Faculty of Sciences

Department of Chemical and Forensic Sciences

# THE EFFECTS OF HEAVY METALS ON THE QUALITY AND YIELD OF VETIVER GRASS (*CHRYSOPOGON ZIZANIOIDES*) ESSENTIAL OIL

by

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14001570

BSc (Chemistry) (BIUST)

A Thesis Submitted to the Faculty of Sciences in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Chemistry of BIUST

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## SUMMARY OF THE CONTENTS

Numerous researches across the world have shown that *Chrysopogon zizanioides* (CZ) can produce essential oil which is stored in the roots. Since CZ roots have been proved to produce highly demanded essential oil worldwide, it is significant to assess the possibility of transference of heavy metals absorbed by the roots to the roots extracted essential oil so as to satisfy quality assurance. Remediation strategies by plants have been suggested for regulation of heavy metals especially in industrial areas such as mines, however there are certain soil amendments that can be employed to speed up the process. Additionally, plants with phytoremediation features and are also essential oil producers are encouraged to be planted since they can be environmentally and economically productive to Botswana. This research study investigated the phytoremediation capability with six soil amendments (varying fungi; *Arbuscular mycorrhizal fungi*, AMF and chelating agent; ethylene diamine tetra-acetic acid, EDTA) on CZ cultivated in Selibe-Phikwe, Botswana mine tailing. The research also focused on the effects of absorbed toxic multi heavy metals (As, Cu, Ni, Zn, Mn and Pb) on CZ roots essential oil. Many studies have shown that CZ has a natural ability to survive in heavy metal polluted soil and accumulate high amount of toxic heavy metals (HM). This led to an interest on application of fungi and EDTA to enhance the phytoremediation strategy which is normally a slow process. Fungi, particularly AMF helps the plant by boosting plant nutrition and for the plant to be resistant and tolerant to any distress while the chelating agent, EDTA speed up the accumulation of heavy metals from the soil to the plant roots by binding to the heavy metals. This was done through an experimental design which followed a two-factor complete randomized design; AMF inoculation factor with two levels and EDTA application factor with three levels. This helps in understanding the individual and collective interaction of AMF-EDTA-HM-CZ in terms of bioaccumulation of heavy metal. The pollution risk factor, bioaccumulation factor was used to quantify the ability of CZ to absorb multi heavy metals per treatment.

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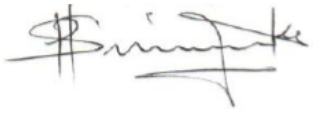

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## LIST OF ABBREVIATIONS

**CZ:** *Chrysopogon zizanioides*

**CZO:** *Chrysopogon zizanioides* essential oil

**US:** Ultra sonication

**USHD:** Ultrasonic -assisted hydro distillation

**MAAD:** Microwave acid assisted digestor

**GC-FID:** Gas Chromatography Flame Ionization Detector

**ICP-OES:** Inductively coupled plasma - optical emission spectrometer

**GC-MS:** Gas chromatography- mass spectrometer

**ICP-MS:** Inductively coupled plasma- mass spectrometer

**NMR:** Nuclear magnetic resonance spectrometer

**HM:** Heavy metals

**CM:** Cow manure

**AMF:** *Arbuscular mycorrhizal fungi*

**EDTA:** Ethelene Diamine Tetra-Acetic Acid

**MTCM:** Mine Tailings plus Cow Manure

**SD:** Standard Deviation

**WHO:** World Health Organisation

**FDA:** Food and Drug Administration

**T1:** Treatment 1

**T2:** Treatment 2

**T3:** Treatment 3

**T4:** Treatment 4

**T5:** Treatment 5

**T6:** Treatment 6

## ABSTRACT

Vetiver grass (*Chrysopogon zizanioides;*CZ) is a well-known grass that can both remediate various heavy metals in soil as well as producing essential oil that is commonly used in premier perfumery, food and pharmaceutical industries. Due to this versatility property, this study was aimed at extracting essential oil from *Chrysopogon zizanioides* roots cultivated in Selibe-Phikwe mine tailings and study the quality and quantity (chemical composition and yield) of the *Chrysopogon zizanioides* essential oil in relation to toxic multi heavy metals absorbed by the grass assisted by *Arbuscular Mycorrhizal Fungi* (AMF) and ethelene Diamine Tetra-Acetic Acid (EDTA) chelating agent. The experimental set-up was designed in a complete randomized design with triplicates. The ultrasonic -assisted hydro distillation (UAHD) extracted CZ essential oil was analysed in each treatment using Gas Chromatography Flame Ionization Detector (GC-FID). Cumulatively fifty-nine constituents were identified from the seven treatment samples, however few constituents which mostly were in trace amount were not identified since they were not reported in similar studies. The essential oil was found to contain mainly sesquiterpenes class of compounds. Control, T1, T2, T3, T4, T5, T6 shows the total identified oils as 99.5%, 97.0%, 100%, 94.6%, 100%, 98.1% and 97.2% respectively. The predominating constituents are alcohols (>50%) across all the treatments. The principal constituents vary in all the treatments but the 'finger print' constituents; khusimol, ά-vetivone, β-vetivone appears in > 50% of the treatment samples. The percentage yield ranged between 0.26% to 0.95%. Two factor ANOVA in complete Randomised Design showed that statistically, the percentage yields across all the treatments are very highly significant ($P = 0.000$, $P < 0.05$).

Microwave acid assisted digestor (MAAD) was used to extract heavy metals from raw CZ roots and extracted essential oil and The heavy metal analysis by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) showed that across all the six treatments with CZ grass grown in mine tailing containing multi heavy metals, the accumulation level is as follows: Ni>Cu>Mn>Zn>Pb>As for raw CZ roots but the cumulative essential oil contained low to negligible amount of heavy metals, as the concentration were; Cu ($0.150 \pm 0.007$ mg $kg^{-1}$), Ni ($0.063 \pm 0.001$ mg $kg^{-1}$), Pb ($0.022 \pm 0.002$ mg $kg^{-1}$), As ($0.003 \pm 0.003$ mg $kg^{-1}$), Zn ($0.109 \pm 0.002$ mg $kg^{-1}$), Mn ($0.043 \pm 0.001$ mg $kg^{-1}$). It shows that the heavy metals were not co-extracted with essential oil. There has been a significant increase in the number of constituents produced by CZ grass for the treatments with CM only and inoculation of AMF with 5 mmol EDTA single and a significant decrease for treatments with AMF and 5 mmol EDTA split, EDTA single, 5 mmol EDTA split as well as AMF only as a result of high metal stress and low metal respectively. The results showed that the amount of heavy metal content does not all the time and it's not the only factor influencing the production of chemical constituents. The integrated effect of Ni, Cu, Mn, Zn, Pb and As in overall did not significantly alter the essential oil composition of most of the CZ constituents across most of the treatments as compared to the control treatment since precision was high.

The extracted light brown viscous CZ essential oil showed percentage yield of 0.26% (C), 0.36% (T3), 0.54% (T5), 0.86% (T1), 0.94% (T6) and 0.95% (T2). The trend shows that T1, T2, T4, T6 had precise and better yield while for C, T3 and T5 the yields dropped with C being the least. The treatments with no heavy metals nor AMF + EDTA (C), the yield was low due to no or less metal stress triggering the production of essential oils.

# CHAPTER 1. INTRODUCTION

## 1.1. BACKGROUND INFORMATION

### 1.1.1. VETIVER GRASS

Vetiver grass, scientifically known as *Chrysopogon Zizanioides (CZ)* is a perpetual grass which can grow up to about 2 m in height and it is from *poaceae* family, originating from India [1]. It is also characterised with good natured earthy flavour, linear stem, paper-thin stiff leaves, lacy fibrous strong roots [2]. Planting of the grass is typically done using seedlings [3]. It is unchallenging to grow as it can resist harsh environmental conditions and cost effective in maintenance [4]. It can be effectively planted in sands, mine tailings as well as toxic soils (high acidity and alkalinity, high concentration of toxic heavy metals, extremely low and high temperatures) [5]. The grass is usually grown for its piquant roots which perform two main duties which are a source of essential oil and the other value that the grass is grown for is that, the roots are good for phytoremediation [1] [6]. Due to the grass' various versatile features, it can also be used in different activities; combat soil erosion, fragrance, medicinal applications, agriculture; botanical pesticides, construction; roof thatch, containers; water containers, energy sources; green fuel, industrial products; pulp and paper [2]. Therefore, it can be concluded that the grass has aromatic, medicinal and environmental features [7].

### 1.1.2. *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

Globally, there has been high demand of (CZO) extracted from plants due to its use as they possess diverse properties [8]. Of all the industries associated with essential oil extracted from different plants, (CZO) is mainly used in premier perfumery and cosmetics (i.e., Diors, Opium) mostly due to its persistent scent [9]. The scent is mainly as a result of different constituents, i.e., presence of ά-vetivone, β-vetivone and khusinol [8]. Other than fragrance, it can be used to add flavour in food industry (i.e., Ice Cream, Beverages) [4]. In addition, the confirmed biological activities of CZO are anti-cancer, anti-bacterial, anti-fungal, anti-oxidant as well as anti-inflammatory activities [1].

Essential oil is a matrix of volatile organic compounds found in specialised secretory tissues in fragrant medicinal plants. This are secondary metabolites which are produced by plants for their own defences in response to stress from external invading factors (i.e., bacterial diseases) and attraction for pollination [10]. CZO is physically characterised by light brown colour, woody odour, high viscosity (low evaporation rate), high volatility but in liquid state at room temperature, low density compared to water, miscible in most organic solvents like dichloromethane and but immiscible in water [9] [11]. CZO are usually extracted by different common conventional distillation methods due to their volatility in nature; steam- distillation, hydro-distillation though other conventional methods exist, with each method displaying certain benefits and determining the essential oil's biological and physicochemical properties [10]. Distillation is regarded as the best method to get the purest essential oil [10]. The distillation method needs more time and employ elevated pressure [12].

CZ roots do not give out their essential oils easily because the essential oils are naturally stored at inaccessible root tissues. To extract essential oil, the oil has to diffuse to the surface from within the fibrous root tissues and the physical process is slow. In addition, CZO contains more of sesquiterpenes which needs more extraction time to be successfully extracted since sesquiterpenes (i.e; vetivones, vetiverol) have high molecular mass with low vapour pressure [13]. This results in sesquiterpenes having high boiling points and being distilled late but distilling at a greater volume [12]. The efficiency of essential oil distillation is predominantly determined by age, quality and stage of root harvest [12]. Both fresh and dried roots can give out essential oil via distillation but exposing the roots in open area for almost 3 days after harvesting at 25° C pay off essential oil of high standard [12]. This result due to natural evaporation of unwanted non polar low boiling point constituents. To enhance the quality and life span of essential oil, the newly distilled essential oil has to be anhydrous and stored in a dull or amber coloured bottle to allow it to be fully-fledged as it oxidizes due to the trapped oxygen in the storage bottle. The oxidation should not be in excess to avoid the formation of malodour. To meet the standard of high-quality essential oil, the oil has to be very stable, residue free, more polar compounds, good odour as this enables the oil to easily solubilise in alcohol and be miscible while incorporated with other perfume substances [12]. There are evident geographical variances in quality and perfumery note of essential oil acquired from different geographic regions of the world. In overall sense, the essential oil of CZ having high specific gravity, negative optical rotation, high vetiverol concentration and higher ester value is considered worthier from perfumery view point [12]. The essential oil yield can be increased by storing the dried roots for a period of 12-24 months, the increase in oil yield is due to enzymatic activities [13].

The constituents from the extracted essential oil can be classified in four groups;

**a. Terpenes**

i. Terpene hydrocarbons:

This are the most common class of chemical constituents found in essential oils (they are also found in abundance in CZO) [14]. They are made from polymerisation/ addition of isoprene units ($C_5H_8$) and combined in cytoplasm of a plant cell *via* mevalonic acid pathway mechanism [14]. The hydrocarbon skeleton can be reconstructed into acyclic and cyclic. The ring sizes determine the type of cyclic terpene; monocyclic, bicyclic and tricyclic. These chemical constituents can be oxidised easily due to expeditious reaction to air and heat sources [14].

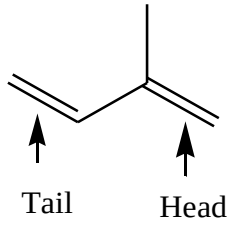
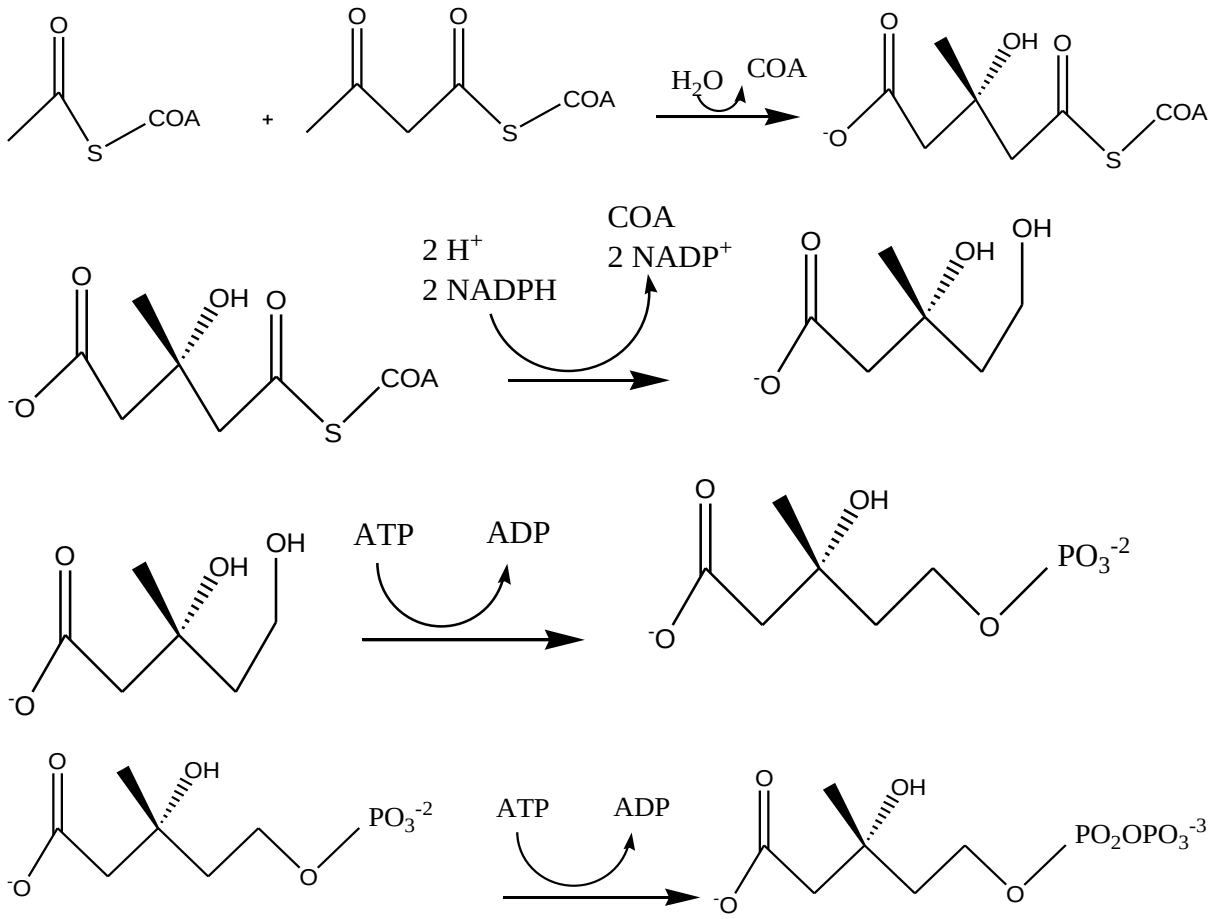



**Figure 1.** Isoprene Unit- $C_5H_8$ (2-methyl-1,3-butadiene)

**Mevalonic Pathway Mechanism**

The mechanism involves the reaction of two molecules of Acetyl -CoA through Acetyl-CoA transferase to make Acetoacetyl-CoA. Acetyl-CoA then condenses with Acetoacetyl-CoA to produce 3-hydroxy-3-methyl glut aryl CoA (HMG-CoA). NADPH reduces HMG-CoA to mevalonate. Mavelonate undergoes ATP dependent phosphorylation with the aid of mevalonate kinase enzyme and further catalysis by phosphomevalonate to produce 5-phosphomevalonate. This reaction leads to the production of isopentyl diphosphate (IPP) which in turn isomerizes to dimethyl allyl diphosphate (DMAPP). The DMAPP undergoes isoprene synthase catalysis to form isoprene [15].



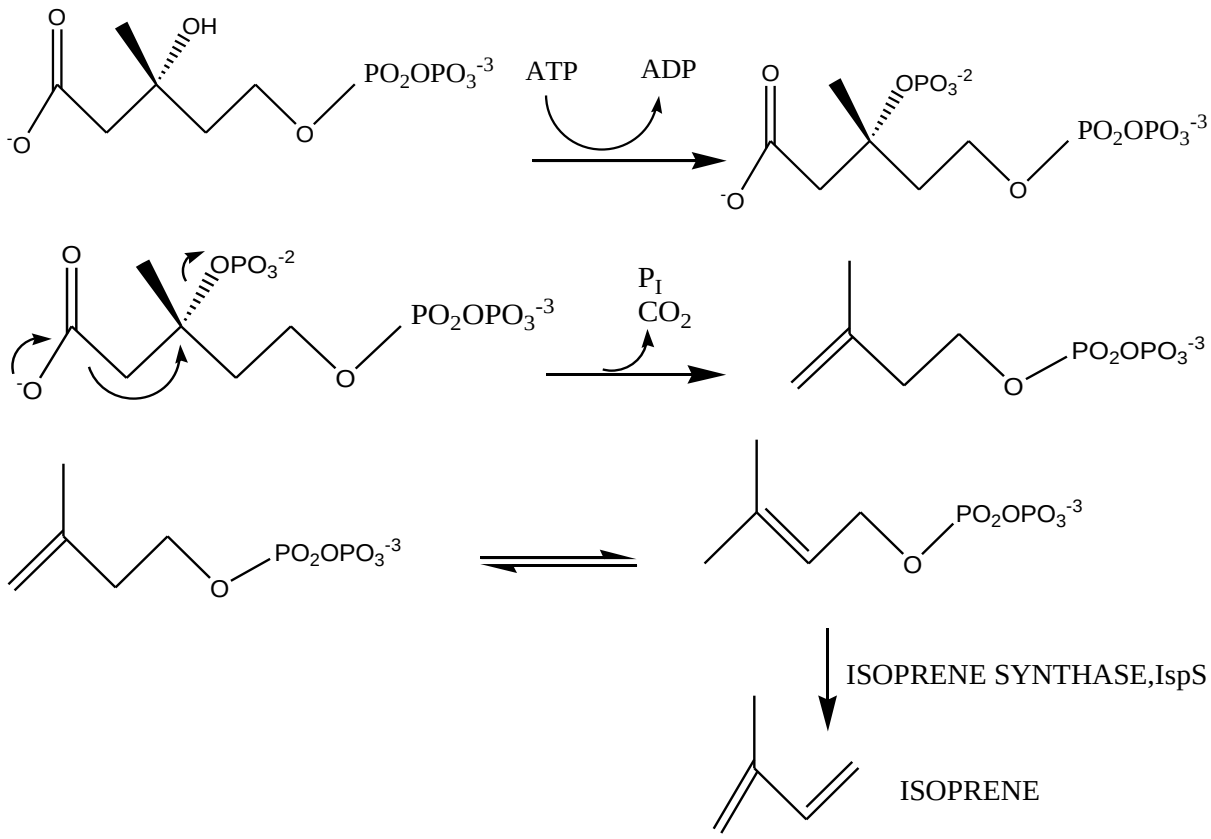


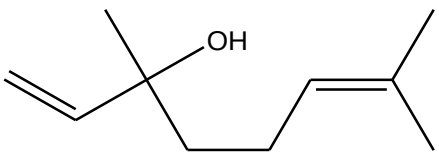

**Figure 2.** Mevalonic pathway mechanism

The categorization of terpenes based on isoprene unit's polymerisation; monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), sesterterpenes (5 units), triterpenes (6 units) and polyterpenes (many units) [14].

ii. Terpenoids

This are oxygenated compounds which are very aromatic. They are grouped into aldehydes, ketones, esters, ethers, alcohols, phenols and epoxides.

a. Monoterpenes

| Acyclic | |
|---|---|
| Hydrocarbon- Myrcene | Oxygenated- Linalool   |

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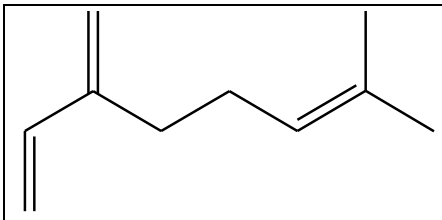
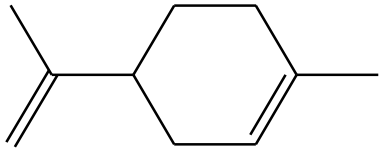
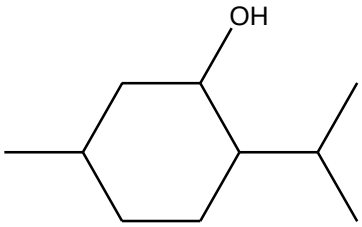
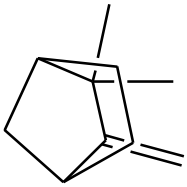
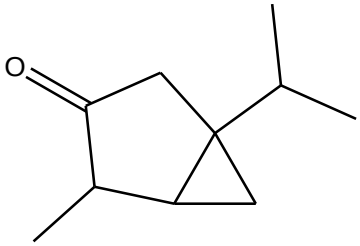
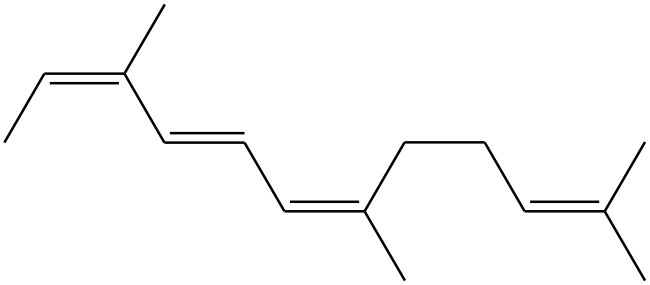
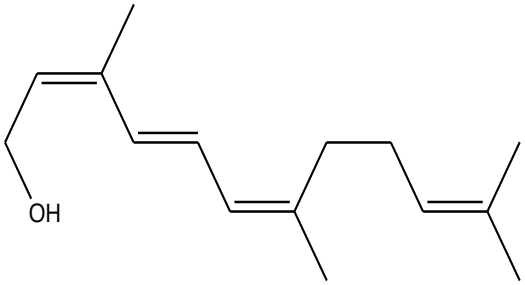
|---|---|
|  | |

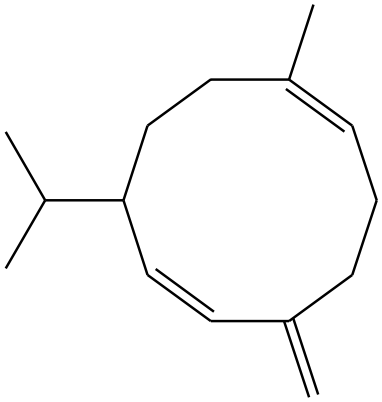
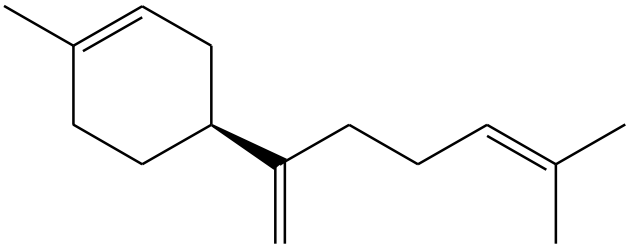
| Monocyclic | |
|---|---|
| Hydrocarbon- Limonene  | Oxygenated- Menthol   |

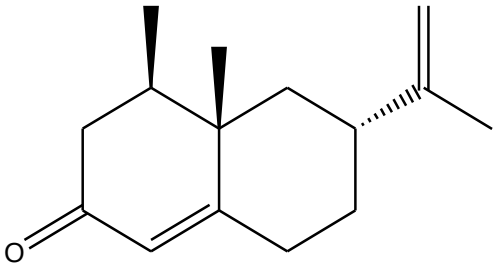
| Bicyclic | |
|---|---|
| Hydrocarbon- Camphene  | Oxygenated- Thujone   |

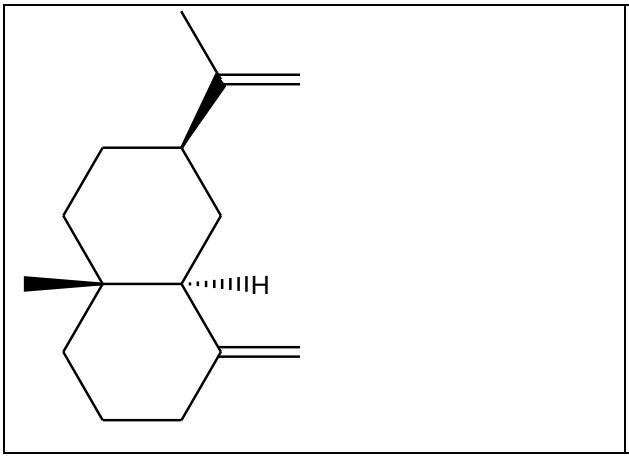
b. Sesquiterpenes

| Acyclic |
|---|

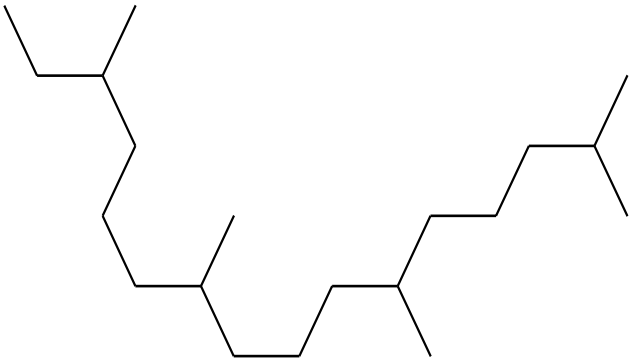
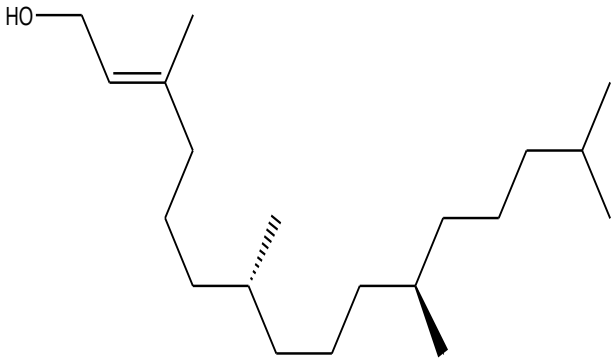
| Hydrocarbon- Farnesene | Oxygenated- Farnesol |
| --- | --- |
|  |  |

