Heavy metal content absorption and medicinal potential of *Egeria densa* (Planch.) Casp.

By

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Submitted in fulfilment of the requirements for the degree of
Master of Science in Botany

Faculty of Science and Agriculture

UNIVERSITY OF FORT HARE, SOUTH AFRICA

YEAR: 2014

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ABSTRACT

The contamination of heavy metals in the environment is a looming concern worldwide. *Egeria densa* (Planch) (Submerged aquatic plant) from two ponds: Site A with co-ordinates (32º 48’22.04”S; 26°48’58.79” E) and Site B with co-ordinates (32°48’33.25”S; 26°48’33.25”S) in Alice (Eastern Cape) was evaluated for its ability to absorb heavy metals, phytochemical constituents, antimicrobial activity and ultra-structure using standard analytic procedures. Cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), and zinc (Zn) were measured in water, sediments and plant. The concentrations of these metal elements were determined with use of Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES). In sediments, the heavy metals (mg/kg) decreased in the order of their average concentration as follows: Fe (40.320) > Zn (1.259) > Pb (0.564) > Mn (0.186) > Cu (0.037) in Pond 1 whereas in Pond 2 Fe (61.527) > Cd (0.999) > Mn (0.648) > Pb (0.586) > Zn (0.156) > Cu (0.045). The highest concentration of Fe was detected in both sites and Cu being the least. The concentrations of the metals in the plants sample (from Pond 1) were found in order of Mn > Pb > Cu > Fe whereas cadmium and zinc were not detected, while the concentration in Pond 2 decreases in order of Zn > Mn > Pb > Cd > Fe > Cu.

In the water samples, concentrations of heavy metals (mg/L) decreased in the order of their average concentrations as follows: Pb (35.36) > Fe (3.07) > Mn (0.238) > Cu (0.104), both cadmium and zinc were below the limit of detection in Pond 1, whereas in Pond 2 the

concentrations decreased as follows: Pb (13.033) >Fe (1.69) > Cu (0.270) > Mn (0.248) > Cd (0.004) and Zinc was not detected.

Phytochemical analyses of the plant extracts revealed the presence of phenols, flavonoids, proanthocyanidin, flavonols, saponins, alkaloid and tannins in all the extracts (water, acetone and n-hexane). Both acetone and water extracts, showed high concentration of proanthocyanidin, while tannin was the lowest in acetone extract.

Antimicrobial evaluation using, Gram positive (*Staphylococcus aureus*, *Bacillus pumilus*, *Bacillus cereus*, *Streptococcus pyogenes*, *Enterococcus faecalis*) and Gram negative (*Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Serratia marcescens*) bacteria showed negative results for all the strain, except *Streptococcus pyogenes* which was inhibited at MIC of 0.1 mg/ml. Scanning electron microscopy (SEM) of ultra-structure of *Egeria densa*, showed that certain bacteria attached to the leaf, However more work has to be done on *E. densa* to verify the mechanism by which it accumulates heavy metals. The study shows that *E. densa* has a potential of accumulating heavy metals especial Manganese in plant.

DECLARATION

No element of the work described in this dissertation, except where otherwise acknowledged, has been previously submitted for a degree at this or any other institution. The work in this dissertation has been performed entirely by author.

Signed by…Mgobozi Vuyokazi at the University of Fort Hare this ……14th……..day of the month of……April……..2014

Signature ……

ACKNOWLEDGEMENTS

I am most grateful to the Most High God, the Creator and the source of life for giving me strength and who has seen me through this journey despite all odds. First and foremost, I wish to express my unalloyed and unreserved gratitude to my Supervisor, Prof AJ Afolayan. Despite your very busy schedule, you still found time to provide very sound, logical and constructive criticisms that made this project to see the light of the day. I really appreciate your guidance and mentorship and I say God bless you. My special thanks go to Dr. Gloria Otunola for her invaluable assistance and excellent skills in proof reading as well as providing sound advice in structuring my dissertation.

I would also like to thank Mr Thando Ngqagweni of the Department of Soil science, for his assistance in elemental analysis and for spending his precious time helping me run my samples. I also appreciate his efforts in helping me with instrumental difficulties and for providing analytical advice. My profound gratitude also goes to the members of Phytomedicine Research Centre and to Botany Department at large for their support, input and contributions. I gratefully acknowledge the funding by the GMRDC (Govan Mbeki Research and Development Centre) that enabled me to do my postgraduate studies at the University of Fort Hare.

Finally, I would like to thank my family for their continuing support, understanding, encouragement and love.

DEDICATION

To my late father Mr S Mgobozi.

His words of inspiration and encouragement in pursuit of excellence, still lingers on.

List of figures

TITLE **PAGE**

LIST OF TABLES

Title **Page**

Table of contents

CHAPTER ONE

1.0 Introduction

One of the major environmental and human health problems worldwide is the increasing contamination of freshwater systems with thousands of industrial and natural chemical compounds (Ensley, 2000; Schwarzenbach *et al*., 2006). Although heavy metals, the major contaminants of the environment are generated by natural activities, almost all human activities also have the potential to produce heavy metals. Removal of these contaminants into non-contaminated areas as dust or leachates through the soil and spreading of heavy metals containing sewage sludge are a few examples of events contributing towards contamination of the ecosystems (Gaur and Adholeya, 2004).

Numerous methods are already in use to clean up the environment from heavy metal contamination, but most of them are costly and not too effective. Remediation of contaminants in the environment involves either on-site management or excavation and subsequent disposal to landfill site. According to Iqbal and Gupta (2009), this method of disposal shifts the contamination problem elsewhere along with the hazards linked with transportation of contaminated soil and migration of contaminants from landfill into adjacent environment. Soil washing for removing contaminated soil is an alternative way to excavation and disposal to landfill, but this method is also very costly and produces a residue rich in heavy metals which will require further treatment (Gaur and Adholeya, 2004).

Recently there is a huge interest in developing cost effective and environmentally friendly technologies for the remediation of soil and water polluted with toxic heavy metals (Zayed *et al*., 1998). Values of metal-accumulating plants to wetland remediation

have recently been realized (Black, 1995). Some of these techniques are environmentally friendly, cost-effective and aesthetically pleasing. Below is the definition of different phytoremediation processes.

Definition of terms

Phyostabilization: Plants reduce mobility and phytoavailibilty of contaminants in the environment. This process does not remove pollutants from contaminated sites but reduces m **Phytostabilization**: Plants prevent contaminants from migrating by reducing runoff, surface erosion, and ground water flow rates (Dary et al., 2010). obility and excludes metal from plant uptake (Vamerali *et al*., 2010).

Phytoextraction, also called phytoaccumulation, refers to the plant uptake of metal contaminants through the roots into the upper portion of the plant (stems and the leaves) (Salt *et al*., 1995; Saraswat and Rai, 2009; Maine *et al*., 2004; Ampiah-Bonney *et al*., 2007).

Phytovolatilization; Hyperaccumulating plants uptake pollutants from soil and water, and translocate to the aerial parts of the plants, and volatilize the pollutants in the air.

Phytovolatization: is the uptake and transpiration of a contaminant by a plant, with release of the contaminant or a modified form of the contaminant to the atmosphere from the plant (Di Lonardo *et al*., 2011).

Phytodegradation also called phytotransformation, is the breakdown of contaminants taken up by plants through metabolic processes within the plant.

Rhizosphere biodegradation: The breaking of contaminants in the soil through microbial; activity which is enhanced by the presence of rhizosphere. This is sometimes referred to as enhanced rhizosphere biodegradation, phytostimulation or plant-assisted bioremediation/degradation.

Rhizofiltration is the adsorption or precipitation onto plant roots or absorption of contaminants into the roots which are in solution surrounding the root zone (Walton and Anderson, 1990).

The use of aquatic plants as wastewater phytoremidiants has been investigated by many authors. Some examples of promising floating aquatic plants that have been used as phytoremidiants include *Lemna minor*, *Eichhornia crassipes*, *Pistia stratiotes* and *Salvinia herzogii* (Zayed *et al*., 1998; Zhu *et al*., 1999; Maine *et al*., 2001, 2004). Submerged plants are completely inundated and have the ability to take up metals directly from the water; therefore, they can be useful species in reducing heavy metal concentrations in storm water and secondarily treated wastewaters (Fritioff *et al*., 2005; Keskinkan, 2005). According to Guilizzoni (1991) and Keskinkan (2005) the use of submerged aquatic macrophytes for wastewater treatment and heavy metal uptake with species such as *Potamogeton* sp., *Ceratophyllum demersum* and *Myriophyllum spicatum* have already been undertaken (Goulet *et al*., 2005; Fritioff and Greger, 2006; Rai *et al*., 1995, Keskinkan, 2005). This study therefore, investigates the potentials of using *Egeria densa* (a submerged plant) for phytoremediation using two ponds located at different points in Alice, Eastern Cape Province, South Africa.

1.2 Justification and Significance of the study

The presence of heavy metals in the environment is undesirable even in low quantities. Heavy metals occur naturally in the environment as part of the earth's crust and pose a threat to both human health and the natural environment. This is due to the fact that unlike many substances, metals are not biodegradable and hence accumulate in the environment.

Anthropogenic activities such as mining and smelting of ores, burning of fossil fuels, disposal of industrial wastes and the processing of raw materials for manufacturing result in the subsequent deposition of heavy metals to the environment (Lobban and Harrison, 1997). Runoff water is the major transporter of heavy metals in the environment and metals are eventually deposited on land or other water bodies including dams and oceans. Furthermore rainwater carries vast amounts of cadmium (Cd), copper (Cu), Zinc (Zn) and lead (Pb) from the land to water bodies (Lobban and Harrison 1997).

Survey carried out since the 1970s indicate that aquatic macrophytes play a vital role in heavy metal accumulation from the environment. They can be reliable indicators of metal pollution in freshwater ecosystems (Dietz, 1973; Flip *et al* 1979; Czuba and Mortimer, 1975; Tremp and Kohler, 1995). Macrophytes take up pollutants via their roots submerged in sediments and also absorb chemicals from the water column through their leaves (Mal *et al*., 2002). According to Duarte *et al*., 1994, submerged plants have major effects on productivity and biogeochemical cycling in aquatic

environments because they represent a link between the sediments and the overlying water.

Egeria densa is a popular aquarium plant that is often sold as ‘Anacharis’. *E. densa* is a perennial that is rooted and grows underwater; however, flowers are borne above the water surface. *E. densa* is characterized by its slender stems and whorls of strap-like leaves that have fine serrations along the margins.

E. densa, a rooted submerged plant can absorb nutrients from both water and the sediments, but it is generally considered that sediments are the main source of both phosphorus and nitrogen (Carpenter and Lodge, 1986; Barko *et al*., 1991). It is therefore the purpose of this research to determine the potential use of *E. densa in* bio-monitoring environmental concentrations of metals in the two selected ponds. This information will become a guide to the people using the water from the ponds, especially for domestic and irrigation purposes, because the presence of these elements in the water can cause contamination vegetables and harm humans through direct ingestion of contaminated material or through accumulation in the tissues of other organisms that are eaten by humans.

