DEVELOPMENT OF PRESSURISED HOT WATER EXTRACTION (PHWE) FOR ESSENTIAL COMPONENTS FROM *MORINGA OLEIFERA* AND *OVALIFOLIA* LEAVES

By

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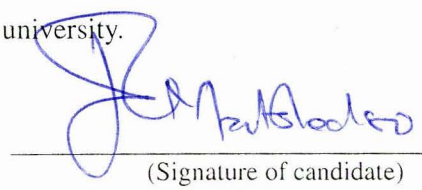
Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.



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Abstract

Medicinal plants have always been used and preferred to allopathy medicine particularly in developing countries due to their accessibility and affordability. These plants have phytochemicals which can be manufactured in their systems to ensure the survival against harsh conditions such as ultra violet (UV) radiation. Some of these substances include phenolic compounds which when ingested can be utilised to boost the immune system of an individual. The challenge however is the best way to extract these substances from the plants.

Conventional methods which use organic solvents are often used for the extraction of the essential compounds such as flavonoids, micro and macro elements. Distillation and soxhlet extraction for instance have mostly been used for the extraction of these essential compounds. These result in higher volumes of the organic solvents which are not only expensive but also environmentally unfriendly. In this study, a novel technique based on pressurised hot water extraction (PHWE) was developed for the extraction of the essential compounds from *Moringa sp* leaves extracts. PHWE is a "green" approach which utilises only pressurised water at elevated temperature to extract essential compounds. PHWE was used for the extraction of essential compounds such as flavonols and minerals.

Moringa Oleifera samples were obtained from Polokwane and Atteridgeville in South Africa while *Moringa Ovalifolia* was obtained from five different sampling sites in Namibia. These are Okahandja (site 1), Okaukuejo also known as Ghost forest (site 2), Halali (site 3) which is situated in Etosha national park, Tsumeb (site 4) and Keetmanshoop (site 5) in the Karas region. No research work has been done regarding this particular Namibian *Moringa* species.

In this study, Flavonols were investigated for their phenolic compounds. Antioxidant properties were investigated using three indicators being the reducing activity, DPPH radical scavenging activity and the total phenolic content. *Moringa Oleifera* is highly dominated by kaempferol. The extraction temperature of 100^0C was found to be optimal for the release of kaempferol as about 3 500 mg kg^{-1} was recovered. 100^0C extraction temperature was also optimal for myricetin as the highest concentration of 2699 mg kg^{-1} was found. An optimal concentration of 1488 mg kg^{-1} for quercetin was found at 150^0C extraction temperature. Extraction temperature influence the extraction efficiency of the flavonols which often exist as glycosides. These glycosides need to be cleaved by acid hydrolysis to release the aglycones of these flavonols which can then be quantified. Myricetin unlike the other two flavonols did not survive hydrolysis and hence was injected directly into the High Performance Liquid Chromatography (HPLC). Reverse phase high performance liquid chromatography with UV detector set at a wavelength of 254 nm was used for the identification and quantification of these flavonols. Myricetin was however found to survive acid hydrolysis with *Moringa Ovalifolia*.

Elemental composition of the leaves was determined using the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The metals behaved differently. In both *Moringa sp*, Na as compared to Ca, K and Mg increased with increasing temperature. It is possible that for Ca, K and Mg, the metals are loosely bound to the active sites as such temperature does not contribute significantly to their desorption. Among the macro nutrients extracted from the *Moringa Oleifera*, potassium was found to have highest concentrations than other metals at all extraction temperatures while in *Moringa Ovalifolia* Na was found to be highest in only sites 3,4 and 5. In the other two sites K was found to be dominant. These variations could be influenced by geographical location, soil conditions, climatic conditions and others.

Moringa Ovalifolia was found to have high concentrations of As. This was observed with samples from site 4 where mining occurs. It is thus possible that the soils are contaminated as such *Moringa Ovalifolia* accumulates these metals. The

total phenolics content (TPC) for *Moringa Oleifera* increased with temperature until 150^{0}C and then decreased while in *Moringa Ovalifolia* the temperature was not affected by temperature. The reducing activity of the *Oleifera* leaf extracts was found to increase with increasing temperature while for *Ovalifolia* it remained constant through out various temperatures. The DPPH radical scavenging activity of *Oleifera* leaf extracts indicated that extraction temperature of 100^{0}C was optimal. Its minimal with fractions collected at 25^{0}C and almost nil with fractions collected at 200^{0}C. When comparing the efficiency of the two species it was concluded that *Moringa Oleifera* is much more effective as it reduced the DPPH radical by almost 30% while *Moringa Ovalifolia* reduced it by 12%. The UV-Vis set at 515 nm was used for the DPPH analysis while for the total phenolic contents and the reducing activity was set at 740 and 700 nm respectively.

The above mentioned parameters were tested when the powder obtained from the leaves was boiled at different times to evaluate the extractability of essential compounds. This information would be useful for people living in communities that would benefit by using *Moringa* powder as a supplement in tea and other foods to enhance their nutritional values.

The PHWE technqiue was found to be efficient in the extraction of the essentials from medicinal plants. The concentration values for minerals obtained using PHWE compared very well with those obtained by microwave extraction.

To my dear family, you are simply the best and love you always. May God bless you abundantly.

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First and foremost I would like to thank the Lord my saviour for seeing me through until this day. Surely His goodness and mercies endureth forever.

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Finally, I would like to thank my sponsors Department of Science and Technology for funding. This work was made possible by their support.

Phatsimo Gimamah Matshediso

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List of Symbols and Abbreviations

The following are the symbols and acronyms used in this dissertation.

DPPH	diphenylpicrylhydrazyl
TPC	Total Phenolic Content
HPLC	High Pressure Liquid Chromatography
ICP -OES	Inductively Coupled Plasma Optical Emission Spectroscopy
UV -VIS	Ultra violet light
PHWE	Pressurised Hot Water Extraction
MAE	Microwave Assisted Extraction
SPME	Solid Phase micro extraction
UAE	Ultrasonication Assisted Extraction
RSD	Relative Standard Deviation
LOD	Limit of Detection
LOQ	Limit of Quantification
PLE	Pressurised Liquid Extraction
FC	Folin-Ciocalteu
BHA	butylated hydroxy anisole
BHT	butylated hydroxy toluene
TBHQ	tertbutylhydroxyquinone
ε	Dielectric constant
PHZ	pre heating zone
NAZ	Normal Analytical Zone

C_6	Hexyl silane bonded silica
IRZ	Initial Radiation Zone
sp	species
RF	radio frequency
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
O_2^-	Peroxy radical
$ONOO^-$	Peroxynitrite
CF	Correlation Factor

Chapter 1

Introduction

Summary

In this chapter, polyphenolic compounds found in medicinal plants are discussed and the advantages of using medicinal plants such as *Moringa Oleifera*. The traditional and conventional methods of extraction are discussed. Finally the need for green extraction techniques especially those whose extracts can be used for value additions in various foods is discussed.

1.1 Essential nutrients in plants

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease [1]. Many plants, citrus fruits and leafy vegetables are the source of ascorbic acid, vitamin E, caratenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body [2]. The health-promoting properties of fruits and vegetables are due to the presence of some vitamins (A, C, E, and folates), dietary fiber, and non-essential phytochemicals in these food products [3]. Plants contain many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which are deposited in their specific parts such as leaves, flowers, bark, seeds, fruits, root and others [4]. There are more than 1,000 known naturally occurring bioactive molecules or phytochemicals that are non-nutritive plant chemicals and yet, have protective or disease preventive properties for human beings [5]. Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases [1]. To recover bioactive compounds from plant materials, efficient and reliable novel extraction techniques should be employed. Selection of the appropriate solvent for extraction of these bioactive compounds is a very important aspect that needs to be addressed in a judicious way so as to achieve the maximum concentration of desired phytoconstituents in plant extracts [6].

1.2 Some functions of other chemicals in plants

Micronutrients play a central role in metabolism and in the maintenance of tissue function [7]. The trace elements together with other macro nutrients are necessary for growth, normal physiological functioning and maintaining of life [8]. Plants like all other living creatures need nourishment to sustain life. Plants require 16 essential elements. Carbon, hydrogen and oxygen are derived from the atmosphere and soil water while the remaining 13 essential elements (nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, zinc, manganese, copper, boron, molybdenum and chlorine) are supplied either from soil minerals and soil organic matter or by organic or inorganic fertilizers [9, 10]. These essential compounds can thus be accessed by human kind if the plants are ingested. The quantitative estimation of various trace elements concentrations are necessary for the effective determination of medicinal plants for the treatment of various diseases and also for the understanding of their pharmacological actions [11].

1.3 Traditional methods of extraction of plant materials

Traditional extraction techniques include solid–liquid extraction, soxhlet extraction, sonication and blending [12, 13]. A broad spectrum of solid-liquid extraction (SLE) techniques are widely used for the purification of natural products from plant materials and micro-organisms [12]. The disadvantage of these is that they are not selective to the analytes of interest which can results in compromised accuracy of the results as they can either be inhibited because of interference or not maximally extracted

because of minimal selectivity. Another drawback of these techniques is that the obtained final extracts often require subsequent concentration and clean-up prior to analysis [14]. This is not only tedious but can pose a health threat if the extract is for food and medicines. They have long extraction times and high solvent consumption [14, 13]. In addition to that, traditional methods of extraction may not be suitable for bioactive compounds that are sensitive, thermolabile and in minute concentrations. According to Roudsari *et al* 2009, [15] these methods may have undesirable effects on the environment and on food components hence "green" technologies would be more desirable.

1.4 Modern methods of extraction of plant materials

Examples of modern methods of extraction include microwave assisted extraction (MAE), ultrasonication assisted extraction (UAE), supercritical fluid extraction (SFE) and solid phase micro extraction (SPME) [4]. These are used in the determination of organics in either solids or aqueous samples depending on the particular technique. MAE can be used in solids while SPME can be used for aqueous samples. The advantages of modern methods include the reduction in the use of organic solvent and in minimizing sample degradation. They are faster as compared to traditional methods such as soxhlet which could run up to 24 hours. They also result in the elimination of undesirable and insoluble components from the extract [4]. However, as research has advanced, better techniques such as pressurised hot water extraction (PHWE) has been developed. PHWE is perceived as a modification of pressurised

liquid extraction (PLE) and it was first introduced in 1994 [5]. It is a "green" technique because it is environmentally friendly as it uses only water for extraction.Table 1.1 below which is an excerpt from literature shows a comparison of the extraction efficiency of PHWE to traditional methods. The amount of baicalein, a chemical from the medicinal plant was extracted using PLE, soxhlet and PHWE.

Table 1.1: Comparison of some traditional methods with PHWE when extracting baicalein in medicinal plants [16].

Phytochemical	Number of plant	PLE (mg/g)	PHWE (mg/g)	Soxhlet (mg/g)
baicalein	1	20.21 ± 0.94	24.28 ± 2.85	24.44 ± 0.22
	2	20.81 ± 0.49	22.81 ± 1.66	25.45 ± 0.04
	3	18.27 ± 0.84	-	-

Chapter 2

Literature Review

Summary

This chapter highlights literature on *Moringa sp.* pertaining to its medicinal, nutritional and other uses. Flavonoids, roles and sources of antioxidants, formation and degradation of radicals, synthetic and natural antioxidants, mineral content and green chemistry are discussed. Pressurised hot water extraction technique and analytical method used for quantification are also discussed.

2.1 *Moringa species* and its benefits

Moringa Oleifera Lam, a member of the family *Moringacea* is a small medium sized tree that grows ranging between 10 and 15 m in height. It is widely cultivated in East and Southeast Asia, Polynesia and the West Indies [17]. *Moringa Oleifera* is indigenous to north western India [18, 19, 20]. The tree is widely distributed in India, Egypt, Philippines, Ceylon, Thailand, Malaysia, Burma, Pakistan, Singapore, West Indies, Cuba, Jamaica and Nigeria [19]. It is also cultivated in African countries such as Ghana, Kenya, Malawi and South Africa. It is also known by the names of drumstick plant [21] kelor tree and horse radish tree [17, 18, 22, 23].

There are thirteen species that are found in *Moringacea*. These include *Moringa Oleifera, Moringa Arborea, Moringa Borziana, Moringa Concanensis, Moringa Drouhardii, Moringa Hildebrandtii, Moringa Longituba, Moringa Ovalifolia, Moringa Peregrina, Moringa Pygmaea, Moringa Rivae, Moringa Ruspoliana, Moringa Stenopetala*. Two of these were studied being *Moringa Oleifera* and *Moringa Ovalifolia*. *Moringa Oleifera* is now grown in many parts of the world including South Africa and is the most studied. *Moringa Ovalifolia* is found naturally mainly in Namibia and very little studies have been done on it. Figures 2.1 and 2.2 below show pictures of the trees. *Moringa Oleifera* thrives in sandy loamy soils while *Moringa Ovalifolia* grows naturally in rocky places and mountains.

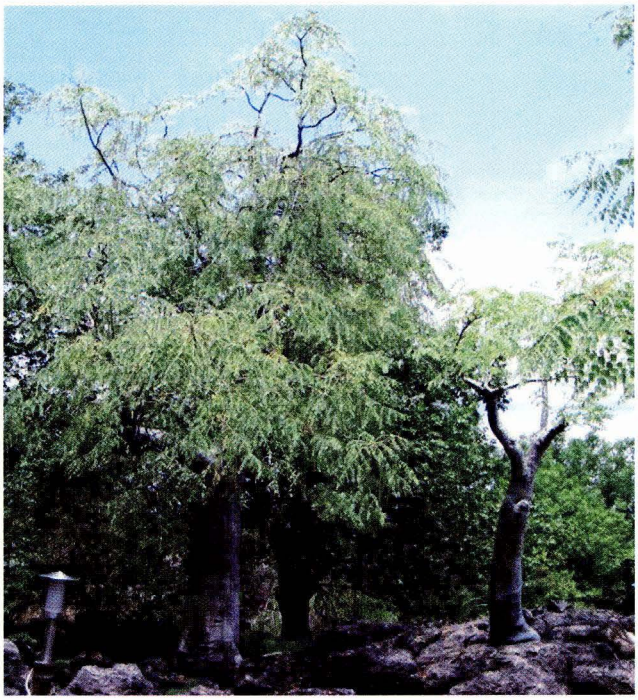

Figure 2.1: *Moringa Ovalifolia.*

Moringa Oleifera is coming to the forefront as a result of scientific evidence that it is an important source of naturally occurring phytochemicals and this provides a basis for future viable developments [24]. Phytochemicals are chemicals extracted from plants [25, 26]. These chemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc [25]. Earlier studies have also shown strong antioxidant and free radical scavenging activities of *Moringa Oleifera* leaves [17] which were also investigated in this study.

Figure 2.2: *Moringa Oleifera.*

2.1.1 Medicinal value

Medicinal plants have a long history of use in therapy throughout the world and still make an important part of traditional medicine [27, 28]. Several hundred genera are used medicinally, mainly as herbal preparations in the indigenous systems of medicine in different countries and are sources of very potent and powerful drugs which have stood the test of time and modern chemistry has not been able to replace most of them [29]. The use of plants as food and medicinal remedies since ancient times is partially attributed to the biological efficacy of secondary metabolites that possess antioxidant activities and other properties [30]. Examples include phenolic

compounds, vitamins C and E, carotenoids and others. Plant extracts rich in phenolics are a potent source of antioxidants in the defence against free radicals [31]. The past decade has seen a significant increase in the use of herbal medicine due to their minimal side effects, availability and acceptability to the majority of the population of third world countries [32]. Among these plants, *Moringa Oleifera* has its great contribution from ancient time with exceptional medicinal and nutritional properties which can resolve the health care needs in several situations [22]. *Moringa Oleifera* commonly known as a "miracle tree" has been researched on and literature shows that it has both medicinal and nutritional properties. It is called a "miracle tree" because of its high content of nutritional chemicals in its parts.

Quite often researchers and scientists use indigenous knowledge to advance in their respective fields. Even though there has been modern drug discovery and screening techniques, traditional knowledge systems have given clues to the discovery of valuable drugs [33]. Therefore, phytotherapy has been integrated into all systems of traditional medicine, often as the main source of healthcare in low- and middle-income countries [28]. Herbal medicines have been focused as new sources of antioxidants with limited complications [34]. Research has therefore geared towards the identification of potential natural sources of antioxidants. *Moringa Oleifera* is considered as one of the world's most useful trees, as almost all parts of this plant have been used for various treatments of ascites, rheumatism and venomous bites, and also as cardiac and circulatory stimulants [35]. Leaves of *Moringa Oleifera* are known to have various biological activities, including hypolipidaemic,

antiatherosclerotic, prevention of cardiovascular diseases and antioxidant [17]. Antimicrobial compounds of *Moringa Oleifera* have been validated after the discovery of inhibitory activity against several microorganisms [22]. *Moringa* leaf extracts were shown to contain compounds with wide-spectrum antibacterial activity, capable of inhibiting the growth of gram-positive and negative bacteria [36]. A paste of the leaves is used as an external application for wounds [20].

The seed kernels of *Moringa Oleifera* also showed promising effect in the treatment of bronchial asthma [22]. Chumark *et al* (2008) [17] found that *Moringa Oleifera* leaf extract suppresses the initiation and propagation of lipid peroxidation, and owing to its phenolic content, it may help suppress atherosclerosis by scavenging hydrogen oxide radicals. Atherosclerosis (or arteriosclerotic vascular disease) also known as "hardening of the arteries" in simpler terms, is a condition whereby the blood vessels particularly arteries become narrowed and hardened as a consequence of build up of plaque around the arteries wall. This results in the heart pumping blood at high pressure and this may weaken the heart, posing serious cardiovascular complications. The potential therapeutic values against cancer, diabetes, rheumatoid arthritis and other diseases have earned this plant name of "wonder tree" in Thailand [17].

2.1.2 Nutritional value

According to Horwath and Benin (2011), [37], *Moringa* shows great promise as a dietary supplement in areas with minimal access to healthcare, due to both high vitamin content and documented antibacterial and anti-carcinogenic properties. All

parts of the *Moringa* tree are edible and have long been consumed by humans [26]. Flowers and young leaves are eaten as vegetables [20, 22, 23] . The tender pods are cooked or pickled and used in culinary preparations [38]. Leaves can be eaten fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value [18, 26]. The seed can be consumed fresh as peas or pounded, roasted, or pressed into sweet, non-desiccating oil, commercially known as Ben oil of high quality [24]. The dry leaves are reported to have four times the calcium of milk, thrice the potassium of bananas and seven times the vitamin C of oranges [18].

Moringa trees have been used to combat malnutrition, especially among infants and nursing mothers [26]. The leaves are rich in iron and therefore highly recommended for expecting mothers [22]. *Moringa* leaves contain more Vitamin A than carrots, more calcium than milk, more iron than spinach, more Vitamin C than oranges, and more potassium than bananas, and that the protein quality of *Moringa* leaves rivals that of milk and eggs [26]. These leaves are also eaten commonly as a food by infants and children in south India, because the high content of b-carotenes helps to prevent the development of vitamin A deficiency blindness [39]. Even though in developing nations, *Moringa* is used as an alternative to imported food supplements to treat and combat malnutrition, especially among infants and nursing mothers by virtue of its chemical constituents coupled with their ubiquitous distribution in the tree, reports still show a high rate of malnutrition in the world. Figure 2.3 below shows the distribution of the *Moringa* in the world and the countries with malnutrition.

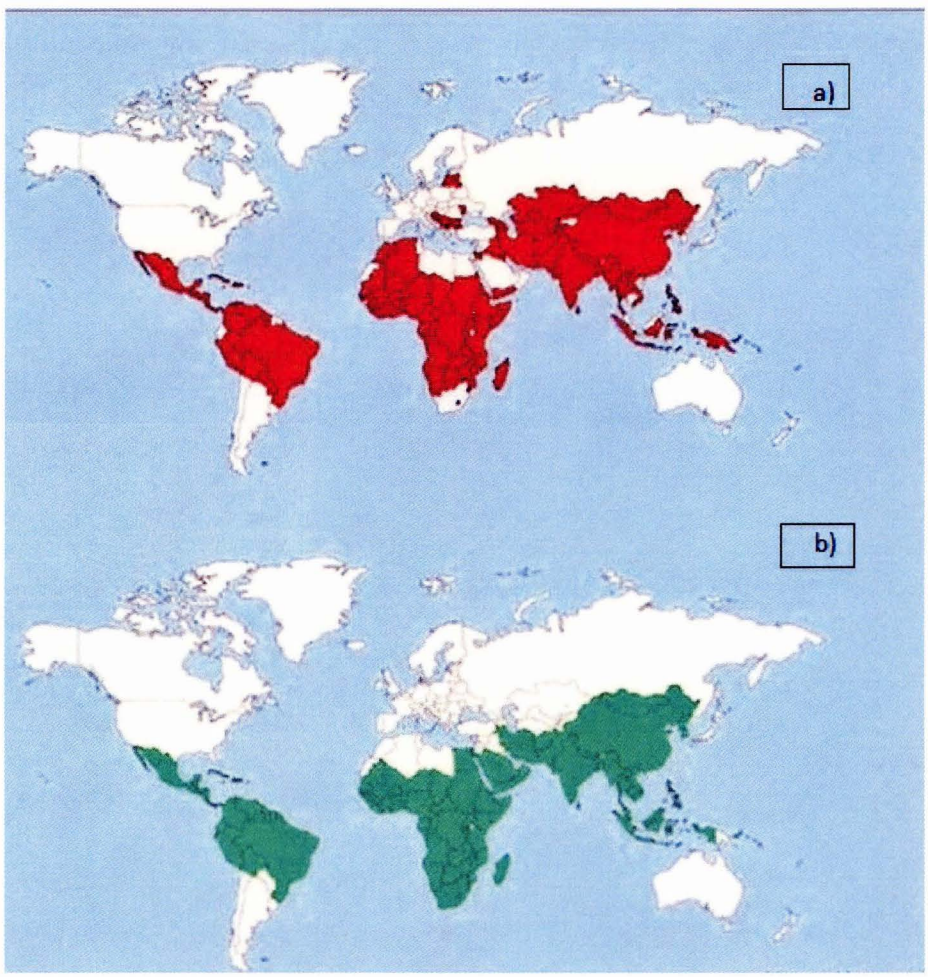

Figure 2.3: Countries with malnutrition (a) and distribution of *Moringa Oleifera* [40].

2.1.3 Purification of water

Contamination of ground water remains a major challenge because of human activities such as mining, industrialization, agriculture to mention a few, that result in deposit of toxic substances in water streams. For example, there is acid mine drainage at coal mines that have ceased to operate resulting in substantial quantities of sulfuric acid and iron hydroxide being released to water streams. This often contains a lot of heavy metals which are toxic.

Moringa seeds can be used to purify water as it is cheaper and accessible to

use other than other purification agents. Aluminium and iron salts are often used for purification of water but they also have undesirable effects on the environment. Aluminium makes water unsafe for hemodialysis because it accumulates in dialysis patients leading to significant morbidity and mortality [41].

Moringa Oleifera also has the potential to be used in the treatment of hardwaters for domestic use in tropical developing countries [42]. *Moringa* seeds are used for purification of turbid water because of their cationic charge. The protein in *Moringa* seeds powder is positively charged compared to particles in the water such as clay, bacteria, and other toxic materials which are negatively charged, hence attract the negatively charged particles that could be present in the water. The flocs formed by the flocculation process can then be easily removed by allowing the water to settle, or removed by filtering. Figure 2.4 compares water with and without treatment using *Moringa* protein seeds.

Figure 2.4: *Moringa* for purification of turbid water [43].

2.1.4 Other benefits of *Moringa Oleifera*

Other benefits that have been mentioned in literature are its antifertility effect [44] and its enhancement of male sexual desire and performance [45]. Actual compounds responsible for these are not fully known. As already highlighted the plant is very useful as all parts are of use. Other than its medicinal, nutritional and the water purification, *Moringa* can be used to prevent soil erosion. Figure 2.5 below summarises the functions of *Moringa*.

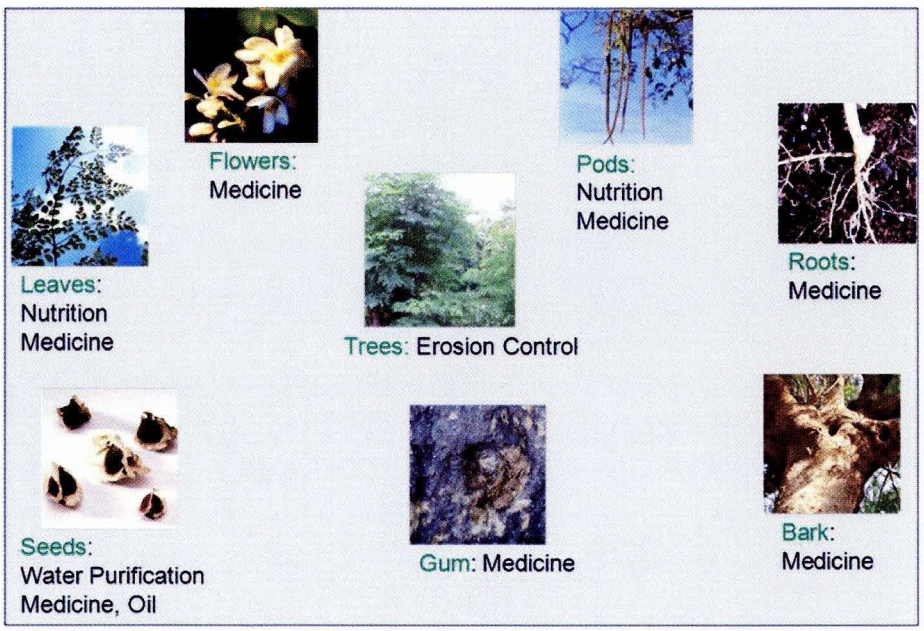

Figure 2.5: Various uses of different parts of *Moringa sp.*

2.2 Plant polyphenols

Plant polyphenols are ubiquitous, essential and located throughout most plant tissues which contribute significantly to plant physiology [46]. Polyphenols may be

classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another [47]. Primarily they, occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist [47]. The chemical properties of polyphenols in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers predicts their antioxidant activity [48]. The main groups of polyphenols are flavonoids, phenolic acids, tannins, stilbenes and lignans [49] which will be discussed in the following paragraphs.

