Phytochemical and Elemental Analysis of Nettles found in KwaZulu-Natal, South Africa

By

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2016

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A thesis submitted to the School of Chemistry and Physics, College of Agriculture,

Engineering and Science, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy.

This thesis has been prepared according to **Format 4** as outlined in the guidelines from the

College of Agriculture, Engineering and Science which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an overall introduction and final discussion where one (or all) of the chapters have already been published. Typically, these chapters will have been published in internationally recognized, peer- reviewed journals.

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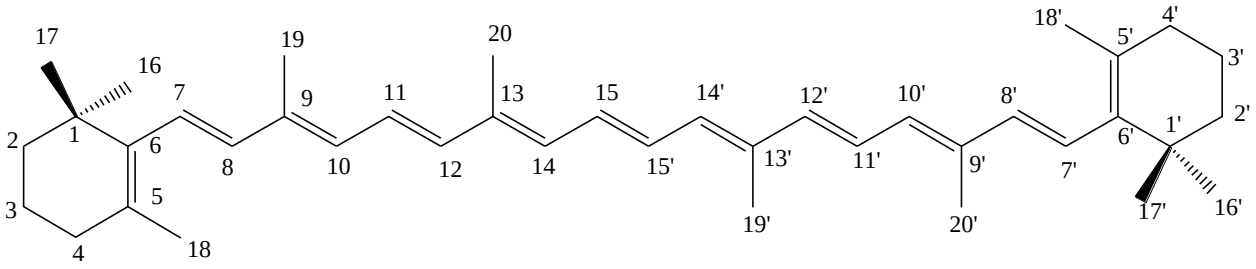
ABSTRACT

There is a rich diversity of indigenous edible plants in South Africa. Rural communities have for years, been collecting indigenous edible medicinal plants for food and for their medicinal properties. However, a combination of a shortage of food and lack of diversity in the diet has resulted in many South Africans suffering from malnutrition. Malnutrition, food insecurity and nutrient deficiencies which help propogate non-communicable diseases are amongst the top concerns in South Africa. Knowledge on the elemental composition, nutritional and medicinal value of medicinal plants would allow for safe consumption of these plants and improve overall health. The aim of this study was to investigate the secondary metabolites in the *Laportea* and *Obetia* nettles found in KwaZulu-Natal (South Africa) and to conduct an elemental investigation into the nutritional composition of these nettles to determine their suitability for consumption and their contribution to recommended dietary allowances. The study showed that cooked and raw leaves of nettles (*L. peduncularis*, *L. alatipes*, and *O. tenax*) were rich sources of macronutrients and essential elements which are comparable to common vegetables. The nettles, *L. alatipes* and *O. tenax*, have higher macronutrient content than elemental content relative to the nettles, *L. peduncularis* and *U. dioica*, after cooking. Soil quality indicators (geo-accumulation indices and enrichment factors) showed moderate to no contamination of nettle growth soils around KwaZulu-Natal. Statistical analysis showed the association of these metals in the different sites. Phytochemical analysis of the nettles showed that the nettles were rich in β-carotene and sterols owing to their use as natural anti-diabetic agents. This study provides information on the nutritional value of nettles and shows that they can serve as an affordable alternative to commercially available herbs and it also lends scientific credence to the ethno-medicinal use of nettles.

SUMMARY OF ISOLATED COMPOUNDS



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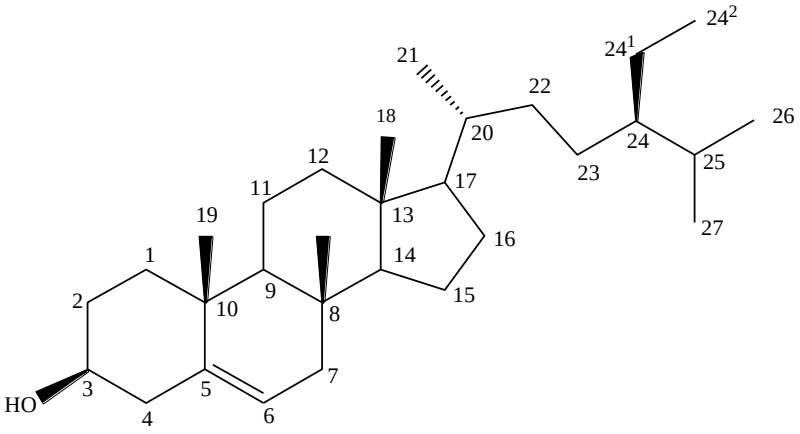

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ABBREVIATIONS

^{13}C NMR - C-13 nuclear magnetic resonance spectroscopy

^{1}H NMR - proton nuclear magnetic resonance spectroscopy

ANOVA - analysis of variance

BAF- bioaccumulation factor

CA- cluster analysis

CEC- cation exchange capacity

CRM- certified reference material

d - doublet

dd - double doublet

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DRI - dietary reference intake

EDTA- ethylenediaminetetraacetic acid

Ex – exchangeable

EF- enrichment factor

FRAP – ferric reducing antioxidant potential

GC-MS - gas chromatography-mass spectrometry

Hz - Hertz

ICP-OES- inductively coupled plasma-optical emission spectrometry

Igeo- geo-accumulation factors

IR- infrared

NIPI- Nemerow integrated pollution index

r- correlation coefficient

PC- principal component

PI- pollution index

RDA- recommended dietary allowance

RI- response index

SD- standard deviation

SOM- soil organic matter

TF- translocation factor

TLC- thin-layer chromatography

UL- tolerable upper intake level

UV- ultraviolet

DECLARATIONS

Declaration 1: Plagiarism

I, Mahlangeni Nomfundo Thobeka declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections

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Declaration 2: Publications & Conferences

Publication 1

***Title*:** The distribution of macronutrients, anti-nutrients and essential elements in nettles, *Laportea peduncularis* susp. *peduncularis* (river nettle) and *Urtica dioica* (stinging nettle)
***Authors*:** *Nomfundo T. Mahlangeni*, Roshila Moodley, Sreekantha B. Jonnalagadda

***Journal*:** Journal of Environmental Science and Health, Part B, **2016**, 51(3): 160-169.

Publication 2

Title: Heavy metal distribution in *Laportea peduncularis* and growth soil from the eastern parts of KwaZulu-Natal, South Africa

Authors: *Nomfundo T. Mahlangeni*, Roshila Moodley, Sreekantha B. Jonnalagadda

Journal: Environmental Monitoring and Assessment, **2016**, 188(2): 1-14.

Publication 3

Title: Elemental analysis of edible mountain nettle (*Obetia tenax*) and the influence of soil on its chemical composition

Authors: *Nomfundo Thobeka Mahlangeni*, Roshila Moodley, Sreekantha Babu Jonnalagadda

Journal: Manuscript accepted for publication: Analytical Letters

Conference

Title: 29th EFFoST International Conference, Food Science Research and Innovation: Delivering sustainable solutions to the global economy and society, Athens, Greece, 10-12 November, 2015

Poster*:* The distribution of macronutrients, anti-nutrients and essential elements in nettles, *Laportea peduncularis* susp. *peduncularis* (River nettle) and *Urtica dioica* (Stinging nettle). (*Nomfundo T. Mahlangeni*, Roshila Moodley, Sreekantha B. Jonnalagadda)

In all of the publications I have performed all the experimental work and written the manuscripts. The co-authors were involved in discussion of the results and were responsible for verifying the scientific content and accuracy of the results as well as editing the manuscripts. I have been the corresponding author on two of the manuscripts contained in this thesis.

Signed: **……**

ACKNOWLEDGEMENTS

Firstly, would like to thank God for giving me the strength, patience and confidence to embark on such a journey. I would also like to convey my sincere gratitude to my supervisor, Prof. S.B. Jonnalagadda, for the opportunity to study under him and for the knowledge and wisdom, support and constant encouragement. And also my co-supervisor, Dr. R. Moodley, for her being a teacher, nurturer and a great mentor from my early stages of being a researcher. It has been a long journey on which she gladly walked with me.

I would also like to acknowledge the following individuals who encouraged me, gave me words of wisdom and helped me through my research; my best friend Bongisiwe, Kimona, Olumuyiwa, Olusola, Samuel, Victoria, Judie, Esther, Gracious; my brothers and sisters Nerena, Letticia, Lydia, Mr and Mrs Lodder. I would like to thank my colleagues in the natural products research group for moral support, Lamla, Saba and the rest of the team. I would like to acknowledge, the technical team in the School of Chemistry & Physics for their assistance: Vashti Reddy, Anita Naidoo, Greg Moodley, Dilip Jagjivan, Unathi Bongoza and Edward Khathi (School of Life Sciences). I would also like to acknowledge the National Research Foundation (NRF) for financial support.

Lastly, I would like to thank my family; my mother, Mrs T.F. Mahlangeni, for her love, support, wisdom and encouragement throughout the research, my sister Phakamile, and my brother Andile for their support and patience and my nieces and nephews (Senamile, Aphiwe, Asanda, Sandiselwe, Nosipho, Minenhle and Lwanele) for their love and support.

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CHAPTER ONE

INTRODUCTION

The application of indigenous medicinal plants for medical purposes has been in practice since the early ages with many discoveries on the medicinal properties of these plants being purely accidental (Petrovska, 2012). In developing countries, there is currently a lack of resources for the treatment and management of infectious and chronic diseases. Knowledge on the traditional system of medicine and its exploration and exploitation may lead to the discovery and management or treatment of such diseases. This may also lead to the identification of new bioactive molecules (Rai & Kon, 2013). In South Africa alone, an estimated 80% of the population prefers traditional medicine to modern mainstream medicine (Mbatha et al., 2012). Research has shown that the focus is now moving towards ethno-medicine, derived from natural growing herbs due to accessibility, affordability and potential.

Studies have shown that 140 million children under the age of five are underweight and this will persist if malnutrition is not addressed, especially in developing countries in sub Saharan Africa, which are leading countries that suffer with this affliction (Smith & Haddad, 2000; Bouner et al., 2007). Malnutrition is seen to hinder the intellectual progression, growth and development of many children. In South Africa, studies have indicated that malnutrition does not only cause nutritional problems amongst children and infants but also leads to serious infections and diseases. These include diarrhoea, pneumonia, malaria as well as HIV and AIDS (Ismail & Suffla, 2013). Access to foods that contain sufficient nutrients for a balanced diet is becoming increasingly more difficult especially for people living in rural areas due to a lack of affordability and availability so the

potential of indigenous, locally grown foods needs to be assessed and promoted as a possible solution to help alleviate this problem.

The collection of herbs and leafy green vegetables from the wild has been practiced in South Africa for more than 2 000 years (van Rensburg et al., 2007). These leafy vegetables are known for their high nutritional and medicinal value. Though this is so, there is not enough information on the potential toxicities of these plants. Hence the matter on safety of wild food plants for human consumption needs to be addressed.

The simultaneous study of plant-soil and plant-human relationships is imperative in order to monitor for heavy metal contamination and accumulation. The most common cause of heavy metal contamination in plants and soil is human and industrial activities. Soil serves as a reservoir for these heavy metals. Plants absorb these heavy metals from contaminated soil and transfer them through the food chain to humans and, if at elevated levels, these heavy metals may cause possible health risks including cancer (Street et al., 2008; Liu et al., 2013). Examining the anti-nutrient content of edible plants is also important in order to assess for possible toxic effects to humans. Similar to heavy metals, anti-nutrients can be detrimental to human health if at elevated levels in edible plants. Since herbs and leafy green vegetables are used medicinally and as a food source, constant monitoring of these plants for contaminants is crucial.

AIMS AND OBJECTIVES OF THE STUDY

The aim of the study was to analytically and phytochemically investigate the nettles (*Laportea peduncularis* susp. *peduncularis* (river nettle), *Laportea alatipes* (forest nettle), *Obetia tenax* (mountain nettle) and *Urtica dioica* (stinging nettle)) found in KwaZulu-Natal, South Africa to validate their ethno-medicinal use, to determine their nutritional value and to evaluate the impact of soil quality parameters on elemental uptake by nettles.

The research objectives were:

- To determine the distribution of macronutrients, anti-nutrients and essential elements in nettles, *Laportea peduncularis* and *Urtica dioica*.
- To determine the distribution of macronutrients, anti-nutrients and essential elements in nettles, *Obetia tenax* and *Laportea alatipes*.
- To determine the proximate chemical composition namely carbohydrate, protein, ash and oil content of the leaves of nettles.
- To determine the heavy metal distribution in *Laportea peduncularis* and *Obetia tenax* and corresponding growth soil, sampled from eight to ten different sites in KwaZulu-Natal and to assess for potential toxicities.
- To determine the uptake, translocation and bioaccumulation of elements in *Laportea alatipes*.
- To determine the nutritional value of nettles (*Laportea peduncularis*, *Laportea alatipes*, *Obetia tenax* and *Urtica dioica*) by comparing to dietary reference intakes.
- To extract, isolate and identify the secondary metabolites in nettles and to test the crude extracts and isolated compounds for biological activity (antioxidant and anti-diabetic).

LITERATURE REVIEW

Soil-Metal-Plant Relationships

Soil is a heterogeneous material consisting of a mixture of fragments of rocks and organic matter. There are three phases of soil namely solid, liquid and gas. All of these phases are responsible for the supply of nutrients to the plant (Mengel et al., 2001). The formation of soil is attributed to soil forming factors which include parent material, climate, topography, living organisms and time. The nutrient distribution in the soil is mainly due to the parent material. The nature of the parent material also influences the amount of nutrient present in the soil (Whitehead, 2000). Regional climate influences the rate at which processes such as chemical weathering occurs, where the mineral composition of the bedrock is altered. Topography which incorporates the relief and aspect (geographic coordinates) also influences the type of soil formed. The amount of time for soil development is dependent on the action of the other soil forming factors (Jenny, 1994).

The essential nutrients of the plant exists in mineral form that is soluble in water, as ions adsorbed on exchange sites of soil colloids and as constituents in soil organic matter. The availability and mobility of nutrients for uptake by plants is influenced by soil factors such as pH, cation exchange and organic matter (Jones, 2012). Additionally, soil pH affects microbiological activities that release nutrients into the soil and the potential toxic effects of elements (Figure 1). Soil has organic matter and colloids that have permanent negative charges where adsorption and desorption of cations occur by exchange. At these sites substitution of cations take place. The organic matter can further decompose, producing weak acid groups that can adsorb cations. These acid groups are greatly affected by a change in soil pH, where low soil pH may result in protons being tightly held by the group. An increase in soil pH increases the exchange capacity of cations at the acid groups.

The cationic exchange capacity also relies on the amount of clay in the soil. Clay and organic matter are known for their high surface area thus, high water and nutrient holding capacities (UNIDO & IFDC, 1998).

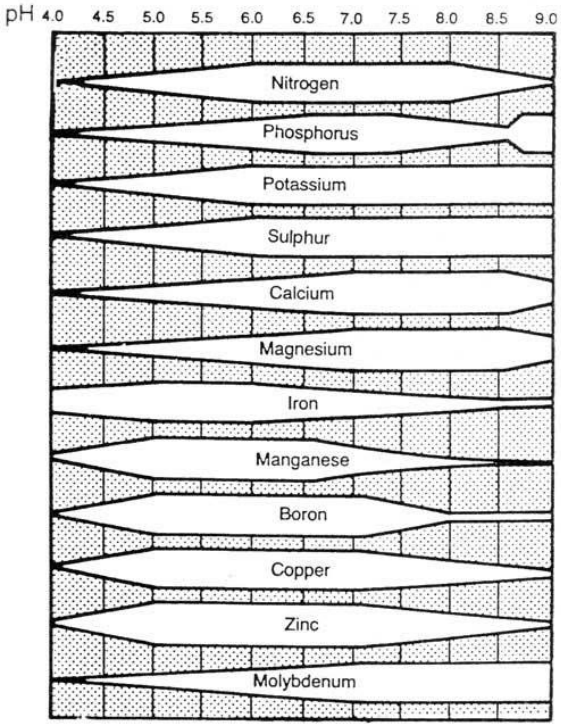

Figure 1: Availability of some essential nutrient in the soil as influenced by soil pH.

(http://www.cogs.asn.au/organic-principles/soil-basics/)

The distribution of metals in the fractions of the soil solid and liquid phases is different and exists in different forms in the soil. Metals can either be in soil solution form, readily exchangeable, sorbed in inorganic colloids, complexed to organic colloids or incorporated into the crystal lattice of clay minerals. Extraction methods have been developed to assess the mobility and availability of metals in soil; these include single extraction and sequential methods (Sungur et al., 2015). Single extraction methods are one step extraction procedures that evaluate the available amounts of metals in water soluble, exchangeable or organically bound fractions. Exhaustive research has been conducted on the use of different extractants including neutral inorganic salts such as $CaCl_2$

and KNO_3. Synthetic chelating agents such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) are also used for the estimation of organically bound metals (Romic, 2012). Research has shown that the assessment of bioavailable metals predict the amount of metal available for absorption by the plant (Xu et al., 2013).

Bioavailable or exchangeable metal fractions contain free metal ions readily accessed by plant roots. In soil solutions a free metal ion may interact simultaneously with more than one metal ion. Interaction of Cu and Zn occurs such that one reduces the uptake of the other by the plant. Zinc also affects the uptake of Fe by plants. Antagonistic and synergistic effects of elements are different at different biochemical processes (Mehra & Farago, 1994). This may cause deficiencies or toxicities in the plant. An excess or shortage of nutrients in the plant may result in impaired growth and development. If the concentration of a particular nutrient in soil solution is relatively low and there is a shortage of this nutrient in the plant, accumulation of the nutrient in the plant can likely occur.

The plant draws up nutrients from the soil by various mechanisms with the main mode of transportation to the roots being mass flow and diffusion. Mass flow occurs when nutrients are transported to the root surface by the movement of water in soil. The amount of nutrients transported to the root surface is dependent on the rate of water flow. Diffusion describes the movement of nutrients along a gradient. Nutrients are transported from a region of high concentration (eg. soil solution) to a region of low concentration (root surface). Diffusion is particularly important when the concentration of nutrients in the soil solution is low and cannot be transported by mass flow to the root surface (Mengel et al., 2001).

The plants' ability to manage toxic elements lies in three main strategies which classifies the plant as an excluder, indicator or accumulator. Excluders maintain constant elemental concentrations of metals in plant tissue over a wide range of soil-metal concentrations. This is achieved by either sequestering the uptake of the metal in the roots or by active efflux pumps (van Hoof et al., 2001). In indicators plants, there is a linear relationship between uptake of metals by the plant and metal concentrations in the soil. Accumulator plants tend to accumulate metals above ground levels regardless of the soil-metal concentration (Baker, 1981). Accumulators have the tendency to translocate metals from the root to the shoot so that the metal concentrations of the aerial parts of the plant are higher than that of the roots (Baker & Walker, 1990). Hyper-accumulators can tolerate metal concentrations at percentages higher than normal in dry plant mass (van der Ent et al., 2013).

Essential nutrients supplied by the soil are required for normal growth of the plant. The essential nutrients are classified as either macro-elements or micro-elements. Macro-elements are minerals that are required in large amounts whilst micro-elements are required in smaller amounts by plant tissue. Non-essential nutrients (As, Cd, Hg and Pb) can cause toxic effects in plants if present in high concentrations.

Nutrients and Anti-nutrients in Edible Plants

The World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) have for every decade reviewed and gathered new evidence on the major nutrient requirements and recommended intakes for individuals in a population of all age groups. Trace elements, fats and oils, carbohydrate and, recently, vitamins and minerals in human nutrition are research materials that have been updated.

Nutrients are required in the diet for growth, normal functioning of cells and the maintenance of good health. They are categorised as either macronutrients or micronutrients. Macronutrients are required by the body in large amounts; these include carbohydrates, proteins and lipids. Carbohydrates in the diet supply energy to brain cells, nervous system and blood. They consist of simple and complex sugars, most dietary sources of fibre and alcohol sugars. Proteins in food provide the body with amino acids used to build and maintain tissues, regulate water and help in growth and supply of energy. Fats or lipids supply energy and insulation for internal organs and also provide a medium for absorption of fat-soluble vitamins (Brown et al., 2014; McCormack-Brown et al., 2002).

Vitamins and minerals are required in smaller amounts and are referred to as micronutrients. Vitamins promote specific chemical reactions within cells. The B-complex vitamins and vitamin C are water-soluble whilst vitamins, A, D, E and K are fat-soluble and are present in fat soluble portions of food. Minerals help regulate bodily functions and aids in growth and maintenance. Macro-elements (Ca, Cl, K, Mg, Na and P) are required in excess of 100 mg/day whilst micro-elements (Cr, Co, Cu. Fe, I, Mn, Mo, Se and Zn) requirements are less than 100 mg/day (McCormack-Brown et al., 2002). Consuming foods rich in vitamin C can increase the bioavailability of Cr, Cu or Fe as vitamin C reduces them in the gastrointestinal tract (McGuire & Beerman, 2007). Apart from essential elements, there are also toxic elements that can be found in foods. These include As and heavy metals such as Hg, Pb and Cd. Mercury poisoning is caused by food containing organomercury compounds. Vegetables with large surface areas grown in Pb contaminated soil accumulate the metal thereby entering the food chain. Certain types of foods (wild mushrooms) accumulate Cd and prolonged intake of Cd results in the metal accumulating in the liver and kidneys (Belitz et al., 2004).

The dietary reference intake (DRI) is the general term used for the nutrient intake standards for healthy individuals. It accounts for age, gender, growth, pregnancy and lactation. Recommended dietary allowances (RDAs) describe the level of nutrient intake judged to be adequate to meet the known nutrient needs of all healthy individuals (Tables 1 and 3). Tolerable upper intake levels (ULs) are the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects amongst individuals in a population (Tables 2 and 4) (Institute of Medicine, Food and Nutrition Board, 2011).

Table 1: Dietary Reference Intake (DRI): Recommended Dietary Allowances (RDAs) for Individuals for Essential Elements.

Life Stage (years)	Ca (mg d^{-1})	Cr (µg d^{-1})	Cu (µg d^{-1})	Fe (mg d^{-1})	Mg (mg d^{-1})	Mn (mg d^{-1})	Se (µg d^{-1})	Zn (mg d^{-1})
Children								
1-3	700	11	340	7	80	1.2	20	3
4-8	1000	15	440	10	130	1.5	30	5
Females/Males								
9-18	1300	25-35	700-890	8-11	240-410	1.9-2.2	40-55	8-11
19-70	1000	35	900	8	400-420	2.3	55	11
>70	1200	30	900	8	420	2.3	55	11

Table 2: Dietary Reference Intake (DRI): Tolerable Upper Intake Levels (ULs) for Individuals Essential Elements.

Life Stage (years)	Ca (mg d^{-1})	Cr (µg d^{-1})	Cu (µg d^{-1})	Fe (mg d^{-1})	Mg (mg d^{-1})	Mn (mg d^{-1})	Ni (mg d^{-1})	Se (µg d^{-1})	Zn (mg d^{-1})
Children									
1-3	2500	ND	1000	40	65	2	0.2	90	7
4-8	2500	ND	3000	40	110	3	0.3	150	12
Females/Males 9-18	3000	ND	8000	40-45	350	6-9	0.6-1	400	23-34
19-70	2500	ND	10000	45	350	11	1	400	40
>70	2000	ND	10000	45	350	11	1	400	40

ND: Not determinable.

Table 3: Dietary Reference Intake (DRI): Recommended Dietary Allowances (RDAs) for Individuals for Macronutrients and Vitamins.

Life Stage (years)	Carbohydrates (g d^{-1})	Total Fibre (g d^{-1})	Fat (g d^{-1})	Protein (g d^{-1})	Vitamin A (µg d^{-1})	Vitamin C (mg d^{-1})	Vitamin E (mg d^{-1})
Children							
1-3	130	19	ND	13	300	15	6
4-8	130	25	ND	19	400	25	7
Female/Male 9-18	130	31-38	ND	34-52	600-900	45-75	11-15
19-70	130	30-38	ND	56	900	90	15
>70	130	30	ND	56	900	90	15

ND: Not determinable.

Table 4: Dietary Reference Intake (DRI): Tolerable Upper Intake Levels (ULs) for Individuals for Vitamins.

Life Stage (years)	Vitamin A (µg d^{-1})	Vitamin C (mg d^{-1})	Vitamin E (mg d^{-1})
Children			
1-3	600	400	200
4-8	900	650	300
Females/Males 9-18	1700-2800	1200-1800	600-800
19-70	3000	2000	1000
>70	3000	2000	1000

Apart from the nutritional information on plants, knowledge on the anti-nutritional properties of plants is also important since they may reduce the availability of nutrients. Anti-nutrients are natural or synthetic compounds that interfere with the absorption of nutrients thereby preventing digestion and absorption by the body. An anti-nutritional factor is not the character of the compound but the effect it has on the digestive system of humans when consumed in large quantities (Kumar, 1992). Polyphenolic compounds, phytates and tannins are deemed anti-nutrients or inhibitors of Fe absorption in the diet (Somsub et al., 2008). High intake of soluble oxalates present in plant tissue can pose a risk of hyperoxaluria (excessive urinary excretion of oxalates). This could lead to the recurrence of calcium oxalate kidney stones (Chai & Liebmann, 2005; Massey et al., 1993). Previous research revealed that an increase in the content of saponins from *Lucerne* plant fed to young male rats decreased Fe absorption in the rats (Southon et al., 1988). Consumption of lower amounts of cyanide is not lethal, but the FAO/WHO (1991) recommends intakes of cyanide in foods to be < 10 mg per kg, dry matter, to prevent acute toxicity.

A variety of plants are utilized for their dual nature, they are used for their medicinal value and consumed as food. These plants are either consumed raw or cooked. Cooking of plants or vegetables has been shown to destroy some of the nutrients, but some will be easier to absorb in the body. Vitamin C and some minerals may leach into the water whilst cooking; other vitamins and enzymes may be destroyed by high temperatures (Kala & Prakash, 2006; Kimura & Itokawa, 1990; Yuan et al., 2009). On the other hand, cooking of the plant may increase the availability of some nutrients and facilitate the breakdown of starch and carotenoids thus allowing the digestive enzymes in our body to easily breakdown these molecules further. Brief cooking with minimal water is therefore more effective (McGee, 2004; Tull, 1996).

Cooking also deactivates anti-nutrients present in raw plants and some are heat-labile. Reports from previous research by Ilelaboye et al. (2013) on seven leafy vegetables species of Nigeria where the effects of two cooking methods were investigated showed that both methods reduced the content of cyanides, phytates, oxalates, saponins and tannins in the vegetables. Further studies by Oulai et al. (2014) on five different leafy vegetables consumed in the northern parts of Côte d'Ivoire revealed that cooking decreased the content of anti-nutrients (oxalates and phytates) in the vegetables.

Nutritional Status in South Africa

Malnutrition results from a lack of proper nutrition and this occurs when there is an imbalance of nutrients required for growth and development. Malnutrition is a major health problem amongst South African children from low income families (Faber & Wenhold, 2007; Iversen et al., 2011). Some of the main micronutrient deficiencies as highlighted by the United Nations International Children's Emergency Fund (UNICEF) are Ca, Fe, vitamin A and Zn deficiencies (UNICEF,

2015). Research by Labadarios et al. (2008) showed that one out of five South African women and one out of seven South African children have a poor Fe status whilst 45.3% of children between the ages of 1-9 had inadequate Zn levels. Statistical analyses done by De Onis et al. (2012) showed that there will be a slow decrease in the percentage of stunted pre-school children in South Africa in the period from 1990 to 2015, from 35.4% to 32.3%, respectively. A nationwide survey conducted in 2005 by the National Food Consumption Survey-Fortification Baseline (NFCS-FB) revealed that almost 20% of children in the age group of 1-9 years were stunted and 10% were underweight. Socio-economic conditions, such as poverty and unemployment, were found to be the main cause of malnutrition (Iversen et al., 2011). High food prices also contribute to food insecurity in South Africa. Food insecurity describes a state in which there are insufficient nutritional and safe foods for consumption.

On the other hand, the global obesity rates have doubled and are accompanied by the growth of non-communicable diseases (diet-related illnesses) including cardiovascular diseases and diabetes. The increasing availability of fast food outlets in South Africa has contributed to the consumption of food with high calories and low-nutritional value (Barilla Center for Food and Nutrition, 2013). These are processed foods that are high in trans fats, sugar and salt, which are harmful when consumed in large amounts. Although processed foods are deemed to be unhealthy, they are found to be more convenient, since preparation time is minimal (Reavley, 1998; Rissman, 2016). A study done by Vorster et al. (2013), in South Africa in 2000, on the nutrition-related health outcomes, revealed some of the causes of premature mortality to be diabetes (4.3%), low fruit and vegetable intake (3.2%), vitamin A deficiency (0.6%) and Fe deficiency (0.4%). South Africa is the second largest country in sub-Saharan Africa with the highest number of people with type 2 diabetes. This increase in diabetes in the low income groups in developing countries is of great concern due to

the combined effect of diabetes with other diseases such as HIV/AIDS and tuberculosis (Mendenhall & Norris, 2015). In these countries, such as in South Africa, there is the double burden of malnutrition where both over and under nutrition occurs. Availability and accessibility to adequate food that is nutritious and safe is therefore crucial (Labadarios et al., 2011).

Edible and Medicinal Plants

South Africa has a rich agro-biodiversity with nearly 22 000 species of native plants, accounting for almost 10% of the world's higher plant species (Street & Prinsloo, 2013). Most of these plants are medicinal and are used to treat different kinds of ailments. There are 80 000 edible species in the 300 000 higher plant species that are present worldwide and 25% have proven medicinal application (Duke, 2001).

Plants in this Study

Four nettles are discussed in this study (*Laportea peduncularis* susp. *peduncularis* (river nettle), *Laportea alatipes* (forest nettle), *Obetia tenax* (mountain nettle) and *Urtica dioica* (stinging nettle)) from the Urticaceae family. These plants are found in the coastal regions of KwaZulu-Natal, South Africa and they are known as imbati in isiZulu. Plants from the Urticaceae family are known for their medicinal and nutritional value.

The Urticaceae family

The Urticaceae family is also known as the nettle family and consists of 49 genera of herbs, shrubs and trees (Watson & Dallwitz, 1992). The family is from the order *Urticles* with 1300 species (Wang et al., 2012) in the wet tropical regions. In South Africa, 11 genera and 22 species exist

(Schmidt et al., 2002). Many of the species have stinging hairs on their stems and leaves (Grieve, 1971).

Traditional medicinal uses and phytochemicals of plants from the Urticaceae family

In Europe, nettles are used extensively in herbal preparations; they are used as diuretics, anti-inflammatory agents, astringents, for stress reduction and scalp conditioning. The leaf is known to be an alkalinizer that helps in eliminating acid-waste product build-up in the body. They are also known to help with rheumatism, osteoarthritis, gout, and other problems often associated with a heavy protein diet. In Fiji, nettles are used medicinally to treat various ailments including, urinary and menstrual disorders, rheumatoid arthritis, diabetes, influenza, and infective hepatitis. The nettles are also enjoyed as tea and consumed as food (Figure 2). The traditional medicinal uses of nettles from different genera show a similar utilisation. The leaf infusion of the genus *Laportea* and *Urtica* are used to treat and control bleeding, the decoction of roots from *Boehmeria*, *Obetia* and *Urtica* are used to treat urinary infections, coughs and colds (Daniel, 2006; Karakaya & Kavas, 1999; Konrad et al., 2000; Nalumansi et al., 2014; Peteros & Uy, 2010; Xu et al., 2011). A common medicinal use of one of the *Obetia* species, *O. rodula*, is in the treatment of infertility with the aid of the root decoction (Beentje, 1994; Friis, 1983; Friis, 1989). Phytocompounds isolated from some genera in the Urticaceae family include carotenoids, fatty acids, flavonoids, polyphenols, sterols and triterpenes (Abdeltawab et al, 2012; Ghaima et al, 2013; Maobe et al, 2013; Peteros & Uy, 2010).

Figure 2: Leaves of *Urtica dioica* are commercially available as tea in the United Kingdom.

The genus Laportea

There are 22 species in the *Laportea* genus which are mainly found in the tropical and subtropical regions of the world, including Africa, eastern Asia and North America (Allaby, 2012). All of the *Laportea* species contain stinging hairs. The fresh hairs are known to contain chemicals such as histamine, serotonin and acetylcholine. The plants in this genus have numerous documented medicinal uses (Table 5); *L. aestuans*, *L. interrupta* and *L. ovafolia* leaves have analgesic properties (Etukudo, 2003; Pullaiah, 2006; Essiett et al., 2011). The main classes of compounds isolated from *Laportea* include cardiac glycosides, flavonoids, saponins, sterols, tannins and triterpenes (Table 5).

Table 5: List of *Laportea* species used as traditional medicine with their active compounds.

Plant material	***Traditional uses***	***References***	***Active compounds***	***References***
L. aestuans (Linn.) Chew	Leaves: enhances childbirth and stomach-ache	Lans, 2007; Etukudo, 2003	Cardiac glycosides, flavonoids, phlobatanins, tannins, saponins,	Essiett et al., 2011
L. interrupta (L.) Chew	Whole plant: gout & rheumatism Leaves: hepatitis, malaria, analgesic Roots: diuretic, fever	Jain, 1994; Pullaiah, 2006; Cambie & Ash, 1994	Alkaloids, flavonoids, glycosides, phenols, steroids, tannins, triterpenes	Deepa, 2014
L. ovalifolia (Schumach.) Chew	Leaves: arthritis, enema, analgesic, diabetes, diuretic	Essiett et al., 2011; Hughes, 2006; Momo et al., 2006	Cardiac glycosides, flavonoids, saponins, tannins, laportoside A and laportomide B	Essiett et al., 2011; Tazoo et al., 2007
L. peduncularis (Wedd) Chew	Leaves: anti-inflammatory	Pooley, 1998	N.D	
L. alatipes Hook. f.	Whole plant: stomach-ache, antiviral, antibacterial, bruises, hepatitis	Quattrochi, 2012; Karhagomba et al., 2013	N.D	

N.D Not determined

Laportea peduncularis subsp. peduncularis

Figure 3: *Laportea peduncularis* subsp. *peduncularis* (river nettle).

Laportea peduncularis subsp. *peduncularis* (Figure 3) commonly known as river nettle or imbati yasemfuleni in isiZulu is a perennial herb distributed along the coastal zone of KwaZulu-Natal, Eastern Cape and some parts of the Western Cape, South Africa. It is also located in Malawi, Mozambique, Swaziland, Tanzania and Zimbabwe. It can be a scrambling herb, the stems can grow up to 1.5 m long and are greenish to brownish in colour. The leaves are ovate and the apex is acuminate. The base of the leaves is broadly rounded or subcordate with serrated margins. The margins have 15-25 teeth on each side; the flowers are unisexual and borne in small cymose clusters (Friis, 1989). The leaves and stem are covered with stiff and stinging hairs. So far, there is no documentation on the compounds found in this plant.

***Laportea alatipes* Hook. f.**

Figure 4: *Laportea alatipes* (stinging forest nettle).

Laportea alatipes (Figure 4) is commonly known as stinging forest nettle; whilst in isiZulu it is known as imbati yasehlathini. It is a perennial herb, distributed in East and West tropical Africa. In South Africa, it is located in the forest and forest edges in the Eastern Cape, KwaZulu-Natal and Limpopo. The plant can grow up to 2 m tall and the stems and leaves contain sparse to dense stinging hairs. The leaves are broadly lanceolate to ovate whilst the base is cordate. The margins are coarsely serrated. The male flowers are in the axils of lower leaves whilst female flowers are in fan-shaped clusters (Friis, 1989; Quattrochi, 2012). There is a lack of detailed information of the bioactive compounds responsible for the medicinal use of this plant.

The genus Obetia

Obetia is a new comer to the Urticaceae family; documentation on the genus is not yet available but there is a listing of the seven species. The genus is distributed in Eastern and Southern Africa, Madagascar and surrounding islands. *Obetia rodula* is the most widely spread of the species; *O. madagascariensis* is endemic to Malagasy whilst *O. tenax* is recorded in Zimbabwe, eastern South Africa, southern Botswana and Mozambique. The genus consists of shrubs and trees which adapt to dry habitats (Burston et al., 1997; Friis, 1983). Like *Laportea*, the species in this genus have stinging hairs.

Obetia tenax (N.E.Br.) Friis

Figure 5: *Obetia tenax* (mountain nettle).

Obetia tenax (Figure 5) is commonly known as mountain nettle whilst in isiZulu it known as imbati yasentabeni or uluzi. The stems and branches are thick with soft wood whilst the leaves are large and deciduous (Burston et al., 1997). The leaves are alternate; the apex has a long-acuminate and is rounded, acute or broadly acuminate. The base is broadly cordate, truncate or rounded. The margins are serrated with stinging hairs; the stems are pinkish-bronze to grey, the flowers are borne in clusters and greenish yellow to white in colour (Boon, 2010; Schmidt et al., 2002). The plant is covered with long stinging hairs which cause burns and itchiness on contact with the skin. The bark yields a fibre used for thatching and the leaves are cooked as vegetables (Brink & Achigan-Dako, 2012). Documentation on the bioactive compounds of the plant is lacking and there is minimal information on the species.

The genus Urtica

Urtica comprises of 80 species and grows in Asia, Europe, North America and North Africa (Jakubczyk et al., 2015). The main varieties under *Urtica* include *Urtica dioica*, *Urtica urens* and *Urtica pilulifera*. *Urtica* species have been used in herbal remedies for the treatment of eczema, asthma, coughs, kidney infections, anaemia and diabetes (Wetherilt, 1992). Previously isolated compounds from *Urtica* include chlorogenic acid, caffeic acid, sterols, sterol glycosides and triterpenes (Gorzalczany et al., 2011; Kraus & Spiteller, 1990). *Urtica* products are commercially available; these include topical creams for eczema, daily food supplements, constituents of Prostamed®, *Urtica* capsules for prostate inflammation and nettle tea.

***Urtica dioica* L.**

Figure 6: *Urtica dioica* L. (stinging nettle).

Urtica dioica (stinging nettle) (Figure 6) is a well-known nettle from the Urticaceae family. Like the *Laportea* and *Obetia* species, it is also referred to as imbati in IsiZulu. It is a perennial shrub that grows up to 2 m high. The leaves have serrated margins, with a cordate base and an acuminate apex. The stems have stinging hairs whilst the leaves may occasionally possess stinging hairs. The flowers are yellowish-brown in colour. Numerous studies have been done on the chemical composition of *U. dioica*. Isolated compounds from the nettle include β-sitosterol, stigmasterol, dotriacotane, erucic acid, ursolic acid, scopoletin, rutin and β-carotene (Motawe et al., 2013; Ji et al., 2007). Owing to the use of *U. dioica* in traditional medicine to treat diabetes, numerous studies on the nettle extracts have been done (Ahangarpour et al., 2012; Kianbakht et al., 2013; Rahimzadeh et al., 2014; Ranjbari et al., 2016). Additionally, *U. dioica* has been found to have antioxidant, antimicrobial, anti-ulcer and anti-inflammatory activity (Gülçin et al., 2004).

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CHAPTER TWO

The distribution of macronutrients, anti-nutrients and essential elements in nettles, Laportea peduncularis susp. peduncularis (river nettle) and Urtica dioica (stinging nettle)

ABSTRACT

Laportea peduncularis and *Urtica dioica* popularly known as "nettles" belong to the plant family *Urticaceae* and are consumed as a vegetable or used for its medicinal benefit in many countries in Africa, Asia, Europe and America. This study aimed at investigating the effect of cooking on the macronutrient, anti-nutrient and elemental composition of *Laportea peduncularis* and *Urtica dioica* leaves. The results showed a decrease in the crude fat, ash, carbohydrate, and vitamin C content with cooking of the leaves, but an increase in the vitamin E content. The anti-nutrient content (cyanides, phytates and saponins) increased slightly with cooking, whilst the oxalate content decreased. The concentration of essential elements in cooked *L. peduncularis* leaves were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Cr > Ni > Co. Both cooked and raw leaves of nettles were found to be rich sources of macronutrients and essential elements and may be used as an alternative to commercially available nutrient supplements. Statistical analyses (principal component analysis and correlations) indicated that certain elements taken up by these plants were from common sources. Positive and negative relationships existed between nutrients, anti-nutrients and elements in the plant.

Keywords: Nettles, *Laportea peduncularis*, *Urtica dioica*, nutrients, anti-nutrients, elements, edible vegetation.

INTRODUCTION

The Food and Agricultural Organization (FAO) has reported that 842 million people around the world experience chronic hunger and about 98% of these people are from underdeveloped countries (FAO, 2013). Although the food security crisis has been a key focal point in recent years there is little progress to alleviate this crisis. Food security refers to the ease of access and availability to sufficient amounts of nutritious foods for a staple diet (Smith et al., 1992). Lack of nutrients in the diet may lead to malnutrition, which is a condition caused by the imbalanced intake of nutrients. Asia and Africa were found to be two of the continents with the highest rates of child malnutrition in the world (Smith & Haddad, 2000). Whilst in Southern Africa, child malnutrition is already a major public health concern, it is expected that number of malnourished children in Sub-Saharan Africa will rise in the next few years (Vorster et al., 2013).

An increase in urbanization is deemed to be one of the factors that have propelled dietary changes amongst the South African black population which has led to adverse health effects (Kruger et al., 2003; Vorster, 2002). Unhealthy diet increases the risk of nutrition based non--communicable diseases (NCDs) (Puoane et al., 2008). Low fruit and vegetable intake, childhood and maternal underweight, Fe deficiency anaemia and vitamin A deficiency have been listed as risk factors which cause death from NCDs amongst the black population in South Africa (Norman et al., 2007). NCDs include obesity, cardiovascular diseases and diabetes mellitus.

In under-developed countries, the foods that are nutritious, accessible and also affordable are scarce and alternate sources of such foods need to be identified. Rural communities still rely on indigenous plants as a source of food for their daily living (van der Hoeven et al., 2013). Most of these plants are found and harvested in the wild (Department of Agriculture, Forestry and Fisheries, 2013). These indigenous plants are known for their nutritional value; these are either leafy vegetables or fruits. Leafy vegetables are cooked or eaten raw (Modi et al., 2006). Plants

from the nettle family (Urticaceae) are native to Asia, Africa, Europe and Americas and are often consumed by people due to their availability. These include *Laportea peduncularis* subspecies *peduncularis* (LPP) (river nettle) and *Urtica dioica* L (UD) (stinging nettle). LPP is found mostly in the coastline of Mozambique and KwaZulu-Natal (KZN), South Africa. It is known as Imbati by the people in KZN. The leaves and young shoots of the plant are cooked and eaten as a vegetable. LPP is an annual herb with stiff and stinging hairs on the surface of the leaves and stem of the plant. The leaves have serrated margins with 15-25 teeth on each side (Friis, 1989). UD is found in the cool regions of America, Asia, British Isles and some parts of Africa (Weigend, 2006). The UD plant is covered with stinging hairs; the young leaves are cooked as a vegetable and added to soups (Rutto et al., 2013). Nettles are known for their high nutritional value, with significant amounts of minerals and vitamins (Upton, 2013).

Anti-nutrients are compounds that interfere with the absorption of certain nutrients. Some examples of anti-nutrients are cyanates, oxalates, phytates and saponins. Cyanates are salts or esters of cyanic acid; as a defence mechanism the plant breaks the cyanates down releasing hydrocyanic acid. High levels of the acid are toxic to humans and animals, since it inhibits cytochrome oxidase, a protein which acts as the terminal enzyme of respiratory chains (Enneking & Wink, 2000). Large amounts of oxalates are found in plant food. Calcium oxalate formation in the bladder is known to cause kidney stones. This occurs during the combination of oxalate with calcium in urine (Voss et al., 2006).

Phytates are cationic salts of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphates) bound to minerals. These are natural chelators with negatively charged sites that bind metal cations (mostly polyvalent) since these bind more strongly than monovalent cations (Lott et al., 2000). Though phytates reduce Fe absorption in food, depending on levels in the plant, they possess beneficial anticancer and antimicrobial properties (Hurrell et al., 2003; Khokhar & Apenten, 2003). Saponins are natural defence compounds found in plants. They contain a carbohydrate

moiety attached to a triterpernoid or steroid (Shi et al., 2004). Saponins are capable of increasing the permeability of cell membranes in the body, thus allowing substances to enter the bloodstream, causing destruction of red blood cells (Hoffman, 2003).

Previously, we reported on the elemental distribution and nutritional value of nuts and indigenous fruits and vegetables found in South Africa (Jonnalagadda et al., 2008; Mahlangeni et al., 2012; Moodley et al., 2007; Moodley et al., 2013; Reddy et al., 2011). In this study, the distribution of nutrients and anti-nutrients in LPP and UD leaves was investigated. This was done on both the raw and cooked forms of the leaves to determine the effect of heating on the anti-nutrient content (Lott et al., 2000). The elemental concentrations in different nettles was also investigated and assessed for their nutritional value.

MATERIALS AND METHODS

Sample collection and preparation

LPP leaves were collected from ten different sites in KZN. The sampling sites were, S1- Umbilo Park, S2 - Umhlanga, S3 - Eshowe, S4 - Stanger, S5 - Mona, S6 - Maphumulo, S7 - Umzumbe, S8 - Amahlongwa, S9 - Gingindlovu, and S10 - Ndwedwe (Fig. 7).

Figure 7: Map of selected sampling sites in KwaZulu-Natal, South Africa.

Sampling was done in July and temperatures were typically 25 ºC. Samples of UD leaves (dried and crushed) were obtained from Cotswold Health Products Ltd., Gloucestershire, United Kingdom. The LPP plant samples were washed with double distilled water then oven dried at 50 ºC to constant mass. Dried LPP samples were crushed using a food processor (Braun range). A portion of the crushed raw samples (both LPP and UD) were stored in polyethylene bags and refrigerated until analysed. A portion of the raw samples (both LPP and UD) were cooked by boiling in double distilled water at 70 ºC on a hot plate for 15 min, cooled, and then sieved. The sieved samples were dried then crushed in a food processor and stored in polyethylene bags and kept aside until analysed.

Reagents and chemicals

All chemicals used were supplied by Merck (Kenilworth, USA) and Sigma (St. Louis, USA) Chemical Companies and were of analytical-reagent grade.

Macronutrient, energy and moisture content

The moisture content was determined by the AOAC method (AOAC, 1990). Two grams of leaf samples were oven dried at 105 ºC for 24 hr. Samples were cooled in a desiccator then weighed to constant mass. The ash content was determined by incinerating the dried leaf samples (2 g) in a muffle furnace at 600 ºC for 12 hr (Elhassan & Yagi, 2010). Crude protein was determined by the Kjeldahl method (Skoog et al., 2004). Total protein was calculated by multiplying the nitrogen content by a factor of 6.25. The total fat content was obtained by exhaustive extraction of leaf samples (2 g) with n-hexane using a soxhlet apparatus. For crude fibre, fat-free samples were digested with 0.128 M H_2SO_4 and 0.313 M NaOH. The insoluble residue was washed with hot water and dried at 130 ºC, then weighed to constant mass. The dried residue was then incinerated at 600 ºC for 3 hr and the ash was weighed to determine the crude fibre content (FAO, 1994). Total carbohydrate was obtained by difference and the energy value was determined using Equation 1 (FAO, 2003).

$$\text{Energy value}\left(\text{kJ } 100\ \text{g}^{-1}\right) = [(37 \times \%\text{lipids}])+(17 \times \%\text{carbohydrates})+(17 \times \%\text{protein})] \quad (1)$$

The vitamin C content was determined by the iodometric method (Igwemmar et al., 2013). A sample of ground leaves (0.1 g) was repeatedly extracted with 10 mL of deionized water. The extracts were poured into 100 mL volumetric flasks and made up to the mark. An aliquot of 20 mL was placed into a conical flask, to which, 50 mL of distilled water, 5 mL 0.6 M KI solution, 5 mL 1M HCl solution and 1 mL starch indicator was added. This was titrated against a 0.002 M KIO_3 solution. The endpoint was marked by the first trace of a dark-blue colour.

The vitamin E content was determined by the method of Phatak and Hendre (2014) with some modifications. The hexane extract (0.1 mL) was mixed with 1 mL of reagent (0.6 M H_2SO_4, 28 mM Na_2SO_4 and 4 mM ammonium molybdate) and incubated at 37 ºC for 1.5 h with vigorous shaking. The absorbance of the aqueous phase was measured at 695 nm against a blank

containing 0.1 mL pure hexane with 1 mL reagent, treated under the same conditions. The vitamin E content was estimated from a standard curve of α-tocopherol acetate at various concentrations.

Anti-nutrient content

The alkaline titration method of AOAC (1990) was used for the determination of hydrocyanic acid. A sample of ground leaves (0.1 g) was placed into a Kjeldahl flask, to which, 100 mL of water was added and left to stand for 2 hr. The mixture was steam distilled and 60 mL of distillate was collected in 2.5% NaOH solution and diluted to 250 mL. An aliquot (100 mL) was taken, to which, 8 mL of NH_4OH and 2 mL of 5% KI was added and titrated against a 0.02 M $AgNO_3$ solution until a permanent turbidity appeared. Equation 2 was used to determine the HCN content.

1 mL $AgNO_3$ = 1.08 mg HCN (2)

The oxalate content was determined by the method of Day and Underwood (1986). A sample of ground leaves (0.1 g) was mixed with 50 mL of 3 M H_2SO_4 in a conical flask and stirred for 1 h with a magnetic stirrer. The mixture was filtered and a 25 mL aliquot of the filtrate was then titrated against a 0.05 M $KMnO_4$ solution until a faint violet colour persisted for at least 30 seconds. Equation 3 was used to determine the oxalate content.

1mL 0.05 M $KMnO_4$ = 2.2 mg oxalate (3)

The phytate content was determined by the method of Reddy and Love (1999) with some modifications. A sample of ground leaves (0.1 g) was soaked in 100 mL of 2% HCl for 5 h then filtered. To a 25 mL aliquot of the filtrate, 5 mL of 0.3% potassium ferricyanide solution was added. The mixture was titrated against a $FeCl_3$ solution until a blue-green colour persisted for 5 min.

The saponin content was determined by the method of Hudson and El Difrawi (1979). A sample of ground leaves (10 g) was mixed with 20 mL of 20% aqueous ethanol and agitated with a magnetic stirrer for 12 h at 55 ºC. The solution was filtered and the residue was re--extracted with 200 mL of 20% aqueous ethanol. The extract was reduced to 40 mL under vacuum and 20 mL of dichloromethane was added and separated in a separating funnel. The aqueous layer was recovered and the DCM layer discarded. The pH of the aqueous layer was adjusted to 4.5 by the addition of NaOH. The solution was then shaken with 60 mL of n--butanol. The n--butanol extract was washed twice with 10 mL of 5% NaCl and evaporated to dryness in a fume hood to produce the crude saponin.

Elemental analysis

Digestion of samples (LPP leaves, UD leaves and certified reference material (CRM)) was performed using the CEM MARS (CEM Corporation, Matthews, North Carolina, USA) microwave reaction system with patented Easyprep^{TM} plus technology. Ground leaf samples (0.2 g) were accurately weighed into liners, to which 10 mL of 70% HNO_3 was added. For digestion, the power was set at 100% at 1600 W and the temperature was ramped to 180 ºC (15 min) where it was held for 15 min. Digested samples were transferred into 25 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles for elemental analysis.

All samples were analysed for the following elements As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn. Elemental analysis was carried out by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). The accuracy of the elemental determination was measured by use of the CRM, White Clover (BCR 402), from the Community Bureau of Reference of the Commission of the European Communities. All samples were analysed in quintuplicate (n = 5).