1.3 Objectives of the study

Water is one of the most important resources, which covers almost three-quarters of the planets, and upon which all life depends. Indiscriminate use and misuse of water sometimes makes it unfit for human consumption. A survey of past literature reveals that many absorption media have been used successfully to remove contaminants from

potable and wastewater. Therefore the aim of the study is to evaluate the heavy metal absorption potential of *E. densa* (Planch.) Casp.

The specific objectives are:

i. To view the ultra-structure of *E. densa* using the Scanning Electronic Microscope

ii. To investigate the phytochemical constituents of *E. densa*

iii. To determine the antimicrobial activity of *E. densa* in any

iv. To analyze *E. densa* whole plant, water and sediments from the selected two ponds for heavy metal concentrations using the Inductively Coupled Plasma – Optical Omission Spectrometry (ICP- OES) Varian 710-ES series

Figure 1.1 (a) *Egeria densa* in water source: Mgobozi,2013

Figure 1.1 (b) Egeria densa Source: Mgobozi, 2013

CHAPTER TWO (Literature review)

2.1 Introduction

Pollution of the natural environment by heavy metals is a worldwide problem which has been reported to be one of the major threats to aquatic ecosystems (Apsimon *et al*. 1990; Baeyens *et al*., 1998). Aquatic environments are more susceptible to the harmful effects of heavy metal pollution because aquatic organisms are in close and prolonged contact with the soluble metals. Aquatic sediments serve as a reservoir for heavy metals in aquatic environment and can be sensitive indicators for monitoring contaminants in aquatic environments. Sediments therefore deserve special consideration in the planning and design of aquatic pollution research studies. Phytoremediation, a plant based green technology is increasingly receiving attention because of the discovery that certain plants accumulate, high concentration of certain toxic element in their above ground harvested parts and therefore could be used as a biological toxicants removal from the environment.

2.2 Heavy Metals

Heavy metals are groups of metallic elements with atomic weights greater than 40 and are characterized by similar electronic distribution in their outer shell (Rand, 1985). They are found throughout the earth, in rocks, soils and sediments in stable forms and are able to be in circulation as a result of natural process such as weathering and erosion. Some of the heavy metals are essential in enzymatic activities and are considered as nutrients for example

: Iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn), while some are non-essential and are highly toxic e.g. cadmium (Cd) and lead (Pb). It is important to note that essential metals can be toxic at elevated concentrations (Clark, 1997). A number of heavy metals are not only toxic but

also tend to bio accumulate and persist in living systems (Clark, 1997). Langston and Spence (1995) reported that the toxic effects vary from one organism to the other and from one metal to the other. Unlike organic wastes, heavy metals are not easily biodegraded by bacteria and even if they are, the rate is so slow that they could as well be considered as permanent additives to the marine environment (Clark, 1997).

2.3 Heavy Metal Pollution in Water

Metals are introduced into marine ecosystems as a result of natural physical and chemical processes such as weathering, volcanic activities, erosion, leaching of soils, wind-blown dust and forest fires (Clark, 1997). It is a well-known fact that in the absence of anthropogenic sources, heavy metals are maintained and distributed in constant ratios in time in the geological cycles (Nieboer and Richardson, 1980; Kruus *et al*., 1991). This stability becomes destabilized once there is an increase in the loading of heavy metals into the environment from anthropogenic sources.

In the recent past, anthropogenic sources such as metal ore refining, plating and vehicle industries, food processing, domestic waste discharge and sewage treatment discharges have played a leading role in the introduction of metals to marine ecosystems. According to several authors, the introduction of heavy metals into the environment and aquatic systems by anthropogenic sources has been so extensive that it has surpassed natural sources (Salomons and Forstner, 1984; Nriagu, 1990).

All heavy metals exist in surface waters in colloidal, particulate, and dissolved phases, although dissolved concentrations are generally low (Kennish, 1992). The colloidal and particulate metal may be found in 1) hydroxides, oxides, silicates, or sulfides; or 2) adsorbed to clay, silica, or

organic matter. The soluble forms are generally ions or unionized organometallic chelates or complexes. The solubility of trace metals in surface waters is predominately controlled by the water pH, the type and concentration of ligands on which the metal could adsorb, and the oxidation state of the mineral components and the redox environment of the system (Connell *et al*., 1984).

Transport of heavy metals is primarily controlled by the water column while their distribution is controlled by sediments. Whether a metal will be transported as colloidal, dissolved ionic or dissolved complex forms will depend on the physico-chemical composition of the water column (Luoma, 1996). Whether metals will be mobilized from sediments or not usually depends upon the physical texture and chemical nature of the sediments, which as reported by Kramer and Duinker, (1983) determines the amount and the strength of metal binding. Once the metals are released into aquatic environments, they are distributed in the various compartments. The target compartment depends on the affinity of the metal for that compartment and the prevailing environmental parameters.

2.4 Heavy Metal Pollution in Sediments

Sediment is a very important compartment in regard to accumulation of heavy metals. Carvalho et al. (1998) reported that sediment is the compartment where natural and anthropogenic chemicals accumulate while Förstner and Patchineelam, (1981) reported that there is usually higher concentration of heavy metals in sediments than in water. Aquatic sediments are known to act both as a sink and a source of metals in aquatic environment. As a sink, previous studies have shown that sediments eliminate pollutants from the aqueous phase as a result of settling down of suspended particles thus reducing the potential toxicity to pelagic aquatic organisms but this

exposes the benthic organisms to a higher risk Van Ryssen *et al*. (1998) ; (Huh *et al*., 1992). As a source, sediment acts like an enriched pool of metals that can potentially be accumulated by the benthic organisms (Campbell *et al.,* 1988) especially during high storms or when environmental conditions change from rainy to extremely dry.

Even though heavy metals are present in sediments throughout the coasts, these pollutants are generally significantly higher in number and concentration in sediments and embayment that are adjacent to the most populated and industrialized areas (Salomons and Föster, 1984) than in sediments that have little or no history of contamination (Hornberger et al., 1999). In natural dynamic environments, sediments continuously interact with the aqueous and other particulate compartments (i.e. suspended particles) through such processes as settling, adsorption, desorption and resuspension of chemicals. Of these processes, adsorption and desorption influences the fate, transport, bioavailability and toxicity of heavy metals in aquatic environments. Changes in pH and salinity in the water may also result into processes such as coagulation, flocculation or co-precipitation, which can lead into removal of dissolved heavy metals from the water column to sediments (Boyle et al., 1977).

2.5 Heavy Metal Accumulation in Plants

Numerous plants are capable of growing in soil with very high concentration of metals, extraction of these metals through their roots and concentrating to extremely high levels of metals in their tissues (Rascio and Flavia, 2011) Plants with exceptional metal-accumulating capacity are known as hyperaccumulator plants. Roots of the hyperaccumulator extract the metals from the soil at a higher rate, transfer it more quickly to their shoot, and store large amounts in the leaves and root (Hossner *et al*., 1998). The ability to hyperaccumulate toxic

metals compared to related species has been shownto be due to differential gene expression and regulation of the same gene in both plants. Over 500 species of flowering plants have been identified as having the ability to hyperaccumulate metals in their tissues. Extensively investigation of hpyeraccumulation of metals in various plant species has been made. It becomes clear that different mechanisms of metal accumulation, exclusion and compartmentation exist in various plant species.

Puschenreiter *et al* (2003) investigated chemical changes in the rhizosphere of hyperaccumulators *Thlaspi goesingense* and *Thlaspi caerulescens* and the metal excluder *Trifolium*. *arvense* with a rhizosphere bag experiment on the contaminated and non-contaminated soils. Hyperaccumulation and depletion of labile Zn in the rhizosphere were observed for *Thlaspi*. *goesingense* grown on the contaminated soil. In the non-contaminated soil, Zn was accumulated but labile Zn in the rhizosphere was not changed

Whiting et al. (2000) found that the plants from *Thlaspi*. *carerulescens* population that accumulated Cd also showed increased root biomass and root length after allocation into Cd-enriched soil, whereas plants from the population that did not accumulate Cd showed no such increase

2.6 Phytoremediation of Toxic Elements by Aquatic Macrophytes

Metals from the aquatic environment can be absorbed by aquatic macrophytes and they can be reliable indicators of metal pollution in freshwater ecosystems (Dean *et al*., 1972; Dietz, 1973; Erikson and Mortimer, 1975; Tremp and Kohler, 1995). Macrophytes take up pollutants via their roots submerged in sediments (Biernacki *et al.*, 1996; Salt, 1998) and also absorb chemicals from the water columnthrough their leaves (Biernacki et al., 1996). According to Mal *et al*.

(2002) the final metal concentration in plants is usually significantly larger than in the water column. Furthermore, measuring components of basic plant traits, such as growth, survival, or reproduction is typically quite simple and repeatable. Therefore macrophytes are potentially useful for biomonitoring environment concentrations of metal (Biernacki *et al*, 1996; Salts, 1998). The degree of metal uptake by some plants is largely dependent on the type of metal and the plant species involved (Mortimer, 1985).

2.7 Potential Aquatic Macrophytes for Heavy Metal Phytoremediation

Several studies have described the performance of heavy metals uptake by macrophytes. It is reported that phytoremediation technology is an alternative to treat heavy-metal contaminated side which will be more admitted in order to remediate the environment. Table 1 is a list of some aquatic plants that have been used to remediate trace element from water.

Table 1: List of some aquatic plants reported to have the potential for uptake of trace elements.

