EFFECTS OF MICRONUTRIENTS ON GROWTH AND QUALITY OF BUSH TEA (*ATHRIXIA PHYLICOIDES* DC.)

By

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(*Athrixia phylicoides* DC.)

DECLARATION

By submitting this thesis, I declare that the entire work of **Effects of micronutrients on growth and quality of Bush tea (*Athrixia phylicoides* DC.)** is my own work and all the sources that I used or quoted have been indicated and duly acknowledged by complete references. Furthermore, I took reasonable care to ensure that the work is original and, to the best of my knowledge, does not breach copyright law and has not been taken from other sources except where such work has been cited and acknowledged within the text. The turnitin report has been attached in line with the College of Agriculture and Environmental Sciences undertakings.

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ABSTRACT

Bush tea (*Athrixia phylicoides* DC.) is a herbal beverage and medicinal plant indigenous to South Africa. A trial was conducted to determine the effect of micronutrients on the plant growth and quality of bush tea. The trial was laid out in a completely randomized block design with five replicates. Treatments consisted of single applications of Zinc (Zn), Copper (Cu), Boron (Bo), Iron (Fe) and Magnesium (Mg) at three levels (50ml/l, 100ml/l and 150ml/l) and a combination of all micronutrients. A control treatment with no spray was also included. Leaf analysis was conducted using Varian Liberty series II instrument. Total polyphenols were determined using the Folin Ciocalteau method and tannins were determined using Vanillin HCl method. Bush tea samples (one leaf per sample) were analysed using head space solid phase micro-extraction gas chromatography (HS-SPME-GC-MS).

Results of this study demonstrated that application of micronutrients increased the total polyphenols, tannins and total flavonoids in bush tea, with most of the increase in total polyphenols (77.5-93.7 mg/g) occurring in combination B + Zn + Fe + Cu + Mg treatment, increase in tannins (87.3-99.5 mg/g) occurring in copper treatment and increase in total flavonoids (164.6-176.6 mg/g) occurring in mixture (B + Zn + Fe + Cu + Mg) treatment.

Results also show a significant increase in the quality and plant growth of bush tea. Five major compounds were identified (>80% identification probability) namely alpha-pinene, beta-pinene, myrcene, beta-caryophyllene and caryophyllene oxide. Linear relationship between percentage leaf tissues and treatments levels of micronutrients in bush tea was also observed. Boron and copper treatments showed strong linear correlation with a positive relationship between treatments levels and leaf percentage. Therefore, for improved total polyphenols content in bush tea leaves, a combination of (B + Zn + Fe + Cu + Mg) is recommended. Tannin content in bush tea leaves were significantly increased at Cu50 ml/l, Cu100 ml/l and Cu150 ml/l. For improved total flavonoids content in bush tea leaves, a combination of foliar spray of (B + Zn + Fe + Cu + Mg) is recommended. The LC-MS observations from the study showed no significant qualitative difference between control and micronutrient treatments with these treatments showing similar number of peaks. There was a significant quantitative difference between control and where magnesium peaks

applied at adequate rates at (50 ml/l and 100 ml/l) and combination of (B + Zn + Fe + Cu + Mg) applied at (10 ml/l and 20 ml/l).

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CHAPTER 1

1.1 INTRODUCTION

Bush tea (*Athrixia phylicoides* DC.) is an aromatic perennial shrub that has medicinal properties (Maudu *et al*., 2012). There are 14 species of bush tea and 9 of these are found in South Africa. This shrub has leafy stems that grow up to a metre in height. The plant belongs to the Asteraceae family (Maudu *et al*., 2012). Bush tea grows in rocky areas, grasslands and forests across South Africa (Nchabeleng, Mudau and Mariga, 2012). Traditionally, bush tea has been used in cleansing blood, treating headaches, boils, bad acne, sore throat and loss of voice (Maudu *et al*., 2012). Various tribes and cultures in South Africa use bush tea in various ways for different purposes. For instance Vhavenda people use bush tea as an aphrodisiac (Mabogo, 2012; Mudau, Soundy and DU Toit, 2007b) and Sothos also use strong brew as a calming wash for sore feet (Roberts, 1990; Mudau, Soundy and DU Toit, 2007b). Zulu people in KwaZulu-Natal use dried stems of bush tea to make brooms (Maudu *et al*., 2012). A herbal tea that is rich in non-toxic flavonoids, antioxidants and tannin levels are very low (Maudu *et al*., 2012), making it a favourable healthy beverage. Moreover, the absence of caffeine is a desirable feature of a healthy beverage, as is the presence of antioxidants which may have health benefits (McGaw *et al*., 2007).

Bush tea has many potential uses that have not yet been harnessed commercially *inter alia*; bush tea has inhibitory effects against micro-organisms such as bacteria *Staphylococcus aureus*, *Bacillus coreus*, *Enterococcus*, *Escherichia coli* and *Mycobacterium smegmantis* (Negukhula, 2010). Antioxidants help boost the immune system and assist in detoxification of contaminants and pollutants thereby reducing inflammation (Maudu *et al.*, 2012). Most herbal teas have a bitter taste owing to the high tannin levels; however, bush tea lacks this bitter taste owing to low tannin levels. Tannins bind iron and reduce the absorption of non-heme iron. They also precipitate protein, inhibit digestive enzymes and affect utilization of vitamins and minerals (Tabasum *et al.*, 2001). Therefore, this desirable characteristic of bush tea renders it a good beverage alternative for people with difficulty in digesting tannin-rich beverages.

Despite available literature on the effect of micronutrients on the quality of certain tea species, information on the effect of micronutrients on bush tea quality is lacking. To bridge this void, this study thus aimed to investigate the effect of foliar application of micronutrient fertilisers on the chemical composition (total polyphenols, tannins and antioxidants) as well as growth of cultivated bush tea. The study focused on foliar application of Zinc (Zn), Magnesium (Mg), Copper (Cu), Boron (B), Iron (Fe) and various combinations of these micronutrients on bush tea.

1.2 PROBLEM STATEMENT

Drinnan (2008) reported that tea crop yields are largely determined by planting material, soil type, climate, pruning, harvesting patterns and fertilizer inputs. Many of these factors are fixed in tea production. However, nutritional management is one of the factors that can be manipulated by the grower to influence yield. In the quest for commercially producing bush tea, for achieving good quality and higher yields, the most vital pre-requisite is ideal crop nutrition. Pot trials done on nitrogen (N), phosphorus (P) and potassium (K) have already reported optimum with P trial, total polyphenols increased in response to P nutrition regardless of season. Again, the highest concentration of total polyphenols (46.8 mg/g) was observed in winter with the optimum P level being 300 kg/ha. In the K trial, regardless of season, total concentration of polyphenols reached a maximum at 400 kg/ha with most of the total polyphenols occurring between 0 and 200 kg/ha. Therefore, for improved concentration of total polyphenols, 300 kg/ha N and P and 200 kg/ha K are recommended. Total polyphenols reached a maximum at 400 kg/ha K with most of the total polyphenols occurring between 0 and 200 kg/ha K quantities for maximized shoots growth and total polyphenols as well as level of tannins (Mudau *et al*., 2007b). The effect of Mg, Fe, Zn, Cu and Boon as micronutrients on bush tea quality and growth has not yet been studied. Micronutrients, also referred to as trace elements, are required in small quantities by the plant. Trace elements enhance enzyme synthesis and activation, production of chlorophyll, disease resistance, stem strength and shoot growth (Drinnan, 2008).

1.3 MOTIVATION

Owing to the several medicinal attributes of bush tea, the viability of commercial production of bush tea rests upon obtaining high yields and good quality tea. Therefore in bush tea production the vital component is appropriate and adequate supply of plant nutrients. Study on the effect of micronutrients on bush tea growth and quality is necessary to establish plant nutrition requirements for bush tea production.

1.4 AIM OF THE STUDY

This study sets out to investigate the effect of applying micronutrient fertilisers on chemical composition (total polyphenols, tannins and antioxidants) as well as growth of cultivated bush tea. The study focused on individual application of Zinc, Magnesium, Copper, Boron, Iron and various combinations of the aforementioned micronutrients.

1.5 OBJECTIVES

1. To determine the effect of micronutrient fertilisation on bush tea foliar nutrient composition.
2. To determine the effects of micronutrient application on bush tea antioxidant activity, polyphenols, flavonoids and tannins compositions.
3. To determine the effect of micronutrient application on bush tea phytochemicals and chemotypic variations.

1.6 HYPHOTHESES

1. Micronutrient fertilisation does not have an effect on bush tea nutrient composition.
2. Micronutrient application does not have an effect on bush tea antioxidant activity, polyphenols, flavonoids and tannins compositions.
3. Micronutrient application does not have an effect on bush tea bioactive phytochemicals and chemotype variations.

1.7 REFERENCES

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CHAPTER 2

LITERATURE REVIEW ON BUSH TEA

2.1 INTRODUCTION

Bush tea (*Athrixia phylicoides* DC.) is a plant used in the processing of popular beverages indigenous to South Africa (Maudu *et al*., 2012). Bush tea is a South African indigenous herbal plant. This perennial leafy shrub grows up to one metre in height. Bush tea leaves are bright and dark green in colour (Nchabeleng *et al*., 2012).

Athrixia phylicoides has been used widely by indigenous people of South Africa as a medicinal herb, documented to be used for curing boils, decontaminate infected wounds and cuts, cleansing blood, acne, treating colds and cough as well as headaches (Nchabeleng *et al*., 2012).

The Vhavenda use bush tea as an aphrodisiac, they also benefit from the extractions from soaked roots and leaves to destroy or eliminate parasitic worms, especially human intestinal helminthes (Maudu *et al*., 2012). The Zulu people extract the essence from the roots by boiling and utilize this as a wheeze remedy and a laxative (Maudu *et al*., 2012). Van Wyk and Gericke (2000) reported that Khoi and Zulu people use leaves that are dry and fine twigs of bush tea as herbal tea and a medicinal decoction.

Many studies have reported bush tea having the prospect to be commercialised as a medicinal herbal beverage. The medicinal significance of bush tea as reported by Mudau (2007) could be linked to the essential oils found in cells in the glandular trichomes present on the surface of leaves. The glandular trichomes are peltate, multi-cellular structures with an apical subcuticular cavity where the secreted products are stored and the essential oil released Mudau *et al*., 2007).

Today, herbal tea cultivation is a rapidly growing business in many parts of the world and the composite industry is now producing a diversity of teas. South Africa produces indigenous herbal teas like honey-bush tea *(Cyclopia intermedia)* and

rooibos tea *(Aspalathus linearis)* (Marnewick *et al*., 2000). Like bush tea, rooibos and honey-bush teas have been used for decades as herbal teas or medicinal teas by people of South Africa (Van Wijk, 1986).The progress of domestication and preparation for market production of bush tea depends on development of the production system as a herbal beverage commercially. Herbal tea quality is one of the serious factors in commercialization that determine the price of tea for either domestic use or export (Mudau *et al*., 2007c). Thus, methods of improving the quality of bush tea produced are of paramount importance when exploring commercial production of bush tea in South Africa.

2.2 ORIGIN AND DISTRUBUTION

The *Athrixia* genus belongs to the Asteraceae family, tribe *Inuleae* and subtribe *Athrixiinae*. There are 14 species, which are predominantly located in Tropical Africa, Madagascar and Southern Africa, of which 9 are endemic to Southern Africa (Herman *et al*., 2000). The most common ones in South Africa are *A. angustissina*, *A. elata*, *A. gerrardii*, *A. heterophylla* and *A. phylicoides*. *Athrixia phylicoides* is widely distributed in the eastern part of South Africa from the Soutpansberg Mountains in Limpopo to Queenstown, King William's Town and East London in Eastern Cape Province and throughout KwaZulu-Natal from the coast to the Drakensberg Mountains (Herman *et al*., 2000; Nchabeleng *et al*., 2012)

2.3 HISTORY AND BOTANICAL INFORMATION

In South Africa the plant is commonly known as bushman's tea (English); Boesmanstee (Afrikaans); Icholocholo, Itshelo and Umshanelo (Zulu). It is an attractive shrub, about 50 cm to 1 m in height, branched, with thin, woolly stems. Leaves are direct to broadly lanceolate, made to a sharp point, shortly stalked, auriculate (shaped like an ear) at the base, light grey-green, smooth on the upper surface and white-woolly below and with the margins totally or slightly revolute (Roberts, 1990). In large panicles, the inflorescence head is usually sub-sessile or sessile and terminal axillary (Herman *et al*., 2000). Flowering period in the coastal areas happens throughout May to June and inland flowers appear throughout mid-summer (Roberts, 1990). Flowers varying in colour starting from pink to all shades of pink and an eye-catching purple, liable on edaphic factors and geographical area (Van Wyk and Gericke, 2000). Bush tea is well adapted to open grassland and in

thick forest margins of South Africa, especially in Limpopo, Free State, KwaZulu-Natal and some parts of the Eastern Cape, and also occurs in Swaziland. Propagation is normally through ripening seeds, mostly collected at the end of summer (Roberts, 1990). It can also be positively propagated by cuttings (Mudau *et al*., 2007b). For good establishment, plants need well-drained soil with full sunlight and enough space for spreading their branches (Roberts, 1990).

2.4 HERBAL TEAS

Throughout history herbal teas have played an important role in everyday living in many societies, not for their flavour but for their medicinal value. They were taken quite often medicinally to cure coughs, sore throats, fever and aches and headaches. A few of the more popular plants commonly used as herbal tea include chamomile, marjoram, peppermint, rosemary, sage, rose, lemon verbena and thyme (Pietta, 2000).

Herbal tea quality is vital as it determines the price of tea (Mudau *et al*., 2007b). Chemical compounds have been reported to influence the quality of herbal teas (Owour *et al*., 2000). Medicinal potential indicators in herbal teas are attributed to presence of compounds such as polyphenols, flavonols and tannins. Other components of herbal teas are amino acids, carbohydrates, organic acids, vitamins and volatile compounds which provide sensory quality attributes such as bitterness, sweetness, aroma and astringent taste (Mudau *et al*., 2007b). Part of the health benefits of herbal tea are characterized by its antioxidant content (Mogotlane *et al*., 2007). Bush tea leaves contain polyphenols, tannins and flavonols (Nchabeleng *et al*., 2012).

2.4.1 Medicinal value of herbal tea

The uses of herbs are as old as mankind and people have used them as medicine, cosmetics and for culinary purposes for thousands of years. Archaeological researches also report that herbal concoctions had been used as an indulgence to bodily grievances for many years. In every civilization, herbs had their place and were used throughout history, with their usage changing very little as the centuries passed (Araya, 2005). Flowers, leaves and bark, used for improving the taste of

food, preparing tea or making medicines (Manteiga *et al*., 1997; Dufresne and Farnworth, 2001).

One of the most popular and long-term uses of herbs is the making of herbal tea. Herbal teas have a long history in keeping people healthy. The indigenous cultures of South Africa for many years used bush tea as a medicinal tea to decontaminate or cleanse blood, treating sores, as well as for headaches, cuts, infected wounds and some cultures used it as a foam bath (Mabogo, 2012). The foam bath brew can also be used as a lotion that can be applied to cuts, boils or skin eruptions (Roberts, 1990). Traditionally, the roots are used as an aphrodisiac in some parts of South Africa (Mabogo, 2012).

In Venda culture, it is known as the tree which is not supposed to be consumed by bachelors (Mudau *et al*., 2007). In Vhembe District of Limpopo Province bush tea extracts from soaked roots and leaves are used as anthelmintic (Mudau *et al*., 2007). Xhosa and Sotho people chew the tea to heal sore throats and coughs. The tea is very helpful when it comes to loss of voice and throat infections as a gargle (Mabogo, 2012).The Sotho people use strong brew preparations as a calming wash for sore feet and then bandage the washed feet synergistically with castor oil leaves (Roberts, 1990; Marnewick *et al*., 2000). Customarily, the common way to prepare bush tea is to boil water together with the leaves for 10 to 15 minutes, and then served. Depending on age of consumers, some prefer to drink sweetened bush tea with fresh milk (Mudau *et al*., 2007). Herbalists often dry the leaf samples in the shade and dispense the samples to people who are diagnosed with diabetes (Mudau *et al*., 2007).

2.4.2 Phenological stage of herbal tea

Phenological stage of tea can be described as tea growth flush. First spring leaf buds, called the first flush, are considered as the highest-quality leaves. When the first flush leaf bud is picked, another one grows, which is called the second flush, and this continues until an autumn flush. Ellis and Grice (1983) reported that the finer the plucking standard, which involves plucking only the first two leaves and bud, the higher the quality. Flush is followed by accumulation of carbohydrate reserves towards the production of total polyphenols (Roberts, 1990). In the fresh first tea flush there exists a wide variety of non-volatile compounds, namely polyphenols,

flavonols, flavones, phenolic acids and depsides, amino acids, chlorophyll and other pigments (Hart, 2009). The chemical composition of the tea leaves depends upon leaf age, the clone being examined, soil and climate conditions and agronomic practices (Hart, 2009). Gulati and Ravindranath (1996) reported maximum content of theaflavins and thearubigins during early flush and a gradual decline with progress in season, showing a minimum during main flush and slight improvement through back end flush.

2.4.3 Chemical composition of herbal teas

Compounds such as polyphenols, flavonols, and tannins are compounds which are the main indicators of the medicinal potential of herbal teas because of their antioxidant (Hirasawa *et al*., 2002; Mudau *et al*., 2007c). Sensory quality attributes such as bitterness, aroma, astringent and sweetness are provided by amino acids, carbohydrates, organic acids, vitamins and volatile flavour compounds (Mudau *et al*., 2007c).

2.4.3.1 Antioxidant activities of herbal tea

Oxidation reactions can produce free radicals. These radicals can start chain reactions in a cell that lead to cell damage or apoptosis. Antioxidant molecules inhibit the oxidation of other molecules, thus terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols.

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants or inhibition of the antioxidant enzymes cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell structure and cell functions by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a very vital role in many human diseases, including cancers. Uses of antioxidants include treatments for stroke and neurodegenerative diseases.

Antioxidant content is broadly or widely used for health benefits, in terms of it being a parameter to characterize different materials (Mogotlane *et al*., 2007). Antioxidant content is associated with those compounds which have the ability to protect biological systems against the harmful effects of extreme oxidation reactions, which involves nitrogen and oxygen species reaction (ROS) (Mogotlane *et al*., 2007). Antioxidant properties of tea are a result of extensive or wide range phenolic compounds that are found or fall under amphipathic molecules (Ivanova *et al*., 2004). The phenolics activity as antioxidant are mainly due to their redox functions enabling them to be reduction agents, oxygen singlet quenches, metal chelators and donators of hydrogen (Rice-Evans *et al*.,1997). The most important chemicals which are common in bush tea leaves are polyphenols, tannins and flavonols (Ivanova *et al*., 2004).

2.4.3.1.1 Uses of antioxidants

Antioxidants are used as food additives in the food industry to help guard against food deterioration. Exposure to oxygen and sunlight are two main factors in the oxidation of food, thus food is preserved by keeping in the dark and sealing it in containers. Oxygen is also important for plant respiration; storing plant material in anaerobic conditions produces unpleasant flavors and unappealing colours (Mogotlane *et al*., 2007). Antioxidants are an especially important class of preservatives as, unlike bacterial or fungal spoilage, oxidation reactions still occur relatively rapidly in frozen or refrigerated food. Fatty foods such as olive oil are protected from oxidation by their natural content of antioxidants. Antioxidant preservatives are also added to fat-based cosmetics such as moisturizers and lipstick to prevent rancidity (Mudau *et al*., 2007c).

2.4.3.2 Polyphenolic compounds

Polyphenols are a structural class of mainly natural and organic chemicals characterized by the presence of large multiples of phenol structural units. Polyphenols always have heteroatom substituents other than a hydroxyl group; ether and ester linkages are common. Application of nitrogen, phosphorus, and potassium fertilizers has been documented to increase the total polyphenols in bush tea (Mudau *et al*., 2007). Bush tea contains high concentrations of polyphenols that are the primary indicator of antioxidant potential in herbal teas (Mudau *et al*., 2007).

Tea polyphenols are natural antioxidants and chosen to control the anticarcinogenic and antimutagenic properties of tea (Reza *et al*., 2007). Herbal teas have a wide range of polyphenols (Owour *et al*, 2000), which have favourable biochemical and physiological properties for human health (Hirasawa *et al*., 2002). The major polyphenol antioxidant reported in green tea is epigallocatechin-3-gallate (EGCG). Epigallocatechin-3-gallate is reported to reduce the amount of free radicals and inflammatory prostaglandins in skin cells (Katiyar and Mukhtar, 1996). Tea leaves are reported to be rich sources of polyphenolic substances (Benzie *et al*., 1999), and account for one third of dry mass of dried leaves (Liang, Lu and Shang, 1996). According to Benzie *et al*., (1999), the colour of the beverage and taste, especially astringency, is attributed to these polyphenolic compounds.

2.4.3.2.1 Chemical properties

Polyphenols are molecules owing their UV/V absorptivity to aromatic structures with large conjugated systems of pi electron configurations; they also have auto-fluorescence properties, especially lignin and the phenolic part of suberin. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) may be used to characterise polyphenol oxidation products. Polyphenols also characteristically possess a significant binding affinity for proteins, which can lead to the formation of soluble and insoluble protein-polyphenol complexes.

