Heavy metals in South African medicinal plants with reference to safety, efficacy and quality

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Abstract

The trend in commercialization of medicinal plant products reflects the excessive exploitation of medicinal plants from the wild populations. Due to widespread soil pollution, there is a likelihood that medicinal plants could be harvested from heavy metal-contaminated soils and thus pose a potential health threat to consumers. Unregulated procurement coupled with the unhygienic trading environment, poor post-harvest handling and processing, represent major routes of heavy metal contamination in medicinal plant products.

A comparative screening was carried out to assess the levels of heavy metal contamination in some frequently used South African medicinal plants obtained from out-door traditional medicinal markets and *muthi* shops. Plant samples were digested using a microwave-assisted acid digestion system and the elemental content determined using inductively coupled plasma optical emission spectrophotometry (ICP-OES). There was multi-elemental contamination in the investigated medicinal plants with elevated levels of Fe, Al and Mn detected in most of the samples and levels of As and Hg were above the World Health Organization limits of 1 mg kg^{-1} and 2 µg kg^{-1} respectively. The high levels of metal contaminations in some of the investigated medicinal plants is a health concern and urgent measures are needed to protect the health of consumers. Samples were quantified for their total phenolic and flavonoid contents as well as screened for antibacterial activity. Variable phenolic and flavonoid composition and antibacterial activity showed that the quality and efficacy of medicinal plants sold at traditional medicine markets is compromised. Data obtained from elemental analysis was subjected to hierarchical cluster analysis which categorized samples into four main groups with samples within a group having relatively similar metal analyte compositions. Hierarchical cluster analysis proved to be a valuable tool in this preliminary screening of heavy metal contamination in medicinal plants and can potentially be used to develop a large database for easy monitoring of plant species with hyperaccumulative potentials. Information such as site of collection, plant species and plant part could be a valuable approach to ensure safety, efficacy and quality of medicinal plants sold at traditional medicine markets.

Exposure to Cd and Al for six weeks in a pot trial induced responses in *Bulbine natalensis, Drimia elata* and *Hypoxis hemerocallidea* and these included variations in heavy metal uptake, growth parameters and physiological changes. Generally, application of Cd and Al at low concentrations (2 and 500 mg L^{-1} respectively) enhanced growth parameters in the three plant species compared to the control plants. However, at the highest concentrations of Cd 10 and Al 1500 mg L^{-1} respectively, there was significant growth inhibition. *Hypoxis hemerocallidea* exhibited good tolerance to Al exposure up to 1000 mg L^{-1} compared to the other plant species. Some of the physiological changes such as accumulation of free-proline increased progressively with increasing heavy metal treatments in all the investigated plant species. The combined treatment of Cd 5:Al 1000 mg L^{-1} exhibited synergistic effects on the uptake and accumulation of Cd and Al with values of about 83 and 918 mg kg^{-1} respectively in the bulbs of *D. elata*. In *B. natalensis,* the combined treatment of Cd 10:Al 1500 mg L^{-1} resulted in the highest amount of Cd (67 mg kg^{-1}) in the bulb samples while the highest amount of Al (1607 mg kg^{-1}) was recorded after treatment with Cd 5:Al 1000 mg L^{-1}. There was an antagonistic effect on the uptake and accumulation of Cd in *H. hemerocallidea* in the combined treatments. Energy dispersive X-ray analysis of the abaxial leaf surface indicated that more Al was translocated to the shoot in *H. hemerocallidea* compared to Cd. The bulbs and corms of the investigated medicinal plants are the most extensively utilized plant parts in traditional medicine. High levels of Cd and Al in the bulbs and corms raise public health concerns.

Analysis of photosynthetic pigments showed total chlorophyll progressively decrease with increasing heavy metal stress in all three plant species. The effect of Cd and Al on chlorophyll fluorescence in *H. hemerocallidea* was investigated. Non-photochemical quenching (NPQ) was adversely affected in most of the heavy metal-treated plants indicating a photoinactivation of photosystem II (PSII) reaction centres. In the present study, increasing heavy metal treatment resulted in the inability of *H. hemerocallidea* to utilize the absorbed light energy leading to oxidative stress. Exposure to Cd and Al treatments for six weeks induced several ultrastructural changes in *H. hemerocallidea* including damage to the cortical cells and an increase in xylem size. Transmission electron microscopy revealed a complete breakdown of the thylakoids at the highest Cd treatment and the application of Al at moderate and the highest treatment significantly reduced the size of the chloroplasts. These

ultrastructural changes could possibly explain the reduced chlorophyll fluorescence and the amounts of total chlorophyll recorded at the higher levels of heavy metal treatments.

Biosynthesis and accumulation of secondary metabolites under heavy metal stress were variable in the investigated plants. The moderate Cd treatment at Cd 5 mg L^{-1} up-regulated the synthesis of total phenolics slightly compared to the controls in *B. natalensis.* All the other heavy metal treatments down-regulated the synthesis of total phenolics and flavonoids compared to the control plants in *B. natalensis*. Application of Cd and Al at the lowest concentrations, 2 and 500 mg L^{-1} respectively up-regulated the synthesis and accumulation of both phenolics and flavonoids in *D. elata* compared to the control plants. In *H. hemerocallidea,* the highest amounts of total phenolics and flavonoids were recorded at the moderate Cd treatment (5 mg L^{-1}). High performance liquid chromatography showed a significant decrease in the levels of hypoxoside, a bioactive compound in *H. hemerocallidea* after heavy metal exposure. The lowest amount of hypoxoside was recorded at the highest concentration of the combined treatment (Cd 10:Al 1500 mg L^{-1}). These variable responses to heavy metal stress indicated the need for in-depth research on changes of secondary metabolites in medicinal plants exposed to heavy metals in order to ensure ultimate quality and efficacy of medicinal plant products.

There was a progressive decrease in antioxidant activity as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging in the bulb extracts of *B. natalensis* and *D. elata.* The lowest treatment of Al (500 mg L^{-1}) had slightly higher DPPH activity compared to the positive control (ascorbic acid). Extracts of *H. hemerocallidea* exhibited a progressive increase in DPPH activity with increasing heavy metal treatments. There was a significant decrease in the DPPH activity at the highest Cd application (10 mg L^{-1}) compared to the control plants indicating a loss in the biosynthesis of important bioactive compounds at high levels of heavy metal exposure.

Cadmium applied at low and moderate concentrations enhanced antibacterial activity (0.78 mg mL^{-1}) against *Staphylococcus aureus* in *B. natalensis* compared to the control plant extracts. However, there was poor antibacterial activity against *Escherichia coli* in all the heavy metal-treated plants in *B. natalensis.* Application of

Cd and Al at low concentration in *D. elata* enhanced good antibacterial activity (0.78 mg mL^{-1}) against *E. coli* which is less susceptible to antibiotics than *S. aureus.* Extracts from all Cd-treated plants as well as low and moderate Al-treated *H. hemerocallidea* plants exhibited the good antibacterial activity against *S. aureus* compared to the control plants. Plants treated with the combined Cd 2:Al 500 mg L^{-1} treatment also had good activity against *S. aureus.* However, all the extracts of *H. hemerocallidea* exhibited poor activity against *E. coli.*

The responses of plants to Cd and Al varied depending on the species. Their ability to accumulate elevated levels of heavy metals raises concerns not only on the safety of these products but also the issues regarding the quality and efficacy of plants grown on heavy metal contaminated soils. The findings presented in this thesis highlight the need for stringent monitoring of heavy metal contamination in medicinal plant material sold at traditional medicine markets and the need for safe and sustainable cultivation of important medicinal plants. This will ensure that medicinal plant products are of a standard quality, safe from toxic contaminants and consistent in terms of phytochemical compositions.

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Declaration by Supervisors

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

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Signed at **UKZN Pietermaritzburg campus** on the 17th day of **November, 2014**.

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Publications from this Thesis

1. A. Okem, C. Southway, W.A Stirk, R.A. Street, J.F. Finnie, J. Van Staden, 2014. Heavy metal contamination in South African medicinal plants: A cause for concern. South African Journal of Botany 93: 125-130.

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3. A. Okem, C. Southway, A.R. Ndhlala, J. Van Staden, 2012. Determination of total and bioavailable heavy and trace metals in South African commercial herbal concoctions using ICP-OES. South African Journal of Botany 82, 75-82.

4. A.R. Ndhlala, B. Ncube, **A. Okem,** R.B. Mulaudzi, J. Van Staden, 2013. Toxicology of some important medicinal plants in southern Africa. Food and Chemical Toxicology 62, 609-621.

Conference Contributions from this Thesis

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Declaration 2 - Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

Contributions: AO conceptualized the research ideas, carried out all the laboratory experiments and prepared the manuscript. JVS partially funded the project and edited the manuscript. CS assisted with elemental analysis using ICP-OES. WAS, RAS and JFF provided supervision and editing of manuscript before submission.

Publication 2

Contributions: AO conceptualized the research ideas, carried out all the laboratory experiments and prepared the manuscript. JVS partially funded the project and edited the manuscript. CS assisted with elemental analysis using ICP-OES. WAS, RAS and JFF provided supervision and editing of manuscript before submission.

Publication 3

Contributions: AO conceptualized the research ideas, carried out all the laboratory experiments and prepared the manuscript with the assistance from ARN. JVS partially funded the project and edited the manuscript. CS assisted with elemental analysis using ICP-OES.

Publication 4

Contributions: AO contributed sub sections on heavy metal contaminations in some important South African medicinal plants. JVS edited the manuscript.

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List of Abbreviations

ADRs	adverse drug reactions
AERs	adverse event reactions
Al	aluminum
$Al(NO_3)_3$	aluminum nitrate
ANOVA	analysis of variance
As	arsenic
ATCC	American type culture collection
BA	benzylaminopurine
BDW	bulb dry weight
BFW	bulb dry fresh weight
C	carbon
Ca	calcium
Cd	cadmium
$Cd(NO_3)_2$	cadmium nitrate
CDW	corm dry weight
CFW	corm fresh weight
Chl	chlorophyll
Cl	chlorine
CP	cortex parenchymatic cell
Co	cobalt
CO_2	carbon dioxide
CTE	catechin equivalent
Cu	copper
Cr	chromium
CW	cell wall
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	dry weight
E	epidermis
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive x-ray spectroscopy

EPA	environmental protection agency
ETR	electron transfer rate
Fe	iron
F_m	maximum fluorescence
F_o	initial fluorescence
F_v	variable fluorescence
FW	fresh weight
GACP	good agricultural and collection practices
GAE	gallic acid equivalent
HCA	hierarchical cluster analysis
HCl	hydrochloric acid
Hg	mercury
HIV/AIDS	human immune deficiency virus/acquired immunodeficiency syndrome
HNO_3	nitric acid
HPLC	high performance liquid chromatography
HS	Hoagland's nutrient solution
ICP-OES	inductively coupled plasma-optical emission spectrophotometry
INT	*p*-iodonitrotetrazolium chloride
IS	intracellular space
K	potassium
LM	light microscopy
M	membrane
MDA	malondialdehyde
Mg	magnesium
mg g-1 FW	milligram per gram fresh weight
mg kg^{-1}	milligram per kilogram
mg L^{-1}	milligram per litre
MIC	minimum inhibitory concentration
MH	Mueller-Hinton
mmol g^{-1} FW	millimole per gram fresh weight
Mn	manganese
MT	mitochondria
MTS	*muthi* shop

MS	Murashige and Skoog
MX	metaxylem
$NaNO_2$	sodium nitrite
NBT	nitroblue tetrazolium
ND	not detected
Ni	nickel
nm	nanometer
NPQ	non-photosynthetic quenching
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	oxygen
O_2^-	superoxide radical
OD	optical density
$^{\cdot}OH$	hydroxyl radical
OSM	outdoor street market
p	p value
P	phosphorous
PH	phloem
PX	proxylem
PAR	photosynthetic active radiation
Pb	lead
PPF	photosynthetic photon flux
PSII	photosystem II
PT	plastoglobuli
rETR	relative electron transfer rate
RDW	root dry weight
RFW	root fresh weight
RL	root length
ROS	reactive oxygen species
RSA	radical scavenging activity
S	sulphur
SD	standard deviation
SDW	shoot dry weight
SEM	scanning electron microscopy

SFW	shoot fresh weight
SG	starch grain
Si	silicon
SL	shoot length
SOD	superoxide dismutase
TBARS	2-thiobarbituric acid reaction substances
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid
TCM	traditional Chinese medicine
TEM	transmission electron microscopy
THPs	traditional health practitioners
TLC	thin layer chromatography
UP	ultra pure
USA	United States of America
UV	ultraviolet
WHO	World Health Organization
X	xylem
Zn	zinc
µmole g^{-1} FW	micromole per gram fresh weight

Chapter 1

Introduction and literature review

1.1 Introduction

The high demand for traditional and alternative medicines has led to a rapid increase in the medicinal plant product market worldwide (**WHO, 2007**). Natural products from plants and their derivatives represent more than 25% of all drugs currently in clinical use (**GURIB-FAKIM, 2006**). The global market value of medicinal plant products is predicted to reach a value of US$107 billion by 2017 (**POSADZKI *et al.,* 2013**). The reason for the rapid growth in the medicinal plant products industry includes marketing strategies such as rigorous advertisements and campaigns by medicinal plant products manufacturers, the perceived effectiveness of medicinal plant remedies by consumers, and the increasing scientific understanding of the health benefits of bioactive components from plants and dietary supplements which have been extensively communicated to the general public (**SCHILTER *et al.,* 2003**). However, there is a misconception that "natural" always means "safe", and that remedies from a natural origin are harmless and carry no risk. But "natural" plant-based medicines are not always safe. Some plant species are inherently toxic and there is the possibility that plant samples may be contaminated with hazardous substances such as toxic heavy metals, agrochemicals and pathogenic microorganisms (**WHO, 2004**). The World Health Organization (WHO), in an effort to promote safe, effective and good quality traditional medicines, has recently developed strategies to support member states to harness the potential contribution of traditional medicine to health, wellness and people-centred health care; and to promote the safe and effective use of traditional medicine by regulating, researching and integrating traditional medicinal products, practitioners and practice into healthcare systems (**WHO, 2013**).

1.2 South African traditional medicine: an overview

It is estimated that over 70% of South Africans are reliant on traditional medicine for their primary healthcare (**MANDER *et al.,* 2007**). There are over 200 000 traditional health practitioners (THPs) that are actively involved in providing healthcare services for millions of South Africans (**GQALENI *et al.,* 2007**). The reasons that individuals

consult THPs include the high cost of Western medicine, availability and accessibility of natural products and cultural beliefs (**DAUSKARDT, 1990; COCKS AND MØLLER, 2002**). Although the cost of consulting THPs may be similar as for private health facilities, payment for the THPs is more flexible. For example, in rural areas, THPs may accept payment in cash or kind (usually livestock), while other traditional healers may follow a 'no cure, no pay' practice (**GALOOBA-MUTEBI AND TOLLMAN, 2007; THORNTON, 2009**). In some instances, a once-off payment may be accepted for multiple services that extend over a period of time (**THORNTON, 2009**).

In South Africa, trade of medicinal plant materials encompasses a network of role-players including collectors, transporters, hawkers, wholesalers, retailers and traditional healers (**MANDER *et al.,* 2007**). Medicinal plant gatherers collect their materials throughout the year in order to meet the constant demand. In some cases, young plants are harvested if the mature ones are not available, thereby resulting in inconsistency in plant material of the same species (**VON ALHLEFELDT *et al.,* 2003**). The harvesting of medicinal plant materials was formally the domain of trained THPs, renowned for their skills as herbalists and diviners (**CUNNINGHAM, 1991**). The customary conservation practices were strictly adhered to, which regulated plant collection times/seasons and the quantities collected. However, the recent trend in urbanization and the subsequent commercialization of traditional health care has led to a high demand for medicinal plant products. As a result, harvesting of medicinal plants has now become the domain of untrained, commercial gatherers with limited sources of income. Thus, harvesting and the provision of medicinal plants to meet the urban demand has become an environmentally destructive activity (**WILLIAMS *et al.,* 2000a**).

1.2.1 Trade of medicinal plant products in South Africa

Trade of medicinal plant products is an old tradition that is deeply rooted in South African society. The commercialization of medicinal plant products in South Africa became popular at the beginning of the 20th century with a traditional medicine market established in 1915 at eMatsheni in Durban. During this period, the Natal Native Medical Association was formed by licensed herbalist in the Durban area of KwaZulu-Natal Province (**CUNNINGHAM, 1988**). A similar association called the African Dingaka Association was formed in 1928 by a group of herbalist near Pretoria.

Although neither of the associations was recognized by the government, they were actively involved in promoting the traditional medicine industry as well as influencing any policy that hindered the trade of indigenous medicinal plant materials (**CUNNINGHAM, 1988**). To date, very little policy has been developed to support and promote the marketing of indigenous plants in South Africa. In addition, most of the proposed policies regarding traditional medicines have not been adequately enforced to create a better trading environment (**MBATHA *et al.,* 2012**).

One of the contributing factors sustaining the trade of medicinal plant products in South Africa is its vast floral biodiversity. South Africa is one of the "biodiversity hotspots" in the world and is home to over 30 000 flowering plant species (**LOUW *et al.,* 2002**), accounting for almost 10% of the world's vascular plant species (**VAN WYK AND GERICKE, 2000**). Over 700 indigenous plant species have been recorded in the South African traditional medicinal market plant trade (**MANDER *et al.,* 2007**). The trade in traditional medicinal plants and products in South Africa is estimated to be worth over R2.9 billion per annum (**MANDER *et al.,* 2007**). For instance, the annual trade of medicinal plant materials in KwaZulu-Natal and Eastern Cape Provinces is valued at R60 million and R27 million, respectively (**MANDER, 1998; DOLD AND COCKS, 2002**). The increasing economic values of medicinal plant trade has created job opportunities for thousands of South Africans, most of whom are marginalized black rural women. In KwaZulu-Natal, it was estimated that over 30 000 people derived their livelihood in the trade of indigenous medicinal plant materials (**MANDER, 1998**). Other commercial opportunities to further develop the medicinal plant market also exist. For instance, in 1998, the San people of South Africa sold the right of ownership of the *Hoodia* plant to a British Company (Phytopharm) for about US$20 million. In 2003, Phytopharm sub-licensed the product to Pfizer for US$21 million (**HOLT AND TAYOR, 2006**).

1.2.1.1 Trade of medicinal plant products in outdoor-street markets

In urban areas, outdoor-street markets for indigenous plant products are usually positioned in the hub of the city where commuters catch taxis or busses for intra- and inter-city destinations. Comparatively, in rural areas, informal markets for traditional medicines are located at trading sites, such as cattle sale lots and administrative

centres. Most of the urban street traders usually visit the city for a few days or weeks at a time to trade their goods, and then return to their rural homes (**MANDER, 1998**). It is common practice that most traders simply trade their goods from the pavements or bare surfaces adjacent to busy roads as shown in **Figure 1.1A**, thereby exposing the products to various forms of contamination. Some traders may erect wooden sheds on the pavement for trading and storage purposes and sometimes plastic sheets are used to cover their products at night and from rain (**MANDER, 1998**).

Typical modern outdoor-street market in Pietermaritzburg, South Africa

One of the earliest records of street trading of medicinal plant products in Durban, South Africa (Courtesy: 'Karen E. Flint. Healing Traditions: African Medicine, Cultural Exchange, & Competition in South Africa, 1820-1948. Athens, OH: Ohio University Press, 2008. Project MUSE).

Figure 1.1 Typical modern (A) and old (B) outdoor-street market in KwaZulu-Natal, South Africa.

In the KwaZulu-Natal Province, it was estimated that over 16 000 medicinal plant gatherers are actively involved in the supply of plant materials to more than16 000 people practicing as healers and street traders of medicinal plant products (**MANDER *et al.,* 2007**). Street traders to a large extent, play the role of wholesalers to healers and shop owners as well as retailing products to end consumers. On-going observations of the medicinal plant trade indicates that the markets continue to be buoyant, with street markets remaining well patronised as the demand for medicinal plant products increase (**MANDER *et al.,* 2007**). This is evident by a visible growth in the street trade of medicinal plants in both urban and rural markets South Africa.

1.2.1.2 Trade of medicinal plant products in indoor *muthi* shops

Medicinal plant products are also traded from indoor traditional medicine *muthi* shops (*muthi* is a Zulu term that refers to a herbal remedy) usually located close to transport nodes and busy parts of town. The trading environment in the *muthi* shops are more organized, relatively attractive and seemingly more hygienic than the open street markets. Medicinal plant products in *muthi* shops are neatly arranged on shelves and labelled (**Figure 1.2A**).

Plant samples arranged on shelves and properly labelled

Manufactured herbal products sold at *muthi* shops

Figure 1.2 Typical medicinal plant products sold at indoor *muthi* shops

The rate of contamination of medicinal plant products sold at indoor *muthi* shops is less compared to the outdoor-street markets as the plant products are not directly exposed to continuous atmospheric deposits. Consumers generally prefer the modernized and relatively hygienic trading environment which is offered by the *muthi* shop owners (**MANDER *et al.,* 2007**). There are approximately 110 *muthi* shops in KwaZulu-Natal. This low number of *muthi* shops could be due to competition from the street traders (**INSTITUTE OF NATURAL RESOURCES, 2003**). Pricing is one of the driving forces that influence consumers to patronize street traders more than *muthi* shops (**INSTITUTE OF NATURAL RESOURCES, 2003**).

One of the more recent advances in South African traditional medicine is the manufacturing, packaging and selling of medicinal plant products as herbal concoctions over the counter (**Figure 1.2B**). This ranges from general tonics and herbal teas, to snuff and herbal cigarettes (**NDHLALA *et al.,* 2009**). These medicinal

plant products are generally available in *muthi* shops rather than street markets. Herbal concoctions are mixtures of medicinal plants or plant parts that are used in treating specific ailments. Herbal concoctions are prepared using different methods, which vary from simple brewing processes to more complex techniques that use alcohol and other organic solvents to extract bioactive compounds (**PUJOL, 1990**).

1.3 Medicinal plant product contamination

Contamination of medicinal plant products is defined as "the undesired introduction of impurities of a chemical or microbiological nature, or of foreign matter, into or onto a starting material, intermediate product or finished medicinal plant product during production, sampling, packaging or repackaging, storage or transport" (**WHO, 2012**). As depicted in **Figure 1.3**, there are a number of potential contaminants of medicinal plant products such as heavy metals (**ERNST, 2000; SAAD *et al.,* 2006; GABARDI *et al.,* 2007**), agrochemicals (**McLAREN *et al.,* 2008**), pharmaceutical drugs (**ERNST, 2002; ERNST, 2003**) and pathogenic microorganisms (**McLAREN *et al.,* 2008; ULBRICHT *et al.,* 2011**). Accumulation of toxic metals in medicinal plant products is becoming a major concern in traditional medicine.

One of the major means of heavy metal accumulation in medicinal plants is through the uptake and accumulation of toxic metals and trace elements that are inherently present in the soil (discussed in Section 1.4). Medicinal plant products sold at outdoor-street markets can become contaminated with toxic heavy metals during open-air drying or when displayed on pavements by street traders. This exposes plant products to atmospheric deposits arising from urban pollution such as industrial and vehicular emissions. Most *muthi* shop-owners buy the bulk of their plant materials from street traders, hence some of the medicinal plant products sold at *muthi* shops may have been exposed to contaminants prior to being housed indoors. Furthermore, contamination may occur when *muthi* shop-owners fumigate their shops to control pests and by doing so, expose plant materials to potentially toxic residues which in the long term, can compromise the health of consumers (**FENNELL *et al.,* 2004**). In addition, heavy metals may be purposefully added to medicinal plant products purported to increase therapeutic properties (**ERNST, 2002; HAIDER *et al.,* 2004; OKEM *et al.,* 2012**). The presence of pathogenic microorganisms in medicinal plant

products could be due to unhygienic conditions in the *muthi* market environment such as unregulated urination in streets by some pedestrians, uncollected garbage and general untidiness from the vendors themselves. Studies have shown that bacteria and mycotoxin-producing moulds are widespread in the atmosphere and these are some of the most common microbial contaminants of medicinal plant products (**CVETNIĆ AND PEPELJNJAK, 1999**).

1.3.1 Cases of heavy metal contamination in South African traditional medicine

Previous studies have shown elevated levels of toxic heavy metals in some important South African medicinal plants obtained from *muthi* markets. For instance, **STEENKAMP *et al.*** (**2006**) reported high levels of manganese (Mn) and chromium (Cr) in some medicinal plant materials used in South African traditional medicine. In a similar study, **STREET *et al.*** (**2008**) reported high levels of arsenic (As) and cadmium (Cd) above the WHO recommended levels in bulbs of some frequently used South African medicinal plants obtained from outdoor-street markets. *Hypoxis hemerocallidea* accumulated high levels of aluminum (Al) which indicated that it could be a hyperaccumulator of this element (**JONNALAGADDA *et al.*, 2008**). On the contrary, **MTUNZI *et al.*** (**2012**) reported levels below the recommended safety limits of metal concentrations in some medicinal plants used in South African traditional medicine.

There have been a number of cases of heavy metal poisoning associated with the use of South African traditional medicine. For example a case of metal poisoning was reported among the Indian community in South Africa. The leading cause was associated with heavy metal contamination in the medicinal products (**KEEN *et al.*, 1994**). **NRIAGU *et al.*** (**1997**) investigated the high rate of lead poisoning among children in KwaZulu-Natal and the leading causes were linked to food and traditional medicine contaminated with lead. **STEENKAMP *et al.*** (**2002**) reported a severe case of multiple heavy metal poisoning in a child treated with a South Africa traditional remedy resulting in several clinical manifestations of gastrointestinal and neurological disorders.

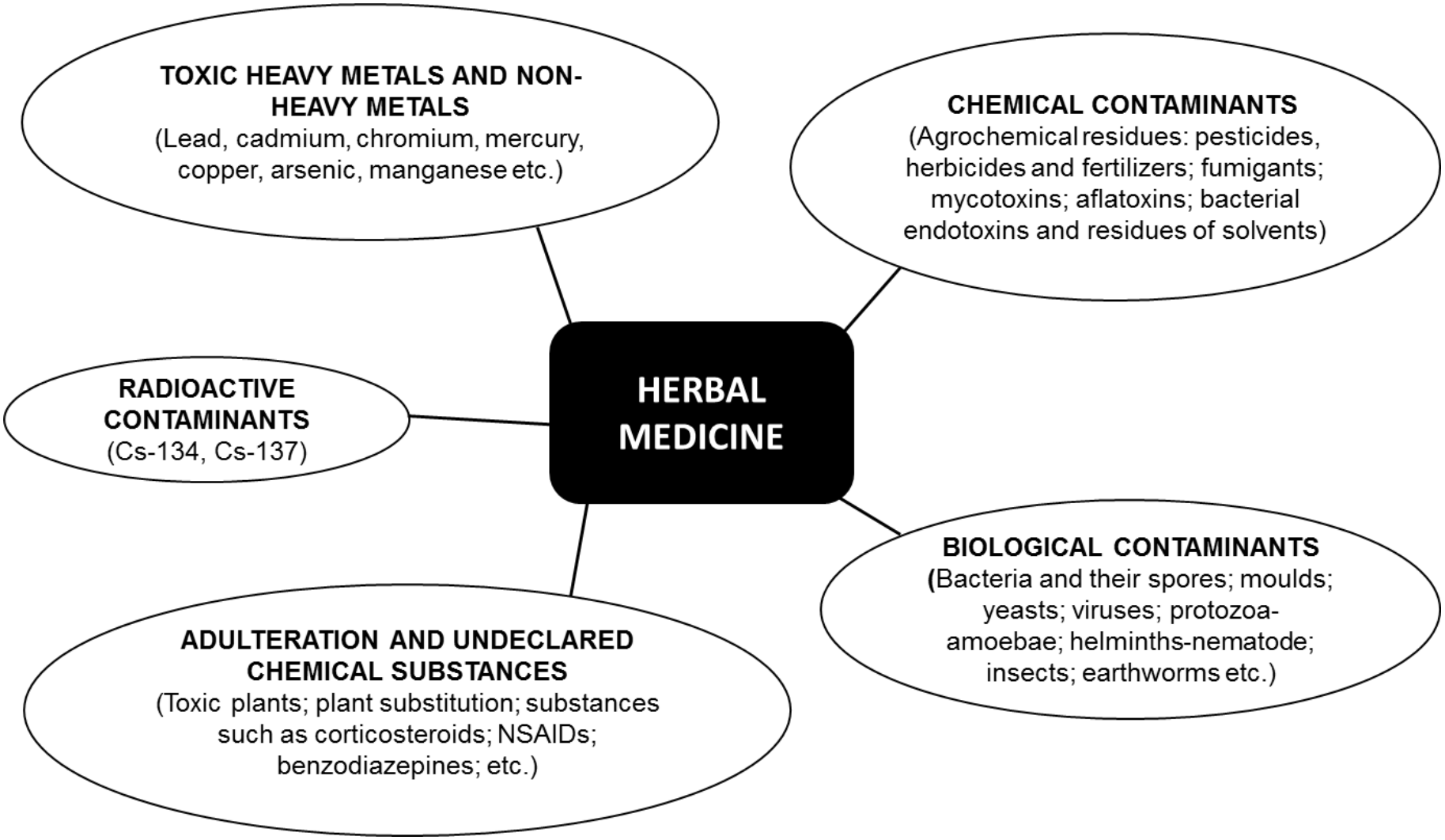

Figure 1.3 The most common contaminants and adulterants in medicinal plant products (Modified from **KOSALEC *et al.,* 2009**). NSAIDS: Non-steroidal anti-inflammatory drugs.

In 2008, 13 members of a family were reported to have died after consuming a medicinal plant concoction in their home near Port Shepstone on the KwaZulu-Natal south coast. The medicinal plant concoction was reported to contained unknown substances (**IOL NEWS, 2008**).

1.4 Heavy metal uptake in plants

Although, in recent decades, the phrase "heavy metals" has been used widely to refer to a group of metals (and semi-metals) that are associated with contamination and potential toxicity, there is no clear and unique scientific definition "heavy metal" **(DUFFUS, 2002)**. In this thesis, "heavy metals" will be used to refer to those elements with metallic properties and an atomic number >20. Metals are natural components in soil (**LASAT, 2000**) and some of these metals are micronutrients necessary for plant growth, such as zinc (Zn), copper (Cu), manganese (Mn), nickel (Ni), and cobalt (Co), while others such as aluminum (Al), cadmium (Cd), lead (Pb), and mercury (Hg) have unknown biological functions (**GAUR AND ADHOLEYA, 2004**). Metals exist in soils in a variety of chemical species that are in dynamic equilibria governed by physical, chemical and biological properties of the soil (**CHANEY, 1988**). Increasing anthropogenic activities have led to high levels of toxic heavy metal contamination of agricultural soils. This has become a critical environmental concern due to their potential adverse ecological effects, their widespread occurrence, and acute and chronic toxic effect on plants (**YADAV, 2010**). Heavy metals do not undergo biodegradation and are accumulated in living organisms, thus causing various diseases and disorders even in relatively low concentrations (**PEHLIVAN *et al.,* 2009**). Plants usually take up trace elements and heavy metals that are dissolved in soil solutions in either ionic, chelated or complexed forms (**KABATA-PENDIAS, 2011**). Uptake and accumulation of heavy metals by plants involve a series of mechanisms such as the use of specific genes as transporter, efflux pumps and chelating agents (**RASCIO AND NAVRI-IZZO, 2011**).

Numerous abiotic factors influence the availability of metals to plants including pH, redox potential, cation exchange capacity and soil organic matter (**GREGOR, 2004**).

Furthermore, interactions of soil-plant roots-microorganisms play a vital role in regulating uptake of heavy metals from the soil to edible plant parts (**ISLAM *et al.,* 2007**). Uptake and accumulation of toxic metals in medicinal plants represent the principal route of toxic metal entry into the human body (**McLAUGHLIN *et al.,* 1999**). Concentration of toxic heavy metals in medicinal plants is of great concern and this requires urgent measures to safeguard public health. According to the **WHO** (**2007**), the concentration of trace elements must be controlled in medicinal plants in order to improve the quality assurance and safety of medicinal plant products.

1.4.1 Mechanisms of heavy metal toxicity and tolerance strategies in plants

Phytotoxicity of heavy metals may result from alterations of numerous physiological processes caused at the cellular/molecular level and these may include inactivation of certain enzymes, blocking functional groups of metabolically important molecules, displacement or substitution of essential elements and disruption of membrane integrity. One of the common consequences of heavy metal toxicity is to enhanced the production of reactive oxygen species (ROS) due to interference with electron transport activities, especially that of chloroplast membranes (**SCHÜTZENDÜBEL AND POLLE, 2002; PAGLIANO *et al.,* 2006; La ROCCA *et al.,* 2009**). Exposure to heavy metal stress also leads to oxidative damage through indirect mechanisms such as interaction with antioxidant defence systems, disruption of the electron transport chain, induction of lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage (**QUARTACCI *et al.,* 2001; SCHÜTZENDÜBEL AND POLLE, 2002**). Heavy metal toxicity also alters the content of essential mineral nutrients and decreases photosynthesis as a consequence of a decline in the chlorophyll content as well as stomatal closure (**VÁZQUEZ *et al.,* 1992**).

Metallophyte species exhibit tolerance mechanisms to toxic heavy metals by using chemically suitable ligands to form stable non-toxic complexes which are then taken up and sequestrated into vacuoles (**SALT *et al.,* 2002**). Some plant species express tolerance by compartmentalization and detoxification of the toxic metals in their root cells by complexation with amino acids, organic acids or metal-binding peptides

(**WILLIAMS *et al.,* 2000b; HALL, 2002**). Alternatively, plant species may use excluding mechanisms by hindering uptake of heavy metals into root cells through entrapment in the apoplastic environment (**WATANABE AND OSAKI, 2002**) or by binding to anionic groups of cell walls (**VECCHIA *et al.,* 2005; RASCIO *et al.,* 2008**). This mechanism restricts translocation of heavy metals to the above-ground organs thus protecting the leaf tissues, and particularly the metabolically active photosynthetic cells from heavy metal toxicity. Some plant species exude certain organic acids into the rhizosphere to chemically reduce or inhibit uptake of toxic metals by forming complexes outside the root (**PINEROS AND KOCHIAN, 2001**). For instance, barley plants exposed to Al exuded high amounts of malic, citric and succinic acids and these enhanced tolerance in the plant compared to Al-sensitive plants (**GUO *et al.,* 2007**). Hyperaccumulator plant species are known to accumulate extremely large amounts of heavy metals in shoots compared to non-accumulator species (**KIRKHAM, 2006**). Plant species that can accumulate heavy metals above threshold values, including 10 000 mg kg^{-1} dry weight of shoots for Zn and Mn, 1 000 mg kg^{-1} for As, Co, Cu and Ni, and 100 mg kg^{-1} for Cd are regarded as hyperaccumulators (**McGRATH AND ZHAO, 2003**).

1.4.2 Physiological and biochemical responses of plants to heavy metal toxicity

The symptoms of metal toxicity in plants have been studied under various conditions. One of the most extensively investigated metals in this regard is Cd. Symptoms of Cd toxicity in plants includes growth inhibition, leaf chlorosis, impaired water uptake, decreased respiration, disruption of photosynthetic apparatus and nitrogen metabolism (**CLEMENS, 2006; TRAN AND POPOVA, 2013**). The phytotoxic effect of Cd on photosynthesis has been studied in various plant species (**KRUPA *et al.,* 1993; OUZOUNIDOU *et al.,* 1997; VARALAKSHMI AND GANESHAMURTHY, 2013**). At toxic concentrations, accumulation of Cd damaged leaf organelles, particularly the chloroplasts (**PENG *et al.,* 2005**). The impact of heavy metals on the chloroplast ultrastructure has been used to explore the physiological alterations induced by heavy metal toxicity in plants (**SRIDHAR *et al.,* 2005; GRATÃO *et al.,* 2009**). The relationship between chloroplast structure, photosynthetic ability, and plant growth is crucial in the plants ability to produce secondary metabolites. Oxidative stress induced

by heavy metals triggers signalling of certain biosynthetic pathways leading to the production of specific plant metabolites (**NASIM AND DHIR, 2010**). In particular, ROS generated during heavy metal stress may cause lipid peroxidation that stimulates the formation of highly active signalling compounds capable of triggering production of bioactive compounds (secondary metabolites) that enhance the medicinal value of the plant (**NASIM AND DHIR, 2010**). Plants exposed to heavy metal stress may either up-regulate or down-regulate the production of therapeutically active compounds (**MURCH *et al.,* 2003; RAI *et al.,* 2004**). For instance, heavy metal stress induced the accumulation of phenolic compounds in plants (**MICHALAK, 2006)**. Nickel toxicity was found to induce phenolic compound biosynthesis in wheat (**DIÁZ *et al.,* 2001**) and maize in response to Al toxicity (**WINKEL-SHIRLEY, 2002**). On the contrary, heavy metals can also reduce the production of certain plant secondary metabolites (**ZHELJAZKOV AND NIELSEN, 1996; XIONG *et al*., 2013**). In addition, heavy metal toxicity can lead to nutrient deficiencies and this may result in starch accumulation within leaves (**SIEDLECKA, 1995**).

Plants have evolved a number of strategies to counteract the toxic effects in the event of heavy metal stress by activating certain intermediary metabolic activities and making physiological adjustments as defence mechanisms. These physiological adjustments include the exudation of phenolic compounds and the accumulation of free-proline as well as antioxidant enzymes (**SHARMILA AND SARADHI, 2002; MICHALAK, 2006**). Accumulation of antioxidant enzymes, proline and phenolics under abiotic stress are part of the mechanisms to protect plants against free radicals, which can disrupt cellular metabolism, as well as cause oxidative damage to biomolecules such as lipids, proteins and nucleic acids (**ASADA, 1994; GILLE AND SIGLER, 1995**).

1.5 Safety assessment of medicinal plant

Safety assessment is a fundamental principle required for the provision of safe medicinal plant products and medicinal plant products. Safety assessment covers all relevant aspects of safety such as toxicological studies (genotoxicity and cytotoxicity),

adverse event reactions (AERs) and post-market surveillance of medicinal plant products (**WHO, 2003**). There is a misconception that medicinal plants are safe due to accumulated knowledge about their uses. However, studies have shown that some medicinal plant products produce serious side effects (**MARKMAN, 2002**). As the demand for medicinal plant products increased, so too have the reports of suspected toxicity and AERs (**SHAW *et al.,* 2012**). Assessment of the safety of medicinal plant products is complicated by the complex nature of ingredients used (**JORDAN *et al.,* 2010**). In most cases, adverse events arise from the use of wrong medicinal plant species, incorrect dosing and the use of products contaminated with potentially hazardous substances, such as toxic metals, agrochemical residues and pathogenic microorganisms (**WHO, 2004**). The increasing body of evidence emerging on hazards and the associated risk profile and the widespread use of medicinal plant products represents a mandate to the scientific community to improve the relatively weak scientific understanding of both the health benefits and the harmful effects of medicinal plant products.

There is a growing concern about the lack of professionalism of some THPs (**PEFILE, 2005**). This is evident in some of the unethical practices such as 'testing' their medicines inappropriately through trial and error with neither ethical approval from ethics committees nor informed consent from the patients concerned (**NYIKA, 2007**). There are also cases of indiscriminate harvesting of plant material from the wild as well as poor post-harvest handling, storage and processing of medical plant materials, thereby compromising the quality and safety of medicinal plant products. Studies have also shown adulteration with pharmaceuticals and excessive contamination of heavy metals and pesticides in traditional medicinal plant products (**ERNST, 2002; OKEM *et al.,* 2012**). Concerns about the safety, claimed efficacy of many medicinal plantl products, and lack of proper scientific evaluation are major challenges facing the growth of medicinal plant product industry. Hence, issues regarding safety and quality assessment, and regulatory control of medicinal plant products from the starting material to the finished products are now a major concern for health authorities, pharmaceutical industries and the general public (**WHO, 2007**).

1.6 Adverse event reactions associated with medicinal plant products

Adverse event reactions resulting from the use of medicinal plant products may lead to severe cases of life-threatening conditions such as hepatic injury, neurological and gastrointestinal disorders and sometimes death (**STEWART *et al.,* 1998; DESMET *et al.,* 1997**). Unexpected toxicity associated with the use of medicinal plant products are numerous. The presence of heavy metal contamination in medicinal plant products is a safety concern for the consumers. Contaminants in medicinal plant products may lead to a variety of adverse conditions ranging in severity from mild (allergic reactions, respiratory complaints, pain, nausea, fatigue, gastrointestinal upset, mood disturbances or muscle weakness) to moderate (vomiting, confusion, lethargy or seizures, sensory disturbances, compression fractures, leucopoenia, convulsions, persistent hypoglycaemia, Cushing's syndrome, burns, dermatitis) to severe (multi-organ failure, perinatal stroke, carcinomas, metabolic acidosis, nephrotoxicity rhabdomyolysis, renal or liver failure, cerebral oedema, coma, intracerebral haemorrhage or death) (**POSADZKI *et al.,* 2013**).

The earliest records of toxicity in some important traditional medicines were documented in the Chinese Pharmacopoeia (25-220 AD) (ShenNong BenChao Jing) in the East Han dynasty (**LIANG AND GAO, 1992; LIU *et al.,* 2003a**). Cases of AERs resulting from the use of traditional medicine is on the increase. A retrospective review of 5 563 cases of AERs received by the National Poisons Unit, London, showed that 19.3% of events were associated with medicinal plant products (**DREW AND MYERS, 1997**). The United State of America Poison Control Centre recorded 21 533 cases of poisoning between 1993-2002 and botanical products were the only substance implicated as the leading cause of severe clinical manifestations leading to high morbidity and death (**WOOLF *et. al*., 2005**). Safety of traditional medicine has been identified as one of the top research priorities in the medicinal plant products industry, starting from raw materials to the finished products (**POPAT *et al.,* 2001**).