Evans *et al.*, (1996) [48] reported that plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. This has motivated researchers to investigate the plant compounds called polyphenols, including flavonoids that are now believed to form an important part of our dietary intake of antioxidants. Studies on the free radical-scavenging properties of flavonoids have permitted characterization of the major phenolic components of naturally occurring phytochemicals as antioxidants [48].

2.2.1 Phenolic acids

Phenolic acids are found abundantly in foods and divided into two classes being derivatives of benzoic acid and derivatives of cinnamic acid [47]. Phenolic compounds are important due to their ability to serve as antioxidants which are widely found in secondary products of medicinal plants [50, 51]. Phenolics are chemical compounds characterized by at least one aromatic ring (C6) bearing one or more

hydroxyl groups [52]. Phenolic acids and other polyphenolic compounds that is, flavonoids, present in fruits, vegetables and other parts of the plants are known to possess antioxidant activity due to presence of hydroxyl groups in their structures [53] as demonstrated by Figure 2.6 below.

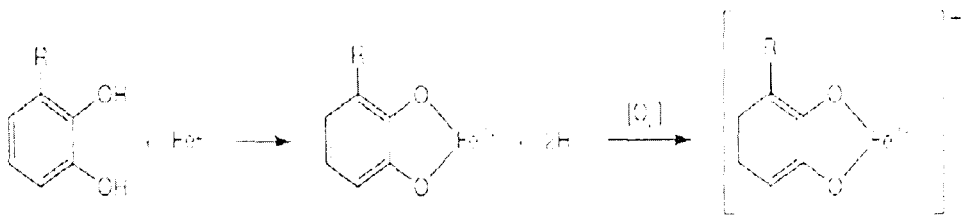

Figure 2.6: Phenolic compound binding with a free Fe^{2+} to inhibit radical formation.

Several investigations have shown that many of these plants have antioxidant activities that could be therapeutically beneficial and it has been mentioned that the antioxidant potential of the plants could be due to their phenolic components [54]. Plant phenolic compounds such as flavonoids and lignin precursors are potent antioxidants [52]. Solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other food constituents and formation of insoluble complexes [55]. According to He *et al*, [56] the conventional methods used currently for extraction of compounds from herbal plants are distillation and solvent extraction which have low extraction efficiencies and toxic solvent residues.

The antioxidant capacity of phenolic compounds is determined by their structure, in particular the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical and the ability of an aromatic compound to support

an unpaired electron as the result of delocalization around the M-electron system [57]. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers [58]. Some phenolic compounds with dihydroxy groups can conjugate transition metals, preventing metal - induced free radical formation [59] as seen in Figure 2.6 above.

$$H_2O_2 + Cu^{+}/Fe^{2+} \longrightarrow Cu^{2+}/Fe^{3+} + OH + \cdot OH^{-} \tag{2.2.1}$$

Extraction of phenolic compounds in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions, as well as presence of interfering substances [55].

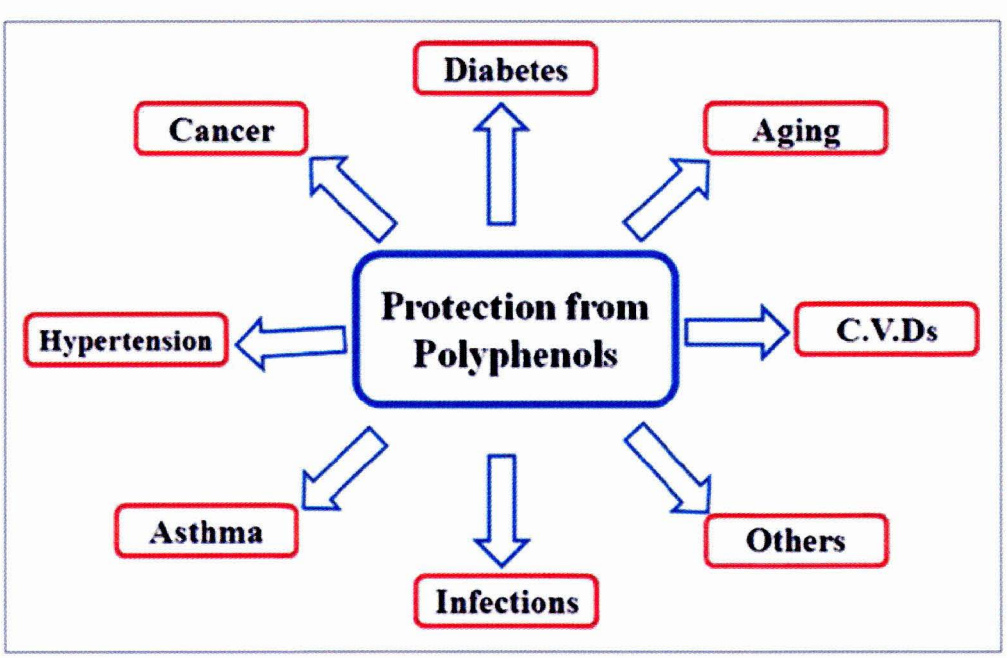

Figure 2.7: Health beneficial effects of dietary plant polyphenols [47].

Table 2.1: Antioxidant activity (Ferric Reducing Antioxidant Power (FRAP)) and Total Phenolic Content (TPC) of 12 Indian medicinal plants [31]

Name of plant (tested part)	FRAP (μmol 100 g $^{-1}$ DW)	TPC (g 100 $^{-1}$ DW)
C. gigantea (root)	185.71	1.29
C. gigantea (flower)	186.13	0.69
C. gigantea (leaf)	93.07	0.69
G. sylvestre (leaf)	93.04	1.02
L. reticulata (leaf)	185.98	0.83
O. esculentum (leaf)	93.01	0.79
P. nivalis (leaf)	93.03	0.53
P. daemia (leaf)	92.99	0.68
S. brevistigma (stem)	185.84	0.72
T. indica (leaf)	92.87	1.09
T. ovata (leaf)	185.95	1.71
Wattakaka volubilis (leaf)	92.98	1.12
Decalepis hamiltonii (leaf)	557.01	3.84
H. indicus (leaf)	278.71	1.57
H. indicus (root)	371.30	2.23

2.2.2 Stilbenes

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge [47]. They are distributed in higher plants and exist in the form of oligomers and in monomeric form (e.g., resveratrol, oxyresveratrol) and as dimeric, trimeric, and polymeric stilbenes or as glycosides [60]. Resveratrol, a stilbenic compound deriving from the phenyalanine/polymalonate route, being stilbene synthase (STS) the last and key enzyme of this pathway, recently has become the focus of a number of studies in medicine and plant physiology, and has emerged as a promising molecule that potentially affects human health [61].

2.2.3 Lignans

Lignans are diphenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues [47].

2.2.4 Flavonoids

Flavonoids are the largest class of polyphenols [62] , with a common structure of diphenylpropanes (C6-C3-C6) [59, 52], consisting of two aromatic rings linked through three carbons as seen below in Figure 2.8. Flavonoids are built upon C6-C3-C6 skeleton in which the three carbon bridge between phenyl groups is commonly cyclised with oxygen [63]. The major flavonoid classes include flavonols, flavanones, flavones, anthocyanidins and isoflavones [64, 63, 48]. Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation [59] as seen in Figure 2.8 below.

Flavonoids are formed in the plants from the aromatic amino acids phenylalanine and tyrosine and malonate [62]. Flavonoids commonly accumulate in epidermal cells of plant organs such as flowers, leaves, stems, roots, seeds and fruits, being found in glycosidic forms (glycosides) and non-glycosidic forms (aglycones)[52]. Flavonoids may trap chain-initiating radicals at the interface of the membranes, thus preventing the progression of the radical chain reaction [57]. The most rational way to inhibit carcinogenesis is by interfering with modulation steps (initiation, promotion, and progression) as well as the associated signal transduction pathways of which the polyphenols can do by virtue of their electron donating capacity [65]. In this study, myricetin, kaempferol and quercetin which are flavonols were studied and

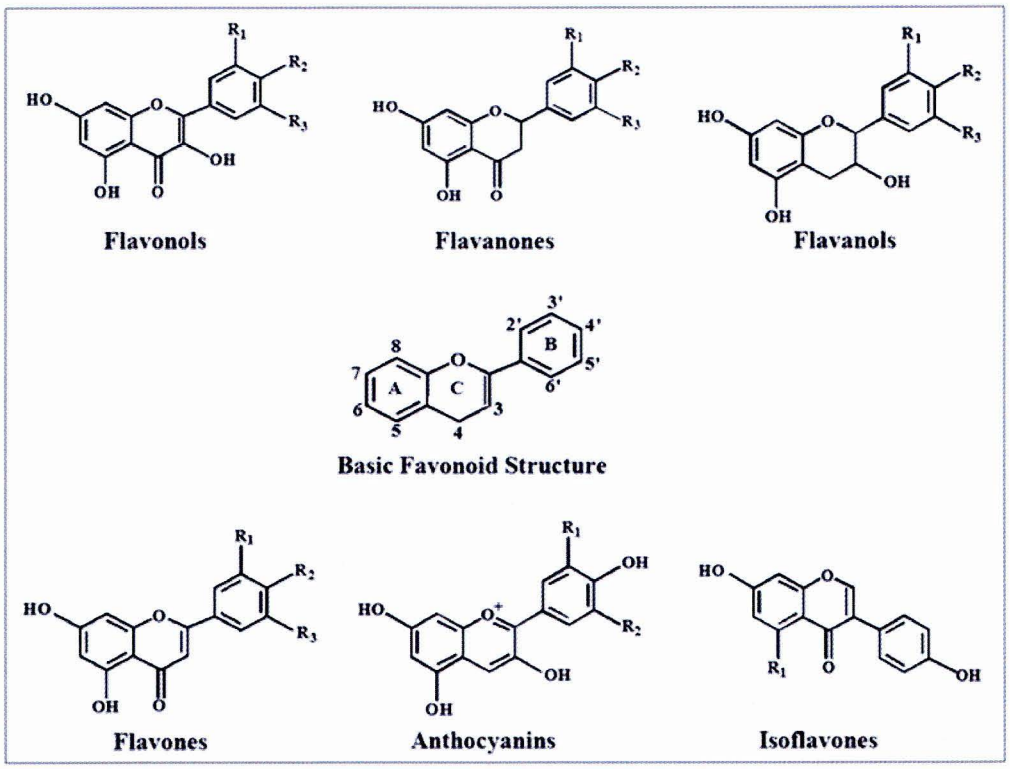

Figure 2.8: Chemical structures of sub-classes of flavonoids. Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation [47].

their basic structure is shown below.

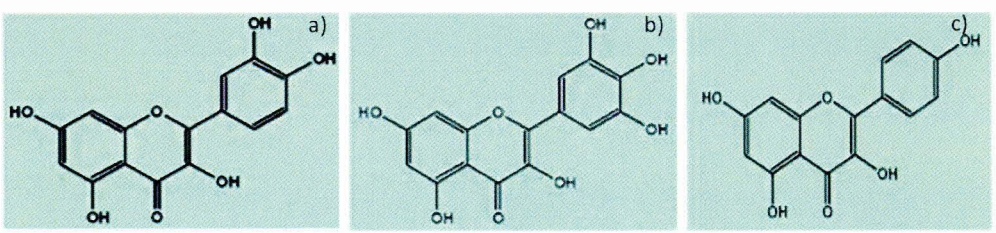

Figure 2.9: Chemical structure of a) quercetin, b) myricetin and c) kaempferol.

Table 2.2: Phytochemicals of *Moringa Oleifera* [66]

Parts	Phytochemical constituents
Roots	4-((α − *L*)-rhamnopyranosyloxy)-benzylglucosinolate and benzylglucosinolate
Stem	4-hydroxymellein, vanillin, β-sitosterone
	coctasanic acid and β-sitosterol
Bark	4-((α − *L*)-rhamnopyranosyloxy)-benzylglucosinolate
Whole	L-arabinose, D-galactose, D-glucuronic acid
gum exudates	L-rhamnose, D-mannose, D-xylose and leucoanthocyanin
Leaves	Glycosideniazirin, niazirin and three mustard oil glycosides
	4-[4-O-acetyl-α-L-rhamnosyloxybenzyl]isothiocynate niaziminin A and B
Mature flowers	D-mannose, D-glucose, protein, ascorbic acid, polysaccharide
Whole pods	Nitriles, isothiocyanate, thiocarbanates
	O-[2-hydroxy-3-(2-heptenyloxy)]-propylundecanoate O-ethyl-4-[(α-l-rhamnosyloxy)-benzyl] carbamate, methyl-p-hydroxybenzoate and β-sitosterone 4-[4-O-acetyl-α-L-rhamnosyloxybenzyl]isothiocynate niaziminin A and B
Mature seeds	crude protein, crude fat, carbohydrate, methionine, cysteine
	4-((α − *L*)-rhamnopyranosyloxy)-benzylglucosinolate benzylglucosinolate, moringyne, mono-palmitic and di-oleictriglyceride
Seed oil	Vitamin A, beta carotene, precursor of Vitamin A

Table 2.3: Traditional use of *Moringa Oleifera* [66]

Parts and its form	Pharmacological activities
Crude ethanolic extract of dried seeds	Anti-inflammatory
Hot water infusion of flowers, leaves, roots, seeds and bark	
Crude methanolic extract of the roots	
Oil from dried seed	Antioxidant
Methanol and ethanol extract of free dried leaves	
Deffated and shell free seeds	Antimicrobial
Fresh leaves juice	
Roots and bark	
Aqueous extract of stem bark	Cardiovascular
Ethanolic extract of leaves	
Ethanolic and aqueous extracts of whole pods and their parts namely; coat, pulp and seed	
Leaves and fruits	Antihyperlipidemic
Methanolic extract of roots	CNS depressant
Aqueous or ethanolic extract	Antiinfertility
of bark and roots	
Paste of leaves	Anticancer
Ethanolic extract of seeds	
Paste of leaves	Anticancer
Ethanolic extract of seeds	
Aqueous and ethanolic extract	Antihepatotoxicity
of roots and flower	
Ethanolic extract of seeds	
Methanolic extract of leaves	Antiulcer
Ethanolic extract of seeds	
Hot water infusion of flowers , leaves, roots,	Antispasmodic
seeds and bark seed infusion	
	Diuretic
	produces Vitamin A
	rises blood haemoglobin
	Increases blood glucose level
	regulate hyperthyroidsm

2.3 Oxidative stress

Oxygen is a necessity for sustaining animal life. The body system of all animals relies on oxygen metabolic production of energy. Oxygen is nevertheless a highly reactive molecule that can produce chemicals called free radicals. Free radicals, also referred to as reactive oxygen species or ROS, are chemical entities that contain one or more unpaired electrons in their outer orbit. They are highly unstable and become very reactive towards any near by molecule in their quest to attain molecular stability. There are several different types of ROS such as hydroxyl radical, superoxide radical, the nitric oxide radical and the lipid peroxyl radical.

During the process of metabolism, oxygen is reduced to water, producing several oxygen-derived free radicals or reactive oxygen species which play an important role in various diseases. They can also be produced externally by exposure to radiation, toxic chemicals, cigarette smoking and alcohol consumption, and by eating oxidized polyunsaturated fats [67]. An imbalance between the formation of free radicals and their destruction by antioxidants is the cause for oxidative stress. Oxidative stress is due to either overproduction of free radicals and our body is not able to scavenge them, or inadequate intake of the nutrients that contribute to the defence system. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins [68].

Reactive oxygen species (ROS) are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA [69]. These by-products are generally reactive oxygen species (ROS) such

as super oxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process [70].

Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases [71]. Oxidative stress can cause oxidative damage to large biomolecules such as proteins, DNA and lipids, resulting in an increased risk of cancer and cardiovascular disease [72]. Oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders such as neurodegenerative diseases, cancer, cardiovascular disease, atherosclerosis and inflammation [18]. The most important macromolecules (lipids, proteins and nucleic acids) in human body as well as in food materials have been reported to undergo oxidative damage caused by free radicals such as superoxide and hydroxyl radicals generated in certain biochemical processes [73].

The main causes of the aging process seem to be related to reactive oxygen species and free radicals, such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen [74].

2.3.1 Formation and degradation of radicals

Heavy exercise, sunshine, air pollution and smoking are known to substantially increase the amount of free radicals being produced. These free radicals oxidize randomly biologically essential molecules such as lipids, proteins, sugars, and nucleic acids, which results in the loss of their physiological functions and induction of deleterious effects [75]. Our bodies have mechanisms of antioxidants to neutralize the

free radicals before they can cause any damage. The body has the capacity to produce its own antioxidants from nutrients obtained from food or the nutrients can be used directly as antioxidants. Vitamins C and E are known to supply the body with direct antioxidants. An antioxidant enzyme system consisting of catalase, superoxide dismutase and glutathione peroxidase in human body plays an important role in protecting the macromolecules from oxidative damage caused by these oxidant species [76]. Superoxide dismutase is an enzyme that converts the superoxide radical to hydrogen peroxide, which is in turn metabolized by another enzyme, catalase, to produce water and oxygen.

Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins [68].

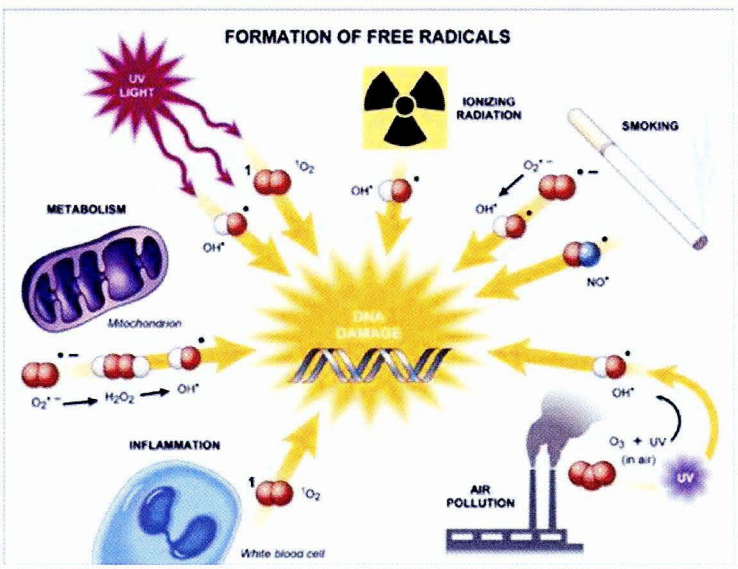

Figure 2.10: Factors that contribute to the formation of free radicals in the body.

2.4 Types of antioxidants

2.4.1 Synthetic antioxidants

The interest in natural antioxidants has increased considerably in recent years because many antioxidants exhibit beneficial biological effects, including antibacterial, antiviral, anti-allergic, anti-thrombotic and because they are linked to lower incidence of cardiovascular disease and certain types of cancer disease [77]. Due to the benefits of antioxidants, food and pharmaceutical products are normally supplemented with synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertbutylhydroxyquinone (TBHQ) [30]. Synthetic antioxidants have been commonly used to prevent undesirable oxidation in many foods. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters, have been suspected to cause or prompt negative health effects [68].

2.4.2 Natural antioxidants

Earlier studies have also shown strong antioxidant and free radical scavenging activities of *Moringa Oleifera* leaves [17]. *Moringa Oleifera* leaf extracts can act as effective modulators in reducing the toxicity by enhancing the stimulation of enzymes in the cells under stress [18]. Treatment with the leaf extract significantly increased the levels of the antioxidant enzymes superoxide dismutase and catalase [18]. The antioxidant properties of phenolic compounds are mainly because of their redox potential, which allow them to act as reducing agents, hydrogen donators, metal chelators and singlet oxygen quenchers [48, 6].

2.4.3 Enzymatic antioxidants

The damage produced by ROS may be prevented by enzymatic antioxidants such as preventive antioxidants which will inhibit the initial production of free radicals; e.g. catalase which is localised in the peroxisome. The following mechanisms depicts this;

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \tag{2.4.1}$$

Chain breaking antioxidants inhibit the propagation stage after the peroxy radicals are generated. These include superoxide dismutase (SOD), vitamin E, etc. The following mechanism elaborates how SOD prevents exponential growth of radicals.

$$M^{(n+1)+} - SOD + O_2^- \longrightarrow M^{n+} - SOD + O_2 \tag{2.4.2}$$

$$M^{n+} - SOD + O_2^- + 2H^+ \longrightarrow M^{(n+1)+} - SOD + H_2O_2 \tag{2.4.3}$$

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2)

The produced H_2O_2 in the overall reaction is then cleaved by the catalase as seen in (2.4.1). Superoxide dismutase (SODs) then puts together the oxygen radical and the metal ion to give oxygen (2.4.2) and further breakdown the metal ion - SOD complex to give hydrogen peroxide (2.4.3). This remains a continous cycle as hydrogen peroxide is cleaved as discussed above. A set of these enzymes, located in the cytosol and mitochondria, require a metal ion cofactor, copper (Cu), zinc (Zn)

or manganese (Mn). Glutathione peroxidases are a large family of enzymes that reduce H_2O_2 to water. They are found both in the cytoplasm and extracellularly in almost every human tissue. Peroxiredoxin catalyses the reduction of H_2O_2, organic hydroperoxides and peroxynitrite ($ONOO^-$).

2.5 Mineral Content in plants

In the biosphere as a whole and in particular ecosystems, there are interrelationships between contents of trace elements in air, soil and plants [9]. Certain trace elements are essential in plant nutrition (micronutrients), but plants growing in a polluted environment have the potential to accumulate trace elements at high concentrations, causing a serious risk to human health when plant-based foodstuffs are consumed [78]. Subsequent problems may be toxicity to the plant growing on the contaminated soil and uptake by the plants resulting in high metals levels in plant tissues [79]. However in the right concentration they are useful. Trace elements have both a curative and a preventive role in combatting diseases [80]. They are also called micro elements and their examples include, iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo), chloride (Cl), nickel (Ni), silicon (Si), cobalt (Co) and selenium (Se) [81]. Macro elements are a number of minerals essential for human nutrition are accumulated in different part of plants as it accumulates minerals essential for growth from the environment [80]. Examples of the macro nutrients are, calcium (Ca), magnesium (Mg), potassium (K), sodium (Na) and lithium (Li). The Table 2.4 below shows the average concentrations of the nutrients needed for the plant.

Table 2.4: Average concentration of macro and micro nutrients in plants [82]

Element	Average concentration in tissue(mg kg^{-1})
Iron	100
Mn	50
Zn	20
B	20
Cu	6
Mo	0.1
Cl	100
Na	15 000
K	10 000
Ca	5 000
Mg	2 000
P	2 000
S	1 000
Zn	20

The table shows that the average concentration of the micro nutrients in plant tissues are lower while the macro nutrients are higher.

2.6 Green chemistry

Green chemistry is the utilization of a set of principles that will help reduce the use and generation of hazardous substances during the manufacture and application of chemical products [83]. The practice of green chemistry begins with recognition that the production, processing, use, and eventual disposal of chemical products may cause harm when performed incorrectly [84]. As a result of industrialization, mining and other activities, pollution of the environment occurs either in the air, land or even in the waters. Depending on the pollutant, the degree of pollution, effects of pollution to animal and human life can be detrimental. Toxic substances polluting

the environment can be passed on to food chain and sometimes these are accumulative in body systems of people or animals for instance. These can result in mutation of cells resulting in diseases such as cancers and others. In this study,"green" chemistry approach was used in the extraction of the phytochemicals from the *Moringa sp.* The use of water as a solvent is is inline with the principles of "green" chemistry stated below because water is environmentally friendly.

The following twelve basic principles of green chemistry have been formulated by Anastas and Warner 1998 [85].

- It is better to prevent waste than to treat or clean up after it is created.
- Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
- Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment at large.
- Chemical products should be designed to affect their desired function while minimizing their toxicity.
- The use of auxiliary substances (e.g., solvents, separation agents, and many others) should be made unnecessary wherever possible and innocuous when used.
- Energy requirements of chemical processes should be recognized for their environmental and economic impacts and therefore should be minimized. If

possible, synthetic methods should be conducted at ambient temperature and pressure.

- A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
- Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
- Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
- Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
- Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
- 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.

In this study, it can thus be concluded that the "green" approach is being utilised considering the fact that an environmetally friendly solvent (water) is used and the raw material (plant) used is replenishable.

2.7 Extraction Techniques for plant materials

2.7.1 Pressurised Hot water extraction system (PHWE)

PHWE is a form of pressurised liquid extraction (PLE) and it was first introduced in 1994 [5]. In pressurised hot water extraction, as water is heated at high temperature and pressure it behaves as an organic solvent, hence become less polar with a dielectric constant that compares to that of organic solvents [86]. Water is a highly polar solvent with a high dielectric constant (ε) at room temperature and atmospheric pressure due to the presence of extensive hydrogen-bonded structure [87]. Dielectric constant indicates the strength of the polarity of water, the higher it is the more polar and vice versa. The dielectric constant (ε) of water at 25^{0}C is 80 [16, 64]. However when heated, the properties of water change markedly as the hydrogen - bonded lattice is disrupted as thermal motion increases [86] leading to a decrease in the dielectric constant [64]. Consequently, the capability of water to dissolve less polar compounds is enhanced because the polarity of water, itself, is decreased [15]. Also at high temperature the physco-chemical properties of water especially the relative permittivity (ε) are favorable of the less polar compounds [88]. By merely adjusting the extraction temperature, water can thus be turned into a suitable solvent for extraction of polar to medium polar solutes [64]. The Table 2.5 below compares the dielectric constant of water to those of organic solvents.