2.4.3.3 Flavonoids

Flavonoids are a class of plant secondary metabolites. Flavonoids were referred to as vitamin P probably because of the effect they had on permeability of vascular capillaries (Mudau *et al*., 2007b). Chemically, they have a general structure of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and a heterocyclic ring (C). Flavonoids are the most important plant pigments for flower coloration, producing yellow or red/blue pigmentation in petals designed to attract pollinators. Flavonoids are involved in UV filtration and symbiotic nitrogen fixation. They also act as chemical messengers, physiological regulators and cell cycle inhibitors. Flavonoids are potential quality indicators of herbal teas since they are antioxidants

in nature (Mudau *et al*., 2007b). Bush tea has been reported to contain 5-hydroxy-6, 7, 8, 3', 4, 5'-hexamethoxy-flavon-3-ol as a major flavonoid (Mashimbye *et al*., 2006).The wide range of physiological and medicinal properties found in flavonoids

enhances any traditional beverage or remedy. Flavonoids are known for their cyclic adenosine monophosphate (cAMP) diphosphoesterase reserve activities, growth inhibition activities and cytotoxicity activities (Mashimbye *et al*., 2006).

2.4.3.3.1 Uses of flavonoids

Flavonoids reduce the risk of atherosclerosis, inhibit coagulation formation or platelet aggregation and reduce arterial blood pressure and reduce the risk of hypertension. They modify vascular inflammatory mechanisms and improve endothelial and capillary function as well as to modifying blood lipid levels.

2.4.3.4 Tannins

Tannins are phenolic compounds that are typically astringent and found in a variety of herbal products. Tannins may be grouped into hydrolysable and condensed tannins (Bokuchova and Skobeleva, 1980). Condensed tannins are polymers of 2 to 5 or more flavonoid units that are joined by carbon-carbon bonds, which are not susceptible to hydrolysation (Van Wyk and Gericke, 2000). Hydrolysable tannins are hydrolyzed by weak acids or bases to produce carbohydrates and phenolic acids (Haslam, 1996). Tannins found in herbal teas are reported to prevent cancers and heart problems. They reduce the propensity of blood platelets to join together (Stensveld *et al*., 1992). Herbal teas are reported to generally contain low tannin levels with a lot of variation seasonally. In the study of seasonal variation of tannins in wild bush tea, Mudau *et al*. (2007c) found that the highest concentrations of condensed tannins were in autumn (4.8%) compared with winter (2.4%), spring (2.7%) and summer (3.0%). Hydrolysable tannins were on the other hand found to be least abundant during summer (0.10%) compared to autumn (0.14%), winter (0.14%) and spring (0.13%) (Mudau *et al*., 2007c).

2.4.3.4.1 Uses of tannins

Foods rich in tannins can be used in the treatment of human hemochromatosis (HFE). Hereditary hemochromatosis, a disease characterized by excessive absorption of dietary iron resulting in a pathological increase in total body iron stores. Other tannin uses involve being most vital ingredient in tannery, as they are pivotal in the process of tanning leather. Tanbark from oak, mimosa, chestnut and quebracho tree has traditionally been the primary source of tannery tannin. Tannins can be used

for production of anti-corrosive primers. The use of resins containing tannins is utilized in the removal of mercury and methyl mercury from solution. Immobilized tannins are also used to recover uranium from seawater, playing a vital bio-remediatory role.

2.5 BUSH TEA PROPAGATION

Bush tea (*Athrixia phylicoides*) is commonly propagated by ripen seeds which are mostly collected at the end of summer (Roberts, 1990). However, there are certain factors that affect the germination ability of bush tea seeds. Successful transplanting survival of bush tea can be attained with apical cuttings and it is also important to transplant cuttings with a higher number of roots for successful establishment. In addition, apical cuttings with more number of roots could be obtained by propagating them in pine bark with Seradix No. 2 hormone. Furthermore, the germination (light and temperature) requirement of bush tea seed is not known (Araya, 2005). Plant propagation is the procedure of generating new plants from an assortment of sources, which include: seeds, cuttings, bulbs and other plant parts. Plant propagation can also refer to the artificial or natural dispersal of plants. Propagation by cuttings is one of the most important means for clonal regeneration of many horticultural crops, ornamental shrubs, and deciduous species as well as broad and narrow-leaved types of evergreens. This method of propagation is reported to be an extensively practiced and economical method of vegetative propagation (Hartmann *et. al*., 1997). Unlike other vegetative propagation techniques such as grafting, budding and micro-propagation, the cutting technique is relatively easy, inexpensive and quick.

A cutting is defined as a part of a vegetative plant that if detached from the parent, has the potential to regenerate again. In vegetative propagation, high root number and longer roots are produced best in apical cuttings than in basal cuttings.

Propagation in pine bark with hormone application increases root number as well as shoot and root length after transplanting. Apical cuttings propagated in pine bark with hormone develop more number of roots while cuttings propagated in sand with hormone and in pine bark without hormone also produce longer shoots after transplanting (Araya, 2005).

In propagating using stem cuttings, individual plants with superior performance in growth characteristics such as volume and form, field resistance to pests, diseases, or frost can be selected for these traits (Radke, 2005). A well organised cutting technique is needed to develop new clones and bring new genetic material into breeding programs as well as for multiplying limited amounts of selected material. It avoids the graftage problems associated with rootstocks and poor graft union formation. Greater uniformity is obtained due to the absence of variation which sometimes appears as a result of variable seedling rootstocks grafted on plants. The parent plant is usually reproduced exactly with no alterations in the genetic material (Hartmann *et al*., 1997).

2.6 HARVESTING AND TRADING

Bush tea is traditionally harvested during early autumn and midwinter during flowering. This essentially is desirable, because flowers are said to improve sweetness after harvesting, when the leaves are dried under a shade (Mudau *et al*., 2007). There are different harvesting techniques e.g. of young shoots or by cutting other branches as low as possible from the ground with sickle or pruning shears. The approximate cutting length measures 1 m but depends on the size of the plant. Excessive cutting helps the re-sprouting for future harvesting and it reduces the occurrence of shoot dieback after harvesting. Bushes previously harvested give better materials for processing, as the stems are softer (Mudau *et al*., 2007). Fire also aids a more vigorous shoot development in the following season.Trading of bush tea depends on the purpose of harvesting the plant. When the plant is not harvested for brewing, bundles of bush tea stems are tied well together to make brooms and people sell them on a small-scale at vendor's markets in Limpopo, Mpumalanga and KwaZulu-Natal Provinces (Mudau *et al*., 2007). Productivity in tea, just like any other crop, is a function of a number of factors such as genotype, soil and cultural practices. Fertilisation is one of the major determinants of yield in tea besides planting material, pruning and harvesting patterns (Drinnan, 2008). A balanced nutrition with both macronutrients and micronutrients is a requirement for tea to produce satisfactory yields and products of desired quality. Micronutrients, although are required in minute quantities, play important roles in plant growth and development. Zinc is indispensable for the healthy development of tea shoot that is plucked for manufacture (Drinnan, 2008).

2.7 PLANT MINERAL NUTRITION

Large scale production of Bush tea guarantees both availability of the product and consistency in quality. The commercialization of bush tea is unlikely to be viable if the plant is harvested from the wild. In agriculture, optimal crop nutrition is an important prerequisite for obtaining high yields and good quality produce. Nutrients are obtained by plants from soil reserves and external nutrient sources such as fertilizers, organic manure and the atmosphere. In light of increasing biomass synthesis of bush tea, an adequate and appropriate supply of nutrients is a vital component of the bush tea production system. The knowledge of the properties and functions of plant nutrients is essential for their efficient management. The aim of plant nutrition management is to optimize the productivity of the crop in a sustainable and economic way (Drinnan, 2008). Plant growth and hence final yield is restricted by the most limiting factor, for example, when growth is restricted by a particular nutrient deficiency, growth responses to irrigation applied will be weak.

Tea is a perennial plant with shoots repeatedly plucked at regular intervals. Harvesting shoots takes away nutrients from the plant. It is therefore important to supplement soil nutrients by applying fertilizers.

A study on nutritional requirements of bush tea reported by Mudau *et al*., (2007b) revealed that nitrogen (N), phosphorus (P) as well as potassium (K) improved or increased bush tea plant height, fresh and dry shoot mass, number of leaves, leaf area and number of branches. The optimum growth of bush tea was achieved by applying 300 kg/ha N and 300 kg/ha P and 200 kg/ha K.

Mudau *et al*., (2007b) in seasonal nutritional requirements study of bush tea reported an increase of total polyphenols in response to N the highest being 51.1 mg/g observed in winter at 300 kg/ha N. The results of the P trial indicated an increase of total polyphenols to 46.8 mg/g observed in winter at 300 kg/ha P. Total polyphenols reached a maximum at 400 kg/ha K with most of the total polyphenols occurring between 0 and 200 kg/ha K. These may be utilized as the standard optimal values for N, P and K application.

Mudau *et al*., (2007b) reported in another trial that was done to investigate N, P and K combinations on the chemical composition and growth of bush tea. The results concluded that the optimum nutrient requirement for bush tea is 300 kg/ha N or P and 200 kg/ha K. At these nutrient rates, new and dry shoot mass, leaf area, number of leaves and concentration of total polyphenols increased. Chabeli *et al*., (2008) conducted a study to investigate the response of tannin content of bush tea grown under 50% shaded nursery to N, P and K. In that study they reported that the N trial, condensed and hydrolysable tannins increased when N was applied at 300 kg/ha. A peak value of 4.5% condensed tannins and 0.06% hydrolysable tannins were observed. The tannins also increased in response to P nutrition at 300 kg/ha. The highest concentration was 5% condensed tannins and 0.02% hydrolysable tannins. At 200 kg/ha K, 5% condensed tannins and 0.041% hydrolysable tannins were observed. Mogotlane *et al*., (2007) reported that the application of N, P and K fertilizers increased the total content of antioxidants and that the greatest increase occurred at 300N, 300P and 100K kg/ha.

2.8 CRITICAL NUTRIENT LEVELS

In modern agriculture, plant nutrition management is of interest to researchers, farm advisers, fertiliser manufacturers and plant laboratory analysis as well as soil laboratory analysis. The main purpose of all these groups is to improve and enhance crop production in a sustainable and economic way. Plant and soil analysis serves to disseminate information concerning the nutrient status of plants as a guide to nutrient or fertiliser management. There are many ways in which soil and plant analysis data can be used. Four common ways include:

- Identifying nutrient problem
- Prediction of nutrient problems that may occur
- Crop nutrition status monitor with a view to enhancing production
- Fertiliser or nutrient programme development (Drinnan, 2008).

2.9 ROLE OF MICRONUTRIENTS IN PLANT GROWTH AND DEVELOPEMENT

Although information on micronutrient effect on the quality of certain tea species is available, data on the effect of micronutrients on bush tea quality is lacking. The effect of micronutrients such as zinc (Zn), boron (Bo), iron (Fe), copper (Cu) and magnesium (Mg) on bush tea growth and quality has not yet been evaluated. The

role of micronutrients in plants in general and their effect on plant growth and quality is discussed in the following section.

2.9.1 Zinc (Zn)

Zinc is a vital essential mineral due to its public health importance. About two billion people are affected by Zn deficiency in the developing countries in the world and are associated with numerous diseases (Roy *et al*, 2006). Most diseases such as diarrhoea, growth retardation and infection susceptibility lead to contribution of death of about 800,000 children worldwide per year. Enzymes containing zinc atom in the reactive centre are widespread in biochemistry which can be alcohol dehygrogenase in humans. Consumption of excess Zn can cause copper deficiency, ataxia and lethargy.

Zinc is taken up by plants as a divalent cation Zn^{2+} (Roy *et al*., 2006). Zn is required by several enzyme systems, for example; it is a component of proteinase, dehydrogenase and peptidase (Roy *et al*., 2006). Zinc promotes RNA synthesis which in turn is needed for protein production. Zinc is vital in seed production and rate of maturity. It is used in chlorophyll formation and nitrogen metabolism. Zinc is not very mobile in the soil. The rate of Zn mobility to younger tissue is particularly depressed in Zn deficient plants (Roy *et al*, 2006). Because, Zn lacks mobility in the soil, it is accessible only in soil tests but not available to the plant (Drinnan, 2008).

Zinc deficiency causes poor tillering; stunted plant growth; development of light green, bleached-yellowish spots; chlorotic bands on either side of the midrib in monocots; brown rusty spots on leaves in some crops, shoots may fail to extend in fruit trees and leaves group together at the tip. Diminutive-leaves develop and internodes are short. Zinc deficiency delays flowering, fruiting and maturity. Shoots may die-off and leaves may fall prematurely (Roy *et al*., 2006). Zinc deficiency is common in high pH soils that are sandy. Zinc is not readily absorbed by tea (*Camellia sinensis*) from the soil and its deficiency cannot be corrected by ground application of Zn compound (Drinnan, 2008). Foliar application of Zn is effective. Zinc can become unavailable when soil P is high (Drinnan, 2008). Organic matter is an important source of Zn sulphate at 20 kg/ha and Zn chelate 1% as a foliar spray are used to correct deficiency (Drinnan, 2008).

2.9.2 Iron (Fe)

Iron is the most abundant of the micronutrients. It is absorbed by plant roots as Fe^{2+} and to a lesser extent as Fe chelates. Iron plays a role in chlorophyll synthesis and carbohydrate production (Roy *et al*., 2006). Iron is used in many biochemical processes such as chemical reduction of nitrate and sulphate as well as N assimilation. Iron deficiency begins to appear on younger leaves first. Leaves exhibit interveinal chlorosis. In severe Fe deficiency, leaves become almost pale white owing to loss of chlorophyll. Complete leaf fall can occur and shoots can die (Roy *et al*., 2006). Foliar (1% chelate) or ground (2-5 kg/ha) application are used to correct Fe deficiencies. If root growth is restricted after harvest/pruning, Fe can be deficient (Drinnan, 2008). When manganese (Mn) levels are high in the plant and this leads to reduction of Fe levels in the plant (Drinnan, 2008). High P levels can lock up Fe (Drinnan, 2008). Iron is freely available in acidic soils therefore in such soils Fe deficiency is rare (Drinnan, 2008).

2.9.3 Copper (Cu)

Copper is essential to all living organisms as a dietary trace element because it is a key constituent of the respiratory enzyme complex cytochromes oxidase (Drinnan, 2008). Copper compounds are used as bacteriostatic substances, fungicides and wood preservatives (Drinnan, 2008).

Copper (Cu) is taken up by the plant as divalent cation Cu^{2+}. The formation of protein and chloroplast in the plant is done by Cu. It is used in respiration and carbohydrate metabolism (Drinnan, 2008). Copper is an essential constituent of several enzymes such as polyphenol oxidase. Polyphenol oxidase is a vital enzyme for fermentation of tea. As much as 70% of the Cu in plants may be present in the chlorophyll largely bound to chloroplasts. It is vital in lignin formation and is required for symbiotic N fixation (Drinnan, 2008). Copper is a part of plastocyanin which forms a link in the electron transport chain involved in photosynthesis (Roy *et al*., 2006).

Copper is not readily mobile in the plant. Its movement depends on the status of the plant. Deficiency symptoms are first visible in the form of narrow, twisted leaves and pale white shoot tips. Die back of terminal growth occurs in the severe deficiency cases (Roy *et al*., 2006). Copper deficiency is corrected by applying copper sulphate

at 10-20 kg/ha or foliar sprays of copper chelate (1%). Deficiencies can lead to reduced cupping quality. Copper is deficient in high pH and soils that are sandy and leached. Excess Cu induces Fe deficiency (Drinnan, 2008).

2.9.4 Boron (B)

Boron is absorbed from the soil by plants as undissociated boric acid (H_3BO_3). Boron in plants binds similar growing cells together e.g. meristems (Roy *et al*., 2006). Boron is used in protein synthesis and regulates metabolism of carbohydrates (Roy *et al*., 2006). Drinnan, (2008) summarizes the key roles of B as; membrane integrity and cell-wall development which affect permeability, cell division and extension. Also boron aids pollen tube growth which affects seed fruit set and hence yields (Drinnan, 2008). Deficiencies of boron are rarely seen because adequate boron is available in most tea growing areas (Roy *et al*., 2006). Boron is relatively immovable in plant. Boron deficiency usually appears on growing points of roots, shoots and youngest leaves. Young leaves are deformed; successive die-back of growing point, reduced bud and flower and seed production, development of auxiliary shoots, and shortened internodes. Roy *et al*., (2006) advises application of borax to the soil (2-4 kg/ha) or application of boric acid (0.1%) to the leaves when there are deficiencies. Boron toxicity can arise where irrigation water is rich in B content, resulting in yellowing of leaf tips followed by gradual necrosis of the tip and leaf margins. Leaves become scorched and may drop early hence reducing yield. Excess boron induces Zn deficiency (Drinnan, 2008).

2.9.5 Magnesium (Mg)

Magnesium is the eleventh-most-abundant element by mass in the human body. Its ions are essential to all living cells, where they play a major role in manipulating vital biological polyphosphate compounds like ATP, DNA, and RNA.

Hundreds of enzymes, thus, require magnesium ions to function. Magnesium compounds are used medicinally as common laxatives, antacids (e.g., milk of magnesia), and in a number of situations where stabilization of abnormal nerve excitation and blood vessels spasm is required. Magnesium ions are sour to the taste and in low concentrations they help to impart a natural tartness to fresh mineral

waters. In vegetation, magnesium is the metallic ion at the centre of chlorophyll and is, thus, a common additive to fertilizer.

Magnesium is the most vital or significant element and chlorophyll formation or development and is involved in carbon dioxide assimilation and carbohydrate partitioning. Common Mg deficiencies are found in the acid soils. Dolomite or Magnesium sulphate (50 kg/ha) are best when it comes to Mg deficiency corrections (Drinnan, 2008). The quantity required by tea is relatively small and deficiencies have rarely been found to affect crop yields (Bonheure and Willson, 1992). Magnesium is easily moved from the older leaves to the new leaves and even when older leaves show a deficiency, no yield responses have been observed (Willson, 1974). Rates of 125 kg of dolomite/ha are used in Sri Lanka to supply magnesium. Deficiency symptoms include interveinal and marginal chlorosis; leaves that are older turn yellow and end up being reddish in colour.

2.10 SUMMARY

This review has examined the important attributes of herbal teas and how micronutrients affect the growth and quality of bush tea. Herbal properties and quality of herbal teas are determined by their active chemical compounds. The chemical compounds have antioxidant properties and therefore are an indicator of the medicinal potential of bush tea. The review also highlighted the role played by micronutrients; sulphur (S), Zn, B, Cu and Fe in bush tea plants.

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CHAPTER 3

Effect of micronutrients fertilisation (Zn, Mg, Cu, B, Fe and their combination) on bush tea nutrient composition.

3.1 INTRODUCTION

Bush tea should guarantee both quality consistency and product availability when grown on a large scale. Viability of bush tea commercialization is doubtful if the plant is harvested from the wild. Optimal crop nutrition in agriculture is a vital prerequisite for attaining good quality produce and high yields. Plants obtain nutrients from external nutrient sources and soil reserves such as atmosphere, fertilizers and organic manure. Plant adsorbs nutrients as dissolved salts that have an electrical charge from the soil solution (Van Scholl and Nieuwenhuis, 2004). Appropriate and adequate supply of nutrients in bush tea production system is a vital component for increasing bush tea biomass synthesis. Knowledge of functions and properties of plant nutrients is essential for their efficient management. The aim of managing plant nutrition is to optimize the crop productivity in an economic and sustainable way (Drinnan, 2008). Plant growth and final yield are limited by the most limiting factor. For example, if growth is restricted because of a particular nutrient deficiency, growth responses to irrigation will be weak.

Tea is a perennial plant with shoots repeatedly plucked at regular intervals. Harvesting shoots takes away nutrients from the plant. It is therefore important to supplement soil nutrients by applying fertilizers. The yield and quality of tea can be manipulated by changing levels of soil plant nutrients (Ruan and Hardter, 2001).

A nutritional requirements study of bush tea reported by Mudau *et al*., (2005) revealed that potassium (K), nitrogen (N) and phosphorus (P) increased bush tea plant height, fresh and dry shoot mass, number of branches, leaf area and number of leaves. Bush tea optimum growth was achieved by applying 200 kg/ha K and 300kg/ha N and P. Chabeli *et al*., (2008) reported on a study to investigate the response of bush tea tannin content grown under 50% shaded nursery to N, P and K. Hydrolysable and condensed tannins increased when 300 kg/ha of N was applied. Peak values observed were condensed tannins with 4.5% and hydrolysable tannins with 0.06%. At 300 kg/ha the tannins also increased in response to nutrition of P.

The highest concentration of condensed tannins was 5% and hydrolysable tannin was 0.02%. At 200 kg/ha K, 0.041% hydrolysable tannins and 5% condensed tannins were observed. Mogotlane *et al.*, (2007) reported that total antioxidant content was increased by application N, P, and K fertilizers with the most increase occurring at 0-300N, 300P and 100K kg/ha.

Leaf analysis (also called foliar analysis, stem leaf analysis or tissue analysis) is the most precise method of plant nutrient level monitoring. The aim of plant analysis is to relate the content of mineral of the plant or part with its growth rate and yield, quality of the harvested product or physical appearance. This technique requires precise sampling procedures in respect of the selected plant part and the growth phase (time of sampling). The interpretation of results depends on the assumption that a significant biological relationship exists between the elemental content of the plant and its growth and/or production. Therefore, the aim of this study was to determine the effect of micronutrient fertilisation on bush tea foliar nutrient composition.

