MICROPROPAGATION AND MEDICINAL PROPERTIES OF *BARLERIA GREENII* AND *HUERNIA HYSTRIX*

BY

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TABLE OF CONTENTS

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PUBLISHED ARTICLES FROM THIS THESIS

- **AMOO, S.O., FINNIE, J.F. and VAN STADEN, J. 2009.** Effects of temperature, photoperiod and culture vessel size on adventitious shoot production of *in vitro* propagated *Huernia hystrix*. *Plant Cell, Tissue and Organ Culture* 99: 233-238
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- **AMOO, S.O., FINNIE, J.F. and VAN STADEN, J. 2009.** *In vitro* propagation of *Huernia hystrix*: an endangered medicinal and ornamental succulent. *Plant Cell, Tissue and Organ Culture* 96: 273-278
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- **AMOO, S.O., FINNIE, J.F. and VAN STADEN, J. 2009.** Micropropagation of an endangered valuable succulent: *Huernia hystrix*. 35th Annual Conference of the South African Association of Botanists (SAAB) and International workshop on “Phosphate as a limiting resource”. Stellenbosch University, South Africa. (Oral presentation by the first author)

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LIST OF FIGURES

Chapter 4

Chapter 5

LIST OF TABLES

LIST OF ABBREVIATIONS

[3G]BA	6-Benzylamino-3-β-D-glucopyranosylpurine
[7G]BA	6-Benzylamino-7-β-D-glucopyranosylpurine
[9G]BA	6-Benzylamino-9-β-D-glucopyranosylpurine
[9R]BA	6-Benzylamino-9-β-D-ribofuranosylpurine
2,4-D	2,4-Dichlorophenoxyacetic acid
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ATCC	American type culture collection
ATCI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
B5	B5 medium (GAMBORG *et al*., 1968)
BA	6-Benzyladenine
BHT	Butylated hydroxytoluene (2,6-Di-*tert*.-butyl-*p*-cresol)
CAM	Crassulacean acid metabolism
CNS	Central nervous system
COX	Cyclooxygenase
DCM	Dichloromethane
DHZ	Dihydrozeatin
DIF	Difference in photoperiod and dark period temperatures
DMRT	Duncan's Multiple Range Test
DPM	Disintegrations per minute
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTNB	5,5-Dithiobis-2-nitrobenzoic acid
DW	Dry weight
ET	Electron transfer
EtOH	Ethanol
Folin C	Folin-Ciocalteu
FRAP	Ferric ion reducing power assay
FSA	Flora of southern Africa

GAE	Gallic acid equivalents
GST	Glutathione *S*-transferase
HAT	Hydrogen atom transfer
HE	Harpagoside equivalents
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
iNOS	Inducible nitric oxide synthase
INT	*p*-Iodonitrotetrazolium chloride
iP	N^6-Isopentenyladenine
IUCN	International Union for the Conservation of Nature and Natural Resources
LOX	5-Lipoxygenase
LTs	Leukotrienes
MBC	Minimum bactericidal concentration
Me*m*TR	*meta*-Methoxytopolin riboside
MeOH	Methanol
MFC	Minimum fungicidal concentration
MFD	Minimum fungicidal dilution
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MID	Minimum inhibitory dilution
MS	MURASHIGE and SKOOG (1962)
*m*T	*meta*-Topolin
*m*TR	*meta*-Topolin riboside
NAA	α-Naphtalene acetic acid
NADPH	Reduced adenine dinucleotide phosphate
ND	Not determined
NF-κB	Nuclear factor-κB
NSAIDs	Nonsteroidal anti-inflammatory drugs
*O*G	*O*-glucosides
OH	Hydroxyl
ORAC	Oxygen radical absorbance capacity

PE	Petroleum ether
PEPC	Phosphoenolpyruvate carboxylase
PGR	Plant growth regulators
PPF	Photosynthetic photon flux
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSA	Radical scavenging activity
SH	Schenk and Hilderbrandt (1972)
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin-layer-chromatographic
US	United States
WWF	World Wildlife Fund
YM	Yeast Malt
Z	*trans*-Zeatin

ABSTRACT

The crisis of newly emerging diseases and the resistance of many pathogens to currently used drugs, coupled with the adverse side-effects of many of these drugs have necessitated the continuous search for new drugs that are potent and efficacious with minimal or no adverse side-effects. The plant kingdom is known to contain many novel biologically active compounds, many of which could potentially have a higher medicinal value when compared to some of the current medications. Indeed, the use of plants in traditional medicine, especially in African communities, is gaining more importance due to their affordability and accessibility as well as their effectiveness. Exponential population growth rates in many developing countries has resulted in heavy exploitation of our plant resources for their medicinal values. In addition, plant habitat destruction arising from human developmental activities has contributed to the fragmentation or loss of many plant populations. Owing to these factors, many plant species with horticultural and/or medicinal potential have become either extinct or are threatened with extinction. These threatened species cut across different taxonomic categories including shrubs, trees and succulents. Without the application of effective conservation strategies, the medicinal and/or horticultural potential of such threatened species may be totally lost with time. The extinction of such species could lead to the loss of potential therapeutic compounds and/or genes capable of being exploited in the biosynthesis of new potent pharmaceutical compounds.

The overall aims of this study were to establish efficient regeneration protocols and explore the medicinal properties of two threatened South African species belonging to different taxonomic categories: *Barleria greenii* (a shrub) and *Huernia hystrix* (a succulent). *Barleria greenii* is a perennial ornamental and a critically endangered shrub, endemic to a small area in KwaZulu-Natal province of South Africa. Its conventional propagation is hampered by high seed parasitism and difficulty in rooting. *Huernia hystrix* is a dwarf perennial stem succulent heavily exploited for traditional medicine among the Zulu people in South Africa. It is considered vulnerable in KwaZulu-Natal province, an endangered endemic to the flora of the southern Africa region and is vulnerable in its global conservation

status. Being a dwarf species, very limited cuttings can be taken from the mother plant for conventional propagation.

In developing a micropropagation protocol for *B. greenii* using shoot-tip explants (5 mm length), the effects of BA with or without NAA combinations, in MS medium were evaluated. Concentrations of 0.0, 0.5, and 1.0 µM of NAA were combined with 1.0, 2.0, 3.0, 4.0 and 5.0 µM BA in a 3 × 5 completely randomised factorial design. The treatments with BA alone gave higher adventitious shoot production both after four and six weeks of culture when compared to the BA treatments supplemented with NAA concentrations. These results imply that the exogenous application of NAA is neither required nor beneficial for adventitious shoot induction or proliferation from the shoot-tip explants of this species. In evaluating the effects of different types and concentrations of cytokinins on shoot production, the treatments with kinetin generally gave a low shoot production whereas BA treatments gave increased adventitious shoot production with the optimum at a concentration of 3 µM. High abnormality indices were however, observed in all the treatments with BA. At higher concentrations (5 and 7 µM), the treatments with *m*TR and Me*m*TR gave increased adventitious shoot production with abnormality indices less than that of the control. These results indicated that the topolins (*m*TR and Me*m*TR) are less toxic and more effective in the micropropagation of this plant species. The abnormality indices recorded in the topolin treatments could possibly be carry-over effects of BA since the explants used were obtained from BA-treated cultures. Furthermore, cultures maintained under a 16 h photoperiod gave a significantly higher production of adventitious shoots with lengths greater than 10 mm than those placed under continuous light. Regenerated shoots were *ex vitro* rooted after an IBA pulse-treatment for five hours and acclimatized successfully with 65% survival. This developed protocol could potentially produce over 60,000 transplantable shoots per year from a single shoot-tip explant.

An efficient and rapid micropropagation protocol was successfully developed for *H. hystrix*. Adventitious shoots were regenerated from stem explants (10 mm length) cultured on MS medium supplemented with a range of NAA (0.00, 2.69, 5.37 and 8.06 µM) and BA (4.44, 13.32 and 22.19 µM BA) concentrations. The treatments with a combination of BA and NAA demonstrated a synergistic effect on

adventitious shoot production. A 100% shoot regeneration frequency with a production of four adventitious shoots per explant was obtained on MS medium containing 5.37 µM NAA and 22.19 µM BA. Callus produced at the base of the explant on the same medium showed root organogenic potential. The effects of photoperiod and temperature were further evaluated in optimizing this micropropagation protocol. Significant increases in shoot proliferation were observed with increased temperature in cultures maintained under a 16 h photoperiod. Slow growth observed at low temperatures (15 and 20°C) offers a potential strategy for cost-effective *in vitro* storage of *H. hystrix* germplasm. The maximum number of adventitious shoots produced per explant and shoot regeneration frequency were observed in cultures maintained at 35ºC, the optimum temperature for photosynthesis in plants possessing CAM. The nocturnal accumulation of organic acids in cultures incubated under a 16 h photoperiod further suggest the presence of CAM in this species. On the other hand, cultures kept under continuous light appear to shift to a C-3 photosynthetic pathway. With an increase in temperature under continuous light, there was a significant decrease in the fresh weight of adventitious shoots regenerated per explant. The use of larger culture vessels further increased the shoot proliferation to 5.6 shoots per explant with a potential production of 3429 shoots per m^2 in the growth room compared to 2750 shoots per m^2 using culture tubes. Regenerated shoots produced roots when transferred to half strength MS medium with or without auxin. The micropropagated plants were easily acclimatized within two months under greenhouse conditions when potted in a soil and sand mixture (1:1; v/v) treated with a fungicide (Benlate, 0.01%). More than 95% survival with no observable morphological variations was obtained. The developed protocol provides a simple, cost-effective means for the conservation of endangered *H. hystrix* by clonal propagation within a short time.

The dual biological and chemical screening approach was used to evaluate the medicinal properties of three *Barleria* species (including *B. greenii*) and *H. hystrix*. Different extracts of these species demonstrated antibacterial, antifungal, anti-inflammatory, antioxidant and AChE inhibition activities. The observed pharmacological activities might be largely due to their relatively high flavonoid content, with a contributing effect from their iridoid and tannin compounds. The

activities shown by *H. hystrix* extracts might possibly explain its heavy exploitation in traditional medicine. Extracts from *Barleria* species generally had comparatively higher pharmacological activities and phytochemical content. The concept of substituting plant parts (such as leaves and stems for roots) for sustainable exploitation was found to be dependent on the species and/or biological activity evaluated. The substantial high activities observed with some *B. greenii* extracts in the pharmacological assays used further highlight the need to conserve our 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 treatment-resistant infections.

Chapter 1 General introduction

1.1 Use of plants in horticulture and traditional medicine

Worldwide, there is an ever-growing demand for valuable plants many of which are used for horticultural and medicinal purposes. Flowering plant species that can be easily potted, are frost-hardy, cold or drought tolerant and requiring little maintenance are sought after by collectors due to their high ornamental value. Some of these ornamental species are also known to be medicinal, and medicinal plants are important in meeting the human need for good healthcare. A report by the World Health Organization, for example, shows that about 80% of the world's population depends on traditional medicines to meet at least some of their primary healthcare needs **(WHO, 2004)**. The use of plants in indigenous or traditional medicine is more affordable and accessible to most of the population especially in African communities, and it is generally believed to be effective. **FENNELL (2002)** observed that about two-thirds of the South African population still use plants for traditional medicines. The majority of plants traditionally used for medicinal purposes in South Africa are harvested from the wild and are yet to be fully analyzed for their bioactive compounds **(TAYLOR and VAN STADEN, 2001)**. In addition, the exponential population growth rates in developing countries since the latter half of the twentieth century has resulted in increased demand for plant resources **(JÄGER and VAN STADEN, 2000)**. This increasing growth rate has also resulted in plant habitat destruction to allow for agricultural and settlement land among other developmental activities **(JÄGER and VAN STADEN, 2000)**. Owing to their over-exploitation, coupled with destructive harvesting methods, habitat loss, habitat change and other human activities, many plant species with horticultural and/or medicinal potentials have become either extinct or are threatened with extinction.

1.2 The need for conservation of plant species

According to **SARASAN *et al*. (2006)**, more than eight thousand plant species were added to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species during the period 1996 -2004.

During this same period, these authors noted that the number of plants recorded as ‘critically endangered’ increased by over 60%. The International Union for Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) estimated that up to 60,000 higher plant species could become extinct or nearly extinct by the year 2050 if the current trends of utilization continue **(ETKIN, 1998)**. Despite increased governmental regulation, destructive and indiscriminate harvesting of medicinal plants continues unabated especially in Africa, where the collection of medicinal plants mainly from the wild has become a form of rural self-employment **(AFOLAYAN and ADEBOLA, 2004)**. The rising rate of unemployment coupled with the recent global economic recession might even worsen the current situation. Although South Africa is very rich in floral biodiversity, many of the species are highly endemic, heavily exploited and are thus facing the risk of becoming extinct. Added to this problem is the fact that some of the species involved are slow-growing, not readily cultivated and with a high habitat specificity. These threatened species cut across different taxonomic categories including shrubs, trees and succulents.

In the light of the current increasing demands (which far exceeds supply), the medicinal and/or horticultural potentials of such species may be totally lost if efforts are not geared towards their conservation. 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)**. It would also mean the loss of interesting chemical compounds **(RATES, 2001)** with pharmaceutical or nutraceutical potential. **CUNNINGHAM (1993)** observed that the majority of plants used in traditional medicines have not been adequately screened for active ingredients. He therefore suggested that conservation efforts be directed at all species vulnerable to being over-exploited. Moreover, the conservation of plant species is crucial to the survival of other life-forms since plants contribute to the integrity of our environment **(ABOEL-NIL, 1997)**. For instance, it has been estimated that a disappearing plant (due to extinction) can take with it ten to thirty other species such as insects, higher animals, and even plants that depend directly or indirectly on it **(WOCHOK, 1981)**.

1.3 Distribution, morphology, uses and conservation status of the studied plant species

1.3.1 *Barleria greenii*

The genus *Barleria*, belonging to the Acanthaceae family, is a large genus of herbs and shrubs comprising about 300 species worldwide **(MAKHOLELA *et al*., 2003)**. The richest representation is in Africa where there are two centers of diversity, one in tropical east Africa (about 80 species) and the other in southern Africa (about 70 species) **(BALKWILL and BALKWILL, 1998)**. Most species in this genus show a high degree of regional endemism. For example, the Indian subcontinent, West Africa, southern Africa and East Africa are reported to have 75, 72, 65 and 56% endemism, respectively **(BALKWILL and BALKWILL, 1998)**. *Barleria greenii* M.-J. Balkwill & K. Balkwill is one such species endemic to South Africa. The first population of *B. greenii* was discovered in 1984 by Dave Green, a farmer and amateur botanist from the Estcourt district of Natal **(BALKWILL *et al*., 1990)**. It is extremely localized and extremely restricted in distribution, occurring in eight localities on three farms near Estcourt, South Africa **(MAKHOLELA *et al*., 2003)**. Plants belonging to this species are found in open, rocky areas on moderately sloping north-facing aspects, mostly between the 1200 m and 1260 m contours **(BALKWILL *et al*., 1990)**. The ballistic seed dispersal occurring over short distances further affects its distribution such that long-range dispersal to new suitable habitats occurs rather rarely **(BALKWILL *et al*., 1990)**.

Barleria greenii is a perennial, profusely branched woody shrub (Figure 1.1) up to 1.8 m high **(BALKWILL *et al*., 1990)**. Its growth form is affected by light intensity and the frequency with which its habitat area is burnt **(BALKWILL *et al*., 1990)**. **BALKWILL *et al*. (1990)**, for example, observed that plants growing in the shade are much less robust and have broader leaves than those growing in full sun. They noted that plants burnt less often are extremely robust, woody, attaining heights of almost 2 m, whereas those burnt often are less robust, attaining a greater diameter with more vigorous branching but a height of only 0.8 m. *Barleria greenii* flowers from mid-to late summer, usually over a period of a few weeks **(MAKHOLELA *et al*., 2003)**. The attractive flowers, ranging from pure white to

dark pink with magenta streaks on the corolla lobes, emit a strong, sweet fragrance at night and produce large quantities of nectar **(MAKHOLELA *et al*., 2003)**. The fruits, which are produced from early to late autumn, are a 4-seeded capsule, green when young, black when mature (Figure 1.1) and dehiscing explosively **(BALKWILL *et al*., 1990)**. The seeds are discoid, greyish-black, and covered in hygroscopic hairs **(BALKWILL *et al*., 1990)**.

Barleria greenii is a beautiful garden plant, flowering prolifically. It grows under a wide range of conditions and is frost hardy **(TURNER, 2001)**. **SCOTT-SHAW (1999)** described it as a successful and popular garden plant with attractive flowers. Other *Barleria* species grown as ornamentals include *B. cristata*, *B. repens* and *B. prionitis*. Although *B. greenii* has no recorded usage in traditional medicine, many *Barleria* species have been reportedly used in folk medicine and validated to contain compounds possessing biological effects such as anti-inflammatory, analgesic, antileukemic, antitumor, antihyperglycemic, anti-amoebic, virucidal and antibiotic activities.

Figure 1.1: *Barleria greenii*. (**A**) The plant during flowering (**B**) Calyx bearing the unopened, mature fruits (capsules).

Out of eighteen *Barleria* species listed by **HILTON-TAYLOR (1996)** in the Red Data List of Southern Africa plants, thirteen are endemic to the flora of southern Africa (FSA) region. These include *Barleria natalensis* (extinct in its global conservation status), *B. argillicola* and *B. greenii* (global conservation status: vulnerable), as well as *B. dolomiticola* and *B. solitaria* (global conservation status: rare). **SCOTT-SHAW (1999)** described *B. greenii* as a very rare species with narrow distribution, low abundance and high habitat specificity, and endangered in its conservation status. Currently, *B. greenii* is listed as 'critically endangered' in the National Red List of South African plants **(SANBI, 2009)**.

1.3.2. *Huernia hystrix*

Huernia (Family: Asclepiadaceae) is a genus of about sixty-four species found in eastern and southern Africa, Ethiopia and Arabia **(HODGKISS, 2004)**. **HILTON-TAYLOR (1996)** listed sixteen *Huernia* species including *H. hystrix* as endemic to the FSA region. *Huernia hystrix* has three varieties, which are *hystrix, nova,* and *parvula*. This succulent species is reported to be very rare, of narrow distribution and low abundance, especially the *parvula* variety **(SCOTT-SHAW, 1999)**.

The plants are dwarf, perennial stem succulents which are normally mat forming or creeping, rarely pendulous **(LIEDE-SCHUMANN and MEVE, 2006)**. The grey-green, five-ridged, prickled stems often branch from the base (Figure 1.2) forming large clusters. Their attractive flowers with short stalks have a five-angled margin or are five-lobed with a characteristic small lobe in the angle between the main lobes **(HODGKISS, 2004)**. The plants are free-flowering in late summer; the flowers being quite frilly and particularly attractive **(HODGKISS, 2004)**.

Huernia hystrix is in the category of Cactus and succulent plants. Being drought-tolerant, it is suitable for xeriscaping and can easily be grown in small containers. **AL-TURKI (2002)** described it as an excellent ornamental for rock gardens. *Huernia* species (likely *H. hystrix*) are reportedly consumed as typical famine-food plants in southern Ethiopia **(GUINAND and LEMESSA, 2000)**. According to **SCOTT-SHAW (1999)**, the whole plant of *H*. *hystrix* is heavily exploited for

traditional medicine (*muthi*) among the Zulu people in South Africa, though an assessment of its medicinal usage has to be done. It is traded in traditional medicine system and commonly known as toad plant or *ililo elinsundu* (Zulu). The infusions of the plant are reportedly used as protective charms **(HUTCHINGS *et al*., 1996)**. In Swaziland, the stem of *H*. *hystrix* is said to be used for sexual stimulation **(LONG, 2005)**. Other *Huernia* species reported to be medicinal include *H*. *stapelioides* and *H. zebrina*, though their medicinal usages were not specified **(LONG, 2005)**.

Of the sixteen endemic *Huernia* species in the Red Data List of southern African plants, two are extinct, one endangered, two vulnerable, and five rare **(HILTON-TAYLOR, 1996)**. *Huernia hystrix* is considered vulnerable in KwaZulu-Natal, endangered endemic to FSA region and vulnerable in its global conservation status **(HILTON-TAYLOR, 1996; SCOTT-SHAW, 1999)**. It is threatened by its heavy exploitation for traditional medicine. Its collection or harvesting is destructive since the whole plant is often used. Furthermore, the plants are susceptible to stem and root mealy bugs, and damage from these may well facilitate fungal attack **(HODGKISS, 2004)**.

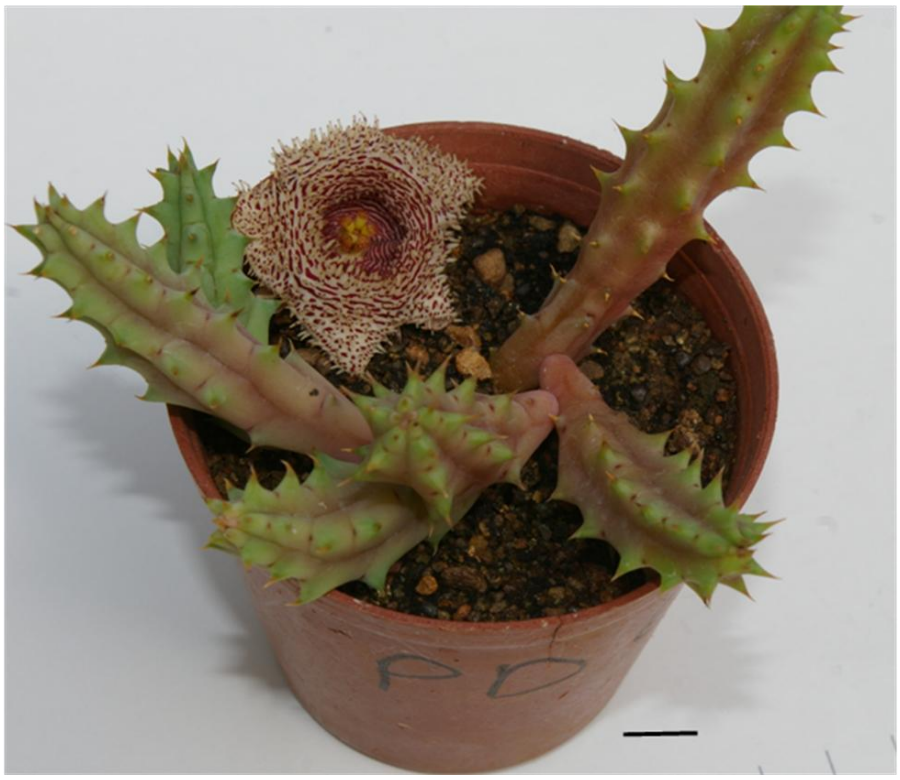


Figure 1.2: Flowering *Huernia hystrix* potted in a small container. Bar = 10 mm

1.4 Value of tissue culture

The application of tissue culture as a biotechnological tool in the conservation of threatened economic plants has gained tremendous impetus in the last two decades. The tissue culture technique is a powerful tool for plant germplasm conservation and can be a viable alternative to conventional propagation of slow growing species or species that produce recalcitrant or few viable seeds **(ABOEL-NIL, 1997)**. Rapid and mass propagation of plant species and their long-term germplasm storage are achievable in a small space and short time, with no damage to the existing population. Plant material can be produced throughout the year without any seasonal limitation. Due to the aseptic nature of tissue culture technique, large numbers of uniform and disease-free plants can be produced from very small portions of the mother plant. The sterile nature of *in vitro* cultures facilitates the exchange of germplasm or plant materials even at international level **(SALIH *et al*., 2001)**.

Furthermore, plant tissue culture systems often serve as model systems in the study of physiological, biochemical, genetic and structural problems related to plants **(TORRES, 1989)**. The use of *in vitro* culture technique has found applications in genetic breeding and improvement, production of new hybrids and overcoming incompatibility during crosses. Somaclonal variation, often referred to as 'off-types' is sometimes considered to be an unwanted side-effect of *in vitro* culture technique. However, depending on the research objective, this could be advantageous in increasing the pool of variability. The variability can result in superior plants or plants with adaptive advantage that can be selected and further exploited in genetic improvement programs. Moreover, the control of chemical and physical conditions of *in vitro* cultures makes it possible to optimize conditions needed for enhanced production of secondary metabolites in target cells or tissues **(ABOEL-NIL, 1997)**. *In vitro* production of secondary metabolites, in turn, offers a steady and reliable source of supply for pharmaceutical or nutraceutical industries **(GIULIETTI and ERTOLA, 1999)**.

1.5 Aims and objectives

Endemic and threatened South African species fall in different taxonomic categories including succulents, shrubs and trees. The overall aims of this study were to establish efficient regeneration protocols and explore the medicinal properties of two threatened South African species belonging to different taxonomic categories: *Barleria greenii* (a shrub) and *Huernia hystrix* (a succulent). The specific objectives were to:

- Determine the appropriate chemical (nutritional) and environmental conditions for *in vitro* propagation of each of the two species;
- Investigate the acetylcholinesterase inhibition, antimicrobial, anti-inflammatory and anti-oxidant activities of different extracts of these species;
- Investigate the possibility of plant-part substitution as a conservation strategy against destructive harvesting of these species for medicinal purpose;
- Explore the phytochemical properties of different parts of these species.

1.6 General overview of the thesis

Chapter 2 provides background information from literature, relating to micropropagation techniques and major factors affecting the success of the technique. It also provides an insight into the fundamental principles underlying the pharmacological activities evaluated in this study.

Chapter 3 gives an insight into the development of the micropropagation protocol established for *B*. *greenii*. The results suggest that exogenous application of NAA is neither required nor beneficial for *in vitro* shoot induction or multiplication from shoot-tip explants of this species. The effects of types and concentrations of aromatic cytokinins as well as the effects of different light periods were investigated and will be discussed.

Chapter 4 describes the simple, rapid and cost-effective clonal propagation protocol developed for *H*. *hystrix*. The findings indicate that both BA (cytokinin) and NAA (auxin) have a synergistic effect on shoot multiplication from stem explants of this species. The chapter further highlights the need to investigate the effects of environmental conditions when developing efficient micropropagation protocols, especially for commercial purposes. Optimizing environmental conditions could increase growth rate, reduce labour costs and thus subsequent production costs.

Chapter 5 presents the pharmacological and phytochemical properties of extracts from different parts of *B. greenii* and *H. hystrix*. For comparison purpose, extracts from two other *Barleria* species (*B. albostellata* and *B. prionitis*) were included. The results indicate the medicinal potential of the studied plants. Different extracts of different plant parts show different potencies in the activities evaluated. The possibility of plant part substitution as a conservation measure is discussed.

Chapter 6 summarizes the major conclusions drawn from this study.

The section '**References**' provides an alphabetical list of publications or materials cited in this thesis.

Chapter 2 Literature review

2.1 Micropropagation

2.1.1 Introduction

The term ‘micropropagation’ also known as ‘*in vitro* propagation’ generally involves aseptic culturing of excised small plant parts (explants) in an artificial medium under appropriate physical conditions for the purpose of producing clonal plants capable of surviving in a natural environment. Although it is not different in principle from the conventional propagation of some species by cuttings, the use of smaller propagules, provision of aseptic and controlled environmental conditions, heterotrophic development, and faster plant multiplication makes the micropropagation technique more effective **(MURASHIGE, 1978)**. The process of micropropagation is often accomplished in different but sequential stages. **MURASHIGE (1978)** proposed four widely accepted stages (I – IV) while **DEBERGH and MAENE (1981)** suggested an additional stage 0. These stages are often used worldwide in research and commercial laboratories for plant propagation through tissue culture. The objectives of each of the stages and what they involve are discussed in the following sections.

2.1.2 Stage 0: Selection and preparation of mother plants

The objective of this first stage in micropropagation is to select mother (stock) plants that could provide healthy explants capable of being uniformly initiated into culture **(DEBERGH and MAENE, 1981)**. Plants that are healthy, apparently free from any disease symptom and vigorously growing are often selected as stock plants **(CONSTABEL and SHYLUK, 1994)**. In some cases, however, selected stock plants are preconditioned by growing them in the greenhouse at a relatively low humidity and avoiding overhead watering **(DEBERGH and MAENE, 1981)**. In addition, subjecting the stock plants sometimes to antibiotic, fungicidal and antiviral treatments could help reduce the contamination level of explants taken subsequently from them **(MURASHIGE, 1978)**. In any case, the careful selection

and preparation of stock plants would not only allow for the availability of standardized and healthy explants, but also increase the rate of explant survival during culture initiation **(DEBERGH and MAENE, 1981)**.

2.1.3 Stage I: Initiation and establishment of aseptic culture

Owing to the inherent totipotentiality of plant cells **(MURASHIGE, 1978)**, explants obtained from different plant tissues have a potential to regenerate plantlets when given appropriate environmental and nutrient conditions. It is important that the explants be able to grow well in an aseptic *in vitro* environment free from any obvious infection **(MURASHIGE, 1974)**. In order to achieve this objective, the choice of explants and their decontamination are given serious attention during this stage.

MURASHIGE (1974) listed five important factors to be considered when choosing suitable explants. These include (i) the organ serving as explant source; (ii) the physiological or ontogenic age of the organ; (iii) the season in which the explant is obtained; (iv) the size of the explant; and (v) the overall quality of the stock plant.

Nearly all plant organs or tissues can serve as a source of explants in micropropagation. However, explants taken from different plant parts often manifest different regeneration and morphogenic responses. **KOMALAVALLI and RAO (2000)**, for example, reported different propagation rates and morphogenic responses in axillary node, shoot-tip, cotyledonary node, leaf, petiole, root and internode explants of *Gymnema sylvestre*. They observed that only axillary node, cotyledonary node and shoot-tip explants readily regenerated multiple shoots while other explants produced only callus. These observations were attributed to the variation in endogenous levels of plant growth regulators in the explants at the time of excision. Similar results have been reported by other researchers in different plant species **(NHUT *et al*., 2004; ISLAM *et al*., 2005; CONDE *et al*., 2008)**.

The regenerative and morphogenic capacities of explants are sometimes affected by the physiological age of the explant and the degree of differentiation among

their constituent cells **(MURASHIGE, 1974)**. **SUDHA *et al*. (1998)**, while investigating the regeneration potential of shoot-tip, terminal and basal node explants in *Holostemma annulare*, observed more rapid multiple shoot formation from basal node explants. They concluded that this variation in regenerative response might be due to the differences in endogenous growth regulator level, nutrient availability and physiological status of the explants. In general, explants obtained from juvenile plants often have a better regenerative capacity compared to those from older plants, especially in trees and shrubs **(NHUT *et al*., 2007)**, perhaps due to their meristematic cell types and other endogenous factors **(FENNELL, 2002)**. **BECERRA *et al*. (2004)** reported an inverse linear regeneration capacity in relation to the age of donor plants in *Passiflora edulis*. They noted that leaf explants taken from juvenile plants (1- to 6-month-old) showed a statistically significant higher shoot regeneration compared to explants taken from reinvigorated adult plants (shoots emerging next to the base after severe pruning of 1-year-old adult plants). Even among the juvenile explant sources, the same inverse linear regeneration capacity with age was observed. In *Gerbera jamesonii*, flower bud age was reported to significantly affect the survival rate and shoot regeneration capacity of the receptacle transverse thin cell layers, with the optimum observed in 10-day-old flower buds **(NHUT *et al*., 2007)**. The authors further observed that the position of receptacle transverse thin cell layers significantly affected the receptacle morphogenic ability. They noted that shoot production and regeneration frequency decreased from the middle to exterior receptacle layers, possibly due to more nutrient reserves in the middle layers.

The regenerative capacity of explants in tissue culture could also be affected by the season in which the explant is obtained due to seasonal influences on the plant developmental stages. **PRAKASH and VAN STADEN (2008)** observed that offshoots of *Searsia dentata* collected during the vegetative stage (April - May) exhibited a better morphogenic response compared to those collected during the flowering stage (October – November). Similarly, young offshoots of *Hoslundia opposita* collected during the months of March and April were reported to elicit a better morphogenic response compared to those collected during other months of the year **(PRAKASH and VAN STADEN, 2007)**. Other researchers have reported

similar differential seasonal responses in explants of different plant species **(LITZ and CANOVER, 1981; RAZDAN, 2003)**.

The size of explants has been reported to influence their survival and growth rates *in vitro*. Very small shoot-tip explants such as meristem tips are said to have a low survival and initial growth rates **(RAZDAN, 2003)** and are therefore not practical for achieving rapid clonal multiplication **(MURASHIGE, 1974)**. Nevertheless, these authors noted that very small explants (such as submillimetre shoot-tips) are very useful in obtaining virus-free plants from an infected individual. **STROSSE *et al*. (2008)** observed that 5 mm sized explants of banana produced two to three times more shoots in 3-month-old cultures than 1 mm sized shoot-tips. On the other hand, **OTHMANI *et al*. (2009)** reported a decrease in embryogenic callus frequency in date palm with an increase in explant size. They observed that leaf explants (5 – 10 mm) had the highest embryogenic callus frequency while in larger explants (15 – 20 mm), the peripheral parts either turned brown or produced hyperhydric non-embryogenic callus. A similar observation was reported in *Triticum turgidum* with small explants showing a higher morphogenic capacity **(BENKIRANE *et al*., 2000)**. According to the authors, larger explants have more normal tissue interactions than the smaller ones and such interactions have a tendency to inhibit cell division.

As regards the overall quality of the donor plants, explants obtained from healthy plants are generally known to respond better than those from diseased plants **(MURASHIGE, 1974)**. **LIU and PIJUT (2008)** observed that only selected leaf explants from *Prunus serotina* shoots in an active state of growth and with no sign of chlorosis regenerated adventitious shoots with a high frequency. **MURASHIGE (1974)** noted that an explant taken soon after heavy fertilization of its source plant may produce a different response in the same culture medium compared with explants taken later from the same plant or from an unfertilized plant. Such differential responses could be largely due to the differences in the physiological status of the parent plants.

In addition to making a good choice of explants, the decontamination treatments given to the explants are crucial for their successful initiation into culture. Since the

media are sterile, the explants mainly need to be free of contaminants in order to establish an aseptic culture. Culture contamination often results in explant death as contaminants rapidly outgrow the cultured explants, exploit the nutrient-rich medium, starve the explants, or even produce phytotoxic substances. Surface-decontamination of explants is often done by soaking the explants in 'sterilants' such as sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, silver nitrate and mercuric chloride solutions for some seconds or minutes, followed by thorough rinsing with sterile distilled water under aseptic conditions **(TORRES, 1989)**. **MURASHIGE (1978)** highlighted other measures that could enhance the effectiveness of the disinfectant. These include the addition of small quantities of detergent (such as Tween 20 which acts as a surfactant), performing the disinfestations under gentle vacuum or with constant and relatively vigorous agitation and prewashing the plant material with alcohol or detergent. In a situation where the explant is internally infested by bacteria, fungi or viruses, the inclusion of antibiotics, fungicides or anti-viral agents respectively in the preliminary culture media has been suggested **(MURASHIGE, 1978; PEIXOTO *et al*., 2006)**.

Another problem usually experienced during culture initiation of some explants is explant browning, which often results in necrosis or tissue death. Browning of explants is most severe in high-tannin or other hydroxyphenol containing species **(TORRES, 1989)**. The browning is attributed to the action of copper-containing oxidase enzymes (such as polyphenol oxidase and tyrosinase), which are produced and/or released due to wounding during the excision of the tissue **(TORRES, 1989)**. The incorporation of antioxidants (such as ascorbic or citric acid), and/or absorbents (such as activated charcoal or polyvinylpyrrolidone) into the culture medium as well as regular subculturing to a fresh medium, culturing in the dark, or excising the explant tissues under sterile distilled water are various suggested measures to minimize or prevent this problem **(RAZDAN, 2003)**. Although the use of activated charcoal is said to be inhibitory in some cultures due to its absorption of plant growth regulators, its stimulatory effect has nevertheless been reported in some other cultures **(TORRES, 1989; RAZDAN, 2003)**.

2.1.4 Stage II: Proliferation or multiplication of propagules

This stage aims at optimizing the production of propagules which can potentially give rise to whole plants. Multiplication can be brought about by axillary shoot production, adventitious shoot proliferation as well as asexual or somatic embryogenesis. The latter two regeneration methods can occur either directly from the cultured explants or indirectly via callus production. Although a particular predetermined regeneration pathway may be inherent within a specific tissue, the type and concentration of exogenous plant growth regulators as well as the culture environmental conditions often affect and can modify the expression of a regeneration method. In deciding the most appropriate multiplication method, however, factors such as the rate and frequency of multiplication as well as the probability of producing aberrant plants should be seriously considered **(MURASHIGE, 1978)**.

During axillary shoot proliferation, axillary and terminal buds (quiescent or active) from the nodal or shoot-tip segments cultured on appropriate media grow into axillary shoots. The newly produced shoots with buds along their axis can, in turn be used to regenerate more shoots until a satisfactory number is achieved. Although the axillary shoot proliferation method is considerably slower than other multiplication methods, it allows a yearly multiplication rate that is much faster than conventional propagation by cuttings **(MURASHIGE, 1978)**. In addition, it has great potential for shoot multiplication in woody plant species that could not be successfully regenerated through adventitious shoot proliferation and somatic embryogenesis **(TORRES, 1989)**. Regeneration through axillary shoot production has been reported in many plant species such as *Opuntia* spp. **(GARCÍA-SAUCEDO *et al*., 2005)**, *Cedrela fissilis* **(NUNES *et al*., 2002)** and *Chimonanthus praecox* **(KOZOMARA *et al*., 2008)**, which are succulents, a tree and a shrub, respectively.

Adventitious shoots can be regenerated from nearly all plant tissues or organs either directly or indirectly (via callus production). They often develop from sites where meristems do not exist **(RAZDAN, 2003)**. Whereas this regeneration method is more rapid, the rate of producing genetically or epigenetically altered

plants is higher **(MURASHIGE, 1978)** especially when the regeneration is indirect through callus production. Minimizing the number of subcultures, especially on media containing plant growth regulators, is therefore encouraged since both the loss of morphogenic capacity and abnormality or variation frequency increase progressively with each subculture **(MURASHIGE, 1978; TORRES, 1989; BAIRU *et al*., 2008)**. Nevertheless, the maintenance of regenerative ability, for example, of a 5-year-old callus subcultured at 6-weekly intervals has been reported **(SATO *et al*., 1995)**. Some researchers have recorded simultaneous shoot multiplication through axillary and adventitious pathways in some cultures **(PIERIK, 1987; DE FÁTIMA *et al*., 1996; NUNES *et al*., 2002; DÁVILA-FIGUEROA *et al*., 2005; PRAKASH and VAN STADEN, 2008)**.

