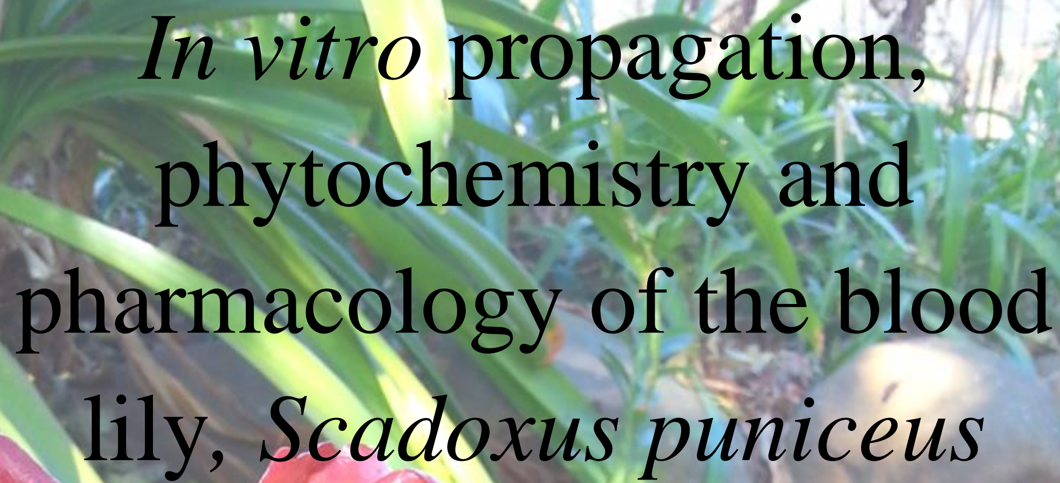

In vitro propagation, phytochemistry and pharmacology of the blood lily, *Scadoxus puniceus*

Devashan Naidoo

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal, Pietermaritzburg

In vitro propagation, phytochemistry and pharmacology of the blood lily, *Scadoxus puniceus*

By

Devashan Naidoo

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg

November, 2016

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In vitro propagation, phytochemistry and pharmacology of the blood lily, *Scadoxus puniceus*

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Student Number: 209506613

Thesis Title: *In vitro* propagation, phytochemistry and pharmacology of the blood lily, *Scadoxus puniceus*

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

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PUBLICATIONS FROM THIS THESIS

1. D. Naidoo, A.O. Aremu, J. Grúz, B. Ondřej, K. Doležal, J. Van Staden, J.F. Finnie, (manuscript under review - Phytochemistry). UHPLC-MS/MS quantification of phenolic compounds and pharmacology of *Scadoxus puniceus*, a highly traded South African medicinal plant.

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DECLARATION 2 - PUBLICATIONS

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

Publication 1

Contributions: AOA, JG, BO and KD performed the ultra high performance liquid chromatography. DN designed and performed pharmacological assays, and prepared the manuscript under the supervision of JFF and JVS.

Publication 2

Contributions: DN designed and performed all experimental work and prepared the manuscript under the supervision of JFF and JVS. AOA assisted with experimental design.

Publication 3

Contributions: DN designed and performed all experimental work and prepared the manuscript under the supervision of JFF and JVS. LPS interpreted NMR spectra and provided spectroscopic data.

Author's abbreviation

DN	Devashan Naidoo
AOA	Adeyemi O. Aremu
JG	JiřÍ Grúz
BO	Bíba Ondřej
KD	Karel Doležal
JFF	Jeffrey F. Finnie
JVS	Johannes Van Staden
LPS	Lenka Poštová Slavětínská

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TABLE OF CONTENTS

STUDENT DECLARATION .... **II**

DECLARATION BY SUPERVISORS .... **III**

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION 1 - PLAGIARISM .... **IV**

PUBLICATIONS FROM THIS THESIS .... **V**

CONFERENCE CONTRIBUTIONS .... **VI**

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION 2 - PUBLICATIONS .... **VII**

ACKNOWLEDGEMENTS .... **VIII**

TABLE OF CONTENTS .... **IX**

LIST OF FIGURES .... **XIV**

LIST OF TABLES .... **XVII**

LIST OF ABBREVIATIONS .... **XIX**

ABSTRACT .... **XXII**

CHAPTER 1: LITERATURE REVIEW **1**

1.1. The value of medicinal plants 1

1.2. Traditional medicine in South Africa 1

1.3. Why conserve plants? 3

1.3.1. Drug discovery from medicinal plants 3

1.3.2. The commercial importance of medicinal plants in South Africa 5

1.4. The contribution of micropropagation to the conservation of plants 6

1.5. The Amaryllidaceae 7

1.5.1. The Amaryllidaceae in South African traditional medicine 7

1.5.2. *In vitro* propagation of the Amaryllidaceae 9

1.5.3. The genus *Scadoxus* 13

1.5.4. Distribution and morphology of *Scadoxus puniceus* 13

1.5.5. Traditional uses of *Scadoxus puniceus* 15

1.5.6. Pharmacological studies on *Scadoxus puniceus* 15

1.5.7. Propagation of *Scadoxus puniceus* 15

1.6. Aims and objectives 16

CHAPTER 2: MICROPROPAGATION OF *SCADOXUS PUNICEUS* 17

2.1. Introduction 17

2.1.1. Requirements for successful tissue culture 17

2.1.2. Micropropagation of *Scadoxus puniceus* 19

2.2. Materials and Methods 20

2.2.1. Source of Plant growth regulators and media supplements 20

2.2.2. Micropropagation from vegetative explants 20

2.2.3. Propagation from seeds 21

2.2.4. The effect of photoperiod on *in vitro* shoot multiplication 23

2.2.5. The effect of adsorbents and antioxidants on oxidative browning 23

2.2.6. Data analysis 23

2.3. Results and Discussion 24

2.3.1. Micropropagation from vegetative explants 24

2.3.2. Micropropagation from seeds 28

2.3.3. The effect of photoperiod on *in vitro* bulblet multiplication 31

2.3.5. The effect of adsorbents and antioxidants on oxidative browning 35

2.4. Conclusions 37

CHAPTER 3: ALLEVIATION OF THE PHYSIOLOGICAL DISORDERS IN MICROPROPAGATED *SCADOXUS PUNICEUS* 38

3.1. Introduction 38

3.1.1. Hyperhydricity 38

3.1.2. Oxidative browning 38

3.1.3. Liquid culture systems 40

3.2. Materials and Methods....41
3.2.1. Source of plant growth regulators and media supplements 41
3.2.2. The influence of cytokinins on the proliferation and development of *Scadoxus puniceus* 41
3.2.3. The influence of organic compounds on the development of *Scadoxus puniceus* and *Merwilla plumbea* 41
3.2.4. Liquid culture system....42
3.2.5. Data analysis 43
3.3. Results and Discussion 43
3.3.1. The influence of cytokinins on the proliferation and development of *Scadoxus puniceus* 43
3.3.2. The effect of gallic acid (GCA) on development of *Merwilla plumbea* and *Scadoxus puniceus* 48
3.3.3. The effect of phloroglucinol (PG) on the *in vitro* development of *Scadoxus puniceus* 54
3.3.4. The growth and development of *Scadoxus puniceus* in a liquid culture system ...57
3.4. Conclusions....63
CHAPTER 4: PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF *SCADOXUS PUNICEUS* 65
4.1. Introduction....65
4.2. Materials and Methods....67
4.2.1. Preparation of plant extracts 67
4.2.2. Ultra high performance liquid chromatographic (UHPLC) analysis of phenolic acids 68
4.2.3. Antioxidant activity of plant extracts....69
4.2.4. Data analysis 72
4.3. Results and Discussion 72
4.3.1. Phenolic acid profile 72

4.3.2. Antioxidant activity 77
4.4. Conclusions 81
CHAPTER 5: *IN VITRO* ANTIMICROBIAL AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF *SCADOXUS PUNICEUS* EXTRACTS 82
5.1. Introduction 82
5.1.1. Infectious diseases and human health 82
5.1.2. Neurodegenerative disorders 83
5.1.3. Drug discovery from plants 84
5.2. Materials and Methods 85
5.2.1. Preparation of extracts 85
5.2.2. Antibacterial microdilution assay 85
5.2.3. Antifungal microdilution assay 86
5.2.4. Microplate assay for determination of acetylcholinesterase (AChE) inhibitory activity 87
5.2.5. Data analysis 88
5.3. Results and Discussion 88
5.3.1. Antimicrobial activity 88
5.3.2. Acetylcholinesterase inhibitory activity 92
5.4. Conclusions 96
CHAPTER 6: ISOLATION AND IDENTIFICATION OF THE BIOACTIVE COMPOUNDS FROM *SCADOXUS PUNICEUS* 98
6.1. Introduction 98
6.2. Materials and Methods 100
6.2.1. General 100
6.2.2. Collection of plant material 101
6.2.3. Extract preparation and isolation of alkaloids 101
6.2.4. Physical and spectroscopic data for haemanthamine 102
6.2.5. Physical and spectroscopic data for haemanthidine 102

6.2.6. Physical and spectroscopic data for metolachlor 103

6.2.7. Antibacterial microdilution assay 104

6.2.8. Antifungal microdilution assay 104

6.2.9. Microplate assay for determination of acetylcholinesterase (AChE) inhibitory activity 104

6.3. Results and Discussion 105

6.3.1. Isolation of the bioactive compounds 105

6.3.2. Antimicrobial activity of isolated compounds 110

6.3.3. Acetylcholinesterase inhibitory activity of isolated compounds 111

6.4. Conclusions 114

CHAPTER 7: GENERAL CONCLUSIONS 115

7.1. Micropropagation of *Scadoxus puniceus* 115

7.2. Phenolic profile and pharmacology of *Scadoxus puniceus* 116

7.3. Isolation, identification and biological activity of compounds from *Scadoxus puniceus* 118

REFERENCES 120

APPENDIX 1 – COMPOSITION OF THE MURASHIGE AND SKOOG (1962) NUTRIENT MEDIUM 151

APPENDIX 2 – CHEMICAL STRUCTURES OF GROWTH SUPPLEMENTS USED IN THE CURRENT STUDY 152

APPENDIX 3 – CHEMICAL STRUCTURES OF PHENOLIC ACIDS QUANTIFIED IN *SCADOXUS PUNICEUS* 154

APPENDIX 4 – KEY COSY AND HMBC CORRELATIONS FOR COMPOUNDS ISOLATED FROM *SCADOXUS PUNICEUS* 156

LIST OF FIGURES

Figure 1.1: *Scadoxus puniceus* during the flowering season (Spring). A; Emerging leaves after the flower has developed. B; Formation of the inflorescence stalk. C; Scarlet flowers from which the common name 'blood lily' is derived. 14

Figure 2.1: Three-month-old seedling of *Scadoxus puniceus*. (1) Root segment, (2) root segment attached to the basal plate, (3, inset) bulb halves, (4) stem segment. Bar = 1 cm. 22

Figure 2.2: *In vitro* culture response of single-scale (SS), single-scales attached to a segment of the basal plate (SS+BP) and twin-scale explant (TS). Different letters indicate significant differences at $p = 0.05$, n = 20.. 26

Figure 2.3: The fate of leaf explants of *Scadoxus puniceus* supplemented with various cytokinins (A) and benzyladenine in combination with naphthaleneacetic acid (B). Bar = 1 cm. 28

Figure 2.4: The development of immature embryos of *S. puniceus* on callus induction medium. (a) Hyperhydric seedling, (b) phenolic exudation, (c) non-regenerable callus. Bar = 1 cm. 29

Figure 2.5: Proliferation of *Scadoxus puniceus* explants cultured on different combination of benzyladenine and 2,4-dichlorophenoxy acetic acid (µM). 31

Figure 2.6: The influence of different light regimes (24 h light, 16/8 h light/dark, 24 h dark) on *in vitro* regeneration of *Scadoxus puniceus*. (A) The number of bulblets produced on leaf explants (mean ± SE). (B & C) The shoot length and fresh weight (mean ± SE) of regenerated bulb halves respectively. (D) Phenolic ratings based on a hedonic scale of bulb halves. 32

Figure 2.7: The influence of photoperiod (1, 24 h light; 2, 16/8 h light/dark; 3, 24 h dark) on regeneration of *Scadoxus puniceus* leaf (A) and bulb-half (B) explants. (A4)

Crystalline callus (arrow) formed on leaf explants in dark conditions. (B4) Phenolic exudation (arrow) by bulb halves into the medium. Bars = 10 mm.......33

Figure 2.8: The influence of phenolic exudates on bulblet production on leaf explants of *Scadoxus puniceus* based on a hedonic scale. 34

Figure 3.1: The effect of benzyladenine (BA) and *meta*-topolin (*m*T) on the proliferation of *Scadoxus puniceus*. Bars represent the mean with SE. n = 20. Different letters represent significant differences at the 5% significance level according to Duncan's Multiple Range Tests. 44

Figure 3.2: The morphology of shoots of *Scadoxus puniceus* cultured in MS medium supplemented with various cytokinins. A, B; hyperhydric shoots derived from plant growth regulator-free and benzyladenine supplemented medium, respectively. C, D; improved development of shoots from topolin treatments (*meta*-topolin and *meta*-topolin riboside, respectively). Scale bar = 10 mm. 48

Figure 3.3: The effect of gallic acid (GCA) combination with equi-molar concentrations (5 μM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development of *Scadoxus puniceus*. A; MS-only (control), B; MS + 1 μM GCA, C; MS + 5 μM BA, D; MS + 5 μM BA + 10 GCA, E; MS + 5 *m*T, F; MS + 5 μM *m*T + 5 μM GCA. Bar = 10 mm. MS; Murashige and Skoog nutrient medium. 54

Figure 3.4: Regeneration of *Scadoxus puniceus* from inflorescence explants; pedicel without (**1**) and with (**2**) the attachment to the ovary, anthers and filament on plant growth regulator-free medium (**A**) or Murashige and Skoog (MS) nutrient medium supplemented with benzyladenine and naphthaleneacetic acid (5μM; **B**). Shoot clusters further developed on solid (**C**) or in liquid (**D**) MS medium supplemented with 6% sucrose. Scale bar = 1 cm. 59

Figure 3.5: Healthy plantlets produced in liquid media supplemented with benzyladenine and naphthaleneacetic acid (5: 1 μM). Bar = 10 mm. 60

Figure 3.6: Root development of liquid culture-derived *Scadoxus puniceus* plantlets on various concentrations of indole-3-butyric acid. In each group, different letters represent significant differences between samples at the 5% significance level. n = 9. 61

Figure 3.7: Root development of liquid culture-derived *Scadoxus puniceus* plantlets on various concentrations of indole-3-butyric acid (IBA). A: PGR-free, B: 1 µM IBA, C: 5 µM IBA, D: 10 µM IBA, E: 15 µM IBA. Bar = 10 mm. 62

Figure 4.1: The proposed biosynthesis of phenolic acids which are present in *Scadoxus puniceus* via the shikimate pathway (adapted and modified from: **HELENO *et al*., 2015**). 76

Figure 4.2: The dose dependent ferric ion-reducing power of extracts of *Scadoxus puniceus*. BHT; butylated hydroxyltolulene. Different letters indicate significant differences between samples at the highest concentration tested (5 µg/ml). n = 3....................78

Figure 5.1: The dose dependent acetylcholinesterase inhibitory activity of different organs of *Scadoxus puniceus*. (A) 80% ethanol extracts, (B) dichloromethane extracts, (C) petroleum ether extracts. In each graph, different letters indicate significant differences among samples (within each solvent) at the 5% significance level according to Duncan's Multiple Range Tests. n = 3....................95

Figure 6.1: The structure of compounds isolated from *Scadoxus puniceus*. Haemanthamine (**1**); Haemanthidine (**2**); Metolachlor (**3**). 109

LIST OF TABLES

Table 1.1: Modifications to *in vitro* propagation protocols for the improvement of regeneration in various Amaryllidaceae species. 10

Table 2.1: Plant growth regulator (PGR) combinations of benzyladenine and 2,4-dichlorophenoxy acetic acid that supplemented Murashige and Skoog medium for regeneration from seedling sections. 22

Table 2.2: The influence of antioxidants (citric acid and ascorbic acid) and adsorbents (polyvinylpyrrolidone and charcoal) on bulblet induction and oxidative browning of *Scadoxus puniceus* twin-scale explants. 35

Table 3.1: The effect of different cytokinins on the development and morphology of *Scadoxus puniceus* shoots 47

Table 3.2: The effect of gallic acid (GCA) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development and phenolic content of *Merwilla plumbea*. 52

Table 3.3: The effect of gallic acid (GCA) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development of *Scadoxus puniceus*. 53

Table 3.4: The effect of phloroglucinol (PG) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or naphthaleneacetic acid (NAA) on *in vitro* development of *Scadoxus puniceus*. 56

Table 3.5: The development of *Scadoxus puniceus* maintained in liquid media supplemented with different combinations of benzyladenine (BA) and naphthaleneacetic acid (NAA). 60

Table 4.1: The sequence of linear gradients and isocratic flows of solvents in the mobile phase of the reversed phase UHPLC. 69

Table 4.2: The composition of phenolic compounds in leaves, stems, roots and bulbs of *Scadoxus puniceus* 75

Table 4.3: 2,2-Diphenyl-1-picryhydrazyl radical scavenging activity (% RSA) of different parts of *Scadoxus puniceus* methanolic extracts. 77

Table 4.4: Prevention of the coupled oxidation of *β*-carotene and linoleic acid by extracts from different parts of *Scadoxus puniceus*..79

Table 5.1: *In vitro* antibacterial activity (expressed as MIC mg/ml) of different extracts of organs of *Scadoxus puniceus* determined using the microdilution technique. 89

Table 5.2: *In vitro* antifungal activity (expressed as MIC mg/ml) of different extracts of organs of *Scadoxus puniceus* against *Candida albicans* determined using the microdilution technique 90

Table 5.3: The IC_{50} (mg/ml) acetylcholinesterase inhibitory values of extracts of *Scadoxus puniceus*. 96

Table 6.1. ^{1}H and ^{13}C NMR data for haemanthamine (**1**) and haemanthidine (**2**)[a] in Methanol-d_4...107

Table 6.2. ^{1}H and ^{13}C NMR data for **3**[a] in Methanol-d_4...108

Table 6.3: The antimicrobial MIC values of isolated compounds from *Scadoxus puniceus* 110

Table 6.4: The inhibition (%) and IC_{50} μM/ml acetylcholinesterase inhibitory values of pure compounds isolated from *Scadoxus puniceus*...112

LIST OF ABBREVIATIONS

^{1}H	NMR proton spectra
2,4-D	2,4-Dichlorophenoxy acetic acid
2iP	N^6-isopentenyladenine or N^6-(2-isopentenyl) adenine
^{13}C	NMR carbon spectra
AA	Antioxidant activity
AC	Activated charcoal
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AIDS	Acquired immune deficiency syndrome
AIP	2-aminoindane-2-phosphoric acid
ANOVA	Analysis of Variance
ANT	Antioxidant activity
ASC	Ascorbic acid
ATCC	American type culture collection
ATCI	Acetylthiocholine iodide
BA	6-Benzyladenine or *N*-(Phenylmethy)-7*H*-purin-6amino)
BCA	*Beta*-carotene/linoleic acid model
BChE	Butyrylcholinesterase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytolulene
CA	Citric acid
CD_3OD	Deuterated methanol
CFU	Colony forming units
CGA	Chlorogenic acid or 5-caffeoyl-D-quinic acid
CNS	Central nervous system
COSY	Correlation spectroscopy
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DIP	Direct injection probe
DMRT	Duncan's multiple range tests

DMSO	Dimethylsulfoxide
DMSO-$_{d6}$	Deuterated dimethylsulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DW	Dry weight
EC_{50}	Half maximum effective concentration
EIMS	Electron impact mass spectrometry
ESI	Electrospray ionisation
EtOH	Ethanol
FA	Fatty acid
FDA	Food and Drug Administration
Folin-C	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GCA	Gallic acid or 3,4,5-trihydroxybenzoic acid
HBA	Hydroxybenzoic acid
HCA	Hydroxycinnamic acid
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
IAA	Indole-3-acetic acid or 2-(1H-indole-3-yl) acetic acid
IBA	Indole-3-butyric acid or 1H-indole-3-butanoic acid
IC_{50}	Half maximum inhibitory concentration
IKS	Indigenous knowledge system
INT	*p*-iodonitrotetrazolium chloride
Me*m*T	*meta*-Methoxytopolin or 6-(3-methoxybenzylamino) purine
Me*m*TTHP	*meta*-Methoxy 9-tetrahydropyran-2-yl or 2-[6-(3-Methoxybenzylamino)-9-(tetrahydropyran-2yl) purine]
MH	Meuller-Hinton
MIC	Minimum inhibitory concentration
Min	Minutes

MS	Murashige and Skoog
MS/MS	Tandem mass spectrometry
*m*T	*meta*-Topolin
*m*TR	*meta*-Topolin riboside
NAA	α - Naphthaleneacetic acid
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
ORR	Oxidation rate ratio
PAL	Phenylalanine ammonia lyase
PDA	Photo diode array
PE	Petroleum ether
PG	Phloroglucinol or 1,3,5-trihydroxybenzene
PGR	Plant growth regulator
PIC	Picloram or 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
POD	Peroxidase
PPFD	Photosynthetic photon flux density
PPO	Polyphenol-oxidase
PVP	Polyvinylpyrrolidone or 1-ethenylpyrrolidin-2one
ROS	Reactive oxygen species
RR	Reaction rate
RSA	Radical scavenging activity
Sec	Seconds
SS	Single-scale
SS + BP	Single-scale and basal plate
TBHQ	*Tert*-butylhydroquinone or 2-(1,1-Dimethy)-1,4-benzenediol
TLC	Thin layer chromatography
TS	Twin-scale
UHPLC	Ultra high performance liquid chromatography
UKZN	University of KwaZulu-Natal
UV	Ultra-violet
YM	Yeast Malt

ABSTRACT

Plants have formed the basis of traditional medicine systems worldwide; their use has been documented for thousands of years. The use of plants in South African traditional medicine has been documented in rock paintings by the San people and today comprises an effective alternative to modern medicine. Given the shortage of medical doctors, coupled with the affordability, availability and cultural importance of traditional medicines, an estimated 27 million South Africans depend on this form of medicine for their primary healthcare needs. However, as a result of their continuous exploitation, and unsustainable harvesting for use in traditional medicines, several medicinal plant species have become threatened with extinction. Owing to the high cost of drugs, multi-drug resistance and treatment failure, or the lack of treatment for chronic disorders, there has been a renewed interest in natural products obtained from plants and other natural resources. Several drugs on the market for treatment of various disorders are natural products or derived from the structure of natural products and include; galanthamine, codeine, vincristine and vinblastine. Thus the extinction of medicinal plant species will equate to the loss of valuable biodiversity together with the potential for the discovery and synthesis of novel compounds and drugs. Plant tissue culture has been widely accepted as a means for mass production and proliferation of healthy, disease-free medicinal plants to reduce the impact of unsustainable harvesting of wild material.

