

**IN VITRO PHARMACOLOGICAL AND SYNERGISTIC EFFECTS OF HERBAL
CONCOCTIONS SOLD IN GA MAJA, LIMPOPO PROVINCE**

by

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DECLARATION

I **Mashilo Mash Matotoka** declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not been previously submitted by me for the degree at this or any other University, and that this is my own work in design and execution. All the materials contained therein have been duly acknowledged.

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Date

DEDICATION

To my parents Mahlodi Nelson and Ramadimetja Josephine Matotoka

“If I have seen further than others, it is by standing upon the shoulders of giants” - Isaac Newton

To my brother Motlhatlego Dennis and sister Thobile Nsimbini

“Float like a butterfly, sting like a bee” - Muhammad Ali

To my nephew Mahlodi Ayabonga Matotoka

“Education is the most powerful weapon which you can use to change the world” - Nelson Mandela

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ABTS	2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt
AlCl ₃	Aluminium chloride
ATCC	American Type Culture Collection
ATM	African traditional medicine
BAW	Butanol/ acetic acid/ water
BEA	Benzene/ethanol/ammonium hydroxide
BHA	Hydroxyanisole
BHT	Butylated hydroxytoluene
CAM	Complementary or alternative medicine
CEF	Chloroform/ethyl acetate/formic acid
CFU	Colony forming units
CGs	Cardiac glycosides
COX	Cyclooxygenase
Cu	Copper
DE	<i>Drimia elata</i>
DIG-POD	Digoxigenin-peroxidase
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2,2, diphenyl-1-picrylhydrazyl
EC ₅₀	Half maximal effective concentrations
EMW	Ethyl acetate/methanol/ water
FRAP	Ferric reducing antioxidant power
GC	Gas chromatography
GIT	Gastrointestinal tract
HAART	Highly active antiretroviral therapy
HC1	Herbal concoction 1
HC2	Herbal concoction 2
HC3	Herbal concoction 3
HC4	Herbal concoction 4
HC5	Herbal concoction 5

HCCA	α -Cyano-4-hydroxycinnamic acid
HCl	Hydrochloric acid
HH	<i>Hypoxis hemerocallidea</i>
HIV	Human Immuno-deficiency Virus
HIV-1 RT	Human immunodeficiency virus type 1 reverse transcriptase
ICP-AES	inductively coupled plasma atomic emission spectrometry
INT	ρ -Iodinitrotetrazolium salts
KwT	<i>Kirkia wilmsii</i> twigs
KwL,	<i>Kirkia wilmsii</i> leaves
KwC	<i>Kirkia wilmsii</i> corm
LC	Lab standard
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MA	<i>Monsonia angustifolia</i>
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mg QE/g	Milligram of quercetin equivalence per gram of extract
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NA	Nutrient agar
NaNO ₂	Sodium nitrite
NCCLS	National Committee for Clinical Laboratory Standards
Ni	Nickel
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
PBS	Phosphate Bovine Saline
PDA	Potato dextrose agar
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SIM	Sulfide, indole and motility
SV	<i>Sarcostemma viminale</i>
TBHQ	Tertbutylhydroquinone

THP	Traditional health practitioner
THP	Traditional health practitioners
TLC	Thin layer chromatography
TLR	Toll-like receptor
USD	United States Dollar
UV/ VIS	Ultraviolet/Visible
v/v	Volume per volume
VC	<i>Vahlia capensis</i>
WHO	World health organisation

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CONFERENCE PRESENTATIONS AND PUBLICATIONS

Paper presentations

Matotoka, M.M. and Masoko, P. 2017. Evaluation of herbal concoctions sold at Ga Maja (Limpopo Province) in South Africa and *in vitro* pharmacological evaluation of plants used to manufacture the concoctions. Indigenous Plant Use Forum (IPUF) (Pretoria).

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ABSTRACT

Informal street traders and traditional health practitioners at Ga Maja (Limpopo Province) primarily offer consumers semi-processed herbal concoctions that are indicated to have blood-cleansing, immune-boosting, detoxifying, antidiarrheal, anti-HIV/AIDS and pain-relieving therapeutic properties. The focus of this study was to validate the pharmacological effects and safety of these herbal concoctions as indicated by the traders. Five herbal concoctions and their medicinal plant recipes used for their preparation were purchased from five independent traders. A laboratory concoction was prepared according to the traders' instructions. Possible microbial contaminants were identified using Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and VITEK 2. Nutritional content of the concoctions was determined by a plasma atomic emission spectrophotometer. Qualitative phytochemical analysis was determined using standard chemical tests and thin layer chromatography. Total polyphenol content was quantified calorimetrically. Antioxidant activity was quantified using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing power assays. Antimicrobial activities were determined using a broth micro-dilution assay and bioautography. Anti-HIV-1 reverse transcriptase activity was used to determine the antiviral potential of the concoctions. Anti-inflammatory activity was determined using the cyclooxygenase inhibition assays and possible cytotoxic effects of the concoctions were evaluated using MTT cell viability assay. Growth on potato dextrose and nutrient agar plates indicated heavy microbial contamination. Sodium, potassium and zinc were most abundant in the concoctions. The concoctions had similar phytochemical profiles, and together with plant decoctions consisted primarily of polyphenolic constituents. Generally, some plant decoctions had higher antioxidant (EC_{50} : 15.71 – 40.87 $\mu\text{g}/\text{mL}$) and antibacterial (MIC: 0.04 – 0.63 mg/mL) activities. The antimicrobial activities of the concoctions were as a result of synergistic effects of the compounds because no single compound was observed to have antimicrobial activities on the bioautograms. Combination studies showed that the combination of crude decoctions from different plant species resulted in reduced antioxidant, antibacterial and antifungal activities. The laboratory concoction had higher anti-HIV reverse transcriptase activity (EC_{50} : 2.90 $\mu\text{g}/\text{mL}$) than the positive controls used (EC_{50} : 36.65 – 40.90 $\mu\text{g}/\text{mL}$). The concoctions also demonstrated considerable anti-inflammatory activity. The lack of standardisation of phytomedicines reduces their

pharmacological potential and consistency. However, this study validated the ethnopharmacological use of the different plant species and herbal concoctions towards the treatment of human immune-deficiency virus, pain caused by inflammation, infectious diarrhoea and for immune-boosting. This study also highlighted the complexities associated with combinations of aqueous extracts and the importance of appropriate knowledge on which plant species are to be mixed to enhance their therapeutic properties. Although the concoctions did not exhibit cytotoxic effects, toxicities may arise from endotoxins produced by the microbial contaminants. Hygienic processing and packaging are essential to ensure that consumers receive quality products that are safe to consume.

CHAPTER 1

1. Introduction

Since the dawn of time, humans have been predisposed to depend on nature for survival and early human beings recognised their reliance on nature as a source of food through plant resources. With this recognition came along a growing dependence on plant resources for other uses such as clothing, shelter and remedies to manage and/or cure ill-health (Barnes *et al.*, 2007).

Traditional medicine is defined by the World Health Organisation as “The sum of knowledge, skills, and practices based on the theories, beliefs, and experiences different cultures use to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses” (WHO, 2008). Traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed complementary or alternative medicine (CAM) (Gurib-Fakim, 2006).

In recent years, many products were prepared using indigenous knowledge from African traditional medicine (ATM). Such remedies are made of mixtures of different plant parts of various species and are designed not only to manage medical problems but also to incapacitate fears and anxieties in everyday life (Mander and Le Breton, 2006). It was estimated that 80% of people in developing countries still depend on traditional medicine (Mahomoodally, 2013). Furthermore, the Limpopo Province of South Africa is dominated by rural settlements that are associated with substantial rates of unemployment among the locals. The latter socio-economic condition results in persistent and prevalent poverty. It is thus, the locals are heavily reliant on ATMs and use medicinal plants and other processed herbal products to manage health conditions (Semenya *et al.*, 2012).

Traditional medicine reaches a considerable number of the South African population and it is in that regard that quality control for the efficacy and safety of such medicines becomes paramount. This is because without effective regulation, any unsubstantiated claims of efficacy, possible toxicity and adulteration can simply go unchecked. The result of this, is an adverse effect on the health of the patients who consume such products to alleviate their ill health.

Africa is a biogeographic region with an enormous floral biodiversity that for centuries has provided its natives and more profoundly, traditional healers with a myriad of medicinal plants. Numerous species of medicinal plants have been in use as therapeutics and as ingredients for the preparation of various herbal remedies. Medicinal plants therefore serve as the corner stone for the development of African traditional medicine and the indigenous methods of curing diseases (Ahmed *et al.*, 2012). It is common-practice across Africa that medicinal plants are used in traditional medicine because they are widely abundant and are easily accessible (Gurib-Fakim and Mahomoodally, 2013).

Medicinal plants have been hailed for their biological activities such as antioxidant, anti-inflammatory, antidiabetic, antibacterial and antifungal activities to mention but a few (Ahmed *et al.*, 2012; Masoko and Makgapeetja, 2015). The pharmacological properties of plants are attributed to the presence of a myriad of secondary metabolites that are synthesized by the plant. Secondary metabolites in plants are also known as phytochemicals and they can either act individually, additively or synergistically to exert a therapeutic effect. Unlike pharmaceutical drugs which mainly consist of only one active compound, the mixture of the phytochemicals brings about a combined effect that results in a greater biological activity than the individual compounds (Gurib-Fakim, 2006; Chintamunnee and Mahomoodally, 2012).

Phytochemicals have been suggested to participate in the relationship between the plant and the environment in which it grows. That is, these compounds can be used to shield the plant from predators, parasites and/or offer resistance towards pathogenic attacks (Nunkoo and Mahomoodally, 2012). It is therefore possible that a plant extract can possess multiple biological activities. As such the crude extracts of plants have commonly been used by traditional healers (Shohawon and Mahomoodally, 2013).

In recent years, there has been a growing demand for plant-derived remedies and therapeutics due to easy accessibility and cheap prices. This has led to the manufacture of complex plant concoctions in an effort to increase the level of efficacy and therapeutic effect of a particular medicine. Herbal concoctions are commonly prepared as decoctions of extracts from different plant species. The formulas to these concoctions range from simple home remedies that are prescribed for minor ailments

such as insomnia, diarrhoea and fever to more complex medicines used to treat life-threatening diseases (Cano and Volpato, 2004). The trade of herbal medicines is a rapidly expanding multi-million Rand industry in South Africa, however, it is still a gruelling task to regulate the manufacture, formulation and proper labelling of these commercial herbal products (Ndhlala *et al.*, 2010).

Integrity, quality, dosage regulations and standardization of herbal products need to be enforced to assure that the herbal remedies meet efficacy, safety and health standards. It is an archaic mentality to simply view herbal remedies as safe, the truth is that some of these herbal preparations can be extremely toxic. It is important to remember that most of the phytochemicals are noxious and are produced to confer the plant with protection against pathogenic, viral or predatory attacks (Ndhlala and van Standen, 2012). There is inadequate documentation regarding poisonings due to the consumption of herbal products. This is because the use of herbs is deeply rooted in indigenous cultures that the people are reluctant to divulge such information with a fear that such communication would incite strong laws and regulations towards their herbal products (Steenkamp *et al.*, 2006).

However, without scientific credence or appropriate quality control measures, the use of herbal remedies poses a potential health hazard towards consumers. Moreover, the question to whether the preparations are effective against a particular ailment will remain unsubstantiated, controversial and lacking in proper dosage prescriptions.

It is therefore the purpose of this study to evaluate the pharmacological properties and combinational effects of the phyto-constituents present in the herbal concoctions sold at Ga Maja, Limpopo Province in order to provide scientific credence regarding their use. The medicinal plants that are used to prepare these concoctions are also evaluated for their biological activities. These concoctions are sold by traders stationed on the side of the road intersecting R37 (24.1631° S, 29.5543° E). The plant species used to prepare the concoctions include; *Monsonia angustifolia*, *Drimia elata*, *Hypoxis hemerocallidea*, *Kirkia wilmsii*, *Vahlia capensis*, *Sarcostemma viminale* and two kinds of powders locally known as “*Tšhikwana*” (White powder), “*Tšhikwana/moroto wa tšhwene*” (black powder).

Due to a lack of weighing equipment, with every batch of the brew, the traders approximate the proportions of each plant to be added to the concoctions. Therefore,

the concentration of chemicals in different batches vary. The cooled preparations are packaged in recycled 2 L and 500 mL plastic bottles from soda beverages and energy drinks. These concoctions are prescribed for the relief of pain, diarrhoea, human immune-deficiency virus and were claimed to have blood purifying and immune boosting properties. They are also prescribed for libido enhancement in men, the treatment of erectile dysfunction, coughs and stomach aches. The appropriate dosages of these concoctions are not precise because the traders loosely prescribe the 2 L bottle of herbal concoction to be consumed within 2 days with morning and night servings. The 500 mL bottle is prescribed to be consumed with a single serving. It was however also noted that although they do give dosages, they claimed that there cannot be an over-dose from drinking the concoctions and that frequent urination is an indication that the medicine is working. The lack of use of standard/measured doses, and the large volumes of the doses used are difficult to manage. This may be dangerous as some of the species could have a high degree of toxicity and cause serious health problems.

There are several approaches that can be taken in order to determine pharmacological properties such as efficacy and toxicity of drugs. Exploring epidemiology, engaging in human clinical studies, animal studies, and *in vitro* studies all contribute to the number of different ways the efficacy and toxicity of drugs can be evaluated. Each of these methods have their strengths and limitations, hence for a safety and efficacy assessment to be reliable and accurate, a combination of the approaches to solve a problem would be advantageous (Devlin *et al.*, 2005).

In this study, *in vitro* assessment was chosen to investigate the pharmacodynamics of the herbal concoctions and the plants used in their preparation. This was because *in vitro* studies minimize costs and enable a direct access and observation of the drug performance. Furthermore, *in vitro* studies are less likely to conflict with ethical considerations (Polli, 2008).

In vitro studies are functional, convenient and helpful in assessing the underlying mechanisms of a biological activity. In addition, *in vitro* studies can help evaluate the cellular pathways and biochemical processes that are initiated by the cells as a response to a toxicant. Another benefit of such studies is that many samples can be screened rapidly, thus this approach is also time-efficient. The prime limitation of *in*

vitro studies is that the cells that are used are removed from their natural environment (inside a living body) where they were surrounded by and interact with neighbouring cells and have blood transporting potentially essential factors and nutrients (Devlin *et al.*, 2005).

The increasing costs of medicine, the low number and unavailability of professional health practitioners and public primary healthcare establishments contribute to the disadvantages of the sole reliance on modern medicine. Consequently, this creates a huge gap of inaccessibility of pharmaceutical drugs to impoverished communities. This serves as motivation to investigate biological activities of plant-based products and validate their claimed pharmacological effects as well as safety. The benefit of such research is the distribution of evidence-based herbal medicines that are not toxic to consumers. The validation of the pharmacological activities of both the concoctions and the plant species in this study would also add value to the Bapedi pharmacopeia in the Limpopo Province.

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CHAPTER 2

2. Literature review

2.1. Traditional medicine

Since the dawn of time, humans have been predisposed to depend on nature for survival and early human beings recognised their reliance of nature as a source of food through plant resources. With this recognition came along a growing dependence of plant resources for other uses such as for clothing, shelter and remedies to manage and/or cure ill-health (Barnes *et al.*, 2007).

Trial and error have been the approach used by the prehistoric men and women to distinguish between plants with medicinal value from those that were poisonous or which in one way or another would adversely affect health. Therefore, the proper diagnosis and treatment of ill-health was conducted based on information gathered through experience (Kunle *et al.*, 2012).

Herbal remedies are usually prepared by mixing different plant portions or extracts with the intention to increase the medicinal value of the remedy as opposed to using one plant species (Cano and Volpato, 2004). This expected increase in therapeutic efficacy of the remedies is attributed to occurring synergistic interactions between the diverse phytochemicals. These synergistic interactions may either increase the effectiveness towards a single or a combination of ailments (Cano and Volpato, 2004).

The use of traditional medicine and the utilisation of traditional health practitioners (THP) is not only limited to rural settlements, on the contrary, people who reside in urban locations are also accustomed to use traditional medicine over western medicine (Marsland, 2007). This kind of paradigm could be attributed to distrust in the ability of western medicine to be efficacious in treating illnesses that are associated with psycho-social factors (Mulaudzi *et al.*, 2015). There is further scepticism towards the use of western medicine due to existing beliefs that the manifestation of an illness is due to supernatural influences and/or the uneasiness of ancestral gods or evil spirits (Kgoatia, 1997). In addition, herbal products can easily be accessed from THP or be bought over the counter from herbal stores without the requirement of a prescription. This makes the use of herbal products more convenient for most consumers.

The use of herbal remedies is common in the Limpopo Province (South Africa) due to the increase in the cost of conventional modern medicine and the lack of accessibility of healthcare providers (Semenya *et al.*, 2012). Subsequently, the role of traditional health practitioners (THP) becomes vital towards the people who desire to utilise herbal remedies rather than modern pharmaceuticals.

2.2. Traditional health practitioners

The World Health Organisation (WHO) has interpreted a THP as “a person who is recognised by the community where he or she lives as someone competent to provide health care by using plant, animal and mineral substances and other methods based on social, cultural and religious practices” (WHO, 1978). In South Africa, there is a high utilisation of THP, whereby it was suggested that about eight in ten black South Africans at some point have used herbal remedies either alone or coupled with western medicine (Ross, 2010). The Limpopo Province has a high distribution of Bapedi THPs and they are distributed mainly in the Capricorn, Sekhukhune and Waterberg districts, which collectively include more than 17 municipalities in which they are operational (Semenya and Potgieter, 2015).

The approach undertaken by THPs to treat ill-health is holistic and relies on addressing the psychosocial aspects of an illness which include the emotional, physical, mental and spiritual attributes. The outcome of this approach of treatment involves the use of herbal remedies accompanied by the performance of peculiar traditional customs which are believed to enhance the pharmacological effect of the therapy (Zuma *et al.*, 2016).

The principal source of medicine for THP is medicinal plants, and this includes the use of different plant parts: flowers, fruits, leaves, twigs, stems, barks, whole plants, rhizomes, resins, seeds and bulbs (Cano and Volpato, 2004). The Bapedi THPs are primarily reliant on medicinal plants as a source of treatment towards ailments. Their predominant mode of preparation of herbal remedies is a decoction and maceration of a single or combination of different plant parts (Semenya *et al.*, 2013; Semanya *et al.*, 2012).

Medicinal plants used in African traditional medicine (ATM) make up an integral source of new potent bioactive compounds (Hutchings *et al.*, 1996). In turn, these bioactive

compounds are integrated into traditional and folk medicine as food supplements and therapeutic agents and further in modern medicine as pharmaceutical intermediates of prospective drugs (Hammer *et al.*, 1999).

2.3. Medicinal plants

The aggregate of all the different phytochemicals provide the potential of a single plant to possess multiple pharmacological properties. A plant may produce bitter chemicals that encourage digestion, and compounds that are able to reduce oxidative stress and the ability to reduce pain and swelling (Nunkoo and Mahomoodally, 2012).

Plants produce a myriad of assorted compounds which vary not only in their function but further differ with their chemical structures and the quantity produced. They are produced by the plant to support its existence and endurance in a particular surrounding (Bernhoft, 2010).

The compounds produced by the plant are classified into two groups based on function and the particular interval in which they are produced. The primary metabolites are vital towards the growth, development and procreation of the plant. These group of chemical substances include carbohydrates, amino acids, proteins, nucleic acids and lipids (Harborne, 1993).

Plants produce another group of compounds, the secondary metabolites. These are enormously diverse in their chemical structures and are not strictly indispensable towards growth. Although these compounds have no obligatory function towards growth, they are understood to be involved in maintaining the good health of the plant. Moreover, they increase the plants all-round capability to survive and triumph against the challenges imparted by its local surrounding. Some of the secondary metabolites are used by the plant to defend itself against bacteria, fungi, viruses and herbivores (Bourgaud *et al.*, 2001). Plants also use these compounds to inhibit and overpower the growth of neighbouring plant species (Ismail *et al.*, 2016).

Medicinal plants that produce phytochemicals with pharmacological properties have the potential of enabling the population that is economically and geographically disadvantaged to access quality healthcare. This is because these secondary metabolites have biological activities that range from antibacterial, antifungal,

antioxidant, anti-inflammatory, anti-cancer, anti-aging and anti-atherosclerotic (Mulaudzi *et al.*, 2012).

The biological activities mentioned here have enabled plants to be used in wound healing, clearing off wound infection, diarrhoea, cholera, decreasing irritable bowel syndrome and ulcers to mention but a few (Begum *et al.*, 2002).

2.4. Phytochemicals

The chemical nature of phytochemicals is diverse; however, a great number of these compounds have similar characteristics and are grouped based on their structural properties owing to the manner and pathway in which they were biosynthesised (Azmir *et al.*, 2013).

The synthesis of the secondary metabolites follows four key biosynthetic pathways, mainly the shikimic acid pathway, malonic pathway, mevalonic pathway and the non-mevalonate pathway (Tiaz and Zeiger, 2006). The phenolic compounds are synthesised with derivatives produced in the shikimic acid pathway and the malonic acid pathway. Alkaloids are produced from derivative aromatic amino acids that are synthesised from the shikimic pathway coupled with aliphatic amino acids that are derived from the tricarboxylic acid cycle (Azmir *et al.*, 2013).

Within this group of secondary metabolites, some of the compounds consist of functional groups which allow them to exert pharmacological or toxicological effects on higher-order organisms such as human and animals (Bernhoft, 2010).

Alkaloids that occur naturally are nitrogenous compounds. The native alkaloids which occur in nature are derived from amino acids and consist of a heterocyclic ring into which a nitrogen moiety is incorporated, for example, nicotine (Ncube *et al.*, 2008). Quinine is an alkaloid known for anti-amoebal activity against the parasitic protozoans (Iwu *et al.*, 1999).

Terpenoids or terpenes as they are alternatively called, are important compounds in plants which range from simple hydrocarbon chains to more complex cyclic structures. They mediate pathogen attacks on plants. These compounds including their oxygenated isoprenoid derivatives and various analogues form a group of

phytochemicals of medicinal and aromatic plants which make up a large proportion of essential oils (Back and Chappell, 1996).

The pentose phosphate, shikimate and phenylpropanoid pathways in plants are responsible for the production of phenolics (Randhir *et al.*, 2004). The latter play vital roles in the growth and reproduction and render protection against pathogens and predators in plants. Phenolics can exist as simple phenolic compounds or as highly polymerised compounds and consist of an aromatic ring with one or more incorporated hydroxyl groups. Although there are significant structural differences between these groups, these compounds are generally referred to as polyphenols (Bravo, 1998).

The study of phenolics has led to the discovery of the biological and physiological properties, which include anti-microbial, antioxidant, anti-inflammatory, anti-allergic, anti-atherogenic, anti-thrombotic, cardio-protective and vasodilatory activities (Benavente-Garcia *et al.*, 1997; Middleton *et al.*, 2000; Puupponen-Pimia *et al.*, 2001; Manach *et al.*, 2005).

The flavonoids have subclasses consisting of flavonols, flavones, flavanones, catechins, isoflavanols and anthocyanidins which all result from variations in the substitution patterns to the carbon ring of the flavonoid structure (Hollman and Katan, 1999). Flavonoids have been shown to have anti-microbial activities with greater potency against Gram-negative bacteria (Martini *et al.*, 2004).

Tannins are soluble in a range of polar solvents such as water, alcohol and acetone. These compounds have been designated to have a broad anti-microbial effect, host-mediated tumour activity and play a role in the functioning of the immune system by having the ability to stimulate scavenger cells (phagocytic cells) (Basri and Fan, 2005). Conventionally as therapeutic agents, the tannins have been used in the intervention of diarrhoea, haemorrhoids, wounds and the inflammation of the surface of the mouth (Ogunleye and Ibitoye, 2003).

Saponins have therapeutic validation on the basis that anti-microbial, anti-inflammatory and cytotoxic activities have been attributed to their presence in plant extracts (Abbas *et al.*, 2015). When dissolved in water to form aqueous solutions,

these compounds have the characteristic of forming a persistent mass of bubbles, commonly referred to as a froth upon vigorous shaking (Sparg *et al.*, 2004).

Cardiac glycosides (CGs) are distributed naturally in various animals and plants (Prassas and Diamandis, 2008). Their medicinal use can be dated back 1500 years ago whereby these compounds were used as arrow toxicants, agents to induce nausea or vomiting and as heart restoratives (Newman *et al.*, 2008).

2.5. Herbal concoctions

A herbal concoction is a mixture of different plant species or plant parts to treat various health ailments. Informal street merchants and traditional health practitioners primarily offer consumers semi-processed herbal preparations that are commonly prepared in small batches. In preparing the herbal concoctions, fresh or dry plant material can be used. The plant material can either be macerated in water for several days or generally boiled in hot water (Ndhlala and van Staden, 2012). In South Africa, herbal products that are sold by informal traders are usually claimed to be immune and energy boosters, blood cleansers, detoxifiers and aphrodisiacs (Ndhlala *et al.*, 2009).

Some of these formulations are unstable, vary in strength and generally have short shelf lives. The poor physical conditions employed in preparation, such as unsterile working environment, contribute to the short expiry period. Unfortunately, the production of such remedies leads to the exhaustion and over exploitation of some plant species as a result of excessive harvesting (Nwankwo *et al.*, 2012). An estimated 20 000 tons of plant material has been suggested to be harvested and used in South Africa to produce herbal medicines (Mander *et al.*, 2007). There also seemed to be a proportional high demand for plant-based medicines, in that the estimated annual market value of phyto-medicines stood at 75 to 150 million USD (Mander and Breton, 2006; Mander and McKenzie, 2005).

The complexity of the formulations is dependent on the severity of the ailment. Simple home remedies can be prepared for common ailments such as diarrhoea, coughs, pains and gastrointestinal disorder. However, more elaborate procedures of preparations are required for life-threatening conditions (Cano and Volpato, 2004).

In preparing herbal remedies by using various plant parts from different sources, one can realise the extent of the number of compounds which are present in these remedies and the possibility for the constituents to chemically interact (van Vuuren and Viljoen, 2011). This presents a challenge that is not commonly encountered in western medicine. The mechanisms and modes of action of single-compound-orientated modern medicine can easily be elucidated. But due to the numerous compounds and their less understood initial interactions in most traditional herbal mixtures, there lies a large possibility of a phyto-medicine to exert more than one mode of action. These modes of actions can be a result of either additive, antagonistic and/or synergistic interactions of the compounds (van Vuuren and Viljoen, 2011).

Furthermore, because these compounds were synthesised for plant protection against various pathogenic and predatory attacks as indicated in Section 2.3, the compounds can also be highly toxic to humans and their consumption is cautioned. This demonstrates the importance of the labelling of herbal products.

Labels on herbal products provide the consumer with information about its contents, that is; the list and quantity of the active ingredients (inclusive of plant species used), the mode and frequency of administration and potential side effects. Moreover, the labels should include details about the expiry date, any additives such as preservatives and appropriate methods for storing and maintaining the product (Ndhlala and van Staden, 2012).

The design and presentation of herbal preparations sold by informal street traders and THP have become modernised in that the overall end-product encompasses the combination of ATM and western medicine features. The formulae (recipes and administration) of the preparations are of ATM origin and are based on concepts of traditional philosophies and spiritual aspects. Nevertheless, the packaging and presentation of these preparations are seemingly adopting modern characteristics. These informal traders usually use recycled bottles and attach hand-written labels that are mainly written in South African languages (Ndhlala *et al.*, 2009).

It is noteworthy to mention that through observation, in rural locations labels are generally not attached to the bottled products and information regarding the ingredients and administration are based on verbal instruction by the traders (Figure

2.1A). On the other hand, herbal concoctions that are commercially available in shops are proficiently produced, packaged and labelled by professional manufacturers (Figure 2.1B) (Ndhlala *et al.*, 2009). Although there are obvious differences in the production of herbal remedies, the common demerit the products suffer from, is that there lies an absence of scientific credence, that is, majority of herbal mixtures lack safety and quality controls to ensure purity, standardisation and pharmacological efficacy.



Figure 2.1. Herbal concoctions prescribed for various health ailments. A: herbal concoctions without labels sold at Ga Maja by informal traders and traditional healers. B: labeled concoctions manufactured by various large-scale companies (Ndhlala and van Staden, 2012).

The broad use of traditional herbal remedies has encouraged manufacturers, private traders and street merchants to capitalise on this upsurge by increasing the availability of herbal remedies to those who desire them (Ndhlala and van Staden, 2012). The signs of urbanisation are witnessed by the increase in herbal shops, informal street traders and the wide distribution of herbal remedies in pharmacies and supermarkets (Ndhlala *et al.*, 2011). Furthermore, the use of media outlets such as internet, television, radio, newspapers, pamphlets and the formation of social networks has provided entrepreneurs with a broad spectrum of a marketing platform to adapt to modernisation and globalisation (Bonora, 2001).

For the different parties or entrepreneurs (industrial manufacturers, plant gatherers, street traders and local THP) involved in the supply of plant-based products or related services, this industry provides job opportunities and thus a much-needed source of income. This benefit brings about an intense competition that may motivate

adulteration. In addition, opportunists take advantage of the ignorance of unsuspecting and desperate patients and offer them products with unsubstantiated claims them (Ndhlala and van Staden, 2012). In attempts to convince the general public concerning the integrity of these bogus products, some herbalists distribute pamphlets in which they attach fake titles such as “Dr” or “Prof” (Ndhlala *et al.*, 2011). The circumstances described above attach a negative stigma around the use of herbal remedies. Therefore, to secure the integrity of ATM from those con-artists or those without knowledge about the principles of herbalism, scientific evaluation of herbal mixtures is vital. Pharmacological evaluation of herbal mixtures also serves to elucidate their biological activities and confirm their claimed efficacies towards ailments indicated. Furthermore, the safety regarding their use would serve to protect the public consumers from potentially toxic and life-threatening concoctions.

2.6. Toxicity of herbal medicines

The toxicity of plant species is not new to primitive African natives. These early human beings honed their knowledge and skill towards the usage of such plants. This is evident because some poisonous plant species were used for euthanasia, abortions and assassinations. Some were applied onto arrow heads to be used in hunting and in combat (Wink and van Wyk, 2008). The normal functioning of the central nervous system and some critical organs such as kidneys, liver, heart and lungs can be affected. The extent to which the phyto-toxins can affect an individual is largely dependent on the amount of the toxins exposed to, the way they were administered, the portion of the plant that was used and the susceptibility of the individual towards the toxin (Botha and Penrith, 2008).

Although it is true that some plants are toxic and have an extreme potential to adversely affect the normal biochemistry and lead to life threatening conditions, a vast variety of toxic plants are still in use for medicinal purposes (Wink and van Wyk, 2008). Ndhlala *et al.* (2013) documented toxic medicinal plants that are used in southern Africa by natives to treat various medical conditions such as eye infections, ulcers, intestinal wounds, abdominal pain, headache, syphilis, gonorrhoea, leprosy, tuberculosis, choking, respiratory paralysis, purgation, heart failure, treatment of burns, snake bites, treatment of colds, fever, hallucinogenic, relieve problems of mental disturbance etc. On a separate study, Ndhlala *et al.* (2012) reported the toxicity

of herbal concoctions commercially available at KwaZulu Natal (South Africa) were, of the thirteen herbal remedies studied, four were found to be mutagenic and the rest were shown to have varying degrees of cytotoxicity.

The toxicity resulting from herbal remedies is linked to the misidentification of plant material and adulteration of the products with other assorted herbal materials. Unregulated prescriptions, dosages, improper methods of preparations and administration contribute to toxicity resulting from utilising herbal remedies (Ndhlala *et al.*, 2013).

2.7. Standardisation of herbal medicines

The absence of efficient health care systems, including the high cost of pharmaceutical medication and limited access to health care providers forces people undergoing such socio-economic conditions to look for alternative routes to manage ill-health. These alternative routes follow an approach to use medicinal plants or the utilisation of cheap road-side herbal remedies. The consequence of this approach is that the consumers are predisposed to health effects triggered by some of the unknown compounds present in the remedies (Ndhlala *et al.*, 2009).

Quality control of phyto-medicines is essential because without regulation, the uniformity, reliability and market value of the products would dwindle. In undertaking quality control, the product formulation, mode of preparation, method of extraction and the source of raw material should be assessed and managed in uniform procedures (Gurib-Fakim and Mahomoodally, 2013).

Gurib-Fakim and Mahomoodally, (2013) reported that the growth of contemporary African medicine is impacted negatively by the absence or limited validation of the knowledge and techniques used. Moreover, the lack of infrastructure, and consistency in standardisation methods contribute to this ordeal. The consequence of these influences is that customers (both national and international) have a challenging task to be able to identify batches that are uniform (Gurib-Fakim and Mahomoodally, 2013).

The quality of hygiene in the manufacture of phyto-medicines is of great importance because contamination of herbal products by microorganisms poses a health risk towards the consumers (Adeleye *et al.*, 2005). However, some of these products are

traded on streets and pavements where hygiene is compromised. This conflicts with parameters set for quality pharmaceutical production (Ndhlala *et al.*, 2012). The poor physical conditions and the lack of appropriate storage methods promote microbial contamination that can also lead to rapid spoilage of the products. As such the batches of the remedies will have to be prepared in short intervals (Nwankwo *et al.*, 2012).

There has been a growing demand for medicinal plants and their various derivatives, and the gap between ATM and scientific credence has been realised (Mahomoodally, 2013). Literature on the ethno-pharmacological use of African medicinal plants has experienced an increasing number of publications. These publications are aimed to assess the efficacy and safety of traditional medicines (Ndhlala *et al.*, 2013).

The quality and purity of herbal remedies can be analysed using a range of modern physical and chemical methods which include the vast techniques of chromatography and spectrometry. Chromatography techniques include the use of thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC). Spectrometry procedures include mass spectrometry and ultraviolet/visible (UV/VIS) spectrometry. In some instances, the combination of these techniques, such as mass spectrometry and nuclear magnetic resonance is used in assessing chemical constituents in herbal remedies (Razmovski-Naumovski *et al.*, 2010).

To analyse the phytochemical make-up of crude extracts and complex mixtures, thin layer chromatography (TLC) becomes a preferable technique. It is time efficient, cost effective and a quick resolution towards challenges involved with being able to discriminate and develop fingerprints for major chemical classes that are present in mixtures (Zeng *et al.*, 2008).

