

**Quantitative and qualitative optimization of antimicrobial bioactive constituents of
Helichrysum cymosum using hydroponics technology**

by

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DECLARATION

I, Yonela Matanzima declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

12 December 2014

Signed

Date

DEDICATION

This work is dedicated:

- To my husband Bathandwa Nduku and my children Kwakhanya Qhawe and Buqaqawule Lumi-Uthando.
- My father (Mboneli William Matanzima) for his love and support, you always encouraged us to study.

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LIST OF ACRONYMS

°C	degrees Celsius
ANOVA	Analysis of Variance
B	Boron
Ca	Calcium
CaCl ₂	Calcium chloride
CuSO ₄	Copper Sulphate
Cu	Copper
CPUT	Cape Peninsula University of Technology
ESI-MS	Electrospray Ionisation Mass Spectrometry
Fe	Iron
Fe-EDTA	Ferric Salts of Ethylenediaminetetraacetic acid
HSD	Honest Significant Difference
INT	<i>p</i> -iodonitrotetrazolium
K	Potassium
KCl	Potassium chloride
KH ₂ PO ₄	Monopotassium phosphate
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
MIC	Minimum Inhibitory Concentration
Mg	Magnesium
MgSO ₄	Magnesium sulfate
MM	Molecular Mass
Mn	Manganese
MnCl ₂ .4H ₂ O	Manganese (II) Tetrahydrate
MoO ₃ .H ₂ O	Molybdic acid
m/z	mass-to-charge ratio
N	Nitrogen
NH ₃ NO ₄	Ammonium nitrate
PPM	Parts Per Million
RH	Relative Humidity
SANBI	South African National Biodiversity Institute
TA	Total Activity

T_R	Retention Time
P	Phosphorus
Zn	Zinc
$ZnSO_4$	Zinc Sulphate

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Abstract

The high demand for medicinal plants has favoured over-exploitation of wild plants. The search for alternative and sustainable methods of medicinal plant cultivation is imperative and desirable. Biotechnological approaches particularly hydroponic technology has the potential for large scale plant cultivation and production of secondary metabolites. The current study aims at optimizing the production of antimicrobial secondary metabolites by an indigenous South African medicinal plant species (*Helichrysum cymosum*) through hydroponics N and K fertilization. In Chapter 1, the conceptual framework and justifications of the study are presented. In Chapter 2 the research objective was to discern the optimal potassium (K) supplement level for *H. cymosum* by evaluating the effects of different hydroponic K levels on growth, K-leaf content, and anti-*Fusarium oxysporum* f.sp.glycines (Ascomycota: Hypocreales) and total activities. Six weeks old seedlings of *H. cymosum* were treated with varied concentrations of K in the form of potassium chloride, potassium nitrate and monopotassium phosphate (58.75, 117.5, 235 and 470 ppm). These concentrations were based on a modification of Hoagland's hydroponic nutrient formula. Plants were maintained under greenhouse conditions and growth parameters (plant height and number of leaves) were recorded weekly. At 8 weeks post treatment, plants were harvested and fresh weights were recorded and tissue nutrient content analysed. Sub-samples of the aerial parts of plants grown in the different treatments were air dried, extracted with acetone and tested against *F. oxysporum*. Plants exposed to 235 ppm K showed a marked increase in leaf number, plant height and fresh weight. Overall there was no significant difference ($P > 0.05$) among the treatments with respect to tissue nutrient content; K ranged from 3.56 ± 0.198 to 4.67 ± 0.29 %. The acetone extraction yield increased with increasing K fertilization: 58.75 ppm (16.67 ± 2.35 mg), 117.5 ppm (22.5 ± 4.79 mg), 235 ppm (210 ± 38.5 mg) but dropped to 40 ± 4.08 mg at 470 ppm K. Results from the anti-*F. oxysporum* bioassay showed that 58.75 and 235

ppm K treatments produced the most bioactive acetone extracts; MIC values of 0.49 and 0.645 mg/l, respectively. Acetone extracts obtained from plants exposed to 235 ppm K yielded the highest total activity, comparatively ($P < 0.05$). In conclusion, the optimum nutrient K level for growing *H. cymosum* hydroponically was 235 ppm.

Chapter 3 focused on another important macro nutrient N and the objective was to determine the optimum nutrient requirements for growing the medicinal plant, *Helichrysum cymosum* (L.) (Asteraceae), hydroponically. Experiments were conducted to assess the effects of varied nitrogen (N) concentrations supplied as nitrate and ammonium on growth, tissue nutrient content, antimicrobial and total activities of acetone extracts of aerial parts. Treatments were based on a modified Hoagland's nutrient formula. Six week old rooted cuttings were treated with 52.5 ppm, 105 ppm, 210 ppm and 420 ppm of N. Leaf number and stem height (cm) were recorded at weekly intervals and leaf analysis conducted. The effects of N treatments on plant growth parameters varied significantly among treatments; 52.5 ppm of N yielded the tallest plants (height) [19.4 ± 0.7 cm], while 105 ppm N yielded the maximum leaf number (68.1 ± 6.2) as well as maximum fresh weight of aerial parts was obtained with 105 ppm (15.12 ± 1.68 g). Nitrogen content of plant tissue ranged between 0.53 ± 0.03 and $4.74 \pm 0.29\%$ (d, f, 3, 12; $f=14$; $P \leq 0.002$) depending on treatments. Powdered aerial parts (5 g) of *H. cymosum* obtained from the different N treatments were extracted with 100 ml of acetone. N treatment significantly affected the yield of crude extracts, which ranged from 87.5 ± 15.5 (52.5 ppm) to 230 ± 23.5 mg (105 ppm). Acetone extracts of plants that were exposed to varied N treatments were screened for anti-*Fusarium oxysporum* activity using minimum inhibitory concentration (MIC) method. The MIC value (0.073 ± 0.014 mg/ml) obtained with acetone extracts of plants exposed to 52.5 ppm N was significantly lower compared to the MICs of the other N treatments (105 [0.47 ± 0 and 0.705 ± 0.135 mg/ml], 210 [0.234 and 0.47 mg/ml] and 420 ppm [0.29 ± 0.101 mg/ml]) at 24 and 48 hours respectively. However, the

total activities of extracts obtained among the four N treatments, which ranged from 0.062 ± 0.02 to 0.26 ± 0.06 ml/g was not statistically different at 24 or 48 hours ($P > 0.05$). LC-MS analysis of acetone extracts of *H. cymosum* plants obtained from the four treatments hinted that known anti-microbial agents such as apigenin, quercetin, kaempferol, helihumulone and quinic acids were present in the extracts and the quantity of helihumulone increased with increased nutrient N level.

These results suggest that *H. cymosum* may be cultivated hydroponically and that the antimicrobial activity and/or the phytochemical profile of the crude acetone extracts is affected by nutrient nitrogen levels. Hydroponic cultivation of plants may be able to alleviate to an extent the pressure on wild medicinal plants.

Chapter One

Introduction, background to research problem and literature review

1.1. Introduction

In developing countries including South Africa, medicinal plants are widely used for the treatment of many human and animal diseases. Exploitation of plant resources for the treatment of human and animal diseases has placed significant pressure on plant biodiversity. Demand for plant-derived medicines has created a large business in indigenous plants in South Africa, currently estimated to be worth R270 million annually (Dold & Cocks, 2002; Wiersum et al., 2006). Well-known examples of plants that are currently traded in South Africa include; *Artemisia afra* (Asteraceae), *Melianthus comosus* (Melianthaceae), *Aloe ferox* (Asphodelaceae), *Aloe arborescens* (Asphodelaceae), *Salvia Africana-caerulea* (Lamiaceae), *Helichrysum cymosum* (Asteraceae) (van Wyk et al., 2009). *H. cymosum* is used in South Africa as a medicinal plant for the treatment of animal and human diseases (Heyman, 2009). This plant is rich in secondary metabolites including helihumulone, helichromanochalcone and 5-hydroxy-8-methoxy-7-prenyloxyflavone (Bohlmann et al., 1979; Heyman, 2009). These compounds have antibacterial, antioxidant, anti-inflammatory, anti-allergic or anti-diabetic properties (Eroglu et al., 2009; Karimi et al., 2011).

Plant secondary metabolites are thought to be responsible for the antimicrobial and insecticidal activities of plant extracts (Kumar et al., 2005). Even though these plant-derived extracts or compounds are exploited for the treatment of human and animal diseases, in nature, plants use these secondary metabolites primarily for defence against pests and microbial pathogens. Regardless of adequate knowledge on the function of plant secondary metabolites and their mechanism of action against plant microbial pathogens, astonishingly, present-day plant disease control depends primarily upon

application of synthetic chemical fungicides, microbicides or insecticides. These substances are not easily biodegradable and have a potential to exert toxic effects on humans and wildlife.

Large scale production of secondary metabolites by plants is crucial for sustainability of medicinal plant use. However, the production of secondary metabolites in plant depends on endogenous and exogenous factors (Vu et al., 2006). The use of innovative agronomic technologies such as hydroponics can optimize the manufacturing of natural molecules that have pharmaceutical and cosmetic significance. Plants grown hydroponically are usually within boundaries of a glasshouse/greenhouse which are characterized by meticulous control of environmental conditions. Furthermore, hydroponic plant cultivation has an advantage over field-grown plants in that production is all year round. Hydroponics makes it possible to manipulate the micronutrients of plant growth media and evaluate the effects of different nutrient mixtures, thus avoiding the inherent biases such as variations in pre-existing soil nutrients levels and physical properties that are common with soil cultivation of plants.

According to Amtman and Armangaud (2009) the lack of nutrients such as nitrate, potassium, phosphorus and sulphate has an effect on the quantity and type of metabolites produced by a plant. Nitrogen is required for the synthesis of nucleic acids and proteins, phospholipids and secondary metabolites (Toor et al., 2005; Amtman & Armengaud, 2009). The plants take up nitrogen either as inorganic ions (NH_4^+ or NO_3^-) or as organic N (Toor et al., 2005; Amtman & Armengaud, 2009). According to Toor (2005), when nitrogen is easily accessible, plants will make compounds that are high in nitrogen content (e.g. proteins for growth) and when nitrogen is inadequate, metabolic process changes to the direction of carbon-containing compounds such as starch, cellulose and non-N-containing secondary metabolites such as phenolic and terpenoids. Nutrients can affect both the variety and the levels of alkaloid in the plant. For example, inoculation of a hydroponic nutrient device with *Agrobacterium*

rhizogenes (wild strain soil bacterium) led to a 4-fold increase of alkaloid productivity in *Datura innoxia* plants compared to control treatments after 32 days of culture (Vu et al., 2006). Pyrrolizidine alkaloid (PA) production takes place in the root and total PA content of plant are influenced by root biomass and nitrogen-phosphorus-potassium (NPK) fertilizers (Hol, 2011), the addition of hormones that influence root growth in a growth medium can also influence production of alkaloids.

This study could have substantial benefits for animal and plant health by developing methods to optimize and guarantee production of high quality plant extracts from medicinal plants. Further, the research project may contribute to the conservation of some rare and endangered species and the preservation of cultural practices especially the traditional use of plants for the treatment of diseases.

1.2. Structure of dissertation

The study comprises three chapters, which are briefly described.

Chapter One: Introduction, problem statement and literature review

Chapter One presents the conceptual framework of the research, provides scientific justification of the study and the aims and specific objectives of the study.

Chapter Two: The effects of varied potassium concentration on growth and anti-*Fusarium oxysporum* activities of leaf extracts of hydroponically-cultivated *Helichrysum cymosum* (L.) D. Don (Asteraceae)

The main objective of this chapter was to determine optimal potassium supplement level for growing *H. cymosum* by evaluating the effects of the different K levels on growth, K-leaf content, and anti-*F. oxysporum* and total activities. The research justification, materials and methods, results and discussion are presented.

Chapter Three: Effect of varying levels of nitrogen on plant growth and anti-*Fusarium oxysporum* (Ascomycota: Hypocreales) activity and secondary metabolite profiles of foliage extracts of hydroponically cultivated *Helichrysum cymosum* (L.) D. Don subsp. *cymosum* (Asteraceae).

Chapter Three focuses on another macro nutrient, nitrogen. The objective of this chapter was to evaluate the effects of nitrogen levels on growth parameters, secondary metabolite profile and *in vitro* anti-*F. oxysporum* activity of crude extracts of hydroponically-cultivated *H. cymosum*. The research justification, materials and methods, results and discussion are presented. Importantly results of the chemical analysis of effects of N on the production of secondary metabolites are presented and discussed.

1.3. Background to the research problem

Medicinal plants have an extensive contribution to the lives of rural people. Besides traditional healers who routinely use plants for healing, more people are involved in collecting, trading and utilizing medicinal plants. Medicinal plants have been used for centuries in developed and developing countries (Revathi & Parimelazhagan, 2010; Street & Prinsloo, 2013). They are traded in the formal and the informal markets. Recent studies state that 70 to 80% of the world's inhabitants use medicinal plants for primary health care purposes (Roussis *et al.*, 2000; Loundou, 2008; Magoro, 2008; Heyman, 2009; Nahashon, 2013; Sobiecki, 2014). Medicinal plants are also exploited for their protective properties against plant diseases (Tomas-Barberan *et al.*, 1990; Okemo, 2003; Shoby & El-Feky, 2007; Bissa *et al.*, 2011; Mahlo *et al.*, 2011; Pal & Kumar, 2013). The use of medicinal plants varies from country to country, culture, history, philosophy and personal beliefs (Magoro, 2008). South Africa is one of the countries with a lot of people who use medicinal plants. The trade for medicinal plants is estimated to be worth

R270 million, annually (Dold & Cocks, 2002). According to Sobiecki (2014), globally, products that are derived from traditional medicine are estimated to be worth R2.9 billion per year.

Over-exploitation of plant resources for the treatment of human and veterinary diseases could accelerate extinction of some plant species and exert significant pressure on plant biodiversity. Demand for plant derived medicines has created a huge business for medicinal plants in South Africa and globally. South Africa has an abundance of medicinal plants that are used in traditional treatments of various diseases. Examples of such plants include *Pelargonium reniforme* (Geraniaceae) and *Helichrysum cymosum* (Bougastos *et al.*, 2004; Albarak *et al.*, 2010a & b; Brendler & van Wyk, 2008). Scientific investigation and information on the therapeutic potential of some medicinal plants are limited (Mativandlela *et al.*, 2006). Due to the high demand for medicinal plants and the realization that some wild plants are threatened because of over-exploitation, commercial cultivation of medicinal plants is being recommended; albeit that commercial cultivation may also lead to environmental degradation and loss of genetic diversity as well as loss of incentives to conserve wild populations (Schippmann *et al.*, 2002).

The search for alternative and sustainable methods of medicinal plant cultivation is imperative and desirable. Biotechnological approaches particularly hydroponic technology has the potential for large scale plant cultivation and production of secondary metabolites. Therefore, the use of hydroponics as one of the strategies for cultivating some of the rare and endangered medicinal plant species might favour a decline in the rate of species extinction by reducing the extent to which wild plant populations are being harvested. Also, plants grown hydroponically can be manipulated to optimize production of secondary metabolites (Hayden, 2006). Environmental factors such temperature, light intensity and nutrient blends

can be controlled. Studies have shown that changes in nutrient concentrations can lead to secondary metabolites production (De La Rose *et al.*, 2002; Le Bot *et al.*, 2009). It is therefore hypothesized that hydroponic technology can be used to increase the quantity and quality of secondary metabolites that are needed for treatment of microbial diseases of plants and humans.

1.4. Statement of the research problem

Due to increasing demand and over-exploitation of wild medicinal plants, there is need to implement efficient and sustainable agro-technologies such as hydroponic cultivation of medicinal plants, which are convenient platforms for manipulating and controlling plant growth and for quality standardization. Nutrient composition is one of the most important factors that influence plant growth and secondary metabolite production in plants. Hydroponic systems can be used to evaluate the effects of macro nutrients (nitrogen and potassium) supplementation on growth, antifungal activities and secondary metabolite profiles of some indigenous South African plants such as *H. cymosum*.