The PHWE system comprises of a heating oven, an HPLC pump, a stainless steel extraction cell and connecting lines. A continous stainless steel wire connects from the pump passing through the extraction cell placed in the oven to the collecting flask outside the oven. A pump is joined to a solvent system using small tubings

Table 2.5: Comparison of dielectric constant of water with of organic solvents [5]

Solvent	Normal boiling point (^{0}C)	Temperature (^{0}C)	Dielectric constant (ε)
Water	100	25	80
Water	100	205	33
Methanol	68	25	33
Ethanol	78	25	24.3
Acetone	56	25	20.7

and a back pressure regulator is situated between the collecting flask and the oven. Figure 2.11 below shows the system.

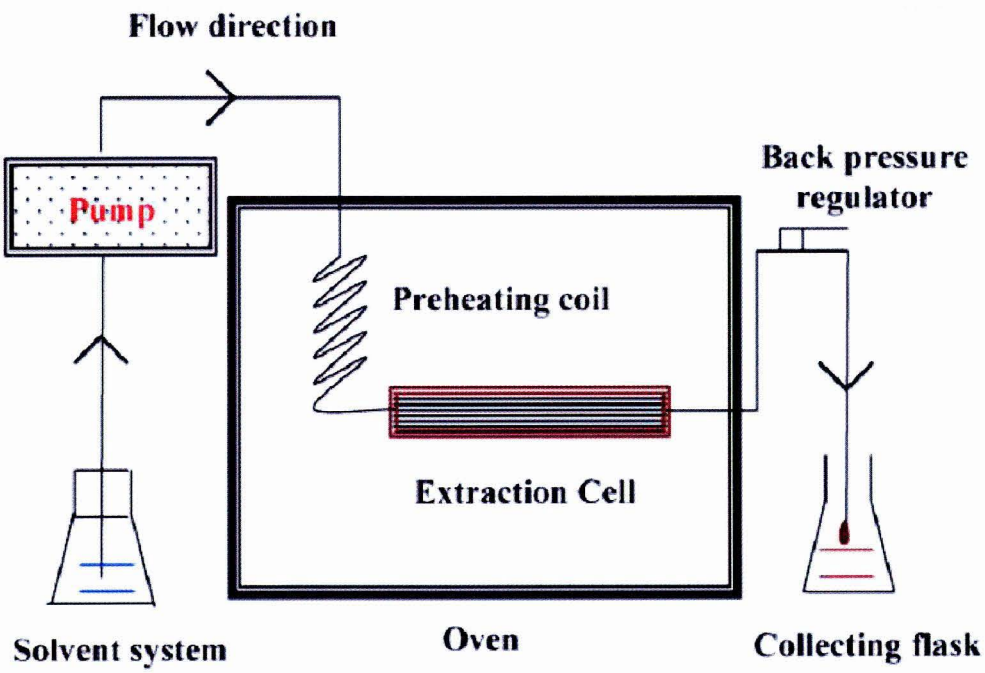

Figure 2.11: Schematic digram of of pressurised hot water extraction [89]

Extraction mechanism

Optimum conditions for pressurised water depend on various factors such as the properties of water, chemical properties of the solute and extraction kinetics. The

extraction mechanism in PHWE is proposed to involve four sequential steps (Figure 2.12) which take place in the extraction cell [87]. Upon the rapid introduction of the pressurised hot water, cells of the *Moringa* break open and the target molecule is desorbed from the active site of the matrix. Thus the first kinetic step is dominated by the intermolecular adhesive and cohesive forces between the solute, matrix and solvent [90].

The second step is the diffusion of the water in to the organic matrix. At higher temperature water becomes less viscous and its surface tension is lowered making it a much more diffusive solvent. This enhances extraction because water becomes more efficient in the extraction of the solutes from the organic matrix.

The third step of extraction is characterised by the dissolution of the analyte into the water. By diffusion gradient the target molecules flow into the bulk solution where eventually the are collected. The driving force is the concentration gradient between that exist between the extract in the water and the extract in the matrix.

Factors affecting extraction efficiency

- **Chemical structure of the extract**.

One of the factors that directly influence the extraction efficiency is the partitioning of the extract between the active sites of the matrix and the mobile phase which is the pressurised hot water as discussed above. The chemical structure of the extract however influences the polarity, the adhesion of the solutes to the active sites, their desorption and hence the solubility of the solutes. The partition coefficient is directly influenced by the solubility of the material in the water phase [90].

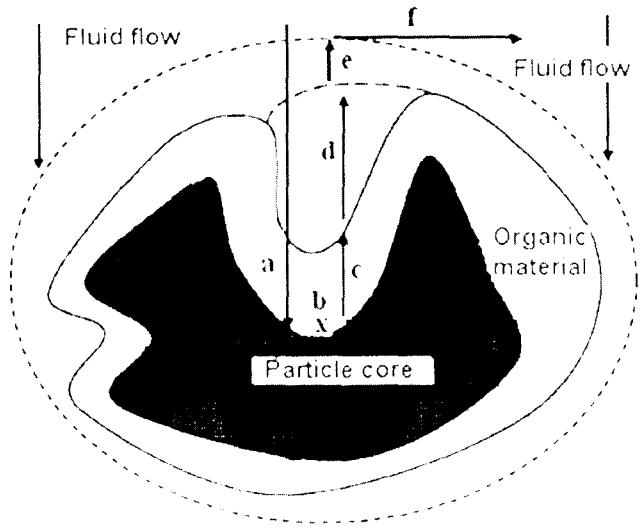

Figure 2.12: Schematic representation showing proposed extraction steps in PHWE: (a) rapid fluid entry; (b) desorption (c) diffusion; (d) diffusion; (e) diffusion [16]

- **Temperature and pressure of the PHWE.**

Temperature of the water plays a tremendous role in the efficiency of the extraction. At elevated temperatures, the polarity of the water is lowered which favors less polar substances. The hydrogen bonds are weakened leading to a decrease in the dielectric constant, reduced viscosity and subsequently an enhanced extraction efficiency. The importance of pressure is to maintain the water in the liquid state. It also forces the solvent into the sample.

2.7.2 Microwave digestion

This technique involves the use of magnetic radiation for the extraction of both metal ions and organic compounds. Microwaves are electromagnetic waves made up of two oscillating perpendicular fields, electrical field and magnetic field [91]. Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz

to 300 GHz and positioned between the X- ray and infrared rays in the electromagnetic spectrum [92]. The vessel used must be transparent to microwave such as teflon and polysterene. The basis of microwave assisted extraction is improvement in the extraction kinetics provided by heating [63]. In the case of extraction, the advantage of microwave heating is the disruption of weak hydrogen bounds promoted by the dipole rotation of the molecules [12].

Solvent composition and the volume, extraction temperature and matrix characteristics are critical parameters needed for efficient extraction of compounds from organic materials. In microwave, absorption occurs owing to the reorientation of permanent dipoles by the electric field, the amount of energy absorbed is proportional to the dielectric constant (ε) of the solvent. The technique can be used for both metal ions and organic compounds depending on the extraction solvent. In this study nitric acid was used to extract total metals in the leaf powder.

The advantages of closed-vessel systems are listed below:

- They can reach elevated temperatures than open vessel systems because the higher pressures inside the vessel raises the boiling point of the solvents used resulting in minimal time.

- The possibility of the loss of volatile substances during microwave irradiation is eliminated.

- Minimal volumes of the solvent is required which is economical and also environmentally friendly.

- The fumes produced during an acid microwave extraction are contained within

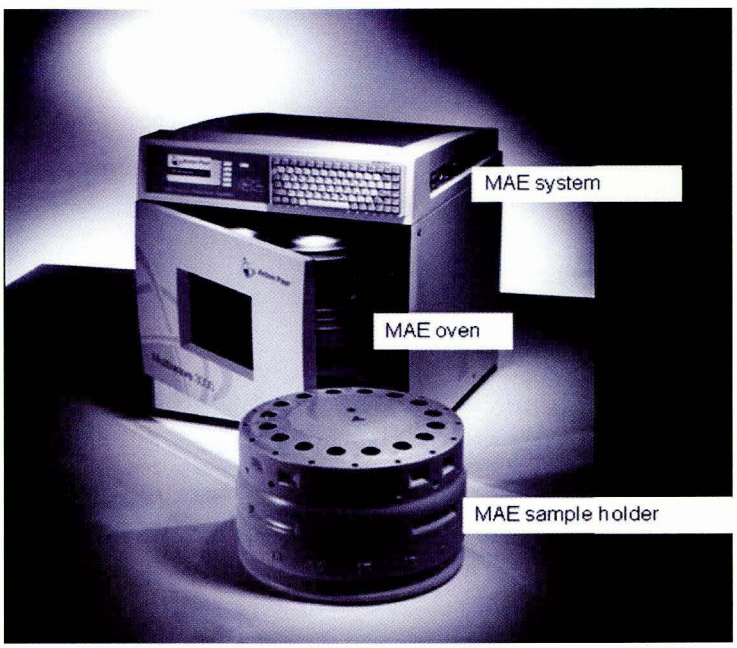

Figure 2.13: Schematic diagram of Microwave Assisted Extraction instrument

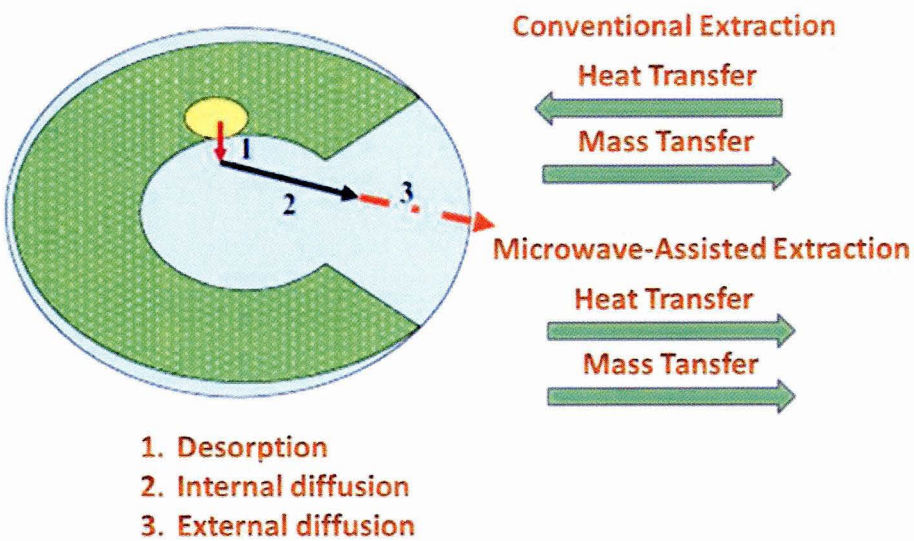

Figure 2.14: Heat and mass transfer mechanisms in microwave [93]

the vessel as such analyst do not handle potentially hazardous fumes released.

Nonetheless the disadvantages of the closed vessels include the following;

- The high pressures can results in explosion
- The amount of sample that can be processed is limited
- The vessels are made up of PTFE (polytetraflouro ethylene) which does not allow high solution temperatures.
- The single-step procedure excludes the addition of reagents or solvents during digestion.
- The vessel must be cooled down before it can be opened after the treatment to avoid loss of volatile constituents which can results in erroneous results and health hazards.

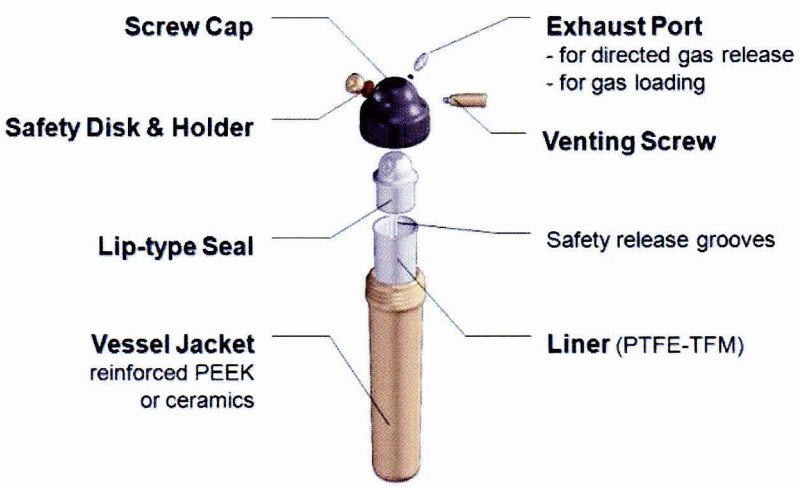

Figure 2.15: Typical diagram of MAE vessels [94].

2.8 Determination Techniques

2.8.1 High Performance Liquid Chromatography (HPLC)

Separation of mixtures in microgram to gram quantities by passage of the sample through a column containing a stationary solid by means of a pressurized flow of a liquid mobile phase, components migrate through the column at different rates due to different relative affinities for the stationary and mobile phase based on adsorption, size or charge [95].

HPLC is the most widely used separation technique and it finds application in various fields of science such as agriculture, forensics, medicine, environment, pharmaceutical, to mention a few. The technique is suitable for the analysis of non-volatile, thermally labile and compounds of high molecular weight such as carbohydrates, proteins, polymers and many others. Below is Figure 2.16 adapted from Clark (2007), [96].

Mobile phase

The mobile phase pushes the sample with the help of the pressure along the analytical column where separation occurs. The eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components [95]. The solvent must have lower viscocity so that a minimal pressure is used to push through to facilitate separation. It must also have low boiling point to facilitate solvent removal from collected fractions Safety i.e. low toxicity and no unpleasant odour. The solvent must be compatible with the detector. It is also critical to know the UV cut off and refractive indices of solvents

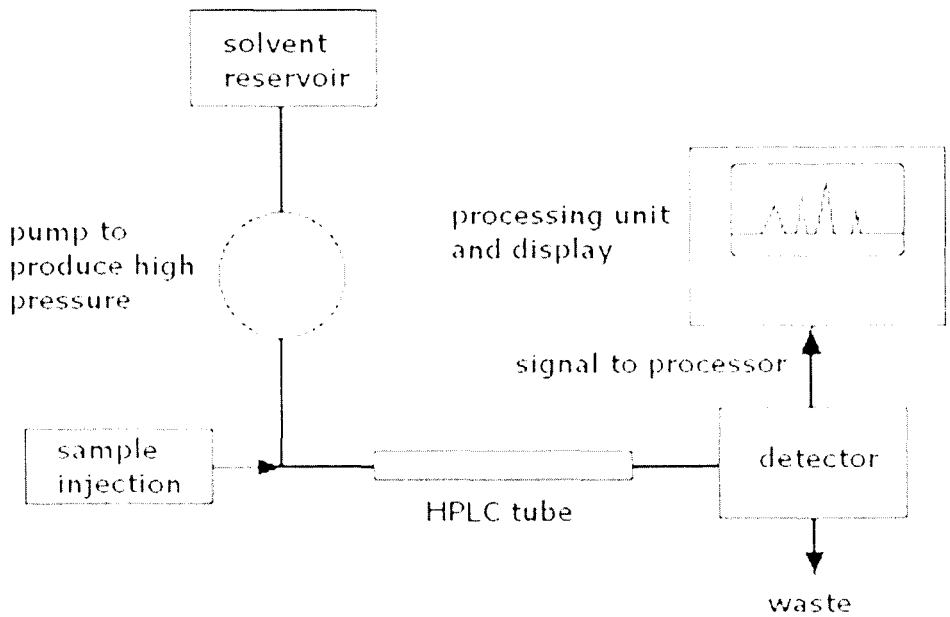

Figure 2.16: Schematic diagram of an HPLC system [96].

particularly that the commonly used detectors are based on absorbance of UV radiation and on refractive index. Table 2.6 adapted from Fifield and Kealey, (1999) [95] illustrates this.

The mobile phase must be filtered and degassed prior to use to avoid any particulate matter which can result in the blockage of the system while degassing is done to remove air bubbles. Separation can be done under isocratic or gradient elution. In isocratic elution only one composition mixture is used to separate the analytes in the column. This elution is suitable where the polarity of the constituents of a sample are almost within the same range. In cases where the polarity of the constituents is far apart, gradient elution is recommended. In here, the polarity of the mobile phase is adjusted during the run by varying its composition. Two or more mixtures with varying polarity are employed. Varying the volume of different solvents periodically varies the polarity of the mobile phase which will result in further separation of the

Table 2.6: Various solvents used in HPLC with their UV cut off and RI values [95]

Solvent	UV Cut off(nm)	RI 25^0C
n-hexane	190	1.372
cyclohexane	200	1.423
carbon tetrachloride	265	1.457
toluene	285	1.494
benzene	280	1.498
methylene chloride	233	1.421
n-propanol	240	1.385
tetrahydrofuran	212	1.405
ethyl acetate	256	1.370
iso-propanol	205	1.384
chloroform	245	1.443
acetone	330	1.356
ethanol	210	1.359
acetonitrile	190	1.341
methanol	205	1.326
water		1.333

analytes.

There are two existing phases of separations which depend on solvent polarity. These are normal phase and reverse phase. Separation can occur under reversed phase where the stationary phase is non polar and the mobile phase is polar. In reversed phase primary attraction of analyte is between nonpolar stationary phase and more polar solvent. The order of elution is hydrophilic to hydrophobic (polar to nonpolar). In other words, analytes that are polar have affinity for the mobile phase and hence eluted first while the less polar substances have higher affinity for the non polar stationary phase. Its widely used. Acetonitrile or methanol can be used as a mobile phase. Often a minute volume of volatile acids such as acetic acid or formic acid is added to the mobile phase. These are called ion-pairing agents. They bind by ionic interaction to the solute molecules to increase the hydrophobicity of the solute

molecule and change selectivity also to maintain the pH.

Normal phase however has a polar stationary phase and a non polar mobile phase and hence the order of elution is from hydrophobic to hydrophilic. Its application is found in isomer separation.

Pumping System

A high pressure pump is needed to ensure a reproducible constant flow pulse free mobile phase at flow rates between 0.1 and 5 ml min^{-1}. Constant flow reciprocating pumps are now the mostly widely used type but because their mechanical action inherently produces a pulsating delivery of the mobile phase the flow must be smoothed so as to eliminate the pulsations [95]. Often a pulse damper is incorporated in the system to remove pulses. A pump must must be chemically inert to the solvents to avoid corrosion of the pumps. It must also have a small hold up volume to facilitate rapid change of the composition of the mobile phase particularly for gradient elution.

Sampling injection system

Samples in HPLC may be injected using either a syringe or a valve injector. The sample is loaded into a stainless steel loop that is incorporated into the valve body. The sample loop is connected to two ports of the three-way valve injector. One port is connected to the column while the other is connected to the mobile phase and two ports for sample loop waste during loading and injection. The valve can with stand high pressures of about 500 bar and give reproducible results of high precision.

Sample loops of different volumes can be used depending on the injection volume. Often they range from 2 to over 100 μL. The knob is set at the "LOAD" position for sample injection as seen below in Figure 2.17. The sample can be injected into the sample loop, which is separated from the flow path. Once the knob is turned to the "INJECT" position, the eluent travels through the loop from the pump then delivers the sample to the column. Figure 2.17 below depicts how the valves are interchangeable during loading and injection of sample in HPLC.

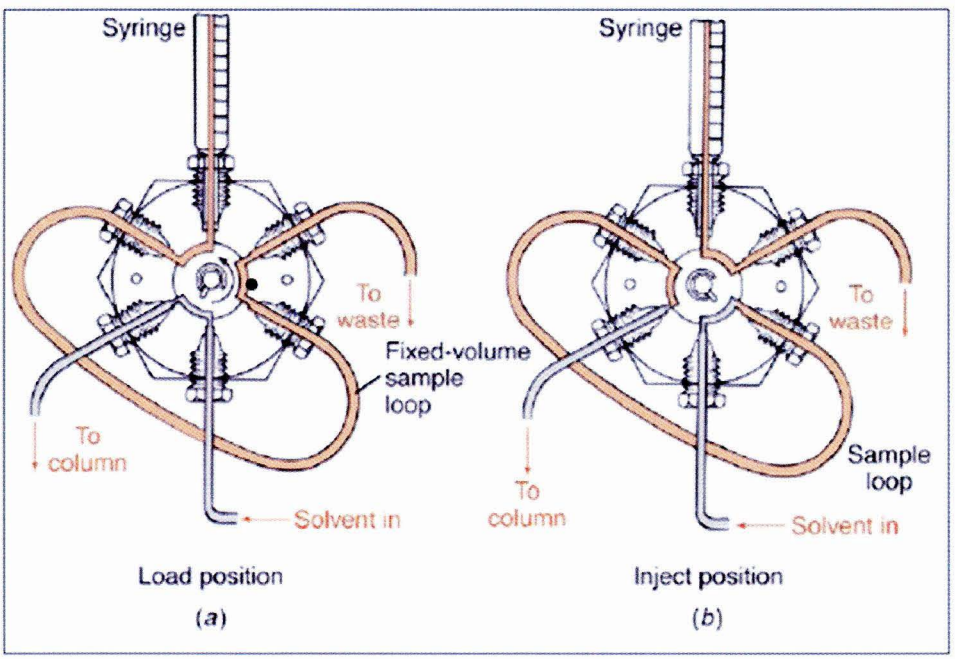

Figure 2.17: a) load and b) inject positions

Analytical, Guard Columns and detector

Columns are made from straight lengths of precision bore stainless steel tubing [95]. Typically they are 10 - 25 cm long and 4 to 5 mm i.d [95]. The stationary phase is made of silica.

Guard column is situated in between the sample introduction port and the analytical column. The whole idea is to prolong the life span of the analytical column as it traps particulate matter before it reaches the analytical column. Since the primary function of a guard column is to retain materials that the mobile phase will not elute from the analytical column, it should contain the same packing as the analytical column to ensure that the retention capacity of both columns is the same.

The ideal detector in HPLC must be rapid and reproducible in response to analytes during analysis. It must be highly sensitive to be able to detect minute concentrations of target analyte and to give a linear response to that effect. UV detector is the most widely used detector and simple to use for compounds that absorb UV radiation. The problem is that it is not selective and not ver sensitive.

2.8.2 Inductively Coupled Optical Emission Spectrometry

The technique is based upon the spontaneous emission of photons from atoms and ions that have been excited in a Radio Frequency (RF) discharge [97]. Liquid and gas samples may be injected directly into the instrument, while solid samples can be injected as slurry by extraction or acid digestion so that the analytes will be present in a solution [97].

The components of ICP-OES comprises of a peristaltic pump, nebulizer, spray chamber, drain assembly, the gas supply, ICP torch and the plasma (Figure 2.19) as shown below.

Firstly the sample is aspirated into the instrument by the help of a peristaltic pump. The sample is then converted into an aerosol (very fine droplets of the sample)

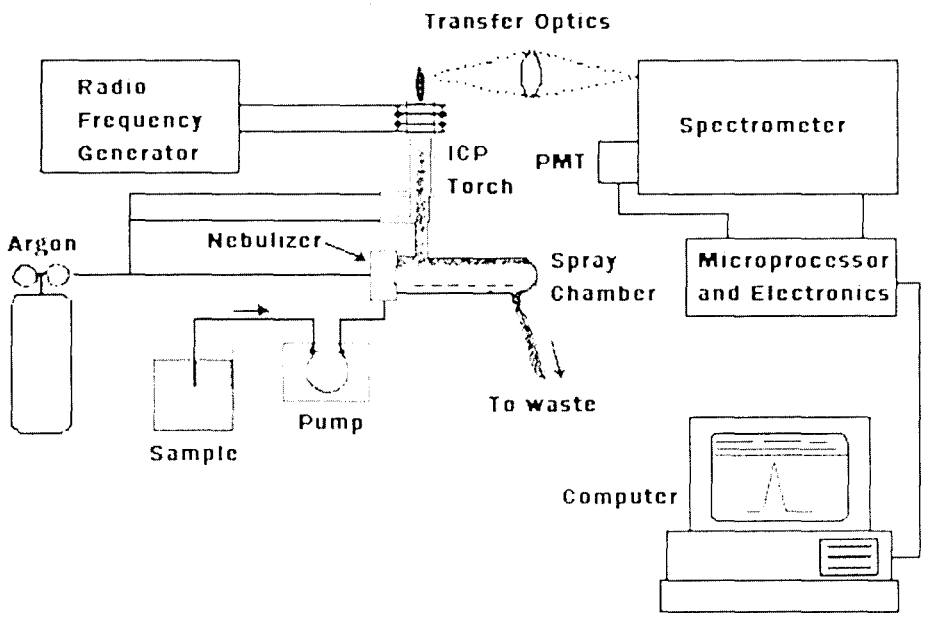

Figure 2.18: Schematic diagram of Inductively Coupled Plasma Optical Emission Spectrometry [98]

by a process called nebulization. The sample aerosols are then carried into the center of the plasma by the help of the flow of the Argon gas. At its core, the inductively coupled plasma (ICP) sustains a temperature of approximately 10 000 K, so the aerosol is quickly vaporized [97]. In here, a process of desolvation occurs. This is to say that high temperature thus removes the solvent leaving behind only the microscopic salt. The solid salt sample further vaporize to form gaseous molecules which then dissociate to form atoms in a process called atomization. All these steps mentioned above happen in the preheating zone as depicted in the Figure 2.19 below which is followed by a summary of the processes that occur in the plasma in Figure 2.20.