Common Names	Scientific names	Tracy elements	References
Duckweed	*Lemna gibba L.*	As, U, Zn	Fritioff and Greger (2003); Mkandawire *et al*. (2004a,b); Mkandawire and Dudel (2005)
Lesser duckweed	*Lemna minor L.*	As, Zn, Cu, Hg	Fritioff and Greger (2003); Kara (2004); Miretzky *et al.* (2004), Robinson *et al*. (2005), Alvarado *et al*. (2008), and Mishra *et al*. (2008)
Star duckweed	*Lemna trisulca L.*	Zn	Huebert and Shay (1992)
Water hyacinth	*Eichhornia crassipes*	.As, Fe, Cu, Zn, Pb, Cd, Cr, Ni, Hg	Wolverton and McDonald (1978), Muramoto and Oki (1983), Delgado *et al*. (1993), Vesk *et al*. (1999), Cordes *et al*. (2000), Chandra and Kulshreshtha (2004), Odjegba and Fasidi (2007), Alvarado *et al*. (2008), Espinoza-Quiñones *et al*. (2008), Junior *et al*. (2008), Mishra *et al*. (2008), Dixit and Dhote (2010)
Water-starwort	*Callitriche cophocarpa*	Cr(V)	Augustynowicz *et al*. (2010)
Petries starwort	*Callitriche petriei*	As	Robinson *et al*. (2005)
Common reed	*Phragmites australis*	Cr, Cu, Ni, Pb, S, V, Zn, Cd	Deng *et al*. (2004), Ghassemzadeh *et al*. (2008),Baldantoni *et al*. (2009)
Butterfly fern	*Salvinia rotundifolia*	Pb(II)	Banerjee and Sarker (1997); Dhir (2009)
	Salvinia natans	As, Ni, Cu, Hg(II)	Sen and Mondal (1987, 1990), Sen and Bhattacharyya (1993), Rahman *et al*. (2008c)
	Salvinia minima	As, Pb, Cd, Cr	Olguin *et al.* (2003), Hoffmann *et al*. (2004), Sanchez-Galvan *et al*. (2008)
		Cd, Cr	
Eared watermoss	*Salvinia herzogii*	Zn, Hg, Cr	Maine et al. (2004);Suñe *et al*. (2007)
	Salvinia auriculata	Cu, Zn, Mn,Cr, Pb	Molisani *et al*. (2006), Espinoza-Quiñones *et al.* (2008), Wolff *et al*. (2009)

Greater duckweed	*Spirodela intermedia* *Spirodela polyrhiza L.*	As, Hg	Miretzky *et al.* (2004)
Indian/sacred lotus	*Nelymbium speciosum* *Ludwigia perennis L.*	Cr, Cu, Ba, Ti, Co, Pb	Rahman et al. (2007, 2008b); Mishra *et al*. (2008)
Arrowhead	*Sagittaria sagittiflia L.* *Nymphoides ceristatum*		
Shoreline	*Sasuvium portulacastrum L.* *Nymphae stellata*	As, Cd, Pb, Hg, Cu, Zn	Vardanyan and Ingole (2006)
Water spinach	*Ipomoea aquatic*	Cu, Cd, Hg	Lee *et al*. (1991), Gothberg *et al*. (2002, 2004),
Eelgrass/eelweed	*Vallisneria spiralis L.*	As, Pb, Zn, Cr	Hu *et al*. (2008), Wang *et al*. (2008);
Esthwaite	*Hydrilla verticillata*	As	Rai and Tripathi (2009); Wang *et a*l. (2010)
waterweed	*Azolla caroliniana*	As, Hg, Cd	Lee *et al*. (1991); Dixit and Dhote (2010);
Mosquito fern	*Azolla filiculoides*		Zhang *et al*. (2008)
Water fern	*Azolla pinnata*	Cd, Cu, Pb, Zn	Rahman *et al*. (2008a), Rai (2008), Zhang *et al*.(2008); Rai and Tripathi (2009)
	Colocasia esculenta		
Elephant's ear	*Cyperus eragrostis*		
Umbrella sedge	*Eleocharis equisitina*		Cardwell *et al.* (2002)
Spike rush	*Myriophyllum aquaticum*		
Parrot's feather		As	Robinson *et al*. (2005)
	Myriophyllum propinquum	Cd, Cu, Pb, Zn	Cardwell *et al*. (2002)
Miriophyllum	*Nymphaea violacea*	Cd	Schor-Fumbarov *et al*. (2003)
Water lily	*Nymphaea aurora*		
	Nymphoides germinate	Cd, Cu, Pb, Zn	Cardwell *et al*. (2002)
Marshwort	*Persicaria attenuatum*		
Knotweeds	*– Persicaria orientalis*		
	– Persicaria subsessilis	As	Robinson *et al*. (2005)
	– Potamogeton orchreatus	Cd, Cu, Pb, Zn	
	Persicaria lapathifolium		
Willow smartweed	*Potamogeton javanicus*	Cd, Pb, Cr, Ni, Zn, Cu	Cardwell *et al*. (2002)

	Potamogeton pectinatus	Cd, Cu, Pb, Zn	Demirezen and Aksoy (2004)
Fennel pondweed	*Rumex crispus*		Cardwell *et al*. (2002)
Curled dock	*Schoenoplectus validus*		
River clubrush	*Typha domingensis*		
Cumbungi	*Typha orientalis*	Cd, Pb, Cr, Ni, Zn, Cu	
Cumbungi	*Typha angustifolia*		
Lesser Bulrush		Cr, As, Zn, Pb, Cd, Cu. Ni	Chandra and Kulshreshtha (2004); Demirezen and Aksoy (2004)
Bulrush	*Typha latifolia*		Ye *et al*. (1997, 1998), Hozhina *et al*. (2001), Blute *et al*. (2004), Chandra and Kulshreshtha (2004), Deng *et al*. (2004), Pratas *et al*. (2007), Sasmaz et al. (2008)
Waterweed/pondweed	*Elodea Canadensis*	As, Pb, Cr, Zn, Cu, Cd	Mayes et al. (1977), Mal *et al*. (2002), Fritioff and Greger (2003); Chandra and Kulshreshtha (2004), Robinson *et al*. (2005), Dogan *et al*. (2009)
Brazilian waterweed	*Veronica aquatica*	As	Robinson *et al*. (2005)

Source: Rahman and Hasegawa (2011)

2.8 Egeria densa

E. densa Planch (Brazilian elodea or Brazilian waterweed) is a South American submerged perennial in the family Hydrochariyaceae. In countries like North America, Australia, New Zealand, South Africa, Chile and part of Asia and Europe, this aquatic plant has become weed (Gassmann *et al*. 2006; Yarrow *et al*. 2009), where it was introduced, presumably through the aquarium trade. *E. densa* crowds out other plants species by forming dense, nonspecific stands that negatively affect the native biota, as well as impede water sports, fishing, navigation, delivery of irrigation water and hydropower production (Howard-Williams, 1993; Dutartre *et al*., 1999; EDCP, 2006; Yarrow *et al*., 2009).

E. densa has bright green leaves and short internodes, giving the plant a very leafy appearance. The stems are erect, cylindrical, and simple or branched, and grow until they reach the surface of the water where they form dense mats. The leaves are minutely serrated and linear, 1–3 cm long, and up to 5 mm broad, and grow in whorls of four towards the water surface, and occasionally three at the base. It has white flowers, 18–25 mm wide, that rise above the water surface on thread-like hypanthiums produced from apical double nodes. Adventitious roots are produced from double nodes on the stem. It is not considered weedy throughout its range in Argentina, where it rarely grows in large stands or interferes with commercial or recreational use of water. However, in Brazil where it is also naturally found in streams and rivers of the coastal plains (Camargo *et al.* 2006),

2.9 Potential of Using *Egeria densa* in Phytoremediation

E. densa is considered a nuisance in reservoirs used for hydropower production (Borges and Pitelli, 2004). In its adventive stage, the plant has established across wide climatic zones, even wider than warranted by its native distribution, thanks to its capacity to store energy in its basal stems and root crown (Pennington and Sytsma 2009) that allows it to recover from winter senescence, and rapidly reinvade water bodies. It has been reported to be able to survive in superficially frozen waters, providing the plant itself is not trapped in the ice (Haramoto and Ikusima, 1988).

According to Duarte et al. (1994), submerged plants have major effects on productivity and biogeochemical cycling in aquatic environments because they represent a link between the sediments and the overlying water. *E. densa* a rooted submerged plant can absorb nutrients from both the water and the sediments, but it is generally considered that sediments are the main source of both phosphorus and nitrogen (Carpenter and Lodge, 1986; Barko *et al*., 1991).

According to Lara *et al*. (2002), *E. densa* behaves as an ecosystem engineer by preventing the re-suspension of sediments and controlling the growth of phytoplankton by removing nutrients from the water column". Aquatic plants not only add beauty but also improve the water quality and add colour to the aquarium (Feijoo` *et al*, 2002). These plants absorb carbon dioxide and nitrates, and provide shelter and security for fish. It is an efficient plant for nitrogen removal, and fish and plant products can be combined to allow for water renovation and reuse on an aquaculture farm (Dillon *et al*, 1988).

Maurice et al. (1983) reported that *E. densa* can be used as a potential feed ingredient for broiler chicks, chemical analyses of the sun-dried *E. densa* meal was superior to corn and soybean meal with respect to calcium, phosphorus and trace elements.

2.10 Nutrient Uptake and Content

Nitrogen and phosphorus are generally considered the most important limiting nutrients for macrophytes (Barko and Smart, 1981; Hung *et al.*, 2007) primarily because these plants are able to extract nutrients from both the sediment and the water bodies. Feijoó *et al*. (2002) looked at Phosphorous uptake of *E. densa* in nutrient rich streams in Argentina and found that between 8-10 % of phosphorous incorporated in the plant biomass was derived from the sediment. Also, *E. densa* biomass gain was closely related to soluble reactive phosphorus (SRP) concentration in the water bodies. In addition *E. densa* has been reported to show a clear preference for ammonium over nitrate and that most Nitrogen was absorbed from the water column. These authors' results suggest that phosphorus may be more of a limiting factor for *E. densa* than nitrogen, given that phosphorous, not Nitrogen, was tied to increased growth *(Feijoó et al*., 2002).

In general, iron and other metals are required in low concentrations by aquatic plants. In saturated sediments, iron and manganese are reduced and become readily available, often to the degree where, if adsorbed, they can become toxic to plant tissues (Mitsch and Gosselink, 2000; Sinha *et al.* 2008). Submerged macrophytes often release oxygen through their roots in order to oxidize iron, creating iron crusts around roots. Heavy metals, such as iron, stress plants by inducing reactive oxygen species (i.e. free radicals) within plant cells and by decreasing

antioxidants (Sinha *et al.*, 2008). These free radicals can damage membranes, proteins, pigments and nucleic acids resulting in a reduction of plant growth and potential death.

CHAPTER 3

3. 0. Materials and Methods

3.1 Materials

3.1.1 Study Sites and Collection of Plant Materials

The research work was carried out in Alice, 32°42' S Latitude and 26º50' E Longitude in the Eastern Cape of South Africa (Figure 3.1 -3.4) in two selected ponds. Pond 1, is located near the Alice dumping site and not far from the residential areas, while Pond 2 is located near a grazing land. The soil material of the areas consist mainly of mudstones and dolorites made up of fine sand and silt but contain significant amount of clay (Averbeke and Mariais, 1991). The area is semi-arid and has a mean annual rainfall of 571 mm in summer (Mariais and Brutsch, 1994).

Fresh *E. densa* were collected from these two ponds in Alice, located about 1km away from University of Fort Hare, which falls within latitude 32° 48' 8.80" S and longitude of 26° 49' 6.90" E. The plant was identified by Prof. D. S. Grierson of the Department of Botany. A voucher specimen of the plant was prepared and deposited in the Griffen Herbarium, University of Fort Hare. The plant was air dried completely at room temperature, milled into powder and stored in airtight glass bottles at 4°C until required for analysis.