In South Africa, cases of AERs associated with the use of traditional medicine are numerous, with many resulting in significant morbidity and mortality (**POPAT *et al.,* 2001; STEWART *et al.,* 1998; STEENKAMP *et al.,* 1999; JOUBERT AND SEBATA, 1982; IOL NEWS, 2008; MALANGU AND OGUNBANJO, 2009**). **STEWART *et al.*** (**1999**) investigated 206 cases of poisoning in South Africa in which 43% were associated with the use of traditional medicinal plants. The number of poisoning cases from traditional medicine may be far greater than the reported statistics due to the fact that data on mortality among South Africans is lacking, especially for rural areas where deaths are not always registered (**STUART, 2000**). Many poisoning case are thought to remain undiagnosed due to the fact that the majority of people are reluctant to admit to the use of medicinal plant products as a result of a negative view towards traditional medicine by Western medical practitioners. The issue of cultural secrecy surrounding the use of medicinal plant products is a major hindrance to access information on AERs. The lack of analytical techniques required to make a confident diagnosis is also another stumbling block to the analysis of cases of poisoning associated with the use of medicinal plant products (**BHAT AND JACOBS, 1995; STEWART *et al.,* 1998; STEENKAMP *et al.,* 1999**). Thus, the paucity of information on adverse effects of medicinal plant products, inadequate reporting schemes, fear of professional liability, and inadequate diagnostic systems are stumbling blocks in the integration of traditional medicine into the national healthcare system.

1.7 Quality assurance of traditional medicinal plant products

There is a need for a series of reliable authentication procedures as well as strict adherence to regulatory standards at all stages of phytomedicine production to ensure safety and quality of medicinal plant products. There are several protocols and guidelines on safety and toxicity testing of medicinal plant products published by the International Life Sciences Institute (summarised by **SCHILTER *et al.,* 2003**), the Institute of Medicine/National Research Council (2004) and the International Union of Pure and Applied Chemistry (**MOSIHUZZAMAN AND CHOUDHARY, 2008**). These documents discuss the guidelines for the assessment of the safety of medicinal plant materials for use in both food and medicine as a means to protect consumer health.

Medicinal plant products must meet all the basic pharmacopeia definitions in order to ensure quality of the product. These include identity, purity, and consistency of chemical compositions and pharmacological properties of the medicinal plant product (**BANDARANAYAKE, 2006**). The quality criteria for medicinal plant products are based on a clear scientific definition of the raw material (**BANDARANAYAKE, 2006**). Quality assurance of botanicals and herbal preparations is the prerequisite for clinical trials (**SAHOO *et al.,* 2010**). For any medicinal plant material to be classified as a drug, it must fulfil the basic requirements of being efficacious and safe, and this can be achieved by suitable clinical trials (**CALIXTO, 2000**). Variation in chemical contents of medicinal plant products is mostly caused by changes in the growth conditions during the day-night rhythm, seasonal changes, age, polymorphisms within plant species and soil contaminated with toxic heavy metals (**ZHU, 1998; CHANG *et al.,* 2006; NCUBE *et al.,* 2011**). The quantity and quality of safety and efficacy data on traditional medicine are far from meeting the criteria needed to support their use world-wide. The reasons for the lack of such data include not only national health care policies, but also inadequate or not widely accepted research methodologies for evaluating traditional medicines (**WHO, 1978**). The majority of AER reports resulting from the use of medicinal plant products are attributed to the poor quality of raw medicinal plant materials (**WHO, 1998**).

The establishment of a quality control system in South African traditional medicine for clinical effect and safety assessment, which would be accepted by international pharmaceutical industries is still lacking. Hence, the need to carry out proper standardization and quality control of raw medicinal plant materials and their preparations is of paramount importance. These will facilitate the development of national standards based on local market conditions.

1.7.1 Approaches to ensure quality of medicinal plant products

1.7.1.1 Safe cultivation of medicinal plants

The quality of medicinal plant material depends mainly on intrinsic factors (genetic) and extrinsic (environmental and ecological) differences, hence wild harvested plants are likely to vary in terms of quality and consistency (**BOPANA AND SAXENA, 2007**).

Safe cultivation of important medicinal plants is one strategy to overcome this problem that is inherent in medicinal plant products and which compromises the quality of medicinal plant products (**WHO, 2003**). Generally, there is greater consistency in chemical compositions of medicinal plant material obtained from cultivation. The problem of plant identity and misidentification is also minimized. Cultivation of medicinal plants has been proposed in response to the combined impacts of dwindling supplies due to overexploitation of natural resources, increasing demands due to population growth and the growing global markets for medicinal plants (**WIERSUM *et al.,* 2006**). Additional advantages with the cultivation of medicinal plants include low risk of toxicity arising from heavy metal contamination or misidentification of herbs as well as to improve uniformity of medicinal plant material (**WHO, 2003**).

One of the major means by which cultivated plants can be contaminated with toxic heavy metals is through agricultural practices such as the application of pesticides, herbicides and the use of fertilizers containing Cd. These kinds of agricultural practices must be avoided in order to obtain clean and safe medicinal plant products. Generally, cultivation of medicinal plants requires intensive management techniques as well as good plant husbandry and good agricultural and collection practices (GACP) (**WHO, 2003**). There is a growing trend in Europe, China and India that most of the highly valued medicinal plants used commercially are cultivated on a large scale (**CANTER *et al.,* 2005**). Some African countries including South Africa, have also accepted the ideal of cultivating medicinal plants (**CUNNINGHAM, 1993**). To date, cultivation of medicinal plants in South Africa is minimal and some of the conservative traditional healers believe that cultivated medicinal plants using typical agricultural practices will not have the same medicinal properties as those harvested from the wild (**WEIRSUM *et al.,* 2006**).

1.7.1.2 Good agricultural and collection practices

A set of guidelines for GACP was issued by the **WHO** (**2003**) to promote sustainable cultivation, harvesting and conservation of medicinal plants. A brief outline of this guideline includes; selection, identification and authentication of cultivated medicinal plant species, seed propagation and cultivation practices using appropriate quality

techniques free from possible contamination and diseases in order to promote healthy plant growth. Medicinal plants should be harvested during the optimal season and age to ensure the best quality of the medicinally important bioactive compounds. Care must be taken to avoid contaminants during harvesting with personnel having sufficient knowledge for GACP. These guidelines were aimed at enforcing compliance with quality assurance measures which can be achieved through regular auditing visits to cultivation or collection sites and processing facilities by expert representatives as well as inspection from the national and/or local regulatory authorities. Regular inspections will ensure that the correct plant material is collected and/or cultivated on a medium free of harmful heavy metals, pesticides, herbicides or other hazardous substances. The source and quality of raw medicinal plant materials as described in the GACP and good manufacturing processes are essential steps for quality control of medicinal plant products (**WHO, 2003**).

Good agricultural and collection practices for medicinal plants is only the first step in quality assurance, on which the safety and efficacy of medicinal plant products directly depend, and will also play an important role in the protection of natural resources of medicinal plants for sustainable use. Only a few countries e.g. China, Japan and the European Union have developed regional and national guidelines for GACP for medicinal plants (**WHO, 2003**).

1.7.1.3 Analytical techniques for quality assurance of medicinal plant products

With rapid globalization and modernization of the medicinal plant products industry, the issue of consistency and quality control of medicinal plant products is becoming increasingly important. Thin layer chromatography (TLC) is one of the traditional methods that is used to identify one or a few biomarkers as a quick quality check of medicinal plant products. This is a simple, cost-effective and relatively rapid method of identifying medicinal plant products (**JIANG *et al.,* 2010**) and can be used to provide valuable information confirming the identity of the plant material (**BANDARANAYAKE, 2006**). **WAGNER AND BLADT** (**1996**) carried out pioneering research by providing a "TLC atlas" of various medicinal plant products with colour photographs of the chromatograms of plant extracts. However, TLC has many shortcomings, such as not

providing a complete profile of the medicinal plant product and it cannot be used to distinguish medicinal plant products with similar chemical profiles nor identify chemical components in medicinal plant products (**JIANG *et al.,* 2010**).

Nowadays, advanced techniques such as high performance liquid chromatography (HPLC), metabolomics, liquid chromatography and mass spectroscopy and ultra performance liquid chromatography are used to accurately quantify and qualitatively identify complex mixtures of biological materials. Of these techniques, HPLC remains relatively cheap and less complex in terms of sample handling and processing. HPLC has been used to rapidly separate, fingerprint qualitatively and to identify and/or quantify molecules from complex mixtures as in the case of plant extracts and medicinal plant supplements (**FAN *et al.,* 2006; GONG *et al.,* 2006; ESTEVE *et al.,* 2012**).

1.7.1.4 Macroscopic and microscopic analysis of medicinal plant products

Macroscopic examination of medicinal plant products involves visual screening of medicinal plant materials as a quick quality check to determine the presence of foreign matter. This approach helps to keep medicinal plant products free from moulds or insects, including excreta and visible contaminants such as unwanted plant materials, sand, stones, and poisonous and harmful foreign matter (**WHO, 2003**). In some cases, a quick microscopic examination is used to determine that the correct species and/or the correct part of the plant species is being used. For example, pollen morphology has been used in the case of flowers to identify plant species, and the presence of certain microscopic structures such as leaf stomata have been used to identify the plant parts in medicinal plant materials.

1.8 Pharmacovigilance in traditional medicine

A pharmacovigilance system was developed to focus on monitoring, detecting and preventing adverse drug reactions (ADRs) and monitoring the safety of marketed drugs under the practical conditions of clinical usage in large communities (**MANN AND ANDREWS, 2002; ZHANG *et al.,* 2012**). Pharmacovigilance is an active

surveillance system which covers the entire life-cycle of a drug, starting from raw materials to manufacturing and post-market safety monitoring (**ZHANG *et al.,* 2012**). Pharmacovigilance practices and tools were developed in the context of conventional medicine and have rarely considered the complexities of monitoring the safety of medicinal plant products (**RODRIGUES AND BARNES, 2013**). However, in recent years, several issues emerged leading to greater awareness on the need to monitor the safety of medicinal plant products and the understanding of their possible toxicity as well as potential benefits (**RODRIGUES AND BARNES, 2013**). The inclusion of medicinal plant products in pharmacovigilance systems is becoming increasingly important given their growing use globally.

The use of systematic pharmacovigilance in medicinal plant products is an essential approach that will help to collect reliable information for the development of appropriate guidelines for safe and effective use of medicinal plant products (**SHAW *et al.,* 2012**). Pharmacovigilance systems in medicinal plant products is aimed at safeguarding public health by taking measures for the intensive supervision of undesirable effects of authorised medicinal plant products so as to ensure the rapid withdrawal from the market of any medicinal product presenting a negative risk-benefit balance under normal conditions of use (**MANN AND ANDREWS, 2007**). There is a stringent regulatory and pharmacovigilance network of medicinal plant products among the European Union member states. These consist of competent bodies such the European Commission and the European Medicines Agency that are actively involved in monitoring and enforcing relevant policies in the medicinal plant industries (**EUROPEAN MEDICINES AGENCY, 2013**). In the United Kingdom, there are two main methods of monitoring and reporting of ADRs associated with the use of medicinal plant products. These include a spontaneous reporting system of suspected adverse drug reaction cases and prescription event monitoring of medicinal plant products (**BARNES, 2003; SHAW *et al.,* 2012**). Traditional Chinese medicines (TCM) has an effective pharmacovigilance system which involves systematic training of clinicians, pharmacists and physicians as watchdogs for reporting ADRs of TCM. There is also an active publicity awareness campaign involving the general public in reporting events of adverse drug reactions of medicinal plant products (**ZHANG *et al.,***

2012). In the USA, medicinal plant products are classified as dietary supplements and the system of reporting cases of AER of dietary supplements is inadequate. Health professionals and consumers can report suspected AER to the Food and Drug Agency Medwatch Scheme (**SHAW *et al.,* 2012**). Limited coordination exists between the national and international surveillance programs for evaluating signals that may indicate potential public health risks. These constitute major limitations for safety monitoring of dietary supplements (**GARDINER *et al.,* 2008**).

1.8.1 Pharmacovigilance of South African traditional medicine

The South African medicinal plant products industry is growing very rapidly with several innovative approaches including large scale manufacturing, packaging and selling of medicinal plant concoctions as well as a range of over the counter products (**NDHLALA *et al.,* 2009**). Although medicinal plant resources have great potential as natural drugs and are of commercial importance, they are often procured and processed with little or no scientific evaluation. These products are launched onto the market without any mandatory safety and toxicology evaluations, because there is no effective system to regulate the manufacturing practices and quality standardization of the products (**BANDARANAYAKE, 2006**). South Africa is one of the African countries that has incorporated traditional medicine into the national healthcare system. Some of the herbal medicine currently marketed are regulated by the Medicine Control Council (**PHARMACEUTICAL SOCIETY OF SOUTH AFRICA, 2014**). Sick certificates issued by South African traditional health practitioners are now being recognized nationwide (**MBATHA et al., 2012**). However, South Africa is still lacking a national surveillance agency as well as proper enforcement of the current regulation to ensure the quality, safety, monitoring of adverse drug reactions and standardization of phytomedicinal products.

In South Africa, the use of a pharmacovigilance system in safety monitoring of medicinal plant products is very difficult because there is no system of reporting ADRs of medicinal plant products. Most cases of poisoning and toxicity arising from the use of traditional medicine remain undiagnosed because some patients will not acknowledge that the toxicity is from the use of medicinal plant products and patients

residing in rural areas may die before reaching a hospital (**STEENKAMP *et al.,* 1999**). There is also little or no regulatory control on the procurement of medicinal plant products as patients can obtain herbs from street vendors, relatives or neighbours, and from traditional medicine practitioners (**BANDARANAYAKE, 2006**). Some of these practices have devastating consequences with regards to ADR (**MEHTA, 2011**). Hence, post-market surveillance of medicinal plant products in South African traditional medicine is very difficult. The country needs a coherent programme of action for pharmacovigilance which should work closely with the National Drug Monitoring Authority for effective exchange of safety information, maintenance of a database on adverse drug reaction reports and the provision of guidelines on monitoring medicinal plant products safety.

1.9 South African medicinal plants investigated in this study

1.9.1 *Adenia gummifera* (Harv.) Harms

Figure 1.4 *Adenia gummifera*

Adenia gummifera is a robust, semi-woody climber which belong to the family Passifloraceae. Stems are striped bluish-green, while older stems are often covered with a whitish powder. This plant species is not endemic to South Africa. It has a widespread distribution from the Eastern Cape, KwaZulu-Natal and Limpopo to Mpumalanga. It also has a widespread distribution in east Africa (**McKEAN, 1993**). Powdered leaves of this plant are used as a snuff to treat headaches and in infusions for abdominal pains, convulsions and septicaemia. Root decoctions are used against anthrax and tapeworm. Stems are used for treating leprosy and malaria (**VAN WYK *et al.,* 2002**). The high demands for this plant material in southern Africa traditional medicine has led to a more destructive harvesting regime than as previously reported (**WILLIAMS, 2003**). According to the **RED LIST OF SOUTH AFRICAN PLANTS** (**2014**), this species is declining very rapidly.

1.9.2 *Alepidea amatymbica* Eckl. & Zeyh.

Alepidea amatymbica belongs to the family Apiaceae. It is a robust, erect plant that grows up to 2 m tall with a wide distribution range in grasslands of the northern and southern Drakensberg Mountains in the Eastern Cape, KwaZulu-Natal, Mpumalanga and Northern Province. It is also found in neighbouring countries e.g. Swaziland, and Zimbabwe (**De CASTRO AND VAN WYK, 1994**). *Alepidea amatymbica* is commonly known as kalmoes and *ikhathazo* in Isizulu (**HUTCHINGS *et al.,* 1996**).

Figure 1.5 *Alepidea amatymbica*

This species is highly regarded as a remedy for respiratory tract infections, asthma, sore throats, gastrointestinal complaints, fever, rheumatism, bleeding wounds and headaches. *A. amatymbica* extracts were active against HIV (**LOUVEL *et al.,* 2013**). This plant species is under threat and local extinction has been reported due to the high demands for medicinal purposes and hence there is an urgent need for conservation (**WILLIAMS *et al.,* 2013; RED LIST OF SOUTH AFRICAN PLANTS, 2014**).

1.9.3 *Bulbine natalensis* A. Rich

Figure 1.6 *Bulbine natalensis*

Bulbine natalensis belongs to the family Asphodelaceae and is locally known as *ibhucu* (Zulu), *rooiwortel* (Afrikaans), and *ingcelwane* (Xhosa). It is widely distributed in the eastern and northern parts of South Africa (**VAN WYK *et al.,* 1997**). *B. natalensis* has been reported to possess aphrodisiac properties (**YAKUBU AND AFOLAYAN, 2010**). The leaf sap is widely used in the management of wounds, burns, rashes, itches, ringworm, and cracked lips. An infusion of the roots is taken orally to quell vomiting, diarrhoea, convulsion, venereal diseases, diabetes, rheumatism, back pain and kidney diseases as well as to purify blood (**VAN WYK *et al.,* 1997; VAN WYK *et al.,* 2008**).

1.9.4 *Cassine transvaalensis* (Burtt Davy) Codd

Cassine transvaalensis belongs to the family Celastraceae and it is commonly known as Transvaal saffron wood and *ingwavuma* (Isizulu). *C. transvaalensis* is a multi-branched evergreen tree that grows to a height of 10 m or more. It is widely distributed in the north-eastern parts of South Africa. The tough fresh roots are ground, boiled in beer and drunk warm for relief from stomach aches and fevers.

Figure 1.7 *Cassine transvaalensis*

The pulverized dry material is also mixed with the roots of *Ozoroa paniculosa* and the resulting mixture taken for relief from menstrual pains and for the control of high blood pressure (**MOTLHANKA *et al.,* 2008**). Bark extracts are used against fungal infection of *Candida albicans,* and for treating inflammed hemorrhoids (piles), venereal diseases, diarrhoea, kidney and bladder infections, killing parasitic worms (anthelmintic), easing bowel defecation (laxative drug agent), relieving stomach aches and coughs and in the management of HIV/AIDS (**MOTLHANKA *et al.,* 2008; SAMIE *et al.,* 2010**).

1.9.5 *Drimia elata* (Jacq.)

Figure 1.8 *Drimia elata*

Drimia elata belongs to the family Hyacinthaceae and is locally known as *isiklenama* (Isizulu) and *brandui* (Afrikaans) *Drimia elata* is the third most popular bulbous medicinal plant used in South African traditional medicine (**WILLIAMS *et al.,* 2000a**) and is one of the top ten most wild-harvested species used in the informal economy trade in Cape Town, South Africa (**PETERSEN *et al.,* 2012**).). This plant species is not endemic to South Africa and its geographic distribution is in almost all the South African Provinces except the Western Cape. *D. elata* is used for treating and managing pain and inflammatory conditions, infections and various ailments including HIV/AIDS-related conditions (**DOLD AND COCKS, 1999; WILLIAMS *et al.,* 2000a; SEMENYA *et al.,* 2013**). The

shoot and bulb materials of *D. elata* exhibited potent antibacterial, anti-inflammatory and anticancer activities (**LUYT *et al.,* 1999; FOUCHE *et al.,* 2008**). This plant species is listed as an endangered plant in the South African red database due to over harvesting (**RAIMONDO *et al.,* 2009**).

1.9.6 *Hypoxis hemerocallidea* Fisch. & C.A. Mey.

Figure 1.9 *Hypoxis hemerocallidea*

Hypoxis hemerocallidea belongs to the family Hypoxidaceae. The plant, commonly known as the African potato (English), *moli-kharatsa* (Sotho), *Inkomfe* (Isizulu), *sterblom* (Afrikaans), or starflowers, is one of the most popular medicinal plants in South African traditional medicine and one of the highly researched medicinal plants in Africa (**OJEWOLE, 2006; DREWES *et al.,* 2008**). The geographical distribution of this species in South Africa is in five Provinces, namely, KwaZulu-Natal, Eastern Cape, Mpumalanga, Limpopo, and Gauteng. The species is also found in the neighbouring countries of Lesotho, Swaziland, Mozambique, and Zimbabwe (**SINGH, 2007**). Dubbed the 'miracle *muthi*', *H. hemerocallidea* is a medicinal plant that is used to treat a wide range of human and veterinary diseases in South Africa (**OJEWOLE, 2006**). Medicinally, *Hypoxis* has been used by different cultures for many ailments such as cardiac diseases, impotency, apprehension, infertility, and intestinal parasites. Today it is used to treat cancer, headaches, urinary tract infections, dizziness, testicular cancer, prostate hypertrophy, burns, ulcers, hypertension, adult - onset diabetes, psoriasis, testicular tumours, HIV/AIDS-related symptoms, some central nervous system disorders as well as an immune booster (**VAN WYK et al, 1997; BUWA AND VAN STADEN, 2006; OJEWOLE, 2006; DREWES *et al.,* 2008; NCUBE *et al.,* 2013**). *H. hemerocallidea* is one of the top ten most popular medicinal plants sold in South African traditional medicine markets with approximately 11000 kg $year^{-1}$ sold and valued at R322 500 in 2002 (**DOLD AND COCKS, 2002**). Owing to its enormous medicinal potential, *H. hemerocallidea* is listed as declining due to overharvesting (**KATERERE AND ELOFF, 2008; RED LIST OF SOUTH AFRICAN**

PLANTS, 2014). The importance of the plant in traditional medicine coupled with the high demand for it has necessitated sustainable harvesting, conservation and there is potential for large scale commercial cultivation.

1.9.7 *Lycopodium clavatum* L.

Figure 1.10 *Lycopodium clavatum*

Lycopodium clavatum belongs to the family Lycopodiaceae and is commonly known as club moss and *inwele* (Isizulu). It is an evergreen spore-bearing vascular plant, growing mainly prostrate along the ground with stems up to 1 m long. The stems are much branched and are densely covered with small, spirally arranged "leaves". This plant species is found in most African countries including South Africa (**PROTA FOUNDATION, 2008**). The spore of *L. clavatum* are used for treating many conditions in homeopathy ranging from upset stomachs, food poisoning, kidney problems, and muscle cramps to more serious conditions such as hepatitis and pneumonia (**PROTA FOUNDATION, 2008**). The plant's spores are also used as a treatment for skin conditions such as eczema. When the spores are processed into a homeopathic remedy, the benefits include treatments for conditions affecting soft tissue, the liver and heart, as well as bones and blood vessels. Minor ailments like earaches, sore throats, and constipation are also relieved by remedies made from the spore (**PROTA FOUNDATION, 2008**). Decoctions from the whole plant are used to treat diarrhoea and dysentery, to induce vomiting after food poisoning or acute stomach pain, as a diuretic in oedema as well as to treat rheumatism and gout in children. It is also smoked for the treatment of headaches. Infusions are taken to treat diseases of the urinary tract (**HUTCHINGS *et al.,* 1996; PROTA FOUNDATION, 2008**).

1.9.8 *Momordica foetida* Schumach.

Momordica foetida is a medicinal plant belonging to the family Cucurbitaceae and is commonly known as gifappel and *intshungu* (Isizulu). The plant has both male and

female flowers (**JEFFREY, 1990**). It is widely distributed in tropical Africa, South Africa and is also available in the flora of west tropical Africa (**JEFFREY, 1990**). Aqueous leaf extracts of the plant are used to treat malaria in East and Central Africa (**HAKIZAMUNGU *et al.,* 1992**).

Figure 1.11 *Momordica foetida*

Plant extracts have also been used to treat hypertension, peptic ulcers, diabetes mellitus and as a purgative (**HAKIZAMUNGU *et al.,* 1992; FROELICH *et al.,* 2007**). The leaves of *M. foetida* are collected from the wild and eaten after boiling as a vegetable (**OLANIYI, 1975**). Their nutritional composition per 100 g edible portion is; energy 92 kj (22kcal), protein 3.3 g, fibre 3.2 g, calcium (Ca) 1.1 mg, iron (Fe) 3.4 mg, Zn 0.4 mg, *β*-carotene 5.4 mg, foliate 40 µg, ascorbic acid 20.6 mg (**NESAMVUNI *et al.,* 2001**).

1.9.9 *Ocotea bullata* (Burch.) E. Mey.

Figure 1.12 *Ocotea bullata*

Ocotea bullata belongs to the family Lauraceae. It is a large evergreen tree that is indigenous to the high forests of South Africa, from Table Mountain in the south, to the afro-montane forests of Limpopo in the north (**ARNOLD AND de WET, 1993**). This plant species is commonly known as black stinkwood (English), *stinkhout* (Afrikaans) and *unukani* (Xhosa, Isizulu). Finely ground bark of *O. bullata* is used as a snuff or the smoke is inhaled to relieve headaches. It is also used as a local application to the area of the bladder in urinary infections. Bark infusion is used to treat headaches, nervous disorders, stomach pain and urinary infections (**VAN WYK *et al.,* 2009**). The stem bark is becoming increasingly expensive as a result of its popularity in traditional medicine and the shortage of supply. The demand for bark for medicinal use has resulted in extensive illegal stripping of trees by commercial harvesters. It has been declared endangered and has special

protection in KwaZulu-Natal. The conservation status indicated that this species is declining (**RED LIST OF SOUTH AFRICAN PLANTS, 2014**).

1.9.10 *Rapanea melanophloeos* (L.) Mez

Figure 1.13 *Rapanea melanophloeos*

Rapanea melanophloeos belongs to the family Myrsinaceae and is commonly known as Cape beech and *ikhubalwane* (Isizulu). It is an evergreen tree that grows up to 18 m high and is widely distributed throughout southern Africa from the southern Cape to Zambia, and the east coast to the tropics. It is found along the damp areas of mountain and coastal forests or swamps and bush clumps (**POOLEY, 1993**). The grey bark or sometimes the roots are used medicinally for respiratory diseases, stomach, muscular and heart complaints and as expectorants and emetics. The plant species population is declining due to bark harvesting for medicinal plant trade, especially in KwaZulu-Natal (**VAN WYK *et al.,* 1997; RAIMONDO *et al.,* 2009**).

1.9.11 *Schizocarphus nervosus* (Burch.) van der Merwe

Figure 1.14 *Schizocarphus nervosus*

Schizocarphus nervosus belongs to the family Hyacinthaceae and commonly known as wild squill (English) and *ingcino* (Isizulu). This plant species has a widespread distribution in southern Africa from the Eastern Cape of South Africa to tropical Africa. It forms rounded clumps with large buried bulbs with fibrous sheaths. Although toxic to livestock, it plays an important role in traditional medicine within the region. The bulbs are used by the Zulu to treat dysentery and nervous conditions in children as well as to treat rheumatic fever. In Botswana the bulbs are used to enhance fertility and to treat infections (**DU TOIT *et al.,* 2011**).

1.10 Aims and objectives

This study aimed to investigate heavy metal and trace element compositions in some frequently used South African medicinal plants and to determine the safety, efficacy and quality of these products. This was achieved through a series of experiments based on specific objectives as listed below:

- To determine the extent and type of heavy metal contamination in commonly used medicinal plant products obtained from traditional medicine markets;
- Assess the extent of heavy metal contamination in medicinal plant products by quantifying heavy metals in some commonly used South African medicinal plants obtained from both *muthi* shops and outdoor-street markets;
- Correlate the phenolic and flavonoid content and antimicrobial activity as potential methods to monitor consistency of phytochemical composition and biological activity;

- Classify the samples based on their elemental composition using chemometric techniques to assess the suitability of such methods to establish a safety database of medicinal plant samples.
- To determine the interactive effects of Cd and Al on their uptake and the physiological changes induced in the selected plant species;
- To investigate the tolerance mechanisms of *H. hemerocallidea* to various concentrations of Cd and Al by screening for a number of physiological and ultrastructural changes as well as investigating chlorophyll fluorescence;
- To investigate the effect of heavy metal stress on total phenolics and flavonoids in selected medicinal plants as well as to quantify hypoxoside, a secondary metabolite in *H. hemerocallidea*.
- To determine the effect of heavy metal stress on biological activity by screening for antibacterial and antioxidant scavenging activity.

The three aspects of this study namely; safety (heavy metal content), efficacy of plant extracts in bioassays and quality of medicinal plant are addressed in the chapters as outlined in **Figure 1.15**.

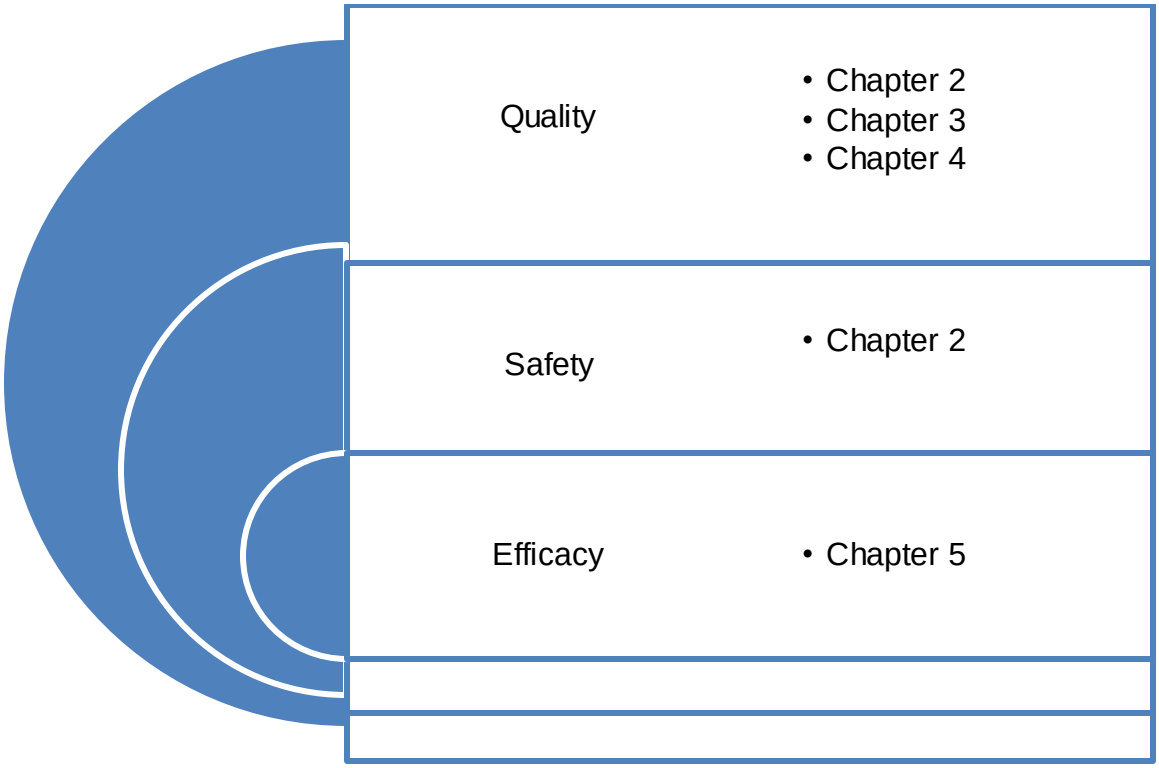

Figure 1.15 Aspects of safety, efficacy and quality of medicinal plant investigated in thesis chapters.

Chapter 2

Heavy metals in medicinal plants obtained from outdoor-street markets and *muthi* shops

2.1 Introduction

High levels of heavy metal contamination in the environment are a public health concern. The rate of migration of contaminants, either as dust or leachates, resulting from high anthropogenic activities into non-contaminated soil is on the increase (**SAMI AND DRUZYNSKI, 2003; ZHANG *et al.,* 2010; WUANA AND OKIEIMEN, 2011**). In South Africa, medicinal plants are primarily harvested from the wild. Thus their growing locations and conditions cannot be verified and contact with heavy metal contaminated soil is a strong possibility. This increases the potential health risk to the majority of the population who rely on medicinal plant products for their primary healthcare. The presence of heavy metal contamination in medicinal plants may also affect the efficacy and quality of the medicinal plant products (**STREET *et al.,* 2008**). Thus, the screening of traditional medicines for potentially harmful components has been recommended to protect consumers (**CHANG, 1995; WHO, 2011**).

2.1.1 Techniques for assessing heavy metals in plant material

There is an increasing interest in routine monitoring as well as risk assessment studies of heavy metals and trace elemental composition in plant samples. This is as a result of increasing industrialization coupled with the commercialization of plants for therapeutic purposes, as nutriceuticals and as crop and food products (**RODUSHKIN *et al.,* 1999**). Bio-monitoring of elements in complex biological samples such as plant material requires a sensitive analytical method with outstanding precision and high sample throughput (**MOMEN *et al.,* 2013**). Nowadays, analysis of heavy metals in environmental samples involve the use of specialized spectroscopic techniques, such as flame atomic absorption spectroscopy, atomic absorption spectroscopy and inductively coupled plasma-optical emission and -mass spectrometry (ICP-OES and ICP-MS). ICP-OES is a powerful technique that allows rapid and precise multi-element

determination in a single solution, with a low detection limit, wide dynamic range and high accuracy (**HOU AND JONES, 2000; US EPA, 2007; SHIRDAM *et al.,* 2008**). However, analysis of metal compositions using the highlighted techniques require that the solid samples first be transformed into a solution. Sample preparation is done either by ashing or wet digestion. Sample digestion by a wet procedure is carried out using a heated mixture of mineral acids. Different heating systems can be used for digestion such as a sand-bath (**McGRATH, 1996**), heating plate (**KOWALEWSKA *et al.,* 1998**) and aluminum blocks (**ANSORENA *et al.,* 1995**). The introduction of a microwave digestion system with both open and closed pressurised systems, has allowed considerable reduction in the total time for analyses as well as in the risk of sample loss or contamination during digestion (**TAM AND YAO, 1999**). The Environmental Protection Agency (EPA) recommends the use of the microwave-assisted acid digestion method with nitric acid for biological samples (**US EPA, 2007)**. Nitric acid a is strong mineral acid that can solubilize metal from plant materials as well as soils with an organic carbon content up to 38% (**XING AND VENEMAN, 1998; MEERAVALI AND KUMAR, 2000**).

2.1.2 Multivariate analysis – hierarchical cluster analysis

Multivariate analysis techniques have been widely used for evaluation and characterisation of analytical results (**da SILVA *et al.,* 2008**). One of the most popular multivariate techniques is hierarchical cluster analysis (HCA) (**WILLIAMS *et al.,* 2009**) which is a group of multivariate techniques used to identify common patterns in data distribution. It assembles data based on the characteristics they possess as well as sorting cases (monitoring points) into groups (clusters) so that the degree of association is strong between members of the same cluster and weak between members of different clusters. The resulting clusters of objects exhibit high internal (within-cluster) homogeneity and high external (between clusters) heterogeneity. Each cluster is thus described, in terms of the data collected and the class to which its members belong. This description may be abstracted from the particular to the general class or type (**EINAX *et al.,* 1997**). HCA is the most applied method for environmental analysis (**TOKALIOĞLU, 2012**). The HCA technique has recently been applied to data on mineral and trace elements in medicinal plants. It provides an interesting and

promising approach for the classification of commercially available medicinal plant products and has application in quality control in the pharmaceutical industry (**ARUMUGAM *et al.,* 2012**).

The aims of the present study were to assess the extent of heavy metal contamination in some of the frequently used South African medicinal plants obtained from both outdoor-street markets and *muthi* shops. To correlate the phenolic and flavonoid content and antimicrobial activity as potential methods to monitor consistency of phytochemical composition and biological activity. To classify the samples based on their metal analyte composition using chemometric techniques.

2.2 Materials and Methods

2.2.1 Sample collection and preparation

Eleven plant species were obtained from both outdoor-street markets and *muthi* shops in Pietermaritzburg, KwaZulu-Natal Province between January and February, 2012. All plant samples were identified by Dr C. Potgieter and voucher specimens were prepared and deposited at the John Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. Plant material was washed under running tap water to remove soil deposits and then oven dried at 50°C for 7 days. The dried plant material was milled into powders using a Retsch® ZM 200 ultra-centrifugal mill. The powdered samples were stored in the dark at room temperature.

2.2.2 Elemental analysis

2.2.2.1 Reagents and standard solutions

All reagents used were of analytical grade. Ultra-pure (UP) water was used for preparing the solutions and dilutions. Stock solutions of metals (1000 mg L^{-1}) were prepared from their nitrate salts. Standard solutions were prepared from 1 000 mg L^{-1} stock solutions and made to volume with UP water. The stock solutions were of analytical grade (BDH Spectrosol®, Fluka Chemika®). Calibration standards were in the range of 0.00125-5 mg L^{-1} for arsenic (As), Cd, Cu, Mn, Ni, Pb and Zn, and 0.0125-50 mg L^{-1} for Al and Fe. Standard calibration was carried out for each set of analyses.

2.2.2.2 Microwave acid-assisted digestion

A microwave assisted system (MARS 5) was used to digest the plant material. Plant samples [0.5 g dry weight (DW)] were placed in Teflon vessels and 10 mL 55% HNO_3 added. The samples were pre-digested for 30 min. Thereafter, the vessels were sealed and heated in a microwave system operating at 1200 W. The temperature of the microwave system was programmed as follows: 1st stage, 2 min heating from ambient temperature to 170°C; 2nd stage, 3.30 min heating to 180°C and 3rd stage, 9.30 min held at 180°C. A blank containing 10 mL 55% HNO_3 was used. After complete digestion, the clear solutions were transferred to 50 mL volumetric flasks and made to volume with UP water. These samples were stored in high density polyethylene bottles until analysis.

2.2.2.3 Elemental analysis using ICP-OES

Elemental analysis was carried out using an ICP-OES (Varian 720-ES, Varian Inc, Palo Alto, CA, USA) instrument (**Table 2.1**). The operating conditions for the ICP-OES instrument were: RF power 1.0 kW; viewing geometry was axial; Argon gas was used: plasma gas flow at the rate of 15.0 L min^{-1}; auxiliary gas flow rate 1.50 L min^{-1}; nebulizer gas flow rate 0.75 L min^{-1}; replicate reading time 9.0 s. All analyses were performed in triplicate.

2.2.3 Antibacterial and phytochemical screening

2.2.3.1 Sample preparation

The ground material (1 g DW) was extracted with 10 mL 70% acetone in a sonication bath (Julabo GmbH sonicator) for 1 h. The plant extracts were then filtered using Whatman No. 1 filter paper. The filtrates were concentrated by a rotary evaporator and then transferred into pre-weighed pill vials. The samples were air-dried under a stream of cold air. The dried extracts were kept in the dark at 10°C until analysis.

2.2.3.2 Determining total phenolics

The amounts of total phenolic compounds in plant samples were determined using the Folin Ciocalteu (Folin C) assay as described by **MAKKAR** (**1999**) with slight modifications.

Table 2.1 Metal elements investigated in this study by ICP-OES

Element	Analytical Wavelength (nm)	Analytical Wavelength (nm)	
Al	237.312	Hg	194.164
As	193.696	Mn	259.372
Cd	226.502	Ni	231.601
Cr	267.716	Pb	220.353
Cu	324.754	Sn	283.998
Fe	238.204	Zn	213.857

In triplicate, 50 µL of each plant extract were transferred into test tubes and was made up to 1 mL by adding 950 µL of distilled water followed by 500 µL of 1 N Folin C phenol reagent and 2.5 ml of 2% sodium carbonate. A blank that contained aqueous methanol instead of the plant extracts was also prepared. The test mixtures were incubated for 40 min at room temperature. The absorbance was then read at 725 nm using a UV–vis spectrophotometer (Varian Cary 50, Australia). Total phenolic concentration was expressed as gallic acid equivalents (GAE) determined from a standard curve.

2.2.3.2 Screening for flavonoids

Flavonoid content was determined using the aluminum chloride colorimetric assay as described by **ZHISHEN *et al.*** (**1999**) and **MARINOVA *et al.*** (**2005**). In triplicate, 500 µL plant extracts were pipetted into test tubes and 2 mL distilled water added, followed by 150 µL 5% $NaNO_2$. After incubating for 5 min, 150 µL 10% $AlCl_3$ was added to all the test tubes. At the 6^{th} min of incubation, 1 mL 1M NaOH was added to all the test tubes and the volume was made to 5 mL by adding 1.2 mL distilled water. Thereafter, the mixture was vortexed and the absorbance was read at 510 nm using a UV–vis spectrophotometer against a reagent blank containing 50% methanol instead of the

plant extract. Total flavonoid content was expressed as catechin equivalents (CTE) as determined from a standard curve.

2.2.3.3 Antibacterial microdilution assay

Evaluation of the plant extracts for antibacterial activity was achieved using the minimum inhibitory concentration (MIC) technique as described by **ELOFF** (**1998**). Overnight culture of a Gram-negative bacterium *Escherichia coli* American type culture (ATCC) 11775 and a Gram-positive bacteria *Staphylococcus aureus* ATCC 12600 were diluted 1:100 with sterile Mueller-Hinton (MH) broth (Oxoid). The plant extracts were resuspended to a concentration of 50 mg mL^{-1} in 70% acetone. A two-fold serial dilution of 100 µL of the resuspended extracts was prepared with sterile water in a 96-well microtitre plate (Greiner Labortechnik). A similar two-fold serial dilution of neomycin (100 µg mL^{-1}) was used as the positive control while 70% acetone, water and bacteria-free broth were used as the solvent and negative controls respectively. From the diluted bacteria cultures, 100 µL was added to all the wells containing the samples and control solutions. The final concentration for each extract resulting in concentrations ranging from 12.5 mg mL^{-1} to 0.098 mg mL^{-1}. The microtitre plates were then covered with parafilm and incubated at 37 °C for 24 h. The MIC values were obtained by adding 50 µL 0.2 mg mL^{-1} *ρ*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Steinham, Germany) and incubated for another 1 h at 37°C. The bacterial growth was indicated by a pink-red colour, while clear wells indicated growth inhibition by the plant extracts. The MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT. The assay was repeated twice with replicates for each extract.

2.2.4 Statistical analysis

The data sets for elemental concentrations and phytochemicals amongst plant samples were subjected to one-way analysis of variance (ANOVA) and Tukey's confidence level ($p<0.05$) using SPSS® statistical package version 21.0 for Windows and GraphPad Prism. Results are presented as mean±SD (n=3).

2.2.5 Hierarchical cluster analysis (HCA)

This is an unsupervised technique that involves classification and measurement of similarity between samples to be clustered. The HCA technique was applied to the data sets using SPSS statistical package using Ward's method with Euclidean distance to calculate the sample interpoint distance.

2.3 Results

2.3.1 Elemental analysis

The amounts of heavy metals and non-essential elemental in the investigated medicinal plants are presented in **Table 2.2**. Samples of *B. natalensis* obtained from outdoor-street market and *A. amatymbica* obtained from MS exhibited extremely high levels of Al (5559 and 4392 mg kg^{-1} DW respectively). The concentrations of Cd was above the WHO permissible limits (0.3 mg kg^{-1}) in *A. amatymbica* samples obtained from outdoor-street market (**Table 2.2**). All the other plant samples analyzed had low levels of Cd and Pb below the WHO limits (0.3 and 10 mg kg^{-1} respectively). High levels of As and Hg were recorded in most of the samples analyzed to be above the WHO permissible limits (0.2 mg $kg^{-1}$and 2 µg kg^{-1} respectively). The highest amounts of As were recorded in samples of *B. natalensis* and *Rapanea melanophloeos* (5.22 and 5.19 mg kg^{-1} respectively).