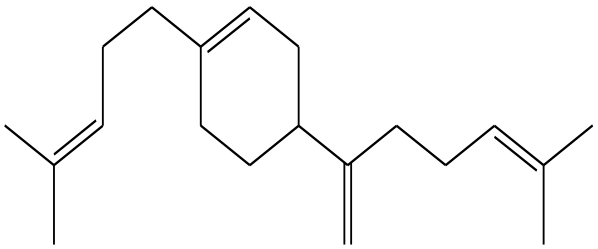
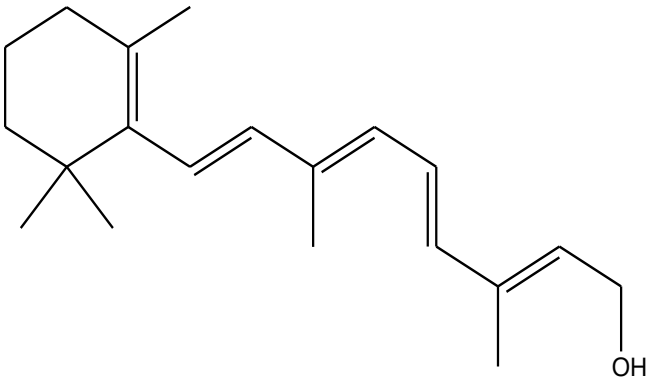
| Monocyclic | |
| --- | --- |
| Hydrocarbon- Germacrene D | Oxygenated- β Bisabolene |
|  |  |

| Bicyclic | |
| --- | --- |
| Hydrocarbon- β Selinene | Oxygenated- Nootkatone |
| |  |

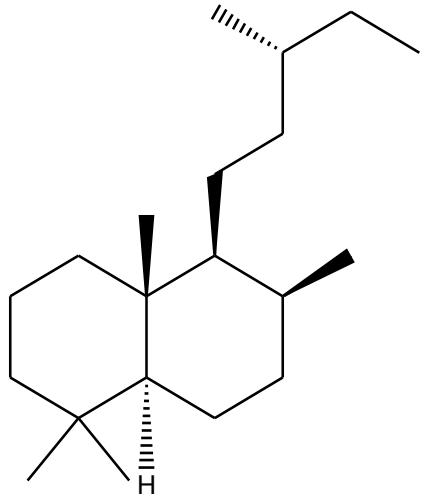



c. Diterpenes

| Acyclic | |
|---|---|
| Hydrocarbon- Phytane | Oxygenated- Phytol |
|  |   |

| Monocyclic | |
|---|---|
| Hydrocarbon- Camphorene | Oxygenated- Vitamin A |
|  |   |

| Bicyclic | |
|---|---|
| Hydrocarbon- Labdane | Oxygenated- Manool |



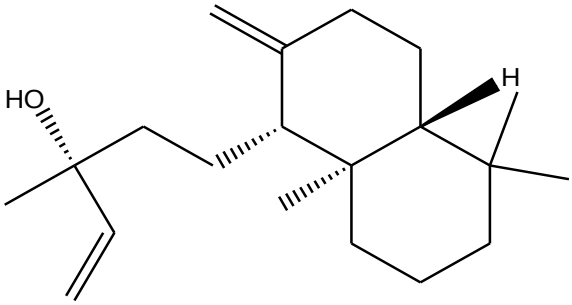




**Figure 3.** Examples of terpenes

**b. Straight Chain Compounds**

This group does not contain any side branches, contains only normal/straight chain non-terpenoids and their oxygen derivatives (Alcohols, Aldehydes, Ketones, Carboxylic acids, Ethers and Esters). These compounds are synthesized via octadecenoic pathway, from carbon 7 to carbon 35 [14].

i. 3-hexene-1-ol (Leaf alcohol)

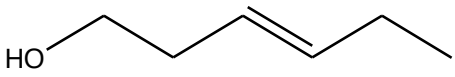



**Figure 4.** Example of straight chain compound

**c. Phenylpropanoids**

These compounds are also known as benzene derivatives. This non-terpenoid group consist of chemical constituents derived from normal propyl benzene and even so, the cyclic ring may attach hydroxy, methoxy and methylene dioxy groups whereas the propyl side chain may attach hydroxyl or carboxyl group [14]. The constituents under this group exist in small percentages of the essential oils but contribute to the fragrance of the essential oil. The subclass of phenylpropanoid being phenylpropene is synthesized from amino acid phenylalanine and L-tyrosine through the shikimic acid path way [14].

i. Estragole

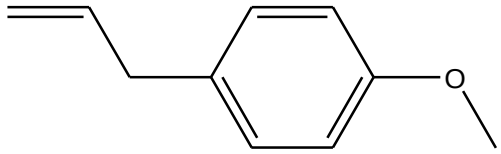



**Figure 5.** Example of phenylpropanoid

**d. Miscellaneous group**

The compounds or chemical constituents of this group usually contain sulphur and nitrogen containing compounds. They mainly occur as glucosinolates or their disintegration products such as isothiocyanates [14]. Allicin and other thiosulphinates breakdown diallyl disulphide, ajoene while the Y-glutamylcysteines are transformed to S-allyl cysteine via a non-alliin/allicin pathway [14]. The sulphur-containing compounds are accountable for the aroma and taste features. Nitrogen-containing compounds exists in limited essential oils, i.e., pyridines exist in *Chrysopogon zizanioides* oils [14].

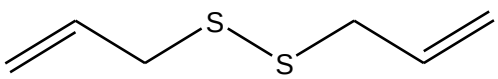
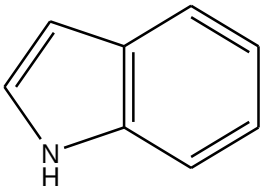
| Diallyl disulphide | Indole |
|---|---|
|  |  |





**Figure 6.** Examples of miscellaneous group compounds

## 1.1.3. PHYTOREMEDIATION POTENTIAL OF *CHRYSOPOGON ZIZANIOIDES*

The rapid rising industrialised global economy (Mining, Industry, Agriculture, Military Activities) is leading to the increase of different pollutants in soil which can be transferred to human beings via food chain as a result of soil to plant transfer [16]. Soil pollutants can be categorized in two groups as organic and inorganic. The main constituents of inorganic pollutants are heavy metals (i.e., Copper, Nickel, Lead) and continuously accumulate in the environment as a result of daily pollution associated activities [17].

Treatment of soil pollution is usually done by conventional methods such as soil vapour extraction and incineration which are expensive and somehow irrevocably affect soil original chemical properties and the substitute method called Phyto-remediation has been put into place, though the process is time consuming, however it is environmentally friendly [16]. Phyto-remediation is the use of plant to clean up pollutants from the environment [18]. This green technology is simple, cost effective and environmentally friendly as compared to other

methods. It is mainly restricted to the place occupied by the root system, so for high efficiency, big plants with high yield and too much network of roots are recommended. Perennial plants with high biomass production require a short-term adherence for the remediation [17]. The environmental factors such as extreme temperatures, high toxicity and the general soil conditions also govern the success of remediation since they affect the survival and the growth of the plant [17].

CZ is among the top plants that have the potential to meet all the required basis of phytoremediation. The grass has a $C_4$ route of photosynthesis, the $C_4$ plants have the ability to defeat the photorespiration restrictions found in $C_3$ plants [17]. This gives the $C_4$ plants (i.e., CZ grass) more advantages as they show high photosynthesis rate at elevated light intensity and temperatures as a result of accelerated efficiency of photosynthetic carbon reduction cycle [17]. CZ just like other $C_4$ plants has high growth rate which is much higher than that of the $C_3$ plants. The grass can be cultivated across different parts of the world due to its ability to keep high activities of major enzymes required in photosynthesis (NADP-NDH and NADP-MET), making it to still perform well when cultivated in fair climates [17]. Even so, CZ grass grows rapidly and it has very high biomass in tropical climate. The numerous unique features the grass has, make the growth of the grass to not be negatively affected by the elevated threshold levels of various toxic heavy metals (i.e., As, Cd, Cr, Cu, Ni, Pb) in soils as compared to most vascular plants [17]. It can also be noted that combination of heavy metals can result in synergistic and antagonistic interactions thus causing either a significant increase or decrease of metal toxicity of individual metal [19].

### 1.1.4. ENHANCED PHYTOREMEDIATION OF *CHRYSOPOGON ZIZANIOIDES*

To speed up the Phyto-remediation process, chemical modifications such as application of chelating agents is practiced to boost metal availability and uptake by plants [20]. The commonly used chelating agent is ethylene diamine tetra acetic acid (EDTA) as it said to be more efficacious due to its powerful chelating ability to many different metals and increases uptake of the heavy metals by the plant as well as bioavailability enhancement in the soil [20]. The use/application of fungi, specifically *Arbuscular mycorrhizal fungi* (AMF) inoculation is also practiced due to its ecological and biotechnological possibility [21]. AMF belonging to a family *Glomales* and class *zygomycetes* has various benefits to the Phyto-remediation by associated plants as it augments nutritional condition by improving nutrient uptake from the soil to the plant, especially those with low mobility such as zinc [19] [21]. It also has capability to endure various abiotic stress factors [22]. The outcome of AMF colonisation on clean-up of soil contaminants relies on the interaction between plant, fungi and heavy metal as well as soil state such as soil pH. The fungus execute approach that is similar to those of its hosts, i.e., immobilisation of the metals by constituents secreted by the fungus and chelation of the metals within the fungus [19]. The organic amendments (cow manure) increase the survival rate, growth and biomass of the plants planted in heavy metal contaminated soil, but it can either increase, decrease or not have any effect on the metal uptake depending on the nature of the metal [17].

### 1.1.5. INTERACTIVE EFFECTS OF TOXIC HEAVY METALS, CHELATING AGENT (EDTA) AND MICROBES (AMF) ON *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

The ability of CZ roots to tolerate and absorb high concentration of toxic heavy metals (non-threshold elements) and the use of AMF-EDTA in CZ can have effect on the essential oil extracted from the roots, in a case where the grass grows in a heavy metal polluted area [23]. AMF and EDTA may independently or collectively have influence on the production of secondary metabolites (essential oil) and may have impact on the composition of the main constituents of essential oils [21]. The interactive effect of CZ root and microbes in essential oil biogenesis shows that the quantity and composition of essential oil constituents varies and too dependent on the microbial community linked to the roots [24]. Some microbes contain keto-synthase gene which is associated with production of the essential oil. In comparison with the AM fungi inoculated CZ and uninoculated CZ, the uninoculated CZ grass showed enhancement in overall essential oil production (increase concentration of the constituents) inclusive of signature constituents as a result of improved nutritional status [24] [21]. These microbes i.e.; AMF perform internal microbial transformation of chemical constituents [24]. However, there is possibility of low essential oil yield in fungi associated compared to non-cleansed plant [25]. Besides CZ roots associated microbes, heavy metals do have impact on the yield and essential oil constituents, trace amounts of Pb and Ni significantly elevate the essential oil content including CZ characteristic constituents like khusimol [24]. The influence of chelating agent on essential oil production is through enhancing the bioavailability and bioaccumulation of toxic heavy metals since heavy metals are one the abiotic factors that enhance the production of essential oil as a result of metal stress [21].

## 1.2. PROBLEM STATEMENT

Essential oils have various important applications i.e., medicine, perfumery, cosmetics, aromatherapy, disinfectants and flavours in food. This makes the demand for essential oil products to keep on gradually increasing worldwide hence the prices keeps on rising. Most of the countries like Botswana highly import this essential oil value added products from international market at a very exorbitant price.

Botswana as one of the countries with Mining industries (i.e., Selibi-Phikwe Copper and Nickel Mine, Morupule Coal Mine) and Agricultural practices (i.e., Use of fertilizers in crop production) contribute to the rise of air and soil pollutants such as toxic heavy metals. Different environmentally friendly and cost-effective methods have to be put in place to try to minimise the amount of toxic heavy metals in the atmosphere and soil such as intensified phytoremediation (phytoremediation improved with biological method-employ micro-organisms for pollutant removal and Chemical methods-use of chelators to wash polluted soil). There are plants which are used to remediate this toxic heavy metal across other countries, *Chrysopogon zizanioides* is one of the plants that successfully absorb and withstand high concentration of these toxic heavy metals [6].

Since *Chrysopogon zizanioides* is a multifunctional grass; essential oil production and phytoremediation, it is vital to examine the quantity and quality of its essential oil. This is due to various applications of *Chrysopogon zizanioides* roots essential oil as it is mainly used in perfumery and cosmetics industry (as a fixative as well as fragrance ingredient) and food industry (for flavour). Bioaccumulation of some toxic heavy metals in the human body interferes with proper functioning of some vital organs. In addition, the fundamental properties and economic value of the essential oil is provided by the corresponding composition of the essential oil.

This research project can be a feasible business idea and it has not been carried out in Botswana and it is worth implementing due to interesting rare features that *Chrysopogon zizanioides* possess. Finally, this research study is much concerned about quality assurance control.

## 1.3. OBJECTIVES

### 1.3.1. MAIN OBJECTIVE

The major objective of the study is to:

- Extract essential oil from potential essential oil producing plant with Phytoremediation properties (i.e., Vetiver grass/ *Chrysopogon zizanioides* roots) and study the quality and quantity (chemical composition and yield) of the *Chrysopogon zizanioides* essential oil in relation to heavy metal content.

### 1.3.2. SPECIFIC OBJECTIVES

The specific objectives are to:

- Extract essential oil from *Chrysopogon zizanioides* grown in heavy metals polluted area.
- Determine the presence of heavy metals from both the *Chrysopogon zizanioides* essential oil and raw *Chrysopogon zizanioides* roots.
- Characterise the *Chrysopogon zizanioides* essential oil.
- Analyse the level of toxic heavy metals in the *Chrysopogon zizanioides* essential oil as well as in direct *Chrysopogon zizanioides* roots.
- Compare the heavy metals level in extracted *Chrysopogon zizanioides* essential oil with heavy metals level in the raw plant that was used for extraction of the *Chrysopogon zizanioides* essential oil.
- Study the effects of fungi (AMF) and chelating agent (EDTA) on the absorption of heavy metals as well as on the quality and quantity of extracted *Chrysopogon zizanioides* essential oil.

## CHAPTER 2. LITERATURE REVIEW

Presently many plants have been discovered which produce essential oil [9]. CZ is found in various countries as shown in table 1 and CZO has been successfully produced in these different countries other than native country via CZ commercial cultivation [9]. CZ is indigenous to India, even though it is being cultivated in different countries worldwide [13]. India, China, Indonesia, Haiti and Reunion are the main producers of CZO for commercial purposes and the main consumers are USA, Europe, India and Japan [13]. The highest volume of CZ essential oil is exported from Indonesia which is competitive with that of Haiti but the superlative CZ essential oil is claimed to be from Reunion Islands [12]. In a year, around 300 tons of vetiver grass essential oil is produced worldwide of which the native country of CZ (India) contributes 20-25 tons, while most of the vetiver essential oil is produced at Haiti, Indonesia (Java) and Reunion [4]. African countries cumulatively contribute 1% of world production due to inability to meet the international market requirements [4].

**Table 1.** Countries where CZ is currently known to be available [4]

| AFRICA | ASIA | CARIBBEAN | AMERICA | PACIFIC | OTHERS |
|---|---|---|---|---|---|
| Algeria | Bangladesh | Antigua | Argentina | Fiji | France |
| Angola | Burma | Barbados | Brazil | Cook Island | Italy |
| Burundi | China | Cuba | Columbia | New Caledo | Spain |
| Comoro | India | Haiti | Costa Rica | Wester | USA |
| Central Africa republic | Indonesia | Dominica republic | French Guiana | America | USSR |
| Ethiopia | Nepal | Jamaica | Guatemala | Samoa | |
| Gabon | Japan | St. Lucia | Guyana | New guinea | |
| Ghana | Malaysia | St. Vincent | Honduras | Tonga | |
| Kenya | Pakistan | Martinique | Paraguay | | |
| Madagascar | Philippines | Puerto Rico | Suriname | | |
| Malawi | Singapore | Trinidad | | | |
| Mauritius | Sir Lanka | Virgin Island | | | |
| Nigeria | Thailand | | | | |
| Rwanda | | | | | |
| Reunion | | | | | |
| Seychelles | | | | | |

**Table 2.** Characteristics of a normal soil which CZ can optimally grow on [8] [26]

| Soil chemical properties | Optimum limits |
|---|---|
| Soil type | Sandy loam soil |
| Soil PH | 3.0 to 10.5 |
| Cation exchange capacity (CEC) | High to very high CEC in respective cations |
| Salinity | 8 $dsm^{-1}$ |
| Organic nutrients | Nitrogen, Phosphorus |

## 2.1. EFFECTS OF SOIL CHEMICAL PROPERTIES ON CZ AND CZ ESSENTIAL OIL

The chemical properties of soil result in a sequence of chemical changes together with growth, yield and quality of the plant biomass [27]. The pH value is a measure of nutrient cycling thus raising the pH will elevate mineral adsorption. In terms of CEC there is direct correlation with organic matter. Organic nutrients ameliorate plant growth by enhancing soil structure, water penetration, and soil biological activities. Salinity regulates the $CO_2$ absorption rate and delay plant growth, consequently resulting in low plant yield [27]. CZ can grow at its best level at specific optimum soil chemical properties shown in table 2; however, it can also grow well an any soil with various chemical properties (soil pH, CEC and salinity level) [4] [5]. Plant nutrition is the main aspect that plays a significant role in influencing the growth and development of CZ grass just like any other plant. Limited supply of major nutrients (N, P, and K) can have negative impact on the yield therefore it is essential to use organic manures and inorganic fertilisers to sustain soil fertility and consequently leading to feasible yield [27]. The secondary metabolites (essential oil) produced by different plants species or same species can show variation in chemical compositions [28]. These difference in chemical profile are linked with various abiotic factors (non-living factors) that influence the production of essential oil. This includes pH, salinity and climate but the major factor is the micro-climate in which the CZ grass is growing on as they can alter the enzymatic package [28].

## 2.2. *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL COMPOUNDS WITH CORRESPONDING PERCENTAGE COMPOSITIONS GROWN IN NORMAL SOIL

CZO constituents are more complicated compared to other essential oils. Across different countries, more than 150 constituents have been discovered, even so the chemical composition is tremendously complex [9] [12].

In the study carried out in India, it has been concluded that the chemical constituents which are mostly found in the light yellow and viscid CZ roots essential oil are classified as Sesquiterpene hydrocarbons and their oxygenated derivatives [9]. The essential oils extracted from CZ roots collected from four locations in south India were analyzed by Gas Chromatography Flame Ionization Detector (GC-FID) and Gas chromatography- mass spectrometer (GC- MS) [9]. Eighty compounds, representing 94.5-97.8% of the oils were identified. The oils from Bangalore, Hyderabad, Kundapur, and Mettupalayam had high quantity of sesquiterpenes and

oxygenated sesquiterpenes with cedrane, bisabolane, eudesmane, eremophilane, and zizaane skeletons [9]. The principal compounds of the four essential oils were: eudesma-4,6-diene (δ-selinene) + β-vetispirene (3.9-6.1%), β-vetivenene (0.9-9.4%), 13-nor-trans-eudesma-4(15),7-dien-11-one + amorph-4-en-10-ol (5.0-6.4%), trans-eudesma-4(15),7-dien-12-ol (vetiselinenol) + (E)-opposita-4(15),7(11)-dien-12-ol (3.7-5.9%), eremophila-1(10),11-dien-2α-ol (nootkatol) + ziza-6(13)-en-12-ol (khusimol) (16.1-19.2%), and eremophila-1(10),7(11)-dien-2α-ol (isonootkatol) + (E)-eremophila-1(10),7(11)-12-ol (isovalencenol) (5.6-6.9%). The key compounds that imparted the characteristic vetiver odor were: khusimene, δ-selinene, β-vetivenene, cyclocopacamphan- 12-ol (epimers A and B), vetiselinenol, khusimol, isovalencenol, khusimone, α-vetivone, and β-vetivone. The chemical profiles of the oils were comparable to Haitian [9]. In a different study carried out in India, it was noted that the roots of the CZ through steam distillation yielded an essential oil predominantly comprising of sesquiterpenes (3-4 %), sesquiterpenols (18-25 %) and sesquiterpenones (7-8 %) [29]. Out of these, the major economically important active compounds were khusimol, α-vetivone and β-vetivone which form 35 % of oil [29].

The research which was carried out in Australia found that the principal compounds of CZ essential oil are; sesquiterpene hydrocarbons (γ-cadenene, clovene, α-amorphine, aromadendrine, junipene) and their alcohol derivatives; vetiverols (khusimol, epiglobulol, spathulenol, khusinol) and carbonyl derivatives; vetivones (α- vetivone, β-vetivone, khusimone; and ester derivative) [12]. Out of the compounds found, three carbonyl constituents; α-vetivone, β-vetivone and khusimone are considered as the prime aroma-influencing constituents. β-vetivone has the finer scent and is considered the most valuable whereas the isomer nordihydro β-vetivone has a strong, rich, woody-peppery scent [12]. All these constituents independently and mutually contribute to the characteristic odor of the vetiver essential oil. Therefore, they, α-vetivone, β-vetivone and khusimone, can be regarded as the 'finger print' of CZ essential oil [12].

In South Africa the composition of the CZ oil extracts was analysed via gas-chromatography techniques and this indicated that a large percentage of nootkatone was present when using the hydro distillation technique, whereas a large percentage of zizanoic acid was present when using the solvent extraction technique [8]. However, a minimal percentage of undesired zizanoic acid with higher percentages of valuable nootkatone and khusimol were present in the SCE extracts [8].

In Indonesia, analysis of vetiver essential oil using GC-MS determined 19 constituents after extraction using microwave hydrodistillation [5]. The principal compounds of CZ oil were $\beta$-Gurjunene (30.12%), $\alpha$-Vetivone (20.12%), 4-(1-cyclohexenyl)-2-trimethylsilymethyl-1-buten-3-yne (13.52%) and $\delta$-Selinene (7.27%) [5].

**Table 3.** Additional findings on the CZ compounds and percentage compositions in research studies carried out in other countries

| Country of study | CZ compounds identified with percentage compositions | References |
|---|---|---|

| | | |
|---|---|---|
| Turkey | -Twenty-eight constituents were identified.<br>-Major constituents were found to be khusinol (19.15% and 15.67%), β-vetivenene (9.76% and 8.16%) and dehydro-aromadendrene (7.34% and 9.66%). | [30] |
| China | - Twenty-three constituents were identified.<br>- Three major constituents; Valerenol (18.48%), Velerenal (10.21%), β-Cadinene (6.23%). | [31] |
| France | -110 compounds were identified.<br>- The major compunds were β-vetispirene (1.6-4.5%), khusimol (3.4-13.7%), vetiselinenol (1.3-7.8%) and ά-vetivone (2.5-6.3%). | [32] |

## 2.3. PERCENTAGE YIELD OF *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL GROWN IN NORMAL SOIL

There are lot of external factors that can affect the essential oil yield including age of the plant, extraction method, extraction time, temperature and particle size but the essential oil yield is mainly directly affected by the type of extraction method [4] [8].

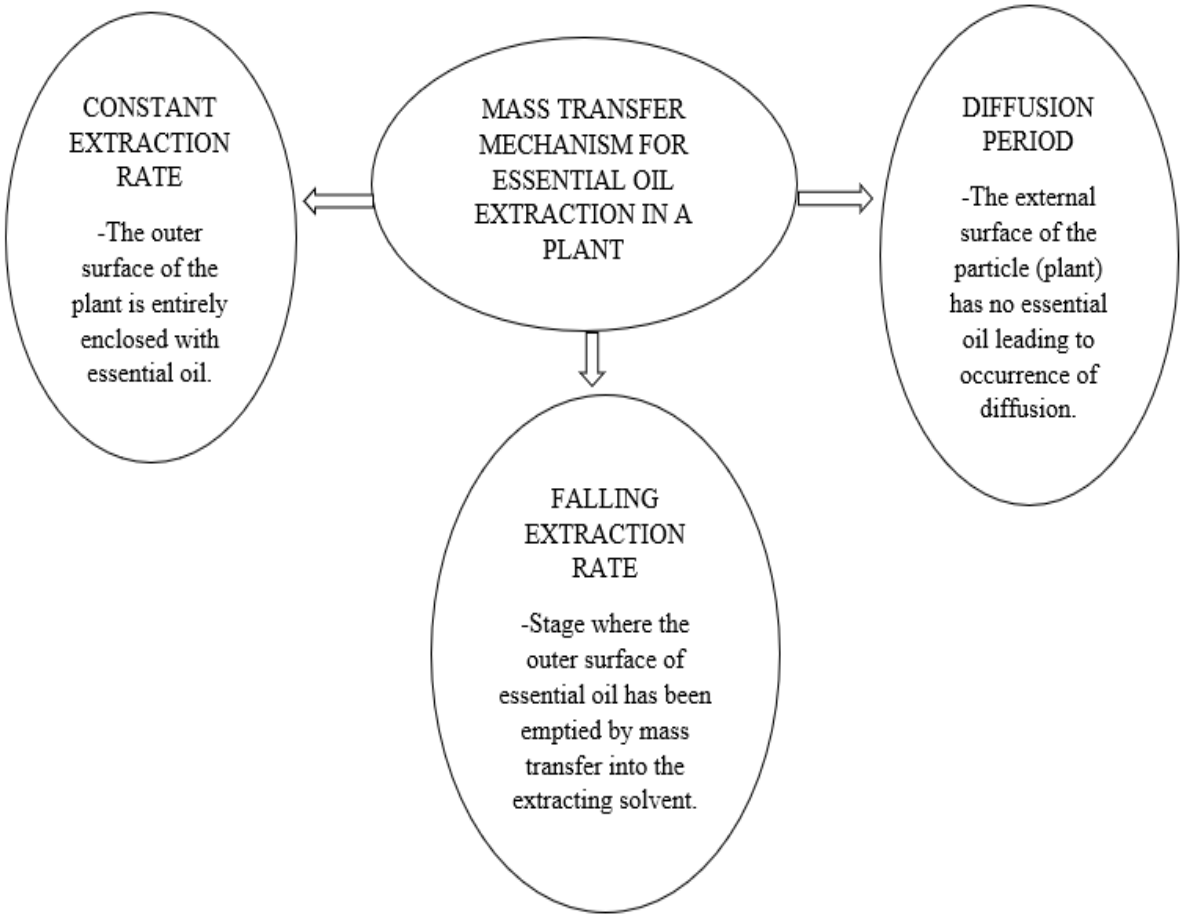



**Figure 7.** Mass transfer mechanism for essential oil extraction in a plant [8]

Most of the extracted essential oils (around 70%) are extracted in the constant and falling rate extraction phases presented in figure 7. However, CZ roots essential oil is not easily extractable because EO is situated in the very inside root tissue leading to slow diffusion rate. CZ essential oil is mainly composed of high molecular weight sesquiterpenes which causes long extraction time of the EO [8].