Figure 3.1 Map of South Africa showing Eastern Cape Province

Figure 3.2 Map of Eastern Cape showing Nkonkobe

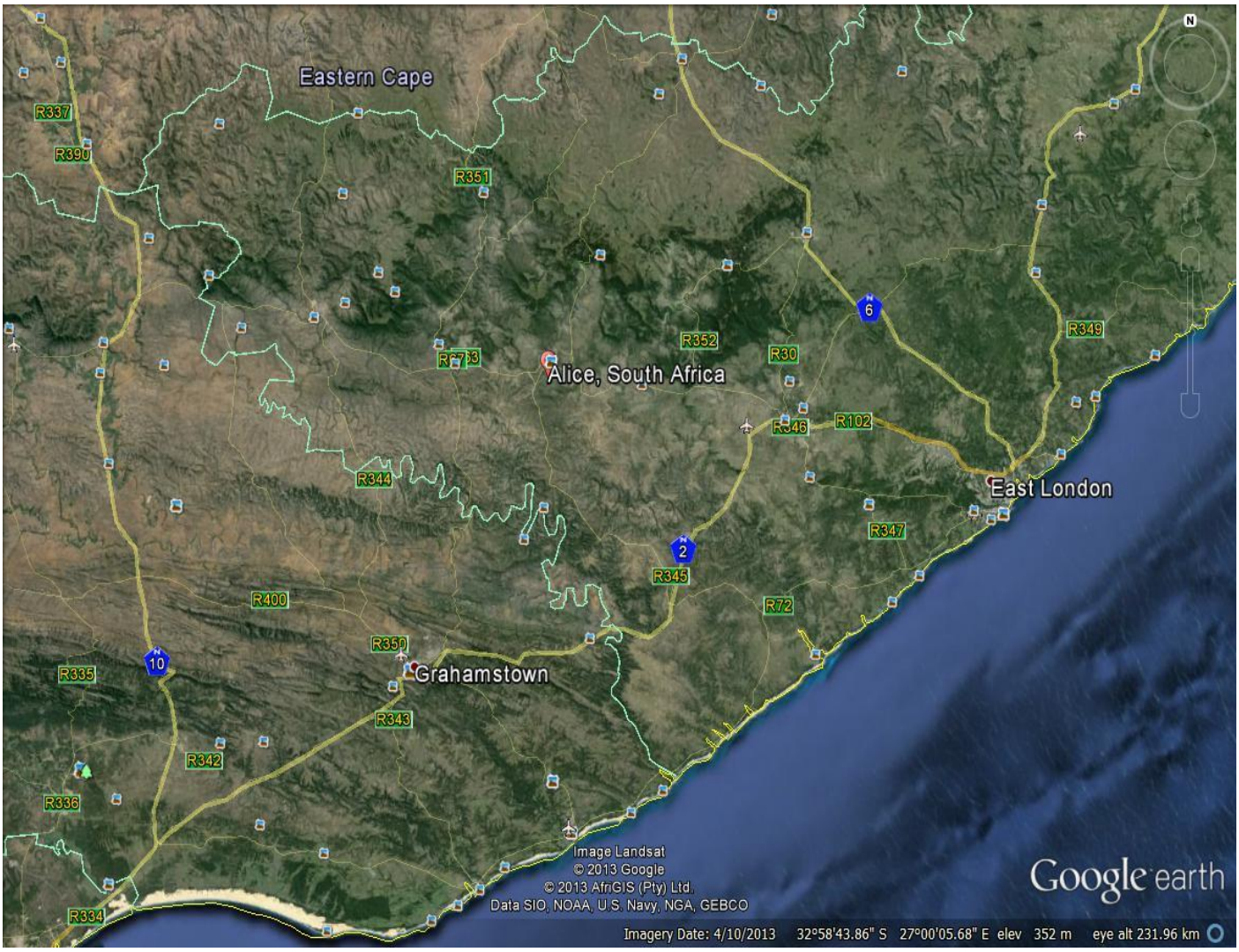

Figure 3.3 Map of Nkonkobe highlighting Alice

Figure 3.4: Enlarged map of Alice with yellow pegs /Pin indicating collection sites

Figure 3.5: Sample site (Pond 1) with co-ordinates (32º 48’22.04”S; 26°48’58.79” E)

Figure 3.6: Sample site (Pond 2) with co-ordinates (32°48’33.25”S; 26°48’33.25”S)

3.*1.2. Chemicals, Reagents and Equipment*

The chemicals, reagents and equipment used in this study are listed in Table 3.1. All other reagents are of analytical grade.

3.1.3 Source of Bacteria Strains

Ten strains of bacteria were used in the study. They were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare.. The bacterial strains include five Gram positive (*Staphylococcus aureus, Bacillus pumilus, Bacillus cereus, Streptococcus pyogenes* and *Enterococcus faecalis*) and five Gram negative (*Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and *Serratia marcescens*)

3.2 Methods

3.2.1 Ultra Structure of Microorganism Associated to *Egeria densa*

Small sections of the plant sample were fixed using the method of Suk *et al* (2009). The specimen was immersed in 6% buffered glutaraldehyde solution for 18 h and dehydrated. Prior to dehydration sample washed 2 to 3 times with distilled water and then rinsed with 0.1 M phosphate buffer pH = 7.2. Plant sample were dehydrated with series of acetone wash (10%, 20 %, 30%, 40%, 50 % 60% 70%, 80%, 90% and 100%) two times. The dehydrated plant sample was mounted on the stub, allowed to dry before being sputter coated with small amount of Gold/palladium (Ion coater: EIKO 1B3) to avoid charging in the microscope. Microscopy was performed with JEOL JSM -6390 LV Scanning Electron Microscope (Japan).

Table 3.1: Chemicals, reagents and instruments used for the experiments

Chemicals and reagent used	**Instruments used**
For ultrastructure of *Egeria densa*	
6% glutaraldehyde	Scanning Electron Microscope (Japan) (JEOL JSM -6390 LV)
0.1 M phosphate buffer	
Acetone	
For phytochemicals analysis	
N-hexane (MERCK)	Orbital shaker (Stuart Scientific Orbital shaker, UK)
Water	
Acetone (MERCK)	Whatman No.1 (90mm) filter paper
5% w/v $FeCl_3$	Freeze dryer (Savant Refrigerated vapor Trap, RVT41404, (USA).
10% Sodium hydroxide (w/v	Hewlett Packard, UV/visible light spectrometer.
10% CH_3CH_2H	Water bath
C_2H_5OH	250 ml separatory funnel
Folin-Ciocalteu	
2% $AlCl_3$	
Ethanol	
Vanillin- methanol	

Hydrochloric acid	
10 % acetic acid	
20% aqueous ethanol	
60 ml n- butanol	
20 ml diethyl ether	
17% aqueous NA_2CO_3	
For antimicrobial activity	
Mueller-Hinton Broth (CM0405) Oxoid	
Nutrient Agar (Merck)	

3.3 Phytochemical screening

3.3.1 Plant Preparation

Homogenized powdered plant materials (60 g) were soaked separately in acetone, n-hexane and water, and shaken using an orbital shaker (Stuart Scientific Orbital shaker, UK) for 24 hours. The extracts were filtered using a Buchner funnel and Whatman No.1 (90 mm) filter paper. The filtrates were evaporated in a pre-weighed flask at 40 °C, using the rotary evaporator. The aqueous extract of about 100 ml was filtered and frozen at -40 °C and dried for 24 hours using a freeze dryer (Savant Refrigerated vapor Trap, RVT41404, (USA), the yield of the filtrate was 5 g. The extract obtained from each of the solvents were stored at 4 °C in the fridge and reconstituted as needed for phytochemical

3.3.1.1 Qualitative Tests

3.3.1.2 Test for Phenolic

Two drops of 5% w/v FeCl3 was added to 1ml of plant extract. A greenish precipitate indicates the presence of phenolic (Awe and Sodipo, 2001).

3.3.1.3 Test for Flavanoids

One milliliter of 10 % Sodium hydroxide (w/v) was added to 3 ml of the extract. A yellow colouration indicated the presence of flavonoids (Yadav and Agarwala, 2001)

3.3.1.4 Test for Alkaloids

Five grams of plant sample was prepared in a beaker and 200 ml of 10% CH_3CH_2H in C_2H_5OH was added to the plant sample nearly 0.5 g. Turbidity or precipitation was taken as indicative of the presence of alkaloid (Edeaga *et al.*, 2005)

3.3.1.5 Test for Saponins

Into 0.2 ml of sample, 4.8 ml distilled water was added, and boiled. Frothing indicate the presence of saponins (Awe and Sodipo, 2001)

3.3.1.6 Test for Tannin

Zero point five grams of powered sample of each plant extract was boiled in 20 ml of distilled water in test tubes and filtered. 0.1% FeCl3 was added to the filtrate and observed for brownish green or a blue black colouration which shows the presence of tannin (Akinpelu and Kolawole 2004)

3.3.2. Quantitative Determination of Phytochemical Constituents

3.3.2.1 Determination of Total Phenols

Total phenolic contents in the plant extract were determined spectrophotometrically using modified FolinCiocalteu method as described by (Wolfe *et al*., 2003). An aliquot of the extract (0.5) was mixed with 2.5ml of 10% Folin-Ciocalteu reagent and 2ml of Na_2CO_3 (75% w/v).The resulting mixture was vortexed for 15 sec and the absorbance of the sample was measured at 765 nm using a Hewlett Packard, UV/visible light spectrometer. The phenolic content was expressed as mg/g tannic acid equivalent using the following equation from the calibration curve: Y= 0.1216X, R^2 =0.9365, where X = was the absorbance and Y = tannic acid equivalent (mg/g).the experiment was conducted in triplicate, and the results were recorded as mean ± standard deviation.

3.3.2.2 Determination of Total Flavonoids

The total flavonoid contents were determined based on the formation of complex flavonoid-aluminium using the method of Ordonez *et al* (2006). A 0.5 ml of 2% $AlCl_3$ solution was added to 0.5 ml extract solution. After 1hr of incubation at room temperature, the absorbance was measured at 420 nm using UV-VIS spectrophotometer. A yellow colour indicated the presence of flavonoid at the final concentration of 0.1 mg/ml. The total flavonoids assay was made in triplicate and were calculated from calibration curve obtained from quercetin using the following equations: $Y= 0.0255\ x$, $R^2 = 0.9812$, where x is the absorbance and Y the quercetin equivalent (mg/g)

3.3.2.3 Determination of Total Flavanols

The method described by Kumaran and Karunakaran (2007) was used to estimate the total flavonol in the plant extract. The reacting mixture of 2 ml of the sample with 2 ml of 2 % $AlCl_3$ were prepared in ethanol and 3 ml of (50g/L) sodium acetate solution was added. The absorption at 440 nm was read after 2.5 hr. at 20 $^{\circ}$C. Extracts of the sample were evaluated at a final concentration of 0.1 mg/ml. Total flavonol content was calculated as quercetin equivalent (mg/g) using the equation obtained from the calibration curve: $Y= 0.0255\ x$, $R^2 = 0.9812$, where x was the absorbance and Y was the quercetin equivalent in (mg/g).