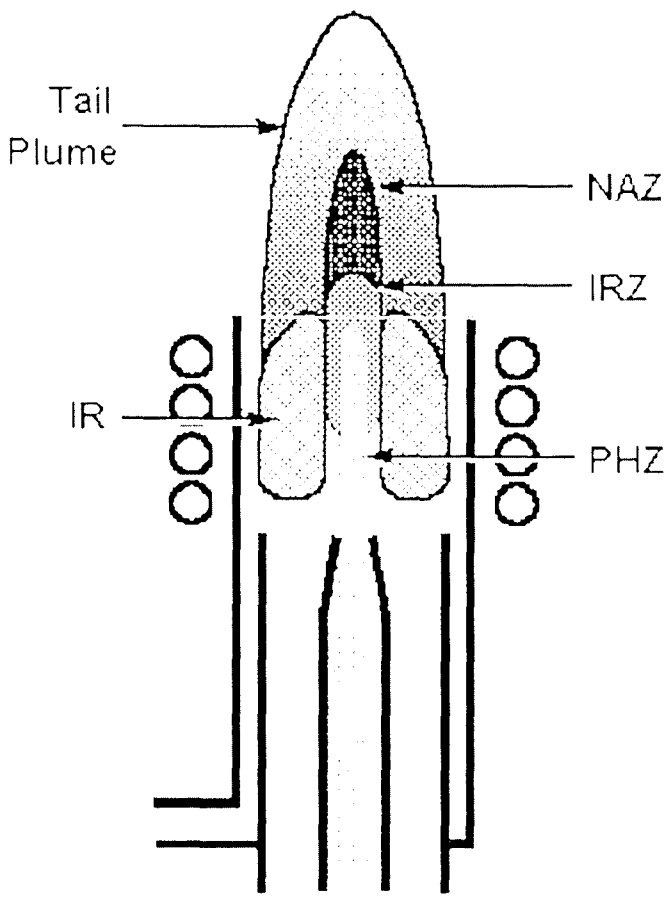

Figure 2.19: Schematic diagram of different zones of plasma; IR - Induction Region, PHZ - Preheating Zone, IRZ - Initial Radiation Zone, NAZ - Normal Analytical Zone [98]

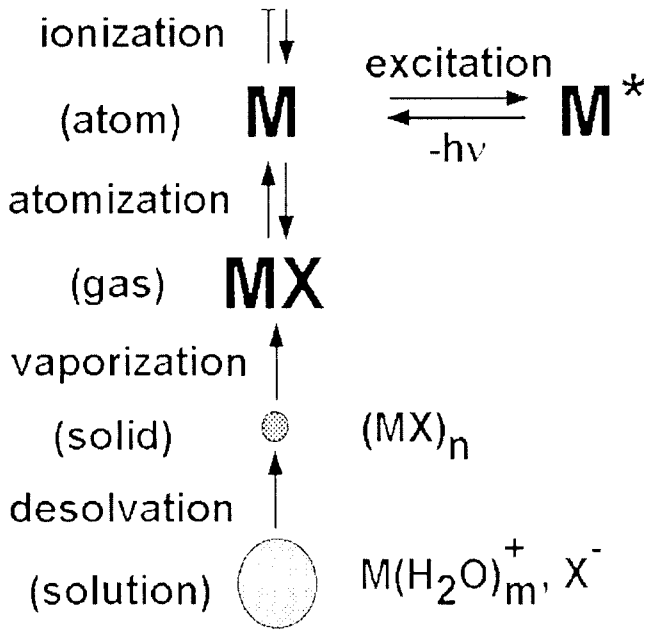

Figure 2.20: A summary of the processes that occur in the plasma [98]

The excitation and ionization processes occur predominantly in the initial radiation zone (IRZ) and the normal analytical zone (NAZ) where temperatures are slightly higher than the PHZ. The NAZ is the region of the plasma from which analyte emission is typically measured [98]. The excited atoms and ions in the plasma emit light at different wavelengths. This results in emission of light of unique frequencies which can then be measured using a polychromator. This polychromatic radiation must be separated into individual wavelengths so the emission from each excited species can be identified and its intensity can be measured without interference from emission at other wavelengths [98]. The particular wavelength exiting the monochromator / polychromator is converted to an electrical signal by a photodetector [97].

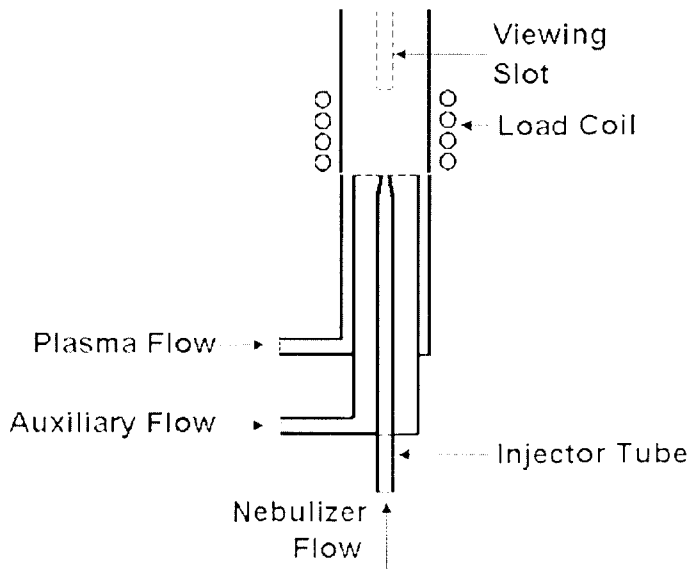

Figure 2.21: Schematic diagram of a torch used in ICP-OES [98]

The main analytical advantages of the ICP over other excitation sources originate from its capability for efficient and reproducible vaporization, atomization, excitation, and ionization for a wide range of elements in various sample matrices [97]. The high temperature of the ICP also makes it capable of exciting refractory elements, and renders it less prone to matrix interferences [97].

Source

The main function of the source is to produce emission spectra characteristic of the elements to be looked for in the sample. The source must be able to excite lines of all the elements of interest as well as to be reproducible when exciting ion moving from one sample to the other. It must also provide sufficient line intensity to achieve the minimum required accurate detection limits with minimal low spectral background. It is important also for the source to provide uniform and reproducible

sample vaporisation and efficient atomisation to avoid variation of replicates.

Radio frequency

The radio frequency (RF) generator is the device that provides the power for the generation and sustainment of the plasma discharge [98].

Nebulizer

Nebulizers are used to break down solution particles into fine aerosol as already discussed above. The solution mixes with both the fuel and oxidant gas to break the large solution particles. The commonly used nebulizers are; concentric, criss-cross, fritted disk and babington nebulizers.

The concentric nebulizer is the most common nebulizer, and it is used in many instruments. The solution is sucked through a capillary tube and the high pressured gas (mixture of fuel and oxidant) enters on the side of the nebulizer and passed on the outside of the capillary that has the solution. On exit, the solution mixes with the high pressured gas to break the solution into fine aerosol.

The criss cross nebulizer allows both the solution and high pressured gas to enter at 90 degrees or at right angles to each other and the gas blows or breaks the solution into fine aerosol.

Fritted Disk Solution is sucked from one side of a fritted disk / membrane and the high pressured gas from the other side so that the gas passes through the fritted disk to break the large particles of solution on the other side of disk or membrane. A very fine aerosol is produced by the nebulizer. The nebulizer has the highest efficiency but it is prone to blockages, especially with high salt content solutions.

The babington nebulizer is used with high salt content solutions, as the solution flow down on the side of a glass bulb with a small orifice, the high pressured gas is passed within the glass bulb and forced out through the small orifice breaking the flowing solution into fine aerosol.

spray chamber

Spray chamber (Figure 2.22) is placed between the nebulizer and the torch in the instrument. Its main function is to remove large droplets from the aerosol because only minute droplets are suitable for injection into the plasma. Generally only droplets of a diameter of about 10 μm or less are permitted into the plasma. The large droplets are taken into the drain. Spray chamber is also used to smooth out pulses that may occur during nebulization due to pumping of the solution.

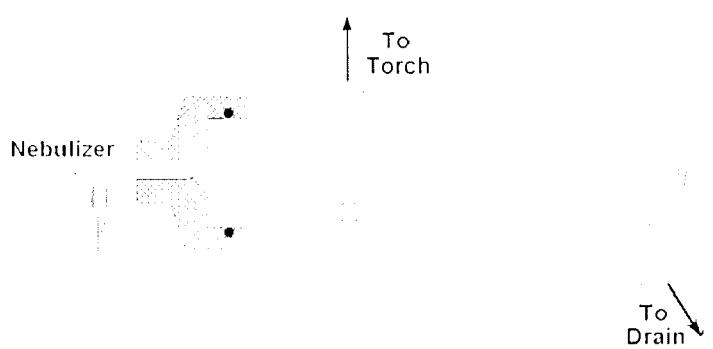

Figure 2.22: Schematic diagram of a spraychamber used in ICP-OES [98]

2.8.3 UV Visible Spectroscopy

UV/Vis spectroscopy is used in analytical chemistry for the quantitative determination of analytes, such as transition metal ions, highly conjugated organic compounds,

and biological macromolecules. Spectroscopic analysis is mainly done in solutions but solids and gases may be studied also.

Ultravioletvisible spectroscopy or ultraviolet-visible spectrophotometry (UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. In this region of the electromagnetic spectrum, molecules undergo electronic transitions.

Molecules containing *pi*-electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. Many organic molecules absorb ultraviolet/visible radiation and due to the presence of a specific functional group. These groups that absorb the radiation are called chromophores.

Factors affecting absorption

The solvent

It is necessary to know the properties of the solvent to be used during UV/Vis spectroscopy as many solvents absorb in the UV or visible regions. The polarity and pH of the solvent can affect the absorption spectrum of an organic compound. When a polar solvent is used the dipole-dipole interaction reduces the energy of the excited state more than the ground state, hence the absorption in a polar solvent such as ethanol will be at a longer wavelength. The excited states of most *Pi-Pi** electronic transitions are more polar than their ground states levels. This is because there is a greater charge separation is observed in the excited state.

Degree of conjugation

Conjugated organic compounds with high degree of conjugation absorb light in the UV or visible regions of the electromagnetic spectrum. Organic compounds that contains double or triple bonds generally display useful absorption peaks in the ultraviolet region since the electrons in unsaturated bonds are relatively loose and hence easily excited. Two types of electrons are responsible for the absorption of the ultraviolet and visible radiation by organic molecules which are, (1) shared electrons that participate directly in bond formation and thus associated with more than atom and (2) unshared outer electrons that are largely localized about such atom as ocyegen , the halogens, sulfur and nitrogen [99]. Therefore how tightly the electrons are bound directly influence the wavelength at which the organic molecule absorbs the radiation. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds as organic solvents may absorb resulting in erroneous results.

The method used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

$$A = log_{10}\,(I_0/I) = abc \tag{2.8.1}$$

where A is the measured absorbance, in Absorbance Units (AU), I_0 is the intensity of the incident light at a given wavelength I is the transmitted intensity, a is a proportionality constant called the absorptivity, b the pathlength through the sample and c the concentration of the absorbing species.

2.9 DPPH radical scavenging activity

DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm [100]. The DPPH antioxidant assay is based on the ability of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical, to decolourize upon the addition of antioxidants. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolourized which can be quantitatively measured from the changes in absorbance [101]. Antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecules tested [102]. This also allows us to make fair projections of the efficiency of the plant extracts in the presence of radicals in the body as the experiments are done at room temperature. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers [103].

Chapter 3

Objectives of the study

Summary

This summarizes the general and specific objectives of the study. It also includes the justification and novelty of the study.

3.1 General and specific objectives

3.1.1 General objectives

Develop a PHWE method for extraction of essential and non essential chemicals from *Moringa Oleifera* and *Moringa Ovalifolia* leaf powder for possible additions in food products.

3.1.2 Specific objectives

- To optimise the PHWE method by varying extraction temperature and sample amount.
- To evaluate the efficiency of the PHWE system on *Moringa* as compared to boiling sample, total digestion for elements and acid hydrolysis for flavonols.
- To determine the total phenolic and flavonoid contents in the leaf powder of two *Moringa* species extracted using the PHWE technique.
- To determine mineral substances in *Moringa* extracts obtained using PHWE technique.
- To determine antioxidant activity and reducing activity in *Moringa* extracts obtained using the PHWE technique.

3.1.3 Hypothesis and Research Questions

Hypothesis

Pressurised hot water extraction can be used to extract essential compounds from *Moringa Oleifera* and *Moringa Ovalifolia* leaves.

Research Question

- Specifically this research project seeks to answer the following; What kind of essential compounds can be extracted in *Moringa Oleifera* and *Moringa Ovalifolia* leaf powder by PHWE system?

- At what temperature and pressure can PHWE efficiently extract various chemicals compounds from *Moringa Oleifera* and *Moringa Ovalifolia* leaf powder?

3.1.4 Justification

The South African government is pushing communities to grow *Moringa* trees for poverty and malnutrition eradication. It is true that consumption of *Moringa* tree parts such as leaves and roots can be a significant source of nutrients some of which are good in the medicinal sense. It is also true that supplementation of other foods other than the direct consumption of *Moringa* parts could even be more beneficial. This can be achieved by extracting these nutrients from *Moringa* and formulating concentrates that can be spiked in other foods such as baby foods. To this end, novel extraction technologies that can be effectively implemented in this avenue such as PHWE being explored in this study should be pursued for the parameters enumerated under objectives.

3.1.5 Novelty

Although PHWE extraction has been described for the extraction of chemicals in plants, no optimization has been performed for isolating targeted nutrients for value addition in food products. This requires that all essential chemicals are preserved

and isolated with extremely high yields

Chapter 4

Materials and Methods

Summary

The main focus of this chapter is to give details of materials and to describe procedures used to generate data. The chapter also elaborates on sample preparation methods, techniques employed for extraction as well as preparation of reagents used for quantification. Analytical techniques are also described.

4.1 Chemicals and Reagents

All reagents used were of analytical grade. Quercetin, myricetin, kaempferol, methanol (HPLC grade), formic acid, sodium hydrogen phosphate anhydrous, citric acid, Folin - Ciocalteu reagent, potassium ferricyanide, ferric chloride, gallic acid, sodium carbonate, diatomaceous earth, sodium nitrite, trichloro acetic acid and 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·), were purchased from Sigma Aldrich (Johannesburg, South Africa). Ultra-pure water from a purification system, Milli-Q, Millipore (MA, USA) was used in all the experiments. Quercetin, myricetin and kaempferol standards were prepared as stock solutions at 100 mg L^{-1} in methanol and stored in an opaque container at -18^{0}C.

Stock solution (100 mg mL^{-1}) containing a mixture of kaempferol, myricetin and quercetin was prepared by dissolving an appropriate amount of each compound in 100 mL methanol. This stock solution was used for the preparation of intermediate solutions that were used for calibration curves during analysis. When not in use the stock was stored at -18^{0}C.

0.02 M Na_2HPO_4 was prepared by dissolving 2.8392 g of the buffer, molecular weight 141.96 g mol^{-1} in water of about 800 mL and topped up to 1.0 L while adjusting the pH to 2.5 by citric acid. 19.21 g of citric acid monohydrate $C_6H_8O_7$ of molecular weight 192.12 g mol^{-1} was dissolved in 50 mL water to make 2.0 M.

4.2 Sample preparation and extraction

4.2.1 Plant material

Leaf samples of *Moringa Oleifera* were collected from a Limpopo farm near Polokwane and an Atteridgeville farm near Pretoria. Both of these farms are in South Africa. The samples were rinsed with distilled water immediately after harvest and left to air dry in a sterile environment in a shade. The leaves were pounded with a pestle and mortar and sieved with a 25 μm sieve and stored in an opaque container for later extraction in PHWE system or MAE. *Moringa Ovalifolia* leaf samples were collected from five different sites where the tree naturally grows in Namibia. These are Okahandja, Okaukuejo also known as Ghost forest, Halali which is situated in Etosha national park, Tsumeb and Keetmanshoop in the Karas region. The leaf samples were treated in the same way.

4.2.2 Pressurised Hot Water Extraction Procedure

0.5 g of *Moringa Oleifera* powder and 0.5 g of diatomaceous earth were both weighed into an extraction cell which is then connected to the pressurised extraction system. An old gas chromatography (GC) oven was used for programmed heating of the sample in the cell. A typical procedure entails pumping of deionised water at a flow rate of 1.0 mL min^{-1}. Typical pressure was 1000 - 3000 psi. An oven was programmed to operate at 100^{0}C for 20 minutes. Prior to pumping, the oven and the extraction cell are allowed to equilibrate to the desired temperature for at least 10 minutes while the deionised water was heated to 80^{0}C to minimise temperature gradient. Continuous pumping was done at appropriate temperature and the extract

collected at the end. The valve placed between the oven and the extraction bottle (container) was used to regulate pressure. A thermometer was used to monitor the temperature of the water. The collected extract was taken for further analysis. As part of optimisation, temperature of the extraction cell was varied from 25^0C to 200^0C.

0.1 g of *Moringa Ovalifolia* was mixed with 0.9 g of the diatomaceous earth and extraction done as elaborated above.

4.2.3 Microwave digestion of plant leaves for elemental analysis

8.0 mL of concentrated nitric acid was added to 0.1 g of the leaf powder into the self-regulating pressure control digestion vessel with a vent plug and Teflon liners. 2.0 mL of the hydrogen peroxide was added and the digestion done for 30 minutes. The samples were then filtered with a 0.45 μm filter paper into a 25 mL volumetric flask and filled up to the mark with deionised water. Samples were taken for elemental analysis on the ICP-OES.

4.2.4 Hydrolysis of extracts

To 1.0 mL of the extract, 1.0 mL of 60% 2.8 M HCl in methanol was added and incubated in a water bath at 90^0C for 2.5 hours. After hydrolysis, the extracts were allowed to cool, filtered with a 0.45 μm filter paper prior to injection in HPLC.

4.3 Measurement of Reducing Power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [104]. In the reducing power assay, the presence of reductants (antioxidants) in tested samples result in reducing Fe^{3+} / ferricyanide complex to the ferrous form (Fe^{2+}) [105]. The reducing power of the *Moringa Oleifera* extracts were determined using the method described by Siddhuraju and Becker, 2003 [20]. 1 mL of the extract was mixed with 0.2 M phosphate buffer (5 mL, pH 6.6), 1% potassium ferricyanide (5 mL), and then incubated at 50°C for 20 minutes. 10% trichloroacetic acid (5 mL) was added to the mixture to stop the reaction and centrifuged at 3000 rpm for 10 minutes. The supernatant (5 mL) was mixed with distilled water (5 mL) and 0.1% ferric chloride (1 mL) and the absorbance measured at 700 nm using UV - visible spectrophotometer. Absorbance measured is directly proportional to reducing power of the extract.

4.4 Determination of Total Phenolic Content (TPC)

Total phenolic contents were determined by the Folin-Ciocalteau method [20]. The extract samples were prepared using the method of Moraes *et al*, 2008 [106]. 0.5 mL of extract in distilled water (1:10) was mixed with 2.5 mL of Folin-Ciocalteu reagent diluted in distilled water (1:10 v/v). The mixture was hand shaken and after 5 minutes of rest, 2 mL of sodium carbonate 4% (v/v) were added. Samples were incubated for 2 hours in the dark and absorbance measured at 740 nm using UV/Vis spectrometry. The calibration curve was prepared by four data points ranging from 10 to 100 $\mu g\ L^{-1}$ solutions of gallic acid in water.

4.5 DPPH· (2,2 - diphenyl-2- picryl -hydrazyl) Free Radical Scavenging Capacity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (-DPPH) was used for determination of free radical-scavenging activity of the extracts [20]. DPPH radical is used as a substrate to evaluate anti-oxidative activity of the *Moringa Oleifera* leaf extract. The antioxidant activity of different extracts was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH method adapted from [20]. 0.1 mL of extract at various concentrations was added to 3.9 mL (0.025 g L^{-1}) of DPPH· solution. The decrease in absorbance at 515 nm was determined continuously at every minute with a spectrophotometer for 20 minutes. The remaining concentration of DPPH· in the reaction medium was calculated from a calibration curve obtained with DPPH· at 515 nm [20]. The anti-radical activity was calculated using the ratio given below:

$$(A_{control} - A_{sample})/A_{control} x100) = anti - radicalactivity \qquad (4.5.1)$$

where, $A_{control}$ is the absorption of the DPPH at time = 0 minutes and A_{sample} is the absorption of the DPPH solution after the addition of the sample at a particular time. The assays were carried out in triplicate and the results were expressed as mean values ± standard deviations.

4.6 Inductively Coupled Plasma-Optical Emission (ICP - OES) analysis

About 5 mL of plant extracts from the PHWE system were analysed for metal content using ICP - OES. ICP - OES was first calibrated using standard metal solutions. Samples for total analysis of the metals digested by microwave assisted extraction (MAE) were also analysed with ICP - OES in a similar way.

4.7 HPLC analysis of flavonols

The Bischoff HPLC (Metrohm, Johannesburg, RSA) system consisting of a Gemini Phenyl - C_6 column (150 x 4.6 mm, 5 μm i.d) with a UV-Vis detector set at 254 nm was used for the quantification of the components. Samples were eluted under isocratic condition with methanol and 20 mM sodium dihydrogen phosphate buffer adjusted to pH 2.5 with citric acid (55:45 v/v and 0.1% formic acid). The flow rate was optimised to 1.0 mL min^{-1} and the injection volume was 20 μL. Samples were prepared in triplicates. Quantification of myricetin, quercetin and kaempferol were done using a four-point calibration curve of standard solutions at concentrations between 1.0 and 30 μg mL^{-1}.

4.8 Determination of extraction kinetics

The first order of extraction kinetics was calculated by calculating the natural log of the actual concentration of the extract while the second order of extraction kinetics was found by taking the inverse of the actual concentration of the extract.

4.9 Statistical Analysis

All measurements were done in triplicates and data reported as mean ± SD, where SD is the standard deviation. Some calculations were reported as mean ± %RSD

4.10 Instrumentation and Techniques

The following analytical equipments and apparatus were used for the sample preparation, extraction and analysis.

4.10.1 High Performance Liquid Chromatography (HPLC)

Bischoff HPLC (Metrohm, Johannesburg, RSA) system consisting of a Gemini Phenyl - C_6 column (150 x 4.6 mm, 5 μm i.d) with a UV-Vis detector set at 254 nm was used for the quantification of the compounds.

4.10.2 Inductively coupled plasma-optical emission spectroscopy (ICP-OES)

An Inductively Coupled Plasma-Optical Emission Spectroscopy (Spectro Genesis, Spectro, Germany) was used. The parameters used in ICP-OES are shown in Table 4.1 below;

4.10.3 UV-VIS spectroscospy

A UV/Vis spectroscopy (Varian, Cary 50 Conc, Germany) and a Varian Cary 1E double beam spectrophotometer (Palo Alto, CA, USA) was used at the wavelengths of interest depending on the particular test conducted.

Table 4.1: Operating conditions of the ICP-OES

Parameter	Settings at 25^{0}C
RF power	1400 W
Coolant gas flow	14.00 L min^{-1}
Auxiliary gas flow	1.00 L min^{-1}
Nebulizer gas flow	1.00 L min^{-1}
Sample pump flow	2.00 L min^{-1}
Sample aspiration rate	2.00 L min^{-1}
Replicates	3
Plasma torch	Quartz
Spray chamber	Single pass
Nebulizer	Cross flow
Processing mode	Area

4.10.4 Microwave digestion

Moringa leaf powder was digested using Multiwave 3000 SOLV Anton Par microwave (Osterreich, Austria).

4.10.5 Ultrasonicate bath

Mobile phase was ultrasonicated on the Transsonic 460 ultrasonic bath (Elma, Singen, Germany) to enhance dissolution and to eliminate air bubbles prior to use in the HPLC.

Chapter 5

Results and discussion

Summary

This chapter is based on the findings of the research work. It gives explanations for certain trends that were observed. The results relate to the following;

- The flavonols found in extracts from the leaves
- The radical scavenging activity of the extracts from the leaves
- The ability of the leaf extracts to reduce ferrous cyanide complex to ferrous form
- The total phenolic content of the extracts from the leaves
- The micro and macro elements contents found in the leaf powder from the two *Moringa* species.

5.1 HPLC analysis of the flavonols

The flavonols were separated in a Gemini C-6 phenyl, 5 μm, column. Myricetin is eluted first followed by quercetin and lastly kaempferol which is less polar hence has more affinity for the stationary phase. This is shown in Figure 5.1.

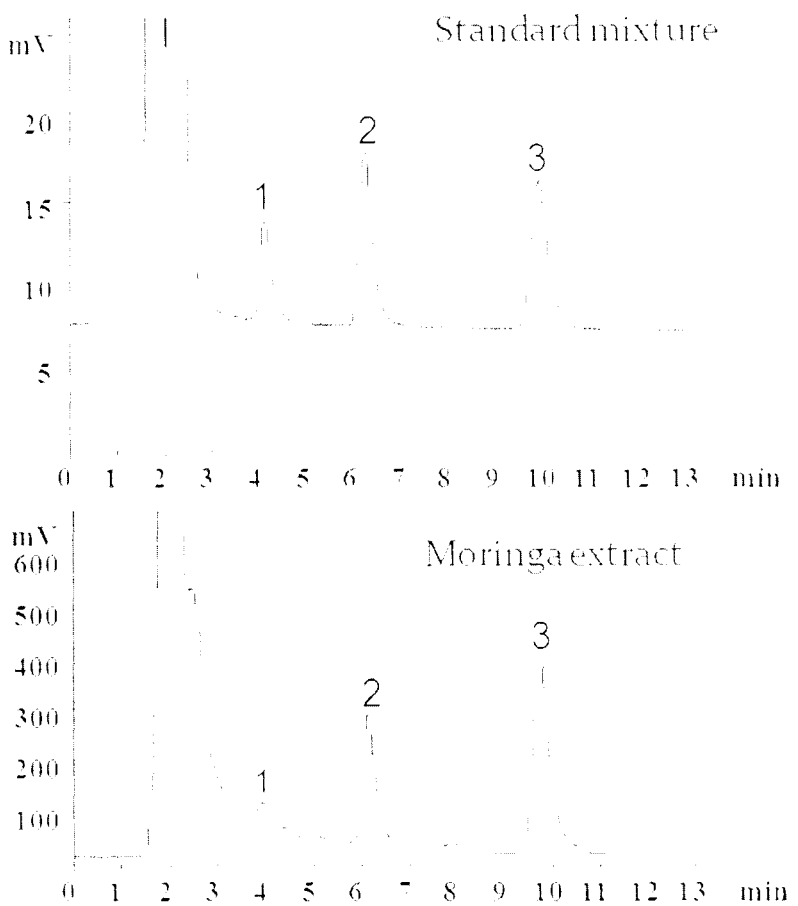

Figure 5.1: Chromatograms of 1) myricetin, 2) quercetin and 3) kaempferol in the extract compared to the ones in the standard mixture.