The results for essential element concentrations in the investigated medicinal plant samples are presented in **Table 2.3**. Extremely high levels of Fe (4465and 4164 mg kg^{-1} DW) were recorded in *A. amatymbica* and *B. natalensis* respectively. Generally, the amounts of Fe in all the evaluated plant samples were more than the other essential elements screened in the present study. Samples of *B. natalensis* obtained from outdoor-street market had the highest levels of Mn and Cr (479 and 237mg kg^{-1} respectively) compared to all the other samples analyzed.

Table 2.2 Heavy metals and non-essential elemental concentrations in medicinal plants (mg kg^{-1}) obtained from outdoor-street market and *muthi* shops in Pietermaritzburg. Results are presented as mean±SD (n=3). MTS = *muthi* shops, OSM = outdoor-street market, ND = Not detected, Bold = significantly high values between MTS and OSM samples.

Plant species/ **Voucher No**.	Source of collection	Elemental concentration in plant samples (mg kg^{-1})					
		Al	As	Cd	Hg	Pb	Sn
WHO and NRC limits		50	0.2	0.3	2 µg	10	-
Adenia gummifera	MTS	**42±3.50**	ND	0.04	0.79±0.07	ND	77±1.36
A Okem 20 NU	OSM	22±7.38	ND	ND	**3±0.46**	0.72±0.03	76±0.44
Alepidea amatymbica	MTS	4392±173	2±0.41	0.27±0.01	ND	0.37±0.06	34±6.19
A Okem 16 NU	OSM	3954±266	2±0.03	0.39±0.10	0.04	5±0.80	85±6.88
Bulbine natalensis	MTS	773±88	5±0.12	0.24±0.07	10±0.18	5±0.61	**58±18**
A Okem 18 NU	OSM	**5559±220**	3±0.06	0.28±0.02	0.48±0.08	4±0.16	4±0.04
Drimia elata	MTS	560±71	ND	ND	0.76±0.08	0.22±0.06	**80±0.41**
A Okem 19 NU	OSM	**1595±73**	2±0.05	0.06	0.04	1±0.37	31±6.39
Cassine transvaalensis	MTS	**42±1.76**	0.06	ND	4±0.55	ND	40±2.13
A Okem 10 NU	OSM	27±5.90	ND	ND	8±0.66	1±0.08	42±0.33
Hypoxis helmerocallidea	MTS	**938±109**	0.55±0.02	0.04	0.15	2±0.35	38±5.97
A Okem 21 NU	OSM	267±13	0.47±0.04	ND	**14±0.0**	0.58±0.07	**86±10**

Table 2.2. *Continued*

Lycopodium clavatum	MTS	**2496±143**	2±0.03	0.11±0.03	0.41±0.01	**4±0.01**	**40±2.29**
A Okem 8 NU	OSM	1253±57	5±0.05	0.15±0.01	1±0.11	ND	27±1.43
Momordica foetida	MTS	382±24	2±0.31	ND	3±0.43	4±0.80	**182±7.47**
A Okem 13 NU	OSM	216±26	3±0.62	ND	2±0.40	3±1.02	25±7.38
Ocotea bullata	MTS	**223±0.30**	1±0.10	0.05f	2±0.03	ND	34±5.61
A Okem 9 NU	OSM	30±2.92	2±0.38	ND	1±0.21	ND	25±6.67
Rapanea melanophloeos	MTS	**93±0.99**	**5±0.15**	ND	0.74±0.11	2±0.03	39±5.69
A Okem 14 NU	OSM	50±5.66	0.40±0.09f	0.04e	0.84±0.03	0.24±0.03	**83±1.81**
Schizocarphus nervosus	MTS	459±23	3±0.80	ND	2±0.02	0.1	26±5.41
A Okem 15 NU	OMS	**809±111**	2±0.26	ND	0.90±0.05	0.42±0.09	26±11.07

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Table 2.3 Essential elemental concentrations in medicinal plants (mg kg^{-1}) obtained from OSM and MTS in Pietermaritzburg. Results are presented as mean ± SD (n=3). MTS = *muthi* shops, OSM = outdoor-street market, ND = Not detected, Bold = significantly high values between MTS and OSM samples.

Plant species	Source of collection	Concentrations of essential elements in plant samples (mg kg^{-1})					
		Cr	Cu	Fe	Mn	Ni	Zn
Alepidea amatymbica	MTS	17±0.68	19±0.81	4465±1.08	249±2.51	12±1.71	56±4.10
	OSM	**33±7.06**	13±1.31	3345±390.5	238±36	**30±4.95**	45±3.90
Adenia gummifera	MTS	6±1.34	4±0.98	**166±56.6**	24±2.06	3±0.19	28±11
	OSM	6±**0.87**	4±0.99	70±10.13	33±2.06	7±0.83	34±5.10
Bulbine natalensis	MTS	9±0.26	**20±1.39**	730±26	169±21.38	4±0.27	**107±40**
	OSM	**237±4.36**	5±0.50	**4164±513**	**479±20**	6±1.02	52±1.60
Cassine transvaalensis	MTS	5±0.23	3±0.10	59±11.06	12±2.84	3±0.94	4±0.03
	OSM	6±0.64	4±0.55	**206±1.66**	13±2.47	2±0.05	4±0.21
Drimia elata	MTS	8±0.09	6±0.12	593±50	69±4.72	4±0.52	34±4.60
	OSM	12±1.98	11±0.43	**1634±78**	**146±4.18**	10±0.78	**103±50**
Hypoxis helmerocallidea	MTS	74±0.36	8±1.18	**698±82**	235±8.50	3±0.36	41±2.90
	OSM	6±0.27	12±0.49	342±54	183±5.25	3±0.16	27±1.70

Table 2.3. ***Continued***

Lycopodium clavatum	MTS	21±2.61	7±1.24	231±38	**315±18**	7±0.64	25±5.2
	OSM	12±1.89	6±1.51	**641±21**	144±44	5±0.71	21±5.1
Momordica foetida	MTS	**73±4.02**	6±0.08	**446±20**	45±1.59	6±1.03	35±9.1
	OSM	6±0.18	7±0.29	267±21	55±2.09	3±0.13	36±1.8
Ocotea bullata	MTS	10±0.55	**19±6.05**	**400±44**	**52±1.35**	9±0.22	**39±4.7**
	OSM	6±0.18	3±0.55	60±4.67	23±1.26	3±0.12	6±0.82
Rapanea melanophloeos	MTS	6±1.09	2±0.38	**114±3.75**	55±1.65	4±0.15	4±0.01
	OSM	5±0.12	4±0.17	78±8.23	**163±4.27**	3±0.41	5±0.04
Schizocarphus nervosus	MTS	10±0.52	4±0.89	435±24	50±1.04	5±0.14	25±3.4
	OMS	10±1.33	3±0.48	**793±154.92**	42±0.54	4±0.04	20±2.4

2.3.2 Phytochemical compositions

There was significantly higher amounts of total phenolics in *B. natalensis* and *S. nervosus* obtained from *muthi* shops compared to samples of the same species obtained from the outdoor-street markets (**Figure 2.1A**).

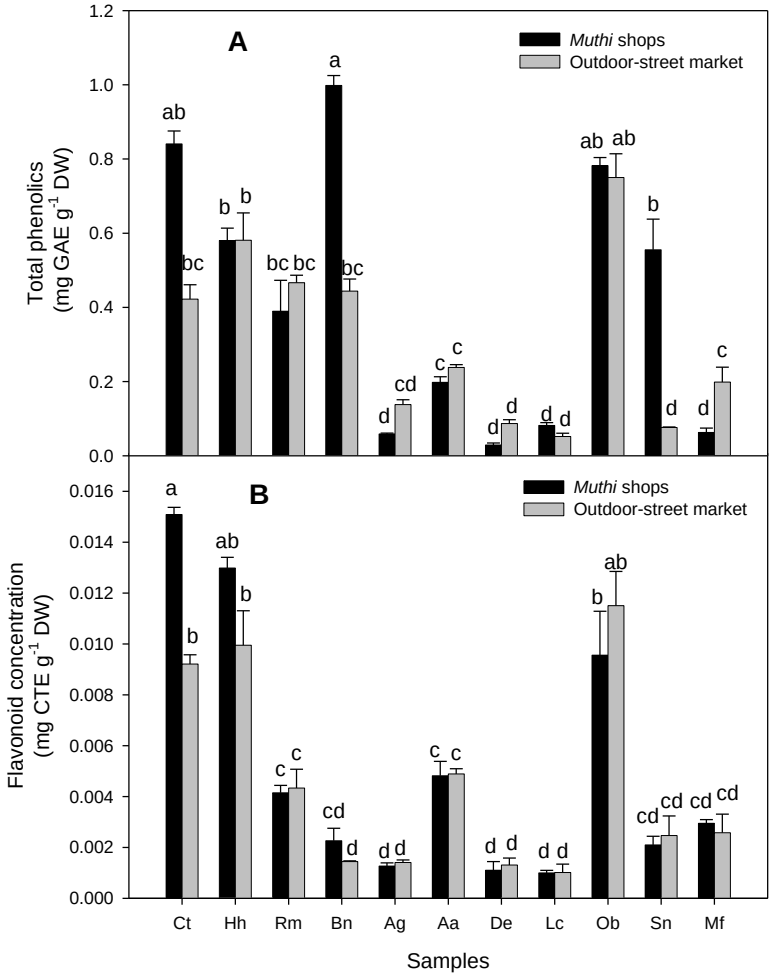


Figure 2.1 Total phenolic composition expressed as gallic acid equivalents (A). Flavonoid concentration as catechin equivalents (B) for medicinal plants obtained from outdoor-street market and *muthi* shops. Results are presented as mean ± SD (n=3). Mean values with dissimilar letter(s) are significantly different (p<0.05). Ct = *Cassine transvaalensis*; Hh = *Hypoxis hemerocallidea*; Rm = *Rapanea melanophloeos*; Bn = *Bulbine natalensis*; Ag = *Adenia gumnifera*; Aa = *Alepidea amatymbica*; De = *Drimia elata*; Lc = *Lycopodium clavatum*; Ob = *Ocotea bullata*; Sn = *Schizocarphus nervosus*; Mf = *Momordica foetida*.

High levels of phenolics was also recorded in *C. transvaalensis* obtained from *muthi* shops compared to outdoor-street market. All the other species have relatively similar amounts of phenolics in both outdoor-street market and *muthi* shops samples. There were high amounts of flavonoids in samples of *C. transvaalensis, H. hemerocallidea,* and *O. bullata* compared to all the other investigated plant samples (**Figure 2.1B**). Low amounts of phenolics and flavonoids were recorded in *A. gummifera, D. elata* and *L. clavatum.*

2.3.3 Antibacterial activity

The results for antibacterial activity in the investigated medicinal plants are presented in **Table 2.4**. Plant extracts that exhibited MIC values <1 mg mL^{-1} (highlighted in bold) were considered as having good antibacterial activity (**GIBBONS, 2005**).

Table 2.4 Antibacterial activity in medicinal plant extracts obtained from *muthi* shops (MTS) and outdoor-street markets (OSM). MIC values was expressed as mg mL^{-1} (n = 3). Standard = Neomycin (2 µg mL^{-1})

Plant name	Source	*E. coli*	*S. aureus*
Cassine transvaalensis	MTS	3.13	**0.78**
	OSM	3.13	1.56
Hypoxis helmerocallidea	MTS	3.13	3.13
	OSM	**0.78**	1.56
Rapanea melanophloeos	MTS	1.56	1.56
	OSM	6.25	**0.78**
Bulbine natalensis	MTS	**0.78**	**0.39**
	OSM	3.13	1.56
Adenia gummifera	MTS	6.25	**0.78**
	OSM	12.5	**0.39**
Alepidea amatymbica	MTS	**0.20**	**0.39**
	OSM	**0.20**	**0.39**
Drimia elata	MTS	12.5	6.25
	OSM	12.5	12.5
Lycopodium clavatum	MTS	6.25	**0.78**
	OSM	6.25	1.56

Ocotea bullata	MTS	1.56	**0.78**
	OSM	**0.20**	**0.39**
Schizocarphus nervosus	MTS	**0.20**	**0.39**
	OMS	**0.78**	**0.39**
Momordica foetida	MTS	1.56	**0.78**
	OSM	**0.20**	**0.39**
Neomycin		**0.20**	**0.09**

Of the 22 samples tested, only eight samples showed good antibacterial activity against *E. coli* compared to 14 samples that had good activity against *S. aureus*. Plant samples obtained from *muthi* shops which had significantly higher amounts of total phenolics, exhibited better antibacterial activity compared to outdoor-street market extracts with lower amounts of total phenolics. However, *S. nervosus* exhibited good activity in both *muthi* shops and outdoor-street market extracts despite the high disparity in the amounts of total phenolics between outdoor-street market and *muthi* shops samples.

2.3.4 Hierarchical Cluster analysis

The data on elemental compositions were subjected to hierarchical cluster analysis and the results obtained is presented as a dendrogram (**Figure 2.2**). This pictographic representation allows for a better explanation in terms of comparing metal compositions between samples obtained from outdoor-street market and *muthi* shops with closely related samples in terms of metal compositions clustered in the same group. The variables were initially separated into two main groups to a distance of approximately 25. Finally, all the samples were separated into four groups except for *D. elata* obtained from outdoor-street market and *L. clavatum* obtained from *muthi* shops which did not belong to any of the clusters. These groups were:

Group 1: *Cassine transvaalensis* MTS, *C. transvaalensis* OSM, *Ocotea bullata* OSM, *Adenia gummifera* MTS, *A. gummifera* OSM, *Rapanea melanophloeos* OSM, and *R. melanophloeos* MTS.

Group 2: *O. bullata* OSM, *Momordica foetida* OSM, *M. foetida* MTS, *Hypoxis hemerocallidea* OSM, *Schizocarphus nervosus* MTS, *Drimia elata* MTS.
Group 3: *Bulbine natalensis* MTS, *S. nervosus* OSM, *H. hemerocallidea* MTS, *L. clavatum* OSM.
Group 4: *Alepidea amatymbica* MTS, *A. amatymbica* OSM, *B. natalensis* OSM.

Where: OSM = outdoor-street market and MTS = *muthi* shops

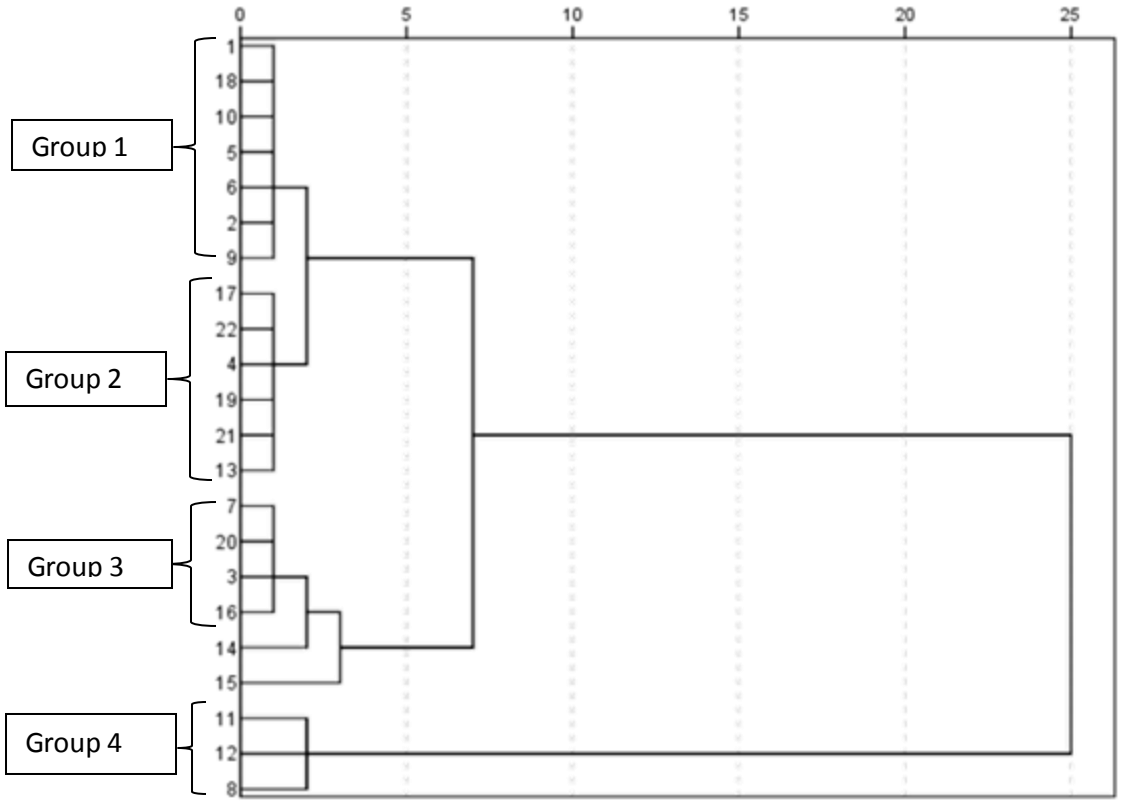


Figure 2.2 Dendogram obtained using HCA based on metal analyte compositions in some highly utilized South African medicinal plants obtained from outdoor-street market and *muthi* shops. Group1: 1 = *Cassine transvaalensis*; MTS, 2 = *C. transvaalensis* OSM, 18 = *Ocotea bullata* OSM, 9 = *Adenia gummifera* MTS, 10 = *A. gummifera* OSM, 5= *Rapanea melanophloeos* OSM, 6 = *R. melanophloeos* MTS. Group 2: 17 = *O. bullata* MTS, 21 = *Momordica foetida* MTS, 22 = *M. foetida* OMS, 4

= *Hypoxis helmerocallidea* OSM, 19 = *Schizocarphus nervosus* MTS, 13 = *Drimia elata* MTS. Group 3: 7 = *Bulbine natalensis* MTS, 20 = *S. nervosus* OSM, 3 = *H. helmerocallidea* MTS, 16 = *Lycopodium clavatum* OSM. Group 4: 11 = *Alepidea amatymbica* MTS, 12 = *A. amatymbica* OSM, 8 = *B. natalensis* OSM. 14 = *D. elata* OSM, and 15 = *L. clavatum* MTS.

2.4 Discussion

One of the most ubiquitous elements in the environment is Al and this could be the reason for the high levels detected in most of the plant samples analyzed. The daily allowance for Al ingestion in humans has been estimated to range from 10-50 mg day^{-1} (**NATIONAL RESEARCH COUNCIL, 1982**). Ingestion of high levels of Al could impair cognitive and speech functions, and may lead to neurodegerative diseases (**BOLLA *et al.,* 1992**). The exact role of Al in the development of neurodegenerative disease is still a subject of intense research and debate (**SHAFER AND MUNDY, 1995; YOKEL, 2000; CAMPBELL, 2002; COUETTE *et al.,* 2009**). High levels of Fe recorded in the present study raise safety concerns as intake of high amount of Fe may be toxic, causing severe damage in the stomach and haematemesis leading to gastric discomfort, nausea, vomiting and diarrhoea. It may also lead to necrosis of mucosal cells and perforation of the gut wall (**BOURMAN AND RAND, 1980**).

Contrary to a previous study (**MTUNZI *et al*., 2012**), high levels of multi-elements was recorded in *A. amatymbica. Hypoxis hemerocallidea* has the ability to take up and accumulate high amounts of Al (**JONNALAGADDA *et al.,* 2008**) and this could be the possible explanation for the high levels of Al recorded in corms of *H. hemerocallidea* obtained from *muthi* shops. The levels of Cd and Pb detected in the present study raise safety concerns as these elements are toxic at very low concentrations (**NIES, 1999**). The levels of As and Hg were high in most of the samples analyzed (**Table 2.2**), with concentrations above the recommended safety limits of 0.2 mg kg^{-1} and 2 µg kg^{-1} respectively (**CODEX ALIMENTARIOUS COMMISSION, 1991; WHO, 2010**). In a previous study, **STREET *et al.*** (**2008**) reported high levels of As and Cd in bulbs and tubers of some South African medicinal plants obtained from outdoor-street

market in Pietermaritzburg. Arsenic at low concentrations causes clinical manifestations such as chronic obstructive pulmonary disease and bronchiectasis, liver disease, cancer of the skin, lung and bladder (**GUHA, 2008**).

In an earlier study, **STEENKAMP *et al.*** (**2006**) reported high levels of Mn in South African medicinal plants. A similar pattern in Mn concentrations was found in the present study alongside Cr and other toxic heavy metals (**Table 2.3**). Toxic levels of chromium (Cr) can induce skin tumors, damage to kidneys, liver, the circulatory system and nerve tissues (**COSTA AND KLEIN, 2006**). Some of the elements analyzed in this study have beneficial roles in plant growth (**JEFFREY, 1987**). For example, Zn, Cu Mn and Fe are important co-enzymes in antioxidant processes and deficiency in any of these essential elements may impair the overall function of the oxidation systems (**LEMBERKOVICS *et al.,* 2002**). However, extremely high levels of these essential elements can be toxic to both humans and plants (**SANDSTEAD, 1995**).

There was no uniform pattern in terms of elemental compositions between samples obtained from outdoor-street market and *muthi* shops. In a number of cases extremely high levels of some of the elements were found in the *muthi* shops samples compared to outdoor-street markets samples as highlighted in bold (**Table 2.2**). For example, *O. bullata* obtained from *muthi* shops had higher amounts of all the twelve evaluated elements compared to outdoor-street market samples. This is an indication that the *muthi* shops samples might have been exposed to different sources of contaminants or been collected from a different locality. Medicinal plant traders were reluctant to give information on possible source and locality where their products were obtained. Hence, heighten the difficulty in explaining the disparity in the levels of metal compositions between outdoor-street market and *muthi* shops. There is a perception that *muthi* shops samples are more hygienic than outdoor-street markets. However, the results of the present study showed that some of the samples obtained from *muthi* shops had increased levels of heavy metals compared to outdoor-street market samples. This could be as a result of post-harvest contamination such as the use of indoor residual spray e.g. pesticides by the *muthi* shop owners. These results highlight

the need to establish guidelines with regards to the use of pesticides in area where medicinal plant products are cultivated and/or stored.

Cluster analysis showed that samples in each group had similar patterns in their elemental concentrations which implies that they might have either been harvested from the same locality, have similar strategies for heavy metal accumulation, or were exposed to the same kind of post-harvest contamination. The plant part analyzed in this study provide additional information in explaining the reasons for the high levels of heavy metals in each group. For instance, group 4 comprises only rhizomes and bulbs; whilst group 3 includes bulbs, corms and whole plants. Such plant parts are in direct contact with the soil when the plants are growing. Hence, there is a higher probability that plants in this group will contain more of the elements, due to the fact that most plants tend to accumulate elements in their underground parts as compared to translocation to above ground parts (**MULLER *et al.,* 2000**). Group 2 comprises a mixture of plant parts and group 1 comprises mainly of stems and bark. Samples of *D. elata* obtained from the outdoor-street market and *L. clavatum* from the *muthi* shops did not belong to any of the clusters, indicating that these plant samples might have come from different localities, and have been exposed to contaminants after harvesting.

This study shows that HCA can be used in preliminary screening to group medicinal plants based on their metal analyte content. The potential to develop HCA techniques as a useful tool to improve the safety of medicinal plants needs to be further investigated. This can be achieved by developing a large database of medicinal plant species collected from known sites and then comparing their metal content. It should be possible to identify plant species that are relatively safe in terms of their metal content. For instance, in the present study all plant samples in group 1 have low concentrations of metals and therefore do not pose a health risk in terms of heavy metal whereas plant samples in group 4 have extremely high levels of heavy metals and need to be more closely monitored to safeguard the consumer's health. Such analyses could also be used to identify potential candidates of medicinal plant species for small scale farming using good agricultural and collection practices (GACP).

The results of the present study highlight that heavy metal contamination is a problem that can impact negatively on the quality and safety of South African medicinal plant products. The high levels of Al, As, Hg and Fe in most of the samples analyzed in the present study are a cause for concern. It is therefore important that a comprehensive elemental profile of frequently used medicinal plants is carried out to establish which medicinal plant species are safe to use and which species and plant parts generally accumulate high concentrations of heavy metals. Additional data on collection sites would also provide valuable information to improve the quality of the medicinal plant products. There is also a need for safe and sustainable cultivation (as discussed in section 1.7.1.1) of valued medicinal plants to prevent heavy metal accumulation and this approach will improve consistency in terms of quality and efficacy of medicinal plant products.

Variability in the phytochemical composition in the present study between outdoor-street market and *muthi* shops samples show that the plant materials probably came from different sources as medicinal plant traders buy the products in bulk from different gatherers and pool them together. The variation in phytochemical compositions recorded in the present study cannot be linked to heavy metal compositions as other factors such as age, locality and time of harvest could have influenced the phenolic and flavonoid contents. This highlight the need for urgent measures to ensure quality of medicinal plants products sold at traditional medicine markets. In a previous study, heavy metal stress was found to induce the accumulation of phenolic compounds in plants (**MICHALAK, 2006)**. For instance, Ni stress induced phenolic compound biosynthesis in wheat (**DIÁZ *et al.,* 2001**) but inhibited the synthesis of anthocyanins in lettuce (*Lactuca sativa* L.) (**HAWRYLAK *et al.,* 2007**). Moderate levels of Al stress was found to increase the biosynthesis of phenolics in maize (**WINKEL-SHIRLEY, 2002**). However, in the present study the amounts of heavy metals in the investigated plants do not translate to high amounts of phenolics. For example, *D. elata* and *L. clavatum* had high amounts of heavy metals but exhibited low amounts of total phenolics. The possible explanation is that the heavy metal contamination was post-harvest rather than the plant being exposed to heavy metals while growing *in situ*. Plant phenolic compounds are known to possess a number of pharmacological

properties such as antioxidant, antimicrobial, anticancer and anti-inflammatory activities (**GALATI AND O'BRIEN, 2004**). Phytochemical screening is a rapid and relatively inexpensive method which can be used to evaluate the quality and authenticity of medicinal plant products (**JIANG *et al.,* 2010**).

In general, plant samples with high levels of heavy metals had good antibacterial activity. **NOORI *et al.*** (**2012**) reported that aqueous extracts of *Verbascum speciosum* obtained from heavy metal contaminated land exhibited potent antibacterial activity against *Salmonella paratyphi* at different concentrations. In a similar study, **STREET *et al.*** (**2009**) reported increased antibacterial activity in *Merwilla plumbea* treated with high levels of Cd. When a plant is exposed to heavy metal stress, it adjusts certain biochemical and physiological processes for survival. These adjustments sometimes favour the production of secondary metabolites (**MURCH *et al.,* 2003**). This could be one of the reasons for the good antibacterial activity noted in this study in plant extracts with high levels of heavy metals. In the present study, *D. elata* samples obtained from both *muthi* shop and outdoor-street market; *B. natalensis* obtained from outdoor-street market, and *L. clavatum* obtained from outdoor-street market which exhibited poor antibacterial activity despite having high levels of heavy metals. The poor antibacterial activity in these plant extracts might be due to loss in certain biosynthetic pathways or down-regulation in the production of important bioactive compounds. Previous studies have shown that some plants species down-regulate the production of secondary metabolites when exposed to high levels of heavy metal stress (**ZHELJAZKOV AND NIELSEN, 1996; RAMAKRISHNA AND RAVISHANKAR, 2011**).

2.5 Conclusions

The high levels of metal compositions especially Al, As, Fe and Hg in the plant samples analyzed in the present study highlight the need for further investigation into the safety of South African medicinal plants sold at informal traditional markets. The use of inorganic pesticides by *muthi* shops owners could also be a potential source of heavy metal contamination and requires further monitoring. There was no consistency in terms of phytochemical composition and these could be possible reasons for variability

in antibacterial activity recorded in the present study. There is need for urgent measures to ensure quality and efficacy of medicinal plant material sold at traditional medicine markets. The results of HCA provide valuable information on the multi-elemental composition in all the evaluated plant samples. The HCA demonstrated which plant species and plant parts tend to have a high elemental content and which have a low elemental content. Building up such a database, including data on harvest locality, will enhance easy identification of which species are 'safe' and which require closer monitoring to ensure high quality medicinal plant products. There is also a need to encourage safe and sustainable cultivation of important medicinal plants using GACP to ensure safety, quality and efficacy of medicinal products.

Chapter 3

Effects of Cd and Al uptake and distribution on physiochemical parameters in *Drimia elata* and *Bulbine natalensis*

3.1 Introduction

Some of the commonly found non-essential and toxic heavy metals in South African agricultural soils include Al, As, Cd and Cr (**DAVIES AND MUNDALAMO, 2010**). Cadmium has received considerable attention due to its toxic effects even at very low concentrations, not only to plants but also to humans and animals (**NIES, 1999**). Cadmium uptake and translocation mechanisms in plants are influenced by several factors such as soil pH and application of phosphate fertilizer. Moderate amounts of Al in the growth media leads to increased phytoavailability of Cd (**KABATA-PENDIAS, 2011**). Tolerance mechanisms in the uptake and accumulation of Cd in plants vary considerably between species (**De La ROSA *et al.,* 2004**). Cadmium toxicity reduces photosynthetic activity in plants due to reduced chlorophyll content and enzymatic activity involved in CO_2 fixation and diminishes water and nutrient uptake (**GREPER AND ÖGREN, 1991; GRATÃO *et al*., 2005**). Exposure to toxic concentrations of Cd leads to production of ROS by auto-oxidation, blocking of essential functional groups in biomolecules, and displacement of essential metal ions from biomolecules (**SCHÜTZENDÜBEL AND POLLE, 2002**). Physiological disorders caused by Cd toxicity are evident in visible symptoms of injury in plants, such as chlorosis, growth inhibition, browning of root tips, and plant necrosis (**KAHLE, 1993**).

Aluminum is a ubiquitous element in the earth's crust. One of the factors that increases Al phytoavailability is soil acidity (**SILVA, 2012**). The primary symptom of Al toxicity in plants is inhibition of root growth resulting in a variety of nutrient-deficient symptoms and decreased yield (**SHAMSI *et al.,* 2007**). The toxic effects of Al can also lead to oxidative stress resulting from excessive production of ROS, including superoxide radicals (O_2^-), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (**ALIA *et al.,* 1995; GILLE AND SIGLER, 1995; SILVA, 2012**). Aluminum toxicity occurs when it

interferes with the uptake, transport and utilization of essential mineral nutrients including Ca, magnesium (Mg), potassium (K), P, Cu, Fe, Mn and Zn (**GUO *et al.,* 2003; GUO *et al.,* 2004; SILVA, 2012**). It also interferes with the plasma membrane phospholipids and proteins by binding to charges associated with these groups of molecules, thereby reducing the negative charge and shielding the membrane surface potential (**KINRAIDE *et al.,* 1992**).

The growing support for cultivation of medicinal plants is an important goal to meet the increasing demands for medicinal plant products. Cultivation of medicinal plants is an income generating venture and is attracting several small- and large-scale farmers. However, small-scale cultivation of medicinal plants by local farmers is hindered by several technical constraints, such as lack of water for irrigation, difficulties with plant propagation and lack of experience with proper cultivation requirements with respect to soil. The selection of medicinal plants for cultivation is based on criteria such as high demand, financial value and ease of cultivation. In South Africa, among the ten most frequently cultivated medicinal plants are *Alepidea amatymbica, Bulbine natalensis, Drimia elata* and *Hypoxis hemerocallidea* (**WIERSUM *et al.,* 2006**). In Chapter 2, these species were found to have elevated levels of Al (**Table 2.2**) indicating that they could be potential hyperacculators of Al.

It is important to monitor the levels of toxic elements in medicinal plant products available to consumers to ensure that the elemental composition is within the permissible limits and that they meet all the safety and quality criteria. These can only be achieved through strict adherence to good agricultural and collection practices (GACP) stipulated by the **WHO** (**2003**), which states that extra caution be taken to avoid risk of contamination caused by environmental pollutants in medicinal plant products. According to the **SOUTH AFRICAN DEPARTMENT OF AGRICULTURE** (**2007**), many South African soils are acidic due to increasing concentrations of Al. Studies have shown that Cd availability increases with decreased soil pH (**WU AND ZHANG 2002**). Soil pollution by heavy metals is a multi-element problem, and several studies have been conducted to investigate the synergistic effect of heavy metals on some plant species. As high levels of Al in the soil can significantly increase phytoavailability of Cd, it is important to monitor levels of Cd in plants species grown on contaminated soil with high levels of Al. Currently, there is no research on the

combined effects of Cd and Al accumulation on South African medicinal plants. These interactions need to be understood in order to improve the quality, efficacy and safety of cultivated medicinal plant products. Thus, the aim of the experiment presented in this Chapter, was to evaluate the interactive effects of Cd and Al on growth, heavy metal accumulation and physiochemical parameters in *D. elata* and *B. natalensis.*

3.2 Materials and Methods

3.2.1 Pot trials

Seeds of *D. elata* and *B. natalensis* were obtained from Silverhill Seeds, South Africa. Seeds were surface decontaminated with 0.1% mercuric chloride for 2 min and then rinsed thoroughly with distilled water. The seeds were germinated on disposable Petri dishes (9 cm), each containing two layers of Whatman No. 1 filter paper. Petri dishes containing seeds were placed in a plant growth chamber at 25 °C under 16:8 h light:dark conditions. The photosynthetic photon flux density of tubes in the growth chamber was 80.4 ± 3.5 µmol m^{-2} s^{-1}. After three weeks, young seedlings (n = 3) of equal size of both species were transplanted into pots (15 cm) containing acid washed quartz sand. There were 6 pots per treatment which were arranged in a randomized block design. The plants were grown in the shade house at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus (29˚ 37.55 S; 30˚ 24.13 E). To each pot, 100 mL 50% Hoagland's nutrient solution (HS) (**Appendix A)** (**HOAGLAND AND SYNDER, 1933**) was added once a week for 7 months until the seedlings were well established. Concentrations of heavy metals in the form of $Cd(NO_3)_2 \cdot 4H_2O$ and $Al(NO_3)_3 \cdot 9H_2O$ both singly and in combination were prepared in HS (**Table 3.1**). The solutions were added once a week from week 29. Hoaglands solution (50%) was used for the control treatment. The experiment was terminated after a further 6 weeks.

3.2.2 Sample preparation and data collection

Plant samples were harvested after 6 weeks of treatment. They were washed under running water to remove any particles of sand. Growth parameters including leaf and root length, and the mean fresh (FW) and dry weights (DW) of the bulb and leaf biomass were recorded. Plant samples were sliced into small pieces and oven dried at 50˚C for 72 h. The dried plant samples were ground into powders using a Retsch®

ZM 200 ultra-centrifugal mill. The powders were stored in air-tight containers in the dark at room temperature until analysis.

Table 3.1 Composition of Cd and Al treatment solutions.

Heavy metal treatment (mg L^{-1})	pH
Control 50% HS	5.4
Cd 2	5.4
Cd 5	5.4
Cd 10	5.4
Al 500	3.3
Al 1000	3.2
Al 1500	3.1
Cd 2:Al 500	3.4
Cd 5:Al 1000	3.3
Cd 10:Al 1500	3.2

3.2.3 Elemental analysis

Dried powdered samples (0.5 g DW) from each treatment for both *D. elata* and *B. natalensis* were digested using a microwave acid-assisted system as described in Section 2.2.2.2.The digested samples were analysed for elemental composition using ICP-OES as described in Section 2.2.2.4.

3.2.4 Physiochemical screening

3.2.4.1 Determining chlorophyll content

The amounts of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl a + b) of leaf samples of *D. elata* and *B. natalensis* were determined quantitatively as described by **LICHTENTHALER** (**1987**). Fresh leaf samples (0.2 g FW) from each treatment were homogenised in 5 mL acetone with the addition of a few fine grains of acid-washed quartz sand (BDH Chemicals, England). The finely homogenised mixture was filtered through Whatman No. 1 filter paper and centrifuged for 5 min at 5 000 *g* at room temperature. The absorbance of the resultant filtrate was measured at 644.8, and 661.6 nm. The pigment content was expressed as mg g^{-1} FW. Chlorophyll

concentrations (Chl a, Chl b and total chlorophyll) were expressed using the equations below:

$$\text{Chl a} = 11.24A_{661.6} - 2.04A_{644.8}$$

$$\text{Chl b} = 20.13A_{644.8} - 4.19A_{661.6}$$

$$\text{Chl a + b} = 7.05A_{661.6} + 18.71A_{644.8}$$

3.2.4.2 Determining free-proline content

The amount of free-proline was determined using the ninhydrin reagent method described by **BARNETT AND NAYLOR** (**1966**) with slight modifications. Fresh shoot samples (1 g FW) of *D. elata* and *B. natalensis* respectively were homogenised using a pestle and mortar by adding 10 mL 3% (w/v) aqueous sulfosalicylic acid solution with the addition of a few grains of fine acid-washed quartz sand (BDH Chemicals, England). The homogenised solution was filtered through two layers of glass-fibre (Schleicher and Schüll, GF6, Germany), and the filtrate was then used in the assay. To each test tube containing 1 mL of filtrate, 1 mL glacial acetic acid and 1 mL ninhydrin reagent (2.5 g ninhydrin 100 mL^{-1} of a solution containing glacial acetic acid, distilled water and 85% ortho-phosphoric acid at a ratio of 6:3:1) were added. The closed test tubes with the reaction mixture were placed in a boiling water bath for 1 h, after which the reaction was terminated by immersing the tubes in a water bath for 5 min at room temperature. Absorbance was measured at 546 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia) against a blank that contained 3% (w/v) aqueous sulfosalicylic acid instead of sample extract. The proline concentrations were determined from a standard curve and calculated on a fresh weight basis (mmol g^{-1} FW).

3.2.4.3 Analysis of lipid peroxidation rate in *B. natalensis*

The rate of oxidative damage to leaf lipids was evaluated by analysing the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA).TBARS content was estimated by the method of **CAKMAK AND HORST** (**1991**) with slight modifications. Due to shortage of plant material, only *B. natalensis* was evaluated in this assay. Fresh leaf samples (0.5 g) were homogenized in 5 mL 0.1% (w/v) trichloroacetic acid (TCA), at 4˚C. Samples were centrifuged at 12000 *g* for 5 min. An aliquot 1 mL of supernatant was added to 4 mL 0.5% (w/v) TBA in 20% (w/v) TCA. Samples were incubated at 90˚C for 30 min

thereafter the reaction was stopped in an ice bath. The sample was centrifuged at 10 000 *g* for 5 min, and absorbance of the supernatant was read at 532 nm on a UV–vis spectrophotometer (Varian Cary 50, Australia). The reading was corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The value obtained was used to calculate the TBARS content using the formula:

$$\text{TBARS content } (\text{mmol g}^{-1}\text{ FW}) = \frac{(A_{532} - A_{600})\text{V X } 1000}{\varepsilon \text{ X W}}$$

where ε is the specific extinction coefficient (=155 mM cm^{-1}), *V* is the volume of crushing medium, *W* is the fresh weight of leaf, A_{600} is the absorbance at 600 nm wavelength, and A_{532} is the absorbance at 532 nm wavelength.

3.2.4.4 Estimation of carbohydrate content in *B. natalensis*

Total carbohydrate content was determined using the method described by **DUBOIS *et al.*** (**1956**) and **BUYSSE AND MERCKX** (**1993**) with slight modification. Due to shortage of plant material, only *B. natalensis* was evaluated in this assay. Dried powdered shoot and root plant samples (25 mg DW) were transferred into a test-tube and 10 mL 80% ethanol added. The extract was heated on a heating block at 95˚C for 60 min and then centrifuged at 2000 *g* for 15 min at room temperature. The supernatant was made up to 10 mL with distilled water. Thereafter, 500 µL supernatant was transferred into a pill vial and 3 mL Anthrone reagent added. Glucose (100 µg mL^{-1}) was used to prepare the standard solution. The mixture was heated for 10 min in a boiling water bath. The reaction was stopped by cooling on ice. Thereafter, absorbance was measured at 620 nm. The amounts of glucose in the samples was calculated based on the glucose standard curve and was expressed as µg mg^{-1} DW.

3.2.5 Statistical analysis

Statistical analyses were carried out using SPSS for Windows by one-way ANOVA using the Student's *t*-test to test the different significance levels.

3.3 Results

3.3.1 *Drimia elata*

3.3.1.1 Effect of heavy metals on biomass in *Drimia elata*

The lowest concentrations of Al (500 mg/l) and Cd (2 mg/l) had no effect on the bulb biomass of *D. elata* while the Cd 2 mg L^{-1} treatment significantly decreased the shoot biomass. There was a significant reduction in both shoot and bulb dry weight at the higher concentrations of Cd and Al treatment (**Table 3.2**). Visible symptoms of Cd toxicity including chlorosis, growth inhibition, browning of root tips and death were recorded in the present study.

Table 3.2 Effect of Cd and Al on the biomass of *D. elata.* Results are presented as mean±SD (n=6). Different letters within a column indicate significant differences (p<0.05).

Treatments	Fresh weight (g)		Dry weight (g)	
(mg L^{-1})	Shoot	Bulb	Shoot	Bulb
Control	9±2.10a	30±5.80a	7±1.80a	13±4.60a
Cd 2	7±1.90b	26±9.20b	4±0.40b	10±2.80b
Cd 5	6±1.10bc	13±3.70bc	3±0.80cd	6±1.90bc
Cd 10	4±0.90cd	10±1.80bc	2±0.20cd	3±0.80cd
Al 500	10±3.40a	29±7.70a	7±2.10a.	11±3.70a
Al 1000	9±2.80b	13±4.20bc	5±0.90bc	5±1.30bc
Al 1500	3±1.00d	4±1.50d	1±0.10d	1±0.50d
Cd 2:Al 500	7±1.70bc	10±3.90c	2±0.60cd	5±1.10cd
Cd 5:Al 1000	5±0.80c	6±1.60cd	2±0.30cd	1±0.70d
Cd 10:Al 1500	6±0.40d	5±1.00d	2±0.90cd	1±0.20d

3.3.1.2 Uptake and accumulation of Cd and Al in *Drimia elata*

The highest amounts of Cd in the bulbs and shoots were recorded in the combined Cd 5:Al 1000 mg L^{-1} treatment (**Table 3.3**). The high levels of Cd recorded in the combined treatments could be as a result of low pH which favoured the uptake of Cd as phytoavailability of Cd increases with reduce pH (**Table 3.1**) compared to when Cd was used alone. Combined application of Cd 5:Al 1000 mg L^{-1} exhibited synergistic effects with significant increase in the uptake of both elements in bulbs and shoots

compared to when Cd and Al were used alone in the treatment. Although, at the highest combined heavy metal treatment (Cd 10:Al 1500 mg/l) there was a significant decrease in the uptake of both Cd and Al.