Somatic embryogenesis involves the production of embryos from vegetative or somatic cells. These embryos could, in turn develop into whole plants. Direct somatic embryogenesis occurs when an embryo develops directly from a cell or tissue without the callus phase. The cells giving rise to the embryos, in this case, are said to be pre-embryonic determined **(PIERIK, 1987)**. On the other hand, an unorganized mass of relatively undifferentiated cells (callus) with proembryogenic masses may be formed from an explant **(SRIVASTAVA, 2002)**. When subcultured on media without auxin, these proembryogenic masses could develop through different embryo stages into whole plants. Somatic embryogenesis is said to have the greatest potential for achieving rapid clonal micropropagation **(TORRES, 1989)**. It has major application in genetic transformation and biolistic gene transfer **(OTHMANI, *et al*., 2009)**. In addition, the formation of synthetic seeds through somatic embryogenesis could facilitate the development of compact storage, packaging and distribution methods for superior varieties **(PAREEK and KOTHARI, 2003)**. However, somatic embryogenesis is not often used in practice as a means of propagation due to some problems as listed by **PIERIK (1987)** and **OTHMANI *et al*. (2009)**. These problems include the recalcitrance of some species to this method, the high probability of developing mutations, abnormal development, low maturation and germination frequencies of somatic embryos, and the increasing probability of losing regenerative capacity with repeated subculture. Nevertheless, many researchers have reported successful regeneration through direct or indirect somatic embryogenesis from explants of

different plant species **(GILL *et al*., 1995; GOGATE and NADGAUDA, 2003; PAREEK and KOTHARI, 2003; GOMES *et al*., 2006; RAI *et al*., 2007; AZAD *et al*., 2009; OTHMANI *et al*., 2009)**.

2.1.5 Stage III: Preparation of propagules for transfer to soil

Regenerated shoots or plantlets produced during the immediate previous stage (stage II) are often too small or of low vigour to be able to survive in soil. The objective of this third micropropagation stage is therefore to prepare regenerated shoots or plantlets for successful transfer to soil **(MURASHIGE, 1978)**. This stage involves the *in vitro* rooting of individual regenerated shoots, hardening of plants to impart some disease resistance and tolerance to moisture stress, as well as rendering the plants capable of autotrophic development **(MURASHIGE, 1978)**.

Although *in vitro* rooting is generally an expensive, labour-consuming process, especially from a commercial perspective, it may be the only practical method of rooting plantlets in some species **(TORRES, 1989)**. **DE KLERK (2002)** reported that *in vitro* rooting of apple microcuttings strongly increased plantlet size during the rooting treatment, enhanced acclimatization by improved water uptake during early acclimatization and facilitated the addition of extra nutrients and protective compounds that may strongly improve performance. *In vitro* rooting is often achieved by culturing shoot cuttings on medium with half-strength concentrations of macro- and micronutrients, with or without auxins **(TORRES, 1989; MONCOUSIN, 1991)**. This however, is often dependent on the plant species. **SUN *et al*., (2008)**, for example, observed a significantly higher rooting frequency in *Zygophyllum xanthoxylon* shoots cultured on full-strength MS medium compared to those cultured on half-strength medium. On the other hand, **PATIL (1998)** reported an inverse relationship between media strength and rooting frequency in three different species of *Ceropegia*. Indeed, many other researchers have reported better *in vitro* rooting on low-strength media compared to full-strength **(SAXENA and BHOJWANI, 1993; PUROHIT *et al*., 1994; ANDRADE *et al*., 1999; BEENA *et al*., 2003).** The beneficial effect of low medium strength on rooting has been reported to be possibly due to a reduced requirement of nitrogen by the plantlets for root formation **(HYNDMAN *et al*., 1982)**. In some plant species,

adventitious roots are produced without the application of exogenous auxin. This response is reportedly triggered by the endogenous auxin produced in the shoot apex and transported basipetally to the cut surface **(DE KLERK *et al*., 1999)**. **NORDSTRÖM and ELIASSON (1991)** observed that the removal of the apex in pea cuttings resulted in a reduction of both the endogenous auxin level in the basal part of the cutting as well as the number of roots produced.

In situations where auxins are exogenously applied for rooting purposes, the auxins frequently added to the media include IAA (0.1 – 10.0 mg l^{-1}), IBA (0.5 – 3.0 mg l^{-1}) and NAA (0.05 – 1.0 mg l^{-1}) **(TORRES, 1989)**. **BEENA *et al*. (2003)** observed that IBA was the best for inducing roots in *Ceropegia candelabrum*, followed by IAA whereas treatments with NAA showed callus formation and poor root induction. Similarly, **CHOFFE *et al*. (2000)** reported that IAA was less effective than IBA for induction of root organogenesis from *Echinacea purpurea* hypocotyl explants, while treatments with NAA were ineffective. The superiority of IAA and IBA over NAA was also reported in *Metrosideros excelsa* **(IAPICHINO and AIRÒ, 2008)**. According to **FOGAÇA and FETT-NETO (2005)**, the efficacy of IBA in inducing adventitious rooting in *Eucalyptus globulus* and *E. saligna* might be due to its capacity to be converted to IAA **(EPSTEIN and LAVEE, 1984)**, thus serving as a slow release reservoir of a more easily metabolized auxin. Conversely, these authors attributed the negative response of NAA to its longer persistence or stability in plant tissues, resulting in inhibition of root growth. **DE KLERK *et al*. (1997)** suggested that NAA might be the preferable auxin (particularly when applied as a short term initial treatment) for rooting plants with a high activity of auxin-oxidase, due to the non-destruction of NAA by auxin-oxidase. High auxin concentrations are generally known to stimulate root induction but inhibit root initiation or emergence **(DE KLERK *et al*., 1997, 1999)**. Ultimately, the effectiveness of auxins in rooting processes depends on the affinity of the auxin receptor involved in rooting and the concentration of free auxin that is reached in the target cells **(DE KLERK *et al*., 1999)**. The authors stated further that the concentration reached in the target cells depends on the amount of endogenous auxin as well as the uptake, transport and metabolism of the exogenously applied auxin.

Other medium additives such as phloroglucinol and activated charcoal could be beneficial in the rooting of some species. **BOPANA and SAXENA (2008)**, for instance, observed that the inclusion of phloroglucinol was critical in the rooting of *Asparagus racemosus*, as it enhanced the rooting frequency considerably. According to these authors, phloroglucinol acts as an auxin synergist during the auxin sensitive phase of root induction. The root-promoting effects of activated charcoal have been extensively reviewed by **PAN and VAN STADEN (1998)**.

In addition to reducing nutrient level, hardening and preparation of plants for autotrophic development can be accomplished by the use of growth retardants, by reducing relative humidity as well as increasing the photosynthetic photon flux and the carbon dioxide concentration in cultures **(MURASHIGE 1978; DEBERGH, 1991; HAZARIKA, 2003)**. Measures for reducing relative humidity as listed by **HAZARIKA (2003)** in his review include the use of desiccants, opening the culture containers, adjusting culture closures or using special closures that facilitate water loss.

2.1.6 Stage IV: *In vivo* rooting and acclimatization for soil establishment

DEBERGH and MAENE (1981) listed four problems associated with *in vitro* rooting of regenerated shoots:

(i) The process of rooting regenerated shoots *in vitro* is the most labour-intensive stage in micropropagation, and has been estimated to be responsible for approximately 35 to 75% of the total cost of micropropagation in different species;

(ii) The root system produced *in vitro* is usually non-functional in a normal substrate and thus require formation of new roots after *in vivo* planting. The production of such new roots is accompanied by a cessation of growth;

(iii) Optimal root formation requires an auxin concentration for the induction phase. This auxin concentration could, when present beyond the induction phase, inhibit subsequent root elongation; and

(iv) Damage to the root system during the transplanting process to the soil creates openings for the entrance of micro-organisms, resulting in root and/or stem diseases.

DEBERGH and MAENE (1981) therefore proposed *in vivo* rooting of tissue cultured shoots as a viable alternative. *In vivo* rooting is often accomplished either by pulse-treating the shoots for some hours, days or weeks with an auxin concentration followed by direct planting on normal substrate, dipping the shoots in an auxin powder or planting the shoots directly in a rooting mixture previously saturated with an auxin solution **(DEBERGH and MAENE, 1981; DEBERGH, 1991)**. Rooting mixtures include such materials as peat, vermiculite, perlite, bark, pumice, rockwool, sand, soil, or their mixtures, with or without supplementation with small amount of lime or fertilizer **(DEBERGH and MAENE, 1981; TORRES, 1989)**. **MAENE and DEBERGH (1983)** observed that the choice of a substrate, the concentration and duration of IBA treatment, and the size of the shoots could affect *in vivo* rooting of regenerated shoots as demonstrated in *Cordyline terminalis* and *Ficus benjamina*. **SHARMA *et al*. (1999)** reported that the optimization of harvesting time of microshoots, shoot size, soil pH, plant growth regulator treatment, CO_2 enrichment and light conditions in specially designed hardening chambers significantly affected the success rate of hardening *Camellia sinensis* microshoots.

The success of micropropagation ultimately lies not only in the production of large numbers of plantlets but also on their survival rate in field conditions. As stated by **RAZDAN (2003)**, tissue cultured plants generally manifest some structural and physiological abnormalities which make them vulnerable to transplantation shocks. The author listed such abnormalities to include noticeable decrease in epicuticular wax, abnormal leaf morphology and anatomy, poor photosynthetic efficiency, malfunctioning of stomata, and hypolignified stems. These abnormalities often lead to high water loss, high susceptibility to leaf scorch by sun and poor survival of micropropagated plants. **SANTAMARIA *et al*. (1993)** however, reported that high rates of water loss from micropropagated *Delphinium* leaves were not due to high rates of cuticular transpiration but linked to a lack of stomatal control. In a similar vein, **SANTAMARIA and KERSTIENS (1994)** strongly argued and concluded that the lack of control of water loss shown by various micropropagated plants is not associated with poor cuticular development, but rather related to the failure of stomata to close in response to leaf dehydration. Nevertheless, micropropagated plants are often placed in conditions of high relative humidity,

partial shade and good even temperatures for acclimatization before their transfer to field conditions **(TORRES, 1989)**. A condition of high relative humidity could be created by placing the plants under a transparent plastic tunnel or an intermittent mist system **(DEBERGH and MAENE, 1981)**. The relative humidity could then be reduced gradually by gradually slitting the plastic bag or reducing the amount of mist received by the plants **(TORRES, 1989)**. **MURASHIGE (1978)** suggested the use of foliar feeding until an effective rooting system is developed. According to **RAZDAN (2003)**, partial defoliation and the application of transpirants (1% Acropol, v/v) in the initial stages of transpiration have been reported to improve the survival frequency by reducing water loss. On the other hand, the use of antitranspirants to reduce water loss during acclimatization, though suggested as a positive measure by some researchers, is said to have mixed results (see the review by **HAZARIKA, 2003)**.

From the above review of the stages in micropropagation, it is clear that success in micropropagation depends on a host of factors. The factors could possibly be classified into three aspects, which are: (i) the chemical composition and physical qualities of the medium **(MURASHIGE, 1974)**, (ii) the culture environment qualities **(MURASHIGE, 1974)**, and (iii) the physiology of the plant species. A suitable *in vitro* medium contains all essential nutrients (macro and micro) in appropriate proportions, a carbohydrate source (such as sucrose and glucose), vitamins (like nicotinic acid and pyridoxine) and plant growth regulators (mainly auxins and cytokinins). Some standard basal media formulated and commonly used include MS medium **(MURASHIGE and SKOOG, 1962)**, B5 medium **(GAMBORG *et al*., 1968)**, White's medium **(WHITE, 1963)**, as well as SH medium **(SCHENK and HILDERBRANDT, 1972)**. Of these, MS medium remains the most widely used. **MURASHIGE (1974)** observed that the types and concentrations of auxin and cytokinin are the most critical organic components of plant propagation media. The active but hidden role of tissue endogenous growth regulators in plant growth and development must however, not be ignored since the exogenously applied growth regulators often interact with the endogenous ones, which may even be of a different group (such as abscisic acid, ethylene and gibberellins) **(GASPAR *et al*., 1996)**. **MURASHIGE (1974)** further observed that light and temperature are the major environmental factors affecting tissue culture. The effects of auxins and

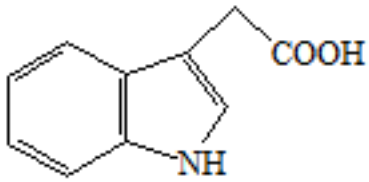
cytokinins as well as these major environmental factors (light and temperature) on *in vitro* plant regeneration are reviewed in the next two sections of this Chapter.

2.1.7 Effects of auxins and cytokinins on plant regeneration in micropropagation

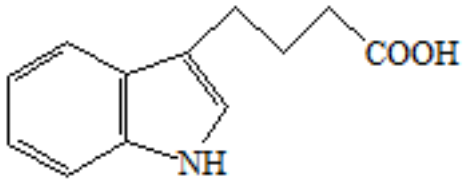
Auxins and cytokinins are usually required for growth and morphogenesis and are therefore frequently added to the culture media. According to **DAVIES (2004)**, the regulatory role of plant growth regulators in growth and development depends on three main factors. These are: (i) the amount present – a factor that is regulated by biosynthesis, degradation and conjugation (ii) the location of the growth regulator – a factor affected by movement or transport (iii) the sensitivity (or responsiveness) of the tissue – a factor which involves the presence of receptors and signal-transduction chain components. The exogenous application of many growth regulators may however modify the synthesis, degradation, activation, sequestration or translocation of, as well as sensitivity to endogenous growth regulators of the same or different type **(GASPAR *et al*., 1996; KAMÍNEK *et al*., 1997)**.

Figure 2.1 presents the structure of some frequently used auxins in plant tissue culture. IAA is regarded as the most common naturally occurring auxin in plants **(MUDAY and DELONG, 2001)**. In general, auxins exist in plant cells either as free acids or in conjugated forms. They can be conjugated to sugars (such as monosaccharides and high molecular weight polysaccharides) and sugar alcohols (such as myo-inositol) through ester linkages or to single amino acids, peptides and proteins through amide linkages **(NORMANLY *et al*., 2004; BAJGUZ and PIOTROWSKA, 2009)**. The formation of auxin conjugates is a mechanism involved in the homeostatic control of auxin levels in plant cells **(BAJGUZ and PIOTROWSKA, 2009)**. Free auxins, when in excess, can be stored and transported in conjugate forms. Such conjugated molecules are protected from oxidative breakdown and may be later hydrolysed enzymatically to release free auxin when required **(GASPAR *et al*., 1996)**. The formation and hydrolysis of auxin conjugates as well as the conjugate profile of endogenous auxin differ among plant tissues and are controlled by the developmental stage of the plant **(RAMPEY *et al*., 2004; BAJGUZ and PIOTROWSKA, 2009)**. The exogenous

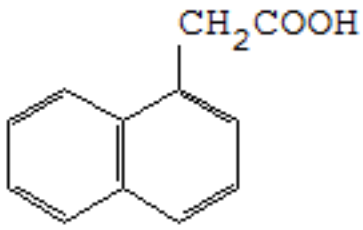
application of different auxins may lead to a distinct physiological and/or morphogenic response in different explants due to variation in their endogenous growth regulator profile at the time of excision, determined by their growth and developmental stage.



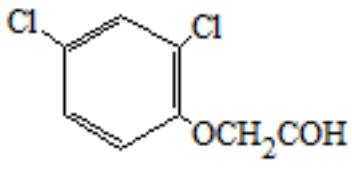
Indole-3-acetic acid (IAA)



Indole-3-butyric acid (IBA)



α-Naphtalene acetic acid (NAA)



2,4-Dichlorophenoxyacetic acid (2,4-D)

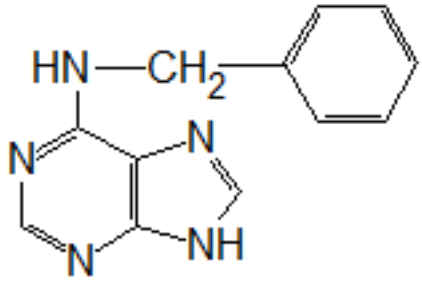
Figure 2.1: Molecular structures of some auxins commonly used in plant tissue culture.

Auxins are known to play a crucial role in regulating or influencing growth and developmental processes such as cell expansion, cell wall acidification, initiation of cell division, differentiation of vascular tissue and organization of meristems giving rise to either unorganized tissue (callus) or defined organs **(GASPAR *et al*., 1996; MUDAY and DELONG, 2001)**. Responses such as callus production, induction of somatic embryogenesis, shoot production, rooting and secondary metabolite production in growth media supplemented with different auxin concentrations have been reported by many researchers **(NIGRA *et al*., 1987; DE KLERK *et al*., 1997; SUN *et al*., 2008; AZAD *et al*., 2009; BASKARAN *et al*., 2009)**. Depending on the objective, **PIERIK (1987)** encouraged limiting the use of 2,4–D due to its ability to induce mutations and/or inhibit photosynthesis. **GASPAR *et al*. (1996)** noted that, in addition to the modifying effect of other growth regulators **(ALONI, 1995; LIU and REID, 1992)**, the activity of auxins can be affected by the free availability of boron. They noted that both the translocation of IAA and nuclear RNA synthesis in response to auxin treatment can be inhibited in boron-deficient plants. Furthermore, the endogenous level of IAA can be regulated by its photo-oxidation **(RAY, 1958)** when cultures are over-exposed to light. The rapid metabolism of auxins such as IAA and IBA within plant tissues may be useful in automatically changing the auxin:cytokinin ratio in culture (for example, during callus production followed by organogenesis) or when less auxin is required for a subsequent developmental phase in the same culture (for example, during root elongation following root induction) **(GASPAR *et al*., 1996)**.

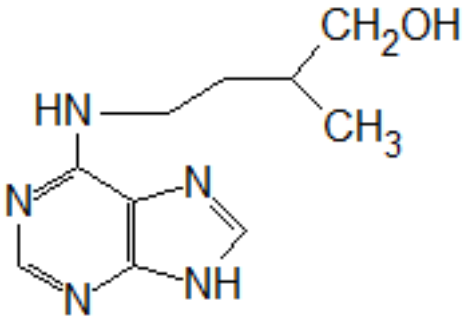
The structures of some commonly used cytokinins in tissue culture are shown in Figure 2.2. Cytokinins such as zeatin and isopentenyladenine with isoprenoid derived chains attached at their N^6 terminal are often described as isoprenoid cytokinins. The aromatic cytokinins (such as benzyladenine and topolins) have an aromatic derivative side chain at their N^6 terminal. Cytokinins can be found in different forms such as free bases, ribosides, nucleotides, *N*-glucosides and *O*-glucosides (*O*G) in plant cells **(LETHAM and PALNI, 1983)** and their endogenous levels often regulate their physiological activities **(KAMÍNEK *et al*., 1997)**. The exogenous application of cytokinins, their uptake and metabolism in turn influence the endogenous cytokinin levels in plant cells **(KAMÍNEK *et a*l., 1997)**. **STRNAD (1997)** classified the metabolism of cytokinins from a physiological perspective into

four main categories. These include: (i) their interconversion, (ii) hydroxylation, (iii) conjugation, and (iv) degradation.

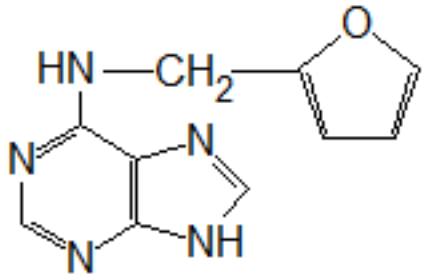
According to **LETHAM and PALNI (1983)**, exogenous cytokinin bases are converted by plant tissues into various types of metabolites. Such metabolites include products of ring substitution (ribosides, nucleotides, *N*-glucosides), and products of isoprenoid side chain cleavage (adenine, adenosine, adenosine-5'-monophosphate) and substitution (*O*-glucosides) **(LETHAM and PALNI, 1983)**. The authors suggested that these metabolites could be active forms of cytokinin, translocation forms, storage forms (releasing free cytokinin when needed), or detoxification products (formed when exogenous levels become too high and toxic), amongst others. According to **STRNAD (1997)**, there is indirect evidence suggesting that the free cytokinin bases are most likely the biologically active forms of cytokinins. That being the case, the interconversion of cytokinin bases, nucleosides and nucleotides is an important process in the regulation of cytokinin activity **(CHEN, 1997; BAJGUZ and PIOTROWSKA, 2009)**. The stereo- and/or regiospecific hydroxylation of isoprenoid and aromatic cytokinins, respectively, is another factor that could regulate cytokinin activity **(KAMÍNEK *et al*., 1979; KAMÍNEK *et al*., 1987a; STRNAD, 1997)**. **KAMÍNEK *et al*. (1987a)** observed that the hydroxylation of the phenyl ring of N^6-benzyladenosine in *meta*-position increased cytokinin activity in tobacco callus and wheat leaf chlorophyll retention bioassays. On the other hand, they noted that the hydroxylation of the phenyl ring in *ortho*- and *para*-positions significantly decreased cytokinin activity, suggesting a possible regulation of cytokinin biological activity by a position specific hydroxylation of the side chain.



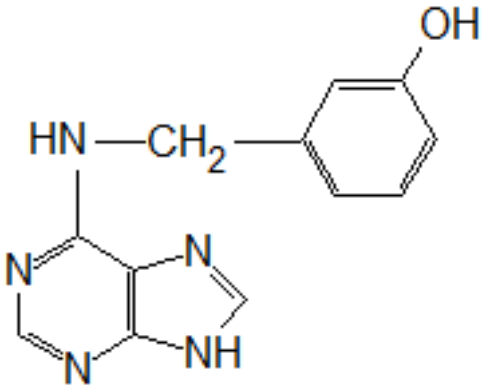
6-Benzyladenine (BA)



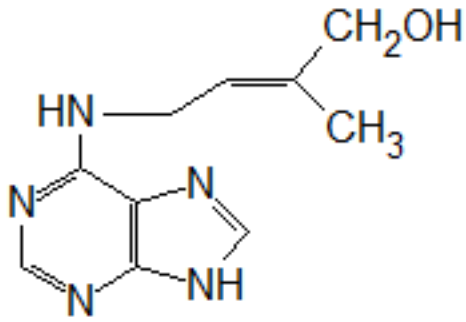
Dihydrozeatin (DHZ)



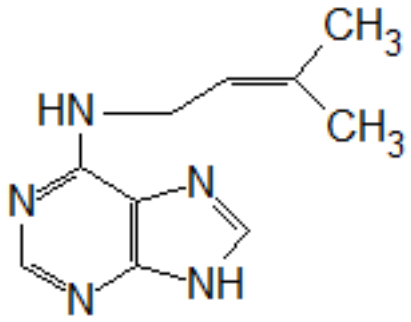
6-Furfurylaminopurine (Kinetin)



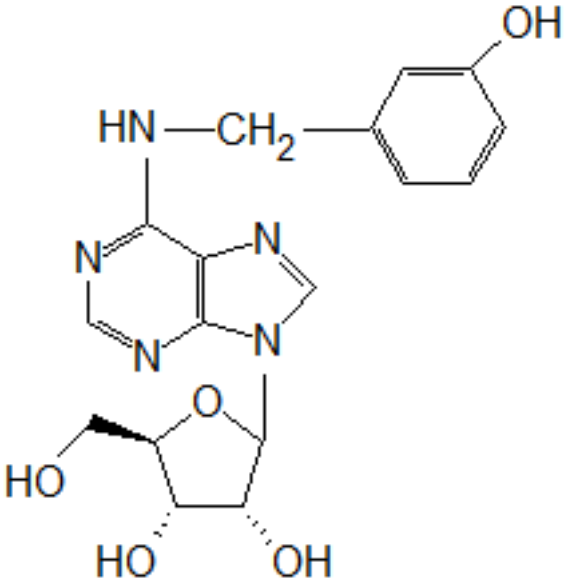
meta-Topolin (*m*T)



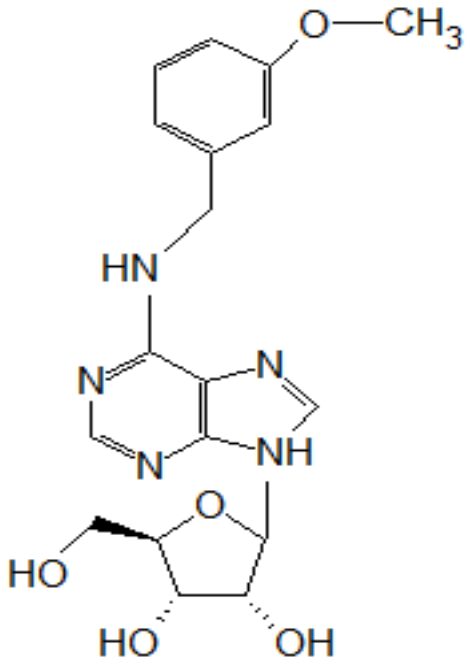
trans-Zeatin (Z)



N^6-Isopentenyladenine (iP)



meta-Topolin riboside (*m*TR)



meta-Methoxytopolin riboside (Me*m*TR)

Figure 2.2: Molecular structures of some cytokinins commonly used in plant tissue culture.

STRNAD (1997) noted that the glucosides, unlike the ribosyl and alanyl conjugates, are not limited to the N^9 position of the purine ring, hence they appear to be the most prominent among the cytokinin conjugates. Owing to this, according to the author, cytokinin glycosides are widespread in the plant kingdom and exist in different metabolic forms. They play indispensable roles in plant growth and developmental processes. In many experiments involving the exogenous application of BA, for example, *N*-glucoside metabolites such as [3G]BA, [7G]BA and [9G]BA have been identified in plant cells and tissues, the latter two usually being the major ones **(LETHAM and PALNI, 1983; WERBROUCK *et al*., 1995; VAN STADEN and CROUCH, 1996)**. BA is an aromatic cytokinin most widely used in the micropropagation industry due to its effectiveness and inexpensiveness **(BAIRU *et al*., 2007)**. The [3G]BA metabolite reportedly exhibited a markedly greater cytokinin-like activity in different bioassays compared to [7G]BA and [9G]BA, but a weak activity when compared to its corresponding base (see the review by **VAN STADEN and CROUCH, 1996)**. As demonstrated in radish cotyledons **(LETHAM and GOLLNOW, 1985)** and soybean callus **(VAN**

STADEN and DREWES, 1992), the relatively high activity of [3G]BA is likely due to cleavage of the 3-glucoside moiety to release free BA **(VAN STADEN and CROUCH, 1996)**. On the other hand, the metabolites [7G]BA and [9G]BA are said to be products of irreversible inactivation of cytokinins which regulate levels of the active free bases **(STRNAD, 1997)**. They are extremely stable in plant tissues and biologically relatively inactive, especially [9G]BA **(LALOUE, 1977; AUER *et al*., 1992)**. Indeed, the accumulation of [9G]BA in the basal part of *Spathiphyllum floribundum* was reported to be responsible for heterogeneity in growth and inhibition of rooting during the acclimatization of this species **(WERBROUCK *et al*., 1995)**. **BAIRU *et al* (2007)** were of a similar opinion that the negative effect of BA on *ex vitro* growth of *Aloe polyphylla* could be due to the accumulation of the stable derivative, [9G]BA.

WERBROUCK *et al*. (1996) suggested two alternatives to the use of BA in micropropagation systems due to the problems associated with its exogenous application. One is the use of BA derivatives which are already conjugated at the N^9 position (such as [9R]BA) as they are better protected against N^9-glucosylation. The second alternative is the use of hydroxylated BA analogues, mainly the topolins. Many researchers have indeed demonstrated the superiority of certain topolins over some commonly used cytokinins in micropropagation of different species. **KUBALÁKOVÁ and STRNAD (1992)**, for example, observed a high shoot formation frequency in sugar beet on medium containing *meta*-topolin (*m*T) compared to BA and zeatin. **WERBROUCK *et al*. (1996)** reported improved rooting in *Spathiphyllum floribundum* with the use of *m*T compared to BA. The use of *m*T improved shoot multiplication of *Curcuma longa* compared to BA, kinetin and zeatin **(SALVI *et al*., 2002)**. Other reported effects of topolins include improved survival rate of *in vitro* grown potato **(BAROJA-FERNÁNDEZ *et al*., 2002)**, higher multiplication rates of plantain by *m*T **(ESCALONA *et al*., 2003)**, histogenic stability of *Petunia* meristem and anti-senescing activity of rose cultures by Me*m*TR **(BOGAERT *et al*., 2006)**, improved multiplication and control of hyperhydricity in *Aloe polyphylla* by *m*T **(BAIRU *et al*., 2007)**, superior multiplication rates by *m*T and *m*TR in two banana cultivars **(BAIRU *et al*., 2008)** and lower percentages of necrotic shoot-tips in *Harpagophytum procumbens* in treatments containing *m*TR **(BAIRU *et al*., 2009)**.

In general, the effectiveness of topolins has been attributed to its distinct metabolism from that of BA **(STRNAD, 1997)**. Their metabolites in plant tissues are mainly the *O*-glucosides, a glucosylation made possible by the hydroxy group on the benzyl ring **(WERBROUCK *et al*., 1996; MALÁ *et al*., 2009)**. **STRNAD (1997)** stated that these metabolites appear to be biologically active intrinsically or as products of β-glucosidase activity **(LETHAM and PALNI, 1983)** and are not clear substrates for cytokinin oxidase. Furthermore, studies have revealed that the *O*-glucosides may be storage forms of cytokinin, stable under certain conditions and rapidly converted to biologically active forms when required **(PARKER *et al*., 1978; WERBROUCK *et al*., 1996; STRNAD, 1997)**. **STRNAD (1997)** therefore concluded that the reversible sequestration of aromatic cytokinin *O*-glucosides may confer stability and continuous availability over an extended period of time at a physiologically active level, resulting in improved shoot multiplication in plants cultured *in vitro*.

Many developmental processes in plants such as cell growth, cell division and differentiation as well as organogenesis in tissue and organ cultures are controlled by an interaction between cytokinins and auxins **(GASPAR *et a*l., 1996)**. For instance, it is well known that a high cytokinin to auxin ratio generally favours shoot production whereas a high auxin to cytokinin ratio favours root formation. It should be noted however, that the cytokinin levels could be regulated by auxin levels **(KAMÍNEK *et al*., 1997)**. These authors noted that auxin may down regulate cytokinin levels on one hand, by inhibiting cytokinin biosynthesis. On the other hand, auxin may promote cytokinin metabolic inactivation by *N*-glucosylation or degradation by cytokinin oxidase. Either way, auxin can strikingly decrease cytokinin to auxin ratio and induce certain physiological events **(KAMÍNEK *et al*., 1997)**.

2.1.8 Environmental factors affecting micropropagation

2.1.8.1 Light

Light duration (photoperiod), intensity (photosynthetic photon flux, PPF) and spectral quality are among the important environmental factors affecting plant

growth and development throughout the culture period **(MURASHIGE, 1974)**. They regulate photomorphogenetic processes in tissue cultures and can lead to improved plant quality and growth rate **(ECONOMOU and READ, 1987)**. Their influence on the levels of endogenous plant growth regulators may elicit different morphological responses in different plant species **(MACHÁČKOVA *et al*., 1992; TAPINGKAE and TAJI, 2000)**. **KAMADA *et al*. (1995)** reported adventitious bud formation in root explants of horseradish treated with auxin alone and cultured under a 16 h photoperiod. No adventitious buds were produced when the cultures were incubated under continuous darkness. They further observed that treatment with cytokinin alone induced bud formation under both continuous dark and 16 h light conditions. They attributed their results to light inducing either the activation of cytokinin biosynthesis or reduction of cytokinin degradation, resulting in an increased endogenous cytokinin level relative to that of auxin.

Shoot multiplication reportedly improved in shoot-tip cultures of *Pelargonium* species **(DEBERGH and MAENE, 1977)** and leaf explants of *Peperomia scandens* **(KUKLCZANKA *et al*., 1977)** maintained under continuous light. On the other hand, **ECONOMOU and READ (1986)** observed a decrease in the shoot length and quality during the first reculture (transfer without subdivision), and in number of shoots during the second reculture of azalea (*Rhododendron* species) placed under continuous light. **JO *et al*. (2008)** noted that the shoot length, root length, number of roots, leaf area, plant fresh weight, dry weight, chlorophyll and carotenoid content in *Alocasia amazonica* increased under shorter day (8/16 h) and equinoctial (12/12 h) (light/dark cycle) photoperiods. **MORINI *et al*. (1991)** however, observed that although shoot production in plum (*Prunus cerasifera*) was not statistically different between 12 and 16 h photoperiods, the 8 h photoperiod gave a much lower shoot production rate. In addition, **PATERSON and ROSE (1979)** reported better shoot production in leaf explants of *Crassula argenta* cultured under constant darkness. It is therefore evident that different photoperiods may elicit different morphogenic response in different species or explants.

ECONOMOU and READ (1987), in their review, showed that root formation may be inhibited by light duration in some species. They listed some factors likely to be responsible for such responses which include insufficient IAA synthesis or IAA

breakdown, photo-inactivation of factors promoting root formation, inhibition of rooting cofactor synthesis (phenolic compounds) or stimulation of cofactor breakdown as well as production of anatomical or histological barriers.

JO *et al*. (2008) observed that the shoot length, bulb size, leaf area, as well as fresh and dry matter yields in plants regenerated from *Alocasia amazonica* leaf explants, cultured under very low PPF (15 and 30 µmol m^{-2} s^{-1}) were more or less the same. These values however decreased in plants cultured at higher PPF (60 and 90 µmol m^{-2} s^{-1}). Conversely, **ECONOMOU and READ (1986)** reported increased number, length and quality of shoots obtained from *in vitro*-derived shoot explants of *Rhododendron* species cultured under PPF of 30 and 75 µmol m^{-2} s^{-1} compared to those cultured under a 10 µmol m^{-2} s^{-1} PPF. In a similar vein, **TAPINGKAE and TAJI (2000)** recorded a significant increase in the dry weight of *Anigozanthos bicolor* roots with an increase in PPF (up to 200 µmol m^{-2} s^{-1}). They further observed the highest number of roots under a white light while yellow and red lights significantly increased root length, suggesting the participation of the phytochrome system in the rhizogenesis of this species. The authors were of the opinion that the rate of auxin breakdown in the media over time may be faster under red and yellow light conditions, resulting in increased root elongation.

2.1.8.2 Temperature

In order to derive the most benefits from the tissue culture propagation of a particular plant species, its precise temperature needs must be met **(MURASHIGE, 1974)**. This is particularly important as the activities of the enzymes involved in many biochemical reactions increase at optimum temperatures. On the other hand, low temperature could modify the levels of plant growth regulators by stimulating the activity of cytokinin oxidase **(VESELOVA *et al*., 2005)**, prolong cell cycle process and activate cold acclimation signalling pathways **(XIA *et al*., 2009)**. In general, *in vitro* cultures are often maintained at a constant temperature of approximately 25°C in many micropropagation protocols. In an experiment carried out on four accessions of mint plants (*Mentha* spp.), **ISLAM *et al*. (2005)** observed that both the apical and nodal explants cultured at 25°C exhibited better *in vitro* growth compared to those cultured at 20°C.

HASEGAWA *et al* (1973) reported a constant temperature of 27°C as the optimum temperature for *in vitro* plant formation of *Asparagus officinalis* through shoot apex culture. Although many annual and tropical species whose life cycles are completed during a period of relatively uniform temperature conditions may respond well under constant temperatures, some other species such as those adapted to temperate and desert climates may respond better under periodically varied temperature conditions **(MURASHIGE, 1974)**. **KOZAI *et al* (1995)** reported increases in stem length and internode length of *Solanum tuberosum* cultures as the difference in photoperiod and dark period temperatures (DIF) increased. An increase in plantlet growth with increased DIF was similarly reported in *Rehmannia glutinosa* **(CUI *et al*., 2001)**.

2.1.9 Tissue culture of the families: Acanthaceae and Asclepiadaceae

With the exception of published articles from this research, the available literature reveals no published reports on the *in vitro* propagation of *Barleria* species and *Huernia* species. However, successful micropropagation has been reported in the families Acanthaceae and Asclepiadaceae (Table 2.1), to which the genera *Barleria* and *Huernia* belong. As Table 2.1 shows, different parts of a plant can serve as explant sources and regeneration can be accomplished through axillary shoot proliferation, adventitious shoot production and/or somatic embryogenesis. In addition, the production of valuable secondary metabolites was reported in induced callus in some cases.

Table 2.1: List of some tissue cultured plant species in the Acanthaceae and Asclepiadaceae families

Plant species	Explant	Response	Reference
Acanthaceae			
Adhatoda beddomei	Node	Axillary shoots, callus	**SUDHA and SEENI (1994)**
Adhatoda vasica	Shoot-tip	Axillary shoots	**ANAND and BANSAL (1998)**
Adhatoda zeylanica	Leaf, petiole	Callus, somatic embryos, roots	**JAYAPAUL *et al*. (2005)**
Andrographis paniculata	Leaf, internode	Callus, somatic embryos	**MARTIN (2004a)**
Aphelandra sinclairiana	Anther, root, leaf	Callus	**NEZBEDOVÁ *et al*. (1999)**
Aphelandra tetragona	Petiole, leaf	Callus	**NEZBEDOVÁ *et al*. (1999)**
Crossandra infundibuliformis	Bud	Axillary shoots	**GIRIJA *et al*. (1999)**
Justicia gendarussa	Leaf, node	Callus, adventitious shoots	**RAGHUVANSHI *et al*. (1994), AGASTIAN *et al*. (2006)**
Nilgirianthus ciliatus	Leaf, node, internode	Callus, adventitious shoots	**DEVI and KAMALAM (2007)**
Asclepiadaceae			
Asclepias curassavica	Node	Shoots, roots, callus	**PRAMANIK and DATTA (1986)**
Calotropis gigantean	Hypocotyl, immature embryo, stem, leaf	Callus, adventitious shoots	**ROY *et al*. (2000), ROY and DE (1990)**
Calotropis procera	Cotyledon	Callus, laticifer differentiation	**SURI and RAMAWAT (1996)**

Table 2.1 continued

Plant species	Explant	Response	Reference
Ceropegia bulbosa var. bulbosa	Node, stem, leaf, bud	Axillary shoots, adventitious shoots, callus, flowering, microtubers, somatic embryos	**PATIL (1998), NAIR *et al*. (2007), BRITTO *et al.* (2003)**
Ceropegia bulbosa var. lushii	Node, stem, leaf	Axillary shoots, callus, microtubers, somatic embryos	**PATIL (1998)**
Ceropegia candelabrum	Node, leaf, internode	Axillary shoots, callus, somatic embryos	**BEENA *et al*. (2003), BEENA and MARTIN (2003)**
Ceropegia hirsuta	Bud	Axillary shoots, adventitious shoots, flowering	**NAIR *et al*. (2007)**
Ceropegia jainii	Node, stem, leaf	Axillary shoots, callus, microtubers, somatic embryos, flowering	**PATIL (1998)**
Ceropegia juncea	Node, internode	Axillary shoots, callus, adventitious shots, roots	**NIKAM and SAVANT (2009)**
Ceropegia lawii	Bud	Axillary shoots, adventitious shoots, flowering	**NAIR *et al*. (2007)**
Ceropegia maccannii	Bud	Axillary shoots, adventitious shoots, flowering	**NAIR *et al*. (2007)**
Ceropegia oculata	Bud	Axillary shoots, adventitious shoots, flowering	**NAIR *et al*. (2007)**
Ceropegia sahyadrica	Bud	Axillary shoots, adventitious shoots, flowering	**NAIR *et al*. (2007)**

Table 2.1 continued

Plant species	Explant	Response	Reference
Cryptolepis buchanani	Leaf, internode	Callus	**VENKATESWARA *et al*. (1987)**
Decalepis hamiltonii	Leaf, shoot-tip	Callus, somatic embryos, adventitious shoots	**GIRIDHAR *et al.* (2004, 2005)**
Gymnema sylvestre	Node, hypocotyl, cotyledon, leaf	Axillary shoots, callus,somatic embryos	**KOMALAVALLI and RAO (2000), ASHOK KUMAR *et al*. (2002)**
Hemidesmus indicus	Leaf, root, node, shoot-tip, root	Axillary shoots, callus, somatic embryogenesis, adventitious shoots	**PATNAIK and DEBATA (1996), SARASAN *et al.* (1994), MISRA *et al*. (2005), RAMULU *et al.* (2003)**
Holostemma ada-kodien	Node, internode, leaf	Axillary shoots, callus, adventitious shoots, somatic embryos	**MARTIN (2002), MARTIN (2003)**
Holostemma annulare	Shoot-tip, node	Axillary shoots, callus	**SUDHA *et al*. (1998)**
Leptadenia reticulata	Node, leaf, internode, shoot tip	Axillary shoots, callus, somatic embryos	**ARYA *et al*. (2003), MARTIN (2004b)**
Pergularia daemia	Shoot-tip, node	Shoots	**KIRANMAI *et al*. (2007)**
Sarcostemma brevistigma	Node	Axillary shoots, callus, adventitious shoots	**THOMAS and SHANKAR (2009)**
Tylophora indica	Stem, leaf, node, petiole	Callus, somatic embryos, axillary shoots, adventitious shoots	**RAO and NARAYANASWAMI (1972), MANJULA *et al*. (2000), SHARMA and CHANDEL (1992), THOMAS and PHILIP (2005), FAISAL *et al*. (2005)**

2.2 Pharmacological and phytochemical investigation of plant extracts

2.2.1 Introduction

The use of natural products in disease prevention and control as well as in drug development has received increased attention in recent times. According to **RATES (2001)**, about 25% of the globally prescribed drugs are obtained from plants. The author observed that 11% of the 252 drugs considered as basic and essential by the World Health Organisation are solely of plant origin. This growing interest in the therapeutic use of drugs of plant origin has been attributed to some factors such as (i) the side effects and ineffective therapy of conventional medicine, (ii) the incorrect and/or abusive use of synthetic drugs resulting in undesired side effects and other problems, (iii) the inaccessibility of a large part of the world's population to conventional pharmacological treatment, and (iv) the suggestion created by folk medicine and ecological awareness that natural products are often harmless **(RATES, 2001)**. In many parts of the world, especially in Africa, the increasing use of plants in traditional medicines by a growing population, among other reasons, has resulted in the over-exploitation of many of our plant resources.