The results in Figure 5.1 confirms the presence of the flavonols myricetin, quercetin and kaempferol. The two peaks for quercetin and kaempferol in the plant extract are

clearly resolved with reasonable peak height while for myricetin the peak height is small and not resolved from the other polar substances that are eluted early. This is because myricetin unlike the other two flavonols does not survive the harsh conditions of acid hydrolysis of which the other flavonols survive so that they are liberated to form aglycones. Acid hydrolysis breaks the glycosidic bonds to realease the flavonol aglycones which can then be detected and quantified. This is because in nature, flavonoids can occur either in the free or conjugated forms, often being esterified to one or two sugar molecules, through at least one hydroxyl group [65].

Among flavonols, hydroxylation decreases retention owing to increasing polarity (hydrogen bond formation ability) and the elution pattern is affected by the number of OH-groups [63]. Therefore myricetin is retained less than quercetin and finally than kaempferol just as the order of OH- substituents decrease.

5.2 Linearity, LOD and LOQ

The calibration curve used for quantification is shown in Figure 5.2. The calibration curve was linear from 1.0 mg L^{-1} to 30 mg L^{-1} concentration range with acceptable correlation factors of 0.99 for all the flavonols. The limit of detection LOD is defined as the lowest concentration that can be detected but not necessarily be quantified. One way of determining this concentration is by determining the concentration that is three times greater than the background noise (3 x S/N). The LOQ was calculated as 10 times the limit of detection . Both LOD and LOQ are shown in Table 5.1.

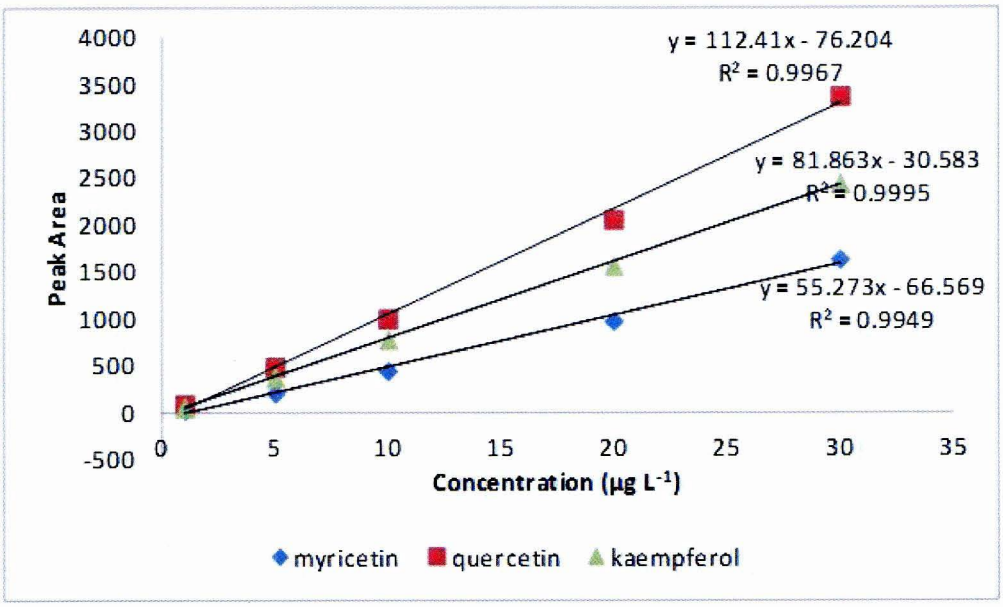

Figure 5.2: Typical calibration curve of myricetin, quercetin and kaempferol.

Table 5.1: LOD and LOQ for flavonols

Flavonol	LOD μg mL^{-1}	LOQ μg mL^{-1}
Myricetin	0.016	0.16
Quercetin	0.028	0.28
Kaempferol	0.038	0.38

5.3 Effect of filtering the extract before hydrolysis on quercetin and kaempferol

The flavonols were lower in concentrations when the extracts were filtered prior to hydrolysis and significantly higher when filtering was done after hydrolysis as seen below in Figure 5.3. It is possible that some compounds were lost when filtering before hydrolysis since such minimal amount of aglycones were only liberated during hydrolysis. This therefore implies that it is of prime importance to only filter the extracts after hydrolysis prior to injection. Myricetin unlike the other two flavonols

of interest, did not need to be hydrolysed as it degrades under hydrolysis condition.

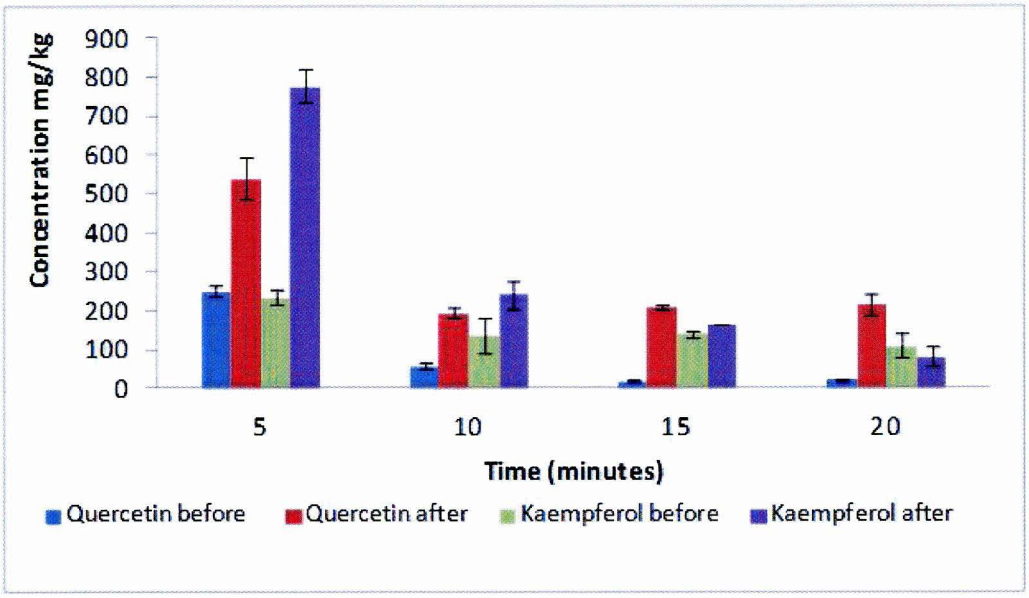

Figure 5.3: Significance of filtering after hydrolysis in sample fractions collected at different extraction times. Extraction temperature was at 25°C and sample flow rate of 1.0 mL min^{-1}

Figure 5.3 shows that the first 5 mL extract collected for the first 15 minutes, before hydrolysis, the amount of flavonols was almost the same while after hydrolysis kaempferol is almost one and half times more of qurcetin. This means before hydrolysis the behaviour of the two flavonols is the same. Hydrolysis conditions however favour kaempferol because the same extract subjected to hydrolysis conditions resulted in significantly higher concentrations of kaempferol.

Figure 5.3 also shows that the concentration of the flavonols decreases with collection time. Extracts collected in the first 5 minutes were more concentrated than the ones collected in the next 5 minutes and so the trend was the same with subsequent fractions. This was expected because during extraction, the first fractions were

highly concentrated because as the water was introduced into the cell in the PHWE system, the effect of pressure and temperature resulted in the opening of the cells reaching out to the active sites where the target molecule was bound. Because of this rapid entry, there is desorption of the target molecules which are then eluted at high concentrations while the subsequent extracts would have lower concentrations because the active sites could be depleted of the target molecules. Nonetheless these last fractions had fairly high amounts of flavonols.

Quercetin after hydrolysis in the last three fractions, seemed to be constant unlike kaempferol which was decreasing with time. It could possibly mean that while the temperature was maintained at 25^0C, the rate of desorption of quercetin from the active sites of the organic matter remained the same. As for kaempferol, the decrease suggested that the active sites were being depleted of the analyte. It is from this experiment that a compromise volume of extraction was deduced to be 20 ml.

5.4 Influence of extraction temperature on the amount of quercetin and kaempferol extracted

The results of the influence of extraction temperature are shown in Figure 5.4. All the figures show that within the first few minutes, there are higher concentrations of the flavonols which then decrease with collection time. This is expected and has been discussed already under Section 5.3. The results also show that increasing the temperature results in increased concentrations of the flavonols extracted. Figure 5.4a shows a value of about 1700 mg kg^{-1} for kaempferol when extraction was done at 25^0C. When extraction was done at 100^0C, the value almost doubled.

This implies that with increased temperature the mass transfers are enhanced and the cells break open more to release the target analyte. There is reduced viscosity and improved diffusivity of water to allow better penetration through the matrix particles [87]. The same pattern was observed with quercetin which was found to be less dominant than kaempferol. In general, extractions at higher temperature give increased mass transfer rates and higher extraction yields as a result of improved solute desorption from matrix active sites [64]. However, there is a significant loss of kaempferol at 150^0C while for quercetin more is relatively extracted. It could be due to structural differences that the loss exhibited by kaempferol defied general observations of higher yields at higher temperatures.

Kaempferol is seen to be highly predominant at all temperatures. No concrete reason can account for this discrepancy because generally for the hydrolysis process, although from literature optimum compromise is to achieve complete release of aglycones and to minimize degradation reactions of compounds involved [107]. Different plant materials contain different flavonoids and phenolics compounds in different forms, resulting in variable amount and also susceptibility to degradation [108]. Many factors influence the distribution of flavonols in the plant. Distribution of phenolics in plants at the tissue, cellular and subcellular levels is not uniform [55]. The impact of geographical location on the extraction of total phenol content is also supported by the fact that variety of diverse factors such as worldwide changes in seasonal patterns, weather episodes, temperature changes, biotic and abiotic stresses may affect the production of secondary metabolites in plants [6]. Other contributing factors could be due time and period of collection, geographical origin and climatic

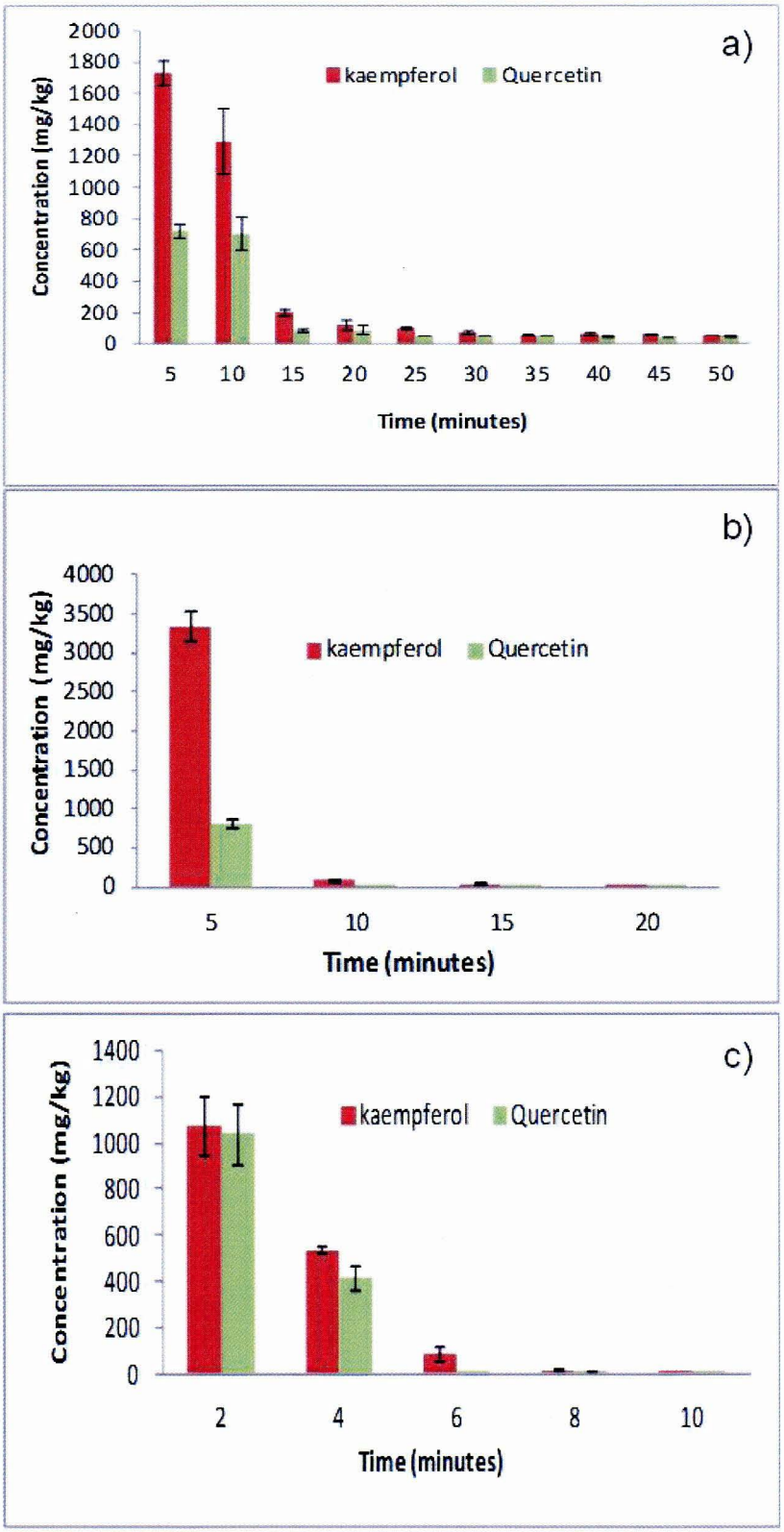

Figure 5.4: Variation of quercetin and kaempferol concentrations from 0.5 g of *Moringa Oleifera* leaf powder at 1.0 mL min^{-1} flow rate (a) extraction at 25^0C (b) extraction at 100^0C (c) extraction at 150^0C.

conditions [6]. Therefore optimum hydrolysis conditions for kaempferol may not necessarily be optimum for quercetin. What matters is that all the target compounds are extracted and quantified. The time taken for collection of extracts at 25, 100 and 150^{0}C extraction temperatures were 50, 20 and 10 minutes respectively. This allowed to collect all the compounds from the system.

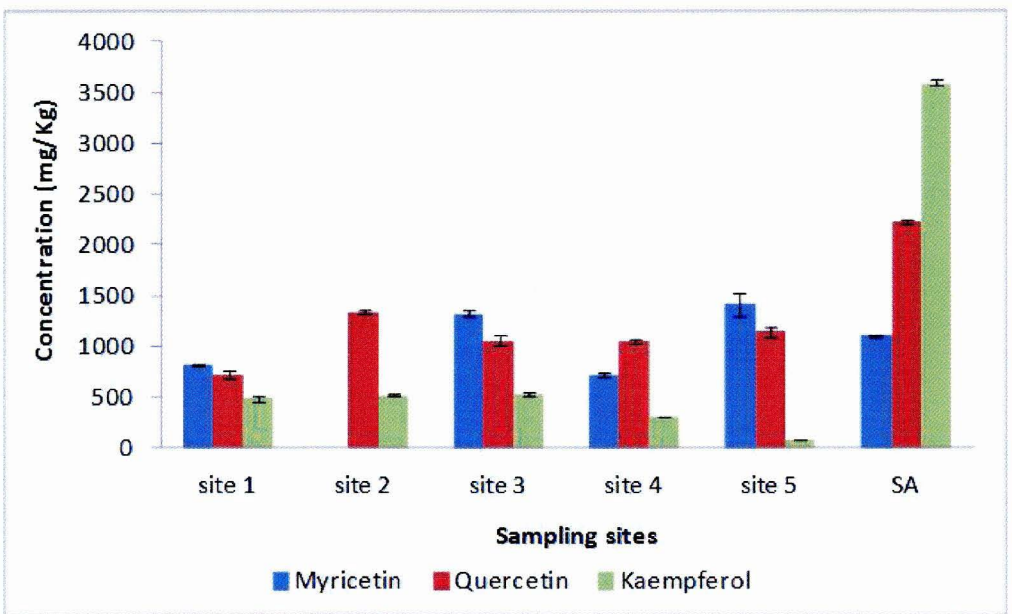

Figure 5.5: Variation of myricetin, quercetin and kaempferol concentrations in 0.1 g of *Moringa Oleifera* and *Moringa Ovalifolia* leaf powder at 1.0 mL min^{-1} flow rate and extraction temperature of 25^{0}C.

5.5 Total Phenolic Content (TPC) studies

5.5.1 Effect of incubation time on Total Phenolic Content

During the process of oxidation of phenol, Folin-Ciocalteu reagent which is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) [6]. This

reaction was optimised by varying the incubation time. As seen in Figure 5.6, the longer the incubation time more of the phenolics are released. In this study however, incubation period of 2 hours was used in order to save time.

The extraction temperature and the incubation time directly influence the phenolics released. The incubation process is also a time dependent process. The more time is allowed, the more the available phenolic compounds are oxidized and in the process Folin -Ciocalteu solution is reduced.

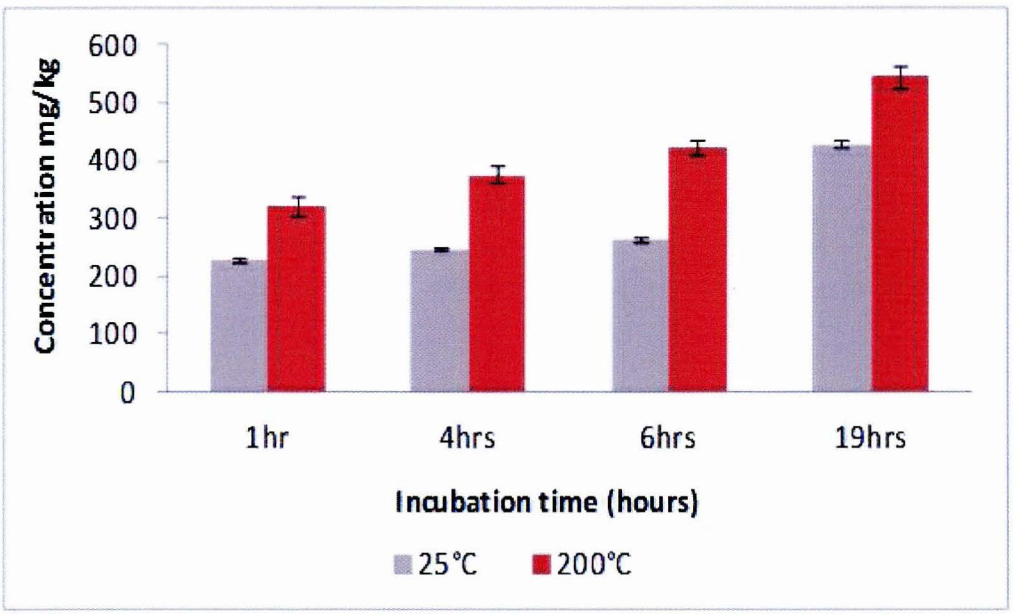

Figure 5.6: The effect of incubation time on the total phenolic content.

5.5.2 Variation of total phenolic contents of extracts with extraction temperature

Table 5.2 shows that with increasing extraction temperature, the phenolic contents do increase. As already discussed above, at higher extraction temperature the water becomes less polar and hence dissolves more compounds which are less polar. More

phenolics would therefore be extracted at moderately high temperatures. However, too high extraction temperatures degrade the phenols as seen by a drastic drop of the phenolic contents at 200^0C. In addition to that the recovery of the phenolic contents at 200^0C is not reproducible as seen by the high percentage relative standard deviation.

Table 5.2: Variation of total phenolic contents of the 0.5 g leaf extracts of *Moringa Oleifera* with extraction temperature at incubation time of 2 hours

Temperature (^{0}C)	Total Phenolic Content (mg kg^{-1}) $\pm$ %RSD
25	1 432 $\pm$ 1.21
50	1 507 $\pm$ 0.15
100	1 757 $\pm$ 0.35
150	1 984 $\pm$ 0.58
200	77 $\pm$ 36.36

Table 5.6 shows the same pattern. Boiling the powder from the leaves for fewer minutes resulted in higher concentrations of the phenolic contents. Table 5.5 shows concentrations that compare very well with the ones in the study. Two of the sampling sites in (Balokok and Chakwaal) showed concentrations of about 30 000 mg kg ^{1} as the values obtained in this study for *Moringa Ovalifolia*. Composition of phenolic compounds varies widely with other factors such as the stage of maturity, variety, part of the plant analyzed, post-harvest handling, processing and storage [109]. Many factors could have attributed to this. The values for *Moringa Oleifera* are however low. However boiling for longer times resulted in less phenolic contents recovered. This implies that when exposed for long at high temperatures they do degrade.

Table 5.3: Variation of total phenolic contents of 0.1 g leaf powder of *Moringa Ovalifolia* extracted at 25^{0}C and incubated for 2 hours

Sampling site	Total Phenolic Content (mg kg^{-1}) ± %RSD
site 1	34 090 ± 0.04
site 2	40 096 ± 0.077
site 3	31 483 ± 0.12
site 4	30 483 ± 0.078
site 5	31 296 ± 0.036

Table 5.4: Variation of total phenolic contents of 0.1 g leaf powder of *Moringa Ovalifolia* of site 4 extracted at different temperatures and extracted and incubated for 2 hours

Temperature (^{0}C)	Total Phenolic Content (mg kg^{-1}) ± %RSD
25	30 488 ± 0.078
100	31 260 ± 0.092
150	31 376 ± 0.044

5.6 Total flavonol Content of the *Moringa sp* leaf extracts

A summation of the respective flavonols of the first 10 ml (different fractions making up 10 ml) of the fractions of the leaf extracts was done and the amounts compared. The concentrations of flavonols increase with increasing temperature. Highest concentrations of myricetin and kaempferol was recorded at 100^{0}C but do decline at 150^{0}C. The results for kaempferol are in agreement with the results above in Figure 5.4 which shows an decrease in concentration even at 150^{0}C. This is in agreement with literature that states that extraction techniques may either cause degradation of the targeted compounds due to high temperature and long extraction times [63]. The

Table 5.5: Total phenolic content of methanolic extracts of *Moringa Oleifera* leaves grown in Pakistan [110]

Sampling site	Total Phenolic Content (mg kg^{-1}) $\pm$ %RSD
Balakot	31 260 $\pm$ 0.31
Chakwaal	30 488 $\pm$ 0.38
Nawabshah	88 200 $\pm$ 0.34
Jamshoro	88 900 $\pm$ 0.33
Mardaan	127 900 $\pm$ 0.29

Table 5.6: Variation of total phenolic contents of the 0.5 g leaf powder of *Moringa Oleifera* when boiled for different times at incubation time of 2 hours

Boiling Time (minutes)	Total Phenolic Content mg kg^{-1} $\pm$%RSD
0	842 $\pm$ 1.03
5	924 $\pm$ 8.00
10	984 $\pm$ 6.61
15	939 $\pm$ 7.14
20	164 $\pm$ 2.79

slight decrease at 100^{0}C for quercetin could be due to experimental errors. In general, extractions at higher temperature give increased mass transfer rates and higher extraction yields as a result of improved solute desorption from matrix active sites [64].

However as for quercetin there is no consistency when comparing Table 5.7 and Figure 5.4. It is important to note that several factors might affect the contents of the phenolic compounds such as the way of preparation (plant processing, concentration, time and temperature), the cultivation characteristics (soil, climate stresses) and the method of analysis [106].

Sreelatha and Padma 2010 [18], determined quercetin and kaempferol content

in the mature leaves of *Moringa Oleifera* and found 795 and 216 mg kg^{-1} for the respective flavonols. These are significantly lower than the amount found in this study which are 1488 and 3440 mg kg^{-1} for the highest concentration of quercetin and kaempferol respectively. Despite the fact that the Folin Ciolcalteu method is routinely used in various laboratories, the specific details of the method differ considerably making it more difficult to make fair comparison. Therefore, when the comparison of results from different laboratories are done, the comparability of the values obtained by each analyst is doubtful even if each relative value can be informative [111].

Table 5.7: Variation of myricetin, quercetin and kaempferol in the first 10 ml extracts with extraction temperature where myricetin was analysed prior to hydrolysis while the other flavonols were analysed after hydrolyis

Temperature (^{0}C)	Myricetin (mg kg^{-1})	Quercetin (mg kg^{-1})	Kaempferol (mg kg^{-1})
25	406	1429	3030
100	2699	840	3440
150	1496	1488	1730

N.B: No percentage relative standard deviation was done as the values are the summation of different fractions.