Statistical analysis

Analysis of variance (ANOVA) was performed on data and the means were separated by Tukey's Post-hoc test to determine significant differences. Principal component analysis (PCA) was performed using the principal component method on the dataset, and the cluster analysis (CA) was applied to the standardized matrix of samples using Ward's method. Pearson's correlation coefficients were obtained using the Statistical Package for the Social Sciences (SPSS) (PASW Statistics, Version 22, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Macronutrient, energy and moisture content

Moisture content of raw LPP leaves was 51.4% whilst that in raw UD leaves was 18.4%. The macronutrient and energy content of LPP and UD leaves are presented in Table 6. The crude fat content of raw LPP leaves (8.2%) was relatively low compared to that of UD leaves (11.8%). There was no significant decrease in the crude fat content of LPP leaves from raw to cooked. Both plants were observed to possess high ash content, which generally indicates the plants high mineral content. The ash content of LPP leaves decreased in cooked sample relative to raw by 9.1% and by 2.9% in UD leaves. This could be due to the leaching of minerals into the water during the cooking process (Oulai et al., 2014).

Table 6: Proximate chemical composition (crude fat, ash, crude protein, crude fibre and carbohydrate (Carb)), energy content and vitamin C and E content of *L. peduncularis* (LPP) and *U. dioica* (UD) leaves, based on a dry mass.

		Crude fat (%)	Ash (%)	Crude protein (%)	Crude fibre (%)	Carb (%)	Vitamin C (mg 100 g^{-1})	Vitamin E (mg 100 g^{-1})	Energy (kJ 100g^{-1})
LPP	Raw	8.2 (0.7)[a*]	44.4 (0.6)[a]	2.8 (0.3)[a]	0.23 (0.1)[a]	44.4	18.1 (2.5)[a]	17.9 (1.0)[a]	1105
	Cooked	6.9 (0.6)[a]	35.3 (4.3)[b]	3.7 (0.24)[b]	1.7 (0.2)[b]	52.5	13.0 (1.4)[b]	23.2 (2.5)[b]	1208
UD	Raw	11.8 (0.5)[b]	20.9 (0.8)[c]	1.81 (0.2)[c]	0.3 (0.1)[a]	65.2	22.0 (0.7)[c]	26.2 (1.1)[b]	1576
	Cooked	4.9 (0.4)[c]	18.0 (1.3)[c]	2.2 (0.3)[c]	0.6 (0.1)[a]	74.3	14.0 (1.4)[b]	25.5 (1.1)[b]	1483

[*]Values represented as mean (S.D.), n=5. Different letters in a column indicate significantly different means (Tukey post hoc comparisons, $P<0.05$).

There was a significant increase in the crude protein and crude fibre content of the plants with the cooking of leaves. The crude protein content of raw LPP leaves increased by 0.9% after cooking in contrast to the crude protein content of raw UD leaves which increased by 0.4% after cooking. Increase in the crude protein content could be a result of denaturing of the protein during cooking which exposes the interior parts of the structure thus facilitating the accumulation of broken down protein (Ma & Boye, 2015). Heating or cooking of vegetables promotes breakdown of indigestible cellulose, complex starch and tough fibres, thus an increase in crude fibre and carbohydrate content after cooking is expected (Underkoffler, 2003). High carbohydrate content generally indicates high energy content.

Vitamin C, a water soluble vitamin, is important for the proper functioning of the immune system, and also the manufacturing of collagen (Combs, 2008; Hughes, 2002). Both vitamin C and E were higher in UD than LPP leaves. However, in both plant species the vitamin C content decreased significantly from raw to cooked leaves. In LPP leaves the decrease was by 5.1% and in UD leaves, it was by 8%. Previous studies have also shown loss of vitamin C after cooking (Yuan et al., 2009). Vitamin E is a fat-soluble vitamin, and is known to be an antioxidant,

preventing cell membrane damage (Comeaux, 2007). There was a significant increase in the vitamin E content of LPP leaves from raw to cooked. Heat has been found to be effective in extracting vitamin E bound to protein membranes or linked to phospholipids by breaking the bonds (Ko et al., 2003). The study shows LPP and UD leaves to be richer sources of vitamin C and E compared to lettuce (13.0 and 1.2 mg 100 g^{-1}, respectively) and cabbage (13.6 and 0.69 mg 100 g^{-1}, respectively) (Chun et al., 2006; Ogunlesi et al., 2010). The study indicates that cooking reduces the vitamin C content but not the vitamin E content.

Anti-nutrient content

The results for the anti-nutrient composition of the raw and cooked leaves are presented in Table 7. The results reveal a decrease in oxalate content of LPP leaves from raw to cooked (1.46 to 0.89 mg 100 g^{-1}). Cooking reduces the amount of oxalates in LPP leaves through leaching of soluble oxalates into the water (Akhathar et al., 2011). There was a slight increase in the cyanide content of LPP and UD leaves after cooking (0.30 to 0.45 mg 100 g^{-1}, 0.37 to 0.85 mg 100 g^{-1}, respectively). The phytate content in both plant species showed a minor increase in its content with cooking. This trend was also observed for the saponin content of LPP leaves where it increased from 17.1 to 17.6 mg 100 g^{-1}, after cooking. However, a slight reduction in the saponin content was observed in UD leaves with cooking.

Table 7: Anti-nutrient composition of *L. peduncularis* and *U.dioica* leaves (raw and cooked).

		Cyanides (mg 100 g^{-1})	Oxalates (mg 100 g^{-1})	Phytates (µg 100 g^{-1})	Saponins (mg 100 g^{-1})
L. peduncularis	Raw	0.30 (0.02)^{a*}	1.46 (0.13)^{a}	3.30 (0.40)^{a}	17.1 (1.6)^{a}
	Cooked	0.45 (0.05)^{b}	0.89 (0.02)^{b}	8.30 (0.50)^{b}	17.6 (1.6)^{a}
U. dioica	Raw	0.37 (0.05)^{b}	2.32 (0.17)^{c}	5.60 (0.20)^{c}	18.9 (1.8)^{a}
	Cooked	0.85 (0.12)^{c}	0.97 (0.11)^{b}	6.00 (0.60)^{c}	24.3 (2.1)^{b}

*Values represented as mean (S.D.), n=5. Different letters in a column indicate significantly different means (Tukey post hoc comparisons, $P<0.05$).

A correlation analysis was done to establish relationships that existed between the macronutrient and anti-nutrient content in LPP leaves (Table 8). The correlations between macronutrients and anti-nutrients in the plant were evaluated by obtaining correlation coefficients (r) where r values ranged from -1 to +1. An r value of -1 indicated a strong negative linear relationship, an r value of +1 indicated a strong positive linear relationship and an r value of 0 indicated no relationship. The concentration of one variable that causes an increase in the concentration of another indicates a positive or synergistic relationship. There were positive correlations between fats and energy (0.9) indicating that these two variables are related. There was a 3-way synergy between the content of cyanides and oxalates (0.9), vitamin C and oxalates (0.8), and cyanides and vitamin C (0.9), indicating that an increase in the content of one increases the content of the other two entities. These variables were negatively related to crude fibre and phytates as indicated by the negative correlations (-0.9).

Table 8: Inter-item correlation matrix between macronutrients and anti-nutrients in *L. peduncularis* leaves.

	Ash	Fats	CP	CF	Carbs	E	Sap	Ox	Phy	CN	Vit C	Vit E
Ash	1											
Fats	0.8*	1										
CP	-0.7*	-0.4	1									
CF	-0.8	-0.5	0.8	1								
Carbs	-0.7	-0.1	0.4	0.6	1							
E	-0.2	0.9	0.7	0.6	0.0	1						
Sap	-0.3	-0.5	0.0	0.1	-0.6	-0.6	1					
Ox	0.8*	0.6	-0.9**	-0.6	0.7	-0.5	-0.1	1				
Phy	-0.9**	-0.8*	0.9**	0.7	0.5	-0.5	0.2	-0.9**	1			
CN	0.9**	0.7*	-0.9**	-0.8	-0.9**	0.1	-0.1	0.9**	-0.9**	1		
Vit C	0.7*	0.5	-0.9**	-0.9**	-0.8	-0.6	0.1	0.8*	-0.9**	0.9**	1	
Vit E	-0.9**	-0.8*	0.8*	0.7	0.5	-0.6	0.2	-0.8*	0.9**	-0.9**	-0.9**	1

*,**: correlations significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.
CP: crude protein, CF: crude fibre, Carbs: carbohydrates, E: energy, Sap: saponins, Ox: oxalates, Phy: phytates, CN: cyanides, Vit. C: vitamin C, Vit. E: vitamin E.

Elemental analysis

Table 9: Comparison of measured values to certified values (mean (S.D) in µg g^{-1}, dry mass) at the 95% confidence interval (n=5) for the certified reference material, White clover, BCR 402.

Element	Wavelength (nm)	Measured value (mg kg^{-1})	Certified value (mg kg^{-1})
Se	196.0	7.02 (1.26)	6.70 (0.25)
Fe	259.9	250 (16)	244
Ni	231.6	8.00 (0.35)	8.25
Zn	213.9	30.9 (6.7)	25.2

The elemental composition of the CRM (White Clover, BCR 402) was used to ensure accuracy of the method of determination and the results are represented in Table 9. The measured values compared well with certified values.

The metal content of LPP leaves obtained from different sites in KZN is shown in Table 10. Concentrations of metals in raw leaves were compared to concentrations in cooked leaves from different locations. For the toxic element, arsenic, its concentrations in raw leaves ranged from 1.16 to 4.02 µg g^{-1}, but only samples containing As after cooking were from S2 and S9 sites. The concentration of the major elements, Ca and Mg, were also seen to decrease with cooking. The Ca content in leaves was found to be the highest of all the elements studied. The concentration of Fe was shown to increase with cooking in some sites (S1, S2, S4, S5 and S6).

If determined, the average percentage of Co remaining after cooking was found to be 88%. Chromium concentration ranged from 1.27 to 9.25 µg g^{-1} in raw leaves and from 0.26 to 7.50 µg g-1 in cooked leaves, indicating a 56% reduction in Cr. The concentration of Cu in raw leaves ranged from 11.5 to 67.8 µg g^{-1} and decreased after cooking (0.11 to 39.6 µg g^{-1}). Manganese concentrations in raw leaves were high (49.4 to 228 µg g^{-1}) with an average of 75% remaining after cooking. The Ni concentration in leaves ranged from 2.07 to 11.0 µg g^{-1} and this was reduced by an average of 55% after cooking. Lead, which is a toxic element, was found in the leaves obtained from 6 of the 10 sites. The concentration of Pb in raw leaves ranged from 0.41 to 4.11 µg g^{-1} and an average reduction of 34.2% was observed after cooking. On average, 68% of Zn remained in the leaves after cooking. This indicates a reduction in the concentration of essential elements after cooking. At the same time, cooking can also be beneficial as it enables the leaching of toxic elements into the cooking water. In this study, the concentration of essential metals in LPP leaves were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Cr > Ni > Co.

Table 10: Concentration (µg g^{-1}, mean (S.D), n=5) of essential and toxic elements in *L. peduncularis* leaves (raw and cooked) from ten different sites in KwaZulu-Natal.

Element	Site*	LPP leaves (raw)	LPP leaves (cooked)
As	S1	ND	ND
	S2	2.21 (0.27)[a]	2.02 (0.68)[b]
	S3	2.34 (0.18)[a]	ND
	S4	3.48 (0.56)[b]	ND
	S5	2.09 (0.33)[a]	ND
	S6	1.16 (0.49)[c]	ND
	S7	4.02 (0.18)[b]	ND
	S8	3.20 (0.17)[b,d]	ND
	S9	3.75 (0.54)[b]	0.66 (0.03)[a]
	S10	2.39 (0.20)[a,d]	ND
Ca	S1	36 867 (528)[a]	15 941 (423)[a]
	S2	28 787 (467)[b]	12 773 (186)[b]
	S3	15 633 (1345)[c]	5 211 (230)[c]
	S4	36 438 (3296)[a]	15 294 (1562)[a,d]
	S5	12 589 (957)[c]	5 735 (167)[c]
	S6	13 632 (279)[c]	8 443 (131)[e]
	S7	22 753 (1557)[d]	6 386 (528)[c]
	S8	22 790 (1393)[d]	7 963 (472)[e]
	S9	36 392 (1835)[a]	14 136 (661)[b,d]
	S10	22 125 (991)[d]	11 683 (545)[b]
Co	S1	ND	ND
	S2	ND	ND
	S3	ND	ND
	S4	ND	ND
	S5	ND	ND
	S6	1.29 (0.07)[a]	1.12 (0.29)[b]
	S7	ND	ND
	S8	0.18 (0.04)[b]	0.16 (0.03)[a]
	S9	0.18 (0.03)[b]	0.16 (0.11)[a]
	S10	ND	ND
Cr	S1	9.25 (0.65)[a]	7.50 (0.40)[a]
	S2	4.88 (0.32)[b]	4.03 (0.17)[b]
	S3	1.57 (0.20)[c]	0.26 (0.07)[c]
	S4	1.60 (0.15)[c]	0.89 (0.25)[d]

	S5	1.94 (0.08)[c]	0.96 (0.12)[d]
	S6	4.13 (0.20)[b,d]	3.07 (0.22)[e]
	S7	1.27 (0.19)[c]	0.90 (0.03)[d]
	S8	4.18 (0.35)[b,d]	1.20 (0.07)[e,f]
	S9	4.21 (0.33)[b,d]	1.51 (0.15)[f]
	S10	3.56 (0.48)[d]	2.22 (0.08)[g]
Cu	S1	62.6 (3.7)[a]	39.6 (4.0)[a]
	S2	11.5 (2.0)[b]	6.09 (0.89)[b,e]
	S3	53.7 (4.3)[a,c]	ND
	S4	15.4 (3.4)[b]	0.11 (0.02)[c]
	S5	30.5 (5.9)[b,c]	7.80 (0.73)[b,e]
	S6	67.8 (7.4)[a]	18.4 (1.5)[d]
	S7	28.0 (3.4)[b,c]	3.77 (0.26)[b,c]
	S8	19.7 (1.9)[b]	6.22 (0.61)[b,e]
	S9	25.2 (4.1)[b,c]	7.99 (0.63)[e]
	S10	35.3 (6.0)[b,c]	15.0 (0.5)[d]
Fe	S1	406 (23)[a,b,c]	824 (52)[a]
	S2	123 (17)[a,b,c]	185 (10)[b]
	S3	445 (48)[a,b,c]	116 (12)[b]
	S4	334 (59)[a,b]	417 (55)[b]
	S5	789 (70)[a]	803 (31)[a]
	S6	3 707 (76)[d]	4 230 (305)[c]
	S7	380 (27)[a,b,c]	161 (33)[b]
	S8	1 215 (59)[e]	471 (38)[a,b]
	S9	1 205 (171)[c,e]	823 (117)[a]
	S10	2 938 (687)[f]	2 111 (286)[d]
Mg	S1	5 069 (119)[a]	2 255 (75)[a]
	S2	5 415 (119)[a,b]	2 144 (73)[a]
	S3	5 173 (259)[a]	1 621 (82)[b]
	S4	6 837 (285)[c]	2 283 (107)[a]
	S5	1 957 (114)[d]	1 070 (18)[c]
	S6	2 266 (65)[d]	1 393 (38)[d]
	S7	6 137 (545)[b,c]	1 425 (95)[d]
	S8	5 728 (421)[a,b]	2 054 (57)[a,b]
	S9	10 787 (556)[e]	2 663 (43)[f]
	S10	6 898 (434)[b,c]	2 517 (53)[f]

Mn	S1	49.4 (2.2)[a]	48.1 (2.12)
	S2	116 (1.0)[b]	112 (2.38)[b,f]
	S3	74.6 (5.8)[a]	20.3 (2.17)[c]
	S4	186 (17.5)[c]	183 (28.5)[d]
	S5	201 (5.8)[c,d]	174 (5.53)[e]
	S6	182 (1.5)[c]	171 (7.58)[e]
	S7	177 (12.0)[c]	76.4 (3.22)[a,f]
	S8	134 (11.8)[b]	54.1 (8.98)[a]
	S9	130 (11.8)[b]	106 (3.89)[b,f]
	S10	228 (31.1)[d]	188 (15.03)[d,e]
Ni	S1	2.46 (0.14)[a,b,e]	2.37 (0.22)[a]
	S2	2.28 (0.23)[a,b,e]	1.34 (0.24)[a,b]
	S3	3.03 (0.69)[a,b,e]	ND
	S4	2.07 (0.30)[b]	1.79 (0.16)[a,b]
	S5	5.02 (0.61)[c]	1.05 (0.35)[b,c]
	S6	5.68 (0.30)[c]	2.52 (0.53)[d]
	S7	11.0 (1.1)[d]	0.16 (0.01)[c]
	S8	3.57 (0.48)[e]	1.07 (0.85)[a,b]
	S9	3.11 (0.42)[a,b,e]	1.50 (0.13)[a,b]
	S10	7.66 (0.44)[f]	1.62 (0.47)[a,b]
Pb	S1	ND	ND
	S2	ND	ND
	S3	ND	ND
	S4	0.41 (0.02)[a,b]	0.40 (0)[a]
	S5	1.31 (0.07)[b,c]	1.24 (0.12)[b]
	S6	0.57 (0.07)[a,b]	0.48 (0.04)[a]
	S7	4.11 (1.25)[d]	0.44 (0.16)[a]
	S8	2.22 (0.23)[c]	1.09 (0.41)[b]
	S9	2.28 (0.37)[c]	1.32 (0.57)[b]
	S10	ND	ND
Zn	S1	87.0 (6.0)[a]	86.5 (8.1)[a]
	S2	26.6 (1.1)[b,e,f]	22.4 (2.7)[b,c]
	S3	27.1 (3.2)[b,e,f]	22.0 (1.7)[b,c]
	S4	27.1 (2.4)[b,e,f]	20.0 (1.3)[c]
	S5	60.2 (2.8)[c]	46.6 (1.3)[d]
	S6	43.1 (1.4)[d]	30.3 (1.5)[e]

S7	21.9 (1.9)[e]	13.0 (0.9)[f]
S8	63.4 (4.1)[c]	27.0 (1.6)[b,e]
S9	27.9 (3.9)[b,e,f]	12.5 (2.1)[f]
S10	34.0 (0.6)[f]	15.4 (0.6)[c,f]

* Site: S1--Umbilo Park, S2--Umhlanga, S3-Eshowe, S4--Stanger, S5--Mona, S6--Maphumulo, S7--Umzumbe, S8--Amahlongwa, S9--Gingindlovu, S10—Ndwedwe.
Different letters in a column indicate significantly different means (Tukey post hoc comparisons, $P<0.05$).
ND: Not determinable.

Contribution to the diet

The results in Table 11 show the estimated contribution of LPP and UD leaves (raw and cooked, based on dry mass) to the RDA. An average daily serving of LPP and UD leaves is approximately one cup, which is equivalent to 100 g (dry mass). Nettles are a good source of minerals, as depicted by the results (Institute of Medicine, Food and Nutrition Board, 2011). Raw LPP and UD leaves are estimated to contribute more than 100% towards the RDA for the elements Ca, Cr, Cu, Fe, Mg and Mn. Cooked leaves, which is the form in which they are consumed, had lower concentrations of most elements studied than raw leaves. Too much of the leaves should not be consumed due to high amounts of Fe and Mn, however, this can be beneficial to those suffering from Fe deficiency anaemia. Statistics on the Fe status in South Africa reveals that there is a prevalence of anaemia in women and children; about 9.7% of women and 1.9% of children aged five and under have Fe deficiency anaemia (Visser et al., 2013). These leaves can be taken as a substitute to the commercially available beetroot, green onions or spinach that is generally recommended to help alleviate anaemia. Also, Zn deficiency amongst young South African children has resulted in their stunted growth and weakened immune system (Buhl, 2010). Therefore, LPP and UD leaves, due to their rich nutrient content, if consumed, can contribute to the reduction of Zn deficiency as well. In Figure 8, a comparison between the Ca and Mg content in a commercial supplement with LPP and UD leaves is shown.

The results clearly indicate that both LPP and UD leaves can be used as an alternative to nutrient supplements that are generally inaccessible and unaffordable. Both LPP and UD leaves appear to be rich sources of the essential elements studied.

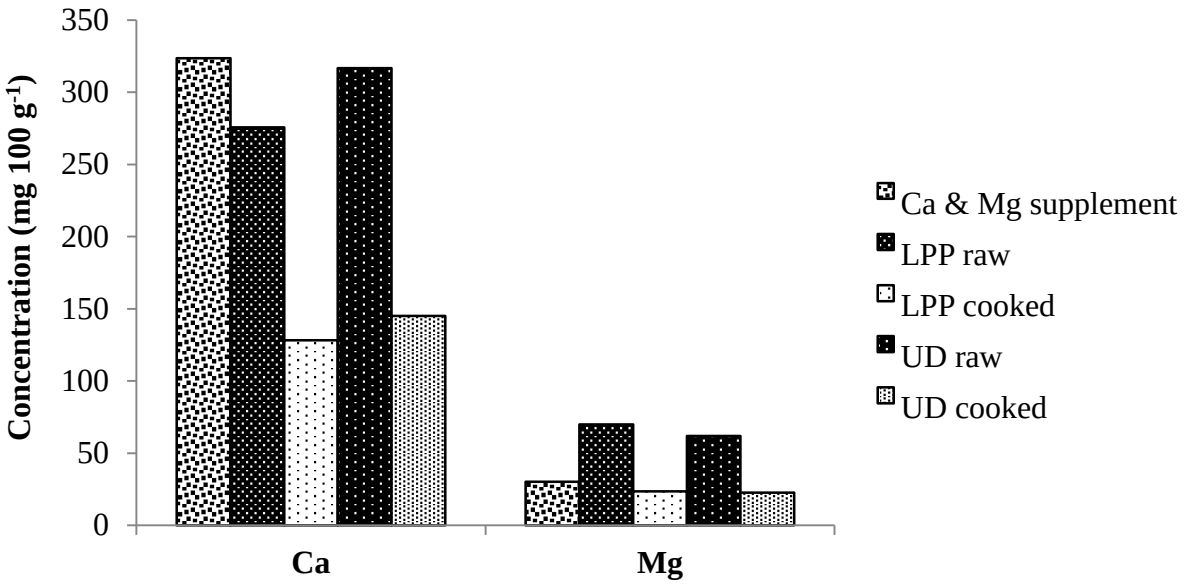

Figure 8: Comparison of Ca and Mg concentration in a nutrient supplement with LPP and UD leaves (raw and cooked).

Table 11: Dietary Reference Intakes (DRIs) (Recommended Dietary Allowance (RDAs) and Tolerable Upper Intake Levels (ULs)) of elements for most individuals and average concentration of elements (n=5) in *L. peduncularis* (LPP) and *U. dioica* (UD) leaves (raw and cooked).

Elements	Average concentration (mg day^{-1}, dry mass)				DRI[a]		Estimated contribution to RDA (%)			
	LPP raw	LPP cooked	UD raw	UD cooked	RDA	UL	LPP raw	LPP cooked	UD raw	UD cooked
Ca	2757	1283	3166	1452	1000-1300	3000	>200	99	>200	112
Cr	0.310	0.678	0.106	0.166	0.02-0.035	ND	>200	1937	>200	474
Cu	2.30	0.637	1.76	1.62	0.7-0.9	10	>200	71	>200	180
Fe	131	101	20.8	31.9	8-18	45	>200	561	>200	177
Mg	700	237	619	227	240-400	350	>100	59	>200	57
Mn	152	17.4	2.56	4.88	1.6-2.3	11	>200	757	>100	212
Ni	0.479	0.201	0.24	0.048	ND	1.0	ND	ND	ND	ND
Zn	3.75	2.52	3.08	2.60	8-11	40	24	23	28	24

[a]Institute of Medicine of the National Academies: Dietary Reference Intakes, 2011.
ND: Not determinable.

Table 12: Dietary Reference Intakes (DRIs) (Recommended Dietary Allowances (RDAs) and Tolerable Upper Intake Levels (ULs)) of macronutrients for most individuals and average concentration of macronutrients (n=5) in *L. peduncularis* (LPP) and *U. dioica* (UD) leaves (raw and cooked).

	Average concentration (g $100g^{-1}$, dry mass)				DRI[a] (g day^{-1})		Estimated contributions to RDA (%)			
	LPP raw	LPP cooked	UD raw	UD cooked	RDA	UL	LPP raw	LPP cooked	UD raw	UD cooked
Carbs	21.6	46.1	53.3	61.6	130	ND	17	35	41	47
Protein	1.37	1.78	1.48	1.83	34-56	ND	2	3	3	3
Total fibre	0.11	0.82	0.23	0.47	21-38	ND	0.3	2	0.6	1
Vitamin C	0.0181	0.0130	0.0220	0.0140	0.045-0.075	1.2-2.0	24	17	29	19
Vitamin E	0.0179	0.0232	0.0262	0.0255	0.011-0.015	0.6-1.0	>100	155	>100	170

[a]Institute of Medicine of the National Academies: Dietary Reference Intakes, 2011.

The results in Table 12 indicate that cooked LPP and UD leaves contributed a higher percentage of carbohydrates, proteins and total fibre to the diet than raw leaves. The contribution of cooked LPP leaves to the RDA for carbohydrates, proteins and total fibre is 35.3 and 2%, respectively. The vitamin E content in both plants exceeded its RDA. Cooked leaves from both of the plants would provide adequate amounts of vitamin C to the diet (17 to19%). The results indicate that LPP and UD leaves are vitamin rich and have high amounts of vitamin E which is essential for the protection of low density lipoproteins (LDLs) from oxidation and the formation of red blood cells and muscles as well as to maintain normal arterial wall flexibility (Cotter et al., 2007).

Principal component analysis and cluster analysis

The results for the principle component loading of heavy metals in LPP leaves are shown in Table 13, and the scatter plot shown in Figure 9. Principal component analysis is a multivariate technique that estimates the correlation structure of the variables (sites and metal concentration) by finding new variables (principal components), describing the data in a simplified way. It allows identifying the main direction in which the data varies; component 1 usually points to the direction where there is a larger variation (Fig. 9).

Table 13: Principal component loadings of heavy metals in *L. peduncularis* leaves.

	Pattern matrix	
	PC 1	PC 2
Eigenvalue	3.435	2.914
Percentage of total variance	38.165	32.378
Percentage of cumulative variance	38.165	70.543
Pb	0.889	0.016
Co	0.835	0.088
Ni	0.811	-0.024
Fe	0.752	0.133
Mn	0.686	-0.591
As	-0.077	-0.938
Cr	-0.199	0.919
Zn	0.185	0.901
Cu	0.043	0.407

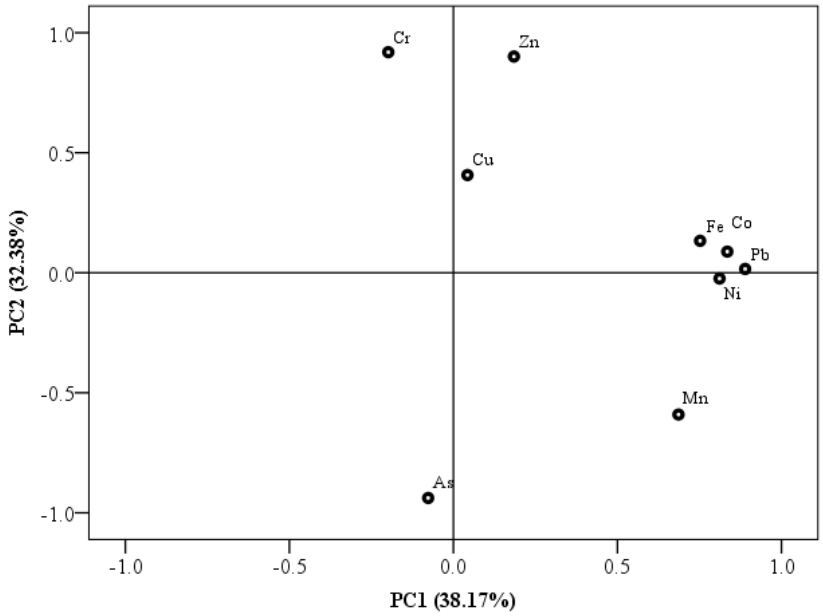

Figure 9: Principal component scatter plot of the nine elements studied in LPP leaves (constructed for all ten sampling site)**.**

A component loading that is greater than 0.4 is considered to belong to that component. Two components were extracted explaining the majority of total variance (70.5%). The metals Pb, Co, Ni, Fe and Mn were associated with the first loading. The high loading and close association (Fig. 9) of heavy metals Pb (0.889), Co (0.835), Ni (0.811) and Fe (0.752) could suggest common anthropogenic inputs. Most sampling sites were close to roads where high vehicular emissions could produce similar effects. The separation of Mn from the other metals, as indicated by Figure 9, could suggest that it came from a different source i.e. soil parent material. The second loading consisted of Cr, Zn and Cu. Higher loadings of Cr and Zn suggest that they have a common origin. The cluster analysis using Ward's method indicates the degree of association between metals in the plant, depicted by the Euclidean distance (Fig. 10). The shorter the distance the more significant is the association (Gupta & Sinha, 2007). Three main clusters corresponded with the PCA results. The first cluster showed close associations between Cr, Zn and Cu, indicating the same soil parent material and similar anthropogenic inputs. The

second cluster showed close associations between As and Mn, indicating that they originate from the same soil parent material and the third cluster showed close associations between Co, Pb, Fe and Ni which suggested similar anthropogenic inputs.

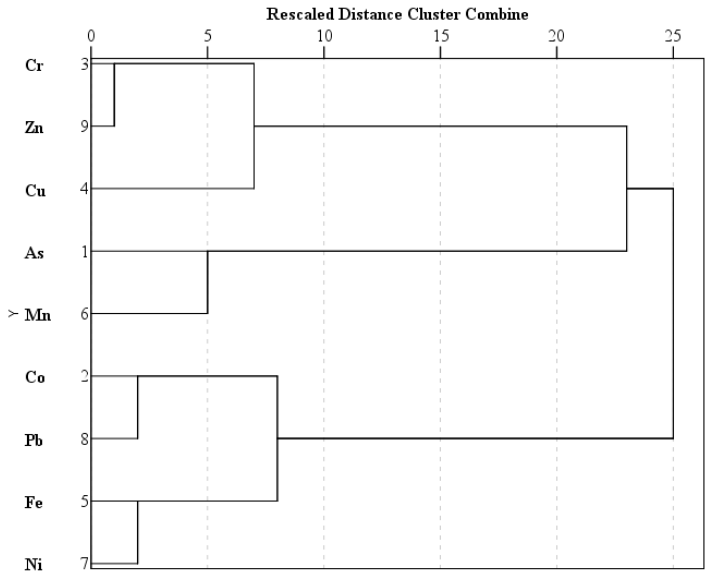

Figure 10: Dendrogram of heavy metals in LPP leaves showing distance between elements, by Ward's method.

Correlation analysis

Table 14 represents the significant correlations between macronutrients and anti-nutrients with elements that have been extracted from an inter-item correlation matrix. The study showed positive correlations between phytates and Ni (0.8), phytates and Pb (0.8). Phytates are known to strongly bind to micronutrients such as Ca, Mg, Fe and Zn thus enabling trace elements, Ni and Pb, to build up in the leaves (Bohn et al., 2008). Positive correlations were observed between vitamin E and Ni (0.8), vitamin E and Pb (0.9) whilst negative correlations were observed between vitamin C and Mn (-0.8), vitamin C and Pb (-0.8). Higher levels of vitamin C are known to decrease Mn levels (Pfeiffer & Bacchi, 1977). The results indicate that both vitamin C and E have the opposite effect on uptake of Pb with vitamin C being antagonistic

and vitamin E being synergistic. Negative correlations were also observed between energy and Cu (-0.9), Fe (-0.8) and Ni (-0.8).

Table 14: Correlation matrix of macronutrients, anti-nutrients and elements.

	As	Ca	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Ash	-0.1	0.7	0.4	-0.2	-0.5	0.6	-0.7*	-0.8*	-0.6	0.1
Fats	0.2	0.7	0.1	-0.5	-0.5	0.8*	-0.4	-0.6	-0.4	-0.4
CP	0.6	-0.6	-0.5	-0.2	0.4	-0.2	0.6	0.7*	0.8*	-0.3
CF	0.3	-0.4	-0.5	0.0	0.7	-0.5	0.9**	0.7	0.7	-0.1
Carbs	0.7	-0.4	-0.1	-0.1	0.5	0.5	0.5	0.3	0.6	-0.6
E	0.7	0.4	-0.3	-0.9**	-0.8	0.7	0.9*	-0.8	0.6	-0.6
Sap	-0.6	0.0	0.5	0.8*	0.5	-0.3	-0.1	0.3	0.0	0.4
Ox	-0.3	0.6	0.2	0.1	-0.5	0.5	-0.6	-0.7	-0.7	-0.1
Phy	0.3	-0.7	-0.4	0.0	0.5	-0.4	0.6	0.8*	0.8*	0.0
CN	-0.3	0.7	0.4	0.0	-0.6	0.5	-0.7	-0.7*	-0.7*	-0.1
Vit. C	-0.7*	0.4	0.7	0.3	-0.3	0.1	-0.8*	-0.7*	-0.8*	0.3
Vit. E	0.4	-0.5	-0.4	0.0	0.2	-0.3	0.6	0.8**	0.9**	0.1

*, **: correlations significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.
Carbs: carbohydrates, CF: crude fibre, CN: cyanides, CP: crude protein, E: energy, Ox: oxalates, Phy: phytates, Sap: saponins, Vit. C: vitamin C, Vit. E: vitamin E.

CONCLUSION

The results indicate that both the macronutrient and anti-nutrient content of the leaves of nettles are affected by cooking. The essential elements in cooked LPP leaves were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Cr > Ni > Co. Statistical analyses, PCA and CA, indicated that certain elements taken up by the plants were from common sources. Correlation analyses revealed relationships between macronutrients, anti-nutrients and elements in the leaves; significant amongst these being the antagonistic and synergistic relationship between Pb and vitamin C and E, respectively. Both LPP and UD leaves appear to be rich sources of essential elements and vitamins and, in South Africa, can be used as a cheaper, more organic and more accessible alternative to commercially available nutrient supplements.

Acknowledgements

The authors like to thank the National Research Foundation (NRF), South Africa for financial support and the School of Chemistry and Physics, UKZN for the research facilities.

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CHAPTER THREE

The distribution of macronutrients, anti-nutrients and essential elements in nettles, Laportea alatipes (forest nettle) and Obetia tenax (mountain nettle)

ABSTRACT

Nettles are commonly consumed in South Africa, Europe and Asia for their nutritional benefits and are also used in traditional medicine to treat a variety of ailments. In this study, the nutritional, anti-nutritional and elemental composition of the leaves of nettles, *Laportea alatipes* (forest nettle) and *Obetia tenax* (mountain nettle) in the cooked and raw state were compared. The contribution of the nettles to the diet was also evaluated. The results show a significant decrease in the crude fat, crude protein, vitamin C and E content and a significant increase in carbohydrate and crude fibre content with cooking, in both nettles. Also, a decrease in the vitamin A content was observed in *L. alatipes*. The anti-nutrient (cyanide, oxalates, saponins and phytates) and toxic element (Cd and Pb) content decreased with cooking. The nettles, *L. alatipes* and *O. tenax*, have higher macronutrient content than elemental content relative to the nettles, *L. peduncularis* (river nettle) and *U. dioica* (stinging nettle), after cooking. Statistical analysis showed positive correlations between the ash content and minor elements in *L. alatipes* leaves whilst positive correlations between phytates, vitamin E and vitamin C were observed. Negative correlations between crude fibre and ash in *L. alatipes* leaves and saponins with Cd and Fe in *O. tenax* leaves were attributed to the complexing ability of crude fibre and saponins with minerals in the plant. Principal component analysis and cluster analysis confirmed the correlation between ash content and the presence of elements as well as the common origin of the nutrients taken up by the nettles.

Keywords proximate chemical composition, macro-nutrients, micro-nutrients, vitamins

INTRODUCTION

Malnutrition results from an inadequate intake of food or improper diet and it can affect normal functioning of the human body as well as growth and development in children (Rubatzky & Yamaguchi, 1997). Malnutrition is a serious concern in developing countries such as Asia, Africa, Latin America and the Middle East with one in five people being malnourished and experiencing the conditions associated with micronutrient deficiencies (FAO, 1997). For proper nourishment, the human body needs foods rich in macronutrients (carbohydrates, fats, proteins and vitamins) and micronutrients (minerals) but low in sugar. An unhealthy diet consists of high amounts of sugars and calories and low amounts of fruits and vegetables; this can be detrimental to human health and was reported to be the number one cause of premature death in the United States (Murray et al., 2013). It can also increase the risk of non-communicable diseases (NCDs) such as cardiovascular diseases, chronic respiratory diseases and cancer. In South Africa, NCDs account for 37% of deaths (Puoane et al., 2013); the World Health Organization (WHO) has therefore suggested a daily intake of 400 g of fruits and vegetable per day to reduce the risk of NCDs (World Health Organization, 2015).

Food insecurity also plays a role in malnutrition as nutritious foods are inaccessible, unaffordable or unavailable. Leafy green vegetables are important in developing countries because they are cheap, readily available, nutritious and easy to cook (Gupta & Wagle, 1988). Vitamin A (a fat soluble vitamin), can be found as retinoids or carotenoids in plants and the carotenoid (β-carotene (provitamin A)) is present in leafy green vegetables. Vitamin A is required by the human body for normal vision, growth and immune function and its deficiency may compromise the immune system which can lead to infectious illnesses such as diarrhoea and respiratory diseases (D'Ambrosio et al., 2011).

Vitamin C (ascorbic acid) is a water soluble vitamin synthesized by plants and animals (not humans) to meet their physiological requirements; humans are only able to obtain it from the diet (Naidu, 2003). Vitamin C functions as an antioxidant and plays an important role in the immune system. It also improves the body's absorption of Cr, Cu and Fe from plants into the gastrointestinal tract (Bobroff & Valentín-Oquendo, 2014; McGuire & Beerman, 2007). Vitamin E, which is only synthesized by plants, is a fat soluble vitamin which functions as a chain breaking antioxidant. It consists of tocopherols and tocotrienols, of which, α-tocopherol has high nutritional importance and is found in leafy green vegetables (Brigelius-Flohé & Traber, 1999).

Minerals are the elements that regulate the metabolic activities in the body and consist of macro-elements and micro-elements. Macro-elements are needed in amounts greater than 100 mg day^{-1} and include Ca, Mg and P. Calcium is essential for the formation of bones and nerve conduction, Mg stabilizes the structure of ATP in ATP-dependent enzyme reactions important for neuromuscular transmission and activity, and P is found in nucleic acids and is critical in the cells' transfer of energy as part of ATP. Micro-nutrients are needed in smaller amounts in the body and include Co, Cr, Cu, Fe, Mn, Ni and Zn. Micro-nutrients may be components of many enzymes; Cu is required for the absorption of Fe and forms part of many enzymes, Fe is a constituent of red blood cells and Mn is required for several essential enzymes (Driskell, 2000).

Plant anti-nutrients are compounds that reduce the body's ability to absorb essential nutrients from the digestive system by disturbing enzymatic processes (Ong, 2008). The presence of anti-nutrients in the diet is of concern mostly to vulnerable communities that suffer from malnutrition or who base their diet on grains, legumes and wild herbs. Phytates reduce the bioavailability of essential nutrients and alter the solubility of proteins (Deshpande, 2002). High amounts of cyanide in plant food can cause cyanide poisoning, oxalates can cause renal

stones and convulsions, and saponins can cause haemolysis (Deshpande, 2002). Anti-nutrients cannot be removed from food completely but cooking for several minutes or hours can denature them. Anti-nutrients are only considered harmful if at elevated levels; low levels of anti-nutrients are deemed beneficial to human health.

The consumption of nettles is recommended to help alleviate vitamin C, Vitamin A and Fe deficiencies as these plants are known to be high in these vitamins and nutrients (Kohlstadt, 2009). Nettles are consumed for their nutritional value either steamed or cooked, similar to spinach, or served as a salad. In traditional medicine, nettles are used to treat arthritis and gout. Previously, we reported on the distribution of nutrients and anti-nutrients in the nettles, *Laportea peduncularis* susp. *peduncularis* (river nettle) and *Urtica dioica* (stinging nettle). In this study, we investigate the concentrations of nutrients and anti-nutrients in the nettles, *Laportea alatipes* (forest nettle) and *Obetia tenax* (mountain nettle) also found in KwaZulu-Natal, South Africa and compare these values to those obtained from the previously studied nettles. The impact of cooking on nutritional value is also evaluated.

MATERIALS AND METHODS

Sample collection and preparation

Laportea alatipes and *Obetia tenax* leaves were collected from KwaZulu-Natal, South Africa and oven-dried at 50 °C. Dried leaves were divided into two parts, one part was crushed using a food processor and placed in polyethylene bottles (uncooked) and the other was subjected to conventional cooking on a hotplate at 70 °C by boiling in double distilled water for 15 min. Leaves were cooled, sieved, dried and crushed using a food processor then placed in polyethylene bottles.

Nutrient analysis

Moisture content was determined by the loss of weight after heating to constant mass in the oven at 105 °C for 24 h (AOAC, 1992). The protein content was calculated using a factor of 6.25 after estimating the nitrogen content using the Kjeldahl distillation method (Skoog et al., 2004). The ash content was determined by igniting the leaves in a muffle furnace at 600 °C for 12 h (Elhassan & Yagi, 2010). Fat content was determined by the soxhlet method using n-hexane. For crude fibre, fat-free samples were digested with 0.128 M H_2SO_4 and 0.313 M NaOH. The insoluble residue was washed with hot water and dried at 130 ºC, then weighed to constant mass. The dried residue was incinerated at 600 ºC for 3 h and the ash was weighed to determine the crude fibre content (FAO, 1994). Total carbohydrate was obtained by difference and the energy value was determined using Equation 7 (FAO, 2003).

$$\text{Energy value}\left(\text{kJ } 100\ \text{g}^{-1}\right) = [(37 \times \%\text{lipids}) + (17 \times \%\text{carbohydrates}) + (17 \times \%\text{protein})] \quad (4)$$

The vitamin C content was determined by the iodometric method (Igwemmar et al., 2013). The vitamin A content (β-carotene) was determined by the methods of Neeld and Pearson (1963) and Besler et al. (2002) with some modifications. Raw and cooked leaves (0.2 g) were mixed with 1 mL cold ethanol then extracted with 2 mL hexane followed by vortex mixing for 2 min and centrifugation at 1500 rpm for 10 min. Beta-carotene was determined spectrophotometrically (Biochrom Libra S11, Cambridge, England) at 450 nm. Vitamin E content was determined as described by Desai (1984) with some modifications. Raw and cooked leaves (0.2 g) were extracted with 1 mL hexane, mixed with 0.5 mL ethanol and 0.25 mL of 25% ascorbic acid then pre-incubated at 70°C for 5 min. Thereafter, 0.3 mL potassium hydroxide was added and the mixture was further incubated for 30 min. The mixture was cooled in an ice bath. Hexane (4 mL) was added, and the mixture centrifuged at 1500 rpm for 10 min. The separated hexane (supernatant) was used to estimate vitamin E content using a UV

spectrophotometer. A calibration curve of α-tocopherol at various concentrations (emission at 330 nm) was used to determine the vitamin E content in the leaves.

Elemental analysis was determined by accurately weighing powdered leaf samples into liners then adding 10 mL of 70% HNO_3. For digestion, the power was set at 100% at 1600 W and the temperature was ramped to 180 ºC (15 min) where it was held for 15 min. Digested samples were transferred into 25 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles until analysed.

All samples were analysed for the following elements As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, Se and Zn. Elemental analysis was carried out by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). The accuracy of the elemental determination was measured by use of the certified reference material (CRM), White Clover (BCR 402), from the Community Bureau of Reference of the Commission of the European Communities. All samples were analysed in quadruplicate (n = 4).

Anti-nutrient analysis

The alkaline titration method of AOAC (1990) was used for the determination of hydrocyanic acid and calculated as: 1 mL $AgNO_3$ = 1.08 mg HCN (5)

The oxalate content was determined by the method of Day and Underwood (1986) and calculated as: 1mL 0.05 M $KMnO_4$ = 2.2 mg oxalate (6)

The phytate content was determined by the method of Reddy and Love (1999) with some modifications. A sample of ground leaves (0.1 g) was soaked in 100 mL of 2% HCl for 5 h then filtered. To a 25 mL aliquot of the filtrate, 5 mL of 0.3% potassium ferricyanide solution was added. The mixture was titrated against $FeCl_3$ solution until a blue-green colour persisted for 5 min. The saponin content was determined by the method of Hudson and El Difrawi

(1979). A sample of ground leaves (10 g) was mixed with 20 mL of 20% aqueous ethanol and agitated with a magnetic stirrer for 12 h at 55 ºC. The solution was filtered and the residue was re-extracted with 200 mL of 20% aqueous ethanol. The extract was reduced to 40 mL under vacuum and 20 mL of dichloromethane (DCM) was added and separated in a separating funnel. The aqueous layer was recovered and the DCM layer discarded. The pH of the aqueous layer was adjusted to 4.5 by the addition of NaOH. The solution was then shaken with 60 mL of n-butanol. The n-butanol extract was washed twice with 10 mL of 5% NaCl and evaporated to dryness in a fume hood to produce the crude saponin.

Statistical analysis

All data was generated using the Statistical Package for the Social Sciences (PASW Statistics, Version 23, IBM Corporation, Cornell, New York). Analysis of variance (ANOVA) was performed on data and the means were separated by Tukey's Post-hoc test to determine significant differences. Principal component analysis (PCA) was performed using the principal component method on the dataset and cluster analysis (CA) was applied to the standardized matrix of samples using Ward's method. Correlations amongst nutrients and anti-nutrients were determined by Pearson's correlation analysis.

RESULTS AND DISCUSSION

Quality Assurance

Measured values (in µg g^{-1}) of the CRM, White Clover (BCR 402), for Fe (250 ± 18.0), Se (6.81 ± 1.40) and Zn (30.3 ± 6.37) were within certified values (in µg g^{-1}) for Fe (244), Se (6.70 ± 0.25) and Zn (25.2).

Nutrient and anti-nutrient analysis

The macronutrient, energy and vitamin content of raw and cooked leaves of the nettles *L. alatipes* and *O. tenax* are presented in Table 15. The moisture content of raw leaves was 9.7% (*L. alatipes*) and 9.6% (*O. tenax*). After cooking, the ash, crude fat and crude protein content decreased significantly in leaves of both plants. However, there was a significant increase in crude fiber (178% in *L. alatipes* and 233% in *O. tenax*) and carbohydrate (1.4% in *L. alatipes* and 27% in *O. tenax*) similar to our previous study on *Laportea peduncularis* and *Urtica dioica* (Mahlangeni et al., 2016). Previous studies conducted by Pérez-Hildago et al. (1997) showed an increase in dietary fibre of chickpeas with cooking. This could be due to the formation of resistant starch together with condensed tannin-protein products that increases the fibre content (Mongeau & Brassard, 1995). Heat during cooking may modify the cell walls and complex sugars thereby reducing the solubility of dietary fibre. Carbohydrates are more bioavailable as a result of cooking (Joshi, 2002). The energy content of *L. alatipes* leaves decreased similar to our previous study on *U. dioica* leaves whilst that of *O. tenax* leaves increased similar to our previous study on *L. peduncularis* leaves (Mahlangeni et al., 2016).

Vitamin A is essential for proper functioning of the retina and for the integrity of epithelial tissue (van Boxtel, 2008). There was a slight increase (2.6%) in the vitamin A content of *O. tenax* leaves form raw to cooked whilst there was a significant decrease (95%) in *L. alatipes* leaves. A significant decrease in vitamin C (7.2% in *L. alatipes* and 0.7% in *O. tenax*) and E (60% in *L. alatipes* and 69% in *O. tenax*) was observed in leaves of both plants. Vitamins are heat sensitive thus susceptible to loss during cooking. The vitamin C content in leaves of both plants (in mg 100 g^{-1}) (39 in *L. alatipes* and 38.7 in *O. tenax*) was higher than that of *L. peduncularis* (18.1), *U. dioica* (22), *Bidens pilosa* (23) and *Spinacia oleracea* (28) (Kruger et al., 1998; Mahlangeni et al., 2016; Steyn et al., 2001).

Table 15: Proximate chemical composition (dry weight basis) (Ash, crude fats, crude fiber, crude protein, carbohydrates), vitamin A, C, E content and energy content of raw and cooked nettles.

Nettles		Ash	Crude Fats	Crude Fiber	Crude Protein	Carbs	Vitamin A (μg 100 g^{-1})	Vitamin C (mg 100 g^{-1})	Vitamin E (mg 100 g^{-1})	Energy (kJ)
Laportea alatipes	Raw	17.9 ± 2.4 [a*]	1.7 ± 0.21 [a]	8.9 ± 1.4 [a]	6.6 ± 0.01 [a]	64.9	99 ± 18 [b]	39 ± 0.1 [a]	98 ± 20 [a]	1278
	Cooked	3.3 ± 0.63 [b]	0.86 ± 0.09 [b]	24.7 ± 4.3 [b]	5.4 ± 0.01 [b]	65.8	4 ± 0.9 [a]	36 ± 0.7 [b]	39 ± 6 [b]	1239
Obetia tenax	Raw	28.1 ± 4.7 [c]	3.95 ± 0.76 [c]	5.9 ± 1.0 [a]	6.5 ± 0.14 [a]	55.6	106 ± 17 [b]	39 ± 0.2 [a]	111 ± 12 [a]	1199
	Cooked	2.4 ± 0.48 [b]	1.4 ± 0.19 [ab]	19.7 ± 1.6 [b]	5.7 ± 0.05 [c]	70.8	109 ± 15 [b]	39 ± 2 [a]	35 ± 5 [b]	1353

[*]Values are represented as mean ± S.D., n=4.

Values in the same column with different superscript letters are significantly different (Tukey's post hoc comparison, $P \leq 0.05$).

The results of the anti-nutrient content of *L. alatipes* and *O. tenax* leaves are presented in Table 16. Anti-nutrients are known to decrease the bioavailability of nutrients. The results reveal a decrease in the anti-nutrient content of leaves of *L. alatipes* and *O. tenax* for all anti-nutrients studied (cyanide, oxalate, phytate and saponin) after cooking. This was contrary to our previous study on *L. peduncularis* and *U. dioica* leaves that showed an increase in the cyanide, phytate and saponin content after cooking. This proves that thermal processing of plants alters the anti-nutrient content (Arinola & Adesina, 2014; Rehman & Shah, 2005).

Table 16: Anti-nutrient composition of raw and cooked nettles.
*Values represented as mean ± S.D., n=4.

		Cyanides (mg 100 g^{-1})	Oxalate (mg 100 g^{-1})	Phytate (µg 100 g^{-1})	Saponin (g 100 g^{-1})
Laportea alatipes	Raw	932 ± 125 ^{*a}	332 ± 292 ^{a}	3.2 ± 0.1 ^{a}	12.8 ± 2.3 ^{a}
	Cooked	401 ± 93 ^{b}	218 ± 14 ^{b}	2.6 ± 0.2 ^{b}	11.73 ± 0.73 ^{a}
Obetia tenax	Raw	1069 ± 177 ^{a}	290 ± 50 ^{a}	2.6 ± 0.1 ^{bc}	13.0 ± 1.9 ^{a}
	Cooked	598 ± 81 ^{b}	191 ± 29 ^{b}	2.3 ± 0.2 ^{c}	12.2 ± 1.7 ^{a}

Values in the same column with different superscript are significantly different (Tukey post hoc comparison, $P \leq 0.05$.