1.5. Literature review

The genus *Helichrysum* (Miller) from the Asteraceae family consists of 500-600 species found all over the world, with 245-250 found in South Africa (Van Vuureen *et al.*, 2006; Lourens *et al.*, 2008; Ncube, 2008; Street *et al.*, 2008; Heyman, 2009; Albayrak *et al.* 2010a & b; Guinoiseau *et al.*, 2013). The species of this genus are aromatic, woolly shrubs which have yellow flowers and are about 60 cm tall. Species of the genus *Helichrysum* possess diuretic, anti-inflammatory, antioxidant, biological or antimicrobial properties and are used in folk medicine for treatment of snake bites, urinary disorders, sciatica, hemias, headache, inflammation, respiratory ailments such as allergies, asthma, bronchitis and coughs or harmful fungi and microorganisms (Tomas-Barberan *et al.*, 1990; Meyer & Dilika, 1996;

Swartz, 2006; van Vuuren *et al.*, 2006; Lourens *et al.*, 2008; Albayrak *et al.*, 2010[a & b]; Karimi *et al.* , 2011; Otang *et al.*, 2012; Omaruyi *et al.*, 2012; Esmaeili, 2013;Viegas *et al.*, 2014) (Table 1.1).

Table .1.1: Ethnobotanical use of *Helichrysum* species in different regions

Species	Traditional use	Region	Referencs
<i>H. odoratissimum</i>	Treat female sterility, Eczema, menstrual pain Abdominal pains, heartburn,	Rwanda	Swartz, 2006
	Coughs, colds and wounds	southern Africa	Swartz, 2006
	<i>H. arenarium</i>	Bile regulatory, diuretic effects	
	Chlorectic, hepaprotective	Europe	Lourens, 2008
	Detoxifying activities, Antifever, anticold, wound healing, Treat skin problems, diuretic effects Disinfectant	Mediterranean	Viegas et al., 2013
<i>H. italicum</i>	Bileregularity, diuretic effects	Europe	Swartz, 2006
	Allergies, colds, cough, skin		Viegas et al., 2013

	Inflammation, infections		
	and sleeplessness, toothache		
<i>H. stoechas</i>	Antifever, anticold, wound		Swartz, 2006
	Healing and anti-infectious		
	Kidney disorders, toothache		
<i>H. foetidum</i>	Poltice for infected sores	Eastern Cape	Swartz, 2006
			Viegas et al., 2013
<i>H. appendiculatum</i>	Circumcision rites	Eastern Cape	Swartz, 2006
<i>H. pedunculare</i>	Circumcision rites	Eastern Cape	Swartz, 2006
<i>H. nitens</i>	Fungal disease	Malawi	Swartz, 2006
<i>H. melaleucum</i>	Cough, pharyngitis	Europe	Viegas et al., 2013
	Bronchitis, cardio-		
	tonic		
<i>H. pendunculatum</i>	Inflammation and	Eastern Cape	Viegas et al., 2013
	Wound healing	South Africa	

1.5.1. Taxonomic classification and general characteristics of the species *H. cymosum*

H. cymosum (L.) D. Don subsp. *cymosum* (synonyms: *Gnaphalium cymosum* L., *Helichrysum cymosum* (L.) D. Don, *Lepiscline cymosum* (L.) Cass) common name: Impepho is a plant species that grows up to 60 cm tall, it has tinny greyish-white woolly twigs compactly concealed with leaves (Fig 1.1). *H. cymosum* has a ring of modified bracts that are brownish, tawny, straw-coloured or yellow. The tips of the inner ring of modified bracts are yellow. The leaves are linear-elongated to oval-elongated, short narrow points or rarely acuminate, the upper surfaces of the leaves are covered in paper-like hairs (Heyman, 2009). It flowers through summer, between September and April, but mostly in late summer and autumn, with cheerful canary-yellow flowers in flat-topped flower heads that look like masses of small circles. Each flower head is a mass of 6–20 flowers. The flowers normally have even tips, and a whorl of numerous hairs. *H. cymosum* can be further divided into subspecies which include; *Helichrysum cymosum* subsp. *calvum*, *H. cymosum* subsp. *cymosum* and *H. cymosum* subsp. *forskalii*. *H. cymosum* subsp. *calvum* and *cymosum* are the two that are well-recognised. *H. cymosum* subsp. *calvum* occurs in the Savannah along the Drakensberg (Eastern Cape), Kwa-Zulu-Natal and Lesotho border to the low Berg (Mpumalanga) (Zenze, 2012).



Fig.1.1: Photograph of *H. cymosum* showing yellow inflorescence (Source: <http://www.ekapa.ioisa.org.za/ekapaaf/module10/M10perennialsandcovers.htm>).

1.5.2. Geographical Distribution of *H. cymosum* subsp.*cymosum*.

H. cymosum subsp. *cymosum* develops in large trailing clusters, frequently in humid regions such as the basins amongst banks, among bushes in Cape thicket and on forest boundaries. It arrays from the Western Cape, as well as the Cape Peninsula, eastwards beside the coastal mountain ranges of the Eastern Cape and as far as Lake St Lucia in KwaZulu-Natal (Heyman, 2009; Zenze, 2012) (Fig 1.2).

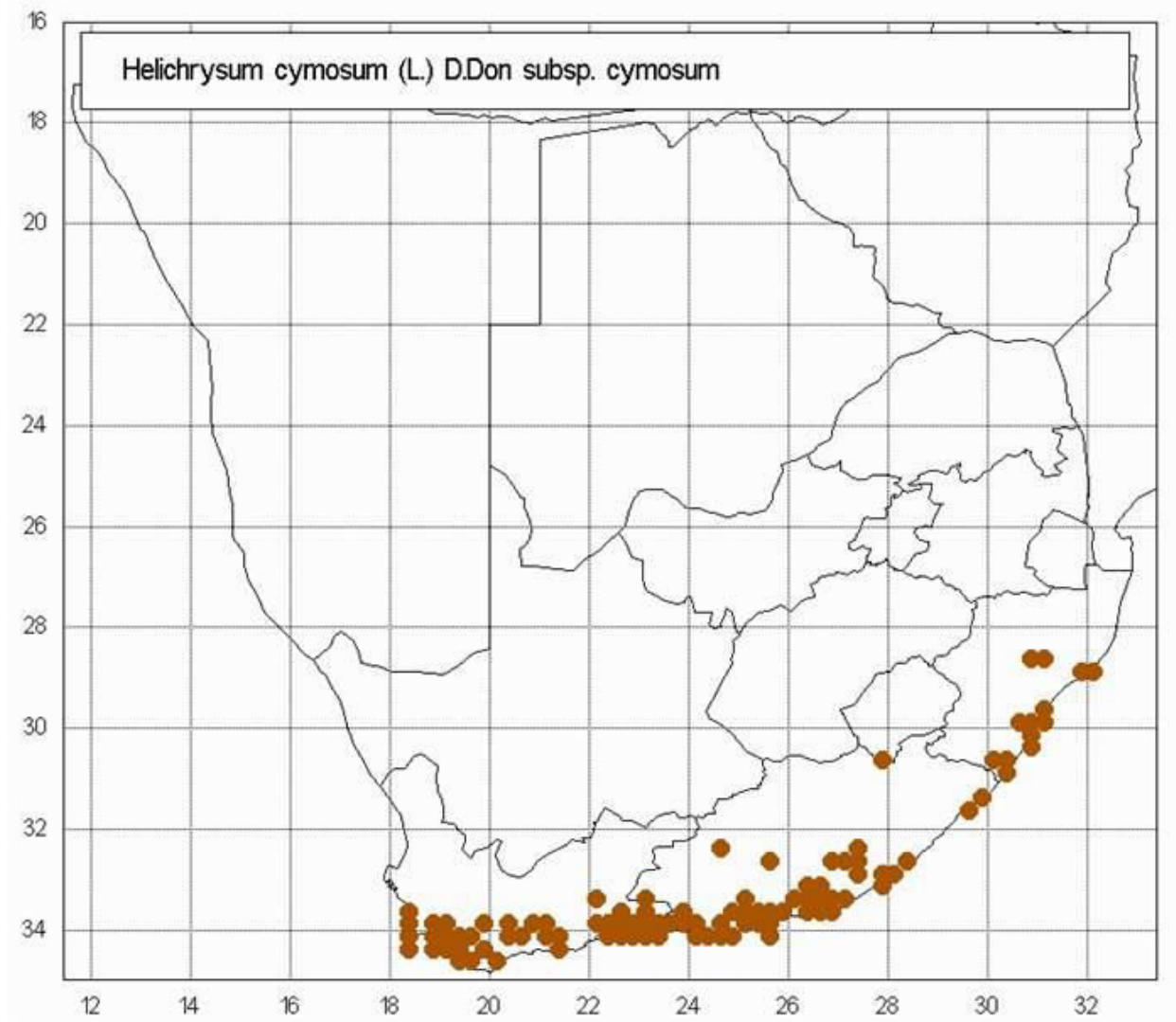


Fig.1.2: Map showing distribution of *H. cymosum* subsp. *cymosum* (source: <http://www.capecontours.co.za/chapmans-peak-fynbos-search-rescue/>).

1.5.3. Propagation

H. cymosum can be propagated through seeds and cuttings. Seeds can be sown in autumn or spring and cuttings can be rooted in sand-based medium with fertilizer and compost (Zenze, 2012).

1.5.4. Some examples of bioactive secondary metabolites from the genus, *Helichrysum*

Previous studies have indicated that the genus *Helichrysum* possesses secondary metabolites which are responsible for the anti-inflammatory, antioxidant, biological and antimicrobial properties. These include apigenin, kaempferol, helihumilone, quercetin, and quinine (Bohlmann et al., 1979; Süzgeç et al., 2004; van Vuuren et al., 2006; Shoby & El-Feky, 2007; Albayrak et al., 2010a & b; Orhan et al., 2014).

Apigenin (5, 7, 4'-trihydroxyflavone) is a flavonoid which is abundant in different fruits, vegetables, medicinal plants, parsley, onion, oranges and paper mulberry. It can reduce high blood pressure and also possesses antioxidant, antitumor and spasmolytic properties (Gradolatto et al., 2004; Babcook & Gupta, 2012; Liu et al., 2013). Apigenin was previously isolated from methanolic extracts of *H. obconicum* (Gouveia & Castilho, 2011). Nayaka et al. (2014) have demonstrated that apigenin has antimicrobial activity against *P. aeruginosa*, *K. pneumonia*, *S. typhimurium*, *P. mirabilis* and *E. aerogenes*.

Kaempferol is a flavonoid, which can be found in tea, broccoli, grapefruit, strawberries, apples and beans. Kaempferol has cardio tonic activity, antiulcer activity and antioxidant activity (Chen and Chen, 2013; Yoshida et al., 2008; Monach et al., 2013; Waghmare et al., 2010; Yu et al., 2013). Siegelin et al. (2008) stated that kaempferol has anticancer activity. Kaempferol and kaempferol-3-glucoside were previously identified in solvent extracts of *H. obconicum*, *H. italicum* and *H. stochas* (Sobhy & El-Feky, 2007; Gouveia & Castilho, 2011; Guinoiseau et al., 2013).

Quercetin is a flavonol which is abundant in medicinal plants, green tea, green apple, vegetables, red wine, lemon, seeds, flowers and bark (Phani et al., 2010). It is known to possess antioxidant, anti-inflammatory, anti-aggregatory, anti-carcinogenic, and vasodilating

activities (Ferguson, 2001; Wiczowski et al., 2003; Erlund, 2004; Murakami et al., 2008; Phani et al., 2010; Gregory & Kelly, 2011). Phytochemical analysis of ethanol extracts of *H. stochas* revealed that the compounds (kaempferol, apigenin glucosides and quinine derivatives [caffeoylquinic acid and dicaffeoylquinic acid]) are present in the plant (Shoby & El-Feky, 2007). Quercetin was previously isolated from *H. melanacme* (Lall et al., 2006).

Quinine is an alkaloid which is used as a salt, sulphate, bisulfate, hydrochloride and dichloride for the prevention and treatment of malaria, to relieve leg cramps (Ncube et al., 2008; Aiyegoro, 2010). Van Vuuren et al. (2006) reported that quinine and chloroquine has biological activity. Quinine isomers (isomers of caffeoylquinic acid [37 %] and 2 isomeric dicaffeoylquinic acid [26.3 %], were identified in *H. stochas* (Sobhy & El-Feky, 2007). Quinic acid, its isomers and derivatives were identified from *H. obconicum* (Gouveia & Castilho, 2011).

Helihumulone is a phloroglucinol derivative with proven antimicrobial activities (van Vuuren et al., 2006; Choi et al., 2012).

1.6. Commercial production of medicinal plants in South Africa

The demand for medicinal plants globally is on the increase and it is estimated up to 700,000 tonnes of plant material is consumed annually to the value of about 150 million US dollars (Wiersum et al., 2006). In a review paper, Street & Prinsloo (2013) presented 10 prominently used South African medicinal plants; *Agathosma betulina* (Rutaceae), *Aloe ferox* (Asphodelaceae) *Aspalathus linearis* (Fabaceae), *Harpagophytum procumbens* (Pedaliaceae), *Hypoxis hemerocallidea* (Hypoxidaceae), *Merwillia natalensis* (Hyacinthaceae), *Pelargonium sidoides* (Geraniaceae) *Siphonochilus aethiopicus* (Zingiberaceae), and *Sutherlandia frutescens* (Fabaceae) and discussed their traditional uses, scientific validation, current cultivation and commercialisation developments. Both potential opportunities and setbacks

with regards to future research and development were also discussed. Recent studies on the perception of cultivation of medicinal plant species indicated that very high proportions (over 69%) of respondents are willing to buy and make use of cultivated medicinal plants (Loundou, 2008). This trend suggests that developing efficient and sustainable agrotechnology should be one of the focal areas for research.

1.7. Challenges associated with soil cultivation of medicinal plants

There are many constraints to the cultivation of medicinal plants. For example, not all plant species are adapted to growing in a wide range of environmental conditions. Factors such as pathogens, heavy metals, pesticides can restrict plant geographical distribution (Bourgard et al., 2001; Zheng et al., 2006; Montanari et al., 2007). Some plants may take too long to grow compared to when they are grown hydroponically, therefore, these plants may not produce sufficient medicinal materials to meet demand. In some species, seed dormancy may seriously hamper continuous productivity (Zheng et al., 2006). Also, geographical variation in the profile of the chemical constituents among plant populations of the same species is common (Smadja & Butlin, 2009; Wason et al., 2013; Gratani, 2014). Duplication of results of experiments involving soil of different geographical locations is not easily achievable because soil samples are inherently unique in their biotic and abiotic characteristics (Andrew et al., 2012), which can potentially influence the standardization and uniformity of the quality of medicinal materials. According to Singh (2009), information on the propagation of medicinal plants is available for less than 10% and agrotechnology is available only for 1% of the total known plants globally. Other constraints to cultivation of medicinal plants include market uncertainty, and the sourcing of permits from government agencies for cultivation of medicinal plants.

1.8. Different methods of optimizing secondary metabolites

1.8.1. Genetic engineering

Genetic engineering has allowed the production of plants with modified composition of secondary metabolites (Siebert et al., 1996; Verpoorte & Memelink, 2002). Plant secondary metabolites that are found in small quantities may be produced through genetic engineering (Breiting et al., 2013). Genetic manipulation gives opportunities to identify secondary metabolites that have never been seen before (Chaudhary et al., 2013). Genes can be used to alter certain metabolic pathways, and enhance the production of desired secondary metabolites in plants (Verpoorte & Memelink, 2002). For examples, 4-Hydroxybenzoate was expressed in tobacco plants as a result of the bacterial gene manipulation (Siebert et al., 1996) and the concentration and enzyme action of scopolamine improved in genetically engineered roots of *Hycosyamus muticus* (Hussain et al., 2012).

1.8.2. Endophytes

Endophytes are the fungus or bacteria which live within a plant without causing harm to the plant (Maheshwari, 2006; Schulz & Boyle, 2006). Endophytes produce chemical substances that defend the plant they are in and increase the survival value of the plant. They are the basis of hypothetically imperative medicinal compounds (Stroubel, 2003; Yu et al., 2010). Their presence in plants may stimulate the production of secondary metabolites by host plants, in other words they may act as vaccines. Schulz et al. (2002) stated that in 12 years, ±6500 endophytic fungi were isolated from herbaceous plants and trees. The medicinal compounds that have been isolated from endophytes include: alkaloids, peptides, steroids, terpenoids, phenols, quinones and flavonoids. These compounds have antimicrobial, anticancer, antioxidant, antidiabetic, antiviral and anti-alzheimer properties (Yu et al., 2010; Pimental et al., 2011; Shukla et al., 2014).

