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A study of the elemental analysis and the effect of the pressurised hot water extraction method (PHWE) on the antibacterial activity of Moringa oleifera and Moringa ovalifolia plant parts

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A Study of the Elemental Analysis and the Effect of the Pressurised Hot Water Extraction Method (PHWE) on the Antibacterial Activity of Moringa *oleifera* and Moringa *ovalifolia* plant parts



By

Charlene Makita

A Masters Dissertation submitted to the Faculty of Science

University of the Witwatersrand

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DECLARATION

I declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Masters of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of Candidate)
----------30^{th} ----------Day of--------JULY----------2014

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Abstract

Heavy metal pollution is an increasing phenomenon and contamination of these heavy metals has detrimental effects on the environment and humans. The concentrations of metals in the soil, leaves, stem bark and flowers of Moringa *ovalifolia* sampled from Okahandja, Okaukuejo, Halali and Tsumeb in Namibia were investigated. Acid digestion of all samples was performed utilising the microwave technique. Determination of elements from the extracts were analysed by inductively coupled plasma - optical emission spectroscopy (ICP-OES). No lethal amounts of heavy metals were found in the soil, leaves, stem bark and flowers of Moringa *ovalifolia*. The results from Moringa *ovalifolia* was compared to spinach from another similar study. On average, Moringa leaves contained more or less similar amounts of Zn (29.4 mg kg^{-1}) and Cr (13.2 mg kg^{-1}) but higher higher amounts of Fe (263.8 mg kg^{-1}) concentrations than spinach, with values of Zn (30.0 mg kg^{-1}), Cr (20.0 mg kg^{-1}) and Fe (190 mg kg^{-1}) from a previous study. In this study, selected statistical methods such as correlation analysis and principal component analysis were used to identify the origin of these metals in the soil, leaves and stem bark collected from the Moringa farm, national parks and the road side in Namibia. From the correlation analysis, it was found that Co, Mg, Cr and Mg pairs were highly significant to each other. The principal component analysis revealed that heavy metals gave 53.41% of the total variance in factor 1. Sources of Mg and Ni could be from vehicle emissions. Factor 2 revealed As, Co, Al, and Si contributing to a total variance of 25.15% Co suggesting that the metals present could be as a result of geologic sources. This

study proves that statistical analysis may provide a scientific foundation for the observation of heavy metal accumulation in samples.

Pressurised hot water extraction (PHWE) was used for the extraction of bioactive compounds from the leaves and stem bark of Moringa *ovalifolia* and Moringa *oleifera* samples. The aim of the study was to assess the effect of temperature and extraction process of the Moringa extracts on *Staphylococcus aureus (S. areus)*, *Enterococcus faecalis (E. faecelis)* and *Pseudomonas aeruginosa (P. aeruginosa)*. The antibacterial activities of the extracts at varying temperatures were evaluated using the disk diffusion method where the zones of inhibition were recorded. ANOVA, which was calculated at a significance level of 0.05, was used to analyse the data. Antimicrobial activity of the stem bark extracts and leaf extracts from different sites extracted at 25˚C and 80˚C showed significant difference. As temperature increased, a decrease in inhibition was observed for stem bark extracts from different sites tested againt *S. areus*, *E. faecelis* and *P. aeruginosa*. When the PHWE stem bark extracts collected in Tsumeb, were compared with ethanol and cold water extractions, the PHWE had more activity than other two methods. This proves that PHWE may be used as a novel extraction to get essential compounds from indigenous plant materials.

To my dad, mom and sisters whom I love dearly, thank you so much for your prayers and continuous support

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Not forgetting My Lord and Saviour who made this all possible

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List of Abbreviations and Symbols

ICP-OES	Inductively coupled plasma optical emission spectroscopy
PHWE	Pressurised hot water extraction
MAE	Microwave assisted extraction
SPME UAE	Solid phase micro extraction
LLE	Liquid-liquid extraction
SPE	Solid phase extraction
LOD	Limit of detection
LOQ	Limit of quantification
ε	Dielectric constant
S. areus	*Staphylococcus areus*
E.faecelis	*Enterococcus faecelis*
P. aeruginosa	*Pseudomonas aeruginosa*
UTI	Urinary tract infection
HIV	Human immunodeficiency virus
CAPE	Caffeic acid phenyl ester
AAS	Atomic absorption spectroscopy
SLE	Solid liquid extraction
PLE	Pressurised liquid extraction
SFE	Supercritical fluid extraction

Chapter 1: Introduction

Summary

This chapter gives the introduction of the research topic. It discusses the uses of traditional plants by humans and the techniques used to extract essential chemicals from plants are briefly discussed.

1.1 Importance of traditional medicinal plants

South Africa is one of the most diverse countries in the African continent and holds the position as the world's third most bio diverse country. It contributes more than 80% of its vascular plant species (Siegfried, 1989). Medicinal plants locally referred to as "muthi plants" form the foundation of customary traditional herbal medicine (Fyhrguist *et al*., 2002). South Africa has a substantial assortment of medicinal plants utilised in the treatment of vast diseases. It is suspected that there are over 27 million consumers of medicinal plants in South Africa (Lin *et al*., 1999; Mander, 1998).

In African societies, medicinal and poisonous plants contain compounds that act as healing and antibacterial agents such as polygoidal, anethole and safrole (Fabry *et al*., 1998). The healing use of herbal plants is mainly used in many rural communities in the form of remedies to cure illnesses from skin disorders to chronic bronchitis (Ahmad *et al*., 1998). In South Africa alone, approximately 20,000 tonnes of more than 700 medicinal plants are traded annually (George *et al*., 2007). The need for therapeutic use of medicinal plants is not limited to rural communities but it is needed in a great demand in urban communities (Matsiliza and Baker, 2001). Scientific research is important as it can lead to the progress in developing new and better plant-based medicine.

1.2 Antibacterial resistance

The number of diseases caused by various pathogens has increased dramatically in the past 20 years (Lister, 2000). Bacterial infections causing these diseases have been one of the leading causes of death in developing countries. Nevertheless, despite the many advances in pharmaceutical drugs, the misuse of antibiotics has led to decreasing susceptibility of microorganisms in the 20th century. A popular newspaper in the United States reported an incident of a new species of bacteria that has the ability to suppress even the strongest antibiotics, making the patient susceptible to various infections (www.usatoday.com). The increasing problem of multi-resistant bacteria has led researches to try and develop new strategies to fight antibacterial resistance (Höjgård, 2012). According to the World Health Organization (WHO), approximately 70-80% of the world's population use medicinal plants as antibiotics due to its low costs and lesser side effects (Uprety, 2012).

For many years, plants have co-evolved with pathogens. Therefore, they have developed various chemical pathways protecting themselves against attacks by the parasitic organisms. Hence, it's expected that various plant compounds would possess a variety of compounds that have potential antibacterial and antifungal activity (Debasis, 2012).

1.3 Traditional methods of extraction vs. Modern methods of extraction

Extracting phytochemicals from solid plant samples is a crucial step in preparation of pharmaceutical products, food and medicine. In the past, organic solvent extractions were the most commonly used procedure in preparation of

extracts from plant materials (Shotipruk *et al.*, 2004). Traditional extraction techniques such as soxhlet extraction and liquid-liquid extraction (LLE) are subject to a lot of errors and consume vast amounts of organic solvents, thus, rendering them harmful to the human and the environment (Andersson, 2007). Reports have shown that using this method tends to leave residual solvent in the product (Shotipruk *et al.*, 2004). Also, due to their non-efficient, and non-environmentally friendly properties, researchers are looking for alternative solvents and extraction techniques that are efficient and more environmentally friendly. Examples of such techniques for solids include supercritical fluid extraction (SFE), pressurised hot water extraction (PHWE), ultra sound assisted extraction and microwave assisted extraction (Andersson, 2007). These extraction techniques are referred to as modern extraction techniques. Pressurised hot water extraction is efficient because it uses water as a solvent (Dai and Mumper, 2010). During the extraction process, analytes are removed from the matrix and transferred into the fluid medium (Andersson, 2007). Factors may affect the extraction process, therefore, optimization is necessary for maximum recovery of target analytes. Due to the amount of hazard waste removal, researchers are seeking automated and environmentally friendly methods of extraction that require little to no use organic solvents.

Chapter 2: Literature review

Summary

This chapter focuses on the literature review while highlighting the following topics in detail: the nutritional and medicinal value of Moringa *oleifera*, the bacteria studied in this project as well as their classification, mineral content and green chemistry. Extraction methods such as the PHWE as well as analytical methods were used for quantification and determination.

2.1 Anthropogenic sources of heavy metals

Heavy metal pollution is a world-wide predicament because it is toxic and carcinogenic even at relatively low concentrations and can't be biologically or chemically degraded (Graphat and Yusuf, 2006). Some heavy metals may accumulate in living organisms causing various diseases and disorders. Heavy metals are generally discharged to the environments via automobile emissions, mining activities, battery industry, fossil fuels, metal plating and electronic industries. The process of biomagnification is where some metals are capable of bioconcentrating from the bottom of the food chain to the top making them toxic. (Pakade *et al.*, 2012). Trace nutrient metals (micronutrients) are inorganic elements that occur at low levels but are essential for human and plant processes (Liaghati, 2004). Major inorganic nutrients are needed in plants and humans in large amounts which include nutrients such as calcium (Ca), sodium (Na) and magnesium (Mg) (Ajasa *et al*., 2011). Examples of micronutrients are (Fe), copper (Cu), zinc (Zn), manganese (Mn) and Boron (B) and Cobalt (Co) (Ajasa *et al.*, 2011). Trace elements are mostly those that are not needed by humans and include lead (Pb), cadmium (Cd) and mercury (Hg).

2.2 Elements in soil

Metals in soil are derived by weathering of geologic material but tend to be immobile (Alloway,1995). In essence, factors determine the fate of metals in soil such as: pH, cationic exchange capacity (CEC), oxide content and type, and redox potentials. These factors also determine the bioavailability of metals in the soil (Alloway, 1995). At low pH's, the soil tends to be acidic, therefore making

metals more soluble and bioavailable for uptake (Pepper *et al.*, 1996). However, if pH is too low, metals can leach out from the soil, making them unavailable to plant roots (Pepper *et al.*, 1996).

Essentially, there are certain factors that affect metal uptake of plants from soil (Pepper *et al.*, 1996). These are:

(i) The concentration and speciation of metals in soil solution

(ii) The movement of the metal from bulk soil to the root surface

(iii)The transport of the metal from the root surface to the root

(iv)It's translocation from the root to the shoot

2.2.1 Maximum allowed values of metals in soil

Table 2.1 and 2.2 shown below demonstrate allowable limits of heavy metals in soil set by the USEPA and other Western countries. No set guidelines are provided by the South African Department of Environmental Affairs, Tourism and Agriculture. These standards help to regulate the content of these metals in soil and thus prevent pollution of the soil. Cadmium is regarded as one of the most toxic elements and thus has the lowest maximum allowed limits. The maximum allowed standards also does not include other metals such as mercury (Hg) and uranium (U) that are toxic. Further, standards do not take into account the various forms (species) of those which are more toxic like chromium (Cr) (Kabata-Pendias, 1995).

Table 2.1 Normal content intervals and maximum allowable limits of heavy metals in soils (Lacatusu, 1999)

Chemical Element	Normal Content interval ($mg\ kg^{-1}$)	Maximum allowable limits
Cd	0.1	3
Cr	2-50	100
Pb	0.1-20	100
Zn	3-50	300
Cu	1-20	100

Table 2.2 Values of maximum allowable limits (M.A.L) for heavy metals in soil ($mg\ kg^{-1}$) used in different countries (Kabata-Pendias, 1995)

Chemical Element	Austria	Canada	Poland	Japan	Great Britain	Germany
Cd	5	8	3	-	3	2
Cr	100	75	100	-	50	200
Pb	100	200	100	400	100	500
Zn	300	400	300	250	300	300
Cu	100	100	100	125	100	50

2.2.2 Soil-plant coefficient

Uptake of metal ions vary with different ions, in fact, competition between ions is very common (Pepper, 1996). For example, Zn^{2+} absorption is inhibited by Cu and H^+, while Cu absorption is inhibited by Zn, NH_4^+, Ca^{2+} and K^+ (Pepper, 1996).

A way of quantifying the relative differences in the bioavailability of metals in the plant is by utilising the transfer coefficient. This is a calculation of the metal concentration in plant tissue above the ground divided by the total concentration in soil (Alloway, 1995). Table 2.3 below demonstrates the general transfer coefficients of metals between plant tissue and soil (Pepper, 1996). It can be seen from Table 2.3 that Cd, Ti, Zn and Se seem to have the highest maximum soil-

plant coefficients. These transfer coefficients are for plants where no metal accumulation occurs in the leaves. Any transfer coefficients that are much higher than reported in Table 2.3 may suggest hyper accumulation of the metal (Pepper, 1996).

Table 2.3 The transfer coefficients of metals between plant tissue and soil (Pepper, 1996)

Element	Soil-plant coefficient
Cd	1-10
Cr	0.01- 0.1
Cu	0.01-0.1
Hg	0.1-10
Ni	0.01-0.1
Pb	0.01-0.1
Ti	1-10
Zn	1-10
As	0.01-0.1
Be	0.01-0.1
Se	0.1-10
Sn	0.01-0.1

2.3 Ethnobotanical

The definition of ethnobotany is the investigation of the relationship between plants and people. “Ethno” means ethnic while “botany” means the study of plants (Choudray, 2008). The primary focus of ethnobotany is how plants are managed and handled in human cultures such as the use of plants in food, medicine, cosmetics, fabrics, ceremonies and even music. The association of plants with human society is not only limited to the catering of food or shelter, nonetheless. It also includes its use in health care (Choudhary, 2008).

2.3.1. Medicinal plants under investigation

The Moringa plant belongs to the family *Moringaceae.* It is one of the 13 species native to India. The plant has many different names by different countries. The 13 species of Moringa include Moringa *arborea*, Moringa *borziana* Mattei, Moringa *concanensis*, Moringa *drouhardii*, Moringa *hildebrandtii*, Moringa *longituba*, Moringa *oleifera* Lam., Moringa *ovalifolia*, Moringa *peregrina*, Moringa *pygmaea*, Moringa *ruspoliana* and Moringa *stenopetala* (Tropics.org, 2012). Moringa *oleifera* is known to thrive and tolerate harsh drought conditions due to its efficient tubular root system (Coppin, 2008). The tree is slightly lean with flaccid branches that grow roughly about 10 m in height. When the tree is needed for commercial purposes, it is cut down to 1 m to allow the leaves and pods to be within arm's reach.

The plant is known for its exceptional nutritional value and healing, hence it has been used for different purposes (Shindano and Kasase, 2009). Moringa *oleifera* is the most cultivated species. It is cultivated in many African countries such as Ghana, Senegal, Malawi and South Africa. In South Africa, Moringa is consumed as a nutritional supplement in the form of capsules or leaf powder (Coppin, 2008). The Moringa tree is known as the miracle tree because all its parts have nutritional and medicinal benefits (Fahey, 2005). The uses of Moringa are very diverse and most parts of the plant such as the roots, leaves, flowers, green pods are edible and can be consumed (Amaglo *et al.*, 2010). The seeds are used for water purification for contaminated water while the roots and tree bark are used as tanning agents (Amaglo *et al.*, 2010). The leaves are consumed in either powder form or in its

fresh state, however, in its dried state, it is higher in nutrition consisting of calcium (4 times more calcium than milk), protein (2 times more protein than yoghurt), vitamin A (4 times more vitamins than carrots) and vitamins C (7 times more vitamins than oranges) which is rich in antioxidants. Figure 2.1 below illustrates the importance of Moringa compared to other common foods.

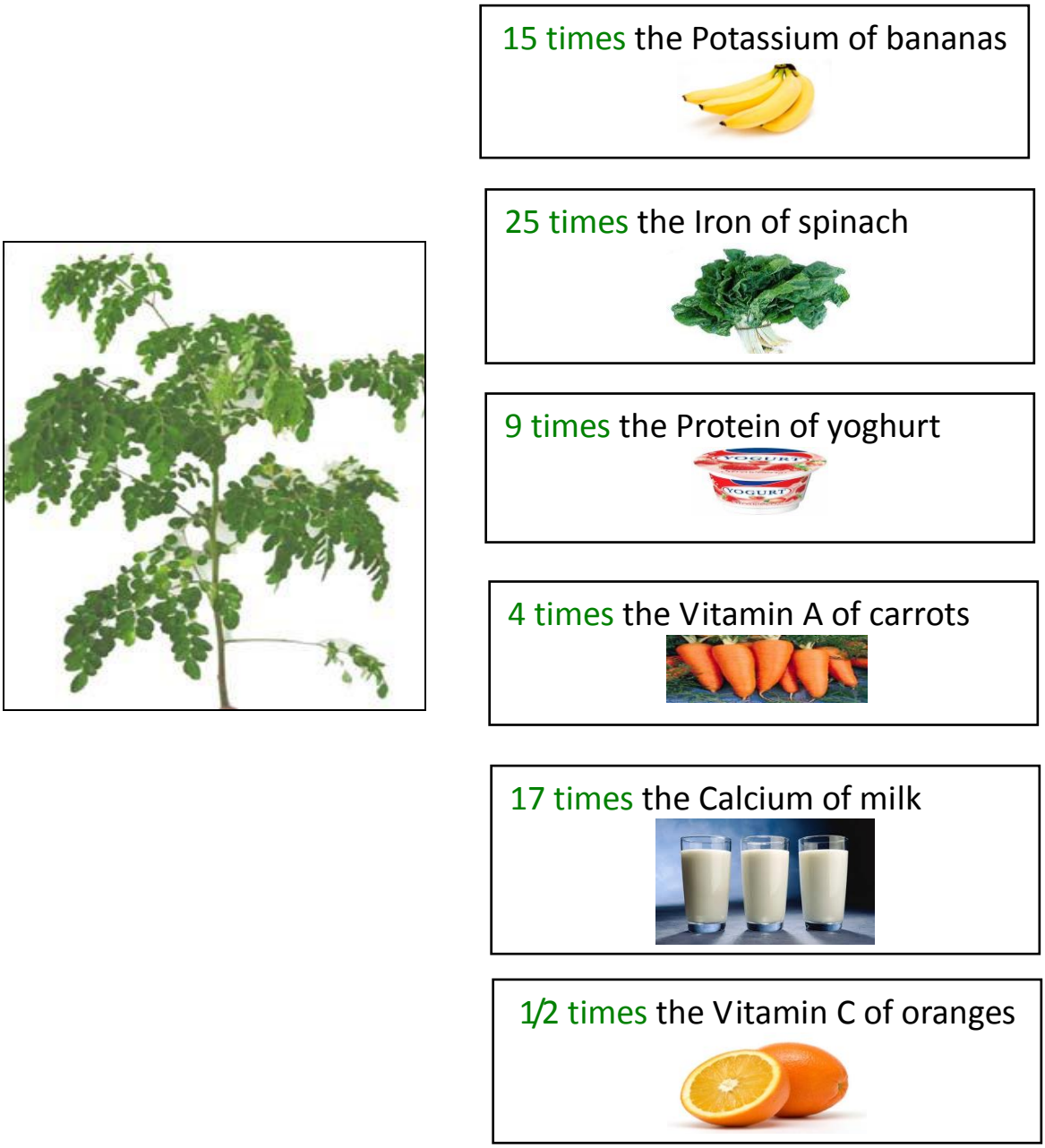


Figure 2.1 Estimation of Moringa contents (Moringa4life, 2011).

This study is the first in series of planned studies to investigate another species called Moringa *ovalifolia*, a species endemic to Namibia. Moringa *ovalifolia* (also known as Phantom or Ghost tree), is restricted in its distribution in the wild where it mainly grows on rocky hills.

The Figures 2.2 and 2.3 illustrate the pictures of Moringa *oleifera* and Moringa *ovalifolia*. The trees differ in size of the stems and leaves of Moringa *ovalifolia.* They are somewhat broader compared to Moringa *oleifera.* The bigger size of Moringa *ovalifolia* allows it to survive in much harsh environmental conditions than Moringa *oleifera*.



Figure 2.2 Moringa *oleifera*, picture taken from Pretoria West, Gauteng, South Africa



Figure 2.3 Moringa *ovalifolia*, picture taken from Tsumeb, Namibia.

2.3.2 Medicinal aspect of Moringa

In a study by Anwar *et al*. (2007), Moringa *oleifera* is the most cultivated amongst the *Moringacea* family. It's known as a nutritious vegetable in many parts of the world. In a study by Fugile *et al.*, (1999), the many uses of Moringa were reported such as ally cropping, animal forage, biogas, domestic cleaning agent, fertilizer, foliar nutrient, green manure, gum, honey- and sugar cane juice- clarifier, honey, medicine, biopesticide, pulp, rope, tannin for tanning hides and water purification (Fugile *et al*, 1999). The root are used as an anti-inflammatory agent against rheumatism and reduces back and kidney pain, the leaves cure and relieve flu like symptoms like sore throats fevers and is said to have anti-cancer properties

(Fugile *et al.*, 1999). Reports have revealed the leaves of the plant being a hypocholesterolemic agent, anti tumor and hypotensive agent (Williams *et al.*, 2013). Reports have presented the leaves of the plant being a hypocholesterolemic agent, anti-diabetic agent, anti tumor agent and hypotensive agent (Williams *et al.*, 2013). The stem bark has antibacterial and anti–tubercular activities, the flowers are said to decrease tumors and seeds have anti-tumor activity (Anwar *et al.*, 2007). A study by Chumark *et al*., (2008) reported the reduction of cholesterol levels found in white rats after the exposure of Moringa *oleifera* leaf extracts (Chumark *et al.*, 2008).

Table 2.4 below summarises the medicinal properties of the Moringa species found in literature.

Table 2.4 Summary of the medicinal uses of the different plant parts of Moringa *oleifera* (Anwar *et al.*, 2007).

Parts of Moringa *oleifera*	**Medicinal properties**
Roots, bark, fresh leaves and defatted and shell free seeds	Antimicrobial
Leaves, roots, bark and seeds	Anticancer
Leaves, flowers and roots	Antihepatotoxic
Leaves and flower buds	Antiulcer
Roots, dried seeds,	Antiinflammatory
Flowers, seeds, leaves, bark and roots	Antiinflammatory
Dried leaves and oil from dried seeds	Antioxidant
Leaves and fruits	Antihyperlipidemic
Roots and bark	Antifertility
Roots	CNS depressant

2.4 Antibacterial activity

The misappropriate use of antibiotics have caused an increase in resistant bacteria (Franklin, 1992). The notion that antibiotics are the ultimate cure has lead physicians to routinely prescribe antibiotics to patients for any infections observed (Franklin, 1992). The overuse of antibiotics, such as penicillin, increased the probability for any the infection to return again because after administering the antibiotics, bacteria are left behind causing them to multiply and develop a resistance gene (Franklin, 1992).

Although most prescribed antibiotics administer bacterial resistance, according Ankril and Mirelman, 1999, some antibiotics prevent bacterial resistance such as

allicin because it inhibits thiol containing enzymes found in the bacteria (Ankril and Mirelman, 1999).

The alteration of enzymes structure, based on the lock and key model, is the cause of bacterial resistance (Ankril and Mirelman, 1999). Modification of the enzymes active site prevents the substrate from fitting into the active site. Due to this process, biochemical enzyme reactions are administered therefore preventing bacterial respiration and lack of survival (Ankril and Mirelman, 1999).

Therapeutic plants possess antioxidant properties that lead to antibacterial activities. The application of plant extracts and bioactive compounds, both with known antibacterial properties, can be of great usefulness in remedial treatments. In previous years, investigations have been conducted in various countries to prove such efficiency (Nascimento *et al*., 2000). In a study by Saadabi and Abu, (2011), the authors reported aqueous extracts of Moringa *oleifera* having inhibitory effects against gram pathogenic bacteria such as gram positive bacteria *S. areus*, *B. subtilis* and gram negative bacteria *P. aeruginosa* and *E. coli*. Table 2.5 below summarises the antibacterial effects of Moringa plant found in literature.