3.2 MATERIALS AND METHODS

3.2.1 Site description

The study was conducted at the University of South Africa (Florida Science Campus) Latitude: S26 ^{o}9.501 and Longitude: E27 ^{o}54.113, during the 2013/2014 summer and winter seasons (Johannesburg, South Africa). The site has a humid subtropical climate (hot, usually humid summers and mild to cool winters). Site experiences minimum temperatures of 13.1 ^{o}C to 15.0 ^{o}C, maximum temperatures of 29.1 ^{o}C to 32.1^{o}C with average rainfall of 6 to 63 mm and 25 to 100 mm respectively between the two seasons. Two locations within the site, (net shading and greenhouse) were chosen for the cuttings and different fertilization practices were put in practice over one year.

3.2.2 Bush tea collection

All bush tea samples were collected while they were still fresh from the wild at Muhuyu village (February) (22 ^{o}53’60S and 30 ^{o}25E, 724) (Figure 1) in Limpopo province, South Africa. Disease and insect free true to type samples were selected and kept under shade prior to preparation of cuttings.

Figure 1 Collection of bush tea at Muhuyu village

3.2.3 Plant material preparations

Two different propagation media, namely sand and pine bark were used in the experiment. The two media were randomly assigned to seedling trays with 5 x 3 x 4.5 cm (width, breadth and depth) cells. To get the medium moist before planting the filled trays were put under a mist system set to come on at 2 min intervals for 8 seconds. A mercury thermometer was inserted to a depth of 2 to 4 cm to measure the misting bed temperature. The measured temperature for a 48 hr period immediately prior to propagation varied between 17 to 29 ^{o}C in the four seasons. Shoots of 16 to 32 cm long were cut from the stock plants early in the morning (between 06:30 and 07:30), and wrapped with wet tissue paper followed by immediately placing them in plastic bags in order to keep them cool and turgid until taken to the working area (Araya, 2005).

To stimulate rapid and prolific rooting of cuttings, they were treated with Seradix® No.2, containing 0.3% Indole-3-butyric acid (IBA) (Bayer (Pty, Ltd) Pretoria, South Africa. Prepared cuttings were planted in trays with growth media composed of pine bark and sand in a ratio of 2:1. The trays were kept in shade netting and thereafter transferred to the greenhouse in winter.

Each seedling tray contained 45 cuttings (Figure 2). The measured minimum and maximum temperatures in the greenhouse varied from 16.4 to 34.3^{0}C and in the

shade netting varied from 14.5 to 32.7^{0}C, measured in the summer and winter seasons with maximum humidity (98.1%) and minimum humidity (39.1%).

Irrigation water was applied through misting drippers and the application frequency was determined by solar radiation, while the amount per irrigation was increased with increase in plant size to keep the soil moist, without excessive drainage.

Figure 2 Bush tea cutting trays

3.2.4 Transplanting

After 35 days, cuttings were transplanted into 4 litre plastic bags with the same media composition (pine bark and sand in 2:1 ratio) Figure 3. After transplanting, plants were kept in the shade net for 40 days. Thereafter, uniform plants were selected for fertility trial layout. These were transplanted to 4 litre bags with the same media, and were allowed to recover from shock for a week before resuming treatments under greenhouse conditions.

Figure 3 Transplanted bush tea

3.2.5 Treatment details and experimental design

The experiment was laid out in a completely randomized design (CRD) with five replications. Treatments were composed of: 0 ml. 50 ml, 100 ml and 150 ml of each micronutrient (Zn, Fe, Cu, B, and Mg) and a mix of all five micronutrients applied as foliar feeding. N, P, K fertilizers were applied across all treatments according to the recommended rate (Mudau *et al*., 2007). Application was done twice for two months, applied twice per month and the trial was terminated after one month and two weeks of the application. Harvested plants were freeze dried for 1-2 weeks for chemical composition (total polyphenols, tannins and antioxidant content) (Figure 4).

Figure 4 Harvested bush tea

3.3 LEAF ANALYSIS

3.3.1 Method for Perchloric + Nitric Acid Sample Digestion

Half a gram of sample was digested (in duplicate) with 7 ml HNO_3 (conc. Nitric acid) and 3 ml $HClO_4$ (perchloric acid) at temperature up to 180 ^{0}C and brought to volume in a 100 ml vol. flask (Zasoski and Burau, 1977).

3.3.2 ICP-OES Determination of Eleven Elements

An aliquot of each digest solution was used for the ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometric) determination of Mg, Fe, Zn, B and Cu. The ICP-OES is a multi-element instrument. The Varian Liberty Series ll is a sequential instrument, where the elements are determined almost simultaneously, with only a few seconds between each element.

Each element was measured at an appropriate emission wavelength, chosen for high sensitivity and lack of spectral interferences. The wavelengths used: Mg: 383.826 nm; Fe: 259.94 nm; Zn: 213.856 nm; Cu: 324.754 nm & B: 249.678 nm. No background correction was used. The instrument was set up and operated according to the recommended procedures in the instrument manual. It was calibrated against a series of standard solutions, containing all the elements of interest in the proportions found in typical leaf samples, with all samples within the calibration range for all elements. (Unpublished method developed by Mike Philpott at ARC-ISCW, based on the recommended procedures in the instrument manual (Varian, 1997).

3.4 STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) using the PROC GLM (General linear model) procedure of SAS version 8.0. (SAS Institute Inc., 1999). Mean separation for significant differences was done using the least significant difference (LSD).

3.5 RESULTS AND DISCUSSION

3.5.1 Results

3.5.1.1 Effects of Boron on bush tea leaves after foliar application

With B (Figure 5), a significant difference was observed between (B50 ml/l and B100 ml/l) treatment applications. The B content in bush tea samples increased slightly from B50 ml/l application to B100 ml/l application, however, it continued to increase at a higher rate with B150 ml/l application. Average means between three applications showed a marked difference, directly proportional to the application concentration, thus increase with boron concentration. Significant differences were observed on the effects of different boron treatments on the leaves of bush tea. Treatment (B150 ml/g) resulted in the highest yield of 1207 mg/kg. Treatment (B50 ml/g) produced the lowest yield of 14.73 mg/kg. Results show that there is strong linear correlation. Figure 5 indicates a perfect, positive relationship with a positive slope between boron treatment levels (x) and boron leaf percentage (y).

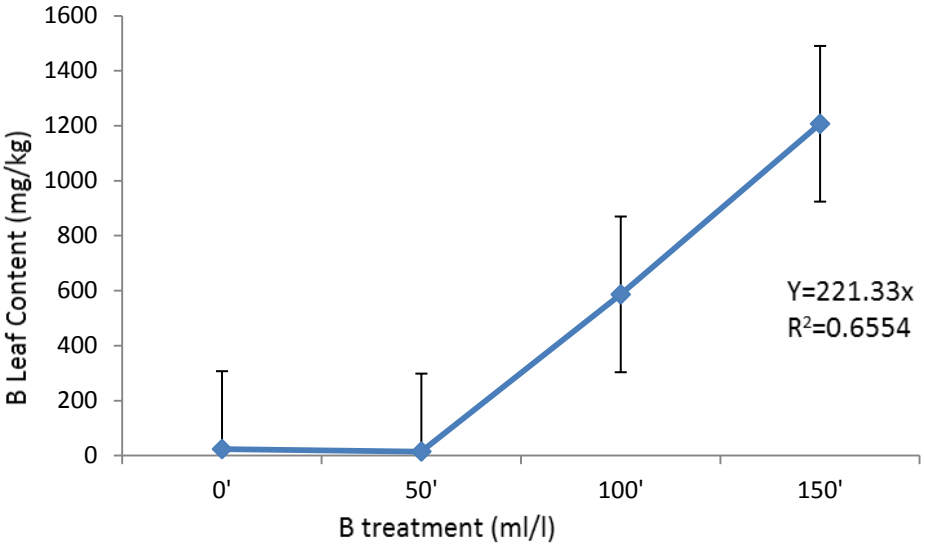

Figure 5 Percentage leaf B of bush tea after foliar application of boron

3.5.1.2 Effects of Iron foliar application on bush tea leaves

Iron, zinc and copper are usually compared with regard to their easily soluble forms and total contents (Matsuura *et al*., 2001).The results showed that with Fe (Figure 6), significant ($p<0.05$) differences exist between (Fe50 ml/l, Fe100 ml/l and Fe150 ml/l) treatment applications. The Fe content in bush tea increased from Fe50 ml/l application to Fe100 ml/l application but the Fe content dropped at Fe 150 ml/l application.

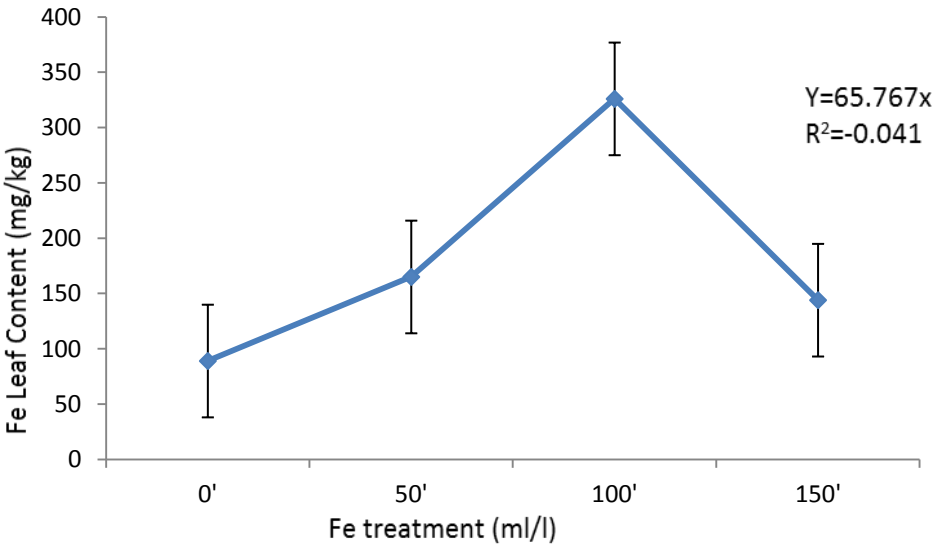

Figure 6 Percentage leaf Fe of bush tea after foliar application of iron

Results in Figure 6 showed that there has been significant quadratic response on leaf tissue Fe. The highest leaf tissue Fe was 326 mg/kg and lowest was 89 mg/kg. The difference between the highest and the lowest was 237 mg/kg reaching maximum 100 ml/l.

3.5.1.3 Effects of Zinc on bush tea leaves

The results from this study exhibited significant differences between (Zn50 ml/l, Zn100 ml/l and Zn150 ml/l) treatment applications (Figure 7). The Zn content increased from Zn50 ml/l application to Zn100 ml/l application, however, dropped significantly with Zn150 ml/l application.

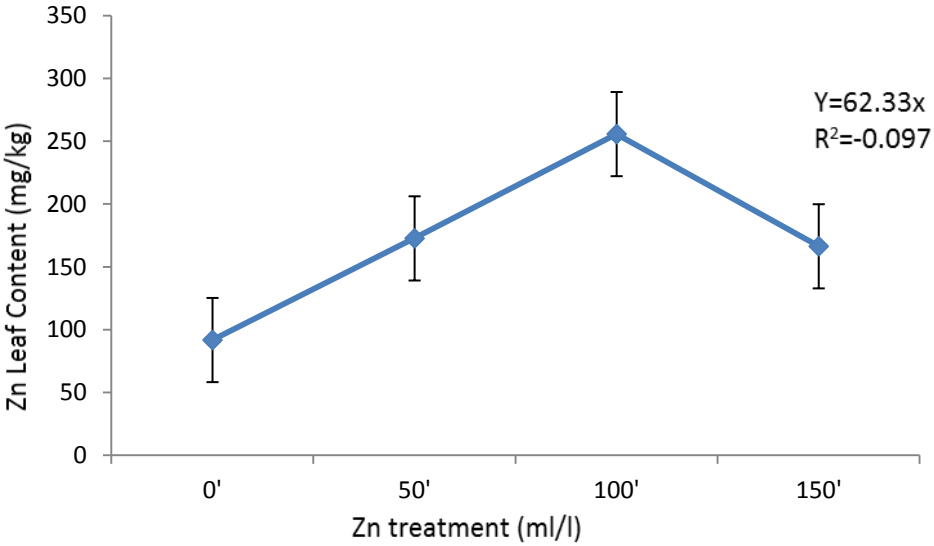

Figure 7 Percentage leaf Zn of bush tea after foliar application of zinc

Results in Figure 7 showed that there was a significant quadratic response on leaf tissue Zn. The highest leaf tissue Zn was 255.7 mg/kg and 91.8 mg/kg was the lowest. The difference between the highest and the lowest was 163.9 mg/kg reaching maximum 100 ml/l. Results show that there is no linear correlation. Figure 7 indicates a negative relationship with a negative slope between zinc treatment levels (x) and zinc leaf percentage (y). This trend was similar to what was observed in Fe (Figure 6).

3.5.1.4 Effects of Copper on bush tea leaves

Figure 8 indicated the significant differences observed between (Cu50 ml/l, Cu100 ml/l and Cu150 ml/l) treatment applications. The Cu content increased slightly from Cu50 ml/l application to Cu100 ml/l then increased at a higher rate with the Cu150 ml/l application.

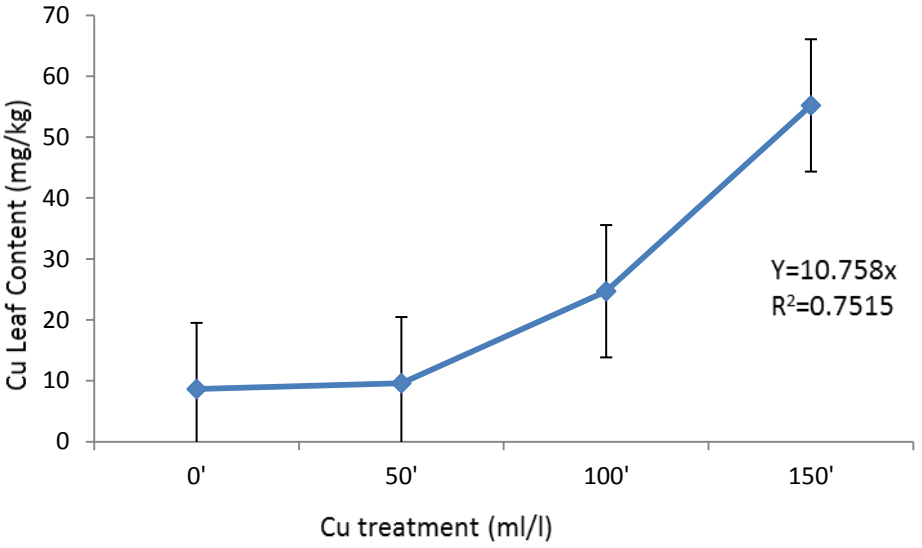

Figure 8 Percentage leaf Cu of bush tea after foliar application of copper

Results in Figure 8 indicated that there was a significant quadratic response on Cu in leaf tissue. The highest concentration of Cu in leaf tissue was 55.2 mg/kg and lowest was 8.64 mg/kg. The difference between the lowest and highest was 46.56 mg/kg reaching maximum at 150 ml/l. Yields from both treatments (Cu50 ml/g and Cu100 ml/g) were statistically similar. Results show strong linear correlation. Figure 8 indicates a perfect, positive relationship with a positive slope between copper treatment levels (x) and percentage of Cu in leaf tissue (y). This particular trend was also observed with the application of boron (Figure 5).

3.5.1.5 Effects of Magnesium foliar application on bush tea leaves

With Mg (Figure 9), treatment applications (Mg50 ml/l, Mg100 ml/l and Mg150 ml/l) showed significant ($p<0.05$) differences. The Mg content increased slightly from Mg50 ml/l application to Mg100 ml/l application but it continued to gently increase during the Mg150 ml/l application. The yields were higher than in the control treatments. The average between three applications is more or less similar to each other

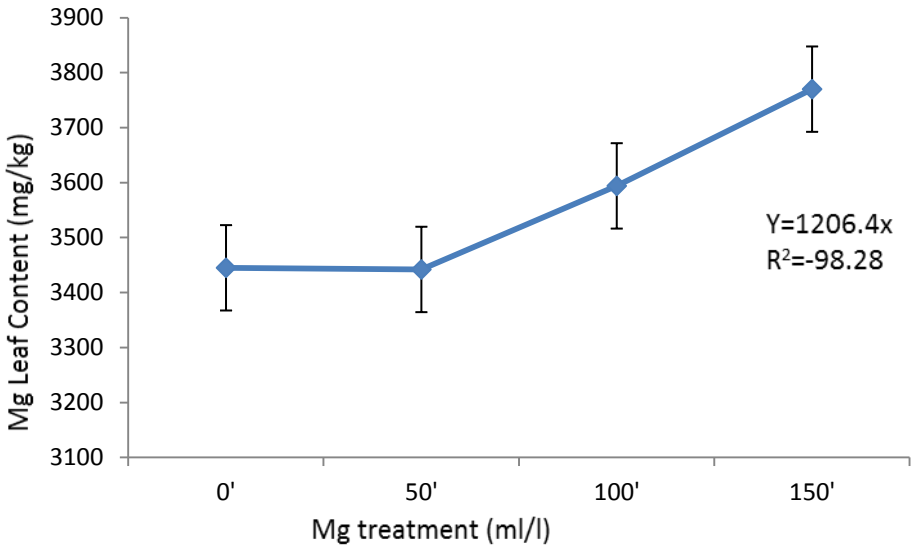

Figure 9 Percentage leaf Mg of bush tea after foliar application of magnesium

Results in Figure 9 showed that there was significant quadratic response on leaf tissue Mg. The highest leaf tissue Mg was 3770 mg/kg and lowest was 3442 mg/kg. The difference between the highest and the lowest was 328 mg/kg reaching maximum at 150 ml/l. Results show that there is no linear correlation. Figure 9 indicates a huge negative relationship with a negative slope between magnesium treatment levels (x) and magnesium leaf percentage (y).

3.5.2 Discussion

3.5.2.1 Effects of Boron on bush tea leaves

Sufficient application of B treatment (B50 ml/l and B100 ml/l) in bush tea content led to a significant increase in the content of B, while high B application treatment (B 150 ml/l) resulted in high significant increase of B content in bush tea. Gupta and Cutcliffe (1985) reported that boron levels (32-103 Mg/Kg) were sufficient in carrot leaf. Gupta *et al*., (1985) also reported that boron levels less than 28 mg/kg are related to B deficiency and are similar to those of Kelly *et al*., (1952) who found boron levels of 16 mg/kg to be deficient. The results of this study indicated that sufficient range of applications were B50 ml/l and B100 ml/l while B150 ml/l was too high. The results are in agreement with Uddin *et al*., (2008) who indicated that boron application (37.54-234.5 mg/kg) significantly increased the number of tillers. Nadim *et al*., (2012) reported that B concentrations (2.68-37.54 mg/kg) increased photosynthesis and more formation of chlorophyll in the leaf tips and leaves.

3.5.2.2 Effects of Iron on bush tea leaves

Adequate application of Fe treatment (Fe 100 ml/l) in bush tea led to a significant increase in the content of Fe, while sufficient Fe application treatment (Fe50 ml/l) resulted in similar but slight increase of Fe content in bush tea. Higher foliar application of Fe (Fe150 ml/l) to bush tea led to a significant decrease in the content of Fe. Results in Figure 6 showed that Fe150 ml/l application had a significant decrease of 182 mg/kg. These results were consistent with those indicated by Street *et al.,* (2006) who found slight increase but similar Fe content level as a result of sufficient application of treatments (103 mg/kg-523 mg/kg).

Iron in living organisms is an essential trace element. The estimated daily minimum requirement for Fe ranges from about 10-50 mg and depends on sex, age, iron bioavailability and physical status (WHO, 1998). The inhibitory effect is greatly reduced an hour after eating if tea is not consumed (Hurrell *et al*., 1999). These results also agree with Olivier *et al.,* (2012) who reported that Fe mineral content are similar in both leaves and tea infusions in bush tea (225 mg/kg). Results also agree with Ferrara *et al*., (2001) who reported that Fe mineral content are similar in black tea and green tea. All these results are in agreement with the results showed in this study (Figure 6).

3.5.2.3 Effects of Zinc on bush tea leaves

Zinc is an essential trace element. Zinc content in tea infusions is very low, therefore cannot be seen as a major dietary source (Street *et al*., 2006). Intake of 150-450 mg of Zn per day have been associated with low Cu status, altered Fe functions, reduced high-density lipoprotein levels and reduced function of immune system (Hamilton *et al*., 2000). In the current study, the highest foliar application of Zn (Zn150 ml/l) to bush tea led to a significant decrease in the content of Zn, while the other Zn applications (Zn50 ml/g and Zn100 ml/g) resulted in slightly increase in Zn content. These results were same to those indicated by Narin *et al*., (2004) who observed the same Zn content level as a result of sufficient application of treatments. Narin *et al*., (2004) reported that Zn levels were 109.9 mg/kg -146.1 mg/kg in tea and tea infusions. These results do not agree with Street *et al*., (2006) who reported higher increase of Zn levels by 256 mg/kg (511 mg/kg-2220 mg/kg) in tea and tea infusions. The current results also disagree with Olivier *et al*., (2012) who reported that zinc mineral contents are lower in both leaves and tea infusions in bush tea (12.7 mg/kg-33.1 mg/kg).

3.5.2.4 Effects of Copper on bush tea leaves

With regards to human health and Cu, the average daily intake of Cu is 1.2 mg (WHO, 1998). Significant dietary source of Cu is not tea infusions; however, drinking tea actually enhances the absorption and solubilisation of Cu from the gut increase the storage of the metal in the liver (Vaquero *et al*., 1994).