1.8.3. Tissue culture

Plant tissue culture is a method that has been used in various countries (Rout et al., 2006; Gahakwa et al., 2012). Plant tissue culture could be used to increase production of secondary metabolites which can be used as food additives, nutraceuticals, pharmaceuticals and pesticides (Smetanska, 2008; Shipla et al., 2010). Example of plants and secondary metabolites that have been produced through tissue culture include: *Panax notoginseng* (Ginseng saponin), *Coptis japonica* (Berberine), *Anchusa officinalis* (Rosmanic acid), *Capsicum frutescens* (Capsaicin), *Matricaria chamomilla* (Herniarin), *Scutellaria baicalensis* (glucoside, 5,7,2',6'-Tetrahydroxyflavone 2-O- β -glucopyranoside) (Chattopadhyay et al., 2002; Jedinak et al., 2004; Shipla et al., 2010).

Plant tissue culture gives an opportunity to produce extensive secondary metabolites. The conditions in which the plants are cultured in are easily controlled (Chattopadhyay et al., 2002; Gaosheng & Jingming, 2012; Dave et al., 2014) and reduce the need for excessive use of pesticides (Chattopadhyay et al., 2002). Tissue culture and cell culture can be used to propagate plants that are endangered (Al-Jabary & Arafah, 2010). However, tissue culture has many disadvantages compared to hydroponics. The plants have different root systems so by growing them in tissue culture the roots might not expand or grow as required. In tissue culture there are high chances of contamination (Conn *et al.*, 2013). Tissue cultured plants are small, weak and may have unwanted characteristics. In addition, the growing chambers or facilities are expensive (Thiart, 2003).

1.8.4. Hydroponics technology

According to Jehnson (1999) and Hayden (2006), hydroponics technology is a technique of growing plants in a nutrient solution (water and fertilizers) with or without the use of artificial medium (e.g. sand, rockwool, vermiculite, gravel, peat moss, coir, sawdust etc) to

provide a mechanism of support. Liquid hydroponics systems have no other supporting medium for the roots of the plants. Aggregate hydroponics have a solid medium of support (Jenson, 1999). The advantages of using hydroponics include high-density maximum crop yield, crop production in areas where good soil for production is not available, plants that are off-season can be grown while they are off-season and temperature can be manipulated (Jenson, 1999; Koochakan et al., 2004). Factors such as seasonal changes, development stages, levels of pathogens, geographical differences, and nutrient status of the soil have an effect on the amount of secondary metabolites the plants produce (Johanna , 2007; White et al., 2008).

Horticulturally, hydroponic technology can be used to increase secondary metabolites (Gontier et al., 2002). Hydroponics can favour plant vigour, decrease poisonous levels of plant toxins, increase uniformity and probability of obtaining bioactive extracts (Canter et al., 2007). In hydroponic cultivation of medicinal plants, the target compounds include secondary metabolites, which for the plant regularly serve to mitigate against negative effects caused by varying temperatures and light conditions (e.g. antioxidants), stress (e.g. proline), infection (e.g. flavonoids), or herbivory (e.g. alkaloids)(Canter et al., 2007). Umckalin production in *P. reniforme* has been hypothesized to be induced by water stress (White et al., 2008). Hydroponics technology may reduce the need to exploit *H. cymosum* in the wild as the production of target compounds might be optimized. The broader goal of this study is to improve the quality of secondary metabolites produced by *H. cymosum* through hydroponics technology.

Hydroponics has been used for centuries for growing vegetables and ornamental plants. Recently many researchers are using hydroponics to grow medicinal plants and to manipulate their secondary metabolites (Economakis et al., 2002; Stewart & Lovett-Doust, 2003; Hassanpouraghdam et al., 2010; Kiferle et al., 2011; Sugumaran et al., 2013). There are different types of hydroponic systems that can be used. They include aeroponics, deep culture

system, ebb and flow system, drip system, Nutrient Film Technique (NFT). The drip system is the type of system that was used in this study. There are many advantages of growing plants hydroponically compared to growing them in soil. The plants grow faster, the plants are disease free (Letchamo et al., 2002; Pagliarulo et al., 2004; Sugumaran et al., 2013), insects are easy to control, it is easy to manage what goes in the plant (nutrients) (Sugumaran et al., 2013), less plant death, sterile environment (Zheng et al., 2006), all year round plant production and environmental conditions are manageable (Stewart & Llovet-Doust, 2003). Hydroponics makes it easy to manipulate the quality and quantity of secondary metabolites. In addition genetically modified plants or tissue cultured plants can be mass-produced hydroponically. *H. cymosum* is a very popular medicinal plant in South Africa amongst the community and traditional healers. The plant is harvested from the wild at a very high rate; hydroponics can be used to produce the plant in large quantities.

1.9. Plant macronutrients (nitrogen and potassium)

Major macronutrients such as nitrogen (N), phosphorus (P) and potassium (K) are essential for growth and development (Singh & Mishra, 2012; Haghghi et al., 2011) and often are applied to the soil at high levels in various forms including; ammonium nitrate, urea, ammonium sulphate, potassium nitrate (Shea et al., 2013). Nitrogen is one of the most important nutrients that limit plant development (Zhao et al., 2005; Britto & Kronzucker, 2002). It is involved in many physiological and biochemical processes in plants and in the production of nucleic acids, amino acids, proteins and chlorophyll a well as cell wall (Haghghi et al., 2011). Many studies have demonstrated that increasing the levels of potassium in plants give best vegetative growth and leaf number (Pal & Ghosh, 2010; Shabani et al., 2012) and high vegetative growth and leaf number could equate to high medicinal materials. The medicinal value of a plant is strongly influenced by the chemical profiles of its secondary metabolites.

1.9.1. The influence of nitrogen and potassium on plant secondary metabolites

Nitrogen plays an important role in the production of plant secondary metabolites (Nguyen & Niemeyer, 2008; Babalar et al., 2010). Recent studies have stated that nitrogen deficient plants tend to produce secondary metabolites that are phenolic (Chishaki & Horiguchi, 1997; Hakulinen, 1998; Bénard et al., 2011), whilst abundance of nitrogen suppress the production of phenolic compounds (Chishaki & Horiguchi, 1997). Previous studies have indicated that when plants have N deficiency they tend to have increased concentration of C-based secondary metabolites (Lou & Baldwin, 2004; Le Bot *et al.*, 2009). Ibrahim et al. (2012) stated that the amount of K application influences secondary metabolites. Potassium is important in regulating the production of secondary metabolites.

1.10. Antimicrobial activity of *Helichrysum*

The Asteraceae family is a very diverse and big family. Members of the family are known to possess antimicrobial properties. Previous studies have indicated that some of the plants found in this family can control *F. oxysporum* and other known diseases (Rani & Murty, 2006; Bissa et al., 2011; Llondu, 2012; Llondu, 2013; Pal & Kumar, 2013). Llondu (2013) found that *Aspilia africana* (Asteraceae) methanol extract had high bioactivity against *Curvularia lunatus* and *Collectotrichum gloeosporioides*. Crude extracts of *H. stoechas*, *H. decumbens* and *H. italicum* have anti-microbial activities against *E. coli*, *Candida albicans*, *Penicillium sp*, *Cladosporium herbarum* and *Phytophthora capsicii* (Tomas-Barberan et al., 1990; Mastelic et al., 2005; Shoby & El-Feky, 2007). Crude extracts of *H. pedunculatum* exhibited bioactivities against all Gram-positive bacteria as well as two Gram-negative bacteria, *Enterobacter cloacae* and *Serratia marcescens* (Dilika et al., 2000; Ncube, 2008). *H. cymosum* compound, helihumone, a phloroglucinol-derived compound has shown

significant antimicrobial activities, ranging from 125 µg/ml (*Staphylococcus aureus*) to 16 µg/ml (*Pseudomonas aeruginosa*) (Heyman, 2009; Paiva et al., 2010). According to Paiva et al. (2010), this species can inhibit *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsilla pneumonia*, *Candida albicans*, *C. neoformans*.

1.11. *Fusarium oxysporum* (Hypocreales)

The genus *Fusarium* comprises a diverse group of fungi which include many soil-borne pathogens that can cause plant diseases such as wilts, root, crown, tuber, and bulb rots (Roncero et al., 2003; Song et al., 2003; Kochman, 2007). The fungi of the *F. oxysporum* species complex inhabit soil and plant. Pathogenic strains are responsible for a wide range of destructive and economically important diseases in plants (Roncero *et al.*, 2003). The crops that are often attacked by this group of organisms include tomato (*Lycopersicon esculentum* Mill.), lentils (*Lens culinaris*), sweet potato (*Ipomoea batatas*), wheat (*Triticum aestivum*), yam (*Dioscorea alata*), Cotton (Strange & Scott, 2005; Rajput et al., 2006; Ignjatov et al., 2012). Conventionally, synthetic fungicides are employed for control of these pathogens (Rajput et al., 2006; Haidukowski et al., 2012; Anjorin et al., 2013). However, the setbacks associated with the use synthetic fungicides are many, for examples; methyl bromide which are used to fumigate soil against fungal pathogens and phytophagous insects is known to deplete the ozone layer and development of resistance to synthetic fungicides by fungal pathogens as well the fact that only a small number of effective fungicides are available for control of fungal pathogens warrant the search for alternative anti-fungal agents. Research on the use of medicinal plants for control of plant pests has intensified in recent years (Tomas-Barberan et al., 1990; Llondu, 2010; Aye, 2001; Okemo et al., 2003; Mastelic et al., 2005; Suleiman & Emua, 2009; Nchu et al., 2010; Bissa et al., 2011; Mahlo, 2011; Llondu, 2012; Llondu, 2013; Pal and Kumar, 2013).

1.12. Hypotheses

1. *H. cymosum* can be cultivated hydroponically.
2. Variations of nitrogen and potassium levels will affect growth and yield of *H. cymosum* grown hydroponically.
3. Variations of nitrogen and potassium levels will affect antimicrobial activities and composition of bioactive constituents of extracts of hydroponically cultivated *H. cymosum*.

1.13. The overall aim of the study

The overarching goal of the study is to optimize the production of antimicrobial secondary metabolites by an indigenous South African medicinal plant species (*H. cymosum*) through hydroponics nitrogen and potassium fertilization.

1.14. Specific objectives of the research

- To evaluate the effects of hydroponics N and K with respect to plant growth parameters (height, number of leaves and fresh weight).
- To evaluate the effects of nutrient content (N and K) variations on anti-fungal activities of the plant extracts and production of active secondary metabolites.
- Assess the total activity of *H. cymosum* against *F. oxysporum*.

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Chapter Two

The effects of varied potassium concentration on growth and anti-*Fusarium oxysporum* activities of leaf extracts of hydroponically-cultivated *Helichrysum cymosum* (L.) D. Don (Asteraceae).

2.1. Introduction

Demand for plant-derived medicines has created a large trade in indigenous plants in South Africa which was estimated to be worth approximately R520 million per year in 2006 (Sobiecki, 2014). One medicinal plant that is widely used in South Africa is *H. cymosum*. Members of the genus *Helichrysum* have many medicinal and traditional uses including treatment of respiratory illnesses and gall bladder disorders, removal of kidney stones, use as diuretics, and for wound dressings in circumcision and to invoke the goodwill of ancestors (van Vuuren et al., 2006; Albayrak et al., 2010 a & b; Esmaeli, 2013). Currently, the plant is heavily harvested from the wild and it is common to find traditional healers selling these plants in urban areas in South Africa. Phytochemical analyses have revealed that *Helichrysum* contains secondary metabolites including hemihumulone, quarcetin, kaempferol, apiginin, quinine (Süzgeç et al., 2004; van Vuuren et al., 2006). Secondary metabolites such as anthocyanins, coumarins, fatty acids, emodins, leucoanthocyanins, tannins, terpenoids, and saponins are responsible for the healing properties of medicinal plants (Savithamma et al., 2011). Current research efforts on the optimization of plant secondary metabolite production have intensified in recent years (Vanisree et al., 2004; Naguib et al., 2012; Ibrahim et al., 2012a & b; Ibrahim & Jaafar, 2012). Naguib et al. (2012) argued that the type and value of fertilizer and the level of application directly influence the level of nutrients available to plants, which may directly or indirectly influence plant physiology and the biosynthesis of secondary compounds in plants. Findings from recent studies suggest that K fertilization affects plant growth and secondary metabolite production, for example, Ibrahim et al.

(2012b) observed that in *Labisia pumila* Benth var. *Alata*, the production of total phenolics, flavonoids, ascorbic acid and carbohydrate content was affected by K fertilization and K deficiency induced decreased production of secondary metabolites by the tested plants. Potassium promotes adaptation and resistance of plants to environmental stresses such as insect infestations and diseases (Al-Moshileh et al., 2005; El-Latif et al., 2011; Garlet et al., 2013; Wang et al., 2013). Plants that are deprived of potassium tend to be more vulnerable to infections than those that receive an adequate amount of potassium (Troufflard et al., 2010; Wang et al., 2013). Young and actively growing plants have high potassium requirement, this high demand can limit potassium supply thereby limit crop yield even when the N fertilizers are applied (Armengaud et al., 2010).

Hydroponic fertilization of plants is gaining traction among horticulturists, farmers, researchers (Economakis et al., 2002; Urbanczyk-Wochniak & Fernie, 2005; Nxawe et al., 2009). Hydroponic plants are mostly grown within the confines of a green house, which is characterized by controlled environmental conditions and all year round production. A simple hydroponic system like the one we used in this study, which consist of flat surface, gutters, irrigation pipe, submissile pump, a reservoir, tubing, fluorescent lights with 15 cm plastic pots could cost roughly R20, 000 depending on the choice of environmental equipment installed. Although the start-up cost for setting hydroponics is expensive, the returns are high (Sardare and Admane, 2013). Furthermore, in hydroponics, it is possible to manipulate the micronutrients and assess the effects of different nutrient mixtures while avoiding the inherent biases such as pre-existing soil nutrients and soil physical and chemical properties that are common with soil study. Exposing plants to different hydroponic nutrient levels can affect yield and phytochemical profiles of plant (Hayden, 2006; Mugundhan et al., 2011). Even though commercial hydroponic cultivation of vegetable is growing steadily, very few

studies have been undertaken on the application of hydroponic technology to improve the medicinal value of indigenous African medicinal plants. Therefore, the objective of the present study was to discern the optimal K supplement level for *H. cymosum* by investigating the effects of different K levels on growth, K-leaf content, anti-*F.oxysporum* activity and total activity.

2.2. Materials and Methods

2.2.1. Plant material

Young stem cuttings were obtained from actively growing *H. cymosum* stock plants at the Cape Peninsula University of Technology (CPUT) (Cape Town, Campus), South Africa, -33° 55' 58.27''S, 18° 25' 57.04 E. The stock plants were originally acquired from a single population of *H. cymosum*, which has established on the CPUT Cape Town campus. The cuttings were rooted in a sterile soil mixture (3 parts river sand and 1 part compost) and placed under mist propagation conditions. After 6 weeks, the rooted cuttings were each transplanted into separate pots (12.5 cm) containing Light Expanded Clay Aggregate (LECA®). Cuttings were maintained in the nursery of the Department of Horticultural Sciences, CPUT Cape Town Campus.

2.2.2. Nutrients Preparation

Analytical grade chemicals were purchased from Merck (Pty) Ltd (South Africa); potassium nitrate [KNO₃], calcium nitrate [Ca(NO₃)₂.4H₂O], potassium dihydrogen phosphate [KH₂PO₄], magnesium sulphate [MgSO₄.7H₂O], boric acid [H₃BO₃], manganese chloride [MnCl₂.4H₂O], zinc heptahydrate sulphate [ZnSO₄.7H₂O], copper sulphate [CuSO₄], molybdic acid [H₃MoO₄.H₂O] and iron-EDTA (supplied by Stark Ayres, Observatory, Cape Town, South Africa) were used to prepare the nutrient solutions. Treatment were based on the Hoagland's solution; Macro nutrients (210 ppm of N, 235 ppm of K, 200 ppm of Ca, 48 ppm

of Mg, 31 ppm of P and 64 ppm of S) and micro nutrients (0.01 ppm B, 0.5 ppm Mn, 0.05 ppm Zn, 0.02 ppm Cu, 0.01 ppm Mo and 2 ppm Fe). In test nutrient solutions, the levels of potassium were adjusted to obtain the following concentrations; 58.75, 117.5, 235 and 470 ppm, and these adjustments were based on modifications of Hoagland's solution as shown in Table.2.1. Complete nutrient solutions were prepared by dissolving appropriate amounts of salt and acid combinations in 50 l of deionised water.