Table 2.5 Ethnobotanical data of the Moringa species in literature

Scientific name	Plant origin	Solvent/ Bioassay	Antimicrobial activity	References
Moringa *Oleifera*	Seeds, chitosan	Nacl	*B. subtilis*	Chen, 2009
	Seeds	Aqueous	*P. aeruginosa, S. pyogenes, S. areus*	Suarez *et al.*, 2005
	Seeds	Aqueous, methanol	C. *albicans, S. areus, B. subtilis, E. coli, P. aeruginosa, A. niger*	Fluck, 1955
	Leaves	Petroleum ether	*P. mirabilis*	Devendra *et al.*, 2011
	Root bark	Ethyl acetate, Acetone	*S. areus, E. coli, S. gallinarum, P. aeruginosa*	Dewangan *et al.*, 2010; Renu *et al.*, 2010
	Stem bark	Petroleum ether	*B. megalenum, B. subtilis, B. cereus, S. pyogenes, S. areus, E. coli, P. aeruginosa, S. dysenteriae, S. typhi, V. cholerae*	Das *et al*., 2012
Moringa *stenopetala*	Leaves, seeds	Bioassay fractionation	*S. areus, S. tyohi, S.*	Catauro *et al.*, 2004

	shigella, C. albicans

2.4.1 Bacteria types

Clinical isolated bacteria used in the study are: *Staphylococcus aureus, Enterococcus Faecelis and Pseudomonas aeruginosa*.

2.4.1.1 *Staphylococcus areus* its classification, morphology and identification

Figure 2.4 shows the staphylococcus areus bacteria. Members of the genus Staphylococcus (staphylococci) are Gram-positive cocci that are usually arranged in clusters (grape-like) (Ryan and Ray, 2004). Table 2.6 shows the classification of bacteria.



Figure 2.4 Gram stain of *Staphylococcus areus* cells, images from CDC Public Health Image Library, PD US HHS CSC, Scanning electron microscopic images of bacteria. 10 µm

Table 2.6 Classification of *S. areus*

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*Areus*

The genus *staphylococcus* contains 37 species, 16 of which are found in humans. The most virulent of these species for man in the genus include *S. areus* (Jia *et al.*, 2005). The cells of Staphylococci appear in the microscope as spherical shaped cells about 0.5 to 1.7 μm arranged in irregular clusters. The term "clusters" is derived from Greek term "staphyl" and aureus due to its distinct golden colour. The golden colonies produced by the bacteria produce triterpenoid carotenoids (Gumbo *et al.*, 2004). These pigments located in their cell membrane which shields the bacteria from toxic oxidants such as polymorphonuclear cells (PMN) and phagocytes (Clauditz *et al.*, 2006). *S. aureus* is a facultative anaerobe that grows by aerobic respiration. On 5% sheep agar, some of the colonies may appear to have a dirty opaque colour, slightly raised and haemolytic. The bacteria can grow at elevated temperatures of up to 37ºC and at an optimum pH of 7,5 (Maritza, 2007).

S. areus is a highly successful colonizer of humans and animals. The *S.* pathogenic bacterium resides in skin, hair follicles, and warm blooded animals and resides in moist areas such as nasal membranes and the groin (Maritza, 2007). Their ability to survive in the air, on objects and in environments such as hospitals is due to their ever evolving multi-resistant and hypervirulent strains (Maritza, 2007). These strains provide *S. areus* the ability to spread rapidly over wide

geographical areas leading to the cause of human infections worldwide beyond the hospital environment but also including communities (Maritza, 2007).

The *S. areus* pathogenic bacterium is in fact a major human pathogen causing *a* series of infections on the skin, soft tissues, bone, lung, heart, brain or blood making humans its natural reservoir (Irving, 2005). These infections can lead to skin abscess, and cellulitis to invasive bacteraemia, endocarditis, and septic arthritis (Emily, 2010). The infections caused by *S. areus* are primarily associated with its complex genome and it's capability to adapt to various environments including living creatures, antibiotics and the human immune system (Goerke and Wolz, 2006; Takeuchi *et al.*, 2006). Even outside of a host, *S. areus* has the ability to withstand environmental stresses such as osmotic stress, starvation, temperatures as high as 50˚C. Due to its high resistance, it can be found even in the cleanest facilities, however, as more patients are treated outside hospital surroundings, it's starting to become an escalating concern in communities (Furuya and Lowy, 2006; Lowy, 2003).

S. aureus has a noteable capability of developing resistance against antibiotics which commenced shortly after the clinical use of the penicillin, a beta- lactam antibiotic. This ongoing bacterial resistance can be traced to bacteriophages that have the capability of converting non tolerant organisms to tolerant organisms (Lowy, 2003). Other antibiotics responsible for antibiotic resistance include vancomycin which causes cross-tolerance to the killing effects of other cell wall inhibitors (Irving, 2005; Lyon and Skurray, 1987). Some strains become resistant to multiple antibiotic such as erythromycin and gentamicin (Irving, 2005).

2.4.1.2 *Enterococcus faecelis its* classification, morphology and identification

Figure 2.5 shows *Enterococcus faecelis* cells. Table 2.8 shows its classification.

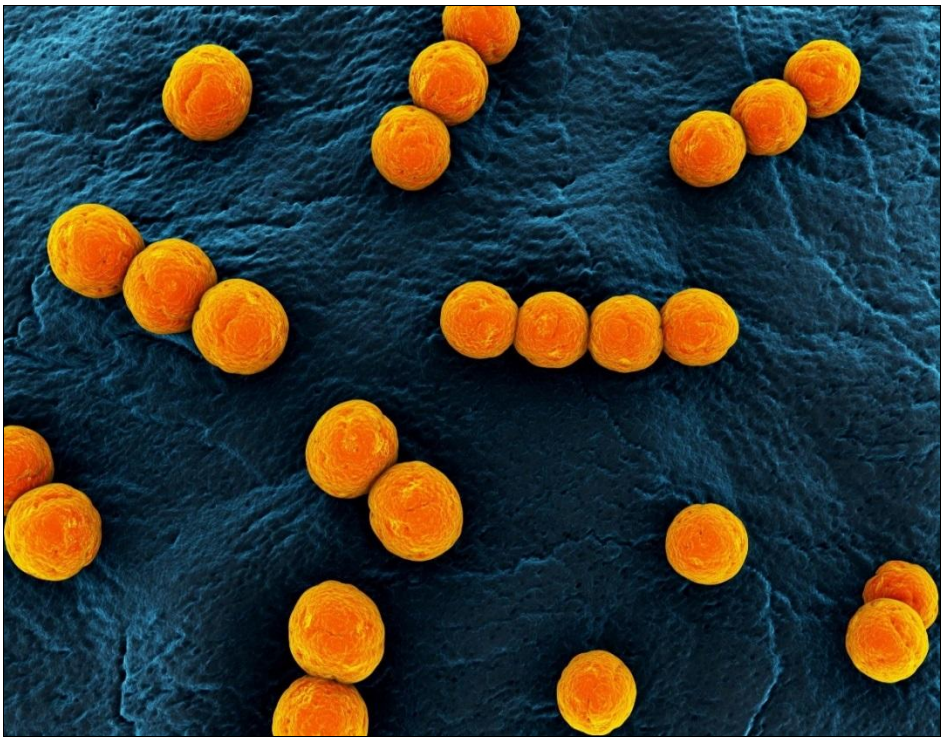


Figure 2.5 Gram stain of *E. faecelis* cells (www.bioquell.com), 10 µm

Table 2.7 Classification of *E. faecelis*

Domain	Bacteria
Phylum	Firmicutus
Class	Bacilli
Order	Lactobacillus
Family	Enterococcaceae
Genus	*Enterococcus*
Species	*Enterococcus faecelis*

Enterococcus faecelis (E. faecelis) is a gram positive, facultative immobile anaerobe. This microbe can withstand harsh environments such as high alkaline conditions (pH 9.6) and salt concentrations. They have the ability to grow and

survive at temperatures ranging from 10°C to 45°C (Stuart *et al.*, 2006). In nutrient broth, it grows as a facultative anaerobe. Many strains catabolize sugars such as carbohydrates, glycerol and keto acids (Stuart *et al.*, 2006).

The derivation of *E. faecelis* is derived from human and animal origin. It has been isolated from food such as cheese and sausage (Foulqui *et al.*, 2006). It was long supposed that *E. faecelis* infections were from urinary tract infections, but recent studies confirm that hospital environment contributed to the *E. faecelis* pathogenicity (Ryan and Ray, 2004).

Enterococcus infections include (Lopes, 2005):

- Urinary tract infections (UTI)
- Surgical site infections
- Cholangitis
- Endocarditis
- Meningitis

The exact number of UTI in South African hospitals is currently unknown, however, hospitals in the USA acquire close to 12 % of UTI by the Enterococcus species (Lopes, 2005). Virulence factors are responsible for *E. faecelis* infections: The cytolysin is a toxin that contributes to the animal modes of infection. Cytolysin in combination with gentamicin resistance is accountable for the five-fold increase of death in human patients (Huycke *et al.*, 1991).

Within the community, *E. faecelis* strains are commonly susceptible to all antibacterial agents. Antibacterial agents include aminoglycosides, penicillin, oxacillin and vancomycin (Amyes, 2007; Courvalin, 2006). However, because of

the frequent occurrence of vancomycin, resistance to this antibiotic is becoming more common. Treatment for the resistance include linezolid and daptomycin, however, if susceptibility arises, ampicillin is the better choice (Arias *et al.*, 2010).

2.4.1.3 *Pseudomonas aeruginosa* its classification, morphology and identification

Figure 2.6 shows the *Pseudomonas aeruginosa* (*P. aeruginosa*) cells. *P. aeruginosa* is a classic pathogen belonging to the genus Pseudomas (Mackie and McCartney, 1989). Its classification is shown in Table 2.7.

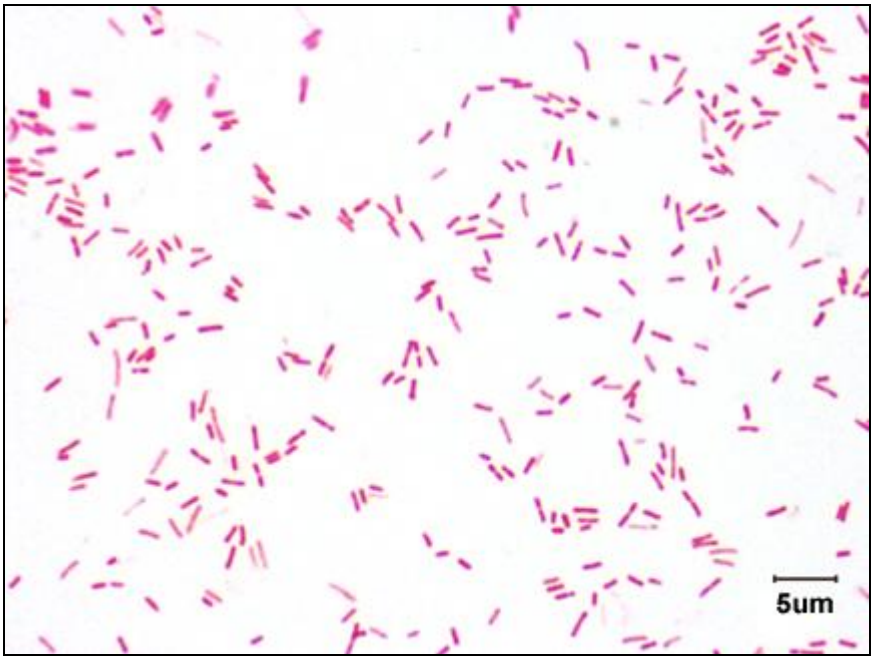


Figure 2.6 Gram stain of *Pseudomonas aeruginosa* cells (www.textbookofbacteriology.net/pseudomonas)

Table 2.8 Classification of *Pseudomonas aeruginosa*

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Pseudomonadecaue
Genus	*Pseudomonas*
Species	*areuginos*

P. aeruginosa is a gram negative obligate anaerobe measuring approximately 0.6 x 2 µm. It is usually found as a single bacteria, pairs and on occasion , it's found in short chains usually discharging a grape like fruity odour and has the ability to grow at temperatures reaching as high as 42 ˚C (Brooke *et al* ., 2007). Like many species belonging to the *Pseudomanadaceae* family, *P. aeruginosa* measures around 1.5-µm to 3.0µm in width and moves around with its flagellum. Under ultra- violet light it emits a yellow pigment known as fluorescin and a blue pigment known as pyocyanin. When fluorescin and pyocyanin are combined, a bright green colour is produced which in turn diffuses through the medium (Ryan and Ray, 2004).

P. aeruginosa is known as an opportunistic pathogen because of its ability to infect individuals with a weakened immune system. It typically inhabits soil, water, skin flora, vegetation, hospital food, man-made and natural environments. They can often spread from individual to individual via contact with fomites or by even touching hospital sinks, taps, mops etc. because they colonize those areas efficiently. They can also spread by ingestion of contaminated food and water (Baron, 1996).

As mentioned earlier, *P. aeruginosa* is an opportunistic pathogen that "preys" on both healthy and immunocompromised patients. This bacterium is notoriously known for its mode of infection. Once it enters the host, it releases an exotoxin which is comprised of two units: A and B. This is known as the A and B toxin which is shown in Figure 2.6. The A-B subunit which has toxic activity is internalised (Pier *et al.*, 2004). The following infections arise as a result of this toxin (Trautmann *et al.*, 2004):

- Respiratory and urinary tract infection
- Pneumonia
- Ocular infection
- Ear infections such as otitis media
- Surgical site infection

HIV infected patients, diabetes, cystic fibrosis are more prone to acquiring an infection. One of the worst characteristics associated with *P. aeruginosa* is its low susceptibility to commonly used antibiotics. This frequent resistance is attributed to its efficient efflux pump chromosomally encoded with antibacterial resistance genes (Poole, 2004). Many of its strains are susceptible to antibiotics such as gentamicin, amikacin and colistin as a result, resistance has developed making susceptibility testing vital (Baron, 1996).

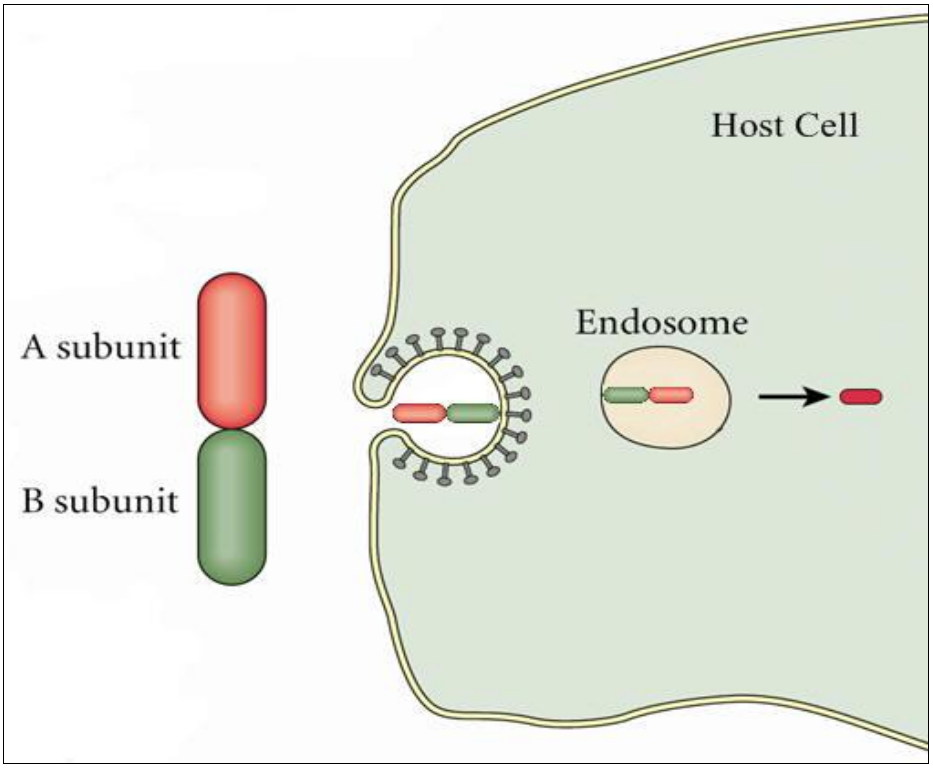


Figure 2.7 A-B toxin entry into host cell (Pier *et al.*, 2004)

2.5 Phytochemical studies

In the last decade, there has been a renewal of interest in natural medicine. Due to increasing costs of pharmaceutical drugs, consumers are seeking cheaper, natural and more affordable plant medicines (Newman and Cragg, 2007). Natural products as a possible source for new medicines have been witnessed among academic circles as well as pharmacological corporations (Newman and Cragg, 2007). According to Cowan *et al.*, (1999), 60–80% of antibacterial and anticancer drugs were from natural origins and in 2000, nearly 60% of all drugs in scientific trials for the range of cancers had natural origins. Natural products have led to the development and discovery of new drugs by producing derivatives of natural products such as synthetic analogues like penicillin. There are approximately

250 000 known plant species and only a small fraction has been investigated for the presence of antimicrobial and antifungal compounds (Cowan, 1999). Therefore, the need to access these natural products, their functions and usefulness is the driving force behind natural product research leading to the development of new drugs intended to fight infectious and other endemic diseases (Cowan, 1999).

What defines a natural product? According to Samuelsson (1999a), natural product can be accessed from plant materials or organisms such as microorganism that haven't undergone processing and standardisation (Samuelsson,1999).With that, wholesome compounds such as alkaloid, sugars, coumarin, glycosides, lignin, steroids, flavonoids, terpenoids are isolated from plants, animals or microorganisms. Plants produce these wholesome compounds known as secondary metabolites (Samuelsson, 1999).

The use of plant extracts with known antibacterial and anti-cancerous properties can be of great significance in future treatments. Plants contain a rich source of nutrients for many organisms like bacteria, fungi and insects. Secondary metabolites have served as a template for many drug discoveries. Unlike primary metabolites, secondary metabolites are not involved in plant growth or reproduction, but are involved as the plants defense mechanism (Craney, 2012).

Secondary Metabolites main role can be defined as:

- Defence against fungi, bacteria, viruses , insects and vertebrates (herbivores)
- Transporting metals

- Ability to protect the plant against physical stress and UV light
- Acts as signals between symbiotic microbes and plants (Craney, 2012)

According to Salim *et.al,* (2008) , plant secondary metabolites have been utilized as drug precursors, drug protoypes and pharmological probes to aid in drug discovery research and clinical trials (Salim, 2008). Due to increasing cases of drug- resistant pathogens, attention has been drawn to identify compounds in secondary metabolites responsible for antibacterial and antifungal properties (Ncube et.al 2008.)

The plant structure contains different phytochemicals that aid in the defense against pathogens. Distribution of phenolics in the plant are very tissue specific. The external layer of the plant contains a waxy cuticle leaving it home to a lot of lyphophilic phenolic compounds such as flavones and flavanol methyl ethers (Lattanzio *et al.,* 2006). Phenolic compounds such as alkaloids, diterpenoids, steroid glykoalkaloids and various suberin, cutin, other phenolic compounds are also found in the external layer of the plant which hinders fungi and bacterial growth. Thionins, an s-rich protein which is found on the cell wall of plants, are known to inhibit the growth of fungi and bacteria by accumulating after antimicrobial attacks (Mazid *et al.*, 2011).

Preliminary studies have shown that Moringa is found to contain a variation of phytochemicals and biological activities including antioxidants. The role of the antioxidants is to minimise the progression of chronic diseases by directly weakening oxidative damage of a tissue by scavenging free radical species (Williams, 2013). Phytochemicals found in Moringa *oleifera* include carotenoids, vitamins, minerals, amino acids, sterols, glycosides, alkaloids, flavonoids and

phenolics as reported by Anwar *et al.*, (2007). The particular function of phytochemicals still remain unclear however as a considerable number of studies have shown that they serve a specific function in plants, pests and diseases. Therefore, screening of plant extracts that possess antibacterial activity and the phytochemicals responsible for the activity, represent a starting point for antibacterial drug discovery. Phytochemical analyses have attracted the attention of plant scientists due to the advancement of the development of new and advanced practices. These procedures play a significant role in the pursuit for supplementary resources for the pharmaceutical industry (Mann, 1994)

2.5.1 Useful antibacterial phytochemicals in Moringa

2.5.1.1 Alkaloids

Alkaloids are known for their medicinal uses and have been documented to possess pharmacological effects such as antiprotozoal, cytotoxic and anti-inflammatory properties (Akumu et al., 2014). Alkaloids such as quindoline and cryptolepine isolated from Sida *Acuta* are found to possess antibacterial activities (Karou *et al.*, 2006). Alkaloid salts such as berkelium, specifically berbenium chloride, a member of protoberberine class of isoquinoline alkaloids not only eliminates bacterial plasmids, but also blocks transcription and replication in bacteria by binding to extra-chromosomal DNA (Davidson *et al.*, 1977). Figure 2.8 shows the general structure of an alkaloid.

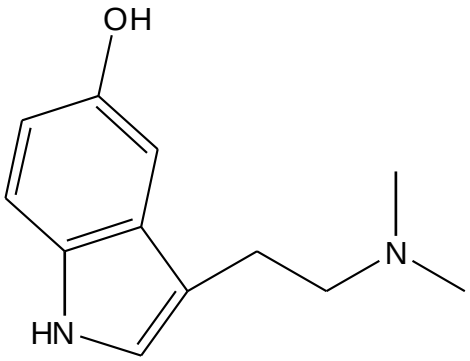


Figure 2.8 A general structure of an alkaloid, image taken from www.shekhar-alk.blogspot.com

2.5.1.2. Caffeic acid

Caffeic acid is a hydroxycinnamic acid and a classified polyphenylpropanoid. Yellow in colour, it contains phenolic and acrylic functional groups. It is known to possess anti- cancerous and anti-tumor properties (Chung *et al.,* 2004). Caffeic acid and its derivative caffeic acid phenyl ester (CAPE) has been used as anti-cancer drug eliminating liver metastasis (Chung *et al.,* 2004). This was performed by suppressing the tumor of HepG2 tumor xenografts in nude mice in vivo. It also eliminated MMP-9, an enzyme responsible for metastasis and cancer (Chung *et al.*, 2004). It's found in every plant species because it's used as an intermediate in the biosynthesis of lignin (Eckardt, 2002). Another study also portrayed the antibacterial effects of caffeic acid. When researches compared membrane damaging abilities of caffeic acid, chlorogenic acid and cinnamic acid, they found that these polyphenylpropanoids damaged gram negative bacteria compared to the other compounds (Hemaiswarya and Doble, 2010)

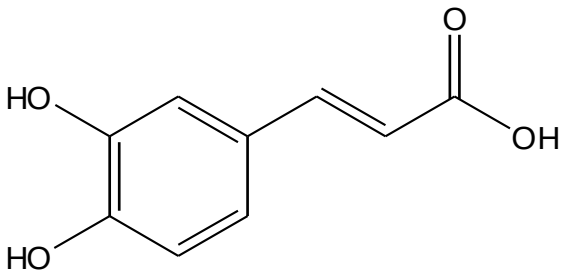


Figure 2.9 The structure of caffeic acid (Oboh *et al.,* 2013).

2.5.1.3. Tannins and gallic acid

Tannic acid and gallic acid are part of a variety of polyphenols which are bioactive compounds containing secondary metabolites (Chung, 1998). They are found specifically in leaves, roots and flowers of plant parts. They contain an aromatic ring and a hydroxyl group (Figure 2.10). Tannic acid is subdivided into two groups: hydrolyzable tannins and condensed tannins. In the presence of hydolysis, tannic acids will hydrolyze into carbohydrates such as gallic acid and its stable form egallic acid (Chung, 1998).