Scadoxus puniceus (L.) Friis & Nordal (Amaryllidaceae) is a robust bulbous plant that is frequently traded in *Muthi* stores across South Africa for the treatment of various illnesses. Although the current conservation status of *S. puniceus* is least concern, the species is ranked amongst the most traded plants in South Africa. As such, the current study was undertaken to establish an efficient *in vitro* propagation protocol for the species taking into consideration the major factors that are necessary for successful propagation *in vitro*. In addition, given its importance in traditional medicine, the species may possess valuable potential for further development in western medicine. For this reason, the study also evaluated the antioxidant, antimicrobial and acetylcholinesterase inhibitory activities of different organs of the species. Secondary metabolites have been advocated as the primary reason for the efficacy of medicinal plants. As such, ultra high performance liquid chromatography (UHPLC) was employed to determine the phenolic acid (major secondary metabolites) profile of *S. puniceus*. Gravity column chromatography was used to separate active fractions of *S. puniceus* and purify the active components. The application of 1 and 2D NMR analysis and

mass spectroscopy aided in the identification of the compounds.The first attempt at the *in vitro* propagation of *S. puniceus* was met with several shortcomings. Leaf explants exhibited seasonal growth patterns while the exudation and subsequent oxidation of phenols into the growth medium hampered the propagation from bulb scales. Propagation from seeds was successful although further proliferation was hampered by phenolic exudation. The addition of antioxidants into the growth medium failed to improve growth. However, leaf explants responded to *in vitro* growth conditions when incubated in the dark. In the current study, light stimulated the production and exudation of phenols into the growth medium. When cultured under a 24 h dark photoperiod, a significantly improved proliferation rate was obtained. Several of the plantlets obtained from these experiments were hyperhydric.

The study then focused on alleviating common physiological disorders (oxidative browning, hyperhydricity and recalcitrance of explants) that presented themselves in the *in vitro* propagation of *S. puniceus*. The application of cytokinins (benzyladenine; BA) improved the proliferation of *S. puniceus*. However, when maintained on media containing BA, plantlets became hyperhydric. Topolins (*meta*-topolins in particular) improved the development of the species by alleviating hyperhydricity. Two organic phenolic compounds, phloroglucinol (PG) and gallic acid (GCA; phenyl-ammonia lyase inhibitor) were evaluated for their role in reducing oxidative browning in *S. puniceus* and *Merwilla plumbea* (a model species). Both compounds improved plant development and also exhibited interactions with cytokinins and auxins. A synergistic relationship of GCA with benzyladenine and an antagonistic relationship with *meta*-topolin were demonstrated. An investigation of the phenolic acid content of *in vitro* grown plantlets revealed that the incorporation of GCA into the medium had no effect on the total phenolic content. This suggests that the exudation rather than the production of phenols was alleviated. The relationship of PG with an auxin (naphthaleneacetic acid, NAA) was particularly strong, producing a six-fold improvement in plant development. A liquid culture system was also developed to improve on the regeneration of leaf explants reducing the burden of recalcitrance. Liquid media proved ineffective for bulblet formation (which can be achieved on solid media). Nevertheless, significantly larger, healthier plantlets (6-8 fold improvement relative to solid media) developed when shoot clusters were maintained in liquid media supplemented with BA and NAA.

The current study accentuates the necessity to mitigate the burden of common physiological disorders such as oxidative browning, hyperhydricity and recalcitrance in tissue culture.

Based on the results of the study, the use of GCA may provide an avenue for reducing oxidative browning of several species by limiting the exudation of phenols into the medium. With regard to *S. puniceus*, propagation in the dark, liquid media and the addition of cytokinins or organic supplements that reduce the exudation of phenols into the medium are essential for the *in vitro* propagation of this species.

UHPLC revealed the presence of 13 phenolic acids distributed non-uniformly throughout the different organs of *S. puniceus*. In aerial organs, hydroxycinnamic acids were more concentrated than the hydroxybenzoic acids while the opposite held true for below ground organs. The study reveals, for the first time, the presence of chlorogenic (CGA), gallic, sinapic and *m*-hydroxybenzoic acids in an Amaryllid species. Chlorogenic acid accumulated more intensely in the leaves and stems of the species, suggesting a functional role against herbivory from the Amaryllis leaf borer which infested many of the stock plants. The phenolic content of the different organs influenced their antioxidant activity, however, the type of phenolic acid rather than its quantity had a greater effect. Leaf extracts possessed the strongest activity, accounted for by the high concentrations of CGA, *p*-coumaric, and protocatechuic acids. Despite the accumulation of CGA in stems, roots possessed the better activity given the presence of *p*-coumaric and vanillic acids in roots.

Pharmacological screening revealed broad spectrum antibacterial activity of dichloromethane and petroleum ether bulb extracts exhibiting minimum inhibitory concentrations ranging between 0.39 and 1.56 mg/ml. Leaf extracts exhibited potent antifungal activity ranging between 0.05 and 0.20 mg/ml against *Candida albicans*. The potent antifungal activity may once again be attributed to the heavy accumulation of CGA (an antifungal agent) in the leaves. Furthermore, all extracts of *S. puniceus* exhibited strong dose dependent AChE inhibitory activity all of which exceeded 90% at the highest concentration tested. The activity may be due to the presence of alkaloids in the various organs. Bulb extracts exhibited the lowest IC_{50} value (0.07 mg/ml) suggesting that alkaloids are more concentrated in this organ.

Chromatographic separation of an ethanolic extract of *S. puniceus* yielded three compounds, two from a methanolic extract of the bulbs (Compounds **1** and **2**) and one from an earlier ethyl acetate fraction of the leaves (Compound **3**). NMR and mass spectroscopy identified compound **1** and **2** as two known alkaloids, haemanthamine and haemanthidine. Compound **3** was a rare chlorinated amide, metolachlor, the naturally occurring structure of which was identified for the first time. In addition, the current study is the first report of the presence of

a chlorinated amide in the Amaryllidaceae. The bioactivity of these compounds was evaluated with regards to their antimicrobial and AChE inhibitory activities in comparison to the crude ethanolic extract. The broad spectrum antimicrobial activity of the isolated compounds against both Gram-positive and Gram-negative bacterial strains and a fungus was demonstrated. Isolated compounds displayed stronger activity when compared to the crude extract, exhibiting MIC values ranging between 0.062 – 0.250 mg/ml. Isolated compounds also displayed potent activity in inhibiting the biotransformation of acetylcholine by eel acetylcholinesterase. The alkaloids, haemanthamine and haemanthidine exhibited similar IC_{50} values of 23.7 and 23.1 μM/ml despite the hydroxyl substitution at position C(6) in haemanthidine. This structurally-related AChE inhibitory activity is of significance representing activity that was absent within the 5,10b-ethanophenanthridine series of alkaloids. Metolachlor displayed even stronger activity effecting an IC_{50} value of 11.5 μM/ml. This activity is unprecedented for a chlorinated amide, highlighting the potential for the use of this group of compounds in medicine.

The current study thus, in addition to providing an *in vitro* propagation protocol for *S. puniceus*, validated the extensive use of this plant in traditional medicine. It also isolated compounds of interest that may hold promise for drug development and signifies the importance of conservation efforts and drug discovery from plants.

CHAPTER 1: LITERATURE REVIEW

1.1. The value of medicinal plants

Since the existence of mankind, humans have made use of natural resources for their basic needs such as food production, shelter, clothing, transportation, fertilisers, fragrances and flavours **(NEWMAN *et al.*, 2000)**. Plants for instance, have provided much potential for socio-economic development in many developing countries **(VAN WYK, 2008)** and have also provided mankind with sophisticated traditional medicine systems **(NEWMAN *et al.*, 2000)**. It is well documented that plants are a source of medicinally active compounds **(BALUNAS and KINGHORN, 2005)**. As such, they have been used as ingredients in traditional medicines for thousands of years and have been used by all cultures and ethnic groups **(HOAREAU and DA SILVA, 1999)**. Traditional medicine systems initially made use of crude extracts such as tinctures, teas, poultices, powders and other herbal formulations. Nowadays, they can be phytopharmaceuticals or herbal mixtures or isolated compounds **(RATES, 2001)**. These preparations were, and still are, used to treat numerous ailments ranging from common stomach concerns, influenza and minor wounds to more serious diseases like malaria, cancer and HIV/ AIDS **(HOAREAU and DA SILVA, 1999)**.

As a result of their potential therapeutic and pharmaceutical applications, there has been an increase in the demand for medicinal plants, despite significant improvements to modern medicine over decades. It has been estimated that 80% of the population in rural areas all over the world subscribe to the use of traditional medicine **(BANQUAR, 1993)**. With half of the world's population surviving on less than two U.S dollars per day, pharmaceuticals are seen as an expensive luxury **(DA SILVA *et al.*, 2002)**. Thus the rationale for the extensive use of traditional medicines is that this form of medicine is more affordable and accessible to the population and is accompanied by extensive knowledge and expertise among local communities **(MANDER, 1998)**.

1.2. Traditional medicine in South Africa

Medicinal plants are found throughout the world and are commonly used within the Chinese, Indian, Japanese, and African traditional customs **(MUKHTAR *et al.*, 2008)**. The Indian Ayurvedic system is considered the oldest while the Chinese system is considered the most developed. African traditional medicine however, is promoted by the rich biological and cultural diversity amongst the flora and the people of the continent.

South Africa has a dual healthcare system, one is based on western medical practices and the other; a traditional medicine system. While western medicine has a scientific and chemical basis providing cause and effect units, traditional medicine is plant and animal-based and provides holistic treatments **(CUNNINGHAM, 1993; BODEKER, 2004)**. Holistic treatment considers various factors when addressing symptoms and diagnosing ailments, including factors such as an individual's mental, social, spiritual, physical and ecological states. As a result of the shortage of western-trained medical doctors and inadequate healthcare facilities and medical equipment, traditional medicine systems are an informal alternative to the sometimes poor rural healthcare systems present in South Africa **(LIGHT *et al*., 2005; VAN WYK *et al*., 1997)**. While 85% of the South African population is covered by 8000 doctors within the state healthcare system and the private healthcare system serving the remaining 15%, with 12 000 doctors, the traditional medical system outnumbers the Western doctors by at least 10 to 1 **(MORRIS, 2001)**. Given the aforementioned shortcomings of the rural healthcare system coupled with the affordability, availability and cultural importance of traditional medicines, 27 million South Africans depend on traditional medicine for their primary health concerns **(MANDER, 1998)**. The extensive range that traditional medical systems cover is evident.

Although traditional medicines can contain both animal and plant materials, within the African systems, plants are the main ingredients used **(CUNNINGHAM, 1993)**. South African traditional medicine is supported by the rich and diverse variety of plants inhabiting the region, accounting for 25% of the total number of higher plants in the world **(VAN WYK, 2008)**. According to the Flora of southern Africa database, a total of 22 755 plant species are found in southern Africa, thus endorsing the extensive use of plants in traditional medicines. In South Africa, a total of 3481 plant taxa are used as traditional medicinal plants, of which 2942 are administered to humans only **(ALMOND and COHEN, 2002)**. In KwaZulu-Natal alone an estimated 1032 plant species from 147 families are reportedly used in traditional medicine **(LIGHT *et al*., 2005)**.

Some species (and their uses) that are considered important in the South African traditional medicine industry include; *Hoodia gordonii* (appetite and food suppressant), *Aloe ferox* (laxative and wound healing), *Sutherlandia frutescence* (traditional cancer tonic) and *Pelargonium sidoides* (tuberculosis and diarrhoea) **(WEBB and WRIGHT, 1986; VAN WYK *et al*., 1997)**. The native Zulu people made frequent use of *Siphonochilus natalensis* and *Alepedia amatymbica* **(WEBB and WRIGHT, 1986)**.

1.3. Why conserve plants?

In recent times, South Africa has documented a drastic decrease in medicinal plant biodiversity. In the Eastern Cape Province, a minimum of 166 plant taxa are associated with traditional medicine, of the 60 most frequently used species, 93% are harvested unsustainably (**DOLD and COCKS, 2002**). Legislation against the harvesting of wild resources passed by the South African Government in an attempt to promote sustainable harvesting yielded no positive results (**MANDER and MCKENZIE, 2005**). Furthermore, an increase in both the formal and informal markets which deal in the trade of these plants has been noted (**MANDER and MCKENZIE, 2005).** It has also been suggested that the demand for medicinal plants may already be too high to be met by sustainable harvesting, owing to the exponential growth of populations in developing countries (**VAN STADEN, 1999; KULKARNI *et al*., 2005**).

Conservation is a process which involves the preservation of the environment and careful management of natural resources as well as to prevent neglect, over-exploitation and destruction of these resources (**OKIGBO *et al*., 2008**). Medicinal plants are just one example of a natural resource that has been placed under strain. Plants are faced with several stressors within their environments and these include; pollution (air, land and water), farming practices, and livestock grazing (**MARSHALL, 1998**). In addition to this, the threat to medicinal plant biodiversity is compounded by increasing urbanization, and the increased demand by the ever growing population (**ZSCHOCKE and VAN STADEN, 2000**). The over-exploitation and the harvesting of non-renewable plant material such as roots, bulbs, and bark are considered to be major contributors to the worldwide decline of medicinal plants in the wild (**MARSHALL, 1998**).

Despite efforts to conserve plants based on their aesthetic, cultural and horticultural uses, a greater effort needs to be placed on plants with commercial and medicinal value (**MABBERLEY, 1987; HOYLES, 1991**).

1.3.1. Drug discovery from medicinal plants

Owing to the high cost of drugs, multi-drug resistance and treatment failure, or the lack of treatment for chronic disorders (**AFOLAYAN and ADEBOLA, 2004**), there has been a renewed interest in natural medicines obtained from plant extracts. Ethnopharmacological research in South Africa has followed this trend (**LIGHT *et al*., 2005**). Several authors have

reported on the ethnobotanical usage of plants as medicines all across South Africa **(BHAT and JACOBS, 1995; HUTCHINGS *et al.*, 1996; THRING and WEITZ, 2006)**. Subsequently, a greater number of investigations have been carried out to determine the potential bioactivity of many plant species. Several investigations successfully validated their traditional uses and isolated bioactive compounds with antibacterial **(KELMANSON *et al.*, 2000)**, antifungal **(MASOKO *et al.*, 2007)**, anti-inflammatory **(IWALEWA *et al.*, 2007; JÄGER *et al.*, 1996)** and anthelmintic **(McGAW *et al.*, 2000)** activities.

A pharmacologically active compound which exerts its activity as a component of medicine may be referred to as a drug despite its derivation, being either of natural, biotechnological or synthetic origin and is used for the diagnosis, prevention and treatment of diseases **(BALUNAS and KINGHORN, 2005; RATES, 2001)**. Drug discovery is a multi-disciplinary process, an endeavour which relies upon the integration of botany, chemistry and pharmacology **(RATES, 2001)**. Several methods have been used to attain compounds with potential for drug development; these include the isolation of compounds from natural sources such as plants and animals, synthetic chemistry, combinatorial chemistry and molecular modelling **(BALUNAS and KINGHORN, 2005)**. The development of organic chemistry during the industrial revolution and the recent increase in interest in molecular modelling and combinatorial chemistry has resulted in a preference for synthetic products for pharmacological treatment **(NEWMAN *et* al., 2000; RATES, 2001)**.

However, the importance of natural products cannot be underestimated. It is estimated that 25% of drugs prescribed worldwide are derived from plants **(RATES, 2001)**. Approximately about 11% of the drugs considered basic and essential by the World Health Organisation are exclusively derived from plants. Furthermore, synthetic drugs are often obtained from naturally occurring precursors **(RATES, 2001)**. Important drugs that were isolated from plants include digoxin from *Digitalis* spp., quinine and quinidine obtained from *Cinchona* spp., codeine from *Papaver somniferum* and vincristine and vinblastine from *Catharanthus roseus* **(RATES, 2001)**. The process of drug discovery from plants is associated with high costs, prolonged research and a relatively low average yield of isolated compounds; most of which are insufficient for lead optimization, development and clinical trials **(REICHERT, 2003; BALUNAS and KINGHORN, 2005)**. However, in light of the fact that several of these compounds have been isolated from plants and have been used successfully in modern medicines, the isolation of natural products from plants still remains a crucial component of drug discovery **(NEWMAN *et al.*, 2000)**. Thus the extinction of plants would equate to the

loss of resources with potential for the synthesis of new compounds and drugs **(RATES, 2001)**.

1.3.2. The commercial importance of medicinal plants in South Africa

A significant proportion of South African communities rely upon and often show a preference to traditional medicine as opposed to the western medicinal systems for their primary health care needs **(WILLIAMS *et al.*, 2013)**. **MANDER *et al.* (2007)** estimated that 72% of the population of Black South Africans, in both urban and rural areas make use of traditional medicine consuming more than 70 000 tonnes of plant material annually. This profound dependence on traditional medicine and the trade thereof has created at least 134 000 income-earning opportunities for local communities **(MANDER *et al.*, 2007, WILLIAMS *et al.*, 2013)**.

Urban *muthi* markets are present throughout South Africa and consist of three large prominent markets, two of which are situated in Durban (the Warwick Triangle and Ezimbuzini markets) and one in Johannesburg (the Faraday market) while smaller urban centres and street traders are scattered throughout the country **(DOLD and COCKS, 2002; BOTHA *et al.*, 2004)**. Formal *muthi* stores are also common in almost all urban areas, such stores in the Eastern Cape and Witwatersrand regions trade in large numbers of medicinal plant species although their contribution to health care and resource exploitation is not always clearly known **(COCKS, 1996; WILLIAMS *et al.*, 2001)**. These commercial endeavours have been cited in the trade of 2062 taxa, 10.1% of South Africa's national flora **(WILLIAMS *et al.*, 2013)**.

WILLIAMS *et al.* (2013) recently examined the current threat status of South African medicinal plants. The study revealed that two medicinal plant species are currently extinct in the wild (*Warburgia salutaris* and *Siphonochilus aethiopicus*) while 82 species are threatened with extinction at a national level. Fourteen species have been listed as critically endangered, 19 endangered and 49 vulnerable **(WILLIAMS *et al.*, 2013)**. Furthermore, 37 species are near threatened, 36 declining and 21 species are either rare or critically rare. In addition, 74, 94 and 81% of the vulnerable, declining and near threatened species respectively, have been recorded in *muthi* markets, thus suggesting that the traded species are at a greater risk of becoming extinct than non-traded species **(WILLIAMS *et al.*, 2013)**.

The continuous increase in the South African population amidst the unrelenting HIV/AIDS epidemic will undoubtedly place a greater strain on medicinal plant resources. Further commercial exploitation without proper conservation intervention could result in the upgrade to Red List status (**WILLIAMS *et al*., 2013**).

1.4. The contribution of micropropagation to the conservation of plants

In order to reduce the decline of these valuable resources, conventional agricultural cultivation methods are often considered. However, the propagation of plants, based on these methods is dependent on external factors which may constrain its success. Several factors may be responsible for this, namely; land and water availability, micro- and macro-climate, season, pathogens, pests, and the slow growth of plants (**PIERIK, 1987; ARIKAT *et al*., 2004**). By contrast, micropropagation has been accepted as a biotechnological tool that can reduce the decline of medicinal plants in South Africa (**AFOLAYAN and ADEBOLA, 2004**).

Micropropagation can be defined as a multipart developmental process that is controlled and regulated by complex interactions of both intrinsic and environmental stimuli (**MOYO *et al*., 2011**). The process involves the aseptic manipulation of plant tissues growing in heterotrophic conditions on an artificial carbohydrate and nutrient-enriched basal medium. These conditions coupled with micro-environmental stimuli, are considered some of the major factors which influence the growth of plantlets *in vitro* (**MOYO *et al*., 2011**). The characteristics of the micro-environment should include a constant temperature, high relative humidity, and low photosynthetic photon flux density (PPFD), as well as optimised concentrations of sugars, salts, and plant growth regulators (PGRs) (**KOZAI *et al*., 1997**). **ABOEL-NIL (1997)** suggested that the use of tissue culture techniques for the conservation of plant germplasm can be a viable alternative to conventional propagation methods. Micropropagation encompasses several advantages including: (1) the year-long production of plant material independent on the season or climate. (2) The rapid propagation of plant species and long-term germplasm storage which are achievable in a small space and short time. (3) The aseptic tissue culture technique aids in producing large numbers of uniform and disease free plants and (4) the sterile nature of *in vitro* cultures makes it possible for across border germplasm or plant material exchanges (**ABOEL-NIL, 1997; KOZAI *et al*., 1997; MOYO *et al*., 2011**).

