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The Impact of different levels of organic plant-based compost on the macro and micro elements, secondary metabolites and water soluble vitamins content of *Moringa oleifera* leaves

by

Nkazimulo Ngwenya

(2177032)

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Supervisor: Dr. I. M. Risenga

Co-supervisor: Prof. L. Chimuka

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Declaration

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

(Signature of candidate)

5th day of October 2023 at APES/WITS UNIVERSITY

Abstract

Moringa oleifera leaves are a high source of phytochemicals and nutrients inclusive of macro and micro elements, secondary metabolites and water-soluble vitamins. All the *M. oleifera* plant parts inclusive of its leaves have been widely utilised for nutritional and/ medicinal properties because of its high levels of minerals and phytochemicals. Studies have been conducted targeting the improvement of the quantity of *M. oleifera* nutrient content. However, with increased consumer awareness of high quality organic, consumer and environmentally safe products there is increased need to improve the quality of *M. oleifera* products. One of the major factors impacting the quality and quantity of *M. oleifera* leaf nutrients is cultivation practices. Particularly soil amendments applied to improve plant biomass have also been found to significantly improve nutrient content of *M. oleifera* leaves. However, the popularly used soil amendments which are in the form of synthetic fertilisers or animal based compost have raised environmental and product safety concerns. They contain, among other contaminants, traces of heavy metals and other toxins which are harmful to both the environment and consumers of the produce. Producers of herbal plants such as *M. oleifera* are looking for sustainable, environmental safe ways of improving the quantity and quality of the produce. Application of organic plant based compost was therefore investigated as a potential soil amendment source in improving *M. oleifera* nutrients in an organic, clean sustainable way, and thus improving the quality of the produce.

The use of plant based compost to improve the nutritional content of *M. oleifera* grown in South Africa was reported in this thesis. The primary objective of the study was to assess the impact of the plant-based organic compost on the macro and micro elements composition, secondary metabolite accumulation and distribution and water soluble vitamins content in *M. oleifera* leaves. This work is novel and worth exploring as it seeks to investigate for the first time the correlation between the use of plant-based organic compost and quality improvement in terms of nutrient content of *M. oleifera* leaf biomass. Furthermore, this research is the first of its kind which

looks at the impact of compost on the nutritional content encompassing macro and micro nutrients, secondary metabolites and water soluble vitamins in *M. oleifera* grown in South Africa. In addition, the developed and validated high performance liquid chromatography (HPLC) method for the simultaneous quantification of five B vitamins was successfully used in the identification and quantification of the vitamins.

Clay soil and *M. oleifera* seeds used in the study were obtained from the *M. oleifera* community farm in Hammanskraal, Gauteng, South Africa. The clay soil was amended with plant-based organic compost purchased from the local nursery store. The amendments achieved four treatment levels namely 15 % compost / soil amendment, 30 % compost / soil amendment, 45 % compost / soil amendment and 60 % compost / soil amendment. *Moringa oleifera* seeds used were obtained from the same farm. Plant samples were grown in the School of Animal, Plant and Environmental Sciences (APES), University of the Witwatersrand, greenhouse under ambient temperatures. Harvesting of the leaf biomass was done after six months and nutrient analysis was carried out. Analytical techniques such as Spectroscopy and Chromatography were used for the nutrient analysis. Presence and quantity of macro and micro elements in the soil and leaves was analysed using the Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES). Whilst, the Ultra high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS) was used to determine plant metabolites, and for the quantification of water-soluble vitamins by coupling it with the diode array detector (DAD).

The nutrient analysis revealed that the use of organic plant-based compost for the amendment of clay soil improved the quality of the soil and *M. oleifera* leaves harvested from each soil amendment. The addition of the organic plant-based compost improved the macro and micro nutrients. Comparison of the different compost levels revealed that addition of plant based compost increased the bioavailability of macro and micro nutrients in the soil and increased their accumulation in *M. oleifera* leaves. Metabolic

fingerprinting of *M. oleifera* leaf samples using UHPLC-ESI-QTOF-MS followed by untargeted compound analysis exposed variation in the identified metabolites. Further use of multivariate analysis in the form of PCA clustered the samples into five distinct clusters indicating diversity in the distribution of secondary metabolite as influenced by the addition of plant-based compost to the soil. The developed HPLC method was suitable for the simultaneous quantification of five B vitamins based on the low LOD and LOQ values, recovery of 97.8- 99.58% and good linearity. Application of the validated method revealed that, the addition of plant based compost significantly improved the quantity of the tested vitamins (Vitamin B1, B2, B3, B6 and B9). The 30% plant-based organic compost and clay soil combination was identified and recommended as the best compost-soil combination in improving nutrients in *M. oleifera* leaf biomass. It had the highest level of macro and micro nutrients. Leaves harvested from this soil/compost combination had higher concentration of Ca (45 042.5 mg/Kg), Mg (17 430 mg/Kg), P (8802. 5 mg/Kg). In addition leaves, harvested from 30% compost treatment exhibited the highest number of identified secondary metabolites and had the highest concentration of two of the five tested water soluble vitamins.

This knowledge will make great contribution in the *M. oleifera* industry in South Africa and worldwide particularly for farmers who are into organic *M. oleifera* production.

Dedication

A dedication with sincere feelings of gratitude to my dear husband Silethemba Dube and my lovely children Amaris Makabongwe and Siyabonga Dube who have been with me throughout this work, I am truly grateful for your support and sacrifice. I also want to dedicate the work to my grandparents, my parents and my siblings, I appreciate you all.

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List of abbreviations

APES	Animal, Plant and Environmental Sciences
BCR	European Community Bureau of Reference
CEC	Cation exchange capacity
DAD	Diode array detector
DIM	Daily intake of metal
EC	Electrical conductivity
FAO	Food and Agriculture Organization
GC	Gas Chromatography
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass Spectrometry
MVA	Multivariate analysis
NMR	Nuclear Magnetic Resonance Spectroscopy
PCA	Principal Component Analysis
PLS-DA	Partial least squares discrimination analysis
UHPLC-ESI-QTOF-MS	Ultra high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry
WSV	Water soluble vitamins

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CHAPTER 1

1. Introduction

The use of plant based products for nutrition and medicinal purposes has increased worldwide (Ogundola *et al.*, 2018). This follows wide research based awareness which reveals that plants offer nutrients which are essential for optimal health and they have no potential side effects when used as medicine (Tuso *et al*., 2013). Therefore due to the increased use of plants, studies centered on the cultivation and improvement of plants have increased. Significant attention has been directed to plants with abundant nutrients, which are easy to cultivate and can tolerate different climatic and soil conditions (Bita and Gerats, 2013). *Moringa oleifera* (*M. oleifera*) is an herbal plant fitting the above description (Gopalakrishnan *et al*., 2016).

Moringa oleifera L. also commonly known as horseradish, miracle tree or drumstick, is a soft stem indigenous and cultivated plant which belongs to the monogeneric family Moringaceae under the Brassicales order. Its nativity can be traced back to the Himalayan Mountains of India, Pakistan and Nepal (Parrotta, 2004). With vast presence in African countries such as South Africa, Zambia, Namibia, Angola, Kenya, Ethiopia, Egypt, Madagascar and in Afghanistan (Konmy *et al.*, 2016; Fahey, 2005) and has been introduced and cultivated in many places around the world (Sánchez-Machado *et al.*, 2010) with notable presence in tropical and subtropical areas. Notable the widely known and researched on species of the genus Moringa (Castro-López *et al*., 2017).

The cultivation of *M. oleifera* worldwide is established by that it is drought and mild frost resistant. It is also fast growing and is tolerant to a wide range of climatic and soil conditions (sand to clayey soils) (Tshabalala *et al*., 2019; Gopalakrishnan *et al*., 2016; Shih *et al*., 2011), though optimal growth can be achieved when grown in loam to clay loam soils of pH 5-9 (Adebayo *et al.*, 2011). The *M. oleifera* tree can be cultivated from both seeds and cuttings, with the former method being the most preferred (Mubvuma *et al.*, 2016). It has an average height and trunk diameter of 5-12 m and 45 cm respectively (Tufarelli, 2019; Padayachee and Baijnath, 2012). Seed germination and

seedling growth are promoted by temperatures of 20/30ºC, and the tree can produce fruits after 6-12 months from the planting date (Muhl, 2009).

Figure 1: *Moringa oleifera* a) shrub; b) pods and c) leaves

Moringa oleifera is a well sort after multipurpose plant with notable economic importance, as all the plant parts (flowers, seeds, pods, leaves, bark, twigs) (figure) are useful as medicine, nutritious vegetable, spice, forage, nectar for bees and cosmetics (Tufarelli, 2019; Kleimanet *et al*., 2008). Figure 1 shows *M. oleifera* leafy shrub, b) pods and c) flowers.

It has been extensively used as a nutraceutical (Khattak *et al*., 2020). Industrially, *M. oleifera* seeds are used as an effective coagulant in water purification (Adline, 2014), animal feed, green fertiliser and pesticide (Abd El-Hack *et al.*, 2018). Due to its highly nutritive content (contains higher than usual levels of carbohydrates, vitamins, proteins and minerals in comparison with other fruits and vegetables) and its medicinal properties, it is the most researched and studied specie among the 13 species of the

genus (Ranie *et al*., 2018) and it is consumed by a significant population in South Africa and the world at large as a dietary supplement (Pakade *et al.*, 2013).

Moringa oleifera leaves are the widely utilised part of the plant, being an above range storehouse of Vitamin C and A, more than 10 times higher than found in oranges and carrots respectively (Barminas *et al.*, 1998). It also contains protein which surpasses that in yoghurt, potassium 15 times higher than banana and iron 25 times higher than the level found in spinach (Rockwood *et al.,* 2013) and essential amino acids (Mahmood *et al*, 2010). The leaves are also a good source of vitamins such as C, B, D ,E, nicotinic acid and pyridoxine (Siddhuraju and Becker, 2003) and minerals including copper (Cu), zinc (Zn), iron (Fe), magnesium (Mg) and potassium (K) (Aslam *et al*., 2005). In addition, *M. oleifera* leaves are rich in phytochemicals like sterols, flavonoids, tannins, saponins, alkaloids, terpenoids, anthraquinones as well as anti-cancer agents namely glycerol-1-9-octadecanote, glucosinolates, isothiocyanates and glycoside compound (Leone *et al*., 2015; Sholapur and Patil, 2013).

The nutrient content of *M. oleifera* leaves makes them a rich nutraceutical which is widely used in vulnerable African communities in countries like South Africa, to boost the immune system (Horn *et al*., 2022) and treat malnutrition in babies and also improve the quality and quantity of breast milk in lactating mothers in Uganda, Pakistan and Senegal to mention but a few (Kasolo *et al.*, 2010; Mahmood *et al.,* 2010; Sambou, 2001). Use of *M oleifera* leaf powder to treat malnutrition is vastly promoted by national health institutions in countries like Benin and Senegal (Kasolo *et al.*, 2010). *Moringa oleifera* leaves are widely studied for their antioxidant levels which were discovered to be higher than in vegetables such as spinach, cabbage and lettuce (González-Romero *et al*., 2020). The leaves are thus used to treat degenerative and or chronic diseases like hypertension, inflammation, coronary heart disease, diabetes, cancer (Vergara-Jimenez *et al*., 2017). The Moringa leaf extract of the plant is also used as a plant growth promoter to stimulate vegetative growth of vegetables and field crops (Tufarelli, 2019).

Vitamins are unquestionably required for the proper function of the human body. However, humans lack the ability to synthesize these vitamins naturally thus a balanced plant based dietary vitamin intake is absolutely necessary (Fitzpatrick *et al.*,

2012). As earlier stated *M. oleifera* contains most essential vitamins which are crucial for human health. As minimal as 20g dried *M. oleifera* leaf supplies the daily vitamin A and C requirement for a child (Fuglie, 2000) and 70g per day has been recommended for adults (Asiedu-Gyekye et al., 2014). Medical research has revealed that insulin production is potentially increased by consuming foods containing Vitamin C, B1, B2 and B12 (Dhakar *et al.*, 2014). *Moringa oleifera* leaves are an integral ingredient African and Indian dishes to make salad, soups, spices and also as cooked vegetables (Coppin *et al.*, 2013; Arabshahi *et al*, 2007).

Although the presence of *M. oleifera* products has significantly increased in South African markets its production is still minimal and unstable (Mabapa *et al.*, 2017). However there has been a steady growth in production in the past couple of years, with notable cultivation ranging from small plots around the farmers' homesteads to big farms set up for commercial production (Mabapa *et al.*, 2017). Research targeting the improvement of *M. oleifera* quality and its utilization is gradually increasing in South Africa as the population becomes more and more aware of its usefulness (Pakade *et al.*, 2013).

Moringa oleifera products namely the leaf powder, oil and Moringa fortified drinks are amongst the most sought nutritious foods and herbal medicine whose trade is steadily gaining momentum globally (Thurber and Fahey, 2009). In order to meet the demands of *M. oleifera* there is need for intensive cultivation and moreover, knowledge of factors affecting the nutrient composition of *M. oleifera* is critically required for the improvement of both yield and nutrient content. Researchers in West Africa discovered that the nutritional content of *M. oleifera* varies based on geographical location (William *et al.*, 2014., Iqbal and Bhanger, 2006) also discovered that *M. oleifera* antioxidant activity varies with temperature with high temperatures decreasing the antioxidant activity by half (Babagana *et al*., 2018). The soil as a growth medium is also one of the critical factors determining the vigour and nutrient content of the plant and thus improvement of the soil structure and nutrient composition cannot be ignored. Organic and inorganic fertilisers mainly chicken manure and NPK respectively have been used to provide nutrients which are essential for optimal plant growth, they have been used worldwide to improve growth and nutritional quality of *M. oleifera* (Sarwar *et al*., 2018; 2019).

Organic farming has been on the rise worldwide and has attracted the interest of researchers, producers and consumers (Singh *et al*., 2013). It employs natural, organic based inputs to enhance plant growth and performance. It ensures the sustainability of the natural soil and the rightful balance of useful soil microorganisms. In addition it excludes the use of artificial fertilizers, pesticides and growth accelerators (Raey, 2018). Organically grown plant products are of a higher quality (contain high concentrations of antioxidants, minerals and high mass of dry matter), and are free from any artificial chemicals residues such as pesticides compared to conventionally grown plants (Györéné et *al.*, 2006; Winter and Davis, 2006). They are thus more expensive when compared to those produced conventionally thus making it a better option economically for farmers. Organic cultivation has improved the growth, morpho-physiological traits and biochemical properties of medicinal plants (Raey, 2018).

The current study focused on the effect of different levels of organic plant-based compost on the secondary metabolites, macro and micro elements and water soluble vitamins content in *M. oleifera* were investigated under greenhouse conditions. To date compost amendments in the form of manure and other forms of organic soil amendments have been used in the cultivation of *M. oleifera* (Adebayo *et al*., 2017) Organically grown *M. oleifera* in Nigeria performed better vegetative and nutritional compared to the control which had nothing added to it (Adebayo et al. 2017). Therefore use of sustainable methods of cultivation such as the application of organic fertilisers cannot be overemphasized.

There are however, no known studies to date which have been conducted to test the effect of organic plant-based compost on the nutrient composition of *M. oleifera* leaves grown in South African clay soils. Furthermore, information on the improvement of clay soil using plant based compost is scarce. Therefore this study is novel because for the first time plant based organic compost will be used to improve the nutrient content of *M. oleifera* looking not only at one nutrient aspect but various aspects of the plant's chemical composition which form the basis of its nutrient content. The study will look into the in-depth interaction of plant-based organic compost with compost in enabling the *M. oleifera* plants to accumulate and synthesize key nutrients which make it marketable as a high quality herbal plant. The results from the high tech analysis of

the plant biomass using key analytical tools are to assist organic *M. oleifera* farmers who usually do not have a lot of options of soil amendments with the benefits of using organic plant based compost.

CHAPTER 2

2. Literature Review

2.1 *Moringa oleifera* and its cultivation

Cultivation of *M. oleifera* has improved across the world due to increased utilization however, intensive cultivated of this plant has been hindered by insufficient knowledge of certain growth factors such as its germplasm, genetic improvement, nutrient requirements (Gadzirayi *et al*., 2013). In South Africa production is still mainly on a small scale done by farmers in small plots around their homesteads mainly for family consumption (Mabapa *et al.*, 2017). However, increase in research work on the nutritional content, utilization and the factors affecting the growth of *M. oleifera* has resulted increased production of the plant (Pakade *et al.*, 2013).

2.2 *Moringa oleifera* as a medicinal and nutritional plant

Moringa oleifera is taken in as a herbal medicine because of its anti-inflammatory, anticancer, antihelmintic and antiurolithiatic properties, it also helps to cure cardiovascular ailments (Farooq *et al.*, 2012). Literally all the plant parts have been used to treat various ailments but its use has been under-recognized for a while (Coppin *et al.*, 2013). However, of late *M. oleifera* products are finding their way into modern medicine (Anwar *et al.*, 2017). *M. oleifera* seeds are especially higher in antimicrobial activity (Seleshe and Kang, 2019), in addition the plant has also been noted for its antifungal properties (Zaffer *et al.*, 2015). The plant is popular for its ability to reduce both acute and chronic inflammation, it's also useful in the treat of conditions such as asthma and arthritis (Mahdi *et al.*, 2018; Farooq *et al.*, 2012). Furthermore, *M. oleifera* is effective as antipyretic (Belangoy and Mariano, 2017), antitumor, antiulcer (Hamid *et al.*, 2015), diuretic (Tahkur *et al.*, 2016), antihypertensive, and also reduces cholesterol levels (Vergara-Jimenez *et al.*, 2017).

In addition to the medicinal properties, *M. oleifera* has significant amounts of nutrients essential for good growth and maintenance. Even though the nutrient content of *M. oleifera* varies with change in geographic location (Gopalakrishnan *et al.*, 2016; Toba *et al.*, 2010), it is generally among the "super foods" because of its high nutrient content. Its leaves are highly recommended for expectant mothers because of its high

iron content (Farooq *et al.*, 2012). In addition the leaves and the pods are also a source of minerals which are essential to human beings, these notable minerals are Carbon (C), Mg, Fe, Manganese (Mg), K, Phosphorous (P) and Cu (Kumssa *et al.*, 2017; Melesse and Berihun, 2013). Zinc which is a key component in the growth of sperm cells and the synthesis of DNA and RNA (Barminas *et al.*,1998). The nutrient content of *M. oleifera* and its healing properties are intertwined, nutrients are the enablers of the plant's healing properties for example polyunsaturated acids like linoleic acid and linolenic acid are key in the reduction of cholesterol (Leone *et al.*, 2016).

2.3 Essential compounds in *Moringa oleifera*

Moringa oleifera is densely packed with bioactive compounds which are essential for nutritional and medicinal properties of the plant, the most noteworthy being flavonoids, vitamins, phenols and antioxidants (Vergara-Jimenez *et al.*, 2017).

2.3.1 Macro and micro elements content of *Moringa oleifera* and their function

Moringa oleifera contains macro and micro elements which are essential for optimum human health (Islam *et al*., 2021). Macro elements are required in higher concentration and they include Sodium (Na), K, Calcium (Ca) and Mg. The micro elements are required in smaller concentration and they include elements like Zn, Fe, Mn, P and Cu. These minerals are involved in key functions in the body responsible for good health. Sodium's function in the body is to regulate and maintain the blood pressure, it also helps in keeping the water and electrolyte balanced in and around the body cells (Strazzullo and Leclercq, 2014). Calcium on the other hand helps in the buildup and health of bone, teeth and the muscle system, regulating fluids within cells and works hand in hand with Mg in transmitting nerve impulses (Pravina *et al.*, 2013).

Potassium is responsible for preventing cardiac arrest by lowering the blood pressure and maintaining a normal heart beat. Phosphorus is responsible for protein phosphorylation, DNA, ATP and membrane synthesis whereas Mg helps fight depression by calming the nervous system. Iron is a vital element in the human body as it facilitates the transportation of oxygen and electrons, DNA and hormone synthesis (Abbaspour *et al*., 2014). Manganese assists in the activation of enzymes (Li an Young, 2018), Zn kick starts the body' s immune defense system and is crucial

in wound healing and Cu works hand in hand with Fe in the formation of red blood cells (Cartwright *et al*, 1957).

Moringa oleifera leaves contain macro and micro minerals essential for the development and metabolism of the human body (Yaméogo *et al*., 2011; Mbailao *et al*., 2014). The abundance of these minerals is part of the reason why it is termed the 'miracle tree' based on its use as a nutraceutical (Oyeyinka and Oyeyinka, 2016). In clinical trials on iron deficiency it was discovered that taken as the only source of Fe *M. oleifera* can reverse iron deficiency (Saini *et al*., 2014). In addition to them being useful in improving human health, the macro and micro elements found in moringa make *M. oleifera* a highly effective bio-organic fertilizer (Abdalla, 2013). Mineral composition of *M. oleifera* seems to vary greatly from one plant to the other (Oyeyinka and Oyeyinka, 2016). Research outputs have indicated that this variation is highly influenced by the soil conditions (Yaméogo *et al*., 2011), biotic factors of the environment, maturity of the plant (Melo *et al*., 2013) and geographic location (Mulyaningsih *et al*., 2018).

2.3.2 Antioxidants

Antioxidants are natural or man-made "free radicals scavengers" due to the fact that they scavenge excess free radicals and inhibit oxidative damage to the cells in the body (Koppula *et al*., 2012). They are also used to preserve food from going bad by delaying the oxidative process (Shaidi, 2015). Antioxidants exist at equilibrium with free radicals in a healthy human body in the form of primary metabolites (Kurutas, 2016). However when the body is under oxidative stress, external sources of antioxidants need to be administered to the body to maintain the equilibrium, these are the secondary metabolites which are further grouped into manmade and natural occurring (Demain and Fang, 2000; Thirumurugan *et al*., 2018). Due to repeated exposure to pathological and physiochemical conditions, the human body may undergo various metabolic reactions which produce free radicals (Lobo *et al*., 2010), when the production of free radicals exceed the level required by the body, they induce oxidative damage to DNA, lipids and proteins resulting in the triggering of degenerative diseases as mentioned above (Thanan *et al.*, 2014).

Research and use of natural oxidants has increased and continues to gain popularity directly proportional to the understanding of the role of free radicals in in damaging human cells leading to degenerative diseases (Aliyu *et al*., 2019). Increased research and use of natural antioxidants is therefore due to the fact that these compounds are vital in the prevention and treatment of degenerative diseases such as Alzheimer's disease, coronary heart disease and cancer (Sridevi *et al*., 2018; Choudyhury *et al*., 2017; Mark and Retail., 1999). Natural antioxidants are preferred over synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) because of the detrimental toxic effects that synthetic antioxidants have on the human body (Lidon and Silva, 2016; Parke and Lewis, 1999).

Antioxidants exist in two forms based on their nature and mode of action, the first class being primary metabolites which are enzymatic in nature and the second non enzymatic secondary metabolites (Moharram and Youssef, 2014). Classification of antioxidants based on their mode of action, size, solubility and structure is further illustrated in Figure 2.1.

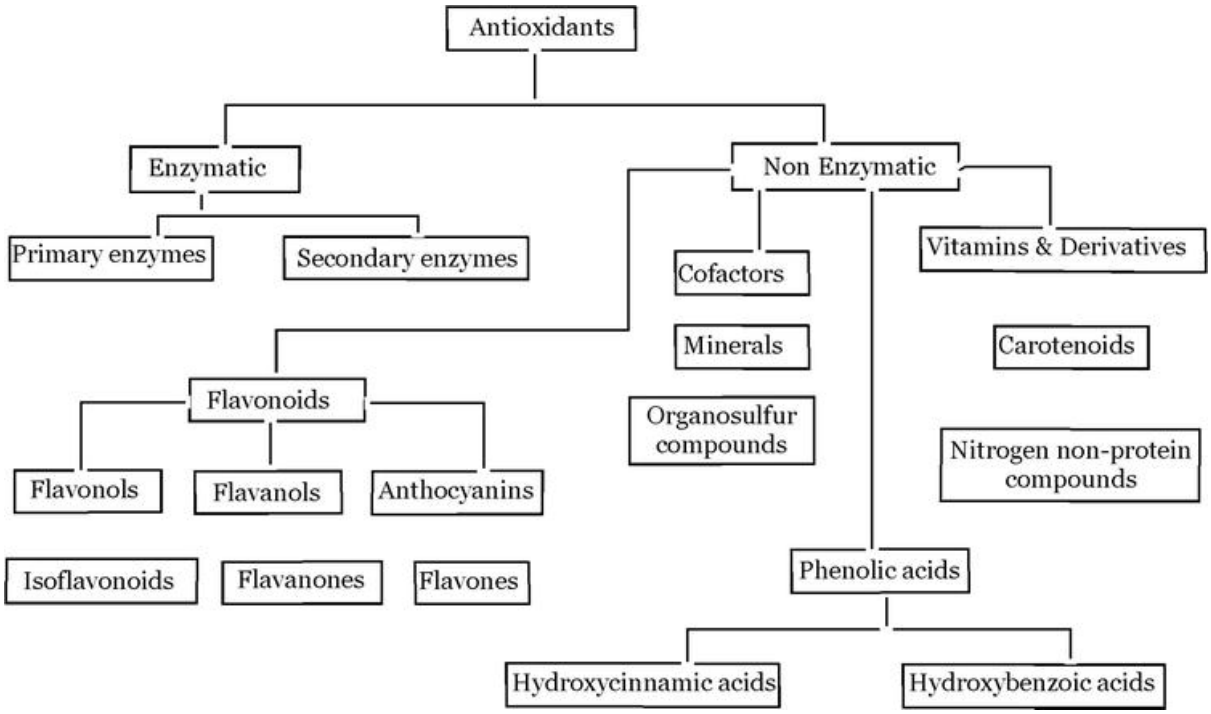

Figure 2.1: Antioxidant classification by Carocho and Ferreira (2013).

Epidemiological studies state that plants contain high levels of antioxidant compounds which are taken by humans as food or dietary supplements (Pandey and Rizvi, 2009).

A large population worldwide use plants as herbs to treat various elements due to their antioxidant levels (Mahomoodally, 2013). *Moringa oleifera* is a 'powerplant' with high levels of natural antioxidants (Adebayo *et al*., 2018), containing over 40 types including notable ones such as ascorbic acid, carotenoids, flavonoids and phenolics (Mahmood *et al.*, 2010). As a result of its high antioxidant levels it has been used in India and Philippines to preserve the shelf life of fatty foods (Iqbal and Bhanger, 2006; Siddhuraju and Becker, 2003). Falowo *et al*. (2017) also recommended the use of *M. oleifera* leaves as nutraceuticals and food preservatives (Castro-López *et al*., 2017). Antioxidant levels in *M. oleifera* are affected by external forces like temperature, being higher in winter than in warmer seasons (Shih *et al*., 2011; Iqbal and Bhanger, 2006), variety and leaf maturity (Castro-López *et al*., 2017).