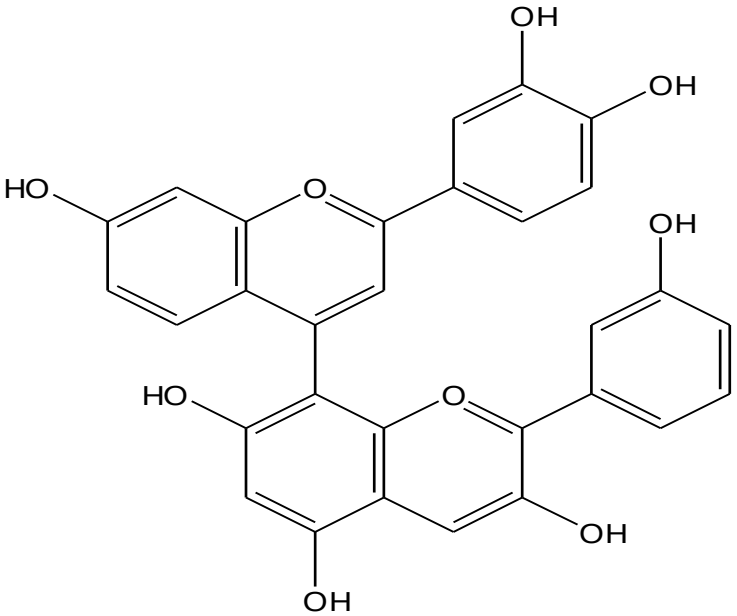


Figure 2.10 General structure of tannins (www.phytochemicals.info)

These polyphenols are widely recognized for their anticarcinogen activities and the reduction of mutagens in cells (Gülçin *et al*., 2010). It's reported that these anticarcinogen and antimutagen activities are linked to their antioxidant activities (Gülçin *et al*., 2010). The antioxidants prevent free radical formation that is generated in the process of carcinogenesis. This scavenging activity leads to a decrease in oxidative stress (Gülçin *et al*., 2010). The antibacterial activity of tannins has been reported in a recent article by Vinoth *et al*., (2012). The phytochemical screening of *M. oleifera* leaves, revealing the presence of tannins, gallic acid and other phytochemicals, the growth of gram positive bacteria *S.aureus* was inhibited utilizing the disk diffusion method (Vinoth *et al.,* 2012).

2.5.1.4 Glucosinolates

Glucosinolates are a class of secondary metabolites (Polat, 2010). These are stored in vacuoles in plants and contain sulphur and nitrogen compounds as shown in Figure 2.11. They are found in the leaves, stem, roots and bark of Moringa *oleifera* (Nouman, 2012). It is reported that glucosinolates, especially sinigrin, is responsible for the bitter taste found in Moringa leaves (Nouman, 2012).

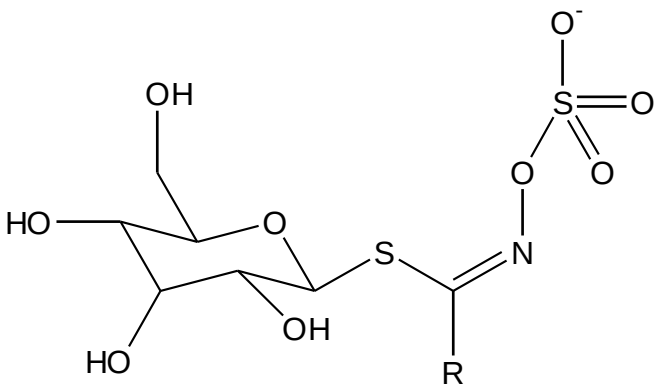


Figure 2.11 A general structure of glucosinolates (Redovnikovic *et al.,* 2008)

The R groups found on the compounds lead to a wide variation in polarity and biological activity of the natural products (Hayes *et al.,* 2008). The plants contain an enzyme called myrosinase, a thioglucosidase which, in the presence of water, catalyses glucosinolates resulting in substrates such as isothiocyanes, thiocyanates and nitriles (Hayes *et al.*, 2008).

The enzyme acts as a plant defence mechanism and only interacts with the substrates once the plant undergoes pathogen attack or wounding (Amaglo *et al.*, 2010). In Moringa, the most abundant of these glucosinolates is is 4-*O*-(α-l-rhamnopyranosyl-oxy)-benzylglucosinolate, otherwise known as glucomoringin (Amaglo *et al.*, 2010).

According to literature, glucosinolates such as 4-(4'-O-acetyl-α-L rhamnopyranosyloxy) benzyl isothiocyanate, benzyl glucosinolates, 4-(4'-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocy-anate**,** benzyl glucosinolates, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate, niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α-L-rhamnopyranosyloxy) are known for their bacteriacide, fungicide and anticancer properties (Fahey, 2005).

When Moringa seeds were tested for their tumor preventative potential, the hydrolysis product isothiocynate, niacimin and 4- (α- rhamnopy-ranosyloxy) benzyl glucosinolate were isolated and tested against the Epstein Barr virus antigen against the Epstein Barr virus antigen, usually known to cause Burkitts lymphoma, in an *in vivo* assay (Guevera *et al.*, 1999). The results showed total inhibition against the Epstein Barr virus cells which was achieved by preventing

carcinogen molecules from the target site and activating enzymes for the protection against carcinogen molecules (Guevera *et al.*, 1999).

2.5.1.5 Chlorogenic acid

Chlorogenic acid, known to be an ester of caffeic acid, is an abundant polyphenolic acid and antioxidant found in the leaves of Moringa (Amaglo *et al.*, 2010). This compound has many medicinal properties such as inhibiting glucose-6-phosphatase an enzyme responsible for raising blood glucose levels. It is also known to inhibit carcinogenic cells from on opportunistic fungal pathogen *C. albicans* which is responsible for morbidity in patients that have a compromised immune system due to HIV, chemotherapy and bone marrow transplant. Results indicated that chlorogenic acid was able to minimize the membrane potential of the pathogen (Sung, 2010).

It's also known for its antibacterial properties by inhibiting microorganisms in combination with other bioactive compounds (Hemmerle, 1997). Figure 2.12 shows the general structure of chlorogenic acid.

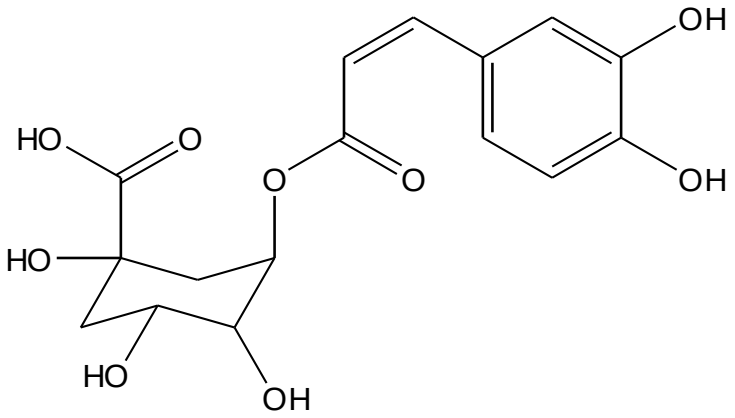


Figure 2.12 General structure of chlorogenic (Natarajan *et al.*, 1996)

2.6 Metal analysis

2.6.1 ICP-OES

The inductively coupled plasma (ICP-OES) is increasingly becoming a preferred source in analytical atomic adsorption (Greenfield *et al.*, (1964); (Wendt and Fassel, (1965). This is due to its efficiency and ability to analyse multi-elements in solution for a variety of samples which range from biological, ecological environmental and organic samples. It was first utilised by Greenfield *et al.*, (1964), followed by Wendt and Fassel, (1965). Since then, it has been updated and recommended as the best analysis method compared to its older spectroscopic counterparts such as atomic adsorption and x-ray fluorescence because of its high precision, accuracy and adaptability (Christian, 1980; Greenfield *et al.*, 1964; Wendt and Fassel, 1965).

2.6.2 Instrument

The basic components associated with ICP-OES is the sample introducing system which consists of a peristaltic pump, nebulizer, spray chamber and drain assembly, the gas supply, ICP torch and the plasma; transfer optics and optical spectrometer; detectors and computer all of which are shown in Figure 2.13.

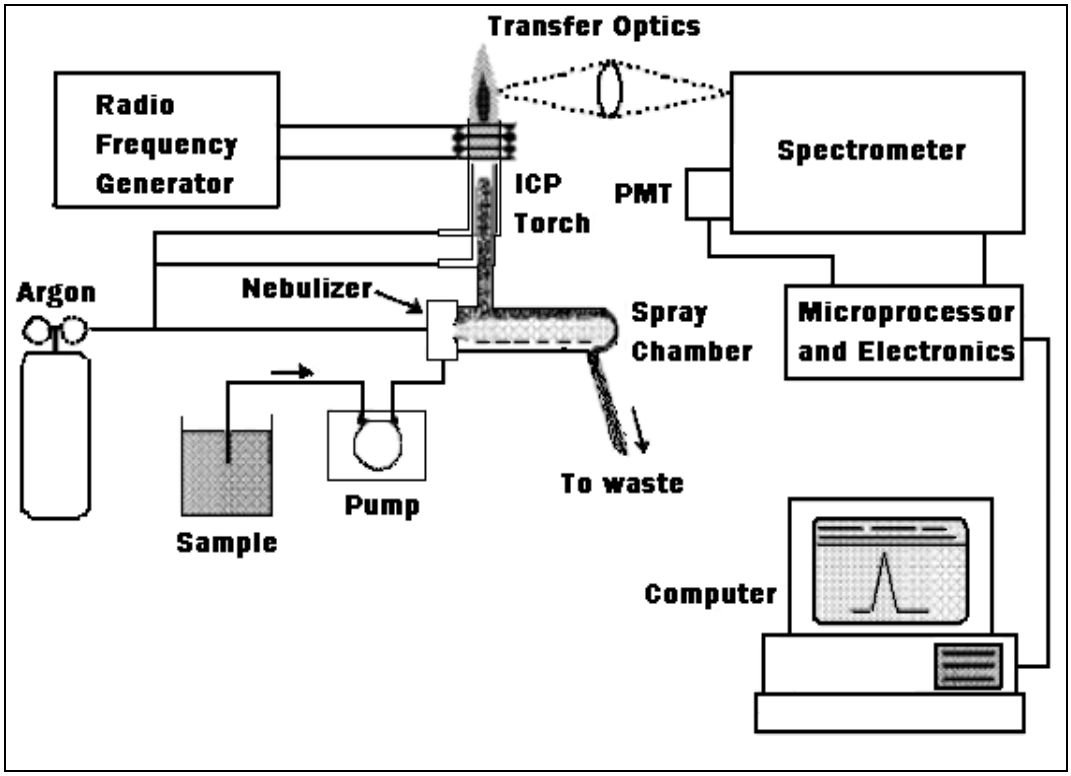


Figure 2.13 Schematic diagram showing major components and layout of a conventional ICP- OES instrument (Boss and Fredeen, 1997).

2.6.3 Nebulizer

In the sample introduction component, the sample is transported into the instrument in the form of a liquid which is pumped from a sample container into the nebulizer which is the most vital device (Boss and Freeden, 1997). The nebulizer has the ability to vaporise the sample and convert it into an aerosol (small droplets), a process which is referred to as "nebulization". The forces responsible for the breakdown of organic samples into aerosol droplets are known as pneumatic forces and ultrasonic mechanical forces (Boss and Freeden, 1997).

2.6.4 Spray chamber and drain assembly

The spray chamber, typically located between the torch and plasma, functions to remove droplets less than 10 µm from the aerosol of which is transported to the

plasma (Boss and Freeden, 1997). The pumping of the solution during the nebulizer process, pulses tend to occur (Boss and Freeden, 1997). The other task associated with the spray chamber is the smoothing out of these pulses so that there is a clean flow reaching the plasma (Boss and Freeden, 1997). The drain assembly's main function is to prevent the introduction of bubbles into the instrument which may interrupt the flow of the sample to the plasma, therefore, providing false results (Boss and Freeden, 1997). The other feature of the drain is to provide back-pressure moving the aerosol sample from the plasma into the torch and finally into the plasma (Boss and Freeden, 1997).

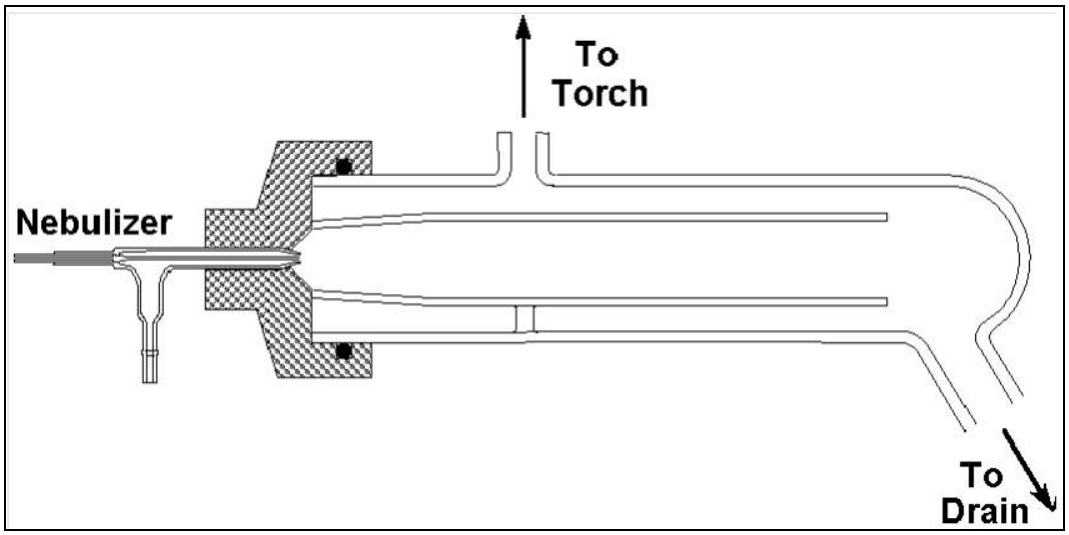


Figure 2.14 A schematic diagram of a spray chamber positioned in the ICP-OES (Boss and Freeden, 1997).

2.6.5 The gas supply and plasma

The plasma torch consists of the three quartz tubes: the inner quartz tubing contains the aerosol sample, the subsequent tubing allows for the sample to collide with the flow of the inert gas present in the tube, which is conventionally Ar. Lastly, the outer tubing contains an "outer flowing gas" responsible in keeping the tubes cool (Boss and Freeden,1997).

Once the organic sample reaches the plasma it is broken down into charged ions which occurs by the sample entrained with the electrons and charged ions situated in the plasma (Boss and Freeden, 1997). The charged ions proceed to break into their individual molecules of which electrons are lost and recombine repeatedly. While all this is transpiring in the plasma, radiation from wavelengths is emitted from the respective elements which is categorised by a certain device in the instrument. The radiation is then translated into an electronic signal which is then converted into concentrations that the analyst can interpret (Boss and Fredeen, 1997).

2.6.6 Interferences in ICP-OES

The interference on ICP-OES is minimal compared to Atomic Absorption Spectroscopy (AAS). Due to the technological advancement of the ICP-OES, the major interferences obtained from AAS outweighed the little interferences from the ICP-OES (Baloyi, 2006).

The interferences undergone from the ICP-OES are as follows:

- Spectral inferences: The overlapping of lines from elements causing the poor resolution of lines (Baloyi, 2006)
- Non–spectral inferences: This involves high background resolution emitted from the plasma, restricting detection limits , nebulizer properties and changes in sample transport, volatilization interferences, automization interferences and ionization interferences (Baloyi, 2006)

These interferences may be corrected by doing the following

- Matrix matching by using a calibration curve (Baloyi, 2006)
- Using an internal standard to calibrate (Baloyi, 2006)
- The utilisation of ionization buffers (Baloyi, 2006)

2.7 Extraction techniques for elements

There is a widespread scale of solid liquid extraction (SLE) techniques used for the refinement of natural products from plant matrices and microorganisms (Kauffman and Christen, 2012).

Customary, the procedure includes soxhlet extraction, marceration, high speed mixing and sonication (Kauffman and Christen, 2002). Although these methods have been around for decades they are time consuming and demand the use of large amounts of solvents which are sources of vast amounts of pollutants (Kauffman and Christen, 2012). Economical and effective extraction techniques such as microwave-assisted extraction (MAE) and pressurised hot water extraction (PHWE) are preferred methods for extracting secondary metabolites from plant material (Kauffman and Christen, 2002). Others include supercritical fluid extraction, pressurised liquid extraction and accelerated solvent extraction.

2.7.1 Microwave assisted extraction (MAE) for metal studies

An efficient technique for the extraction of metal elements organic in solid matrices in the soil, leaves, seeds, stem bark and flowers of a plant is the utilisation of the microwave extraction technique (Kaufmann and Christen, 2002).

The use of microwaves has dated back as far as World War II succeeding the advancement of radar technology and was later used as a domestic oven (Ganzler and Salgo, 1987; Ganzler *et al.*, 1986). In 1986, the introduction of extraction of

compounds from the soil, seeds, food and feed was first reported by Ganzler and fellow researchers (Ganzler and Salgo, 1987; Ganzler *et al.*, 1986).

Microwave-assisted extraction is a method that involves the introduction of microwave energy to a heating source and applying it to acid digestion (Kaufmann and Christen, 2002).

Microwaves are electromagnetic radiation with a frequency from 0.3 to 300 GHZ which, on the electromagnetic spectrum, is situated between infrared and radio frequency (Camel, 2000). Electric fields and magnetic fields perpendicular to each other are present in the microwave which is due to the electromagnetic nature of the microwave. Due to the electric field of the microwave, two mechanisms are implicated: dipolar rotation and ionic conduction (Thuery, 1992; Demesmay and Olle, 1993; Sinquin *et al.*, 1993). Unlike traditional conductive heating techniques, microwaves heat the entire sample instantaneously through the disruption of hydrogen bonds stimulated by dielectric rotation of the molecules (Sinquin *et al.*, 1993; Camel and Bermond, 1999).

2.7.2 Instrument

There are two types of microwave instruments that are presently available: closed vessel system which regulates under controlled pressure and temperature and open vessel systems that uses atmospheric pressure (Kauffmann and Christen, 2002). When comparing the two systems, the open system is simple and safe to use compared to the former which runs the risk of possible explosions (Kauffmann and Christen, 2002). The system utilised for the identification and quantification

of elements located in the soil, leaves, stem bark, seeds and flowers of Moringa *oleifera* and Moringa *ovalifolia* is the closed system.

2.7.3 Closed vessel system

Closed vessel systems are generally used for the digestion or acid mineralisation using solvents under controlled pressure and temperature. Under this system, extraction speed and competency are enhanced and the solvents used are heated up to 100°C above its bp. (Barnabas *et al*., 1995; Jassie *et al*., 1997). Some of the disadvantages of using this system is the risk of an explosion due to the heating of flammable solvents, however, security systems such as exhaust fans, solvent vapour detectors or pressure-burst safety membranes located on each vessel may overcome these unsafe hazards (Demesmay and Olle, 1993; Jassie *et al*., 1997). To evenly distribute the non- homogeneity of the field, the vessels, or commonly known as "liners", are placed on a rotating carousel in the microwave oven (Demesmay and Olle, 1993; Jassie *et al*., 1997). The rotating carousel is placed in the microwave oven and allows many samples to be extracted at the same time (Demesmay and Olle, 1993; Jassie *et al*., 1997). The liners are made of Teflon which are opaque in colour do not absorb energy from the microwave but rather from the extracted samples through conduction. The solvents added to the sample mix can be varied, and the pressure in the liners during the run time principally is dependent on the volume and boiling point of the solvents used (Demesmay and Olle, 1993; Jassie *et al*., 1997). Generally, the addition of botanical materials increases the pressure by the production of gaseous products from the sample. The rate of the reaction and efficiency of extraction is achieved at high temperatures. The temperature of the system can be set at a fixed value by adjusting the

microwave power (Demesmay and Olle, 1993; Jassie *et al*., 1997). The ability of the samples to cool before the extraction time is terminated is of importance because it minimizes volatization of the solutes and prevents contact of some of the volatile solutes with the inner head space of the liner (Demesmay and Olle, 1993; Jassie *et al*., 1997).

Other disadvantages associated with the use of the closed vessel system which includes:

(i) Sample sizes are not very large (0.5-1.0g) because the heating rate of the solvent may run the risk of being reduced because the energy that closed system outputs is split between the many extraction vessels (Armstrong, 2009). This can be a problem where large sizes are needed.

(ii) The instrument is allowed to cool and depressurize before opening it thus losing time (Armstrong, 2009).

MAE has been used for the extraction of plant extracts for the assessment of antibacterial and antioxidant properties (Kothari and Seshadri, 2010; Kothari, 2010; Kothari, 2011; Dai and Mumper, 2010; Kothari *et al*., (2010); Kothari and Seshadri, 2010; Kothari, 2010). In a study by Sin *et al*., (2014), MAE was used for the extraction of phenolic compounds from processed dry leaves from Moringa *oleifera.*

2.8 Green Chemistry

A general definition of green chemistry is the eradication of hazardous substances by the unique invention of chemical products (Badami, 2008).

In modern age, current protocols from manufacturers in the industry have requested the reduced use of petrochemical and organic volatile substances be it in extraction organic synthesis or other manufacturing processes. The purpose of this urgent request is due to the fact that most organic solvents are flammable, volatile and toxic and account for environmental pollution and greenhouse effect (Lyon and Skurray, 1987). The research conducted in this study seeks to isolate both elemental and phytochemical compounds from the plant materials of Moringa *oleifera* and Moringa *ovalifolia* utilizing an efficient and environmentally friendly method. This method seeks to apply the green chemistry principle which can be summarized as follows (Anastas and Warner, 1987):

- The prevention of waste is a better method as opposed to recycling it or cleaning it up (Anastas and Warner, 1987).
- Synthetic methods should be considered to increase the incorporation of materials used in the chemical process into the final product (Anastas and Warner, 1987).
- Man-made methods should be produced that have little to no toxicity that could pose a threat to humans and the environment (Anastas and Warner, 1987).
- The production of reagents should serve for a specific purpose whilst reducing toxicity (Anastas and Warner, 1987)
- Substances that could pose a threat to human health, such as auxiliary substances, should be prevented from use (Anastas and Warner, 1987).
- Before the use of any procedure, energy requirements and the impact of chemical processes should be noted. The use of man-made methods should

be conducted at high pressures and temperatures (Anastas and Warner, 1987).

- Recycling feedstock rather than discarding it would be economically feasible (Anastas and Warner, 1987).
- Prevention of derivitization is recommended due to the additional use of reagents which can accumulate waste (Anastas and Warner, 1987).
- Catalytic reagents are recommended than stoichiometric reagents because a catalytic reaction has a higher activation energy (Anastas and Warner, 1987)
- Methods should include chemical products that break down at the end of reactions so that the end products don't immerse into the environment.
- Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions and fires. Substances produced during experimental procedures should be environmentally safe and free from fire hazards (Anastas and Warner, 1987)

2.8.1 Instrument and extraction mechanisms

The basic PHWE instrument set-up consists of a stainless steel extraction cell, a Gas Chromatography oven, a pump system, back pressure regulator and a sample collecting cell (Teo *et al*., 2007). Currently, there is no commercial supplier of the PHWE system. One has to build different components together. The typical set-up used in this study is shown in Figure 2.15.

The extraction mechanism is propositioned to comprise of four sequential steps that occur in the extraction cell such as that illustrated in Figure 2.16. These are discussed below:

At high temperature and pressure, this helps the rapid entrance of water into the sample matrix, thus disrupting any interaction between the analyte and matrix such as Van der Waals forces, hydrogen bonding and dipole interactions. This transfers the analytes into the extraction solvent rapidly (Kronholm, 2007).