Thus the implementation of tissue culture methods may provide a more feasible means for rapid propagation from a conservation perspective while also maintaining the genotype of threatened plants **(AFOLAYAN and ADEBOLA, 2004)**. What may be worthy to note considering medicinal plants, is the ability to control the chemical and physical conditions of *in vitro* cultures, allowing for the optimization of conditions required for the production of secondary metabolites **(ABOEL-NIL, 1997)**. Plant tissue culture techniques have improved significantly over the past 50 years with a myriad of protocols being established for many plant species. However despite the improvements, the field still faces several challenges relating to the developmental and physiological processes of plant tissue culture. Challenges associated with tissue culture include somaclonal variation, hyperhydricity and oxidative browning of plant tissue.

1.5. The Amaryllidaceae

The Amaryllidaceae is a family of monocotyledonous, perennial or biennial bulbous flowering plants **(DAHLGREN *et al.*, 1985; MEEROW and SNIJMAN, 1998)**. The family consists of an estimated 1000 species classified within 60 genera **(MEEROW and SNIJMAN, 1998)**. Amaryllidaceae exhibit a pan-tropical distribution and are particularly common in the southern hemisphere including locations such as South America, the Mediterranean and southern Africa **(MEEROW and SNIJMAN, 1998)**. Approximately one-third of the global population of Amaryllids are concentrated in South Africa, the bulk of which inhabit the Cape floral kingdom of the Western Cape **(MEEROW and SNIJMAN, 1998)**. The species present in South Africa are classified almost exclusively within three of the fourteen recognized tribes in the Amaryllidaceae; these are the tribes Amaryllideae (*Amaryllis belladonna*), Haemantheae (*Clivia miniata*) and Crytantheae (*Cyrtanthus breviflorus*) **(SNIJMAN and LINDER, 1996; MEEROW and CLAYTON, 2004; OLIVIER, 1980; NAIR AND VAN STADEN, 2013)**. Given the aesthetic value of several varieties such as the daffodils (*Narcissus*), snowdrops (*Galanthus*), and snowflakes (*Leucojum*), these plants are important commodities in the floriculture industry **(NAIR and VAN STADEN, 2013)**.

1.5.1. The Amaryllidaceae in South African traditional medicine

Despite their aesthetic value, species within the family have been and still are important components in traditional medicine systems around the world **(VILADOMAT *et al.*, 1997; BASTIDA *et al.*, 2006)**. In South Africa, rock paintings of *Brunsvigia* species by the San, the

regions first inhabitants, demonstrate the age old cultural importance of the Amaryllidaceae (**DYER, 1950**). In addition, the local Khoi-San tribes were cited by early European settlers to the Cape, for the use of *Gethyllis* species to treat various ailments (**WATT and BREYER-BRANDWIJK, 1962**). Currently, species within the family rank amongst the most frequently traded bulbous plants in Zulu and Xhosa *muthi* markets (**HUTCHINGS *et al*., 1996; DOLD and COCKS, 2002; LOUW *et al*., 2002**). The majority of the 300 known species of South African Amaryllids are used in traditional medicines and are traded as concoctions, decoctions, extracts and herbal preparations with several species (**WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996; VILADOMAT *et al*., 1997; VAN WYK *et al*., 2005; NDHLALA *et al*., 2011**).

The traditional use of several Amaryllidaceae species has been substantiated by a recent surge in ethnopharmacological studies. Several authors report on the antibacterial activity of species such as *Boophone disticha*, *Cyrtanthus* spp., and *Gethyllis ciliaris* (**ELGORASHI and VAN STADEN, 2004; HEYMAN *et al*., 2009; CHEESMAN *et al*., 2012**). **VILADOMAT *et al*. (1997)** demonstrated the antifungal or anti-yeast activity of *Amaryllis belladonna*, *Crinum macowanii* and *Crinum moorei*. Furthermore, alkaloids isolated from the Amaryllidaceae, lycorine and vittatine, possess significant activity against *Candida albicans* (inhibitory concentrations of 39 and 31 μg/ml, respectively) (**EVIDENTE *et al*., 2004**). Similarly, **HUSSON *et al*. (1991; 1994)** revealed the antiviral activity of *Haemanthus albiflos* when a concentration of 50 μl/ml of bulb extracts inhibited viral replication of rotavirus within 4 h of application. Other species cited for their antiviral activity include *Clivia miniata* and *Crinum macowanii* (**VAN DEN BERGE *et al*., 1978; DURI *et al*., 1994**).

Certain Amaryllids are known for their use to treat inflammatory disorders including pain, swelling, asthma and arthritis. **ELGORASHI and VAN STADEN (2004)** made use of the cyclo-oxygenase (COX-1 and COX-2) assay to reveal the anti-inflammatory activity (70.5-100%) of six South African Amaryllid species (*Cyrtanthus falcatus*, *Cyrtanthus mackenii*, *Cyrtanthus suaveolens*, *Gethyllis ciliaris*, *Gethyllis multiflora* and *Gethyllis villosa*).

Several members of the Amaryllidaceae are also used in traditional medicine to treat mental health problems such as epilepsy, convulsions, depression and dementia (**NEUWINGER, 2000; BAY-SMIDT *et al*.., 2011**). Certain Amaryllids are known for their hallucinatory properties. The San and Sotho of southern Africa documented the use of *Boophone disticha* as a psychoactive plant and the species is used widely to induce hallucinations to treat

headaches and anxiety as well its use as a sedative **(DE SMET, 1996; NEERGAARD *et al.*, 2009).**

Despite the fact that two potent anticancer drugs, pancratistatin and narciclasine, have been isolated from the Amaryllidaceae, very little is known about the use of Amaryllids for the treatment of cancer in southern Africa **(KORNIENKO and EVIDENTE, 2008; NAIR and VAN STADEN 2013)**. Species used traditionally for cancer treatment include *Amaryllis belladonna*, *Boophone disticha* and *Crinum delagoense* **(PETTIT *et al.*, 2001; NAIR *et al.*, 1998; BOTHA *et al.*, 2005)**. The activity of these plants was attributed to the presence of the antiproliferative agent, lycorine **(LAMORAL-THEYS *et al.*, 2009; NAIR and VAN STADEN, 2012)**.

1.5.2. *In vitro* propagation of the Amaryllidaceae

Various *in vitro* propagation protocols have been established for several members of the Amaryllidaceae. However, tissue culture of the Amaryllidaceae pales in comparison to other families because the techniques involved are often more difficult for several reasons. Most species of the Amaryllidaceae are bulbous and it is not always easy to establish decontamination protocols for these species. Seasonal variation also restrains tissue culture to particular seasons and explants. The accumulation and exudation of toxic phenolic compounds often inhibits *in vitro* regeneration resulting in reduced growth or tissue death. As a result of these drawbacks, researchers are continuously searching for methods to improve the regeneration and multiplication rates of Amaryllid species **(Table 1.1)**.

Table 1.1: Modifications to *in vitro* propagation protocols for the improvement of regeneration in various Amaryllidaceae species.

Species	Explant	Basal medium	PGR	Modification	Effect on development	Reference
Amaryllis belladonna	Bulb scales	MS	3 mg/l NAA + 0.5 mg/l BA 3 mg/l BA 1 mg/l BA + 0.5 mg/ml NAA -	1 g/l Activated charcoal 1 g/l Activated charcoal 1 g/l Activated charcoal	Callus initiation Increased shoot production Increased number and shoot length	**SARATHE *et al*., 2014**
Boophone disticha	Twin-scales	MS	4.44 µM BA + 26.85 µM NAA	2 g/l Activated charcoal + 150 mg/l ascorbic acid	Prevented browning, inducing bulblet production	**CHEESMAN, 2013**
Brunsvigia undulata	Twin-scales	MS	2 mg/ml NAA or 1 mg/ml BA	5 g/l Activated charcoal	Increased number of bulblets	**RICE, 2009**
Crinum variabile	Twin-scales	MS	-	5 g/l Activated charcoal	Increased bulblet size and increased production of bulblets from shoots	**FENNELL *et al*., 2001**
Crinum moorei	Twin-scales	MS	-	5 g/l Activated charcoal	Increased bulblet size and formation	**FENNELL, 2002**

Cyrtanthus clavatus *Cyrtanthus spiralis*	Twin-scales	MS	-	5 g/l Activated charcoal Liquid medium	Increased shoot formation Increased bulblet diameter and fresh weight	**MORAN *et al*., 2003**
Eucrosia stricklandii	Twin-scales	MS	0.54 µM NAA + 4.44 µM BA	0.5% (w/v) charcoal	Increased shoot induction	**COLQUE *et al*., 2002**
Galanthus spp.	Bulb chips	MS	-	1-5 g/l Activated charcoal & 60 g/l sucrose	Increased fresh weight, root production and elongation	**STAIKIDOU *et al*., 2006**
Galanthus ikariae	Bulb-scales	Half-strength MS	0.5 mg/l NAA	0.5% Activated charcoal	Highest *ex vitro* success	**TIPIRDAMAZ, 2003**
Hippeastrum vittatum	Twin-scales	MS	16 mg/l 2iP + 4 mg/l NAA	80 mg/l spermine 4 mg/l methyl jasmonate 20 mg/ml progesterone	Improved multiplication rate Increased fresh weight Increased leaf length	**ZAYED *et al*., 2011**
Narcissus papyraceus	*In vitro* derived shoot clusters	MS	-	Liquid shake culture	Greater shoot proliferation and size	**BERGOÑÓN *et al*., 1992**
Narcissus tazetta	Twin-scales	MS	10 µM BA + 5 µM NAA	Addition of 2.5 mM sodium phosphate,	Efficient bulblet induction	**CHEN and ZIV, 2005**

				0.8mM adenine sulphate and 5 g/l activated charcoal		
Nerine sarniensis	*In vitro* derived bulblets	MS	1 µM BA + 1 µM NAA	Cultured in the dark for 11 weeks	Enhanced bud and bulblets regeneration	**VISHNEVETSKY *et al*., 2003**
Nerine. x *mansellsi*	*In vitro* derived nodular tissue	MS	0.25 µM NAA + 10 µM 2iP	Liquid shake culture	Enhanced somatic embryogenesis	**LILIEN-KIPNIS *et al*., 1992**

MS; Murashige and Skoog (1962) nutrient medium. BA; 6-Benzyladenine. NAA; Naphthaleneacetic acid. 2iP; N6-(2-isopentenyl) adenine.

1.5.3. The genus *Scadoxus*

The genus *Scadoxus* belongs to the Amaryllid tribe; Haemantheae Pax (Hutchinson) along with *Haemanthus*, *Clivia*, *Gethyllis* and *Apodolirion* (**MEEROW and CLAYTON, 2004**). *Scadoxus* was formerly included in the genus *Haemanthus*. However due to variation within the genus based on the stems of the species; they were divided into two genera. *Scadoxus* now includes the species that exhibited an elongated stem while *Haemanthus* species exhibit broad stemless leaves (**POOLEY, 1998**). The South African genus consists of three species of *Scadoxus* including, *Scadoxus puniceus*, *Scadoxus multiflorus*, and *Scadoxus membranaceous*.

1.5.4. Distribution and morphology of *Scadoxus puniceus*

Scadoxus puniceus (L.) Friis & Nordal (Amaryllidaceae) (**Figure 1.1**) is a robust bulbous plant that grows up to 1 m in length (**POOLEY, 1998**). The name *Scadoxus puniceus* originates from the words “doxus” which means glory and “puniceus” which means crimson, scarlet or purple. The species is commonly referred to as the “blood lily”, “snake lily”, “royal paintbrush” or the “king-of-Candida” (**VAN WYK *et al*., 1997**). The species grows naturally in shaded areas in coastal bush, ravines and forests, distributed throughout the Free State, KwaZulu-Natal and the Eastern Cape, South Africa (**POOLEY, 1998**). During Spring and early Summer (August-October), the species exhibits a large inflorescence of 10-15 cm in diameter which consists of small scarlet flowers with bright yellow anthers (**BATTEN, 1986**). The leaves of *S. puniceus* usually appear after the flowers and are dark green, thin, wavy and grow up to 30 cm long. The flower stem which is separate from the base may reach up to 50-60 cm and consists of reddish/ purple speckles at the base (**BATTEN, 1986**). The fruit of *S. puniceus* are fleshy, round, shiny red berries of 1 cm in diameter and enclose a single soft pearl-like seed (**BATTEN, 1986**). The bulb of *S. puniceus* may be up to 10 cm in length and contains a thick stem at the base from which the fleshy roots grow.

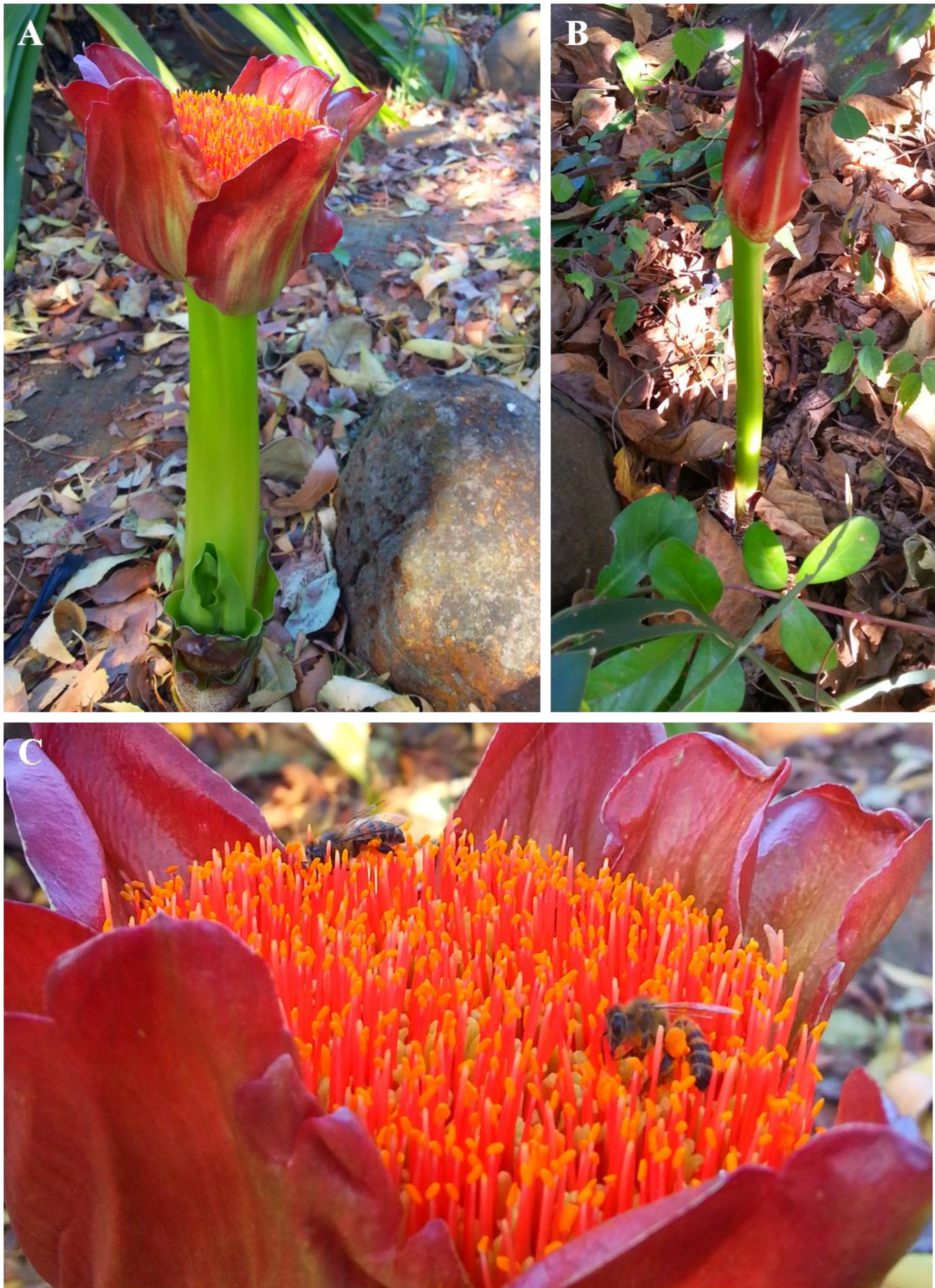


Figure 1.1: *Scadoxus puniceus* during the flowering season (Spring). A; Emerging leaves after the flower has developed. B; Formation of the inflorescence stalk. C; Scarlet flowers from which the common name ‘blood lily’ is derived.

1.5.5. Traditional uses of *Scadoxus puniceus*

Despite the fact that the bulb of *S. puniceus* is poisonous and ingestion has resulted in human death, decoctions of the bulb and the root are used to treat coughs, gastro-intestinal problems, febrile colds, asthma, leprosy, sprains and bruises; it has also been taken as an antidote to poisons and is used as a diuretic **(VEALE *et al.*, 1992; DOLD and COCKS, 1999; KOORBANALLY *et al.*, 2006)**. The leaves of the plant are used as an antiseptic on sores and ulcers. *Scadoxus puniceus* is also known to cause central nervous system (CNS) excitation or depression and visual disturbances **(VEALE *et al.*, 1992)**. *Scadoxus puniceus* is also one of the 21 identified medicinal plants used in the traditional herbal tonic *Imbiza ephuzwato* **(NDHLALA *et al.*, 2011)**. The tonic is used frequently by the Zulu community as a detoxifying and energising agent as well as to clear skin conditions, treat kidney and urinary infections, cure tonsillitis, treat pneumonia, and is used as a pain reliever amongst others **(NDHLALA *et al.*, 2011)**.

1.5.6. Pharmacological studies on *Scadoxus puniceus*

ADEWUSI and STEENKAMP (2011) reported on the strong acetylcholinesterase (AChE) inhibitory activity of *S. puniceus* bulb extracts with an IC_{50} value of 0.3 µg/ ml. **BAY-SMIDT *et al.* (2011)** upon phylogenetic analyses of 37 taxa of the Amaryllidaceae showed that *S. puniceus* exhibited the lowest IC_{50} value against AChE (18 µg/ ml) along with *Clivia miniata* and *Haemanthus sanguineus*. **SEOPOSENGWE *et al.* (2013)** determined the cytotoxicity of extracts of *S. puniceus* and rotenone, a pesticide which induces Parkinson's disease like symptoms in neurons and measured the intracellular redox state. The authors revealed that of all extracts tested, *S. puniceus* was the most cytotoxic and while rotenone reduced intracellular reactive oxygen species levels, pre-treating cells with *S. puniceus* extracts reversed the effects of rotenone, thus suggesting that the species may possess neuroprotective properties **(SEOPOSENGWE *et al.*, 2013)**.

1.5.7. Propagation of *Scadoxus puniceus*

Scadoxus puniceus may be propagated from seed however; this is a slow process taking up to five years before flowering. The species may be planted in well-drained soil with plenty of shade. The plant should be watered well during Spring and Summer and kept dry in Winter. The Amaryllidaceae are often difficult to propagate. Characteristically, the family exhibits long generation times, taking up to seven years to mature and flower **(KOOPOWITZ, 1986;**

DU PLESSIS and DUNCAN, 1989). In addition, much like many other Amaryllid species, the seeds of *S. puniceus* are recalcitrant and thus need to be sown fresh and cannot be stored. Several species are also vulnerable to diseases and pests. The Amaryllis lily borer for instance is a major pest of *S. puniceus* and cause damage to the entire plant, snails and slugs on the other hand damage the foliage extensively **(TURNER, 2001)**.

1.6. Aims and objectives

The aim of the current research was to establish an *in vitro* propagation protocol for the extensively traded medicinal plant species *Scadoxus puniceus*. The species use in traditional medicine also prompted pharmacological evaluations.

The specific objectives were to:

- Establish an *in vitro* propagation protocol for efficient regeneration and development of *S. puniceus*. In so, considering various factors including the type of explant, photoperiod, the use of plant growth regulators and several media supplements to optimize the protocol and alleviate physiological disorders.
- Investigate the antioxidant, antimicrobial and acetylcholinesterase inhibitory activities of this species.
- Explore the phytochemical properties of this species by determining the phenolic acid composition with the use of ultra performance liquid chromatography (UHPLC).
- Isolate the bioactive constituents of *S. puniceus* with the use of column chromatographic, nuclear magnetic resonance and mass spectroscopic techniques.
- Evaluate the ability of isolated compounds to inhibit microbial growth and inhibit the activity of acetylcholinesterase.

CHAPTER 2: MICROPROPAGATION OF *SCADOXUS PUNICEUS*

2.1. Introduction

Micropropagation, also referred to as tissue, *in vitro*, axenic or sterile culture is an important multipart developmental process that is controlled and regulated by complex interactions of both inherent and environmental stimuli **(MOYO *et al.*, 2011)**. Based on the concept of totipotency proposed by Gottlieb Haberlandt in 1902, the process encompasses the aseptic manipulation of cells, tissues and organs as well as their components in heterotrophic conditions on an artificial carbohydrate and nutrient-enriched basal medium **(THORPE, 2006; MOYO *et al.*, 2011)**. These conditions coupled with the micro-environmental stimuli, are considered some of the major factors which influence the growth of plantlets *in vitro* **(MOYO *et al.*, 2011)**.