2.3.3 Metabolite content of *Moringa oleifera* leaves

Secondary metabolites are phytochemicals naturally produced by plants to make them cope with changes and be competitive in their environment. These substances have an influence on the plant's own functions and on the health of other organisms (Teoh, 2016). Unlike primary metabolites which are responsible for the survival of plants by playing a key role in processes such as photosynthesis, secondary metabolites impact have a lasting effect on the survival of the plant (Raghuveer *et al*., 2015). Based on their biosynthesis secondary metabolites are divided into four major groups namely: polyphenols or phenolic compounds, nitrogen-containing alkaloids, terpenoids and sulfur containing compounds (Divekar *et al*., 2022).

Moringa oleifera is an abundant source of polyphenols such as flavonoids, phenolic acids and tannins (Ma *et al*., 2020). The content and function of these polyphenols and other phythochemicals present in *M. oleifera* are discussed further in the sub-sections below.

2.3.3.1 Flavonoids

Flavonoids are a large group of natural polyphenols varying in chemical structure and characteristics (Kumar and Pandey, 2013). Figure…. illustrates the basic flavonoid structure.

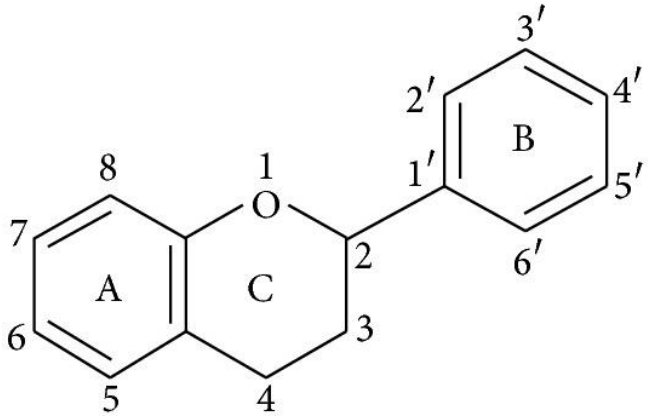

Figure 2.2: General structure of Flavonoids (Kumar and Pandey, 2013)

Flavonoids are found in a number of edible plants, human beings however are unable to naturally synthesize them (Harborne *et al.*, 2013 ;Cook and Samman, 1996). Their name is derived from the latin word 'flavus' meaning yellow (Anand David *et al*, 2016), they are responsible for pigmentation. More than 3000 flavanoids have been found and they are classified according to their chemical structure into 6 groups namely flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Panche *et al*., 2016). Plant based biomolecules such as antioxidants have gained significant attention recently, however, there are still some gaps to be filled with more information on their accumulation in plants and their role in disease prevention and treatment (Sharifi-Rad *et al*., 2020). Even though their function is not known in totality, results have revealed that they have unquestionable health benefits. Flavonoids found in plants such as *M. oleifera* are responsible for reducing oxidative stress and fight degenerative diseases (Kou *et al.*, 2018). Several medical research outputs have revealed that diets rich in flavonoids and other antioxidants have been effective in the protection and treatment of atherosclerosis and related diseases such as coronary heart disease, Alzhemier's disease and certain cancers (Lako *et al*., 2007; Mahungu *et al*., 1999). This is due to the anti-inflammatory, antiatherogentic and lipid lowering activity of the flavanoids (Salvamani *et al.*, 2014).

A comparative study on flavonoids content between *M. oleifera* and another Moringa species *Moringa ovalifolia* revealed that *M. oleifera* has more flavonoids in number and also have flavonoids with broad pharmacological range compared to the other species (Makita *et al.*, 2016). A total of 14 flavonoids were extracted from *M. oleifera* plant leaves harvested from South Africa and Namibia (Makita *et al.*, 2016) whilst 12

were discovered from plant leaves from Sub- Saharan Africa (Coppin *et al.*, 2013). The difference in these values could be a result of different environmental conditions. The flavonoids found in *M. oleifera* have been linked to increased death of cancer cells preventing damage of normal cell DNA leading to better chances of fighting the disease (Lin *et al.*, 2018). *M. oleifera* has high levels of important antioxidant flavonoids such as quercetin and kaempferol which have exhibited a higher performance as antioxidant when compared to conventional vitamins (Coppin *et al.*, 2013). Quercetin is a flavanol that has been extensively used in protecting and repairing body cells damaged by drug induced oxidative stress and toxicity (Anand David *et al.*, 2016).

2.3.3.2 Phenolic acids

Phenolic acids are a major class of phenolic compounds derived from benzoic and cinnamic acid, naturally present and widely distributed in plants (Leone *et al*., 2015). They are one of the most researched and popular plant derived antioxidants because of their potential health benefits (Leone *et al*., 2015; Sultana *et al.*, 2009). They are largely found in fruits and vegetables and numerous researches have revealed their presence in *M. oleifera* leaves (Konmy *et al*, 2016; Leone *et al*., 2015).

Use of *M. oleifera* as a medicinal plant has been largely attributed to the presence of phenolic acids and other phytochemicals (Saini *et al*., 2016a). Phenolic acids are abundantly found in different parts of the plant (Coppin *et al.*, 2013), together with flavonoids they are major antioxidants found in *M. oleifera* leaves (Leone *et al*., 2015). The phenolic extracts have been identified as potential treatment for erectile dysfunction (Oboh *et al*., 2015) and other degenerative diseases such as diabetes ,arthritis (Xu *et al*, 2019; Konmy *et a*l., 2016). Studies on the effect of season on the phenolic levels in *M. oleifera* revealed that total phenolics are high in winter (Shih *et al*., 2011; Bhanger, 2006). Other factors influencing phenolic acids content in *M. oleifera* leaves include plant genetics, agro-climatic location (Bhanger, 2006), extraction method (Rocchetti *et al*., 2020), age of the leaf and the drying method (Leone *et al*., 2015).

2.3.3.3 Glucosinolates

Moringa species has a high level of glucosinolates which is predominantly present in the Brassicaceae family (del Carmen Martínez-Ballesta *et al*., 2013). Glucosinolates fall under the sulfur containing group of the secondary metabolites (Divekar *et al*., 2022). These compounds are highly present in young leaves and are highly affected by environmental conditions (del Carmen Martínez-Ballesta *et al*., 2013). Glucosinolates have been associated with the detoxification of carcinogenic chemicals in animals (Johnson, 2002). In addition it has been noted to have anti-inflammatory, anti-fungal and anti-microbial properties (Melrose, 2019).

The most common glucosinolates in *M. oleifera* are glucomoringin and isothiocyanate moringin (Fahey *et al*., 2019; Abd Rani *et al*., 2018), these two are popularly known for their anti-inflammatory and antioxidant activity (Fahey *et al*., 2019). Glucosinolates rich *M. oleifera* extract significantly reduced human colon cancer cells (Cuellar-Núñez *et al*., 2020). The environmental influence of glucosinolates in *M. oleifera* was observed in the variation of these phytochemicals in wild and cultivated plants (Chodur *et al*., 2018). Higher germination temperatures also increased the concentration of glucosinolates in *M. oleifera* (Coello *et al*., 2020).

2.3.4 Vitamin content of *Moringa oleifera* and their function

Vitamins together with amino acids, organic acids and enzymes fall under the primary metabolites (Singh *et al*., 2017). *Moringa oleifera* is a treasure store of vitamins, it contains both water and fat soluble vitamins. The fat soluble vitamins are stored in fat upon absorption in the body and thus stay longer however water soluble vitamins are soluble in nature and are easily washed out during food preparation or through the excretory system upon entering the human system (Lykstad and Sharma, 2019). These vitamins are found in high concentration in the leaves of this species with notable presence of B vitamins such as folic acid, vitamin A as beta-carotene, vitamins K and C (Abbas *et al*., 2018). Vitamin C is in higher quantity per equal weight compared with most citrus fruits and it was found to be of higher content when the plants are harvested in dry winter than in summer rainy season (Yang *et al.*, 2006). Higher concentrations of vitamin C in winter is linked to its ability to fight colds and flu in human beings (Mahmood *et al*., 2010). In addition Vitamin C enhances the absorption of iron (Richardson and Lane, 2014). Vitamin Bs on the other hand are

water soluble and are vital in the proper function of the mitochondria (Depeint *et al.*, 2006). Vitamin K, a fat soluble vitamin is key element in the regulation of calcification, thus is essential to both infants and adults for good bone strength (Shearer *et al*., 2012).

2.3.4.1 Water soluble vitamins

Water soluble vitamins as the name suggests are vitamins which are soluble in water. These vitamins are naturally not synthesized by the human body and thus need to be taken in through the diet or as supplements. A constant supply of the water soluble vitamins is essential as they easily lost through urination and food preparation (Clifford and Curley, 2020). They comprise Vitamin C and Vitamin B complex which encampases all the B vitamins such as Thiamine (B1), Riboflavin (B2), Niacin (B3), Pantothenic acid (B5), Pyridoxine (B6), Biotin (B7), Folate (B9) and Cobalamin (B12) (Lykstad and Sharma, 2019). High levels of B vitamins (B1, B2, B3, B5, B6, B7 and B9) have been recorded in *M. oleifera* leaves, which increases the nutritive value of the plant (Abbas *et al*., 2018). Water soluble B vitamins are the most abundant form of vitamins found in *M. oleifera* (Peñalver *et al*., 2022). Testing of *M. oleifera* seeds revealed high levels of vitamin B2 (Coello *et al*., 2020). The leaves are also abundant in vitamins B3, B6 and B9 (Mbikay, 2012). Consumption of *M. oleifera* could therefore help supply and relieve water soluble deficiencies in the body (Peñalver *et al*., 2022).

The chemical structure of water-soluble vitamins affects their distribution in different foods and is also highly essential in their analysis and quantification (Said and Nexo, 2018). This is due to the fact that the chemical structure of vitamins influences their solubility and boiling point.

Vitamin B1

Vitamin B1 also known as Thiamine ($C_{12}H_{17}N_4OS^+$) (Figure 2.3) is composed of a pyrimidine and thiazole rings connected by a carbon bond (Jurgeson *et al*., 2009). It is usually supplied as a chloride salt (Thiamine hydrochloride). Thiamine hydrochloride is highly soluble in water and quite stable and not easily oxidized in slightly acidic conditions of pH 5 and below. Thiamine naturally occurs in food in small amounts with its concentration in most vegetables being less than 0.1 mg/100 g (Gupta *et al*., 2005). Upon entrance to the human body, it is absorbed by the small intestine, a lot of it is lost through excretion. However, a remainder of it is stored in the liver and one of its

major functions are in carbohydrate metabolism where it facilitates the breakdown of carbohydrates into energy (Frank, 2015). Deficiencies of vitamin B1 in the body lead to a disease known as beriberi and mental confusion among other symptoms (Polegato *et al*., 2019).

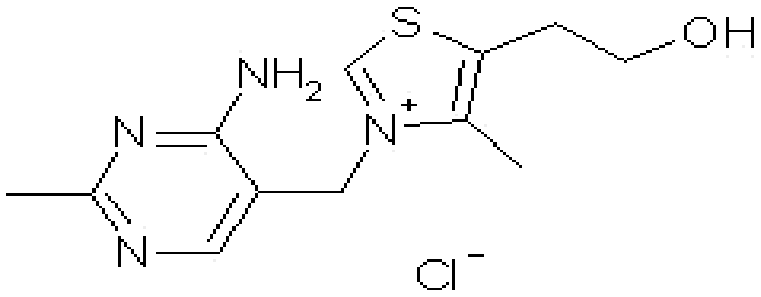

Figure 2.3: Chemical structure of Vitamin B1.

Vitamin B2

Vitamin B2 or Riboflavin ($C_{17}H_{20}N_4O_6$) (Fig 2.4) is a chemical compound consisting of an isoalloxazine molecule conjugated to a sugar, alcohol and ribityl (Northrop-Clewes and Thurnham, 2012). Although classified as a water-soluble vitamin, Vitamin B2 is partially soluble in water and highly soluble in acidic or alkaline conditions. It is a yellow substance involved in many metabolic pathways in the body. Absorbed by the small and stored it is chiefly stored in the liver and kidneys. It facilitates the conversion of amino acid tryptophan into niacin. Riboflavin has antioxidant properties, it is a key component in maintaining the levels of the molecule glutathione protecting the body from free radicals. It also essential in the normal developmental processes involving reproduction and lactation (Mahabadi, 2022). Vitamin B2 deficiencies lead to skin disorders and reproductive problems (Clifford and Curley, 2020).

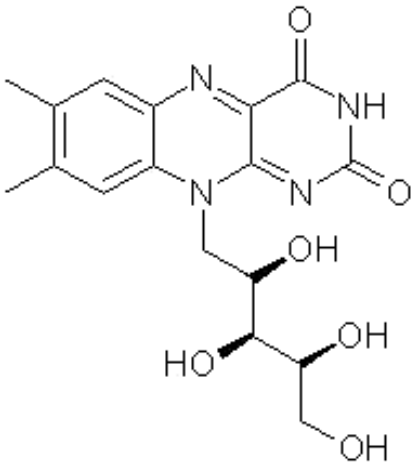

Figure 2.4: Chemical structure of Vitamin B2.

Vitamin B3

Niacin ($C_6NH_5O_2$) which is Vitamin B3 (Figure 2.5) is made up of two related compounds namely Niacin (A) and its amide, Niacinamide (B). Niacin is the common name used for both forms of Vitamin B3. Niacin acts as precursor for the nucleotide in two coenzymes Nicotinamide Adenine Dinucleotide (NAD+ and NADP+), it facilitates the transportation of hydrogen in intermediary metabolism. It is chiefly involved in critical cellular functions by being a co-enzyme for most enzymes, it is also involved in the production of energy (Clifford and Curley, 2020). The deficiency of this vitamin leads to a disease known as pellagra which involves diarrhea and dementia (Redzic and Gupta, 2020).

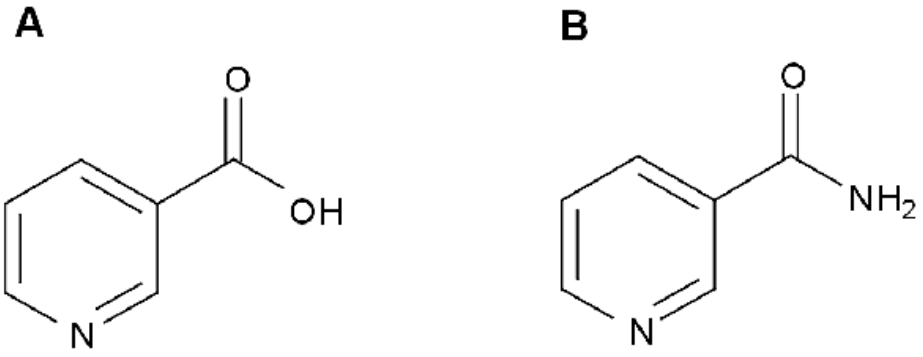

Figure 2.5: Chemical structure of Vitamin B3.

Vitamin B6

Vitamin B6 also known as Pyridoxine ($C_8H_{11}NO_3$) (Figure 2.6) it is also found in different forms such as pyridoxal, and pyridoxamine. All the forms of Vitamin B6 are

stable in acidic conditions. It acts as a coenzyme for a number of enzymes. Together with Vitamin B2, Vitamin B6 are cofactors in the synthesis of tryptophan essential for the synthesis of Vitamin B3. Vitamin B6 works in association with B9 and B12 and its deficiency leads to reduced concentrations of these vitamins. It plays a major role in the formation of red blood cells and also acts as an antioxidant (Clifford and Curley, 2020).

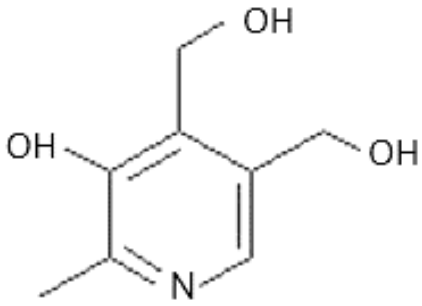

Figure 2.6: Chemical structure of Vitamin B6.

Vitamin B9

Folic acid ($C_{19}H_{19}N_7O_6$) also known as Vitamin B9 (Figure 2.7) is composed of the pterin ring, joined to para-aminobenzoic acid (PABA), which is then joined to a glutamic acid via a peptide bond (Ly *et al*., 2012). In the body folic acid is transformed into tetrahydrofolic acid (THFA) in the tissues (Arsic *et al*., 2016). Folic and or folate together with vitamin B6 and B12 is responsible for the synthesis of nucleotides (purine and pryrimidine) bases, it is also involved in the synthesis, methylation, stability and repair of the DNA (Szczuko *et al*., 2018; Salini *et al*., 2016). Folate is essential in the formation of red blood cells and is highly needed by pregnant and lactacting mothers, its deficiency leads to anemia. The bioavailability of folate is high in *M. oleifera*, making the plant a good source of vitamin B9 in the body (Salini *et al*., 2016).

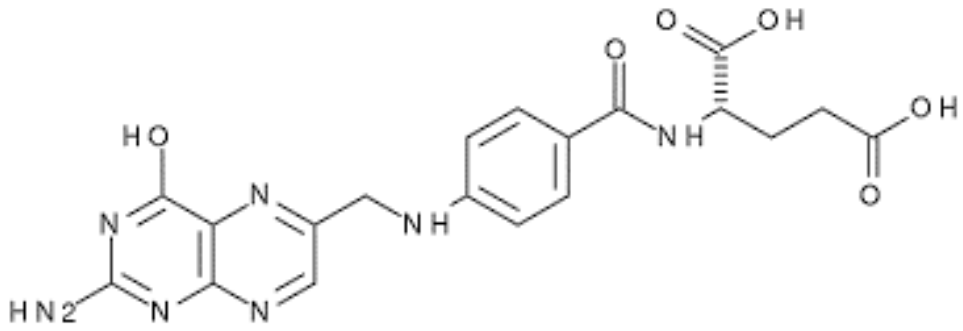

Figure 2.7: Chemical structure of Vitamin B9.

2.4 Factors affecting the nutrient content of *Moringa oleifera*

As already alluded afore chemical and or nutrient constituents of *M. oleifera* are influenced by various factors such as the growth environment, genetic makeup, harvesting techniques and post-harvest handling (Yang *et al.*, 2006).

2.4.1 Climatic and geographic location

Change of climatic and geographic location of a place has an impact on the growth and chemical composition of plants because of varying conditions of rainfall, humidity, daylight and temperature. A study investigating the effect of planting location on the nutrient content of *M. oleifera* leaves revealed noteworthy changes in the nutrient content as the location changes (Agunbiade *et al*., 2017). Asante *et al*., (2014) also reported major differences in the nutrition content of *M. oleifera* leaves grown in different agro-ecological regions with different climates, the plants grown in a region with higher temperatures and low rainfall had lower nutrients in comparison to those grown in a high rainfall, colder area. Similar results were obtained from *M. oleifera* plants grown in 2 different continents (Africa and Asia) (Gopalakrishnan *et al*., 2016).

2.4.2 Plant maturity

Plant physiological age is one of the key factors influencing its chemical composition (Bates 1971). Results from a trial investigating the effect of leaf age on the nutritional and or chemical composition of *M oleifera* revealed that these properties significantly change in each leaf stage (Yang *et al*., 2006). Sreelatha and Padma, (2009) also reported nutrition and chemical variation including antioxidant activity between you young and mature *M. oleifera* leaves. Mature leaves have higher nutrient content than

younger leaves although the later are more edible and thus preferred as a vegetable (Price, 2007). However, in the work done by Ndubuaku *et al*., (2015) younger succulent *M oleifera* leaves had higher vitamins content compared to the older ones.

2.4.3 Genetic and environmental factors

The genetic makeup of the plant has an impact content on the accumulation of nutrients, it's also noted that different accessions of *M. oleifera* have exhibited different chemical compositions (Leone *et al.*, 2015). In addition, harvesting time, techniques, post-harvest handling and food preparation have an impact on the nutrient content of *M. oleifera* (Sangeetha *et al.*, 2017). Planting location factors also give rise to chemical composition variations within the *M. oleifera* species (Gopalakrishnan *et al.*, 2016). Studies done by other researchers have also showed that harvesting season affected the nutrient content of *M. oleifera* (Shih *et al.*, 2011; Yang *et al.*, 2006). A study done in Nigeria on the nutrient content of *M. oleifera* grown in four different locations with variations in soil factors revealed that soil type affect the nutrient content of the plant (Lamidi *et al.*, 2017). Thus altering the soil nutrients through the addition of organic fertilizers undoubtedly influences the nutrient content and chemical composition of the plant.

2.4.3.1 Organic farming

The drive to have future sustainable futures have lead researchers around the world to focus on the environmental benefits of organic soil amendments (Okazaki *et al*., 2012). Mineral fertilizers are regarded as one of the best sources in improving plant nutrition but organic fertilizers are a preferred safer source (Rady *et al.*, 2016). Extensive application of artificial fertilizers and other anthropogenic activities associated with urbanization and industrialization are major sources of heavy metal soil contamination (Zhang *et al.*, 2010). Presence of heavy metals and metalloids in agricultural soils is a major constraint to sustainable agriculture (Manzoor *et al.*, 2018). Heavy metals turn out to be persistence in the soil as they are neither bio nor chemical degradable leading to toxic levels. Heavy metal contamination of plants eventually enter the human and animal system through the food chain. Their presence in toxic level in the human body may lead to kidney failure, disability in children, vision disturbances , depression amongst many defects (Jan *et al.*, 2015). *Moringa oleifera* has also been susceptible to heavy metal contamination. This has lead producers to

try safer ways of production which avoid toxicity but improve quality and amount of produce. Organically grown *M. oleifera* in Nigeria performed better vegetative and nutritional compare to the control which had nothing added to it (Adebayo *et al*., 2017). Therefore use of sustainable methods of cultivation such as the application of compost cannot be overemphasized.

2.4.3.2 Composting

Compost is one of the organic fertilizers which support sustainable agriculture improving the physiochemical composition, organic matter content and nutrient content of the soil (Adugna, 2016). This leads to improvement of plant production and nutrient content. Application of compost helps to produce “safe and green” and at the same time meet the desired expectations of the produce (Paulin and Peter, 2008). In a research done in Korea on the effect of compost and NPK on the growth of *M. oleifera*, plants grown under the treatment of compost showed higher levels of phenolics and flavonoids content when compared with those under the NPK treatment (Sarwar *et al.*, 2019). Haouvang *et al*., (2019) also recommended the use of compost improve the vigor and chemical composition of *M. oleifera* seedlings, the same was reported by Dania *et al*., (2014). Health soils lead to healthy plants and ultimately healthier human beings (Balfour, 1950).

2.5 Types of Compost

2.5.1 Animal based compost

Organic fertilizers are a safer good source of plant nutrients, however there are still some constraints in their use. Previous research has revealed that organic fertilizers based on animal waste pose a health risk to the environment and plants due to microbial contamination (Sorensen and Thorup-kristensen, 2010). Foodborne pathogenic bacteria like *Salmonella* and *Eschericia coli* spp were discovered in agricultural soils and in vegetables grown organically and fertilized by animal manure, some strains of this bacteria can cause severe effects to the human body such as vomiting and diarrhea (Machado *et al.*, 2006; You *et al.*, 2006). In addition to microbial contamination, compost obtained from sewage sludge and livestock excretion can contain traces of heavy metal contamination (Liu *et al*., 2006).

2.5.2 Plant based compost

Compost obtained from plant based material then becomes an even safer fertilizer in organic farming because it is not only free from heavy metals but fecal coliforms also. Plant-based compost also suppress plant diseases caused by pathogenic formae speciales of *Fusarium oxysporum* (Yogev *et al.*, 2006). Compost is not only safe to the environment, it is used in soil amendment programs to reduce heavy metal contamination (Angelova *et al.*, 2010). The use of compost for soil remedial is increasingly attracting attention because it is cost effective (Gul *et al.*, 2015). It contains elements like humus, microbes and mineral ions, these react with the heavy metals by forming insoluble bonds with and thus make them immobile and unavailable to plants (Belyuchenko, 2016). Angelova *et al.,* (2010) investigated the use of compost to detoxify agricultural soils and discovered that its application resulted in the reduction of heavy metals like Cd and Zn content in potato tubers, it also increased plant growth, development and quality of potatoes (Angelova *et al.*, 2010) Compost amendment also reduced the concentration of Lead (Pb), Zinc (Zn), Fe and Mn in *Chenopodium album L* (Walker *et al*., 2004).

2.6 Effect of composting on the soil

Compost is preferred to other fertilizers because it contains both nutrients and organic matter, it is useful in the reintegration of organic matter lost due to intensive farming (Petruzzelli, 1996). Organic matter is essential in the soil as it positively alters soil properties (biological, chemical and physical properties) and mineral deposition. It facilitates the availability of macro and micronutrients to the plant through the process of mineralization (Griffin *et al*., 2007) which is essential for plant growth (Galbiatti *et al*., 2012). In South African soil a decline in soil organic matter directly impacts the degradation of soils negatively affecting the physiochemical and biological properties of the soil (Barnard and Du Preeze, 2004). Application of organic matter further binds carbon to the soil and thus reducing the amount released to the air to form carbon dioxide and increase the greenhouse effect (Van der Merwe *et al.*, 2000). Humus released when organic matter decomposes completely is essential in making cations available to plants, being negatively charged it forms a bond with the positively charged ions. Organic matter is also a hub of microorganism which help in nitrogen fixation and aeration of the soil, it also helps in water retention, and in the formation of soil aggregates thereby increasing aeration.

2.6.1 Effects of composting on plant biomass

Yield reduction has been observed in plants grown in soils with limited organic matter (Madeleine *et al*., 2005). Application of compost in pre sowing soil increased the biomass of wheat and tomato plants (Ngo and Cavagnaro, 2018). Studies on effect on compost on the biomass of Rye grass also revealed that addition of compost increases biomass, fresh compost had a higher effect than mature compost (Keeling *et al*., 1995). Compost amendments on the soil increased growth and yield in in lettuce (Trupiano *et al*., 2017) and wheat plants (Duong, 2013). Vegetative growth of *M. oleifera* seedlings increased when grown in soil amended with compost in comparison with seedlings grown under the control (Sarwar *et al*., 2018; Haouvang *et al*., 2017).

2.6.2 Effects of composting on macro, micro and trace elements in the soil and plant

Moringa oleifera like any other plant requires mineral nutrients for physiochemical processes in the plants (Ai *et al*, 2017), these minerals also have an impact on the accumulation of nutrients in the plant. They are grouped into two main groups according to their requirements namely macro (required in large quantities) and micro (required in small quantities) nutrients (Morgan and Connolly, 2013). Knowledge of the factors affecting the bioavailability and the uptake of the mineral nutrients is essential in order to improve the accumulation of the nutrients in the leaves.

