…..…...	Least concerned
LCE……..	Leucocyanidin equivalents
MFC………	Minimum fungicidal concentration
MH…….....	Mueller-Hinton
MIC…..….	Minimum inhibitory concentration
MLC………	Minimum lethal concentration
MPM.....	microtitre plate modules
MRC….....	Medical Research Council
NSAIDs....	Nonsteriodal anti-inflammatory drugs
NG…….....	Nematode growth
NI……..	Neutral red inhibition
NRU.....	Neutral red uptake
OD.....	Optical density
ORR.....	Oxidation rate ratio
PE.....	Petroleum ether
PGE.....	Prostaglandins
RCPGD....	Research Centre for Plant Growth and Development
RT.....	Reverse transcriptase
SAAB.....	South African Association of Botanists
SASSB.....	Southern African Society for Systematic Biology

HepG2….	Human hepatocellular liver carcinoma cell line 2
STD.....	Sexually transmitted diseases
TLC.....	Thin layer chromatography
TRAMED	The Research Group on Traditional Medicine
UCT.....	University of Capetown
UKZN.....	University of KwaZulu-Natal
UV.....	Ultraviolet
SDS.....	Sodium dodecyl sulphate
USD.....	United States Dollar
UWC.....	University of the Western Cape
WHO.....	World Health Organisation
WOCMAP	World Congress on Medicinal and Aromatic Plants
YM.....	Yeast medium
ZAR.....	South African Rand

CHAPTER 1
Introduction

1.1. Traditional Medicine

Throughout history people had to take measures to cope with health challenges such as respiratory ailments, coughs, colds, intestinal disorders, headaches, skin infections, wounds, burns, cuts, snakebites and insect bites using available resources such as herbs, marine organisms and other animal products. The way these communities prepared healing remedies formed an information reservoir that was passed from generation to generation **(PUJOL, 1990; LIU, 2005)**. A large number of studies have so far been conducted on the traditional usage of plants and resulted in the isolation of bioactive compounds for use in western medicine **(GILANI and RAHMAN, 2005; PATWARDHAN, 2005; VAN VUUREN, 2008)**.

The emergence of drug-resistant infectious bacteria, viruses and parasites has resulted in the growing interests in medicinal plant research to search for new leads for modern medicine. The numerous plant extracts that have been and are currently used safely by communities throughout the world have become a source of hope in the discovery of new and effective remedies **(GRAYBILL, 1996; BODEKER, 2004; LEUNG, 2004)**.

Traditional medicine encompasses health practices, approaches, knowledge, and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singly or in combination, to treat, diagnose and prevent illnesses or maintain well-being **(WHO, 2001)**.

1.2. African Traditional Medicine in South Africa

In southern Africa, the local communities, mostly those who dwell in communal homesteads are truly the masters of nature, deriving most of their medicine, nutritional and material resources from indigenous vegetation **(PUJOL, 1990)**. They

make sleeping mats and baskets from grass and reeds. They believe strongly that plants are powerful sources of healing remedies.

According to the **DRAFT NATIONAL POLICY on African Traditional Medicine for South Africa (ATMSA) (2008)**, 'African Traditional Medicine (ATM) is a body of knowledge that has been developed and accumulated by Africans over tens of thousands of years, which is associated with the examination, diagnosis, therapy, treatment, prevention of, or promotion and rehabilitation of the physical, mental, spiritual or social wellbeing of humans and animals.' While Chinese Traditional Medicine (CTM) is centred on the '*yin-yang*' theory which states that every human being is under three different axes of balance, i.e. balance between 'heat and cold', between the 'surface and depth,' between 'emptiness and fullness' **(LEUNG, 2004)**, ATM is driven by the spirit of '*ungumuthu ngabantu*' i.e. the essence of being human, open and available to others, affirming of others, and not being threatened.

The application and philosophy of ATM in different tribal communities of Africa, including South Africa is essentially the same. Sickness or diseases are believed to be caused by supernatural powers through spiritual entities, ancestral spirits, living people, animals, plants and 'pollutants'. Ingredients obtained from animals, plants and administered by a chosen medium can restore health and therefore have medicinal properties **(KALE, 1995)**. In ATM, the term 'disease' refers more to symptoms than a disease entity. The healer controls symptoms with different means but not for a specific disease. In most cases, the healer lacks the exact pathology and sequence of events in illness. The healer can therefore treat only symptoms **(LEUNG, 2004)**.

In Zulu communities, the traditional herbalist (*Inyanga*), who has the knowledge of herbal healing, is the one who takes care of the health of the community. Modern herbalists now spend most of their time learning and acquiring knowledge in herbal medicines while dealing with both human and livestock ailments. In traditional communities, an ill person generally has four options for their health care **(MARSLAND, 2007)**. Firstly, they can opt for self care which involves self treatment using information that is passed on from generation to generation within families and communities. Secondly, they can ask help from family heads where the elders will

use their experience in treating several conditions. The third option is to consult a traditional healer such as an *Inyanga* while the fourth option is to go to a modern clinic or hospital **(PUJOL, 1990)**.

In these communities, modern clinics and hospitals are in many cases the last option when dealing with illness. Most people when they cannot self-treat seek relief from an *Inyanga* and when 'bewitchment' is suspected, the patient is referred to a spiritual healer (*Sangoma*). Bewitchment is the unusual illness that is believed among black African cultures as inflicted by a person who has been offended by a victim's behaviour and/or success **(DOUGLAS, 1999)**. The *Sangoma* uses various forms of divination to find the root cause and ultimately the cure of the illness. These include 'throwing of bones' or going into a trance and in some cases rituals and sacrifices can be performed to appease ancestors (*amadlozi*) who are also believed to cause illness in the case of sexual infidelities, violations of taboos, lack of ritual observances, and general breakdown in traditional family life that accompany urbanisation **(PUJOL, 1990)**.

This intricate bond shows that traditional medicine is part of African culture, enabling the local people to guard themselves against diseases and alleviating them from suffering.

1.3. Modernization and commercialization of ATM in South Africa

In the late nineteenth century, during colonialism, the conversion to Christianity and the rise in capitalism saw the introduction of modern medicine, which makes use of health care systems based on science and technology **(PRETORIUS *et al*., 1993)**. Since then, *Inyangas* and *Sangomas* were regarded as 'witchdoctors' who took advantage of the ignorance of the uneducated rural communities **(RAPPAPORT, 1980)**. Despite this, modern medicine has not managed to completely replace ATM. Traditional medicine will continue to be the priority for most African populations mainly because it is accessible, affordable, acceptable and dependable **(NCHIDA, 1976; WHO, 2001)**. Furthermore, modern medicine is in some cases regarded as

culturally irrelevant and ill-suited in dealing with some complicated illnesses amongst African populations **(CHAVUNDUKA, 1994)**.

In South Africa, there exist about 200 000 practising traditional healers, compared to 25 000 certified doctors practicing in modern medicine. About 80% of the black population in South Africa use the services of these traditional healers, while 60% of babies are delivered by traditional attendants **(KARIM *et al*., 1994)**. Thus one can understand how traditional healers can withstand the competition from modern medicine. Traditional healers are enshrined in the minds of the people and respected in their community **(KALE, 1995)**.

However, according to the **SUPPRESSION OF WITCHCRAFT ACT (1957)** (amended in 1970), traditional healers were banned in South Africa but this represents an example of a law that exists only on the statute books as many organizations freely exist. These organizations include: the Southern African Traditional Healers Council, the Association of Traditional Healers of Southern Africa, the Congress of Traditional Healers of South Africa, the African Dingaka Association, and the African Skilled Herbalists Association **(ASHFORTH, 2005)**.

It was expected that the use of traditional healers will diminish as people becomes urbanized and the youth become westernised. Instead the influence of traditional healers runs deep within the black people's cultures **(SHERRIFS, 1996)**. **DE JONG, (1991)** reported that educated black people living in urban areas continue to use traditional healers for their primary health care. It is surprising to note that the demand for traditional healers could even increase with modernization since they become skilled in helping people to cope with the stress associated with current globalization **(HARDON *et al*., 2008)**. Despite western based modernization and urbanization, **MARSLAND (2007)**, reported that ATM is not merely a rural phenomenon but also an urban phenomenon increasing in frequency in both rural and urban black communities.

However, with rapid globalization there have been notable changes in the operation of ATM. With education, as people get involved in modern occupations, particularly the youth, traditional healers and herbalists are also evolving in order to

accommodate such social changes. Furthermore, the widespread use of internet, televisions, radios, newspapers and formation of social groups has offered traditional healers and herbalist a new platform for commercialization of ATM **(BONORA, 2001)**.

The evidence of commercialization of ATM is found in the prevalence of medicinal plant (*muthi*) trade in urban areas of South Africa. The ATM being practiced in urban areas does not differ with the practices used in rural areas as it also deals with both the so-called natural diseases (colds and headaches) and the supernatural diseases (witchcraft related and ancestry displeasure) **(BONORA, 2001)**. The term *'muthi'* refers to substances, in most cases plant material, prepared and administered as an aid to patients in distress by an experienced or trained traditional healer. Most healers or herbalists claim to possess secret knowledge regarding the mixing and administering of effective *muthi* to achieve positive healing powers **(ASHFORTH, 2005)**.

Several activities in the name of ATM can be identified in almost every town or city in South Africa, including traditional medicinal plant gatherers, herbal shops (*muthi* shops), herbal pharmacists, herbal hawkers, herbal wholesalers **(DAUSKARDT, 1990; BONORA, 2001)**, and recently herbal product processors and distributors. Figure 1.1A shows a typical herbal shop commonly found in both rural and urban areas; Figure 1.1B represents an urbanised herbal shop with mostly processed herbal preparations instead of the plants while Figures 1.1C and D represent a traditional open market and a modernized open market respectively.

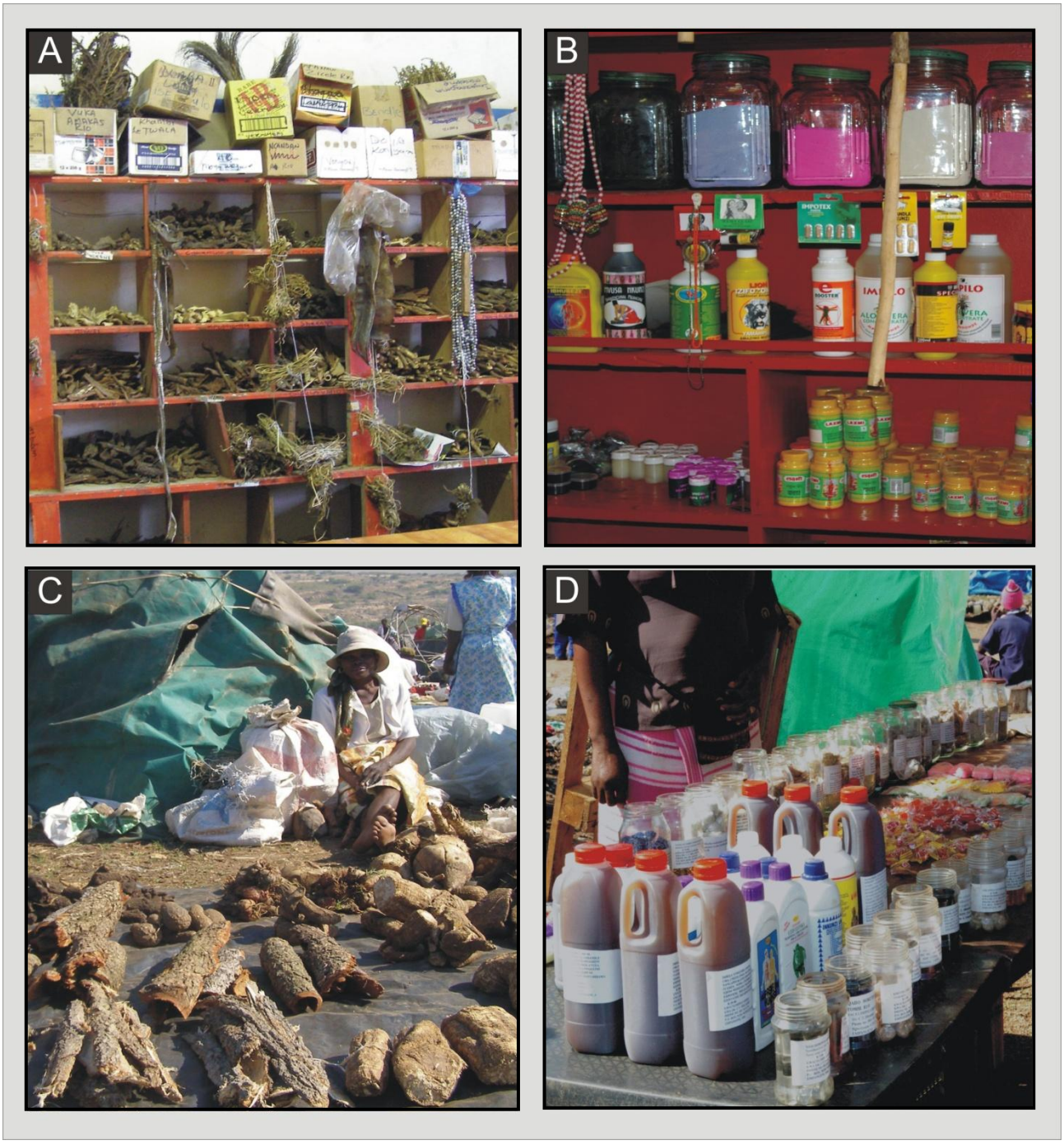


Figure 1.1: (A) Traditional herbal shop with dry plant materials on the shelf, (B) traditional urbanized herbal shop with plant material replaced by commercial herbal preparations alongside statues of Gods, candles decorated with lucky charms, and other items showing the blending of African Traditional Medicine (ATM) and Hinduism in Pietermaritzburg, (C) traditional open herbal market, (D) modernized herbal market (Nongoma, Zululand) with packaged herbal preparations dominating the products on sale.

It is estimated that over half a million people in both rural and urban areas are involved in the herbal trade in South Africa resulting in 525 tonnes of plant materials valued at 3.4 million USD being traded annually **(MANDER, 1998; DOLD and COCKS, 2002)**. In the province of KwaZulu-Natal only, about 6 million people are known to be involved in the trade of indigenous medicinal plants either by selling or buying **(MANDER, 1998)**.

It can be deduced that for such a huge traditional medicine trade to be present within urban areas, there has to be a great demand for such services. Urbanization and movements of people to informal settlements has introduced a range of traditional activities in the cities which subsequently became informal employment **(DAUSKART, 1990)**. Most households in South Africa spend between 4 to 6% of their annual income on ATM and services **(MANDER, 1998)**. As a result, the herbal trade has become a way of generating an extra income for most households. However, this has caused what is now an illegal plant trade and smuggling problem.

Such demand has also brought about competition amongst herbalists. The competition is so intense that even in the streets of urban towns, for example Pietermaritzburg, the capital of KwaZulu-Natal, different posters and pamphlets adverting herbal products and services are handed out to people in the streets. Figure 1.2 shows examples of some of the pamphlets. Besides pamphlets, advertisements of herbal products in newspapers, television and radio have also increased as a result of the competition. The pamphlets advertise services such as the protection of houses and cars as well as lucky charms for increasing chances of winning gambling games such as lotto and others offered at social establishments such as casinos using herbal medicines. Besides lucky charms, the pamphlets advertise services such as business attraction, job recovery and even promotion at work. There are however, some potentially dangerous and harmful services that are also advertised, such as abortion, '*short boys for money*' (*tokoloshes*- zombie like creatures made from dead human beings) and '*hiring of magic sticks*' (Figure 1.2). Such services have led to a negative stigma that is now being associated with traditional medicine in South Africa. This is unfortunate as these services are most likely the work of a group of money-hungry con-artists (*umthakathi*) who are

responsible for the numerous '*muthi*-motivated' killings (the use of human remains) in ATM. The *umthakathi's* intention is not to heal but rather to destroy.

The pamphlets have the contact information of the service provider such as the address where the consultations are done, business phone and cellular-phone numbers and/or street directions. Most of the pamphlets have names of the herbalists and in most cases with dubious titles such as *'Dr'* or *'Prof.'* (Figure 1.2). Some of the names on the pamphlets are not of Zulu origin. For example one of the pamphlets in Figure 1.2 has '*Dr Shaban* and *Mama Aminah*' as the herbalists, both of which are not Zulu names. This also shows that ATM is slowly being blended with other cultures such as Hinduism and Muslim practices as well as other North African cultures.

Figure 1.3 shows a newspaper cutting advertising a special sale of two commercial herbal mixtures (*Stameta* and *Uzifozonke*) in KwaZulu-Natal. Internet websites also offer much information about herbal products, but most are directed toward marketing and advertising of such products.

Figure 1.2: Examples of pamphlets handed to people in the streets of Pietermaritzburg, KwaZulu-Natal. The pamphlets advertise a range of services offered by commercial and modernized 'traditional' herbalists.

Figure 1.3: A newspaper cutting advertising special offers for two popular herbal mixtures amongst other products in KwaZulu-Natal: *Stameta* and *Uzifozonke* (highlighted), both commercialized multipurpose herbal mixtures. Values of products are in ZAR (South African Rand).

1.4. General aims and objectives

This project was aimed at documenting and evaluating claimed efficacy and safety of herbal mixtures manufactured by private entrepreneurs and commonly sold in herbal shops in KwaZulu-Natal. Commercial herbal mixtures, known locally as *muthi* concoctions provide a new facet and a monumental step in modernization and commercialization of ATM in South Africa. The doubtful efficacy, hygiene and safety of herbal products used in ATM are a concern as they are manufactured and sold on pavements and in open markets where the materials are often exposed to contaminants, in contrast with pharmaceutical manufacturing standards which are necessary for the production and packaging of such products. However, such mixtures may contain considerable amounts of active/lead pharmacological compounds. The finding of this study will help in the documentation and the long term understanding of traditional herbal mixtures as well as their efficacy and safe usage. The project will also offer a stepping stone for policy makers on the way towards regulating the manufacturing and selling of herbal mixtures/preparations in South African, a country undergoing rapid social changes and trying to keep pace with rapid globalization.

CHAPTER 2

Commercial Herbal Mixtures/Preparations

2.1. Introduction

The evolution of traditional medicines reflects a paradigm shift from using single agents (herbs, minerals and animals) to combining them to generate new herbal mixtures **(KONG *et al*., 2009)**. Initially, several centuries ago, traditional remedies consisted of only single agents **(ZHANG, 1993)**. With the accumulation of therapeutic experience, traditional healers realized that combining diverse natural medicines to constitute a mixture could effectively enhance the therapeutic effects **(KONG *et al*., 2009)**. This chapter examines and documents the prevalence of commercial herbal mixtures and preparations common in Pietermaritzburg, KwaZulu-Natal (Table 2.1). Different types of herbal mixtures and their claims will be discussed. Methods of preparation, advantages and disadvantages as well as the way forward in ensuring safety and efficacy of this new aspect of South African traditional medicine will be highlighted.

The use of herbal mixtures as part of traditional medicine has increased over the last decade. In 1997, an estimated 12% of the world population used over-the-counter herbal products and herbal therapy has since become popular. This has resulted in an estimated world market of 10 billion USD in the commercial herbal industry, with an annual growth of 6.5% **(RATES, 2001; ASHAR and DOBS, 2004)**. The WHO has since incorporated phytotherapy in its health programmes and calls for basic procedures for the validation of plant derived products **(WHO, 2001)**. In ATM, many healers rely not only on single plant extracts for healing but often combine various plant parts and even different species to make herbal mixtures, in the belief that efficacy may be increased.

Herbal preparations, in most cases are mixtures of selected medicinal plants or plant parts used to treat specific heath conditions including natural and spiritual diseases. Plant parts such as leaves, flowers, tubers, bulbs and/or roots from different plant species in specific proportions based on the desired function of the herbal product

are used. The mixtures could be simple, commonly known home remedies used to treat minor illnesses (such as colds, headaches, stomach pains and menstrual pains) or could be complex preparations often used for life threatening diseases **(PUJOL, 1990; RATES, 2001; CANO and VOLPATO, 2004)**.

2.2. Herbal mixtures in South Africa

Apart from the small scale use of herbal mixtures in ATM, there has recently been appearance in shops, pharmaceuticals and supermarket shelves of different informally 'patented' preparations, manufactured by private entrepreneurs making numerous claims to the efficacy of their products. The mixtures usually take the form of dark brown brews, in 500 ml to 1 litre labelled bottles, coloured solids or capsules. The mixtures represent something in-between ATM and western medicine. The recipes and preparations are of ATM origin while the packaging and presentations are western, but they lack safety and quality controls.

Consumers seek natural therapies mostly with the hope that plant products will sustain or restore health, even though health care practitioners and health authorities do not always believe in such products **(RATES, 2001)**. The other reasons for acceptance of herbal preparations include the perception that natural products represent purity, simplicity and safety which in some cases may be far from the truth **(TANKO *et al*., 2005)**.

Some herbal mixtures are manufactured in small quantities by informal street traders, market traders or individual traditional healers and are mostly packaged in recycled bottles. Most of the herbal mixtures on sale in shops are professionally manufactured in large quantities by private entrepreneurs in factories to produce packaged and labelled products. Table 2.1 documents some of the common herbal mixtures sold in Pietermaritzburg, KwaZulu-Natal including the manufacturer's details, medicinal uses and other information printed on the labels. Table 2.2, taken from **MANDER *et al*. (2007)**, lists numbers and types of formal as well as informal sector role players in the ATM industry in South Africa. Figure 2.1 presents some of the common herbal mixtures/preparations sold in shops around Pietermaritzburg.

Table 2.1: Information* on commercial herbal mixtures commonly found in Pietermaritzburg, KwaZulu-Natal

Product name **Trade name** Batch number	Manufacturer details	Medicinal uses and ingredients (as listed on the label)	Directions of use **Packaging** *Shelf life*
African potato extract- South Africa's miracle herb 6008306000506	Ngwenya quality product P.O. Box 747, Germiston 1400	Used to boost the immune system and treatment of HIV/AIDS symptoms.	1 cup daily **1 L**
Amandla esambane No. 1	Dr L. Bhayu C 188 Umzomusha, Danisa Rd, Inanda 4310 Mobile: 0768026451	Used to relieve: Blood pressure, period pain, HIV/AIDS, swollen body or legs, kidney infections, back pain, sores, rash, diarrhoea and chickenpox. Also helps to improve appetite.	4 teaspoons every morning, afternoon and evening. **1L**
Ibhubezi™	Pharmachem Pharmaceuticals, Private Bag X12, Pretoria West 0117	Used for wounds, fungal infections, STDs, treatment of influenza, to reverse impotence, clean the body system and stimulate blood production.	¼ cup twice a week. Not for children under 14 years of age and pregnant women. Shake well before use. **500 ml** *6 months*
Imbiza ephuzwato	Kwa Nyanga Yezizwe, 117 Retief Street, Pietermaritzburg Tel: +2733 3942570	A detoxifying and energising tonic used to increase sexual prowess, relieve constipation, reduce stress, reduce high blood pressure, clear skin conditions, boost energy, boost vitality, helps to prevent arthritis, kidney problems and relieve general body pains.	¼ cup in the morning after meals twice a week. **1 L**
Ingungumbane Mahlabizifo	Mobile: 0826905449 Fax: +27866119353	Used to relieve: 'hangover', ulcers, skin eruptions, blood pressure, asthma, gout and stress. Also used to increase CD4 count, reduce viral load, clean blood and increase sex drive. Contains *Sutherlandia*, *Aloe* extracts, African potato, fortified with vitamin B and C. Also contains brewer's yeast.	6 teaspoons in the morning and afternoon before meals. Not for pregnant women. Shake well before use. **1L**
Ingwe muthi mixture **Ingwe®** AMM 0011	Guideline Trading, P.O. Box 136701, Alberton North 1456 Tel: +2711 9070707	A traditional African mixture for chest Infections, STDs, arthritis, heart burn, relieving constipation and increasing sexual prowess.	3 tablespoons every morning. **500 ml** *6 months*

Table 2.1: continued

Product name **Trade name** Batch number	Manufacturer details	Medicinal uses and ingredients (as listed on the label)	Directions of use **Packaging** *Shelf life*
	sales@guidelinetr.com www.guidlinetrading.co.za		
Ingwe special muti **Ingwe®** AMM 003	Guideline Trading, P.O. Box 136701, Alberton North 1456 Tel: +2711 9070707 sales@guidelinetr.com www.guidlinetrading.co.za	Used for alleviating menstrual pain, general pain.	Take one 5 ml teaspoon in hot water or with tea every morning until the course is finished. Not for children under 14 years of age and pregnant women. **40 g**
Lion izifozonke **Ingwe®** AMM 003	Guideline Trading, P.O. Box 136701, Alberton North 1456 Tel: +2711 9070707 sales@guidelinetr.com www.guidlinetrading.co.za	Used for chest infections, STDs, arthritis, heart burn, relieving constipation and increasing sexual prowess.	¼ cup three times a day after meals. Not to be taken by children and pregnant women. **500 ml** *6 months*
Mvusa ukunzi	Kwa Nhlanhla, Indlu Yemithi, Pietermaritzburg	A 'man tonic' for increasing sexual prowess and can be used as an energiser.	Not on label. **500 ml**
Ngoma herbal tonic 710001001	Ngoma Natal, P.O. Box 11730, Dorpspruit 3200, South Africa Tel: +27797001370	Used as an immune booster, against diabetic and blood pressure conditions. *Ngoma* is also used for the relief of stomach ailments, arthritis, hypertension, stress and influenza. Contains *Sutherlandia, Echinacea* sp.*, Dandelion* sp.*, Alfalfa* sp.*, Aloe ferox, Harpagophytum* sp. and 13.5% alcohol.	1 tablespoon in the morning, evening after meals. Shake well before use. Not to be taken by children under 6 years of age, pregnant and breastfeeding women. **500 ml** *1 year*
S'mangaliso herbal mixture 6000528154548	P.O. Box 48292, Qualbert 4087 Tel: +27319095126	Used for treatment of HIV Aids symptoms, high blood pressure, period pain and cancer.	5 tablespoons per day for persons aged 13 years and over. **500 ml**
Sejeso herbal mixture **Ingwe ®** AMM 005	Guideline Trading, P.O. Box 136701, Alberton North 1456 Tel: +2711 9070707	Used to relieve heartburn, constipation, stomach ache, stomach cramps and indigestion.	¼ cup three times a day after meals. Not to be taken by children and pregnant women.

Table 2.1: continued

Product name **Trade name** Batch number	Manufacturer details	Medicinal uses and ingredients (as listed on the label)	Directions of use **Packaging** *Shelf life*
	sales@guidelinetr.com www.guidlinetrading.co.za		**500 ml** *6 months*
Stameta™ **BODicare®** 04020207	BODicare Products P.O. Box 2545 Florida 1710 South Africa	Used for nervous disorders, skin conditions, boosts sexual performance, poor blood quality, high blood pressure. Chest, lung and kidney infections. Fever and flu. Heart problems, back pain, persistent tiredness. Menstrual pain, cleans out bile, bleeding gums, body sores. Strengthens bones and boosts the immune system.	½ or ¼ cup three or four times a week. Not for children under 14 years of age and pregnant women. Drink water after using *Stameta™*. **500 ml**
Supreme one hundred **BODicare®** 05020207	BODicare Products P.O. Box 2545 Florida 1710 South Africa	Used for nervous disorders, skin conditions, stimulates blood production, boost sexual performance, treats back pains, fights influenza and strengthens the body.	¼ cup every night before sleeping after meals. Not for children under 14 years of age and pregnant women. **500 ml** *6 months*
Umpatisa inkosi	Kwa Nhlanhla, Indlu Yemithi, Pietermaritzburg	An 'adult tonic' used for increasing sexual prowess, as an energiser also used to treat sexually transmitted diseases (STDs), to stop menstrual pains, increase appetite, treat high blood pressure and fight arthritis.	Not on label. **500 ml**
Umuthi wekukhwehlela ne zilonda	Kwa Nhlanhla, Indlu Yemithi, Pietermaritzburg	Used as a cough mixture, to treat chest infections and difficulty in breathing.	Not on label. **500 ml**
Umzimba omubi	Kwa Nhlanhla, Indlu Yemithi, Pietermaritzburg	Used to treat wounds, skin rashes, fungal infections and boils.	Not on label. **500 ml**
Uvukahlale 100901713	Herbal solutions, P.O. Box 37219, Overport 4067, KwaZulu-Natal. Tel: +27312021082 Fax: +2732022147	Natural sex enhancer for men. The natural herbs contained in the mixture have long been used as tonics and aphrodisiacs. The mixture is a natural testosterone booster and increase sex drive and red blood cell production. Contains *Aloe ferox*, vitamins, trace elements, natural *tribulus* and is preserved in	½ a cup twice a day before meals. Use on regular basis. **500 ml** *6 months*

Table 2.1: continued

Product name **Trade name** Batch number	Manufacturer details	Medicinal uses and ingredients (as listed on the label)	Directions of use **Packaging** *Shelf life*
		sodium benzoate & potassium sorbate.	
Vuka Uphile herbal remedy 6009814670007	44 Milner street, Durban Tel: +27313687934 e-mail: vukaphile@webmail.co.za	Used to treat diabetes, TB, kidney infections, arthritis, back pains, influenza, period pains, high blood pressure, ulcers, painful eyes, painful ears and diarrhoea. Also used for treatment of inflammation related conditions. Also used to boost erection and purify blood.	¼ cup every morning and evening. Can safely be used after 3 months of giving birth.
Vusa umzimba **Ingwe®** AMM 0016	Guideline Trading, P.O. Box 136701, Alberton North 1456 Tel: +2711 9070707 sales@guidelinetr.com www.guidlinetrading.co.za	Used to treat wounds, rashes, fungal infections, boils and chest infections, stop menstrual pains, increase stamina and fight against influenza virus.	4 tablespoons twice a day. Not for children under 14 years of age and pregnant women. **500 ml** *6 months*

* Information in the Table was obtained through informal surveys from herbal shops in Pietermaritzburg in order to document the commonly sold herbal preparations.

Table 2.2: Formal and informal sectors of herbal product industry in South Africa

Formal/informal producers	Number of traders	Products sold
Retail herbal '*muthi* ' shops. Informal market.	Between 300 to 400 in South Africa.	These shops sell similar products to the informal trade: plants traded as raw material, chopped or as simple mixtures. They also trade quality tested and packaged traditional medicines supplied by manufacturers.
Health shops specializing in traditional herbal medicines. Formal market.	Number unknown for South Africa, but there are at least 5 in Durban.	Popular medicinal plants are sold in various processed and packaged forms (creams, mixtures, tablets). Products are manufactured by pharmaceutical companies and by private entrepreneurs. They also trade a number of non-traditional chemical mixtures.
Pharmaceutical manufacturers. Formal market.	5 to 10 in South Africa.	Usually single species products containing safe (non toxic) plants. Products include tablets, creams, tinctures or mixtures without any claims of efficacy. These products are manufactured using industrial Good Manufacturing Practice standards.
Private entrepreneurs. Informal market.	50 to 100 in South Africa.	Usually complex mixes of species (with claims of up to 50 species per single product) in various forms (creams, mixtures and tablets), with numerous unproven claims of efficacy. The safety of these products is not proven.

Table taken from **MANDER *et al*. (2007)**.

Figure 2.1: Examples of some herbal mixtures and preparations common in herbal shops and markets in Pietermaritzburg, KwaZulu-Natal. *Tokoloshe Salts*- protective charm against evil spirits and witchcraft; *Tshepe*- general tonic consisting of herbs fortified with vitamin supplements; *Impotex forte* and *Sex sugar*- herbal supplements for sexual dysfunction. Last insert shows a Pietermaritzburg bulk shopping store with an assortment of herbal mixtures and preparations.

The herbal mixtures are in most cases made up of combinations of plant species that have been used in ATM for centuries thus they represent a social heritage, and their ethnobotanical investigation can add much to the understanding of traditional medicine **(CANO and VOLPATO, 2004)**. Despite the obvious differences in the degree of 'modernization' of these herbal preparations, they share a common market and their formulation and conception are ATM. These herbal preparations are becoming extremely popular in South Africa and are becoming an important component of the medicinal plant trade and ATM. Most of the products have African names, dosages and indications are mostly written in South African languages, most commonly Zulu.

There exist a range of herbal preparations with different traditional uses. These include lucky charms, to drive away evil spirits. Such preparations are usually carried about on the person in the form of a protective talisman such as the *Tokoloshe* salts (Figure 2.1). The *Tokoloshe* salts are sprinkled (*intelezi*) around the homestead, added to bathing water or chewed and are often carried around the neck (*imfingo*) and the waist on ones person to drive away evil spirits. In rural areas, the *intelezi* is usually made of a mixture of bulbous plants. It can also be made using leaves and barks that are placed in a container (normally a broken used clay-pot) infused in cold water and sprinkled around the homestead or on the person. Plant species used for making *intelezi* and *imfingo* are mainly strong-smelling or strongly scented plants.

Other products include aphrodisiacs, used to increase sexual prowess, sexual appetite or stamina. These products include Fire-BarkTea® (*Bangala*) (Figure 2.2), mainly advertised on the internet as a 'love reed'. *Bangalala*, also known as *'African Viagra'*, is prescribed as an aphrodisiac, notably to enhance male potency. *Bangalala* is a product of a mixture of *Corchorus aspleniifolius* and *Eriosema cordatum* roots, both plants have a reputation of being powerful sexual tonics **(HUTCHINGS *et al*., 1996)**. Other species that are likely to be used for the same purpose includes *Securidaca longepeduculata* (Polygalaceae). The instructions on the packaging states that the roots are either boiled in water and the decoction is imbibed, or can be whisked into milk. The packaging of Bangalala (Figure 2.2) professes its love powers: "*It was once a closely guarded secret of a few Xhosa and Zulu witchdoctors but because of its potency and popularity it is now available to all*

who wish to enjoy its benefits". The packaging also declares it to be the most powerful tonic known to *Muthi* practitioners, prescribed throughout Africa for centuries. Apparently you cannot overdose on it; according to most herbalists, "*if you overdo this love drug, it will simply upset your stomach*". '*Sex sugar*' (Figure 2.1) is also another popular aphrodisiac product available on the market.

Another important group of herbal mixtures include the 'general tonics'. The names of these mixtures mainly mimic the strength of animals, for example '*Ingwe* (leopard) *izifozonke*' and 'Lion *izifozonke*' presented in Figure 2.3A and B. The word '*izifozonke*' means 'all diseases', thus the mixtures are used for many conditions. General tonics are in most cases administered orally by drinking a warmed portion or by adding the mixture to a traditional beer (*umcomboti*) or the lagers, fermented corn (*amageu*), traditional porridge (*uphutu*) and/or in tea. *Umcomboti* is an integral part of everyday Zulu life thus it is widely used in administering herbal preparations.

Mixtures used as general tonics may also have names that describe how they are administered or what they cure. An example includes a general tonic called '*Imbiza ephuzwato*' which literally means '*a medicine to drink'*. Some names describe the use of the tonic for example '*Umzimba omubi*' refers to bad skin, and is used to treat skin conditions such as rashes. The general tonics mainly consists of a mixture of up to 50 or 100 plant species used to treat a wide range of conditions.

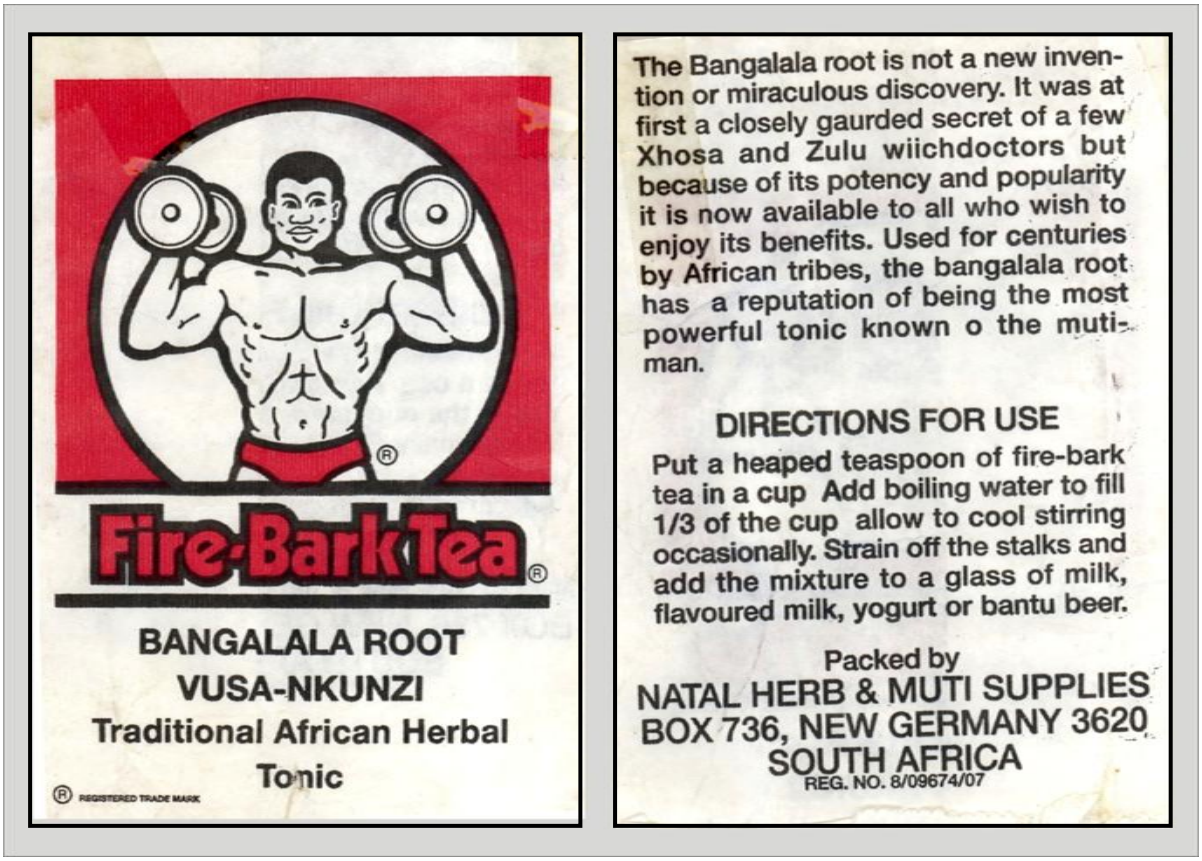


Figure 2.2: Fire-BarkTea® herbal tonic for men. Bangalala is claimed to be the most powerful aphrodisiac used in KwaZulu-Natal. It is sometimes referred to as '*African Viagra*' and is usually prescribed to men to enhance potency, increase sexual appetite and stamina; however women are also known to use the sexual tonic. The product is widely advertised on the internet.