In South Africa, solvent extraction in a soxhlet apparatus with hexane as the extracting agent presented an average yield of + 1.6% of the essential oil for a 5-hour run which is faintly lower than the yield of 1.91% for a 5-hour run stated in literature [8]. The slight difference in yield was that for 1.91% experiment (higher yield experiment), the hexane extract incorporated solid particles that were insoluble in hexane solvent and the particles were high molecular weight constituents and the experiment was carried out at high boiling rate, consequently increasing the yield [8]. In accordance with the experimental results, yields of up to about 2% for hexane extraction can be attained by increasing the extraction time to 12 hours [8]. The CZ roots were also hydro-distilled in a Clevenger apparatus for 16 hours (extraction time); this resulted in a yield of approximately 0.18 to 0.35% [8]. The yield in hydro-distillation (Clevenger apparatus) was lower compared to solvent extraction (hexane extraction) because of narrow boiling point (63-69 ºC), easy oil recovery and a great solubilizing ability, also higher molecular weight constituents were not extracted in hydro-distillation and loss of the extract during arm removal

in hydro-distillation [8]. According to literature hydro distillation of CZ roots in a similar apparatus resulted in an average yield of 1.8% for a 16-hour run [8]. This big variation indicated that high molecular weight vetiver essential oil constituents were not released for hydro distillation experiment that yielded 0.18-0.35%. In comparison between Soxhlet extraction and hydro-distillation, the results showed that the denser constituents of the CZ oil were not released during the hydro-distillation extraction hence lower yield. Research has indicated that supercritical carbon dioxide extraction (SCE) generates the highest yields ranging from 2.9 to 3.74% when using the recommended parameters of 190 bar and 50 °C [8]. Experimentally a yield of approximately 2.3% was attained by SCE at 180 bar and 40 °C [8]. This yield is less than that seen in literature due to the lower operating temperature and pressure; however, SCE gives a higher yield than the other methods tested in this investigation such as solvent extraction and hydro-distillation [8]. This is due to low viscosity and high diffusivity of supercritical fluids which makes them to diffuse easily through solid particles (in plant) hence resulting in quick and good extraction yields [8]. The low viscosity and high diffusivity happen because at supercritical state, the liquid and gas phases are indistinguishable therefore the supercritical fluid adopts the liquid phase density and the gas phase viscosity [8]. The solvent extraction technique produces high yield with more of non-valuable zizanoic acid while hydro-distillation gives very low yields but no zizanoic acid with high percentages of valuable nootkatone and khusimol. It was therefore inferred that SCE would be the best extraction method for these particular CZ roots [8].

Other studies conducted in Ethiopia using steam distillation revealed a maximum average yield of (1.72 percent) after 6 hours of extraction time, particle size range of (0.425-0.75mm), and temperature of (95 ºC), with a minimum average yield of (0.15 percent) at 2 hours of extraction time, temperature of (75 ºC), and particle size range of (1.075-1.4mm) [4]. According to this discovery, increasing the extraction time, temperature, and decreasing particle size will enhance the percentage yield of oil extraction [4].

Furthermore, the extraction of essential oil from CZ roots has been performed in Indonesia using microwave hydro distillation [5]. It was found that microwave hydro distillation method offered great advantages over hydro distillation, such as shorter extraction time (3 h vs. 24 h respectively); better or equivalent yields (0.49% vs. 0.46% respectively); and environmental impact (energy cost is considerably higher for performing hydro distillation than that required for extraction using microwave hydro distillation) [5].

**Table 4.** Additional findings on the yields of CZ essential oil in research studies carried out in other countries

| Country of study | Yield of CZ essential oil from normal soil (%) | References |
|---|---|---|
| France | -Supercritical carbon dioxide extraction method produced high yield of about 3.2% in notably less time in comparison with other extraction methods. | [33] |

| | | |
|---|---|---|
| China | -Carbon dioxide expanded ethanol extraction method yielded 5.12% to 7.42% which was higher than that of hydro-distillation and steam distillation. | [31] |

## 2.4. PHYTOREMEDIATION POTENTIAL OF *CHRYSOPOGON ZIZANIOIDES* GROWN IN POLLUTED SOIL

CZ is tolerant to both individual heavy metals as well as combinations of different heavy metals. CZ can grow in mine tailings that have Cu, Cr, Pb, Zn with levels as high as 3921 mg $kg^{-1}$, 2290 mg $kg^{-1}$, 10750 mg $kg^{-1}$, 4377 mg $kg^{-1}$ respectively [17]. Plants usually accumulate heavy metals primarily by their roots, therefore more concentration of heavy metals are found in the root system as compared to the aerial part [17]. The accumulation of Cu by the plant is very low and the translocation ratio of Cu from the roots to the leaves is normally less than 10%. In comparison CZ accumulates high amount of Cr but the translocation ratio is also quite low.CZ grass is said to be a hyperaccumulator of Pb and Zn, which means it can accumulate high concentrations of these two heavy metals however the translocation depends on the proportion of water soluble Pb/Zn [17].

**Table 5.** Threshold measure of heavy metals to CZ growth based on single element experiment [17]

| Heavy metals | Soil level (mg $kg^{-1}$) | Shoot level (mg $kg^{-1}$) |
|---|---|---|
| Arsenic | 100-250 | 21-72 |
| Cadmium | 20-60 | 45-48 |
| Copper | 50-100 | 13-15 |
| Chromium | 200-600 | 5-18 |
| Lead | >1500 | >78 |
| Mercury | >6 | >0.12 |
| Nickel | 100 | 347 |
| Selenium | >74 | >11 |
| Zinc | >750 | 880 |

The pilot research in India looked at the potential of CZ to rehabilitate iron ore waste dumpsites from the Joda East Iron mine in Odisha [6]. S2 (diploid variety), S4 (tetraploid derivative of S2), TH (originating in Thailand), and BL (a broad leaf) genotypes of CZ were grown for a year to study their individual growth performance, metal tolerance, and metal absorption. The shoot/root length, photosynthetic pigments – chlorophyll, carotenoid, and biomass output of plants grown on iron mine soil were initially decreased when compared to control plants grown on regular garden soil. Nonetheless, at the end of a year, the plants showed typical development

and appeared healthy. Furthermore, heavy metals such as Zn, Mn, Cr, and Cu were identified in plant tissues. Metal builds up in plant tissues resulted in oxidative damage caused by reactive oxygen species (ROS). As a result, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPOD), glutathione reductase (GR), and glutathione peroxidase (GPX) increased in activity. Based on the findings, it is possible to conclude that CZ can withstand high amounts of Fe as well as other heavy metals in its tissues. Such potential varies between the four genotypes, with genotype BL ranking highest in terms of phytoremediation, followed by genotype S4. As a result, CZ may be utilized to efficiently rehab and stabilize soil in places polluted with high amounts of heavy metals, including Fe, Mn, Zn, and Cr [6].

In Australia a glassware and field studies were conducted which showed that CZ can mass produce high biomass (>100t/ $tha^{-1}year^{-1}$) [17]. CZ also demonstrated that it can withstand great climatic variation such as protracted drought, flood, submergence, and temperatures (−15 ◦C–55◦C), soils high in acidity and alkalinity (pH 3.3–9.5), soil salinity (ECse 47.5 dS $m^{-1}$), sodicity (ESP 48 percent), and a wide spectrum of heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, and Zn) [17]. According to the findings, it may collect large quantities of Al (85 percent saturation percentage), Mn (578 mg $kg^{-1}$), Pb (shoot 0.4 percent and root 1%), and zinc (shoot and root 1 percent). Since the majority of heavy metals concentrate in roots, phytostabilization and phytoextraction using chelating agents are viable options [17]. Despite the fact that CZ is not as effective as some other species in heavy metal accumulation, few plants in the literature have a wide range of resistance to severely harsh climatic and growth medium conditions (soil, sand, and tailings) coupled into one plant like CZ [17]. All of these unique characteristics make CZ an ideal plant for heavy metal phytoremediation [17].

**Table 6.** Additional research studies on phytoremediation potential by CZ in other different countries

| Country of study | Heavy metals accumulated (mg $kg^{-1}$) | References |
|---|---|---|
| Indonesia | - The research study was aimed at evaluating uptake rate and elimination rate of heavy metals by CZ on metal polluted water.<br>- The Cr and Ni highest uptake rates were: 127.21 mg $kg^{-1}$ $day^{-1}$ and 15.60 mg $kg^{-1}$ $day^{-1}$ respectively whereas the Cr and Ni elimination rates were 1.09 mg $kg^{-1}$ $day^{-1}$ and 12.24 mg $kg^{-1}$ $day^{-1}$. | [34] |

| | | |
|---|---|---|
| | - The CZ BCF, BAC, TF on Cr and Ni contaminated water were greater than 1; CZ phyto-remediate through phyto-extraction and phyto-stabilization. | |
| Iran | -The research study was aimed at investigating the potential of vetiver phytoremediation of heavy metals contaminated soils.<br><br>-The mean and adsorption percentage of Pb, Cd, Ni and Mn were 282.45 mg $kg^{-1}$ (83.4%), 248.3 mg $kg^{-1}$ (53, 2%), 69.4 mg $kg^{-1}$ (65.5%) and 63.29 mg $kg^{-1}$ (61%).<br><br>- Pb had the highest uptake rate.<br><br>-Vetiver roots absorbed more heavy metals as compared to shoots.<br><br>- The BCF was more than one and the TF was close to one, concluding that vetiver grass can be used as a phyto-stabilization plant. | [35] |

## 2.5. EDTA AND AMF ON PHYTOREMEDIATION OF *CHRYSOPOGON ZIZANIOIDES* GROWN IN POLLUTED SOIL

The implementation of chemical additives and organic chelates can be used as a suitable approach extraction of heavy metals from polluted soils [36]. Some micro-organisms such as AMF earn attention since they are one of the key functional groups of soil microbes which can make a symbiotic relationship with various plant species including CZ. AMF help ameliorate plant growth, boost plant nutrition absorption and intensify CZ tolerance to abiotic stress such as availability of high concentrations of toxic heavy metals [36]. Some research studies have found that AMF can immobilise heavy metals in CZ roots and thus preventing root to shoot metal translocation. Even so, other various studies have been concerned on the probable of the AMF in escalating the heavy metals availability in the contaminated soils [36]. Additionally, various research studies have recommended that chemical amendments such EDTA may be

used to ameliorate either phyto-stabilisation or phyto-extraction processes [36]. EDTA can improve the metals mobility in soil matrix thus enhancing metal bioavailability ad consequently improving the ability of CZ to absorb metals. However, EDTA as a synthetic agent can be toxic to soil micro-organisms (including AMF) that play a major role in plant growth and development [36].

The effect of application of EDTA on Cu uptake by CZ is very effective as the accumulation of Cu by the roots triples as compared to the control due to an increase in Cu solubilities [17]. Even so, introduction of organic matter (cow manure) significantly reduces the concentration of Cu in the roots as well in shoots since they lower the Cu solubility rate and immobilise Cu via complexation with organic matter components [17] [37]. The effect of AMF on the uptake of Pb by CZ depends on the level of Pb in the soil since it increases the uptake of Pb if Pb level in soil is low and decreases the Pb uptake if the Pb level in soil level is high as it offers protective effect at high Pb level [17] [38]. At high Pb concentration there is possibility of occurrence of retention of Pb by the fungal mycelium implying adsorption to cell wall and fixation by polyphosphate granules [38]. The reduction or delay in colonising ability of AMF can result in sensitivity of AM endophytes to escalated amount of Pb [38]. EDTA can also be added as it shows efficiency in mobilizing Pb from soil and therefore increasing the level of Pb on roots and shoots as well as translocation ratio [17]. Although EDTA works effectively with so many heavy metals, with Zn it does not show any significant effect in terms of the Zn uptake and translocation ratio [17].

A pot experiment was conducted in China to study the take up and transport of Pb by CZ from Pb-contaminated soils using EDTA [39]. The findings revealed that CZ may withstand high Pb concentrations in soils. The use of EDTA significantly boosted the Pb translocation ratio from CZ roots to shoots. After 14 days of 5.0 mmol EDTA $kg^{-1}$ soil treatment, the shoot Pb concentrations were 42, 160, 243 mg $kg^{-1}$ DW and the roots Pb concentrations were 266, 951, and 2280 mg $kg^{-1}$ DW in the 500, 2500, and 5000 mg Pb $kg^{-1}$ soils, respectively [39]. After 5.0 mmol EDTA $kg^{-1}$ of soil application and approximately 126 mm of rainfall irrigation, approximately 3.7, 15.6, 14.3 and 22.2% of the soil Pb, Cu, Zn, and Cd were leached from the artificially contaminated soil profile in the short soil leaching column (9.0-cm diameter, 20-cm height) experiment [39]. In the long soil leaching experiment, soil columns (9.0-cm diameter, 60-cm height) were packed with uncontaminated soils (to simulate the subsoil under contaminated top layers) and CZ was planted. The heavy metals leachate from the short column experiment was put to the surface of the long soil column, the fake rainfall was filtered, and the final leachate was collected at the soil columns' bottoms. The results demonstrated that soil matrix with planted CZ could re-adsorb 98, 54, 41 and 88% of the previously applied Pb, Cu, Zn, and Cd, reducing the probability of heavy metals running downwards and entering groundwater [39].

The effect (AMF - *Glomus spp* on the development of CZ grass and it's As absorption from polluted hydroponic and soil systems has been studied [40]. An ameliorative impact of AMF inoculation in increasing plant growth was discovered in a study conducted in the United States, mostly via encouraging the development of their root system [40]. Furthermore, AMF-inoculated plants absorbed more As from both polluted systems than non-inoculated plants. It was also shown that As absorption by CZ grass from tainted hydroponic solutions was more efficient than spiked soils, owing to the former's increased Phyto availability. Hydroponically grown plants translocated more As from their roots to their shoots. According to the findings

of this study, the application of CZ grass technology in conjunction with AMF would be more suited to disinfect As-contaminated water than soils [40].

**Table 7.** Additional research studies on effects of AMF and/or EDTA on CZ phytoremediation in other countries

| Country of study | AMF/EDTA | Effects on HM accumulation | References |
|---|---|---|---|
| Switzerland | EDTA | - The research study evaluated the Phyto assessment of vetiver grass in both single and multi-heavy metal (Cd, Pb, Cu, and Zn) - EDTA enhanced contaminated soil.<br>- Cd + Pb + Cu + Zn + EDTA treatments showed higher accumulation of heavy metals as compared to the control.<br>- In single element treatments, Zn + EDTA treatment showed high accumulation of Zn (8068 ± 407 mg $kg^{-1}$) however in multi element treatment (Cd + Pb + Cu + Zn + EDTA), Cu and Pb showed to have been the highest accumulated metals with concentrations of 1977 ± 293 mg $kg^{-1}$ and 1096 ± 75 mg $kg^{-1}$ respectively.<br>- For all the treatments, the heavy metal | [41] |

| | | | |
|---|---|---|---|
| | | accumulation trend was Zn >>> Cu > Pb >> Cd. | |
| Iran | AMF | - The research study evaluated the effectiveness of AMF in phytoremediation of Pb contaminated soil by vetiver grass.<br>- Shoot and root dry weights showed to be increased by mycorrhizal inoculation.<br>- Pb uptake of shoot and root significantly increased by mycorrhizal inoculation and increasing Pb levels.<br>- Phosphorus uptake as well as concentration was notably high in shoot of vetiver inoculated with AMF. | [42] |

## 2.6. HEAVY METALS EFFECTS ON *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

The quality of CZ essential oil is a vital aspect since the essential oil is mainly used in perfumery and food industries [8]. The CZ essential oil must be toxins free to avoid causing major harm to the public [8]. However, many research studies have showed that as a results of high molecular weight of the metal, the metals are denser to evaporate with essential oil during extraction [8]. However, the high toxic heavy metal level in the plant can alter the composition and yield of the essential oil [43].

In a research study carried out in Bulgaria, the content of various heavy metals (Cd, Pb, Cu, Mn, Zn) and other trace elements in different plant extracts and essential oil was very low [44]. Cd concentration was below the detection limit, Pb concentration was less than 0.1 mg $kg^{-1}$, Cu concentration was less than 0.1 mg $kg^{-1}$, Mn concentration was below 0.02 mg $kg^{-1}$, Zn concentration was below 0.9 mg $kg^{-1}$, As concentration was below 0.08 mg $kg^{-1}$ [44].

In India, the pot experiment showed that Cr, Ni and Pb had a detrimental effect on CZ essential oil yield [43]. The injection of a little quantity of Cr into the soil had no discernible effect on the CZ essential oil content, whereas a large dose of Cr lowered the CZ essential oil content. The administration of tiny to moderate doses of Cd, Pb, and Ni considerably increased the CZ essential oil content as compared to the control. However, successive additions of Cd, Pb, and Ni had no discernible effect on essential oil content [43]. This change might be attributed to the impact of metallic elements on enzyme activity and C metabolism [43]. Small levels of Cr and Ni, on the other hand, had no effect on CZ essential oil yield, but large dosages of Cr and Ni drastically reduced output [43]. The drop in essential oil output caused by increased Cr and Ni administration is due to a decrease in root biomass and essential oil concentration. [43]. The injection of modest to moderate doses of Cd and Pb led in a considerable rise in CZ essential oil output, whereas a significant increase in Cd and Pb levels had no effect on CZ essential oil yield. The increased CZ essential oil output is related to increased root biomass as well as an increase in CZ essential oil concentration [43]. One of the major constituents of CZ, khusimol showed a significant content increase as a result of application of Pb and Ni and small to moderate amounts of Cr and Cd. However, an additional increase in Cr and Cd levels had no significant impact on khusimol content [43]. The significant alteration in khusimol content can be associated with the uptake of essential and phytotoxic metals [43]. Metals are vital for the catalytic activity in some enzymes because they make enzyme-substrate-metal complex hence metal enzymes execute a major role in synthesis of monoterpene compounds [43]. The chemical composition of CZ essential oil could be regulated by the application of metallic salts [43]. Moreover, the CZ essential oil yield and composition (content) could also be influenced by various factors including geographical origin, cultivation methods as well as presence of micro-organisms [43].

**Table 8.** Additional research studies on effects of heavy metals on CZ essential oil in different other countries

| Country of study | Effects of HM on CZ essential oil | Reference |
|---|---|---|
| Indonesia | - The research study focused on acquiring the aromatic plants chosen as phytoremediation of Cd and studying their association with production of essential oil.<br><br>- The results showed that there was an improvement | [45] |

| | | |
|---|---|---|
| | of 101.56% in CZ essential oil content. | |

## 2.7. EDTA AND AMF ON *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

Microbes (arbuscular mycorrhizal) symbiosis have exhibited varying effects on the secondary metabolites inclusive of essential oil quality and quantity [46]. Specific inocula are tactical in boosting the essential oil chemical profile [46]. Various research studies have discovered that AMF increase the production of essential oil (yield) and improve essential oil composition [47]. The increase in essential oil yield by AMF result from the fact that mycorrhization enables the root system to utilize large volume of soil by elongating the root zone thus accessing soil pores that the root hairs cannot reach. Consequently, obtaining the organic phosphate via synthesis of extracellular acid phosphatases [47]. The symbiotic associations by AMF influence the plant to easily absorb phosphorus which in turn impact the biosynthesis of essential oil [46]. On the other hand, the concentration and composition of essential oils present a very vital ecological part since that is influenced by plant defensive response to micro-organisms colonization; various essential oils possess antimicrobial properties [47]. The microbes and bacteria available in the soil with CZ grass roots react with essential oil within the grass roots, therefore sesquiterpenes go through bio-conversion into oxygenated sesquiterpenes [8]. The unique odour of CZ essential oil is as a result of oxygenated sesquiterpenes which some were formed as a result of microbial bioconversion [8]. The microbes are killed by synthetic fertilisers and pesticides thus stopping the bioconversion and as a result the grass yield less valuable essential oil [8].

In Thailand a research study was carried out where CZ roots were planted in three different cultivation systems (normal soil, normal soil with added microbes and semi hydroponically) [3]. The essential oil was extracted using a simultaneous steam distillation and solvent extraction apparatus and the extracted essential oil was brown yellow and viscous. The yields of the essential oils obtained in normal soil, normal soil with added microbes and semi-hydroponically were 0.18, 0.27 and 0.06%, respectively [3]. The higher yield achieved by cultivation in normal soil with microbes disclosed the intracellular bacteria activity that were engaged with the essential oil cells inside the glands of CZ roots. There could be possibility of biotransformation of the essential oil precursors by these microbes hence increasing the yield [3]. The cultivation method also has significant impact on the composition of the CZ essential oil [3]. In comparison between all the three cultivation methods, the method employing microbes had higher content of some low molecular weight constituents including 2-norzizaene and γ-vetivenene, and quantitative variance (essential oil profile) was observed in major constituents [3]. This can result from an unidentified biotic factor that can interact with the microbes [3].

In the research study carried out in USA, tissue cultured cleansed (bacteria and fungus free) and non-cleansed CZ plants were cultivated for five months in pots with identical conditions except for sterilized soil for cleansed CZ plant and unsterilized soil for non-cleansed CZ plant [25]. The percentage yields of the steam distilled (24 h) oil of the roots from both the tissue

cultured cleansed CZ as well as non-cleansed CZ yielded 0.02% with clear colour and 0.35% with light-yellow oil colour respectively. CZ has been scientifically proved to contain AMF and intracellular bacteria which could be involved in biotransformation of CZ essential oil hence high yield in non-cleansed CZ grass. Furthermore, GC/MS analyses of the oils indicated that the non-cleansed (normal) CZ had the normal vetiver oil profile, while the tissue cultured (cleansed) vetiver produced many of C19–C29 alkanes, several alkanols along with normal CZ oil constituents, but it was in deficit of presumed fungal metabolites such as b-funebrene, prezizaene, a-amorphene, and b-vetispirene [25]. It shows that there was unavailability some essential oil production stimulant in tissue cultured cleansed plant since both plants were cultivated in the same soil properties and conditions [25].

As pre-mentioned before, EDTA chelating agent has normally been used in phytoremediation to ameliorate the bioavailability of heavy metals in the soil for uptake by plants [41]. The availability of EDTA molecules improve the metal extraction from exchangeable sites and eventually creating soluble metal-EDTA complexes [41]. However chelating metal ions expose plants to toxic metal stress which triggers the plant to produce more of certain components including phenolic components [48]. Such components can play an antioxidant role so that they can defend themselves against toxic metal damages [48]. It is worth noting that plants respond to stressors differently since they are capable of synthesizing different components. The synthesis of such constituents is cautiously regulated process in plants and the plants" defensive response to various environmental conditions is complex [48]. Since little to no research has been carried out on the effect on the interaction between EDTA-heavy metals-CZ essential oil profiles it is challenging to discuss the variance in CZ essential oil profile. More research studies have to be done across different essential oil producing plants including CZ to understand the mechanism for difference in essential oil profile induced by chemical amendments.

## CHAPTER 3. METHODOLOGY

### 3.1. MINE TAILINGS SAMPLING AND POT PREPARATION

The sampling of mine tailings used in the experiment was carried out at BCL mine in Selibe-Phikwe, Botswana (figure 8).


**Figure 8.** Map of Botswana showing the mining tailing sampling site at Selibe-Phikwe

Mine tailings were collected, air dried and sieved to pass through 5 mm and 2 mm mesh for cultivation of plants and chemical analysis, respectively. Plastic pots were filled with 8 kg tailings, or a mixture of mine tailing (MT) + cow manure (CM) before mycorrhiza inoculation (AMF), ethylene diamine tetra-acetic acid application (EDTA) and plant establishment.

### 3.2. DESCRIPTION OF THE TREATMENTS AND PLANT CULTURE

A two-factor experiment was established on the response on CZ to mycorrhiza inoculation and EDTA amendments in a heavy metal contaminated mine tailings in the presence of cow manure. As shown in table 9, for AMF, there is either AMF inoculation or non-AMF inoculation and for EDTA, there is either no EDTA or EDTA applied in a split style or single application but accumulating to the same concentration. The variations are described as treatments (T1-T6): T1- mine tailings + cow manure (MTCM), without AMF and EDTA); T2- MTCM with 5 mmol EDTA split application; T3- MTCM with 5 mmol EDTA single dose application; T4- MTCM with AMF; T5- MTCM with AMF and 5 mmol EDTA split

application; T6- MTCM with AMF and 5 mmol EDTA single dose application and control - media with no heavy metals as well as chemical amendments. Cow manure equivalent to 10% of the total weight of soil was mixed thoroughly before potting. The slips of the CZ were obtained from seedlings grown under nursery conditions and were selected for visual uniformity. These CZ slits were cleaned with tap water and surface sterilized before replanting at the center of each pot. For the mycorrhiza treatments, five grams of sterile (121 °C, 30 min for three consecutive days) or non-sterile AM inoculants (MYKOVAM®, National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Banos, Philippines) were mixed with the soil at the center of pot just below the roots of the CZ slips of the without AMF and with AMF treatments, respectively. Three replicates were used for each treatment laid out in a complete randomized design. The plants were irrigated regularly and monitored for survival and growth inside the shade house (80% exposure) for 40 weeks as displayed in figure 9.

### 3.3. EXPERIMENTAL DESIGN LAYOUT

The Experimental Design followed a**: 2-factorial in Complete Randomized Design**:The factor 1 entails of AM inoculation which is in two levels (with AMF or with no AMF) and factor 2 entails of EDTA application which is in three levels (no EDTA, split 5 mmol; 2.5 mmol *2 EDTA application and single application of 5 mmol EDTA). All the treatments/ pots had cow manure. The total number of pots including the control pot were 21; 7 pots * 3 replicates.

**Figure 9.** A picture of *Chrysopogon zizanioides* growing in pots

**Table 9.** Experimental Design layout

| Treatment | AM inoculation | Cow Manure Application | EDTA application |
|---|---|---|---|
| 1 | 0 AMF | + Cow manure | 0 EDTA |
| 2 | 0 AMF | + Cow manure | 5 mmol EDTA split |
| 3 | 0 AMF | + Cow manure | 5 mmol EDTA single |
| 4 | + AMF | + Cow manure | 0 EDTA |
| 5 | + AMF | + Cow manure | 5 mmol EDTA split |
| 6 | + AMF | + Cow manure | 5 mmol EDTA single |
| Control (0 HM) | - | + Cow manure | - |

### 3.4. SOIL SAMPLE PREPARATION AND CHEMICAL ANALYSIS

Representative soil samples from each pot were collected after harvest of plants. The samples were passed through a 2 mm sieve, air dried and stored at 4 °C until chemical analysis. The soil pH and electric conductivity (EC) were measured in water suspension (1:2) after shaking for 1 hour. Total heavy metal contents were measured following the reported method; wet acid digestion in an open vessel at 120$^{o}$C for 6 hours using 1 $HNO_3$: 3 HCl mixture (manufactured by Rochelle chemicals and Minema chemicals respectively) in a hot plate followed by

quantification using inductively coupled plasma - optical emission spectrometer (ICP-OES) [49]. The available fraction of heavy metal in soil was assessed by determining the water-soluble (WS) and ammonium acetate exchangeable. Soil samples were shaken with distilled water (1:100) for 2 h at room temperature to obtain a water-soluble (WS) fraction. The exchangeable fraction (Exch) was determined by using 1N ammonium acetate (pH 7) at 1:100 ratio for 2 hours at room temperature. The WS and the Exch. fractions were analyzed for different heavy metals using ICP-OES. Samples were analyzed in duplicates and bioaccumulation factor for each metal was calculated as a ratio of the roots divided by the soil concentration.

### 3.5: PLANT SAMPLING AND CHEMICAL ANALYSIS

During the 40-weeks cultivation period, plants were periodically monitored for survival and growth. At harvest, the shoots were excised, and the roots were carefully separated from the soil, and their fresh weights were determined. A portion of the fresh roots, 1.0 g from each plant was subjected to trypan blue staining for microscopic examination of mycorrhizal infection [50]. The root length colonized by AMF was quantified using the gridline intersection method [51]. The remaining roots and all the shoots were air dried for 3 days and their weights were recorded. A portion of roots and shoots (5 g each) were oven dried at 70$^{o}$C for 3 days and ground for chemical analysis. The remaining roots were subjected to essential oil extraction.