Organic compost has been used in soil amendment programs in order to influence the bioavailability of heavy metal and mineral nutrients. Research outputs have revealed that organic compost has been useful in treating heavy metals contaminated agricultural soils where by reducing the bioavailability of heavy metals (Gul *et al.*, 2016). The addition of compost in the soil increased the plant available P, N levels were also increased which in turn increased NO_3^- ions through the nitrification process (Ngo and Cavagnaro, 2018; Keeling *et al*., 1995). In addition to increasing P and N levels, addition of compost also improves Carbon (C), Nitrogen (N), pH and cation exchange capacity (CEC) levels in the soil (Premamali *et al*., 2019; Trupiano *et al*., 2017). The addition of compost yielded better results in in improving the soil nutrients in comparison with some inorganic fertilizers (Lok and Suarez, 2014).

2.6.3 Effects of composting on plant metabolites

Plants generally respond to the external environmental stimuli by changing their metabolome, the composition of plant metabolites changes with growth conditions variation (Commisso *et al*., 2013). Compost significantly alters the soil physical and chemical properties, mineral absorption by the plant and eventually impacts the level of phytochemical accumulation in the plant. Organically grown medical plants had increased effectiveness as medicine in comparison with organically grown plants (Trisilawati *et al*., 2019). In an experiment conducted on the vegetable pak choi (*Brassica rapa* cv. *chinensis*), compost extracts increased antioxidant levels in the vegetable (Mohd Din *et al*., 2017). Total phenolics and flavonoids were also more enhanced in Kacip Fatimah (*Labisia pumila* Benth) grown under organic fertilizer in comparison with the one grown under inorganic fertilizers (Ibrahim *et al*., 2013). Similar results were obtained in *M. oleifera* where addition of compost to the soil increased the antioxidant levels and antioxidant activity of the plant (Sarwar *et al*., 2019, 2017; Gopalakrishnan *et al*., 2016). Dania *et al.*, (2014) also reported that addition of compost in the soil resulted in an increase in the concentration of nutritive components in *M. oleifera* seedlings. Macro and micro elements of *M. oleifera* were also increased in by the addition of compost to the soil (Gad *et al*., 2019). Total flavonoids and total phenolic activity in *M. oleifera* leaves and seed also increased with addition of compost to the soil (Attia *et al*., 2014). The chemical composition of *M. oleifera* bioactive compounds is largely influenced by biotic and abiotic factors (Saini *et al*., 2016b). Although, the impact of organic soil amendments on plant metabolites and quality of the produce there is still limited or no documented information on the effect of plant based organic soil amendments on plant metabolites.

2.6.4 Effects of composting on plant vitamin content

There are key components of a balanced diet responsible for the proper function of the nervous system, respiratory system, production of fatty and nucleic acid and the formation of blood cells (Kennedy, 2016) their deficiency causes different ailments. Deficiency of vitamins in the body may lead to physiological challenges such as the disruption of DNA which leads to phenotypic changes (Ames and Wakimito, 2002).

Pak choi vegetables grown in soil amended with compost showed increased levels of carotenoids which help prevent vitamin A deficiency (Neugart *et al*., 2018). Vitamin C

levels of Kacip Fatimah plants increased when the plant was grown in soil amended with organic fertilizer (Ibrahim *et al*., 2013). The increase in Vitamin C content was also observed in *Capsium Chinese* grown in composted soil (Premamali *et al*., 2019), Tortosa *et al*., 2018 also reported similar findings in Pepper (*Capsicum annuum* L.). The assessment of vitamin content in *M. oleifera* grown under composted soil indicated that compost increased carotenoids and ascorbic acid levels (Gad *et al*., 2019).

2.7 Organic cultivation of *Moringa oleifera* and its impact on the local and international market

Cultivation of *M. oleifera* has been on a steady increase in the last decade attracting farmers, researchers and consumers (Mashamaite *et al*., 2021). *Moringa oleifera* production in South Africa mainly focuses on leaf powder production as the main product (Mabapa *et al*., 2017). Due to the high commercial value of *M. oleifera* products, its cultivation and marketing of the product locally and internationally potentially improves the livelihood of the local farmers (Mabapa *et al*., 2017). *Moringa oleifera* growers in Africa face challenges in marketing their products internationally as they compete with Indian and Chinese markets who offer the same products at cheaper prices. This could be solved by focusing on niche markets which focus on higher grade certified organic products (IDC, 2019). *Moringa oleifera* farmers has sighted lack of market and limited knowledge in sustainable Moringa production as some of the constraints they face (Mabapa *et al*., 2017). Therefore, improving the quality of the product through the use of organic soil amendments will broaden the market bracket of *M. oleifera* growers as they will cater for consumers seeking for chemical free, organic products.

Positive outcomes have been identified in growing *M. oleifera* organically using compost as a fertiliser. A continuous supply of organic compost has increased shoot productivity in *M. oleifera* (Amaglo *et al*., 2006). Compost in the form of manure has also been used in *M. oleifera* plantations to increase production (Palada and Chang, 2003). The use of chemical free plant organic fertilisers has however not been researched thus far in *M. oleifera* grown in South Africa (Mashamaite *et al*., 2021).

2.8 Introduction to plant metabolomics

Metabolomics is a comprehensive analytical tool focusing on the identification and quantification of endogenous small molecules commonly known as metabolites within a biological system (Zhang *et al.*, 2012). It seeks to identify and quantify a wide range of unique chemical fingerprint of small molecules (<1,000 Da) affected by physiological factors and involved in cellular reactions (Ziangchin *et al*., 2021; Roberts *et al*., 2012). It focuses on the organism's entire metabolites unlike genomics, transcriptomics, preteomics which focus on the organisms' DNA, RNA and proteins respectively (Figure 2.8). Changes in metabolites profile remains a major challenge affecting the use of herbal plants such as *M. oleifera* (Managa *et al*., 2021).

Metabolomics employs the use of techniques such as the Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS) which is usually coupled with separation techniques such as Liquid chromatography (LC) and Gas Chromatography (GC) in order to detect any changes in the plant metabolic profile (Managa *et al*., 2021).

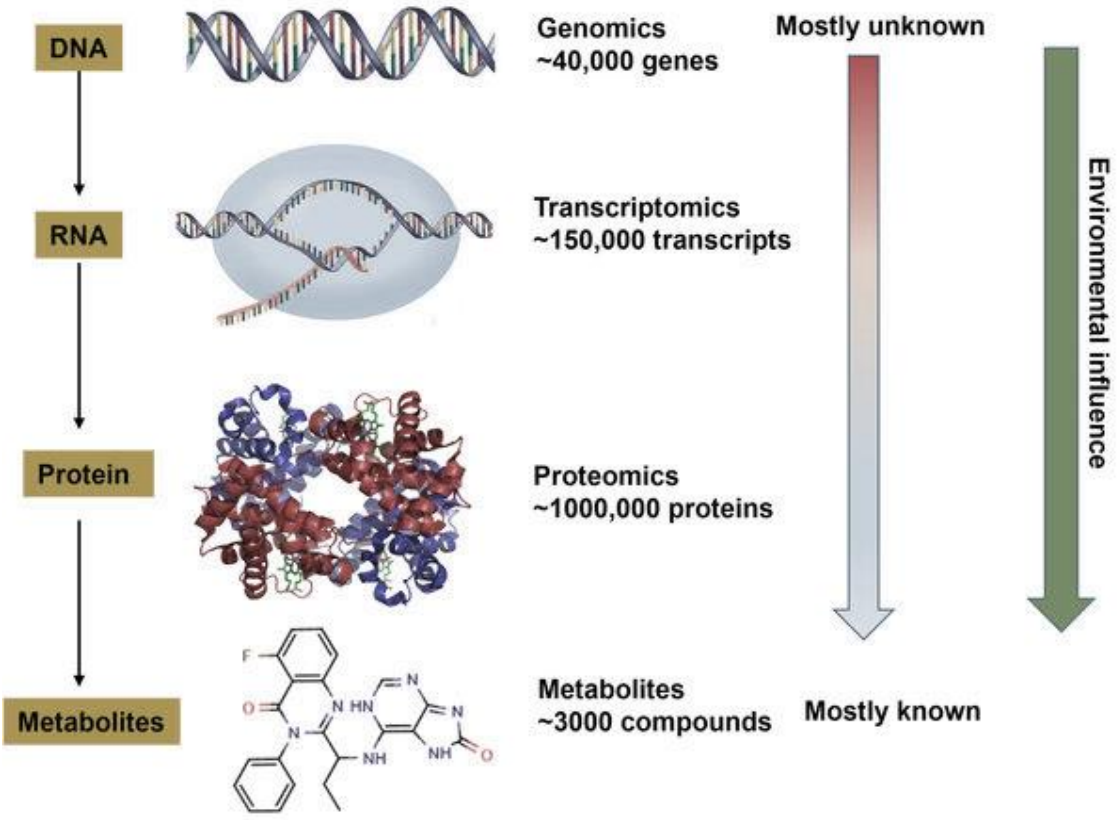

Figure 2.8: Difference between Metabolomics and other omics studies (Yu *et al*., 2017).

2.9 Chemical analysis of phytochemicals in plants

High powered analytical techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy, Liquid Chromatography Mass spectrometry (LC-MS) and Gas Chromatography Mass spectrometry (GC-MS) are used in the analysis of plant phytochemicals (Lammerhofer and Weckwerth, 2013). The combination of Mass Spectrometry (MS) and chromatography brings about a powerful tool in the analysis of metabolites. This is due to the fact that chromatography is an exceptional technique in the separation of complex molecules, it is however not capable to identify the molecular structure and composition which are then provided by the MS (Bouziani *et al*., 2021).

There are advantages and limitations to every technique used for metabolite analysis thus the use of these technique in combination is recommended (Zhang *et al*., 2012). The NMR is ideal for multidimensional experiments which give detailed structural information of compounds. The analysis is also non-destructive, thus the same sample may be re-used in another analysis (Gathungu *et al*., 2020). With the many advantages, it has however low sensitivity compared to MS and concentration of biomarkers could be below detectable limits. Mass spectrometry (LC-MS and GC-MS) is the preferred analytical technique by biologist over NMR. It is able to detect very low detection limits, furthermore the tandem mass spectrometry (MS/MS) has the ability to give structural information based on compound fragmentation (Neagu *at al*., 2022). The GC-MS only focuses on volatile compounds and is unable to analyse non-volatile compounds. In addition limitation of both LC-MS and GC-MS is that without the use of authentic standards they fail to provide absolute structural identification of compounds (Gathungu *et al*., 2020).

Technologies associated with the MS are rapidly developed and constant training by users is necessary for optimal results. The choice of the analytical technique to use depends chiefly on the objectives of the study.

2.9.1 Targeted analysis using HPLC-MS

Targeted metabolomics focuses on relatively small and specific chemical group of molecules, it is also commonly known as biased/ directed metabolomics or metabolic

profiling (Wang *et al*., 2010). The chemical and biochemical characteristics of the targeted metabolites are known at the beginning of the analysis (Roberts *et al*., 2012). This type of analysis requires the use of an authentic chemical standard and quantification is achieved by constructing a calibration curve (Broadhurst *et al*., 2018). This analysis is more selective and sensitive compared to the untargeted and semi-targeted approaches, it is used to identify and validate metabolites from the untargeted analysis (Xiao *et al*., 2012). The sample preparation for this analysis focuses on maximising the content of the target metabolites by removing other biological interferences (Dettmer *et al*., 2007). The output data interpretation is much simpler for targeted analysis.

2.9.1 Semi-targeted analysis using HPLC-MS

Semi-targeted approaches are in between the targeted and the untargeted (Billet *et al*., 2020). Semi-targeted techniques are hypothesis based, they focus on identifying a wide range of metabolites including unknown chemicals in order to provide a metabolomics pattern essential for the classification of phenotypes (Wang *et al*., 2010).

2.9.2 Non targeted analysis using HPLC-MS

Non targeted analysis of metabolites is also known as the unbiased or undirected metabolomics or metabolomics fingerprinting (Wang *et al*., 2010). Non targeted just as semi-targeted techniques are hypothesis based analysis (Wang *et al*., 2010). Untargeted techniques are ideal in observing unexpected changes in the detected metabolites (Lelli *et al*., 2021).

The untargeted analysis sample preparation extract the metabolites from the biological matrix into a solvent ideal for analytical analysis (Macioszek *et al*., 2021). The sample is then analysed with appropriate analytical tools. The peak area of each targeted metabolite is used for quantification, this process is known as relative quantification as no standard was used (Kapoore *et al*., 2016). The data acquired at the end of the analysis is used to identify each metabolite using the chemical structure and mass. The identification of metabolites remains a major challenge for the untargeted analytical technique (Schrimpe-Rutledge *et al*., 2016). There could be challenges in identifying the metabolites highlighted by the statistical packages (Lelli *et al*., 2021).

They quantify a number of metabolites which are known at the beginning of the analysis. A large number of metabolites are analysed and not one specific chemical standard is used but a number of standards catering for metabolites with similar chemical structures (Xia *et al*., 2012). Quality control of herbal plants remains a challenge around the world necessitating the understanding of changes in plant metabolites which can be achieved by untargeted metabolomics (Ziangchin *et al*., 2021). Untargeted metabolomics analysis have been employed to determine variation in *M. oleifera* cultivars from different locations (Lin *et al*., 2019, Makita *et al*., 2017). Untargeted and targeted NMR-based metabolomics was used to detect changes in *M. oleifera* influenced by harvesting frequency (Managa *et al*., 2021). A summary of targeted and untargeted metabolomics analysis in *M. oleifera* is summarised in Table 2.1.

Table 2.1: Summary of Targeted and untargeted analysis in *M. oleifera studies.*

Reference	Type of analysis	Extraction methods	Analytical techniques used
Managa *et al*., 2021	**Targeted:** Chlorogenic acid, Ferulic acid, Vanillic acid, Niazirin, Wogonin, Esculetin, and Gamma-aminobutyric acid (GABA)	ultrasound-assisted extraction, using the following solvents (deuterated Methanol (CD_3OD), potassium dihydrogen phosphate (KH_2PO_4).	NMR chemical shifts coupled with Chenomx profiler and the Human Metabolome Databases Standards were further used to confirm Chlorogenic acid and GABA
Zhu *et al*., 2020	**Targeted:** Gallic acid, Chlorogenic acid, Vanillin, Ferulic acid, Gallogen, Rutin, Isoquercetin, Quercitrin, Baicalin, Quercetin, and Kaempferide	ultrasound-assisted extraction using Methanol as the solvent	Ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS)
Ralepele *et al*., 2021	**Targeted:** Gallic acid, Catechin, Chlorogenic acid, Quercetin, Kaempferol, Quercetin 3,4-diglycoside, Quercetin 3-O-glucoside	ultrasound-assisted extraction using Methanol as the solvent	UPLC-DAD-QTOF-MS/MS analysis
Lin *et al*., 2019	**Untargeted:** 122 total metabolites tentatively determined	Methanol was used for ultrasound-assisted extraction	Ultra-high-performance liquid chromatography (UPLC) quadrupole time-of-flight tandem mass spectrometry (QTOF-MS)
Rocchetti *et al*., 2019	**Untargeted:** 262 phenolic compounds were annotated	Maceration, homogenizer-assisted extraction, rapid solid-liquid dynamic extraction, microwave-assisted extraction and	UHPLC-ESI/QTOF

		ultrasound-assisted extraction	
Makita *et al*., 2017	**Untargeted**	ultrasound-assisted extraction using Methanol as the solvent	UPLC qTOF-MS

2.10 Use of Chemometric to process data in HPLC-MS analysis

Large amounts of complex data are generated from the MS untargeted metabolomics analysis. A number of multidimensional and multivariate statistical packages have been developed to filter the large number of metabolites from the untargeted MS data (Katajamaa *et al*., 2007). Chemometric data processing is one of the techniques used for analysis. It involves the use of multivariate analysis (MVA) such as principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) (Worley and Powers, 2013).

PCA is an unsupervised chemometric model that reduces the complexity and the high-dimensionality of the data sets, providing a comprehensive and qualitative illustration of similarities or dissimilarities among and within the sample (rephrase) (Djande *et al*., 2018). PCA technique is used to reduce dimension, it also deals with multi-collinearity problems (Maitra and Yan, 2008). The PLS-DA model on the other hand is a supervised discriminant analysis of the data (Tock *et al*., 2021). Both the PCA and PLS models are able to differentiate variations between samples ignoring variations within each sample. They also convert correlated variables to a set of independent variables (Maitra and Yan, 2008)

The metabolomics analytical tool could be divided into four experimental stages: 1) Sample preparation (extraction of metabolites) and acquisition, 2) data acquisition, 3) data mining and extraction and lastly 4) data analysis (Wang *et al*., 2010). Figure 2.9 below illustrates the experimental stages of untargeted metabolomics analysis.

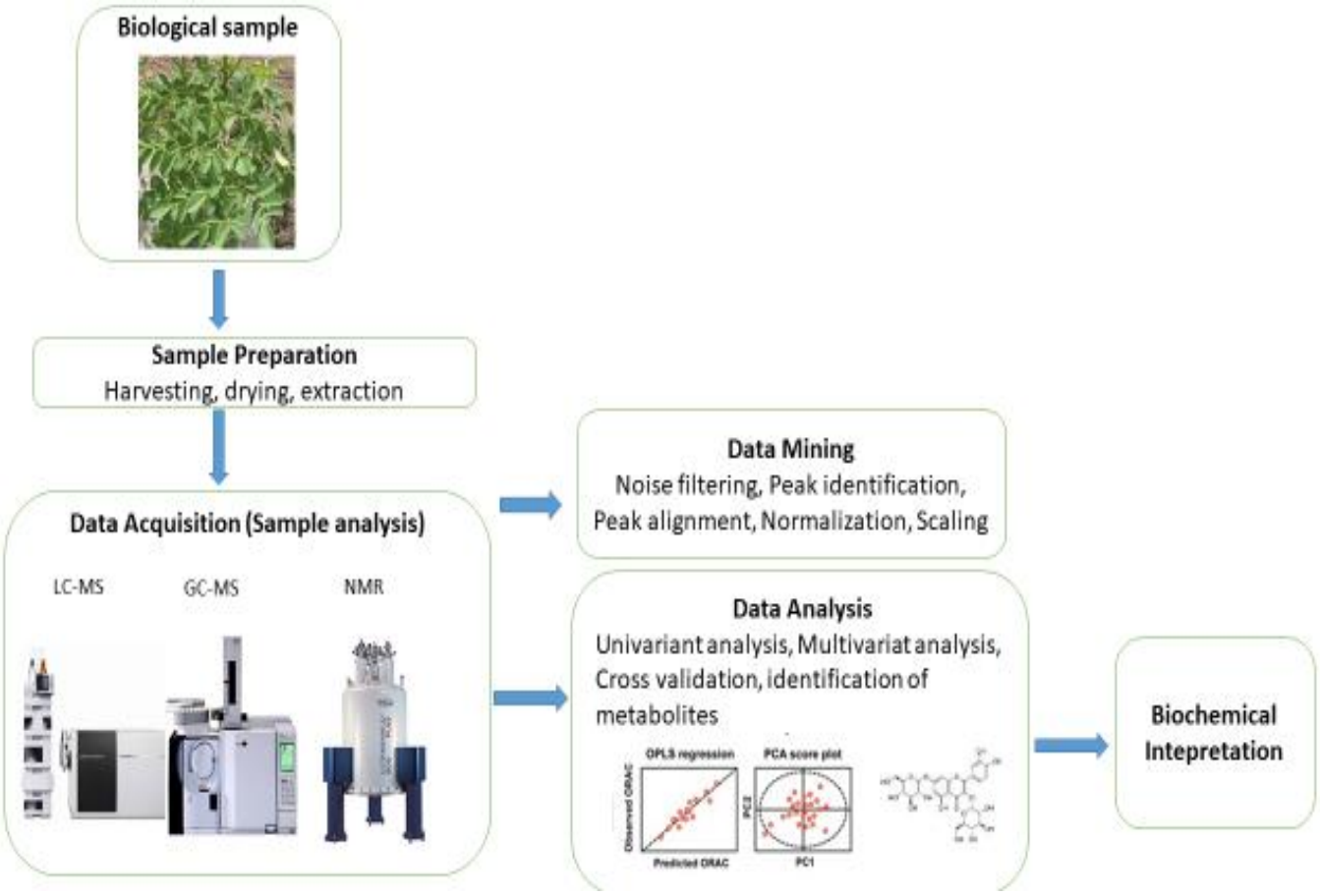

Figure 2.9: Schematic Workflow of the untargeted metabolomics analysis.

2.10.1 Data Preprocessing

Data preprocessing or pretreatment is essential in reducing variations in the huge data output produced by the mass spectrometry and NMR (Karaman *et al*., 2017). Preprocessing minimizes and extracts features from the raw data, which is technically and structural complex to analyze with statistical packages (Vettukattil, 2015). There are a number of different software tools which have been developed for data preprocessing, these are either commercial or free (Gürdeniz *et al*., 2012). Popular data preprocessing software are MZmine, XCMS, MarkerLynx^{TM} , metAlign and MS-DIAL. MZmine is a user friendly free software widely used for the preprocessing of MS data for untargeted metabolomics analysis (Tkalec *et al*., 2022).

In comparison with other preprocessing tools such as XCMS and MarkerLynx^{TM} data preprocessed by MZmine yielded the same biological information (Gürdeniz *et al*., 2012).

2.11 Research Aims and Objectives

2.11.1 General aim

The aim of the study was to assess the influence of plant based compost on chemical composition of *M. oleifera* grown in the green house and in the field. Further, the study determined the influence of plant based compost on frost resistance of *M. oleifera* in the field.

2.11.2 Specific objectives

- To determine the content and bioavailability of macro and micro nutrients (Al, Ca, Co, Cr, Fe, K, Mg, Mn, Na, P, S, and Zn) in clay soil amended with different levels of plant-based organic compost.

- To determine macro and micro nutrients (Al, Ca, Co, Cr, Fe, K, Mg, Mn, Na, P, S, and Zn) content of *M. oleifera* leaf powder harvested from plants grown in clay soil amended with different levels of plant-based organic compost.

- To use an LC-MS based metabolomics approach to determine plant-based organic compost impact on the metabolite profile of *M. oleifera* leaves using (UHPLC-ESI-QTOF-MS) followed by untargeted analysis of compound search.

- To develop an HPLC method for simultaneous determination of five water-soluble B complex vitamins (Vitamins B1, B2, B3, B6 and B9) and use the method to quantify for the five vitamins in *M. oleifera* leaf biomass of plants grown in different levels of plant-based organic compost

2.11.3 Novelty

To date the effect of plant-based organic compost on the nutrients and phytochemicals found in *M. oleifera* leaves has never been investigated. Previous researchers have focused on the animal based compost and not but never on organic plant based compost. The study also reports for the first time, the development and use of an HPLC

method for the simultaneous quantification of five water-soluble vitamins in *M. oleifera* grown under organic plant-based compost amendment.

2.12 Study approach and thesis structure

The thesis is divided into five sections. The overview of each section is given in full details below bringing to view how each section fulfils the research objectives of the current study and thus meeting the aim of the research.

Chapter 1 is the general introduction of the research which outlines the background of the study and the rationale.

Chapter 2 is the detailed literature review of the study it expounds the targeted research components and the analytical methods in detail. The chapter also has the aims and objectives of the study and the thesis outline.

Chapters 3, 4 and 5 are written in scientific paper format, the chapters are to be or have been submitted for publication to scientific. With that being the case unavoidable repetition on some sections such as introduction are to be noted. In addition there are variations in the design of the papers according to each Journal requirement, these affect the topics, subtopics and the referencing style.

Chapter 3 gives an insight on the impact of plant based compost on the uptake of metals also known as minerals.

Chapter 4 focuses on the impact of plant based compost on the accumulation and distribution of secondary metabolites in *M. oleifera* leaves.

Chapter 5 is based on the quantification of water soluble vitamins in *M. oleifera* leaves using an altered quantification method.

The general discussion and conclusion chapter (Chapter 6) encompasses findings from all the chapters, it gives an overview of the overall findings and how the study impacts the production and nutritional value of *M. oleifera.*

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Chapter 3

3. Influence of Plant-based Compost on Soil Chemical and Nutrient content and their availability in *Moringa oleifera* leaf biomass grown on Clay soil in South Africa

Ngwenya, N [1], Naupia, Y[2], Risenga, I[1], Chimuka, L.[3]

3.1 Abstract

The objective of the present study was to investigate the effect of plant-based organic compost on the availabilty and accumulation of macro and micronutrients in *Moringa oleifera* leaves. Clay soil from the *M. oleifera* community farm in Hammanskraal, South Africa was mixed with four different levels of plant-based compost (15%, 30%, 45% and 60%) in 5 L pots. *Moringa oleifera* seedlings were planted in each treatment and in the control in four replicates. Results showed that addition of compost increased the concentration of micro and macro-nutrients in the soil with the exception of Mg. Correspondingly, nutrient concentration of *M. oleifera* leaves increased with the addition of compost to the soil. Correlation between availability of nutrients in the soil and accumulation in plant leaves was also noted The recommended soil-compost combination was 30% compost soil amendment which had the highest concentration of Ca (45 042.5 mg/Kg), Mg (17 430 mg/Kg), P (8802. 5 mg/Kg) in *M. oleifera* leaves. The study concluded that the addition of compost improves the availability of nutrients in the soil and their concentration in *M. oleifera* leaves while keeping trace elements below the maximum allowed limits. Analysis is recommended before applying any plant-based compost in *M. oleifera* plantations to maximise the nutritional content in the biomass and also to make sure that toxic elements do not exceed their maximum allowed limits.

Key Words

Moringa, plantbased-compost, nutrients, organic, bioavailability

3.2 Introduction

Moringa oleifera L. commonly known as “horseradish”, “drumstick” or “miracle tree” is a soft stem plant which falls under the monogeneric family Moringaceae, (Gopalakrishnan *et al.*, 2016). It is native to the Himalayan Mountains of India,

Pakistan and Nepal (Leone *et al*. 2015) and also grows naturally in African countries (Namibia, Angola, Kenya, Ethiopia and Egypt) (Konmy *et al*., 2016). It is the widely known, cultivated and researched species of the genus *M. oleifera* (IDC, 2019).

Moringa oleifera is well-known for its anti-inflammatory, anticancer, antihelmintic and antiurolithiatic properties, it also helps to cure cardiovascular ailments (Bhattacharya *et al*., 2018). The leaves are the most used part of the plant, containing Vitamin C (400 mg) higher than oranges (30 mg) per 100 g biomass (Mahmood *et al*., 2010). In addition, the leaves also contain vitamins such as B, D, E, nicotinic acid and pyridoxine (Siddhuraju and Becker, 2003) and minerals like copper, zinc, iron, magnesium and potassium (Aslam, 2005). They are also rich in phytochemicals such as sterols, flavonoids, tannins, saponins, alkaloids, terpenoids, anthraquinones as well as anti-cancer agents such as glycerol-1-9-octadecanote, glucosinolates, isothiocyanates and glycoside compounds (Leone *et al*., 2016).