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 **(BALUNAS and KINGHORN, 2005)**. However, a large percentage of the estimated 350, 000 plant species on earth is yet to be investigated for their pharmacological and phytochemical potential **(HOSTETTMAN and MARSTON, 2002)**. **SHAI *et al*. (2008)** for example, noted that some South African indigenous plant species are at risk of becoming extinct before the investigation and application of their potential as sources of therapeutic drugs can be carried out. **HOSTETTMANN and MARSTON (2002)** recommended that urgent attention be given to the exploration of this 'green inheritance' for their pharmacological and phytochemical potential, especially due to the rapid disappearance of our tropical forests and other important vegetation areas. Some of these plant resources (including those rapidly disappearing) could contain novel, active chemotypes that can serve as leads for effective drug development **(CRAGG *et al*., 1997)**.

The dual chemical-biological screening approach has been recommended as the fastest method of discovering important plant-derived bioactive compounds **(HOSTETTMAN and MARSTON, 2002)**. The localisation of such active compounds however, requires the use of simple but sensitive target-specific bioassays, since plant extract often contains low concentrations of active compounds **(RATES, 2001; HOSTETTMAN and MARSTON, 2002)**. The main targets for biological tests can be lower organisms (bacteria, fungi and viruses), invertebrates (insects, crustaceans and molluscs), isolated subcellular systems (enzymes and receptors), animal or human cell cultures, isolated organs of vertebrates, or whole animals **(HOSTETTMAN and MARSTON, 2002)**. Furthermore, since plants often contain a large number of promising compounds, there is a need to use a variety of test systems in order to detect other potential bioactivities **(RATES, 2001; HOUGHTON *et al*., 2007)**. The use of solvents of increasing polarity during extraction processes has also been recommended, especially when the chemical composition is unknown **(WILLIAMSON *et al*., 1996**; cited by **RATES, 2001)**.

2.2.2 Antimicrobial activity

Some bacteria and fungi have been implicated in the pathology of many diseases. For example, the fungus *Candida albicans* is known to be a pathogenic organism causing candidosis **(SHAI *et al*., 2008)**, while bacteria such as *Eschericha coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* are known to be involved in gastroenteritis or respiratory infections **(MOISE and SCHENTAG, 2000; SUN *et al*., 2006)**. The occurrence of new and re-emerging infectious diseases with no effective therapies as well as the development of resistant pathogen strains to some currently used drugs necessitate the urgent and continuous search for potent and efficacious antimicrobial compounds **(CRAGG *et al*., 1997)**.

In the biological screening of plants for antibacterial activity, **COS *et al.* (2006)** recommended the use of a panel of test organisms consisting of, at least, a Gram-positive (such as *S. aureus* and *Bacillus subtilis*) and a Gram-negative (such as *E. coli* and *K. pneumonia*) bacterium. The use of yeasts (such as *C. albicans*), dermatophytes (such as *Trichophyton mentagrophytes*) and opportunistic

filamentous fungi (such as *Fusarium solani*), are among the recommended fungi in the screening for antifungal activity **(COS *et al*., 2006)**. These authors further suggested the use of American type culture collection (ATCC) strains as they are well-characterized.

Besides the choice of test organisms, of vital importance is the choice of an appropriate screening method. The three commonly used antimicrobial screening methods are (i) agar-diffusion, (ii) bio-autographic, and (iii) dilution assays **(COS *et al*., 2006; VAN VUUREN, 2008)**. The agar-diffusion method is said to be simple and can be used to screen a large number of test samples against one micro-organism **(RÍOS and RECIO, 2005; VAN VUUREN, 2008)**. However, the differences in physical properties of test samples, such as the solubility, volatility and diffusion characteristics in agar, make it difficult to effectively compare the antimicrobial activity of different samples using the agar-diffusion method **(COS *et al*., 2006)**. The bio-autographic assay, although highly sensitive, requires the complete removal of residual low volatile solvents and is applicable mainly to micro-organisms that easily grow on thin-layer-chromatographic (TLC) plates **(COS *et al*., 2006)**. It is therefore mainly used in the isolation of bioactive antimicrobial compounds **(VAN VUUREN, 2008)**. The dilution assay allows for the quantitative determination of the minimum inhibitory concentrations (MIC) for comparing the antimicrobial potency of test samples **(VAN VUUREN, 2008)**. In addition to its usefulness in assaying polar and non-polar extracts as well as isolated compounds, the dilution assay method can be used to determine whether an extract or a compound has a lethal (-cidal) or static action at a particular concentration **(COS *et al*., 2006)**. In general, as benchmarks for determining the potency of extracts, extracts with MIC values below 8 g/ml are considered as having some antimicrobial activity **(FABRY *et al*., 1998)** while those with MIC values less than 1 mg/ml are regarded as exhibiting interesting activity **(RÍOS and RECIO, 2005)**.

Antimicrobial activity has been reported in some *Barleria* species. For example, **MAREGESI *et al*. (2008)** reported a MIC value of 1 mg/ml in *B. eranthemoides* root methanolic extract tested against *Bacillus cereus*. **CHOMNAWANG *et al*. (2009)** reported MIC values of 5.0, 2.5 and 5 mg/ml in *B. lupulina* ethanolic extract

tested against *S. aureus*, *S. epidermidis* and methicillin-resistant *S. aureus*, respectively. Unspecified extracts of *B. lupulina* were reported to have minimum bactericidal concentration (MBC) values of 1.25 and 5.0 mg/ml against *Propionibacterium acnes* and *S. epidermidis*, respectively **(CHOMNAWANG *et al*., 2005)**. **KOSMULALAGE *et al*. (2007)** reported antibacterial activity of crude ethanolic extract of the aerial part of *B. prionitis* against *S. aureus* and *Pseudomonas aeruginosa* using the paper disk diffusion method. The authors further reported the antibacterial activity of three compounds isolated from this ethanolic extract against *Bacillus cereus* and *P*. *aeruginosa* (25 µg/disk) using the same method. As far as can be ascertained from the available literature however, no report has been made (with the exception of published materials from this thesis) on the antibacterial and antifungal activities of the different parts of the three *Barleria* species in the present study as well as of any *Huernia* species.

2.2.3 Anti-inflammatory activity

Inflammation is a process implicated in the pathogenesis of many diseases like Alzheimer's disease, asthma and auto-immune diseases such as rheumatoid arthritis, multiple sclerosis and ulcerative colitis **(HOWES and HOUGHTON, 2003; POLYA, 2003)**. It is an immunological response often elicited by tissue injury from bacterial and viral infection, wounding and other sources of damage **(POLYA, 2003; BYEON *et al*., 2008)**. Currently, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in the symptomatic treatment of inflammation **(VIJI and HELEN, 2008)**. In fact, NSAIDs are reported to be among the most widely used drugs worldwide **(FIORUCCI *et al*., 2001; STEINMEYER and KONTTINEN, 2006)**. Nevertheless, the unwanted side effects such as gastric intolerance, water and electrolyte retention, bone marrow depression and cardiovascular risks associated with the use of these drugs **(XIAO *et al*., 2005; STEINMEYER and KONTTINEN, 2006)** have necessitated further search for 'classical' anti-inflammatory drugs with reduced or no side effects.

Inflammation is a complex process involving the release of highly active pro-inflammatory mediators such as prostaglandins, leukotrienes, histamine, cytokines and free radicals **(ZSCHOCKE *et al*., 2000a)**. Prostaglandins, such as PGE_2,

PGI_2, $PGF_{2\alpha}$, are known to sensitize primary afferent nociceptive nerve fibers, which contributes to inflammatory pain **(KONTTINEN *et al*., 1994)**. The release of prostaglandins from arachidonic acid is catalysed by the action of the cyclooxygenase (COX) enzyme (also known as prostaglandin-H_2-synthase, PGH_2 synthase) **(STEINMEYER, 2000)**. The bi-functional COX enzyme catalyses the oxygenation of arachidonic acid (hydrolytically released from membrane phospholipids) to form the cyclic prostaglandin endoperoxide PGG_2, followed by a peroxidase reaction which results in the formation of PGH_2 **(FRÖLICH, 1997; STEINMEYER, 2000)**. PGH_2 is the precursor of other bioactive prostaglandins and thromboxanes **(STEINMEYER, 2000)**. NSAIDs are known to inhibit the production of prostaglandins mainly by inhibiting COX activity **(VANE, 1994)**.

The COX enzyme is known to exist mainly in two isoforms (COX-1 and COX-2), although the possible existence of a third isoform (COX-3) has been hypothesized **(BERENBAUM, 2004)**. The COX-1 and COX-2 isoenzymes are said to have approximately 60% homology in their nucleic acid and amino acid structures **(VANE, 1994)**. COX-1 is identified to be constitutively expressed in most tissues with prostaglandin synthesis for maintaining some physiological functions such as gastric mucosa protection and renal perfusion maintenance **(LI *et al*., 2006)**. On the other hand, COX-2 was thought to be induced only under pathological conditions and thus is solely responsible for the production of inflammatory prostanoid mediators **(STEINMEYER and KONTTINEN, 2006)**. This concept led many researchers to start focussing on selective COX-2 inhibitors. However, recent published studies have shown that COX-2 is also constitutively expressed in some body parts such as the brain, spinal cord, kidney and uterus **(STEINMEYER and KONTTINEN, 2006)**. As illustrated in Figure 2.3, COX-2 isoform has both physiological and pathophysiological roles. Furthermore, the use of some selective COX-2 inhibitors have been reported to carry a risk of gastro-intestinal toxicity in some patients **(MACAULAY and BLACKBURN, 2002; BERTIN, 2004; WARNER and MITCHELL, 2008)**. **STEINMEYER and KONTTINEN (2006)** further stated some undesirable side effects such as water and electrolyte retention, delayed wound healing (in animal experiments) and cardiovascular risks reported with the use of selective COX-2 inhibitors. According to these authors, several selective COX-2 inhibitors have already been withdrawn

from the market due to their cardiovascular risk and at present, all COX-2 selective NSAIDs are surmised to be likely causing heart attacks, strokes and thromboses. They therefore recommended the classical combination of a non-selective NSAID with a proton pump inhibitor (such as omeprazol) or misoprostol as a viable, low-cost alternative to COX-2 selective NSAIDs in the prophylaxis of the NSAID-gastropathy.

On the other hand, the inhibition of COX enzyme is known to up-regulate the production of leukotrienes (LTs) from arachidonic acid through the 5-lipoxygenase (LOX) enzyme pathway **(FIORUCCI *et al*., 2001)**. Leukotrienes are involved in the pathophysiology of chronic inflammation and allergic diseases such as rheumatoid arthritis, asthma as well as skin diseases like psoriasis **(ZSCHOCKE *et al*., 2000a)**. The leukotriene LTB_4, in particular, has been reported to have a pathophysiological function in the development of gastrointestinal ulcers **(ASAKO *et al*., 1992; cited by FIORUCCI *et al*., 2001)**. Many authors have therefore suggested that the use of compounds that are dual inhibitors of COX and LOX enzymes could enhance anti-inflammatory effects with reduced undesirable side effects **(ZSCHOCKE *et al*., 2000a; FIORUCCI *et al*., 2001; LI *et al*., 2006; VIJI and HELEN, 2008)**. These authors were of the opinion that dual inhibitors of COX and LOX enzymes could be a potential viable alternative to standard NSAIDs and selective COX-2 inhibitors.

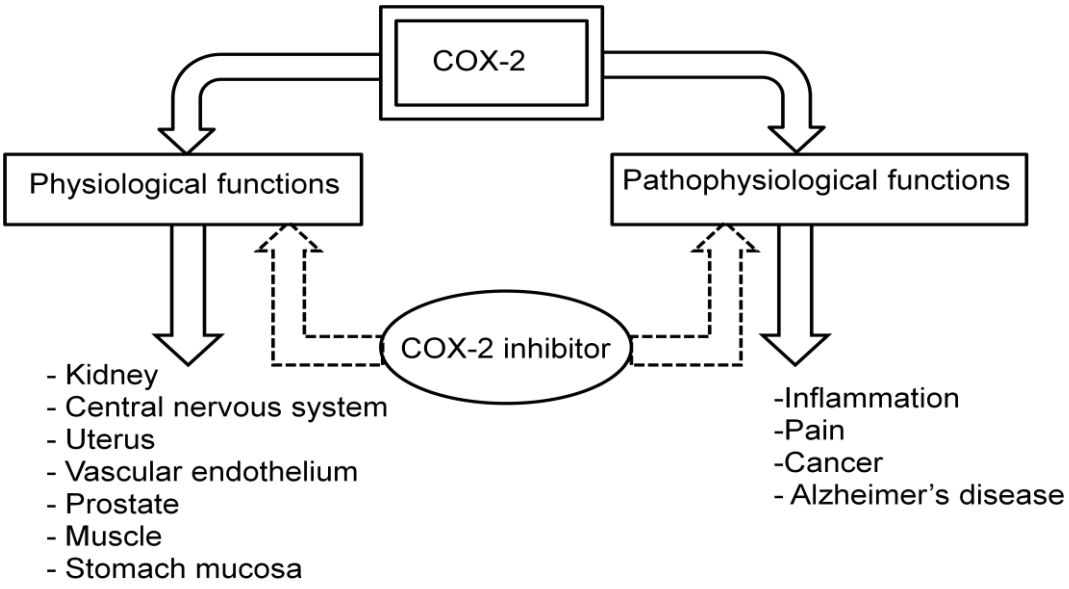


Figure 2.3: The physiological and pathophysiological functions of COX-2 enzyme **(STEINMEYER, 2000).**

Many researchers have reported anti-inflammatory activity in plant extracts using different *in vivo* animal models in their evaluation. **SINGH *et al*. (2003)** demonstrated the anti-inflammatory activity of the *n*-butanol and aqueous fractions of the whole *B. prionitis* plant against carrageenan-induced oedema in rats. **WANIKIAT *et al*. (2008)** reported the anti-inflammatory effect of *B. lupulina* methanolic extract in carrageenan-induced paw oedema and ethyl phenylpropiolate-induced ear oedema in rats. However, according to **TALHOUK *et al*. (2007)**, studies reporting anti-inflammatory activities of crude plant extracts in whole animal models demonstrate gross anti-inflammatory activities but typically fall short of identifying possible intracellular targets. Some of the common key molecules at the cellular level in the inflammatory cascade that are targeted by plant extracts include nuclear factor-κB, cytokines and COX **(TALHOUK *et al*., 2007)**. Assays involving enzymes are generally known to be highly specific, very sensitive and mechanism-based, and they are valuable in screening large numbers of samples as they are relatively easy and small amounts of material are required **(HOSTETTMANN and MARSTON, 2002)**.

2.2.4 Acetylcholinesterase inhibition

Acetylcholinesterase (AChE) is an enzyme involved in the hydrolysis of acetylcholine into a choline and acetyl group **(DOHI *et al*., 2009)**. Acetylcholine is a neurotransmitter at all preganglionic autonomic, parasympathetic and some sympathetic postganglionic nerve endings, neuromuscular junctions and at some central nervous system (CNS) synapses **(KOSMULALAGE *et al*., 2007)**. Neurotransmitter disturbances and low cholinergic function are among the pathological features involved in CNS disorders **(HOWES and HOUGHTON, 2003)**. The inhibition of the AChE enzyme, leading to the maintenance of acetylcholine level and enhanced cholinergic function has become the standard approach in the symptomatic treatment of Alzheimer's disease (AD) **(HOWES and HOUGHTON, 2003; VINUTHA *et al*., 2007)**. AD is the most common type of neurodegenerative disease, characterized primarily by impaired memory and cognitive dysfunction, and at advanced stages, language deficit, depression, agitation, mood disturbances and psychosis **(HOUGHTON *et al*., 2007)**. The undesirable side effects such as hepatotoxicity in the therapeutic use of some

AChE inhibitors (like tacrine) coupled with their limitation to symptom alleviation has necessitated further search for more potent drugs **(LÓPEZ *et al*., 2002; HOWES and HOUGHTON, 2003; FERREIRA *et al*., 2006)**.

KOSMULALAGE *et al*. (2007) recently reported moderate AChE inhibitory activities of four compounds isolated from ethanolic extract of *B. prionitis*. These compounds were identified to be balarenone, pipataline, lupeol and 13,14-*seco*-stigmasta-5,14-diene-3-α-ol. They also reported a significant AChE inhibitory activity with an IC_{50} value of 36.8 µM in the compound 8-amino-7-hydroxypipataline, a synthetic derivative of pipataline. **ATA *et al.* (2009)** reported the isolation of a new phenylethanoid glycoside (barlerinoside) along with six known iridoid glycosides (shanzhiside methyl ester, 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester, barlerin, acetylbarlerin, 7-methoxydiderroside and lupulinoside) from the ethanolic extract of *B. prionitis* aerial parts. These compounds were reported to exhibit weak AChE inhibitory activities with IC_{50} values of 175.9, 133.5, 150.0, 100.0, 145.8, 190.0 and 178.9 µM, respectively. The possibility of a synergistic AChE inhibition of two or more of these compounds cannot be completely ruled out. Synergistic interaction was reported to be responsible for stronger AChE inhibition in *Salvia lavandulaefolia* essential oil compared with that of its constituent terpenes **(SAVELEV *et al*., 2003)**. The authors also reported both synergistic and antagonistic responses in some combinations of this plant terpene constituent. Taken together however, the reported results on *B. prionitis* perhaps suggest the possibility of discovering potential effective AChE inhibitors or leads from *Barleria* species. At present, there is no report available on the AChE inhibitory activities of the individual plant parts of *B. prionitis* and other *Barleria* species.

2.2.5 Antioxidant activity

Free radical reactions have been implicated in the pathology of a large number of disease states such as cancer, AD, diabetes, inflammation and several cardiovascular diseases **(HOUGHTON *et a*l., 2007)**. According to **HOUGHTON and HOWES (2003)**, the use of antioxidants has been shown to slow AD progression and neuronal degeneration. **HOUGHTON *et al*. (2007)** suggested the

inclusion of antioxidant and free radical scavenging tests, among others, in the biological screening of plants used for wound healing. This is due to the fact that the wound healing process is impaired by microbial infection and destruction of cells and tissues by reactive oxygen species (ROS) **(HOUGHTON *et al*., 2007; PATTANAYAK and SUNITA, 2008)**. ROS can damage cellular components and act as secondary messengers in the inflammation process **(FERREIRA *et al*., 2006)**. Antioxidants however, have the ability to scavenge ROS, attenuate inflammation pathways, reduce cancer, heart disease and other degenerative problems associated with aging **(HENRY *et al*., 2002; FERREIRA *et al*., 2006)**. The realization of antioxidant roles in the management of some disease states has led to the inclusion of antioxidant tests in many pharmacological screenings of plant extracts and isolated compounds. According to **HUANG *et al*. (2005)**, the major antioxidant assays can be divided into two categories, based on the chemical reactions involved. These two categories are (i) hydrogen atom transfer (HAT) reaction based assays and (ii) single electron transfer (ET) reaction based assays. In the HAT based assays, a synthetic free radical generator, an oxidizable molecular probe and an antioxidant are generally involved, and the assays measure the competitive kinetics of the reaction **(HUANG *et al*., 2005)**. The ET based assays measure the antioxidant capacity of an extract or a compound in the reduction of an oxidant, a probe indicating the reaction endpoint **(HUANG *et al*., 2005)**. Some examples of HAT based assays include inhibition of linoleic acid oxidation, ORAC (oxygen radical absorbance capacity) and Crocin bleaching assays while the ET based assays include FRAP (ferric ion reducing power assay), DPPH (1,1-diphenyl-2-picrylhydrazyl) and TEAC (Trolox equivalent antioxidant capacity) assays **(HUANG *et al*., 2005)**. The use of the DPPH assay in antioxidant screening is very common due to its simplicity and reproducibility **(KATSUBE *et al*., 2004)**. Nevertheless, due to the fact that antioxidant processes are complex, the use of at least two different types of assays in antioxidant activity study has been recommended **(MOON and SHIBAMOTO, 2009)**. These authors recommended the combination of assays for scavenging electrons or radicals with assays associated with lipid peroxidations.

2.2.6 Phytochemical property

Plants often contain secondary metabolites, many of which are known to demonstrate good biological activity in pharmacological screening. The common groups of secondary metabolites in plants include the phenolics, terpenes and alkaloids. Phenolic compounds are a class of secondary metabolites generally having an aromatic ring with one or more hydroxyl substituents **(ROBARDS *et al*., 1999)**. **ROBARDS *et al*. (1999)** defined phenolics based on their metabolic origin as substances derived from the shikimate pathway and phenylpropanoid metabolism. They are extremely diverse both in their structures and functions **(POLYA *et al*., 2003)**. The presence of -OH groups in the phenolic ring system allows for the polarity and water-solubility of phenolic compounds as well as their capacity for hydrogen bonding **(POLYA, 2003)**. The capacity of the phenolic group to be deprotonated and oxidised explains their biologically important antioxidant properties **(POLYA, 2003)**. Through covalent reaction with free radicals, especially the ROS, many phenolics can serve as good anti-inflammatory and antioxidant agents **(POLYA, 2003)**. Some common phenolics in plants include tannins (hydrolysable tannins and condensed tannins) and flavonoids (the most widespread and diverse of the phenolics) **(ROBARDS *et al*., 1999; POLYA, 2003)**.

Tannins are widely distributed defensive compounds in plants **(POLYA, 2003)**. According to **NIEMETZ and GROSS (2005)**, they are commonly classified into two categories: (i) condensed tannins (also known as proanthocyanidins), and (ii) hydrolysable tannins. Condensed tannins are essentially of flavonoid origin while hydrolysable tannins are further divided into two subclasses: (i) gallotannins, which involve a glucose esterified to gallic acid, and (ii) ellagitannins, which are ellagic acid-derived hexahydroxydiphenic acid **(POLYA, 2003)**. The distribution of gallotannins is rather limited in nature while ellagitannins are commonly found in many plant families **(NIEMETZ and GROSS, 2005)**. **OKUDA (2005)** stated that the profile of hydrolysable tannin in a plant species is generally stable throughout the year. **AKIYAMA *et al*. (2001)** reported the antibacterial activity of several tannins against 18 strains of *S. aureus*, including 11 strains that are methicillin-resistant. **ENGELS *et al*. (2009)** and **TIAN *et al*. (2009a, b)** observed antimicrobial

activity of gallotannins from *Mangifera indica* and *Galla chinensis*, respectively. The antimicrobial activity of hydrolysable tannins have been linked to (i) their ability to interact with proteins and inhibit enzyme activities, (ii) the damage of lipid bilayer membranes as demonstrated in *Helicobacter pylori*, and (iii) their ability to complex metal ions (such as iron) **(ENGELS *et al*., 2009)**. Furthermore, **OKUDA (2005)** stated that tannins are generally not mutagenic (based on Ames' test), but instead, they showed antimutagenic activity against certain mutagens. Their radical scavenging ability as well as the inhibition of lipid peroxidation and enzyme activities (such as LOX) are said to be the activities underlying the antioxidant and anti-inflammatory effects of tannins **(OKUDA, 2005)**.

Flavonoids are well characterized in plant extracts as the main compounds possessing anti-inflammatory activity **(TALHOUK *et al*., 2007)**. They inhibit a variety of molecules such as nuclear factor-κB (NF-κB), inducible nitric oxide synthase (iNOS), cytokines, COX, LOX and matrix metalloproteinases **(TALHOUK *et al*., 2007)**. **CHI *et al*. (2001)** reported various degrees of inhibition of COX-1, COX-2, 5-LOX and/or 12-LOX by 19 naturally occurring flavonoids isolated from medicinal plants. **TUNALIER *et al*. (2007)** reported both antioxidant and anti-inflammatory activities in flavonoid rich polar extracts of *Lythrum salicaria*. **PATTANAYAK and SUNITA (2008)** observed antibacterial and antifungal activities of *Dendrophthoe falcata* flavonoid-containing extracts, using 12 and 5 different bacterial and fungal strains, respectively. They concluded that the marked activity recorded was due to the presence of flavonoids and terpenes in the extracts.

Many authors have reported the isolation of iridoids in some *Barleria* species. **ATA *et al*. (2009)** reported the isolation of seven iridoids from the aerial parts of *B. prionitis*, with different levels of biological activity in glutathione *S*-transferase (GST) inhibition, AChE inhibition and free radical scavenging assays. **CHEN *et al.* (1998)** reported the isolation of some iridoids from *B. prionitis* with antiviral potency against the respiratory syncytial virus. **SINGH *et al*. (2004)** observed a significant and concentration-dependent hepatoprotective potential of the iridoid enriched fraction from *B. prionitis* extract. The isolation and characterization of iridoids have also been reported in other *Barleria* species such as *B. strigosa*

(KANCHANAPOOM *et al*., 2004) and *B. lupulina* **(KANCHANAPOOM *et al*., 2001)**. The anti-inflammatory activity of some plant extracts has been attributed to the presence of iridoids **(LEVIEILLE and WILSON, 2002)**. **BOLZANI *et al*. (1997)** have reported the antifungal property of certain iridoids such as gardiols. Iridoids generally are monoterpenes deriving biosynthetically from geranylpyrophosphate and can be found in free or glycoside form in plant extracts **(POLYA, 2003)**.

Chapter 3 *In vitro* propagation of *Barleria greenii*

3.1 Introduction

The application of tissue culture techniques in the propagation of ornamental plants has recorded a big stride in recent years and has become widely accepted as a standard practice in the horticultural industry **(READ and PREECE, 2009)**. At present, more than one billion traded ornamental plants are reportedly produced through tissue culture **(PRAKASH, 2009)**. A large percentage of this figure focuses on cut flowers and pot plants whereas ornamental perennials and garden plants account for only 2% of the total ornamental plants produced through tissue culture **(PRAKASH, 2009)**. It has been estimated that the global trade of ornamental perennials alone is about 8 billion US dollars per year compared to 90 and 60 billion US dollars for cut flower and pot plants, respectively **(PRAKASH, 2009)**. There is indeed a great need as well as a great potential for the application of tissue culture techniques in propagating ornamental perennials and garden plants.

The major challenges or setback in the application of tissue culture techniques to ornamental perennials and garden plants include the recalcitrant nature of plant tissues, often related to seasonal dormancy, establishment of clean explants, slow multiplication rate, poor rooting frequency, morphological aberrations and high cost of production **(PRAKASH, 2009)**. The choice of plant growth regulators has however been reported to play a crucial role in solving or alleviating most of these problems **(WERBROUCK *et al*., 1995, 1996; BAIRU *et al*., 2007, 2008, 2009)**. The use of topolins, for example, has been reported to increase shoot multiplication, maintain histogenic stability, improve rooting efficiency and subsequently reduce the production cost **(KUBALÁKOVÁ and STRNAD, 1992; WERBROUCK *et al*., 1996; BOGAERT *et al*., 2006)**. It is therefore important that the choice of appropriate plant growth regulators, amongst others, be given close attention when developing micropropagation protocols especially suitable for commercial application.

Barleria greenii (Family: Acanthaceae) is a perennial ornamental shrub, endemic to a specific area in the KwaZulu-Natal province, South Africa. **SCOTT-SHAW (1999)** described it as a successful garden plant with horticultural potential. This plant species was discovered in 1984 **(BALKWILL *et al*., 1990)** and is known to be found only in eight localities on three farms near Estcourt, South Africa **(MAKHOLELA *et al*., 2003)**. It is currently listed in the National Red List of South African plants as critically endangered **(SANBI, 2009)**. *Barleria greenii* can be grown from seed as well as from cuttings. The propagation from seed is often affected by low seed viability as a result of high seed parasitism. **MAKHOLELA *et al*. (2003)** observed that in instances where there might have been successful pollination and seed production, survival of the seed into the next generation is often prohibited by seed parasites, resulting in a highly reduced number of viable seeds. **SCOTT-SHAW (1999)** therefore suggested that where viable seeds are not available, propagation by cuttings, although difficult, is the best option.

This study was aimed at developing an effective micropropagation protocol, which can potentially provide a conservation measure for this endangered plant species. The effects of different cytokinins and photoperiods were also investigated in this study. As far as can be ascertained from the available literatures, there is no report to date on micropropagation of any *Barleria* species.

3.2 Materials and methods

3.2.1 Explant decontamination, selection and bulking

Shoot-tip and nodal explants were excised from stock plants (Figure 3.1A) maintained in the shade house in the University of KwaZulu-Natal Botanical Garden. The explants were thoroughly rinsed under running tap water before they were subjected to surface decontamination treatments. The treatments involved soaking the plant materials in 70% ethanol for 60 sec followed by 3.0% sodium hypochlorite solution for 20 or 30 min, or 3.5% for 20 min. In each solution, a few drops of Tween 20 were added as a surfactant. The surface decontaminated explants were cut into 10 mm lengths and cultured individually on 10 ml (in culture tubes, 100 mm × 25 mm, 40 ml volume) of full strength MS medium supplemented

with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 3.0 µM BA and solidified with 8 g l^{-1} agar (Bacteriological agar–Oxoid Ltd., Basingstoke, Hampshire, England). The pH of the medium was adjusted to 5.7 with KOH or HCl before autoclaving at 121^{o}C and 103 kPa for 20 min. Cultures were incubated in a growth room with 16 h photoperiod and PPF of 60 µmol m^{-2} s^{-1} at 25 ± 2^{o}C. The frequency of decontaminated surviving explants, expressed in percentage, in each decontamination treatment was recorded. Shoot-tip explants exhibited a high decontamination frequency and were therefore selected for all the subsequent experiments. Due to the low availability of explants as a result of dormancy imposed during the winter season, plant materials were bulked up by subculturing on the same medium using screw cap jars (110 mm × 60 mm, approximately 300 ml volume). Shoot-tip explants obtained from these cultures were used in the next two experiments (sections 3.2.2 and 3.2.3)

3.2.2 Effects of BA and NAA on shoot multiplication

After bulking up sufficient plant material, an experiment investigating the effects of BA with or without NAA supplementation on shoot multiplication was conducted using shoot-tip explants (5 mm length). Concentrations of 0.0, 0.5, and 1.0 µM of NAA were combined with 1.0, 2.0, 3.0, 4.0 and 5.0 µM BA in a 3 × 5 completely randomised factorial design. MS medium without any plant growth regulator was included as a control. Three explants were cultured on 30 ml of medium contained in each screw cap jar, with a total replicate (observation unit) of twenty-seven explants per treatment. Cultures were incubated in a growth room with 16 h light/8 h dark conditions and 60 µmol m^{-2} s^{-1} PPF at 25 ± 2^{o}C. The total number of adventitious shoots produced per cultured explant, number of adventitious shoots greater than 10 mm in length, number of adventitious shoots 5 – 10 mm long, and percentage of cultured explants producing shoots were recorded after four and six weeks of culture.

3.2.3 Effects of types and concentrations of cytokinins on shoot multiplication

Concentrations of 1.0, 3.0, 5.0 and 7.0 µM of different aromatic cytokinins (BA, Kinetin, *m*T, *m*TR, and Me*m*TR) were used in a completely randomised design.

BA and Kinetin were purchased from SIGMA (USA) while *m*T, *m*TR, and Me*m*TR were obtained from the Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, Czech Republic. Three explants were cultured on 30 ml medium contained in each screw cap jar, with a total replicate (observation unit) of twenty-four explants per treatment. Cultures were incubated in a growth room with 16 h photoperiod and 60 µmol m^{-2} s^{-1} PPF at 25 ± 2^{o}C. The total number of adventitious shoots produced per cultured explant, number of adventitious shoots greater than 10 mm in length, number of adventitious shoots 5 – 10 mm long, and percentage of cultured explants producing shoots were recorded after four and six weeks of culture. In addition, abnormality index, calculated as the ratio of abnormal (hyperhydric shoots and shoots with shoot-tip necrosis) to normal shoots **(BAIRU *et al*., 2008)**, was recorded after six weeks of culture.

3.2.4 Effects of photoperiod on shoot multiplication

Shoot-tip explants used for this experiment were excised from regenerated shoots in cultures containing MS medium supplemented with 7 µM Me*m*TR. Based on the results obtained from the preceding experiment (section 3.2.3), three shoot-tip explants were cultured on 30 ml of MS medium supplemented with 7 µM Me*m*TR in screw cap jar. The cultures were placed at 25°C under two different photoperiods: continuous light and 16 h light (60 µmol m^{-2} s^{-1} PPF in each case). Each treatment had a total of twenty-four replicates. The total number of adventitious shoots produced per cultured explant, number of adventitious shoots greater than 10 mm in length, number of adventitious shoots 5 – 10 mm long, and percentage of cultured explants producing shoots were recorded after six weeks of culture.

3.2.5 *In vitro* rooting of regenerated shoots

For *in vitro* rooting of individual regenerated shoots, shoot clusters produced in the shoot multiplication stage from each optimal cytokinin concentration were carefully separated and cultured on half-strength MS medium with or without 2.5 µM IBA supplementation, contained in screw cap jars. The cultures were completely

randomised and maintained in a growth room at 25°C under a 16 h photoperiod (60 µmol m^{-2} s^{-1} PPF). After four weeks of culture, the number of roots produced per shoot, root length and percentage of cultured shoots producing roots were recorded.

3.2.6 *Ex vitro* rooting and acclimatization

Regenerated shoots obtained from MS medium supplemented with 7 µM Me*m*TR were used in this experiment. *Ex vitro* rooting of regenerated shoots was investigated by pulsing regenerated shoots in different IBA concentrations for five hours in the light at 25°C. The IBA concentrations used were 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M while distilled water served as the control. After pulsing, the shoots were potted in vermiculite and placed in a mist house with about 90% relative humidity for three weeks. The acclimatized plants were subsequently transferred to a greenhouse and the percentage of surviving plants was recorded in each of the treatments after another two weeks.

For the purpose of acclimatization, agar was carefully washed off the *in vitro* rooted shoots (regenerated on medium with 7 µM Me*m*TR) and the plants then potted in a mixture of sand:soil:vermiculite (1:1:1, v/v). The potted plants were maintained in the mist house for two weeks before being transferred to a greenhouse. The percentage of surviving plants was recorded after an additional two weeks.

3.2.7 Data analyses

Mean values of the various treatments were subjected, as appropriate, to either one way analysis of variance (ANOVA) or student's *t*-test using SPSS version 15.0 or SigmaPlot 8.0, respectively. The significance level was determined at $P = 0.05$. Where there were significant differences, the means were separated using Duncan's Multiple Range Test (DMRT).

3.3 Results and discussion

Figure 3.1 shows the different stages involved in the successful micropropagation of *Barleria greenii*. These include explant selection from the stock plant, culture initiation, adventitious shoot regeneration, rooting of regenerated shoots and their acclimatization.