The technique affords the biotechnological field several advantages which include the year-long production of plants independent of season, climate or dormancy of initial plant material, rapid propagation and long term germplasm storage which is achievable in a small space and short time period, and the rapid multiplication of new cultivars, species and recalcitrant species **(HUSSEY, 1982; ABOEL-NIL, 1997; KIM & DE HERTOGH, 1997; KOZAI *et al.*, 1997)**. The aseptic nature of the technique aids in producing large numbers of uniform and disease-free plants which makes it possible for across border germplasm or plant material exchanges **(ABOEL-NIL, 1997; KOZAI *et al.*, 1997; CHANG *et al.*, 2000)**. Micropropagation also plays an important role in the conservation of plant species where diminishing populations such as medicinal plants, can be saved from becoming extinct **(WALA and JASRAI, 2003; AFOLAYAN and ADEBOLA, 2004; AMOO *et al.*, 2012)**. The extended maintenance of *in vitro* material via cryopreservation also provides an effective system for the establishment of germplasm collections **(FAY, 1994)**. The technique also has implications in genetic engineering and plant breeding. Furthermore, the ability to control the chemical and environmental conditions in which plants are maintained is not only advantageous in promoting plant growth and development, but also in optimising secondary metabolite production **(ABOEL-NIL, 1997)**.

2.1.1. Requirements for successful tissue culture

The success of any tissue culture endeavour depends on several factors including the media components, environmental conditions and the choice of explant.

2.1.1.1. Tissue culture media components

Early attempts at plant tissue culture made use of nutrient formulations that consisted of a complex mixture of micro- and macro-elements (salts) which are absorbed by plant cells as cations (**GEORGE *et al.*, 2008**). The micro-elements added to plant tissue culture media consist of manganese, copper, boron, iron, molybdenum, zinc and iodine (**GEORGE, 1993**). The macro-elements include calcium, magnesium potassium, phosphorous, and sulphur (**GEORGE, 1993**). Several formulations exist however; the most preferred media were that of Knop and of Uspenski and Uspenkia (**WHITE, 1963; THORPE, 2006**). In 1962, **MURASHIGE and SKOOG** (MS) developed a new medium that contained 25 times the concentration of some salts present in Knops' formulation. The higher concentrations of NO_3^- and NH_4^+, and a greater range of micro-elements allowed for a drastic increase in the number of plant species that could be cultured *in vitro* (**MURASHIGE and SKOOG, 1962; THORPE, 2006**). Currently, the MS salt formulation is the most widely used nutrient medium in plant tissue culture.

In addition to the micro- and macro-nutrients, plant tissue culture media also requires a carbon source, vitamins and plant growth regulators (PGRs). Sucrose is the primary source of carbohydrates, while the most frequently used vitamins are thiamine, nicotinic acid and pyridoxine (**THORPE, 2006; GEORGE *et al.*, 2008**). Plant growth regulators including the cytokinins, auxins, abscisic acid, ethylene and gibberellins; are important determinants and regulators of cellular development and physiological processes in plants. Cytokinins and auxins are valuable components in plant tissue culture media. Cytokinins are involved in several processes *in vitro* including cell division, morphogenesis, delaying senescence and reproductive competence. They also promote the release of apical dominance, leaf expansion and the conversion of etioplasts into chloroplasts (**MAUSETH, 1991; RAVEN *et al.*, 1992; SALISBURY and ROSS, 1992; DAVIES, 1995a; GAN AND AMASINO, 1995**). Auxins on the other hand promote cell enlargement, bud formation and root initiation and development.

2.1.1.2. Environmental conditions

Environmental conditions such as temperature, illumination and carbon dioxide concentration are also important factors to consider for *in vitro* plant development. **ASCOUGH *et al.* (2008)** suggested that in addition to regulating plant growth rates, temperature is also an important signal for the transition between vegetative and reproductive phases of

development. Typically, explants for tissue culture are incubated at 25 ± 2 °C for the duration of the culture period. Light, more specifically its quality, intensity and duration influence the morphology of plants at different levels. Generally, the quality of light affects cell elongation, the development of axillary shoots and leaf anatomy. The intensity of light has implications for the size of leaves and stems while the effect of duration or photoperiod varies between species (**GEORGE, 1993**).

2.1.1.3. Choice of explant

The preferred choice of explant for tissue culture is dependent upon the plant species and the desired outcome. A variety of explants have been used for regeneration and growth of the Amaryllidaceae. **ZIV and LILIEN-KIPNIS (2000)** revealed the highly regenerative potential of scape explants of young, unemerged inflorescences of *Narcissus* bulbs. The authors showed that the junction between the pedicels and peduncle of *Nerine*, *Eucrosia* and *Haemanthus* inflorescences regenerated several bulbs. **SARATHE *et al.* (2014)** used leaf disks of *Amaryllis belladonna* in addition to PGRs for the induction of callus. The use of leaves and stems for tissue culture has also been documented **(HUSSEY, 1980)**. Despite this, the most common and most successful explant used in Amaryllid tissue culture is twin-scales. Twin-scales are two bulb scales which are taken from the basal region of the bulb and are joined together by a segment of the basal plate. Several species have been clonally propagated via twin-scaling; these include *Hippeastrum hybridum*, *Amaryllis belladonna*, *Crinum variabile*, *Crytanthus clavatus* and *Crytanthus spiralis* **(Table 1.1) (FENNELL *et al.*, 2001; MORAN *et al.*, 2003; SULTANA *et al.*, 2010; SARATHE *et al.*, 2014)**.

2.1.2. Micropropagation of *Scadoxus puniceus*

The Amaryllidaceae are known for their production of biologically active alkaloids. Amaryllids were amongst the first five plants studied *in vitro* for the production of alkaloids and given their success, several studies have followed this trend **(STABA, 1980; BASTIDA *et* al., 1992; FENNELL *et al*,. 2003; CHEESMAN *et al.*, 2012)**. Similarly, the pharmacological activities of *Scadoxus puniceus* has been examined and given its potential, an investigation of its alkaloid content was undertaken. However, no reports on the *in vitro* propagation or *in vitro* production of alkaloids have been documented. The ultimate aim of the current study was to consider the major factors (media components, environmental conditions and explant choice) necessary for *in vitro* propagation and establish a successful *in vitro* propagation protocol for *S. puniceus*.

2.2. Materials and Methods

Young *S. puniceus* plants were collected between April and September 2013, from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg. A voucher specimen (Naidoo 02) was deposited at the Bews Herbarium, UKZN Pietermaritzburg. Unless otherwise stated, the medium used for *in vitro* culture consisted of full strength MS basal nutrient medium (**MURASHIGE and SKOOG, 1962**; See Appendix 1) supplemented with 3% sucrose and 0.1 g/l *myo*-inositol (Sigma-Aldrich). The pH of the medium was adjusted to 5.8 using 1.0 N potassium hydroxide (KOH) or 1.0 N hydrochloric acid (HCl) and solidified with 8 g/l agar (Agar bacteriological-Agar No. 1, Oxoid Ltd., England). The medium was sterilised by autoclaving at 121 °C and 103 kPa for 20 min and cultures were grown at 25 ± 2 °C under a 16/8h light/dark photoperiod with a PPFD of 30 µmol m^{-2} s^{-1}.

2.2.1. Source of Plant growth regulators and media supplements

Benzyladenine (BA), naphthaleneacetic acid (NAA), picloram (PIC) and 2-isopentenyladenine (2-iP) were acquired from Sigma-Aldrich (Steinheim, Germany). 2,4-Dichlorophenoxy acetic acid, activated charcoal (AC), polyvinylpyrrolidone (PVP) and ascorbic acid (AA) were acquired from BDH Biochemicals Ltd. (Poole, England). *meta*-Topolin and *meta*-topolin riboside were prepared as described by **DOLEŽAL *et al.*, (2006)**. Citric acid (CA) was purchased from MERCK (Darmstadt, Germany).

2.2.2. Micropropagation from vegetative explants

2.2.2.1. Bulb scale cultures

Bulbs (5-10 cm) of *S. puniceus* were collected in March, 2013 from the UKZN Botanical Garden. The leaves, roots and three quarters of the lower basal plate were removed along with the outer scales of the bulb. The bulbs were washed with tap water for 5 min prior to decontamination. The bulbs were then divided into two halves and soaked in 70% ethanol for 60 sec followed by 1% benomyl (Benlate, Sigma-Aldrich, Steinheim, Germany) for 15 min and 0.3% mercuric chloride ($HgCl_2$) for 15 min. Tween 20® (BDH Biochemicals Ltd. Poole, England) was used as a surfactant. The bulb halves were rinsed three times with sterile distilled water and cut into explants. Explants (1 cm x 1 cm) from bulbs consisted of (1) a single scale, (2) a single scale attached to a segment of the basal plate and (3) twin-scales

(two adjacent scales connected by a segment of the basal plate). Explants were inoculated on 20 ml solid MS media without PGRs for three months.

2.2.2.2. Leaf cultures

Young leaves of *S. puniceus* were collected in Summer, Autumn, and Spring 2013 from the UKZN Botanical Garden. The leaves were washed with tap water for 5 min prior to decontamination. The leaves were then soaked in 70% ethanol for 60 sec followed by 0.2% Benlate for 15 min and 0.1% $HgCl_2$ for 10 min. Tween 20® was used as a surfactant. Leaves were rinsed three times with sterile distilled water containing 100 mg l^{-1} ascorbic acid (ASC). The leaves were cut into 1 x 1 cm explants and inoculated on 20 ml solid MS media containing various types (BA, *m*T, *m*TR, 2iP) and concentrations (0.0, 2.5, 5.0, 10.0, 15.0 μM) of PGRs and maintained at 25 ± 2 °C under a 16/ 8 h light/ dark photoperiod for three months. For the induction of callus from leaf material, leaf explants were also inoculated on MS medium supplemented with PIC or 2,4-D at various concentrations (0.0, 5.0, 10.0, 15.0 and 20.0 μM).

2.2.3. Propagation from seeds

2.2.3.1. Seed collection and germination

Mature fruits of *S. puniceus* were harvested during November and December 2013 from the UKZN Botanical Garden. The seeds were dehisced from the fruit and were pre-treated with 70% ethanol for 60 sec and surface decontaminated with 1.5% sodium hypochlorite for 5 min. Tween 20® was used as a surfactant. The seeds were then rinsed three times with sterile distilled water and inoculated on $1/10^{th}$ strength MS medium for two months.

2.2.3.2. Embryo cultures

In an attempt to produce embryogenic material, seeds of *S. puniceus* were prepared as per **Section 2.2.3.1**, however, after decontamination they were cut in two, exposing the immature embryo. The embryos were inoculated on MS medium containing PIC or 2,4-D at various concentrations (0.0, 5.0, 10.0, 15.0 and 20.0 μM).

2.2.3.3. Micropropagation from seedling sections

Three-month-old *in vitro* grown seedlings were divided into segments **(Figure 2.1)** and used as explants as a source of meristematic tissue. Sections were cultured on MS medium

supplemented with 6% sucrose, 0.1% *myo*-inositol and various concentrations of PGRs (BA and 2,4-D) singly or in combinations (**Table 2.1**).

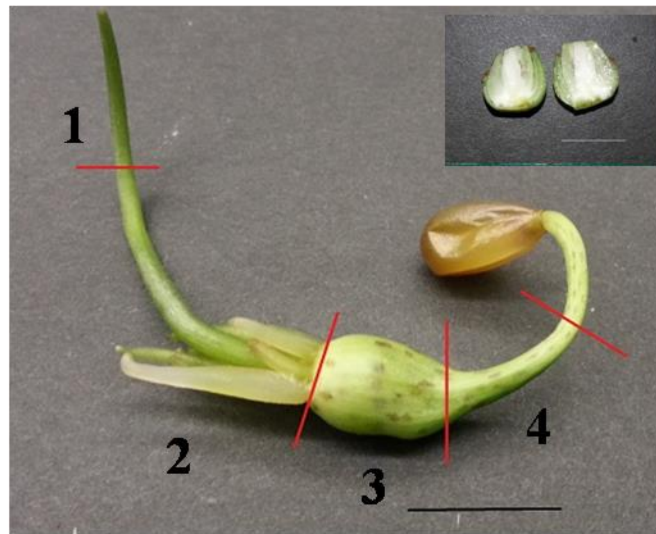


Figure 2.1: Three-month-old seedling of *Scadoxus puniceus*. (1) Root segment, (2) root segment attached to the basal plate, (3, inset) bulb halves, (4) stem segment. Bar = 1 cm.

Table 2.1: Plant growth regulator (PGR) combinations of benzyladenine and 2,4-dichlorophenoxy acetic acid that supplemented Murashige and Skoog medium for regeneration from seedling sections.

PGR Combinations	
Benzyladenine (µM)	**2,4-Dichlorophenoxy acetic acid (µM)**
0	0
0	5
0	10
0	15
5	5
5	10
5	15
10	5
10	10
10	15

2.2.4. The effect of photoperiod on *in vitro* shoot multiplication

Due to the lengthy periods of time required for bulb scale and leaf culture, photoperiod experiments were carried out on *in vitro* grown material. Leaves of *S. puniceus* were collected during Spring 2013 and surface decontaminated as in **Section 2.2.2.2**. Leaves were cut into 1 x 1 cm explants and inoculated on MS media supplemented with equi-molar concentrations (5 µM) of BA and NAA. Cultures were maintained at 25 ± 2 °C under a 16/ 8 h light/dark photoperiod. After five months of culture, the *in vitro* grown shoots, bulbs and leaves were harvested. The shoots and leaves were divided into 1 x 1 cm explants while the bulbs were divided into two halves and sub-cultured on MS media containing BA and NAA at 5 µM. The cultures were then maintained at 25 ± 2 °C under different light regimes (24 h light, 16/8 h light/dark and 24 h dark) for three months. After three months, explants were evaluated for symptoms of browning using a hedonic scale ranging from 0 to 10; of which 0 being no observable signs of browning and 10 being dead, dark brown tissue. Plant growth parameters including number of bulblets, shoot length, and fresh weight were also recorded.

2.2.5. The effect of adsorbents and antioxidants on oxidative browning

Bulbs of *S. puniceus* were collected during Spring 2013 and pre-treated as in **Section 2.2.2.1**. Twin-scales were excised from bulbs and placed on MS media supplemented with different types of adsorbents (activated charcoal and polyvinylpyrrolidone) and antioxidants (ascorbic acid and citric acid) at various concentrations (0, 100, 200, 500 and 1000 mg/l) in an attempt to reduce the oxidative browning of explants *in* vitro. Cultures were maintained at 25 ± 2 °C under a 16/ 8 h light/ dark photoperiod for 6 months after which , growth parameters including the percentage bulblet induction, number of bulblets, and the rate of browning was recorded.

2.2.6. Data analysis

Experiments were conducted in a completely randomized design. Data pertaining to the culture of bulb scales was subjected to a One-Way-Analysis of Variance (ANOVA), where there were differences at $p = 0.05$, the means were separated using Duncan's Multiple Range Tests (DMRTs). A student's *t*-test was used to compare the regeneration from stem segment and bulb halves ($p = 0.05$). A chi-square analysis was performed to determine the induction

rate of leaf and bulb explants maintained at different photoperiods. Growth parameters were compared using a One-Way-ANOVA, and the means were separated using DMRTs. A linear regression analysis was performed to determine the relationship between explant browning and light intensity. In the case of the effect of adsorbents where the assumption of normality was not met, a non-parametric test was performed. All statistical analysis was conducted on IBM SPSS software, version 21 for Windows.

2.3. Results and Discussion

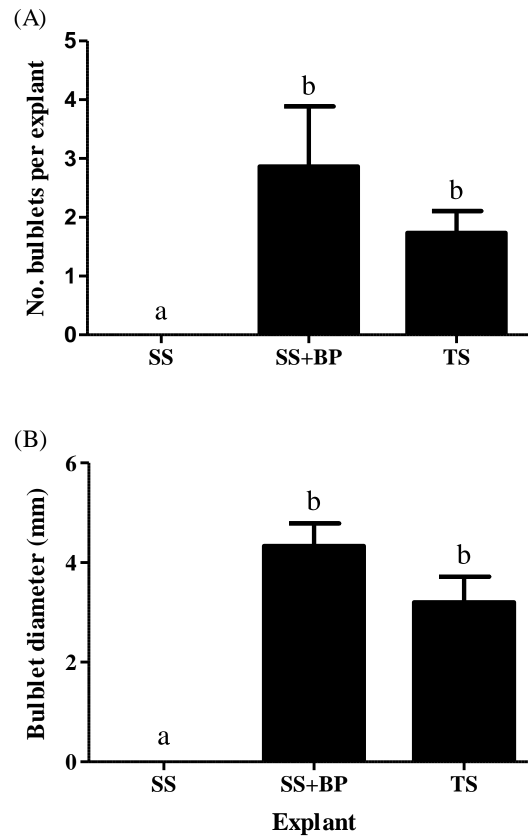
2.3.1. Micropropagation from vegetative explants

2.3.1.1. Bulb scale cultures

The current study investigated the potential of three types of bulb scales for *in vitro* propagation of *S. puniceus*. Single scale explants turned brown or black within four weeks of culture and failed to regenerate or induce a growth response **(Figure 2.2)**. These data are in agreement with previous studies in that the regeneration of several Amaryllids was unsuccessful when a single bulb scale explant was used. Limited growth was observed when bulb scales of *Crinum* spp. were cultured in the presence of coconut milk and 6 mg/l 2,4–D **(MULLIN, 1970)**. **FENNELL (2002)** reported the failure of *Crinum moorei* bulb scales to regenerate when cultured in the absence or presence of various concentrations of cytokinins and auxins. Several authors suggest that meristematic activity of Amaryllids is restricted to the basal plate thus endorsing the lack of success from bulb scales **(PIERIK and IPPEL, 1977; CHOW *et al.*, 1993; FENNELL, 2002)**. It has been suggested that without the attachment to a portion of the basal plate, bulb scales will not regenerate *in vitro* **(FENNELL and VAN STADEN, 2004)**. A comprehensive examination of the literature by **RICE (2009)** demonstrated the popularity of twin-scales in Amaryllidaceae tissue culture thus suggesting its importance. The current data revealed no significant difference in regeneration between single scales attached to a segment of the basal plate and twin scale explants. Single scale explants attached to the basal plate developed bulbs at the base of the scale above the basal plate while twin-scale explants developed bulbs on the basal plate in between the two scales. Although not significant, on average, single scale explants attached to a segment of the basal plate produced more and larger bulbs than twin-scale explants **(Figure 2.2)**.

However, despite the successful regeneration of bulblets from bulb scales explants, regenerated plantlets exhibited an extremely slow growth rate taking up to a year to reach a

height of 30-40 mm. Furthermore, the majority (52 and 40%, respectively) of single scale explants attached to a segment of the basal plate and twin-scale explants succumbed to oxidative browning drastically reducing the regeneration potential. The exudation and oxidation of phenols releases toxic quinines and polymerized material into tissue culture media, plant tissue then turns black or brown and this inhibits any further growth **(OZYIGIT *et al.*, 2007)**. Oxidative browning depends on several factors including the plant genotype, the source of explant and the period in which it was acquired **(SATHYANARAYANA and MATHEWS, 2007)**. Severe oxidative browning occurs in species which contain high concentrations of tannins and hydroxyphenols. Several species of the Amaryllidaceae have proven difficult to culture as a result of oxidative browning. **RICE (2009)** suggested that the release of phenolic substances into the growth medium inhibited the growth of callus cultures of *Brunsvigia undulata*. **CHEESMAN (2013)** reported on the reduced growth of *Boophone disticha in vitro* as a result of oxidative browning of twin-scale cultures. Similarly, the exudation of phenolics into the growth medium by bulb scales of *S. puniceus* drastically reduced explant regeneration. Explants exude several different types of phenolic compounds into the growth medium that may affect *in vitro* plant growth differently, or not at all **(SATHYANARAYANA and MATHEWS, 2007)**. Explants of *S. puniceus* turned red early in culture and advanced to brown after two to three months. Exuded substances resulted in the discolouration of the culture medium which turned yellow, brown or red **(Figure 2.4)**. Several strategies have been employed with varying success to combat oxidative browning. These will be discussed in detail later.



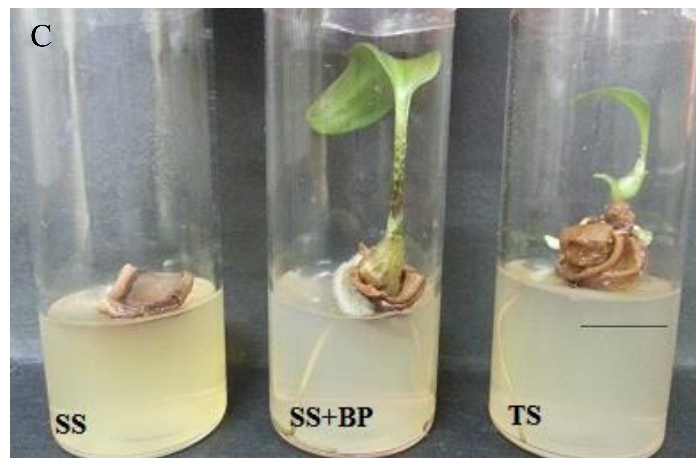


Figure 2.2: *In vitro* culture response of single-scale (SS), single-scales attached to a segment of the basal plate (SS+BP) and twin-scale explant (TS). Different letters indicate significant differences at $p = 0.05$, n = 20.