Global awareness of *M. oleifera* as a nutracetrical has increased, which has led its increased utilisation and production (Brilhante *et al*., 2017). Production is expected to grow steadily as demand for a healthier lifestyle, organic foods and nutritional supplementation grows (Akinola *et al*., 2020). In South Africa *M. oleifera* production for local and international markets is increasing steadily with noticeable farms present in Limpopo, Northwest, Gauteng, KwaZulu-Natal, Western Cape and Mpumalanga provinces (Mashamaite *et al*., 2021). Various factors such as cultivation, climate, location and soil physiochemical properties affect the production and nutrient content of *M. oleifera* (Grosshagauer *et al*., 2021; Su and Chen. 2020). Improving quality and quantity is vital for herbal plants like *M. oleifera* used as nutraceuticals.

The nutrient content of *M. oleifera* and its herbal properties are intertwined, nutrients are enablers of plants' herbal properties (Ahmadi *et al*., 2020). Factors affecting nutrient content are soil-physiochemical properties, nutrient chemical nature and the plant species (Kidd *et al*., 2007). Growth enhancers in the form of artificial fertilisers have been used to increase production and nutrient content of *M. oleifera* (Sarwar *et al*., 2017). Though increasing growth, artificial fertilisers result in the accumulation of toxins such as heavy metals in plant leaves (Chibuike and Obiora, 2014). Further, as consumers are gradually embracing a heathier lifestyle, demand for organically grown food has increased (Meemken and Qaim, 2018). Thus many *M. oleifera* farmers are

avoiding the use of artificial fertilisers. Organic certification of *M. oleifera* plantations also rejects the use of artificial fertilisers.

Compost is one of the organic fertilisers which support sustainable agriculture. It improves the physiochemical, organic matter and nutrient contents of the soil (Adugna, 2016). Organically grown plant products are of a higher quality (contain higher levels of antioxidants, minerals and dry matter), and are free from any artificial chemicals residues Crinnion (2020). Studies done in Nigeria revealed that organically grown *M. oleifera* performed better in biomass and nutritionally compared to the control which had nothing added to it (Adebayo *et al*., 2017).The use of compost in the production of *M. oleifera* improves the nutritional content of the plant and also helps evade soil degradation (Ngakou *et al*., 2020). Compost widely used by farmers is generally from animal manure. However, previous research on compost has revealed that organic fertilisers based on animal waste pose a health risk to the environment and plants due to microbial contamination and some chemicals added in animal feed (Nygaard *et al*., 2008). Foodborne pathogenic bacteria like *Salmonella* and *Eschericia coli* spp were discovered in agricultural soils and in vegetables grown using animal manure (You *et al*., 2006). In addition compost obtained from sewage sludge and livestock excretion can contain traces of heavy metal contamination (Liu *et al*., 2006).

Compost obtained from plant-based material has been a safer fertiliser in organic farming because it contains less or no heavy metals (Bożym (2017) and faecal coliforms. Plant-based compost also suppress plant diseases caused by pathogenic formae speciales of *Fusarium oxysporum* (Yogev *et al*., 2006). Compost is not only safe to the environment, it is used in soil amendment programs to reduce heavy metal contamination (Angelova *et al*., 2010). The use of compost for soil remedial is increasingly attracting attention, compost contains constituents like humus, microbes and mineral ions.

Soil amendments are essential in *M. oleifera* farms around South Africa (Mashamaite *et al*., 2021) as about 50% of the land is deemed less suitable and or not suitable for Moringa production based on climate and soil physiochemical properties (Tshabalala *et al*., 2020). This study focused on organic soil amendment in *M. oleifera* to improve the nutrient content of the leaves. Dark clay soil obtained from the *M. oleifera* community farm in Hammanskraal, Gauteng, South Africa was used. The dark clay

soils are nutrient-rich when compared to other soil types. However, the soils pose a challenge to *M. oleifera* growth as they are prone to water logging. The soil also cracks when it is dry, this is a serious challenge during winter as the cracked soil exposes the *M. oleifera* tubers to frost and thus drying up the plants. The plant-based compost amendment is expected to improve the soil physiochemical properties and nutrient quality of *M. oleifera*. In our best knowledge, there has not been any scientific research or literature on the use of plant-based compost to increase the nutrient quality of *M. oleifera* leaves especially grown in clay soil. Studies conducted in South Korea reveal that a combination of compost and NPK improves the biochemical content of *M. oleifera* (Sarwar *et al*., 2020). However, the investigation of the influence of compost especially plant based compost on the *M. oleifera* grown in South Africa has not been investigated.

3.3 Materials and Methods

3.3.1 Reagents

Hydrochloric acid (HCl), nitric acid (HNO_3), hydrogen fluoride (HF), hydrogen peroxide (H_2O_2), all purchased at Sigma Aldrich (Johannesburg, South Africa).

3.3.2 Greenhouse experiment

Moringa oleifera seeds used to grow the plant samples and the clay soil were obtained from a community *M. oleifera* farm in Hammanskraal. The soil is clay soil, top soil (0-15 cm) was collected using a spade. Appendix 1, Figure 3.4 and 3.5 show the map of Hammanskral and the clay soil in the Moringa farm. The soil was air-dried, homogenised and sieved in order to remove gravel, debris and clogs. The seeds were primed for 24 hours and thereafter planted into germination trays containing potting soil and watered every two days for four weeks. Healthy seedlings were transferred into 2 L pots containing clay soil as control and clay soil amended with four different levels (15%, 30%, 45% and 60%) of organic compost made from diverse plant material as treatments. The percentages for each compost-soil combination was by volume of the total mixture. Each soil treatment was replicated four times and the pots were laid out in a randomised complete block design (RCBD). Appendix 1, Figure 3. 6 is a picture of Moringa plants in the greenhouse. After transplanting, the plants were watered for two weeks with approximately 100 mL of water per day. Thereafter

watering was done twice a week, and plant growth was monitored for six months. The plant growth experiment was carried out in the greenhouse at the School of Animal, Plant and Environmental Sciences (APES), University of the Witwatersrand under ambient temperatures.

3.3.3 Sample preparation

The collected leaves were washed, both the leaf and soil samples were air dried under the shade then ground and sieved. Processed samples were stored in amber glass bottles to avoid continual exposure to the son and degradation of nutrients. Soil pH and electrical conductivity (EC) were determined by a pH and conductivity meter after suspending the soil in deionised water in a ratio of 1: 2 soil (20 g) and deionised water (40 mL) respectively).

3.3.4 Metal content in soil and in *M. oleifera* leaves

3.3.4.1 Total digestion and metal content analysis

A microwave digester (Anton Paar, Switzerland) was used for digestion. A mass of 0.1 g of the sieved soil was weighed into Teflon® perfluoroalkoxy resin polymer (TPFA) digestion vessels, 9 mL concentrated HCl, 3 mL concentrated HNO_3, 1 mL concentrated HF and 6 drops of concentrated H_2O_2 were added. Digestion was then done at 200 °C for 30 minutes.

To a mass of 0.1 g dried leaf powder in microwave digestion vessel, 8 mL concentrated HNO_3 and 2 mL concentrated H_2O_2 was added. Microwave digestion was carried out for 30 minutes. Digestions were carried out in triplicates.

The digested solution for both soil and leaf samples were transferred into 50 mL volumetric flask and made up to the mark with deionized water. The digested samples were then filtered and dilutions were made before analyses for metal content using the inductively coupled plasma – optical emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro, Germany). The Carbon, Hydrogen, Nitrogen and Sulphur analyzer (CHNS) was used to determine percentages of Carbon (C), Hydrogen (H), Nitrogen (N) and Sulphur (S).

3.3.4.2 BCR sequential extraction of metals

Bioavailability of metals in the soil was determined by slightly modifying the modified BCR sequential extraction method by Alan and Kara, (2019). The procedure was

applied to 1 g of each soil sample placed in 50 mL centrifuge tubes, each soil sample was replicated thrice. The summarized procedure is as follows:-

The first step (Exchangeable fraction) extracted free and acid-soluble fractions-bound to carbonates by adding to the sample, 40 mL of 0.11 moL^{-1} acetic acid. The sample was shaken at 30 ± 10 rpm for 16 hours at room temperature, 25 ± 1°C. The extract was separated from the solid by centrifuging at 3000 rpm for 20 minutes and collected and stored in polythene bottles at 4°C. Washing was done with 20 mL deionized water and shaking for 15 minutes followed by centrifuging at 3000 rpm. The supernatant was discarded.

The second step focused on the reducible fraction i.e. extraction of metals bound to Fe and Mn oxides. To the residue from step one, 40 mL of freshly prepared 0.5 moL^{-1} hydroxy ammonium chloride ($NH_2OH.HCL$) at pH 1.5 (adjusted by adding 25 ml of 2 moL^{-1} HNO_3) was added. The mixture was shaken for 16 hours at room temperature. Separation of the extract and washing was done as in step one.

Third step (oxidizable fraction, bound to sulphides and organic matter), 10 mL of 8.8 moL^{-1} of concentrated H_2O_2 of pH 2-3 (pH adjusted by adding HNO_3) was added to step 2 residue. The mixture was digested for an hour at room temperature. Digestion continued for another hour by heating the vessel at 85 °C in a water bath until the residue was reduced to 2-3 mL. After which 50 mL of 1 moL^{-1} ammonium acetate ($C_2H_7NO_2$ (pH 2 adjusted by HNO_3) was added and the mixture shaken for 16 hours at room temperature. Extraction and washing of the residue was carried out as described in step 1.

The residual step subjected the residue to aqua regia digestion. The total metal content was analyzed using the (ICP-OES) (Spectro Genesis, Spectro, Germany).

3.3.5 Data analysis

All measurements were done in triplicate, data was then analysed using One-way analysis of variance (ANOVA) with SAS statistical package version 17.0.

Significance within the means was compared using Fisher's test.

3.4 Results and Discussion

3.4.1 Soil pH and Electrical conductivity

The pH of the control clay soil was 7.68 whilst that of the compost was 8.01 (Table 3.1). However, addition of compost to the soil reduced the pH of the soil slightly but no major changes in pH within various compost amendment treatments (Table 3.1). Likewise, the EC of compost (249.67 µS/cm) was higher than that of the control clay soil (14. 62 µS/cm). This can be attributed to anions such as carbonates, nitrates and sulphates in compost that are part of the metal content. Total C and N content in the soil increased with addition of compost as expected (Hu *et al*., 2018). Though C and N content increased simultaneously, the C to N (C/N) ratio decreased with the addition of compost to the soil. The C/N ratio value was lower than 20 for all soil amendments indicating very stable organic matter (Guzmán-Albores *et al*., 2019) and ability of plants to assimilate N. These values are ideal for nutrient uptake as N can only be assimilated when C/N value is less than 20 (Khwairakpam and Bhargava, 2009). High soil electrical conductivity (EC) has been known to increase absorption and accumulation of metals in plant shoots (Salimi *et al*., 2012). Similar results were reported by Pakade *et al*. (2013) as high EC corresponded with high levels of cations and anions in the soil. The EC of compost was very high at 249.67 µS/cm compared to that of clay soil (14.62 µS/cm) which might be an indication of high anions that balance cations (Adugna (2016), Duong *et al*., 2012). In the current study, compost had the highest pH of 8 which is an indication of mature compost. The addition of compost to clay soil decreased clay soil pH slightly. This trend was also reported by Mohammad and Athamneh (2004), which can be due to the buffering effect of the compost. The same results were obtained by Duong *et al*. (2012). However, addition of compost to the clay soil increased conductivity levels because of its high cation exchange capacity (CEC) (Sung *et al*., 2011).

Table 3.1: Soil pH and Conductivity.

Sample	pH	Conductivity/ µS/cm*	TOC (%)	TN (%)	CN Ratio
Compost	8.01*	249.67*	34.49	1.53	22.5
Control clay soil	7.68	14.62*	1.50	0.08	18.4
15% Compost	7.59	25.2*	1.78	0.12	15.3
30% Compost	7.43	45.1*	4.98	0.36	13.9
45% Compost	7.49	77.6*	2.78	0.19	14.6
60% Compost	7.48	81.5*	6.44	0.42	15.2

TOC (total organic carbon); TN (total nitrogen); CN (carbon to nitrogen ratio). *Significant difference at $P<0.05$

3.4.2 Total metal concentration of clay soil amended with plant-based compost

Macro and micro metal concentration in control clay soil and in organic compost amended clay soil is shown in Figure 3.1 (a, b and c) and Table 3.2. Figure 3.1 (a, b and c) displays that compost had higher metal content of Zn, K, P, S, Na and Ca than clay soil. The metal concentration of K, P, S, Na and Ca was almost double in amount in compost compared to clay soil. This is expected as compost was made up of plant material and these nutrients are major nutrients found in plants in relatively large amounts (Hocking, 1994). On the other hand metals needed in trace amounts by plants such as Al and Fe were slightly more in clay soil and addition of plant-based compost reduced the presence of these elements in the soil

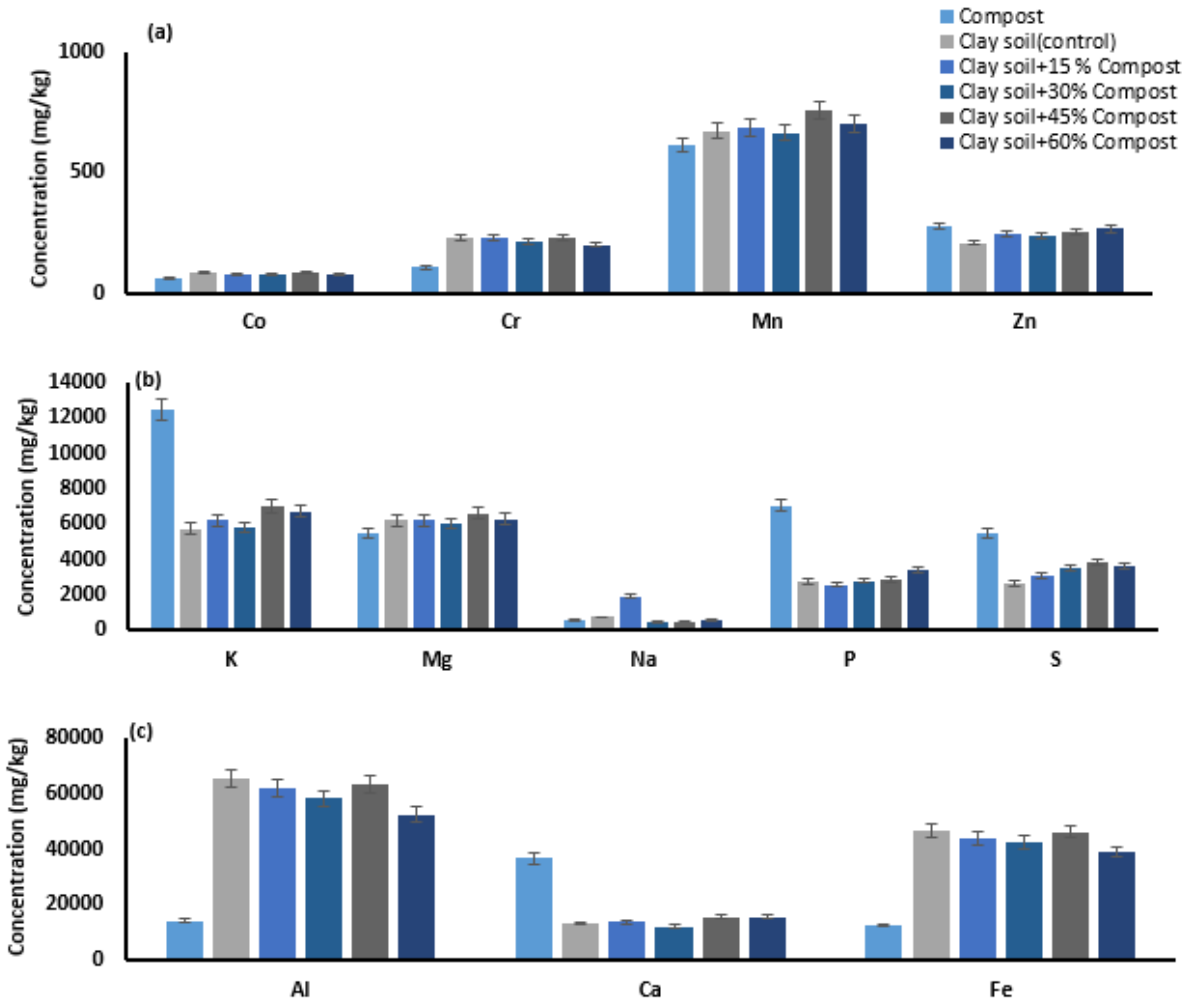

Figure 3.1: Metal concentration of Clay soil amended with compost with a) lowest metal concentration, (b): medium metal concentration; (c) high metal concentration.

Table 3.2 reveals significant difference P< 0.05 for the concentration of metals in the clay soil (except Co and Cr) with the addition of plant-based compost. High levels of metals in compost which were observed were also reported by Madeleine *et al*., (2005). The high increase of P in the soil upon addition of compost is due to the high P levels in plant compost this is synonymous to results by Verma, (2013). Thus the addition of compost to clay soil generally increase the concentration of metals that were in high amounts in the compost. The concentration of Al, Ca, Co, Cr, K, Mn, P, S and Zn in the different soil compositions currently studied was within the range of soil mineral composition from South African *M. oleifera* farms as presented by IDC, (2019). Whilst Chromium, Fe and Mg concentrations were higher in the current study when equated to concentrations from other *M. oleifera* farms in South Africa based on the concentrations presented by IDC, (2019).

Table 3.2: Metal concentration of clay soil amended with different levels of plant-based organic compost.

	Compost	Control (clay soil)	Clay soil + 15% compost	Clay soil + 30% compost	Clay soil + 45% compost	Clay soil + 60% compost
Al	13962.5±56.79^{a}	65700±1396.95^{b}	62075±1438.64^{c}	52520±1223.22^{d}	63687.5±1683.68^{bc}	58225±1213.76^{e}
Ca	36607.5±907.5^{a}	13162.5±555^{b}	13730±109.29^{b}	11980±381.27^{c}	15355±442.52^{d}	15432.5±596.8^{d}
Co	59,3±0.76^{a}	83,5±1^{b}	81,7±0.58^{c}	79,3±1.15^{d}	82,83±0.29^{bc}	79,5±0.5^{d}
Cr	106.3±1.76^{a}	227,5±2.6^{b}	226,5±2.3^{b}	216,2±5.35^{c}	227,3±0.76^{b}	200,5±2.18^{d}
Fe	12425±65.53^{a}	46487,5±638.2^{b}	43937,5±809.7^{c}	42497,5±967.3^{d}	46265±990.04^{b}	39070±657.45^{e}
K	12460±321.92^{a}	5755±114.56^{b}	6215±351.17^{c}	5835±216.98^{b}	7015±341.26^{d}	6700±371.79^{cd}
Mg	5444,8±26.1^{a}	6209,7±61.78^{b}	6210,8±58.11^{b}	6018±161.63^{c}	6626,8±8.98^{d}	6273±76^{b}
Mn	613,5±4.5^{a}	672±1.32^{b}	687±1.8^{bc}	665,3±16.8^{d}	757,7±3.69^{e}	699,5±11.38^{c}
Na	1904,8±26.14^{a}	479±69.72^{b}	500,7±3.33^{b}	599,2±51.01^{c}	745,8±14.78^{d}	591±34.41^{c}
P	7052,5±45.2^{a}	2765±4.33^{b}	2565±45.62^{c}	2805±79.02^{b}	2890±30.31^{d}	3422,5±38.49^{e}
S	5457,5±126.02^{a}	2630±104.01^{b}	3040±99.87^{c}	3557,5±232.54^{d}	3832,5±41.76^{e}	3615±136.45^{de}
Zn	277,8±1.04^{a}	209,3±7.52^{b}	243,8±0.76^{c}	234,7±6.11^{d}	256,3±2.93^{e}	266,5±5.07^{f}

One way ANOVA test carried out to determine significant difference at $p<0.05$. Means for each row with different letters were significant different using Fisher's LSD post hoc test.

3.4.3 Total metal concentration of metals in dried *M. oleifera* leaves

The results of metal concentration in dried *M. oleifera* leaves are shown in Figure 3.2 (a) and (b). The leaf biomass of plants grown in clay soil amended with 30% compost had the highest concentrations of metals in about 50% of the studied metals than other composition. What is interesting is that the high concentration in 30% compost was for major nutrients such as Ca, Mg, P and S. Adebayo *et al*. (2017) reported an increase *M. oleifera* leaf metal content upon addition of different forms of compost, synonymous with our current findings. The clay soil amended with 45% compost gave higher amounts of Zn. Sodium (inserted on figure 3.2a) was highest in 30% and 45% clay soil compost amendment. Clay soil amended with 60% compost gave higher amounts of Fe while Al was highest in the control. Similar trends were reported in other plants after the addition of compost Nguyen (2013). These variations in the uptake of metals by *M. oleifera* plants grown in different soil amendments reflect the ability of plant-based compost to alter the soil physiochemical properties which alter metal uptake by the roots (Celik *et al*., 2004). Addition of compost to clay soil improves aeration, water

holding capacity and cation exchange capacity (CEC) and thus improving the uptake of metals (Azeez *et al*., 2021). Haouvang *et al*. (2017) reported that high levels of compost in the soil may lead to toxicity due to over accumulation of metals such as Fe and Mn which justifies the decline in nutrients in 60% compost compared to 30% compost.

a)

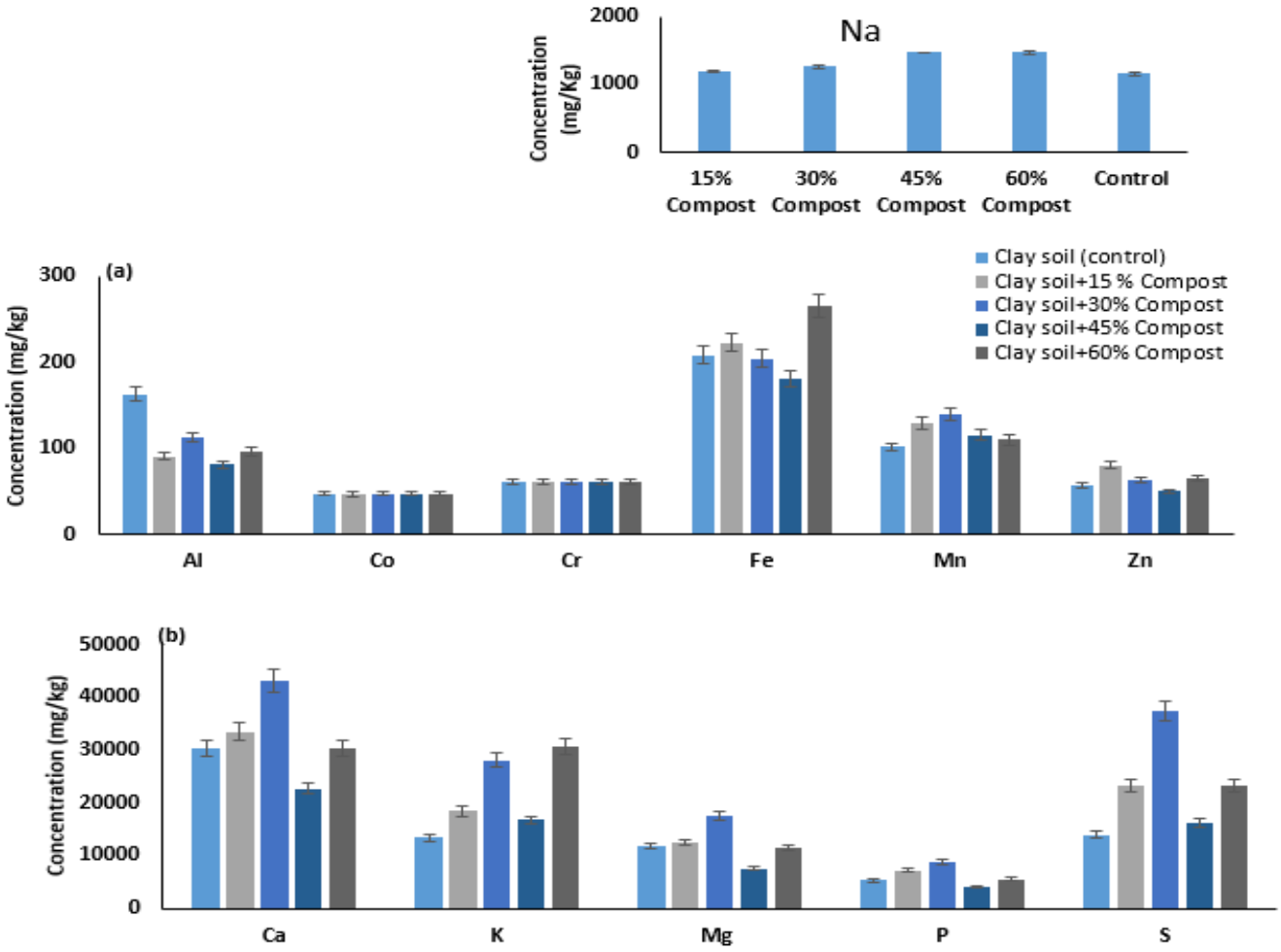

Figure 3.2: Metal content in *Moringa oleifera* leaves, (a) low concentration metals (b) high concentration metals

The concentration of the metal macro and micro nutrients in *M. oleifera* leaf powder varied significantly P< 0.05, with the addition of compost in the soil (Table 3.3). Variation was also noted within the different plant based compost levels (Table 3.3). Calcium, Cr, Mn, Na and Zn concentrations found in *M. oleifera* leaves in the current study were higher than concentrations of the same metals found in other studies of *M. oleifera* grown in South Africa (IDC, 2019). Iron, K and P in the leaves were within the

range of the concentration found in *M. oleifera* grown across South Africa whilst Mg had a lower concentration. The high concentration of Ca and Na in the leaves is due to their high concentrations in the clay soil and various compost treatments as also reported by Marosz (2012). Although higher than in other *M. oleifera* leaf studies in South Africa, Na concentration in the current study was still lower than the Na toxic concentration of 2500 mg/kg, which affects the growth and quality of the produce (FAO, 1995). Calcium and Mg concentrations were higher than those of most vegetables (Pakade *et al*., 2013). The high concentration of macro and micro nutrients in the current study is attributed to the addition of compost which increases conductivity and absorption and accumulation of nutrients in plant leaves (Gondek *et al*., 2020). Van Herwijnen *et al*. (2007) discovered that application of compost decreased the uptake of Zn and Cu by Greek Cress. Likewise in this study Zn concentration decreased in *M. oleifera* leaves with increased soil compost levels.