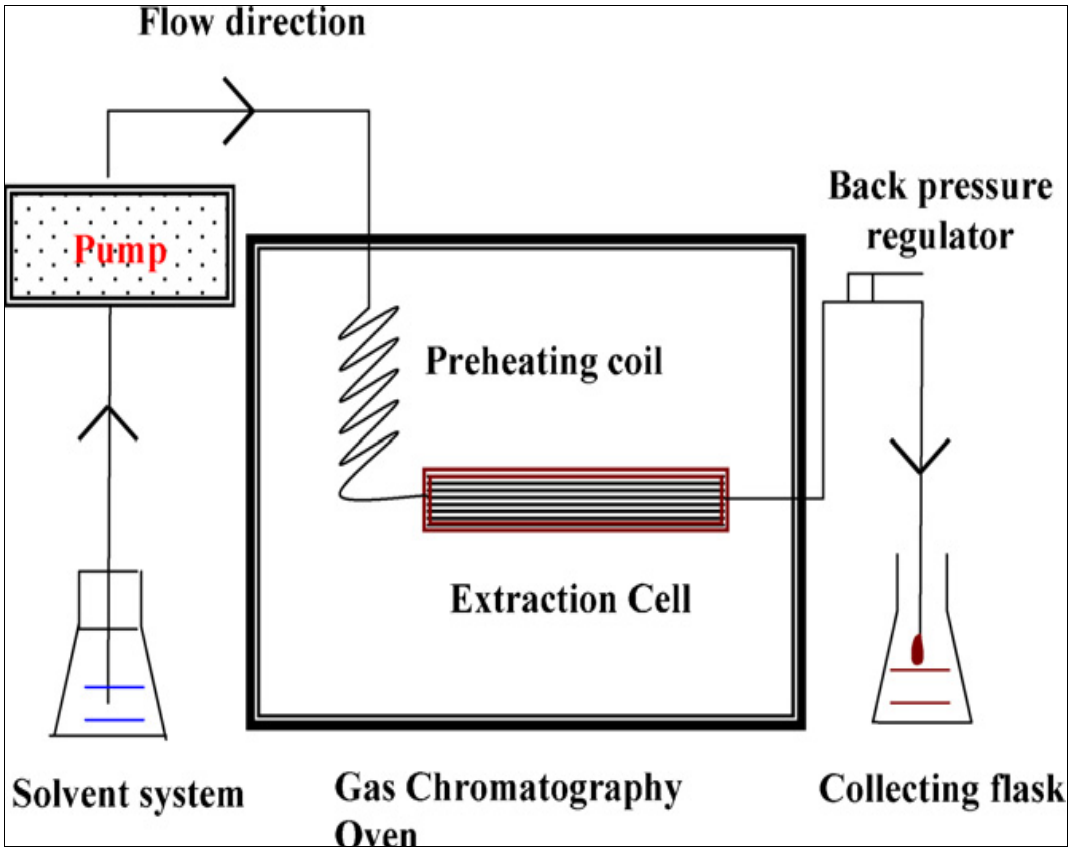


Figure 2.15 Pressurised Hot Water Extraction system setup (Kronholm, 2007).

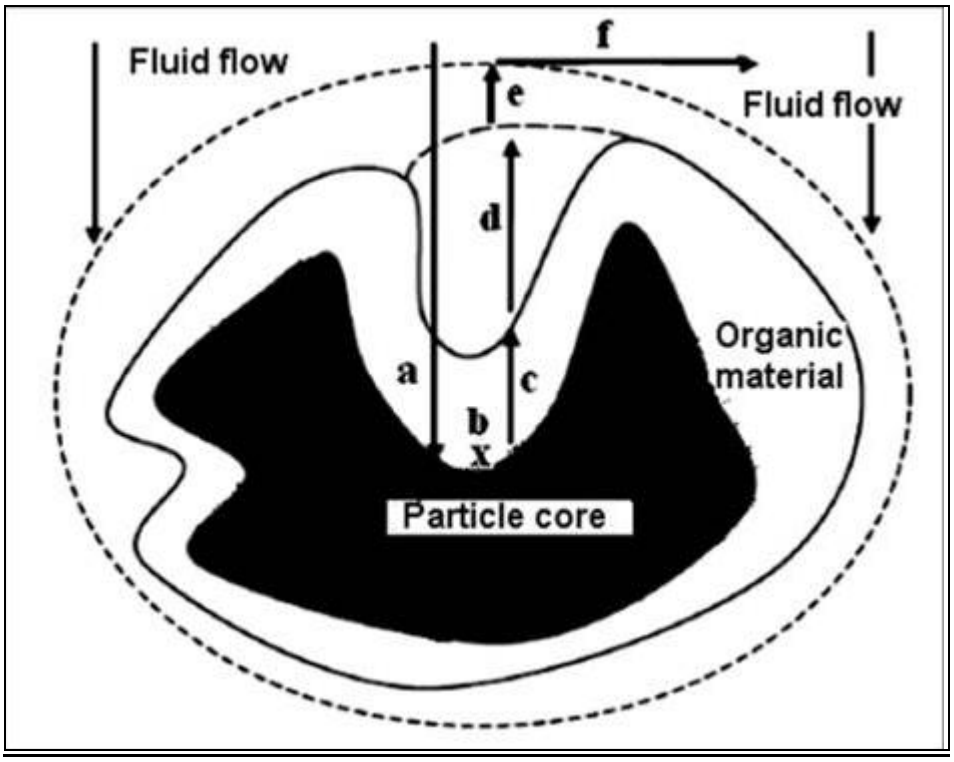


Figure 2.16 Schematic representation showing proposed extraction steps in PHWE: (a) rapid fluid entry; (b) desorption of solutes from matrix active sites; (c) diffusion of solutes through organic materials; (d) diffusion of solutes through static fluid in porous materials; (e) diffusion of solutes through layer of stagnant fluid outside particles; and (f) elution of solutes by the flowing bulk of fluid (Ong *et al*., 2006).

2.8.2 Temperature and pressure as main features in PHWE

According to literature, utilising high temperature and pressure during extraction ensures higher extraction efficiency of soluble compounds from the matrix (Teo *et al.,* 2010). For effective extraction efficiency, the polarity of water needs to be low which is only achieved at elevated temperatures and it keeps the pressurised water in a liquid state above its boiling point. The pressure of the extraction instrument is requested to be high as it improves the recovery of the extract as well. The high pressure forces the liquid and pressure into the sample matrix thus disrupting any analyte and matrix interactions (Teo *et al.,* 2010). .

Non polar organic compounds may only be extracted from temperatures up to 200 ˚C. Extractions higher than 200 ˚C result in the degradation of thermolabile compounds due to the sensitivity and lack of stability of phytochemicals. Phytochemicals are said to be sensitive to temperature, oxygen and light (Teo *et al.,* 2010; Junior *et al.,* 2010).

The effect of temperature and time on deodorized thyme was reported by Vergara-Salinas *et al.,* (2012). The crude extracts were extracted utilising PHWE at temperatures (50-200°C) for the antioxidant and polyphenol activity. The report indicated that the crude extracts extracted at 200°C yielded the highest extraction yield and the highest antioxidant activity, however, total polyphenols extraction yield was detected at 100°C (Vergara-Salinas *et al.,* 2012). Nonetheless, degradation of thermally labile compounds is of concern at elevated temperatures. If a liquid like water is used above its BP, pressure needs to be exerted in order to keep water in its liquid state. Elevated pressure provides a highly efficient extraction for polyphenols compounds trapped in the matrix pores because it forces the liquid into the pores, a process that wouldn't occur if the pressure of the instrument was low (Rickter, 1996; Vergara-Salinas *et al.,* 2012).

2.8.3 Loading the cell

To ensure that most of the compounds from Moringa are extracted, upon loading the cell, the addition of diatomaceous earth (sand) to the plant material is recommended. This allows any voids in the extraction cell to be filled and prevents the absorption of water from plant materials (Ong *et al*., 2003). This method is feasible as it also prevents the blocking of the extraction system (Ong *et al*., 2003).

2.8.4 Pressurized hot water extraction (PHWE) for metal studies

PHWE is a suitable alternative to traditional extraction methods not only does it remove heavy metals and organic compounds it is also said to retain the structure of the matrix (Teo *et al.,* 2010). Water is used in place of organic solvents for extraction purposes. Due to water's polar nature it allows extraction of water-soluble products such as proteins, sugars and organic acids, as well as inorganic substances (Teo *et al.,* 2010). The polarity of water is in part due to its dielectric constant. At room temperature, water has a high dielectric constant of 80 which is due to its hydrogen bond structure (Teo *et al.,* 2010). The higher the dielectric constant the more polar it is (Ong *et al*., 2006). PHWE is one of the most promising modern green extraction techniques due to its feasibility, its fast extraction, small solvent consumption, and automated extraction procedures (Petersson *et al.,* 2010).

The exceptional feature of using the PHWE is its ability to manipulate the dielectric constant at high temperature and pressure (Teo *et al.,* 2010). For instance, at high temperatures and pressures, the properties of subcritical water changes resulting in a decrease in its permittivity and surface tension and an increase in its diffusivity characteristics. The high temperature leads to the disruption of the hydrogen bond – lattice (Smith, 2002). At 250 °C the dielectric constant value decreases to that of 27, which allows water to mimic the dielectric constants to that of the solvents like ethanol (ε=24) and methanol (ε=33) at 25°C. This makes it suitable for extraction of low-polarity compound (Smith, 2002).

In the study conducted by Matshediso, (2013), water was used as extraction solvent for the extraction of metal in both Moringa *oleifera* and Moringa

ovalifolia leaves at varying degrees (Matshediso, 2013). A comparison study was conducted between PHWE and MAE to assess the efficiency of extraction of both methods. The results indicated that although both methods were efficient in extraction, the amount of metals extracted was higher in the MAE than in PHWE. A plausible reason for this difference is because extraction is conducted in a closed vessel system, the loss of heat to the environment is prevented and loss of sample by degradation is also prevented due to irradiation supplied in the instrument (Matshediso, 2013). However, quantitative extraction of metal ions besides other organic compounds is also achieved in PHWE (Matshediso, 2013).

2.8.5 Co**mparison with other extraction techniques**

Table 2.9 below illustrates a comparative list of the PHWE method with other modern extraction techniques including traditional Soxhlet extraction. The techniques were compared in terms of extraction time selectivity, solvent consumption, solvent type and energy needed for consumption counter to the components in the sample. According to the other extraction methods, PHWE is advantageous and eco-friendly because it utilises only water as a novel extraction solvent (Boss *et al*., 1997). Likewise, application of the supercritical fluid extraction (SFE) is also considered eco-friendly provided CO_2 is used as the extraction solvent because CO_2 is non-flammable, naturally occurring and less likely to cause pollution (Boss *et al*., 1997). According to Table 2.9, the extraction time for all methods of extraction are similar, except for Soxhlet extraction which is very time consuming and can take up to 2 or three days (Hartonen *et al*., 2000). It also requires the use of large quantities of organic solvents. Other methods like pressurised liquid extraction (PLE), MAE and supercritical extraction (SFE) use

organic solvents but not as large as Soxhlet (Hartonen *et al*., 2000). All techniques in Table 2.9 are non-selective except for SFE whose parameters can be fined tuned to get good selectivity. PHWE and Soxhlet can give some selectivity but this depends on analyte and sample matrices. When comparing eco- friendly extraction techniques such as PHWE and SFE, it offers corresponding information. In terms of selecting an extraction technique that is suitable for obtaining compounds of interest with a wide range of polarities, the PHWE is a more fitting method compared to SFE, however, when choosing an extraction technique that caters to compounds that can withstand really high temperature conditions and non-polar compounds, SFE is the most suitable extraction technique (Hartonen *et al*., 2000).

Hydrodistillation, which is not accounted for Table 2.9, has been frequently used in the extraction of essential oils from plant materials, however, compared to the PHWE technique, the PHWE has the ability to extract more essential oils from plant materials with a higher more oxygenated compounds present. It is also a quicker and economical method than hydro distillation and should be favoured (Jiménez-Carmona, 1999).

The relevant advantages and disadvantages of each technique is listed in Table 2.9 below. However, among the advantages of the PHWE, no detrimental organic solvents are needed and analyte selectivity is high. The availability of the PHWE equipment is very limited, however, commercial availability is not critical because construction of the instrument in the laboratory is not difficult.

Table 2.9 Comparison of Soxhlet, PHWE, SFE, PLE and MAE extraction techniques (Andersson, 2007)

Traditional Techniques		**Instrumental extraction techniques**			
Extraction technique	Soxhlet	PHWE	SFE	PLE/ASE	MAE
Typical extraction time	4-8 hrs	5-30 min	30-90 min	12-20 min	30-60 min
Typical solvent	Acetone-hexanre, acetone-dichloro-methane, dichloromethane, toluene, methanol	Water	CO_2, CO_2 + modifier	Acetone-hexane, acetone-dichloromethanee	Acetone- water
Typical solvent consumption (mL)	300	A few millitres for elution of analytes	8-50	15-40	25-50
Selectivity for compound classes	Non-selective	Selective	Slightly selective	Non- selective	Non- selective
Selectivity for sample matrix	Some selectively	Some Selective	Selective	Non- selective	Non –selective
Benefits	Automated, cheap equipment	No use of organic solvents	Automated, little or no use of organic solvent	Automated	Automated, 14 vessels used concurrently
Disadvantages	Time consuming, large utilization of organic solvents	High temperature,no commercial instruments available	Expensive equipment, modifiers needed when CO_2	Expensive equipment, frequent blockages, additional clean	Additional clean up required for removal of the matrix

		is used	up required

Chapter 3: Research objectives

Summary

This chapter highlights the main questions and issues in this dissertation by addressing the main objectives, specific objectives and novelty of this study.

3.1 Research Questions

This research project seeks to answer the following research questions:

- What optimum temperature and pressure can we use to extract these compounds efficiently from PHWE that has antibacterial properties.
- Are the elements found in Moringa *ovalifolia* similar to Moringa *oleifera?*

3.2 Main and specific objectives

3.2.1 Main objectives

The main purpose of this study was to optimize the PHWE method in order to acquire extracts with bioactive properties, specifically antibacterial from Moringa *oleifera* and Moringa *ovalifolia*.

3.2.2 Specific objectives

- To determine and compare the elemental composition of the soil, bark, leaves and flowers of Moringa *ovalifolia* collected at different sites in Namibia by using microwave digestion followed by Inductively Coupled Plasma-Optical Emission Spectrometry.
- Comparative assessment of the the heavy metal content in Moringa *ovalifolia* from Namibia with Moringa *oleifera* grown in Limpopo Province, South Africa.
- To determine and compare the elemental composition of the soil, bark, leaves and flowers of Moringa *ovalifolia.*
- Optimise PHWE method to extract the various parts of Moringa *oleifera* and Moringa *ovalifolia* at varying temperatures.

- To evaluate *in vitro* antibacterial activity in Moringa extracts utilising the PHWE technique.
- To compare the antibacterial properties of PHWE of extracts to those extracted by organic solvents and water.

3.3 Hypothesis and Research Questions

3.3.1 Hypothesis

H_1. PHWE at varying temperatures can be used to extract essential compounds from Moringa *olefeira* and Moringa *ovalifolia*.

H_2. PHWE can be employed as a cheap but effective alternative techniques to extract phytochemicals from Moringa *oleifera* and Moringa *ovalifolia* with antibacterial activities

3.4 Novelty

Although many studies have been done extracting bioactive compounds using PHWE, only one report has been published on Moringa *oleifera.* No studies has been done on PHWE of Moringa *ovalifolia*. PHWE has the advantage to extract essential compounds

There have been reports on Moringa *oleifera* with regards to its nutritional and medicinal benefits (Fahey, 2005), however, there is no information on Moringa *ovalifolia* and its medicinal properties. It is therefore worth investigating these two species and comparing them to validate its effectiveness in combating bacterial resistance and even as a source of essential nutrients.

Chapter 4 Materials and Methods

Summary

The main emphasis of this chapter is to provide information on the materials used and methods developed to generate data. This chapter also focuses on techniques used to generate reliable and reproducible data such as extraction methods and analytical techniques used for quantitation.

4.1 Part A: Elemental analysis

4.1.1 Chemicals and reagents

All stock solutions were purchased from Sigma-Aldrich (Johannesburg, South Africa) and were of analytical grade. These include formic acid, concentrated nitric acid, hydrofluoric acid, boric acid and hydrogen peroxide. Ultrapure water (MilliQ, USA) from a purification system was used in all experiments and chemicals were used as received.

4.2. Preparation and extraction of the plant extract

4.2.1 Plant material

Fresh leaf samples of Moringa *oleifera* were sampled from a Moringa farm located in Polokwane, Limpopo. The leaf samples were rinsed with deionised water for approximately a minute with the intention to remove any contaminants that may have been lodged on the surface of the leaves. Following which, the leaves were air dried in a sterile environment under a shade. The leaves were then ground into a fine powder utilizing pestel and motor and sieved with a 25 µm sieve. The sample was stored in an amber container in a cool and dark place until further use.

Moringa *ovalifolia* samples were collected from five different locations as shown in Table 4.1 in February 2013 and were all treated as mentioned above.

Table 4.1 Sample collection sites

Site	Location	Sample types
1	Rocky hills in Moringa Safari Farm about 60 km from Okahandja to Karibib on a D2156 route in Otjozondjupa Region, central Namibia	Stem bark, leaves, flowers, soil
2	Well-known "Sprokieswoud" or 'enchanted forest to the west of Okaukuejo in Etosha National Park	Stem bark, leaves, soil
3	Dolomite hills near Halali in Etosha National Park	Stem bark, leaves, flowers, soil
4,5	3 km south of Tsumeb along B1 road side	leaves, soil

A sample preparation method from Pakade *et al*., (2012) with slight modifications was used. The fresh leaf, flower and stem bark samples collected were air dried as mentioned. After drying, the samples were crushed into a fine powder utilizing liquid nitrogen and stored in separate tubes which were then placed in a cool dark place until further use.

The soil samples collected were placed in separate tubes and labelled accordingly. The samples were placed in an oven for 24 hours and left in a desiccator to cool. The pH and electric conductivity of the soil was measured utilizing a mixture that contained soil suspension and deionized water in a 1:3 ratio.

4.2.2 Pressurised hot water extraction procedure

In this procedure a ratio of 0.5 g of Moringa *ovalifolia* and Moringa *oleifera* powder and 0.5 g of diatomaceous earth (1:1) was were weighed and placed in an extraction cell. The extraction cell was connected inside a GC oven which is programmed for heating the sample contained inside the stainless steel cell.

Deionised water was pumped through the cell at a flow rate of 1.0 mL min^{-1}. The pressure was between 1000-3000 psi and was regulated by a valve. Prior to the start of the extraction procedure, the oven is allowed to preheat for approximately 10 minutes until equilibration is reached. Deionised water was also heated prior to extraction to 80˚C so as to minimise the temperature gradient and is regulated by a thermometer. After pumping of the system was completed, the extract was collected in 10 mL tube and was taken for further analysis. With regards to optimization studies, the extraction cell was heated at temperatures ranging from 25˚C-100˚C.

4.2.3 Total digestion of plant samples using MAE

For digestion of soil samples, 0.1 g dry soil was placed in a microwave digestion vessel. 6 mL of concentrated hydrochloric acid, 2 mL concentrated nitric acid and 1 mL hydrofluoric acid was then added. After a digestion period of 50 minutes elapsed, 6 mL boric acid was added to each vessel to neutralise the hydrofluoric acid. The solution was transferred then to a 25 mL volumetric flask and made up to the mark with deionized water. All digestions were carried out in triplicates.

A mass of 0.1 g of leaf, stem bark and flower samples was placed in a microwave digestion vessel. To this, 8 mL concentrated nitric acid and 2 mL hydrogen peroxide was added. Digestion was carried out for 30 minutes in a microwave. After digestion, the leaf samples were transferred to a 50 mL volumetric flask whereas the flower and bark samples were transferred to a 25 mL volumetric flask. All solutions were made up to the mark with deionised water. The flower and stem bark sampled were filtered prior to being diluted. All digestions were carried out in triplicate.

4.3 Analytical determination

4.3.1 Inductively Coupled Plasma-Optical Emission Spectrometry (ICP - OES) analysis

The total metal content was analysed using inductive coupled plasma optical emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro, Germany). All measurements of samples were done in triplicate and here only the mean of the measured values are reported. Error bars (RSD) are omitted because they do not add value at the scales for the figures.

4.3.2 pH and electric conductivity meter

Calimatic pH meter (Knick, Berlin, Germany) was used for pH measurements at 25.5°C. Before use, the pH was calibrated at pH's 4 and 10. The electrical conductivity was measured in the same solution was used for pH measurement.

4.4 Part B: Antibacterial studies

4.4.1 Bacteria strains and culture conditions

Three bacterial strains (Table 4.2) were used in this study. These were selected from our strain collection which included Gram-negative bacteria *Pseudomonas aeruginosa* and Gram- positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis*. Cultures were incubated at 35°C for 24 hrs.

Table 4.2 Representative bacterial strains from different species

Strain group	Strain ID and serotype
Gram-negative	
P. aeruginosa	ATCC 27853
Gram- positive	
S. areus	ATCC 25923
E. faecelis	ATCC 19433

4.5 Antimicrobial susceptibility testing

4.5.1 Preparation of inocula

For antimicrobial susceptibility methods, bacterial suspensions were prepared by transferring a loopful of 3-5 colonies of the strains into Mueller Hinton autoclaved broth. Mueller Hinton broth which was prepared in grams/ liter purified water and was incubated overnight at 37°C. Table 4.3 shows the composition of the broth.

Table 4.3 Composition of the broth

Component	Amounts (g)
Beef Extract	2.0
Acid Casein Hydrolysate	17.5
Starch	1.5

After the incubation period, the broth was compared against a 0.5 McFarland standard. A McFarland standard is used for visual comparison against bacterial density. Preparation of a McFarland standard includes 0.5 mL aliquot of 0.048 mol L^{-1} which was added to 99.5 mL of 0.18 mol L^{-1} H_2SO_4 with constant stirring. The density of the turbidity standard was determined by using a spectrophotomer

with a 1 cm light path and matched cuvette. At an absorbance of 625 nm, the 0.5, the McFarland standard absorbance ranges between 0.08 to 0.13. To further test the accuracy of the broth, the bacterial suspensions were diluted in Mueller Hinton media at different dilutions and absorbance determined so that it fell within the McFarland standard range.

4.5.2 Preparation of Agar

In Table 4.4 Mueller Hinton Agar was be prepared in grams/ litre purified filtered water as shown.

Table 4.4 Composition of the agar

Component	**Amounts (g)**
Beef Extract	2.0
Acid Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0

38 g of Mueller Hinton dehydrated media was be suspended in 1 L filtered water. The media prepared was autoclaved and mixed gently and dispensed into sterile Petri dishes.

4.5.3 Paper disk diffusion assay

Before inoculation, the bench was wiped with 95% ethanol. For the disk diffusion method as show in Figure 4.1, 10:990 of the bacterial solution was prepared and 100 µl of this suspension was spread on the entire surface of a Mueller-Hinton agar with a sterile bent glass rod to form an even lawn by turning the plate at 60˚ every time as shown in Figure 4.1. 3 sterile paper disks 6 mm in diameter (Sigma

Aldrich, Johannesburg) were impregnated with plant extracts and placed on the surface of each plate using a sterile pair of forceps. The plates were incubated aerobically at 37 ˚C for 24 h. The diameter of the inhibition zone was measured after the incubation period using a ruler. Each experiment was repeated three times and average values were taken.