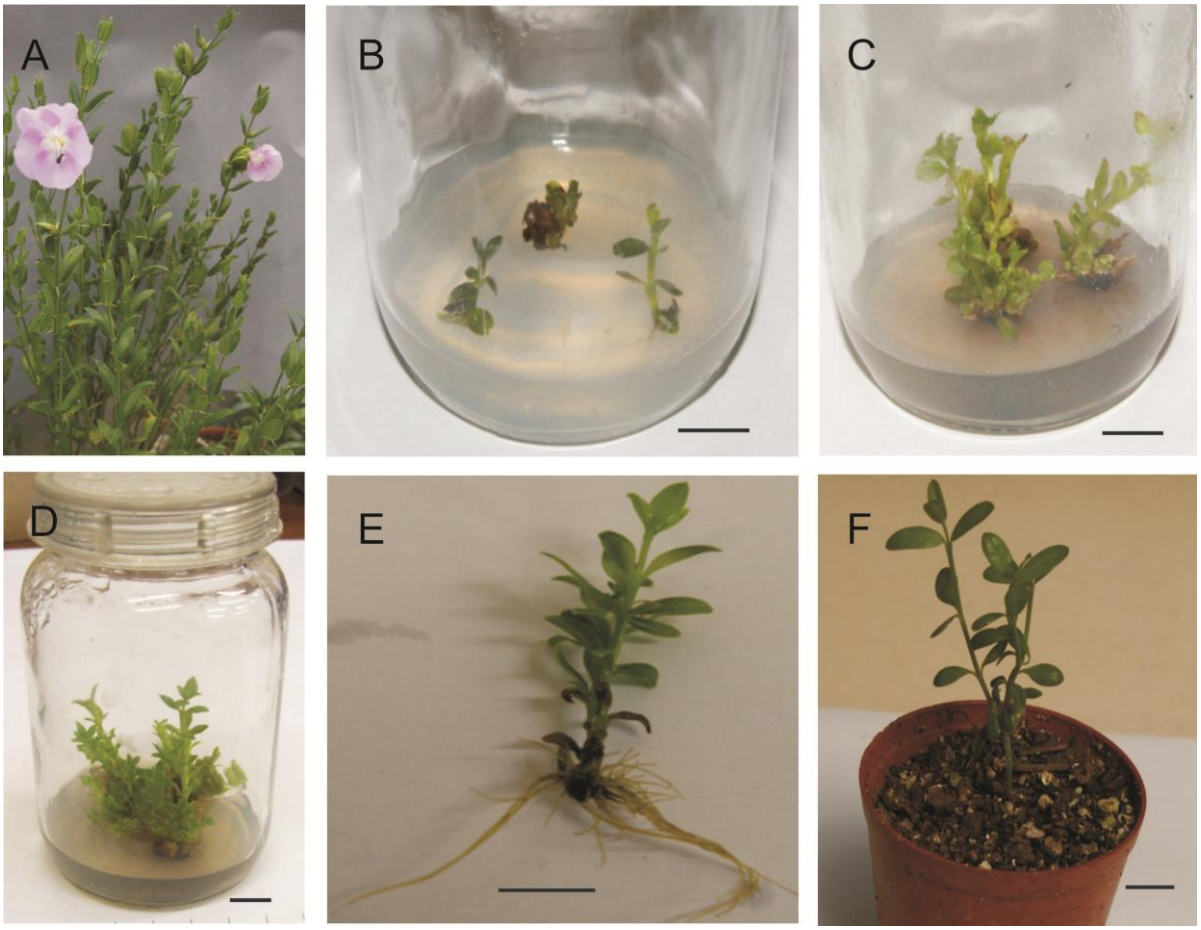


Figure 3.1: *In vitro* propagation of *Barleria greenii*. (**A**) Stock plant. (**B**) Control (MS medium without PGR). (**C**) Shoot multiplication on MS medium supplemented with 3 µM BA. (**D**) Shoot multiplication on MS medium supplemented with 7 µM Me*m*TR. (**E**) *In vitro* rooted regenerated shoot ready for acclimatization. (**F**) Three-month-old fully acclimatized plant. Bars = 10 mm.

3.3.1 Explant decontamination

Figure 3.2 shows the effects of different sodium hypochlorite solution treatments on decontamination of nodal and shoot-tip explants. The decontamination frequency recorded in the nodal explants was generally low, the highest being 47% decontamination. On the other hand, shoot-tip explants treated with 3.0% NaOCl for 30 min and those treated with 3.5% NaOCl for 20 min showed high decontamination frequencies of 72 and 74%, respectively. The soaking of the shoot-tips in 70% ethanol for 60 sec followed by 3.5% for 20 min proved to be the best surface decontamination treatment.

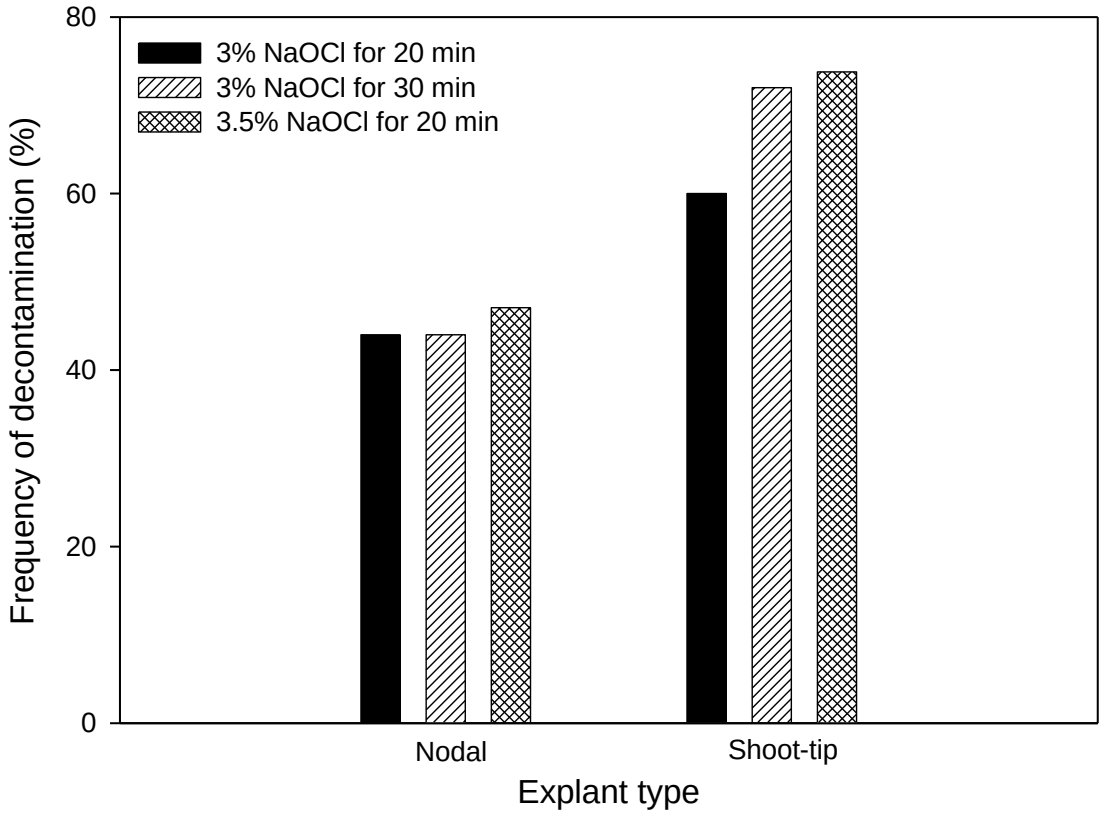


Figure 3.2: Effects of sodium hypochlorite (NaOCl) solution treatments on explants decontamination.

3.3.2 Effects of BA and NAA on shoot multiplication

The effects of different combinations of BA and NAA concentrations on adventitious shoot production after four and six weeks of culture are presented in Figures 3.3 and 3.4, respectively. The number of adventitious shoots produced per cultured explant, adventitious shoots with a length greater than 10 mm and adventitious shoots 5 – 10 mm in length were higher in all the treatments, compared to the control. The treatments with BA alone showed higher adventitious shoot production when compared to the BA treatments supplemented with NAA concentrations. This trend was observed both after four and six weeks of culture. In cultures with BA alone, there was an increase in shoot production with increased BA concentration, reaching the optimum at 3 µM BA. At supra-optimal concentrations, there was a decrease in shoot production. At equimolar supra-optimal BA concentrations, an increase in NAA concentration generally gave a reduced shoot production. In a similar vein, the frequency of explants producing shoots was generally lower in cultures supplemented with NAA compared to cultures with BA alone. These results imply that the exogenous application of NAA is neither a requirement for adventitious shoot induction nor shoot proliferation from shoot-tip explant of this species. In fact, the addition of NAA tends to show an antagonistic effect on adventitious shoot production. Auxins are generally known to be produced in shoot-tips of plants, from where they are mainly transported basipetally. Hence, in this study, the endogenous auxin content in the excised shoot-tip explant appears sufficient for shoot induction and growth. A similar significant inhibition of NAA on shoot proliferation and growth was reported by **NUNES *et al*. (2002)** in *Cedrela fissilis* cultures. According to these authors, the addition of NAA to medium containing BA strengthened apical dominance at the expense of shoot proliferation. Furthermore, it is possible that the exogenous application of NAA in the current study down-regulated cytokinin levels by promoting BA metabolic inactivation through *N*-glucosylation **(KAMÍNEK *et al*., 1997)**. The lowering effect of exogenous auxin application on active cytokinin levels has also been reported by other researchers **(HANSEN *et al*., 1987; BEINSBERGER *et al*., 1991; ZAŽÍMALOVÁ *et al*., 1996)**.

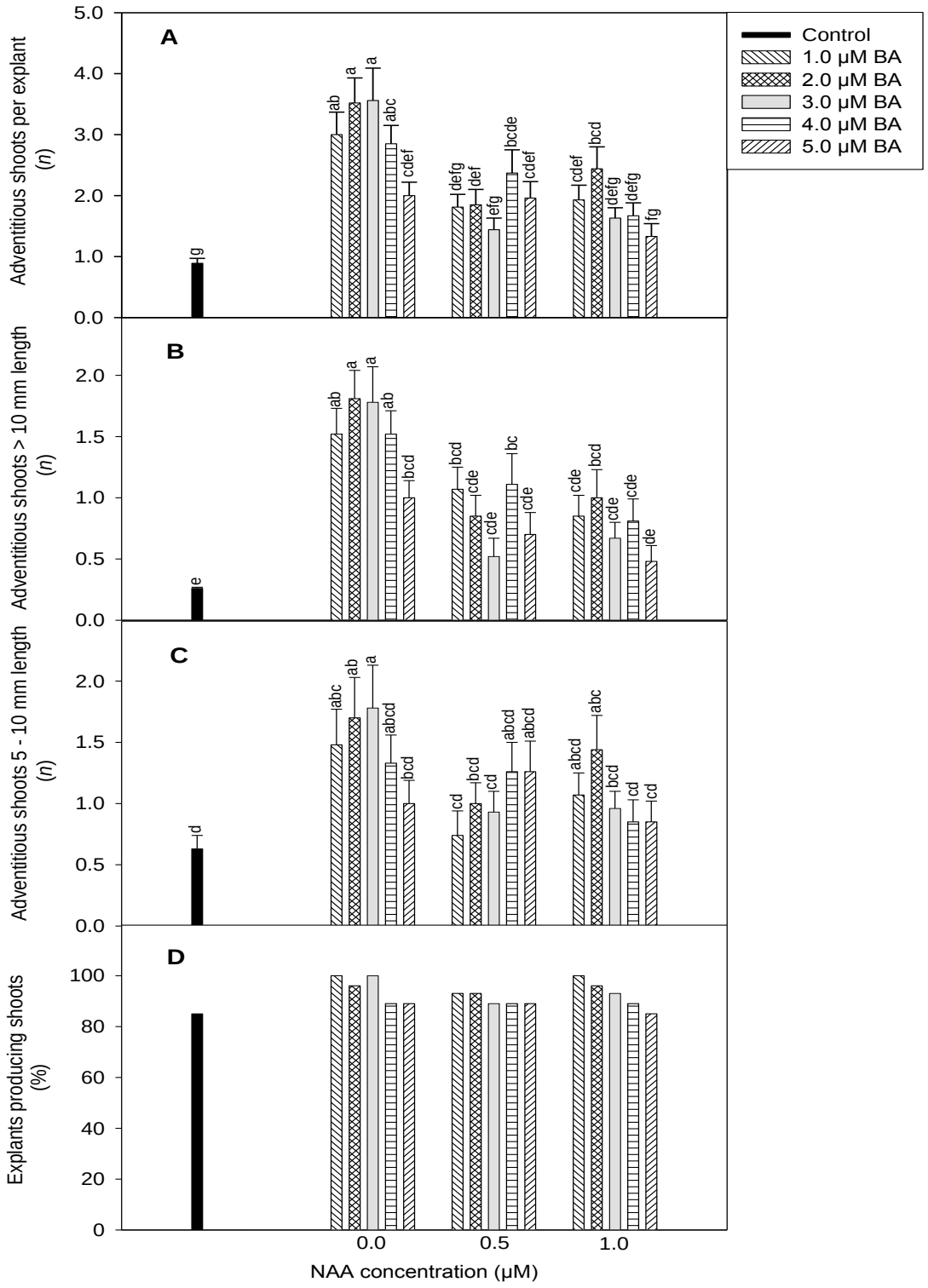


Figure 3.3: Effects of BA and NAA on adventitious shoot production of *Barleria greenii* after four weeks of culture. Bars with different letters are significantly different ($P = 0.05$) according to DMRT.

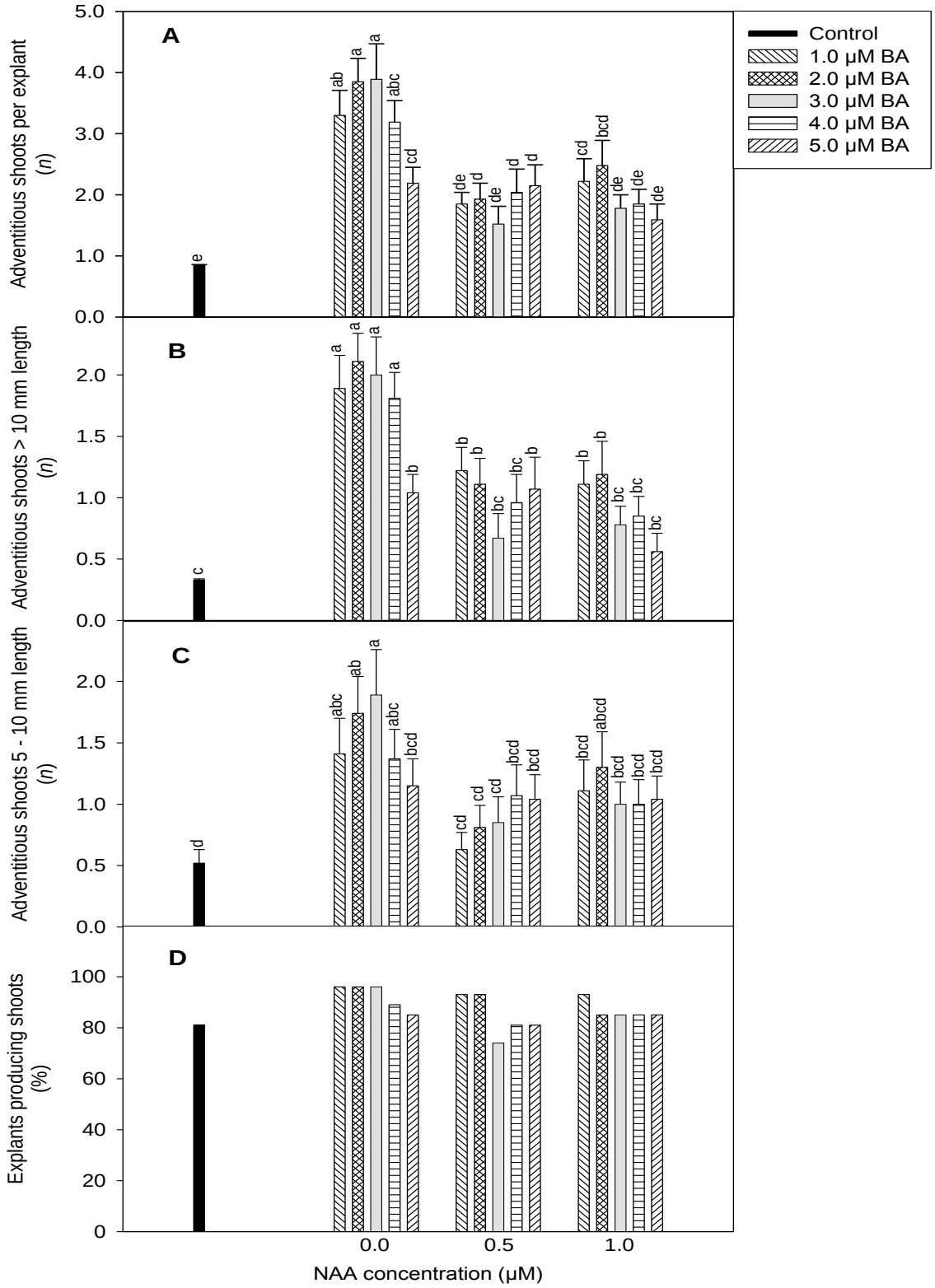


Figure 3.4: Effects of BA and NAA on adventitious shoot production of *Barleria greenii* after six weeks of culture. Bars with different letters are significantly different (P = 0.05) according to DMRT.

3.3.3 Effects of types and concentrations of cytokinins on shoot multiplication

Table 3.1 shows the effects of different types and concentrations of aromatic cytokinins on adventitious shoot production. The lowest and highest adventitious shoot production were observed in the control (Figure 3.1B) and the treatment with 7 µM Me*m*TR (Figure 3.1D) respectively, both after four and six weeks of culture. With the exception of BA treatments, an increase in concentration generally resulted in an increase in adventitious shoots produced per cultured explant as well as in adventitious shoots with a length greater than 10 mm. All the kinetin concentrations (including the highest concentration tested) gave a comparatively low adventitious shoot production, which was not significantly different from the control. This indicates that kinetin is a relatively weak cytokinin (especially when compared with BA), perhaps requiring a much higher concentration to achieve a significant shoot production in this species. The low effectiveness of kinetin in producing a high shoot proliferation compared to BA has also been reported in *Crossandra infundibuliformis* (another Acanthaceae species) and *Citrullus lanatus* by **GIRIJA *et al*. (1999)** and **COMPTON *et al*. (1993)**, respectively. Nevertheless, **BOGAERT *et al*. (2006)** observed that kinetin (a weak cytokinin) is useful in slowly and safely micropropagating some valuable ornamentals such as chimeras, without compromising their histogenic stability.

The plant growth regulator, BA is reported to be among the most effective and affordable cytokinins widely used in micropropagation techniques **(BAIRU *et al*., 2007)**. The optimum BA concentration for adventitious shoot production was reached in the current study at 3 µM (Figure 3.1C; Table 3.1). The abnormality index recorded at this concentration was higher than the abnormality index recorded in all concentrations of cytokinins (except 5 µM Kinetin, 1 µM BA and supra-optimal BA concentrations) used in this study. At supra-optimal BA concentrations, adventitious shoot production decreased while the abnormality index increased considerably. This response possibly reflects the toxic nature of BA in this species. Similar observations with respect to BA toxicity have been reported by many researchers for different plant species **(KUBALÁKOVÁ and STRNAD, 1992; BOGAERT *et al*., 2006; BAIRU *et al*., 2007, 2008, 2009)**. This toxicity is often attributed to the more stable nature of BA and its metabolites such

as [9G]BA compared to other cytokinins. **DOLEŽAL *et al*. (2006)** however, reported that growth inhibition by excessive cytokinin concentrations in tobacco callus is at least partly due to the cytokinin inhibition of cyclin-dependent kinase activity. Nevertheless, some recent investigations have focussed on evaluating other cytokinins (mainly the aromatic ones) with a view to finding a replacement for the use of BA in the micropropagation industry. Such replacement would be expected, among other things, to essentially improve multiplication frequency while maintaining genetic stability of regenerated plants.

The use of *m*T and its derivatives has been advocated by some researchers **(WERBROUCK *et al*., 1996; BOGAERT *et al*., 2006; BAIRU *et al*., 2007)** as a potential replacement for BA in the micropropagation industry. Some of these topolins were evaluated in this study. Unlike in BA treatments, an increase in adventitious shoot production per cultured explant and adventitious shoots with length greater than 10 mm was generally observed with an increase in the concentration of the topolins evaluated (Table 3.1). It is particularly noteworthy that the abnormality index recorded in all the topolin treatments (including the higher concentrations evaluated) was much lower than the abnormality index recorded at the lowest BA concentration. These findings indicate the effectiveness and less toxic nature of these topolins at higher equimolar concentrations compared to BA. Some factors responsible for the superiority of *meta*-topolins over BA have been discussed by some researchers. As stated by **KAMÍNEK *et al*. (1987b)**, the localised accumulation of *m*T is prevented by its faster translocation in plant tissues. The metabolites of *m*T and *m*TR are said to be easily degradable **(BAIRU *et al*., 2009)**. The hydroxyl group in the side chain of *meta*-topolins makes possible the formation of *O*-glucoside metabolites **(WERBROUCK *et al*., 1996)**. The *O*-glucosides are considered to be cytokinin storage forms, stable under certain conditions but rapidly converted to active cytokinin bases when required **(PARKER *et al*., 1978; WERBROUCK *et al*., 1996)**. The reversible sequestration of the *O*-glucosides in turn, allows for the continuous availability of cytokinins at a physiologically active level over a prolonged period of time, resulting in high shoot formation in *in vitro* cultures **(STRNAD, 1997)**.

Table 3.1: Effects of types and concentrations of cytokinins on adventitious shoot production of *Barleria greenii*

Cytokinin (µM)	4th Week of culture				6th Week of culture				
	Adventitious shoots/explant (*n*)	Adventitious shoots (*n*)		Explants producing shoots (%)	Adventitious shoots/explant (*n*)	Adventitious shoots (*n*)		Explants producing shoots (%)	Abnormality index (x 10^{-1})
		5 – 10 mm length	> 10 mm length			5 – 10 mm length	> 10 mm length		
Control	0.92 ± 0.06^{h}	0.42 ± 0.10^{cd}	0.50 ± 0.10^{h}	92	0.96 ± 0.07^{g}	0.33 ± 0.12^{de}	0.63 ± 0.10^{h}	92	0.45
1 BA	2.46 ± 0.24^{cdef}	0.79 ± 0.17^{bcd}	1.67 ± 0.27^{cdefg}	100	2.79 ± 0.30^{bcde}	0.92 ± 0.16^{bcd}	1.88 ± 0.29^{cdef}	100	1.55
3 BA	3.04 ± 0.50^{bc}	1.21 ± 0.27^{b}	1.83 ± 0.38^{bcde}	96	3.79 ± 0.62^{b}	1.38 ± 0.25^{ab}	2.42 ± 0.48^{bcd}	96	1.52
5 BA	2.83 ± 0.27^{bcd}	1.04 ± 0.19^{bc}	1.79 ± 0.26^{bcdef}	100	3.13 ± 0.28^{bc}	1.17 ± 0.21^{bc}	1.96 ± 0.27^{cdef}	100	4.42
7 BA	2.13 ± 0.24^{cdefg}	0.71 ± 0.20^{bcd}	1.42 ± 0.18^{defg}	100	2.33 ± 0.29^{cdef}	0.79 ± 0.20^{bcde}	1.54 ± 0.26^{defgh}	100	4.36
1 Kin	1.00 ± 0.06^{h}	0.46 ± 0.10^{cd}	0.54 ± 0.10^{h}	96	1.04 ± 0.07^{g}	0.33 ± 0.10^{de}	0.71 ± 0.09^{h}	96	0.87
3 Kin	1.17 ± 0.13^{gh}	0.58 ± 0.17^{bcd}	0.58 ± 0.10^{h}	92	1.17 ± 0.10^{fg}	0.54 ± 0.13^{cde}	0.63 ± 0.10h	96	0.38
5 Kin	1.25 ± 0.12^{gh}	0.38 ± 0.12^{d}	0.88 ± 0.14^{gh}	96	1.25 ± 0.09^{fg}	0.38 ± 0.10^{de}	0.88 ± 0.13^{gh}	100	1.54
7 Kin	1.58 ± 0.20^{fgh}	0.63 ± 0.18^{bcd}	0.96 ± 0.17^{fgh}	96	1.63 ± 0.18^{efg}	0.58 ± 0.15^{cde}	1.04 ± 0.11^{fgh}	100	0.26
1 *m*T	1.67 ± 0.21^{efgh}	0.21 ± 0.10^{d}	1.46 ± 0.19^{defg}	100	1.67 ± 0.21^{efg}	0.21 ± 0.10^{e}	1.46 ± 0.19^{efgh}	100	0.86
3 *m*T	2.67 ± 0.34^{bcde}	0.46 ± 0.18^{cd}	2.21 ± 0.23^{bcd}	100	2.96 ± 0.44^{bcd}	0.58 ± 0.21^{cde}	2.38 ± 0.31^{bcd}	100	0.29
5 *m*T	2.71 ± 0.30^{bcde}	0.46 ± 0.19^{cd}	2.25 ± 0.26^{bcd}	100	3.08 ± 0.36^{bcd}	0.58 ± 0.20^{cde}	2.50 ± 0.28^{bc}	100	0.72
7 *m*T	2.83 ± 0.45^{bcd}	0.75 ± 0.24^{bcd}	2.08 ± 0.31^{bcde}	96	3.08 ± 0.48^{bcd}	0.67 ± 0.23^{cde}	2.42 ± 0.36^{bcd}	96	0.72
1 *m*TR	1.21 ± 0.15^{gh}	0.25 ± 0.09^{d}	0.96 ± 0.14^{fgh}	88	1.42 ± 0.16^{fg}	0.33 ± 0.12^{de}	1.08 ± 0.16^{fgh}	92	0
3 *m*TR	1.90 ± 0.23^{defgh}	0.29 ± 0.12^{d}	1.62 ± 0.22^{cdefg}	100	1.90 ± 0.21^{defg}	0.19 ± 0.09^{e}	1.71 ± 0.22^{defg}	100	0.53
5 *m*TR	3.17 ± 0.45^{bc}	0.71 ± 0.19^{bcd}	2.46 ± 0.35^{bc}	100	3.71 ± 0.56^{b}	0.75 ± 0.22^{cde}	2.96 ± 0.42^{b}	100	0.23
7 *m*TR	2.46 ± 0.26^{cdef}	0.46 ± 0.15^{cd}	2.00 ± 0.26^{bcde}	100	2.92 ± 0.30^{bcd}	0.63 ± 0.13^{cde}	2.29 ± 0.27^{bcd}	100	0.29
1 Me*m*TR	1.42 ± 0.22^{fgh}	0.17 ± 0.08^{d}	1.25 ± 0.24^{efgh}	100	1.46 ± 0.26^{fg}	0.21 ± 0.08^{e}	1.25 ± 0.28^{fgh}	100	0.29
3 Me*m*TR	3.08 ± 0.61^{bc}	1.04 ± 0.36^{bc}	2.08 ± 0.39^{bcde}	100	3.38 ± 0.67^{bc}	1.13 ± 0.31^{bc}	2.25 ± 0.41^{bcd}	100	0
5 Me*m*TR	3.63 ± 0.43^{b}	1.04 ± 0.20^{bc}	2.58 ± 0.38^{ab}	100	3.88 ± 0.50^{b}	1.13 ± 0.28^{bc}	2.75 ± 0.42^{bc}	100	0.33
7 Me*m*TR	5.04 ± 0.62^{a}	1.75 ± 0.32^{a}	3.29 ± 0.43^{a}	100	5.88 ± 0.73^{a}	1.88 ± 0.31^{a}	4.00 ± 0.53^{a}	100	0.37

Means within the same column followed by different letter(s) are significantly different (P = 0.05) according to DMRT.

Furthermore, **BOGAERT *et al*. (2006)** reported the histogenic stability and anti-senescing activity of Me*m*TR in *Petunia hybrida* and *Rosa hybrida* cultures respectively. **BAIRU (2008)** reported increased callus yield with Me*m*TR > *m*TR > *m*T in the soybean callus bioassay. In the current study, cultures containing 7 µM Me*m*TR (Figure 3.1D) gave both the highest adventitious shoot production per cultured explant and adventitious shoots with length greater than 10 mm (5.88 ± 0.73 and 4.00 ± 0.53 shoots per cultured explant, respectively) after six weeks of culture. These values were significantly different from every other treatment and the control. In addition, the treatment with 7 µM Me*m*TR gave an abnormality index of 0.37, a value lower than that observed in the control. In fact, almost all the abnormality indices recorded with *m*TR and Me*m*TR concentrations were lower than that of the control, thus suggesting that the observed abnormalities in *m*TR and Me*m*TR treatments could be carry-over effects of BA since the explants were originally taken from cultures containing BA. On one hand, the superiority of *m*TR and Me*m*TR might be due to the presence of a ribose at their N^9 position, which better protects them against N^9-glucosylation **(WERBROUCK *et al*., 1996)**. On the other hand, the overall superiority of Me*m*TR might be partly due to the presence of the methyl group in its molecular structure. As shown by **SCHIMTZ *et al*. (1972)**, the presence and position of the methyl group could greatly influence the activity of a cytokinin. They observed that a shift of the methyl group from the 3- to the 2-position in going from dihydrozeatin to dihydroisozeatin resulted in a 70-fold decrease in activity, whereas the complete removal of the methyl group as in the case of *cis*-norzeatin resulted in a significant loss of activity (less than a fifth as active as *cis*-zeatin). **BAIRU (2008)** reported that the addition of a methoxy group to *ortho*-topolin prevents the formation of a hydrogen bond between the OH group on the benzyl ring and the nitrogen at the N^1 position, a bond responsible for the intermolecular inhibition of cytokinin activity. The author further mentioned that the prevention of hydrogen bonding between the N^1 and H^{16} atoms by the methyl group serves to increase cytokinin activity by maintaining the dihedral angle between least-square planes fitted through the purine and phenyl ring.

3.3.4 Effects of photoperiod on shoot multiplication

Cultures maintained under a 16 h photoperiod gave a higher (though not statistically significant) adventitious shoot production than those under continuous light (Table 3.2). The production of adventitious shoots with length greater than 10 mm was significantly (P = 0.01) higher in cultures maintained under a 16 h photoperiod than those under continuous light. This implies an improved growth rate in cultures placed under a 16 h photoperiod. **ECONOMOU and READ (1986)** similarly observed increased shoot length and quality in azalea (*Rhododendron* sp.) shoot-tip cultures grown under a 16 h photoperiod compared to continuous illumination, which inhibited shoot elongation. While the number of shoots produced during the first subculture was the same for both 16 and 24 h photoperiods, they further observed that continuous light significantly suppressed shoot proliferation during the second subculture. The authors gave two likely reasons for their observed response. Firstly, the dark periods (following the 16 h photoperiod) might promote the synthesis or accumulation of substance(s) which stimulate growth during the ensuing 16 h light periods. Secondly, the reduced growth in cultures under continuous light could be due to photo-oxidation of endogenous IAA resulting from over-exposure to light, which might affect the endogenous balance of the growth regulators. It is very likely that the response observed in the current study may be due to change in endogenous balance of growth regulators as a result of IAA photo-oxidation or synthesis of growth promoting substance(s).

Furthermore, a high accumulation of CO_2 in culture vessels/tubes has been reported during the dark period of a light: dark cycle **(FUJIWARA *et al*., 1987; cited by SERRET *et al*. 1997)**. Such a high availability of CO_2 could result in an increased rate of photosynthesis and reduced photorespiration, at least during the early phase of the subsequent light period in cultures maintained under a 16 h photoperiod. Photorespiration, a light dependent process, is known to reduce net CO_2 fixation and subsequent growth rates in C-3 plants **(SALISBURY and ROSS, 1992)**. The increased CO_2 concentration could result in decreased photorespiration by increasing the ratio of CO_2 to O_2 available in a reaction involving the enzyme ribulose bisphosphate carboxylase (rubisco), leading to a

faster or an improved net photosynthesis **(SALISBURY and ROSS, 1992)**. In addition to the improved growth rate observed in cultures maintained under a 16 h photoperiod, the reduced energy consumption can greatly cut down the cost of propagating this species *in vitro*.

Table 3.2: Effects of photoperiod on adventitious shoot production of *Barleria greenii* after six weeks of culture

	Photoperiod	
Parameter measured	24 h light	16 h light
Adventitious shoots per explant (*n*)	3.71 ± 0.52	5.38 ± 0.81$^{\#}$
Adventitious shoots with length > 10 mm (*n*)	1.81 ± 0.26	3.52 ± 0.53**
Adventitious shoots 5 – 10 mm in length (*n*)	1.90 ± 0.32	1.86 ± 0.33$^{\#}$
Explants producing shoots (%)	100	100

Mean values followed by the asterisk (**) indicate a significant difference at P = 0.01 while # indicates a non-significant difference at P = 0.05 according to *t*-test.

3.3.5 *In vitro* rooting of regenerated shoots

Figure 3.5 shows the effects of half strength MS medium with or without IBA supplementation on the *in vitro* rooting of regenerated shoots. Generally, the number of roots produced per shoot significantly increased with IBA supplementation, although there was no significant difference in the root length. Similarly, the frequency of shoots producing roots increased in cultures supplemented with IBA. There was however, no significant difference both in root length and number of roots produced by shoots from different cytokinin treatments. The low production of roots in the medium without auxin supplementation might possibly be attributed to the low endogenous auxin concentration produced in the shoot apex and transported in a basipetal manner to the basal cut surface **(DE KLERK *et al*., 1999)**. On the other hand, the effectiveness of exogenous application of IBA in root induction and elongation of many plant species has been reported **(CHOFFE *et al*., 2000; FOGAÇA and FETT-NETO, 2005; IAPICHINO and AIRÒ, 2008)**. The auxin IBA can be converted to IAA and is therefore known

to be a slow release reservoir of a more easily metabolized auxin **(EPSTEIN and LAVEE, 1984)**.

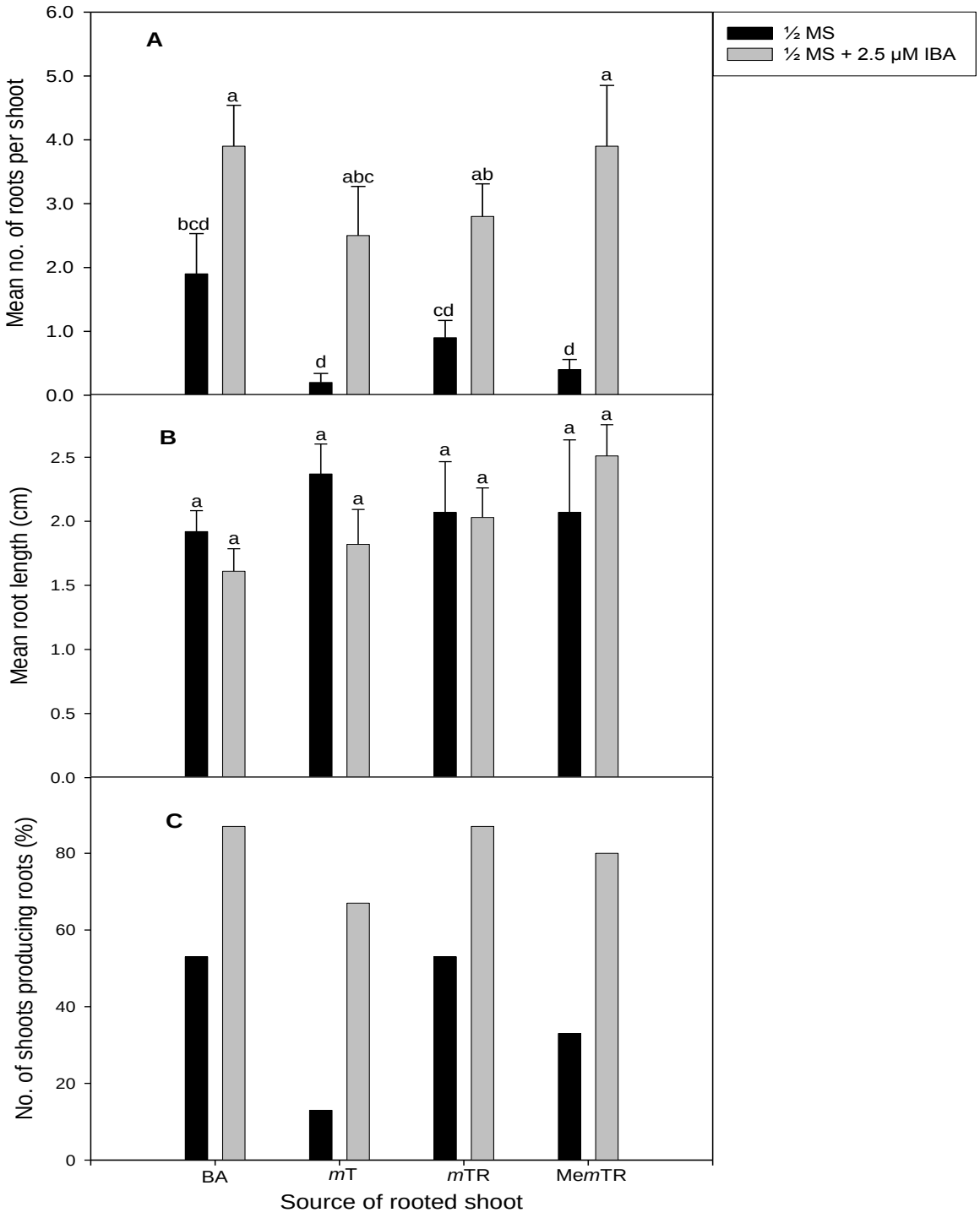


Figure 3.5: Effects of half strength MS medium with or without IBA treatment on *in vitro* rooting of regenerated shoots. Bars with different letters are significantly different (P = 0.05) according to DMRT.

3.3.6 *Ex vitro* rooting and acclimatization

The effects of pulsing regenerated shoots in different IBA concentrations on *ex vitro* percentage survival is presented in Figure 3.6. It appears that the percentage survival increased with increased IBA concentration. The best percentage survival (65%) was achieved in the treatment with 10^{-3} M IBA. An increase in the pulsing period or IBA concentration might likely improve the survival rate recorded in this study. Nevertheless, the survival rate recorded in the treatment with 10^{-3} M IBA was higher than that recorded in the case of acclimatized *in vitro* rooted shoots with 52% survival. In addition, the direct acclimatization of regenerated shoots following the pulse treatment with IBA concentration is time-saving and less labour-intensive when compared to *in vitro* rooting followed by acclimatization. It is possible that some of the roots produced *in vitro* are not functional in the normal substrate to which they were transplanted, resulting in a cessation of growth and subsequent death of some potted plants **(DEBERGH and MAENE, 1981)**. The low survival rate recorded during the acclimatization of *in vitro* rooted shoots might also be due to the fragile nature of the roots produced, which are susceptible to damage during transplanting and can lead to plant death **(DEBERGH and MAENE, 1981)**. In general, however, there was no morphological variation observed in any of the acclimatized plants even after 3 months under greenhouse conditions (Figure 3.1F). This developed protocol has a potential of producing more than 60,000 transplantable shoots per year from a single shoot-tip explant.