2.3.1.2. Leaf cultures

Generally, leaf material is considered a more suitable explant than bulb scales because the use of bulbs in tissue culture requires destructive harvesting of plant material **(DREWES and VAN STADEN, 1994)**. However, ideal leaf explants should be young material and given the

seasonal availability of leaves, this is not always possible thus limiting successful propagation from leaf explants. The current data revealed that the addition of cytokinins to the medium at various concentrations failed to promote a growth response from leaf explants harvested throughout the year. The inability of leaf explants to regenerate may be the result of a lack of meristematic activity within the tissue. The totipotency of leaf explants from three Amaryllid species was evaluated by **HUSSEY (1975)**. Leaf explants of *Hippeastrum hybridum*; *Narcissus pseudonarcissus*; *Narcissus* hybrids and *Ipheion uniflorum* failed to regenerate on MS medium supplemented with sucrose, *myo*-inositol and thiamine under 24 h dark conditions. Similarly, **CHEESMAN (2013)** reported the lack of regeneration from leaf explants of *Boophone disticha* supplemented with various types and concentrations of PGRs. Basal regions of leaves produced shoots although only with the addition of 2.22 µM BA. Upper regions produced non-regenerable callus or failed to respond at all the applied PGR combinations. Leaf explants of *Crinum moorei*, although harvested during the growing season, failed to regenerate in MS media supplemented with various combinations of BA and NAA **(FENNELL, 2002)**. Given the lack of success with leaf cultures in general, the inability of *S. puniceus* leaf explants to regenerate in the presence of cytokinins is thus no surprise.

However, further experimentation revealed the importance of auxins for the *in vitro* propagation of *S. puniceus*. Leaf explants harvested during Spring 2013 were cultured on MS medium supplemented with BA and NAA at equi-molar concentrations (5 µM). This combination proved to be successful in initiating shoot development producing, on average, 6 shoots per explant **(Figure 2.3)**. Auxin and cytokinin combinations are frequently used in plant tissue culture to initiate shoot and root production **(SKOOG and MILLER, 1957; BAJAJ, *et al*., 1998; ROUT and DAS, 1997)**. Auxin and cytokinin combinations control several developmental processes in plants including cell growth and division, differentiation as well as organogenesis **(GASPAR *et al*., 1996)**. It has thus been suggested that an appropriate balance between auxins and cytokinins will initiate shoot development *in vitro*. Thus the addition of the auxin (NAA) to the culture media may have promoted the development of shoots on explants which are otherwise dormant.

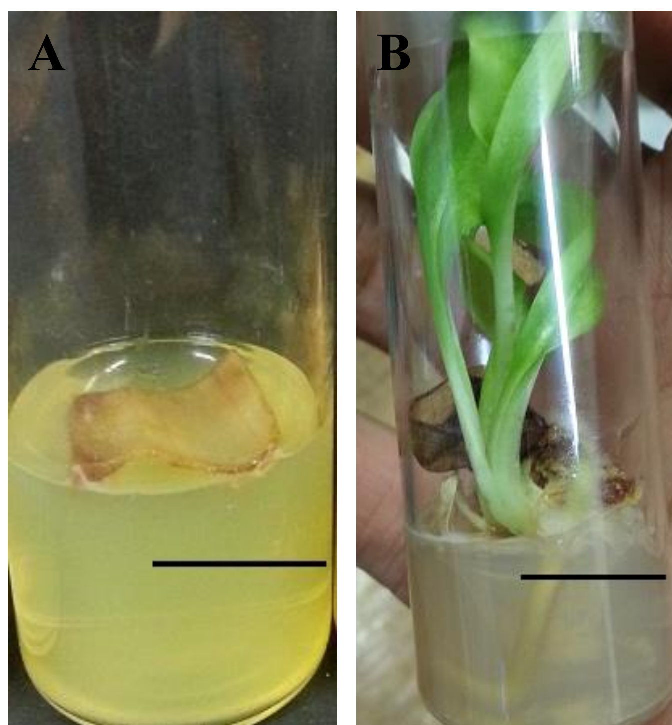


Figure 2.3: The fate of leaf explants of *Scadoxus puniceus* supplemented with various cytokinins (A) and benzyladenine in combination with naphthaleneacetic acid (B). Bar = 1 cm.

2.3.2. Micropropagation from seeds

2.3.2.1. Embryo cultures

The culture of *S. puniceus* embryos for the production of embryogenic material was not successful. Instead, embryos developed into seedlings, producing roots and non-regenerable callus and showed signs of hyperhydricity **(Figure 2.4)**. No clear morphological differences were observed between the type and range of auxins tested. The current study made use of immature embryos which were excised from fresh seeds prior to germination; the lack of callogenesis on these explants is an example of the typical recalcitrant nature of Amaryllidaceae embryos **(SELLÈS *et al.*, 1999)**.

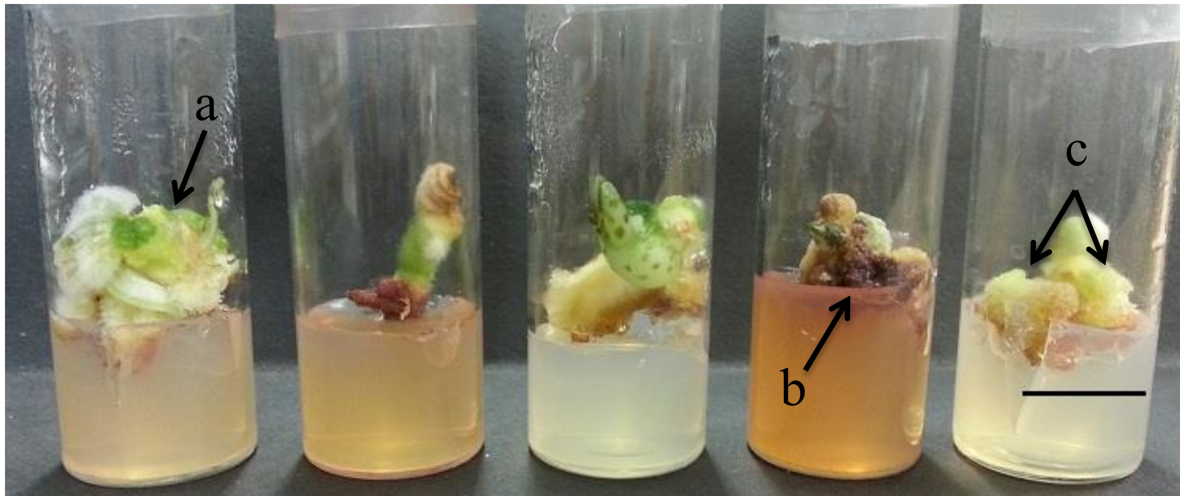


Figure 2.4: The development of immature embryos of *S. puniceus* on callus induction medium. (a) Hyperhydric seedling, (b) phenolic exudation, (c) non-regenerable callus. Bar = 1 cm.

2.3.2.2. Culture of seedling sections

As an alternate source of explants, the regeneration of *in vitro* grown seedling sections was evaluated. Seedlings were divided into four segments including roots, roots attached to a segment of the basal plate, bulb halves and stem sections **(Figure 2.1)**. The interaction between seedling sections and certain PGRs significantly influenced the regeneration rate and thus the meristematic potential of explants **(Figure 2.5)**. Root segments failed to respond to the range of PGR combinations tested. Similarly, root segments of *Brunsvigia undulata* and *Boophone disticha* were unsuccessful in forming meristematic tissue **(RICE, 2009; CHEESMAN, 2013)**. Despite this, root segments attached to a portion of the basal plate did regenerate although producing very few (0.50 - 0.70 ± 2.60) bulblets in the absence of PGRs or with high concentrations of BA. The inclusion of a portion of the basal plate may have promoted the development of bulblets. The above results are in agreement with the reports of confinement of meristematic tissue to the basal region of the bulb. However, the highest number of bulblets (3.30 ± 2.98) formed on stem segments cultured on 15 µM 2,4-D. Apart from the poor bulblet production (0.30 ± 0.15) on bulb half explants, stem segments were the only plant parts to respond to a PGR combination of 0 µM BA and 15 µM 2,4-D. These results reveal the increased meristematic activity of stem explants at higher concentrations of 2,4-D in the absence of BA. **FENNELL (2002)** suggested that explants that possess meristematic tissue are often the most successful explants *in vitro*. However, **BOONEKAMP (1997)** revealed that not all explants form meristematic tissue at the same frequency. Stem

segments may have been able to metabolize 2,4-D at a faster rate and thus produce meristematic tissue faster and more efficiently than bulb halves. All explants (except roots) regenerated on MS medium supplemented with 10 µM BA and 5 or 10 µM 2,4-D, with bulb halves producing the highest number of bulblets (2.30 ± 0.51). While higher concentrations of the auxin, 2,4-D seem to be conducive to bulblet formation on stem segments, improved bulblet formation on bulb halves was restricted to higher concentrations of BA.

Embryogenic callus was induced with equi-molar concentrations (10 µM) of BA and 2,4-D on all explants except roots (data not shown). Callus and shoot clusters were transferred to MS medium containing 6% sucrose without PGRs for bulblet formation and further development and rooting, respectively. The initial explant (stem segment or bulb half) that the callus formed on significantly influenced bulblet production ($t = 3.127$, $df = 38$, $p = 0.003$). The highest number of bulblets (4.60 ± 0.51) were formed on callus from stem segments while callus derived from bulb halves produced an average of 2.25 ± 0.55.

The majority of Amaryllid *in vitro* protocols for bulblet formation make use of bulb halves or twin-scales. **VISHNEVETSKY *et al.* (2003)** used *in vitro* derived bulb halves of *Nerine sarniensis* to induce bud formation. The authors reported on a prolific seven to nine bulblets that formed on bulb halves supplemented with BA and NAA. However, the bulb halves were incubated in darkness which promoted improved regeneration rates. Similar results were recorded for *Amaryllis belladonna* and *Hippeastrum* hybrids where bulb halves regenerated on semi-solid MS medium supplemented with low hormonal concentrations **(DE BRUYN *et al.*, 1992; PIERIK *et al.*, 1990)**. Of the factors responsible for the reduced regeneration of bulb halves in the current study, two (PGRs and photoperiod) seem more likely. Bulb halves were supplemented with relatively high concentrations of PGRs in relation to previous studies. However, the lack of regeneration reported at lower concentrations of PGRs suggests the inadequate concentrations of endogenous PGRs to allow for the formation of bulblets. Bulb halves were also exposed to a 16/8 h light/ dark photoperiod, which may have stressed the explants resulting in the exudation of toxic phenolic compounds into the medium. Despite this, the results of the current study reveal the ability of *in vitro* derived stem sections of *S. puniceus* to form meristematic tissue which was not seen with *ex vitro* material.

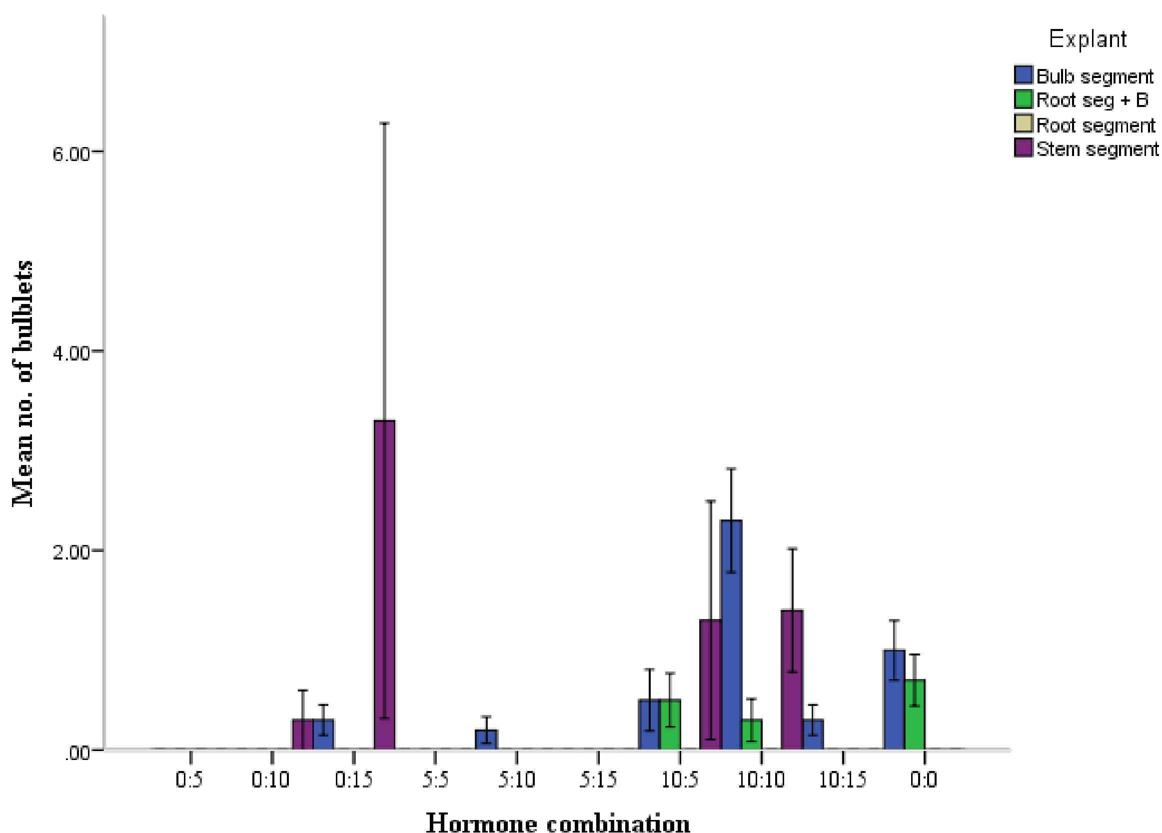


Figure 2.5: Proliferation of *Scadoxus puniceus* explants cultured on different combination of benzyladenine and 2,4-dichlorophenoxy acetic acid (µM).

2.3.3. The effect of photoperiod on *in vitro* bulblet multiplication

The induction rate of leaf and bulb explants did not differ between the three light regimes tested (χ^2 = 3.360; p = 0.186). However, photoperiod significantly affected the number of bulblets that formed on leaf explants and the shoot length and fresh weight of regenerated bulb halves **(Figure 2.6; A and 2.6; B, C**, **D**, respectively). The highest number of bulblets (8.8 ± 1.1 bulblets per explant) was formed on leaf explants that were maintained under a 24 h dark photoperiod. This light regime produced on average three to four times as many bulblets as the 24 h light and 16/8 h light/dark regimes. Instead of bulblets, a single shoot developed from the centre meristematic region of the bulb half maintaining apical dominance **(Figure 2.7 B2**). Apical dominance is broken by wounding the basal plate just below the bulb scale, which was not done in the current study. **CHOW *et al.* (1992)** revealed that meristematic zones were larger in shoot clump cultures where apical dominance was broken. Significantly longer shoots (104 ± 17 mm) and increased fresh weight (1.81 ± 1.13 g) was produced by bulb halves maintained under 24 h dark conditions (F = 6.079, p = 0.007, **Figure**

2.6). Furthermore, bulb halves maintained in darkness produced callus from which more shoots developed **(Figure 2.7 B3)**.

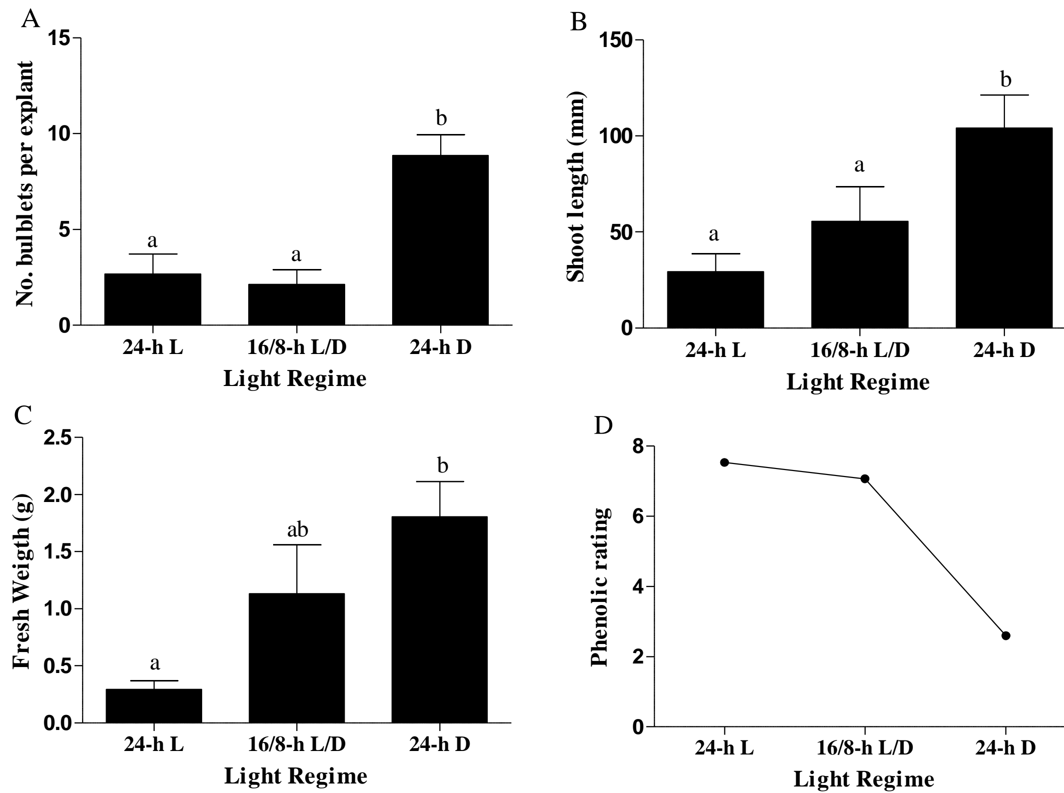


Figure 2.6: The influence of different light regimes (24 h light, 16/8 h light/dark, 24 h dark) on *in vitro* regeneration of *Scadoxus puniceus*. (A) The number of bulblets produced on leaf explants (mean ± SE). (B & C) The shoot length and fresh weight (mean ± SE) of regenerated bulb halves, respectively. (D) Phenolic ratings based on a hedonic scale of bulb halves.

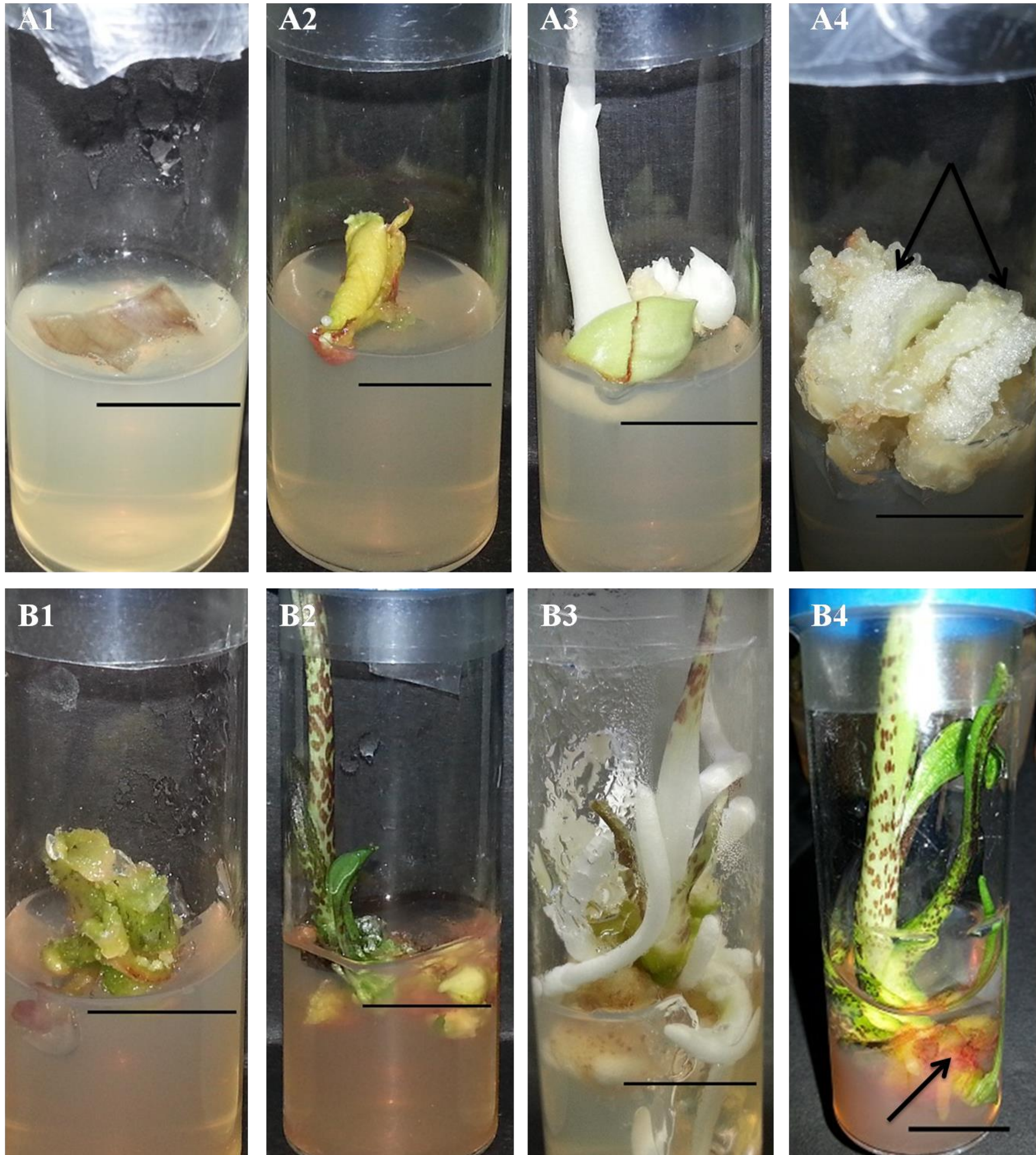


Figure 2.7: The influence of photoperiod (1, 24 h light; 2, 16/8 h light/dark; 3, 24 h dark) on regeneration of *Scadoxus puniceus* leaf (A) and bulb-half (B) explants. (A4) Crystalline callus (arrow) formed on leaf explants in dark conditions. (B4) Phenolic exudation (arrow) by bulb halves into the medium. Bars = 10 mm.