Figure 2.3: Multipurpose mixtures ‘for all diseases’ with names mimicking strong and vicious animals. *Ingwe*- Leopard. *Izifozonke*- all types of diseases.

‘*Umckaloabo*’ is a multipurpose Zulu herbal remedy widely used as a tonic as well as treatment of tuberculosis, fever and cough. The remedy is also used to treat colds, influenza, pneumonia and malaria **(BRENDLER and VAN WYK, 2008)**. The term *umckaloabo* is derived from *isiZulu*, with a rough translation of ‘*a stabbing pain*’ or ‘*a stitch in the side*’. It is however, believed that the term may be an invention based on the Zulu language, intended to create a mysterious image of the remedy as a market strategy. Figure 2.4 shows an antique advertisement of *umckaloabo* referred to as “*The Doom of 150 000 People*”.

The plant ingredients of *Umckaloabo* were identified in the 1970s and to date the product is supported with data on efficacy and safety, provided through scientific research. *Umckaloabo* consists of the rhizome of *Pelagonium sidoides*. Various metabolites have been isolated and characterized from the rhizome of *Pelagonium sidoides*. These include phenolic compounds such as tannins, flavonoids and coumarins as well as essential oils. Antimicrobial activities against pathogens involved in infection of the respiratory tract as well as the immunomodulatory potential of the product have been conducted. Clinical trials with ethanolic extracts of the rhizome referred to as EPs® 7630 (Umcakaloabo®) have confirmed its efficacy in conditions such as acute bronchitis. *Umcakaloabo* is now popular in the EU and is fully licensed **(BRENDLER and VAN WYK, 2008)**.

Other types of herbal mixtures include sedatives, herbal teas, resins, snuffs, and herbal cigarettes. From personal observation, general sales shows that herbal mixtures/preparations for abortion and those used as aphrodisiacs are by far the most consumed products in urban societies and rural communities.

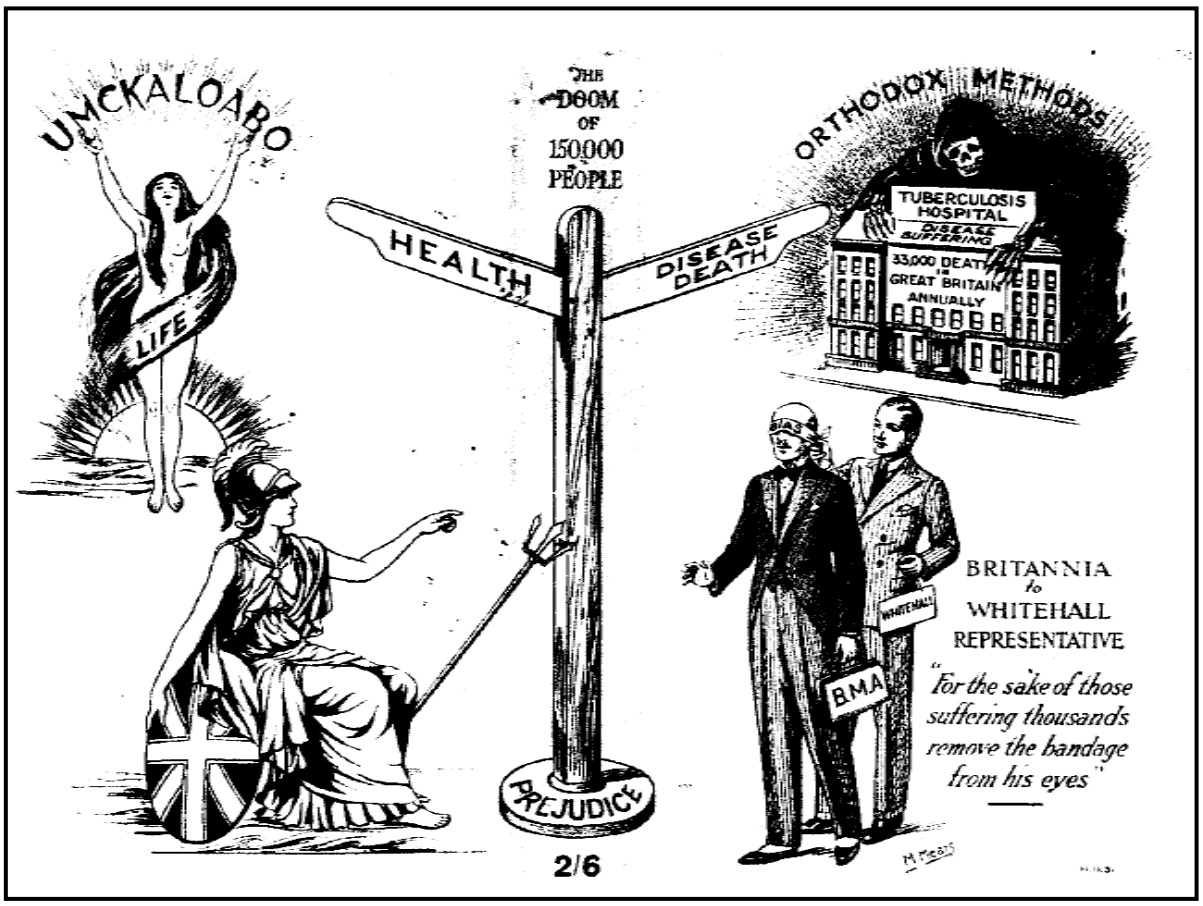


Figure 2.4: An ancient advertisement of *Umckaloabo* (*Pelagonium sidoides*) – '*The Doom of 150 000 People*' from the Dust jacket of Anonymous (1931). Taken from **BRENDLER and VAN WYK (2008)**.

2.3. Preparation of commercial herbal mixtures

Methods of preparing herbal mixtures varies from simple brewing processes to more complicated procedures that make use of alcohol and other organic solvents to dissolve the “essences” of the plant **(PUJOL, 1990)**. In some cases, addition of accepted western medicines, such as aspirin, has been recorded for some herbal mixtures **(CANO and VOLPATO, 2004)**. In other cases, clay has been reportedly detected in some herbal mixtures **(OATES, 1978)**. Clay binds and neutralizes the toxicity of chemical compounds such as alkaloids and tannins as well as heavy metals **(JOHNS, 1990)**. Therefore adding clay from termite mounds to herbal decoctions could represent a practical attempt to deal with toxic chemicals present in some medicinal species at the same time benefiting from their therapeutical properties **(CANO and VOLPATO, 2004)**.

Herbal preparations used as stomachic and sedative mixtures mainly contain aromatic plant species, rich in therapeutically-active essential oils **(PUJOL, 1990)**, which exert their antispasmodic, antibacterial and stomach-soothing properties. Mixtures for stomach ulcers and diarrhoea are characterized by plant species with high tannin content, popularly used as astringents, antimicrobials and anti-inflammatory **(CANO and VOLPATO, 2004)**.

Some herbal mixtures are medicinal-food formulae rather than herbal preparations. Fruits such as *Cocos nucifera* L. (Arecaceae) (Coco) and vegetables such as *Solanum americanum* Mill. (Solanaceae) (*Yerba mora*) are traditionally consumed in Cuba as food but are also reported to have specific pharmacological properties such as an anthelmintic function. The fruits of *Capsicum frutescens* L. var*. frutescens* (Solanaceae) (*Aji guaguao*) and seeds of *Myristica fragrans Houtt.* (Myristicaceae) (*Nuez moscada*), along with eggs, milk and wine are macerated together and taken as a tonic to reverse impotence and sterility **(ESQUIVEL *et al*., 1992)**.

Ingestion is the preferred means of taking herbal mixtures and topical application as a lotion is used to treat skin problems (rashes and boils) and inhaling is often used for mental conditions **(PUJOL, 1990)**. Decoctions are sometimes administered as

enemas (*uchatho* in Zulu). In urban areas, they are administered using enema syringes or tubes. However in rural areas, a lubricated, truncated cow's horn is often used **(VAN WYK *et al*., 1997)**.

There is however, very little regulation and scientific backing of these products in South Africa, posing a potential threat to consumer health. Many of these private entrepreneurs seem not to conform to industry Good Manufacturing Practice (GMP) standards leaving only a few certified pharmaceutical manufacturers producing formalized traditional herbal medicines **(MANDER *et al*., 2007)**. Despite the existence of the Medicines and Related Substances Control Act 101 of 1965 and its amendments of 2002 **(DEPARTMENT OF HEALTH, 1965)** it is still difficult to regulate commercially labelled medicines, herbal formulations and nutritional supplements. Medicine regulation, is in the public interest, and comprises of three integral aspects: quality, safety and efficacy. In terms of quality the hygiene and potential contamination of herbal products used in traditional medicines are a concern as they are sold on pavements and in markets where the materials are often exposed to sputum, urine and faeces, contrasting with the pharmaceutical manufacturing standards which are necessary for production and packaging of other medicines **(RATES, 2001; STEENKAMP *et al*., 2006)**. It should however be noted that in ATM, it still remains a challenge to convince herbalists to reveal their ingredients as well as the recipes of their products as it is a guarded secret amongst themselves.

The lack of standardization of these commercial products can also be as a result of high investment requirements for the development of clinically tested and certified medicines especially those derived from medicinal plants. Intellectual property rights regarding products from natural resources and the high cost of resolving such issues prior to investing in product development can impact directly to the consumer through higher prices. An increase in the price of these products will make them inaccessible to the majority of current users. Also there are risks associated with sustainability of raw plant material supplies, as the majority of indigenous medicinal plants cannot be cultivated in local gardens **(MANDER *et al*., 2007)**.

In a country like South Africa, it is difficult to enforce any quality and safety controls on natural products that are not listed as registered pharmaceuticals because there exist parallel bodies within the government with different views on the use of natural products. One group is advocating for the use of ATM and the other putting in stringent laws regulating the use of certain plants. The National Policy on African Traditional Medicine seeks to address this dichotomy.

2.4. Mode of action of herbal preparations

The use of herbal mixtures presents unique challenges not encountered by western single compound medicines. Since these preparations are mostly complex mixtures of different plant species with different bioactive compounds, the indications and use criteria for western single compound medicines may not be applicable. Compared to single compound pharmaceuticals, traditional herbal mixtures may have more than one mode of action **(YONG and LOH, 2004)**.

2.4.1. Combinational or additive effects

The healing effects of herbal mixtures could be as a result of the total sum of different classes of compounds having diverse mechanisms of action. An example of an herbal preparation that could simulate combinational effects is *Gingko biloba*, commercially known as ginkgo. The herbal preparation is used in western countries for brain dysfunction, to improve memory and cognition **(YONG and LOH, 2004)**. The bioactive compounds in the preparation are known to be flavonoids and diterpenes called ginkgolides. These compounds are potent inhibitors of the actions of platelet-activating factors, which are important for platelet activation and clotting. In addition, ginkgo has also been shown to exhibit antioxidant properties. Platelet activating and antioxidant effects could combine to reduce inflammation and increase microcirculation of blood flow and improve brain function. Gingko has also been reported to have membrane stabilising effects and smooth muscle relaxation properties which could also contribute to the total vasodilatation and microvascular effects of the preparation **(YONG and LOH, 2004)**.

2.4.2. Synergistic effects

Synergism in drugs occurs when compounds interact in ways that enhance or amplify one or more healing effects of mixtures of drugs than when they are administered individually **(BECKER *et al*., 2004)**. Therefore, a partially purified extract of a plant offers advantages over a single isolated ingredient. This is the concept that underpins the philosophy of herbal mixtures **(YONG and LOH, 2004)**. The presence of synergistic properties in medicinal plants is an old concept put forth by Hippocrates (an ancient Greek physician) and reinforced by Ibn Sina (the great Persian Physician). However, this concept lacks sufficient scientific evidence **(GILANI and RAHMAN, 2005; VAN VUUREN, 2008)**.

Apart from the studies on combinational interactions of commercial oils in South Africa by **VAN VUUREN *et al*. (2007)**, the importance of the concept of synergism can clearly be reflected in the evolution of traditional Chinese medicine (CTM). The history of CTM dates back 4000 years and is still popular and becoming even more accepted by western countries. The initial CTM prescriptions consisted of mainly single agents. With accumulation of experience, the Chinese traditional healers realized that combining diverse natural medicines to form an herbal mixture could efficiently enhance the therapeutic effect **(KONG *et al*., 2009)**.

To help understand the concept of synergism, the CTM compounds within a herbal mixture were usually assigned with different roles and names, such as ‘ master (*jun*)’, ‘adviser (*chen*)’, ‘soldier (*zuo*)’ and ‘guide (*shi*)’ **(KONG *et al*., 2009)**. The mission of the master compound is to treat the principal symptom of the disease. The ‘adviser’ compound potentiates the effect of the master compound or treats accompanying symptoms. The role of the soldier compound is to enhance and modulate the effects of the master and adviser compounds. The task of the guide is to lead the active ingredients to specific organs (drug delivery system) and to harmonise the actions of these compounds **(KONG *et al*., 2009)**.

Although at that time, the theory of synergism was not scientifically proven, it was justified for a Chinese formula (*Fu Fang Qing Dai Pian*) created by Dr Huang Shi-Lin 20 years ago. The mixture consists of *Realgar* (tetraarsenic tetrasulfide) as the

master, *Salvia miltiorrhiza* as the adviser/guide, *Indigo naturalis* as the soldier/guide and *Radix pseudostellariae* also as adviser but not essential for the mixture. The mixture is used in the treatment of human acute promyelocytic leukaemia (APL). The synergistic mechanism of the mixture was recently elucidated **(KONG *et al*., 2009)**. First the tetraarsenic tetrasulfide directly attack the promyelocytic leukaemia (PML)-retinoic acid receptor α (RAR α) oncoprotein and promotes APL cell differentiation, therefore it behaves like a 'master'. Secondly the principal components of *Salvia miltiorrhiza* and *Indigo naturalis*, is tanshinnone IIA and indirubin, respectively, which increases tetraarsenic tetrasulfide-induced degradation of PML -RAR α, thus serving as 'adviser' and 'soldier'. Finally, indirubin and tanshine IIA which also works as a 'guide' enhances the expression of aquaglyceroporin 9, which helps transport tetraarsenic tetrasulfide into APL cells where it carries out its effect **(KONG *et al*., 2009)**.

Another theory categorises the components of herbal mixtures into four roles: role of an 'emperor,' of an 'adviser,' of a 'minister' and of an 'ambassador.' The philosophy demands a balanced system that includes effectiveness, efficacy, reinforcement, moderation and safety. Based on this philosophy, poisonous herbs can be used when there is need to forcibly bring about a effective treatment, while toxicity is being checked and controlled by other herbs that possess different effects or that act as antidotes, low doses on the other hand are supplemented by a variety of other herbs present in the mixture **(LEUNG, 2004)**. This clears the mystery that surrounds the use of poisonous herbs in herbal mixtures. Most of the mixtures presented in Table 2.1 contain at least one toxic plant. These includes potentially poisonous herbs such as *Gomphocarpus fruticosus* (Apocynaceae), *Scadoxus puniceus* (Amaryllidaceae) and *Drimia robusta* (Hyacinthaceae) which forms components of '*Imbiza ephuzwato*', a popular Zulu herbal multipurpose mixture.

2.4.3. Dose response relationships

Sometimes dose-dependent effects of combinational medicine are encountered in efficacy studies, where higher doses used in traditional medicine have therapeutic effects. This has been shown by the extracts of goldenrod (*Solidago virgaurea*) which in low doses have no diuretic effect, whereas in traditional medicine where

higher doses (6-12 g dried herb per cup) are prescribed, it has diuretic effects **(YONG and LOH, 2004)**.

2.5. Advantages of using herbal mixtures

Western medicine is based on the lock and key theory, which uses one agent to treat one disease. However, as the mechanisms of many diseases involve multiple factors, a one-agent-one-target approach normally fails. In addition, as the human body is a complex network, inhibiting a single target (organ or receptor) usually has very little therapeutic effect **(KONG *et al*., 2009)**.

Furthermore, inhibiting a single target can exert unexpected side effects because of the breaking of the balance of the complex body network. It is assumed that herbal mixtures modulate the biological networks in a holistic way and in this way may be efficient in controlling disease systems **(YEH and KISHONY, 2007)**.

Herbal mixtures containing multiple and complex compounds thus can deal with drug resistance that is being observed for most antibiotics, antimalarial, antiviral and anticancer drugs **(KEITH *et al*., 2005)**.

Herbal treatments cost much less than western prescribed drugs. Herbal mixtures are also more convenient as they can be purchased over the counter from any herbal or food store without the need for a prescription. Herbal mixtures are not yet categorized as drugs, they are considered as food and supplements, thus they are not subjected to the same strict scientific inspection as prescription medication **(CANO and VOLPATO, 2004)**.

In China, hospitals have begun treating the current outbreak of influenza virus (*H1N1*) infected patients with an herbal remedy instead of the WHO-recommended *Tamiflu*. The herbal treatment is a mixture of four locally-used medicinal herbs: *Jin yin hua* (*Lonicera japonica*), *Da qing ye* (*Lsatis indigodica*), *Bo he* (*Mentha haplocalyx*) and *Sheng gan cao* (*Glycyrrhiza glabra*). Doctors in Beijing first began using the remedy on May 15, 2009, treating patients with a combination of the herbal

remedy and *Tamiflu*. After successful trials, doctors have since begun prescribing only the herbal treatment. Wang Yuguang, head of the Centre of Integrated Chinese and Western medicine at Ditan Hospital, said that treatment time for the herbal remedy was comparable with that of *Tamiflu* treatments. The big difference, he said, was the cost of the two treatments. The CTM remedy only costs between 10 and 13 Yuan (between 1.50 and 1.90 USD), while *Tamiflu* costs 56 Yuan (8.19 USD) **(KRISHNAN, 2009)**.

The Chinese Ministry of Public Health has expressed concern about the virus' increasing resistance to *Tamiflu* and fears of the drug's side-effects. However, the Chinese government appears to have given full-backing to promoting the herbal remedy **(KRISHNAN, 2009)**. Professor Zhitao Tu, dean of the research and education department at the Beijing Administration of CTM confirmed that "*in China*, *Tamiflu is not sufficient, and it also weakens the body and has adverse effects on body tolerance*". Prof. Tu said the essential difference was that while the CTM remedy works to build up the body's resistance in a holistic way, the *Tamiflu* drug focuses on attacking the infection **(KRISHNAN, 2009)**.

2.6. Problems associated with use of herbal mixtures

Despite the popularity of botanical supplements, many herbal mixtures on the market are of low quality and doubtful efficacy. Researchers and clinicians are usually concerned about safety, effectiveness and consistency of herbal mixtures and preparations. The low quality of herbal products is usually due to several parameters. These include a variety of poorly controlled factors such as collection of raw herbs, processing methods and complex heterogeneity of compounds, and unpredicted consequences when herbs are mixed with western medicines as well as a lack of scientific validation **(McINTYRE, 1998)**.

2.6.1. Problems due to identification, harvesting and manufacturing

Plant species used in herbal preparations often have very similar appearances, especially between subspecies or even varieties within the same family. These could

be even harder to separate when the materials are dried. This poses a great threat as different plants, even when in the same genus, can have very different chemical constituents. Inappropriate use of herbs through misidentification is one of the major causes of morbidity and mortality in local communities **(RATES, 2001; TANKO *et al*., 2005)**.

Between 1992 and 1993, an outbreak of a rapidly progressing renal failure was recorded in Belgium and was linked to a slimming Chinese herbal mixture containing *Stephania tetrandra* and *Magnolia officinalis*. Chemical analysis of the herbal mixture sold in Belgium at that time pointed to a misidentification between *Stephania tetrandra* and another potentially nephrotoxic Chinese herb, *Aristolochia fangchi* **(YONG and LOH, 2004)**.

Currently, in South Africa, manufacturers are not bound by any law to have good manufacturing practices. Therefore, inconsistent methods used by the manufacturers can result in different quality levels between batches of a product. Different therapeutic potentials in herbal products result from different factors such as the age of plants, season of harvesting, geography of plant habitat and post harvest handling. Furthermore, herbal mixtures may not be pure and it is possible they may contain pollen grains or heavy metal contaminants which could induce sickness. Currently, introduction of standardized manufacturing and testing protocols are much needed in the herbal industry **(MANDER *et al*., 2007)**.

2.6.2. Lack of clinical efficacy and safety evidence

The risk and benefits of most herbal mixtures are not supported by scientific or clinical testing. Randomised control trials are the standard for clinical efficacy and currently they have been done on a handful of herbal products which are now acceptable in the European Union (EU) and other western countries. These products include gingko, St John's wort (*Hypericum perforatum*), ginseng, saw palmetto and kava **(YONG and LOH, 2004)**. Little or no information exists for the vast majority of the herbal mixtures flooding today's market **(YEH and KISHONY, 2007)**.

2.6.3. Herb-drug and herb-herb interactions

There is sparse documentation regarding the interaction between western medicines and herbal products. However, research on the common herbs indicates that significant herb-drug interactions exist. St John's wort, a common herbal product is known to interfere with cyclosporine, digoxin, warfarin and theophyline and has been shown to cause intermenstrual bleeding when used together with oral contraceptives (ethinylestradiol/desogestrel). It also causes serotonin syndrome when used together with selective serotonin-reuptake inhibitors (sertaline, paroxetine and nefazodone). Ginkgo interactions include bleeding when combined with warfarin and raised blood pressure when combined with a thiazide diuretic. Garlic (*Allium sativa*) changes the pharmacokinetic variables of paracetamol and produces hypoglycaemia when taken with chlorpropamide **(YONG and LOH, 2004)**. Thus interactions between herbal products and western synthetic drugs exist and can have fatal consequences **(YEH and KISHONY, 2007)**.

2.6.4. Inadequate government regulations

In many countries, including South Africa, herbal remedies are sold for stimulating, maintaining, supporting, regulating and promoting health rather than treating disease. In South Africa, such supplements are required to carry a label that describes the ingredients intended to affect the structure or functions within humans in line with Act 101 of 1965 and amendments (2002) **(DEPARTMENT OF HEALTH, 1965)**. However, this represents another example of a law that exists only on the statute books as many herbal mixtures are being sold without ingredient information on their labels. It is also regrettable that this law does not require product standardization for uniformity between batches. It is an important factor in the future to investigate batch to batch pharmacological activities in order to understand the effects of storage and other manufacturing practices. Furthermore, the use of different processing methods of a particular herb could result in inconsistencies in chemical constituents **(YONG and LOH, 2004)**.

2.6.5. Insufficient consumer education

Many consumers have the belief that herbal mixtures are safe and natural. It is however, important for such consumers to be told that even though herbal mixtures have been in use for a long time, they still bring with them risks of side effects **(STEENKAMP *et al*., 2006)**. An example of a herbal product that has been used for a long time but still have a lot of side effects is *ma huang* (*Ephedra sinica*), commonly sold as a weight loss product. *Ephedra sinica* was traditionally used to treat a variety of conditions, including asthma, hypotension and depression. The traditional communities have, over the years come to recognise that *ma huang* is not a safe herbal product because it has side effects. The side effects include tremors, nervousness, insomnia, headache, gastrointestinal distress, high blood pressure, irregular heart beat and kidney stones **(TINSLEY, 1999)**.

2.7. When do herbal mixtures become poisonous?

Poisoning due to plant products (herbal poisoning) is not well documented. This is mainly because people are unwilling to admit poisoning by herbal products because of the fear that their cultural heritage will be put under strong laws and regulations **(STEENKAMP *et al*., 2006)**.

The largest numbers of acute poisoning occur in the age group 1-5 years and according to descriptions on most of the labels of the herbal mixtures (Table 1), most preparations are not recommended for children under the age of 14 years. In east Africa, the widespread use of *Crotalaria* sp. in the treatment of measles amongst children has resulted in a number of cases of liver lesions **(STREET *et al.*, 2008)**.

Adult poisoning occurs usually as a result of mislabelling of products, or products which are not stored in their original containers. Occasionally, poisoning is through confusing a toxic plant with something that is thought to be edible **(VAN WYK *et al*., 2002)**. In some instance, adults could be poisoned by taking incorrect doses thus it is also important for the manufacturers to clearly state the directions for use and possible side effects.

A pharmaceutical product is defined as being stable if it has five basic properties which are: physical, chemical, microbial, toxicological and therapeutic stability. Many natural products readily oxidise and degrade, giving products with variable properties. Carotenoids such as *β*-carotene and lycopene may have antioxidant properties as intact molecules yet their degradation products can be toxic **(YEH and HU, 2001; HALLIWELL, 2004)**.

Microbial contamination from soil, during handling of plants and storage is a potential threat to the consumer's health. Aflatoxins are among the deadly products that are generated by bacterial/fungal infections **(AMES and GOLD, 2000; HALLIWELL, 2004)**. Heavy metal contamination and pesticide and herbicide residues from the soil are also a common cause of herbal poisoning. Heavy metal contamination can be introduced during sample preparation, for example when extraction of herbal products is done in lead containers **(ONG *et al*., 1999)**. Dry mixtures are often coloured with mineral compounds which contain heavy metals such as copper, iron and cadmium.

2.8. Research and development in herbal mixtures

Despite the difficulties of quality control on herbal products because of their heterogeneous constituents, different mechanisms of action and unusual dose-response relationships, scientific efficacy and safety evaluation are still possible **(YONG and LOH, 2004)**. This is best reflected in the evolution of CTM and of traditional medicine native to India (Ayurveda medicine), from which South Africa may be able to learn a lot. While CTM has a history of more than 4000 years, it is still popular in China and becoming more and more accepted by western countries, Ayurveda remains an influential system of medicine in South Asia **(KONG *et al*., 2009)**.

The most ancient CTM book of prescriptions for fifty two diseases, compiled around 300 BC, recorded 247 agents and 150 formulae (herbal mixtures and preparations). In another survey published two thousand years later, the number of single CTM

agents had only risen seven-fold, from 247 to 1892, while the quantity of herbal mixtures increased more than 400 times, from 150 to 61,739. To date, there are about 500 combinational agents used to tackle a wide range of diseases in CTM **(KONG *et al*., 2009)**.

Research into herbal medicine is urgently needed and if this need is to be met, dedicated funding will be required to be allocated by the government. Research will ensure a rational basis for the use of herbal medicines. Examples of areas that still need attention include:

- Research aimed at the safe and effective production of herbal products. The pharmacodynamics, pharmacokinetics, safety and efficacy of bioactive herbal mixtures can be examined in animal models;
- Education and training for both traditional and orthodox medicinal practitioners. There is a concurrent need for self-regulation by senior traditional health practitioners, focusing on peer supervision and enforcement of standards of professional practice and conduct; and
- Research policies that promotes clinical and quality assurance studies. National testing centres are needed and should be appropriately funded.

In South Africa, an initiative has now been made by the formation of research groups that are devoted to the research on herbal medicines. ATM and its pharmacopoeia form the core business of a new research group formed by the Medical Research Council (MRC), the University of Cape Town (UCT) and the University of Western Cape (UWC). The Research Group on Traditional Medicine (TRAMED) is based at UCT and is headed by Professor Peter Folb of the Department of Pharmacy and Professor Peter Eagles of the School of Pharmacy at UWC. The establishment of the Indigenous Knowledge Systems (Health) Office (IKS) at the Medical Research Council, headed by Dr Motlalepula Matsabisa has contributed greatly in terms of research of herbal medicines in South Africa. The IKS office is actively involved with the Drugs for Neglected Disease initiative (DNDi) project. The DNDi project aims at developing new drugs for neglected diseases such as malaria. Other research groups include the Research Centre for Plant Growth and Development (RCPGD), headed by Professor van Staden, based at the University of KwaZulu-Natal (UKZN)

Pietermaritzburg, University of Pretoria research group headed by Professor J.J.M. Meyer, Tshwane University of Technology group headed by Professor A. Viljoen, University of Johannesburg group headed by Professor B.E. van Wyk and the Pytomedicine programme, University of Pretoria, headed by Professor J.N. Eloff are also actively involved in conservation and testing for efficacy of medicinal plants and their products. The major aims of all these research groups include documenting traditional medicines and herbal products derived from indigenous medicinal plants while attempting to identify and isolate their therapeutically active components, thereby establishing a database of ATM in South Africa.

Both scientific and political will are needed to support the development of a data base of ATM. In poor countries, the search for effective and affordable treatments for epidemics such as malaria and opportunistic infections associated with HIV/AIDS is driving renewed policy interests in herbal medicine research. Substantial increases in research funding are therefore needed and new directions in clinical evaluation must be forged by researchers who are able to transcend limitations in research orthodox in the interests of providing sound information to the public on what constitute good health care **(BODEKER, 2004)**.

A vital requirement in ensuring that policies such as the **National Policy on ATMSA (2008)** which is still at a draft level and the Medicines and Related Substances Control Act 101 of 1965 become enacted is an adequate level of development. Most South African policies only exist in the statutory books and are never enacted or are not adequately policed/enforced. This trend suggests that the way forward in policy development should be via a combination of concerted local consultation with all relevant interest groups, matched by an exchange of information and experience with international partners who have developed some perspective or have experience in one or more areas of herbal medicines such as in China and Germany.

Some recipes and validation of claimed properties of herbal mixtures have been reported in China, Japan, Cuba and Syria **(CANO and VOLPATO, 2004; CARMONA *et al*., 2005; LEE *et al*., 2006)**. In China, there exists a number of government supported groups responsible for quality and safety of CTM. In

Germany, The Ministry of Health runs a commission (Commission E) comprising of doctors, pharmacists, scientists and herbalists to evaluate quality, safety and efficacy of herbal products **(VALLI and GIARDINA, 2002)**. The regulations are designed so that manufacturers have to provide proof of quality according to pharmaceutical standards for herbal medicines. This includes statutory declarations for herbal ingredients, dosages, application/administering instructions and intended use. On the other hand, safety and effectiveness are relegated to monographs published by Commission E **(YONG and LOH, 2004)**.

The setting up of government supported groups responsible for quality and safety of CTM has led to the advancement in their traditional medical systems so much that the Chinese government has sanctioned hospitals to launch CTM-based treatments for *H1N1* on a large scale. The herbal mixture is now undergoing clinical trials "*to show concrete evidence that the treatment is scientific*." The Chinese government has invested 10 million Yuan (6.83 million USD) in conducting a comparative study of herbal and *Tamiflu* treatments. Mao Yue, president of the Ditan Hospital, said, "*There was a serious possibility of a large-scale outbreak of the flu in the autumn and winter of this year, and that the government has already begun procuring enough herbs to treat a possible 2 million patients*" **(KRISHNAN, 2009)**.

2.9. Conclusions

Due to the relatively recent development of herbal preparations very little is known by the scientific community about the epistemology influencing herbal preparation design, the production process as a whole and knowledge management. Are there quality assurance measures in place (i.e. why and how quality is ensured; are there tests for quality, validation of standards and specifications)? Can we observe an 'experimental process' that has lead to the development of these herbal formulae from single component remedies?

It cannot be denied that herbal mixtures harbour many potential lifesaving bioactive compounds. The isolation of many microbial compounds including antiplasmodial diterpenoids, sesquiterpene lactones, antibacterial anthraquinones, anti-

inflammatory isoeugenitols and (S)-naringenin from different plant species observed to be the commonly used in commercial herbal mixtures and preparations in South Africa is a case in point **(CHUKWUJEKWU *et al*., 2005; ELGORASHI *et al*., 2007; STAFFORD *et al*., 2007; PEDERSEN *et al*., 2009)**. The modernization and acceptance of herbal mixtures into mainstream medical practice will depend on the outputs of scientific research aimed at providing the rational for their usage. In this way, with time, herbal mixtures will achieve the same recognition as western pharmaceutical drugs.

CHAPTER 3

Pharmacological, Phytochemical and Safety Evaluation of Fourteen Commercial Herbal Preparations Used in KwaZulu-Natal, South Africa

3.1. Introduction

As herbal products become popular, their safety and efficacy become an important issue **(YEH and KISHONY, 2007)**. The study of ethnobotanical systems as therapeutic agents is of paramount importance to addressing health problems of traditional communities. A large number of studies have been conducted in the past few decades on the traditional pharmacopoeia of indigenous peoples and rural communities throughout the world **(CANO and VOLPATO, 2004)**. Nevertheless, these studies are rarely focused on commercial herbal preparations and mixtures. The botanical and pharmacological aspects of such complex preparations have often been overlooked and very little attention has been paid to them.

In this study, *in vitro* tests, frequently known as 'bioassays' were used as ethnopharmacological research, to measure biological activity in undefined substances, determination of toxicity and measurement of the concentration of known substances in herbal products. The bioassays most commonly utilize microbial cells and enzymes as targets for the substances under test. In some cases, nematodes can be used, for example, the use of the *Caenorhabditis elegans* for anthelmintic studies **(HOUGHTON *et al*., 2007)**. This chapter will answer several questions relating to the efficacy and safety of fourteen commercial herbal preparations currently sold in KwaZulu-Natal, South Africa.

3.1.1. Aims and objectives

The project was aimed at investigating the pharmacological properties of fourteen commercial herbal preparations common in KwaZulu-Natal. The bioassays used for

the evaluation of the pharmacological properties included the antibacterial, antifungal, anthelmintic assays, the inhibition of the cyclooxygenase (COX-1 and COX-2), HIV-1 reverse transcriptase (RT) and the acetylcholinesterase (AChE) enzymes and antioxidant assays, as well as the cytotoxicity and mutagenicity assays.

3.1.2. Sample procurement

Fourteen commercial herbal preparations, *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe*® traditional *muthi mixture*, *Ibhubezi*™, *Supreme one hundred*, *Sejeso herbal mixture Ingwe*®, *Lion izifozonke Ingwe*®, *Stameta™ BODicare*®, *Ingwe*® *special muti* and *African potato extract*™ were bought from herbal shops around Pietermaritzburg, KwaZulu-Natal. Figure 3.1 presents the pictures of the fourteen herbal preparations. The information of the commercial herbal preparations including the manufacturers' details, claims and directions of use are presented in Table 2.1 (Chapter 2).

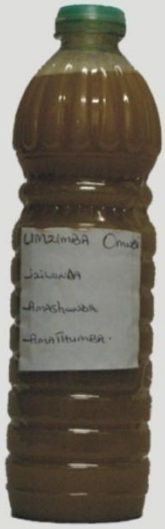
3.1.3. Sample preparation

The herbal preparations (200 ml) were filtered through Whatman No. 1 filter paper and freeze dried. The dried material was weighed and resuspended in water and filtered through a sterile 0.22 µm filter unit (Millex® GV, Molsheim, France) to obtain a sterile 50 mg/ml starting concentration, subsequently diluted to lower concentration depending on the assay. *Ingwe*® *special muti* was obtained from the herbal shop as a powdered material, therefore the extract was prepared following the directions on the packaging. The powdered sample (5 g) was extracted in boiling water (200 ml) with stirring for five minutes (obtaining a tea-like solution) and left to stand until cold. The solution was filtered through Whatman No. 1 and the filtrate was treated as described for the other herbal preparations.

The resuspended extracts of the herbal preparations, were evaluated for antibacterial, antifungal, anthelmintic properties and the inhibitory activities against

the cyclooxygenase (COX-1 and COX-2), the HIV-1 reverse transcriptase (RT), acetylcholinesterase (AChE) enzymes. The herbal preparations were also evaluated for cytotoxicity and mutagenicity using the neutral red uptake assay (NRU) and the Ames test. The antioxidant potentials as well as the phytochemical constituents of the herbal preparations were also investigated.

1



2

3

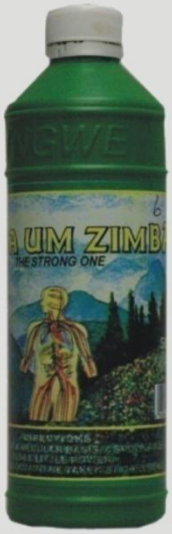
4


HERBAL TRADINGS

5


KWA NYANGA YEZIZWE

6


THE STRONG ONE

7


INGWE
IZIFOZONKE
Traditional African Muti
THE STRONG ONE
AMAZING MIXTURE
FOR ALL DISEASES

8


BODY CLEANSING LIQUID
IBHUBEZI
SUSA ZONKE No 1
500 ml

9


New Formula
SUPREME ONE HUNDRED
REAL HERBS, REAL GOOD
All POWER of the POWDER NOW INSIDE

Figure 3.1: Commercial herbal preparations commonly found in traditional herbal shops, pharmacies and supermarkets in KwaZulu-Natal, South Africa. (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred™*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract™*.

3.2. Antimicrobial and anthelmintic evaluation

3.2.1. Introduction

Bacteria form a heterogeneous and the most abundant group of unicellular organisms on earth and are able to adapt to almost any living condition. They are both useful and harmful to humans. Bacterial infections are very common and are amongst the leading causes of death worldwide **(SLEIGH and TIMBURY, 1998)**. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant microorganisms **(PAGE *et al*., 1997)**.

