DEVELOPMENT OF CONSERVATION METHODS FOR *Gunnera perpensa* L.: AN OVEREXPLOITED MEDICINAL PLANT IN THE EASTERN CAPE, SOUTH AFRICA

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DECLARATION

I, the undersigned, declare that this thesis, submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Botany in the Faculty of Science and Agriculture, is my original work; and that this work has not been submitted at any other University for the award of any degree. This thesis does not contain other personsô data, pictures, graphs or other information, unless specifically cited/acknowledged as being sourced from other persons.

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Signatureé é é é é é é ..

Date é é 31/07/2014é é é é é é é é é é é é é .

DEDICATION

I dedicate this work to my Lord and Saviour Jesus Christ, in whom I live and move and have my being.

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LIST OF ABBREVIATIONS

2, 4-D	2,4-Dichlorophenoxyacetic acid
Abs	Absorbance
ABTS	2,2ô-azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid
AC	Activated charcoal
AIDS	Acquired immune deficiency syndrome
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
ATCC	American type culture collection
BAP	Benzyl-aminopurine
BHT	Butylated hydroxytoluene
DNA	Deoxy-ribonucleic acid
DPPH	Scavenging activity of 2, 2-Diphenyl-1-Picrylhydrazyl
ECSECC	Eastern Cape socio economic consultative council
EN	Endangered
GA_3	Giberrellic acid
$HgCl_2$	Mercuric chloride
HIV	Human immune deficiency virus
IUCN	International Union for Conservation of Nature and Natural resources
KNO_3	Potassium nitrate
KZN	Kwazulu- natal
LC	Least Concern
MFC	Minimum fungicidal concentrations
MGT	Mean germination time
MIC	Minimum inhibitory concentration
MS	Murashige and Skoog
Na_2CO_3	Sodium carbonate
NAA	Ŭ-Naphtalene acetic acid
NaOCl	sodium hypochlorite
ND	Not determined
NO	Nitric oxide
NT	Near threatened
PDA	Potato dextrose agar
PPM	Plant preservative mixture
PPO	polyphenol oxidase
PVP	Polyvinylpyrrolidone
QE	quercetin equivalent
ROS	Reactive oxygen species
SANBI	South Africa National Biodiversity Institute
SPAD	Special Product Analysis Division
SSA	Statistics South Africa
TCA	Trichloracetic acid
TDZ	Thidiazuron
TE	tannic acid equivalent
TTC	2,3,5-triphenyltetrazolium chloride

VU	Vulnerable
WHO	World Health Organisation

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ABSTRACT

Abstract

In South Africa, many plants which are used in traditional medicines are collected from wild populations. The high demand for trade and use of these medicinal plants place an enormous pressure on their natural populations, especially because they are indiscriminately harvested. The most affected of these plant species are those harvested from their underground parts, among which is *Gunnera perpensa* L.

Gunnera perpensa is of considerable ethnobotanical interest in traditional medicine because of its wide usage. The rhizomes are widely used and indiscriminately collected in large quantities from the wild to meet the ever increasing demand in traditional medicine markets. As a result, this valuable medicinal plant species is being endangered. According to the Red List of South African Plants, the conservation status of *G. perpensa* has been listed as ódecliningô. The ethnobotanical survey conducted as part of this research confirms the plant species as threatened. It is, therefore, important to develop propagation and conservation strategies for this medicinal plant.

Clonal propagation of *G. perpensa* was conducted using varying lengths of the rhizome (1, 2, 3, 4 and 5 cm) segments as propagules. While regeneration was possible with all the rhizome lengths, most of the growth parameters were significantly higher in the 5 cm rhizomes than the other rhizome segments. The appropriate planting depth for the rhizomes was also determined and 4 or 5 cm planting depths were found appropriate. No significant difference was observed in the growth parameters amongst the planting depths; nevertheless, 4 cm depth gave higher growth and yield. The results of this study show that regenerating *G. perpensa*

through rhizome segments is an efficient method for obtaining plant material for medicinal purposes.

Seed germination is an important determinant in the distribution and survival of a plant species. It therefore becomes essential to study the germination requirements of *G. perpensa* seeds. The effects of light, temperature conditions, leaching, scarification, pre-chilling and chemical substances such as gibberellic acid and KNO_3 were investigated in the germination of the seeds. The optimum temperature for germination in this study was 25^oC under light conditions. Overall, germination of *G. perpensa* seeds was poor and irregular, but mechanical scarification significantly improved the percentage germination from 4% to 32% compared to untreated seeds (control), with a mean germination time (MGT) of 56 days. This was followed by 7 day leaching with 26% germination and MGT of 44 days.

Antimicrobial and antioxidant activities were evaluated between two different plant parts of *G. perpensa,* for their potential in plant part substitution. The plant parts (roots and leaves) were extracted in differing polarity solvents. These were screened for antibacterial activity against ten bacterial species including *Bacillus subtilis* and *Staphylococcus aureus*. The plant extracts presented some activity against the bacterial strains with zones of inhibition varying from 8 to 25 mm and MIC values ranging from 2.5 to 10 mg/ml. The highest activity against the tested bacteria was obtained with the methanol leaf extract with inhibition zone of 25 mm against *Serratia mercescens*. *Gunnera perpensa* extracts were also screened for antifungal activity against four fungal species. All the plant extracts exhibited high antifungal activity against *Candida krusei* and *Absidia corymbifera* with MIC values ranging from 0.15 to 0.62 mg/ml. The ability of the extracts of *G. perpensa* to inhibit the growth of several bacteria and

fungi is an indication of the broad-spectrum antimicrobial potential of this plant that further validates its use for the treatment of various ailments. The phytochemical evaluation of the studied plant species indicated that the observed activities might largely be due to their high flavonoid and phenol contents, with a contributing effect from their alkaloids and saponins. The highest flavonoid content (434.09 mg Quercetin/g) was recorded in acetone rhizome extract, followed by methanol leaf extract (432.22 mg Quercetin/g). In the antibacterial and some of the antioxidant assays, the leaves of this plant demonstrated higher activity than the rhizomes, while in the antifungal assay the two plant parts exhibited similar activities suggesting their potential in plant part substitution. The harvesting of leaves as a conservation strategy is certainly more sustainable than the destructive use of the roots of this threatened plant species.

Micropropagation is a useful technique for rapid clonal multiplication of plant material which could alleviate the pressure on the wild plant populations as well as potentially provide plant materials for use. This study did develop a successful decontamination method and the efficient means for eliminating detrimental browning of *G. perpensa* explants and media. The result demonstrated that ascorbic acid or activated charcoal was required as media supplements to reduce the browning effect. This information provides an important starting point for the development of a successful micropropagation protocol for the conservation of *G. perpensa*.

This research further highlights the need to conserve our indigenous plant resources before they become extinct, since some of them could be pharmacologically active and perhaps contain novel compounds that are biologically active against some diseases.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER ONE

General Introduction

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1.1 Use of plants in traditional medicine

Plants have always played a major role in manôs life. In fact, medicinal and food uses are two of the most popular uses of plants. They are among the most persistent ones, even in cultures that are steadily losing their close relationships with nature (Bonet and Vallès, 2007). The vast majority of people in the world rely on traditional medicinal plants for their everyday health care needs (Azaizeh *et al.*, 2003). The World Health Organisation estimated that up to 80 percent of the people, mostly from the developing world, use traditional medicine for this purpose (WHO, 2001). Besides their cultural significance, the remedies from medicinal plants are generally more accessible and affordable to most of the population especially in African communities, and are generally believed to be effective (Zhang, 2004). The primary beneýt of using plant-derived medicines is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits (Agrawal, 2005). As a result, there is an increasing trend worldwide to integrate herbal medicines with primary health care (Fennell *et al.*, 2004).

1.2 Medicinal plants in South Africa

South Africa has a rich plant biodiversity, with many of the plants having medicinal uses. Nowadays, most of the populations in urban South Africa as well as smaller rural communities are reliant on herbal medicines for their health care needs (Fennell *et al.*, 2004). The use of herbal remedies in traditional medicine is an integral part of South African cultural life and it is not likely to change much in years to come (Van Vuuren, 2008). Diverse groups of people depend on several species of medicinal plants for the treatment of various ailments in both humans and domestic animals (Masika and Afolayan, 2002).

In the Nkonkobe Municipality of the Eastern Cape Province, medicinal plants are locally used by the sangomas, herbalists and rural dwellers for the treatment of a wide range of diseases such as *diabetes mellitus* (Oyedemi *et al.*, 2009); HIV and AIDS related sicknesses (Otang *et al.*, 2012). Indigenous medicinal plants are used to enhance the personal well-being of many inhabitants in the province. These medicinal plants are also used as protection against evil spirits, for ritual purification, for luck in the general sense as well as in relationships and in the courtroom, and for removing poison inflicted through witchcraft (Dolds and Cocks, 2012).

Plant species are used in Zulu traditional medicine for the treatment of various illnesses. For example, an infusion of the powdered þowers of *Aloe maculata* is used as a remedy for colds and fever in children; as a remedy for overindulgence in food and alcohol (Hutchings *et al.*, 1996). Cape aloes are most widely used for its potent laxative and cathartic effects, attributed to anthraquinones and in particular aloe-emodin in the leaf exudate (Steenkamp and Stewart, 2007). *Pentanisia prunelloides* has many uses in Zulu medicine, but most commonly root decoctions are used for swellings, sore joints, rheumatism and chest pain (Watt and Breyer-Brandwijk, 1962).

The Mpumalanga, Zulu and the Swazi people drink decoctions of the *Alepidea amatymbica* root to treat influenza, while the Xhosa people use the plant to cure abdominal disorders and stomach pains (Hutchings, 1989). *Bulbine frutescens* is well known in households of South Africa where the fleshy, jelly-like leaves are applied to burns, acne, insect bites, blisters, rashes and chapped lips to help soothe and heal the area (Van Wyk *et al.*, 2000).

According to Mander *et al.* (2007), seventy two percent of the black African population in South Africa is estimated to use herbal medicine, accounting for some 26.6 million consumers. The authors indicated that many of these people do not consider herbal medicine as an inferior alternative to western medicine, but as a desirable and necessary means for treating a range of health problems that western medicine does not treat adequately. The consumption of herbal medicines in South Africa is widespread and growing. The demand for better quality medicinal plant products is increasing. On the other hand, the supply of wild medicinal plant stocks is declining and highly valued species are becoming inaccessible due to extinction and the rapid rise in market prices (Mander *et al.*, 2007).

1.3 The need for conservation of medicinal plants

The intensive harvesting of plant materials from wild populations for medicinal purposes and commercial trade in South Africa poses a threat to many plant species (Mander 1998; Keirungi and Fabricius, 2005). In many developing countries, over-exploitation of natural plant resources for medicinal purposes is closely linked to rapid population growth and increasing urbanization (Nzue, 2009). These have resulted in the decline of certain species, extinction in others, and a general decrease in biodiversity of high use areas of such plant species. Many researchers have reported destructive and/or unsustainable harvesting of some of these species, since a very large proportion harvested are the underground parts (Cunningham, 1990; Dold and Cocks 2002). In some instances, the entire plant is harvested. Rukangira (2001) also noted that the demand for medicinal plants in developing countries, such as South Africa, has resulted in unsustainable harvesting which in turn, reduces the biodiversity of natural vegetation. For the medicinal plant gatherers, sustainable harvesting

methods are not a priority. The short-term financial incentives for plants traded are their main aim, rather than the long-term benefit of conserving wild populations (Fennell *et al.*, 2004).

In the light of the current increasing demand for medicinal plants which far exceed supply, the medicinal potentials of such species may be totally lost if efforts are not geared towards their conservation (Amoo, 2009). Their extinction would mean, amongst other things, the loss of genes which could be used for plant improvement or in the biosynthesis of new compounds (Rates, 2001). Conservation of medicinal plants is therefore, important to guarantee continuous supply of plant materials in the future, for medicinal purposes. The ultimate goal of conservation process is to preserve the natural habitats of vulnerable medicinal plant species and to achieve sustainable exploitation and utilization in less vulnerable areas (Cunningham, 1993). Effective conservation of biodiversity can only be achieved through the sustained efforts of all, but most importantly rural communities, who rely directly on local biodiversity for their livelihoods (Hunde, 2007).

1.4 Conservation through cultivation

In order to meet the current demand for medicinal plants, several efforts have been attempted to conserve the diversity of medicinal plants. One of such efforts is stimulation of cultivation as a means to relieve over-exploitation (Wiersum *et al.*, 2006). Cultivation can reduce the threat to wild populations by making propagated plants more readily available. An example is the successful cultivation of some South African aloes as well as other medicinal plants such as *Siphonochilus aethiopicus* (Donaldson, 2006). Cultivation offers the opportunity to optimize yield and to achieve uniform, high quality production of medicinal plants (Canter *et al.*, 2005). Cultivation was also reported to have an advantage over wild collection, in that it

greatly reduces the possibilities of misidentification and adulteration (Amujoyegbe *et al.*, 2012). Therefore, the movement toward domestication as a vehicle for genetic preservation and conservation is an important strategy (Franz, 1993).

Cunningham (1993) recommended that there should be rapid development of alternative supply sources through cultivation in large enough quantities and at a low enough price to compete with prices obtained by gatherers of wild stocks. The author affirmed that the approach would satisfy market demands, result in more secure jobs, provide fewer incentives to gather from the wild and prevent key plant species from disappearing in the wild. The success of this approach will prevent the local medicinal plant resource from decline.

1.5 Conservation through plant-part substitution

In southern Africa the most frequently used medicinal plants are slow-growing forest trees, bulbous and tuberous plants, with bark and underground parts being mostly utilized (Zschocke *et al.*, 2000). A strategy which would satisfy the requirements of sustainable harvesting, and at the same time provide for primary health care needs, would be the substitution of bark or underground parts with the aerial parts of the same plant (Zschocke *et al.*, 2000). A study by Cunningham (1988) has shown that leaf and fruit harvesting does not damage plants in the same way as debarking. For this strategy to work effectively, it was recommended that every investigation into a medicinal plant should always include an investigation of leaves and other aerial plant parts, even though they might not be the parts traditionally used (Zschocke *et al.*, 2000). Findings from such investigations communicated to herbal healers for implementation and the cultivation of medicinal plants may protect more

species from extinction, and allow the recovery of threatened medicinal plants (Zschocke *et al.*, 2000).

1.6 Conservation through Micropropagation

In vitro propagation methods offer highly efficient tools for conservation and large-scale propagation of medicinally important and endangered plants (Afolayan and Adebola, 2004). The technique of tissue and organ culture is used for rapid multiplication of plants in limited space and time, for genetic improvement of crops, for obtaining disease- free clones and for preserving valuable germplasm (Bhojwani and Razdan, 1992). Plant material can also be produced throughout the year without any seasonal limitation.

Although micropropagation protocols have been established for many indigenous medicinal species, these techniques are labour-intensive, costly and therefore only feasible for high-value species (Ziv *et al.*, 1995). The standardization of a propagation protocol could, sometimes be time consuming. However, successful micropropagation would reduce harvesting of natural populations and provide a reliable source of plants to small scale farmers, thus conserving such plant species.

1.7 Distribution, morphology, uses and conservation status of *Gunnera perpensa*

The genus *Gunnera*, the only member of Gunneraceae family, is a genus of herbaceous flowering plants comprising about 40ï 50 species (Hinkley, 2007). The species vary enormously in leaf size and some of them are gigantic. The genus *Gunnera* has numerous unique characteristics, some of which indicate that the plants have been in existence for the past 95 million years (Bergman *et al.* 1992). They belong to one of the oldest angiosperm

families and are among the largest herbs on earth (Bergman *et al.* 1992). *Gunnera perpensa*, the only African member of the genus is widespread in tropical Africa from Sudan, Ethiopia, Zaire, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe and Mozambique, including Lesotho and Swaziland (Hinkley, 2007). In South Africa, it is distributed in the following provinces: Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West, Western Cape (Williams *et al*., 2008).

Gunnera perpensa is a perennial, robust, erect herb up to 1 m tall, with petioles that vary from 150 to 750 mm in length (Figure. 1.1). They have large, dark bluish green, kidney-shaped leaves that are covered with hairs on both surfaces, and arise from a central tuft near the top of the apex, just above the soil level (Mendes, 1978). The margin of the leaves is irregularly toothed. The flowers are numerous, small, tiny pinkish reddish brown and not very noticeable, borne on a long slender spike which is taller than the leaves (Mendes, 1978). The plant flowers between September and February. The roots are up to 300 mm thick, creeping in black, muddy soil.

Figure 1.1: *Gunnera perpensa* L. plant

Gunnera perpensa is used as a source of traditional medicine in Southern Africa. A decoction of the roots or rhizomes is used to facilitate labour, expel the placenta after birth or relieve menstrual pains (van Wyk and Gericke 2000; Ngwenya *et al*., 2003; Von Ahlenfeldt *et al*., 2003). They have also been used for rheumatic pains, cold, wound-dressing, stomach ailments, treatment of female infertility and male impotence (Grierson and Afolayan, 1999; Iwalewa *et al.*, 2007). Infusions of the rhizome have been used topically and orally for psoriasis, while, tinctures have been used for urinary stones (van Wyk *et al.*, 1997). It is used as an antibacterial medication, in addition to other uses (Hutchings, 1996).

Widespread harvesting has placed *G. perpensa* under threat of overexploitation. Its collection or harvesting is destructive since the roots are often used. Dold and Cocks (2002) described this plant species as scarce and heavily traded. Furthermore, recent assessment showed that large volumes of this species are traded in traditional medicine markets and local extirpations have been noted (Williams *et al*., 2008). The current Plant Red List of South Africa enlists *G. perpensa* as declining (Raimondo *et al.*, 2009).

1.8 The choice of *Gunnera perpensa* for this study

An ethnobotanical survey conducted in Nkonkobe municipality of the Eastern Cape to identify and document scarce medicinal plants in the study area revealed that *Gunnera perpensa* was one of the frequently cited as threatened (Chapter 2). The local communityôs perception of its conservation status was in agreement with the Red Data List of Southern Africa, where the current conservation status of this plant species was recorded as declining (Raimondo *et al.*, 2009). Despite the conservation status of *G. perpensa*, little work has been done on its propagation and conservation, unlike other highly threatened species listed along

with it. Conventional propagation and Micropropagation work has been carried out on most of the plants. *Gunnera perpensa* are species with specific habitat requirements and are intensively exploited for their roots. Its habitat is reported to be degrading and declining due to wetland mismanagement (Von Ahlenfeldt *et al*., 2003). Following Struhsakerôs (1996) definition of the term ósustainableô, it can be established that this plant species is harvested unsustainably, as they are either entirely or partially removed, resulting in the loss of the plant. As a result, urgent conservation attention is needed for *G. perpensa*. Furthermore, this plant has ethnopharmacological data indicating their traditional utilisation in the treatment of various diseases (van Wyk *et al.*, 1997; Grierson and Afolayan, 1999; Buwa and van Staden, 2006). Despite the widespread use of *G. perpensa* in treating various illnesses, there is dearth of information regarding the propagation, cultivation and conservation; yet these are necessary for the scientific conservation and preservation of this species. Hence, the choice of *Gunnera perpensa* as a case study plant.

1.9 Objectives of the study

The broad objective of this study was to develop propagation and conservation techniques for *Gunnera perpensa*, an over-exploited medicinal plant in the Eastern Cape Province, South Africa.

The specific objectives were to:

- Conduct an ethnobotanical survey to identify and document scarce medicinal plants in Nkonkobe municipality of the Eastern Cape Province, South Africa. From the survey, *Gunnera perpensa* was chosen.

- Propagate the plant chosen from the survey data (*G. perpensa*), using varying lengths of their rhizomes as propagules.
- Determine the appropriate planting depth for vegetative propagation of *G. perpensa* using rhizome.
- Examine the growth and establishment of the plant from rhizome to maturity.
- Carry out germination studies and seedling growth on *G. perpensa* seeds.
- Investigate the possibility of using the aerial part of *G. perpensa* for antimicrobial formulation, as a conservation strategy, against the unsustainable use of the underground part for medicinal purposes.
- Evaluate the phytochemical and antioxidant properties of the leaf and root of this plant species for their potential in plant part substitution.
- Investigate the possibility of establishing *G. perpensa* through *in vitro* propagation technique.

1.10 The structure of this thesis

This thesis contains 9 chapters. Chapter 1 is the general introduction. Chapter 2 is the ethnobotanical survey of scarce medicinal plants in Nkonkobe Municipality, Eastern Cape Province, South Africa. Chapter 3 contains the clonal propagation of *Gunnera perpensa* using varying lengths of rhizome. Determination of the appropriate planting depth for *G. perpensa* rhizome is presented in Chapter 4. Chapter 5 contains seed germination studies and seedling growth on *G. perpensa*. Chapter 6 contains the investigations of the antibacterial and antifungal activities of the leaf and rhizome extracts of *G. perpensa* for the possibility of plant part substitution. Chapter 7 contains the evaluation of the phytochemical constituents and the antioxidant properties of the leaf and root extracts for their potential in plant part

substitution. Chapter 8 is the micropropagation of *G. perpensa*. The general discussion, recommendations and contributions emanating from the entire study are presented in Chapter 9.

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CHAPTER 2

ETHNOBOTANICAL SURVEY OF SCARCE MEDICINAL PLANTS IN NKONKOBE MUNICIPALITY, EASTERN CAPE, SOUTH AFRICA

CHAPTER TWO

Ethnobotanical survey of Scarce Medicinal Plants in Nkonkobe Municipality, Eastern Cape, South Africa

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2.1 Introduction

Plants are universally regarded as a vital part of the world biological diversity and an essential resource for the planet earth. Many plant species have great economic and cultural importance; as sources of food, medicine, fuel, clothing and shelter for humans around the world (SCBD, 2009). The use of plants for traditional medicine and cultural purposes is an age-old practice in South Africa which continues to thrive in the present day. For example, Mander (1998) estimated that 27 million South Africans make use of indigenous medicinal plants for the treatment of various ailments in man and animal, with thousands of herb gatherers supplying the demand. It is also a common practice in the Eastern Cape Province of South Africa, where a very large proportion (80%) of the people (the inhabitants of the study area) depends on traditional medicine for their health care (Hirst, 1990; Dold and Cocks, 2000). The Eastern Cape Province of South Africa has high plant diversity, many of which are medicinally useful.

Most of the plants used in traditional medicine in the province are collected from the wild. The intensive harvesting of the wild stock is causing a decline of valuable medicinal plant species, resulting in a serious threat to the biodiversity of the region (Dold and Cocks, 2002). According to the authors, the continuous increase in human population and high level of poverty has increased the demand for herbal medicines and the pressure on natural resources have become even greater. Because of this trend, many plant species are seriously threatened with extinction and there is no guarantee that humanity will continue to benefit indefinitely from this valuable resource (Kambizi and Afolayan, 2006). According to the Red List Statistics, the Eastern Cape is third in the list of provinces in South Africa where threatened species occur most, with 295 threatened taxa (SANBI, 2011).

The plant species are threatened by factors that include urbanization, agricultural expansion, habitat transformation, medicinal plant harvesting, illegal collecting, over-grazing, invasive alien species, pollution and forestry (Victor and Dold, 2003). Rather than leave natural vegetation intact, these factors prompt increased scarcity and even extinction in some cases. Victor and Dold (2003) reported that illegal collecting has been a serious threat to certain plant taxa in this region. Plants of medicinal importance such as *Alepidia amatymbica* and *Hypoxis hemerocallidea* are facing serious threats from collectors. According to Williams *et al.* (2008), *Hypoxis hemerocallidea* has been declining in population because of intensive harvesting for medicinal plant trade throughout the country since 1997.

It has become very difficult to stop the destruction of plant diversity that is essential to meet the present and future needs of humankind. This disappearance of many valuable medicinal plant species has compounded the problem of loss of indigenous knowledge on herbal medicine in many ethnic groups in South Africa and elsewhere. Therefore, there is an urgent need to identify and document this unique traditional knowledge of medicinal plant use and status at the community level before it vanishes.

The purpose of this chapter was to identify and document the medicinal plants used by herbal healers in Nkonkobe Municipality, Eastern Cape Province, which are becoming scarce and under threat of extinction and thus need conservation attention. The knowledge of these plants is very important as this can serve as leads in the campaign for sustainable utilisation and critical studies leading to the conservation of these valuable plant resources. This will help to optimize their use in the primary health care system. The failure to pass indigenous knowledge from one generation to the next, from a cultural perspective necessitates the need

to document such knowledge for future generations (Thring and Weitz, 2006). This chapter presents an inventory of scarce medicinal plants used in Nkonkobe municipality; parts of the plant used, mode of harvesting, conservation status and factors threatening the various species.

2.2 Materials and methods

2.2.1 Description of the study area

Nkonkobe Local Municipality is an administrative area in the Amathole District of the Eastern Cape (Fig. 2.1). It is located within 32°47' S, 26°50' E. The area is enclosed by the sea in the east and drier Karroo in the west (Erasto *et al.*, 2005). Eastern Cape, on the other hand covers approximately 170,500km^2 in area. An estimated 6,562,053 people live in the province and this constitutes about 13.5% of the total population of people living in South Africa (SSA, 2011). The altitude is approximately 1300 m above sea level and the vegetation is the Veld type (Masika and Afolayan, 2003). Nkonkobe Local Municipality covers 3,725 km^2, and constitutes 16% of the surface area of the Amathole District Municipality. According to ECSECC (2012), Nkonkobe Municipality has an estimated total population of 135,660 with 27,716 households. The majority of the population (72%) resides in villages, farms and scattered settlements while 28% resides in urban settlements, mostly Alice and Fort Beaufort, where the main concentration of businesses occurs (ECSECC, 2012). The dominant group is the Black Africans (95%) of the population, followed by the mixed race (4%) and 1% white (ECSECC, 2012). The population of Nkonkobe is dominated by youth and women, while the majority ages are between 15 and 34. The major ethnic group in the area is Xhosa, with farming as their main occupation. The high cultural value of traditional medicine, coupled with deep-seated rural life (ECSECC, 2012) suggests that a large

proportion of this population makes use of traditional methods of health care with high demand on the natural plant resource.

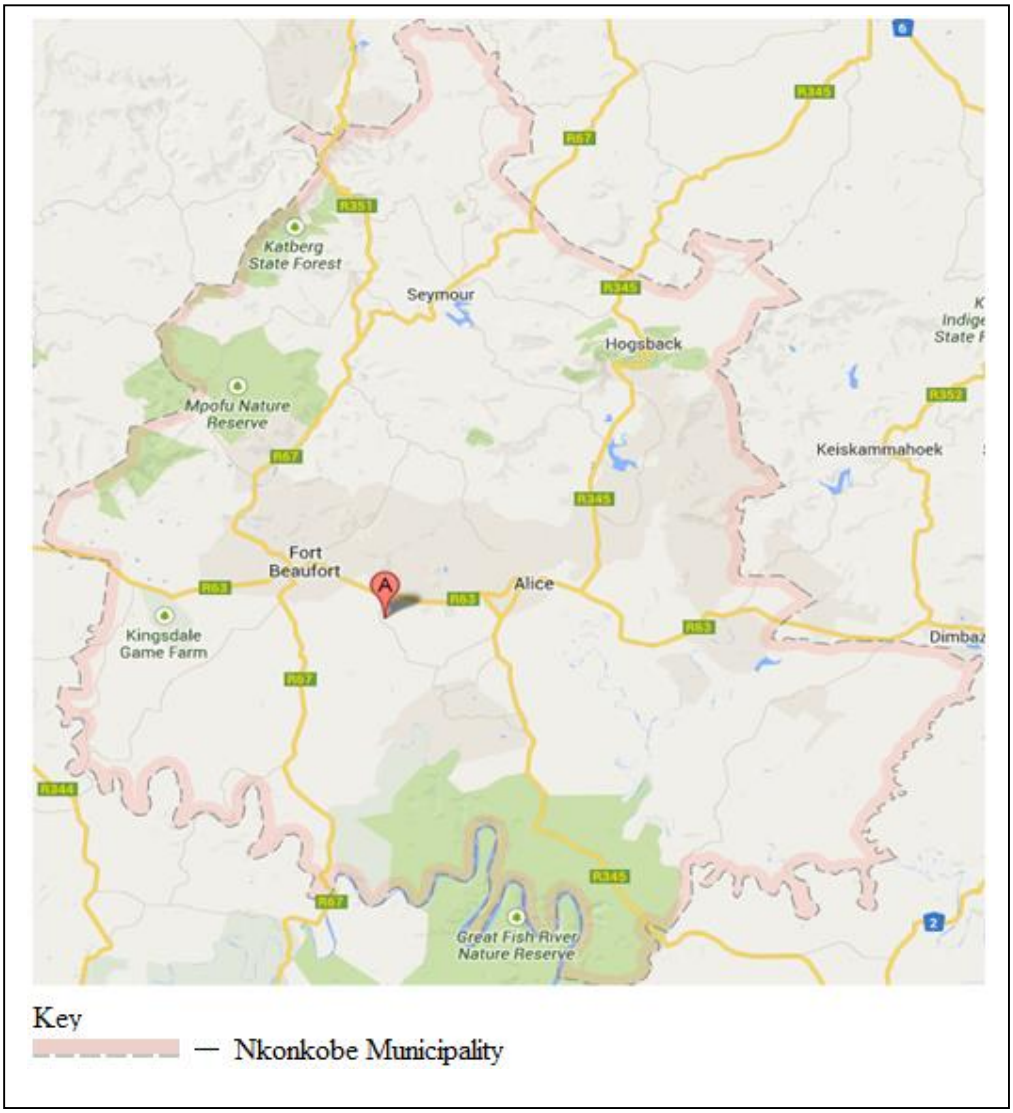

Figure 2.1: Map of Nkonkobe Municipality showing Alice and Fort Beaufort

(Source: Google Map, 2014)

2.2.2 Ethnobotanical data collection

The survey was conducted from January to September 2013, and the study sites included villages around Alice and Fort Beaufort. Information was collected from 65 persons aged between 34 and 74, who had traditional knowledge of plants. The investigation was carried out using interviews among herbal healers, herbal sellers and knowledgeable local elders who use medicinal plants to treat common illnesses. Structured questionnaires were administered through personal contact discussions (Bisi-Johnson *et al.*, 2010). Interviews were conducted in the local language of the informants (isiXhosa) and were later translated to English. In most cases the interviews often started in the form of informal discussions to gain the confidence of the interviewees. Detailed notes were taken on the medicinal uses of the plants, following the methodology of Abassi *et al.* (2010). The survey aimed to identify medicinal plants that are becoming scarce in the study area, their growth form, parts of plant used, frequency of harvest and factors threatening the plant resources. The mention of each plant species was accepted as valid, only if it was mentioned by at least 3 independent respondents.