Table.2.1: Macro and micro-nutrient concentration in Modified Hoagland's hydroponics solution: 58.75 ppm, 117.5 ppm, 235 ppm and 470 ppm of K.

Nutrients	58.75ppm	117.5ppm	235ppm	470ppm
KNO ₃			70 (N)	70 (N)
			195.5 (K)	195.5(K)
NH ₄ NO ₃	210 (N)	210 (N)	140 (N)	140 (N)
KH ₂ PO ₄	39.1 (K)	39.1 (K)	39.1 (K)	39.1 (K)
	30 (P)	30 (P)	30 (P)	30 (P)
CaCl ₂	100(Ca)	100(Ca)	100 (Ca)	100 (Ca)
KCl	19.5 (K)	78.2 (K)		235 (K)
MgSO ₄	48 (Mg)	48 (Mg)	48 (Mg)	48 (Mg)
	64 (S)	64 (S)	64 (S)	64 (S)
CuSO ₄	0.02 (Cu)	0.02 (Cu)	0.02 (Cu)	0.02(Cu)
ZnSO ₄	0.05 (Zn)	0.05 (Zn)	0.05 (Zn)	0.05 (Zn)
H ₃ BO ₃	0.05 (B)	0.05 (B)	0.05 (B)	0.05 (B)
MnCl ₂ .4H ₂ O	0.01(Mn)	0.01(Mn)	0.01(Mn)	0.01(Mn)
MoO ₃ .H ₂ O	0.01(Mo)	0.01(Mo)	0.01(Mo)	0.01(Mo)
Fe-EDTA	2 (Fe)	2 (Fe)	2 (Fe)	2 (Fe)

2.2.3. Greenhouse experiment

The experiment was conducted in the greenhouse of the Department of Horticultural Sciences, CPUT, Cape Town Campus. Inside, the greenhouse was equipped with a 40% Alunet shade cloth suspended at 2 m above ground. Steel tables (2.5 x 1 m) were used as flat surfaces for plastic gutters (2 x 0.15 m). Sixteen white plastic gutters (2 x 0.15 m) were placed on four steel tables; each steel table had four gutters which were held in place by cable ties (cable ties and plastic gutters were supplied by Builders Warehouse [Pty] Ltd, Cape Town). Each gutter had 10 plastic pots (12.5 cm) filled with LECA®. On commencement of the experiment, individual pots received a single 6-week old *H. cymosum* rooted stem cuttings grown on river sand and compost corresponding to 10 plants per gutter. Beneath the steel table, four fish tanks (65 L) each containing one submersible water pump which recirculates water through a 20 ml black plastic pipe to one gutter, only. In order to avoid pseudo-replication, data from each of the 10 pots per row were pooled and the means functioned as replicates. Each potassium treatment had a total of 4 gutters corresponding to 40 plants and 4 replicates per treatment. Nutrient solution from the fish tank was continually supplied to the plants by spaghetti tubing with drippers held in place by stakes at a rate of 4 L/h. All the gutters were wrapped with black polyethylene sheets in order to avoid evaporation and algae build-up. The drainage water was collected and recycled. The experiment was arranged in a randomized block design. To avoid build-up of salts in the clay pebbles due to crystallization of salts, nutrient solutions were replaced every 2 weeks and the greenhouse experiment was allowed to run for 8 weeks (Fig. 2.1). The following experimental conditions were maintained in the greenhouse; temperature; day (16-36 °C) and night (10-18 °C), relative humidity range (35-65%) and natural day/night regime. The pH range of the growth media were maintained at 5-6.5.



Fig. 2.1: Hydroponics setup of *H. cymosum* treated with potassium.

2.2.4. Tissue analysis

Leaves were washed with Teepol solution, rinsed with de-ionised water and dried at 70 °C overnight in an oven. The leaves were ashed and milled at 480 °C, shaken up in a 50:50 HCl (32%) solution for extraction through filter paper (Campbell & Plank, 1998; Miller, 1998). Total N content of the ground leaves was determined through total combustion in a Leco N-analyser. For K analysis, the plant tissue procedures as described in Campbell & Plank (1998) and Miller (1998) were adopted. Samples were ashed at 480 °C, shaken up in a 50:50 HCl (32%) solution for extraction through filter paper.

2.2.5. Extraction of plant material

Fresh aerial plant materials were harvested at 8 weeks post-treatment and air dried at 28 ± 2 °C under shade. Dried plant materials were cut into smaller pieces and ground using a Jankel and Kunkel Model A 10 mill into fine powder. Powdered foliage material (5 g) was extracted with 100 ml of acetone in glass beaker with the aid of a vortex mixer for 15 min and the supernatant filtered using Whatman No.1 filter paper. The extracted material was left to dry over-night at room temperature (22 ± 2 °C) and the dried acetone extracts were weighed.

2.2.6. Antimicrobial activities of extracts

The microdilution method described by Eloff (1998) was employed with slight modifications in determination of the minimum inhibitory concentration (MIC) for the extracts. *H. cymosum* extracts were diluted into acetone to obtain a starting concentration of 6 mg/ml. The starting concentration was diluted two fold in each successive serial dilution. *F. oxysporum* was sub-cultured from stock ager plate and grown in Nutrient Broth (Merck, South Africa) for 4 hours. The fungal culture (100 ml) containing (10^5 cells/ ml) was transferred into single wells of a 96-well microplates. Mancozeb (80 mg/10 ml) was prepared using sterile distilled water as a positive control and sterile distilled water was used as a negative control. Forty micro litre (40 μ l) of 0.2 mg/ml of *p*-iodonitrotetrazolium (INT) (Sigma) dissolved in sterile distilled water was added to each microplate well and incubated at 37 °C 100% RH (sealed in a plastic bag). The MIC values were recorded after 24 to 48 hours. The antifungal bioassay (MIC) consisted of 4 replicates per row of plants times 4 replicates per treatment.

2.2.7. Total activity (TA)

The total activity is a good criterion for comparing biological activities among plant species or cultivars because its formula takes into account the yield and antimicrobial activities of test extracts (Eloff, 2000; Eloff, 2004). Precisely, it is the volume to which the mass in mg extracted from one gram of dried plant material can be diluted and still kills the microorganisms. It is calculated by dividing the amount in mg of extract from 1 mg plant material by the MIC in mg/ml of the same extract or compound isolated and is expressed in ml/g.

Total Activity (ml/g) = Extract per gram dried plant part in milligramme/ MIC of extract (mg/ml)

2.2.8. Statistical Analysis

Growth parameters (leaf number, plant height and fresh weight of aerial parts), MIC and total activity were analyzed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means at a level of significance, $P < 0.05$. These analyses were performed using STATISTICA software (StatSoft, 2013). Sigma Plot 10.0 package was used for plotting graphs.

2.3. Results

2.3.1. Plant fresh weight

K level significantly influenced (df, 1, 3; $F=9.965$; $P =0.0014$) fresh weight (aerial parts) of *H. cymosum* at 8 weeks post treatment. The highest fresh weight yield of aerial parts (2.64 ± 0.265 g) was obtained from plants treated with 235 ppm of N while 117.5 ppm yielded the lowest fresh weight (0.45 ± 0.037 g) at 8 weeks post-treatment (Table 2.2). When the means of fresh weights of the aerial parts of plants were compared, 235 ppm was statistically different ($P < 0.05$) from 58.75 and 117.5 ppm with the exception of 470 ppm of K (Table 2.2).

Table.2.2: Effect of potassium levels on plant fresh weight (aerial parts) of *H. cymosum* at 8 weeks post-treatment.

Treatments (ppm)	Fresh weight
58.75	1.169 ± 0.47 A
117.5	0.45 ± 0.037 A
235	2.62 ± 0.25 B
470	1.5 ± 0.19 AB

The same mean followed by same uppercase letters in the same column are not significantly different ($P > 0.05$) following comparison using Tukey test.

2.3.2. Plant height

Plant growth increased significantly ($df = 5, 234, P < 0.05$) in all treatments overtime following repeated measure ANOVA analysis; from week 3 post treatment: (58.75 ppm [3.54 ± 0.244 cm], 117.5 ppm [3.095 ± 0.127 cm], 235 [4.44 ± 0.15 cm], and 470 [4.22 ± 0.19 cm]). At 8 weeks post treatment, nutrient K applied at 235 ppm produced the highest plant height (20.98 ± 0.401 cm) (Fig.2.2), which was statistically different ($P < 0.05$) compared to the other treatments (58.75 ppm [15.51 ± 0.83 cm], 117.5 ppm [16.35 ± 0.467 cm], and 470 ppm [17.89 ± 0.59 cm]) in which instances plant growths were visibly stunted and in addition, a few of these plants even died during the course of experiment.

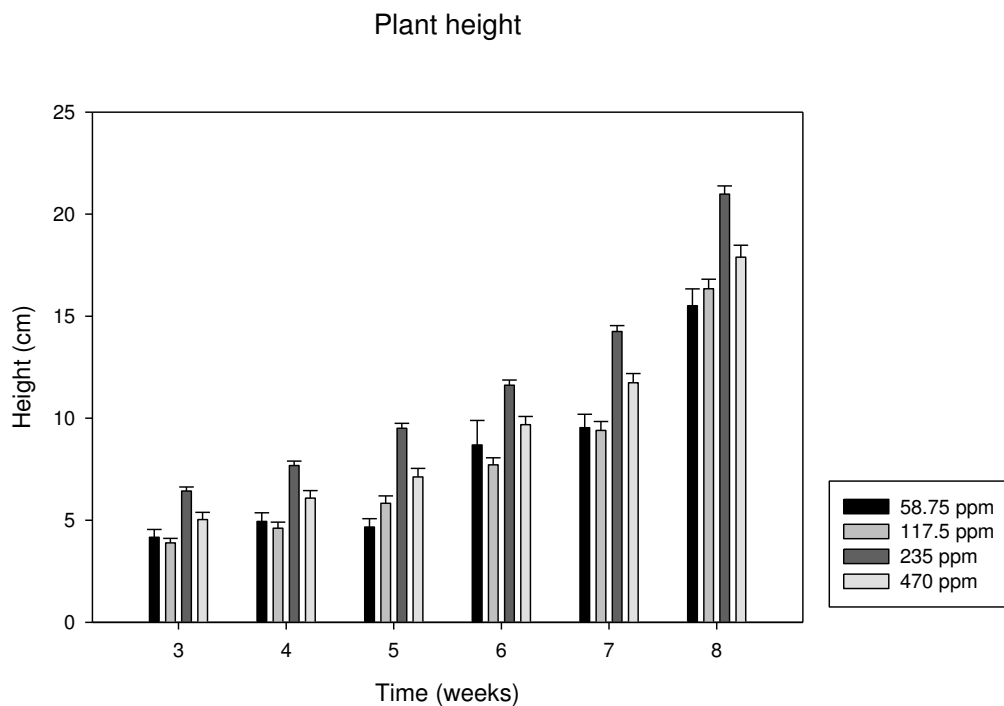


Fig. 2.2: Effect of potassium treatments (58.75 ppm, 117.5 ppm, 235 ppm and 470 ppm) on plant (*H. cymosum*) height at weeks 3-8.

2.3.3. Number of leaves

Three (3) weeks after treatment with K fertilizer, 235 ppm (2.73 ± 0.199) was significant different ($P < 0.05$) from the other three (3) treatments (58.75 ppm [1.43 ± 0.1], 117.5 ppm [1.65 ± 0.12], and 470 ppm [1.78 ± 0.121]) (Fig. 2.3), when means were separated using Tukey HSD test. At 8 weeks post treatment, leaf numbers produced by *H. cymosum* varied significantly among treatments 235 K (28.4 ± 2.76), 58.75 (10.77 ± 1.4), 117.5 (2.97 ± 0.22) and 470 ppm (15.4 ± 1.26). At lower K levels (58.75 and 117.5 ppm), scorching of plant leaves were observed among tested plants.

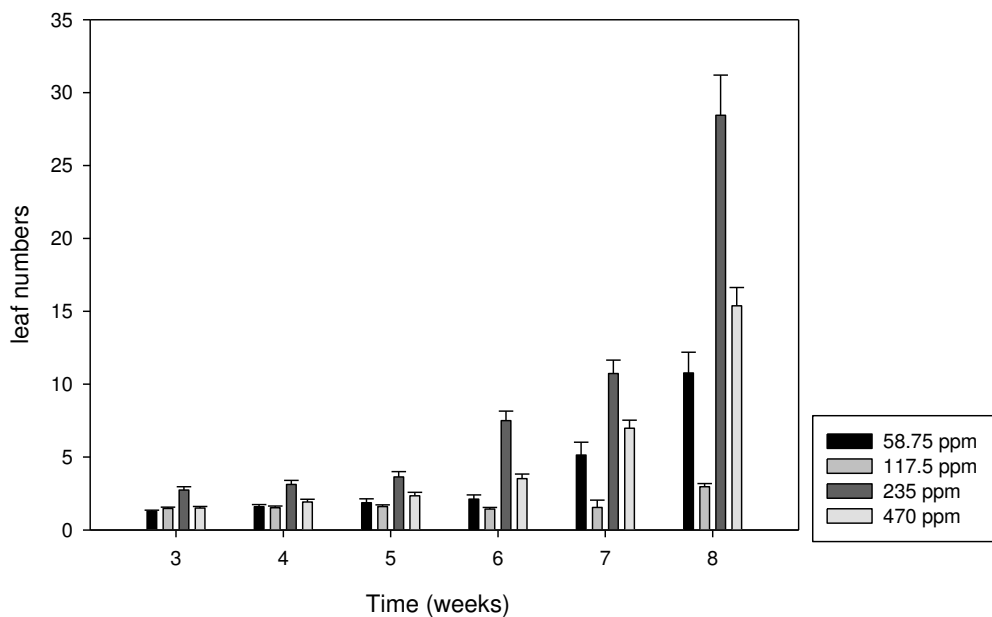


Fig. 2.3: Effects of different potassium supplies on the number of leaves produced by *H. cymosum* under hydroponics conditions from 3-8 weeks post-treatment.

2.3.4. Tissue content

Plant tissue phosphorus level which ranged from 0.69 - 0.78% was not significantly different ($P < 0.05$) among plants exposed to the different nutrient K treatments. Generally, K and N tissue content varied significantly ($P > 0.05$) among treatments and ranged from 2.95 to 3.15

and 3.56 to 4.67 %, respectively (Table 2.3). Tissue nutrient content analysis for plants exposed to treatment 117 ppm K could not be carried out due to unavailability of sufficient plant materials. Some plants among those supplied with lower nutrient concentrations (58.75 or 117 ppm) of K showed symptoms of K deficiency, which included stunted growth, scorching and mortality.

Table 2.3: Leaf nutrient (N, P, K) content (%) following treatments of *H. cymosum* with different hydroponics potassium levels (58.75 ppm, 235 ppm and 470 ppm) at 8 weeks post treatment.

Treatment (ppm)	N (%)	P (%)	K (%)
58.75	3.15 ± 0.25 A	0.69 ± 0.15 A	3.56 ± 0.198 A
117	-	-	-
235	3.13 ± 0.026 A	0.78 ± 0.01 A	4.32 ± 0.05 AB
470	2.95 ± 0.086 A	0.76 ± 0.01 A	4.67 ± 0.29 B

Means followed by the same uppercase letters in the same column means not significant different ($P > 0.05$) following comparison using Tukey test. Due to the presence of symptoms such as including stunted growth, scorching among plants exposed to lower concentrations of K, there were insufficient plant materials of 117 ppm K treatment for tissue content analysis

2.3.5. Acetone extraction yield

The yield of acetone extracts of powdered aerial part of *H. cymosum* increased significantly ($P < 0.05$) with increasing nutrient K level; 235 ppm (210 ± 38.5 mg) 58.75 ppm (16.67 ± 2.35 mg), 117.5 ppm (22.5 ± 4.79 mg), however, the yield dropped at 470 ppm K to 40 ± 4.08 mg (Table 2.4).

2.3.6. Minimum inhibitory concentration

The MIC of acetone extracts of *H. cymosum* plant from the four K treatments; (58.75 ppm [0.47 ± 0.25 mg/ml and 0.76 ± 0.41 mg/ml], 117.5 ppm [2.46 ± 1.68 and 2.64 ± 1.65 mg/ml], 235 ppm [0.645 ± 0.14 mg/ml], and 470 [1.88 ± 0.66 and 2.82 ± 1.6 mg/ml] at 24 and 48 hours post treatment, respectively were not statistically different ($P \geq 0.05$) (Table 2.4).