For the herbal concoctions to be reputable, maintain quality, reliability and be marketable, they must meet quality health standards. However, investigating herbal products is accompanied by the challenge that some herbalists, traditional healers and/or traders are reluctant to divulge the ingredients and formulae to some of their products. This underscores research efforts aimed at standardising and providing evidence-based pharmacological effects of the remedies.

2.8. Antioxidants

Essential biomolecules such as nucleic acids (deoxyribonucleic acid and ribonucleic acid), proteins (enzymes) and lipids (cell membrane) are adversely affected by the presence of free radicals (Sarikurkcu and Tepe, 2015). Free radicals are tremendously reactive oxygen species (ROS) that have either one or more unpaired electrons. They are produced by living organisms through numerous biological activities such as aerobic respiration, nutrient metabolism, the progress of aging, peroxisomes, stimulation of macrophages and inflammatory cells (Klaunig and Kamendulis, 2004). Moreover, the exposure to environmental elements such as ionizing radiation, atmospheric pollution (inclusive of tobacco smoke) and contact with organic solvents and/or pesticides contribute towards the production of these free radicals (Chanda *et al.*, 2013).

These reactive oxygen species are destructive in that they are involved in direct oxidation of biomolecules and important cellular structures. Lipid peroxidation occurs when hydrogen peroxide (H_2O_2) and hydroxyl ions (OH^\cdot) produced from superoxide (O_2^\cdot) interact with the cell membrane. The resulting chain reactions weaken and damage the cell membrane. The detection of such damage stimulates an inflammatory response that results in the production of mediators and chemotactic aspects (Govindappa *et al.*, 2011).

Living systems have natural mechanisms to defend against reactive oxygen species (ROS). The synthesis of catalytic enzymes such as superoxide dismutase, catalase and glutathione peroxidase confer protection against oxidative damage towards tissues, cells and biomolecules. However, this biological approach to eliminating ROS can become overwhelmed (Javan and Javan, 2014).

Epidemiological studies on the occurrence of ROS, their effects and how to reduce them have suggested that foods that are abundant in chemical species that can retard or remove these ROS may contribute towards the reduction of development of cancer, coronary heart diseases, cataracts, neurodegenerative disorders, atherosclerosis and inflammatory conditions (Keser, 2014; Sakat *et al.*, 2010). Inflammatory disorders are associated with the inconsistent activation of phagocytes, excessive accumulation of free radicals (O_2^\cdot and OH^\cdot) and non-free radicals (H_2O_2) (Govindappa *et al.*, 2011).

The antioxidant activity of a compound is related to its ability to either donate a hydrogen atom or an electron, to scavenge free radicals and chelate reactive metal cations (Balasundram *et al.*, 2006).

Synthetic antioxidants such as hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ) have been broadly used in the food industry as food additives (Balasundram *et al.*, 2006). However, their use has been met with discontent and criticism mainly due to safety concerns over their consumption (Balasundram *et al.*, 2006). Restrictions in the use of TBHQ have been enforced due to reports of carcinogenicity and liver toxicity associated with consumption (Grice, 1986; Wichi, 1988). These safety concerns motivate the search for more safe and powerful natural antioxidant compounds to confer therapeutic benefits.

In complex herbal mixtures, numerous phytochemicals possess diverse degrees of antioxidant potential. Therefore, the choice of an appropriate solvent for extraction is vital. This is because the polarity of each solvent enables it to discriminate between compounds it can extract from plant material or leave behind. When assessing the antioxidant potential of extracts, no one single assay can provide definite information about an extract's antioxidant activity. This is because antioxidant compounds have dissimilar chemical structures and modes of actions and as such can react differently towards various ROS (Park *et al.*, 2014).

One of the most basic ways to determine the antioxidant potential of medicinal plants is by determining their free radical scavenging activity (Huang *et al.*, 2005; Chigayo *et al.*, 2016). Other numerous assays have been frequently used to estimate antioxidant capacities of plant extracts. These include: ferric reducing antioxidant power (FRAP), chelating effect and phosphomolybdenum assays (Sarikurkcu and Tepe, 2015).

2.9. Antimicrobials

There are a variety of antibiotics that are aimed to treat microbial infection and prevent the development of septic wounds. Some antibiotics primarily function by inhibiting the synthesis of peptidoglycan, a fundamental molecule used to build a bacterial cell wall. Whilst others can target and interfere with protein synthesis, which ultimately alters the synthesis of constitutive enzymes required for essential pathways (Miller *et al.*, 2014).

Over time, the use of most antibiotics has been associated with negative connotations in that, they have been linked to side effects that make patients reluctant to complete the full course of treatment. Allergic reactions and immunosuppression are some of the side effects experienced during the use of antibiotics (Sagbo *et al.*, 2017).

Although during the antibiotic era, the use of antibiotics was an effective approach to treating infections, clinicians recognised that microbes were exhibiting reduced susceptibility towards commonly administered antimicrobial agents (Miller *et al.*, 2014). The loose prescription and extensive usage of antibiotics to treat even benign infections has resulted in the reduction of the efficacy of most antibiotics (Miller *et al.*, 2014).

Microorganisms can evolve and survive. Within this process of evolution, they develop defences against antimicrobial agents which they were previously exposed to. These defences are in the form of multidrug resistance pumps and enzymes (membrane translocases). They confer protection to the bacteria by forcing out structurally distinct and foreign chemical substances from the cell wall. This benefits the microorganisms by increasing their chances of remaining viable even after exposure from antibiotics (Ncube *et al.*, 2008).

Plants are not spared from infection by disease causing microorganisms. Key phytopathogens are oomycetes, nematodes, viruses, fungi and bacteria (Mansfield *et al.*, 2012). The latter consists of more than 200 pathogenic species from the genera of *Pseudomonas*, *Ralstonia*, *Agrobacterium*, *Xanthomonas*, *Erwinia*, *Xylella*, *Pectobacterium*, and *Dickeya* (Mansfield *et al.*, 2012).

As a defence mechanism against such infections, plants secrete phenolic compounds, alkaloids, diterpenoids, steroids, alkaloids and other complex compounds to obstruct the proliferation of the pathogens such as the abovementioned (Angeh, 2006). It is therefore not entirely startling that traditionally; many medicinal plants were and still continue to be used for the treatment of microbial and parasitic infections.

On a recent ethnobotanical study carried out in Venda (Limpopo Province, South Africa), Masevhe *et al.* (2015) reported a remarkable number of plants and herbs that were used by the traditional healers to treat infections such as diarrhoea, tuberculosis,

pneumonia, ringworm, malaria and sexually transmitted infections. In addition, 45 plant species that belong to 24 different families were recorded to be used by Venda traditional healers to treat candidiasis (*Candida* infections). On another ethnobotanical survey carried out in the Limpopo Province, Semenya *et al.* (2013) reported 47 plant species (belonging to 32 families) used by Bapedi traditional healers to treat sexually transmitted infections in the Capricorn district, Sekhukhune district and Waterberg district.

Southern Africa has a rich diversity of flora that forms part of the culture of the population for many generations. Due to the differences in geographical locations, some communities may rely on different plant species to treat similar pathogenic infections (Luseba *et al.*, 2007). Moreover, they may even rely on different combinations of plant species to treat infections (van Vuuren and Viljoen, 2011). The ethnopharmacological properties and antimicrobial screening of undocumented plants need to be pursued to broaden the knowledge on the types of medicinal plants used. This will ensure that regardless of location, there will be knowledge on an alternative species to treat an ailment (Semenya *et al.*, 2013).

The search for novel antimicrobial agents from natural sources has prompted the expansion and improvements in bioassays to equip them with the optimal sensitivity to detect even small amounts of bioactive compounds. This is due to the varied concentrations in which each plant genera can produce secondary metabolites (Lampinen, 2005).

Antimicrobial susceptibility assays can be appropriately classified in to two categories, namely diffusion and dilution methods. The former includes agar disk diffusion, agar well diffusion and bioautography whilst the latter commonly refers to broth micro/macro dilution assays (Ncube *et al.*, 2008). The application of the agar diffusion (Figure 2.2) methods has gradually lost popularity due to shortcomings of not always producing reproducible results for some plant extracts and its lack of quantitative analysis (Eloff, 1998). Moreover, the drawback of the diffusion techniques lies in the inability to discriminate between cidal or static properties of plant extracts. The mentioned demerits are addressed using dilution techniques (Figure 2.3) because they are more sensitive to small amounts of extracts, adequately determine cidal and static

properties and quantify the concentrations of samples required to produce inhibitory effects towards test microbes (Eloff, 1998; Langfield *et al.*, 2004; Masoko *et al.*, 2005).



Figure 2.2: Agar diffusion method based on the diffusion of antimicrobial chemicals into the agar to produce a clear zone of inhibition (Shami *et al.*, 2013).

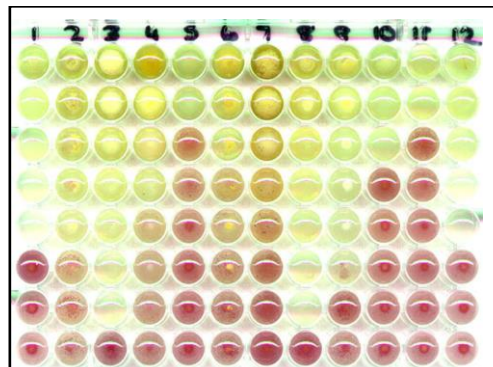


Figure 2.3: Broth micro-dilution method based on the conversion of tetrazolium salts to purple formazan complexes by active microbes, unchanged wells indicate inhibition (Adamu *et al.*, 2012).

2.10. Microorganisms used in this study

Microorganisms used in this study were selected because they are common causative agents of infectious diarrhoea. The bacterial species represent two major types of cell wall conformations, the Gram-negative and Gram-positive bacteria.

2.10.1. *Staphylococcus aureus*

Humans are natural harbours of *Staphylococcus aureus* (Figure 2.4) and there lies a commensal relationship between the two (Tong *et al.*, 2015). Even so, *S. aureus* is Gram-positive human pathogen and is part of the leading cause in the development

of bacteraemia and infective endocarditis, skin, soft-tissue, catheter-related, bone, joint, pulmonary, and central nervous system infections (Tong *et al.*, 2015).

Virulence factors that are associated with the pathogenicity of *S. aureus* are mainly reliant on the production of cell wall molecules such as capsular polysaccharides. These molecules link to form protective capsules around the bacterial cell (Archer, 1998). Extracellular proteins; coagulases, haemolysins, enterotoxins, toxic-shock syndrome toxin and Panton-Valentine leukocidin may also be synthesised for invasive infections (Archer, 1998).

The prevalence of antibiotic resistant strains of this species makes it more problematic to treat. The emergence of methicillin and vancomycin resistant *S. aureus* strains are of public concern (Kirst *et al.*, 1998). This public concern is further compounded by the increased rate of hospital-acquired infections and the occurrence of these strains in local communities (van Hal *et al.*, 2012). *Staphylococci* are also associated with food poisoning which results from oral ingestion of food that has been exposed to these bacteria and their enterotoxins (Hennekinne *et al.*, 2012). Shared symptoms of the host include nausea, vomiting and diarrhoea which can occur after an average incubation time of 3.7 – 4.7 hr (Wakabayashi *et al.*, 2018).

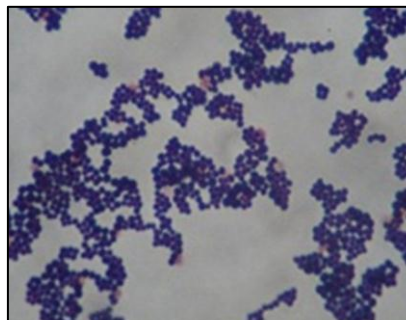


Figure 2.4: Gram stain of *S. aureus* visualised under a light microscope with a 100X objective (Jahan *et al.*, 2015).

2.10.2. *Enterococcus faecalis*

Enterococci are Gram-positive and facultative anaerobes that live in the gastrointestinal tract (GIT) of humans and other animals. Under normal biochemical conditions, *Enterococci* have a commensal relationship with the host and thus, thrive without compromising the host (Murray, 1990).

However, multidrug resistant *Enterococci* present in the GIT proliferate hastily, especially in the hospital surroundings leading to various nosocomial infections. *Enterococci* infections are of a great concern and are the second, behind *staphylococci*, as causative agent towards Gram-positive bacterial infections (Miller *et al.*, 2014). Infections associated with *Enterococci* bacteria include: urinary tract, bloodstream, meningitis, wounds and the biliary tract, among others (Murray, 1990).

E. faecalis (Figure 2.5) has been reported to be resistant to a wide range of β -lactam and aminoglycoside antibiotics such as cephalosporin, ampicillin, penicillin, vancomycin, tetracyclines and glycylicyclines (Miller *et al.*, 2014). These bacteria can synthesise a catalytic enzyme (β -lactamase) that facilitates antibiotic resistance by cleaving the β -lactam ring (Miller *et al.*, 2014).

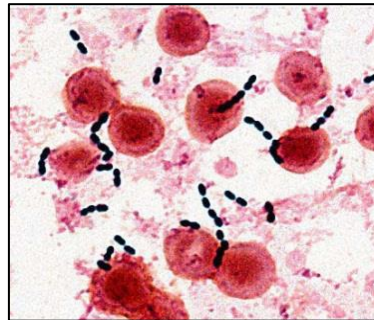


Figure 2.5: The Gram stain of the bacterium *Enterococcus faecalis* visualised under a 100X magnification with a light microscope (Todar, 2009).

2.10.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped, Gram-negative, non-fermenting bacterium that is ubiquitous in numerous environmental conditions and sources such as animals, flora and humans (Figure 2.6). The minimal nutritional requirement of the organism allows it to withstand a variety of physical conditions, which in turn is able to persevere in local communities and in hospital surroundings (Lister *et al.*, 2009).

This bacterium has the ability to become an opportunistic pathogen in immune-compromised patients. This usually leads to the development of pneumonia, urinary tract infections, and bacteremia; and chronic lung infections in patients with cystic fibrosis (Tran *et al.*, 2014).

P. aeruginosa is remarkably resistant to a wide variety of antimicrobial agents (Mesaros *et al.*, 2007). The resistance of this species is conferred by its use of enzymes and mutational mechanisms. The organism is able to constitutively express efflux pumps that have a diverse specificity towards various substrates (Livermore, 2001). Moreover, resistance can be developed by the transfer of vectors that have resistance genes e.g. plasmids. Mutational mechanisms that modify the expression and/or the function genes also provide resistance (Lister *et al.*, 2009). These resistance mechanisms can either occur singularly or simultaneously to help the bacterium acquire resistance towards antimicrobial agents (McGowan, 2006).

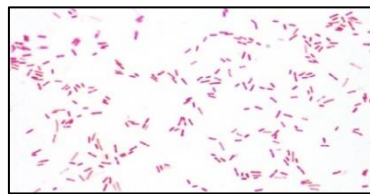


Figure 2. 6: *Pseudomonas aeruginosa* cells visualised with a conventional microscope with a 100X light microscope (Todar, 2009).

2.10.4. *Escherichia coli*

Escherichia coli is a Gram-negative bacterium with a rod-shaped cell wall and a facultative anaerobe (Figure 2.7). This bacterium is commonly harmless and colonises the gastrointestinal tract of both animals and humans as normal flora (Nataro and Kaper, 1998; Kaper *et al.* 2004). Nevertheless, there are several strains of *E. coli* that have evolved to become infectious due to the receipt of virulence factors contained within plasmids, transposons, bacteriophages and/or pathogenicity islands (Kaper *et al.* 2004).

Infections observed in humans are due to the intake of drinking water that has been contaminated with human faeces or animal droppings, the consumption of undercooked meat and infected vegetables or fruits. Poor hygiene is also implicated in the transmittance of the bacterium through person-to-person contact (Berger *et al.* 2010).

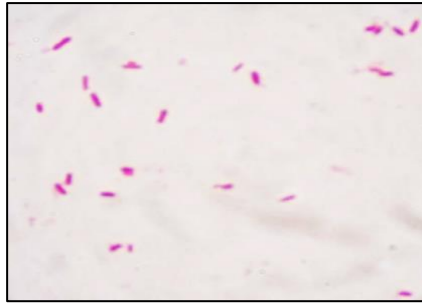


Figure 2. 7: *Escherichia coli* cells visualised under a light microscope at a 100X objective (Todar, 2009).

2.11. Plants used in this study

The plants used in this study were selected based on their traditional use as ingredients in the herbal concoctions.

2.11.1. *Kirkia wilmsii*

Kirkia wilmsii belongs to the *Kirkiaceae* family, it has a rounded crowning (van Wyk and van Wyk, 1997). The leaves are used as coarse food for livestock such as goats and sheep by rural communities in South Africa (Plantzafrica, 2013). This plant can produce multiple underground corms (Figure 2.8) that store large amounts of water to allow it to survive during the winter. In turn the corms are used by indigenous people to quench thirst whilst the remaining fibrous bark and young roots are used to weave ornamentals (Plantzafrica, 2013).

The extent of the use of *K. wilmsii* in the Limpopo Province is not adequately documented. However, the ethno-medicinal use of the plant in treatment of diabetes mellitus was reported by Semenya *et al.* (2012). In another ethno-medicinal survey regarding this plant, Semenya and Potgieter, (2015) reported that the dominant ethnic group, Bapedi, which is 57% of the Limpopo population (Limpopo Provincial Government, 2013) refer to *K. wilmsii* as *Legaba* or *Modumela* in their vernacular and also use it to treat hypertension. From this survey, some of the interviewed healers indicated that they solely used 5-6 uncooked freshly skinned tuberous roots of the plant and administered it orally after meals whilst others pound the tuberous roots and prescribe three teaspoons with warm water. The Chuene and Moletjie mountains in the Limpopo Province were the fundamental locations where *K. wilmsii* is harvested by Bapedi traditional healers (Semenya and Potgieter, 2015).

The anti-microbial potential of extracts of *K. wilmsii* was demonstrated by Eloff *et al.* (2010) using hexane, acetone, methanol and dichloromethane extracts of its leaves against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* nosocomial bacterial strains and pathogenic fungal species of *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporium canis* and *Sporothrix schenckii*. The leaf extracts of *K. wilmsii* obtained with acetone were reported to have good antifungal activities with an average MIC value of 0.5 mg/mL across the different fungal species.



Figure 2.8: The underground corm of *K. wilmsii* collected from Ga Maja.

2.11.2. *Hypoxis hemerocallidea*

Hypoxis hemerocallidea Fisch. & C.A. Mey is a medicinal plant of southern Africa that forms part of the numerous *hypoxis* species that belong to the family *Hypoxidaceae* (van Wyk *et al.*, 1997). In Zulu, the plant is commonly known as “*Inkomfe*” or “*Lilabatseka*”, in Swati it is called “*Zifozonke*” and in English it is loosely called African potato, *hypoxis* or yellow star flower (van Wyk *et al.*, 1997). In Sepedi, the plant is called “*Monna maledu*” (Semenya *et al.*, 2012). This perennial plant propagates by a large underground corm (Figure 2.9) that is characterised by a dark-brown to black shade on the outside and bright-yellow colour inside upon its cross-section. The longitudinal leaves protrude outwards from the centre of herb, spreading in a circular pattern being stacked on top of each other. The star-shaped flowers also exhibit a bright-yellow colour (van Wyk *et al.*, 2002; van Wyk *et al.*, 1997).

Indigenously, various African ethnic groups use the decoction of the *Hypoxis* genus to cure and manage a variety of health conditions and disorders such as cancer,

headaches, testicular tumours, impotency, cardiac diseases and intestinal parasites (Drewes *et al.*, 2008). The widespread use of this plant has led to its inclusion as an integral ingredient in a number of herbal mixtures (Hutchings *et al.*, 1996). Urinary tract infections, dizziness and mental disorders have been reported to be alleviated by the infusion of the corm of the plant rather than the decoction (Hutchings *et al.*, 1996).

The widespread traditional use of *H. hemerocallidea* sparked *in vitro* ethnopharmacological investigations which confirmed that the infusions of the corm indeed have a range of pharmacological properties (Owira and Ojewole, 2009). Unfortunately, the broad usage of the corm has led to the depletion and unsustainable harvesting of the plant. Katerere and Eloff, (2008) reported significant anti-microbial activity of crude ethyl acetate leaf extracts against *Staphylococcus aureus* (MIC of 0.31 mg/mL) and *Enterococcus faecalis* (MIC of 0.63 mg/mL). This was in addition to evidence of season-dependent anti-bacterial activity of various organic solvent extracts against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* and *Candida albicans*.



Figure 2.9: The underground corm of *H. hemerocallidea* collected from Ga Maja.

2.11.3. *Monsonia angustifolia*

Monsonia angustifolia E. Mey. ex. A. Rich is classified under the Geraniaceae family and commonly known as Crane's bill, *Alsbos*, *Angelbossie*, or *Teebossie*. The family is distributed from Australia to America, Asia, Europe and widely distributed in Africa (Hutchinson, 1969). In South Africa it is distributed through the Eastern Cape, Limpopo, KwaZulu-Natal and Mpumalanga. The National Assessment Red List of South Africa has adopted *Monsonia angustifolia* into the list of threatened plant species (Foden and Potter, 2012).

The leaves of this plant are generally narrow with the broadest width in the middle ending off with narrow ends (Figure 2.10). The margins of the leaves are irregularly toothed with cuneate bases. The Flowers produced are small with outward protruding stamens and overlaying toothed petals. The stems are thick, woody, red and covered with gland-tipped hairs (Lyimo *et al.*, 2003).

The ethnopharmacological application of *Monsonia angustifolia* as a medicinal plant include its use as a blood cleanser, aphrodisiac and to enhance libido (Khorombi, 2006). Further investigations of this plant by Fouche *et al.* (2015) demonstrated the effect of the aqueous extract of the aerial parts on the sexual behaviour of male wistar rats. The results obtained showed that when the extracts were given to the male rats at 300 mg/kg body weight, the extracts had stimulatory effects on their sexual behaviour, thus validating the traditional use of the plant by indigenous cultures in the intervention of libido, erectile dysfunction and premature ejaculatory disorders. Other documented ethnopharmacological properties range from the management of anthrax to treatment of heartburn and diarrhoea (SANBI, <http://pza.sanbi.org/monsonia-angustifolia>).



Figure 2.10: *Monsonia angustifolia* (SANBI, <http://pza.sanbi.org/monsonia-angustifolia>).

2.11.4. *Drimia elata* Jacq

Drimia elata belongs to the family Hyacinthaceae and is commonly known as *Sekanama* in Sepedi (Semenya *et al.*, 2013). The bulb of the plant (Figure 2.11) is used in combination with *E. transvaalense*, *E. elephantina* (roots), *S. birrea* (bark), *Z. capense* (root) and *S. viminale* (twigs) by Bapedi traditional healers in treatment of sexually transmitted infections such as Human immunodeficiency virus and acquired immunodeficiency syndrome (Semenya *et al.*, 2013). Furthermore, Semanya *et al.*

(2015) documented the use of *D. elata* by Bapedi traditional healers in the Waterberg district as an alternative species used in the treatment of hypertension.

The cardiotoxic bufadienolides present in *Drimia elata* and *Bowiea volubilis* species have also been implicated in human poisoning. This plant is also used by traditional healers as blood purifiers and for treating headaches, oedema, infertility and bladder complaints (van Wyk *et al.*, 1997).



Figure 2.11: Bulb of *Drimia elata* collected at Ga Maja, Limpopo Province.

2.11.5. *Vahlia capensis*

Vahlia capensis is a small perennial shrub that is used by people in Botswana to treat sore eyes mainly in small children (Figure 2.12). This medicinal plant is also found in Lesotho, Namibia, Zimbabwe and South Africa. It belongs to the family *Vahliaceae* (Staugard and Hedberg, 1989).

Majinda *et al.* (1995) detected coumarins umbelliferone, scopoletin and scoparone, and the terpenoids sitosterol, cycloartenol, 24-methylenecycloartanol, cycloart-23-en-3 β ,25-diol, glutinol and a seco ring-A triterpene acid, 3,4-seco-olean-4,(23),18-dien-3-oic acid in the n-hexane and ethyl acetate extracts of this plant. In addition, the n-butanol extract of *V. capensis* was reported to consist of kaempferol, quercetin, afzelin, astragalin, quercitrin, isoquercitrin, rutin, gallic acid, chiro-inositol, dulcitol, and a novel biflavonoid, vahlia biflavone (Majinda *et al.*, 1997). The gallic acid and vahlia biflavone from *V. capensis* were reported to have antibacterial activities against *Staphylococcus aureus* and *Bacillus subtilis*.



Figure 2.12: Flowering *Vahlia capensis* ([www. Mozambiqueflora .com/speciesdata/image - display.php?species_ id=183740&image_id=5](http://www.Mozambiqueflora.com/speciesdata/image-display.php?species_id=183740&image_id=5)}).

2.11.6. *Sarcostemma viminale*

Sarcostemma viminale (L.) R.Br. (Figure 2.13) belongs to the family *Asclepiadaceae*. The family of this plant includes herbals, stem succulents and vines (Endress and Bruyns, 2000).

Semenya *et al.* (2013) reported that at the Waterberg district in the Limpopo Province, Bapedi traditional healers use *S. viminale* or the treatment of sexually transmitted infections. In the Venda region (Limpopo Province), the aerial parts of the plant are crushed and applied directly on an open wound. It was reported to have high efficacy against maggots (Luseba *et al.*, 2007). The latex from the whole plant may also be applied to treat ulcers and skin lesions (Luseba *et al.*, 2007). Farmers utilise a variety of plants such a *S. viminale* in ethnoveterinary medicine for the treatment of wounds, sores and lumpy skin diorders in livestock due to its irritant nature (van der *Merwe et al.*, 2001).



Figure 2.13: Flowing *Sarcostemma Viminale* collected at Ga Maja.

2.12. Purpose of the study

2.12.1. Aim

To evaluate pharmacological and synergistic effects of herbal concoctions sold at Ga Maja and selected plants used in their preparation.

2.12.2. Objectives

The objectives of this study were to:

- I. Evaluate possible microbial contamination of the commercial herbal concoctions;
- II. Evaluate the nutritional content of the herbal concoctions;
- III. Prepare a laboratory standard as per the instructions of the manufacturers;
- IV. Phytochemical analysis of the herbal concoctions and the plants used in their preparation;
 - a. Qualitative (thin layer chromatography and phyto-constituent screening)
 - b. Quantitative (total phenolics, flavonoids and tannins)
- V. Determine the antioxidant potential of the herbal concoctions and the plants used as ingredients in their preparation using qualitative and quantitative methods which involve 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing assays;
- VI. Investigate the antimicrobial activity of the plant constituents and the herbal concoctions against diarrheagenic pathogens using broth micro-dilution and bioautography;
- VII. Investigate the combinational effects of the mixture of different plant extracts towards a biological activity (antioxidant and antimicrobial);
- VIII. Determine the anti-human immune-deficiency virus (HIV) reverse transcriptase inhibitory activity of the concoctions;
- IX. Determine the anti-inflammatory activity of the concoction.

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CHAPTER 3

3. Isolation and identification of microbial contaminants and nutrient evaluation of herbal concoctions

3.1. Introduction

Pharmaceutical medications and plant-based traditional medicines have two important aspects put in place in order to regulate their authenticity and quality. One is safety and refers to the probability of a medicines' inability to induce harm towards the consumer under the conditions proposed for their usage. The other important aspect is efficacy, and this refers to the capacity of a prescribed medicine to confer a therapeutic benefit regarding its indicated pharmacological actions (Moreira *et al.*,

2014). Thus, in principle, a medicine has no clinical relevance if it is active against the indicated ailment, but its ingestion or application is unsafe and vice versa (Moreira *et al.*, 2014).

Manufacturing conditions of herbal medicines such as; quality, quantity and type of plant species used, the hygienic handling, packaging and storage are primarily under the control of the manufacturer (Ndhlala *et al.*, 2009). In the case of rural locations such as the Ga Maja area, it is the sole responsibility of the traders and traditional healers. Because of African traditional medicine (ATM) becoming a lucrative business in South Africa, the quality and safety of commercialised herbal preparations has been overlooked in an exchange for profit gains (Nair *et al.*, 2012).

It is important to note that quality control is in the interest of the public. As briefly mentioned above, hygiene during the manufacturing processes and storage is integral. This is because under unsterile conditions, pathogenic microorganisms can easily contaminate the products. Microbial contaminations in consumables are a concern as they potentially jeopardise the health of consumers. Contamination of phyto-medicines by microbes may further persist because of the locations in which they are sold, such as pavements and road-sides which are generally unhygienic (Steenkamp *et al.*, 2006).

Precise microbial (bacteria and fungi) identification plays a vital role in the event of infectious disease outbreaks, diagnosis and relevant treatment of such cases. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a current technology that is routinely utilised in clinical microbiology laboratories to efficiently identify microorganisms (Bizzini and Greub, 2010). MALDI-TOF-MS can detect a great range of proteins and therefore is able to distinguish closely related microorganisms and can categorise them at the species level (Murray, 2010). In a study by Bizzini and Greub, (2010), it was reported that the accuracy of identification of bacterial isolates was improved at a species level from 70.3% to 93.2% when a preliminary protein extraction step was performed.

Metals are native constituents of the Earth's crust and therefore cannot be completely removed from consumables such as food, beverages and plant material. These consumables can contain a variety of trace metals such as sodium (Na), potassium (K), iron (Fe), calcium (Ca), boron (B), magnesium (Mg), aluminium (Al), copper (Cu),

zinc (Zn), molybdenum, cobalt (Co), manganese (Mn), silicon (Si) and nickel (Ni) (Hecht and Kumpulainen, 1995; Alka, 2000; Garcia-Rico *et al.*, 2007). Not only are they present in products due to their natural occurrence in the environment and the product itself, but human activities such as agricultural and industrial processes contribute to their accumulation (Iwuoha *et al.*, 2013). Some of the metals are regarded as essential micro-nutrients because of their functionality in biological systems but are required only in trace amounts (Iwuoha *et al.*, 2013).

Micro-nutrients are required in trace amounts because they are toxic at excessive levels and can be damaging to cells and their related biological processes. Therefore, the knowledge of their concentrations in foods, beverages and medicines is essential so that safe daily dietary intakes can be recommended, and consumers can also know how much they consume per measurement of the product (Soliman and Zikovski, 1999).

The quantification of micro-nutrients in the herbal concoctions was carried out using multi-element techniques of spectrometry. Particularly, the inductively coupled plasma atomic emission spectrometry (ICP-AES). In this technique, argon is used to excite the atoms of a test sample, during this excitation phase, light is emitted and directed to a detector. The optical signals from the detector processes values to produce the concentration of present elements (Bolann *et al.*, 2007).

3.2. Methodology

3.2.1. Sample procurement

Five herbal concoctions were randomly purchased from five available independent traders from Ga Maja. The five samples were purchased based on the common pharmacological properties claimed by the traders. The traders were also traditional healers who were responsible for the preparation of the herbal concoctions. The traders have set up a market-place alongside the road leading to Lebowakgomo (24.1631° S, 29.5543° E). The environmental conditions that the herbal concoctions were found to be exposed to included alternating daily temperatures, exposure to

sunlight and wind that randomly carries dust particles. The collection was conducted during the late summer of 2017.

3.2.2. Isolation of microbial contaminants

The herbal concoctions were cultured under aseptic conditions on nutrient agar (NA) and potato dextrose agar (PDA) plates immediately on the day of collection, using the spread-plate technique. Afterwards the bottles of the concoctions were placed in a refrigerator until they were freeze dried for use in subsequent assays. A 100x serial dilution of each concoction was made and 100 μ L of dilutions were spread-plated on the abovementioned media using a sterile bend glass rod. For bacterial growth, the NA plates supplemented with cycloheximide were incubated at 37 $^{\circ}$ C for 24 hrs. The NA was supplemented with the antifungal in order to suppress fungal growth on the media so that only bacterial species could grow. For fungal species isolation, the PDA was supplemented with the antibiotic chloramphenicol which suppresses bacterial growth. Chloramphenicol is a broad-spectrum antibiotic that inhibits protein synthesis due to its high affinity towards the peptidyl transferase in bacteria (Schwarz *et al.*, 2004). The PDA plates were incubated at 25 $^{\circ}$ C for 48 hrs.

Following incubation, viable colonies were counted using a colony count. The viable cell count was used to determine the colony forming units per millilitre (CFU/mL) and was calculated using equation 3.1. To enable purification, the isolates were sub-cultured on fresh agar plates and subjected to multiple Gram staining procedures. A conventional light microscope was used to visualise the isolates for a swift purification.

$$\frac{CFU}{mL} = \frac{\text{number of colonies}}{mL \text{ of sample plated}} \quad (3.1)$$

3.2.3. Identification of microbial isolates

The identification of pure isolates was carried out using MALDI-TOF-MS and the VITEK 2 system.

3.2.3.1. Identification of pure isolates using the MALDI-TOF-MS

For an increased accuracy of identification, formic acid extraction of proteins from the isolates was performed prior to using the MALDI biotyper. Briefly, 300 μ L of deionised water was pipetted into an Eppendorf tube. To this Eppendorf tube, a single colony

was transferred followed by thorough vortexing. Ethanol (99% v/v) was added to the mixture and vortexed. The mixture was then centrifuged at a speed of 13 000 rpm for 2 minutes. The supernatant was decanted and the tube was centrifuged again as described above. The residual supernatant was carefully pipetted out of the tube without disturbing the pellet. The pellet was allowed to dry at room temperature for 3 minutes. Fifteen microliters of 70% formic acid was added to the pellet and thoroughly mixed by a vortex. This was followed by the addition of 15 μ L of acetonitrile to the mixture in the tube. The mixture was centrifuged at 13 000 rpm for 2 minutes. The supernatant (1 μ L) was pipetted onto a MALDI target plate and allowed to dry at room temperature (25 °C). After drying, the entire spot on the MALDI plate was overlaid with α -Cyano-4-hydroxycinnamic acid (HCCA) solution and allowed to dry. The target plate was inserted into the MALDI and analysed with the biotyper's library to identify the isolates.