Darkness promoted the formation of bulblets in many Amaryllidaceae species including *Eucrosia stricklandii*, *Narcissus* spp., *Lilium* spp. and *Nerine sarniensis* with the use of twin scale explants (**STEINITZ and YAHEL, 1982; BERGOÑÓN *et al*., 1992; COLQUE *et al*., 2002; VISHNEVETSKY *et al*., 2003**). The success of these species *in vitro* was

attributed to the simulation of dark conditions much like those that bulbs grow in, *in vivo*. However, this does not explain the increased formation of bulblets on leaf material given the previous reports of their inability to regenerate as a result of the lack of meristematic tissue. The production of bulblets on leaf explants and the success of bulb explants in the dark over the other light regimes may be attributed to the inhibition of oxidative browning of explants in dark conditions. A reduction in oxidative browning was closely correlated to an increase in bulblet production **(Figure 2.7 & 2.8)**. Illumination at different photoperiods significantly influenced the number of bulblets that formed on leaf explants (t = -7.876; p = 0.005, R^2 = 0.591, **Figure 2.8**) suggesting that an increase in illumination intensifies phenolic exudation and reduces development *in vitro*. Similarly, **BIRMETA and WELANDER (2004)** overcame the exudation of phenolics in *Ensete ventricosum* by incubating explants in a continuous dark photoperiod.

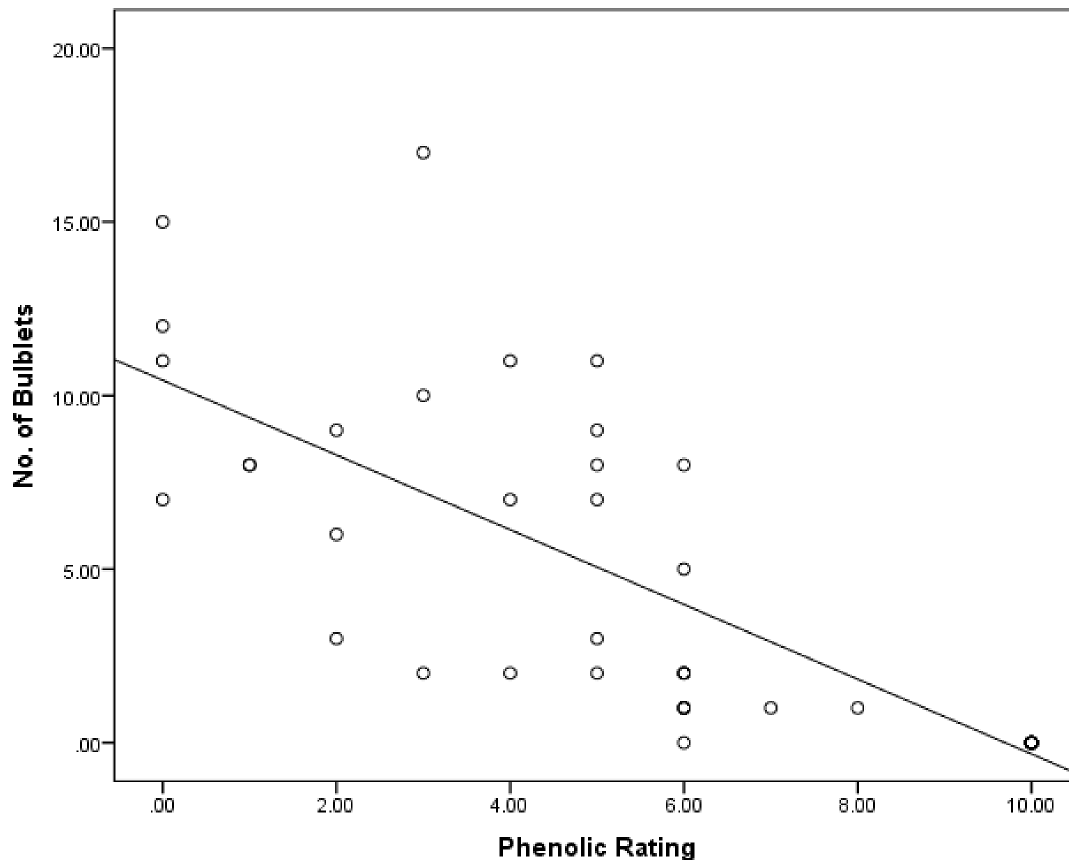


Figure 2.8: The influence of phenolic exudates on bulblet production on leaf explants of *Scadoxus puniceus* based on a hedonic scale.

2.3.5. The effect of adsorbents and antioxidants on oxidative browning

Table 2.2: The influence of antioxidants (citric acid and ascorbic acid) and adsorbents (polyvinylpyrrolidone and charcoal) on bulblet induction and oxidative browning of *Scadoxus puniceus* twin-scale explants.

Adsorbent/ Antioxidant	Concentration mg/l	% Bulblet induction	No. Bulblets/ twin scale	No. Bulblets > 10 mm	Browning rating
MS only	-	52	0.84 ± 0.22	0	8.24 ± 0.46
PVP	100	48	0.80 ± 0.20	1	6.72 ± 0.47
	200	52	0.76 ± 0.18	1	7.96 ± 0.43
	500	44	0.92 ± 0.25	0	6.68 ± 0.59
	1000	32	0.56 ± 0.25	0	6.80 ± 0.46
Activated Charcoal	100	48	0.48 ± 0.10	3	6.80 ± 0.41
	200	68	0.80 ± 0.14	3	6.96 ± 0.58
	500	32	0.44 ± 0.14	0	4.00 ± 8.44
	1000	60	0.76 ± 0.14	1	5.56 ± 0.73
Citric acid	100	48	0.60 ± 0.14	0	8.20 ± 0.46
	200	56	0.80 ± 0.16	0	7.64 ± 0.55
	500	28	0.40 ± 0.14	0	8.60 ± 0.38
	1000	20	0.48 ± 0.28	3	8.84 ± 0.48
Ascorbic acid	100	24	0.24 ± 0.08	0	7.56 ± 0.70
	200	24	0.40 ± 0.15	0	7.00 ± 0.82
	500	24	0.40 ± 0.20	0	8.48 ± 0.49
	1000	8	0.20 ± 0.16	0	9.00 ± 0.55

MS; Murashige and Skoog (1962) nutrient medium. PVP; Polyvinylpyrrolidone.

The incorporation of antioxidants and adsorbents to the nutrient medium was investigated in an attempt to overcome the exudation of phenolic compounds by twin-scale explants of *S. puniceus*, as mentioned previously in this chapter. The addition of activated charcoal (AC), polyvinylpyrrolidone (PVP), citric acid (CA) and ascorbic acid (ASC) significantly affected bulblet induction and the number of bulblets that formed on twin-scales (χ^2 = 41.195, p = 0.001, **Table 2.2**). However, no significant improvements beyond that of the control (MS-only) were recorded. Activated charcoal has been used to improve the initiation and growth of several Amaryllid species *in vitro* **(Table 1.1)**; likewise, the inclusion of AC (200 mg/l)

promoted the highest induction rate of explants (68%) as compared to the control (52%). Tissue culture media are frequently supplemented with AC for the reason that they absorb inhibitory substances such as phenolic exudates while also providing an environment much like bulbs would grow in *in vivo*. However, multiple genotypes of a species make it difficult to predict the possible influence of AC, either positively or negatively and thus despite the substantial increase in bulblet induction, AC failed to promote any further improvements beyond that of the control.

The highest number of bulbs per explant (0.92 ± 0.25) was induced on media supplemented with PVP (500 mg/l). Similar to AC, PVP is an adsorbent that binds to phenolic compounds essentially reducing their toxicity. The current study however, revealed a decrease in bulblet production with an increase in PVP concentration beyond 500 mg/l. **WEATHERHEAD *et al.* (1979)** suggested that the decrease in induction with the addition of adsorbents could be due to the absorption of essential media components other than the inhibitory compounds. This may be evidenced by the phenolic ratings based on a hedonic scale recorded in the current study. Activated charcoal and PVP at higher concentrations improved the visual browning scores of explants (5.56 ± 0.73; 6.80 ± 0.465 respectively), this improvement was however not translated into increased bulblet regeneration but rather a reduction was noted.

The antioxidants CA and ASC were unsuccessful in reducing visual browning scores of the explants and reduced both the induction and number of bulblets per explant **(Table 2.2)**. These results contradict previous studies in which CA and ASC have been found to improve productivity of explants by preventing oxidative browning. **KOMALAVALLI and RAO (2000)** found that the incorporation of CA (100 mg/l) significantly improved shoot production and bud regeneration in *Gymnema sylvestre* while adsorbents reduced shoot development. Ascorbic acid was found to promote multiple shoot induction in *Tylophora* spp. and *Gymnema elegans* **(NEELAM and CHANDEL, 1992; KOMALAVALLI and RAO, 2000)**. These contrasting results further emphasise the atypical influence of antioxidants and adsorbents in plant tissue culture media for improving plant development. Furthermore, it is worth mentioning that the addition of antioxidants and adsorbents to the medium failed to improve the response rate and productivity of twin-scale explants used in previous experiments **(Section 2.3.1.1)**. Rather a drastic decrease was observed.

2.4. Conclusions

The current study reports on the first attempt at the *in vitro* propagation of *Scadoxus puniceus*. The propagation of *S. puniceus in vitro* proved to be rather difficult considering the slow growth rate of twin-scale explants and the inability of leaf explants and embryos to regenerate which was exaggerated by the exudation of phenolics. Although improved propagation of several Amaryllid species was achieved by incorporating antioxidants and adsorbents into the media, the current study demonstrated the recalcitrance of *S. puniceus* to respond successfully to these treatments.

Nevertheless, a significantly improved regeneration rate was induced when *in vitro* grown leaf explants were incubated under a 24 h dark photoperiod. Exudation of phenolics may be remedied by incubating explants in the dark or with the use of liquid culture systems. However, as highlighted by the varied success of the current protocol, there exists a need to establish a method for reducing the exudation of phenolic compounds into tissue culture media in order to develop more efficient protocols.

CHAPTER 3: ALLEVIATION OF THE PHYSIOLOGICAL DISORDERS IN MICROPROPAGATED *SCADOXUS PUNICEUS*

3.1. Introduction

The optimisation of plant tissue culture protocols is essential for producing a significant number of healthy plants. The initial attempt at the *in vitro* propagation of *Scadoxus puniceus*, although successful, was riddled with several shortcomings. Identified drawbacks include hyperhydricity, oxidative browning and the recalcitrance of initial explants. The current **Chapter** focuses on the alleviation of the aforementioned factors, thus promoting improved regeneration and development of *S. puniceus.*

3.1.1. Hyperhydricity

Hyperhydricity in micropropagated plants occurs in response to stress factors associated with the *in vitro* growth and culture conditions and often results in reduced lignin and oxidative stress (**ROGERS and CAMPBELL, 2004**). Hyperhydricity may be caused by a passive diffusion of water into the tissue or by metabolic stressors (**PÂQUES, 1991**). *In vitro* stress conditions that may result in hyperhydricity include high humidity, high concentrations of plant growth regulators (PGRs), gas accumulation and high light intensity (**KEVERS *et al*., 2004; SAHER *et al*., 2004; TEIXEIRA da SILVA *et al*., 2013**). Several methods have been employed to reduce hyperhydricity in plant tissue culture. These include the manipulation of PGR concentrations, the pH, the gelling agent or gaseous enrichment (CO_2) (**ASCENCIO-CABRAL *et al*., 2008; WU *et al*., 2011**). External factors such as light and temperature conditions and the choice of culture vessel are also important factors to consider when attempting to prevent hyperhydricity (**TEIXEIRA da SILVA *et al*., 2005; ASCENCIO-CABRAL *et al*., 2008**). Topolins in plant tissue culture have also been successful in alleviating several physiological disorders including hyperhydricity (**AREMU *et al*., 2012**). The ability of the topolins to reduce hyperhydricity and improve proliferation and development was explored with *S. puniceus*.

3.1.2. Oxidative browning

Oxidative browning is an unwanted, yet common challenge associated with micropropagation (**KRISHNA *et al*., 2008; UCHENDU *et al*., 2011**). The phenomenon results in reduced growth and regeneration and can ultimately lead to cell/ tissue or plant death (**TOTH *et al*.,**

1994; LAUKKANEN *et al*., 2000; ALIYU, 2005; TABIYEH *et al*., 2006; KRISHNA *et al*., 2008). Browning is caused by the oxidation of phenolic compounds (that accumulate in the culture medium) which are produced and released in greater abundance in response to biotic or abiotic stress such as pests, pathogens and wounding **(LAWSON *et al*., 1996, BECKMAN, 2000)**.

Tissue culture protocols primarily rely on wounding plant material to obtain an explant from the parent material and then to elicit a growth response. In addition, explants are cultured in stressful conditions including high concentrations of PGRs and high or low light intensities. As a result of these stress factors, the explant produces and exudes toxic phenolic compounds into the growth medium that reduces regeneration and promotes tissue death. Browning in plant cell cultures is a consequence of the oxidation and polymerization of accumulated phenolic compounds which is mediated by the phenylalanine ammonia-lyase (PAL), polyphenol-oxidase (PPO) and peroxidase (POD) enzymes. Phenolic compounds are synthesised via the phenylpropanoid pathway whereby PAL converts $_{L}$-phenylalanine into trans-cinnamic acid which acts as a substrate for the synthesis of phenolic compounds **(DIXON and PAIVA, 1995)**. On the other hand, PPO is associated with plastids and its phenolic substrates are concentrated within the vacuole **(LANDRIGAN *et al*., 1996)**. The polymerization of quinones mediated by the oxidation of phenols and catalysed by PPO, produces brown pigments frequently seen on plant material **(BUCHELI and ROBINSON, 1994)**. Browning of explants occurs after wounding, when cells are damaged and intracellular compartmentalisation is disrupted **(LANDRIGAN *et al*., 1996)**. Similarly, POD is also associated with injury, wound repair and disease resistance **(PRÈSTAMO and MANZANO, 1993)**.

Given the importance of the *in vitro* production of plants, an increasing effort has been placed into developing efficient methods that prevent oxidative browning and/or the consequences thereof **(BHAT and CHANDEL, 1991; TOTH *et al*., 1994; MADHUSUDHANAN and RAHIMAN, 2000; TANG *et al*., 2004a; TANG *et al*., 2004b; KRISHNA *et al*., 2008; THOMAS, 2008)**. PAL is the first dedicated enzyme in the phenylpropanoid pathway, and its activity has been shown to increase prior to the onset of tissue browning. As such, research suggests that the alteration of the tissue culture environment may reduce oxidative browning often by lowering PAL activity. Several studies have shown that culturing plant material in a 24 h dark photoperiod significantly reduces browning. **TOTH *et al*. (1994)** demonstrated that the pre-treatment of explants or alteration of the growth medium with compounds specifically

used to reduce browning alleviated browning in oak explants. Amendments to the medium that have shown some success in reducing oxidative browning include the addition of: (1) antioxidants such as ascorbic acid, melatonin or citric acid which prevent the oxidation of phenolic compounds or (2) adsorbents such as activated charcoal or polyvinylpyrollidone (PVP) which bind to phenolic compounds reducing their toxicity.

However, as previously demonstrated in **Chapter 2**, the addition of antioxidants and adsorbents to the culture medium failed to improve the development of *S. puniceus.* Various studies have demonstrated the growth-regulating property of phloroglucinol (PG; 1,3,5-trihydroxybenzene), an organic phenolic compound produced from the degradation of phloridzin. A recent review by **TEIXEIRA da SILVA *et al.* (2013)** revealed the broad spectrum beneficial effects of PG for inducing or improving *in vitro* plant development. Among these included the prevention or reduction of browning in explants of *Ficus carica* with the incorporation of PG (500 μM) and 2-(N-morpholino) ethanesulfonic acid to the culture medium **(KIM *et al.*, 2007)**.

Competitive inhibitors of PAL synthesis have been suggested to reduce browning caused by the oxidation of accumulated phenolics. Gallic acid (GCA), the most common phenolic acid found in plants, is a strong inhibitor of PAL in higher plants solidifying its status as a competitive inhibitor of phenylalanine ammonia-lyase **(SATO *et al.*, 1982)**. The incorporation of gallic acid to culture media may provide a means for the elimination of browning *in vitro*. As such, PG and GCA were evaluated for their ability to reduce oxidative browning. In addition, their interaction with cytokinins was also determined.

3.1.3. Liquid culture systems

Liquid media were traditionally used for suspension cultures and today, have become more common for the *in vitro* propagation of bulbous and cormous plants **(BERGOÑÓN *et al.*, 1992)**. Liquid culture affords several advantages over solid medium. The technique reduces costs as a gelling agent is not required. Agar in tissue culture medium creates a static environment giving rise to gradients of nutrients in the medium. The constant movement of liquid cultures on a shaker eliminates gradients and improves bud regeneration **(BERGOÑÓN *et al.*, 1992)**. Agitation of a liquid medium also improves aeration, reduces the effect of plant polarity and distributes nutrients and PGRs uniformly **(SATHYANARAYANA & MATHEWS, 2007)**. Of particular interest with regard to *S. puniceus* is the ability of liquid media to dilute toxic phenols that have accumulated in the

medium **(SATHYANARAYANA & MATHEWS, 2007)**. As such, a liquid culture system was developed for *S. puniceus*.

3.2. Materials and Methods

3.2.1. Source of plant growth regulators and media supplements

Benzyladenine (BA), naphthaleneacetic acid (NAA) and indole-3-butryric acid (IBA) were obtained from Sigma-Aldrich (Steinheim, Germany). The topolins including *meta*-topolin (*m*T), *meta*-topolin riboside (*m*TR), *meta*-methoxytopolin (Me*m*T) and *meta*-Methoxy-9-tetrahydropyran-2-yl topolin (Me*m*TTHP) were prepared as described by **DOLEŽAL *et al.*, (2006)**. Gallic acid was purchased from Sigma Aldrich and phloroglucinol from UNILAB.

3.2.2. The influence of cytokinins on the proliferation and development of *Scadoxus puniceus*

For initiation, leaves of *S. puniceus* were collected during Spring (September) 2013 and surface decontaminated as in **Section 2.2.2.2**. Leaves were then cut into 1 x 1 cm explants and inoculated on MS medium supplemented with equi-molar concentrations (5 µM) of BA and NAA. Cultures were maintained at 25 ± 2 °C under a 16/ 8 h light/ dark photoperiod. In order to determine the effect of *m*T on the multiplication of *S. puniceus*, the *in vitro* grown shoots were harvested after five months of culture and divided into 1 x 1 cm explants after which these were inoculated on MS medium supplemented with BA or *m*T (0, 5 and 10 µM).

To determine the effect of cytokinins on the development of shoots, shoots (≥ 10 mm, but not greater than 15 mm) from the initiation stage were separated from the explant and embedded in MS medium supplemented with various types (BA, *m*T, *m*TR, Me*m*T, and Me*m*TTHP) and concentrations of cytokinins **(Table 3.1)**. Cultures were maintained at 25 ± 2 °C under a 24 h dark photoperiod for three months after which the growth parameters and physiological changes were recorded.

3.2.3. The influence of organic compounds on the development of *Scadoxus puniceus* and *Merwilla plumbea*

Seeds of *Merwilla plumbea* (Lindl.) Speta (model species), and *S. puniceus* were obtained from the University of KwaZulu-Natal Botanical Garden (Pietermaritzburg, South Africa) and decontaminated as per **BASKARAN *et al.* (2012)** and **Section 2.2.3** of the current thesis, respectively. Leaf explants derived from *M. plumbea* seedlings were sub-cultured on 10 ml of

one-tenth strength MS medium supplemented with 30 g/l sucrose. Leaf explants (1 x 1 cm) of *M. plumbea* derived from the subculture experiment and bulb halves of *S. puniceus* seedlings were used for further experimentation. The concentrations and combinations of (1) GCA and cytokinins (BA and *m*T), and (2) PG and auxin-cytokinin (BA, NAA) interactions are presented in **Tables 3.2**, **3.3** and **3.4** respectively. Media consisted of full strength MS basal nutrient medium supplemented with 3% sucrose and 0.1 g/l *myo*-inositol. The pH of the medium was adjusted to 5.8 using 1.0 N potassium hydroxide (KOH) or 1.0 N hydrochloric acid (HCl) and solidified with 8 g/l agar (Agar bacteriological-Agar No. 1, Oxoid Ltd., England). The medium was sterilised by autoclaving at 121 °C and 103 kPa for 20 min and cultures were grown at 25 ± 2 °C under a 16/8 h light/dark photoperiod with a photosynthetic photon flux density (PPFD) of 30 µmol m^{-2} s^{-1}. Regenerated plants were harvested and growth parameters assessed after 12 weeks of culture.