2.3.7. Total activity

In this study, the extracts of plants grown hydroponically at 235 ppm recorded the highest values of TA against *F. oxysporum* (0.08 ml/g [24 and 48 hours]) compared to the other K treatments (58.75 ppm [0.024 and 0.013 ml/g], 117.5 ppm [0.006 and 0.008 ml/g], and 470 ppm [0.006 and 0.005 ml/g]) at 24 and 48 hours respectively (Table 2.4).

Table 2.4: Yield from 1 g of plant material, minimum inhibitory concentration and total activities of acetone extracts obtained from aerial parts of hydroponically-cultivated *H. cymosum* following exposure to a range of K levels (58.75-470 ppm).

Treatments (ppm)	Yield \pm SE (mg)	MIC \pm SE (mg/ml)	Total activity (ml/g)
58.75 (24H)	16.67 \pm 2.35	0.49 \pm 0.25	0.02 \pm 0.01 AB
(48H)		0.76 \pm 0.41	0.01 \pm 0.01 b
117.5 (24H)	22.5 \pm 4.79	2.46 \pm 1.68	0.006 \pm 0.00 B
(48H)		2.64 \pm 1.65	0.008 \pm 0.00 b
235 (24H)	210 \pm 38.5	0.645 \pm 0.14	0.08 \pm 0.00 A
(48H)		0.645 \pm 0.14	0.08 \pm 0.00 a
470 (24H)	40 \pm 4.08	1.88 \pm 0.66	0.006 \pm 0.00 B
(48H)		2.82 \pm 1.6	0.005 \pm 0.00 b

- Means followed by the same uppercase or lowercase letters in the same column indicate no significant difference ($P > 0.05$) at 24 and 48h post treatment, respectively, following comparison using Tukey test. MIC values in all treatments were not significantly different ($P > 0.05$) at 24 or 48 hours.

2.4. Discussion

The leaf number produced by plants exposed to 235 ppm K was significantly higher than other levels of K (58.75, 117.5 and 470 ppm) (Fig 2.3). These findings are in agreement with findings of Hossain et al. (2010), which showed that treatment with 100 mg/L K was effective in increasing the leaf numbers of *Hibicus cannabinus* L. in contrast to lower

concentrations that had fewer leaf numbers. Reduced amount of potassium can inhibit plant growth (Jouyban, 2012). Inadequate supply reduces the rates of cell division, cell expansion and cell permeability (Hossain et al., 2010). Potassium is involved in the process of photosynthesis, transfer of N, protein synthesis, carbohydrates, starch development, helps plant to improve tolerance to drought situations and improves the capability to counterattack attacks of pests and diseases, producing strong stems and reducing lodging (Tahir et al., 2008; Haghghi et al, 2011; Bhuvanewari et al., 2013; Vafaie et al., 2013). Potassium plays a role in oxidative metabolism and balancing the electric charge of plant cells (Vafaie et al., 2013). Also, K affects the stimulation of certain enzyme in extracts of higher plants and guideline of enzyme activity (Haghghi et al, 2011; Ibrahim et al., 2012b).

Whilst evidence suggest that reduced K treatment can negatively influence plant growth, on the other hand, it appears excessive application of K, beyond a tolerable threshold can negatively affects growth and yield. For example in this study the tallest and highest number of leaves was produced by *H cymosum* plants that were exposed to 235 ppm K treatment. This argument is corroborated by results published by Inthichack et al. (2012); highest plant yield was obtained in celery plants treated with 321.8 mg/l of KCL compared to corresponding plants that were grown in 643.7 mg/l KCL.

The highest concentration of nutrient (K) was observed in plants treated with 470 ppm K, however, this was not significantly different to 235 ppm K. Mudau et al. (2007) reported that the potassium leaf content of bush tea increased with the increased application of potassium. Previously, Shaibur et al. (2008) reported that where rice seedlings had higher leaf content when seedlings were exposed to 30 mM of KCl compared to 10 and 20 mM.

Symptoms (stunted growth, scorching and death) of K deficiency were observed among plants exposed to lower concentrations of K. Based on the results of this study; it is plausible

to assume that K may potentially have an influence on the growth and establishment of *H. cymosum* plants in nature. It is worth mentioning that one of the natural habitats of the *H. cymosum* in South Africa is the Cape Peninsula, and this region is characterised by acidic and potassium rich soils from the weathering of granites (Bargmann, 2005). It should be interesting to investigate the ecological factors that influence the distribution of *H. cymosum*.

In the antifungal bioassay, acetone extracts of *H. cymosum* plants that were treated with 58.75 ppm K was the most bioactive against *F. oxysporum* compared to plants exposed to 117.5 ppm, 235 ppm and 470 ppm K. Previous studies have indicated that potassium deficiency increase phenolic concentrations (Lehman & Rice, 1972; Du et al., 2011; Ramakrishma & Ravishankar, 2011). Recently, Chen et al. (2013) observed that K starvation led to an increase in rosmarinic acid, ursolic acid, oleanolic acid and flavonoid content in *Prunella vulgaris* plants and high concentration of K lead to a decrease in RA. Phenolic compounds are bioactive against microbial pathogens (Puupponen-Pimiä et al., 2005). In this study, the extracts of plants grown hydroponically at 235 ppm recorded the highest values of TA against *F. oxysporum* (81.73 ml/g [24 and 48 hours]) compared to the other K treatments. Potassium treatments (117.5 and 470 ppm) recorded the least TA activities, 0.005 and 0.006 ml/g, respectively. The total activity is a very good criterion for comparing biological activities among plant species or cultivars because its formula takes into account the yield and antimicrobial activities of test extracts.

In conclusion, K supplementation affects bioactivity and yield of hydroponically-grown *H. cymosum* plants. The optimum nutrient K level for growing *H. cymosum* hydroponically was 235 ppm. Knowledge of nutrient requirements can be used to increase yield and quality of the medicinal properties.

Due to the presence of symptoms including stunted growth, scorching among plants exposed to lower concentrations of K there were insufficient materials to pursue phytochemical analysis and therefore comparison of the effects of treatments on the chemical profiles of secondary metabolites produced by *H. cymosum* was not possible. The next Chapter focuses on the effect of nutrient N levels on growth and tissue nutrient content, anti-fungal activity and growth chemical profiles of crude extracts of aerial parts of *H. cymosum*.

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Chapter Three

Effect of varying levels of nitrogen on plant growth and anti-*Fusarium oxysporum* (Ascomycota: Hypocreales) activity and secondary metabolite profiles of foliage extracts of hydroponically cultivated *Helichrysum cymosum* (L.) D. Don (Asteraceae)

3.1. Introduction

Hydroponic plant cultivation presents interesting opportunities for growers and researchers to manipulate nutrient feed content (Maggini et al., 2012 a & b; Kiferle et al., 2013; Conn et al., 2013), for purposes of influencing plant growth parameters and production of secondary metabolites (Maggini et al., 2012b). In hydroponics, it is possible to manipulate environmental factors (temperature, water and nutrient availability), which also influence production of secondary metabolites (Gontier et al., 2002; Maggini et al., 2012a) plant growth and development (Pagliarulo et al., 2004; Maggini et al., 2012a). Thus, hydroponics presents opportunities to develop optimum protocols for standardizing secondary metabolite production in a targeted plant. For example sweet basil plants that were grown hydroponically had higher levels of rosmarinic acid and total phenolic compared to those that were grown in soil (Kiferle et al., 2011). The content of essential oils of *Ocimum basilicum* L. was superior in plants that were grown hydroponically than in plants that were cultivated in soil (Economakis et al., 2002)

Secondary plant metabolites, which are sometimes, referred to as phytochemicals may be divided into many classes, which include phenolic compounds (e.g. flavonoids and phenolic acids), terpenes (e.g. monoterpenes, sesquiterpenes etc), nitrogen (alkaloids, non-protein amino acids etc.) and sulphur-containing compounds (e.g. glucosinolates) (Wink, 2004; Mazid et al., 2011). Secondary metabolite production may be triggered by stressful conditions such as presence of pathogens (Bénard et al., 2011), high UV radiation (Crozier et al., 2006) and

herbivory (Lavola & Julkenen-Titto, 1994; Nelson & Kursar; 1999; Pedneault et al., 2002; Kennedy & Wightman, 2011; Mazid et al., 2011). Furthermore, many recent studies have demonstrated that nutrient content in a plant growth medium affects secondary metabolite production (Van Alstyne & Pelletreau, 2000; Economakis et al., 2002; Maggini et al., 2012; Sugumaran et al., 2013), which in turn might influence the medicinal properties of extracts derived from such plants (Cowan, 1999). Nitrogen is known to be one of the important elements needed by plants; its levels in plant growth media influence the production of secondary metabolites, and plant growth and development (Beatty et al., 2010; Fan et al., 2010; Farag et al., 2013). The deficiency of N in a plant growth medium may increase plant production of secondary metabolites whereas high concentration of N may decrease the plant production of secondary metabolites (Bénard et al., 2011). According to Ibrahim et al. (2012), low application of nitrogen increases the amount of flavonoids by 14% in tomato plant while increased application of N decreases the concentration of flavonoids in grapefruit. Gayler et al. (2008) reported that polyphenols of *Picea sitchensis* (Bong.) Carr. (Family: Pinaceae) were higher in low nitrogen concentration. On the other hand, inadequate or excessive application of N may lead to poor plant growth, increased plant stress as well as increased susceptibility of a plant to diseases (Bénard et al., 2011; Amanullah & Stewart; 2013). In order to overcome shortage of nitrogen, farmers have tended to use more inorganic nitrogen fertilizers and unfortunately, under field conditions, application of nitrogen at higher rates may lead to contamination of groundwater (Zhao et al., 2005; Beatty et al., 2010; Fan et al., 2010; Ibrahim et al., 2011).

There are many studies, which have been conducted on the tissue phytochemical contents of plants grown in soil; however, very little research has been conducted on the tissue contents, growth and antimicrobial activities of hydroponically-grown indigenous southern African plants. *Helichrysum cymosum* subsp.*cymosum* L. (Asteraceae) or gold carpet (Eng) or

Impepho (Xhosa) is a well-branched, spreading groundcover plant with involucre bracts that are brownish, tawny, straw-coloured or yellow (Heyman, 2009). Member species of the genus *Helichrysum* has been used in folk medicine due to remedial properties such as pain relieving, stomach-ache relief, wound dressing, anti-infective, hepatoprotective, detoxifying, cholagogic and choleric property; stimulation of emission of gastric juices, and treatment of coughs, erythematic, and diabetes mellitus as well as to invoke the goodwill of ancestors and induce trances (Omaruyi et al., 2003; Lourens et al., 2008; Demir et al., 2009; Albayrak et al., 2010 a and b; Otang et al., 2012). Members of the genus *Helichrysum* are known to be rich in secondary metabolites, including; apigenin, quercetin, kaempferol, helihumulone and quinic acid derivatives (Matic et al., 2013). Extracts of the plants possess antimicrobial activities (van Vuuren et al., 2006; François et al., 2010). *H. cymosum* is extensively harvested from the wild and is mostly propagated using cuttings and seeds and it grows well in sandy soil. Identification of optimum conditions for growing *H. cymosum* hydroponically could increase efficacy of the extracts and minimize excessive exploitation of plants from the wild. The objective of this study was to evaluate the effects of nitrogen levels on growth parameters, secondary metabolite profile and *in vitro* anti-*F. oxysporum* activity of crude extracts of hydroponically-cultivated *H. cymosum*. To our knowledge, this is the first investigation on hydroponics cultivation of *H. cymosum*.

3.2. Materials and methods

3.2.1. Plant material

Young stem cuttings were obtained from actively growing *H. cymosum* stock plants at the Cape Peninsula University of Technology (Cape Town, Campus), South Africa, -33° 55' 58.27''S, 18° 25' 57.04 E. The stock plants were originally acquired from a single population of *H. cymosum*, which has established on the CPUT Cape Town campus. The cuttings were

rooted in a sterile soil mixture (3 parts river sand and 1 part compost) and placed under mist propagation conditions. After 6 weeks, the rooted cuttings were each transplanted into separate pots (12.5 cm) containing Light Expanded Clay Aggregate (LECA®). Cuttings were maintained in the Nursery, Department of Horticultural Sciences, and Cape Peninsula University of Technology Cape Town Campus in February 2013.

3.2.2. Nutrients preparation

Analytical grade chemicals were purchased from Merck (Pty) Ltd (South Africa); potassium nitrate [KNO₃], calcium nitrate [Ca(NO₃)₂·4H₂O], potassium dihydrogen phosphate [KH₂PO₄], magnesium sulphate [MgSO₄·7H₂O], boric acid [H₃BO₃], manganese chloride [MnCl₂·4H₂O], zinc heptahydrate sulphate [ZnSO₄·7H₂O], copper sulphate [CuSO₄], molybic acid [H₃MoO₄·H₂O] and iron-EDTA (supplied by Stark Ayres, Observatory, South Africa) were used to prepare the nutrient solutions. Treatments were based on the Hoagland's solution; Macro nutrients (210 ppm of N, 235 ppm of K, 200 ppm of Ca, 48 ppm of Mg, 31 ppm of P and 64 ppm of S) and micro nutrients (0.01 ppm B, 0.5 ppm Mn, 0.05 ppm Zn, 0.02 ppm Cu, 0.01 ppm Mo and 2 ppm Fe). In test nutrient solutions, the levels of nitrogen were adjusted to obtain the following concentrations; 52.5 ppm, 105 ppm, 210 ppm and 420 ppm, and these adjustments were based on modifications of Hoagland's solution as shown in Table 3.1. Complete nutrient solutions were prepared by dissolving appropriate amounts of salt and acid combinations in 50 l of deionised water.

Table.3.1: Macro and micro-nutrient concentrations in modified Hoagland's hydroponic solutions; 52.5 ppm, 105 ppm, 210 pmm and 420 ppm of N.

Nutrients	52.5ppm	105ppm	210ppm	420ppm
KNO ₃			70 (N) 195.5(K)	70 (N) 195.5(K)
Ca (NO ₃) ₂ .4H ₂ O		70 (N) 100 (Ca)	56 (N) 80 (Ca)	84 (N) 120 (Ca)
NH ₄ NO ₃	52.5 (N)	35(N)	84 (N)	266 (N)
CaCl ₂	200 (Ca)	100(Ca)	120 (Ca)	80 (Ca)
KH ₂ PO ₄	39.1 (K) 30.97 (P)	39.1 (K) 30.97 (P)	39.1 (K) 30.97(P)	39.1 (K) 30.97 (P)
KCl	195.9 (K)	195.9 (K)		
MgSO ₄	48 (Mg) 64(S)	48 (Mg) 64 (S)	48 (Mg) 64(S)	48 (Mg) 64 (S)
CuSO ₄	0.02 (Cu)	0.02 (Cu)	0.02 (Cu)	0.02 (Cu)
ZnSO ₄	0.05 (Zn)	0.05 (Zn)	0.05 (Zn)	0.05 (Zn)
H ₃ BO ₃	0.05 (B)	0.05 (B)	0.05 (B)	0.05(B)
MnCl ₂ .4H ₂ O	0.01 (Mn)	0.01 (Mn)	0.01 (Mn)	0.01 (Mn)
MoO ₃ .H ₂ O	0.01 (Mo)	0.01 (Mo)	0.01(Mo)	0.01 (Mo)
Fe-EDTA	2 (Fe)	2 (Fe)	2 (Fe)	2 (Fe)

3.2.3. Greenhouse experiment

The experiment was conducted in the greenhouse of the Department of Horticultural Sciences, Cape Peninsula University of Technology (Cape Town, Campus). Inside, the greenhouse was equipped with a 40% Alunet shade cloth suspended at 2 m above ground.