3.2.3.2. Identification of pure isolates using the VITEK 2 system

The VITEK 2 instrument was used for identification of the pure cultures of the bacterial isolates. The instrument is housed at the Limpopo agro-food technology station (LATS) at the University of Limpopo. The manufacturer's protocol was followed for analysis. The VITEK 2 system is a fully automated microbiology system utilizing growth-based technology system and operates *in vitro*. A suspension of a pure culture was prepared by suspending isolated colonies in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tubes using a sterile swab. The test kit card with transferred suspensions were placed in the VITEK incubator. The VITEK system analyses the card as growth of the organism which occurs and gives an identity of the organism (Pincus, 2006).

3.2.4. Evaluation of the micro-nutrient content of the concoctions

The composition of nutrients, metals present in the concoctions was determined with a plasma atomic emission spectrophotometer (Model ICPE-900, Shimadzu).

3.3. Results

3.3.1. Isolation of microbial contaminants

The colonies were enumerated using a colony count to obtain the colony forming units per volume (CFU/mL) of the concoctions. The large CFU/mL values indicated a heavy contamination of different bacterial species in all the five concoctions (Table 3.1).

3.3.2. Identification of bacterial isolates by MALDI-TOF-MS

Purified bacterial isolates were identified using MALDI-TOF. Sulfide, indole and motility (SIM) agar medium was used to evaluate motility and biochemical tests. Majority of the isolates were found to be pathogenic motile members of the Enterobacteriaceae family (Table 3.2).

3.3.3. Bacterial identification by VITEK 2 system

Bacterial isolates that were not suitable to be identified by the MALDI-TOF due to the texture of their colonies were identified using the VITEK 2 system. Nosocomial pathogens such as the *Klebsiella* genus were identified with a high identification score (Table 3.3).

3.3.4. Fungal species identification by the VITEK 2 system

The yeast contaminants of the concoctions were cultured on potato dextrose agar and were purified by multiple steps of microscopic evaluation of wet mounts. The purified colonies were identified by the VITEK 2 system. Two coccus budding yeasts were identified with a high identification percentage score (Table 3.4).

Table 3. 1: The different number and types of distinct bacterial colonies that appeared after 24 hr incubation of nutrient agar plates

Morphologically distinct colonies	Concoction 1		Concoction 2		Concoction 3		Concoction 4		Concoction 5	
	CFU/mL	CFU/500 mL bottle	CFU/mL	CFU/500 mL bottle	CFU/mL	CFU/500 mL bottle	CFU/MI	CFU/500 mL bottle	CFU/mL	CFU/500 mL bottle
Cream, circular and elevated	4.09×10^7	2.09×10^{10}	6.1×10^6	3.05×10^9	4.52×10^6	2.26×10^9	3.54×10^6	1.77×10^9	TNTC	TNTC
Transparent, circular and flat	6.09×10^7	3.045×10^{10}	2.8×10^8	1.4×10^{11}	TNTC	TNTC	-	-	-	-
Cream, circular and flat	-	-	-	-	1.44×10^6	7.2×10^8	2.73×10^4	1.365×10^7	3.5×10^4	1.75×10^7
Yellow, circular and elevated	-	-	-	-	-	-	-	-	4.1×10^6	2.05×10^9

Key: TNTC: Too numerous to count, (-): Not present

Table 3. 2 Bacterial species isolated from herbal concoctions identified with MALDI-TOF and their associated human infections

Identified Bacterial species	H ₂ S production	Indole production	Motility	Infection	Reference
<i>Enterobacter cloacae</i>	-	-	+	Nosocomial pathogen: intra-abdominal, endocarditis and septic arthritis	Fata <i>et al.</i> , 1996
<i>Enterobacter aerogenes</i>	-	+	+	Respiratory, bacteremia and urinary tract	Phillippe <i>et al.</i> , 2015
<i>Escherichia coli</i>	-	+	+	Diarrheal diseases and urinary tract infections	Wanke, 2001
<i>Leclercia adecarboxylata</i>	-	+	+	Blood, wounds, urinary tract infections in immunocompromised hosts	Tamz and nayak, 2012
<i>Citrobacter braakii</i>	+	-	+	Bacteremia	Hirai <i>et al.</i> , 2016
<i>Klebsiella pneumoniae</i>	-	-	-	Urinary tract infections, septicemia, pneumonia, bacteremia and meningitis	Ko <i>et al.</i> , 2002; Cheng <i>et al.</i> , 1991
<i>Pantoea agglomerans</i>	-	-	-	Uncommon pathogen	Cruz <i>et al.</i> , 2007
<i>Bacillus subtilis</i>	-	-	-	No known human infection	Guo <i>et al.</i> , 2017

Key: (+): positive; (-): Negative

Table 3. 3: Bacterial contaminants of the concoctions identified using the VITEK 2 system

Identified species	Identification score (%)	Infection	Reference
<i>Klebsiella pneumoniae</i>	91	Nosocomial infections (Urinary tract, wound and lung)	Chung, 2016
<i>Pseudomonas fluorescense</i>	94	-	Scales <i>et al.</i> , 2014
<i>Klebsiella oxytoca</i>	99	Nosocomial (urogenital tract infections and pneumonia)	Bleich <i>et al.</i> , 2008
<i>Pantoea agglomerans</i>	95	Nosocomial septicemia	Dutkiewicz <i>et al.</i> , 2016
<i>Burkholderia cepacia</i>	87	Cystic fibrosis and granulomatous	Matthaiou <i>et al.</i> , 2011
<i>Burkholderia mallei</i>	33	Glanders and melioidosis	Gilad <i>et al.</i> , 2007

Key: (-): Generally, not considered human pathogen

Table 3. 4: The cellular morphology and reproductive characteristics of the yeast contaminants isolated from the concoction and their associated human infections

Identified species	Characteristics	Percentage identification score (%)	Infections	References
<i>Stephanoascus ciferrii</i>	Coccus, budding yeast	98	Ear diseases, non-insulin heart disease and most especially with cases of onychomycosis	De Gentile <i>et al.</i> , 1991
<i>Cryptococcus laurentii</i>	Coccus, budding yeast	96	Fungemia	Johnson <i>et al.</i> , 1998

3.3.5. Micro-nutrient content of the herbal concoctions.

Prior to analysis, the concoctions were stored at room temperature. The quantification was performed in duplicates for each sample using multi-element standards. The concentrations of the analysed elements in each concoction sample were expressed in mg/L (Figures 3.1 and 3.2). Each concoction exhibited variable amounts of trace elements, with sodium, potassium and zinc being the most abundant in the samples. copper and nickel were not present in the concoctions.

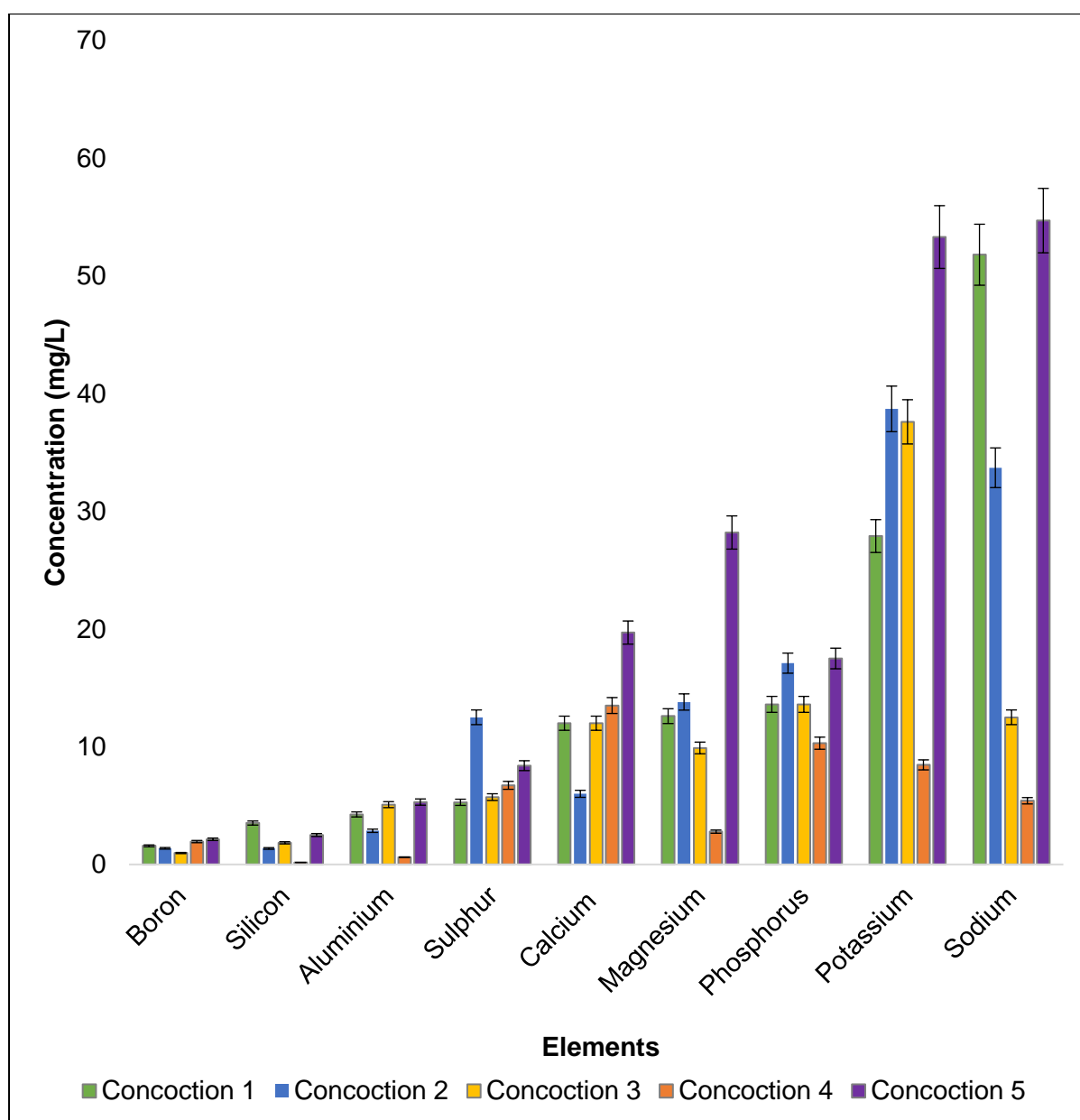


Figure 3. 1: The concentrations of elements present in the herbal concoctions.

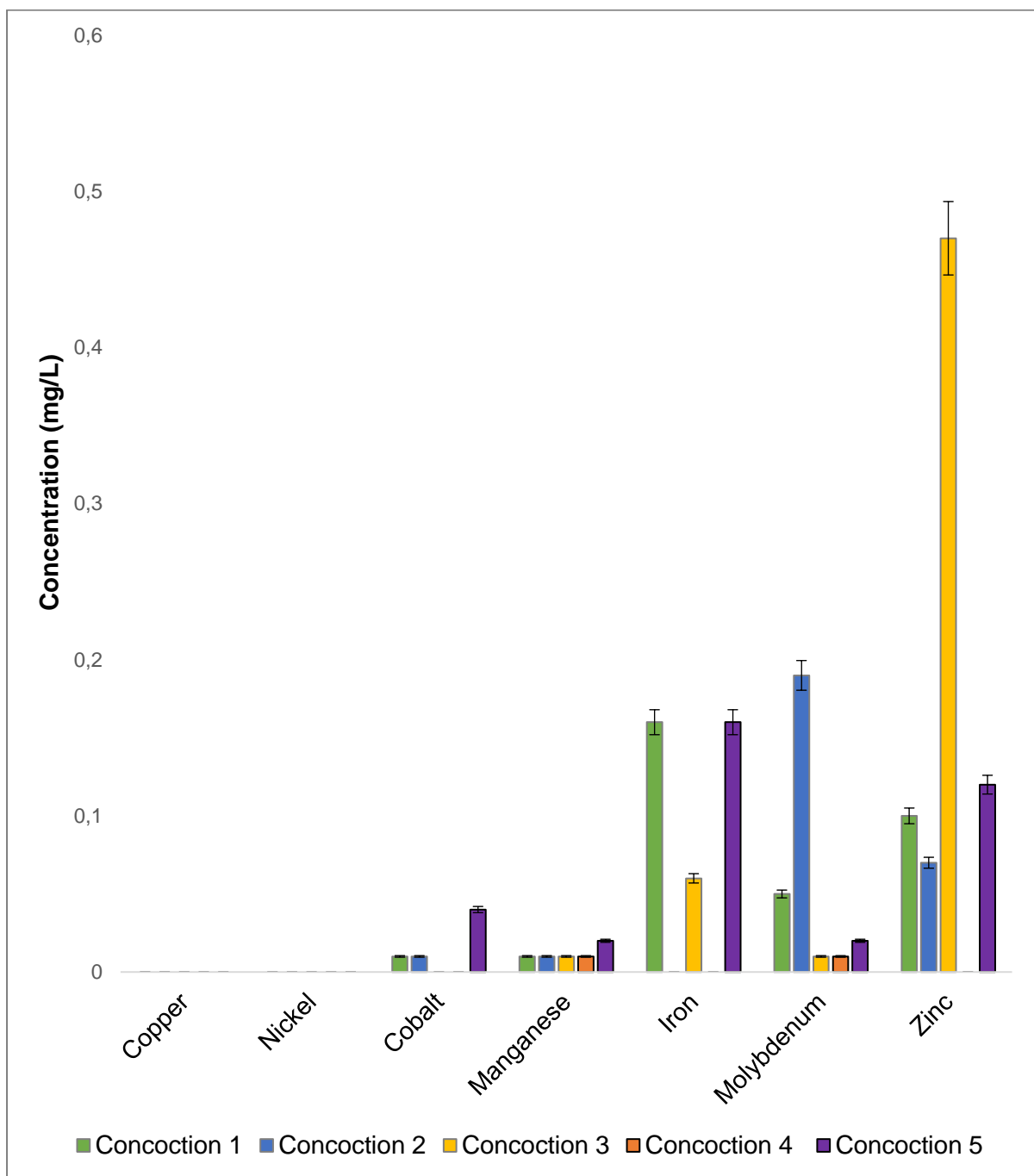


Figure 3. 2: Concentrations of various transition metals present in the different herbal concoctions.

3.4. Discussion

South Africa is one of the countries in the world with abundant cultural and flora diversities. A large number of medicinal plant products are commonly sold to the general populace, either as unprocessed material or in the form of herbal mixtures of semi-processed or processed material (Ndhlala *et al.*, 2009).

The increase in commercialisation of such products is lucrative and highly competitive, that in the efforts of making profits, the quality of manufacturing and packaging process is often neglected. This means that consumers have a greater chance of getting products that are sub-standard and may pose a threat to human health.

The traders at this marketplace utilise previously used bottles from sodas, water and energy drinks to store their herbal concoctions. In this chapter, safety of the concoctions was investigated by evaluating possible microbial contamination. This was motivated by the un-informed and poor attempts by the handlers to decontaminate the recycled bottles and the plant material during processing.

Microbial contamination was evaluated by spread-plating the concoctions on nutrient agar and potato dextrose agar to isolate bacteria and fungi respectively. After a 24-hr incubation of the concoction-inoculated nutrient agar plates, morphologically distinct bacterial colonies appeared in all the agar plates. All the concoctions were heavily contaminated by different bacterial species. Some of the bacterial species in concoctions 3 and 4 were too numerous to count (Table 3.1).

Of the plant parts used to prepare the concoctions, some were underground parts such as *Kirkia wilmsii* (corm), *Hypoxis hemerocallidea* (corm), *Drimia elata* (corm) and “*Tšhikwana/Moroto wa tšhwene*” (powdered root mixture) (Section 2.11). The large number and varieties of microorganisms that are associated with the soil are consequently carried onto these plant parts. In addition, other plant parts such as the leaves and stems can harbour a variety of microorganisms. The use of water is the only means by which the traders and traditional healers clean the plant material and bottles. This approach of sterilisation is inefficient to decontaminate the bottles, because water alone does not have the strength to remove all microbes.

The bacterial isolates were identified using MALDI-TOF. All the isolated bacteria except for *Bacillus subtilis*, belonged to the Enterobacteriaceae family (Table 3.2). This

family of bacteria are Gram-negative rods and some members secrete endotoxins (lipopolysaccharides) that occur on the outer membrane of the bacterial cell (Ramachandran, 2014). Bacterial isolates identified with the Vitek 2 biotyper were also detected to be Gram-negative pathogens (Table 3.3). In Lagos, Nigeria, several commercially available concoctions were screened for microbial contamination by Adeleye *et al.* (2005). Similar heavy microbial contamination was detected. These studies demonstrate the need for hygienic approach towards the preparation of the concoctions and a continuous monitoring of contamination levels in herbal products.

Motility is a beneficial characteristic, and motile bacteria are more advantaged in that they are able to adjust to vast environmental conditions. These bacteria are capable of propagating towards favourable conditions or move away from threats and this gives them the advantage over non-motile bacteria (Duan *et al.*, 2011). This could explain the larger number of viable motile bacteria in the concoctions.

Majority of the bacteria; *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Leclercia adecarboxylata* and *Citrobacter braakii* were found to be motile (Table 3.2). Motility was observed as cloudy growth or protrusions moving away from the stab line in the tube. Motility was further validated by visualising wet mounts of the bacteria using a light microscope at 100x objective.

Motile bacteria are more damaging, owing to their capability to colonise cells and propagate through vast host cells, tissues and vital organs (Duan *et al.*, 2011). The intake of these concoctions suggests that consumers would be predisposed to bacterial infections that would effectively deteriorate their health status and may be more debilitating to immune compromised consumers. It is therefore appropriate and essential that more effective sterilisation methods be used to ensure the purity of herbal products.

The contamination of the herbal concoctions by fungal species (Table 3.4) presents a health hazard to consumers. Fungi produce low-molecular weight secondary metabolites known as mycotoxins and exposure to these toxic metabolites leads to the onset of diseases in animals and humans generally known as mycotoxicoses (Bennett, 1987). Dietary exposure remains the largest risk factor leading to mycotoxicoses because a large proportion of these diseases infect human beings by the consumption of contaminated foods (Wild *et al.*, 1992).

The potential toxins produced both by the bacterial and fungal isolates may differ in their chemical structures and modes of actions. Furthermore, a single microbial species may be able to produce more than one toxin or have more than one mode of pathogenicity. The secretion of these chemical substances can lead to sepsis and haemolysis to mention a few (Ramachandran, 2014). Therefore, one is tempted to deduce the possible synergistic interactions of present microbial species and their toxins which can accumulatively enhance their toxicity towards the patient.

One of the consequences of inadequate regulation is that consumers purchase herbal remedies from these informal markets without evidence-based prescriptions or proper counselling and monitoring. The traders recommend different consumption of each herbal concoction, i.e. the first trader suggested that the herbal concoction should be taken three times daily, second trader recommended that a 2 L bottle should be finished in two days and others suggested that the 2 L bottles should be consumed every day in 500 mL increments.

An elementary look into the element profiles showed that Potassium (K) and sodium (Na) were generally higher in the concoctions with the exception of concoction 4, which contained relatively low concentrations (figure 3.1). Other essential macro-nutrients detected at relatively higher levels were calcium (Ca) and magnesium (Mg). Manganese (Mn) and zinc (Zn) were also detected and are micro-nutrients valuable for their role in catabolising macromolecules (fats, carbohydrates and proteins) and serve as cofactors to numerous enzymes (Soriano *et al.*, 2007).

Copper (Cu) and nickel (Ni) were not present in detectable levels in all the tested concoctions (Figure 3.1B). This reflected good on the element concentrations in the products because although copper (Cu) is an essential micronutrient, it is only required at minute concentrations and higher doses [1.5-3 mg per day for adults, 0.4–0.6 mg per day for infants and 0.7–2 mg per day for children (National academy of Science, 1989)] have been associated with liver damage (Gomez *et al.*, 2004). In addition, nickel is one of the bioavailable heavy metals associated with skin allergies, lung fibrosis, variables degrees of kidney and cardiovascular system poisoning (Denkhaus and Salnikow, 2002).

The concentrations of the elements in the five herbal concoctions were inconsistent and varied considerably. The difference in the concentrations could be due to the heterogeneousness of the samples as a result of being collected from different traditional healers. The consequence of the latter is that the traditional healers may have dissimilar sources of raw materials and different methods of preparing and packaging of their products. Of the raw materials, tap water is the main solvent of choice for the preparation of the concoctions and as such the concentrations of the elements could be related to the purity of the water used (Iwegbue, 2010).

Conclusion

The degree of microbial contamination in the concoctions was overwhelming and a cause for concern because many of the microbial contaminants were found to be pathogenic. Moreover, this contamination poses a threat to the health of patients that are dependent on the medicines. This highlights a lack of quality control over the manufacture and packing of the products. This could be related to the poor quality of utensils used and unhygienic processing and packaging employed during the overall manufacture of the concoctions.

Although there was no uniformity regarding the concentrations of the elements detected in the concoctions, their levels such as those for Cu and Ni were in trace amounts. Overall, it seems less probable that potential toxicities of the herbal concoction could be attributed to the concentration of the elements. The disadvantage is that since the amounts of these elements are not indicated on the bottles, it then becomes difficult for patients to adequately regulate their daily dietary limits when they use the concoctions in conjunction with pharmaceutical medicines.

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CHAPTER 4

4. Phytochemical analysis of the herbal concoctions and the plants used as ingredients in their preparation

4.1. Introduction

Phytochemicals are naturally occurring plant secondary metabolites that are biologically active but non-nutritive in the human diet (Chang *et al.*, 2016). They are produced by the plant to protect itself against environmental threats and to maintain its survival in the event of nutrient scarcity (Bourgaud *et al.*, 2001). The pharmacological effects exhibited by medicinal plants are typically accredited to the presence of diverse phytochemicals and they can be unique to a plant genus or family (Akindele and Adeyemi, 2007). Phytochemicals include compounds such as flavonoids, alkaloids, tannins, saponins, terpenoids, phenolics and steroids (Akindele and Adeyemi, 2007).

Some important biological activities associated with phytochemicals include antibacterial, antifungal, antioxidant, anti-inflammatory, anti-cancer, anti-aging and anti-atherosclerotic (Akindele and Adeyemi, 2007). The pharmacological screening of plants is an important step towards exploring and identifying novel phytochemicals that could serve as new sources for pharmaceutical agents and therapies (Akindele and Adeyemi, 2007). Moreover, the ethnopharmacological use of phytochemicals needs to be researched to validate the proposed therapeutic effects (Mulaudzi *et al.*, 2012).

Thin layer chromatography (TLC) is a time efficient separation technique that can be able to resolve and develop major phytochemical profiles of plant crude extracts (Masoko *et al.*, 2005) and herbal remedies (Ndhlala *et al.*, 2013).

The choice of an appropriate solvent for the extraction of compounds in a plant material is an important step to access them and to accurately evaluate and ascertain their associated biological activities. Solvents that are preferred by researchers have low toxicity, they are easily removable from the plant extracts at low temperatures (Masoko *et al.*, 2005) and do not induce the formation of complexes, precipitates and/or disintegration of phyto-constituents in the extracts (Ncube *et al.*, 2008). Moreover, the solvents used have the properties of being able to extract a high yield

of compounds of different chemical structures and properties therefore increasing the opportunity of obtaining bioactive compounds in the extract (Ncube *et al.*, 2008; Eloff, 1998). It is noteworthy to mention that in the preparation of the concoctions, all the traders used water as a means of extraction of their plant material. It is for this reason that for this study, water was the solvent of choice for extraction.

4.2. Methodology

4.2.1. Plant collection

The plant species in this study were selected for evaluation because they were claimed by the traders and traditional healers to be the ingredients used for the preparation of the herbal concoctions. The plant species were collected under the guidance of the traders. Table 4.1 shows the list of the collected plant specimens. The identity of the plants species was confirmed by the University of Limpopo's Larry Leach herbarium.

The plant material was stored at room temperature away from sunlight and left to completely dry. The corms were cut into smaller pieces to increase the surface area of the parts and a quicker drying period. A commercial blender was used to grind the dried plant material to fine powder. For the course of this study, the powdered plant material was stored in air-tight glass containers and kept away from sunlight.

4.2.2. Laboratory standard preparation

To prepare the herbal concoctions, the traders boil water in a large pot on wood fire. Once the water begins to boil, the different plant species mentioned in Table 4.1 are all added to the pot and boiled between 5 – 10 minutes. The traders rely on visual observation to try and keep the amount of plant material added constant. For the corms, the traders have a designated number that they add to the mixtures and depends on the size of the pot used on the day of preparation (personal communication, Ga Maja traders and traditional healers).

A laboratory herbal concoction was prepared in a similar procedure followed by the traders. One gram of each of the plants was added to 140 mL of boiling distilled water and boiled for 5 minutes. The laboratory and purchased concoctions were separately filtered through a Whatman No.1 filter paper and freeze dried.

Table 4. 1: Plant species used by traders as ingredients for herbal concoctions. The concoctions were produced by preparing water decoctions of the different plant parts. Generally, the combinations of the plant parts used by the traders was similar.

Plant material	Voucher number	Vernacular name	HC1	HC2	HC3	HC4	HC5
<i>Kirkia wilmsii</i> (leaves)	SS 94	<i>Legaba/modumela</i>	+	+	+	+	-
<i>Kirkia wilmsii</i> (corm)	SS 94	<i>Legaba/modumela</i>	+	+	+	+	+
<i>Kirkia wilmsii</i> (twigs)	SS 94	<i>Legaba/modumela</i>	-	-	-	+	+
<i>Hypoxis hemerocallidea</i> (corm)	SS 115	<i>Monna maledu</i>	+	+	+	+	+
<i>Monsonia angustifolia</i> (leaves)	121393	<i>Tee ya thaba</i>	+	+	+	+	+
<i>Drimia elata</i> (corm)	S 18	<i>Sekanama</i>	+	+	+	-	-
<i>Sarcostemma viminale</i>	121404	<i>Moema</i>	-	-	+	+	+
<i>Vahlia capensis</i>	121394	<i>Makgonatsohle</i>	+	+	+	+	+
“ <i>Tšhikwana/Moroto wa tšhwene</i> ” (Powder)*			-	+	+	+	+

Key: (+): Included, (-): Not included, HC1-sample 1; HC2- sample 2; HC3-Sample 3; HC4-Sample 4; HC5-Sample 5

*To the best of our knowledge, *Tšhikwana/Moroto wa tšhwene* is a powdered plant mixture consisting of *D. elata*, *S. viminale*, *V. capensis* and roots of unspecified plant species. The traditional healers were reluctant to divulge all the components of this mixture citing that secrecy contributed efficacy of these powders because intervention by their ancestral gods enhanced the therapeutic action of the treatment.

4.2.3. Phytochemical analysis

The chemical properties of the phytochemicals present in the decoctions of the plant species and herbal concoctions were evaluated using qualitative and quantitative analytical methods.

4.2.3.1. Qualitative phytochemical fingerprint profiles

All the extracts were reconstituted with distilled water to a concentration of 10 mg/mL. Using a micropipette, 10 μ L of each of the reconstituted extracts was loaded onto the thin layer chromatography (TLC) plates (Merck, Silica gel F₂₄₅). The TLC plates were developed in saturated TLC tanks using mobile phases which varied in polarities, particularly butanol/ acetic acid/ water (BAW) (3:2:2) (polar) (Merk, technical grade), ethyl acetate/methanol/ water (80:11:10) (EMW) (polar/neutral) (Merk, technical grade), chloroform/ethyl acetate/formic acid (25:20:5) (CEF) (intermediate polarity/acidic) (Merk, technical grade) and benzene/ethanol/ammonium hydroxide (BEA) (90:10:1) (non-polar/basic) (Merk, technical grade) (Kotze and Eloff, 2002). Once the mobile phases had reached the solvent-front, the chromatograms were removed from the tanks and air-dried in a fume hood.

To detect separated compounds, ultraviolet (UV) light 254 nm was used to visualize compounds which have the ability to fluoresce under electromagnetic spectrum. The TLC plates were sprayed with vanillin-sulphuric acid [0.1 g vanillin (Sigma Aldrich), 28 mL methanol (Merk, technical grade), 1 mL sulphuric acid (Sigma Aldrich)] (Stahl, 1969) and heated for 5 minutes at 110 °C for optimal colour development of compounds which had not been visualized under UV light.

4.2.3.2. Qualitative phyto-constituent screening

The plant species and herbal concoctions under investigation were screened for the presence of various classes of phyto-constituents such as terpenoids, flavonoids, cardiac glycosides, phlobatannins, saponins, tannins and alkaloids.

4.2.3.2.1. Tannins

Ground plant material (0.5 g) was resuspended in 5 mL of distilled water and 0.25 g of the dried concoctions was resuspended in 2.5 mL of distilled water. The solutions

were boiled then cooled to ambient temperature. Of these solutions, 1 mL was transferred into a clean test tube and a few drops of 1% aqueous ferric chloride solution was added to the test tube. A blue-black colouration of the solution indicated the presence of tannins (Trease and Evans, 1989).

4.2.3.2.2. Phlobatannins

The plant material (0.2 g) and the dried concoctions (0.2 g) were suspended in 10 mL of distilled water. The solutions were filtered with Whatman's filter paper No. 1. The filtrates were boiled with 2% (hydrochloric acid) HCl solution (2 mL) and the development of a coloured precipitate was indicative of the presence of the phlobatannins (Borokini and Omotayo, 2012).

4.2.3.2.3. Cardiac glycosides

The Keller-Killani test was used to evaluate the presence of cardiac glycosides whereby 2 mL of glacial acetic acid was added to 5 mg of ground plant material and concoctions in test tubes. A few drops of 1% aqueous ferric chloride was added followed by the careful and steady addition of 1 mL of concentrated sulphuric acid. The appearance of a brown ring at the interface between two layers served as an indication for the presence of cardiac glycosides (Borokini and Omotayo, 2012).

4.2.3.2.4. Flavonoids

Dilute ammonia solution (34%) (5 mL) was added to a portion of the (10 mg) aqueous filtrate of each plant species materials and herbal concoction sample. Concentrated (99%) sulphuric acid (1 mL) was added carefully against the wall of the test tube. A yellow colouration of the solution that fades on standing served as an indication of the presence of flavonoids (Borokini and Omotayo, 2012).

4.2.3.2.5. Saponins

The froth test outlined by Odebiyi and Sofowora, (1978) was used to screen for saponins. Aqueous extracts of the plants (0.5 g) and concoctions (0.25 g) were resuspended in 30 mL and 15 mL of tap water respectively. The mixture was vigorously shaken and heated to about 100 °C. The formation of a persistent mass of bubbles, a froth, indicated the presence of saponins.

4.2.3.2.6. Alkaloids

Plant materials and the concoctions (0.2 g) were extracted with 2 mL of 95% ethanol. The ethanol extracts were dried by evaporating the solvent under a stream of air from a bench-top fan. The residue was resuspended in 5 mL of Hydrochloric acid (HCl) followed by addition of 5 drops of Drangendoff's reagent. Following the addition of the indicator, a colour change to orange red indicated the presence of alkaloids. (Harborne, 1973).

4.2.3.2.7. Terpenoids

The Salkowski test described by Borokini and Omotayo, (2012) was employed for the screening of terpenoids. Briefly, 0.5 g of ground plant material and the concoctions were extracted using 5 mL of 95% ethanol and the extracts filtered using a Whatman's No. 1 filter paper and dried. Chloroform (99%) (2 mL) was used to resuspend the extracts followed by the diligent addition of 3 mL of concentrated sulphuric acid that resulted in the development of a layer. A red-brown colouration at the interface of the layer was indicative of the presence of terpenoids.

4.2.3.2.8. Steroids

Acetic anhydride (2 mL) was added to 0.5 g ground plant material and concoctions, followed by the addition of 2 mL of (99%) sulphuric acid. The sample was observed for a colour change to blue-greenish indicated the presence of the steroids (Borokini and Omotayo, 2012).

4.2.3.3. Quantitative phytochemical analysis

The total phenolic, flavonoid and tannin contents of the plants and the concoctions were determined. The choice of the selected phyto-constituents was because the above mentioned phyto-constituents are associated with several key biological activities such as antimicrobial, antioxidant and anti-inflammatory activities (Akindele and Adeyemi, 2007).

4.2.3.3.1. Determination of phenolic content

The quantity of phenolics present in each concoction was determined by the Folin-Ciocalteu reagent method (Humadi and Istudor, 2008) with minor modifications. Ten

microliters of 10 mg/mL of the concoctions and aqueous plant extracts were diluted with 490 μ L of distilled water, followed by the addition of 0.25 mL of Folin-Ciocalteu reagent in each test tube. To stop the reaction, 1.25 mL sodium carbonate (Na_2CO_3) was added and the mixtures were incubated in the dark at room temperature (25 $^\circ\text{C}$) for 30 minutes. An ultraviolet/visible (UV/VIS) spectrophotometer was used to determine the absorbance of the mixtures at 725 nm. A blank and the standard curve were prepared in a similar manner, except that the plant extracts were replaced by distilled water for the blank and various concentration of tannic acid (1.25, 0.63, 0.31, 0.16, 0.08 mg/mL) were prepared for the standard. The results obtained from the linear regression formula of the tannic acid standard curve were expressed as milligram of tannic acid equivalence/gram of extract (mg of TAE/g extract). The experiment was conducted in triplicates and independently repeated three times.

4.2.3.3.2. Total tannin content determination

The Folin-Ciocalteu method described by Tambe and Bhambar, (2014) was used to determine the tannin content in the concoctions. Briefly, 100 μ L of 10 mg/mL of the concoctions and aqueous plant extracts were added to a clean test tube containing 7.5 mL of distilled water. The Folin-Ciocalteu reagent (0.5 mL) was added to the mixture and vortexed. Ten millilitres of a 35% solution of sodium carbonate (Na_2CO_3) was added to the mixture. The mixture in the tube was transferred to a 10 mL volumetric flask and the volume of the mixture made up to 10 mL with distilled water. The mixture was shaken and kept at room temperature (25 $^\circ\text{C}$) for 30 minutes in the dark. Gallic acid was used as a standard and reference standard solutions (1.0, 0.5, 0.25, 0.125, 0.625 mg/mL) were prepared. The absorbance for the solutions was measured at 725 nm using a UV/VIS spectrophotometer against a blank that was prepared in the same manner as the test solutions without adding any extract. Tannin content was expressed as milligram gallic acid equivalence/gram of extract (mg GAE/g extract). The experiment was conducted in triplicates and independently repeated three times.