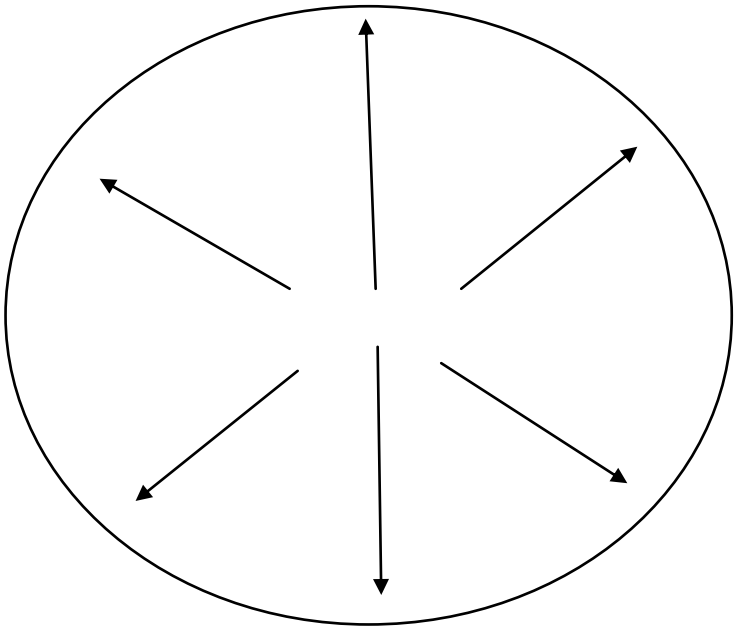


Figure 4.1 Swabbing direction of nutrient agar

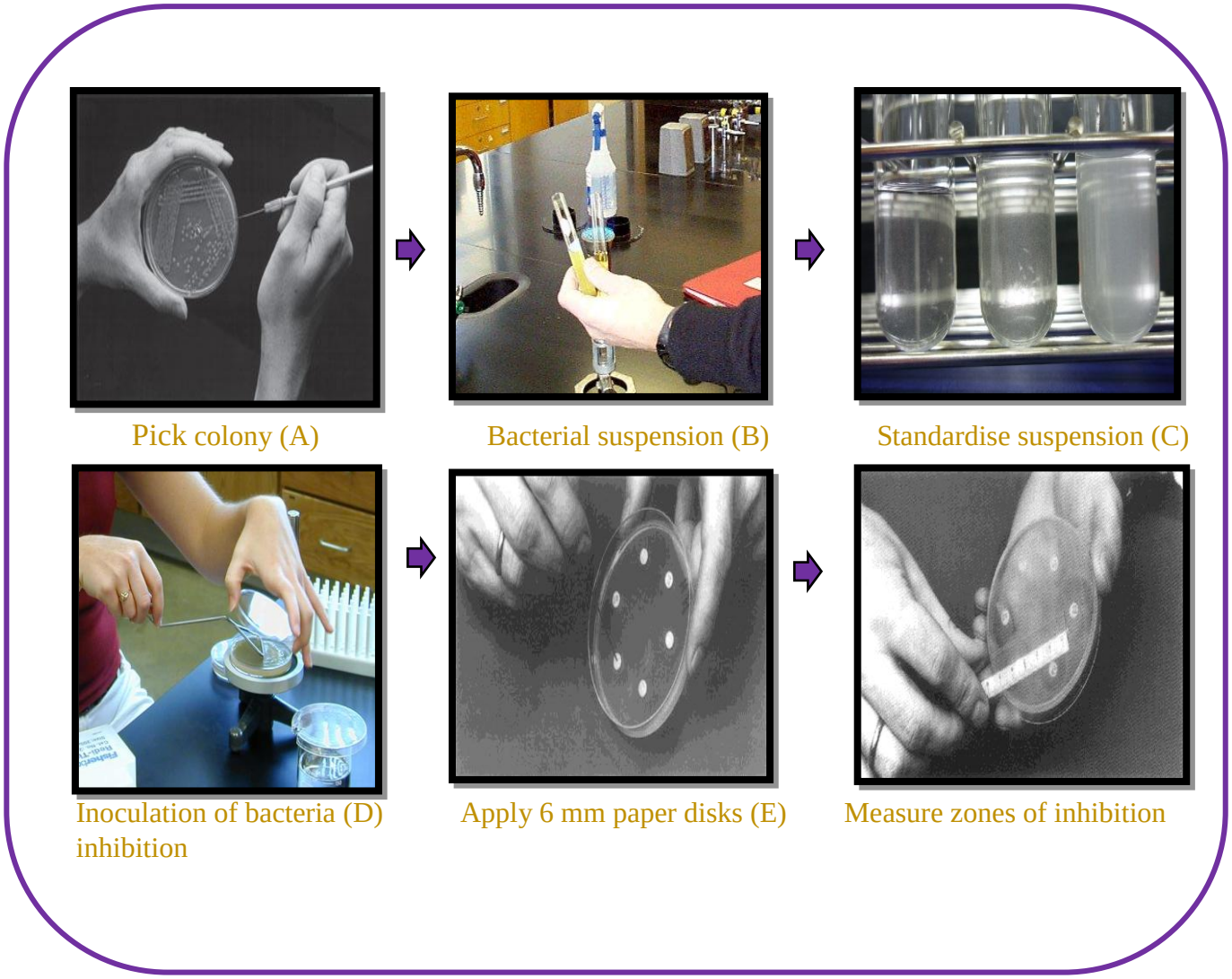


Figure 4.2 Disk diffusion method procedure (www./braukaiser.com)

Chapter 5: Results and Discussion

Summary

This chapter is subdivided into two chapters. Section 5.1 main emphasis is on the major and minor elements located in the soil, leaves, stem bark and flowers of Moringa *ovalifolia* samples. Section 5.2 specifically deals with the antibacterial effect of Moringa *oleifera* and Moringa *ovalifolia* PHWE extracts on pathogenic bacteria. In order to see trends and patterns within the data, relevant statistical analysis was performed.

5.1 Part A: Elemental analysis: Comparison of metals of Moringa *ovalifolia*

5.1.1 Calibration table

The calibration curves used for quantification for all metals and elements are illustrated in the table below. The linearity of all the graphs were suitable as correlation factors were above 0.99. The results were found to be precise. The limits of detection and limit of quantification are found below in Table 5.2. The limit of detection (LOD) ranged from 0.014 µg L^{-1} for Na to 63 µg L^{-1} for Na. The limit of quantification (LOQ) ranged from 0.05 µg L^{-1} to 210 µg L^{-1} for Na. These values were enough to quantify most elements in the digested samples.

Table 5.1 The limit of detection (LOD) for certain elements

Element	Slope (x)	Intercept (y)	R^2
Ca	7E+06x	-6e=E+06	0.9964
Mg	89266	-102658	0.9975
Al	89266	-102658	0.9666
Mn	640595	-541179	0.9977
Co	48170	-50709	0.9516
As	11939	-12354	0.9628
Cu	24571	-26148	0.9466
Cr	61329	-64370	0.9478
Fe	125475	-130849	0.9493
Ba	54596	-52382	0.9561
K	37290	-42422	0.9395
Na	525878	-616332	0.8293
Zn	165731	-169102	0.9507

Table 5.2 Typical LOD and LOQ for some metals

Nutrients	LOD µg L^{-1}	LOQ µg L^{-1}
Ca	0.25	0.83
Mg	0.014	0.05
Mn	0.26	0.87
Ni	0.89	2.96
Al	21.9	72.92
As	14.9	49.61
Ba	15.7	52.28
Cd	1.79	5.96
Co	5.84	19.44
Cr	5.57	18.54
Cu	13.7	45.62
Fe	0.30	0.99
K	14	46.62
Na	63	209.79
Si	5.78	19.23
Li	0.127	0.42
Zn	2.51	8.35

5.2 Heavy metals

Plants have the capability of up taking heavy metals from its environment such as soil, water and air (Cui *et al.,* 2004). Mobilization of lethal trace metals may consequently accumulate into the food chain. In the soil, human exposure of these heavy metals frequently occurs through soil-plant transfer of these lethal heavy metals (Cui *et al*., 2004). Generally, there are two stages of metal accumulation into the xylem: passive uptake via the symplast and active uptake via symploplast. The objective of this chapter is to evaluate the uptake of metals found in the leaves, stem bark and flowers of Moringa *ovalifolia* cultivated on the soils of Namibia. These plants grow in the wild and are indigenous to Namibia.

5.2.1 Soil properties

Soil characteristics such as pH and conductivity are one of the most indicative techniques of measuring chemical properties available in the soil. It is also a method utilised to govern heavy metal adsorption in plants and it's for this reason that they were therefore investigated.

The results demonstrated that the mean pH from sites 1-4 ranged from 7.45 and 8.01, indicating, that the soils from all sites were basic in character. Based on a study by Thomas, (1967), pH values ranging from 7.8-8.2 usually signify the presence of calcium carbonate in the soil, an essential component in calcerous soil. Moringa thrives within soils ranging from pH 5 – 9 and evidently all sites fall within that range (Nichols *et al*., 1991). Heavy metal availability is reduced due to the presence of high bicarbonates and organic matter, thus, a lower concentration of heavy metals is expected for heavy metal transfer from the soil to the plant (Degenhardst *et al.,* 1991, Rashad *et al.,* 2000; Masarovičová *et al.,* 2011). Conductivity is an indication of the amount of ions available in the soil. The higher the conductivity, the higher the concentration of ions in the soil therefore allowing more ions in the soil for uptake (Pakade *et al.,* 2013). Ions are nutrients in the form of anions and cations and are available for plants to absorb (Pakade *et al*., 2013). The mean conductivity values of the water extracted soil were between 304 and 1108 µs/cm.

Soils that are highly alkaline contain ions, such as Cl^-, K^+, NO_3^-, and in addition micronutrients such as Fe, Zn, Cu and Mn (Osman, 2013). In acidic soils, ions such as K^+, Ca^{2+}, Na^+, SO_4^{2-} and Cl^- are normally dominant. Therefore, the studied

sites could contain a lot of anions besides metals (Osman, 2013). However, no anions were measured as this was not the focus of this study.

Table 5.3 pH and conductivity values of the soils from which Moringa *ovalifolia* samples were collected

Site	pH	Conductivity (µs/cm)
Moringa safari farm (Site1)	7.45	304
Sprokieswoud (Site 2)	8.01	332
Dolomite Hills (Site 3)	7.78	1012
Road side (Site4)	7.73	1108

5.2.2 Metal content in soil

The study of heavy metals and micronutrients in the soil were investigated. Determination of heavy metals in soil is critical because the plants can accumulate hazardous elements which represent one way in which these metals can enter the food chain (Maboladisoro *et al*., 2004).

Metals found in soil can be divided into major and minor nutrients. Major nutrients such as P, K, Na, Ca, and Mg are required in large quantities in plants and humans whereas, minor nutrients known as trace elements such as Zn, Mn, Cu, Cr, Co and Ni are needed in small amounts (Pakade *et al*., 2013). Heavy metal contaminants in soil such as Pb and U were not detected in the soil in any of the four sites, the plant can be rendered as deficient in these metals.

According to the results (Figure 5.8), site 2 was found to have a higher Ca (6413 mg kg^{-1}) concentration compared to the other sites. A sample sampled from site 3 has the highest concentration of Mg (5264 mg kg^{-1}). Sites 1 and 2 contain close

amounts of Na (5388 mg kg^{-1} and 5464 mg kg^{-1}) and lastly, the concentration of K was the lowest and ranged from 24-51 mg kg^{-1}. On average, Mg and Na were found to be the most abundant in the soil. Site 2 was found to have the highest levels of major nutrients whilst site 4 the least. Site 1 and 3 had almost similar level of nutrients.

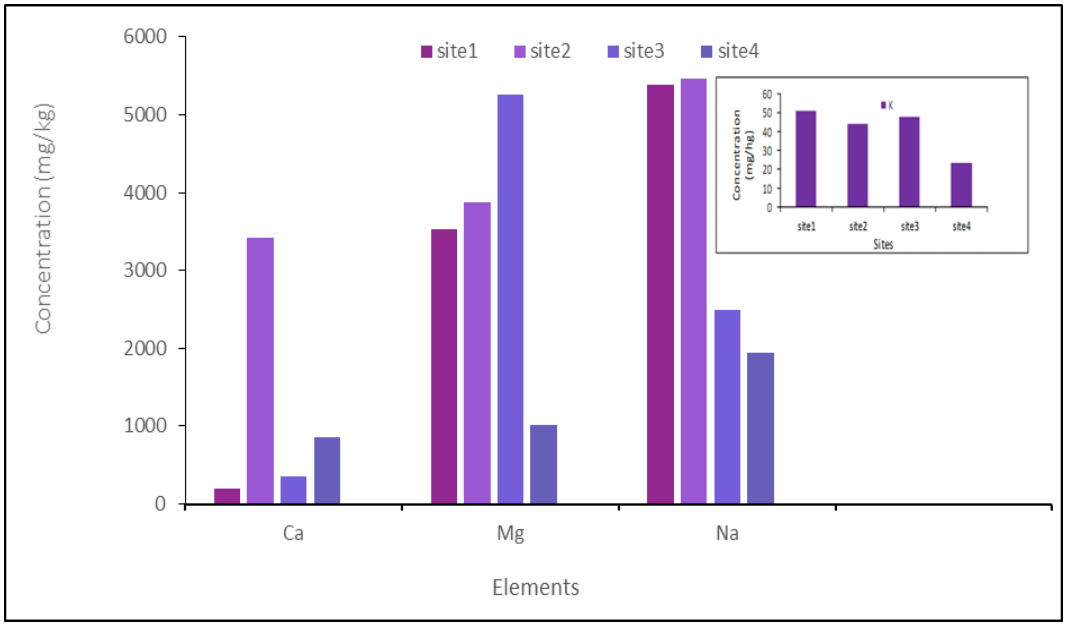


Figure 5.8 Average concentrations of major nutrients in soil samples from the four sampling sites.

Figures 5.9 and 5.10 demonstrate the minor nutrients from all four locations. Site 1 was found to have Fe (66375 mg kg^{-1}) as the most dominant element in the soil samples. Other sites had Fe concentrations not very different from each other. High concentrations of Si (55000-55734 mg kg^{-1}) and Al (32983-8600 mg kg^{-1}) were found in the soil (Figure 5.8). Figure. 5.10 shows that sites 1, 2 and 3 contain high concentrations of Co and Ba relative to the other sites. Highest concentration of Co was 310 mg kg^{-1} from site 3 and highest concentration of Ba was 360 mg

kg^{-1} also from site 3. According to the graph, other minor elements studied were lower than 200 mg kg^{-1}. The level of minor elements in Figure 5.9 could be arranged in this decreasing order: Ba > Co > Zn > Cr > Li > Cu > As > Cd.

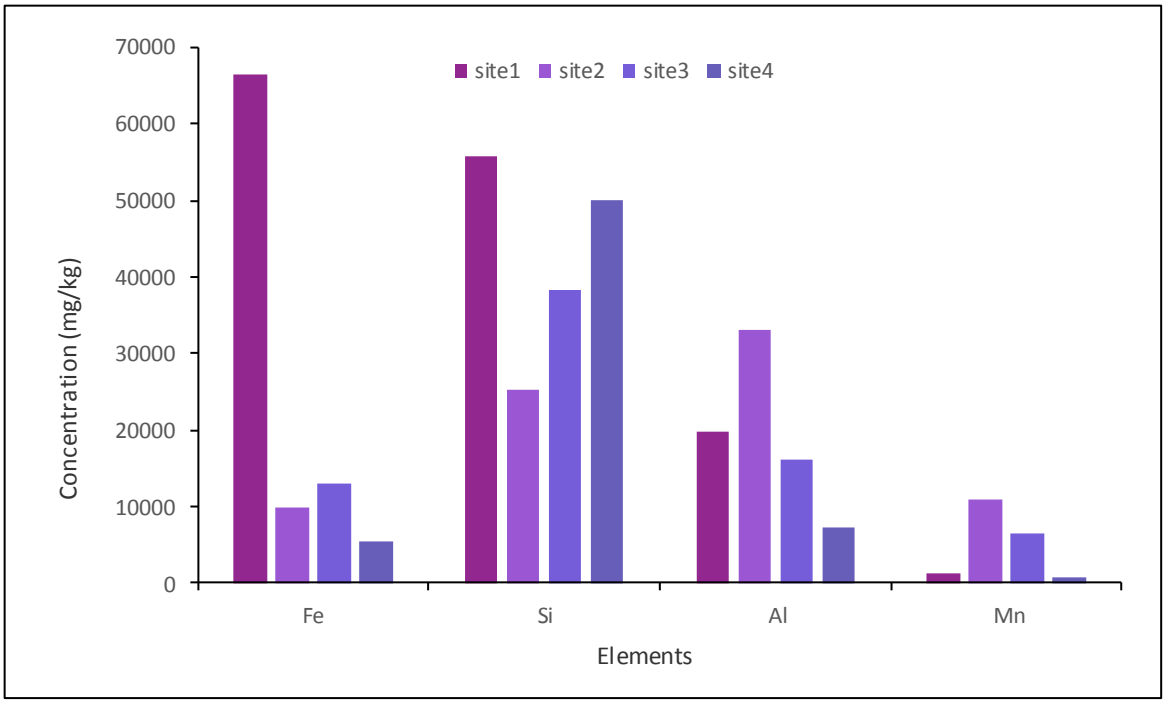


Figure 5.9 Average concentrations of minor elements in soil samples from the four sampling sites

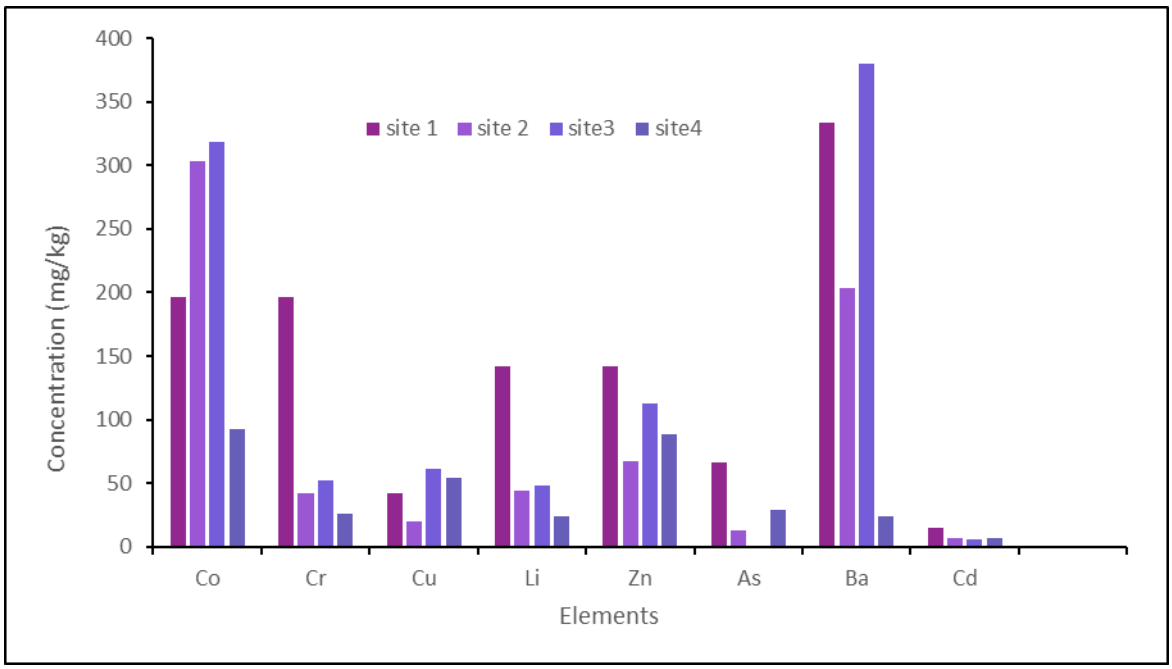


Figure 5.10 Average concentrations of minor elements in soil samples from the four sampling sites.

Concentrations of trace metals such as Cr, Cd, Cu and Zn were determined in this study and were compared with limits of heavy metals in soil in Western countries such as Austria, Canada, Poland, Germany and Great Britain which are illustrated in Table 2.1 and Table 2.2 under section 2.2.2. The concentration range of Cr concentrations for all the soil samples were 197 mg kg^{-1} for site 1, 43 mg kg^{-1} for site 2, 52 mg kg^{-1} for site 3 and 26 mg kg^{-1} for site 4. According to the International standards, the mean Cr levels in the soil samples were below the maximum permissible limits, except site 1 which were above Austria, Germany and Poland standards as well as the normal content intervals of heavy metals. Site 1, which was sampled at the Moringa farm, could mean a possible contamination of the soil from a natural geological source. This could be true because the site is far from industrial sector and Cr might occur naturally in soils. Zn concentrations are considered to be 10 times less hazardous for living organisms compared to lead and cadmium. The Zn concentrations for all four sites were 142 mg kg^{-1}, 67 mg kg^{-1}, 113 mg kg^{-1} and 89 mg kg^{-1} for sites 1, 2, 3 and 4, respectively. All these concentrations fall below the maximum allowed limits indicated in Tables 2.1 and Table 2.2 (Section 2.2.2). The range level of Cu from laboratory results was measured to be 15 mg kg^{-1}, 6.8 mg kg^{-1}, 5.5 mg kg^{-1} and 7.1 mg kg^{-1} for sites 1, 2, 3 and 4, respectively and all fell within the minimum allowable range for local and International standards provided in Table 2.1 and 2.2. It can be concluded, therefore, that the soils from all four sites are not heavily contaminated by heavy metals.

5.3 Metal content in leaves

Plants can accumulate heavy metals from contaminated soil and humans are exposed to them through consumption, therefore, it is necessary to monitor these metals in Moringa *ovalifolia.* Further, it is also important to know the nutrient content from the elemental composition of Moringa *ovalifolia.* The concentrations of the major nutrients and trace elements are shown in Figures 5.11 and 5.12, respectively. According to Figure 5.10, sites 3 and 4 contained high amounts of Ca compared to the other sites with values of (22994 mg $kg^{-1}$and 23864 mg kg^{-1}) respectively. Site 4 contained high amounts of K at (25000 mg kg^{-1}). Mg and Na had the least concentrations present in the leaves of Moringa *ovalifolia* from all five sites. It has been found in the past that Ca and Mg display antagonism where Ca directly influences the transport of Mg to the leaves (Morard *et al*., 1996). The higher the Ca concentration, the less Mg a plant can accumulate and vice versa. On average, Moringa *ovalifolia* was found to have slightly higher concentrations of macronutrients, however, the levels of minor nutrients such as Zn, Cr and Fe had similar values. The concentration of minor elements in the leaves showed high concentrations of Al, Fe and Mn from all four sites as illustrated in Figure 5.12.

Zn and Cu concentrations were fairly low in the leaves of Moringa. Cu, although an essential plant micronutrient, is required in trace amounts in plants (Cio *et al*., 2004). The concentrations of Cu in all four sites were relatively low ranging from 14.7l mg kg^{-1}-15.38 mg kg^{-1}in all plant samples. According to the local and International standards, this range in Cu concentrations is considered non- toxic to plants and animals because it is lower than the critical threshold levels which

range from 15-200 mg kg^{-1} (Alloway, 1995). Zn concentrations were in the ranges of 14.4-22.73 mg kg^{-1}.