2.2.3 Identification of plant materials

The species were initially identified by their vernacular names. Identification of the collected plant specimen was done in the Department of Botany at the University of Fort Hare, with the help of floristic works of South Africa (Van Wyk *et al.*, 1997; Dold and Cocks. 1999). The identification and nomenclature of the listed plants were based on the Flora of South Africa. Voucher specimens of the reported plants were deposited at the Griffin Herbarium of the University.

2.2.4 Conservation status

Literature was surveyed to determine the conservation status of the recorded plant species. Each species was assessed according to version 3.1 of the International Union for Conservation of Nature (IUCN) categories and criteria as listed in the National Red Data List of South African plants (SANBI, 2009). The IUCN has developed a clear set of categories and criteria to indicate the varying degrees of probability of extinction of a species. The national conservation status of the indicated plants as recorded in the Red Data List was subsequently compared with the local communityôs perception of scarce or threatened medicinal plants.

2.2.5 Data analysis

The data were spread on an excel sheet to summarise and to identify various proportions like plant families, growth form, plant parts used as medicine and frequency of citation. Frequency of citation was calculated by following the formula of Singh *et al.* (2012):

$$\text{Frequency of citation}\,(\%) = \frac{\text{Number of informants who cited the species}}{\text{Total number of informants interviewed}} \times 100$$

2.3 Results

2.3.1 Medicinal plants recorded

A total of 61 plant species belonging to 40 families were reported as scarce and declining in the study area (Table 2.1). The family Asteraceae had the highest number (12.5%) of species. The proportions of other families were as follows: Asphodelaceae, Amaryllidaceae, Hyacinthaceae, Asparagaceae and Euphorbiaceae (7.5% each). The families Apiaceae,

Apocynaceae, Rubiaceae, Orchidaceae, Vitaceae, Fabaceae and Rhamnaceae had 5% of the total plant species each. The medicinal plants, their ethnomedicinal uses along with local names, plant parts used, growth forms and national conservation status have been summarized in Table 2.1.

Table 2.1: Ethnobotanical data on scarce medicinal plants in Nkonkobe Municipality

Botanical name voucher no	**Family**	**Freq. of citation (%)**	**Local name**	**Parts used**	**Growth form**	**Medicinal uses**	**2009 Red List status**
Acalypha glandulifolia (CB01)	Euphorbiaceae	14	Umanzanyama	Root	Herb	To treat diarrhoea	LC
Acokanthera oppositifolia (Lam.) Codd (CB02)	Apocynaceae	30	Ubuhlungu	Leaves and roots	Shrub	To treat headaches, abdominal pains and convulsion	LC
Agapanthus africanus (L.) Hoffmanns. (CB03)	Agapanthaceae	36	Isicakathi	Root	Evergreen grass	Augment labour	EN
Alepidea amatymbica Eckl.& Zeyh. (CB04)	Apiaceae	32	Iqwili	Root	Herb	To treat colds, coughs, wound, rheumatism	VU
Arctotis arctotoides	Asteraceae	22	Ubushwa	Leaves	Herb	To treat epilepsy,	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
(L.f.) O.Hoffm. (CB05)						indigestion and catarrh of the stomach	
Artemisia afra Jacq. ex Willd. (CB06)	Asteraceae	40	Umhlonyane	Root, stem and leaves	shrub	Coughs, fever, loss of appetite, cold, colic, earache headache, intestinal worms	LC
Asparagus africanus Lam. (CB07)	Asparagaceae	14	Ubulawu	Root	Climbing shrub	Relief of pains, rheumatism, syphillis	LC
Barleria sp. (CB08)	Acanthaceae	8	Inzinziniba	Leaves, roots	shrub	Pains, swellings	LC
Behnia reticulata (Thunb.) Didr. (CB09)	Behniaceae	36	Isilawu	Root	Climber	For body wash and good fortune in court cases	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Bersama lucens (Hochst.) Szyszyl. (CB10)	Melianthaceae	36	Isindiyandiya	Bark or root	Tree	to relieve menstrual pain, headache, stroke and nervous disorders, cancer	LC
Boophone disticha (L.f.) Herb. (CB11)	Amaryllidaceae	12	Incwadi	Bulb	Herb	To treat constipation, circumcision wound	Declining
Bowiea volubilis Harv. ex Hook.f. (CB12)	Hyacinthaceae	30	Umaqana	Bulb	Herb	To treat male impotence, asthma, lower back pains	VU
Bulbine Latifolia (L.) Willd. (CB13)	Asphodelaceae	22	Umanzabomvu	Root	Succulent	Bladder and kidney problems	LC
Burchellia bubaline (L.f.) Sims (CB14)	Rubiaceae	22	Umfincane	Root	Tree	Body wash	ND

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Carpobrotus edulis (L.) Bolus (CB15)	Mesembryanthemaceae	30	Igcukuma	Leaves and root	Herbaceous perennial	Boost immune system, stomach complaint, skin infection	LC
Centella coriacea Nannfd. (CB16)	Apiaceae	10	Umsolo	Whole plant	Herb	Wound healing and toothache	LC
Cinnamomum camphora (L.) J Presl. (CB17)	Lauraceae	14	uRoselina	Bark and root	Tree	Body and blood cleansing, vomiting	ND
Clivia sp. (CB18)	Amaryllidaceae	14	Ugobeleweni	Root	Perennial herb	High blood pressure, cancer	VU
Combretum apiculatum Sond. (CB19)	Combretaceae	18	Umdubi	Root, leaf and bark	Tree	Chest pain, stomach complaint, eye pain	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Curtisia dentata (Burm.f.) C.A.Sm. (CB20)	Cornacea	24	Umlahleni,	Bark	Tree	Stomach ailments, diarrhoea and as a blood purifier	NT
Cyrtorchis arcuata (Lindl.) Schltr. (CB21)	Orchidaceae	24	Iphamba	Whole plant	Epiphytic herb	Ritual wash and steam treatment to ensure good luck	LC
Dianthus thunbergii Hooper (CB22)	Caryophyllacea e	30	Ungcana	Root	Shrub	Body cleansing, stomach ache	LC
Dioscorea sylvatica (Kunth) Eckl. (CB24)	Dioscoreaceae	36	Isifundo	Tuber	Climber	Chest pain and as a ritual wash	VU
Drimia anomala (Baker) Benth.	Hyacinthaceae	30	Ungcana	Bulb and leaves	Herb	Skin problems, body cleansing, stomach	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
(CB23)						complaint	
Elephantorrhiza elephantina (Burch.) Skeels (CB25)	Fabaceae	22	Intolwane,	Root	Shrub	Diarrhoea, stopping bleeding, heart ailments, treat intestinal disorders, haemorrhoids,	LC
Eucomis autumnalis (Mill.) Chitt. (CB26)	Asparagaceae	50	Isithithibala	Bulb	Bulbous herb	To treat low backache and aid in healing fractures	Declining
Euphorbia bupleurifolia Jacq. (CB27)	Euphorbiaceae	22	Intsema	Whole plant	Succulent plant	Cancerous sores, cracked skin, skin disorders	Declining
Exomis microphylla	Chenopodiaceae	18	Umvenyathi	Leaf	Perennial herbs	Healing of wounds,	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
(Thunb.) (CB28)						convulsion in infant	
Gasteria bicolor Haw. (CB30)	Asphodelaceae	34	Intelezi	leaf	succulent	Various ailments like treating HIV patients	rare
Gunnera perpensa L. (CB29)	Gunneraceae	32	Iphuzi	Rhizome	Herb	Induce labour, rheumatoid pains, stomach ailment	Declining
Haemanthus albiflos Jacq. (CB31)	Amaryllidaceae	36	Umathunga	Bulb	Herb	Chronic cough, healing of broken bones, heal an operation	LC
Helichrysum odoratissum (L.) Sweet (CB32)	Asteraceae	50	Impepho	Leaf or whole plant	Perennial herb	Healing of headache and flu	LC
Helichrysum	Asteraceae	50	Isicwe	Leaves	Herb	To treat circumcision	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
penduculatum Hilliard & Burtt (CB33)						wounds	
Helinus integrifolius (Lam.) Kuntze (CB34)	Rhamnaceae	36	Isilawu	Root	Herbs	Hysteria, backache	LC
Hippobromus pauciflorus (L.f.) Radlk. (CB35)	Sapindaceae	8	Umfazi Onengxolo	Bark	Semi- deciduous tree	Relieves cough, eye problem, dysentery, headaches	LC
Hydnora africana Thunb. (CB36)	Hydnoraceae	30	Umavumbuka	Whole plant	Epiphyte	Kidney problem, used as face wash to treat acne, diarrhoea	LC
Hypoxis	Hypoxidaceae	26	Inongwe	Root	Herb	High blood pressure,	Declining

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
hemerocallidea Fisch. & C.A.Mey. (CB37)						diabetes, arthritis, skin rashes, acne	
Ilex mitis (L.) Radlk. (CB38)	Aquifoliaceae	34	Isidumo	Bark	Tree	Treating colic in children, eczema, as purgative	Declining
Kedrostis africana (L.) Cogn. (CB39)	Curcubitaceae	14	Utuvishe	Leaves and root	Shrub	To treat coughs, colds, fever and bronchitis	LC
Kniphofia drepanophylla Baker (CB40)	Asphodelaceae	26	Ixonye	Bulb and leaves	Herb	Body cleansing, for good fortune	VU
Ledebouria sp (Hook.f.) Jessop	Hyacinthaceae	22	Inqwebeba	Bulb	Succulent	Pregnancy, diarrhoea, influenza, backache,	EN

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
(CB41)						skin irritations	
Malva parviflora L. (CB42)	Malvaceae	22	Ijongilanga	Roots or leaves	Herb	Used as a hair rinse to remove dandruff, to treat swellings and boils	ND
Olea capensis L. subsp. Capensis (CB43)	Oleaceae	32	Umnquma	Bark	Tree	Used as tonic and blood purifier, treat stomach problems	LC
Ornithogalum longibracteatum Jacq. (CB44)	Asparagaceae	14	Umredeni	Bulb	Herb	To treat muscle pain, toothache, rheumatism, head ache	LC
Pachycarpus grandiflorus (CB45)	Apocynaceae	18	Itshongwe	Root	Herb	Treatment of sexually transmitted infections and headache	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Pentanisia prunelloides (Klotzsch ex Eckl. & Zeyh.) Walp (CB46)	Rubiaceae	14	Isicimamlilo	Tuberous root	Herb	Use for swellings, relieve chest pain, sore joints and rheumatism	LC
Pittosporum viridiflorum Sims (CB47)	Pittosporaceae	10	Umkhwenkwe	Stem bark and root	Tree	To treat stomach complaint, fever, ease pain and having a generally calming effect	LC
Polystacha pubescens (CB48)	Orchidaceae	14	Iphamba	Bulb	Epiphyte	Treatment of inflammatory, intestinal disorders	LC
Rapanea melanophloeos	Myrsinaceae	30	Umemezi,	Bark	Tree	For respiratory problems, stomach,	Declining

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
(L.)Mez. (CB49)						muscular and heart complaints	
Rhoicissus digitata (L.f.) Gilg & Brandt (CB50)	Vitaceae	40	Uchithibhunga	Tuber	Climber	To facilitate delivery	LC
Rhoicissus tomentosa (Lam.) Wild & R.B.Drumm. (CB51)	Vitaceae	32	Umphindabam shaye	Tuber	Climber	Taken for acute headache, high blood pressure	LC
Rumex lanceolatus (CB52)	Polygonaceae	32	Idolo Lenkonyana	Leaves and root	Herb	Increase blood cell and treatment of wounds	LC
Sanseviera thyrsiflora (L.)	Dracaenaceae	14	Isikolokotho	Leaves	Herb	To treat ear infection	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Druce (CB53)							
Schotia latifolia Jacq. (CB54)	Fabaceae	32	Umaphipha	Bark	Tree	used for the management of heartburn, diarrhoea	LC
Spirostachys africana Sond. (CB55)	Euphorbiaceae	30	Umthombothi	Bark	Tree	Ease stomach pain and tooth ache	LC
Strychnos henningsii Gilg. (CB56)	Loganiaceae	36	Umnonono	Bark	Small tree	Gastrointestinal disorder, diabetes, ease menstrual pain	LC
Talinum caffrum (Thunb.) Eckl. & Zeyh. (CB57)	Portulacaceae	18	Uphuncuka	Tuber	Shrub	Used as an emetic for protection against evil spirits and bad luck	LC

Table 2.1 Continued

Botanical name voucher no	Family	Freq. of citation (%)	Local name	Parts used	Growth form	Medicinal uses	2009 Red List status
Tulbaghia acutiloba Harv. (CB58)	Alliaceae	30	Isivumbampunzi	Root	Herb	To treat anxiety attacks	LC
Vernonia mespilifolia Less. (CB59)	Asteraceae	22	Uhlunguhlungu	Whole plant	Shrub	High blood pressure, for fertility, treat stomach poisoning	LC
Zantedeschia aethiopia (L.) Spreng. (CB60)	Araceae	14	Inyinyiba	Rhizome and leaves	Herb	For dressing wounds	LC
Ziziphus mucronata Willd. (CB61)	Rhamnaceae	22	Umphafa	Root, bark and leaves	Tree	Chest pain relief, and pain killer	LC

VU: vulnerable, EN: endangered, LC: least concern, NT: near threatened, NE: not evaluated
Frequency refers to the number of respondents out of all the respondents interviewed who listed the plant species amongst their top 3 highly scarce plant species

2.3.2 Growth form, plant part used and collection

The growth forms of scarce medicinal plant species harvested by traditional healers for medicinal purposes were majorly herbs (43%), followed by trees (23%), shrubs (15%), succulents (6%), Climbers (6%), epiphytes (5%) and evergreen grass (2%) as shown in Figure 2.2.

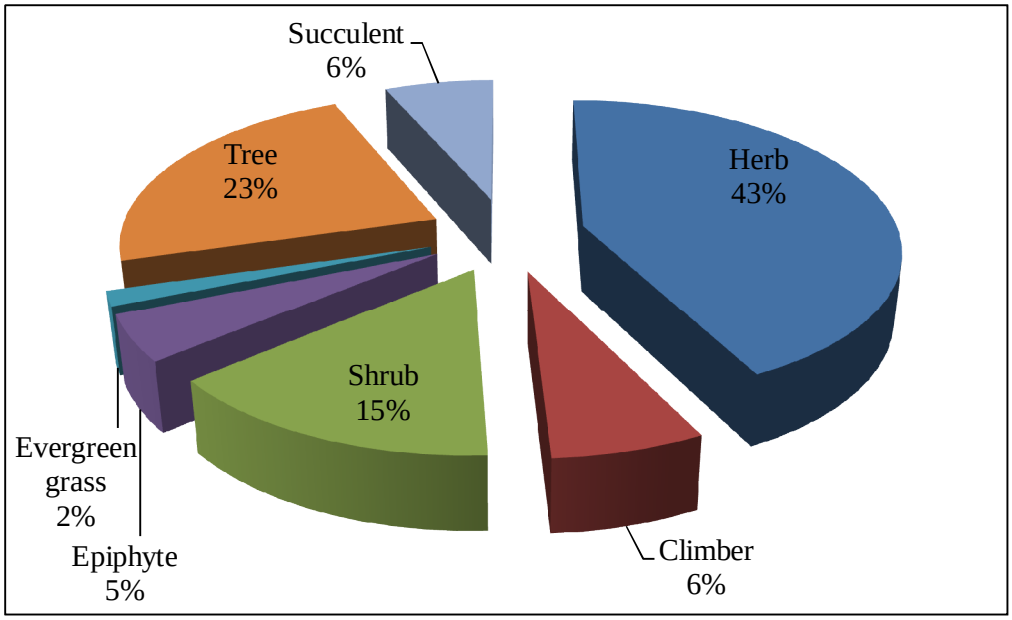

Figure 2.2: Growth forms of the scarce medicinal plant species

Amongst the various plant parts, roots were the most frequently used constituting 31% followed by leaves (25%), bark (16%), bulb (11%), tuber (6%) and rhizome (2%). In 9% of the species, whole plant was used (Fig.2.3). The findings of this survey show that almost all plant parts used for medication are used either singly or in combination with other plants. Plant parts used as medicine are collected by healers themselves (85%) from the wild, while 15% of the healers make use of trainees for the collection of plant material. Generally, fresh parts are collected for use from natural population. Various plant parts are collected in

different seasons at different stages of maturity, dried under shade and stored in dry places for use during off season.

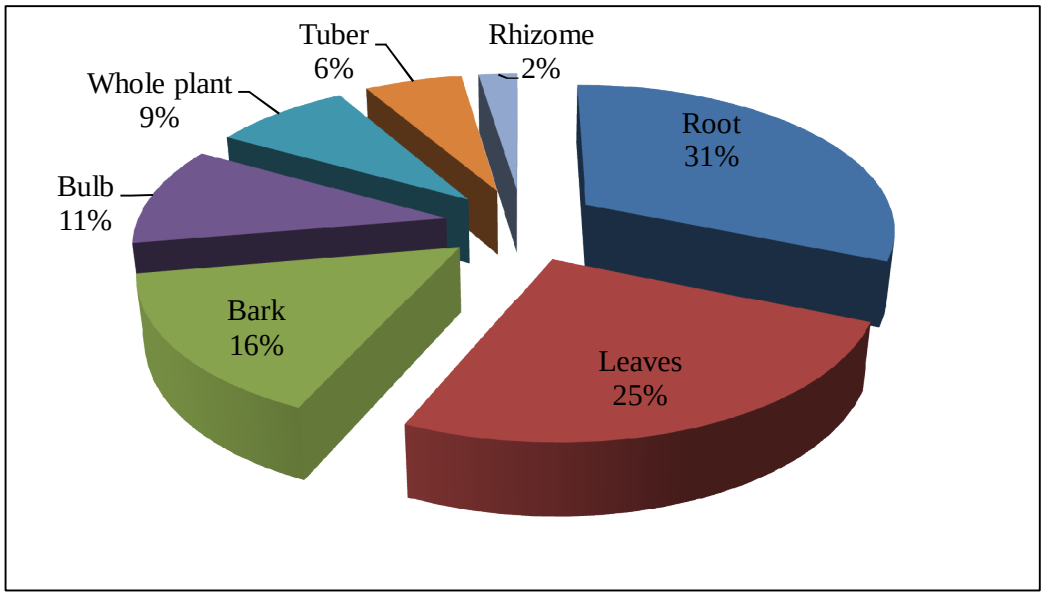

Figure 2.3: Parts of the scarce medicinal plants harvested for use

2.3.3 Source of plant

Regarding the source of medicinal species used by the respondents (healers), a great majority (82.7%) collected plants from the wild. Those obtained from home gardens and around the house were mentioned by 17.3%. Those found within protected areas were quite few, and included *A. afra, A. amatymbica, S. thyrsiflora and T. caffrum*. The harvesting of plant species from the wild and the exchange of locally unavailable ones with other goods was practiced by 10% of the respondents who were mostly herbal sellers. The herbal healers collect some of the preferred species, which were declared scarce in the wild and conserve them in home gardens. These included *E. autumnalis, A. africana, T. acutiloba, O. longibracteatum* and *T. caffrum.* Some of the plant species like *O. capensis* are grown around

the home not necessarily for conservation purposes, but they are believed to prevent lightning strikes and dispel evil spirits.

2.3.4 The degree of scarcity of plants

The scarcity of each medicinal plant was evaluated based on the number of citations made by the respondents as shown in Table 2.1. Initially, 105 plant species reported to be getting scarce in the wild were recorded. After the determination of the frequency of citations for each plant mentioned, 61 frequency categories that varied from 8% to 50% were recorded. *Eucomis autumnalis, H. odoratissum* and *H. penduculatum* had the highest frequency with 50% citations each, followed by *A. afra* and *R. digitata* with 40% citations each.

2.3.5 National conservation status of the plant species

All the reported plant species in the present study appear in the South African National Red Data List of plants, with the exception of *B. bubaline C. camphora* and *M. parviflora*. According to the IUCN Red List Categories and Criteria (version 3.1) the plant species fall under the following categories: near threatened (NT) (1.61%), rare (1.61%), endangered (EN) (3.22%), vulnerable (VU), (8.06%), declining (11.29%) and least concern (LC) (69.35%).

2.3.6 Harvesting frequency of plants

Findings from this study clearly indicate that no ýxed protocol existed, as to how often medicinal plants should be collected. Plants were collected throughout the year, and seasonality only played a role when plant parts such as leaves were to be collected. About 50% of the respondents collected medicinal plants once in two weeks, 33% harvested once a month, 15% twice a week and 2% on a daily basis. It was revealed that healers harvest and

carry as many plants as they can when they go out for collection because of the distance travelled to collect the plants. According to the respondents, collection of small quantity means waste of time and energy. In addition, the scarcity of certain species warrants the collection of larger volumes so as to have enough; an approach that might in the end result in over-exploiting certain wild species.

2.3.7 Method of Harvesting

The underground parts constituted the largest portion of plant parts harvested for medicinal purposes (Fig. 2.3). The roots of herbs or small shrubs are generally uprooted. Harvesting by uprooting plants has a detrimental effect on the plant itself and on surrounding plants as well. Herb gatherers in the study area sometimes dig deep before collecting some of the medicinal plants and in some cases the soils are not refilled after removing the underground parts. Subsequently, this might cause soil erosion which in turn leads to loss of productivity of the land due to leaching of mineral nutrients and soil microorganisms, with eventual death of plant species. Species such as *G. perpensa*, *R. digitata, A. amatymbica, B. volubilis* and *P. prunelloides* were affected by this method of harvesting.

The harvesting of whole plant (reported by 9% of the respondents) was observed on herbs such as *C. coriacea, H. hemerocallidea, C. arcuata* and *H. Africana* (Table 2.1). In this study, the harvesting of whole plant by healers was because they usually use all plant parts together. The leaves which are used for treatment are however harvested by hand-picking or by cutting down a whole branch to obtain the leaves. The practice of cutting down whole branch wastes plant material because most times, all the leaves brought down are not used.

The practice of strip barking was mostly used by plant collectors in harvesting the bark of trees, but in a few cases ring-barking was practised. Barks were harvested from species such as *C. dentata, , Z. mucronata* and *S. henningsii* as presented in Table 2.1.

2.3.8 Red Data species versus local community perception of scarce medicinal plants

The plants indicated by the respondents as declining in the wild and scarce were compared with their national conservation status as recorded in the Red Data List. About 14 plant species that were highly rated by the local respondents and had frequency of citation of 30% and above, were classified as Least Concern (LC), at a national scale (Table 2.1). For example, *Helichrysum* spp. which was ranked second according to the degree of scarcity of the medicinal plants was classified as Least Concern, nationally. Similarly, *A. afra*, *R. digitata*, *B. reticulata, B. lucens, H. albiflos, H. integrifolius, R. tomentosa, S. latifolia, A. oppositifolia, H. africana* and *S. africana* also reported to be highly diminishing in the locality, were classified as of least concern on a national scale. The variation could be a reflection of local distribution patterns, which are not reflected in the Red Data List. In contrast, some of the species cited by the respondents as highly threatened corresponded with their status at the national level as listed in the South African National Red Data List of plants. These species include amongst others *E. autumnalis* (Declining), *A. africanus* (Endangered), *A. amatymbica* (Vulnerable), *B. volubilis* (Vulnerable), *D. sylvatica* (Vulnerable), *G. perpensa* (Declining), *G. bicolor* (rare) and *Ilex mitis* (Declining).

2.3.9 Factors threatening medicinal species

The respondents in this study indicated that the most serious threats faced by plant species in the region are over exploitation and harvesting for medicinal plant trade, accounting for 42%. Plant species most affected by this threat in the present study include *A. amatymbica, H.*

hemerocallidea, G. perpensa, B. volubilis and *E. autumnalis.* Some of the respondents revealed that traditional healers from the Eastern Cape had not lost their cultural roots to the province even though they had settled in other provinces. They were said to travel regularly from where they reside to their homelands in the Eastern Cape to collect the necessary plants for their healing activities or for sale. The plants are mostly collected from the wild. With increasing demand and pressure on the natural resources, many valuable plant species are declining.

Overgrazing is the second most threat to medicinal plants constituting 21%. Overgrazing by livestock is the main driver causing habitat degradation (Raimondo *et al.*, 2009). *Kniphofia drepanophylla* is threatened by habitat degradation brought about by overgrazing and trampling. A number of other species such as *B. reticulata*, *M. parviflora* and *H. odoratissum* are threatened by overgrazing. Threats from agricultural expansion account for about 15 percent of plant loss. Generally, the dependency on agricultural activities results in the destruction of natural forests and consequently depletion in the availability of many species of medicinal importance.

Urban and residential development is another factor that threatens indigenous plant species used in the study area. Respondents revealed that about 11% of plant decline was as a result of development from road construction and building of houses. Notwithstanding that development in this region is at a low rate, valuable plant species are still affected by the transformation. Plant species threatened by this factor include *A. oppositifolia, S. africana* and *R. melanophloes*.

Deforestation is the cutting down of trees without planting others in their place. Some of the reasons trees have been cut down were to obtain land for cultivation of both subsistence and cash crops; to obtain firewood especially those who live in rural areas where electricity and gas are unavailable; and to obtain building materials. Destruction of these forests leads to destruction of medicinal plants that could be used as treatment for various ailments. Only 10% of the respondents acknowledged that deforestation is a threat to wild populations of medicinal species. Plant species in the study area threatened by this factor were *S. latifolia*, *S. henningsii* and *E. microphylla.*

2.4 Discussion

The family Asteraceae accounted for the highest number of threatened medicinal plants, followed by Asphodelaceae, Hyacinthaceae, Euphorbiaceae and the rest. The family Asteraceae was consistently recorded as mostly used in different ethnomedicinal inventories and this could be a probable reason why they constitute the highest number of plant taxa that are getting scarce and thus, facing the threat of extinction. For instance, the Asteraceae and Asphodelaceae were reported to be amongst the most used plant families by Xhosa people in the same study area, for treatment of various diseases (Wintola and Afolayan, 2010; Omoruyi *et al.*, 2012). Plant species from the Asteraceae are most used by Bapedi people of the Limpopo province of South Africa for the treatment of sexually transmitted infections (Semenya *et al.*, 2013). The high use of plant species from Asteraceae family for medicinal purposes were also indicated in studies conducted elsewhere in the world (Giday *et al.*, 2009; Tangjang *et al.*, 2011; Khatun *et al.*, 2012).

The higher usage of herbs more than the other growth forms among the people of this locality could be an indication of their better abundance and high effectiveness in the treatment of ailments as compared to other growth forms. Studies conducted by other researchers (Giday *et al.*, 2009; Singh *et al.*, 2012; Semenya *et al.*, 2013) also indicated the common use of herbs as sources of medicine. This agrees well with the result of this survey that scarce medicinal plants constitute majorly of herbs.

Whole plants and underground parts such as roots, bulbs, tubers and rhizomes constituted the most harvested plant parts (59%) for medicinal purposes in the study area (Fig. 2.3). Following the definition of the term ósustainableô harvesting by Struhsaker (1998), it can be inferred that the practice of harvesting underground parts by uprooting them could be seen as unsustainable. Such harvesting practices have a damaging effect on the plant itself, since the roots which are important for the survival of the plants are implicated. This finding is similar to that of Semenya *et al.* (2012) who also reported on unsustainable harvesting practices by Bapedi healers. Dold and Cocks (2002) concluded that 93% of the species traded from the Eastern Cape were harvested unsustainably, as they are either entirely or partially removed resulting in the death of the plants. It was also noted that harvesting by uprooting herbs and shrubs has a negative impact on surrounding plants by affecting their root systems (Magoro, 2008). Medicinal plant collectors should be encouraged to harvest fewer parts or different parts from a single plant rather than uprooting the entire plant. Sustainable harvesting should be encouraged.

Tree species such as *S. henningsii* are routinely harvested for their bark. Uncontrolled harvesting could result in their extinction since they are slow growing with little wound

recovery (Geldenhuys and Williams, 2005). Bark harvesting practices in the study area were mainly strip-barking, and in few instances, ring-barking. Zschocke *et al.* (2000) highlighted that ring-barking is the most destructive harvesting practice as it often means that the debarked tree has no chance of survival, especially if ring-barking is done by unskilled collectors. On the other hand, it was noted that strip-barking enables the tree to recover faster from wounding (Kambizi and Afolayan, 2001). Cunningham (1989) stated that debarking of species causes more damage to the plant than harvesting its leaves and fruits. The herb gatherers should be encouraged to adopt plant-part substitution, the art of using another plant part that will not endanger the species being used, as this will help in conservation of medicinal plants.

The plant collectors in the study area gather medicinal plants from various sources, but collection from the wild is the most sourced (mentioned by 82.7% of the respondents). This agrees with Street *et al.* (2008) who reported that in South Africa, most medicinal plants are harvested from the wild populations with resultant threat on medicinal plant biodiversity and population stability. Likewise, Nzue (2009) in a survey of medicinal plant use in Cape Peninsula, Western Cape Province of South Africa showed that 51% of the plants used for herbal remedy in that area were harvested from the Eastern Cape wild.

The periods of harvesting reported by the respondents described a non-uniform behaviour of natural resource users. For example, bi-weekly visits to the harvesting areas (50%) dominated, followed by monthly visits (33%). This result reflects the huge pressure exerted on medicinal plants. The situation is similar to the findings of Semenya *et al.* (2013) in Limpopo province where medicinal plants were mostly collected once or thrice a month.

Our findings clearly indicate that the local community recognized that wild supplies of medicinal plants are declining. It also shows that the community people, depending on these resources, would be willing to collaborate with the Government and various conservation agencies to conserve these indispensable plant species.

The local communityôs perception of threatened or scarce medicinal plants was compared with conservation status of the plants as in the Red List. Some species perceived by the local community as declining or rare corresponded to their Red List conservation status and included species like *E. autumnalis*, *D. sylvatica*, *G. perpensa* and *A. amatymbica*. *E. autumnalis* bulb, which rated first in our survey, is in great demand for use in traditional medicine and the declining numbers due to harvesting for this purpose is alarming (Dold and Cocks, 2012). Cunningham (1988) recorded *E. autumnalis* spp. as the second most prevalent species in the KwaZulu-Natal medicine markets. Williams *et al.* (2007) listed the same plant as occurring in 78% of muthi shops in the Witwatersrand. This confirms the relevance and considerable contribution made by this investigation. The plant species claimed by the respondents to be threatened are not limited to this study. They have been documented in other provinces. On the other hand, some species were perceived by the local respondents to be declining from the community, but country-wide they are common species that are listed as of Least Concern and not threatened in the Red Data List (SANBI, 2009). Examples of such species include *R. digitata*, *S. henningsii,* and *H. albiflos. Rhoicissus digitata* was reported to be heavily traded and unsustainably harvested in the Eastern Cape Province (Dold and Cocks, 2001). This plant, sold for only R11.30 per kg, has been ranked 2nd among the 60 most traded plants in the Eastern Cape (Dold and Cocks, 2002). However, conservation measures should still be taken, as this plant was ranked fifth in the scarce medicinal plants

cited by the local respondents in this survey. Furthermore, *S. henningsii*, considered as threatened in the community, has been evaluated as of Least Concern nationally. In a similar study in the Eastern Cape Province, the plant species was mentioned by 27 respondents to be amongst the most commonly sold plant species (Dold and Cocks, 2002). A similar situation where local resource users perceived threatened species differently from the authoritative Red List has been reported (Nzue, 2009). Hence, local community perception regarding plant availability is important, as it would contribute to a better understanding of the plant conservation status at the local level. Semenya *et al.* (2012) expressed the need to include the community people who have good knowledge of medicinal plants, in any initiative focusing on the sustainable utilisation of these plant resources.