Steel tables (2.5 x 1 m) were used as flat surfaces for plastic gutters (2 x 0.15 m). Sixteen (16) white plastic gutters (2 x 0.15 m) were placed on four (4) steel tables; each steel table had four (4) gutters which were held in place by cable ties (cable ties and plastic gutters were supplied by Builders Warehouse (Pty) Ltd, Cape Town). Each gutter had ten (10) plastic pots (12.5 cm) filled with LECA®. On commencement of the experiment, individual pots received a single 6-week old *H. cymosum* rooted stem cuttings grown on river sand and compost corresponding to 10 plants per gutter. Beneath the steel table, four (4) fish tanks (65 L) each containing a submersible water pump, which re-circulated water through a 20 ml black plastic pipe to one gutter, only. In order to avoid pseudo-replication, data from each of the ten (10) pots per row were pooled and the means functioned as replicates. Each nitrogen treatment had a total of 4 gutters corresponding to 40 plants and 4 replicates per treatment. Nutrient solution from the fish tank was continually supplied to the plants by spaghetti tubings with drippers held in place by stakes at a rate of 4L/h. All the gutters were wrapped with black polyethylene sheets in order to avoid evaporation and algae buildup. The drainage water was collected and recycled. The experiment was arranged in a randomized block design. To avoid buildup of salts in the clay pebbles due to crystallization of salts, nutrient solutions were replaced every 2 weeks and the greenhouse experiment was allowed to run for 8 weeks. The following experimental conditions were maintained in the greenhouse; temperature day (16-36° C) and night (10-18°C), relative humidity range (35-65%) and natural day/night regime. The pH range of the growth media were maintained at 5-6.5.

3.2.4. Plant growth

The following parameters were recorded weekly; plant height was determined from the base (LECA® surface) to the highest point (cm) and leaf numbers. The fresh weight was determined by weighing the aerial parts (stem and leaves) in grams after harvest, 8 weeks post-treatment. Four replicates were used per treatment.

3.2.5. Tissue analysis

Plant foliage from subsamples of plants from each treatment were sent to Bemblab (Pty) Ltd (Somerset West, South Africa) for analysis. Leaves were washed with Teepool solution, rinsed with deionised water and dried at 70 °C over night in an oven. The leaves were ashed and milled at 480 °C, shaken up in a 50:50 HCl (32%) solution for extraction through filter paper (Campbell & Plank, 1998 and Miller, 1998). Total N content of the ground leaves was determined through total combustion in a Leco N-analyser. For K analysis the plant tissue procedures as described in Campbell & Plank (1998) and Miller (1998) were adapted. Samples were ashed at 480°C, shaken up in a 50:50 HCl (32%) solution for extraction through filter paper.

3.2.6. Extraction of plant material

Fresh aerial plant material was harvested at 8 weeks post-treatment and air-dried at 28 ± 2 °C. Dried plant material was cut into smaller pieces and ground using a Jankel and Kunkel Model A 10 mill into fine powder. Powdered foliage material (5 g) was extracted with 100 ml of acetone in glass beaker with the aid of a vortex mixer for 15 min and the supernatant filtered using Whatman No.1 filter paper. Acetone is a very useful extractant in that it is less toxic, highly volatile and capable of extracting a wide range of compounds from lipophilic to

hydrophilic compounds (Eloff, 1998). The extracted material was left to dry over-night at room temperature $22 \pm 2^{\circ}\text{C}$ and the dried acetone extracts were weighed.

3.2.7. Antimicrobial activities of extracts

The microdilution method described by Nchu et al. (2010) was employed with slight modifications in determination of the minimum inhibitory concentration (MIC) for the extracts. *H. cymosum* extracts were diluted into acetone to obtain a starting concentration of 7.5 mg/ml. The starting concentration was diluted two fold in each successive serial dilution. The *Fusarium oxysporum* f sp.glycines strain (UPFC no. 21) was obtained through the courtesy of the Phytomedicine Programme, University of Pretoria. The fungus strain was originally isolated by C. Cronje from roots of a maize plant Delmas, Gauteng. *F. oxysporum* was sub-cultured from stock agar plate and grown into Nutrient Broth (Merck) for 4 hours. The fungal culture (100 ml) was introduced to each well of the 96-well microplates (10^5 cells/ml). Mancozeb (80 mg/10 ml) was prepared using sterile distilled water and served as a positive control and sterile distilled water was used as a negative control. Forty micro litre (40 μl) of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT) (Sigma) dissolved in sterile distilled water was added to each microplate well and incubated at 37°C and 100% RH (sealed in a plastic bag). The MIC values were recorded after 24 and 48 h. The antifungal bioassay (MIC) consisted of 4 replicates per row of plants times 4 replicates per treatment.

3.2.8. Total activity (TA)

The total activity is a very good criterion for comparing biological activities among plant species or cultivars because its formula takes into account the yield and antimicrobial activities of test extracts. The unit of TA is ml/g and it indicates the degree to which the active compounds in one (1) g of plant materials can be diluted and still inhibit the growth of

the tested microorganisms (Eloff, 2000; Eloff, 2004). It is calculated by dividing the amount of extract from 1 g of plant material in mg by the MIC in mg/ml of the same extract.

Total Activity (ml/g) = yield of extract per gram of dried plant material in mg / MIC of extract (mg/ml)

3.2.9. Liquid Chromatography-Mass Spectrometry (LC-MS)

Three (3) replicates of material derived from each set of growth conditions with respect to nutrient nitrogen concentration (*viz.* 52.5; 105; 210 and 420 ppm) were subjected to extraction with acetone (analytical grade). In each case powdered plant material (5 g) was suspended in acetone (100 ml) followed by stirring for 18 hours. Each mixture was then filtered through a sintered glass funnel. The filtrate was centrifuged at 3500 rpm. The supernatant was collected and evaporated to dryness. A representative sample from each of three replicates was submitted for LC-MS analysis in the ESI negative mode using the instrument, Waters Synapt G2. The negative mode was chosen for the purpose of targeting the phenolic class of compounds, because they are amenable to this method of analysis and they occur broadly in the *Helichrysum* genus. LC separation was attained using the solvents; water and acetonitrile containing 0.1% formic acid. A constant flow rate of 0.35 ml/min was used for each analysis. Capillary voltage and cone voltage were maintained at 2.5 kV and 15 V, respectively. The entire flow from the LC was directed into the mass spectrometer. LC-MS chromatograms obtained for the crude extracts were analysed by Masslynx to get spectra of various peaks. The initial approach was to extract ion chromatograms of molecular masses corresponding to compounds which had previously been reported for *H. cymosum*. Subsequently, peaks for which the molecular masses corresponded to those of other well known bioactives from the *Helichrysum* genus were similarly targeted. Only major peaks in each chromatogram were analysed for determination of molecular masses and fragmentation data for targeted compounds were compared to known data. Retention time peaks for crude

extracts were compared to the peak obtained with the standards (kaempferol, quercetin, apigenin). From the chromatograms, the observed area under each peak was used to visually estimate the quantity of each of the targeted compounds (kaempferol, quercetin, apigenin and helihumulone) present in the eluted crude plant extract at a fixed scale. Due to unavailability of the standard for helihumulone, only three (3) external standards, apigenin, kaempferol and quercetin was run simultaneously with crude extracts on the analytical column.

3.2.10. Statistical analysis

Growth parameters (leaf number, plant height and fresh weight of aerial parts), MIC and total activity were analyzed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means at a level of significance, $P < 0.05$. These analyses were performed using Statistica (StatSoft, 2013). Sigma Plot 10.0 package was used for plotting graphs.

3.3. Results

3.3.1. Plant fresh weight

Nitrogen levels had a significant effect ($df = 3, 12; F = 5.243; P = 0.01527$) on fresh weight of *H. cymosum* at 8 weeks post treatment. The highest yield (fresh weight) of aerial parts (15.12 ± 1.68 g) was obtained at 105 ppm N treatment while 52.5 ppm yielded the lowest fresh weight (5.53 ± 1.11 g) (Table 3.2). When the means of fresh weights of above ground parts of plants were compared, 52.5 ppm showed a significant difference ($P < 0.05$) compared to 105 ppm of N (15.12 ± 1.68), however it was not significantly different from other treatments (210 [8.2 ± 3.3] and 420 ppm [10.5 ± 0.9]) and 105 ppm of N was not significantly different to 210 and 420 ppm of N (Table 7).

Table.3.2: Effect of nutrient nitrogen levels on plant fresh weight (aerial part) of *H. cymosum* at 8 weeks post-treatment.

Treatment (ppm)	Fresh weight (g)
52.5	5.53 ± 1.11 A
105	15.12 ± 1.68 B
210	7.95 ± 3.1 AB
420	10.5 ± 0.9 AB

The same means followed by same uppercase letters in the same column are not significantly different ($P < 0.05$) following comparison using Tukey test.

3.3.2. Plant height

Plant growth increases significantly ($P < 0.05$) in all treatments overtime; from week 3 post treatment: [52.5 ppm (9.6 ± 0.5 cm), 105 ppm (10.64 ± 0.7 cm), 210 ppm (7.3 ± 0.4 cm), 420 ppm (6.5 ± 0.74 cm)] to week 8: [52.5 ppm (19.4 ± 0.7 cm), 105 ppm (18.7 ± 0.8 cm), 210 ppm (14.9 ± 0.6 cm), and 420 ppm (14.2 ± 0.9 cm)]. At week 8 post treatment, nutrient N applied at 52.5 ppm produced the highest height (Fig 3.1), which was significantly different ($P \leq 0.05$) compared to the other treatments (210 and 420 ppm of N) with the exception of 105 ppm of N. Generally, there was a significant increase (df = 5, 234, $P \leq 0.05$) in plant height over time for all treatments based on repeated measure ANOVA analysis.

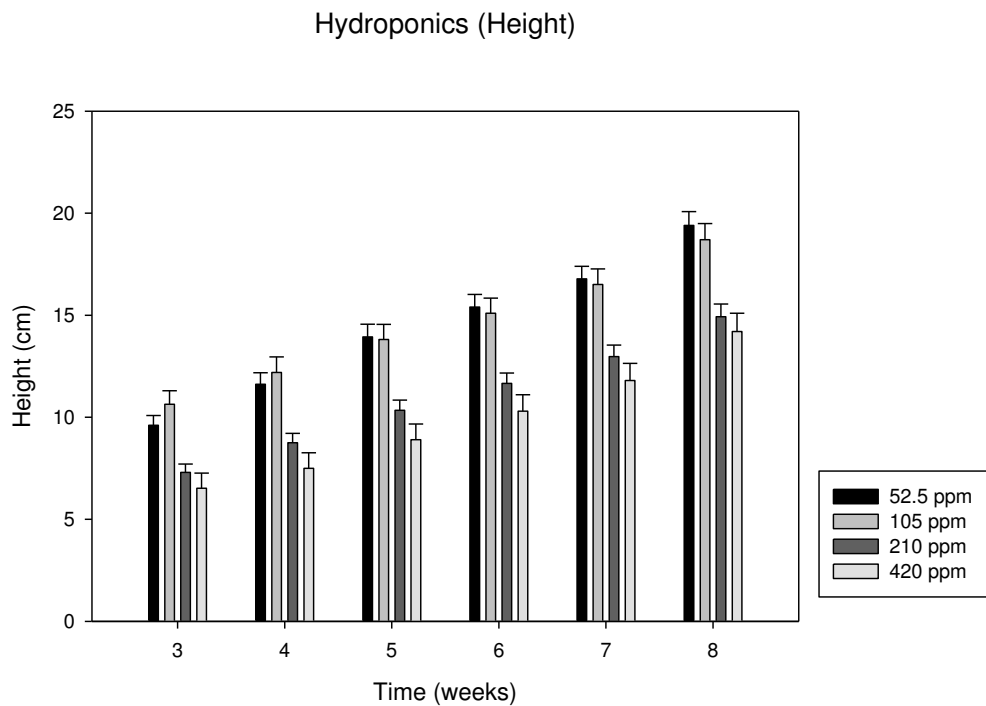


Fig.3.1: Effect of nitrogen treatments (52.5 ppm, 105 ppm, 210 ppm and 420 ppm) on plant (*H. cymosum*) height at weeks 3-8.

3.3.3. Number of leaves

Three weeks after initiation of N fertilizer treatment, there was a significant difference ($P < 0.05$) in leaf numbers between 105ppm [11.35 ± 1.828] and 420 ppm [3.7 ± 0.61], whilst no significant difference in leaf numbers was observed among plants exposed to 52.5 ppm (4.1 ± 0.44), 210 ppm (7.275 ± 0.75) and 420 ppm (3.7 ± 0.61) (Fig 3.2) when means were separated using Tukey HSD test. At 8 weeks post treatment, 105 ppm N concentration had a significant effect ($P \leq 0.03$) on leaf numbers (68.1 ± 6.2) produced by *H. cymosum* compared to the other treatments (52.5 [25.8 ± 1.67], 210 [41.7 ± 3.3] and 420 ppm [35.5 ± 4.5] of N). Generally, there was a significant increase in plant height ($df = 5, 234, P \leq 0.05$) for all treatments based on repeated measure ANOVA analysis.

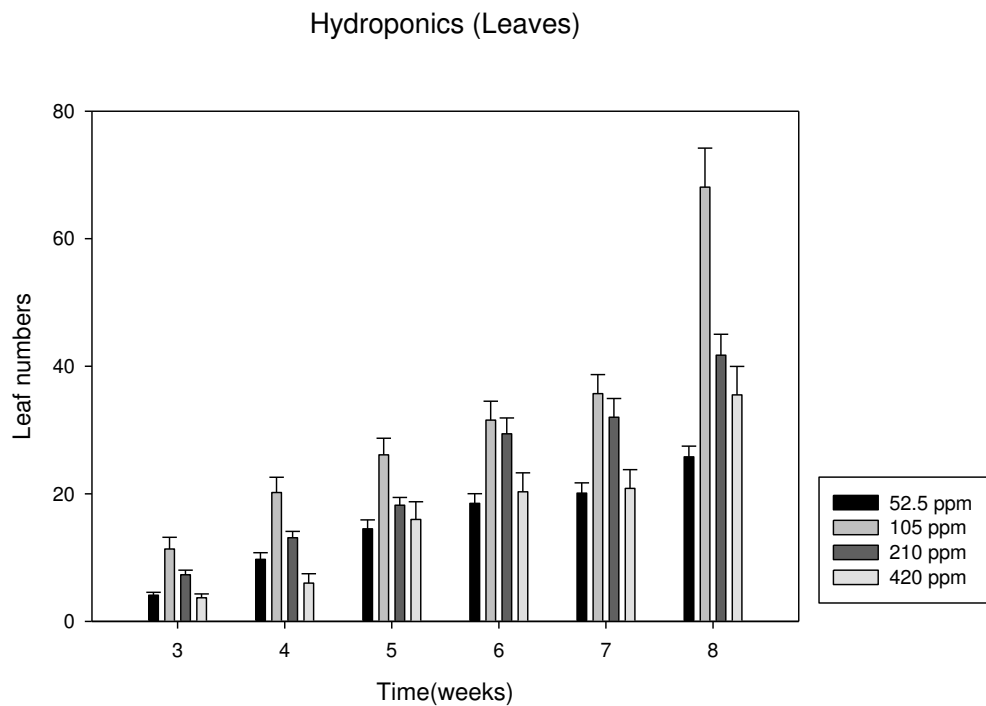


Fig.3.2: Effects of different nitrogen supplies on the number of leaves produced by *H. cymosum* under hydroponics conditions from 3 – 8 weeks post-treatment.

3.3.4. Tissue Nutrient Content

Plant tissue N, K and P contents were significantly higher in plants exposed to the highest nutrient nitrogen level (420 ppm) compared to those exposed to lower levels of N different (df, 3, 12; $F = 14, 78$; $P \leq 0.01$) (Table 3.3).

Table.3.3: Leaf nutrient (N, K, P) content (%) following treatments of *H. cymosum* with different hydroponics nitrogen levels (52.5 ppm, 105 ppm, 210 ppm and 420 ppm) at 8 weeks post treatment.

Treatment	N (%)	K (%)	P (%)
52.5 ppm	3.52 ± 0.154 B	3.52 ± 0.154 B	0.53 ± 0.03 B
105 ppm	3.21 ± 0.22 B	4.177 ± 0.057 B	0.78 ± 0.0263 B
210 ppm	2.86 ± 0.156 B	3.6 ± 0.29 B	0.69 ± 0.126 B
420 ppm	4.32 ± 0.21 A	4.74 ± 0.29 A	1.06 ± 0.09 A

Means followed by the same uppercase letters in the same column means not significant different ($P > 0.05$) following comparison using Tukey test.