Elemental analysis

Food processing such as cooking has an effect on the amount of elements retained in the plant as well as its bioavailability. The raw and cooked leaves of *L. alatipes* and *O. tenax* were assessed for macro, micro and trace elemental content and the results are presented in Table 17. Essential plant macro-elements are needed for healthy plant growth (Roy et al., 2006). There was a significant decrease in the Ca concentration in *L. alatipes* leaves and Mg concentrations in both plants after cooking. There was a 36% increase in P content in *L. alatipes* leaves and a significant increase in Fe concentration in both plants after cooking. Phytic acid (myo-inositol 1,2,3,4,5,6 hexakis-dihydrogen phosphate) is the major P storage compound and

phytates form complexes with Fe. Cooking of the leaves therefore releases the bound P and Fe increasing their bioavailability (Kumar et al., 2010).

Micro-elements are needed by the plant for normal growth and functioning and is a source of nutrition for humans (Bruulsema et al., 2012). A significant decrease in Cr, Mn and Zn whilst a significant increase in Ba and Cu concentrations was observed with cooking, in both plants. The concentration of Ni decreased by 37% in *O. tenax* leaves and increased by 86% in *L. alatipes* leaves with cooking. Cobalt concentrations increased by 84% and 87% in *L. alatipes* and *O. tenax* leaves, respectively, after cooking.

Cadmium is one of the most mobile trace elements and readily taken up by plants from the soil, where it can accumulate (Hajeb et al., 2014). Studies have shown that Pb accumulates in the plant only when present in soil (Chary et al., 2008). Concentrations of toxic elements decreased significantly in *L. alatipes* (57% for Cd and 34% for Pb) and *O. tenax* (74% for Cd and 64% for Pb) leaves. This shows that cooking reduces the levels of toxic elements in the plants.

Table 17: Concentration (µg g^{-1}, mean ± S.D., n=4) of essential and toxic elements in *L. alatipes* and *O. tenax* leaves (raw and cooked). Dietary reference intakes (DRIs) (recommended dietary allowance (RDAs) and tolerable upper intake levels (ULs)) of elements for most individuals and average contribution of nettles (*L. alatipes* (LA), *O. tenax* (OT) *L. peduncularis* (LP) and *U. dioica* (UD)) for essential elements to the diet (in mg per 60 g, dry mass) (n=4) in both raw and cooked leaves (raw/cooked).

	Obetia tenax		*L. alatipes*		DRI[a]		LA	OT	LP[b]	UD[b]
	Raw (µg g^{-1})	Cooked (µg g^{-1})	Raw (µg g^{-1})	Cooked (µg g^{-1})	RDA (mg day^{-1})	UL (mg day^{-1})	Raw/Cooked (mg 60 g^{-1})	Raw/Cooked (mg 60 g^{-1})	Raw/Cooked (mg 60 g^{-1})	Raw/Cooked (mg 60 g^{-1})
Macro-elements										
Ca	34084±1 074[c*]	12652±1598[a]	21990±1 818[b]	24316±1 542[b]	1000-1300	3000	20.5/7.60	13.2/14.6	1654/770	1899/871
Fe	6114±687[c]	39196±5376[a]	12045±1 060[bc]	16807±2 035[b]	8-18	45	3.67/23.7	7.27/10.1	78.7/60.6	12.5/19.1
Mg	12407±432[c]	2023±212[a]	10648±544 [d]	4075±155[b]	240-400	350	7.50/1.21	6.42/2.45	420/142	371/136
P	1264±112[b]	1715±108[a]	2135±153[c]	1340±74.6[b]						
Micro-elements										
Ba	57.7±6.26[c]	77.0±5.22[a]	88.1±5.00[a]	151±7.41[b]						
Co	1.42±0.139[b]	2.61±0.439[a]	6.64±0.430[c]	0.845±0.118[b]						
Cr	12.1±1.32[b]	0.073±0.00[a]	87.7±6.11[c]	0.074±0.001[a]	0.02-0.035	ND	0.007/0	0.053/0	0.186/0.407	0.063/0.099
Cu	19.1±1.02[b]	32.0±1.43[a]	23.9±2.01[c]	35.1±2.11[a]	0.7-0.9	10	0.011/0.019	0.015/0.021	1.38/0.382	1.05/0.97
Mn	260 ± 18.2[c]	180±24.9[a]	206±14.3[a]	76.1±3.34[b]	1.6-2.3	11	0.15/0.11	0.13/0.045	91.3/10.5	1.53/2.93
Ni	6.36±1.39[a]	11.8±0.95[b]	15.8±1.04[c]	8.42±1.19[a]	ND	1.0	0.003/0.007	0.009/0.005	0.287/0.121	0.144/0.029
Zn	60.7±4.51[b]	50.9±4.31[a]	34.3±0.705[c]	47.1±6.76[a]	8-11	40	0.037/0.031	0.021/0.028	2.25/1.51	1.85/1.56
Toxic-elements										
Cd	1.86±0.207[b]	0.806±0.168[a]	2.97±0.177[c]	0.780±0.167[a]						
Pb	2.87±0.250[c]	1.88±0.134[a]	2.21±0.128[a]	0.795±0.157[b]						

The results in Table 17 also show the concentration of elements in, *L. alatipes*, *O. tenax*, *L. peduncularis* and *U. dioica* leaves in comparison to the RDAs (Institute of Medicine of the National Academies, 2011). The concentrations of elements in the nettles (*L. peduncularis* and *U. dioica*) from our previous study were compared to the nettles, *L. alatipes* and *O. tenax* contribution in the current study. Low fruit and vegetable intake by individuals has contributed to micronutrient deficiencies. The World Health Organization recommends consumption of a minimum of 400 g of fruits and vegetables daily (Joint FAO/WHO, 2004). Studies have shown that the average consumption of vegetables is less than 80 g per day (Food and Agriculture Organization of the United Nations, 2003). The serving amount was related to spinach where two cups are equivalent to 60 g, based on dry mass. This will give an estimate of the contribution made by nettles to an individual's diet thereby decreasing the risk of nutrient deficiencies. If nettles are consumed after cooking, a decrease in contribution to the diet is observed due to the leaching of nutrients into the cooking water.

Generally, nettles had high Fe contribution specifically *L. peduncularis* which would be beneficial to individuals who are suffering from Fe deficiency anaemia (Phatlhane et al., 2016). The most common elemental deficiencies in humans are for Fe, Zn and Cu (Bruulsema et al., 2012). *L. alatipes* and *O. tenax* both contribute 3% towards the RDA for Cu and both contribute 0.3% towards the RDA for Zn; this is much lower than the nettles *L. peduncularis* (42% Cu and 108% Zn) and *U. dioica* (14% Cu and 14% Zn). Therefore, with regards to minerals the nettles *L. peduncularis* and *U. dioica* are richer in minerals than *L. alatipes* and *O. tenax*.

The mineral content of the nettles (*L. alatipes*, *O. tenax*, *L. peduncularis* and *U. dioica*) was compared to that of common leafy green vegetables (lettuce, spinach and cabbage) (Table 18). The results show that Co, Cr, Cu, Mn, Ni and Zn concentrations are higher in *L. alatipes* and *O. tenax* leaves compared to lettuce, spinach (except for Zn in South African spinach) and cabbage. Furthermore, amongst the four nettles, *O. tenax* had the highest concentrations of Co,

Cr, Cu, Fe and Ni. Higher concentrations of Mn were observed in *L. alatipes* leaves compared to the other three nettles. This shows that nettles have higher concentrations of micro-elements which can be beneficial to human health.

Lead content in lettuce, spinach and cabbage ranged from 0.013-3.6 µg g^{-1}, 0.134-82.9 µg g^{-1} and 0.4-3.1 µg g^{-1}, respectively. Lead in all nettles was below 3 µg g^{-1}.

Table 18: Average concentration of micro-elements in uncooked leafy green vegetables (lettuce, spinach and cabbage) and nettles (µg g^{-1}).

Vegetables	Country	Co	Cr	Cu	Fe	Mn	Ni	Zn	References
Lettuce	Egypt	-	-	1.97	-	-	-	9.76	Radwan & Salam, 2006
	Greece	-	0.036	0.17	4.04	0.95	0.05	1.01	Stalikas et al., 1997
	Tanzania	-	-	5.8	-	-	-	15.9	Bahemuka & Mubofu, 1999
Spinach	Egypt	-	-	4.48	-	-	-	20.9	Radwan &Salam, 2006
	Greece	0.026	0.130	2.45	21.5	4.42	0.52	2.99	Stalikas et al., 1997
	South Africa	-	10.05	10.64	2 840	140	5.11	70	Lion & Olowoya, 2013
	Tanzania	-	-	13.7	-	-	-	48.1	Bahemuka & Mubofu, 1999
Cabbage	South Africa	-	-	1.18	-	23.56	-	29.6	Bvenura & Afokeyan, 2012
	Tanzania	-	-	5.6	-	-	-	41.8	Bahemuka & Mubofu, 1999
	Zimbabwe	-	0.5	0.2	-	-	0.5	3.2-15	Mapanda et al., 2007
Nettles									
Laportea alatipes	South Africa	1.42	12.1	19.1	6 114	260	6.36	60.7	This study
Obetia tenax	South Africa	6.64	87.7	23.9	12 045	206	15.8	34.3	This study
Laportea peduncularis	South Africa	0.33	3.10	23.0	1 310	152	4.79	26.0	Mahlangeni et al., 2016
Urtica dioica	South Africa	ND	1.06	17.6	208	25.6	2.40	37.5	Mahlangeni et al., 2016

ND – not determined due to concentrations being below the instrument detection limit.

Table 19: Dietary reference intakes (DRIs) (recommended dietary allowance (RDAs) and tolerable upper intake levels (ULs)) of macronutrients for most individuals and average concentration of macronutrients (n=4) in *L. alatipes* (LA), *O. tenax* (OT) *L. peduncularis* (LP) and *U. dioica* (UD) leaves (raw/cooked).

	Average concentration in raw/cooked leaves (g 60 g^{-1}, dry mass)				DRI[a] (g day^{-1})	
	LA	OT	LP[b]	UD[b]	RDA	UL
Carbs	35.1/35.7	30.2/38.4	12.9/27.7	32.0/36.9	130	ND
Protein	3.22/2.94	3.20/3.11	0.82/1.07	0.89/1.10	34-56	ND
Total fibre	4.81/12.1	3.21/9.67	0.067/0.49	0.14/0.28	21-38	ND
Vitamin A[c] (µg 60 g^{-1})	9.93/0.40	10.6/10.9	-	-	600-900	3000
Vitamin C	0.023/0.022	0.023/0.023	0.011/0.010	0.013/0.010	0.045-0.075	1.2-2.0
Vitamin E	0.059/0.023	0.067/0.021	0.011/0.014	0.016/0.015	0.011-0.015	0.6-1.0

[a]Institute of Medicine of the National Academies: Dietary Reference Intakes, 2011.
[b]Mahlangeni et al., 2016.
[c]Vitamin A as retinol; 1 µg β-carotene = 0.167 µg retinol.

The results in Table 19 show the concentration of macronutrients in the different nettles in comparison to the RDA. Cooked leaves of nettles, *L. alatipes* and *O. tenax*, contribute more to protein (5% and 6%, respectively), fibre content (32% and 25%, respectively), vitamin C (29% and 31%, respectively) and vitamin E (153% and 140%, respectively) compared to the nettles, *L. peduncularis* and *U. dioica*. The nettles, *L. alatipes* and *O. tenax*, have higher macronutrient content than elemental content relative to the nettles, *L. peduncularis* and *U. dioica*, after cooking. Vitamin A is one of the most common nutrient deficiencies in South Africa, and this deficiency has been linked to non-communicable diseases. The vitamin A content was only determined in this study for *L. alatipes* and *O. tenax* nettles and contributes 0.04% and 0.1%, respectively to the diet.

Correlation analysis, principal component and cluster analysis

The results in Table 20 and 21 represent the correlation coefficients of macronutrients, anti-nutrients and minerals extracted from an inter-item correlation matrix. Correlation coefficients approaching 1 (0.9 to 1) indicated positive correlation and values approaching -1 (-0.9 to 1) indicated negative correlation. In *L. alatipes* leaves a positive correlation for ash and Cd (1.0) and ash and Co (0.9) was observed, whilst a negative correlation between crude fibre and ash (-1.0), Cd (-1.0) and Co (-0.9) was observed. Crude fibre is known to bind minerals rendering them unavailable (Joshi, 2002). A positive correlation between saponins and vitamin E (0.9), and saponins and crude protein (0.9) was observed. Saponins have the ability to complex with sterols and since vitamin E is fat soluble, an increase in saponin content will increase vitamin E (Waller & Yamasaki, 1996). In *O. tenax* leaves negative correlations between saponins and Cd (-1.0) and saponins and Fe (-1.0) as well as crude fibre and Pb (-1.0), and crude fibre and Zn (-1.0) were observed. Saponins are known to form insoluble complexes with Fe. Studies by Southon et al. (1988) showed a decreased in Fe absorption in rats with an increase in saponin concentration. Positive correlations between phytates and vitamin C (0.9) and vitamin E (1.0) were observed. Decreases in phytates lead to a decrease in the vitamin C and E content.

Table 20: Correlation matrix of macronutrients, anti-nutrients and elements in *L. alatipes* leaves.

	Ash	Crude fibre	Cd	Co
Ash	1			
Crude fibre	-1.0	1		
Cd	1.0	-1.0	1	
Co	0.9	-0.9	1.0	1

	Saponins
Crude protein	0.9
Vitamin E	0.9

Table 21: Correlation matrix of macronutrients, anti-nutrients and elements in *O. tenax* leaves.

	Cd	Fe	Saponins
Cd	1		
Fe	0.9	1	
Saponins	-1.0	-1.0	1

	Crude fibre	Pb	Zn
Crude fibre	1		
Pb	-1.0	1	
Zn	-1.0	1.0	1

	Phytates	Vitamin C	Vitamin E
Phytates	1		
Vitamin C	0.9	1	
Vitamin E	1.0	1.0	1

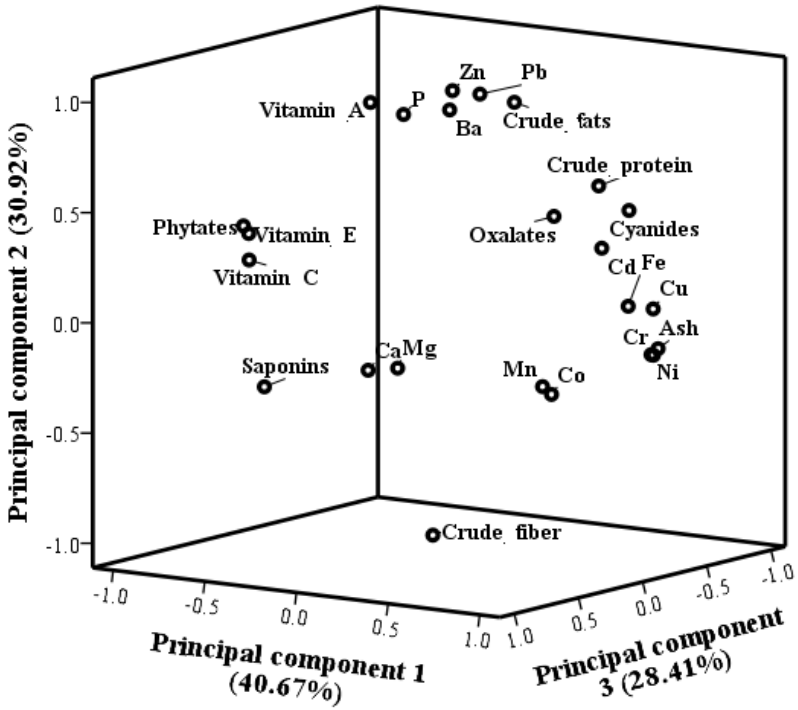
Table 22 presents the rotated component matrix of nutrients, anti-nutrients and elements in *L. alatipes* and *O. tenax* leaves whilst the scatter plots are illustrated in Figure 11. Principal component analysis (PCA) is a statistical tool that examines the interrelations amongst a set of variables in order to identify the underlying structure of those variables (Shippenburg University, 2012). Correlations between variables close to 1 were considered in each principal component (PC). There were three PC's extracted for *L. alatipes* and *O. tenax* leaves which accounted for 100% of the total variance for each plant. In *L. alatipes* leaves, there were high loadings of oxalates (0.879), ash (0.978), Cd (0.968), Co (0.975), Cr (0.965) and Pb (0.878) in the first PC (40.67%), suggesting that the ash content contained high amounts of trace elements Cd, Co, Cr and Pb whilst oxalates functions as chelating agents for these elements. The second PC had high loadings of vitamin C (0.962), Ca (0.980), Mg (0.929) and P (0.924). Calcium, Mg and P are needed in large amounts for cellular communication. The third PC was in agreement with the correlation analysis were high loadings of saponins, vitamin E and crude protein were observed. In *O. tenax* leaves, there were high loadings of ash and the elements Cd, Cr, Fe, Mn, and Ni suggesting that the ash content mostly contained these elements. High loadings of vitamin A (0.952), crude fats (0.938), Ba (0.964), P (0.947), Pb (0.938) and Zn

(0.953) were observed in the second PC. Vitamin A is fat soluble therefore could be found incorporated in the cellular lipids of plants. Again, high loadings of vitamin C, Ca and Mg were observed in the third PC as in the second PC of *L. alatipes*.

Table 22: Principal component loadings of nutrients and anti-nutrients in *L. alatipes* and *O. tenax* leaves.

	Rotated component matrix					
	L. alatipes			*O. tenax*		
	1	2	3	1	2	3
Eigenvalue	9.761	7.420	6.819	8.693	7.776	7.531
Percentage of total variance	40.672	30.916	28.412	36.220	32.399	31.381
Percentage of cumulative variance	40.672	71.588	100.000	36.220	68.619	100.000
Cyanides	-0.983	0.149	0.104	0.498	0.402	-0.769
Oxalates	**0.879**	-0.173	0.443	-0.042	0.293	-0.955
Phytates	-0.230	0.618	0.752	-0.587	0.432	0.685
Saponins	0.448	0.200	**0.871**	-0.905	-0.420	0.068
Vitamin A	-0.437	-0.439	0.785	-0.250	**0.952**	0.176
Vitamin C	0.043	**0.962**	0.271	-0.451	0.312	**0.836**
Vitamin E	0.316	0.438	**0.841**	-0.511	0.414	0.753
Ash	**0.978**	0.180	0.109	**0.888**	-0.139	-0.439
Crude fats	0.523	-0.843	0.124	0.227	**0.938**	-0.263
Crude protein	0.279	0.040	**0.959**	0.294	0.484	-0.825
Crude fiber	-0.963	-0.235	-0.130	0.126	-0.966	0.228
Ba	0.669	0.725	-0.165	0.188	**0.964**	0.187
Ca	0.021	**0.980**	0.200	0.282	-0.097	**0.955**
Cd	**0.968**	0.248	0.031	**0.920**	0.390	0.045
Co	**0.975**	0.184	-0.121	0.890	-0.226	0.395
Cr	**0.965**	-0.083	-0.250	**0.924**	-0.153	-0.352
Cu	-0.726	-0.524	-0.446	0.620	-0.035	-0.784
Fe	0.523	0.521	0.675	**0.991**	0.119	-0.059
Mg	0.304	**0.929**	-0.213	0.410	-0.081	**0.908**
Mn	0.418	0.764	0.492	**0.877**	-0.184	0.445
Ni	0.170	0.293	-0.941	**0.940**	-0.142	-0.309
P	0.140	**0.924**	-0.357	0.031	**0.947**	0.321
Pb	**0.878**	0.136	0.459	-0.018	**0.938**	-0.345
Zn	-0.292	-0.547	0.784	-0.122	**0.953**	-0.279

A



B

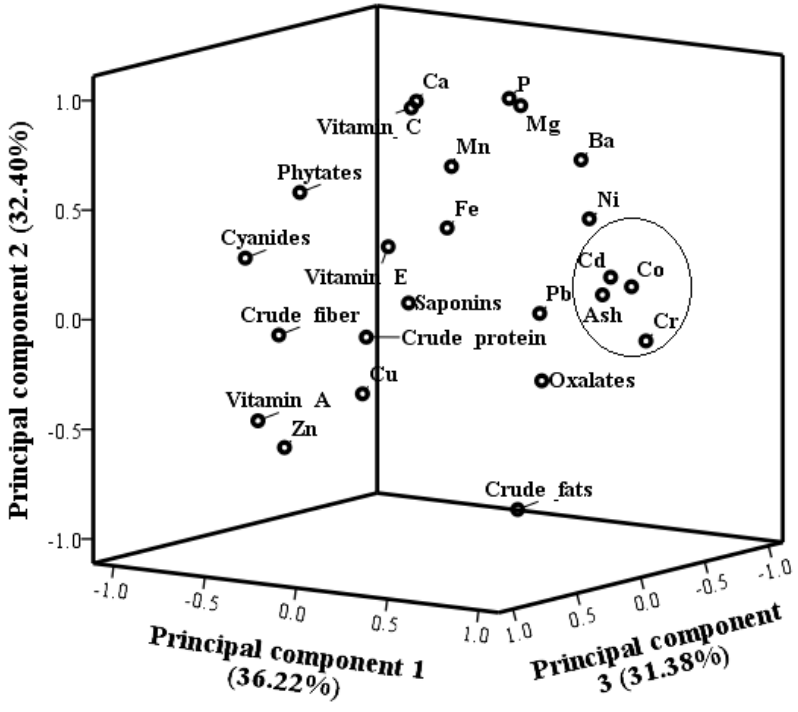


Figure 11: Principal component scatter plots of the nutrients and anti-nutrients studied in *L. alatipes* (A) and *O. tenax* (B) leaves.

Cluster analysis classifies samples on the basis of a set of measured variables (e.g. concentration) into a number of different groups such that similar samples are placed in the same group (Statstutor, 2007). The cluster analysis of nutrients and anti-nutrients in *L. alatipes* and *O. tenax* leaves are represented as dendrograms in Figure 12. There were 3 main clusters in *L. alatipes* leaves; cluster A with Cr, Ni, Cu, Cd and Fe; cluster B with Ba, P and Pb; and cluster C with vitamin A and crude fats. Elements in cluster A could be from the same anthropogenic source such as anti-friction bearings from cars on main roads. Cluster B is associated with elements originating from motor oil additives and wearing of automobile clutches and tyres (Kanu et al., 2015; Warner et al., 2001). Four main clusters were observed in *O. tenax* leaves; cluster A with vitamin C, crude protein, Ca, Mg and phytates; cluster B with cyanides and crude fibre; cluster C with Fe and Mn as well as Ba and P; and cluster D with ash, Cd, Co, Cr and Cd. Unlike the other clusters, cluster C may be from anthropogenic sources.

A **B**

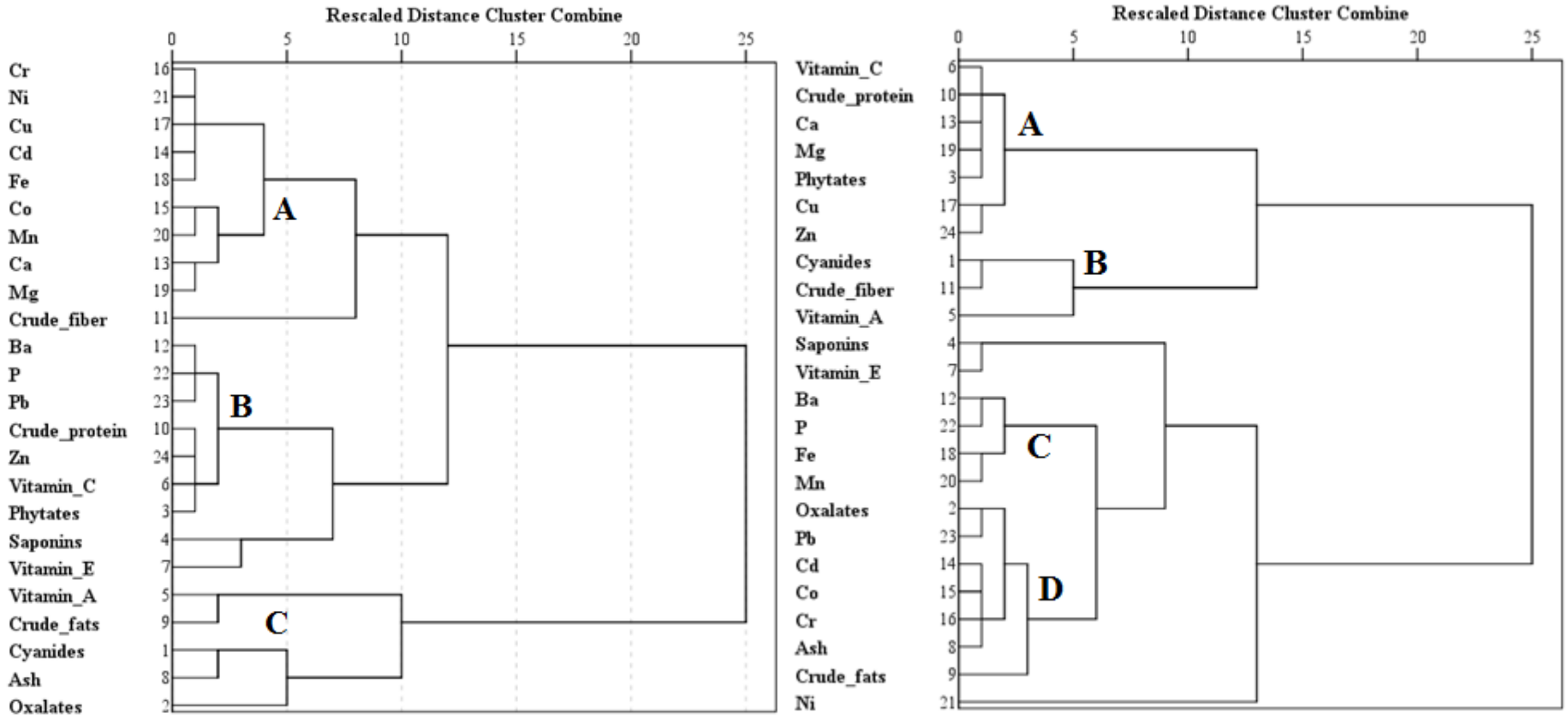


Figure 12: Dendrogram of nutrients and anti-nutrients in *L. alatipes* (A) and *O. tenax* (B) leaves showing the distance between elements, by Ward's method. *A, B, C, D - different clusters.

CONCLUSION

The results showed that cooking has an effect on both nutrients and anti-nutrients in nettles. The findings also indicate an increase in carbohydrates and crude fibre content with a decrease in anti-nutrient content in the nettles, *L. alatipes* and *O. tenax*. A comparison amongst nettles after cooking showed *L. peduncularis* and *U. dioica* to be are richer in macronutrients and *L. alatipes* and *O. tenax* to be richer in essential elements. Correlation analysis showed relationships between elements, macronutrients and anti-nutrients. Crude fibre and saponins had negative correlations with elements due to their complexing ability. An association between ash content and elemental concentrations in the nettles was observed by principal component and cluster analysis. This study confirms the positive contribution of nettles to the diet due to it being rich in macronutrients, essential elements and other minerals therefore its consumption is recommended.

Acknowledgments

We are grateful to the National Research Foundation (NRF) for financial support and the School of Chemistry and Physics at the University of KwaZulu-Natal, South Africa.

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CHAPTER FOUR

Heavy metal distribution in Laportea peduncularis and growth soil from the eastern parts of KwaZulu-Natal, South Africa

ABSTRACT

Laportea peduncularis is a medicinal plant consumed by the local people in South Africa**.** Due to its oral consumption and therefore its potential for harm to human health, the distribution of metals in the leaves of *L. peduncularis* as a function of soil characteristics was evaluated. Broadly, the concentration of metals in the soil were in decreasing order of Fe> Ca> Mg> Mn> Zn> Cr> Cu> Ni> As> Co> Cd> Pb. Low molecular weight organic acid, calcium chloride and ethylenediaminetetraacetic acid extraction methods were employed to assess for exchangeable forms of metals in the soil. Geo-accumulation indices and enrichment factors showed no contamination or enrichment for most of the heavy metals studied except for Cd which showed moderate contamination and significant enrichment at Mona, KwaZulu-Natal. Principal component and cluster analyses revealed that As, Cd, Fe and Ni in the soil came from the same source whilst Cu, Pb and Zn in the soil were from a common origin. Correlation analysis showed significantly positive correlation between heavy metals As, Cd, Fe, and Ni in the soil as well as Cu, Pb and Zn confirming the metals common origin. Concentration of metals in plants and soil were influenced by site but the availability and uptake of the metals solely depended on the plant's inherent controls.

Keywords Heavy metals, enrichment factor, geo-accumulation index, soil quality

INTRODUCTION

Due to the rapid development in industrialization, soil contamination is becoming of serious concern with typical and significant causes being anthropogenic activities (agricultural, chemical and industrial), vehicular emissions and improper waste disposal (Krishna & Govil, 2007). Heavy metals in soil are among the most noteworthy contaminants as they are non-biodegradable, have long-term toxicity effects and originate from both the weathering of parent rock material (natural source) and anthropogenic activities (man-made source) (McLaughlin et al., 2000). Soil is a geochemical reservoir for heavy metals and, if polluted, has a significant impact on environmental health due to its ability to introduce heavy metals into the food chain through plants. The presence of heavy metals at high and toxic levels in food can cause detrimental health effects such as cardiovascular disease, cancer and functioning of internal organs, if consumed (Tahar & Keltoum, 2011). Therefore, metal contamination in the current industrialized climate is of grave concern and soil assessment initiatives with focus on impact on uptake by plants should be undertaken (Iqbal & Shah, 2014).

Metals in soil can be in solution, associated with carbonates, bound to Fe and Mn-oxyhydroxide complexes, bound to organic matter or incorporated into the soil lattice. Several approaches to assessing and estimating exchangeability of heavy metals in soil with regards to plant uptake have been undertaken (Takáč et al., 2009). Single or sequential extraction approaches have been used (Kučak & Blanuš, 1998; McGrath, 1996; Novozamsky et al., 1993; Fuentes et al., 2004; Zhu et al., 2012). Some of these extraction methods may be less specific to certain metals resulting in the uneven extraction of metals in the soil (Ure, 1996). Low-molecular weight organic acids are secreted by plant roots in order to lower soil pH to release metals from their bound state in the soil rhizosphere for uptake by the plant (Violante et al., 2010). Therefore, the use of organic acids may

reasonably depict the amount of metal available for plant uptake. Other chemical methods that may be used to predict metal availability include the use of $CaCl_2$ that extracts weakly adsorbed metal ions from soil (Houba et al., 2000) or the use of the synthetic chelating agent, ethylenediaminetetraacetic acid (EDTA) which forms strong complexes with many heavy metals in soil.

Soil quality also plays a role in the availability of heavy metals. Acidic soils are known to possess trace or heavy metals in soluble or ionic form. This can result in the absorption of the metal by the plant or reduction in the availability of another metal. Soil organic matter (SOM) also influences the availability of metals, high SOM results in metals being bound in organic complexes, therefore rendering them unavailable. Cation exchange capacity (CEC) is the measure of the ability of soil to hold cations; high CEC results in metals being held on the clay and organic matter particles in the soil through electrostatic forces, therefore rendering them unavailable (Oliver et al., 2013; Schoenholtz et al., 2000).

Due to economic reasons, people in rural communities have resorted to consuming indigenous vegetables, which are either cultivated or picked from the wild (Schippers, 2000). These indigenous vegetables, unlike commercial ones, are readily available and inexpensive (van Rensburg et al., 2007). *Laportea peduncularis* subsp *peduncularis*, is an indigenous plant from the Urticaceae family. Its leaves and stems have stinging hairs therefore it is known as stinging nettle by local people in South Africa. In KwaZulu-Natal (South Africa), the plant grows abundantly in the wild and the leaves and shoots are picked for cooking. The *Laportea* species are also used traditionally for their anti-inflammatory properties (Quattrocchi, 2012).

The elemental distribution in indigenous medicinal plant species in South Africa as a function of soil quality has previously been reported (Jonnalagadda et al., 2008; Moodley et al., 2012; Moodley et al., 2013). In this study, the distribution of metals in *L. peduncularis* leaves and associated soil from ten different sites in KwaZulu-Natal was investigated. From the ten sampling sites the thirteen elements selectively investigated were As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn. The impact of soil quality parameters on elemental uptake was determined. Three single extraction methods (low-molecular weight organic acids, $CaCl_2$ and EDTA) were employed to determine the amount of metals available for plant uptake. For each of the selected heavy metals, the Enrichment factor (EF) and geo-accumulation index (I_{geo}) was calculated to assess metal contamination in the soil. Multivariate statistical analyses performed on soil data facilitated the determination of the source of heavy metals.

MATERIALS AND METHODS

Sample collection and preparation

Plant leaves and soil samples were collected from ten different sites in KwaZulu-Natal. The sampling sites were: L1-Umbilo Park, L2-Umhlanga, L3-Eshowe, L4-KwaDukuza, L5-Mona, L6-Maphumulo, L7-Umzumbe, L8-Amahlongwa, L9-Gingindlovu, and L10-Ndwedwe (Fig. 13). Plant and soil samples were collected throughout July; the average temperature on days sampled was 25 ºC, with no rain or wind. Soil was generally sandy or loamy sand in texture. Soil samples were systematically collected from six points around the plants at a depth of 15 cm with the use of a plastic spade. Representative soil samples were composited in a clean plastic bucket to achieve homogeneity and reduced to 500 g by quartering. Soil samples from each site were passed through a 2 mm mesh sieve to remove gravel then air-dried to constant mass. Thereafter, the soil was

crushed to reduce particle size with a mortar and pestle. Plant samples were washed with double distilled water then oven dried at 50 ºC to constant mass. Dried plant samples were crushed using a food processor (Braun range). All samples were stored in labelled polyethylene bags in a refrigerator at 4 ºC until analysed.

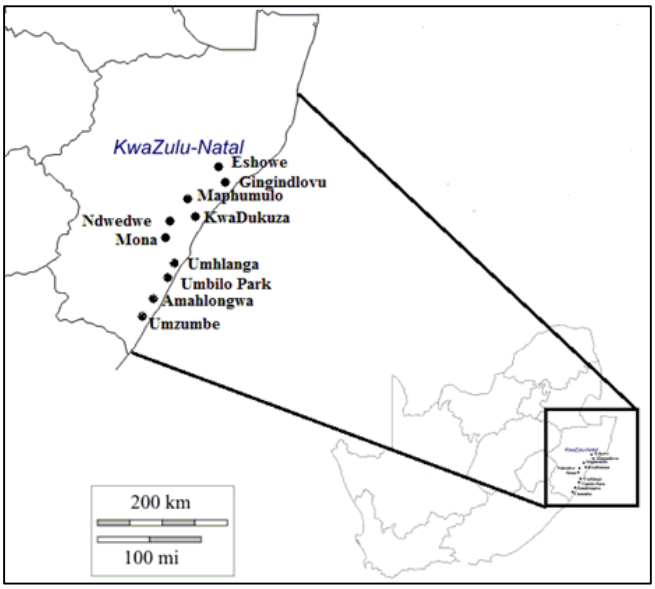

Figure 13: Sampling sites on the eastern parts of KwaZulu-Natal, South Africa.

Reagents and chemicals

All chemicals used were supplied by Merck (Germany) and Sigma Aldrich (USA) and were of analytical-reagent grade. Double distilled water was used throughout the experiments. Working standards were made up with double distilled water and 10 mL of 70% HNO_3 to match the sample matrix. To minimize the risk of contamination all glassware and other equipment were cleaned with 6.0 M HNO_3 and rinsed off with double distilled water.

Extraction of exchangeable metals

Organic acid extractant

The method of Feng et al. (2005), with some modifications, was used for the organic acid extractant. Ground soil samples (2.0 g) were extracted with 20 mL of 10 mM organic acid solution (acetic, citric, formic, malic and oxalic acid) in a 1:1:1:1:1 molar concentration ratio, then two drops of toluene were added to inhibit microbial activity. This mixture was shaken for 16 hr and centrifuged at 3000 x *g* for 10 min. An aliquot of 5 mL of supernatant was removed by a transfer pipette to a 10 mL volumetric flask and diluted to volume with 2% HNO_3.

Calcium chloride extractant

The method of Novosamsky et al. (1993) was used for the $CaCl_2$ extractant. Approximately 1.0 g of soil sample was extracted with 10 mL of 0.01 M $CaCl_2$ and the mixture shaken for 3 hr. The resulting solution was centrifuged at 6000 rpm for 10 min then filtered on Millipore 0.45 µm filter membranes to permit analysis of extracted metals.

EDTA extractant

The method of Quevauriller et al. (1996) was used for the EDTA extractant. Approximately 4.0 g of soil sample was extracted with 20 mL of 0.05 M disodium-EDTA salt adjusted to pH 7 with an ammonia solution and the mixture shaken for 1 hr. The resulting solution was centrifuged at 6000 rpm for 10 min then filtered on Millipore 0.45 µm filter membranes to permit analysis of extracted metals.

Soil organic matter (SOM), cation exchange capacity (CEC) and soil pH

Soil pH was obtained by measuring a 1:2 soil/ 0.01 M $CaCl_2$ suspension using a pH meter calibrated using standard buffer solutions of pH 4 and pH 7. SOM was estimated using the wet chemistry extraction technique of Walkley and Black (1934). Ammonium acetate at pH 7 was used to determine the CEC of the soil (Chapman, 1965). All determinations were done in quadruplicate.

Elemental analysis

The microwave-assisted closed vessel digestion technique was used for digestion of leaves and soil samples. Digestions were performed using the CEM Microwave Accelerated Reaction System (MARS) 6 (CEM Corporation, Matthews, North Carolina, USA) with patented EasyPrep^{TM} Plus technology. Samples (0.2 g for leaf, certified reference material (CRM) and 0.25 g for soil) were accurately weighed into 50 mL liners and 10 mL of 70% HNO_3 was added into each liner. The mixture was allowed to pre-digest for 30 min before microwave digestion. For leaf samples, the power was set to 100% at 1600 W and the temperature was ramped to 180 °C (ramp time 15 min) where it was held for 15 min. For soil samples, the power was set to 100% at 1600 W and temperature was ramped to 200 °C (ramp time 15 min) where it was held for 15 min. All digests were filtered and transferred to 25 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles for elemental analysis. All extracted and digested samples were analysed for As, Ca, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) with axial plasma observation with the exception of Ca and Mg which was analysed using radial plasma observation.

The accuracy of the elemental determinations was measured by use of CRMs that were chosen on the basis of matrix similarities. For soil analyses, Metals in soil (D081-540), from Era

Quality Control was used and for plant analyses, White Clover (BCR 402), from the Community Bureau of Reference of the Commission of the European Communities was used. All determinations were done in quintuplicate.

Bioaccumulation factor (BAF)

Bioaccumulation describes processes of accumulation of an element in a plant organism from the surrounding environment (Ivanciuc et al., 2006). It is defined by the bioaccumulation factor (BAF) which is the ratio of the concentration of an element accumulated inside the plant organism and the concentration of the element in the soil

$$BAF = \frac{C_{plant}}{C_{soil}} \quad (7)$$

Enrichment factor (EF) and geo-accumulation index (I_{geo})

Enrichment factor (EF) describes the magnitude of contamination in the soil. It compares the concentration of an element in the soil to concentrations in the earth's crust. The reference element used in this study is Zn since total baseline concentrations for this element is known in South African (Herselman et al., 2005; Mendiola et al., 2008).

$$EF = \frac{\left[\frac{X}{Zn}\right]_{soil}}{\left[\frac{X}{Zn}\right]_{crust}} \quad (8)$$

Where $\left[\frac{X}{Zn}\right]_{soil}$ is the mean ratio between the concentration of the target element and Zn in the soil whilst $\left[\frac{X}{Zn}\right]_{crust}$ is the mean ratio between the concentrate of the target element and Zn in the earth's crust. EF values were interpreted as follows: EF<1 background concentration, 1>EF<2 depletion

to minimal enrichment, 2>EF<5 moderate enrichment, 5>EF<20 significant enrichment, 20>EF<40 very high enrichment, and EF>40 extremely high enrichment (Sutherland, 2000).

The geo-accumulation index (I_{geo}) describes the extent to which metal contamination has occurred by comparing measured metal concentrations to that of the earth's crust (Müller, 1969). It is depicted by the following equation:

$$I_{geo}=\log_2\left[\frac{C_n}{1.5\ B_n}\right] \quad (9)$$

Where C_n is the measured concentration of the element in the soil sample, B_n is the geochemical background value of the earth's crust (Herselman et al., 2005). The factor 1.5 is introduced to minimize possible variations in the background values due to lithological differences. I_{geo} values were interpreted as follows: $I_{geo}\leq 0$ uncontaminated, $0>I_{geo}<1$ uncontaminated to moderately contaminated, $1>I_{geo}<2$ moderately contaminated, $2>I_{geo}<3$ moderately to heavily contaminated, $3>I_{geo}<4$ heavily contaminated, $4>I_{geo}<5$ heavily to extremely contaminated and $I_{geo}>5$ extremely contaminated soil (Müller, 1969).

Statistical analysis

Multivariate statistical analyses (principal component analysis (PCA) and cluster analysis (CA)) were performed to determine the relationship between input variables. Analysis of covariance (ANCOVA) was performed with concentration in leaves as the dependent variable, element and site as factors and total and exchangeable concentrations in soil as covariates. Significance of plant-soil relationships was established by computing Pearson's correlation coefficients (r) for the relationship between the concentration of the elements in leaves and the total and exchangeable concentrations in the soil. All statistical analyses were performed using the Statistical Package for the Social Science (PASW Statistics, Version 22, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Distribution of elements in the plant and soil

The method for the elemental analysis was validated by the use of CRMs (Table 23). The recorded values for Fe, Mn and Zn in the soil CRM compared well with certified values, whilst those for Cd, Co, Cr and Se were within the acceptable limits. For the plant CRM, measured values compared well with certified values.

The concentrations of elements in the leaves and soil (total and exchangeable) and BAFs are presented in Table 24. If present in leaves, Cd and Se concentrations were below the instrument detection limits (<0.0034 µg g^{-1} for Cd and 0.1150 µg g^{-1} for Se). Soil was rich in Fe, Ca and Mg with concentrations ranging between 3 564 - 29 686 µg g^{-1}; 1 472 - 5 117 µg g^{-1}, 600 - 5 589 µg g^{-1}, respectively. Total soil As at sites L5 and L6 were high whilst no As was detected at site L2. The concentrations of elements in soil were generally in decreasing order of Fe> Ca> Mg> Mn> Zn> Cr> Cu> Ni> As> Co> Cd> Pb.

Table 23: Comparison of measured values (mg kg^{-1} dry mass, mean ± standard deviation, 95% confidence interval, *n=5*) to certified values and acceptable limits for certified reference materials, Metals in soil, D081-540 and White clover, BCR 402.

Element	Wavelength (nm)	Measured value	Certified value	Acceptable limits
		Soil		
Cd	226.5	156.0 ± 4.6	143 ± 5.6	116-159
Co	228.6	238.5 ± 7.8	199 ± 4.1	166-233
Cr	205.6	102.6 ± 2.3	86.8 ± 6.1	69.3-104
Fe	259.9	13 119 ± 608	12 800 ± 18.0	5 380-20100
Mn	257.6	448.5 ± 18.7	425 ± 9.7	347-502
Se	196.0	161.5 ± 5.9	127 ± 4.5	98.4-156
Zn	213.9	136.9 ± 7.9	141 ± 11.5	113-184
		White clover		
Se	196.0	7.02 ± 1.26	6.70 ± 0.25	-
Fe	259.9	250 ± 16	244	-
Ni	231.6	8.00 ± 0.35	8.25	-
Zn	213.9	30.9 ± 6.7	25.2	-

An analysis of extraction results was performed to determine the best extraction method for the various metals studied. Organic acids are capable of forming complexes with metal ions and thus modify the mobility of metals in the soil rhizosphere (Zhang et al., 1999). Exchangeable forms of Cd and Cr in soil were best represented by the organic acid extraction method with exchangeable percentages ranging from 0.3 - 1.9% and 0.1 - 0.7%, respectively. The CaCl2 extraction method best represented exchangeable As which showed an extraction ability of up to 3.6%. EDTA was more representative of the extractable percentage for Co, Cu, Mn, Ni and Zn.

BAFs showed the plants tendency to accumulate Ca and Mg to meet physiological requirement levels, similar to other studies (Mahlangeni et al., 2012; Moodley et al., 2012; Reddy et al., 2014). Although Cd was detected in the soil and was found to be in exchangeable form, no Cd was

detected in the plant. Previous studies have shown that Cd ions are normally retained in plant roots, resulting in small amounts being transported to the shoots (Cataldo et al., 1983). Extremely low or undetectable amounts of Co were found in the leaves. High levels of Cu (BAF>1) were observed in the leaves for 6 of the 10 sites. Although total and exchangeable soil Fe was high, Fe levels in the leaves were low (BAFs<1). High levels of Mn were observed at sites L4 and L5 (186 and 201 $\mu g\ g^{-1}$, BAFs>1). Some of the factors that contribute to low levels of Fe in the plant include high levels of Cu and Mn in the plant and soil (Fageria et al.,1990; Marschner, 1995). These metals compete with the Fe for the same absorption sites in the soil and for the same membrane carrier in the plant during uptake. Nickel was present in the leaves at moderate levels (BAF<1), with accumulation at site L7 only. Lead was present in the leaves at 6 of the 10 sites. Lead forms stable complexes with the hydroxyl group of clay minerals, amorphous silicate minerals, and insoluble humic substances in the soil reducing its availability. Plants release protons and organic acids through the roots to lower the pH of the soil to release essential nutrients from their bound state in the soil which also frees Pb for uptake (Huang & Chen, 2003). Zinc accumulation was only observed at one site (site L5).

Table 24: Concentration of elements (µg g^{-1}dry mass, mean ± standard deviation, 95% confidence interval, *n=5*) in soil (Total-T and Exchangeable-E) and the leaves of *L. peduncularis* with bioaccumulation factors (BAFs).