For chemical analysis of plant tissues, the dried CZ grass roots samples from each 6 treatments (with triplicates) were crushed with a blender and weighed to around 0.5 g for each treatment. The samples were introduced to wet digestion/microwave assisted acid digestion. The method was as follows; the sample was dissolved in concentrated $HNO_3$ and 30% $H_2O_2$ (4:1) (manufactured by Rochelle chemicals and Sigma-Aldrich respectively) and predigested overnight (16 hours) in a fume hood at room temperature. The solution was put in a microwave digestor shown in figure 10 for digestion according to the following optimized program: Power; 290-1800, Ramp time; 20 minutes, Hold time; 10 minutes and Cooling; 15 minutes. After cooling, the entire digest was transferred into 50 mL volumetric flasks and diluted to 50 mL mark with distilled water. Reagent blank was prepared similarly to the samples [52]. All samples were filtered so as they are clear for ICP-OES analysis

All the 6 samples (with triplicates) were analyzed with ICP-OES displayed in figure 11 using the following optimized parameters: RF power; 1150 W, analysis pump rate; 50 rpm, auxiliary gas flow; 0.5 L min$^{-1}$, nebulizer gas flow; 0.70 L min$^{-1}$, coolant gas flow; 12 L min$^{-1}$ and purge gas flow; normal.


**Figure 10.** A picture of the BIUST microwave digestor used for extraction of heavy metals

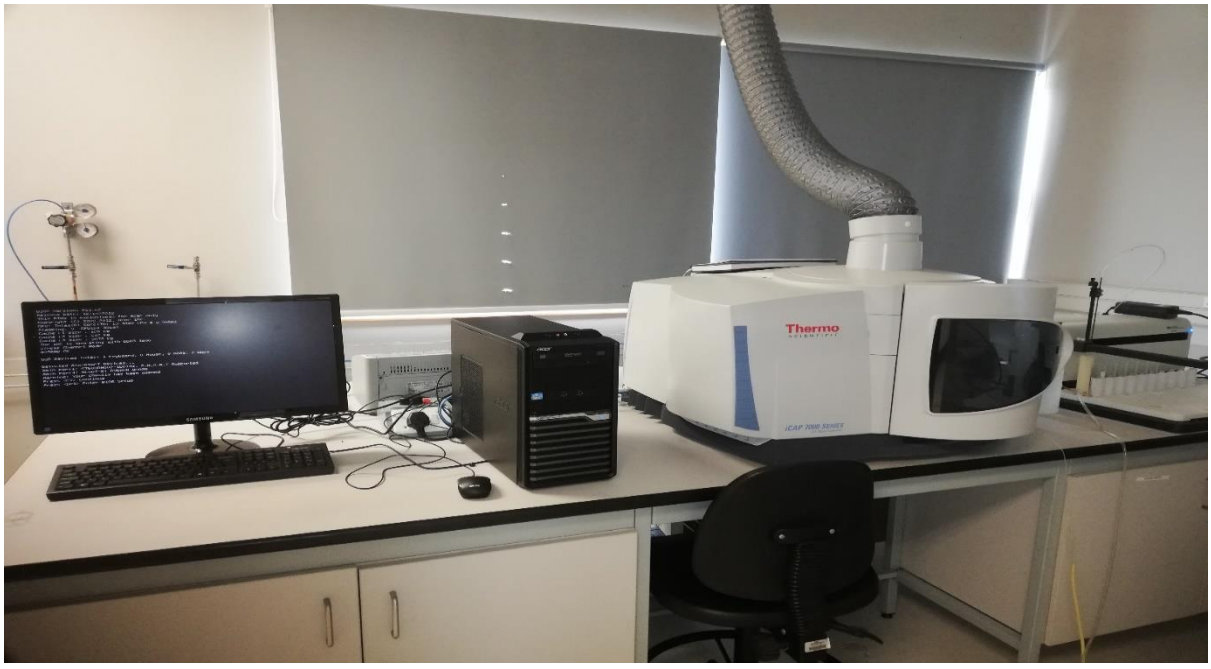



**Figure 11.** A picture of the BIUST ICP-OES instrument used for heavy metals analysis

## 3.6. ESSENTIAL OIL EXTRACTION AND CHARACTERIZATION

The dried CZ grass roots samples from each 6 treatments were crushed with a blender and weighed to around 50 g for each treatment (in duplicates). The samples respectively were then dissolved with 2 L water in a distillation flask and subjected to ultrasonication as in the setup shown in figure 12 at the following optimum parameters: Duration; 25 minutes, Temperature; 70 ºC, High Power (005) and High Frequency. This was followed by Hydro distillation for 5 hours as it appears in figure 13 [8]. After collection of the distillate, the distillate was introduced to liquid-liquid extraction method: water was decanted by separation of water from essential oil using 5 mL pentane / dichloromethane (manufactured by Alpha Chemika and Rochelle Chemicals respectively) thrice consecutively. Then half a spatula sodium sulphate was put in essential oil-pentane/dichloromethane mixture to dry the possible remaining water. To finally get the essential oil, pentane/dichloromethane was evaporated at its boiling point of 40 ºC. The remaining essential oil was weighed for yield recording and stored in a refrigerator for gas chromatography - flame ionization detector (GC-FID) analysis.

All the 6 samples (with duplicates) of extracted essential oils from different treatments were introduced to GC-FID (displayed in figure 14) analysis after being diluted to small amount of hexane with the following optimum parameters: Method running time; 46 minutes, Oven temperature setpoint; 70 ºC, Hold time; 0 minutes, Program rate; 5 ºC/minute, Program value; 300 ºC. Program Hold time; 0 minutes, Injection volume; 0.2 µL, Mode; split, Heater 250 ºC, Split Ratio; 25:1 and Column; HP-5MS.

For identification of compounds, the retention times of the peaks eluted in each sample as well as the peaks of the standard (normal alkanes) were used to calculate the retention indices which was compared with literature to confirm the results.

**Isothermal kovats retention indices formular:**

$I_x = 100*[n+ ((LOG (t_x)-LOG (t_n))/ (LOG (t_{n+1})-LOG (t_n)))]$

$I_x = 100[n +( \log (t_x) – \log (t_n)] / [\log (t_{n+1}) – \log (t_n)]$

Here $t_n$ and $t_{n+1}$ are retention times of the reference n-alkane hydrocarbons standard eluting immediately before and after chemical compound “X”

$t_x$ is the retention time of compound “X”.

n is the number of carbons in the hydrocarbon standard that elute before the compound of interest

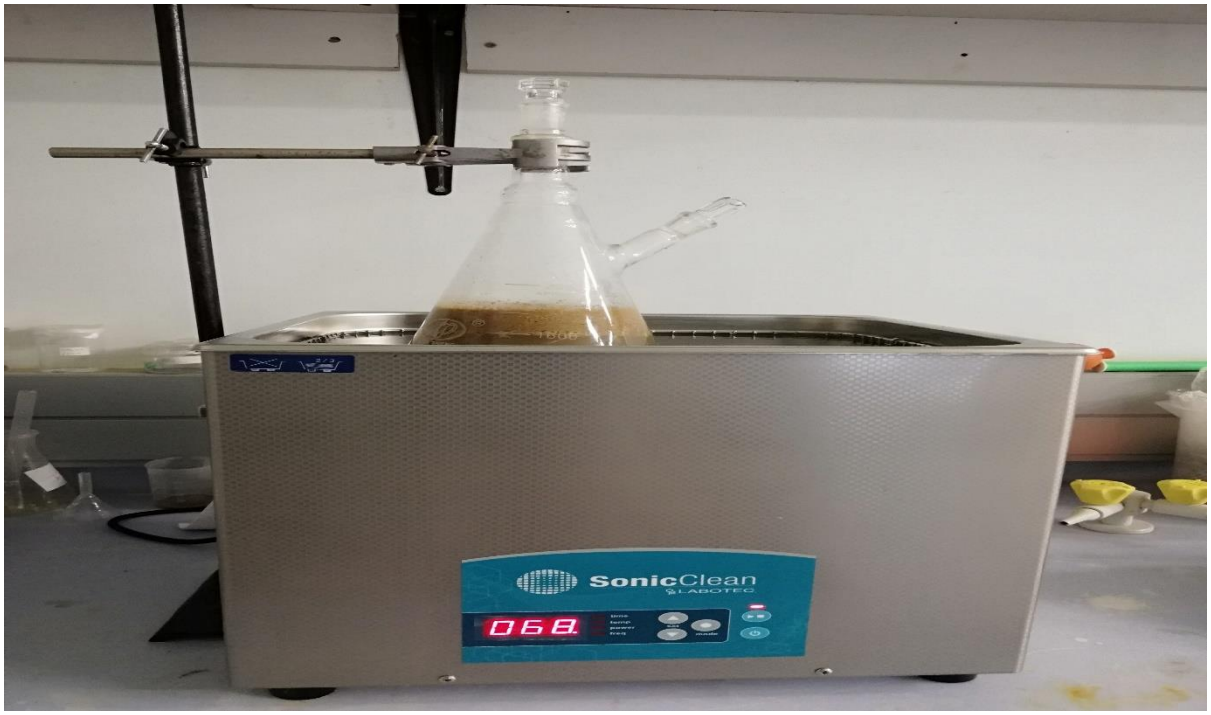



**Figure 12.** The ultrasonication setup for extraction of essential oil

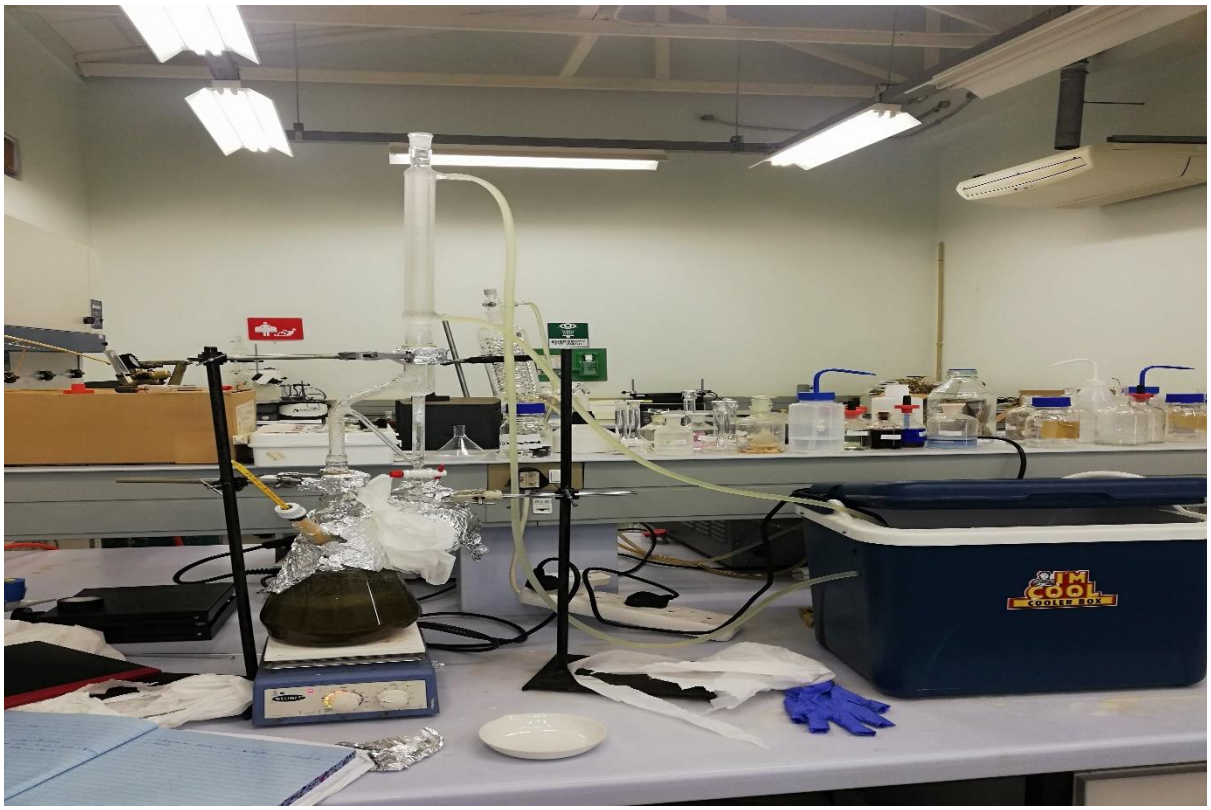



**Figure 13.** The hydro-distillation setup for extraction of essential oil

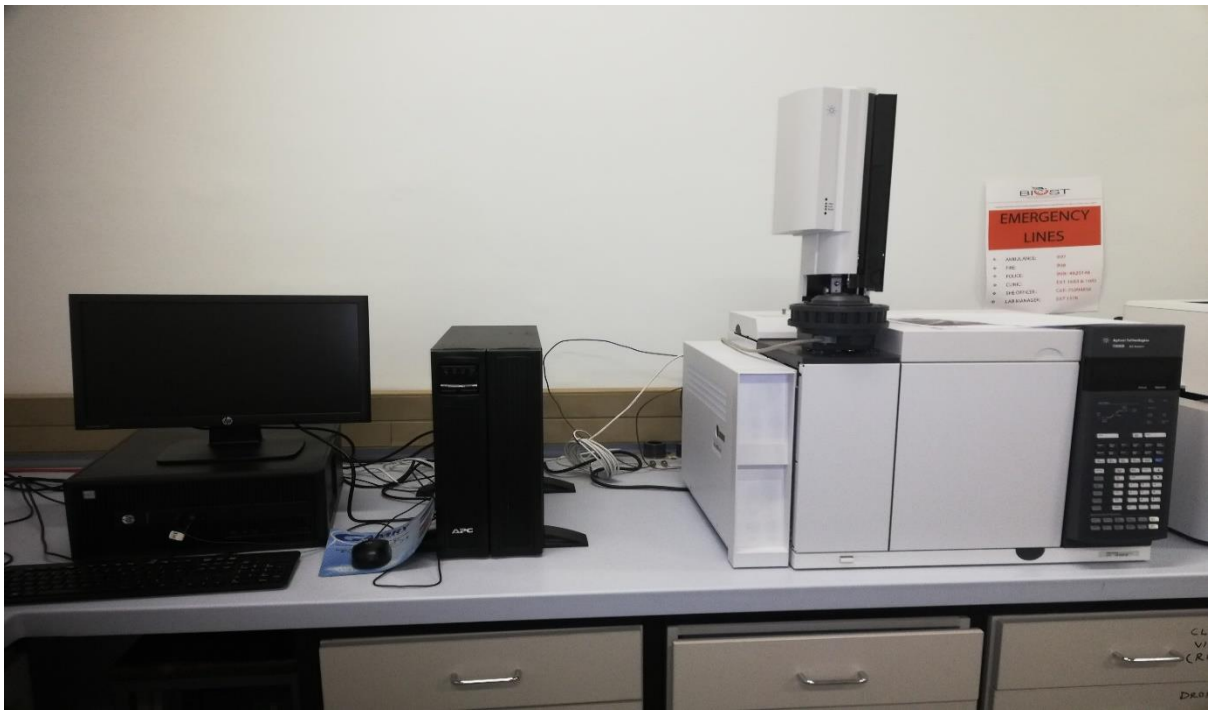



**Figure 14.** A picture of the BIUST GC-FID instrument used for essential oil analysis

### 3.7. CHEMICAL ANALYSIS OF EXTRACTED ESSENTIAL OIL

The extracted essential oils from 6 different treatments were combined in their respective categories (replicate 1 and replicate 2) so as to have better volume that can be introduced to wet digestion/Microwave Assisted Acid Digestion. The method was as follows; the sample was dissolved in concentrated $HNO_3$ and 30% $H_2O_2$ (4:1) (produced by Rochelle Chemicals and Sigma-Aldrich respectively) and predigested overnight (16 hours) in a fume hood at room temperature. The solution was put in a microwave digestor displayed in figure 10 for digestion according to the following optimized program: Power; 290-1800, Ramp time; 20 minutes, Hold time;10 minutes, Cooling; 15 minutes. After cooling, the entire digest is transferred into 50 mL volumetric flasks and diluted to 50 mL mark with distilled water. Reagent blank is prepared similarly to the sample [52]. The sample was filtered so as it is clear for ICP-OES analysis. All the 6 samples (with duplicates) were analyzed with ICP-OES shown in figure 11 using the following optimized parameters: RF power; 1150 W, analysis pump rate; 50 rpm, auxiliary gas flow; 0.5L $min^{-1}$, nebulizer gas flow; 0.70 L $min^{-1}$, coolant gas flow; 12 L $min^{-1}$ and purge gas flow; normal.

### 3.8. STATISTICAL ANALYSIS

The data is expressed as mean ± standard deviation of three or two replicates. The data were analyzed using a 2-factor ANOVA in complete randomized design (CRD) using IBM SPSSv25 (IBM, 2017) statistics software. When significant differences were observed, Tukey's test was performed for treatment mean comparison at 5% level of significance.

# CHAPTER 4. RESULTS AND DISCUSSION

## 4.1. PHYTOREMEDIATION CAPABILITY AND ENHANCED PHYTOREMEDIATION OF *CHRYSOPOGON ZIZANIOIDES* CULTIVATED IN SELIBE-PHIKWE (BOTSWANA) MINE

### 4.1.1. CHEMICAL PROPERTIES OF GROWING MEDIA

The chemical properties of mine tailings plus cow manure (compost) mixture before the start of the experiment is presented in Table 10. The tailings are highly acidic, sandy loam with very low organic matter and nutrient content and has high amounts of various heavy metals. The cow manure used in the experiment was obtained from local farm that contain 3.8% N, 34% organic carbon with pH of 7.9. The compost added to mine tailings significantly reduced EC of As, Cu, Mn, Ni, Pb and Zn compared to pure cow manure. In contrast, pH had a significant increase and the organic matter content as well as total N significantly increased to 3.21% and 0.28% respectively. The water-soluble Cu had also decreased significantly compared to pure cow manure while water-soluble As, Mn, Ni, Pb and Zn had increased significantly.

**Table 10.** Physical and chemical properties and heavy metal contents (mean ± SD) of BCL mine tailings, cow manure and mixture of BCL mine tailings and cow manure

| Properties | Mine Tailings (n=3) | Cow Manure (n=3) | Mine Tailings +Cow Manure (n=3) | P-value |
|---|---|---|---|---|
| pH (1:2, soil:water) | 3.72±0.22c | 7.9±0.12a | 6.24±0.10b | 0.000*** |
| Electrical Conductivity ($\mu Scm^{-1}$ 1:2, soil:water) | 2482±160a | 982±102c | 1643.24±88b | 0.000*** |
| % Organic Matter (Walkley-Black acid digestion) | 0.000±0.00c | 34.00±1.26a | 3.21±0.34b | 0.000*** |
| % Total N (Kjeldahl method) | 0.000±0.00c | 3.80±0.18a | 0.28±0.06b | 0.000*** |
| Particle Size Distribution | | | | |
| %Sand | 65.92 | | | |
| %Silt | 31.29 | | | |
| %Clay | 2.79 | | | |
| Total Heavy metals ($mgkg^{-1}$) | Mine tailings | Cow Manure | Mine Tailings +Cow Manure | |
| As | 47.31±1.40a | 5.48±1.08b | 42.84±2.26a | 0.000*** |
| Cu | 1632.24±38.20a | 62.23±4.36c | 1489.23±28.30b | 0.000*** |
| Mn | 402.68±18.21a | 112.32±6.24c | 367.25±10.48b | 0.000*** |
| Ni | 728.26±46.20a | 72.24±12.64b | 648.24±60.28a | 0.000*** |

| | | | | |
|---|---|---|---|---|
| Pb | 41.26±5.38a | 28.64±2.86b | 40.26±4.26a | 0.048* |
| Zn | 103.97±5.34a | 95.20±8.20a | 102.58±4.60a | Ns |
| Water Soluble Heavy Metals (mg $kg^{-1}$) | Mine Tailings | Cow Manure | Mine Tailings +Cow Manure | |
| As | 1.86±0.12b | 0.68±0.26c | 2.48±0.14a | 0.019* |
| Cu | 81.06±4.24a | 3.28±0.32c | 62.41±3.28b | 0.005** |
| Mn | 8.74±0.38b | 12.43±0.86a | 9.48±0.42b | 0.037* |
| Ni | 42.64±5.60a | 9.18±2.30b | 48.27±6.12a | 0.013* |
| Pb | 6.47±1.20a | 3.24±0.82b | 7.32±1.40a | 0.028* |
| Zn | 4.32±0.84b | 14.28±1.28a | 6.48±1.63b | 0.019* |

Means within the same column followed by the same letter(s) are not significantly different at 5% level based on Tukey's test. ns: non-significant ($p > 0.05$); *significant; **highly significant; ***very highly significant, SD: standard deviation per treatment

### 4.1.2. GROWTH AND DRY MATTER YIELD OF *CHRYSOPOGON ZIZANIOIDES*

*Chrysopogon Zizanioides (CZ)* was able to grow and survive in Selibe-Phikwe Cu and Ni mine tailings (with varying six soil amendments-influence of Arbuscular Mycorrhizal Fungi (AMF) and Ethylene Diamine Tetra Acetic Acid (EDTA)) in the presence of cow manure, withstanding and tolerating the heavy metals that accumulated in the roots of the grass. Literature confirmed that usually in CZ, most of the accumulated heavy metals are concentrated in the roots and the accumulation of heavy metals by CZ roots is greatly enhanced by the chelating agent (EDTA) [17]. The survival rate, growth and biomass of CZ cultivated in mine tailings/soil contaminated with complex combinations of heavy metal is greatly enhanced by organic matters (cow manure) [17]. This proved that indeed CZ is not only tolerant to individual heavy metals but also it can endure the combination of various heavy metals present in the soil as it also produced fair biomass [6]. As presented in Table 11, both the total plant yield as well as the roots yield are affected by introduction of AMF (AMF inoculated samples (T4, T5, T6) > non-inoculated (T1, T2, T3)). This proves that indeed the overall increase in biomass across the treatments can be due to the introduction of AMF, normally the heavy metal contaminated soils are very hostile and low in nutrient contents so fungi improve and optimizes the nutrient uptake by significantly enlarging the effective surface of the plant in contact with the soil and consequently promoting the plant growth [18]. In addition, the mycorrhizal percentage naturally present in CZ significantly decreased as EDTA is introduced regardless of application by splitting or by single application but it significantly increased with AMF inoculation. Even so, comparing AMF + EDTA treatments (T5 and T6) with solo AMF treatment (T4), it shows that EDTA somehow lowers the percentage of mycorrhiza. This is so because EDTA has antifungal activity [53].

**Table 11.** Mycorrhizal infection and dry matter yield (mean ± SD) of CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

| Treatments | | Mycorrhizal Roots (%) n=3 | Shoots (g/plant) n=3 | Roots (g/plant) n=3 | Total (g/plant) n=3 | Shoot/Root Ratio n=3 |
|---|---|---|---|---|---|---|
| T1 | 0 EDTA 0 AMF | 6.20±1.40d | 113.34±3.20c | 79.24±5.84b | 193.75±5.20c | 1.41±0.12a |
| T2 | +EDTA split | 2.10±1.32e | 118.79±6.28c | 72.84±6.90b | 195.01±7.42c | 1.59±0.08a |
| T3 | +EDTA single | 0.00±0.00e | 120.48±4.76c | 72.05±3.74b | 201.64±8.36c | 1.55±0.10a |
| T4 | +AMF | 68.43±6.28a | 164.86±8.24a | 120.37±6.48a | 285.23±6.20a | 1.38±0.16a |
| T5 | +AMF+EDTA split | 46.40±3.72b | 125.29±4.38c | 89.36±8.32b | 214.65±8.20c | 1.39±0.06a |
| T6 | +AMF+EDTA single | 32.70±4.82c | 141.56±5.62b | 122.32±4.50a | 263.88±6.72b | 1.16±0.04b |
| P-value | | $0.000^{***}$ | $0.000^{***}$ | $0.000^{***}$ | $0.000^{***}$ | $0.000^{***}$ |

Means within the same column followed by the same letter(s) are not significantly different at 5% level based on Tukey's test. ns: non-significant ($p > 0.05$); *significant; ***very highly significant, SD: standard deviation per treatment

### 4.1.3. HEAVY METAL CONCENTRATION ABSORBED IN THE ROOTS OF *CHRYSOPOGON ZIZANIOIDES*

CZ, like other remediation operations, implements battling measures to mitigate the severity of elevated metal concentrations and activate physiological tolerance mechanisms that limit hazardous metal build up. In order to mitigate the negative consequences of heavy metal-induced oxidative stress, CZ is normally equipped with an enzymatic and non-enzymatic antioxidant defense mechanism. Heavy metal detoxification through absorption of these elements into plant tissues in their reduced form is the first method of tolerance technology. This is followed by an increase in antioxidant enzyme activity and heavy metal sequestration via glutathione and phyto-chelatins. All of these techniques work together to minimize the amount of oxidative stress in plants in order to protect them from the detrimental effects of reactive oxygen species (ROS) [6]. Furthermore, the soil naturally reduces heavy metal solubility and mobility through sorption, precipitation, and complexation processes. The speed up of this natural process is influenced by organic amendments by increasing cation exchange capacity which increases soils' ability to bind with heavy metals, making them less transportable and microbial immobilization. Despite the fact that organic amendments reduce heavy metal solubility, they increase plant life and production by serving as slow-release fertilizers, which adds to the long-term effectiveness of the phytoremediation method. In summary, this study demonstrated that organic amendments improve plant life, nutrient

availability, and soil characteristics in polluted soils while decreasing metal bioavailability [18]. This is supported by this study since all the treatments with cow manure survived until harvest time. The treatments deprived of organic matter-cow manure did not survive, this is due to an increase in metal bioavailability eventually increasing toxicity to the plant. Consequently, hindering the plant survival and the potential of the plant to absorb and sequester metals, thus ruining the phytoremediation strategy as a heavy metal pollution control method [18].