In the past, conservation officials relied on law enforcement to address the rapidly depleting wild medicinal plant populations. The degree of harvesting from the wild did not reduce, regardless of concerted effort over the years indicating that the enforcement is incapable of curtailing over-exploitation (Cunningham, 1988). As a result, alternative measures like cultivation of threatened species should be employed. Cultivation could be a means of maintaining and increasing the supply of useful plants to the market as well as reducing pressure on the wild population (Keirungi and Fabricius, 2005). However, large scale nursery propagation is equally necessary for the threatened and highly demanded species.

Cultivation by means of conventional propagation or *in vitro* methods would certainly reduce pressure from the wild. Plant *in vitro* regeneration has been reported to be a biotechnological tool that offers a potential solution to the problem of medicinal plants decimation (Afolayan and Adebola, 2004). With this technology, rapid and mass propagation of plant species are

possible in a small space and short time, with no damage to the existing population. As an alternative to the conventional methods, a continuous culture system for *Kniphofia leucocephala,* a highly endangered medicinal species was established as part of a program for the propagation and re-introduction of the species into the wild (McCartan and van Staden, 2003).

A conservation action that fortifies all the others is education and awareness programmes. Educational programmes addressing sustainable harvesting methods and natural resource conservation should be undertaken. Knowledge of biodiversity leads to better understanding, better management, and thus better conservation and protection of our plant resources.

2.5 Conclusion

In this survey, 61 medicinal plants were identified as scarce and threatened with extinction in the study area. It was also found that a high number of plant resource users were aware of the consistent depletion of a number of plant species used for healing purposes. This, in fact, represents an important starting-point to tackle the problem of over-exploitation of medicinal plants in the wild. The current practices of collecting medicinal plants almost entirely from the wild coupled with unsustainable harvesting techniques are gradually reducing the populations of highly valuable plant species. As more and more medicinal plants and the associated knowledge are lost, the potential for the continuous supply of medicinal plant resources for the large numbers of population who still rely on them for their everyday health care is not guaranteed. Therefore, ethnobotanical studies and subsequent conservation measures are urgently needed to salvage the medicinal plants and the associated knowledge from further loss. It is hoped that this documentation may contribute useful knowledge to the

understanding of the status of medicinal plants in the study area. Such knowledge is critical for planning the sustainable management of medicinal plants in the study area.

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CHAPTER 3

CLONAL PROPAGATION OF *Gunnera perpensa* L.

CHAPTER THREE

Clonal Propagation of *Gunnera perpensa* L.

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3.1 Introduction

The intensive harvesting of plant materials for medicinal purposes and commercial trade in South Africa poses a threat to many plant species (Mander, 1998; Keirungi and Fabricius, 2005). Many researchers have reported destructive and/or unsustainable harvesting of some of these species (Cunningham, 1990; Dold and Cocks, 2002). The most affected of these plant species are those harvested for their underground parts (Dold and Cocks, 2002; Rock *et al.*, 2004) among which is *Gunnera perpensa* L. (Fig. 1.1).

Gunnera perpensa (Gunneraceae) is widely distributed in South Africa where it is found in several provinces including the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West and Western Cape (Williams *et al.*, 2008). Natural populations of *Gunnera* are restricted to super-humid areas with heavy rainfall, riverbanks, near waterfalls and close to wells (Bergman *et al.*, 1992).

Gunnera perpensa has long been used in traditional medicine. A decoction of the roots or rhizomes is used to facilitate labour, expel the placenta after birth or to relieve menstrual pains (van Wyk and Gericke, 2000; Ngwenya *et al.* 2003; Von Ahlenfeldt *et al.*, 2003). *G. perpensa* is reported to be one of the top natural remedies for a variety of skin conditions and in the control of gastro-intestinal parasites in village chickens (van Wyk and Gericke, 2003; Mwale and Masika, 2009). Important compounds like Z-venusol, a phenylpropanoid glucoside and two new 1, 4 benzoquinones having antibacterial activity as well as the ability to cause both ileal and uterine smooth muscles contractions were reported to be isolated from *G. perpensa* rhizome (Drewes *et al.*, 2005).

The rhizomes are widely used and indiscriminately collected in large quantities from the wild to meet the ever increasing demand in traditional medicine markets (Williams *et al.*, 2008). This is causing a decline of this valuable medicinal plant species. Considering the large volumes traded, amounting to about 115.6 kg/yr. per trader, continuous harvesting will have more negative impact on their wild population (Dold and Cocks, 2002). The current Plant Red List of South Africa enlists *G. perpensa* as declining (Raimondo *et al.*, 2009). In addition to its unsustainable exploitation, the destruction of *G. perpensa* habitats due to mismanagement of wetlands is another threat to its survival (Von Ahlenfeldt *et al*., 2003; Williams *et al.*, 2008).

The natural means of propagation of *G. perpensa* are by seed and by rhizome. However, the rhizomes are removed during harvesting which threatens the prospect for perennial rejuvenation. The viability of the seeds of this plant is low and germination from seed is quite erratic and difficult. In addition, under natural conditions this plant species is subject to harsh and extended winter conditions, thereby affecting its growth and availability (Mendes, 1978). One of the effective measures for stemming over-exploitation of this plant is to encourage its propagation and cultivation. Successful cultivation would reduce harvesting from natural populations and could help conserve *G. perpensa*. Any improvement in vegetative propagation can substantially help in preservation of this valuable medicinal plant. Despite the conservation status of *G. perpensa*, the demand is still high and there is little or no information on its propagation. The present investigation was, therefore, carried out to examine clonal propagation of *G. perpensa*, using varying lengths of the rhizome segments as propagules.

3.2 Materials and methods

3.2.1 Plant collection and identification

Gunnera perpensa plants were collected during the month of July 2013 from plants growing in their natural habitats around Hogsback mountains (32° 35ôS 26° 57ôE) in Eastern Cape Province. The plant was identified and authenticated by Prof. DS Grierson of the Department of Botany, University of Fort Hare. Voucher specimen (CB29) was deposited at the Giffen herbarium of the University. They were transported in polyethylene bags to the GreenHouse of the Faculty of Science and Agriculture, University of Fort Hare. The rhizomes were washed under running tap water to remove adhering soil particles and subsequently, leaves were removed from them.

3.2.2 Determination of the minimum length of rhizome that could be used as propagules

Healthy rhizomes were cut into sections of 1, 2, 3, 4, 5 and 6 cm to determine the minimum rhizome length suitable for the clonal propagation of *G. perpensa*. The varying rhizome lengths were then planted out in plastic pots of 14 cm in diameter and 24 cm depth filled to three-quarter with Hygrotech potting mix (Hygrotech, South Africa). The experiment was laid out in a randomised design with six treatments replicated five times. Each treatment (1, 2, 3, 4, 5 and 6 cm long rhizomes) comprised 15 rhizome segments. All the pots were irrigated daily with water. The experiment was carried out in glass house which was maintained at 18°C ï 30°C. Data on the number of leaves, length of petiole, height of plants, leaf area, chlorophyll content and petiole girth were taken weekly for 10 weeks. Moisture content, fresh and dry weights of the leaves and rhizomes were also determined.

3.2.2.1 Germination/Seedling emergence

Observations were made daily for six weeks and the number of rhizomes that emerged from each treatment was recorded. The number of days from the date of planting to the first and subsequent shoot emergence was also recorded. Cumulative emergence was taken as the total number of rhizome segments that sprouted from the beginning of the trial.

3.2.2.2 Plant height and number of leaves

A meter rule was used to measure the shortest distance between the upper boundary of the main photosynthetic tissues on the plant and the soil level (Cornelissen *et al.*, 2003). Leaves formed were manually counted from each plant and the average determined.

3.2.2.3 Chlorophyll content determination

The chlorophyll content of leaves was measured with Minolta SPAD 502 Plus Chloropyll meter. SPAD stands for Special Products Analysis Division (a division of Minolta). SPAD meter potentially offers a useful non-destructive, handheld system to help in the evaluation of plant health (Pinkard *et al.*, 2006). It measures the absorbance of the leaf in two wavelengths (400-500 nm and 600-700 nm) and the results are then given in SPAD values (SPAD-502 Plus manual, 2009).

3.2.2.4 Petiole girth

Petiole girth was measured at about 2.5 cm above soil level using a manual vernier caliper.

3.2.2.5 Shoot and root moisture content

At the end of 10 weeks, plants were removed from the pots, washed with water and separated into leaves and rhizomes/roots. The fresh weight and the dry weight of the respective plant parts were determined by weighing the fresh plant material after which it was then dried in an oven at 70°C until there was no further loss in weight. Moisture content was expressed in percentage on the basis of fresh weight using the equation below (Bewley and Black, 1982):

$$\text{Moisture content } (\%) = \left(\frac{\text{Fresh weig ht - Dry weight}}{\text{Fresh weig ht}} \right) \times 100$$

3.2.3 Data Analysis

Where applicable, data were subjected to statistical analysis using MINITAB Release 12. A one way analysis of variance (ANOVA) was used to compare the means of various growth parameters among the treatments. A two way analysis of variance was also used to determine interaction between plant age and treatment on various growth parameters. Means were compared using Duncanôs multiple range tests. The means were treated as significantly different at $P < 0.05$.

3.3 Results

3.3.1 Effects of varying lengths of rhizome on shoot emergence of *Gunnera perpensa*

All the varying lengths of rhizome showed viability, sprouted and developed into new plants (Fig. 3.1). The first shoot emergence from the rhizome buds was observed at day 2 after planting in 3, 4, 5 and 6 cm rhizomes. With 1 cm and 2 cm, the first shoots emerged at day 3 and 5 respectively. Rhizomes of longer lengths were found to emerge quicker than the shorter ones.

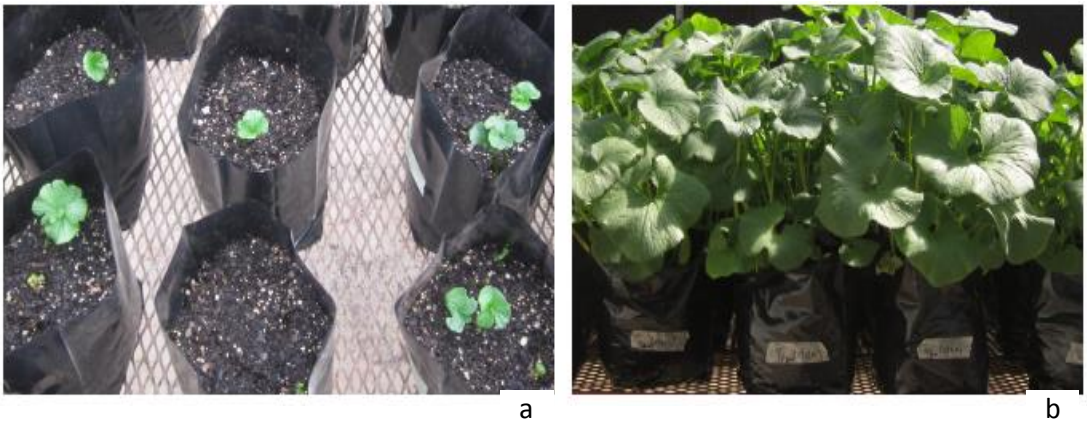

Figure 3.1: Clonal propagation of *G. perpensa* (a) Shoots emerging from the rhizome. (b) Plants raised through rhizome segments growing in the glasshouse.

The viability and percentage cumulative emergence increased with increase in rhizome length. The 1 cm exhibited the lowest shoot emergence of 87%. The 2 and 3 cm had 93% maximum emergence respectively, while the highest emergence of 100% was recorded in the 4, 5 and 6 cm respectively. A higher percentage of shoots emerged from the 4 cm compared to the other lengths, in the first 15 days after planting. From day 16 onwards, the 5 and 6 cm had a greater percentage of shoots emerging (Fig. 3.2). Between day 5 and day 20 after planting, the 6 cm rhizomes had a very low shoot emergence, but the percentage emergence increased sharply after that, outstripping the others with the exception of 5 cm rhizomes. Cumulative emergence increased with time (Fig. 3.2) such that, by the 40th day, emergence across the varying lengths of rhizome was at their maximum. For the 5 and 6 cm, maximum emergence occurred at day 27 after planting, while it occurred at day 38 for the 2, 3 and 4 cm respectively and at day 39 for the 1 cm.

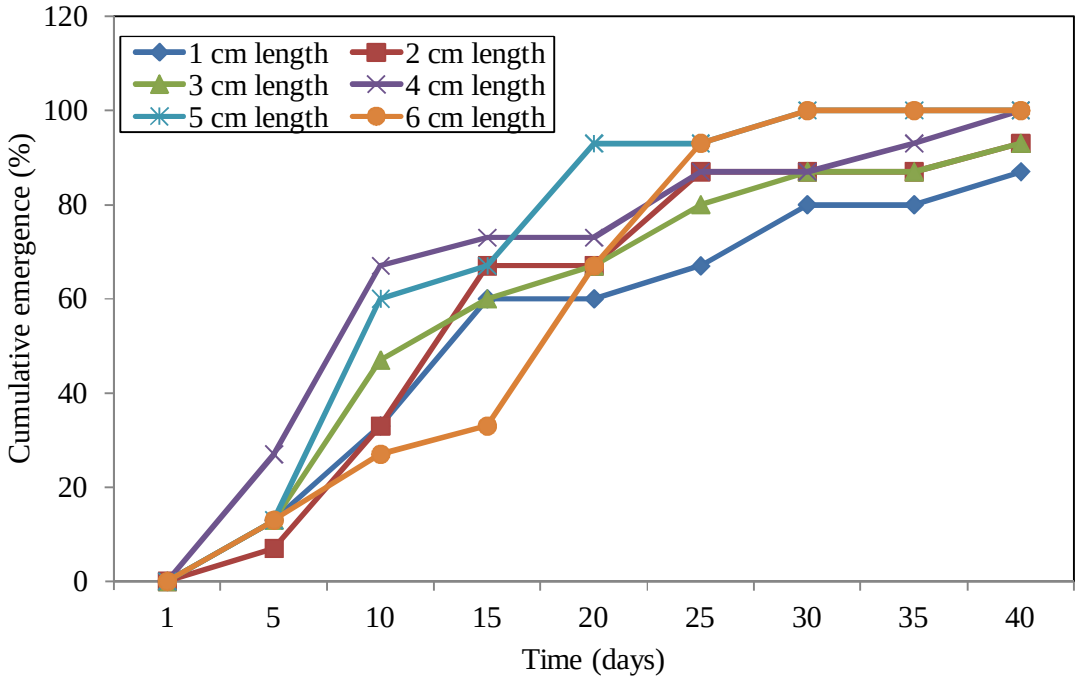

Figure 3.2: Effect of rhizome lengths on cumulative emergence (%) of *G. perpensa* shoots.

3.3.2 Effects of varying lengths of rhizome on number of leaves

The lengths of rhizome planted had significant effect on the number of leaves produced by the plants. The 6 cm rhizomes produced the highest number of leaves, followed by 5 cm, 4 cm, 3 and 2 cm (Fig. 3.3). The least number of leaves were obtained from 1 cm rhizome. Generally, the number of leaves across the varying lengths of rhizome increased with increasing plant age until the termination of the experiment. The 1 cm which gave the lowest number of leaves increased from 0.8 ï 6.2 mean number of leaves per plant, while the 6 cm which produced the highest number of leaves increased from 0.8 - 12.8 mean number of leaves per plant.

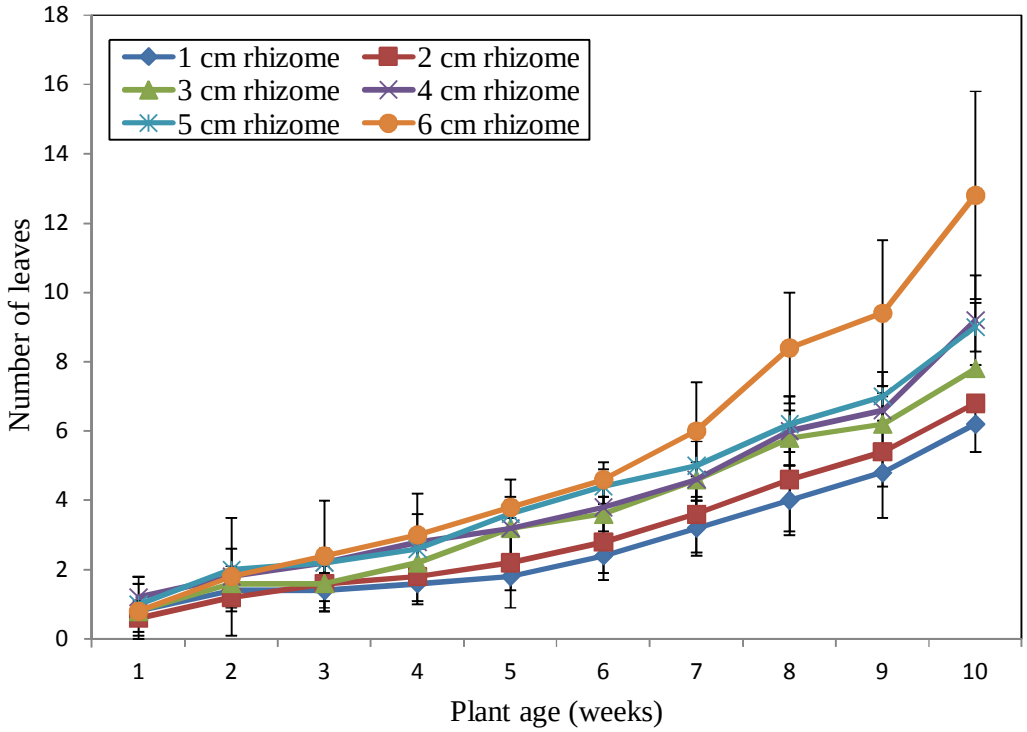

Figure 3.3: Effect of varying lengths of *G. perpensa* rhizome segment on the number of leaves produced. Data is mean ± S.D.

3.3.3 Effect of varying lengths of rhizome on plant height

The effect of varying rhizome lengths on plant height is shown in Figure 3.4**.** The mean plant height for the duration of the trial was highest in 5 cm rhizomes (20.13 cm), followed by 4 cm (19.30 cm) and lowest in 1 cm (7.89 cm). Statistical analysis showed there were significant differences among the treatment means ($P < 0.05$). By the seventh week, a sharp increase in plant height was observed. Plant height increased with plant age. The plant height, which was highest in 5 cm rhizomes increased from 2.1 cm the first week to 42.9 cm when the experiment was terminated at the 10th week.

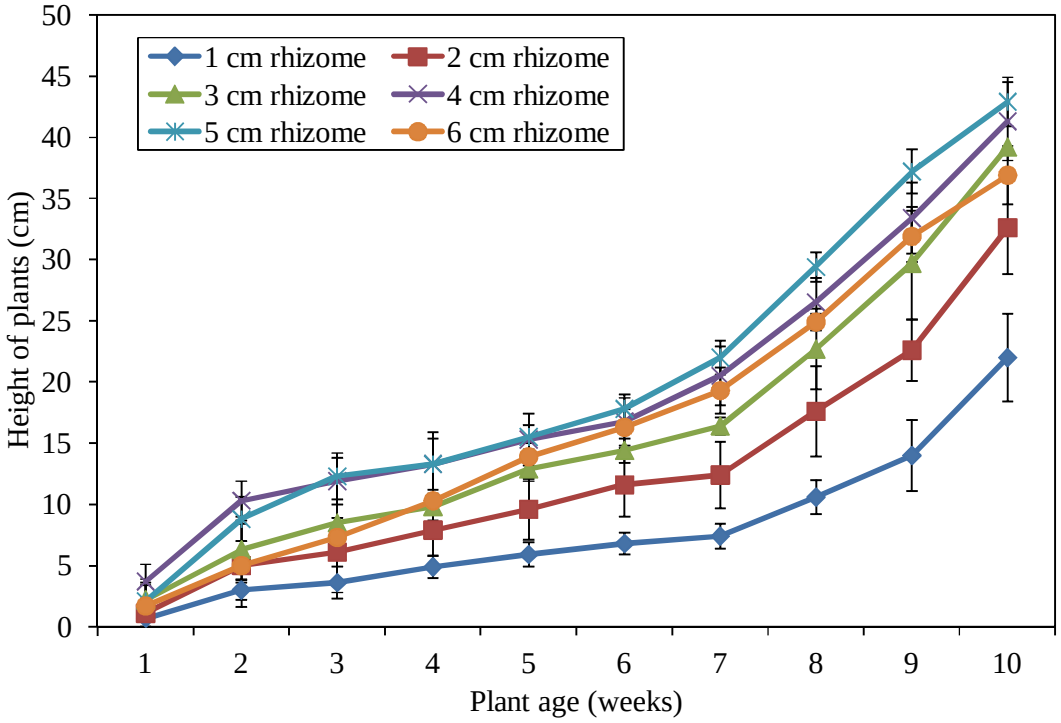

Figure 3.4: Effect of varying lengths of *G. perpensa* rhizome segment on plant height. Data is mean ± S.D

3.3.4 Effects of rhizome lengths on length of petiole and petiole girth

The effects of varying rhizome lengths on petiole length and petiole girth are shown in Figures 3.5 and 3.6 respectively. The mean length of petiole was highest in 5 cm rhizomes, followed by 4 cm and lowest in 1 cm. By the seventh week, a sharp increase in the length of petiole was observed in all the rhizome lengths. Petiole length increased with plant age. In 5 cm rhizome, it increased from 1.4 cm to 34.3 cm at the termination of the experiment. The petiole girths for the trial period were also highest in 5 cm rhizomes and lowest in 1 cm.

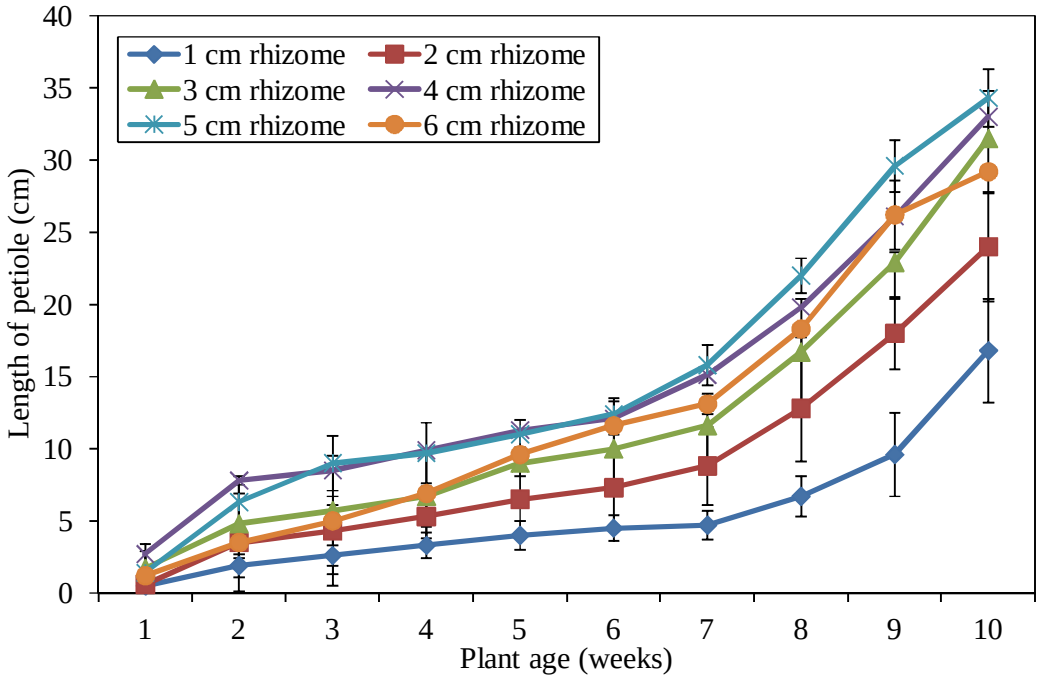

Figure 3.5: Effect of varying lengths of *G. perpensa* rhizome segment on length of petiole. Data is mean ± S.D

The petiole girths for each of the rhizome lengths were observed to drop at particular weeks of the experimental period and it was at the point that the plants elongated more. The petiole girth across the varying lengths of rhizome ranged between 0.1 cm and 0.67 cm. Analysis of variance showed an interaction between plant age and rhizome length on length of petiole and petiole girth.

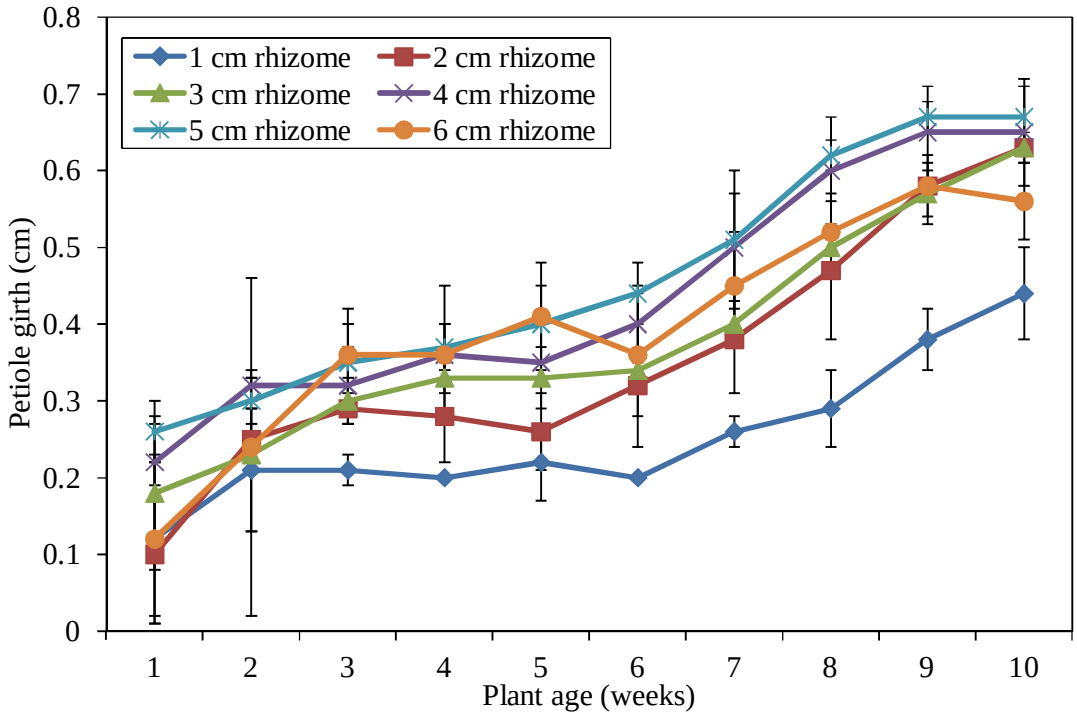

Figure 3.6: Effect of varying lengths of *G. perpensa* rhizome segment on petiole girth.

3.3.5 Effects of rhizome lengths on leaf area

The mean leaf area for the experimental period was highest in 5 cm rhizome segment and lowest in 1 cm (Fig. 3.7). Leaf area was consistently high throughout the experimental period. Statistical analysis showed that significant differences exist among the treatment means across the different rhizome segments ($P < 0.05$). Longer rhizome segments were observed to have larger leaf area than the shorter ones. Analysis of weekly yields of this growth parameter revealed there were significant effects of plant age on leaf area ($P < 0.05$) amongst the varying rhizome lengths.

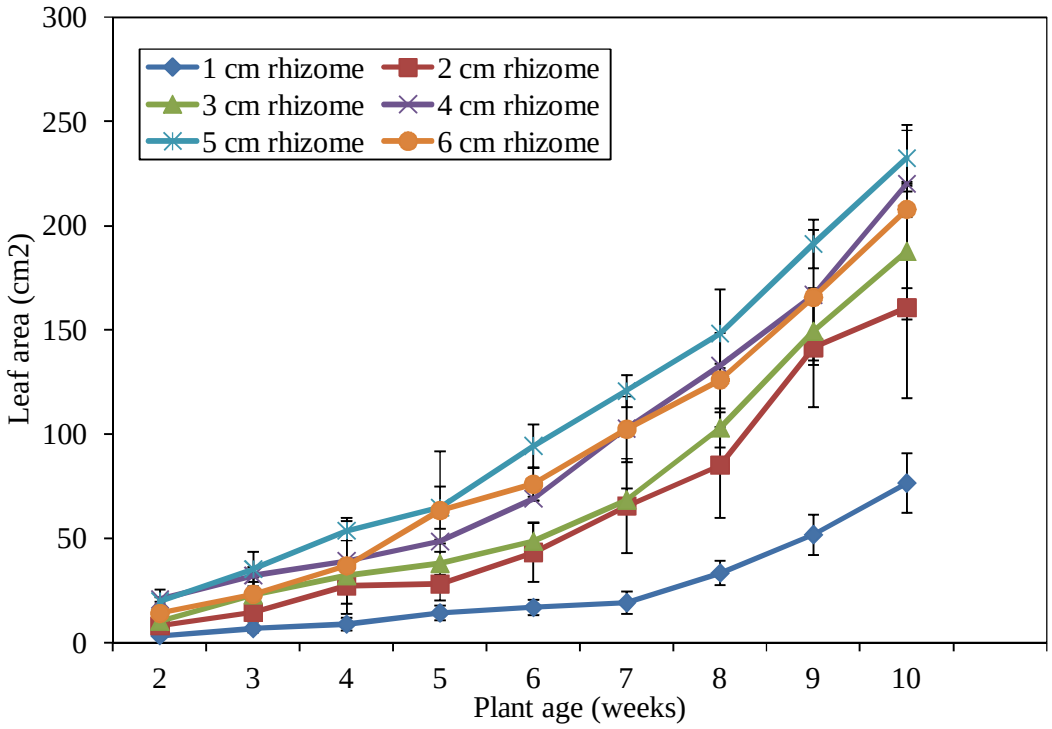

Figure 3.7: Effect of varying lengths of *G. perpensa* rhizome segment on leaf area. Data is mean ± S.D

3.3.6 Effects of rhizome lengths on chlorophyll content

The highest mean chlorophyll content [41.80 SPAD (Special Product Analysis Division) values] was obtained from 4 cm rhizomes followed by 6 cm (40.76 SPAD values) and the least was in 1 cm rhizomes (32.61 SPAD values). The chlorophyll content increased sharply between week one and three, after which it dropped (Fig. 3.8). In 6 cm rhizome, chlorophyll content did not differ significantly after the third week of planting. Statistical analysis showed an interaction between plant age and the rhizome lengths on chlorophyll content.