Table 3.3: Metal concentration of *M. oleifera* leaves harvested from clay soil amended with different levels of plant-based organic compost

	Control (clay soil)	**Clay soil + 15% compost**	**Clay soil + 30% compost**	**Clay soil + 45% compost**	**Clay soil + 60% compost**
Al	163 ±5.5^{a}	91±6.5^{bd}	113±3.61^{c}	81.83±4.072^{d}	96±6.38^{b}
Ca	30405±1102.58^{a}	33400±498.42^{b}	43042.5±1000.52^{c}	22732.5±608.06^{d}	30387.5±1396.14^{a}
Co	52±1.32^{a}	52.83±1.04^{a}	51.33±1.61^{a}	52.33±0.29^{a}	51.33±1.61^{a}
Cr	101.5±1.32^{a}	96±1.73^{b}	101±^{ac}	98±1.32^{bc}	102±0.5^{a}
Fe	208.5±2.18^{a}	223.17±1.76^{b}	204±2.18^{c}	181±0.87^{d}	265±.5^{e}
K	13477.5±679.28^{a}	18392.5±244.91^{b}	28017.5±581.25^{c}	16685±223.79^{d}	30772.5±765.7^{e}
Mg	11837.5±413.95^{a}	12447.5±72.84^{b}	17430±212.66^{c}	7482.5±256.58^{d}	11457.5±303.94^{e}
Mn	101.67±0.76^{a}	129.83±0.57^{b}	140.17±1.04^{c}	116±0^{d}	110.17±0.29^{e}
Na	1155.83±25.96^{a}	1190.67±17^{a}	1261.5±24.74^{b}	1470.5±7.37^{c}	1469.5±17.33^{c}
P	5330±20.31^{a}	7215±83.52^{b}	8802.5±75.87^{c}	4062.5±37^{d}	5507.5±15.61^{e}
S	13965±81.12^{a}	23225±22.91^{b}	37460±69.1^{c}	16302.5±97.88^{d}	23285±172.06^{b}
Zn	57.5±0.87^{a}	80.17±3.05^{a}	62.83±2.02^{c}	50.5±0.5^{d}	65.17±0.29^{c}

One way ANOVA test carried out to determine significant difference at $p<0.05$. Means for each row with different letters were significant different using Fisher's LSD post hoc test

3.4.4 Studying the metal bioavailability in clay soil amended with compost

The results for metal speciation in clay soil and the clay soil amended with 15%, 30%, 45% and 60% plant-based compost are summarised in Figure 3.3 (a) and (b). Distribution and concentration of metals in the four steps varied between the control

soil and the treatments. Increase in the exchangeable forms of Ca, K, Mg, Mn, Na, P and Zn increased with the addition of compost to the soil however there was no significant difference (P> 0.05) in the different soil to compost ratios. Addition of compost increased the availability of Al and Fe by a decrease in the residual fraction and increase in the oxidazable fraction. No significant difference (P> 0.05) was observed among the clay soil and the treatments was noted for Cr and Co. Calcium exchangeable fraction was higher in 30% compost clay-soil amendment, whilst Mg, Mn were high in both 15% and 30% treatments. Potassium exchangeable fraction was highest in 15% compost clay-soil amendment (Figure 3a).

The sequential extraction scheme provides the relationship between the soil characteristics and the metal content (Sungur *et al*., 2014). It further provides information on the metal attraction and extent of binding to the soil matrix and their phytoavailability (Sahito *et al*., 2015; Tokalıoğlu and Kartal, 2006). Sungur *et al*. (2014) used sequential extraction to reveal that there are some metals present in the soil but not accessible to plants. This was also noted in the current study, Al, Fe and Zn concentration was high in the soil but low in *M. oleifera* leaves (Figures 3.1 and 3.2). This could be due to high soil pH in the current study which increases the Al binding functionality reducing its availability to the plants (Zhao and Shen, 2018). Zinc is also known to be largely bound to silicate in the soil (Ptistišek *et al*., 2001). Similarly, Cr was extracted the least in the first two steps indicating that the metal could be bound to silicate and is not easily extractible (Adamo *et al*., 2018), thus its low concentration in *M. oleifera* leaves. Higher concentrations of Cu and Mn in the residual fraction indicate the immobile state of these metals, same trend was reported by Sahito *et al*. 2015).

Furthermore, Ca is highly soluble in water (Van Herck *et al*., 2000) and acid as indicated by its high concentration in Acid-soluble fraction (Figure 3.3) making it accessible to plant uptake leading to high Ca concentrations in the leaves. Jakubus (2016) cited that different soil compositions have an effect on the release and phytoavailability of metals. Additionally organic amendments influence the content, mobility of metals in the soil and the uptake of nutrients by the plants (Herencia, 2008). The current study affirms these findings.

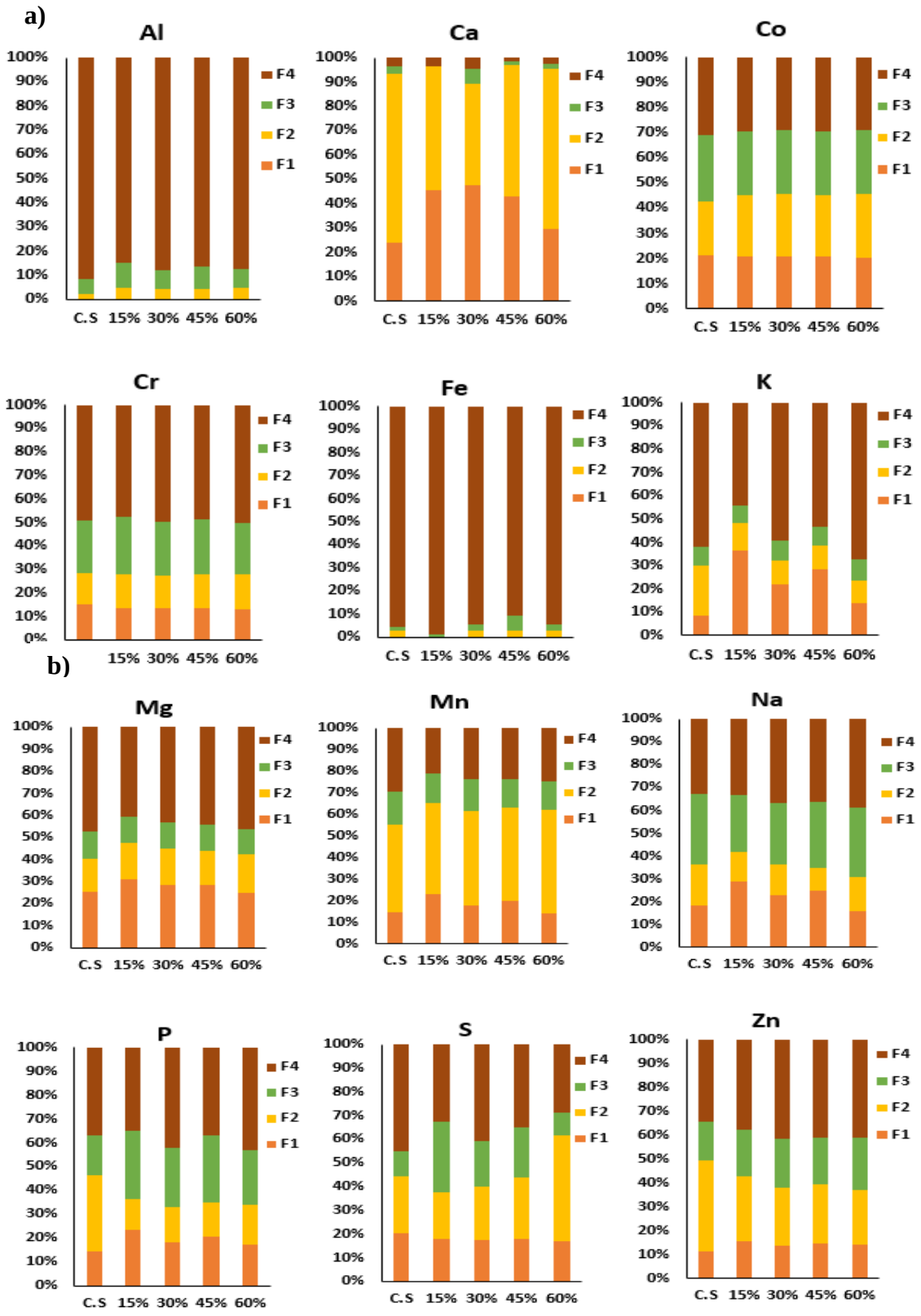

Figure 3.3 (a) and (b): Bioavailability of metals in the clay soil amended with plant-based compost (F1- Exchangeable fraction; F2- Reducible fraction; F3- Oxidizable fraction; F4- Residual fraction). C.S (Control clay soil); 15% (clay soil+15% compost);

30% (clay soil+30% compost); 45% (clay soil+45% compost); 60%(clay soil+60% compost).

3.4.5 Assessment of possible adverse health effects posed by daily intake of *M. oleifera* leaf biomass based on the metal concentrations found in this study

Calculating the daily intake of the metals found in the current study is of paramount importance to avoid cumulative toxicity (Grosshagauer *et al*., 2021). The daily metal intake (DIM) was calculated by multiplying the concentration of metals with the recommended daily dose (40 g) of *M. oleifera* leaf powder (Asiedu-Gyekye *et al*., 2014). The DIM results (Table 3.4) indicate that Al, Fe, Mn and Zn DIM is below their recommended daily intake. However, Co and Cr (Table 3.4) limits were above their recommended daily intake. High levels of Cr could be attributed to the fact that South Africa geologically has high Cr deposits (Naupia *et al*., 2018). Concentration of Cr in *M. oleifera* leaf powder from farms around South Africa reported by (IDC, 2019) was also above the permissible limit. Further investigation need to be done to determine the source of Co and Cr in the current study as addition of compost did not significantly increase their metal content in the soil or their bioavailability (Figures 3.1(a), 3.3). Mineral nutrients in *M. oleifera* leaf powder from the current study makes it a good health supplement. However, metals such as Al, Co, Cr, Fe, Mn and Zn are known to have a toxic effect to the human body therefore, caution must be taken in consuming the leaf powder (Asiedu-Gyekye *et al*., 2014).

Table 3.4: Daily metal intake estimate in *M. oleifera* leaf powder

	DMI (Al)	**DMI (Co)**	**DMI (Cr)**	**DIM (Fe)**	**DIM (Mn)**	**DIM (Zn)**
Clay soil (control)	6.52	1.92	2.44	8.34	4.08	2.3
15% compost	3.64	1.88	2.48	8.928	5.192	3.208
30% compost	4.52	1.92	2.44	8.16	5.608	2.512
45% compost	3.272	1.92	2.44	7.24	4.64	2.02
60% compost	3.84	1.92	2.48	10.6	4.408	2.608
Recommended daily intake (mg day^{-1} $person^{-1}$)*	120	0.011	0.039	19-22	11	40

*****Trumbo *et al*., 2001

3.5 Conclusion and implications of using plant-based compost for clay soil amendments in growing *M. oleifera* plants

The overall outcome of the study affirms that addition of plant-based compost to clay soil increases the content and availability of metals in *M. oleifera* leaf biomass. Nutrient elements like Ca, K, Mg, P and S found in high concentration in *M. oleifera* biomass studied are major nutrients needed for humans and animal. The availability of trace metals is influences by their chemical composition, some soil amendments can alter the physiochemical properties of the trace elements to more available forms (Ukalska-Jaruga *et al*., 2022). However, in the current study it was noted that the addition of plant-based compost did not significantly increase the availability and accumulation of trace metals. Thus plant based compost is recommended for increasing essential elements in the soil and *M. oleifera* leaves and also for the suppression of trace and or heavy metals that could be present in the soil. In addition nutrient concentration of *M. oleifera* leaves grown under plant-based compost soil amendments are recommended for use as nutrient supplement for both humans and animals. In this study it was discovered that addition of 30% plant compost to clay soil gave the highest amount of major nutrients of Ca, K, Mg, P and S. This composition is therefore recommended for improved nutrients in *M. oleifera* leaves at the *M. oleifera* farms grown in clay soils. Careful studies are also needed before plant based compost is added to increase nutrient content of *M. oleifera* biomass as this also depends on the soil and to avoid increasing the concentration of trace elements above maximum permissible limits.

3.6 Appendix 1

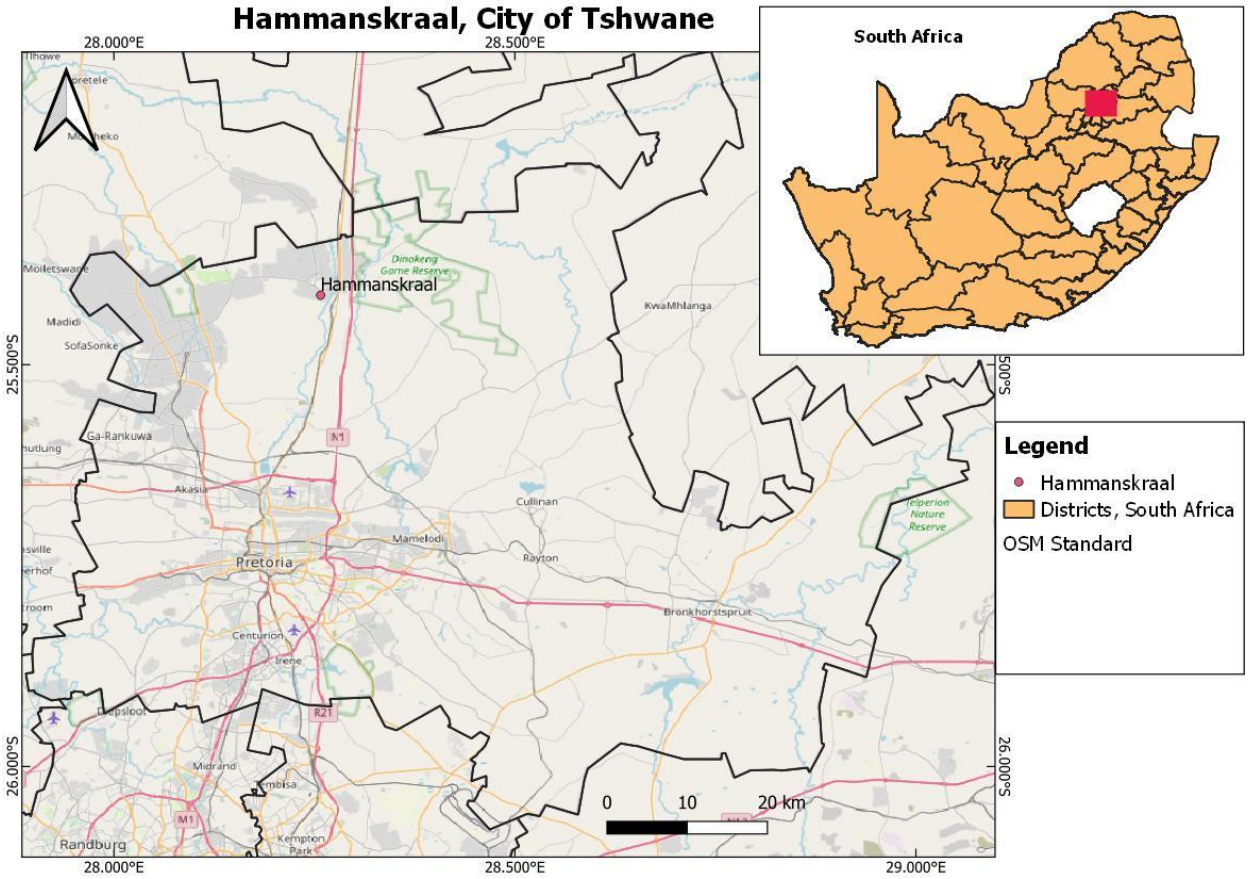

Figure 3.4: Map indicating the collection site of the soil and *M. oleifera* seeds used in the study

Figure 3.5: The clay soil used in the current study with evidence of cracks when dry

Figure 3.6*: Moringa oleifera* plants in the Greenhouse

3.7 References

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Chapter 4

4. Metabolomics profiling of *Moringa oleifera* grown in clay soil amended with different levels of organic plant-based compost: A greenhouse study

Ngwenya, N [1], Naupia, Y[1, 2] Morifi, E, Risenga, I[1], Chimuka, L.[2]

4.1 Abstract

Organic soil amendments are esteemed by scientist and farmers as a healthier and sustainable way of producing nutrient rich organic plants. There is however, still limited scientific information on the influence of plant-based organic soil amendments on metabolites found in medicinal plants such as *Moringa oleifera*. The current work explored the potential of unsupervised machine learning and ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry in analysing the impact of plant-based organic compost on the metabolite profile of *M. oleifera* leaves. Clay soil was mixed with 15%, 30%, 45% and 60% plant-based organic compost by volume in 20cm wide 2 L pots. The plant-based compost was made out of diverse plant materials. *Moringa oleifera* seedlings were planted in each treatment and control in four replicates. Forty-five metabolites were tentatively identified, forty-four of which were identified from all the soil compositions and one (leu/lle) was unique to 30% compost soil composition. Principal component analysis revealed variation in the intensity of selected metabolites in *M. oleifera* leaves among the different plant-based compost amendment levels. Quercetin and Kaempferol diglycoside were high in 30% compost whilst Glucomoringin, and Quercetin glycoside were high in 15% compost and control and respectively. The results indicate that amending the soil with plant-based compost has a positive effect on secondary metabolites distribution of *M. oleifera*. Application of organic plant-based compost to the soil has potential in assisting *M. oleifera* producers with a desire to increase targeted polyphenols organically. The 30% was the preferred soil combination for the clay soil, having the highest number of identified metabolites.

Key words

Moringa oleifera, metabolomics, plant-based, organic compost

4.2 Introduction

Changes in the lifestyle of many societies due to industrialization have resulted in increased scientific research on achieving a healthier lifestyle. This has resulted in increased knowledge of the direct impact of diet on the human health (Guthrie *et al*., 1999). Foods rich in bioactive compounds are essential in promoting and improving good health (Teodoro, 2019). Therefore, identification of the bioactive compounds is essential in the development of nutraceuticals and food functions targeting the improvement of human health (Gil-Chávez *et al*., 2013). Bioactive compounds such as secondary metabolites which are synthesized by plants are understood to have the potential in minimizing risk of a number of chronic diseases such as cancer, Alzhemier's, diabetes and cardiovascular diseases risk (Acquaviva *et al*., 2021).

Commercial cultivation of herbal plants has steadily increased worldwide in order to satisfy consumers constantly requiring products with bioactive compounds (Managa *et al*., 2021). The use of herbal medicine also skyrocketed when the Covid-19 pandemic hit the world as communities and researchers sought for preventative and therapeutic agents to help fight the disease (Lam *et al*., 2021; Ang *et al*., 2020; Nugraha *et al*., 2020). One such plant which has attracted attention because of its bioactive compounds is *Moringa oleifera*.

Moringa oleifera (Moringa) is a member of 13 species of the Moringaceae family, it is popularly referred to as horse radish and is native to India (Guzmán-Albores *et al*., 2019)[a]. Moringa is used in the food and biochemical industry and it is of notable economic importance, as all the plant parts (flowers, seeds, pods, leaves, bark, twigs) are useful as medicine, nutritious vegetable, spice, forage, nectar for bees and cosmetics (Tufarelli, 2019; Kleimanet *et al*., 2008). It has been extensively used as a nutraceutical (Khattak *et al*., 2020) because of its key constituents of minerals, vitamins, proteins and bioactive compounds (Managa *et al*., 2021). Industrially Moringa seeds are used as an effective coagulant in water purification (Adline, 2014), animal feed, green fertilizer and pesticide (Abd El-Hack *et al.*, 2018). Due to its highly nutritive content and its herbal properties, it is the most researched and studied specie

among the 13 species of the genus (Ranie *et al*., 2018) and is consumed by a significant population in South Africa and the world at large as a dietary supplement (Pakade *et al.*, 2013, Asiedu-Gyekye., 2014). The leaves are the high marketable part of the plant in South Africa (Bopape-Mabapa *et al*., 2020).

The above mentioned herbal benefits of Moringa are due to the presence of bioactive compounds in the plant (Managa *et al*., 2021). *Moringa oleifera* is a powerhouse of antioxidants like Vitamin C, flavonoids, flavanols and polyphenols. Flavonoids such as quercetin and kaempferol which are present in abundance in Moringa are responsible for alleviating various ailments including cancer and cardiovascular diseases (Ay *et al*., 2021). Moringa leaves taken orally have shown an ability to cause the death of cancer cells (Kashyap *et al*., 2020) in the human body, this could be attributed to them containing phytochemicals such as isopropyl isothiocynate, D-allose, niazimicin, niazirin, β-sitosterol3-O-β-D-glucopyranoside, and hexadeconoic acid ethyl ester (Guevara *et al*., 1999).

Metabolomics or metabolic profiling is the scientific study of the presence of metabolites in plant tissue (Kumar, 2016). It is essential in determining all the metabolites (sugars, amino acids and fatty acids) and their distribution pattern and thus revealing biological differences between samples (Gonzalez and Pierron, 2015). It is a clear gauge of the interaction between the biological organism and the environment (Lankadurai *et al*., 2013), and noting phenotypic changes (Gomez-Casati *et al*., 2013) and thus resulting in the improvement of yield and quality (Hong *et al*., 2016).

Plant secondary metabolites or phytochemicals content are influenced by genetic and various environmental factors namely season, genetics, post-harvest processing, cultivation practices and climate (Li *et al*., 2020; Borgs *et al*., 2017). These factors have also been noted to greatly influence secondary metabolite content in *M. oleifera* (Managa *et al*., 2021, Lin *et al*., 2020). Agronomic practices such as water availability, soil type and organic soil amendments also have an impact on the quantity of metabolites in Moringa (Förster *et al*., 2015; Guzmán-Albores et al., 2019) [b]. Among the environmental factors affecting metabolite content in plants, soil amendments have revealed a noticeable impact, for example, lettuce grown under different fertilization

had certain sugars being influenced by the type of fertilizers applied to the soil (Matamoros *et al*., 2021). Similarly, bioactive compounds in *Bupleurum L. (Apiaceae)* also increased with the addition of nitrogen (N) to the soil (Sun *et al*., 2021). Addition of biofertilisers in the soil also resulted in changes in metabolite profile for Zea mays (Brusamarello-Santos *et al*., 2017). The afore-mentioned environmental factors can be manipulated to influence secondary metabolites and thus improve the quality of medicinal plants such as Moringa (Ncube *et al*., 2021).

There are various ways of cultivating *M. oleifera* but most strive to increase biomass and nutrition of the leaves (Bopape-Mabapa *et al*., 2020). This is so because leaves are the most used part of the plant due to their high nutritive value (Förster *et al*., 2015). *Moringa oleifera* leaves are the popular part of the plant as they can be used as a vegetable (cooked or raw) and can be stored dry for longer and still retain most of its nutrients (Kashyap *et al*., 2022). Variations in leaf biomass and secondary metabolites have been reported in Moringa cultivars due to location and cultivation practices (Lin *et al*., 2019; Förster *et al*., 2015). Organic soil amendments such as compost are sustainable way of soil amendments that enhance the soil nutrients and physiochemical properties. Application of vermicompost in *M. oleifera* grown in Mexico revealed that in comparison with the control vermicompost positively improved metabolite profiling (Manzano-Gómez *et al*., 2021). These results indicate that high levels of nutrients in the soil and their availability have an impact on the plant metabolism which in turn influences the biosynthesis and accumulation of plant secondary metabolites (Kolton *et al*., 2022).

Moringa oleifera production has steadily increased in the past years with the increased commercialization of its products (Teclegeorgish *et al*., 2021). This has resulted in the formation of organizations dedicated to promoting its production and consumption. Identification of chemical composition in *M. oleifera* particularly secondary metabolites have recently been investigated in South Africa (Teclegeorgish *et al*., 2021; Makita *et al*., 2016). However, according to our knowledge, there has not been any prior investigation conducted to determine the effect of organic compost in boosting the quality and quantity of metabolites in *M. oleifera* leaves. The improvement of the quality and quantity of metabolites is especially critical for a commercial plant like M. oleifera which is marketed for its secondary metabolite content amongst other benefits.

Therefore the focus of the present study was to discover the best plant-based compost/ soil proportion ideal for boosting metabolites in *M. oleifera* leaves.

4.3 Materials and Methods

4.3.1 Plant material, treatments and sowing

The plant growth experiment was carried out in the greenhouse at the School of Animal, Plant and Environmental Sciences (APES), University of the Witwatersrand under ambient temperatures. *Moringa oleifera* seeds and the clay soil were obtained from a community *M. oleifera* farm in Hammanskraal. A stainless steel spade was used to collect the top soil (0-15 cm). The soil was air-dried, homogenised and sieved in order to remove gravel, debris and clogs. The seeds were firstly primed for 24 hours and thereafter planted into potting soil filled germination trays watered every two days for four weeks. Healthy seedlings were transferred into 2 L pots containing clay soil as control and clay soil amended with four different levels (15%, 30%, 45% and 60%) of organic plant-based compost as treatments. The percentages for each compost-soil combination was by volume of the total mixture. Each soil treatment was replicated four times. After transplanting, the plants were watered for two weeks with approximately 100 mL of water per day. Thereafter watering was done twice a week, and plant growth was monitored for six months.

4.3.2 Chemicals

Analytical grade reagents from Sigma-Aldrich were used throughout the study. These are HPLC/Ms grade Methanol. Deionized water was produced through a Millipore DirectQ 3 UV waters system-2345 from Millipore (Massachusetts, USA).

4.3.3 Metabolite extraction

Extraction of metabolites was done according to the method of Makita *et al*., (2016) with a few modifications. Briefly, 2 g of *M. oleifera* leaf powder was extracted with 20 mL of 80% aqueous methanol (MeOH) by sonicating for 30 min using a professional ultrasonic cleaning bath (Eins Sci- United Scientific). The samples were then centrifuged at 5000 rpm for 10 min at room temperatures (25 °C). The supernatant liquid was then filtered through Whatman's No.1 filter paper, the resultant liquid was then decanted into a round bottom flask and the solvent evaporated to complete

dryness using a rotary evaporator under reduced pressure at 45 °C. The dried extracts were reconstituted in 2 mL 50% MeOH and then filtered through 0.22 µm nylon filters. The resulting extracts were then stored in a freezer at −20 °C to avoid degradation until they were analyzed on the UHPLC-QTOF-MS.