Application of Cu treatment of Cu150 ml/l to bush tea led to a significant increase in the content of Cu, while Cu application treatment of Cu50 ml/l and Cu100 ml/l resulted in similar but slight increase of Cu content in bush tea. These results were in agreement to those indicated by Narin *et al*., (2004) who observed the same Cu content level as a result of sufficient application of treatments. Narin *et al*., (2004) reported that Zn levels were 110.4 mg/kg-24.8 mg/kg in tea and tea infusions. Results (Figure 8) showed that Cu150 ml/g application had a significant increase of 30.4 mg/kg. These results were similar to those observed by Street *et al*., (2006) who indicated the same Cu content level as a result of sufficient application of treatments (9.0 mg/kg-65 mg/kg).

Marcus *et al*., (1996) stated that in tea leaves the metal content differs according to geological conditions and the type of tea (black or green tea). Fung *et al*., (2002) reported that in different parts of the tea plant (young leaves, old branches and leaves) the element concentration differs in different locations. These results also agree with Olivier, *et al*., (2012) reported that Cu mineral contents are similar in both leaves and tea infusions in bush tea (9.4 mg/kg-13.5 mg/kg). Results also corroborate with Ferrara, *et al*., (2001) who reported that Cu mineral content are similar in black tea and green tea (7.5 mg/kg-29.6 mg/kg) but results of the current study (Figure 8) had a slight increase by 25.6 mg/kg. Results also agree with Wong *et al*., (1998) observation on Cu content level of 6.25 mg/kg-32 mg/kg in tea. All these results are in agreement with the results shown in Figure 8.

3.5.2.5 Effects of Magnesium on bush tea leaves

Higher application of Mg treatment of (Mg150 ml/l) to bush tea led to a significant increase in the content of Mg, while Mg application treatments of Mg50 ml/l and Mg100 ml/l resulted in similar but slight increases of Mg content in bush tea. These results were consistent with those indicated by Bassel and Erdemoglu (2006) who found similar Mg content level as a result of sufficient application of treatments. Bassel and Erdemoglu (2006) reported that Mg levels in seven herbs and their infusions were in the range of 1643-3378 mg/kg and 610-2078 mg/kg, respectively. Results showed in Figure 9 disagree with Lozak *et al*., (2002) who reported data on Mg present in raw mint leaves as high as 5778-15.331 mg/kg.

The results of current study were similar to those of Nookabkaew *et al*., (2006) who reported that herbal tea products contain high concentration of Mg (783-7739 mg/kg). Nookabkaew *et al*., (2006) also reported that other effective sources of Mg for humans can be G*. pentaphyllum* (2153-9610 mg/kg) and G. *sinensis* (764.3-2152 mg/kg). Nookabkaew *et al*., (2006) also indicated that the average daily intake of Mg is 320-420 mg/day. Bello *et al*., (2004) found that large amounts of nutrients are found in herbal plants and they are also rich in Mg (1540-5470 mg/kg). The abundance of Mg in the result of this study was consistent with findings of Chizzola and Franz, (1996) and Lavilla *et al*., (1999) who reported that Mg abundant metal constituents in many plants.

3.6 CONCLUSION

Micronutrient foliar applications influenced the quality of bush tea. Due to application of micronutrients solutions, treatment of Mg150 ml/g resulted in the highest yield of 3770 mg/kg, treatment of B150 ml/g resulted in the highest yield of 1207 mg/kg, treatment of Fe100 ml/g resulted in the highest yield of 326 mg/kg, treatment of Zn100 ml/l resulted in the highest yield of 255.7 mg/kg and treatment of Cu150 ml/g resulted in the highest yield of 55.2 mg/kg respectively. All levels of treatments indicated highly significant difference from each other. It can be concluded that micronutrient folia application has significant effects on the quality of bush tea at different application rates and foliar feed influenced the leaf content of that micronutrient, with changes in content due to different fertilizer application rates.

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CHAPTER 4

Effect of micronutrient application on bush tea antioxidant activity, polyphenols, flavonoids and tannins compositions.

4.1 INTRODUCTION

Polyphenols are a structural class of organic chemicals, semisynthetic or synthetic but mainly natural, characterized by the presence of large multiples of phenol structural units. Polyphenols are substances that contain anti-mutagenic, anti-oxidant, anti-inflammatory, anti-allergic, anti-viral and anti-bacterial properties (Hirasawa *et al.,* 2002). In green tea, epigallocatechin-3-gallate (EGCG) is reported to be the major anti-oxidant polyphenol which reduces the amount of inflammatory prostaglandins and free radicals (Katiyar and Mukhtar, 1996). Polyphenols are potential quality indicators and rich source of these compounds are bush tea leaves, which in nature are potent antioxidants (Mudau *et al*., 2007b).

Agronomic practices have been indicated to enhance black tea growth and quality (Ogunmoyela *et al*., 1994). The quality of herbal teas have been reported to be influenced by chemical compounds (Owour *et al*., 2000). Medicinal potential indicators are attributed to presence of compounds such as tannins, polyphenols and flavonols in herbal teas. Amino acids, organic acids, carbohydrates, volatile compounds and vitamins are other components of herbal tea which are able to influnce sensory quality attributes such as sweetness, aroma, astringent taste and bitterness (Mudau *et al*., 2007b).

Herbal tea health benefits are characterized by its antioxidant content (Mogotlane *et al*., 2007). Bush tea leaves also contain flavonols, polyphenols and tannins (Nchabeleng *et al*., 2012). In most cases flush of tea has been known to be followed accumulation of carbohydrate reserves which are channelled towards polyphenol production (Roberts, 1990). In tea flush, total polyphenols range from 20% to 35%. Leaf age, the clone being examined, soil, leaf age, and agronomic practices and climate conditions determine the chemical composition of the tea leaves (Hartmann *et al*, 1996). Antioxidant content is broadly or widely used for health benefits, in terms of it being a parameter to characterize different materials (Mogotlane *et al*., 2007). Antioxidant content is associated with those compounds which have the ability to provide protection of biological systems against the effects that are harmful

from reactions of extreme oxidation, which involves nitrogen and oxygen species reaction (ROS) (Mogotlane *et al*., 2007).

Extensive or wide range of phenolic compounds produce antioxidant properties of tea that fall or found under amphipathic molecules (Ivanova *et al*., 2004). The of activity of phenolics as antioxidant are mainly due to their redox functions enabling them to be reduction agents, oxygen singlet quenches, metal chelators and donators of hydrogen (Rice-Evans *et al*., 1997).

In bush tea leaves the most common important chemicals are tannins, flavonols and polyphenols (Ivanova *et al*., 2004). Tea polyphenols are chosen to control the antimutagenic and anticarcinogenic properties of tea and are natural antioxidants (Reza *et al*., 2007). Wide range of polyphenols are found in herbal teas (Owour *et al*, 2000), which contain favourable physiological and biochemical properties for human health (Hirasawa *et al*., 2002). Rich sources of polyphenolic substances are reported to be tea leaves (Benzie *et al*., 1999), and these polyphenolics account for one-third of dry mass of dried leaves (Liang *et al*., 1996). Therefore the aim of this study was to determine the effects of application of micronutrients on bush tea antioxidant activity, polyphenols, flavonoids and tannins compositions.

4.2 MATERIALS AND METHODS

4.2.1 Chemical composition

Bush tea was propagated as reported in Chapter 3 leaf samples were collected and air dried in the shade. There after the samples were finely ground. Approximately, 15 g of finely ground material was sieved (≤1.0 mm Endocotts test sieves) for 5 minutes. From the sieved material, 0.5 g sample with 5 mL acetone was mixed for 2 hours in a shaker, and then centrifuged for 5 minutes at 4000 rpm. The supernatant was carefully decanted and the extraction procedure was repeated three times on residues. Three supernatants were combined and a volume of 15 mL with 75 % acetone was prepared. The residues were discarded.

4.2.1.1 Determination of antioxidant activity

Antioxidant activity of extracts was determined using Trolox Equivalent Antioxidant Capacity (TEAC) assay as described by Awika *et al.,* (2004). The chosen assay, TEAC is a spectrophotometric technique that measures the relative ability of

hydrogen-donating antioxidants to scavenge the 2, 2'-azino-bis3-ethylbenzthiazoline-6-sulfonic (ABTS+) radical cation chromogen in relation to that of Trolox, the water soluble vitamin E analogue, which is used as an antioxidant standard. The ABTS+ was produced by mixing equal volume of 8 mm ABTS with 3 mm potassium persulfates prepared in distilled water and allowed to react in the dark for at least 12 hours at room temperature before use. The ABTS+ solution was diluted with a phosphate buffer solution (pH 7.4) prepared by mixing 0.2 M of NaH_2PO_4, 0.2 M $NaHPO_4$ and 150 mM NaCl in 1 L of distilled water, with pH adjustment using NaOH, where necessary. This solution was made fresh for each analysis. The ABTS+ solution (2900 µL) was added to the methanol extracts of bush tea (100 µL) of Trolox in a test tube and mixed. Absorbance readings (at 734 nm) were taken after 30 minutes (for the samples) and 15 minutes (for the standard) of the initial mixing of the samples and standard respectively. The results were expressed as µM Trolox equivalents per g of sample on a dry weight basis.

4.2.1.2 Determination of polyphenol content

Methanol extracts were utilized for the determination of total phenols. Duplicates of 2 g of selected herbal teas extracted using 30 ml of methanol: Ten millilitres of methanol were added to 2 g of sample in centrifuge tubes and vortex mixed every 10 minutes for 2 hours to improve extraction efficiency. Samples were centrifuged at 3 500 rpm for 10 minutes (25°C) and decanted. Each sample residue was rinsed twice with 10 ml of solvent, vortex mixed for 5 minutes, centrifuged as above, and decanted. The two supernatants were pooled before being used for analysis. The Folin Ciocalteau method (Singleton and Rossi, 1965), modified by Waterman and Mole (1994), was used to determine total phenols in all selected herbal tea extracts. This method is based on the reducing power of phenolic hydroxyl groups (Hahn *et al*., 1984) which react with the phenol reagent to form chromogens that can be detected spectrophotometrically. In brief, methanol extracts (0.5 ml) were added to a 50 ml volumetric flask containing distilled water and mixed. Folin Ciocalteau phenol reagent (2.5 ml) was added and mixed, followed by 7.5 ml sodium carbonate solution (20 g/100 ml) within 1-8 minutes after addition of the Folin Ciocalteau phenol reagent. The contents were mixed and the flask made up to volume with distilled water and thoroughly mixed. Absorbance of the reactants was read after 2 hours at 760 nm using UV-visible a-Genesys 20 Spectrophotometer. Catechin was utilized as

a standard to formulate a standard curve and results expressed as mg equivalents/100 mg of samples on a dry weight basis.

4.2.1.3 Determination of tannins

The Vanillin HCl method of Prince *et al*. (1978) was used for the determination of tannins. This method is based on the ability of flavonoids to react with vanillin in the presence of mineral acids to produce a red colour that is measured spectrophotometrically. The extracts and reagents were maintained at 30°C in a thermostat-controlled water bath before mixing the reactants. The methanolic extracts (1 ml) was added to 5 ml vanillin reagent (4% HCl in methanol and 0.5 ml vanillin in methanol) then mixed. Sample blanks were prepared with 4% HCl in methanol replacing vanillin reagent. The reactants were maintained at 30°C and absorbance read at 500 nm after 20 minutes. Absorbance reading of the blanks was subtracted from those of the samples. Catechin was used as a standard and results were expressed as mg catechin equivalents/100 mg sample on a dry weight basis.

4.3 STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) using the PROC GLM (General linear model) procedure of SAS version 8.0. (SAS, Institute Inc., 1999). Mean separation for significant differences was done using the least significant difference (LSD).

4.4 RESULTS AND DISCUSSION

4.4.1 Results

4.4.1.1 Total polyphenols

Variations in the concentration of total polyphenols among the different treatments applied (Table 1) were shown in this work. Where fertiliser treatments were not applied (control), no significant difference was observed on total polyphenols of bush tea. Different application rates had a significant difference on the total polyphenols of bush tea ranging from 10 ml/l to 30 ml/l of mixture (B + Zn + Fe + Cu + Mg) application, with the highest total polyphenols concentration obtained from both mixture (B + Zn + Fe + Cu + Mg) (10 ml/l, 20 ml/l and 30 ml/l) applications compared to other micronutrient treatments.

The highest total polyphenols concentration was observed in mix 30 ml/l application with 93.70 mg/g. Furthermore, it was observed that iron applications had no significant difference on total polyphenols of bush tea as results were similar to control applications with lowest total polyphenols (34.70 mg/g). The study showed that different application rates of B, Zn and Cu treatments had significant difference on the total polyphenols of bush tea. The optimum rate of polyphenols in B application was obtained at B100 ml/l reaching maximum total polyphenols of 78.70 mg/g. The optimum rate of polyphenols in zinc application was obtained at Zn150 ml/l reaching maximum total polyphenols of 70.20 mg/g. The optimum rate of polyphenols in Cu application was obtained at Cu50 ml/l reaching a maximum of total polyphenols of 78.40 mg/g.

Table 1 Total Polyphenols of bush tea in reaction to different concentrations fertilizers' foliar spray

Fertilizer type	Concentration level	Total phenols
Control	0	31.43[b]
B	50	67.40[a]
	100	78.70[a]
	150	71.20[a]
Fe	50	35.00[b]
	100	45.10[a]
	150	34.70[b]
Zn	50	56.70[a]
	100	60.30[a]
	150	70.20[a]
Cu	50	78.40[a]
	100	68.40[a]
	150	72.00[a]
Mix (B+Fe+Zn+Cu+Mg)	10	89.30[a]
	20	77.50[a]
	30	93.70[a]

Values with the same letter along the row within the same fertilizer treatment type are not significantly different at p<0.05 level of significant, using Duncan Multiple Rage Test (DMRT). All foliar spray of B, Fe, Zn, Cu and the combination of all foliar spray significantly improve total polyphenols.

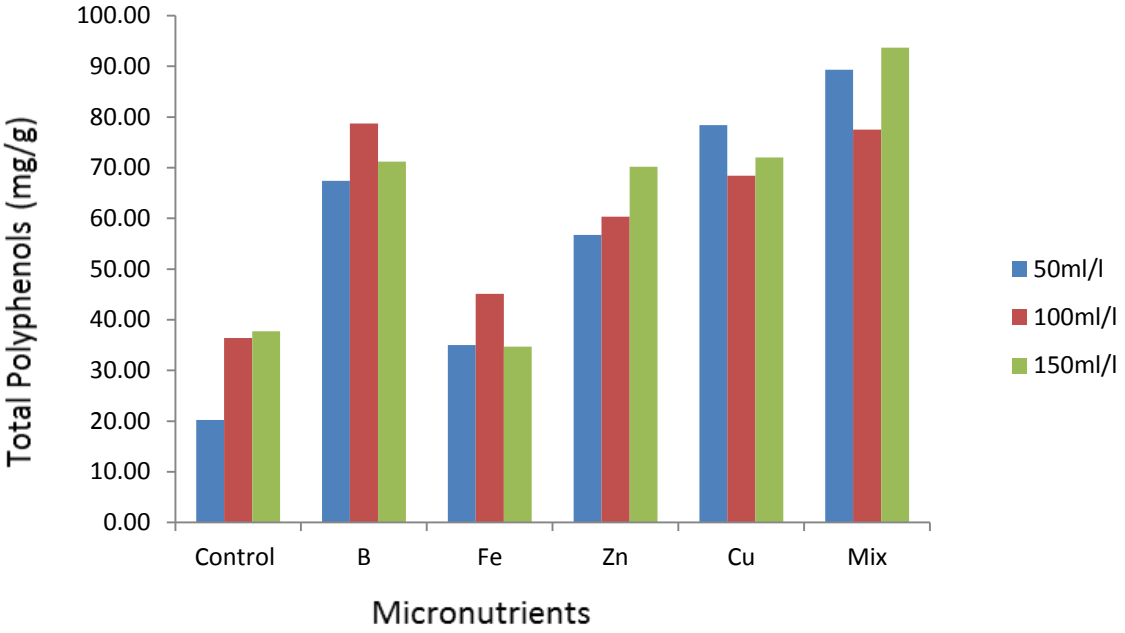

Figure 10 Total Polyphenols content (50 ml/l, y=15.545x, R^2=0.5581; 100 ml/l, y=15.135x, R^2=-1.02 and 150 ml/l, y=16.343x, R^2=0.1168) in bush tea

[1]

[1] Mix treatments were 10ml/l, 20ml/l and 30ml/l

4.4.1.2 Tannins

Results Table 2 showed that all treatments significantly increased tannin content of bush tea compared with the control. Higher tannin concentrations were observed in all copper treatments; however, Cu50 ml/l treatment exhibited the highest tannins content with 99.50 mg/g. There was a drop of tannin content between Cu50 ml/l (89.30 mg/g) and Cu100 ml/l (87.30 mg/g). It was observed that different applications rates of boron, iron, zinc and mixture (B + Zn + Fe + Cu + Mg) treatments had significant difference on the tannins content of bush tea compared with the single foliar elements applications.

The tannin content in boron application increased significantly from B50 ml/l (77.40 mg/g) application to B100 ml/l (88.70 mg/g), however, it slightly dropped with B150 ml/l (88.40 mg/g) application. Table 2, shows the significant difference between (Fe50 ml/l, Fe100 ml/l and Fe150 ml/l) treatment applications. The Fe50 and 150 ml/l applications showed significant accumulation of tannin compared with the control and Fe100 ml/l (Table 2). There was a 20.2 % yield increase of tannin between Zn50 ml/l and Zn100 ml/l, followed by a significant ($p<0.05$) 42.8 % drop in tannin accumulation in the leaves of bush tea at Zn150 ml/l foliar application. The results of the single elements folia application are similar to those of mixture (B + Zn + Fe + Cu + Mg) application.

Table 2 Total tannins of bush tea in reaction to different concentrations fertilizers' foliar spray

Fertilizer type	Concentration level	Total tannins
Control	0	34.76[b]
B	50	77.40[a]
	100	88.70[a]
	150	88.40[a]
Fe	50	77.50[a]
	100	54.40[a]
	150	78.40[a]
Zn	50	78.60[a]
	100	98.50[a]
	150	56.30[a]
Cu	50	99.50[a]
	100	89.30[a]
	150	87.30[a]
Mix (B+Fe+Zn+Cu+Mg)	10	87.30[a]
	20	87.40[a]
	30	71.90[a]

Values with the same letter along the row within the same fertilizer treatment type are not significantly different at p<0.05 level of significant, using Duncan Multiple Rage Test (DMRT). All foliar spray of B, Fe, Zn, Cu and the combination of all foliar spray significantly improve total tannin.

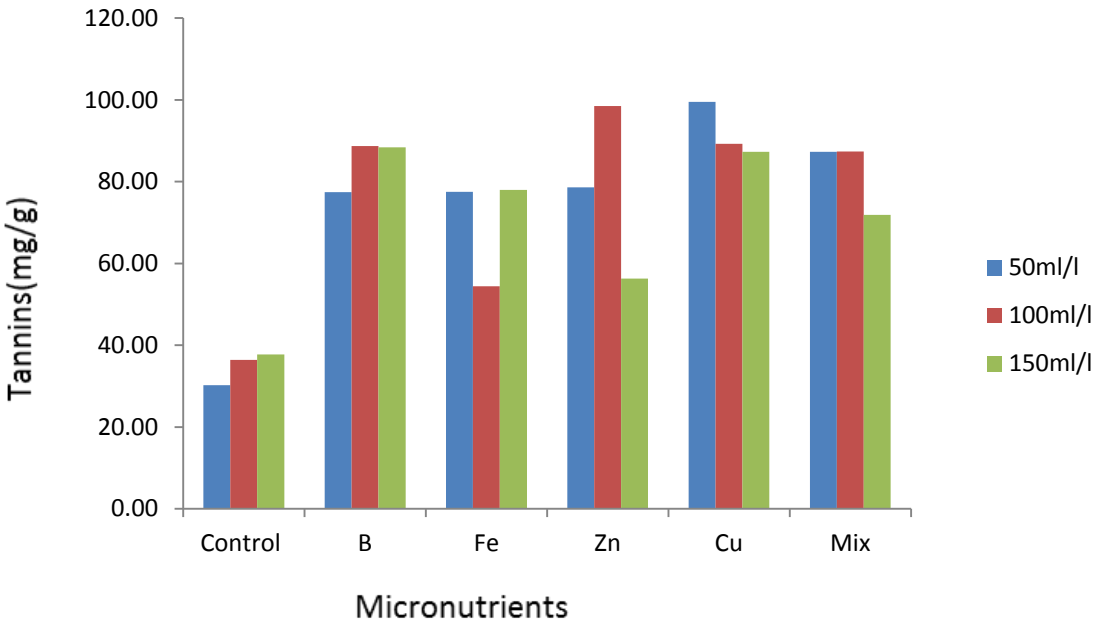

Figure 11 Tannin content (50 ml/l, y=19.266x, R^2=-0.017; 100 ml/l, y=19.142x, R^2=-0.371 and 150 ml/l, y=19.142x, R^2=-0.371) in bush tea

[2]

[2] Mix treatments were 10ml/l, 20ml/l and 30ml/l

4.4.1.3 Total flavonoids

There were variations in the concentration of total flavonoids among the different treatment applications (Table 3). Where fertiliser treatments were not applied (control), total flavonoids in bush tea resulted with no significant differences. Different application rates had significant differences on the total flavonoids of bush tea ranging from 10 ml/l to 30 ml/l of mixture (B + Zn + Fe +Cu + Mg) application. The highest total flavonoids concentrations were obtained from both Cu50 ml/l with 177.90 mg/g and mixture (B + Zn + Fe + Cu + Mg) application mix10 ml/l with 176.60 mg/g compared to other micronutrients treatments.