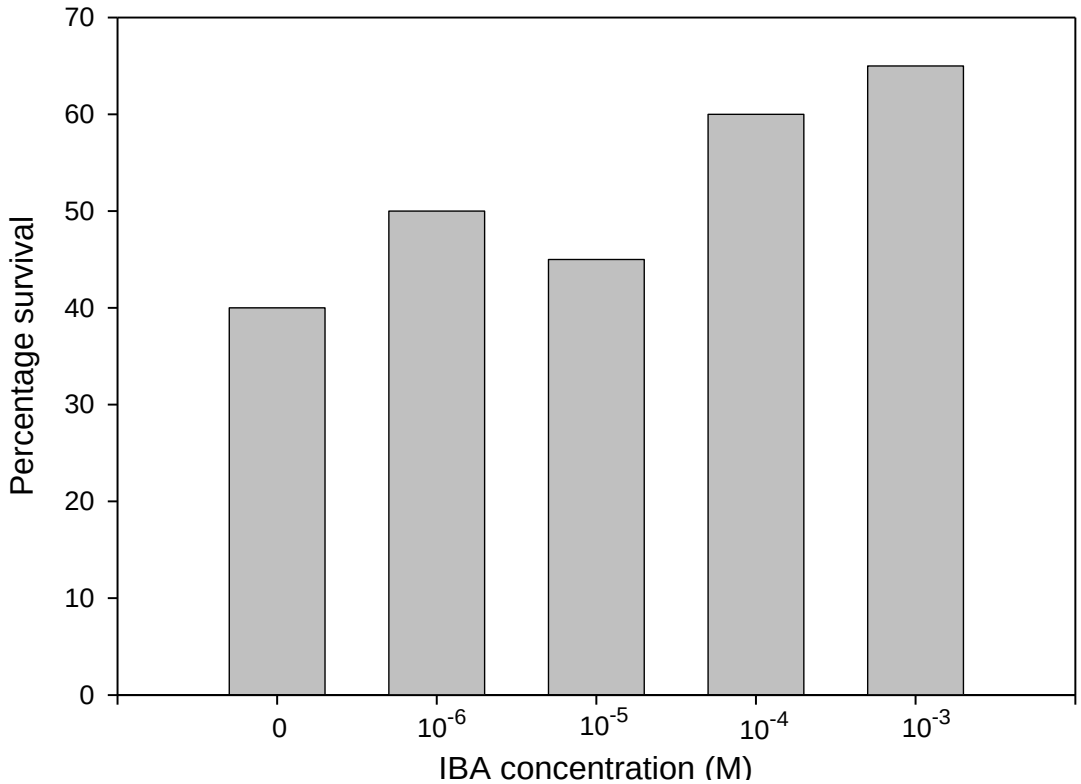


Figure 3.6: Effects of pulsing with different IBA concentrations on *ex vitro* acclimatization of regenerated shoots.

Chapter 4 *In vitro* propagation of *Huernia hystrix*

4.1 Introduction

Huernia hystrix (Hook.f.) N.E.Br. (Family: Asclepiadaceae) is a succulent used as a medicinal plant, destructively harvested as a whole plant and extensively sold and marketed in South Africa. In the recent survey of plants sold by traders at Zululand *muthi* markets, **NDAWONDE *et al*. (2007)** listed *H. hystrix* as one of the threatened plant species that are more scarce (below 20%) than others. They noted that traders reported a high market demand for this species. **OLIVER (1998)** described *Huernia* species (including *H. hystrix*) as interesting container plants for ornamental purposes.

In some cases when the flowers are sterile, this plant does not set seed **(ANONYMOUS, 2007)**. In other situations when the flowers are not sterile and seeds are eventually produced, the plant does not often come true from seed **(ANONYMOUS, 2007)**. The conventional propagation method is therefore by cuttings. Since *H. hystrix* is a dwarf species, very limited cuttings can be taken from the mother plant. Vertical planting of the stem cutting disposes the part in the rooting substrate to rot rather than to root **(HODGKISS, 2004)**. **OLIVER (1998)** noted that asclepiad species are prone to numerous diseases and are perhaps among the most difficult of the succulent group to grow. Owing to these inherent propagation problems, **HODGKISS (2004)** was of the opinion that regular propagation with a few cuttings seems to be the best insurance against the loss of such asclepiad species. Its conventional propagation cannot meet the increasing demand, which will ultimately lead to extinction if no attention is given to its conservation and propagation. The need for an effective propagation method that can possibly be used as a conservation measure for this species is further heightened by it being red-listed and ranked as vulnerable in its global conservation status **(HILTON-TAYLOR, 1996; SCOTT-SHAW, 1999)**.

According to **MALDA *et al*. (1999)**, nearly all succulent plant species are affected by habitat destruction and the collection of wild plants for illicit trade. The

exponential population growth rates in developing countries since the latter half of the twentieth century, leading to an increased demand for medicinal plants, has further exacerbated this problem **(JÄGER and VAN STADEN, 2000)**. The application of micropropagation techniques in the conservation and rapid mass propagation of threatened species is well known and has gained tremendous impetus in the last two decades. According to **SARASAN *et al*. (2006)**, many plant species belonging to different major taxonomic categories are being tissue cultured for propagation and conservation purposes. Nevertheless, the commercial application of micropropagation techniques is generally still hampered by high production costs. These costs are attributed to high labour costs, low growth rates *in vitro* and poor survival of the plantlets during acclimatization **(KOZAI *et al*., 1997)**. Plant growth and development *in vitro* are affected by a host of factors including the environmental and chemical conditions during the culture period. Environmental factors such as temperature, photoperiod, light intensity, humidity, and gaseous environment affect physiological processes in plants and are therefore critical to micropropagation success.

It is well known that the optimal environmental and chemical conditions for plant growth and development often vary between species and sometimes genotypes. This therefore necessitates investigating the effects of these factors on *in vitro* growth and development of individual species while developing a micropropagation protocol especially amenable to commercial application. Up to the point of publishing the results of this study, there is no detailed report, to our knowledge, on the micropropagation of any *Huernia* species. In order to meet the increasing demand for *H. hystrix* while conserving those in the wild, this study was aimed at developing a simple, rapid and cost-effective protocol for its clonal propagation. The effects of temperature, photoperiod and culture vessel size on adventitious shoot production were also investigated in the establishment of the protocol.

4.2 Materials and methods

4.2.1 Source material, decontamination and bulking of explants

Stock plants planted in pots (Figure 4.1A) were maintained in the shade house in the University of KwaZulu-Natal Botanical Garden. Stem explants taken from the stock plants were thoroughly washed under running tap water and dipped in 80% ethanol for 60 sec prior to additional decontamination treatments. The additional decontamination treatments included the use of either 3.5% sodium hypochlorite solution, 0.1 or 0.3% (w/v) mercuric chloride solution for 10, 20 or 30 min. A few drops of Tween 20 were added to the solution as a surfactant in each case. The surface decontaminated stem explants were cut into 10 mm lengths and then inoculated on 10 ml (in culture tubes, 100 mm × 25 mm, 40 ml volume) of full strength MS medium supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 2.69 µM NAA, 22.19 µM BA and solidified with 8 g l^{-1} agar (Bacteriological agar– Oxoid Ltd., Basingstoke, Hampshire, England). The pH of the medium was adjusted to 5.7 with KOH or HCl before autoclaving at 121°C and 103 kPa for 20 min. Cultures were incubated in a growth room under continuous light provided by cool white fluorescent tubes (Osram® L 58 W/640, 30 µmol m^{-2} s^{-1} PPF) at 25 ± 1°C. The frequency of surviving decontaminated explants was recorded in percentage for each of the decontamination treatments. Based on the results obtained from this experiment, stem explants that were surface decontaminated using 80% ethanol followed by 0.3% mercuric chloride solution with a few drops of Tween 20 for 20 min were cultured on the medium described above for the purpose of bulking up more explants.

4.2.2 Effects of BA and NAA on shoot multiplication

Following bulking of sufficient plant material, shoot multiplication experiments were designed using stem explants, excluding terminal portions, and cut into 10 mm lengths. Concentrations of 0.00, 2.69, 5.37 and 8.06 µM of NAA were combined with 4.44, 13.32 and 22.19 µM BA in a 4 × 3 completely randomised factorial design. A control without any plant growth regulator was included. Each treatment, including the control had 20 replicates and the experiment was repeated twice.

Cultures were incubated under the same growth conditions stated above. After nine weeks, the following growth parameters were recorded: the total number of adventitious shoots produced per cultured explant (shoot multiplication rate), number of adventitious shoots 5 – 10 mm long, number of adventitious shoots greater than 10 mm in length, number of adventitious roots per cultured explant, fresh weight of the adventitious shoots regenerated from each explant, fresh weight of the adventitious roots regenerated from each explant, percentage of cultured explants producing shoots and the presence or absence of basal callus.

4.2.3 Effects of temperature and photoperiod on shoot multiplication

Stem explants (10 mm) were cultured individually on 10 ml of medium (full strength MS medium supplemented with 5.37 µM NAA and 22.19 µM BA) contained in culture tubes. The NAA and BA concentrations used were selected because they gave the best shoot proliferation in the experiment described in section 4.2.2. The cultures were maintained in different growth cabinets under two sets of light conditions, defined as follows: (a) 15, 20, 25, 30, and 35°C at 16 h photoperiod; (b) 25, 30, and 35°C at 24 h photoperiod. A PPF of 30 µmol m^{-2} s^{-1} provided by cool, white fluorescent tubes (Osram® L 58 W/640) was used in all the experiments. Each treatment had at least 18 replicates. After eight weeks of culture, the following growth parameters were recorded: the number of adventitious shoots produced per cultured explant, number of adventitious shoots 5 – 10 mm long, number of adventitious shoots greater than 10 mm in length, fresh weight of the adventitious shoots regenerated from each explant and the percentage of cultured explants producing shoots.

4.2.4 Determination of titratable acidity

On the basis of the results obtained from experiments on effects of temperature and photoperiod, nocturnal changes in titratable acidity was determined after eight weeks in shoots regenerated from explants cultured under a 16 h photoperiod at either 25 or 35 °C. The method described by **MARTIN *et al*. (1990)** was followed. The objective was to determine the presence of crassulacean acid metabolism (CAM) in these cultures. Shoot tissues were collected at the beginning (22:00 h),

middle (02:00 h) and end (06:00 h) of an 8 h dark period, weighed and ground in distilled water. The ground tissues were then filtered under vacuum and the filtrate was titrated against 0.01 N NaOH to a pH 7.0. Titratable acidity was expressed in µM H^+ g^{-1} fresh weight. Each determination had four replicates.

4.2.5 Effects of culture vessel size on shoot multiplication

Explants were cultured in two culture vessels of different size. Individual stem explants were cultured on 10 ml of medium contained in culture tubes (100 mm × 25 mm, 40 ml volume) while three stem explants were cultured on 30 ml of medium contained in tightly-closed screw cap jars (110 mm × 60 mm, approximately 300 ml volume). Each culture tube had a headspace volume of 30 ml per explant while the screw cap jar had a headspace volume of 90 ml per explant. The cultures were kept in a growth room maintained at 25°C and continuous photoperiod (30 µmol m^{-2} s^{-1} PPF). A total of 18 replicates (observation unit) per treatment were used. The experiment lasted for eight weeks whereafter the same growth parameters described above (section 4.2.3) were recorded.

4.2.6 Indirect organogenesis

The callus produced at the base of the stem explant during the bulking process was subcultured on the same medium to produce more calli. A completely randomised experiment was designed to determine the organogenic potential of these calli. Concentrations of 0.00, 0.27, 2.69, and 5.37 µM NAA were combined in a factorial manner with 0.00, 2.22, 5.55, and 11.10 µM BA. Cultures were incubated in a growth room under continuous light (30 µmol m^{-2} s^{-1} PPF) at 25°C. Each treatment had 15 replicates. After nine weeks, presence or absence of organogenesis was examined and callus fresh weight recorded.

4.2.7 Rooting and acclimatization

For rooting of individual shoots, shoot clusters produced in the shoot multiplication stage were carefully separated and cultured in PGR free half-strength MS medium as well as half-strength MS medium supplemented with 1 µM IBA contained in

loosely closed screw cap jars (300 ml). After four weeks of culture under continuous light (30 µmol m^{-2} s^{-1} PPF) at 25°C, the rooted shoots were planted in a potting mixture of 1:1 (v/v) ratio of soil:sand, treated with fungicide (Benlate, 0.01%) and transferred to the greenhouse. The greenhouse thermostat was set to regulate the temperature at 15°C minimum and 25°C maximum. After two months of growth in the greenhouse, fresh and dry weights of shoots and roots, number of roots and percentage survival were recorded.

4.2.8 Data analyses

The data collected were subjected to student's *t*-test or one-way ANOVA where appropriate. Where there was a significant difference ($P \leq 0.05$), the means were further separated using Duncan's Multiple Range Test (DMRT). The analysis was done using SigmaPlot version 8.0 (*t*-test) and SPSS software version 15.0 (ANOVA).

4.3 Results and discussion

4.3.1 Explant decontamination

All the treatments with 3.5% sodium hypochlorite solution were contaminated. The frequencies of surviving clean explants obtained in different treatments with mercuric chloride solution are presented in Figure 4.2. The treatment with 0.3% (w/v) mercuric chloride for 20 min gave the highest frequency of 55% clean explants. All the remaining treatments gave a frequency ranging from 0 to 35% decontamination. In general, the low frequency observed was due to a high incidence of internal contamination of the explants.

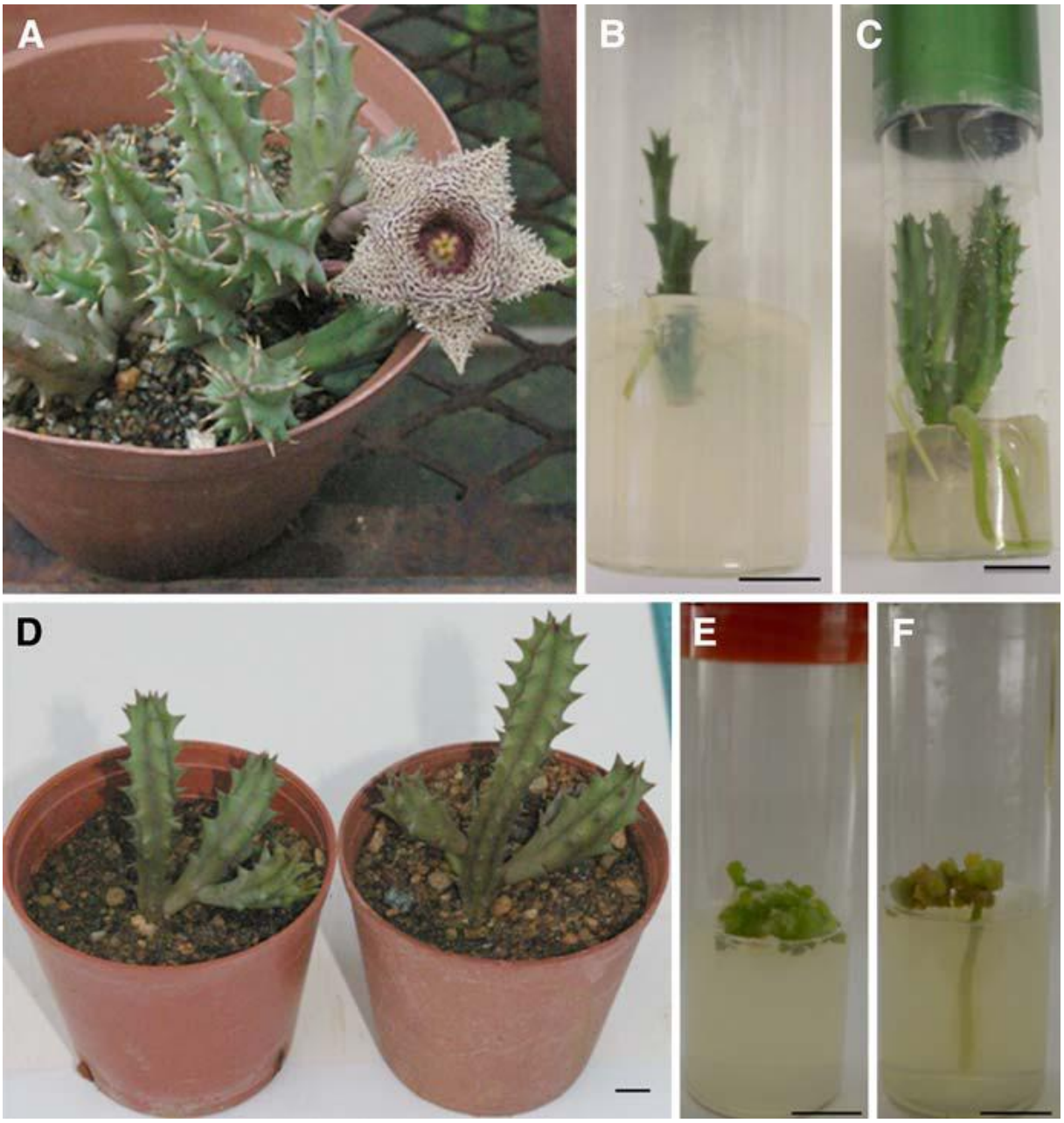


Figure 4.1: *In vitro* propagation of *Huernia hystrix*. (**A**) Stock plant. (**B**) Control with root production (MS medium without plant growth regulators). (**C**) Multiple shoot production accompanied with root formation on MS medium supplemented with 5.37 µM NAA and 22.19 µM BA. (**D**) Two-month-old fully acclimatized plants (**E**) Green callus growth with root hairs on MS medium supplemented with 5.37 µM NAA. (**F**) Root regeneration from callus on MS medium supplemented with 2.69 µM NAA and 2.22 µM BA. Scale bar = 10 mm.

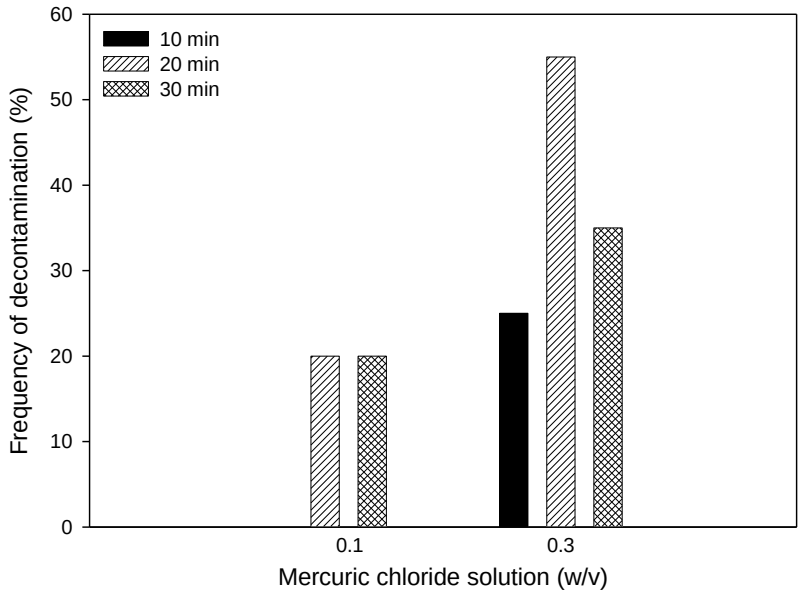


Figure 4.2: Frequencies of explants decontamination in different mercuric chloride solution treatments.

4.3.2 Shoot and root organogenesis

The effects of different combinations of NAA and BA concentrations on root and shoot organogenesis are presented in Table 4.1. All the treatments showed a significantly higher adventitious shoot production compared to the control with an average of 0.71 ± 0.097 shoots per explant (Figure 4.1B). A maximum of 4.11 ± 0.428 adventitious shoots per explant with 100% frequency developed on media supplemented with 5.37 µM NAA and 22.19 µM BA (Tables 4.1and 4.2, Figure 4.1C). The same media also produced the highest number of adventitious shoots with a length greater than 10 mm. At the same concentration of NAA, adventitious shoot production generally increased with increased BA concentrations. Similarly, at the same level of BA concentration, adventitious shoot production generally increased with increased NAA concentration. It therefore appeared that BA and NAA have synergistic effects on shoot multiplication. **SUDHA *et al*. (1998)** observed that supplementation with NAA in addition to BA was necessary for shoot elongation and multiple shoot production from nodal explants of *Holostemma annulare* (an Asclepiadaceae species).

Table 4.1: Effects of different combinations of NAA and BA on shoot and root regeneration of *Huernia hystrix* after nine weeks of culture

PGR combination NAA:BA (µM)	Adventitious shoots per explant (*n*)	Adventitious roots per explant (*n*)	Adventitious shoots (*n*)	
			5 - 10 mm length	> 10 mm length
Control	0.71 ± 0.097^{f}	3.77 ± 0.527^{def}	0.40 ± 0.093^{c}	0.31 ± 0.080^{f}
0.00:4.44	2.11 ± 0.292^{e}	1.57 ± 0.313^{f}	1.17 ± 0.203^{ab}	0.94 ± 0.192^{ef}
0.00:13.32	2.91 ± 0.475^{abcde}	1.31 ± 0.325^{f}	1.26 ± 0.270^{ab}	1.66 ± 0.307^{cde}
0.00:22.19	3.46 ± 0.358^{abcd}	1.17 ± 0.433^{f}	1.80 ± 0.252^{a}	1.66 ± 0.333^{cde}
2.69:4.44	2.40 ± 0.296^{de}	8.51 ± 1.319^{ab}	1.00 ± 0.169^{bc}	1.40 ± 0.253^{de}
2.69:13.32	2.94 ± 0.272^{abcde}	2.69 ± 0.590^{ef}	0.89 ± 0.196^{bc}	2.06 ± 0.275^{abcd}
2.69:22.19	3.77 ± 0.480^{abc}	2.94 ± 1.332^{ef}	1.89 ± 0.317^{a}	1.89 ± 0.350^{bcd}
5.37:4.44	2.83 ± 0.334^{bcde}	7.71 ± 1.338^{abc}	1.34 ± 0.201^{ab}	1.49 ± 0.230^{cde}
5.37:13.32	3.74 ± 0.508^{abc}	5.89 ± 1.234^{bcde}	1.46 ± 0.237^{ab}	2.29 ± 0.375^{abcd}
5.37:22.19	4.11 ± 0.428^{a}	3.91 ± 1.177^{def}	1.29 ± 0.203^{ab}	2.83 ± 0.321^{a}
8.06:4.44	2.60 ± 0.290^{cde}	9.89 ± 1.364^{a}	0.74 ± 0.180^{bc}	1.86 ± 0.210^{bcde}
8.06:13.32	4.06 ± 0.533^{ab}	6.23 ± 1.184^{bcd}	1.37 ± 0.266^{ab}	2.69 ± 0.366^{ab}
8.06:22.19	3.57 ± 0.432^{abcd}	5.03 ± 0.964^{cde}	1.20 ± 0.187^{ab}	2.37 ± 0.343^{abc}

Mean values followed by different letters in a column are significantly different (*P* = 0.05) according to DMRT.

Table 4.2: Frequencies of shoot, root and basal callus production from treatments with different concentration combinations of NAA and BA

PGR combination NAA:BA (µM)	Explants producing shoots (%)	Explants producing roots (%)	Frequency of basal callus (%)
Control	80	100	0
0.00:4.44	95	75	5
0.00:13.32	100	65	10
0.00:22.19	100	35	10
2.69:4.44	100	90	35
2.69:13.32	100	85	20
2.69:22.19	100	30	15
5.37:4.44	95	90	25
5.37:13.32	100	90	40
5.37:22.19	100	70	5
8.06:4.44	100	100	10
8.06:13.32	100	90	25
8.06:22.19	100	90	15

The synergistic effect of BA in combination with an auxin on shoot multiplication has been reported for many Asclepiadaceae medicinal plants such as *Hemidesmus indicus* **(SREEKUMAR *et al*., 2000)**, *Holostemma ada-kodien* **(MARTIN, 2002)**, and *Ceropegia candelabrum* **(BEENA *et al*., 2003)**. Increased NAA concentration resulted in an increase in the number of adventitious roots produced per explant (Table 4.1). However, at the same NAA concentration, an increase in BA concentration caused a reduction in both frequency and the number of adventitious roots produced per explant (Table 4.2). This could be due to a reduction in the NAA:BA ratio since a high auxin:cytokinin ratio is known to promote root production. **PATNAIK and DEBATA (1996)** observed a similar response in *Hemidesmus indicus* when various concentrations of IBA were combined with a fixed concentration of kinetin.

Regenerated shoots remained viable for more than six months when left in the original medium without any subculture. This has potential as a cost-effective

short-term *in vitro* storage of *H. hystrix* germplasm. Shoot regeneration in all treatments with PGR was accompanied by callus formation at the basal cut end of the explant (Table 4.2). Various workers have reported similar observations for other Asclepiadaceae species **(SUDHA *et al*., 1998; MARTIN, 2002)**. The prolific adventitious shoot production coupled with callus formation at the base of the explant may be due to auxin accumulation at the basal cut ends by downward movement, which stimulates cell proliferation especially in the presence of cytokinins **(MARKS and SIMPSON, 1994)**.

Table 4.3 shows the effects of different combinations of NAA and BA concentrations on fresh weights of regenerated shoots and roots per cultured explant. At all NAA concentrations, supplementation with 13.32 µM BA gave the highest fresh weight of the shoots regenerated per explant, which was significantly different in most cases compared to the control. This was however not significantly different from supplementation with 22.19 µM BA. At the same BA concentration, fresh weight of the shoots regenerated per explant generally increased with increased NAA concentrations. On the other hand, the fresh weight of adventitious roots produced per cultured explant was consistently lower with increased concentrations of BA at the same level of NAA but increased with increased NAA concentrations at the same level of BA. This could be due to the fact that BA acts specifically on shoot multiplication while NAA acts directly on cell elongation **(GABA, 2005)**.

4.3.3 Effects of temperature and photoperiod on shoot multiplication

Tables 4.4 and 4.5 show the effects of temperature and photoperiod on adventitious shoot production as well as on shoot fresh weight and percentage of explants producing shoots, respectively. Although there was no significant difference in the mean number of adventitious shoots produced per explant between the different temperature treatments under constant light, the number of adventitious shoots 5 – 10 mm long significantly increased with increased temperature. Conversely, the number of adventitious shoots greater than 10 mm in length as well as the fresh weight of adventitious shoots regenerated per explant decreased significantly with increased temperature in constant light. According to

SALISBURY and ROSS (1992), a temperature rise increases the ratio of dissolved chloroplastic O_2 to CO_2 in C-3 species, such that oxygen fixation by rubisco (ribulose bisphosphate carboxylase) occurs faster and photorespiration indirectly slows growth. The authors further added that at high temperatures, ATP and NADPH are not produced fast enough in C-3 plants to allow increases in CO_2 fixation. This might explain the results observed in the cultures incubated under constant light. Furthermore, 38% of the regenerated shoots in cultures under constant light at 35°C appear bleached. This might be due to solarization, a phenomenon described by **SALISBURY and ROSS (1992)** as a light-dependent inhibition of photosynthesis followed by oxygen-dependent bleaching of chloroplast pigments.

Table 4.3: Effects of different concentration combinations of NAA and BA on fresh weights of adventitious shoots and roots produced per explant

PGR combination	Fresh weight per explant (mg)	
NAA:BA (µM)	Shoot	Root
Control	100 ± 15^{d}	40 ± 4^{ef}
0.00:4.44	130 ± 22^{d}	40 ± 13^{ef}
0.00:13.32	230 ± 84^{d}	30 ± 23^{ef}
0.00:22.19	100 ± 20^{d}	3 ± 2^{f}
2.69:4.44	380 ± 82^{bcd}	330 ± 53^{b}
2.69:13.32	680 ± 148^{ab}	170 ± 37^{cde}
2.69:22.19	410 ± 69^{bcd}	130 ± 47^{def}
5.37:4.44	340 ± 35^{cd}	360 ± 58^{b}
5.37:13.32	680 ± 170^{ab}	290 ± 60^{bc}
5.37:22.19	600 ± 98^{abc}	200 ± 31^{bcd}
8.06:4.44	550 ± 114^{abc}	640 ± 102^{a}
8.06:13.32	820 ± 136^{a}	350 ± 68^{b}
8.06:22.19	550 ± 105^{abc}	330 ± 59^{b}

Mean values followed by different letters in a column are significantly different (P = 0.05) according to DMRT.

Table 4.4: Effects of temperature and photoperiod on adventitious shoot production of *Huernia hystrix* after eight weeks of culture

Temperature (°C)	Adventitious shoots per explant (*n*)		Adventitious shoots (*n*)			
			5 - 10 mm length		> 10 mm length	
	16 h light	24 h light	16 h light	24 h light	16 h light	24 h light
15	0.4 ± 0.20^{c}	ND	0.2 ± 0.10^{c}	ND	0.2 ± 0.13^{b}	ND
20	1.8 ± 0.31^{b}	ND	1.0 ± 0.26^{bc}	ND	0.8 ± 0.22^{ab}	ND
25	2.3 ± 0.40^{b}	$4.2 \pm 0.47^{a**}$	$1.5 \pm 0.26^{b\#}$	1.3 ± 0.25^{b}	0.8 ± 0.25^{ab}	$2.9 \pm 0.37^{a***}$
30	2.7 ± 0.48^{b}	$4.1 \pm 0.53^{a\#}$	1.2 ± 0.42^{b}	$1.8 \pm 0.24^{b\#}$	1.5 ± 0.49^{a}	$2.3 \pm 0.42^{a\#}$
35	4.2 ± 0.74^{a}	$4.2 \pm 0.47^{a\#}$	2.4 ± 0.41^{a}	$3.4 \pm 0.50^{a\#}$	$1.8 \pm 0.58^{a\#}$	0.8 ± 0.22^{b}

ND = Not determined. Mean values followed by different letters in a column are significantly different ($P = 0.05$) according to DMRT. Mean values in the same row per growth parameter followed by asterisks indicate significance at $P = 0.001$ (***) or $P = 0.01$ (**) while $^{\#}$ = non significant effect according to *t*-test.

Table 4.5: Effects of temperature and photoperiod on frequency and fresh weight of regenerated shoots per explant of *Huernia hystrix* after eight weeks of culture

	Shoot fresh weight per explant (mg)		Explants producing shoots (%)	
Temperature (°C)	16 h light	24 h light	16 h light	24 h light
15	ND	ND	28	ND
20	114 ± 33.6[a]	ND	83	ND
25	192 ± 67.0[a]	690 ± 95.6[a***]	89	100
30	249 ± 79.3[a]	569 ± 71.6[a**]	94	96
35	192 ± 44.0[a]	251 ± 34.0[b#]	94	100

ND = Not determined. Mean values followed by different letters in a column are significantly different (P = 0.05) according to DMRT. Mean values in the same row per growth parameter followed by asterisks indicate significance at P = 0.001 (***) or P = 0.01 (**) while # = non significant effect according to *t*-test.

On the other hand, a significant increase in adventitious shoot production with an increase in temperature was observed in cultures placed under a 16 h photoperiod (Table 4.4). At lower temperatures (15 and 20°C), the shoot proliferation rates were low due to slow growth and less differentiation of shoot meristems. Such low shoot proliferation at lower temperatures offers an approach for *in vitro* storage of *Huernia hystrix* germplasm. **ISLAM *et al*. (2005)** similarly reported a significant effect of temperature on *in vitro* growth of mint plants (*Mentha* spp.) with slow growth at lower temperature (20°C). Slow growth at lower temperatures is economically viable especially at times when labour for transplanting or subculturing and greenhouse space are not available **(KOZAI *et al*., 1997)**. However, according to these authors, *in vitro* storage of plantlets at low temperatures for production management should maintain the photosynthetic and regrowth abilities of the plantlets while suppressing growth. We observed in this study that the proliferation rate and percentage of explants producing shoots generally improved when the cultures at low temperature were later incubated at 25°C under constant light. Furthermore, slow growth at low temperature might have a beneficial effect on the genetic stability of the cultures **(BARNEJEE and DE LANGHE, 1985)** since much repeated subculturing on medium containing plant growth regulators could give rise to undesirable somaclonal variation.

In cultures placed under a 16 h photoperiod, the maximum number of adventitious shoots produced per explant and percentage of explants producing shoots (4.2 ± 0.74 and 94% respectively) were observed at 35°C. Percentage of explants producing shoots increased with increased temperature. There was no significant difference in the fresh weight of adventitious shoots regenerated from each explant with increased temperature. Temperature is known to affect photosynthesis with varying optimum levels depending on the species type and the environmental conditions under which the plant is grown. Often plant cultures are maintained *in vitro* at 25°C which is the optimum temperature for photosynthesis in many C-3 plants **(SALISBURY and ROSS, 1992)**. However, plants exhibiting CAM are reported to have a higher optimum temperature of approximately 35°C **(SALISBURY and ROSS, 1992)**. The increased adventitious shoot production at higher temperature (35°C) observed in cultures under a 16 h

photoperiod could therefore be due to the presence of CAM which has been reported for *Huernia* species **(WATSON and DALLWITZ, 1992)**.

Furthermore, a comparison of cultures grown at 25°C under constant light to those under a 16 h photoperiod gives more insight into the physiology of this plant species. A significantly higher adventitious shoot production as well as fresh weight of the shoots regenerated per explant were observed in cultures placed under continuous light compared to 16 h light (Tables 4.4 and 4.5). The frequencies of explants producing shoots in cultures placed under 24 and 16 h light were 100% and 89% respectively. The increases observed in cultures under constant light suggest that *Huernia hystrix* possesses a facultative C-3 photosynthetic pathway requiring a longer photoperiod for best vegetative growth.

On the other hand, the reduction in fresh weight of shoots regenerated per explant and shoot production observed in cultures kept under 16 h light might be due to the formation of malic acid at night or in the dark. Accumulation of malic acid in the dark is characteristic of CAM plants, and is often accompanied by a net loss of sugars and starch **(SALISBURY and ROSS, 1992)**. The fact that some succulents have the ability to shift between the C-3 and CAM photosynthetic pathways based on photoperiod has been reported by some authors **(QUIEROZ, 1974; CHENG and EDWARDS, 1991)**. The similar adventitious shoot production observed in cultures at 24 h light (25°C) compared to those kept at 35°C (16 h light, Table 4.4) suggests that an increased photoperiod could in part compensate for sub-optimal temperature. Even then, the higher number of adventitious shoots longer than 10 mm and higher fresh weight of adventitious shoots regenerated per explant in cultures at 24 h light (25°C) compared to any of the cultures at 16 h light make 24 h photoperiod and 25°C temperature the best environmental conditions for the *in vitro* adventitious shoot production of this species.

4.3.4 Titratable acidity

Figure 4.3 presents the titratable acidity at the beginning, middle and end of the dark periods in cultures incubated at 25 and 35°C. Generally, the levels of titratable acidity at the end of the dark period were significantly higher than at the

beginning. In the case of cultures incubated at 25°C, 35% of the increased titratable acidity was recorded by the middle of the 8 h dark period. In contrast, cultures incubated at 35°C had 92% of the increased titratable acidity by the middle of the dark period. This implies a faster increase in titratable acidity with an increase in temperature. Temperature is said to affect CAM performance in many ways **(LÜTTGE, 2004)**. For instance, as stated by **LIN *et al*. (2006)**, the catalytic activity of phosphoenolpyruvate carboxylase (PEPC) (an enzyme involved in dark fixation of CO_2) increases with increasing temperature. **BRANDON (1967)** reported optimum activity of acid-producing enzymes in CAM at 35°C. However, **FRIEMERT *et al*. (1988)** explained that an increase in temperature increases the rate of malic acid efflux from the vacuole, which could lead to the exposure of PEPC in the cytosol to increasing malic acid concentrations and its subsequent allosteric inhibition. The lack of significant difference of the titratable acidity between 25°C and 35°C at the different times of measurement (Figure 4.3) might be due to increased sensitivity of PEPC to malate at high night temperature **(CARTER *et al*., 1995; LIN *et al*., 2006)**. In any case, the nocturnal synthesis and accumulation of organic acid which is an essential component of CAM **(LÜTTGE, 2004)** further indicates the presence of CAM in cultures kept under a 16 h photoperiod. Although a low night temperature and high day temperature is often indicated as more favourable to CAM, the expression of CAM under constant temperature has been reported **(CHENG and EDWARDS, 1991; LÜTTGE and BECK, 1992)**. A combination of a hot day with lower night temperature (not tested in this study) might likely enhance the expression of CAM in this species.

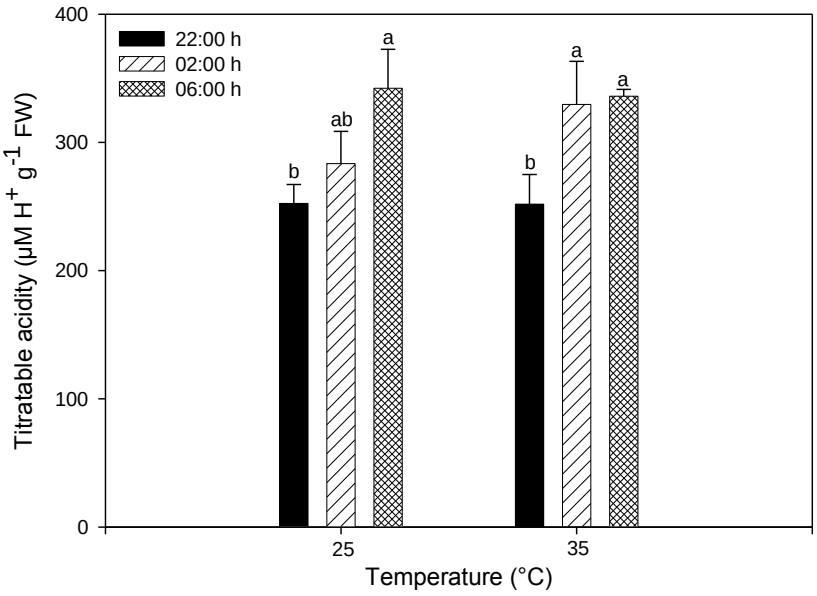


Figure 4.3: Nocturnal titratable acidity in *Huernia hystrix* shoots cultured at different temperatures. 22:00, 02:00 and 06:00 h are the beginning, middle and end of 8 h dark period respectively. Bars with different letters are significantly different (*P* = 0.05) according to DMRT.

4.3.5 Effects of culture vessel size on shoot multiplication

A higher adventitious shoot production (though not statistically significant) of 5.6 ± 0.94 shoots per explant was obtained with cultures in screw cap jars (Table 4.6, Figure 4.4). In the same vein, the number of adventitious shoots longer than 10 mm as well as fresh weight of shoots regenerated per explant were higher (not significantly) in cultures contained in screw cap jars. **ISLAM *et al*. (2005)** were of the opinion that larger vessels with larger air volumes create a better gaseous composition in the headspace by retarding accumulation of unfavourable gases which affect growth and development of plants in cultures. In the current study, the headspace volume of 90 ml per explant in the screw cap jars compared to 30 ml headspace volume per explant in the culture tubes could have created a more favourable gaseous composition in the headspace. However, **KOZAI *et al*. (1997)** stated that the type of vessel closure could also affect the gaseous composition as well as light environment of cultures. Besides the improved adventitious shoot production and fresh weight of shoots regenerated per explant, the use of larger

culture vessels in this study is more economical as it conserves space in the growth room (Table 4.6). Based on the number of adventitious shoots produced per explant in each of the two different vessels (Table 4.6), an additional 679 shoots per square meter could potentially be produced in the growth room with the use of the screw cap jars compared to culture tubes.

Table 4.6: Effects of culture vessel size on adventitious shoot production of *Huernia hystrix* after eight weeks of culture

	Culture vessel	
Parameters measured	Culture tube (40 ml)	Screw cap jar (300 ml)
Adventitious shoots per explant (*n*)	4.4 ± 0.53	5.6 ± 0.94^{ns}
Explants producing shoots (%)	100	100
Adventitious shoots > 10 mm in length (*n*)	2.8 ± 0.48	3.1 ± 0.63^{ns}
Adventitious shoots 5 - 10 mm in length (*n*)	1.6 ± 0.25	2.5 ± 0.48^{ns}
Shoot fresh weight per explant (mg)	560 ± 154	630 ± 168^{ns}
Shoots (*n*) per m^2 space	2750	3429

^{ns} = Non-significant effect (P = 0.05) according to *t*-test.