4.3.4 UHPLC-QTOF-MS/MS analysis

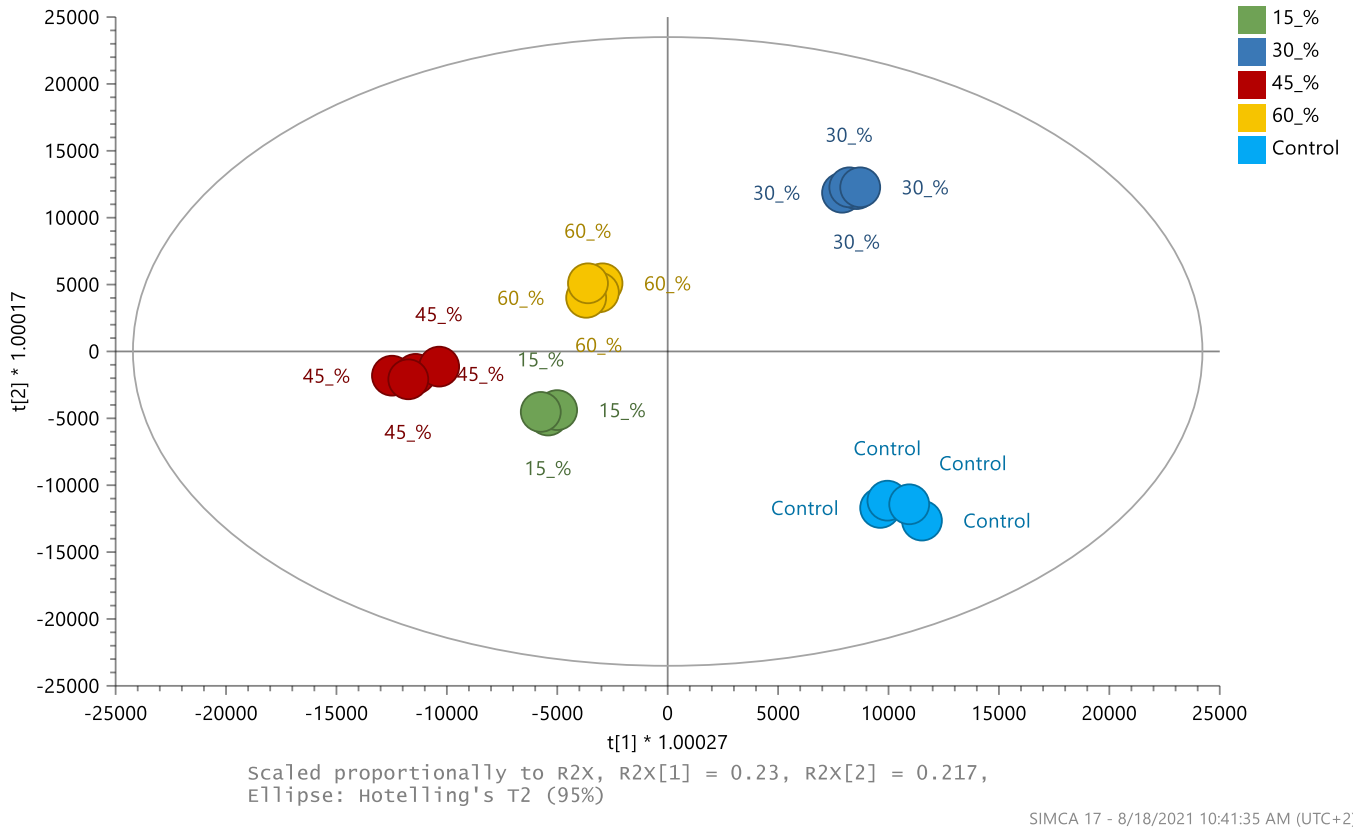
The 3000 Series Rapid Resolution LC system (Ultimate 3000, Thermofisher South Africa), equipped with a vacuum degasser, a binary pump, an autosampler, and a Diode Array Detector (DAD) was used for the separation and analysis of compounds. Ultra-high-performance liquid chromatography method procedure was performed as described by Ralepele *et al*., (2021) with some modifications. Chromatographic separation was performed on an XTerra® C18 (2.1 mm x150 mm, 2.5µm particle size) (Luna, South Africa) using water (0.01% formic acid) and Methanol (0.01% formic acid) as mobile phase A and B, respectively. The flow rate was set at a flow rate of 0.50 mL min^{-1}. The gradient was programmed as follows: 0 min /5% B; 10 min/35% B; 15 min / 95% B; 25 min / 5% B. The injection volume in the UHPLC system was 20 µL. It was coupled to a QTOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with Electrospray Ionization (ESI) operating in negative mode. Detection was carried out in a mass range of 50-1300 m/z and using a capillary voltage of + 4500 V, a dry gas temperature of 210°C, and a dry gas flow of 8.0 L/min, a nebulizer pressure of 2.0 bar, and a spectra rate of 1 Hz. To attain the mass accuracy necessary to identify the compounds, external instrument calibration was used. For this, the calibrant used was sodium formate clusters consisting of 5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% formic acid. This calibrant was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the Ion source, equipped with a Hamilton syringe (Reno, NV, USA). The accuracy of the mass data for the molecular ions was controlled by the Data Analysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormulaTM editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/ maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

4.4 Results and Discussions

4.4.1 Principal component analysis (PCA) comparison of the samples

Principal component analysis (PCA) was performed on the spectral data obtained in the current study to expose any form of clustering of the samples. The accumulated proportion of variance contribution rate was R2X= 0.425, and Hotelling's T2 was at 95% (Figure 4.1(a). The PCA was executed on the control and the different soil amendments (15% compost, 30% compost, 45% compost, 60% compost) using the Simca®17, Umetrics. The PCA 2D score plot clustered the 5 five samples into 5 distinct clusters. The data analysis revealed different metabolite distribution patterns in leaves harvested from 15% compost, 30% compost, 45% compost, 60% compost soil amendments the black soil (as control), giving rise to 5 distinct clusters (Fig 4.1a). This indicated variation in the chemical constitution of metabolites from each soil type. Further analysis was conducted using the supervised discriminant analysis of the data using the OPLS-DA statistics model (Fig 4.1b). This analysis intended to identify the compounds which are responsible for the clustering of the samples into 5 groups. The results from the OPLS-DA loadings plot revealed bulk clustering of compounds at the centre which are shared by all the tested samples. Distinguishing compounds for each sample were located at a distance from the centre (4.1 b). The noted distinguishing compounds were further identified in Table 4.1 according to their mass-to-charge ratio (*m/z*) and their retention time (*Rt*). Three and two compounds were tentatively identified as potential biomarkers for the control soil and the 15% compost amendments respectively, whilst seven and two compounds were noted as potential biomarkers for 30 and 45% soil amendment respectively (Fig 4.1 b). Finally three biomarkers were associated with the 60% soil amendment. The highest number of possible biomarkers were identified under the 30% compost soil amendment cluster. Non target analysis was used in this study, this type of analysis ensures that important variables are not missed when not targeted (Zhang *et al*., 2019).

a)



b)

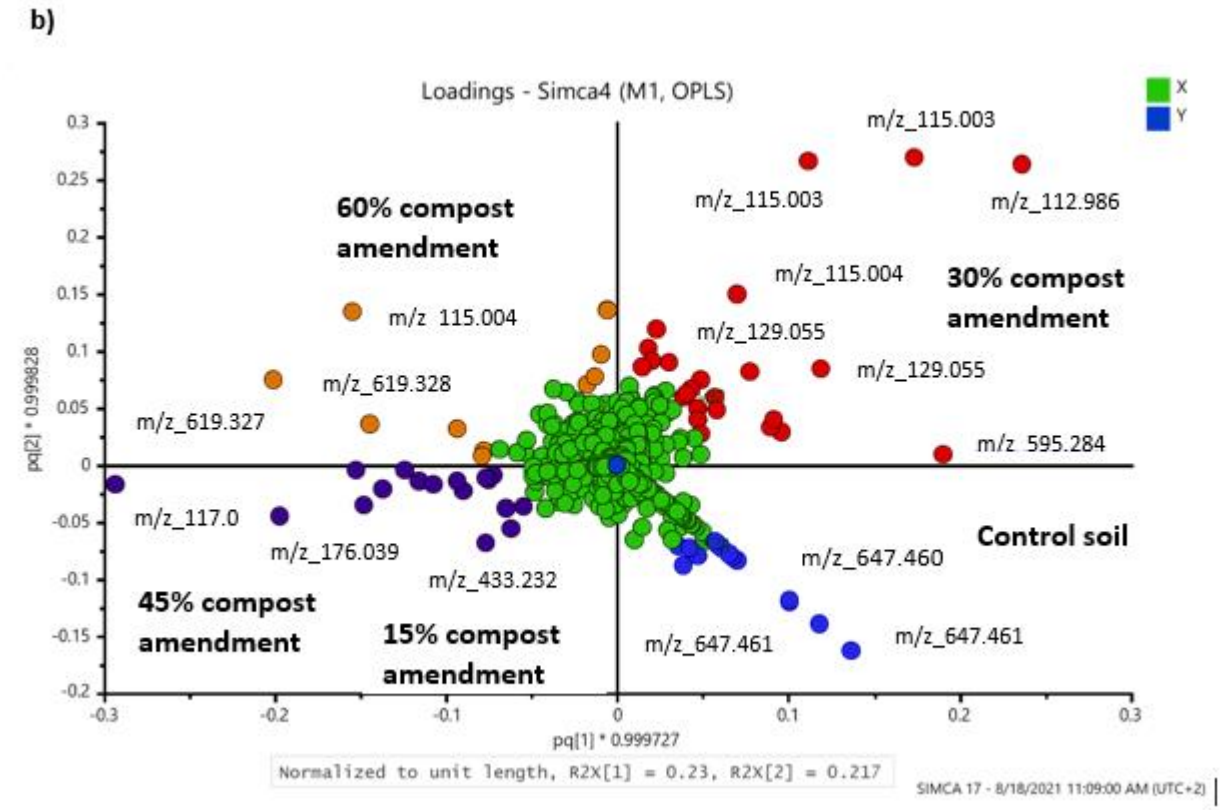


Fig 4.1 (a) and (b): Classification of the secondary metabolites *in* M. oleifera *leaves* based on the similarities within the different soil amendment.

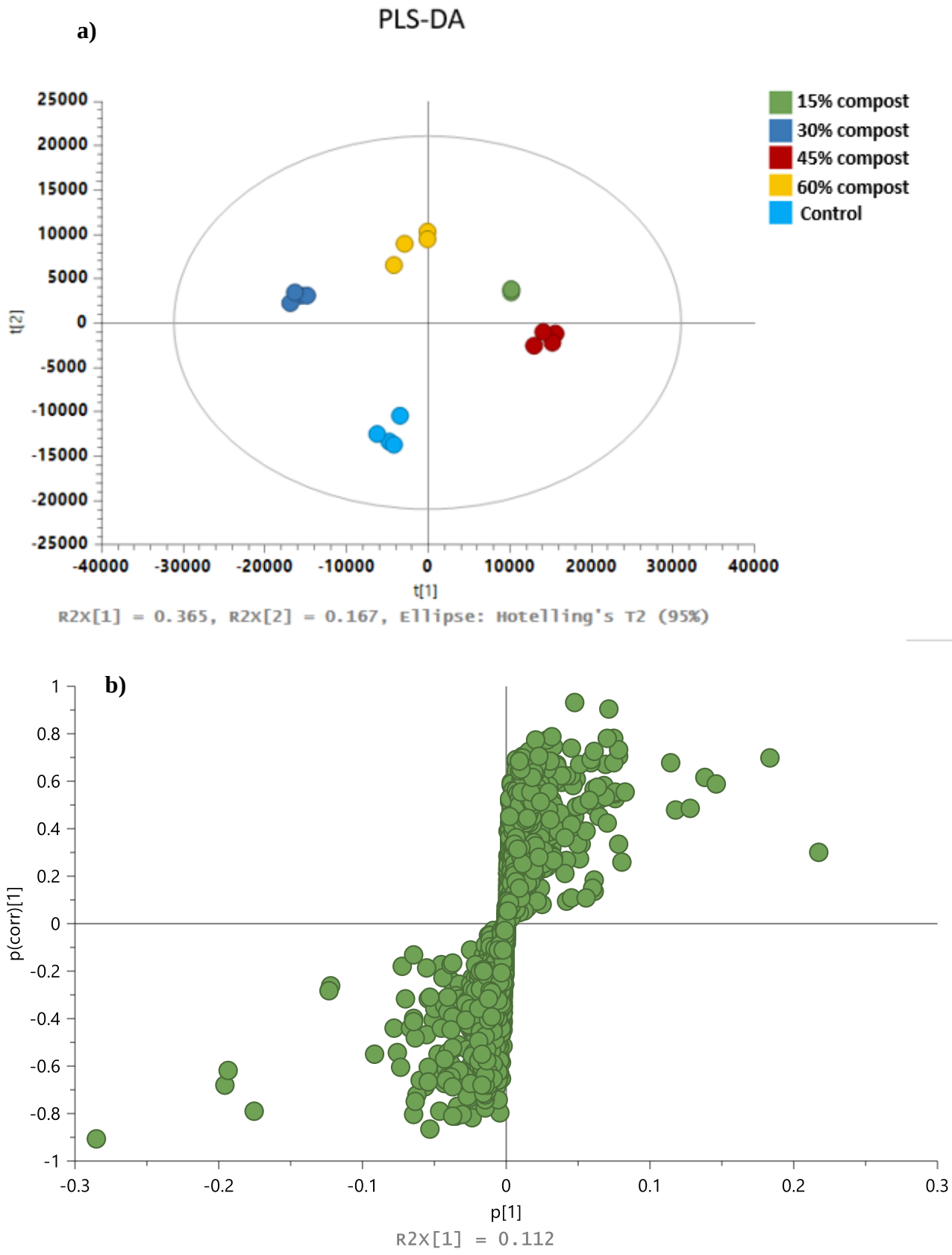


Figure 42.2: OPLS-DA scatter plot (a) and the Loadings S-plot for metabolites in *M. oleifera* leaves harvested from the four treatments and control soil.

The supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) (Figure 4.2 (a)) further revealed separate clusters of the different soil amendment treatments. This is an indication of substantial chemical differences between the various plant-based compost treatments and the control clay soil. The S-plot (Figure 4.2 (b) was then used to reveal the covariance and correlation within OPLS-DA results. The variables which are on each of the extreme end of the S-plot are an indication of potential biomarkers (possible metabolite) influencing the clustering of the samples.

The results from the current study propose that the addition of plant-based compost to the soil alters the metabolite profile of *M. oleifera* leaves which was noted by the different clustering patterns based on the variation in secondary metabolite content (Fig 4.1a). This occurrence was also discovered in *Brassica rapa* L. var. Nipposinica where manure soil amendments altered the metabolite profile of the plant (Okazaki *et al*., 2016). This could be attributed to the N absorption which influences the formation of plant secondary metabolites, organic matter chemical compositions affects the absorption of N (Dahlin *et al*., 2005). Organic soil amendments also increased the total flavonoid and total phenolic content in *M. malabathricum* L. leaves (Rusli *et al*., 2022). It has also been established that addition of organic soil amendments increases elicitors which in turn are involved in the elicitation of plant cells to stimulate the biosynthesis of secondary metabolites (Rusli *et al*., 2022; Naik and Al- Khayri, 2016). The concentration of compost also has an impact on the metabolomics differences in plants, which was observed by the variation in the clustering of *M. oleifera* metabolite from different compost levels, the same results were reported in *Brassica rapa* ssp. *chinensis* (Neugart *et al*., 2018). *Moringa oleifera* leaves are well-known for their high flavonoid content (Saini *et al*., 2016), which is connected with flavonoids being a greater portion of the identified metabolites in the current study.

4.4.2 Identification of Moringa leaf metabolites

The compounds were first identified by interpretation of their MS and MS/MS spectra data determined by UHPLC-ESI-QTOF-MS (Figure 4.3 (a, b, c, d) and Table 4.1). A total of 45 metabolites (most of which have been identified in a similar matrix) listed in Table 4.3 were tentatively identified according to retention time, molecular masses, and fragmentation patterns. The Bruker software was used for the identification of the

parent ion and the fragmentation pattern, Figure 4.3 (a, b, c, d) gives a representation of how the metabolites were identified.

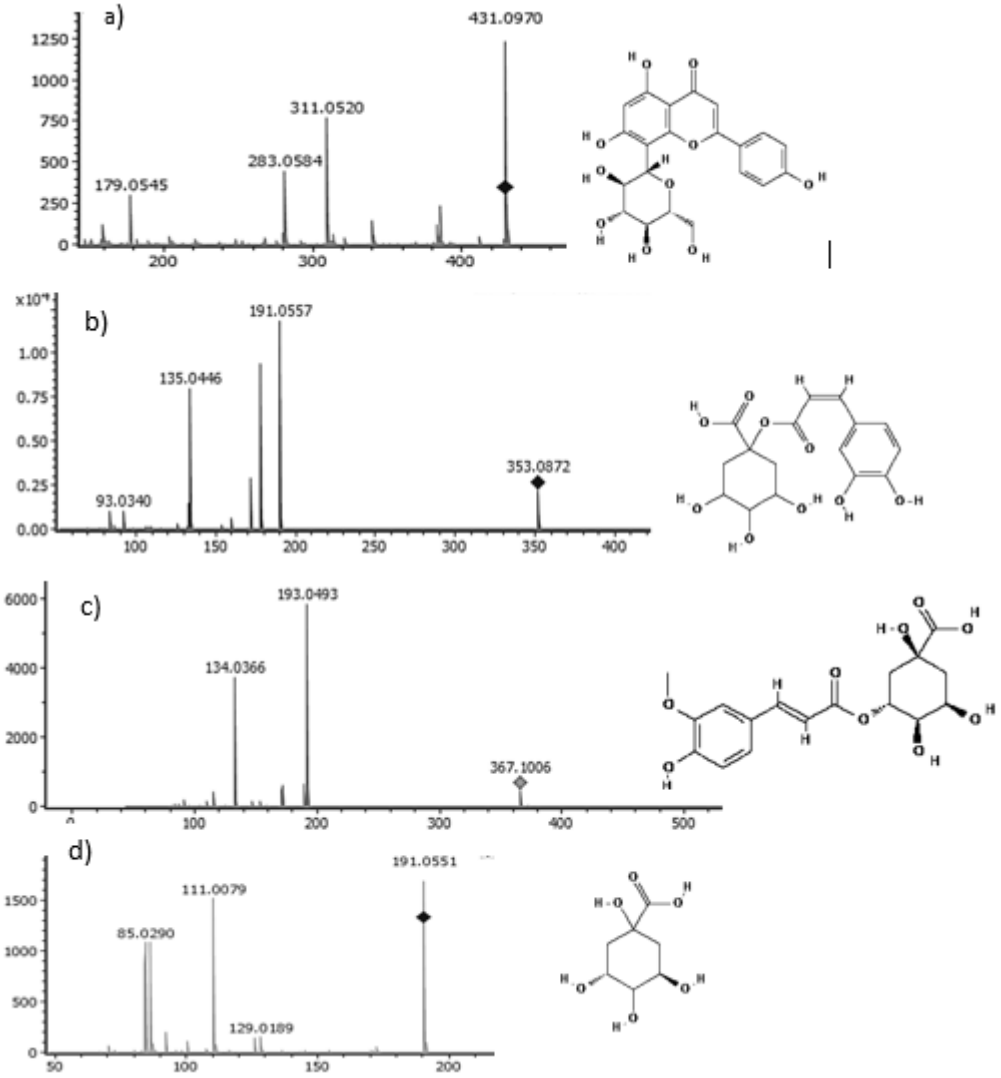


Figure 4.3: Representative UHPLC-MS/MS spectra indicating the parent ion and the fragmentation patterns a) Apigenin glucoside b) Caffeoylquinic acid isomer; c) Feruloylquinic acid; d) Quinic acid

A larger portion of the tentatively identified secondary metabolites belonged to glucosinolates and polyphenolics (flavonoids and chlorogenic acids) (Table 4.1). The compounds were identified by a screening analysis which included the comparison of the current data with previous literature and databases such as MassBank (http://mass bank.jp), Metlin Metabolite Database (http://metlin.scripps.edu) and SciFinder Scholar

(http://scifinder.cas.org). A total of 44 metabolites were tentatively identified in all soil types, whilst 1 compound was only identified in 30% compost (Table 4.1).

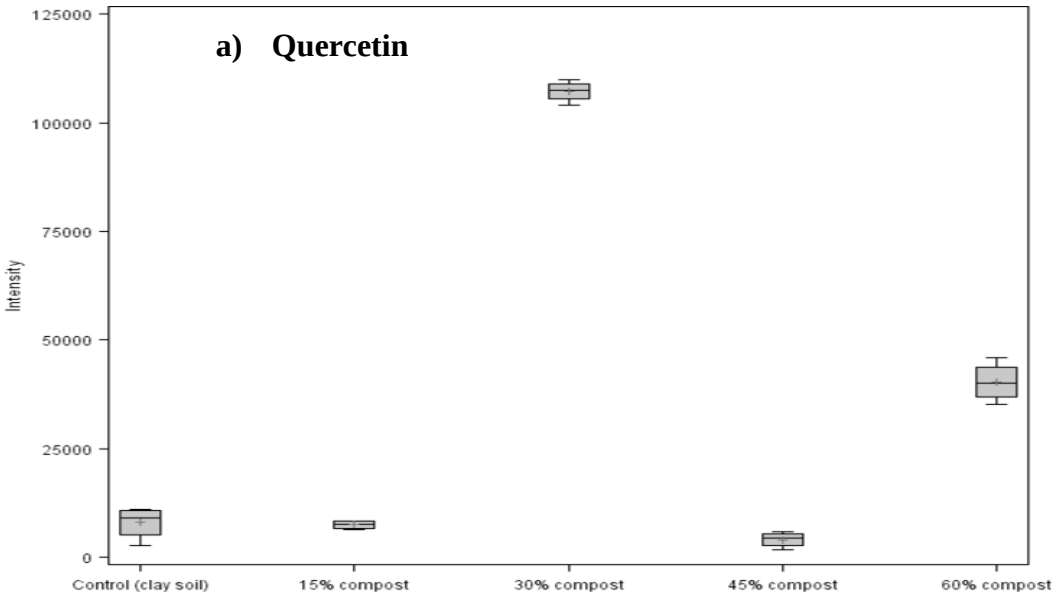
Table 4.1: HPLC-ESI-QTOF-MS data of the compounds identified in *M. oleifera* leaves harvested from clay soil amended with different levels of compost.

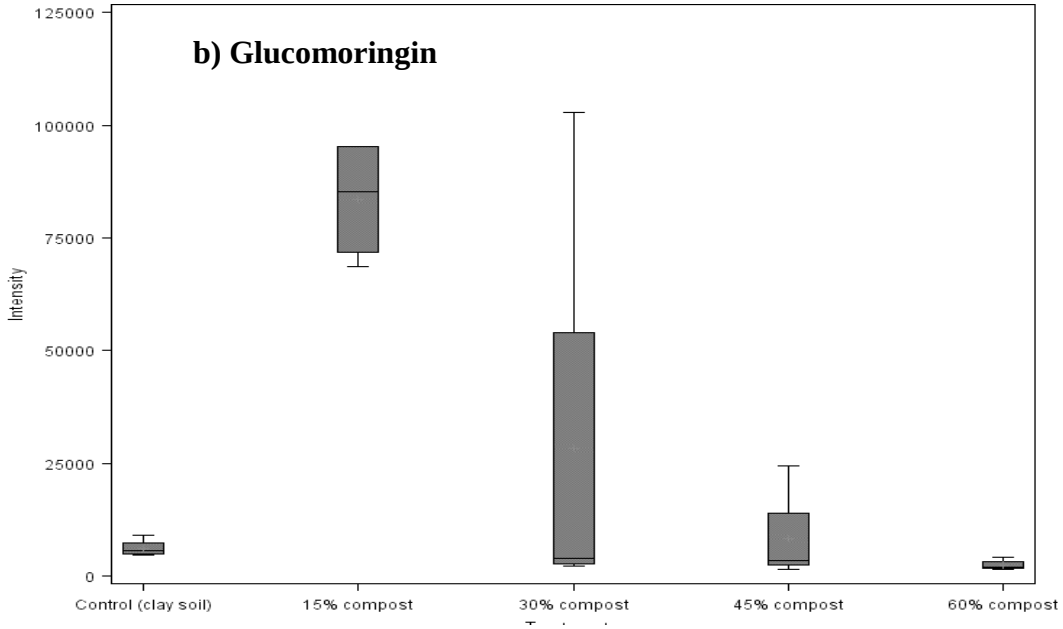
No.	RT Time (min)	Formula	Identification	Mass (m/z)	Fragments	Control	15 %	30 %	45 %	60 %
1	1.25	$C_6H_{12}O_7$	Gluconic acid	195.051	85.0303 (53.93%), 99.010 (30.37%), 159.0345 (27.75%), 173.0515 (24.61%)	×	×	×	×	×
2	1.31	$C_6H_8O_7$	Citric acid	191.020	85.0921 (60.7%), 87.0082 (79.8%), 111.0083 (100%)	×	×	×	×	×
3	1.32	$C_4H_5O_5$	Malic acid	133.014	71.0136(49.4%); 115.0036(100%)	×	×	×	×	×
4	1.34	$C_{16}H_{20}O_{10}$	Dihydroferulic acid 4-O-glucuronide	371.116	353.0865 (20.6%), 191.560 (70%)	×	×	×	×	×
5	1.68	$C_{16}H_{18}O_{35}$ 745057830 320_9	Caffeoylquinic acid isomer	353.086	135. 0449(62.4%), 173.0453 (26%), 179.0347 (71.4%), 191.0560(100%)	×	×	×	×	×
6	1.81	$C_{20}H_{20}$ $N_{14}O_{16}$	Apigenin Acetyl 4-(a-L-rhamnopyranosyloxy) benzyl glucosinolate isomer	612.105	96.959 (81.43%), 612.1047 (100%)	×	×	×	×	×
7	1.89	$C_{20}H_{29}NO_{1}$ $_4S_2$	Glucomoringin	570.094	96.9598 (29.2%), 570.0950 (100%)	×	×	×	×	×
8	1.98	$C_6H_{13}NO_2$	leu/lle	130.087	100.9302 (100%), 130.0871(100%)			×		
9	2.58	$C_{12}H_{22}O_{11}$	α-Maltose	341.108	89.0234 (14.8%), 179.0543 (22.8)	×	×	×	×	×
10	3.11	$C_{16}H_{18}O_8$	3-ρ-Coumarorylquinic acid	337.091	119.0493 (39.8%), 163.0389 (100%), 191.569 (35.5%)	×	×	×	×	×
11	3.20	$C_{11}H_{12}N_2O$ $_2$	L-tryptophan	203.081	74.0258 (30.07%), 116.0506 (93.96%)	×	×	×	×	×
12	3.58	$C_{20}H_{20}$ $N_{14}O_{16}$	Acetyl 4-(a-L-rhamnopyranosyloxy) benzyl glucosinolate isomer 2	612.103	96.9694 (69.95%), 612.1034(100%)	×	×	×	×	×
13	4.16	$C_{17}H_{20}O_9$	Feruloylquinic acid	367.100	134.0368 (65.2%), 193.0493 (100%)	×	×	×	×	×
14	4.31	$C_{16}H_{18}O_9$	Caffeoylquinic acid isomer	353.085	135. 0449(62.4%), 173.0453 (26%), 179.0347 (71.4%), 191.0560(100%)	×	×	×	×	×
15	4.76	$C_{21}H_{20}O_{11}$	Cyanidin hexose	449.105	96.9594(100%), 353.0875 (22.9%), 403.1942 (22.1%)	×	×	×	×	×
16	5.32	$C_{21}H_{21}O_{11}$	Luteolin-7-O-glucoside	447.090	161.0446(25%), 269.1028 (35.1%), 401.1436 (100%), 401.1436 (61.5%)	×	×	×	×	×
17	5.44	$C_{16}H_{17}O_7$	4'-O-Methylepigallocatechin	321.0984	142.0424 (100%)	×	×	×	×	×
18	5.47	$C_{26}H_{34}O_{11}$	Isolariciresinol glycoside/lariceresinol glycoside	521.199	133.0122 (100%), 179.0545 (56.1%), 521.2036 (66.6%), 523.2111 (45.8%)	×	×	×	×	×
19	5.82	$C_{27}H_{30}O_{16}$	Kaempferol diglycoside	609.141	300.0244 (78.5%), 609.1433(100%)	×	×	×	×	×
20	6.12	$C_{20}H_{20}$ $N_{14}O_{16}$	Acetyl 4-(a-L-rhamnopyranosylox	612.102	96.9597 (27.4%), 612.1054 (100%)	×	×	×	×	×