The cellular organisation of bacteria is prokaryotic with rigid cell walls, which determines their shape. Bacteria may be spherical (*cocci*), cylindrical (*bacilli* or rods) or helical (*spirochaetes*). Besides conferring rigidity upon bacteria, the rigid cell wall protects the cell against osmotic damage. It is porous and permeable to substances of lower molecular weight **(SLEIGH and TIMBURY, 1998)**.

Gram's staining technique is used to divide bacteria into two different classes, the Gram-positive and the Gram-negative. Bacterial cells are stained with crystal violet (CV) and after being decolourized with alcohol and treated with safranine and washed in water, bacterial cells that retain the crystal violet complex are Gram-positive and those that do not retain it are Gram-negative. The Gram-negative bacterium consists of an outer membrane which contains lipopolysaccharide, a reduced layer of peptidoglycan separated from the cytoplasm by a periplasm or a periplasmic space. The cell wall also contains specific proteins which include pore-forming proteins through which hydrophilic molecules are transported. The other proteins include the receptor sites for phages and bacteriocins. The peptidoglycan layer of the cell wall of Gram-positive bacteria is much thicker than in Gram-negative bacteria. Gram-positive bacteria lack periplasmic space.

The Gram-staining reaction reflects the structure of the cell wall. In aqueous solutions, CV dissociates into CV^+ and chloride ($Cl^–$) ions. The CV^+ and Cl^- ions can penetrate through the cell walls and cell membranes of both Gram-positive and

Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Addition of Iodine (I) ions to the stained cells results in the interaction in the formation of large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. When alcohol or acetone is added, as a decolourizer, it destabilises the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. Washing with water removes CV–I complexes from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated by an ethanol treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. Therefore, Gram-positive cells remain purple and the Gram-negative cells lose their purple colour **(BEVERIDGE and DAVIES, 1983; DAVIES *et al*., 1983)**.

Some of the medically important bacteria that are known to cause infections include *Bacillus subtilis, Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus. Bacillus subtilis* is a Gram-positive aerobic rod-shaped spore-producing bacterium. *Bacillus subtilis* often occurs in chainlike formations, found primarily in soil and causes food poisoning **(RYAN and RAY 2004)**. *Staphylococcus aureus* is a spherical Gram-positive pyogenic bacterium that causes illnesses such as boils, conjunctivitis, pneumonia, skin infections, meningitis and septicaemia as well as worsening conditions such as wound infections and food poisoning **(FRANKLIN and LOWY, 1998)**. *Staphylococcus aureus* exists in multiple resistant forms, especially in hospitals **(SLEIGH and TIMBURY, 1998)**. *Escherichia coli* is a Gram-negative bacterium normally present as part of the normal flora of the gut and within the intestinal tract of humans and other warm-blooded organisms. The bacterium benefits its hosts by producing vitamin K_2, and prevents the establishment of pathogenic bacteria within the intestinal tract. Most *Escherichia coli* strains are harmless, but sometimes they can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls. Strains of *Escherichia coli* are also responsible for wound and urinary tract infections **(SLEIGH and TIMBURY, 1998)**. *Klebsiella pneumonia* is a genus of non-motile rod-shaped Gram-negative enterobacteria which cause urinary tract infections, septicaemia, meningitis and pneumonia **(RYAN and RAY 2004)**.

Besides bacterial infections, humans are constantly fighting fungal invasions, especially from *Candida albicans*. *Candida albicans* is a member of the yeast family and sub-group fungi or moulds **(CHAITOW, 1996)**. *Candida albicans* forms part of the flora living in the gastrointestinal tract, including the mouth and the gut. The fungus lives in 80% of the human population with no harmful effects but sometimes it is a causal agent of opportunistic oral and genital infections in humans **(WOLSKI and GLOWNIAK, 2003)**. Overgrowth of *Candida albicans* results in candidiasis. Candidiasis is often a common case in immunocompromised individuals such as HIV-AIDS patients. Candidiasis, also known as 'thrush', is a common condition which is usually observed in people who are not immunocompromised. To infect host tissue, the usual unicellular yeast-like form of *Candida albicans* reacts to environmental cues and switches into invasive, multicellular filamentous forms **(ZACCHINO *et al*., 2003)**. "*The introduction of broad-spectrum antibiotics, the use of the contraceptive pill and the widespread proliferation of steroid medication, have played their part in Candida's growth. In addition, the increase in the use of sugar and sugar rich foods has provided the yeast with just the sustenance it loves. This is the unfortunate combination of factors that is the root cause of the problem for many people*" **(CHAITOW, 1996)**.

Intestinal nematodes are also important pathogens of humans. The WHO estimates that 2 billion people harbour worm infections. Helminthic infections are mostly controlled by treatment with synthetic drugs but there is evidence of drug resistance **(BEHNKE *et al*., 2008)**. Anthemintics are grouped into three classes. The first group includes the benzimidazoles, introduced in the early 1960s but resistance was detected after 4 years of usage. The second group, the nicotinic acetylcholine agonists such as levamisol was introduced in the 1970s and resistance was first reported in 1977. The third group, macrocyclic lactones includes ivermectin, were first introduced in the early 1980s and resistance was first reported in South Africa within seven years of use **(BEHNKE *et al*., 2008)**.

Nematodes are not considered ideal for laboratory use because they have complex life-cycles. Activity of the extracts is rated based on the viability or mobility of the worms after incubation thus the method is subjective, often unreliable and time consuming. For this study, a new colourimetric technique was used which utilizes the

metabolic activity of *p*-iodonitrotetrazolium chloride (INT) as a measure of biological activity. *Caenorhabditis elegans* is very different to the other nematodes, because it is easy to culture, making the assay cheap and rapid, thus it has been used in the search for anathematic drugs. *Caenorhabditis elegans* is sensitive to the majority of anthelmintic drugs that are used for the treatment of parasitic worm infections **(BEHNKE *et al*., 2008)**.

Although it appears that many drugs are available for the treatment of microbial and worm infections, there are in fact, a limited number of efficacious microbial drugs **(ZACCHINO *et al*., 2003)**. Many of the drugs currently available lead to the development of resistance or have undesirable side effects as well as being toxic **(LERNER, 1998)**. Therefore, development of antimicrobial and anthelmintic agents for clinical use from natural products could play a crucial role in meeting the demand for new drugs against microbial infections.

Several screening methods have been described for antimicrobial evaluation of botanical extracts. These could be classified into three groups, disc-diffusion, microdilution and bioautographic assays **(RIOS *et al*., 1988)**. Microdilution techniques are used to determine more precisely the antimicrobial activity of the extracts by determining the minimum inhibitory concentration (MIC) or minimum bactericidal/fungal concentration of test extracts for a given microorganism **(ELOFF, 1998)**.The disc-diffusion assay is also a common form of testing for antimicrobial activity, and does not require homogenous dispersion of the extract and has an advantage of allowing multiple screening of plant extracts at the same time. The bioautographic assays allows for rapid detection of bioactive constituents of plant extracts and it is mainly utilised for bioassay-guided fractionation of antibacterial compounds **(FENNELL *et al*., 2004)**.

In order to test for antimicrobial and anthelmintic activities of the herbal preparations, antibacterial, antifungal and anthelmintic evaluation was carried using microdilution techniques against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains and a fungus *Candida albicans* as well as a nematode *Caenorhabditis elegans.*

3.2.2. Materials and methods

3.2.2.1. Antibacterial microdilution bioassay

Minimum inhibitory concentration (MIC) values for antibacterial activity of the fourteen herbal preparations were determined using the microdilution bioassay in a 96-well (Greiner Bio-one GmbH, Germany) microtitre plates **(ELOFF, 1998)**. One hundred microlitres of each resuspended herbal mixture (in water to a concentration of 50 mg/ml) obtained by the process explained in Section 3.1.3 was two-fold serially diluted with sterile distilled water, in duplicate down the microtitre plate for each of the four bacteria used. A similar two-fold serial dilution of neomycin (Sigma) (0.1 mg/ml) was used as a positive control against each bacterium. Water was included as a negative/solvent control. Overnight Mueller-Hinton (MH) broth cultures (incubated at 37 °C in a water bath with shaking) of four bacterial strains: two Gram-positive (*Bacillus subtilis* American type culture collection (ATCC) 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) were diluted with sterile MH broth (1 ml bacteria/50 ml MH) resulting in a final inoculum of approximately 10^6 cfu/ml. One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was tested by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37 °C for 1 h. Bacterial growth in the wells was indicated by a red-pink colour, whereas clear wells indicated inhibition of growth by the tested sample. MIC values were recorded as the lowest concentration of extract showing a clear well. Each assay was repeated twice with two replicates.

3.2.2.2. Antifungal microdilution bioassay

The antifungal activity of the herbal preparations was evaluated against *Candida albicans* (ATCC 10231) using the micro-dilution assay **(ELOFF, 1998)** modified for an antifungal assay **(MASOKO *et al*., 2007)**. An overnight fungal culture was prepared in 10 ml yeast malt (YM) broth. Four hundred microliters of the overnight *Candida* culture were added to 4 ml of sterile saline solution. The absorbance was read at 530 nm and adjusted with sterile saline solution to match that of a 0.5 M

McFarland standard solution. From this prepared stock, a 1:1000 dilution with sterile YM broth was prepared to give an approximately 10^6 cfu/ml culture.

One hundred microlitres of each herbal mixture obtained by the process explained in Section 3.1.3 were resuspended in water to a concentration of 50 mg/ml and two-fold serially diluted with sterile distilled water, in duplicate down a 96-well microtitre plate. A similar 2-fold serial dilution of Amphotericin B (Sigma) (2.5 mg/ml) was used as a positive control. Water and fungal free broth were included as negative controls. To each of the wells containing the test and control solutions, 100 µl of the dilute *Candida* cultures were added and incubated for 24 h at 37 °C. To indicate fungal growth, 50 µl of 0.2 mg/ml INT were added to each well, and the plates were incubated for a further 24 h. The wells which displayed no change in colour represented antifungal activity. The MIC was taken as the lowest concentration of plant extract to inhibit growth of the tested fungus after 48 h. After noting the MIC, 50 µl YM broth was added to the clear wells to determine whether the inhibition was fungicidal. The microplates were re-covered with parafilm and incubated for a further 24 h after which new MFC (minimum fungicidal concentrations) were noted. In the case where there was no growth of fungus in the last clear well even after addition of YM broth to clear wells, the MIC was taken as the MFC.

3.2.2.3. Anthelmintic microdilution bioassay

The anthelmintic activity of the herbal preparations was evaluated against *Caenorhabditis elegans* var. Bristol (N2) using a rapid colourimetric microdilution assay **(JAMES and DAVEY, 2007)** with modifications to obtain minimum lethal concentration (MLC) values.

A nematode growth (NG) agar plate containing 7-day-old *Caenorhabditis elegans* culture was washed with 5 ml M9 (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl and 0.25 g $MgSO_4 \cdot 7H_2O$ per litre) buffer and the liquid part with suspended worms, was transferred into a sterile McCartney bottle. The optical density (OD) at a wavelength of 530 nm of the M9 buffer with the nematodes was measured using a UV-visible spectrophotometer (Varian Cary 50, Australia). Thereafter, 5 ml M9 buffer was

diluted with appropriate volume of the prepared *Caenorhabditis elegans* culture to obtain a mixture with an OD_{530} range of 0.4 - 0.6 (100 worms/50 µl).

One hundred microlitres of each resuspended herbal mixture (in water to a concentration of 50 mg/ml) obtained by the process explained in Section 3.1.3 were two-fold serially diluted with sterile distilled water, in duplicate down the microtitre plate. A similar two-fold serial dilution of levamisole (Sigma) (5 mg/ml) was used as a positive control. 50 µl of the prepared *Caenorhabditis elegans* culture were added to each well. The microtitre plates were covered with parafilm and incubated at 20 ºC for 48 h. After the incubation period, 50 µl of 1.25 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma, Germany) was added to each well and further incubated at 20 ºC for 24 h. Active organisms biologically reduce the colourless INT to a red-pink colour. Nematode survival in the wells was indicated by a red-pink colour, whereas clear wells indicated inhibition of survival by the tested sample. MLC values were recorded as the lowest concentration of extract showing a clear well. Each assay was repeated in duplicate, two times.

3.2.3. Antimicrobial and anthelmintic: Results and discussion

The antibacterial MIC values for the 14 herbal preparations are presented in Table 3.1. Antibacterial MIC values less than or equal to 1 mg/ml **(ALIGIANNIS *et al*., 2001)** is considered to be good activity. *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi* and *Umpatisa inkosi* had low activities against the bacterial strains tested with MIC values above 12.5 mg/ml. High antibacterial activity was expected from *Umzimba omubi* and *Umuthi wekukhwehlela ne zilonda* which are herbal preparations for wounds, boils, chest infections including tuberculosis and general coughs. Wounds are mostly polymicrobial, involving numerous microorganisms that are potentially pathogenic **(LUSEBA *et al*., 2007)**. *Mvusa ukunzi* is a tonic/aphrodisiac that has less to do with microbial infections and accordingly low antimicrobial activity was observed.

The MIC values for *Vusa umzimba*, *Ingwe® muthi mixture*, *Lion izifozonke Ingwe®*, *Stameta™ BODicare®*, *Ingwe® special muti* and *Supreme one hundred* were between 12.5 mg/ml and 1.56 mg/ml. *Vusa umzimba* and *Lion izifozonke Ingwe®*,

however, showed exceptional activity against *Staphylococcus aureus* with MIC of 0.39 and 0.78 mg/ml respectively. *Ingwe*® *muthi mixture*, *Vusa umzimba* and *Supreme one hundred* are frequently used for wounds and boils. However, *Ingwe*® *muthi mixture* showed low activity against the tested bacterial strains. The herbal preparations are prepared by extraction with water. Water extracts have frequently been reported to have low antimicrobial activity **(RABE and VAN STADEN, 1997; LUSEBA *et al*., 2007)**.

Imbiza ephuzwato, *Ibhubezi*™ and *Sejeso herbal mixture Ingwe*® showed good activity against the *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* strains tested. *Imbiza ephuzwato* is a complex multipurpose mixture of 21 plant species consisting of bulbs, leaves and roots. Most of the 21 plant species that constitute *Imbiza ephuzwato* are used by traditional people to treat various conditions. Bioactive compounds with utero-active properties have been isolated from one of the plant species *Gunnera perpensa* or *Ugobo* in Zulu **(BROOKES and DUTTON, 2007)**. Mixing plant extracts can result in synergistic effects and sometimes antagonistic effects which could result in either a more potent and/or less toxic product.

Table 3.1: Antibacterial activity (MIC) of herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	MIC value (mg/ml)			
	Bacteria			
	B.s.	*E.c.*	*K.p.*	*S.a.*
Umzimba omubi	>12.50	>12.50	>12.50	>12.50
Umuthi wekukhwehlela ne zilonda	>12.50	>12.50	>12.50	>12.50
Mvusa ukunzi	>12.50	>12.50	>12.50	>12.50
Umpatisa inkosi	>12.50	>12.50	>12.50	>12.50
Imbiza ephuzwato	**0.78**	**0.78**	1.56	**0.78**
Vusa umzimba	1.56	3.13	3.13	**0.39**
Ingwe® muthi mixture	6.25	6.25	12.50	>12.50
Ibhubezi™	**0.78**	1.56	**0.78**	**0.78**
Supreme one hundred	>12.50	12.50	12.50	6.25
Sejeso herbal mixture Ingwe®	**0.78**	**0.78**	**0.78**	**0.39**
Lion izifozonke Ingwe®	1.56	1.56	3.13	**0.78**
Stameta™ BODicare®	12.50	6.25	3.13	12.50
Ingwe® special muti	1.56	1.56	1.56	1.56
African potato extract™	1.56	3.125	1.56	**0.78**
Neomycin	1.6×10^{-3}	0.8×10^{-3}	0.8×10^{-3}	1.6×10^{-3}

B.s.=Bacillus subtilis; *E.c.=Escherichia coli*; *K.p.=Klebsiella pneumoniae*; *S.a.=Staphylococcus aureus*.

Herbal extracts with MIC values written in bold font are considered to be very active (MIC < 1 mg/ml).

The results for the antifungal activity (MIC) of the herbal concoctions are shown in Table 3.2. *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi* and *Umpatisa inkosi* had low antifungal activity against *Candida albicans* with MIC and MFC values above 12.5 mg/ml. *Umzimba omubi*, *Ingwe® muthi mixture* and *Stameta™ BODicare®* had moderate antifungal activity. *Imbiza ephuzwato*, *Ingwe® special muti*, *Vusa umzimba* and *Supreme one hundred* had good antifungal activities with MIC values of 0.78 mg/ml and MFC values of 1.56 mg/ml. *Ibhubezi™*, *Sejeso herbal mixture* and *Lion izifozonke* exhibited the best antifungal activities against *Candida albicans* each with an MIC value of 0.39 mg/ml and MFC values of 0.78, 3.125 and 6.25 mg/ml respectively.

It is interesting to note that seven products (*Lion izifozonke*, *Imbiza ephuzwato*, *Ingwe® special muti*, *Sejeso herbal mixture*, *Vusa umzimba*, *Ibhubezi™* and *Supreme one hundred*) sold for treating fungal infections and skin conditions showed good activity against *Candida albicans*. However, these seven products also exhibited a fungistatic characteristic which is undesirable for good antifungal drugs. This was shown by the re-growth of the *Candida albicans* after the addition of YM broth to clear wells (MIC). There are certain conditions ideal for a herbal product or compound to be a good antifungal drug. The compound should be fungicidal other than fungistatic and should have a selective mechanism of action **(ZACCHINO *et al*., 2003)**. According to **POLAK (1999)**, "*ideal antifungal drugs are not yet discovered*". It is important to carry out further research on the herbal preparations which showed some promising activity in order to understand their mode of antifungal action.

Table 3.2: Antifungal activity (MIC and MFC) of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	*Candida albicans*	
	MIC (mg/ml)	MFC (mg/ml)
Umzimba omubi	1.56	6.25
Umuthi wekukhwehlela ne zilonda	>12.50	>12.50
Mvusa ukunzi	>12.50	>12.50
Umpatisa inkosi	>12.50	>12.50
Imbiza ephuzwato	**0.78**	1.56
Vusa umzimba	**0.78**	1.56
Ingwe® muthi mixture	1.56	6.25
Ibhubezi™	**0.39**	**0.78**
Supreme one hundred	**0.78**	1.56
Sejeso herbal mixture Ingwe®	**0.39**	3.125
Lion izifozonke Ingwe®	**0.39**	6.25
Stameta™ BODicare®	6.25	6.25
Ingwe® special muti	**0.78**	1.56
African potato extract™	**0.39**	1.56
Amphotericin B	9.77×10^{-3}	7.81×10^{-2}

Herbal preparations with MIC values written in bold font are considered to have good activity (MIC < 1 mg/ml).

The anthelmintic MLC values for the 14 herbal preparations are presented in Table 3.3. In this study, anthelmintic MLC values equal to or less than 1 mg/ml **(ALIGIANNIS *et al*., 2001)** was considered to be good activity. *Sejeso herbal mixture Ingwe*®, *Imbiza ephuzwato*, *Ingwe*® *muthi mixture*, *Lion izifozonke Ingwe*® and *African potato extract*™ exhibited good anthelmintic activity. The rest of the herbal preparations showed low activity.

Most anthelmintic drugs are taken as hot decoctions, exerting their therapeutic effects by cleansing the blood, promoting circulation, clearing toxins and killing the worms as well as their eggs. Hot decoctions can be combined with several other cold infusions to strengthen the detoxification power, thus herbal preparations still remain a hope for the discovery of anthelmintic drugs. Even though *Ingwe*® *special muti* did not show a good anthelmintic activity, it is administered as a hot tea thus may be active in that form.

Moreover, because of the blood cleansing effect of anthelmintic herbs, excessive use or misuse have emaciating effects upon body tissues. In this regard, anthelmintic herbs may reduce sperm count and deplete vitality **(FRAWLEY and LAD, 1986)**. It is therefore, not desirable to have prolonged use of herbal preparations with high anthelmintic activity such as *Sejeso herbal mixture Ingwe*®, *Imbiza ephuzwato*, *Ingwe*® *muthi mixture*, *Lion izifozonke Ingwe*® and *African potato extract*™, as such products should be used with care. However, herbal preparations with high anthelminitic activity can be used synergistically or rather include nutritive or tonic herbs to build up the destroyed tissue **(FRAWLEY and LAD, 1986)**.

Table 3.3: Anthelmintic activity (MLC) of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	*Caenorhabditis elegans*
	MLC (mg/ml)
Umzimba omubi	2.34
Umuthi wekukhwehlela ne zilonda	1.17
Mvusa ukunzi	4.66
Umpatisa inkosi	4.66
Imbiza ephuzwato	**0.59**
Vusa umzimba	9.38
Ingwe® muthi mixture	**0.59**
Ibhubezi™	4.66
Supreme one hundred	2.34
Sejeso herbal mixture Ingwe®	**0.29**
Lion izifozonke Ingwe®	**0.59**
Stameta™ BODicare®	9.38
Ingwe® special muti	1.17
African potato extract™	**0.59**
Levamisole	0.042

Herbal extracts with minimum lethal concentration (MLC) values written in bold font are considered to have good activity (MIC < 1 mg/ml).

As expected, *Lion izifozonke*, *Imbiza ephuzwato*, *Ingwe*® *special muti*, *Sejeso herbal mixture* and *Vusa umzimba* herbal preparations used as multipurpose remedies showed moderate to higher activity against the bacterial and fungal strains used. *Supreme one hundred* exhibited selective activity against the fungus *Candida albicans*. However, this was not the case for *Stameta*™, a multipurpose mixture, fortified with vitamins, as it consistently showed lower activities against both the bacteria and the fungus. Activity cannot be ruled out as there are many mechanisms by which natural products can act when combating microbial and helminthic infections, including immune boosting which will in turn deal with the infection.

Apart from the absence of active compounds within the herbal preparations, other reasons explaining the lack of antimicrobial and anthelmintic activity, includes the parts of the plants used, the solvent used for extraction. Water extracts have often been reported to have poor activity in many bioassays **(LUSEBA *et al*., 2007)**. The location where the plants were collected, and possibly the time of collection as well as the storage of the material can result in lower compounds with antimicrobial activities. Another grave concern is the storage of the manufactured herbal preparations. The herbal preparations are kept in non-refrigerated places. Higher temperatures may lead to rapid degradation of unstable active compounds. It is also possible that the preparatory process of these mixtures for the assays which includes freeze drying and filtration could possibly inactivate or remove active compounds from the extracts.

3.3. Enzyme inhibition bioassays

3.3.1. Introduction

Enzymes are the most attractive targets for drug intervention in the treatment of a number of human diseases. This is because of their involvement in essential catalytic roles in many physiological processes that may be altered during disease progression. The structures of enzymes have lent them well to inhibition by small molecular weight drugs. As a result there is a large and growing interest in the discovery of compounds that may serve as enzyme inhibitors. Enzyme inhibitors represent almost half the drugs in clinical use at the moment **(COPELAND, 2005)**.

Prostaglandins are known to be involved in many physiological processes such as the regulation of blood flow to organs, stimulation of protective mucosal linings in the gastrointestinal tract as well as the participation in the initiation of platelet aggregation in blood clotting, bone resorption, ovulation and the mediation of the classic symptoms of inflammation such as pain, swelling and fever **(MORITA, 2002; COPELAND, 2005)**. Prostaglandins are derived from arachidonic acid by an enzymic reaction catalysed by a single class of enzyme referred to as cyclooxygenase (COX) or prostaglandin synthase. Research studies in the 1990s led to the conclusion that there exist two isoforms of the COX enzymes, having distinct functions in the tissues and organs. This raised a possibility that selective inhibition of either COX isoenzyme may have useful therapeutic outcomes **(HANSEN *et al*., 1999)**. The two isoenzymes are referred to as COX-1 and COX-2.

COX-1 is consistently expressed in a wide variety of tissues while COX-2 is induced in response to pro-inflammatory stimuli. Nonsteriodal anti-inflammatory drugs (NSAID) are used for the treatment of inflammation. Aspirin and Ibuprofen (Brufen) are the mostly used examples of NSAIDs for pain and fever. All the NSAIDs exert their therapeutic function by blocking or inhibiting the two COX isoenzymes. Unfortunately, the use of NSAIDs results in side effects including gastric and renal ulceration from long term usage. It was later elaborated that these side effects were associated with inhibition of COX-1 while the anti-inflammatory activity was due to

inhibition of COX-2 **(COPELAND, 2005)**. It is therefore important to have natural products with COX-2 selective inhibition, in the hope that such products will exhibit good anti-inflammatory efficacy with or without reduced side effects that are common with inhibition of COX-1 as with NSAIDs usage.

HIV-1 reverse transcriptase (RT) is a DNA dependent polymerase that catalyses the synthesis of a double stranded DNA copy from a single stranded HIV-RNA. The enzyme is for that reason, essential for the life cycle of HIV-1 and hence, a target for anti-HIV therapy **(SPALLAROSSA *et al*., 2009)**. The current therapeutic drugs against HIV infections include the inhibitors of RT and protease enzymes. These include the nucleoside RT inhibitors, nucleotide RT inhibitors and the non-nucleoside RT inhibitors. All non-nucleoside RT inhibitors bind to an allosteric binding pocket (i.e. a site other than the enzyme's active site) **(DE CLERCQ, 2009)**. The formation of a RT-non-nucleoside RT inhibitor complex results in short and long range structural changes that make the enzyme inactive **(SPALLAROSSA *et al*., 2009)**.

Presently, the oral prodrug forms of nucleoside RT inhibitors have been approved for the treatment of HIV infections. A prodrug is a substance that is prescribed and taken orally in an inactive or less active form. Once administered, the prodrug is metabolized *in vivo* into an active form. The use of prodrugs generally increases absorption, distribution and metabolism of most oral pharmaceuticals. The prodrugs include zidovudine, didanosine, lamivudine, stavudine, zalcitabine, abacavir, nevirapine, ritonavir and emtricitabine **(DE CLERCQ, 2009)**.

Although anti-retroviral drugs have resulted in an improvement of the quality of life amongst HIV infected humans (i.e. eating and sleeping well, and keeping a positive outlook on life), the development of resistance, appreciable levels of toxicity, high cost, unavailability and lack of curative effect are their major short-comings **(BESSONG *et al*., 2005)**. These short-comings, especially the appearance of drug resistant virus strains have resulted in increased efforts for the search of better anti-HIV agents and much attention is now being directed towards natural products **(WANG *et al*., 2004)**.

The prevalence of HIV in South Africa is a prominent health concern because South Africa is believed to have more people infected than in any other country. HIV/AIDS prevalence in South Africa is an epidemic of shattering dimensions with more than 5 million people infected by 2002 **(BESSONG *et al*., 2005)**. The high prevalence of HIV among poor people in South Africa has resulted in the increase in usage of medicinal plants and herbal products. The beneficial effects of herbal preparations and remedies could be due to direct inhibition of HIV replication by blocking RT, boosting of the immune system or curative effects on the opportunistic infections **(MILLS *et al*., 2005)**.

Besides the COX and RT enzymes, another pharmacologically important enzyme used in drug development is acetylcholinesterase (AChE), that catalyses the hydrolytic degradation of the neurotransmitter acetylcholine, resulting in choline and an acetate group. AChE is found at neuromuscular junctions and cholinergic synapses in the central nervous system (CNS), where its activity serves to terminate synaptic transmission **(VOET and VOET, 1995)**. Alzheimer's disease (AD) that occurs frequently in elderly people is caused by malfunctioning of biochemical pathways associated with the production of the neurotransmitter acetylcholine resulting in progressive memory loss and cognitive impairment **(LIN *et al*., 2008)**. Treatment of AD takes advantage of AChE inhibition. Galanthamine, an alkaloid isolated from snowdrop (*Galanthus nivalis*, in the family Amaryllidaceae), is a well known AChE inhibitor and is widely used for AD treatments **(FERREIRA *et al*., 2006; STAFFORD *et al*., 2008)**.

Apart from the use in treatment of AD, AChE inhibitors are also used in the control of Myasthenia gravis, an autoimmune disorder, in which there is severe muscle weakness caused by circulating antibodies that block acetylcholine receptors at the post-synaptic neuromuscular junction, inhibiting the stimulative effect of acetylcholine **(RAJ *et al*., 2005)**.

There is a wide usage of herbal preparations for pain related conditions, which could involve inflammation responses. The high prevalence of HIV infection has also left a number of patients to rely on herbal products to counteract the infection. Herbal preparations are also widely used for reviving memory loss and stress related

problems amongst the depressed and old people. The fourteen commercial herbal preparations were evaluated for their inhibitory activities against COX-1 and COX-2, HIV-1 RT and AChE.

3.3.2. Materials and methods

3.3.2.1. Cyclooxygenase 1 (COX-1) inhibitory bioassay

The COX-1 bioassay was performed as described by **JÄGER *et al*., (1996)**. The COX-1 enzyme isolated from ram seminal vesicles was obtained from Sigma-Aldrich. A stock solution of COX-1 enzyme was stored at -70 °C until use. The enzyme was activated with 1250 µl of co-factor solution (0.3 mg/ml L-adrenaline and 0.3 mg/ml reduced glutathione in 0.1 M Tris buffer, at pH 8.0) and pre-incubated on ice for 5 min. In 1.5 ml Eppendorf tubes, 60 µl of the enzyme solutions were added to each sample solution (20 µl of 10 mg/ml sample giving a final assay concentration of 2 mg/ml) in duplicate and the mixture was incubated at room temperature for 5 min. Two separate sets of Eppendorf tubes, labelled the background (the enzyme was inactivated with HCl before incubation), solvent blank (containing water instead of sample) and positive control (containing 5 µM indomethacin obtained from Sigma) were included in the test. After 5 min of incubation, the reaction was started by adding 20 µl ^{14}C-arachidonic acid (16 Ci/mol, 30 µM) to each Eppendorf tube. The preparations were incubated in a water bath at 37 ºC for 10 min and afterwards the reaction was stopped by adding 10 µl 2N HCl except in the background tubes. Glass columns were packed with silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pasteur pipettes. Four microlitres (0.2 mg/ml) of unlabeled prostaglandins (PGE_2: $PGF_{2\alpha}$ 1:1) (Sigma-Aldrich) were added to each Eppendorf tube as a carrier solution.

The test solutions were applied to the columns with 1 ml of eluent 1 [hexane: 1, 4-dioxane: glacial acetic acid (70:30:0.2 v:v:v)]. This was done to separate the prostaglandins and unmetabolized arachidonic acid. The arachidonic acid was eluted first with 4 ml eluent 1. The prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15 v:v)] and collected in scintillation vials. To each vial, 4 ml of scintillation fluid were added and the disintegration per minute of the

radioactive material was counted using a scintillation counter (Beckman LS 6000LL scintillation counter). The assay was repeated twice. Inhibition percentage was calculated using the equation below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}\right)\right\} \times 100$$

where DPMsample is the disintegrations per minute for plant extract, DPMbackground is the disintegrations per minute in which the enzyme was inactivated and DPMblank is the disintegrations per minute for the reaction mixture containing water. Results are presented as means ± standard errors of two independent experiments, each experiment in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 4.0) statistical software programme for Windows (GraphPad Software Inc.).

3.3.2.2. Cyclooxygenase 2 (COX-2) inhibitory bioassay

The COX-2 activity was assessed using a method described by **NOREEN *et al*. (1998)**, with slight modifications **(ZSCHOCKE and VAN STADEN, 2000)**. Human recombinant COX-2 enzyme containing a six histidine sequence near the N-terminus isolated from a Baculovirus over expression system in S*f* 21 cells was used (Sigma-Aldrich). Ten microlitre of the enzyme containing 3 units were prepared and stored at -70 ºC until use. The enzyme was activated with 1450 µl co-factor solution (0.6 mg/ml L-adrenaline, 0.3 mg/ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, pH 8.0) and pre-incubated on ice for 5 min. In 1.5 ml Eppendorf tube, 60 µl of the enzyme solution were added to each sample solution (20 µl of 10 mg/ml sample giving a final assay concentration of 2 mg/ml) in duplicate and the mixture was incubated at room temperature for 5 min. Two separate sets of Eppendorf tubes, labelled the background (the enzyme was inactivated with HCl before incubation), solvent blank (containing water instead of sample) and positive control (containing 200 µM indomethacin obtained from Sigma) were included in the test. After 5 min of incubation, the reaction was started by adding 20 µl ^{14}C-arachidonic acid (16 Ci/mol, 30 µM) to each Eppendorf tube. The reaction preparations were incubated in a water bath at 37 ºC for 10 min and afterwards the reaction was stopped by adding 10 µl 2N HCl except in the background tubes. Glass columns were packed with silica gel

(silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pasteur pipettes. Four microlitres (0.2 mg/ml) of unlabeled prostaglandins (PGE_2: $PGF_{2\alpha}$ 1:1) (Sigma-Aldrich) were added to each Eppendorf tube as a carrier solution.

The test solution was applied to the column with 1 ml of eluent 1 [hexane: 1, 4-dioxan: glacial acetic acid (70:30:0.2)]. This was done to separate the prostaglandins and unmetabolized arachidonic acid. The arachidonic acid was eluted first with 4 ml eluent 1. The prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15)] and collected in scintillation vials. To each vial 4 ml of scintillation fluid were added and the disintegration per minute of the radioactive material was counted using a scintillation counter (Beckman LS 6000LL scintillation counter). Inhibition percentage was calculated using the equation below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}\right)\right\} \times 100$$

Where DPMsample is the disintegrations per minute for herbal preparations, DPMbackground is the disintegrations per minute in which the enzyme was inactivated and DPMblank is the disintegrations per minute for the reaction mixture containing water. Results are presented as means ± standard errors of two independent experiments, with each herbal preparation being tested in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 4.0) statistical software programme for Windows (GraphPad Software Inc.).

3.3.2.3. HIV-1 reverse transcriptase (RT) inhibitory bioassay

The effect of the herbal preparations on reverse transcription was evaluated using a non-radioactive HIV-RT colourimetric ELISA kit obtained from Roche Diagnostics, Germany. The protocol supplied together with the kit was followed, under nuclease-free conditions. The reverse transcriptase colourimetric assay, takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly (A) × oligo (dT)15. The kit avoids the use of [3H]- or [32P]-labelled nucleotides which are used for the other classical RT assays. In place of radio-labelled nucleotides, digoxigenin- and biotin-labelled nucleotides are incorporated into one and the same DNA molecule, which is freshly synthesized by the RT. The detection and

quantification of synthesized DNA as a parameter for RT activity is followed in a sandwich ELISA protocol: Biotin-labelled DNA freshly synthesized by the RT, binds to the surface of microtitre plate modules (MPM) with wells that were precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labelled DNA. In the final step, the peroxidase substrate ABTS (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a coloured reaction product which is measured spectrophotometrically.

The following solutions, provided with the kit were prepared according to the manufacturer; Solution 1, HIV-1 reverse transcriptase (final concentration 2 ng/µl, corresponding to 10 mU/µl) stored at -70 ºC. Solution 2, incubation buffer. Solution 3, reaction mixture containing poly (A) x oligo $(dT)_{15}$ (46 mM Tris-HCl, 266 mM potassium chloride, 27.5 mM magnesium chloride, 9.2mM DDT, 10 µM dUTP/dTTP, template/primer hybrid, 750 $_{mA260\ nm}$/ml). Solution 4, lysis buffer. Solution 5, anti-digoxigenin-peroxidase (anti-DIG-POD) (200 mU/ml). Solution 6, washing buffer and solution 7, ABTS substrate solution.

In sterile Eppendorf tubes, 20 µl of resuspended herbal preparations (with final assay concentrations of 0.25, 2.5, 25, 250, 2500 µg/ml) or controls were mixed with 20 µl of recombinant HIV-1-RT (4 ng in lysis buffer) and 20 µl reaction mixture (solution 3) and the tubes were incubated for 1 h at 37 °C. After the 1 h incubation period, the contents of the tubes (60 µl) were transferred into a MPM wells. The MPM were covered with foil and incubated for 1 h at 37 °C after which the contents were removed from the MPM wells completely. The wells were rinsed 5 times with 250 µl of washing buffer (solution 6) per well for 30 s, the washing buffer being removed carefully after each wash. After the wash, 200 µl of anti-DIG-POD (solution 5) was added to each well and the MPM were recovered with foil and incubated for 1 h at 37 °C. After the incubation period, the solution was removed completely from the MPM wells. The MPM wells were rinsed 5 times with 250 µl of washing buffer (solution 6) per well for 30 s, the washing buffer being removed carefully after each wash. After washing, 200 µl of ABTS substrate solution (solution 7) was added to each well and the MPM were incubated at room temperature for 5 minutes (a green

colour appeared in the wells). The absorbance of the reaction mixture was then measured at 405 nm (reference wavelength: 490 nm) using a microplate reader (Opsys MR^{TM}, Dynex Technologies Inc.). Percentage of inhibition was calculated by comparing the absorbance of the sample to the negative control using the equation below:

$$\text{HIV-1 RT inhibition (\%)} = \left\{1 - \left(\frac{\text{Abs}_{405\ \text{nm}}\ \ \text{Sample}}{\text{Abs}_{405\ \text{nm}}\ \ \text{Neg Control}}\right)\right\} \times 100$$

where Abs_{405nm} Sample is the absorbance of the reaction with herbal preparations or positive control at 405 nm and Abs_{405nm} Neg Control is the absorbance of reaction with water instead of sample at 405 nm.

Three tubes containing water instead of sample were used as a negative controls. Another set of three tubes containing lysis buffer and no HIV-1-RT added were included. Combivir® (GlaxoSmithKline) [lamivudine (1.0 mg/ml) + zidovudine (2.0 mg/ml)] and Kaletra® (Abbott) [lopinavir (8.9 mg/ml) + ritonavir (2.2 mg/ml)]. Results were presented as means ± standard errors of two independent experiments; each experiment was done in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 4.0) statistical software programme for Windows (GraphPad Software Inc.).