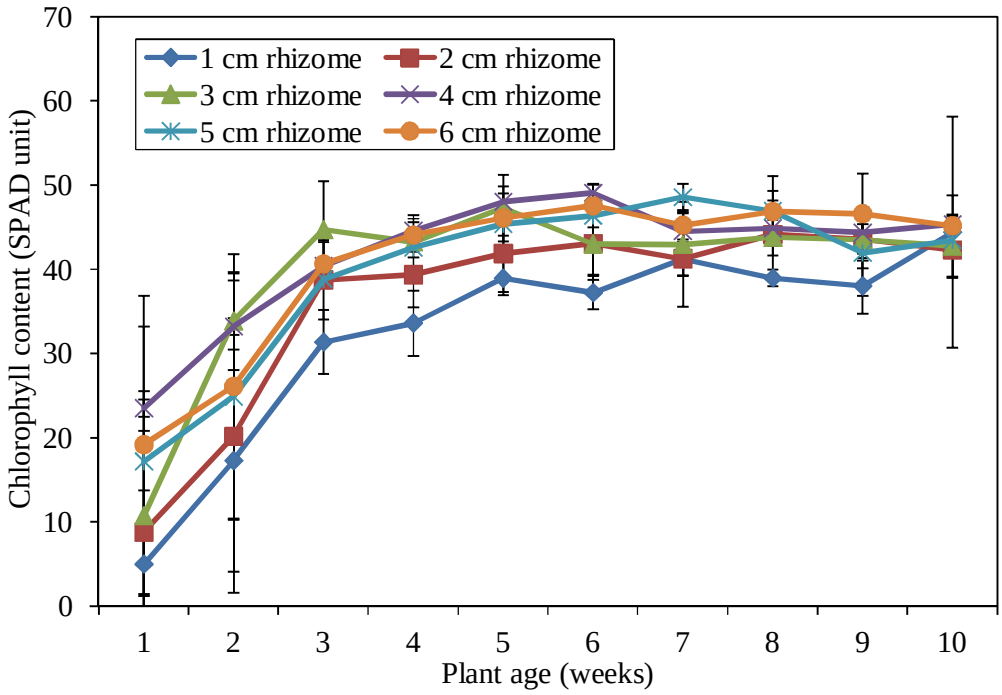

Figure 3.8: Effect of varying *G. perpensa* rhizome lengths on chlorophyll content. Data is mean ± S.D

3.3.7 Effects of rhizome lengths on fresh weight, dry weight and moisture content

The six rhizome lengths used in this study significantly affected shoot fresh weight, shoot dry weight, rhizome fresh weight and rhizome dry weight ($P< 0.05$), but no significant effect was observed on percentage moisture content of both shoots and rhizomes (Table 3.1). At the end of the 10 week experiment, the shoot fresh weight, the rhizome fresh weight, the shoot dry weight and the rhizome dry weight were highest in 5 cm rhizomes, followed by 4 cm (Table 3.1). The lowest shoot and rhizome yield were obtained from the 1 cm rhizomes. Moisture content was consistently high in both shoots and rhizomes. However, moisture content was higher in rhizomes than in the shoots, averaging 92.15% in rhizomes compared to 88.37% in shoots.

Table 3.1: Effect of varying lengths of rhizome on the yield of *Gunnera perpensa* plant

Length (cm)	Shoot fresh weight (g)	Rhizome fresh weight (g)	Shoot dry weight (g)	Rhizome dry weight (g)	Shoot MC (%)	Rhizome MC (%)
1	5.68 ± 1.44a	3.84 ± 0.55a	0.68 ±0.17a	0.30±0.05a	88.14±1.28a	92.29±0.59b
2	17.53 ± 3.93b	11.25 ± 2.75ab	2.05 ±0.53b	0.86±0.26ab	88.64±1.78a	92.63±0.69b
3	27.93 ± 2.95c	15.93 ± 0.61b	3.41 ±0.32c	1.27 ±0.06b	87.77±0.95a	92.01±0.51b
4	36.91 ± 3.86d	21.47 ± 2.79bc	4.08 ±0.50c	1.68±0.24bc	88.99±0.87a	92.17±0.53b
5	47.99 ± 2.94e	27.58 ± 2.77c	5.55 ±0.28d	2.29 ±0.24c	88.39±0.51a	91.70±0.11b
6	30.77 ± 3.15c	27.03 ± 4.45c	3.66 ±0.54c	2.16 ±0.39c	88.29±1.71a	92.11±0.47b

Values shown are mean ± S.D. MC= moisture content

Means with different letters in the same column are significantly different at $P < 0.05$

3.3.8 Effects of varying lengths of rhizome on flowering

Gunnera perpensa plants commenced flowering on the 8th week after planting. The cumulative number of flowers produced from the varying rhizome lengths is shown in Figure 3.9a. The 4 cm rhizome was the first to flower, followed by 5 cm and then 6 cm rhizomes. The 1 cm rhizomes were the last to flower at ten weeks after planting and also produced the least total number of flowers. At the commencement of flowering, the cumulative number of flowers formed was more in 5 cm rhizome, but by the 9th and 10th week the 6 cm rhizome had the highest cumulative number of flowers.

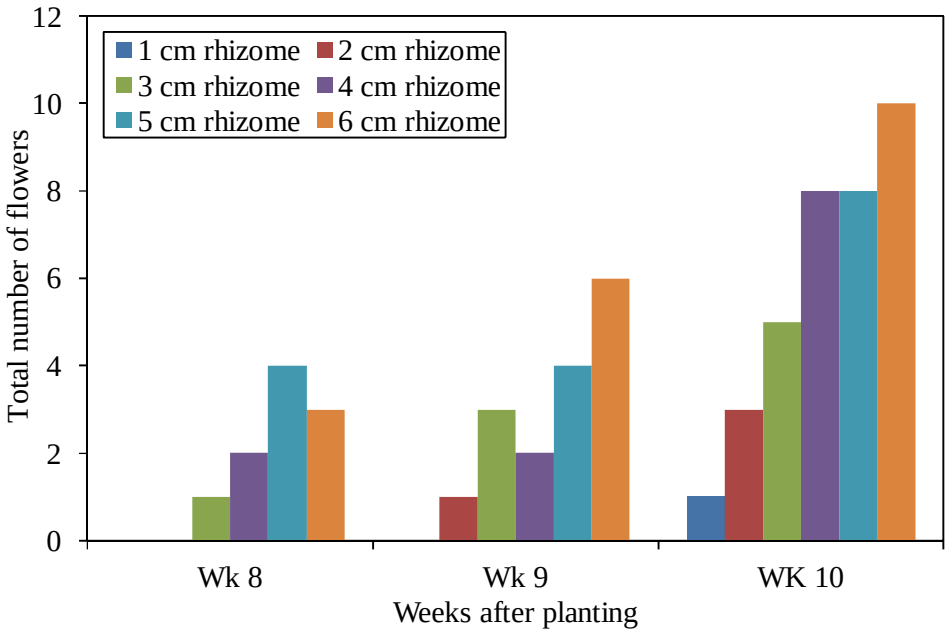

Figure 3.9a: Effect of varying lengths of *G. perpensa* rhizome on number of flowers produced

The number of flowers produced by *G. perpensa* plants is shown in Figure 3.9b.

Figure 3.9b: *G. perpensa* plants flowering in the glasshouse

3.4 Discussion

3.4.1 Effects of varying lengths of rhizome on shoot emergence

Similar results of high viability and high shoot emergence as obtained in this study were also found in a study by Huisman and Kortleve (1994) which reported emergence rates of 70-95% from rhizome pieces planted immediately after harvest from mother plants. However, our result is in contrast with the findings of Kouakou *et al.* (2009) who reported that rhizomes of *L. secundiflorum* with large diameter had the lowest viability percentages, although the specific dimension of the rhizome was not stated. The authors also noted that despite the 50% viability exhibited by the rhizome, it did not develop shoot. The 5 cm rhizome which took less time for maximum emergence is recommended to be used as propagules.

3.4.2 Effects of varying lengths of rhizome on number of leaves

The plant uses the rhizome to store starch, proteins and other nutrients. These nutrients become useful for the plant when new shoots must be formed (Jang *et al.*, 2006). This could be a possible reason why the 6 cm rhizomes gave the highest number of leaves, followed by 5 cm, whereas the lowest number of leaves was obtained from 1 cm. The longer rhizomes could have had more metabolic reserves which were released for the production of more leaves. This could also explain why the longer rhizomes had higher percentage shoot emergence than the smaller rhizome segments. Jonojit and Nirmalya (2002) previously demonstrated that the length of a rhizome played an important role in shoot formation. In this study, rhizome length also played a significant role in the plant growth and development; therefore it should be an essential factor to consider when propagating this plant.

3.4.3 Effects of varying lengths of rhizome on plant height

The 5 cm rhizomes with the tallest plants have the advantage of improved access to light which in turn increases photosynthesis and plants productivity. Height is a crucial component of a plant speciesô ecological strategy. It is central to a speciesô carbon gain strategy, because height is a major determinant of a plantôs ability to capture light. There is also a correlation between plant height and traits such as leaf mass fraction, leaf area ratio, leaf nitrogen per area, leaf mass per area and canopy area (Falster and Westoby, 2003). Plant height is an important part of a coordinated set of life-history traits including seed mass, time to reproduction, longevity and the number of seeds a plant can produce per year (Moles and Leishman, 2008). The 5 cm rhizome length that gave the tallest plant is recommended here, in the light of the mentioned advantages of plant height.

3.4.4 Effects of rhizome lengths on length of petiole and petiole girth

The petiole has the same internal structure as the stem and functions to direct the leaf so that it could get sufficient sunlight to perform photosynthesis. Petiole elongation has been observed to be an indication of the health status of the plant. This study showed that the 5 cm rhizome length produced the highest petiole girth and highest petiole length which would be beneficial in the vegetative propagation of this plant species. The highest petiole girth value (0.67 cm) obtained in the present study is similar to the stem diameter of 6.64 mm reported in *Solanum scabrum* by Ondieki *et al.* (2011).

3.4.5 Effects of rhizome lengths on leaf area

Leaf area measurement is important in monitoring growth and vigour in plants, which in turn has an effect on photosynthesis, storage of nutrients and many other physiological processes such as transpiration and sensing of environmental conditions (Severino and Auld, 2013).

The growth of leaf area is particularly important because it is directly related to the plantôs capacity to intercept light and photosynthesize. It is an important parameter in understanding water and nutrient use, plant growth and yield potential (Pandey and Singh, 2011). In this study, there was a continuous growth in the leaf area until the termination of the experiment which indicates the maintenance of good plant growth and vigour. Sumbele (2012) observed that leaf size alongside other factors, enhanced root elongation in *Treculia africana*. According to Severino and Auld (2013), the plant size is determined by the number of epidermal cells and the mean cell area. Hence, the variation on leaf sizes amongst the rhizome lengths may possibly be due to changes in any of these components in the varying rhizome segments.

3.4.6 Effects of rhizome lengths on chlorophyll content

An increase in SPAD values indicates an increase in chlorophyll content and values above 30 indicate good chlorophyll concentration. At week 10, all the varying lengths of rhizome had SPAD values above 30 which is an indication of good chlorophyll concentration and good health status of cultivated *G. perpensa*. Low chlorophyll concentrations limit photosynthesis while high chlorophyll concentrations increase photosynthesis and therefore plant growth (Pinkard *et al.*, 2006). The amount of light absorbed by a leaf is largely a function of leaf chlorophyll concentration (Filella *et al.*, 1995). Results of this finding showed high chlorophyll concentrations in the various rhizome lengths, with the highest in 4 cm rhizome. This is an indication of enhanced photosynthetic activity with subsequent good plant growth. Monitoring chlorophyll contents may assist in managing plant health for optimal growth (Pinkard *et al.*, 2006).

3.4.7 Effects of rhizome lengths on fresh weight, dry weight and moisture content

The highest fresh weight and dry weight for shoots and rhizomes were obtained from the 5 cm rhizome length. This also gave the best plant height and leaf area. Board (2004) reported that increased leaf area has implications for light interception and dry matter production to support plant growth and yield. Productivity in plants is generally measured in terms of yield or total dry mater. The yield in plant dry weight in this study could be attributed to adequate develpoment in plant height, length of petiole, leaf size and plant fresh weight. The higher plant dry weight in 5 cm rhizome suggested that these plants were relatively healthier and could better withstand stress (Molatudi and Mariga, 2009). This will influence the plant growth as they mature. Mahfouz and Sharaf-Eldin (2007) showed that the highest fresh and dry weights of Fennel plant (*Foeniculum vulgare*) were obtained from the same treatment of bio fertilization that gave the tallest plants and the highest number of branches per plant. The moisture content values were in the range of the moisture content (66.98% to 94.37%) of traditional vegetables reported by Irawan *et al.* (2006). Water is one of the most important chemical substances in higher plants. Water stress restricts transpiration including closure of stomata and less water evaporating from the leaf surface. Further, it reduces efficiency of photosynthesis and limits crop productivity (Zhang *et al.*, 2012). All the rhizome lengths planted in this study indicated sufficient water in them for good plant growth and productivity, with the best in 5 cm rhizome.

3.4.8 Effects of varying lengths of rhizome on flowering

Flower initiation marks an important transition from vegetative development to reproductive development, which is one of the most crucial events in the life cycle of a plant. That the highest number of flowers per plant was obtained from the 6 cm rhizome length may be related to the fact that the highest number of leaves was produced at the same rhizome length.

The fact that even the smallest rhizome length of *G. perpensa* flowered is an indication that clonal propagation will be a useful conservation tool for this plant.

This study has provided useful information on clonal propagation of *G. perpensa,* using varying rhizome segments as propagules. Seedling production by rhizome has been reported by Kouakou *et al.* (2009) on *Laccosperma secundiflorum*, a depleting rattan cane and Pyter *et al.* (2010) on *Miscanthus giganteus*.

Most of the growth parameters were significantly higher in the 5 cm rhizomes than the other rhizome segments ($P<0.05$). Nevertheless, regeneration of *G. perpensa* was still successful with as little as 1 cm rhizome without any pre-plant treatment, unlike in some plants where much longer rhizomes are needed for propagation. Commercial propagation of Myoga in New Zealand through vegetative division of rhizomes required planting rhizome pieces that are approximately 25 cm long (Gracie *et al.*, 2000). The results of this study show that regenerating *G. perpensa* through rhizome segments is an efficient method for obtaining plant material for medicinal purposes. This is advantageous because it eliminates some of the difficulties associated with seed germination and seedling survival and being a clonal method of propagation avoids variation found in the seedling populations (Nadeem *et al.*, 2000).

3.5 Conclusion

Mass-production by means of vegetative propagation using rhizomes is possible for *G. perpensa*. For its regeneration and conservation purposes, it was critical to identify the rhizome size which will optimize the vegetative growth after planting and at the same time minimize the number of individual propagules that can be extracted from the mother plant. The result demonstrated that the optimal rhizome size to be used as propagules and maximize

vegetative growth and yield is 5 cm rhizome length. Nevertheless 1 cm rhizome could still be effectively used as propagules without any preplant treatment and obtain healthy and vigorous plants. Vegetative propagation by rhizome could help meet the current and future demands on *G. perpensa.*

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CHAPTER 4

DETERMINATION OF APPROPRIATE PLANTING DEPTH FOR *Gunnera perpensa* L. RHIZOME

CHAPTER FOUR

Determination of Appropriate Planting Depth for *Gunnera perpensa* L. Rhizome

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4.1 Introduction

The rhizomes of *Gunnera perpensa* are widely used and indiscriminately collected in large quantities from the wild to meet the ever increasing demand in traditional medicine market (Williams *et al.*, 2008). This is causing a decline of this valuable medicinal plant species. Reinforcement of wild plant populations using individuals raised ex-situ is considered a valid means of reducing the risk of extinction of overexploited plant populations (Bowes, 1999).

Clonal propagation of *G. perpensa* was successful, using varying lengths of the rhizome as propagules (Chapter 3). The appropriate planting depth of the rhizome also needs to be determined for successful establishment of the plant species because different plants perform variably in different planting depths with regard to growth and development.

Planting depth is an important factor in crop management practices (Kirby, 1993). It influences the time for seedling emergence, vigour, yield and establishment (Mahdi *et al.*, 1998; Molatudi and Mariga, 2009). Therefore, it is important to plant accurately in order to achieve good germination, emergence and high plant population (Srivastava *et al.*, 2006). When considering water availability for the germination and growth of plants, it is also important to take into account other soil factors such as depth (Rivas-Arancibia, 2006). Too shallow sowing results in poor germination due to inadequate soil moisture at the top soil layer. On the other hand, too deep sowing (e.g. beyond 8 cm) can affect crop emergence and yield (Desbiolles, 2004; Aikins *et al.*, 2006). Rhizome size and depth of planting were found to be critical to successful establishment of *M. giganteus* in the establishment studies conducted in Europe (Christian and Haase, 2001). Pyter *et al.* (2010) also reported that planting depth was important to providing higher and more consistent yield in the establishment of *M. giganteus* using rhizome.

The depth of planting is important in maximising the potential of seedling emergence and plant yield. This study was undertaken to determine the appropriate planting depth of *G. perpensa* rhizome for maximum growth and yield.

4.2 Materials and Methods

4.2.1 Determination of the appropriate planting depth

The 5 cm rhizome segments which gave the highest growth parameters in the clonal propagation study (Chapter 3) were used in this investigation. They were planted at five different depths (1, 2, 3, 4 and 5cm) in potted soils in order to determine the appropriate depth. Plastic pots of 14 cm in diameter and 24 cm depth were used. The experiment was laid out in a randomized design with five treatments replicated four times. Germination/emergence was monitored daily for six weeks and the number of days from the date of planting until the first shoot emergence from the potting medium was recorded. Data on the number of leaves, length of petiole, height of plants and leaf area were recorded on weekly basis for a period of 12 weeks. The experiment was carried out under glasshouse conditions at the University of Fort Hare, Alice, South Africa.

4.2.2 Data analysis

Data were analysed as reported in Chapter 3. The means were treated as significantly different at $P < 0.05$.

4.3 Results

4.3.1 Effects of planting depths on shoot emergence

In this study, rhizome survival as indicated by shoot emergence across all treatments (planting depths) were 100% at 1, 3, 4 and 5 cm planting depths and 83% at 2 cm depth for the 40 days of monitoring emergence (Fig. 4.1). The first shoot emergence from the rhizome buds was observed four days after planting in 1 cm and 4 cm depths respectively. The maximum emergence of 100% was observed first at 5 cm depth on the 35th day after planting, while 100% emergence for 1, 3, and 4 cm depths were observed on the 40th day.

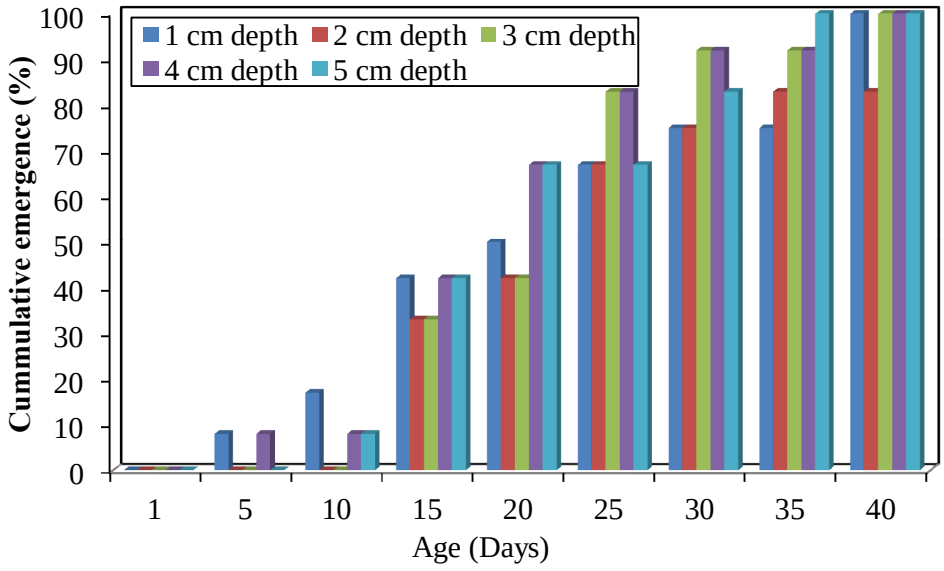

Figure 4.1: Effect of planting depths on shoot emergence of *G. perpensa*

4.3.2 Effects of planting depth on the number of leaves and leaf area

The effect of planting depths on number of leaves and leaf area are shown in Figure 4.2 and Figure 4.3 respectively. There were no significant differences on the number of leaves produced, among the various planting depths. However, at the end of the experimental

period, the highest number of leaves were obtained from the 5 cm depth, followed by the 4 cm while the lowest number of leaves were obtained from the 1 cm depth. The number of leaves increased with the age of the plant from 2.5 to 14 leaves in 5 cm depth. Similarly, the leaf area increased with increasing plant age. The mean leaf area was highest in 4 cm depth and lowest in the 1 cm, although there were no significant differences among the treatment means. Statistical analysis showed an interaction between plant age and planting depth on number of leaves and leaf area. Regression analysis with number of leaves and leaf area as the dependent variables and plant age as the regressor showed a coefficient of determination (R^2) of 95% respectively indicating that plant age had a significant effect on leaf number and leaf area.

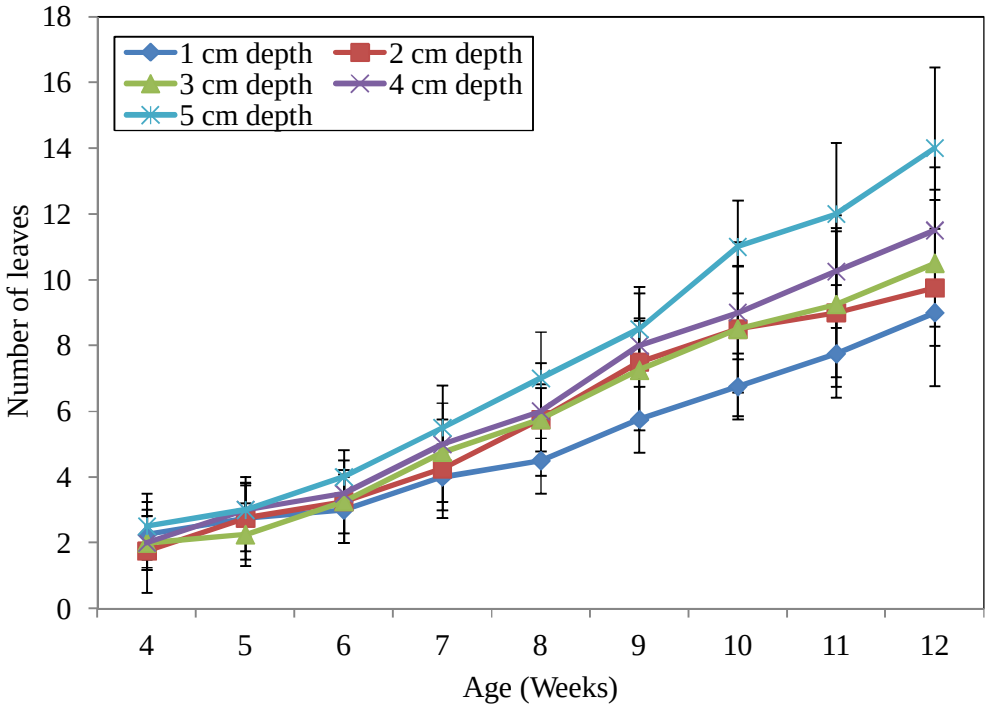

Figure 4.2: Effect of planting depths on number of leaves produced.

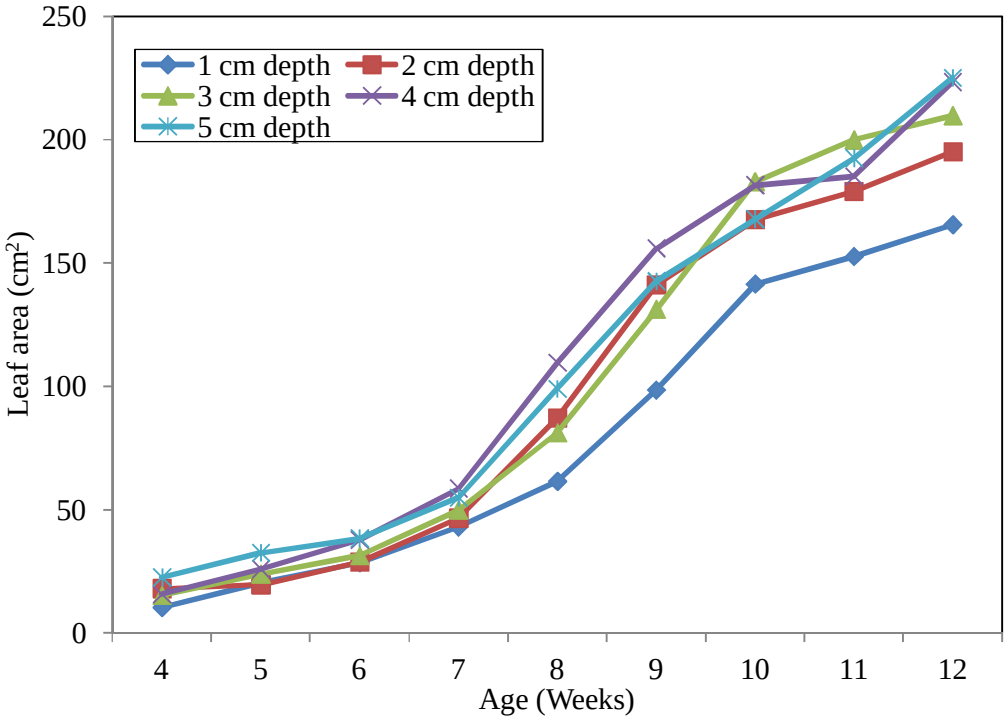

Figure 4.3: Effect of planting depths on leaf area.

4.3.3 Effects of planting depths on petiole length and height of plant

The effects of planting depth on length of petiole and heights of plant are shown in Figure 4.4 and Figure 4.5 respectively. From the 9th week to the 12th week of the experiment, plant heights and petiole lengths were higher at depth of 3 cm. However, the highest mean (22.75 cm) for plant height was observed at 4 cm depth while the lowest mean (18.58 cm) was obtained at 1 cm. Similarly, the highest mean (17.04 cm) for petiole length was observed at 4 cm depth, followed by 3 cm (16.76 cm) and the lowest (13.40 cm) was obtained at 1 cm.

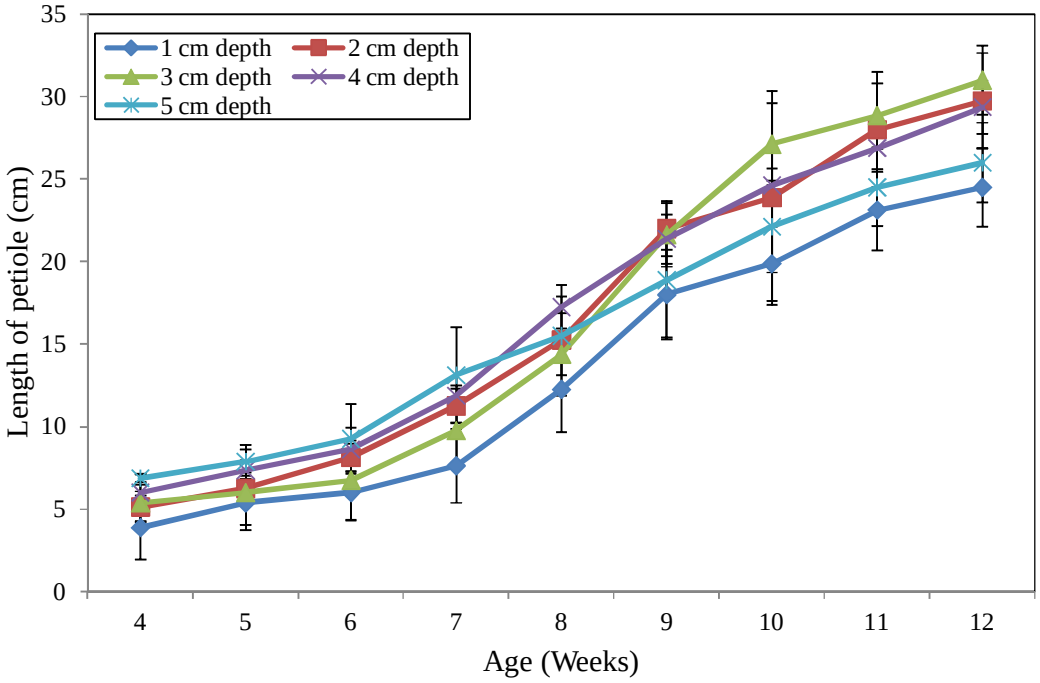

Figure 4.4: Effect of planting depth on length of petiole at different times after planting.

The mean plant heights increased steadily from the time of emergence to the termination of the experiment at the 12th week. The mean petiole length also increased steadily until the termination of the experiment. Statistical analysis showed that plant age had significant effect on plant height and petiole length ($P<0.05$).