The total flavonoids content in B application increased highly from B50ml/l (144.80 mg/g) application to B100 ml/l (167.40 mg/g) application, however, it slightly dropped with B150 ml/l 159.50 mg/g application. Significant difference exists between (Fe50 ml/l, Fe100 ml/l and Fe150 ml/l) treatment applications (Table 3). The total flavonoids content in Fe applications of bush tea increased highly from Fe50 ml/l application with 112.50 mg/g, it continued to drop highly during Fe100 ml/l application with 99.50 mg/g, however, it continued to increase at a high rate at Fe150 ml/l application with 112.70 mg/g. Significant differences were also shown between Zn50 ml/l, Zn100 ml/l and Zn150 ml/l treatment applications (Table 3). The total flavonoids content in zinc applications of bush tea increased considerable from Zn50 ml/l (135.30 mg/g) application to Zn100 ml/l (158.80 mg/g) application, however, dropped significantly with Zn150 ml/l (126.40 mg/g) application. Results indicated that with Cu (Table 3) significant difference exists between (Cu50 ml/l, Cu100 ml/l and Cu150 ml/l) a treatment application. Results also displayed that Cu50 ml/l application had higher total flavonoids content with 177.90 mg/g; however Cu100 ml/l and Cu150 ml/l applications which had similar total flavonoids content of 157.90 mg/g and 159.20 mg/g, respectively.

Table 3 Total Flavonoids of bush tea in reaction to different concentrations fertilizers' foliar spray

Fertilizer type	Concentration level	Total flavonoids
Control	0	66.23[b]
B	50	144.80[a]
	100	167.40[a]
	150	159.50[a]
Fe	50	112.50[a]
	100	99.50[b]
	150	112.40[a]
Zn	50	135.30[a]
	100	158.80[a]
	150	126.40[a]
Cu	50	177.90[a]
	100	157.70[a]
	150	159.20[a]
Mix (B+Fe+Zn+Cu+Mg)	10	176.60[a]
	20	164.90[a]
	30	164.60[a]

Values with the same letter along the row within the same fertilizer treatment type are not significantly different at p<0.05 level of significant, using Duncan Multiple Rage Test (DMRT). All foliar spray of B, Fe, Zn, Cu and the combination of all foliar spray significantly improve total flavonoids.

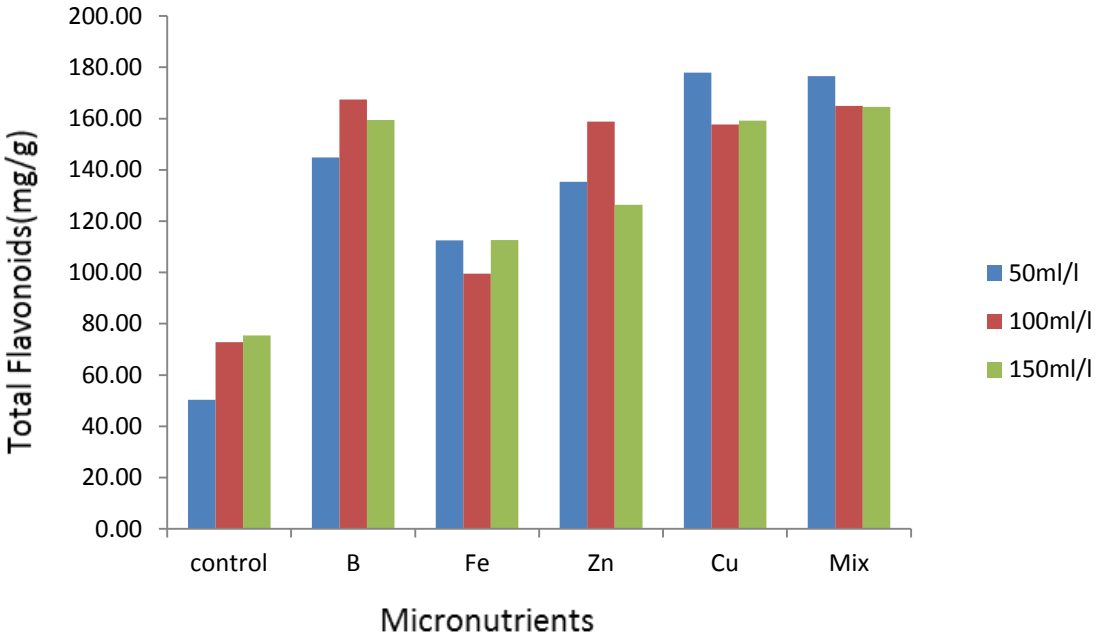

Figure 12 Total flavonoid content (50 ml/l, y=34.811x, R^2=0.378; 100 ml/l, y=34.277x, R^2=-0.67 and 150 ml/l, y=33.207x, R^2=-0.937) in bush tea

3

[3] Mix treatments were 10ml/l, 20ml/l and 30ml/l

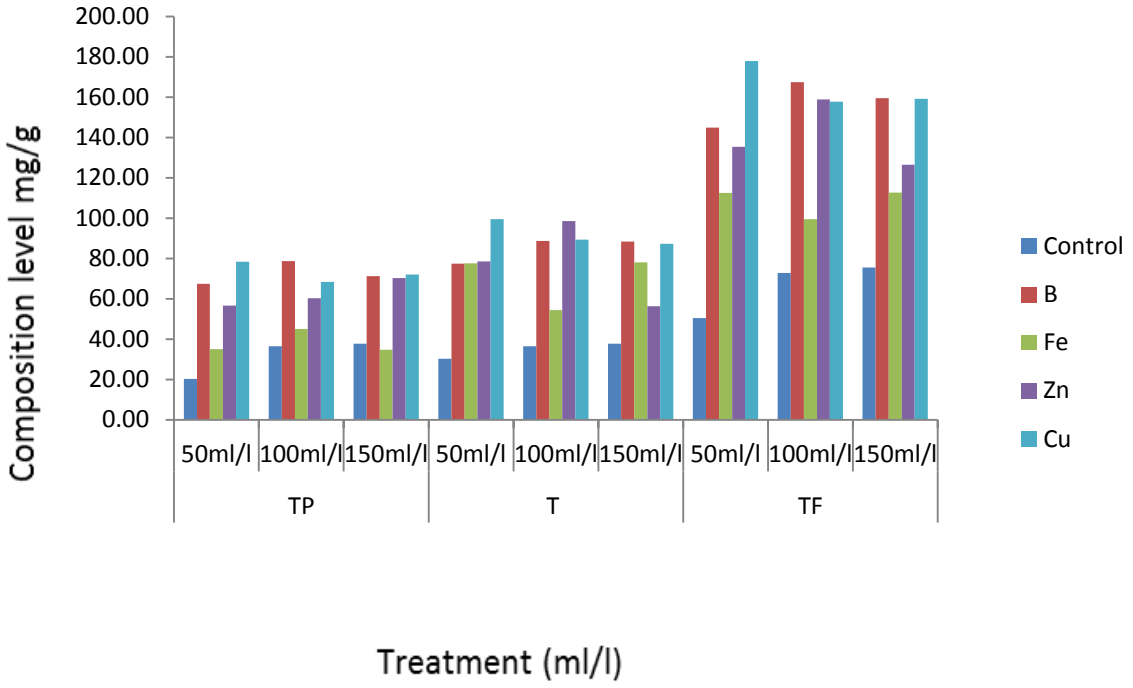

Figure 13 Combined total polyphenols concentration, tannins concentration and total flavonoids concentration

4.4.2 Discussion

4.4.2.1 Total polyphenols

In bush tea leaves the most common important chemicals are polyphenols (Ivanova *et al*., 2004).The results revealed that the control had no significant differences on total polyphenols of bush tea This is in full agreement with a study by Njogu *et al*., (2014) who reported that no fertilizer application had any significant influence on total polyphenols of *Camellia sinensis*. Furthermore, the findings of Mashingaidze *et al*., (2014) were consistent with results of this study who found that the control (no fertilizer application) had the least average score of 15.31 mg/g, therefore the poorest quality. The study showed that different application rates of zinc treatments had significant difference on the total polyphenols of bush tea ranging from 50 ml/l to 150 ml/l. The optimum rate of polyphenols following zinc application was obtained at Zn150 ml/l, reaching a maximum of 70.20 mg/g. The results of this study are consistent with the findings of Venkatesan *et al*., (2005) who reported a highly and positive significant correlation between polyphenols content and zinc levels (31.8 mg/g) of mature leaves of tea. Zinc and Copper in tea leaves have been reported to increase the levels of polyphenol oxidase and flavins. These elements are important elements in the formation of theaflavins and thearubins during fermentation of black tea; this could explain their significant effect on quality and are vital components of polyphenol oxidase. A positive correlation of total phenol content and copper was found in tea samples of Nigerian tea which improves tea quality (Ogunmoyela *et al*., 1994).

4.4.2.2 Tannins

There is no existing literature on the effects of micronutrients to determine tannins on growth and quality of bush tea. The results showed a significant highest tannins concentration in all copper treatments, the results of the accumulates of tannins in response to Cu treatments is consistent with Chiu, (1990) who reported that the increase in hydrolysable tannins during summer was found to be pronounced due to higher temperatures and light intensities. In bush tea leaves the most important chemicals are tannins (Ivanova *et al*., 2004). A study on the seasonal variation of tannins in wild bush tea showed that the highest concentrations of condensed

tannins were 4.8% compared to those found in winter (2.4%), spring (2.7%) and summer (3.0%) Mudau *et al*. (2007c).

4.4.2.3 Total flavonoids

Information is lacking on the effects of micronutrients application on growth and quality of bush tea. The results revealed that highest total flavonoids concentrations were obtained from both Cu50 ml/l with 177.90 mg/g and mixture (B + Zn + Fe + Cu + Mg) application mix10 ml/l with 176.60 mg/g compared to other micronutrients treatments. In bush tea leaves the important chemicals are flavonols (Ivanova *et al*., 2004). Mashimbye *et al*., (2006) found that 5-hydroxy-6, 7, 8, 3', 4, 5'-hexamethoxy-flavon-3-ol is a major flavonoid in bush tea. Flavonoids are known for their growth inhibition activities and cytotoxicity activities and cyclic adenosine monophosphate (cAMP) diphosphoesterase reserve activities (Mashimbye *et al*. 2006). In herbal teas flavonoids are potential quality indicators since in nature they are antioxidants (Mudau *et al*., 2007b).

4.5 CONCLUSION

All levels of treatments were highly significantly different from each other. Due to application of micronutrients solutions, the highest total polyphenols concentration was observed in Treatment (mix30 ml/l) application with 93.70 mg/g and the lowest total polyphenols concentration was observed in the control with 20.20 mg/g. Treatment (Cu50 ml/l) showed the highest tannins content with 99.50 mg/g and control treatment showed the lowest tannins content with 30.20 mg/g. The results showed that the highest total flavonoid concentrations were obtained from treatment (Cu50 ml/l) with 177.90 mg/g and the results showed that lowest total flavonoids concentrations were obtained from (control) with 50.40 mg/g. It can be concluded that micronutrients application had significant effects on the bush tea quality.

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CHAPTER 5

Effect of micronutrient application on bush tea phytochemicals and chemotypic variations.

5.1 INTRODUCTION

Metabolomics is the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind", the study of their small-molecule metabolite profile. There are essentially two approaches: targeted and untargeted methods. The targeted approach focuses on identifying and quantifying selected metabolites, such as substrates of an enzyme, direct products of a protein, a particular class of compound or members of a particular pathway (Lommen *et al*., 2007). It was found that bush tea contained 5-hydroxy-6, 7, 8, 3', 4', 5'-hexamethoxyflavon-3-ol.

Flavonoids such as 5-hydroxy-6, 7, 8, 3', 4', 5'-hexamethoxyflavon-3-ol are found in bush tea. This flavonoid ($C_{21}H_{22}O_{10}$) was characterized and found for the first time in bush tea (Mudau, 2007). Flavonoids are known to be potent anti-oxidants (Schewe and Sies, 2005) and have ability to scavenge lipid peroxy radicals, hydroxyl radicals and superoxide anions. The aim of this experiment was to determine the effect of micronutrient application on bush tea chemotypic variations.

5.2 MATERIALS AND METHODS

5.2.1 Sample preparation

Bush tea was propagated as reported in Chapter 3 leaf samples were collected and air dried in the shade at room temperature. Samples were then pulverized to powder using liquid nitrogen (LN_2) in a mortar and pestle. Samples were kept in sealed glass vials and placed in the dark at 2-8^{o}C until further use (LC-MS). For Gas chromatography linked to mass spectrometry (GC-MS) samples were picked 24 hours prior to being tested. Samples consisted of single leaf per replicate per plant from the greenhouse experiment, that were placed in airtight zip lock® bags. They were later transferred using forceps into GC-MS headspace screw cap vials.

5.2.2 Gas Chromatography linked to mass spectrometry (GC-MS)

Bush tea samples (one leaf per sample) were analysed using head space solid phase micro-extraction gas chromatography linked to mass spectrometry (HS-SPME-GC-MS), a method similar to Musarurwa *et al*., (2010), but oven ramping temperatures and injection ratios were modified. The HS-SPME of leaves was performed with Supelco® SPME fibres [DVB/ Carboxen/PDMS, (Supelco)]. Leaves were placed directly into a 20 ml headspace vial, sealed with an aluminium-coated silicone rubber septum. Volatiles were extracted at 70^{o}C for 15 minutes. The gas chromatography was performed with a Waters GCT Premier AS 2000 instrument coupled to a mass spectrometer, equipped with a HP5 column (30 m, 0.25 mm ID, 0.25 lm film thickness). Temperatures were set at 250^{o}C for both the injection (split-less injection) and the ion source temperature. Helium was used as the carrier gas (1 mlmin^{-1}). The temperature ramp regime was initiated by heating at 50^{o}C for 3 minutes, followed by an oven ramp to 142^{o}C at 5^{o}C min^{-1}; and a second ramp of 10Cmin-1 up until 240^{o}C. A mass scanning range of 40–550 m/z (perfluorotriN-butylamine as mass reference) was employed and mass spectra were recorded at 2 scans s-1. The NIST library was used in the tentative identification of compounds. Kovat indices were also calculated from an alkane series to verify compound names.

5.2.3 Liquid Chromatography linked to mass spectrometry (LC-MS)

Method employed was similar to that used by (Nkomo, 2014). Extracts were resuspended in 1 mL of a 50% (v/v) mixture of acetonitrile and water (H_2O) containing 0.1% (v/v) formic acid. The suspensions were vortexed for 1 minutes then sonicated for 5 minutes, vortexed again for 1 minutes prior to spinning at 10 000 rpm for 10 minutes. Four hundred microliters of the supernatant were pipetted into an LC-MS vial containing a guide. From the vial, the supernatant (3µl) was injected into the LC-MS instrument. Metabolites were separated using a gradient of water (H_2O) with 0.1% formic acid (solvent A) and acetonitrile (solvent B), using a Waters UPLC at a flow rate of 0.4 mlmin^{-1} on a Waters BEH C18, 2.1x50 mm column. Mass spectrometry was obtained on a Waters SYNAPT™ G2 MS (Manchester, England) using electron spray ionization (ESI) running in positive mode with a cone voltage of 15 V. The injections were repeated once to ensure repeatability.

5.3 RESULTS AND DISCUSSION

5.3.1 Results

The volatile compounds in bush tea samples were analysed using GC-MS while the soluble non-volatile compounds were analysed using LC-MS. From the magnesium trial 39 compounds were identified using GC-MS (Figure 14). Compounds detected mostly were monoterpenes, terpenes, cyclic terpenes and cyclic thiothers. The identified compounds have been documented to possess a plethora of biological properties and have been previously identified in some important South African medicinal plants. For instance, sabinene is a major constituent of carrot seed oil. It also occurs in tea tree oil at a low concentration (Shulgin *et al*., 1967). Sabinene is found in all the treatments, in control (32.3%), in mg50 ml/l (38.1%), in mg100 ml/l (32%) and in mg150 ml/l (9%). N-ethyl-1, 3-dithioisoindoline, alpha pinene, beta-pinene, myrcene, limonene and 1, 8-cineole were found in all the treatments.

Compounds such as o-cymene, delta.-3-carene, Delta.-4-carene were found only in the control treatment, While 3-octanone, allo-ocimene, 1 octen 3 ol and gamma.-cadinene were found only in the mg50 ml/l treatment. A general trend revealed that more compounds were detected as the concentration of magnesium was increased in comparison to the control, with only just 3 compounds being exclusively observed in the control only. Compounds in the control were seen at relatively lower concentrations in comparison to any of the other treatments.

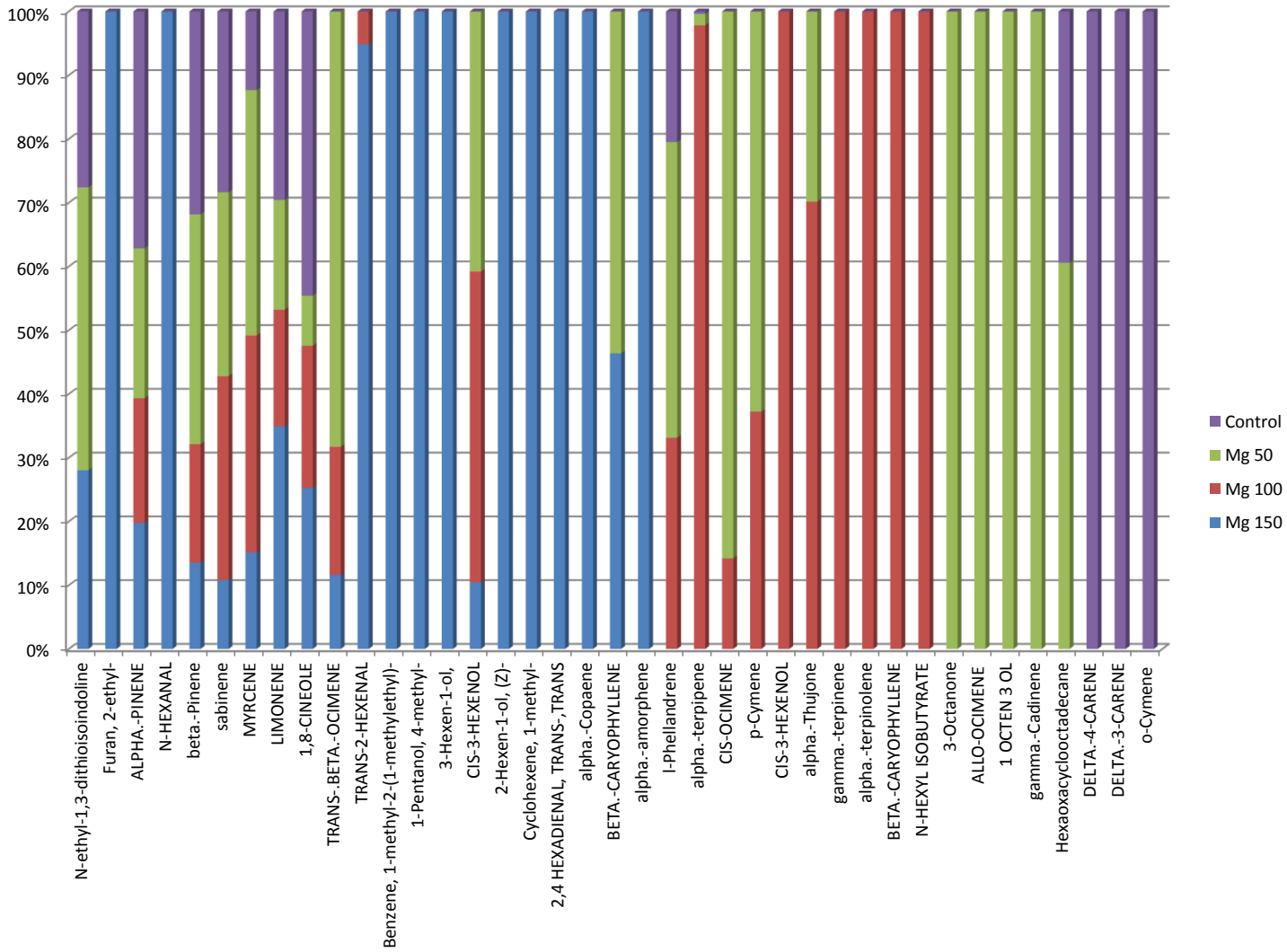

Figure 14 Compounds detected using GC-MS on Magnesium treatment

The results revealed thirty-one compounds in treatment with Fe (Figure 15). The figure also shows a slight decrease in detected compounds found in iron treatments compared to the magnesium treatment. However, compounds detected were similar to those found in both treatments as they were also monoterpenes, terpenes, cyclic terpenes, cyclic thiothersalbeit and also carboxylic acid. More compounds were only detected in Fe50 ml/l (propanoic acid, beta-caryophyllene, is-3-hexenol, 1,3,7-octatriene, 3,7-dimethyl, is-3-hexenol and alpha-terpipene) and Fe100 ml/l (phenylethyl alcohol, butanoic acid, butyl ester, furanone, 3-methyl- , butanal, 2-methyl-, propanal, 2-methyl-, methane, thiobis- and n-hexane) while results revealed a decrease in compounds found only in control and Fe150 ml/l. Generally seemed to be a trend in which compounds exclusively detected in higher concentrations of iron are more, suggesting less compounds are produced at higher iron concentrations when the plant will be less stressed.