Table 3.3 Uptake and accumulation of Cd and Al in *D. elata* bulbs and shoots. Results are presented as mean±SD (n=3). ND = not detected. Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L^{-1})	Cd concentration (mg kg^{-1})		Al concentration (mg kg^{-1})	
	Bulb	Shoot	Bulb	Shoot
Control	ND	ND	ND	ND
Cd 2	24±20e	21±0.30d	ND	ND
Cd 5	69±0.10b	25±2.00c	ND	ND
Cd 10	77±29a	53±18a	ND	ND
Al 500	ND	ND	639±53d	419±8c
Al 1000	ND	ND	735±7.50b	179±29d
Al 1500	ND	ND	778±6.40b	457±0.10b
Cd 2:Al 500	62±1.30c	45±0.50b	704±5.10c	461±3.40b
Cd 5:Al 1000	83±0.10a	58±3.50a	918±14a	495±8a
Cd 10:Al 1500	54±1.50d	21±40d	129±7e	59±5.30e

3.3.1.3 Effect of Cd and Al on the uptake and distribution of essential elements in *Drimia elata*

Application of Cd and Al at highest concentrations antagonized uptake and distribution of all the essential elements investigated in the present study (**Table 3.4**). The lowest and moderate amount of Al treatment enhanced uptake and accumulation of all the evaluated essential elements including Zn, Mn, Fe and Cu (**Table 3.4**) in the bulbs and shoots compared to the control plants. However, at the highest concentration of Al (1500 mg L^{-1}) there was an antagonistic effect in the uptake of Zn, Mn and Cu. Conversely, Cd-treated plants exhibited a marked reduction in the amounts of essential elements in both the bulbs and shoots (**Table 3.4**). All the combined Cd and Al treatment enhanced uptake and accumulation of essential elements.

Table 3.4 Effect of Cd and Al treatments for 6 weeks on the uptake of essential elements in *D. elata*. Results are presented as mean±SD (n=3). Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L^{-1})	Plant part	Metal element concentration (mg kg^{-1})			
		Zn	Mn	Fe	Cu
Control	Bulbs	242 ±8.60bc	130±2.30cd	318±53bc	139±26cd
	Shoots	288±10b	149±8.70cd	325±9.80bc	112±0.20cd
Cd 2	Bulbs	217±0.70bc	109±0.50d	116±32d	127±0.90cd
	Shoots	118±1.50d	91±8.90d	170±0.10cd	131±0.60cd
Cd 5	Bulbs	202±0.70bc	115±30d	145±8.20cd	84±1.30d
	Shoots	219±1.30bc	105±0.70d	195±9.70bc	118±0.30cd
Cd 10	Bulbs	232±17bc	110±3.80d	164±16cd	102±4.10cd
	Shoots	142±7.40d	127±4.20cd	119±79d	127±5.40cd
Al 500	Bulbs	457±2.90a	378±25b	514±8.30b	448±9.70b
	Shoots	545±5.30a	323±6.80b	470±3.50b	225±3.70c
Al 1000	Bulbs	368±4.90ab	480±3.50ab	505±50b	292±24bc
	Shoots	466±2.70a	320±2b	701±12a	397±3.50bc
Al 1500	Bulbs	128±7.60d	96±3.40d	119±28d	76±5.20d
	Shoots	182±0.50c	92±1.70d	103±4.60d	115±1.60cd
Cd 2:Al 500	Bulbs	324±0.60ab	931±6.10a	491±5b	523±9.80a
	Shoots	426±4.90a	624±0.10b	256±5c	394±3.20bc
Cd 5:Al 1000	Bulbs	223±1.60bc	197±25c	271±15c	164±3.80cd
	Shoots	383±5.30ab	236±4.10bc	796±3.60a	391±16bc
Cd 10:Al 1500	Bulbs	293±0.90b	182±5.30c	263±71c	219±23cd
	Shoots	265±57bc	168±21cd	260±50c	182±28cd

3.3.1.4 Effect of heavy metals on chlorophyll content in *Drimia elata*

The physiological response of *D. elata* to heavy metal stress was demonstrated by the significant reduction in Chl a and Chl b and total chlorophylls in all the treatments except at Al 500 mg L^{-1} (**Table 3.5**). The highest Al treatments (1500 mg/l) applied singularly and in combination with Cd resulted in the lowest chlorophyll content that was approximately four times lower than the control.

Table 3.5 Effect of Cd and Al treatments on the chlorophyll content in the shoots of *D. elata*. Results are presented as mean±SD (n=3). Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L^{-1})	Chl a mg g^{-1} FW	Chl b mg g^{-1} FW	Chl a+b mg g^{-1} FW
Control	15.67±0.38a	43.04±1.25a	58.70±0.88a
Cd 2	9.43±1.84b	34.51±2.26b	43.93±4.11b
Cd 5	9.44±1.40b	32.61±2.92b	42.05±4.32b
Cd 10	4.19±2.38cd	22.41±4.02c	26.59±6.41c
Al 500	9.72±2.12b	35.40±1.79a	45.12±3.91ab
Al 1000	7.43±1.80c	29.62±025bc	37.05±1.56bc
Al 1500	3.19±0.04d	12.23±0.64d	15.42±1.04d
Cd 2:Al 500	5.09±1.18bc	23.05±2.01c	28.14±3.19bc
Cd 5:Al 1000	3.90±0.96d	19.98±1.84cd	23.88±2.80c
Cd 10:Al 1500	4.79±1.23cd	15.67±1.80cd	20.46±0.57cd

3.3.1.5 Effect of heavy metals on the accumulation of free-proline in *Drimia elata*

The levels of proline accumulated in *D. elata* was significantly higher in most of the heavy metal-treated plants compared to the control. The highest concentration of proline was recorded at 1500 mg L^{-1} Al which was< a 4-fold increase compared to the control (**Figure 3.1**). *Drimia elata* generally exhibited a metal concentration (dose) dependent increase in the amount of free-proline accumulated indicating that the plants were stressed (**Figure 3.1**). However, the combined Cd and Al treatments had lower amounts of proline compared to when Al was used alone.

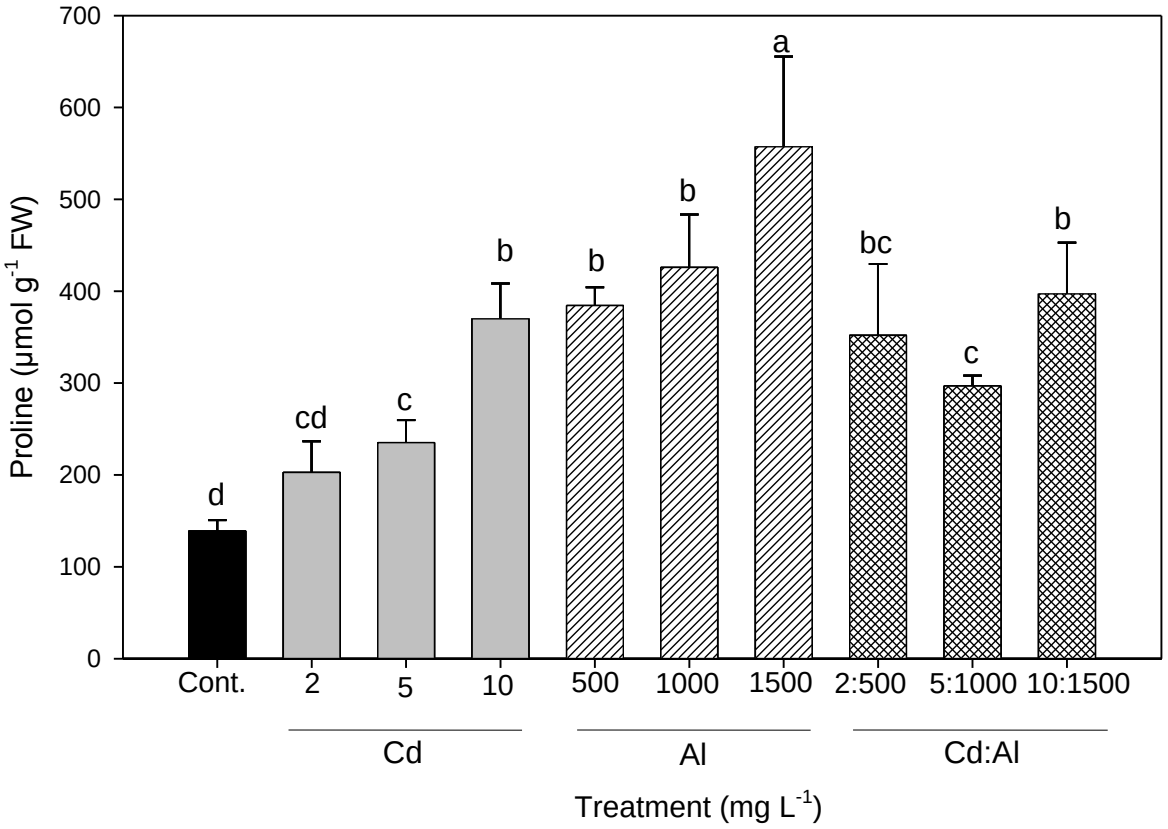


Figure 3.1 Effect of Cd and Al on free-proline content in *D. elata* bulbs and shoots after six weeks treatment. Results are presented as mean±SD (n=3). Different letters indicate significant differences (p<0.05).

3.3.2 *Bulbine natalensis*

3.3.2.1 Effect of heavy metals on biomass in *Bulbine natalensis*

In the present study a significant reduction in different growth parameters such as length of shoot and root, fresh and dry biomass were recorded in *B. natalensis* at high levels of Cd and Al treatment indicating toxic effects of these elements at higher concentrations (**Table 3.6**). In comparison with the control plants, plants treated with Cd 2 mg L^{-1} had a slight positive effect on shoot fresh weight and shoot dry weight. Also, Al 500 mg L^{-1} had a positive effect on shoot length and bulb fresh weight compared to all the other treatments, indicating a stimulating effect of Cd and Al at lower concentrations in *B. natalensis.*

Table 3.6 Effects of heavy metals on the biomass in *B. natalensis.* Results are presented as mean±SD (n=6). Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L^{-1})	SL (cm)	SFW (mg)	RL (cm)	RFW (mg)	BFW (mg)	SDW (mg)	RDW (mg)	BDW (mg)
Control	13±2.60a	24±8.70a	10±1.20a	12±1.80a	27±0.20a	10±2.20a	4±0.12a	10±0.20a
Cd 2	13±3.20a	25±2.30a	9±1.30a	9±0.90ab	18±0.00b	10±3.10a	3±0.00bc	8±1.80ab
Cd 5	9±2.50ab	7±1.00c	5±1.00c	7±0.01b	13±0.78bc	3±0.20cd	2±1.00bc	6±2.40b
Cd 10	6±1.00b	6±0.72d	4±2.30cd	4±0.40c	9±1.31d	3±0.10d	1±0.00c	2±1.10bc
Al 500	15±1.60a	21±50ab	7±0.00ab	9±0.90a	27±4.20a	8±0.00ab	4±0.10a	9±1.80a
Al 1000	8±1.50ab	10±3.10cd	5±1.20cd	6±0.30b	14±0.20bc	3±0.10cd	3±0.30b	4±0.10bc
Al 1500	5±1.60c	4±1.30d	2±2.30d	3±0.40d	8±0.09d	1±0.00d	1±0.00d	2±0.00c
Cd 2:al500	13±4.40a	17±4.90b	6±1.10b	10±0.70a	20±0.00ab	5±0.30b	2±0.10bc	7±2.20ab
Cd 5:Al1000	9±2.40ab	12±2.70cd	6±2.20b	6±0.00b	13±0.010c	4±0.10c	1±0.00bc	3±1.10bc
Cd 10:Als1500	6±2.10c	10±0.70cd	4±1.50cd	6±0.30b	9±2.810d	3±0.00cd	1±0.10c	2±0.90c

SL = Shoot length, SFW = Shoot fresh weight, SDW = Shoot dry weight, RL = Root length, RFW = Root fresh weight, RDW = Root dry weight, BFW = Bulb fresh weight, BDW = Bulb dry weight

Increasing heavy metal treatments significantly decreased shoot and root lengths as well as plant biomass compared to the control plants. Significant growth inhibitory effects at the highest concentrations of individual treatments with Al followed by Cd. The low pH 3.1 at the highest concentrations of Al 1500 mg L^{-1} treatment could have contributed to the significant growth inhibition recorded in the present study.

3.3.2.2 Interactive effect on the uptake and accumulation of Cd and Al in *Bulbine natalensis*

The highest concentration of Cd was recorded in root samples grown in the highest treatment of the combined Cd 10:Al 1500 mg L^{-1} (**Table 3.7**). The amounts of Al in the roots and bulb samples increased with increasing concentrations of Al treatment. The highest amounts of Al translocated to the shoot was recorded at the combined treatment of Cd 5:Al 1000 mg L^{-1}. Generally, the pattern of Cd and Al uptake and distribution in *B. natalensis* are in the following order: root > bulb > shoot. This is an indication that translocation of heavy metals from the root to shoot is slow in *B. natalensis.* The amounts of Cd in the medicinally used *B. natalensis* bulbs increased with increasing concentrations of treatment. Plants grown on the combined Cd 10:Al 1500 mg L^{-1} contaminated soil accumulated more Cd in the bulb than shoot. The lower pH 3.2 recorded in the combined Cd 10:Al 1500 mg L^{-1} treatment could have been the reason for the significant increase in the amounts of Cd in the bulbs.

Table 3.7 Interactive effects in the uptake and accumulation of Cd and Al in *B. natalensis.* Results are presented as mean±SD (n=3). ND = Not detected. Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L^{-1})	Cd concentration (mg kg^{-1})			Al concentration (mg kg^{-1})		
	Shoot	Bulb	Root	Shoot	Bulb	Root
Control	ND	ND	ND	ND	ND	ND
Cd 2	18±2.20d	22±90e	17±10d	ND	ND	ND
Cd 5	25±1.90c	37±11d	103±35a	ND	ND	ND
Cd 10	38±4.50a	59±3.10b	88±5.10b	ND	ND	ND
Al 500	ND	ND	ND	298±47d	259±48d	129±13e
Al 1000	ND	ND	ND	484±34b	432±10a	1414±47b
Al 1500	ND	ND	ND	199±13e	444±26a	1782±83a
Cd 2:Al 500	26±8.20c	36±7.20d	62.38±4.8c	294±65d	201±39d	420±48d
Cd 5:Al1000	32±9.50b	45±38c	88.72±23b	608±69a	308±46c	1023±39c
Cd 10:Al1500	37±3.70a	68±33a	109.67±27a	424±53c	410±35b	1580±64a

ND = Not detected.

3.3.2.3 Effects of heavy metals on chlorophyll content in *Bulbine natalensis*

Under stress conditions, plants use strategies such as allocation and/or partitioning of resources to balance cellular functions. In the present study, plants exposed to Cd and Al showed a significant decline in the total chlorophyll content (**Table 3.8**). There was a significant decrease in amounts of total chlorophyll content at the highest Cd 10 mg L^{-1}, Al 1500 mg L^{-1} and the combined treatment Cd 10:Al 1500 mg L^{-1} respectively compared to the control plants.

Table 3.8 Effect of Cd and Al on photosynthetic pigments in *B. natalensis.* Results are presented as mean±SD (n=3). Different letters within a column indicate significant differences (p<0.05).

Treatment (mg L-1)	Chl a mg g^{-1} FW	Chl b mg g^{-1} FW	Chl a+b mg g^{-1} FW
Control	35±2.31a	42±6.81a	38±4.33a
Cd 2	21b±1.92c	33±4.94ab	27±3.86ab
Cd 5	15±2.01c	23±5.11bc	19±1.94bc
Cd 10	10±0.87e	16±2.83c	13±2.88d
Al 500	25±3.21b	38±4.22a	31±4.71ab
Al 1000	16±1.48bc	20±3.71bc	18±3.02bc
Al 1500	11±0.91d	16±1.84c	14±1.96cd
Cd 2:Al 500	23±3.81bc	35±2.95ab	29±4.03ab
Cd 5:Al 1000	14±2.19cd	21±3.01bc	17±1.54bc
Cd 10:Al 1500	9±1.73e	12±2.09d	10±1.82e

3.3.2.4 Effect of heavy metals on free-proline in *Bulbine natalensis*

The concentrations of free-proline were found to be linear dose dependent response in all the heavy metal-treated plants (**Figure 3.2**). All the Al-treated plants were not statistically different from the combined Cd and Al treated plants in terms of proline accumulation.

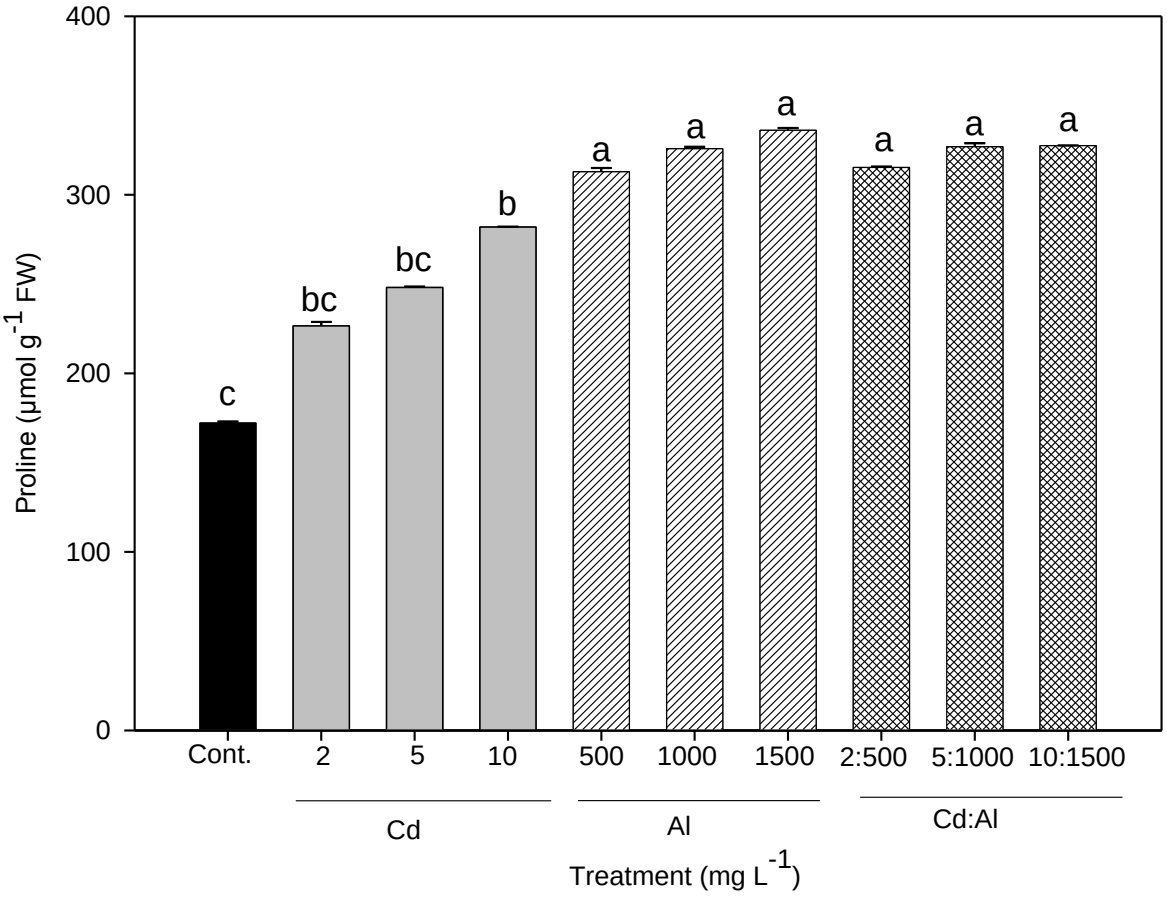


Figure 3.2 Proline concentrations in *B. natalensis*. Results are presented as mean±SD (n=3). Different letters indicate significant differences (p<0.05).

3.3.2.5 Effect of heavy metals on the accumulation of MDA in *Bulbine natalensis*

There was a progressive increase in the amounts of MDA in Cd and Al treated plants, indicating that Cd and Al triggered lipid peroxidation in *B. natalensis.* The final product of membrane lipid peroxidation is MDA, which accumulates when plants are subjected to oxidative stress. Therefore, the concentration of MDA is commonly considered as a general indicator of lipid peroxidation (**XU *et al*., 2009**). Significantly high amounts of MDA were recorded at Cd 10 mg L^{-1} as well as the combined treatment Cd 10:Al 1500 mg L^{-1} (**Figure 3.3**). The lowest amounts of MDA was recorded at the Al 500 mg L^{-1}.

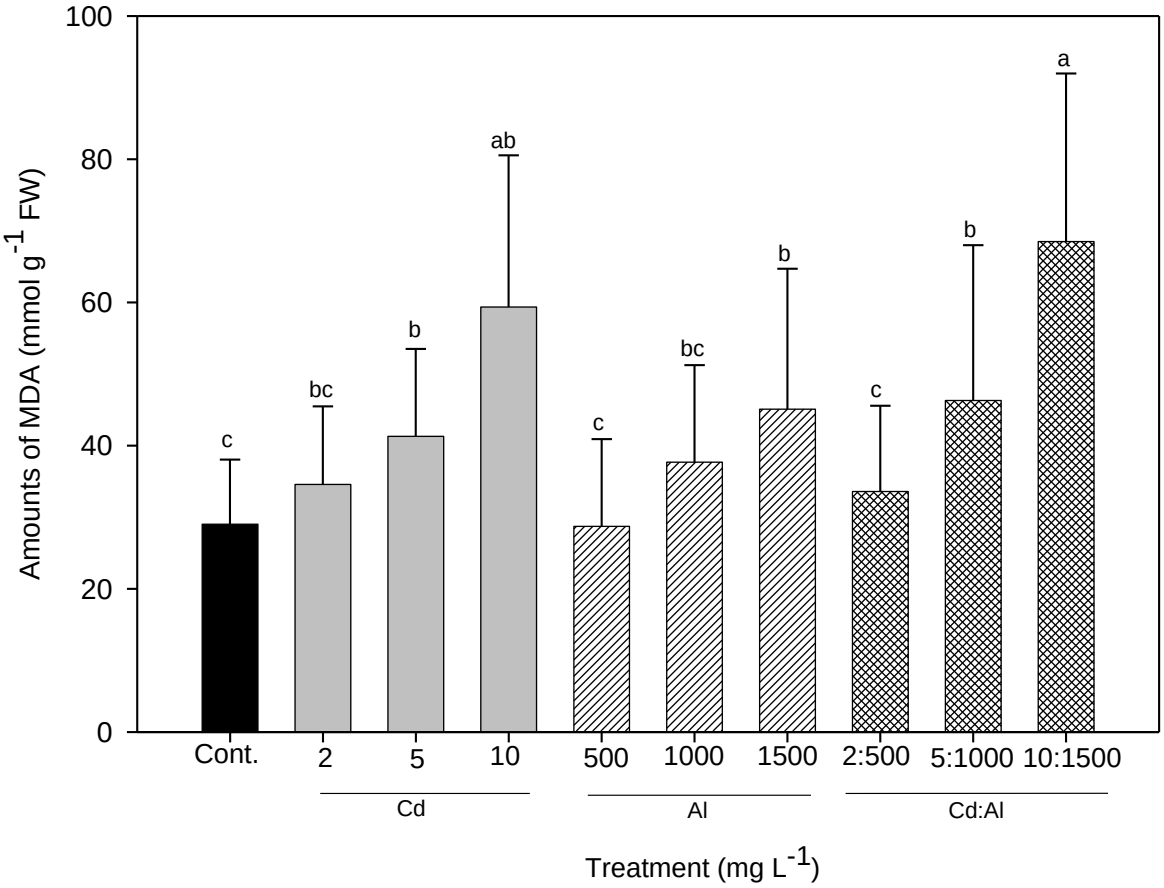


Figure 3.3 Activity of MDA (mmol g^{-1} FW) in leaves of *B. natalensis* exposed to heavy metal treatments. Results are presented as mean±SD (n=3). Different letters indicate significant differences (p<0.05).

3.3.2.6 Effect of heavy metals on biosynthesis of carbohydrates in *Bulbine natalensis*

Heavy metals play an important role in plant metabolism. Of particular interest is the interactions of heavy metals with carbohydrate metabolism which serves as source of energy and carbon skeletons needed for survival under environmental stress. In the present study, a gradual increase in the concentrations of Cd alone slightly increased the amounts of carbohydrate in the leaf sample compared to the control plants (**Figure 3.4**). The highest amounts of carbohydrate content in the leaf sample was recorded at Cd 10 mg L^{-1}. Significantly high amounts of carbohydrate was recorded in the root samples of Al 500 mg L^{-1}.

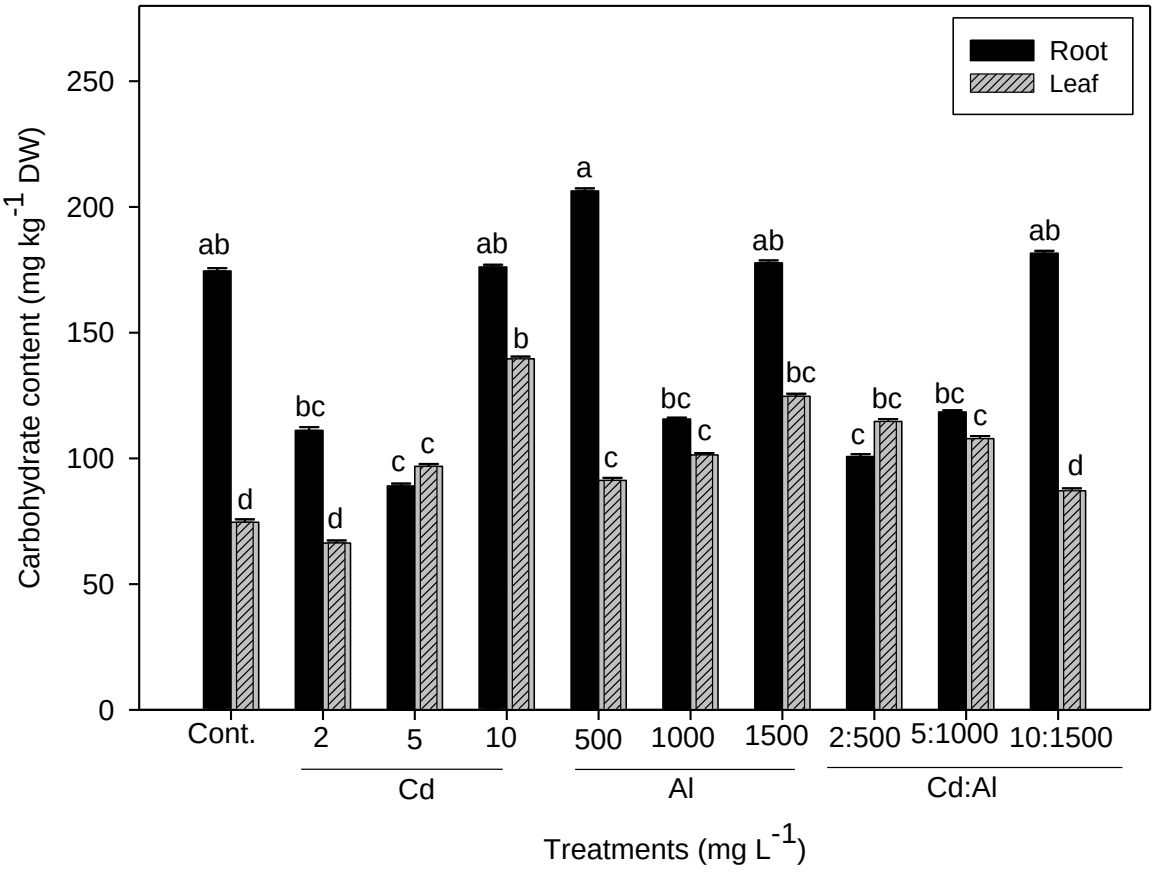


Figure 3.4 Effect of heavy metals on the amount of soluble total carbohydrate in *B. natalensis*. Results are presented as mean±SD (n=3). Different letters indicate significant differences (p<0.05).

3.4 Discussion

In a previous study, **SHAMSI *et al.*** (**2007**) reported that at low levels of Cd and Al (1.0 µmol L^{-1} respectively) there was a positive effect on biomass in soybean while higher levels (150 µmol L^{-1} Al) inhibited plant growth. **WU AND ZHANG** (**2002**) reported a similar trend that low levels of Cd exhibited a positive effects on biomass in barley seedlings. In the present study, the lowest concentrations of Cd (2 mg L^{-1}) exhibited a slight positive effects on shoot fresh weight and root dry weight in *B. natalensis* compared to the control. The results of the combined treatments are not statistically different from the individual Cd and Al treatments indicating that these treatments were not synergistic in terms of toxicity in *B. natalensis.* This was contrary to previous studies which reported that high concentrations of Al in growth media significantly

reduced plant biomass by inhibiting uptake of essential elements in a number of plant species (**LIDON AND BARREIRO, 2002; SHAMSI *et al.,* 2007; HE *et al.,* 2011**). This is due to the fact that acidic soil has many growth constraining factors besides Al toxicity (**GUO *et al.,* 2004**). The result of the present study showed that *B. natalensis* has good tolerance to heavy metal stress. This finding is consistent with a previous study which recorded growth stimulatory effects of Al at lower concentrations in *Camellia sinensis* (**HAJIBOLAND *et al.,* 2013**). On the contrary, application of Cd and Al at low concentration in *D. elata,* exhibited a significant growth inhibition by decreasing the shoot and bulb fresh and dry weight compared to the control plants. Thus, growing *D. elata* on heavy metal contaminated soil could potentially cause slower growth rates and lower biomass. This would potentially decrease the medicinal values of the plant and place more pressure on wild populations.

Uptake and accumulation of Cd and Al in *B. natalensis* and *D. elata* were higher in the roots than the shoots. This was similar to a trend reported in a previous study (**LIU *et al.,* 2003b**) where higher Cd accumulation was recorded in the root than the shoot in different rice cultivars and genotypes. **SHAMSI et al.** (**2007**) reported that low pH increased uptake and accumulation of Cd and Al due to increasing availability of both elements at reduced pH. This could probably be the reason for the relatively high amounts of Cd and Al uptake in the present study. The ability of roots to absorb Cd may depend on both the activity of roots and the interaction between roots and the rhizosphere (**LIU *et al.,* 2003b**). Plant species capable of accumulating high levels of Cd and Al (>100 mg kg^{-1} and >1000 mg kg^{-1} respectively) are regarded as a hyperaccumulators (**BAKER *et al.,* 1994; JANSEN *et al.,* 2002**). From the data presented in this study it appears that the investigated species should not be regarded as a hyperaccumulator of Cd and Al as the amounts of these elements translocated to the shoots are below the recommended amounts for a hyperaccumulator. Non-hyperaccumulators generally tend to store more heavy metals in an inactive form in the root rather than in the aerial plant parts (**RASCIO AND NAVARI-IZZO, 2011**). There was a significant decrease in the accumulation of essential elements at the highest levels of Cd and Al treatments. This is in agreement with previous studies which showed that Cd had an antagonistic effect on the uptake of essential elements (**WU AND ZHANG, 2002; ZORNOZA *et al.,* 2002; LIU *et al.,* 2003b**). Deficiency of

some of the essential elements could lead to breakdown of basic metabolic processes and perturb the biosynthesis of important bioactive compounds.

The bulbs of *B. natalensis* and *D. elata* are the most extensively utilized plant part in traditional medicine and the results of the present study revealed high levels of Cd and Al in bulbs compared to the shoots (**Table 3.3**). This may represent a major route of heavy metal into the food chain. Ingestion of high levels of Al can impair cognitive and speech functions and may lead to neurodegerative diseases (**BOLLA *et al.,* 1992**). Cadmium induces various toxic effects in humans at low doses and the primary target organs are the kidneys and liver (**WHO, 1998; NIES, 1999**). Bulbs of *D. elata* have been reported to be highly toxic and can cause death as a result of cardiac arrest (**VAN WYK *et al.,* 2008; NDHLALA *et al.,* 2013**). The reported toxicity may be further exacerbated by high levels of heavy metal accumulation in the bulb. Accumulation of Cd and Al in the bulbs of *D. elata* harvested from heavy metal contaminated soil raises concern for consumer safety when used as medicine for human consumption.

Exposure to heavy metals induced other aspects of plant physiology such as a decrease in chlorophyll content. Studies have shown that increased concentrations of Cd and Al in growth media induce toxicity by specifically inhibiting the photosynthetic apparatus in many plant species (**AKAYA AND TAKENAKA, 2001; RAI *et al.,* 2004**). One of the most important cellular functions in plants is photosynthesis whereby photoradiation energy is incorporated in a metabolic process to synthesise carbon and nitrogen assimilates. Hence, any environmental factors that interferes with these processes alter the nutrient balance, resulting in toxic effects and sometimes death. Cadmium phytotoxicity has been shown to not only interfere with the normal metabolism of some micronutrients but also exhibit inhibitory effects on photosynthesis as well as disturb the transpiration and fixation of CO_2 (**PRASAD, 2005**). Cadmium induces modifications in chloroplast ultrastructure and chlorophyll biosynthesis (**Di CAGNO *et al.,* 2001**). Al toxicity inhibits the photosynthetic apparatus of many plant species (**AKAYA AND TAKENAKA, 2001**). In the present study, increasing concentrations of Cd and Al both singly and in combination, significantly reduced the total chlorophyll content in *B. natalensis* and *D. elata*.

Accumulation of free-proline is one of the most commonly induced adaptive responses of plants to heavy metal stress (**BALESTRASSE *et al.,* 2005**). High levels of proline accumulation was recorded in the investigated plants in a dose-dependent response. The mechanisms of proline to reduce heavy metal stress in plants may be either osmo- and a redox-regulation, metal chelation or scavenging of free radicals (**PERVEEN *et al.,* 2012**). Free-proline accumulation in plant tissue is due to a decrease in the degradation of free-proline resulting in increasing accumulation of free-proline and a decrease in the utilization of free-proline (**HARE AND CRESS, 1997; ASHRAF AND FOOLAD, 2007**). Cd toxicity has been reported to inhibit various metabolic activities in plants which may result in cellular damage through lipid peroxidation of polyunsaturated fatty acid of the lipid membrane (**KUMAR *et al.,* 2013**). Aluminum is a non-transition metal, hence it cannot catalyze the peroxidation reaction (**DEVI *et al.,* 2003**). However, the rigidification of membranes by Al seems to facilitate the Fe-catalyzed peroxidation of lipids (**YAMAMOTO *et al.,* 1997**), as was observed in tobacco cultured cells (**IKEGAWA *et al.,* 1998**). In *B. natalensis,* analysis of MDA to elucidate the extent of lipid peroxidation revealed a progressive increase in the accumulation of MDA with increasing heavy metal treatment. A moderate concentration of Cd induced relatively low MDA accumulation and this suggests less oxidative stress. The low levels of oxidative damage recorded at 500 mg L^{-1} Al explained the significant positive effects in growth parameters recorded at this concentration in *B. natalensis.*

Metabolic response of *B. natalensis* to heavy metal stress showed a variation in the accumulation of carbohydrate in all the heavy metal treatment. The highest amounts of carbohydrate recorded in leaf samples was at Cd 10 mg L^{-1}. Leaves are the metabolically active part in plants and require energy in order to carry out the basic metabolic activity (**KAPLAN AND GUY, 2004**). Under stress conditions, plants tend to accumulate elevated levels of sugar in leaves compared to the root due to depletion of starch by amylase (**KAPLAN AND GUY, 2004; BASU *et al.,* 2007**). Contrary to previous studies, the highest amounts of carbohydrate was recorded in the root samples of plants treated with Al 500 mg L^{-1} compared to all other treatments. When plants are exposed to abiotic stress conditions the demand for energy may increase owing to initiation of response mechanisms and secondary metabolism (**STOBRAWA AND LORENC-PLUCIŃSKA, 2007**). This is a fundamental counter-acting strategy to

curb abiotic stress by maintaining primary metabolic pathways and the carbohydrate balance.

3.4 Conclusions

Exposure to Cd and Al caused a slower growth rate and lower biomass in *D. elata* and *B. natalensis* in the present study. High levels of Cd and Al treatments induced progressive increase in the accumulation of free-proline but significant decrease in the levels of total chlorophyll. Accumulation of Cd and Al, especially in the bulbs of these plant species, to concentrations above the WHO safety guidelines, exposes the consumer to the risk of heavy metal toxicity. The physiological and biochemical responses of *B. natalensis* to heavy metal stress indicates that high levels of heavy metal treatments perturbed the basic metabolic process and this could lead to inability of the plant to synthesis important bioactive compounds. There is need for in-depth studies of metabolic response of medicinal plant to heavy metal stress in order to ensure quality and efficacy of medicinal plant products. Cultivation of highly valued medicinal plants on heavy metal free-soil should be encouraged to safeguard the health of consumers.

Chapter 4

Effect of heavy metals on physiochemical and ultrastructural changes in *Hypoxis hemerocallidea*

4.1 Introduction

The genus *Hypoxis* in the family Hypoxidaceae, is the most frequently used genus in southern Africa for traditional medicine with *Hypoxis hemerocallidea* the most utilized species (**NCUBE *et al.*, 2013**). Hailed as 'the miracle *muthi'* and 'wonder potato', *H. hemerocallidea* is used to treat various human and veterinary diseases including HIV/AIDS related conditions (**DREWES AND KHAN, 2004**). There is increasing demand for *H. hemerocallidea* due to its purported efficacy. The demand for this plant species has intensified following the isolation and elucidation of a phytosterol diglucoside, namely hypoxoside, with promising antitumor properties (**DREWES *et al.*, 1984; ALBRECHT *et al*., 1995**). This has led to over exploitation of the species in the wild.

Plant species grown on naturally heavy metal-enriched soils have evolved metal resistance mechanisms (**ERNST AND NELISSEN, 2000; MARTÍNEZ-IÑIGO *et al.*, 2009**). The response of plants to heavy metal toxicity is species dependent. Heavy metals elicit a number of toxicities on plants and these include physiochemical and ultrastructural changes leading to an inability of plants to carry out basic metabolic processes. Optimal photochemical potential is the most important strategy for plant survival under any environmental stress condition. For many years, chlorophyll fluorescence has been used to probe the fate of excitation energy within the photosynthetic apparatus and to provide insights into the mechanisms and regulation of photosynthesis *in vivo* (**EDWARDS AND BAKER, 1993**). In photosystem II, the absorbed light from chlorophyll molecules is re-emitted as chlorophyll fluorescence and its measurement provides information on the efficiency of PSII (**EDWARDS AND BAKER, 1993**). Heavy metal toxicity perturbs the chlorophyll fluorescence ability of many plant species by inhibiting efficient electron transfer to PSII which leads to oxidative stress and physiological defects.

The aim of this study was to investigate the tolerance mechanisms of *H. hemerocallidea* to various concentrations of Cd and Al by screening for a number of physiological and ultrastructural changes as well as investigating the chlorophyll fluorescence.

4.2 Materials and Methods

4.2.1 Micropropagation of *H. hemerocallidea*

Hypoxis hemerocallidea corms were harvested from the University of KwaZulu-Natal Botanical Garden and washed thoroughly under running tap water. The corms were cut horizontally into slices approximately 2 cm thick after which the outer epidermis of each slice was removed. The slices were then washed in running tap water for 3 h to remove excess phenolic exudates. Following the procedure described by **PAGE AND VAN STADEN** (**1984**) and **APPLETON *et al.* (2012**), the slices were decontaminated with absolute ethanol (5 min), followed by 1% (w/v) Benlate® (15 min) and 0.1% (w/v) mercuric chloride (20 min). The sliced corms were rinsed three times in sterile distilled water, cut into approximately 1 cm^3 cubes (from the cambial region) and placed onto Murashige and Skoog (MS) medium (**MURASHIGE AND SKOOG, 1962**) supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and 4.44 M 6-benzylaminopurine (BA) for culture initiation. The pH of the medium was adjusted to 5.8 with NaOH or HCl before solidification with 8 g L^{-1} agar. Aseptic cultures were maintained at 25±2˚C, in a 16:8 h light:dark photoperiod with a photosynthetic photon flux (PPF) of 40–50 µmol m^2 s^{-1} provided by cool white fluorescent lamps (Osram L 58W/640, Germany) as measured at the culture jar level. Regenerated shoots were bulked up by sub-culturing three times at 6 week intervals on fresh MS medium of the same composition.

4.2.2. Rooting and acclimatization of *in vitro*-derived plants

Rooting was achieved by culturing adventitious shoots (>20 mm) on half strength MS medium supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} myo-inositol but devoid of plant growth regulators. The pH was adjusted to 5.8 before solidification with 8 g L^{-1} agar. The cultures were incubated in growth conditions as described earlier for a period of 4 weeks. Rooted *in vitro*-derived *H. hemerocallidea* plantlets were washed under running tap water to remove agar, potted in plastic containers (15 cm) with

sterilized acid-washed quartz sand and transferred to the greenhouse under natural photoperiod conditions, constant temperature (25±2˚C) and midday PPF of approximately 1000 µmol m^2 s^{-1}. Plants were kept moist by adding 100 mL 50% HS (**Appendix A**) every two days for 7 months to achieve well established plants.

4.2.2.1 Pot experiment

Healthy plants were selected for heavy metal treatment using the solution as described in Chapter 3 (**Table 3.1)** for six weeks. There were 10 replicates per treatment. Plants were watered with the treatment solutions 100 mL per pot every two days. The experiment was terminated after six weeks and growth parameters such as root and shoot lengths and biomass were recorded.

4.2.3 Elemental analysis

Dried powdered samples (0.5 g DW) from each treatment were digested using a microwave acid-assisted system as described in Section 2.2.2.2.The digested samples were analyzed for elemental composition using ICP-OES as described in Section 2.2.2.4.

4.2.4 Determining free-proline

The amounts of free-proline in *H. hemerocallidea* was determined every week starting from week 3 of the treatment using the method described in Section 3.2.4.2.