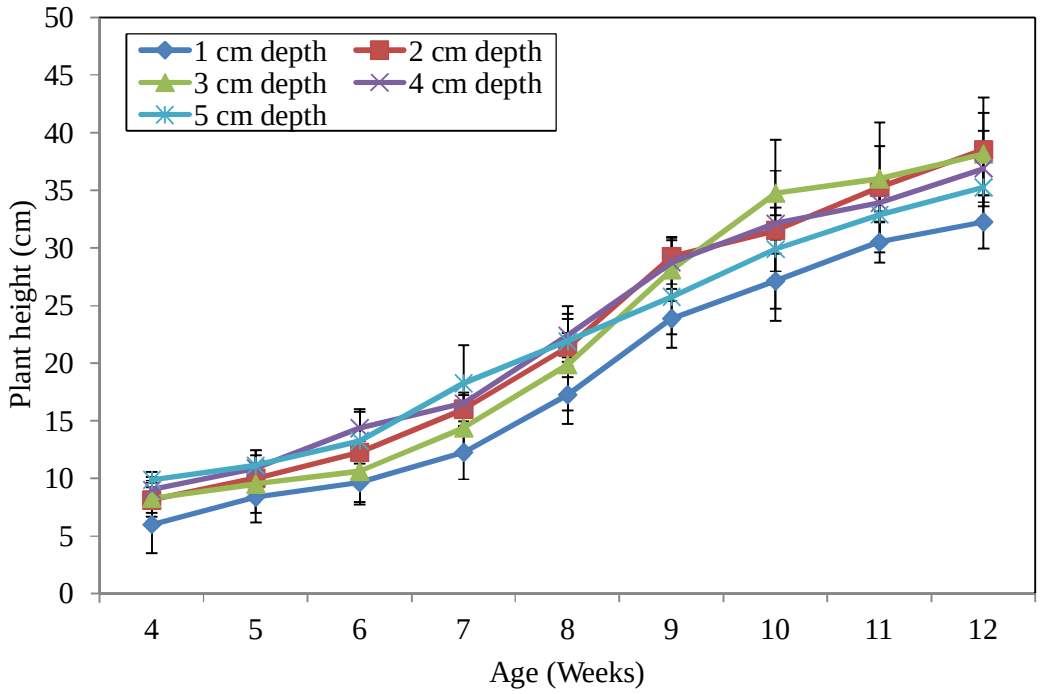

Figure 4.5: Effect of planting depth on plant height

4.3.4 Effect of planting depths on flowering

Flowering commenced on the 10th week after planting, with plants at 5 cm depth producing the highest number of flowers (Fig. 4.6). At week eleven, 4 cm and 5 cm planting depths produced the same number of flowers. By the 12th week of the study, the highest cumulative number of flowers produced was from 4 cm planting depth.

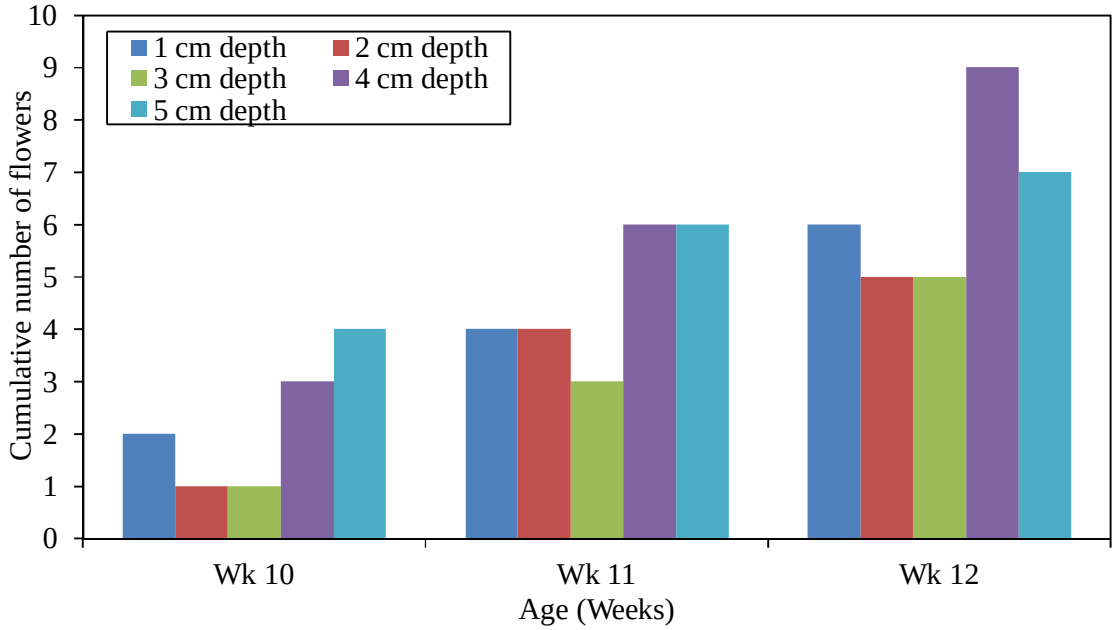

Figure 4.6: Effect of varying planting depths of rhizome on the number of flowers produced.

4.4 Discussion

High percentage shoot emergence was recorded at all the varying depths (1, 2, 3, 4 and 5 cm). A high percentage germination and emergence is the key to controlling stand establishment. Similarly, vigorous early growth is often associated with greater yields (Carter *et al.*, 1992). Delayed emergence can reduce the subsequent relative growth rate of the seedling. In general, healthy plants with well-developed root systems can withstand adverse conditions better than plants whose development and growth have been interrupted at an early stage (Harris, 1996). The first shoot emergence was observed 4 days after planting at depths of 1 cm and 4 cm. The plants from 1 cm planting depth also took less time to emerge than the varying depths. This finding is in agreement with the report of Harris (1996) that seedlings emerged more quickly from shallow sowings when conditions allowed them. Planting depth is one of the major factors that influence the time of seedling emergence (Soltani *et al.*,

2006). Rapid, uniform and complete emergence of vigorous seedlings leads to high plant yield and provides plant with time and spatial advantages to compete with weeds (Soltani *et al.*, 2001).

Planting at 5 cm depth resulted in more number of leaves per plant than those of the other planting depths. Similar result on depth of planting has been reported for soybean (Aikins *et al.*, 2011). According to Asare *et al.* (2011), leaves are the site of photosynthetic activities through which biomass are produced, partitioned among various parts of plants and stored for plant productivity. Plant productivity depends on the interaction of light intercepting the leaf area of a plant and the intensity of the CO_2 assimilation process taking place in those leaves. Thus, leaf area is a very important determinant of photosynthetic rate and biomass productivity/growth (Barigah *et al.*, 1994). The reduction of leaf area reduces carbon assimilation, while the increase also increases carbon assimilation and subsequently growth. Therefore, planting depths of 4 cm and 5 cm which gave the highest leaf area and highest number of leaves respectively are recommended for high productivity in cultivated *G. perpensa* plant. Heilman *et al.* (1988) reported that larger leaf number and leaf area index were among the characteristics of the poplar hybrids that favour growth.

Results showed that the different planting depths influenced growth and development in *G. perpensa*, but did not have significant effect on the number of leaves, leaf area, plant height and length of petiole. In some other studies, planting depth showed significant effect in the plant establishment. Christian and Haase (2001) reported that successful establishment of *M. giganteus* was achieved by planting rhizome pieces of 200 mm length at a soil depth of 200 mm, but when the same size of rhizome was planted at a depth of 100 mm, winter survival

rates were low. Nevertheless, in this study the highest mean leaf area, plant height and petiole length were obtained at 4 cm planting depth while the least was observed at 1 cm depth. This is in agreement with the report that deeper sowing was associated with a faster rate of leaf production and taller plants (Harris, 1996). The author established that seedlings from shallow sowings often do not grow as vigorously as those from deeper sowings. This suggests that 4 cm planting depth, having produced the largest leaf area, highest length of petiole and plant height, would be appropriate for high yield and better plant quality when compared to the other depths studied. The higher productivity at this depth can be explained by their larger leaf area which could have enhanced photosynthetic activity.

Flower produces the reproductive cells of a plant and then produces seeds, the dormant young plant of the next generation. The more the number of flowers produced per plant, the higher the chances of producing more seeds. The seed enables the plant embryo to survive until seed maturation and seedling establishment, thereby ensuring the initiation of the next generation of plants (Koornneef *et al.*, 2002). The depth of 4 cm which gave the highest number of flowers is recommended for planting of *G. perpensa* rhizome.

The depth of 4 cm may be optimum because it is deep enough to cushion the effect from fluxes in temperature and moisture availability, yet shallow enough to avoid delayed emergence as earlier described by Pyter *et al.* (2010). Planting depth was one of the factors reported to affect plant establishment and survival (Pyter *et al.*, 2010).

4.5 Conclusion

Planting depth did not have a significant effect on leaf number, plant height, length of petiole nor leaf area of *G. perpensa* planted using rhizome. However, a planting depth of 5 cm gave the highest number of leaves while 4 cm planting depth gave the best plant height, best petiole length, leaf area and highest number of flowers. Plant age over time has significant effect on the growth parameters, amongst the various planting depths. The results of this investigation indicate that planting depths of 4 and 5 cm could be appropriate for clonal propagation of *G. perpensa*, but 4 cm depth would be most preferred for maximum plant growth and development.

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CHAPTER 5

SEED GERMINATION STUDIES AND SEEDLING GROWTH ON *Gunnera perpensa* L.

CHAPTER FIVE

Seed Germination Studies and Seedling Growth on *Gunnera perpensa* L.

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5.1 Introduction

Gunnera perpensa is under increasing exploitation in Eastern Cape due to its wide medicinal usage. Thus, there is an urgent need to conserve this species. Suggested practical means of addressing over exploitation of wild plant species is to develop propagation techniques and encourage their cultivation thereby reducing pressure on the wild populations (Lewu *et al.*, 2006). According to some reports, the successful cultivation of medicinal plants is determined to a large extent by the germinability of the seeds (Kulkarni *et al.*, 2005). It is desirable to standardise techniques for efficient cultivation practices. It is therefore critical to study and understand the various factors that could affect the germinability of the seeds of any plant species before embarking on its propagation and conservation (Lewu *et al.*, 2006). In any case, different species have peculiar requirements for seed germination as a means of adapting to changing environmental conditions (Schütz and Milberg, 1997).

Different seeds have a range of temperatures at which germination will occur optimally, below and above which germination is delayed but not prevented (Takahashi *et al.*, 1996; Kumar and Sharma, 2012). The optimal temperature may be taken to be that at which the highest percentage of germination is attained in the shortest time (Takahashi *et al.*, 1996). Temperature is one of the most important environmental factors controlling seed germination (Gairola *et al.*, 2011).

Light is an important environmental factor that interacts with temperature to regulate seed germination in many plant species (Baskin and Baskin, 1998), but light requirement for germination may vary with temperature (El-keblawy and Al-rawai, 2005). Light stimulus can break dormancy, but it can also inhibit germination (Bian et al., 2013). Several studies have

reported on seed germination with different temperatures, light conditions and different treatments for seeds of medicinal plants of southern Africa (Kambizi *et al.*, 2006; Adebola and Afolayan, 2006).

There are other factors such as cold stratification, mechanical scarification and leaching that can influence the germination percentage. Baskin *et al.* (2001) reported seed dormancy-breaking and increase in the germination percentage of *Drosera anglica* after storage of seed under cold stratification (4°C). Ochuodho and Modi (2013) observed improvement in seed germination of *Cleome gynandra* after they were mechanically scarified. Plant regulators such as gibberellic acid (GA_3) (Vakeswaran and Krishnasarny, 2003; Ochuodho and Modi, 2013) and chemicals such as KNO_3 (Rehman and Park, 2000; Kouakou *et al.*, 2009) have been recommended to break dormancy and enhance germination. However, seed germination of some plant species does not respond to these regulators (Dewir *et al.*, 2011). Effects of these chemicals are often a function of the concentration and treatment durations.

Understanding the germination requirements of *G. perpensa* seed will be useful to develop a technique that will maximise its germination in an effort to bring this plant under cultivation. This experiment was carried out to determine the optimum conditions for seed germination of *G. perpensa* as well as to examine the seedling growth and establishment.

5.2 Materials and Methods

5.2.1 Seed collection

Seeds of *G. perpensa* were obtained from Silverhill seeds Cape Town in March 2013. They were kept in airtight envelopes at 15°C until used for these experiments.

5.2.2 Viability test

Samples of 100 seeds from each seed lot were tested for viability using the tetrazolium technique. Following the method described by Peters (2000), the seeds were imbibed in water overnight, cut along the margin without damaging the embryo and soaked in colourless 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 16 h at a temperature of 20-25°C in the dark. The seeds were removed from the TTC solution, washed in distilled water and observed under a stereo microscope. The red-stained embryos were considered as viable while those that did not stain as non-viable (Baskin and Baskin, 1998).

5.2.3 Seed moisture content and imbibition

Moisture content of the seeds was determined by weighing them, drying them at 40°C in an oven until there was no further loss in seed weight. Moisture content, expressed as percentage, was calculated on the basis of fresh weight using the equation below (Moyo *et al.*, 2009):

$$\text{Moisture content}(\%) = \left(\frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \right) \times 100$$

In imbibition studies, the procedure was the same as described by Kulkarni *et al.* (2007). Seeds were placed in 9 cm sterile Petri dishes on two layers of filter paper (Whatman No.1) moistened with sterile distilled water and allowed to imbibe at room temperature (25 ± 2°C). The increase in seed mass was determined after 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h. At each interval, the seeds were blotted dry, weighed and then replaced on the wet filter papers. The amount of water imbibed by the seed was graphically represented as the percentage increase over the initial mass. There were four replicates of 25 seeds each.

5.2.4 Germination tests

Seeds were surface sterilized using 1% sodium hypochlorite solution for 2 min, with several rinses of distilled water to prevent fungal attack. The germination was conducted in 9 cm Petri dishes lined with two discs of Whatman No 1 filter paper. Seed treatments consisted of soaking in cold water for 1, 3 and 7 days with intermittent rinses (leaching) and 1, 3 and 7 days of cold treatment of imbibed seeds (pre-chilling) at 4°C. Other treatments included continuous darkness, l6 h light/8 h dark photoperiod, mechanical scarification, hot water treatment and chemical treatments. For each experiment, there were four replicates of 25 seeds each. Germination was recorded daily and considered complete once the radicle protruded about 2 mm in length. Experiments were terminated after 120 days. Mean germination time (MGT) was calculated by using the equation:

$$MGT = \sum \frac{(n \times d)}{N}$$

Where n = number of seeds germinated on each day, d = number of days from the beginning of the test and N = total number of seeds germinated at the termination of the experiment (Kulkarni *et al.*, 2007).

5.2.4.1 Effects of temperature

To evaluate the effects of different temperature regimes, the seeds were incubated at constant temperatures of 10, 15, 20, 25, 30 and 35°C.

5.2.4.2 Effects of Photoperiod

To evaluate the effects of photoperiod, seeds were germinated under alternating light (16/8 h photoperiod) and constant dark where Petri dishes were covered in aluminium foil and placed

in the dark room. Germination was recorded under green safe light (0.3µmol/m^2/s) at 25 ± 2 °C daily.

5.2.4.3 Effects of cold stratification

For stratification treatments, seeds were placed between two sheets of moist paper towels in plastic bags at 4°C for 1, 3 and 7 days. Subsequently, the seeds were germinated under light conditions (16/8 h photoperiod) and at the different temperatures. Non-stratified seeds were considered as the control.

5.2.4.4 Mechanical scarification

Mechanical scarification was achieved by gently rubbing the seeds between two sheets of fine-grained sand paper to remove the seed coat without injuring the embryo (Pérez-García and González-Benito, 2006). The seeds were placed in Petri dishes lined with two layers of Whatman filter paper and incubated at the various temperatures and light conditions investigated. Filter papers were kept moistened throughout the duration of the experiments.

5.2.4.5 Effect of chemical substances

Chemical substances tested on seed germination were gibberellic acid (GA_3) at 100 µM (0.035 mg/l) and 250 µM (0.086 mg/l) concentrations and potassium nitrate (KNO_3) at a concentration of 1 g/l under light conditions at the varying temperatures investigated. The seeds were soaked and swirled in 50 ml of the different chemical solutions for 24 hr at room temperature. Following treatment, seeds were rinsed thoroughly with sterile distlled water.

5.2.4.6 Hot water treatment

Seeds were soaked in hot water at 80°C and 60°C respectively and stirred continuously until cooled.

5.2.4.7 Early seedling growth

Seeds were sown into small size polythene pots, three-quarter filled with Hygrotech mix. Routine watering was carried out. The polythene pots were laid out in the glasshouse maintained at 18°C ï 30°C. The seeds germinated five weeks after sowing and data on seedling growth were taken. Three selected seedlings were tagged and used for growth investigation. Growth parameters studied were plant height, length of petiole, petiole girth, number of leaves and leaf area.

5.2.4.8 Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA). The one way ANOVA test was used to determine if there was any statistically significant difference in the germination percentage obtained from the different pre-treatments on seed.

5.3 Results

5.3.1 Seed moisture content, viability and imbibition

The average mass of one *G. perpensa* seed was 0.69 ± 0.01 mg. The mean moisture content of the seeds was found to be 7.54% while the viability of the seed lot was 49.6%. Imbibition of water by the seeds reached maximum by the 12th h, after which the water uptake began to decline (Fig. 5.1).

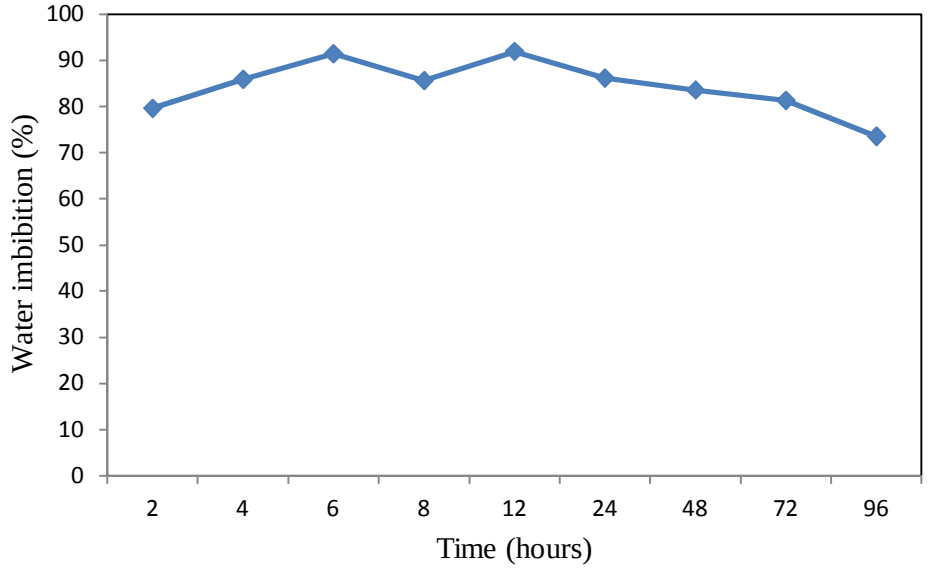

Figure 5.1: Water uptake by seeds of *G. perpensa* at 25 ± 2°C

5.3.2. The effect of temperature and photoperiod on seed germination

At a temperature of 25°C, seeds of *G. perpensa* exhibited maximum germination which was obtained from pre-treated seeds, whereas at 20°C, very low germination (5.3%) was recorded. Seeds failed to germinate at 10°C, 15°C, 30°C and 35°C respectively. *G. perpensa* seeds did not germinate when exposed to continuous darkness. On the other hand, very poor germination (5.3%) was recorded under a regime of 16/8 h photoperiod. Most of the germination was obtained from pre-treated seeds incubated under light at 25°C, with the highest germination percentage as 32%. Germination of untreated seeds exposed to the same light regime and temperature condition was 4%.

5.3.3. Effect of pre-chilling, leaching and hot water treatment on seed germination

When the seeds were subjected to leaching for different duration, the seven day treatment gave the highest percentage germination (26.7%) with the shortest mean germination time

(MGT) of 44 days, followed by three day treatment (Table 5.1). The lowest percentage germination (16%) was obtained from one day leaching, with longer MGT (80 days) compared to the seven and three day treatments. Leaching improved the germination of *G. perpensa* seeds when compared with the control.

Table 5.1: Effect of pre-treatments on seed germination of *Gunnera. perpensa* at 25°C

Treatment	Germination (%)	MGT (days)
Control (untreated)	4.0 ± 0.1^{cd}	56
Leaching (1day)	16.0 ± 0.0^{c}	80
Leaching (3days)	24.0 ± 2.0^{b}	47
Leaching (7 days)	26.7 ± 0.0^{b}	44
Mechanical Scarification	32.0 ± 1.0^{a}	56
Pre-chilling (1 day)	8.0 ± 1.0^{cd}	90
Pre-chilling (3 days)	0.0	Not applicable
Pre-chilling (7 days)	0.0	Not applicable
Hot water (80°C)	4.0 ± 0.0^{cd}	52
Hot water (60°C)	1.3 ± 0.0^{d}	59
GA_3 (0.086g/l)	2.7 ± 0.0^{d}	84
GA_3 (0.035g/l)	0.0	Not applicable
KNO_3 (1.0mg/l)	0.0	Not applicable
KNO_3 (scarified)	8.0 ± 1.0^{cd}	77

MGT = mean germination time. Percentage germination values (± SE) in a column with different letters are significantly different ($p < 0.05$). Data is in means of 3 replicates.

There was no record of germination when the seeds were subjected to pre-chilling treatment for three and seven days respectively. However, very low germination (8%) occurred at one day treatment with the longest MGT (Table 5.1). In this study, subjecting the seeds to low temperature (4°C) prior to germination resulted in a pronounced negative effect on their germination, except for the one day treatment. Hot water treatments (60 and 80°C) did not improve germination when compared to untreated seeds (Table 5.1). However, germination was slightly higher (4%) in 80°C with shorter MGT than in 60°C (1.3% germination).

5.3.4. The effect of mechanical scarification on seed germination

Mechanical scarification significantly increased the germination percentage of *G. perpensa* seeds when compared to the untreated seeds. Amongst the various pre-treatments given to the seeds in this study, mechanical scarification using sandpaper produced the highest germination percentage (32%), with the same MGT as the untreated seeds (Table 5.1).

5.3.5. Effect of chemical substances on seed germination

Treatment of seeds of *G. perpensa* with GA_3 did not have any positive effect on germination when compared with the untreated seeds (Table 5.1). Also the treatment of seeds with KNO_3 significantly inhibited germination at a constant temperature of 25°C ($P<0.05$), but at 20°C only 2.67% germination was obtained. However, when the seeds were scarified before treating with KNO_3 and incubated at 25°C, a little improvement in germination (8%) was observed compared to the control, but the MGT was high (Table 5.1).

5.3.6. Seedling growth performance

The first seedling emergence occurred at 5 weeks after planting. Generally, the growth of *G. perpensa* seedling was slow and poor. The seedlings attained maximum growth at the 5th

week, after which growth declined significantly as shown in Table 5.2. This is quite different from the seedlings raised using the rhizome which showed increasing trend in growth until the termination of the experiment at 10th week (Chapter 3). The seedlings from seed produced numerous number of leaves but their leaf area was small (Table 5.2). The highest leaf area, obtained at the 5th week was 3.33 cm^2 as against 232.46 cm^2 leaf area obtained from the rhizome-raised plants.

Age also played significant role in the growth of the seedlings. After the 5th week, the seedlings started dying, that by the 10th week the plant height and petiole length was greatly reduced from what it was at the first week. The seedlings could not maintain their leaves for a longer period. Longer leaf retention has been reported as an important component of growth (Nelson and Isebrands, 1983). Petiole girth did not increase appreciably throughout the trial period. Establishment of *G. perpensa* through seedling was difficult and may be improved by addition of nitrogen source.

Table 5.2: Growth parameters of seedlings of *Gunnera perpensa* raised from seeds.

Growth Parameters	Age (weeks)									
	1	2	3	4	5	6	7	8	9	10
Height of plants (cm)	3.00c	7.50bc	10.67b	11.83a	11.83a	11.50a	9.67b	7.67bc	6.00bc	2.00c
Length of petiole (cm)	2.50c	6.83bc	9.33b	10.00a	10.00a	9.67a	8.67b	6.83bc	4.50bc	1.50c
Petiole girth (cm)	0.05a	0.05a	0.10a	0.10a	0.10a	0.10a	0.10a	0.05a	0.05a	0.05a
No of Leaves	4.00d	10.00d	24.00c	42.00b	63.00a	60.00a	56.00a	15.00cd	9.00d	9.00d
Leaf area (cm^2)	0.49d	1.32c	2.65b	3.27a	3.33a	3.13a	1.77c	0.56d	0.25d	0.25d
Chlorophyll content	7.70cd	12.60c	26.80b	27.90b	31.90a	28.60b	19.50bc	6.10cd	2.70d	0.90d

Means followed by different letters in the same row are significantly different

5.4 Discussion

Seed moisture content is the most important factor that regulates the longevity and storability of seeds. High moisture in seeds enhances seed deterioration, which ultimately reduces the planting value of seeds in the field (Nahar *et al.*, 2009). The moisture content (7.54%) obtained for the seeds of *G. perpensa* in this study is an indication that it can remain viable for a long time. Justice and Bass (1978) noted that if the moisture content of seed in storage condition exceeds 8-9%, then the risk of insect, fungal and bacterial attack increases. On the other hand, very low moisture content below 4% may also damage seeds due to extreme desiccation or cause hard óseedednessô in some crops.

Baskin and Baskin (2003) suggested that if seed mass increases greater than or equals 20% during water uptake which is the case in this study, then the seed coat should be assumed to be permeable to water. The authors further explained that the failure of permeable seeds to germinate in 2 to 4 weeks at several temperature regimes shows the seeds are dormant. Seeds of *G. perpensa* imbibed water, but failed to germinate within 4 weeks in all temperature conditions evaluated indicating that the seed is dormant. The rate of imbibition was not consistent though. Imbibition differs greatly between seeds in relation to seed size and weight, structure, permeability, chemical composition, variation in temperatures and seed-water contact areas (Obroucheva and Antipova, 1997). Seed germination is initiated by imbibition. Hence, seeds will not germinate unless both minimal soil water potential and seed hydration are attained during imbibition (Hadas and Russo, 1974). Water is therefore an essential component for the rehydration and germination of seeds (Bewley and Black, 1978). Sivritepe and Dourado (1995) explained that in low viability, low-moisture content pea seed

lot, percentage of normal germination decreased significantly after 12 h soaking. This is similar to the result of low viability and low moisture content obtained in this study.

The result of this study clearly shows that the seeds of *G. perpensa* cannot tolerate high or low temperatures. High temperatures may prevent germination because proteins may be denatured and the permeability of the membranes altered. At low temperatures there is reduced metabolic activity which may alter the germination processes (Delachiave and De pinho, 2003).

The results also show that seeds of *G. perpensa* are temperature and light dependent for germination to occur. The optimum condition for the germination of the seeds in this study was 25°C under continuous light. Kulkarni *et al.* (2007) reported similar finding for *Dioscorea dregeana*. Temperature and light play a significant role in germination of seeds, but seeds of many plant species respond differently to these two vital environmental factors (Fenner and Thompson, 2005). Temperature could serve as a good indicator of the timing of germination in seasonal climates (Fenner and Thompson, 2005). *Gunnera perpensa* seeds germinated under light regime, but not in the dark which is an indication that exposure of the seeds to light is required to trigger germination. This condition is typical of small-seeded plant species (Clarke *et al.*, 2000). As a result, deep sowing is not recommended for *G. perpensa* seeds.

Leaching improved the germination of *G. perpensa* seeds when compared with the control. The seeds of *G. perpensa* were characterized by a long delay in germination, which was shortened when subjected to leaching treatments. The physiological role behind these

phenomena is usually associated with the breakdown of germination inhibitors (Tucker and Gray, 1986). Overall, mean germination time was shortest in seeds leached for seven days. Mean germination time has been shown to be a high indication of emergence performance in seed lots of pepper (Demir *et al.*, 2008) and of maize in soil (Matthews and Khajeh-Hosseini, 2006). Matthews and Khajeh-Hosseini (2007) pointed out that the MGT of a seed lot in maize was also the mean of the lag period from the start of imbibition to physiological germination.

Pre-chilling treatment has been reported to break dormancy of viable seeds and enhance germination in many species (Baskin *et al.*, 2001). In contrast, it has also caused lethal effect on some viable seeds (Ren and Tao, 2004). The latter might have been the case with *G. perpensa* seeds when they were subjected to pre-chilling treatment, because no germination was recorded.

Hot water scarification did not stimulate germination in *G. perpensa* seeds. Similar results were reported by Long *et al.* (2012) where only 1.3% to 8% of *Astragalus arpilobus* seeds germinated when treated in hot water of temperatures 70, 80 or 90°C, regardless of the exposure time. In contrast, dormancy was broken in 70% of *Astragalus hamosus* seeds exposed to 10 min of wet heat at 80°C (Patane and Gresta, 2006).

Mechanical scarification improved germination in dormant seeds of *Cleome gynandra* (Ochuodho and Modi, 2003). Previous reports (Addis, 2003; Long *et al.*, 2012) have recommended mechanical scarification in breaking dormancy imposed on seeds due to water-impermeable seed coat, to improve imbibition rate and ultimately enhance germination.

Patane and Gresta (2006) reported dormancy breaking of 100% in *Astragalus hamosus* seeds scarified with sandpaper.

The use of chemical compounds such as plant growth regulators to influence uniform and consistent seed germination is well documented (Nadeem *et al.*, 2000). In the present study, the stimulatory effect of GA_3 was not observed; germination was rather very low. Potassium nitrate (KNO_3) did not give any germination at 25°C, except when the seeds were scarified before treatment with KNO_3. In a similar study, GA_3 did not influence germination of *Podophyllum hexandrum* seeds (Choudhary *et al.*, 1996) and KNO_3 did not either (Nadeem *et al.* 2000). On the other hand, treatments using GA_3 and KNO_3 improved germination percentage in *E. macrocarpa* and *L. secundiflorum* (Kouakou *et al.*, 2009). According to Rehman and Park (2000), application of GA_3 and KNO_3 showed positive effect in overcoming dormancy caused by inhibitors.

Proper seed germination and growth are indispensable for the continued existence of any plant. The small leaf area in this study is an indication of poor photosynthesis and poor growth. The reduction of leaf area also reduces carbon assimilation and subsequent growth. According to Pandey and Singh (2011), leaf area is an important parameter in understanding water and nutrient use, plant growth and yield potential. The growth of leaf area is important because it is directly related to the plantôs capacity to intercept light and photosynthesize (Barigah *et al.*, 1994). Low chlorophyll concentrations limit photosynthesis and therefore plant growth (Pinkard *et al.*, 2006). The chlorophyll content in the seed-raised seedlings was also low (Table 5.2) that they may not produce sufficient food for the plant to utilise for good growth.

5.5 Conclusion

The highest percentage germination (32%) in this experiment, obtained from pre-treated seeds is very low. This result is consistent with the percentage of viable seeds obtained from viability test, which is equally low. This factor coupled with the practice of over exploitation may be part of the reasons for poor germination in nature with consequent declining populations of *G. perpensa* in the wild. From this study, it is evident that germination of seeds of this plant would be largely dependent on light and temperature conditions, the optimum temperature being 25°C. These environmental conditions and seed pre-treatment should be taken into consideration in the cultivation of this valuable plant. The germination of *G. perpensa* seeds is irregular and poor. The low germination is possibly the result of dormancy; however mechanical scarification and pre-soaking of seeds in water prior to germination improved its germination responses. Seedling growth was very poor, but pre-sowing seed treatments can be used to enhance a faster and more uniform germination, thereby improving seedling vigour.