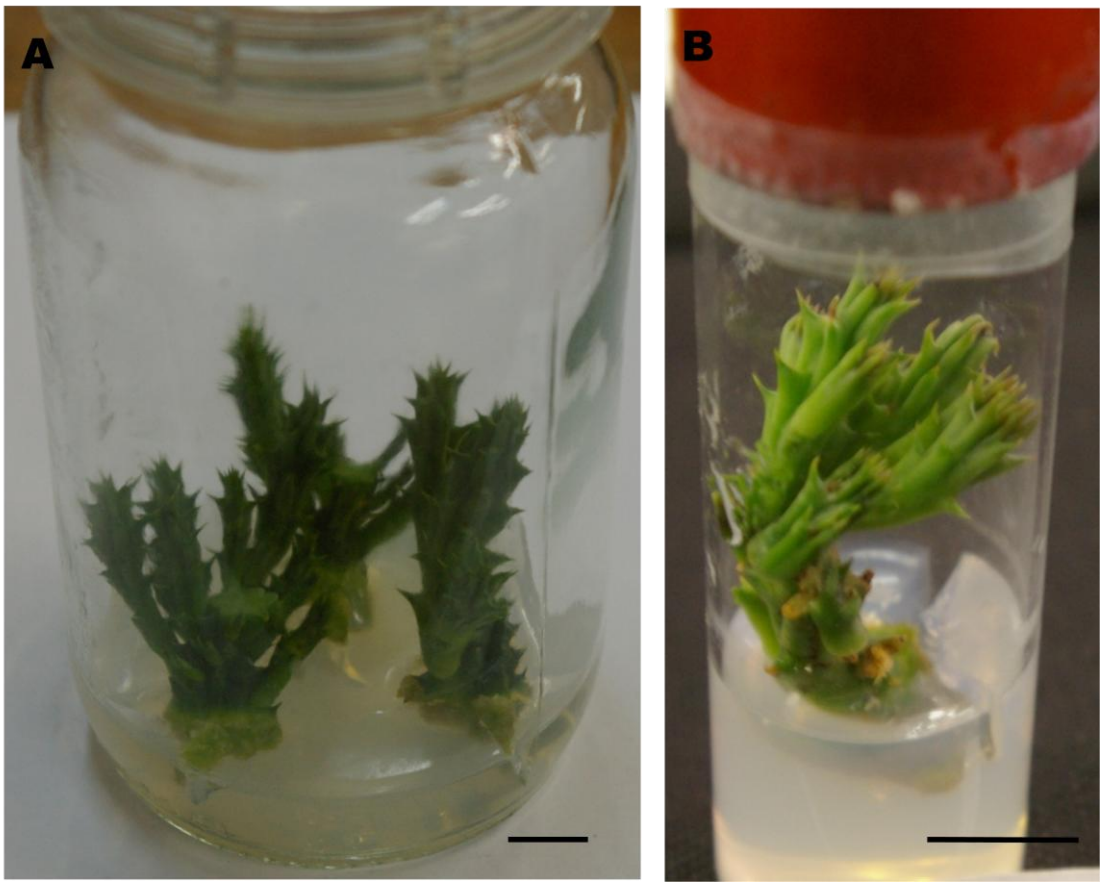


Figure 4.4: *Huernia hystrix* adventitious shoot production from explants cultured in different culture vessels. (**A**) Screw cap jar [300 ml]. (**B**) Culture tube [40 ml]. Scale bar = 10 mm.

4.3.6 Indirect organogenesis

The effects of different combinations of BA and NAA on callus growth are presented in Figure 4.5. Generally, callus fresh weight increased (significantly in many cases) with increased BA concentration at the same NAA concentration. Similarly, there was a significant increase in most cases with increased NAA concentration at the same level of BA concentration. This suggests that both NAA and BA have synergistic effects on callus growth in *H. hystrix*. **MARTIN (2002)** reported shoot organogenesis from callus developed at the basal cut ends of the node and internode explants of *Holostemma ada-kodien* on MS medium fortified with 1.0 - 2.5 mg l^{-1} BA. On the other hand, **PATNAIK and DEBATA (1996)** reported no shoot formation from *Hemidesmus indicus* callus. In this study, there was no evidence of shoot induction in all the combinations evaluated. However,

the root induction observed in some cases (Figures 4.1E and F) demonstrates the organogenic capacity of the induced callus. There may be a need to test other growth regulators for shoot induction from the derived callus and evaluate the possibility of inducing somatic embryos. Meanwhile the established callus cultures could be a potential alternative source of bioactive secondary metabolite production **(GIULIETTI and ERTOLA, 1999)**.

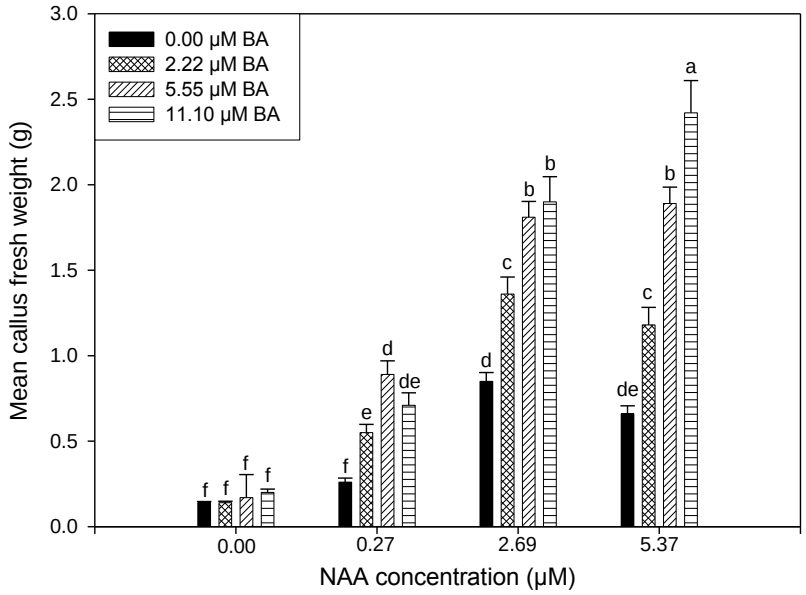


Figure 4.5: Effects of combinations of NAA and BA concentrations on callus growth. Bars with different letters are significantly different (*P* = 0.05) according to DMRT.

4.3.7 Rooting and acclimatization

The efficiency of half strength MS medium with or without IBA supplementation in rooting has been reported for many Asclepiadaceae plants **(KOMALAVALLI and RAO, 2000; SREEKUMAR *et al*., 2000; MARTIN, 2002; BEENA *et al*., 2003)**. **PATNAIK and DEBATA (1996)** observed that IBA was more effective than IAA for the induction of rooting in *Hemidesmus indicus*. Based on the results from the multiplication experiments, regenerated shoots were rooted individually on half strength MS medium with or without IBA. Table 4.7 shows the effects of half

strength MS medium with or without supplementation with IBA concentration on rooting and acclimatization of regenerated shoots. The PGR-free medium produced an average of 7.5 roots per shoot, higher than the IBA treatment, although with no significant difference. Similarly, the treatment with IBA did not produce a significant difference in both the fresh and dry weights of the roots and shoots. Rooted plantlets acclimatized successfully in greenhouse conditions with more than 95% survival and no observable morphological abnormalities (Figure 4.1D). Best root induction and survival of *Holostemma annulare* (another Asclepiadaceae species) on half strength MS medium supplemented with 1.48 µM IBA has been reported **(SUDHA *et al*., 1998)**. **KOZAI *et al*. (1997)** stated that the relatively high production costs of micropropagation, resulting mainly from high labour costs, low growth rate *in vitro* and poor survival rates of the plantlets during acclimatization limits its commercial application. The high number of roots per shoot produced on half strength PGR-free medium in *H. hystrix* with subsequent high survival rate (after direct transfer to a greenhouse environment) makes this regeneration protocol particularly attractive from an economic point of view as it reduces labour and cost of production.

Table 4.7: Effects of half-strength MS medium with or without IBA supplementation on rooting and acclimatization of regenerated plants

Parameter	Medium	
	½ MS	½ MS + 1 µM IBA
Total plantlets potted (*n*)	60	60
Survival frequency (%)	96.7	100
Roots produced per shoot (*n*)[#]	7.50 ± 0.764[ns]	6.75 ± 0.827
Root fresh weight per plant (g)[#]	0.10 ± 0.011[ns]	0.10 ± 0.018
Shoot fresh weight per plant (g)[#]	2.47 ± 0.270[ns]	2.52 ± 0.333
Root dry weight per plant (mg)[#]	20 ± 1[ns]	20 ± 2
Shoot dry weight per plant (mg)[#]	100 ± 10[ns]	90 ± 12

[ns] = Non-significant effect ($P = 0.05$)

[#]Twelve fully acclimatized 2-month-old plants were used for this analysis.

The successful micropropagation system described here provides an effective means for the conservation and rapid clonal propagation, within a short time, of endangered *H. hystrix*. The observations from this study provide insight into the physiology of this species when cultured at different temperatures and photoperiods. This study highlights the need to investigate the effects of environmental conditions when developing efficient micropropagation protocols, especially for commercial purposes. Optimizing environmental conditions could increase growth rate, reduce labour costs and thus subsequent production costs. The increased multiplication rate and cost-effective, easy acclimatization process makes this protocol highly advantageous.

Chapter 5 Pharmacological and phytochemical evaluation of *Barleria* species and *Huernia hystrix*

5.1 Introduction

The development of new diseases and resistance of many pathogens to currently used drugs coupled with the negative side-effects of many of these drugs have necessitated the continuous search for potent and efficacious new drugs or drug leads with minimal or no side-effects **(CRAGG *et al*., 1997)**. The plant kingdom has been appropriately described as a reservoir of many novel biologically active molecules of medicinal value **(TALHOUK *et al*., 2007)**. The exploration of plant resources for their phytochemical and pharmacological potential could lead to identifying such novel biologically active compounds. According to **CRAGG *et al*. (1997)**, the continuing threat to biodiversity and the proven record of natural products in drug discovery, among others, give compelling reasons for expanding the exploration of nature to identify novel active agents as leads for effective drug development.

Southern Africa is known to have a highly diverse and bioresource-rich flora estimated to be approximately one-tenth of global plant diversity **(ELOFF, 1998a)**. This region is also known to have a high level of endemic plant species **(VAN STADEN, 2008)**. The rich floral diversity provides a great bioprospecting opportunity for the discovery of potentially new pharmacologically active compounds. **ELOFF (1998a)** reported however, that only a relatively small number of plants in this flora have been evaluated for their pharmacological and phytochemical potential. Yet, many plants in this region are facing the risk of extinction even before they are evaluated for their medicinal properties **(SHAI *et al*., 2008)**. The extinction of such species could lead to the loss of potential therapeutic compounds and/or genes capable of being exploited in the biosynthesis of new potent pharmaceutical compounds or drugs **(RATES, 2001)**. **HOSTETTMANN and MARSTON (2002)** recommended dual biological and chemical screening as the fastest approach in the exploration for new lead compounds from plant species. They suggested the use of simple, sensitive and

target-specific pharmacological assays with the capacity to quickly localise the chosen activity in plant extracts. Plant extracts often contain different chemicals with different pharmacological activities **(HOUGHTON *et al*., 2007)**. Thus, the use of a series of pharmacological tests has been recommended in order to get a complete bioactivity spectrum of a plant extract **(HOUGHTON *et al*., 2007)**. Furthermore, since the aetiology of many disease states is often due to more than one factor, the use of a series of pharmacological tests might be helpful in understanding the mechanism of action involved in the therapeutic effect of a particular plant extract **(HOUGHTON *et al*., 2007)**.

This study was aimed at exploring the medicinal value of two endangered and endemic southern Africa species belonging to different taxonomic categories, *B. greenii* (a shrub) and *H. hystrix* (a succulent). For comparison purposes, extracts from two other Barleria species (*B. prionitis* and *B. albostellata*) were included. *B. albostellata* is mainly a horticultural species while *B*. *prionitis* is widely used in folk medicine to treat infection-related ailments **(KOSMULALAGE *et al*., 2007)**. The leaves of *B. prionitis* are reportedly used in the treatment of fever, toothache, liver ailments, against ulcers and piles as well as in irritation control. The aerial parts are used in inflammation treatments and the roots to disperse boils and glandular swellings **(SINGH *et al*., 2003; VERMA *et al*., 2005)**. Different extracts from different parts of these plant species were evaluated in a number of pharmacological assays with a view to investigating the possibility of plant-part substitution as a conservation strategy against destructive harvesting of these species for medicinal purpose. The phytochemical properties of different parts of these species were also evaluated.

5.2 Materials and methods

5.2.1 Collection of plant materials

Plant materials were collected during the summer in 2007. *Barleria greenii* was bought from the Indigenous Nursery at Natal Botanical Gardens, Pietermaritzburg, *B. prionitis* from Val Lea Vista Nursery, Pietermaritzburg while *B. albostellata* and *Huernia hystrix* were collected from the University of KwaZulu-Natal Botanical

Garden, Pietermaritzburg, South Africa. Voucher specimens (S. Amoo 02 NU, S. Amoo 04 NU, S. Amoo 03 NU, and S. Amoo 05 NU, respectively) were deposited in the University of KwaZulu-Natal Herbarium, Pietermaritzburg. Plant parts were dried at 50°C, ground and stored in airtight containers at room temperature in the dark.

5.2.2 Pharmacological evaluation

5.2.2.1 Preparation of extracts

Dried, ground plant materials were extracted sequentially with petroleum ether (PE), dichloromethane (DCM) and 80% ethanol (EtOH) (20 ml/g) using a sonication bath containing ice water for 1 h each. Methanolic (MeOH) extracts (used in antioxidant and acetylcholinesterase assays) were obtained by extracting plant materials with 50% methanol (20 ml/g) using a sonication bath containing ice water for 20 min. In each case, the crude extracts were filtered through Whatman No. 1 filter paper and concentrated *in vacuo* at 40°C using a rotary evaporator. The concentrated extracts were air-dried at room temperature.

5.2.2.2 Antibacterial activity

Minimum inhibitory concentration (MIC) of extracts for antibacterial activity was determined using the micro-dilution bioassay in 96-well microtitre plates as described by **ELOFF (1998b)**. Overnight cultures (incubated at 37°C in a water bath with orbital shaking) of four bacterial strains: two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacteria were diluted with sterile Mueller-Hinton (MH) broth (1 ml bacteria/50 ml MH). Each crude plant extract was re-dissolved in ethanol to make a concentration of 50 mg/ml. One hundred microlitres of each extract were two-fold serially diluted with 100 µl sterile distilled water in a 96-well microtitre plate for each of the four bacteria. A similar two-fold serial dilution of neomycin (0.1 mg/ml) was used as a positive control against each bacterium. One hundred microlitres of each bacterial culture were added to each well. The ethanol solvent, distilled water and MH broth

were included as negative controls. The plates were covered with parafilm and incubated overnight at 37°C. To indicate bacterial growth, 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) were added to each well and the plates were further incubated at 37°C for at least 30 min. Bacterial growth in the wells was indicated by a change in colour, whereas clear wells indicated inhibition by the tested extracts. MIC values were recorded as the lowest concentrations of extracts showing clear wells. The minimum inhibitory dilution (MID) (ml/g) indicating the volume to which the extract derived from 1 g can be diluted and then still inhibit bacterial growth **(ELOFF, 2004)** was also determined for each extract. The assay was repeated twice with two replicates each.

5.2.2.3 Antifungal activity

Antifungal activity against *Candida albicans* (ATCC 10231) was performed using the micro-dilution assay **(ELOFF, 1998b)** modified for fungi **(MASOKO *et al*., 2007)**. Four milliliters of sterile saline were added to 400 µl of a 24-h-old *Candida* culture prepared in Yeast Malt (YM) broth. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 M McFarland standard solution. From this stock, a 1:1000 dilution with sterile YM broth was prepared. One hundred microlitres of each extract dissolved in 80% ethanol at 50 mg/ml were two-fold serially diluted with sterile distilled water in a 96-well microtitre plate. One hundred microlitres of the dilute fungal culture were added to each well. Amphotericin B was used as a positive control while the 80% ethanol solvent, distilled water and YM broth were included as negative controls. The plates were covered with parafilm and incubated at 37°C overnight after which 50 µl of INT (0.2 mg/ml) were added to each well as a growth indicator. The wells remained clear where there was inhibition. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48 h. To determine whether the activity is fungistatic or fungicidal, 50 µl of YM broth were added to the clear wells and further incubated for 24 h after which the minimum fungicidal concentration (MFC) was recorded as the last clear well. The MID (ml/g) as well as minimum fungicidal dilution (MFD) (ml/g), indicating the volume to which the extract derived from 1 g can be diluted and still inhibit the growth of or kill the fungal cells **(ELOFF,**

2004) respectively, were also determined. The assay was repeated twice with at least two replicates each.

5.2.2.4 Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the enzyme-based cyclooxygenase assays (COX-1 and COX-2) as described by **JÄGER *et al*. (1996)** as well as **ZSCHOCKE and VAN STADEN (2000)**. Plant extracts were re-dissolved in ethanol at a concentration of 10 mg/ml to give a concentration of 0.25 µg/µl in the final assay. The cofactor solution was prepared by the addition of 10 ml of 0.1 M Tris buffer (pH 8.0) and 100 µl of hematin solution to 3 mg of L-epinephrine (6 mg for COX-2) and 3 mg of reduced glutathione. The COX-1 (from ram seminal vesicles) or COX-2 (human recombinant) enzyme (Sigma-Aldrich) was, in each assay, activated with the co-factor solution and pre-incubated on ice for 5 min. Enzyme/co-factor solution (60 µl) was added to 20 µl of plant extract solution (2.5 µl of plant extract + 17.5 µl distilled water) and pre-incubated for 5 min at room temperature. Twenty microlitres of [^{14}C] arachidonic acid (16 Ci/mol, 3 mM) were added to this enzyme-extract mixture and incubated at 37°C in a water bath for 10 min. After incubation, the reaction was terminated by adding 10 µl of 2 N HCl. Unlabelled prostaglandins (PGE_2:PGF_2, 1:1 v/v) (4 µl, 0.2 mg/ml) were then added to each sample as a carrier solution.

The labelled prostaglandins synthesized in the assay were separated from the unmetabolized arachidonic acid by column chromatography using silica columns. Silica gel (0.063-0.200 mm particle size, Merck) in eluent 1 (hexane:1,4-dioxan:acetic acid, 350:150:1 v/v/v) was packed to a height of 3 cm in Pasteur pipettes stoppered with glass wool. One millilitre of eluent 1 was added to each of the assay mixtures and the mixtures were then applied to the columns. The unmetabolized arachidonic acid was eluted with 4 ml of eluent 1 (1 ml at a time) and discarded. The labelled prostaglandins were then eluted into scintillation vials with 3 ml (1 ml at a time) of eluent 2 (ethyl acetate:methanol, 85:15 v/v). Four millilitres of scintillation fluid were added to each vial and the radioactivity was counted after 30 min in the dark using a Beckman LS6000LL scintillation counter. In each assay, four controls were run. Two were backgrounds in which the

enzyme was inactivated with HCl before adding [^{14}C] arachidonic acid and which were kept on ice, and two were solvent blanks. Indomethacin was used as a positive control at a concentration of 5 µM for COX-1 and 200 µM for COX-2. The percentage inhibition by the extracts was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank, using the following equation:

$$\text{Inhibition } (\%) = \left[1 - \left(\frac{\text{DPMsample} - \text{DPMbackground}}{\text{DPMblank} - \text{DPMbackground}}\right)\right] \times 100$$

where DPM is the disintegrations per minute. Results presented are the mean values of two experiments (each experiment in duplicate).

5.2.2.5 Acetylcholinesterase (AChE) inhibition

A microtitre plate assay based on the colorimetric method described by **ELLMAN *et al*. (1961)** and outlined by **ELDEEN *et al.* (2005)** was used to determine the AChE inhibition activity by the plant extracts. The following buffers were prepared: Buffer A (50 mM Tris-HCl, pH 8), Buffer B (50 mM Tris-HCl, pH 8, containing 0.1% bovine serum albumin) and Buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$). Twenty-five microlitres of each plant methanolic extract at a known concentration were two-fold serially diluted with millipore distilled water in a 96-well microtitre plate. Twenty-five microlitres of acetylthiocholine iodide (ATCI) (15 mM in millipore distilled water), followed by 125 µl of 3 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (dissolved in Buffer C) and 50 µl of Buffer B were then added to each microtitre plate well. A similar procedure was followed with galanthamine (20 µM) used as a positive control in place of plant extract. The absorbance was read every 45 s (three times) at 405 nm using a microtitre plate reader (Opsys MR^{TM}, Dynex Technologies). After the readings, 25 µl of 0.2 U ml^{-1} AChE enzyme (from electric eels) was added to each well and the absorbance was read again every 45 s (five times). The absorbance readings before the addition of enzyme were subtracted from the absorbance readings taken after the addition of enzyme, to correct any increase in absorbance due to spontaneous hydrolysis of the substrate. The rate of reaction was calculated for

each of the plant extracts, the blank and positive control. The percentage inhibition by the plant extracts was calculated using the formula:

$$\text{Inhibition (\%)} = \left[1 - \frac{\text{Sample reaction rate}}{\text{Blank reaction rate}}\right] \times 100$$

5.2.2.6 Antioxidant activity

5.2.2.6.1 DPPH radical-scavenging activity

Fifteen microlitres of methanolic extracts at different concentrations were diluted with methanol to a final volume of 750 µl. The diluted extracts were then added to an equal volume of DPPH (1,1-diphenyl-2-picrylhydrazyl) (100 µM in methanol) **(SHARMA and BHAT, 2009)** and the mixtures incubated in the dark at room temperature for 30 min. The negative control had methanol in place of the extract while ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls. The absorbance was read at 517 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia). Blank solutions with methanol in place of DPPH were included for each extract, in order to correct any absorbance due to extract colour. The spectrophotometer was zeroed with methanol and tests were carried out in triplicate. The radical scavenging activity (RSA) was calculated using the equation:

$$\text{RSA (\%)} = \left[1 - \left(\frac{A_{\text{extract}} - A_{\text{blank}}}{A_{\text{control}}}\right)\right] \times 100$$

where A_{extract}, A_{blank} and A_{control} are the absorbances of the extract, blank solution and negative control, respectively. The EC_{50}, which is the concentration of the extract required to scavenge 50% of DPPH radical, was determined for each extract using GraphPad Prism software (version 4.03). The data were log-transformed, normalized and fitted into a nonlinear regression for EC_{50} determination.

5.2.2.6.2 Ferric reducing power activity

The reducing power of the extracts was determined according to the method described by **KUDA *et al*. (2005)** with slight modification. Thirty microliters of each extract were two-fold serially diluted with methanol in a 96-well microtitre plate, followed by the addition of 40 µl each of 0.2 M potassium phosphate buffer (pH 7.2) and 1% (w/v) potassium ferricyanide. The mixture was incubated in the dark at 50°C for 20 min after which trichloroacetic acid (40 µl, 10% w/v), distilled water (150 µl) and $FeCl_3$ (30 µl, 0.1% w/v) were added. The positive controls were similarly prepared using ascorbic acid and BHT, while methanol was used in place of extract as the negative control. After incubating at room temperature for 30 min, the absorbance was read at 630 nm **(JONFIA-ESSIEN *et al*., 2008)** using a microtitre plate reader.

5.2.2.6.3 β-Carotene linoleic acid assay

The method described by **AMAROWICZ *et al*. (2004)** was followed with slight modification. β-Carotene (10 mg) was initially dissolved in chloroform (10 ml) and the chloroform was removed by evaporation under vacuum, followed by the addition of linoleic acid (200 µl) and Tween 20 (2 ml). The mixture was then made up to 500 ml (with aerated distilled water) with vigorous shaking to form an emulsion. An aliquot (4.8 ml) of the emulsion was added to 200 µl of plant extract (6.25 mg/ml) or BHT (6.25 mg/ml), used as a positive control. Each sample was prepared in triplicate and the test systems were incubated in a water bath at 50°C for 120 min. Initial absorbance (before incubation) and absorbance at every 30 min (during incubation) were read using a UV-visible spectrophotometer (Varian Cary 50, Australia) set at 470 nm. A Tween 20 solution was used to blank the spectrophotometer **(PAREJO *et al*., 2002)**. Antioxidant activities were expressed as ANT (%), ORR and AA (%) at t = 60 or 120 min **(AMAROWICZ *et al*., 2004; PAREJO *et al*., 2002)** using the following equations:

$$\text{ANT}\ (\%) = \left(\frac{R_{\text{control}} - R_{\text{sample}}}{R_{\text{control}}}\right) \times 100$$

$$\mathrm{ORR} = \frac{R_{\mathrm{sample}}}{R_{\mathrm{control}}}$$

$$\mathrm{AA}\ (\%) = \left[1 - \left(\frac{A_E^{t=0} - A_E^{t=t}}{A_W^{t=0} - A_W^{t=t}}\right)\right] \times 100$$

where ANT (%) is the overall antioxidant activity by the extract based on the average β-carotene bleaching rate by the extract relative to the aqueous control, R_{sample} and R_{control} are the average bleaching rates of β-carotene in the emulsion with and without the plant extracts respectively, ORR is the oxidation rate ratio, AA is the antioxidant activity at t = 60 or 120 min, $A_E^{t=0}$ and $A_W^{t=0}$ are the absorbances of extract and aqueous control respectively at t = 0 min, $A_E^{t=t}$ and $A_W^{t=t}$ are the absorbances of extract and aqueous control respectively at *t* = 60 or 120 min.

5.2.3 Phytochemical evaluation

5.2.3.1 Preparation of extracts

The method described by **MAKKAR (2000)** was followed with slight modifications. Two grams of ground, dried plant samples were extracted with 10 ml of 50% aqueous methanol by sonication in cold water for 20 min. The extracts were then filtered under vacuum through Whatman No.1 filter paper. The filtrates were kept on ice for subsequent analysis.

5.2.3.2 Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu (Folin C) method as described by **MAKKAR (2000)** with slight modifications. Distilled water (950 µl) was added to 50 µl of each plant sample, followed by the addition of 1 N Folin C reagent (500 µl) and 2% (w/v) sodium carbonate (2.5 ml). A blank containing 50% (v/v) aqueous methanol in place of plant extract was similarly prepared. After incubation at room temperature for 40 min, the absorbance was read at 725 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia). Standards for the calibration curve were prepared using gallic acid (Sigma). Each

sample was tested in three replicates and the results were expressed in mg or µg gallic acid equivalents (GAE) per gram dry weight (DW).

5.2.3.3 Total iridoid content

The total iridoid content was determined following the method described by **LEVIEILLE and WILSON (2002)**, which was adapted from **HAAG-BERRURIER *et al*. (1978)**. The method was based on the characteristics of glucoiridoids to form a fulvoiridoid complex when reacted with aldehydes (such as vanillin) in an acidic medium **(LEVIEILLE and WILSON, 2002)**. A vanillin-sulphuric acid reagent was prepared by the addition of methanol (82 ml), vanillin (100 mg) and concentrated sulphuric acid (8 ml). A blank reagent containing methanol (82ml) and concentrated sulphuric acid (8 ml) was also prepared. In triplicate, the vanillin-sulphuric acid reagent (1.35 ml) was added to 150 µl of each extract. A blank was prepared for each extract by adding 1.35 ml of the blank reagent to 150 µl of extract. The reaction occurred immediately at room temperature and the absorbance was read at 538 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia). HPLC-grade harpagoside (Extrasynthèse, France) was used as a standard for the calibration curve. The total iridoid content, expressed in µg harpagoside equivalents (HE) per gram DW was determined for each extract using their differential absorbance values correlated with the calibration curve.

5.2.3.4 Flavonoid content

The vanillin assay as described by **NDHLALA *et al*. (2007)** was used to determine the flavonoid content of the samples. Fifty microlitres of each extract were diluted with distilled water to 1 ml before the addition of 2.5 ml of methanol-HCl (95:5 v/v) and finally 2.5 ml of vanillin reagent (1% w/v). The mixtures were incubated for 20 min at room temperature after which the absorbance was read at 500 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia) against a blank containing methanol instead of plant extracts. Each extract had three replicates. Standards for the calibration curve were prepared using catechin and the flavonoid content was expressed in mg catechin equivalents per gram DW.

5.2.3.5 Gallotannin content

The rhodanine assay method as described by **MAKKAR (2000)** was used to determine the gallotannin content. Fifty microlitres of each plant sample were diluted with distilled water to 1 ml. One hundred microlitres of 0.4 N sulphuric acid and 600 µl of rhodanine solution (0.667% w/v in methanol) were added to the diluted extract. Two hundred microlitres of 0.5 N KOH were then added after 5 min of incubation at room temperature, and 4 ml distilled water was added after a further 2.5 min. The mixtures were further incubated at room temperature for another 15 min after which the absorbance was read at 520 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia) against a blank prepared similarly but containing methanol instead of plant extracts. Each extract had three replicates. Gallic acid was used to prepare the standards for the calibration curve and gallotannin content was expressed in µg gallic acid equivalents (GAE) per gram DW.

5.2.3.6 Condensed tannin (proanthocyanidin) content

This was determined using the butanol-HCl method as described by **MAKKAR (2000)**. Three millilitres of butanol-HCl (95:5 v/v) were added to 500 µl of each sample, followed by 100 µl of ferric reagent (2% w/v ferric ammonium sulphate in 2 N HCl). The mixtures were placed in a boiling water bath for 60 min. The absorbance was then read at 550 nm using a UV–visible spectrophotometer (Varian Cary 50, Australia) against a blank prepared in a similar way but without heating. Each sample had three replicates. Condensed tannins (% per dry matter) as leucocyanidin equivalents were calculated using the formula described by **PORTER *et al*. (1986)**:

$$\text{Condensed tannins (\% per dry matter)} = \frac{A_{550\text{nm}} \times 78.26 \times \text{Dilution factor}}{\text{\% dry matter}}$$

where A_{550nm} is the absorbance value at 550 nm.

5.2.4 Data analyses

The percentage inhibitions were log-transformed before they were subjected to statistical analysis. Data were subjected to one-way ANOVA. Where there were significant differences (P = 0.05), the mean values were further separated using DMRT. The analysis was done using SPSS software (version 15.0).

5.3 Results and discussion

5.3.1 Yield of plant extracts

Tables 5.1 and 5.2 show the percentage yield obtained from different extracts of *Barleria* species and *Huernia hystrix*, respectively. Both the EtOH and MeOH plant extracts (polar extracts) have higher yields compared to the PE and DCM extracts (non-polar extracts). The yields obtained from the EtOH and MeOH extracts of *Barleria* species leaves were higher compared to other parts of the same plant. In *H. hystrix*, the yield from MeOH extracts were greater than that obtained with EtOH extract.

5.3.2 Pharmacological evaluation

5.3.2.1 Antibacterial activity

The MIC and MID of extracts from different parts of the studied *Barleria* species are presented in Table 5.3. All the extracts showed a broad-spectrum antibacterial activity. The DCM extract of *B. greenii* roots gave the best antibacterial activity against *B. subtilis* and *S. aureus* with MIC values of 59 µg/ml and 234 µg/ml respectively. The PE, DCM and EtOH root extracts of *B. greenii* had lower MIC values (in most cases) against all the bacterial strains compared to its stem extracts. However, the harvesting of the roots of this endangered plant is destructive and could spell more problems for the survival of this species if no effective propagation measure is put in place. The observed antibacterial activity (especially in the roots) highlights the need for the propagation of this species in order to fully explore its potential therapeutic antibacterial agents. In all the

Barleria species, the EtOH extracts had the highest MID values. The DCM and EtOH extracts of *B. prionitis* leaves had lower MIC values against all the bacterial strains compared to similar extracts from its stems. This observation suggests the potential of substituting the leaves of this plant for its stems. The observed activity in *B. prionitis* is in agreement with the findings of **KOSMULALAGE *et al.* (2007)** who reported antibacterial activity of ethanolic extracts of aerial parts of *B. prionitis* against *S. aure*us and *Pseudomonas aeruginosa*. They reported the isolation of a new compound, balarenone along with other known natural products, pipataline and 13,14-*seco*-stigmasta-5,14-diene-3-β-ol from the ethanolic extract, all of which showed antibacterial activity against *Bacillus cereus* and *P. aeruginosa*. The observed antibacterial activity in *B. greenii* and *B. albostellata* could be due to the presence of these or other compounds. According to **JÄGER *et al*. (1996)**, when active compounds are found in one species, it is likely that more species of the same genus contain active compounds of a similar nature.

Table 5.4 presents the MIC and MID recorded in different extracts of *H*. *hystrix*. Although a broad spectrum activity with MIC values ranging from 0.39 to 6.25 mg/ml was observed in all the extracts, only the root extracts showed good activity (< 1 mg/ml) against the two Gram-positive bacteria used. The PE extract of the roots had lower MIC values against the two Gram-positive bacteria compared to the stem and whole plant extracts, indicating its better antibacterial activity. The root PE extract also had high MID values against the two Gram-positive bacteria. In general, with some exception in root extracts, the MID recorded in ethanol extracts was generally higher than all the other extracts.

Table 5.1: Yield (% w/w) of extracts prepared from different parts of three *Barleria* species in terms of starting crude material

Plant species	Plant part	Extract	Yield (% w/w)
B. prionitis	Leaves	PE	0.706
		DCM	0.312
		EtOH	9.404
		MeOH	31.82
	Stems	PE	0.362
		DCM	1.578
		EtOH	5.592
		MeOH	9.84
	Roots	PE	0.224
		DCM	0.332
		EtOH	4.28
		MeOH	12.05
B. greenii	Leaves	PE	2.163
		DCM	0.773
		EtOH	20.565
		MeOH	29.07
	Stems	PE	1.756
		DCM	0.758
		EtOH	8.504
		MeOH	4.53
	Roots	PE	0.312
		DCM	0.492
		EtOH	13.293
		MeOH	12.35
B. albostellata	Leaves	PE	5.54
		DCM	1.866
		EtOH	23.306
		MeOH	24.38
	Stems	PE	0.398
		DCM	0.35
		EtOH	5.306
		MeOH	4.56

Table 5.2: Yield (% w/w) of extracts prepared from different parts of *Huernia hystrix* in terms of starting crude material

Plant part	Extract	Yield (% w/w)
Stems	PE	1.288
	DCM	1.896
	EtOH	18.655
	MeOH	34.49
Roots	PE	2.728
	DCM	1.298
	EtOH	5.428
	MeOH	15.83
Whole plants	PE	1.66
	DCM	1.895
	EtOH	19.577
	MeOH	33.72

The comparatively lower activity against Gram-negative bacteria by some extracts might be as a result of the thick murein layer in these bacteria structures preventing the entry of inhibitors **(MATU and VAN STADEN, 2003)**. The low potency values generally observed in some of the extracts could be due to the fact that the extracts are still in an impure form and thus there could be some compounds having an antagonistic effect on the active principle(s). In addition, it could be as a result of low concentrations of active compounds present in the extracts **(RABE and VAN STADEN, 1997)**. The weak activities observed *in vitro* with some extracts however does not necessarily translate to a similar low activity *in vivo*. Some of these plant extracts, as with some drugs, may be more potent *in vivo* due to metabolic transformation of their components into highly active intermediates or their interaction with the immune system **(GARCIA *et al*., 2003; NGEMENYA *et al*., 2006)**.

Table 5.3: Antibacterial activity (MIC and MID) of crude extracts from different parts of three *Barleria* species

Plant species	Plant part	Extract	Minimum inhibitory concentration (MIC) (mg/ml)				Minimum inhibitory dilution (MID) (ml/g)			
			B. s[#]	*S. a*	*E. c*	*K. p*	*B. s*	*S. a*	*E. c*	*K. p*
B. prionitis	Leaves	PE	3.125	3.125	3.125	3.125	2.259	2.259	2.259	2.259
		DCM	**0.781***	1.563	1.563	1.563	3.995	1.996	1.996	1.996
		EtOH	3.125	3.125	3.125	3.125	30.093	30.093	30.093	30.093
	Stems	PE	1.563	3.125	1.563	3.125	2.316	1.158	2.316	1.158
		DCM	3.125	6.250	3.125	3.125	5.050	2.525	5.050	5.050
		EtOH	6.250	6.250	6.250	6.250	8.947	8.947	8.947	8.947
B. greenii	Stems	PE	3.125	6.250	3.125	3.125	5.619	2.810	5.619	5.619
		DCM	1.563	1.563	3.125	3.125	4.850	4.850	2.426	2.426
		EtOH	6.250	6.250	6.250	3.125	13.606	13.606	13.606	27.213
	Roots	PE	**0.780**	3.125	3.125	1.563	4.000	0.998	0.998	1.996
		DCM	**0.059**	**0.234**	1.875	3.750	83.390	21.026	2.624	1.312
		EtOH	**0.390**	**0.781**	6.250	3.125	340.846	170.205	21.269	42.538
B. albostellata	Leaves	PE	**0.781**	3.125	3.125	1.563	70.935	17.728	17.728	35.445
		DCM	**0.195**	1.563	3.125	**0.781**	95.692	11.939	5.971	23.892
		EtOH	1.563	1.563	3.125	3.125	149.111	149.111	74.579	74.579
	Stems	EtOH	1.563	3.125	3.125	3.125	33.948	16.979	16.979	16.979
Neomycin (µg/ml)			0.098	1.563	3.125	1.563				

[#]*B*. s = *Bacillus subtilis, S. a = Staphylococcus aureus, E. c = Escherichia coli*, *K. p = Klebsiella pneumonia*.

*Values boldly-written are considered very active (< 1 mg/ml).

Table 5.4: Antibacterial activity (MIC and MBC) of crude extracts from different parts of *Huernia hystrix*

		Minimum inhibitory concentration (MIC) (mg/ml)				Minimum inhibitory dilution (MID) (ml/g)			
Plant part	Extract	*B. s*[#]	*S. a*	*E. c*	*K. p*	*B. s*	*S. a*	*E. c*	*K. p*
Stems	PE	1.56	3.13	3.13	3.13	8.256	4.115	4.115	4.115
	DCM	3.13	1.56	3.13	3.13	6.058	12.154	6.058	6.058
	EtOH	6.25	6.25	3.13	3.13	29.848	29.848	59.601	59.601
Roots	PE	**0.78***	**0.39**	6.25	3.13	34.974	69.949	4.365	8.716
	DCM	**0.78**	1.56	6.25	6.25	16.641	8.321	2.077	2.077
	EtOH	6.25	6.25	6.25	3.13	8.685	8.685	8.685	17.342
Whole plants	PE	3.13	3.13	6.25	6.25	5.304	5.304	2.656	2.656
	DCM	3.13	1.56	3.13	3.13	6.054	12.147	6.054	6.054
	EtOH	6.25	6.25	3.13	3.13	31.323	31.323	62.546	62.546
Neomycin (µg/ml)		0.195	0.78	3.13	1.56				

[#]*B*. s = *Bacillus subtilis, S. a = Staphylococcus aureus, E. c = Escherichia coli*, *K. p = Klebsiella pneumonia*.

*Values boldly-written are considered very active (< 1 mg/ml).