**Table 12.** Heavy metal concentration (mean ± SD) in the roots of CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

| Treatments | | Ni($mgkg^{-1}$) n=3 | Cu($mgkg^{-1}$) n=3 | Mn($mgkg^{-1}$) n=3 | Zn($mgkg^{-1}$) n=3 | Pb($mgkg^{-1}$) n=3 | As($mgkg^{-1}$) n=3 |
|---|---|---|---|---|---|---|---|
| T1 | 0 EDTA 0 AMF | 525.88±0.92c | 178.26±5.54a | 98.67±2.55b, c | 22.41±0.08c | 2.13±0.22b, c | 0.55±0.43a |
| T2 | +EDTA split | 495.98±5.58b | 193.26±5.95a | 103.29±3.52c | 20.08±0.19b | 1.47±0.31a, b | 0.17±0.07a |
| T3 | +EDTA single | 749.78±10.84e | 411.26±11.07b | 147.39±4.15d | 26.84±0.35d | 2.22±0.08c | 0.60±0.54a |
| T4 | +AMF | 1093.98±11.14f | 372.26±0.40c | 155.39±1.90e | 29.38±0.32e | 2.09±0.11b, c | 0.34±0.29a |
| T5 | +AMF+EDTA split | 605.68±4.20d | 213.76±4.29d | 93.61±2.30b | 20.28±0.12b | 1.25±0.20a | 0.54±0.42a |
| T6 | +AMF+EDTA single | 434.88±3.65a | 192.16±0.70a | 84.26±0.96a | 17.53±0.17a | 1.51±0.47a, b, c | 0.40±0.33a |
| P-value | | $0.000^{***}$ | $0.000^{***}$ | $0.000^{***}$ | $0.000^{***}$ | $0.002^{**}$ | ns |

Means within the same column followed by the same letter(s) are not significantly different at 5% level based on Tukey's test. ns: non-significant ($p > 0.05$); *significant; ***very highly significant, SD: standard deviation per treatment

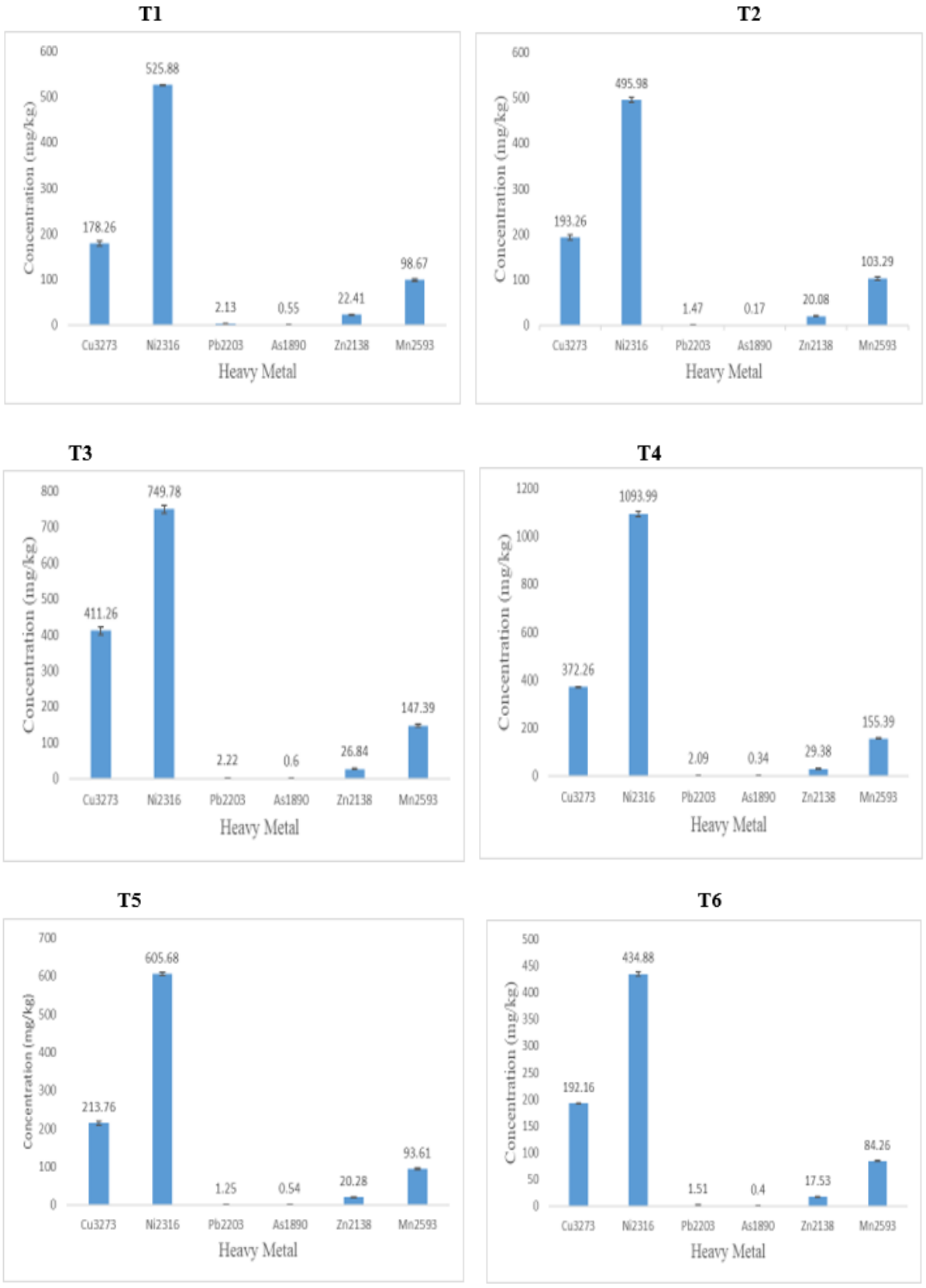
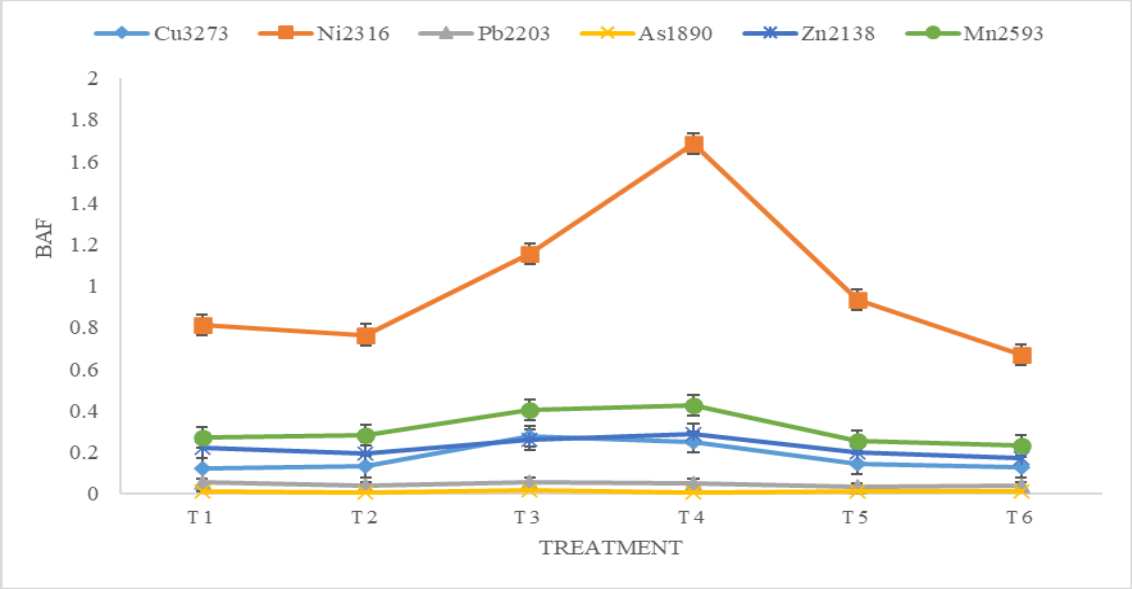



**Figure 15.** Heavy metal concentration (mean ± SD) in the roots of CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA




Bioaccumulation Factor = (Heavy metal in Plant roots/Heavy metal in Soil)

**Figure 16.** Bioaccumulation factors (mean ± SD) of detected heavy metals of CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

### 4.1.3.1. Ni concentrations on different treatments

Figure 15 and Table 12 shows the detected heavy metals with varying concentration across all the treatments, it has been observed that; T4, T3, T5, T1, T2 and T6 had the concentrations of Nickel (Ni) in descending order as 1093.99 mg $kg^{-1}$, 749.78 mg $kg^{-1}$, 605.68 mg $kg^{-1}$, 525.88 mg $kg^{-1}$, 495.98 mg $kg^{-1}$ and 434.88 mg $kg^{-1}$ respectively. Based on two factor ANOVA in CRD, the concentrations across all the treatments are very highly significant ($P = 0.000$, $P < 0.05$). The bioaccumulation factor (BAF) can moderately indicate plants' ability to remove heavy metals from soils. BAF assesses the potential of plants to acquire heavy metals in their various components in relation to metal concentrations in the soil [6]. The BAF data in Figure 16, reveal that for the six different treatments, Ni shows better BAFs as compared to other heavy metals in comparison with it. The BAF range is between 0.67-1.68, with T3 and T4 showing more 1 while the rest of the treatments showing lower but closer to 1. More of Ni was greatly absorbed by CZ across all the other treatments as compared to other detected heavy metals. Even so, CZ showed high Ni content in T3 (with CM and 5mmol EDTA single) and T4 (with CM and AMF) and low Ni content on T6 (with CM, AMF and 5mmol EDTA single).

The overall high Ni content in CZ roots can be influenced by the fact that Ni is among other heavy metals that remain attached to the cell wall or are stored in the root's vacuole due to their ability to reduce water transfer to the aerial part, therefore small portion of Ni is transferred to the shoots. In addition, it can be due to the sequestering of Ni in roots vacuole and the cells, this mechanism makes plants to tolerate heavy metal toxicity since organs engaged in the plant metabolism are fully protected from the heavy metal damage [54]. This makes CZ the best hyperaccumulator of Ni and that is evident since BAF values were found to be more than one or close to 1, such results entails that the plant is a hyperaccumulator of that particular heavy metal [55]. The independent AMF inoculation and EDTA (single) application treatments show

high Ni content in comparison with other treatment, whereas combination of the two amendments (AMF inoculation + EDTA-single application) show lower Ni content among other treatments. This show that even though Ni can be absorbed with ease, stabilization and accumulation of Ni can be improved by either AMF or EDTA as they both commonly improve Ni bioavailability. Recent studies have also highlighted that, Ni stress or excess of Ni level considerably reduces and inhibit the uptake and absorption of many nutrients like Cu, Zn and Mn in many plant species since Ni competes with other nutrients for uptake and translocation, thus affecting the concentration of many other nutrients up to deficiency levels [56].

#### 4.1.3.2. Cu concentrations on different treatments

T3, T4, T5, T2, T6 and T1 had the concentrations of copper (Cu) in descending order as 411.26 mgkg$^{-1}$, 372.26 mg kg$^{-1}$, 213.76 mg kg$^{-1}$, 193.26 mg kg$^{-1}$, 192.16 mg kg$^{-1}$ and 178.26 mg kg$^{-1}$ respectively. The analysis of variance found that $P = 0.000$, $P < 0.05$ indicating that the Cu concentrations are very highly significant or the Cu concentrations are significantly different. Even though Cu BAFs in all the treatments are below 1, T3 (with CM and 5mmol EDTA single) and T4 (with CM and AMF) treatments absorbed more of Cu whereas T1 (with CM only) shows the least concentration of Cu. Cu is the second heavy metal to be highly accumulated by CZ in comparison with other heavy metals detected in this study. This relates well with other researches that also found that CZ tolerates high level of Cu, noting that CZ is a hyperaccumulator of Cu [17]. With reference to the research carried out at Chile, La Africana mine, CZ can grow well in mine tailing containing the total Cu level of around 3921 mg kg$^{-1}$ [17]. From the results, the AMF inoculated as well as EDTA single treatments had the highest level of Cu whereas the non-chemically enhanced treatment had the lowest Cu content. The AMF inoculation in to CZ notably enhanced the biomass as well as nutrient uptake hence giving CZ an advantage to improve uptake of Cu. EDTA also effectively increased the contents of Cu, similar reports have been found as the contents of Cu increased in roots 2-3 times compared to the non EDTA modified soil treatments [17].

#### 4.1.3.3. Mn concentrations on different treatments

T4, T3, T2, T1, T5 and T6 had the concentrations of Manganese (Mn) in descending order as 155.39 mg kg$^{-1}$, 147.39 mg kg$^{-1}$, 103.29 mg kg$^{-1}$, 98.67 mg kg$^{-1}$,93.61 mg kg$^{-1}$ and 84.26 mg kg$^{-1}$ respectively. The concentrations are very highly significant ($P = 0.000$, $P < 0.05$) which means these concentrations are significantly different. Even so, the Mn BAFs all the treatments are below 1, however T3(with CM and 5mmol EDTA single) and T4 (with CM and AMF) are the treatments with the highest of level Mn. T6 (with CM, AMF and 5mmol EDTA single) have the lowest Mn concentration. Similarly, as Ni, Mn is usually stored in CZ root vacuole and it is translocated in a smaller portion due to its ability to reduce water transfer to the aerial part [54]. In addition, the results show that the utilisation of AMF and EDTA separately elevated the Mn level in CZ roots as compared to when they are used together. This entails that the AMF-EDTA interaction lowers the absorption of Mn or does not have effect on Mn accumulation by CZ roots.

#### 4.1.3.4. Zn concentrations on different treatments

The Zinc (Zn) concentrations for T4, T3, T1, T5, T2 and T6 in descending order are 29.38 mg $kg^{-1}$, 26.84 mg $kg^{-1}$,22.41 mg $kg^{-1}$, 20.28 mg $kg^{-1}$, 20.08 mg $kg^{-1}$ and 17.53mg $kg^{-1}$ respectively. Statistical analysis shows that the Zn concentrations across the treatments are very highly significant (P= 0.000, P< 0.05), the concentrations are significantly different. The Zn BAFs in all the treatments are below 1. The T3 (with CM and 5mmol EDTA single) and T4 (with CM and AMF) are the treatments with the highest level of Zn. T6 (with CM, AMF and 5mmol EDTA single) have the lowest concentration of Zn. This entails that vetiver grass managed to uptake more of Zn with the application of 5mmol EDTA only (single; 5mmol * 1) and inoculation of AMF only in the presence of cow manure in both cases. The soil amendments managed to work optimally when they are applied separately and that shows that AMF and EDTA can produce almost same results independent of each other.

CZ is regarded as Zn hyperaccumulator, in the similar experiment carried out it is shown that CZ can accumulate Zn concentration as high as 10000 mg $kg^{-1}$ DW (over 1%) in the roots [17]. In this case, the level of Zn absorbed by CZ across all the treatments was not good enough since low concentration (6.48 mg $kg^{-1}$) of soluble Zn was used, this is confirmed by literature that in general, studies employing high levels of soluble Zn produces higher plant uptakes than studies using non-readily available fractions of Zn. Normally, most of Zn accumulated in CZ is retained in the roots. The application EDTA chelating agent can be utilised to enhance Zn bioavailability, however in this case it didn't make a significant difference. Similar results were found, where the use of EDTA had no effect on Zn accumulation of CZ as compared to control [17].

#### 4.1.3.5. Pb concentrations on different treatments

The lead (Pb) concentrations for T3, T1, T4, T6, T2 and T5 in descending order are 2.22 mg $kg^{-1}$, 2.13 mg $kg^{-1}$, 2.09 mg $kg^{-1}$, 1.51 mg $kg^{-1}$, 1.47 mg $kg^{-1}$ and 1.25 mg $kg^{-1}$ respectively. Turkey's test has shown that this Pb concentrations are highly significant, they are statistically different (P = 0.002, P < 0.05). Even though Pb BAFs are way below 1 and the Pb concentrations are very low, T3 (with CM and 5 mmol EDTA single), T1 (with CM only) and T4 (with CM and AMF) were observed to be having a bit higher level of Pb (>2 $mgkg^{-1}$) and T5 (with CM, AMF and 5 mmol EDTA split) had the lowest Pb level. Generally, CZ can accumulate high level of Pb and very tolerant to Pb as seen in literature that it can accumulate up to 10750 $mgkg^{-1}$ [17]. The reason that may have led Pb level to be lower in CZ roots in all the treatments case may be due to addition of organic matter (cow manure) into Pb contaminated soils as it has the ability to decrease the total and extractable lead in soil. This is consistent with several studies that demonstrated that growing CZ in soil treated with organic matter, such as pig dung, sewage sludge, manure compost, and household garbage, reduced Pb absorption [17]. The conclusion reached is that organic matter has shown to be very efficacious in reducing Pb bioavailability, hence lowering Pb uptake of plants [17]. CZ in Pb contaminated

area showed better Pb accumulation when there is introduction of CM only, CM with 5 mmol EDTA single and CM with AMF but the combination of CM, AMF and EDTA split made CZ to accumulate less of Pb. In support of the findings, literature mentioned that mycorrhizal colonization increases Pb uptake by plants cultivated in soils with low Pb concentrations (at 0 and 10 mg Pb $kg^{-1}$) whereas at higher heavy metal concentrations (100 and 1000 mg Pb $kg^{-1}$) it decreases Pb uptake. In addition, the accumulation of Pb by CZ has been proved to be enhanced by the addition of chelating agent (EDTA) since for Pb mobilization, EDTA has been shown to be the most effective chelating agent. This in turn results in an increase of Pb concentration in the roots [17].

Even so, the groundwater may be polluted by the leaching of EDTA and EDTA–heavy metal complexes causing further environmental pollution in surrounding areas. Nonetheless, this metal leaching can be prevented since CZ has a long (3-4 m), huge and complex root system which can easily penetrate to the deeper layers of the soil. Furthermore, CZ can retain Pb in the plant, thus minimizing the possibility of spreading the contaminant [17].

CZ is considered Pb hyper accumulator and most of the absorbed Pb in CZ tends to accumulate in the roots and normally a bit of Pb is translocated to the shoots. When Pb reaches the plant roots, it is immediately exposed to a fluid with high phosphate concentrations, a relatively high pH, and high carbonate-bicarbonate concentrations. Pb precipitates out of solution in phosphates or carbonates conformation at these circumstances, as observed in electron micrographs of hydroponically grown plant roots in Pb solutions. Consequently, the formation of insoluble Pb compounds lowers Pb translocation in plants hence CZ is a useful plant for Phyto stabilization [17].

#### 4.1.3.6. As concentrations on different treatments

The concentrations of As in descending order are 0.60 mg $kg^{-1}$, 0.55 mg $kg^{-1}$, 0.54 mg $kg^{-1}$, 0.40 mg $kg^{-1}$, 0.34 mg $kg^{-1}$ and 0.17 mg $kg^{-1}$ for T3, T1, T5, T6, T4, T2 respectively. However, all of this results statistically showed no significant difference ($P > 0.05$). The BAFs across all the treatments are way below 1. In comparison of heavy metals that can be successfully absorbed by CZ, CZ is not an As hyper accumulator but it can successfully absorb other heavy metals from contaminated sites and can be safely disposed of, thus gradually minimising the contaminant levels [17]. The contributing factors for low As concentration in all treatments can be that, the As accumulation rate decreases with plant growth as well as low mobility [17]. The As uptake of CZ can be remarkably boosted by using organic amendments and EDTA to enhance mobility rate. In this research better concentration was observed when cow manure was coupled with single 5 mmol application of EDTA (0.60 mg $kg^{-1}$), treatment with only cow manure (0.55 mg $kg^{-1}$) as well as treatment with cow manure coupled with splitted 5 mmol EDTA and AMF inoculation (0.54). These results are somehow in agreement with the reports of the present study since in the research in which AMF was added to the growing medium, after six months the As concentration that accumulated in CZ roots was 185.4 mg $kg^{-1}$ DW and in overall, CZ extracted up to 62.1% As from the contaminated soils. This brings the conclusion that the elevated As concentration accumulated in CZ was due to the introduction

of organic substances to nurture the maturing of microbial population in soils that, which in turn, may play an major role in As hyper accumulation [17].

The heavy metal analysis results of vetiver grass roots in Figure 15 and Table 12 indicate that across all the six treatments in terms of the concentration's comparison: Ni>Cu>Mn>Zn>Pb>As. Even though Cu, Mn, Zn, Pb and As has been found across all the treatments, there have been an observation that for these metals the BAFs is way below 1 as compared to Ni, the other factors that can also contribute to such results is that most of these heavy metals were either left in the mine tailing (mobility rate; lower mobility rate) or most probably there is a chance that these heavy metals translocated from the CZ roots to other parts of such as the aerial part (leaves) and most probably synergistic or antagonistic interactions may have occurred due to combination of different heavy metals.

### 4.1.4. Comparison of heavy metals level found in CZ roots and essential oil with WHO/FDA permissible plants limits

Because there are no rules in Botswana governing the permitted quantity of heavy metals in medicinal and aromatic plant extracts, the maximum allowable and safe concentration of each metal is desperately needed. In comparison to the most recent WHO allowed limits in medicinal plants, the concentrations of all heavy metals accumulated by CZ roots surpassed the regulatory limits, with the exception of Zn, Pb, and As, but heavy metal levels in essential oil are lower, as indicated in Table 13.

**Table 13.** WHO/FDA permissible/regulatory limits for heavy metals in medicinal plants with the comparison to the obtained results [57]

| Heavy metals | Permissible limit of plants ($mgkg^{-1}$) | Concentrations of heavy metals found in CZ raw roots ($mg\ kg^{-1}$) | Concentrations of heavy metals found in CZ roots essential oil ($mg\ kg^{-1}$) |
|---|---|---|---|
| Ni | 1.63 | 434.88-1093.98 | 0.063 |
| Cu | 20.0 | 178.26-411.26 | 0.150 |
| Mn | - | 84.26-155.39 | 0.043 |
| Zn | 50.0 | 17.53-29.38 | 0.109 |
| Pb | 10.0 | 1.25-2.22 | 0.022 |
| As | 10.0 | 0.17-0.60 | 0.003 |

WHO; World Health organisation, FDA; Food and Drug administration (USA)

## 4.2. *CHRYSOPOGON ZIZANIOIDES* AS THE ESSENTIAL OIL PRODUCING PLANT CULTIVATED IN SELIBE-PHIKWE (BOTSWANA) MINE TAILINGS WITH SOIL AMENDMENTS

### 4.2.1. EXTRACTION TECHNIQUE EMPLOYED TO EXTRACT *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

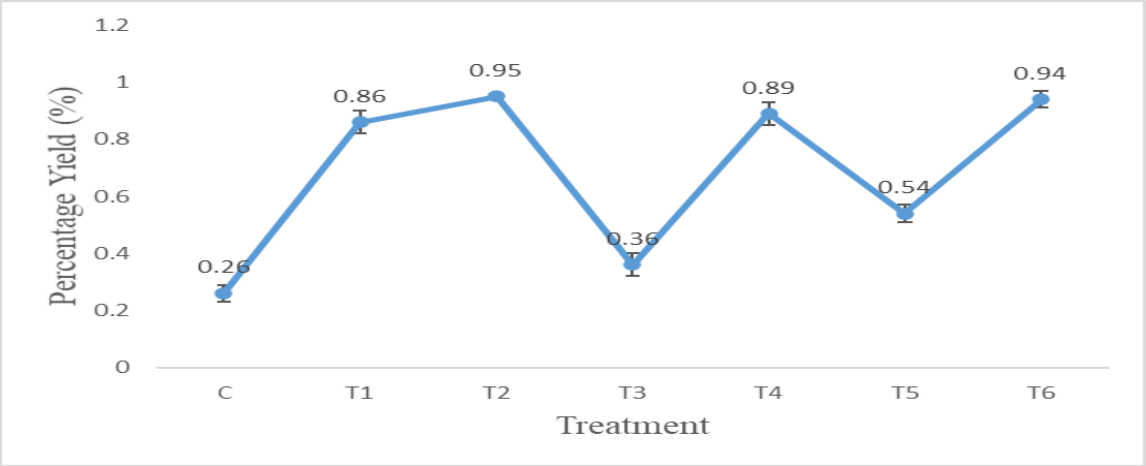
CZ roots essential oils are not easily extractable since they are stationed inside hard to reach root tissues and the presence of high percentage of high molecular weights with low vapor pressure constituents (i.e., sesquiterpenes) [13]. To be successfully extracted, the essential oil has to be extracted by relatively fast process to improve diffusion of the oil from inside fibrous root tissues to outward and to the surface. Ultrasonic-assisted hydro distillation (US-HD) was employed in this study across all the sample treatments at optimised parameters so as to crush the cell wall (cause cell lysis) or increase the diffusion rate thus allowing the oil to be exposed out of the oil glands / sacs fast in a short extraction time. The ultra-sonication (US) concept states that during sonication, ultrasonic waves cause cavitation bubbles, which allow for more penetration of the extraction solvent through the plant cell wall than conventional procedures, resulting in efficient release of the plant's intracellular product [58].

When compared to traditional extraction methods, the ultra-sonication (US) methodology in the extraction of CZ roots essential oil overcomes the disadvantages of degradation and loss of thermolabile components, as well as reduced active compound damage (hydro distillation). It proved to be efficient in this study, since more constituents including most valuable constituents (those with high boiling points-late eluters, i.e; vetivone) in some treatments were reported at shorter extraction time;5 hours of extraction as compared to literature which need 24 hours in hydro distillation (HD) to achieve similar results [5]. US also provides better yield, the percentage yield across the all the treatment ranged between 0.26% to 0.94%, which is a good yield that is comparable to other extraction methods. Other reports on current study found that steam distillation usually produces around 0.3 - 1.0 wt% on dry root basis of oil [16] [59]. The percentage yield of CZ roots essential oil extracted by ultrasonic-assisted hydro distillation extraction process obtained between 0.26% to 0.95% as shown in Figure 17 and Table 14. The essential oils were concentrated with sesquiterpenes alcohol, similar results were obtained by related research [4]. Two factor ANOVA in CRD showed that statistically, the percentage yields across all the treatments are very highly significant ($P = 0.000$, $P < 0.05$). Even so, in Indonesia (the world's largest producer of CZO), yearly on 5 t/ha of dried root, an average oil content of 1.5% is achieved, the range being 0.7-2.3% [13]. The lower yield attained in this study can also be as a result of dissimilarities in the maturity of the vetiver roots used. Literature has noted that essential oil production reaches a peak after at least two growing seasons but due to time constraints, the harvest was carried out after a single growing season [7]. In addition, US provides superior consistency (viscosity, homogenization), so the extracted CZ roots essential oil retained its viscosity property since it was denser and the colour was light brown which is classified as a regular quality while clear yellow colour indicate that it is a premium quality [59] [60]. The freshly extracted CZO had an earthy woody and sweet odour which correlates well with related research [13].