4.2.3.3.3. Total flavonoid content determination

Total flavonoid content was determined by the aluminium chloride colorimetric assay described by Tambe and Bhambar, (2014). Briefly, 100 μ L of 10 mg/mL of the concoctions and aqueous plant extracts were added to 4.9 mL of distilled water in a

clean test tube. To this reaction mixture, 300 μL of 5% sodium nitrite (NaNO_2) dissolved in distilled water was added and the mixture was left at room temperature (25 $^\circ\text{C}$) for 5 minutes. After the 5 minutes, 300 μL of 10% aluminium chloride (AlCl_3) (dissolved in distilled water) was added to the reaction mixture. The reaction was then allowed to stand for 5 minutes at room temperature, after which 2 mL of 1 M sodium hydroxide (NaOH) was added to the solution. The mixture in the test tube was then made up to 10 mL with distilled water. Quercetin was used as a standard. Different concentrations (500, 250, 125, 62.5, 31.5 $\mu\text{g}/\text{mL}$) of the quercetin were prepared in the same method as the extracts. The absorbance of the experimental samples and the standard were determined using a UV/VIS spectrophotometer at a wavelength of 510 nm. The blank was prepared in the same manner as the experimental and standard samples, however, 100 μL of distilled water was added instead of the extracts. The total flavonoid content of the samples was expressed as milligram quercetin equivalence/ gram of extract (mg QE/g extract). The experiment was conducted in triplicates and independently repeated three times.

4.3. Results

4.3.1. Qualitative phytochemical fingerprint profiles

Thin layer chromatography (TLC) was used to analyse the phytochemical composition of the extracts. Fluorescent compounds on the chromatograms (Figure 4.1–4.4) were observed under ultraviolet (UV) light. Vanillin-sulphuric acid was used to visualise non-fluorescent compounds (Figures 4.5 and 4.6). The plant decoctions and the concoctions had dissimilar profiles which majorly consisted of polar compounds.

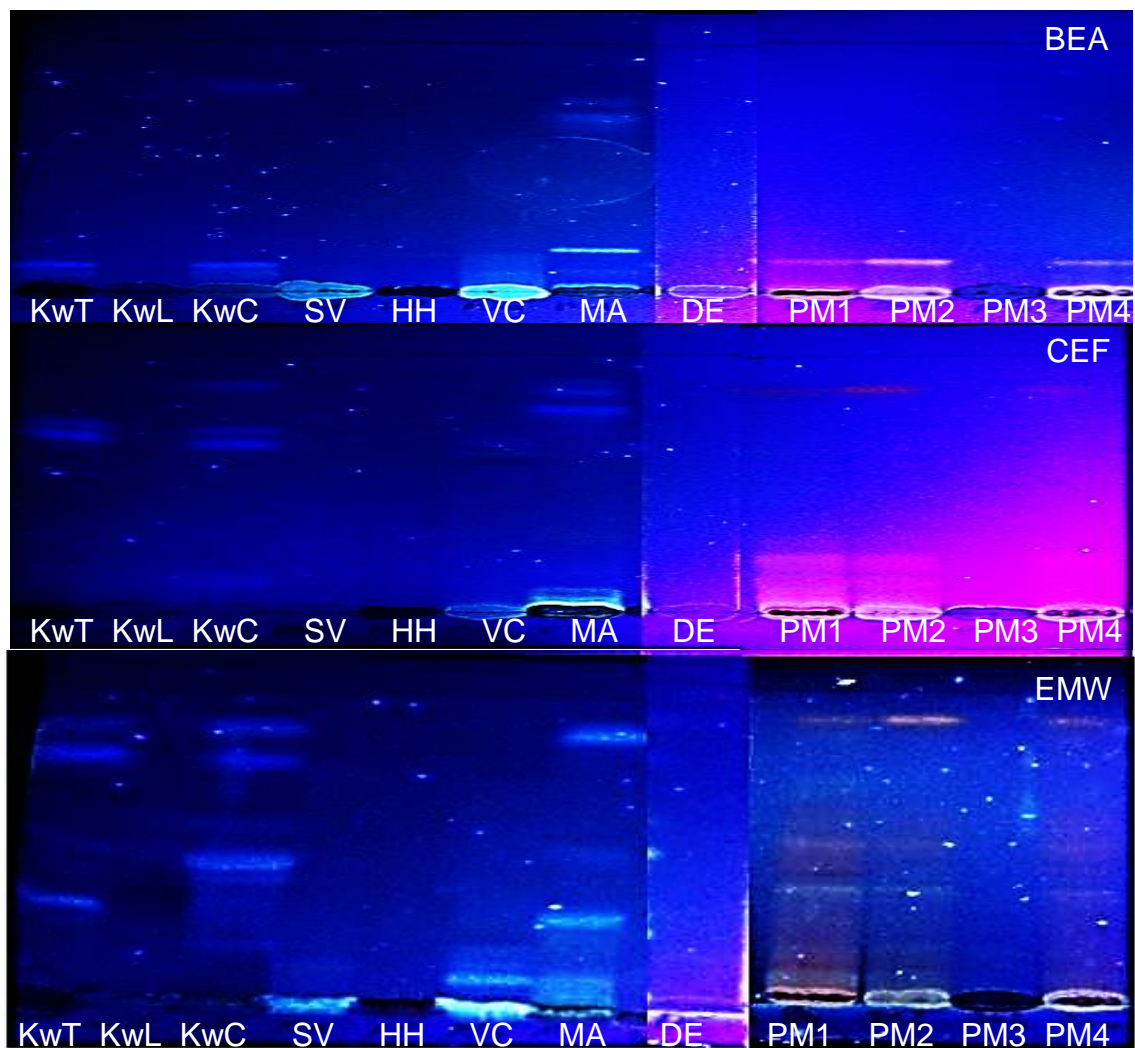


Figure 4.1: Chromatograms showing a variety of 245 nm UV light fluorescent compounds present in the various plant decoctions. The extracts were eluted with non-polar to polar mobile systems.

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tšhikwana* powders.

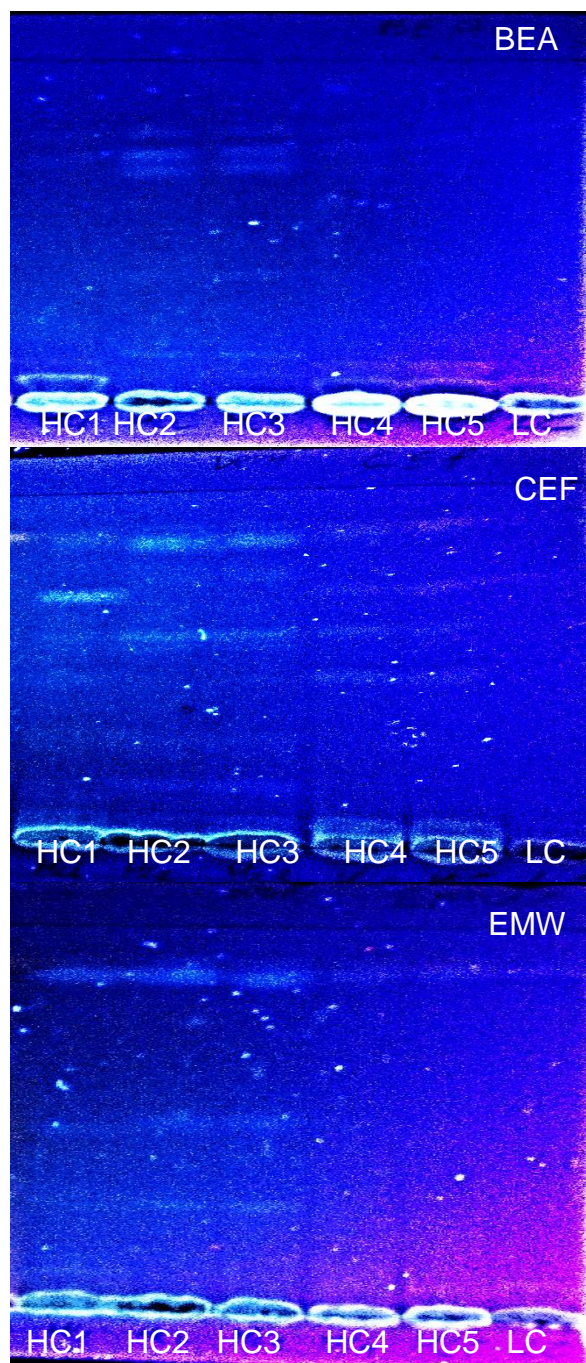


Figure 4.2: The chromatograms of the concoctions were developed with BEA, CEF and EMW. UV light of 245 nm was used to visualise fluorescent compounds.

Key: HC1: concoction 1; HC2: concoction 2; HC3: concoction 3; HC4: concoction 4; HC5: concoction 5; LC: Lab standard

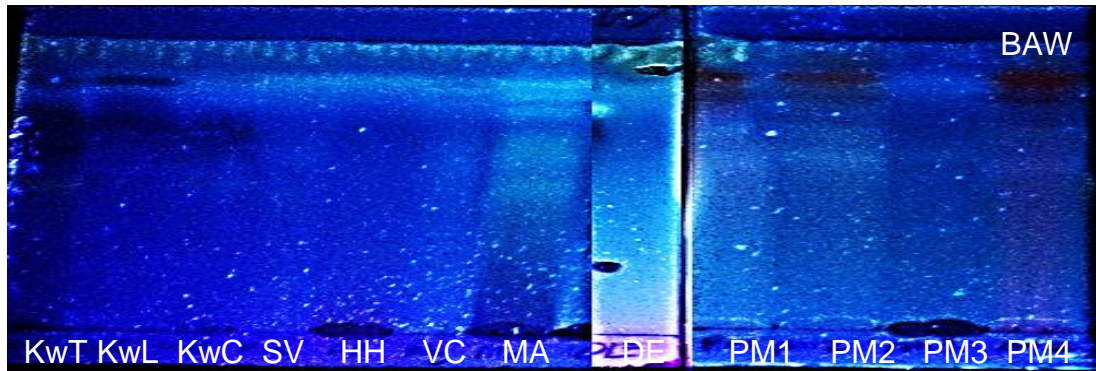


Figure 4.3: Chromatogram showing compounds in the plant decoctions that fluoresced at 245 nm UV light. The extremely polar mobile phase (BAW) was used to develop the profiles.

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

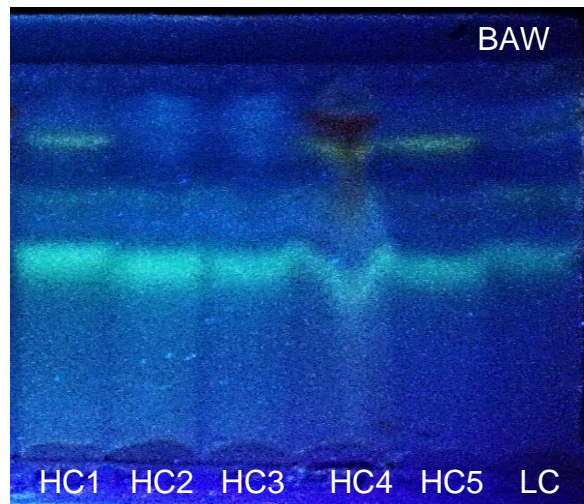


Figure 4.4: Chromatogram developed with polar BAW mobile system, shows numerous distinct polar compounds in the concoctions that fluoresced under 245 nm UV light.

Key: HC1: concoction 1, HC2: concoction 2, HC3: concoction 3, HC4: concoction 4, HC5: concoction 5, LC: Lab standard

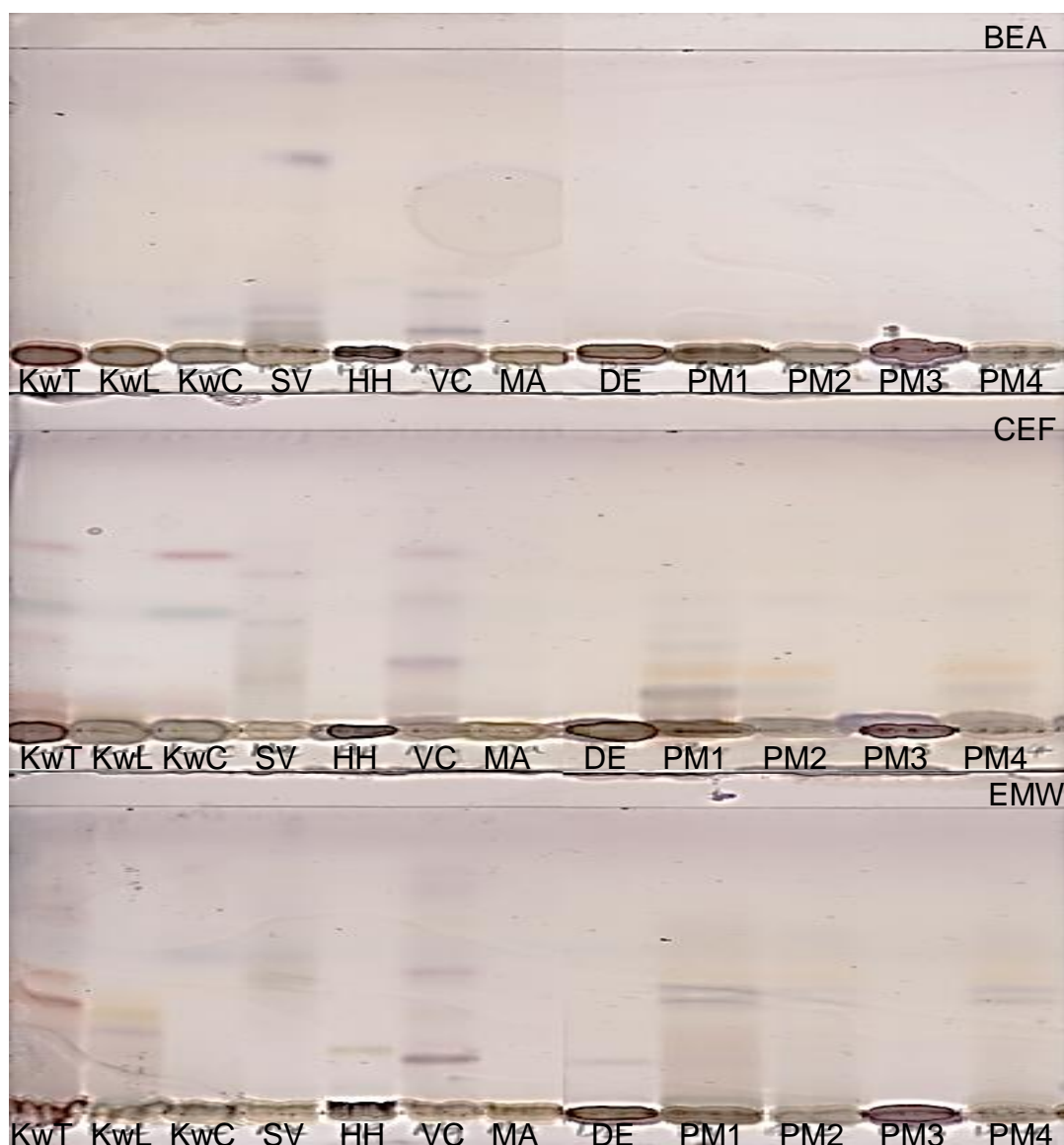


Figure 4.5: Chromatograms of plant decoctions showing various coloured compounds that reacted with vanillin-sulphuric acid reagent. Different colours developed after heating the sprayed chromatograms at 110 °C.

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

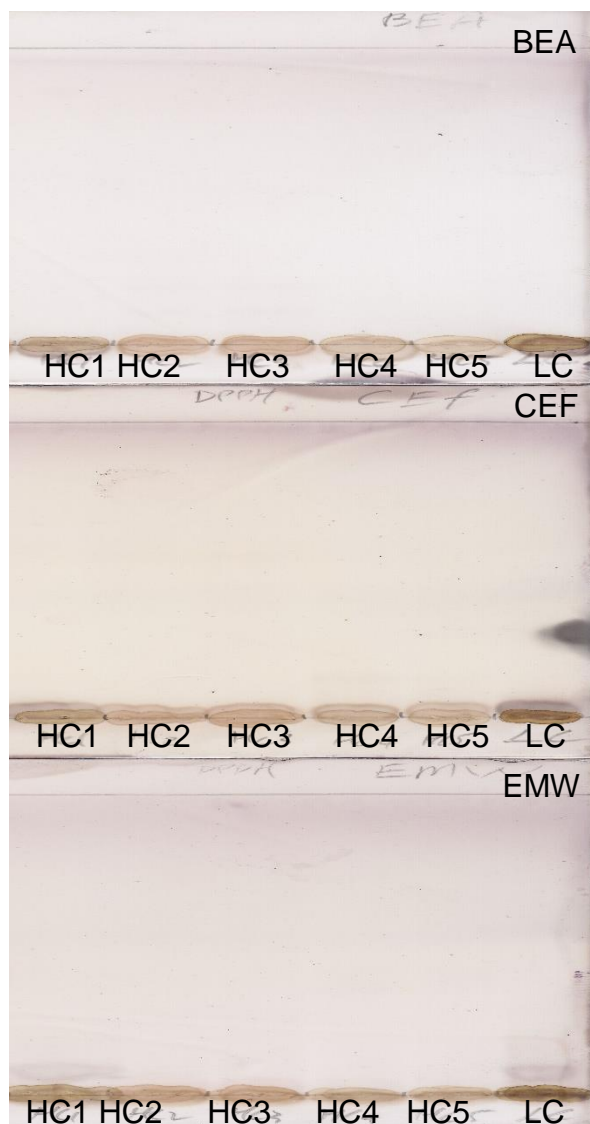


Figure 4.6: Chromatograms loaded with the concoctions were developed in various mobile systems (BEA, CEF and EMW) and sprayed with vanillin-sulphuric acid reagent.

Key: HC1: concoction 1; HC2: concoction 2; HC3: concoction 3; HC4: concoction 4; HC5: concoction 5; LC: laboratory standard.

4.3.2. Qualitative phyto-constituent screening

Phyto-constituents present within both the plant decoctions and concoctions were screened using standard chemical tests. Tannins and steroids were present in the all the extracts. The different *Tshikwana* powders varied in their chemical compositions (Table 4.2). The concoctions possessed all the tested phyto-constituents except for the phlobatannins which were also absent in the plant decoctions (Table 4.3).

Table 4.2: Phyto-constituents present in the decoctions of plant species used to prepare the herbal concoctions

Phyto-constituent	Plant species											
	KwT	KwL	KwC	SV	HH	VC	MA	DE	PM1	PM2	PM3	PM4
Terpenoids	+	+	+	+	+	+	+	+	+	-	-	-
Alkaloids	-	-	-	-	-	+	-	-	-	+	-	-
Saponins	+	-	+	-	+	+	-	+	+	+	+	+
Flavonoids	+	+	+	+	-	+	-	-	+	+	-	-
Phlabatannins	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	+	+	+	-	+	+	+	+	+	-	+	+
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+	+	+

Key: (+): present, (-): absent, Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

Table 4.3: Various phyto-constituents present in herbal concoctions

Phyto-constituents	HC1	HC2	HC3	HC4	HC5	LC
Terpenoids	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Phlbatannins	-	-	-	-	-	-
Cardiac glycosides	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+

Key: (+): present, (-): absent, HC1: concoction 1; HC2: concoction 2; HC3: concoction 3; HC4: concoction 4; HC5: concoction 5; LC: laboratory standard.

4.3.3. Quantitative phytochemical analysis

Detectable concentrations of phyto-constituents provide a preliminary indication of possible pharmacological effects of the extracts. *K. wilmsii* twigs contained the most total phenolics, “*Tšhikwana*” 1 possessed most flavonoids while tannins were most abundant in *V. capensis* decoction (Table 4.4). Concoction 1 and 4 contained higher total phenolic content than the other concoctions (Table 4.5)

Table 4.4: Quantified total phenolic, flavonoid and tannin content of the plant decoctions

Plant species	Phenolic content (mg TAE/g extract)	Flavonoids (mg QE/g extract)	Tannins (mg GAE/ g extract)
<i>K. wilmsii</i> (leaves)	61.51 ± 0.07	1.68 ± 0.12	16.62 ± 0.28
<i>V. capensis</i>	53.65 ± 0.08	0.46 ± 0.01	47.02 ± 2.57
<i>S. viminalis</i>	52.84 ± 0.05	0.01 ± 0.04	3.85 ± 0.16
<i>K. wilmsii</i> (Twigs)	88.84 ± 0.11	4.90 ± 0.09	22.76 ± 0.22
<i>H. hemerocallidea</i>	65.69 ± 0.01	4.57 ± 0.21	8.08 ± 0.31
<i>k. wilmsii</i> (Corm)	63.06 ± 0.08	1.45 ± 0.10	6.85 ± 0.09
<i>D. elata</i>	53.29 ± 0.06	1.19 ± 1.36	6.97 ± 0.87
<i>M. angustifolia</i>	66.84 ± 0.06	4.22 ± 0.05	8.21 ± 0.13
<i>Tšhikwana</i> 1	50.18 ± 0.11	9.44 ± 0.49	16.75 ± 0.16
<i>Tšhikwana</i> 2	50.83 ± 0.06	1.23 ± 0.07	8.28 ± 0.09
<i>Tšhikwana</i> 3	63.16 ± 0.15	3.70 ± 0.05	8.46 ± 0.04
<i>Tšhikwana</i> 4	52.5 ± 0.19	1.94 ± 0.20	-

Key: (-): No detectable concentration; mg TAE/g: milligram tannic acid equivalence per gram of plant extract; mg QE/g: milligram quercetin equivalence/gram of plant extract; milligram gallic acid equivalence/ gram of plant extract. All values are presented as mean of triplicates ± standard deviation (SD) (n=3).

Table 4.5: Concentrations of phenolics, flavonoids and tannins present in the herbal concoctions

Herbal concoctions	Phenolic content (mg TAE/g extract)	Flavonoid content (mg QE/g extract)	Tannin content (mg GAE/ g extract)
Concoction 1	65.551 ± 0.164	1.561 ± 0.036	7.754 ± 0.094
Concoction 2	61.677 ± 0.064	2.402 ± 0.076	9.567 ± 0.142
Concoction 3	52.636 ± 0.031	0.536 ± 0.024	4.505 ± 0.217
Concoction 4	64.257 ± 0.187	1.841 ± 0.002	3.120 ± 0.062
Concoction 5	55.761 ± 0.026	1.754 ± 0.024	7.175 ± 0.155
Lab concoction	60.970 ± 0.103	1.416 ± 0.036	28.858 ± 0.471

Key: mg TAE/g: milligram tannic acid equivalence per gram of plant extract; mg QE/g: milligram quercetin equivalence/ gram of plant extract; milligram gallic acid equivalence/ gram of plant extract. Values are mean of triplicates ± standard deviation (SD) (n=3).

4.4. Discussion

Plants produce a diverse assortment of phytochemicals that are essential towards their development and endurance in the environment. Associated biological activities of these compounds have enabled plants to be used as therapeutic agents against various ailments. The plants analysed in this study were selected because their ethnopharmacological applications involved their use by traditional healers at Ga Maja to prepare herbal concoctions that were claimed to have blood-cleansing, detoxifying, antidiarrheal, anti-Human immune-deficiency virus (HIV) and pain-relieving properties.

Thin layer chromatography (TLC) was used to examine the phytochemical profiles of both the plant decoctions and the herbal concoctions. In addition, TLC was used to assess the chemical purity of the herbal concoctions. The TLC plates were loaded with the crude extracts and developed using mobile phases of varying polarity, mainly BEA (non-polar), CEF (intermediate), EMW (polar) and BAW (extreme). The latter approach was taken because by using the different mobile phases, one is able to separate and observe the chemistry of the majority of compounds in the crude extracts. Thin layer chromatography separates the compounds in a mixture by size through the matrix of the stationary phase (silica). This meant that smaller compact compounds were able to migrate longer distances and than larger molecules.

The compounds separated on the chromatograms were visualised under ultraviolet (UV) light at 254 nm wavelength. Under UV light, electrons in compounds transition from a ground state to an excited state. When the electrons de-excite to the ground state, they release energy in the form of an electromagnetic radiation. The wavelength of the electromagnetic radiation has a lower energy (within the visible spectrum) than UV radiation. The latter results in structurally diverse compounds to fluoresce and be visualised in different colours.

The different colours shaped as linear bands in Figures 4.1–4.4 represent the separated compounds in the crude extracts. Therefore, the number of coloured bands on a chromatogram is representative of the number of compounds present in the extracts. Moreover, the chromatogram in which the compounds are observed is representative of their polarities. The decoctions of the plants contained a larger number of polar compounds because many of them were visible in the chromatogram developed with the polar mobile phase (EMW) (Figure 4.1). Several other compounds

were mostly visible in the intermediate polar-developed chromatogram (CEF). The non-polar BEA developed chromatogram exhibited the least number of compounds. This observed trend suggested that water extracted particularly polar compounds from the plant material. Water has an extreme polarity and as such, it was expected to extract majority of polar compounds and discriminate against non-polar compounds. Masoko *et al.* (2008) also reported the same principle.

The fluorescence of the separated compounds at 245 nm UV light indicated that on their chemical structure, the compounds consisted of conjugated double bonds and/or extended pi (π) electron configurations (Ahmed *et al.*, 2014). Polyphenols have been reported to have lengthy conjugated aromatic systems in their chemical structures (Dai and Mumper, 2010). Therefore, although not entirely exclusive, the various fluorescing compounds in figures 4.1-4.4 could be due to the presence of polyphenolic compounds and/or their analogues. In addition, phenolics consist of polar hydroxyl functional group(s) (Dai and Mumper, 2010), hence their high concentrations in the water samples was a reasonable outcome.

Majority of the compounds in the concoctions were distributed in the chromatogram developed with the intermediate mobile system (CEF) followed by the EMW and BEA developed chromatograms (Figure 4.2). Although, the EMW was a good mobile phase to resolve compounds in the plant decoctions it was not a good solvent system for the concoctions because most of the compounds in the crude extract had not migrated from the spotting region on the chromatogram. Therefore, an extremely polar solvent system was selected to better resolve the phytochemicals in the concoctions and compare them with those of the plants.

The phytochemical profiles established using the polar solvent system (BAW) also demonstrated that the profiles of the plant decoctions and the herbal concoctions were different (Figure 4.3 and 4.4). For the plant decoctions the phytochemicals aggregated at the solvent front. There was good separation of compounds from the concoctions. This further showed the differences in the chemistry of the compounds in the concoctions and the plant extracts.

In the preparation of the plant decoctions, the individual dried material for each plant species was added to a specified volume of boiling water and boiled for 5 minutes. The same was done for the concoctions whereby, weighed amounts of all the dried

material of the plant species were collectively added to a specified volume of boiling water and also boiled for 5 minutes. Since the manner of preparation of the samples was similar, the differences in the chemical profiles of the herbal concoctions and the plant decoctions, could be attributed to chemical reactions that occurred when all the plant species were added altogether to make the concoction. These reactions could have also resulted in the synthesis of different compounds. The potential biological activities of the herbal concoctions and the plant decoctions may be a result of heat tolerant phytochemicals. The plant material is boiled at 100 °C, therefore, a large number of volatile compounds evaporated or were destroyed during preparation of the extracts.

Herbal concoctions 1, 2 and 3 shared a closely related phytochemical profile (Figure 4.4). The same was observed for concoction 4, 5 and the laboratory standard. This indicated that the traders indeed used similar plant species to prepare the herbal concoctions. They may also share common knowledge regarding the pharmacological properties of plants around their environment.

The chromatograms were sprayed with vanillin-sulphuric acid reagent. The sprayed chromatograms were heated at 110 °C until sufficient colour development. Numerous structurally diverse compounds in the plant decoctions were observed in the EMW developed chromatogram, followed by the CEF and the BEA chromatograms (Figure 4.5). The visible compounds on the chromatograms have steroidal chemical structures because the vanillin-sulphuric reagent detects steroidal compounds (Taganna *et al.*, 2011). Moreover, other chemical classes were detected, such as terpenoids (purple or bluish-purple bands), flavonoids (pinkish, yellow or orange) and proanthocyanidins (pink) (Taganna *et al.*, 2011; Ahmed *et al.*, 2014). The compounds in the concoctions did not react with vanillin-sulphuric acid, as such, no bands were observed on their chromatograms (Figure 4.6).

Thin layer chromatography was an efficient tool to help better understand the phytochemical profiles of the plants and the concoctions and to further establish their relationship. Unfortunately, this technique could not provide sufficient identification of the types of phyto-constituents the bands represented. Therefore, various chemical tests were conducted to screen for the various classes of phyto-constituents present in both the herbal concoctions and the plants decoctions.

Terpenoids have been reported to have been applied in the treatment of microbial infections (Krzyzanowska *et al.*, 2010). Flavonoids and tannins have been documented to possess antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties (Krzyzanowska *et al.*, 2010). The biological activities associated with saponins include anti-inflammatory, antimicrobial and cytotoxic effects (Sarikurkcu and Tepe, 2015). The presence of such compounds in the water extracts could be the basis for the pharmacological effects claimed by the traders.

There were variations in the phyto-constituents amongst the different plant species. However, tannins and steroids were detected in all the plant decoctions (Table 4.2). The herbal concoctions possessed all the tested phyto-constituents (Table 4.3) except for the phlobatannins which were also absent in the plant decoctions. All the plant extracts had no phlobatannins and therefore could not be contributed to the concoctions.

The screening of the types of phyto-constituents has the potential to provide valuable information of the types of bioactive compounds that could result in potential human clinical effects. Phenolics are classified into various groups such as the non-soluble compounds which include condensed tannins, lignins, and hydroxycinnamic acids, and soluble phenolics such as phenolic acids, flavonoids and quinones (Krzyzanowska *et al.*, 2010).

The twigs of *Kirkia wilmsii* (KwT) had the greatest amount of total phenolics followed by *Hypoxis hemerocallidea* (corm) and *Monsonia angustifolia* (leaves). The flavonoids were highest in “*Tšhikwana*” 1 (PM1), followed by the twigs of *Kirkia wilmsii* and *Hypoxis hemerocallidea* (corm). Tannin content was greatest in the twigs of *Kirkia wilmsii*, followed by “*Tšhikwana*” 1 (PM1) and the leaves of *kirkia wilmsii* (KwL) (Table 4.4).

The herbal concoctions generally had high content of total phenolics, flavonoids and tannins (Table 4.5). It could be that the plants used in their preparation, additively deposited a certain amount of the constituents they possessed. Although the concoctions demonstrated similar phytochemical profiles (Figure 4.4), they varied in the concentrations of polyphenols. These variations suggested that although similar plants may have been used in their preparation (Table 4.1), the amount of each species added may have been different and unique to each trader.

Higher extraction temperatures were reported to enhance solubility and mass transfer rate of phytochemicals into a solvent. In addition, under such conditions, the solvent is able to penetrate the cell membrane and matrices which improves the rate of extraction (Dai and Mumper, 2010). Henceforth, the choice by the traders to use boiling water as an extractant enables them to obtain considerable concentrations of bioactive compounds (phenolics, flavonoids and tannins) across the different parts of the various plant species. The presence of these different phyto-constituents in the concoctions and the plants specifies a host of potential beneficial ways they can be used to improve health.

Conclusion

The herbal concoctions had similar phytochemical profiles and suggested that the traders generally used the same plants for their preparation. However, when compared to those of the plant species, they were distinctly different. This difference could be brought about because of possible chemical reactions that may have occurred during the preparation of the herbal concoctions or degradation of compounds overtime due to either light or solvent exposure. Regarding the interactions, they may be synergistic, additive or antagonistic towards a biological activity. This chemical diversity of the concoctions and the individual plant extracts therefore prompts an investigation to understand the effect these chemicals have on the biological activity.

The different phyto-constituents present in the plant extract and the concoctions has basis for their use in various health conditions. Therefore, the claimed pharmacological effects of these medicines need to also be investigated to validate their use towards the indicated ailments.

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CHAPTER 5

5. Antioxidant potential of the herbal concoctions and their individual plant constituents

5.1. Introduction

The herbal concoctions in this study were claimed to have therapeutic benefits of blood-cleansing, immune-boosting, act as an antidiarrheal and used for pain-relief. Therefore, to investigate such claims, the antioxidant potential of the herbal concoctions and the plant decoctions was determined.

Free radicals such as the hydroxyl radical, superoxide anion radical, hydrogen peroxide, singlet oxygen and nitric oxide are extremely reactive chemical species that are produced by either normal biochemical processes or by diseases and environmental factors. These chemical species can participate in chain reactions with cellular membrane lipids resulting in chain reactions that lead to lipid peroxidation and cellular damage. These oxidants are also capable of oxidising nucleic acids, proteins, enzymes and other small molecules in the body (Shivaprasad *et al.*, 2005). The deleterious chain reactions of free radicals are associated with inflammation. The recognition of cellular damage stimulates inflammatory responses that lead to the production of various mediators that are involved in the regulation of pain (Govindappa *et al.*, 2011).

Antioxidants are chemical substances that confer protection against the action of free radicals. They can scavenge them and inhibit their cell-damaging oxidising chain reactions (Pietta, 2000). In addition, antioxidants can further act as reducing agents and can also be complexes that readily bind to pro-oxidant metals and are capable of quenching singlet oxygen formation (Pietta, 2000). Epidemiological studies have reported a link between the treatments of serious health conditions with the intake of antioxidants (Frie, 1995; Halliwell, 1997; Liu, 2003).

The use of synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, and tertiary butyl-hydro-quinone has encountered disapproval. This response arises from their reported low solubilities, moderate antioxidant activity, toxicity and associated carcinogenic effects (Jayaprakasha *et al.*,

2003; Ghafar *et al.*, 2010). Therefore, novel and more potent natural antioxidants that are safe to use by humans are needed and their properties need to be studied.

Plants are a rich source of free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, lignins, tannins, flavonoids, quinones, coumarins, alkaloids, amines, and other metabolites which are rich in antioxidant activity (Aiyegoro and Okoh, 2010). Furthermore, the antioxidant effects of a large number of polyphenols has been associated with anti-inflammatory activities and have been used in the treatment of diabetes, cancer and capillary fragility (Lamson *et al.*, 2000; Thring *et al.*, 2011).