3.3.2.4 Determination of Total Proanthocyanidins

Total proanthocyanidins was determined as described by Sun *et al* (1998). A volume of 0.5 ml of 1mg/ml extract solution was mixed with 3ml of vanillin- methanol (4 % w/v), and 1.5 of hydrochloric acid was added and then vortexed. The mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Proanthocyanidin

content was expressed as catechin equivalent (mg/g) using the equation based on the calibration curve: Y= 0.5825 x, R^2= 0.9277, where x was the absorbance and Y the catechin equivalent (mg/g)

3.3.2.5 Determination of Total Alkaloids

The method described by Harborne (1973) was used to estimate alkaloid content. A 5g of the sample was weighed into a 250 ml beaker and 200 ml of 10 % acetic acid in the ethanol was added and covered and allowed to stand for 4 hr. the sample was filtered and the extract was concentrated on a water bath to ¼ of the original volume. Drops of concentrated ammonium hydroxide were added to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and the filtered. The residue was the alkaloid, which was dried and weighed

3.3.2.6 Determination of Total Saponin

Saponin was estimated using the method of Obadoni and Ochuko (2001). A 20g of each ground sample were weighed into a conical flask and 100 cm^3 of 20% aqueous ethanol were added. The samples were heated over water bath at 55 ºC for 4hr with continuous stirring. The mixture was filtered and residue was extracted with another 200 ml of 20 % ethanol. The combined extracts were reduced to 40 ml over water bath at 90 ºC. The concentrate was transferred into a 250 ml separatory funnel; 20 ml diethyl ether layer was added and shaken vigorously. The aqueous was recovered while the ether layer was discarded. The purification process was repeated using 60 ml n- butanol. The remaining solution was heated in the water bath, to evaporation. The samples were dried in the oven to a constant weight and saponin content was calculated as percentage using the equation:

% Saponin = weight of residue/weight of sample X100.

3.3.2.7 Determination of Total Tannin

Tannin determination was done according to the method of AOAC (1990) with some modifications. To 0.20 g of the sample was added 20 ml of 50 % methanol. This was shaken thoroughly and placed in the water bath at 80 °C for 1hr to ensure uniform mixing. The extract was filtered into a 100 ml volumetric flask, followed by the addition of 20 ml of distilled water, 2.5 ml of folin-Denis reagent and 10 ml of 17% aqueous NA_2 CO_3 and was thoroughly mixed. The mixture was made up to 100 ml with distilled water, mixed and allowed to stand for 20 min. The bluish- green colour developed at the end of the reaction mixture of different concentrations ranges from 0 to 10 ppm. The absorbance of the tannic acid standard solution as well as sample was measure after colour development at 760 nm using the UV-VIS spectrophotometer. Results were expressed as (mg/g) of tannic acid equivalent using the calibration curve: Y =0.0593x – 0.0485, R^2 = 0.9826, where X is the absorbance and Y is the tannic acid equivalent.

3.5 Antimicrobial Assay.

3.5.1 Preparation of Bacterial Culture

Cultures of the 10 strains of bacteria were prepared using Muller Hinton broth (MHB) according to the method described by Jani *at el*, (2011). Briefly 0.8 g of MHB was dissolved in 500 ml of distilled water; 50 ml was poured into ten 100ml conical flasks one for each species of bacteria used and was autoclaved at 121 °C for 15 min, in order for the bacteria to be sub-cultured. Using aseptic technique, the prepared broth was inoculated with each bacterial strain.

Muller Hinton agar plates were used to determine the minimal inhibitory concentration (MIC) of the extracts against the test microorganisms according to CLSI (2012). A 3.1 g Nutrient agar was suspended in 100 ml distilled water, stirred and warmed at 45 °C to completely dissolve the agar. About 4.5 ml of the Nutrient agar was poured into 18 test tubes (three replicates for each concentration of extract). The tubes were sterilized by autoclaving at 121 °C for 15 min. Thereafter, the liquid agar was cooled and kept in a water bath at 60-70 °C to avoid solidification. In order to determine the MIC values, the variuos concentrations, 5.0, 1.0, 0.5 and 0.1 ml were prepared using micro-dilution technique and 0.5 ml of each extract was added to test tubes containing 4.5 ml of sterile Muller Hinton media. The tubes were vortexed, poured into sterile plates (90mm diameter) and allowed to cool at room temperature. The plates with nutrient agar were labelled and streaked radially with 10 bacteria each (Fugure 6). The cultured plates were incubated at 37 °C for 48 hours. Each treatment was in triplicates and two plates without the extract were used as control. The concentration at which there was complete inhibition of bacteria growth was recorded as MIC of the extract.

3.6. Extraction of Heavy Metals from the Sediments Sample

The sediments samples were determined by the ethylenediaminetetraacetic acid (EDTA) extraction method (Okalebo *et a*l., 2002). A reagent solution of 1% EDTA was prepared by dissolving 10g of disodium salt and dilution to 1000 ml in a volumetric flask with deionized water. Five grams (5g) of air dried sediments was placed in clean 250 ml plastic bottle fitter with air tight screw cap. 50ml of the 1% EDTA solution was added and the suspension mixed using an orbital shaker (LABOTEC orbital shatter, South Africa) for 60 min. The suspension was filtered through a Whatman fiter paper Number 1 and analysed for heavy metals using the ICP OES, Varian 710-ES series.

3.6.1 Digestion of Plant Samples and Sediments

The sediment samples were digested using aqua solution, HCl: HNO_3 (3:1). Five gram of sediment was weighed and placed into a digestion tube (Figure 6) with 20 ml digestion mixture. Samples were digested for three hours at 110°C. After evaporation to near dryness, the tubes were removed and allowed to cool. The samples were diluted with 20 ml of 2 % (v/v with H_2O) nitric acid. It was then quantitatively transferred into a100 ml volumetric flask after filtering through Whatman No.1 filter paper and diluted to volume with distilled water. About 0.5 g of plant material was accurately weighed into a silica crucible. The crucible was then placed in a muffle furnace and heated to 400 °C, until the samples became a white ash. After cooling the ash was dissolved in 4 ml of hydrochloric acid and warm water. This was then filtered and diluted to volume in a 100 ml volumetric flask, with a final hydrochloric acid concentration of 1 %.

3.6.2 Preparation of Standard Solutions

The 0.2 mol.l^{-1} Nitric acid reagent for standard solution was prepared, by diluting 5 ml concentrated Nitric acid in 1000 ml de ionised water. The working standards for each element were prepared for ICP-OES analysis as follow

3.6.2.1 Standard for Manganese

A working standard solution of 100 ppm was prepared by pipetting 10 ml of 100 ppm stock solution into a 100 ml volumetric flask and marking the 100 ml mark with 0.2 mol.l^{-1} nitric acid reagent. To make 0,1,2,4,6,8,and 10 mg Mn kg^{-1} standard solution; 0,1,2,4,6,8 and 10 ml of the already prepared 100 ppm manganese working standard were pipette into a set of clean 100 ml volumetric flasks

3.6.2.2 Standard for Solution for Copper

A standard solution of 100 ppm was prepared by pipetting 10 ml of 1000 ppm stock solution into a 100 ml volumetric flask and making up to the mark with de-ionised water. To make 0, 1, 2, 4, 6, 8,and 10 mg Cu kg^{-1} standard solution; 0, 1, 2, 4, 6, 8 and 10 ml of the already prepared 100 ppm copper working standard were pipette into 100 ml volumetric flasks were the manganese standard solution were prepare.

3.6.2.3 Standard Solution for Zinc

A standard solution of 100 ppm was prepared by pipetting 10 ml of 1000 ppm stock solution into a 100 ml volumetric flask and making up to the mark with de-ionised water. To make 0, 1, 2, 4, 6, 8 and 10 mg Zn kg^{-1} standard solution; 0, 1, 2 ,4, 6, 8 and 10 ml of the already prepared 100 ppm zinc working standard were pipette into 100 ml volumetric flasks were the manganese and copper standard solution were prepare.

3.6.2.4 Standard Solution for Cadmium

A standard solution of 100 ppm was prepared by pipetting 10 ml of 1000 ppm stock solution into a 100 ml volumetric flask and making up to the mark with de-ionised water. To make 0, 1 ,2, 4, 6, 8 and 10 mg Cd kg-1 standard solution; 0, 1, 2, 4, 6, 8 and 10 ml of the already prepared 100 ppm cadmium working standard were pipette into 100 ml volumetric flasks were the manganese, copper and zinc standard solution were prepare.

3.6.2.5 Standard Solution of Lead

A standard solution of 100 ppm was prepared by pipetting 10 ml of 1000 ppm Zinc stock solution into a 100 ml volumetric flask and making up to the mark with de-ionised water. To

make 0, 0.5, 1, 2, 3, 4 and 5 mg Pb kg^{-1} standard solution: 0, 0.5, 1, 2, 3 ,4 and 5 ml of the already prepared 100 ppm lead working standard were pipette into 100 ml volumetric flasks were the manganese, copper, zinc and cadmium standard solution were prepare. The standard solution were topped up to the 100 ml mark with the 0.2 nitric acid reagent

3.6.3 Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) detection

The concentration of the metals in the plants sample was determined using the ICP-OES, Varian 710- ES series. The instruction was set at the following conditions: power 1.00 KW, plasma flow 15.0 L/min, auxiliary flow 1.50 L/min, nebulizer flow 200KPa, sample uptake delay 30 s, rinse rate 10 s pump rate 15 rpm replicate read time 5.00 s, instrument stabilising delay 15s and 3 replicates. The following wavelengths were used: cadmium 228.802 nm, manganese 259.372 nm, copper 324.754 nm, lead 182.143 nm and zinc 206.200 nm

3.6.3.1 Data Conversion

Heavy metal contents from ICP-OES in mg/L were converted into mg/kg using the formula: $\frac{(a-b)x\,v}{w}$

where ‘a’ is the concentration of the heavy metal in the sample (mg/L) b is the concentration of heavy metal in the blank (mg/L), v is the total volume of digest (m/L) and w is the weight of the plant material (g) (Temminghoff and Houba, 2004)

3.7 Statistical Analysis

One-way Analysis of Variance (ANOVA) was used to evaluate the significant difference in the concentration of different studied metals with respect to different ponds. A probability at level of 0.05 or less was considered significant (Bailey, 1981). Standard errors were also estimated.