**Table 14.** Average yields (mean ± SD) of extracted CZ roots essential oils (via 5 hours ultra-sonic assisted hydro distillation) grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

| Treatments | | CZO yield (%) n=2 |
|---|---|---|
| C | Control (0 HM) | 0.26 ± 0.03a |
| T1 | 0 EDTA 0 AMF | 0.86 ± 0.04c |
| T2 | +EDTA split | 0.95± 0.01c |
| T3 | +EDTA single | 0.36 ± 0.04a |
| T4 | +AMF | 0.89 ± 0.04c |
| T5 | +AMF+EDTA split | 0.54 ± 0.03b |
| T6 | +AMF+EDTA single | 0.94 ± 0.03c |
| P-value | | 0.000*** |

Means within the same column followed by the same letter(s) are not significantly different at 5% level based on Tukey's test. ns: non-significant (p > 0.05); *significant; ***very highly significant, SD: standard deviation per treatment




%Yield= (Mass of Essential Oil/Mass of Starting Plant Sample) * 100%

**Figure 17.** Average yields (mean ± SD) of extracted CZ roots essential oils (via 5 hours ultra-sonic assisted hydro distillation) grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

### 4.2.2. CHEMICAL COMPOSITION OF *CHRYSOPOGON ZIZANIOIDES*

CZ essential oil constituents were identified on the basis of their calculated retention indices based on the retention times of the peaks eluted in each sample treatment from GC-FID as well as the peaks of the standard (normal alkanes), and their mass spectra by comparison of literature data. Qualitative and quantitative data are shown in Table 15, in total fifty-nine compounds were identified accounting across all the seven samples but similar researches has reported that there are over 250 compounds [16]. The essential oils are found to be mainly constituted with sesquiterpenes, however few of the constituents were not identified and mostly the longer carbon chain (C19-C29) compounds which are present in trace amounts and some of them have never been observed in the vetiver oil compositions reported in early studies hence unable to be identified. Control, T1, T2, T3, T4, T5, T6 shows the total identified oils as 99.5%, 97.0%, and 100%, 94.6%, 100%, 98.1% and 97.2% respectively. The predominating constituents are alcohols (>50%) across all the treatments. The percentages of other class of constituents (Hydrocarbons, Ketones, Aldehydes, Esters, Ethers, and Carboxylic Acids) significantly varied across the treatment samples. The chromatograms showing the main constituents are shown in Figure 18: The individual primary constituents for Control; Cis-eudesm-6,11-dien-3b-ol (4.56), Aromadendrene <epoxide-allo-> (7.36), Khusinol (10.33), Eudesm-7(11)-en-4α-ol (juniper camphor) (4.11), Ziza-6(13-en-12-ol (khusimol) (9.31), Preziza-7(15)-en-12-ol (18.06), Vetivone <β-> (14.44), Pentadecanoic acid (5.35), For T1; Cis-eudesm-6,11-dien-3b-ol (4.17), Khusinol (7.10), Eudesm-7(11)-en-4α-ol (juniper camphor) (5.85), Ziza-6(13-en-12-ol (khusimol) (8.23), Valencen-12-ol (16.39), Vetivone <β-> (12.14), Eremophila-1(10),7(11)-dien-2-one(isonootkatone.a-vetivone) (4.01), (E)-eremophila-1(10),7(11)-dien-12-yl acetate (9.36), For T2; Cis-eudesm-6,11-dien-3b-ol (6.80) , Khusinol (8.30), 2-epi-ziza-6(13)-en-12-al (7.38), Ziza-6(13-en-12-ol (khusimol) (11.64), Preziza-7(15)-en-12-ol (19.48),Eremophila-1(10),11-dien-2-one (nootkatone) (16.50), Methyl-(E)-eremophila-1(10),7(11)-dien-12-ether (4.69), Ziza-6(13)-en-12-yl acetate (5.15), Pentadecanoic acid (10.43), For T3; Cis-eudesm-6-en-11-ol (4.82), Cis-eudesm-6,11-dien-3b-ol (9.26), Khusinol (10.76), 2-epi-ziza-6(13)-en-12-al (11.64), Ziza-6(13-en-12-ol (khusimol) (7.46), Preziza-7(15)-en-12-ol (21.67), Eremophila-1(10),11-dien-2-one (nootkatone) (4.42), Pentadecanoic acid (9.36), Unidentified (5.37), For T4; Preziza-7(15)-en-12-ol (100.00), For T5; Cis-eudesm-6,11-dien-3b-ol (8.11), Khusinol (10.11), 2-epi-ziza-6(13)-en-12-al (10.93), Ziza-6(13-en-12-ol (khusimol) (7.29), Preziza-7(15)-en-12-ol (21.58), Eremophila-1(10),11-dien-2-one (nootkatone) (4.89), Pentadecanoic acid (9.42), For T6; Cis-eudesm-6,11-dien-3b-ol (4.98), Khusinol (4.92), Eudesm-7(11)-en-4α-ol (juniper camphor) (5.52), Ziza-6(13-en-12-ol (khusimol) (7.61), Valencen-12-ol (15.78), Vetivone <β-> (8.78), Eremophila-1(10),7(11)-dien-2-one (isonootkatone. α-Vetivone) (6.34), (E)-eremophila-1(10),7(11)-dien-12-yl acetate (9.41).

The characteristic constituents of CZ roots essential oil in this study have been found to be khusimol, valencen-12-ol, α-vetivone, and β-vetivone among other constituents regarded as "fingerprint" of CZ essential oil as shown in Table 16. Eventhough the pleasant scent of the CZ essential oil is extremely complex, the principal alcohols; khusimol and isovalencenol contribute to the woody note, and the ketone α-vetivone influences a strong grapefruit aroma. β-Vetivone present grapefruit and woody notes. It can be noted that the highly complex scent of CZ essential oil is due to the presence of various organoleptically valued compounds [9].

**Table 15.** Compounds and corresponding percentage compositions of essential oil extracted from CZ roots grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

| Class | Compounds | RI | C (%) | T1 (%) | T2 (%) | T3 (%) | T4 (%) | T5 (%) | T6 (%) |
|---|---|---|---|---|---|---|---|---|---|
| Hydrocarbon | Cycloisosativene | 1370.145812 | _ | _ | _ | _ | _ | _ | 0.193629299 |
| Hydrocarbon | Trans-2-nor-zizaene | 1386.896271 | 0.18202592 | 0.216898868 | _ | _ | _ | 1.150109916 | 0.702193421 |
| Hydrocarbon | cyperene | 1401.913579 | 0.11656990 3 | 0.173592436 | _ | _ | _ | _ | 0.145210562 |
| Hydrocarbon | acoradiene II | 1411.640903 | _ | _ | _ | _ | _ | _ | 0.085896848 |
| Hydrocarbon | β-cedrene | 1424.695113 | 0.09716449 7 | _ | _ | _ | _ | _ | 0.093398557 |
| Hydrocarbon | Gurjunene <β-> | 1434.309543 | 0.05977415 | _ | _ | _ | _ | _ | 0.043640773 |
| Hydrocarbon | Acora-3,9-diene | 1443.992199 | 0.11485159 7 | 0.394175844 | _ | _ | _ | _ | 0.467205816 |
| Hydrocarbon | Ziza-6(13)-ene (khusimene) | 1453.739816 | 0.06460831 9 | 0.357051055 | _ | _ | _ | _ | 0.349537024 |
| Hydrocarbon | Selina-4,7-diene | 1469.796363 | 0.24626766 7 | 0.17200337 | _ | _ | _ | _ | 0.239263427 |
| Hydrocarbon | Amorpha-4,7(11)-dien | 1474.566266 | 0.53494313 4 | 0.389740427 | _ | _ | _ | _ | 0.492647717 |
| Hydrocarbon | β-vetispirene | 1486.179311 | 0.26060979 7 | 0.433832661 | _ | _ | _ | 0.931465748 | 0.480231095 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Hydrocarbon | δ-selinene (eudesma-4,6-diene) | 1494.039717 | 0.944839388 | 1.334903308 | 1.610175905 | _ | _ | 1.762373002 | 1.972355975 |
| Hydrocarbon | δ-amorphene | 1499.920257 | 0.155884753 | _ | _ | _ | _ | _ | 0.298470625 |
| Hydrocarbon | 7β,10b-epoxy-4β-eremophila-1,11(12)-diene | 1508.623895 | 1.052932313 | 0.833072563 | _ | _ | _ | _ | 0.740036726 |
| Hydrocarbon | Isocalamenene | 1515.992243 | _ | _ | _ | _ | _ | _ | 0.031802376 |
| Hydrocarbon | γ-vetivenene | 1524.757285 | 0.200950201 | _ | _ | _ | _ | _ | 0.134406884 |
| Hydrocarbon | α-calacorene | 1529.360109 | 0.326959333 | 0.451364781 | _ | _ | _ | _ | 0.440577032 |
| Hydrocarbon | Selina-3,7(11)-diene or eudesma-3,7(11)-diene | 1540.601214 | 0.572104373 | 0.354798423 | _ | _ | _ | _ | 0.252319139 |
| Hydrocarbon | Vetivenene <β-> | 1554.979335 | 0.085823675 | _ | _ | _ | _ | _ | 0.058796355 |
| Alcohol | cis-eudesm-6-en-11-ol | 1564.601305 | 1.267674786 | 2.365805942 | 2.356324639 | 4.820566816 | _ | 3.694670548 | 2.076329963 |
| Ketone | 13-nor-eudesm-5-en-11-one (epimer A) | 1570.375613 | 0.170547633 | 0.342225587 | _ | _ | _ | _ | 0.028028697 |
| Alcohol | cis-eudesm-6,11-dien-3b-ol | 1583.370087 | 4.564325715 | 4.171946093 | 6.803566004 | 9.255067123 | _ | 8.106173133 | 4.981210736 |
| Ketone | 12-nor-preziza-7(15)-en-2-one | 1592.650948 | 0.854639759 | 0.458244916 | _ | _ | _ | _ | 0.26914853 |
| Ketone | 15-nor-funebran-3-one | 1597.682596 | 0.354223127 | 0.104599003 | _ | _ | _ | _ | 0.0591159 |
| Alcohol | 12-nor-ziza-6(13)-en-2b-ol | 1611.7757 | 0.653666648 | 0.967008182 | _ | _ | _ | _ | 1.097516737 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ketone | 13-nor-eremophil-1(10)-en-11-one | 1616.882558 | 0.400113362 | 0.642594111 | _ | _ | _ | _ | 0.637051807 |
| Alcohol | 10-epi-γ-eudesmol | 1629.249147 | 1.548308579 | 1.428501081 | _ | 3.162235676 | _ | 2.875646129 | 1.602413694 |
| Ether | Aromadendrene <epoxide-allo-> | 1640.229214 | 7.362163765 | _ | 3.157835806 | _ | _ | 1.907343592 | 0.81537336 |
| Aldehyde | Eremophila-1(10),6-dien-12-al | 1646.33263 | 0.60768477 | 3.011071778 | _ | _ | _ | _ | 2.205791502 |
| Alcohol | β-eudesmol | 1654.576433 | 0.295365407 | 0.435089945 | _ | _ | _ | _ | 0.745073371 |
| Alcohol | Eudesm-11-en-4-ol (intermedeol) | 1659.642775 | 0.154624661 | 0.618548564 | _ | _ | _ | 0.716683501 | 0.687053055 |
| Alcohol | Cyclocopacamphan-12-ol (epimer B) | 1666.471465 | _ | _ | _ | _ | _ | _ | 0.037112916 |
| Alcohol | Khusinol | 1677.525569 | 10.32708992 | 7.097522593 | 8.30395012 | 10.76248135 | _ | 10.10828234 | 4.921318791 |
| Aldehyde | Prezizaan-15-al | 1683.668113 | _ | _ | _ | _ | _ | _ | 2.761724425 |
| Aldehyde | 2-epi-ziza-6(13)-en-12-al | 1689.869045 | _ | 1.89408015 | 7.381761293 | 11.63815039 | _ | 10.93443646 | 1.041565858 |
| Alcohol | Eudesm-7(11)-en-4α-ol (juniper camphor) | 1702.365383 | 4.109364014 | 5.849633781 | 2.483430148 | 3.736070896 | _ | 3.328976294 | 5.522611946 |
| Alcohol | Ziza-5-en-12-ol | 1713.033879 | 1.833616167 | 1.63041732 | _ | _ | _ | 2.837918127 | 2.115268853 |
| Alcohol | Trans-eudesm-4(15),7-dien-12-ol | 1724.064889 | 3.047611062 | 2.052759807 | _ | _ | _ | _ | 2.312245771 |
| Alcohol | Eremophila-1(10),7(11)-dien-2β-ol | 1737.753631 | 1.40076334 | 0.79882905 | _ | _ | _ | _ | 0.8774869 |
| Alcohol | Ziza-6(13-en-12-ol (khusimol) | 1751.354608 | 9.313976498 | 8.225236055 | 11.64186934 | 7.463367553 | _ | 7.286851643 | 7.609228228 |

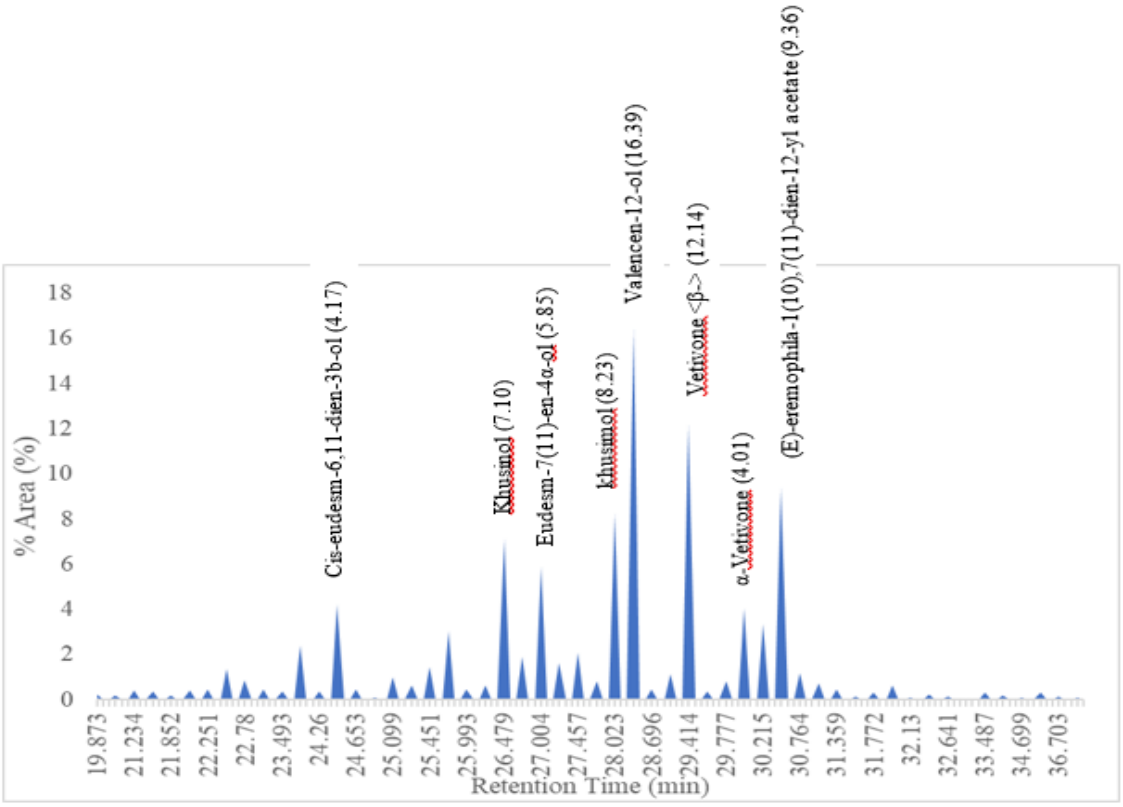
| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Alcohol | Preziza-7(15)-en-12-ol | 1767.555942 | 18.06315715 | _ | 19.48415484 | 21.66710538 | 100 | 21.57833759 | _ |
| Alcohol | Valencen-12-ol | 1776.775621 | 0.354085663 | 16.38502352 | _ | _ | _ | _ | 15.77831522 |
| Alcohol | (E)-eremophila-1(10),7(11)-dien-12-ol(isovalencenol) | 1781.575653 | _ | 0.440119079 | _ | _ | _ | _ | _ |
| Alcohol | Eremophila-1(10),7(11)-dien-2α-ol(isonootkatool-vetiselinenol) | 1791.78462 | 0.77172575 | 1.122527168 | _ | _ | _ | _ | 1.084400158 |
| Alcohol | Cadinene <14-hydroxy-δ-> | 1801.102923 | _ | _ | _ | _ | _ | _ | 0.117242731 |
| Ketone | Eremophila-1(10),11-dien-2-one (nootkatone) | 1807.024069 | _ | _ | 16.50349955 | 4.418706677 | _ | 4.892608876 | 0.01760543 |
| Ketone | Vetivone <β-> | 1819.05744 | 14.43865337 | 12.13596404 | _ | _ | _ | _ | 8.77680149 |
| Aldehyde | (E)-eremophila-1(10),7(11)-dien-12-al | 1829.915247 | 0.408109215 | 0.351340893 | _ | 2.370799333 | _ | 2.203374725 | 0.135304655 |
| Ether | Methyl-(E)-eremophila-1(10),7(11)-dien-12-ether | 1837.726382 | 3.235960349 | 0.819958398 | 4.687041562 | _ | _ | 1.502733052 | 1.174283716 |
| Ketone | Eremophila-1(10),7(11)-dien-2-one(isonootkatone. a-vetivone) | 1848.605506 | _ | 4.006071981 | _ | _ | _ | _ | 6.341804634 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ester | Ziza-6(13)-en-12-yl acetate | 1860.115069 | 2.88961852 | 3.329199432 | 5.152231137 | 2.883214881 | _ | 2.845493435 | 3.257232834 |
| Carboxylic Acid | Pentadecanoic acid | 1869.331005 | 5.34961463 | _ | 10.43415966 | 9.362112837 | _ | 9.417592538 | _ |
| Ester | (E)-eremophila-1(10),7(11)-dien-12-yl acetate | 1879.539421 | 0.203264186 | 9.364911173 | _ | 3.090286918 | _ | _ | 9.407081978 |
| Carboxylic Acid | Prekhusenic acid | 1888.093845 | _ | 1.156438898 | _ | _ | _ | _ | 0.78363185 |
| _ | _ | 1903.010555 | _ | 0.727198814 | _ | _ | _ | _ | 0.496527911 |
| Hydrocarbon | Nonadecane <n-> | 1919.55529 | 0.213482382 | 0.446021326 | _ | _ | _ | _ | 0.397803597 |
| _ | _ | 1927.929401 | 0.292570295 | 0.129168419 | _ | _ | _ | _ | 0.102756673 |
| _ | _ | 1941.24283 | 0.150890209 | 0.294710748 | _ | 5.369834167 | _ | 1.120848434 | 0.150886297 |
| _ | _ | 1948.07373 | _ | 0.618408866 | _ | _ | _ | _ | 0.44418333 |
| Carboxylic Acid | Hexadecanoic acid | 1960.120863 | 0.139228636 | 0.070809509 | _ | _ | _ | _ | 0.069173972 |
| _ | _ | 1967.087218 | _ | 0.204849901 | _ | _ | _ | _ | 0.410904959 |
| _ | _ | 1986.671712 | _ | 0.113347601 | _ | _ | _ | _ | 0.101250244 |
| Hydrocarbon | Eicosane <n-> | 2000.570516 | _ | 0.057869966 | _ | _ | _ | _ | 0.07488014 |
| _ | _ | 2032.862994 | 0.05274055 | 0.298936618 | _ | _ | _ | _ | 0.188881768 |
| _ | _ | 2048.497685 | _ | 0.15363306 | _ | _ | _ | _ | 0.109117148 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| _ | _ | 2068.905289 | _ | _ | _ | _ | _ | _ | 0.078714685 |
| _ | _ | 2082.661312 | _ | _ | _ | _ | _ | _ | 0.075367066 |
| Hydrocarbon | Heneicosane <n-> | 2098.484497 | 0.053427872 | 0.102119361 | _ | _ | _ | _ | 0.1112931 |
| _ | _ | 2130.834063 | _ | _ | _ | _ | _ | _ | 0.038528046 |
| _ | _ | 2164.901185 | 0.048227132 | 0.287865538 | _ | _ | _ | _ | 0.186690599 |
| _ | _ | 2191.494211 | _ | _ | _ | _ | _ | _ | 0.030052485 |
| _ | _ | 2215.672348 | _ | 0.112020469 | _ | _ | _ | _ | 0.112312602 |
| _ | _ | 2397.972025 | _ | 0.065937535 | _ | _ | _ | 0.798080922 | 0.322679994 |
| Hydrocarbon | Tetracosane | 2827.92716 | 0.053175854 | _ | _ | _ | _ | _ | _ |
| | | | | | | | | | |
| Hydrocarbons | | | 5.336395126 | 5.71744439 | 1.610175905 | _ | _ | 3.843948666 | 7.805596488 |
| Alcohols | | | 57.70535537 | 53.58896818 | 51.07329509 | 60.8668948 | 100 | 60.5335393 | 51.56482907 |
| Ketones | | | 16.21817725 | 17.68969963 | 16.50349955 | 4.418706677 | _ | 4.892608876 | 16.12955649 |
| Aldehydes | | | 1.015793985 | 5.256492822 | 7.381761293 | 14.00894972 | _ | 13.13781118 | 6.144386439 |
| Esters | | | 3.092882706 | 12.6941106 | 5.152231137 | 5.973501799 | _ | 2.845493435 | 12.66431481 |
| Ethers | | | 10.59812411 | 0.819958398 | 7.844877368 | _ | _ | 3.410076644 | 1.989657076 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Carboxylic Acids | | | 5.488843266 | 1.227248407 | 10.43415966 | 9.362112837 | _ | 9.417592538 | 0.852805822 |
| Total Identified Compounds | | | 99.45557181 | 96.99392243 | 100 | 94.63016583 | 100 | 98.08107064 | 97.15114619 |

Compounds: Volatile constituents listed in order of elution from an HP 5MS column with reference to literature [32],[61]. RI: Retention Indices calculated relative to C6-C28 n alkanes on HP 5MS. C%, T1%, T2%, T3%, T4%, T5%, T6%: Percentage compositions of control, treatment 1, treatment 2, treatment 3, treatment 4, treatment 5, treatment 6 respectively. _: not detected/not identified



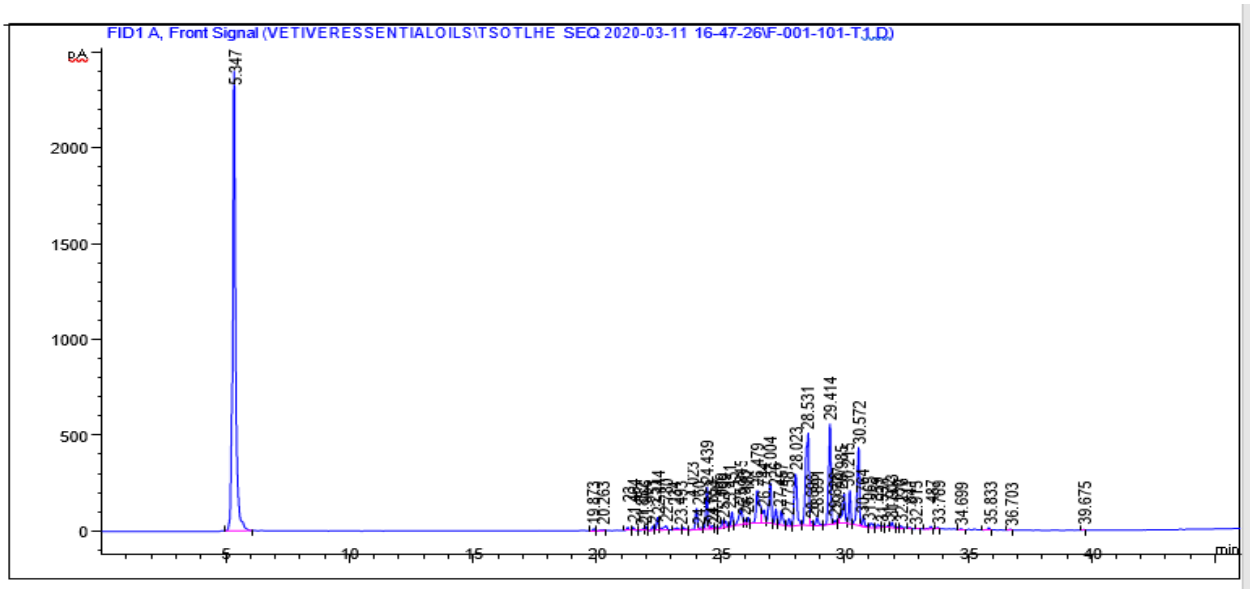
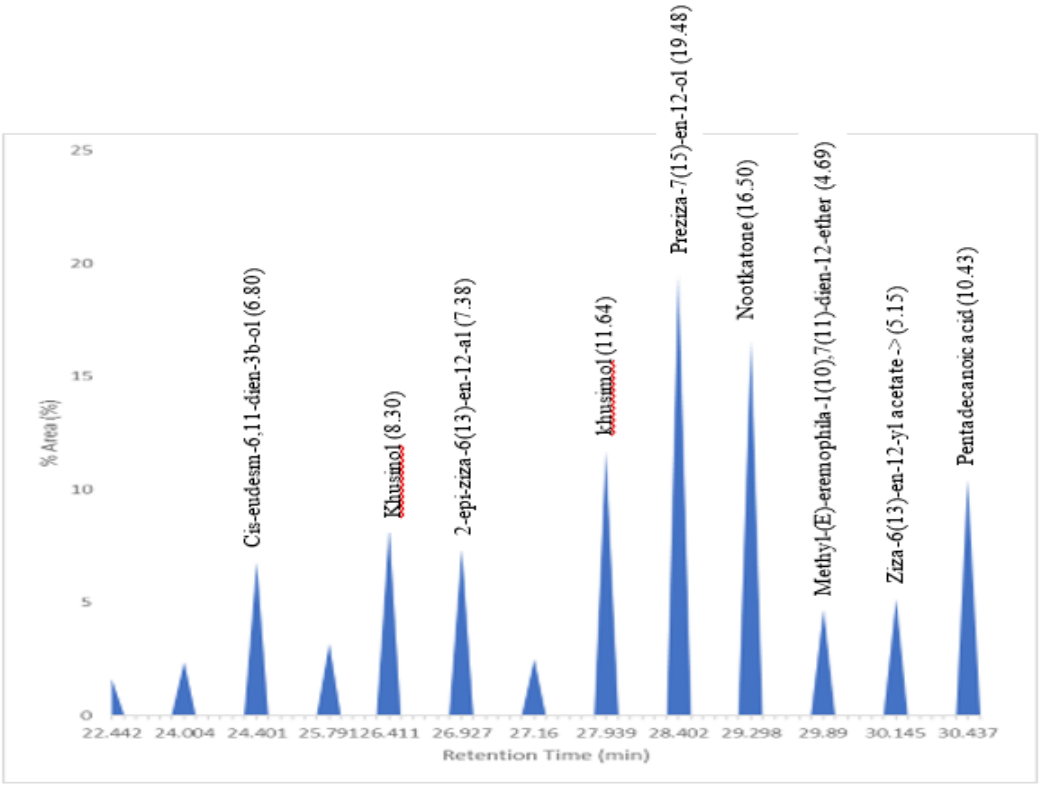