3.3.5. Yield, Minimum Inhibitory Concentration (MIC) and Total Activity (TA)

3.3.5.1. Yield

The yield of acetone extracts of *H. cymosum* treated with 52.5 ppm (87.5 ± 15.5 mg) of N was significantly different ($P \leq 0.05$) compared to 105 ppm (230 ± 23.4 mg) but not statistically different ($P > 0.05$) to 210 ppm (145 ± 41.33 mg) and 420 ppm (175 ± 27.8 mg) of N.

3.3.5.2. Minimum inhibitory concentration

The MIC value of acetone extracts of plants treated with 52.5 ppm of N was 0.073 ± 0.014 mg/ml at 24 post treatment and remained unchanged at 48 hours post treatment. This MIC

value was significantly lower (df, 1, 3 ; F= 10.14; $P < 0.05$) compared to those obtained with acetone extracts of plants that were exposed to 105 ppm N (Table 3.4) with exception of 210 and 420 ppm at 24 hours. The MIC value of acetone extracts exposed to plants treated with 105 ppm was not statistically different ($P > 0.05$) to plants treated with 210 ppm at 48 h post treatment. Acetone extracts of plants exposed to nutrient solution containing 105 ppm of N exhibited the weakest anti-fungal activities MIC values were 0.47 ± 0 mg/ml and 0.705 ± 0.136 mg/ml at 24 h and 48 h post-treatment. Acetone extracts of plants that were grown in growth media containing 210 ppm of N recorded; 0.23 mg/ml and 0.47 mg/ml in the anti-*F. oxysporum* bioassay at 24 and 48 h respectively (Fig. 3.3). MICs of acetone extract at 420 ppm of N were (0.29 mg/ml) at 24 and remained unchanged at 48 hour. The antifungal activities reduced over time significantly ($P < 0.05$) in two treatments (105 and 210 ppm N). The antifungal activities of the extracts were significantly lower than mancozeb.

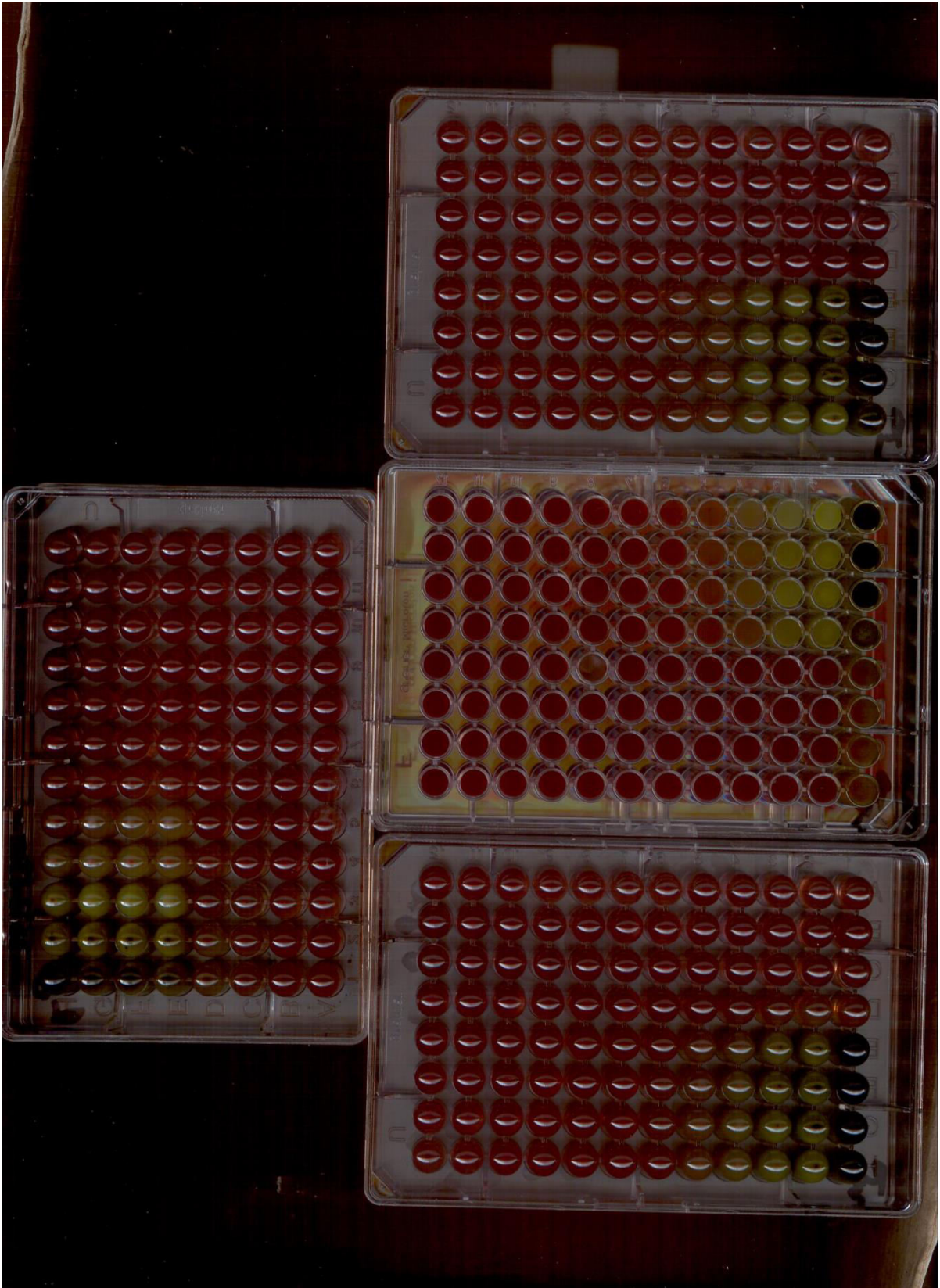


Fig 3.3: Anti-*Fusarium* activity of acetone extracts of *H. cymosum*; photograph showing activity of plant extract (light to dark green colouration).

3.3.5.3. Total activity (TA)

Total activity shows the quantity at which the extract may be diluted with a solvent, while retaining the capacity to eradicate a microorganism (Table 3.4). In this study, acetone extract of plants grown hydroponically at 52.5 ppm (0.26 ± 0.06 ml/g [24 and 48 hours]) recorded the highest values of TA against *F. oxysporum* compared to the other N treatments (105 ppm [0.1 ± 0.01 and 0.08 ± 0.0 ml/g], 210 ppm [0.12 ± 0.04 and 0.062 ± 0.02 ml/g] and 420 ppm [0.02 ± 0.06 and 0.18 ± 0.06 ml/g]) at 24 hours and 48 hours respectively. At 24 h post-treatment, there was no significant difference the calculated TA for all four treatments.

Table.3.4: Results on yield, minimum inhibitory concentration and total activities of acetone extracts obtained from aerial parts of hydroponically-cultivated *H. cymosum* following exposure to a range of N nutrient levels (52-420 ppm).

Treatments (ppm)	Yield \pm SE (mg)	MIC \pm SE (mg/ml)	Total Activity (ml/g)
52.5(24 H)	87.5 \pm 15.5 A	0.073 \pm 0.014 A	0.26 \pm 0.06
(48H)		0.073 \pm 0.0014 a	0.26 \pm 0.06
105 (24 H)	230 \pm 23.5 B	0.47 \pm 0 C	0.1 \pm 0.01
(48 H)		0.705 \pm 0.136 c	0.08
210 (24 H)	145 \pm 41.33 AB	0.234 \pm 0 AB	0.12 \pm 0.04
(48 H)		0.47 \pm 0 bc	0.062 \pm 0.02
420 (24 H)	175 \pm 27.8 AB	0.29 \pm 0.102 BC	0.02 \pm 0.06
(48 H)		0.29 \pm 0.102 ab	0.18 \pm 0.06
Positive control (24 H)		0.02 \pm 0.002 A	
(48 H)		0.02 \pm 0.002 a	
Negative control (24 H)		no effect	
(48 H)		no effect	

- Means followed by the same uppercase letters in the same column indicate no significant difference ($P > 0.05$) at 24 hours post treatment following comparison using Tukey test
- Means followed by same lowercase letters in the same column are not significantly different at 48 hours post treatment.
- Total activities in all treatments were not significantly different ($P > 0.05$) at 24 or 48 hours

3.3.5.4. LC-MS

The LC-MS results showed what may be described as distinct fingerprints of each extract (Fig.3.4). From a qualitative perspective, the profiles are largely similar in that they show the presence of mostly the same peaks, but quantitatively (following visual assessment of chromatographic peaks) they are different. Thus, while the earlier peaks (around the retention times (T_R) of approximately 8.0 and 9.0 min) are more prominent relative to the later peaks (from about 15.0 to 23.0 min), for the sample associated with the lowest N concentration (52.5 ppm), the pattern is reversed at higher N concentrations, with the later peaks predominating over the earlier ones. This is more profound where the different profiles have been overlaid on top of each other using different colour coding (Fig.3.5). To further interrogate the LC-MS data, extracted mass ion chromatograms were determined, using the molar mass (MM) values of the compounds which have been previously reported as natural product constituents of the genus *Helichrysum* (Bohlmann et al., 1979) (Fig.3.6). These include helihumulone (MM = 424) (Figs. 3.8 & 3.9), a major compound, as well as others (quercetin, kaempferol and apigenin) whose structures are presented in Fig. 3.7. Given that these compounds elute within the later T_R interval of 15-23 min, it is logical to expect that they are present in somewhat higher concentrations where the N levels (105 - 420 ppm) in the nutrients were higher. More importantly it is noteworthy that helihumulone was detected in minute amount in the sample corresponding to the lowest concentration of 52.5 ppm. On the other hand, the three earlier peaks which eluted at about T_R 8-9 min, and so consistently at all N levels, were identified as dicaffeoyl quinic acids. The ESI-MS fingerprints of the acetonitrile *H. cymosum* extracts suggest that quinine and caffeic acid derivative, dicaffeoylquinic acid (m/z 515) was present in the extracts.

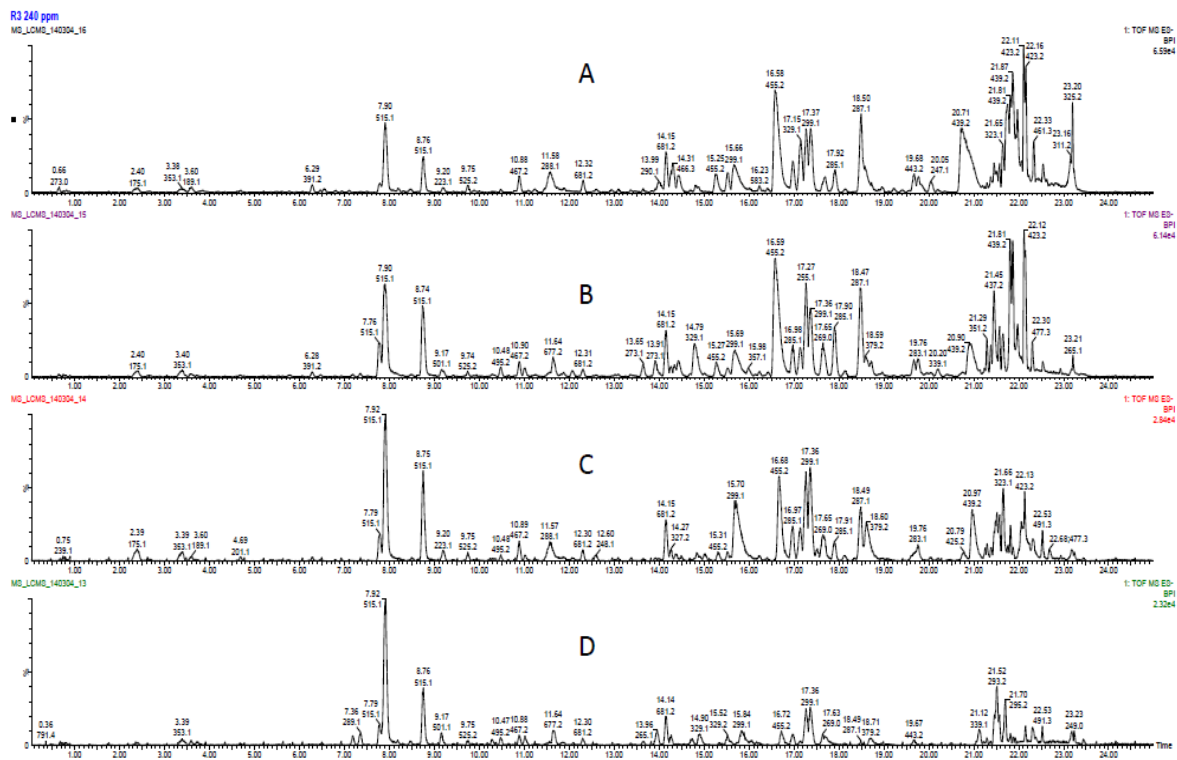


Fig.3.4: LC-MS profiles (TIC = total ion current) of extracts corresponding to N concentrations of A (420), B (210), C (105) and D (52.5) ppm respectively, read from top to bottom.

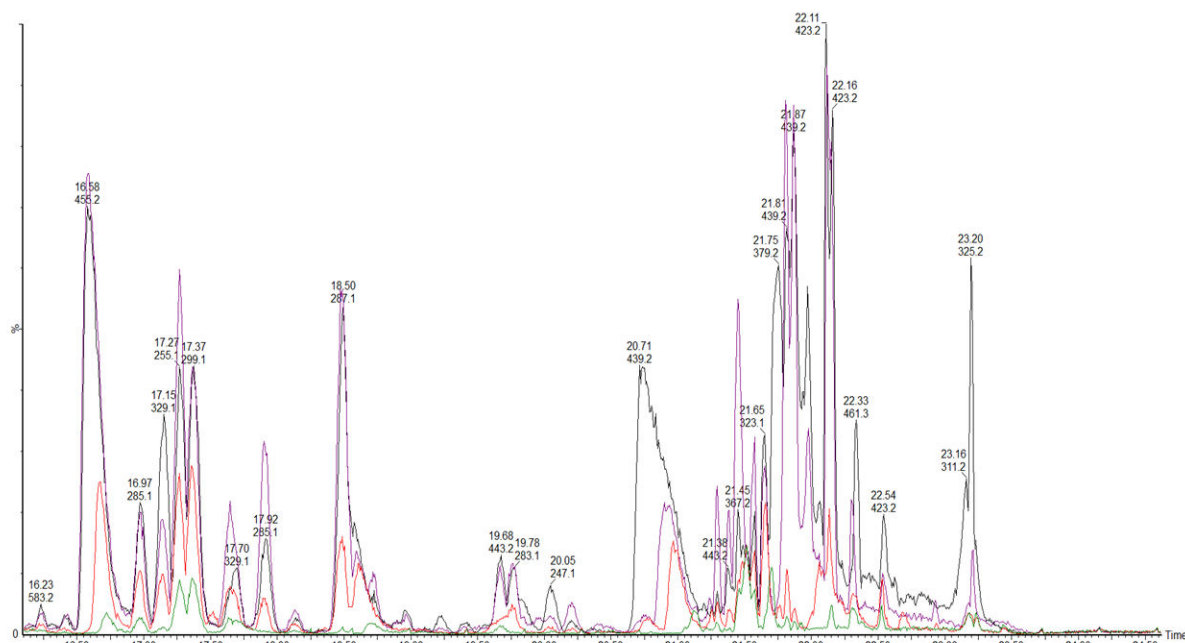


Fig.3.5: An overlay of the different LC-MS profiles for the retention time interval of 16.0 to 23.5 min; 420 ppm-black, 210 ppm-purple, 105 ppm-red, 52.5 ppm-green.