3.3.2.4. Acetylcholinesterase (AChE) enzyme inhibitory bioassay

Inhibition of AChE by the herbal preparations was done as described by **ELLMAN *et al.* (1961)** with some modifications. The acetylcholinesterase enzyme activity was measured by spectrophotometric observation of the increase in a yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. The following buffers were used; Buffer A: 50 mM Tris-HCl, pH 8; Buffer B: 50 mM Tris-HCl, pH 8, containing 0.1% bovine serum albumin (BSA); Buffer C: 50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$. Acetylthiocholine iodide (ATCI), galanthamine, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and AChE (isolated from electric eels) (type VI-S lyophilized powder) were obtained from Sigma-Aldrich.

In a 96-well plate, 25 µl of herbal preparations at an initial concentration of 10 mg/ml were added to the first well and serially diluted two-fold down the plate. To the diluted sample, 25 µl of 15 mM ATCI in water, and 125 µl of 3 mM DTNB in buffer C were added, followed by 50 µl of buffer B. The absorbance of the reaction mixture was then measured three times at 405 nm every 45 s using a microplate reader (Opsys MR^{TM}, Dynex Technologies Inc.). After the third reading, 25 µl of 0.2 U/ml AChE in buffer A was added. The absorbance was measured again every 45 s for a further five times. The final concentration of the herbal preparations in the first well containing the highest concentration was 1.0 mg/ml. Galanthamine at 0.12, 0.23, 0.46, 0.92, 1.84, 3.68 and 7.37 µg/ml concentrations and water were used as positive and negative controls respectively. The increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the ratio of reaction before adding the enzyme from the rate after adding the enzyme. Percentage of inhibition was calculated by comparing the reaction rates for the sample to the negative control. Results were presented as means ± standard errors of the experiment in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 4.0) statistical software programme for Windows (GraphPad Software Inc.).

3.3.3. Enzyme inhibition: Results and discussion

Figure 3.2 represents the percentage inhibition of COX-1 and COX-2 enzymes by the fourteen herbal preparations. Four levels of activity are defined in the COX assay with activity below 20% being considered insignificant, 20–40% low, 40–70% moderate, and 70–100% high **(TAYLOR and VAN STADEN, 2001)**. The level to which the herbal preparations could inhibit the COX-1 enzyme can consequently be considered high for *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe® muthi mixture*, *Ibhubezi™*, *Lion izifozonke Ingwe®*, *Stameta™* and *African potato extract*^{TM} and moderate for *Umzimba omubi*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Sejeso herbal mixture Ingwe®*, and *Ingwe® special muti.* COX-1 inhibitory activity was insignificant for *Umuthi wekukhwehlela ne zilonda* and *Supreme one hundred* although both remedies are used for purposes related to inflammation responses.

Imbiza ephuzwato, *Vusa umzimba*, *Ingwe® muthi mixture*, *Ibhubezi™*, *Sejeso herbal mixture Ingwe®* and *African potato extract™* showed high COX-2 inhibitory activity while *Lion izifozonke Ingwe®* and *Ingwe® special muti* exhibited moderated activity. The rest of the preparations showed low activity and none had insignificant activity.

The lower doses of herbal preparations assayed for COX-1 and COX-2 were used to calculate IC_{50} values (concentration effective to cause a 50% enzyme inhibition) The IC_{50} values are presented in Table 3.4. IC_{50} values less than 1 mg/ml were considered to represent good COX inhibitory activity. The order of the active herbal preparations with respect to the IC_{50} values for COX-2 were *Imbiza ephuzwato* > *African potato extract™* > *Vusa umzimba* > *Sejeso herbal mixture* > *Ibhubezi™.* For COX-1, the order was as follows; *Imbiza ephuzwato* > *Lion izifozonke Ingwe®* > *African potato extract™* > *Ingwe® special muti* > *Sejeso herbal mixture*. The IC_{50} value for *Sejeso herbal mixture* was lower in COX-2 compared to COX-1, suggesting the mixture could be a COX-2 selective inhibitor. *Lion izifozonke* and *Ingwe® special muti* showed lower IC_{50} values for COX-1 than COX-2 thus the preparations are COX-1 selective inhibitor.

Non-steroidal anti-inflammatory drugs (NSAIDs) that exert their therapeutic effects by blocking the COX enzymes are still being used in the treatment of inflammation, fever, pain and thrombosis although they have a tendency to cause significant damage in the gastrointestinal tract, resulting in ulcers **(KIM *et al*., 2005)**. Therefore, plant remedies are becoming popular and are now often preferred to synthetically derived drugs. COX-1 catalyses the production of prostaglandins involved in prostanoid-mediated physiological functions such as gastric cytoprotection, maintenance of renal homeostasis, and normal platelet functions and its inhibition could is associated with serious side effects. Because of the side effects such as the damage to the gastrointestinal tract, it is undesirable to have a remedy that has high COX-1 activity **(LUSEBA *et al*., 2007)**. Prolonged use of products with high COX-1 inhibitory activity such as *Lion izifozonke* and *Ingwe® special muti*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe® muthi mixture*, *Ibhubezi™* may be associated with damage in the gastrointestinal tract. It is therefore important to develop remedies with specific COX-2 activity. This remains a considerable challenge.

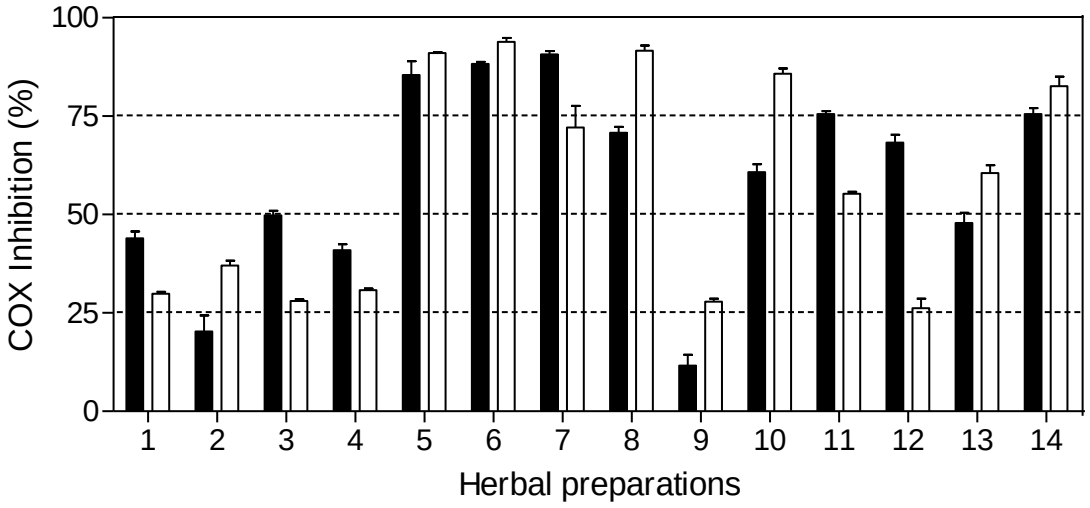


Figure 3.2: Percentage inhibition of COX-1 (■) and COX-2 (□) by commercial herbal preparations at the highest concentration (2 mg/ml); (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi*™, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract*™. Herbal preparations with inhibitory activity above 70% were considered to be highly active. Percentage inhibition by indomethacin was 64.18 ± 3.10 and 68.50 ± 2.57 for COX-1 and COX-2 respectively. Indomethacin® concentrations were 5 µM and 200 µM for COX-1 and COX-2 respectively.

Table 3.4: IC_{50} values for the inhibition of COX-1 and COX-2 enzymes by the fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	IC_{50} Values (mg/ml)	
	COX-1	COX-2
Umzimba omubi	2.14 ± 0.02	4.44 ± 0.06
Umuthi wekukhwehlela ne zilonda	4.23 ± 0.06	2.89 ± 0.05
Mvusa ukunzi	1.99 ± 0.21	4.74 ± 0.14
Umpatisa inkosi	2.33 ± 0.05	3.41 ± 0.09
Imbiza ephuzwato	**0.41 ± 0.03**	**0.43 ± 0.02**
Vusa umzimba	**0.64 ± 0.02**	**0.61 ± 0.01**
Ingwe® muthi mixture	1.53 ± 0.08	1.43 ± 0.12
Ibhubezi™	**0.87 ± 0.13**	**0.66 ± 0.04**
Supreme one hundred	4.51 ± 0.05	2.55 ± 0.01
Sejeso herbal mixture Ingwe®	**0.89 ± 0.01**	**0.65 ± 0.04**
Lion izifozonke Ingwe®	**0.65 ± 0.04**	1.81 ± 0.04
Stameta™ BODicare®	1.91 ± 0.04	4.71 ± 0.79
Ingwe® special muti	**0.87 ± 0.08**	1.54 ± 0.05
African potato extract™	**0.66 ± 0.06**	**0.55 ± 0.12**

IC_{50} values less than 1 mg/ml were considered to represent good COX inhibitory activity.

The HIV-1 RT inhibitory activity of the herbal preparations is shown in Figure 3.3. Only four of the fourteen herbal preparations (*Imbiza ephuzwato*, *Ingwe*® *muthi mixture*, *Sejeso herbal mixture Ingwe*® and *African potato extract*™) showed good activity (> 50%) at 2.5 mg/ml. The rest of the preparations showed moderate to low activity.

The IC_{50} values presented in Table 3.5 show that *Ingwe*® *muthi mixture* > *Imbiza ephuzwato* > *African potato extract*™ > *Sejeso herbal mixture Ingwe*®, in that order, are potent inhibitors of the HIV-1 RT. The mechanisms of action of these four herbal preparations could be through a conformational change on the HIV-1 RT thereby rendering it inactive. It is also possible that the herbal preparations may contain compounds that may act as competitive inhibitors of the HIV-1 RT. Since these preparations are made of a mixture of plant species, there is a possibility that the compounds may be novel. It is therefore important to carry out bioassay guided fractionation in order to isolate the active principles.

In South Africa, there is a rapid proliferation of the consumption of plant based decoctions and herbal preparations by HIV infected people **(BESSONG *et al*., 2005)**, and this is likely to increase as more people become infected and are desperate to get well. The preparation of herbal preparations is cheap and simple; hence they remain a hope for the infected people who cannot access the government sponsored antiretroviral (ARV) programmes.

Basing on the results presented in Figure 3.3 and Table 3.5, herbal mixtures such as *Imbiza ephuzwato*, *Ingwe*® *muthi mixture*, *Sejeso herbal mixture Ingwe*® and *African potato extract*™ have great potential for use as anti-HIV-1 RT inhibitors. However, more studies are still required to confirm such activity both *in vitro* and *in vivo*. It is therefore, important to determine the plant species used to make these four herbal preparations as well as to isolate the active compounds from the mixture. Upon isolation of active compounds, the mechanisms of action of the herbal mixture as HIV-1 RT inhibitors will be better understood.

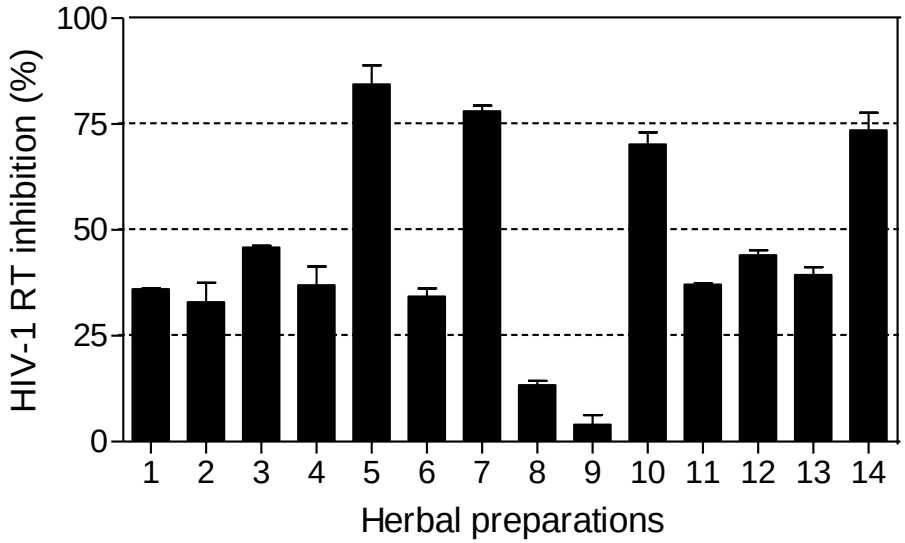


Figure 3.3: Percentage inhibition of HIV-1 RT by commercial herbal preparations (2.5 mg/ml); (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract™*. Herbal preparations with inhibitory activity above 70% were considered to be highly active. Percentage inhibition by Combivir® (0.5 mg/ml) was 79.80 ± 0.12 and 62.50 ± 0.31 for Kaletra® (0.5 mg/ml).

Table 3.5: HIV-1 RT inhibitory activity (IC_{50} mg/ml) of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	HIV-1 RT inhibitory activity
	IC_{50} (mg/ml)
Umzimba omubi	2.628 ± 0.645
Umuthi wekukhwehlela ne zilonda	2.762 ± 0.400
Mvusa ukunzi	3.500 ± 1.605
Umpatisa inkosi	2.177 ± 0.100
Imbiza ephuzwato	**0.152 ± 0.001**
Vusa umzimba	3.493 ± 1.008
Ingwe® muthi mixture	**0.095 ± 0.008**
Ibhubezi™	3.983 ± 0.100
Supreme one hundred	4.320 ± 0.336
Sejeso herbal mixture Ingwe®	**0.367 ± 0.083**
Lion izifozonke Ingwe®	2.389 ± 0.309
Stameta™ BODicare®	3.025 ± 0.026
Ingwe® special muti	2.332 ± 0.764
African potato extract™	**0.364 ± 0.022**
Combivir®	0.065 ± 0.003
Kaletra®	0.330 ± 0.105

Herbal preparations with IC_{50} values in bold are considered potent inhibitors of HIV-1 RT.

The results of AChE inhibitory activity are presented in Figure 3.4. Nine out of fourteen herbal preparations showed inhibitory activity above 50%. *Imbiza ephuzwato*, *Umpatisa inkosi*, *African potato extract*™, *Sejeso herbal mixture Ingwe*® and *Vusa umzimba* showed good AChE inhibitory activity (> 80%). *Ingwe*® *muthi mixture*, *Ibhubezi*™, *Lion izifozonke Ingwe*® and *Ingwe*® *special muti* exhibited moderate AChE inhibitory activity (between 50% and 70%). The rest of the herbal preparations exhibited very low activity (< 20%).

The IC_{50} values for herbal preparations with dose dependent activity are shown in Table 3.6. Five of the herbal preparations (*Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Supreme one hundred* and *Stameta*™ *BODicare*®) did not show dose dependant activity, making it impossible to calculate IC_{50} values. The order of potent activity with respect to IC_{50} values was: *Imbiza ephuzwato* > *Sejeso herbal mixture Ingwe*® > *African potato extract*™ > *Vusa umzimba* > *Umpatisa inkosi* > *Ibhubezi*™ > *Ingwe*® *special muti* > *Ingwe*® *muthi mixture* > *Lion izifozonke Ingwe*®.

Umpatisa inkosi, a male tonic used as an aphrodisiac and lucky charm showed good activity against AChE, as expected. Herbal preparations used as lucky charms (*Umpatisa inkosi*) are likely to contain plants with psychoactive effects, exerting their therapeutic effects by blocking AChE. This is also possible for the rest of the herbal preparations which showed activity against AChE as they are multipurpose products. Such herbal preparations are likely to contain high levels of alkaloids, known potent inhibitors of AChE. The results of this study indicate that nine out of fourteen herbal preparations offer great potential for the treatment of AD and other psychosis related conditions.

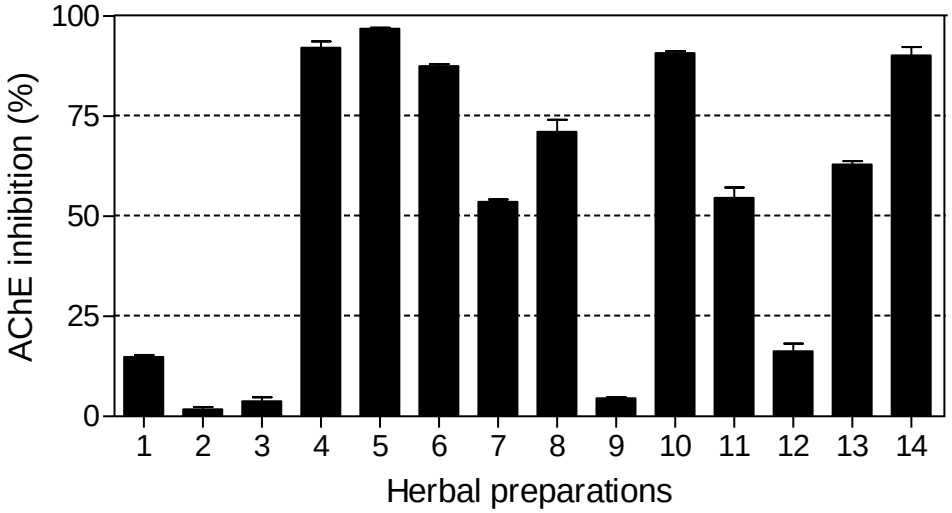


Figure 3.4: Percentage inhibition of AChE by commercial herbal preparations (1 mg/ml); (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract™*. Herbal preparations with inhibitory activity above 70% were considered to be highly active. Percentage inhibition by galanthamine (20 µM) was 89.90 ± 0.32.

Table 3.6: AChE inhibitory activity (IC_{50} mg/ml) of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	AChE inhibitory activity
	IC_{50} (mg/ml)
Umzimba omubi	NC
Umuthi wekukhwehlela ne zilonda	NC
Mvusa ukunzi	NC
Umpatisa inkosi	297.00 ± 5.00
Imbiza ephuzwato	**0.48 ± 0.01**
Vusa umzimba	26.50 ± 0.10
Ingwe® muthi mixture	950 ± 30.00
Ibhubezi™	367.35 ± 35.85
Supreme one hundred	NC
Sejeso herbal mixture Ingwe®	21.50 ± 9.50
Lion izifozonke Ingwe®	**0.96 ± 0.01**
Stameta™ BODicare®	NC
Ingwe® special muti	NC
African potato extract™	**0.023 ± 0.01**
Galanthamine	1.6 ± 06 (µM)

Herbal preparations with IC_{50} values in bold are considered potent inhibitors of AChE.

NC- IC_{50} could not be calculated because the activity was less than 50% at highest concentration.

The use of enzymes in drug discovery is on the increase, and it coincides with the widespread use of commercialized herbal products that still need quality control **(COPELAND, 2005)**. Based on IC_{50} values, *Imbiza ephuzwato*, *Sejeso herbal mixture Ingwe*®, *Lion izifozonke Ingwe*®, *Ingwe*® *muthi mixture* and *African potato extract*™ showed consistent potent activities against the three enzymes (COX, RT and AChE) used in this study. The observed activities validate the use of these herbal preparations in treating pain related conditions, eradicating HIV/AIDS symptoms and mental conditions.

Poor or lower inhibition of enzymes in these bioassays by some herbal preparations does not rule out activities as some plant compounds exert their therapeutic effects through other pathways, resulting also in relief of symptoms. During the anti-inflammation process, some compounds act as inhibitors of the nuclear factor κB (NF κB) mediated signalling pathways in immune cells that leads to the production of the inducible COX enzymes themselves and other pro-inflammatory cytokines **(POLYA, 2003)**. Thus, such compounds will not inhibit COX enzymes but would rather suppress its production, achieving the same anti-inflammatory activity as inhibitors of COX.

3.4. Antioxidant potential of the fourteen commercial herbal preparations

3.4.1. Introduction

Free radicals produced from oxygen to form reactive oxygen species such as the singlet oxygen, superoxide, peroxyl, hydroxyl and peroxynitrite radicals, are constantly produced within living cells for specific metabolic purposes **(WAFFO-TÉGUO *et al*., 2008)**. Living cells have complex mechanisms that act as antioxidant systems to counteract the damaging effects of reactive species. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Apart from enzyme systems, macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules, including ascorbic acid, alpha-tocopherol, carotenoids, polyphenols, ubiquinol, reduced glutathione (GSH), methionine, uric acid, and bilirubin also act as protective systems of living cells against oxidation **(KATALINIC *et al*., 2005)**.

Overproduction of free radicals (Oxidative stress), which occurs when the cell's natural antioxidant systems are overwhelmed, results in severe damage to biological molecules, especially to DNA, proteins and lipids. Oxidative stress has been associated with the progression of chronic conditions such as cancer, aging, atherosclerosis, inflammation, cardiovascular disease, diabetes, and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases **(ORHAN *et al*., 2009)**.

Natural compounds, which are present in herbal products, vegetables, fruits and grains, possess the ability to reduce oxidative damage by acting as antioxidants **(SIDDHURAJU and BECKER, 2007)**. An antioxidant is a substance that, even in small quantities is able to prevent, or greatly delay, the oxidation of an oxidizable substance **(BECKER *et al*., 2004)**. The oxidation process proceeds as a chemical reaction that transfers electrons from a reducing substance to an oxidizing agent, forming chain reactions that can be difficult to contain. Antioxidants terminate these

chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by getting themselves oxidized **(BECKER *et al*., 2004)**.

The consumption of herbal products, vegetables, fruits and grains has been linked to reduced risk of several conditions, including cancer and cardiovascular diseases. The fourteen commercial herbal preparations were evaluated for their antioxidant potentials including the DPPH (2,2–diphenyl–1–picryl hydrazyl) radical scavenging activity, ferric-reducing power (FRAP) and the ability to delay or halt the bleaching of *β*-carotene-linoleic acid in a model system.

3.4.2. Materials and methods

3.4.2.1. Diphenyl–1–picryl hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging assay was done as described by **KARIOTI *et al*. (2004)** with modifications. Fifteen microlitres of each resuspended herbal preparation (0.065, 0.26,0.52, 1.04, 6.25, 12.5, 25 and 50 mg/ml, in triplicate), was diluted in methanol (735 µl) and added to freshly prepared methanolic DPPH solution (750 µl, 50 µM) to give a final volume of 1.5 ml in the reaction mixture. The reaction preparations were prepared under dim light and incubated at room temperature for 30 min in the dark. Absorbance was read at 517 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia), with methanol as the blank solution. A standard antioxidant, ascorbic acid (5, 10, 20, 40, 80 µM) was used as a positive control. A solution with the same chemicals without the herbal preparations or standard antioxidants served as the negative control. The assay was repeated twice. The free radical scavenging activity (RSA) as determined by the decolouration of the DPPH solution was calculated according to the formula:

$$\text{RSA (\%)} = \left\{1 - \left(\frac{\text{Abs}_{517\,\text{nm}}\ \ \text{Sample}}{\text{Abs}_{517\,\text{nm}}\ \ \text{Neg Control}}\right)\right\} \times 100$$

where Abs_{517} sample is the absorbance of the reaction mixture containing the resuspended herbal preparation or positive control solution, and Abs_{517} Neg control is the absorbance of the negative control. The EC_{50} values, representing the amount

of extract required to decrease the absorbance of DPPH by 50% was calculated from the percentage radical scavenging activity.

3.4.2.2. Ferric-reducing power (FRAP) assay

The ferric reducing power of the commercial herbal preparations was determined based on the method by **LIM *et al*. (2009)** with modifications. The ferric-reducing power assay involves the reduction of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. Thirty microlitres of each resuspended herbal preparation (6.25 mg/ml) or the positive control, ascorbic acid dissolved in methanol was added to a 96 well microtitre plate in triplicate and two-fold serially diluted down the wells of the plate. To each well, 40 µl potassium phosphate buffer (0.2M, pH 7.2) and 40 µl potassium ferricyanide (1% in phosphate buffer, w/v) were added. The microtitre plate was covered with foil and incubated at 50 °C for 20 min. After the incubation period, 40 µl trichloroacetic acid (10% in phosphate buffer, w/v), 150 µl distilled water and 500 µl $FeCl_3$ (0.1% in phosphate buffer, w/v) were added. The microtitre plate was re-covered with foil and incubated at room temperature for 30 min. Absorbance was measured at 630 nm using a microtitre plate reader (Opsys MR^{TM}, Dynex Technologies Inc.). The ferric-reducing power of the commercial herbal preparations and ascorbic acid were expressed graphically by plotting absorbance against concentration. The assay was repeated twice.

3.4.2.3. *β*-Carotene-linoleic acid model system (CLAMS)

The delay or inhibition of *β*-carotene and linoleic acid oxidation was measured according to the method described by **AMAROWICZ *et al*. (2004)** with modifications. The antioxidant assay measures the ability of a test solution to prevent or minimize the coupled oxidation of *β*-carotene and linoleic acid in an emulsified aqueous system. In the reaction, the emulsion loses its orange colour due to the reaction with radicals, but this process can be inhibited by antioxidants.

β-carotene (10 mg) was dissolved in 10 ml chloroform in a brown Schott bottle. The excess chloroform was evaporated under vacuum, leaving a thin film of *β*-carotene near to dryness. Linoleic acid (200 µl) and Tween 20 (2 ml) were immediately added

to the thin film of *β*-carotene and mixed with aerated distilled water (497.8 ml), giving a final *β*-carotene concentration of 20 µg/ml. The mixture was further saturated with oxygen by vigorous agitation to form an orange coloured emulsion. The emulsion (4.8 ml) was dispensed into test tubes to which the resuspended herbal preparation or butylated hydroxytoulene (BHT) (200 µl, 6.25 mg/ml) were added, giving a final concentration of 250 µg/ml in the reaction mixtures. Absorbance for each reaction was immediately (*t = 0*) measured at 470 nm and incubated at 50 °C, with absorbance of each reaction mixture being measured every 30 min for 180 min. Tween 20 solution was used to blank the spectrophotometer. The negative control consisted of 50% methanol in place of the sample. The rate of *β*-carotene bleaching was calculated using the following formula:

$$\text{Rate of bleaching (R)} = \left\{\ln\left(\frac{A_{t=0}}{A_{t=t}}\right)\right\} \times \frac{1}{t}$$

where $A_{t=0}$ is the absorbance of the emulsion at 0 min; and $A_{t=t}$ is the absorbance at time *t* (90 min). The calculated average rates were used to determine the antioxidant activity (ANT) of the respective herbal preparations, and expressed as percent inhibition of the rate of *β*-carotene bleaching using the formula:

$$\%\ \text{ANT} = \left(\frac{R_{\text{control}} - R_{\text{sample}}}{R_{\text{control}}}\right) \times 100$$

where R_{control} and R_{sample} represent the respective average *β*-carotene bleaching rates for the control and herbal preparation, respectively. Antioxidant activity was further expressed as the oxidation rate ratio (ORR) based on the equation:

$$\text{ORR} = \frac{R_{\text{sample}}}{R_{\text{control}}}$$

3.4.3. Antioxidant: Results and discussion

Figure 3.5 demonstrates the percentage DPPH radical scavenging activity of the fourteen herbal preparations commonly sold in Pietermaritzburg, KwaZulu-Natal. Only one of the herbal preparations (*Ibhubezi*™) had DPPH radical scavenging activity above 70% while eleven (*Umzimba omubi*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe® muthi mixture*, *Supreme one hundred*,

Sejeso herbal mixture Ingwe®, *Lion izifozonke Ingwe*®, *Stameta*™ *BODicare*®, *Ingwe*® *special muti* and *African potato extract*™) had moderate activity between 40% and 70%. One of the preparations (*Umuthi wekukhwehlela ne zilonda*) had poor activity, lower than 25%.

For this study EC_{50} values less than or equal to 2 mg/ml was considered good activity. The radical scavenging activity of the herbal preparations against DPPH radicals according to the respective EC_{50} values (Table 3.7) that were less than 2 mg/ml was in the following order: *Ibhubezi*™ > *Lion izifozonke Ingwe*® > *Umzimba omubi* > *African potato extract*™ > *Stameta*™ *BODicare*® > *Sejeso herbal mixture Ingwe*® > *Vusa umzimba* > *Umpatisa inkosi* > *Imbiza ephuzwato*.

It was interesting to note that some herbal preparations that showed poor activities in the antibacterial, antifungal, HIV-1 RT, AChE and COX assays, i.e. *Umzimba omubi* and *Umpatisa inkosi* showed good potentials as antioxidants by having higher DPPH radical scavenging percentages as well as lower EC_{50} values. This would validate the use of such herbal preparations as antioxidants in traditional medicine.

A fresh preparation of DPPH radical solution has a deep purple colour which disappears when an antioxidant is added to the medium. Thus, the DPPH radical scavenging assay detects the ability of substances to transfer hydrogen (H) atoms or electron donation via a radical attack (on the DPPH radicals) and convert them to colourless products **(ROMANO *et al*., 2009)**. Therefore, *Ibhubezi*™, *izifozonke Ingwe*®, *Umzimba omubi*, *African potato extract*™, *Stameta*™ *BODicare*®, *Sejeso herbal mixture Ingwe*®, *Vusa umzimba*, *Umpatisa inkosi* and *Imbiza ephuzwato* may act as antioxidants by donating H-atoms to radicals, thereby terminating free radical chain reactions.

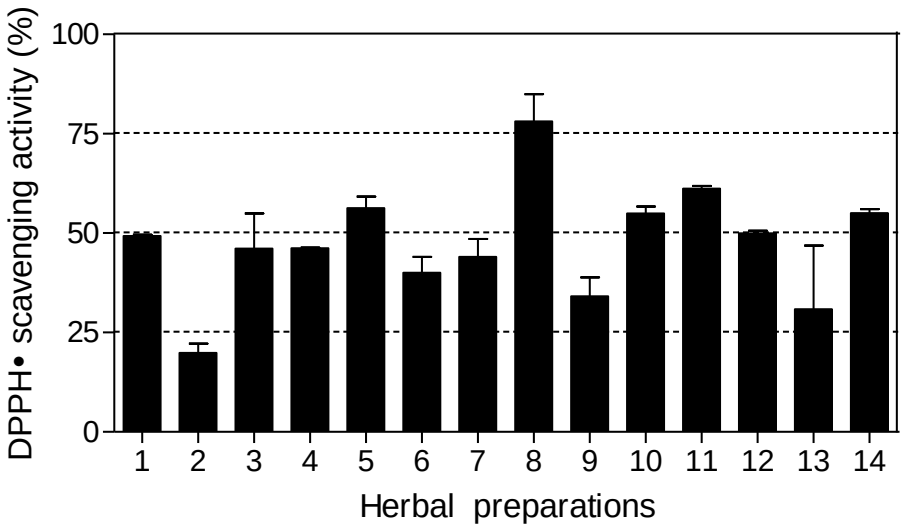


Figure 3.5: Percentage DPPH radical scavenging activity by commercial herbal preparations (1 mg/ml); (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi*™, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract*™. Herbal preparations with scavenging activity above 70% were considered to be highly active. Percentage inhibition by ascorbic acid (0.6 mg/ml) was 62.4 ± 1.42.

Table 3.7: DPPH radical scavenging activity (EC_{50} mg/ml) of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	DPPH scavenging activity
	EC_{50} (mg/ml)
Umzimba omubi	**1.24 ± 0.2**
Umuthi wekukhwehlela ne zilonda	13.22 ± 0.72
Mvusa ukunzi	4.52 ± 1.12
Umpatisa inkosi	**1.86 ± 0.66**
Imbiza ephuzwato	**1.89 ± 0.90**
Vusa umzimba	**1.86 ± 0.06**
Ingwe® muthi mixture	5.48 ± 1.02
Ibhubezi™	**0.40 ± 0.03**
Supreme one hundred	21.76 ± 5.46
Sejeso herbal mixture Ingwe®	**1.58 ± 0.08**
Lion izifozonke Ingwe®	**0.94 ± 0.05**
Stameta™ BODicare®	**1.41 ± 0.09**
Ingwe® special muti	4.38 ± 0.02
African potato extract™	**1.38 ± 0.02**
Ascorbic acid	0.07 ± 0.01

Herbal preparations with EC_{50} values (< 2 mg/ml) in bold are considered potent DPPH radical scavengers.

The lower the EC_{50}, the more rapidly the colour of DPPH radical was being bleached and hence the more potent the antioxidant.

Figure 3.6 depicts the reducing powers of the fourteen herbal preparations at varying concentrations. The ability of the herbal preparations to reduce the Fe^{3+} solution increased with an increase in concentration of the preparation. Depending on the reducing power of the herbal preparation, the initial yellow colour of the reaction mixture changes to various shades of green and blue. A strong antioxidant (reductant) reduces the Fe^{3+}/ferrcyanide complex to a green/blue ferrous form, exhibited by higher absorbance values at λ 630 nm. At the highest concentration (6.5 mg/ml) of the herbal preparations, the reducing powers of *Umuthi wekukhwehlela ne zilonda*, *Umpatisa inkosi* and *Mvusa ukunzi* were higher than that of all the other preparations while *Umzimba omubi*, *Vusa umzimba* and *Ingwe*® *special muti* were weaker than the rest.

As with the DPPH radical scavenging results, herbal preparations which had previously showed poor activities, exhibited higher reduction powers towards the Fe^{3+}/ferrcyanide complex, i.e. *Umuthi wekukhwehlela ne zilonda*, *Umpatisa inkosi* and *Mvusa ukunzi*. Another interesting observation was that at all concentrations tested, *Imbiza ephuzwato*, which was consistently showing higher activities in all the other bioassays, exhibited moderate reducing power.

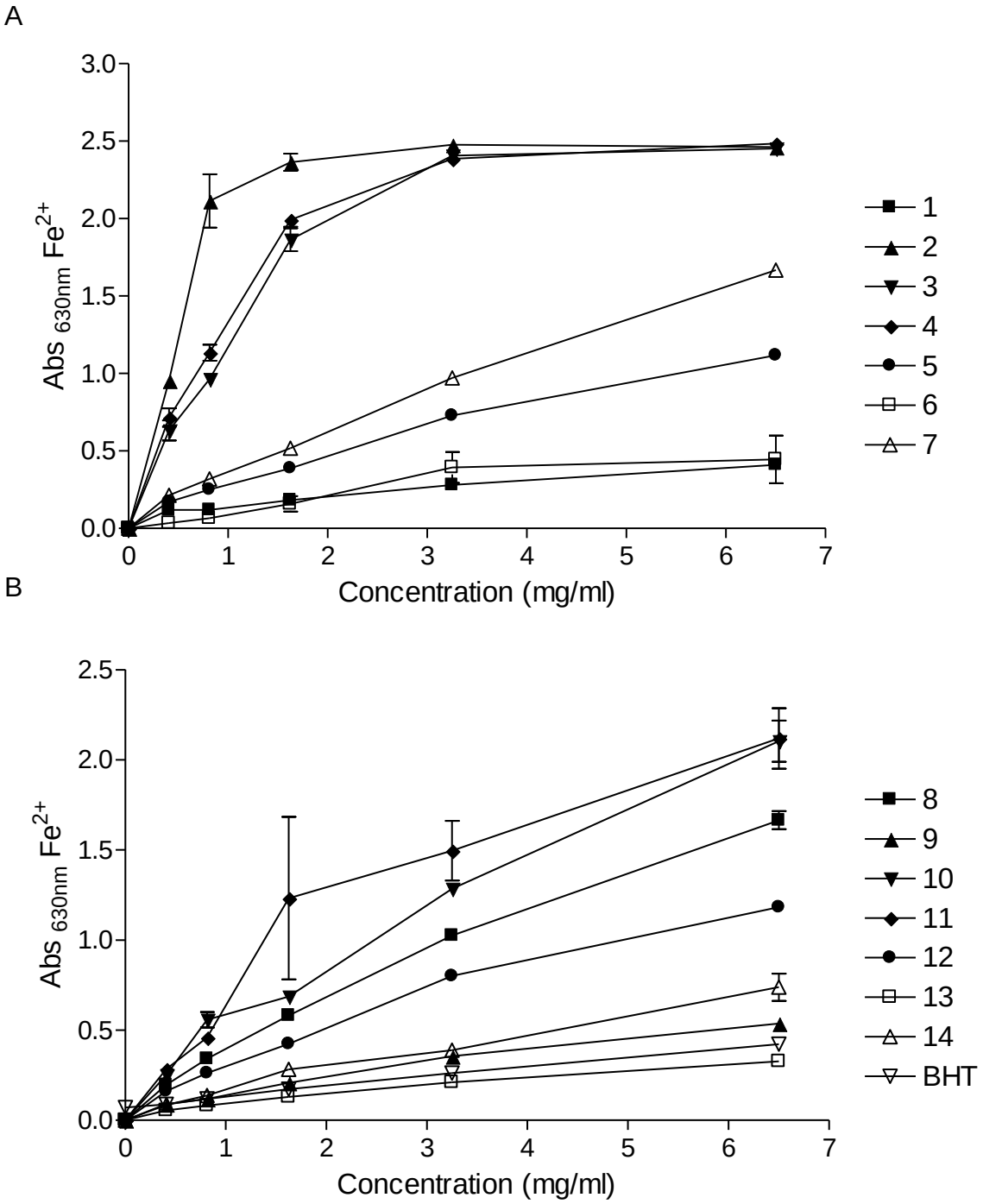


Figure 3.6: Ferric reducing activity of commercial herbal preparations (A; 1-7 and B; 8-14); (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti*, (14) *African potato extract™* and (BHT) butylated hydroxytoulene. Increase in absorbance of the reaction mixture indicates the increase in reducing power.

The results of the prevention of the heat-induced oxidation of *β*-carotene and linoleic acid in a model system by the herbal preparations are presented in Figure 3.7. In the presence of an active antioxidant, the rate of *β*-carotene bleaching is reduced. Heat (50 °C) induced oxidation involves the subtraction of a H-atom from an active methylene group of linoleic acid, forming a linoleate free radical. The linoleate radical then viciously attack the highly unsaturated *β*-carotene in an effort to regain lost H-atoms. As the *β*-carotene is attacked, it loses its orange colour. The presence of a good antioxidant can prevent the attack on *β*-carotene by neutralizing the linoleate radical.