5.7 Extraction Kinetics

The order of the extraction kinetics for all the flavonols at 150^{0}C were found to be first order. Quercetin unlike others also followed second order at 150^{0}C. At 100^{0}C, quercetin and kaempferol both follow second order while myricetin followed first order. Kaempferol, at the same temperature also followed first order. At 25^{0}C

myricetin followed first order while the other followed second order of extraction. These differences arise due to variability of the way the target molecules are bound to the active sites. The following table summarises the correlation factors that were found in the plots below;

Table 5.8: Comparison of correlation factors of extraction kinetics for quercetin and kaempferol

Temperature	Flavonols	1st order	Conclusion	2nd order	Conclusion
(^{0}C)		(R^2)		(R^2)	
25	myricetin	0.65	√	0.26	x
	quercetin	0.66	x	0.81	√
	kaempferol	0.77	x	0.94	√
100	myricetin	0.99	√	0.87	x
	quercetin	0.75	x	0.83	√
	kaempferol	0.96	√	0.99	√
150	myricetin	0.99	√	0.74	x
	quercetin	0.90	√	0.94	√
	kaempferol	0.98	√	0.87	x

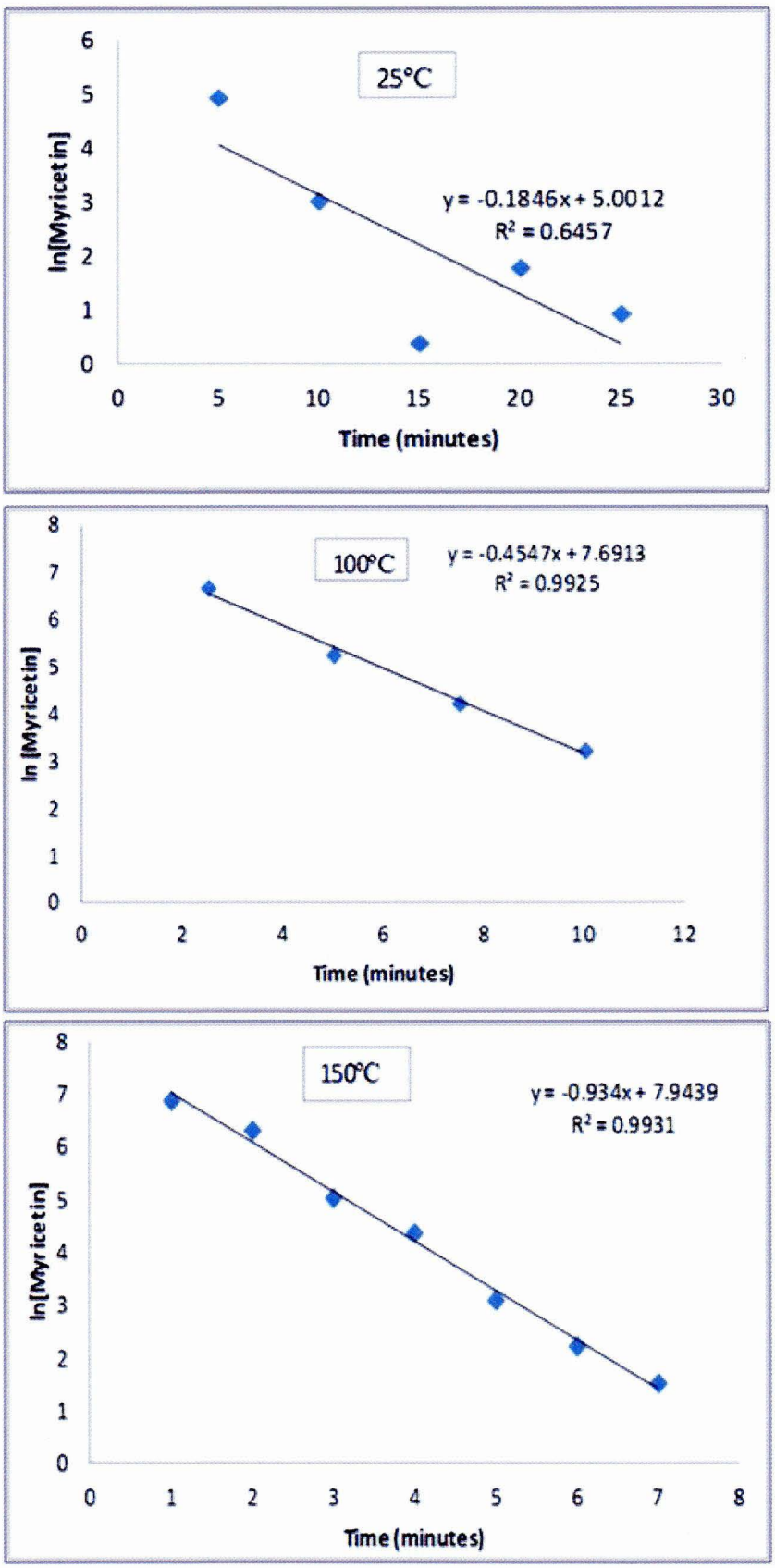

Figure 5.7: Kinetics first order extraction of myricetin when extracted at; a) 25 b) 100 c) 150^{0}C.

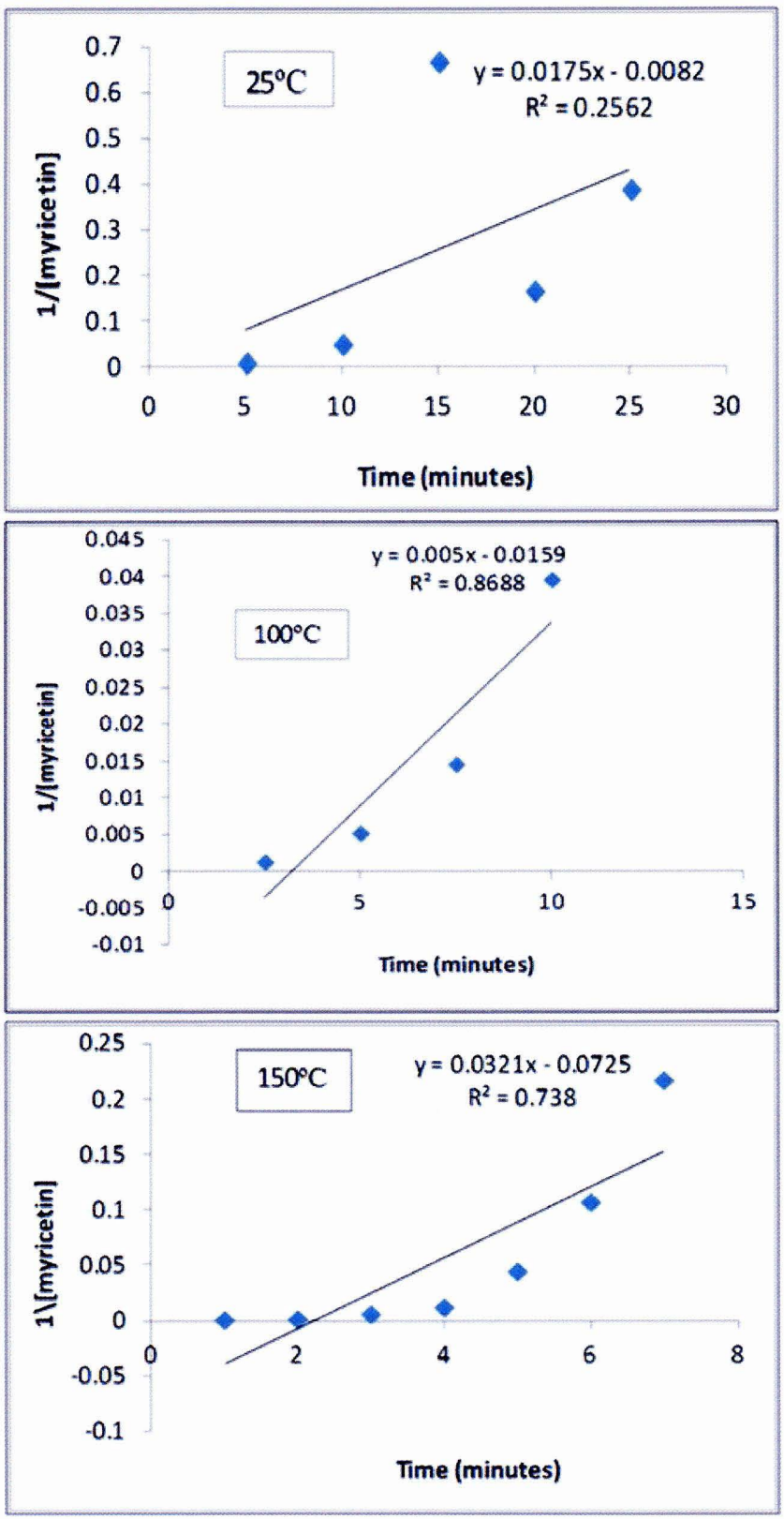

Figure 5.8: Kinetics second order extraction of myricetin when extracted at; a) 25 b) 100 c) 150^0C.

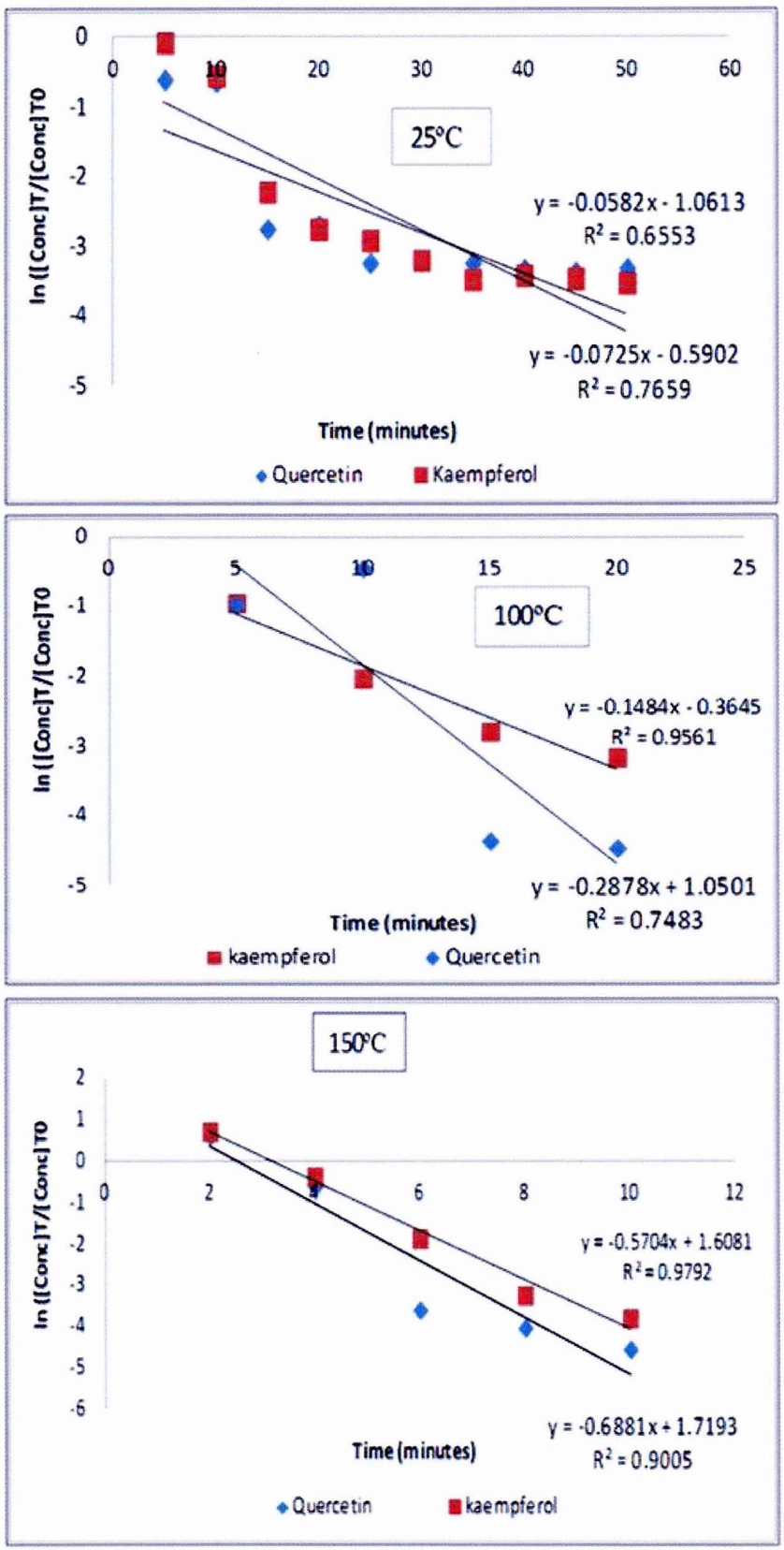

Figure 5.9: Kinetics first order extraction of quercetin and kaempferol when extracted at; a) 25 b) 100 c) 150^0C.

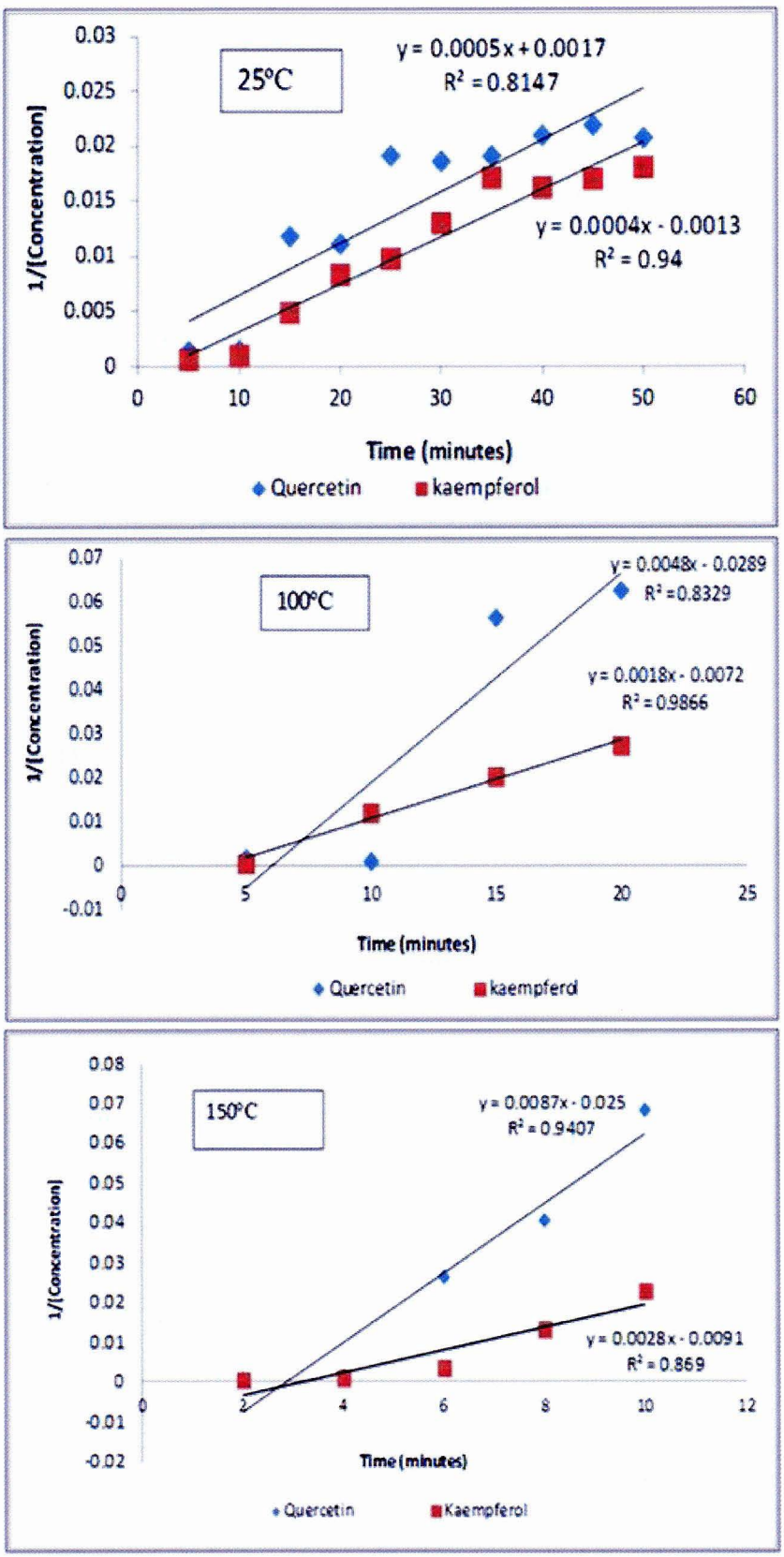

Figure 5.10: Kinetics second order reaction of quercetin and kaempferol when extracted at; a) 25 b) 100 c) 150 ^{0}C.

5.8 The Ferric reducing activity of *Moringa* leaf samples

The reducing power of the aqueous *Moringa Oleifera* leaf extracts was determined spectrophotometically through their absorbance at 700 nm. In the reducing power assay, the presence of reductants (antioxidants) in tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) [105] (Figure 5.11).

The results show that with increasing temperature, the extracts have the capability to reduce more of the ferricyanide to the ferrous form. This is consistent with the results obtained during evaluation of flavonols. At higher temperature analytes of interest are released more from the active sites of the matrix as such more antioxidants are extracted. In addition to that, the solubility of these antioxidant is enhanced at higher temperature. The differences for the reducing activity of different fractions of extracts could be attributed to the varied quantity of the phytochemical contents in a particular fraction of the plant. Figure 5.11b shows that even with boiling *Moringa* powder one is able to extract the antioxidants. However boiling is not as efficient as PHWE. When the powder is boiled for fewer minutes less is extracted.This should justify why supplementation of other foods after PHWE is necessary. The figure also shows that at 10 and 15 minutes there is not much difference with what is extracted. It is possible that at these times, extraction had reached maximum.

Figure 5.12 shows that *Ovalifolia sp* has the higher reducing activity as compared to the *Oleifera sp*. This was anticipated because the *Ovalifolia sp* was collected from Namibia where it grows naturally without any cultivation or irrigation done. This meant the physiological stress it was subjected to was quite intense and very

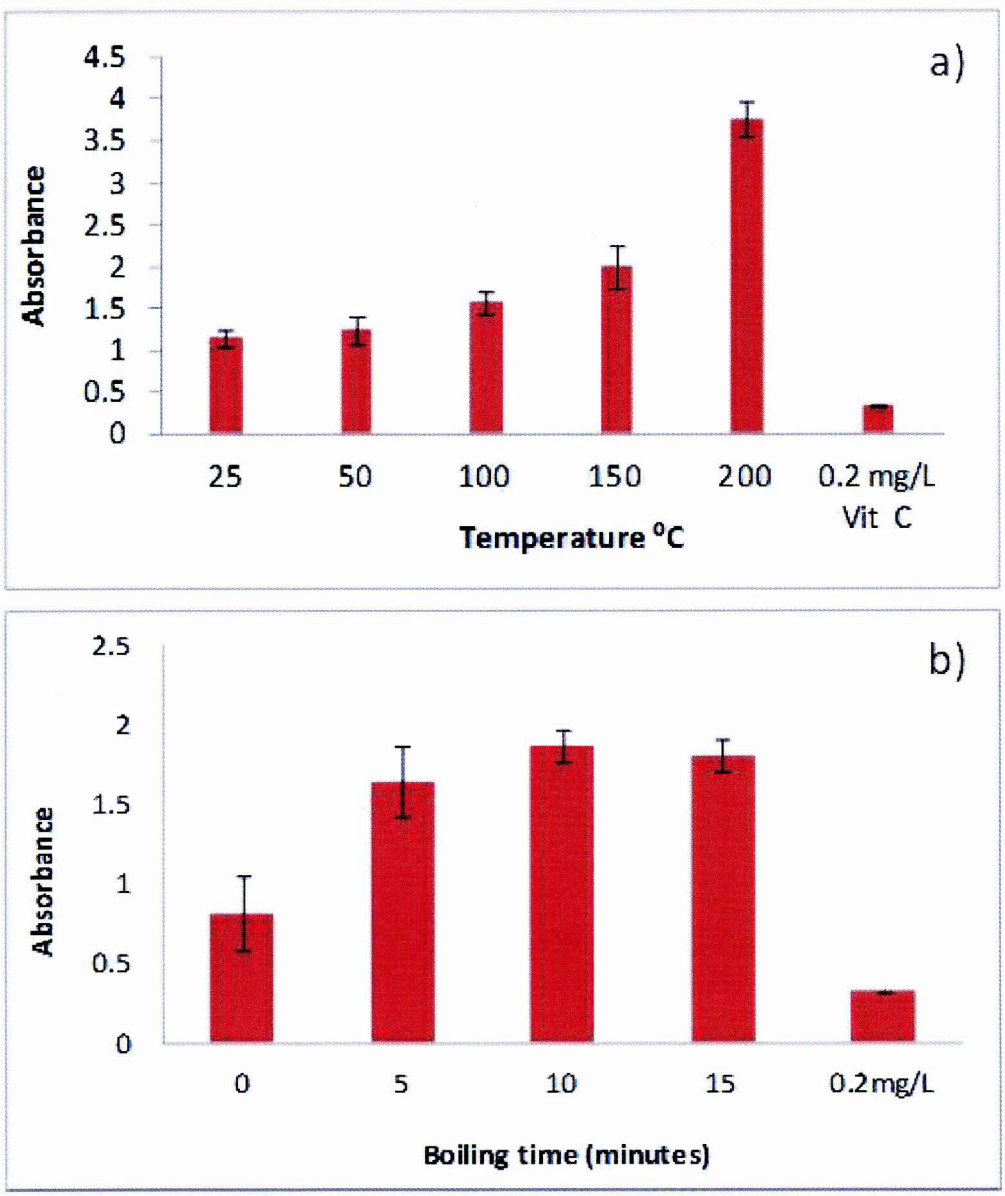

Figure 5.11: Reducing activity of *Moringa Oleifera* a) variation with extraction temperature b) variation with boiling time.

different as compared to that of the *Oleifera sp.* On that note, one would expect more production of the phenolic compounds in the plant to counteract the effects of stress. This plant stress could be due to little or no rain, insects, too much heat and others.

Figure 5.12 also shows that temperature influences the reducing activity of *Moringa Oleifera* but not *Ovalifolia.* As discussed in the preceding paragraph, since *Moringa Ovalifolia* exist naturally in the wild, the phenolic compounds are readily released whenever the plant is subjected to any form of stress. However, *Moringa Oleifera* behaves in a different way most probably because the higher the extraction temperature, which can be taken as a stress parameter triggers more release of the phenolics which alleviate the effects of stress.

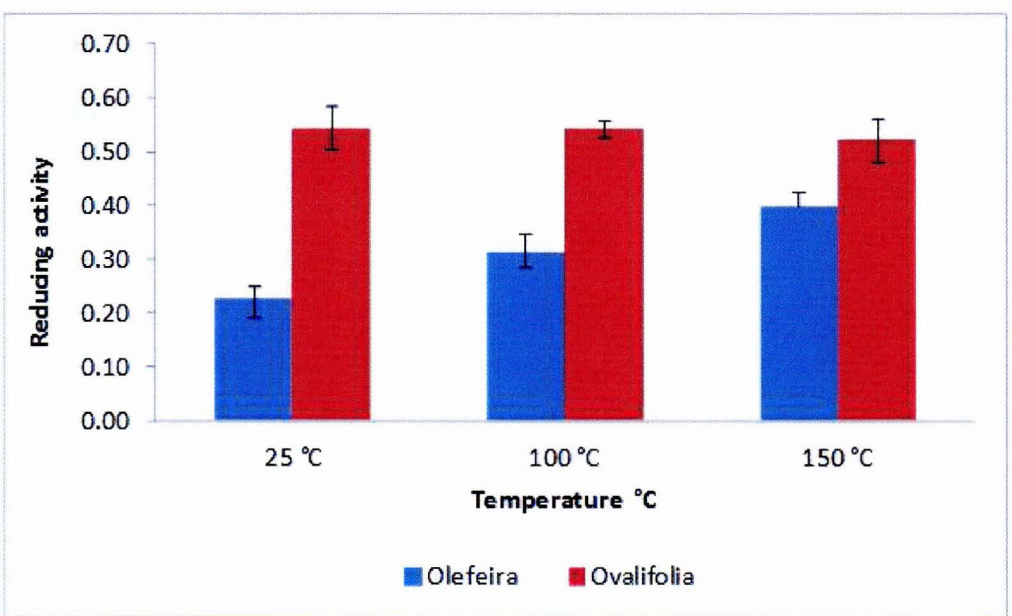

Figure 5.12: Comparison of reducing activity of two *Moringa* species at different temperatures.

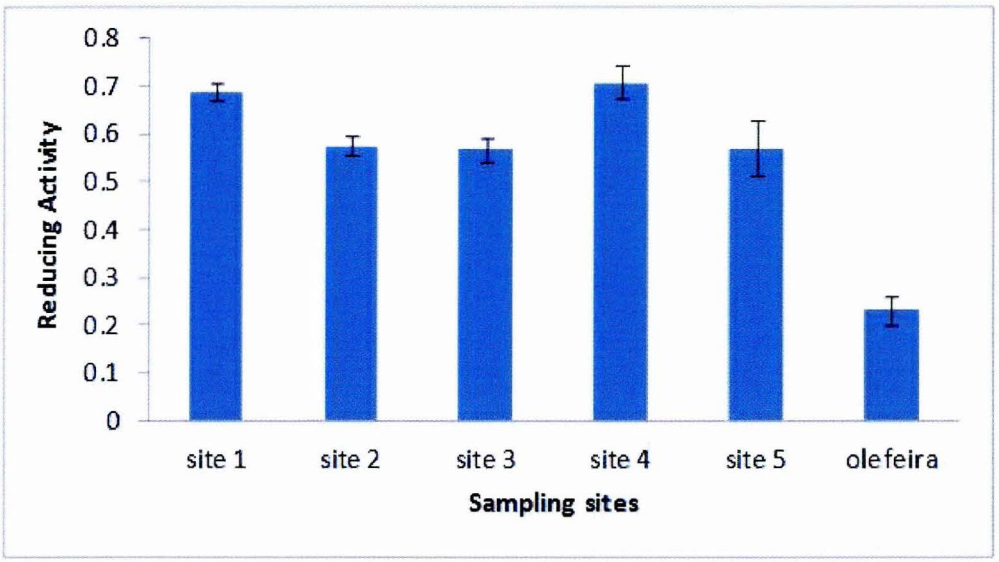

Figure 5.13: Comparison of reducing activity of *Moringa Ovalifolia* from different sites and with *Oleifera* at 25 ^{0}C.

5.9 DPPH Radical scavenging activity of *Moringa*

In this study, the *Moringa Oleifera* leaf extracts were found to have antioxidant properties as upon the addition of the extracts to the DPPH radical, colour changed from blue to yellow. Figure 5.14 shows the variation of the DPPH radical scavenging activity of the fraction collected every 5 minutes.