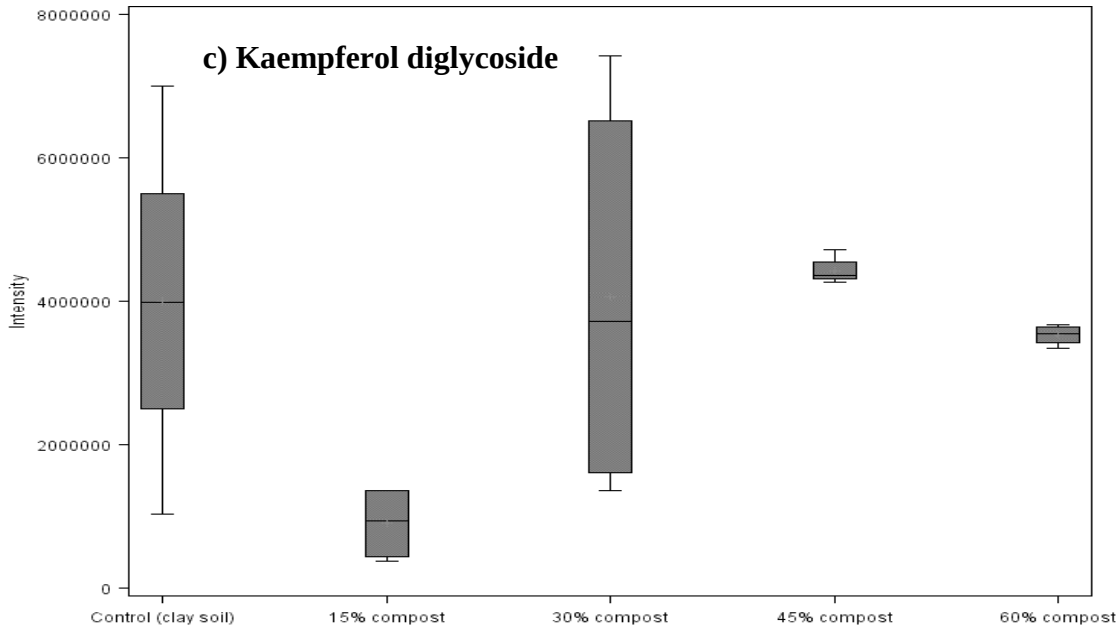
			y) benzyl glucosinolate isomer 1							
21	6.14	$C_{27}H_{28}O_{16}$	Quercetin-hydroxyl-methylglutarorly glycoside	607.126	503.3189 (53.50%), 521.3292 (15.89%), 563.3238 (74.30%), 607. 1255 (49.48%)	×	×	×	×	×
22	6.15	$C_{24}H_{22}O_{15}$	Quercetin 3-O-malonylglucoside	549.085	300.0286 (55.5%), 505.1010 (92.11%), 506. 0904 (21.17%)	×	×	×	×	×
23	6.38	$C_{27}H_{30}O_{15}$	Apigenin- 6.8 C-dihexose	593.147	383.0750 (24.4%), 593.1489 (100%)	×	×	×	×	×
24	6.43	$C_9H_{16}O_4$	Azelaic acid	187.096	125.0961 (100%), 187.0996 (24.3%)	×	×	×	×	×
25	6.58	$C_{27}H_{28}O_{15}$	Kaemperol glycoside-hydroxy-methylglutarate	591.131	284.0316 (87.4%), 285. 0375 (77.9%), 591.1335 (100%)	×	×	×	×	×
26	6.64	$C_{24}H_{22}O_{14}$	Kaempferol malonylglucoside glycoside	533.090	284.0299 (54.3%), 285.0385 (37.3%), 489.1005 (100%), 490.1081 (23.1%)	×	×	×	×	×
27	6.74	$C_{26}H_{32}O_{11}$	Pinoresinol/epipinoresinol glycoside	519.111	299.0171 (69.98%), 519.1069 (100%)	×	×	×	×	×
28	6.83	$C_{13}H_{16}O_8$	Benzoic acid 4-O-β-glucoside	300.085	183.1369 (100%), 227.1274 (23.7%), 255.2308 (77.4%)	×	×	×	×	×
29	6.9	$C_{21}H_{20}O_{10}$	(Vitexin) Apigenin glucoside	431.096	96.9588(61%), 311.0554(65.4%), 387.1997 (68.5%), 431.0975 (100%)	×	×	×	×	×
30	7.26	$C_{21}H_{20}O_{12}$	Quercetin glycoside	463.085	149.0440 (27.1%), 415.1580 (100%)	×	×	×	×	×
31	7.28	$C_{21}H_{20}O_{11}$	Kaempferol 3-O-glucoside	447.091	227.0335 (40.5%), 255.0279 (42.8%), 447.0901 (100%)	×	×	×	×	×
32	7.34	$C_{15}H_{18}O_{10}$	Dihydroca_eic acid 3-O-glucuronide	358.078	358.078	×	×	×	×	×
33	7754.31	$C_{23}H_{22}O_{13}$	Quercetin-acetyl-glycoside	505.095	96.96877(44.4%), 300.0269 (72.1%), 505.0996 (100%)	×	×	×	×	×
34	7.33	$C_{15}H_{16}O_{10}$	Cafeic acid 3-O-glucuronide	356.078	356.078 (100%)	×	×	×	×	×
35	7.34	$C_{28}H_{28}O_{11}$	Isorhamnetin glycoside	621.153	575.2727 (10.61%), 619.2737 (100%), 620.2909 (32.24%)	×	×	×	×	×
36	7.91	$C_{23}H_{22}O_{12}$	Kaempferol 3-O-acetyl- glycoside	489.101	255.0282 (27.6%), 284.0313 (32.5%), 443.1967(29.4%), 489.1009 (100%)	×	×	×	×	×
37	7.99	$C_{15}H_{10}O_7$	Quercetin	301.163	255.2319 (100%),	×	×	×	×	×
38	8.09	$C_{16}H_{18}O_{10}$	Ferulic acid 4-O-glucuronide	369.084	369.084	×	×	×	×	×
39	8.53	$C_{18}H_{32}O_5$	Trihydroxyoctadeca dienoic acid	327.215	171.0996 (12.6%), 327.2154 (100%)	×	×	×	×	×
40	8.93	$C_{10}H_{20}O_6$	n-Butyl-β-D-fructopyranoside	236.128	65.9975 (23.8%), 151.628 (23.4%), 208.1339 (20.4%), 236.1270 (100%)	×	×	×	×	×
41	9.2	$C_{12}H_{15}O_4$	5-(3'-Methoxy-4'-hydroxyphenyl)-gamma - valerolactone	223.097	177. 1022 (93.55); 136.0761(8.69)	×	×	×	×	×
42	9.28	$C_7H_{12}O_6$	Quinic acid	191.055	85.0289(39.1%), 87.0079 (34.8%), 111. 0081 (48.6%), 191.0557 (100%)	×	×	×	×	×
43	9.78	$C_{18}H_{34}O_5$	Tianshic acid	329.231	211.1329(15.3%), 229.1430 (14.4%), 328.2190 (14.5%), 329.2308 (100%)	×	×	×	×	×
44	13.18	$C_{27}H_{31}O_{16}$	Cyanidin 3,5-O-diglucoside	611.1521	611.1521	×	×	×	×	×
45	21.08	$C_{18}H_{30}O_2$	α-Linolenic acid	278.219	277.2160 (100%)	×	×	×	×	×

4.4.3 Relative quantification of selected secondary metabolites

Box and Whisker plots were used to determine the variation in the quantity of selected compounds in different soil amendments (Fig 4.4). The quantification was based on the peak intensity of each of the four selected metabolites namely Quercetin glycoside, Glucomoringin, Quercetin and Kaempferol diglycoside. A significant difference ($p < 0.05$) of the mentioned metabolites was noted within the samples harvested from the control soil and the soil amended with different levels of plant-based compost for each selected key metabolites (Fig 4.4). The presence of Quercetin glycoside, Glucomoringin and Quercetin were higher in 15%, 45%, 45% and 30% compost soil amendment respectively whilst Kaempferol diglycoside presence was lower in 30% compost and generally higher with no significant difference in all other soil samples (Fig 4.4). Variation in metabolite content and distribution in plants has been linked to geographic location and cultivar/genotype diversity (Makita *et al*., 2017; Zhang *et al*., 2014).


a) Quercetin
125000
100000
75000
50000
25000
0
Intensity
Control (clay soil)
15% compost
30% compost
45% compost
60% compost


b) Glucomoringin
125000
100000
75000
50000
25000
0
Intensity
Control (clay soil)
15% compost
30% compost
45% compost
60% compost



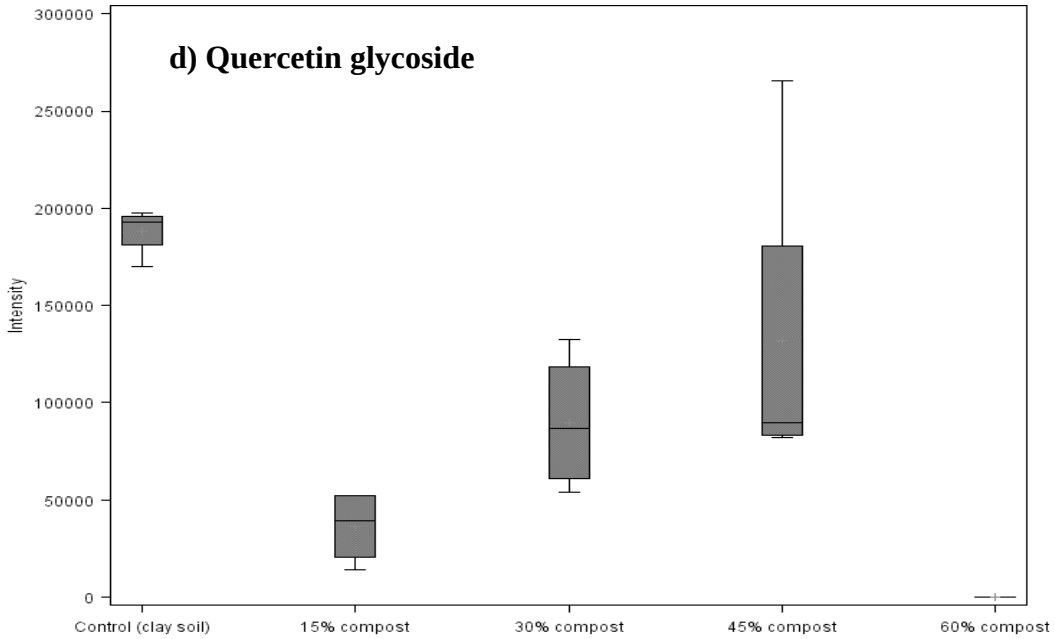


Figure 4.4 (a-d): Box and whisker plots for the distribution of four metabolites based on their intensity across *M. oleifera* leaves harvested from clay soil amended by different levels of plant based compost

As aforementioned, application of compost increases the availability of N in the soil (Lim *et al*., 2018). Changes in nitrogen content have reportedly affected changes in phenolic compounds in leafy vegetables (Groenbaek *et al*., 2014), phenolic compounds are precursors of several primary and secondary metabolites such as phenolic acids and flavonols (Okazaki *et al*., 2016). The same trend was observed in the current study where metabolites from the control soil varied from the samples with compost added on them (Table 4.1). The presence of other essential minerals in the soil such as Potassium (K) and Phosphorous (P) has an impact on plant metabolomics pathways and thus influences the quality and quantity of metabolites (Sung *et al*., 2015) consequently variations noted in the current study could be attributed to differences in mineral composition of the soil amendments.

4.5 Conclusion

The results from the study revealed that organically grown *M. oleifera* was rich in natural compounds, as the presence of identified metabolites increased with the addition of plant based compost. The type of compost in addition to the concentration has a bearing on secondary metabolites (Neugart et al., 2018) and thus it is expected that compost made from different plant materials will yield give rise to different metabolite profiles. Therefore further studies may be conducted to determine the metabolite profile of *M. oleifera* leaves grown in different forms of organic compost.

The tested *M. oleifera* plants in the current study were grown in the Greenhouse with controlled temperature and irrigation to rule out any other environmental factors such as climate and water availability. Genetic variation was also excluded since the same species were tested for the presence of metabolites. As a consequence the variations in metabolites noted in this study are then attributed to the different soil compositions from the different levels of plant-based compost. The outcome of the current study suggests 30% plant-based compost soil amendment as the recommended soil type for organically growing *M. oleifera*, the leaves harvested from this soil type exhibited the second highest percentage of selected metabolites. In addition, this soil amendment had the highest number of potential biological markers linked to its cluster. Findings in this study affirm that plant-based soil amendments have the capacity to enhance *M. oleifera* secondary metabolites.

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Chapter 5

5. A Rapid High-performance Liquid Chromatography method for the simultaneous quantification of five Water-soluble Vitamin in *Moringa oleifera* plants grown in clay soil amended with different levels of plant based compost

5.1 Abstract

Moringa oleifera is identified as a good source of water-soluble vitamins. A reverse phase liquid chromatography (HPLC) photodiode array detector and mass spectrometry method was developed and validated for the simultaneous quantification of the water-soluble vitamins on a C18 column. Limit of detection (LOD) and limit of quantification (LOD) values were within the ranges of 0.2-0.89 µg/mL) and 0.6-2.71µg/mL) whilst percentage recovery was above 97% for all analytes. Addition of compost increased the concentration of all tested vitamins except for Vitamin B2.

Highest concentrations were found for Vitamin B1 (9.6 mg/100g)-, B3 (42.1 mg/100g) and B6 (5.9 mg/100g) in 45% plant-based compost-soil amendment whilst B9 (9.6 mg/100g) was highest in 30% plant-based compost-soil amendment. The findings support using plant-based compost for improving the nutrient content of *M. oleifera* in a sustainable and environmentally friendly way.

Key words

Water-soluble vitamins, *Moringa oleifera*, plant-based compost, HPLC-UV

5.2 Introduction

Vitamins are essential micronutrients, organic in nature taken in smaller quantities for the proper physiological function of the body, they are key components of a balanced diet (Kennedy, 2016). Most of these essential nutrients cannot be naturally synthesized by human beings and are supplied to the body through diet to meet daily requirements (Drouin *et al*., 2011). They are responsible for the optimum function of chemical and physiological processes in the body, and thus maintaining good health

(Clifford and Curely, 2020, Abibu *et al*., 2019, Seal and Chaudhuri, 2017). Vitamins are classified into two major groups, namely fat-soluble and water-soluble vitamins (WSV). As the name suggests, fat-soluble vitamins are soluble in fat, they are mainly absorbed in the fatty filled intestines (Albahrani and Greaves, 2016). Fat soluble vitamins consists of vitamins such as A, D, E, K and some carotenoids with elements of Vitamin A**.** Water soluble vitamins on the other hand are soluble in water and are composed of Vitamin C and eight B complex Vitamins (B1 to B6, B9, B12) (Clifford and Curely, 2020). Vitamin B complex functions as a cofactor in many biochemical reactions in the body, each component has a specific role in the growth and development of a healthy body (Lykstad and Sharma, 2019).

Vitamin B1 (Thiamine) is a cofactor for enzymes responsible for carbohydrate and amino acid metabolism (Hrubša *et al*., 2022) deficiency may lead to disorders of the nervous system (Fitzpatrick and Chapman, 2020). Vitamin B2 (Riboflavin) on the other hand is responsible for the production of red blood cells and assist in energy release from proteins (Mahabadi *et al*., 2022). In addition, Vitamin B2 in conjunction with Vitamin B3 (Niacin) and Zinc enables the function of Vitamin B6 (Pyridoxine) which in turn acts as a coenzyme for numerous reactions in the body such as the production of neurotransmitters, metabolism of fats, carbohydrates and proteins (Stach *et al*., 2021). Vitamin B9 (Folate) is involved in the synthesis of DNA (Knyazev *et al*., 2016) its deficiency leads to various cancers and neurological diseases such as the fetus neural tube defects (Ebara, 2017).

The human body is able to produce water soluble vitamins with the exception of niacin which can be synthesized by the liver from the amino acid tryptophan (Said, 2011). In addition the body is unable to store water soluble vitamins for more than a day due to their nature, they are also easily destroyed during food preparation (Khaneghah *et al*., 2019). They therefore, need to be taken daily in form of a diet or supplement to meet the daily body requirements (Kennedy, 2016).Plant based diets and supplements have been a great source of these nutrients, more so organically grown plants as they are of a better nutritional quality (Hever and Cronise, 2017). *Moringa oleifera* is a treasured herbal plant widely gaining popularity around the world because of its therapeutic and nutritional properties (Abdull Razis *et al*., 2014). Among many of its beneficial properties it is identified as a source of water-soluble vitamins (Witt, 2013). The

present study was conducted to investigate the concentration of five water soluble vitamins (Vitamin B1, B2, B3, B6 and B 9) in organically grown *Moringa oleifera*.

Cultivation of *Moringa oleifera* has increased over the years as more and more consumers are becoming aware of its therapeutic properties (Nwafor, 2020) Organically grown plants and vegetables are popularly known to be of high quality as they are grown without use of artificial fertilisers and chemical which may potentially be hazardous to health (Trisilawati *et al*., 2020). Although the yield and productivity of organically grown plants is lower than that of conventionally grown ones (Abou-Sreea *et al*.,2021; Premamali *et al*., 2019), organically grown plants generally have a higher nutrient content which gives rise to the efficacy of their products (Trisilawati *et al*., 2020).

Moringa oleifera is a highly nutritive herbal tree native to India which has been naturalized and extensively cultivated across Asia and Africa (Sujatha and Patel, 2017). *Moringa oleifera* plant has a high concentration of minerals, phytochemicals and vitamins (Islam *et al.,* 2021). The leaves are particularly recommended for their high Vitamin content (Grosshagauer *et al*., 2021). Water soluble vitamins were reported to be in impressive levels in *M. oleifera* leaves (Sultana, 2020).

The juggle between feeding the ever increasing world population projected to be 9.8 billion by 2050 while simultaneously preserving the environment from degradation is a tough one (Fitzpatrick and Chapman, 2020). In order to satisfy the need of consumers, farmers are more interested in producing plants designed at meeting the daily requirements in terms of calories and essential micronutrients. Organic farming is a sustainable way of food production which preserves the environment (Golijan and Sečanski, 2021). The nutritional content of edible plants is largely influenced by various agronomy practices encompassing soil and plant management. The soil nutrients are directly proportional to the accumulation of nutrients in plant leaves (Premamali *et al*., 2019).

Comparing B vitamins content between plants growing under organic fertilisation and inorganic fertilisation revealed that organic soil amendments gave higher levels of vitamins such as Vitamin B1 (thiamine) and Vitamin B 12 (cyanocobalamin) (Mozafar,

1994). Vitamin C content in tomato plants significantly improved in plants grown in soil mixed with plant residue compost (Khan *et al*., 2017). The concentration of B vitamins concentration is affected by several environmental factors such as light, temperature, soil type and nutrients (Kolton *et al*., 2022). Soil type have been recorded to have a direct impact on B-complex content in plants, for example melons growing in different types had varying levels of Vitamin B9 (Lester and Crosby, 2002). Addition of compost to the soil increases the abundance of nutrients such as nitrogen, phosphorous and boron in the soil, abundance of these nutrients led to the increase of the B vitamins in plants (Mozafar, 1994). Nitrogen has been identified as one of the building blocks of niacin (Vitamin B3).Organic soil amendments were also reported to have improved the synthesis of B-complex vitamins (Kolton *et al*., 2022). Addition of organic manure during the growth of carrots increased their concentration of B-complex vitamin (Lester, 2006).

Moringa oleifera has been produced for a while across the globe. However, there is no known plant based organic fertilization formula existing for this species. In addition, no known studies have been conducted to examine the influence of different doses of plant based organic fertilizers on the quantity of water soluble vitamins in *M. oleifera* leaves. A number of HPLC methods have been developed for the quantification of Vitamin C, a water soluble vitamin in *M. oleifera*. Yet, methods designed for the simultaneous quantification of a number of B vitamins in the plant are very scarce. Essentially, there has not been any recorded methods for the simultaneous quantification of B vitamins in *M. oleifera* using HPLC-UV and mass spectrometry for detection. Based on that ground the current study therefore reports for the first time the HPLC-UV method for the simultaneous quantification of B vitamins in Moringa and the impact of plant based compost on plant vitamin content. In addition, the developed HPLC quantification method was also used for the first time to simultaneously quantify water soluble vitamins from *M. oleifera* leaf biomass.

5.2.1 Chemicals

The analytical grade vitamin standards thiamine (Vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pyridoxine (vitamin B6) and folic acid (Vitamin B9) were purchased

from Sigma- Aldrich. HPLC grade Acetronitrile and Triflouroacetic acid (TFA) were also purchased from Sigma-Aldrich.

5.2.2 Instrument

The LC system used for the separation and analysis of compounds was the Thermo Scientifc Dionex Ultimate 3000 from ThemoFisher Scientifc (Waltham, MA, USA). The LC was coupled to the diode array detector (DAD) and the compact quadrupole time-of-fight mass spectrometer system (QTOF) from Bruker Daltonics Inc. (Billerica, MA, USA) equipped with Electrospray Ionization (ESI) operating in positive mode. Detection was carried out in a mass range of 50-1300 m/z and using a capillary voltage of + 4500 V, a dry gas temperature of 220°C, and a dry gas flow of 9.0 L/min, a nebulizer pressure of 1.8 bar. The accuracy of the mass data for the molecular ions was controlled by the Data Analysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormulaTM editor.

The UV detector was used to detect the vitamins and the mass spectrometer was used to identify the compounds. To attain the mass accuracy necessary to identify the compounds, external instrument calibration was used. The calibrant used was sodium formate clusters consisting of 5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% formic acid. This calibrant was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the Ion source, equipped with a Hamilton syringe (Reno, NV, USA).The B-vitamins were identified by time, however for precision and reference the mass spectrometry was further used for identification. Bruker Compass Quant Analysis version 4 was used for collection and processing of quantitative data. Chromatographic separation of the B vitamins was achieved on a *Synergi* C18, 150 × 4.6, 4 µm particle size was from Phenomenex (California, USA).

Figure 5.1 exhibits the product ion spectra for each tested water soluble vitamin.

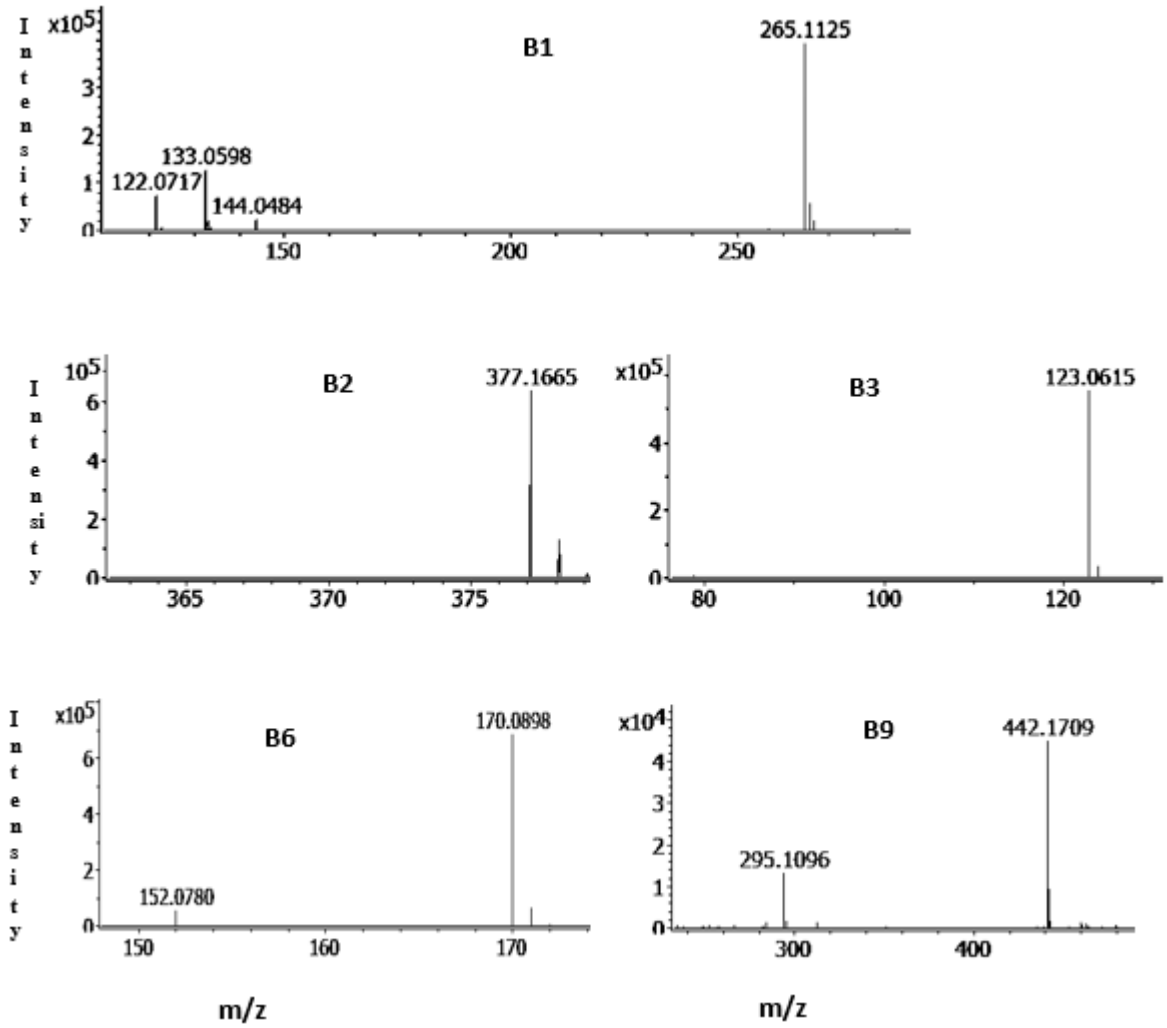

Figure 5.1: MS/MS spectra of water-soluble vitamins, indicating the precusor ion and the fragments.