4.2.4.1 Determining antioxidant activity

4.2.4.1.1 Superoxide dismutase (SOD)

SOD activity was determined in leaf samples after six weeks of Cd and Al treatment using the nitroblue tetrazolium (NBT) method described by **SADASIVAM AND MANICKAM** (**1996**) with modifications. Fresh leaf samples (1g) were homogenized in 10 mL ice cold potassium-phosphate buffer (pH 7.8) in a pre-chilled mortar. The homogenate was centrifuged at 10 000 *g* for 10 min at 4˚C. The supernatant was saved as the enzyme source and used in the assay. The reaction mixture (3 mL) contained 50 mM potassium-phosphate buffer, pH 7.8, 13 mM methionine, 75 mM NBT, 0.1 mM ethylenediamine tetra acetic acid (EDTA), 2 mM riboflavin and 50 µL enzyme extract. Riboflavin was added last, and the volume was made to 3 mL with distilled water. Test tubes were shaken and placed under fluorescent lamps (48 mmol

photon m^2 s^{-1}). The reaction was initiated by switching-on the lights. The reaction was terminated after 15 min by switching off the lights. The absorbance was measured at 560 nm. One unit of SOD activity was defined as that being contained in the volume of extract that caused 50% inhibition of the SOD-inhibitable fraction of the NBT reduction in the presence of methionine. The enzyme activity was expressed as mg protein. Enzyme activity was calculated using the following formula:

$$\text{SOD activity} = \frac{\text{OD}_\text{b} - \text{OD}_\text{s}}{50\%\ \text{OD}_\text{b}}\ \text{X dilute aliquot of sample}$$

Where: OD_b = optical density of the blank, OD_s = optical density of sample

4.2.4.1.2 Determining the activity of MDA

The amounts of MDA was evaluated using fresh leaf samples after six weeks of treatment using the method described in Section 3.2.4.3.

4.2.5 Determining total carbohydrate content

The amounts of total carbohydrate in leaf, corm and root samples were determined after six weeks of Cd and Al treatment using the method described in Section 3.2.4.4.

4.2.6 Amylase assay

The activity of *α*-and *β*-amylase (starch degrading enzymes) was determined in leaf samples after six weeks of Cd and Al treatment using the method described by **SADASIVAM AND MANICKAM** (**1996**) with slight modifications. Amylase enzymes were extracted from freshly cut leaf samples by homogenizing 1 g of plant material in 10 mL ice-cold 10 mM CaCl solution that was stored overnight at 4 ˚C. The homogenate was centrifuged at 54 000 *g* at 4 4˚C for 20 min. The supernatant was used as the enzyme source for *α*-amylase. Enzyme enriched supernatant 1 mL and 1 mL starch solution (1%) were transferred into a test tube and incubated at 27˚C for 15 min. The reaction was stopped by adding 2 mL dinitrosalicyclic acid reagent. The mixture was heated in boiling water for 5 min and 1 mL potassium sodium tartrate solution was immediately added. The tubes were then cooled in running tap water and the volume was made to 5 mL with distilled water. Absorbance was read at 560 nm.

Both *α*- and *β*-amylase activity was expressed as mg of maltose produced during 5 min incubation.

4.2.7 Determining photosynthetic pigments

The amounts of total chlorophyll in all the treatments were assessed every week starting from week 3 of treatment initiation using the method described in Section 3.2.4.1. Total carotenoids was measured using the method described for total chlorophyll but the OD was measured at 470 nm.

4.2.8 Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in leaf samples after plants were exposed to Cd and Al for six weeks. Chlorophyll fluorescence measurements were carried out to examine the effects of heavy metals on the photosynthetic apparatus in *H. hemerocallidea.* The chlorophyll *a* fluorescence was measured using a FMS 2 modulated fluorometer (Hansatech Instruments, King's Lynn, U.K.). Fully expanded leaves from 10 plants in each treatment were clamped in standard Hansatech leaf-clips. The measurements were performed on non-detached leaves adapted to the dark for 10 min to allow for oxidation of the photosynthetic electron transport system, and the minimum fluorescence (F_o) and maximum (F_m) were recorded. An adaptation time of 10 min was selected because this was the minimum time for fast-relaxing non-photosynthetic quenching (NPQ) to disappear in unstressed plants (control plants). The fluorescence intensity was measured by switching on the actinic light with a saturation light pulse of 3,000 µmol m^2 s^{-1} and maximum fluorescence (F_m') were measured after 5 min. Fluorescence parameters were calculated as described by **SCHREIBER AND BILGER** (**1993**) and **SCHREIBER *et al.*** (**1995**). Briefly, F_v/F_m = $(F_m - F_o)/F_m$; ΦPSII = $(F_m' - F)/ F_m'$; and relative electron transfer rate (rETR) = ΦPSII x 0.42 x PFD. NPQ was estimated as the Stern-Volmer quotient ($(F_m' - F)/ F_m'$), and its components using the method described by **THIELE *et al.*** (**1997**).

4.2.9 Ultrastructural analysis of plant samples

4.2.9.1 Sample preparation

Leaf sample 1 mm^2 was excised from the middle portion of the leaf and root samples approximately 1 mm length were excised from the corm from each treatment after six weeks of Cd and Al treatment. All the samples were immediately fixed in 2.5%

glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.1) for 8 h (**SASS, 1958**). The samples were then prepared for light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis.

4.2.9.2 Light microscopy (LM)

Root samples were fixed in 3% glutaraldehyde buffered in 0.2 M sodium cacodylate buffer for 8 h. Samples were washed twice in 0.5 M sodium cacodylate buffer (pH 7.2) for 30 min. Samples were fixed in 2% osmium tetroxide for 2 h and then dehydrated in an ethanol series (10-100%) at 10 min intervals. Thereafter, samples were embedded in Spurr's epoxy resin and polymerized in an oven at 70 ˚C for 16 h. Ultrathin sections (1 µm) from each sample were obtained using a Leica UC7 RT ultramicrotome with glass knives which were produced on an LKB 7800 knifemaker. Samples were picked up on glass slides which were allowed to dry on a hot plate at 60 ˚C, stained with toluidine blue and imaged using an Olympus AX70 Fluorescent Compound Light Microscope under Brightfield mode (with a Nikon Digital Camera + NIS Elements Imaging Software) at various magnifications.

4.2.9.3 Scanning electron microscopy (SEM)

Leaf samples were fixed as described above and then washed twice in cacodylate buffer (pH 7.2) and dehydrated in an ethanol series. Fixed leaflets were dried in a Quorum K850 Critical Point Drier with liquid CO_2. The lower surfaces of the leaflets were mounted on aluminum stubs with double-sided sticky carbon tape. Material on the stub was coated with a 10 nm conductive film of gold, in an ion sputter coater (Eiko IB-3 Ion Sputter Coater). Thereafter, coated specimens were examined and photographed at different magnifications using a Zeiss EVO LS15 VP SEM 10 kV.

4.2.9.4 Visualizing the distribution of elements on abaxial leaf surface using SEM/EDX

Leaf samples from *H. hemerocallidea* were air-dried and mounted on aluminum stubs with double-sided sticky carbon tape. Material on the stubs were coated with a 20 nm conductive film of carbon, in a Quorum Q150RS Carbon Coater. EDX analysis was performed on the Zeiss EVO LS15 VP SEM with the use of the Oxford X-Max EDX 80 mm SDD (silicon drift detector). This EDX system can detect elements with 0.1% detectability limit and heavy elements with better than 0.1% detectability limit. The

software used for the EDX analysis was INCA v4.14 from Oxford Instruments. The EDX scan time was set for 60 s, and scan depth was approximately 1 µm.

4.2.9.5 Transmission electron microscopy (TEM)

The same set of resin blocks from light microscopy was used for TEM. Ultrathin sections (100 nm) from each sample were obtained using an ultramicrotome (Leica UC7 RT) with a diamond knife. Sections were picked up on a copper grid, air-dried and positively stained with aqueous uranyl acetate for 5 min and rinsed with distilled water. The grids were allowed to air-dry and were subsequently imaged on a JEOL JEM 1400 120kV TEM (**JOHANSEN, 1940**).

4.2.10 Statistical analysis

Statistical analyses were carried out using SPSS for Windows by one-way ANOVA using the Student's *t*-test to test the different significance levels.

4.3 Results

4.3.2 Uptake and distribution of Cd and Al in *Hypoxis hemerocallidea*

Root to shoot translocation of Cd in *H. hemerocallidea* was poor. Low amounts of Cd were recorded in the leaf samples of all the Cd-treated plants compared to the concentrations in the roots and corms (**Table 4.2**). The highest amount of Cd translocated to the shoot were recorded at 5 mg L^{-1} Cd treatment. High amounts of Cd was recorded in the root samples. There was a progressive increase in the uptake and translocation of Al from the root to shoot at the lowest and moderate Al-treated plants but there was a decrease in the translocation of Al to shoot at the highest treatment (**Table 4.2**). The highest amount of Al was recorded in the combined treatments Cd (5:Al 1000 mg L^{-1}) indicating synergistic effects on the uptake of Al (**Table 4.2**).

Table 4.1 Interactive effects in the uptake of Cd and Al in *H. hemerocallidea* after six weeks of exposure. Results are presented as mean±SD (n=3).

Treatment (mg L^{-1})	Cd concentration (mg kg^{-1})			Al concentration (mg kg^{-1})		
	Shoot	Corm	Root	Shoot	Corm	Root
Control	ND	ND	ND	ND	ND	ND
Cd 2	22±2.20d	35±90c	152±10b	ND	ND	ND
Cd 5	34±1.90a	47±11a	195±35a	ND	ND	ND
Cd 10	18±4.50d	25±3.10d	88±5.10d	ND	ND	ND
Al 500	ND	ND	ND	898±53c	559±48d	429±13e
Al 1000	ND	ND	ND	1584±34a	932±10b	1814±47a
Al 1500	ND	ND	ND	809±13d	444±26e	1182±83c
Cd 2:Al 500	26±8.20c	34±7.20c	78±4.80d	981±65b	675±39c	1026±48d
Cd 5:Al 1000	32±9.50b	40±38b	107±23c	1608±69a	908±46b	1123±39c
Cd 10:Al 1500	26±3.70c	48±33a	110±27c	723±53e	1010±3a	1410±64b

ND = Not detected

4.3.1 Physiochemical screening

4.3.1.1 Effect of heavy metals on growth parameters

Growth parameters such as biomass, root and shoot length are used as indicators of heavy metal toxicity in plants (**DONG *et al*., 2005**). The results of the present study showed a significant reduction in the shoot and root length, fresh and dried biomass with increasing Cd concentrations (**Table 4.1**). The highest concentration of Cd treatment decreased the root length and biomass significantly compared to the control plants (**Figure 4.1**). The lowest treatment with Al (500 mg L^{-1}) had a significant positive effect on root length and biomass but the highest treatment (1500 mg L^{-1} Al) significantly reduced biomass as well as shoot and root length (**Table 4.1 and Figure 4.1**) compared to the control plants. The results of the combined treatments were significantly lower compared to Al-treated plants but similar to the Cd-treated plants. Visible symptoms of metal toxicity were observed at the highest concentrations of the combined treatment which included stunted growth, chlorosis and cell death (**Figure 4.1J**).

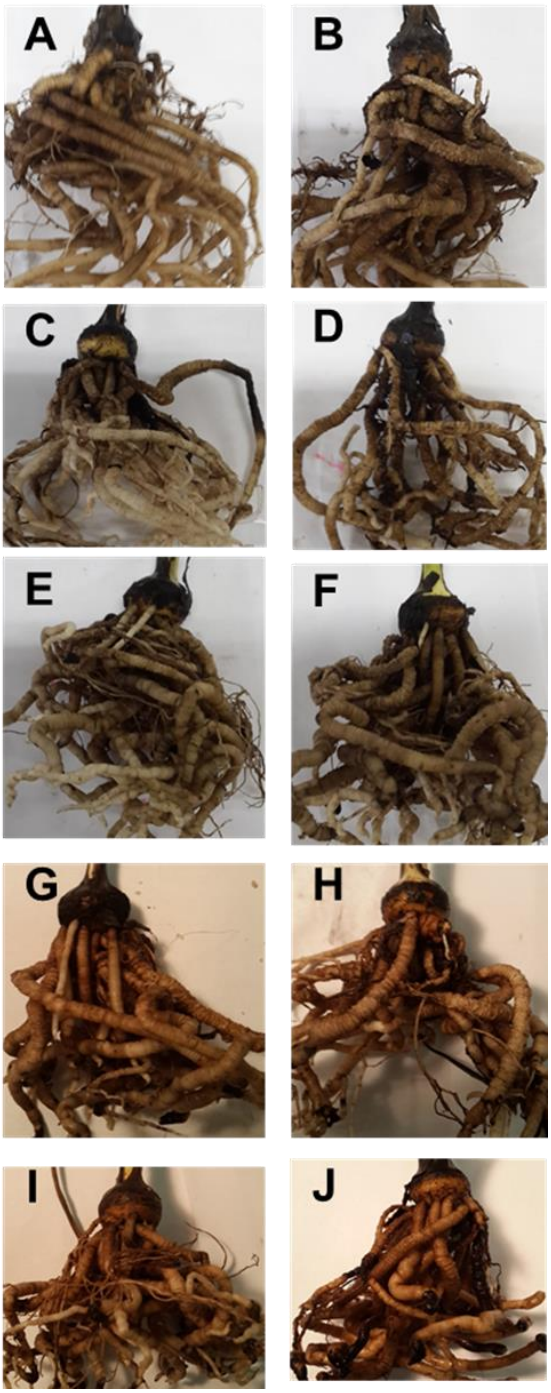


Figure 4.1 Effect of Cd and Al on root morphology of *H. hemerocallidea* after exposure for six weeks. A = Control; B = Cd 2 mg L^{-1}; C = Cd 5 mg L^{-1}; D = Cd 10 mg L^{-1}; E = Al 500 mg L^{-1}; F = Al 1000 mg L^{-1}; G = Al 1500 mg L^{-1}; H = Cd 2:Al 500 mg L^{-1}; I = Cd 5:Al 1000 mg L^{-1}; J = Cd 10:Al 1500 mg L^{-1}.

Table 4.2 Effect of Cd and Al on shoot and root length and biomass after six weeks of treatment in *H. hemerocallidea*. Results are presented as mean±SD (n=10).

Treatment (mg L^{-1})	SL (cm)	RL (cm)	RFW (g)	CFW (g)	SFW (g)	RDW (g)	CDW (g)	SDW (g)
Control	29.40a	16.70b	59.19±1.46b	38.15±0.70b	23.57±0.82c	30.8±3.98b	24.5±4.51b	16.61±1.38c
Cd 2	23±1.70b	12±2.40bc	48±2.39b	34±4.68b	22±3.38d	26±6.06c	17±4.83c	9±1.35d
Cd 5	18±3.20bc	9±1.90c	35±4.51c	23±0.63cd	14±2.62d	19±1.03d	13±0.32d	7±2.10de
Cd 10	13±0.80d	7±2.30d	22±3.26d	13±0.7ab	10±0.26e	7±2.98e	6±1.37e	3±1.03e
Al 500	32±4.70a	21±0.50a	72±4.43a	54±3.27a	49±0.17a	49±3.18a	32±2.15a	30±3.33a
Al 1000	27±2.10ab	17±1.50b	66±5.24ab	47±5.98ab	34±1.05b	35±3.91ab	28±5.10ab	19±4.83b
Al 1500	24±2.90b	15±2.10bc	34±6.84cd	26±3.40c	22±5.02c	21±2.13cd	15±1.09d	10±3.81d
Cd 2:Al500	23±1.80bc	16±1.30bc	27±1.74cd	18±0.71d	13±1.27d	17±3.38d	13±2.82d	7±1.04de
Cd 5:Al1000	17±0.90c	13±1.50c	22±1.84d	18±1.04d	14±3.89d	14±1.33d	12±0.92d	6±1.63de
Cd 10:Al1500	15±1.80d	10±2.20c	15±1.56e	11±0.66e	10±0.52e	9±0.84e	6±1.25e	41±0.48e

L = Shoot length, RL = Root length, RFW = Root fresh weight, CFW = Corm fresh weight, SFW = Shoot fresh weight, RDW = Root dry weight, CDW = Corm dry weight, SDW = Shoot dry weight.

4.3.3 Effect of Cd and Al on free-proline *Hypoxis hemerocallidea*

The levels of free-proline measured at weekly intervals from week 4 to week 6 increased in a stress-dependent pattern in all heavy metal treatments (**Figure 4.2**). At week 4, the levels of free-proline in all the treatments were not statistically different from the controls. The stress pattern in both low and moderate concentrations of Cd treatments were similar from week 4 to week 6. The highest amounts of proline were recorded at 10 mg L^{-1} Cd treatment at week 6 followed by 1500 mg L^{-1} Al and Cd 10:Al 1500 mg L^{-1}. In comparison with the control, the amounts of proline in most of the heavy metal treated plants increased significantly from week 4 to week 6.

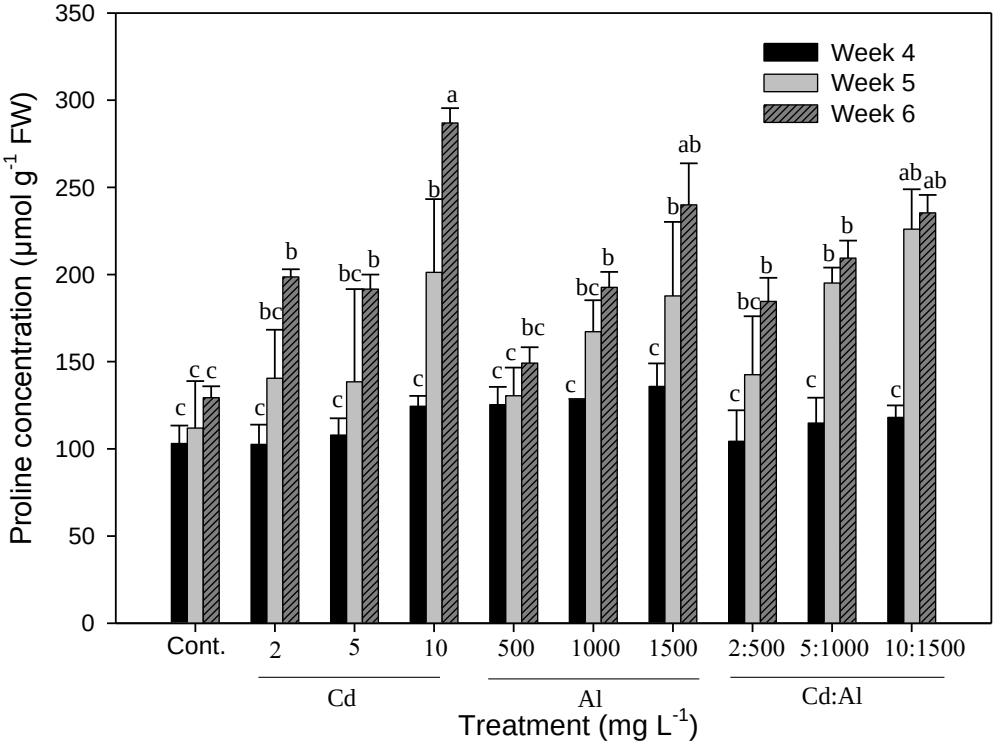


Figure 4.2 Changes in the levels of free-proline (µmol g^{-1} FW) in *H. hemerocallidea* treated with various Cd and Al concentrations for six weeks. Results are presented as mean±SD (n=3). Different letters on each bar indicates significant differences (p<0.05).

In the present study, at Al 500 mg L^{-1} treatment the increment in proline accumulation over the three weeks of sampling were not significantly different. The highest levels of Cd and Al applied alone, triggered high proline biosynthesis particularly at week 6.

Accumulation of proline at weeks 5 and 6 in the moderate and highest concentrations of the combined Cd and Al treatments were not significantly different.

4.3.4 Effect of Cd and Al on antioxidant enzymes *Hypoxis hemerocallidea*

4.3.4.1 SOD activity

In the present study, plants exposed to increasing concentrations of Cd and Al treatments showed a significant increase in the SOD activity (**Figure 4.3**). The highest SOD activity was recorded at Al 1000 mg L^{-1}. SOD activity in the moderate and highest Cd-treated plants were similar, indicating that both treatments had similar effects on SOD. Relatively low concentrations of Cd and Al (2 and 500 mg L^{-1} respectively) had less effect on the accumulation of SOD.

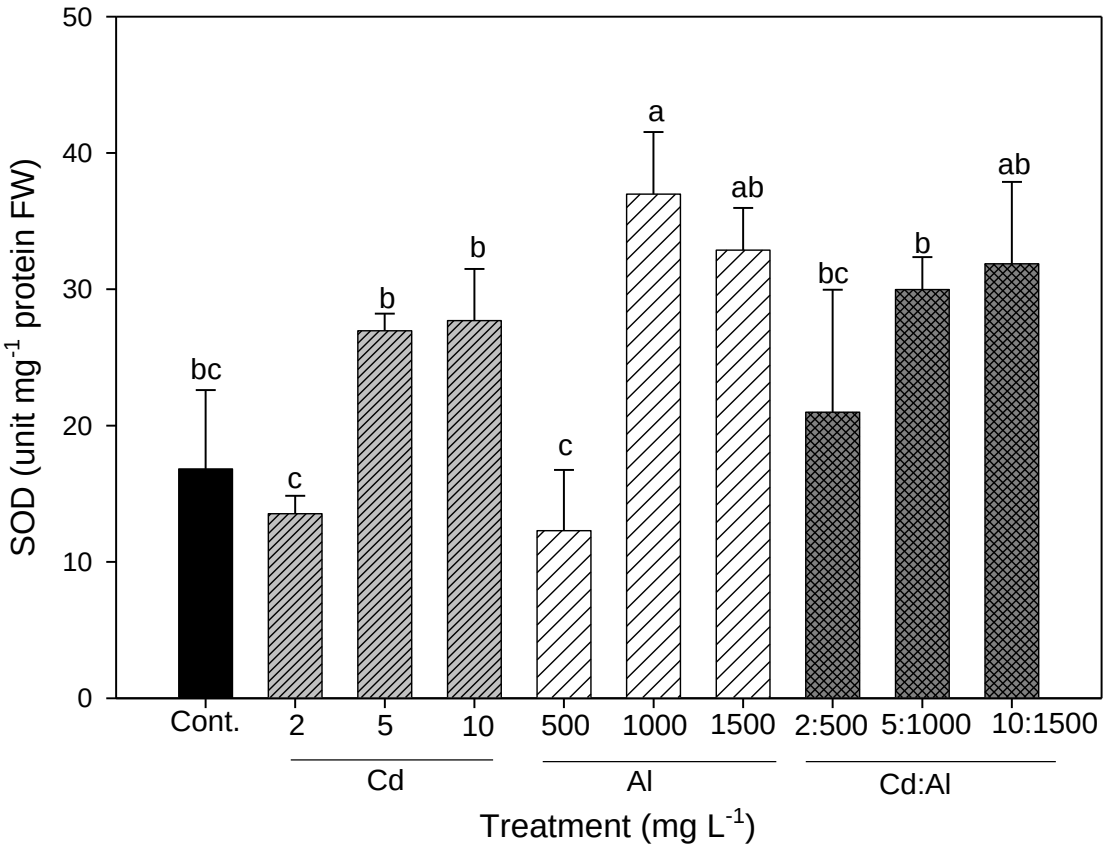


Figure 4.3 Activity of SOD in *H. hemerocallidea* exposed to different concentrations of Cd and Al after six weeks. Results are presented as mean±SD (n=3). Different letters on each bar indicates significant differences (p<0.05).

4.3.4.2 MDA accumulation *Hypoxis hemerocallidea*

There was a dose-dependent response in the accumulation of MDA in all the treatments (**Figure 4.4**). The concentrations of MDA in the present study significantly increased, particularly in the combined treatment of Cd 10:Al 1500 mg L^{-1} compared to the rest of the treatments (**Figure 4.4**). The amount of MDA at Al 500 mg L^{-1} was slightly lower than all the other heavy metal-treated plants.

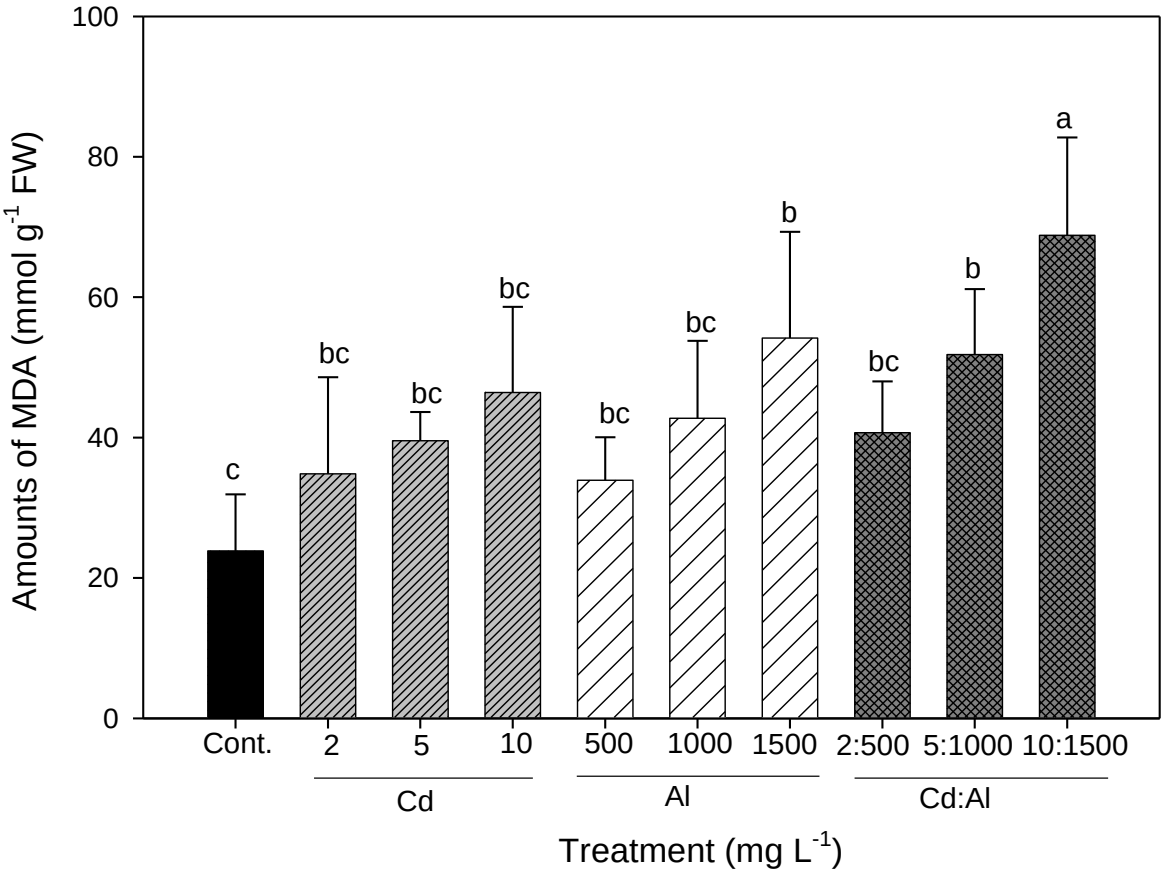


Figure 4.4 Accumulation of MDA in *H. hemerocallidea* exposed to Cd and Al after six weeks. Results are presented as mean±SD (n=3). Different letters on each bars indicates significant differences (p<0.05).

4.3.5 Effect of Cd and Al on total carbohydrate content *Hypoxis hemerocallidea*

The amounts of carbohydrate in the corm of the control plants was slightly higher than all the heavy metal-treated plants. There was a marked decrease in the amounts of carbohydrate in the leaf samples at moderate and high concentrations of Cd-treated plants compared to the control plants (**Figure 4.5**). The pattern of carbohydrate

accumulation in the corms decreased in a dose-dependent pattern with increasing concentrations of heavy metals. The lowest concentrations of the combined Cd and Al treatments increased the accumulation of sugar in the leaf samples compared to all heavy metal-treated plants.

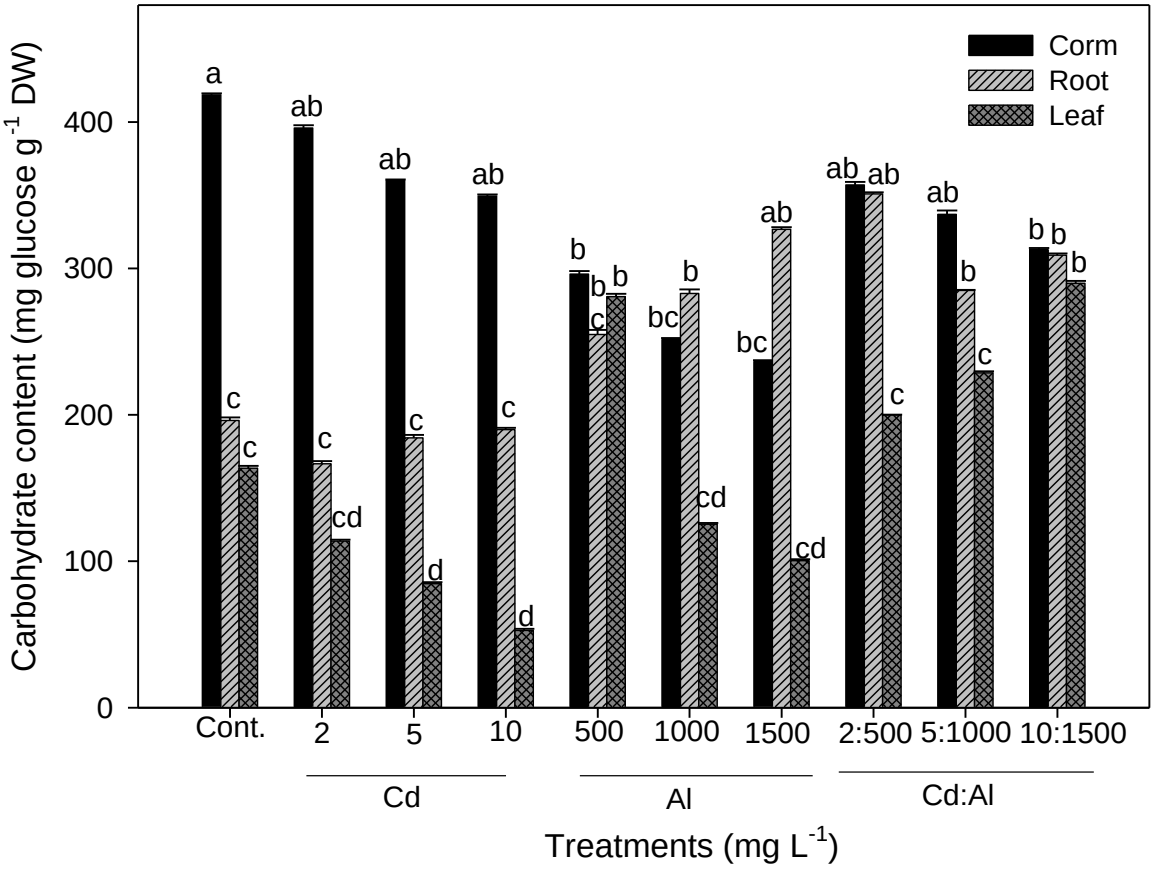


Figure 4.5 Accumulation of total carbohydrate content in *H. hemerocallidea* exposed to Cd and Al stress for six weeks. Results are presented as mean±SD (n=3). Different letters on each bar indicates significant differences (p<0.05).

4.3.6 Amylase activity in *Hypoxis hemerocallidea*

The activity of hydrolytic enzymes (*α-, β*-amylases) were significantly higher in the controls and at low concentrations of Cd compared to all other treatments (**Figure 4.6**). Increasing concentrations of Cd and Al treatment significantly reduced the activity of the hydrolytic enzymes in a dose-dependent manner. The lowest amounts of amylases were recorded at the Cd 10:Al 1500 mg L^{-1} treatment.

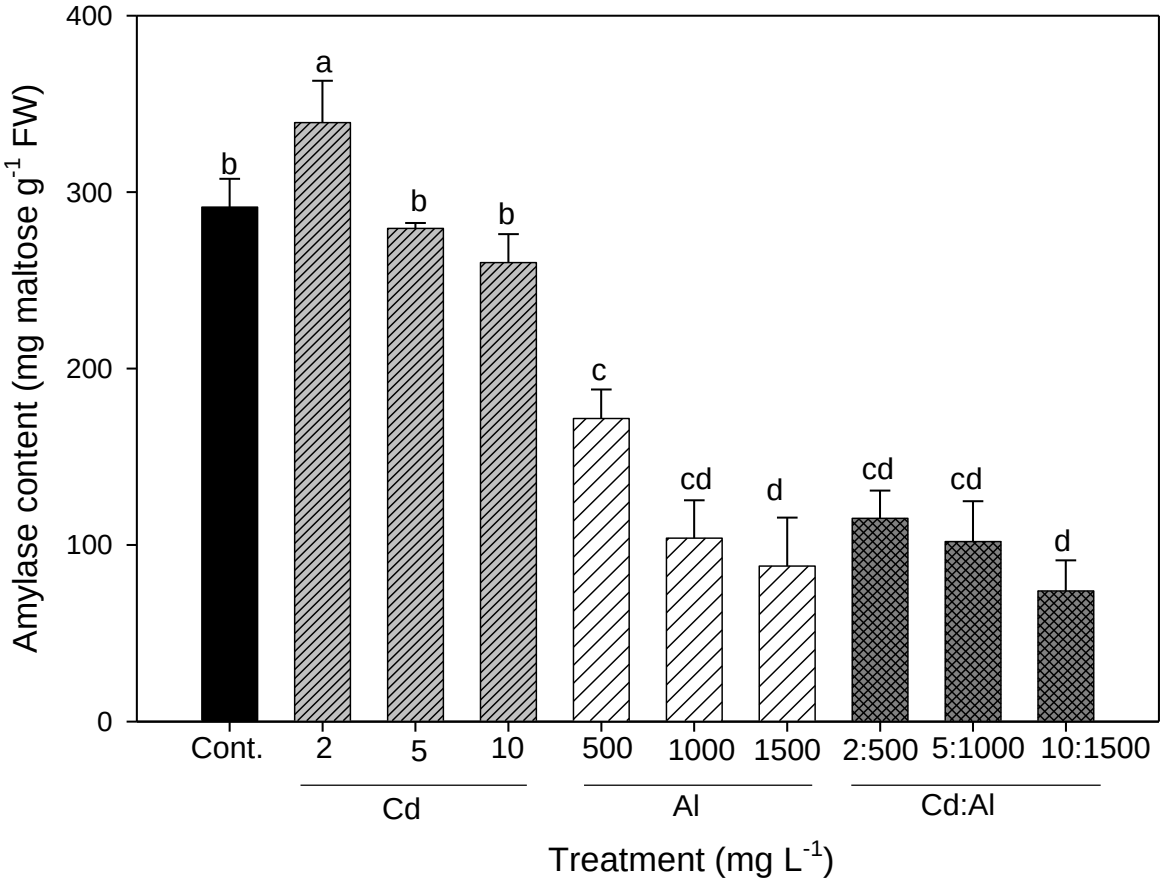


Figure 4.6 Effect of Cd and Al exposure on the activity of *α*- and *β*-amylase in fresh leaf samples of *H. hemerocallidea* after six weeks. Results are presented as mean±SD (n=3). Different letters on each bar indicate significant differences (p<0.05).

4.3.7 Effect of Cd and Al on photosynthetic pigments *Hypoxis hemerocallidea*

In comparison with the control, all the heavy metal-treated plants exhibited lower amounts of total chlorophyll (**Figure 4.7**). The decrease in the amounts of total chlorophyll in Cd-treated plants were in a stress-dependent pattern and the lowest amounts of chlorophyll recorded in the present study was at 10 mg L^{-1} Cd at week 6. The levels of chlorophyll in the Al-treated plants as well as the combined treatments were not in the stress-dependent pattern. For instance, at 500 mg L^{-1} Al there was a decrease in the amounts of total chlorophyll at week 5 but this increased by week 6. At 1000 mg L^{-1} Al there was a progressive decrease in the amounts of total chlorophyll from week 4 to week 6. In the combined Cd and Al treatments, there was a decrease in the amounts of total chlorophyll at week 5 both at the low and moderate

concentrations but at the highest concentrations of treatment there was a decrease in a dose-dependent pattern from week 4 to week 6.

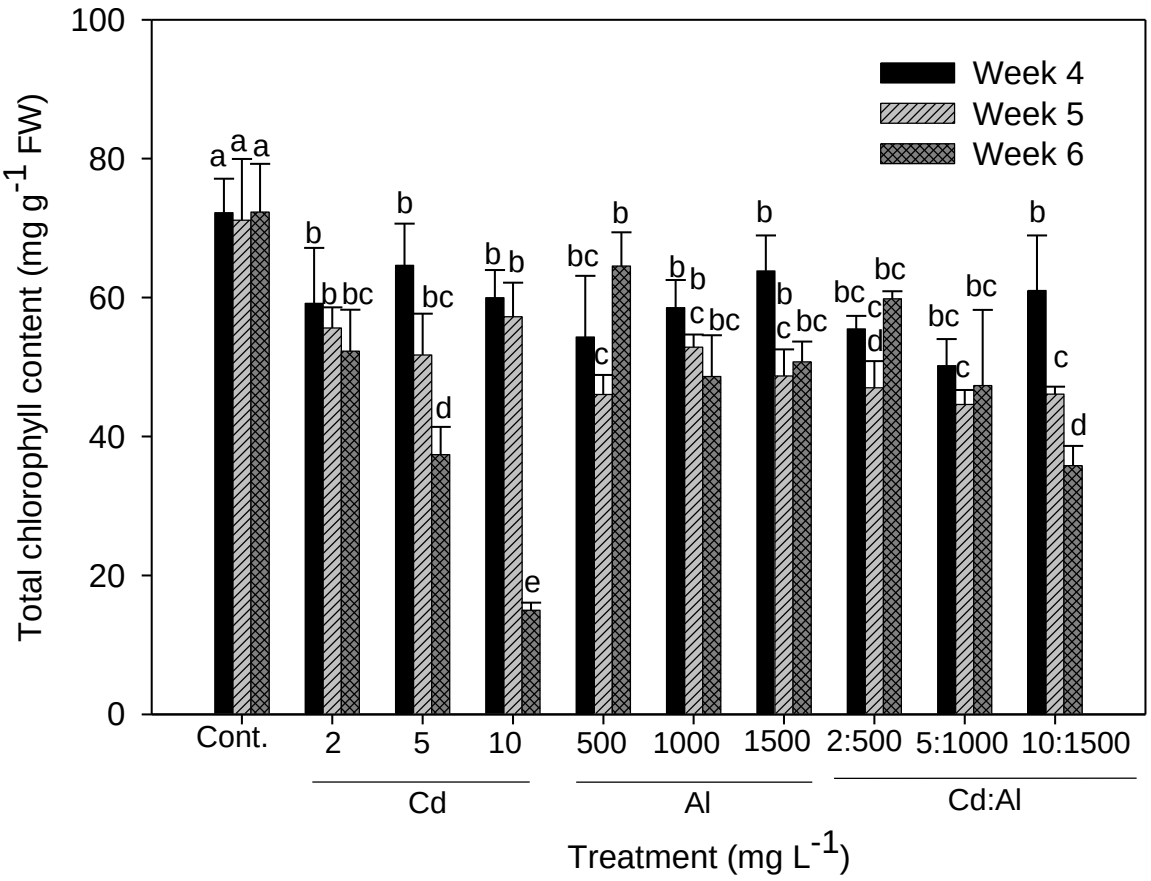


Figure 4.7 Effect of Cd and Al exposure on total chlorophyll content (mg g^{-1} FW) in leaf samples of *H. hemerocallidea* examined on a weekly basis from week 4 to week 6. Results are presented as mean±SD (n=3). Different letters on each bar indicate significant differences (p<0.05).

The levels of total carotenoids measured increased from week 4 to week 6 in all the treatments in a stress-dependent pattern (**Figure 4.8**). The lowest amounts of carotenoids was recorded at low concentrations of Cd and Al at week 4. There was a progressive increase at week 5 to week 6 in all the heavy metal-treated plants in a dose-dependent pattern. In Al-treated plants at 500 mg L^{-1} the increase in carotenoids from week 4 to week 6 were slightly lower than in the control plants. The highest amounts of total carotenoids were recorded in the combined treatment Cd 10:Al 1500 mg L^{-1} at week 6 followed by the single treatment of at Al 1500 mg L^{-1}.

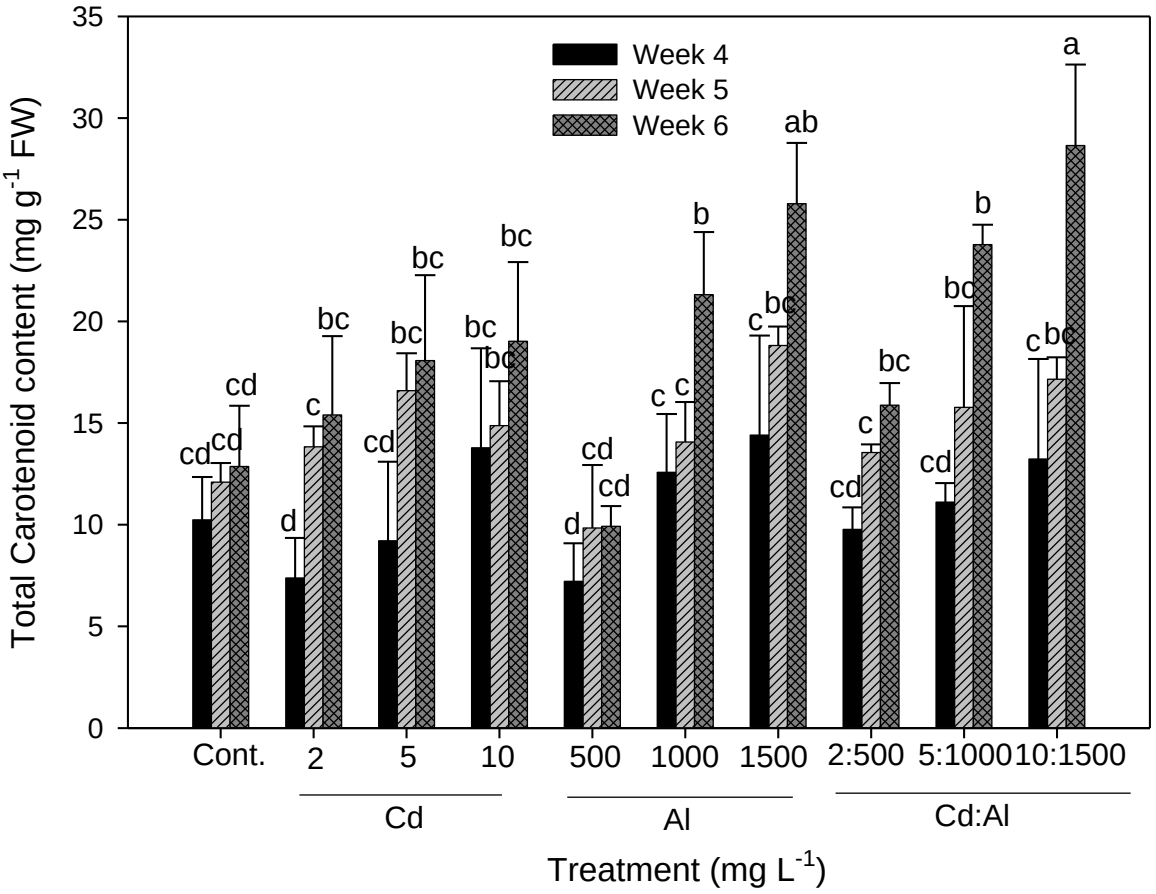


Figure 4.8 Effect of Cd and Al exposure on total carotenoids (mg g^{-1} FW) in fresh leaf samples in *H. hemerocallidea* examined on a weekly basis from week 4 to week 6. Results are presented as mean±SD (n=3). Different letters on each bar indicates significant differences (p<0.05).