To determine the effect of GCA on the total phenolic acid content of *in vitro* grown shoots, shoots were frozen with liquid nitrogen and freeze dried for 48h. Dried material were extracted with 50% methanol (10 mg/ml) in a sonication bath containing ice for 40 min thereafter, the samples were filtered under *vacuo* and used immediately in the assay. The Folin-Ciocalteu (Folin-C) assay as described by **MAKKAR *et al.* (2007)** was used to determine the total phenolic content of plant material. The reaction mixture contained 50 µl of extract, 950 µl of distilled water, 500 µl Folin-C reagent (1 N) and 2.5 ml of 2% sodium carbonate (Na_2CO_3). The reaction mixture was incubated at room temperature for 30 min in the dark. The absorbance was then read using a UV-visible spectrophotometer (Varian Cary 50, Australia). Gallic acid (Sigma-Aldrich, USA) was used as a standard and 50% methanol was used as a blank instead of the sample extracts. Total phenolic acid concentrations were expressed as mg gallic acid equivalents (mg GAE). The experiment was repeated twice with three replicates each.

3.2.4. Liquid culture system

Whole inflorescences of *S. puniceus* were collected during Spring 2013 prior to fruiting. Flowers were removed and surface decontaminated with 70% ethanol for 60 sec followed by a 5 min soak in 3% sodium hypochlorite. Initially, explants consisting of mature pedicels that were attached to the ovary, anthers and filaments were excised from flowers and inoculated on MS medium only or MS medium supplemented with BA and NAA at equi-molar (5µM) concentrations.

The callus attained from the initiation stage (above) was subcultured in either solid or liquid MS medium supplemented with 6% sucrose for bulblet development (**BERGOÑÓN *et al.*, 1992**). For liquid culture, callus was cultured in 100 ml conical flasks (30 ml per flask), sealed with a cotton plug and maintained on a rotary-shaker at 60 rpm while callus in solid medium were maintained in 250 ml culture jars (30 ml per jar). Cultures were maintained at 25 ± 2 °C under a 16/8 h light/dark photoperiod for 1 month. Thereafter, the length and weight of shoots derived from clusters, and the number of bulblets that formed on callus in liquid and solid medium were compared. To determine the influence of cytokinins in liquid culture, clusters were separated into individual shoots and cultured in liquid media supplemented with combinations of BA and NAA (5 µM BA: 1 µM NAA; 5 µM BA: 5 µM NAA). Rooting of shoots that developed in liquid media was carried out on MS medium supplemented with various concentrations (1.0, 5.0, 10.0 or 15.0 µM) of IBA.

3.2.5. Data analysis

All experiments were carried out in a completely randomized design. Data pertaining to **Section 3.2.1**, **3.2.2** and rooting experiments were subjected to a One-Way-Analysis of Variance, where there were significant differences ($p = 0.05$); the means were separated using a Duncan's Multiple Range Test. A non-parametric Mann Whitney U-test was used to compare the regeneration of inflorescence explants while a Student's *t*-test was used to compare regeneration between solid and liquid culture.

3.3. Results and Discussion

3.3.1. The influence of cytokinins on the proliferation and development of *Scadoxus puniceus*

The effect of BA and *m*T, on proliferation of leaf explants of *S. puniceus* is presented in **Figure 3.1**. Explants failed to regenerate on MS medium without PGRs. The highest number (3.80 ± 1.10) of bulblets was formed on BA (5 µM) followed by BA (10 µM; 3.48 ± 0.85). BA was superior to the topolin treatments at both concentrations tested. *m*T induced a response at 10 µM, however an equi-molar concentration of BA still produced more bulbs than *m*T.

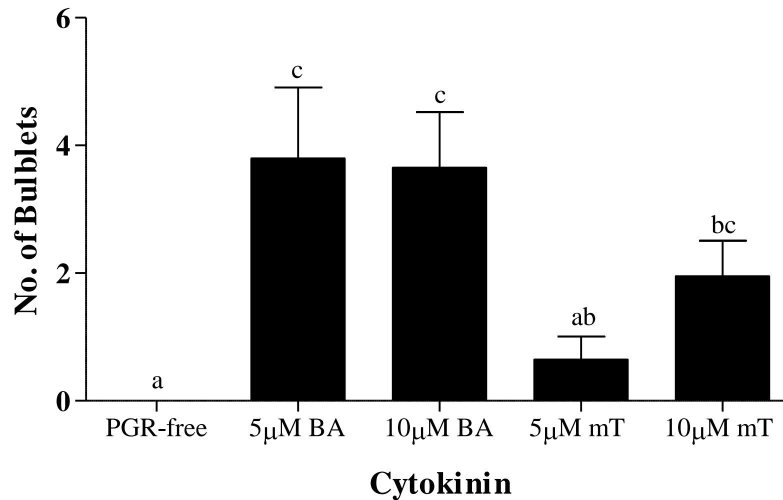


Figure 3.1: The effect of benzyladenine (BA) and *meta*-topolin (*m*T) on the proliferation of *Scadoxus puniceus*. Bars represent the mean with SE. n = 20. Different letters represent significant differences at the 5% significance level according to Duncan's Multiple Range Tests.

The developmental parameters and morphological changes of shoots associated with the cytokinin treatments are presented in **Table 3.1**. Regardless of the fact that the greatest development occurred when shoots were maintained on MS medium supplemented with 10 µM *m*T, shoot development of plants maintained in PGR-free medium exhibited good development in terms of shoot length (43.0 ± 12.9, 33.0 ± 10.8 mm, respectively). Although not statistically significant from the control, the greatest plant development occurred on 10 µM *m*T > 10 µM *m*TR > 5 µM BA > 5 µM *Mem*T. Furthermore, plantlets maintained on PGR-free (control) and BA-supplemented media produced greater fresh weights than topolin treatments (with the exception of *m*T). The greater fresh weight of these plantlets may be attributed to excessive hydration as a direct result of hyperhydricity **(Figure 3.2)**. Thus despite their development, control and BA-treated plantlets were hyperhydric. The structure activity relationship of the topolins was evident as plant development varied between treatments. Nevertheless, this group of cytokinins reduced hyperhydricity, improved shoot development and maintained a similar fresh weight to those of the control (10 µM *m*T specifically; **Figure 3.2**).

In vitro proliferation of the Amaryllidaceae is based on organogenesis rather than embryogenesis due to the recalcitrant nature of the seeds and certain explants. As previously discussed, the *in vitro* proliferation of *S. puniceus* was influenced by seasonality of explants,

slow growth rates and hyperhydricity. The use of cytokinins in plant tissue culture is universally seen as an effective way of promoting growth by limiting or improving these factors. Cytokinins play a significant role in organogenesis and regulate several physiological and developmental processes **(PIERIK, 1991; MALÁ *et al.*, 2009)**. Endogenous cytokinins occur in different metabolic forms such as free bases, ribosides, *N*-glucosides, *O*-glucosides and nucleotides **(LETHAM and PALNI, 1983)**. However, the inability of leaf explants to regenerate without the application of exogenous cytokinins suggests that the explants lack sufficient concentrations of endogenous cytokinins to allow for cell division and proliferation **(Figure 3.1)**.

Recent literature has demonstrated the improvement of morphogenetic activity of explants treated with topolins and since their discovery, they have been branded as potential replacements to commonly used cytokinins, such as BA and kinetin, in several plant tissue culture protocols **(AREMU *et al.*, 2012)**. The primary metabolite (*m*T) of the naturally occurring BA-analogue degrades more rapidly, improving shoot proliferation, rooting and acclimatization **(WERBROUCK *et al.*, 1996; STRNAD, 1997; AREMU *et al.*, 2012)**. However, in rare cases, conventional cytokinins seem to hold the upper hand against the topolins. **MALÁ *et al.* (2009)** investigated the effect of BA and topolin derivatives on the proliferation of *Sorbus torminalis* and found that the highest regeneration rate occurred when explants were cultured with BA. In *Aloe barbadensis*, optimum proliferation was obtained when explants were cultured on 6 µM BA while *m*T induced proliferation at 10 µM **(ADELBERG and NAYLOR-ADELBERG, 2012)**. Similarly, the current study revealed that BA was more effective for shoot proliferation of *S. puniceus*. The superiority of BA over *m*T may be a result of the quicker uptake and translocation of BA in plant tissue. However, organogenesis in explants is influenced by both endogenous and exogenous stimuli **(CENTENO *et al.*, 1996)**. Cytokinins interact with auxins, polyamines and phenolic acid derivatives **(ALTAMURA *et al.*, 1993; CVIKROVÁ and HRUBCOVÁ, 1999; MALÁ *et al.*, 2009)**. The interaction of BA with these endogenous substances may promote improved organogenesis. Further studies quantifying endogenous PGR concentrations, polyamines and phenolic acid constituents are required to elucidate the possible mode of action of BA in *S. puniceus*.

Conversely, with regard to shoot development, the study demonstrates the ability of the topolins to alleviate the physiological disorders often associated with *in vitro* cultured plants. Unlike PGR-free and BA-treated plantlets, topolin treated plantlets exhibited no indication of

abnormality or hyperhydricity. Several authors have recommended the use of topolins to alleviate hyperhydricity. The topolins completely alleviated hyperhydricity in shoots of *Aloe polyphylla* (**BAIRU *et al*., 2007**), and *Beta vulgaris* (**KUBALÁKOVA and STRNAD, 1992**), and reduced most of the abnormality indices of *Barleria greenii* (**AMOO *et al*., 2011**). The authors further postulated that the abnormality that was noted for these treatments was a result of the carry over effect of BA. Factors that may be responsible for the superiority of the topolins over BA include its faster translocation time in plant tissue, their metabolites are more easily degraded, and the side chain of *meta*-topolins allows for the formulation of *O*-glucoside metabolites which are cytokinin storage forms that convert to active cytokinin bases when these are required (**PARKER *et al*., 1978; KAMINEK *et al*., 1987; WERBROUCK *et al*., 1996; BAIRU *et al*., 2009**). However, the present study identified strong toxicity of Me*m*T and Me*m*TTHP to *S. puniceus* as evidenced by a reduced regeneration rate when plantlets were maintained on MS medium supplemented with these cytokinins.

Despite this, the less toxic nature of *m*T and *m*TR coupled with their ability to reduce hyperhydricity encourages their use to improve the *in vitro* development of *S. puniceus* after proliferation with BA.

Table 3.1: The effect of different cytokinins on the development and morphology of *Scadoxus puniceus* shoots

Cytokinin	Concentration (µM)	Response (%)	Shoot length (mm)	Fresh weight (g)	Morphological appearance
PGR-free	-	90	33.00 ± 10.83 [ab]	0.60 ± 0.20 [ef]	Hyperhydric shoots
BA	5	100	37.00 ± 7.70 [b]	0.45 ± 0.21 [de]	Hyperhydric shoots
	10	90	22.50 ± 5.12 [ab]	2.01 ± 0.31 [f]	Hyperhydric cluster
*m*T	5	60	20.00 ± 5.22 [ab]	0.20 ± 0.14 [bcd]	Normal
	10	80	43.00 ± 12.89 [b]	0.51 ± 0.27 [cde]	Normal
*m*TR	5	50	15.00 ± 6.19 [a]	0.07 ± 0.04 [abc]	Normal
	10	80	37.78 ± 8.08 [b]	0.11 ± 0.04 [bcde]	Normal
*Mem*T	5	90	35.50 ± 5.45 [b]	0.18 ± 0.64 [bcde]	Normal
	10	30	14.50 ± 3.98 [a]	0.09 ± 0.52 [ab]	Failed to respond
*Mem*TTHP	5	10	12.00 ± 2.00[a]	0.09 ± 0.01 [a]	Failed to respond
	10	50	31.50 ± 10.17 [ab]	0.29 ± 0.14 [bcd]	Normal

Values represent mean ± (SE) of 10 replicates. Different letters in the same column represent significant differences at the 5% significance level according to Duncan's Multiple Range Tests. BA: Benzyladenine; *m*T: *meta*-Topolin; *m*TR: *meta*-Topolin Riboside; *Mem*T: *meta*-Methoxytopolin; *Mem*TTHP: *meta*-Methoxy-9-tetrahydropyran-2-yl topolin

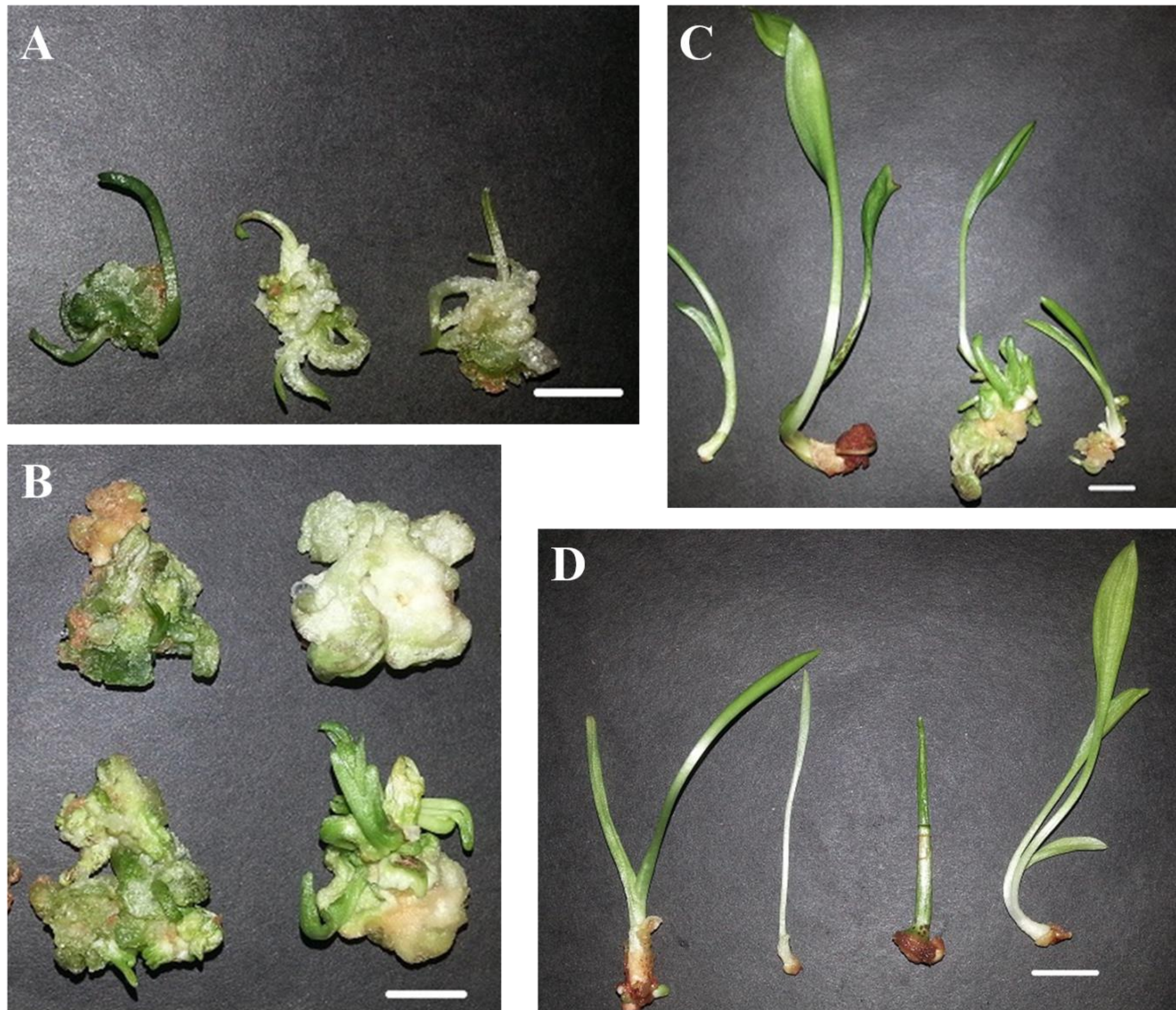


Figure 3.2: The morphology of shoots of *Scadoxus puniceus* cultured in MS medium supplemented with various cytokinins. A, B; hyperhydric shoots derived from plant growth regulator-free and benzyladenine supplemented medium, respectively. C, D; improved development of shoots from topolin treatments (*meta*-topolin and *meta*-topolin riboside, respectively). Scale bar = 10 mm.

3.3.2. The effect of gallic acid (GCA) on development of *Merwilla plumbea* and *Scadoxus puniceus*

The effect of GCA and its interaction with BA and *m*T on the development of *M. plumbea* is presented in **Table 3.2**. An improved number of shoots per explant was obtained with all treatments when compared to the control (PGR-free). When only GCA was used, the highest number of shoots (7.60 ± 1.25) formed with the highest concentration tested (10 µM). Gallic acid treated plantlets developed faster than control plantlets after 12 weeks of incubation considering the significantly higher number of shoots that were greater than 10 mm in length

and improved overall plant development (shoot length and bulb width). Root development and overall fresh weight of plantlets cultured on MS medium supplemented with GCA (1 and 10 µM) alone were also significantly larger/ greater than control and cytokinin-treated plantlets.

Gallic acid improved the effect of BA given that 1 and 10 µM GCA induced significantly greater (10.65 ± 1.57; 8.55 ± 1.60, respectively) shoot regeneration compared to the control (PGR free; 3.55 ± 0.81, BA only; 6.35 ± 0.93). Despite this, a decrease in all other growth parameters was recorded. On the other hand, rather unsatisfactory results were recorded for plantlets treated with *m*T and GCA. The inclusion of GCA significantly reduced shoot regeneration while simultaneously improving plant development (shoot length).

With regard to *S. puniceus*, explants inoculated on MS medium containing GCA only, succumbed to systemic bacteria that were contained in the seed of the three-month-old seedlings; as a result the effect of GCA on development could not be determined. The effect of GCA in combination with BA and *m*T is presented in **Table 3.3**. The combination of GCA (at all concentrations) and BA significantly improved the development (shoot number, diameter and fresh weight) of *S. puniceus* **(Figure 3.3)**. Despite a non-significant decrease in the number of shoots produced with the addition of 10 µM GCA and BA, the longest shoots were recorded with this combination (66.00 ± 9.03 mm). The combination of 5 µM GCA and *m*T, produced the greatest number of shoots (3.30 ± 0.61), despite this the *m*T - GCA combinations failed to significantly improve all other growth parameters. Upon visual inspection, explants of *S. puniceus* that were treated with GCA were healthier than control explants. Red pigmentation on explants that was characteristic of the exudation of phenols from previous experiments was reduced.

The results of the current study reveal the synergistic relationship of GCA with BA and its antagonistic relationship with *m*T with regard to shoot proliferation while the reverse relationship is evident with regard to plant development. **BASKARAN *et al.* (2012)** determined the effect of various cytokinins on shoot regeneration of *M. plumbea*. These authors recorded a significantly higher regeneration rate with the addition of topolins (*m*T and *meta*-Topolin riboside) when compared to BA. Recently, **AREMU *et al.* (2013)** revealed that the addition of BA to the culture medium resulted in the accumulation of phenolic acids such as protocatechuic, 4-hydroxybenzoic, *p*-coumaric, and ferulic acids in shoots of *M. plumbea*. Generally, growth *in vitro* and phenolic metabolism are inversely related,

substantiating the previous and current results. However, the current study also recorded an increase in regeneration with regard to BA and an increase in development with regard to *m*T with the addition of GCA. Gallic acid has been identified as a strong inhibitor of PAL and is also a competitive inhibitor of phenylalanine, the primary metabolite from which *trans*-cinnamic acid and an array of phenolic acids are produced **(SATO *et* al., 1982; SHARAN *et al.*, 1998)**. As such, the increase in growth recorded with the addition of GCA may be a result of GCA inhibiting PAL and further phenolic acid metabolism as well as accumulation. **JONES and SAXENA (2013)** revealed that the addition of a competitive inhibitor of the PAL enzyme, 2-aminoindane-2-phosphoric acid (AIP), into tissue culture medium significantly reduced tissue browning of *Artemisia annua* callus cultures. Reduced tissue browning was also associated with reduced total phenolic content and improved callus development.

It should however be noted that AIP improved the development of callus cultures and that phenolic compounds do have a stimulatory effect on *in vitro* morphogenesis of whole plantlets. Phenolic compounds influence root development **(HAMATT and GRANT, 1997; ROMAIS *et al.*, 2000; CEASAR *et al.*, 2010)**, shoot proliferation **(SARKAR and NAIK, 2000)**, shoot organogenesis **(LORENZO *et al.*, 2001)** and somatic embryogenesis **(HANOWER and HANOWER, 1984)**. In transgenic tobacco plants, the inhibition of phenolic acid metabolism by the transcription factor AmMYB308 resulted in abnormal leaf palisade development and promoted premature cell death in mature leaves **(TAMAGNONE *et al.*, 1998)**. The authors further revealed that the resultant changes were due to a lack of phenolic intermediates which are important signalling molecules in the final stages of leaf palisade formation and also suggested that they play a role in tissue senescence **(TAMAGNONE *et al.*, 1998)**. Various authors demonstrated the importance of phenolics in rooting. **DE KLERK *et al.* (2011)** revealed that phenolic compounds significantly improved rooting of cut apple stem slices in the presence of indole-3-acetic acid (IAA). They suggested that *ortho*- and *para*-diphenols, methylated orthodiphenols and triphenols act by inhibiting the decarboxylation of IAA **(WILSON and VAN STADEN, 1990; BANDURSKI *et al.*, 1995; DE KLERK *et al.*, 2011)**. **DE KLERK *et al.* (2011)** also recorded a reduction in phenolic exudation as a result of wounding when exogenous phenolics were applied. The inhibition of phenolic metabolism may also reduce the *ex vitro* acclimatisation of *in vitro* cultured plants.