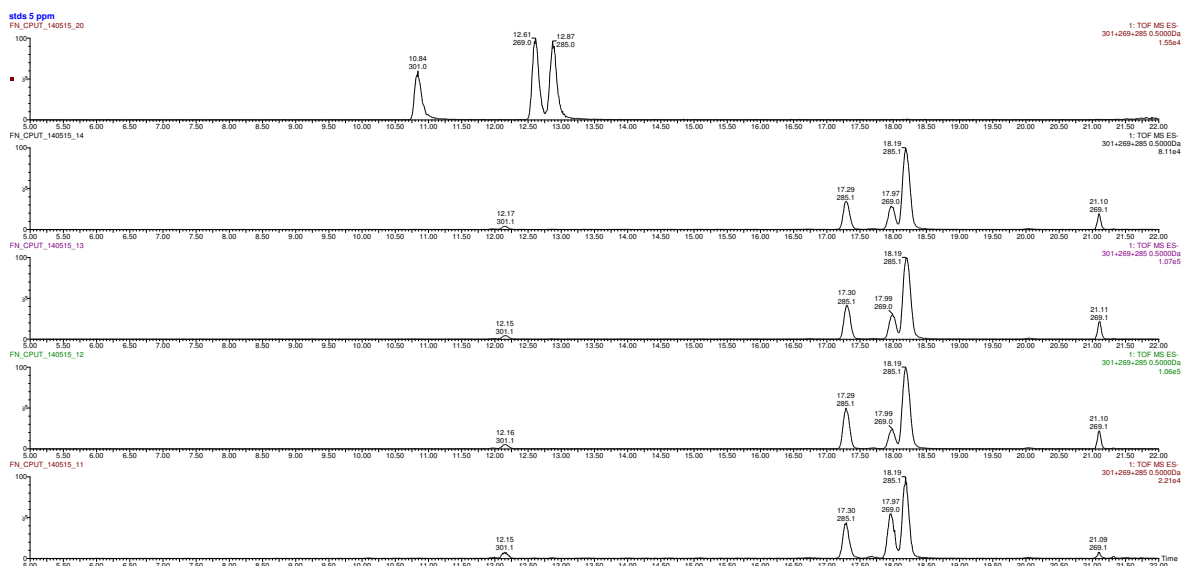
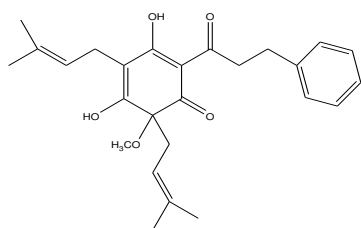
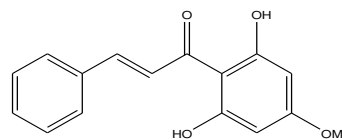


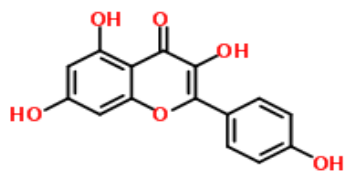
Fig.3.6: Extracted ion chromatograms for standards used at 5 ppm; A. Kaempferol (m/z 285, peak at 12.9 min); Apigenin (m/z 269, peak at 12.6 min); Quercetin (m/z 301; peak at 10.9 min) and crude acetone extracts and acetone crude extracts of *H. cymosum* in different N nutrient treatments; A (5 ppm), B (210 ppm), C (105 ppm), D (420 ppm) and E (52.5 ppm).



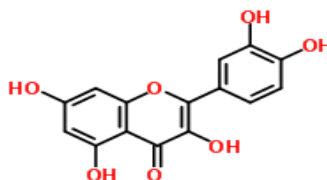
A: Helihumulone (MM=424)



B: Apigenin (MM=270)



Kaempferol



Quercetin

Fig.3.7: Chemical structures of the four phenolic compounds that are thought to be present in acetone extracts of *H. cymosum*.

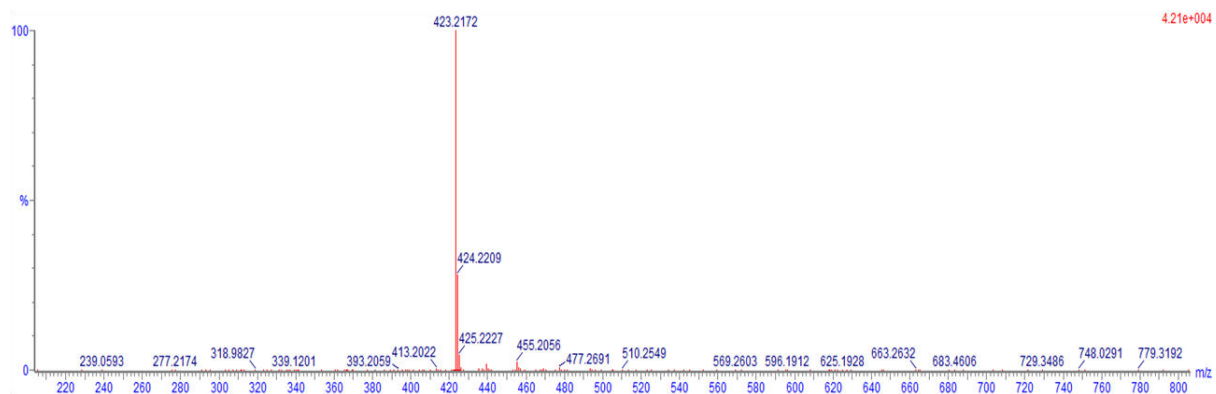


Fig. 3.8: ESI negative mass spectrum of helihumulone.

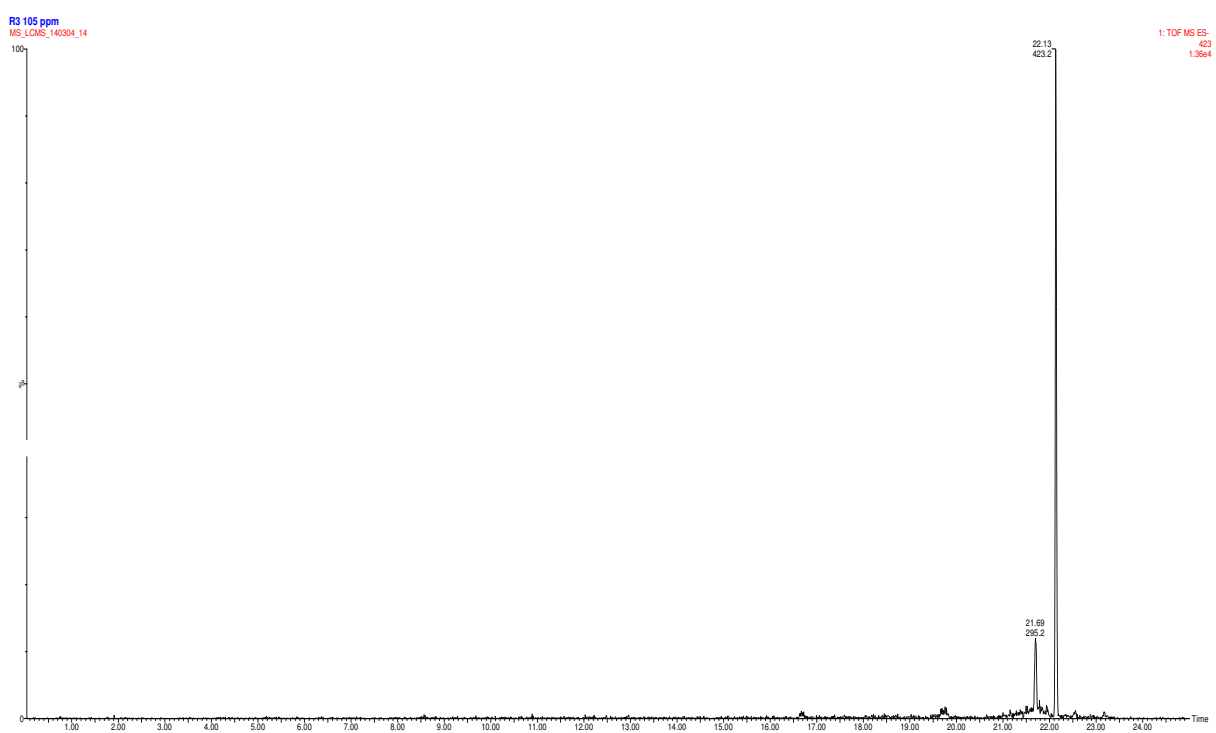


Fig. 3.9: Extracted mass ion chromatogram of helihumulone.

3.4. Discussion

3.4.1. Plant growth

The leaf number produced by plants exposed to the lowest level of N (52.5 ppm) was significantly lower than other N levels (105, 210 and 420 ppm) (Fig 2). According to Ma et al. (1997) N shortage at some stage of growth interferes with formation and development of leaves. Giorgi et al. (2009) found that in yarrow (*Achillea collina* Becker ex Rchb.), more shoots were produced by control-treated plants than in nitrogen-starved plants. Although results from most previous studies suggest a positive influence of increasing nutrient nitrogen level on plant growth parameters (Wahle & Masiunas, 2003; Moniruzzaman et al., 2009), increasing nutrient nitrogen levels does not always translate to increased vegetative growth in all instances. Najm et al. (2013) and Khan et al. (2014) stated that too much nitrogen application may decrease plant yield. In the current study, *H. cymosum* supplied hydroponically with relatively moderate N level (105 ppm) concurrently produced higher leaf numbers and fresh weights than those exposed to lower or higher N levels; 52.5, 210 or 420 ppm. These results are in agreement with previously published results by Farag et al. (2013), wherein treatments with 100 and 150 ppm of N were equally effective in inducing the production of higher leaf numbers in *Lactuca sativa* L. It is possible that the ideal range of nitrogen required by *H. cymosum* for optimum leaf production lies between 52.5 – 210 ppm. At the lowest treatment level of N, it was observed that plants actually grew tallest compared to the other treatments. These results further strengthened the argument that *H. cymosum* does not grow better beyond the optimum concentration of nitrogen, instead growth is reduced at very high levels of N. This argument is also corroborated by results published by Hussain et al. (2006); Asparagus highest spear height was reached in plants treated with 90 kg N ha⁻¹ whereas plants that were grown in 120 kg N ha⁻¹ had a height that was less than those grown in 90 kg ha⁻¹. Savvas et al. (2006) indicated that growth of plants may be limited if the rate of

N goes beyond the verge. Results from the present study show an increase in plant biomass production in *H. cymosum* plants supplied with hydroponics nutrient containing 105 ppm N.

3.4.2. Tissue content

The highest tissue concentrations of nutrients (N, P & K) were observed in plants treated with 420 ppm of N while no significant difference in concentrations of tissue nutrients occurred amongst the other lower N treatments. Application of N at high rate may lead to high nitrate concentration in plant tissues (Lastra et al., 2009) and may even increase the demand for other nutrients such as K, P by plant. In the present study, the percentage of phosphorus was elevated in plants treated with 420 ppm of N, these results are similar to results published by Savvas et al. (2006), N level positively correlated with P concentration in lettuce. Kane et al. (2006) reported that the increase in hydroponics N elevated the N and K content in onion plants.

3.4.3. Antimicrobial activity and total activity

Results obtained from this study indicated that acetone extract of plants treated with 52.5 ppm of N was the most bioactive against *F. oxysporum* compared to extracts obtained from plants exposed to 105 ppm, 210 ppm as well as 420 ppm of N treatments. Generally, acetone extracts of aerial parts of *H. cymosum* were found to be bioactive against *F. oxysporum* in the antifungal assay. When acetone extracts of plants exposed to the lowest level of Nitrogen (52.5 ppm) was compared to the other treatments (105 ppm, 210 ppm and 420 ppm), it gave the best MIC value (0.102 ± 0.044) in anti-*F. oxysporum* activity assay suggesting that the extracts of plants supplied with the lowest N concentration (52.5 ppm) was more bioactive than the plant extracts of plants exposed to higher concentrations. Furthermore, no statistical difference in the antifungal activities was observed between 52.5 ppm and mancozeb. In previous studies, *H. cymosum* was shown to have antimicrobial activity against the following

pathogens; *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *C. neoformans* (van Vuuren et al., 2006; Paiva et al., 2010). MICs of crude acetone extracts of *H. cymosum* tested against 10 pathogens in the study conducted by van Vuuren et al. (2006) ranged from 0.156-0.313 mg/ml. These results are comparable to the current results obtained in our anti-*F. oxysporum* bioassay; MIC values =0.078-03.31 mg/ml. It is worth-mentioning that the plant materials that van Vuuren et al. (2006) used were harvested from wild *H. cymosum* while the one that was used in this study was hydroponically-grown. Some of the challenges associated with sourcing medicinal plant materials from the wild is the variability in the profiles of phytochemicals in extracts obtained from different geographical region, probably due to variations in biotic and abiotic factors (Khan et al., 2011; Shalajan et al., 2013). These variations can be minimized in hydroponics cultivation of plants (Stewart & Lovett-Doust, 2003; Kiferle et al., 2011; Kiferle et al., 2013; Sugumaran et al., 2013).

According to the LC-MS analysis, the molecular masses of four compounds (MM 424; MM 270; MM 285; 301) matched previously isolated compounds from the genus, *Helichrysum* ; helihumulone, apigenin, kaempferol, quercetin, respectively (van Vuuren et al., 2006; Lourens et al., 2008; Albayrak et al., 2010; Matic et al., 2013). These compounds, including helihumulone were found to elute within the later T_R interval of 12-23 min, and judging by the corresponding area under each of the peaks it is logical to expect that they are present in somewhat higher concentrations where the N levels in the nutrients were higher (Fig. 4). More importantly it is noteworthy that the major active compound, helihumulone was detected in minute quantity in the sample corresponding to the lowest concentration of 52.5 ppm. High nutrient N treatment may have favoured plant production of bioactive principles as well as some compounds with no antimicrobial activity. Results from a previous study by Mudau et al. (2007) indicated that application of N, K and P fertilizers increased

quadratically the total polyphenols in bush tea (*Athrixia phyllicoides*). In the same study, linear relationships between percentage leaf tissue and N, P and K and with total polyphenols were observed. Increased tissue N, P and K contents were recorded among plants exposed to high hydroponics nitrogen fertilization in this study (Table 4). These macro elements are associated with the manufacturing of primary and secondary metabolites (Wanyoko, 1983; Mudau et al., 2007; Ibrahim et al., 2011). Our findings are in disagreement with the wide held view that the enhancement of total flavonoids and phenolics usually occur when plant is deficient in nitrogen (Koricheva et al. 1998; Felgines et al., 2000). According to recent studies, N has an influence on the amount of secondary metabolites; when plants are deprived of N, they tend to produce secondary metabolites (Giorgi et al., 2009; Le Bot et al., 2009; Bénard et al., 2011; Lingua et al., 2013). In a study conducted by Ibrahim et al. (2011), it was found that the total phenolics decreased in the leaves with the increase of N and plants that had limited amount of N had an increase in total flavonoids and phenolics.

On the basis of this data obtained in the LC-MS analysis it may be concluded that growing the subject species under different nutrient N levels affects the natural product profile of the plant. However, it should be noted that proposal of chemical structures on the basis of MM is only tentative, as in certain case the possibility of the presence of structural isomers of the suggested structures cannot be excluded. For example, the MM of 270 for compound B is identical to that of apigenin, another common phenolic compound in plants. The lack of correlation between bioactivity of extracts and increased N supply could be explained by the increased production of less bioactive molecules alongside bioactive principles to the extent that when extracts are proportionately compared, the bioactivity of the extracts obtained from plants exposed to high nutrient N treatment is more masked than in plants grown in low nutrient N treatments. Also, the ESI-MS fingerprints of the acetonic *H. cymosum* extracts suggest that the quinine and caffeic acid derivative, dicaffeoylquinic acid (m/z 515) was

present in the extracts (Cliford et al., 2005; Bastos et al., 2007) and have been isolated from *Helichysum* genus previously (Gouveia & Castilho, 2011; 2012). This compound which seemed to occur in relatively large quantity in plants exposed to 52.5 ppm N possesses antimicrobial activities (Ooi et al., 2006; Salomão et al., 2008; Chen, 2013). The high anti-*F. oxysporum* activity of 52.5 ppm of N might have been due to the deprivation of N in the *H. cymosum* plants. N deprivation favours production of carbon based secondary metabolites while high nutrient nitrogen favours nitrogen based compounds by plants (Ibrahim et al., 2011). In the current study, even though 52.5 ppm showed higher anti-*F. oxysporum* activity, the high yield of acetone extracts produced by plants exposed to high nutrient N resulted in no significant difference in total activities when treatments were compared. Eloff (2004) demonstrated the usefulness of this approach and equates total activity to terms like efficacy and potency, which are commonly used in pharmacology.

3.5. Conclusion

In conclusion, antifungal activities were maintained among *H. cymosum* plants grown hydroponically and plants exposed to 52.5 ppm N nutrient content in the growth medium yielded the most bioactive acetone extracts, while 105 ppm N yielded the highest plant biomass. The total activity suggests that there is no difference among plant extracts obtained from plants grown under different N fertilization. This study demonstrated that hydroponic cultivation of medicinal plants presents opportunity to improve quantity and quality of secondary metabolites produced, and may potentially reduce the over exploitation of certain plant species from the wild.

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