Element	Sites[a]	Soil-T	Soil-E			*L. peduncularis*	BAF
			EA[b]	EB[c]	EC[d]		
As	L1	2.77 ± 0.56 a*	0.021 ± 0.004 ab	0.065 ± 0.008 ab	ND	ND	-
	L2	ND	ND	ND	ND	2.21 ± 0.27 a	-
	L3	7.86 ± 1.93 a	0.010 ± 0.003 c	0.065 ± 0.003 ab	ND	2.34 ± 0.18 a	0.31
	L4	1.91 ± 0.85 a	0.053 ± 0.012 bc	0.068 ± 0.002 bc	ND	3.47 ± 0.56 b	1.82
	L5	30.9 ± 2.1 b	0.009 ± 0.003 abc	0.060 ± 0.001 ad	ND	2.09 ± 0.33 a	0.07
	L6	32.4 ± 15.5 b	ND	0.064 ± 0.002 ad	ND	1.16 ± 0.49 c	0.04
	L7	3.23 ± 0.52 a	ND	0.059 ± 0.008 d	0.048 ± 0.004 a	4.02 ± 0.18 b	1.24
	L8	4.39 ± 1.74 a	ND	0.064 ± 0.004 abd	ND	3.20 ± 0.17 bd	0.73
	L9	6.90 ± 1.40 a	ND	0.063 ± 0.002 acd	ND	3.75 ± 0.54 b	0.54
	L10	5.86 ± 1.75 a	ND	0.062 ± 0.002 ad	ND	2.39 ± 0.20 ad	0.41
Ca	L1	2 527 ± 135 abc	53.8 ± 1.3 a	234 ± 2 a	384 ± 25 ab	36 867 ± 528 a	14.6
	L2	2 867 ± 272 b	40.6 ± 1.7 b	221 ± 3 b	416 ± 41 bc	28 787 ± 467 b	10
	L3	5 117 ± 345 d	82.1 ± 1.7 c	194 ± 3 c	651 ± 15 d	15 633 ± 1345 c	3.06
	L4	1 941 ± 367 ce	41.7 ± 1.2 b	215 ± 4 b	196 ± 24 e	36 438 ± 3296 a	18.8
	L5	1 472 ± 92 c	20.4 ± 0.8 d	220 ± 5 b	179 ± 12 e	12 589 ± 957 c	8.55
	L6	1 769 ± 458 ce	22.2 ± 0.3 d	199 ± 3 c	252 ± 10 f	13 632 ± 279 c	7.71
	L7	2 776 ± 174 abf	52.0 ± 0.7 a	194 ± 5 c	453 ± 15 c	22 753 ± 1557 d	8.2
	L8	5 030 ± 204 d	65.4 ± 2.0 e	195 ± 4 c	723 ± 13 g	22 790 ± 1393 d	4.53
	L9	4 002 ± 209 g	73.8 ± 1.3 f	177 ± 5 d	703 ± 12 dg	36 392 ± 1835 a	9.09
	L10	2 130 ± 177 aef	52.4 ± 1.1 a	212 ± 5 b	426 ± 4 abc	22 125 ± 991 d	10.4
Cd	L1	3.44 ± 0.04 a	0.031 ± 0.002 a	0.002 ± 0.001 a	0.056 ± 0.013 a	ND	-
	L2	1.29 ± 0.06 b	0.024 ± 0.001 a	ND	0.020 ± 0.005 a	ND	-
	L3	8.44 ± 0.22 c	0.029 ± 0.002 a	ND	0.017 ± 0.003 a	ND	-
	L4	2.97 ± 0.29 a	0.031 ± 0.011 a	ND	ND	ND	-
	L5	13.4 ± 0. 3 d	0.009 ± 0 a	ND	ND	ND	-
	L6	13.7 ± 1.8 d	0.012 ± 0.001 a	ND	ND	ND	-
	L7	3.74 ± 0.23 a	0.015 ± 0.001 a	ND	ND	ND	-
	L8	5.22 ± 0.33 e	0.024 ± 0.001 a	0.001 ± 0 b	0.149 ± 0.010 a	ND	-
	L9	6.87 ± 0.34 f	0.034 ± 0.002 a	ND	0.023 ± 0.001 a	ND	-

	L10	6.19 ± 0.25 ef	0.036 ± 0.001 a	ND	0.019 ± 0.002 a	ND	-
Co	L1	7.50 ± 0.30 ab	0.225 ± 0.013 a	0.009 ± 0.001 a	0.687 ± 0.063 a	ND	-
	L2	3.83 ± 0.48 c	0.095 ± 0.004 b	0.001 ± 0 bc	0.498 ± 0.108 b	ND	-
	L3	8.87 ± 0.13 bd	0.123 ± 0.001 c	ND	0.659 ± 0.025 ab	ND	-
	L4	5.20 ± 0.45 ce	0.129 ± 0.004 c	0.001 ± 0 bc	0.055 ± 0.025 c	ND	-
	L5	9.14 ± 0.04 bd	0.032 ± 0 d	0.003 ± 0.001 cd	0.054 ± 0.004 c	ND	-
	L6	11.8 ± 1.9 fg	0.127 ± 0.004 c	0.007 ± 0.001 a	0.218 ± 0.015 c	1.29 ± 0.07 a	0.11
	L7	10.4 ± 0.9 df	0.280 ± 0.004 e	0.018 ± 0.002 d	1.10 ± 0.09 d	ND	-
	L8	11.3 ± 1.4 f	0.190 ± 0.009 f	0.002 ± 0.001 d	1.14 ± 0.12 d	0.176 ± 0.039 b	0.02
	L9	13.6 ± 0.8 g	0.268 ± 0.007 e	0.011 ± 0.001 a	1.50 ± 0.06 e	0.177 ± 0.032 b	0.01
	L10	6.73 ± 0.40 ae	0.188 ± 0.008 f	0.004 ± 0.001 c	0.645 ± 0.045 ab	ND	-
Cr	L1	43.0 ± 2.2 a	0.085 ± 0.002 a	0.003 ± 0 a	0.020 ± 0.005 ac	9.25 ± 0.65 a	0.22
	L2	28.3 ± 0.8 bcd	0.191 ± 0.006 b	0.005 ± 0 a	0.176 ± 0.048 b	4.88 ± 0.32 b	0.17
	L3	21.6 ± 1.0 cde	0.033 ± 0.001 cd	0.002 ± 0 a	0.003 ± 0.001 a	1.57 ± 0.20 c	0.07
	L4	25.7 ± 6.0 bcde	0.038 ± 0.001 de	0.002 ± 0 a	ND	1.60 ± 0.15 c	0.06
	L5	84.0 ± 1.7 f	0.126 ± 0.003 f	0.005 ± 0.001 a	0.046 ± 0.008 c	1.94 ± 0.08 c	0.02
	L6	23.8 ± 5.4 de	0.030 ± 0.001 c	0.002 ± 0 a	ND	4.13 ± 0.20 bd	0.17
	L7	18.7 ± 1.3 e	0.032 ± 0.001 cd	0.004 ± 0.001 a	ND	1.27 ± 0.19 c	0.07
	L8	33.0 ± 3.3 b	0.040 ± 0.002 e	0.013 ± 0.005 b	0.006 ± 0.002 a	4.18 ± 0.35 bd	0.13
	L9	52.8 ± 3.5 g	0.071 ± 0.004 g	0.010 ± 0.001 b	0.030 ± 0.005 ac	4.21 ± 0.33 bd	0.08
	L10	23.8 ± 1.2 e	0.047 ± 0.003 h	0.009 ± 0.002 b	0.003 ± 0 a	3.56 ± 0.48 d	0.15
Cu	L1	49.5 ± 4.5 a	0.471 ± 0.024 a	0.036 ± 0.005 a	6.36 ± 0.55 a	62.6 ± 3.7 a	1.26
	L2	14.3 ± 3.2 b	0.242 ± 0.008 b	0.024 ± 0.003 bc	3.39 ± 0.47 b	11.5 ± 2.0 b	0.8
	L3	21.1 ± 0.2 cd	0.153 ± 0.020 c	0.031 ± 0.003 ab	3.08 ± 0.28 b	53.7 ± 4.3 ac	2.55
	L4	16.0 ± 1.5 bd	0.187 ± 0.010 d	0.026 ± 0.006 bc	ND	15.4 ± 3.4 b	0.96
	L5	26.8 ± 1.7 c	0.102 ± 0.002 e	0.021 ± 0.002 cd	1.14 ± 0.09 de	30.5 ± 5.8 bc	1.14
	L6	23.6 ± 4.6 c	0.113 ± 0.008 e	0.015 ± 0.003 d	0.875 ± 0.112 ce	67.8 ± 7.4 a	2.87
	L7	15.3 ± 0.6 bd	0.122 ± 0.015 ce	0.023 ± 0.002 bc	1.39 ± 0.10 de	28.0 ± 3.3 bc	1.83
	L8	40.6 ± 2.0 e	0.253 ± 0.007 b	0.044 ± 0.002 a	9.60 ± 0.75 f	19.7 ± 1.9 b	0.49
	L9	38.5 ± 1.4 e	0.172 ± 0.009 cd	0.040 ± 0.002 a	6.18 ± 0.22 a	25.2 ± 4.1 bc	0.65
	L10	17.2 ± 0.8 bd	0.153 ± 0.016 c	0.026 ± 0.003 bc	1.85 ± 0.03 d	35.3 ± 6.0 bc	2.05
Fe	L1	9 893 ± 109 a	63.9 ± 0.8 a	ND	2 655 ± 215 a	406 ± 23 abc	0.04
	L2	3 564 ± 166 b	53.8 ± 2.6 b	ND	2 139 ± 156 a	123 ± 17 abc	0.03
	L3	20 885 ± 385 c	67.4 ± 0.8 a	ND	7 193 ± 825 c	445 ± 48 abc	0.02
	L4	8 299 ± 539 a	74.6 ± 2.0 c	0.031 ± 0.012 bc	565 ± 179 d	334 ± 59 ab	0.04

	L5	28 715 ± 537 d	12.6 ± 0.2 d	0.348 ± 0.026 d	2 383 ± 71 a	789 ± 70 a	0.03
	L6	29 686 ± 2523 d	20.0 ± 0.2 e	ND	805 ± 16 d	3 707 ± 76 d	0.12
	L7	9 923 ± 477 a	31.3 ± 1.6 f	0.008 ± 0.002 a	2 172 ± 143 a	380 ± 27 abc	0.04
	L8	13 045 ± 921 e	46.0 ± 2.7 g	0.051 ±0.014 c	4 404 ± 366 e	1 215 ± 59 e	0.09
	L9	17 309 ± 731 f	85.6 ± 4.5 h	0.052 ± 0.005 c	8 438 ± 108 f	1 205 ± 171 ce	0.07
	L10	15 863 ± 583 f	83.2 ± 2.2 h	0.027 ± 0.002 b	4 311 ± 86 e	2 938 ± 687 f	0.19
Mg	L1	1 237 ± 114 ab	13.1 ± 0.5 a	10.0 ± 0.4 a	30.5 ± 1.8 ab	5 069 ± 119 a	4.1
	L2	600 ± 151 c	13.1 ± 0.4 a	10.9 ± 0.3 ab	33.6 ± 4.5 b	5 415 ± 119 ab	9.03
	L3	1 562 ± 38 d	39.6 ± 0.5 b	33.4 ± 0.4 c	18.1 ± 4.4 c	5 173 ± 259 a	3.31
	L4	957 ± 43 b	14.5 ± 0.2 c	12.5 ± 0.3 b	6.93 ± 1.86 d	6 837 ± 285 c	7.14
	L5	1 262 ± 34 a	9.09 ± 0.23 d	10.8 ± 0.5 b	27.2 ± 2.1 abe	1 957 ± 114 d	1.55
	L6	5 589 ± 189 e	6.46 ± 0.04 e	7.7 ± 0.4 d	24.2 ± 1.0 acef	2 266 ± 65 d	0.41
	L7	2 126 ± 175 f	23.7 ± 0.3 f	19.9 ± 1.5 e	32.0 ± 6.2 ab	6 137 ± 545 bc	2.88
	L8	2 291 ± 130 f	45.2 ± 0.6 g	32.8 ± 1.3 c	21.2 ± 2.6 ce	5 728 ± 421 ab	2.5
	L9	2 142 ± 109 f	56.7 ± 1.0 h	44.0 ± 0.9 f	16.3 ± 0.5 c	10 787 ± 556 e	5.04
	L10	1 130 ± 83 ab	22.4 ± 0.5 i	21.7 ± 1.2 e	20.1 ± 1.7 cf	6 898 ± 434 bc	6.1
Mn	L1	186 ± 8 a	7.44 ± 0.28 a	1.00 ± 0.02 a	35.1 ± 2.9 a	49.4 ± 2.2 a	0.27
	L2	156 ± 17 ab	8.65 ± 0.25 a	1.67 ± 0.06 b	80.2 ± 14.2 b	116 ± 1 b	0.74
	L3	386 ± 22 c	11.2 ± 0.2 b	1.62 ± 0.04 b	91.0 ± 4.3 b	74.6 ± 5.8 a	0.19
	L4	123 ± 12 ab	4.55 ± 0.10 c	0.390 ± 0.020 c	3.21 ± 1.07 c	186 ± 18 c	1.51
	L5	81.0 ± 3.2 b	1.38 ± 0.03 d	0.370 ± 0.020 c	5.04 ± 0.41 ac	201 ± 6 cd	2.48
	L6	211 ± 19 a	2.88 ± 0.06 cd	0.520 ± 0.040 c	10.7 ± 0.4 ac	182 ± 2 c	0.86
	L7	407 ± 27 c	14.5 ± 0.1 e	3.19 ± 0.32 d	90.0 ± 6.7 b	177 ± 12 c	0.43
	L8	1 105 ± 114 d	20.6 ± 2.4 f	1.27 ± 0.07 a	194 ± 36 d	134 ± 12 b	0.12
	L9	335 ± 21 c	10.7 ± 0.5 b	1.89 ± 0.08 b	93.2 ± 2.3 b	130 ± 12 b	0.39
	L10	404 ± 18 c	18.5 ± 0.4 g	3.21 ± 0.20 d	104 ± 4 b	228 ± 31 d	0.56
Ni	L1	16.0 ± 2.6 a	0.157 ± 0.007 a	0.024 ± 0.002 a	0.674 ± 0.056 a	2.46 ± 0.14 abe	0.15
	L2	6.44 ± 0.41 b	0.081 ± 0.001 b	0.013 ± 0.001 bcde	0.470 ± 0.104 b	2.28 ± 0.23 abe	0.35
	L3	9.65 ± 1.37 bc	0.025 ± 0.002 c	0.011 ± 0 bcd	0.188 ± 0.025 c	3.03 ± 0.69 abe	0.31
	L4	9.66 ± 2.29 bc	0.071 ± 0.001 bd	0.015 ± 0.002 ce	0.050 ± 0.008 d	2.07 ± 0.30 b	0.21
	L5	37.7 ± 1.1 d	0.018 ± 0.002 c	0.010 ± 0.001 d	0.029 ± 0.005 d	5.02 ± 0.61 c	0.13
	L6	19.9 ± 2.5 e	0.030 ± 0.008 c	0.011 ± 0.001 bd	0.035 ± 0.005 d	5.68 ± 0.30 c	0.29
	L7	6.82 ± 0.85 b	0.060 ± 0.013 d	0.017 ± 0.004 ef	0.204 ± 0.010 c	11.0 ± 1.1 d	1.61
	L8	14.7 ± 1.0 af	0.109 ± 0.005 e	0.022 ± 0.003 ag	0.811 ± 0.094 e	3.57 ± 0.48 e	0.24
	L9	17.7 ± 1.6 ae	0.084 ± 0.005 b	0.023 ± 0.001 ag	0.867 ± 0.047 e	3.11 ± 0.42 abe	0.18

	L10	11.7 ± 0.7 cf	0.073 ± 0.003 bd	0.019 ± 0.003 fg	0.397 ± 0.008 b	7.66 ± 0.44 f	0.65
Pb	L1	32.8 ± 4.1 a	0.231 ± 0.005 a	0.012 ± 0.003 ab	2.23 ± 0.09 a	ND	-
	L2	11.2 ± 0.9 b	0.058 ± 0.006 b	0.012 ± 0.001 ab	0.621 ± 0.109 b	ND	-
	L3	ND	ND	ND	ND	ND	-
	L4	5.14 ± 0.83 c	0.096 ± 0.006 c	0.011 ± 0.001 a	0.080 ± 0.023 c	0.406 ± 0.019 a	0.08
	L5	ND	ND	ND	ND	1.31 ± 0.07 b	-
	L6	ND	ND	ND	ND	0.574 ± 0.07 a	-
	L7	ND	ND	ND	ND	4.11 ± 1.25 a	-
	L8	21.8 ± 5.6 d	0.045 ± 0.003 d	0.012 ± 0.002 ab	1.99 ± 0.25 d	2.22 ± 0.23 b	0.1
	L9	ND	ND	ND	ND	2.28 ± 0.37 b	-
	L10	ND	ND	ND	ND	ND	-
Zn	L1	247 ± 20 a	10.9 ± 0.5 a	2.43 ± 0.17 a	61.1 ± 3.7 a	87.0 ± 6.0 a	0.35
	L2	61.2 ± 5.2 b	1.06 ± 0.01 b	0.129 ± 0.03 b	6.16 ± 1.03 b	26.6 ± 1.1 bc	0.43
	L3	55.0 ± 5.9 b	0.410 ± 0.030 b	0.109 ± 0.033 b	3.84 ± 0.17 b	27.1 ± 3.2 bc	0.49
	L4	43.5 ± 7.5 b	0.920 ± 0.010 b	0.077 ± 0.009 b	3.80 ± 0.02 b	27.1 ± 2.4 c	0.62
	L5	50.4 ± 3.4 b	0.320 ± 0.010 b	0.100 ± 0.022 b	1.78 ± 0.15 b	60.2 ± 2.8 d	1.19
	L6	97.9 ± 7.2 b	0.460 ± 0.040 b	0.089 ± 0.018 b	1.97 ± 0.01 b	43.1 ± 1.4 e	0.44
	L7	35.8 ± 2.9 b	0.420 ± 0.030 b	0.077 ± 0.009 b	2.55 ± 0.31 b	21.9 ± 1.9 f	0.61
	L8	439 ± 170 c	11.4 ± 1.2 a	0.290 ± 0.011 c	111 ± 13 c	63.4 ± 4.1 be	0.14
	L9	39.9 ± 1.0 b	0.480 ± 0.030 b	0.052 ± 0.004 b	4.28 ± 0.79 b	27.9 ± 3.9 f	0.7
	L10	54.8 ± 4.4 b	0.540 ± 0.020 b	0.058 ± 0.012 b	2.78 ± 0.09 b	34.0 ± 0.6 cf	0.62

[a]Sites - L1-Umbilo Park, L2-Umhlanga, L3-Eshowe, L4-KwaDukuza, L5-Mona, L6-Maphumulo, L7-Umzumbe, L8-Amahlongwa, L9-Gingindlovu, L10-Ndwedwe.
[b]EA - soil exchangeable (organic acids), [c]EB - soil exchangeable ($CaCl_2$), [d]EC - soil exchangeable (EDTA), BAF- [leaves]/[Soil-T]. ND - not determinable.
*Different letters within columns indicate mean separation by Tukey's Post-hoc test at the 5% level.

Values for soil parameters (pH, SOM and CEC) for each site are presented in Table 25. The pH of the soil at all sites proved to be acidic by nature (pH<6) and ranged from 4.47 (site L2) to 5.56 (site L5). SOM ranged from 0.8 (site L4) to 30.5% (site L2). The CEC of soil ranged from 7.9 to 23.1 meq 100 g^{-1}.

Table 25: Soil pH, soil organic matter (SOM) and cation exchange capacity (CEC) (mean ± standard deviation, *n=4*) of soil samples from ten different sites.

Sites[a]	Soil pH ($CaCl_2$)	SOM (%)	CEC (meq.100g^{-1})
L1	5.36 ± 0.01 a*	29.4 ± 1.7 a	9.7 ± 0.0 a
L2	5.56 ± 0.03 b	30.5 ± 1.1 a	11.8 ± 0.1 b
L3	5.44 ± 0.01 c	7.3 ± 0.2 bc	23.1 ± 0.2 c
L4	5.44 ± 0.01 c	0.8 ± 0.0 d	9.8 ± 0.1 a
L5	4.47 ± 0.01 d	8.1 ± 0.3 bc	17.1 ± 0.1 d
L6	4.58 ± 0.02 e	1.3 ± 0.0 d	13.6 ± 0.5 e
L7	4.86 ± 0.01 f	5.9 ± 0.1 b	14.4 ± 0.2 f
L8	5.10 ± 0.02 g	8.4 ± 0.0 bc	13.5 ± 0.3 e
L9	5.21 ± 0.02 h	10.7 ± 0.1 e	18.0 ± 0.3 g
L10	5.19 ± 0.02 h	6.2 ± 0.1 b	7.9 ± 0.1 h

Enrichment factors (EFs) and geo-accumulation indices (Igeos)

The EF and Igeo values of heavy metals in soil samples from different sites are shown in Table 26. Both the EF and Igeo values are used to quantify the degree of metal enrichment or contamination in the soil (Zhou et al., 2014). The normal or natural concentrations of metals in the soil are described as background concentrations. Background concentrations for heavy metals in South African soils were as follows (in μg g^{-1}): 2.7 for Cd, 69 for Co, 353 for Cr, 117 for Cu, 159 for Ni, 65.8 for Pb and 115 for Zn (Herselman et al., 2005). EF is the relative abundance of heavy metals compared to background concentrations. EFs are designated to distinguish natural and anthropogenic involvements. EF values above 1.5 indicate anthropogenic contributions; the higher

the EF the more severe the contribution (Zhang & Liu, 2002). EFs for selected heavy metals (Co, Cr, Cu, Ni, Pb and Zn) in soil at different sites were below 1 indicating no enrichment. Cadmium enrichment was found to vary with site. Sites L1, L2 and L8 showed no Cd enrichment; these sites were along river banks. Site L4 showed minimal Cd enrichment whereas sites L3, L6, L7, L9 and L10 showed moderate Cd enrichment. Site L5 indicated significant enrichment with an EF value of 11.3. Sites L3 to L7 were located next to main roads whilst sites L9 and L10 were located in the forest. Studies have shown that petroleum, diesel soot, tire rubber and asphalt contribute to heavy metal pollution of Cd, thus causing high enrichment of the heavy metal in the soil next to main roads (Al-Dousari et al., 2012). Cadmium enrichment of forest soil can be attributed to atmospheric deposition and the air-filtering effect of the vegetation (Hernandez et al., 2003; Nickel et al., 2015; Zaccherio & Finzi, 2007).

Table 26: Enrichment factors (EF) and geo-accumulation indices (I_{geo}) of metals in soil from ten different sites.

Site	Cd		Co		Cr		Cu		Ni		Pb		Zn
	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	I_{geo}
L1	0.6	-0.2	0.1	-3.8	0.1	-3.5	0.2	-1.8	0.1	-3.9	0.2	-1.6	0.5
L2	0.9	-1.7	0.1	-4.7	0.2	-4.2	0.2	-3.6	0.1	-5.2	0.3	-3.1	-1.5
L3	6.5	1.1	0.3	-3.5	0.1	-4.6	0.4	-3	0.1	-4.6	ND	ND	-1.6
L4	2.9	-0.4	0.2	-4.3	0.2	-4.3	0.4	-3.4	0.2	-4.6	0.2	-4.2	-2
L5	11.3	1.7	0.3	-3.5	0.5	-2.6	0.5	-2.7	0.5	-2.6	ND	ND	-1.8
L6	6.0	1.8	0.2	-3.1	0.1	-4.5	0.2	-2.9	0.2	-3.6	ND	ND	-0.8
L7	4.5	-0.1	0.5	-3.3	0.2	-4.8	0.4	-3.5	0.1	-5.1	ND	ND	-2.3
L8	0.5	0.4	0	-3.2	0	-4	0.1	-2.1	0	-4	0.1	-2.2	1.3
L9	7.3	0.8	0.6	-2.9	0.4	-3.3	0.9	-2.2	0.3	-3.7	ND	ND	-2.1
L10	4.8	0.6	0.2	-3.9	0.1	-4.5	0.3	-3.3	0.2	-4.3	ND	ND	-1.6

ND-Not determinable.

The Igeo value provides the degree or levels of contamination of metals in the soil as described by Müller (1969). Igeo values for Co, Cr, Cu, Ni and Pb in soil were less than 0 indicating no contamination by the metals. Sites L1, L2, L4, L7, L8, L9 and L10 showed no Cd contamination, whilst sites L3, L5 and L6 showed moderate Cd contamination. This further suggests that Cd is the main pollutant in soils from sites next to main roads. Similarly, most sites showed no Zn contamination. Site L8 indicated moderate Zn contamination.

Statistical analysis

Site, element and their interaction affected elemental concentrations in *L. peduncularis* leaves. Therefore, elements in the leaves were analysed separately (Table 27). For the elements As, Ca, Co, Cr, Fe, Mg, Ni and Pb, total or exchangeable soil concentrations had no effect on the elemental concentrations in the leaves. On the other hand, total and exchangeable soil concentrations of Zn, and total soil Cu and Mn had a significant effect on the elemental concentrations in the leaves. This indicates that soil concentrations of these metals are good predictors of metal concentrations in the plant. There was also a significant difference ($P \leq 0.001$) in the elemental concentrations at the different sites. This could be due to soil quality parameters pH, SOM, CEC, environmental conditions such as temperature, moisture and wind at the different sites.

Table 27: Analysis of covariance of all sites involving the presence of elements in *L. peduncularis* leaves.

	Element										
Source	As	Ca	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Soil (T)[a]	ns	ns	ns	ns	*	ns	ns	*	ns	ns	*
Soil (EA)[b]	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns
Soil (EB)[c]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
Soil (EC)[d]	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	***
Site	***	***	***	***	*	***	***	***	***	***	***

ns - not significant; *,***, significant at $P \leq 0.05$ and $P \leq 0.001$
[a]T - Total
[b]EA - Exchangeable (organic acids), [c]EB - Exchangeable ($CaCl_2$), [d]EC - Exchangeable (EDTA)

To determine if heavy metals in the soil were from a common source, multivariate PCA and CA analysis was performed. The PCA loadings of the metals in the soil are given in Table 28 and the corresponding loading scatter plots are presented in Figure 14. The PCA reduces the data by extracting new variables (principal components, PCs) from previous variables (metal concentration and site) that will describe the data in a simplified way. The first component points to the direction in which the larger variation is obtained. A component loading higher than 0.71 was considered excellent (Nowak, 1998). Three PCs were extracted with eigenvalues >1 explaining 89% of the total variance. The first PC (44.9% of the variance) indicated high loadings of As, Cd, Fe and Ni. The second PC showed significant loadings of Cu, Pb and Zn (29.3% of the variance). The third PC (14.6% of the variance) revealed higher loadings of Mn and Co could be from natural sources (soil mineral forming processes).

Table 28: Principal component loadings of heavy metals in soil.

	PC1	PC2	PC3
Eigenvalues	4.498	2.931	1.460
Percentage of total variance	44.979	29.314	14.603
Percentage of cumulative variance	44.979	74.292	88.895
As	0.940	-0.043	0.031
Cd	0.959	0.000	0.186
Co	0.525	0.439	0.547
Cr	0.638	0.287	-0.586
Cu	0.103	0.901	-0.217
Fe	0.947	0.017	0.222
Mn	-0.259	0.638	0.627
Ni	0.877	0.264	-0.348
Pb	-0.465	0.731	-0.398
Zn	-0.228	0.912	0.114

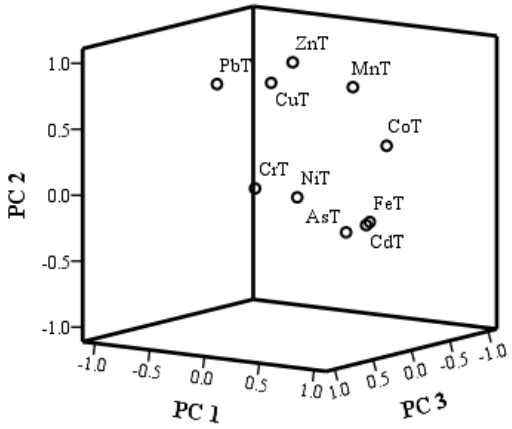

Figure 14: Three dimensional PCA loading plot for ten heavy metals in the soil (constructed for ten sampling sites).

Cluster analysis was done to identify relatively homogenous groups of heavy metals (Hu et al., 2013). Ward's method was used to indicate the degree of associations between metals in the soil, shown by the Euclidean distance (Fig. 15). In the dendrogram, the shorter the distance the more significant is the association. The metals were grouped into two main clusters (A and B) with cluster A having two sub-clusters (A1 and A2). Sub-cluster A1 consisted of As, Cd, Cr, Fe and Ni whilst A2 consisted of Co and Cu. This indicates that these metals have similar distribution patterns in the soil. In cluster B there was close association between Mn, Pb and Zn. PCA and CA yielded similar results indicating three different factors responsible for the distribution of heavy metal concentrations in the soil. Arsenic, Cd, Fe and Ni in soils around industries originate from an anthropogenic source. Lead and Zn could be due to vehicular emissions.

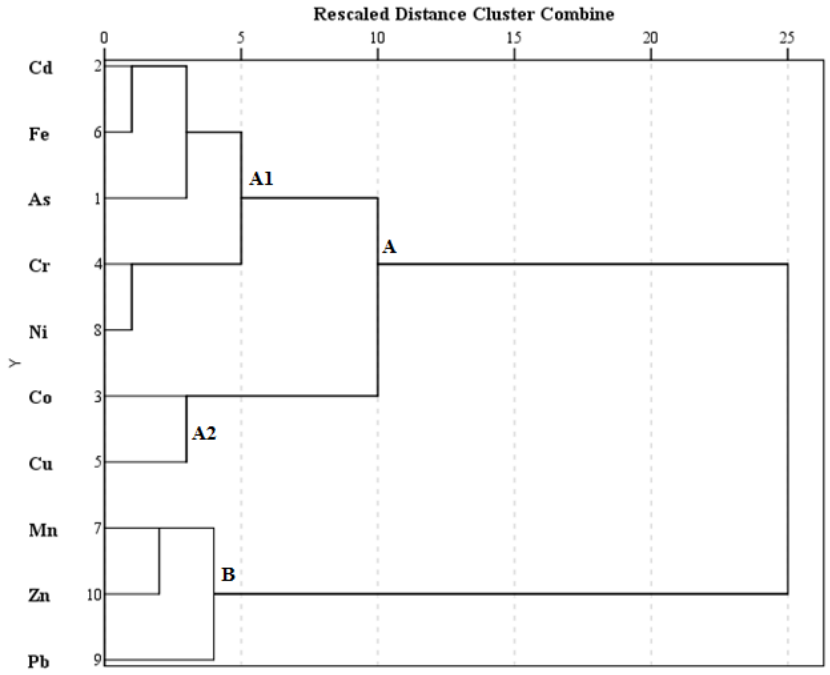

Figure 15: Cluster analysis using Ward's method of heavy metals in soil measured by Euclidean distance.

The correlations between elements in the plant and soil were evaluated by obtaining correlation coefficients (r) where r values ranged from -1 to +1. An r value of -1 indicated a strong negative linear relationship, an r value of +1 indicated a strong positive linear relationship and an r value of 0 indicated no relationship. An inter-correlation matrix between the soil (total and exchangeable) and plant concentrations of elements, soil pH, SOM and CEC was done. Only the strong correlations were extracted and are presented in Table 29.

Table 29: Correlation matrix for concentration of elements in soil, total (T) and exchangeable (E).

	AsT	CdT	FeT	CrT	CuT	PbT
CdT	0.9**	1	1.0**	ns	ns	ns
FeT	0.9**	1.0**	1	ns	ns	ns
NiT	0.8**	0.7*	0.7*	0.9**	ns	ns
PbT	ns	ns	ns	ns	0.7*	1
ZnT	ns	ns	ns	ns	0.7*	0.8**
	CuE	ZnE				
PbT	ns	0.8**				
ZnT	0.8**	1.0**				

*,** - correlations significant at P≤0.05 and P≤0.01.
ns - not significant.

There was a four way synergy between As, Cd, Fe and Ni in the soil, indicating that these elements have a common origin as confirmed by PCA and CA. This trend has been observed and reported in other studies (Wu et al., 2013). There was also a three way synergy between Cu, Pb and Zn, indicating that these elements come from a common origin as confirmed by PCA (Oze et al., 2008). There was a positive correlation between total soil Cr and total soil Ni (r=0.9), which was also observed in the CA.

There was a significantly positive correlation between total soil Zn with exchangeable Cu (r=0.8) and total soil Pb with exchangeable Zn (r=0.8) indicating a synergistic effect between these metals in soil. There was no significant correlation between soil concentration (total and exchangeable) and plant concentrations thereby indicating that uptake was not dependent on soil concentrations and that the plant controlled uptake to meet physiological needs.

CONCLUSION

Heavy metals in soil can threaten human health if introduced into the food chain at toxic levels. However, the results indicate that though there was moderate contamination and significant enrichment of Cd in the soil, the plant tended to exclude this metal, therefore posing no risk of Cd toxicity if consumed. PCA and CA revealed the dominance of As, Cd, Fe and Ni in the soil, possibly adsorbed onto iron oxide minerals. Correlations between the concentrations of As, Fe, Cd and Ni in soil further indicated the metals common origin. Although site had an effect on the metal concentration levels in the plant and soil, the plant exhibited control on the uptake amounts to meet its physiological requirement levels, as evidenced by the selective accumulation and exclusion of different elements.

Acknowledgements

The authors would like to acknowledge the National Research Foundation (NRF) for financial support and the School of Chemistry and Physics at UKZN.

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CHAPTER FIVE

Elemental analysis of edible mountain nettle (Obetia tenax) and the influence of soil quality on its chemical composition

ABSTRACT

Trace element toxicity due to soil pollution has been implicated as a causative factor in a number of health-related conditions such as cancer. Its greatest impact is in developing countries, where the dietary intake of essential elements is largely dependent on the consumption of wild edible fruits and leafy vegetables. Therefore, the aim of this study was to investigate the distribution of elements in the indigenous edible plant, *Obetia tenax* (mountain nettle), as a function of soil quality, from eight different sites in KwaZulu-Natal, South Africa. The results show concentrations of elements in the leaves to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cr > Cu > Ni > Pb > Co > As > Cd > Se, and in the stems and roots to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Ni > As > Pb > Co > Cd > Cr > Se. The quality and pollution status of soil was evaluated by applying pollution indices to soil data. Soil quality indicators (geo-accumulation indices and enrichment factors) indicated moderate Cd contamination at Msinga which was confirmed by the pollution index and ecological risk levels of single factor pollution. An assessment of overall contamination of soil using Nemerow pollution index showed moderate pollution by Cd whilst the potential toxicity index indicated low-grade risk for all elements at all sites. Principal component and cluster analysis revealed two groups of elements with similarities, As, Cd, Co, Cr, Cu, Mn, Ni and Fe, suggesting a lithogenic source and an anthropogenic source for Pb and Zn. Correlation analysis showed significantly positive correlations between As, Co, Cr, Cu, Fe and Ni/Cd in the soil, confirming the elements common origin.

Keywords Toxicity, plant nettles, soil pollution, metal contamination

INTRODUCTION

Soil formation occurs through the weathering of parent material in the earth's crust which is influenced by biota and climate (Schaetzl & Anderson, 2005). Soil minerals are inorganic solids with physical, chemical and crystalline properties that are incorporated into the soil. As part of the development of soil, chemical and physical weathering of the parent material results in the transformation of solid-state primary minerals (eg. quartz, amphiboles and pyroxenes) into more stable secondary minerals (Chesworth, 2008). The most common secondary mineral classes are clay minerals, hydrous Al, Fe and Mn oxides, carbonates and sulfates. Trace elements are normally found co-precipitated with secondary minerals in the soil. Trace elements include trace metals and micronutrients (Sparks, 1995). Co-precipitation is defined as the simultaneous precipitation of an element in conjunction with other elements by any mechanism at any rate (Sposito, 1983). Weathering of these minerals can release soluble ions that are then transported into the groundwater or removed through surface run off processes (Osman, 2013). Considering the presence of metal ions in the soil at different concentrations and their implication in soil pollution, concentrations (total and bioavailable) of these elements should be assessed. This is important since the exchangeable form of a metal ion in the soil is known to correspond to its level in the plant.

Quantification of the exchangeable form of metal ions will disclose their mobility and is essential to determine potential toxic effects if levels are above those that are permissible. There are several well documented methods for the extraction of exchangeable metal ions and these are based mainly on extraction with dilute acids, buffered and unbuffered inorganic

salts, organic salts and chelating agents (Adamo & Zampella 2008). Extraction with inorganic salt solutions at a neutral pH results in the extraction of exchangeable or easily mobile metal ions (Meers et al., 2007a; Meers et al., 2007b; Sager, 1992). The change in the ionic composition could cause competition between sorbed and excess ions for the adsorption sites. Weak acids such as acetic acid (0.43 M and 0.11 M) render the carbonates, hydroxides and trace elements exchangeable (Abedin et al., 2012; Rauret et al., 1999; Ure et al., 1993; Zhang et al., 1998). Synthetic compounds such as ethylenediaminetetraacetic acid (EDTA) desorb the exchangeable forms of elements and carbonates and partially desorb the organic matter complexes (Rauret, 1998) and, as such, have been used in the United Kingdom to estimate exchangeable Cu in soil (Ministry of Agriculture, Fisheries and Food, 1981).

Plant roots have the ability to alter soil pH and redox potential by releasing organic exudates and chelating agents thereby extracting metal ions from soil. Soils polluted with potentially toxic elements such as As, Cd, Hg and Pb, pose an environmental and health risk as they can accumulate in plants and move to the human food chain (Singh, 2007). Such soil contamination may arise from industrial activities, mining, agricultural practices and atmospheric deposition. These elements, although non-essential to plant growth, due to their chemical similarity to essential elements under the altered soil conditions, are taken up by plant transporters and integrated into the plant.

Rural communities have for generations consumed wild edible fruits and traditional leafy vegetables, due to geographic availability and financial accessibility (Mahlangeni et al., 2016a). *Obetia tenax* N.E. Br, a traditional leafy vegetable commonly known as mountain nettle, is located on the rocky hillsides and forests of Southern Africa. In South Africa, it grows in six of the nine provinces. The branches and leaves are densely covered in stinging hairs, which is a common trait

in the Urticaceae family. *O. tenax* leaves are used multi-contextually as a green vegetable for their nutritional value (usually prepared with maize meal porridge) and for their medicinal properties (van Wyk & van Wyk, 1997).

Previously, we reported on the distribution of trace elements in some indigenous, edible and medicinal plant species found in South Africa (Jonnalagadda et al., 2008; Mahlangeni et al., 2016a; Moodley et al., 2012). We have also reported on the distribution of nutrients in *Laportea peduncularis* subspecies *peduncularis* (river nettle) and *Urtica dioica* (stinging nettle) (Mahlangeni et al., 2016b). In this study, we report on the concentration and distribution of elements in the mountain nettle (*O. tenax* leaves, stems and roots) and associated growth soil, collected from eight different sites in KwaZulu-Natal, South Africa. The soil quality was assessed by obtaining geo-accumulation indices, enrichment factors, pollution indices and potential ecological risk. Multivariate statistical analysis of data was performed to reveal potential sources of elements in the soil and correlation analysis revealed relationships between elements in soil and plant

MATERIALS AND METHODS

Sample collection and sample preparation

Plant samples (leaves, stems and roots) and associated soil samples were collected from eight different sites in KwaZulu-Natal, South Africa (Fig. 16).

Figure 16: Map showing eight selected sampling sites in KwaZulu-Natal, South Africa.

Sampling sites are classified as either forest land, urban (next to a main road) or suburban areas (Table 30). Plant and soil samples were collected in May when temperatures ranged from 23 ºC to 27 ºC (in autumn). Soil samples were taken from a depth of 15 cm and the sample size was reduced by coning and quartering. Soil was generally loamy sand in texture. The soil was then passed through a 2 mm mesh sieve to remove gravel, air-dried to constant mass then crushed with a mortar and pestle to reduce particle size. Plant samples were washed with double distilled water to remove debris, oven dried at 50 ºC to constant mass then crushed using a food processor. All samples were stored in labelled polyethylene bags in a refrigerator at 4 ºC until analysed.

Table 30: Site description and geographical coordinates, in decimal degrees, for the eight different sites.

Site description	Sites	Latitude	Longitude
Forest land	Montebello	-29.822382	30.946485
	Msinga	-28.796667	30.495556
	Opongola	-27.276730	31.275310
	Tongaat	-29.569330	31.085177
Urban area	Bhambayi	-29.703003	30.977200
	Westville	-29.822382	30.946485
Suburban area	Goqokazi	-29.690948	30.927423
	Nkwazi	-29.281758	31.361105

Soil analysis

Soil pH, soil organic matter and cation exchange capacity

Soil pH was measured using a $CaCl_2$ solution (0.01 mol L^{-1}) in a 1:2 ratio (dry wt/v). Soil organic matter was determined by the Walkley-Black wet extraction technique (Walkley & Black 1934). Cation exchange capacity of soil was determined by the Chapman method (1965) using ammonium acetate at pH 7.

Extraction of exchangeable elements

The exchangeable form of elements in soil was estimated using three methods which were briefly described: (i) as per method of Quevauriller et al. (1997). 1.0 g of soil in 10 mL of 0.11 mol L^{-1} acetic acid was shaken for 16 h; (ii) as per method described by Novosamsky et al. (1993), 1.0 g of soil in 10 mL a combined unbuffered salt solution of 0.01 mol L^{-1} $CaCl_2$, $NH_4C_2H_3O_2$ and NH_4NO_3 was shaken for 2 h and (iii) as per method of Quevauriller et al. (1996), 1.0 g of soil in 10 mL 0.05 mol L^{-1} disodium-EDTA salt was shaken for 2 h. All determinations were done in quadruplicate and samples were stored in the refrigerator until elemental analysis.

Digestion of soil

The microwave-assisted closed vessel digestion technique (Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, North Carolina, USA) with patented Xpress Plus technology™, was used for digestion of samples (Mahlangeni et al., 2016a). Soil samples and soil certified reference material (CRM, 0.25 g) were placed in 50 mL liners with 10 mL of HNO_3 and digested. Digests were filtered into 25 mL volumetric flasks and made up to the mark with double distilled water, transferred into polyethylene bottles and stored in a refrigerator until elemental analysis. All determinations were done in quadruplicate.

Digestion of plant material

Plant samples and plant CRM (0.20 g) were digested with 10 mL of HNO_3 as above and filtered into 25 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles (Mahlangeni et al., 2016a). All determinations were done in quadruplicate and samples were stored in the refrigerator until elemental analysis.

Elemental analysis

All elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn) were analysed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) and reported as $\mu g\ g^{-1}$, dry matter. Elemental standards (1000 mg L^{-1}) were supplied by Sigma Aldrich (St Louis, USA) and were of analytical-reagent grade. Working standards were made up with double distilled water and 10 mL of 70% HNO_3 to match the sample matrix.

Indicators of soil quality

The bioaccumulation factor is the ratio of the concentration of element in the edible part of the plant organism (C_{plant}) and the concentration of the element in the soil (C_{soil})

$$BAF = \frac{C_{plant}}{C_{soil}} \quad (10)$$

The enrichment factor (EF) gives the ratio of the concentration of an element in the soil to its concentration in the earth's crust. This factor is used to assess for elemental contamination in soils. In this study, Zn is used as reference metal, since total baseline concentrations for Zn in South Africa are known (Herselman et al., 2005; Mendiola et al., 2008).

$$EF = \frac{\left[\frac{X}{Zn}\right]_{soil}}{\left[\frac{X}{Zn}\right]_{crust}} \quad (11)$$

where $\left[\frac{X}{Zn}\right]_{soil}$ is the mean concentration ratio between the element and Zn in the soil whilst $\left[\frac{X}{Zn}\right]_{crust}$ is the mean concentration ratio between the element and Zn in the earth's crust. Background concentrations for elements in South African soils were obtained from Herselman et al. (2005) and are as follows (in $\mu g\ g^{-1}$): 2.7 for Cd, 69 for Co, 353 for Cr, 117 for Cu, 159 for Ni, 65.8 for Pb and 115 for Zn. The EFs were interpreted as suggested by Zhang and Liu (2002) where $0.5 < EF \leq 1.5$ indicate that the element is from crustal minerals or natural processes, and $EF > 1.5$ indicates sources are more likely to be anthropogenic.

The geo-accumulation index (I_{geo}) is another soil quality indicator that describes the extent to which elemental contamination has occurred by comparing measured elemental concentrations to that of the earth's crust (Müller, 1969). It is determined by the following equation:

$$I_{geo}=\log_2\left[\frac{C_n}{1.5\ B_n}\right] \quad (12)$$

Where C_n is the measured concentration of the element in the soil sample, B_n is the geochemical background value of the earth's crust and 1.5 is the factor used to minimise possible variations in background values due to lithological differences (Herselman et al., 2005). The geo-accumulation value (degree of metal contamination) in soil as described by Müller (1969) are: $I_{geo} \leq 0$ (uncontaminated), $0 < I_{geo} \leq 1$ (uncontaminated to moderately contaminated), $1 < I_{geo} \leq 2$ (moderately contaminated), $2 < I_{geo} \leq 3$ (moderately to heavily contaminated), $3 < I_{geo} \leq 4$ (heavily contaminated), $4 < I_{geo} \leq 5$ (heavily to extremely contaminated), and $I_{geo} > 5$ (extremely contaminated).

The extent of elemental pollution in the soil was evaluated by assessing the degree of elemental pollution by a single pollution index (PI) and Nemerow integrated pollution index (Yang et al., 2011).

$$PI=\frac{C_i}{S_i} \quad (13)$$

Where C_i is the concentration of the element and S_i is the background concentration of the element in the earth's crust. Pollution index is classified as non-polluted if $PI < 1$, lowly polluted if $1 < PI \leq 2$, moderately polluted if $2 < PI \leq 3$, strongly polluted if $3 < PI \leq 5$ and very strongly polluted if $PI > 5$. The Nemerow integrated pollution index (NIPI) can be expressed as

$$NIPI=\sqrt{\frac{PI_{iave}^2+PI_{imax}^2}{2}} \quad (14)$$

where PI^2_{iave} and PI^2_{max} refer to mean and maximum pollution index values of each element, respectively. Nemerow integrated pollution indices are classified as non-polluted if $NIPI < 0.7$,

warning of pollution if $0.7 < NIPI \leq 1$, lowly polluted if $1 < NIPI \leq 2$, moderately polluted if $2 < NIPI \leq 3$, and highly polluted if $NIPI > 3$ (Jiang et al., 2014).

The potential ecological risk is an assessment of the harmful effects of elements in the environment which includes water, soils and sediments (Hakanson, 1980; Du et al., 2015). Considering the toxic response and the total risk index, actual pollution conditions of seriously polluted sediments are exhibited (Li et al., 2014). The potential toxicity response index (RI) is calculated as the sum of the risk factors (E_i) of elements:

$$RI = \sum E_i \quad (15)$$

where E_i is the single risk factor for element *i*, and is defined as

$$E_i = T_i f_i = T_i \frac{C_i}{B_i} \quad (16)$$

where T_i is the toxic-response factor for element *i*, which accounts for the toxic and sensitivity requirements. The T_i values for Cd, As, Ni, Cu, Pb, Cr, and Zn are 30, 10, 5, 5, 5, 2, and 1, respectively (Hakanson, 1980). The ratio f_i is the elemental pollution factor calculated from the measured concentration C_i and the background concentration B_i of the elements in the earth's crust. The ecological risk level of single-factor pollution is classified as low if $E_i < 40$, moderate if $40 < E_i \leq 80$, higher if $80 < E_i \leq 160$, high if $160 < E_i \leq 320$; and serious if $E_i > 320$. Potential toxicity index (RI) is classified as low-grade, if $RI < 150$, moderate $150 < RI \leq 300$, severe if $300 < RI \leq 600$, and serious if $RI > 600$ (Guo et al. 2010).

Statistical analysis

Multivariate statistical analyses (principal component analysis and cluster analysis) were performed to determine the relationship between input variables. An analysis of covariance was performed to assess for significant differences between plant and soil at the different sites. Correlation analysis was performed by computing Pearson's correlation coefficients (r) for the relationships between the concentrations of the elements in leaves and total and exchangeable concentrations in the soil. All statistical analyses were performed using the Statistical Package for the Social Science (PASW Statistics, Version 23, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

The accuracy of the method for elemental analysis was measured by comparing experimental results obtained with certified values (Table 31). The recorded values for Cd and Pb in the soil CRM compared well with certified values, whilst those for As, Co, Cr, Cu, Ni and Zn were within the acceptable limits. For the plant CRM, measured values compared well to certified values.

Table 31: Measured values (mg kg^{-1}, dry mass, mean ± standard deviation, 95% confidence interval, n=4*)* compared to certified values for certified reference materials (Elements in soil - D081-540 and White clover - BCR 402).

Element	Certified value	Accepted limits	Measured value
		White Clover	
Cr	5.19	-	5.10 ± 0.170
Fe	244	-	237 ± 18.0
Se	6.70 ± 0.25	-	6.81 ± 1.40
Zn	25.2	-	30.3 ± 6.37
		Soil	
As	101 ± 5.92	61.0-116	63.6 ± 5.25
Cd	143 ± 5.60	104-182	131 ± 11.4
Co	232 ± 4.10	148-250	170 ± 13.2
Cr	86.8 ± 6.1	60.0-104	69.0 ± 5.65
Cu	268 ± 4.72	204-332	210 ± 6.2
Ni	236 ± 4.17	175-302	189 ± 14.0
Pb	97.9 ± 11.3	69.3-126	89.7 ± 12.5
Zn	130 ± 11.5	87-173	104 ± 10.4

Elemental distribution in plants and soil

The concentrations of elements in soil (total and exchangeable), leaves, stems, roots and bioaccumulation factors are presented in Table 32. The analysis showed 12 of the 13 elements to be present in the soil (total and exchangeable) and plants. If present, Se was found to be below the instrument detection limit (0.1150 µg g^{-1}). Exchangeable forms of elements were predicted using different extraction methods (acetic acid, combined inorganic salts and EDTA). For most elements, the extraction efficiency by EDTA was generally higher than acetic acid and combined inorganic salts. For Cr, Mg and Mn, exchangeable forms were best predicted by acetic acid with

exchangeable percentages ranging between 0.1 - 1.9%, 1.3 - 5.8% and 2.6 - 7.6%, respectively. For As and Ca, exchangeable forms were best predicted by the combined inorganic salts. Exchangeable forms of Co, Cu, Fe, Ni, Pb and Zn were best predicted by EDTA. These elements are known to have greatest affinity for carbonates (Kabata-Pendias & Pendias, 1984).

All soils were rich in Fe, Ca and Mg with concentrations ranging between 4664 - 60733 μg g^{-1}, 1220 - 7788 μg g^{-1} and 302 - 3189 μg g^{-1}, respectively. Although total soil Fe was high, only 0.2 - 3.2% was in exchangeable form and bioaccumulation factors were less than one, similar to previous studies (Jonnalagadda et al., 2008; Mahlangeni et al., 2012; Moodley et al., 2012). The toxic elements, As and Cd were only present in soil at Msinga and Opongola, yet present in plant roots at all sites. Plant roots have the ability to increase the solubility of As and Cd in the rhizosphere by the formation of complexes with inorganic ligands (eg. Cl^-, SO_4^{2-} and NO_3^-) and soluble organics (eg. low molecular organic acids) thus allowing these elements to diffuse into the root surface (Mengel et al., 2001; Welch & Norvell, 1999).

Table 32: Concentration of elements in µg g^{-1} (mean ± standard deviation, 95% confidence interval, n=4) in leaves, stems, roots of *Obetia tenax* and soil (total and exchangeable) samples with bioaccumulation factors (BF) and Exchangeable percentages (%Ex).