The whole idea behind this experiment was to evaluate the effective plant extract against a radical. The first 5 mL was not reproducible. This could be an experimental error especially that it was a preliminary analysis as such fewer replicates were done. There was no trend established. The second fraction (10 minutes extract) reduced the concentration of the radical by about 30% while the third fraction (15 minutes extract) reduced the radical by 10%. This means that the most effective extract was the one that was collected after 10 minutes. It was concluded that throughout the experiments 20 mL of the extract would be collected. The subsequent fractions are

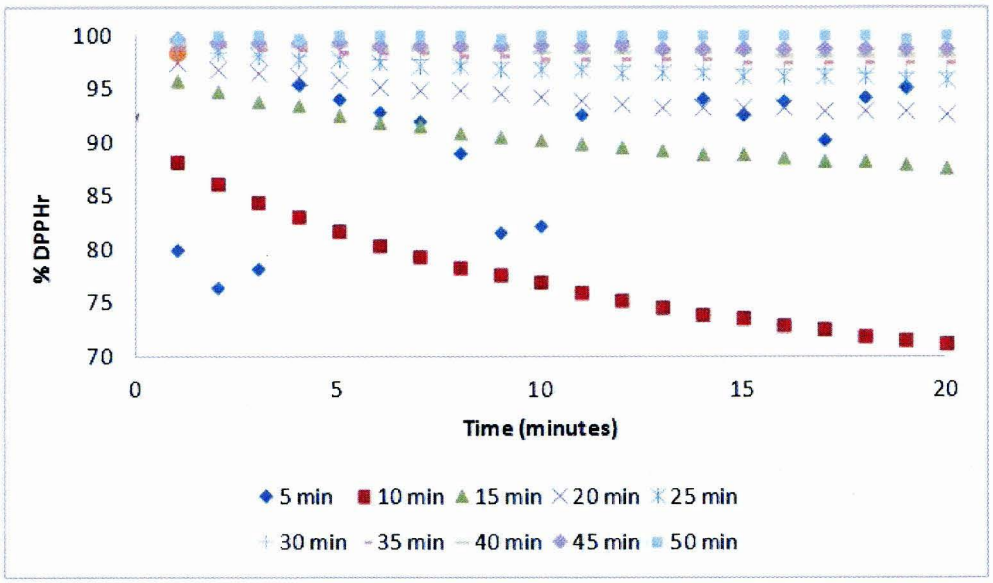

Figure 5.14: DPPH radical scavenging activity of different fractions collected at 5 minutes interval at 25^0C.

clearly ineffective against the radical because the radical concentration is still very high.

Figure 5.15 below shows the behaviour of different extracts collected at differen temperatures in the presence of the radical. The most effective fraction that reduced the radical is the fraction that was collected at 100^0C since it reduced the radical by about 45% (Figure 5.15). The fraction collected at 25^0C was relatively good as it was found to reduce the radical by about 35% while fractions extracted at 150^0C and 200^0C were found to be ineffective against the radical. The extract collected at 50^0C showed a reduction of the radical concentration but the challenge was the fact that the error bars were fairly high. This could be an experimental error. The results are generally in agreement with literature because too high temperature do degrade phenols while at the same time moderately high temperatures facilitate the break opening of the cells, enhanced mass transfers which ultimately results in the

liberation of the phenolics compounds hence 100^0C is optimum. Since the solvent is water which is polar, one would expect the extraction of the phenolic compounds to be relatively lower at lower temperatures and hence less effective against a radical. This is because at lower temperatures, the water has minimal capacity to dissolve substances because it has a relatively high surface tension and hence not as diffusive as when heated. Minimal target molecules will be liberated from the organic matter at lower temperatures.

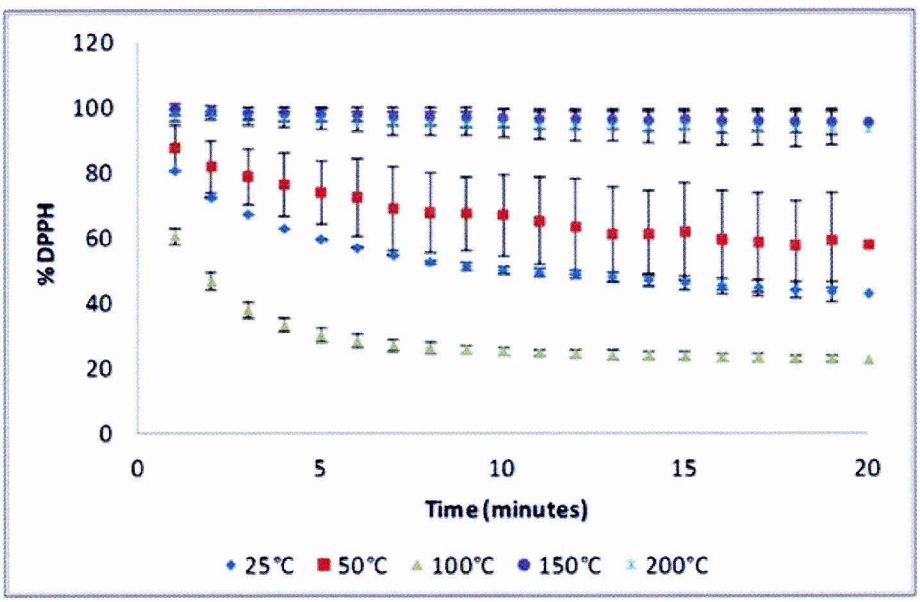

Figure 5.15: DPPH radical scavenging activity of fractions extracted at different temperatures.

Figure 5.16 below shows the efficiency of the *Moringa Ovalifolia* against the DPPH radical. Samples were collected from five different sites in Namibia to evaluate the radical scavenging activity and the geographical effects on the plant. The results show that all the five Namibian sites are not different as the response of the plant extracts against the DPPH radical was almost the same. However, site 4 is slightly different from the rest of other sites. The ability of the plant from site 4 to

reduce DPPH is slightly lower. It is possible that since Site 4 is a mining site, some of the phenolics compounds are not readily available in the presence of radicals as they could be already bound because phenolic compounds with dihydroxy groups can conjugate transition metals [59] hence possible active sites to donate a hydrogen to the radical minimal.

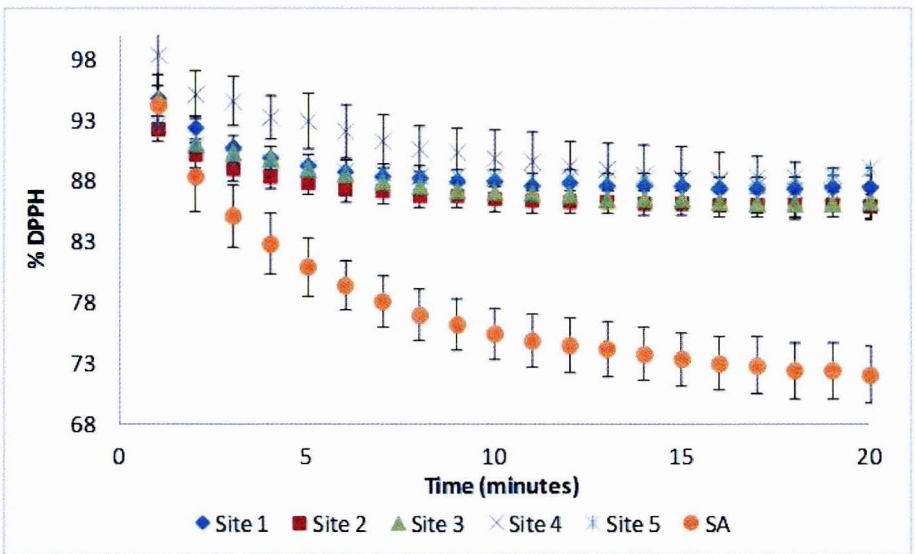

Figure 5.16: Comparison of DPPH radical scavenging activity of *Moringa Ovalifolia* from five sampling sites of Namibia and *Moringa Oleifera* of South Africa.

Figure 5.16 shows a comparison of the effectivenes of the two *Moringa* species against the DPPH radical. *Moringa Oleifera* is significantly effective against the DPPH radical as compared to *Moringa Ovalifolia*. The former reduces the radical by about 30% while the latter reduces it by about 12%.

5.10 Metal content of the aqueous *Moringa sp* leaf extracts

5.10.1 Calibration curves

The calibration curves used for quantification for all metals are shown below. The calibration curve was linear from 0.1 mg L^{-1} to 1.0 mg L^{-1} concentration range. The linearity was good as all the correlation factors were above 0.99. The results were found to be reproducible and the limits of detection are found below in Table 5.9.

Table 5.9: LOD and LOQ for nutrients

Nutrients	LOD $\mu g\ L^{-1}$	LOQ $\mu g\ L^{-1}$
Ca	1.07	10.67
K	0.12	1.20
Mg	0.04	0.4
Na	0.18	1.80
Li	0.06	0.61
Al	0.08	0.8
Ba	0.15	1.49
Co	0.04	0.37
Cu	0.04	0.37
Fe	0.02	0.21
Mn	0.06	0.62
Ni	0.01	0.12
Si	0.01	0.10
Zn	0.07	0.66

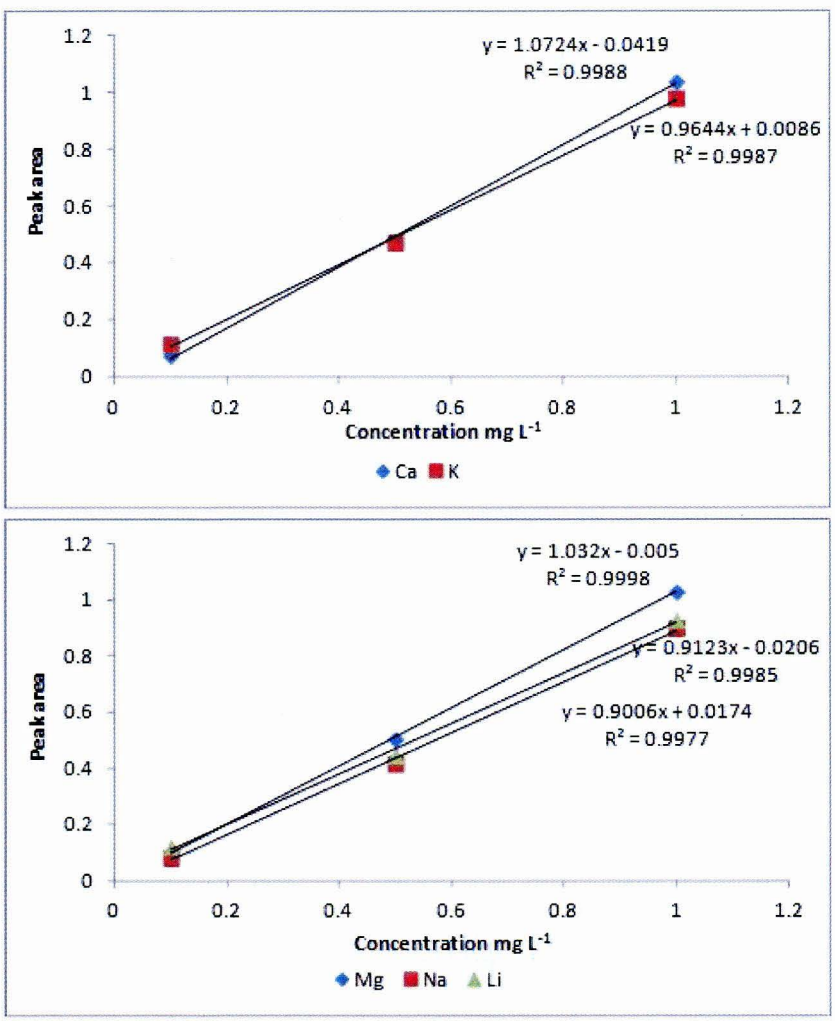

Figure 5.17: Typical calibration curve of Ca, K, Mg, Na, amd Li.

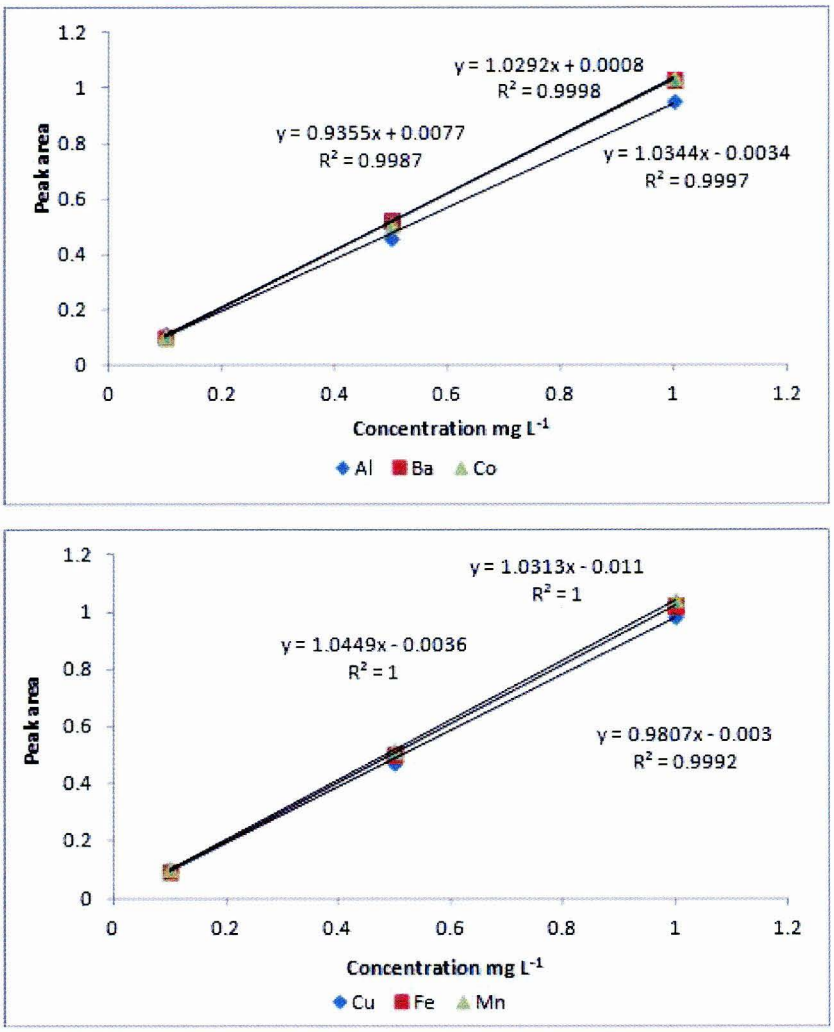

Figure 5.18: Typical calibration curve of Al, Ba, Co, Cu, Fe and Mn.

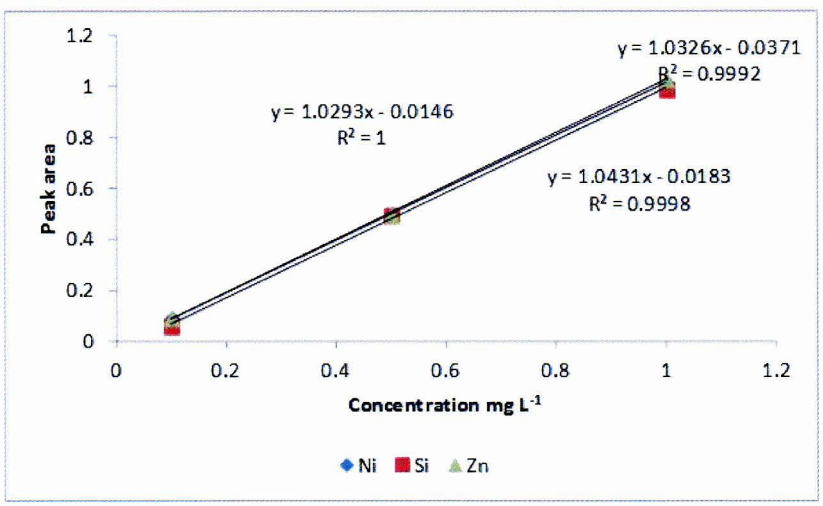

Figure 5.19: Typical calibration of Ni, Si and Zn curve.

5.10.2 Metal content in the PHWE extracts, microwave digested and boiled samples

In this study, the metal content of both *Moringa Ovalifolia* and *Moringa Oleifera* were investigated. Comparison was made between powder obtained from the leaves that was digested in the microwave and powder that was subjected to pressurised hot water extraction (Figures 5.20, 5.21, 5.22, 5.25). Further comparison was made with boiled leaf powder for *Moringa Oleifera*. The two plant species were found to have minerals which are classified as macro and micro elements. Just as the name suggests, the former are minerals that are needed in large quantities while the latter are needed in minute concentration.

When evaluating the extraction of the macro elements of *Moringa Oleifera* using PHWE, the metals were extracted differently. Na as compared to Ca, K and Mg increased with increasing temperature. The same trend was observed in *Moringa Ovalifolia*. Sodium is vital in maintaining turgidity within the plant stem. The higher the concentrations of sodium in the stem, the higher the osmotic pressure, and water flows into the stem to maintain concentration equilibrium. The lowest concentration of about 500 mg kg^{-1} was found at 25^{0}C while the highest concentration of almost 6 000 mg kg^{-1} was detected at 200^{0}C. It is possible that the Na are tightly bound to the active sites and so when the temperature increases they become loosely bound. It has been recognized that the amount of trace elements extracted depends principally on whether the compound is strongly bound to the matrix or more soluble in the solution employed [112]. Since the other metals are just the same across different temperatures, it probably means that the metals of interest are loosely bound to the

active site as such any temperature can successfully facilitate their desorption. Na concentration from the microwave digested extract was approximately 500 mg kg^{-1}. According to the study by Pakade *et al*, (2012) [113] the average concentration of Na in *Moringa Oleifera* from the same farm was previously determined on a PHWE extract and found to be less than 1 000mg kg^{-1}. The variation between this result and the microwave result is understandable because the uptake of the mineral would have increased slightly. However the discrepancy between the microwave, reference result and PHWE is too big. The expectation is that the microwave results are higher. This therefore implies that there could have been contamination.

Potassium in *Moringa Oleifera*, was consistently higher in all the three applied extraction techniques with values ranging between 15 000 to 22 000 mg kg^{-1}. Potassium is one of the most abundant elements in the leafy materials [114] and hence the high values are consistent with literature. The results are in agreement as they are all high implying that potassium is quantitatively extracted by boiling as well as PHWE. Potassium is highly concentrated in the plant leaves and it plays pivotal roles in the plants. It transports water and nutrients throughout the plant in the xylem vessels. When potassium supply is reduced, translocation of nitrates, phosphates, calcium, magnesium and amino acids is depressed [115]. The presence of K in the plants activates various enzymes to catalyse their respective reactions eg. It is vital for the enzymes nitrate reductase, the starch synthetase that catalyzes the formation of proteins and starch respectively. Potassium is also needed to regulate the opening and closing of stomata to allow exchange of carbon dioxide, water vapor, and oxygen

with the atmosphere during transpiration process. Stomata are essential for photosynthesis, water and nutrient transport and plant cooling and thus the general upkeep of the plant. These reasons highlight that potassium is indispensable to plant life and hence high concentrations are detected. *Moringa Ovalifolia* from five sampling sites in Namibia showed concentrations varying from about 4 000 mg kg^{-1} to about 13 000 mg kg^{-1}. The variation is acceptable because geographic patterns of leaf minerals composition may be related to climatic variables, including temperature, precipitation, length of growth season and climatic variability [116].

Calcium is a major element needed in higher concentrations in the plants. Calcium as according to Figure 5.20b and c is the second most abundant in *Moringa Oleifera*. In microwave digested powder from the leaves, calcium was found to be about 8 000 mg kg^{-1} while in PHWE extracts it was found to be about 9 000 mg kg^{-1}. In the study done by Pakade *et al*, (2012) [113] the amount of calcium obtained was 18 500 mg kg^{-1} which is significantly higher. The results of calcium in PHWE leaf powder extracts of *Moringa Ovalifolia* were on average found to be 2 000 mg kg^{-1}. While the MAE results were very high, both results are accepted because the solubility of calcium under different conditions changes. Calcium in boiling is significantly lower as compared to the other two extraction techniques. This means that when boiling, the conditions are not suitable for efficient extraction of calcium as, the movement of Ca^{2+} relies upon the rate of water movement and the Ca^{2+}-binding capacity (absorption - desorption) of the intervening matrix [117]. Calcium is needed to maintain a strong and thick cell wall. It is also critical for producing strong structural rigidity by forming cross-links within the pectin

polysaccharide matrix [118]. Calcium is thought to influence the development of heat shock proteins that enable the plant to tolerate the stress of prolonged heat. With rapid plant growth, the structural integrity of stems that hold flowers and fruit, as well as the quality of the fruit produced, is strongly coupled to calcium availability [119].

Magnesium is a major constituent of the chlorophyll molecule and hence it is actively involved in photosynthesis. Across all the three extraction techniques used, the amount extracted from *Moringa Oleifera* is almost the same at about 4 000 mg kg^{-1}. This shows that PHWE is highly efficient in the extraction of Mg as it compares well with MAE which is considered as a highly efficient extraction technique. The results from boiling extracts showed that when *Moringa Oleifera* is considered for value addition in food, nutrients like Mg can be released when the leaf powder is taken as tea. In PHWE of *Moringa Ovalifolia* from site 4, magnesium concentration does not change with increasing temperature. An average value of about 5 000 mg kg^{-1} was found with MAE while for PHWE the value is around 2 000 mg kg^{-1}. Extraction was done at 25, 100 and 150^{0}C.

The results are slightly lower than 5 500 mg kg^{-1} that Pakade *et al.*, (2012) [113] found in a study of *Moringa Oleifera* in South Africa. The variation could be due to the stage of maturity of the leaves at the time of harvest [120]. *Moringa Ovalifolia* from all the five sampling sites, showed values ranging from 4 000 to 8 400 mg kg^{-1} of Mg. Even though no reports have been documented on the mineral composition of *Moringa Ovalifolia* these results compare well with *Oleifera* species which ranges around 4 000 mg kg^{-1}. The results also showed great variation among themselves.

This plant grows naturally as such many factors such as the soil composition and climatic conditions can influence the mineral compositions.

Generally the amount of metals extracted using PHWE were significantly lower than the amount recovered using the microwave assisted extraction. This is consistent with literature because MAE is much more efficient. In MAE, heating occurs in a targeted and selective manner with practically no heat being lost to the environment as the heating occurs in a closed system [92]. One other reason microwave extraction is efficient is because, there is a synergistic combination of the heat and mass transfers working in the same direction as seen in Figure 2.14 as opposed to conventional extractions where the mass transfer occurs from inside to the outside yet the heat transfer occurs from the outside to the inside of the substrate. The great advantage of microwave heating is that all of the sample fluid is heated, allowing the extraction solution (solvent and sample) to reach the desired temperature more rapidly and avoiding a thermal gradient caused by conventional heating [63]. One more reason of good efficiency of the microwave could be attributed to the fact that, the use of closed systems reduces the risk of losses and the fact that microwave irradiation reduces overheating problems which could also minimise the degradation of analytes [63].

Micro elements concentration were also evaluated as shown in Figure 5.22. Trace elements have both a curative and a preventive role in combatting diseases [80, 8, 32]. It is very important to know the level of macro- and micro-elements in medicinal plants and herbal medicaments and to estimate their role as sources of these components in the human diet because, at elevated levels, these metals can

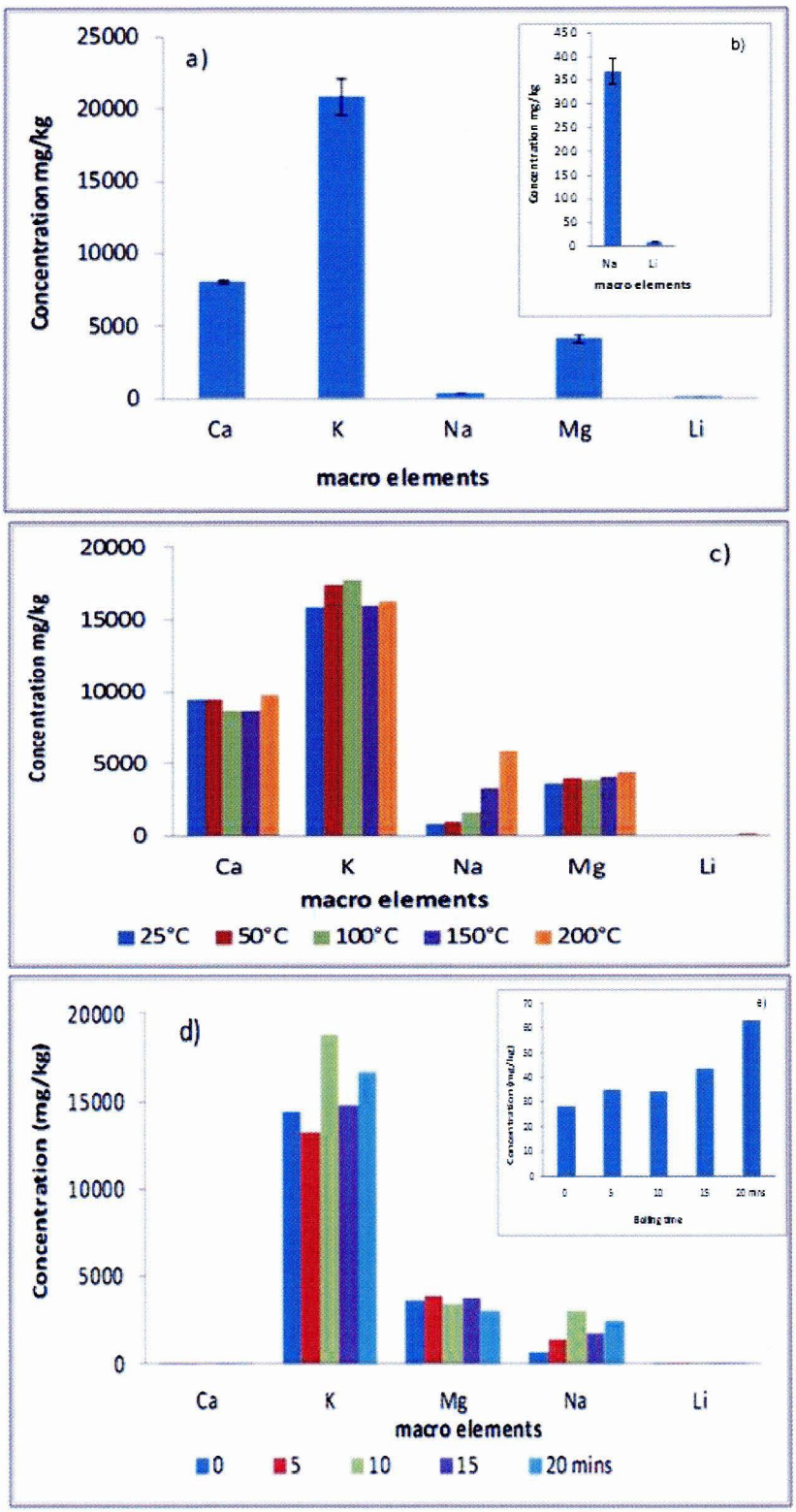

Figure 5.20: Variation of macro elements, a) when the *Moringa Oleifera* leaf powder was digested, b) an insert of Na and Li, c) when the extraction was done at various temperatures, d) when the powder was boiled, e) an insert of Ca.