Nine herbal preparations (*Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Ibhubezi*™, *Sejeso herbal mixture Ingwe*® and *Stameta*™ *BODicare*®) exhibited the best potentials to delay the oxidation of *β*-carotene throughout the incubation period. *Umpatisa inkosi*, *Ingwe*® *muthi mixture* and *African potato extract*™ exhibited the poorest potentials to delay the oxidation of *β*-carotene.

Variable antioxidant percentages (ANT), shown in Table 3.8, were observed for the herbal preparations with *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Supreme one hundred*, *Sejeso herbal mixture Ingwe*® and *Ingwe*® *special muti* showing moderate activity, between 35% and 70%. *Stameta*™ *BODicare*® and *African potato extract*™ exhibited low activity (15% to 35%) while *Ibhubezi*™ and *Lion izifozonke Ingwe*® showed poor activity (< 15%).

The lower oxidation rate ratio (ORR) values, just like EC_{50} values, denote better antioxidant potentials. Based on ORR, the order of antioxidant capacity with respect to the protection of *β*-carotene against bleaching was as follows; *Mvusa ukunzi* > *Ingwe*® *special muti* > *Umzimba omubi* > *Umuthi wekukhwehlela ne zilonda* > *Imbiza ephuzwato* > *Sejeso herbal mixture Ingwe*® > *Vusa umzimba* > *Supreme one hundred* > *Stameta*™ *BODicare*® > *Ingwe*® *muthi mixture* > *Lion izifozonke Ingwe*® > *Ibhubezi*™ > *African potato extract*™ > *Umpatisa inkosi*.

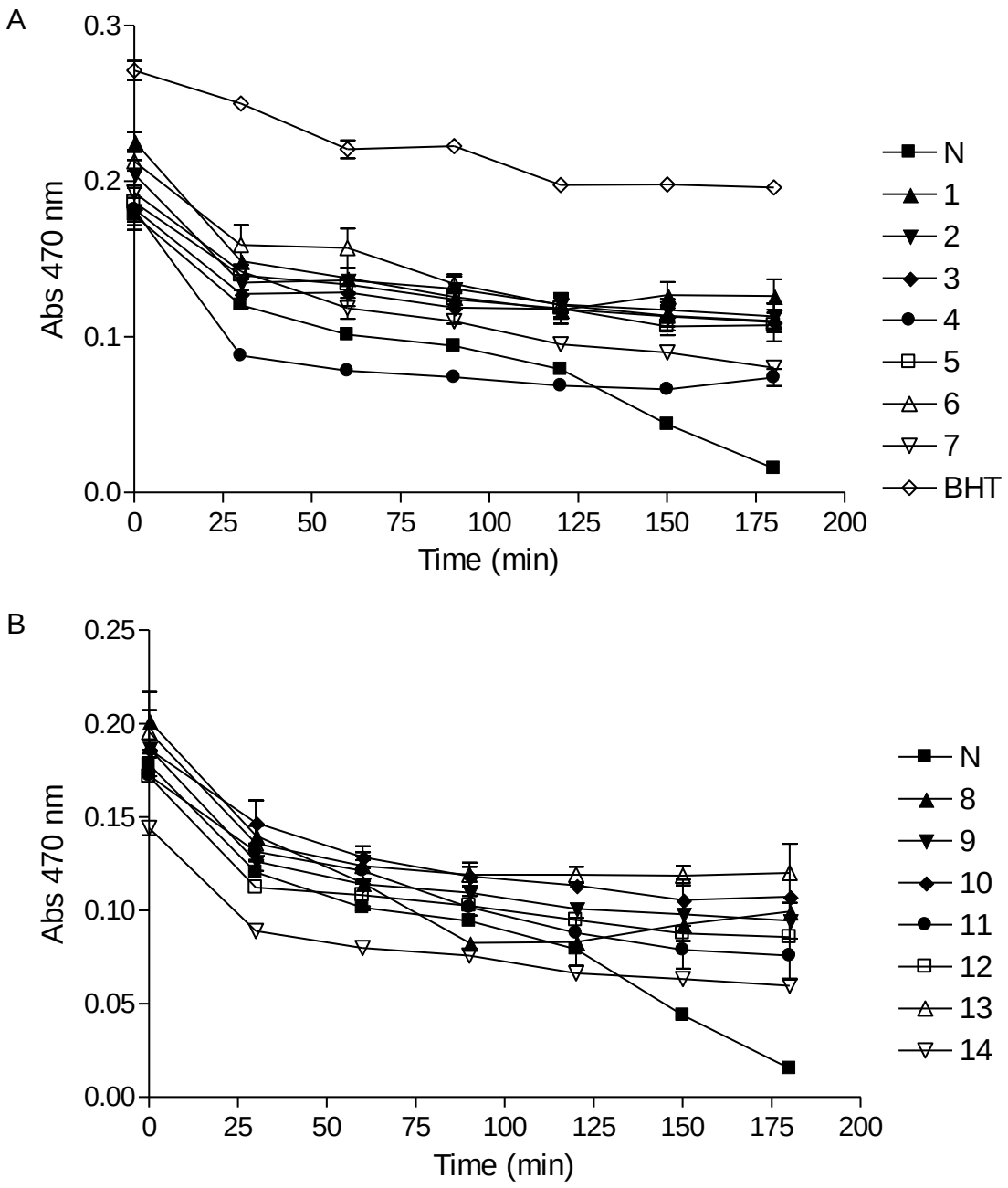


Figure 3.7: Antioxidant activity of commercial herbal preparations (A; 1-7 and B; 8-14) as determined by the *β*-carotene-linoleic acid coupled oxidation model system; (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti*, (14) *African potato extract™*, (N) negative control (water) and (BHT) butylated hydroxytoulene. Slow decrease in absorbance signifies protection of *β*-carotene; hence the test preparation is a potent antioxidant.

Table 3.8: Antioxidant activity as determined by the *β*-carotene-linoleic acid model system of the fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	Antioxidant capacity	
	ANT (%)	ORR
Umzimba omubi	57.14 ± 6.77	0.43 ± 0.07
Umuthi wekukhwehlela ne zilonda	55.90 ± 4.19	0.44 ± 0.04
Mvusa ukunzi	60.90 ± 3.62	0.39 ± 0.04
Umpatisa inkosi	39.16 ± 1.24	1.39 ± 0.10
Imbiza ephuzwato	51.33 ± 5.79	0.48 ± 0.06
Vusa umzimba	48.41 ± 5.31	0.52 ± 0.05
Ingwe® muthi mixture	21.53 ± 8.80	0.78 ± 0.09
Ibhubezi™	1.69 ± 0.41	1.02 ± 0.39
Supreme one hundred	39.13 ± 7.40	0.61 ± 0.07
Sejeso herbal mixture Ingwe®	50.31 ± 6.01	0.49 ± 0.06
Lion izifozonke Ingwe®	9.04 ± 2.40	0.91 ± 0.02
Stameta™ BODicare®	29.08 ± 6.67	0.71 ± 0.07
Ingwe® special muti	61.15 ± 4.58	0.39 ± 0.05
African potato extract™	19.4 ± 2.26	1.19 ± 0.02

ANT (%) - Antioxidant activity calculated on the basis of the rate of *β*-carotene bleaching at *t* = 30, 60 and 90 min.

ORR - Oxidation Rate Ratio at *t* = 90. The lower the ORR value, the more protective the herbal preparation against *β*-carotene bleaching.

Many plant secondary metabolites are known to possess antioxidant activity **(STEENKAMP *et al*., 2006)**. Flavonoids, one of the largest groups of plant secondary metabolites have been given much credit as being responsible for the antioxidant properties of most plant extracts. Herbal preparations with plant species that are known to contain flavonoids will therefore show high antioxidant potentials. Flavonoids reduce free radicals by quenching, up-regulating or protecting antioxidant defences and chelating radical intermediate compounds. Flavonoids have also been shown to inhibit the enzymes responsible for free radical production, for instance xanthine oxidase, cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione *S*-transferase, mitochondrial succinoxidase, NADH oxidase and protein kinase C **(JOVANOVIC *et al*., 1994)**.

However, some compounds could behave as both antioxidants and prooxidants, depending on the specific set of conditions such as concentration of the antioxidant and whether oxygen or transition metals are present. In the presence of transition metals, in aqueous medium, flavonoids autooxidize to form highly reactive hydroxyl radicals. Also, some phenolic compounds may act as substrates for xenobiotic metabolizing enzymes such as the cytochrome P450s (CYP450) and peroxidases and other metallo-enzymes, yielding quinone- or quinomethide-type prooxidant and alkylating products **(SERGEDIENE *et al*., 1999)**.

Therefore, it is important to use herbal products that exhibit high antioxidant properties such as *Umzimba omubi*, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Supreme one hundred*, *Sejeso herbal mixture Ingwe*® and *Ingwe*® *special muti* at correct doses and with a controlled diet, without a lot of transition metals. There are so many compounds that are responsible for antioxidant properties displayed by the plant extracts, that it becomes important to characterize them.

3.5. Determination of the phenolic compounds of the fourteen commercial herbal preparations

3.5.1. Introduction

Plants produce a wide variety of secondary metabolites which form a large and heterogeneous group of biologically active non-nutrients **(HARBORNE, 1998)**. Plant secondary metabolites are mainly involved in the adaptation of plants to their environments; ultra violet ray protectants, defence against herbivores and pathogens as well as regulators of seed germination **(MAKKAR *et al*., 2007)**. Common plant secondary metabolites, often referred to as phytochemicals, include alkaloids, saponins, tannins, protease inhibitors, lectins and glycosides **(MAKKAR, 2003)**.

Phytochemical compounds are attracting interest among researchers because of their use as natural drugs, antibiotics, insecticides, herbicides, oils, waxes and dyes **(CROZIER *et al*., 2006)**. Depending on their biochemical synthesis, phytochemical compounds are grouped into three major groups: (i) the phenolic compounds including flavonoids and tannins, (ii) the terpenoids, and (iii) the alkaloids **(MITHEN, 2006)**.

Most phytochemical compounds exhibit bioactivity. Many bioactive compounds with known effects on human physiology and diseases have been isolated and identified mainly from traditional medicines **(MUELLER-HARVEY, 2001; CHRISTENSEN and BRANDT, 2006)**. Plant phenolic compounds are hydroxylated derivatives of benzoic acid and cinnamic acids. Phenolic compounds are of possible pharmacological value and have been reported to have antioxidative and anti-carcinogenic effects. Flavonoids make up the largest group of phenolic compounds. The group include flavonols, flavones, catechins, proanthocyanidins, anthocyanidins and isoflavonoids. They have long been recognised to possess anti-inflammatory, antiallergenic, antiviral and anti-proliferative activities **(HARBORNE, 1994; KUDA *et al*., 2005)**.

Tannins are tentatively separated into two classes: condensed tannin and hydrolysable tannin. Tannins are known to have both beneficiary and diverse effects,

depending on their concentration and nature. Although research on tannins has been carried out for a long time, additional studies must be carried out to investigate the benefits of incorporating plant species with high tannin levels into herbal preparations and/or agro-industrial products such as animal feed **(MAKKAR, 2003)**.

A number of methods are available for quantification of phytochemical compounds. The mostly utilized methods are the Folin-Ciocalteu, Folin-Denis or Prussian blue methods, the vanillin-HCl assay, the acid butanol method, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) **(MUELLER-HARVEY, 2001)**.

The fourteen herbal preparations were evaluated for their phenolic constituents including total phenolic compounds, condensed tannin, hydrolysable tannin and flavonoids using colourimetric methods. TLC profiles were obtained to demonstrate the characteristic constituents of each herbal preparation. The TLC profiles can be used for monitoring the identity and purity of the preparations as well as detecting adulterations and substitutions of plant materials initially used.

3.5.2. Materials and methods

3.5.2.1. Determination of total phenolics

The Folin Ciocalteu (Folin C.) assay for determination of total phenolic compounds was done as described by **MAKKAR *et al*., (2007)** with modification **(NDHLALA *et al*., 2008)**. In triplicate, 50 µl of herbal preparations were transferred into test tubes and were made up to 1 ml with distilled water (950 µl). Folin C. reagent (500 µl, 1 N) and 2% sodium carbonate (2.5 ml) were added to the dilute sample. Similarly, a blank that contained 50% aqueous methanol instead of the herbal preparation was also prepared. The test mixtures were incubated for 40 min at room temperature and the absorbance was measured at 725 nm using a spectrophotometer (Varian Cary 50, Australia). Total phenolic concentrations were expressed as gallic acid (GAE) equivalents, derived from a standard curve.

3.5.2.2. Determination of condensed tannins (proanthocyanidins)

The butanol-HCl assay for the determination of condensed tannin was done as described by **PORTER *et al*. (1985)**. In triplicate, 3 ml of butanol-HCl reagent (95:5 v/v) was added to 500 µl of the herbal preparation, followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test tube contents were mixed using a vortex. The tubes were transferred into a water bath set at 100 ºC for 60 min. A blank test was prepared for each herbal preparation by mixing the herbal preparation (500 µl) with butanol-HCl reagent (3 ml) and ferric reagent (100 µl), but without heating. Absorbance was then read at 550 nm using a spectrophotometer (Varian Cary 50, Australia) against a suitable blank. Condensed tannin (% in dry matter) was calculated as equivalent amount of leucocyanidins using the formula:

$$\text{Condensed tannin (\%)} = \left(\frac{A_{550\ nm} \times 78.26 \times \text{dilution factor}}{\text{\% dry matter}} \right)$$

where $A_{550\ nm}$ is the absorbance of the herbal preparation at 550 nm and 78.26 is the molecular weight of leucocyanidin.

3.5.2.3. Determination of hydrolysable tannin

The determination of hydrolysable tannin as gallotannins was done according to **MAKKAR (1999)** with modifications **(NDHLALA *et al*., 2007)**. In triplicate, herbal preparations (50 µl) were made up to 1 ml with distilled water. Sulphuric acid (0.4 N, 100 µl) and 600 µl rhodanine were added to the diluted extracts. After 5 min incubation at room temperature, 200 µl of potassium hydroxide (0.5 N) was added followed by the addition of distilled water (4 ml) after a further 2.5 min. The mixtures were incubated for an additional 15 min at room temperature, after which the absorbance at 520 nm was read using a spectrophotometer (Varian Cary 50, Australia) against a blank that contained 50% aqueous methanol instead of the herbal mixture. Gallotannin concentrations were expressed as gallic acid (GAE) equivalents, derived from a standard curve.

3.5.2.4. Determination of flavonoids

The assay was performed as described **HAGERMAN (2002)** with modifications. In triplicate, 50 µl of herbal preparation was made up to 1 ml with methanol in a test tube before adding 2.5 ml of 8% HCl in methanol and 2.5 ml of 1% vanillin reagent in methanol. A blank that contained methanol instead of the herbal preparation was made. After 20 min of incubation at 30 °C, absorbance at 500 nm was read using a spectrophotometer (Varian Cary 50, Australia). The amounts of flavonoids in the herbal preparations were expressed as catechin equivalents (CTE), derived from a standard curve.

3.5.2.5. Thin Layer Chromatography

The crude herbal preparations (10 µl, 50 mg/ml) were applied to Silica gel 60 on polyester plates, 10 x 20 cm, (Merck, Germany) and developed with ethyl acetate:methanol:water (100:16.5:13.5 v:v:v) in a pre-saturated chromatographic chamber **(SHEN *et al*., 2001)**. Several other solvent systems that include chloroform:ethyl acetate:formic acid (6:3:1), benzene:acetone (1:1), chloroform:acetone:diethylamine (5:4:1), benzene:1,4-dioxane:acetic acid (90:25:4) and ethyl acetate:propan-2-ol (9:1) were run but with poor compound separation. The developed plates were dried under a stream of cold air and visualized under visible (VIS) and ultraviolet (UV) light (λ = 254 and 366 nm). Photographic pictures were taken at both wavelengths. The plates were then sprayed with a universal anisaldehyde spray reagent, Folin C. phenol reagent and Dragendorff reagent.

3.5.3. Determination of phenolic compounds: Results and discussion

Plants with high phenolic composition, including tannins have been regularly used as astringents, antimicrobials, anti-inflammatory and antioxidants **(POLYA, 2003; CANO and VOLPATO, 2004)**. Such plants are usually used in making herbal preparation for stomach ailments, infectious diseases and general pain. The herbal preparations were therefore, subjected to quantitative phytochemical analysis for identification of the major classes of active phenolic constituents. The phytochemical analysis carried out included colourimetric reactions for detection of total phenolic

compounds, condensed tannins, gallotannins and flavonoids. Table 3.9 presents the phenolic composition of the fourteen herbal preparations.

Phytochemical analysis revealed high total phenolic compounds, gallotannins and condensed tannins and less flavonoids in *Ingwe*® *special muti*, *Ibhubezi*™ and *Stameta*™ *BODicare*®. Surprisingly, these three herbal preparations were not active in most bioassays except for *Ingwe*® *special muti* which showed moderate activity against COX-1 and AChE. On the other hand, *Imbiza ephuzwato* exhibited high flavonoid concentrations and less total phenolic compounds, condensed tannin and gallotannin. Generally, *Supreme one hundred*, *Sejeso herbal mixture Ingwe*®, *Mvusa ukunzi* and *African potato extract*™ showed lesser amounts of total phenolic compounds, gallotannin, condensed tannin and flavonoids.

At lower concentrations, phytochemical compounds have beneficiary effects such as antioxidant effects. Condensed tannin levels of up 4% in dry matter have been shown to contribute positively in diet. However, the beneficiary effects still depend on the nature and type of phenolic compound. This explains why *Ingwe*® *special muti*, *Ibhubezi*™ and *Stameta*™ *BODicare*® showed no activities in the bioassays evaluated even though they contain high amounts of phenolic compounds.

In some instances, depending on the chemistry of the phenolic compounds, phytochemicals at higher concentrations (> 4% in dry mater) may have negative physiological effects such as neurological problems, reproductive failure, goiter, gangrene and in lower animals may lead to death **(MAKKAR *et al*., 2007)**. In this case, all the herbal preparations had condensed tannin far less than 4% (Table 3.9). "*Poisoning from the consumption of oak* (*Quercus* sp.) *leaves and yellow-wood toxicity from the leaves of Terminalia sp., Clidemia sp., and Ventilago sp. has been attributed to the presence of hydrolysable tannins, in particular gallotannins*" **(MAKKAR *et al*., 2007)**. The toxicity is due to higher levels of hydrolysable tannins and other phenolic compounds in the blood stream, which will be beyond the levels of the liver to detoxify.

However, tannins isolated from various other plants have been shown to have anti-carcinogenic activity and also protect some ruminates from bloat and have

anthelmintic effects **(KAHN and DIAZ-HERNANDEZ, 2000)**. The use of herbal preparations with beneficiary effects provides cheaper alternatives than synthesized compounds.

The challenge that still remains is the bioavailability of the phytochemical compounds. However, some studies have recently reported on the absorption of flavonoids like catechin, quecetin and isoflavones **(MAKKAR *et al*., 2007)**. The bioavailability of flavonoids has been reported to be about 10 to 50% **(KING, 2000)**. Consumption of herbal preparations with higher levels of tannins may lead to decreased absorption of glucose and amino acids. This is because tannins bind and precipitate proteins.

Table 3.9: Total phenolics, gallotannin, flavonoid and condensed tannin contents of fourteen herbal preparations sold in Pietermaritzburg, KwaZulu-Natal

Herbal preparations	Phytochemical composition			
	Total phenolic[s] (mgGAE/ml)	Gallotannin[a] (µgGAE/ml)	Condensed Tannin[b] (% LCE)	Flavonoids[c] (mgCTE/ml)
Umzimba omubi	228.80 ± 4.13	1.59 ± 0.61	0.01 ± 0.00	0.25 ± 0.06
Umuthi wekukhwehlela ne zilonda	73.35 ± 1.41	1.82 ± 0.05	0.01 ± 0.00	0.10 ± 0.01
Mvusa ukunzi	303.40 ± 9.34	6.53 ± 2.27	0.23 ± 0.02	0.27 ± 0.01
Umpatisa inkosi	158.00 ± 1.60	8.41 ± 2.21	0.02 ± 0.018	0.37 ± 0.01
Imbiza ephuzwato	155.90 ± 13.95	14.13 ± 1.55	0.01 ± 0.01	1.03 ± 0.14
Vusa umzimba	55.30 ± 1.35	6.23 ± 0.88	0.01 ± 0.00	0.22 ± 0.01
Ingwe® muthi mixture	189.60 ± 41.79	2.918 ± 0.85	0.14 ± 0.06	0.39 ± 0.17
Ibhubezi™	629.10 ± 30.37	16.12 ± 0.17	0.45 ± 0.02	0.99 ± 0.02
Supreme one hundred	141.50 ± 6.02	2.24 ± 0.81	0.05 ± 0.04	0.08 ± 0.01
Sejeso herbal mixture Ingwe®	87.49 ± 4.07	1.10 ± 0.05	0.01 ± 0.01	0.25 ± 0.01
Lion izifozonke Ingwe®	115.00 ± 8.72	7.54 ± 0.12	0.05 ± 0.00	0.87 ± 0.01
Stameta™ BODicare®	658.30 ± 3.11	6.82 ± 0.80	0.43 ± 0.01	0.19 ± 0.01
Ingwe® special muti	832.90 ± 2.23	16.14 ± 0.41	0.48 ± 0.01	0.10 ± 0.03
African potato extract™	56.44 ± 0.5	7.66 ± 0.33	0.01 ± 0.01	0.08 ± 0.01

[a]Values expressed as gallic acid equivalent (GAE) per 1 ml of commercial herbal preparation.
[b] Values expressed as percentage leucocyanidin equivalents (LCE) per 1 ml of commercial herbal preparation.
[c] Values expressed as catechin equivalents (CTE) per 1 ml of commercial herbal preparation.
All data represented as mean ± SE of three separate measurements.

Thin layer chromatography (TLC) profiles were performed to investigate the chemical profiles of the fourteen herbal preparations and the chromatograms are presented in Figure 3.8. It was not surprising to note that *Ingwe® muthi mixture*, *Sejeso herbal mixture Ingwe®*, *Lion izifozonke* and *Ingwe® special muti* showed similar chemical profiles as exhibited in their TLC profiles. The four herbal preparations are manufactured by the same company (Guideline Trading) but are sold in different packaging and under different names. This is also confirmed by the almost similar activities observed for *Lion izifozonke Ingwe®* and *Ingwe® special muti* in all the bioassays. Despite similarities in the TLC profiles of *Ingwe® muthi mixture*, *Sejeso herbal mixture Ingwe®*, *Lion izifozonke* and *Ingwe® special muti*, there were variation in their phenolic contents. *Ingwe® special muti*, had almost ten-fold amounts of total phenolic compounds when compared with *Sejeso herbal mixture Ingwe®* (Table 3.9). Therefore, the similarities in the TLC profiles could be due to some common plant species and/or additives that make up the four herbal preparations.

Under UV (λ = 254 nm), all compounds containing at least two conjugated double bonds appear as dark bands/zones against a green fluorescent background of the TLC plate (Figure 3.8B). Phenol (C_6H_5OH, benzene with hydroxyl group) derivatives have this property **(WAGNER *et al.*, 1984)**. Other examples of compounds with conjugated double bonds include vitamins A and D. However, with the rigorous processing methods used to make these preparations, it is highly unlikely that vitamins A and D could be responsible for the dark bands observed on the TLC plates. However, some other stable compounds such as anthraquinone derivatives, anethole, eugenol, cinnamic aldehydes and thymol also contain conjugated double bonds **(WAGNER *et al.*, 1984)** thus, could be the quenching compounds.

Under UV (λ = 366 nm), intense blue fluorescence is observed (Figure 3.8C), characteristic of alkaloids, flavonoids and terpene alcohols. Anisaldehyde spray result in strong red and brown colouration to detect terpenoids, propylpropanoids, and saponins **(SPANGENBURG, 2008)**. Figure 3.9 represents a TLC chromatogram produced in an attempt to separate the compounds that makes up the herbal mixtures using a combination of ethyl acetate:propan-2-ol (9:1 v/v) and spraying with anisaldehyde universal spray. The pencil marks represent fluorescence observed under UV light. After spraying with anisaldehyde, no useful bands were detected as

the compounds remained stationed at the spotting point. Several other solvent systems including chloroform:ethyl acetate:formic acid (6:3:1), benzene:acetone (1:1), chloroform:acetone:diethylamine (5:4:1) and ethyl acetate:propan-2-ol (9:1) were run but with poor compound separation. Figure 3.10A represent TLC profiles after spraying with Folin C. phenol reagent. Distinct amounts of phenolic compounds were detected for the mixtures tested. Figure 3.10B represents TLC profiles after spraying with Dragendorff reagent to detect alkaloids. *Supreme one hundred* and *Sejeso herbal mixture Ingwe*® showed some trace amounts of compounds that can be suspected to be alkaloids. Further confirmatory tests using quantitative methods needs to be carried out for a more solid conclusion.

Further research needs to be done, aimed at identifying the chemicals shown on the TLC profiles. These TLC profile profiles recorded here, forms an important component of quality control for the herbal mixtures. Besides quality control, the TLC profiles will aid in bioassay-guided fractionation leading to isolation of active compounds.

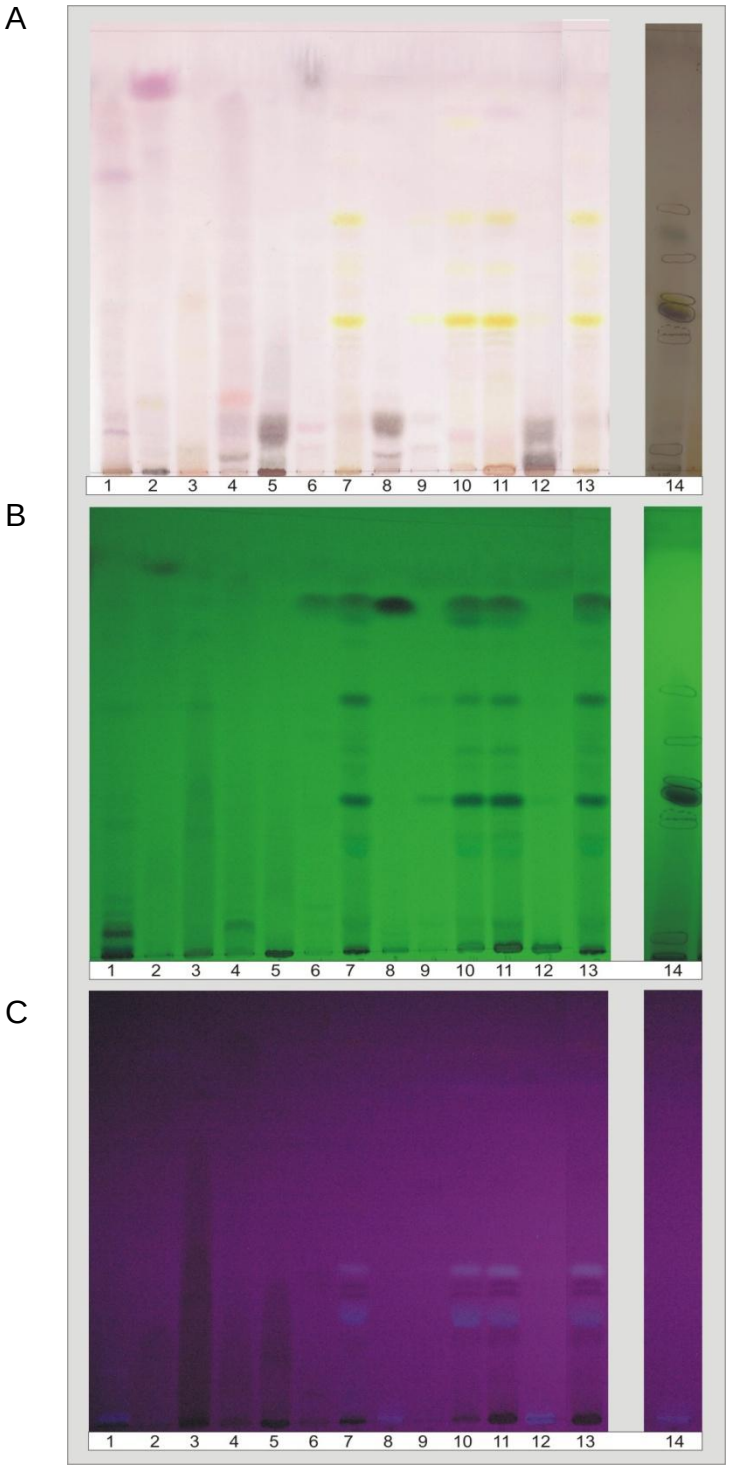


Figure 3.8: TLC profiles of the fourteen commercial herbal preparations commonly found in herbal shops, pharmacies and supermarkets in Pietermaritzburg, KwaZulu-Natal; (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*, (13) *Ingwe® special muti* and (14) *African potato extract™*. (A) Anisaldehyde universal spray, (B) UV λ = 254 nm and (C) UV λ = 366 nm fluorescence. The solvent system used consisted of ethyl acetate:methanol:water (100:16.5:13.5).

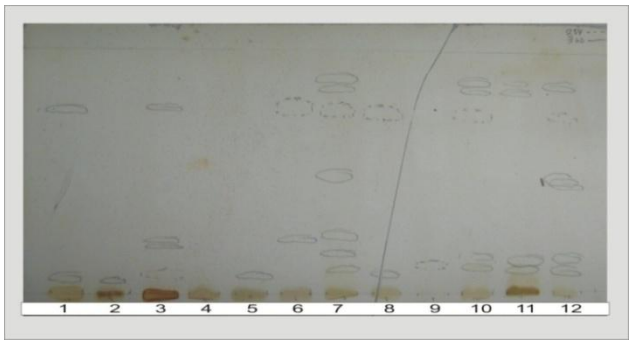


Figure 3.9: TLC profiles of herbal preparations commonly found in herbal shops herbal mixtures in Pietermaritzburg, KwaZulu-Natal; The solvent system used consisted of ethyl acetate:propan-2-ol (9:1) after spraying with anisaldehyde universal spray. (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®*. Dotted pencil marks represents bands observed at UV λ = 366 nm and solid lines represents UV λ = 254 nm fluorescence.

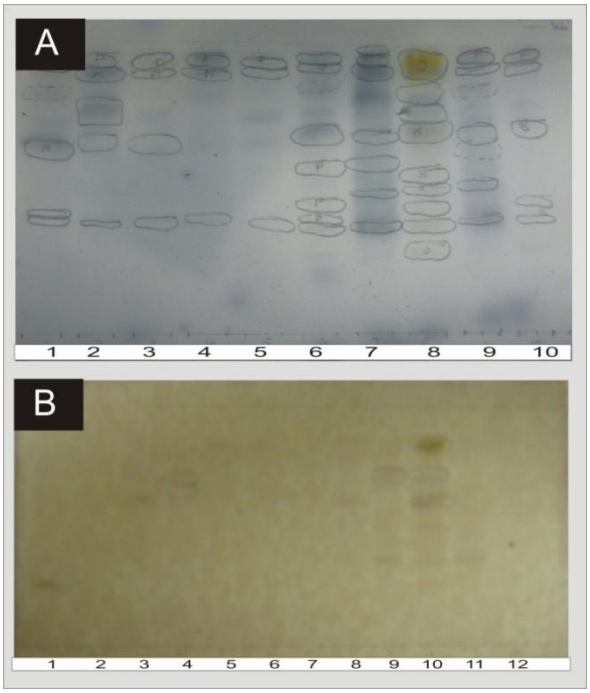


Figure 3.10: TLC profiles of herbal preparations commonly found in herbal shops herbal mixtures in Pietermaritzburg, KwaZulu-Natal; (A) after spraying with Folin C. phenol reagent and (B) Dragendorff reagent. (1) *Umzimba omubi*, (2) *Umuthi wekukhwehlela ne zilonda*, (3) *Mvusa ukunzi*, (4) *Umpatisa inkosi*, (5) *Imbiza ephuzwato*, (6) *Vusa umzimba*, (7) *Ingwe® muthi mixture*, (8) *Ibhubezi™*, (9) *Supreme one hundred*, (10) *Sejeso herbal mixture Ingwe®*, (11) *Lion izifozonke Ingwe®*, (12) *Stameta™ BODicare®.* The solvent system used for plate (A) consisted of benzene:1,4-dioxane:acetic acid (90:25:4) while for plate (B) the system consisted of chloroform:acetone:diethylamine (5:4:1). Dotted pencil marks represents bands observed at UV λ = 366 nm and solid lines represents UV λ = 254 nm fluorescence.

3.6. *In vitro* cytotoxic and mutagenic evaluation of fourteen commercial herbal preparations sold in KwaZulu-Natal, South Africa

3.6.1. Introduction

Techniques involving *in vitro* tests are increasingly used as alternatives to whole animal toxicity tests due to their reduced use of experimental animals, their lower cost, higher specificity and rapidity of performance **(ASENSIO *et al*, 2007)**. Cell line methods such as the neutral red uptake (NRU) assay are used as screening tests for new therapeutic products to assess acute and chronic toxicity **(CASTELL and GÓMEZ-LECHÓN, 1996)**. The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane leads to lysosomal fragility and other changes that gradually becomes irreversible. Such changes brought about by the action of xenobiotics result in decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which form the basis of this assay **(BARILE, 1994; REPETTO *et al*., 2008)**.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition **(REPETTO *et al*., 2008)**.

Damage of the genetic material by environmental mutagens can lead to mutations in many organisms, including humans. Mutations are associated with the development of most cancers and various degenerative disorders and genetic defects in offspring **(CARIÑO-CORTÉS *et al*., 2007)**. To prevent mutagenic risk, it is important to identify

the involved environmental mutagens and minimize human exposure to them. Short-term genetic bioassays like the Ames assay have been used as important tools in mutagenic studies because of their simplicity, sensitivity to genetic damage, speed, low cost of experimentation and small amount of sample required **(MATHUR *et al*., 2007)**.

Poisoning due to plant products (herbal poisoning) is not well documented because of the unwillingness of people to admit using traditional medicine derived from plant material and because of the fear that the cultural heritage of the people will be put under strong laws and regulations **(STEENKAMP *et al*., 2006)**. To date several studies have looked at the mutagenic effects of individual medicinal plants from South Africa **(ELGORASHI *et al*., 2003; VERSCHAEVE *et al*., 2004; REID *et al*., 2006; VERSCHAEVE and VAN STADEN, 2008)**, but not at the toxicity and mutagenic potential of commercial herbal preparations. This study was undertaken to evaluate cytotoxic and mutagenic effects of fourteen herbal preparations that are commonly sold in herbal shops in Pietermaritzburg, KwaZulu-Natal.

3.6.2. Materials and methods

3.6.2.1. The neutral red uptake (NRU) assay

The NRU test was done by Professor L. Verschaeve at the Toxicology, Scientific Institute of Public Health (Brussels, Belgium) according to **BORENFREUND and PUERNER (1985)**. Cell suspensions of human hepatocellular liver carcinoma cell line 2 (HepG2) in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% foetal calf serum was seeded into each well of a 96-well microtitre plate such that the cell density was forty thousand cells/well. Plates were incubated overnight at 37 ºC, 5% CO_2 and humidity was maintained using a water bath (milli-q water) inside the incubator. After the 24 h incubation, the cells were treated with dilutions of the herbal preparations and a positive control sodium dodecyl sulphate (SDS). Cells were kept in the presence of the test mixture (0, 0.0005, 0.005, 0.05, 0.5, 5 and 50 mg/ml of each herbal mixture initially and thereafter suitable concentrations were used depending on the preliminary toxicity results) for another 24 h. At this point the medium was removed and cells were

washed with PBS solution. Medium (200 µl) containing 0.05 mg/ml neutral red dye (NR) was added to each well using a multichannel pipette. The microtiter plates were incubated for 3 h in a humidified 5% CO_2 incubator at 37 °C. The medium was removed and the cells were rapidly washed with 0.2 ml of PBS solution. An acetic acid-ethanol mixture (0.2 ml) was used to extract the dye from the cells. The plates were agitated on a microtiter plate shaker for at least 1 h (or until a homogeneous stained medium was obtained) and then absorbance against a blank reference was measured at 540 nm using a micro plate spectrophotometer.

For all wells optical density (OD) values were calculated as the measured value minus the control value (Vc). Results were expressed as percentage of the OD determined from the average of the blank control culture read at 540 nm and set at 100%. The NI_{50}, (50% inhibition of NRU) was determined from the dose response curve of the mean OD values of the seven concentrations as indicated.

For the positive control a separate plate was used where cells were treated with different concentrations of SDS and the NI_{50} was determined as for the herbal extracts described above. The NI_{50} was kept within limits that were determined from 10 independent experiments from which the average NI_{50} values and standard deviations were calculated. The calculated NI_{50} for the positive control in an experiment should be within ± 2.5 SD of the historical data for SDS. If this is not the case the results cannot be accepted and the test should be repeated.

3.6.2.2. Ames test

Mutagenicity was tested using the *Salmonella* microsome assay based on the plate-incorporation procedure with *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activation **(MARON and AMES, 1983; MORTELMANS and ZEIGER, 2000)**. TA98 strain has a -1 frameshift mutation at *hisD3052* which affects the reading frame of a repetitive base pair –C-G-C-G-C-G-C-G- sequence, TA100 and TA1535 has a *hisG46* marker resulting from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC). The *hisG46* mutation can be reverted to a wild type by mutations that cause base pair substitution at the GC site. TA102 contains AT base pairs at the *hisG428* mutant

site. The *hisG428* can be reverted by mutagens that cause oxidative damage. TA1537 carries a +1 frameshift mutation *hisC3076* located near the repetitive site –C-C-C- sequence and can be reverted by frameshift mutagens that are not readily detected by the *hisD3052* (TA98).