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CHAPTER 6

SUBSTITUTING *Gunnera perpensa* L. ROOTS WITH LEAVES IN TREATMENT OF BACTERIAL AND FUNGAL INFECTIONS

CHAPTER SIX

Substituting *Gunnera perpensa* L. Roots with Leaves in Treatment of Bacterial and Fungal Infections

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6.1 Introduction

Some bacteria and fungi have been implicated in the pathology of many diseases. For example, *Candida albicans* is known to be a pathogenic fungus causing candidiasis (Ryan and Ray, 2004), while bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* are known to be involved in gastroenteritis or respiratory infections (Sun *et al.*, 2006). Rapid increase in diseases associated with fungal infections such as candidiasis is related with the increase rate of HIV infection in many communities (Mcneil *et al.*, 2001; Reichart, 2003). Due to the rapid increase in the rate of infections, antibiotic resistances in microorganisms and side effects of synthetic antibiotics, a great deal of interest has grown in the use of medicinal plants over synthetic antibiotics in the treatment of microbial infections (Babu and Subhasree, 2009). In contrast to other types of medication, antibiotics ultimately lose their effectiveness as resistant strains of bacteria develop (Lerner, 1998). An example is the Gram-positive, methicillin-resistant *Staphylococcus aureus* (Lesse, 1995). However, natural products could play a vital role in meeting the demand for new drugs against infectious diseases (Cragg and Newman, 2001), while simultaneously mitigating against many of the side effects that are often associated with synthetic antimicrobials (Iwu, 1999). Plants, in addition to their therapeutic use in herbal preparations, can serve as important sources of new drugs, new drug leads and new chemical entities (Saklani and Kutty, 2008).

During the ethnobotanical study of this project, it was discovered that mostly the rhizomes of *Gunnera perpensa* were collected for herbal preparations. This is a destructive method of plant harvesting. It reduces the prospect for natural rejuvenation and could negatively affect plant population for future use. A number of strategies to solve the problem of over

harvesting have been suggested among which is the use of leaves and stems as alternatives to tubers and roots for medicinal purposes. A pre-requisite for achieving this objective is to assess the differences and similarities between the various parts of the same plant in their chemical and pharmacological properties. Lewu *et al.* (2006) reported that the leaves of *Pelargonium sidoides* may substitute for its roots in the treatment of bacterial infections.

In this chapter, the antimicrobial properties of methanol, acetone and water extracts of the rhizomes and leaves of *G. perpensa* were studied; first to provide the rationale for the use of the plant in the treatment of various infectious ailments and secondly to determine which part of the plant is most active. The comparison of the activity of the leaves and rhizomes was aimed at assessing the differences and similarities in the antimicrobial actions of the aerial and underground parts of the plant, in order to encourage the use of an alternative (aerial) part of the herb rather than the underground part.

6.2 Materials and Methods

6.2.1 Extract preparation

Leaf and rhizome samples of *G. perpensa* were separately chopped, dried in an oven at 40°C and homogenized. The homogenized samples were put into separate conical flasks containing acetone, methanol and water and shaken for 24 h on an orbital shaker. After filtering with a Buchner and Whatman No. 1 filter paper, the acetone and methanol filtrates were concentrated to dryness under reduced pressure at a maximum of 40°C using rotary evaporator. Aqueous filtrates were freeze-dried. Each extract was resuspended in the respective solvent of extraction to yield a 50 mg/ml stock solution.

6.2.2 Test organisms

Ten bacterial species were used in this study and included (1) *Escherichia coli* (ATCC 8739), (2) Bacillus *cereus* (ATCC 10702), (3) *Serratia mercescens* (ATCC 9986), (4) *Enterococcus faecalis* (KZN), (5) *Staphylococcus aureus* (OK3), (6) *Pseudomonas aeruginosa* (ATCC 19582), (7) *Salmonella typhi* (ATCC 13311), (8) *Vibrio cholera* (KZN), (9) *Bacillus subtilis* (KZN) and (10) *Streptococcus faecalis* (ATCC 29212). The organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. They were maintained on nutrient agar plates and were revived for bioassay by culturing them in fresh nutrient broth (Biolab, South Africa) and incubated for 24 h before use. Four fungal species namely *Candida krusei*, *Candida albicans*, *Candida glabrata* and *Absidia corymbifera* were also obtained from the same Department and University. The fungal cultures were maintained on potato dextrose agar (PDA) and were recovered for testing by sub-culturing on fresh PDA for 3 days prior to bioassay.

6.2.3 Antibacterial assay

6.2.3.1 Susceptibility test and Determination of Minimum inhibitory concentration

The agar diffusion method was used to carry out the susceptibility screening of the test bacteria to the various extracts of *G. perpensa*. Residues of the plant extracts were re-dissolved with the extracting solvents at a concentration of 50 mg/ml for the susceptibility screening and ciprofloxacin was used as control. The activity of the extracts and minimum inhibitory concentration (MIC) against the test organisms were also determined using agar diffusion method as described by Olajuyigbe and Afolayan (2012). The inoculum of each test bacterial strains was standardized at 10^{-2} cfu/ml using McFarland Nephelometer standard (NCCLS, 1993). Sterile nutrient agar plates were seeded with each adjusted test bacterial

strains and allowed to stand at 37°C for 1 h. Wells were then bored into the agar media using a sterile 5 mm cork borer. The wells were filled with the various extracts at concentrations of 2.5, 5.0, 7.5 and 10.0 mg/ml for MIC, taking care not to allow spillage of the solutions onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 30 min to allow proper diffusion of the extracts and antibiotics before being incubated at 37°C for 24 h. After 24 h incubation period, antimicrobial activities were determined by measuring the inhibition zones against the test organisms using a calibrated transparent meter rule. Experiments were carried out in triplicates.

6.2.4 Antifungal test

The activity of the extracts and minimum inhibitory concentrations were determined by the macrobroth dilution methods (Olajuyigbe and Afolayan, 2012). Stock solutions (40 mg/ml) of plant extracts were prepared in suitable solvents and filtered using 0.45 µm sterile filters. Sabouraud dextrose broth was used for serial dilution. Nine serial dilutions were prepared, ranging from concentration of 20 mg/ml to 0.07 mg/ml. Each broth concentration was inoculated with 100 µl of the prepared fungal sporesô solution. Two control tubes were included: One with spores and broth but no plant extract, and one with broth and plant extract but no spores. The fungal containing tubes were incubated at 37°C for 24 h to 3 days (depending on the fungi) and the MIC determined. The first tube in the series with no visible growth after incubation period was taken as the MIC.

6.2.5 Determination of minimum fungicidal concentrations (MFC)

For the determination of the MFC, fresh potato dextrose agar plates were inoculated with one loopful of culture taken from each of the first three broth cultures that showed no growth in

the MIC tubes (Olajuyigbe and Afolayan, 2012). The MFC assay plates were incubated for 3 to 5 days. After the incubation periods, the lowest concentration of the extract that did not produce any fungal growth on the solid medium was regarded as MFC values for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of spore germination for the fungi after incubation.

6.2.6 Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA) and the mean values were separated at ($p<0.05$) using Duncanôs multiple range test. The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of the zones of inhibition obtained from the different concentrations of the extract tested against the microorganisms.

6.3 Results

6.3.1 Antibacterial activity

The leaf and root parts of *G. perpensa* were evaluated for their antimicrobial potential against 10 bacterial species. The results obtained for the plant extracts presented some activity against the microorganisms with zones of inhibition varying from 8 to 25 mm (Fig. 6.1). The methanol leaf, methanol root and aqueous leaf extracts were active against all the ten bacteria species, while the aqueous root extract was active against nine out of the 10 bacteria. The inhibitory activity of the active extracts based on the overall mean inhibition diameters was in the order: methanol leaf > methanol root > aqueous leaf > aqueous root (Fig. 6.2). The highest activity against the tested bacteria was obtained with the methanol leaf extract with inhibition zone diameters of 25, 24, 24 and 22 mm against *Serratia mercescens*,

Staphylococcus aerus, *Bacillus cereus* and *Pseudomonas aeruginosa* respectively (Fig 6.1). The lowest activity was obtained with the aqueous root extract with inhibition zone diameter of 8 mm against *Streptococcus faecalis* and no activity against *Bacillus subtilis*. The most susceptible bacteria based on the overall mean diameter of growth inhibition were *S. mercescens, B. cereus, S. aureus* and *P. aeruginosa*.

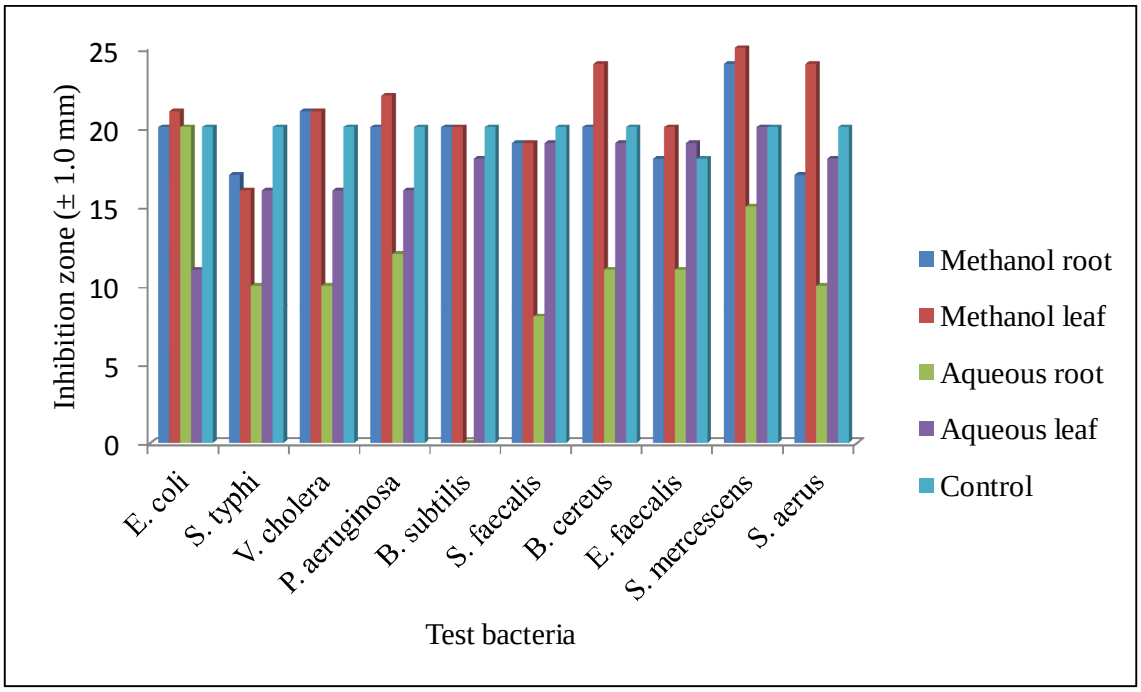
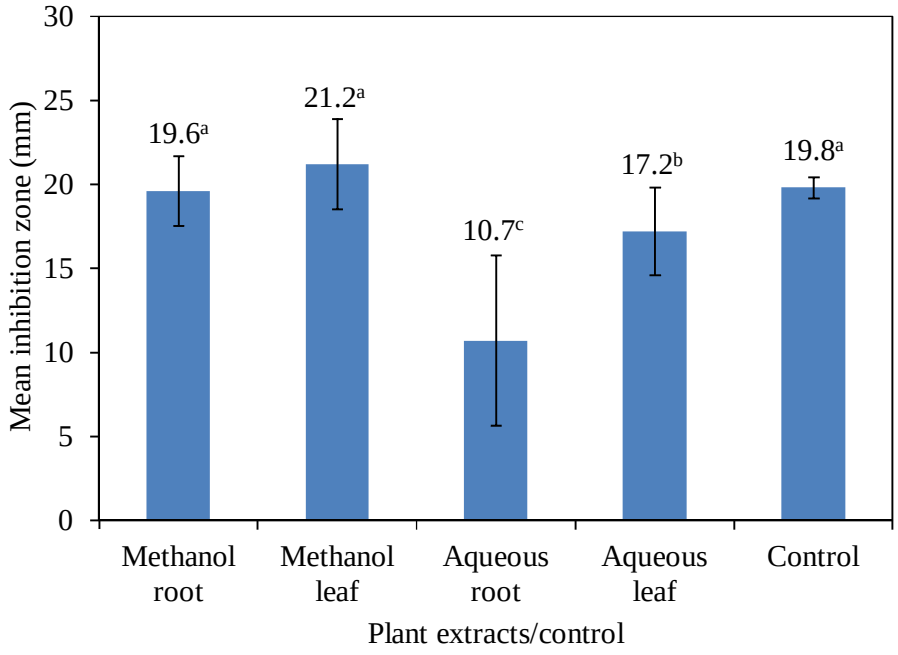


Figure 6.1: Antibacterial activity of the extracts of *G. perpensa* and the control against tested bacteria.

The inhibitory activity of the extracts based on the overall mean inhibition diameters was in the order: methanol leaf > methanol root > aqueous leaf > aqueous root (Fig. 6.2). The antibacterial activities of the extracts against the test organisms were very close and similar to that of the standard antibiotic (ciprofloxacin) (Fig. 6.2). The methanol leaf extract had slightly higher activity than the ciprofloxacin against some of the bacterial species.



(Inhibition zones are the means of all the fungi tested. Mean inhibition zones with the same superscript letters are not significantly different from each other. $P< 0.05$)

Figure 6.2: Inhibitory activity of the plant extracts against the 10 bacteria species

6.3.2 Determination of the minimum inhibitory concentration of *G. perpensa* extracts

The degree of the antibacterial activity of this plant was determined by MIC of its extracts. With the exception of the aqueous root extract, other extracts from both root and leaf showed activity against all the Gram-positive and Gram-negative bacteria, with MIC values ranging from 2.5 to 10 mg/ml (Table 6.1). However, these values were slightly higher in the leaf than in the root especially against *Streptococcus faecalis, Bacillus cereus*, and *Enterococcus faecalis*.

Table 6.1: Minimum inhibitory concentration (MIC) of *Gunnera perpensa* extracts on bacterial species

Bacterial species	Gram +/-	MIC (mg/ml)			
		Methanol		Aqueous	
		Root	Leaf	Root	Leaf
Escherichia coli	-	5.0	5.0	ni	10
Salmonella Typhi	-	5.0	5.0	ni	7.5
Vibrio cholera	-	10	10	ni	10
Pseudomonas aeruginosa	-	10	10	ni	10
Serratia mercescens	-	2.5	2.5	10	2.5
Bacillus subtilis	+	10	7.5	ni	10
Streptococcus faecalis	+	5.0	10	ni	10
Bacillus cereus	+	7.5	10	ni	10
Enterococcus faecalis	+	7.5	10	ni	10
Staphylococcus aureus	+	7.5	5.0	ni	10

ni = no inhibition.

6.3.3 Antifungal activity

All the *G. perpensa* plant extracts exhibited high antifungal activity against *Candida krusei* and *Absidia corymbifera* with MIC values ranging from 0.15 to 0.62 mg/ml (Table 6.2). The extracts showed moderate activity against *Candida glabrata* and *Candida albicans*. The highest antifungal activity was observed with methanol root and acetone leaf extracts. Extracts from both root and leaf of this plant showed similar activity against the fungal species, though the values were slightly higher in the leaf extract than in the root extract especially in the methanol extracts.

After 48 h, once the MIC values had been recorded, more broth was added to determine whether the activity was fungistatic (a state where fungal growth is inhibited but not dead) or fungicidal (the fungus is destroyed/killed). Extracts which initially had an MIC value of 0.15 mg/ml, after additional broth and further incubation had MFC values greater than this value all showed fungistatic activity. Extracts which had an MIC value of 0.15 mg/ml and a MFC value of 0.15 mg/ml indicated fungicidal activity. The extracts are fungicidal when MIC=MFC. It was noted that all the four extracts were fungicidal against *Candida krusei*, at lower concentrations. The acetone root extract also showed fungicidal effect against *Absidia corymbifera.*

Table 6.2: Antifungal activity of *Gunnera perpensa* expressed as MIC and MFC (mg/ml)

Fungal species	Methanol root		Methanol leaf		Acetone root		Acetone leaf	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida krusei	0.15	0.15	0.31	0.31	0.31	0.31	0.15	0.15
Candida albicans	1.25	2.5	2.50	-	2.5	2.5	5.0	5.0
Absidia corymbifera	0.31	2.5	0.62	1.25	0.31	0.31	0.31	0.62
Candida glabrata	1.25	1.25	1.25	1.25	1.25	-	1.25	1.25

‘’-” = not tested

Generally, the root and leaf of this plant were observed to have antimicrobial properties. There was no significant observable difference between the MIC of extracts from the two plant parts. This may also be true with their medicinal values, yet our survey revealed that mostly roots are used for medicinal purposes. The choice of the roots by the traditional healers over the leaves may be arbitrary.

6.4 Discussion

In this study, the results obtained indicated that the methanol leaf, methanol root and aqueous leaf extracts of *G. perpensa* were active against all the ten bacteria species. This, therefore shows that the extract contains substance(s) that can inhibit the growth of some microorganisms.

Staphylococcus aureus is an opportunistic organism which only becomes pathogenic when the immune system of a patient is compromised. They contribute significantly to the initiation of infection and are commonly associated with skin and wound infections (Bannister *et al*., 2000). The results indicate that the extracts are effective against *S. aureus* and therefore can be utilized in the treatment of the conditions associated with it.

The plant extracts also exhibited promising activity against *P. aeruginosa* which is naturally resistant to many antimicrobial agents (Konning *et al*., 2004) and also associated with a dysentery-like enteric infection and pneumonia (Bannister *et al*., 2000).Thus, the isolation of compounds from the most active extracts of *G. perpensa* may produce compounds that are active against *P. aeruginosa* making it useful for these resistant organisms.

In addition, *G. perpensa* extracts were found to produce good antifungal activity against *Candida krusei* and *Absidia corymbifera* and may, thus, be useful as an antifungal agent. Antifungal agents are amongst the most expensive antibiotics. Thus, readily accessible and inexpensive alternative remedies for treatment of fungal infections, is required (Salie et al., 1996).

The result suggests that bioactive compounds present in the roots of the plant may be similarly present in the leaves but at different concentrations. Lewu *et al.* (2006) reported similar results on the roots and leaves of *Pelargonium sidoides*. The leaves of *G. perpensa* may, as well, substitute for its roots especially in the treatment of bacterial and fungal diseases. The methanol leaf extract had slightly higher activity than the standard (ciprofloxacin) against some of the bacterial species. This result points to the fact that if the crude extracts were subjected to purification, the active components may exhibit same or even higher zones of inhibition than what was obtained for the standard antibiotic. Similar observations were made by Ogundare (2011).

6.5 Conclusion

The ability of the extracts of *G. perpensa* to inhibit the growth of several bacteria and fungi is an indication of the broad-spectrum antimicrobial potential of this plant that further validates its use for the treatment of various ailments. In the antibacterial assay, the leaves of this plant demonstrated higher activity than the roots, while in antifungal assay the two plant parts exhibited similar activities suggesting their potential in plant part substitution. The harvesting of leaves as a conservation strategy is certainly more sustainable than the destructive use of the roots of this threatened plant species.

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CHAPTER 7

PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF THE LEAF AND RHIZOME OF *Gunnera perpensa* L.

CHAPTER SEVEN

Phytochemical Constituents and Antioxidant Activities of the Leaf and Rhizome of *Gunnera perpensa* L.

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7.1 Introduction

Living cells may generate free radicals and other reactive oxygen species by-products as a result of physiological and biochemical processes. Free radicals are chemically unstable atoms that can cause damage to cell lipids, proteins and DNA as a result of an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes (Manian et al., 2008). Lipids are highly prone to free radical damage resulting in lipid peroxidation that affects membrane structure and function, often resulting in tissue damage or death of cells. Free radical damage to protein can result in loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis (Valko et al., 2006). Hence, free radicals are known to be the underlying cause of oxidative stress which has been implicated in the etiology of various diseases such as cancer, diabetes, cardiovascular diseases, ageing and metabolic syndrome (Hosseinimehr, 2007; Raghuveer and Tandon, 2009).

ROS include superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide and singlet oxygen (Asada, 2006). These radicals can be scavenged by the protective role of natural and synthetic antioxidant agents. Several synthetic antioxidants such as Rutin and Butylhydroxytoluene (BHT) are commercially available, but they have been reported to be toxic (Madhavi and Salunkhe, 1995). In recent years, however, there has been a worldwide trend towards the use and ingestion of natural antioxidants present in different parts of plants due to their phytochemical constituents and their consideration to be safer than synthetic antioxidants (Mathew and Abraham, 2006). Dietary and other components of plants form major sources of antioxidants (Devasagayam et al., 2004). Most phytochemicals possess

antioxidant activities and can protect the cells against damage induced by free radicals acting at various levels (Basile et al., 2005).

The realization of roles played by antioxidants in the management of some diseases has led to the inclusion of antioxidant tests in many pharmacological screenings of plant extracts and isolated compounds. In the present study we quantitatively determined phytochemical constituents and evaluated the free radical scavenging and antioxidant activities of *Gunnera perpensa* leaf and rhizome extracted in different solvents. The activities of the two plant parts were compared in order to determine their differences and similarities for the possibility of plant part substitution in conserving this highly valued, but threatened plant species.

7.2 Materials and methods

7.2.1 Phytochemical analysis

7.2.1.1 Determination of total phenolic contents

Total phenol contents in aqueous, acetone and methanol extracts of *G. perpensa* leaf and rhizome were determined with Folin-Ciocalteu reagent following the modified method described by Wolfe *et al*. (2003). A volume of 0.5 ml of the extract (1 mg/ml), was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent and 2ml of Na_2CO_3 (75% w/v). The resulting mixture was vortexed for 15 sec and incubated at 40°C for 30 min for colour development. The absorbance of the samples were read at 765 nm using Hewlett Packard, UV/visible light spectrophotometer. Total phenolic content was expressed as mg/g tannic acid equivalent (TE) using the expression from the calibration curve: $Y = 0.1216x$, $R^2 = 0.9365$, where x was the absorbance and Y was the tannic acid equivalent in mg/g. The experiment was conducted in triplicate and the results were expressed as mean ±standard deviation.

7.2.1.2 Determination of total flavonoids

Total flavonoid was determined using the method described by Ordonez *et al.* (2006) based on the formation of a complex flavonoid-aluminium. A volume of 0.5 ml of 2% $AlCl_3$ prepared in ethanol was mixed with 0.5 ml of the various solvent extracts (1 mg/ml). The resultant mixture was incubated for 1 h at room temperature for yellow colour development which indicated the presence of flavonoids. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. Total flavonoid content was calculated as quercetin equivalent (mg/g) using the equation obtained from the curve, $Y = 0.255x$, $R^2 = 0.9812$, where x is the absorbance and Y is the quercetin equivalent (mg/g).

7.2.1.3 Determination of total flavonols

Total flavonols content was determined using the method described by Kumaran and Karunakaran (2007). Two millilitre of the plant sample (1 mg/ml) was mixed with 2 ml of $AlCl_3$ prepared in ethanol and 3 ml of (50 g/l) sodium acetate solution. The mixture was incubated at 20^oC for 2.5 h after which the absorption was measured at 440 nm. Total flavonol content was calculated as quercetin (mg/g) using the following equation based on the calibration curve, $Y = 0.0255x$, $R^2 = 0.9812$, where x is the absorbance and Y is the quercetin equivalent (mg/g).

7.2.1.4 Determination of proanthocyanidins

Total proanthocyanidins was determined using the procedure reported by Sun *et al.* (1998). A mixture of 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid was added to 0.5 ml of 1 mg/ml of each extract solution and vortexed. The resulting mixture was kept undisturbed for 15 min at room temperature followed by measurement of the absorbance at

500 nm. Total proanthocyanidin content was expressed as catechin (mg/g) using the equation from the curve, Y = 0.5825x, R^2 = 0.9277, where x is the absorbance and Y is the catechin equivalent (mg/g).

7.2.1.5 Determination of tannins

Tannin content of the samples was determined according to the AOAC method (2002) with some modifications. To 0.20 g of the sample was added 20 ml of 50% methanol. The mixture was thoroughly shaken and placed in a water bath at 80°C for 1 h to ensure a uniform mixture. 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% aq. Na_2CO_3 was also added and thoroughly mixed together. 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 ranging from 0 to10 ppm. The absorbance of the tannic acid standard solutions as well as the sample was measured after colour development at 760 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent using the calibration curve, Y = 0.0038x + 0.0432, R^2 = 0.9627 where x is the absorbance and Y is tannic acid equivalent.

7.2.1.6 Determination of saponins

The determination of saponins was done following the method described by Obadoni and Ochuko (2001). Five grams of fine powder (plant sample) was added in 50 ml of 20% (v/v) ethanol prepared in distilled water and the mixture was heated over water bath at 55°C for 4 h with continuous stirring. The mixture was then filtered and the residue collected after filtration was re-extracted with another 50 ml of 20% ethanol. The combined extracts were

evaporated to about 20 ml in water bath at 100°C. The concentrated solution obtained was shaken vigorously with 10 ml of diethyl ether and extracted using a separating funnel. The ether layer was discarded, while the aqueous layer was collected for purification process and repeated. Twenty millilitre of but-1-ol was added to the filtrate and then washed with 10 ml of 5% w/v aqueous sodium chloride. The whole mixture was evaporated on a hot water bath and oven dried at 40°C to a constant weight. The saponin content of the samples were calculated as percentage using the formula:

$$\%\ \text{Saponins} = \frac{\text{Weight of final filtrate}}{\text{Weight of samples}} \times 100$$

7.2.1.7 Determination of alkaloids

Alkaloids were quantitatively determined according to the method of Harborne (2005). One hundred millilitres of 10% acetic acid in ethanol was added to 2.5 g powdered plant sample, covered and allowed to stand for 4 h. The filtrate was then concentrated on a water bath to 1/4 of its original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was completed and the whole solution was allowed to settle. The collected precipitates were washed with diluted ammonium hydroxide before being filtered. The residue was dried and weighed. The alkaloid content was determined using this formula:

$$\%\ \text{Alkaloid} = \frac{\text{Final weight of sample}}{\text{Initial weight}} \times 100$$

7.2.2 Antioxidant Assays

7.2.2.1 Scavenging activity of 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical

The method described by Shen *et al*. (2010) was used for the determination of scavenging activity of DPPH radical in the extract solution. One millilitre of 0.135 mM DPPH prepared in methanol was mixed with 1 ml of each of the extracts ranging from 0.2 ï 1.0 mg/ml concentration of the plant extracts and standard controls (BHT and Vitamin C) separately. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract on DPPH was calculated using the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard. IC_{50} values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals.

7.2.2.2 Scavenging activity of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid (ABTS)

The scavenging activity of plant extract against ABTS radical was determined following the method described by Adedapo et al. (2008). Two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal volumes were allowed to react in the dark for 12 h at room temperature. The resultant $ABTS^+$ solution was diluted by mixing 1 ml of freshly prepared $ABTS^+$ solution with 60 ml methanol to obtain an absorbance of (0.706 ± 0.001) units at 734 nm after 7 min using a spectrophotometer. BHT and Vitamin C were used as control. The percentage inhibition of $ABTS^+$ by the plant extracts was calculated using the equation:

$$ABTS^{+}\ \text{Scavenging activity} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control is the absorbance of $ABTS^{+}$ radical + methanol; Abs sample is the absorbance of $ABTS^{+}$ radical + sample extract or standard.

7.2.2.3 Nitric oxide scavenging activity

The scavenging radical of nitric oxide was based on the procedure reported by Ebrahimzadeh et al. (2010). A volume of 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of each plant extract or BHT or Vitamin C at various concentrations (0.2 - 1.0 mg/ml). The mixture was incubated at 25°C for 2.5 h. An aliquot of 0.5 ml of the solution was withdrawn and mixed with 0.5 ml of Griess reagents [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The resultant mixture was incubated at room temperature for 30 min, after which absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated using the equation:

$$\%\ \text{Inhibition of NO} = \frac{A_0 - A_1}{A_0} \times 100$$

Where NO is nitric oxide, A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

7.2.2.4 Determination of reducing power of *Gunnera perpensa* extracts

The reducing power of the leaf and rhizome extracts was evaluated as described by Aiyegoro and Okoh (2010). The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of the various extracts and standards (0.2-1.0

mg/ml) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 ml of TCA (10% w/v), and centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was measured at 700 nm against blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

7.2.2.5 Statistical analysis

Where applicable, the data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncanôs Multiple Range test using the Minitab program (version 12 for windows). Values were considered significant at $P < 0.05$.

7.3 Results

7.3.1 Phytochemical evaluation

Phytochemical analysis conducted on the leaf and rhizome extracts of *G. perpensa* revealed presence of phenols, flavonoids, flavonols, proanthocyanidins, tannins, alkaloids and saponins. Due to the vast differences in the nature of the phytochemical constituents found in a plant, there is no particular solvent that is known to extract all the compounds on its own from the plant (Ordonez *et al*., 2006). Therefore in this study, aqueous, methanol and acetone solvents were used for extraction. Quantification of phytochemical compounds obtained from the leaf and the rhizome extracts varied greatly among the three solvents used, as shown in Table 7.1. This is an indication that solvents have different extracting capacities for phytochemical compounds present in a plant. The results also revealed variations in the phytochemical constituents within the different plant parts.

Table 7.1: Phytochemical analysis of different solvent extracts of the leaf and rhizome of *Gunnera perpensa*

Phytochemical constituents (mg/g)	Aqueous Leaf	Aqueous Rhizome	Acetone Leaf	Acetone Rhizome	Methanol Leaf	Methanol Rhizome
Total Phenols (TE/g)	234.81±0.24^{a}	235.27±0.31^{a}	235.37±0.31^{a}	234.53±0.19^{a}	235.50±0.13^{a}	235.58±0.14^{a}
Total Flavonoids (QE/g)	414.21±0.64^{a}	301.92±0.25^{c}	313.39±0.00^{c}	434.09±0.63^{a}	432.22±0.00^{a}	376.21±0.52^{b}
Total Flavonols (QE/g)	21.39±0.03^{ab}	16.01±0.08^{b}	31.12±0.02^{a}	27.70±0.01^{ab}	24.63±0.02^{ab}	16.69±0.00^{b}
Proanthocyanidins(CE/g)	46.02±0.58^{c}	64.08±0.58^{bc}	153.78±0.58^{a}	127.38±0.34^{b}	69.12±0.89^{bc}	56.50±0.58^{c}
Tannins (ND)	0.1±0.0^{b}	0.17±0.00^{b}	0.40±0.00^{a}	0.27±0.05^{b}	0.25±0.00^{b}	0.17±0.00^{b}
Alkaloids (%)	47.50±0.0^{b}	18.67±0.11^{c}	25.24±0.02^{bc}	59.38±0.02^{a}	14.37±0.03^{c}	32.08±0.00^{bc}

Data expressed as mean ± SD; Values along a row with the same superscripts are not significantly different ($P < 0.05$). ND: not detected; TE: Tannic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalen

7.3.1.1 Total phenolic content

The total phenolic content of the leaf and rhizome of *G. perpensa* extracted in different solvents are shown in Table 7.1. The concentration of phenol in the different solvent extracts is in the following decreasing order: methanol rhizome > methanol leaf > Acetone leaf > Aqueous rhizome > aqueous leaf > acetone rhizome. In all three solvents, total phenolic contents observed in the leaves was similar to that obtained in the rhizomes. There were no significant differences in the phenolic contents between the two plant parts. High concentration of phenolic compounds was observed in this plant.