**Figure 18a.** Chromatograms of T1 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA



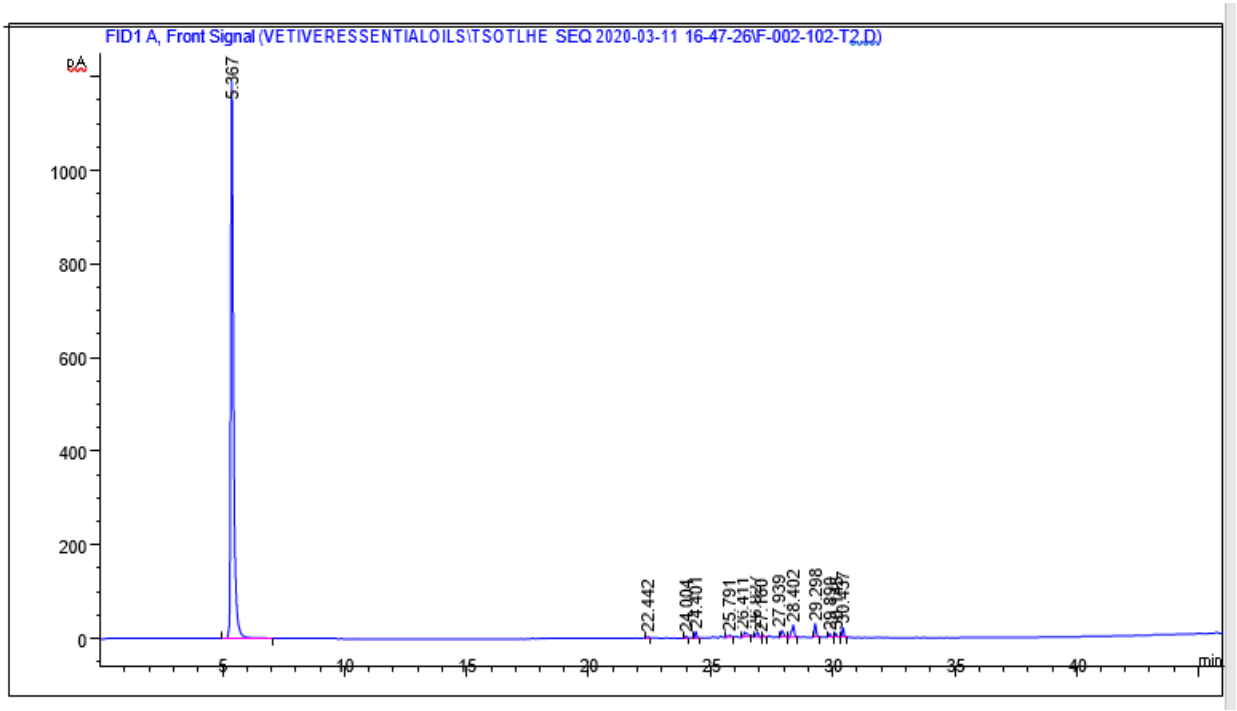
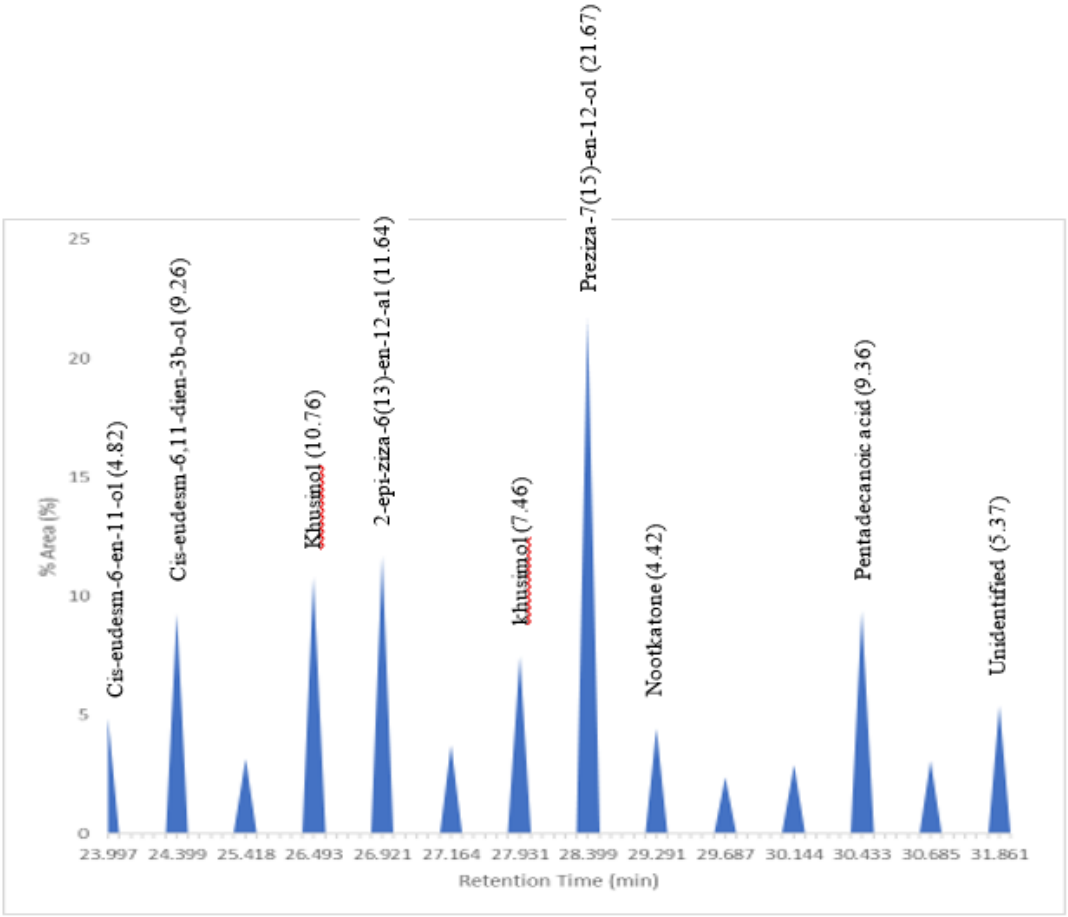




**Figure 18b.** Chromatograms of T2 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA



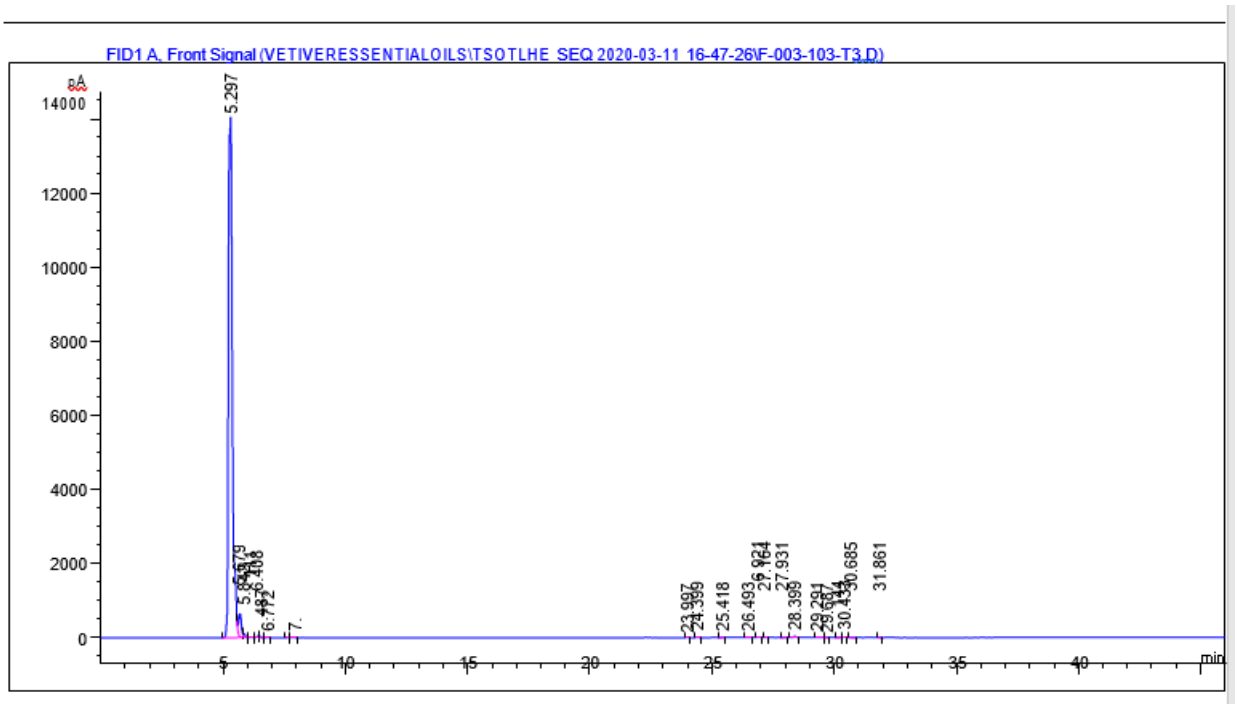
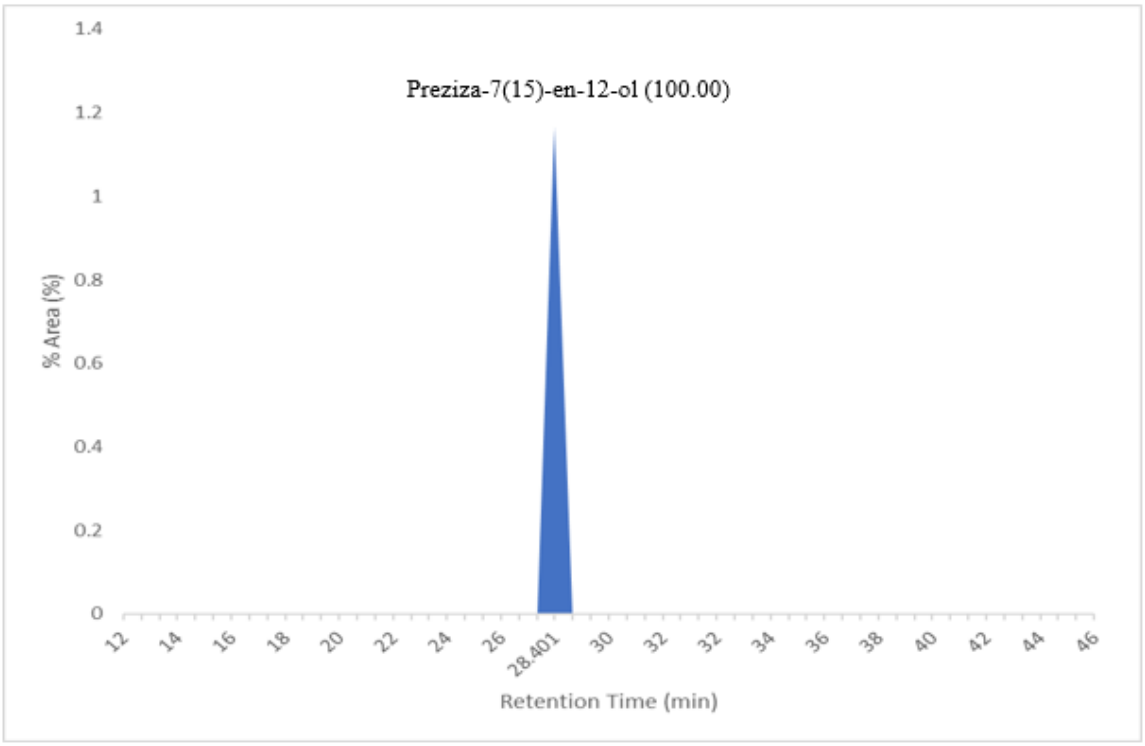




**Figure 18c.** Chromatograms of T3 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA



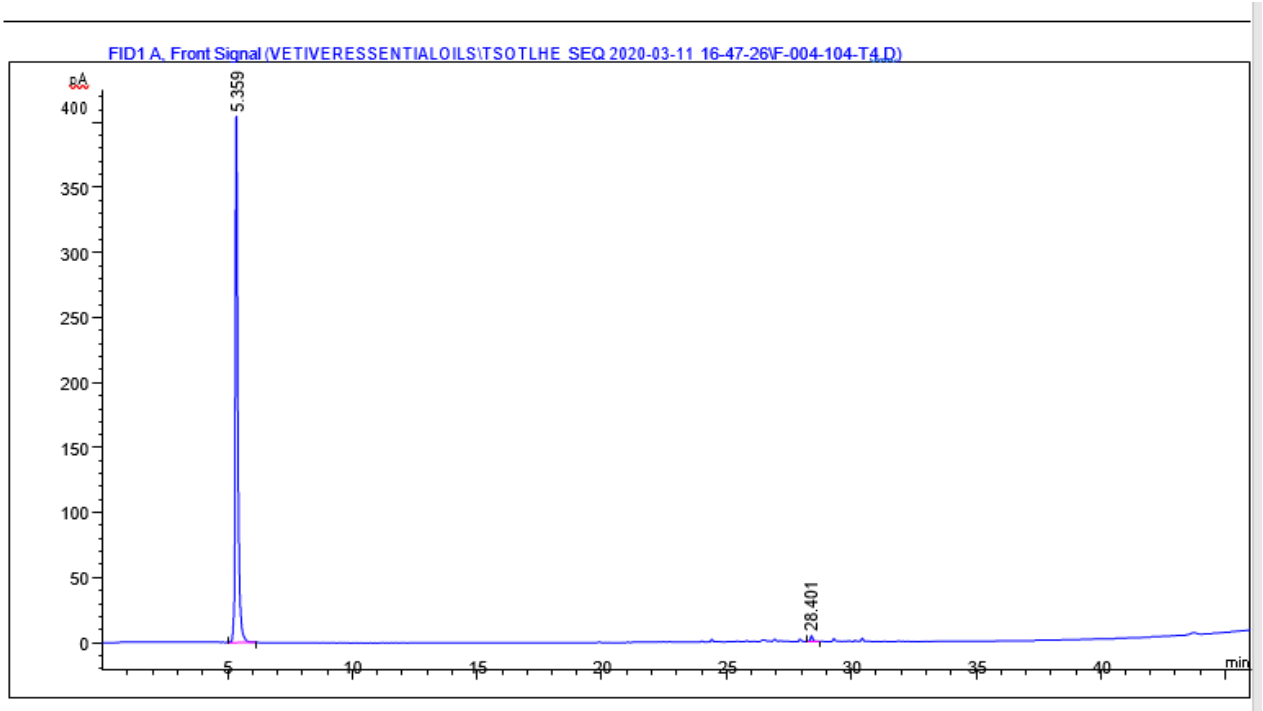
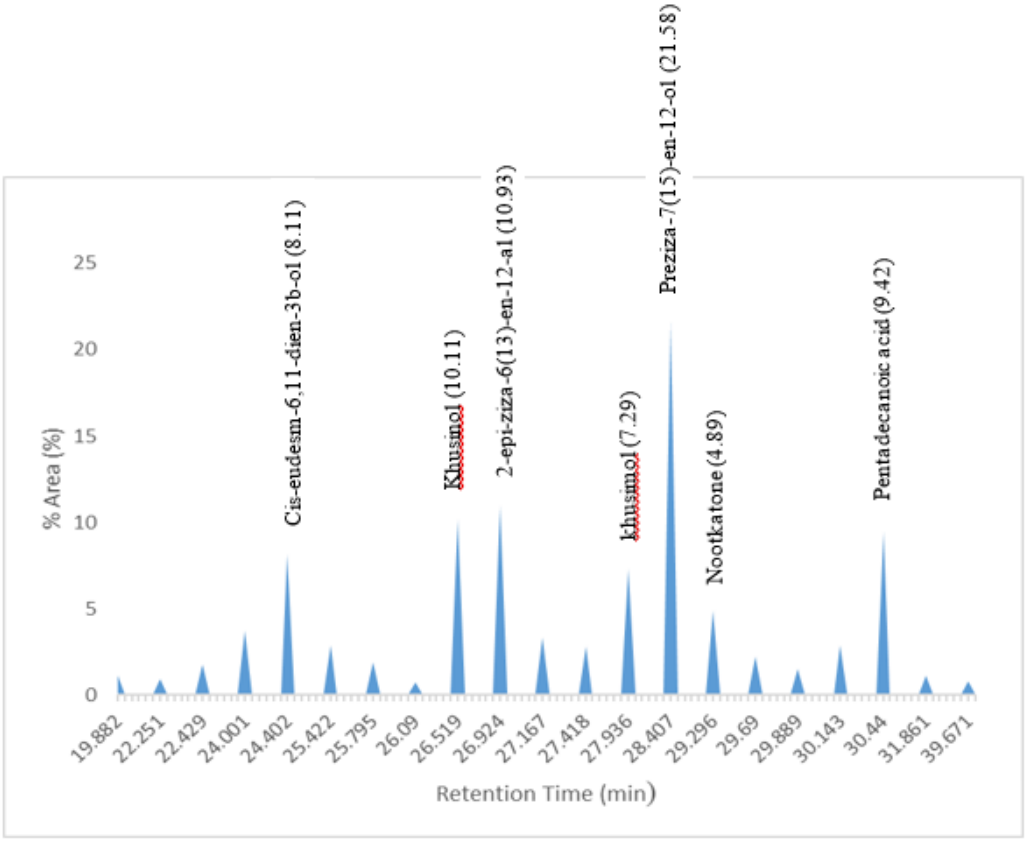




**Figure 18d.** Chromatograms of T4 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA



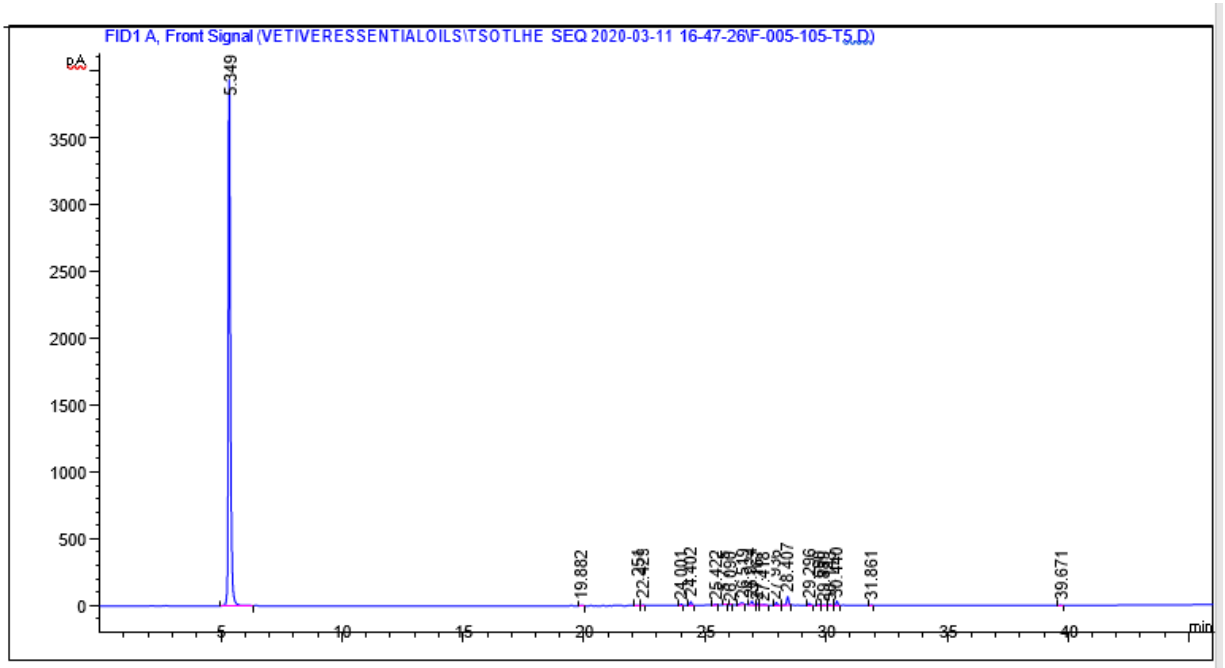
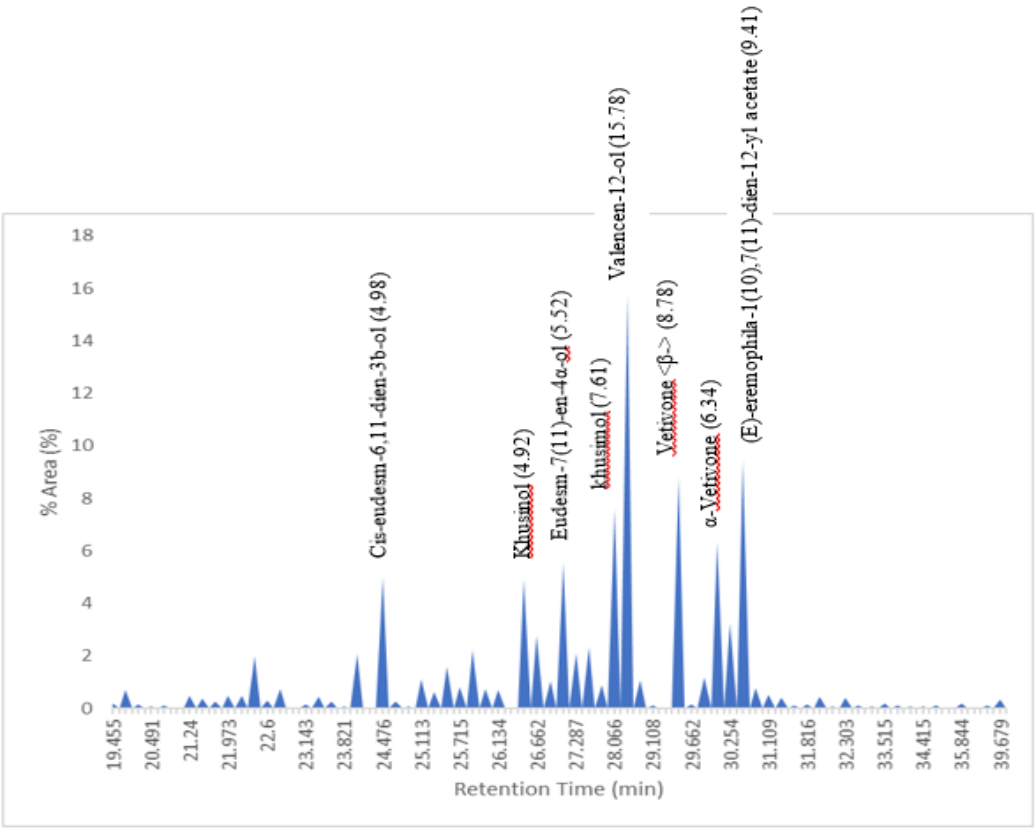