Overnight bacterial tester strains were grown in 10 ml Oxoid nutrient broth No. 2 for 16 h at 37 °C to obtain a density of 1-2 x 10^9 colony forming units (cfu/ml). The metabolic activation mixture (S9 mix) was prepared freshly before the assay and kept on ice throughout the assay procedure. The S9 mix consisted of 5% (v/v) S9 fraction (Sigma-Aldrich, Co., St Louis) pooled from Sprague-Dawley male rats in mixed enzymic cofactors containing NADP. At the beginning of the assay, top agar supplemented with 0.5 mM histidine and biotin was melted and kept in a 50 °C water bath. To sterile glass tubes, in triplicate, 100 µl of three dilutions (50, 500, 5000 µg/ml) per sample were added, followed by 500 µl phosphate buffer (0.1 mM, pH 7.4) or S9 mix. To the mixture, 100 µl of the overnight bacterial culture were added followed by 2 ml of the melted top agar. The contents of the tubes were then mixed and poured onto labelled minimal agar plates. As soon as the top agar had hardened (2-3 min), the plates were inverted and incubated at 37 °C for 48 h. The colonies were then counted with the aid of a binocular microscope. The assay was repeated twice for each bacterial strain and the results were expressed as the mean (± standard error) number of revertant colonies per plate. 4-Nitroquinoline-N-oxide (4NQO) (2 µg/plate) was used as a positive control for the assay without metabolic activation while 2-aminoanthracene (2-AA) (2 µg/plate) was used where the assay was carried out with S9 metabolic activation. Sterile distilled water was used as a negative control in both assays. The test herbal mixture/compound was classified as a 'mutagen' if the results satisfied two criteria (1) a dose dependent increase in the number of revertants is observed and (2) the number of histidine (His^+) revertants is equal to or greater than two times that of the negative control.

3.6.3. Neutral red uptake (NRU) and Ames test: Results and discussion

The neutral red uptake inhibition in human liver (HepG2) cells was used to assess potential toxicity of the fourteen commercial herbal preparation sold in South Africa. The NRU assay is important for ranking of toxic components according to their

potencies and structure-toxicity relationship studies. The NI_{50} values of the fourteen preparations are summarised in Table 3.10. The results revealed that the most toxic herbal preparation was *Umpatisa inkosi*, with an NI_{50} value of 0.016 mg/ml and a yield of 2.2 X 10 10^{-4} mg/ml residue which implies that 72.72 ml of the herbal preparation will result in the stated NI_{50} value (Table 3.10). The least toxic herbal preparation was *Stameta*™ *BODicare*® with an NI_{50} value of 28.00 mg/ml and a yield of 1.8 x 10^{-4} mg/ml which implies that 115.56 litres of the mixture is required to reach the NI_{50} value of the mixture. The rest of the preparations exhibited moderate toxicity with NI_{50} values ranging within the two highlighted values.

Umpatisa inkosi showed consistently low IC_{50} values in the other assays but showed a potent NI_{50} value in the NRU assay. *Stameta*™ *BODicare*® had the least toxic value in terms of NI_{50} as well as in the other assays where it did not perform well, basing on its IC_{50} values for COX assays and HIV-1 RT assay.

HepG2 cells used were from a highly differentiated human hepatoma cell line that retains many of the cellular functions often lost by cells in culture. This cell line also has the enzymes involved in phase I (mixed function oxidases) and phase II (glucuronic acid and sulphate conjugation) metabolism of xenobiotics, and it has been used as an *in vitro* system instead of human normal hepatocytes to study drug metabolism and toxicity **(NAKAMA *et al*., 1995)**.

The standard plate incorporation test methods for the Ames test using *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 exposed to three dilutions with and without S9 metabolic activation of the herbal preparations was performed. Ames test without S9 metabolic activation can only detect direct mutagens while with S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems. Table 3.11 presents the spontaneous reversion response of the *Salmonella typhimurium* tester strain to the different dilutions of the herbal preparations. The results revealed that all fourteen herbal preparations were non-mutagenic towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 for the assay without metabolic activation. The average His^+ revertants observed for all the tester strains caused by the herbal preparations at all the concentrations without

metabolic activation did not satisfy the criteria for mutagenicity. There were no notable dose dependent increase in the number of revertants and the numbers of revertants were all not equal to or greater than two times that of the negative control **(BULMER *et al*., 2007)**. There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion) which could also be classified as toxic.

The Ames test with metabolic activation was carried out by addition of the S9 mix so as to detect indirect mutagenic effects caused by metabolites of the test herbal preparations. The S9 fraction contains a mixture of xenobiotic metabolizing enzymes such as the cytochrome P450s and sulfotransferase. Five of the fourteen herbal preparations (*Umpatisa inkosi*, *Imbiza ephuzwato*, *African potato extract*™, *Vusa umzimba* and *Stameta*™ *BODicare*®) showed indirect mutagenic effects towards the tester strain TA98 after metabolic activation but not in the other tester strains (TA100, TA102, TA1535 and TA1537). *Umpatisa inkosi*, induced 219.6, 70.6 and 32.3 TA98 revertant colonies from the highest to the lowest concentration, with colonies increasing as the concentration of the mixture increases. However, the revertant colonies (32.3) induced at the lowest concentration (50 µg/ml) by *Umpatisa inkosi* did not satisfy the criteria for mutagenicity. *Imbiza ephuzwato*, a multipurpose Zulu herbal tonic induced a high number of TA98 revertant colonies (227.7, 71.5 and 45.3) while *Vusa umzimba*, also a multipurpose herbal mixture induced 217.0, 106.0 and 52.7 TA98 revertant colonies with decreasing concentration. The number of revertant colonies for *Imbiza ephuzwato* and *Vusa umzimba* satisfy the criteria of mutagenicity. *Stameta*™ *BODicare*® exhibited a weak mutagenicity potential against TA98. At the highest concentration (5000 µg/ml), *Stameta*™ *BODicare*®, induced 131.1 revertant colonies and 48.0 at 500 µg/ml. *African potato extract*™ also exhibited a weak mutagenic potential against TA98 with about 174, 65 and 40 revertant colonies at 5000, 500, and 50 µg/ml. The rest of the preparations did not show any mutagenic potential against all tester strains after metabolic activation.

TA98 has a -1 frameshift mutation *hisD3052* which affects the reading frame of a repetitive -C-G-C-G-C-G-C-G- sequence **(MORTELMANS and ZEIGER, 2000)**. Therefore, the five herbal preparations (*Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba African potato extract*™, and *Stameta*™ *BODicare*®) cause a reversion of

the *hisD3052* mutation back to the wild-type state. The herbal preparations could contain various aromatic nitroso-derivatives of amine carcinogens that have been shown to cause such a reversion of *hisD3052* mutation back to the wild-type state.

Some carcinogenic compounds such as aromatic amines or polycyclic aromatic hydrocarbons like benzo-[a]-pyrene are biologically inactive unless they are metabolized to active forms. In humans, xenobiotic metabolizing enzymes present in the liver, lungs and kidneys, are constantly carrying out conjugation reactions and in some cases the compounds result in bioactive metabolites capable of damaging DNA **(MORTELMANS and ZEIGER, 2000)**.

Imbiza ephuzwato contains plants that are known from previous investigations to possess mutagenic and toxic constituents **(VERSCHAEVE and VAN STADEN, 2008)**. *Imbiza ephuzwato*, consists of a mixture of 21 plant species consisting of bulbs, leaves and roots. Most of the 21 plant species that constitute *Imbiza ephuzwato* are used by traditional people to treat various conditions. For example, *Gunnera perpensa* or *Ugobo* in Zulu, one of the plant constituents of *Imbiza ephuzwato* contains bioactive compounds with uteroactive properties **(BROOKES and DUTTON, 2007)**. Other plant species in the same mixture include toxic plants species such as *Scadoxus puniceus*, *Gomphocarpus fruticosa*, *Gnidia kraussiana* and *Drimia robusta* **(VAN WYK *et al*., 2002)**. *Gnidia kraussiana* contains diterpenoids which causes fatalities in both humans and livestock in various parts of Africa including South Africa. The plant has been used as a fish poison. However, diterpenoids also have antitumor and antileukaemic activities **(VAN WYK *et al*., 2002)**.

A great deal of research has already been done on the curative effects of African potato extracts, '*South Africa's miracle muthi*' which originates from the plant *Hypoxis hemerocallidea* (formerly *Hypoxis rooperi*) and locally known in Zulu as '*inkomfe*'."*A study on the safety and efficacy of the Hypoxis plant (African potato) extract in HIV-positive patients was terminated prematurely, and reported to the Medicines Control Council, because most of the patients who received the extract showed severe bone marrow suppression after eight weeks*," and as such "*At best, therefore, HIV/Aids patients should avoid any such supplements, until such time as*

their safety and efficacy, or otherwise, has been fully documented" **(TERREBLANCHE, 2003)**.

The evaluation of bacterial mutagenicity is important as an initial test for complex mixtures because of the possibility that one or more of their components can be a mutagen **(LEE *et al*., 2005; DÉCIGA-CAMPOS *et al*., 2007)**. The fact that some of the tested herbal preparations showed no mutagenic effects against all the tester strains could be due to antagonism on potential toxic compounds which could be a result of mixing a number of herbs **(LEUNG, 2004)**.

Recently, there have been reports on increased global demands for herbal products that act as energy boosters, detoxifiers, immune boosters and aphrodisiacs **(CHERDSHEWASART *et al*., 2008)**. The result of this study provides evidence to support the safe consumption of some herbal preparations at low and medium doses and also have alerted us to preparations that were mutagenic.

Table 3.10: NI_{50} values (mg/ml) after 24 h treatment of HepG2 with thirteen commercial herbal preparations

Herbal preparations	*Yield (mg/ml)	NI_{50} (mg/ml)
Umzimba omubi	2.4×10^{-6}	1.220
Umuthi wekukhwehlela ne zilonda	2.3×10^{-6}	1.010
Mvusa ukunzi	2.9×10^{-6}	1.680
Umpatisa inkosi	2.2×10^{-4}	0.016
Imbiza ephuzwato	2.1×10^{-5}	1.150
Vusa umzimba	5.5×10^{-6}	5.890
Ingwe® *muthi* mixture	5.5×10^{-6}	3.610
Ibhubezi™	1.1×10^{-4}	15.200
Supreme one hundred	9.2×10^{-5}	17.900
Sejeso herbal mixture *Ingwe*®	0.5×10^{-5}	2.630
Lion *izifozonke* Ingwe®	5.3×10^{-6}	4.280
Stameta™ BODicare®	1.8×10^{-4}	28.000
Ingwe® *special muti*	6.2×10^{-6}	2.080
African potato extract™	NT	NT
SDS (positive control)		0.082

* Yield was obtained by filtering and freeze drying 100 ml portions and the residue was weighed and expressed as yield (mg/ml).

NT – Not tested.

Table 3.11: Number of His$^+$ revertants in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 produced by fourteen commercial herbal preparations with and without S9 metabolic activation

Herbal preparations	µg/ml	Number of His+ revertants									
		TA98		TA100		TA102		TA1535		TA1537	
		S9$^-$	S9$^+$	S9$^-$	S9$^+$	S9$^-$	S9$^+$	S9$^-$	S9$^+$	S9$^-$	S9$^+$
Umzimba omubi	5000	22.3 ± 0.9	29.0 ± 1.7	157.0 ± 12.6	183.3 ±10.1	349.3 ± 20.7	277.0 ± 5.8	13.3 ± 1.2	21.3 ± 1.8	6.0 ± 1.7	9.3 ± 0.3
	500	21.7 ± 1.7	24.3 ± 3.8	195.0 ± 2.9	234.3 ± 15.7	374.0 ± 26.4	269.0 ± 9.1	14.3 ± 4.5	18.0 ± 0.5	6.7 ±0.6	8.0 ± 0.5
	50	26.7 ± 0.9	24.7 ± 0.9	182.7 ± 8.3	202.3 ± 6.6	326.3 ± 31.6	262.3 ± 4.2	12.0 ± 1.5	13.3 ± 0.8	7.0 ± 0.5	7.7 ± 0.6
Umuthi wekukhwehlela ne zilonda	5000	26.0 ± 0.6	21.3 ± 0.7	168.7 ± 4.4	209.7 ± 5.9	331.7 ± 12.8	284.7 ± 6.8	17.0 ± 1.2	33.0 ± 0.5	9.0 ± 0.5	7.7 ± 1.4
	500	25.0 ± 1.5	23.0 ± 1.0	173.3 ± 8.9	187.7 ± 5.5	321.6 ± 17.1	275.0 ± 6.1	16.3 ± 0.3	21.7 ± 1.8	8.3 ± 0.3	7.0 ± 0.5
	50	23.3 ± 0.9	19.3 ± 0.9	183.0 ± 6.5	188.3 ± 6.1	301.0 ± 2.1	269.3 ± 3.7	14.3 ± 2.4	18.3 ± 0.6	7.7 ± 0.8	6.7 ± 1.2
Mvusa ukunzi	5000	22.7 ± 0.7	19.3 ± 0.3	163.0 ± 4.6	191.0 ± 6.6	306.3 ± 10.1	287.0 ± 1.2	12.0 ± 0.6	21.0 ± 4.5	7.0 ± 0.0	10.0 ± 0.5
	500	23.0 ± 0.3	21.0 ± 2.1	178.3 ± 11.4	191.0 ± 7.1	319.0 ± 13.3	257.0 ± 5.0	14.0 ± 0.6	21.3 ± 2.1	8.0 ± 1.0	10.0 ± 0.5
	50	25.0 ± 2.0	26.0 ± 4.4	200.3 ± 6.0	198.0 ± 0.6	320.0 ± 2.0	257.0 ± 2.1	17.7 ± 2.6	21.3 ± 0.8	7.7 ± 1.3	8.7 ± 0.3
Umpatisa inkosi	5000	23.3 ± 2.6	**219.6 ± 16.3**	180.7 ± 8.0	207.0 ± 2.6	281.3 ± 9.3	274.0 ± 1.5	17.0 ± 4.0	96.3 ± 1.7	6.3 ± 0.8	8.3 ± 0.6
	500	24.7 ± 0.9	**70.6 ± 1.5**	183.0 ± 7.6	198.3 ± 0.7	291.3 ± 7.7	264.3 ± 1.2	14.0 ± 0.6	71.0 ± 2.5	6.3 ± 1.4	7.7 ± 0.3
	50	25.7 ± 1.2	32.3 ± 4.1	195.3 ± 5.7	200.0 ± 2.1	289.3 ± 6.9	272.3 ± 7.9	15.7 ± 0.9	47.3 ± 2.0	5.7 ± 0.8	7.3 ± 0.3
Imbiza ephuzwato	5000	22.0 ± 0.9	**227.7 ± 23.1**	171.6 ± 10.1	230.7 ± 1.5	329.6 ± 19.7	256.3 ± 1.9	14.7 ± 1.5	52.0 ± 3.0	7.3 ± 0.3	8.0 ± 1.0
	500	24.0 ± 1.6	**71.5 ± 5.5**	173.7 ± 2.9	194.7 ± 1.8	320.7 ± 50.3	236.3 ± 3.9	15.3 ± 1.5	34.0 ± 1.5	5.0 ± 0.5	9.3 ± 0.3
	50	19.7 ± 1.2	**45.3 ± 1.8**	180.5 ± 4.4	203.7 ± 1.8	299.3 ± 7.2	231.7 ± 0.7	14.0 ± 0.6	22.0 ± 0.5	5.7 ± 0.6	9.7 ± 1.7
Vusa umzimba	5000	23.7 ± 2.7	**217.0 ± 26.2**	167.7 ± 3.2	191.0 ± 1.2	305.3 ± 1.7	320.3 ± 4.4	13.3 ± 0.9	41.3 ± 0.6	5.7 ± 0.3	8.0 ± 0.1
	500	19.3 ± 0.7	**106.0 ± 18.0**	173.6 ± 9.1	181.3 ± 14.8	314.3 ± 26.5	223.7 ± 1.2	17.0 ± 0.6	32.0 ± 0.5	7.0 ± 0.5	6.7 ± 0.3
	50	18.0 ± 1.2	**52.7 ± 3.0**	182.6 ± 11.5	181.3 ± 9.8	288.7 ± 12.7	209.7 ± 0.7	11.0 ± 0.6	20.3 ± 6.0	6.0 ± 0.5	8.7 ± 0.3
Ingwe® muthi mixture	5000	25.0 ± 1.0	32.7 ± 5.2	197.0 ± 9.7	209.0 ± 3.2	350.3 ± 14.1	288.0 ± 0.6	10.3 ± 2.4	21.3 ± 1.2	7.3 ± 0.8	7.3 ± 0.3
	500	19.3 ± 1.7	24.0 ± 5.5	185.3 ± 7.2	200.7 ± 0.3	311.3 ± 6.1	269.7 ± 4.2	16.0 ± 4.5	20.7 ± 1.8	7.3 ± 1.2	9.7 ± 0.3
	50	20.0 ± 1.0	33.7 ± 8.0	179.7 ± 6.3	207.3 ± 3.8	288.3 ± 5.7	215.7 ± 0.9	12.3 ± 0.9	15.7 ± 2.0	6.0 ± 0.5	8.3 ± 0.6
Ibhubezi™	5000	23.3 ± 1.3	36.0 ± 2.1	184.6 ± 6.4	179.3 ± 1.8	337.0 ± 7.1	301.3 ± 0.3	16.0 ± 2.5	27.0 ± 0.5	6.0 ± 0.5	7.7 ± 0.3
	500	23.0 ± 1.0	39.7 ± 2.7	177.0 ± 5.2	180.3 ± 14.8	319.3± 3.9	266.0 ± 6.1	13.7 ± 4.8	19.7 ± 1.7	6.0 ± 0.5	8.0 ± 0.5

Table 3.11: continued

Herbal preparations	µg/ml	Number of His+ revertants									
		TA98		TA100		TA102		TA1535		TA1537	
		$S9^-$	$S9^+$	$S9^-$	$S9^+$	$S9^-$	$S9^+$	$S9^-$	$S9^+$	$S9^-$	$S9^+$
	50	22.0 ± 1.2	35.3± 0.9	181.3 ± 5.9	176.7 ± 11.7	305.0 ± 5.0	258.7 ± 6.9	12.0 ± 1.0	17.0 ± 0.5	6.3 ± 0.8	7.3 ± 0.3
Supreme one hundred BODicare®	5000	28.0 ± 1.5	31.6 ± 6.7	178.0 ± 9.9	223.3 ± 1.5	388.7 ± 12.7	244.0 ± 5.8	12.3 ± 0.3	26.0 ± 0.5	8.0 ± 1.5	7.7 ± 0.8
	500	26.0 ± 3.2	31.3 ± 2.3	179.6 ± 11.4	213.3 ± 1.8	322.7 ± 13.3	219.3 ± 6.8	17.0 ± 0.5	25.3 ± 0.3	7.3 ± 0.3	7.3 ± 0.3
	50	26.0 ± 1.5	31.0 ± 1.7	176.7 ± 4.1	204.0 ± 2.1	305.0 ± 2.5	206.3 ± 1.8	13.0 ± 2.0	24.3 ± 0.6	6.0 ± 1.5	9.3 ± 1.2
Sejeso herbal mixture *Ingwe*®	5000	20.0 ± 0.7	23.3 ± 0.9	163.3 ± 25.4	211.7 ± 5.3	307.0 ± 19.4	277.7 ± 6.1	14.3 ± 0.6	25.0 ± 1.5	5.3 ± 0.8	8.3 ± 0.3
	500	22.0 ± 0.6	25.0 ± 3.5	155.7 ± 1.8	203.7 ± 0.3	302.3 ± 12.3	255.3 ± 5.8	14.0 ± 1.5	18.3 ± 0.8	5.0 ± 0.5	8.7 ± 1.3
	50	20.4 ± 0.2	21.3 ± 1.2	172.6 ± 2.2	203.3 ± 1.2	304.0 ± 4.0	312.3 ± 1.2	17.7 ± 0.6	17.7 ± 0.6	6.3 ± 1.2	8.7 ± 0.3
Lion *izifozonke Ingwe*®	5000	22.7 ± 1.0	23.7 ± 0.9	177.7 ± 10.1	255.7 ± 4.9	319.0 ± 12.9	213.0 ± 0.5	24.3 ± 0.3	24.3 ± 0.3	7.0 ± 2.0	7.7 ± 0.6
	500	23.1 ± 1.7	25.3 ± 3.5	177.7 ± 13.3	235.7 ± 2.9	298.0 ± 13.2	216.0 ± 2.5	22.0 ± 1.5	22.0 ± 1.5	6.3 ±0.8	7.7 ± 0.8
	50	21.0 ± 0.2	23.7 ± 0.9	188.6 ± 16.5	203.7 ± 1.5	284.0 ± 3.6	256.0 ± 6.4	13.3 ± 0.3	15.3 ± 1.2	6.7 ± 0.8	8.3 ± 0.3
Stameta™ BODicare®	5000	22.0 ± 2.0	**131.3 ± 6.9**	172.6 ± 6.9	187.0 ± 15.0	395.3 ± 9.3	257.0 ± 5.5	13.0 ± 0.5	28.0 ± 1.5	6.3 ± 0.3	8.7 ± 0.3
	500	22.3 ± 2.0	**48.0 ± 3.2**	166.3 ± 1.9	135.0 ± 1.0	341.3 ± 10.1	259.0 ± 9.6	13.0 ± 0.5	24.0 ± 0.5	6.3 ± 0.8	8.7 ± 0.3
	50	19.0 ± 2.0	39.3 ± 8.0	197.7 ± 4.9	125.3 ± 3.3	325.7 ± 9.5	255.3 ± 5.8	13.3 ± 0.8	17.7 ± 0.6	7.3 ± 1.2	8.7 ± 0.3
Ingwe® special muti *Ingwe*®	5000	21.3 ± 0.3	23.6 ± 2.4	175.0 ± 5.7	173.3 ± 4.2	320.3 ± 6.9	262.0 ± 3.1	11.3 ± 0.3	19.0 ± 0.5	6.7 ± 0.8	10.3 ± 1.2
	500	19.0 ± 1.0	24.3 ± 1.9	188.3 ± 2.2	167.0 ± 1.2	344.3 ± 11.3	256.0 ± 6.4	16.0 ± 2.0	18.7 ± 1.4	7.3 ± 0.8	7.7 ± 0.3
	50	21.0 ± 0.9	28.0 ± 1.5	204.3 ± 4.5	163.0 ± 3.5	318.7 ± 18.2	247.7 ± 8.8	12.7 ± 1.8	18.3 ± 0.8	7.7 ± 0.6	7.0 ± 0.5
African potato extract™	5000	22.0 ± 0.9	**174.1 ± 3.4**	191.3 ± 0.6	204.1 ± 1.2	319.7 ± 0.3	328.3 ± 12.2	26.7 ± 2.3	24.3 ± 2.4	8.7 ± 1.3	10.3 ± 0.2
	500	23.3 ± 0.3	**65.7 ± 1.4**	164.3 ± 0.3	190.3 ± 3.9	323.3 ± 17.2	311.7 ± 21.3	20.3 ± 4.3	21.7 ± 4.5	7.3 ± 2.2	11.7 ± 1.1
	50	19.3 ± 2.3	40.3 ± 2.2	190.1 ± 2.2	218.3 ± 1.9	295.3 ± 23.2	299.3 ± 10.3	21.5 ± 0.9	17.7 ± 1.4	8.3 ± 0.1	11.3 ± 1.8
4NQO	2	208.0 ± 0.6		931.7 ± 145.2		2436.3 ± 120.1		1415.7 ± 62.3		65.0 ± 6.9	
2-AA	2		218.0 ± 13.4		985.3 ± 13.9		1659.0 ± 51.5		162.3 ± 4.0		128.7 ± 31.4
Water (-ve cont)		22.0 ± 1.6	25.3 ± 1.0	199.6 ± 2.3	207.0 ± 4.9	296.0 ± 10.6	266.7 ± 7.6	18.7 ± 1.4	21.7 ± 1.4	6.7 ± 1.2	9.3 ± 0.8

Number of His^+ revertants/plate: mean values of three triplicates, the assay was repeated two times. $S9^-$ refers to the assay without metabolic activation; $S9^+$ refers to the assay with metabolic activation. 4-NQO; 4-nitroquinoline-oxide was the positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene was the positive control for $S9^+$ assays.

3.7. Conclusions

In the present study, the fourteen herbal preparations showed promising pharmacological properties including antibacterial activity against two Gram-negative and two Gram-positive bacterial strains as well as antifungal properties against *Candida albicans*. The herbal preparations also exhibited potential use as anthelmintics. Besides activities in antimicrobial and anthelmintic bioassays, more than 50% of the herbal preparations showed high inhibitory effects against COX, HIV-1 RT and AChE. Most of the herbal preparations which showed poor and low activities in the antibacterial, antifungal, anthelmintic and inhibitory properties against COX, HIV-1 RT and AChE, exhibited higher antioxidant potentials. *Imbiza ephuzwato*, *Sejeso herbal mixture* and *African potato extract*™ showed consistently moderate to high activities in the bioassays used in this study. The observed activity therefore validates the use of these commercial herbal preparations in both urban and rural communities. However, further studies to investigate the botanical composition of the herbal preparations are urgently needed.

The herbal preparations were all not mutagenic towards the Ames test when *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 exposed to three dilutions without S9 metabolic activation were used. However, five of the fourteen herbal preparations showed indirect mutagenic effects towards the tester strain TA98 after metabolic activation but not in the other tester strains (TA100, TA102, TA1535 and TA1537).

Table 3.12 summarizes the various activities observed for the herbal mixtures for all the bioassays used in this study. Based on the summarised results, *Imbiza ephuzwato*, *Ibhubezi*™, *Sejeso* herbal mixture, and *African potato extract*™ were the best mixtures for bacterial infections. *Imbiza ephuzwato*, *Ingwe muthi* mixture, *Sejeso* herbal mixture, *Lion izifozonke* and *African potato extract*™ were the best mixtures for fungal infections. For pain and other conditions related to inflammation and COX enzymes, it will be best to use herbal mixtures such as *Imbiza ephuzwato*, *Vusa umzimba*, *Ingwe muthi* mixture, *Ibhubezi*™, *Sejeso* herbal mixture, and *African potato extract*™. For the use as antiretroviral medication, *Imbiza*

ephuzwato, *Ingwe muthi* mixture, *Sejeso* herbal mixture, and *African potato extract*™ will be the best herbal mixtures to take. *Umpatisa inkosi*, *Vusa umzimba*, *Ibhubezi*™, *Sejeso* herbal mixture, and *African potato extract*™ are the best herbal mixtures for psychosis and mental related conditions such as AD. Herbal mixtures that worked well as antioxidants include *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Ingwe muthi* mixture, *Ibhubezi*™, *Sejeso* herbal mixture and Lion *izifozonke*. Overally, *Imbiza ephuzwato*, *Ingwe muthi* mixture, *Sejeso* herbal mixture and *African potato extract*™ are best mixtures to use. However, *Imbiza ephuzwato* and *African potato extract*™ showed mutagenic effects after bioactivation, therefore they cannot be recommended for prolonged use until the sources of the toxins are identified and eliminated.

Table 3.12: Summary of activities observed in bioassays for the fourteen herbal preparations

Herbal preparations	Efficacy										Safety	
	Antimicrobial bioassays			Enzyme inhibition bioassays				Antioxidants bioassays			Cytotoxicity/mutagenicity	
	Antibacterial	Antifungal	Anthelmintic	COX-1	COX-2	HIV-1 RT	AChE	DPPH	FRAP	CLASMS	NRU	Ames test
Umzimba omubi	–	++	++	++	+	+	–	++	–	+	+	–
Umuthi wekukhwehlela ne zilonda	–	–	++	–	+	+	–	–	++++	+	+	–
Mvusa ukunzi	–	–	+	++	+	++	–	++	++++	+	–	–
Umpatisa inkosi	–	–	+	++	+	+	++++	++	++++	–	++++	TA98 $S9^+$ TA135 $S9^+$
Imbiza ephuzwato	++++	++++	++++	++++	++++	++++	++++	++	++	+	+	TA98 $S9^+$
Vusa umzimba Ingwe®	+	++++	–	++++	++++	+	++++	+	–	+	–	–
Ingwe muthi mixture Ingwe®	–	++	++++	++++	++++	++++	++	+	++++	+	–	–
Ibhubezi™	++++	++++	+	++++	++++	–	++++	++++	++++	–	–	–
Supreme one hundred BODicare®	–	++	++	–	+	–	–	+	–	+	–	–
Sejeso herbal mixture Ingwe®	++++	++++	++++	++	++++	++++	++++	++	++++	+	–	–

Table 3.12: continued

Herbal preparations	Efficacy										Safety	
	Antimicrobial bioassays			Enzyme inhibition bioassays				Antioxidants bioassays			Cytotoxicity/mutagenicity	
	Antibacterial	Antifungal	Anthelmintic	COX-1	COX-2	HIV-1 RT	AChE	DPPH	FRAP	CLASMS	NRU	Ames test
Lion *izifozonke* Ingwe®	++	++++	++++	++++	++	+	++	++	++++	+	–	–
Stameta™ BODicare®	–	–	–	++	+	+	–	++	+	+	–	–
Ingwe special muti Ingwe®	++	++++	++	++	++	+	++	+	–	–	–	–
African potato extract™	+++	++++	++++	++++	++++	++++	++++	++	–	+	NT	TA98 $S9^{+}$

Antimicrobial bioassays
++++ = MIC values < 1 mg/ml
++ = MIC values > 1 – 3.125 mg/ml
+ = MIC values > 3.125- 6.25 mg/ml
– = MIC values > 6.25- >12.5 mg/ml

Enzyme inhibition/DPPH bioassays
++++ = % inhibition 70-100
++ = % inhibition 40-69
+ = % inhibition 20-39
– = % inhibition 0-19

FRAP (Ferric reducing assay)
++++ = Absorbance above 1.5
++ = Absorbance between 1.0-1.5
+ = Absorbance between 0.5-1.0
– = Absorbance between 0 – 0.5

CLAMS (*β*-caroten-linoleic acid model)
++++ = Absorbance above 0.2
++ = Absorbance between 0.15- 0.2
+ = Absorbance between 0.10- 0.15
– = Absorbance between 0 – 0.1

NRU (Neutral red uptake assay)
++++ = NI_{50} values < 0.5 mg/ml
++ = NI_{50} values between 0.5 – 1.0 mg/ml
+ = NI_{50} values between 1.0 – 1.5 mg/ml
– = NI_{50} values > 1.5 mg/ml

NT = Not tested

++++ = High activity
++ = Moderate activity
+ = Low activity
– = Insignificant activity

CHAPTER 4

Plant Composition and Pharmacological Properties of a Commercial Zulu Herbal Mixture: *Imbiza ephuzwato*

4.1. Introduction

Imbiza ephuzwato is a traditional herbal tonic containing extracts of roots, bulbs, rhizomes and leaves of 21 medicinal plants. The herbal mixture is prepared using plants that has been used by the Zulu people for decades. The herbal tonic is manufactured by a Pietermaritzburg herbalist trading as '*KwaNyanga Yezizwe*' which translate '*a place of a great herbalist, for all nations*'. The herbal tonic is sold in *muthi* shops around Pietermaritzburg, KwaZulu-Natal. *Imbiza ephuzwato* is a detoxifying and energising tonic used to increase sexual prowess, relieve constipation, reduce stress, reduce high blood pressure, clear skin conditions, boost energy, boost vitality, prevent arthritis, cure kidney problems and relieve general body pains. *Imbiza ephuzwato* showed consistent bioactivities in all the assays reported in Chapter 3, therefore the mixture was ideal for further studies. This chapter will examine the botanical constituents of *Imbiza ephuzwato* as well as their pharmacological efficacy and significance in the herbal mixture.

The dried materials (chopped) of the 21 plant species are mixed in equal portions and extracted with water by boiling for 1 h. The mixture is then cooled and subsequently sieved through a cloth. The cold brew is then packaged into 1 litre labelled plastic containers (Figure 4.1). Figure 4.1C shows the final stage of the production line, where the labels are put on to the containers while Figure 4.1B represents the label of *Imbiza ephuzwato* and Figure 4.1A presents the packaged *Imbiza ephuzwato* product, ready for sale. The label is written in isiZulu and lists 16 medical conditions *Imbiza ephuzwato* is claimed to improve together with the directions of use. After packaging, *Imbiza ephuzwato* is placed on the shelf, without refrigeration.

Figure 4.1: (A) Packaged product. (B) *Imbiza ephuzwato* label with 16 conditions the product is claimed to cure as well as directions for use. (C) Final step in the production line of *Imbiza ephuzwato* where labels are put on to the containers and then placed onto the shelf for sale.

The herbal tonic's name, *Imbiza ephuzwato- ichathe ilapha lezizifo*, gives a brief description of how the tonic is taken. The translation of the herbal tonic's name is: *Imbiza* (herbal pot) *ephuzwato* (to drink) – *ichathe* (to use as an enema) *ilapha lezizipho* (cures many diseases).

4.1.1. Plant constituents of *Imbiza ephuzwato*

The number of plant species in *Imbiza ephuzwato*, and the level of knowledge of plant choice and time needed to collect the plants represent a complicated and sophisticated process. Amongst the Zulu people, the ability to recognize medicinal plants seems to be an innate gift. The herbalists, *Inyangas* and *Sangomas* who are gifted in terms of traditional medicine form an integral part of the society. This explains the extraordinary ability amongst such herbalists to select plant medicines and the degree to which they can match them as well as understanding their ability to cure diseases **(PUJOL, 1990)**.

The mixture consists of 21 plant species, belonging to 17 families. Table 4.1 lists the plant constituents of *Imbiza ephuzwato* according to their families. The information about the plant constituents was provided by the manufacturer through an oral informal interview. Voucher specimens were collected and deposited in the Herbarium of the University of KwaZulu-Natal. The medicinal, aromatic and toxic wild plants that make up *Imbiza ephuzwato* are collected by the rural population and professional herbalists, who in turn sell them to the manufacturer. The plants are also sold in the herbal shops and markets of KwaZulu-Natal.

Hyacinthaceae is the most represented family (three species) in the herbal tonic, followed by Amaryllidaceae and Asteraceae (two species each) and the rest had one species each. We can speculate that the high species representation of the Hyacinthaceae family may be due to either the fact that the species in the family are abundant in KwaZulu-Natal or the bulbous plants are highly utilized in ATM **(PUJOL, 1990; HUTCHINGS *et al*., 1996)**.

The plant parts used to prepare the mixture are mostly roots (ten species). The other plant parts include bulbs (four species), leaves (three species), corms (one species), rhizomes (one species), stems (one species) and whole plant (one species).

The plant species in the mixture represents a broad range of therapeutical uses (Table 4.1), giving *Imbiza ephuzwato* a variety of medicinal uses **(PUJOL, 1990; HUTCHINGS *et al*., 1996; NEUWINGER, 1996; VAN WYK *et al*., 1997)**. According to the ethnobotanical records of the plant species listed in Table 4.1, the uses are consistent with the medicinal claims on the label of *Imbiza ephuzwato*. However, several questions still remains as to whether the extracts retain their therapeutical strength after a rigorous extraction process involving boiling for 1 h and after mixing extracts of several plant species to make a tonic. A lot of chemical changes and alterations are likely to take place during the manufacturing process of herbal mixtures. It should also be noted that beneficial effects related to synergism, combinational and additive effects of active compounds could also result. However, antagonistic actions amongst different compounds resulting in minimised therapeutic effects of the mixture are also possible.

According to the National Red List of South African Plants, *Imbiza ephuzwato* contains 14 plant species listed as 'least concern' (LC), one species listed as 'declining' and one species listed as 'data deficient-taxonomy problems' (DDT) **(NATIONAL RED LIST OF SOUTH AFRICAN PLANTS, 2001)**. Despite the fact that most of the species used to make up the herbal mixture are listed as LC, implying that the taxa are widespread and abundant, conservation strategies still need to be implemented i.e. substitution of bulbs, roots and/or rhizomes with aerial parts.

Despite the widespread use of *Imbiza ephuzwato* to treat these conditions, no scientific evidence exists to support its uses. The aims and objectives of this study were to investigate the bioactivity of the plant species used in the preparation of *Imbiza ephuzwato* herbal tonic. A compilation of the therapeutic validations for each single plant component could help to demonstrate the medicinal benefits from *Imbiza ephuzwato* herbal mixture.

Table 4.1: Information on the 21 plant species used to manufacture *Imbiza ephuzwato* herbal mixture

Family name	Botanical name **Plant parts used** Red Data List Status *Voucher specimen number*	Traditional medicinal uses
Amaryllidaceae	*Scadoxus puniceus* (L.) Friis & Nordal **Bulbs** LC AR NDHLALA 06	Traditionally used to treat coughs and gastro-intestinal problems. Used to ensure easy birth. Also used for wound therapy, asthma and ulcers. **(VAN WYK *et al*., 1997)**.
Amaryllidaceae	*Cyrtanthus obliquus* (L.f.) Aiton **Bulbs** Declining AR NDHLALA 28	Used to treat chronic coughs and the dried bulb scales are used as a snuff for the relief of headaches **(HUTCHINGS *et al*., 1996)**.
Apocynaceae	*Acokanthera venenata* G. Don. **Roots** NODL -	Root decoction used to treat headache, toothache, anthrax, colds and as antidotes to snake bites **(VAN WYK *et al*., 1997)**.
Asclepiadaeceae	*Asclepias fruticose* L. **Roots** NODL AR NDHLALA 29	Used for chest infections including tuberculosis and can be used as an emetic to strengthen the body. Relieves stomach pain and general body pains **(VAN WYK *et al*., 1997)**.
Asteraceae	*Aster bakeranus* Burtt Davy ex C.A. Sim. **Roots** LC AR NDHLALA 05	Powdered roots are used as a snuff for relieving headache and may be mixed with water to clean the nostrils **(PUJOL, 1990)**. Used also as an antidote for snake bites. Used for venereal diseases and can be administered as an enema to treat severe stomach pain and intestinal parasites **(VAN WYK *et al*., 1997)**.