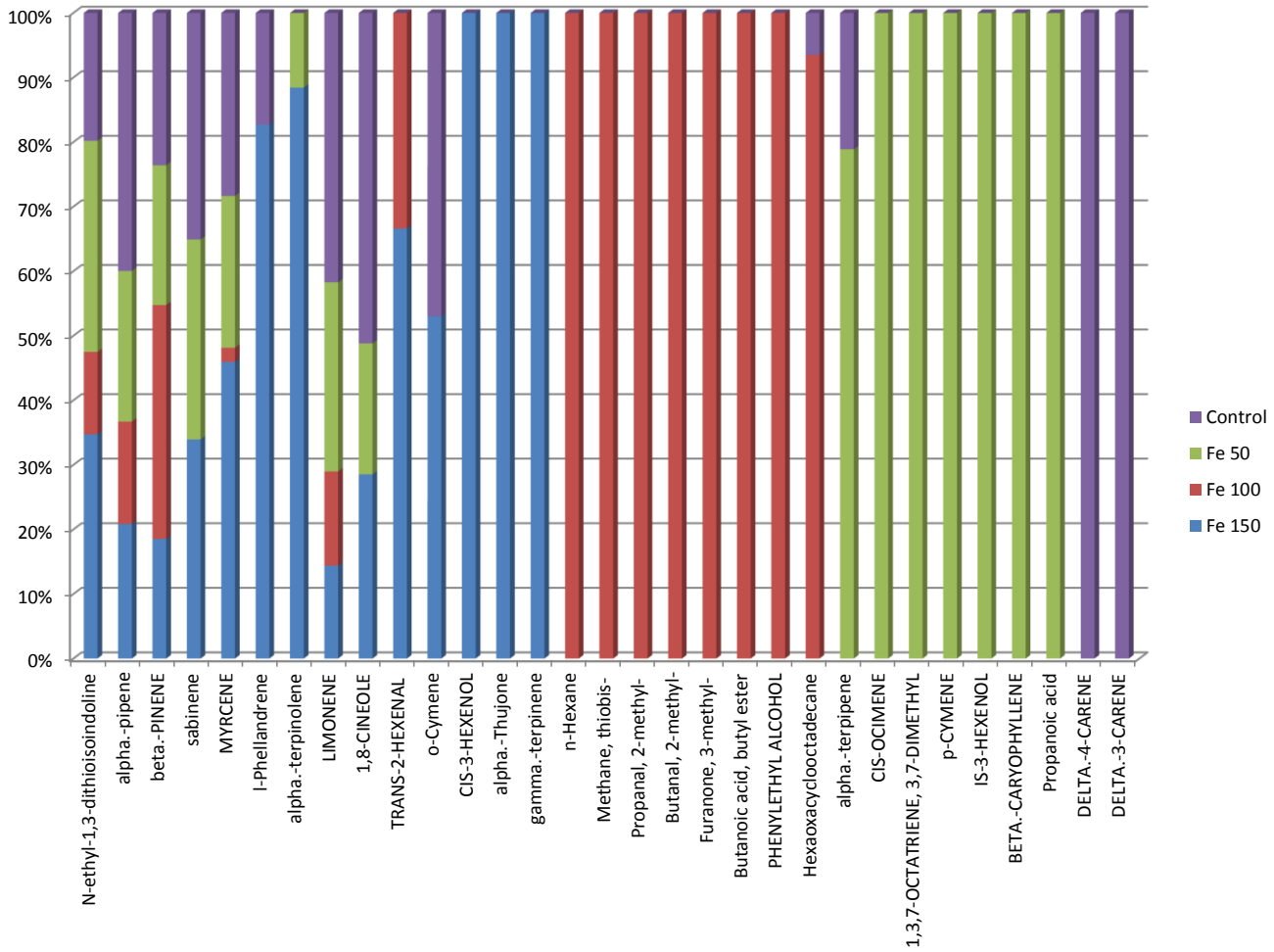

Figure 15 Compounds detected using GC-MS on Iron treatment

Twenty-eight compounds were detected from samples treated with different copper levels (Figure 16). The results also showed a slight decrease in compounds found in copper compared to iron and magnesium treatments. Compounds found were similar to those found in both latter treatments. The largest number of compounds was detected only in Cu150 ml/l (alpha-copaene, cyclohexene, 1-methyl-, cis-3-hexenol, o-cymene, 3-octanone, trans-beta-ocimene and trans-2-hexenal), these were also found in the control.

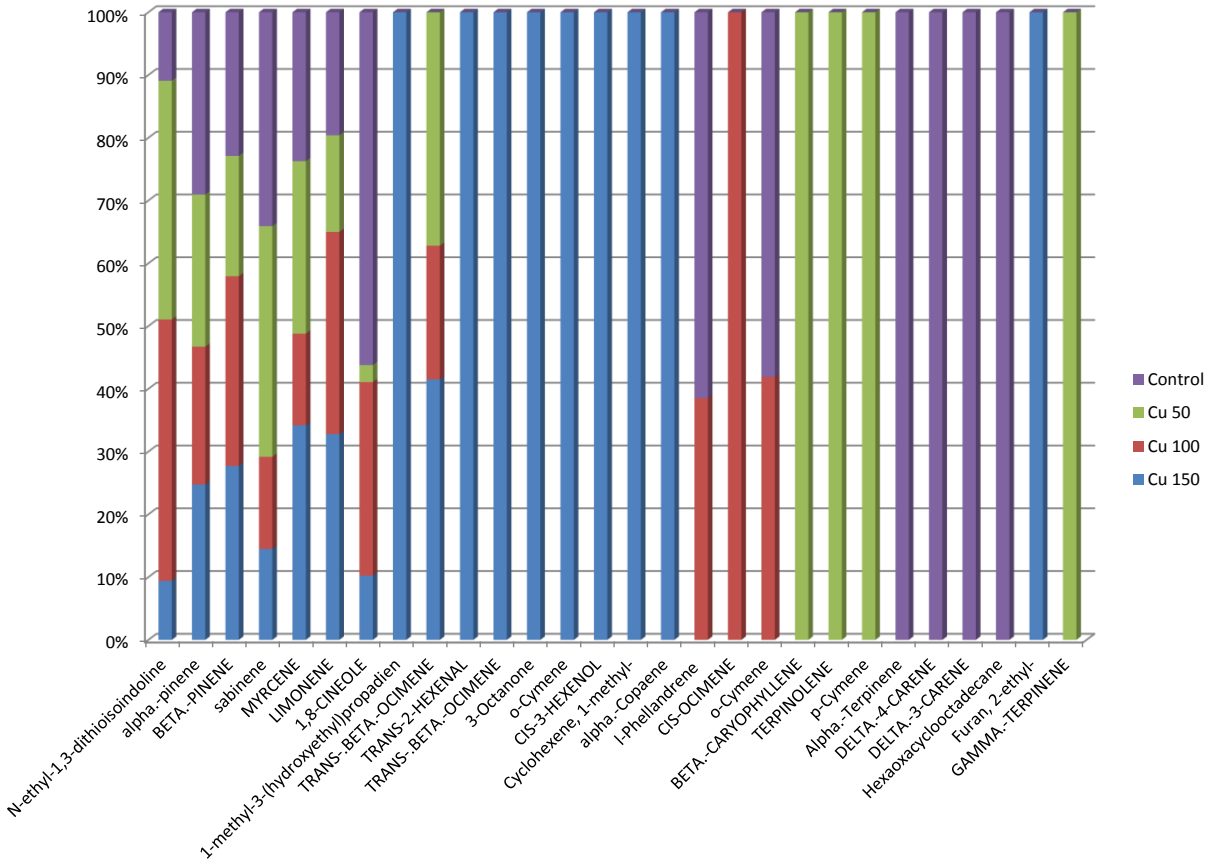

Figure 16 Compounds detected using GC-MS on Copper treatment

The results revealed twenty-nine compounds representing 99% of the constituents in boron treatment (Figure 17). The results also show a slight difference in compounds found in boron compare to iron treatments and magnesium treatment. Compounds found are similar to those found in both treatments as they are also monoterpene, terpene, cyclic terpene and cyclic thiother. More compounds were found only in B150 ml/l (tetradecanoic acid, benzeneethanol, neoalloocimene and trans-beta-ocimene) and B50 ml/l.

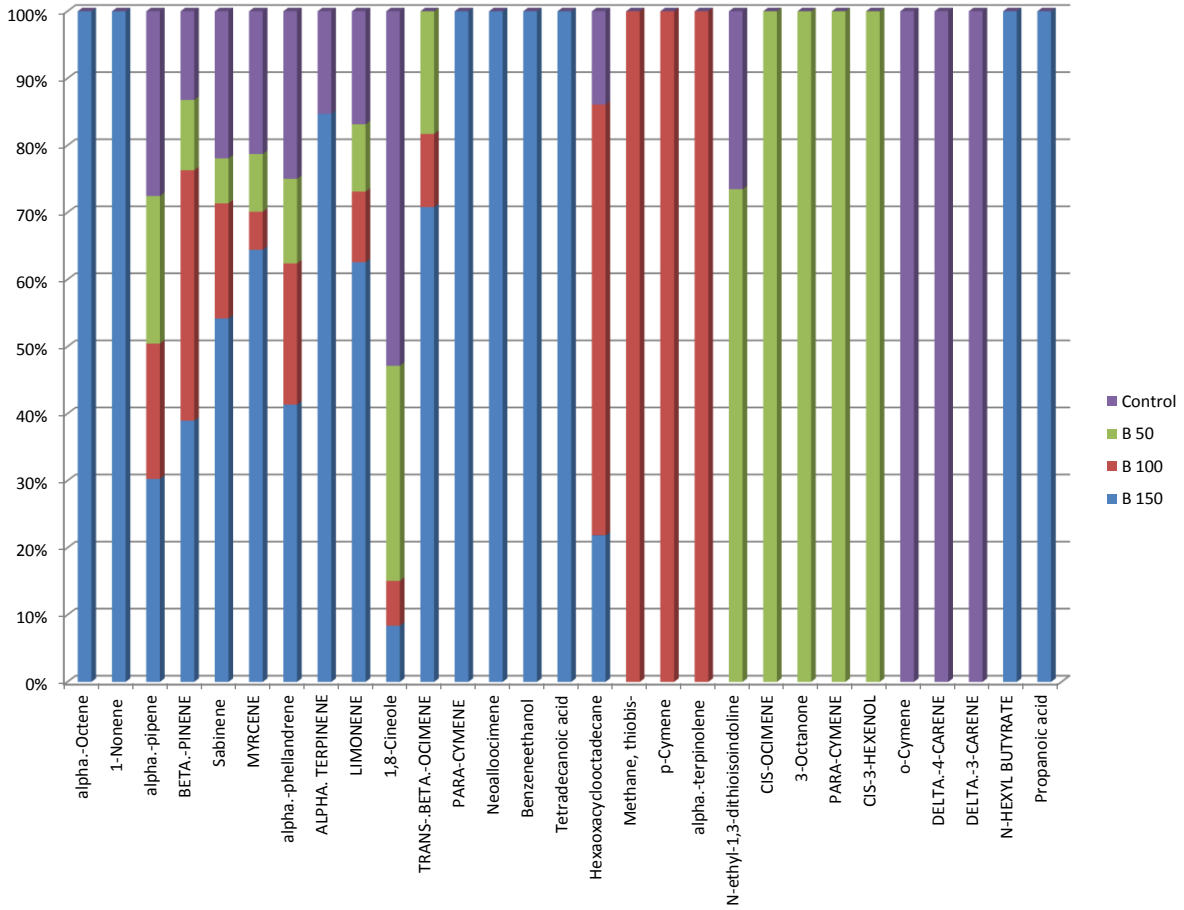

Figure 17 Compounds detected using GC-MS on Boron treatment

Twenty-five compounds were detected in Zn treated oil sample (Figure 18), a slight different number of detected compounds found in zinc in comparison to all the other micronutrient treatments were observed. Compounds found were similar despite being fewer in number. Most compounds were found only in the control (hexaoxacyclooctadecane, delta-3-carene, delta-4-carene and alpha-terpinene).

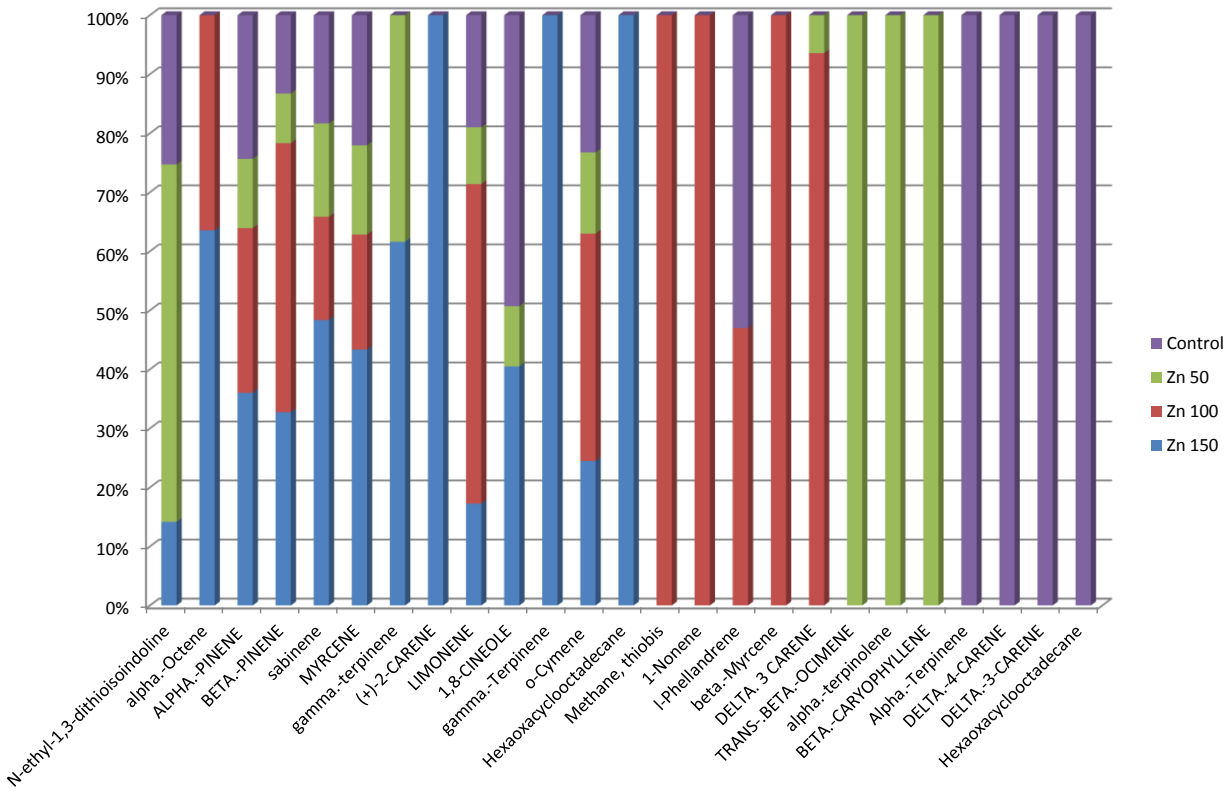

Figure 18 Compounds detected using GC-MS on Zinc treatment

Results from the mix treatment, detected thirty compounds, mostly monoterpenes, terpenes, cyclic terpenes and cyclic thiothers. Sabinene, beta-pinene, alpha-pinene and N-ethyl-1-3-dithioisoindoline were found in all the treatments levels (Figure 19). Compounds such as gamma-terpinene, 3-octanone, p-cymene, cis-3-hexenol and beta-thujone were detected in mix10 ml/l, while dodecane, trans-beta-ocimene, p-cymol, 3-hexen-1-oi and hexaoxacyclooctadecane were detected only in mix20 ml/l, (Figure 19) also shows a slight increase in compounds found in mix treatments compared to the zinc, boron and copper treatments. However, compounds found are similar to those in all the individual treatments.

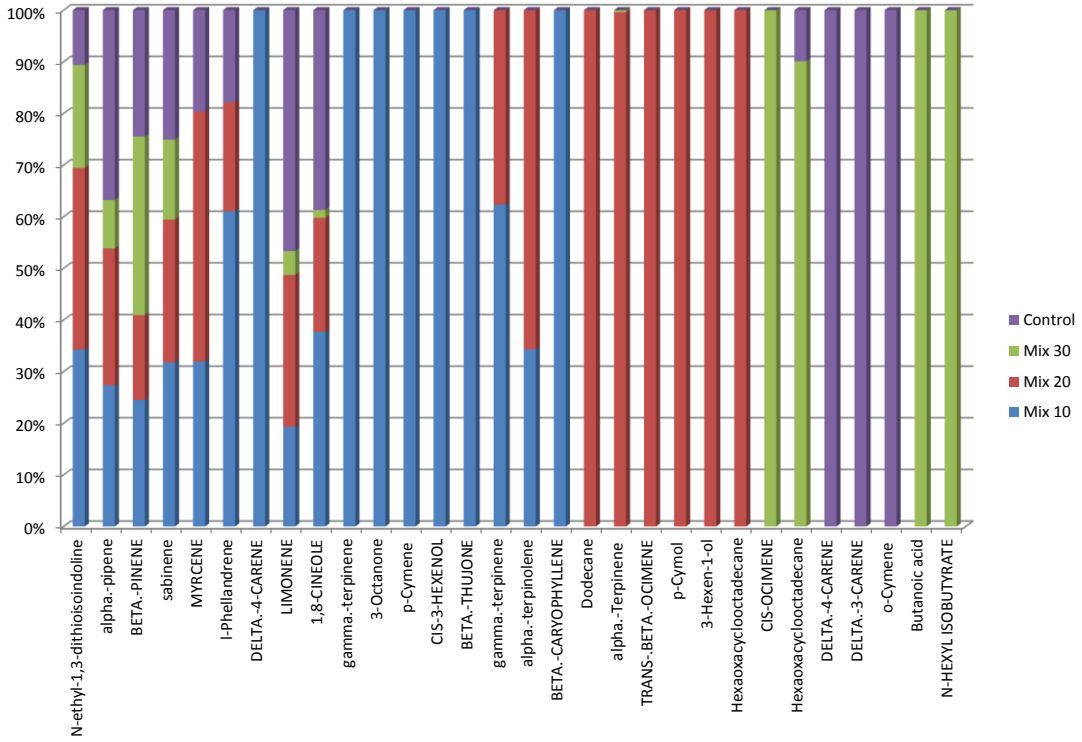

Figure 19 Compounds detected using GC-MS on Mixture (Mg+Fe+Cu+B+Zn) treatment

Table 4 Retention Index of selected compounds detected using GC-MS in bush tea

Retention index	Compound
2288.00	N-ethyl1,3dithioisoindoline
958.00	beta-myrcene
948.00	alpha.pinene
1564.00	propanoic acid
990.00	beta.pinene
962.00	sabinene
995.00	myrcene
1020.00	limonene
1032.00	1,8-cineole
976.00	trans.beta.ocimene
1061.00	trans-2-hexenal
890.00	1-nonene
966.00	3-octanone
868.00	cis-3-hexenol
969.00	l-phellandrene
976.00	cis-ocimene
1012.00	p-cymene
1084.00	alpha.terpinolene

The LC-MS observations from the study showed no significant qualitative difference between control and Mg treatments (100 ml/l and 150 ml/l) with these treatments showing similar number of peaks. However, the total ion chromatograms (TIC) indicated a significant difference between control and Mg50 ml/l treatment. Higher application of Mg at 150 ml/l, showed a decrease in the number of peaks with 21 peaks which are similar to zero application (Figure 20). The study indicated that no application of Mg (control) and where Mg was applied at high rate (150 ml/l), the height of peak had similar retention time at 3.41 minutes. However, where magnesium peaks applied at adequate rates of 50 ml/l and 100 ml/l there was a significant quantitative difference as compared to the control (Figure 20). Main peak in the chromatography was reached at 3.41 retention time. All applications of Mg treatments ranging from 0-150 ml/l showed no difference in the main peak as they differ by few seconds of retention time (Figure 21). The main peak (Figure 20) has mass accurate of 515.1 m/z at 3.41 minutes with an A+2 isotope of 516.1281 m/z, this compound may potentially have an elemental composition of $C_{36}H_{19}O_4$ (Figure 22).