CHAPTER 4

4. 0. RESULTS

4.1 Ultra- Structure of *Egeria densa*

The ultrastructure of the abaxial surfaces of *E. densa.* is characterized by multicellular trichomes that are sparsley distributed all over the leaves (Fig 4.1)

4.2 Phytochemical Analysis of *E.densa*

The acetone, hexane and water extracts of *E. densa* revealed the presence of phenols, flavonoids, proanthocyanidins, flavonols, saponins, alkaloid and tannins (Table 4.1). Total proanthocyanidin was the highest 250.833 mg/g followed by total phenols 105.867 mg/g while tannin was lowest 2.820 mg/g in hexane extract. Proanthocyanidin content of the acetone extract of *Egeria densa* was highest followed by phenols(197.967), total flavonoids (50.763), flavonols (43.323), saponin (32.100), alkaloids (2.00) and tannin(0.158). Water extract followed almost a similar trend with the acetone extract.

Egeria densa has considerable amount of total phenols and total proanthocyanidin, while the tannin content was very low in acetone extract. The results indicated that acetone extract had high proanthocyandin content of total (901.53mg g^{-1}), follows by water extract.

The lowest tannin value in the experiment was recorded in acetone extract of the plant, followed by alkaloid which also shows lowest value in acetone extract. There were no significant differences in tannin content between three solvents the mean concentrations of tannin content of 3.33 mg/g. However, overall comparison revealed that the water extract had the highest poly phenols, saponin, alkaloid and tannin.

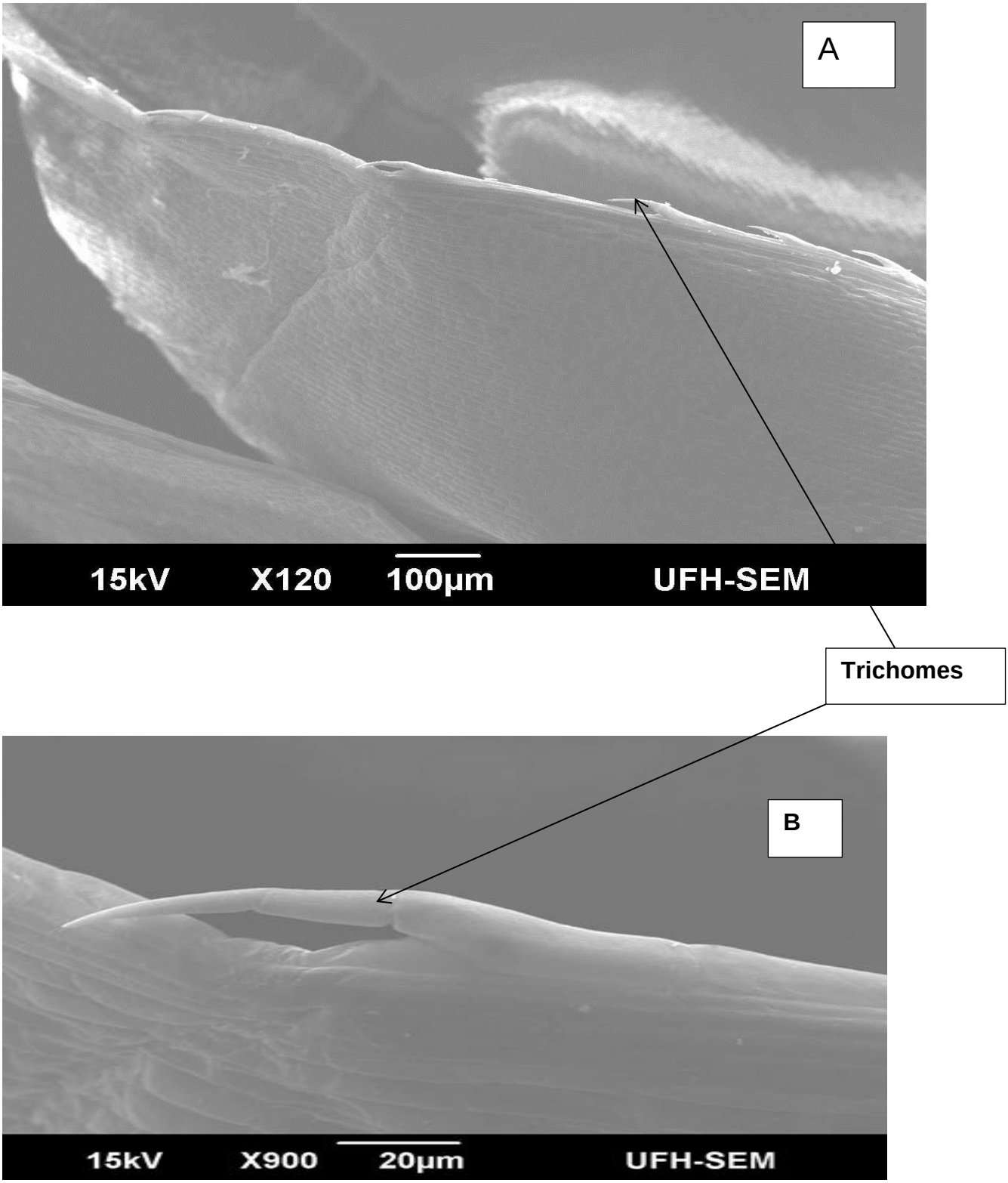

Figure 4.1 SEM ultrastructure of abaxial leaf surface of E. densa showing trichomes in A and B

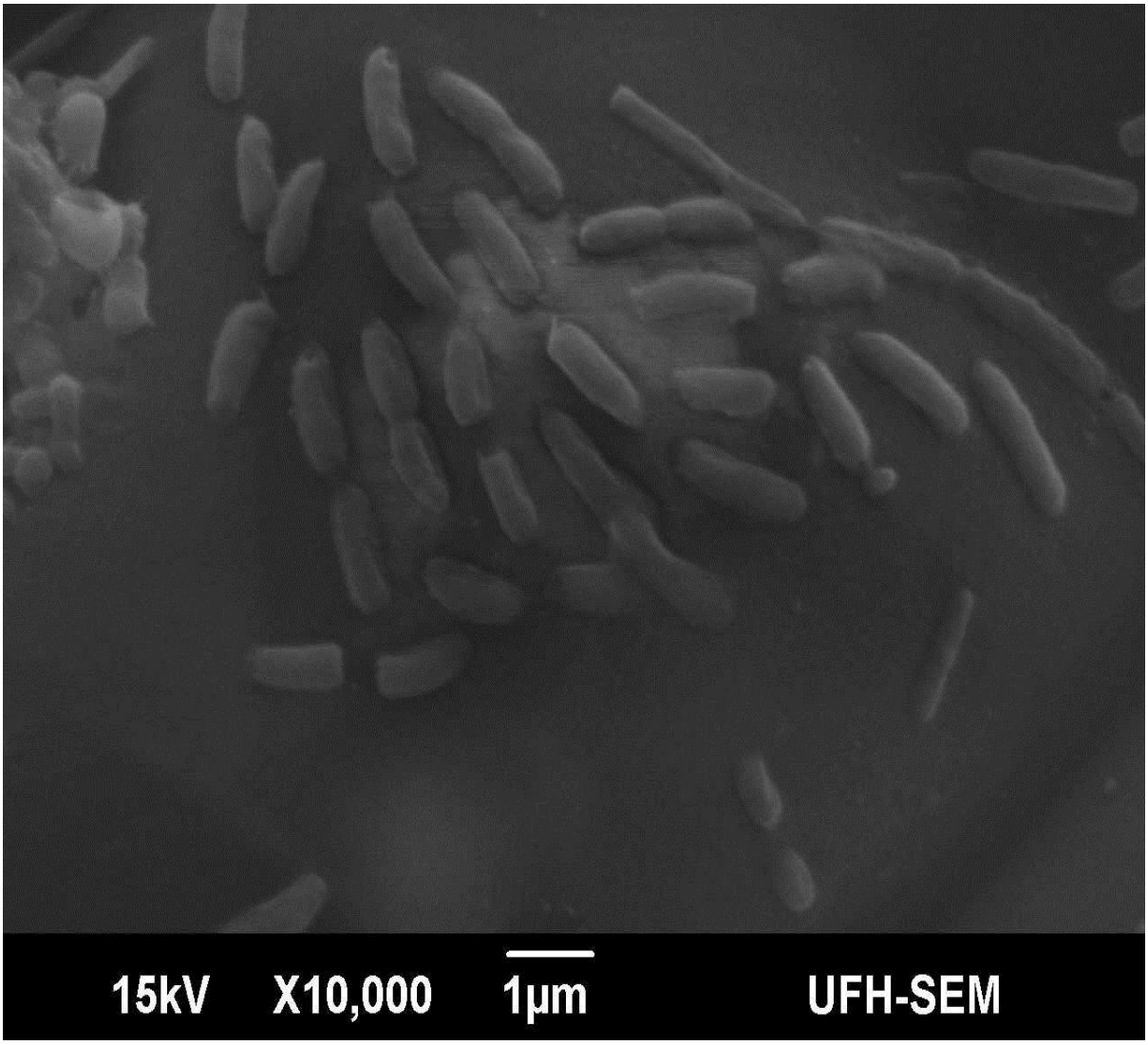

Figure 4.2: SEM ultrastructure of *E. densa* whole plant with associated bacteria

4.3 Antibacterial Assay

The antibacterial assay of acetone, hexane and water extracts of *Egeria densa* (Tables 4.2-4.4) showed that none of the extracts possess antibacterial activity against both Gram positive and Gram negative bacteria except the acetone extract which showed some inhibitory activity against *S. pyogenes* (Table 4.3)

4.4 Elemental Analysis

4.4.1 Sediments

The results of heavy metals concentration of the sediments of the two ponds under study are presented in Table 4.5. Heavy metal concentration of sediments from the two ponds revealed significant differences in the metals investigated. Pond 2 had a significantly higher concentration ($P<0.05$) of Cd, Fe, Pb, Cu, Mn and Mg while Pond 1 had the highest concentration of Zn.

4.4.2 Water Sample

Analysis of the water sample from the Pond for heavy metals (Table 4.6) showed cadmium at undetectable amount in Pond 1, while zinc was also not detected in both ponds. Iron lead and Manganese were significantly higher ($P <0.05$) in water column from Pond 1 compared to Pond 2. The Mn content of water from both Ponds was not significantly different in that they both have minimum values of 0.238 and 0.248 respectively.

4.2.3 Plant Sample (*E. densa*)

Egeria densa sample from Pond 1 exhibited a significantly ($P <0.05$) higher concentrations of Fe, Pb, Cu, Mn and Mg than that from pond 2 (Table 4.3), while cadmium and zinc were not

detected at all in the plant samples . Pond 2 showed concentrations of 0.26 and 229.0 mg/kg for Cd and Zn respectively.