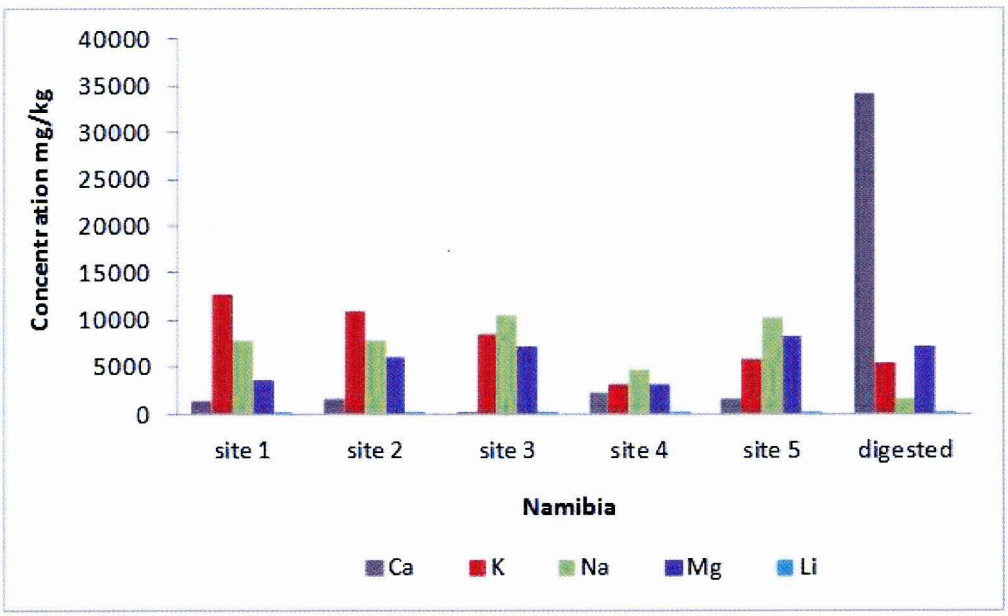

Figure 5.21: Comparison of macro elements of PHWE extracts (25^0C) and MAE digested (site 4) of *Moringa Ovalifolia*.

also be dangerous and toxic [8]. Comparison of the micro nutrients in MAE and PHWE shows the highest concentration to be of iron in *Moringa Oleifera*. A value of about 290 mg kg^{-1} was found with MAE while the highest in PHWE is around 90 mg kg^{-1}. In boiling, only about 4 mg kg^{-1} was recovered. This explains why the plant is given to nursing mothers in Phillipines [20] so that the lron lost during delivery can be restored. In the same farm a study done previously by Pakade *et al* was found to have 390 mg kg^{-1} in the year 2006, 610 mg kg^{-1} in 2010 and 440 mg kg^{-1} of iron. While the previous study shows variation of the iron concentration in the same farm within the years, it showed that the concentration of minerals may vary depending on various factors. This therefore means variations of results in any given study at any given time may show different trends to the previous studies and hence the criteria of acceptance of results should not be influenced by previous results. According to the Tolerable Upper Limit (TUP) as seen below in Table 5.10,

the plant extracts can be used for value addition into various food products. The fairly high content of the various metals in the extracts compares well with Tolerable Upper Limit in Table 5.10. A TUP of Fe is 45 mg day^{-1}. Generally all the limits do not exceed what is found in the plant species.

Table 5.10: Tolerable upper limit intake of minerals [121]

Minerals	Tolerable Upper Limit mg day^{-1} for adults
Mn	11
Zn	40
Fe	45
Se	10
Ca	2500
Mg	350

The second highest concentration in MAE was silicon at about 225 mg kg^{-1} followed by manganese at about 145 mg kg^{-1}, aluminiun at about 130 mg kg^{-1} and then barium, cobalt, copper, nickel and zinc all under 50 mg kg^{-1}. The efficiency of the PHWE is depicted by Figure 5.22b as it compares well with the trend in Figure 5.22a. In PHWE Fe was extracted in highest amount followed by by Mn and Si. The PHWE results also show that almost all micro elements increase with increasing temperature. Barium however is constant with increasing temperature but copper decreases with increasing temperature.

The results obtained by extraction by boiling showed that Mn is the highest concentration with values closer to that of the MAE. Manganese is naturally occurring in many surfaces, ground water sources and in soils and may increase levels of Mn in drinking water. The water used for boiling was tap water as such this could have

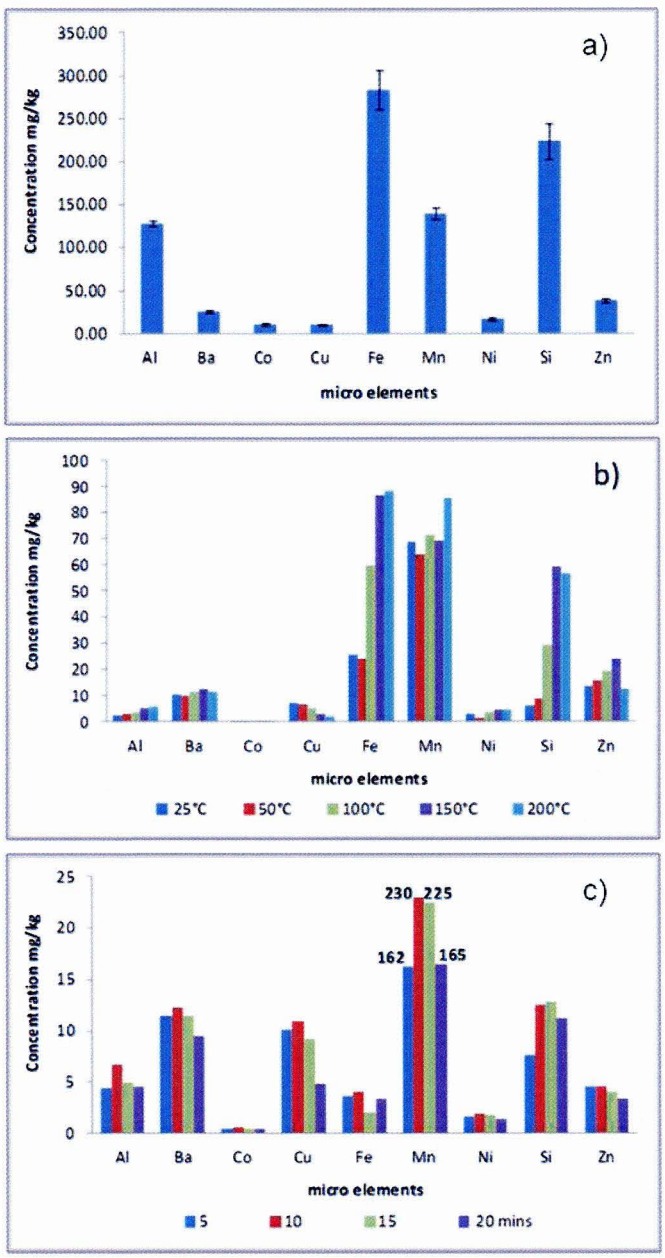

Figure 5.22: Variation of micro elements a) when the *Moringa Oleifera* leaf powder was digested b) when the extraction was done at various temperatures c) when the powder was boiled.

contributed to the positive error. The concentrations were found to decrease in this order, Mn > Si > Ba > Cu > Al > Zn > Fe > Ni > Co. There is no permissible limit prescribed in local food law or by World Health Organization (WHO), however, range of 4-15 ppm for Cu and 15-200 ppm for Zn is considered to be safe [11]. Even though some of the essential elements do not have the recorded permissible levels, the values in this plant are comparable to other medicinal plants.

When comparing the micro elements of all the sites from where *Ovalifolia sp* was obtained as seen in Figure 5.25, the amount of arsenic is very high for site four. This is where mining occurs. It is possible that the soils of which the plant is growing in are contaminated as such the minerals are uptaken by the plant. Most plants that survive in toxic soils do so by either, avoiding heavy metals, or, hyperaccumulating them in their tissues [122]. Metal hyperaccumulating species have been identified in at least 45 plant families and individual species (or even populations) can accumulate different metals such Zn, Cd, Cu, Co, Ni, Se and As or particular combinations of these [122]. Metal hyperaccumulation is a phenomenon generally associated with species endemic to metalliferous soils and it is found in only a very small proportion of such metallophytes [122]. The relationship between the concentration of heavy metals in soil may lead to increased uptake by plants [123].

The uptake of chemical species by plants in soils is dependent on various plant factors. These include; physical processes such as root intrusion, water, and ion fluxes and their relationship to the kinetics of metal solubilization in soils; biological parameters, including kinetics of membrane transport, ion interactions, and metabolic fate of absorbed ions; and the ability of plants to adapt metabolically to

changing metal stresses in the environment [124]. In other words, uptake of minerals by roots depend on the movement of the mineral elements to the roots surface due to the concentration gradient. In addition to that, root interception where soil volume is displaced by root volume due to growth and [125]. Trace element uptake by roots depends on both soil and plant factors (e.g. source and chemical form of elements in soil, pH, organic matter, plant species, plant age, etc.) [9]. Arsenic compared to other chemical species may be highly soluble in the soils and highly mobile in the plant reaching up to the leaves. Having said that, it is worth noting that Arsenic is a toxic metal especially in high concentrations. Fe concentration is generally high with values ranging between 100 and 200 mg kg^{-1} for all the sampling sites in Namibia. Figure 5.24 shows that the concentration of arsenic and other micro nutrients is constant with temperature while for iron and silicon increase with increasing temperature temperature.

In general almost all the metals in the plant leaf powder were quantitatively extracted by the PHWE system, microwave digestion and boiling. This is very important for value additions of *Moringa* extracts into various food products.

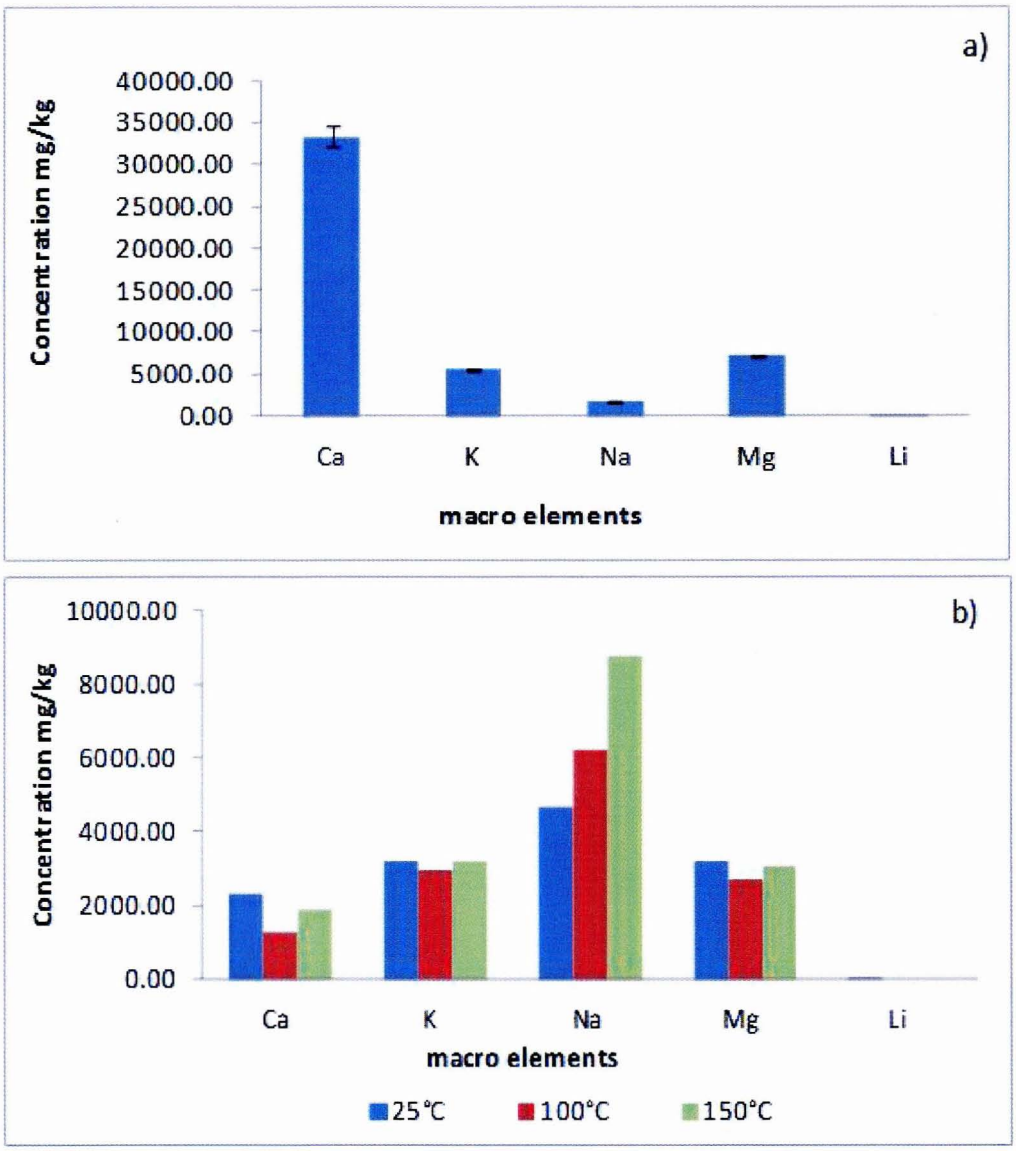

Figure 5.23: Variation of macro elements a) when the *Moringa Ovalifolia* (site 4) leaf powder was digested b) when the extraction was done at various temperatures.

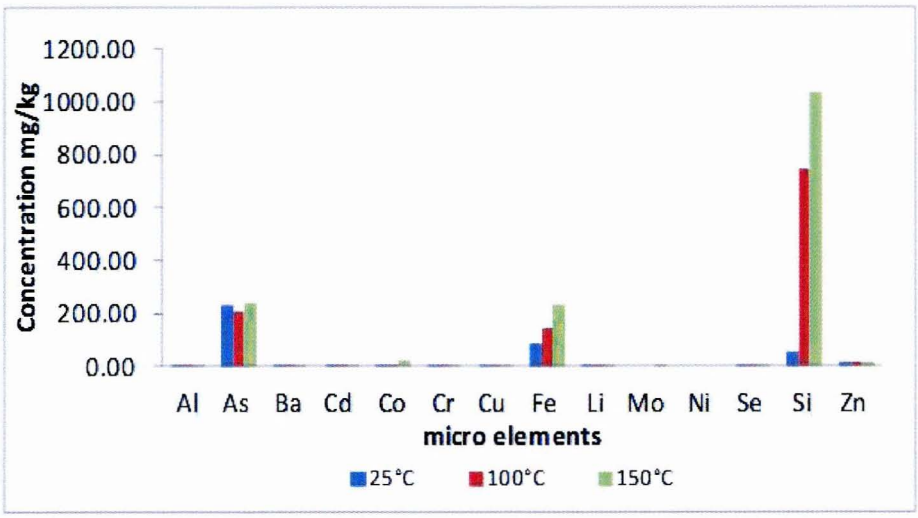

Figure 5.24: Variation of micro elements of *Moringa Ovalifolia* leaf powder at different extraction temperatures from site 4.

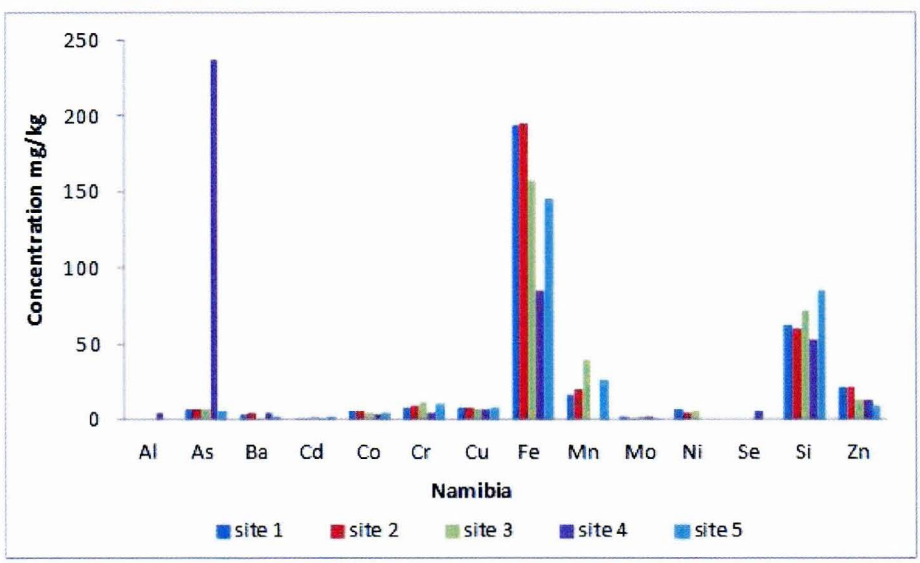

Figure 5.25: Variation of micro elements with different sampling sites when *Moringa Ovalifolia* leaf powder when the extraction was done at 25^0C.

Chapter 6

Conclusions and Recommendations

Summary

This chapter gives a summary of the findings of the study. It compares trends of *Moringa Oleifera* and *Moringa Ovalifolia* obtained with antioxidant properties based on the radical scavenging activity, flavonol content and reducing ability indicators. It further discusses the efficiency of PHWE of essential compounds from two *Moringa* plant species. It summarises the optimum conditions for the extraction of the investigated essential compounds and further highlights the most abundant minerals in the plant species. It also gives a brief summary of the order of reaction kinetics.

6.1 Conclusions

In this study a novel technique of extracting essential compounds from *Moringa sp* using pressurised hot water was successfully developed. Properties of water were manipulated to make it suitable for dissolving less polar substances. Water at ambient temperature and atmospheric pressure is polar and thus dissolves only polar substances. However, upon heating water at high pressures of ranging between 1 000 and over 2 000 psi resulted in the weakened hydrogen forces of attraction making water less polar. It became less viscous with decreased surface tension and ultimately more diffusive. It thus resulted in the enhanced capacity of water to dissolve less polar substances.

The antioxidant properties of *Moringa Oleifera* and *Moringa Ovalifolia* leaf extracts were evaluated using two indicators. The ability of the plant leaf extract to reduce potassium ferricyanide and to donate hydrogen and scavenge for the DPPH radical. The two *Moringa sp* were found to have antioxidant properties. For the *Moringa Oleifera*, the optimal extraction temperature for DPPH analysis was found to be 100^0C. The fraction collected at 100^0C had the ability to reduce the radical by 45%. Higher temperatures degrade the phenolic compounds that have the ability to reduce the radicals while lower temperatures do no sufficiently facilitate the desorption of those phenolic compounds. Even though *Moringa Ovalifolia* has radical scavenging activity, it is not as effective as the *Moringa Oleifera*. At the same extraction temperature of 25^0C, *Moringa Oleifera* had the ability to reduce the radical by 30% while *Moringa Ovalifolia* could only reduce by 12%.

The reducing activity results show that the two *Moringa* species have the ability

to reduce ferricyanide to the ferrous form. The results of *Moringa Oleifera* show that the efficiency of the ability to reduce the ferricyanide is dependant on the temperature of extraction. At high extraction temperatures the PHWE leaf extracts are more effective. However, *Moringa Ovalifolia*'s ability to reduce ferricyanide is not influenced by extraction temperature. The results also show that boiling the *Moringa Oleifera* leaf powder liberates the compounds that reduce ferricyanide. Unlike the DPPH study which shows that *Moringa Oleifera* is better, *Moringa Ovalifolia* is far much better in the reducing activity.

Three flavonols namely kaempferol, myricetin and quercetin were extracted and quantified using the HPLC. Myricetin being more polar was eluted first followed by quercetin and lastly kaempferol. Kaempferol was found to be more abundant than the other flavonols in *Moringa Oleifera*. In *Moringa Ovalifolia*, quercetin is much more abundant. Of the five sampling sites in Namibia it was found to be dominant in three sites. An extraction temperature of 100^0C was found to be optimal for the release of quercetin and kaempferol. Myricetin does not survive harsh conditions of acid hydrolysis as such no peaks were found after hydrolysis. However, myricetin was detected after hydrolysis for *Moringa Ovalifolia*.

The kinetics order of extraction was found to be first order for all flavonols at 150^0C. Quercetin was found to be both first and second order of extraction at 150^0C while kaempferol followed both first and second order at 100^0C. At 100^0C, quercetin followed second order while myricetin followed first order. At 25^0C, quercetin and kaempferol were found to be second order while myricetin was found to be first order.

Essential minerals were also quantitatively extracted in the *Moringa Oleifera and Moringa Ovalifolia* extracts from leaves. Macro nutrients extracted were Ca, K, Na and Li while the micro nutrients detected were Al, Ba, Ca, Cu, Fe, Mn, Ni, Si and Zn. The PHWE system was found to be efficient as the values for the metals extracted were reasonably high as those of the MAE. The study revealed that boiling the powder from the leaves does extract some minerals too. Potassium followed by calcium are the most abundant macro elements in *Moringa Oleifera*. In *Moringa Ovalifolia*, potassium is the most abundant at sites 1 and 2 while 3, 4 and 5 sites are higher in calcium. Iron is the most abundant micro element in both *Moringa* species. Arsenic was found to be in high concentrations in site 4.

Plants grown in contaminated areas have a high risk of having heavy metal concentrations beyond the permissible limit for each of them as compared to the less contaminated areas. *Moringa Ovalifolia* growing naturally in site 4 around the mining site was found to accumulate arsenic. Arsenic is a toxic metal particularly in high concentrations. Symptoms of long-term exposure of high levels of arsenic (e.g. through drinking-water and food) are observed in the skin, and include pigmentation changes, skin lesions which may later develop into skin cancer.

The physical properties of the two species are different. Generally the *Ovalifolia sp* is fairly big, the leaves are bigger and broader and so is the stem. When the powder from the leaves is compressed under pressure and temperature it becomes a sticky paste while *Oleifera sp* is non sticky. The *Ovalifolia sp* grows even in the rocky places and mountains while its counterpart thrives in sandy loamy soils. It grows to become a thick tall bush while *Oleifera* is tall and tiny.

The method of Folin-Ciocalteu procedure used for the total phenolic contents is good as it indicates the presence of phenolic constituents in the plant extracts. However it has its disadvantages as it does not give a full picture of the quantity or quality of the phenolic constituents. It is difficult to find a specific and suitable standard for quantification of phenolics due to the complexity of plant phenolics as well as existing differences in the reactivity of phenols towards reagents used for their quantification [55]. There is also a likelihood of interference by proteins, nucleic acids and amino acids which are UV-absorbing substances.

6.2 Recommendations

- This study was targeted for the three flavonols (myricetin, quercetin and kaempferol). Further studies on other classes of flavonoids such as flavanols, flavones, isoflavones and flavonones should be done to have detailed understanding of polyphenols present in the *Moringa* species.

- The PHWE technique has proved to be an efficient and effective extraction technique. Because it is also environmentally friendly, it should be used for the extraction of analytes from plant material as compared to conventional methods.

- In this study, temperature was varied while pressure and sample size were maintained to evaluate the efficiency of the temperature during extraction. A similar study can be done varying pressure and sample size to evaluate the best kinetics of extraction.

- Development of phytoremediation technologies of contaminated soils could be considered particularly for where medicinal plants like *Moringa Ovalifolia* grow.

Emanating from this research are the following;

- A patent of file number; PCT/IB2013/058765 of the title "Extract from *Moringaceae* and a method to prepare the extract" has been filed.
- A manuscript entitled "Development of Pressurised Hot Water Extraction (PHWE) for essential compounds from *Moringa Oleifera* leaves" is submitted to Food Chemistry.

List of conference and seminar presentations

1. Matshediso PG., Cukrowska E., Chimuka L. Pressurised Hot Water Extraction of Essential Chemicals from *Moringa Oleifera* Leaves for Value Additions in Food Products, 4th Cross - Faculty Post Graduate Symposium, 19th and 22nd October 2012, University of the Witwatersrand, Johannesburg, South Africa, poster presentation.

2. Matshediso PG., Cukrowska E., Chimuka L. Pressurised Hot Water Extraction of Essential Chemicals from *Moringa Oleifera* Leaves for Value Additions in Food Products, ChromSAAMS conference 7th - 10th October 2012, Dikhololo, North West Province, South Africa, oral presentation.

3. Matshediso PG., Cukrowska E., Chimuka L. Pressurised Hot Water Extraction of Essential Chemicals from *Moringa Oleifera* Leaves for Value Additions in Food Products, 12th International Chemistry Conference for Africa (ICCA) 2013, 7th - 12th July, University of Pretoria, South Africa, oral presentation.

4. Matshediso PG., Cukrowska E., Chimuka L. V Parkade. *Moringa* leaves as a source of antioxidants and a potential source to combat malnutrition. 5th Cross -

Faculty Post Graduate Symposium, 1st - 2nd August 2013, University of the Witwatersrand, Johannesburg, South Africa, poster presentation.

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