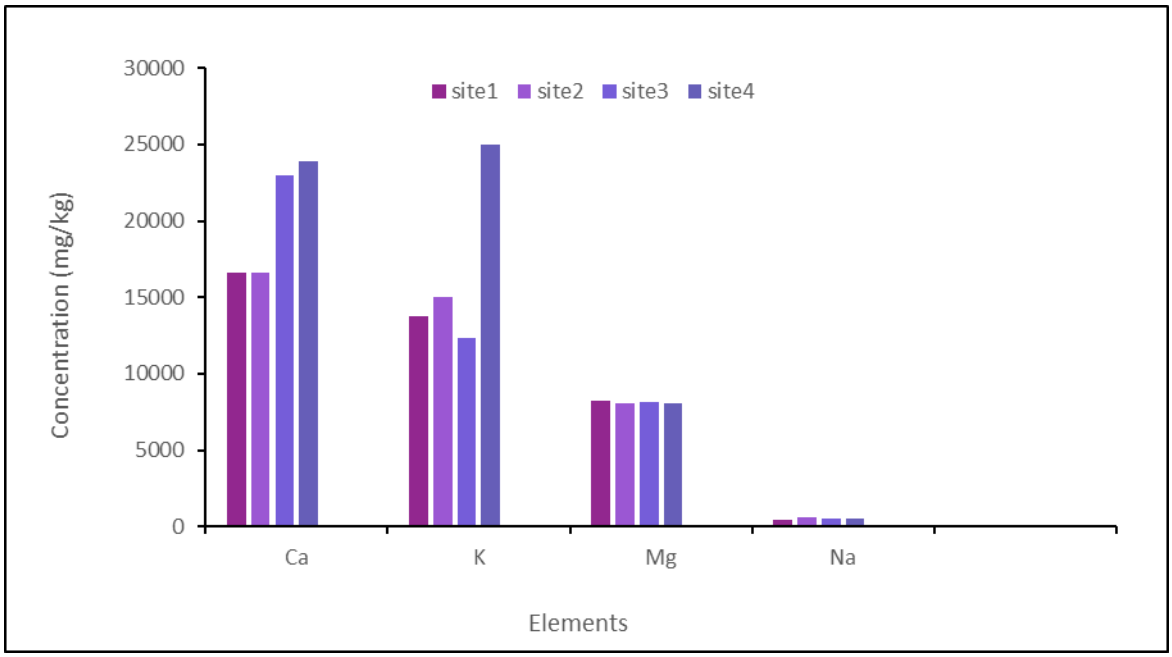


Figure 5.11 Average concentration of major elements in leaf samples from the four sampling sites

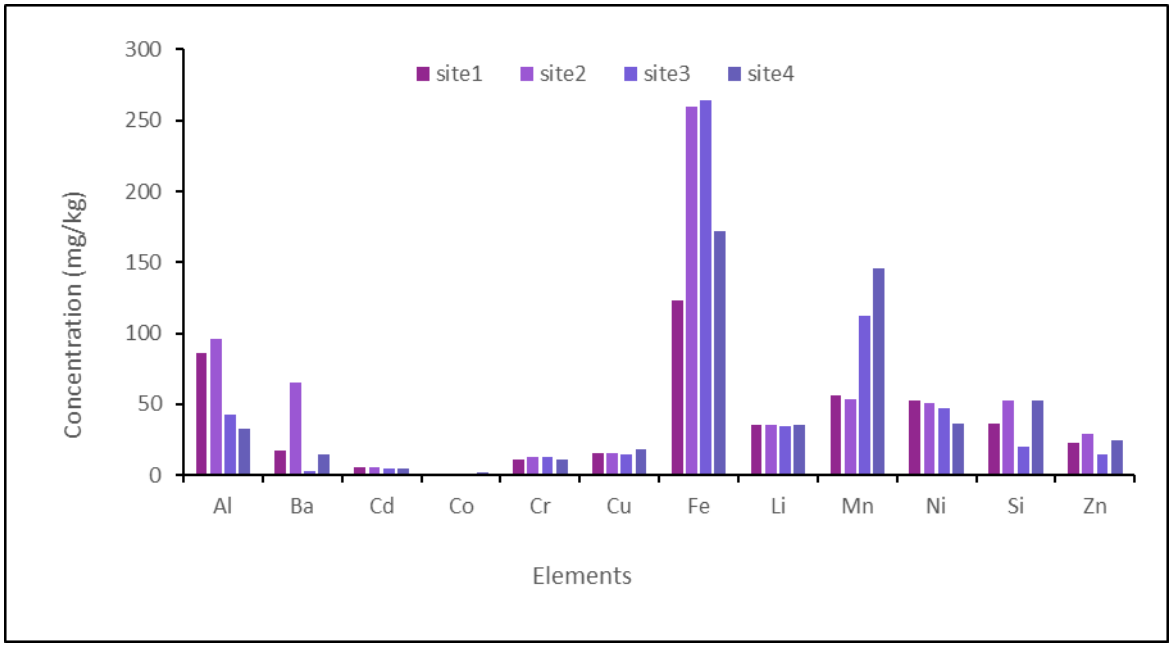


Figure 5.12 Average concentration of minor elements in leaf samples from four sampling sites

Average concentration of minor elements in leaf samples from the four sampling sites. The concentration of Al in Moringa *ovalifolia* leaves were in the range 32.9-95.6 mg kg^{-1} from all sites**.** A study conducted by Ondo *et al*., (2012) found Al in edible parts of plants such as leaves, flowers and tubers sampled from Moanda in Haut-Ogooue province of eastern Gabon. The concentration of Al in leaves, for instance, was determined to be in the range 63-300 mg kg^{-1} (Ondo *et al.*, 2012). Al stress or toxicity causes growth retardation. However, plants counteract this toxicity and develop an Al tolerance by secreting an organic acid which detoxifies it (Ondo *et al*., 2012).

Fe was found to be the dominant minor nutrient in the leaves of Moringa *ovalifolia* from all five sites (Figure 5.12) with the highest concentration of 250 mg kg^{-1} from site 3. This high concentration in Fe is a good indication because it is needed by both plants and humans. In humans, it is vital for metabolic processes such as DNA synthesis and oxygen transport to cells (Ogbe *et al*., 2012). Daily intake of Fe supplements reduces the risk of anaemia. Fe concentrations in Moringa *ovalifolia* from all five sites ranged from 124 mg kg^{-1} - 264 mg kg^{-1}. The minor differences in these values may be due to different geographical locations and maturity of the plant prior to harvesting (Ogbe *et al*., 2012).

Manganese (Mn) is needed for fat and carbohydrate metabolism. Nevertheless, toxic effects of Mn can have adverse effects on the brain and lungs (Smith, 1990). The plant toxic level of Mn in leaves investigated by Kabata-Pendias and Mukherjee is 500 mg kg^{-1} (Kabata- Pendias, 1995). In all four sites, the Mn

concentration was lower than the acceptable limiting value making Moringa *ovalifolia* safe to consume as far as this trace element is concerned (Smith, 1990).

5.3.1 Comparison of the elemental composition of Moringa *ovalifolia* to Moringa *oleifera* and Spinach

Information pertaining to the Moringa *ovalifolia* species, to our knowledge, has never been recorded, therefore, it is of necessity to compare the concentrations of metals in Moringa *ovalifolia* to Moringa *oleifera* sampled South Africa (Table 5.4).

Table 5.4 Comparison of metal concentrations found in Moringa *ovalifolia* to that of Moringa *oleifera* and spinach

Chemical Element	Moringa *oleifera*[1] ($mg\ kg^{-1}$)	Moringa *ovalifolia*[2] ($mg\ kg^{-1}$)	Spinach[1]
K	5000-8000	13733-25000	25000
Na	1000-2000	451-620	8000
Ca	12000-16000	16643-23863	12000
Mg	5000-6000	8024-8249	9000
Zn	20-30	14-25	30
Cr	20-30	11-13	20
Fe	170-210	124-264	190

Note: 1: Pakade *et al*., (2013); 2: This study

In the study conducted by Pakade *et al.,* (2013), major, minor and trace elements in Moringa *oleifera* were also compared to a variety of common vegetables. In this comparison, spinach was found to have metal elements much closer to

Moringa *oleifera*. Thus the metal elements in Moringa *ovalifolia* were also compared to those of spinach reported by Pakade *et al*., (2012). It can be seen, from the Table 5.4, that the average range of metal concentrations of Ca, K and Na from the sites collected were significantly higher in Moringa *ovalifolia* than Moringa *oleifera*. The results suggest that Moringa *ovalifolia* has higher amounts of macronutrients. It could also be due to differences in age of the plants (Pakade *et al.,* 2013). Concentrations of Zn, Cr and Fe, however, were found to be slightly higher in Moringa *oleifera*. There are a number of factors that may influence uptake of these metals from these species such as physical properties of the soil and climate issues besides the age of the plant themselves (Cook *et al.*, 1991). Moringa *ovalifolia* grows in mountainous and rocky terrains and generally has a large trunk with big leaves compared to Moringa *oleifera* which grow in loamy soils and with a thinner trunk and leaves. Just like Moringa *oleifera*, Moringa *ovalifolia* had relatively large abundance of Fe compared to the other minor nutrients. However, it did not contain more Fe than did the spinach. Due to the complications correlated with the absorption of non haem by the intestines it is strongly suggested that these vegetables be consumed with foods rich in vitamin C (Hurrel *et al.*, 2010), however, due to the high Fe content already present in the Moringa leaves, co-consumption is not mandatory.

5.4 Metals content in the flowers

Figures 5.13 and 5.14 illustrate the average concentrations of major and minor elements in Moringa *ovalifolia* for flowers from two sites. No samples were available from other sites. The results show that the flowers from site 3

accumulated the most heavy metals, compared to site 1, and is rich in major nutrients such as Ca, K and Mg. Possible causes of variation discussed in plant leaves could be responsible such as the age of the plant and environmental and climatic conditions (Dunbabin *et al.,* 1992; Runyoro *et al*., 2010). The results in this study show that Moringa *ovalifolia* flowers have less major and minor nutrients compared to Moringa *oleifera* (Pakade *et al.,* 2013). Most of the minor nutrients and elements were not consistently found in flowers from the two sites. Amongst all the minor elements analysed in the flowers, the Fe content (50 mg kg^{-1}) from site 3 was the highest. Medicinal-food flowers from medicinal plants such as Moringa *oleifera* and *Lagerstroemia speciosa* followed a similar trend although the Fe concentrations are relatively lower in Moringa *ovalifolia* (Pakade *et al*., 2013).

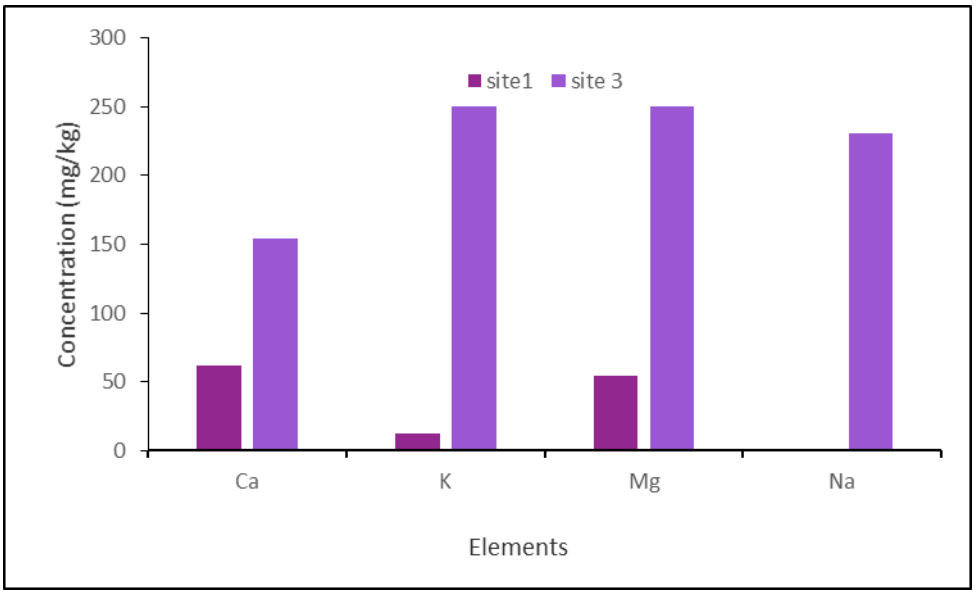


Figure 5.13 Average concentration of major and minor elements in flower and stem bark samples from Moringa *Ovalifolia*

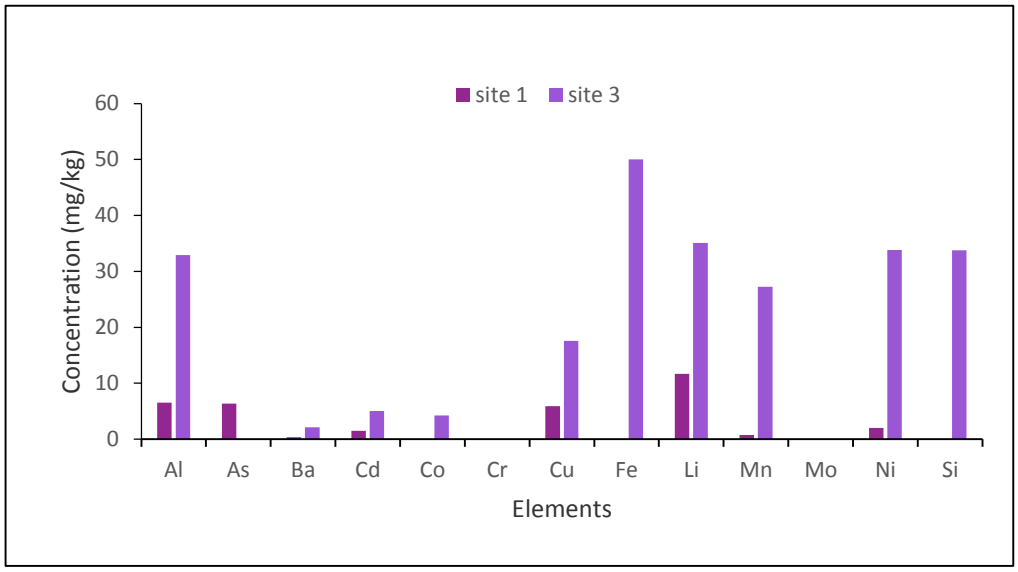


Figure 5.14 Average concentration of minor elements in flower samples from the four sampling sites in Namibia

Moringa *oleifera* and *Lagerstroemia speciosa* flower samples have been found to have 150 and 216 of Fe, respectively (Pakade *et al.*, 2013). Site 3 showed increased amounts of Mn, Ni, Al and Si. Ni concentration from site 3 was slightly higher. The necessary Ni dietary intake is about 0.025 mg kg^{-1} a day and the WHO limit is 0.02 μg g^{-1} (Smith, 1990). According to the results obtained in this study, the flower samples were above this range. Manganese (Mn) is a vital element associated with fat and carbohydrate metabolism. Nevertheless, toxic effects of Mn can have adverse effects on the brain and lungs (Smith, 1990). The daily recommended dietary intake for adults is 11 mg/day (Smith, 1990).

5.4.1 Metal content in flowers and leaves compared to normal standards

The Cd, Cu, Pb and Zn analysis in the flowers and the leaves were compared to the deficient normal and phytotoxic levels (Table 5.5). The concentrations of these heavy metals in Moringa *ovalifolia* leaves and flowers were within the

normal levels (Table 5.5). Therefore, according to this analysis, the Moringa *ovalifolia* plants in this study are fit for human consumption as reported. Although levels of trace elements in the soil are much higher, there is little accumulation in the biomass to reach phytotoxic levels. This is good news for human consumption of Moringa parts.

Table 5.5 Concentrations of trace elements in plants to the deficient, normal and phytotoxic levels (Pugh et al., 2002)

Level	**Concentration** (mg kg^{-1})			
	Cd	**Cu**	**Pb**	**Zn**
Deficient	-	<1.5	-	<10
Normal	0.05-2	30-Mar	0.5-10	10-150
Phytotoxic	5-700	20-100	30-300	>100

5.5 Metal content in stem bark

The major and minor elements in the stem bark are presented in Figures 5.15 and 5.16. These results demonstrate the high concentrations of Ca from site 1 (334 mg kg^{-1}) and site 2 (192 mg kg^{-1}). Ca concentration accumulated in Moringa *ovalifolia* may be therapeutic because it is essential in the body as it is responsible for metabolic processes such as cell division and the regulation of cell proliferation (Whitfield *et al.,* 1979). It is also vital in regulating and strengthening bone mass especially for individuals suffering from osteoporosis and osteopenia caused by chemotherapy (Lipkin and Newmark, 1999). The determined amounts of Na from sites 1 and 2 (300 mg $kg^{-1}$and 240 mg kg^{-1}, respectively) were higher compared than site 3 of the flower samples and also than in the medicinal plant *C. nigricans* which has been found here to about 40 µg

g^{-1} (Ayo *et al*., 2013). Na has no specific function in higher land plants and is unavoidable during root uptake, therefore, it is present as consequence of uptake of other important elements (Seasterdt and Crossley, 1981). The stem bark showed low accumulation of K and Mg which were below 100 mg kg^{-1} from all four sites (Figure 5.15 and Figure 5.16). The major elements were higher in the leaves compared to the stem bark. This is as a result of the plant storing and utilizing these elements for vital processes such as photosynthesis (Usman *et al*., 2011).

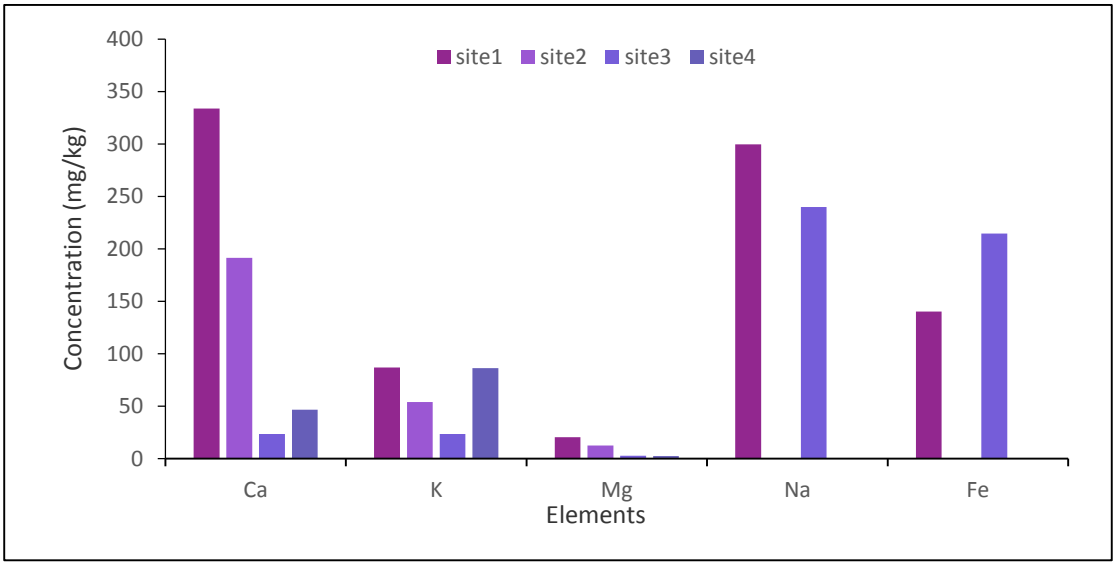


Figure 5.15 Average concentration of major and minor elements in stem bark samples from the four sampling sites in Namibia

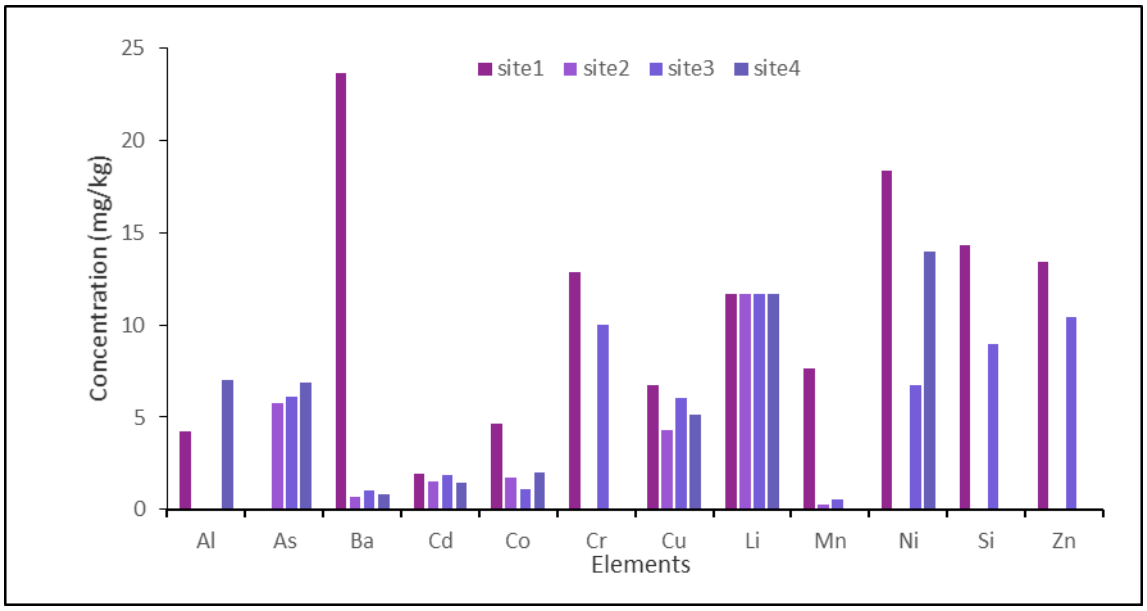


Figure 5.16 Average concentration of minor elements in stem bark samples from the four sampling sites in Namibia

The decreasing order of minor elements in the bark samples was found to be Fe > Cr > Cd. The decreasing trend similar was reported in the bark of Moringa *oleifera* tree sampled in Nigeria. However, the Moringa *ovalifolia* samples showed a higher heavy metal accumulation from all four sites compared to the bark samples sampled from Nigeria (Fowotade and Abdallah, 2012). High concentrations of As obtained from three sites with concentrations above 5 mg kg^{-1} exceeded WHO permissible limit of 0.01 μg g^{-1} (Wenser *et al*., 2012). The high concentration found in Ba (24 mg kg^{-1}) from site 1 is higher than the other sites. This high concentration could be a possible error.

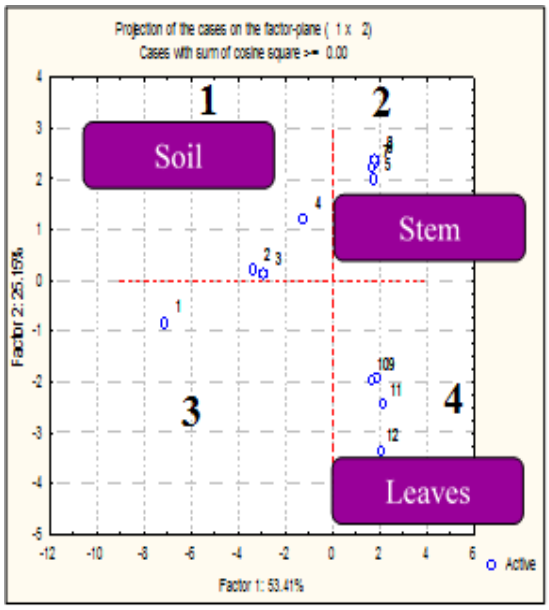
5.6 Statistical analysis

In this study, selected statistical methods such as principal component analysis and correlation analysis were implemented to assist in the interpretation of

elemental data as well as the inter-elemental association and origin between the different sites in the soil, leaves and stem bark of Moringa *ovalifolia*. All statistical data were applied using the Minitab® 16, © 2010 Minitab Inc. software using the significance level of 0.05.

5.6.1 Principal component analysis

The principal component analysis (PCA) is a powerful tool in classifying different groups of metals that correlate with each other, and as a result, can be considered as having similar behaviour patterns and common origins (Tahri *et al.*, 2005). The PCA scatter plots of the various sites and the corresponding metals are shown in Figures 5.16a and 5.6b.



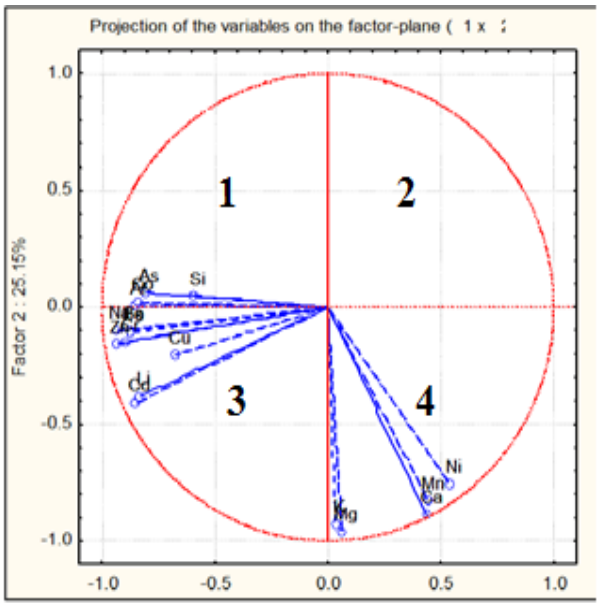


Figure 5.17a Loading plots of PCA analysis of the soil, stem bark and leaves sampled from different sites. **1**- Site 1 soil **; 2**- Site 2 soil; site **3**- Site 3-soil; **4**- Site 4 soil; **5**- Site 1 leaves; **6**- Site 2 leaves; **7**- Site 3 leaves ; **8**- Site 4 leaves; **9**- Site 1 stem bark; **10**- Site 2 stem bark; **11**- Site 3 stem bark; **12**- Site 4 stem bark

Figure 5.17b Loading plots of PCA analysis of heavy metals for the soil, stem bark and leaves of Moringa *ovalifolia*

The score plot (Figure 5.17a) obtained revealed a distinct partition of the soil, stem bark and leaves into four different quadrants, while the soil sampled from site 1 seems as an outlier. This information strongly implies that there is no major significant difference between the various sites of the sampled plants but rather, the comparative differences of heavy metal accumulation is dependent on the geographical location and physiographical conditions. However, spreading of the sites within similar quadrants may still suggest some differences.