Table 4. 1: Phytochemical constituents of *E. densa*

Phytochemicals (Mg/g)	Extracts		
	N-hexane	Acetone	Water
Total phenols	105.867 ± 0.153^{b}	197.967 ± 0.058^{b}	232.567 ± 0.351^{b}
Total flavonoids	28.387 ± 0.819^{d}	50.763 ± 0.475^{c}	37.0567 ±0.644^{d}
Total proanthocyanidin	250.833 ± 1.069^{a}	901.533 ± 0.404^{a}	320.067 ± 0.208^{a}
Total flavonols	43.270 ± 0.000^{c}	43.323 ±0.046^{c}	43.237 ±0.104^{d}
Saponins	21.367 ± 0.058^{e}	32.100 ± 0.100^{cd}	66.167 ± 0.153^{c}
Alkaloid	3.633 ± 0.208^{f}	2.000 ± 0.100^{d}	7.033 ± 0.058^{e}
Tannins	2.820 ±0.031^{f}	0.158 ± 0.078^{d}	7.029 ± 0.265^{e}

Data expressed as means ± SD (n=3); Value along the same row having different superscripts significant differences ($p<0.05$)

Table 4.2 Minimum inhibition concentration (MIC) of different extract of *Egeria densa* against gram positive and negative

	Test organism	Gram	Extracts		
			Acetone	Aqueous	n-hexane
1	*Staphylococcus aureus*	+	Na	Na	Na
2	*Bacillus purinitis*	+	Na	Na	Na
3	*Bacillus cereus*	+	Na	Na	Na
4	*Streptococcus pyogenes*	+	> 0.1	> 5	> 5
5	*Enterococcus faecalis*	+	>0.1	>5	-
6	*Klebseilla pneumoniae*	-	Na	Na	Na
7	*Escherichia coli*	-	Na	Na	Na
8	*Pseudomonas aeruginosa*	-	Na	Na	Na
9	*Proteus Vulgaris*	-	Na	Na	Na
10	*Serratia Marcescens*	-	Na	Na	Na

Na=not active, Gram positive = (-), Gram negative = (+)

Table 4.3: Heavy metal content of sediment of two investigated ponds (mg/kg)

	Site	
Metal Concentration (mg/kg)	Pond 1	Pond 2
Cd	0.52 ± 0.04^{a}	0.999 ± 0.237^{b}
Fe	40.320 ± 1.261^{a}	61.527 ± 3.340^{b}
Zn	1.259 ± 0.005^{a}	0.156 ± 0.001^{b}
Pb	0.564 ± 0.042^{a}	0.586 ± 0.15^{b}
Cu	0.037 ± 0.042^{a}	0.045 ± 0.49^{b}
Mn	0.186 ± 0.012^{a}	0.648 ± 0.056^{b}

Values along the same row having different superscripts indicate significant differences ($p < 0.05$). Data are mean ± SD (n = 3)

.

Table 4.4: Heavy metal content of water sample of the two investigated ponds (mg/L)

	Site	
Concentration (mg/kg)	Pond 1	Pond 2
Cd	ND	0.004 ± 0.003^{a}
Fe	3.07 ± 0.59^{a}	1.69 ± 0.260^{b}
Zn	ND	ND
Pb	35.36 ± 3.53a	13.03 ± 3.18b
Cu	0.104 ± 0.05a	0.270 ± 0.27b
Mn	0.238 ± 0.08	0.248 ± 0.016

Values along the same row having different superscripts indicate significant differences ($p < 0.05$). Data are mean ± SD (n = 3).

ND- Not Detected

Table 4.5: Heavy metal content *Egeria densa* whole plant sample (mg/Kg)

	Site	
Concentration (mg/kg)	Pond 1	Pond 2
Cd	ND	0.26 ± 0.01
Fe	0.03 ± 0.03	0.014 ± 0.03
Zn	ND	229.0 ± 26.00
Pb	24.32 ± 27.14	12.29 ± 0.25
Cu	0.10 ± 0.18	0.02 ± 0.01
Mn	620.80 ± 95.61	104.99 ± 15.69

Values along the same row having different superscripts indicate significant differences ($p < 0.05$). Data are mean ± SD (n = 3).

ND - Not Detected

CHAPTER 5

Discussion

Ultrastructural observations of *E. densa* whole plant indicated the presence of trichomes and associated bacteria. Trichomes have been reported to help in protecting the aerial shoot of plants against water loss due to evaporation, attack by pests such as insects and airborne fungal particles (Pendota *et al*., 2008). Also, these trichomes may possess secretory glands which may have medicinal potentials. The associated bacteria observed could be one of those that have been known to be good bioremediants of metal pollution since the microbial remediation of metals differs from that of organic pollutants as metals are not degraded into what are ultimately innocuous products (Moore *et al*., 2011). Also these associated bacteria on their own, may have the capacity to accumulate heavy metals, or facilitate their uptake by the plant. According to (Caslake *et al*., 2005; Crump and Roch 2008), some bacteria associated with aquatic or submerged plants have the capacity to accumulate heavy metals such as mercury and iron or to oxidize them.

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve a defence mechanism against predation by many microorganisms, insects and other herbivores (Shihabudeen *et. al*.2010). Proanthocyanidins have been shown to have very potent antioxidant activity (Ayoola *et al.,* 2008). The present study indicated that in n- hexane extract, the content of proanthocyanidin and total phenols was found to be (250.833± 1.069 and 105.86 ±0.153) which were more than the content of flavonoids, saponins, alkaloids and tannin. According to Li (2010), phenolic compounds possess multiple biological properties such as

antitumor, antibacterial properties and these activities might be related to their antioxidant activity. The high content of total phenols in the study indicates that *E. densa* may have antioxidant properties.

None of the extracts in this study showed strong antimicrobial activity; however, negative results do not necessarily mean absence of bioactive constituents in the plant. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose level employed (Taylor *et al*; 2001). Lack of activity can thus only be proven by using large doses (Farnsworth, 1993)

Reports from various authors have shown that *S. aureus* and *Bacillu*s were the most sensitive isolates to the estuarine submersed aquatic plants extracts, and that due to the cell wall structure of Gram positive bacteria (that consists of a single layer), while Gram negative bacteria cell wall have a double layer membrane, that makes the cell membrane resistant to the antimicrobial agent(s) and hence delay the osmotic lysis of a bacterial cell (Bushmann and Ailstock, 2006; Wang *et al.,* 2007).

Analyses of metal accumulation in plants are important in outlining the deposits of various metals. The concentration of heavy metals in the two ponds under study is especially important as the water from these two ponds is used for domestic and irrigation purposes. The high accumulation of heavy metals observed in water from pond 1 could be attributed to a increased pollution and contamination from various sources or leaching from the dumpsite beside which it is located. The low accumulation in Pond 2 could be as a result of its location as well as the presence of other aquatic plants such as algae which absorb metals from both water and sediments

Heavy metal component of water column, sediments and *E densa* from Pond 1 indicated that cadmium was present in the sediment from both ponds and it was absent from both water column and *E densa*. Cadmium is a highly toxic non-essential element whose concentration in normal plants from uncontaminated source range from 0.05 to 0.2 mg/kg (Kebith- and Pendous 1992). The absence of this element in the water column and *E. densa* could be attributed to the bioaccumulation of Cd by other tolerant of aquatic environments (Fonkou *et al* 2005) and that *E. densa* does not have affinity for Cd therefore not taking it up from the sediments. In contrast, Cd was present in all three parameters of Pond 2, though the high concentration was exhibited by the sediment, followed by the plant and least by water column. According to Loizeau et al (2007), accumulation of heavy metal in the sediments is important in self-purification of aquatic environments, but it is a reversible process which could be a threat of secondary water pollution.

In the present study concentration of Cd ranged 0.26, 0.52 and 0.999 mg/kg in *E. densa* from Pond 2 and sediment from ponds 1 and 2 respectively. This agrees with reports of Cardwell et al (2002) on the concentration of Cd in the leaves of 13 plant species from contaminated urban streams.

The high concentration of Mn observed in *E. densa* from both ponds compared with the water column and sediment from the same sources could be a indication that *E densa* is a very good accumulator of Mn. It is observed *that E. densa* from Pond 1 accumulated more than 2605 times Mn from the water column and 337 times from the sediment while *E. densa* from Pond 2 showed 423.3 times accumulation in water and sediment respectively. Two could be an indication of phytoremediation

potential of *E densa* as reported by Podar *et al*. (2004), which makes it a good candidate for the remediation of Mn polluted water

Concentration of lead (Pb) was observed to be very high in the water column from pond, 1 and 2 (35.36 -13.03)). In contrast Pb concentration was low in *E densa* from both pond 24.32 and 12.29 mg/kg respectively. The sediments from the two sources exhibited a very low concentration of Pb this could be an indication that *E densa* is not a good accumulator of Pb.

The non-detection of Zn metal in water column of Pond 1 and 2 as well as *in E densa* from Pond 1 could be an indication that Zn was not a pollutant of these 2 ponds. According to Peng *et al*. (2008) concentration of Zn could range from 72-6590 mg/kg in plants. The observation in the present study was that concentration of Zn in sediments was 1.259 and 0.15 mg/kg in *E. densa* from pond 2 this could indicate that *E.densa a* would be a good accumulator of Zn if present as a pollutant.

Copper is an essential element for plant growth and a important constituent of many enzymes in living organism. In the present study, copper concentration ranged from 0.02- 0.245 mg/kg in sediment *and E. densa* samples from Pond 1 and 2. According to Aksoy *et al*. (2005) Cu concentrations above 5-20ug/g are regards as poisonous in plants. In this study, the highest concentration of copper was found in water column of pond 2 and could be attributed to herbicides, pesticides or fertilizers used from agricultural purpose in the surrounding areas.

Iron is a major content of many sites with a range of 3 to 200 mg/l. In this study the observed high concentration of iron in the sediments from both Pond 1 and 2

compared to its low concentration in water column and *E densa* could be a indication of precipitation of iron- hydroxides in the sediments (Woelfl *et al*., 2006).

Conclusion

Based on results from this study on the heavy metal accumulation potential of E densa, conclude I conclude that

i. *E. densa* possess the ability to accumulate heavy metals especially Manganese

ii. *E. densa* has associated bacteria which may also possess remediative properties

iii. *E. densa* has a high content of phytochemicals which could prompt further investigation into its potential medicinal properties

iv. In the present study*, E.densa* exhibited no antimicrobial action against both gram positive and negative bacteria.

v. That *E.densa* has the potential to improve the economic development of the Eastern Cape if properly harnessed for use in remediation.

Further work need to be done on *E. densa* to verify the mechanism by which it accumulates the heavy metals it has the greatest affinity for. In addition, investigation of the associated bacteria as well as its function and mechanism of action should be investigated. Also, the effect of the dried and powdered form of *E densa* on the removal of metals should further be investigated to quantify its economic value in phytoremediation.

REFERENCES

Akinpelu D.A.and Kolawole D.O. (2004). Phytochemical and antimicrobial activity of leaf extract of *Piliostigma thonningii* (Schum.). Science. Focus Journal. 7: 64-70.

Alvarado, S., Guédez, M., Lué-Merú, M.P., Nelson, G., Alvaro, A., Jesús, A.C. and Gyula, Z., (2008). Arsenic removal from waters by bioremediation with the aquatic plants Water Hyacinth (*Eichhornia crassipes*) and Lesser Duckweed (*Lemna minor*). *Bioresour. Technol*., 99(17): 8436-8440.