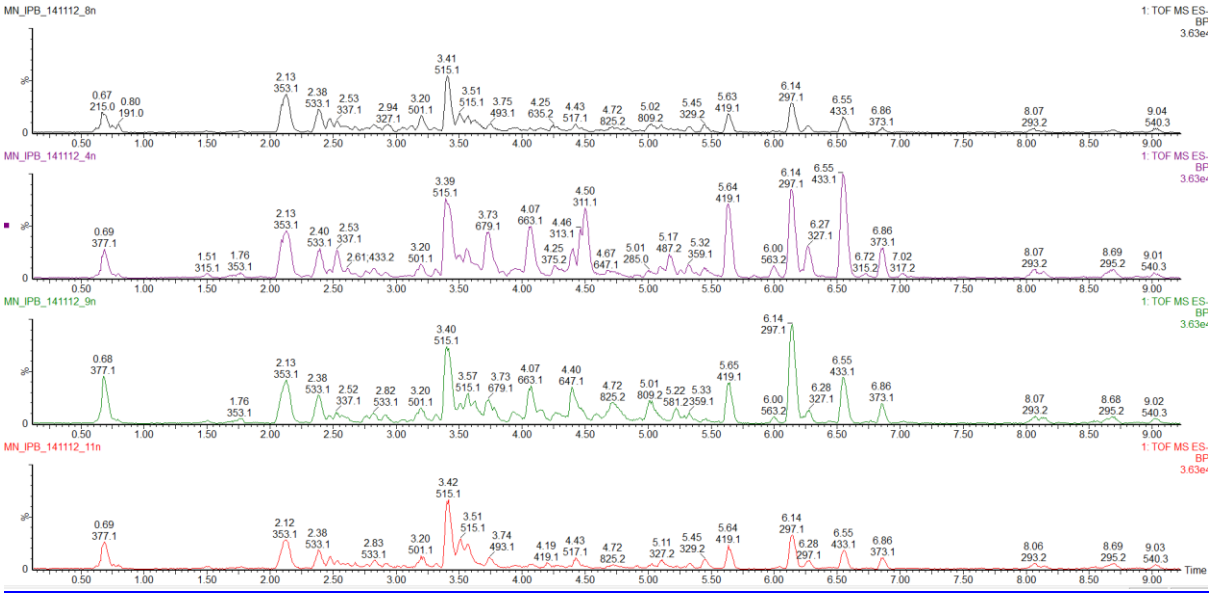

Figure 20 LC-MS (TIC) of spectra of Mg treatment (overlay of the control, Mg 50ml/l, Mg100 ml/l and Mg150 ml/l respectively)

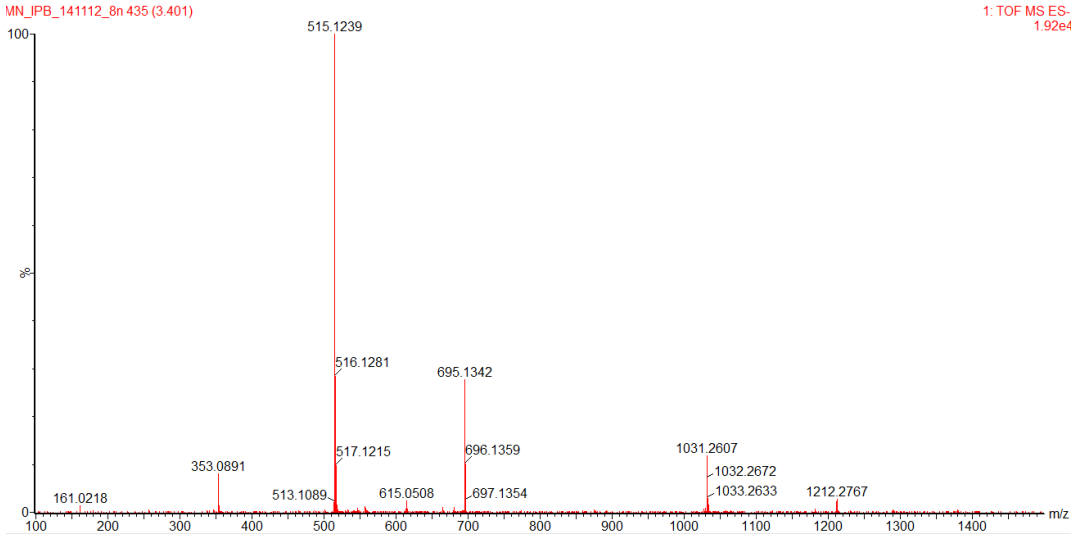

Figure 21 MS-MS ion fragmentation of 515.1 ion

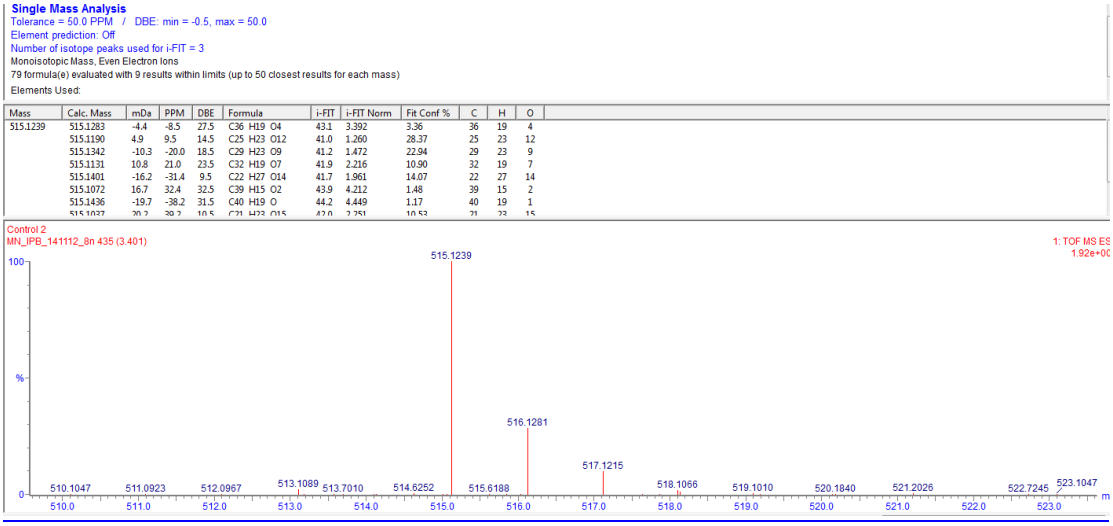

Figure 22 Elemental composition and ion fragmentation of the 515.1 ion

For the zinc treatments, no qualitative differences between Zn treatments (50 ml/l, 100 ml/l and 150 ml/l) and control with these treatments showing similar number of peaks. However, the number peaks indicated a slight difference between control reaching 21 peaks and the highest number of peaks (22) reached at Zn150 ml/l. Lower application of Zn at 50 ml/l, showed a decrease in the number of peaks with 15 peaks which showed a difference to zero application (Figure 23). However, there were quantitative differences between the control and Zn applied at (100 ml/l), (Figure 23). Apart from the main peak at 3.41 minutes (Figure 21) the peak at 4.51 minutes with an accurate mass of 311 m/z was observed in varying intensities amongst the overlayed TIC_S (Figures 23, 24 and 25).

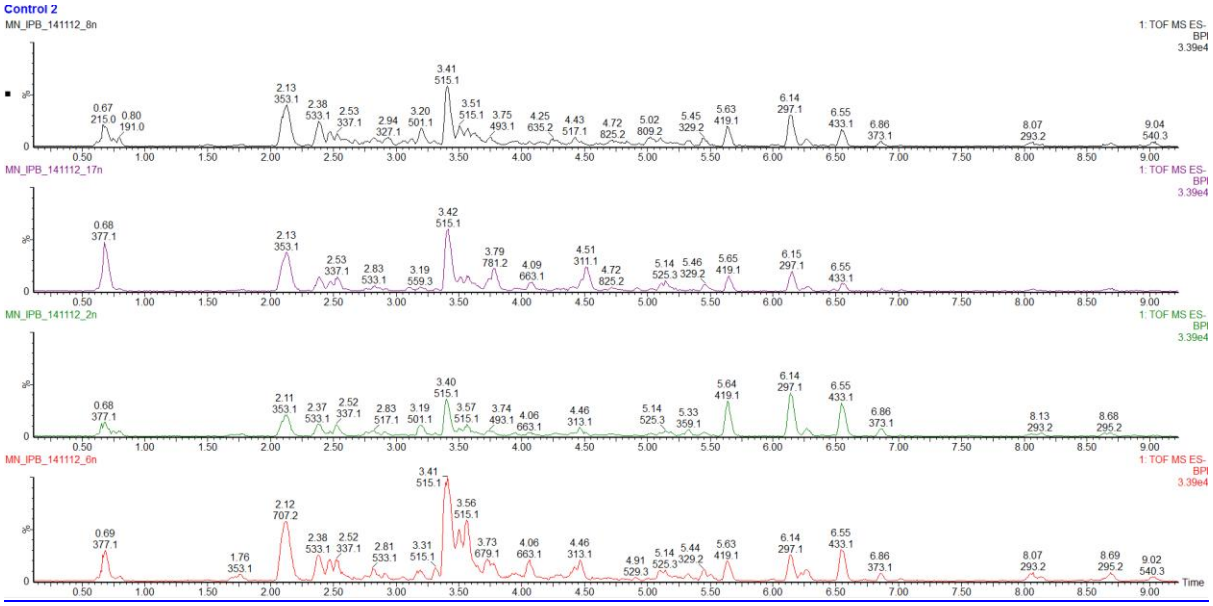

Figure 23 LC-MS (TIC) spectra of the control and Zn treatments (overlay of control, Zn50 ml/l, Zn100 ml/l and Zn150 ml/l respectively)

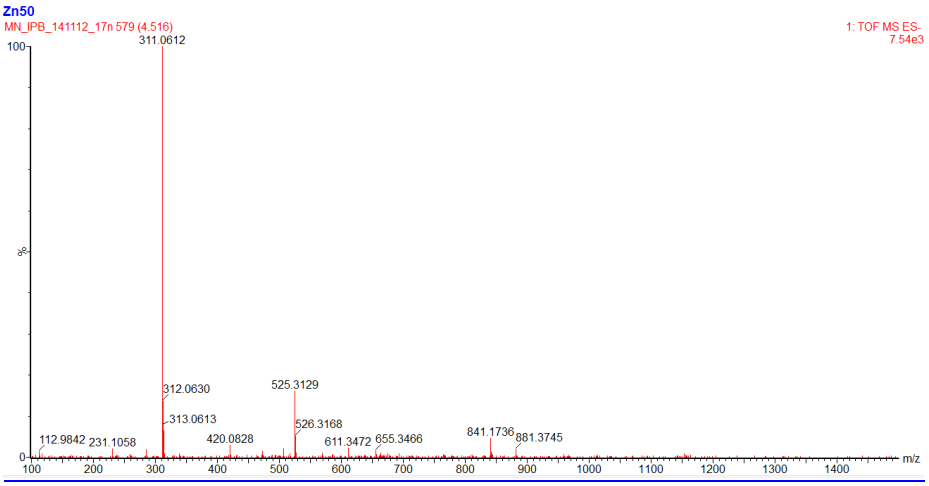

Figure 24 MS-MS fragmentation of the 311 ion

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
311.0612	311.0614	-0.2	-0.6	3.5	C10 H15 O11	37.6	0.791	45.35	10	15	11
	311.0556	5.6	18.0	12.5	C17 H11 O6	39.0	2.183	11.28	17	11	6
	311.0708	-9.6	-30.9	16.5	C21 H11 O3	40.4	3.584	2.78	21	11	3
	311.0497	11.5	37.0	21.5	C24 H7 O	41.3	4.491	1.12	24	7	1
	311.0462	15.0	48.2	-0.5	C6 H15 O14	38.7	1.878	15.29	6	15	14
	311.0767	-15.5	-49.8	7.5	C14 H15 O8	38.3	1.419	24.19	14	15	8

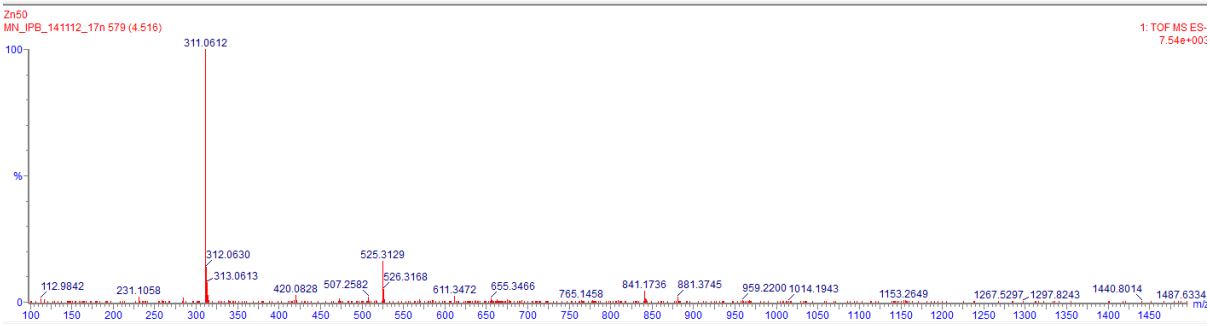

Figure 25 Elemental composition and fragmentation of the 311 ion

No qualitative differences were observed between the control and 50 ml/l, 100 ml/l and 150 ml/l Cu treatments application. However, quantitative differences exist between the control and Cu treatments (Figure 26).This particular trend was also observed with the application of magnesium (Figure 20).The main peak in the chromatography was reached at 515.1 m/z. All applications of Cu treatments ranging from 0-150 ml/l showed no difference in the main peak as they differ by few seconds of retention time (Figure 21). The main peak at 3.41 minutes (Figure 20) has an A+2 isotope of m/z 516.1281 with accurate mass m/z of 515.1 with (Figure 22).

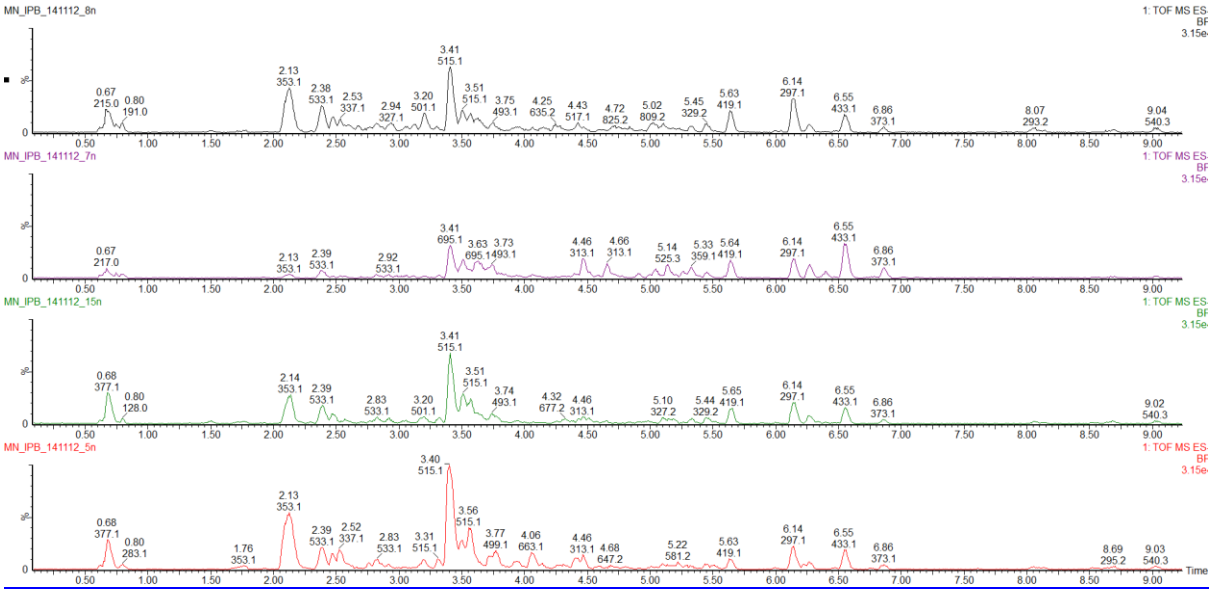

Figure 26 Total ion chromatograms (TIC) of the control and Cu treatments (control, Cu50 ml/l, Cu100 ml/l and Cu150 ml/l)

A similar trend was observed as with the Cu, Zn and Mg treatments. No qualitative differences were seen between Fe treatments (50 ml/l, 100 ml/l and 150 ml/l) and control; however, there were quantitative differences (Figure 27).

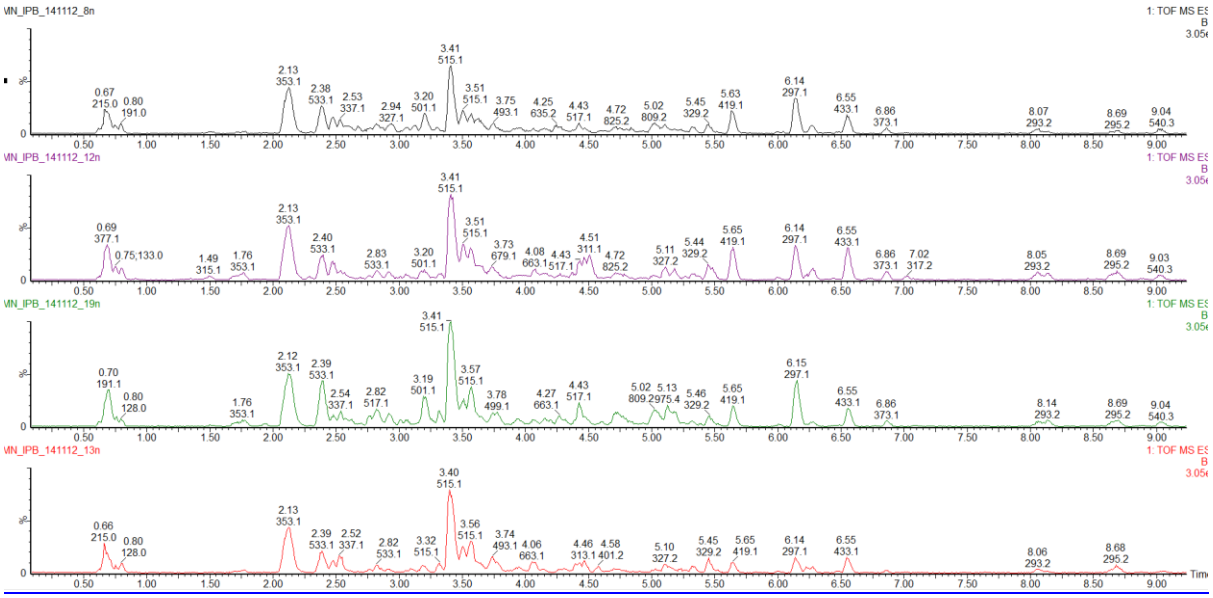

Figure 27 LC-MS (TIC) spectra of the control and Fe treatments (overlay of control, Fe50 ml/l, Fe100 ml/l and Fe150 ml/l respectively)

For the boron treatments, between control and B treatments no qualitative differences were observed (50 ml/l and 100 ml/l) with these treatments showing similar number of peaks. However, the number peaks indicated a slight difference between control reaching 22 peaks and the highest number of peaks (23) reached at B50 ml/l and B100 ml/l. Higher application of B at 150 ml/l, showed a decrease in the number of peaks with 13 peaks which showed a difference to zero application (Figure 28). However, there were quantitative differences between the control and B applied at 150 ml/l (Figure 28).

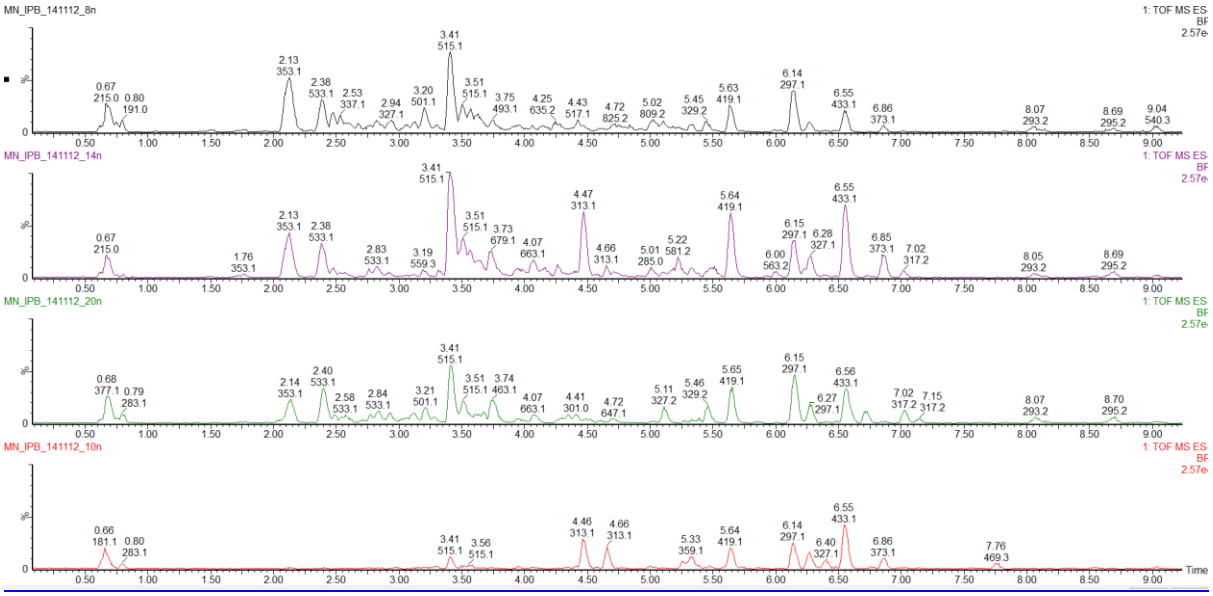

Figure 28 Total ion chromatograms (TIC) of the control and B treatments (control, B50 ml/l, B100 ml/l and B150 ml/l)

A similar trend was observed as with the Cu, Zn, B and Mg treatments. No qualitative differences were seen between the control and mixture (B + Zn + Fe + Cu + Mg) (10 ml/l, 20 ml/l and 30 ml/l), however, there were quantitative differences (Figure 29). Higher application of mixture (B + Zn + Fe + Cu + Mg) at 30 ml/l, showed a decrease in the number of peaks with 16 peaks which showed a difference to zero application (Figure 29).