The phytochemicals mentioned above are structurally diverse and because of this, their modes of antioxidant activity may intersect, be similar or different. That is, the chemical interactions of these myriad of compounds may lead to additive, synergistic, or antagonistic effects toward a therapeutic action (Wang *et al.*, 2011).

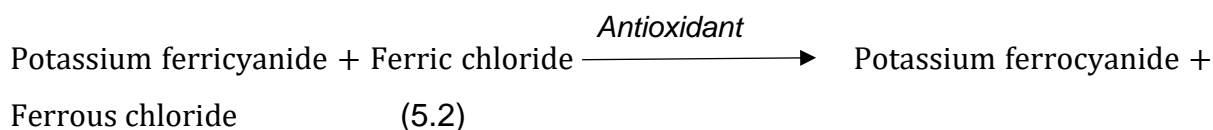
5.2. Methodology

5.2.1. Qualitative free radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a stable free radical due to its unpaired electron delocalization over the whole molecule. The donation of a proton (H^+) to this radical makes a colour change from violet to pale yellow in a solution (Figure 5.1). During this conversion of colour, a proportionate decrease in absorbance at 517 nm occurs (Irshad *et al.*, 2012).

Thin layer chromatography (TLC) sprayed with DPPH was used to screen for possible antioxidant compounds in the concoctions and the plant decoctions. The chromatograms were prepared and developed identical to phytochemical fingerprinting (Section 4.2.3.1). DPPH solution (0.2% w/v) was prepared by dissolving 0.2 g of the DPPH (Sigma) in 100 mL of methanol. This solution was sprayed onto the air-dried chromatograms. The presence of antioxidant activity was indicated by the development of yellow bands against a purple background (Deby and Margotteaux, 1970).

and blue depending on the reducing power of each extract. Higher absorbance values indicate good reducing activity. Therefore, measuring the formation of Pearl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration.



Five different concentrations of the samples (625 – 39 $\mu\text{g}/\text{mL}$) were prepared by serially diluting a stock solution of 1 250 $\mu\text{g}/\text{mL}$. The different concentrations (2.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of (1% w/v in distilled water) potassium ferricyanide (Rochelle) in test tubes respectively. The mixtures were vortexed after addition of solutions. The mixtures were incubated at 50 °C for 20 minutes. Two millilitres of trichloroacetic acid (Sigma) (10% w/v in distilled water) was added to the test tubes after incubation. The mixtures were centrifuged at 3000 rpm for 10 minutes and 5 mL of the resulting supernatant was transferred to a clean test tube. To this solution, 5 mL of distilled water and 1 mL ferric chloride (0, 1% w/v in distilled water) were added consecutively with thorough vortexing after each addition. A UV/VIS spectrophotometer was used to read the absorbance of solutions at 700 nm wavelength. The blank for this procedure was prepared in the same manner, however, the extracts were replaced by an equal amount of distilled water. L-Ascorbic acid (Sigma) (625–39 $\mu\text{g}/\text{mL}$) was used as a positive control and was prepared similar to the concoctions. The experiment was conducted in triplicates and independently repeated three times.

5.2.4. Synergistic, additive and antagonistic interactions

The effects of the combination of structurally different bioactive phytochemicals from the various plant decoctions were studied. The combinational effects were studied because to prepare the concoctions, the plants were mixed together in a solution. The same *in vitro* methods as described in Sections 5.2.2 and 5.2.3 were used to assess the combinational effects of the plant decoctions on antioxidant activity. The ratio used for each mixture was a 1:1 of the extracts.

The fractional inhibitory concentration (FIC) of each plant extract was calculated in order to determine types of chemical interactions that occur when they are mixed. The method by van Vuuren and Vijoer, (2011) for determination of FIC values was

adopted. However, this method was modified to optimise the analysis of the extract combinations towards antioxidant activity. The FIC value for each extract in a combination was calculated by dividing the EC₅₀ value of the combination by the EC₅₀ value of each plant decoction placed in the combination (equation 5.3). The fractional inhibitory index ($\sum FIC$) was then calculated by adding the two FIC values of the plant extracts in a combination (equation 5.4).

The interpretations of the fractional inhibitory index to explain the effect of the mixture of the decoctions were adopted from van Vuuren and Vijoën, (2011) as detailed in Table 5.1.

$$FIC = \frac{EC_{50} (combination\ a,b)}{EC_{50} (a)} \quad (5.3)$$

$$FIC\ index = \sum FIC = FICA + FICB \quad (5.4)$$

Where EC₅₀ is the concentration of the extract that inhibits at least 50% of the DPPH free radicals from a DPPH solution. FICA is the FIC for the first extract in the combination and FICB is of the second extract.

Table 5. 1: The definitions on the interpretation of the combinational effects of plant extracts at a 1:1 ratio

FIC index value	Outcome of combination
$\sum FIC \leq 0.5$	Synergistic
$\sum FIC > 0.5 - 1.00$	Additive
$\sum FIC > 1.00 - \leq 4.00.$	Indifferent
$\sum FIC > 4.00$	Antagonistic

Key: $\sum FIC$: Fractional inhibitory index

Additive interactions occur when the therapeutic effect is of the sum of the individual components. The synergistic effects of a combination refer to when the therapeutic effect of the combination is greater than that of the individual constituents. Antagonistic interactions mainly refer to chemical interactions which result in the reduction or loss of a biological activity compared to the individual phyto-constituents (Wang *et al.*, 2011). Indifferent interactions indicate the non-interactive reaction of a combination

that results in neither an additive nor antagonistic effect (van Vuuren and Viljoen, 2011).

5.3. Results

5.3.1. Qualitative DPPH assay on TLC

The screening of antioxidant compounds in the plant decoctions and concoctions was conducted using DPPH. Yellow zones on the chromatograms against a purple background (Figure 5.2 and 5.3) indicated antioxidant compounds. Numerous antioxidant compounds from *K. wilmsii* (Figure 5.2) had good separation on polar (EMW) and intermediate (CEF) chromatograms. All the concoctions did not possess discrete free radical scavenging compounds (Figure 5.3)



Figure 5. 2: Antioxidant screening of the phytochemicals present in the different plant decoctions. The chromatograms were developed in BEA, CEF and EMW mobile systems. The yellow colour is an indication of antioxidant activity

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

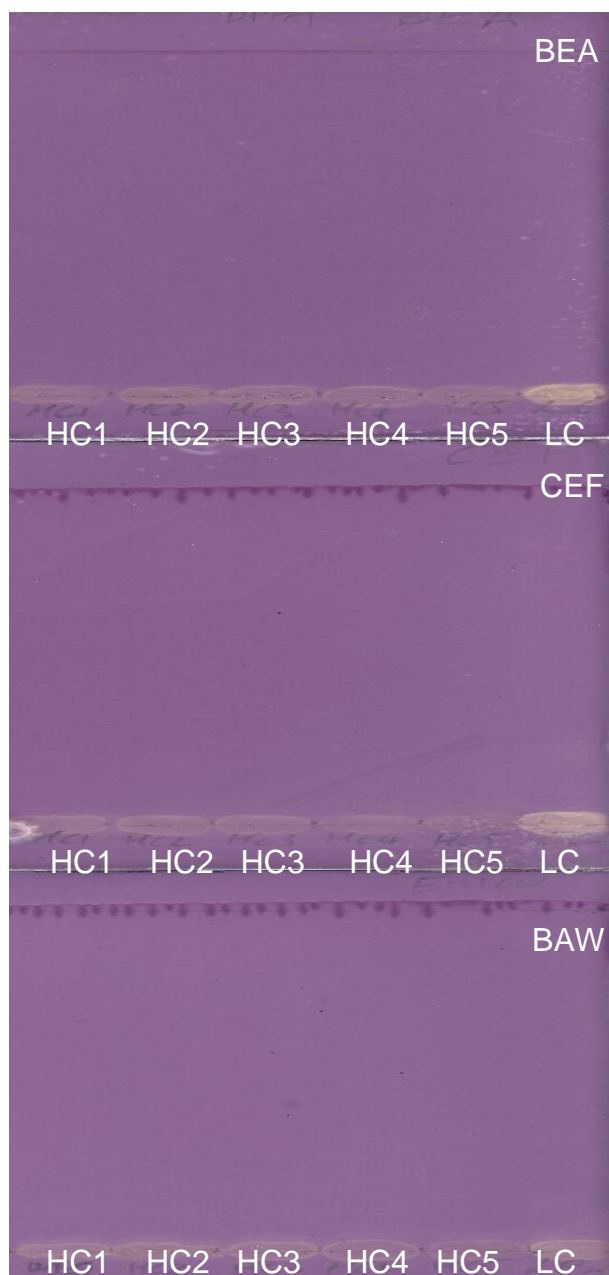


Figure 5.3: Antioxidant screening of the herbal concoctions. DPPH was used as a free radical. The chromatograms were developed in the BEA, CEF and BAW.

Key: HC1: concoction 1, HC2: concoction 2, HC3: concoction 3, HC4: concoction 4, HC5: concoction 5, LC: Lab standard

5.3.2. Quantification of Antioxidant activity

The antioxidant activity of the herbal concoctions and the plant decoctions was quantified using two assays. The free radical scavenging assay determined the proton (H^+) donating ability of the extracts to the radical DPPH. The ferric reducing power assay was used to determine the electron (e^-) donating ability of the extracts to reduce Fe^{3+} to Fe^{2+} .

5.3.2.1. Free radical scavenging activity assay

DPPH was used to determine the antioxidant potential of the plant extracts and the herbal concoctions. Tšhikwana 2 and *V. capensis* had the lowest activity (Figure 5.4a). *K. wilmsii* and *M. angustifolia* had comparable activity to L-ascorbic acid standard (Figure 5.4b). Of all the herbal concoctions, concoction 1 had the highest free radical scavenging activity (Figure 5.5). Ascorbic acid was the most potent.

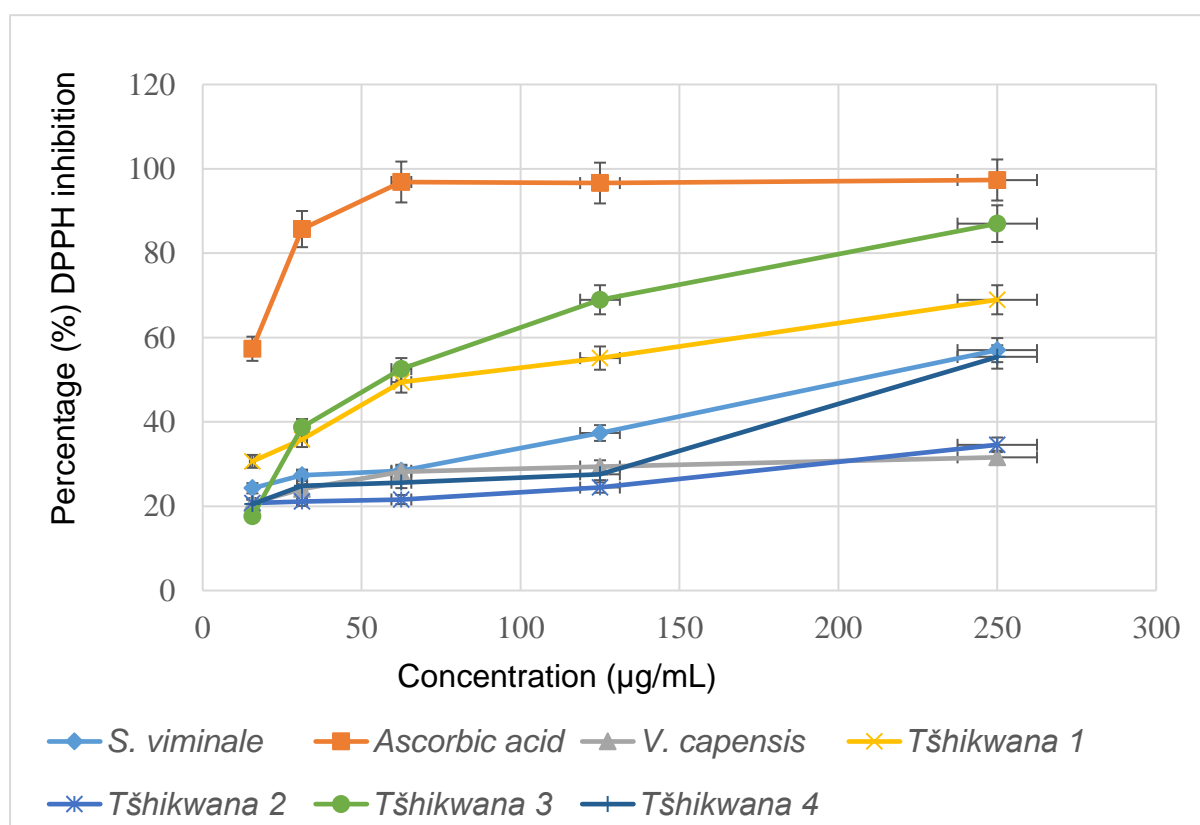


Figure 5.4a: Percentage free radical (DPPH) inhibition by plants and the “Tšhikwana” mixtures. L-ascorbic acid was used as a standard to which the activity of the extracts was compared. Values used are means of triplicates \pm standard deviation.

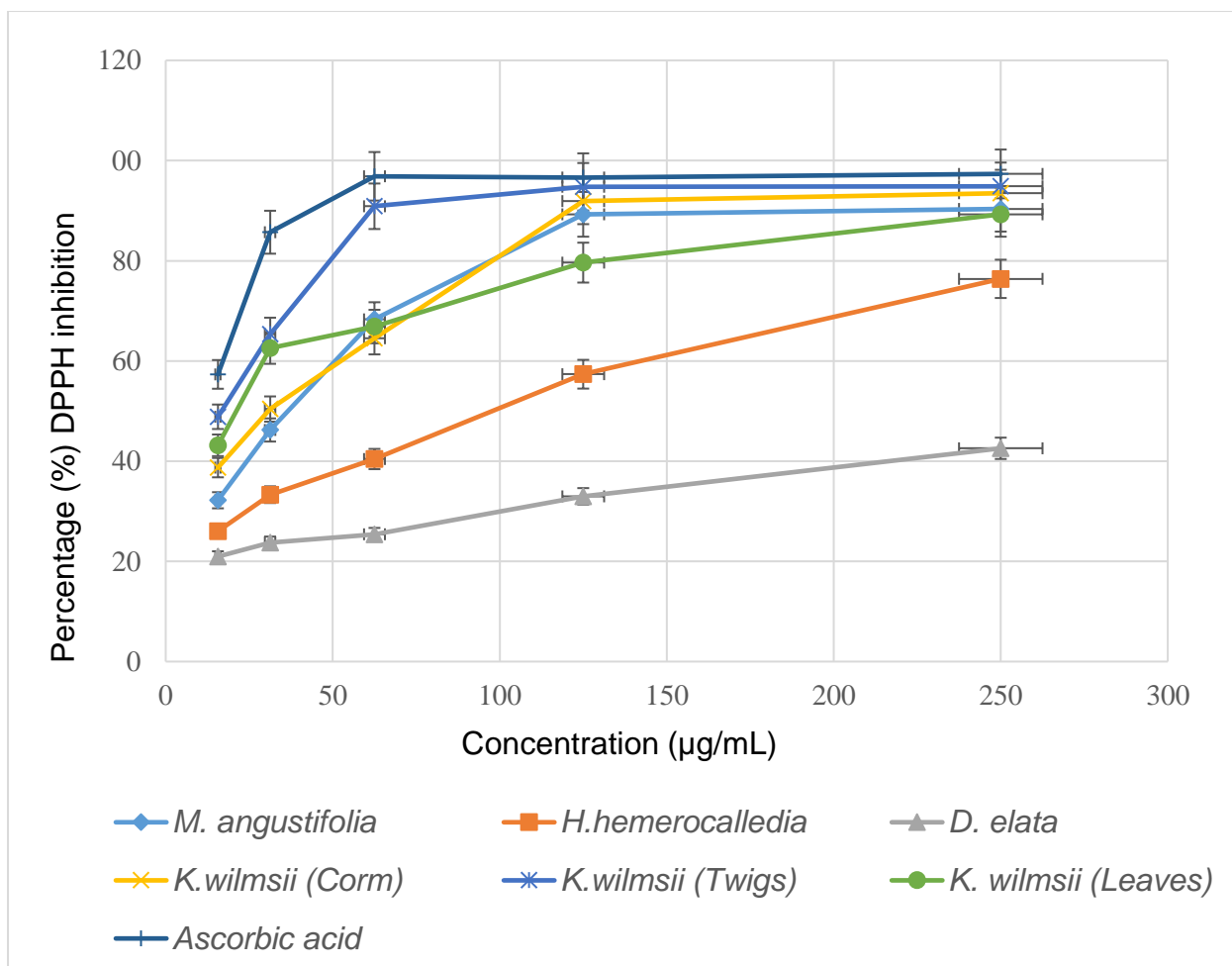


Figure 5.4b: DPPH inhibition of plant species with higher free radical scavenging activity. Values used are means of triplicates \pm standard deviation

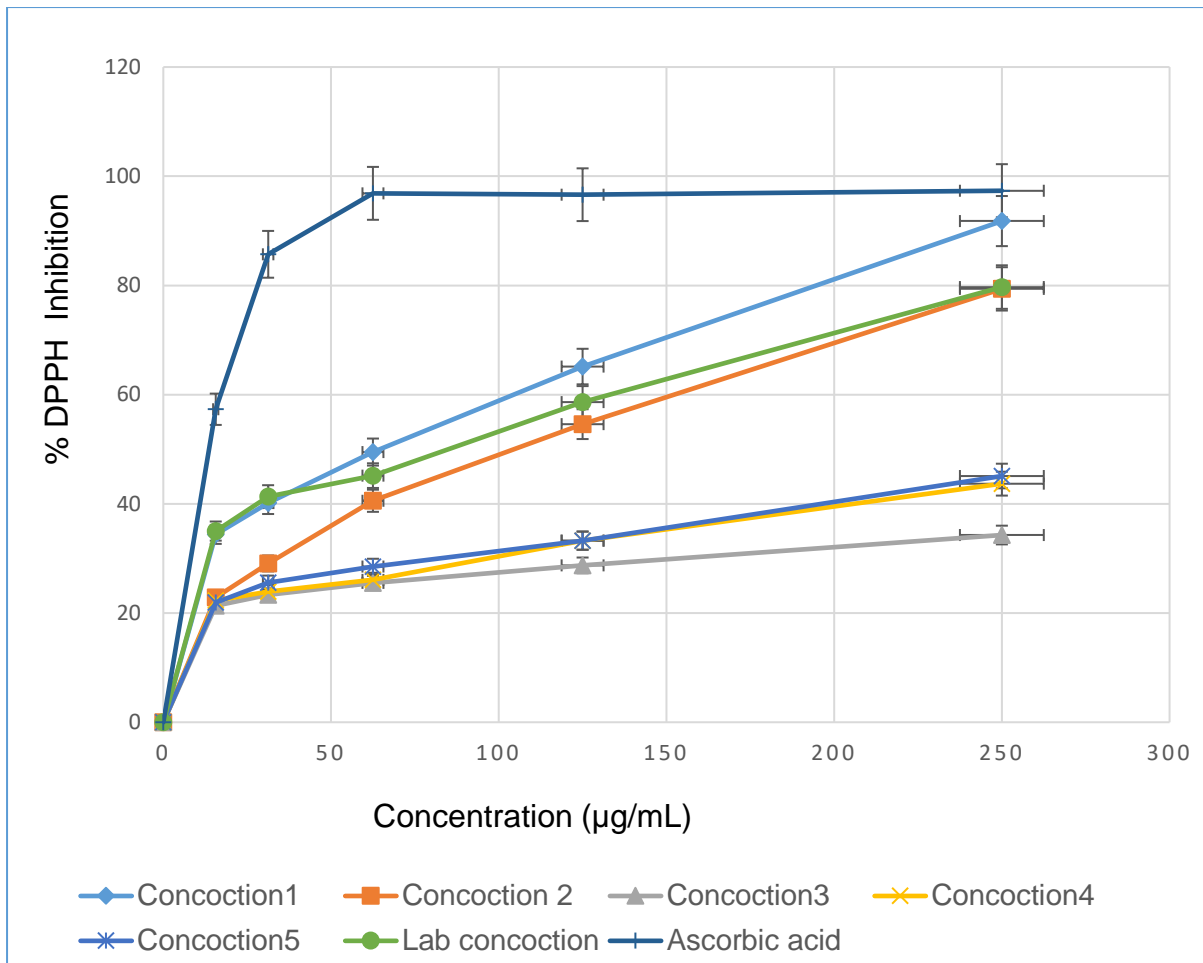


Figure 5. 5: Free radical (DPPH) scavenging activity of the herbal concoctions at different concentrations expressed as percentage inhibition. L-Ascorbic acid was used as a standard for comparison of activity with the concoctions.

5.3.2.2 Half maximal effective concentrations (EC₅₀) towards DPPH inhibition

The antioxidant activity of the herbal concoctions and the plant decoctions was further quantified and assessed by determining the concentrations of the extracts that was required to inhibit 50% of the DPPH control solution. Lower EC₅₀ values indicate good scavenging activity. The twigs of *K. wilmsii* had the best antioxidant activity (Table 5.2). Of all the herbal concoctions, concoction 1 had the highest free radical scavenging activity (Table 5.3).

Table 5. 2: Half maximal effective DPPH scavenging concentration (EC₅₀) of the plant decoctions.

Samples	EC₅₀* (µg/mL)
<i>M. angustifolia</i>	40.87 ± 0.03
<i>H. hemerocalledia</i>	112.19 ± 0.78
<i>D. elata</i>	329.04 ± 2.12
<i>K. wilmsii</i> (Corm)	34.45 ± 0.32
<i>K. wilmsii</i> (Twigs)	15.71 ± 0.25
<i>K. wilmsii</i> (Leaves)	18.50 ± 0.24
<i>S. viminalis</i>	207.25 ± 4.78
<i>V. capensis</i>	203.47 ± 4.74
"Tšhikwana" 1	109.67 ± 0.15
"Tšhikwana" 2	535.91 ± 0.37
"Tšhikwana" 3	85.85 ± 0.14
"Tšhikwana" 4	237.53 ± 0.21
Control	
Ascorbic acid	4.87 ± 0.03

*Values used are means of triplicates ± standard deviation

Table 5. 3: Half maximal effective DPPH scavenging concentration (EC₅₀) of the herbal concoctions

Samples	EC50* (µg/mL)
Concoction 1	71.33 ± 0.47
Concoction 2	117.02 ± 0.73
Concoction 3	586.37 ± 2.45
Concoction 4	320.45 ± 15.21
Concoction 5	294.72 ± 0.30
Laboratory concoction	86.4 ± 0.75

*Values used are means of triplicates ± standard deviation.

5.3.2.3. Ferric reducing power of the plants and the herbal concoctions

The ferric ion reducing power was used to elucidate another alternative mechanism of antioxidant activity of the plants and the concoctions. Tšhikwana 1 and 2 had the lowest activity (Figure 5.6a). *K. wilmsii* twigs had comparable reducing power with L-ascorbic acid (Figure 5.6b). The concoctions possessed weaker reducing power (Figure 7). The control (Ascorbic acid) was higher than all plants and concoctions.

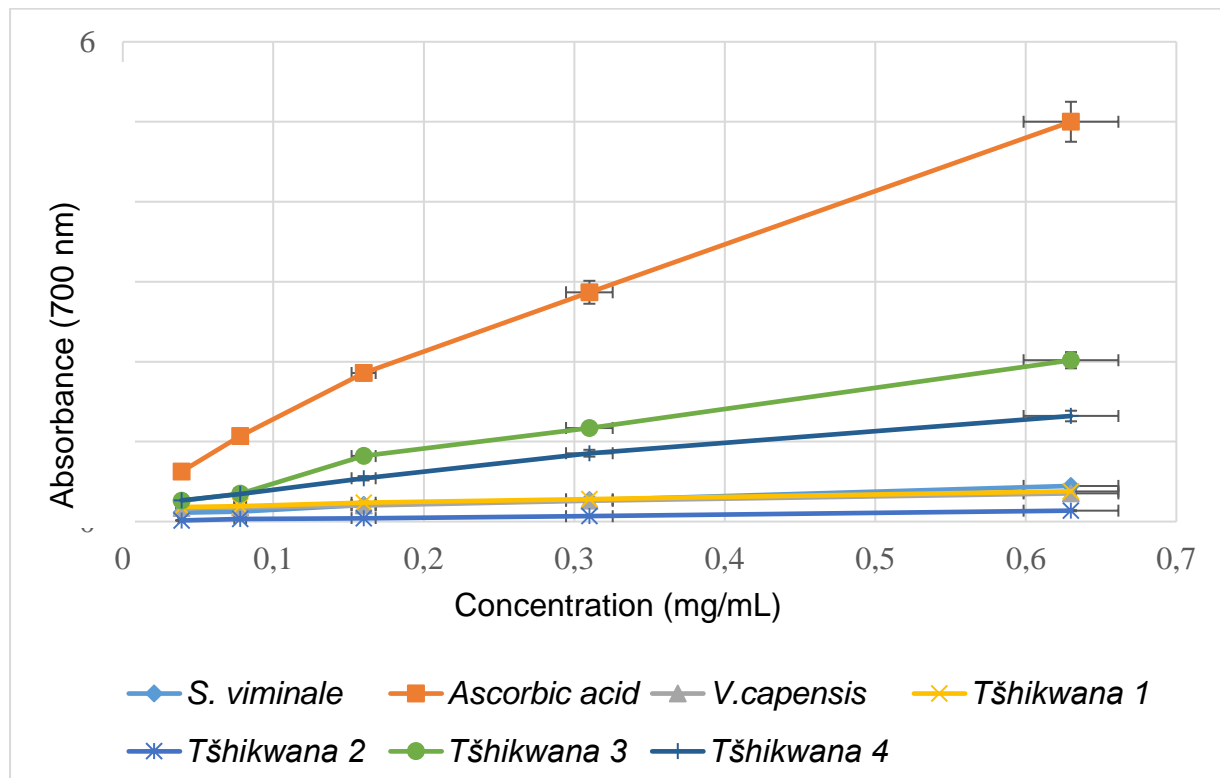


Figure 5.6a: The ferric reducing power of plants extracts at different concentrations expressed as absorbance at 700 nm wavelength.

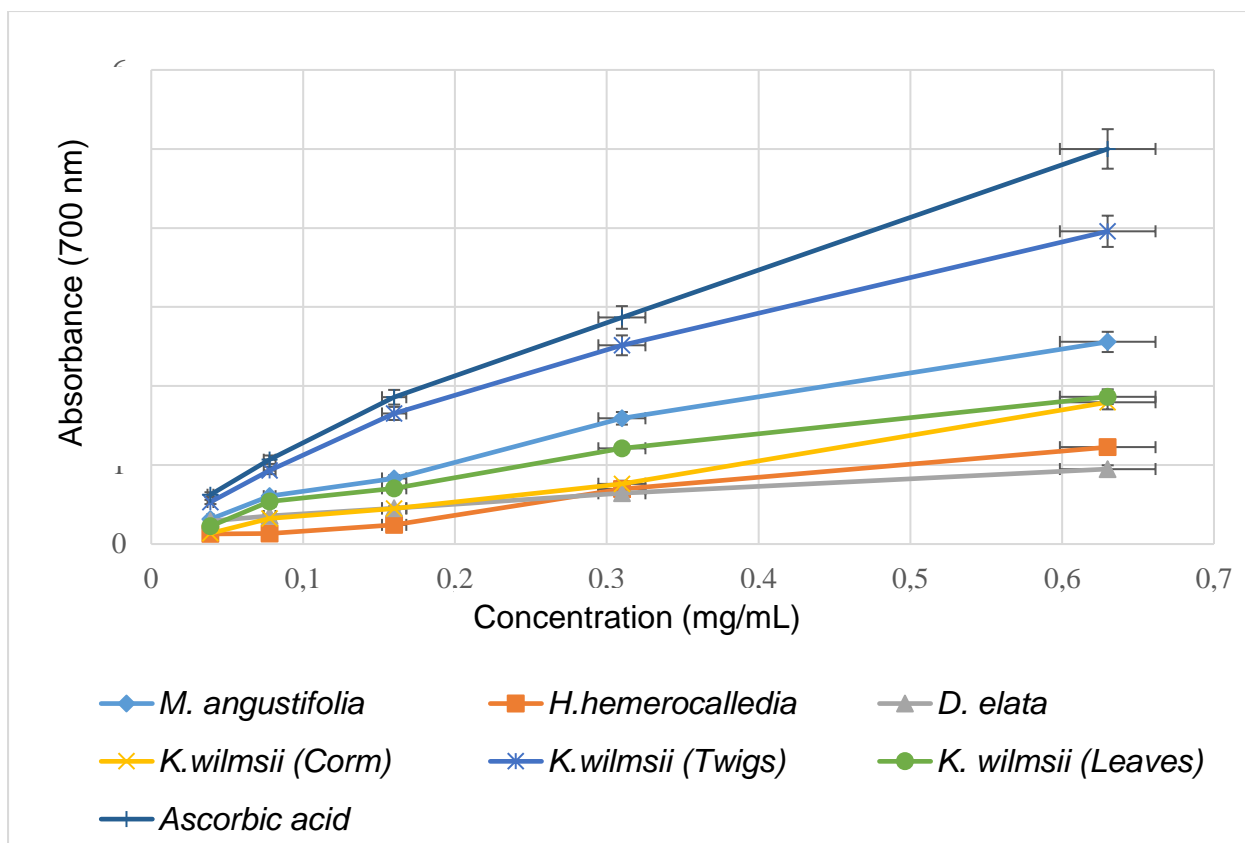


Figure 5.6b: The ferric reducing power of the plant decoctions compared with L-ascorbic acid.

*Values used are means of triplicates \pm standard deviation.

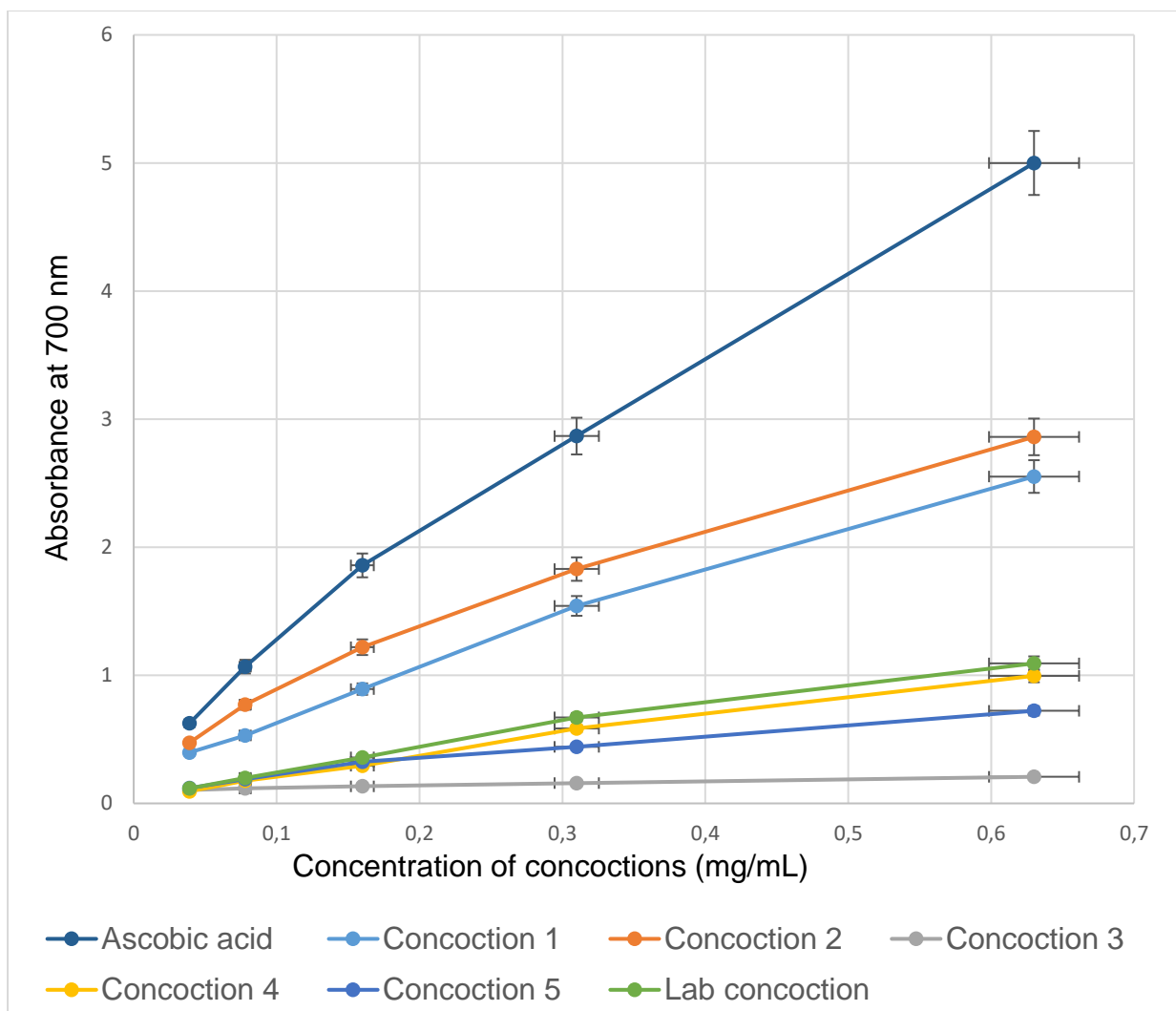


Figure 5. 7: The ferric reducing power of the different herbal concoctions at various concentrations. Ascorbic acid was used as a standard.

*Values used are means of triplicates \pm standard deviation.

5.3.3. Combinational effects of different plant decoctions on free radical scavenging activity

The possible outcomes of mixing different plant decoctions on free radical activity were investigated. No synergistic effects were detected. All the plant combinations yielded either indifferent or antagonistic interactions (Table 5.4).