The factor analysis applied on the data accounts for 79.56% of the total variance (Figure 5.17a and b). Mg, Ca, Mn and Ni are placed together within Factor 1 accounting for 53.41% of the total variance. This factor may be considered a geological factor. This is because Moringa *ovalifolia* is growing in remote areas away from industrial activities. Anthropogenic sources are therefore minimal. According to Figure 5.17b, these elements found in factor 1 exhibited high correlation coefficients. Factor two, which explains 25.15% of the total variance is mainly composed of As, Co, Al, and Si. This factor comprises of a mixture of human contact and anthropogenic sources (Harami *et al*., 2003). When comparing the PCA plot of the samples with the heavy metals, we find that no sources that are associated with the variables. However, looking at the fourth quadrant, we see that the soil from sites 2 and 3 are associated with the elements As, Co, Al and Si. These results are justified because the soil from sites 2 and 3 were sampled in different areas in Namibia's National Park with different geological properties.

5.6.2 Correlation studies

The results of correlation studies are presented in Table 5.6. The data suggests that some correlation pairs such as K/Al (r=0.976), Si/Al (r=0.908), As/Fe (r=0.906), Ca/Mg (r=0.872), Cd/Cr (r=0.908), Co/Fe (r=0.996), Cr/Li (r=0.967), Cr/Fe (0.997), Fe/Li (0.957) all have very strong and significant correlations between each other. These strong correlations suggest that these paired elements have common sources of contamination or they were similar to each other due to natural circumstances, topography, soil conditions and parent material (Soffianian *et al*., 2014). Similarly, strong positive correlations were found between Ca/Mg (r=0.872). This could be because Mg and Ca possess similar properties because they are found in the same group on the periodic table, therefore they may directly influence the phytochemistry of the leaves of the plant which would explain the presence of these elements in high concentrations in the leaves of Moringa *ovalifolia*. Other metals did not show strong correlations. Further, these results, differ to those reported by Fakankun *et al.*, (2013), whereby, upon comparison of the heavy metals in the leaves , root and stem of Moringa *oleifera* using the Pearson correlation coefficient, researchers found that Mn and Fe showed strong correlation. This is contrary to what has been found in Table 5.6.

Table 5.6 Mean correlation between different components of Moringa *ovalifolia* in different sites

	Al	As	Ba	Ca	Cd	Co	Cr	Cu	Fe	K	Li	Mg	Mn	Na	Si	Zn	Ni
Al	1																
As	0.483																
Ba	0.777	0.480	1														
Ca	-0.350	-0.391	-0.330	1													
Cd	0.613	0.837	0.671	-0.07	1												
Co	0.928	0.369	0.390	-0.389	0.543	1											
Cr	0.594	0.888	-0.276	-0.268	0.908	0.539	1										
Cu	0.488	0.438	-0.143	-0.143	0.629	0.908	0.509	1									
Fe	0.560	0.906	0.722	-0.299	0.886	0.996	0.996	0.469	1								
K	0.976	-0.180	0.076	-0.842	0.279	0.009	0.009	0.076	-0.035	1							
Li	0.558	0.811	0.732	-0.367	0.941	0.967	0.967	0.495	0.957	0.230	1						
Mg	-0.005	-0.227	0.127	0.872	0.293	0.020	0.020	0.182	-0.020	0.896	0.265	1					
Mn	-0.392	-.361	-0.346	0.955	-0.05	-0.257	-0.257	-0.117	-0.277	0.808	-0.05	0.766	1				
Na	0.954	0.791	0.790	-0.298	0.808	0.782	0.782	0.536	0.753	0.128	0.754	0.04	-0.337	1			
Si	0.908	0.265	0.46	-0.243	0.376	0.280	0.280	0.225	0.248	0.137	0.258	-0.034	-0.308	0.809	1		
Zn	0.730	0.719	0.868	-0.290	0.864	0.823	0.824	0.879	0.793	0.095	0.793	0.107	-0.302	-0.827	0.433	1	
Ni	-0.498	0.458	-0.399	0.897	-0.1000	-0.289	-0.323	-0.286	0.353	0.645	-0.117	0.792	0.771	-0.432	-0392	-0.391	1

5.7 Soil - plant transfer coefficient

Normally, heavy metals are found in numerous soils in various forms, of which, are involved in the movement from soil to plant (Zurerea *et al.*, 1989). A way of quantifying the relative differences in the bioavailability of metals in the plant is by utilising the transfer coefficient. This a calculation of the metal concentration in plant tissue above the ground divided by the total concentration in soil (Zurerea et al., 1989). The accumulation of the metals available in the plant when accumulated from the soil is determined as factor (f), which is also known as the transfer coefficient. This is calculated as the formula: (Zurera *et al*., 1989).

$f = [M]_p / [M]_s$

where:

[M] = metal concentration. p, s subscripts refer to plant and soil respectively.

The calculated transfer coefficients are given in Table 5.7.

Table 5.7 Comparison of the transfer coefficients of metals between soil and plant tissue and from literature (Cui *et al.,* 2004)

Element	Soil-plant coefficient[1]	Transfer coefficient Site 1	Transfer coefficient Site 2	Transfer coefficient Site 3	Transfer coefficient Site 4
Cd	1-10	0.4	0.8	0.8	0.6
Cr	0.01- 0.1	0.05	0.29	0.25	0.44
Cu	0.01-0.1	0.36	0.76	0.24	0.33
Zn	1-10	0.16	0.43	0.12	0.28
Be	0.01-0.1	-	-	-	-
Se	0.1-10	-	-	-	-

Note 1: Transfer coefficient obtained from Cui *et al.,* (2004).

All calculated transfer coefficients of these metals fell below the lowest limits except for Cr and Cu. Even for this, the increase was not pronounced. The principle factors which influence the behaviour of metals present in the soil are pH, amount of clays, oxides, organic matter and humic substances in the soil (Basta *et al*., 2005; Wahba *et al.,* 2007). Dynamics affecting solubility and exchange behaviours of metals in the clay and organic matter in the soil are usually low to medium and this might have accounted for the low exchangeable concentrations from the soil to the plant despite the higher values in the total concentrations in soil (Basta *et al.,* 2005).

The other reason for the low plant transfer coefficients could possibly be because of the high soil pH causing a lower availability in the soil for plant uptake and also. The metals are bound tightly to the soil colloids, thus preventing efficient uptake from the roots to the plant (Pepper *et al*., 1996).

Chapter 6 Part B: Antibacterial studies

Summary

Medicinal plants play a pivotal role not only as traditional medicines but also as commercial commodities meeting the demand of consumers. In order to contend with the expanding market, there is a growing need to introduce and scientifically confirm the use of medicinal plants. With the ever increasing drug resistance, more effort is being made to find alternative antibacterial components. Therefore, this study has been undertaken with the aim of determining the antibacterial activities of PHWE extracts of Moringa *ovalifolia* and Moringa *oleifera* against *Staphylococcus areus, Enterococcus faecelis and Pseudomonas aeruginosa.* These microbes are routinely responsible for oral diseases, wound infections, skin diseases and gastro-intestinal infections. The study was aimed at providing scientific evidence for the traditional use of the plants. Other studies reviewed in literature review section (Chapter 2) have shown that Moringa *oleifera* extracts, both aqueous and organic possess some of the mentioned properties. However, no such studies in Moringa *ovalifolia* have been reported. Furthermore, no PHWE extracts have been reported for antibacterial properties, including studies of the influence of temperature.

6.1 Results

Antibacterial activity against *S. areus* in the leaves of Moringa *oleifera* and Moringa *ovalifolia*

By disk diffusion method, the effectiveness of the Moringa *ovalifolia* extracts from site 1 and 3 at 100˚C (10 mm and 12 mm respectively), site 2 at 80˚C and 100˚C (8 mm and 10 mm respectively), site 3 and 5 at 100˚C (12 mm), as well *as* Moringa *oleifera* extracts extracted at 80˚C (12 mm) showed the highest inhibition zone against *S. areus.* Plant extracts from site 2, 3, and 5 extracted at 25˚C showed the least antibacterial activity with inhibition zones of 6 mm (Figure 6.1- Figure. 6.6).

Antibacterial activity against *E. faecelis* in the leaves of Moringa *oleifera* and Moringa *ovalifolia*

Results indicate that the plants extracts collected from sites 4 from Moringa *ovalifolia* and from Moringa *oleifera* extracts exhibited high zones of inhibition of 14 mm and 11 mm respectively at 25˚C. Site 1, 3, 4 and 5 showed the least activity at 6 mm. The inhibition diameter was 10-11 mm at 100˚C (Figure 6.1- Figure. 6.6).

Antibacterial activity against *P. aeruginosa* in the leaves of Moringa *oleifera* and Moringa *ovalifolia*

The plant extracts from Moringa *oleifera* collected from sites 4 and 5 were found to be the most active against *P. aeruginosa* at temperatures of 100˚C with inhibition zones of 11 mm and 14 mm. The least zone of inhibition was from all

the sites extracted at 25˚C from both species with inhibition zones as low as 6 mm at all 25˚C (Figure 6.1- Figure 6.6).

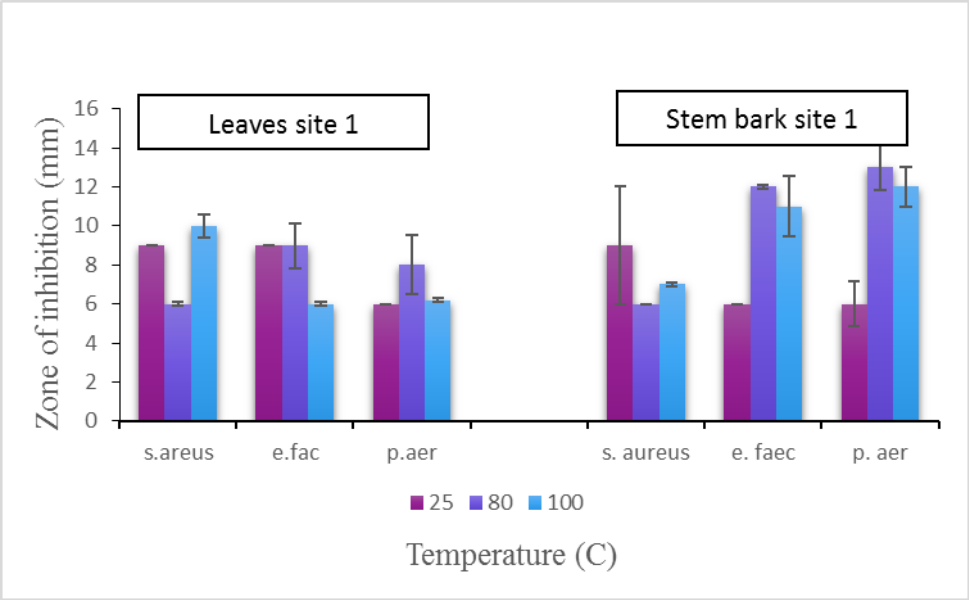


Figure 6.1 Thermostability of morphological parts of Moringa *ovalifolia* site 1. Each variable denotes the mean zone of inhibition (n=3).

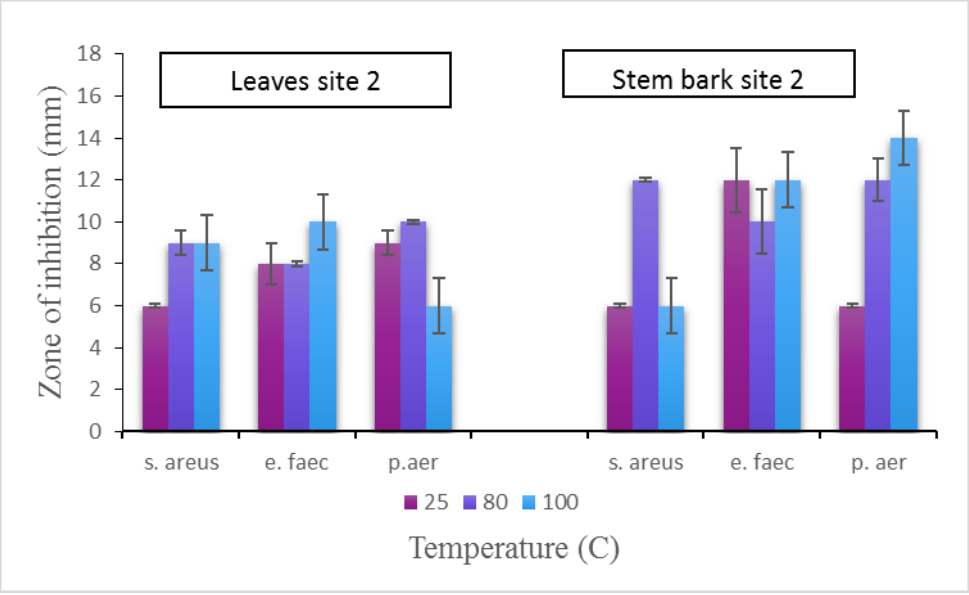


Figure 6.2 Thermostability of morphological parts of Moringa *ovalifolia* site 2. Each variable denotes the mean zone of inhibition from (n=3)

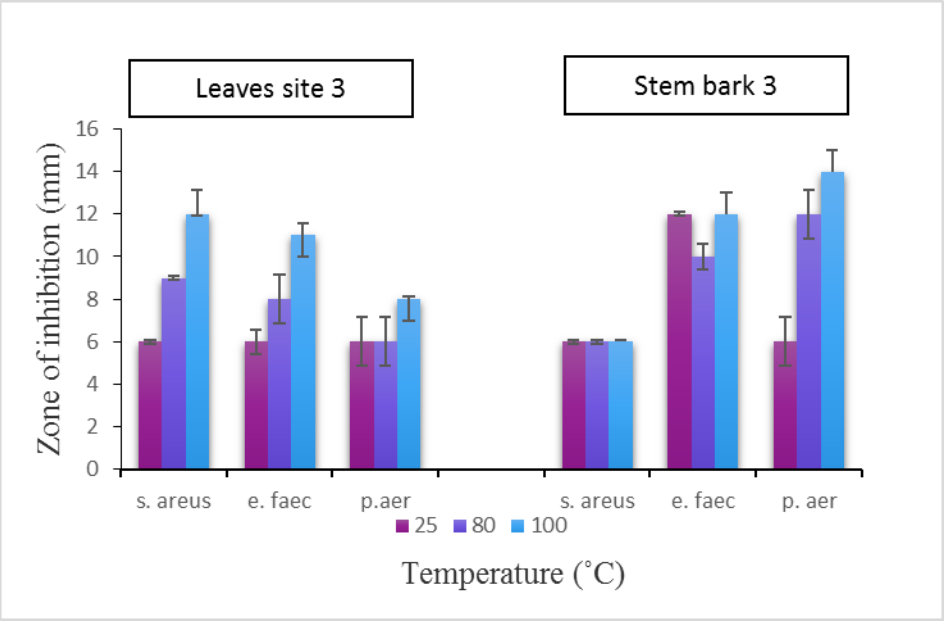


Figure 6.3 Thermostability of morphological parts of Moringa *ovalifolia* site 3. Each variable denotes the mean zone of inhibition from (n=3)

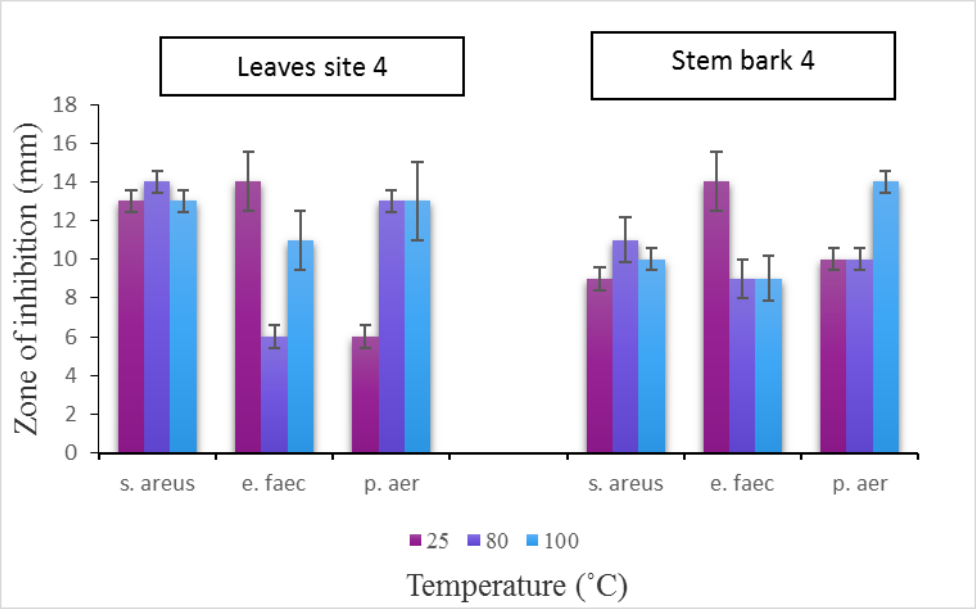


Figure 6.4 Thermostability of morphological parts of *Moringa ovalifolia* site 4. Each variable denotes the mean zone of inhibition from (n=3)

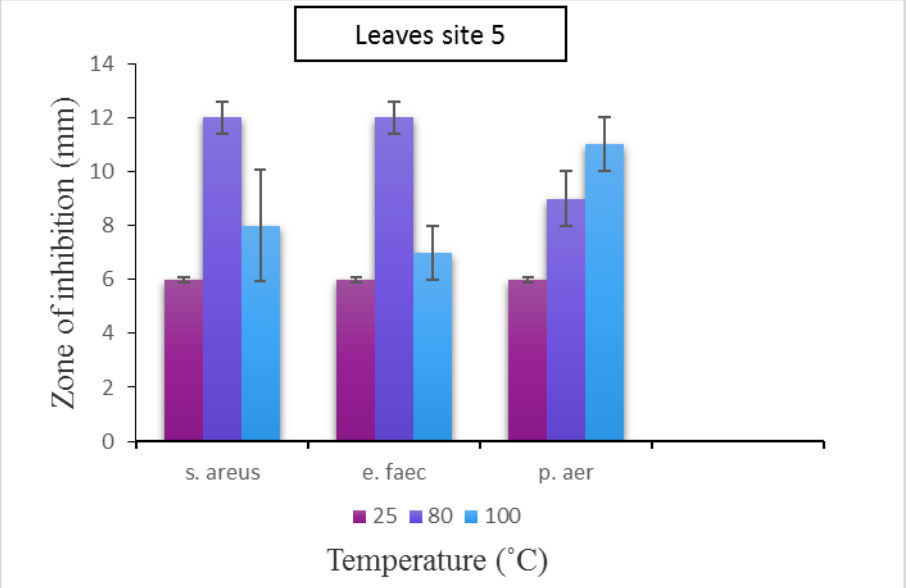


Figure 6.5 Thermostability of Moringa *ovalifolia* leaves site 5. Each variable denotes the mean zone of inhibition from (n=3)

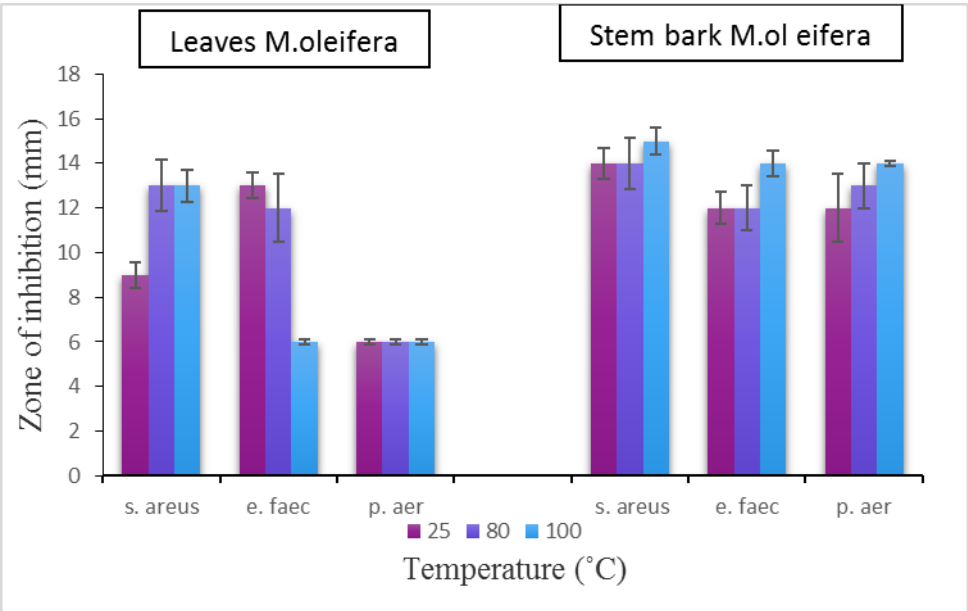


Figure 6.6 Thermostability of Moringa *oleifera* leaves. Each variable denotes the mean zone of inhibition from (n=3)

Antibacterial activity against *S. areus* in stem bark extracts in Moringa *oleifera* and Moringa *ovalifolia*

The results indicated that most sites exhibited the antibacterial activity against *S. areus* at temperatures 80˚C and 100˚C with maximum inhibition zones of up to 11 mm. The antibacterial activity from sites 2 and 3 had a low antibacterial activity of 6 mm at 25˚C. Site 1, however, exhibited a high zone of inhibition at 9 mm at 25˚C (Figure 6.1- Figure 6.6). The ethanol and aqueous extracts showed the least effect against *S. areus* with a minimum inhibition zone of 6 mm (Figure 6.7).

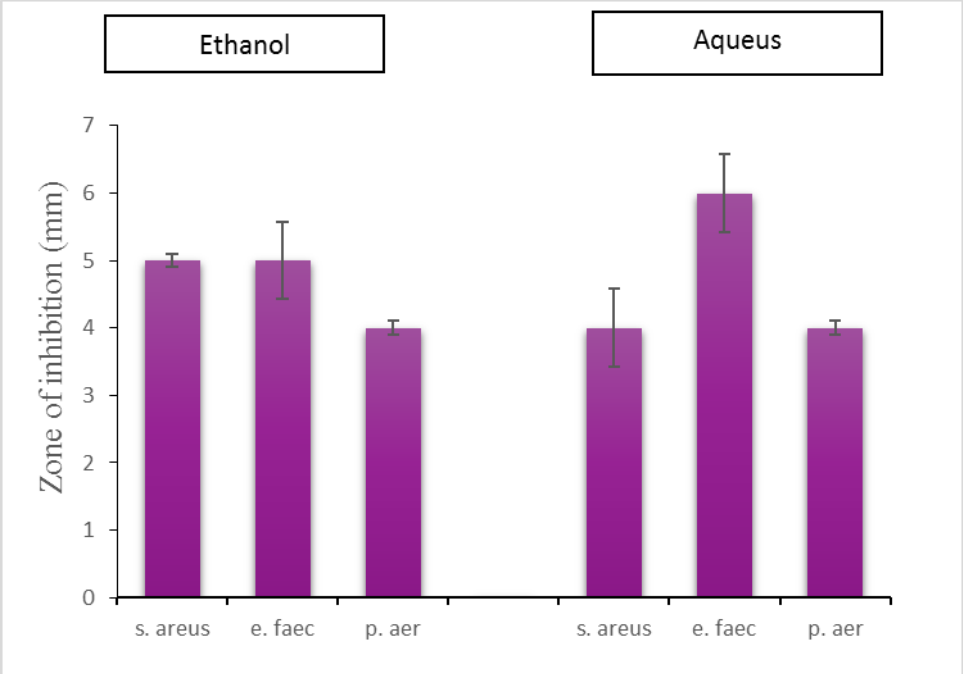


Figure 6.7 Antibacterial properties of *M. Ovalifolia* site 4. Each variable denotes the mean zone of inhibition from (n=3)

Antibacterial activity against *E. faecelis* in stem bark extracts in Moringa *oleifera* and Moringa *ovalifolia*

The largest zone of inhibition against *E. faecelis* was observed at 25˚C from site 4 from Moringa *ovalifolia* at 14 mm as well as 100˚C from Moringa *oleifera* extracts with a zone of inhibition at 14 mm. Site 2 at 25˚C, site 4 extracted at 80˚C and site 1 extracted at 100˚C showed the least antibacterial activity as low as 6 mm (Figure 6.1- Figure 6.6). Ethanol and aqueous extracts from site 4 of Moringa *ovalifolia* exhibited antibacterial activity slightly lower than the PHWE extracts at 8 mm and 10 mm (Figure 6.7).