	Sites	Soil-total	Soil-exchangeable			%Ex	Leaves	Stem	Roots	BF
			Method 1	Method 2	Method 3					
As	Bhambayi	ND	ND	ND	ND	-	4.39 ± 0.585 a	3.86 ± 0.537 a	4.07 ± 0.523 ab	-
	Msinga	11.9 ± 2.24 a*	ND	0.216 ± 0.0411 a	0.0458 ± 0.0084 a	1.8	5.95 ± 0.917 ab	4.88 ± 0.886 ab	3.48 ± 0.306 b	0.41
	Opongola	10.4 ± 1.68 a	ND	0.262 ± 0.0500 a	0.0564 ± 0.0056 b	2.5	5.10 ± 0.864 a	ND	4.13 ± 0.779 ab	-
	Nkwazi	ND	ND	ND	ND	-	4.54 ± 0.776 a	ND	4.12 ± 0.133 ab	-
	Westville	ND	ND	ND	ND	-	4.70 ± 0.640 a	3.94 ± 0.388 a	4.07 ± 0.900 ab	-
	Montebello	ND	ND	ND	ND	-	5.09 ± 0.973 a	5.95 ± 1.12 b	3.69 ± 0.567 b	-
	Tongaat	ND	ND	ND	ND	-	4.86 ± 0.850 a	ND	3.93 ± 0.601 ab	-
	Goqokazi	ND	ND	ND	ND	-	7.63 ± 1.25 b	ND	5.15 ± 0.628 a	-
Ca	Bhambayi	3 821 ± 229 a	280 ± 6.99 a	397 ± 22.4 a	198 ± 43.9 ad	10.4	25 759 ± 792 a	31 896 ± 1 950 a	15 552 ± 610 a	8.35
	Msinga	3 952 ± 99.6 a	259 ± 9.38 a	360 ± 13.1 ab	239 ± 29.1 a	9.1	16 433 ± 828 bc	23 207 ± 1 708 b	17 664 ± 3 385 a	5.87
	Opongola	7 788 ± 501 b	649 ± 24.6 b	362 ± 26.7 ab	432 ± 65.0 b	4.7	18 768 ± 1 033	30 739 ± 996 a	17 554 ± 2 239 a	3.95
	Nkwazi	1 220 ± 95.7 c	76.0 ± 6.38 c	337 ± 11.4 b	48.6 ± 4.88 c	27.6	12 979 ± 1 575	15 284 ± 3 516 c	5 942 ± 363 b	12.5
	Westville	3 656 ± 177 a	196 ± 10.4 d	361 ± 13.5 ab	159 ± 25.2 de	9.9	14 330 ± 856	23 631 ± 1 021 b	16 554 ± 2 175 a	6.46
	Montebello	1 965 ± 94.7 d	146 ± 10.4 e	351 ± 38.3 ab	104 ± 2.13 ce	17.9	13 770 ± 475	13 719 ± 1 819 c	14 934 ± 724 a	6.98
	Tongaat	1 301 ± 76.3 c	95.8 ± 15.6 cf	339 ± 26.7 b	71.3 ± 3.36 c	26.1	26 339 ± 3 395	23 759 ± 1 019 b	10 610 ± 1 555 c	18.3
	Goqokazi	1 699 ± 186cd	134 ± 23.4 ef	352 ± 28.5 ab	98.6 ± 5.28 ce	20.7	21 051 ± 1 104	15 940 ± 1 232 c	9 248 ± 643 bc	9.38
Cd	Bhambayi	ND	ND	ND	ND	-	0.409 ± 0.0700 a	ND	ND	-
	Msinga	9.83 ± 0.915 a	ND	ND	ND	-	2.49 ± 0.306 b	2.97 ± 0.177 a	0.453 ± 0.0720 ac	0.30
	Opongola	1.95 ± 0.528 b	ND	ND	ND	-	1.00 ± 0.151 cd	ND	ND	-
	Nkwazi	ND	ND	ND	ND	-	0.400 ± 0.0627 a	ND	ND	-
	Westville	ND	ND	ND	ND	-	1.12 ± 0.379 c	1.88 ± 0.100 b	0.878 ± 0.205 b	-
	Montebello	ND	ND	ND	ND	-	1.11 ± 0.0925 c	1.68 ± 0.203 b	0.398 ± 0.0646 ac	-
	Tongaat	ND	ND	ND	ND	-	0.643 ± 0.105 ad	0.633 ± 0.115 c	0.292 ± 0.0554 c	-
	Goqokazi	ND	ND	ND	ND	-	1.80 ± 0.0711 e	0.688 ± 0.108 c	0.566 ± 0.0772 a	-
Co	Bhambayi	10.3 ± 1.24 a	0.340 ± 0.0287 a	ND	0.702 ± 0.127 a	6.8	0.886 ± 0.0486 ad	0.726 ± 0.0564 a	0.602 ± 0.0658 ad	0.07
	Msinga	29.3 ± 2.81 b	0.827 ± 0.0291 b	ND	2.09 ± 0.246 b	7.1	3.54 ± 0.377 b	6.64 ± 0.431 b	1.37 ± 0.0767 b	0.23
	Opongola	21.8 ± 0.900 c	0.192 ± 0.0172 c	ND	1.05 ± 0.134 ce	4.8	1.58 ± 0.0297 ce	1.28 ± 0.0512 ac	0.693 ± 0.0230 acd	0.06
	Nkwazi	ND	ND	ND	ND	-	0.562 ± 0.0699 d	0.669 ± 0.0757 a	0.467 ± 0.0529 d	-
	Westville	2.20 ± 0.428 d	0.173 ± 0.0067 c	ND	0.344 ± 0.0358 d	15.6	1.35 ± 0.392 ac	3.15 ± 0.207 d	0.966 ± 0.0539 ce	1.43
	Montebello	6.93 ± 1.46 e	0.524 ± 0.0316 d	ND	0.870 ± 0.0502 ac	12.6	1.66 ± 0.126 ce	6.11 ± 1.00 b	0.760 ± 0.0738 ae	0.88

	Tongaat	11.4 ± 0.690 a	0.338 ± 0.0082 a	ND	1.26 ± 0.108 e	11.1	1.28 ± 0.226 ac	1.54 ± 0.253 ac	0.786 ± 0.0765 ae	0.14
	Goqokazi	ND	ND	ND	ND	-	2.20 ± 0.504 e	1.72 ± 0.0777 c	2.30 ± 0.286 f	-
Cr	Bhambayi	61.8 ± 3.21 a	0.203 ± 0.0178 a	0.0703 ± 0.0019 ab	0.0988 ± 0.0067 ab	0.3	0.0044 ± 0.0007 a	7.38 ± 0.896 ac	0.0101 ± 0.0014 a	0.12
	Msinga	361 ± 19.5 b	0.172 ± 0.0032 b	0.0688 ± 0.0019 ab	0.0858 ± 0.0028 b	0.1	0.0430 ± 0.0067 b	87.7 ± 6.11 b	0.0070 ± 0.0016 b	0.24
	Opongola	308 ± 32.9 c	0.172 ± 0.0032 b	0.0713 ± 0.0025 ab	0.0978 ± 0.0044 ab	0.1	0.0179 ± 0.0018 c	12.8 ± 0.613 c	0.0019 ± 0.0002 c	0.04
	Nkwazi	7.54 ± 0.624 d	0.142 ± 0.0016 c	0.0695 ± 0.0019 ab	0.0853 ± 0.0046 b	1.9	0.0018 ± 0.0004 a	5.48 ± 1.22 a	0.0013 ± 0.0003 c	0.73
	Westville	52.3 ± 3.92 ae	0.159 ± 0.0083 bc	0.0700 ± 0.0020 ab	0.0903 ± 0.0039 b	0.3	0.0184 ± 0.0045 c	144.1 ± 3.00 d	0.0093 ± 0.0008 a	2.76
	Montebello	27.7 ± 2.69 de	0.159 ± 0.0105 bc	0.0738 ± 0.0067 a	0.112 ± 0.0165 a	0.6	0.0116 ± 0.0013 c	28.6 ± 3.03 e	0.0026 ± 0.0004 c	1.03
	Tongaat	48.3 ± 2.53 ae	0.145 ± 0.0039 c	0.0660 ± 0.0023 b	0.0850 ± 0.0029 b	0.3	0.0028 ± 0.0005 a	6.23 ± 1.02 a	0.0022 ± 0.0008 c	0.59
	Goqokazi	48.7 ± 2.23 ae	0.155 ± 0.0068 bc	0.0685 ± 0.0033 ab	0.0925 ± 0.0013 b	0.3	0.0136 ± 0.0023 c	10.2 ± 1.53 ac	0.0054 ± 0.0013 b	0.21
Cu	Bhambayi	28.4 ± 1.30 a	ND	ND	0.965 ± 0.121 a	3.4	5.25 ± 0.828 a	14.0 ± 1.08 ac	12.8 ± 2.05 a	0.49
	Msinga	101 ± 2.56 b	ND	ND	1.80 ± 0.252 b	1.8	29.6 ± 5.63 b	23.9 ± 2.02 b	8.19 ± 1.95 ab	0.24
	Opongola	53.3 ± 1.35 c	ND	ND	0.870 ± 0.105 a	1.6	14.6 ± 1.41 c	10.2 ± 1.82 c	8.65 ± 1.76 ab	0.19
	Nkwazi	8.12 ± 2.59 d	ND	ND	0.338 ± 0.0382 c	4.2	7.83 ± 1.55 a	9.19 ± 1.80 c	7.33 ± 1.06 b	1.13
	Westville	26.5 ± 1.81 a	ND	ND	0.826 ± 0.0705 a	3.1	16.4 ± 2.96 cd	44.2 ± 4.86 d	12.5 ± 1.81 a	1.67
	Montebello	12.6 ± 1.24 e	ND	ND	0.419 ± 0.0420 c	3.3	29.5 ± 2.45 b	17.9 ± 1.66 a	6.69 ± 1.47 b	1.42
	Tongaat	15.2 ± 1.42 e	ND	ND	0.434 ± 0.0132 c	2.9	8.79 ± 1.45 ac	10.4 ± 0.888 c	5.12 ± 0.836 b	0.68
	Goqokazi	13.6 ± 1.52 e	ND	ND	0.324 ± 0.0170 c	2.4	21.1 ± 2.86 d	9.23 ± 1.51 c	21.3 ± 3.61 c	0.68
Fe	Bhambayi	16 505 ± 1 372 ae	19.5 ± 3.15 a	ND	158 ± 34.8 a	1.0	559 ± 59.8 a	697 ± 59.8 a	397 ± 69.7 ae	0.04
	Msinga	60 733 ± 1 634 b	9.62 ± 1.24 bc	ND	131 ± 19.5 a	0.2	9 229 ± 1 284 b	12 045 ± 1 060 b	918 ± 74.6 b	0.20
	Opongola	38 900 ± 1 940 c	11.7 ± 2.18 c	ND	237 ± 33.2 b	0.6	3 139 ± 373 c	1 407 ± 100 ac	261 ± 52.1 ac	0.04
	Nkwazi	4 664 ± 570 d	22.5 ± 0.440 a	ND	130 ± 10.9 a	2.8	270 ± 50.7 a	410 ± 31.0 a	138 ± 13.2 c	0.09
	Westville	18 760 ± 524 e	18.2 ± 2.81 ac	1.37 ± 0.219 a	163 ± 19.5 a	0.9	3 983 ± 703 c	6 346 ± 397 d	1 602 ± 161 d	0.34
	Montebello	11 538 ± 849 f	32.5 ± 6.70 d	2.45 ± 0.361 b	373 ± 9.77 c	3.2	3 598 ± 346 c	6 059 ± 668 d	539 ± 93.1 e	0.53
	Tongaat	15 539 ± 769 a	3.42 ± 0.731 b	ND	178 ± 16.3 a	1.2	1 427 ± 244 a	2 468 ± 125 c	213 ± 12.4 ac	0.16
	Goqokazi	17 956 ± 1 705 ae	20.6 ± 1.20 a	ND	80.1 ± 5.15 d	0.5	8 041 ± 1 316 b	1 961 ± 327 c	1 469 ± 93.6 d	0.11
Mg	Bhambayi	1 865 ± 106 a	42.6 ± 0.966 af	27.9 ± 1.48 a	19.0 ± 3.21 ae	2.3	5 336 ± 173 a	4 333 ± 156 a	6 591 ± 658 ab	2.33
	Msinga	2 702 ± 77.4 b	103 ± 2.84 b	79.2 ± 4.82 b	53.6 ± 5.60 b	3.8	8 090 ± 409 b	10 648 ± 544 b	8 214 ± 1 028 bc	3.94
	Opongola	3 189 ± 146 c	186 ± 6.81 c	86.2 ± 3.98 c	68.0 ± 9.72 c	5.8	10 157 ± 509 c	1 563 ± 468 c	11 881 ± 1 494 d	0.49
	Nkwazi	302 ± 20.3 d	11.7 ± 0.854 d	8.07 ± 0.466 d	6.38 ± 0.386 d	3.9	3 876 ± 467 d	4 064 ± 897 a	2 189 ± 171 e	13.5
	Westville	3 074 ± 127 c	38.9 ± 3.34 ef	34.9 ± 1.69 e	25.9 ± 3.01 e	1.3	6 946 ± 186 e	7 457 ± 160 d	9 256 ± 831 c	2.43
	Montebello	1 034 ± 53.1 e	42.2 ± 5.61 af	33.8 ± 3.26 ae	21.7 ± 0.316 e	4.1	5 812 ± 269 af	3 809 ± 316 a	4 910 ± 229 a	3.68
	Tongaat	1 044 ± 35.4 e	29.2 ± 9.37 e	20.7 ± 0.515 f	13.3 ± 1.36 ad	2.8	6 599 ± 223 ef	7 474 ± 391 d	2 568 ± 368 e	7.16
	Goqokazi	1 510 ± 96.1 f	53.8 ± 3.38 a	38.5 ± 2.77 e	26.1 ± 1.60 e	3.6	6 032 ± 318 af	9 194 ± 593 e	8 357 ± 981 bc	6.09
Mn	Bhambayi	335 ± 23.2 a	17.3 ± 0.709 a	2.47 ± 0.197 a	16.4 ± 2.07 a	5.2	20.6 ± 2.55 a	58.2 ± 3.16 ac	39.3 ± 1.73 ab	0.17

	Msinga	605 ± 22.3 b	32.8 ± 1.56 b	4.25 ± 0.492 b	36.7 ± 4.25 b	5.4	132 ± 11.5 b	207 ± 14.5 b	47.3 ± 6.62 b	0.34
	Opongola	805 ± 33.7 c	54.5 ± 2.93 c	1.55 ± 0.134 ac	41.7 ± 6.51 b	6.8	109 ± 7.50 bc	85.3 ± 0.766 c	32.1 ± 4.64 abc	0.11
	Nkwazi	73.9 ± 10.0 d	4.55 ± 0.346 d	1.27 ± 0.0827 ac	3.01 ± 0.310 c	6.2	34.8 ± 6.04 a	30.8 ± 5.99 a	13.5 ± 1.11 c	0.42
	Westville	317 ± 25.6 a	16.0 ± 1.55 a	5.47 ± 0.460 b	17.8 ± 2.26 a	5.1	101 ± 22.7 ac	142 ± 3.86 d	51.5 ± 2.57 b	0.45
	Montebello	648 ± 72.6 b	49.5 ± 1.76 ce	17.2 ± 1.94 d	38.6 ± 1.52 b	7.6	347 ± 33.0 ac	330 ± 27.4 e	78.0 ± 9.24 d	0.06
	Tongaat	802 ± 57.7 c	43.0 ± 7.34 e	12.4 ± 0.501 e	63.9 ± 4.89 d	5.4	146 ± 9.28 ac	371 ± 19.5 f	86.4 ± 19.9 d	0.08
	Goqokazi	43.2 ± 2.30 d	1.14 ± 0.106 d	0.178 ± 0.0126 c	0.670 ± 0.0730 c	2.6	51.1 ± 4.60 d	28.7 ± 2.70 a	26.7 ± 6.00 ac	0.66
Ni	Bhambayi	10.4 ± 1.41 a	0.317 ± 0.0188 a	0.0938 ± 0.0022 a	0.317 ± 0.0402 a	3.1	2.51 ± 0.243 a	2.14 ± 0.149 a	4.06 ± 0.342 a	0.21
	Msinga	84.4 ± 4.62 b	0.410 ± 0.0090 b	0.0985 ± 0.0037 a	0.542 ± 0.0505 b	0.6	11.4 ± 1.22 b	15.8 ± 1.04 b	8.54 ± 0.671 a	0.19
	Opongola	122 ± 2.38 c	0.299 ± 0.0178 a	0.109 ± 0.0031 b	1.16 ± 0.155 c	1.0	9.07 ± 0.489 bc	4.90 ± 0.224 c	6.79 ± 0.474 a	0.04
	Nkwazi	ND	ND	ND	ND	-	1.82 ± 0.0990 a	1.54 ± 0.140 a	1.73 ± 0.101 a	-
	Westville	11.7 ± 1.23 a	0.224 ± 0.0038 c	0.0985 ± 0.0037 a	0.216 ± 0.0129 ad	1.9	4.37 ± 0.504 ac	6.31 ± 0.402 c	2.93 ± 0.304 a	0.54
	Montebello	ND	ND	ND	ND	-	3.43 ± 0.259 ac	4.98 ± 0.684 c	1.74 ± 0.0837 a	-
	Tongaat	3.68 ± 0.512 d	0.293 ± 0.0105 a	0.0895 ± 0.0047 a	0.286 ± 0.0169 a	7.8	4.63 ± 0.981 ac	19.5 ± 1.79 d	3.69 ± 0.219 a	5.30
	Goqokazi	7.96 ± 0.932 ad	0.201 ± 0.0057 c	0.0913 ± 0.0083 a	0.118 ± 0.0068 d	1.5	38.3 ± 7.48 d	4.94 ± 0.683 c	79.6 ± 9.04 b	0.62
Pb	Bhambayi	19.8 ± 3.10 a	0.0096 ± 0.0014	ND	1.34 ± 0.282 a	6.8	0.627 ± 0.0759 ab	1.22 ± 0.260 a	0.148 ± 0.0205 a	0.06
	Msinga	2.63 ± 0.311 b	ND	ND	0.340 ± 0.0499 bc	12.9	1.07 ± 0.0836 b	2.17 ± 0.181 a	ND	0.82
	Opongola	2.09 ± 0.325 b	ND	ND	0.177 ± 0.0322 c	8.5	0.0520 ± 0.0095 a	0.860 ± 0.150 a	0.196 ± 0.0273 ab	0.41
	Nkwazi	ND	ND	ND	ND	-	ND	0.817 ± 0.0695 a	0.354 ± 0.0715 ab	-
	Westville	42.9 ± 4.58 d	0.144 ± 0.0013	ND	3.09 ± 0.349 d	7.2	4.26 ± 0.610 c	19.6 ± 2.26 b	3.68 ± 0.297 c	0.46
	Montebello	4.31 ± 0.891 b	ND	ND	0.442 ± 0.0129 bc	10.3	4.34 ± 0.484 c	8.61 ± 1.19 c	0.742 ± 0.124 de	2.00
	Tongaat	6.40 ± 0.971 b	ND	ND	0.579 ± 0.0681 b	9.1	0.454 ± 0.0420 ab	0.798 ± 0.123 a	0.507 ± 0.0763 be	0.12
	Goqokazi	2.45 ± 0.337 b	ND	ND	0.403 ± 0.0207 bc	16.4	2.23 ± 0.324 d	1.65 ± 0.194 a	1.05 ± 0.261 d	0.67
Zn	Bhambayi	170 ± 19.2 a	3.72 ± 0.24 a	ND	4.67 ± 0.966 a	2.8	15.3 ± 2.72 a	35.2 ± 5.98 a	55.0 ± 5.08 a	0.21
	Msinga	89.6 ± 7.93 b	0.420 ± 0.0700 be	ND	0.656 ± 0.0770 b	0.7	41.1 ± 7.24 a	34.4 ± 0.705 a	53.5 ± 4.93 a	0.38
	Opongola	84.0 ± 6.38 b	0.410 ± 0.0577 be	ND	1.67 ± 0.305 c	2.0	30.3 ± 2.30 a	35.5 ± 4.64 ab	40.1 ± 7.19 ab	0.42
	Nkwazi	88.7 ± 11.3 b	7.13 ± 0.538 c	0.523 ± 0.0461 a	5.62 ± 0.431 a	6.3	51.9 ± 8.28 a	55.7 ± 3.81 c	42.8 ± 7.31 ab	0.63
	Westville	101 ± 5.69 b	2.61 ± 0.315 d	0.161 ± 0.0295 b	3.39 ± 0.399 d	3.4	60.9 ± 9.08 a	127 ± 3.65 d	50.7 ± 6.31 ab	1.26
	Montebello	43.9 ± 4.58 c	0.918 ± 0.0235 e	ND	0.598 ± 0.0255 b	1.4	58.5 ± 6.71 a	50.2 ± 11.2 bc	37.0 ± 4.39 b	1.14
	Tongaat	54.7 ± 7.48 c	0.302 ± 0.0499 b	ND	0.195 ± 0.0331 b	0.4	34.0 ± 4.23 a	38.9 ± 1.16 abe	41.7 ± 8.08 a	0.71
	Goqokazi	62.0 ± 4.89 c	1.52 ± 0.142 f	ND	1.12 ± 0.0873 bc	1.8	663 ± 130 b	51.7 ± 10.6 ce	91.7 ± 10.6 c	0.83

ND - not determinable

*Different letters within columns indicate mean separation by Tukey's Post-hoc test at the 5% level.

Method 1 - 0.11 mol L^{-1} CH_3COOH, Method 2 - 0.01 mol L^{-1} $CaCl_2$, $NH_4C_2H_3O_2$, NH_4NO_3, Method 3 - 0.05 mol L^{-1} EDTA.

These elements can also enter by competing with essential elements of the same radii for specific-ion transporters of the plant tissue (Saxena et al., 1999). Lead was observed to be present in soil at all sites except in Nkwazi. Total soil Pb was highest at Westville (42.9 μg g^{-1}) with 7.2% being in exchangeable form; however, bioaccumulation factors were less than one.

Msinga and Opongola had higher concentrations of the elements Co, Cr, Cu and Ni however there was no accumulation of these elements in the leaves (BAFs < 1) indicating the plants control on elemental uptake by adaptive mechanisms to meet physiological needs. Higher Cr concentrations in the leaves compared to stems and roots across all sites indicated higher mobility of Cr to the leaves. Chromium is not essential for plant growth but it is important for animal and human nutrition (Rodionava, 2001). At Goqokazi, total soil Zn was 62.0 μg g^{-1} and Zn in the roots was 663 μg g^{-1} (ten times higher) showing the plants ability to accumulate high concentrations of Zn in the roots to balance storage and transport of this metal to other parts of the plant. The study shows concentrations of elements in *O. tenax* leaves to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cr > Cu > Ni > Pb > Co > As > Cd, and *O. tenax* stems and roots to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Ni > As > Pb > Co > Cd > Cr.

The soil parameters (pH, soil organic matter and cation exchange capacity) for each site are presented in Table 33. Soil pH ranged from 3.94 (Montebello) to 4.73 (Msinga). Higher availability of micronutrients is observed between pH 5 and 7; above and below these pH values micronutrients become less available for uptake by plants (Strawn et al., 2015). Soil organic matter is a major source of carbon and plant nutrients; soil organic matter ranged from 2.18% (Goqokazi) to 18.24% (Opongola). Cation exchange capacity throughout the sites were relatively low with values ranging from 3.04 meq 100 g^{-1} (Nkwazi) to 5.35 meq 100 g^{-1} (Montebello).

Table 33: Soil pH, soil organic matter and cation exchange capacity (mean ± standard deviation, n=4) of soil samples from eight different sites.

Site	pH	Soil organic matter (%)	Cation exchange capacity (meq $100g^{-1}$)
Bhambayi	4.54 ± 0.06 a*	5.41 ± 0.37 a	3.38 ± 0.42 a
Msinga	4.73 ± 0.06 b	10.01 ± 0.84 b	4.86 ± 0.20 b
Opongola	4.70 ± 0.08 b	18.24 ± 1.48 c	3.35 ± 0.35 a
Nkwazi	4.23 ± 0.04 c	2.83 ± 0.63 d	3.04 ± 0.29 a
Westville	4.12 ± 0.02 c	11.24 ± 1.19 be	3.64 ± 0.53 a
Montebello	3.94 ± 0.07 d	13.05 ± 0.53 e	5.35 ± 0.68 b
Tongaat	4.16 ± 0.04 c	3.72 ± 0.32 ad	4.83 ± 0.06 b
Goqokazi	4.20 ± 0.02 c	2.18 ± 0.12 d	5.04 ± 0.20 b

*Different letters within columns indicate mean separation by Tukey's Post-hoc test at the 5% level.

Indicators of soil quality

Soil enrichment factor and geo-accumulation index values were determined to assess the level of elemental contamination and enrichment in the soil. Soils are considered contaminated if levels are above background levels (Wu et al., 2014). Enrichment factor and geo-accumulation index values of elements in soil samples from different sites are shown in Table 34. For elements, Co, Cr, Cu, Ni and Pb, enrichment factors (EF) were below 1 for all sites indicating no enrichment. There was enrichment of Cd at Msinga with an EF value of 4.6. EF values greater than 1.5 suggest that the sources are more likely to be anthropogenic (Zhang & Liu, 2002). Geo-accumulation index values for elements Co, Cr, Cu, Ni, Pb and Zn indicated non-contamination by these elements and moderate contamination by Cd at Msinga (along the roadside) possibly due to deposition of airborne particles from vehicular emissions.

Table 34: Enrichment factors and geo-accumulation indices of soil from eight different sites.

Sites	Elements	Enrichment factor	Geo-accumulation index
Bhambayi	Cd	ND	ND
	Co	0.1	-3.3
	Cr	0.1	-0.6
	Cu	0.2	-2.6
	Ni	0.0	-4.5
	Pb	0.2	-2.3
	Zn	1	0.0
Msinga	Cd	4.6	1.3
	Co	0.5	-1.8
	Cr	1.3	-0.6
	Cu	1.1	-0.8
	Ni	0.7	-1.5
	Pb	0.1	-5.2
	Zn	1	0.9
Opongola	Cd	1.0	-1.1
	Co	0.4	-2.2
	Cr	1.2	-0.8
	Cu	0.6	-1.7
	Ni	1.1	-1.0
	Pb	0.0	-5.6
	Zn	1	-1.0
Nkwazi	Cd	ND	ND
	Co	ND	ND
	Cr	0.0	-6.1
	Cu	0.1	-4.4
	Ni	ND	ND
	Pb	0.7	-1.2
	Zn	1	-1.0
Westville	Cd	ND	ND
	Co	0.0	-5.6
	Cr	0.2	-3.3
	Cu	0.3	-2.7
	Ni	0.1	-4.3
	Pb	0.2	-4.5
	Zn	1	-0.8
Montebello	Cd	ND	ND
	Co	0.3	-3.9
	Cr	0.2	-4.3
	Cu	0.3	-3.8
	Ni	ND	ND
	Pb	0.2	-4.5
	Zn	1	-2.0
Tongaat	Cd	ND	ND
	Co	0.3	-3.2
	Cr	0.3	-3.5
	Cu	0.3	-3.5
	Ni	0.0	-6.6
	Pb	0.2	-3.9
	Zn	1	-1.7

Goqokazi	Cd	ND	ND
	Co	ND	ND
	Cr	0.3	-3.4
	Cu	0.2	-3.7
	Ni	0.1	-4.9
	Pb	0.1	-5.3
	Zn	1	-1.5

ND - not determinable.

Pollution is defined as contamination in the soil that can cause adverse biological effects (Wu et al., 2014). The pollution index (PI) values of elements at different sites and Nemerow integrated pollution indices (NIPI) for each element are shown in Table 35. Pollution indices indicated no pollution by elements Co, Cu, Ni and Pb; low levels of pollution for Cr (Msinga) and Zn (Bhambayi); and high levels of pollution for Cd (Msinga). NIPIs, which assessed overall contamination of soils, indicated no pollution by Co, Cu, Ni and Pb, gave a warning-line of pollution for Cr and Zn and moderate levels of pollution for Cd.

Table 35: Single pollution indices and Nemerow integrated pollution indices of soil from eight different sites.

Sites	Pollution index						
	Cd	Co	Cr	Cu	Ni	Pb	Zn
Bhambayi	ND	0.15	0.18	0.24	0.07	0.30	1.48
Msinga	3.59	0.42	1.02	0.86	0.53	0.04	0.78
Opongola	0.71	0.32	0.87	0.46	0.77	0.03	0.73
Nkwazi	ND	ND	0.02	0.07	ND	ND	0.77
Westville	ND	0.03	0.15	0.23	0.07	0.65	0.88
Montebello	ND	0.10	0.08	0.11	ND	0.07	0.38
Tongaat	ND	0.17	0.14	0.13	0.02	0.10	0.48
Goqokazi	ND	ND	0.14	0.12	0.05	0.04	0.54
Nemerow integrated pollution index	2.96	0.33	0.76	0.64	0.57	0.48	1.06

ND - not determinable.

The potential ecological risk assessment reflects the effects of various contaminants and reveals the extensive influence of multiple contaminants in an environment (Wang et al., 2013). The single risk factor and potential ecological risk factor for elements in soils are presented in Table 36. The ecological risk levels of single factor pollution indicated low pollution for all elements (less than 40) except Cd at Msinga (single risk factor = 108) where it indicated higher pollution similar to pollution index. The potential ecological risk of elements at the eight sites were in decreasing order of Cd > Cu > Ni > Pb > Zn > Cr. The potential toxicity index indicated low-grade risk for all elements at the eight sites.

Table 36: Single risk factor and potential ecological risk factor of soil from eight different sites.

Sites	Single risk factor						Potential ecological risk factor
	Cd	Cr	Cu	Ni	Pb	Zn	
Bhambayi	ND	0.35	1.21	0.33	1.50	1.48	4.87
Msinga	108	2.05	4.32	2.65	0.20	0.78	118
Opongola	21.4	1.75	2.28	3.84	0.16	0.73	30.1
Nkwazi	ND	0.04	0.35	ND	ND	0.77	8.75
Westville	ND	0.30	1.13	0.37	3.26	0.88	5.93
Montebello	ND	0.16	0.54	ND	0.33	0.38	1.40
Tongaat	ND	0.27	0.65	0.12	0.49	0.48	2.00
Goqokazi	ND	0.28	0.58	0.25	0.19	0.54	1.83

ND - not determinable

Statistical analysis

The significance of principal component analysis is the reduction of the dimensionality of a set of datasets consisting of a large number of related variables, whist retaining as much as possible, the variation present in the dataset. This is done by extracting new sets of variables called the principal

components from the original dataset. Principal components point to the direction where there is the most variance (Jolliffe, 1986). Component loadings higher than 0.71 are deemed to be excellent (Nowak, 1998). The component matrix and rotated component matrix of the elements in the soil and roots are given in Table 37 and the resultant loading scatter plots are presented in Figure 17. The components were rotated using Varimax rotation. The components of minor elements (As, Cd, Co, Cr, Cu, Fe, Mn and Ni) present in the soil and roots were obtained to deduce their possible origin. In soil, two principal components were extracted with eigenvalues >1 explaining 83% of the total variance. The first principal component (66.1% of the variance) had high loadings of As, Cd, Co, Cr, Cu, Fe, Ni and partially Mn which could be from natural sources (eg. Fe and Mn oxides and hydroxides). The second principal component (16.5% of the variance) had high loadings of Pb and Zn, indicating that these elements could be from a common source such as automobile emissions. Zinc could be emitted from the erosion of automobile tyres (Brimblecombe, 1996; Dale & Freedman, 1982). In roots, three components were extracted with eigenvalues >1 explaining 91% of the total variance. The first principal component (40.7% of the variance) had high loadings of Cd, Co, Cr, Cu and Fe, similar to soil. The second principal component (29.7% of the variance) had high loadings of As, Ni and Zn. The third principal component (20.3% of the variance) had high loadings of Mn and Pb. Minor elements enter the roots from the soil solution into the xylem and are generally bound by organic chelates upon entry into the plant cells. Non-essential elements such as Cd and Pb are transported via the apoplastic pathway which aids in the transportation of water and ions from soil through roots into the plant (White, 2012).

Table 37: Component matrices for elements in the soil and roots.

Elements	Rotated component matrix				
	Soil		Roots		
	Principal component 1	Principal component 2	Principal component 1	Principal component 2	Principal component 3
Eigenvalues	6.607	1.651	4.065	2.972	2.028
% Total variance	66.069	16.505	40.652	29.718	20.279
% Cumulative variance	66.069	82.574	40.652	70.370	90.649
As	**0.967**	-0.166	0.356	**0.786**	0.043
Cd	**0.888**	-0.047	**0.913**	0.351	0.155
Co	**0.949**	-0.150	**0.949**	0.223	0.114
Cr	**0.985**	-0.101	**0.974**	-0.031	0.061
Cu	**0.977**	0.087	**0.673**	0.176	0.650
Fe	**0.976**	-0.023	**0.836**	0.504	0.165
Mn	0.505	-0.445	0.164	-0.251	**0.868**
Ni	**0.893**	-0.134	0.275	**0.933**	-0.064
Pb	-0.176	**0.772**	0.036	0.170	**0.882**
Zn	0.154	**0.876**	0.022	**0.966**	0.029

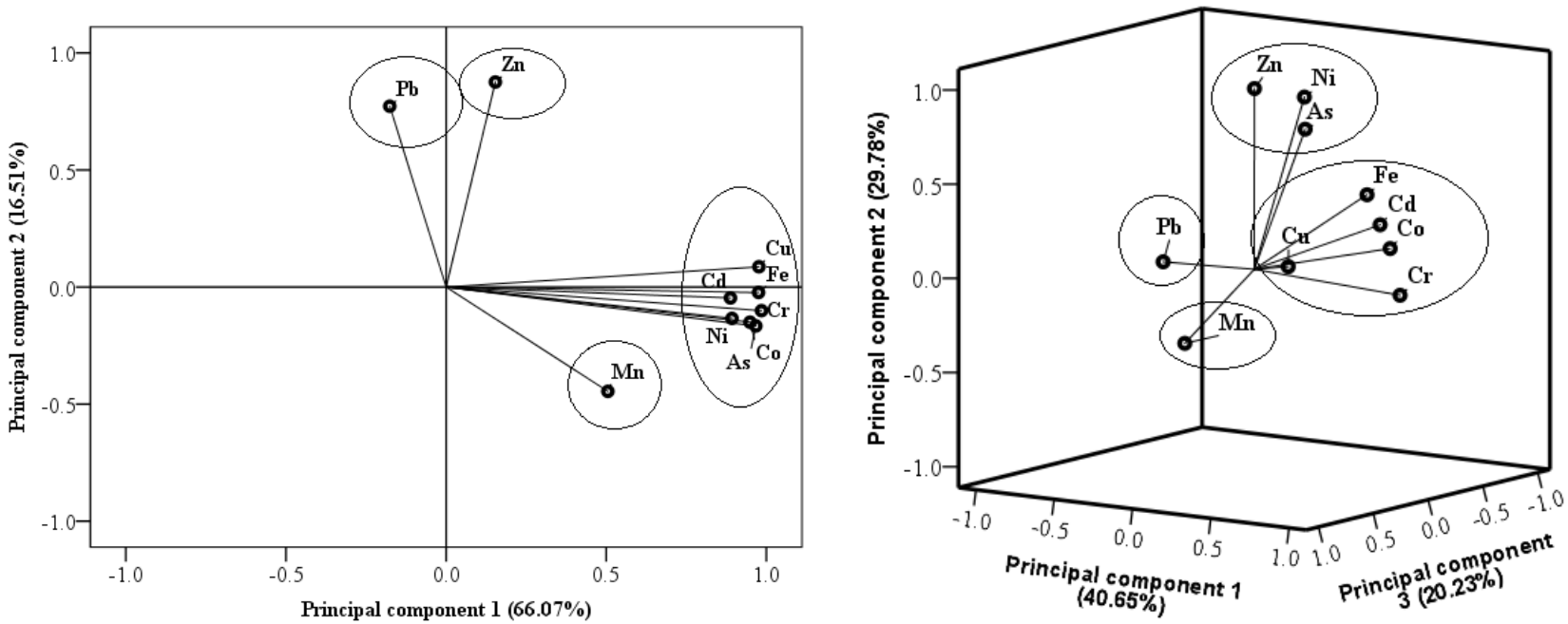

Figure 17: Principal component analysis loading plot for ten elements in the soil and roots (constructed for eight sampling sites).

Ward's method was used to indicate the degree of association between elements in the soil and roots, shown by the Euclidean distance (Fig. 17). In this study, cluster analysis was used to further analyse the possible source of elements. In soil, there were two main clusters, A and B, where A showed close associations amongst As, Cd, Co, Cr, Cu, Fe and Ni, similar to principal component analysis, and B showed close associations between Pb and Zn, also similar to principal component analysis of soil, thereby confirming that these elements are from a common source. Three clusters were observed in the roots; cluster A showed close associations between Ni and Zn, Cluster B showed close associations amongst As, Cd, Co, Cr and Fe, and cluster C showed close associations between Mn and Pb, similar to principal component analysis of roots.

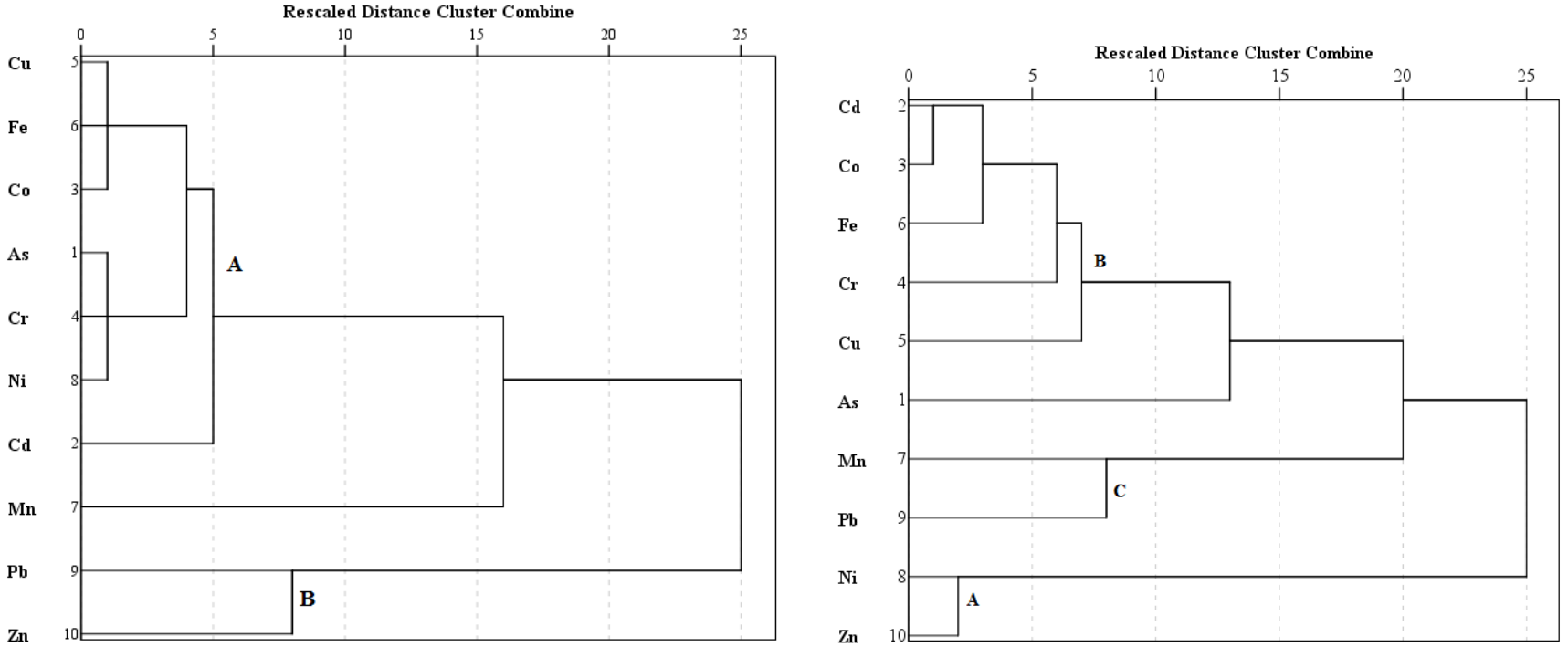

Figure 18: Cluster analysis using Ward's method of elements in soil and roots measured by Euclidean distan.

Site, element and their interaction affected elemental concentrations in *O. tenax* leaves. Therefore, elements in the leaves were analysed separately (Table 38). For all elements analysed, total and exchangeable soil concentrations alone had no effect on plant concentrations but there were significant differences between the concentrations found at the different sites ($P \leq 0.001$). This could be due to factors such as soil quality parameters (pH, soil organic matter and cation exchange capacity) and environmental conditions such as temperature, moisture and wind at the different sites (Mahlangeni et al., 2016a).

Table 38: Analysis of covariance at all sites involving the presence of elements in *O. tenax* leaves.

Element											
Source	As	Ca	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Soil-total	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant
Soil-exchangeable	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant	not significant
Site	significant	significant	significant	significant	significant	significant	significant	significant	significant	significant	significant

significant at calculated probability values ≤ 0.001.

As described previously in our work (Mahlangeni et al., 2012; Mahlangeni et al., 2016a; Moodley et al., 2012; Reddy et al., 2014), the correlations between elements in the plant and soil were evaluated by obtaining correlation coefficients (r) where r values ranged from -1 to +1. Correlation coefficients >0.8 indicates a strong positive linear relationship, < -0.8 indicates a strong negative linear relationship and 0 indicates no relationship. An inter-item correlation matrix between the soil (total and exchangeable) and plant concentrations of elements, soil pH, soil organic matter and cation exchange capacity was obtained and only the strong correlations are presented in Table 39 and 40.

A significant positive correlation was observed between soil pH and exchangeable As, Cu and Ni and a significant negative correlation was observed between cation exchange capacity and exchangeable Zn. Soil organic matter correlated positively with Ca in the stem and Mg in the roots. There was a six-way synergy between total soil As, Co, Cr, Cu, Fe and Ni/Cd, indicating that these elements have a common origin as indicated by principal component analysis and cluster analysis (Table 40). Iron oxides and hydroxides have high sorption capacities, particularly for trace elements, which can accumulate at the Fe rich points. Singh and Gilkes (1992) reported that major portions of Co, Cr, Cu and Ni, amongst other elements in the soils of South-Western Australia, were concentrated with the Fe oxides. Studies by Liao et al. (2010) on the sorption of Ni and Cd in single and binary Ni-Cd systems in three different soils showed that from the estimated K_f values of the absorption isotherms, there was significant mutual inhibition of Cd and Ni sorption in soils; the suppression effect was increased with higher concentrations of metal ions in soil. This could explain the six-way synergy with either Cd or Ni as the sixth element.

Table 39: Inter-correlation matrix of soil pH, soil organic matter, cation exchange capacity and elements in soil (exchangeable), in stems and roots of *O. tenax*.

	As-exchangeable	Cu-exchangeable	Ni-exchangeable	Zn-exchangeable	Ca-stem	Mg-roots
pH	0.8*	0.8*	0.8*	not significant	not significant	not significant
Soil organic matter	not significant	not significant	not significant	not significant	0.8*	0.8*
Cation exchange capacity	not significant	not significant	not significant	-0.8*	not significant	not significant

* - correlations significant at calculated probability values ≤ 0.05.

Table 40: Inter-correlation matrix of elements in soil (total and exchangeable), in leaves and stems of *O. tenax.*

	As-total	Cd-total	Co-total	Cr-total	Cu-total	Fe-total
Cd-total	0.8*					
Co-total	0.9**	0.8*				
Cr-total	1.0**	0.8*	0.9**			
Cu-total	0.9**	1.0**	0.9**	0.9**		
Fe-total	0.9**	0.9**	0.9**	1.0**	1.0**	
Ni-total	1.0**	not significant	0.8*	0.9**	0.8*	0.8*

	As-total	Co-total	Cr-total	Cu-leaves	Zn-leaves
Cu-exchangeable	0.8*	0.8*	0.8*	not significant	not significant
Mg-exchangeable	0.9**	0.7*	0.9**	not significant	not significant
Ni-exchangeable	0.8*	0.8*	0.8*	not significant	not significant
Pb-exchangeable	not significant	not significant	not significant	0.9**	0.8*

A synergistic relationship occurs when an increase in total soil concentration of one element increases exchangeability of another, indicating that these elements are competing for the same adsorption sites (Kalavrouziotis et al., 2008). There was a significantly positive correlation between total soil As, Co and Cr with exchangeable Cu, Mg and Ni, indicating a synergistic relationship. A positive correlation was observed between exchangeable Pb with Zn and Cu in the leaves. This indicates that high concentrations of Pb in the soil solution promotes uptake of Cu and Zn. The plasma membrane metal uptake system has a high affinity for certain metal ions, thus Cu and Zn are preferentially absorbed by the plant when ion concentrations increase, leaving more Pb in the soil solution (Alloway, 1995; Krämer et al., 2007).

CONCLUSION

Entry of toxic trace elements into the food web via plant-soil interactions poses a health risk for both animals and humans. In this study, although there was Cd contamination and pollution at Msinga, there was no accumulation of the metal in the plant. Plant roots stored trace elements As, Cd, Pb and controlled the amounts that were transported into the stems and edible leaves. Principal component and cluster analysis revealed that As, Cd, Co, Cr, Cu, Ni and Fe were from the same natural source (Fe and Mn hydroxides and oxides) which was confirmed by correlation data. Site location had influence on elemental concentrations in the plant, however the plant controlled uptake as evidenced by the root and stem elemental concentrations, as well as accumulation and exclusion of elements in the leaves. The plant therefore takes up elements in order to accomplish its normal functions.

Acknowledgements

We are grateful to the National Research Foundation (NRF) for financial support and the School of Chemistry and Physics at UKZN.

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CHAPTER SIX

Uptake, translocation and bioaccumulation of elements in the forest nettle (Laportea alatipes) from KwaZulu-Natal, South Africa

ABSTRACT

Elements found in the edible parts of plants are considered the main source of nutrients for humans and animals. In this study, the distribution of elements in the edible forest nettle (*Laportea alatipes*) was evaluated as a function of geographical region by sampling from eight different locations in KwaZulu-Natal, South Africa. Translocation and bioaccumulation factors were used to assess the transport of elements between roots, stems and leaves. Translocation factors for Co, Cr, Cu, Mn, Ni, Pb and Zn showed effective translocation from stems to leaves. Bioaccumulation factors for Co, Cr, Mn, Ni, Pb and Zn indicated that the plant excluded these elements to meet physiological requirements whilst storing the elements As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn in the roots thereby reducing its translocation to the aerial parts of the plant. Concentrations of minor elements in the leaves were found to be in decreasing order of Fe> Mn> Zn> Cu> Cr> Ni> Co> Pb> Se> As>Cd. Geo-accumulation indices and enrichment factors showed no contamination or minimal enrichment of trace elements in the soil. Principal component and cluster analysis of soil showed Co, Cr, Cu, Fe, Ni, Pb and Zn to come from the same source, whilst Mn was from a natural source, chelated on the soil organic matter. Correlation analysis showed significantly positive correlation between total soil Ni with Co, Cr and Pb as well as total soil Pb with Fe and Zn.

Keywords Trace metals, Bioaccumulation factor, Translocation factor, Nettle

INTRODUCTION

In the plant, elements such as Cu, Cr, Mn, Fe and Zn are essential for various enzymatic processes, structural formation and the functioning of various membranes; their deficiency in the plant may result in metabolic disruptions and their superfluity may cause toxic effects (Marschner, 1983). The source of plant nutrients is exogenous; these nutrients are present naturally in the soil but their levels are affected by anthropogenic inputs (Chibuike & Obiora, 2014).

The distribution and content of elements in the soil as well as their form and availability differs (Lawlor, 1991). The different tissue organs of the plant contain varying amounts of nutrients (Epstein, 1972). Nutrients are taken up or absorbed through the roots of the plant in inorganic form, from where they are transported to the various plant tissues via xylem vessels. Translocation is the movement of elements, in the complexed form, from the roots to the shoots or leaves of the plant through the xylem vessels (Greger, 2004). These nutrients, through various processes in the roots can also become immobile and this restricted uptake may result in nutrient deficiency's (Kabata-Pendias, 2011). Conversely, increased uptake and translocated to the various tissue organs of the plant may result in bioaccumulation. Bioaccumulation is the accumulation of a chemical entity in the tissue of an organism and, in the case of plants, soil characteristics such as elemental soil concentrations influence bioaccumulation (Environmental Protection Agency, 2000). Other soil characteristics that affect bioaccumulation include cation exchange capacity (CEC), pH, organic matter and competition of elements at the uptake sites (Hardiman et al., 1984). An increase in CEC of the soil increases elemental uptake and a decrease in soil pH promotes an increase in elemental uptake (Marschner, 1995).

Wild edible plants have been a source of food for centuries, as they are accessible and affordable but, more importantly, they are a rich source of minerals and nutrients. *Laportea alatipes* Hook. f. (forest nettle) belonging to the Urticaceae family, is found in three discrete areas in South Africa namely Limpopo, KwaZulu-Natal and Eastern Cape (JSTOR, 2015). The local people of these regions eat this plant although it has dense stinging hairs on the surface leaves and stems.

Studies on the translocation and bioaccumulation of elements of edible plant species have shown good correlation between plant and soil concentrations (Nouri et al., 2009). These studies indicate that soil enrichment influences absorption and uptake of these elements by the plant and is the route of human exposure to toxic elements. Previously, we reported on the distribution and bioaccumulation of metals in edible medicinal plant species and the following nettles (*Laportea peduncularis* subspecies *peduncularis* (river nettle) and *Urtica dioica* (stinging nettle)) (Jonnalagadda et al., 2008; Mahlangeni et al., 2016a; Mahlangeni et al., 2016b; Moodley et al., 2012). In this study, we report on the concentration of elements in the plant species *Laportea alatipes* Hook. f. (forest nettle) and corresponding growth soil to evaluate the impact of soil quality on elemental uptake by the plant and to determine its influence on bioaccumulation and translocation by the plant.

MATERIALS AND METHODS

Collection and preparation of soil and plant samples

Plant and soil (0-15 cm depth, cone and quartering method) samples were collected from the different sites in KwaZulu-Natal, South Africa (Umgababa beach, Hluhluwe, Esigedleni, Ndwedwe, Penicuik, Sokhulu, Montebello and Amagcino) (Fig. 19). The samples were from forestlands, rural, suburban and urban areas (Table 41). Plant samples were divided into roots, stems and leaves, washed with double distilled water, oven dried at 50 ºC, and crushed. Soil samples were sieved (2 mm mesh), oven dried at 50 ºC and ground with a mortar and pestle to reduce the particle size. All samples were stored in labelled polyethylene bags in a refrigerator at 4 ºC until analysed.

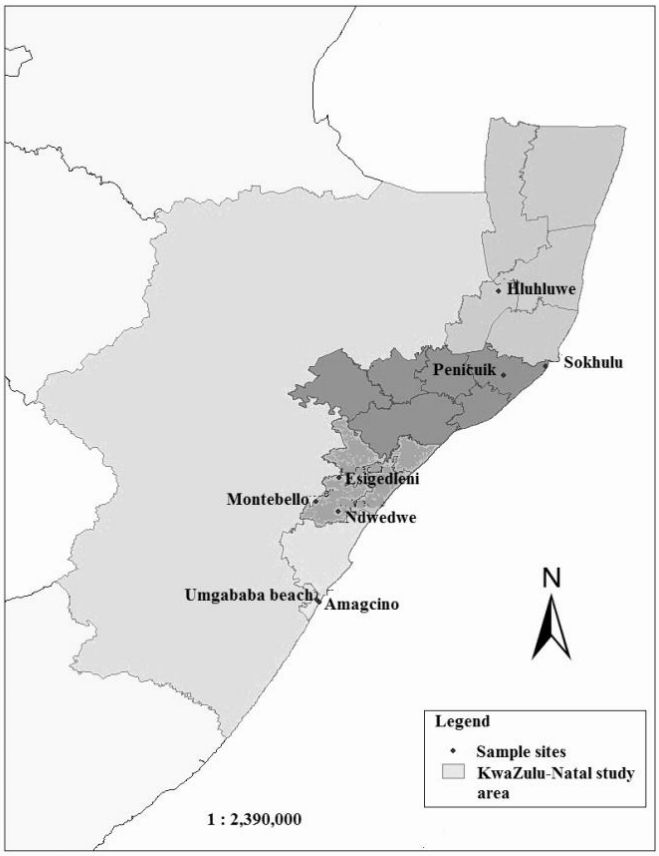


Figure 19: Map showing eight selected sampling sites in KwaZulu-Natal, South Africa.

Table 41: Sampling area description, geographical coordination, in decimal degrees, and altitude for eight different sites.

Sampling area	Sites	Site description	Latitude	Longitude	Altitude (m)
Forest land	Amagcino	-Brown loamy soil in forest next to main road (220m)	-30.129913	30.822694	9
	Hluhluwe	-Blackish loamy soil in forest next to main road (5m)	-28.002701	32.073632	468
	Montebello	- Dark brown loamy soil in deep forest	-29.448814	30.813846	868
Rural area	Esigedleni	-Brown loamy soil in a vegetable garden	-29.2832458	30.9719488	293
	Ndwedwe	-Brown loamy soil in a vegetable garden	-29.518063	30.9652310	557
	Sokhulu	-Light grey beach sand	-28.518145	32.3962810	94
Suburban area	Penicuik	-Greyish beach sand in controlled plantation next to N2 highway road (100m)	-28.578557	32.103398	115
	Umgababa beach	-Reddish beach sand, next to R102 main road (60m)	-30.141663	30.837976	14

Instrumentation

All extracted and digested samples were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Optima 5300 DV, Perkin Elmer, Shelton, Conn.)

Analytical methods

The pH of the soil was obtained using a 1:2 ratio of dry soil: 0.01 mol L^{-1} $CaCl_2$ solution (wt/v). The Walkley-Black wet extraction technique was used for the determination of soil organic matter (SOM) (Walkley & Black, 1934). The cation exchange capacity (CEC) was determined by using

ammonium acetate at pH 7 (Chapman, 1965). Three extractant solutions were used to represent the exchangeable form of elements in the soil. These were an acetic acid (0.11 mol L^{-1} CH_3COOH) extractant (1:10 ratio of soil: extractant) (Quevauriller et al., 1997), combined unbuffered salt solution (0.01 mol L^{-1} $CaCl_2$, $NH_4C_2H_3O_2$, NH_4NO_3) (1:10 ratio of soil: extractant) (Hall, 1998; Novosamsky et al., 1993), EDTA extractant (0.05 M Na_2EDTA) (1:10 ratio of soil: extractant) (Quevauriller et al., 1996).

For both plant and soil, digestions were performed using the Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, North Carolina, USA) with patented Xpress Plus technology™ (Table 42). Thereafter, digests were filtered into 25 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles for elemental analysis. The analytical technique was validated by use of a certified reference material (CRM). Samples (extracted and digested) were analysed for As, Ca, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn by ICP-OES in quadruplicate. All chemicals used were supplied by Merck (Kenilworth, USA) were of analytical-reagent grade. Working standards were prepared in double distilled water and 10 mL of 70% HNO_3 to match the sample matrix. To prevent contamination, glassware and other equipment was soaked in 20% HNO_3 and rinsed off with double distilled water, prior to use.

Table 42: Microwave digestion programs with parameters for soil and plant.

Type of sample, mass (g)	Reagent	Power, W	Temperature, °C	Ramp time, min	Hold time, min
Soil and soil CRM, 0.25	70% HNO_3	1600	200	15	15
Plant and plant CRM, 0.20	70% HNO_3	1600	180	15	15

Bioaccumulation and translocation factor

The bioaccumulation factor (BAF) is the ratio of the concentration of element accumulated inside the edible parts of the plant organism (C_{plant}) and the concentration of the element in the soil (C_{soil}). For plant analysis, BAF values <1 indicates an excluder, from 1-10 indicates an accumulator and >10 indicates a hyper-accumulator (Ma et al., 2001)

$$BAF=\frac{C_{plant}}{C_{soil}} \quad (17)$$

The translocation factor is the ratio of the concentration of metal in the leaves (C_{leaves}) or stems (Cstems) to roots (C_{roots}) (Rezvani & Zaefarian, 2011). An effective translocation between the various plant parts is indicated by a TF value >1 (Baker & Brooks, 1989).

$$TF=\frac{C_{leaves}}{C_{roots}} \text{ or } TF=\frac{C_{stems}}{C_{roots}} \quad (18)$$

Soil pollution indices

The presence, level and extent of anthropogenic based contamination in the soil may be assessed by the Enrichment factor (EF) and geo-accumulation index (I_{geo}) (Barbieri, 2016). These factors are obtained by comparing the concentration of the element in the soil to the background concentration in the earth's crust.

EF values are calculated using the following equation:

$$EF=\frac{\left[\frac{X}{RE}\right]_{soil}}{\left[\frac{X}{RE}\right]_{crust}} \quad (19)$$

Where $\left[\frac{X}{RE}\right]_{soil}$ is the mean ratio between the concentration of the target element and RE is the reference element in the soil whilst $\left[\frac{X}{RE}\right]_{crust}$ is the mean ratio between the concentration of the target element and RE is the reference element in the earth's crust. Zinc is the reference element used in South Africa since total baseline concentrations is known (Herselman et al., 2005; Mendiola et al., 2008).