5.2.3 Preparation of standards

The preparation of standards was according to the method by Seal et al., (2018) with few modifications. Briefly, the stock standard solutions of Vitamin B1, B3 and B6 and were prepared by dissolving 25 mg of the each standard in 1 ml 0.1 M hydrochloric acid (HCL) in 25 ml standard volumetric flask and topped up to mark with double distilled water. Whilst for Vitamin B9 and B2, 25 mg of each standard were dissolved in 1 ml 0.1 M sodium hydroxide (NaOH) in 25 ml standard volumetric flask and made up to mark with double distilled water. The standard solutions were stored in amber glass bottles at 4°C. The working standards were prepared from the stock standard solutions.

5.2.4 Sample preparation

The method of Seal *et al*., (2018) was also adopted for sample preparation. One g of finely ground *M. oleifera* leaf powder was soaked in 10 ml water. One ml 0.1 M NaOH and 10 ml phosphate buffer (1M, pH 5.5) was added to it and the solution was kept in the dark for 24 hours. The solution was first filtered through a Whatman No. 1 filter paper and the resulting filtrate was taken in a 25 ml volumetric flask and solution was topped up to the mark with HPLC grade water. The sample solution was filtered through 0.22 µm membrane filter before injection into LC system.

5.2.5 Linearity

To ascertain the linearity, the stock solution of the standard (1 mg/ml) was diluted to six different concentrations (10, 20, 30, 40, 50 and 60µg/ml) which were injected and analyzed by the HPLC in triplicate. A calibration curve was then obtained by plotting peak area versus concentration for each vitamin standard. The coefficients of determination were used to ascertain linearity, where $R^2 > 0.99$ indicates the degree of linearity.

5.2.6 Accuracy

The matrix matching method was used to determine accuracy. Two samples of *Moringa oleifera* leaf powder were prepared simultaneously. One sample was spiked with a known standard concentration derived from the standard cocktail before sample preparation whilst the other sample was spiked with the known standard concentration after sample preparation. This method considers the matrix effect of the sample.

Recovery was calculated using the following formula by Abibu *et al*., (2019) was used:

$$\textbf{Recovery} = \frac{Concentration\ in\ PreSP}{Concentration\ in\ PoSP} \times 100$$

Where PreSP and PoSP refer to the sample spiked before sample preparation and after sample preparation respectively.

5.2.7 Limit of detection (LOD) and quantification (LOQ)

The limit of detection for each vitamin and limit of quantification were calculated using the following formula by Seal and Chaudhuri (2017),

LOD = 3.3* $\frac{\sigma Intercept}{S}$

LOD = 10* $\frac{\sigma Intercept}{S}$

Where (σ) = standard deviation of response (peak area) and S = slope of the calibration curve. The % relative standard deviation (RSD) of the retention time and the peak area was used to define precision. Intra and inter day % RSD was calculated for retention time and peak area using 5 replications, the following equation by Abibu *et al*., (2019) was used:-

% RSD = $\frac{Standard\ deviation}{Mean} \times 100$

5.2.8 Data analysis

Quantification of vitamins was done in triplicates, significant difference between each vitamin was determined by the Kruskall-Wallis test. In cases where normality was assumed, a One-way analysis of variance (ANOVA) was used. Dunn's test was employed at significant level of P<0.05. The analysis was done using the R-studio version 4. 1. 2 statistical package.

5.3 Results and Discussion

5.3.1 Optimization of chromatographic conditions and recovery of extraction

The optimised gradient elution program was used in the separation of five water soluble vitamins. The solvents used were 0.01% Triflouroacetic acid as solvent A and solvent B as Acetonitrile. A flow rate of 5 was also maintained throughout the run. The gradient elution program was executed by varying solvent A and B as follows: In 1 minute, solvent A was 99% and solvent B was 1%. Solvent B increased to 5% in 5 minutes, at 7 minutes solvent B increased to 15% and further increased to 45 % at 15 minutes. The mobile phase composition returned to initial rates (99% solvent A and 1% solvent B at 16 minutes).

The HPLC chromatogram of the standard mixture of five water soluble vitamins was recorded at wavelength 254 nm and 300 nm (Figure 5.2). The eluent of the vitamins mixture produced good peaks under these wavelengths and were used throughout the study to detect the vitamins in *M. oleifera* samples. Vitamins B1, B2 and B3 were quantified using the 254 nm wavelength whilst vitamins B6 and B9 were detected by the 300 nm wavelength. A flow rate of 0.5 mL/min was maintained throughout.

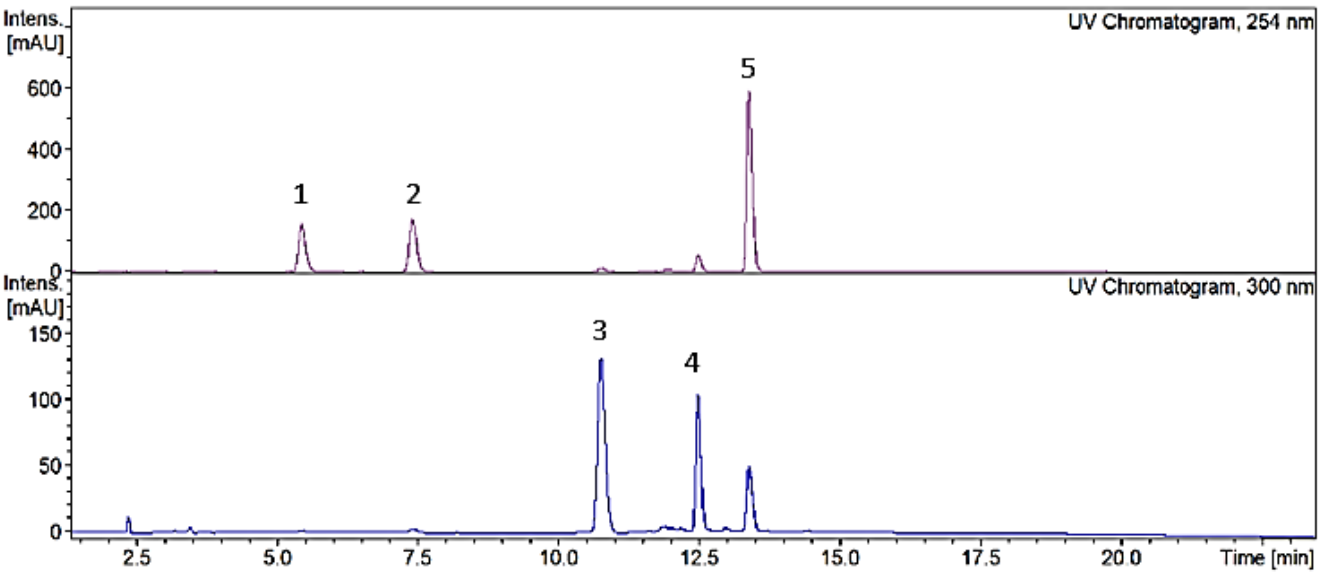

Figure 5.2: Chromatogram of the vitamin mixture (1= B3; 2= B1; 3= B6; 4= B9; 5= B2)

The regression equation, R^2 values, %RSD for retention time and peak are, LOD, LOQ and the percentage recovery values obtained for method evaluation are recorded in Table 5.1. The R^2 values recorded were all in the range of $R^2 > 0.99$ indicating good linearity. The LODs of the vitamins ranged from 0.2 to 0.89 µg/mL whilst the LOQs ranged from 0.6 to 2.71 µg/mL. These values were a bit higher for all the tested vitamins (except for Vitamin B6) than those reported by Seal and Chaudhuri (2017) who used the same solvents for water soluble vitamin quantification. This variation could be due to the instrument used and the vitamin mixture composition. Percentage recovery for all vitamins was above 97.8%. The values obtained deems the method accurate and reproducible.

Table 5.1: Retention time and parameters of calibration curve, limit of detection, limit of quantification, and percentage recovery study of standard water-soluble vitamins.

Analyte	Retention time(tR)	Regression equation	Correlation coefficient (R^2)	RSD(%) of the retention time	RSD(%) of the peak area	LOD (µg/mL)	LOQ (µg/mL)	Percentage of recovery (%)
B1	5.03	y=270.44x-24.519	0.998	1.8	4.23	0.89	2.71	98.03
B3	6.69	y=174.93x+26.899	0.999	0.9	4.06	0.47	1.43	98.86
B9	12.18	y=97.694x+12.226	0.999	0.16	2.76	0.56	1.71	98.72
B2	13.24	y=218.28x+28.362	0.999	0.16	5.84	0.55	1.70	99.58
B6	7.31	y=162.64x+139.28	0.999	0.55	14	0.2	0.6	97.8

Simultaneous quantification of seven water soluble vitamins in *M. oleifera* was conducted by Abibu *et al*., 2021 using a similar column to the one used in this study on an *Agilent* 1260 HPLC. The same researchers used 0.01% forming acid and methanol as mobile phases, their total run time was 14 minutes. The current method had all the analytes eluted at 13 minutes compared to 12 minutes in the method of Abibu *et al*., 2021. The current method remains superior in that it used an affordable and yet effective sample preparation and in addition the identification of the vitamins was quicker as the ms/ms detected and displayed the *mz* value of each vitamin. To our knowledge the current study is the first study to report the simultaneous quantification of B vitamins in Moringa using both LC-UV and mass spectrometry for determination.

5.3.2 Identification and quantification of water soluble vitamins in *Moringa oleifera* samples

Figure 5.3 shows a representative 20 minute chromatogram from one of the tested *M. oleifera* samples with all the five vitamins identified. Figure 5.4 indicates the identification of the tested water-soluble vitamins in the sample using the Mass spectrometer compartment of the instrument.

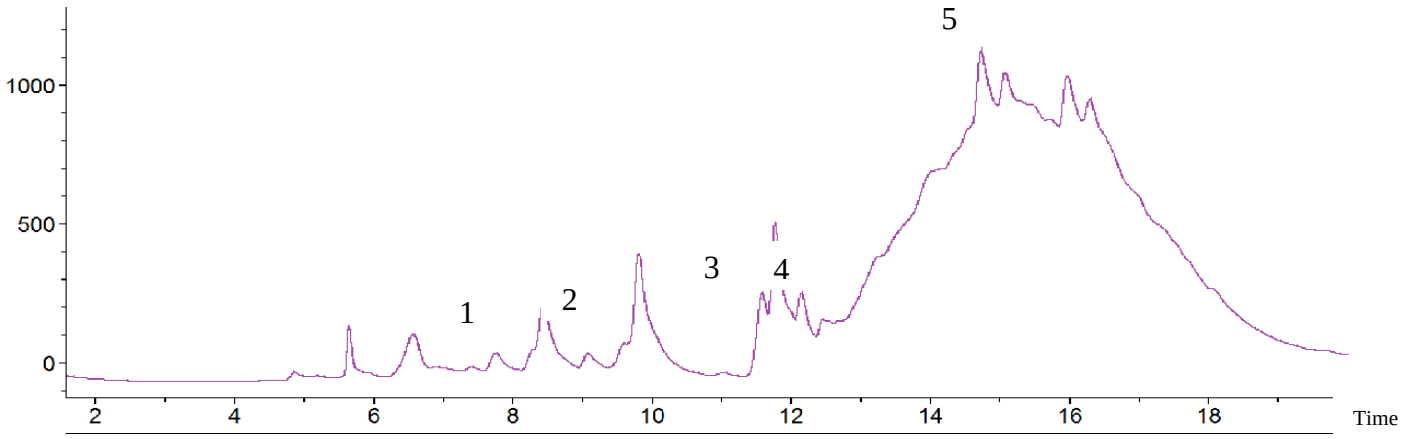

Figure 5.3: Chromatogram of *M. oleifera leaf* powder (1= B1; = B3; 3= B6; 4= B9; 5= B2).

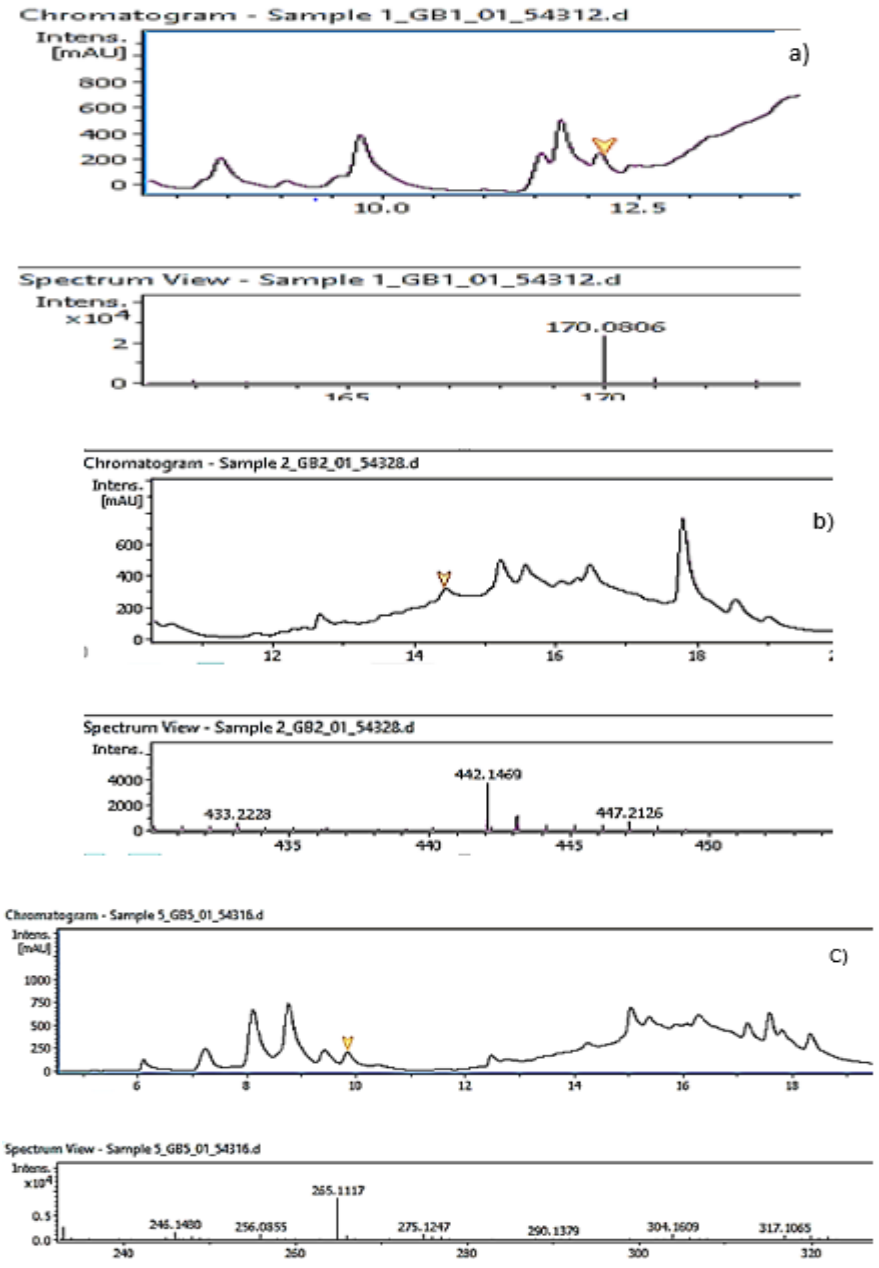

Figure 5.4: Identification of Vitamins from the M*. oleifera* leaf sample using Mass spectrometry (Ms): a= B6, b= B9, c= B1).

Table 5.2 indicates the individual vitamin concentration in five *M. oleifera* samples harvested from different soil compositions altered by the plant-based compost.

There is significant difference p< 0.05 on the addition of compost and various levels in *M .oleifera* leaves vitamin content (Table 5.2).The concentration of Vitamin B1 (Table 5.2) improved with the addition of plant-based compost to the clay soil. It also increased directly proportional with the added compost levels except at 60% compost

amendment where a decline was noted. The same trend was reported in Vitamin B6 and Vitamin B9 although in Vitamin B9 Vitamin B2 concentration in *M. oleifera* leaves decreased with the addition of plant-based compost to the soil, the highest concentration of the vitamin was detected in the control soil (Table 5.2). The amount of Folate (Folic acid) also known as Vitamin B9 in the study ranged from (1.13 to 9.77 mg/100g) in all *M. oleifera* leaf samples (Table 5.2). The highest content was noted in 30% compost soil amendment. Vitamin B3 and B9 were not detected (nd) in the control clay soil and in the 60% plant-based compost soil amendment respectively.

Table 5.2: Concentration of water-soluble vitamins in *M. oleifera* leaves.

	Control (clay soil)	**Clay soil + 15% compost**	**Clay soil + 30% compost**	**Clay soil + 45% compost**	**Clay soil + 60% compost**	**P-value**
Vitamin B1	1.1 ± 0.2	1.67 ± 0.25	8.2 ± 0.46	8.63 ± 0.87	1.4 ± 0.99	0.02
Vitamin B2	25.1 ± 0.7	15.9 ± 0.21	22 ± 0.5	13.3 ± 0.5	15.6 ± 0.67	0.01
Vitamin B3	nd	13.5 ± 0.6	33.6 ± 0.7	40.6 ± 1.45	6.2 ± 0.42	0.01
Vitamin B6	1.87 ± 0.47	1.87 ± 0.32	2.9 ± 0.2	5.77 ± 0.51	3.83 ± 0.21	0.01
Vitamin B9	3.73 ± 0.57	7.17 ± 0.31	9.77 ± 0.29	1.13 ± 0.45	nd	0.01*

Kruskall wallis test carried out to test for significant differences in vitamins content of different soil compositions. * One way ANOVA was carried out where the data met the assumption of the test

The increase in Vitamin B1 concentration in plants in response to organic soil amendments was also reported by Mozafar (1994). Organic soil amendments have resulted in the increase of water-soluble vitamins in plants which is why an increase in vitamin content was noted in Vitamins B1, B3, B6 and B9 upon addition of compost. Water soluble vitamins such as Vitamin C were also noted to increase significantly in organically grown leafy vegetables in comparison with conventionally grown ones (Premamali *et al*., 2019). Theunissen *et al*., (2010) also reported the same trend. In addition, application of organic soil amendment led to higher concentrations of Vitamins B1, B2, B6 and C (Akachukwu *et al*., 2018). Organic soil amendments in the growth of pineapple fruits revealed an increased in Vitamin B6 content in comparison with the control (Kpéra *et al*., 2019).

This increase of vitamins with the addition of compost observed in this study could also be attributed to the increase in soil micro-organism upon addition of compost, these organisms increase the availability of plant nutrients essential in the formation of vitamins (Chauhan *et al*., 2006). Fruit trees (apples, pineapples and strawberries) who were organically grown did not significantly differ with those grown conventionally (Woese *et al*., 1997; Alvarez *et al*., 1993). A decline in Vitamin B2 concentration in organically grown *M. oleifera* leaves was reported in this study, no known data is currently available on the increase of Vitamin B2 content in plants due to organic soil amendments.

Excessive levels of nitrogen in the soil increase the concentration of nitrates which in turn reduce the synthesis of water-soluble vitamins such as Vitamin C and other essential nutrients in plants and in turn reduce their concentration (Bourn and Prescott, 2002). There have been some contradictory views on Vitamin B1 and B2 content in organically grown plants (Golijan and Sečanski, 2021), this can be due to the fact that organic components differ from lot to lot. Therefore, more studies need to be conducted using different types of plant-based compost.

Vitamin B1 content in the current study was in the range of the Vitamin B1 content found in other *M. oleifera* and herbal plants studies (Abibu *et al*., 2021; Abibu *et al*., 2019; Datta *et al*., 2019; Ali *et al*., 2017; Fuglie, 2005). Higher concentration of Vitamin B2, B3 and B9 were detected in the current study which above those detected in other *M. oleifera* studies (Abibu *et al*., 2021; Ali *et al*., 2017 El Sohaimy *et al*., 2015; Fuglie, 2005). Vitamin B3 was also high in *M. oleifera* leaf biomass from the compost amended soils in comparison with other herbal plants such as Sutherlandia frutescens and Amaranthus (Abibu *et al*., 2019; Datta *et al*., 2019). Whilst Vitamin B6 content in the current study were a bit lower than it's concentration in *M. oleifera* leaves studied by Abibu *et al*., 2021 it was however in the concentration range of selected herbal plants (Abibu *et al*., 2019; Datta *et al*., 2019).

Comparing the water soluble vitamins content in the current study with quantities of the same vitamins determined in other studies of *M. oleifera* studies could be attributed to the addition of plant based compost. However, other factors such as cultivation,

processing and storage techniques which affect vitamin content could have impacted the variation (Grosshagauer *et al*., 2021). High levels of vitamins such as the ones found in this study are also an indication of good plant health as vitamins are vital enablers of plant pathways. For example Vitamin B1, is a cofactor of plant metabolic pathways (Subki *et al*., 2018).

5.4 Conclusion

The concentration of the five water soluble vitamins studied was dependent on the application and application rate of the plant-based compost on clay soil. High concentration of vitamins in this study are an indication that *M. oleifera* leaves can provide varied amounts of vitamins essential for human health and have a higher potential of being used as nutritional supplements. This study also confirms that the use of organic plant-based compost alone is ideal in increasing the vitamin content and thus nutritional content of *M. oleifera* leaf biomass. The variation noted in the distribution of the vitamins amongst the different soil amendments reveal 30% plant-based compost soil amendment as the recommended soil amendment, this soil combination exhibited a balanced distribution of all the studied vitamins this is in comparison with other treatments which were higher in certain vitamins and very low in some. Furthermore, the developed method was successfully employed in the simultaneous quantification of B vitamin in *M. oleifera* leaf biomass. The LC-DAD-MS method developed and used in the current study is recommended for the simultaneous quantification of B-vitamins in Moringa. A shorter HPLC column can be used to further reduce the run time.

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Chapter 6

6. Conclusions and Recommendations

6.1 Conclusions

This chapter summarizes the main findings of the current research and its contribution in the *M. oleifera* production industry in South Africa. There are many factors affecting the production of *M. oleifera* which impact the quantity and quality of the produce. Environmental factors such as season, soil type, temperature and rainfall have been found to influence the nutrient content of the plant. In addition, agronomic practices such as spacing, fertilization, harvesting frequency and storage employed during the production of *M. oleifera* have an ability to alter its quality and quantity.

The quality of *M. oleifera* leaves is of paramount importance as they are the most used and marketable part of the plant. Popularity of *M. oleifera* amongst consumers is increasing rapidly based on its medicinal and nutritional properties, its unique taste is also favoured (Ponnuswami and Rani, 2019). The use of *M. oleifera* leaf powder increased rapidly in the last two years during the COVID-19 pandemic as the world was looking for medicinal herbs to boost the immune system. The leaf powder is nutrient dense, a high source of essential nutrients such as Zinc and Iron, metabolic compounds such as chlorogenic acids and flavonoids and numerous vitamins which are all responsible for its health properties. Soil amendments have been used to improve the nutrient content of the leaves, organic soil amendments have been favoured as they are safer and result in products free of toxins. Therefore this research used organic plant based compost to enhance the nutritional quality of *M. oleifera* leaf biomass.

The application of plant-based compost to the control clay soil proved to be a powerful way to increase the concentration of nutrients in *M. oleifera* leaf biomass making the plant marketable both locally and in top international organic markets. The concentration and bioavailability of macro and micro nutrients (Al, Ca, Co, Cr, Cu, Zn, K, P, S, Na, Mn, and Fe) in the clay soil significantly increased with the addition of plant-based compost. The same trend was noted in the leaves harvested from each

treatment, improved concentration of macro and micro nutrients was observed in leaves harvested from each compost treatment in comparison to the control.

Of the tested water soluble vitamins (Vitamin B1, B2, B3, B6 and B9), the application of plant-based compost significant increase the concentration of Vitamin B1, B3, B6 and B9. The high concentration of the water-soluble vitamins indicate the increased nutrient content of the leaves as vitamins are enablers of essential metabolic pathways. The use of high profile analytical techniques and analytical tools such as UHPLC-ESI-QTOF-MS and multivariate analysis models effectively identified nutritionally important secondary metabolites. The classification of the metabolites according to soil treatments revealed an increase in the identified secondary metabolites with the addition of plant-based compost to the control soil.

It could therefore be concluded that the current research study provided an in-depth insight on the positive effect of plant-based compost in improving the quality of *M. oleifera* leaves by increasing the nutrient content. Generally the 30% plant based compost- soil amendment exhibited the highest impact on the leaf nutrient content. The leaves that were harvested from this soil composition had higher levels of macro and micro elements, they were also had high concentration of the two out of the five tested water soluble vitamins, second to 45% plant-based compost soil amendment whose levels were high in the remaining three vitamins.

In addition to directly influencing the content of macro and micro nutrients in the soil, application of plant based compost also improves the physical properties of the soil and thereby increasing the bio-availabity of the nutrients. Clay soil in particular as much as it is considered generally fertile soil is prone to water logging conditions. In drier conditions this soil type cracks-up exposing *M. oleifera* tubers, this is a challenge to *M. oleifera* farmers in regions which experience very cold conditions such as Gauteng, South Africa. The cracked soil exposes the tubers to very low temperatures and resulting to frost damage and drying up of the plant. This is a challenge for small scale farmers with limited resources to protect the plants from frost. Therefore application of plant-based compost will also help alleviate these challenges.
In conclusion based on the findings from the current study it is highly noted that the plant-based compost application could enhance the nutrient properties, secondary

metabolites, and vitamin content of *M. oleifera* grown in clayey soil conditions. The 30% plant-based compost to clay soil ratio by volume is the recommended rate, its application resulted in overall increased concentration of nutrients and biomolecules in *M. oleifera* leaf biomass.

6.2 Recommendations

It should be noted that clay soil was used in the current study and thus the recommended plant-based 30% compost/soil combination reported in the current research should only be applied to this particular soil type. Testing of each soil and compost to be used is recommended prior to planting in order to get the optimum combination for improving the nutrient content of *M. oleifera* leaves.

Care must be taken in the application of plant based compost as higher levels of compost in the current study did not significantly increase the nutrient content of *M. oleifera* leaves beyond the optimum levels (30% compost –soil amendment). Therefore the use of very high compost levels could just be unnecessarily costly.

Additionally, a field study is recommended in order to observe the impact of the greenhouse findings under real environmental conditions. This is due to the fact that variations may arise between a greenhouse and field experiment as greenhouse conditions are controlled to optimal levels. The use of plant based compost will be an affordable option for beginner *M. oleifera* farmers with limited income as they can make the compost from plant matter which is readily available. A targeted metabolomics approach can also be carried out in addition to the analysis done in order to determine the exact concentration of metabolites of interest present in *M. oleifera* leaves grown in different levels of organic plant-based compost.