Table 4.1: continued

Family name	Botanical name **Plant parts used** Red Data List Status *Voucher specimen number*	Traditional medicinal uses
Asteraceae	*Hypericum aethiopicum* Thunb. **Leaves and stems** LC -	Applied to cuts, swellings, burns and sores to promote healing. Treating of skin eruptions, swollen gums and chest pains **(PUJOL, 1990)**.
Cucurbitaceae	*Momordica balsamina* L. **Leaves** LC AR NDHLALA 10	Purgative, for worms, fever, syphilis, skin diseases, anti-inflammation, liver diseases, jaundice, gonorrhoea, arthritis, skin allergies, measles, chicken pox, scabies and anti-malaria **(NEUWINGER, 1996)**.
Fabaceae	*Eriosema cordatum* E.Mey. **Roots** LC AR NDHLALA 22	Roots used for infertility and as an aphrodisiac when mixed with *Corchorus asplenifolius* Burch. **(HUTCHINGS *et al*., 1996)**.
Gunneraceae	*Gunnera perpensa* L. **Rhizomes** Declining AR NDHLALA 02	To induce or augment labour, antenatal medication to tone the uterus, used to assist the expulsion of the uterus, treats stomach pains, rheumatic fever, swellings, menstrual pain and stomach bleeding **(PUJOL, 1990; HUTCHINGS *et al*., 1996)**.
Hyacinthaceae	*Ledebouria* sp. **Bulbs** NODL AR NDHLALA 01	Used for the treatment of diarrhoea, flu and headache **(POOLEY, 1998)**.
Hyacinthaceae	*Drimia robusta* Bak. **Bulbs** DDT AR NDHLALA 08	Treat diseases of the uterus and to clean the bladder **(PUJOL, 1990)**.

Table 4.1: continued

Family name	Botanical name **Plant parts used** Red Data List Status *Voucher specimen number*	Traditional medicinal uses
Hyacinthaceae	*Urginea physodes* (Jacq.) Baker **Bulbs** NODL AR NDHLALA 23	Bulbs are used as some of the ingredients to make infusions known in isiZulu as *isihlambezo* and *inembe* which are taken during pregnancy to facilitate easy delivery **(HUTCHINGS *et al*., 1996)**.
Iridaceae	*Watsonia densiflora* Bak. **Corms** LC AR NDHLALA 07	Corms are used for treatment of diarrhoea **(HUTCHINGS *et al*., 1996)**.
Lamiaceae	*Tetradenia riparia* (Hochst.) Codd **Leaves** LC AR NDHLALA 27	Leaves are used for treating respiratory ailments (Coughs, colds, sore throat), mouth ulcers, stomach ache, diarrhoea, influenza, fever, malaria, swollen legs and headaches **(PUJOL, 1990; HUTCHINGS *et al*., 1996)**.
Lycopodiaceae	*Lycopodium clavatum* L. **Whole plant** LC AR NDHLALA 13	The whole plant is used for magical purposes, smoked for the treatment of headache and infusions are taken to treat diseases of the urinary tract **(HUTCHINGS *et al*., 1996)**.
Menispermaceae	*Stephania abyssinica* (Dill. & Rich.) Walp. **Roots** LC -	Decoction of the chopped root, mixed with *Momordica foetida* Schum. are used for treatment of boils. The root infusions are also used as enemas for children. The plant is used as a protective charm **(HUTCHINGS *et al*., 1996)**.
Rubiaceae	*Rubia cordifolia* L. **Roots** LC AR NDHLALA 24	The roots are used to lower the blood pressure, used internally in the treatment of abnormal uterine bleeding, internal and external haemorrhage, bronchitis, rheumatism, stones in the kidney, bladder and gall and dysentery. Roots are made into paste for ulcers, inflammations and skin

Table 4.1: continued

Family name	Botanical name **Plant parts used** Red Data List Status *Voucher specimen number*	Traditional medicinal uses
		conditions **(HUTCHINGS *et al*., 1996)**.
Rutaceae	*Zanthoxylum capense* (Thunb.) Harv. **Roots** LC AR NDHLALA 26	Used to relieve flatulent colic, stomach ache and fever. Also used for treating toothache and for general mouth wash **(VAN WYK *et al*., 1997)**.
Sapotaceae	*Vitellariopsis marginata* (N.E. Br.) Aubrév **Roots** LC AR NDHLALA 16	Roots are used for the treatment of indigestion and blood cleansing (PUJOL, 1990). Root infusion is taken twice daily for *idliso* (poisoning caused by sorcery) **(HUTCHINGS *et al*., 1996)**.
Thymelaeaceae	*Gnidia kraussiana* Meisn. var. *kraussiana* **Roots** LC AR NDHLALA 25	Treatment of burns, snake bites, stomach complaints, used to ensure easy birth **(HUTCHINGS *et al*., 1996)**.
Tilliaceae	*Corchorus asplenifolius* Burch. **Roots** LC AR NDHLALA 21	Roots used for infertility and as an aphrodisiac when mixed with *Eriosema cordatum* **(HUTCHINGS *et al*., 1996)**.

LC-least concerned (the taxa are widespread and abundant).

Declining- species not endangered or threatened but there are threatening processes causing a continuing decline in the population.

DDT- A taxon is DDT when taxonomical problems hinder its distribution range and habitat from being well defined, so that an assessment of risk of extinction is not possible.

NODL- Not on data list.
Status on red data list taken from the **NATIONAL RED LIST OF SOUTH AFRICAN PLANTS (2001)**.

4.2. Materials and methods

4.2.1. Sample procurement

Plant materials were obtained from the herbal shop where *Imbiza ephuzwato* is manufactured. Voucher specimens were collected and deposited in the Herbarium of the University of KwaZulu-Natal, Pietermaritzburg. The plant materials were completely dried in an oven at 50 °C. After the materials were completely dry, they were ground and stored in airtight containers at 10 °C in the dark.

4.2.2. Sample preparation

Dried, ground plant parts were extracted sequentially with 20 ml/g of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water using a sonication bath for 1 h, the temperature being kept low by adding ice to the water bath. The crude extracts were then filtered through Whatman No. 1 filter paper. PE, DCM and EtOH were concentrated *in vacuo* at 40 °C using a rotary evaporator. The concentrated extracts were then dried under a stream of cold air. Water extracts were freeze dried and kept in airtight containers at 10 °C in the dark.

4.2.3. Antibacterial microdilution bioassay

Minimum inhibitory concentration (MIC) values for antibacterial activity of the plant extracts was determined using the microdilution bioassay in 96-well (Greiner Bio-one GmbH, Germany) microtitre plates **(ELOFF, 1998)** as described in section 3.2.2.1 (Chapter 3) except that PE, DCM and EtOH extracts were resuspended in 80% ethanol and wells consisting of 80% ethanol as solvent control were included in the microtitre plate.

4.2.4. Antifungal microdilution bioassay

The antifungal activity of the plant extracts was evaluated against *Candida albicans* using the micro-dilution assay **(ELOFF, 1998)** modified for an antifungal assay

(MASOKO *et al*., 2007) as described in section 3.2.2.2 (Chapter 3) except that PE, DCM and EtOH extracts were resuspended in 80% ethanol and wells consisting of 80% ethanol as solvent control were included on the microtitre plate.

4.2.5. Cyclooxygenase (COX-1 and COX-2) inhibitory bioassays

The COX-1 and -2 bioassays were performed as described by **JÄGER *et al*. (1996)** and **ZSCHOCKE and VAN STADEN (2000)** as described in Sections 3.3.2.1 and 3.3.2.2 (Chapter 3) respectively except that 2.5 µl of 10 mg/ml sample in 17.5 µl giving a final assay concentration of 250 µg/ml were used for PE, DCM and EtOH and 80% ethanol was used for the background and solvent blanks.

4.2.6. Acetylcholinesterase (AChE) inhibitory bioassay

The microtiter plate assay for the inhibition of AChE by the water extracts of the plants used to make *Imbiza ephuzwato* was done as described by **ELLMAN *et al.* (1961)** with some modifications, as outlined in Section 3.3.2.4 (Chapter 3).

4.2.7. *In vitro* mutagenic evaluation

4.2.7.1. Ames test

Mutagenic properties of the plant extracts were tested using the *Salmonella* microsome assay based on the plate-incorporation procedure with *Salmonella typhimurium* tester strains TA98 with and without metabolic activation **(MARON and AMES, 1983; MORTELMANS and ZEIGER, 2000)** as outlined in Section 3.6.2.2 (Chapter 3).

4.3. Results and discussion

The antibacterial activity (MIC values) for the extracts of the 21 plant species used to manufacture *Imbiza ephuzwato* are presented in Table 4.2. The plant extracts with high antibacterial activity (< 1 mg/ml) are highlighted in bold. Out of the 21 plants

used to manufacture *Imbiza ephuzwato*, 13 species had at least one extract active against *Bacillus subtilis*; 7 against *Escherichia coli*; 9 against *Klebsiella pneumoniae* and *Staphylococcus aureus* respectively.

Extracts of *Corchorus asplenifolius* (PE), *Eriosema cordatum* (DCM), *Tetradenia riparia* (PE and EtOH) and *Zanthoxylum capense* (PE, DCM and EtOH) showed the best antibacterial activity against *Bacillus subtilis* with MIC values of 0.195 mg/ml each. *Corchorus asplenifolius* (PE) and *Tetradenia riparia* (PE) also had MIC values of 0.195 mg/ml each against *Klebsiella pneumoniae*. *Corchorus asplenifolius* (PE), *Eriosema cordatum* (DCM), *Gunnera perpensa* (EtOH) and *Tetradenia riparia* (DCM) and *Zanthoxylum capense* (DCM) also exhibited good activity with MIC values of 0.195 mg/ml each against *Staphylococcus aureus*. The best activity against *Escherichia coli* was exhibited by *Lycopodium clavatum* (EtOH) extract.

Gunnera perpensa and *Rubia cordifolia* were the only plant species used to manufacture *Imbiza ephuzwato* that had water extracts which showed good antibacterial activity with MIC values of 0.78 mg/ml against some of the bacterial strains tested. The rest of the water extracts from the other plants showed poor activity. The MIC values (0.78 mg/ml) exhibited by the water extracts of *Gunnera perpensa* were comparable to the MIC values (0.78 mg/ml) shown by *Imbiza ephuzwato* against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. *Rubia cordifolia* water extracts showed MIC values (0.78 mg/ml) comparable to *Imbiza ephuzwato* herbal mixture's MIC values only for *Bacillus subtilis* and *Staphylococcus aureus*. However, the water extract of *Gunnera perpensa* was more active (0.78 mg/ml) against *Klebsiella pneumoniae* than to *Imbiza ephuzwato* (1.56 mg/ml). This could probably be due to a dilution effect in *Imbiza ephuzwato* on the compounds that inhibits growth of *Klebsiella pneumoniae*. Apart from synergistic effects, interaction of secondary metabolites due to mixing of different plant species may also result in compounds with lower pharmacological effects compared to individual plant species.

Table 4.2: Antibacterial activity (MIC) of different extracts of the plant species used to manufacture *Imbiza ephuzwato* herbal mixture

Plant name	Plant Part	Extract	Bacterial MIC (mg/ml)			
			B.s.	*E.c.*	*K.p.*	*S.a.*
Acokanthera venenata	R	PE	3.125	3.125	3.125	3.125
		DCM	1.56	1.56	3.125	1.56
		EtOH	3.125	3.125	1.56	3.125
		Water	12.5	12.5	12.5	12.5
Asclepias fruticosa	R	PE	1.56	1.56	1.56	1.56
		DCM	1.56	1.56	3.125	1.56
		EtOH	1.56	1.56	1.56	3.125
		Water	12.5	12.5	12.5	12.5
Aster bakeranus	R	PE	**0.78**	3.125	6.25	6.25
		DCM	**0.78**	3.125	3.125	**0.78**
		EtOH	1.56	3.125	3.125	3.125
		Water	>12.5	>12.5	6.25	6.25
Corchorus asplenifolius	R	PE	**0.195**	**0.39**	**0.195**	**0.195**
		DCM	**0.78**	**0.78**	**0.78**	**0.78**
		EtOH	1.56	1.56	3.125	1.56
		Water	1.56	6.25	3.125	>12.5
Cyrtanthus obliquus	B	PE	**0.39**	6.25	3.125	**0.39**
		DCM	**0.39**	1.56	1.56	**0.78**
		EtOH	**0.78**	1.56	1.56	**0.78**
		Water	12.5	6.25	3.125	3.125
Drimia robusta	B	PE	3.125	3.125	**0.78**	12.5
		DCM	3.125	3.125	3.125	6.25
		EtOH	3.125	3.125	3.125	3.125
		Water	>12.5	6.25	3.125	6.25
Eriosema cordatum	R	PE	6.25	1.56	1.56	6.25
		DCM	**0.195**	**0.39**	**0.39**	**0.195**
		EtOH	**0.39**	**0.39**	**0.39**	**0.39**
		Water	1.56	3.125	6.25	1.56
Gnidia kraussiana	R	PE	1.56	1.56	3.125	3.125
		DCM	1.56	1.56	1.56	3.125
		EtOH	3.125	6.25	6.25	3.125
		Water	1.56	1.56	1.56	3.125

Table 4.2: Continued

Plant name	Plant Part	Extract	Bacterial MIC (mg/ml) *B.s.*	*E.c.*	*K.p.*	*S.a.*
Gunnera perpensa	Rh	PE	6.25	3.125	6.25	12.5
		DCM	3.125	3.125	3.125	6.25
		EtOH	**0.39**	**0.39**	**0.39**	**0.195**
		Water	**0.78**	**0.78**	**0.78**	**0.78**
Hypericum aethiopicum	L	PE	**0.78**	3.125	3.125	1.56
		DCM	**0.78**	1.56	1.56	1.56
		EtOH	**0.39**	3.125	**0.78**	1.56
		Water	6.25	>12.5	>12.5	6.25
Ledeboria sp	B	PE	1.56	3.125	3.125	3.125
		DCM	**0.78**	3.125	1.56	1.56
		EtOH	1.56	3.125	3.125	1.56
		Water	6.25	6.25	12.5	6.25
Lycopodium clavatum	WP	PE	**0.78**	1.56	**0.78**	**0.39**
		DCM	**0.39**	**0.78**	1.56	**0.78**
		EtOH	**0.39**	**0.195**	**0.39**	**0.39**
		Water	1.56	3.125	6.25	6.25
Momordica balsamina	L	PE	3.125	6.25	3.125	12.5
		DCM	**0.78**	3.125	1.56	12.5
		EtOH	**0.78**	1.56	1.56	3.125
		Water	3.125	6.25	12.5	12.5
Rubia cordifolia	R	PE	3.125	**0.78**	**0.78**	3.125
		DCM	1.56	**0.78**	**0.39**	3.125
		EtOH	**0.39**	1.56	**0.78**	**0.39**
		Water	**0.78**	1.56	3.125	**0.78**
Scadoxus puniceus	B	PE	6.25	3.125	3.125	3.125
		DCM	3.125	3.125	1.56	3.125
		EtOH	6.25	6.25	6.25	6.25
		Water	6.25	6.25	6.25	6.25
Stephania abyssinica	R	PE	3.125	3.125	3.125	6.25
		DCM	6.25	1.56	3.125	6.25
		EtOH	3.125	3.125	6.25	6.25
		Water	>12.5	>12.5	>12.5	>12.5

Table 4.2: Continued

Plant name	Plant Part	Extract	Bacterial MIC (mg/ml) *B.s.*	*E.c.*	*K.p.*	*S.a.*
Tetradenia riparia	L	PE	**0.195**	**0.39**	**0.195**	**0.39**
		DCM	**0.39**	**0.39**	**0.39**	**0.195**
		EtOH	**0.195**	**0.78**	**0.78**	**0.78**
		Water	>12.5	6.25	6.25	3.125
Urginea physodes	B	PE	1.56	3.125	3.125	3.125
		DCM	6.25	3.125	1.56	6.25
		EtOH	1.56	3.125	6.25	1.56
		Water	>12.5	12.5	12.5	12.5
Vitellariopsis marginata	R	PE	6.25	6.25	3.125	>12.5
		DCM	1.56	1.56	1.56	1.56
		EtOH	**0.78**	6.25	1.56	3.125
		Water	1.56	6.25	6.25	3.125
Watsonia densiflora	C	PE	1.56	3.125	3.125	3.125
		DCM	1.56	1.56	1.56	1.56
		EtOH	1.56	3.125	1.56	1.56
		Water	12.5	12.5	12.5	12.5
Zanthoxylum capense	R	PE	**0.195**	1.56	1.56	1.56
		DCM	**0.195**	**0.78**	0.78	**0.195**
		EtOH	**0.195**	1.56	**0.78**	1.56
		Water	3.125	3.125	1.56	3.125
Imbiza ephuzwato		Water	**0.78**	**0.78**	1.56	**0.78**
Neomycin (+ve control)			1.6×10^{-3}	0.8×10^{-3}	0.8×10^{-3}	1.6×10^{-3}

B.s.=Bacillus subtilis; *E.c.=Escherichia coli*; *K.p.=Klebsiella pneumoniae*; *S.a.=Staphylococcus aureus.*

B- bulbs; C- corms; L- leaves; R- roots; Rh- rhizomes; WP- whole plant; +ve- positive.

Plant extracts with MIC values written in bold font are considered to be very active (MIC < 1 mg/ml).

The observed antibacterial activity of the water extracts of *Gunnera perpensa* and *Rubia cordifolia* justifies their inclusion in the makeup of *Imbiza ephuzwato* herbal drink. Besides possible synergistic effects of compounds within the heterogeneous mixture, *Gunnera perpensa* and *Rubia cordifolia* could be contributing a lot to the observed activity in *Imbiza ephuzwato*. However, further studies using techniques such as HPLC to ascertain the involvement of *Gunnera perpensa* and *Rubia cordifolia* are much needed.

Gunnera perpensa contains a bioactive bitter principle, celastrin while several active compounds including anthraquinones, sistosterols, triterpenoids and fatty acids have been isolated from *Rubia cordifolia* **(HUTCHINGS *et al*., 1996)**. These and other compounds could be responsible for the activity observed in the plant extracts as well as *Imbiza ephuzwato* herbal mixture. Further studies aimed at identifying the active compounds also needed.

The PE, DCM and EtOH extracts of *Tetradenia riparia* exhibited high antibacterial activity. *Tetradenia riparia* contains diterpenoids which have antibiotic activity. However, the water extract did not show activity, suggesting that the active compounds were not extracted by the water phase. The other extracts (PE, DCM and EtOH) showing a wide range of activity amongst the test bacteria were from *Lycopodium clavatum* and *Zanthoxylum capense* which contains benzophenanthridine alkaloids which have various biological activities including antibiotic effects. The genus *Hypericum* contains flavonoids and a well known antibacterial compound called hyperforin **(VAN WYK *et al*., 1997)**.

Surprisingly, plant species such as *Scadoxus puniceus* and *Watsonia densiflora* which exhibited poor antibacterial activity in this study, have been reported to contain alkaloids with potent antibiotic activity **(VAN WYK *et al*., 1997)**. However, several factors also contribute to loss of activity in plant extracts including storage conditions, geographical location of plant growth and environmental stress.

Basing on the antibacterial results of the plant species investigated, *Imbiza ephuzwato* can be used against bacterial infections caused by both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*

and *Klebsiella pneumoniae*) and possibly related bacteria. However, these results only offer supporting evidence for effective use of these extracts but still needs to be confirmed through other assays including *in vivo* tests. Such infections include stomach upsets due to *Escherichia coli* and food poisoning due to products of *Bacillus subtilis*. Although not included on the *Imbiza ephuzwato* label, it can be speculated that the mixture can also be used for wound healing. Wounds are mostly polymicrobial, involving numerous microorganisms that are potentially pathogenic **(LUSEBA *et al*., 2007)**. The mixture can also be applied topically for skin infections, such as pimples and boils and, for to life-threatening diseases such as pneumonia, meningitis and osteomyelitis caused by *Staphylococcus aureus*.

The antifungal activity (MIC and MFC values) for the extracts of the 21 plant species used to manufacture *Imbiza ephuzwato* are presented in Table 4.3. The plant extracts with high antifungal activity (< 1 mg/ml) are highlighted in bold. Out of the 21 plants used to manufacture *Imbiza ephuzwato*, 13 species were highly active against *Candida albicans*.

Just as in the antibacterial assay, the water extract of *Gunnera perpensa* showed good activity (0.78 mg/ml) which was comparable to that of *Imbiza ephuzwato* (0.78 mg/ml). It can also be tentatively concluded that *Gunnera perpensa* could be contributing to the observed antifungal properties exhibited by *Imbiza ephuzwato*. The observed activity in *Gunnera perpensa* water extracts also justifies the inclusion of this plant species in the herbal mixture. The water extracts of the rest of the plant species exhibited poor activity. Water extracts have frequently been reported to have low antimicrobial activity **(RABE and VAN STADEN, 1997; LUSEBA *et al*., 2007)**.

A biologically active bitter principle, celastrin is reported to be present in *Gunnera perpensa* **(VAN WYK *et al*., 1997)**. Celastrin could be the compound responsible for the observed activity in the water extract of *Gunnera perpensa* as well as in *Imbiza ephuzwato*. Interaction of the biological molecules in the mixture can also result in compounds or complexes with high biological activity. However, further research aimed at isolating and identifying the active principles is needed.

Table 4.3: Antifungal activity (MIC and MFC) of extracts from the plant species used to manufacture *Imbiza ephuzwato* herbal mixture

Plant name	Plant Part		*Candida albicans*	
		Extract	MIC	MFC
Acokanthera venenata	R	PE	3.125	6.25
		DCM	**0.195**	1.56
		EtOH	**0.78**	6.25
		Water	3.125	6.25
Asclepias fruticosa	R	PE	3.125	6.25
		DCM	3.125	>12.5
		EtOH	3.125	3.125
		Water	6.25	12.5
Aster bakeranus	R	PE	3.125	6.25
		DCM	1.56	6.25
		EtOH	3.125	6.25
		Water	12.5	>12.5
Corchorus asplenifolius	R	PE	3.125	3.125
		DCM	**0.39**	3.125
		EtOH	**0.78**	1.56
		Water	6.25	12.5
Cyrtanthus obliquus	B	PE	1.56	6.25
		DCM	**0.195**	3.125
		EtOH	1.56	1.56
		Water	12.5	>12.5
Drimia robusta	B	PE	6.25	12.5
		DCM	3.125	6.25
		EtOH	3.125	6.25
		Water	6.25	12.5
Eriosema cordatum	R	PE	1.56	12.5
		DCM	1.56	12.5
		EtOH	3.125	6.25
		Water	12.5	>12.5
Gnidia kraussiana	R	PE	3.125	6.25
		DCM	3.125	6.25
		EtOH	3.125	6.25
		Water	>12.5	>12.5

Table 4.3: Continued

Plant name	Plant Part	*Candida albicans*		
		Extract	MIC	MFC
Gunnera perpensa	Rh	PE	3.125	6.25
		DCM	3.125	6.25
		EtOH	**0.093**	**0.78**
		Water	**0.78**	**0.78**
Hypericum aethiopicum	L	PE	3.125	6.25
		DCM	**0.093**	**0.78**
		EtOH	3.125	6.25
		Water	12.5	12.5
Ledeboria sp	B	PE	3.125	12.5
		DCM	6.25	12.5
		EtOH	1.56	1.56
		Water	12.5	>12.5
Lycopodium clavatum	WP	PE	1.56	1.56
		DCM	1.56	1.56
		EtOH	**0.195**	1.56
		Water	12.5	>12.5
Momordica balsamina	L	PE	3.125	3.125
		DCM	**0.195**	**0.195**
		EtOH	**0.39**	**0.39**
		Water	6.25	>12.5
Rubia cordifolia	R	PE	1.56	3.125
		DCM	**0.78**	**0.78**
		EtOH	1.56	1.56
		Water	6.25	12.5
Scadoxus puniceus	B	PE	3.125	6.25
		DCM	3.125	12.5
		EtOH	3.125	6.25
		Water	12.5	>12.5
Stephania abyssinica	R	PE	3.125	6.25
		DCM	3.125	3.125
		EtOH	3.125	6.25
		Water	12.5	>12.5

Table 4.3: Continued

Plant name	Plant Part	*Candida albicans*		
		Extract	MIC	MFC
Tetradenia riparia	L	PE	1.56	3.125
		DCM	**0.78**	**0.78**
		EtOH	3.125	3.125
		Water	>12.5	>12.5
Urginea physodes	B	PE	3.125	6.25
		DCM	1.56	>12.5
		EtOH	**0.093**	**0.78**
		Water	1.56	6.25
Vitellariopsis marginata	R	PE	**0.78**	6.25
		DCM	1.56	6.25
		EtOH	3.125	3.125
		Water	12.5	>12.5
Watsonia densiflora	C	PE	3.125	12.5
		DCM	3.125	12.5
		EtOH	**0.78**	**0.78**
		Water	0.78	3.125
Zanthoxylum capense	R	PE	**0.78**	6.25
		DCM	**0.78**	1.56
		EtOH	**0.39**	**0.78**
		Water	3.125	6.25
Imbiza ephuzwato		Water	**0.78**	1.56
Amphotericin B (+ve control)			9.77×10^{-3}	7.81×10^{-2}

B- bulbs; C- corms; L- leaves; R- roots; Rh- rhizomes; WP- whole plant; +ve- positive.

Plant extracts with MIC and MFC values written in bold font are considered to be very active (MIC < 1 mg/ml).

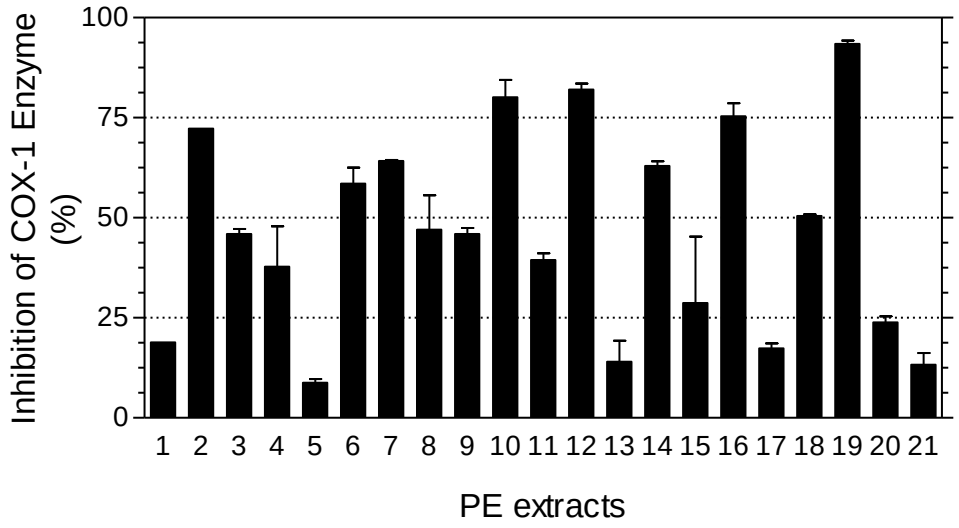
The extracts of *Gunnera perpensa* (EtOH), *Hypericum aethiopicum* (DCM) and *Urginea physodes* (EtOH) showed the best antifungal activity with MIC values of 0.093 mg/ml each. All the extracts of *Asclepias fruticose*, *Aster bakeranus*, *Drimia robusta*, *Eriosema cordatum*, *Gnidia kraussiana*, *Ledeboria* sp, *Scadoxus puniceus* and *Stephania abyssinica* showed poor activity. The rest of the plants had at least one extract active against *Candida albicans*.

The antifungal assay was modified to test if the active extracts were fungistatic or fungicidal. As previously stated, there are certain conditions ideal for an extract or compound to be a good antifungal drug. The extract should be fungicidal rather than fungistatic **(ZACCHINO *et al*., 2003)**. From the extracts which showed good antifungal activity (< 1 mg/ml), it was observed that the extracts of *Gunnera perpensa* (water), *Momordica balsamina* (DCM and EtOH), *Rubia cordifolia* (DCM), *Tetradenia riparia* (DCM) and *Watsonia densiflora* (EtOH) were fungicidal as there was no growth of fungal cells after addition of broth to the clear wells.

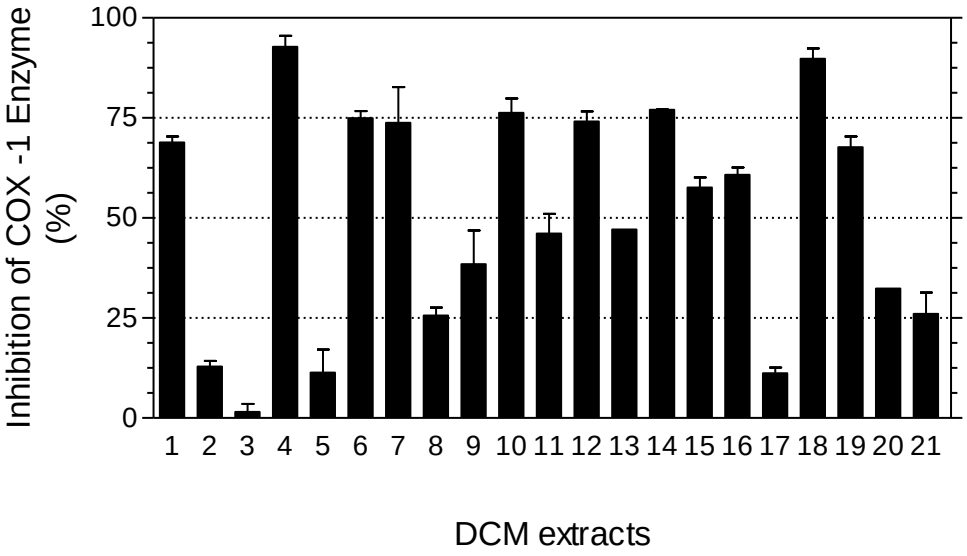
Based on the results of the plant species investigated as well as the observed activity of *Imbiza ephuzwato* against *Candida albicans*, it is expected that the mixture can be successfully used for fungal infections including candidiasis (in blood, oral and genital) although these conditions are not listed on the label. Further studies aimed at investigating the effects of *Imbiza ephuzwato* against clinical strains of *Candida albicans* as well as isolating the active compounds are much needed.

The inhibitory effects on COX-1 and COX-2 enzymes by extracts of the 21 plant used to manufacture *Imbiza ephuzwato* are presented in Figures 4.2 and 4.3 respectively. As previously mentioned, four levels of activity are defined in the COX assay with activity below 20% being considered insignificant, 20– 40% low, 40–70% moderate and 70–100% high **(TAYLOR and VAN STADEN, 2001)**. The level to which the plant extracts could inhibit the COX-1 enzyme can consequently be considered high for 5 PE, 7 DCM, 3 EtOH, 6 water extracts, moderate for 6 PE, 5 DCM, 5 EtOH, 2 water extracts and low for 4 PE, 4 DCM, 5 EtOH and 2 water extracts. COX-1 inhibition activity was insignificant for 5 PE, 4 DCM, 8 EtOH and 11 water extracts.

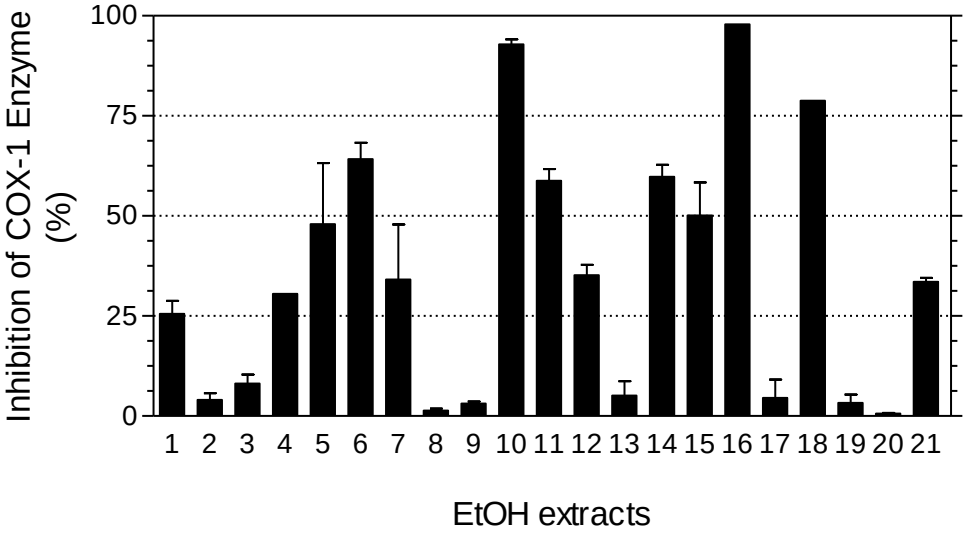
A


Inhibition of COX-1 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
PE extracts

B


Inhibition of COX -1 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DCM extracts

C


Inhibition of COX-1 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EtOH extracts

D

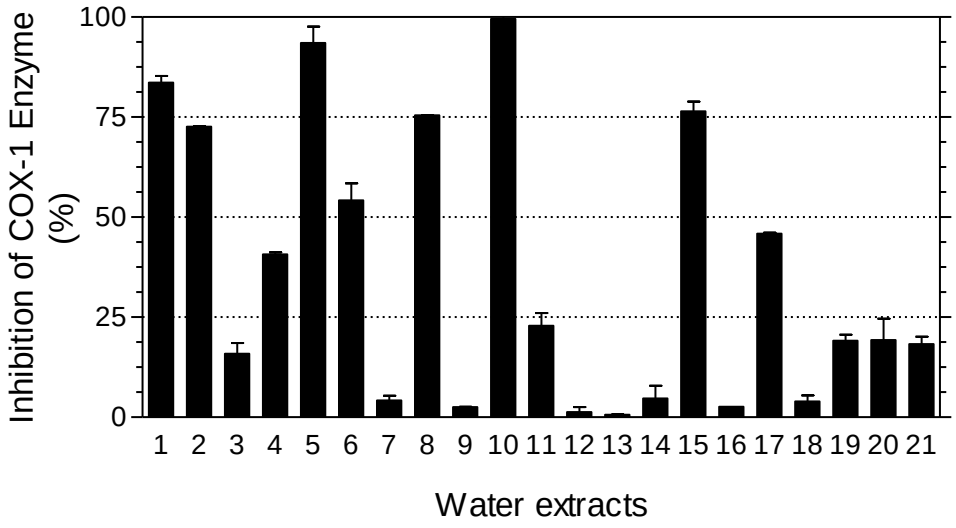


Figure 4.2: Percentage inhibition of COX-1 enzyme by (A) PE, (B) DCM, (C) EtOH and (D) water extracts of the plants used to make *Imbiza ephuzwato* (250 µg/ml and 2 mg/ml for organic and water extracts respectively); (1) *Hypericum aethiopicum*, (2) *Gunnera perpensa*, (3) *Zanthoxylum capense*, (4) *Corchorus asplenifolius*, (5) *Drimia robusta*, (6) *Cyrtanthus obliquus*, (7) *Aster bakeranus*, (8) *Vitellariopsis marginata*, (9) *Asclepias fruticosa*, (10) *Scadoxus puniceus*, (11) *Watsonia densiflora*, (12) *Gnidia kraussiana*, (13) *Stephania abyssinica*, (14) *Urginea physodes*, (15) *Momordica balsamina*, (16) *Acokanthera venenata*, (17) *Ledeboria sp*, (18) *Tetradenia riparia*, (19) *Lycopodium clavatum*, (20) *Rubia cordifolia* and (21) *Eriosema cordatum*. Percentage inhibition by *Imbiza ephuzwato* (2 mg/ml) and indomethacin (5 µM) was 82.23 ± 2.22 and 64.18 ± 3.10 respectively. Plant extracts with inhibitory activity above 70% were considered to be highly active.

The water extracts of *Hypericum aethiopicum*, *Gunnera perpensa*, *Drimia robusta*, *Vitellariopsis marginata*, *Scadoxus puniceus* and *Momordica balsamina* showed percentage inhibition of COX-1 that was over 70%. These plant species could have contributed to the COX-1 inhibition observed for *Imbiza ephuzwato* (82.23%) through additive or synergistic effects.

The extent to which the plant extracts could inhibit the COX-2 enzyme can consequently be considered high for 6 PE, 5 DCM, 1 EtOH, 4 water extracts, moderate for 5 PE, 5 DCM, 4 EtOH, 2 water extracts and low for 4 PE, 3 DCM, 8 EtOH and 2 water extracts. COX-2 enzyme inhibition activity was insignificant for 6 PE, 6 DCM, 8 EtOH and 13 water extracts.

The water extracts of *Gunnera perpensa*, *Cyrtanthus obliquus*, *Momordica balsamina* and *Tetradenia riparia* exhibited inhibitory activity above 70% against the COX-2 enzyme. *Imbiza ephuzwato* exhibited 84.10% inhibition against the COX-2 enzyme. These four species could jointly have contributed to the observed COX-2 inhibitory activity shown by *Imbiza ephuzwato*.

The water extracts of *Gunnera perpensa* and *Momordica balsamina* showed higher inhibitory activity in the COX-2 bioassay when compared to the COX-1 bioassay which suggests that these extracts could be selective towards the COX-2 enzyme. It is unfeasible to have selective COX-1 inhibitory activity as the main criterion because of the side effects associated with COX-1 enzyme inhibition, including damage to the gastrointestinal tract, resulting in ulcers **(KIM *et al*., 2005)**.