4.3.8 Effect of Cd and Al on chlorophyll fluorescence *Hypoxis hemerocallidea*

There was a progressive decrease in dose-dependent response in F_v/F_m in Cd-treated plants (**Figure 4.9**). Application of Al at lowest concentrations slight increase F_v/F_m in *H. hemerocallidea* compared to the control (**Figure 4.9**). The combined Cd and Al treatments at moderate and high concentrations significantly reduced the F_v/F_m compared to the controls. A similar pattern in the activity of F_v/F_o (**Figure 4.10**) were also recorded in all the treatments.

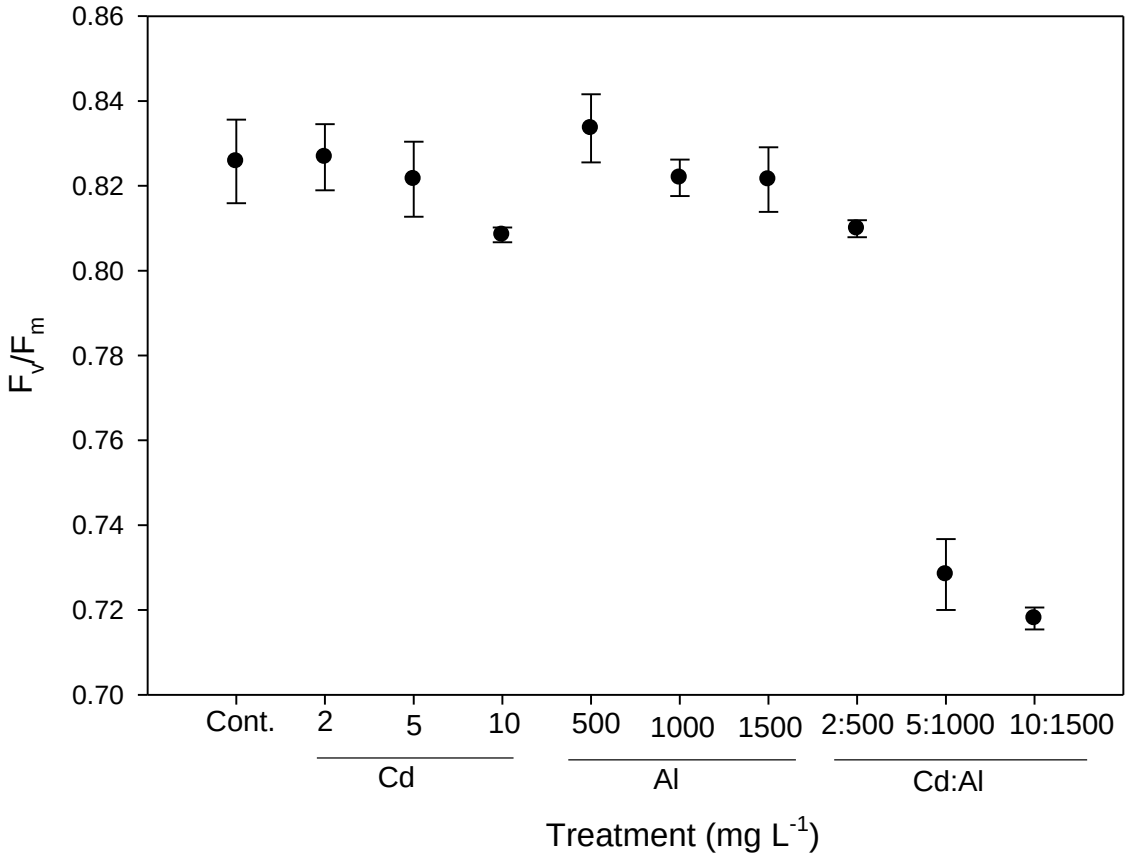


Figure 4.9 Effect of Cd and Al on the ratio of variable fluorescence and maximum fluorescence (F_v/F_m) in *H. hemerocallidea* after six weeks. Results are presented at mean±SD (n=10).

There was a significant increase in NPQ in all the Cd-treated plants compared to the control (**Figure 4.11A**). Application of Al at low concentrations exhibited a lower NPQ activity compared to the controls but increasing Al concentrations significantly increased NPQ activity (**Figure 4.11B**). The combined Cd and Al treatments significantly increased the activity of NPQ at the highest concentrations of treatment compared to the controls (**Figure 4.11C**).

In the present study, increasing concentrations of Cd and Al significantly decreased the rETR (**Figure 4.12A and B**). The efficiency of light captured is represented as α which is proportional to the initial slop of line at low PAR values created by relating ETR to PAR (**RALP AND GADEMAAN, 2005**).

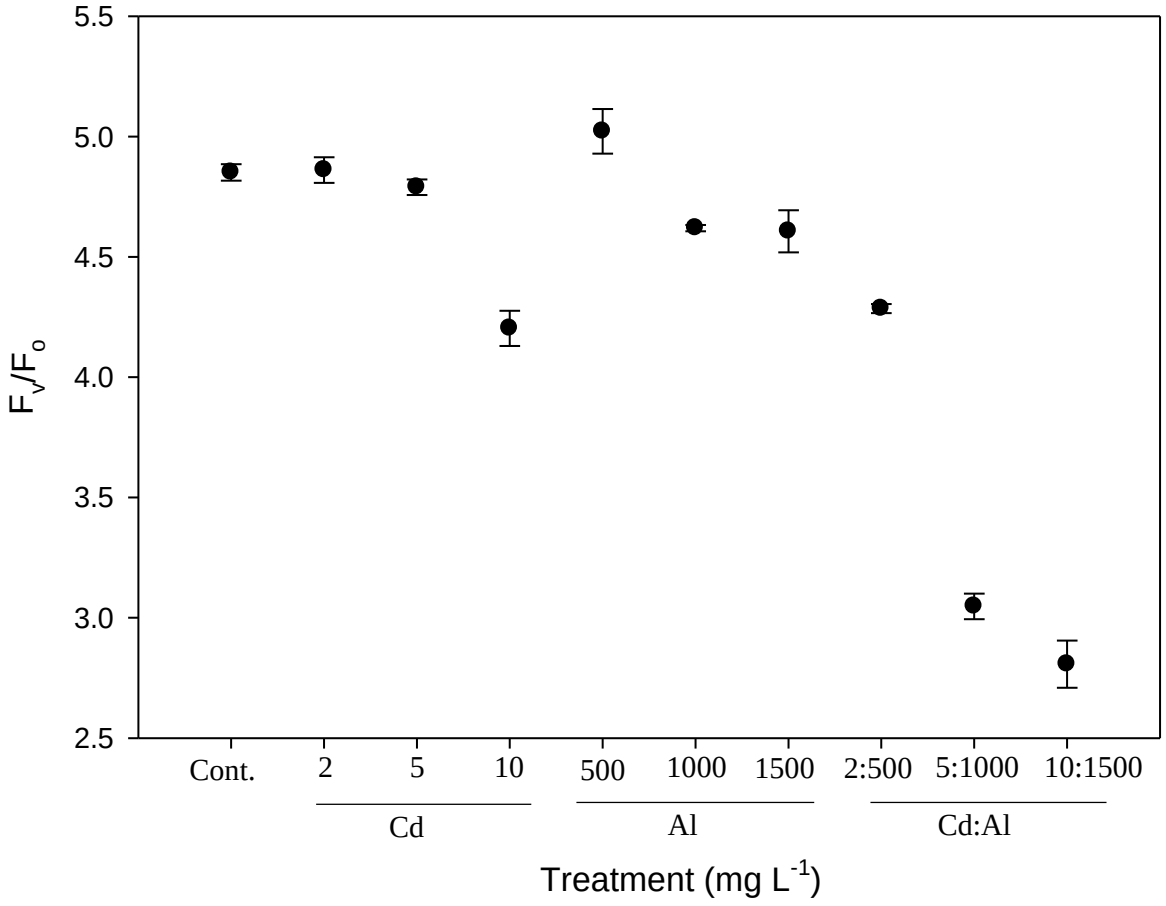


Figure 4.10 Effect of Cd and Al treatments on variable fluorescence and initial fluorescence (F_v/F_o) in *H. hemerocallidea* after six weeks. Results are presented as mean±SD (n=10).

The lowest concentrations of Al had positive effects on rETR similar to the control plants (**Figure 4.12B**). All the concentrations in the combined Cd and Al treatments significantly reduced the rETR when compared to the controls (**Figure 4. 12C)**

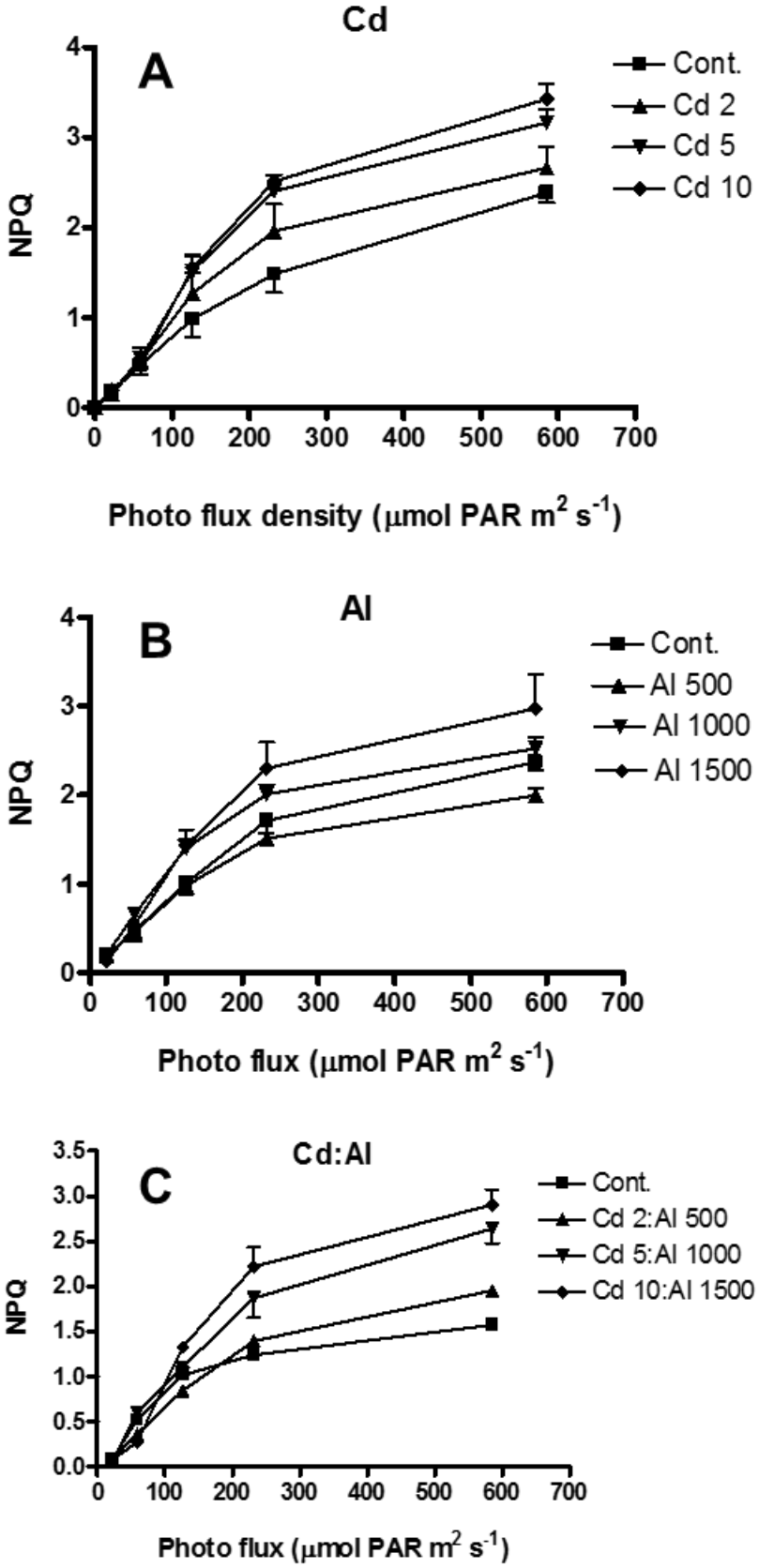


Figure 4.11 Effect of Cd and Al on photosystem II and non-photosynthetic quenching in *H. hemerocallidea* after six weeks. Results are presented as mean±SD (n=10).

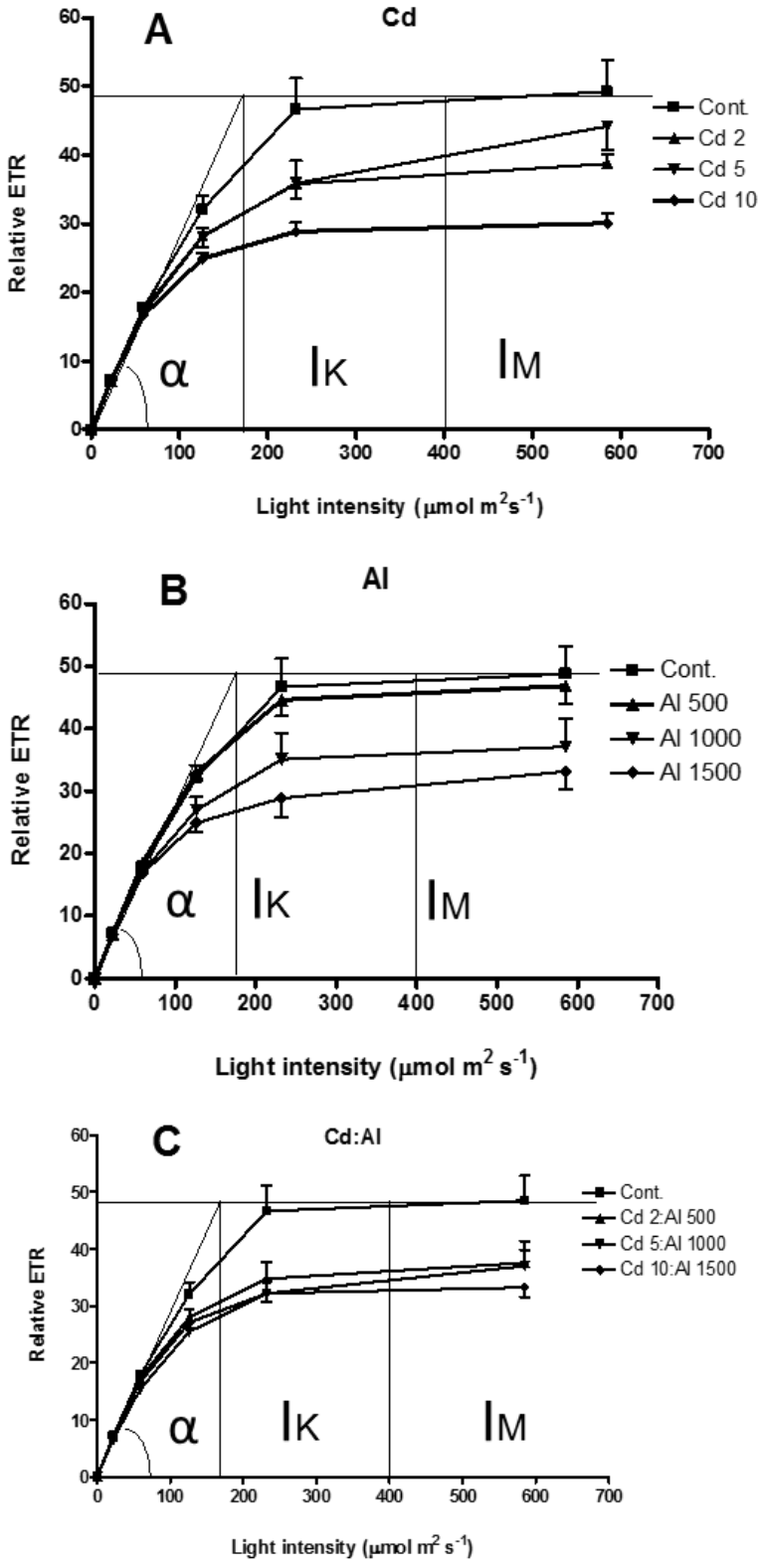


Figure 4.12 Effect of Cd and Al on relative electron transfer rates (rETRs) in *H. hemerocallidea.* Results are presented as mean±SD (n=10). α = efficiency of light capture, IK = minimum light saturation level and IM = light irradiation intensity.

4.3.9 Effects of Cd and Al on ultrastructure *Hypoxis hemerocallidea*

4.3.9.1 Anatomical analysis of root cells using light microscopy

Light microscopic analysis presented in this study gives an indication of anatomical changes in the root structures of *H. hemerocallidea* upon exposure to Cd and Al, some of the changes include an increase in the size of the xylem, phloem, cortex and epidermis (**Figure 4.13**).

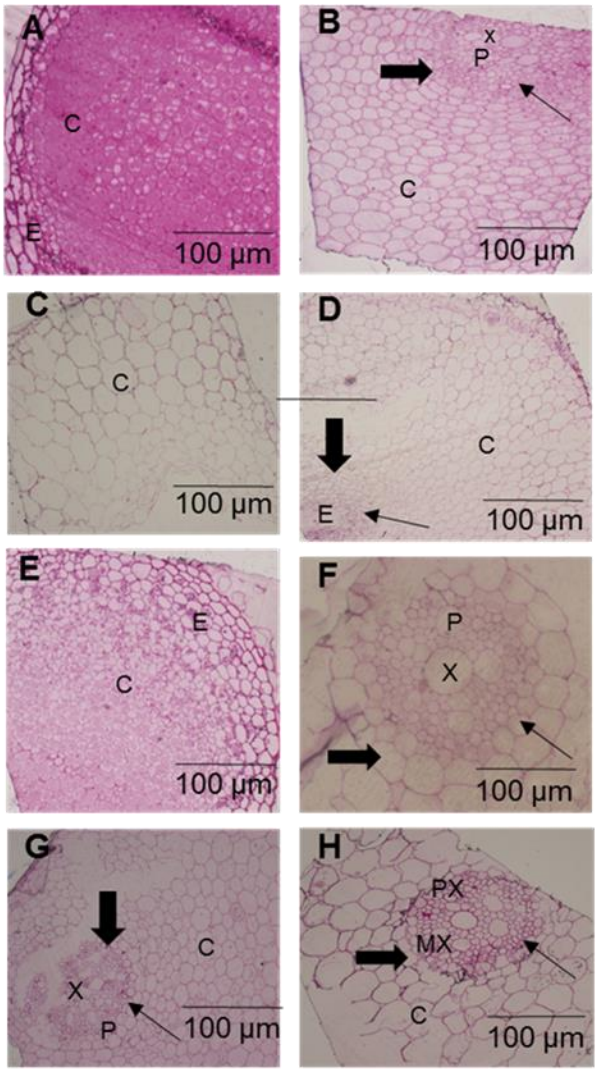


Figure 4.13 Light microscopy analysis of anatomical changes in root structures of *H. hemerocallidea* exposed to Cd and Al after six weeks. (A) = Control; (B) = Cd 2 mg L^{-1}; (C) = Cd 5 mg L^{-1}; (D) = Cd 10 mg L^{-1}; (E) = Al 500 mg L^{-1}; (F) = Al 1000 mg L^{-1}; (G) = Al 1500 mg L^{-1}; (H) = Cd 2:Al 500 mg L^{-1}. E = Epidermis, C = Cortex parenchymatic cell, X = Xylem, PH = Phloem, PX = Proxylem, MX = Metaxylem, small arrrow pointing at endodermis, big arrow pointing at pericycle.

The diameter of the root, and the size of xylem and phloem increased with increasing Cd and Al concentrations. The epidermis consisted of small cells in the control plants but in the Cd- and Al-treated plants, the cells were larger and disordered. Application of Al at moderate and highest concentrations induced anatomical changes such as conspicuous xylem and phloem strands, increasing cortical parenchyma layers of approximately 20 cells with increasing pit size compared to controls (**Figure 4. 13F and G**). A similar pattern in anatomical changes was also observed at the lowest concentrations of the combined treatment of Cd 2:Al 500 mg L^{-1} (**Figure 4. 13H**).

4.3.9.2 Scanning electron microscopic analysis

Scanning electron micrographs presented in **Figure 4.14** are representative of approximately 10 micrographs per treatment. The micrographs presented showed that most of the stomata from the control and all the heavy metal-treated plants were open at the time of sampling (during the light period). There was a greater proportion of open stomata with increasing concentrations of Cd compared to the control plants (micrographs not shown). The stomata of Al-treated plants at 500 mg L^{-1} Al were slightly open at the time of sampling compared to the other treatments. There was a significant decrease in the density of guard cells in all the heavy metal-treated plants compared to the controls. The number of trichome increased with increasing heavy metal concentrations and more trichomes were found on the abaxial leaf surface rather than at the margin. The highest density of trichomes were recorded on the abaxial leaf surface at 5 mg L^{-1} Cd and 1500 mg L^{-1} Al treatments (**Figure 4.14C and G**).

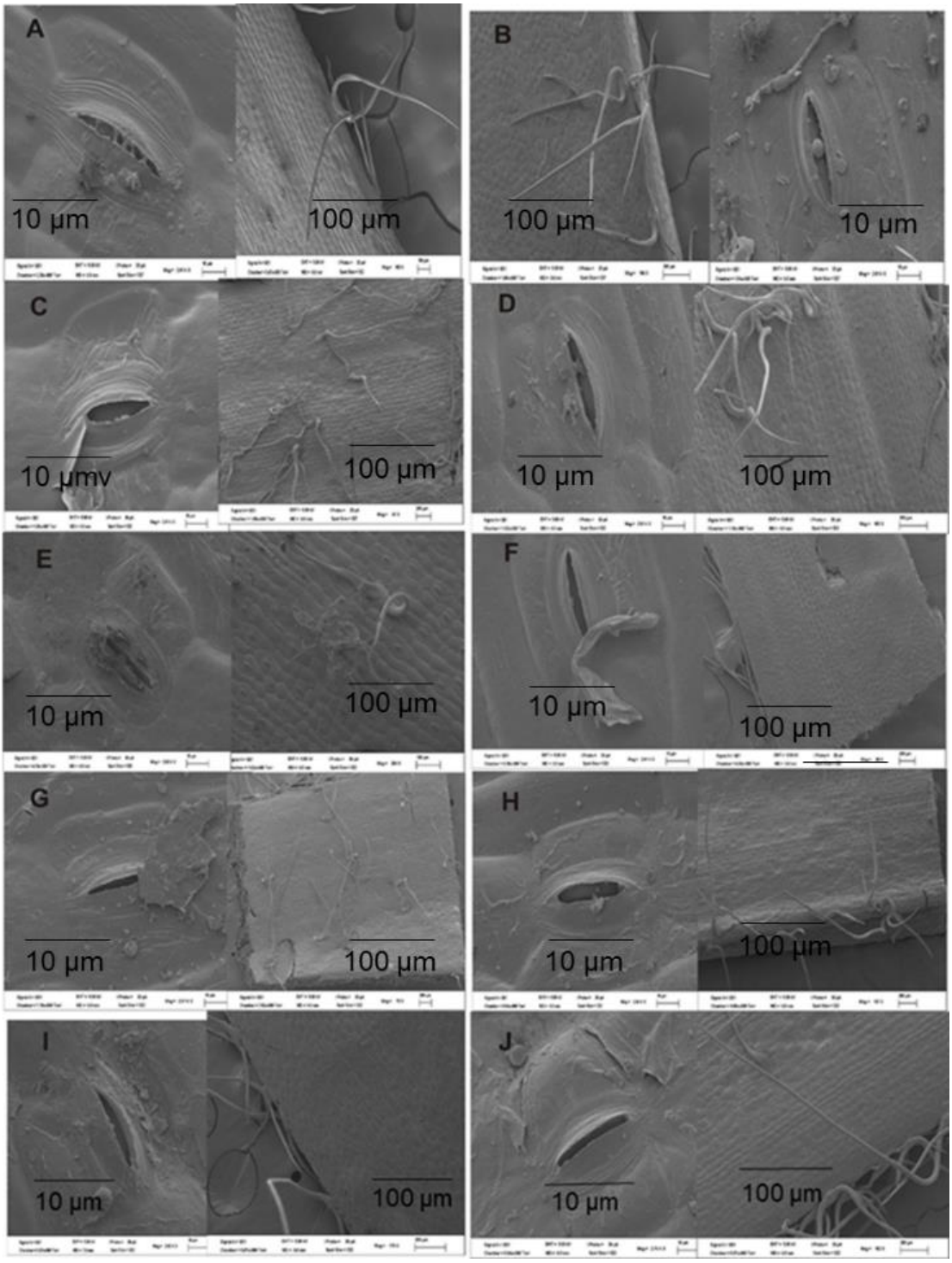


Figure 4.14 SEM micrograph of abaxial leaf surfaces of *H. hemerocallidea* exposed to Cd and Al treatments after six weeks. (A) = Control; (B) = Cd 2 mg L^{-1}; (C) = Cd 5 mg L^{-1}; (D) = Cd 10 mg L^{-1}; (E) = Al 500 mg L^{-1}; (F) = Al 1000 mg L^{-1}; (G) = Al 1500 mg L^{-1}; (H) = Cd 2:Al 500 mg L^{-1}; (I) = Cd 5:Al 1000 mg L^{-1}; (J) = Cd 10:Al 1500 mg L^{-1}.

4.3.9.3 EDX micrographs of elemental distributions in leaf samples of *H. hemerocallidea*

Energy dispersive X-ray analysis of elemental distribution in abaxial leaf samples of *H. hemerocallidea* revealed nine essential elements in the control plants. These were C, O, Na, Mg, P, S, K, Ca, as well as a non-essential element Si (**Figure 4.15**). Plants treated with Cd alone showed reduced amounts of detectable essential elements in the leaf samples and at the highest Cd concentration (10 mg L^{-1} Cd) only six essential elements were detected (**Figure 4.16-18**). This could be due to inhibitory effects of Cd on the uptake of essential elements. Cd was only detected on the abaxial leaf surface of *H. hemerocallidea* at 5 mg L^{-1} treatment in very minute quantities (**Figure 4.17**). EDX analysis indicates poor distribution Cd in the shoot compared to Al. Analysis of crystal deposits on the leaf of all the Al-treated plants showed high amounts of Al (**Figure 4.19-21**). The highest amounts were detected at 1000 mg L^{-1} Al (**Figure 4.20**). A similar pattern in the distribution of Al was also recorded in all the combined treatments of Cd and Al (**Figure 4.22-24**). This clearly showed that Al translocation and distribution to the shoot in *H. hemerocallidea* is high.

Element	Weight (%)	Atomic (%)
C	30.54	42.35
O	43.67	45.47
Na	0.18	0.13
Mg	0.24	0.17
Si	0.68	0.40
P	2.05	1.10
S	9.09	4.72
K	2.07	0.88
Ca	11.48	4.77
Totals	100.00	

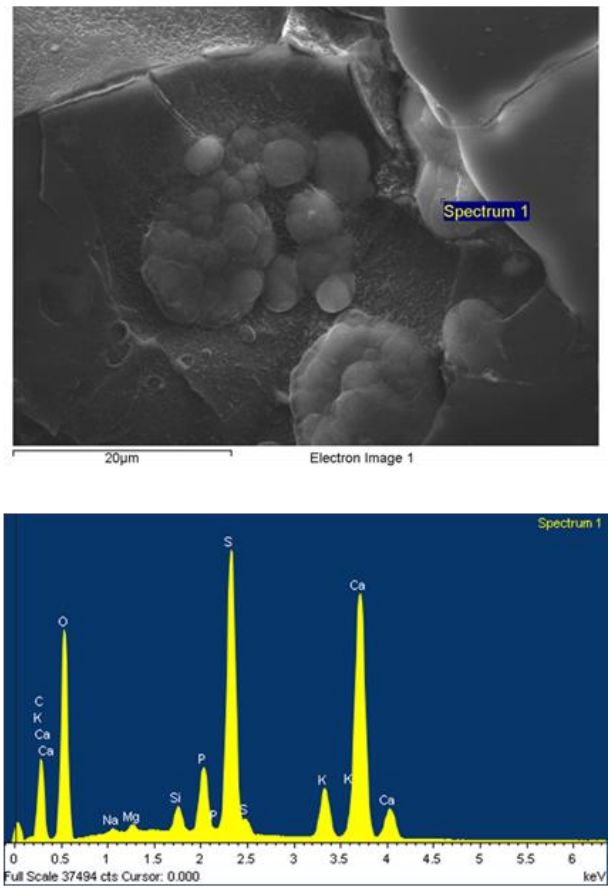


Figure 4.15 Energy-dispersive spectral analysis of essential elemetal distribution in the abaxial leaf surface of the control plants of *H. hemerocallidea* after six weeks of treatment Hoagland's solution (n=6).

Element	Weight (%)	Atomic (%)
C	62.74	70.58
O	33.05	27.91
Mg	0.19	0.11
Si	0.13	0.06
S	0.33	0.14
K	2.18	0.75
Ca	1.24	0.42
Cu	0.12	0.03
Zn	0.02	0.00
P	-	-
Na	-	-
Totals	100.00	

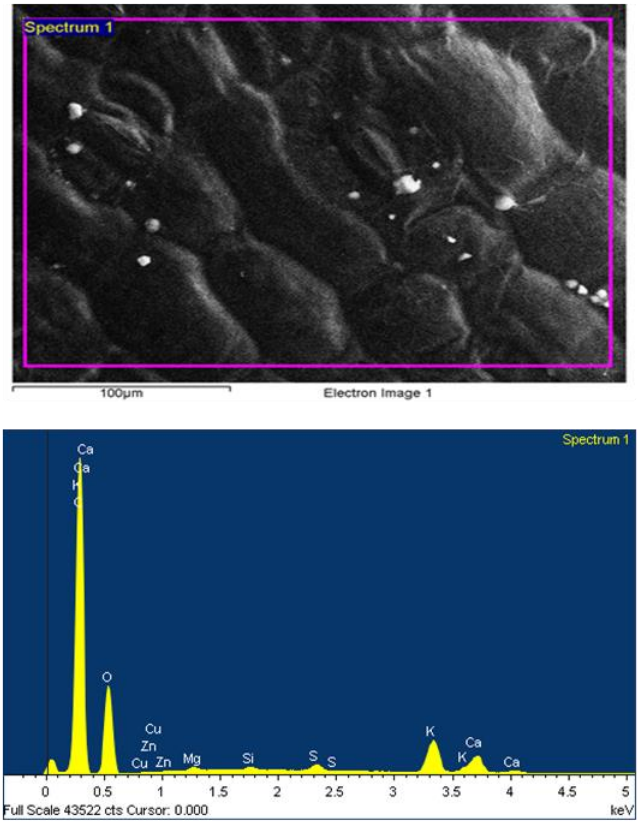


Figure 4.16 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 2 mg L^{-1} Cd-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	65.67	73.06
O	30.63	25.59
Mg	0.21	0.12
Si	0.10	0.05
P	0.02	0.01
S	0.33	0.14
Cl	0.07	0.03
K	2.07	0.71
Ca	0.88	0.29
Cd	0.01	0.00
Na	-	-
Totals	100.00	

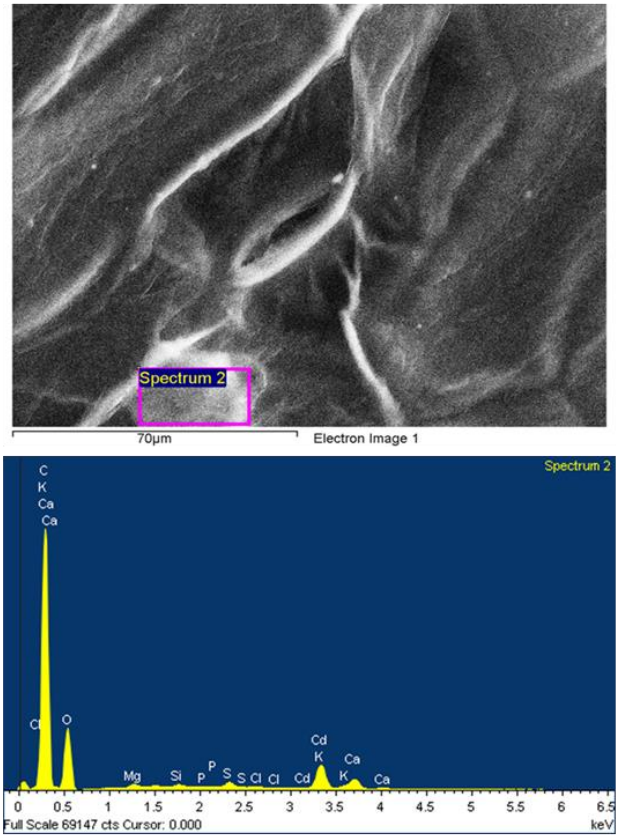


Figure 4.17 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 5 mg L^{-1} Cd-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	65.14	72.59
O	31.23	26.12
Mg	0.21	0.11
S	0.22	0.09
K	2.11	0.72
Na	-	-
Ca	1.09	0.36
Si	-	-
P	-	-
Totals	100.00	

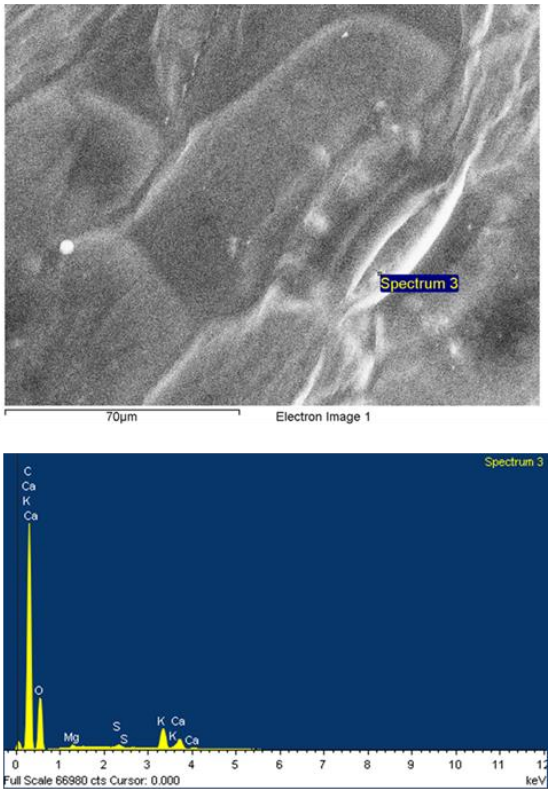


Figure 4.18 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 10 mg L^{-1} Cd-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	62.93	71.41
O	30.00	25.56
Na	0.10	0.06
Mg	0.19	0.11
Al	0.75	0.38
Si	2.72	1.32
S	0.17	0.07
Cl	0.08	0.03
K	2.48	0.86
Ca	0.58	0.20
Totals	100.00	

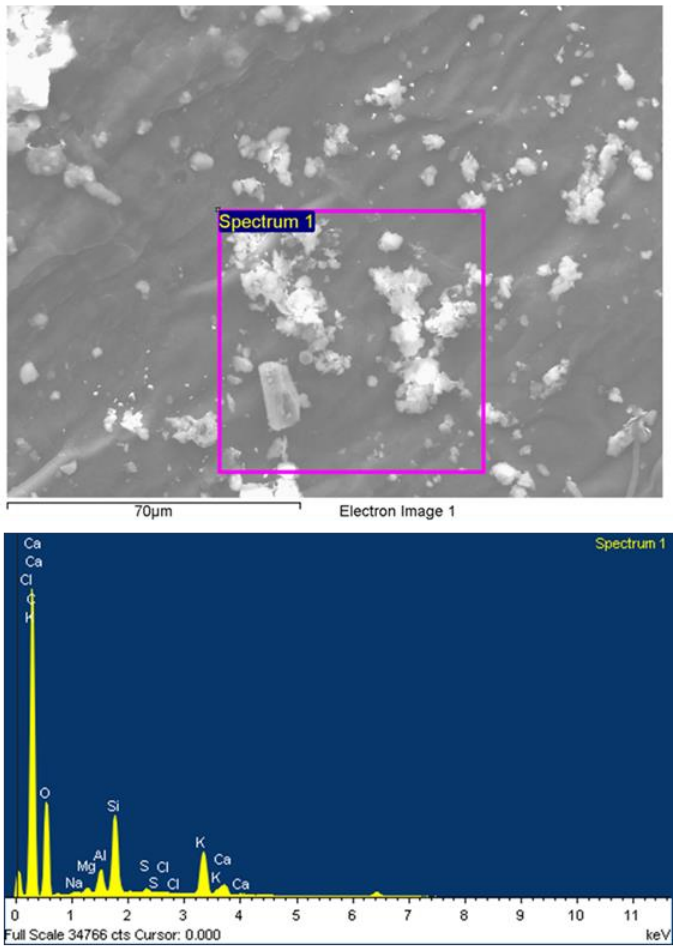
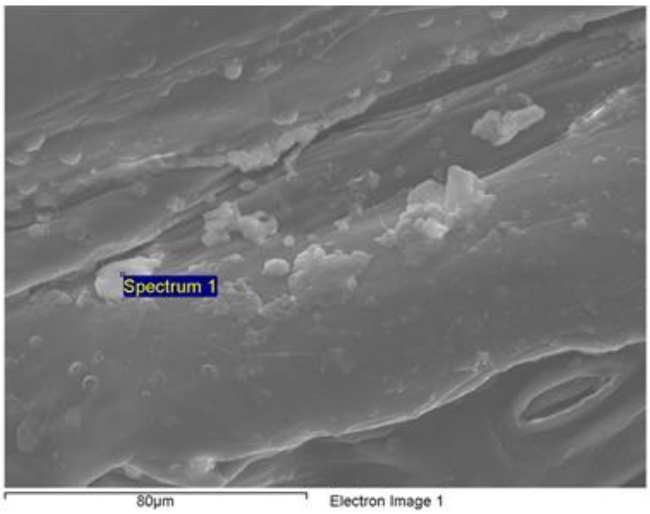


Figure 4.19 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 500 mg L^{-1} Al-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	64.20	73.32
O	26.43	22.66
Mg	0.28	0.16
Al	2.00	1.02
Si	1.81	0.88
P	0.48	0.21
S	1.22	0.52
Cl	0.11	0.04
K	0.96	0.34
Ca	2.40	0.82
Fe	0.11	0.03
Totals	100.00	



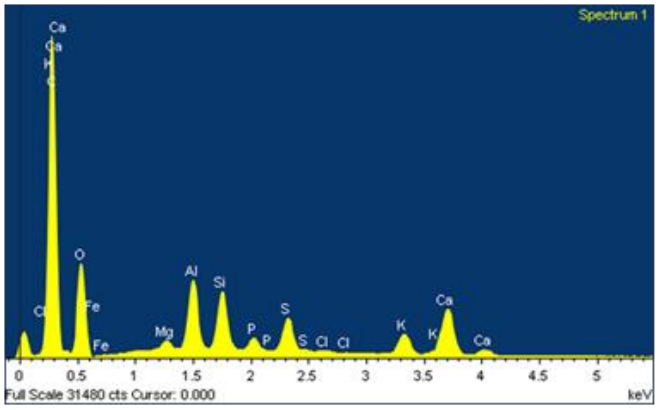
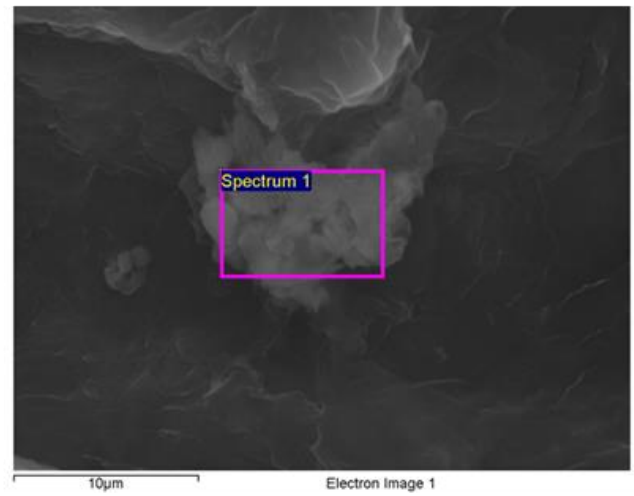


Figure 4.20 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 1000 mg L^{-1} Al-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	68.86	76.35
O	25.59	21.30
Mg	0.27	0.15
Al	1.37	0.68
Si	1.45	0.69
P	0.18	0.08
S	0.25	0.10
Cl	0.07	0.03
K	1.15	0.39
Ca	0.49	0.16
Fe	0.32	0.08
Zn	0.01	0.00
Totals	100.00	



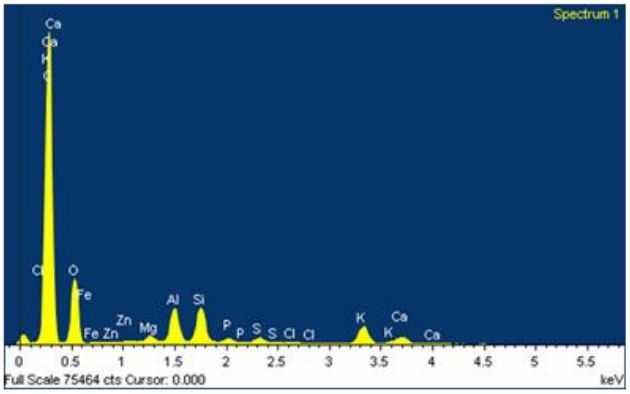


Figure 4.21 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface of 1500 mg L^{-1} Al-treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	52.72	64.46
O	30.40	27.90
Mg	0.17	0.10
Al	3.95	2.13
Si	7.48	3.91
S	0.10	0.05
Cl	0.07	0.03
K	0.79	0.28
Ca	0.33	0.12
Fe	3.99	1.02
Totals	100.00	