Table 5.4: EC₅₀ and combinational effects of various plant mixtures

Combination	EC ₅₀ (µg/mL)	A	B	FIC index	Outcome
KWT + KWL	32.89 ± 0.32	2.09	1.78	3.87	Indifferent
KWT + KWC	38.61 ± 0.08	2.46	1.12	3.58	Indifferent
KWT + MA	13.30 ± 0.27	0.85	0.32	1.17	Indifferent
KWT + HH	57.40 ± 0.30	3.66	0.51	4.17	Antagonistic
KWL + KWC	58.42 ± 0.07	3.16	1.70	4.86	Antagonistic
KWL + MA	45.68 ± 0.06	2.47	1.12	3.59	Indifferent
KWL + HH	64.77 ± 0.09	3.50	0.58	4.08	Antagonistic
KWC + MA	46.76 ± 0.10	1.36	1.14	2.50	Indifferent
KWC + HH	69.89 ± 0.07	2.03	0.62	2.65	Indifferent
MA + HH	63.91 ± 0.16	1.56	0.57	2.13	Indifferent
SV + VC	156.21 ± 0.09	0.75	0.77	1.52	Indifferent
SV + DE	295.64 ± 0.54	1.42	0.90	2.32	Indifferent
SV + KWT	95.12 ± 0.67	0.46	6.05	6.51	Antagonistic
SV + KWL	66.83 ± 0.40	0.322	3.61	3.93	Indifferent
SV + KWC	75.62 ± 1.06	0.36	2.20	2.56	Indifferent
SV + MA	85.21 ± 1.21	0.41	2.08	2.49	Indifferent
SV + HH	102.39 ± 0.83	0.49	0.90	1.39	Indifferent
VC + DE	198.92 ± 0.96	0.98	0.60	1.58	Indifferent
VC + KWT	68.97 ± 0.45	0.34	4.39	4.73	Antagonistic
VC + KWL	79.42 ± 0.05	0.39	4.29	4.68	Antagonistic
VC + KWC	91.68 ± 0.07	0.45	2.66	3.11	Indifferent
VC + MA	104.75 ± 0.48	0.50	2.56	3.06	Indifferent
VC + HH	96.34 ± 0.54	0.47	0.86	1.33	Indifferent
DE + KWT	151.20 ± 0.09	0.46	9.62	10.08	Antagonistic
DE + KWL	165.10 ± 0.78	0.50	8.92	9.42	Antagonistic
DE + KWC	134.29 ± 0.31	0.41	3.90	4.31	Antagonistic
DE + MA	109.62 ± 0.45	0.33	2.68	3.01	Indifferent
DE + HH	121.31 ± 0.55	0.37	1.07	1.44	Indifferent

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*

5.4. Discussion

The Herbal concoctions were claimed to alleviate symptoms of pain and diarrhoea and were also claimed to be immune boosters and blood cleansers. To validate the mentioned pharmacological effects of the phyto-medicines, their antioxidant properties were investigated. The motivation behind this approach was that the presence of reactive oxygen species has been associated with immune-deficiency diseases (Lee *et al.*, 2004), inflammation (Maeda and Omata, 2008) and the pathogenesis and progression of liver diseases (Cichoż-Lach and Michalak, 2014). The use of antioxidants in the treatment of oxidative stress related pathologies is a possible remedial strategy and would validate the use of the herbal concoctions.

The antioxidant activity of the extracts was evaluated using thin layer chromatography (TLC), by separating the extracts in different solvent systems (BEA, CEF and EMW). The chromatograms were air-dried and sprayed with 0.2% free radical (DPPH) solution. The presence of antioxidant activity was observed by the development of a yellow colour against a purple background formed by the DPPH solution upon spraying.

The decoctions of *K. wilmsii* (twigs, leaves and corm) and *M. angustifolia* (leaves) had singular compounds in the crude extracts that are responsible for antioxidant activity (Figure 5.2). The BEA solvent system was unable to separate the antioxidant compounds of the all the extracts. This was indicated by the yellow bands at the base of the spotting region of the chromatogram showing that the antioxidant compounds had not migrated. The polarity of the compounds could be too polar to be separated with a non-polar solvent system. This deduction is further supported by the good migration (and separation) of the compounds of *K. wilmsii* on the polar (EMW) and intermediate (CEF) developed chromatograms, indicating that the solvent systems was able to migrate the compounds through the stationary phase matrix. The antioxidant compounds in *M. angustifolia* were unable to be separated by the mobile systems. Perhaps and even more polar solvent would be suitable to separate the compounds.

The herbal concoctions did not show any compounds with antioxidant activity (Figure 5.3). This observation indicated that the antioxidant activity of the concoctions was not dependent on single compounds but the collective interaction of the compounds in the

crude extract. Since TLC separates the compounds in the crude extracts, the combinational effects that confer biological activity may have been disrupted and thus, the loss of activity.

Free radical scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a widely used technique to assess antioxidant activity due to its time efficiency and accuracy in quantification. The deep violet colour of DPPH is neutralised to a yellow coloured α , α -diphenyl- β -picryl hydrazine. The intensity of discolouration of the solution is indicative of the antioxidant power of the test extract (Huang *et al.*, 2005). Therefore, when measuring the free radical scavenging activity of the extracts by a UV/VIS spectrophotometer, lower absorbance values indicated higher scavenging activity.

The decoctions of *D. elata*, *V. capensis*, *S. viminalis*, “*Tšhikwana*” 2 and 4 had the lowest antioxidant activity (Figure 5.4a). However, the twigs of *K. wilmsii* exhibited the greatest activity among the extracts and was comparable to that of L-ascorbic acid. This high activity was also observed in its corm and the leaves of *M. angustifolia*. Of the plants, *Kirkia wilmsii* displayed noteworthy antioxidant activity (Figure 5.4b). The species of *Kirkia* has been reported to consist of high levels of fatty acids, flavonols, isocoumarin, lignans, neolignans, carotenoids, phenols and tannins in its different parts such as the leaves, bark of the stem and roots (Maroyi, 2016). The mentioned classes of identified phytochemicals could be responsible for the high antioxidant activity observed.

Concoction 1, 2 and the lab standard demonstrated good free radical scavenging activity. However, the activity was weaker than that of ascorbic acid (Figure 5.5). The concoctions were found to have similar phytochemical profiles (Section 4.3.1), therefore, the difference observed in their antioxidant activity could be as a result of the concentration of bioactive phytochemicals in the samples being different.

The mechanism of antioxidant activity of phytochemicals has a relationship with the reducing power. This reducing power emanates from the ability of the phytochemicals to donate an electron to free radicals and/or their radical intermediates. The benefit of the reducing capability is in the limitation of the occurrence of cell and tissue damage associated with oxidative stress (Oktay, 2003; Chanda and Dave, 2009). The reducing power of the plant decoctions and the concoctions was measured by their ability to reduce a ferricyanide complex (Fe^{3+}) to a ferrous (Fe^{2+}) complex. Therefore, it was

possible to determine the reducing power with spectroscopy by measuring the formation of Pearl's Prussian blue at 700 nm.

The ferric reducing power of the extracts was compared to that of L-ascorbic acid. As shown the Figures 5.6 – 5.7, greater absorbance was indicative of higher antioxidant activity due to the colour intensity of the solutions. Moreover, the reducing power of the samples showed a concentration-dependent relationship. The twigs of *K. wilmsii* demonstrated the highest activity amongst the plant extracts followed by *M. angustifolia* (Figure 5.6b). Herbal concoction 1 and 2 also exhibited good reducing capacity (Figure 5.7). “*Tšhikwana*” 1 and 2, *S. viminalis*, *D. elata* and herbal concoctions 3, 4 and 5 had the lowest reducing powers (Figures 5.6–5.7). These observations demonstrated that an extract can have more than one mode of action of antioxidant activity. Furthermore, they showed that the strengths of the modes of action can differ for a single extract, for example, an extract can have excellent free radical scavenging activity but low reducing power and vice versa.

Observations from Sections 4.3.1–3 demonstrated that the phytochemical profiles of all the extracts (plant decoctions and concoctions) were dominated by the presence of polar compounds. Moreover, it was also shown that phenolic constituents e.g. flavonoids and tannins, attributed to the number of these polar compounds in the extracts. Polar solvents such as methanol, water, acetone and ethyl acetate are commonly used to extract polyphenols from plant material (Masoko *et al.*, 2008; Dai and Mumper, 2010). Henceforth, the plant extracts and concoctions showing the antioxidant activity could reflect the presence of these highly polar polyphenols. This study demonstrated a relationship between phenolic content and antioxidant activity, mainly, that the high concentration of phenolic compounds is associated with and may be responsible for high levels of antioxidant activity.

In addition, natural antioxidants in plants that have these defence effects have been arranged in three major groups, the vitamins, carotenoids and phenols. The carotenoids are lipophilic whilst the vitamins and the phenols are hydrophilic antioxidants (Thaipong *et al.*, 2006).

All of the samples used in this study underwent thermal treatment. During the preparation of the concoctions, all the plant species were added to boiling water. The

heat treatment as a means of extraction has been reported to be responsible for the reduction of polyphenols in food products (Kaur and Kapoor, 2001; Mphahlele *et al.* 2016). However, the antioxidant activity observed in all the extracts could be due to polyphenols that were not thermo-sensitive because not only did they not get degraded, numerous compounds still maintained antioxidant activity as visualised on the chromatograms in Figure 5.2. In accordance with these results, a study by Ranilla *et al.* (2009) on the effects of different cooking conditions also found and suggested that thermal treatment increased both the phenolic and antioxidant activity of the certain bean cultivars.

Under high temperatures phenolic compounds were reported to easily undergo hydrolysis and/or oxidation. The extraction time of the plants (5 minutes) had not altered the bioactivity of the phyto-constituents (Dai and Mumper, 2010). This demonstrated that the manner of preparation of the extracts (boiling for a short time in water) was an efficient and inexpensive approach by the traders to gain excess to compounds of pharmacological value. Other researchers have also reported the thermo-stability of some phenolic compounds such as gallic acid, ellagic acid, quercetin and isoflavone at temperatures of upto 100 °C (Liazid *et al.*, 2007).

The half maximal effective concentration (EC_{50}) to inhibit DPPH was calculated to further elucidate the effectiveness of the free radical scavenging activity of the extracts. Tables 5.2 and 5.3 represent the EC_{50} values of the plant decoctions and the concoctions respectively. Low EC_{50} values indicate that for an extract, a small amount is required to inhibit half the amount of total DPPH in a solution. Higher EC_{50} values indicate the converse. For example, only 15.71 $\mu\text{g/mL}$ of *K. wilmsii* twigs was required to inhibit 50% of DPPH in a solution compared to “*Tšhikwana*” 2 (535.91 $\mu\text{g/mL}$) and *D. elata* (329.04 $\mu\text{g/mL}$) (Table 5.2). The lab concoctions exhibited the highest activity 86.4 $\mu\text{g/mL}$) among the concoctions (Table 5.3). This could be because all the plants species were present in the concoction whilst some species were excluded by the traders in their commercial products.

The effect of the combination of decoctions of different plant species, chemical interactions were studied. The combinations of the different plant decoctions resulted in decreased free radical activity. This was because the EC_{50} values of the combinations (Table 5.4) were generally higher than those of the individual plant

decoctions (Table 5.2). The fractional inhibitory indexes (ΣFIC) indicated that the chemical reactions between the mixed decoctions resulted in a majority of indifferent and antagonistic outcomes. No synergism was observed. This observation explained the reason behind the lower activity observed in the combinations compared to individual plant extracts.

An interesting pattern was realised when comparing the antioxidant activity (EC_{50} values) of the concoctions, the plant decoctions and the 1:1 combination (Tables 5.2–4). In general, the plant decoctions had higher activity, followed by the combinations and lastly the concoctions. This trend suggested that activity of a single plant decoction decreased when numerous decoctions from different plant species were added to it. Although synergistic effects were sought after, the combination of more than two plant species eventually resulted in the concoctions attaining reduced activity. This study demonstrated the complexities that are associated with mixing plant decoctions. Moreover, adequate knowledge is required to know which species of plants should be mixed to enhance antioxidant activity because diverse chemical groups are prone to negative combinations.

Similar findings were reported by other researchers regarding the lack of synergistic interactions in phenolic compounds. When using the ABTS antioxidant assay, Heo *et al.*, (2007) did not observe any synergistic outcomes from mixing different phenolics, they reported only additive effects. Moreover, when using the DPPH method, Pinelo *et al.* (2004) reported antagonistic interactions between phenols when they reacted at different temperatures. Hidalgo *et al.* (2010) reported the occurrence of multiple antagonistic interactions that resulted from the combination of different flavonoids.

The free radical scavenging activity of phenolic compounds is dependent on their chemical structures and the arrangement of the hydroxyl groups. It was reported that higher antioxidant activity was associated with phenolic compounds that have an *ortho* 3, 4, -dihydroxyl structure at the B ring and hydroxyl groups in *meta* position e.g. 5, 7, dihydroxy at ring A. The presence of a double bond between the C2 and C3 hydroxyl group at ring C is also responsible for antioxidant activity (Rice-Evans *et al.* 1996; Kondo *et al.* 2000). The heat treatment that these electron rich compounds undergo during the preparation of the decoctions seemed to have induced chemical reactions between their functional groups to produce chemical derivatives with weakened free

radical scavenging and reducing activity. Therefore, with regard to the plant species used in this study, it would appear to be disadvantageous to mix their decoctions in an effort to increase therapeutic effect towards oxidative stress related diseases.

Conclusion

Although the traders have knowledge of which plant species to use to reduce oxidative stress related diseases, the manner in which they prepare the concoctions reduced the potential of antioxidant activity. This section highlighted the importance of choosing the proper combination of plant species that should be borne in mind when designing new aqueous phyto-medicines. Polyphenolic compounds present in a mixture can interact, and their interactions can affect the total antioxidant capacity.

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CHAPTER 6

6. Antimicrobial activities of the herbal concoctions and the plants used in their preparations

6.1. Introduction

South Africa is rich in diverse flora that serves as a renewable source of bioactive chemical structures which can be used for their therapeutic effect (Mander *et al.*, 2007). In the last three decades, the need for antimicrobial agents from natural resources has been recognized and literature has witnessed an increase in reported screening of medicinal plants and isolation of phytochemicals with antimicrobial activities. Masoko *et al.* (2007) reported the antifungal activity of twenty-four southern African *Combretum* species and Shai *et al.* (2008) reported both antifungal and antibacterial activity of seven traditionally used South African plant species.

Due to the outbreak of resistant strains of microorganisms, two or more antimicrobials may be combined to improve inhibitory effects. The principle behind this type of approach is synergism. Many antimicrobials are singularly effective, however, when combined with other agents of a different mechanism of action, they can work together and result in improved efficacy (Beringer, 1999). This approach of combining various chemical constituents is also recognised in African traditional medicine regarding the treatment of infectious diseases. Herbal concoctions are prepared by mixing various plant species together to increase the therapeutic value of phyto-medicine (Cano and Volpato, 2004).

Traders at Ga Maja prepare their concoctions guided by the same rationale mentioned above. The chemical reactions of the diverse compounds need to be investigated to understand their overall effect on the biological activity of the mixture. In doing so, claimed efficacies of therapeutic action towards infectious diseases can be validated.

Several socio-economic conditions such as the lesser number of professional health practitioners and public primary healthcare establishments in local rural areas influence the sole reliance on phyto-medicines. Compounding to this is the annual

increase of pharmaceutical medicine (Shai *et al.*, 2008). This serves as motivation to investigate and screen for plants that synthesise antimicrobial active compounds. The benefit of such research is the distribution of more effective drugs than those currently on the market and may provide rural areas with an alternative healthcare (Shai *et al.*, 2008)

The broad use of traditional herbal remedies has encouraged manufacturers, private traders and street merchants to capitalise on this upsurge by increasing the availability of herbal remedies to those who desire them at a cost (Ndhlala and van Staden, 2012). The signs of urbanisation are witnessed by the increase in herbal shops, informal street traders and the wide distribution of herbal remedies in pharmacies and supermarkets. Furthermore, the use of media outlets such as internet, television, radio, newspapers, pamphlets and the formation of social networks has provided entrepreneurs with a broad spectrum of marketing platforms to adapt to modernisation and globalisation (Bonora, 2001).

Some of the claims made by the traders regarding the pharmacological effects of the concoctions included their use as anti-diarrhoeal agents. Diarrhoea is a condition that is characterised by clinical signs of the excessive release of watery stool. The consequence of this is the massive loss of fluid and electrolytes (Baldi *et al.*, 2009). Non-infectious and infectious elements are involved in the progression of diarrhoea. Numerous microorganisms such as bacteria, fungi and parasites are responsible for the manifestation of diarrhoea in humans and animals (Ahmed *et al.*, 2012). Diarrheagenic pathogens rely on the production of enterotoxins or the invasion of enterocytes from the intestine. The result of the latter is an inflammatory response that leads to mediators that can affect epithelial cell functionality (Ahmed *et al.*, 2012).

There are currently several methods that are employed in the screening of antimicrobial agents. For this study, the micro-broth dilution technique was adopted with special emphasis on antibacterial and antifungal studies. The micro-broth dilution enables the categorisation of the effects of antimicrobial agents as either bacteriostatic or bactericidal. Moreover, it enables reproducible results by determination of the minimum inhibitory concentration (MIC), that is; the lowest concentration of an extract that can inhibit the growth of pathogenic micro-organisms (Masoko *et al.*, 2005). This

approach to screening has been shown to be more accurate and effective than other commonly used techniques such as the agar disc diffusion (Eloff, 1998).

Bioautography is a variation of anti-microbial screening assays and involves the adsorption of compounds onto the surface of a thin layer chromatography (TLC) plate (Nostro *et al.*, 2000). This method has an advantage of defeating the difficulty of identifying and isolating compounds with observed antimicrobial properties based on polarities and uses only a small amount of sample (Runyoro *et al.*, 2006).

6.2. Methodology

6.2.1. Microorganisms used in this study

American type culture collection (ATCC) specimens of *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) and *Enterococcus faecalis* (ATCC 21212) were the bacterial strains used in this study. The microorganisms were maintained on nutrient agar plates at 4 °C. They were recommended for antibacterial activity testing by the United States National Committee for Clinical Laboratory Standards (NCCLS, 1990). In addition, the abovementioned bacterial strains and the fungus *Candida albicans* (ATCC 10231) are economically important causative agents of diarrhoea (Ahmed *et al.*, 2012). The *C. albicans* strain was maintained on potato dextrose agar plates at 4 °C.

6.2.2. Micro-dilution assay

The antimicrobial activity of the plant decoctions and the herbal concoctions was evaluated by determining the minimal inhibitory concentration against; *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*. The method adopted was the broth micro-dilution assay developed by Eloff, (1998) and as modified by Masoko *et al.* (2005) to suit fungal growth requirements. The extracts were reconstituted to a concentration of 10 mg/mL using sterile distilled water. Sterile distilled water (100 µL) was added to each well of a 96 well microtitre plate. The water extracts (100 µL) were serially diluted with distilled water in the 96 well microtitre plates. Each microorganism culture (100 µL) was separately added to each well aseptically. The antibiotic Amphotericin B was used as a positive control for fungi and Ampicillin was used for bacteria and sterile distilled water as the negative control. The microtitre plates were covered with a sterile

laboratory plastic wrap and incubated for 24 hrs at 37 °C for bacteria and for 48 hrs at 25 °C for fungi. After incubation, 40 µL of 0.2 mg/mL of *p*-iodonitrotetrazolium chloride (INT) (Sigma) dissolved in sterile distilled water was added to each well of the microtitre plates and further incubated for 30 minutes (bacteria) and 2-3 hrs (fungi). INT, served as a growth indicator, whereby the growth of the microorganism reduced the tetrazolium salt to a purple formazan. The MIC was determined as the lowest concentration of the plant extract that was able to inhibit bacterial growth *i.e.* MIC values were recorded as the lowest concentrations of clear wells for each extract. Microbial growth in the wells was indicated by a violet-purple colour, whereas clear wells indicated growth inhibition. The assay was repeated three times in duplicate.

6.2.3 Bioautography on TLC

The antimicrobial activities of the concoctions and the plant decoctions were qualitatively determined using bioautography on TLC as described by Begue and Kline, (1972) with modifications by Masoko and Eloff, (2005). The various extracts were re-dissolved in water to a concentration of 10 mg/mL and 20 µL of this resuspension was loaded onto the TLC plates. The TLC plates were prepared and developed in chambers with solvents of varying polarity as described in Chapter 4, Section 4.2.3.1. The chromatograms were air-dried for two weeks to completely remove any possible lurking residue of the mobile system. The chromatograms were sprayed with overnight cultures of bacterial and fungal species using a spray-gun, until the plates were moistened. The moist chromatograms were incubated for 24 hrs at 37 °C (bacteria) and at 25 °C (fungi) in 100% relative humidity. After incubation, 2.0 mg/mL of *p*-iodonitrotetrazolium chloride (INT) dissolved in distilled water was sprayed onto the chromatograms. The chromatograms were incubated at 37 °C (bacteria) and at 25 °C (fungi) in 100% relative humidity for 30 minutes (bacteria) and 2-3 hrs (fungi). Clear zones against a purple background served as an indication of the presence of compounds in the extract that inhibited the growth of the microorganisms used. The purple colour develops during the occurrence of microbial growth.

6.2.4. Synergistic, additive and antagonistic effects

The effects of the combinations of different plant decoctions were studied. The micro-dilution assay described in Section 6.2.2 was used to determine the combinational effects on the antimicrobial activity of various mixtures.

The mixture ratio was at 1:1 for the extracts. In order to determine these effects, the fractional inhibitory concentration (FIC) of the extracts was used. The method by van Vuuren and Vijoen, (2011) for determination of FIC values was adopted.

The FIC value for each extract in a combination was calculated by dividing the MIC value of the combination by the MIC value of each plant decoction placed in the combination (Equation 6.1). The fraction inhibitory index ($\sum FIC$) was then calculated by adding the two FIC values of the plant extracts in a combination (Equation 6.2).

The value of the fractional index provides information regarding the outcome of a combination. Briefly, $\sum FIC$ values ≤ 0.5 demonstrate synergistic interactions. For additive effects of the combinations, $\sum FIC$ are $> 0.5 - 1.00$. The interpretation for indifference was $\sum FIC$ values $> 1.00 - \leq 4.00$. Antagonistic interactions were interpreted as $\sum FIC$ values >4.00 . The summary of the interpretations is tabulated in table 6.1.

$$FIC = \frac{MIC (combination a,b)}{MIC (a)} \quad (6.1)$$

$$FIC\ index = \sum FIC = FICA + FICB \quad (6.2)$$

Where MIC is the minimum concentration of the extract that was able to inhibit microbial growth. FIC_A is the FIC for the first extract in the combination and FIC_B is of the second extract.

Table 6. 1: The definitions on the interpretation of the combinational effects of plant extracts at a 1:1 ratio

FIC index value	Outcome of combination
$\sum FIC \leq 0.5$	Synergistic
$\sum FIC > 0.5 - 1.00$	Additive
$\sum FIC > 1.00 - \leq 4.00$.	Indifferent
$\sum FIC >4.00$	Antagonistic

6.3. Results

6.3.1. Micro-dilution assay

The micro-dilution assay was used to determine the lowest amount of the extracts (mg/mL) that was able to inhibit microbial growth. The lowest concentration of an extract capable of inhibiting growth was taken as the minimum inhibitory concentration (MIC). The MIC values that were below 1.0 mg/mL were considered to have noteworthy antimicrobial activity (Ndhlala *et al.*, 2015).

6.3.1.1. Antibacterial activity against diarrheagenic pathogens

The antibacterial activity of the concoctions and plant decoctions was determined against common causative agents of diarrhoea. *V. capensis* (5 mg/mL), *S. viminale* (3.75 mg/mL) and *D. elata* (5 mg/mL) had the weakest antibacterial activity due to the high concentrations needed to inhibit the growth of the pathogens. *K. wilmsii* and *M. angustifolia*, however had more notable inhibitory activity of the pathogens because of the low minimum inhibitory concentrations (Table 6.2). Gram-negative bacteria, particularly *E. coli*, were generally more susceptible to the concoctions (0.63 – 0.31 mg/mL) (Table 6.3).

Table 6.2: Minimum inhibitory concentrations (MIC) of various plant decoctions against diarrheagenic bacteria

Microorganism	MIC (mg/mL)												
	KWT	KWL	KWC	MA	SV	VC	HH	DE	PM1	PM2	PM3	PM4	Amp (µg/mL)
<i>E. coli</i>	0.16	0.31	1.25	0.04	5	5	0.63	> 5	2.5	2.5	1.25	1.25	0.08
<i>P aeruginosa</i>	0.63	1.25	1.25	1.25	2.5	5	2.5	5	2.5	2.5	2.5	0.63	0.16
<i>E. faecalis</i>	0.63	1.25	2.5	0.63	2.5	> 5	0.63	> 5	1.25	2.5	2.5	2.5	0.13
<i>S. aureus</i>	0.63	2.5	2.5	1.25	5	5	1.25	5	2.5	2.5	0.63	1.25	0.16
Average	0.51	1.33	1.88	0.79	3.75	> 5	2.5	> 5	2.19	2.5	1.72	1.41	0.13

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, Del: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders

Table 6.3: Antibacterial activity of herbal concoctions against diarrheagenic pathogens expressed as MIC (mg/mL)

Microorganisms	MIC (mg/mL)						
	HC1	HC2	HC3	HC4	HC5	LC	Amp (µg/mL)
<i>S. aureus</i>	0.63	2.5	2.5	2.5	2.5	1.25	0.08
<i>E. faecalis</i>	0.63	2.5	2.5	2.5	2.5	2.5	0.16
<i>P. aeruginosa</i>	0.63	2.5	2.5	0.63	1.25	1.25	0.13
<i>E. coli</i>	0.31	0.63	0.63	0.63	0.63	0.31	0.16
Average:	0.55	2.03	2.03	1.56	1.72	1.33	0.13

Key: HC1: concoction 1, HC2: concoction 2, HC3: concoction 3, HC4: concoction 4, HC5: concoction 5, LC: Lab standard

6.3.1.2. Antibacterial activity against isolated microbial contaminants

The antibacterial activity of the samples was tested against the microbial contaminants identified using MALDI-TOF-MS, Section 3.3.2. The different parts of *K. wilmsii* (leaves, twigs and corm) and the leaves of *M. angustifolia* had notable antibacterial activity which is indicated by the low MIC values (0.63 - 0.08 mg/mL) (Table 6.4). Herbal concoction 3 had the weakest activity among the concoctions whilst concoction 4 exhibited good inhibitory effects (Table 6.5). In general, the plants had higher activity than the concoctions.

Table 6. 4: Minimum inhibitory concentrations (MIC) of plant decoctions against bacteria isolated from the concoctions

Microorganism	MIC (mg/mL)											
	KWT	KWL	KWC	MA	SV	VC	HH	DE	PM1	PM2	PM3	PM4
<i>E. coli</i>	0.08	0.16	0.31	0.16	2.5	> 2.5	0.63	> 2.5	2.5	2.5	1.25	0.16
<i>K. pneumoniae</i>	0.16	0.63	0.63	0.31	> 2.5	> 2.5	0.63	> 2.5	> 2.5	> 2.5	0.31	0.16
<i>P. agglomerans</i>	0.04	0.31	0.31	0.31	> 2.5	> 2.5	0.63	> 2.5	0.63	1.25	0.31	0.31
<i>E. aerogenes</i>	0.04	0.63	0.63	0.31	> 2.5	2.5	1.25	> 2.5	2.5	2.5	> 2.5	> 2.5
<i>E. cloacae</i>	0.16	0.63	0.63	0.31	> 2.5	> 2.5	1.25	> 2.5	2.5	2.5	> 2.5	> 2.5
<i>L. adecarboxylata</i>	0.16	0.63	0.63	0.63	> 2.5	> 2.5	1.25	> 2.5	2.5	2.5	1.25	0.63
<i>C. braakii</i>	0.04	0.31	0.16	0.16	2.5	> 2.5	1.25	> 2.5	2.5	2.5	1.25	0.31
<i>B. subtilis</i>	0.16	1.25	0.63	0.31	2.5	> 2.5	0.63	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders

Table 6.5: Antibacterial activity of the herbal concoctions expressed as minimum inhibitory concentrations (mg/mL) against isolated species

Microorganism	MIC (mg/mL)					
	HC1	HC2	HC3	HC4	HC5	LC
<i>E. coli</i>	2.5	1.25	> 2.5	0.63	1.25	1.25
<i>K. pneumoniae</i>	2.5	1.25	> 2.5	0.31	0.63	0.63
<i>P. agglomerans</i>	1.25	0.31	> 2.5	0.63	2.5	1.25
<i>E. aerogenes</i>	2.5	2.5	> 2.5	0.63	0.31	2.5
<i>E. cloacae</i>	1.25	0.31	> 2.5	1.25	1.25	1.25
<i>L. adecarboxylata</i>	1.25	2.5	> 2.5	0.63	0.63	1.25
<i>C. braakii</i>	2.5	1.25	> 2.5	0.63	1.25	1.25
<i>B. subtilis</i>	1.25	0.31	2.5	0.31	1.25	0.63

Key: HC1: concoction 1, HC2: concoction 2, HC3: concoction 3, HC4: concoction 4, HC5: concoction 5, LC: Lab standard

6.3.1.3. Antifungal activity against *Candida albicans*

The herbal concoctions and the plant decoctions were tested for antifungal activity against *C. albicans* using the micro-dilution method. The *C. albicans* was susceptible to decoctions of *K. wilmsii*, *M angustifolia*, “*Tšhikwana*” 2 and 3 (Table 6.6). Although, the plants individually demonstrated antifungal activities, none of the concoctions exhibited activity against *C. albicans* at concentrations equal to or less than (\leq) 2.0 mg/mL.

Table 6.6: Antifungal activity of the plant decoctions against *Candida albicans* expressed in mg/mL.

Plant species	MIC (mg/mL)
KWT	0.16
KWL	0.16
KWC	0.31
MA	0.31
HH	1.25
SV	0.63
VC	2.50
DE	2.50
PM1	0.63
PM2	0.31
PM3	0.31
PM4	2.50

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminalis*, AP: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders

6.3.5. Bioautography assay

Dried chromatograms were sprayed with fresh bacterial and fungal culture. To detect antimicrobial compounds, INT was sprayed onto the chromatograms. All the chromatograms did not show bioactive compounds against any of the test microbial cultures (*E. coli*, *S. aureus*, *E. faecalis*, *P. aureginosa* and *C. albicans*). The whole

surface of the chromatograms turned purple after incubation and there were no clear zones. This indicated that the microorganisms were metabolically active and capable of reducing INT to its purple formazan product.

6.3.6. Synergistic, additive and antagonistic effects

Combinational effects of the addition of multiple plant species on antimicrobial activity were investigated. The combinations were of a 1:1 ratio and their minimum inhibitory concentrations were used to assess the fractional inhibitory index values ($\sum FIC$). Tables 6.7-6.11 represent the outcomes of mixing decoctions of two plant species together on antibacterial and antifungal activity. Most of the combinations resulted in either indifferent or antagonistic interactions, particularly for *E. coli* (Table 6.10) and *S. aureus* (Table 6.8). This indicated that the biological activity of the individual plant extracts decreased when they were in combination. On the other hand, there were reoccurring additive effects against *E. faecalis* (Table 6.7) and a synergistic interaction against *P. aeruginosa* (Table 6.9) indicating that the combinations may be efficacious against infectious diseases caused by these pathogens. Antagonistic interactions were predominant against *C. albicans* (Table 6.11) which indicated that the extracts are better used individually to inhibit the proliferation of the yeast than in combination.

Table 6.7: Antibacterial activity of extract combinations against *Enterococcus faecalis*.