**Figure 18d.** Chromatograms of T5 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA



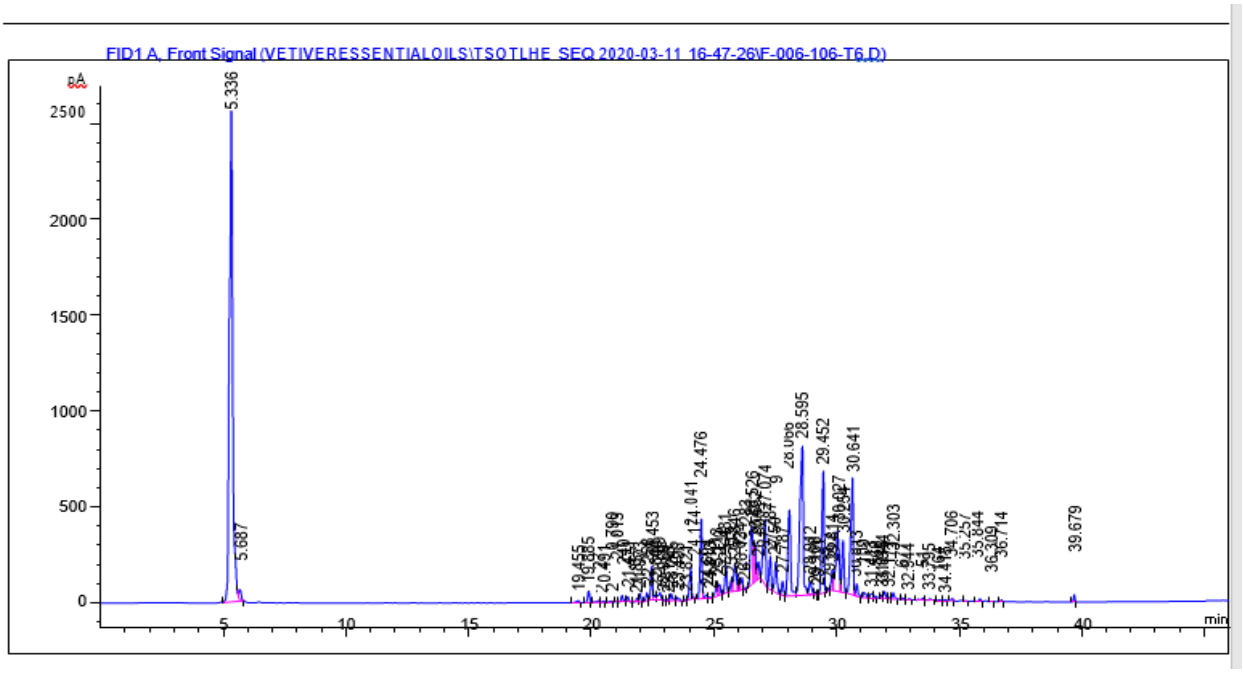




**Figure 18e.** Chromatograms of T6 sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

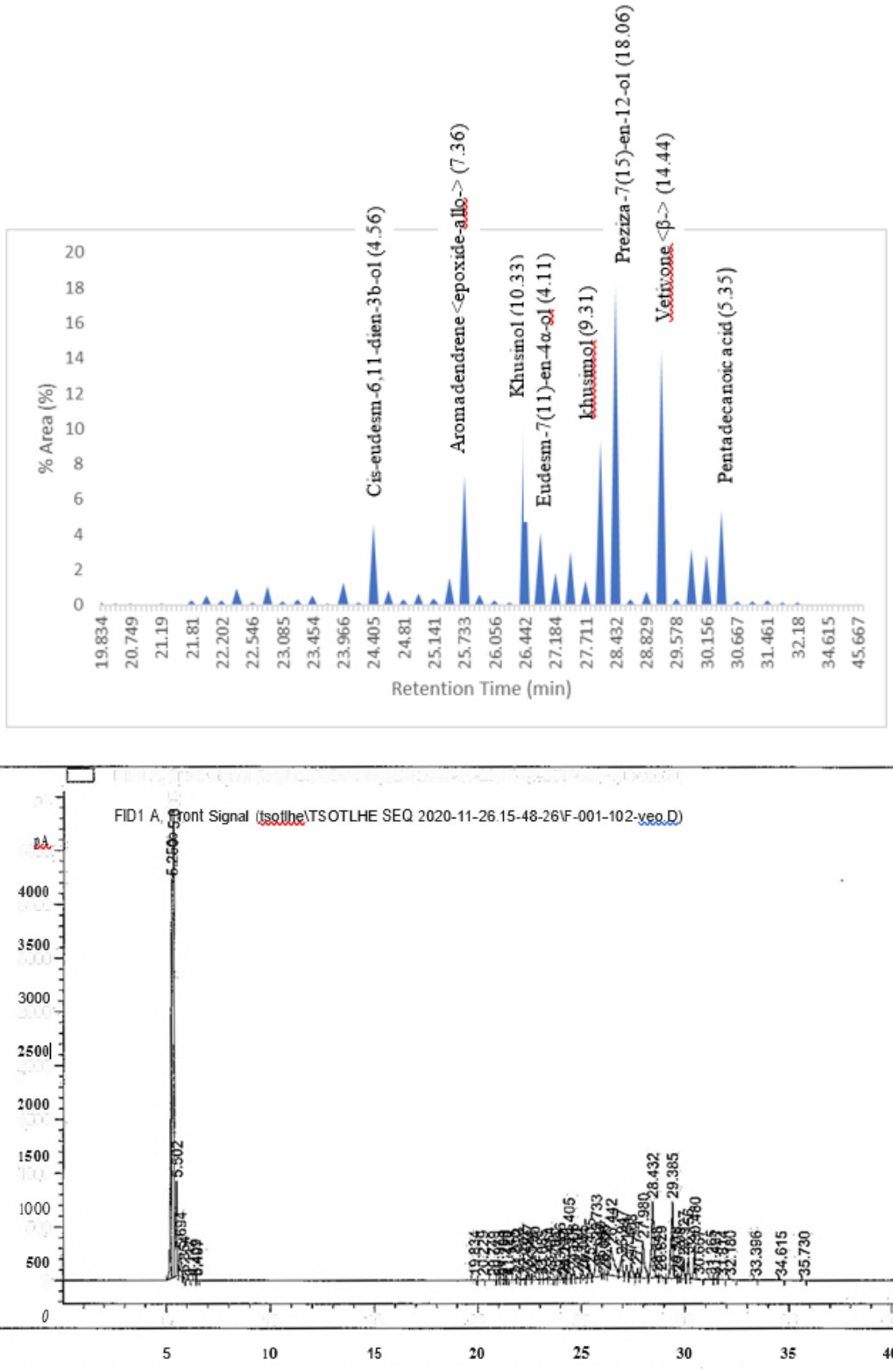
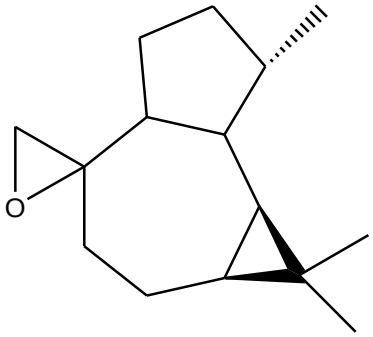
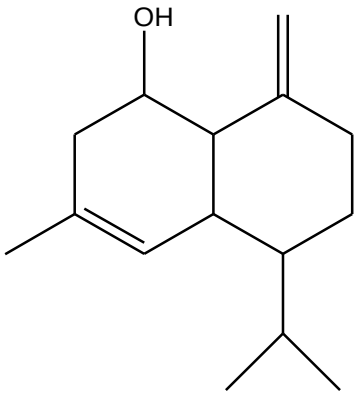
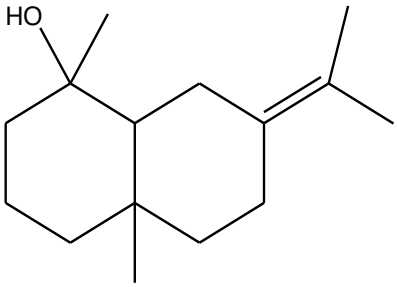
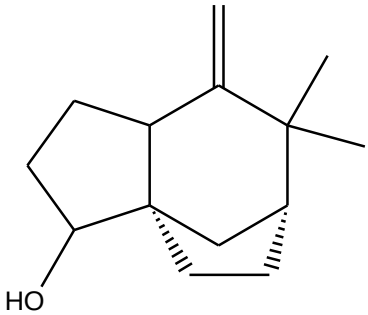
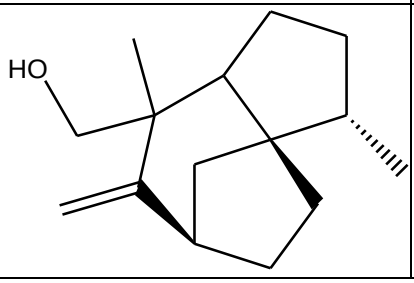
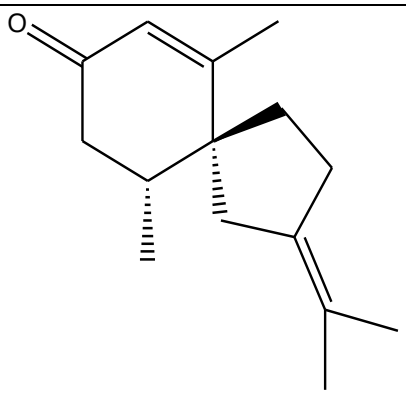
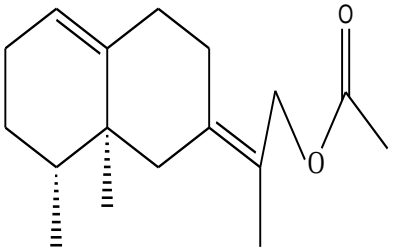
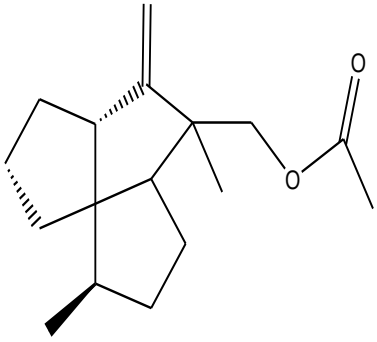
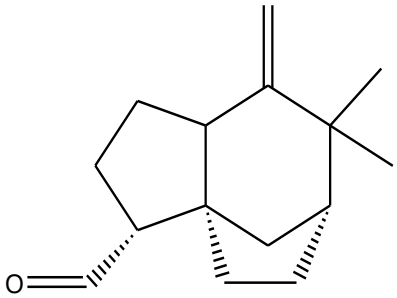
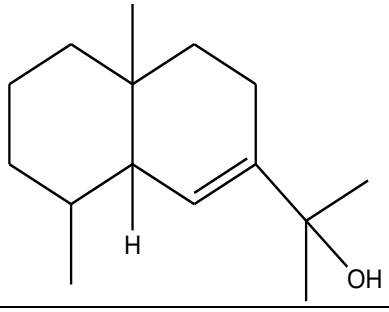
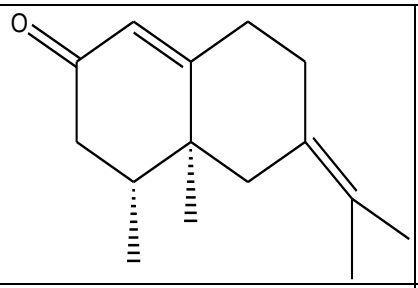
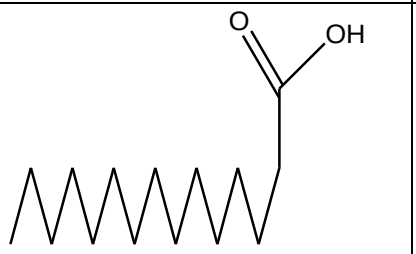
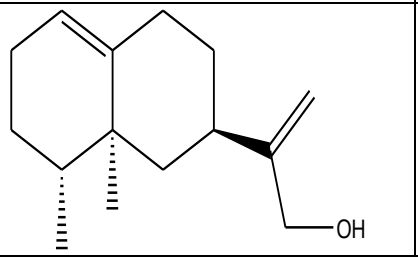



**Figure 18f.** Chromatograms of control sample indicating major constituents (> 4% of percentage composition) and other constituents of CZ essential oil grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

**Table 16.** Variation of principal constituents of CZ essential oil extracted from the CZ roots cultivated in selibe-phikwe Cu-Ni mine in different treatments

| Constituent name | Constituent structure | C | T 1 | T 2 | T 3 | T 4 | T 5 | T 6 |
|---|---|---|---|---|---|---|---|---|
| Aromadendrene<epoxide-allo-> |  | ✓ | × | × | × | × | × | × |
| Khusinol |  | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ |
| Juniper camphor |  | ✓ | ✓ | × | × | × | × | ✓ |
| Khusimol |  | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ |
| Preziza-7(15)-en-12-ol |  | ✓ | × | × | ✓ | ✓ | ✓ | × |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| B-Vetivone |  | ✓ | ✓ | × | × | × | × | ✓ |
| (E)-eremophila-1(10),7(11)-dien-12-yl acetate |  | × | ✓ | × | × | × | × | ✓ |
| Ziza-6(13)-en-12-yl-acetate |  | × | × | ✓ | × | × | × | × |
| 2-epi-ziza-6(13)-en-12-al |  | × | × | × | ✓ | ✓ | ✓ | × |
| Cis-eudesm-6-en-11-ol |  | × | × | × | ✓ | × | × | × |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ά-Vetivone |  | × | ✓ | ✓ | ✓ | × | ✓ | ✓ |
| Pentadecanoic acid |  | ✓ | × | ✓ | ✓ | × | ✓ | × |
| Valencen-12-ol |  | × | ✓ | × | × | × | × | ✓ |

✓; Principal constituent in a specific treatment sample, ×; not a principal constituent in a specific treatment sample

## 4.3. PHYTOREMEDIATION AND SOIL AMENDMENTS EFFECTS ON *CHRYSOPOGON ZIZANIOIDES* ROOTS ESSENTIAL OIL

Studies have shown that CZ roots have intracellular bacteria that link with the cells that produce essential oil. They implement the biotransformation of terpenes derived from the plant for essential oil production, with the oil profiles possibly varying according to the presence of microbial group and other external bio-factors. The production of essential oil is not exclusively dependent on environmental factors, the specific plant genetic make-up is also involved. However, the genetic make-up of CZ is not yet understood, and studies show that generally, the production of essential oil in aromatic and medicinal plants may be linked to both the genotype and environmental factors. Each aromatic plants species has constituents that are produced according to physiological necessity [7].

**Table 17.** Heavy metal concentrations (mean ± SD) in the essential oil extracted from CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

| Treatments | | Ni($mgkg^{-1}$) n=2 | Cu($mgkg^{-1}$) n=2 | Mn($mgkg^{-1}$) n=2 | Zn($mgkg^{-1}$) n=2 | Pb($mgkg^{-1}$) n=2 | As($mgkg^{-1}$) n=2 |
|---|---|---|---|---|---|---|---|
| C | 0 HM | - | - | - | - | - | - |
| T1 | 0 EDTA 0 AMF | - | - | - | - | - | - |
| T2 | +EDTA split | - | - | - | - | - | - |
| T3 | +EDTA single | - | - | - | - | - | - |
| T4 | +AMF | - | - | - | - | - | - |
| T5 | +AMF+EDTA split | - | - | - | - | - | - |
| T6 | +AMF+EDTA single | - | - | - | - | - | - |
| PT | INTEGRATED/POOL TREATMENT(T1+T2+T3+T4+T5+T6) | 0.063±0.001 | 0.150±0.007 | 0.043±0.001 | 0.109±0.002 | 0.022±0.002 | 0.003±0.003 |

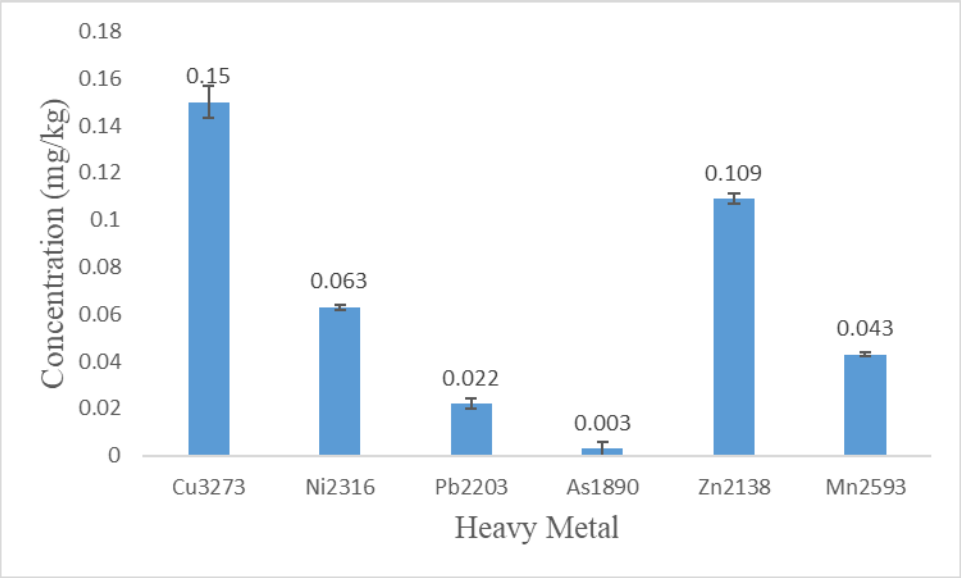



**Figure 19.** Heavy metal concentrations (mean ± SD) in the essential oil (cumulative treatments) extracted from CZ grown in Cu-Ni mine tailings with cow manure as affected by mycorrhizal inoculation and EDTA

In order for CZ to be successfully credited for application in the perfumery and food industries, CZ extracts must not incorporate any toxic substances that may cause a health hazard to the consumers. However, CZ have great capability for accumulating high levels of heavy metals, especially Pb, Zn and Cu, in their roots. This property may cause cross-contamination of CZ oils extracted from the roots with heavy metals [23]. Essential oils of CZ grown on heavy metal contaminated mine tailings extracted by ultra-sonic assisted hydro distillation (UAHD) was further extracted by acid digestion method so as to determine if the heavy metals detected in raw roots (Cu, Ni, Pb, As, Zn, Mn) were not co-extracted with CZ essential oil. The results from cumulative treatments as shown in Figure 19 and Table 17, showed the concentrations as follows; Cu (0.150 mg $kg^{-1}$), Ni (0.063 mg $kg^{-1}$), Pb (0.022 mg $kg^{-1}$), As (0.003 mg $kg^{-1}$), Zn (0.109 mg $kg^{-1}$), Mn (0.043 mg $kg^{-1}$). From comparison of heavy metals level with raw roots, the CZ essential oil contained very low levels of toxic heavy metals. This indicates low to negligible contamination of the extracts. Similar studies found no heavy metals in CZ essential oil extracted by Hydro distillation as heavy metals are left in the plant residues, however, there are no studies reported in literature about heavy metal contents in the CZ essential oil extracted by UAHD [16].

The chemical composition and percentage yield of CZ essential oil is determined by diverse aspects; however, different cultivation methods have significant effect [16]. The Chemical constituents of essential oil with corresponding chemical composition of CZ essential oil under different treatments (varying heavy metal content and varying soil amendments) is presented in Table 15. The number of constituents trend is as follows; T6(70)>T1(54)>C(51)>T5(21)>T3(14)>T2(13)>T4(1). This shows that in comparison with control (not introduced to heavy metals and no soil modification), there have been a tremendous

increase in the number of constituents produced by CZ grass for the treatment with CM only and inoculation of AMF with 5 mmol EDTA single. The AMF inoculated and 5 mmol EDTA applied treatment have managed to absorb multi-elements, even though this treatment absorbed least amount of Ni, Mn and Zn and moderate amount of Cu, Pb and Zn as compared to other treatments, the rise in constituents' number can be due to metal stress caused by heavy metal toxicity enhanced by EDTA since EDTA have eradicated the fungi which can allow the grass to grow well and stress free. The results also show that the non-modified treatment managed to produce more constituents in the presence of organic matter only, this is because CZ can survive and trigger defensive mechanisms (produce essential oil) against metal stress hence many constituents produced as compared to the control, it also did not vary much with the control. On the other hand, there was less metal stress for treatment with AMF + 5 mmol split EDTA, the metal absorbed by this treatment were in moderate level across all the metals. Splitting EDTA delayed Fungi eradication rate hence CZ did not have more metal stress toxicity thus producing less constituents as compared to control. For treatments with absence of AMF but with EDTA single and split applications, the constituents' number are almost equal, the metals absorbed by these treatments were high to moderate due to EDTA application as it improved metal availability but this did not trigger more production of many constituents as compared to control. This can be possibly that the metals of interest were not toxic enough to cause more stress. The AMF inoculated treatment had accumulated greatest levels of heavy metals but only one constituent (an alcohol) was produced as a result of possibility of bio-conversion or breaking down compounds to simpler ones by fungi [61]. GC/FID analysis of the oils revealed that T1 which is fungus free had the typical vetiver oil profile as proved by the results since they have almost similar oil profile, whereas T6 which is inoculated with fungi produced large amounts of C19–C29 alkanes plus several alkanols along with typical vetiver oil compounds, but lacked some secondary metabolites such as Preziza-7(15)-en-12-ol, Isovalencenol and Pentadecanoic acid though fungal metabolites are present which are not available in normal "typical" oil. It seems that the biotic factors enhance the oil production in normal vetiver by both increasing yield and the generation of signature oil compounds [29]. Even so literature advices the use of adapted indigenous fungal strains that are more suitable for phyto-stabilization and extraction purposes than laboratory strains [19].

The combined effect of Ni, Cu, Mn, Zn, Pb and As in overall did not significantly alter the essential oil composition of most of the CZ constituents across most of the treatments as compared to the control treatment. The considered fingerprint of CZ essential oil such as khusimol which is desired for perfumery applications, α and β-vetivone for antioxidant activity [16] are among the major constituents in some treatments and the compositions are precise to each other (no significant difference) in all the treatments including the control treatment except for α-vetivone which showed a notable high composition in T2 which divert from other treatments.CZ essential oil from all treatments including those with high heavy metals levels contained many of the high molecular weight compounds which are classified as the third fraction of oils (alcohol components) with small variance. However, the lower boiling (hydrocarbon fraction) and intermediate fraction (various oxygenated derivatives; Ketones Aldehydes Esters, Ethers Carboxylic Acids) showed strikingly dissimilarities across all the treatments. It can be summarised that the main oil components from the control treatment and all other modified treatments are alcohols. The findings also have demonstrated that the CZ essential oil composition is not affected by the multi heavy metal but also depends on the

individual constituents, however for T2 (ά- vetivone) it can be due to biotic factor that interferes with biosynthesis of essential oil which is not studied in this study.

The resulting extracted CZ essential oils appeared as light brown viscous liquids, obtained as shown in Figure 17 and Table 14 in 0.26%, 0.86%, 0.95%, 0.36%, 0.89%, 0.54% and 0.94% yields from CZ cultivated in C (control; no heavy metals), T1 (soil with mine tailing and added CM only), T2 (soil with mine tailing and added CM with 5 mmol EDTA split), T3 (soil with mine tailing and added CM with 5 mmol EDTA single), T4 (soil with mine tailing and added CM with AMF), T5 (soil with mine tailing and added CM with 5 mmol EDTA split and AMF), T6 (soil with mine tailing and added CM with 5 mmol EDTA single and AMF) respectively. The trend shows that T1, T2, T4, T6 had precise and better yield while for C, T3 and T5 the yields dropped with C being the least. The higher yield produced by cultivating on soil/mine tailings with AMF in T4 and T6 might be attributed to the activities of intracellular bacteria engaged with essential oil cells in CZ root glands. These bacteria are most likely bio-transforming essential oil precursors, resulting in higher production although the frequency and intensity with which this occurs is mostly determined by plant-fungus contact and growing circumstances [3] [21]. In addition, cow manure combined with AMF can improve essential oil quality as well as content [21]. The heavy metals stress in CZ which was increased by increased bioavailability of heavy metals as a result of application of EDTA might have had an impact in the production of more essential oils/secondary metabolites as the defence mechanism to survive the heavy metals stress. For the control treatment, no heavy metals were present hence the yield was so low since the CZ did not have any stimulator factor to trigger it to produce more essential oil.

## 4.4. METHOD VALIDATION FOR ICP-OES

To validate and verify the method of analysis for the heavy metal contents in both the CZ roots and CZ essential oil, the following parameters were carried out; linearity, LOD, LOQ, accuracy (recovery) and precision.

### 4.4.1. Linearity

The five standards (5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm) from multi element standard and blank (0 ppm) were prepared and then analysed by ICP-OES prior to sample analysis.

**Table 18.** The coefficient of determination ($R^2$) of the calibration/standard curve

| Heavy metals | $R^2$ |
|---|---|
| As | 0.9812 |
| Cu | 0.9657 |
| Mn | 0.9660 |
| Ni | 0.9833 |
| Pb | 0.9839 |
| Zn | 0.9838 |

Coefficient of determination ($R^2$) range between 0.9657 to 0.9839 which is close to 1 indicating perfect collinearity.

### 4.4.2. Accuracy (recovery)

The certified reference material (CRM) was prepared and analysed in triplicates using ICP-OES.

**Table 19.** Percentage recoveries of heavy metals of interest

| Heavy metals | Known/actual concentrations ($mgkg^{-1}$) | Found concentrations ($mgkg^{-1}$) n=3 | % Recovery (%) |
|---|---|---|---|
| As | 0.052±0.005 | 0.055±0.007 | 105.2 |
| Cu | 0.022±0.005 | 0.021±0.003 | 95.5 |
| Mn | 0.031±0.005 | 0.030±0.025 | 96.9 |
| Ni | 0.052±0.005 | 0.050±0.001 | 96.7 |
| Pb | 0.027±0.005 | 0.026±0.001 | 96.3 |

| | | | |
|---|---|---|---|
| Zn | 0.054±0.005 | 0.051±0.001 | 94.0 |

%Recovery = (Found Concentrations/ Actual Concentrations) * 100%

All the found metal concentrations are close to the actual concentrations, falling within the uncertainty with overall percentage recovery ranging between 94% to 105%.

### 4.4.3. Precision

5ppm standard was prepared from multi-element standard, then it was analysed using ICP-OES for 3 consecutive days and twice a day (5 replicates in each run). % RSD was calculated from the data obtained.

**Table 20.** Percentage relative standard deviation for intra-day precision (within one day)

| Heavy metals | D1R1 (% RDS) (%) | D1R2 (% RSD) (%) |
|---|---|---|
| As | 0.82 | 0.86 |
| Cu | 0.89 | 1.04 |
| Mn | 0.92 | 1.04 |
| Ni | 0.84 | 0.89 |
| Pb | 0.83 | 0.84 |
| Zn | 0.84 | 0.88 |

% RSD = (SD/$\bar{x}$) * 100, %RSD; percentage relative standard deviation, SD; standard deviation, $\bar{x}$; mean, D1R1; Day 1 Run 1, D1R2; Day 1 Run 2

**Table 21.** Percentage relative standard deviation for inter-day precision (between days)

| Heavy metals | D1R1 (%RSD) (%) | D2R1 (% RSD) (%) | D3R1 (% RSD) (%) | D1R2 (% RSD) (%) | D2R2 (% RSD) (%) | D3R2 (% RSD) (%) |
|---|---|---|---|---|---|---|
| As | 0.82 | 0.85 | 0.98 | 0.86 | 0.87 | 0.83 |
| Cu | 0.89 | 0.77 | 0.61 | 1.04 | 0.98 | 0.80 |
| Mn | 0.92 | 0.79 | 0.63 | 1.04 | 0.97 | 0.84 |
| Ni | 0.84 | 0.85 | 0.88 | 0.90 | 0.88 | 0.88 |
| Pb | 0.83 | 0.85 | 0.86 | 0.84 | 0.89 | 0.89 |
| Zn | 0.84 | 0.85 | 0.90 | 0.88 | 0.88 | 0.88 |

% RSD = (SD/$\bar{x}$) * 100, %RSD; percentage relative standard deviation, SD; standard deviation, $\bar{x}$; mean, D1R1; Day 1 Run 1, D1R2; Day 1 Run 2, D2R1; Day 2 Run 1, D2R2; Day 2 Run 2, D3R1; Day 3 Run 1, D3R2; Day 3 Run 2

Percentage RSD values both intraday and interday were found to be smaller (< 2%). This means that the data set was too close to the means. The smaller the % RSD, the precise the data.

### 4.4.4. Limit of detection and limit of quantification

The data obtained from 5ppm standard that was used to test precision was used together with slopes obtained from calibration standards to calculate LOD and LOQ in respective metals.

**Table 22.** The LODs and LOQs of heavy metals of interest analysed by ICP-OES

| Heavy metals | LOD | LOQ |
|---|---|---|
| As | 0.0014 | 0.3105 |
| Cu | 0.0010 | 0.0034 |
| Mn | 0.0003 | 0.0011 |
| Ni | 0.0063 | 0.0209 |
| Pb | 0.0256 | 0.0853 |
| Zn | 0.0017 | 0.0056 |

LOD = (3*SD)/S LOQ= (10*SD)/S, SD; standard deviation, S; slope, LOD; Limit of detection, LOQ; Limit of quantification

## CHAPTER 5. CONCLUSION

*Chrysopogon Zizanioides (CZ)* managed to successfully grow and survive in Selibe-Phikwe Cu and Ni mine tailings, with acidic condition (pH =6.24±0.10) as well as in complex combination of Ni, Cu, Mn, Zn, Pb and As. The varying six soil amendments through the influence of Arbuscular Mycorrhizal Fungi (AMF) and Ethylene Diamine Tetra Acetic Acid (EDTA)) in the presence of cow manure, assisted the CZ grass to be able to withstand and tolerate the heavy metals that accumulated in the roots of the grass. AMF inoculated samples (T4, T5, T6) had high biomass for both the roots and the total plant as compared to non-inoculated samples (T1, T2, T3) due to the ability of fungi to enhance nutrient uptake and consequently promoting the growth rate of the plant [18]. On the other hand, concerning EDTA-AMF interaction, it has been observed that EDTA has the ability to reduces the mycorrhiza fungi since the treatment (T4) with only AMF and No EDTA showed high mycorrhiza percentage compare to that with the mixture of EDTA AND AMF (T5 and T6) and those with only EDTA (T2 and T3) showed a declined mycorrhiza percentage as compare to the treatment with no soil amendment (T1). It is worth noting that it was observed that addition of organic matter (cow manure) and inoculation of fungi (AMF) enhanced survival rate, growth rate and biomass of CZ grown on mixture of different heavy metals contaminated soils. Even so, application of organic matter may have had an impact in low heavy metal concentrations of this study, literature found out that even though it can increase As absorption, it can reduce metal solubility, decrease Pb, Zn, and Cu availability due to the ability of these heavy metals to complex with organic matter and stay attached to the soil [17] [62]. In overall, CZ roots showed the potential to absorb Ni>Cu>Mn>Zn>Pb>As making it one of the qualifying plants for phytoremediation but the accumulation rate can be influenced by the nature of the metal and its interaction with CZ as well as chemical modifications. In this study based on the concentration levels and BAF, it can be concluded that CZ a hyperaccumulator of Ni and an excluder of As.

The extracted essential oil from *chrysopogon zizanioides* roots grown in Cu-Ni mine in Selibe-Phikwe in consideration of all the different treatment samples, had a total of fifty-nine identified compounds (>94% of the extracted constituents) even though over 250 compounds can be found in the same plant in other countries [16]. These essential oils are found to be mainly constituted with sesquiterpenes, predominantly sesquiterpene alcohols. The major constituents comprise of 'fingerprint' constituents of CZO, being khusimol, isovalencenol, α-vetivone, and β-vetivone among other constituents regarded as "fingerprint" of CZ essential oil. However, they are not major constituents nor available in some treatment samples. The statistically proven highly significant percentage yield attained, ranged from 0.26% to 0.95%.

The CZO extracted via UAHD from the CZ roots contaminated with heavy metals (Ni, Cu, Mn, Zn, Pb, As) showed that heavy metals were not co-extracted with the essential oil. Thus, based on analysis of the key components (i.e., khusimol), the heavy metals did not inflict significant alteration of the composition. Even so, the number of constituents varies per treatment, it has been observed that EDTA is antifungal therefore combining the amendments induce metal toxicity/stress to CZ hence more constituents are produced. Additionally, more constituents can be produced with no AMF inoculation and EDTA application but in the presence of just organic matter. This study showed that splitting EDTA and introducing AMF results in less metal stress and consequently less constituents produced. Introducing EDTA

only regardless of single and splitting applications also result in less constituents possibly metal to metal interactions were not stressful enough to CZ even though such treatments absorbed more of some of the metals. Bio- conversion of one compound to the other by fungi or plant itself in response to metal stress can also be a reason to drastically decreasing number of constituents.

The percentage yield varied across the treatments; it has been observed that AMF improve CZ essential oil yield due this fungi bio- conversions but this can also be governed by the plant-fungus interaction as well as environmental conditions. The combination of organic matter and AMF also increase the oil content. The EDTA application also had an impact in improved oil content since CZ had to initiate defence mechanism for the available heavy metals. As observed with treatment with no heavy metals nor AMF and EDTA (control), the yield was low due to no metal stress triggering the production of essential oils.

# CHAPTER 6. FUTURE RESEARCH WORK

For future work in this research;

- The cultivation procedure can be altered from multi-elements matrix to single elements so as to check how CZ will respond in terms of bioaccumulation of individual heavy metals.
- Different extraction methods should be tried to check if they also have an impact on the attained results especially the CZ essential oil percentage yield and constituents' compositions.
- The analysis of CZ constituents was performed by GC-FID in this study, gas chromatography- mass spectrometer (GC-MS) can be used to as an alternative. Similarly, for heavy metals, in this study ICP-OES was used but inductively coupled plasma- mass spectrometer (ICP-MS) can also be used.
- This study was performed in Palapye, Botswana, the CZ grass can be cultivated in different part of Botswana to observe if there is any variance.

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