5.3.2.2 Antifungal activity

Opportunistic fungal infections such as candidiasis caused by *C. albicans*, have been reported to be common especially among immunocompromised persons with AIDS (acquired immune deficiency syndrome) all over the world **(MOTSEI *et al*., 2003; SHAI *et al*., 2008)**. Table 5.5 shows the antifungal activity of extracts from different parts of *Barleria* species against *C. albicans*. In general, all the extracts demonstrated fungistatic activity against *C. albicans*. The MIC of the extracts ranged from 0.78 to 9.375 mg/ml while the MFC ranged from 1.17 to 12.5 mg/ml. The EtOH extracts had the highest MID and MFD in all cases. The PE, DCM and EtOH extracts of *B. greenii* leaves had higher inhibitory activity compared to similar extracts of the stems. In the same vein, the leaf EtOH extract of *B. greenii* had the highest MID compared to all other extracts and demonstrated a fungicidal activity similar to the EtOH extracts of its roots and stems. The fungistatic activity of *B. greenii* leaf EtOH extract was also higher than the EtOH extract of its stems or roots. These results suggest the potential of *B. greenii* leaves in plant part substitution. The leaves can be sustainably harvested for medicinal purposes without the destructive harvesting of the roots which could threaten the survival of this already rare plant species **(ZSCHOCKE *et al*., 2000b; MATU and VAN STADEN, 2003)**.

The antifungal activity of *H. hystrix* extracts against *C. albicans* is presented in Table 5.6. All the extracts showed inhibitory activity with MIC ranging from 0.78 to 6.25 mg/ml. With the exception of stem EtOH extract (the MFC of which was not observed within the concentration range tested), all the extracts evaluated generally demonstrated a moderate fungicidal activity against *C. albicans*. The EtOH extracts had the highest MID compared to PE and DCM extracts. The EtOH extracts similarly had the highest MFD (except stem EtOH extract) compared to PE and DCM extracts. Although the PE and DCM extracts of the whole plant had the same MFC as the stem extracts, the inhibitory activities of the whole plant PE and DCM extracts were higher than that of the stem or the root. This observation possibly suggests the presence of fungistatic agents, likely acting in an additive manner in the whole plant extract compared to their individual inhibitory action in the stem or root extract.

Table 5.5: Antifungal activity of crude extracts from different parts of three *Barleria* species against *Candida albicans*

Plant species	Plant part	Extract	Minimum inhibitory concentration (MIC) (mg/ml)	Minimum fungicidal concentration (MFC) (mg/ml)	Minimum inhibitory dilution (MID) (ml/g)	Minimum fungicidal dilution (MFD) (ml/g)
B. prionitis	Stems	PE	3.125	4.688	1.158	0.772
		DCM	3.125	4.688	5.050	3.366
		EtOH	6.250	6.250	8.947	8.947
	Roots	PE	4.688	4.688	0.478	0.478
		DCM	2.343	4.688	1.417	0.708
		EtOH	6.250	6.250	6.848	6.848
B. greenii	Leaves	PE	7.813	12.500	2.768	1.730
		DCM	**0.975***	9.375	7.928	0.825
		EtOH	3.515	9.375	58.506	21.936
	Stems	PE	9.375	12.500	1.873	1.405
		DCM	6.250	12.500	1.213	0.606
		EtOH	6.250	9.375	13.606	9.071
	Roots	PE	3.125	4.688	0.998	0.666
		DCM	3.125	3.125	1.574	1.574
		EtOH	6.250	9.375	21.269	14.179
B. albostellata	Leaves	PE	4.688	6.250	11.817	8.864
		DCM	1.170	4.688	15.949	3.980
		EtOH	4.688	6.250	49.714	37.29
	Stems	PE	**0.780**	1.560	5.103	2.551
		DCM	**0.780**	1.170	4.487	2.991
		EtOH	3.125	3.125	16.979	16.979
Amphotericin B (µg/ml)			0.048	0.193		

*Values boldly-written are considered very active (< 1 mg/ml).

Table 5.6: Antifungal activity of different parts of *Huernia hystrix* against *Candida albicans*

Plant part	Extract	Minimum inhibitory concentration (MIC) (mg/ml)	Minimum fungicidal concentration (MFC) (mg/ml)	Minimum inhibitory dilution (MID) (ml/g)	Minimum fungicidal dilution (MFD) (ml/g)
Stems	PE	1.56	3.125	8.256	4.122
	DCM	1.56	6.25	12.154	3.034
	EtOH	6.25	> 12.5	29.848	ND
Roots	PE	6.25	9.375	4.365	2.91
	DCM	6.25	9.375	2.077	1.385
	EtOH	4.688	4.688	11.578	11.578
Whole plants	PE	0.585	3.125	28.376	5.312
	DCM	**0.78***	6.25	24.295	3.032
	EtOH	6.25	6.25	31.323	31.323
Amphotericin B (µg/ml)		0.048	0.193		

ND = Not determined

*Value boldly-written is considered very active (< 1 mg/ml).

5.3.2.3 Anti-inflammatory activity

Figure 5.1 presents the anti-inflammatory activity of different extracts of *Barleria* species as measured by their ability to inhibit COX enzymes. Twelve out of twenty-one crude extracts evaluated showed good activity (> 70%) in the COX-1 assay while ten extracts showed good activity in the COX-2 assay. All the PE extracts (except *B. prionitis* stem) showed good activity in the COX-1 assay. It is noteworthy that all *B. greenii* extracts showed a consistently higher anti-inflammatory activity, significant (P = 0.05) in some cases, compared to *B. prionitis* which is reportedly used in traditional medicine. Both *B. greenii* and *B. albostellata* have no recorded usage in traditional medicine. In addition, only root EtOH extracts of *B. greenii* out of all the EtOH extracts gave a very good (> 80%) anti-inflammatory activity in both COX-1 and COX-2 assays. In general, however, the non-polar extracts (PE and DCM) showed better activity compared to the EtOH extracts.

Figure 5.2 shows the anti-inflammatory activity of extracts from different parts of *H. hystrix*. All the PE (except root PE) and DCM extracts consistently showed a high activity (> 70%) against both COX-1 and COX-2 enzymes. The EtOH extracts generally showed a moderate (40-69%) to poor activity (< 40%) against both enzymes.

COS *et al*. (2006) observed that compounds that are potent inhibitors of enzymes *in vitro* frequently fail to demonstrate similar activity *in vivo* because, among other things, they do not pass through the cell membrane. The activity shown by the non-polar extracts in the current study is therefore of interest since lipophilic compounds extractable by non-polar solvents have better resorption through the cell membrane **(ZSCHOCKE and VAN STADEN, 2000)**. The inhibition of COX enzyme is known to up-regulate the LOX pathway, resulting in the production of leukotrienes, which are suggested to be responsible for many of the undesirable adverse effects associated with the use of NSAIDs **(FIORUCCI *et al*., 2001; LI *et al*., 2006)**. Dual inhibitors of COX and LOX enzymes have been suggested to be potential classical anti-inflammatory agents with reduced adverse effects **(ZSCHOCKE *et al*., 2000a; VIJI and HELEN, 2008)**. Further evaluation of the

inhibitory activity of the extracts tested in this study against LOX enzyme may therefore be needed.

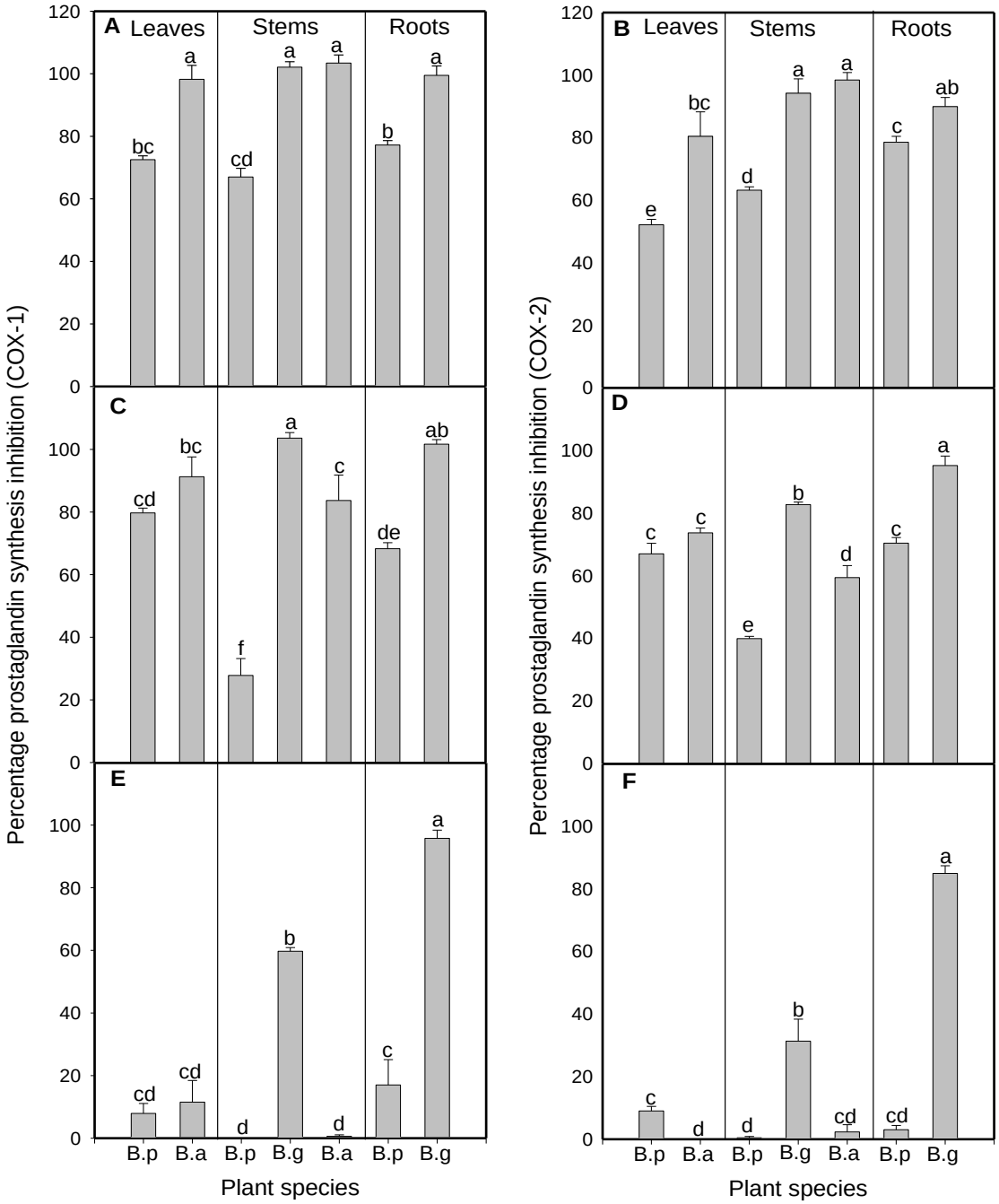


Figure 5.1: Anti-inflammatory activity of extracts from different parts of three *Barleria* species in COX-1 (on the left side) and COX-2 (on the right side) assays. B.p, B.a, and B.g are *Barleria prionitis*, *B. albostellata* and *B. greenii*, respectively. (**A**) and (**B**) are PE extracts. (**C**) and (**D**) are DCM extracts. (**E**) and (**F**) are EtOH extracts. Bars in the same graph bearing different letters are significantly different (*P* = 0.05) according to DMRT. Percentage inhibition by indomethacin in COX-1 was 63.4 ± 1.98 and COX-2 was 73.6 ± 1.47.

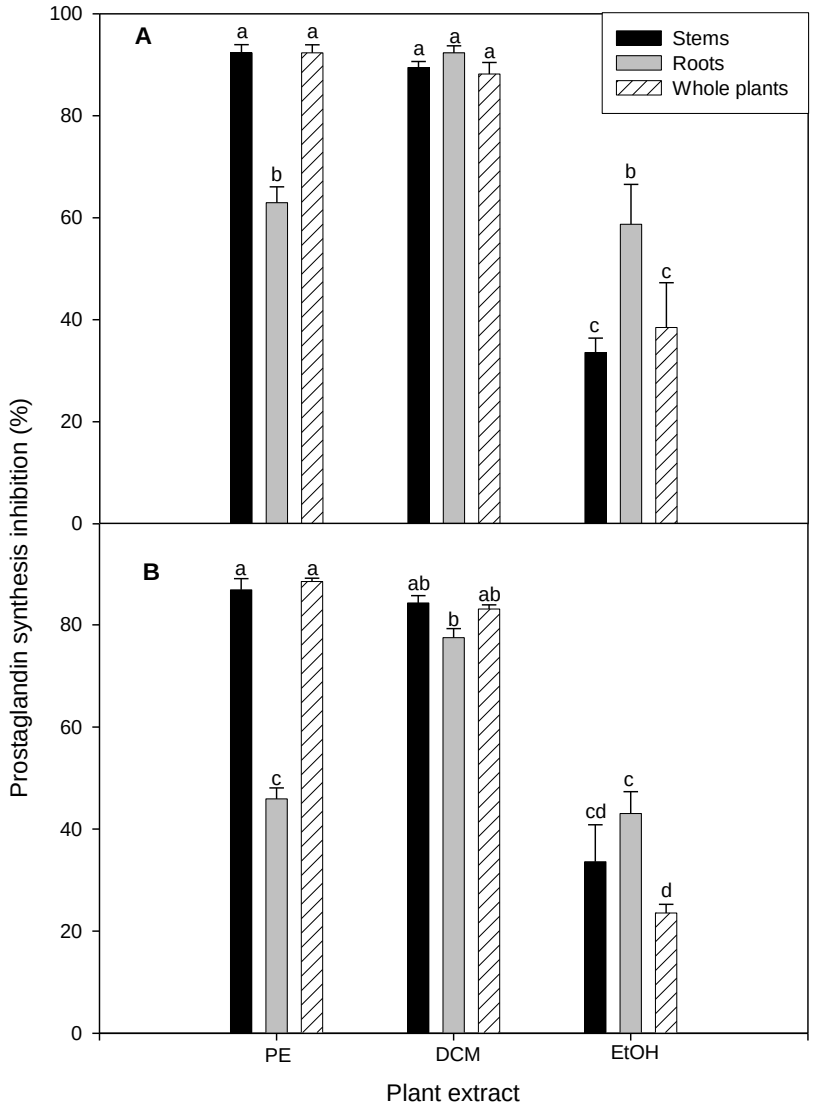


Figure 5.2: Anti-inflammatory activity of different extracts of *Huernia hystrix*. (**A**) COX-1 assay. (**B**) COX-2 assay. Bars with different letters in each graph are significantly different (P = 0.05) according to DMRT. Percentage inhibition by indomethacin in COX-1 was 54.73 ± 2.00 and COX-2 was 63.44 ± 2.52.

5.3.2.4 Acetylcholinesterase inhibition

Figure 5.3 shows the AChE inhibition activity observed in the MeOH extracts of *Barleria* species. All the extracts evaluated showed a dose-dependent inhibition. In general, at the highest extract concentration (0.625 mg/ml), the leaf and stem extracts of both *B. greenii* and *B. prionitis* exhibited higher inhibitory activities than

their root extracts. The leaf extracts of *B. greenii* and *B. albostellata* showed the highest (68%) and lowest (22%) AChE inhibition respectively, at the highest extract concentration evaluated. In the same vein, at the lowest extract concentration (0.156 mg/ml), the leaf extracts of *B. greenii* and *B. albostellata* showed the highest (38%) and lowest inhibition (3%) respectively, compared to other extracts. The inhibitory activity shown by the leaf extract of *B. greenii* at the lowest extract concentration was in fact significantly higher (P = 0.05) than the activities recorded in the stem and root extracts of the same species at the lowest concentration. The findings indicate that *B. greenii* leaves can potentially be substituted for its stems or roots in the inhibition of the AChE enzyme. In *B. albostellata*, the inhibitory activity demonstrated by the stem at the lowest extract concentration was significantly greater than the activity recorded in its leaf extract. This observation suggests that the idea of plant part substitution may be species and/or biological activity dependent.

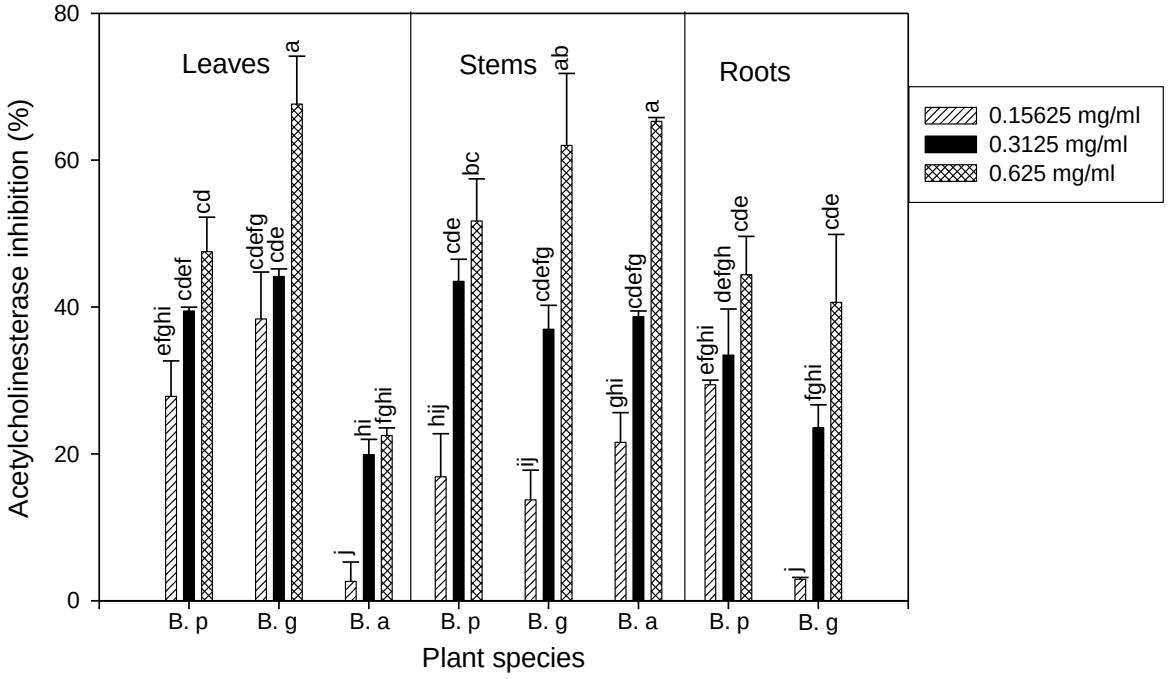


Figure 5.3: Dose-dependent acetylcholinesterase inhibition by different parts of three *Barleria* species. B.p = *Barleria prionitis*, B.g = *Barleria greenii*, B.a = *Barleria albostellata*. Bars bearing different letters are significantly different (P = 0.05) according to DMRT. The AChE inhibition activities by galanthamine at 0.5, 1.0 and 2 µM were 49.24, 59.81 and 77.03%, respectively.

The AChE inhibitory activity of extracts from different parts of *H. hystrix* is presented in Figure 5.4. At all the extract concentrations evaluated, the whole plant extract showed a higher inhibitory activity, statistically significant in some cases, compared to the extracts from the stems or roots. It is likely that the whole plant extract contains some compounds that act synergistically or additively to produce a higher inhibitory activity compared to their acting individually in the stem or root extracts.

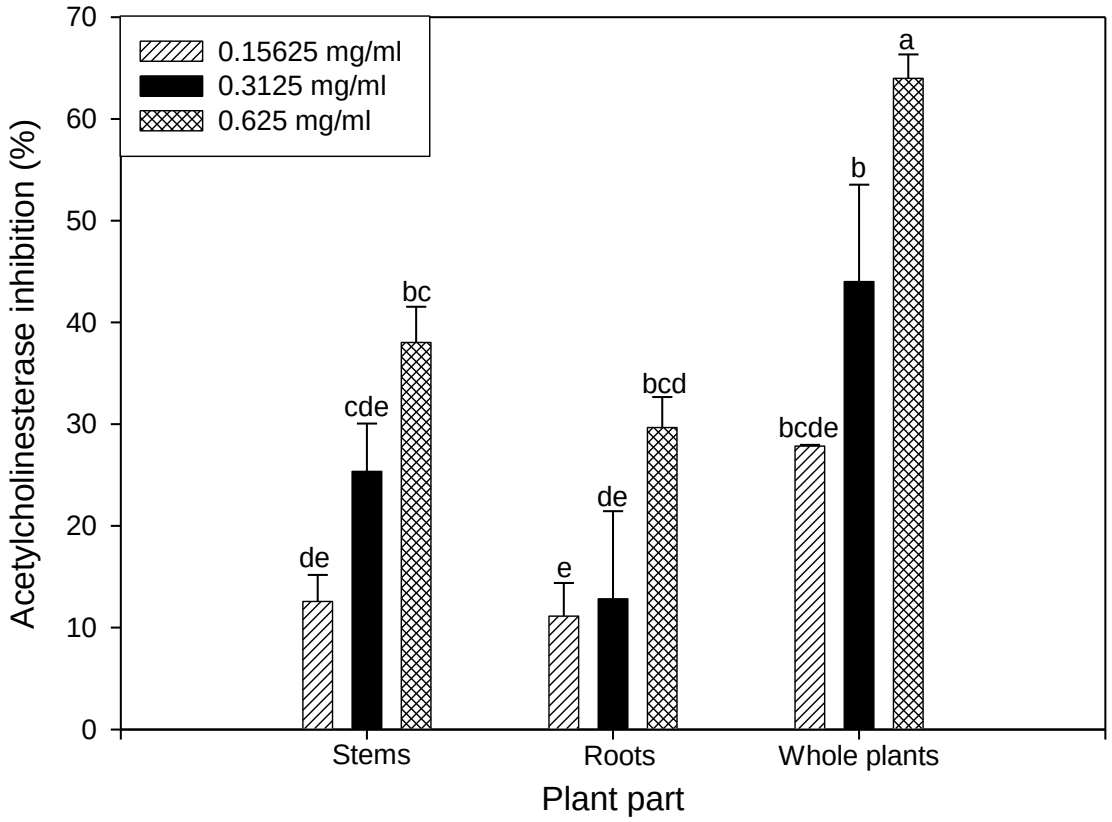


Figure 5.4: Dose-dependent acetylcholinesterase inhibition by different parts of *Huernia hystrix*. Bars bearing different letters are significantly different (P = 0.05) according to DMRT. The AChE inhibition activities by galanthamine at 0.5, 1.0 and 2 µM were 49.24, 59.81 and 77.03%, respectively.

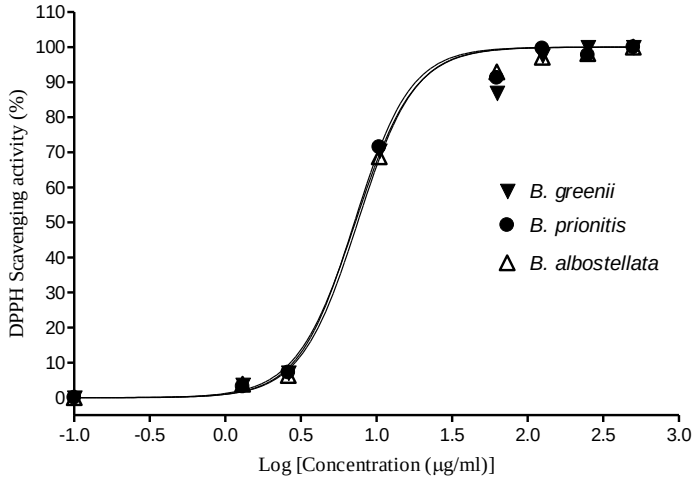
5.3.2.5 Antioxidant activity

5.3.2.5.1 DPPH radical scavenging activity

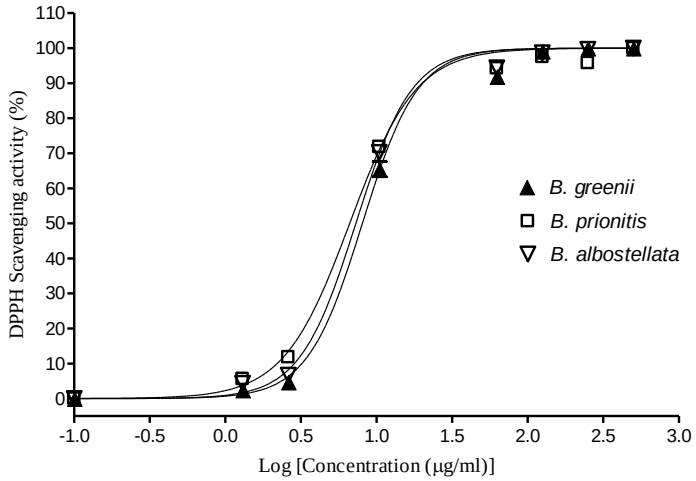
Figures 5.5 and 5.6 show the dose-response radical scavenging activities observed in the MeOH extracts of different parts of *Barleria* species and *Huernia hystrix*, respectively. In all the extracts, there was an increase in the DPPH radical scavenging activity with increasing extract concentration. The dose-dependent curve of *H. hystrix* whole plant extract was however less steep when compared to its root or stem dose-dependent curves (Figure 5.6). From their dose-response activities, their EC_{50} values were obtained and presented in Tables 5.7 and 5.8. The EC_{50} values for the different extracts of *Barleria* speces ranged from 6.65 to 12.56 µg/ml (Table 5.7). The EC_{50} values of *B. greenii* leaf and all *B. prionitis* extracts were in fact not significantly different from the EC_{50} of ascorbic acid, a standard antioxidant agent used as a positive control. In the case of *B. greenii*, the leaf extract showed a lower EC_{50} value compared to the stem and significantly, the root. This observation correlates well with AChE inhibitory activities observed in the leaves, stems and roots of *B. greenii* (Figure 5.3). According to **HOUGHTON *et al*. (2007)**, free radical reactions are involved in the pathology of many diseases like Alzheimer's disease, cancer and inflammation.

The stem and the root extracts of *H. hystrix* showed a good radical scavenging activity as indicated by their EC_{50} values, which were not significantly different from that of the ascorbic acid standard (Table 5.8). In the whole plant however, the radical scavenging activity was significantly lower than any of the individual plant parts. The results suggest the likely presence of certain compounds that are perhaps antagonistic in their radical scavenging activities.

(**A**)



(**B**)



(**C**)

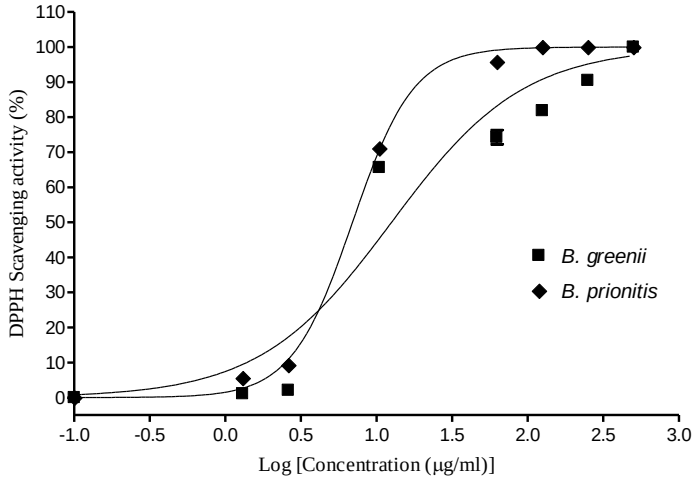


Figure 5.5: Dose-dependent curve of DPPH radical scavenging activity of different parts of three *Barleria* species. (**A**) Leaves (**B**) Stems (**C**) Roots.

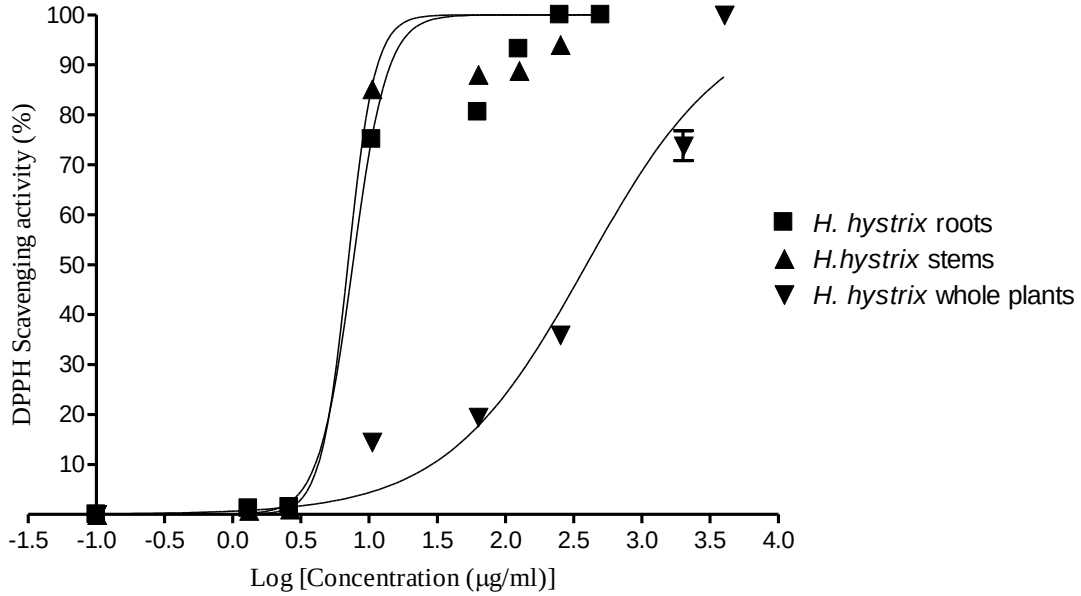


Figure 5.6: Dose-dependent curve of DPPH radical scavenging activity of different parts of *Huernia hystrix*.

Table 5.7: DPPH radical scavenging activity of different parts of three *Barleria* species

		DPPH radical scavenging	
Plant species	Plant part	EC_{50} (µg/ml)	R^2
B. prionitis	Leaves	7.14 ± 0.056^{bcd}	0.9949
	Stems	6.65 ± 0.037^{bc}	0.9965
	Roots	6.94 ± 0.033^{bc}	0.9984
B. greenii	Leaves	7.24 ± 0.326^{bcd}	0.9881
	Stems	8.09 ± 0.266^{d}	0.9954
	Roots	12.56 ± 0.401^{e}	0.9307
B. albostellata	Leaves	7.52 ± 0.169^{cd}	0.9952
	Stems	7.31 ± 0.175^{cd}	0.9973
Ascorbic acid		6.17 ± 0.434^{b}	0.9550
Butylated hydroxytoluene		3.75 ± 0.764^{a}	0.9027

Mean values followed by different letters are significantly different (P = 0.05) according to DMRT.

Table 5.8: DPPH radical scavenging activity of different parts of *Huernia hystrix*

	DPPH radical scavenging	
Plant part	EC_{50} (µg/ml)	R^2
Stems	7.01 ± 0.183[a]	0.9805
Roots	7.63 ± 0.154[a]	0.9724
Whole plants	393.83 ± 7.364[b]	0.9542
Ascorbic acid	6.17 ± 0.434[a]	0.9550
Butylated hydroxytoluene	3.75 ± 0.764[a]	0.9027

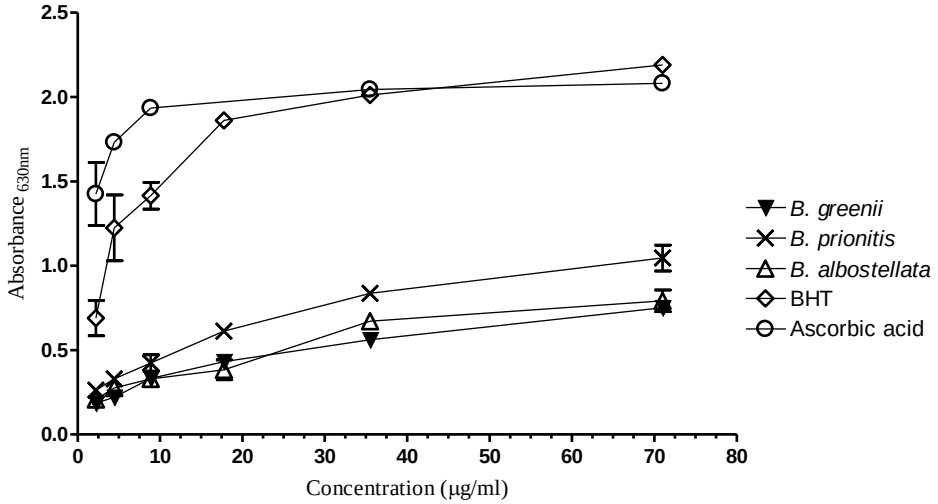
Mean values followed by different letters are significantly different (P = 0.05) according to DMRT.

5.3.2.5.2 Ferric ion reducing power activity

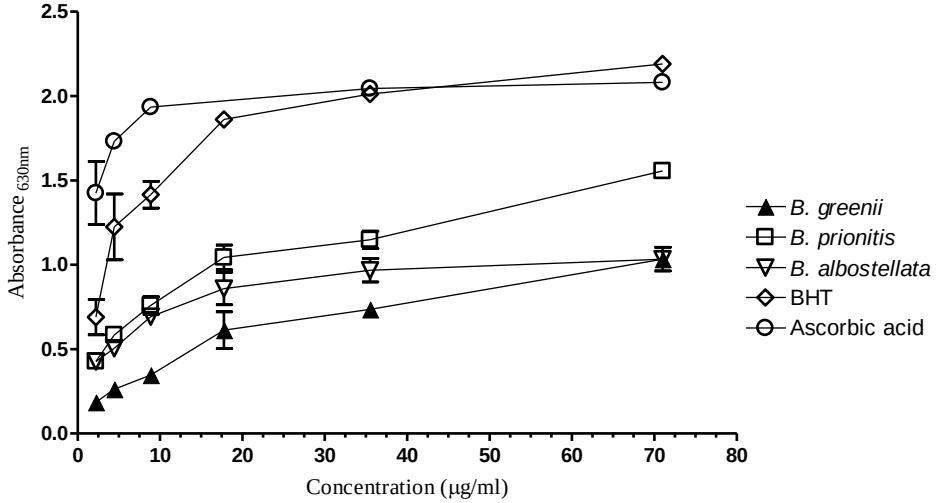
The ferric ion reducing power assay (FRAP) is an assay based on an electron transfer reaction **(HUANG *et al*., 2005)**. In the assay, the presence of reductants (antioxidants) in the tested extracts results in the reduction of the ferric ion/ferricyanide complex to the ferrous form, with a characteristic formation of Perl's Prussian blue, which is measured spectrophotometrically **(CHUNG *et al*., 2002)**. The degree of colour change is directly proportional to the antioxidant concentrations in the extracts **(HUANG *et al*., 2005)**.

Figures 5.7 and 5.8 show the reducing power of different extracts of three *Barleria* species and *H. hystrix*, respectively. All the extracts evaluated showed an increase in reducing power activity with an increase in extract concentration. However, the reducing activities of the extracts were significantly lower than the ascorbic acid and BHT standard controls. The ferric reducing power activity of the leaf and stem extracts of both *B. prionitis* and *B. greenii* were significantly higher than that of their root extracts (Figure 5.7), indicating a potential plant part substitution of the leaves or stems for the roots.

(**A**)



(**B**)



(**C**)

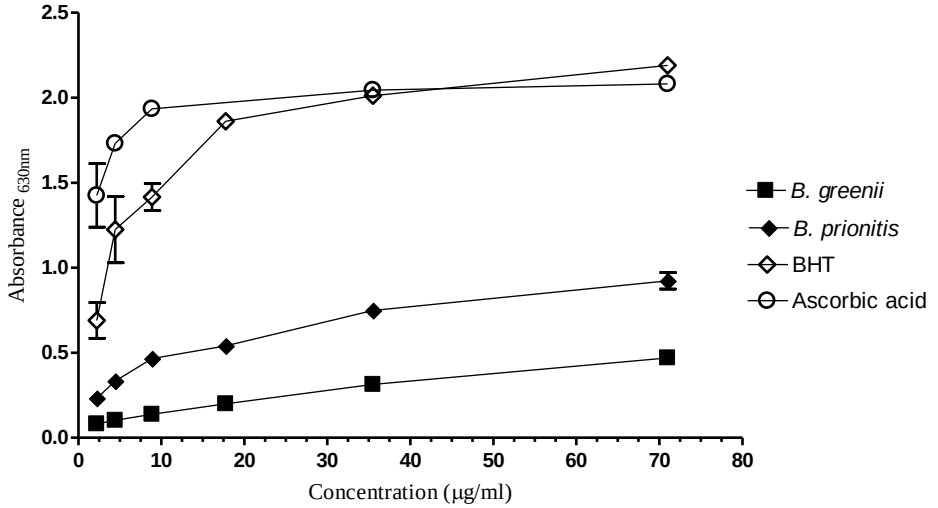


Figure 5.7: Ferric ion reducing power activity of different parts of *Barleria* species. (**A**) Leaves (**B**) Stems (**C**) Roots.

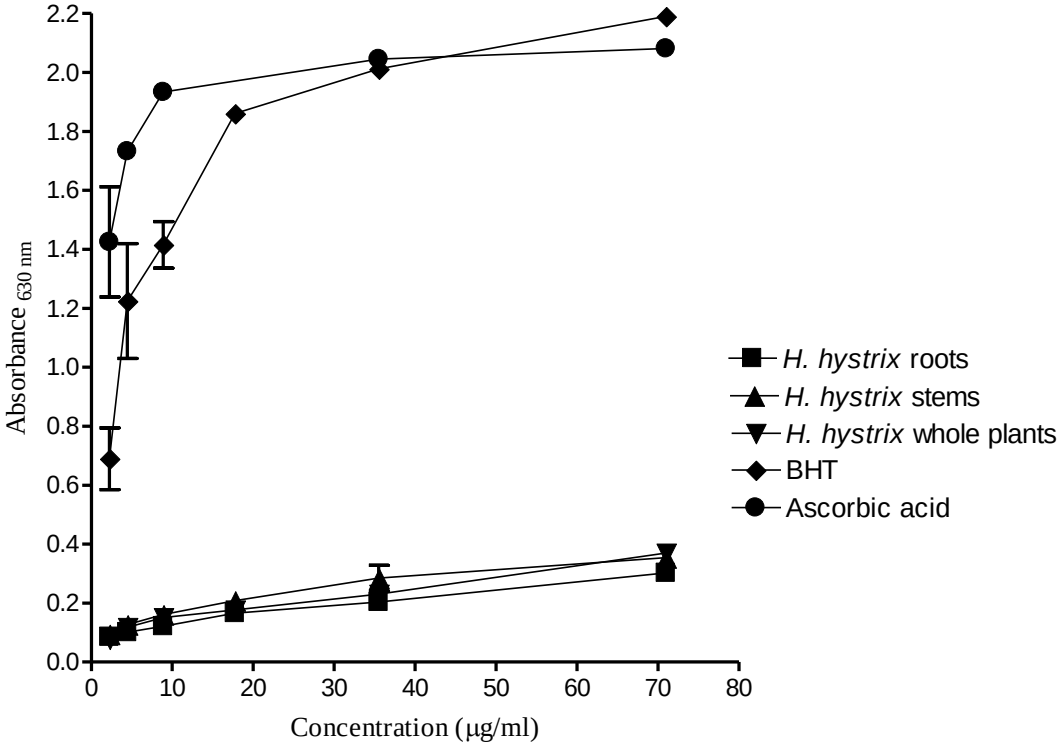


Figure 5.8: Ferric ion reducing power activity of different parts of *Huernia hystrix*.