The high COX-2 inhibitory activity of *Gunnera perpensa* and *Momordica balsamina* makes *Imbiza ephuzwato* a better product when treating inflammation related conditions. This is because it is desirable to have a product with higher COX-2 inhibitory effects than for the COX-1 enzyme. The COX-2 enzyme is specific in treating inflamed tissue, resulting in less gastric irritation as is associated with COX-1 inhibitors, hence decreased risk of gastric ulceration. However, the selectivity of COX-2 does not seem to negate other side-effects, such as increased risk of renal failure. There is evidence which indicates an increase in the risk for heart attack,

thrombosis, and stroke due to persistent use of COX-2 selective drugs **(COPELAND, 2005)**.

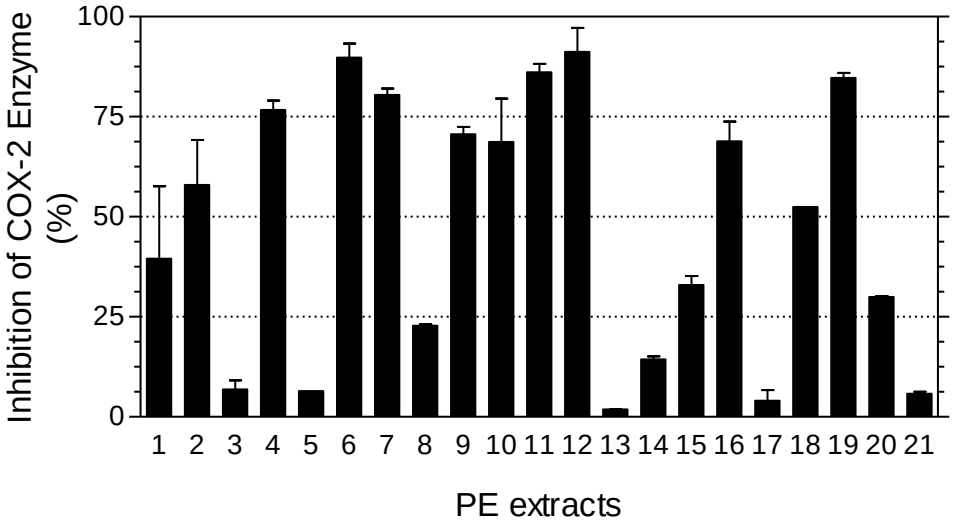
As mentioned previously, the rhizome of *Gunnera perpensa* contains an active bitter principle, celastrin and other compounds that have been reported to show uterotonic activity **(BROOKES and DUTTON, 2007)**. Such compounds could also have anti-inflammatory activities, justifying the inclusion of *Gunnera perpensa* into *Imbiza ephuzwato* herbal mixture. The activity observed can also validate the use of *Gunnera perpensa* in ATM to treat stomach problems, fever, swelling, menstrual pain and for wound dressing.

Tetradenia riparia has been reported to contain Ibozol, a diterpene diol and umuravumbolide **(VAN WYK *et al*., 1997)** which justifies the use of the leaf in ATM to treat stomach ache and swollen legs as well as its inhalation to treat headaches and its inclusion into *Imbiza ephuzwato* herbal mixture.

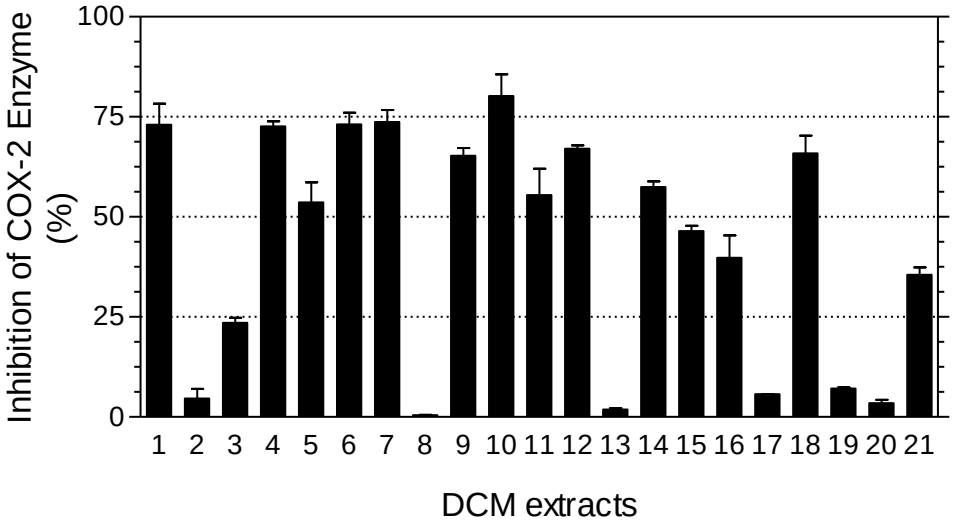
The low inhibitory values in some of the extracts tested in the present study could be due to the impure form and/or low concentration of the active compound(s) in the crude extracts **(RABE and VAN STADEN, 1997)**. Another important point to note is that enzyme inhibition assays involve specific mechanisms and if any compound acts by a mechanism not related to the enzyme being inhibited then the compound will be regarded as being not active **(TAYLOR and VAN STADEN, 2001)**. Therefore, extracts showing low inhibitory activity in COX-1 and COX-2 bioassays can not conclusively be said to lack inhibitory activity as they may contain compounds that are active through other mechanisms linked to the processes of pain and inflammation **(McGAW *et al*., 1997)**.

Imbiza ephuzwato can therefore be used for treating inflammation related conditions listed on the label including back pain, swelling of the feet, menstrual pains, kidney inflammation, sore back and general body pain effectively. However, as mentioned above, persistent use of *Imbiza ephuzwato* may have negative effects associated with damage of the gastrointestinal tract because of the use of plants with high COX-1 inhibitory effects.

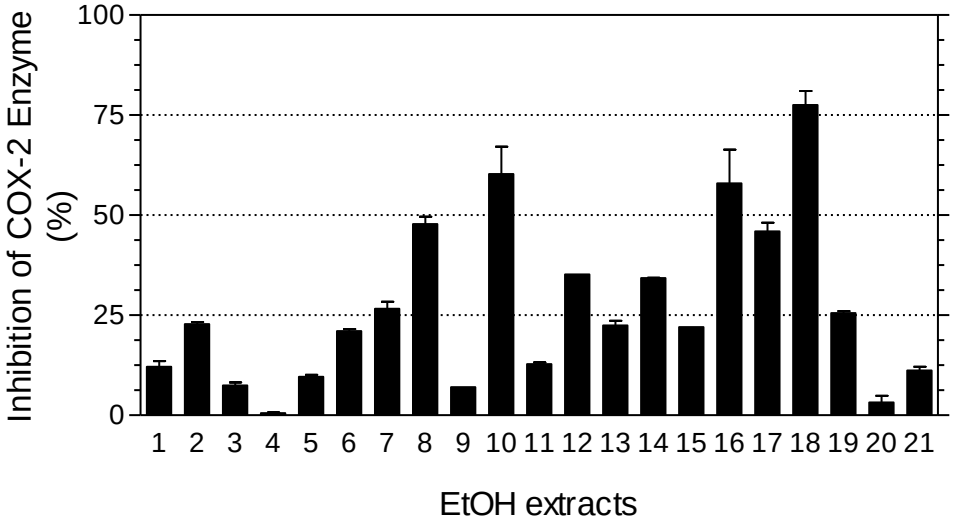
A


Inhibition of COX-2 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
PE extracts

B


Inhibition of COX-2 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DCM extracts

C


Inhibition of COX-2 Enzyme (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EtOH extracts

D

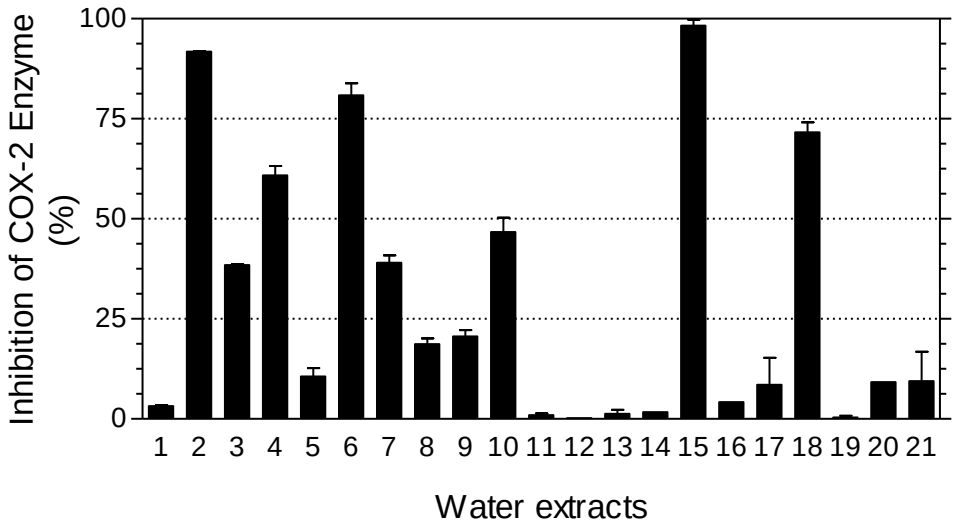


Figure 4.3: Percentage inhibition of COX-2 enzyme by (A) PE, (B) DCM, (C) EtOH and (D) water extracts of the plants used to make *Imbiza ephuzwato* (250 µg/ml and 2 mg/ml for organic and water extracts respectively); (1) *Hypericum aethiopicum*, (2) *Gunnera perpensa*, (3) *Zanthoxylum capense*, (4) *Corchorus asplenifolius*, (5) *Drimia robusta*, (6) *Cyrtanthus obliquus*, (7) *Aster bakeranus*, (8) *Vitellariopsis marginata*, (9) *Asclepias fruticosa*, (10) *Scadoxus puniceus*, (11) *Watsonia densiflora*, (12) *Gnidia kraussiana*, (13) *Stephania abyssinica*, (14) *Urginea physodes*, (15) *Momordica balsamina*, (16) *Acokanthera venenata*, (17) *Ledeboria sp*, (18) *Tetradenia riparia*, (19) *Lycopodium clavatum*, (20) *Rubia cordifolia* and (21) *Eriosema cordatum*. Percentage inhibition by *Imbiza ephuzwato* (2 mg/ml) and indomethacin (200 µM) was 84.10 ± 0.17 and 68.50 ± 2.57 respectively. Plant extracts with inhibitory activity above 70% were considered to be highly active.

The results of the AChE enzyme inhibitory activity are presented in Figure 4.4. Fifteen out of the 20 water plant extracts showed inhibitory activity above 50%. *Gunnera perpensa*, *Cyrtanthus obliquus*, *Vitellariopsis marginata*, *Asclepias fruticosa* and *Watsonia densiflora* showed good AChE inhibitory activity (> 80%). *Corchorus asplenifolius*, *Drimia robusta*, *Aster bakeranus*, *Scadoxus puniceus*, *Stephania abyssinica*, *Acokanthera venenata*, *Ledeboria sp*, *Lycopodium clavatum* and *Eriosema cordatum* extracts exhibited moderate AChE enzyme inhibitory activity (between 50% and 80%). The rest of the plant extracts exhibited low activity (< 50%).

The IC_{50} values for water extracts of the plant species used to make *Imbiza ephuzwato* herbal mixture are shown in Table 4.4. Four of the water extracts of the plant species (*Aster bakeranus*, *Gnidia kraussiana*, *Ledeboria* sp. and *Urginea physodes*) exhibited lower than 50% inhibitory activity at 1 mg/ml, making it impossible to calculate IC_{50} values. The order of potent activity with respect to IC_{50} values for the plant extracts was: *Gunnera perpensa* > *Zanthoxylum capense* > *Momordica balsamina* > *Lycopodium clavatum* > *Corchorus asplenifolius* > *Scadoxus puniceus* > *Asclepias fruticosa* > *Tetradenia riparia* > *Drimia robusta* > *Rubia cordifolia* > *Stephania abyssinica* > *Acokanthera venenata* > *Eriosema cordatum* > *Cyrtanthus obliquus* > *Watsonia densiflora* > *Vitellariopsis marginata* > *Hypericum aethiopicum*.

The family Amaryllidaceae (*Scadoxus puniceus* and *Cyrtanthus obliquus*) contains bioactive alkaloids such as epibuphanisine, epivittatine, haemathamine, 1-*O*-acetyllycorine and cherylline. These alkaloids have been reported to have good AChE enzyme inhibitory effects **(ELGORASHI *et al*., 2001)**. Some or all of these alkaloids in synergism or addition could be attributed to the AChE enzyme inhibitory activity exhibited by *Scadoxus puniceus* and *Cyrtanthus obliquus* in this study.

Low activity observed in some extracts could be due to lower doses used in scientific studies. In ATM, healers seldom weigh plant materials when administering them to patients, resulting in consumption of larger doses of the medicine than may be required **(FRANSWORTH, 1993)**.

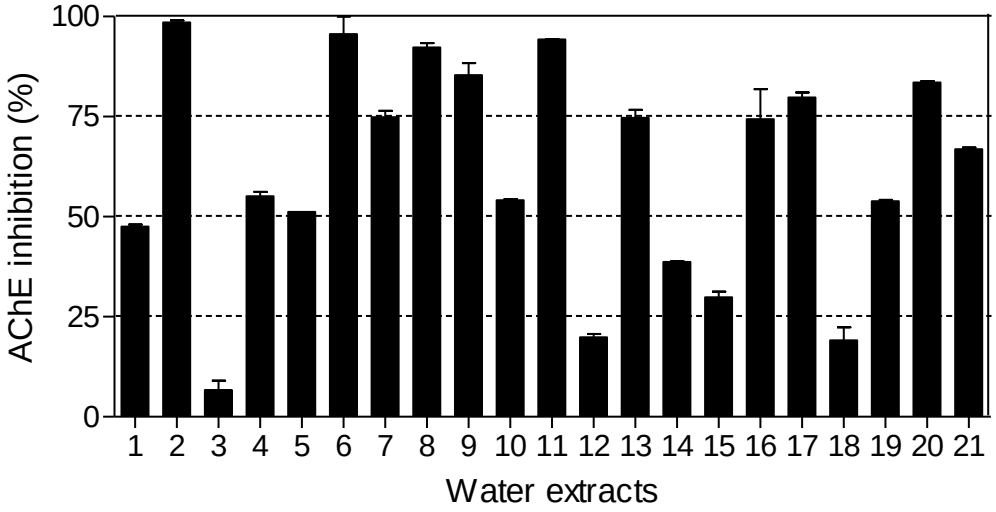


Figure 4.4: Percentage inhibition of the AChE enzyme by water extracts (1 mg/ml) of the plants used to make *Imbiza ephuzwato*; (1) *Hypericum aethiopicum*, (2) *Gunnera perpensa*, (3) *Zanthoxylum capense*, (4) *Corchorus asplenifolius*, (5) *Drimia robusta*, (6) *Cyrtanthus obliquus*, (7) *Aster bakeranus*, (8) *Vitellariopsis marginata*, (9) *Asclepias fruticosa*, (10) *Scadoxus puniceus*, (11) *Watsonia densiflora*,(12) *Gnidia kraussiana*, (13) *Stephania abyssinica*, (14) *Urginea physodes*, (15) *Momordica balsamina*, (16) *Acokanthera venenata*, (17) *Ledeboria sp*, (18) *Tetradenia riparia*, (19) *Lycopodium clavatum*, (20) *Rubia cordifolia* and (21) *Eriosema cordatum*. Plant extracts with inhibitory activity above 70% were considered to be highly active. Percentage inhibition by *Imbiza ephuzwato* herbal mixture (1 mg/ml) and galanthamine (20 µM) was 94.2 ± 0.29 and 89.90 ± 0.32 respectively.

Table 4.4: AChE inhibitory activity (IC_{50} µg/ml) of water extracts of the plant species used to manufacture *Imbiza ephuzwato* herbal mixture

Plant species	Plant Part	AChE inhibitory activity IC_{50} (µg/ml)
Acokanthera venenata	R	590.00 ± 10.22
Asclepias fruticosa	R	372.10 ± 3.44
Aster bakeranus	R	NC
Corchorus asplenifolius	R	118.10 ± 2.65
Cyrtanthus obliquus	B	899.90 ± 4.34
Drimia robusta	B	487.40 ± 7.98
Eriosema cordatum	R	756.56 ± 9.12
Gnidia kraussiana	R	NC
Gunnera perpensa	Rh	**3.249 ± 0.56**
Hypericum aethiopicum	L	1218.0 ± 2.44
Ledeboria sp	B	NC
Lycopodium clavatum	WP	98.23 ± 1.23
Momordica balsamina	L	61.34 ± 0.45
Rubia cordifolia	R	545.60 ± 12.47
Scadoxus puniceus	B	271.90 ± 10.87
Stephania abyssinica	R	560.90 ± 9.44
Tetradenia riparia	L	464.20 ± 16.33
Urginea physodes	B	NC
Vitellariopsis marginata	R	959.10 ± 6.33
Watsonia densiflora	C	911.60 ± 18.55
Zanthoxylum capense	R	**7.52 ± 0.01**
Imbiza ephuzwato		**0.48 ± 0.01**
Galanthamine		1.6 ± 06 (µM)

B- bulbs; C- corms; L- leaves; R- roots; Rh- rhizomes; WP- whole plant.

Plant extracts with IC_{50} values in bold are considered potent inhibitors of AChE.

NC- (Not calculated) IC_{50} could not be calculated because the activity was less than 50% at highest concentration.

Values in bold represents extracts with potent inhibitors of AChE enzymes.

A high activity against the AChE enzyme justifies the use of *Imbiza ephuzwato* for depression. Based on the inhibitory effects against AChE by most of these plant extracts, *Imbiza ephuzwato* can possibly also be used for treatment of Alzheimer's disease (AD) and short term memory lose.

The standard plate incorporation method for the Ames test using *Salmonella typhimurium* tester strains TA98 exposed to three dilutions with and without S9 metabolic activation of the water extracts of the 21 plant species used to make *Imbiza ephuzwato* was performed to investigate the observed mutagenicity observed in the herbal mixture. Ames test without S9 metabolic activation can only detect direct mutagens while the test with S9 metabolic activation allows the detection of indirect mutagens, as was observed in *Imbiza ephuzwato*.

Table 4.5 presents the spontaneous reversion response of the *Salmonella typhimurium* tester strain to the different dilutions of the water extracts of the 21 plant species. The results revealed that all the extracts were non-mutagenic towards the *Salmonella typhimurium* strains TA98 for the assay with and without S9 metabolic activation. The average His^+ revertant colonies observed for the tester strain caused by all the extracts at all the concentrations did not satisfy the criteria for mutagenicity. There were no notable dose-dependent increase in the number of revertants and the numbers of revertants were all not equal to or greater than two times that of the negative control **(BULMER *et al*., 2007)**. There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion) which could also be classified as toxic.

In view of the fact that no plant extract was found to be mutagenic, the mutagenicity observed against TA98 when *Imbiza ephuzwato* was exposed to S9 metabolic activation could therefore be as a result of interaction of biomolecules in the heterogeneous mixture, yielding compounds that are converted to mutagenic agents by xenobiotic metabolizing enzymes. As discussed in Section 3.6.3, such biomolecules includes various aromatic nitroso-derivatives of amine carcinogens that have been shown to cause a reversion of *hisD3052* mutation in TA98 back to the wild-type state **(MORTELMANS and ZEIGER, 2000)**.

Table 4.5: Number of revertant colonies of *Salmonella typhimurium* strain TA98 induced by extracts of the plant species used to manufacture *Imbiza ephuzwato* herbal mixture with and without S9 metabolic activation

Plant species	Plant part	Concentration (µg/ml)	Number of His^+ revertants	
			$S9^-$	$S9^+$
Acokanthera venenata	R	5000	20.3 ± 2.4	26.7 ± 2.2
		500	26.7 ± 0.2	28.3 ± 0.1
		50	22.3 ± 1.1	26.3 ± 1.1
Asclepias fruticosa	R	5000	19.3 ± 4.2	20.7 ± 1.2
		500	26.7 ± 3.3	24.3 ± 0.2
		50	23.7 ± 4.1	22.7 ± 5.1
Aster bakeranus	R	5000	19.3 ± 5.2	26.7 ± 2.2
		500	23.7 ± 3.3	29.3 ± 0.9
		50	24.7 ± 4.4	29.7 ± 1.2
Corchorus asplenifolius	R	5000	26.3 ± 2.2	25.3 ± 3.3
		500	27.0 ± 0.0	29.3 ± 2.2
		50	28.7 ± 0.8	20.3 ± 2.2
Cyrtanthus obliquus	B	5000	21.3 ± 2.1	23.7 ± 4.4
		500	24.3 ± 2.2	25.7 ± 0.9
		50	18.7 ± 3.1	27.3 ± 1.1
Drimia robusta	B	5000	21.3 ± 3.3	22.7 ± 2.7
		500	17.7 ± 2.4	20.7 ± 3.3
		50	20.3 ± 4.4	20.7 ± 1.3
Eriosema cordatum	R	5000	21.3 ± 3.3	25.3 ± 2.2
		500	18.7 ± 4.4	24.3 ± 1.1
		50	25.3 ± 3.3	26.7 ± 2.3
Gnidia kraussiana	R	5000	24.3 ± 4.2	27.3 ± 2.2
		500	25.7 ± 2.0	26.3 ± 1.3
		50	20.3 ± 2.5	27.7 ± 1.0
Gunnera perpensa	Rh	5000	23.3 ± 2.3	24.7 ± 1.2
		500	24.7 ± 1.1	31.3 ± 2.2
		50	22.7 ± 2.1	27.7 ± 3.3

Table 4.5: continued

Plant species	Plant part	Concentration (µg/ml)	Number of His^+ revertants	
			$S9^-$	$S9^+$
Hypericum aethiopicum	L	5000	20.3 ± 1.3	27.3 ± 1.2
		500	26.7 ± 4.5	28.7 ± 2.7
		50	22.7 ± 3.3	23.3 ± 3.4
Ledeboria sp	B	5000	20.3 ± 2.2	28.7 ± 0.2
		500	23.7 ± 3.4	29.3 ± 0.2
		50	31.3 ± 2.3	26.3 ± 1.2
Lycopodium clavatum	WP	5000	27.7 ± 5.2	19.7 ± 3.3
		500	21.7 ± 2.9	18.7 ± 5.5
		50	26.3 ± 3.8	16.7 ± 4.5
Momordica balsamina	L	5000	21.3 ± 2.9	23.3 ± 1.1
		500	28.7 ± 1.8	23.7 ± 0.9
		50	27.3 ± 0.2	28.3 ± 1.5
Rubia cordifolia	R	5000	27.3 ± 1.1	19.7 ± 4.7
		500	20.7 ± 2.7	24.3 ± 3.3
		50	31.7 ± 3.9	23.7 ± 2.2
Scadoxus puniceus	B	5000	24.3 ± 1.2	24.3 ± 2.8
		500	21.7 ± 3.4	23.3 ± 4.4
		50	24.3 ± 2.7	33.7 ± 3.5
Stephania abyssinica	R	5000	20.7 ± 4.7	23.3 ± 1.0
		500	15.3 ± 2.3	24.7 ± 1.7
		50	22.3 ± 0.3	23.7 ± 3.3
Tetradenia riparia	L	5000	21.7 ± 3.7	28.7 ± 1.7
		500	21.3 ± 5.1	28.3 ± 2.7
		50	23.3 ± 3.3	20.7 ± 5.4
Urginea physodes	B	5000	20.3 ± 0.1	43.0 ± 0.0
		500	22.7 ± 1.3	26.3 ± 1.5
		50	23.3 ± 1.7	28.7 ± 3.3
Vitellariopsis marginata	R	5000	19.7 ± 3.3	23.7 ± 2.7
		500	20.3 ± 1.7	25.3 ± 0.1
		50	20.3 ± 3.1	30.7 ± 0.7

Table 4.5: continued

Plant species	Plant part	Concentration (µg/ml)	Number of His^+ revertants	
			$S9^-$	$S9^+$
Watsonia densiflora	C	5000	23.7 ± 1.9	29.7 ± 3.7
		500	20.3 ± 0.7	32.3 ± 2.3
		50	19.3 ± 3.7	33.3 ± 2.2
Zanthoxylum capense	R	5000	19.7 ± 4.3	22.3 ± 3.3
		500	21.7 ± 3.3	30.7 ± 3.7
		50	21.3 ± 1.0	24.3 ± 1.2
Imbiza ephuzwato		5000	22.0 ± 0.9	**227.7 ± 23.1**
		500	24.0 ± 1.6	**71.5 ± 5.5**
		50	19.7 ± 1.2	45.3 ± 1.8
4-NQO		2	208.0 ± 0.6	
2-AA		2	31.3 ± 1.7	218.0 ± 13.4
Water			22.0 ± 1.6	25.3 ± 1.0

B- bulbs; C- corms; L- leaves; R- roots; Rh- rhizomes; WP- whole plant.

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times.

$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation.

4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays and water was used as a negative control.

Values in bold represents plant extracts/herbal mixture with mutagenic activity.

The results obtained here offer supporting evidence for the safe use of these water extracts. However, more confirmatory tests using other assays and/or *in vivo* tests are still required. Studies aimed at investigating the effects of the interactions that occurs in heterogeneous mixtures towards toxicity and mutagenicity are urgently needed.

4.4. Conclusions

The plant species used to make *Imbiza ephuzwato* were investigated for their individual pharmacological properties. Extracts (PE, DCM, EtOH and water) of the plants were evaluated against two Gram-positive and two Gram-negative bacteria, *Candida albicans*, inhibitory effects against COX-1 and -2 as well as AChE enzymes. *Gunnera perpensa* and *Rubia cordifolia* were the only plant species used to manufacture *Imbiza ephuzwato* that had water extracts which showed good antibacterial activity. The extracts of *Gunnera perpensa* (EtOH), *Hypericum aethiopicum* (DCM) and *Urginea physodes* (EtOH) showed the best antifungal activity. The water extracts of *Hypericum aethiopicum*, *Gunnera perpensa*, *Drimia robusta*, *Vitellariopsis marginata*, *Scadoxus puniceus* and *Momordica balsamina* showed percentage inhibition of COX-1 that was over 70%. For COX-2 enzyme, the water extracts of *Gunnera perpensa*, *Cyrtanthus obliquus*, *Momordica balsamina* and *Tetradenia riparia* exhibited inhibitory activity above 70%. Water extracts of *Gunnera perpensa*, *Cyrtanthus obliquus*, *Vitellariopsis marginata*, *Asclepias fruticosa* and *Watsonia densiflora* showed good AChE inhibitory activity (> 80%). The observed activities of the plant extracts (especially *Gunnera perpensa* water extract) justify their inclusion in the makeup of *Imbiza ephuzwato* herbal mixture as well as their use in ATM. The high activities in some of the plant species also validates the claimed medicinal properties listed on the *Imbiza ephuzwato's* label. Several biological potent compounds have already been isolated from several of the 21 plant species used to manufacture *Imbiza ephuzwato*. Further studies aimed at investigating possible synergistic effects are much needed.

The Ames test results revealed that all the water extracts of the 21 plant species used to make *Imbiza ephuzwato* were non-mutagenic towards the *Salmonella*

typhimurium strains TA98 for the assay with and without S9 metabolic activation. To the contrary, *Imbiza ephuzwato* showed mutagenic effects after exposure to the S9 enzyme mix. The observed mutagenicity in *Imbiza ephuzwato* could be as a result of interaction of biomolecules in the heterogeneous mixture, yielding compounds that are converted to mutagenic agents by xenobiotic metabolizing enzymes. It is therefore important to carry out further studies aimed at identifying the mutagenic compounds in the heterogeneous mixture.

CHAPTER 5
General Conclusions

Humans have relied on plants for their basic needs including medicine, food, clothing and shelter for centuries. Plants are used for additional purposes such as poisons, ritualistic purposes associated with magic, religion and culture, stimulants for endurance and for appetite suppression. The plant chemicals responsible are mostly the secondary metabolites, which are classified into three major groups namely alkaloids, terpenoids and phenolics **(SALIM *et al*., 2008)**. In South Africa, recent developments, most likely due to urbanisation, have resulted in a proliferation of commercial herbal preparations, most notable are the liquid preparations of plant material from several species that are sold in informal street markets, herbal (*muthi*) shops, supermarkets as well as some pharmacies.

Fourteen herbal preparations commonly sold in Pietermaritzburg were tested for their pharmacological properties. The commercial preparations, regardless of their uses, were tested for antibacterial, antifungal and anthelmintic effects, the ability to inhibit two COX isoenzymes (COX-1 and -2), HIV-1 RT, AChE enzymes as well as for their antioxidant potentials. Apart from pharmacological tests, the fourteen herbal preparations were also subjected to phytochemical analysis to detect phenolic compounds including total phenolics, hydrolysable tannin, condensed tannin and flavonoids. TLC was used to profile the chemical composition of the fourteen herbal mixtures. Lastly, the fourteen herbal preparations were tested for their cytotoxic and mutagenic effects.

Three of the fourteen herbal preparations (*Imbiza ephuzwato, Ibhubezi*^{TM} *and Sejeso herbal mixture Ingwe*®) showed good activity against the *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* strains tested. Hence these products can be successfully used to treat these bacterial infections. The rest of the preparations had moderate to low antibacterial activity. Seven products (*Lion izifozonke*, *Imbiza ephuzwato*, *Ingwe® special muti*, *Sejeso herbal mixture*, *Vusa umzimba*, *Ibhubezi*^{TM} and *Supreme one hundred*) of all those indicated for treating fungal infections and skin conditions showed good activity

against *Candida albicans*. These seven products also exhibited a fungistatic characteristic which is undesirable for good antifungal drugs. *Sejeso herbal mixture Ingwe*®, *Imbiza ephuzwato*, *Ingwe*® *muthi mixture*, *Lion izifozonke Ingwe*® and *African potato extract*™ exhibited good anthelmintic activity. The rest of the herbal preparations showed low activity against *Candida albicans* with *Stameta*™ *BODicare*® and *Vusa umzimba* showing poor activity.

Based on IC_{50} values, *Imbiza ephuzwato*, *Sejeso herbal mixture Ingwe*®, *Lion izifozonke Ingwe*®, *Ingwe*® *muthi mixture* and *African potato extract*™ showed consistent potent activities against the four enzymes (COX-1, COX-2, RT and AChE) used in this study. The observed activities, though they were mainly moderate especially for *Lion izifozonke Ingwe*® and *Ingwe*® *muthi mixture*, validates the use of these herbal preparations in treating pain related conditions, eradicating HIV/AIDS symptoms and easing mental conditions.

Umzimba omubi, *Umuthi wekukhwehlela ne zilonda*, *Mvusa ukunzi*, *Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Supreme one hundred*, *Sejeso herbal mixture Ingwe*® and *Ingwe*® *special muti* exhibited high antioxidant properties. Phytochemical analysis revealed high total phenolic compounds, gallotannins and condensed tannins and lower levels of flavonoids in *Ingwe*® *special muti*, *Ibhubezi*™ and *Stameta*™ BODicare®. Surprisingly, these three herbal preparations were not active in most bioassays except for *Ingwe*® *special muti* which showed moderate activity against COX-1 and AChE. On the other hand, *Imbiza ephuzwato* exhibited high flavonoid concentrations and less total phenolic compounds, condensed tannin and gallotannin. This suggests that it is the quality rather than quantity of phenolic compounds that matters in terms of pharmacological properties. Thin layer chromatography (TLC) was used to investigate the chemical profiles of each of the fourteen herbal preparations. It was not surprising to note that *Ingwe*® *muthi mixture*, *Sejeso herbal mixture Ingwe*®, *Lion izifozonke* and *Ingwe*® *special muti* showed similar chemical profiles as shown by their TLC profiles. This implies that this particular manufacture is most likely using the same or similar combinations of plants for all its products and that the differences in the presentation and name are marketing ploys.

The neutral red uptake inhibition bioassay in human liver (HepG2) cells was used to assess potential toxicity of the commercial herbal preparations. The results revealed that the most toxic herbal mixture was *Umpatisa inkosi*, with an NI_{50} value of 0.016 mg/ml and a yield of 2.2 X 10^{-4} mg/ml residues which implies that 72.72 ml of the herbal mixture will result in the stated NI_{50} value. The least toxic herbal preparation was *Stameta*™ *BODicare*® with an NI_{50} value of 28.00 mg/ml and a yield of 1.8 x 10^{-4} mg/ml which implies that 115.56 litres of the mixture is required to reach the NI_{50} value of the mixture.

The standard plate incorporation test methods for the Ames test using *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535 and TA1537 exposed to three dilutions with and without S9 metabolic activation of the herbal mixtures was performed to test for mutagenic effects of the fourteen herbal mixtures. The results revealed that all fourteen herbal preparations were non-mutagenic towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 without metabolic activation. However, five of the fourteen herbal mixtures (*Umpatisa inkosi*, *Imbiza ephuzwato*, *Vusa umzimba*, *Stameta*™ *BODicare*® and *African potato extract*™) showed indirect mutagenic effect toward the tester strain TA98 after metabolic activation but not in the other tester strains (TA100, TA102, TA1535 and TA1537). The herbal mixtures could contain various aromatic nitroso-derivatives of amine carcinogens that have been shown to cause such a reversion of *hisD3052* mutation back to the wild-type state. Some carcinogenic compounds such as aromatic amines or polycyclic aromatic hydrocarbons like benzo-[a]-pyrene are biologically inactive unless they are metabolized to active forms.

The observed activity exhibited by some of the herbal mixtures gives some credence to the manufacturers' claims and goes part of the way to validating their use against certain conditions such as microbial infections, pain and some mental conditions. Activity cannot be ruled out in the herbal preparations that showed poor/low activity as they could have different mechanisms of action that were not tested in this study. Furthermore, in traditional medicine, people seldom weigh the plant material when making decoctions or infusions and they may use more of the plant material which could result in consumption of higher doses of active compounds. Another point

which is often overlooked is that traditionally prepared decoctions are only crudely filtered, resulting in a considerable amount of particulate matter. This is often consumed by the user. However, in most laboratory tests extracts are general filtered to remove all solid materials. Little is know about the physiological contribution of ingested solids inconjuction with traditional herbal preparations. It can be assumed that these are partially or totally digested and absorbed and thus may elicit physiological responses.

It is desirable to carry out further studies to determine the effects of mixing plant species/parts in one mixture as well as to try to isolate active constituents. The observed cytotoxic and mutagenic effects against TA98 after S9 metabolic activation of *Umpatisa inkosi*, *Imbiza ephuzwato*, *African potato extract*™, *Vusa umzimba* and *Stameta*™ *BODicare*® raises concerns as to the safety of these herbal products. At present the contents of most of the mixture are not known. Further investigation and confirmation needs to be done with the objective of determining and hopefully eliminate the source of the observed cytotoxic and mutagenic effects. This will remain a challenge as it is difficult to convince the herbalist to reveal their ingredients as well as the recipes of their products. These herbal preparations are produced commercially by private entrepreneurs who guard their recipes in fear of competition and they consider themselves holding unofficial patents.

The 21 plant species used to make *Imbiza ephuzwato* herbal mixture were each investigated for their pharmacological properties. PE, DCM, EtOH and water extracts of the 21 plants were evaluated against two Gram-positive and two Gram-negative bacteria, *Candida albicans*, inhibitory effects against COX-1 and -2 as well as AChE enzymes. *Gunnera perpensa* and *Rubia cordifolia* were the only plant species used to manufacture *Imbiza ephuzwato* that had water extracts which showed good antibacterial activity. The extracts of *Gunnera perpensa* (EtOH), *Hypericum aethiopicum* (DCM) and *Urginea physodes* (EtOH) showed the best antifungal activity. The water extracts of *Hypericum aethiopicum*, *Gunnera perpensa*, *Drimia robusta*, *Vitellariopsis marginata*, *Scadoxus puniceus* and *Momordica balsamina* showed percentage inhibition of COX-1 that was over 70%. For COX-2 enzyme, the water extracts of *Gunnera perpensa*, *Cyrtanthus obliquus*, *Momordica balsamina* and *Tetradenia riparia* exhibited inhibitory activity above 70%. Water extracts of

Gunnera perpensa, *Cyrtanthus obliquus*, *Vitellariopsis marginata*, *Asclepias fruticosa* and *Watsonia densiflora* showed good AChE inhibitory activity (> 80%). The observed activities of the plant extracts (especially *Gunnera perpensa* water extract) justify their inclusion in the makeup of *Imbiza ephuzwato* herbal mixture as well as their use in ATM. The high activities in some of the plant species also validates the claimed medicinal properties listed on *Imbiza ephuzwato's* label. Several biologically potent compounds have already been isolated from several of the 21 plant species used to manufacture *Imbiza ephuzwato*. Further studies aimed at investigating possible synergistic effects as a result of mixing plant extracts are much needed.

The Ames test results revealed that all the water extracts of the 21 plant species used to make *Imbiza ephuzwato* were non-mutagenic towards the *Salmonella typhimurium* strains TA98 for the assay with and without S9 metabolic activation. To the contrary, *Imbiza ephuzwato* showed mutagenic effects after exposure to S9 enzyme mix. The observed mutagenicity in *Imbiza ephuzwato* could be as a result of interaction of biomolecules in the heterogeneous mixture, yielding compounds that are converted to mutagenic agents by xenobiotic metabolizing enzymes. It is therefore important to carry out further studies aimed at identifying and eliminating the sources of the mutagenic compounds in the heterogeneous mixture.

The results obtained in this study reveal high levels of pharmacological activities by most of the herbal mixtures, in some cases providing scientific validation for their use. It can therefore be concluded that these herbal mixtures represents an important component of traditional medicine, not just a money making business. The results also offer a stepping stone towards the documentation of commercial herbal mixtures and information to policy makers on the way towards regulating the manufacturing and validation of herbal/natural products. The modernization and acceptance of herbal mixtures into mainstream medical practice will depend on the outputs of scientific research aimed at providing the rational for their usage. In this way, with time, herbal mixtures will achieve the same recognition as western pharmaceutical drugs.

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