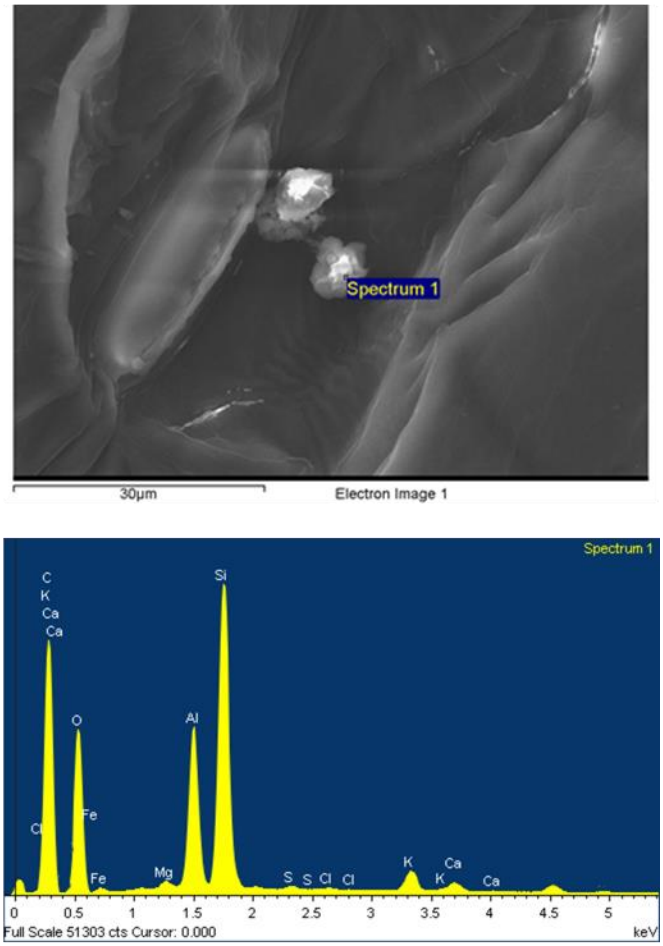
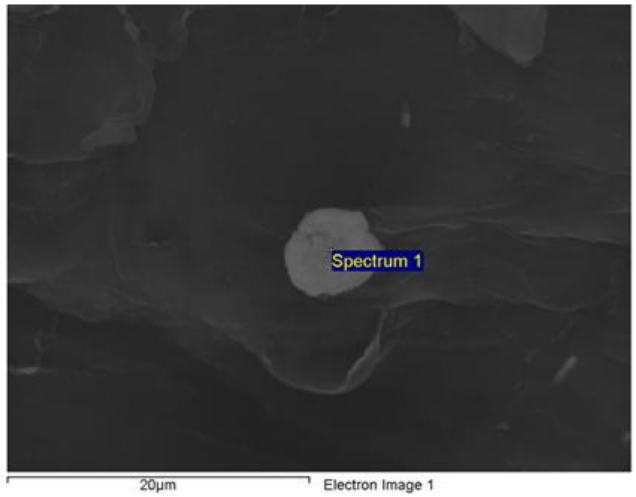


Figure 4.22 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface in the combined Cd 2:Al 500 mg L^{-1} treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	58.02	66.76
O	34.07	29.43
Na	0.09	0.05
Mg	0.14	0.08
Al	5.76	2.95
Si	0.60	0.30
P	0.06	0.03
S	0.07	0.03
Cl	0.04	0.01
K	0.44	0.16
Ca	0.25	0.08
Fe	0.47	0.12
Totals	100.00	



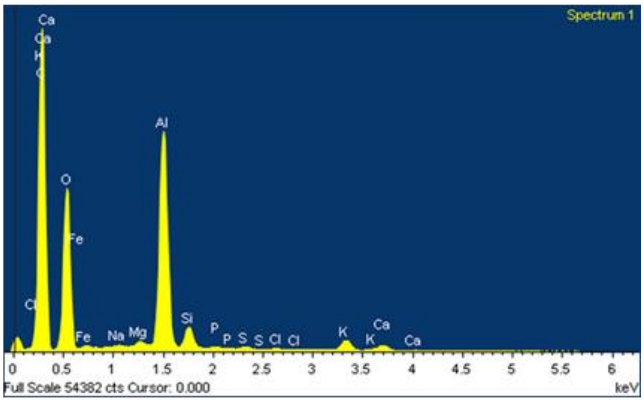


Figure 4.23 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface in the combined Cd 5:Al 1000 mg L^{-1} treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

Element	Weight (%)	Atomic (%)
C	60.81	69.92
O	31.15	26.89
Mg	0.35	0.20
Al	1.39	0.71
P	0.05	0.02
S	0.98	0.42
Cl	0.11	0.04
K	4.41	1.56
Ca	0.60	0.21
Cu	0.15	0.03
Na	-	-
Si	-	-
Totals	100.00	

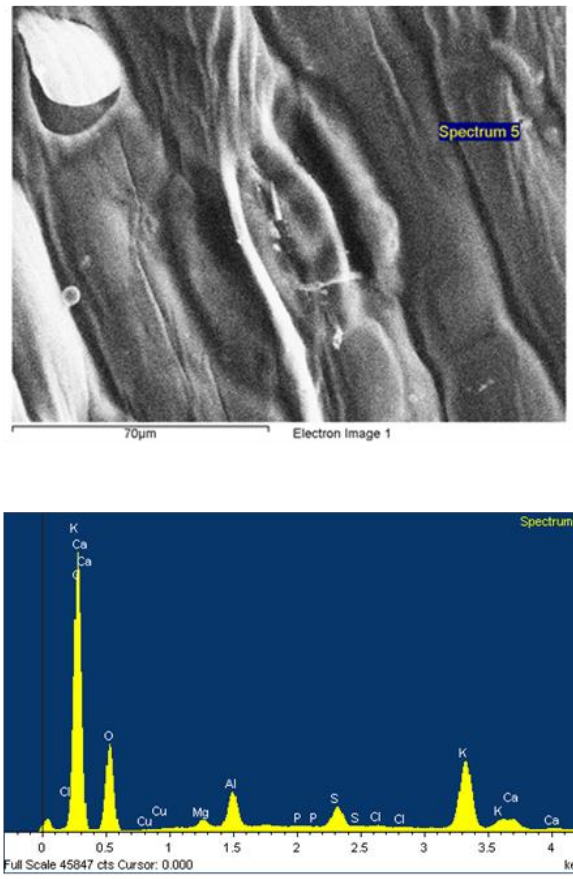


Figure 4.24 Energy-dispersive spectral analysis of elemetal distribution in the abaxial leaf surface in the combined Cd 10:Al 1500 mg L^{-1} treated plants of *H. hemerocallidea* after six weeks of treatment (n=6).

4.3.9.3 Ultrastructure analysis using TEM

Transmission electron microscopic analysis of *H. hemerocallidea* leaf samples from the controls revealed that the normal chloroplasts have a bean-seed shape and contained numerous well compartmentalized grana stacks and evenly spaced thylakoids (**Figure 4. 25A**). In between the thylakoids were electron-dense lipid droplets known as plastoglobuli. These significantly decreased at the highest concentrations of Al treatment and the combined Cd 5:Al 1000 mg L^{-1} (**Figure 4.25G and H**). The chloroplast and thylakoid structures at the lowest treatment with Al were similar to the control plants indicating a positive effects and good tolerance at lowest treatments with Al (**Figure 4.25E**).

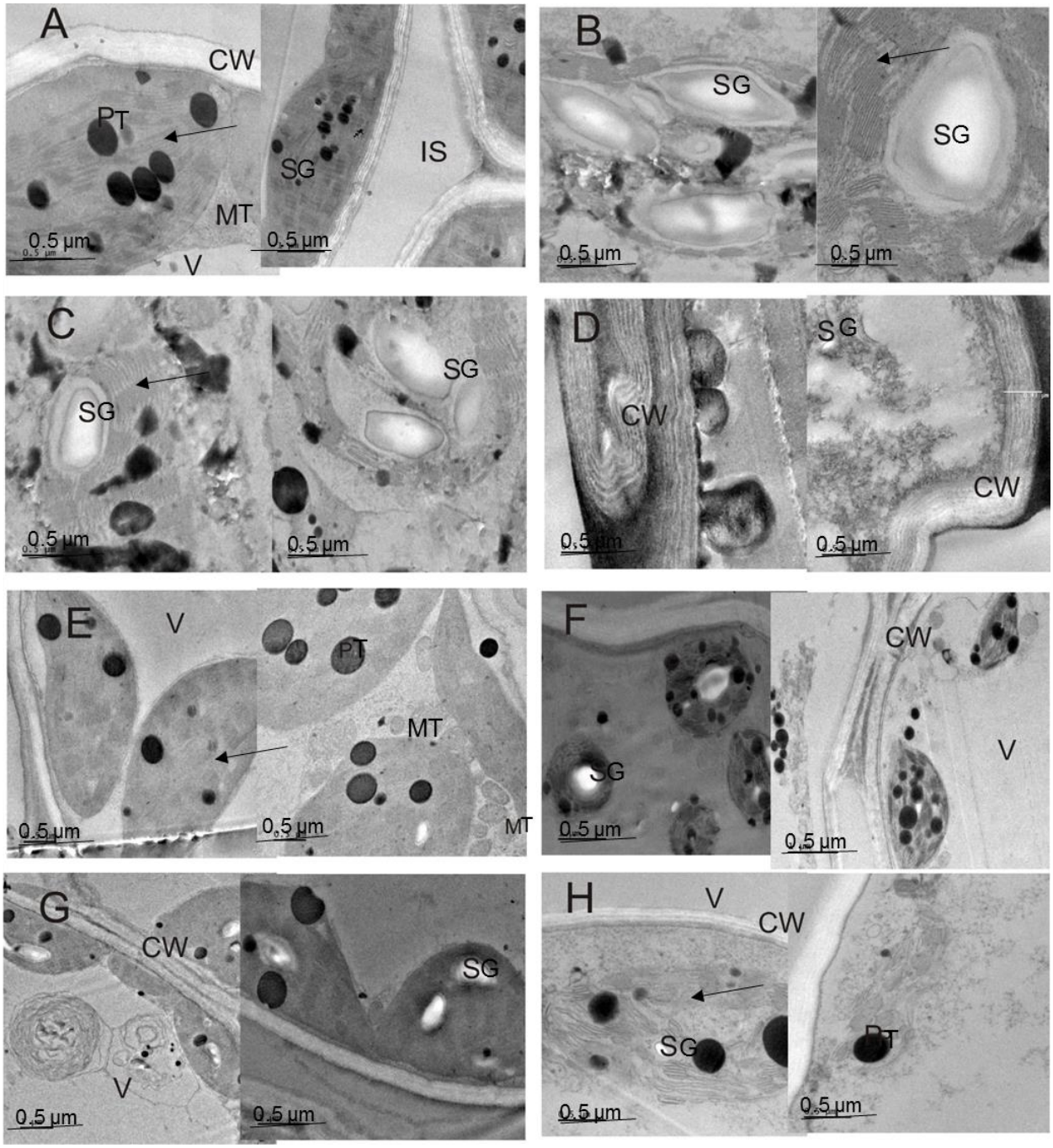


Figure 4.25 Anatomical changes of leaf structures of *H. hemerocallidea* exposed to Cd and Al treatments. (A) = Control; (B) = Cd 2 mg L^{-1}. Note the abnormally swollen chloroplast (C) = Cd 5 mg L^{-1}; (D) = Cd 10 mg L^{-1}; (E) = Al 500 mg L^{-1}; (F) = Al 1000 mg L^{-1}; (G) = Al 1500 mg L^{-1}; (H) = Cd 5:Al 1000 mg L^{-1}. Damaged disintegrating chloroplast. (CW) = Cell wall; (IS) = Intracellular space; (MT) = Mitochondria; (PT) = Plastoglobuli; (SG) = Starch grain and (V) = Vacuole. Arrow pointing at thylakoids.

Increasing concentrations of Al had a negative effect on chloroplast ultrastructure by significantly reducing the size and changing the structure into a disc-like shape compared to the bean-seed shape in the control plants (**Figure 4.25F**). At the highest concentrations of 10 mg L^{-1} Cd cells were greatly disorganized with complete disruption of chloroplast and thylakoids as well as mitochondria (**Figure 4.25D**). There was a large amount of starch deposit in chloroplasts at low and moderate concentrations of Cd-treated plants but these were not detected at higher concentrations of Cd probably due to complete damage to the chloroplast (**Figure 4.25B-D**). In the Al-treated plants at the lowest concentration there was no starch deposit detected but increasing concentration increased the amounts of starch deposited (**Figure 4.25E-G**). The effect is less compared to Cd-treated plants. In the combined Cd and Al treatment starch deposit was negligible (**Figure 4.25H**).

4.4 Discussion

High levels of Cd and Al significantly reduced biomass of *H. hemerocallidea* in the present study. This is consistent with previous studies were Cd and Al inhibited plant growth with both roots and shoots affected negatively (**AIDID AND OKAMOTO, 1992; MOSSSOR-PIETRASZEWSKA, 2001; IRFAN *et al.*, 2014**). Cd toxicity is associated with suppression of cell elongation and growth rate, due to irreversible inhibition exerted on the proton pumps responsible for cell elongation (**AIDID AND OKAMOTO, 1992**). Al toxicity is a serious factor limiting crop productivity in acidic soil. Toxic effects of Al involve blocking of mechanisms needed for cell division leading to poor crop yield (**PANDA *et al.*, 2009**). In the present study low concentrations of Al promoted growth parameters significantly compared to the controls and this indicates that *H. hemerocallidea* is an Al-tolerant species. In a previous study, **JONNALAGADDA *et al.*** (**2008**) showed that *H. hemerocallidea* had the ability to accumulate high levels of Al. High levels of Al exerted toxic effects on root elongation, poor root hair development and caused brittle roots in the present study. The reduction in biomass, shoot and root lengths recorded in the combined Cd and Al treatment could be due to Cd toxicity rather than Al due to the fact that low-moderate Al treatments enhanced growth parameters compared to Cd-treated plants.

Uptake and accumulation of Cd in *H. hemerocallidea* was lower in the shoot compared to the root and corm, indicating a slow translocation of this element from root to shoot. Cd accumulation in roots has been reported in many plant species and the restriction of Cd to the root parts involves a number of processes. These include immobilization inside the vacuoles of root cells, metal precipitation or binding of free metal cations to cell walls which slows down Cd movement into the shoot (**RAMOS *et al.*, 2002**). Recently metal pumps responsible for loading of zinc ions into the xylem have been proposed as essential in the accumulation of Cd into the shoot (**WONG *et al.*, 2009**). These factors could probably play key roles in translocation of Cd to the shoots. Al is a very reactive element with many potential binding sites including the cell wall, plasma membrane surface, cytoskeleton and even the nucleus (**PANDA *et al.*, 2009**). Perhaps, these could be the reasons for the elevated levels of Al detected in the present study. The results of EDX provide valuable information on the chemical analysis at specific locations on the abaxial surface of the *H. hemerocallidea* leaf. EDX analysis revealed a high percentage of Al distribution on the leaf surface in all the Al-treated plants. EDX/SEM technology offers a good approach to instantaneous analyses of elemental composition of plant samples as it requires less sample preparation than the conventional methods for elemental analysis such as ICP-MS and -OES. This technology could have broad applications in the field of biological sciences, such as determination of heavy metal uptake and accumulation by plants, and detection of heavy metal pollution in the environment.

In the event of heavy metal stress, plants exhibit strategies such as enzymatic and non-enzymatic antioxidant processes to curb abiotic stresses. Accumulation of free-proline is a non-enzymatic antioxidant mechanism plants use in the event of stress. The dose-dependent increase in the free-proline recorded in the present study is consistent with previous studies which reported correlations between high proline and the severity of stress, indicating an adaptive response of plants to abiotic stress (**HARE AND CRESS, 1997; ROSHANDEL AND FLOWERS, 2009**). Cd toxicity is usually associated with water stress and this can lead to high proline accumulation in the plant (**SCHAT *et al.*, 1997**). One of the basic function of proline is osmo-regulation in the event of abiotic stress. Proline can also act as an antioxidant and decrease the damage of free radicals by preventing lipid peroxidation. In the present study, the high

levels of free-proline recorded in most of the heavy metal treatments could be a preventive measure against free radicals resulting from heavy metal toxicity.

SOD is one of the important enzymatic antioxidant defence systems in plants. Some of the initial responses of plants to environmental stress are free radical and ROS generation, which are stimulated by metals (**MAKSYMIEC, 2007**), and this can strongly disrupt normal metabolism through oxidative damage to cellular components. In order to repair the damage initiated by ROS, plants have defensive antioxidant systems including SOD. Exposure to Cd toxicity causes a change of oxidant level in plants and these include the generation of ROS such as H_2O_2, OH^- and O_2^- thereby inducing oxidative stress (**CHOUDHARY AND PANDA, 2004**). SOD is considered a key enzyme in the process of ROS scavenging. Therefore, increased SOD activity recorded in the present study indicated that it plays an effective role in controlling the cellular level of these ROS molecules and/or repairing oxidative damage (**MILLER *et al.*, 2008**). Although at the highest concentrations (1500 mg L^{-1} Al) there was a decrease in the SOD activity, this could be due to significant membrane damage at this concentration leading to inability of the plant to activate basic defence enzymes.

When a plant is subjected to oxidative stress the final product of membrane lipid peroxidation is the accumulation of MDA and its levels correlates with the extent of oxidative stress in plants. Therefore, the concentration of MDA is commonly considered as a general indicator of lipid peroxidation (**GRATÃO *et al.*, 2008**). A moderate concentration of Cd and Al induced relatively low MDA accumulation and this suggests less oxidative stress. The low levels of oxidative damage recorded at 500 mg L^{-1} Al explained the significant positive effects in growth parameters recorded at this concentration (**Table 4.1**). In the present study, increasing concentrations of Cd and Al increased the levels of lipid peroxidation as indicated by the high levels of MDA accumulation in all the metal-treated plants compared to the controls. The high levels of lipid peroxidation recorded in the present study suggested that *H. hemerocallidea* accumulated elements in the membrane. Aluminum has been reported to binds to phospholipids within the cell membrane (**PANDA *et al.*, 2009**). The inverse correlation between SOD and MDA in the present study suggest that they balance the production of free radicals thereby increasing tolerance of *H. hemerocallidea* to relatively low concentrations of Cd and Al in contaminated soils.

Exposure to Cd and Al affected some metabolic processes such as starch and sugar metabolism in *H. hemerocallidea*. Starch is a high molecular weight polysaccharide and the chief storage carbohydrate in higher plants, consisting of approximately 80% water soluble amylopectin and 20% water soluble amylose (**MUTHUKUMARAN AND RAO, 2013**). Starch is an assimilate product of metabolisms in chloroplasts and is degraded into simple sugars when the need for energy arises (**MUTHUKUMARAN AND RAO, 2013**). Sugars such as reducing and non-reducing sugars and their associated hydrolysing enzymes play an important role in plant metabolic processes and development (**PFEIFFER AND KUTSCHERA, 1996**). In the present study, a progressive decrease in sugar levels in *H. hemerocallidea* was observed in corms of Cd-treated plants with a significant decrease in the leaves with increasing concentrations of treatment. The decrease in the amounts of sugar in leaf samples correlated with the decrease in the activity of the amylase enzymes. Amylase hydrolyses breakdown reserve metabolic products (starch) leading to increasing amounts of the hydrolyzates e.g. glucose, that are required for growth and other metabolic processes in plants (**KRASENSKY AND JONAK, 2012**). This can lead to an increase in the amounts of soluble sugars and can induce early leaf senescence. Probably, the accumulated soluble sugars may inhibit the amylase expression in a feedback mechanism (**KRASENSKY AND JONAK, 2012; MUTHUKUMARAN AND RAO, 2013**). In support of our results, a previous study reported that glucose and fructose exert a repression on amylase synthesis similar to that of sucrose and carbohydrate starvation in suspension cells of rice (**YU *et al.*, 1992**). Under heavy metal stress, plants accumulate elevated levels of starch but decrease in the amounts of reducing and non-reducing sugars as well as total sugar content in plant species (**NARWAL AND SINGH, 1993; BHATTACHARYA AND CHOUDHARY, 1994; MITHOFER *et al.*, 2004; RAI *et al.*, 2005**). In the present study, Cd-treated plants accumulated more starch than Al-treated plants. The low amounts of amylase enzymes recorded in the present study could have accounted for the significant reduction in plant biomass at the highest concentrations of heavy metal treatment due to an inability to synthesise the basic metabolites needed for various metabolic processes in plants.

Analysis of fluorescence photochemistry of *H. hemerocallidea* showed a number of toxic effects of Cd and Al on chlorophyll fluorescence capacity, particularly at the

highest concentrations of heavy metal treatment. The ratio of F_v/F_m is used as a stress indicator, representing the maximum quantum yield of PSII photochemistry (**HE *et al.*, 2008**). F_v/F_o indicates the size and number of active photosynthetic centers in the chloroplast and this represents the photosynthetic strength of the plant (**ISRAR *et al.*, 2011**). It was suggested that F_v/F_m values higher than 0.8 indicate positive effects on PSII (**BJÖRKMAN AND DEMMING, 1987**). In the present study, a significant decrease in F_v/F_m as well as in PSII at high levels of Cd and Al treatment suggest that the photoactivation of PSII was inhibited as a result of a decrease or partial blocking of electron transport from PSII to PSI (**MALLICK AND MOHN, 2003**).

In many stress situations, an increase in NPQ is often accompanied by photoinactivation of PSII reaction centres, which then dissipate excitation energy within cells as heat rather than as photochemistry. Photoinactivation can lead to oxidative damage and loss of PSII reaction centres, both of which are associated with an increase in F_o (**BAKER, 2008**). In the present study, increasing heavy metal stress resulted in the inability of *H. hemerocallidea* to utilize the absorbed light energy leading to a high carotenoid content. High amounts of carotenoids are associated with an increase in thermal energy dissipation capacity of plants (**ZENGIN, 2013**). Thus heavy metal induced changes in the balance between electron transfer and excitation rates thereby reducing the state of PSII reaction centres, i.e., captured light cannot be effectively used (**HUANG *et al.*, 2013**). Progressive increase in heavy metal treatment significantly affected rETR, suggesting that photosynthetic electron transport via PSII in higher Cd and Al treatments was inhibited. In a previous study **VASSILEV *et al.*** (**2004**) reported a similar patterns in Cd-treated barley plants.

Heavy metal toxicity reduces photosynthetic apparatus by disrupting the thylakoidal and stromal membranes resulting in the inability of plants to effectively use the absorbed light energy (**QURESHI *et al.*, 2005; FUSCO *et al.*, 2005**). These changes in the photosynthetic measurements explains the decrease in photosynthetic ability of *H. hemerocallidea* exposed to high levels of Cd and Al treatment and consequently the availability of photosynthetic apparatus needed for biomass accumulation as recorded in the biomass reduction at high levels of heavy metal treatments.

Analysis of root structures using light microscopy showed that Cd treatment induced severe damage to the piliferous layer and cortex cells in *H. hemerocallidea* compared to the control (**Figure 4.13**). When exposed to high levels of Cd and Al, *H. hemerocallidea* tended to decrease water stress-tolerance by increasing xylem size in most of the stressed plants. The increase in the size of the xylem is due to an increase in the volume of the vascular bundles. These findings are consistent with previous studies which reported a decrease in water stress-tolerance in plants due to an increase in the volume of vascular bundles (**VOLLENWEIDER *et al.*, 2006; BOSABALIDIS *et al.*, 2004; SOARES *et al.*, 2011**). The increase in the size of xylem observed in this study could also be associated with root maturation enhanced by heavy metals. Plants exposed to heavy metals produce stress-related hormones leading to early senescence, which in turn affect tissue morphogenesis as well as aging of cells in plant tissues (**SOARES *et al.*, 2011**).

There was an increase in trichome density on the abaxial leaf surface of *H. hemerocallidea* at high levels of heavy metal treatment. This indicated a significant change in morphogenesis leading to early senescence in *H. hemerocallidea*. Increasing Cd and Al concentrations increased stomata opening but decreased guard cell density and this was consistent with previous studies where there was an increase in stomata opening in Cd-stressed plants in a variety of plant species e.g. *Silene vulgaris* (**CHARDONNENS *et al.*, 1998**), *Brassica napus* (**BARYLA *et al.*, 2001**), and *Arachis hypogaea* (peanut) (**SHI AND CAI, 2009**).

Ultrastructure analysis using transmission electron microscopy showed high amounts of plastoglobuli in *H. hemerocallidea* at moderate heavy metal treatments but decreased when treated with high amounts of heavy metals. High amounts of plastoglobuli have been linked to changes in metabolism, which, in turn, result in a reduction of cell proliferation and a decrease in plant biomass (**dos SANTOS *et al.*, 2012**). Plastoglobuli contain high amounts of carotenoids (**STEINMÜLLER AND TEVINI, 1985**) and this correlates to the high amounts of carotenoids recorded in *H. hemerocallidea* treated with high levels of heavy metals in the present study (**Figure 4.8**). A progressive increase in Cd concentrations exhibited toxic effects at ultrastructure level such as irregular chloroplasts, breakdown of thylakoids and mitochondria as well as changes in cell wall architecture (**Figures 4.25B-D**). The

altered chloroplast architecture at high levels of Cd and Al explains the significant decrease in the amounts of total chlorophyll and fluorescence capacity of *H. hemerocallidea* recorded in the present study. Cd toxicity triggers either necrosis or programmed cell death (**FOJTOVÁ AND KOVARÍK 2000; BEHBOODI AND SAMADI 2004**). Programmed cell death plays an essential role during development and morphogenesis of plants by removing unwanted or misplaced cells in specific structures and organs (**OVERMEYER *et al.* 2003; van DOORN AND WOLTERING 2005**). These could have been responsible for the high levels of necrosis observed at the highest levels of Cd-treated plants and in the combined Cd and Al treatments (**Figure 4.1**).

4.5 Conclusions

The results of the present study showed that *H. hemerocallidea* has good tolerance to lower concentrations of Cd (2 mg L^{-1}), but is more tolerant to Al at moderate concentrations (up to 1000 mg L^{-1} Al) as shown in significant positive effects in growth parameters and other physiological parameters. The positive effects of low concentrations of Al on growth parameters in the present study suggest that *H. hemerocallidea* produces a better yield in the presence of small amounts of Al in the growth media. High levels of Cd and Al induced ultrastructure changes recorded in the present study particularly on the chloroplast structures. This led to increase in the accumulation of plastoglobuli, SOD and carotenoid content. The EDX analysis used in the present study is an ideal and quick method that can be applied in monitoring heavy metal distribution in plants.

Chapter 5

Effect of Cd and Al on the accumulation of secondary metabolites and biological activity in selected medicinal plants

5.1 Introduction

Plant secondary metabolites are often referred to as compounds that have no fundamental role in metabolic processes in plants, but they are important for plant interactions with the environment for adaptation and defence (**BENNETT AND WALLSGROVE, 1994; BOURGAUD *et al.,* 2001**). In higher plants, a wide variety of secondary metabolites are synthesized from primary metabolites (e.g., carbohydrates, lipids and amino acids). Secondary metabolites have significant practical applications in the field of medicine, nutrition and cosmetics (**RAMAKRISHNA AND RAVISHANKAR, 2011**). Accumulation of secondary metabolites often occurs in plants subjected to environmental stresses including heavy metal stress (**OKSMAN-CALDENTEY AND INZÉ, 2004**). Exposure to heavy metal stresses can either up-regulate or down-regulate the synthesis and accumulation of secondary metabolites (**KRUPA *et al.,* 1996; GROPPA *et al.,* 2001; HAWRYLAK *et al.,* 2007**). This is one of the important strategies for plant adaptation to abiotic stress.

The aim of the study was to investigate the effect of heavy metal stress on total phenolics and flavonoids in selected medicinal plants as well as to quantify the levels of hypoxoside, a secondary metabolite in *Hypoxis hemerocallidea*. To determine the effect of heavy metal stress on biological activity.

5.2 Materials and methods

5.2.1 Pot trials and sample preparation

Pot trials and sample preparation for *Drimia elata* and *Bulbine natalensis* are described in Sections 3.2.1, and 3.2.2, and *Hypoxis hemerocallidea* in Section 4.2.1, 4.2.2 and 4.2.2.2.1. Only the medicinally used bulbs and/or corms were tested for phyotochemical analysis, antioxidant properties and antibacterial activity.

5.2.2 Solvent extraction of plant material

Dried powdered samples (1 g DW) were extracted with 10 mL 50% methanol as described in Section 2.2.3.1.

5.2.3 Screening for total phenolics and flavonoids

Screening for total phenolics and flavonoid were carried out as described in Section 2.2.3.2 and 2.2.3.3.

5.2.4 Quantitative analysis of hypoxoside using high performance liquid chromatography (HPLC)

5.2.4.1 Reagents

Acetonitrile and methanol (HPLC grade) were purchased from Romil Ltd., (Cambridge, United Kingdom); butanol, ethyl acetate, and toluene were purchased from BDL Chemicals Ltd. (Poole, United Kingdom); and water was purified in a Milli-Q System (Millipore, Bedford, MA). Millex HV Hydrophilic (PVDF) filters were purchased from the same source. Working solutions comprising a set of five calibration concentrations in the range 10-100 mg L^{-1} hypoxoside standard were used. Hypoxoside was isolated and donated by Prof S.E. Drewes (School of Chemistry and Physics, University of KwaZulu-Natal) using the method described by **DREWES *et al.*** (**1984**). Hypoxoside standards were prepared daily by appropriate dilution with methanol.

5.2.4.2 Extraction and isolation of hypoxoside

Hypoxoside was extracted from fresh *H. hemerocallidea* corms after six weeks of treatment using the method described by **NAIR AND KANFER** (**2005**) with modifications. Fresh corms (10 g) were homogenised with 10 mL methanol using a mortar and pestle. The homogenate was extracted with 50 mL methanol for 30 min using a magnetic stirrer and strained through muslin cloth. The extraction was repeated three times. Aqueous solutions of hypoxoside are photolabile at moderate light intensity, hence the above extraction were carried out under subdued light to prevent photodegradation of hypoxoside. The extracts were subsequently filtered using Whatman no. 44 filter paper. The filtrate was concentrated in a rotary evaporator under vacuum at 45 °C and air-dried under a stream of cold air. The extract (3 g) was then dissolved in 50 mL water by shaking for 10 min. The water-soluble extract was

filtered through Whatman no. 44 filter paper and transferred to a 250 mL separating funnel where it was first partitioned with water-saturated ethyl acetate (50 mL x3) to remove nonpolar impurities. The aqueous portion was further partitioned with water-saturated n-butanol (50 mL x3), and the non-aqueous layer was removed and thoroughly dried under vacuum to obtain a yield of approximately 50 mg (butanolic extract). This procedure facilitated the removal of polar and other mucilaginous impurities.

5.2.4.3 Quantification of hypoxoside using high performance liquid chromatography (HPLC)

Quantification of hypoxoside was determined using HPLC (Varian, Walnut Creek, CA, USA) equipped with a photodiode array detector, a degasser, a column heater, and an autosampler. A Luna C18 (2) (5 µm, 150 mm length, 4.6 mm i.d.) column (Phenomenex) was used at 23±2 °C. Separation was achieved by injecting 25 µL extract diluted in methanol (0.5 mg mL^{-1}) into the HPLC using Spectra SYSTEM P2000 pump equipped with an AS 1000 autosampler and a UV 1000 variable-wavelength UV detector (Thermo Separation Products, Riviera Beach, FL). A mobile phase consisting of acetonitrile:water in isocratic mode (20:80, v/v) was used at a flow rate of 0.5 mL min^{-1}. Samples (25 µL) were injected into the column, and the eluate was monitored at a wavelength of 260 nm. Quantification of sample was done in triplicate. The quantification of hypoxoside was calculated from a standard curve of hypoxoside concentrations (ranging from 10-90 µg mL^{-1}) as a function of peak area (R^2 = 0.97871, R_T = 31.8).

5.2.5 Antibacterial screening

Antibacterial activity was carried out as described in Section 2.2.3.4.

5.2.6 Diphenylpicrylhydrazyl (DPPH) antioxidant activity assay

Antioxidant activities were measured using the DPPH assay for the determination of free radical scavenging activity as described by **KARIOTI *et al.*** (**2004**) with modifications. Dried plant extracts were redissolved in 50% aqueous methanol at 50 mg mL^{-1}. Each plant extract (15 µL) was diluted with methanol (735 µL) and then added to a methanolic DPPH solution (750 µL, 0.1 mM) to give a final volume of 1.5 mL in the reaction mixture, and final concentrations of 0.65 µg mL^{-1} in the assay. The

concentration of DPPH in the final reaction was 50 µM (**SHARMA AND BHAT, 2009**). The DPPH solution was freshly prepared before the assay. The reaction mixtures were prepared under dim light and incubated at room temperature for 30 min in the dark. Decrease in the purple colouration of the reaction mixtures was read at 517 nm in a Cary 50 UV–visible spectrophotometer. A standard antioxidant compound, ascorbic acid (0.88 µg mL^{-1}) was used as a positive control in the assay. A solution containing 50% aqueous methanol instead of sample extracts or the standard antioxidant was used as the negative control. Absolute methanol was used as a blank. Background correction of the sample absorbance (without DPPH) was done by subtracting the absorbance readings of the sample extracts from the corresponding readings obtained in the presence of DPPH (**KARIOTI *et al.,* 2004**). Each sample extract was replicated three times. The free radical scavenging activity (RSA) as determined by the decolouration of the DPPH solution, was calculated according to the formula:

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D)$$

Where A_E is the absorbance of the reaction mixture containing the sample extract or standard antioxidant, and A_D is the absorbance of the DPPH solution only. Radical scavenging activity (%) was plotted against the sample extract for each treatment.

5.3 Statistical analysis

Statistical analyses were carried out using SPSS for Windows by one-way ANOVA using the Student's *t*-test to test the different significance levels. Results are presented as mean±SD (n=3).

5.4 Results

5.4.1 Phytochemical properties

5.4.1.1 Phenolic and flavonoid compositions in selected medicinal plants exposed to Cd and Al after six weeks

Application of Cd at moderate treatment had a positive effect on the synthesis of total phenolics in *B. natalensis*. Exposure to high levels of Cd and Al exhibited a significant decrease in the amounts of total phenolics in *B. natalensis* compared to the control plants (**Figure 5.1A**). Al-treated plants and the combined Cd and Al treatments

exhibited a dose-dependent decrease in the amounts of total phenolics with an increase in heavy metal treatments. The flavonoid content in *B. natalensis* was negatively affected in all the heavy metal-treated plants compared to the control plants (**Figure 5.1B**). There was a progressive decrease in the amounts of flavonoid content with increasing heavy metal treatment. The lowest flavonoid content was recorded in plants exposed to the highest concentrations of the combined Cd and Al treatment.

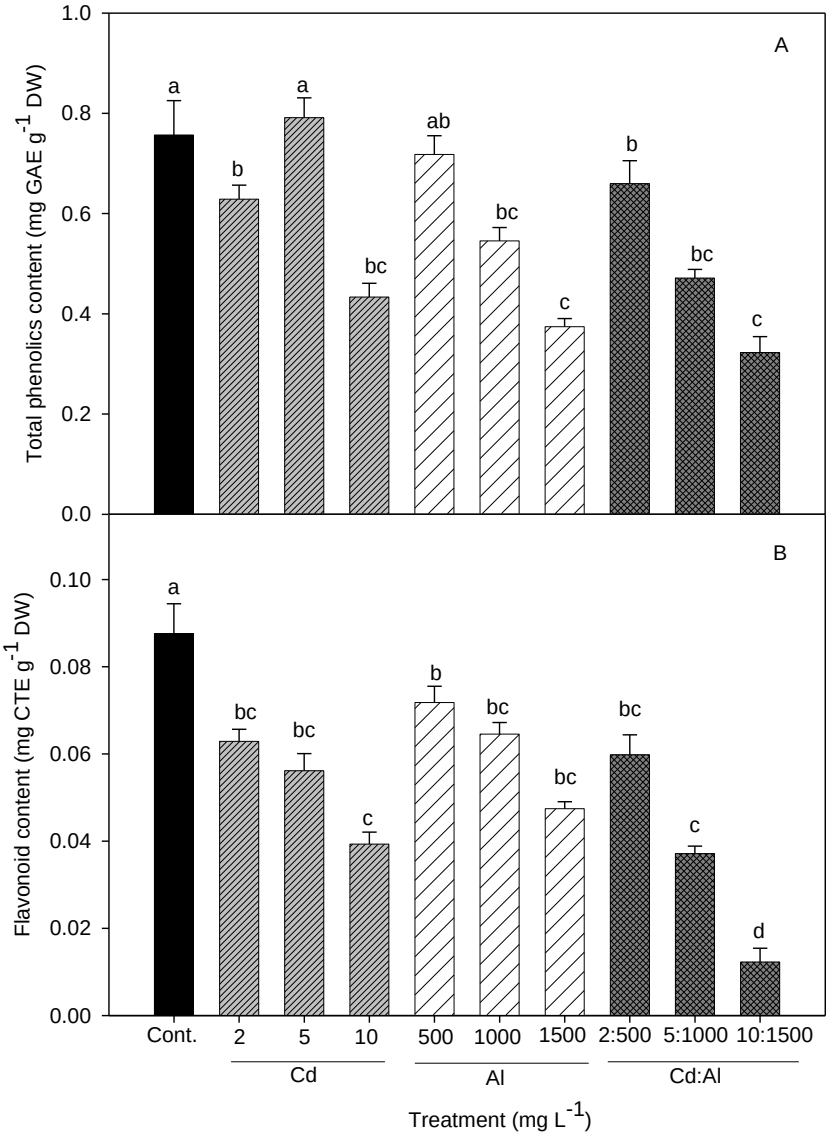


Figure 5.1 Total phenolic (A) and flavonoid (B) compositions in *B. natalensis* bulbs exposed to Cd and Al treatment after six weeks. Results are presented as mean±SD (n=3). Different letters on bars indicate significant difference (p<0.05).

Cd and Al applied singularly at low and intermediate concentrations had a slight positive effect on the total phenolic content in *D. elata* bulbs but the highest Cd and Al treatments significantly reduced the total phenolic content. All the combinations of Cd and Al significantly reduced the amounts of total phenolics (**Figure 5.2A**). A similar trend was observed for the flavonoid content with increasing concentrations of Cd and Al having a significantly negative effect on the flavonoid content, indicating an inhibitory effect of Cd and Al on the synthesis of flavonoids (**Figure 5.2B**).

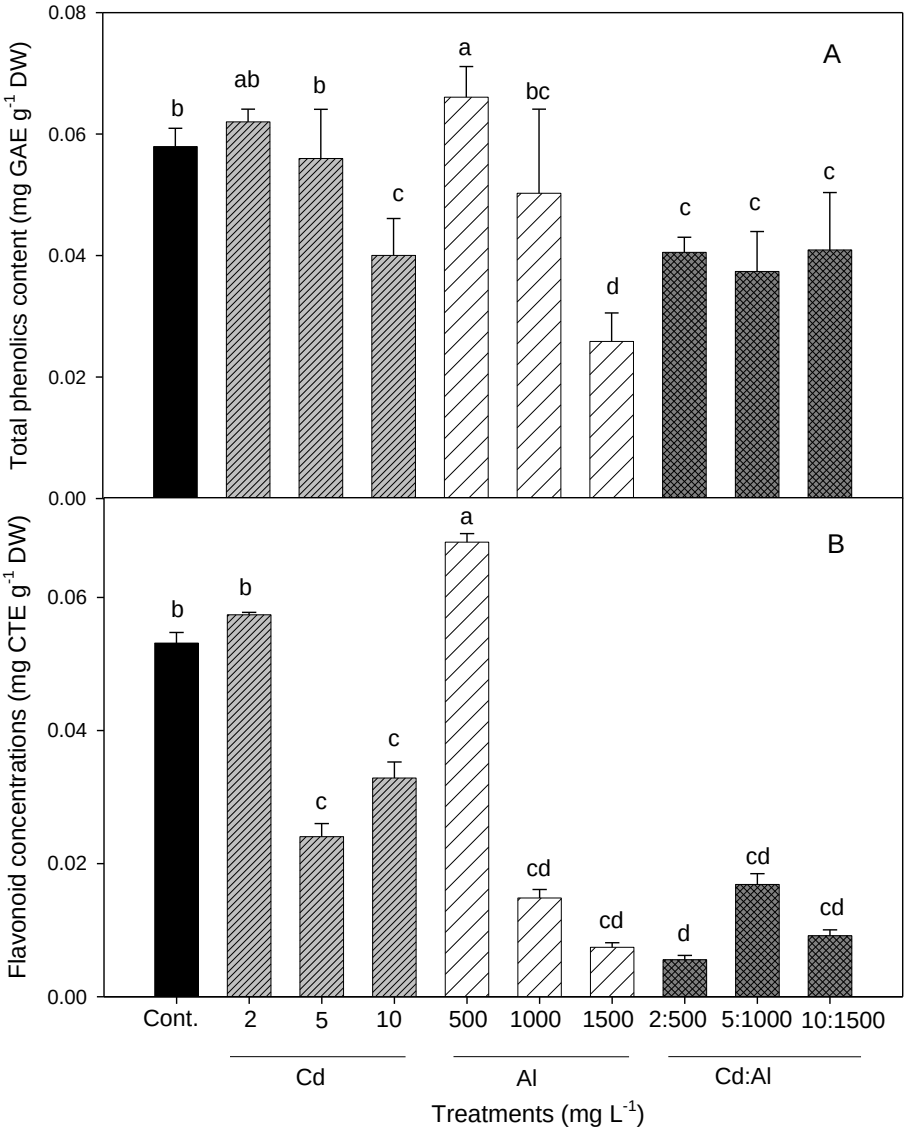


Figure 5.2 Total phenolic (A) and flavonoid (B) compositions in *D. elata* after six weeks of exposure to Cd and Al treatments. Results are presented as mean±SD (n=3). Different letters on bars indicate significant difference (p<0.05).

Exposure to moderate concentrations of Cd and Al significantly enhanced the synthesis and accumulation of total phenolics in *H. hemerocallidea* but at the highest concentrations there was significant decrease in total phenolics (**Figure 5.3A**).