Combination	MIC (mg/mL)	FIC values		FIC index	Outcome
		A	B		
KWT + KWL	0.31	0.5	0.25	0.75	Additive
KWT + KWC	0.63	1	0.25	1.25	Indifferent
KWT + MA	0.63	1	1	2	Indifferent
KWT + HH	0.63	1	1	2	Indifferent
KWL + KWC	0.63	0.5	0.25	0.75	Additive
KWL + MA	0.31	0.5	1	1.5	Indifferent
KWL + HH	0.63	1	2	3	Indifferent
KWC + MA	0.31	0.13	0.5	0.63	Additive
KWC + HH	1.25	0.5	2	2.5	Indifferent
MA + HH	0.63	1	1	2	Indifferent
SV + VC	> 2.5	1	0.5	1.5	Indifferent
SV + DE	> 2.5	1	0.5	1.5	Indifferent
SV +KWT	> 2.5	1	4	5	Antagonistic
SV + KWL	> 2.5	1	2	3	Indifferent
SV + KWC	> 2.5	1	1	2	Indifferent
SV + MA	1.25	0.5	2	2.5	Indifferent
SV + HH	> 2.5	1	4	5	Antagonistic
VC + DE	> 2.5	0.5	0.5	1	Additive
VC + KWT	2.5	0.5	4	4.5	Antagonistic
VC + KWL	> 2.5	0.5	2	2.5	Indifferent
VC + KWC	> 2.5	0.5	1	1.5	Indifferent
VC + MA	> 2.5	0.5	4	4.5	Antagonistic
VC + HH	> 2.5	0.5	4	4.5	Antagonistic
DE + KWT	> 2.5	0.5	4	4.5	Antagonistic
DE + KWL	> 2.5	0.5	2	2.5	Indifferent
DE + KWC	> 2.5	0.5	1	1.5	Indifferent
DE + MA	> 2.5	0.5	4	4.5	Antagonistic
DE +HH	> 2.5	0.5	4	4.5	Antagonistic

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different "Tšhikwana" powders

Table 6.8: Chemical interaction effects towards antimicrobial efficacy against *Staphylococcus aureus*

Combination	MIC (mg/mL)	FIC values		FIC index	Outcome
		A	B		
KWT + KWL	1.25	2	0.5	2.5	Indifferent
KWT + KWC	1.25	2	0.5	2.5	Indifferent
KWT + MA	1.25	2	1	3	Indifferent
KWT + HH	1.25	2	1	3	Indifferent
KWL + KWC	2.5	1	1	2	Indifferent
KWL + MA	2.5	1	2	3	Indifferent
KWL + HH	1.25	0,5	1	1.5	Indifferent
KWC + MA	2.5	2	2	4	Indifferent
KWC + HH	2.5	1	2	3	Indifferent
MA + HH	2.5	2	2	4	Indifferent
SV + VC	> 2.5	0.5	0.5	1	Additive
SV + DE	> 2.5	0.5	0.5	1	Additive
SV +KWT	1.25	0.25	2	2.25	Indifferent
SV + KWL	> 2.5	0.5	1	1.5	Indifferent
SV + KWC	> 2.5	0.5	1	1.5	Indifferent
SV + MA	2.5	0.5	2	2.5	Indifferent
SV + HH	> 2.5	0.5	2	2.5	Indifferent
VC + DE	> 2.5	0.5	0.5	1	Additive
VC + KWT	1.25	0.25	2	2.25	Indifferent
VC + KWL	> 2.5	0.5	1	1.5	Indifferent
VC + KWC	> 2.5	0.5	1	1.5	Indifferent
VC + MA	> 2.5	0.5	2	2.5	Indifferent
VC + HH	> 2.5	0.5	2	2.5	Indifferent
DE + KWT	> 2.5	0.5	4	4.5	Antagonistic
DE + KWL	> 2.5	0.5	1	1.5	Indifferent
DE + KWC	> 2.5	0.5	1	1.5	Indifferent
DE + MA	> 2.5	0.5	2	2.5	Indifferent
DE +HH	> 2.5	0.5	2	2.5	Indifferent

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders

Table 6.9: Outcomes of mixing various plant decoctions on antimicrobial activity against *Pseudomonas aeruginosa*

Combinations	MIC (mg/mL)	FIC values		FIC index	Outcome
		A	B		
KWT + KWL	1.25	4	2	6	Antagonistic
KWT + KWC	1.25	4	2	6	Antagonistic
KWT + MA	1.25	4	2	6	Antagonistic
KWT + HH	0.63	4	1	5	Antagonistic
KWL + KWC	0.63	1	1	2	Indifferent
KWL + MA	2.5	2	2	4	Indifferent
KWL + HH	0.63	0,5	4	4,5	Antagonistic
KWC + MA	1.25	1	1	2	Indifferent
KWC + HH	2.5	2	1	3	Indifferent
MA + HH	0.31	0,25	0,13	0,38	Synergistic
SV + VC	> 2.5	1	0.5	1.5	Indifferent
SV + DE	> 2.5	1	0.5	1.5	Indifferent
SV +KWT	1.25	0.5	2	2.5	Indifferent
SV + KWL	> 2.5	1	2	3	Indifferent
SV + KWC	> 2.5	1	2	3	Indifferent
SV + MA	> 2.5	1	2	3	Indifferent
SV + HH	> 2.5	1	1	2	Indifferent
VC + DE	> 2.5	0.5	0.5	1	Additive
VC + KWT	> 2.5	0.5	4	4.5	Antagonistic
VC + KWL	> 2.5	0.5	2	2.5	Indifferent
VC + KWC	> 2.5	0.5	2	2.5	Indifferent
VC + MA	> 2.5	0.5	2	2.5	Indifferent
VC + HH	> 2.5	0.5	1	1.5	Indifferent
DE + KWT	> 2.5	0.5	4	4.5	Antagonistic
DE + KWL	> 2.5	0.5	2	2.5	Indifferent
DE + KWC	> 2.5	0.5	2	2.5	Indifferent
DE + MA	> 2.5	0.5	2	2.5	Indifferent
DE +HH	> 2.5	0.5	1	1.5	Indifferent

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders

Table 6.10: Combinational outcomes of plant decoctions against antimicrobial activity against *Escherichia coli*

Combinations		FIC values		FIC index	Outcome
		A	B		
KWT + KWL	0.31	2	1	3	Indifferent
KWT + KWC	0.63	4	0,5	4,5	Antagonistic
KWT + MA	0.63	4	16	20	Antagonistic
KWT + HH	0.63	4	1	5	Antagonistic
KWL + KWC	0.63	2	0,5	2,5	Indifferent
KWL + MA	0.31	1	8	9	Antagonistic
KWL + HH	0.63	2	1	3	Indifferent
KWC + MA	0.31	0,25	8	8,25	Antagonistic
KWC + HH	1.25	1	2	3	Indifferent
MA + HH	0.63	16.03	1	17	Antagonistic
SV + VC	> 2.5	0.5	0.5	1	Additive
SV + DE	> 2.5	0.5	0.5	1	Additive
SV +KWT	0.63	0.13	4.01	4.14	Antagonistic
SV + KWL	1.25	0.25	4.03	4.28	Antagonistic
SV + KWC	> 2.5	0.5	2	2.5	Indifferent
SV + MA	0.63	0.13	16.03	16.16	Antagonistic
SV + HH	> 2.5	0.5	3.97	4.47	Antagonistic
VC + DE	> 2.5	0.5	0.5	1	Additive
VC + KWT	1.25	0.25	7.81	8.06	Antagonistic
VC + KWL	2.5	0.5	8.06	8.56	Antagonistic
VC + KWC	> 2.5	0.5	2	2.5	Indifferent
VC + MA	1.25	0.25	32.05	32.3	Antagonistic
VC + HH	> 2.5	0.5	4	4.5	Antagonistic
DE + KWT	1.25	0.25	7.81	8.06	Antagonistic
DE + KWL	1.25	0.25	4.03	4.28	Antagonistic
DE + KWC	> 2.5	0.5	2	2.5	Indifferent
DE + MA	0.63	0.13	16.15	16.28	Antagonistic
DE +HH	> 2.5	0.5	3.97	4.47	Antagonistic

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different "Tšhikwana" powders

Table 6.11: Combinations effects of adding various plant species against *Candida albicans*

Combination	MIC (mg/mL)	FIC values		FIC index	Outcome
		A	B		
KWT + KWL	0.31	2	2	4	Indifferent
KWT + KWC	0.31	2	1	3	Indifferent
KWT + MA	0.31	2	1	3	Indifferent
KWT + HH	0.31	2	0,25	2,25	Indifferent
KWL + KWC	0.31	2	1	3	Indifferent
KWL + MA	0.08	0,5	0,25	0,75	Additive
KWL + HH	2.5	16	2	18	Antagonistic
KWC + MA	0.31	1	1	2	Indifferent
KWC + HH	2.5	8	2	10	Antagonistic
MA + HH	0.31	1	0,25	1,25	Indifferent
SV + VC	> 2.5	3.97	1	4.97	Antagonistic
SV + DE	> 2.5	3.97	1	4.97	Antagonistic
SV +KWT	1.25	1.98	7.81	9.79	Antagonistic
SV + KWL	1.25	1.98	7.81	9.79	Antagonistic
SV + KWC	> 2.5	3.97	8.06	12.03	Antagonistic
SV + MA	0.63	1	2.03	3.03	Indifferent
SV + HH	> 2.5	3.97	2	5.97	Antagonistic
VC + DE	> 2.5	1	1	2	Indifferent
VC + KWT	1.25	0.5	7.81	8.31	Antagonistic
VC + KWL	> 2.5	1	15.63	16.63	Antagonistic
VC + KWC	> 2.5	1	8.06	9.06	Antagonistic
VC + MA	> 2.5	1	8.06	9.06	Antagonistic
VC + HH	> 2.5	1	2	3	Indifferent
DE + KWT	1.25	0.5	7.81	8.31	Antagonistic
DE + KWL	> 2.5	1	15.63	16.63	Antagonistic
DE + KWC	> 2.5	1	8.06	9.06	Antagonistic
DE + MA	> 2.5	1	8.06	9.06	Antagonistic
DE +HH	> 2.5	1	2	3	Indifferent

Key: Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different “*Tšhikwana*” powders.

6.4. Discussion

The traders at Ga Maja claimed that their preparations were potent towards the treatment of diarrhoea. To validate this ethnopharmacological claim, four different strains of common diarrheagenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*) and a yeast isolate (*Candida albicans*) were selected as test organisms for antimicrobial activity of the herbal concoctions and plant decoctions.

The American type culture collection (ATCC) specimens were used to investigate the antimicrobial activity of the extracts. This was done not only to standardise the activity, but to also avoid working with numerous pathogenic strains that can become a health hazard. However, antimicrobial activity against these selected microorganisms has the capacity to highlight the possibility of analogous that can exert inhibitory effects against more pathogenic strains (Clements *et al.*, 2002).

The micro-dilution assay was used to determine the lowest amount of the crude extracts that would be able to inhibit the growth of pathogenic microorganisms. This lowest concentration of the extract responsible for inhibitory effects was taken as the minimum inhibitory concentration (MIC) with units of milligram per millilitre (mg/mL). The INT solution was added to the 96-well microtiter plate to indicate the occurrence of growth. Growth was observed by the development of a purple colour in the wells, therefore signifying cellular activity.

As recommended by Orchard and van Vuuren, (2017), MIC values equal or less than 1.0 mg/mL were considered as noteworthy/significant antimicrobial activity. Noteworthy activity against the four bacterial strains was observed with the decoction of the twigs of *K. wilmsii* (KWT) followed by the leaves of *M. angustifolia* (MA) with average MIC values of 0.51 mg/mL and 0.79 mg/mL respectively across the Gram positive and negative strains (Table 6.2). It was also notable that the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were generally more susceptible to the plant decoctions. The concoctions and decoctions showed effective antibacterial activity against Gram-negative bacteria than the Gram-positive strains. The antimicrobial activity of the extracts against the Gram-negative bacterial strains maybe attributed to

their ability to inactivate microbial adhesion mechanisms, enzymes (peptidyl transferase) and cell membrane transport proteins (Ahmed *et al.*, 2012).

Eloff and Picard, (2007) demonstrated that solvents, such as methanol, dimethylsulphoxide (DMSO) and ethanol had various degrees of toxicity towards test microorganisms. This solvent toxicity thus leads to inaccurate recordings (exaggerated activity) and therefore, ambiguous data. Water is non-toxic to microorganisms (Masoko and Makgapeetja, 2015), as such, there is less probability that the observed antibacterial activity in this study could have been because of the harmfulness/toxicity of the water towards the microorganisms.

In Chapter 4, Sections 4.3.1 and 4.3.3, it was shown that these plant decoctions were mainly constituted by highly polar polyphenols. Polyphenols have been shown to possess concentration-dependent antimicrobial activity against various microbial species (Ahmed *et al.*, 2012). Therefore, the antimicrobial activity of the herbal concoctions and plant decoctions maybe related to the presence of these phytoconstituents.

D. elata, *V. capensis* and *S. viminale* exhibited low antibacterial activity against the used test microorganisms because MIC values as high as 5 mg/mL were recorded (Table 6.2). These observed results suggested that *D. elata*, *V. capensis* and *S. viminale* were not appropriate choices to treat infectious diarrhoea as indicated by Ga Maja traders. Furthermore, the inappropriate use of plants leads to their depletion. Semanya *et al.* (2013) reported that some Bapedi traditional healers in the Limpopo Province boil a mixture of *D. elata* (corn), *S. viminale* (twigs), *E. transvaalense*, *E. elephantina* (root), *S. birrea* (bark), *Z. capense* (root) for 20 minutes for treatment of HIV/AIDS. Whereby one cup of the extract taken orally thrice a day is said to boost the immune system remarkably. Although the need for validation still stands, the plants may possibly possess anti-viral activities rather than antimicrobial activities.

Due to an increase in the occurrence of infectious diseases and associated resistant strains of microorganisms. The traders attempted to increase the therapeutic capacity of an individual plant species. This approach is rooted from the theory that by adding different plant species, the overall biological activity will be enhanced. The concoctions produced from the plant species were also tested for their antibacterial activity against

diarrheagenic microbes because they are sold with indications of anti-diarrhoea effects.

Concoction 1 exhibited the greatest antibacterial activity among the concoctions with an average MIC of 0.55 mg/mL across all the bacterial strains (Table 6.3). The plant decoctions and the concoctions shared similar modes of actions against the bacteria, justifying the composition of concoctions. Even so, it was however also noted that overall, the plant decoctions had better antibacterial activity compared to the concoctions. This could indicate that in this case the individual plant species would be a better approach to obtain therapeutic benefits for diarrhoeal relief.

Although numerous microbial contaminants were present in the concoctions as described in Chapter 3, Sections 3.3.1 and 3.3.2, some of the plant species chosen by traders showed good antibacterial activity. Moreover, the concoctions also exhibited variable activity, in which case, the extracts were tested for antibacterial activity against the bacterial contaminants isolated and identified with MALDI-TOF-MS.

The plant decoctions exhibited excellent activity against the *Enterobacteriaceae* bacterial contaminants, most notably, *K. wimlsii* and *M. angustifolia* (0.08 – 0.63 mg/mL) (Table 6.4). The concoctions (with the exception of concoction 3) demonstrated considerable inhibitory activity (1.25 – 0.31 mg/mL) (Table 6.5). The results indicated that although the concoctions were prepared using plants with antimicrobial activities, the concentrations of the components may have been diluted in the final preparation to inhibit bacterial growth. Dilute concentrations also have negative implications on the efficacy of the concentrations of the concoctions prepared by the traders to treat specified infections.

The lurking challenge is that the traders, especially in impoverished communities, lack efficient measuring systems to ensure that they prepare concentrations that are efficacious towards the intended therapeutic benefit. Other consequences of not preparing concentrations that are adequate to induce a pharmacological effect is that, the consumers will lose confidence in the phyto-medicines due to no healing outcomes. This study demonstrated the importance of standardisation because, as shown in the results of this chapter, when the concentrations are not accurate, even

phyto-medicines that contain potent bioactive compounds may end up not imposing inadequate effects towards a particular ailment.

Candida albicans is an opportunistic pathogen that has been associated with the infection of immune compromised persons and the onset of diarrhoea upon its morphogenesis to form filamentous hyphae (Ahmed *et al.*, 2012; Gow *et al.*, 2002). As such, it was selected as a test pathogen used to evaluate the antimicrobial spectrum of the extracts using the micro-dilution method.

C. albicans was most susceptible to the decoction of *K. wilmsii* twigs and leaves which both gave an MIC value of 0.16 mg/mL (Table 6.6). Generally, there was good fungal inhibitory activity from all the plant decoctions and *Tšhikwanas*, with MIC values ranging from 0.16 to 0.63 mg/mL, with an exception of the decoctions of *H. hemerocallidea*, *V. capensis*, *D. elata* and “*Tšhikwana*” 4 which showed moderate (MIC 1.25 mg/mL) to low (MIC 2.5 mg/mL) antifungal activity.

The antimicrobial profile of the plant decoctions showed that the extracts were active against Gram-negative, Gram-positive bacteria and yeasts. The wide range of activity indicated that the plants possess a broad-spectrum inhibitory activity. These results validated the choice of the some of the plant species for use as antidiarrheal agents.

The herbal concoctions were also tested for anti-candidal activity. The concoctions did not demonstrate antifungal activity against *C. albicans* at concentrations equal or less than 2.5 mg/mL. This lack of good antifungal activity from the concoctions implied that during their preparation, the chemical reactions that occurred between the phyto-constituents of different plant extracts reduced or altered the activity of the final product. *C. albicans* as a contaminant, is in itself evident that the concoctions would not be effective against it, which is true in our case. As to whether the mixing of plants in the concoction renders them inactive warrants investigation by determining such anti-candidal activity from non-contaminated concoctions. Nonetheless, activity from non-contaminated concoctions would not rule out the fact that the strain has developed resistance, only then, consuming unstandardized concoctions would be deemed undesirable, as this imposes serious health implications and could be considered as one of the factors contributing to the emergence of resistant strains.

The antimicrobial activity of these phenolic-rich extracts is influenced by the existence and position of hydroxyl groups on their chemical structure (Taguri *et al.*, 2004). The high temperatures employed could trigger chemical reactions between the various hydroxyl groups to form other structurally different bioactive compounds. The traders add the different plants to the boil in an effort to obtain a mixture that is more potent and broad-based than a single plant species extract.

There was a consistent pattern of weaker antimicrobial activity from the concoctions and strong activity from the single plant decoctions. Therefore, the consequence of the combinational effects of the different plant decoctions on antimicrobial activity were determined. Tables 6.7 –11 represent the antimicrobial activity of the combinations as well as the type of interaction the extracts participate in. It was observed that many combinations resulted in indifferent outcomes. This suggested that when most of plant species are added together, the chemicals in each extract produced non-interactive reactions. The consequence of this leads to the reduction of the activity observed in the plant combined plant species.

The only synergistic interactions that were detected were from the combination of *M. angustifolia* and *H. hemerocallidea* against *P. aeruginosa* (MIC of 0.31 mg/mL) (Table 6.9). It was remarkable to observe that combinations that consisted of *V. capensis*, *S. viminale* and *D. elata* generally resulted in additive and antagonistic interactions across the microorganisms (Table 6.7 – 11). It was shown that individually, the decoctions of these three plant species had weak antibacterial activities against the diarrheagenic pathogens (Table 6.2). Therefore, the presence of these plants species in the concoctions may be responsible for the decrease of the overall antimicrobial activity.

The absence of synergistic interactions between the plant decoctions at a 1:1 ratio explained the observed weaker antibacterial activity in the concoctions compared to individual plant decoctions. Moreover, the type of extraction process may affect the biological activity. Standardisation and quality control over unsubstantiated herbal products are important as it can elucidate and validate the pharmacological effects claimed by the manufacturers.

Bioautography was used to investigate the source of the antimicrobial activities observed in the micro-broth dilution assays, that is, whether the activities were due to the phytochemicals in the crude extracts or singular bioactive compounds. Bioautography was used to localise and discriminate antimicrobial compounds in the extracts. The decoctions of plant species used, and the concoctions did not have singular antibacterial or antifungal compounds (no clear zones against a purple background) against bacteria (*E. coli*, *S. aureus*, *E. faecalis*, *P. aureginosa*) and yeast (*C. albicans*). This finding was contrary to the finding obtained under micro broth dilution assays. It can thus be concluded that the activity imposed by all plant decoction and concoctions was through synergistic effects of bioactive active compounds within an extract and not necessarily the combination of different extracts.

There were no clear bands against the purple background of the bioautograms. This indicated that the separated compounds were unable to inhibit the microbial growth. This observation could be due to the low concentration of antimicrobial phytochemicals in the corms and leaves of the plant species under investigation. The low concentration of the compounds may be unable to inhibit growth. The antimicrobial phytochemicals could be volatile compounds which evaporated during the preparation of the extracts. Moreover, the separation of the compounds on the TLC plates has the disadvantage of diminishing the antimicrobial activity of extracts that depend of synergistic interactions to gain or enhance the activity.

The plant species used by the traders could be possessing antimicrobial compounds, however, water excluded major non-polar compounds in the extracts as seen on the phytochemical profiles (Figure 4.2 and 4.3). Numerous reports have suggested and demonstrated that bioactive compounds that are responsible for antimicrobial activity also included those that are non-polar (Masoko and Eloff, 2005; Masoko and Eloff, 2006; Suleiman *et al.*, 2010; Masoko and Makgapeetja, 2015). Henceforth, due to their exclusion in the water decoctions during extraction, the antimicrobial activity of the plant species could thus be diminished. One other possibility that could have led to no observed antimicrobial compounds on the bioautograms is that the strains of pathogens used in this study are resistant to the antimicrobial effects of the compounds. These observations demonstrated the importance of the choice of an appropriate solvent for the extraction of antimicrobial compounds. That is, polar and

non-polar constituents need to be considered when screening for antimicrobial compounds.

Suleiman *et al.* (2010) detected both antifungal and antibacterial compounds in the methanol, acetone, hexane and dichloromethane leaf extracts of *K. wilmsii*. Using bioautography, these bioactive compounds were localised on chromatograms developed with a non-polar mobile system (benzene/ethanol/ammonia hydroxide) [BEA], this was an indication that the antimicrobial compounds of *K. wilmsii* were non-polar. Henceforth, the absence of the antimicrobial activity of distinct polar compounds on the bioautograms was a reasonable outcome.

Conclusion

The lack of standardisation methods reduces the potential of the products. Although plants with antimicrobial activities were used by the traders, the efficacious concentrations to produce a therapeutic response were not adequately measured and adhered to.

Although the traditional healers had knowledge about which plant to use to treat infectious diarrhoea, the concoctions had reduced antimicrobial activity. This was because majority of the plant combinations resulted in antagonistic and indifferent interactions. While additive interactions were detected, this study recommends the use of single plant decoctions for the treatment of infectious diarrhoea because the single extracts showed lower minimum inhibitory concentrations.

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CHAPTER 7

7. Anti-HIV-1 reverse transcriptase, anti-inflammatory and cytotoxicity of the herbal concoctions

7.1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). To reproduce and propagate inside the host, one of the first mechanisms that the retrovirus (HIV-1) uses after cell invasion is the reverse transcription of its genetic code. During this process, several viral enzymes facilitate the transcription of the ribonucleic acid (RNA) to synthesise deoxyribose nucleic acid (DNA). The HIV-1 reverse transcriptase (HIV-1 RT) is accountable and facilitates the transcription of the viral single stranded RNA to DNA inside the cytoplasm of the host cell. The newly formed viral DNA components will be integrated into the host cell genome to synthesise more viral components. Due to the central role that the HIV-1 reverse transcriptase plays, it henceforth became the primary target to interfere with the HIV-1 life cycle and halt its propagation in the host (Silprasit *et al.*, 2011).

At present, there are two well understood inhibitors of the HIV-1 reverse transcriptase. The first type of inhibitor is called nucleoside HIV-1 RT inhibitors (NRTI) and the second group is known as the non-nucleoside HIV-1 RT inhibitors (NNRTI). The NRTI are nucleoside analogues that bear a resemblance to the substrates of the polymerase. The binding of the NRTI to catalytic sites interferes with the function of the HIV-1 reverse transcriptase. However, the chemical structure of the NRTI (nucleoside-shape) resembles human mitochondrial polymerase substrates as well. As such, the use of NRTI for the treatment of HIV-1 infection has serious toxic side effects towards the patient (Masuda *et al.*, 2004).

The second type of inhibitors, the non-nucleoside HIV-1 inhibitors (NNRTI) function by binding to the HIV-1 RT hydrophobic pocket that is distal from the catalytic region. This allosteric site interferes with the functionality of the HIV-1 RT leading to inhibition (Shen *et al.*, 2003).

Highly active antiretroviral therapy (HAART) is treatment aimed at treating HIV infection. This therapy involves the combination of protease inhibitors, NRTI and/or NNRTI. The general challenge of the use of this abovementioned therapy is the

emergence of drug resistant strains of the HIV-1 due to prolonged use, thus creating opportunities for the virus to evolve new survival mechanisms (Han *et al.*, 2011).

Most ailments at a glance, AIDS included, involves tissue injury which can either be primary or secondary and the body responds to such insults by initiating an inflammatory response. Inflammation is one of the key biological protective mechanisms that respond to cellular and tissue damage and/or microbial infections. During the microbial infections, inflammation can induce cell injury, thus releasing pro-inflammatory proteins or mediators. Several diseases that have been associated with inflammation include rheumatoid arthritis, inflammatory bowel disease, neurodegenerative disorders, cancer and atherosclerosis (Krishnamoorthy and Honn, 2006).

Inflammation and oxidative stress are inseparably connected through complex feed-forward and feedback loops. The reactive oxygen species (ROS) are integral components of pro-inflammatory signalling that is mediated by cytokines (Lee *et al.*, 2017).

Numerous drugs that target the inflammatory responses and their effects are commercial and include steroidal and non-steroidal anti-inflammatory drugs. The disadvantage of using these drugs stems from the deleterious side effects exhibited during their use. Their prolonged usage has garnered disinterest and thus increases the need of alternative therapies from natural sources (Maurent *et al.*, 2018).

The pathogenicity of HIV-1 is associated with ability to select receptors on the membrane of the T-cells. The HIV-1 uses gp120 protein to bind to the membrane of the cells, this results in the stimulation and upregulation of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α). The upregulation of TNF- α takes place through the activation of Toll-like receptor (TLR)2 and TLR4 pathways (Nazli *et al.*, 2013). In addition to pro-inflammatory activity, TNF- α has been shown activate HIV-1 replication in latently infected monocyte and T-cell lines through the HIV-long terminal repeat promoter regions (Chun *et al.*, 1998; Folks *et al.*, 1989).

The inflammation induced by the cytokines mediates the disruption of the cell membrane which upon disruption consequently allows entry of the viral components into the now host cell (Nazli *et al.*, 2010). Numerous research outputs have showed

that the transmission and subsequent acquisition of the HIV-1 is maintained by inflammation (Deeks *et al.*, 2013)

Investigations into inflammation have demonstrated that the lipoxygenase (LOX) and cyclooxygenase (COX-1 and COX-2) pathways are also central towards the stimulation of inflammation and the onset of inflammatory disorder (Steinhilber and Hofmann, 2014). Chemical substances that can inhibit the activity of the COX-1 and COX-2 may assist in treating inflammation and accelerate tissue regeneration (Hayashi *et al.*, 2011).

A cell viability assay was conducted to determine the cytotoxic effect of the samples against normal primary fibroblast Kmst-6 cell line (PC-201-012TM) (human skin cells). *In vitro* toxicological studies employ vast assays to determine cell viability and cytotoxicity that results from the exposure of chemical substances. In turn the determinations from these *in vitro* cytotoxicity assays can be used to predict possible human toxicities (Fotakis and Timbrell, 2006).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is converted to an insoluble purple formazan by metabolically active cells. The tetrazolium ring is cleaved by succinate dehydrogenase present in the mitochondria. Due to the impermeability of the resulting formazan, this purple product becomes collected inside healthy cells. Upon resuspension, the absorbance of the purple colour gives an estimation of the number of viable cells (Mosmann, 1983).

Several plant species have been shown to possess anti-HIV reverse transcriptase activity. Moreover, in some countries, medicinal plant products are being used by people to treat HIV infections and associated co-infections, however, the efficacy of the medicines would not be validated. The traders at Ga Maja had indicated that the herbal concoctions were prescribed for the treatment of HIV/AIDS infections. It was the objective of this section to validate the anti-HIV properties of the phyto-medicines. Furthermore, the potential toxic effects that may arise from the consumption of the concoctions were evaluated through the determination of cell viability after 24 hr exposure.

7.2. Methods and Materials

7.2.1. 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) x 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 °C. 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 m_{A260} 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 hr at 37 °C. After the 1 hr incubation period, the

contents of the tubes (60 µl) were transferred into MPM wells. The MPM were covered with foil and incubated for 1 hr 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 re-covered 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™, 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 $\text{Abs}_{405 \text{ nm}} \text{ Sample}$ is the absorbance of the reaction with herbal preparations or positive control at 405 nm and $\text{Abs}_{405 \text{ nm}} \text{ Neg Control}$ is the absorbance of reaction with water instead of sample at 405 nm.

Three tubes containing water instead of sample were used as negative controls. 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)] were used as positive controls.

Results were presented as means duplicates \pm standard deviations of two independent experiments; each experiment was done in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 5.0).

7.2.2 Cyclooxygenase (COX-1 and COX-2) inhibitor screening assay

The anti-inflammatory activity of the selected plants was evaluated by examining the inhibition of the ovine COX-1 and human COX-2 enzyme using the COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) as described by Boudjou *et al.* (2013). The assay measures the peroxidase activity of ovine COX-1 and human COX-2, by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Briefly, 150 μ L assay buffer (0.1 M Tris-HCl, pH 8), 10 μ L heme and 10 μ L ovine COX-1 enzyme were added to each well of the 96-well microtiter plate. The same procedure was repeated with the human COX-2 enzyme. A volume of 10 μ L plant extract at a concentration of 50, 25, 12.5 and 6.25 μ g/mL was added to each well. The plate was carefully mixed by shaking for 30 sec and followed by incubation at 25 °C for 5 min. After incubation, 20 μ L of TMPD was added to each well of the 96-well microtiter plate and the reaction was initiated by addition of 20 μ L arachidonic acid. The plates were further incubated at 25 °C for 5 min. Reaction mixture containing assay buffer and heme served as the blank and a mixture with either COX-1 or COX-2 enzyme, assay buffer and heme served as the 100% IA. Indomethacin served as a positive control. The absorbance of oxidized TMPD was read at 490 nm using a microplate reader (Optic Iveyment® System, Model 2100-C). The percentage inhibition was calculated using the formula below. The percentage inhibition was plotted against plant extract concentration and the IC₅₀ determined from the normalized logarithmic regression curve. Data was expressed as means of duplicates \pm standard deviations

$$\text{COX \% Inhibition} = [(100\% \text{ IA} - \text{Inhibitor})/100\% \text{ IA} \times 100]$$

Where IA is inhibition absorbance.

7.2.3. Cytotoxicity assay

To determine the toxicological outcomes of the consumption of the concoctions, their effect on cell viability of normal primary fibroblast Kmst-6 cell line (PC-201-012) was assessed. The MTT calorimetric assay described by Mosmann, (1983) was performed with modifications. The cell culture was maintained in a flask with Dulbecco minimal essential medium (DMEM, Whitehead scientific) supplemented with 10% foetal bovine serum (FBS) (Adcock-Ingram). Prior to seeding 96 well microtitre plates, the medium

was discarded, and the cells washed with 5 mL 1X phosphate buffered saline (PBS). The PBS was discarded, and 2 mL of trypsin was used to detach the cells from the surface. Trypan blue was used to dye the cells and an automatic cell counter (model), was used to quantify viable cells. The cells were diluted with DMEM to obtain 1×10^5 cells/mL cell suspension. One hundred microliter of the cell suspension was added into each of the wells of the 96 well microtitre plate using a multi-channel micropipette. The plates were incubated at 37 °C in a 5% carbon dioxide (CO₂) incubator for 24 hours to allow the cells to attach to the bottom surface of the wells.

The stock solutions of the extracts were prepared to a concentration of 200 mg/mL dissolved in (6:4) dimethyl sulfoxide: distilled water (DMSO: dH₂O). The stock solutions were diluted to 10 mg/mL with DMEM. The 10 mg/mL dilutions were then filter-sterilised into sterile 25 mL centrifuge tubes. The 10 mg/mL dilutions were further diluted to 1 mg/mL with DMEM supplemented with 10% FBS in order to maintain the same concentration of FBS during the serial dilution. In a separate sterile 96 well microtitre plate, the 1 mg/mL extracts were serially diluted 50% with DMEM with FBS to obtain a concentration range of (1000 – 31.25 µg/mL). Prior to treating the cells with the prepared concentrations, the DMEM was aspirated from the cells and the cells were washed with 100 µL of 1X PBS. The PBS was then discarded. One hundred microliters of the extracts (1000 – 31.25 µg/mL) prepared in a separate 95 well plate was transferred to the plate containing the cell cultures. Thus, the different concentrations were used to evaluate their effect on cell viability. The microtitre plates were incubated at 37 °C in a 5% carbon dioxide incubator for 24 hours. Following incubation, 50 µL of 1 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dissolved in 1X PBS was added to each well and the plates were further incubated for 3 hours. After incubation, the media was removed from the plates and 100 µL of DMSO was added to each well. The plates were carefully swirled to dissolve the purple formazan crystals. Purple formazan crystals are formed when MTT is reduced by metabolically active cells. Thus, the amount of formed formazan products produced provides an indication of the number of viable cells. A microtitre plate reader (promega) was used to measure the absorbance of the purple colour at 560 nm. Cells treated with the extracts were compared with untreated cells (positive control) and the cells treated with actinomycin (negative control).

7.3. Results

7.3.1. HIV-1 Anti-reverse transcriptase activity

The herbal concoctions were tested for their ability to inhibit the activity of the HIV-1 reverse transcriptase (HIV RT). The inhibition of this transcriptase interferes with the life cycle of reproduction for HIV-1. The IC₅₀ values (Table 7.1) indicated that the laboratory concoction (2.90 µg/mL) and concoction 5 (11.74 µg/mL) had higher anti-HIV RT than known and currently used antiretroviral drugs. Low IC₅₀ values are indicative of good activity.

Table 7.1: HIV-1 reverse transcriptase activity of the concoctions expressed as IC₅₀ (µg/mL)

Sample	IC₅₀ (µg/mL)
HC1	33.77 ± 0.74
HC2	50.35 ± 0.45
HC3	190.55 ± 7.05
HC4	207.5 ± 1.0
HC5	11.74 ± 1.64
LC	2.90 ± 0.97
Positive controls	
Lamivudine	40.90 ± 0.2
Zidovudine	25.45 ± 0.1
Lopinavir	34.25 ± 0.3
Ritonavir	36.65 ± 0.1

Key: HC1-sample 1; HC2- sample 2; HC3-Sample 3; HC4-Sample 4; HC5-Sample 5; LC- Lab Concoction. Data are represented as means duplicates ± standard deviations.

7.3.2. Anti-inflammatory activity by COX-1 and COX-2 inhibition

The anti-inflammatory activity of the herbal concoctions was evaluated by determining their capacity to inhibit the activity of pro-inflammatory enzymes. At the highest tested concentration (1.25 mg/mL), concoctions 2 and 4 (HC2 and HC4) had higher COX-1 inhibition (Figure 7.1). All concoctions had more than 70% inhibition of COX-2 at 1.25 mg/mL (Figure 7.2). IC₅₀ values showed that COX-2 was more inhibited than COX-1 (Table 7.2).

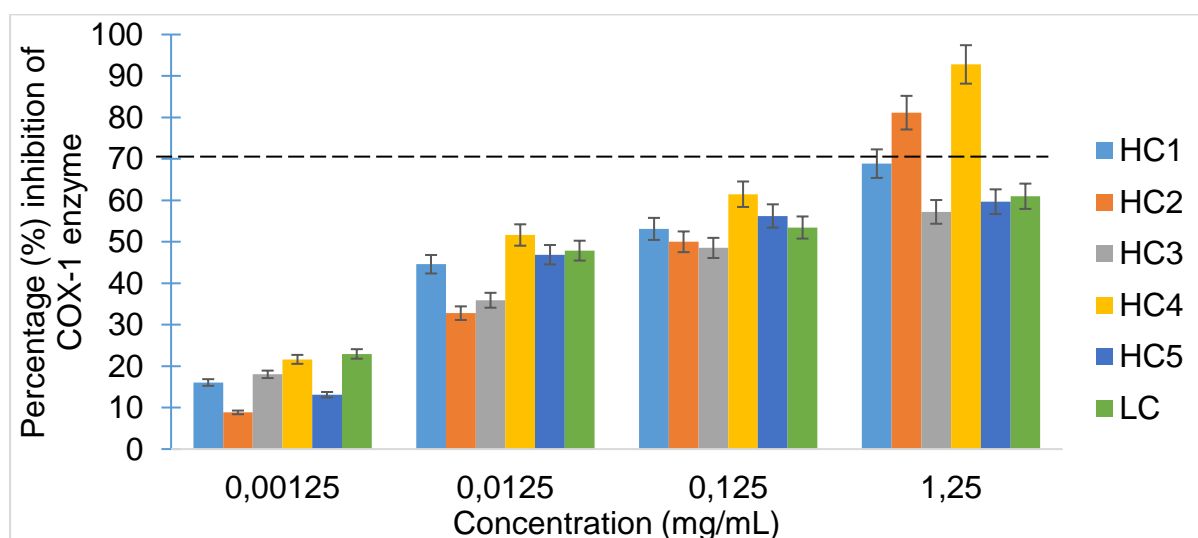


Figure 7.1: COX-1 percentage inhibition of the herbal concoctions.