The geo-accumulation index as described by Müller (1969) measures the extent to which metal contamination has occurred by comparing measured metal concentrations to that of the earth's crust and is calculated by the following equation:

$$I_{geo}=\log_2\left[\frac{C_n}{1.5\ B_n}\right] \quad (20)$$

Where C_n is the measured concentration of the element in the soil sample and B_n is the geochemical background value of the earth's crust (Herselman et al., 2005). The factor 1.5 is introduced to minimise possible differences in the background values due to lithological differences.

Data analysis

Statistically significant differences between the four means was revealed using one-way analysis of variance (ANOVA) and Tukey's post hoc test was applied to determine where these differences occurred at the 5% level. The relationship between input variables was classified using Principal component analysis (PCA) and cluster analysis (CA). PCA transforms a number of correlated variables into a smaller number of uncorrelated variables called principal components; these are rotated (using Varimax rotation) to maximize the total sum of squares of the loadings along the new axis (Brereton, 1990). In this study, component loadings greater than 0.71 was considered excellent (Nowak, 1998). Cluster analysis revealed similarities between elemental concentrations

and physicochemical characteristics of soil from different sites. Correlation analysis, using Pearson's correlation coefficients (r), was performed using elemental concentrations in plant tissue and soil (total and exchangeable) to establish significant plant-soil relationships. All statistical analyses were done using the Statistical Package for the Social Science (PASW Statistics, Version 23, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Quality Assurance

The statistical data obtained from the analysis of the CRMs (metals in soil (D081-540) and White clover (BCR 402)) is presented in Table 43. The data, which was statistically evaluated using the statistical mean and standard deviation, showed that the experimental results for the different elements are within the acceptable ranges of that stipulated for the CRMs, meaning that the method is accurate at the 95% confidence interval.

Table 43: Comparison of measured values (mg kg^{-1} dry mass, mean ± standard deviation, 95% confidence interval, *n=4)* to certified values for certified reference materials, Metals in soil, D081-540 and White clover, BCR 402.

Elements	Certified Value	Accepted value	Measured value
	White clover		
Fe	244	-	237 ± 18.0
Se	6.70 ± 0.25	-	6.81 ± 1.40
Zn	25.2	-	30.3 ± 6.37
	Soil		
Ca	26 200 ± 7.27	5 620-9 440	5 476 ± 241
Co	232 ± 4.10	148-250	170 ± 13.2
Cr	86.8 ± 6.1	60.0-104	69.0 ± 5.65
Cu	268 ± 4.72	204-332	210 ± 16.2
Fe	12 800 ± 18.0	5 380-20 100	9 217 ± 673
Mg	2 850 ± 5.51	1 860-3 840	2 221 ± 166
Ni	236 ± 4.17	175-302	189 ± 14.0
Pb	97.9 ± 11.3	69.3-126	89.7 ± 12.5
Zn	130 ± 11.5	87-173	104 ± 10.4

Soil properties pH, soil organic matter (SOM) and cation exchange capacity (CEC)

The measured soil properties (pH, SOM and CEC) for the various sites are presented in Table 44. The availability of trace elements in soil is influenced by the pH, SOM and CEC (Škrbić & Đurišić-Mladenović, 2013). Soil pH ranged from 4.13 – 4.91, soils with pH less than 5 are considered acidic and low in the nutrients Ca and Mg, as observed at the site, Sokhulu. Soil organic matter ranged from 1.83 – 13.94 %, the lowest being at Sokhulu and CEC ranged from 0.41 – 11.47 meq

$100g^{-1}$. In soil, low pH, low organic matter and low CEC decreases fixation of metals to soil particles and results in leaching of metals.

Table 44: Soil pH, soil organic matter (SOM) and cation exchange capacity (CEC) (mean ± standard deviation, *n=4*) of soil samples from eight different sites.

Sampling area	Site	pH	SOM (%)	CEC (meq $100g^{-1}$)
Forest land	Amagcino	4.9 ± 0.05 a	13.3 ± 0.61 b	4.8 ± 0.57 c
	Hluhluwe	4.9 ± 0.04 a	13.9 ± 0.66 b	6.3 ± 0.27 b
	Montebello	4.4± 0.02 b	5.2 ± 0.47 e	3.3 ± 0.17 e
Rural	Esigedleni	4.4 ± 0.05 b	12.7 ± 1.37 b	4.8 ± 079 c
	Ndwedwe	4.4 ± 0.13 b	7.9 ± 0.44 d	11.5 ± 0.17 d
	Sokhulu	4.1 ± 0.05 d	1.8 ± 0.22 a	3.3 ± 0.50 e
Suburban	Penicuik	4.6 ± 0.05 c	2.0 ± 0.44 a	0.4 ± 0.04 a
	Umgababa beach	4.8 ± 0.05 a	2.8 ± 0.19 a	0.4 ± 0.08 a

Elemental concentrations in soil and plant

Concentration of elements in the plant (leaves, stems and roots) and soil (total and exchangeable) as well as the exchangeable percentage (%Ex) which is the percentage of total element in soil that is in exchangeable form ((Soil Ex/Soil T) x 100), is presented in Table 45. The extraction of mobile or exchangeable fraction of elements from the soil was conducted by three single extraction methods. The results showed acetic acid to best extract exchangeable Cr and Mg, the combined unbuffered salt solution to best extract exchangeable Ca and the EDTA solution to best extract exchangeable Co, Cu, Fe, Mn, Ni, Pb and Zn. Concentrations of the elements Ca (772 – 5 077 µg g^{-1}), Fe (1 859 – 33 112 µg g^{-1}) and Mg (186 – 3 325 µg g^{-1}) were found to be high in the soil. These elements were higher in forestland soils than rural and suburban soils. These findings are similar to findings of previous studies done on elemental concentrations in KwaZulu-Natal soils (Mahlangeni et al., 2012; Mahlangeni et al., 2016a; Moodley et al., 2007; Moodley et al., 2012;

Reddy et al., 2014). Although total soil Fe was high, it was present in mostly insoluble form (Fe^{3+}); on average only about 2.4% was in exchangeable form. Previous studies have indicated a positive relationship between SOM and exchangeable soil Fe and Cu (Zhanbin et al., 2013). The higher the SOM, the higher the adsorption of metals onto soil particles by way of complexing with organic acids. High SOM at sites Amagcino, Hluhluwe and Esigedleni could therefore explain high soil concentrations of Fe and Cu.

Table 45: Concentration of elements in µg g^{-1} (mean ± SD, *n=4*) in leaves, stems and roots of *L. alatipes* and soil (Total (T) and Exchangeable (Ex)) samples and exchangeable percentage.

Elements	Sampling areas	Sites	Soil-T	Soil-Ex	[Soil Ex/Soil T] (%)	Leaves	Stems	Roots
As	Forest land	Amagcino	ND	ND	-	ND	4.69 ± 0.763 b*	6.23 ± 0.970 bc
		Hluhluwe	ND	ND	-	ND	4.00 ± 0.361 ab	4.74 ± 0.580 ab
		Montebello	ND	ND	-	ND	3.07 ± 0.442 a	3.76 ± 0.373 a
	Rural	Esigedleni	ND	ND	-	ND	4.22 ± 0.538 ab	6.99 ± 1.03 c
		Ndwedwe	ND	ND	-	ND	3.65 ± 0.735 ab	6.19 ± 0.739 bc
		Sokhulu	ND	ND	-	ND	4.09 ± 0.827 ab	3.32 ± 0.301 a
	Suburban	Penicuik	ND	ND	-	ND	4.26 ± 0.951 ab	3.79 ± 0.845 a
		Umgababa beach	ND	ND	-	ND	3.66 ± 0.393 ab	4.04 ± 0.829 a
Ca	Forest land	Amagcino	5 077 ± 96.2 e	324 ± 22.1 c	6.5	12 033 ± 211 d	6 731 ± 478 c	13 706 ± 1 749 a
		Hluhluwe	4 374 ± 284 b	455 ± 27.0 b	10.4	23 180 ± 2 452 b	9 621 ± 1 055 b	8 236 ± 432 b
		Montebello	1 875 ± 129 c	337 ± 12.8 ac	18.0	17 215 ± 680 a	6 871 ± 423 c	4 820 ± 522 c
	Rural	Esigedleni	1 962 ± 68.7 c	335 ± 24.7 ac	17.1	35 971 ± 1134 c	6 107 ± 930 c	9 336 ± 1 534 b
		Ndwedwe	1 864 ± 146 c	348 ± 17.7 ac	18.7	9 687 ± 463 d	9 871 ± 1 840 b	5 134 ± 220 c
		Sokhulu	772 ± 40.8 d	362 ± 23.8 ac	46.9	10 974 ± 1 058 d	12 289 ± 386 d	8 019 ± 907 b
	Suburban	Penicuik	939 ± 120 d	373 ± 15.0 a	39.7	18 168 ± 2 573 a	16 314 ± 1 038 a	15 665 ± 1 317 a
		Umgababa beach	3 280 ± 402 a	378 ± 11.1 a	11.5	18 545 ± 771 a	16 776 ± 1 025 a	16 010 ± 1 535 a
Cd	Forest land	Amagcino	ND	ND	-	1.17 ± 0.227 b	0.961 ± 0.133 b	1.91 ± 0.396 d
		Hluhluwe	ND	ND	-	ND	ND	1.14 ± 0.160 b
		Montebello	ND	ND	-	ND	ND	0.486 ± 0.0973 a
	Rural	Esigedleni	ND	ND	-	1.86 ± 0.206 a	ND	2.70 ± 0.233 c
		Ndwedwe	ND	ND	-	ND	ND	1.82 ± 0.204 d
		Sokhulu	ND	ND	-	ND	ND	ND
	Suburban	Penicuik	ND	ND	-	1.86 ± 0.293 a	0.512 ± 0.0726 a	1.09 ± 0.0999 b

		Umgababa beach	ND	ND	-	ND	ND	0.449 ± 0.0303 a
Co	Forest land	Amagcino	11.3 ± 0.221 b	1.06 ± 0.0500 b	9.4	2.45 ± 0.558 c	1.61 ± 0.218 d	2.56 ± 0.498 bc
		Hluhluwe	5.41 ± 1.31 a	1.01 ± 0.0556 b	18.7	0.880 ± 0.149 a	0.683 ± 0.0331 a	2.33 ± 0.270 bc
		Montebello	4.30 ± 1.62 a	0.883 ± 0.0357 c	20.5	1.28 ± 0.0645 a	1.20 ± 0.091 c	2.65 ± 0.453 bc
	Rural	Esigedleni	ND	ND	-	1.42 ± 0.140 a	0.348 ± 0.0069 b	1.65 ± 0.0588 ad
		Ndwedwe	ND	ND	-	0.805 ± 0.162 a	0.553 ± 0.0378 ab	2.87 ± 0.297 b
		Sokhulu	ND	ND	-	0.975 ± 0.138 a	0.806 ± 0.064 a	2.14 ± 0.0893 cd
	Suburban	Penicuik	ND	ND	-	3.65 ± 0.533 b	1.20 ± 0.186 c	0.517 ± 0.0247 a
		Umgababa beach	3.85 ± 0.884 a	0.740 ± 0.0330 a	19.2	1.11 ± 0.050 a	0.751 ± 0.0272 a	1.11 ± 0.117 a
Cr	Forest land	Amagcino	84.6 ± 3.28 e	0.150 ± 0.0042 b	0.2	20.8 ± 1.31 d	0.0098 ± 0.0012 c	0.0231 ± 0.0036 d
		Hluhluwe	12.7 ± 1.27 b	0.156 ± 0.0045 bc	1.2	5.46 ± 1.00 a	0.0012 ± 0.0003 ab	0.0060 ± 0.0010 a
		Montebello	25.6 ± 6.08 bc	0.179 ± 0.0056 ac	0.7	5.43 ± 0.750 a	0.0015 ± 0.0003 ab	0.0039 ± 0.0009 a
	Rural	Esigedleni	29.9 ± 2.35 c	0.166 ± 0.0070 ac	0.6	12.1 ± 1.32 a	0.0006 ± 0.0001 b	0.0127 ± 0.0009 c
		Ndwedwe	18.1 ± 4.34 bc	0.149 ± 0.0044 b	0.8	5.25 ± 0.516 a	0.0009 ± 0 ab	0.0215 ± 0.0047 d
		Sokhulu	14.3 ± 3.74 bc	0.148 ± 0.0096 b	1.0	12.3 ± 5.23 a	0.0067 ± 0.0007 d	0.0013 ± 0.0002 a
	Suburban	Penicuik	47.0 ± 9.87 d	0.146 ± 0.0066 b	0.3	41.8 ± 6.58 c	0.0084 ± 0.0013 c	0.0195 ± 0.0018 d
		Umgababa beach	124 ± 14.6 a	0.175 ± 0.0099 a	0.1	9.48 ± 0.866 a	0.0025 ± 0.0004 a	0.0066 ± 0.0008 a
Cu	Forest land	Amagcino	61.6 ± 10.6 c	1.81 ± 0.0649 f	2.9	14.8 ± 0.619 ace	15.5 ± 1.49 ac	17.4 ± 1.47 bc
		Hluhluwe	39.2 ± 1.48 b	1.38 ± 0.0471 b	3.5	16.3 ± 0.752 ab	19.5 ± 2.29 a	23.9 ± 4.83 b
		Montebello	14.1 ± 2.89 a	0.415 ± 0.0306 d	2.9	10.6 ± 1.78 c	9.84 ± 1.71 b	8.32 ± 0.644 ad
	Rural	Esigedleni	14.8 ± 1.83 a	0.753 ± 0.0324 c	5.1	19.1 ± 1.05 b	7.99 ± 0.782 b	13.8 ± 2.14 ac
		Ndwedwe	12.7 ± 2.57 a	0.565 ± 0.0650 a	4.5	12.0 ± 1.19 ce	8.18 ± 1.45 b	19.5 ± 3.64 bc
		Sokhulu	5.53 ± 0.274 a	0.306 ± 0.0109 e	5.5	15.5 ± 1.08 abe	31.2 ± 4.16 d	5.16 ± 0.921 d
	Suburban	Penicuik	6.83 ± 0.781 a	0.351 ± 0.0219 de	5.1	26.2 ± 3.18 d	10.9 ± 2.55 bc	14.7 ± 3.45 ac
		Umgababa beach	11.8 ± 3.27 a	0.617 ± 0.0415 a	5.2	17.5 ± 2.99 ab	18.4 ± 2.56 a	14.4 ± 2.21 ac

Fe	Forest land	Amagcino	26 256 ± 373 f	247 ± 17.3 d	0.9	3 803 ± 384 c	2 926 ± 424 d	5 935 ± 734 cd
		Hluhluwe	13 387 ± 699 ab	430 ± 28.6 b	3.2	623 ± 133 a	220 ± 46.1 ab	2 949 ± 351 b
		Montebello	12 850 ± 1 894 b	434 ± 24.6 b	3.4	740 ± 81.4 a	183 ± 16.9 b	774 ± 131 a
	Rural	Esigedleni	33 112 ± 824 c	758 ± 15.6 c	2.3	6 114 ± 687 b	180 ± 33.1 b	7 278 ± 1206 c
		Ndwedwe	8 464 ± 1 122 d	262 ± 12.3 d	3.1	725 ± 114 a	115 ± 9.98 b	5 375 ± 805 d
		Sokhulu	1 859 ± 246 e	64.3 ± 3.68 e	3.5	596 ± 99.3 a	556 ± 103 ab	65.0 ± 9.80 a
	Suburban	Penicuik	3 554 ± 713 e	86.4 ± 5.56 ae	2.4	6 034 ± 704 b	1 709 ± 123 c	2 649 ± 545 b
		Umgababa beach	15 698 ± 1 941 a	119 ± 8.31 a	0.8	1 111 ± 906 a	584 ± 124 a	982 ± 146 a
Mg	Forest land	Amagcino	3 305 ± 49.7 e	97.2 ± 6.63 d	2.9	9 492 ± 290 ae	4 717 ± 277 cd	5 130 ± 370 a
		Hluhluwe	990 ± 25.8 b	59.8 ± 4.54 b	6.0	4 959 ± 441 bd	2 582 ± 240 b	1 338 ± 151 b
		Montebello	1 137 ± 128 ab	33.5 ± 1.55 a	3.0	4 529 ± 219 bf	2 228 ± 132 b	1 900 ± 80.5 bd
	Rural	Esigedleni	1 107 ± 36.6 ab	57.4 ± 2.48 b	5.2	12 407 ± 432 c	3 995 ± 380 c	3 784 ± 499 c
		Ndwedwe	908 ± 74.1 b	39.0 ± 3.18 a	4.3	5 894 ± 467 d	2 315 ± 221 b	2 307 ± 70.4 d
		Sokhulu	186 ± 22.6 d	7.33 ± 1.42 c	3.9	3 661 ± 335 f	4 644 ± 184 cd	2 419 ± 148 d
	Suburban	Penicuik	376 ± 36.2 c	11.8 ± 0.263 c	3.1	8 573 ± 1 055 e	5 378 ± 639 d	4 611 ± 418 a
		Umgababa beach	1 229 ± 105 a	34.1 ± 3.31 a	2.8	9 878 ± 468 a	8 111 ± 591 a	5 253 ± 493 a
Mn	Forest land	Amagcino	598 ± 15.8 bc	40.0 ± 1.84 f	6.7	73.3 ± 10.1 a	71.1 ± 6.53 a	147 ± 15.5 ab
		Hluhluwe	576 ± 23.3 b	64.3 ± 3.90 b	11.2	79.4 ± 10.0 a	110 ± 22.8 ab	185 ± 25.5 ab
		Montebello	516 ± 72.8 b	36.9 ± 1.71 f	7.2	211 ± 24.1 c	201 ± 15.2 b	191 ± 65.0 ab
	Rural	Esigedleni	606 ± 9.83 bc	44.0 ± 1.25 cd	7.3	260 ± 18.4 b	71.0 ± 13.1 a	244 ± 31.7 b
		Ndwedwe	693 ± 86.6 c	47.2 ± 1.64 d	6.8	102 ± 7.51 a	58.2 ± 8.51 a	437 ± 100 c
		Sokhulu	53.9 ± 5.32 e	2.25 ± 0.259 e	4.2	88.8 ± 4.26 a	342 ± 10.2 d	208 ± 30.9 b
	Suburban	Penicuik	262 ± 48.4 a	23.6 ± 1.26 a	9.0	283 ± 47.6 b	540 ± 119 c	442 ± 69.6 c
		Umgababa beach	369 ± 43.1 a	26.5 ± 1.68 a	7.2	55.8 ± 2.23 a	50.1 ± 7.27 a	84.9 ± 5.58 a
Ni	Forest land	Amagcino	20.1 ± 1.27 c	0.487 ± 0.0274 c	2.4	8.94 ± 2.07 bd	14.3 ± 1.38 d	9.57 ± 2.05 d
		Hluhluwe	3.78 ± 1.10 b	0.425 ± 0.0231 a	11.2	2.27 ± 0.216 a	1.81 ± 0.280 ab	3.41 ± 0.327 ab
		Montebello	ND	ND	-	2.19 ± 0.136 a	1.96 ± 0.290 ac	2.36 ± 0.285 ac
	Rural	Esigedleni	3.00 ± 0.77 b	0.167 ± 0.0036 b	5.6	6.36 ± 1.39 bc	1.28 ± 0.0827 b	3.97 ± 0.196 bc

		Ndwedwe	ND	ND	-	4.06 ± 0.296 ac	1.27 ± 0.137 b	4.64 ± 0.486 b
		Sokhulu	ND	ND	-	4.09 ± 0.257 ac	3.73 ± 0.638 c	1.71 ± 0.168 a
	Suburban	Penicuik	ND	ND	-	10.0 ± 1.82 d	2.67 ± 0.207 ac	4.87 ± 0.437 b
		Umgababa beach	16.0 ± 1.93 a	0.443 ± 0.0125 a	2.8	2.26 ± 0.0698 a	1.60 ± 0.109 ab	2.45 ± 0.278 ac
Pb	Forest land	Amagcino	13.2 ± 0.920 d	1.12 ± 0.0702 a	8.5	3.23 ± 0.172 e	1.61 ± 0.285 c	3.13 ± 0.502 c
		Hluhluwe	7.20 ± 0.876 b	0.457 ± 0.0149 b	6.4	0.663 ± 0.0378 a	0.705 ± 0.885 ab	1.58 ± 0.298 b
		Montebello	3.88 ± 0.967 c	0.503 ± 0.0452 b	13.0	0.813 ± 0.0768 ac	0.488 ± 0.0986 b	0.798 ± 0.161 a
	Rural	Esigedleni	9.80 ± 0.735 a	0.730 ± 0.0343 c	7.5	2.87 ± 0.250 b	0.473 ± 0.0651 b	3.35 ± 0.247 c
		Ndwedwe	3.70 ± 0.716 c	0.514 ± 0.0293 b	13.9	1.04 ± 0.132 c	0.472 ± 0.0737 b	6.57 ± 0.558 d
		Sokhulu	ND	ND	-	0.918 ± 0.0574 ac	1.00 ± 0.309 a	ND
	Suburban	Penicuik	ND	ND	-	2.39 ± 0.168 d	0.861 ± 0.0955 ab	1.14 ± 0.161 ab
		Umgababa beach	9.88 ± 2.01 a	1.06 ± 0.101 a	10.7	0.620 ± 0.0548 a	1.10 ± 0.280 a	0.738 ± 0.108 a
Se	Forest land	Amagcino	ND	ND	-	ND	ND	ND
		Hluhluwe	ND	ND	-	0.793 ± 0.0873 b	ND	ND
		Montebello	ND	ND	-	1.93 ± 0.356 c	ND	ND
	Rural	Esigedleni	ND	ND	-	ND	ND	ND
		Ndwedwe	ND	ND	-	0.838 ± 0.109 b	ND	ND
		Sokhulu	ND	ND	-	1.98 ± 0.142 c	ND	ND
	Suburban	Penicuik	ND	ND	-	ND	ND	ND
		Umgababa beach	ND	ND	-	0.123 ± 0.022 a	ND	ND

Zn	Forest land	Amagcino	106 ± 12.4 b	1.91 ± 0101 e	1.8	36.2 ± 6.24 c	43.7 ± 8.77 ab	40.8 ± 4.20 a
		Hluhluwe	164 ± 2.72 a	0.971 ± 0.0413 b	0.6	56.2 ± 8.50 ab	55.6 ± 6.86 a	44.1 ± 5.23 a
		Montebello	60.1 ± 7.36 c	0.348 ± 0.0269 c	0.6	33.9 ± 4.93 c	39.9 ± 8.42 abc	23.6 ± 5.70 c
	Rural	Esigedleni	96.3 ± 11.4 b	0.914 ± 0.0499 b	1.0	60.7 ± 4.51 ab	33.4 ± 5.44 bc	44.2 ± 3.58 a
		Ndwedwe	55.7 ± 7.05 c	0.683 ± 0.0356 a	1.2	55.2 ± 5.85 ab	24.8 ± 3.51 c	65.3 ± 8.10 b
		Sokhulu	55.2 ± 8.29 c	1.63 ± 0.183 d	3.0	45.2 ± 5.12 ac	74.8 ± 5.77 d	12.7 ± 2.30 c
	Suburban	Penicuik	34.3 ± 5.99 c	0.333 ± 0.0236 c	1.0	65.6 ± 10.1 b	52.6 ± 8.12 a	51.5 ± 9.21 a
		Umgababa beach	154 ± 32.6 a	0.683 ± 0.0442 a	0.4	54.1 ± 10.1 ab	49.9 ± 5.17 a	40.8 ± 5.50 a

ND – Not determinable.

*Different letters within columns indicate mean separation by Tukey's Post-hoc test at the 5% level.

Studies have shown suburban soils to have higher concentrations of elements relative to rural or forestland soils (Sieghardt et al., 2005). In this study, forestland soils had higher concentrations of minor elements (Cr, Cu, Ni, and Zn) compared to rural and suburban soils. The concentration of the microelements in the soil were compared to the South African maximum permissible levels (MPLs) in order to assess for potential of elements to cause health hazards. The location of the forestland sites (Amagcino and Hluhluwe) next to main roads may cause higher concentrations of elements in the soil due to particulate matter emissions from automobile tyres and exhaust systems (Akbar et al., 2006; Naser et al., 2012; Pagotto et al., 2001; Sieghardt et al., 2005). However, concentrations of minor elements were still below South African MPLs in soil for Cr (350 μg g^{-1}), Cu (120 μg g^{-1}), Ni (150 μg g^{-1}), and Zn (200 μg g^{-1}) (Herselman et al., 2005). Exchangeable Cu was slightly higher in rural (5.0%) and suburban (5.2%) soils compared to forest soil (3.1%) and exchangeable Cr (0.8%) and Zn (1.7%) was higher in rural soils.

For the toxic elements (As, Cd and Pb) concentrations of As and Cd were below the instrument detection limits. Total soil Pb was below the instrument detection limits at sites Sokhulu and Penicuik and highest at Amagcino (13.2 μg g^{-1}) however this did not exceed the MPL for Pb in soil (100 μg g^{-1}). In this study, total soil concentrations of elements were found to be in the decreasing order of Fe > Ca > Mg > Mn > Zn > Cu > Cr > Pb > Ni > Co > As~Cd~Se with concentrations being lowest in rural soil and highest in forestland soil.

In the plant, Ca aids in the stabilization of the cell wall, stimulates root and shoot development and is required in large quantities for nodulation and nitrogen fixation (Camberato & Pan, 2012; Price, 2006). Magnesium is the central atom in the chlorophyll molecule, it also aids in activation of numerous enzyme systems (Price, 2006). Iron is needed in small amounts in the plant however; it plays a role in energy production and redox reactions (Miller et al., 1995). Since humans consume

the leaves of the plant, the concentrations of the minor elements were compared to the South African MPLs for micro and trace nutrients in vegetables (Department of Health, South Africa, 2014). Copper concentration in leaves ranged from 10.6 – 26.2 μg g^{-1}, below South African set maximum permissible limit of 30 μg g^{-1}. For Zinc (33.9-65.6 μg g^{-1}), only two sites were within permissible limits of 40 μg g^{-1} whilst Ni (2.0 μg g^{-1}) and Mn (10 μg g^{-1}) concentrations were above permissible limits for vegetables for all sites.

For the toxic elements (As, Cd and Pb), if present, As in the leaves was below detection limits of the instrument. Cadmium present in leaves of sites Amagcino (1.17 μg g^{-1}), Esigedleni (1.86 μg g^{-1}) and Penicuik (1.86 μg g^{-1}) were above permissible limits for vegetable (0.2 μg g^{-1}). Lead concentrations in the leaves of sites, Amagcino, Esigedleni and Penicuik were above maximum permissible limits for vegetables (1.0 μg g^{-1}).

In this study the elements in the edible leaves were in the decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Cr > Ni > Co > Pb > Se > Cd > As.

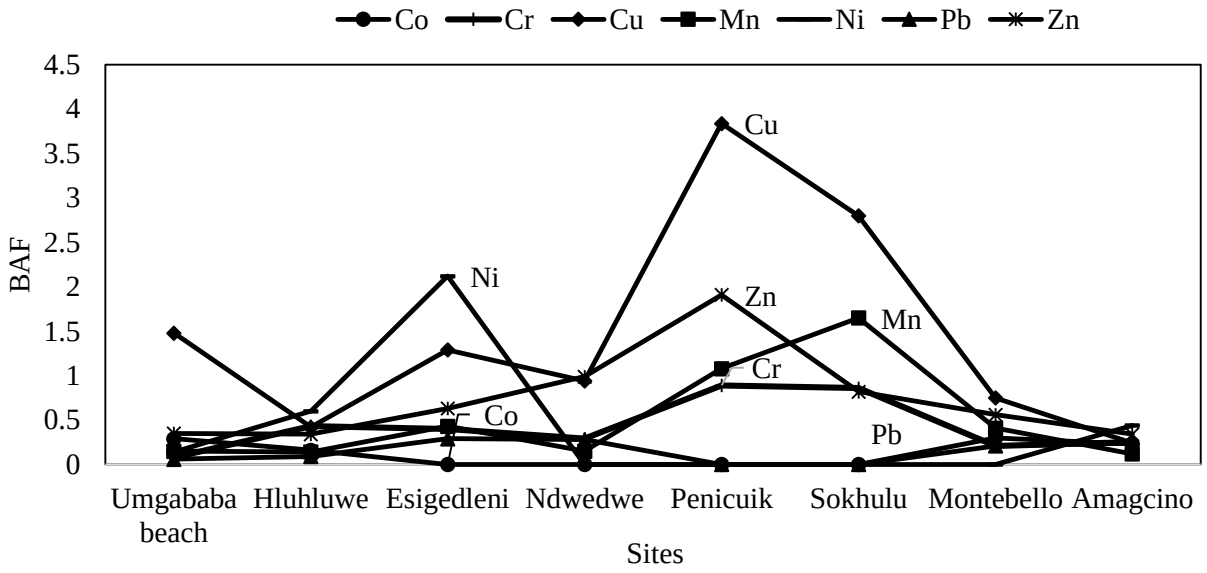
Bioaccumulation and translocation of elements in plant

The bioaccumulation and translocation factors of some of the minor and toxic elements in the plant in different sites are represented in Figure 20. The BAFs for As and Cd could not be determined due to soil concentrations being below detection limits, however Pb was present in soil and leaves. The trace element, Se was observed to be present in the plant organs but not detected in the soil across all sites. Therefore, As, Cd and Se were not included in Figure 20A. Changes in environmental conditions such as climate and soil pH, time together with plant roots and microbial activities may cause metal ions in the soil phase to become available for plant uptake. These elements move along the same pathway as in the plant. Cadmium was present in leaves at

Esigedleni, Penicuik and Amagcino whilst Se was present in leaves at Umgababa beach, Hluhluwe, Ndwedwe, Sokhulu and Montebello. Studies conducted by Cary (1980) on the effect of Se and Cd addition to soil on uptake by lettuce and wheat indicated that increased soil Cd decreased Se uptake by the first crop of lettuce and wheat. According to a study conducted by Feng et al. (2013), Se in soil inhibited uptake of Cd by paddy rice suggesting an antagonistic relationship between Cd and Se. There was no translocation of As and Cd from the roots to the leaves therefore these elements were not included in Figure 20B. Previous studies on the accumulation and translocation of As in mangrove (*Aegiceras corniculatum* L.) grown in As contaminated soil revealed higher accumulation of As in the roots compared to stems and leaves (Wu et al., 2015). Chromium is an essential trace element for humans and animals (Felcman & Bragança, 1988). No Cr accumulation in the leaves was observed across all sites (BAF<1) yet effective translocation of Cr from the root to the leaves ($TF_{L/R}$>100) was observed.

Translocation of Cr from the roots to the stems was below 1, except at site Sokhulu. Dube et al. (2003) observed that the exposure of *Citrullus* to different Cr concentrations (0.05 – 0.4 mM) resulted in low Cr concentration in the roots and high Cr concentration in the leaves. The lowest Cr concentrations were in the stems which was deemed to be due to uptake and translocation. There was an even distribution of Cu, Mn and Zn in the leaves, stems and roots of the plant. There was effective translocation of Co from the root to the leaves at the sites, Penicuik and Umgababa beach ($TF_{L/R} \geq 1$), while no accumulation was observed (BAF<1) (Fig. 20B). There was effective translocation of Ni from roots to leaves (1.60).

A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Cr
Cu
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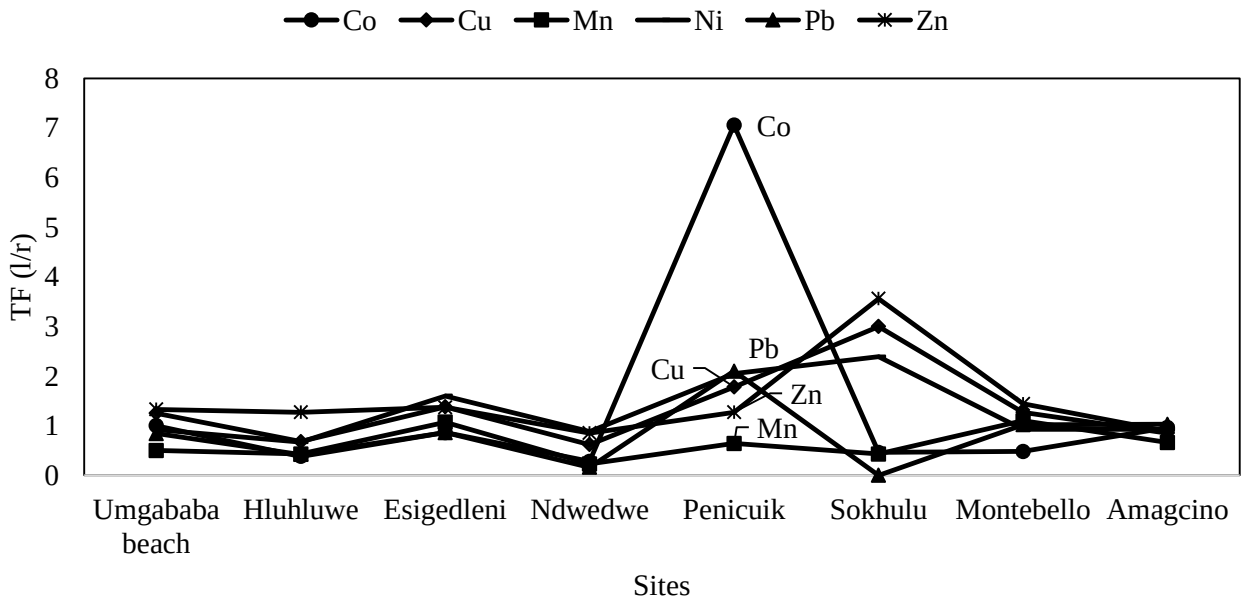
Cu
Ni
Zn
Mn
Cr
Co
Pb
Umgababa beach
Hluhluwe
Esigedleni
Ndwedwe
Penicuik
Sokhulu
Montebello
Amagcino
Sites

B


Co
Cu
Mn
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TF (l/r)
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7
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Co
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Cu
Zn
Mn
Umgababa beach
Hluhluwe
Esigedleni
Ndwedwe
Penicuik
Sokhulu
Montebello
Amagcino
Sites

C

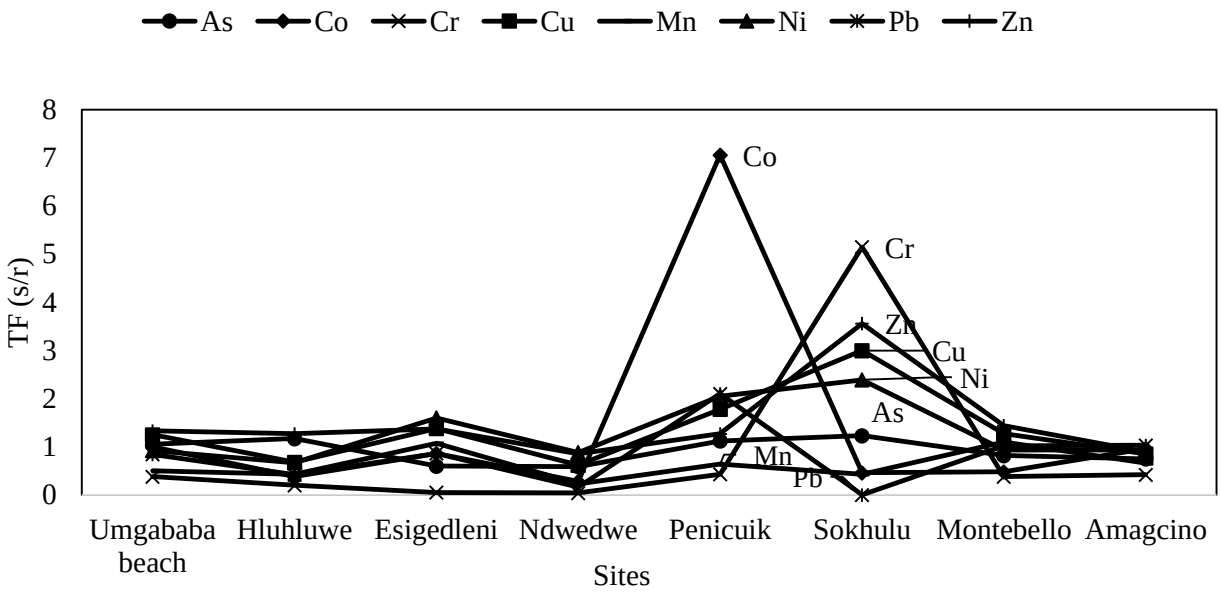

Figure 20: Bioaccumulation factors (BAF) of elements in the edible leaves (A), translocation of elements from the roots to the leaves ($TF_{l/r}$) (B) and from the roots to stem ($TF_{s/r}$) (C) of the eight sites.

BAF plots

The bioaccumulation plots (BAF vs total soil concentrations) for *L. alatipes* for micronutrient Cu and toxic element Pb, are show in figure 21. The BAF of each replicate was show in the graph. In order to determine the essentiality or non-essentiality of an element, relative accumulation plots are obtained, and the resulting curve examined. BAF graphs giving hyperbolic shaped graphs (Cu) indicated essentiality of these nutrients (Timperley et al., 1970). When the soil concentration where below plant requirements, accumulation occurred until the required physiological level was reached. An element is essential when without it the plant cannot complete its life cycle and it is part of an essential plant constituent or metabolite (Mehra & Farago, 1994). A linear graph as in

the case of Pb indicates non essentiality of the element. Lead is considered toxic to plants as it inhibits activities of various enzymes and affects the mineral nutrition (Sharma & Dubey, 2005). High levels of Cu pose no toxic risk to humans but phytotoxic to the plant.

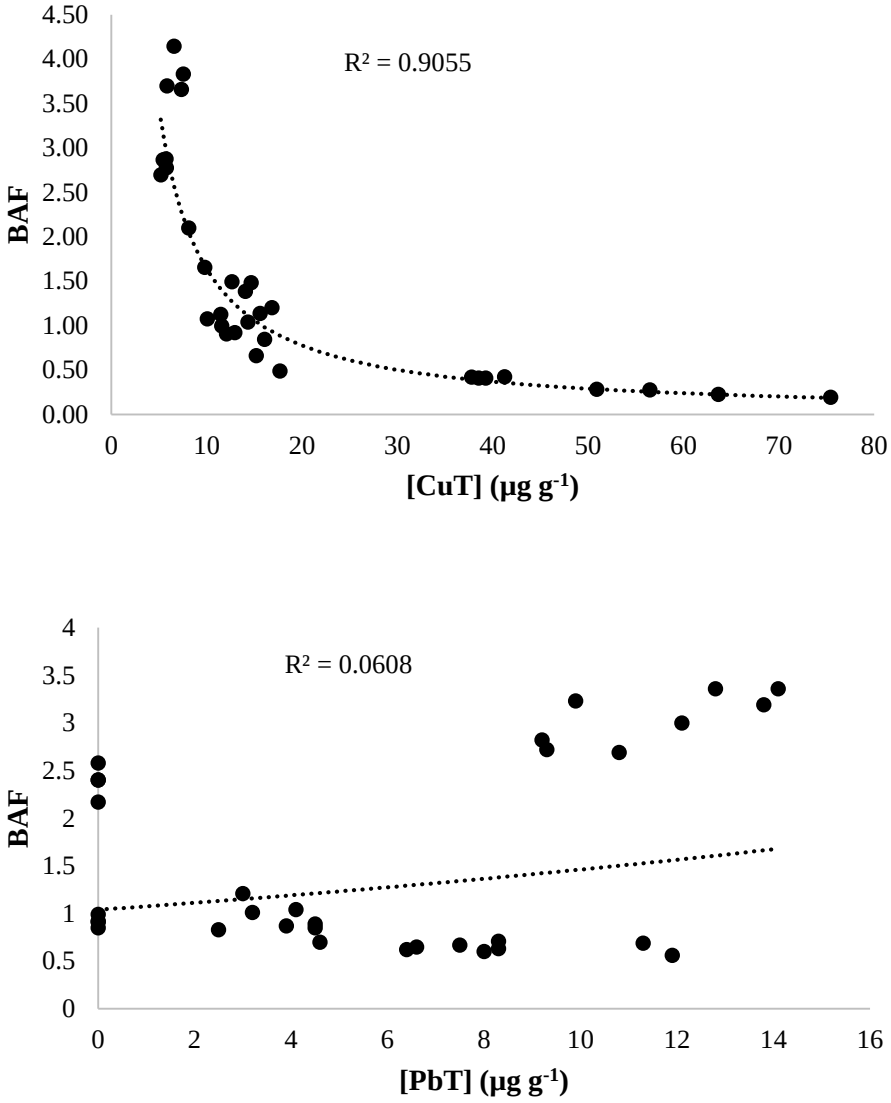

Figure 21: Bioaccumulation factor (BAF) vs total soil concentration in µg g^{-1} for the essential minor element; Cu, and trace and toxic element, Pb.

Enrichment factors and geo-accumulation index

Enrichment factor is used to assess the level of metal contamination and possible anthropogenic impact in the soil (Ghrefat et al., 2011). The background baseline metal concentrations in South African soils are 2.7 µg $g^{-1}$for Co, 353 µg $g^{-1}$for Cr, 117 µg $g^{-1}$for Cu, 159 µg $g^{-1}$for Ni, 65.8 µg g^{-1} for Pb, and 115 µg $g^{-1}$for Zn (Herselman et al., 2005). The EFs were interpreted as suggested by Sutherland (2000) where, EF<2 indicates deficiency to minimal enrichment, 2<EF<5 moderate enrichment, 5<EF<20 significant enrichment, 20<EF<40 very high enrichment, and EF>40 extremely high enrichment. EF values between 0.5 and 1.5 are deemed to be from natural processes whilst above 1.5 indicate anthropogenic sources. The EF and I_{geo} of heavy metals in soil for the various sites are presented in Table 46. The resulting EF values indicated minimal enrichment (EF<1) for all the metals (Co, Cr, Cu, Ni, and Pb) in the soil in the different sites.

Table 46: Enrichment factor and geo-accumulation indices of metals in soil from eight different sites.

Sampling area	Sites	Co		Cr		Cu		Ni		Pb		Zn	
		EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}	EF	I_{geo}
Forest land	Amagcino	0.2	-3.2	0.3	-2.6	0.6	-1.5	0.1	-3.6	0.2	-2.9	1	-0.7
	Hluhluwe	0.1	-4.3	ND	-5.4	0.2	-2.2	ND	-6.0	0.1	-3.8	1	-0.2
	Montebello	ND	-4.6	0.1	-4.4	0.2	-3.6	ND	ND	0.1	-4.7	1	-1.5
Rural	Esigedleni	ND	ND	0.1	-4.1	0.2	-3.6	ND	-6.3	0.2	-3.3	1	-0.8
	Ndwedwe	ND	ND	0.1	-4.9	0.2	-3.8	ND	ND	0.1	-4.7	1	-1.6
	Sokhulu	ND	ND	0.1	-5.2	0.1	-5.0	ND	ND	ND	ND	1	-1.6
Suburban	Penicuik	ND	ND	0.4	-3.5	0.2	-4.7	ND	ND	ND	ND	1	-2.3
	Umgababa Beach	ND	-4.7	0.3	-2.1	0.1	-3.9	0.1	-3.9	0.1	-3.3	1	-0.2

Geo-accumulation index evaluates the possible enrichment of the metal in the soil (Ghrefat et al., 2011). The geo-accumulation index (I_{geo}) classification and degree of metal contamination where described by Müller (1969) as, $I_{geo} \leq 0$ indicates uncontaminated, $0 < I_{geo} < 1$ uncontaminated to moderately contaminated, $1 < I_{geo} < 2$ moderately contaminated, $2 < I_{geo} < 3$ moderately to heavily contaminated, $3 < I_{geo} < 4$ heavily contaminated, $4 < I_{geo} < 5$ heavily to extremely contaminated, and $I_{geo} \geq 5$ extremely contaminated soil. The negative I_{geo} values indicated no contamination of all the metals (Co, Cr, Cu, Ni, Pb and Zn) in the soil in the different sites.

Statistical analysis

To determine if heavy metals in the soil were from a common source, multivariate PCA analysis was performed. The rotated component matrix of the metals in the soil is given in Table 47 and the corresponding loading scatter plots are presented in Figure 22. Two PCs were extracted with eigenvalues >1 explaining 77% of the total variance. The first PC (48.3% of the variance) indicated high loadings of Co, Cr, Cu, Fe, Ni, Pb, pH and Zn. These metals may be from vehicular emission, wearing of brake lining and tyres of vehicles, for sites are situated on local roads (Carrero et al., 2010). The second PC (28.8% of the total variance) showed high loadings of Mn, CEC and SOM. Manganese may be bound to the soil organic matter of the soil therefore from geochemical origin (Zhang & Wang, 2009).

Table 47: Component matrices of elements in soil.

	Rotated Component Matrix	
	1	2
Eigenvalues	5.313	3.173
Percentage of total variance	48.298	28.844
Percentage of cumulative variance	48.298	77.141
Co	**0.817**	0.222
Cr	**0.794**	-0.454
Cu	**0.710**	0.505
Fe	**0.584**	0.510
Mn	0.264	**0.855**
Ni	**0.949**	-0.065
Pb	**0.864**	0.398
Zn	**0.733**	0.181
pH	**0.821**	0.118
SOM	0.397	**0.877**
CEC	-0.288	**0.833**

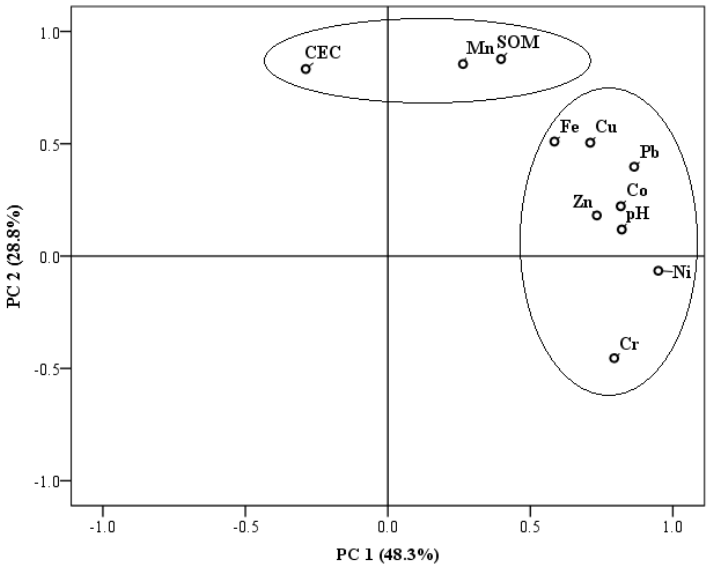

Figure 22: Principal component analysis loading plot for heavy metals, SOM, CEC and pH of the soil (constructed for eight sampling sites).

Ward's method was used to indicate the degree of association between metals in the soil, shown by the Euclidean distance (Fig 23). In this study, CA was used to further analyse the possible source of elements based on the similarities of their chemical properties. There were three main clusters, A, B, and C where A showed close associations amongst Fe-Pb-Zn, B showed close associations between Mn-SOM-CEC, C showed close association between Co-Cr-Cu-Ni which agreed with the PC analysis.

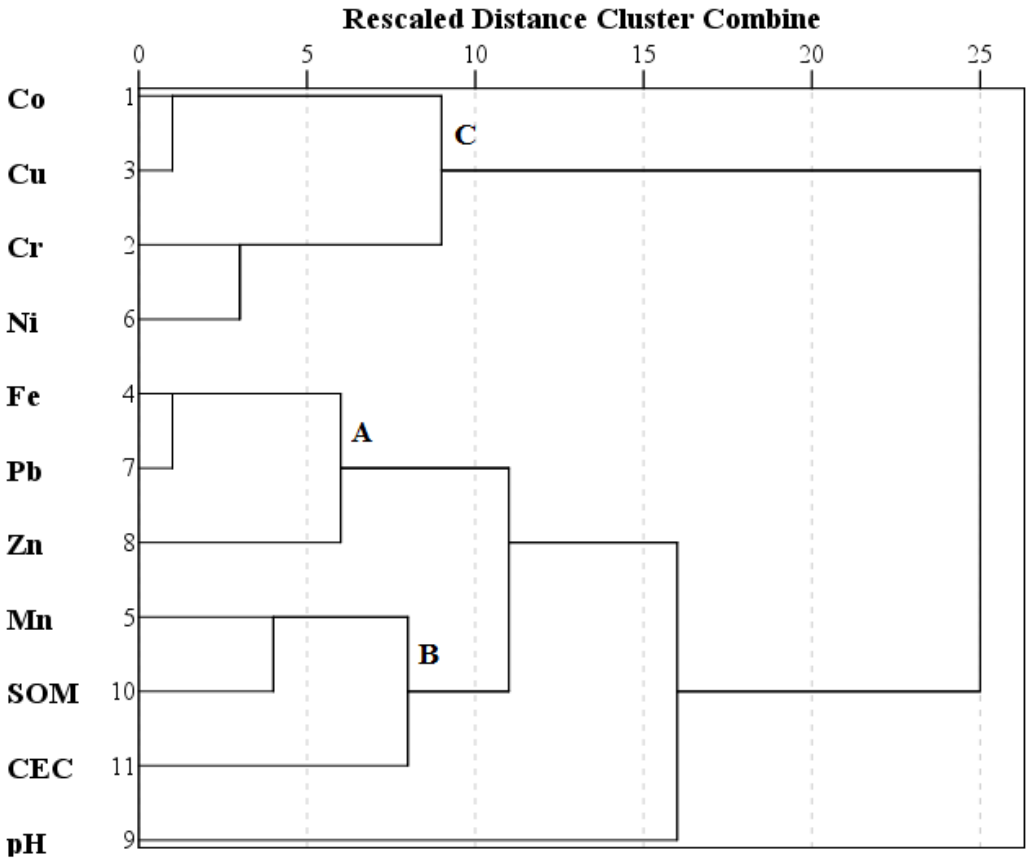

Figure 23: Cluster analysis using Ward's method of heavy metals, SOM, CEC and pH of the soil measured by Euclidean distance.

An inter-correlation matrix between the soil (total and exchangeable) and plant concentrations of elements, soil pH, SOM and CEC was done. Only the strong correlations were extracted and are presented in Table 48 and 49. Correlation coefficients (r) >0.8 indicated a strong negative linear relationship, between 0.8 and 0.7 positive, < -0.8 a strong negative linear relationship, between -0.7 and -0.8 negative and 0 indicated no relationship (Mahlangeni et al., 2016a). There was a significantly positive correlation between total soil Ni, Co, Cr and Pb. Again, a significant positive correlation between total soil Pb, Fe and Zn was observed, indicating their common origin confirmed by PCA and CA. Competition between elements for the same adsorption sites would be synergistic as an increase in total soil concentration of one element would reduce the soil retention capacity of another, increasing its exchangeability (Moodley et al., 2013). This was observed for exchangeable Ni with total soil Co and Cu as well as exchangeable Pb with total soil Fe and Ni. SOM correlated positively with total soil Mn, exchangeable Cu, Mn and Ni. Manganese is readily chelated by organic complexes and adsorbed onto the soil organic matter (Allison, 1973). There was also a positive correlation between SOM and As, Cd and Fe in the roots as well as CEC with Pb in the roots. Plant roots release low molecular mass organic ligands which displace the adsorbed As, Cd and Fe on the soil organic matter thereby allow for their uptake (Mehmood et al., 2009; Violante et al., 2010). Cations can be retained on the negatively charged particles of the soil surface thus allowing for the replacement of Pb ions by other cations on the exchange sites and for the absorption of the Pb ions in the roots (Adriano, 1986).