Antibacterial activity against *P. aeruginosa* in stem bark of Moringa *oleifera* and Moringa *ovalifolia extracts*

The results for the antibacterial activity against *P. aeruginosa* revealed that Moringa *ovalifolia* from all four sites exhibited the highest activity against *P. aeruginosa* at temperatures 80˚C and 100˚C with inhibition zones ranging from 12-15 mm. Moringa *oleifera* extracts showed a similar effect with extracts showing high zones of inhibition of 13 mm and 14 mm at 80˚C and 100˚C (Figure 6.1- Figure 6.6). Ethanolic and aqueous extracts showed the least activity at 6 mm (Figure 6.7).

6.1.1 Discussion of the results

Authors have reported that Moringa plant possess antibacterial properties in the leaves and stem bark (Onsare *et al.*, 2013). In this investigation, PHWE method is utilised using water at varying degrees as a solvent for the preparation of plant extracts from Moringa *oleifera* and Moringa *ovalifolia* for antibacterial studies. The effectiveness of the aqueous crude extracts in *vitro* was quantitatively assessed for its efficacy. Stem bark extracts from Moringa *oleifera* and Moringa

ovalifolia showed more antibacterial activity than the leaf extracts of Moringa *ovalifolia* against all the test organisms except *S. aureus* where the leaves from PHWE were more active. The marginally greater antibacterial activities recorded by the stem bark over the leaf extracts signify that more of the bioactive ingredients are lodged in these parts (Ogu *et al.,* 2012). The stem bark and leaves from site 4 of Moringa *ovalifolia and* stem bark extracts from Moringa *oleifera* collectively showed the highest antibacterial with the average inhibition zone ranging from 6- 16mm. This suggest that environmental factors and differences in species all contribute to antibacterial activities.

It is worth noting that the leaf extracts showed potent antibacterial activity against both gram positive and gram negative bacteria. However, the highest antibacterial activity by the stem bark was demonstrated against *P. aerugonasa* with the inhibition zone of 16 mm. This information is a breakthrough because according to literature, Moringa *oleifera* aqueous and ethanolic leaf extracts did not have any antibacterial effects on *P. aeruginosa* (Rahman *et al*., 2008). This report is in agreement with a study conducted by Valsraj *et al.,* (1997), where extracts of Moringa peel were used to evaluate its effectiveness on infectious diseases. These studies showed that *P. aeruginosa* was inhibited (Valsaraj *et al.,* 1997). The leaves, however, were found to exhibit to a varying degree on both gram positive and gram negative bacteria. Similar effects have been reported in Moringa *oleifera* leaf extracts inhibiting the growth of *S. areus* and *P. aeruginosa* (Cáceres *et al.,* 1991*).* Authors accredited the antibacterial effects to phytoconstituents such as saponins, tannins, phenolic acids and alkaloids (Doughari *et al.,* 2006). The lack of antibacterial activity exhibited by some extracts isn't enough to assume

that the extracts don't contain the necessary phytoconstituents for this action but, the effectiveness and yield of the extracts is dependent on the solvent and extraction method used to obtain the extract, the age the plant is cultivated and the amounts of bioactive constituents available in the plant which various depending on the season (Sofowora, 1982; Rios, 2005).

6.2 Data processing of antibacterial results using ANOVA

Further data analysis was carried out using ANOVA analysis of variance (ANOVA) from Microsoft Excel 2013®. P value < 0.05 was considered as significant. The ANOVA software separates the variance of all parameters within and between each group. The results of ANOVA determine whether the manipulated data, which would be temperature in this case, causes significant difference in the antibacterial activity in the stem bark and leaves from all sites of the Moringa species. A multi comparison test was also determined to provide evidence that the means of the groups are similar. This was also to provide information as to which group is significantly different to the other.

In order to apply ANOVA to our data, three assumptions were made. These are:

- The value of one observation should not be associated with another observation, therefore, making the observations independent of each other.
- The observation in each respective group must be normally dispersed
- Variance of each respective group is equivalent to that of any other group

In order to determine our results statistically, a null hypothesis was assumed, that is, there is no statistical difference between and within the groups. The F ratio was determined as follows:

$$F_{ratio}=\frac{\text{mean square within the groups}}{\text{mean square between the groups}}$$

If the F-ratio is bigger than F-critical, and there is a significant level at P=0.05. Then the null hypothesis is not valid and there is statistical difference between and within groups.

Below are the results of the test analysing the variables in the leaves and stem bark of Moringa *oleifera* and Moringa *ovalifolia* (Tables 6.1 and 6.2).

6.3. Discussion on the ANOVA results

The ANOVA tests shown in Tables 6.1 and 6.2 summarises the influence of temperature on the antibacterial activities of Moringa *oleifera* and Moringa *ovalifolia* extracts. As this section is focused on the influence of temperature on the various extracts, the antibacterial susceptibility and its significant difference between *S. areus*, *E. faecelis* and *P. aeruginosa* is not discussed.

The results are supposed to support what is observed in Figures 6.1-6.6 which has been discussed under Section 6.2. The ANOVA test results show that only the *S. areus* bacteria was influenced by temperature from the leaf extracts in the plant species in Moringa *oleifera* leaves and from Moringa *ovalifolia* leaves from sites 2, 3 and 4. For the stem bark extractions, temperature had an influence on the *E. faecelis*, *P. aeruginosa* and *S. areus* bacterial species like those seen in site 1, 3 and 4. The slightly greater antibacterial activity of the stem bark indicates that more of the phytoconstituents are lodged in these parts and also that the stem bark had matured and developed faster than the leaves. This information also implies

that the phytoconstutients responsible for the inhibition were stable enough to withstand temperatures ranging from 80-100˚C (Fig 6.1- Fig 6.6). The significant differences between species may also attribute to the growing and drying conditions of the two species. Moringa *oleifera* was cultivated in a farm in South Africa while Moringa *oleifera* plants were naturally grown at different sites in Namibia.

Table 6.1 Results of ANOVA from the leaves of Moringa *oleifera* and Moringa *ovalifolia* to determine whether there is a significant difference between mean zones of inhibition extracted at different temperatures (25˚C, 80˚C and 100˚C) on *S.areus, E.faecelis* and *P.aeruginosa.*

Variable	**Bacterial Strain**	**F-value**	**F-critical**	**P-value**
Temperature (25˚C-100 ˚C)	*S. areus*	1.28	4.10	0.31
	E. faecelis	0.9	4.10	0.41
	P. aeruginosa	4.01	4.10	0.05
	S. areus	7.63	3.34	0.003
	E. faecelis	1.93	3.84	0.19
	P. aeruginosa	2.35	3.33	0.12

Table 6.2 Results of ANOVA from the stem bark of Moringa *oleifera* and Moringa *ovalifolia* to determine whether there is a significant difference between mean zones of inhibition extracted at different temperatures (25˚C, 80˚C and 100˚C) on *S.areus, E.faecelis* and *P.aeruginosa.*

Variable	**Bacterial Strain**	**F-value**	**F-critical**	**P-value**
Temperature	*S. areus*	2.53	4.46	0.14
	E. faecelis	3.62	0.07	4.46
	P. aeruginosa	2.24	0.17	4.46
	S. areus	7.1	4.46	0.14
	E. faecelis	1.93	3.84	0.20
	P. aeruginosa	0.49	3.84	0.74

The ANOVA test in Table 6.4-6.6, was also used as a comparison test between Moringa *ovalifolia* plant sites to compare the influence of individual temperatures on the bacteria studied and to provide further evidence to which group is different from each other. These results are intended to support the figures displayed in Figures 6.1-6.6. When using the ANOVA test, only certain coupled sites showed the influence of temperature on antibacterial activity and these are shown in Tables 6.4-6.6. Results showed that the stem bark extracts from Moringa *ovalifolia* from site 2 and 4 extracted between 25˚C and 80˚C as well as 25˚C and 100˚C were significantly different from each other (Table 6.3). Similarly, temperatures extracted from Moringa *ovalifolia* from sites 1 and 3 at 25˚C and

80˚C as well as 25˚C and 100˚C were also significantly different from each other. Results showed that Moringa *ovalifolia* leaf samples from site 3 and 4 extracted at 80˚C and 100˚C showed that they were significantly different from each other. Since there is little activity at 25˚C for sites 2 and 4 of the stem bark (Figures 6.2 and 6.4) and sites 1 and 3 of the leaves (Figures 6.1 and 6.3), you would expect a significant difference in antibacterial activity at 80˚C and 100˚C from 25˚C because of the differences in extraction temperature. When comparing the stem bark extracts this concept does not apply to sites 3 and 4 because antibacterial activity varied for site 4 at different temperatures. Therefore, based on these results, we can therefore confirm that antibacterial activity is site specific.

Table 6.3 Results on the ANOVA test on mean inhibition zone of aqueous Moringa stem bark extracts, from site 2 and 4, extracted at different temperatures on *P. aeruginosa*, to determine which group is significantly different from one another.

Group combinations incubated at different temperatures on *P. aeruginosa* (˚C)		**F-value**	**F-critical**	**P-value**	**Implication**
25	**80**	65535	161.45	0.00	significant difference
80	**100**	1	161.45	0.00	No Significant difference
25	**100**	169	161.45	0.05	significant difference

Table 6.4 Results on the ANOVA test on mean inhibition zone of aqueous Moringa stem bark extracts, from site 1 and 3, extracted at different temperatures on *P. aeruginosa*, to determine which group is significantly different from one another.

Group combinations incubated at different temperatures on *P. aeruginosa* (˚C)		**F-value**	**F-critical**	**P-value**	**Implication**
25	**80**	65535	161.45	0.00	significant difference
80	**100**	1	161.45	0.00	No Significant difference
25	**100**	169	161.45	0.05	significant difference

Table 6.5 Results on the ANOVA test on mean inhibition zone of aqueous Moringa leaf extracts, from site 3 and 4, extracted at different temperatures on *E. faecelis*, to determine which group is significantly different from one another.

Group combinations incubated at different temperatures on *E. faecelis* (˚C)		**F-value**	**F-critical**	**P-value**	**Implication**
25	**80**	1	161.45	0.5	No significant difference
80	**100**	6335	161.45	0.00	Significant difference
25	**100**	25	161.45	0.13	No significant difference

The inconsistency in results from site 3 and 4 could be due to the localisation of the nutrients. The bioactive molecules and phenolic compounds in the leaves may not be scattered uniformly, but rather, confined in the rhizophere and spermosphere of the plant. Therefore, whether the leaf extracts will exhibit antibacterial activity is dependent on the extraction process (Weller *et al.*, 1990; Weller *et al.*, 1993 and Blum *et al.*, 1994). Another factor could be due to the diffusion rate of the active constituents through the agar (Marjorie, 1999; Rioss *et al.*, 1998).

Based on the results from Tables 6.1-6.5, optimum extraction temperature for these sites are site specific and plant part specific (Onsare *et al*., 2013). These results also confirm that the chemistry of the plant as well as environmental factors is a key factor in the antimicrobial activity of the Moringa extracts (Thomas *et al.*, 2012). As mentioned earlier, growing and drying conditions could affect antibacterial activity. When researchers examined the effect the growing and drying conditions had on the phenolic acid composition of medicinal plant *I. paraguariensis*, they found that the plantation grown sample had higher a higher phenolic acid composition compared to its forest grown counterpart therefore concluding that cultivation and processing had a substantial effect on the manufacture and concentration of the phytochemicals present (Heck *et al.*, 2008). Pigmentation may also be a determining factor, the fewer the pigments available in the leaves, a higher antibacterial activity is expected. The phenolic compounds extracted may also hinder the antibacterial effect of the extracts (Doughari, 2006).

The increased antibacterial activity at high extraction temperatures from sites 1, 3 and 4 of the stem bark extracts tested on *S. areus*, *E. faecelis* and *P. aerugunosa* is in agreement with the work of Roudsari *et al.,* (2009) who reported that there was an enhanced effect of the extracts of canola meal extracted with hot water extraction at temperatures 80˚C and above. At room temperatures, the surface tension in water is not as diffusive as it is at elevated temperatures, thus, preventing the cells in the organic matter to break open to release the target analyte (Heck *et al.,* 2008). This information proves that based on the active compounds miscibility, some will have antibacterial action and some extracts won't, which would explain why in most sites at 25ºC, there was a decrease in antimicrobial activity. Similarily, the increase in temperature is in agreement with a previous report conducted by Doughari, (2010), whereby, antibacterial activity of methanol and aqueous extracts increased with increasing temperatures (4˚C-100˚C) from medicinal plant *Erytrina senegalensis* (Doughari, 2010). Since the extraction yield is dependent on the solvent used, the set time, temperature of the extraction as well as the chemical property of the sample, then it is possible that the extraction of phytoconstituents that are thermally stable might have been enhanced at temperatures of 80˚C and 100˚C, producing a higher yield of antibacterial activity (Co *et al.,* 2011).

Overall, for a total extraction time of 20 min, all extracts showed a good antibacterial activity against all of the bacteria regardless of the temperature used. An increase in the extraction temperature from 80ºC – 100ºC produced extracts with a higher antibacterial activity, whereas, 25ºC was too low for the total extraction of phytochemical compounds responsible for antibacterial activity. This

only applied to the stem bark of Moringa *oleifera* and Moringa *ovalifolia* which revealed the antibacterial activity from all four sites increased with increasing temperature with inhibition halos ranging from 12-15 mm. This shows that the PHWE method is a substantial method of extraction.

6.4 Comparison of PHWE with ethanol and water

Based on the results of the screening experiments conducted above, the stem bark from site 4 collected from Moringa *ovalifolia* was selected for further analysis against ethanol and aqueous extraction methods.

In order to determine if the PHWE is the best medium for extraction of active components responsible for antibacterial activity, ethanol and aqueous extracts (Figure 6.7) from site 4 were compared to PHWE technique. Moringa *ovalifolia* and Moringa *oleifera* were extracted utilising the method reported by Peixoto *et al.,* (2012). The variance analysis revealed a statistically significant relation between the variable (temperature) and the size of inhibition of the PHWE, ethanol and aqueous extracts (Table 6.6). However, the relation between the variable and size of inhibition for the ethanol and PHWE extracts was not significant for *S. areus* ($p=0.05$). The comparative study generally showed that the PHWE stem bark extracts were more active than the ethanol and cold water extracts respectively. This is in some way new findings as published articles have been reported that ethanol extracts being more effective than aqueous extracts. Researchers attributed this observation due to the polarity of ethanol which tends to extract more active compounds from the sample than water (Silva *et al.,* 2011; Dutta, 1993). The lower antibacterial activity in the aqueous extracts re-

emphasizes the decreased solubility of bioactive constituent at room temperature and less penetration power since pressure is less. This finding is also in line with the report of Banso (2005), that implicated that hot water extraction expressed resulted into a greater amount of phytochemicals than its cold water counterpart. This hot water extraction gave more antibacterial activities (Banso, 2005). The researchers noted that the stability of these extracts in hot water further justified the efficacy of the plant extract when boiled and utilised as herbal medicine (Banso, 2005).

If most of the phytochemicals and phenolics in the plant are miscible in water, than a high antibacterial activity can be observed. The increase in pressure and temperature in the PHWE method caused water to mimic similar properties to that of the organic solvent such as that of ethanol, which caused water to become less polar allowing for the increased extraction of the biologically active compounds responsible for the elimination of bacteria. This also strongly suggests that the active compounds. This observation is in agreement with the work of Hassas-Roudsari *et al.,* (2009) who reported that there was an enhanced effect of the extracts of canola meal extracted with hot water extraction at temperatures 80˚C and above. At room temperatures, the surface tension in water is not as diffusive as it is at elevated temperatures. This prevents cells in the organic matter to break open to release the target analyte (Hassas-Roudsari *et. al* 2009). This information proves that based on the active compounds miscibility, some will have antibacterial action and some extracts won't, which would explain why in most sites at 25˚C, there was a decrease in antibacterial activity.

Table 6.6 Variance analysis of susceptibility of *S.areus*, *E. faecelis* and *P.aeruginosa of* PHWE, ethanol and water *stem* bark (site 4) extracts at 25^{o}C.

	S.areus					*E.faecelis*					*P. aeruginosa*				
SV	SS	DF	MS	F	P	SS	DF	MS	F	P	SS	DF	MS	F	P
Zone of inhibition	8.17	1	8.17	4.90	0.09	48.1	1	48.17	18.06	0.01	10.67	1	10.67	64.00	0.001

* SV: Sources of variation. SS: Sum of squares. DF: Degree of freedom. MS: Mean square.

6.5 PHWE compared to literature studies

The results of antibacterial activity from this study were compared to others reported in literature (Table 6.7). From the results it can be concluded that PHWE has adequate antibacterial activity. It can be compared to the other solvents. Solid extraction using solvents like the ethanol extraction. The concentrations of samples extracted differs in Table 6.8. Thus, comparison is relative but can give an indication of what method is better. Since the zones of inhibition of the Moringa species at low concentrations are comparable to the ethanol extracts at high concentrations, this information implies that the PHWE method will be more effective at similar concentrations.

Table 6.7 Comparison of the effect of PHWE against human pathogenic bacteria with other conventional methods of extraction from literature.

Solvent	Extraction technique	Extract	Concentration	Bacteria	Zone of inhibition (mm)	References
Cold water	Conical flask with shaking	Moringa *oleifera leaves*	100 g/100 mL	*S. areus*	15	Rahman *et al.*, 2009
Cold water	Conical flask with shaking	Moringa *oleifera leaves*	100 g/300 mL	*S. areus*	15	(Rahman *et al*., 2009)
Cold water	Conical flask with shaking	Moringa *oleifera leaves*	100 µl of 20 g/ 180 mL	*E. faecelis*	30	(Rahman *et al*., 2009)
Cold water	Conical flask with shaking	*Moringa oleifera leaves*	100 g/ 300 mL	*P. aeruginosa*	15.0±0.12	(Rahman *et al*., 2009)
Water	PHWE	Moringa *oleifera* Stem bark	0.5 g/ 10 mL	*S. aureus* (100˚C*)*	15	This study
Water	PHWE	Moringa *oleifera* Stem bark	0.5 g/ 10 mL	*E. faecelis* (100 ˚C)	14	This study
Water	PHWE	Moringa *oleifera* Stem bark	0.5 g/ 10 mL	*P. aeruginosa*	14	This study

Water	PHWE	Moringa *ovalifolia* Stem bark (site2)	0.5 g/ 10 mL	*S. aureus* (*80˚C*)	11	This study
Water	PHWE	Moringa *ovalifolia* (site 2)	0.5 g/ 10 mL	*E. faecelis* (*80˚C*)	12	This study
Water	PHWE	Moringa *ovalifolia* (site 2)	0.5 g/ 10 mL	*P. aeruginosa (80˚C)*	13	This study
Ethanol	Liquid liquid extraction	Moringa *oleifera leaves*	100 g/300 mL	*S. aureus*	13	Rahman *et al.*, 2009
			100 µl of 20 g/180 mL	*E. faecelis*	14.4	(Silver *et al*., 2011)
			100 g/300 mL	*P. aeruginosa*	21	Rahman *et al.*, 2009

Chapter 7 Conclusions and Recommendations

Summary

This chapter gives a brief summary of the work done in determining the elemental composition of Moringa *oleifera* and Moringa *ovalifolia.* Preliminary *in vitro* antimicrobial studies were investigated to discuss the effectiveness of the PHWE compared to conventional methods of extraction. It further discusses optimal conditions for the extraction of essential compounds responsible for the elimination of pathogenic bacteria.

7.1 Conclusions

This study revealed essential elements that were determined in the soil, leaves and stem bark of Moringa *ovalifolia* sampled from four sites. The concentrations of heavy metals in soils were within its accepted maximum allowable concentrations in Germany, UK and Canada. Concentration levels were also within the normal content intervals in soil. Thus, this information implies that the soils where Moringa *ovalifolia* grows naturally is not polluted. According to the soil to plant transfer coefficient, Moringa leaves do not accumulate the metals from the soil and are, therefore, fit for consumption. On average, the study revealed high concentration of macronutrients in Ca, Mg, K and Fe were all present in the leaves but Fe was the most abundant micronutrient. When compared to Moringa *oleifera* sampled from South Africa, results indicated that Ca, K and Na in Moringa *ovalifolia* were higher than Moringa *oleifera*. However, concentrations of Zn, Cr and Fe were found to be higher in Moringa *oleifera* than Moringa *ovalifolia*.

As for the antibacterial results, the PHWE system was successful in extracting bioactive compounds responsible for the elimination of pathogenic bacteria. For the total extraction time of 20 min in various sites, an increase in the extraction temperature from 25ºC to 80ºC and 100ºC produced extracts with a higher antimicrobial activity. When the PHWE technique was compared to other conventional methods of extraction, results showed that PHWE has adequate antibacterial activity and can be compared to that of ethanol extraction. Our results, thereby, indicate the possibility of using these extracts from the PHWE system in the treatment of bacterial infections. Further, the search for active constituents found in both Moringa samples could aid in the developments of

drugs. This study was encouraging, despite the need for clinical studies to determine the real effectiveness and potential toxic effects in vivo. Therefore the active constituents of both Moringa samples could be a source of potential candidates in the search for active constituents that could lead to development of drugs or drug leads of broad antibacterial spectrum. However, it should be noted that research on antibacterial in *vitro* level may not always culminate with the same effects when administered into the human body.

7.2 Recommendations

It is noteworthy to observe how antibacterial strains of *S.areus*, *E. faecelis* and *P. aeruginosa* react to other aerial parts of Moringa *ovalifolia*. viz. root and seeds. Identifying phytochemicals responsible for antibacterial activity using HPLC-MS will prove to be beneficial to the study. Lastly, exploring other medicinal properties of Moringa against cancer, high blood pressure and other lifestyle diseases.

List of conferences and seminar presentations:

1. C. Makita, L. Chimuka, E. Cukrowska, Martha Kandawa-Schulz, Habauka M. Kwaambwa, Hlanganani Tutu and Marius Hedimbi . The Elemental analysis of Moringa *ovalifolia*.The Elemental analysis of Moringa *ovalifolia.* International Chemistry Conference for Africa (ICCA) 2013, 7^{TH} -12^{TH} July, University of Pretoria, SA, Poster presentation.

2. C. Makita, L. Chimuka , E. Cukrowska, Martha Kandawa-Schulz, Habauka M. Kwaambwa, Hlanganani Tutu and Marius Hedimbi . The Elemental analysis of Moringa *ovalifolia*. 5^{th} Cross- Faculty Post Graduate Symposium, 1^{st} -2^{nd} August 2013, University of the Witswatersrand, Johannesburg, South Africa, Poster presentation.

3. C. Makita, L. Chimuka , E. Cukrowska, Martha Kandawa-Schulz, Habauka M. Kwaambwa, Hlanganani Tutu and Marius Hedimbi . The Elemental analysis of Moringa *ovalifolia*. Saci conference, 41^{st} Walter Sisulu University, East London, Oral presentation.

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