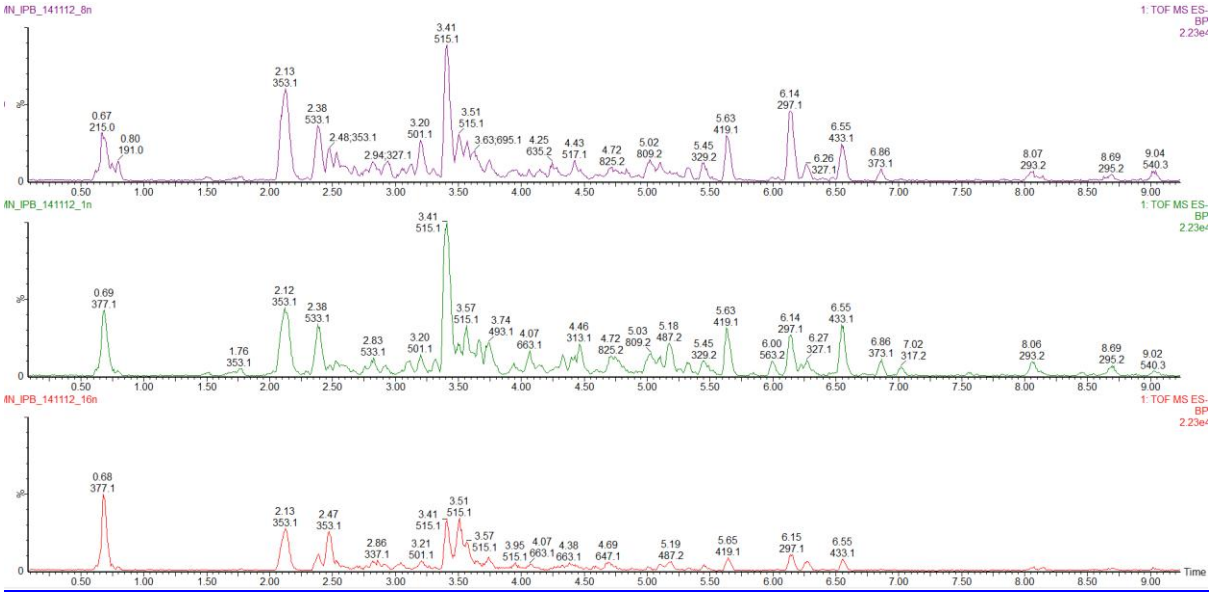

Figure 29 LC-MS (TIC) spectra of the control and mix (B+Cu+Zn+Mg+Fe) treatments (overlay of control, 10 ml/l, 20 ml/l and 30 ml/l respectively)

5.3.2 Discussion

The five major compounds identified in this study (Figure 30) (>80%) using GC-MS were α-pinene, β -pinene, myrcene, β–caryophyllene and caryophyllene oxide (Figure 20 Chemical structure of major compounds identified in bush tea leaves)

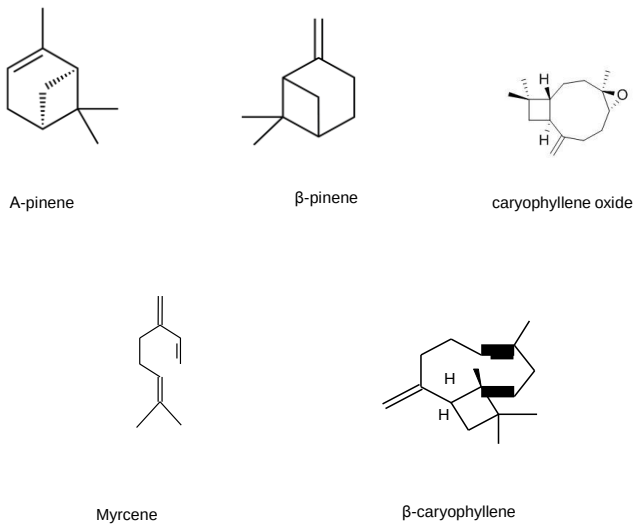

Figure 30 Five major compounds identified in bush tea leaf

The composition percentages of major compounds found in this study showed both quantitative and qualitative differences. These results were consistent with the findings by Padayachee, (2011) who reported that seven major chemical compounds were identified using GC/MS representing 74.4% to 99.8% of total composition (α-pinene, β-pinene, myrcene, β–caryophyllene and caryophyllene oxide). It has been documented that α-pinene and β–caryophyllene exhibit anti-inflammatory activity against the 5-lipoxygenase enzyme (Baylac and Racine, 2003).

Alpha-pinene has been documented to contain *Plasmodium falciparum* which display potent antimalarial activity against the chloroquine-resistant strain (Van Zyl *et al*., 2006). The toxicity of some of major essential oil constituents of bush tea has been investigated. Sibanda *et al*., (2004) reported that caryophyllene oxide had cytotoxicity activity against human tumour cells. Beta-pinene and α-pinene are

reported to display some degree of toxicity against epithelial cells in human kidney (Van Zyl *et al*., 2006). The results indicated that same compounds were found in the different treatments collected from the same location. The composition of the essential oils can be affected by many factors such as location and seasons (Putievsky *et al*., 1986). Temperature, seasons and the conditions of analysis can also affect the composition of the essential oils (Sangwan *et al*., 2001). Results in this study revealed that β-caryophyllene and α-caryophyllene were found to be the main constituents of samples with the highest yield (99%), β-pinene was found to have the middle yield (97%) and α-pinene and myrcene were found to have the lowest yield (96%).

There is no existing literature on the effect of micronutrient application on bush tea phytochemicals and chemotypic variations. All applications of treatments showed no difference in the main peaks from the TIC_S. These results were similar to those found by Nuengchamnong *et al*., (2009) who reported that cafferic acid in *Houttuynia cordata* herbal plant had peaks 3-5 with m/z 179.0 (caffeic acid-H), 191.0 m/z (quinic acid-H) and m/z 371.2 (caffeic acid-H+18) and all these m/z are characteristic of caffeic acid. These results also agree with Beelders *et al*., (2012) who reported that peaks in rooibos tea infusion had good response with similar molecular mass ($C_{26}H_{29}O_{15}$) with same 581 m/z retention times at 5.45, 6.33 and 6.55. The results showed that the main peak in the chromatography was reached at 311 m/z on Zn treatments.

5.4 CONCLUSION

In conclusion, all micronutrients applications contained all five major compounds (α-pinene, β -pinene, myrcene, β–caryophyllene and caryophyllene oxide) with magnesium treatments showing a higher number of compounds while zinc treatments had lowest number of compounds. All applications of treatments had a significant quantitative differences compared to control applications (GC-MS). LC-MS profiling showed no qualitative differences within the treatments and between the treatments to the control, however there were quantitative differences between the control and all other treatments. Application of micronutrients did have an influence on the metabolite quantities as has been reported with most secondary metabolite

fluctuations caused by plant-environment interactions. Application of micronutrients improved the medicinal quality of bush tea

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GENERAL DISCUSSION AND CONCLUSIONS

Experiment to analyse the leaf sample of bush tea was conducted (Chapter 3). It was found that micronutrients foliar applications increased the quality and the growth of bush tea. Due to application of micronutrients solutions, treatment (Mg150 ml/g) resulted in the highest yield of 3770 mg/kg. These results were in agreement with those reported by Bassel and Erdemoglu (2006) who found similar Mg content level as a result of sufficient application of treatments. Bassel and Erdemoglu (2006) reported that magnesium levels in seven herbs and their infusions were 1643-3378 mg/kg and 610-2078 mg/kg in range, respectively. Results of this study disagree with Lozak and Fijalek (1998) who reported that data on magnesium was 5778-15331 mg/kg in raw mint leaves.

Treatment (B150 ml/g) resulted in the highest yield of 1207 mg/kg. Gupta and Cutclife (1985) also reported that boron levels less than 28 mg/kg are related to B deficiency and are similar to those of Kelly *et al*., (1952) who found boron levels of 16 mg/kg to be deficient. Results agree with Uddin *et al*., (2008) who found that boron application (37.54-234.5 mg/kg) significantly increase the number of tillers. Treatment (Fe100 ml/g) resulted in the highest yield of 326 mg/kg. These results also agree with Olivier *et al.,* (2012) who reported that Fe mineral content are similar in both leaves and tea infusions in bush tea (225 mg/kg). Treatment (Zn100 ml/l) resulted in the highest yield of 255.7 mg/kg. These results are in agreement with Street *et al*., (2006) who found increase of Zn levels by 256 mg/kg in tea and tea infusions. Treatment (Cu150 ml/g) resulted in the highest yield of 55.2 mg/kg. These results were slight similar to those reported by Narin *et al*., (2004) who reported the same Cu content level as a result of sufficient application of treatments.

Narin *et al*., (2004) reported that Zn levels were 110.4-124.8 mg/kg in tea and tea infusions. Therefore it can concluded that plant which received no fertilization showed no significant results compared to plants which received fertilization of micronutrients solution that showed high significant results. Micronutrients applications had significant effects on the growth and quality of bush tea by the fertilisers applied and their different rates which led to increase in bush tea yield.

Polyphenols are potential quality indicators and in nature bush tea leaves are potent antioxidants and also are a rich source of polyphenols (Mudau, 2007). Trial to

determine the total polyphenols, tannins and total flavonoids in bush tea were conducted (Chapter 4). Results in this study indicated the highest total polyphenols concentration was observed in treatment (mix30 ml/l) application with 93.70 mg/g and the lowest total polyphenols concentration was observed in control with 20.20 mg/g. The optimum rate of polyphenols in mixture application was obtained at mix30 ml/l reaching maximum total polyphenols of 93.70 mg/g. The results of this study had high significant difference and are consistent with the findings of Venkatesan *et al*., (2005) who found a highly and positive significant correlation between polyphenols content (31.8 mg/g) and zinc levels of mature leaves of tea. Treatment (Cu50 ml/l) treatment showed the highest tannins content with 99.50 mg/g and control treatment showed the lowest tannins content with 30.20 mg/g. These results are similar to Chiu, (1990) who reported that the increase in hydrolysable tannins during summer was found to be pronounced due to higher temperatures and light intensities. The results showed that highest total flavonoids concentrations were obtained from treatment (Cu50 ml/l) with 177.90 mg/g and the results revealed that lowest total flavonoids concentrations were obtained from control with 50.40 mg/g.

An experiment to identify the major compounds of bush tea was conducted (Chapter 5). It was found that bush tea contained five major compounds (β–caryophyllene, myrcene, β -pinene, caryophyllene oxide and α-pinene). All these five compounds were identified as $C_{10}H_{16}$ (α-pinene), $C_{10}H_{16}$ (β –pinene), $C_{10}H_{16}$ (myrcene), $C_{15}H_{24}$ (β-caryophyllene) and $C_{15}H_{24}O$ (caryophyllene oxide). Results were consistent with those of Padayachee, (2011), who reported that seven major chemical compounds were identified in *A. phylicoides* using GC. Möller *et al*., (2006) reported that bush tea glandular trichomes present on the leaf surfaces, which are believed to be responsible for essential oil synthesis, bursts open during late autumn. LC-MS profiling showed no qualitative differences within the treatments and between the treatments to the control, however there were quantitative differences between the control and all other treatments. Application of micronutrients did have an influence on the metabolite quantities as has been reported with most secondary metabolite fluctuations caused by plant-environment interactions.

The following future aspects can be investigated based on the conclusions and results from this study: The effects of multi feeder (Cu + Zn + Zn + B + Mg) on dry roots mass, number of flower buds and branches and plant height.

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APPENDICES

APPENDIX A

Table A-1 Response of foliar spray to leaf tissue Boron of bush tea

Foliar Boron applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
			MEAN		
0	8.64	3445	23.6	89	91.8
50	7.25	3059.10	14.73	136.03	66.69
100	8.65	2849.50	586.32	92.89	69.70
150	8.49	3351	1207	89	88.4

Table A-2 Response of foliar spray to leaf tissue Iron of bush tea

Foliar Iron applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
			MEAN		
0	8.64	3445	23.6	89	91.8
50	7.98	3765	29.9	165	70.3
100	11.08	2836	169.0	326	80.1
150	8.35	2844	17.3	144	77.1

Table A-3 Response of foliar spray to leaf tissue Zinc of bush tea

Foliar Zinc applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
			MEAN		
0	8.64	3445	23.6	89	91.8
50	8.22	2616	34.8	96	172.7
100	7.83	2957	27.7	159	255.7
150	8.69	3344	101.0	106	166.6

Table A-4 Response of foliar spray to leaf tissue Copper of bush tea

Foliar Copper applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
			MEAN		
0	8.64	3445	23.6	89	91.8
50	9.6	3095	30.3	128	84.5
100	24.7	2899	19.9	156	67.2
150	55.2	3195	31.4	157	83.4

Table A-5 Response of foliar spray to leaf tissue Magnesium of bush tea

Foliar Magnesium applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
		MEAN			
0	8.64	3445	23.6	89	91.8
50	8.51	3442	21.9	103	74.5
100	7.41	3594	27.8	109	81.2
150	9.39	3770	27.1	109	75.2

Table A-6 Response of foliar spray to leaf tissue Mixture (B+Fe+Zn+Cu+Mg) of bush tea

Foliar Mixture applied	Cu mg/kg	Mg mg/kg	B mg/kg	Fe mg/kg	Zn mg/kg
		MEAN			
0	8.64	3445	23.6	89	91.8
10	9.42	3404	22.4	85	76.3
20	59.28	3407	25.0	81	83.6
30	8.07	3483	28.4	114	92.6

APPENDIX B

Table B-1 Kovats Indices of control compounds identified using GC-MS

Control				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.6357	4.4252	n-ethyl-1,3-dithioisoindoline	035373-06-9	83
5.8274	10.6933	alpha-pinene	000080-56-8	96
7.3342	1.9118	beta-pinene	000127-91-3	97
7.5945	1.959	sabinene	003387-41-5	96
8.5534	3.6248	myrcene	000123-35-3	96
8.7999	0.2549	l-phellandrene	000099-83-2	90
9.2246	0.0377	alpha-terpinene	000099-86-5	87
9.8136	1.8496	limonene	000138-86-3	99
10.2382	4.9805	1,8-cineole	000470-82-6	98
10.7314	2.7721	delta-4-carene	000554-61-0	92
11.3341	0.721	delta-3-carene	013466-78-9	95
12.2518	0.938	o-cymene	000527-84-4	95
42.6558	0.0207	hexaoxacyclooctadecane	017455-13-9	91

Table B-2 Kovats Indices of Magnesium compounds identified using GC-MS

Magnesium				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.6495	7.1153	n-ethyl-1,3-dithioisoindoline	035373-06-9	83
5.8412	6.7693	alpha-pinene	000080-56-8	96
7.2521	2.1638	beta-pinene	000127-91-3	96
7.4439	1.9919	sabinene	003387-41-5	96
8.1836	11.3075	myrcene	000123-35-3	96
8.4165	0.5769	l-phellandrene	000099-83-2	90
8.8274	0.1723	alpha.terpinene	000099-86-5	98
9.4027	1.0777	limonene	000138-86-3	99
10.0465	0.8775	1,8-cineole	000470-82-6	98
10.3616	4.4988	cis-ocimene	027400-71-1	97
10.9917	2.485	trans-beta-ocimene	003779-61-1	98
11.5396	0.0773	3-octanone	000106-68-3	91
11.9506	0.9379	p-cymene	000099-87-6	95
15.6491	0.248	allo-ocimene	000673-84-7	98
16.2929	2.2141	cis-3-hexenol	000928-96-1	97
18.5881	1.3667	1 octen 3 ol	003391-86-4	86
18.7776	0.1597	alpha-thujone	000546-80-5	96
24.5903	0.7267	beta-caryophyllene	000087-44-5	99
28.8869	0.2297	gamma-cadinene	039029-41-9	95
45.3739	0.0318	hexaoxacyclooctadecane	017455-13-9	83

Table B-3 Kovats Indices of Iron compounds identified using GC-MS

Iron				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.0604	2.3389	n-hexane	000110-54-3	92
3.444	6.0096	methane, thiobis-	000075-18-3	95
3.622	2.8464	n-ethyl-1,3-dithioisoindoline	035373-06-9	83
3.7042	7.8839	propanal, 2-methyl-	000078-84-2	87
4.4713	10.0308	butanal, 2-methyl-	000096-17-3	92
5.8274	4.2435	alpha-pinene	000080-56-8	96
7.252	2.9323	beta-pinene	000127-91-3	94
8.4574	0.2822	myrcene	000123-35-3	96
9.7314	0.6468	limonene	000138-86-3	99
16.7366	0.433	cis-3-hexenol	000928-96-1	95
29.8634	0.3329	furanone, 3-methyl-	022122-36-7	93
33.623	1.7064	butanoic acid, butyl ester	000109-21-7	86
34.7948	1.6625	phenylethyl alcohol	000060-12-8	97
44.0555	0.2972	hexaoxacyclooctadecane	017455-13-9	81

Table B-4 Kovats Indices of Copper compounds identified using GC-MS

Copper				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.8003	3.8105	n-ethyl-1,3-dithioisoindoline	35373-06-9	86
5.8413	9.1053	alpha-pinene	000080-56-8	97
7.2522	2.3151	beta-pinene	000127-91-3	96
7.444	0.832	sabinene	003387-41-5	96
8.1563	5.2224	myrcene	00123-35-3	96
9.3754	3.094	limonene	000138-86-3	99
10.0329	0.9061	1,8-cineole	000470-82-6	99
10.1425	0.786	1-methyl-3-(hydroxyethyl) propadien	5900,"005689-23-6	91
10.3206	1.5302	trans-beta-ocimene	003779-61-1	97
10.7726	3.4695	trans-2-hexenal	000505-57-7	98
10.937	1.3618	trans-beta-ocimene	003779-61-1	98
11.5123	0.053	3-octanone	000106-68-3	92
11.9232	0.5809	o-cymene	000527-84-4	95
16.3066	6.6114	cis-3-hexenol	000928-96-1	96
17.3634	0.1185	cyclohexene, 1-methyl-	000591-49-1	90
20.1797	0.1233	alpha-copaene	003856-25-5	99
4.8413	5.2207	furan, 2-ethyl-	003208-16-0	76

Table B-5 Kovats Indices of Boron compounds identified using GC-MS

Boron				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.8139	7.0773	alpha-octene	000111- 66-0	97
4.6084	6.4948	1-nonene	000124-11-8	91
5.8412	11.747	alpha-pipene	000080-56-8	96
7.2521	5.6338	beta-pinene	000127-91-3	96
7.4987	4.839	sabinene	003387-41-5	97
8.4439	10.9696	myrcene	000123-35-3	96
8.6904	0.4217	alpha-phellandrene	000099-83-2	90
9.1151	0.2088	alpha.terpinene	000099-86-5	95
9.7178	6.8703	limonene	000138-86-3	99
10.184	0.7885	1,8-cineole	000470-82-6	96
11.252	2.0284	trans-beta-ocimene	003779-61-1	98
12.17	1.7706	para-cymene	000099-87-6	97
15.841	0.1138	neoalloocimene	007216-56-0	94
34.925	0.427	benzeneethanol	000060-12-8	93
40.654	0.2702	tetradecanoic acid	000544-63-8	95
45.39	0.0325	hexaoxacyclooctadecane	017455-13-9	87
33.835	0.337	n-hexyl butyrate	002639-63-6	78
33.884	0.1199	propanoic acid	074367-34-3	78

Table B-6 Kovats Indices of Zinc compounds identified using GC-MS

Zinc				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.6358	2.4768	n-ethyl-1,3-dithioisoindoline	035373-06-9	90
3.8139	12.5993	alpha-octene	000111-66-0	80
5.8412	15.817	alpha-pinene	000080-56-8	96
7.3343	4.7187	beta-pinene	000127-91-3	96
7.5946	5.1651	sabinene	003387-41-5	96
8.5397	7.1251	myrcene	000123-35-3	96
8.8	0.5301	gamma-terpinene	000099-85-4	90
9.2247	0.1662	(+)-2-carene		95
9.8137	1.6812	limonene	000138-86-3	98
10.2246	4.0914	1,8-cineole	000470-82-6	99
11.2931	0.6476	gamma-terpinene	000099-85-4	95
12.2519	0.9874	o-cymene	000527-84-4	95
43.2744	0.0431	hexaoxacyclooctadecane	017455-13-9	91

Table B-1 Kovats Indices of Mix (B + Zn + Fe + Cu + Mg) compounds identified using GC-MS

Mix (B + Zn + Fe + Cu + Mg)				
RT	**Area Pct**	**Library**	**Cas**	**Quality**
3.8139	14.2492	n-ethyl-1,3-dithioisoindoline	035373-06-9	86
5.8412	7.9397	alpha-pipene	000080-56-8	86
7.2521	1.9131	beta-pinene	000127-91-3	96
7.4439	2.4796	sabinene	003387-41-5	96
8.1288	5.8849	myrcene	000123-35-3	96
8.3206	0.8701	l-phellandrene	000099-83-2	89
8.7041	0.265	delta-4-carene	000554-61-0	98
9.2657	0.7644	limonene	000138-86-3	99
9.9643	4.8493	1,8-cineole	000470-82-6	98
10.7725	0.3597	gamma-terpinene	000099-85-4	96
11.4711	0.0753	3-octanone	000106-68-3	94
11.8273	1.3212	p-cymene	000099-87-6	95
16.3203	1.0394	cis-3-hexenol	000928-96-1	96
18.7047	0.3321	beta-thujone	000471-15-8	97
19.317	0.1935	gamma-terpinene	000099-85-4	91
22.3605	0.1873	alpha-terpinolene	000586-62-9	91
24.4763	0.7802	beta-caryophyllene	000087-44-5	99