Table 48: Inter-correlation of elements in soil, total (T) and exchangeable (E) of *L. alatipes.*

	CoT	CrT	FeT	PbT
CoT	1	ns	ns	ns
CrT	ns	1	ns	ns
CuT	0.9**	ns	ns	ns
FeT	ns	ns	1	ns
NiT	0.8*	0.9**	ns	0.8*
PbT	ns	ns	0.9**	1
ZnT	ns	ns	ns	0.7*

	CoE	CuE	NiE	PbE
CoT	0.9**	0.9**	0.8*	ns
CuT	0.9**	1.0**	0.7*	ns
FeT	ns	ns	ns	0.8*
NiT	ns	ns	0.9**	0.8*
PbT	ns	0.8*	0.9**	0.9**

*,** - correlations significant at P≤ 0.05 and P≤ 0.01.
ns - not significant.

Table 49: Inter-correlation of elements in soil, total (T), exchangeable (E), SOM, leaves (L), stems (S), and roots (R) of *L. alatipes.*

	CuE	MnE	MnT	NiE	AsR	CdR	FeR	PbR
SOM	0.8*	0.8*	0.8*	0.9**	0.8*	0.7*	0.8*	ns
CEC	ns	ns	ns	ns	ns	ns	ns	0.8*

*,** - correlations significant at P≤ 0.05 and P≤ 0.01.

ns - not significant.

CONCLUSION

Despite high translocation of Cr from the stems to the leaves (>100), no Cr accumulation was observed. The plant roots stored trace and toxic elements, As and Cd, thereby controlling the

amounts translocated into the stems and leaves. Translocation factor values showed effective translocation of minor and trace elements from stem to the leaves. Bioaccumulation plots showed that accumulation of elements occurred when the concentration of the element in the soil was low so that the plant meets physiological needs. There was no metal enrichment or contamination in the soil on all sites. PCA and CA analysis showed that the close association between metals Co, Cr, Cu, Fe, Ni, Pb and Zn was from anthropogenic sources. Manganese in the soil was found to be chelated into the soil organic matter. Correlation analysis further validated the association of these metals in the different sites.

Acknowledgements

The authors would like to convey their gratitude to the National Research Foundation (NRF) for providing funds and the School of Chemistry and Physics at UKZN for laboratory facilities.

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CHAPTER SEVEN

Chemical composition, antioxidant activity and anti-diabetic properties of nettles (Laportea peduncularis, Laportea alatipes and Obetia tenax) found in KwaZulu-Natal, South Africa

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ABSTRACT

Nettles are highly nutritious herbs and are eaten by the local people in KwaZulu-Natal, South Africa. They are also used in traditional medicine to treat a variety of ailments such as rheumatoid arthritis, eczema, fever, diabetes and hyperglycaemia. In this study, a phytochemical analysis on nettles (*Laportea peduncularis*, *Laportea alatipes* and *Obetia tenax)* found in KwaZulu-Natal, South Africa was conducted to identify the secondary metabolites that impart medicinal properties to these herbs. The antioxidant and anti-diabetic activity of the crude extracts and phytocompounds was also investigated. Extracts from *L. peduncularis* and *O. tenax* nettles were found to be rich in β-sitosterol and β-carotene. The methanol extracts of leaves and stems of all nettles showed higher DPPH radical scavenging activity relative to the other extracts but lower activity relative to ascorbic acid and α-tocopherol. ß-carotene had higher DPPH radical scavenging activity relative to the sterol The ferric reducing power (FRAP) assay showed the methanol extract of *O. tenax* to have higher antioxidant activity (250-500 µg mL^{-1}) than α-tocopherol. The anti-diabetic assay showed extracts of nettles to have comparable activity to the known standard, acarbose. This study provides scientific validation for the ethno-medicinal use of nettles.

Keywords radical scavenger, medicinal plant, phytochemicals, sterols

INTRODUCTION

Oxidative stress results when the equilibrium between the production of free radicals and antioxidant defences in the body is disturbed which may result in premature ageing, cardiovascular and neurodegenerative diseases (Bettridge, 2000; Wu & Cederbaum, 2003). The most important class of free radicals are the reactive oxygen species that include superoxide anions (O_2^-), hydroxyl ions (OH^-), singlet oxygen ions (O^-) and hydrogen peroxide (H_2O_2). The reduction of oxygen in the system gives rise to the superoxide anion that is converted to H_2O_2 by superoxidase dismutase. Hydrogen peroxide binds with metals such as Fe^{2+} or Cu^{2+} to release OH^- ions which attack proteins, lipids and DNA thereby causing damage to the body (Noori, 2012; Bogaerts et al., 2008).

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia (a high blood glucose condition) resulting in defects in the secretion of insulin, impaired action of insulin or both (American Diabetes Association, 2004). In the absence of insulin, glucose (from broken down carbohydrates and starch) builds up in blood vessels as it cannot be absorbed into the cells of the body which results in organ and tissue failure. The International Federation of Diabetes (IDF) reported an increase in the number of diabetes cases to 382 million in 2013, with 80% of these cases being from low to middle income countries and 2.6 million being from South Africa (IDF Annual Reports, 2013; IDF Diabetes Atlas, 2013).

There are numerous therapeutic drugs available for the treatment of diabetes, the most common commercial agents being acarbose and miglitol. However, these agents are known to have side effects, which include severe constipation and bowel obstruction. The exploitation of plants for medicinal purposes is one of the oldest practices that still exist throughout the world. Nearly 80% of the population in developing countries such as Africa and Asia use traditional medicine for their

healthcare needs (Hussain et al., 2013; WHO, 2002). Plants found to have medicinal properties are used to treat a variety of conditions from the common cold to cancer. Medicinal plants have also played a key role in modern medicine in the development of breakthrough drugs. Secondary metabolites (phytochemicals) are responsible for the medicinal properties of these plants; these secondary metabolites are being investigated for leads into commercial therapeutic agents.

The growing popularity of nutraceuticals has led to a greater demand for the identification of new plants that are both nutritional and medicinal. *Laportea peduncularis* (Wedd.) Chew subspecies *peduncularis, Laportea alatipes* Hook. f. and *Obetia tenax* (N.E.Br.*)* Friis are from the Urticaceae (nettle) family and are known for their nutritional and medicinal value. These nettles are found in KwaZulu-Natal, South Africa. They are generally known as the river nettle (*L. penducularis*), forest nettle (*L. alatipes*) and mountain nettle (*O. tenax*) or Imbati in isiZulu. In traditional medicine, nettles are used to treat conditions such as rheumatoid arthritis, gout, eczema, benign prostatic hyperplasia, anaemia, influenza, asthma and diabetes (Phillips, 2014; Warren, 2006).

Laportea peduncularis is an annual herb that grows up to 1.5 m tall and is mostly found next to river banks. The leaves are triangular with margins serrated with 15-25 teeth on each side. The leaves and stem are covered with short stinging hairs (Friis, 1989). *Laportea alatipes* is a shrub that grows up to 2 m high and is found in the forest or forest edges. The leaves are broadly lanceolate to ovate, the base is cordate and the margins are coarsely serrated (Friis, 1989). The stems and leaves contain stinging hairs. *Obetia tenax* is a small tree that grows 5-7 m tall and is found mostly on rocky locations. The base of the leaves is cordate or truncate, the apex is acuminate and the margins are serrated (Brink & Achigan-Dako, 2012). The leaves and younger branches are covered with stinging hairs.

The aim of the study was to extract, isolate and identify the secondary metabolites from the leaves and stems of *L. peduncularis, L alatipes and O. tenax* nettles to provide a scientific basis for their ethno-medicinal use. The plant extracts as well as isolated compounds were further assessed for their antioxidant, α-amylase and α-glucosidase inhibitory activity.

MATERIALS AND METHODS

General experiment

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III spectrometer at 400 MHz in deuterated chloroform ($CDCl_3$) at room temperature with tetramethylsilane (TMS) as an internal standard. Infra-Red (IR) spectra were obtained using a Perkin Elmer Spectrum 100 FT-IR spectrometer with Universal ATR sampling accessory. Ultraviolet-Visible (UV-Vis) spectra were obtained on a UV-Vis-NIR Shimadzu UV-3600 spectrophotometer. For gas chromatography - mass spectrometry (GC-MS), the Agilent GCMSD apparatus equipped with a DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 μm film thickness) fused silica capillary column, operated in the EI mode (70 eV) was used. Helium (2 mL/min) was used as a carrier gas and hexane was used to dissolve the samples. The injector was kept at 250 ℃ whilst the transfer line was at 280 ℃. The column temperature was held at 50 ℃ for 2 min, and then ramped to 280 ℃ at 20 ℃/min where it was held for 15 min. Fractions were profiled using thin layer chromatography (TLC) (Merck silica gel 60, 20 x 20 cm F254 aluminium sheets) and visualized using anisaldehyde spray reagent (97: 2: 1; MeOH: conc. H_2SO_4: anisaldehyde). The absorbance was measured using a UV spectrometer (UV Spectrophotometer Biochrom Libra S11, Cambridge, England).

Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium hexaferricyanide [$K_3Fe(CN)_6$], TCA (trichloro acetic acid), porcine pancreatic α-amylase, α-glucosidase from *Saccharomyces cerevisiae*, acarbose and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma Aldrich chemicals, USA. All other chemicals were of analytical grade.

Collection of plant material

Leaves and stems of all plants (*L. peduncularis peduncularis*, *L. alatipes* and *O. tenax*) were collected from various sites in KwaZulu-Natal, South Africa and were identified by curator, Mr E. Khathi, from the School of Life Sciences, University of KwaZulu-Natal, Westville and voucher specimens (Mahlangeni NT1, Mahlangeni NT2 and Mahlangeni NT3) were deposited in the ward herbarium at the university. Leaves and stems, respectively of *L. peduncularis* (474 & 391 g), of *L. alatipes* (196 & 386 g), and *O. tenax* (292 & 474 g) were air-dried, ground and extracted with hexane, dichloromethane (DCM), and methanol (MeOH) in turn by continuous shaking on an orbital shaker for 48 h. The aqueous extract was obtained by boiling leaves and stems (5 g each) in 200 mL for 15 min as per instructions by the herbalist for preparation of the tonic. The aqueous MeOH extract was partitioned with DCM followed by ethyl acetate (EA). All mixtures were filtered thereafter the filtrate (extract) was concentrated to dryness and stored in the fridge until analysed. The extracts were spotted on TLC plates and were subjected to column chromatography (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM).

Extraction and isolation of compounds

The hexane (1.46 g) and DCM extract (7.30 g) of *L. peduncularis* leaves were combined due to similar TLC profiles. The extract was loaded onto the column and separated using a hexane: EA step gradient from 100% hexane to 100% EA. At 100% hexane, fraction 16 yielded compound **C1** (4.12 mg). Fractions 99-131 were combined and re-recrystallized with 100% MeOH affording compound **C2** as a white solid (9.0 mg). Similarly, hexane and DCM extracts (4.28 g) of stem were combined and after elution with 30% hexane: 70% EA, fraction 127-151 afforded compound **C2** (69.1 mg) as a precipitate**.**

In a similar manner, the combined DCM extracts (12.23 g) of *L. alatipes* leaves and stems were separated with 90% hexane: 10% EA solvent system affording fraction 51-72, were recrystallized with 100% MeOH affording compound **C2** (12.1 mg).

The DCM extracts (5.91 g) of *O. tenax* leaves and stems were separated using 90% hexane: 10% EA solvent system and afforded compound **C2** (12.1 mg) in fractions 4-5. The aqueous MeOH extract (150 mL) of leaves and stems (combined) were partitioned with 150 mL DCM in triplicate then subjected to column chromatography using a DCM: MeOH solvent system. Compound **C1** (3.23 mg) was eluted with 99% DCM: 1% MeOH.

Antioxidant activities

DPPH assay

The scavenging activity of the plant phytocompounds on the stable free radical, DPPH, was evaluated according to the method as described by Murthy et al. (2012) with some modifications. A volume of 150 µL of ethanolic solution of plant extracts and compounds at different concentrations

were mixed with 2850 µL of the ethanolic solution of DPPH (0.1 mM). An equal amount of ethanol and DPPH without sample served as a control. After 30 min of reaction at room temperature in the dark, the absorbance was measured at 517 nm against ethanol as a blank using a UV spectrophotometer. Ascorbic acid and α-tocopherol served as positive controls. All procedures were done in triplicate.

$$\% \text{ Scavenging [DPPH]} = \left[\frac{A_c - A_s}{A_c}\right] x100 \qquad (21)$$

where A_C is the absorbance of the control and A_S is the absorbance of the sample.

Ferric reducing antioxidant power (FRAP) assay

The total reducing power of the compounds from plant material was determined according to the FRAP method as described by Murthy et al. (2012) with some modifications. A 2.5 mL volume of different concentrations of the plant extracts or compounds were mixed with 2.5 mL phosphate buffer solution (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] in test tubes. The mixture was placed in a water bath of 50℃, for 20 min. A volume of 2.5 mL of 10% trichloro acetic acid (TCA) was added to the mixture and mixed thoroughly. A volume of 2.5 mL of this mixture was then mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ of 0.1% solution and allowed to stand for 10 min. The absorbance of the mixture was measured at 700 nm using a UV-Vis spectrophotometer; the higher the absorbance of the reaction mixture, the greater the reducing power. Ascorbic acid and α-tocopherol were used as positive controls. All procedures were performed in triplicate.

Enzyme inhibitory activities

Alpha amylase inhibition assay

The alpha amylase inhibition assay was performed as described by Saravanan and Parimelazhagan (2014), with some modifications. Porcine pancreatic α-amylase was dissolved in 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride to give a concentration of 0.5 mg mL^{-1} enzyme solution. Plant extracts or compounds (400 µL) and enzyme solution (400 µL) was incubated at 25 ℃ for 10 min. Thereafter 400 µL potato starch (0.5%; w/v) in 20 mM sodium phosphate buffer (pH 6.9 with 6.7 mM sodium chloride) were added and the reaction mixture was incubated at 25 ℃ for a further10 min. Thereafter, 1.0 mL of dinitrosalicylic acid (DNS) reagent (1 g of 3,5-dinitrosalicylic acid, 30 g sodium potassium tartrate and 20 mL of 2 M sodium hydroxide in 100 mL) was added and the reaction was stopped by incubating in boiling water for 5 min then cooled to room temperature. The reaction mixture was diluted with 10 mL distilled water, and the absorbance taken at 540 nm using a spectrophotometer. Acarbose was used a positive control. All experiments were done in triplicate. A mixture containing all of the reagents except test sample was used as a control. The percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \left[\frac{A_c - A_s}{A_c}\right] x100 \qquad (22)$$

Where A_C is the absorbance of the control and A_S is the absorbance of the sample.

Alpha glucosidase inhibition assay

The alpha glucosidase inhibition assay was performed as described by Saravanan and Parimelazhagan (2014), with some changes. Plant extracts or compounds (200 µL) and 200 µL α-glucosidase (0.5 mg mL^{-1}) with 1 mL 0.1 mM phosphate buffer (pH 6.9) solution were incubated

at 25 ℃ for 10 min. Thereafter, 200 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) solution was added. The reaction mixture was incubated at 25 ℃ for 5 min then diluted with 4 mL distilled water. The α-glucosidase activity was determined by measuring the yellow-colored paranitrophenol released from *p*NPG at 405 nm using a spectrophotometer. Acarbose was used a positive control. All experiments were done in triplicate. A mixture containing all of the reagents except test sample was used as a control. The percentage inhibition was calculated using equation 22.

Statistical analysis

All experiments were done in triplicate and expressed as mean ± standard error (SE). Separation of the means was done by Tukey's Post hoc range test and Pearson's correlation coefficients were obtained using the Statistical Package for the Social Sciences (PASW Statistics, Version 23, IBM Corporation, Cornell, New York). The differences between the means were considered significant for values of $p < 0.05$.

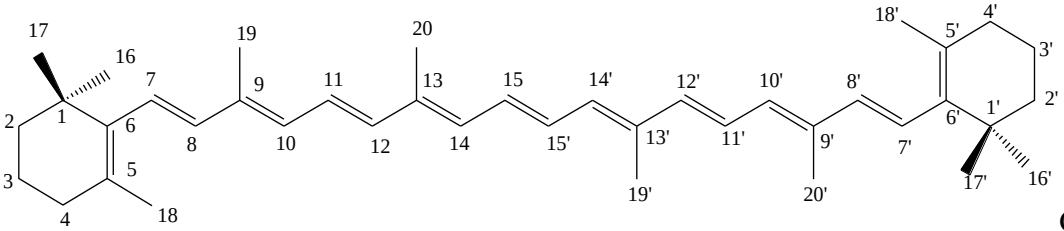
RESULTS AND DISCUSSION

Chemical composition of nettles

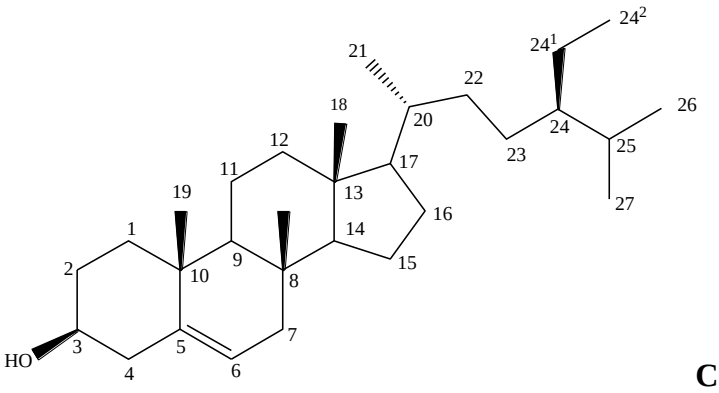
DCM extracts of the nettles yielded known compounds, **C1** (β-carotene) and **C2** (β-sitosterol). Additionally, DCM/MeOH fraction of *O. tenax* leaves/stems also yielded **C1**. β carotene and β-sitosterol were previously isolated from *Laportea* species (Njogu et al., 2011; Zhu et al., 2011) but this is the first report of it being isolated from *Obetia* species. β-sitosterol is a ubiquitous compound found in almost all plant species. The ^{1}H-NMR and ^{13}C-NMR spectral data for compounds **C1-C2** are in agreement with literature data for β-carotene and β-sitosterol, respectively (Chaturvedula & Prakash, 2012; Miglietta & Lamanna, 2006; Moss, 1976) (Fig. 24).

C1: β-carotene: EIMS: $[M]^+$ 536 $C_{40}H_{56}$; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.00 (6H, s, Me-16/17), 1.44 (2H, dd, J=3.28, 3.48 Hz, H-2), 1.61 (2H, m, H-3), 1.69 (3H, s, Me-5), 1.95 (6H, s, Me-19/20), 2.00 (2H, t, J=6.16 Hz, H-4), 6.14 (1H, d, J=5.80 Hz, H-8), 6.17 (1H, d, J=7.16 Hz, H-7), 6.24 (1H, s, H-14), 6.35 (1H, s, H-12), 6.63 (1H, d, J=11.0 Hz, H-15), 6.66 (1H, s, H-11).); ^{13}C-NMR ($CDCl_3$, 400 MHz): 12.66 (C-19), 12.72 (C-20), 19.18 (C-3), 21.66 (C-18), 29.60 (C-16/17), 33.02 (C-4), 34.18 (C-1), 39.57 (C-2), 124.94 (C-11), 126.56 (C-7), 129.28 (C-5), 129.89 (C-15), 132.32 (C-14), 135.92 (C-9), 136.37 (C-13), 137.10 (C-12), 137.67 (C-8), 137.83 (C-6).

C**2**: β-sitosterol: EIMS $[M]^+$ 414 $C_{29}H_{50}O$; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.65 (3H, s, H-18), 0.80 (3H, H-26), 0.82 (3H, H-27), 0.84 (3H, H-29), 0.90 (3H, d, J=6.56 Hz, H-21), 0.98 (3H, s, H-19), 1.06 (2H, m, H-1b), 1.07 (2H, m, H-15b), 1.09 (1H, m, H-17), 1.11 (2H, m, H-22b), 1.15 (2H, m, H-12b), 1.16 (2H, m, H-23), 1.21 (2H, m, H-16b), 1.23 (2H, m, H-28), 1.47 (2H, m, H-2b), 1.48 (2H, m, H-7), 1.49 (2H, m, H-11), 1.54 (2H, H-15a), 1.65 (1H, m, H-25), 1.83 (2H, m, H-1a), 1.84 (2H, m, H-2a), 1.85 (2H, m, H-16a), 1.97 (1H, H-8), 2.00 (2H, m, H-12a), 2.26 (2H, m, H-4), 3.50 (1H, s, H-3), 5.30 (1H, s, H-6); ^{13}C-NMR ($CDCl_3$, 400 MHz): 11.76 (C-18), 11.88 (C-29), 18.68 (C-21), 18.93 (C-27), 19.30 (C-19), 19.72 (C-26), 20.99 (C-11), 22.97 (C-28), 24.20 (C-15), 25.98 (C-23), 28.15 (C-16), 29.05 (C-25), 31.56 (C-2), 31.81 (C-7), 31.81 (C-8), 33.85 (C-22), 36.05 (C-20), 36.41 (C-10), 37.15 (C-1), 39.68 (C-12), 42.20 (C-4), 42.23 (C-13), 45.74 (C-24), 50.04 (C-9), 55.96 (C-17), 56.67 (C-14), 71.72 (C-3), 121.63 (C-6), 140.68 (C-5).



C1



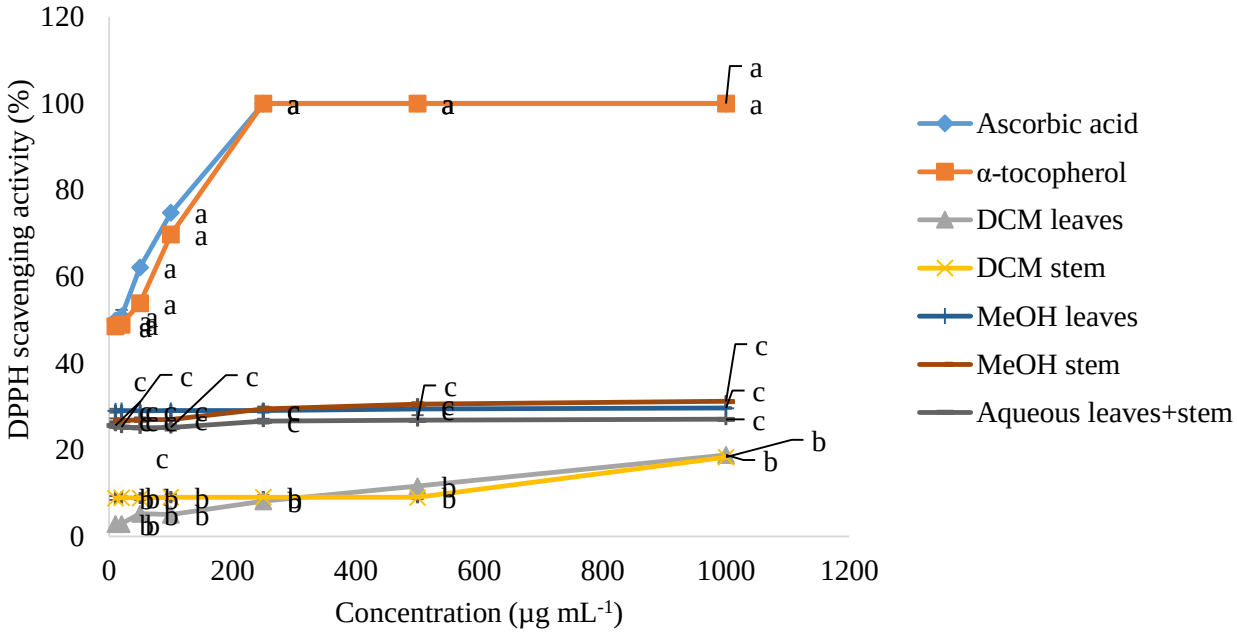
C2

Figure 24: Compounds isolated from the nettles; **C1** (β-carotene) and **C2** (β-sitosterol)

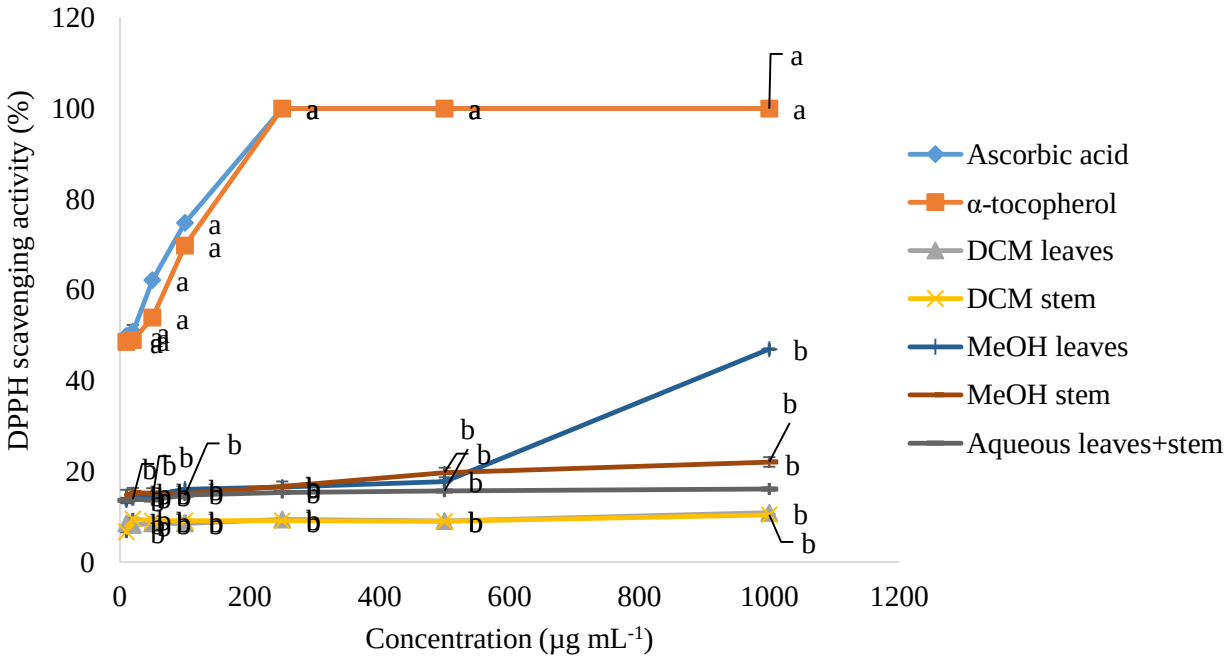
Antioxidant activity

The DPPH assay is based on the measurement of the ability of antioxidants to scavenge the DPPH free radical. When the antioxidant donates a proton to the DPPH radical in solution there is colour change of the solution from deep violet to yellow (Kedare & Singh, 2011). This assay showed that all nettles had moderate inhibition of the DPPH radical when compared to known standards, ascorbic acid and α-tocopherol, as seen in Figures 25 and 26. Previous studies by Krishna et al (2014) on extracts of *L. interrupta* showed similar results. Generally, MeOH extracts from stem and leaves had higher DPPH radical scavenging activity for all nettles, above 20% for *L. peduncularis* and *O. tenax*. These findings are similar to previous studies on plants from the Urticaceae family (*Pilea microphylla*) (Chahardehi et al., 2010) where the MeOH extracts were found to have the highest DPPH radical scavenging activity. β-carotene, known to be an efficient scavenger of radicals, was found to have the highest scavenging activity relative to the other compounds, up to 90% at a concentration of 500 μg mL^{-1}.

A


DPPH scavenging activity (%)
Concentration (µg mL-1)
Ascorbic acid
α-tocopherol
DCM leaves
DCM stem
MeOH leaves
MeOH stem
Aqueous leaves+stem

B


DPPH scavenging activity (%)
Concentration (µg mL-1)
Ascorbic acid
α-tocopherol
DCM leaves
DCM stem
MeOH leaves
MeOH stem
Aqueous leaves+stem

C

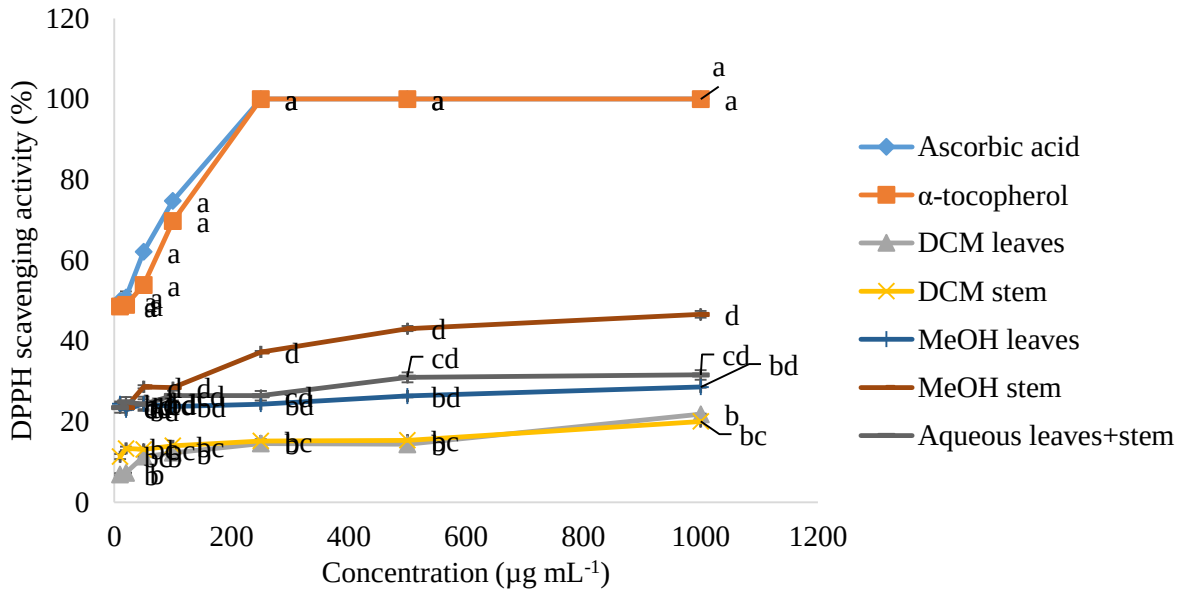


Figure 25: DPPH radical scavenging activity of extracts from *L. peduncularis* (A), *L. alatipes* (B) and *O. tenax* (C) leaves and stem. Different letters indicate mean separation by Tukey's post-hoc test at the 5% level.

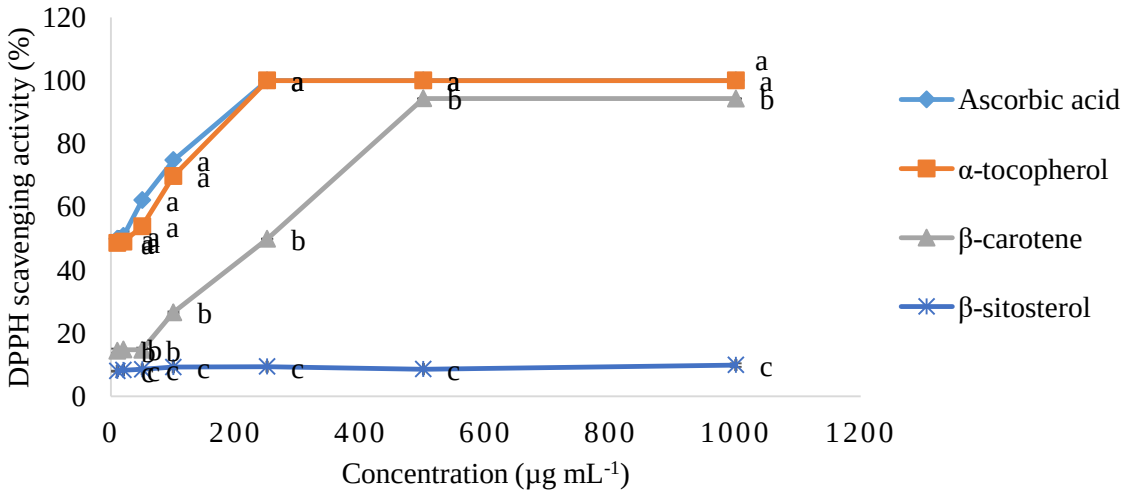
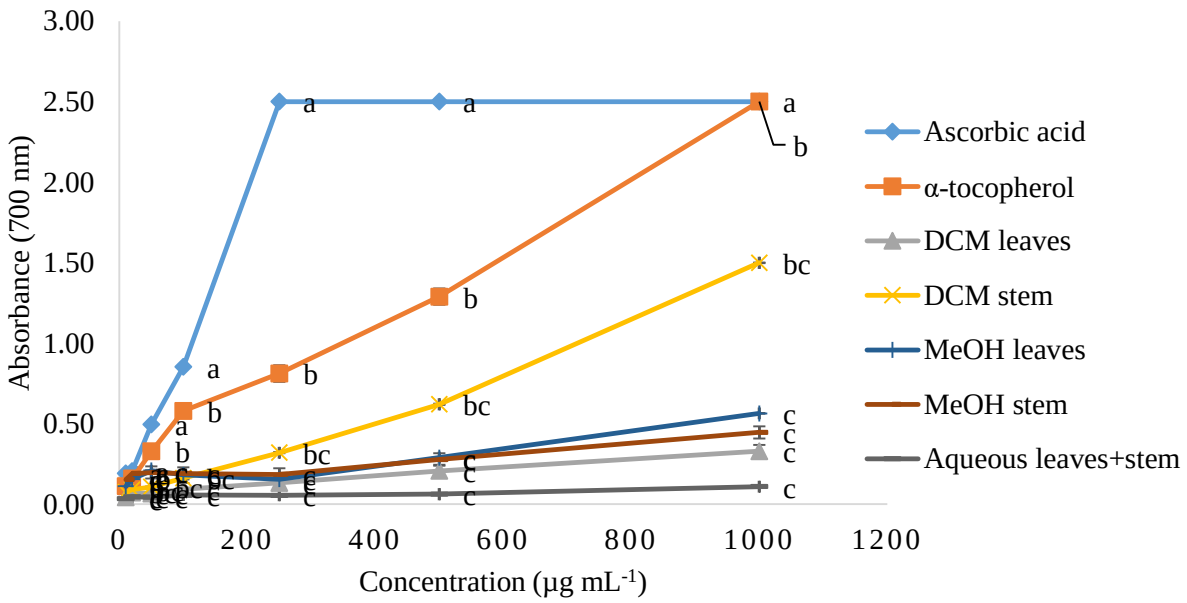


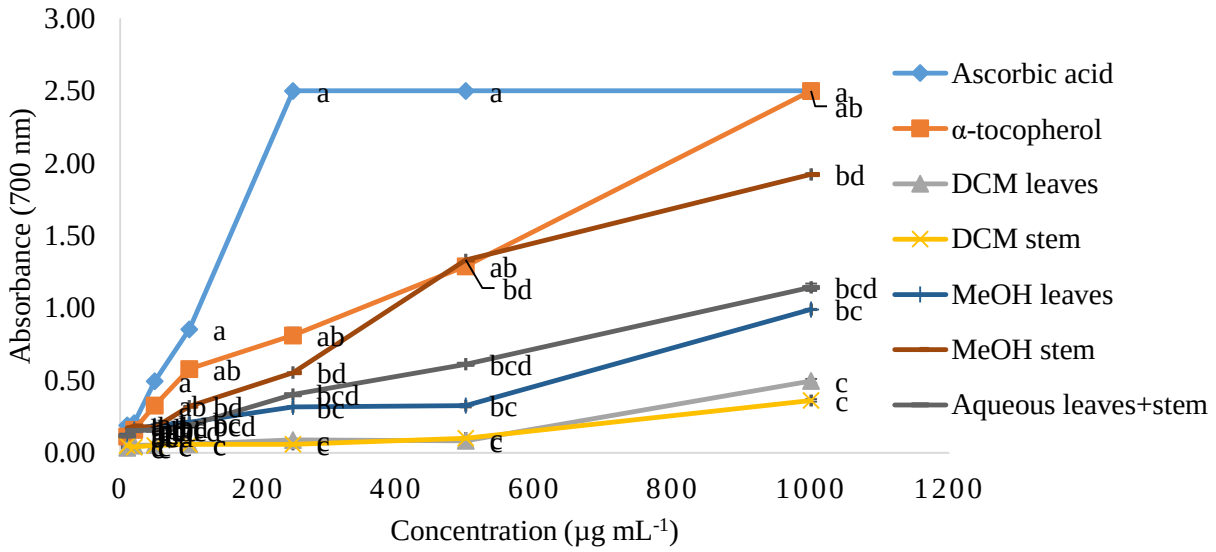
Figure 26: DPPH radical scavenging activity of compounds isolated from the nettles. Different letters indicate mean separation by Tukey's post-hoc test at the 5% level.

The FRAP assay evaluates the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron by the antioxidant (Soumya et al., 2014). There was a positive relationship between the concentration of plant extract or compound with absorbance (Figures 27 and 28). The DCM extract of the stem of *L. peduncularis* had the highest reducing capacity compared to the other extracts but lower than ascorbic acid and α-tocopherol. For both *L. alatipes* and *O. tenax*, the MeOH extracts of the stem had the highest activity compared to the other extracts. *Obetia tenax* and *L. alatipes* showed higher ferric reducing power compared to *L. peduncularis*. Beta -sitosterol exhibited the higher reducing capacity compared β-carotene but lower than ascorbic acid and α-tocopherol.

A



B



C

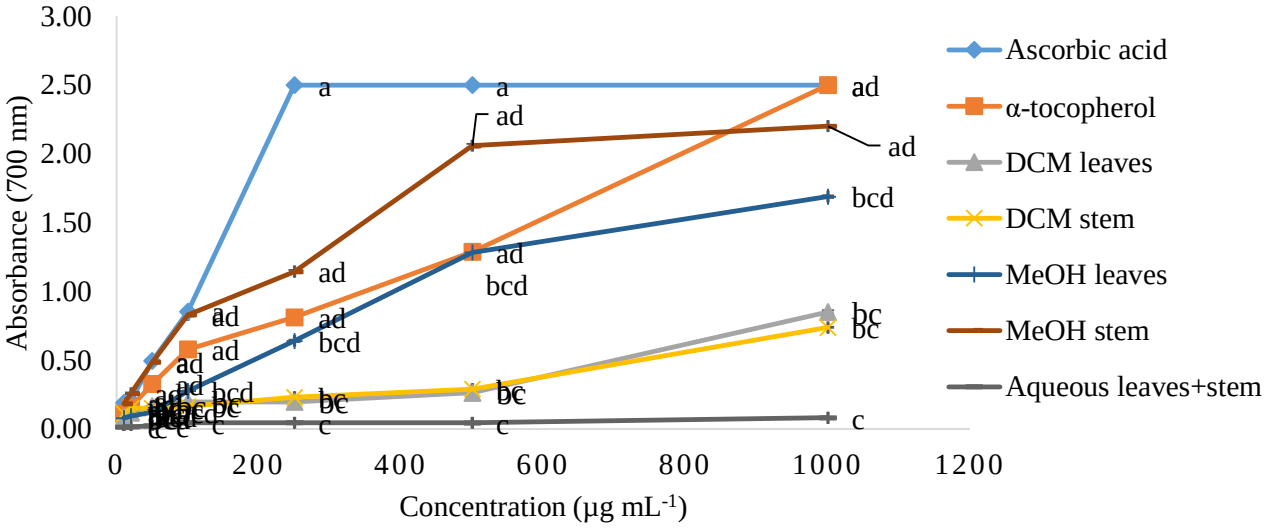


Figure 27: Ferric reducing power of extracts from *L. peduncularis* (A), *L. alatipes* (B) and *O. tenax* (C) leaves and stem. Different letters indicate mean separation by Tukey's post-hoc test at the 5% level.

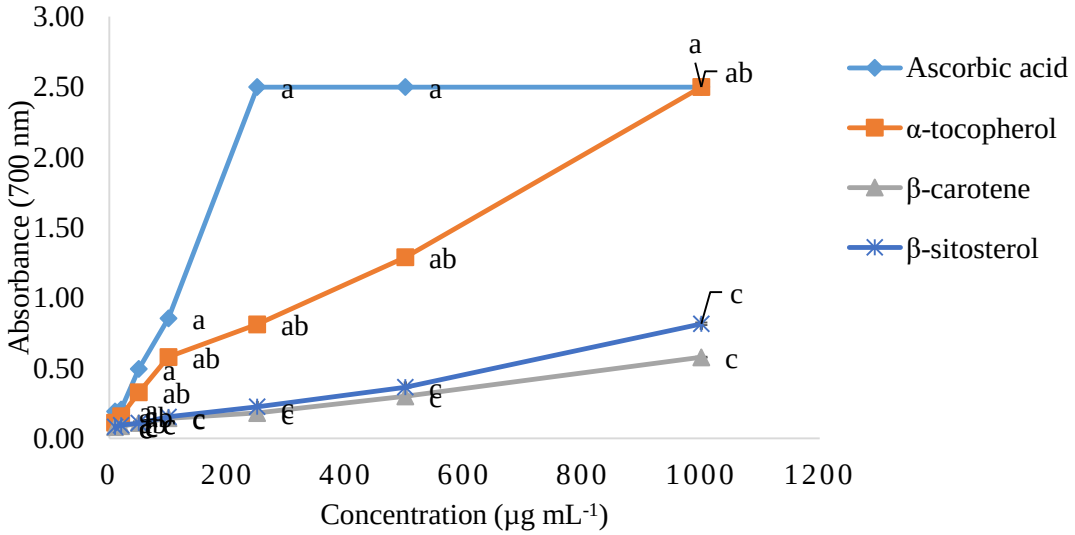


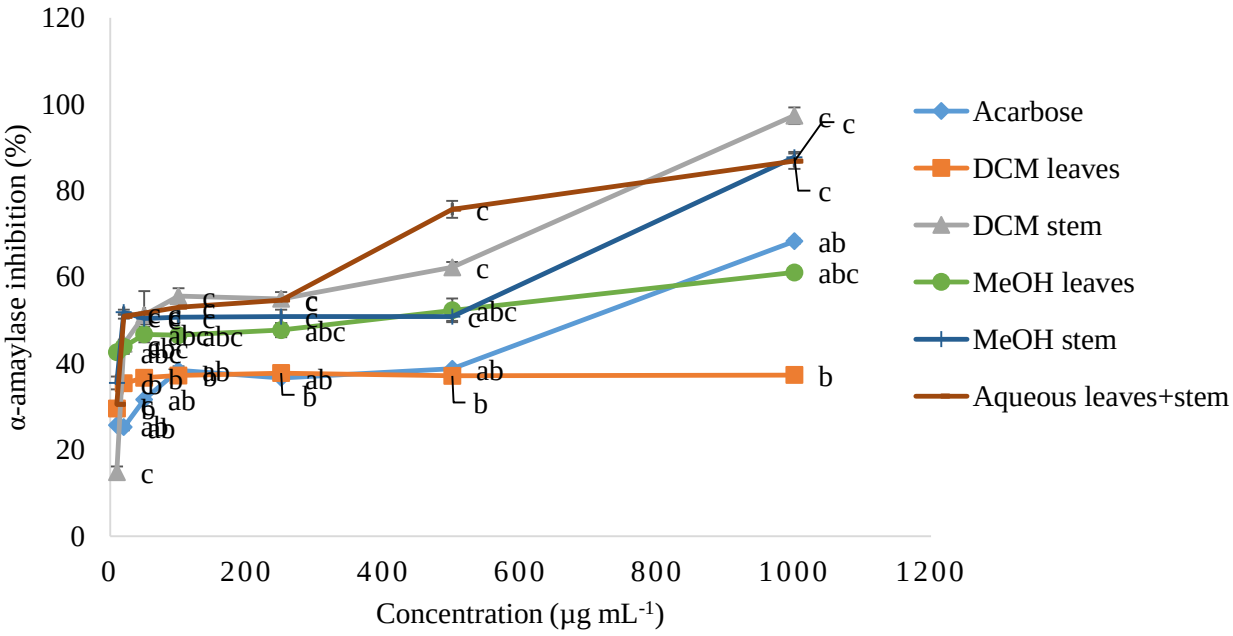
Figure 28: Ferric reducing power of compounds isolated from the nettles. Different letters indicate mean separation by Tukey's post-hoc test at the 5% level.

Enzyme inhibitory activity

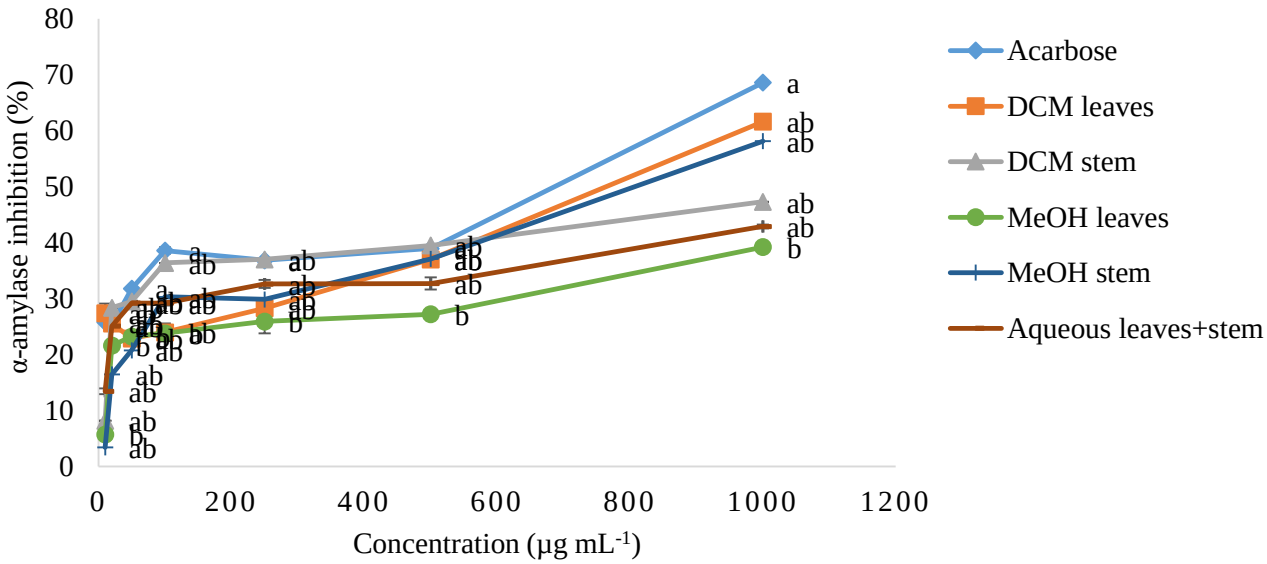
Alpha amylase is responsible for the digestion of dietary carbohydrates in humans. Inhibition of this enzyme slows down the rate of digestion of carbohydrates, glucose absorption and thereby lowers blood glucose levels. High levels of glucose absorption give rise to a condition known as hyperglycemia (Tadera et al., 2006). The effects of plant extracts and isolated compounds on the inhibition of α-amylase are represented in Figures 29 and 30. The IC_{50} values were determined for plant extracts and compounds with inhibition ≥50%. The aqueous extract of *L. peduncularis* had an IC_{50} value of 48.2 µg mL^{-1}, which was lower than that of the reference standard, acarbose (544 µg mL^{-1}). Considering the activity of the aqueous extract, studies on *Urtica dioca*, showed that aqueous extracts had antihyperglycemic activity (Bnouham et al., 2003; Das et al., 2011; Momo et al., 2007; Sasan et al., 2011). Furthermore, studies conducted by Momo et al (2006) showed that the administration of aqueous extract from *L. ovalifolia* on diabetic rats had positive effects on their body weight and the blood glucose levels of the diabetic rats decreased significantly. Extracts

of *L. alatipes* also showed inhibition of α-amylase but this was lower than acarbose. The DCM extract of the stems of *O. tenax* was shown to be the most active with an IC_{50} value of 21.39 µg mL^{-1}. The results suggest that extracts from nettles block the hydrolysis of 1,4 glycosidic linkage of starch into simple sugars (Dutta & Kalita, 2016). *Laportea pedunc*ularis and *O. tenax* are most effective in the inhibition of α-amylase activity. It was observed β-sitosterol (511 µg mL^{-1}) had lowest IC_{50} value, followed by β-carotene (527 µg mL^{-1}) relative to acarbose. These two compounds were both found in both *O. tenax* and *L. peduncularis*.

A



B



C

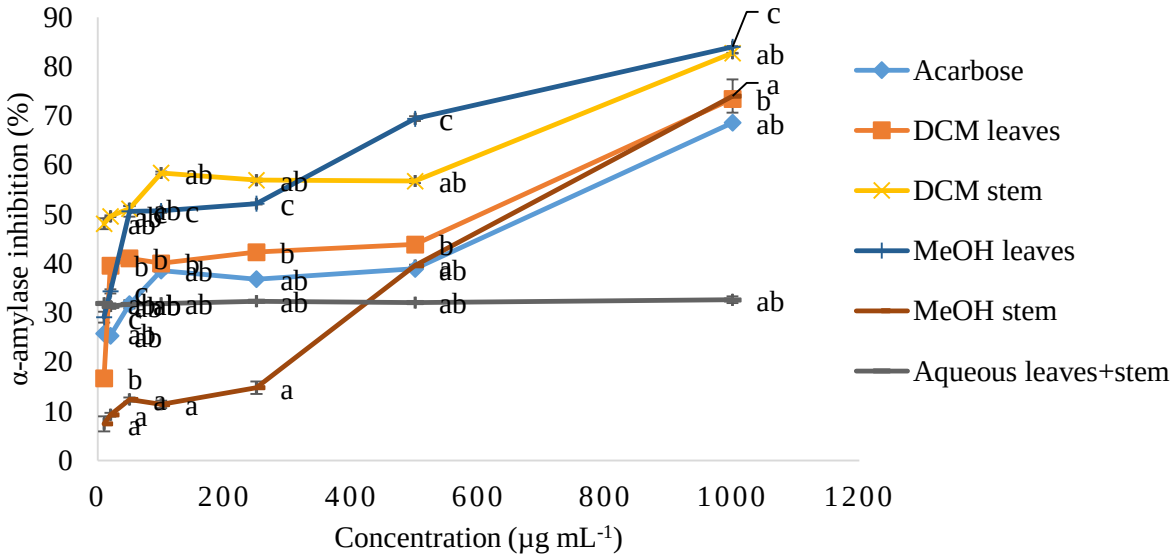


Figure 29: Inhibition of α-amylase activity by extracts from *L. peduncularis* (A), *L. alatipes* (B) and *O. tenax* (C) leaves and stem. Different letters indicate mean separation by Tukey's *post-hoc* test at the 5% level.