7.3.1.2 Total flavonoid content

The flavonoid content of the leaf and the rhizome extracts of *G. perpensa* are presented in Table 7.1. The highest flavonoid content (434.09 mg Quercetin/g) was recorded in acetone rhizome extract, followed by methanol leaf extract (432.22 mg Quercetin/g) while the lowest flavonoid content (301.92 mg Quercetin/g) was recorded in aqueous rhizome extract. In general, the flavonoid content of the leaf was higher than that of the rhizome, although there was no significant difference between the values obtained (Fig. 7.1). In the present study, the flavonoid content was substantially higher compared to other phytochemicals evaluated. Also, methanol had greater extracting capacity for flavonoid as obtained from the present study.

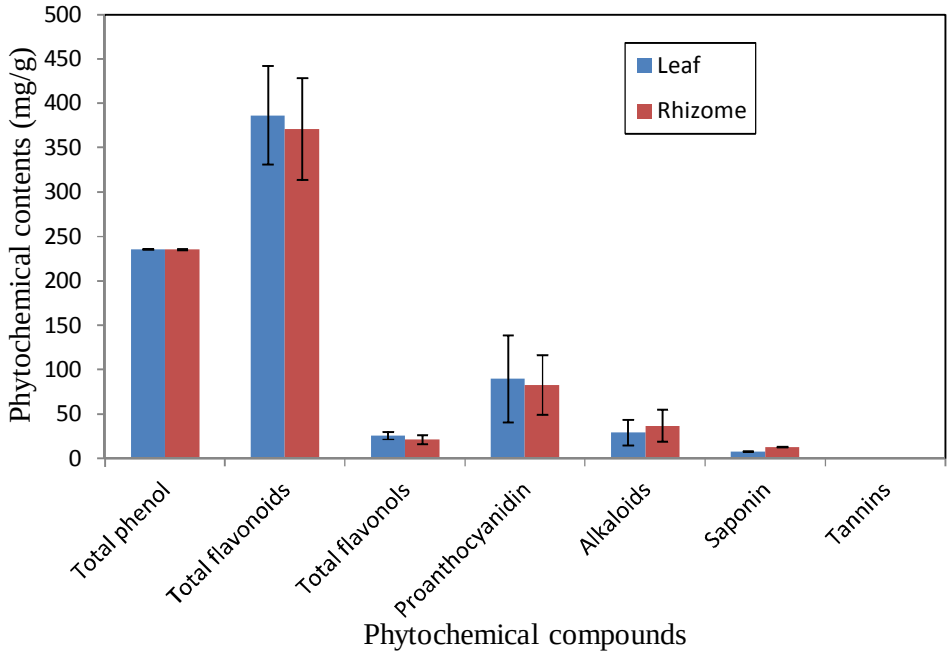

Figure 7.1: Mean phytochemical contents of different parts of *G. perpensa*

7.3.1.3 Total flavonol content

The acetone leaf extract had the highest total flavonol content (31.12 mg/g) followed by acetone rhizome (27.70 mg/g) as shown in Table 7.1. The total flavonol content was lowest (16.01 mg/g) in aqueous rhizome. Overall, the leaf had significantly higher (P<0.05) flavonol content over the rhizome. Acetone exhibited higher extracting capacity for flavonol in this study.

7.3.1.4 Total proanthocyanidin

The highest proanthocyanidin content (153.78 mg/g) was recorded in acetone leaf extract, followed by the acetone rhizome (127.38 mg/g) and they are significantly different (*P<0.05*) from those of the other extracts (Table 7.1). Overall, the proanthocyanidin content of the leaves was higher than that of the rhizomes, although there was no significant difference

between the values (Fig 7.1). In this study, proanthocyanidin content was relatively high in acetone extracts compared to the aqueous and methanol extracts.

7.3.1.5 Tannin content

Tannin content was generally low in all the extracts of *G. perpensa* evaluated, compared to the other phytochemicals. However, tannin content was slightly higher (40%) in acetone leaf extract compared to that of the other extracts (Table 7.1). Overall, the leaf of *G. perpensa* showed higher tannin content than the rhizome (Fig. 7.1).

7.3.1.6 Alkaloid and saponins contents

Quantitative estimation indicated that the saponins content of the rhizome extract (12.8%) was significantly higher ($P<0.05$) than that of the leaf (7.67%). The alkaloid content was highest in acetone rhizome extract (59.38%), followed by aqueous leaf (47.50%), while the lowest (14.37%) was recorded in methanol leaf extract (Table 7.1). There is a significant difference among the values ($p < 0.05$). Overall, the rhizome extract exhibited higher alkaloid content than the leaf (Fig 7.1).

7.3.2 Antioxidant activity

7.3.2.1 DPPH radical scavenging activity

The results showed that acetone and methanol rhizome extracts had good DPPH scavenging activity in a concentration dependent manner, when compared with BHT and vitamin C used as reference drugs (Fig. 7.2). The percentage inhibition of DPPH by the various plant extracts and the standard drugs was recorded in decreasing order as: vitamin C > acetone rhizome > methanol rhizome > BHT > methanol leaf > acetone leaf > aqueous leaf > aqueous rhizome. The concentration required to attain 50% radical scavenging effect (IC_{50}) was determined from the results of a series of concentrations tested. A lower IC_{50} value corresponds to a

larger scavenging activity (Tung *et al*., 2009). The IC_{50} values of 0.27, 0.28, 0.50, 0.67, 0.67 and 0.68 mg/ml were recorded for acetone rhizome, methanol rhizome, methanol leaf, acetone leaf, aqueous leaf and aqueous rhizome extracts respectively. BHT and vitamin C had their activities at 0.31 and 0.15 mg/ml respectively. The scavenging activity of the leaves is lower than that of the rhizomes and the standards. The ability of extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+.

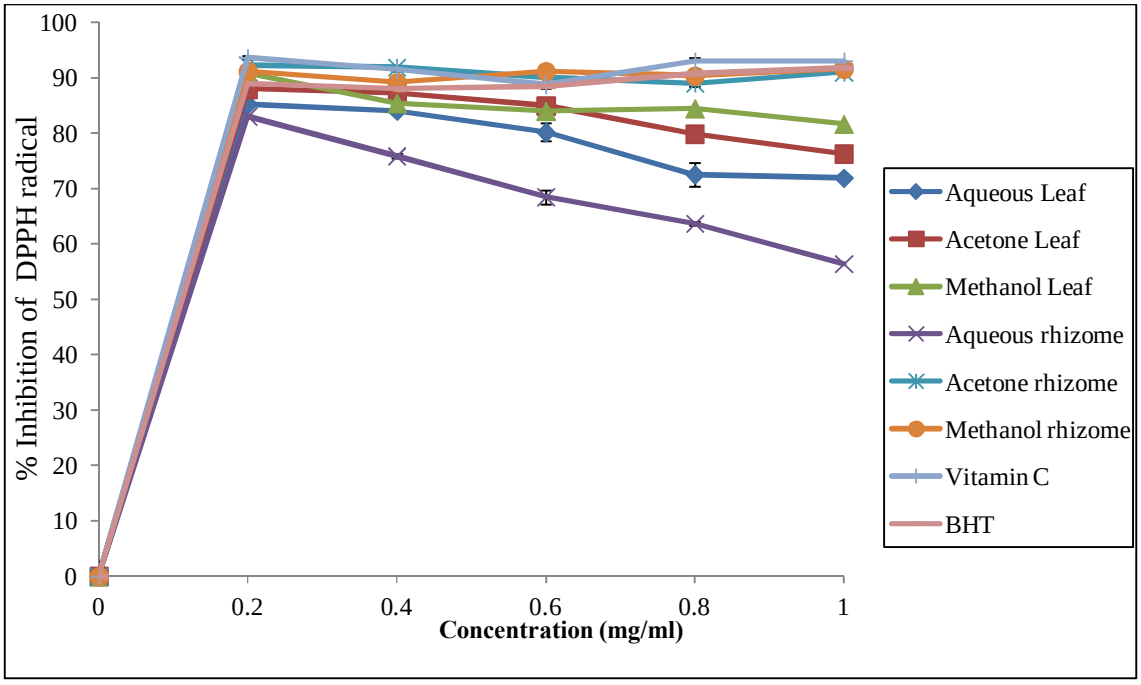

Figure 7.2: DPPH radical scavenging activity of *G. perpensa* leaf and rhizome extracted in different solvents

7.3.2.2 ABTS radical scavenging activity

G. perpensa extracts were effective scavengers of ABTS radicals as shown in Figure 7.3. The scavenging activity in the various extracts was in the order: acetone leaf > vitamin C > BHT > methanol rhizome > acetone rhizome > methanol leaf > aqueous rhizome > aqueous leaf. Acetone leaf extract had the highest inhibitory activity against ABTS, with an IC_{50} value of 0.098 mg/ml. Aqueous leaf, aqueous rhizome, acetone rhizome, methanol leaf, methanol

rhizome, vitamin C and BHT were active with IC_{50} values of 0.59, 0.50, 0.25, 0.30, 0.15, 0.11 and 0.13 mg/ml respectively. With the exception of aqueous leaf and aqueous rhizome extracts, the same trend observed for scavenging activity of the leaf against ABTS was also recorded for the rhizome. There was no significant difference between the antioxidant activities of *G. perpensa* leaves and rhizomes as well as the standards, BHT and vitamin C.

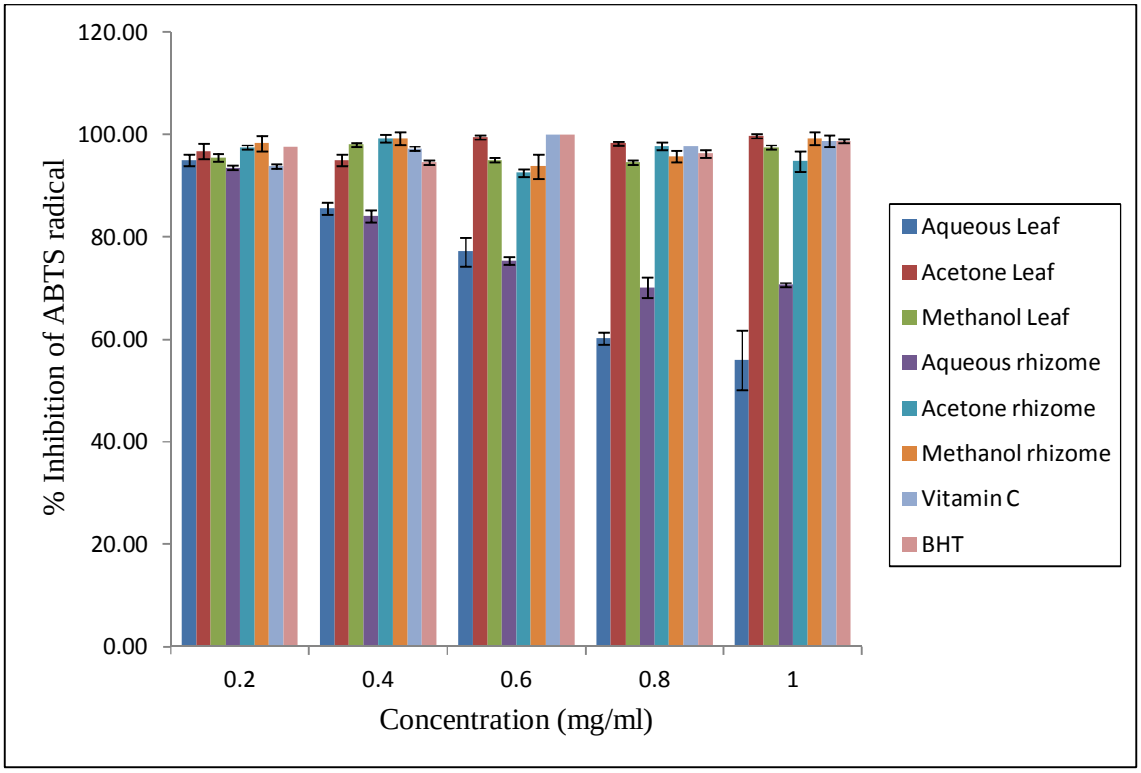

Figure 7.3: ABTS radical scavenging activity of *G. perpensa* leaf and rhizome extracted in different solvents.

7.3.2.3 Nitric Oxide scavenging activity

In this study, Nitric oxide was generated from Sodium nitroprusside in aqueous solution at physiological pH and it reacted with oxygen to form a nitrite and later peroxynitrite when combined with superoxide. Aqueous rhizome, methanol leaf and acetone leaf extracts showed

good nitric oxide scavenging activity which was higher than that of the other extracts and the standard vitamin C at all the tested concentrations (Fig. 7.4). The activity of the aqueous rhizome extract was most pronounced compared to the rest of the extracts.

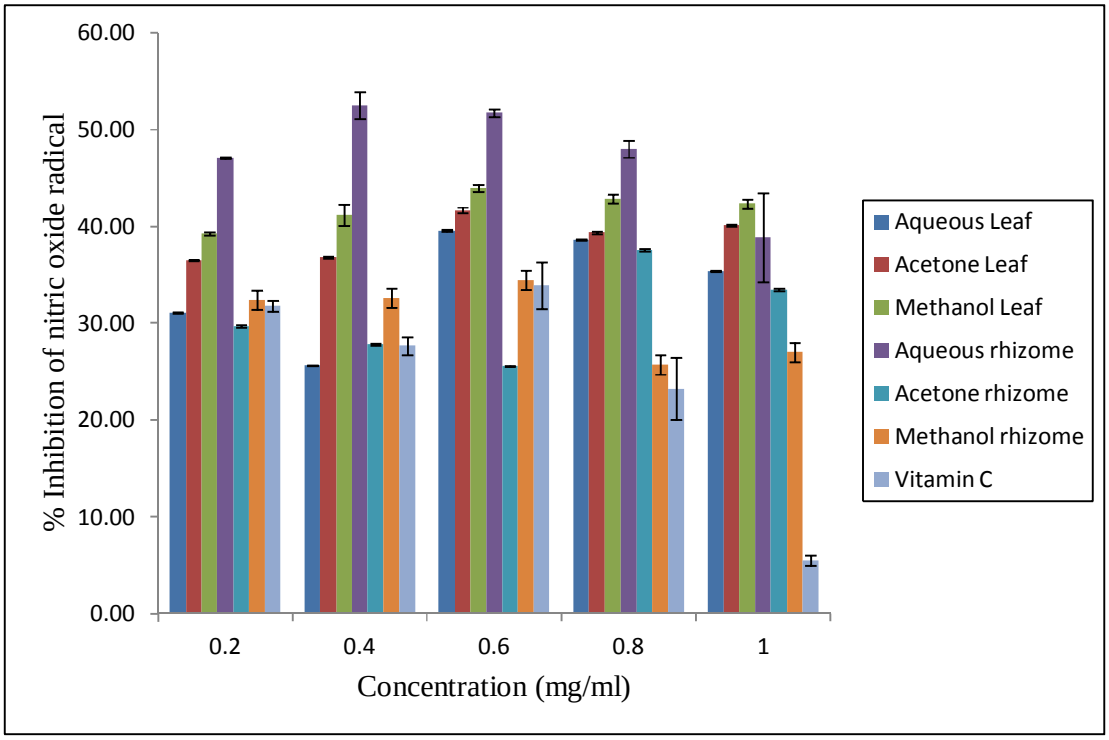

Figure 7.4: Nitric Oxide scavenging activity of *G. perpensa* leaf and root extracted in different solvents

7.3.2.4 Reducing power assay of *Gunnera perpensa* extracts

The dose-response curves for the reducing power of the samples are shown in Figure 7.5. All the extracts evaluated showed an increase in reducing power activity with an increase in extract concentration. The reducing power of the crude extracts and the standards was in the order: aqueous rhizome < BHT < Rutin < methanol rhizome < methanol leaf < acetone rhizome < aqueous leaf < acetone leaf at a concentration of 0.4 mg/ml. With the exception of

aqueous rhizome extract, the reducing abilities of all the other extracts were significantly greater (*P<0.05*) than that of the standards (BHT and Rutin). The reducing power activity of the leaf of *G. perpensa* was slightly higher than that of the rhizome extracts. indicating a potential plant part substitution of the leaves for the rhizome. Plant extracts with reducing properties have been shown to exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom (Erukainure *et al.*, 2011).

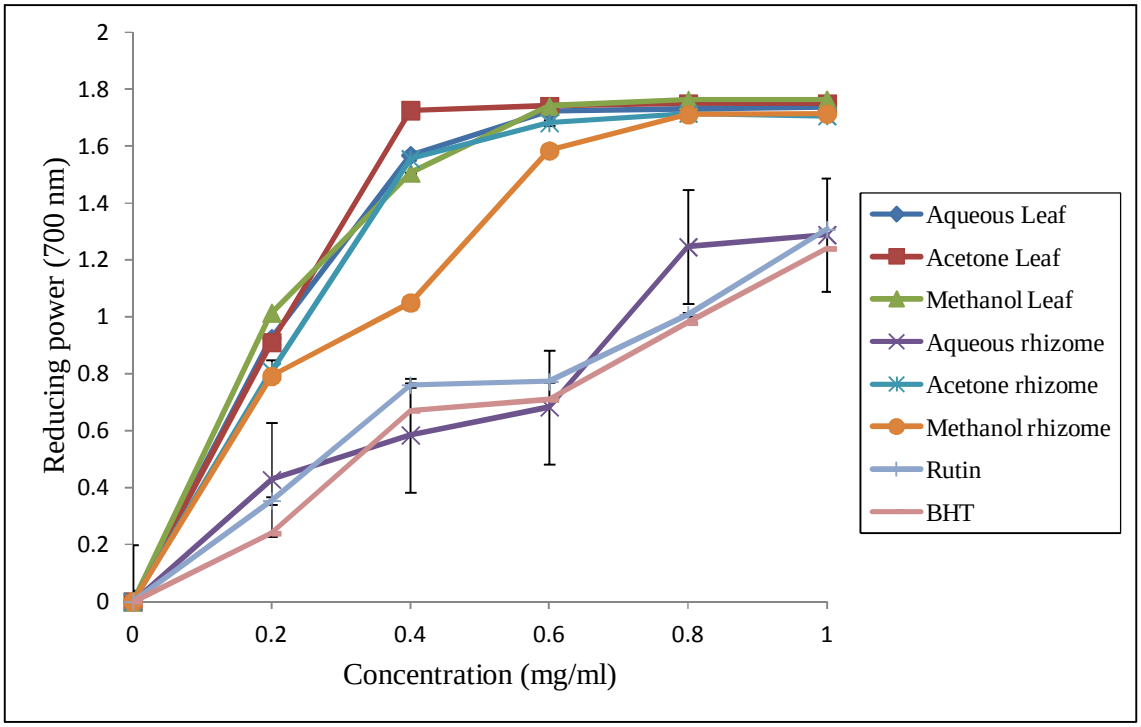

Figure 7.5: Reducing power of *G. perpensa* leaf and rhizome extracts

7.4 Discussion

7.4.1 Phytochemical analysis

Phenolic compounds have been demonstrated to exhibit strong antioxidant activity. This activity is believed to be mainly due to their redox properties which play an important role in

adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Adedapo *et al*., 2008). The results from this study strongly suggest that phenolics are important components of *G. perpensa*, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

Flavonoids are important plant components with significant antioxidant activities and a wide range of biological activities including antibacterial, anti-inflammatory, analgesic and anti-allergic properties (Fergusion, 2001). Many other researchers have reported the antioxidant, anti-inflammatory and antimicrobial activities of flavonoids or flavonoid-rich extracts (Havsteen, 2002; Tunalier *et al*., 2007). In the present study, the flavonoid content was substantially higher compared to other phenolic groups evaluated. It is therefore likely that the pharmacological activities in *G. perpensa* are largely due to their flavonoid content. Flavonols contain quercetin, a particularly potent antioxidant reported to have all the right structural features for free radical scavenging activity (Dwivedi and Flora 2011).

Proanthocyanidins have been shown to help protect the body from tissue damage, cancer, and to improve blood circulation by strengthening the capillaries, arteries and veins (Majo *et al*., 2008; Owolabi *et al*., 2010). Their high antioxidant activity has also been demonstrated (Cos *et al*., 2004). The concentration of this compound in *G. perpensa* could have contributed to the significant antioxidant potency of this plant and may serve as a potential source of bioactive agents in the treatment of cancer and other degenerative diseases (Loots *et al*., 2007).

Tannins have been reported to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention (Li *et al*., 2003). An alkaloid is one of the

secondary metabolites in plants with a potential to protect the cells against foreign organisms due to its toxic nature. Medicinal properties of alkaloids are quite diverse and include local anesthetic properties, anti-malarial activity, analgesic properties, respiratory stimulant and reducing uterine hemorrhage after childbirth (Alkaloid, 2014). The appreciable amount of alkaloids in *G. perpensa* extracts could be contributory to the medicinal values of this plant in management of various diseases. Sodipo *et al.* (2000) demonstrated that saponins are active antifungal agents. Saponins in *G. perpensa* could have been responsible for the antifungal activities exhibited by this plant. Saponins in medicinal plants are responsible for most biological effects related to cell growth and division in humans and have inhibitory effect on inflammation (Liu and Henkel, 2002).

7.4.2 Antioxidant activity

The relatively high level of flavonoids and phenols in *G. perpensa* might account for the strong activity observed in the DPPH radical scavenging assay. Therefore, the data obtained from this study justified the ethnomedicinal use of this plant in the treatment of pathological disease emanating from oxidative stress.

There was no significant difference between the scavenging activities of *G. perpensa* extracts and that of the standard, BHT. BHT is one of the synthetic antioxidants mostly used as a food preservative (Hassas-Roudsari *et al*., 2009). The toxicity of these synthetic antioxidants has however raised concerns about their health safety, resulting in the increased search for naturally occurring antioxidants useful in food and cosmetic industries and as nutraceuticals (Orhan *et al*., 2009). The findings from this study suggest that the leaves and roots of *G. perpensa* possibly contain antioxidant agents with activity equivalent to that of BHT, which

can potentially be exploited as alternatives in the food and cosmetic industries. The purification of the antioxidant agents present in this plant material could perhaps improve their antioxidant capacity. The use of the leaves of *G. perpensa* is more sustainable and can potentially substitute for the rhizomes, owing to their equivalent antioxidant activity in this assay.

Nitric oxide is an important cellular signaling molecule needed in low amounts to protect some vital organs in the human body, especially the liver and heart, from ischemic damage and blood pressure (Shami *et al*., 1995). However, the over production of this molecule has been implicated in the pathogenesis of various physiological and pathological disorders such as ulcerative colitis and arthritis (Mondal, 2005; Hazra *et al*., 2008). The scavenging activity of *G. perpensa* extracts against nitric oxide formation was comparable to the standard vitamin C used in this study. This observation gives an indication of strong antioxidant potential of the extracts. The observed results also suggest the ability of *G. perpensa* to minimize oxidative damage to some vital tissues in the body.

The reducing power activity of the leaf of *G. perpensa* was slightly higher than that of the rhizome extracts indicating a potential for plant part substitution of the leaves for the rhizome. Plant extracts with reducing properties have been shown to exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom (Erukainure *et al*., 2011).

7.5 Conclusion

The results from this study demonstrate the therapeutic potential of *G. perpensa* evaluated. Extracts from the leaf and rhizome of *G. perpensa* demonstrated different levels of phytochemical constituents. The results clearly indicate that the extracts from this plant possess antioxidant properties and could serve as free radical inhibitors or scavengers. The phytochemical evaluation of the studied species indicated that the observed antioxidant activities of the extracts might largely be due to their flavonoid and phenol contents, with a contributing effect from their alkaloids and saponins. In some of the phytochemical evaluation and the antioxidant assays, the leaves of *G. perpensa* demonstrated higher constituents and activities than the rhizomes suggesting their potential in plant part substitution. The concept of substituting plant parts for sustainable exploitation appeared to be dependent on the plant species and/or biological activity of interest. The harvesting of leaves as a conservation strategy would certainly be more sustainable than the destructive use of the roots of this threatened plant species.

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CHAPTER 8

MICROPROPAGATION OF *Gunnera perpensa* L., A THREATENED MEDICINAL PLANT IN THE EASTERN CAPE, SOUTH AFRICA

CHAPTER EIGHT

Micropropagation of *Gunnera Perpensa* L., A Threatened Medicinal Plant in the Eastern Cape, South Africa

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8.1 Introduction

Plant micropropagation, an *in vitro* propagation technique, offers highly efficient tools for conservation and large-scale propagation of medicinally important and endangered plants (Afolayan and Adebola, 2004). This has obvious implications for the mass propagation of exploited species and hence for relief of harvesting pressures on wild populations of these species. Through micropropagation, plant material can also be produced throughout the year without any seasonal limitation and controlling the environmental conditions of plant growth (Sidhu, 2010).

The major challenges in the application of plant tissue culture, particularly micropropagation of medicinal plants, include the recalcitrant nature of plant tissues often related to seasonal dormancy, contamination of tissue cultures by microorganisms, poor rooting frequency, morphological aberrations, phenolic exudation coupled with media and explants browning and high cost of production (Krishna and Singh, 2007; Prakash, 2009). The browning experienced in plant tissue culture is caused by oxidation of phenolic compounds that are released into the medium upon excision of explants (Moyo and Van Staden, 2013). This phenolic compound hinders the regenerative ability of plant cells *in vitro* (Raghuvanshi and Srivastava, 1995). Thus, the use of phenolic attractive substances in regeneration media is reportedly suitable (Ramsay and Gratton, 2000; Abrie and Van Staden, 2001). Explant contamination occurs due to several plant and environmental related factors such as plant species, age of the plant, explant source and prevailing weather condition. A successful *in vitro* culture protocol starts with effective explant sterilization (George, 1993).

The rhizomes of *Gunnera perpensa* are greatly valued in traditional medicine for the treatment of various diseases such as rheumatic pains, cold, wound-dressing, stomach ailments and menstrual pains (van Wyk and Gericke, 2000; Ngwenya *et al.*, 2003). A growing demand for *G. perpensa* has caused a serious reduction in the wild populations as a consequence of over-harvesting. *G. perpensa* can be grown from seed as well as from rhizomes. The propagation from seed is often affected by low seed viability and very poor germination. Thus, this may not be a very efficient means for the conservation of this medicinally important plant. Propagation by rhizome was successful and appears to be the best option. However, considering the high demand of *G. perpensa* for medicinal purposes, conventional methods of propagation alone is insufficient to meet the growing demand. There is need; therefore, to develop an efficient *in vitro* propagation method that can potentially provide a conservation measure for this plant. The need for an effective propagation method for this species is further heightened by it being red-listed and ranked as declining in its national conservation status (Raimondo *et al.*, 2009).

The study was aimed at developing a micropropagation protocol, which can potentially provide a conservation measure for this endangered plant species. Suitable sterilization regime for the explants and the influence of phenolic adsorptive substances in controlling explants and media browning were also investigated.

8.2 Materials and methods

8.2.1 Explant source

The stock plants for this study were collected from their natural population in the Eastern Cape Province, South Africa. Explants were taken from the mother plant collected from the wild, but subsequently, they were taken from the plants maintained for about four months in

the glasshouse. The rhizomes were separated from the leaves and roots were cut out. Rhizome buds about 1 to 2 cm long were selected as the initial explants. Healthy rhizomes, petioles and leaves were taken separately for decontamination.

8.2.2 Surface sterilization and culture condition

For explant decontamination, various surface sterilants such as 70% v/v ethanol, sodium hypochlorite and mercuric chloride were used (Pierik, 1987). The rhizomes were washed under running tap water for 1 hr, surface-sterilized in seven different treatments by immersion in 70% alcohol for 2 min, agitated in benomyl for 15 and 30 min and then washed with sterile distilled water and transferred to laminar air flow cabinet. In the laminar flow chamber, the rhizome cuttings were again agitated in 3% sodium hypochlorite solution for 10 min, 20 min and 30 min. The hypochlorite solution contained 2 drops of Tween-20 per 100 ml solution. The rhizome explants were then washed severally with sterile distilled water. In another batch of rhizome explants, the treatments involved soaking the plant materials in 70% ethanol for 2 min and in benomyl followed by agitation in 0.1% and 0.3% (w/v) mercuric chloride ($HgCl_2$) respectively for 10 min and 20 min respectively.

The leaf and petiole explants were washed under running tap water for 30 min. Surface sterilization entailed swirling the explants in 70% ethanol (1 min), followed by treatment with 2g/l benomyl for 15 and 30 min respectively and gentle agitation in 1% Sodium hypochlorite solution (NaOCl) for 15 and 30 min. All treatments included a few drops of Tween 20. The explants were then washed three more times with sterile distilled water, before they were cultured on a Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The final explants of the trimmed rhizome buds (5 mm), the leaf segments (7 mm×10 mm) and the

petiole (5 mm) length were inoculated on MS medium supplemented with 30 g/L sucrose and 7 g/l agar. The pH of the medium was adjusted to 5.7 before being autoclaved at 121°C under a pressure of 1.5 kg/cm^{2} for 20 min. Most cultures were grown in culture tubes 145 mm high and with a diameter of 16 mm, while some batches were grown in jam bottles (75 mm× 45 mm). The cultures were placed in a growth room under 16 h light/8 h dark photoperiod (cool, white fluorescent light of 40µmol $m^{-2}S^{-1}$ and incubated at 25 ± 2°C. Each treatment had at least 30 observable units. Plant preservative mixture (PPM) at 1 ml/l (v/v) was also incorporated in some of the media to check the effect on decontamination.