As shown in Figure 5.8, all the extracts of *H. hystrix* demonstrated a poor ferric reducing power activity compared to either of the standard controls (ascorbic acid and BHT) or even the *Barleria* species. There was no significant difference in the ferric reducing power activity demonstrated by the different parts evaluated. Taken together however, the results suggest the presence of antioxidant compounds with electron-donating ability in the different plant parts evaluated, which the assay is known to measure semi-quantitatively **(AMAROWICZ *et al*., 2004; RUMBAOA *et al*., 2009)**. The presence of these compounds perhaps in an impure form or in small amounts in the extracts may be responsible for the generally low activity demonstrated by the extracts.

5.3.2.5.3 β-Carotene linoleic acid assay

This assay involves the heat-induced bleaching of carotenoids and is based on hydrogen atom transfer reactions **(HUANG *et al*., 2005)**. According to **AMAROWICZ *et al*. (2004)**, the abstraction of a hydrogen atom from linoleic acid

during oxidation results in the formation of a pentadienyl free radical. This free radical subsequently attacks the highly unsaturated β-carotene molecules, leading to the loss of conjugation of the β-carotene molecules and the characteristic orange colour of the carotenoids **(AMAROWICZ *et al*., 2004)**. The presence of antioxidants, capable of donating hydrogen atoms can however prevent or reduce carotenoid bleaching by quenching free radicals formed within the system **(BURDA and OLESZEK, 2001; AMAROWICZ *et al*., 2004)**.

The antioxidant activities of different parts of three *Barleria* species and *H. hystrix* in the β-carotene-linoleic acid model system are shown in Figures 5.9 and 5.10, respectively. In *Barleria* species, the antioxidant activity based on the average β-carotene bleaching rate ranged from 52 to 77%. The antioxidant activity of *B. prionitis* roots was significantly higher than that of its leaves and stems (Figure 5.9A). There was no significant difference observed in the antioxidant activity (based on average β-carotene bleaching rate) of the different parts of either *B. greenii* or *B. albostellata* evaluated. This finding again suggests the potential to substitute the leaves of *B. greenii* for its stems or roots.

The same trend recorded in the antioxidant activity of the extracts (based on the average β-carotene bleaching rate) was also observed with their oxidation rate ratio (Figure 5.9B). In this case, the lower the oxidation rate ratio, the higher the antioxidant activity of the different plant parts. According to **AMAROWICZ *et al*. (2004)**, the antioxidant activity at 60 or 120 min possibly demonstrates the antioxidant activity of an extract more accurately than the oxidation rate ratio or antioxidant activity based on the average β-carotene bleaching rate. With the exception of *B. prionitis* stem, *B. greenii* stem and *B. albostellata* leaf extracts, the same trend observed for antioxidant activity at 60 min (Figure 5.9C) was also recorded at 120 min for all the extracts (Figure 5.9D). There was no significant difference between the antioxidant activities of *B. greenii* leaves and roots as well as the BHT standard control at either t = 60 or 120 min. 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 concern 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; ABDEL-HAMEED, 2009). The findings from this current study suggest that the leaves and roots of *B. greenii* 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 these plant materials could perhaps improve their antioxidant capacity. The use of the leaves of *B. greenii* is more sustainable and can potentially substitute for the roots, owing to their equivalent antioxidant activity in this assay.

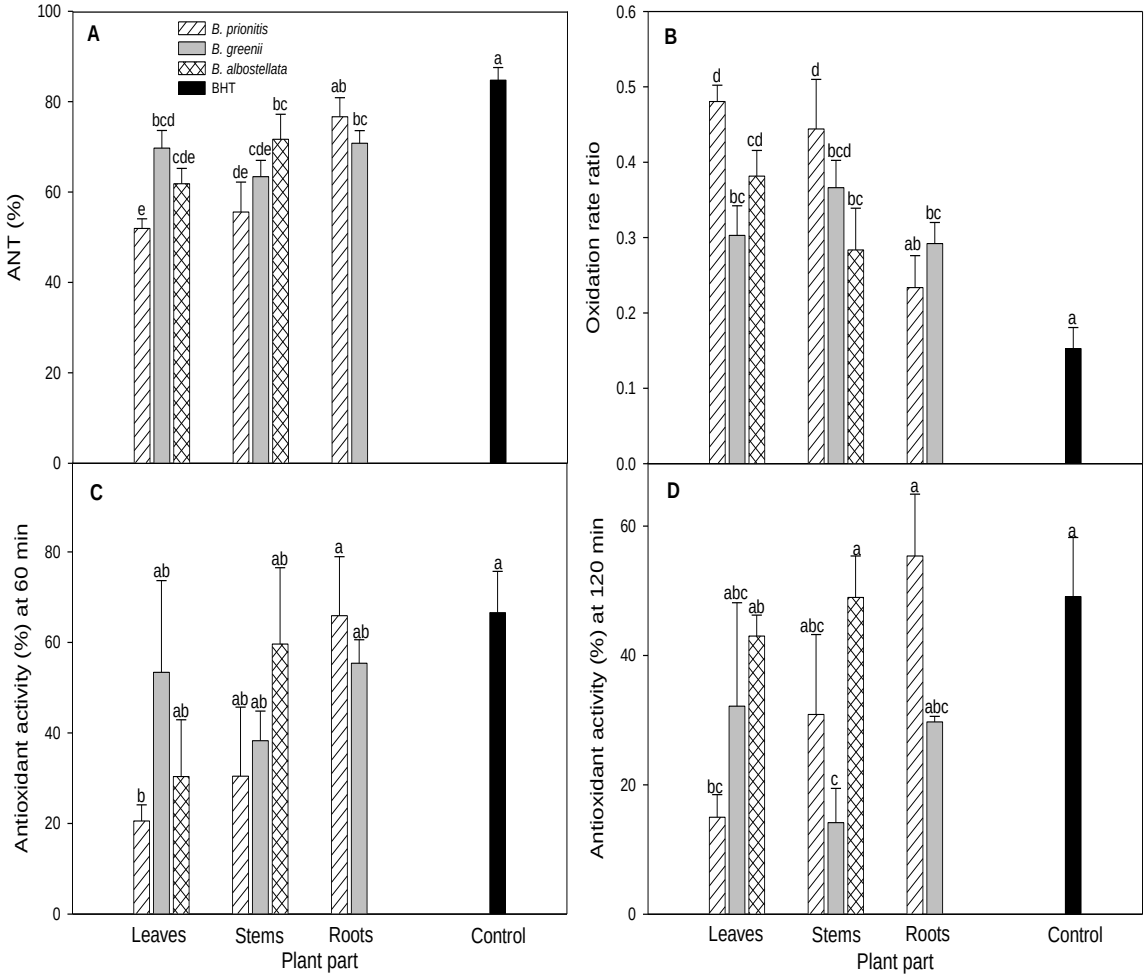


Figure 5.9: Antioxidant activities of different parts of three *Barleria* species in β-carotene-linoleic acid model system. Bars bearing different letters in each graph are significantly different (*P* = 0.05) according to DMRT. (**A**) Antioxidant activity (ANT) based on the average β-carotene bleaching rate. (**B**) Oxidation rate ratio (ORR). (**C**) Antioxidant activity (AA) at t = 60 min. (**D**) Antioxidant activity (AA) at t = 120 min.

In *H. hystrix*, the roots consistently showed a significantly higher antioxidant activity (based on average β-carotene bleaching rate), oxidation rate ratio as well as antioxidant activity at either 60 or 120 min, compared to the stems or whole plant (Figure 5.10A-D). Although there was no significant difference between the antioxidant activity of the roots and the standard synthetic antioxidant (BHT) at 60 or 120 min, the use of the roots of this plant may not be sustainable from a conservation point of view. In general however, the activities recorded in the different parts of all the species evaluated in this study indicate the presence of antioxidant compounds with the ability to donate hydrogen atoms.

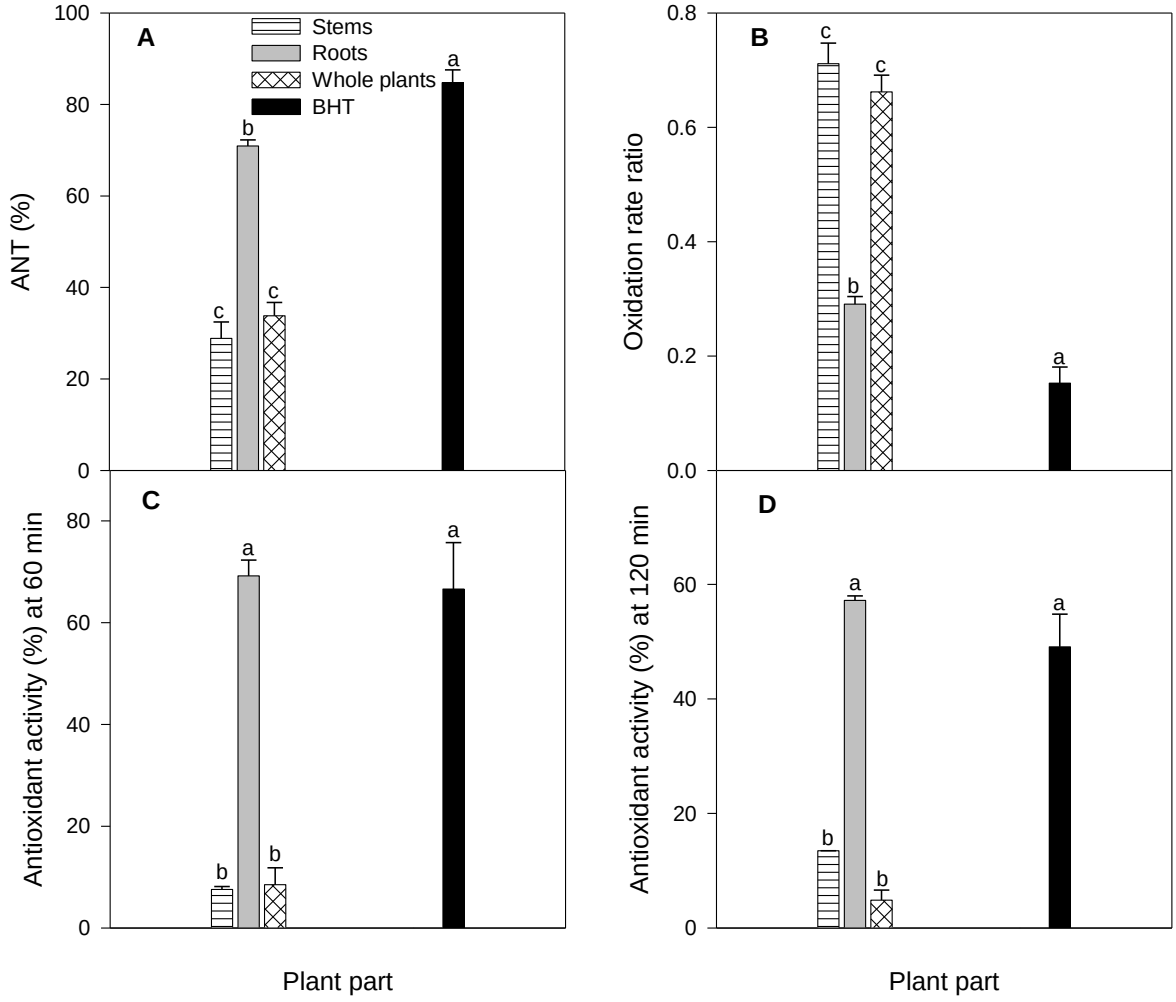


Figure 5.10: Antioxidant activities of different parts of *Huernia hystri*x in β-carotene-linoleic acid model system. Bars bearing different letters in each graph are significantly different (*P* = 0.05) according to DMRT. (**A**) Antioxidant activity (ANT) based on the average β-carotene bleaching rate. (**B**) Oxidation rate ratio. (**C**) Antioxidant activity at 60 min. (**D**) Antioxidant activity at 120 min.

5.3.3 Phytochemical evaluation

5.3.3.1 Total phenolic content

Figure 5.11 presents the total phenolic content of different parts of three *Barleria* species. In all three species, the highest total phenolic content was observed in the leaves, compared to other plant parts. In *B. prionitis* and *B. greenii*, the stem phenolic contents were significantly higher than that of the root. The highest (12.79 mg GAE/g DW) and lowest (1.95 mg GAE/g DW) total phenolic content were recorded in *B. prionitis* leaves and *B. greenii* roots, respectively.

Figure 5.12 shows the total phenolic content of different parts of *H. hystrix*. The roots had the highest total phenolic content (181.22 µg GAE/g), although not significantly different from that of the stems. The total phenolic content of the whole plant was the lowest with 128.73 µg GAE/g DW.

Phenolic compounds include flavonoids, condensed tannins and hydrolysable tannins, many of which are reported to have antimicrobial, anti-inflammatory and antioxidant activities **(MARCUCCI *et al*., 2001; POLYA, 2003)**. According to **SAMAK *et al*. (2009)**, the redox property of phenolic compounds is an important underlying factor for their antioxidant activity, giving them the ability to act as hydrogen donors, reducing agents and singlet oxygen quenchers. In the light of the total phenolic content recorded in the different parts of the plant species in the current study, the amounts of some particular groups of phenolic compounds were further evaluated in the different parts of the studied species. This could possibly help in underpinning the specific phenolic groups likely to be responsible for the observed pharmacological activities.

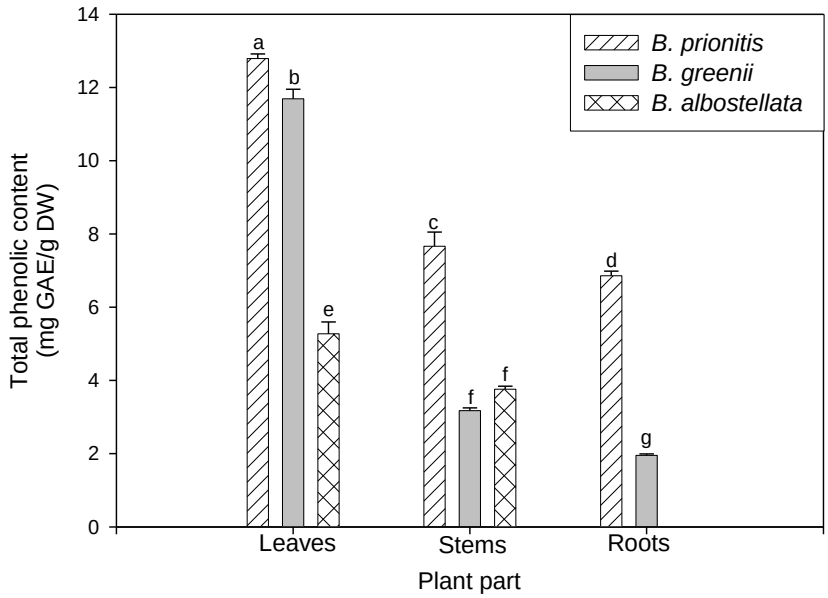


Figure 5.11: Total phenolic content of different parts of three *Barleria* species. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

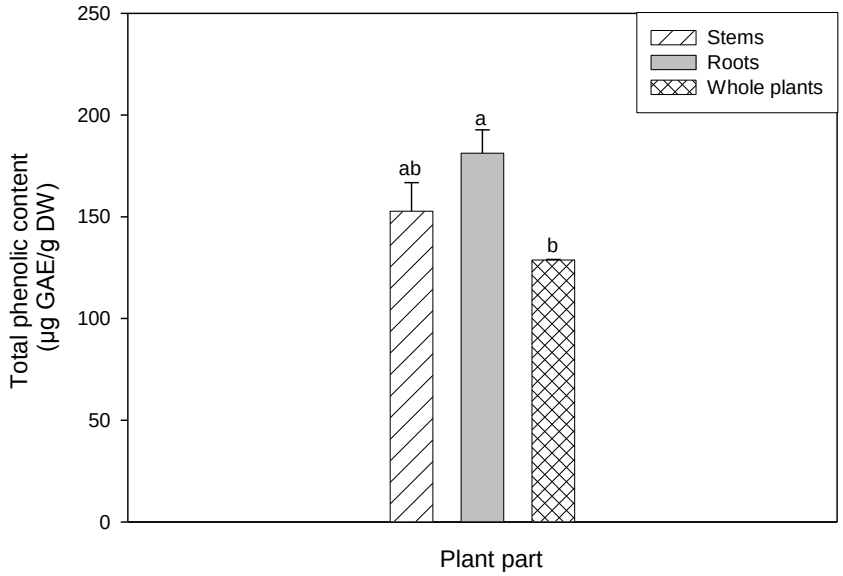


Figure 5.12: Total phenolic content of different parts of *Huernia hystrix*. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

5.3.3.2 Total iridoid content

Figure 5.13 shows the total iridoid content of different parts of the three *Barleria* species. The highest total iridoid content was recorded in *B. albostellata* leaves with 801.4 µg HE/g DW while the lowest was found in *B. prionitis* roots with 30 µg HE/g DW. In each of the *Barleria* species, the total iridoid content of the leaves was significantly higher than that of the other parts. There was no significant difference between the iridoid contents of the stems of the three species. The total iridoid content of *B. greenii* roots was significantly higher than that of *B. prionitis* roots. The isolation of six known iridoids (shanzhiside methyl ester, 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester, barlerin, acetylbarlerin, 7-methoxydiderroside and lupulinoside) with different levels of AChE inhibitory and free radical scavenging activities from aerial parts of *B. prionitis* has been reported **(ATA *et al*., 2009)**. The AChE inhibitory and radical scavenging activities recorded in the *Barleria* species evaluated in this study could be due to the presence of these or other iridoid compounds. The presence of iridoid compounds even at a low concentration in all parts of the three *Barleria* species could perhaps play a role in the pharmacological activities observed in all the extracts evaluated. Since the leaves of each of these *Barleria* species contained more iridoid compounds, two- to twelve-fold higher than their stems or roots, they may possibly be a potential sustainable source for iridoid compounds.

In *H. hystrix*, the roots had the highest total iridoid content of 92.6 µg HE/g DW (Figure 5.14). This value was however, not significantly different from the total iridoid content of the stems or whole plant. Some iridoids or iridoid-rich plant extracts have been reported to demonstrate antimicrobial and anti-inflammatory activities **(BOLZANI *et al*., 1997; WANIKIAT *et al*., 2008)**. The pharmacological activities observed in the studied species could perhaps be attributed in part, to the iridoids present in the different parts of this species.

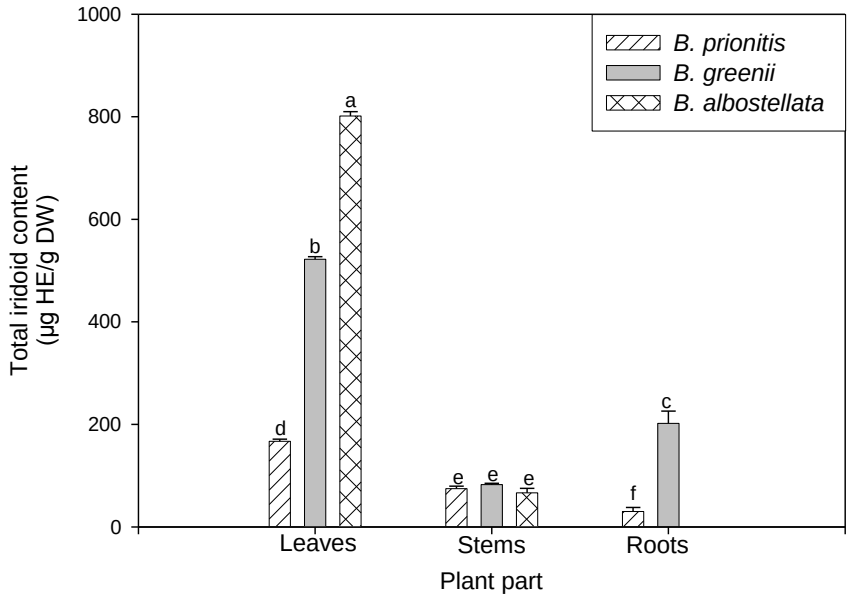


Figure 5.13: Total iridoid content of different parts of three *Barleria* species. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

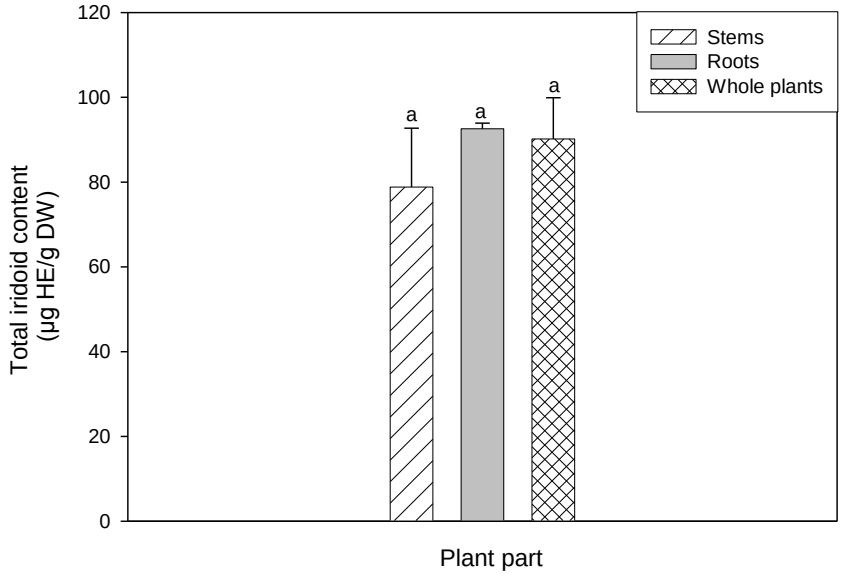


Figure 5.14: Total iridoid content of different parts of *Huernia hystrix*. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

5.3.3.3 Flavonoid content

The flavonoid content of different parts of the three *Barleria* species is presented in Figure 5.15. The highest (3.92 mg Catechin/g DW) and lowest (0.35 mg Catechin/g DW) flavonoid contents were recorded in *B. greenii* leaves and *B. albostellata* stems, respectively. In general, higher flavonoid content was observed in the leaves compared to the other plant parts in each of the species.

Figure 5.16 shows the flavonoid content of different parts of *H. hystrix*. Although there was no significant difference between the values, the flavonoid content of the roots was higher than that of the whole plant or stems.

In an extensive review on the effects of naturally occurring flavonoids on mammalian cells, **MIDDLETON *et al*. (2000)** observed that flavonoids demonstrate a noteworthy array of biochemical and pharmacological actions, most notable being their antioxidant, anti-inflammatory and antiproliferative effects. Many other researchers have reported the antioxidant, anti-inflammatory and antimicrobial activities of flavonoids or flavonoid-rich extracts **(BURDA and OLESZEK, 2001; HAVSTEEN, 2002; TUNALIER *et al*., 2007; PATTANAYAK and SUNITA, 2008)**. In the present study, the flavonoid content in all the different parts of the studied species appeared to be substantially higher compared to other phenolic groups evaluated in this study. It is therefore likely that the observed pharmacological activities in the different plant parts are largely due to their flavonoid content. In addition to their quantity however, the quality or nature of the flavonoid present in the different plant parts could make a difference in their therapeutic potential.

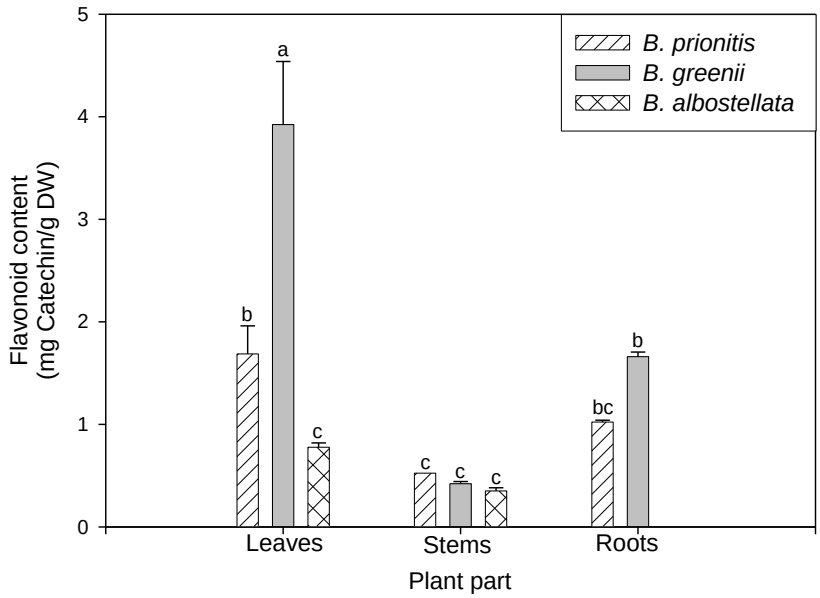


Figure 5.15: Flavonoid content of different parts of three *Barleria* species. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

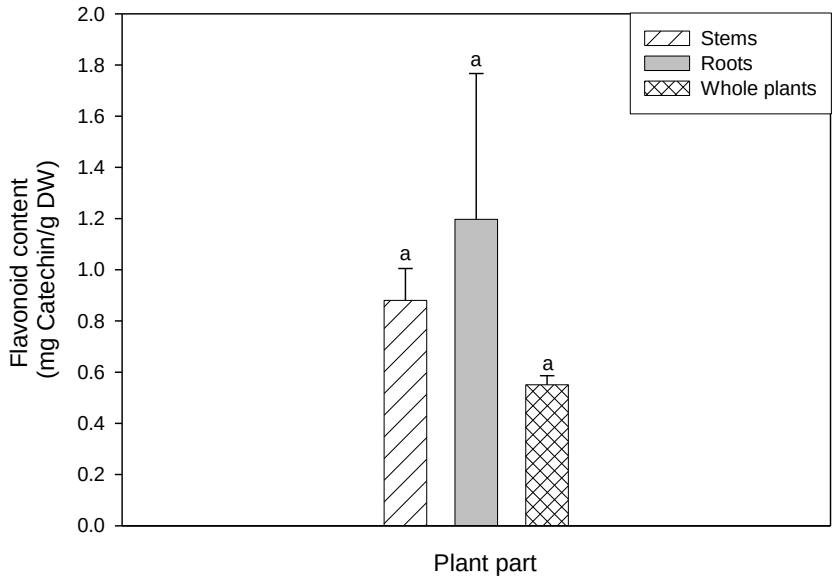


Figure 5.16: Flavonoid content of different parts of *Huernia hystrix*. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

5.3.3.4 Gallotannin content

The roots of *B. greenii* showed the highest gallotannin content with 145.33 µg GAE/g DW compared to all other *Barleria* species parts (Figure 5.17). In all three species, the gallotannin content of the leaves was significantly higher than that of the stems. The gallotannin contents of the leaves and stems of *B. albostellata* were significantly higher than that of the leaves and stems, respectively of other *Barleria* species.

Figure 5.18 shows the gallotannin content of the different parts of *H. hystrix*. The stems had a higher gallotannin content (49.05 µg GAE/g DW) compared to the roots and the whole plant. There was however, no significant difference between the values obtained.

TIAN *et al*. (2009a) reported antioxidant and antibacterial activities of gallotannin-rich extracts from *Galla chinensis*. **ENGELS *et al*. (2009)** also reported antimicrobial activities of gallotannin-rich extracts and gallotannins isolated from *Mangifera indica* kernels. The antibacterial activity of hydrolysable tannins (the group to which gallotannins belong) isolated from medicinal plants used in treating gastric disorders against *Helicobacter pylori* has been demonstrated **(FUNATOGAWA *et al*., 2004)**. Some gallotannins have also been reported to act as inhibitors of particular enzymes such as the COX enzymes involved in the inflammatory pathway **(POLYA, 2003)**. The presence of gallotannins in different parts of the species evaluated in this study could, at least, partly contribute to their antioxidant, anti-inflammatory and antibacterial activities.

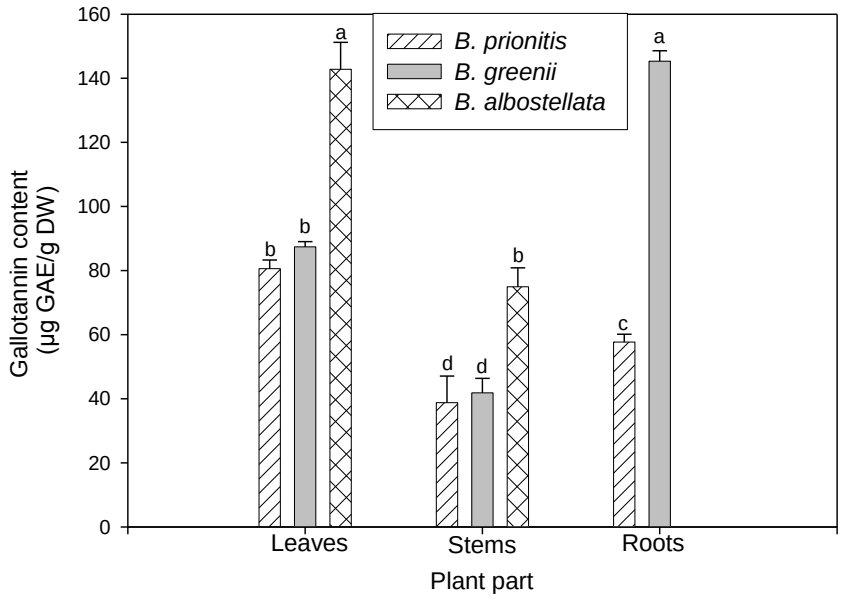


Figure 5.17: Gallotannin content of different parts of three *Barleria* species. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

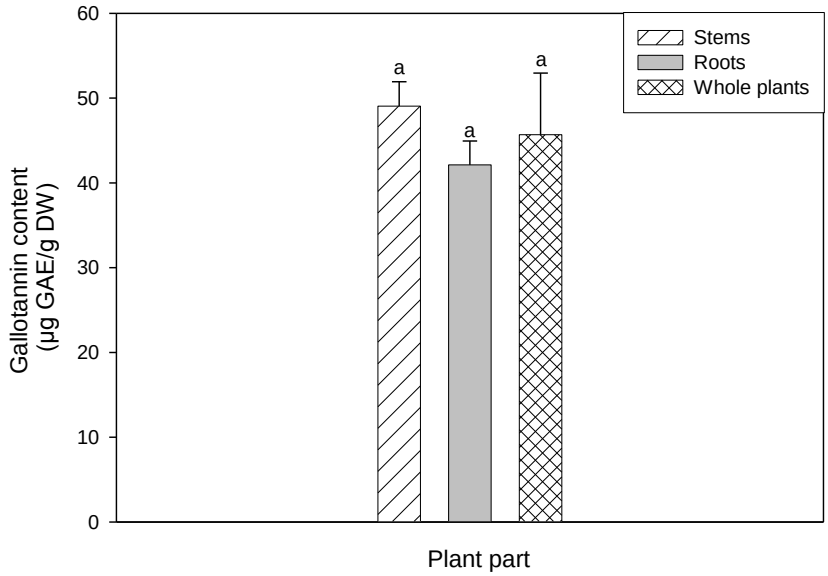


Figure 5.18: Gallotannin content of different parts of *Huernia hystrix*. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

5.3.3.5 Condensed tannin (proanthocyanidin) content

Proanthocyanidins are made up of oligomeric and polymeric flavan-3-ols, and they have been reported to demonstrate strong antioxidant capacity **(SHYU *et al*., 2009)**. **SHAN *et al*. (2007)** reported the significant contribution of proanthocyanidin to the antibacterial activity of *Cinnamomum burmannii*. The condensed tannin content of different parts of the three *Barleria* species evaluated in this study is presented in Figure 5.19. No condensed tannin was detected in *B. prionitis* leaves and *B. albostellata* stems. Where condensed tannin was recorded, the content was generally low (ranging from 0.007 to 1.2% per g DW) in all parts of the three *Barleria* species evaluated. All the different parts of *B. greenii* had a significantly higher proanthocyanidin content compared to any part of *B. prionitis* and *B. albostellata*. There was no condensed tannin detected in *H. hystrix*. These findings indicate that the pharmacological activities recorded in different parts of *H. hystrix* as well as *B. prionitis* leaves and *B. albostellata* stems could not be due to proanthocyanidins. In other parts of *Barleria* species however, their proanthocyanidin content could possibly contribute, at least to a small extent, to their antioxidant and antibacterial activities.

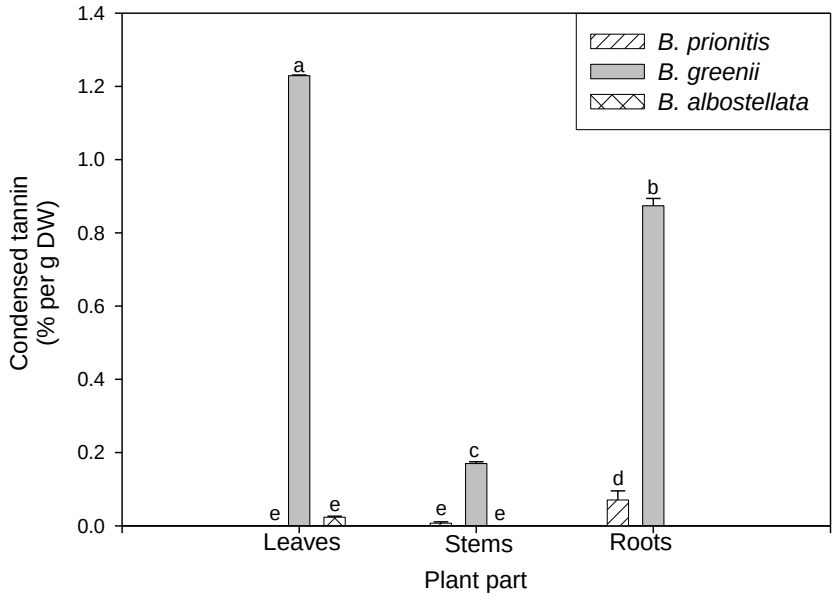


Figure 5.19: Condensed tannin content (as leucocyanidin equivalents) of different parts of three *Barleria* species. Bars bearing different letters are significantly different (*P* = 0.05) according to DMRT.

Overall, the results from this study demonstrate the therapeutic potential of the *Barleria* species evaluated as well as that of *H. hystrix*. As far as can be ascertained and besides the published article from this study, this is the first report on the antimicrobial, anti-inflammatory, AChE inhibition and antioxidant activities of *B. greenii*, *B. albostellata* and *H. hystrix*. The observed activities might largely be due to their flavonoid content, with a contributing effect from their iridoid and tannin compounds. The concept of substituting plant parts for sustainable exploitation appeared to be dependent on the species and/or biological activity evaluated. The substantial better activities observed with *B. greenii* in some cases highlight the need to conserve our 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 treatment-resistant infections.

Chapter 6 General conclusions

An effective micropropagation protocol was developed for *B. greenii*, an endangered and endemic South Africa horticultural shrub. Based on the highest regeneration rate obtained, more than 60,000 transplantable shoots per year can potentially be produced from a single shoot-tip explant using this protocol. Culturing under a 16 h photoperiod was more favourable for adventitious shoot production than culturing under continuous light. Higher adventitious shoot production was obtained in the treatments with BA alone when compared to the BA treatments supplemented with NAA concentrations. These results indicate that the exogenous application of NAA is not a requirement for adventitious shoot production of this plant species. The treatments with BA, commonly used in micropropagation industry, resulted in a comparatively high abnormality index in regenerated adventitious shoots from shoot-tip explants. Increased adventitious shoots and reduced abnormality indices were observed in the treatments with *m*TR and Me*m*TR even at higher concentrations (5 and 7 µM). The topolins (*m*TR and Me*m*TR) are less toxic and more effective than BA in the micropropagation of this plant species. The abnormality indices recorded in treatments with some topolin concentrations were in fact lower than what was observed in the control. It is possible that the observed abnormalities in the treatments with topolins are carry-over effects of BA since the explants used were taken from BA-treated cultures. The use of explants that have not been exposed to BA treatments may be necessary to confirm the effect of the topolin treatments on the induction of abnormalities in this plant species.

Unlike in *B. greenii*, the treatments with a combination of BA and NAA showed a synergistic effect on adventitious shoot regeneration of *H. hystrix*, an endangered ornamental succulent. Regenerated adventitious shoots were successfully acclimatized with a high survival rate. Environmental factors such as temperature and photoperiod significantly affected adventitious shoot production. This study highlights the need to investigate the effects of environmental conditions when developing efficient micropropagation protocols, especially for commercial purposes. Optimizing environmental conditions could increase shoot production,

reduce labour costs and thus subsequent production costs. The observations from this study also provide an insight into the physiology of *H. hystrix* when cultured at different temperatures and photoperiods. The results suggest that this ornamental succulent possibly has the ability to shift between the C-3 and CAM photosynthetic pathways depending on the photoperiod conditions under which the plants are cultured. In cultures maintained at lower temperatures (15 and 20°C) under a 16 h photoperiod, low shoot proliferation rates were observed due to slow growth and less differentiation of shoot meristems. In addition to offering an approach for short-term *in vitro* storage of *H. hystrix* germplasm, such slow growth at low temperatures is economically beneficial when manpower requirement for subculturing is not available.

The pharmacological activities and phytochemical properties of the species studied in this research highlight their therapeutic potential. Extracts from different parts of three *Barleria* species and *H. hystrix* demonstrated different levels of antibacterial, antifungal, antioxidant, anti-inflammatory and AChE inhibition activities. The pharmacological activities observed in some extracts of *H. hystrix* might possibly explain its heavy exploitation in traditional medicine. In general, however, the *Barleria* species evaluated showed better pharmacological activities compared to *H. hystrix*. Although *B. greenii* has no recorded usage in traditional medicine, some of its extracts particularly demonstrated higher pharmacological activities in some cases than other *Barleria* species evaluated. In some of the pharmacological assays, the leaves of *Barleria* species and stems of *H. hystrix* demonstrated higher activities than the other plant parts, suggesting their potential in plant part substitution. The harvesting of leaves or stems as a conservation strategy is certainly more sustainable than the destructive use of the roots of these 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 shown by the extracts could possibly be due to their phenolic (including flavonoids, proanthocyanidins and gallotannin) and iridoid content. The isolation of specific bioactive compounds through bioassay-guided fractionation and their characterization as well as studies

evaluating their safety may be necessary in the exploration of these species for potential new therapeutic drugs or drug leads.

Taken together, the present study highlights the need for the conservation of our indigenous plant resources. *In vitro* propagation methods offer powerful techniques for rapid propagation and germplasm storage of our endemic and threatened plant species.

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