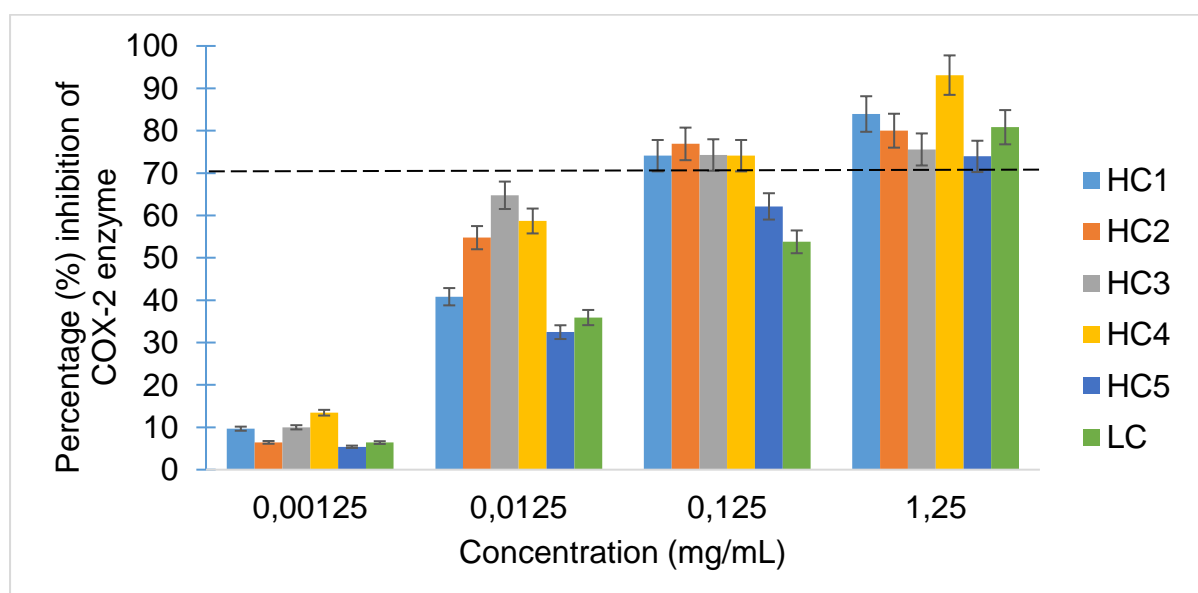


Figure 7.2: Percentage inhibition of COX-2 enzyme by the different concoctions.

*Concoctions with bar graphs above 70% were considered to be highly active against the COX enzymes

Table 7. 2: Cyclooxygenase Inhibitory concentrations of the concoctions expressed as IC₅₀ (µg/mL)

Sample	COX-1	COX-2
HC1	71.86 ± 0.008	27.10 ± 0.0005
HC2	86.30 ± 0.0001	17.3 ± 0.0007
HC3	241.5 ± 0.031	13.6 ± 0.0004
HC4	18.21 ± 0.001	12.3 ± 0.0004
HC5	95.81 ± 0.002	70.2 ± 0.001
LC	78.79 ± 0.001	71.5 ± 0.003
Positive control		
Indomethacin	5.67 ± 0.0002	8.6 ± 0,0002

Key: HC1-sample 1; HC2- sample 2; HC3-Sample 3; HC4-Sample 4; HC5-Sample 5; LC- Lab Concoction. Data is represented as means duplicates ± standard deviations

7.3.3. Cell viability assay

The cytotoxic effects of the herbal concoctions were investigated using the cell viability assay. As the concentration of the concoctions increased, cell viability reduced as shown in Figures 7.3 a and b. Despite the decrease in cell viability, the highest concentration of the concoctions exhibited cell viability more than 50%, making them less toxic when compared to Actinomycin D (negative control).

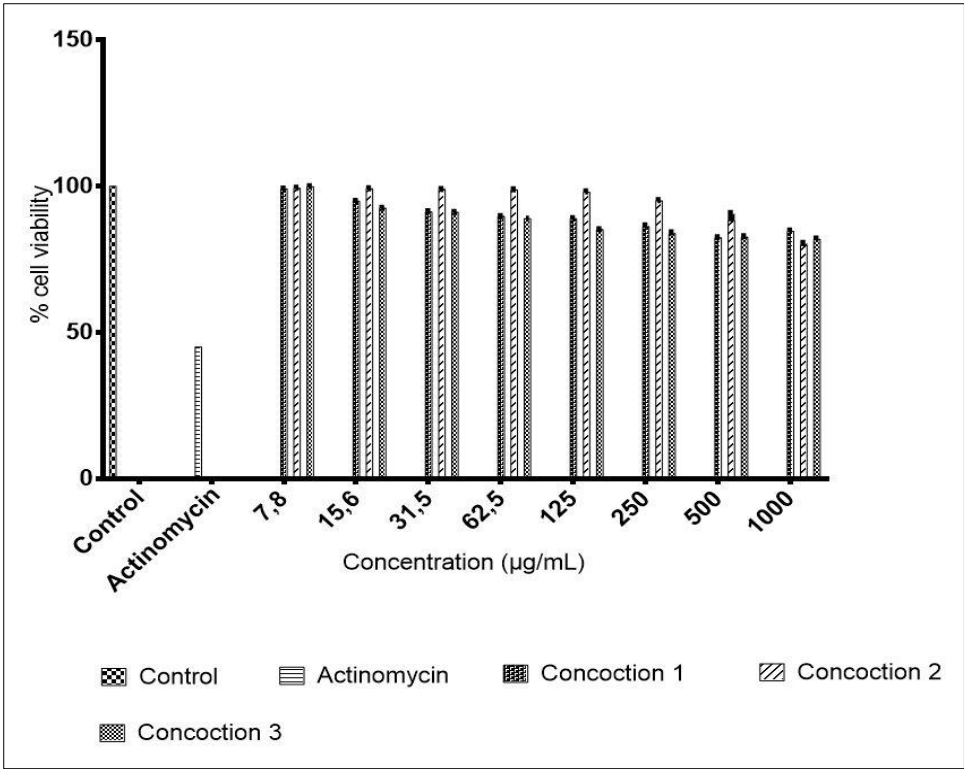


Table 7.3a: Percentage cell viability of the herbal concoctions. Untreated cells were used as a positive control and actinomycin was used as a negative control.

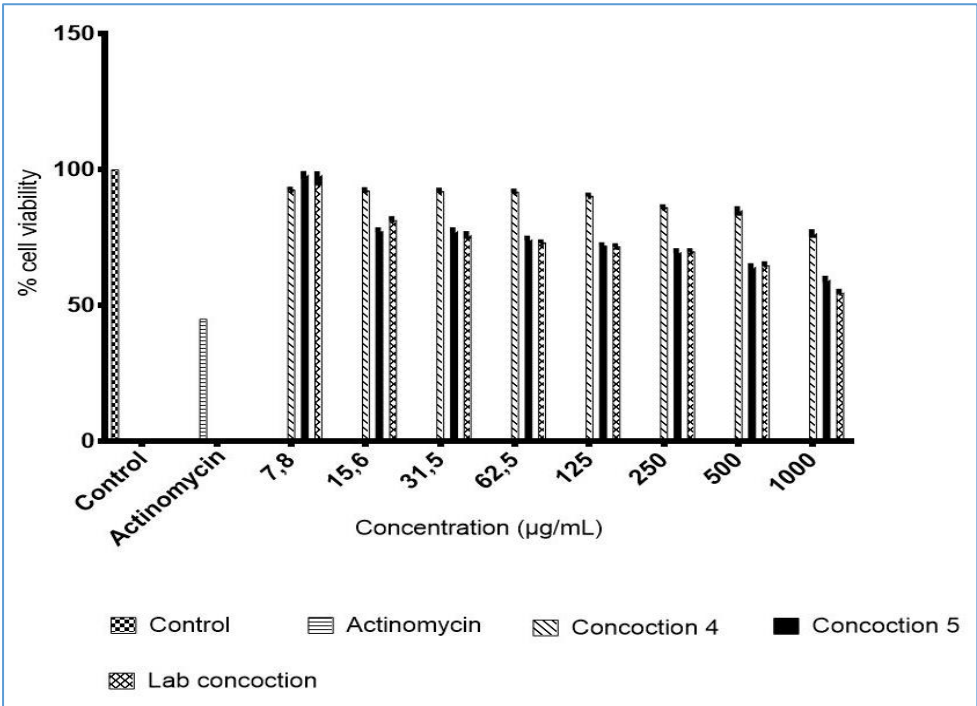


Figure 7.3b: Cytotoxic effects of the herbal concoctions expressed as percentage cell viability. Untreated cells were used as a positive control and actinomycin was used as a negative control. Data represented as means duplicates \pm standard deviations.

7.4. Discussion

The human immune-deficiency virus (HIV) HIV-1 reverse transcriptase is a very important enzyme in the HIV life-cycle, in which it transcribes its ribose nucleic acid (RNA) code to synthesise a viral deoxyribose nucleic acid (DNA) that invades other cells (Chukwujekwu *et al.*, 2014). The role of the HIV RT therefore makes it a crucial target towards the screening and development of anti-retroviral drugs against HIV. The HIV potential of the herbal concoctions was evaluated by determining their capability to inhibit the HIV-1 reverse transcriptase (RT) activity.

The concentration of an inhibitor that can produce half (50%) maximal activity is denoted as IC_{50} . Most of the phytochemicals are foreign to the human body. The use of these bioactive compounds can be accompanied by detrimental toxicities and/or adverse effects that may impact the morbidity and mortality rate of the global populace. Therefore, the smaller the concentration (IC_{50}) of a drug required to inhibit HIV RT whilst exhibiting minimum or no toxicities can be considered for pharmaceutical development.

In this study, the IC_{50} values of the herbal concoctions were represented in microgram/millilitre ($\mu\text{g/mL}$). Herbal concoction 2, 3 and 4 had the lowest activity because their IC_{50} (50.35 – 207.5 $\mu\text{g/mL}$) were relatively higher than the standards used. On the other hand, herbal concoction 1 had better activity than some of the standards, particularly Lamivudine, Lopinavir and Ritonavir.

The results demonstrated that compared to the standard anti-HIV drugs; Lamivudine, Zidovudine, Lopinavir and Ritonavir, herbal concoction 5 and lab concoction (LC) showed the highest inhibition of the HIV-reverse transcriptase with IC_{50} values of 11.74 and 2.90 $\mu\text{g/mL}$, respectively. HC5 and LC were able to inhibit 50% of the HIV-RT activity, whilst the antiviral activity of the standards ranged between 40.90 - 25.45 $\mu\text{g/mL}$. The antiviral activities of the herbal concoctions suggested that they may be able to inhibit the early phases of the HIV-1 replicative cycle that are mediated by the RT.

The antiviral activity of the herbal concoctions may be attributed to the medicinal plants used in their recipe or subsequent chemical reactions that occurred during their preparation. The use of *Sarcostemma viminale* by Bapedi traditional healers to treat

HIV/AIDS was reported by Semenya *et al.* (2013a) and Semenya *et al.* (2013b). *Hypoxis hemerocallidea* consists of compounds such as β -sitosterol and β -sitosterol glycoside which have been reported to effectively reduce plasma viral loads in HIV positive patients (Bouic *et al.*, 1996). In the Limpopo Province. *Drimia elata* was reported to be used by some of Bapedi traditional healers to treat gonorrhoea and HIV/AIDS (Semenya *et al.*, 2013b).

When the body is under biochemical stress or invasion, the production of reactive oxygen species such as the hydroxyl radicals, superoxide anion and hydrogen peroxide become greatly increased. Consequently, this leads to the overload of the body's natural defence mechanisms such as endogenous enzymes (Garcia-Lafuente *et al.*, 2009). Multiple oxidative stress-related effects are involved in inflammatory processes due to the production of free radicals by stimulated immune cells. This is because the highly active free radicals can initiate chain reactions that can impair the normal function of important biomolecules. In addition, the chain reactions involving free radicals can cause cell injury because the cell membranes become weakened and damaged. The overload of the free radicals has a consequence of activating pro-inflammatory gene expression (Li *et al.*, 2001).

The herbal concoctions were indicated to have pain-relieving properties; hence, their anti-inflammatory activity was evaluated. For this study, concentrations with cyclooxygenase (COX) activity inhibition above 70% were considered highly active. Generally, the capacity of the concoctions to inhibit COX was concentration dependent. For the inhibition of COX-1, only herbal concoctions 2 and 4 had the highest activity at a concentration of 1.25 mg/mL (Figure 7.1). Generally, the herbal concoctions achieved higher percentage inhibitions for COX-2 than for COX-1 at lower concentrations. Particularly, at the concentration of 0.125 mg/mL, herbal concoctions 1, 2, 3 and 4 had inhibition concentrations above 70% in COX 2 (Figure 7.2). This meant that the COX-2 enzyme activity was inhibited more than COX-1 at lower concentrations.

The IC₅₀ values for the inhibition of the COX enzymes were expressed as μ g/mL and compared with the positive control, indomethacin (Table 7.2). Herbal concoctions 3 (13.6 μ g/mL) and 4 (12.3 μ g/mL) had comparable IC₅₀ concentration to the positive control (8.6 μ g/mL). The IC₅₀ concentrations of the herbal concoctions also

demonstrated that the COX-2 was inhibited more than COX-1. The positive control had a contrast pattern to the concoctions, in that, indomethacin inhibited more of the COX-1 than COX-2 (Table 7.2). The herbal concoctions demonstrated good COX-1 and COX-2 enzyme activity and as such, their use towards the treatment of inflammatory ailments is justified.

Earlier studies on HIV-1 infections have shown that the exposure of human epithelial cells to the HIV gp120, results in an increased production of inflammatory mediators such as the cytokine TNF- α (Nazli *et al.*, 2010). In a study by Ferreira *et al.* (2015), it was demonstrated that the pre-treatment of human genital epithelial cells with curcumin, a high anti-inflammatory active compound resulted in the dysregulation of pro-inflammatory mediators. These pro-inflammatory factors consequently are responsible for cell membrane impairment and the recruitment of HIV target cells towards the cells (Ferreira *et al.*, 2015).

Inflammation is one of the mechanisms that facilitates the acquisition and spread of the HIV inside the host. Because of this relationship between inflammation and HIV, one can deduce that plant extracts that can inhibit or interfere with inflammatory processes may also contribute towards reducing the accelerated rate at which the HIV can proliferate in a host. The herbal concoctions demonstrated both anti-inflammatory and anti-HIV RT activities. The combined effect of these pharmacological activities described in this section may be partly responsible for the efficacy of the herbal concoctions towards the treatment of HIV and associated biochemical irregularities.

Cell viability assay was conducted to determine the cytotoxic effect of the samples against normal primary fibroblast Kmst-6 cell line (PC-201-012TM) (human skin cells). *In vitro* toxicological studies employ vast assays to determine cell viability and cytotoxicity that results from the exposure of chemical substances. In turn, the determinations from these *in vitro* cytotoxicity assays can be used to predict possible human toxicities (Fotakis and Timbrell, 2006).

The trend that was observed after the 24hr incubation of the treated cell lines was that, at higher concentrations of the concoctions, less cells were viable, and this viability increased as the concentrations decreased (Figures 7.3 a and b). At the highest tested concentration (1000 $\mu\text{g/mL}$) percentage cell viability for samples (1, 2 and 3) was above 60% and at the lowest tested concentrations (78 $\mu\text{g/mL}$) it was above 90%

(Figures 7.3a). Herbal concoction and the lab concoction had a lower percentage cell viability (below 60%) at 1000 µg/mL (Figure 7.3b). Therefore, acute toxicity seems less probable upon the consumption of the concoctions because a large percentage of the cells remain viable after the 24 hr exposure. Nonetheless, cell-based assays are ideal and an *in vivo* model is required to further validate these outcomes and to test for effects arising from prolonged exposure. The fact that there are no reports of toxicity on such concoctions could just arise from poor documentation, and that they are non-toxic to humans cannot be ruled out.

Some of the plants used to prepare the herbal concoctions were reported to possess variant levels of toxicities, such the *Drimia elata* (Ndhlala *et al.*, 2013) and *Sarcostemma viminale* (Botha and Penrith, 2008). However, the final products did not exhibit alarming toxicity. The manner of preparation of the herbal concoctions and the choice of other supplementary medicinal plants to add to the recipe could be responsible for the reducing toxic effects of toxic species. Experience shared through generations and trial and error has equipped the traders with knowledge of how to prepare a non-toxic concoction whilst including toxic plants.

Conclusion

The antiviral activity of some of the herbal concoctions was higher than the pharmaceutical standards used for the treatment of HIV-1. The combined effect of anti-inflammatory and high antiviral activity suggests that the herbal concoctions maybe potent therapies for the treatment of HIV. This study demonstrated two potential mechanisms the herbal concoctions may use to manage HIV; by inhibiting the replication of the virus (anti-reverse transcriptase activity) and propagation of the virus in the host (anti-inflammatory activity). The concoctions also reduce inflammatory responses that are as a consequence of HIV phenotypic outcomes, where pain is a subset, making the use of the concoctions in the treatment of pain justified.

The preliminary toxicological results from this study suggest that the concoctions may be non-toxic to human cells after 24 hr exposure. Although there is validity in the traditional use of the herbal concoctions, animal models are required to investigate the pharma-kinetics of the concoctions.

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CHAPTER 8

8. General discussion, conclusions and recommendations

8.1 General discussion

The present study was conducted to investigate claimed pharmacological activities and combinational effects of five concoctions that were purchased randomly from five different traders. The safety evaluation concerning the consumption of the concoctions was carried out using a cell viability assay and screening for microbial contaminants. The traders primarily boil their plant material when preparing their phyto-remedies, henceforth, to accurately validate the biological activities of the concoctions, only water decoctions were analysed.

Extraction of these bioactive compounds is the first step that allows their access and usage in traditional medicine and scientific study. It is generally known that in part, some of the factors that affect the yield and type of compounds extracted from a plant material include but are not limited to polarity of the solvent, extraction time, temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples (Dai and Mumper, 2010).

The diverse compounds in the plant decoctions and concoctions were observed on thin layer chromatography (TLC) chromatograms that were developed in mobile phases which varied in polarity, primarily, non-polar BEA, intermediate CEF, polar EMW and BAW (Kotze and Eloff, 2002). Some of the separated compounds were visualised by fluorescent quenching of ultraviolet (UV) light of 254 nm wavelength. For the compounds that were unable to fluoresce under the UV light, vanillin-sulphuric acid reagent was used to visualise them.

The phytochemical profiles generally showed that majority of the compounds in the plant extracts and concoctions were primarily polar and intermediate. Other researchers reported the tendency of water to extract polar compounds (Masoko *et al.*, 2008). Although the plant extracts and concoction had a similar pattern regarding the polarities of the compounds, their phytochemical profiles were dissimilar. These differences could be attributed to possible chemical reactions that occurred between the types of compounds from the mixture of the different plant species during the preparation of the concoctions.

The quantification of the phyto-constituents (phenolics, flavonoids and tannins) demonstrated that the concoctions generally contained higher concentrations than the singular plant decoctions. Since the concoctions were made up of all the plant species, each of them seemed to have contributed to the total amount of compounds in the concoctions.

Phytochemicals in plants which have antioxidant, antimicrobial, anti-inflammatory and anti-HIV properties have the potential of being integral components in new pharmaceutical drugs (Fabricant and Fansworth, 2001). It is within this regard that the screening of phytochemicals has paved a way for discovering new potent medicinal drugs that are effective in treating various human diseases.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that was used in this study to evaluate the free radical scavenging activity of the plant decoctions and the concoctions. The stable form of DPPH has a deep violet colour that possesses a characteristic absorption at 517 nm. However, as an antioxidant compound donate protons to this radical, the colour of the solution turns yellow and absorption decreases. The concentration-response curves detailing the percentage inhibition of DPPH indicated the twigs of *Kirkia wilmsii* showed the best scavenging activity (comparable to ascorbic acid) followed by its corm and leaves of *Monsonia angustifolia*.

Antioxidant activity is a multifaceted property and involves numerous mechanism that eventually either scavenge free radicals or reduces destructive metal complexes. The latter was chosen to assess the antioxidant potential of the extracts with respect to their potential to reduce a ferricyanide complex (Fe^{3+}) to a ferrous (Fe^{2+}) complex by the donation of electrons, contrary to the DPPH assay (centred around proton donation). The yellow colour of the solution of the potassium ferricyanide solution was converted to various shades and intensities of green and blue when a reducing extract was introduced. Concoction 1 and 2 showed good reducing potential among the concoctions, however, the twigs of *K. wilmsii* and the leaves of *M. angustifolia* had more superior ferric reducing power than all the extracts.

The total antioxidant activity (free radical scavenging and ferric reducing power) of the abovementioned extracts was a result of phytochemical classes such as terpenoids, flavonoids, steroids and tannins which were all positively detected. Furthermore, this

study reports the direct proportional correlation between high concentrations of total phenolics in the extracts and their high antioxidant activity. Other studies have also found a similar pattern (Baba and Malik, 2015; Lokesh *et al.*, 2016).

The micro-dilution method (Eloff, 1998) and bioautography (Begue and Kline, 1972) were used to study antimicrobial activity of the plant decoctions and concoctions against diarrheagenic microbes (*Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*) and pathogenic bacterial contaminants. The micro-dilution method was used to obtain the minimum concentration of the crude extracts that was able to inhibit microbial growth. In this study, MIC values that were equal or less than 1000 µg/mL were considered as noteworthy antimicrobial activity (Orchard and van Vuuren, 2017). Bioautography was performed to localise possible singular antimicrobial compounds.

The decoctions of some of the plant species had superior antimicrobial activity than the concoctions. Most notable were *K. wilmsii* and *M. angusifolia* which generally had low MIC values and were equal or less than 1.0 mg/mL. This good activity against the Gram-negative and positive bacterial species and a coccus yeast isolate suggested that these extracts have a broad spectrum of antimicrobial activity.

The absence of clear bands on the bioautograms indicated that the determined antimicrobial activities of both the plant decoctions and concoctions were not a result of the single discrete bioactive compound but rather were a result of the collection of all the compounds in the crude water extracts. This implied that these water extracts were dependent on combinational effects of the tested bioactive compounds such as alkaloids, saponins, tannins, terpenoids, cardiac glycosides, steroids and flavonoids to exert antimicrobial action. Tannins are known to bind proline-rich proteins and interfere with protein synthesis, and this interferes with microbial cell division and a deactivation of essential metabolic cell processes (Ahmed *et al.*, 2014). On the other hand, saponins are known to produce an inhibitory effect on inflammation (Ahmed *et al.*, 2014), which in turn their application can alleviate inflammatory responses associated with diarrhoea.

The mixing of different compounds may have the consequence of affecting their biological activity and other chemical properties such as the arrangement of functional groups. It then became important to evaluate the effect of the combinations of extracts

from different plant species on therapeutic action in order to assess the efficacy of the concoctions.

The results of the present study established that binary combinations (1:1 ratios) of phenolic-rich plant decoctions generally had indifferent and antagonistic effects towards the free radical scavenging and antimicrobial activity of the extracts. The indifferent interactions indicated that the combinations of the extracts yielded neither additive nor antagonistic effects, that is, the combinations did not enhance or decrease activity. The antagonistic interactions demonstrated that the combination of the extracts resulted in an activity that was lower than the individual plant extracts.

Hajimehdipoor *et al.* (2014) reported several antagonistic effects of numerous tertiary combinations (combinations of more than two compounds). The researchers concluded that antioxidant activity as measured by ferric antioxidant power (FRAP) was decreased by the combinations of more than two bioactive compounds. The concoctions in this study were prepared with numerous plant species which varied in their pharmacological effects and strengths. This in turn seems to have decreased the biological activity of the singular plant species.

The tendency of polyphenols to react together when they are mixed has been documented and in part concluded that flavonoid interactions reduced total antioxidant activity in plant extracts, foods and fruit Juices etc (Hidalgo *et al.*, 2010). It is thus possible that when the phyto-constituents from the different plant species were mixed, the hydrogen-bonding between the different compounds may occur. The occurrence of such reactions led to the decrease in the availability of the hydroxyl groups, thus reducing the chances of interacting with highly reactive oxygen species.

Concerning phenolic compounds, the number and pattern of hydroxyl substitutions of the B-ring of their structure are linked with activity. Hidalgo *et al.* (2010) demonstrated that flavonoids, quercetin and myricetin, with two and three hydroxyl groups respectively, possessed higher activity than pelargonidin-3-glucoside and kaempferol, with only one hydroxyl group in the B-ring. In addition, the combination of catechol moiety with a double bond at C2–C3 and a hydroxyl group in position 3 makes them extremely active (van Acker *et al.*, 1996). The antimicrobial activity of the plant species could have been compromised and/or weakened when added to produce the concoctions.

The Gram-negative bacterial strains have a complex multi-layered cell wall which is made up of lipopolysaccharides. This complex cellular structure has been documented to serve as a sieve and/or barrier towards outside environmental particles such as synthetic and natural antibiotics (Chanda *et al.*, 2013).

Of the mycotoxins produced by fungal species, aflatoxins are a common class of these chemical groups. Aflatoxins normally contaminate animal feeds, nuts, corn, cereals, oilseeds and dehydrated foods. This contamination by mycotoxins is usually during plant harvest, food manufacturing processes such as productions, handling and storage (Madrigal-Santillan *et al.*, 2010).

The herbal concoctions were tested for their antiviral potential against HIV. The method used focused on the activity inhibition of the reverse transcriptase of the virus. The consequence of this inhibition is that the retrovirus would be unable to continue its replicative life cycle because its viral genetic code (RNA) would not be transcribed to DNA.

The herbal concoctions demonstrated remarkable anti-HIV reverse transcriptase inhibition, particularly herbal concoction 5 and the lab concoction. Of which, the latter demonstrated higher antiviral activity than the positive controls used. This indicated that the concoctions were able to inhibit the activity of the reverse transcriptase, which is to synthesise the viral deoxyribonucleic acid. This significant activity validated the efficacy of the formulation of these concoctions for the treatment of HIV. The anti-inflammatory activity of the concoctions suggested that they may assist in relieving pain and reducing the acquisition of the HIV.

8.2. Conclusion

The herbal concoctions and the plant decoctions demonstrated striking antioxidant, antimicrobial, anti-HIV and anti-inflammatory activities as claimed by the traders. HIV-1 virus uses inflammation as one of its mechanisms to propagate. Therefore, the combination of the anti-inflammatory and anti-HIV RT activities of the concoctions may prove to attain an improved efficacy *in vivo*. While their antioxidant activity could improve the functionality of the immune system due to high concentration of various phenolics. The antimicrobial activities demonstrated the potential of the herbal concoctions in the treatment of infectious diarrhoea. The combined effects of the

various pharmacological properties of the concoctions demonstrated their potential as effective medicinal products based on their ethnopharmacological application. These products further have the potential of bridging the gap between efficient healthcare and the population that is economically and geographically disadvantaged. To ensure that more people can obtain quality healthcare regardless of the inaccessibility to commercial pharmaceutical drugs.

8.3. Recommendations

To improve the validation of the herbal concoctions, *in vivo* studies are required to evaluate the pharmacokinetics of the herbal concoctions on animal models and eventually on clinical trials. Mutagenicity of the concoctions should also be investigated to substantiate the safety of their consumption.

The isolation of the antioxidant compounds in *Kirkia wilmsii* and *Monsonia angustifolia* is required to further develop them into pharmaceutical agents, because they exhibited activity that was proximal to that of L-ascorbic acid (vitamin C). Toxicological studies of the plant species that have excellent biological activity is required.

Anti-HIV reverse transcriptase activity of the plant species is needed to investigate which plant species contributed the antiviral activities to the concoctions.

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Evaluation of Herbal Concoctions Sold at Ga Maja (Limpopo Province) in South Africa and In Vitro Pharmacological Evaluation of Plants Used to Manufacture the Concoctions

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Abstract

The aim of the study was to evaluate the biological activities and safety of commercial herbal concoctions manufactured in Ga Maja (Limpopo province). Microbial contamination was evaluated by spread-plating the concoctions on agar plates. The VITEK 2 instrument was used for identification of the pure cultures. Nutritional content of the concoctions was determined. Thin layer chromatography was used to analyze the chemical constituents of the extracts. The microdilution assay and bioautography were used to evaluate antimicrobial activity against selected microorganisms. Sodium, potassium, and zinc were elements most abundant in the concoctions. Phytochemical screening revealed the presence of various phytoconstituents. Acetone extracts of *Hypoxis hemerocallidea* and *Kirkia wilmsii* extracts had antioxidant activity. The minimum inhibitory concentrations values against test bacteria ranged between 0.02 and 0.63 mg/mL. Further studies are required to isolate bioactive compounds and evaluate their cytotoxicity. Caution in the consumption of the herbal mixtures should be adhered to.

Keywords

alternative medicine, antimicrobial activity, antioxidants

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The majority of people in developing countries, estimated to be about 80% of the world's population, depend on medicinal plants for their principal health care needs.¹ In South Africa, an estimated 65% to 80% of the citizens depend on medicinal plants as the primary health care system to treat various ailments.² The Limpopo province of South Africa is predominantly rural and has high levels of unemployment among its locals, subsequently poverty is prevalent. It is within these factors that the locals are heavily reliant on medicinal plants and use herbal medicines in combination with Western medicines or alone.³

Herbal concoctions have been described as a mixture of different plant species or plant parts to treat various health ailments. The formulas to these concoctions range from simple home remedies to more complex formulas administered by traditional healers to manage life-threatening diseases. Moreover, the latter ranges from simple preparations to more elaborate methods involving organic solvents and alcohols for more detailed extraction of crude plant chemicals.⁴ However, boiling of fresh herbal portions with water as means of extraction, thus a decoction, was a more favored method used traditionally to

prepare herbal concoctions.⁵ Various plant parts such as leaves, flowers, stems, and/or roots from different or the same plant species are used as ingredients for the medicines.⁶

The increase in commercialization of herbal mixtures in South Africa has been attributed to increased globalization and urbanization. Many traditional healers and herbalists have integrated into the system of using media outlets such as radio, television, and newspapers as a marketing platform for their herbal products and these include the Internet whereby social networks are of popular use.⁷

The characteristic(s) of the recipes and the manner of preparation are based on traditional theories and beliefs; however, the packaging and presentation of these medicines rather adopt

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Phytochemical screening and pharmacological evaluation of herbal concoctions sold at Ga Maja Limpopo Province

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ABSTRACT

Informal street merchants and traditional health practitioners at Ga Maja (Limpopo Province) primarily offer consumers semi-processed herbal preparations that are indicated to have blood cleansing, detoxifying, antidiarrheal and pain relieving properties. The focus of this study was to evaluate the phytochemical composition of the concoctions and substantiate the pharmacological effects and safety indicated by the traders. Five herbal concoctions and plant material used in their preparation were purchased from five independent traders and a laboratory concoction was prepared according to the traders' instructions. Possible bacterial and fungal contaminants were isolated and identified using Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Qualitative phytochemical analysis was determined using standard chemical tests and thin layer chromatography. Total polyphenol content was quantified. Antioxidant activity was quantified using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing power. Antimicrobial activities were determined using a broth micro-dilution assay and bioautography. Cell viability assay was used to determine the cytotoxicity of the concoctions. Pathogenic bacteria, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli* and *Klebsiella pneumoniae* were identified as bacterial contaminants. The commercial concoctions and the laboratory standard had similar phyto-constituents and phytochemical fingerprint profiles. The antimicrobial properties of the concoctions were a result of synergistic effects of the compounds because no single compound was observed

to have antimicrobial activities on the bioautograms. The phenolic content, antioxidant and antimicrobial activities varied substantially amongst the concoctions. The lack of standardisation methods reduces the pharmacological potential of the products. This study concludes that while plants with biological activities were used by the traders to prepare the concoctions, the efficacious concentrations to produce a therapeutic response were not adequately measured and adhered to. Furthermore, although the concoctions did not exhibit cytotoxic effects, toxicities may arise from endotoxins produced by the microbial contaminants. Hygienic processing and packaging are essential to ensure that consumers receive quality products that are safe to consume.

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1. Background

Informal street merchants and traditional health practitioners primarily offer consumers semi-processed herbal preparations that are commonly prepared in small batches. In preparing the herbal concoctions, fresh or dry plant material can be used; the plant material can either be macerated in water for several days or generally boiled in hot water (Ndhkala and Van Staden, 2012). In South Africa, herbal products that are sold by informal traders are usually indicated to be immune and energy boosters, blood cleansers, detoxifiers and aphrodisiacs (Ndhkala et al., 2009).

Some of these formulations are unstable, vary in strength and generally have short shelf lives. The poor physical conditions employed in preparation, such as unsterile working environment and storage,

contribute to the short expiry period. Unfortunately, the production of such remedies leads to the depletion and wastage of plant material (Nwankwo et al., 2012).

The plant parts commonly used as ingredients in preparation of the herbal concoctions include leaves, stems, barks, roots, rhizomes, bulbs and/or seeds. The complexity of the formulations is dependent on the severity of the ailment. Simple home remedies can be prepared for trivial ailments such as diarrhoea, coughs, pains and gastrointestinal disorder. However, more elaborate procedures of preparations are required for life-threatening conditions (Cano and Volpato, 2004).

A single plant species can produce numerous bioactive compounds that are neither stringently required for metabolic processes nor do they form part of nutrition. The production of the compounds is subject to the interaction of the plant with the environment in which it is supposed to thrive (Okem et al., 2015). The biological activity of these compounds is attributed to their role in the plants' survival. Some of these compounds are synthesised to effectively shield the plant from

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