8.2.3 Effect of supplements in controlling explant and media browning

Several supplements to MS medium were tested to obtain a successful culture medium. Adsorptive materials such as polyvinylpyrrolidone (PVP) and activated charcoal (AC) were examined for their ability in controlling browning of explants of *G. perpensa* and the resultant effect on media. PVP were used at concentrations of 1 g/l and 5 g/l, while activated charcoal was used at 1 g/l, 2 g/l and 3 g/l concentrations. Ascorbic acid, an antioxidant compound was also examined for its ability to control browning of the explants. Ascorbic acid was filter-sterilized before incorporation into the autoclaved medium because it was heat labile. A concentration of 50 mg/l ascorbic acid was incorporated into the medium. After surface sterilization, the explants were agitated in sterilized distilled water, 150 mg/l ascorbic acid and 7 g/l PVP for 1 hr before inoculation into the media. The degree of browning was determined by physical examination of the culture tubes.

8.2.4 Effect of sucrose concentration and growth hormones

The MS medium used in this study was supplemented with different growth regulators at varying concentrations. Concentrations of 0.5, 1, 2, 3 and 5 mg/l of BAP combined with 0.5 and 1 mg/l NAA; concentrations of BAP alone; Kinetin at concentrations of 0.1, 0.2, 0.5, 1 and 2 mg/l; TDZ at concentrations of 0.2, 0.5 and 1 mg/l and 2, 4-D at concentrations of 1, 2, 2.5, 3, 3.5 and 4 mg/l were used. MS medium without any plant growth regulator was included as a control. Sucrose was added to the medium at concentrations of 10 g/l and 30 g/l respectively.

8.3 Results

8.3.1 Surface sterilization of leaf and petiole explants

The different sterilization regimes for decontamination of the leaf and petiole explants and the effect of sterilization method on percentage decontamination of the leaf and Petiole explants after 2 and 4 weeks in culture are shown in Tables 8.1 and 8.2 respectively. Best sterility results for the leaf explant (100%) was obtained with Treatment 3, which involved the exposure of the leaf segments in 70% ethanol for 1 min; agitation in 2 mg/l benomyl for 30 min and 1% sodium hypochlorite for another 30 min (Table 8. 2). After four weeks in culture, the decontaminated leaf explants still remained 100% contamination free. This was followed by establishment of 100% of the leaf explants after 2 weeks in culture and 97% after 4 weeks in culture when exposed to treatment 1 and 2 (Tables 8.1 & 8.2). The least number of contaminant free cultures (43%) was obtained when 1% sodium hypochlorite only, was used for 15 minutes.

Table 8.1: Sterilants, concentration and exposure time for sterilization of *G. perpensa* leaf and petiole explants

Treatment	Sterilants	Concentrations	Exposure time (min)
T1	Ethanol	70%	1
	Benomyl	2 mg/l	15
	NaOCl	1%	15
T2	Ethanol	70%	1
	Benomyl	2 mg/l	30
	NaOCl	1%	15
T3	Ethanol	70%	1
	Benomyl	2 mg/l	30
	NaOCl	1%	30
T4	NaOCl only	1%	30
T5	NaOCl only	1%	15

Table 8.2: Effect of sterilization method on percentage decontamination of the leaf and Petiole explants after 2 and 4 weeks in culture

Treatment	Leaf segment decontamination (%) after 2 wks	Leaf segment decontamination (%) after 4 wks	Petiole explant decontamination (%) after 2 wks	Petiole explant decontamination (%) after 4 wks
T1	100	97	70	60
T2	100	97	70	70
T3	100	100	83	80
T4	67	67	43	33
T5	43	43	40	27

For the petiole explant, the highest percentage (83%) of contaminant free cultures after 2 weeks and 80% after 4 weeks were established when the explants were exposed to 70% ethanol for 1 min; benomyl for 30 min and 1% sodium hypochlorite for 30 min (T3). The least number of contaminant free cultures after 2 and 4 weeks respectively was obtained at T5. Generally, it was observed that exposure of the leaf and petiole explants to sodium hypochlorite for 30 min produced higher number of contaminant free explants than when exposed for shorter duration of 15 min. The result indicated that time of exposure to sterilants was significant on percentage aseptic plants. Furthermore, higher percentage of contaminant free cultures was obtained with the sterilization treatment that has the fungicide, benomyl, incorporated in it than those treated with sodium hypochlorite alone. This shows that benomyl played a very important role in eliminating contaminants, which were mostly fungi, from *Gunnera perpensa* explants in culture establishment.

8.3.2 Surface sterilization of rhizome explants

The effect of surface sterilants on rhizome explants is summarized in Table 8.3. The result showed that after 2 and 4 weeks of culture, highest contamination free culture (100%) was achieved in the explants treated with T11. This was followed by 90% contamination free cultures after two weeks, in explants treated with T4. The least number of contaminant free cultures (47% and 33%) for 2 and 4 weeks of culture respectively, were obtained in treatment T1. In the range of explants sterilized with sodium hypochlorite, the result showed that after 4 weeks of culture the highest percentage (67%) of contaminant free explants were established when exposed to benomyl for a duration of 30 min and sodium hypochlorite for 30 min. The percentage of contaminant free culture reduced to 57% when the duration of exposure to benomyl was reduced to 15 min. A higher percentage of contaminant free explants were also

obtained in cultures treated with benomyl and sodium hypochlorite together, compared to using sodium hypochlorite alone (Table 8.3). Benomyl and the duration of exposure of explants to it played a vital role in explants decontamination.

Rhizome explants sterilized with sodium hypochlorite had lower percentage of contaminant free cultures than the mercuric chloride treatment. Sodium hypochlorite, even accentuated browning of the explants more than mercuric chloride. However, the decontaminated explants from sodium hypochlorite treatment remained healthy. Although the application of mercuric chloride at different concentrations and time duration for surface sterilization of rhizome explants were very effective in decontamination, a high proportion of the explants got dried up and subsequently died. The incorporation of PPM in culture media also reduced contamination load compared to medium without the supplement of PPM.

Table 8.3: Effects of sterilants on decontamination percentage of *G. perpensa* rhizome

Treatment	Sterilants	Concentration	Exposure time (min)	Decontamination after 2 weeks (%)	Decontamination after 4 weeks (%)
T1	Ethanol	70%	2	47	33
	Benomyl	2 mg/l	15		
	NaOCl	3%	10		
T2	Ethanol	70%	2	73	43
	Benomyl	2 mg/l	30		
	NaOCl	3%	10		
T3	Ethanol	70%	2	67	63
	Benomyl	2 mg/l	15		
	NaOCl	3%	20		
T4	Ethanol	70%	2	90	63
	Benomyl	2 mg/l	30		
	NaOCl	3%	20		
T5	Ethanol	70%	2	73	57
	Benomyl	2 mg/l	15		
	NaOCl	3%	30		
T6	Ethanol	70%	2	70	67
	Benomyl	2 mg/l	30		
	NaOCl	3%	30		
T7	NaOCl	3%	30	60	50
T8	Ethanol	70%	2	71	71
	Benomyl	2 mg/l	30		
	$HgCl_2$	0.1%	10		
T9	Ethanol	70%	2	86	71
	Benomyl	2 mg/l	30		
	$HgCl_2$	0.1%	20		
T10	Ethanol	70%	2	86	71
	Benomyl	2 mg/l	30		
	$HgCl_2$	0.3%	10		
T11	Ethanol	70%	2	100	100
	Benomyl	2 mg/l	30		
	$HgCl_2$	0.3%	20		

8.3.3 Effect of supplements to MS medium in controlling browning

G. perpensa explants secrete phenolic substances that lead to blackening or browning of the media and often death of the explant. The petiole explants were lightly blackened when inoculated into the MS media (Fig. 8.1b). The leaf explants retained their green colour for three days after inoculation in the MS media, thereafter they became moderately blackened (Fig. 8.1c). The rhizome buds were prone to extensive browning in plain MS media and high proportion died (Fig. 8.1d).

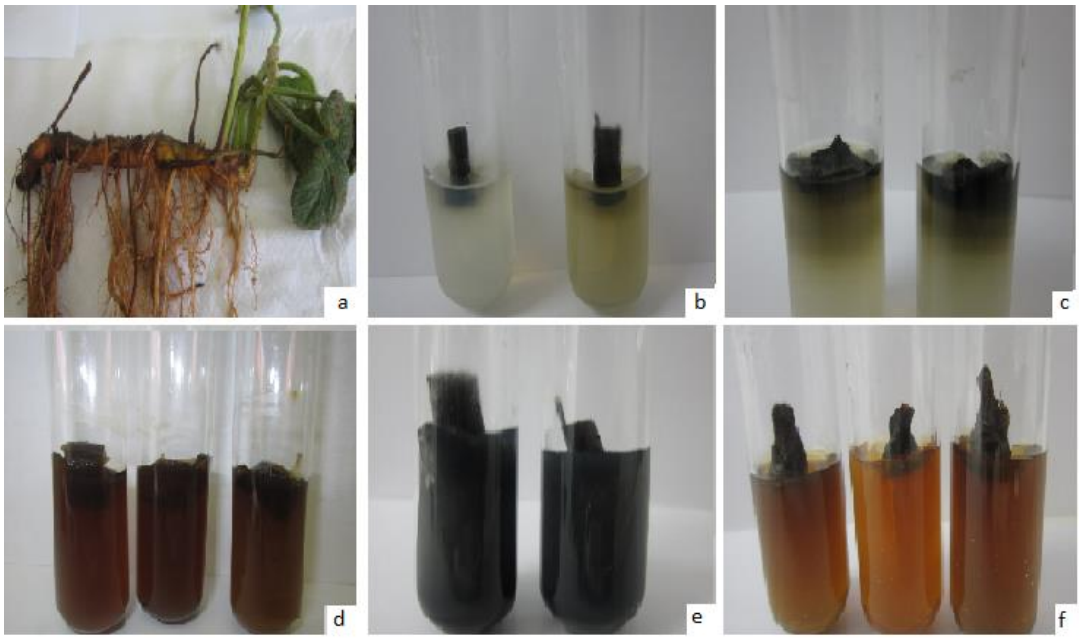

Figure 8.1: *G. perpensa* explants in MS media (a) Plant from the wild (b) Petiole explant in plain MS media (c) Leaf explant in plain MS media (d) Rhizome explant in MS media turned brown (e) Petiole explant in AC media (f) Rhizome explant washed in PVP and inoculated in PVP media.

Several supplements to MS medium were tested to control browning and obtain a successful culture medium. The addition of the anti-oxidant PVP (1 and 5 g/l respectively) to the medium did not visibly reduce the browning in the cultures. The browning increased within

hours of culturing until the medium turned completely brown (Fig. 8.2a). Nevertheless, media containing 1 g/l PVP showed lesser browning than 5 g/l PVP incorporated media (Fig. 8.2b). By adding activated charcoal (AC) to the medium, the build-up of phenolic inhibitors in the medium and explant browning was counteracted (Fig. 8.2c and e). The 2 g/l AC showed sign of shoot formation, but died immediately. The addition of ascorbic acid to the medium was very effective in eliminating browning of the medium. However, the explants washed in a solution of ascorbic acid before inoculation (Fig. 8.2d) showed clearer culture media than those washed in distilled water and PVP (Fig. 8.2f). Sucrose concentration was used at 1% and 3% respectively, but there was no visible change to the browning of the media.

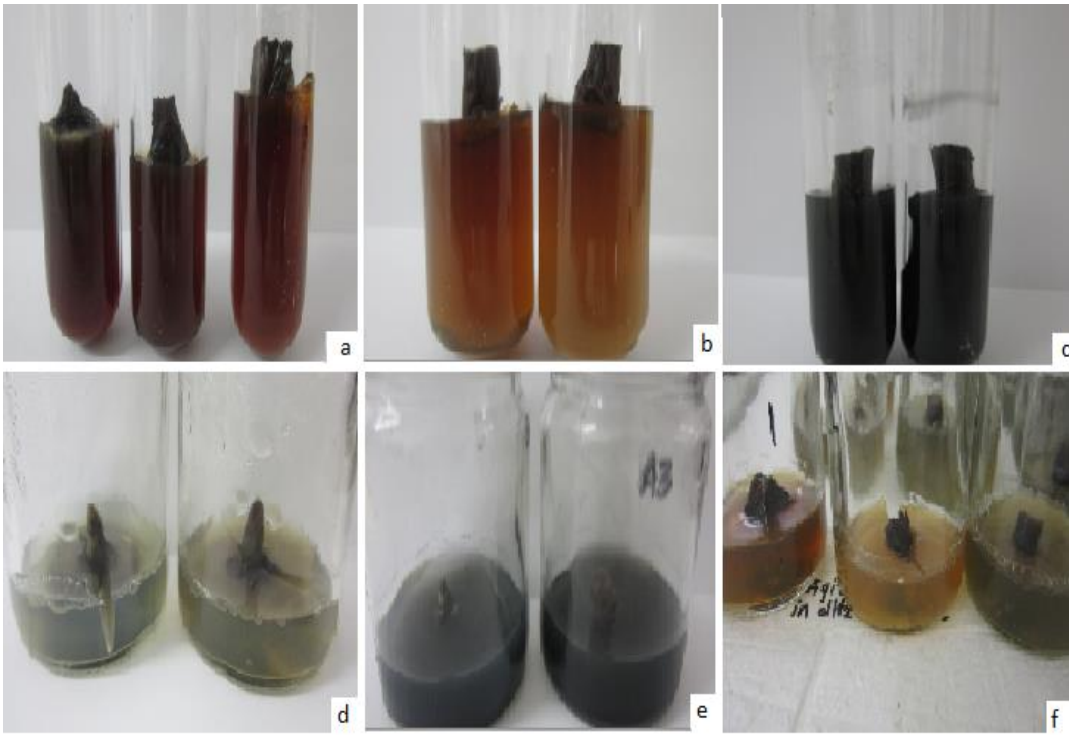

Figure 8.2: *G. perpensa* explants in MS media with supplements (a) Rhizome explant in 5 g/l PVP incorporated media (b) Rhizome explant in 1 g/l PVP incorporated media (c) Rhizome explant in 1 g/l AC (d) Rhizome explant in ascorbic acid incorporated media (e) Rhizome explant in 2 g/l AC (f) Rhizome explant washed in distilled water and inoculated in ascorbic acid containing media

8.3.4 Effect of growth hormones on plant regeneration

Several growth regulators including: BAP alone and in combination with NAA, kinetin alone, Kinetin in combination with NAA, TDZ and 2, 4-D at varying concentrations were used on ascorbic acid incorporated media, as well as on plain MS media for shoot regeneration in all the three explants. The explants were sub-cultured bi-weekly, but no regeneration response was achieved in any of the explants after several trials, except a little sign of shoot from rhizome bud inoculated in activated charcoal media that died immediately.

8.4 Discussion

8.4.1 Explant decontamination

In vitro propagation involves culturing explants under aseptic conditions in which surface sterilization or disinfection of explants is one of the important prerequisites for successful *in vitro* culture. Hence, removing contaminants from the surface of the organ/explant is of prime importance (Hartmann *et al.*, 1997). Explant contamination occur due to several plant and environmental related factors such as the part of plant selected as the explant. Underground organs such as roots, tubers, rhizomes and bulbs of soil grown plants are usually highly contaminated when initiating cultures, but shoots of many plants are less contaminated especially when they are in active growth (Babaei *et al.*, 2013). This was the situation in this study because higher percentage contamination was obtained in rhizome explants of *G. perpensa,* followed by the petiole while the lowest proportion was in leaf explants. When the rhizome buds were sterilized with 1% NaOCl, all the cultures got contaminated unlike the leaf and petiole explants. Higher concentration of NaOCl was needed to decontaminate *G. perpensa* rhizome buds. Hamirah *et al.* (2010) reported that contamination was still a problem after the sterilization of rhizome buds used as explant for the culture of *Zingiber*

montanum, until the incorporation of an antibiotic and biocide PPM into culture medium, which helped to check contamination. High contamination of cultures was also reported when rhizomes or vegetative buds were used as explants for initiation of the culture of *Cornukaempferia aurantiflora*, but the problem was eliminated almost completely by using leaf tissue as explants (Piyaporn, 2011). The contamination of explants of *G. perpensa* was majorly from fungi, moulds and bacteria.

General disinfection procedures have been given by various workers for plant tissues (Srivastava *et al*., 2010). Disinfection requires the use of chemicals that are toxic to microorganisms but non-toxic to plant materials. Tissue culture became possible with the use of convenient and effective disinfectants such as ethanol, sodium hypochlorite, mercuric chloride, calcium hypochlorite and others (Krikorian, 1982). In this study, effect of surface sterilants on reducing contamination rate showed that NaOCl is better sterilant than $HgCl_2$ for the rhizome bud explants. This is because NaOCl did not only reduce contamination, but also produced healthy cultured explants. Although $HgCl_2$ had high level of decontaminated explants, they were toxic to the plant materials. Mercuric chloride is extremely poisonous due to high bleaching action of two chloride atoms and also mercuric ions which combines strongly with protein causing death of organism. The highest proportion of aseptic culture in leaf and petiole explant was obtained using pre-treatment with 1% NaOCl for 30 min, ethanol for 1 min and benomyl for 30 min (T3). In the present investigation, benomyl played a vital role in reducing contamination rate of *G. perpensa* explants. Different authors have reported differential response from rhizomatous crop in obtaining contaminant free cultures using different exposure durations (Habiba *et al.*, 2002; Molla *et al.*, 2004, Titov *et al.*, 2006). In order to find an optimized protocol for sterilization of a specific tissue, three factors have to

be taken into consideration viz., sterilizing chemical, its concentration and the treatment duration (Razdan, 1993). In this study, we recommend for petiole and leaf explants to be swirled in 70% ethanol for 1 min, agitated in 2mg/l benomyl for 30 min and put in 1% sodium hypochlorite (NaOCl) for another 30 min. The explants will be thoroughly rinsed in sterile distilled water before inoculation into the media. The sterilization regime recommended for rhizome explants from the present study is 2 min swirling in 70% ethanol, followed by 30 min agitation in 2 mg/l benomyl solution, and sterilization in 3% NaOCl for 30 min.

8.4.2 Elimination of Browning

Apart from contamination, blackening or browning of excised plant tissues and nutrient media occurs frequently and remains one of the major challenges in plant *in vitro* culture (Krishna and Singh, 2007). The severity of browning has varied according to species, tissue or organ, developmental phase of plant, age of tissue or organ, nutrient medium and other tissue culture variables (Huang *et al.*, 2002). The browning phenomenon is usually imputed to oxidized phenolic compounds by polyphenol oxidase (PPO) that are released into the medium upon excision of explants (Moyo and Van Staden, 2013). The exudation of phenolic compounds from explant wound site hinders the regenerative ability of plant cells and pre-treating with polyvinylpyrrolidone (PVP) and antioxidants like ascorbic acid and citric acid are ways to remove phenols or reduce their accumulation in tissue culture media (Krishna *et al.*, 2008).

The current investigation showed that ascorbic acid and activated charcoal were able to control browning in *G. perpensa* rhizome explants, but addition of PVP did not visibly reduce

browning of the cultures. The effectiveness of ascorbic acid in resolving the lethal browning problem of *G. perpensa* explants suggests that such disorder may be caused by oxidation of phenolic compounds present in the plant organs. Antioxidant assay carried out on *G. perpensa* revealed high phenol content in the plant species. It is conceivable that ascorbic acid may have been absorbed by the plant tissues and prevented the oxidation of phenolic compounds on the target site. The studies of Minas (2007) on 3 types of *Gardenia, Tulip* and *Chrysanthemum* showed that by using of 55.7 mg/l ascorbic acid, none of the cultures of meristem tip showed blackening.

Similar to our findings, control of *in vitro* browning was minimized by the application of activated charcoal in some Anacardiaceae species (Thimmappaiah *et al.*, 2002; Benmahioul *et al.*, 2012). According to Soniya and Sujitha (2006), leaching of polyphenols affected *in vitro* shoot growth in *Aristolochia indica* but addition of AC in the medium coupled with increased sub-culturing frequency reduced the problem considerably. Weatherhead *et al.* (1979) showed that activated charcoal could adsorb inhibitory substances in the culture medium and drastically decrease phenolic oxidation or brown exudate accumulation, and this was later confirmed by Liu (1993). The difýculty in using AC in medium is that in addition to adsorbing unwanted substances, it may adsorb needed hormones (Pan and Van Staden, 1998).

Previous studies indicated that browning could be successfully controlled by adding PVP to the culture medium of *Pistacia vera* (Ananthakrishnan *et al.*, 2002), Chinese aloe (Liao *et al.*, 2004) and *Sclerocarya birrea* (Moyo *et al.*, 2009). In this study, the reverse was the case, as browning increased on PVP containing media.

Carbohydrate is required for the biosynthesis of phenolic compounds and reducing the sucrose concentration to 1% can decrease polyphenols within tissues (Davies, 1972). In the present study, 1% sucrose concentration did not show any visible difference in the medium browning from the 3% concentration.

The presence of the plant growth regulators in media are necessary for shoot and root initiation (Liao *et al.*, 2004). Various growth regulators were employed for the regeneration of *G. perpensa*, but the plant tissues did not respond to *in vitro* culture manipulations. The establishment of primary cultures was difficult, owing to the secretion of the phenolic substances by explant. Nevertheless, the interesting finding in this study was the elimination of the intensive browning of the explants and media by incorporating 50 mg/l ascorbic acid in the culture media and agitating the explants with 150 mg/ l ascorbic acid after sterilization.

8.5 Conclusion

Contamination poses one of the greatest problems in the micropropagation of *G. perpensa*, a problem made even more acute by explant and media browning, especially after the use of sterilizing agents. Although the micropropagation of this plant species had limited success, this study did develop a successful decontamination protocol as well as effective means of eliminating intense browning of the explants and media. This information provides an important starting point for the development of a successful micropropagation protocol for the conservation of *G. perpensa.*

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CHAPTER 9

GENERAL DISCUSSION AND HIGHLIGHTS OF THE STUDY

CHAPTER NINE

General Discussion and Highlights of the Study

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9.1 General discussion

For many years medicinal plants have been used as a primary source of medicines to improve the health of people. It is now well documented that medicinal plants are over-utilised and this could lead to the extinction of many species. *Gunnera perpensa* is one of such medicinal plants under threat of overexploitation. Its harvesting is destructive since the roots are often used. Dold and Cocks (2002) described this plant species as scarce and heavily traded; local extirpations have been noted (Williams *et al.*, 2008). Therefore, there is urgent need for the propagation and conservation of this valuable medicinal plant. The need for an effective propagation method for *G. perpensa* is further heightened by it being red-listed and ranked as declining in its national conservation status (Raimondo *et al.*, 2009). Propagation of medicinal plants is aimed at increasing the supply of the plant materials for use and at the same time conserving them.

The ethnobotanical studies conducted as part of this research revealed that the current practices of collecting medicinal plants almost entirely from the wild coupled with unsustainable harvesting techniques are gradually reducing the populations of highly valuable plant species. As more and more medicinal plants and the associated knowledge are lost, the potential for the continuous supply of medicinal plant resources for the large numbers of population who still rely on them for their everyday health care is not guaranteed. Hence, there is need to develop conservation strategies for the endangered plant species.

Vegetative propagation of *G. perpensa* using varying lengths of the rhizome segment as propagules was successful and should be considered as a key tool in their conservation. The

result demonstrated that for maximum growth and yield, 5 cm rhizome length at a planting depth of 4 or 5 cm is most appropriate.

Seed germination is an important determinant in the distribution and survival of a plant species. Favourable conditions necessary for seed germination of *G. perpensa* were investigated. This was done by subjecting the seeds to different treatments such as temperatures and light conditions. Stimulation of seed germination was attempted using chemical regulators, mechanical scarification, cold and hot water stratification. From the results, it is evident that germination of seeds of this plant would be largely dependent on light and temperature conditions, the optimum temperature being 25°C. These environmental conditions and seed pre-treatment should be taken into consideration in the cultivation of this valuable plant. The germination of *G. perpensa* seeds was irregular and very poor. The low germination is possibly the result of morpho-physiological dormancy; however mechanical scarification and pre-soaking of seeds in water for 3 and 7 days prior to germination improved its germination response. The establishment of *G. perpensa* plant using seeds was difficult. The growth and yield were not as proliferating as those of the rhizome raised plants.

The pharmacological activities and phytochemical properties of *Gunnera perpensa* studied in this research highlight their therapeutic potential. Extracts from two different parts (leaf and rhizome) of this plant species demonstrated different levels of antibacterial, antifungal and antioxidant activities. The pharmacological activities observed in some extracts of *G. perpensa* might possibly explain its heavy exploitation in traditional medicine. In some of the pharmacological assays like the antifungal assay, the leaves and rhizomes of *G. perpensa* exhibited similar activities. In the antibacterial assay and most antioxidant activities, the

leaves demonstrated higher activities than the rhizomes, suggesting their potential in plant part substitution. In general, the leaves showed better pharmacological activities compared to the rhizomes. The harvesting of leaves as a conservation strategy is certainly more sustainable than the destructive use of the roots of this threatened plant species. The results obtained from this study also suggest that the concept of plant part substitution is dependent on the plant species and/or pharmacological activity of interest. The phytochemical evaluation of the studied species indicated that the various activities could possibly be due to their high flavonoid and phenolic content with a contributing effect from their alkaloids and saponins.

Micropropagation is a biotechnological tool that is frequently used for the conservation of threatened plant species. Contamination poses one of the greatest problems in the micropropagation of *G. perpensa*, a problem made even more acute by explant browning, especially after the use of sterilizing agents. Although the micropropagation had limited success in this study, two important factors were achieved. The first point was that an efficient method to eliminate contamination was achieved by pre-treating the leaf and petiole explants in 70% ethanol for 1 min, 2 mg/l benomyl for 30 min and 1% sodium hypochlorite (NaOCl) for 30 min. The sterilization procedure recommended for rhizome explants from the present study is 2 min swirling in 70% ethanol, followed by 30 min agitation in 2 mg/l benomyl solution, then 3% NaOCl for 30 min. The second was that the culture media must be supplemented with either activated charcoal or ascorbic acid in order to prevent browning and to help adsorb phenolics released by the leaf and rhizome explants. Furthermore, the explants need to be rinsed in ascorbic acid before inoculation into the culture media.

9.2 Highlights of this study

- The ethnobotanical survey conducted as part of this study showed that the communityôs perception of the conservation status of *G. perpensa* corresponds with the conservation status as indicated in South Africa Plant Red List.
- The results of this study showed that regenerating *G. perpensa* through rhizome segments is an efficient method for obtaining plant material and reducing pressure from the natural population. This is advantageous because it eliminates some of the difficulties associated with seed germination and seedling survival.
- In vegetative propagation of *G. perpensa,* the optimal rhizome size that can be used as propagules and obtain maximum growth and yield is 5 cm rhizome length.
- The appropriate planting depth for *G. perpensa* rhizomes is 4 cm. Most growth parameters were highest at this depth.
- *G. perpensa* seeds exhibited low viability and poor germination percentage.
- Mechanical scarification and leaching for 3 and 7 days improved seed germination in *G. perpensa.*
- Temperature clearly plays an important role in seed germination of *G. perpensa*, as they only germinated at 25°C. Very negligible germination was observed at 20°C.
- Seedling survival and establishment from seed was difficult.
- The ability of the extracts of *G. perpensa* to inhibit the growth of several bacteria and fungi is an indication of the broad-spectrum antimicrobial and and antifungal potentials of this plant that further validates its use for the treatment of various ailments.
- *G. perpensa* leaf extracts were higher in antibacterial activity than the rhizome extracts, but the extracts were similar in antifungal activity.

- The use of *G. perpensa* leaves may as well substitute the use of its roots especially in the treatment of bacterial and fungal diseases.
- The extracts from this plant possess antioxidant properties and could serve as free radical inhibitors or scavengers.
- In some of the phytochemicals evaluated and the antioxidant assays, *G. perpensa* leaf extracts demonstrated higher constituents and activities than the rhizome extracts suggesting their potential in plant part substitution.
- This study did develop a successful decontamination protocol as well as effective means for eliminating the lethal browning of *G. perpensa* explants and media.
- The result demonstrated that ascorbic acid or activated charcoal was required as media supplements to reduce the browning effect.
- This information provides an important starting point for the development of a successful micropropagation protocol for the conservation of *G. perpensa.*
- The present study highlights the need for the conservation of our indigenous plant resources.

9.3 Recommendations

- Vegetative propagation of *G. perpensa* using varying lengths of the rhizome segment as propagules was successful and should be considered a key tool in their conservation. The result demonstrated that for maximum growth and yield, 5 cm rhizome length at a planting depth of 4 or 5 cm is most appropriate.
- The optimum temperature for germination of *G. perpensa* seeds in this study was 25°C under light conditions. Generally, germination of *G. perpensa* seeds was poor and irregular, but mechanical scarification significantly improved the percentage

germination from 4% to 32% compared to untreated seeds (control). Mechanical scarification is recommended for seed pre-treatment.

- In the antibacterial and some of the antioxidant assays, the leaf extracts of this plant demonstrated higher activity than the rhizome extracts, while in the antifungal assay the two plant parts exhibited similar activities suggesting their potential in plant part substitution. The harvesting of the leaves (instead of roots) of *G. perpensa* is certainly more sustainable and could serve as a conservation strategy.

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APPENDIX

The semi-structured questionnaire used for data collection during the survey of the present study

Questionnaire:

A. General information

1. Location é .

2. Interviewee é .

3. Spoken Language é

4. Respondent professioné .

5. Gender: [M] [F] é .

6. Age group: [10-20] [20-35] [>35]é é é é é é é é é é é é é é é é ..

7. Educational Level: [No Schooling] [Primary] [Secondary] [Tertiary]

8. Status in medicinal plant usage: (1) Trader (2) Healer (3) Collector

B. Study Questions

1. Which medicinal plant(s) is/are getting scarce and threatened in your area?

2. What is/are the local usage of this/these plants?

3. Which part of the plant/s is/are normally harvested for medicinal uses?

4. Do you use a combination of plants in treating patients? [Yes] [No]

5. How do you obtain these plants: [grow] [buy] [collect from wild]?

6. Are these plants easy to find? [Yes] [No]

7. How often do you harvest these plants? [Daily] [Weekly] [Monthly]

8. Has the number of patients changed compared to the past? [Decrease] [Increase] [Stable] [Do not know]

9. How do you describe the demand for medicinal plants in the future? [Decrease] [Increase] [Stable] [Do not know]

10. Do people from outside the area collect these plants in the area?

C. Conservation status of the used plants and alternatives to alleviate pressure on the most popular species:

1. Have you noticed any change in terms of the availability of these plants? [Yes] [No]
2. Are you aware of the reduction of some medicinal plants in the wild? [Yes] [No]
    a. If yes, what do you think are the reasons of this reduction?
    b. Could you please list some of them?
    c. Should we conserve natural plant species? [Yes] [No]