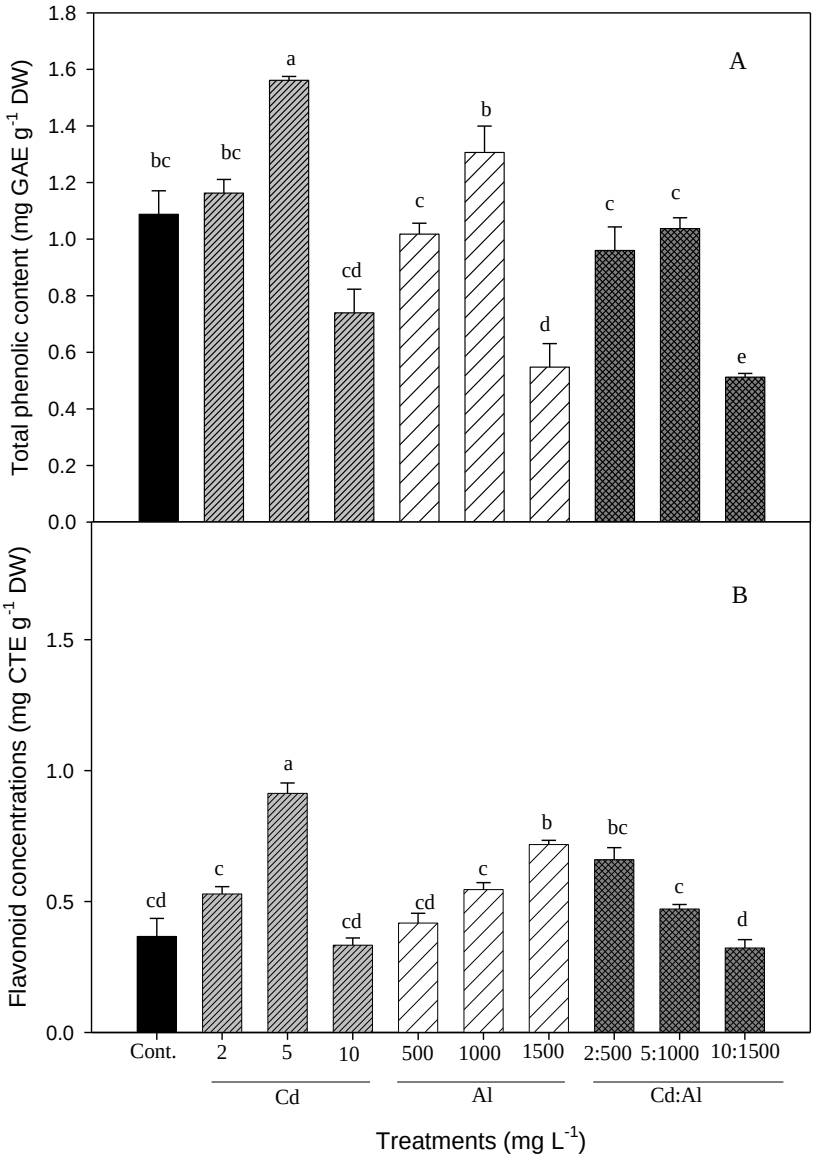


Figure 5.3 Effects of Cd and Al concentrations on the synthesis and accumulation of total phenolic (A) and flavonoid (B) in the corms of *H. hemerocallidea* after six weeks of treatment. Results are presented as mean±SD (n=3). Different letters on bars indicate significant difference (p<0.05).

The moderate treatment of Cd had the highest amount of flavonoid followed by the moderate concentrations of Al compared to all the other heavy metal treatments

(**Figure 5.3B**). There was a progressive positive effect in the accumulation of flavonoids in Al-treated plants in a dose-dependent response but in the combined treatment there was a decrease in flavonoids with increasing concentration of heavy metal treatment (**Figure 5.3B**).

5.4.2 HPLC quantification of hypoxoside

The HPLC chromatogram in the present study showed that the pure compound hypoxoside eluted at 10.2 min (**Figure A of A in Appendix B**). The pure compound hypoxoside used in the present study behaved in a similar pattern in previous studies where hypoxoside was reported to elute at 10 and 12 min (**NAIR AND KANFER, 2006; BOUKES *et al.,* 2008**). In the present study, the peak (and or area) of chromatogram for each treatment that behaved in a similar patterns like the pure compound hypoxoside were taken as hypoxoside (**Appendix B, Figure A, B and C**).

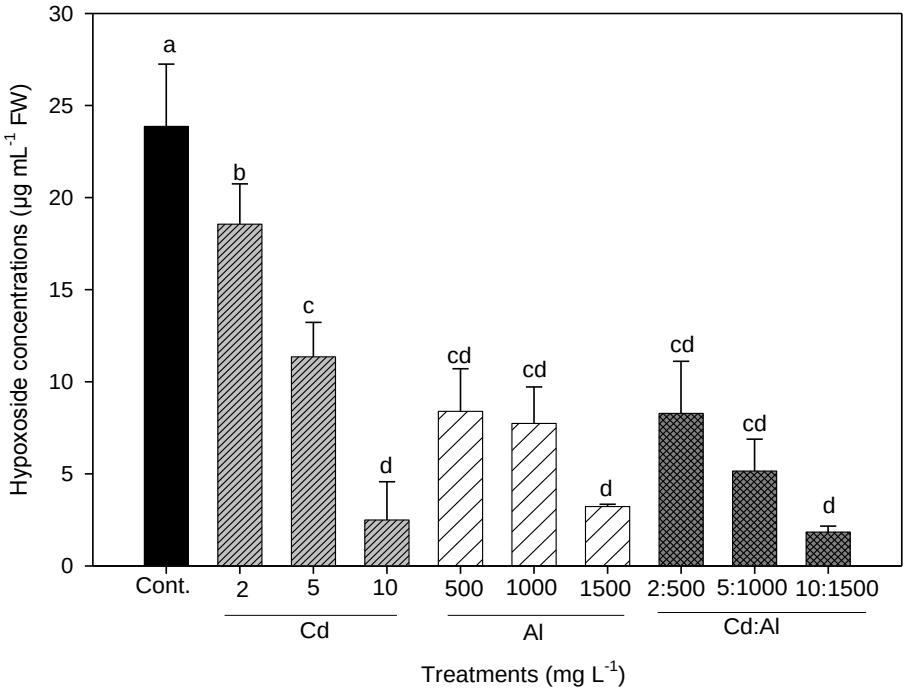


Figure 5.4 HPLC quantification of hypoxoside accumulation in bulbs of *H. hemerocallidea* exposed to Cd and Al after six weeks of treatment. Results are presented as mean±SD (n=3). Different letters on bars indicate significant differences (p<0.05).

There was a significant decrease in the amounts of hypoxoside in all the heavy metal-treated plants compared to the control plants (**Figure 5.4**). The lowest concentrations of Cd treatment had the highest amounts of hypoxoside compared to all the other heavy metal-treated plants. Decrease in the amounts of hypoxoside were dose-dependent and the least amount was recorded at Cd 10:Al 1500 mg L^{-1}.

5.4.3 DPPH radical scavenging activity

The DPPH radical scavenging activity was measured in terms of percentage inhibition of free radicals by antioxidants in bulb extracts of *B. natalensis, D. elata* and *H. hemerocallidea* after six weeks of heavy metal treatment (**Figure 5.5**). In *B. natalensis,* there was a progressive decrease in the DPPH activity with increasing heavy metal concentrations in all the treatments. A similar pattern in DPPH activity was recorded in *D. elata* in most of the heavy metal-treated plants compared to the control plants (**Figure 5.5B**). However, at the lowest treatment of Cd in *D. elata* where DPPH activity was slightly higher than in control plants and the positive control. The activity of DPPH in scavenging free radicals in *H. hemerocallidea* corms was significantly increased in the 5 mg L^{-1} Cd-treated plants compared to all the other treatments. All the heavy metal-treated plants have significantly higher DPPH activity compared to the positive control with the exception of 10 mg L^{-1} Cd which had the least DPPH activity (**Figure 5.5C**).

5.4.4 Antibacterial activity of selected plants exposed to Cd and Al after six weeks

Antibacterial activity varied among the investigated plant species in their responses to heavy metal stress. MIC values <1 mg mL^{-1} (highlighted in bold in **Table 5.1**) were considered as having good antibacterial activity (**GIBBONS, 2005**). *Bulbine natalensis* exposed to low and moderated concentrations of Cd exhibited good antibacterial activity against *S. aureus.* All the other extracts of *B. natalensis* had poor antibacterial activity against both *E. coli* and *S. aureus* (**Table 5.1**). Extracts of *D. elata* exposed to the lowest Cd and Al concentrations had good antibacterial activity that was similar to the control (**Table 5.1**).

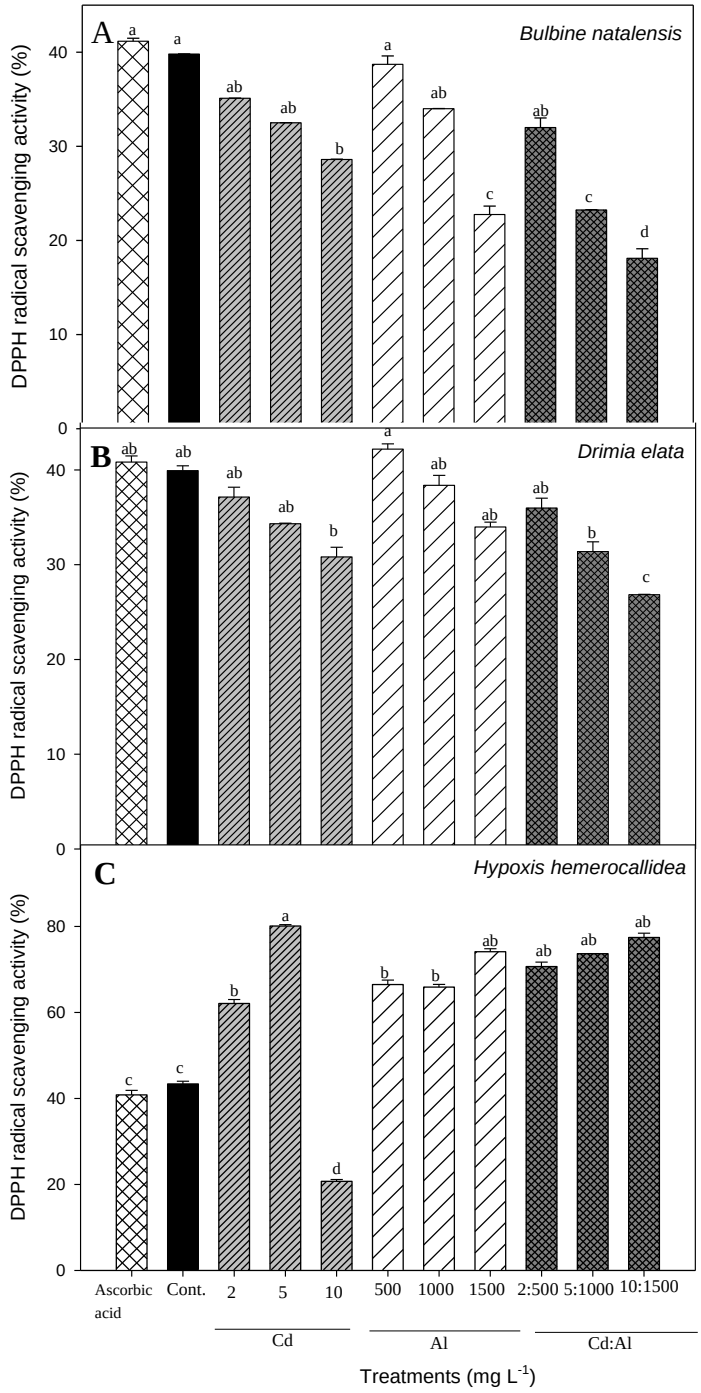


Figure 5.5 Antioxidant scavenging properties of bulb extracts of selected medicinal plants exposed to Cd and Al after six weeks of treatment. (+ve) = Ascorbic acid as positive control. Results are present as mean±SD (n=3). Different letters on bars indicate significant differences (p<0.05).

Table 5.1 Antibacterial activity (MIC mg mL^{-1}) in the bulb/corm extracts of the investigated medicinal plants exposed to Cd and Al treatments after six weeks (n=3). Standard = Neomycin (2 µg mL^{-1}).

Plant species/part	Part	Bacteria strains		Heavy metal treatment (mg L^{-1})								
			Control	Cd			Al			Cd:Al		
				2	5	10	500	1000	1500	2:500	5:1000	10:1500
Bulbine natalensis	Bulb	*E. coli*	3.13	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13
		S. aureus	1.56	**0.78**	**0.78**	1.56	3.13	3.13	3.13	3.13	3.13	3.13
Drimia elata	Bulb	*E. coli*	**0.78**	**0.78**	1.5	6.25	**0.78**	3.13	6.25	3.13	12.5	12.5
		S. aureus	**0.39**	**0.78**	6.25	12.5	1.56	1.56	12.5	1.56	6.25	12.5
Hypoxis hemerocallidea	Corm	*E. coli*	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13
		S. aureus	**0.78**	**0.2**	**0.39**	**0.39**	**0.78**	**0.39**	1.56	**0.39**	3.13	3.13
Neomycin		*E. coli*	**0.39**									
		S. aureus	**0.20**									

E. coli = Escherichia coli, S. aureus = Staphylococcus aureus

Increasing concentrations of Cd and Al had a negative effect on antibacterial activity in *D. elata* as seen by increasing MIC values. There was an additive effect in the combined Cd and Al treatments with the highest MIC values recorded in these plant extracts (**Table 5.1**). *Hypoxis hemerocallidea* exposed to Cd at all the tested concentrations exhibited good activity against *S. aureus* and similar activity was recorded in Al-treated plants although at the highest concentrations there was poor antibacterial activity (**Table 5.1**). In the combined Cd and Al treatments only the lowest concentrations had good activity and increased heavy metal treatments exhibited poor activity.

5.5 Discussion

Plants have the ability to modify metabolic processes by synthesising and accumulating diverse groups of secondary metabolites including phenolics and flavonoids when exposed to heavy metals (**MICHALAK, 2006; XIONG *et al*., 2013**). In the present study, higher concentrations of Cd and Al down-regulated the synthesis and accumulation of phenolics and flavonoids in *D. elata* compared to the control plants. A similar pattern in the compositions of phenolics and flavonoids were recorded in *B. natalensis* although to a lesser extent. These findings are consistent with a previous study which reported a decrease in the accumulation of secondary metabolites in sunflower treated with Cd (**GROPPA *et al.,* 2001**). In *H. hemerocallidea,* there was up-regulation in the synthesis and accumulation of phenolics at moderate treatment with Cd and Al but the highest treatments with heavy metals down-regulated phenolic accumulation.

Phenolic and flavonoid accumulation in the event of abiotic stress is intended to scavenge free radicals induced by metallic ions by preventing deleterious molecules from eliciting oxidative damage to the membrane components, thereby maintaining the membrane integrity (**MICHALAK, 2006**). Flavonoids can directly scavenge molecular species of active oxygen due to its ability to donate electrons or hydrogen atoms (**BORS *et al.,* 1990; RICE-EVANS *et al.,* 1997**). This implies that continuous exposure to heavy metal stress will require more electron donors from flavonoids or phenolics in order to curb the oxidative stress thereby leading to breakdown of these compounds. This could probably be one of the reasons for the lower amounts of

phenolics and flavonoids recorded in *B. natalensis* and *D. elata* with increasing heavy metal concentrations. Plant species investigated in the present study responded differently to heavy metal stress in terms of phenolic and flavonoid accumulation. This is an indication that tolerance to heavy metal stresses are species-dependent. Exposure to Cd and Al concentrations for six weeks could have potentially perturbed the essential biosynthetic pathways or resulted in a loss of specific enzymes which resulted in a decrease in the accumulation of phenolics and flavonoids in *B. natalensis* and *D. elata*. Cd exhibits its phytotoxicity by suppressing the activities of metabolising enzymes in plants (**KABATA-PENDIAS, 2011; HOSSAIN *et al.,* 2012**). Perhaps the decrease in phenolics and flavonoids could be as a result of reallocation of resource to protect the plant against the heavy metal stress and maintain the primary metabolic processes.

This is the first report on quantitative measurement of the effects of heavy metals on the synthesis and accumulation of hypoxoside in *H. hemerocallidea.* HPLC analysis revealed that biosynthesis of hypoxoside was significantly reduced in all the Cd and Al treatments in *H. hemerocallidea* compared to the control plants after six weeks. This finding was consistent with a previous study where exposure to Cd significantly reduced scutellarin content in *Erigeron breviscapus* (**XIONG *et al*., 2013**). The progressive decrease in the amounts of hypoxoside with increasing heavy metal treatments indicated a loss in the biosynthetic pathways or reallocation of resources to sustain the primary metabolic processes. The lowest concentrations of Al stimulated various growth parameters (Section 4.3.1.1) compared to the control but significantly down-regulated the synthesis of hypoxoside compared to the controls. This indicates that the presences of Al at moderate concentrations in the soils can improve biomass of *H. hemerocallidea* but decrease the synthesis of some essential secondary metabolites. Ultrastructural changes including modification in the shape and size of chloroplasts reported in Section 4.3.8.3, provided some possible explanation for the decrease in the biosynthesis of secondary metabolites (hypoxoside) as these alterations have detrimental effects on the overall metabolic processes in plants leading to down-regulation of certain biosynthetic pathways as recorded in the present study. Information on changes of secondary metabolites in medicinal plants exposed

to heavy metal stress is still limited, hence there is need for more data on plant response to heavy metal stress in order to ensure quality of medicinal plant products.

There was a progressive decrease in the DPPH activity with increasing concentrations of Cd and Al in *B. natalensis* and only the lowest concentrations of Al-treated plants had good DPPH activity similar to the positive control. Crude extracts of *B. natalensis* have previously been reported to have poor DPPH activity although the isolated knipholone from this plant species has potent DPPH activity (**LAZARUS, 2011**). A similar pattern in DPPH activity was recorded in *D. elata* where increasing concentrations of heavy metals decreased antioxidant properties. The detoxification potentials of plant antioxidant agents decrease with prolonged exposure to stress conditions (**ABBASI *et al.,* 2011**). The results of the present study indicate that increasing heavy metal concentrations down-regulated the synthesis and accumulation of the antioxidant compounds leading to significant decreases in DPPH activity in *B. natalensis* and *D. elata*.

All the *H. hemerocallidea* extracts showed a propensity in quenching DPPH, as shown by a progressive percentage increase in radical scavenging activity with increasing heavy metal concentrations in all the treatments. All the heavy metal-treated plants showed a significant DPPH activity compared to a known DPPH scavenger (ascorbic acid) and the control plants. However, at the highest concentrations of Cd significantly reduced the activity of DPPH. Good DPPH activity corresponded to a rapid decrease in light absorbance in the presence of a plant extract, indicating high antioxidant potency of the extracts in terms of electron or hydrogen atom-donating capacity (**AMAROWICZ *et al.,* 2004**). In the event of heavy metal stress, plants tend to synthesis a number of secondary metabolites that act as antioxidant agents scavenging free radicals leading to oxidative damages to biomolecules (**DAI *et al.,* 2012**). However, in the present study there was variability in the antioxidant properties in all the investigated plant species.

Crude extracts and isolated compounds from *H. hemerocallidea* have been shown to possess strong antioxidant properties both *in vitro* and *in vivo,* indicating that this plant is a prime candidate as a source of antioxidant agents (**DREWES *et al.,* 2008; NCUBE**

***et al.,* 2013**). Phytochemical analysis showed that glycosides are the main constituents of *Hypoxis* species characterised by a common pent-1-en-4-yne backbone or a slight modification of it (**NCUBE *et al.,* 2013**). The bioactive compound hypoxoside isolated from *H. hemerocallidea* which is converted to rooperol upon enzymatic hydrolysis has a unique structural similarity to nordihydroguaiaretic acid which is a strong antioxidant (**VAN DER MERWE *et al.,* 1993; BOUKES AND VAN DE VENTER, 2012**). In the present study, exposure to Cd and Al stresses decreased the synthesis of hypoxoside in *H. hemerocallidea* but increased the antioxidant properties in a dose-dependent pattern. One of the possible explanations for the increase in DPPH activity recorded in the present study could be due to an increase in the amounts of carotenoids reported in Section 4.3.6. Carotenoids have potent radical scavenging properties and good cooperative effects with other antioxidants (**STAHL AND SIES, 2003**). Synergistic effects between carotenoids and other antioxidant compounds could be the reason for the increasing DPPH activity in the present study in *H. hemerocallidea.*

Variable antibacterial activity was recorded between the three investigated plant species. Of all the extracts tested, only the extracts from the lowest concentrations of Cd and Al treatments in *D. elata* had good activity against *E. coli.* Pharmacological properties of *D. elata* have been linked to the presence of proscillaridin A (**LUYT *et al.,* 1999**). The poor antibacterial activity recorded in most of the extracts of *D. elata* could be due to loss in the synthesis of this bioactive compound, particularly with increasing heavy metal concentrations. In a previous study, **YAKUBU *et al.*** (**2012**) reported good antibacterial activity in crude extracts of *B. natalensis* against a number of microbial strains including *E. coli* and *S. aureus.* The purported antimicrobial activity in *B. natalensis* was linked to the presence of the bioactive compound knipholone (**YAKUBU *et al.,* 2012**). In the present study, extracts of *H. hemerocallidea* exhibited the best antibacterial activity against *S. aureus* in almost all the tested concentrations of heavy metals. Exposure to heavy metals induced the synthesis of various secondary metabolites, either for tolerance or as a by-product of some physiological process (**NOORI *et al.,* 2012**). This could be responsible for the good antibacterial activity recorded in some of the extracts. Previous studies have shown that *S. aureus* is more susceptible to antibiotics than *E. coli* and this could have been the reason for

the good activity recorded against *S. aureus* in most of the tested extracts. Only the extracts from *D. elata* at low concentrations of Cd and Al had good activity against *E. coli.*

5.6 Conclusions

The results of the present study showed that plant response to heavy metal stresses is species-dependent. This was evident in the variable amounts of total phenolics and flavonoids recorded in the present study. This study is the first report to measure the effects of heavy metals on the synthesis and accumulation of hypoxoside in *H. hemerocallidea*. The results of the present study clearly showed that prolonged exposure to Cd and Al stresses perturbed the quality and efficacy of medicinal properties in both *B. natalensis* and *D. elata* but enhanced biological activity in *H. hemerocallidea.* There is an urgent need for safe and sustainable cultivation of these valuable medicinal plants to improve the quality of medicinal products. The **DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES** (**2012**) recommended that agrochemicals should not be used during cultivation of *H. hemerocallidea*. The result of the present study confirm this recommendation that exposure to chemical residues affects the biosynthesis of hypoxoside. Further studies will be needed to investigate the effects of other environmental stresses such as drought, salinity and acidity on the biosynthesis of *H. hemerocallidea* and the other investigated plant species.

Chapter 6

General conclusions

Rapid industrialization and urbanization has led to large-scale pollution of the environment, most especially contamination of heavy metals in the soil. Medicinal plant gatherers harvest plant material indiscriminately in order to meet the high demands for plant products thereby over exploiting the wild population. Thus there is high possibility of harvesting plants from heavy metal contaminated soils. The use of inappropriate methods of collection, storage and processing are additional sources of heavy metal contamination in medicinal plant products. Heavy metal contamination of these plant products compromises the safety, efficacy and quality of medicinal plant products available to consumers.

The trading environment of the traditional medicine market in South Africa is a public health concern with little policy or regulation on good manufacturing practices, procurement and sale of medicinal plant products. It is of paramount importance that National policies standardize the trading environment for traditional medicine markets in South Africa. To date, there is limited data on heavy metal toxicity resulting from the use of medicinal plant products largely because consumers usually dismiss any association between the use of traditional medicine and adverse reactions. This leads to underreporting of adverse reactions. The paucity of scientific data regarding toxicology of locally used medicinal plant products is very high in developing countries (**NEERGHEEN-BHUJUN, 2013**).

This study investigated the levels of heavy metals in some of the most frequently used South African medicinal plants. A comparative multi-elemental screening of medicinal plants sold at outdoor-street markets and *muthi* shops was carried out to elucidate the pattern of heavy metal contamination in plant products.

The composition of elements varied in plant materials obtained from outdoor-street market compared with *muthi* shops products indicating the different source that medicinal plant traders obtained their products from as well as the possibility of the

products being expose to different forms of heavy metal contaminations. High levels of As and Hg were detected in most of the samples, above the WHO safety limits except for *Cassine transvaalensis* which had low amounts of As. Elevated levels of Al and Fe were recorded in most of the samples investigated. Extremely high levels of toxic elements in most of the investigated medicinal plants pose serious safety concern to consumers. The use of medicinal plant products contaminated with high levels of heavy metals can adversely affect consumer's health leading to conditions such as renal or liver failure. For instance, Cd was detected at a very low concentrations in most of the samples however, prolonged use of these medicinal plants by patients could result in accumulative effects of Cd as it can elicit toxicity even at a very low dose in the human body.

One of the limitations of the present study was that samples were only obtained from Pietermaritzburg traditional medicine markets and not countrywide. Hence, it will be difficult to generalize about the trend of heavy metal contamination in medicinal plant products sold in all South African traditional medicine markets. The likelihood is that medicinal plant products sold at both outdoor-street markets and *muthi* shops could have be harvested from different localities hence, the present study cannot conclusively suggest whether it is safer to buy medicinal plant products from outdoor-street markets or *muthi* shops. A more in-depth assessment of heavy metals in medicinal plant products is needed in this regard.

The inconsistency in terms of total phenolic and flavonoid compositions as well as variability in the antibacterial activity in medicinal plants obtained from outdoor-street markets and *muthi* shops indicates that plant material could have been harvested from different locality, at different stages of maturity and/or seasons. There is need to ensure consistency of phytochemical compositions of medicinal plant products sold at traditional medicine markets in order to ensure quality and safe products. Hierarchical cluster analysis used in this study proved to be a valuable tool in preliminary screening of heavy metals in medicinal plants which can be used to develop a large database for easy monitoring of plant species with hyperaccumulative potentials.

Interactive effects of Cd and Al on their uptake and distribution as well as effects of heavy metal stress on quality and efficacy in *Bulbine natalensis, Drimia elata* and *Hypoxis hemerocallidea* were investigated. Plant response to heavy metal stresses appeared to be species-dependent. This is evident as exposure to high levels of Cd and Al induced significant growth inhibition. Thus the decrease in the yield of these plant species when grown on heavy metal contaminated soil would put pressure on wild populations due to high demands for these medicinal plant species. Interactive effects of Cd and Al elicited a number of toxic effects on plant physiology and biochemistry. These include accumulation of free-proline, increase in antioxidant defence enzymes, changes in photosynthetic capacity, alterations in phytochemical composition as well as changes in metabolic processes that were dissimilar in the three medicinal plant species investigated. Ultrastructural changes such as complete breakdown of thylakoids at the highest treatments with Cd, changes in cell organization and cell well architectural were recorded in *H. hemerocallidea*.

The ability of the three medicinal plants to accumulate elevated levels of Cd and Al in their bulbs and/or corms which was found to be above the WHO safety limits is a safety concern. The bulbs and/or corms of the investigated medicinal plants are the most extensively utilized part in traditional medicine. Ingesting of Cd induces various toxic effects in humans at low doses and the primary target organs are the kidneys and liver. Intake of high doses of Al can impair cognitive and speech functions and may lead to neurodegerative diseases. Hence, the high levels of Cd and Al accumulations in the bulbs and/or corms of the investigated medicinal plants harvested from heavy metal contaminated soil raises concern for safety of the products. The findings presented in this thesis showed that accumulation of heavy metals in medicinal plants perturbed metabolic processes thereby leading to reduction of secondary metabolites including phenolic and flavonoid contents in all the investigated plants. In *H. hemerocallidea,* there was a significant reduction in the accumulation of hypoxoside in plants exposed to heavy metal treatments. There is need for an in-depth research on metabolic response of medicinal plants to heavy metal stress in order to ensure the quality and efficacy of medicinal products.

Cultivation of medicinal plants is gaining popularity among small-scale farmers with increasing government support programmes such as “Farm-to-Pharm Grand Challenge” which projected that by 2018, South Africa will be one of the top three emerging economies in the global pharmaceutical industry (**DEPARTMENT OF SCIENCE AND TECHNOLOGY, 2007**). The results of this thesis provide valuable information regarding heavy metal contamination in traditional medicinal plants and the need to improve safe and sustainable cultivation of important medicinal plants through proper site selection of soils free of toxic contaminants as well as effective application of GACP. This will ensure a safe medicinal products devoid of toxic contaminants thereby improving the consistency in terms of phytochemical composition of cultivated medicinal plants

The results of the present study emphasize the need for immediate government interventions to improve the trading environment of traditional medicine markets as well as stringent regulation and monitoring of heavy metals in medicinal plant products sold at traditional medicine markets in South Africa in order to protect the general public.

It is suggested that pharmacovigilance must be done to improve the quality, safety, and efficacy of medicinal plant products not only during their growth, but also during their sampling, processing and storage by traditional healers in order to avoid heavy metal contamination. A periodic assessment of medicinal plant products sold at South African traditional medicine markets should be encouraged as this will assist in predicting the safety assurance and monitoring the trend of heavy metals in medicinal plant products. This can be achieved by developing rapid and user friendly techniques for assessing medicinal plant products to ensure the safety and quality of medicinal plant products sold at traditional medicine market. It is also important to educate everyone involved in traditional herbal medicine about the risk of heavy metal contamination in order to improve the quality, safety, and efficacy of medicinal plant products.

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Appendix A – Hoagland's Nutrient Solution

Hoagland's nutrient solution was prepared with chemicals of analytical grade as presented in Table A. Stock solutions were keep in the dark at 10 ˚C until required. Hoagland's nutrient solution was prepared using 5 mL of each stock and made up to 1.5 L with distilled water to give a 50% Hoagland's nutrient solution.

Table A. Composition of stock solutions used to prepare Hoagland's nutrient solution (**HOAGLAND and SYNDER, 1933**).

Stock	Concentration
$Ca(NO_3)_2 \cdot 4H_2O$	0.75 M
$CuSO_4 \cdot 5H_2O$	22 µM
H_3BO_3	7.0 mM
H_2MoO_4	16 µM
KH_2PO_4	0.15 M
KNO_3	0.75 M
$MgSO_4 \cdot 7H_2O$	0.30 M
$MnCl_2 \cdot 4H_2O$	1.37 mM
NaFeEDTA	2.3 mM
$ZnSO_4 \cdot 7H_2O$	0.12 mM

Appendix B – HPLC Peak Area of hypoxoside

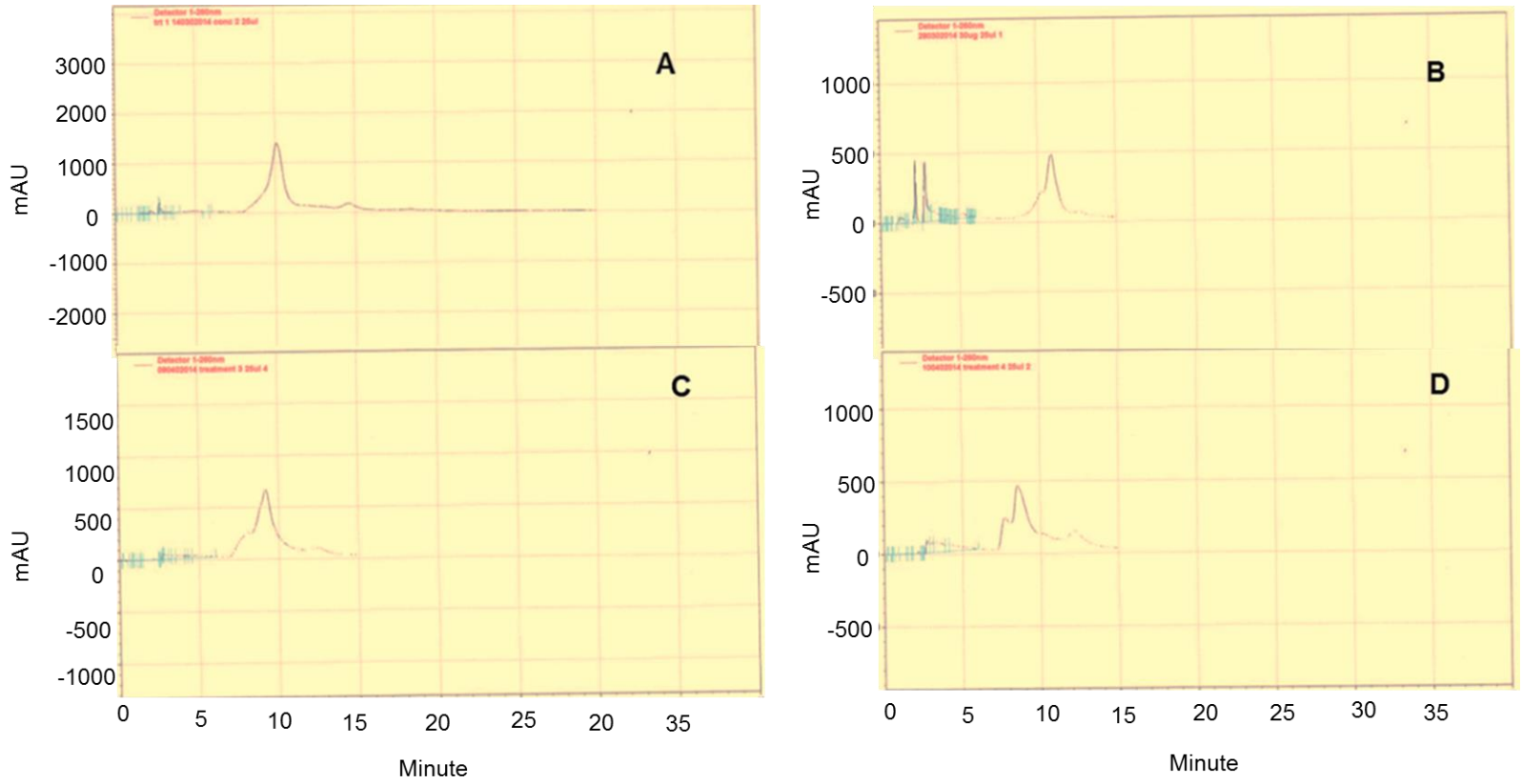

Figure A Peak area of hypoxoside in the extracts of *H. hemerocallidea* after six weeks of exposure to Cd. A = Control plant, B = Cd 2 mg L^{-1}, C = Cd 5 mg L^{-1} and D = Cd 10 mg L^{-1}.

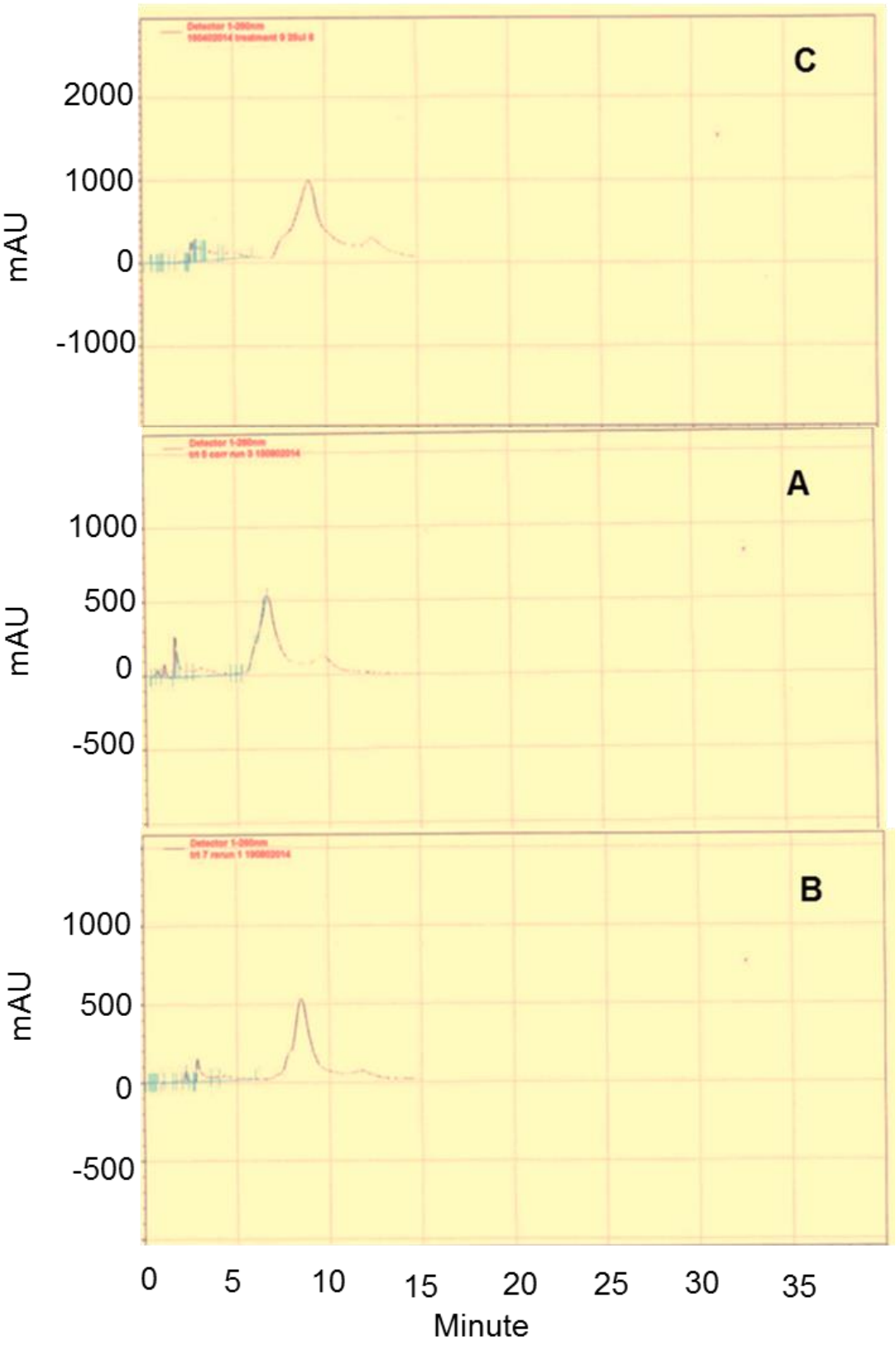

Figure B Peak area of hypoxoside in the extracts of *H. hemerocallidea* after six weeks of exposure to Al. A = Al 500 mg L^{-1}, B = Al 1000 mg L^{-1} and C = Al 1500 mg L^{-1}.

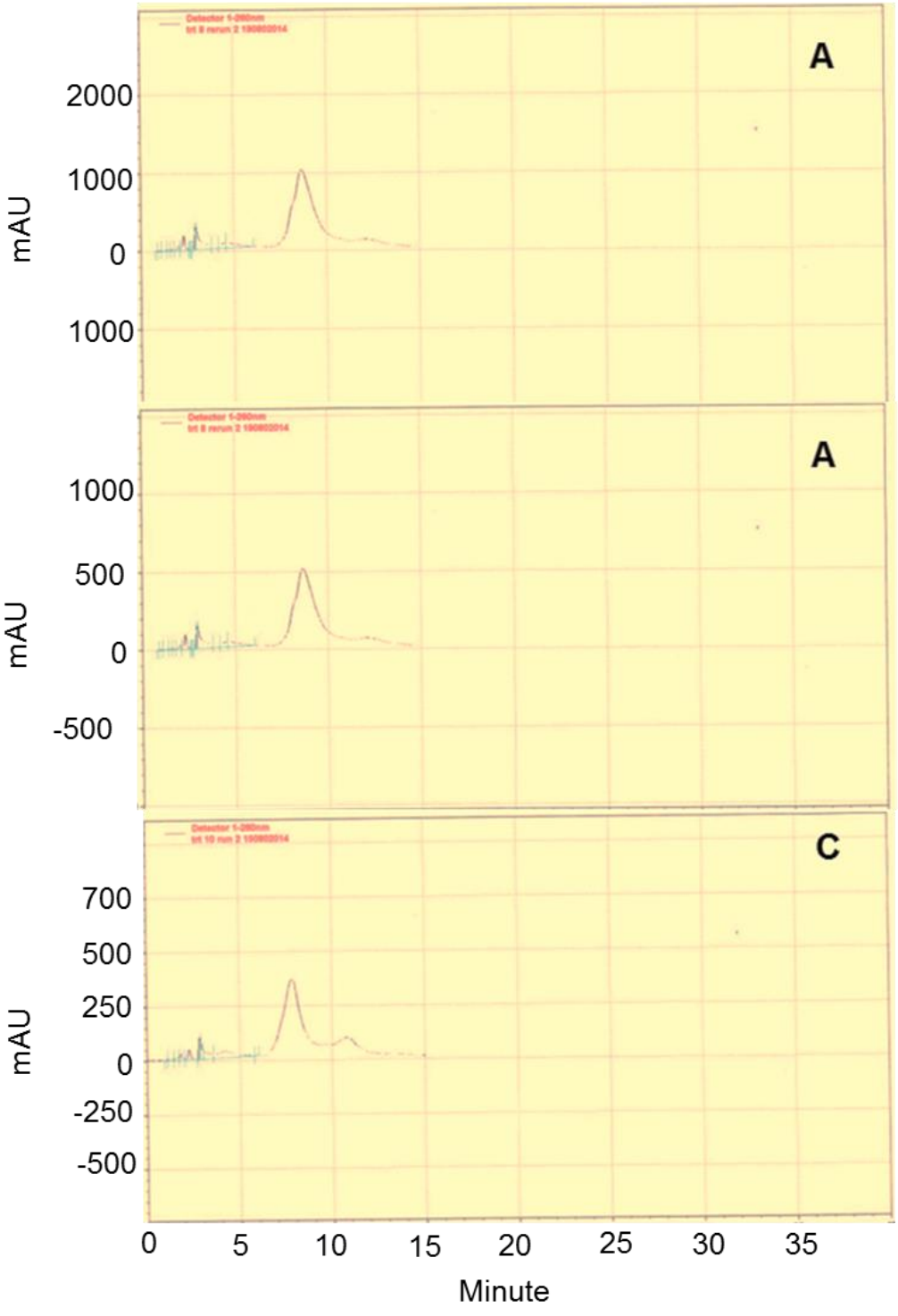

Figure C Peak area of hypoxoside in the extracts of *H. hemerocallidea* after six weeks of exposure to combined treatment of Cd and Al. A = Cd 2:Al 500 mg L^{-1}, B = Cd 5:Al 1000 mg L^{-1} and C = Cd 10:Al 1500 mg L^{-1}.