Ampiah-Bonney, R. J., Tyson, J. F., and Lanza, G. R. (2007). Phytoextraction of arsenic from soil by Leersia orywides. International Journal of Phytoremediation, vol. **9** (1-3): 31-40.

AOAC. (1990). Official methods of analysis (15th Ed.). Association of Official Analytical Chemists, Washington DC, USA

Apsimon, H., Thornton, I., Fyfe, W., Hong, Y., Leggett, J., Nriagu, J.O., Pacyna, J.M., Page, A.L., Price, R., Skinner, B., Steinnes, E., and Yim, W. (1990). Anthropogenically induced global change — Report of working group 3, IUGS workshop on global change past and present. Palaeogeography, Palaeoclimatology, Palaeoecology, vol. **82** (1–2): 97-111.

Awe I. S. and Sodipo, O. A. (2001). Purification of saponins of root of Bhighia sapida Koenig-Holl. Nigerian. Journal of Biochemistry. Molecular. Biology. (Proceedings Supplement). 16: 201-204.

Baeyens, W. and Leermakers, M. (1998.) Elemental mercury concentrations and formation rates in the Scheldt estuary and the North Sea. Marine Chemistry, vol.**60** (3–4): 257-266.

Bailey, N. T. (1981). Statistical Methods in Biology. 2nd Ed. (Biological Science Texts).

Barko, J. and smart, R.M. (1981). Comparative influences of light and temperature on the growth and metabolism of selected submersed freshwater macrophytes. Ecological Monographs 51: 219-235.

Barko, J. W., Gunnison, D., and Carpenter, S.R. 1991. Sediment interactions with submersed macrophyte growth and community dynamics. Aquatic Botany, vol. **41**(1–3): 41-65.

Borges Neto CR, Pitelli RA (2004) Adjuvantes e herbicidas e a infectividade de Fusarium graminearum, agente potencial de biocontrole de Egeria densa e Egeria najas. Planta Daninha 22:77–83

Camargo AFM, Pezzato MM, Henry-Silva GG, Assumpc¸aoAM (2006) Primary production of Utricularia foliosa L., Egeria densa Planchon and Cabomba furcata Schult and Schult.f from rivers of the coastal plain of the State of Sao Paulo Brazil. Hydrobiologia 570:35–39

Carpenter, S.R., and Lodge, D.M. 1986. Effects of submersed macrophytes on ecosystem processes. Aquatic Botany vol **26** (0): 341-370.

Czuba, M., and Mortimer, D.C. 1983. Effects of heavy metals absorbed from water on the laser-induced fluorescence in *Elodea densa*. Ecotoxicology and Environmental Safety **7**(5): 447-450.

Dary, M., Chamber-Perez, M.A., Palomares, A.J., and Pajuelo, E. 2010. "In situ" phytostabilisation of heavy metal polluted soils using Lupinus luteus inoculated with metal resistant plant-growth promoting rhizobacteria. Journal of Hazardous Materials **177**(1-3): 323-330.

Di Lonardo, S., Capuana, M., Arnetoli, M., Gabbrielli, R., and Gonnelli, C. 2011. Exploring the metal phytoremediation potential of three Populus alba L. clones using an in vitro screening. Environmental Science and Pollution Research **18**(1): 82-90.

Dietz, F. 1973. The enrichment of heavy metals in submerged plants. *In* Advances in Water Pollution Research. *Edited by* S.H. Jenkins. Pergamon. pp. 53-62.

Dillon, C. R., D. V. Maurice, J. E. Jones. (1988). Chemical composition of Egeria densa. Journal of Aquatic and Plant Management. 26: 44-45.

Duarte CM, Marba N, Agawin N, Cebrian J, Enriquez S, Fortes MD, Gallegos ME, Merino M, Olesen B, Sand- Jensen K. Uri J, Vermaat J (1994) Reconstruction of sea grass dynamic ages determinations and associated tools for the see grass ecologist. Mar Ecol Pro Ser 107: 195-209

Edeaga H O, Okwu D E and Mbaebie B O. (2005). Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology Vol. 4 (7), pp. 685-688

Ensley, B.D., 2000. Rationale for the use of phytoremediation, phytoremediation of toxic metals: Using plants to clean-up the environment, John Wiley Publishers, New York.

Feijoó C., García M.E., Momo F., Toja J. (2002) Nutrient absorption by the submerged macrophyte Egeria densa Planch.: effect of ammonium and phosphorus availability in the water column on growth and nutrient uptake. Lirnnetica 21: 93-104.

Filip, D.S., Thomas Peters, V., Dean Adams, E., and Middlebrooks, J. 1979. Residual heavy metal removal by an algae-intermittent sand filtration system. Water Research **13**(3): 305-313.

Fritioff, A., Greger, M., 2006. Uptake and distribution of Zn, Cu, Cd, and Pb in an aquatic plant Potamogeton natans. Chemosphere 63, 220–227.

Gassmann A, Cock MJW, Shaw R, Evans HC (2006). The potential for biological control of invasive alien aquatic weeds in Europe: a review. Hydrobiologia 570:217–222

Gaur A and Adholeya A (2004). Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Current Science, vol. 86, no. 4, pp. 528–534, 2004.

Goulet, R.R., Lalonde, J.D., Munger, C., Dupuis, S., Dumont-Frenette, G., Premont, S., Campbell, P.G.C., 2005. Phytoremediation of effluents from aluminum smelters: a study of Al retention in mesocosms containing aquatic plants. Water Res. 39 (11), 2291–2300.

Haramoto T, Ikusima I (1988) Life cycle of Egeria densa Planch., an aquatic plant naturalized in Japan. Aquat Bot 30:389–403

Harborne JB (1973). Phytochemical methods, London. Chapman and Hall, Ltd. pp. 49- 88.

Hung, N. T. asaeda and J Manatunge (2007) Modeling interactions of submersed plant biomass and environmental factors in a stream using structural equation modeling. Hydrobiologia 583: 183-193.

Iqbal M. A and Gupta SG (2009). Studies on the heavy metal Ion pollution of ground water source as an effect of municipal solid waste dumping. African journal of basic & Applied Science

Keskinkan, O., 2005. Investigation of heavy metal removal by a submerged aquatic plant (Myriophyllum spicatum) in a batch system. Asian J. Chem. 17 (3), 1507–1517.

Kumaran, A. and R.J. Karunakaran, 2007. In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. LWT-Food Sci. Technol., 40: 344-352.

Lobban C.S. and Harrison P.J. (1997) seaweed Ecology and physiology, Journal of Applied phycology (9) 295.

Maine, M.A., Sun˜e, N.L., Lagger, S.C 2004. Chromium bioaccumulation: comparison of the capacity of two free-floating macrophytes. Water Research 38, 1494-1501.

Mal, T., Adorjan, P., Corbett, A., 2002. Effect of copper on growth of an aquatic macrophyte, Elodea canadensis. Environment. Pollution. 120, 307–311.

Marchand, L., Mench, M., Jacob, D.L., Otte, M.L., 2010. Metal and metalloid removal in constructed wetlands, with emphasis on the importance of plants and standardized measurements: a review. Environment. Pollution. 158, 3447–3461.

Nriagu, J.O. (1990). Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. Nature **279**(5712): 409-411.

Obdoni BO, Ochuko PO (2001). Phytochemical studies and comparative efficacy of the crude extracts of some Homostatic plants in Edo and Delta States of Nigeria. Global J. Pure Appl. Sci. 8 b: 203-208.

Okalebo J R, Gathua K W and Woomer P L (2002): Laboratory Method of soil and plant Analysis: A working Manual.2nd Edition, TSBF-CIAT and SACRED Africa, Nairobi, Kenya.

Ordonez AAL, Gomez JD, Vattuone MA, Isla MI (2006). Antioxidant activities of *Sechium edule* (Jacq). Food Chem; 97:452–458.

Pennington. T G, Sytsma M D (2009). Seasonol changes in carbohydrate and nitrogen concentrations in Oregon and Calofornia population of Brazilian Egeria (Egeria densa) Invasive plant Sci Manage 2:120-129.

Rai, U.N., Sinha, S., Tripathi, R.D., and Chandra, P. 1995. Wastewater treatability potential of some aquatic macrophytes: Removal of heavy metals. Ecological Engineering **5**(1): 5-12.

Salt, D.E., Blaylock, M., Kumar, N., Dushenkov, V., Ensley, B.D., Chet, I., and Raskin, I. 1995. Phytoremediation - a novel strategy for the removal of toxic metals from the environment using plants. Bio-Technology **13**(5): 468-474.

Saraswat, S., and Rai, J.P.N. 2009. Phytoextraction potential of six plant species grown in multimetal contaminated soil. Chemistry and Ecology **25**(1): 1-11.

Schwarzenbach, R. P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313, 1072–1075.

Suk K.T, Kim H. S, Kim M Y, Kim J. W, uh Y, Jang I. H, Kim M J , Joo J. S & Balk S.K (2009). In vitro antibacterial and Morpholgical effects of the Urushiol components of the Sap of the Korean lacquer tree (Rhus vernicifera stokes)on Helicobacter pylori

Sun, J.S., Tsuang, Y.W., Chen, I.J., Huang, W.C., Hang, Y.S. and Lu, F.J. (1998). An ultraweak chemiluminescene study on oxidative stress in rabbit following acute thermal injury. Burns, 24: 225-231.

Tremp, H., Kohler, A. (1995):The usefulness of macrophytes monitoring-systems, exemplified on eutropication and acidification of running water, Acta. Bot.Gallica 149 (6):541-550.

Vamerali, T., Bandiera, M., and Mosca, G. 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environmental Chemistry Letters **8**(1): 1-17.

Walton, B.T., and Anderson, T.A. 1990. Microbial-degradation of trichloroethylene in the rhizosphere - potential application to biological remediation of waste sites. Applied and Environmental Microbiology **56**(4): 1012-1016.

Wolfe, K., Wu, X., and Liu, R.H. (2003). Antioxidant activity of apple peels. Journal of Agriculture and Food Chemistry 51: 609- 614.

Yadav RNS and Agarwala Munin (2011). Phytochemical analysis of some medicinal plants. Journal of phytology 3(12) 10-14

Yarrow M, Marı´n VH, Finlayson M, Tironi A, Delgado LE, Fischer F (2009). The ecology of Egeria densa Planchon (Liliopsida: alismatales): a wetland ecosystem engineer? Rev

Zeyed, A., S. Gowthaman, N. Terry, (1998). Phytoaccumulation of trace elements by wetland plants: I. Duckweed, Environ. Qual., 27: 715-21.

Zhu, Y.L., Zayed, A.M., Qian, J.-H., de Souza, M., Terry, N., 1999. Phytoaccumulation of trace elements by wetland plants. Part II. Water hyacinth. J. Environ. Qual. 28, 339–344.