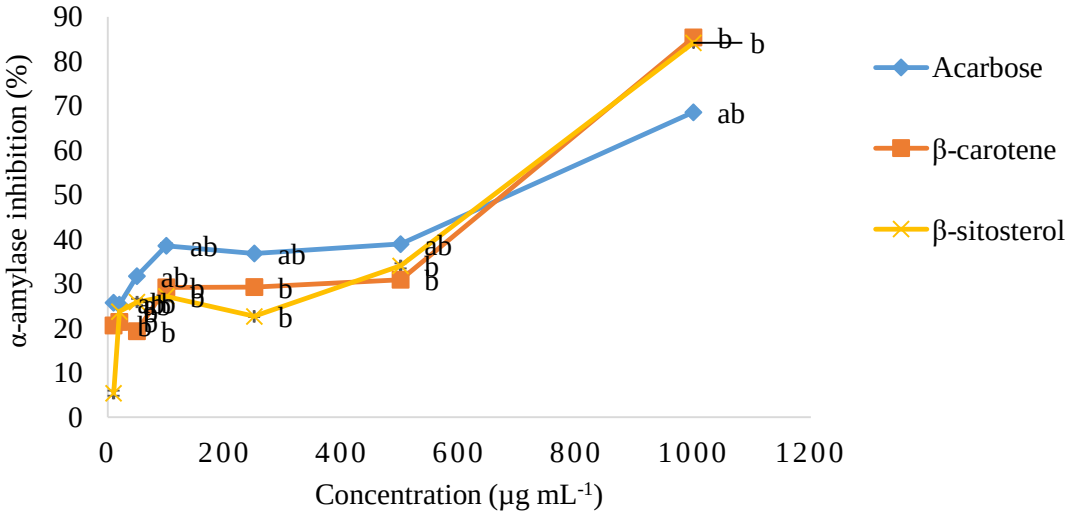
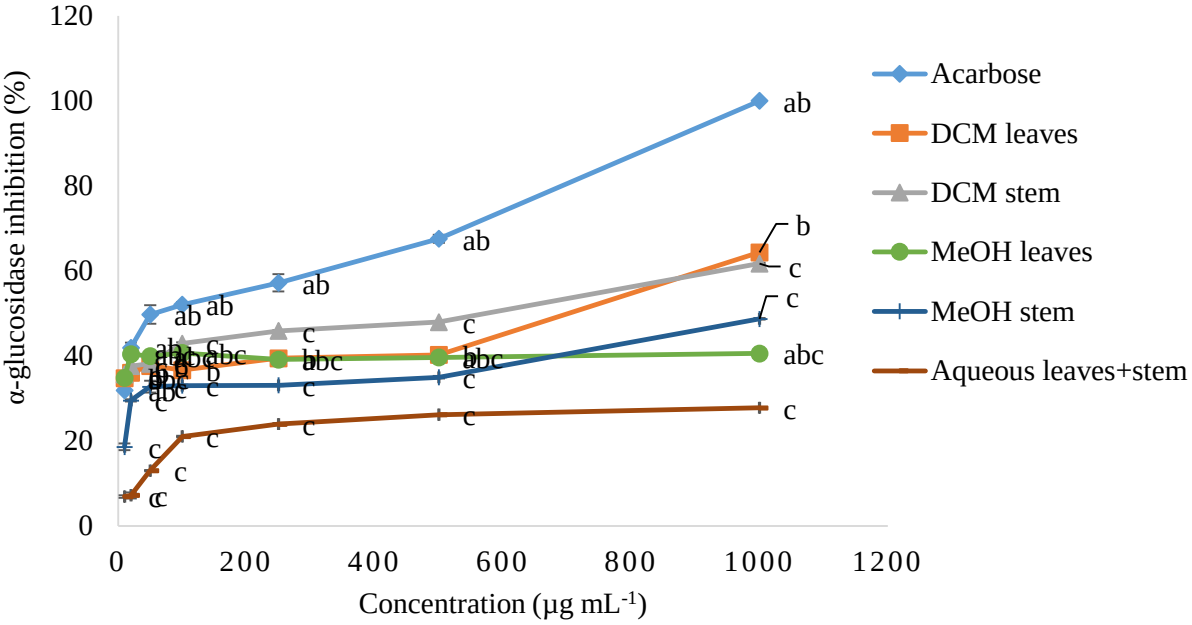


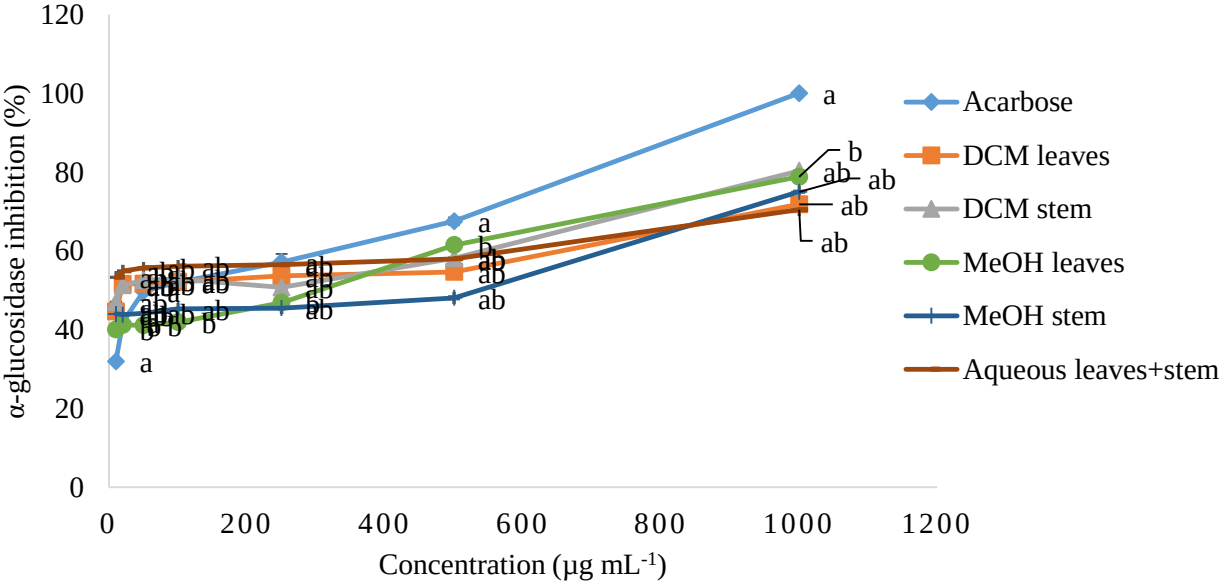
Figure 30: Inhibition of α-amylase activity by compounds isolated from the nettles. Different letters indicate mean separation by Tukey's *post-hoc* test at the 5% level.

The inhibition of α-glucosidase activity of the extracts and compounds from nettles is presented in Figures 31 and 32. There was a milder inhibition of α-glucosidase activity compared to α-amylase activity for *L. peduncularis* and *O. tenax*. DCM extract from leaves (75.0 µg mL^{-1}) and stems (65.2 µg mL^{-1}) of *L. alatipes* had the lowest IC_{50} values compared to acarbose (153 µg mL^{-1}). The results show *L. alatipes* to be the nettle that is most active in the inhibition of α-glucosidase. ß-sitosterol (IC_{50} value of 256 µg mL^{-1}) was observed to be the most active inhibitor of α-glucosidase activity compared to the other compounds but this was still lower than acarbose.

A


α-glucosidase inhibition (%)
Concentration (µg mL-1)
Acarbose
DCM leaves
DCM stem
MeOH leaves
MeOH stem
Aqueous leaves+stem

B


α-glucosidase inhibition (%)
Concentration (µg mL-1)
Acarbose
DCM leaves
DCM stem
MeOH leaves
MeOH stem
Aqueous leaves+stem

C

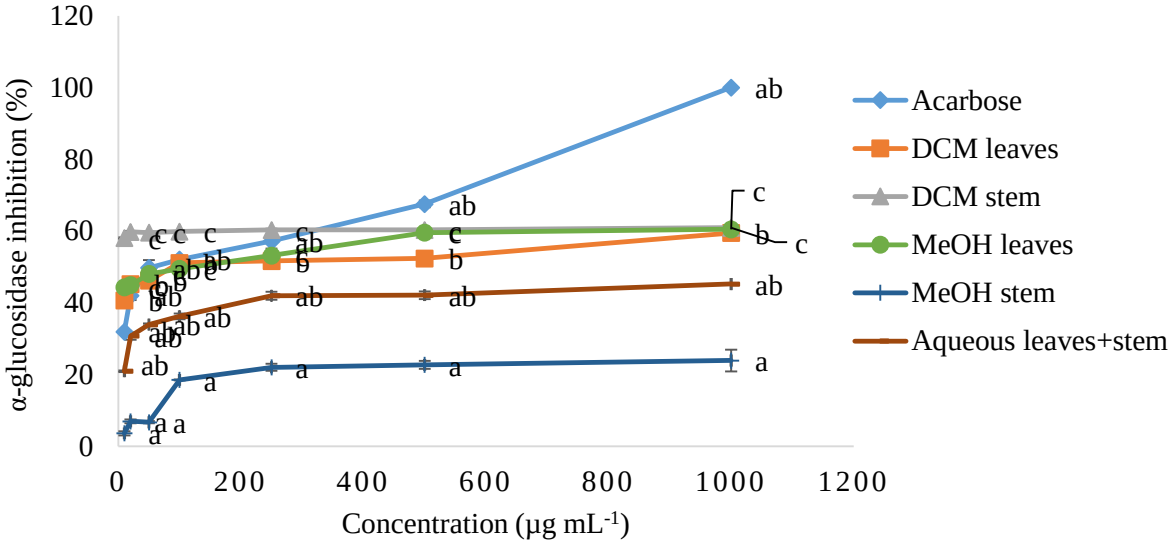


Figure 31: Inhibition of α-glucosidase activity by extracts from *L. peduncularis* (A), *L. alatipes* (B) and *O. tenax* (C) leaves and stem. Different letters indicate mean separation by Tukey's *post-hoc* test at the 5% level.

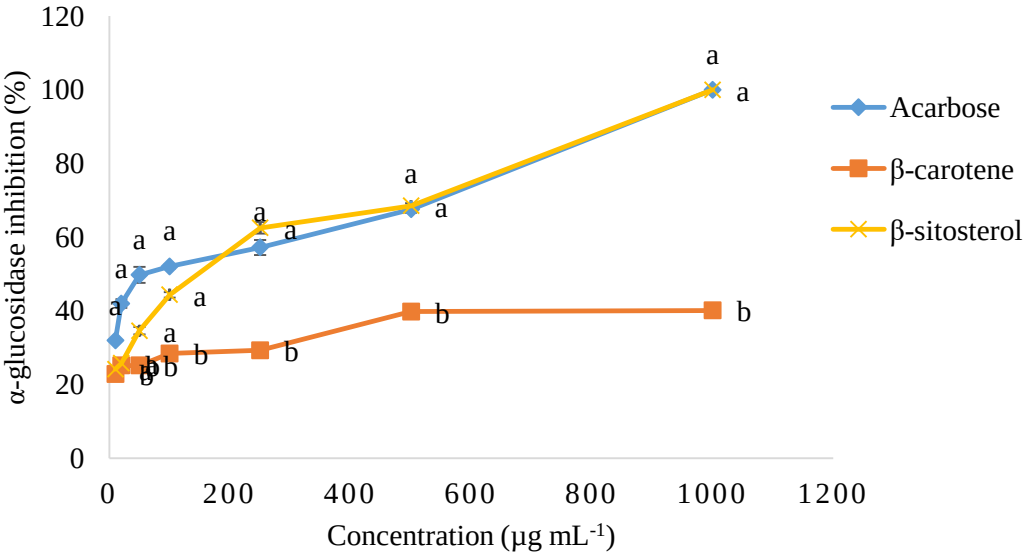


Figure 32: Inhibition of α-glucosidase activity by compounds isolated from the nettles. Different letters indicate mean separation by Tukey's *post-hoc* test at the 5% level.

CONCLUSION

The phytochemical analyses lead to the isolation of β-carotene from *L. peduncularis* and *O. tenax* and β-sitosterol from all three nettles. Extracts from nettles showed moderate DPPH radical scavenging activity. The MeOH extracts of leaves and stems of *O. tenax* had higher ferric reducing antioxidant power compared to α-tocopherol. The aqueous extract of *L. peduncularis* leaves and the DCM extract of the stems of *O. tenax* appeared to have high α-amylase inhibitory activity, whilst β-sitosterol and β-carotene had high α-amylase inhibitory activity. Overall, the α-glucosidase inhibitory activity of *L. alatipes* was higher than the other two nettles studied. ß-sitosterol was observed to have the highest α-glucosidase inhibitory activity. Our study suggests that nettles can be used as natural therapeutic agents for type 2 diabetes and therefore provides a scientific basis for its ethno-medicinal use.

Acknowledgements

The authors like to thank the National Research Foundation (NRF), South Africa for financial support and the School of Chemistry and Physics, UKZN for the research facilities.

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CHAPTER EIGHT

OVERALL SUMMARY

The ongoing food security crisis in South Africa has caused an increase in child malnutrition, undernutrition and negative health effects. The development of non- communicable diseases such as hypertension and diabetes has been linked to nutrient deficiencies (vitamins and minerals). There are numerous indigenous edible plants that are underutilised in South Africa as information on the chemical composition of these plants is lacking; this information would highlight the nutritional potential of these plants which can be exploited to alleviate hunger and malnutrition and would also validate the ethno-medicinal use of these plants. Nettles are indigenous medicinal plants that can be exploited as a food source and for their biologically active compounds; they are comparable to commonly used vegetables such as spinach and cabbage. The aim of this study was to analytically and phytochemically investigate the nettles (*Laportea peduncularis* susp. *peduncularis* (river nettle), *Laportea alatipes* (forest nettle), *Obetia tenax* (mountain nettle) and *Urtica dioica* (stinging nettle)). The distribution of macronutrients, anti-nutrients and essential elements in the four nettles was determined. The heavy metal distribution in *Laportea peduncularis* and *Obetia tenax* and corresponding growth soil was determined to evaluate the impact of soil quality on uptake by nettles and to assess for potential metal toxicities. Additionally, the uptake, translocation and bioaccumulation of elements in nettles were determined by looking at *Laportea alatipes*. The nutritional value of the four nettles was also evaluated by comparing to dietary reference intakes. Finally, the secondary metabolites in nettles were isolated and identified and these were tested for their biological activity (antioxidant and anti-diabetic).

FINDINGS FROM THE STUDY

The concentration of essential elements in cooked *L. peduncularis* leaves were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Cr > Ni > Co. Both cooked and raw leaves of *L. peduncularis* and *U. dioica* nettles were found to be rich sources of macronutrients and essential elements and may be used as an alternative to commercially available vegetables or herbs. According to the findings in *O. tenax*, the results showed that the concentrations of elements in the leaves to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cr > Cu > Ni > Pb > Co > As > Cd > Se, and in the stems and roots to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > Ni > As > Pb > Co > Cd > Cr > Se. Findings on *Laportea alatipes* revealed that concentrations of minor elements in the leaves were in decreasing order of Fe> Mn> Zn> Cu> Cr> Ni> Co> Pb> Se> As>Cd.

Soil quality indicators (geo-accumulation indices and enrichment factors) indicated moderate Cd contamination in nettles. The concentration of metals in the soil were in decreasing order of Fe> Ca> Mg> Mn> Zn> Cr> Cu> Ni> As> Co> Cd> Pb. Principal component and cluster analyses revealed that certain elements were from a common origin.

Findings from the proximate analysis of *L. alatipes* and *O. tenax* showed a significant decrease in the crude fat, crude protein, vitamin C and E content and a significant increase in carbohydrate and crude fibre content with cooking. Also, a decrease in the vitamin A content was observed in *L. alatipes*. The anti-nutrient (cyanide, oxalates, saponins and phytates) and toxic element (Cd and Pb) content decreased with cooking.

Nettles grown near main roads were found to contain higher concentration of toxic elements As, Cd and Pb, yet moderate contamination was observed. It would be therefore recommended that

nettles grown near main roads be cooked in order to decrease the concentration of harmful elements. The nettles were found to be rich in β-carotene and sterols. The nettle extracts were found to have moderate antioxidant activity and higher anti-diabetic activity than the known standard, acarbose.

CONCLUSION

An investigation of the phytochemical composition of the nettles revealed the presence of β-carotene and β-sitosterol. Extracts from the nettles were found to possess anti-diabetic potential which indicates that they can be beneficial to individuals suffering from hyperglycaemia which is one of its uses in traditional medicine. Nettles were found to be rich in macronutrients, essential elements and other minerals, thus they can contribute positively to the diet. Cooking of nettles was found to decrease the levels of toxic elements and anti-nutrients in the plants. Nettles were found to be rich in Fe therefore, they can be used as Fe supplements for individuals suffering from Fe deficiency anaemia. Since nettles are readily available they can serve as an affordable alternative to commercially available herbs. This study lends scientific credence to the ethno-medicinal use of nettles and provides information on their nutritional value.

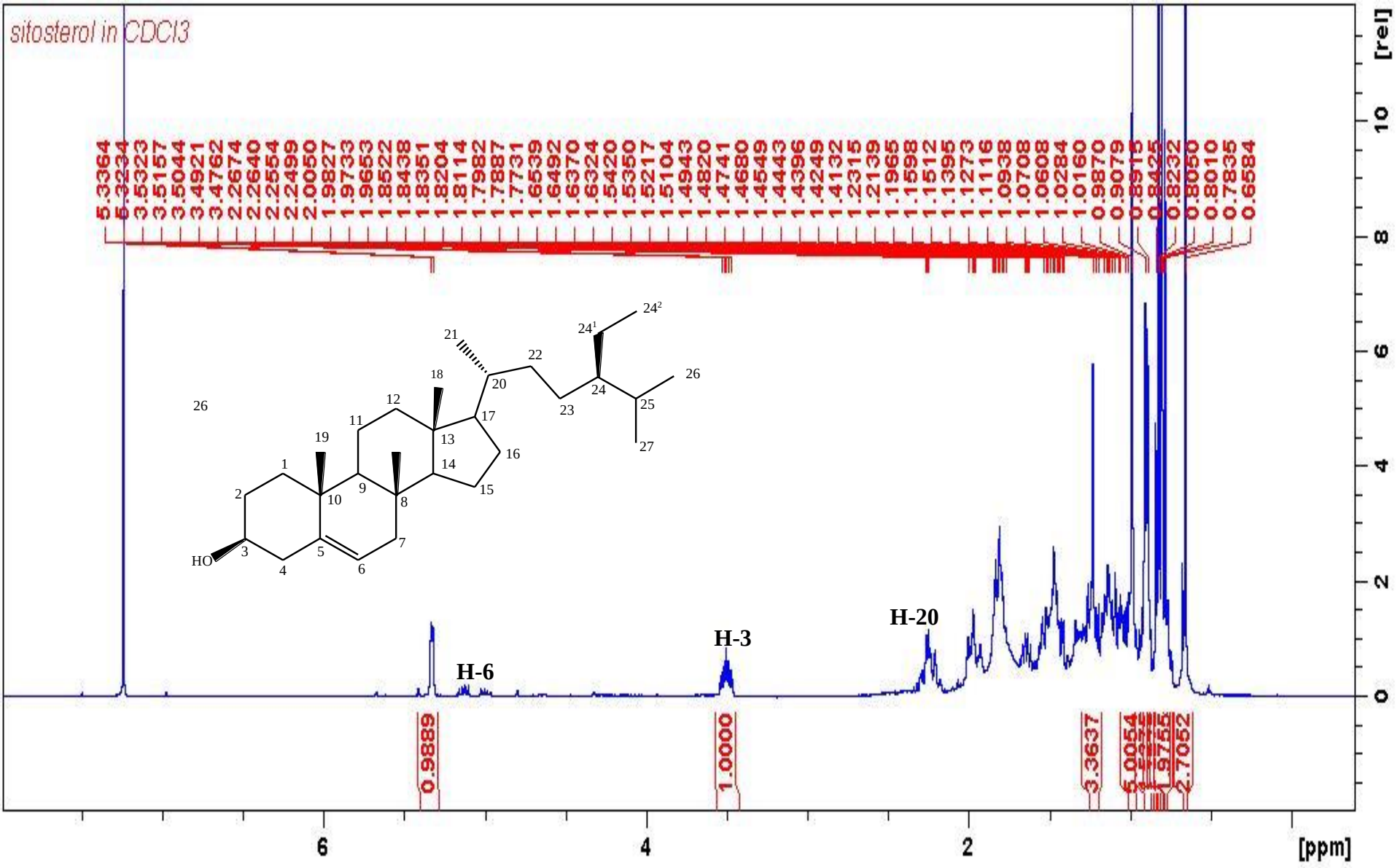
RECOMMENDATION FOR FUTURE WORK

- Isolation and structural elucidation of all phytocompounds in nettles.
- Determination of the fatty acid composition of nettles.
- Further investigation of the anti-diabetic potential of nettles.

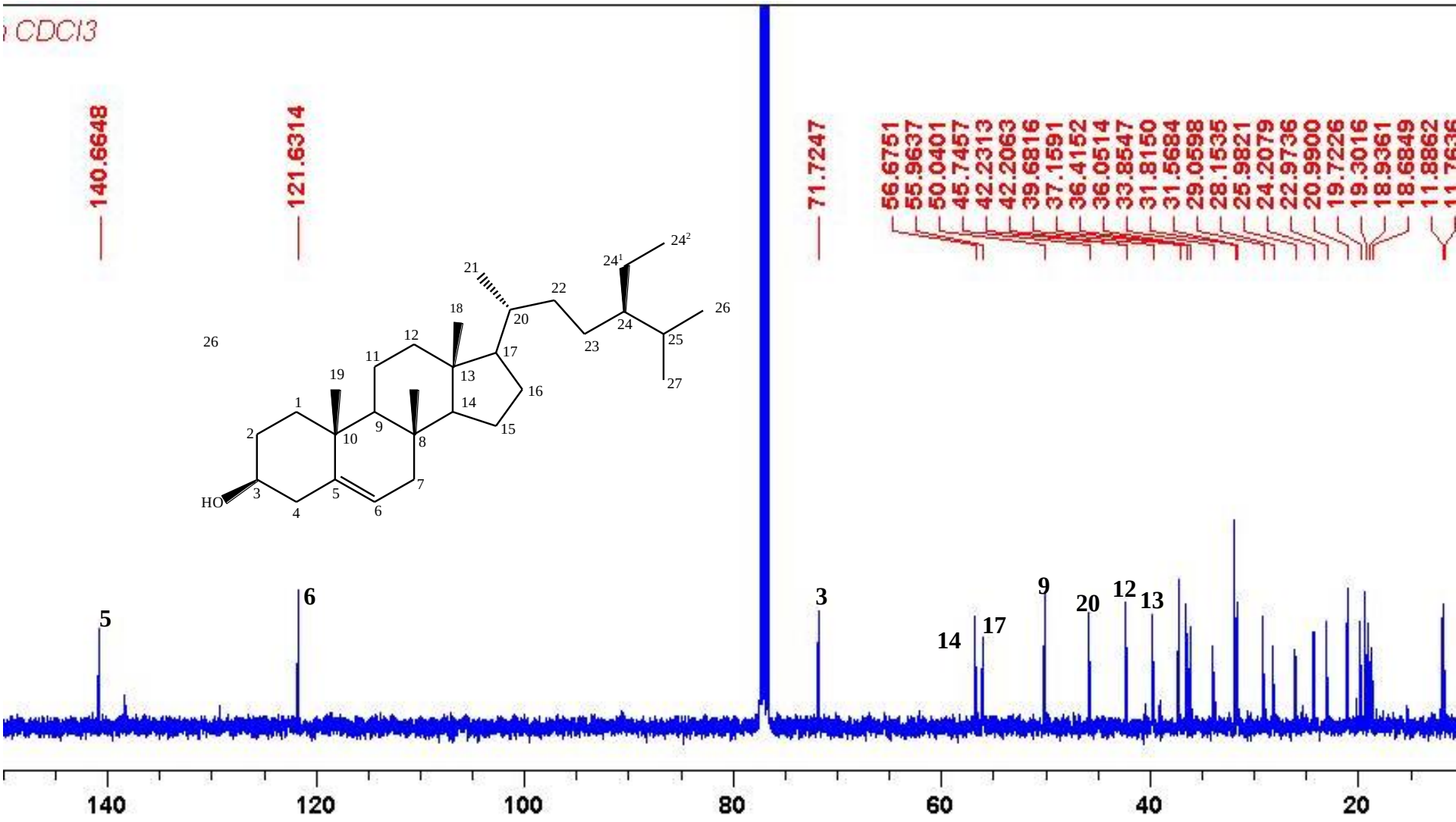
APPENDIX

SUPPORTING INFORMATION

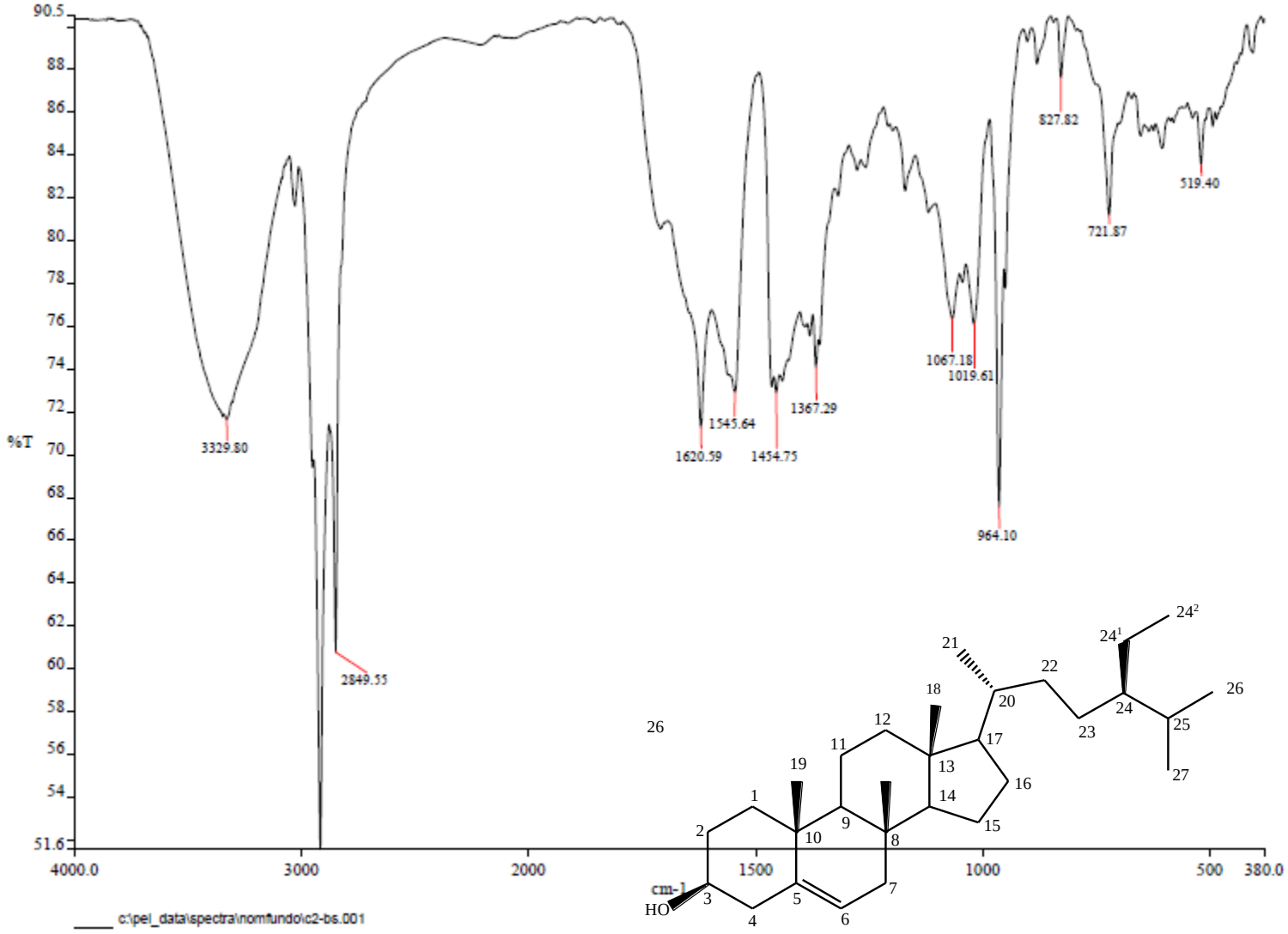
Supporting information include NMR, IR, MS, UV spectra.



[1]H-NMR spectrum of C2(β-sitosterol)



^{13}C-NMR spectrum of C2(β-sitosterol)



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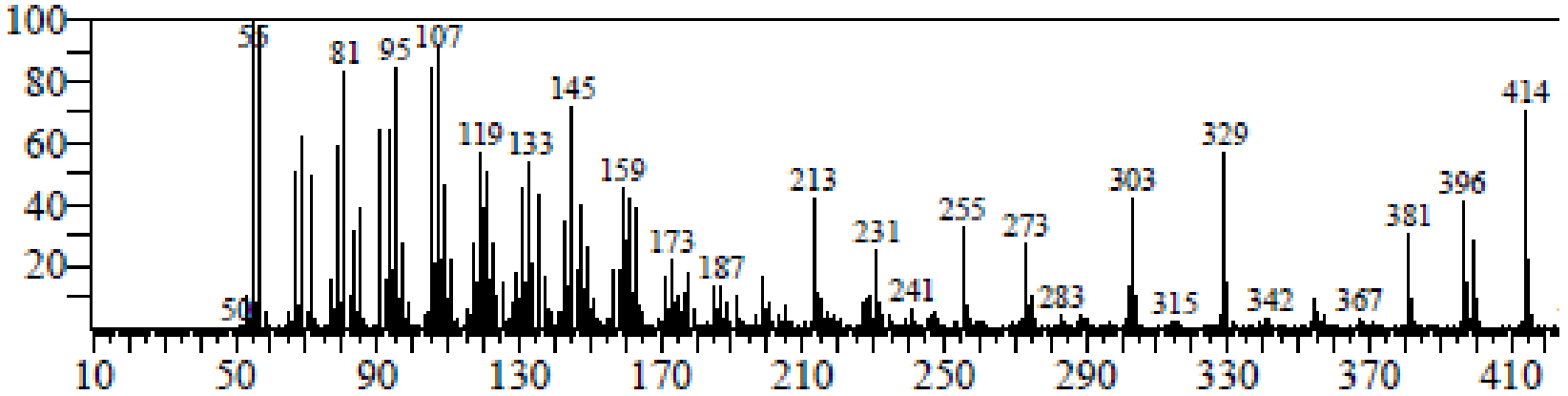

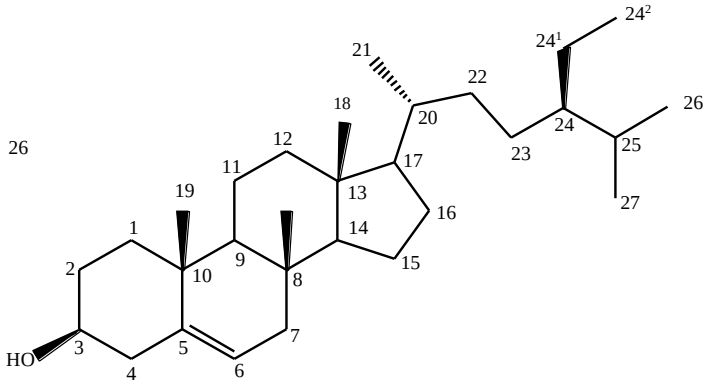

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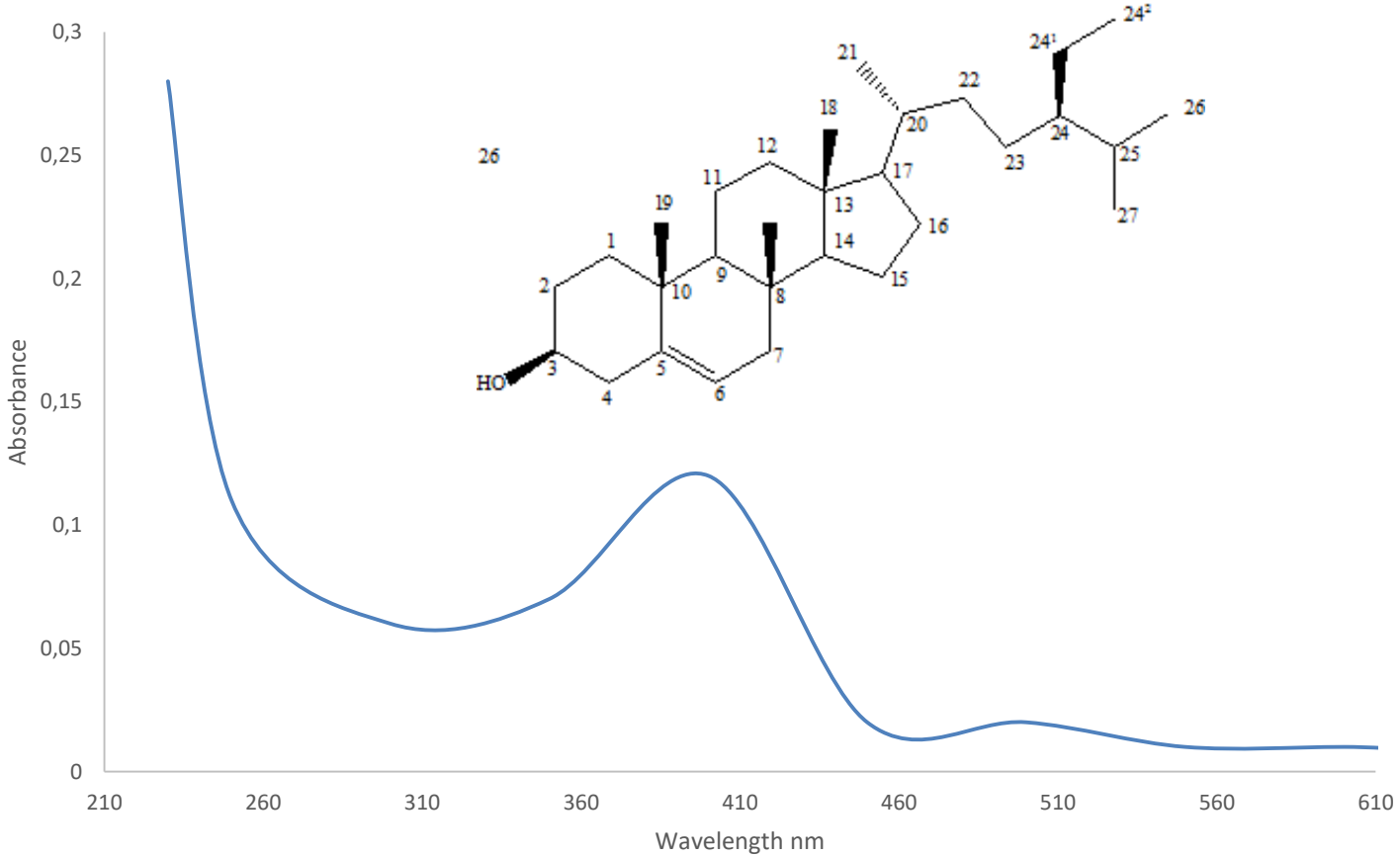
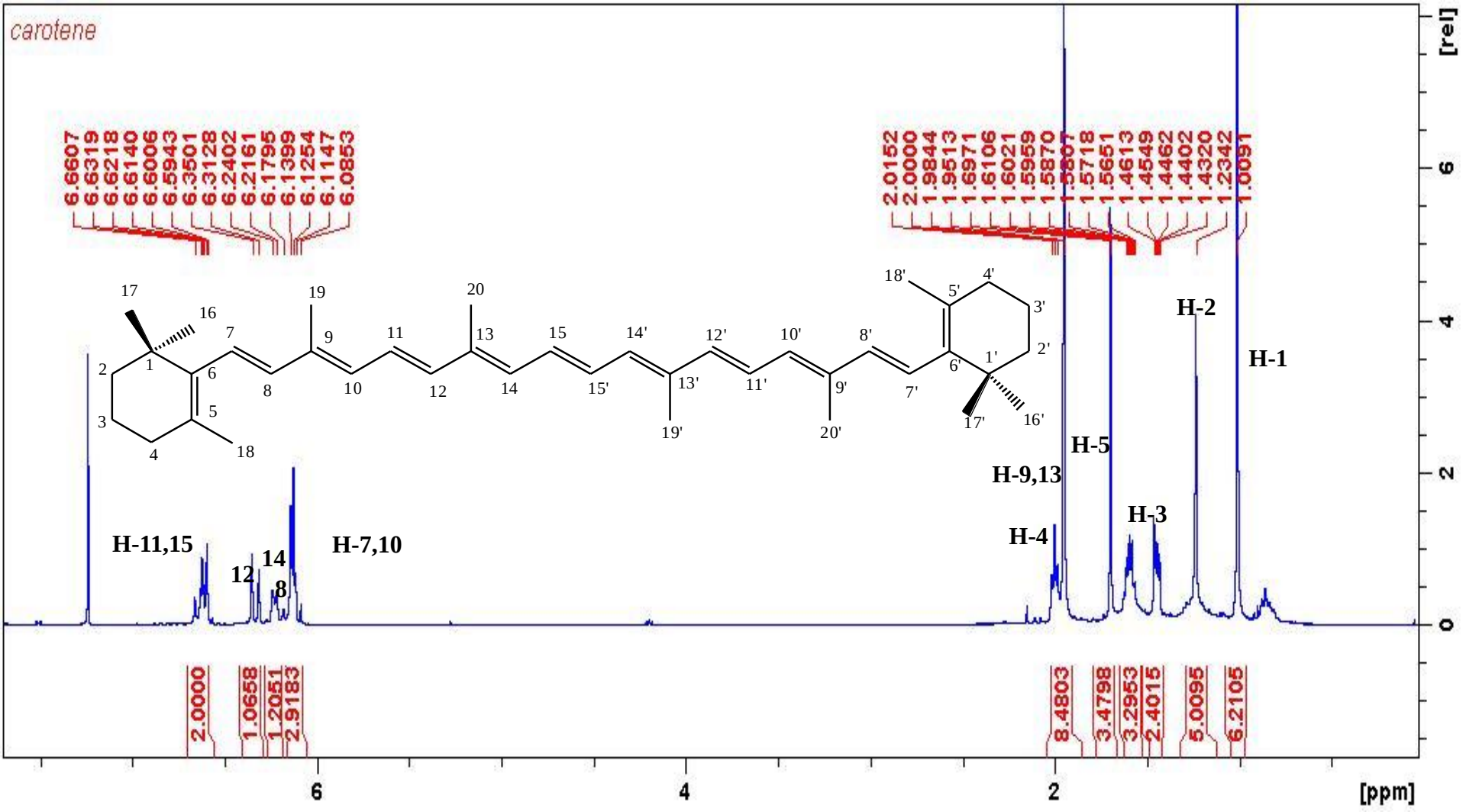


UV-Vis spectrum of C2(β-sitosterol)



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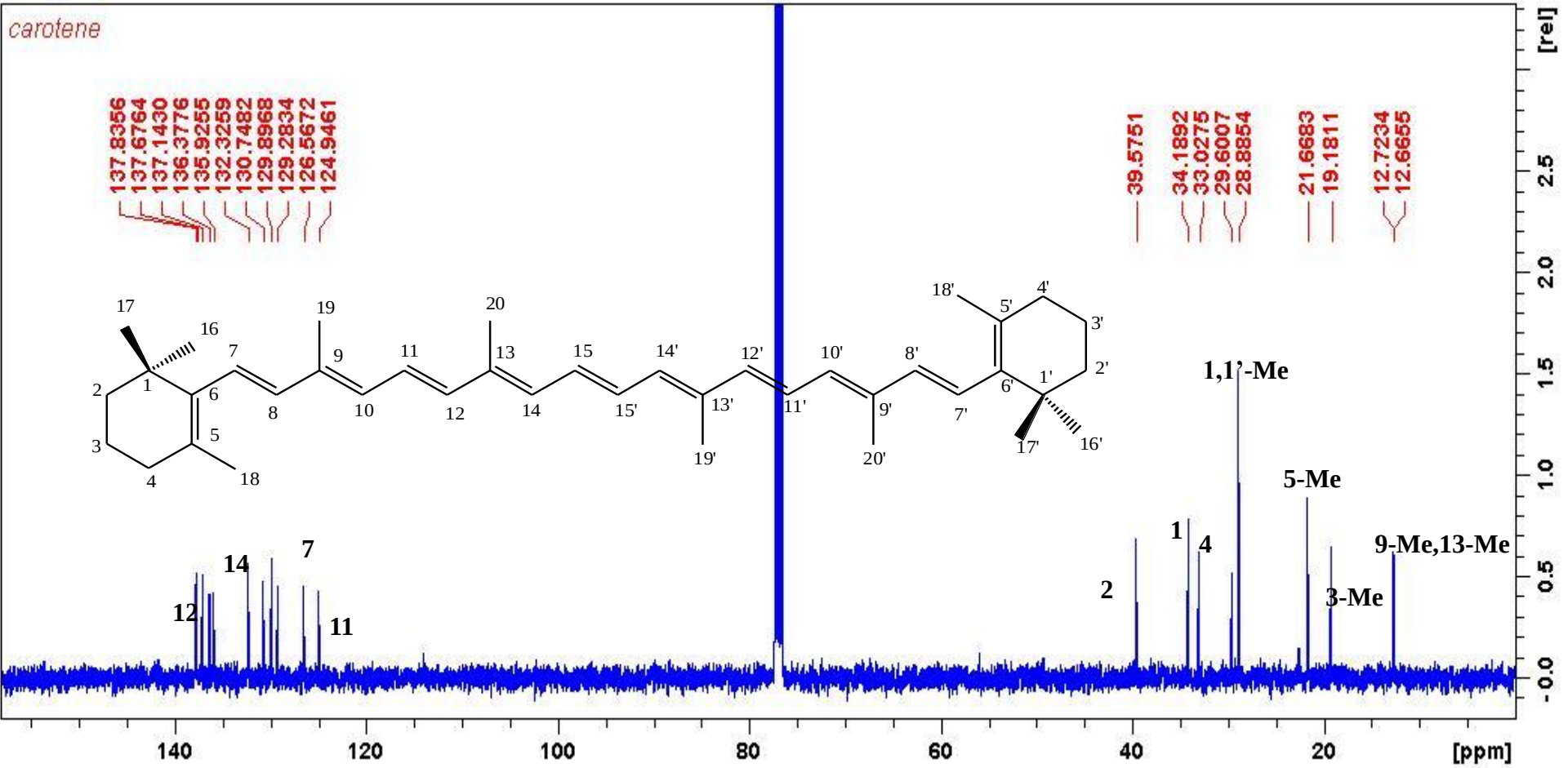

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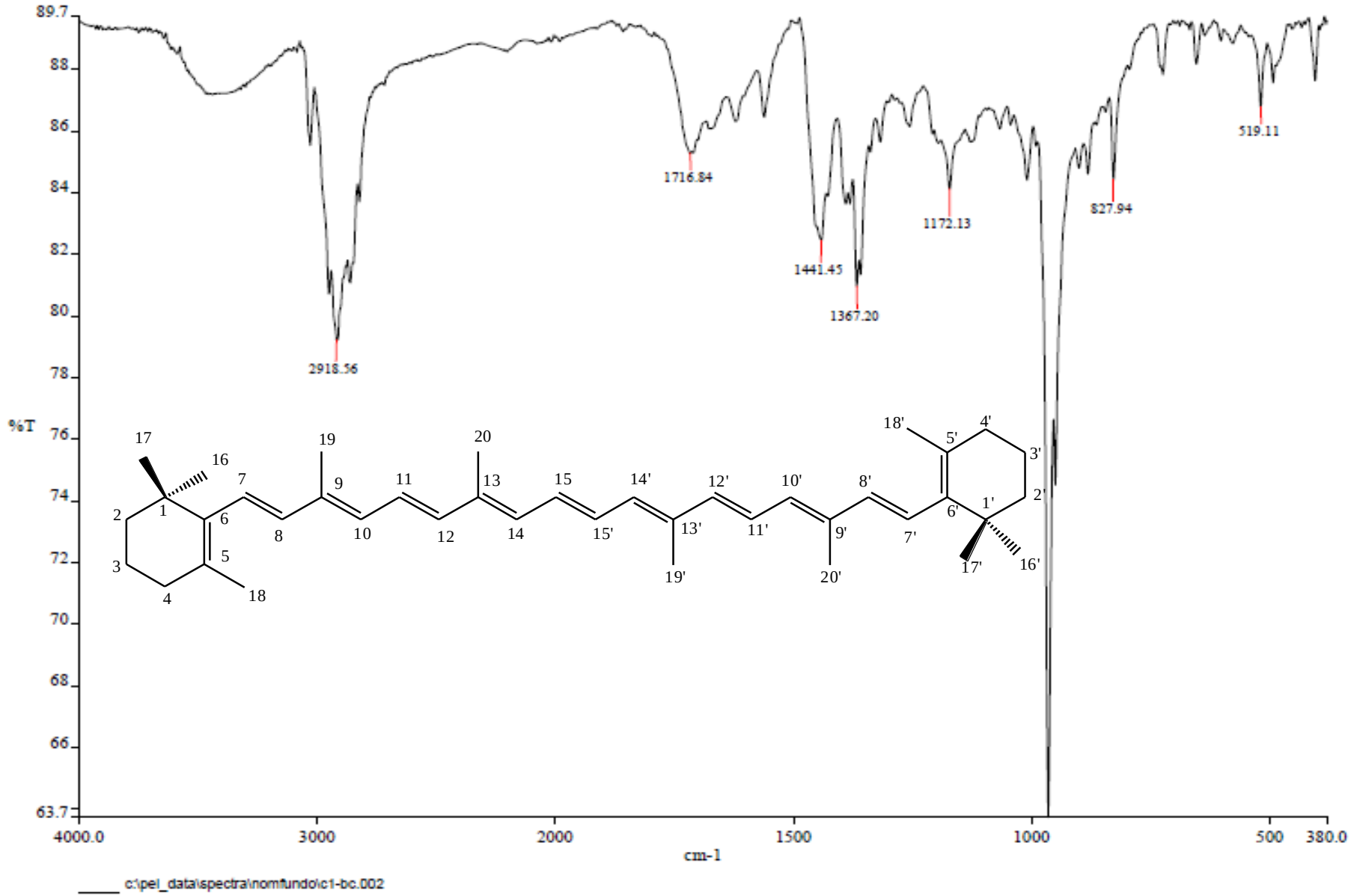

IR spectrum of C1(β-carotene)

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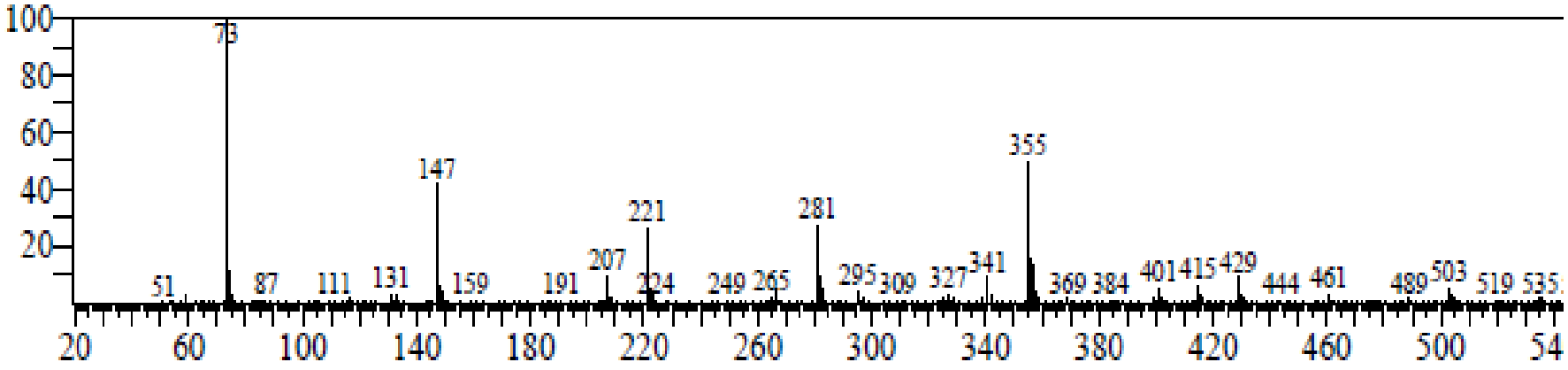
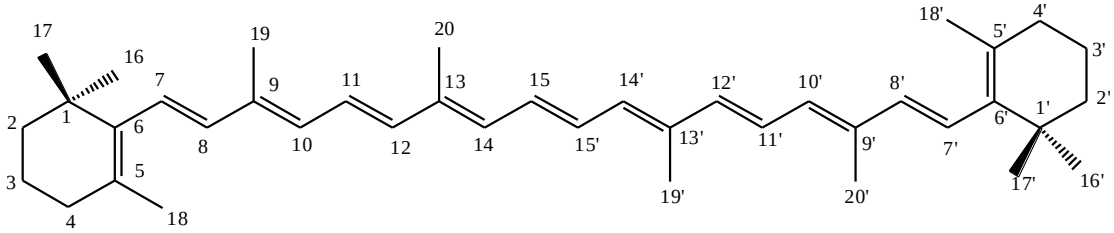
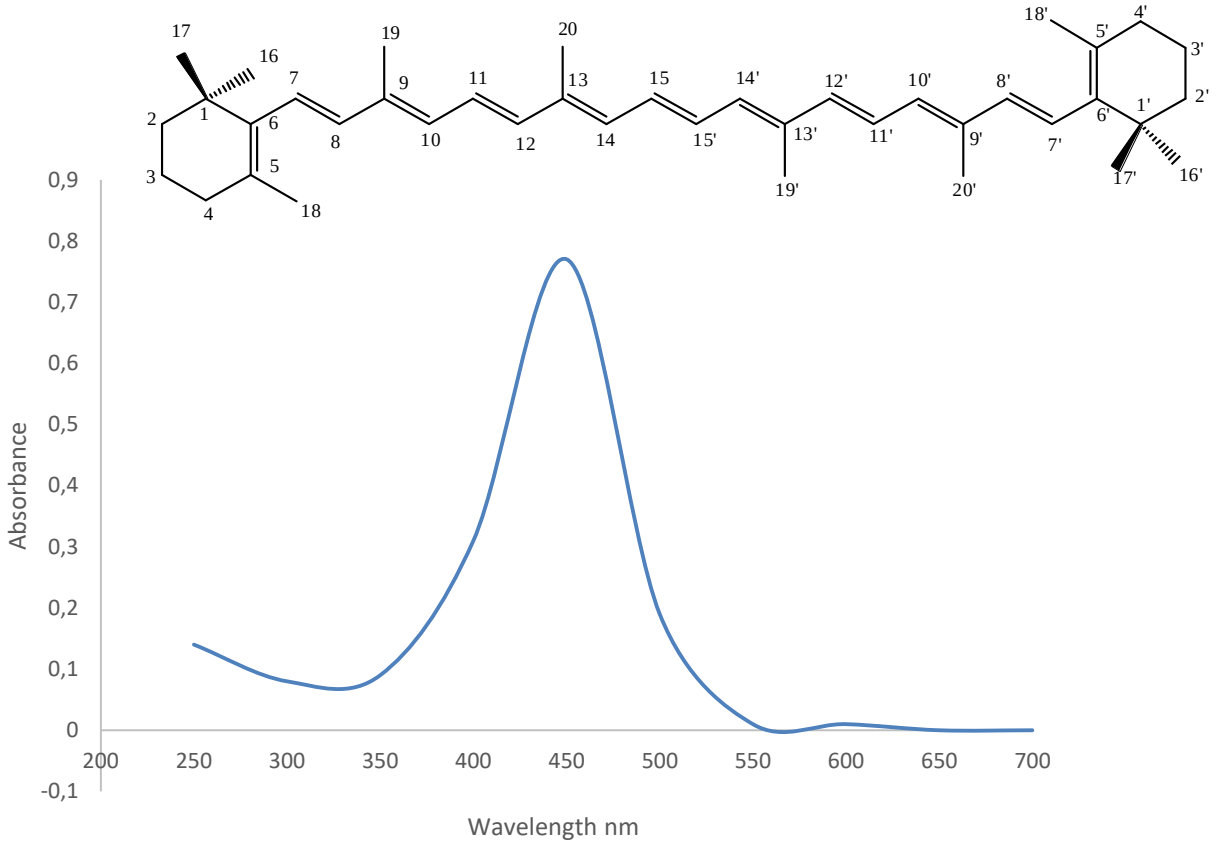




GC-MS spectrum of C1(β-carotene)



UV-Vis spectrum of C1(β-carotene)