The role of phenolics in plant development should not be overlooked and thus the addition of a phenolic compound such as GCA, with the added benefit of the inhibition of PAL provides a means for reducing oxidative stress while also not compromising plant development.

Table 3.2: The effect of gallic acid (GCA) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development and phenolic content of *Merwilla plumbea*.

Medium constituents	No. of shoots	No. of shoots > 10 mm	Shoot length (mm)	Bulb width (mm)	No. of roots	Fresh weight (g)
MS-Only	3.55 ± 0.81 [a]	3.10 ±0.71 [a]	44.00 ± 8.79 [def]	2.45 ± 0.43 [cde]	6.30 ± 1.50 [abcd]	0.38 ± 0.11 [abcd]
1 µM GCA	6.85 ± 1.12 [bc]	6.40 ± 0.97 [bc]	64.5 ± 7.54 [g]	5.58 ±1.85 [f]	14.00 ± 1.42 [f]	0.83 ± 0.14 [f]
5 µM GCA	5.25 ± 1.01 [ab]	5.25 ± 1.01 [abc]	52.00 ± 7.87 [fg]	5.25 ± 2.40 [de]	9.95 ± 1.66 [de]	0.74 ± 0.19 [def]
10 µM GCA	7.60 ± 1.25 [bcd]	7.05 ± 1.20 [bc]	65.25 ± 6.98 [g]	2.65 ± 0.30 [e]	10.00 ± 1.57 [e]	0.54 ± 0.63 [ef]
BA-only (5 µM)	6.35 ± 0.93 [abc]	4.15 ± 0.60 [abc]	25.60 ± 3.51 [cde]	3.95 ± 2.70 [ab]	6.00 ± 0.93 [cde]	0.12 ± 0.04 [a]
1 µM GCA + BA	10.65 ± 1.57 [d]	4.45 ± 0.78 [abc]	13.25 ± 1.37 [ab]	1.45 ± 0.13 [abc]	3.30 ± 1.05 [ab]	0.15 ± 0.04 [ab]
5 µM GCA + BA	6.60 ± 0.89 [bcd]	3.50 ± 0.71 [a]	12.35 ± 2.34 [a]	1.10 ± 0.12 [a]	2.65 ± 0.49 [a]	0.12 ± 0.45 [a]
10 µM GCA + BA	8.55 ± 1.60 [bcd]	3.25 ± 0.74 [a]	12.10 ± 2.66 [a]	1.35 ± 0.23 [ab]	3.65 ± 0.722 [abc]	0.18 ± 0.06 [abc]
*m*T-Only (5 µM)	11.90 ± 1.72 [d]	4.70 ± 0.56 [abc]	20.75 ± 1.93 [bcd]	1.75 ± 0.14 [bcd]	5.10 ± 0.89 [abc]	0.20 ± 0.03 [bcde]
1 µM GCA + *m*T	10.20 ± 1.20 [d]	7.45 ±1.15 [c]	32.75 ± 2.28 [ef]	1.70 ± 0.15 [bcd]	5.60 ± 0.60 [cde]	0.22 ± 0.028 [cde]
5 µM GCA + *m*T	5.40 ± 0.94 [ab]	4.05 ± 0.76 [ab]	20.00 ± 24 [abc]	1.40 ± 0.17 [abc]	5.60 ± 1.00 [bcd]	0.12 ± 0.03 [ab]
10 µM GCA + *m*T	5.35 ± 0.65 [ab]	4.70 ± 0.69 [abc]	29.25 ± 3.43 [cdef]	1.45 ± 1.14 [abc]	4.55 ± 0.59 [abc]	0.12 ± 0.02 [abc]

Values represent mean ± SE, n = 20. Different letters in the same column indicate significant differences between samples at the 5% significance level according to Duncan's Multiple Range Tests. MS: Murashige and Skoog (1962) basal nutrient medium (control).

Table 3.3: The effect of gallic acid (GCA) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development of *Scadoxus puniceus*.

Medium constituents	No. of shoots	No. of shoots > 10 mm	Shoot length (mm)	Bulb width (mm)	No. of roots	Root length (mm)	Fresh weight (g)
MS-Only	1.40 ± 0.22 [a]	0.30 ± 0.15 [a]	8.00 ± 2.65 [a]	2.05 ± 0.19 [a]	0.90 ± 0.35 [ab]	23.50 ± 9.58 [bc]	0.07 ± 0.03 [a]
1 µM GCA	Nd	Nd	Nd	Nd	Nd	Nd	Nd
5 µM GCA	Nd	Nd	Nd	Nd	Nd	Nd	Nd
10 µM GCA	Nd	Nd	Nd	Nd	Nd	Nd	Nd
BA-only (5 µM)	2.10 ± 0.46 [ab]	1.10 ± 0.32 [ab]	19.50 ± 5.50 [a]	2.65 ± 0.24 [ab]	1.80 ± 0.53 [c]	37.50 ± 9.58 [c]	0.28 ± 0.68 [ab]
1 µM GCA + BA	3.10 ± 0.38 [b]	2.20 ± 0.39 [c]	34.00 ± 10.77 [ab]	4.30 ± 0.68 [c]	2.30 ± 0.76 [c]	12.50 ± 2.61 [bc]	0.55 ± 0.14 [b]
5 µM GCA + BA	3.11 ± 0.53 [b]	2.44 ± 0.52 [c]	55.56 ± 13.06 [bc]	4.11 ± 0.35 [c]	0.11 ± 0.11 [a]	1.67 ± 1.67 [a]	0.53 ± 0.14 [b]
10 µM GCA + BA	2.50 ± 0.45 [b]	1.80 ± 0.25 [bc]	66.00 ± 9.03 [c]	3.70 ± 0.30 [cb]	1.10 ± 0.38 [a]	19.00 ± 7.02 [bc]	0.55 ± 0.08 [b]
*m*T-Only (5 µM)	2.80 ± 0.44 [ab]	1.70 ± 0.21 [bc]	47.00 ± 7.35 [bc]	2.90 ± 0.31 [ab]	1.10 ± 0.59 [ab]	6.50 ± 2.99 [ab]	0.38 ± 0.09 [b]
1 µM GCA + *m*T	2.60 ± 0.37 [ab]	1.80 ± 0.33 [bc]	53.00 ± 8.88 [bc]	2.80 ± 0.20 [ab]	0.30 ± 0.15 [a]	8.00 ± 4.42 [ab]	0.42 ± 0.07 [b]
5 µM GCA + *m*T	3.30 ± 0.61 [b]	1.50 ± 0.40 [bc]	31.50 ± 7.85 [ab]	2.40 ± 0.37 [a]	0.50 ± 0.22 [ab]	24.00 ± 11.47 [ab]	0.44 ± 0.15 [b]
10 µM GCA + *m*T	Nd	Nd	Nd	Nd	Nd	Nd	Nd

Values represent mean ± SE, n = 20. Different letters in the same column indicate significant differences between samples at the 5% significance level according to Duncan's Multiple Range Tests. MS: Murashige and Skoog (1962) basal nutrient medium (control). Nd: Not determined due to contamination.

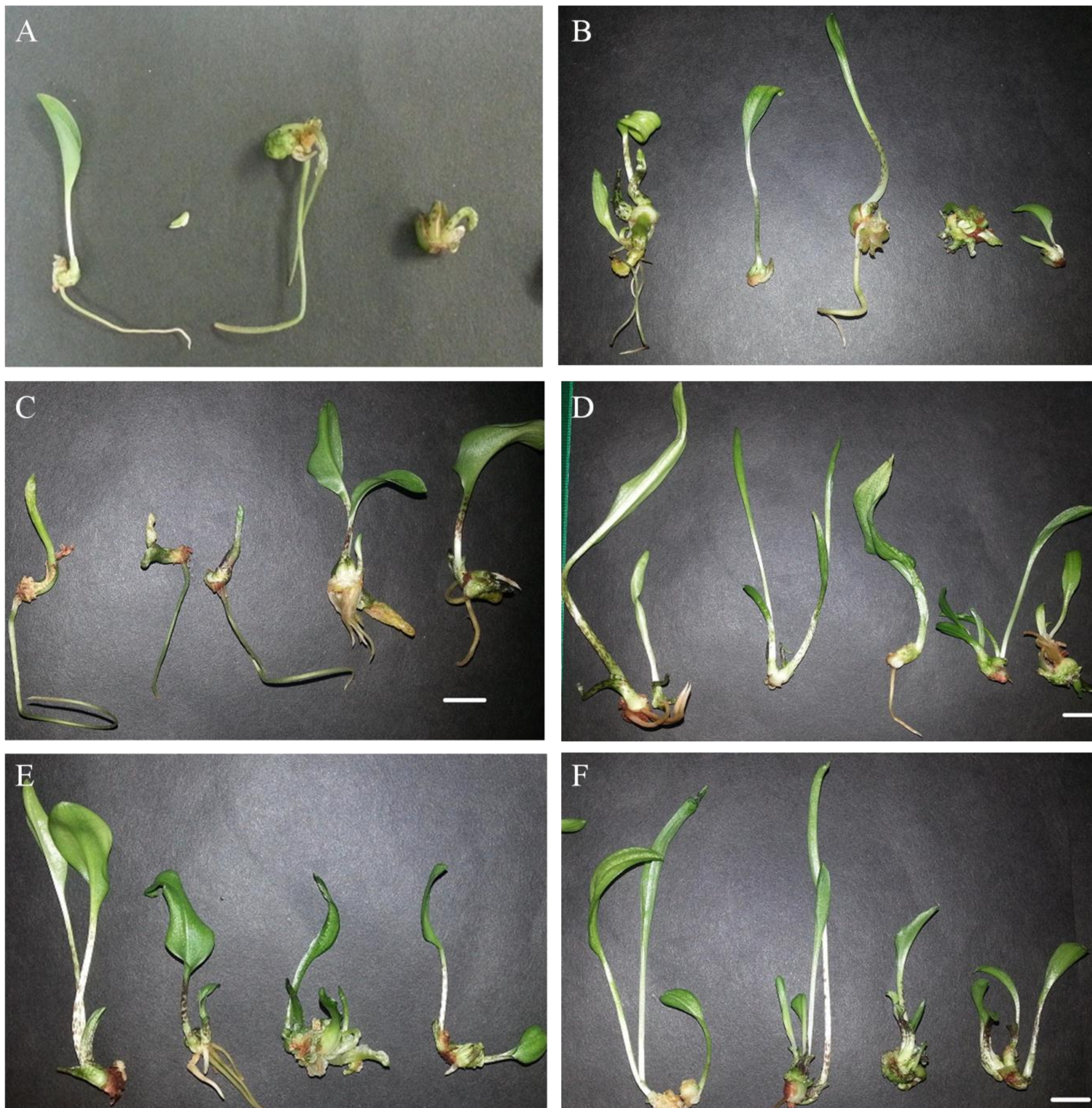


Figure 3.3: The effect of gallic acid (GCA) combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or *meta*-topolin (*m*T) on *in vitro* development of *Scadoxus puniceus*. A; MS-only (control), B; MS + 1 µM GCA, C; MS + 5 µM BA, D; MS + 5 µM BA + 10 GCA, E; MS + 5 *m*T, F; MS + 5 µM *m*T + 5 µM GCA. Bar = 10 mm. MS; Murashige and Skoog nutrient medium.

3.3.3. The effect of phloroglucinol (PG) on the *in vitro* development of *Scadoxus puniceus*

The development of *S. puniceus* cultured on MS media supplemented with PG and BA or NAA is presented in **Table 3.4**. When applied singularly at the various concentrations tested, PG did not significantly improve the developmental parameters of *S. puniceus*. Increases in

the number of bulblets, shoot length and overall fresh weight were noted with the addition of BA or NAA (5 μM) in combination with PG. All BA treatments in combination with PG produced significantly more bulblets that were larger than the control treatments (PGR-free and BA alone). Despite this, BA-treated plantlets exhibited decreases in the number of bulblets, and bulb diameter with increasing concentrations of PG. Furthermore, root development was completely inhibited by these treatments. Conversely, the length of aerial parts of the plant were significantly improved with the addition of PG as compared to the control. The highest number of bulblets occurred with the combination of 12.5 μM PG and 5 μM NAA (2.53 ± 0.75), significantly more than the control treatments (PGR-Free or NAA alone) of singular PG treatments. Given the significantly larger shoot length and fresh weight (**Table 3.4**, 58.67 ± 12.00 mm; 0.90 ± 0.18 mm, respectively) of these plantlets, it is evident that they developed at a faster rate than plantlets from the other treatments. The current data thus reveal the synergistic relationship between PG and cytokinins (BA) or auxins (NAA) for the development of *S. puniceus*.

PG is a naturally occurring phenolic compound present is several plant species. The compound is seldom used as the main focus of any tissue culture endeavour but rather used in combination with other medium supplements **(TEIXEIRA da SILVA *et al*., 2013)**. Several authors have reported on the synergism between PG and cytokinins that enhances shoot proliferation and development. A successful protocol for the *in vitro* propagation was developed for *Minuartia valentina*, an endangered Spanish endemic plant, when supplementing medium with a combination of either PG and BA or PG and kinetin **(IBAÑEZ and AMO-MARCO, 1998)**. Furthermore, the authors recorded a two-fold increase in the shoot length of plantlets with the addition of PG. **GURURAJ *et al*. (2004)** revealed that combinations of BA and gibberellic acid with PG induced the formation of multiple shoots on single-node explants of *Decalepis hamiltoni*. **BUTHUC-KEUL and DELIU (2001)** made use of high concentrations of PG (600 μM) in combination with NAA and kinetin to improve shoot proliferation and development of *Arnica montana*. The current study revealed the ability of PG (12.5 μM) to significantly improve (approximately 6-fold) the shoot length of *S. puniceus* when used in combination with NAA (5 μM).

Table 3.4: The effect of phloroglucinol (PG) in combination with equi-molar concentrations (5 µM) of benzyladenine (BA) or naphthaleneacetic acid (NAA) on *in vitro* development of *Scadoxus puniceus*.

Medium constituents	No. of bulblets	Bulb width (mm)	Shoot length (mm)	No. of roots	Root length (mm)	Fresh weight (g)
MS-only	1.20 ± 0.32 ^{abc}	1.73 ± 0.28 ^{abcd}	10.67 ± 4.73 ^{bcde}	0.67 ± 0.21 ^{cd}	13.00 ± 4.68 ^{cd}	0.07 ± 0.03 ^{abc}
12.5 µM PG	0.75 ± 0.14 ^{abc}	1.63 ± 0.30 ^{abc}	3.31 ± 1.56 ^{a}	0.44 ± 0.22 ^{abc}	9.69 ± 4.71 ^{abcd}	0.05 ± 0.02 ^{ab}
25.0 µM PG	0.60 ± 0.21 ^{ab}	1.27 ± 0.38 ^{a}	13.67 ± 3.34 ^{bc}	0.60 ± 0.25 ^{abcd}	12.1 ± 5.43 ^{bcd}	0.08 ± 0.04^{abc}
37.5 µM PG	1.00 ± 0.17 ^{abc}	1.33 ± 0.25 ^{a}	1.00 ± 1.00 ^{a}	0.20 ± 0.20 ^{ab}	3.00 ± 3.00 ^{ab}	0.02 ± 0.02 ^{a}
50.0 µM PG	1.20 ± 0.11 ^{c}	3.00 ± 0.29 ^{de}	8.00 ± 3.27 ^{bcde}	0.60 ± 0.24 ^{bcd}	10.33 ± 4.40 ^{bcd}	0.72 ± 0.21 ^{abcd}
5 µM BA	1.00 ± 0.09 ^{bc}	2.23 ± 0.27 ^{abcde}	6.60 ± 2.32 ^{abcd}	0.47 ± 0.19 ^{abcd}	10.33 ± 4.40 ^{bcd}	0.06 ± 0.01 ^{abcd}
6.25 µM PG + BA	2.33 ± 0.50 ^{d}	2.80 ± 0.37 ^{cde}	19.67 ± 5.70 ^{def}	0.07 ± 0.07 ^{ab}	1.00 ± 1.00 ^{ab}	0.20 ± 0.56 ^{de}
12.5 µM PG + BA	2.13 ± 0.49 ^{d}	2.53 ± 0.24 ^{bcde}	14.67 ± 3.02 ^{ef}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.12 ± 0.03 ^{cde}
25.0 µM PG + BA	2.00 ± 0.39 ^{d}	2.27 ± 0.46 ^{abcde}	27.33 ± 11.45 ^{cdef}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.41 ± 0.20 ^{cd}
5 µM NAA	0.60 ± 0.34 ^{a}	2.00 ± 0.82 ^{ab}	11.33 ± 6.03 ^{abc}	6.67 ± 0.39 ^{abc}	6.67 ± 3.38 ^{abcd}	0.21 ± 0.09 ^{abcd}
6.25 µM PG + NAA	0.73 ± 0.41 ^{ab}	2.00 ± 0.82 ^{ab}	15.33 ± 9.57 ^{abc}	0.67 ± 0.39 ^{abc}	6.67 ± 3.8 ^{abcd}	0.27 ± 0.12 ^{abcd}
12.5 µM PG + NAA	2.53 ± 0.75 ^{d}	4.00 ± 0.79 ^{e}	58.67 ± 12.00 ^{f}	1.80 ± 5.58 ^{d}	17.00 ± 5.58 ^{d}	0.90 ± 0.18 ^{e}
25.0 µM PG + NAA	2.40 ± 0.96 ^{abc}	2.10 ± 0.57 ^{abcde}	6.73 ± 2.14 ^{bcde}	0.40 ± 0.34 ^{abc}	0.30 ± 2.06 ^{abc}	0.39 ± 0.14 ^{bcd}

Values represent mean ± SE, n = 15. Different letters in the same column indicate significant differences between samples at the 5% significance level according to Duncan's Multiple Range Tests.

3.3.4. The growth and development of *Scadoxus puniceus* in a liquid culture system

The initial experiment was conducted to identify and establish a source of meristematic tissue for further development. Since the 1970s, various studies reported extremely regenerative tissue culture protocols based on floral (inflorescence) explants. The current study made use of pedicels with or without the attachment to the ovary, filament and anther. Pedicels failed to regenerate on MS medium without PGRs and thus were not included in the analyses. On MS media supplemented with BA and NAA at equi-molar concentrations, pedicels with the attachment to the ovary, anther and filament produced significantly more shoots than pedicels without this attachment ($U = 14.0$, $df = 28$, $p = 0.0001$; 10.67 ± 1.85, 1.33 ± 0.94 respectively, **Figure 3.4 A & B**).

To determine the effect of liquid and solid media on bulblet production of *S. puniceus*, callus from leaf explants were transferred to liquid or solid media containing 6% sucrose. At the end of two months, callus maintained in liquid media developed healthy shoots while callus in solid media produced bulblets. Shoot clusters were further subcultured in either liquid or solid media. Clusters maintained in liquid medium were significantly superior in length and fresh weight to those maintained on a solid medium ($t = 4.31$ $df = 18$, $p = 0.002$; **Figure 3.4 C & D**).

Given the superiority of liquid to solid media, the influence of cytokinins in culture was investigated with liquid media only. Clusters were separated into individual shoots and maintained in liquid media supplemented with different combinations of PGRs. Compared to control plantlets (6% sucrose) which produced shoot lengths averaging 10 mm in length, MS medium supplemented with BA and NAA significantly improved plant development. The largest plants developed with the combination of 5 µM BA and 1 µM NAA (**Table 3.5, Figure 3.5**). Plantlets in liquid media were healthy and showed no sign of hyperhydricity. Plantlets were successfully rooted on full strength MS medium supplemented with IBA at varying concentration. The most prominent root development occurred with the addition of 1 µM IBA (**Figure 3.6 & 3.7 B)** while the majority of plantlets transferred to solid medium without IBA died prior to root development.

ZIV and LILIEN-KIPNIS (2000) revealed the highly regenerative potential of the junction between the peduncle and pedicel when compared to bulb or corm explants. Following this, inflorescence explants of several families including the Amaryllidaceae, Asparagaceae, Alliaceae and the Iridaceae produced significantly greater buds than bulb and corm explants.

Bud regeneration of the Amaryllidaceae was established with BA and NAA (5:10 µM) supplemented with sodium phosphate, adenine sulphate and activated charcoal (**CHEN and ZIV, 2005**). **FOSTER and GIFFORD (1959)** suggested that the inflorescence is composed of several meristems which form the florets and, if cultured at the appropriate developmental time, can develop into buds given the appropriate hormones.

The current study highlights the importance of liquid cultures for the *in vitro* production of *S. puniceus*. The regeneration of bulblets from callus explants was not achieved with the use of liquid cultures, but despite this, plantlets produced from callus cultures were healthier and larger than those from the solid medium. Furthermore, plantlets developed at a much faster rate when maintained in liquid medium with a combination of cytokinins and auxins.

The liquid shake culture system is the preferred technique for bulbous species with the advantage that it eliminates gradients of the nutrients that are prevalent in static or solid media. **BERGOÑÓN *et al.* (1992)** used liquid shake cultures to improve the regeneration of *Narcissus* plants and promoted the technique as an alternative to twin-scaling. The method also affords repeated cycles of regeneration by successive subcultures thus increasing proliferation **(HUSSEY, 1982; BERGOÑÓN *et al.*, 1992**). Factors that may have contributed to the success of the current study include the elimination of nutrient gradients and the dilution of toxic phenols which are exuded into the medium. As previously discussed, phenolic exudation and oxidative browning are severe challenges associated with the *in vitro* propagation of *S. puniceus*. Liquid culture thus provides an avenue for the propagation of this valuable medicinal plant and in turn will aid in reducing the pressure placed on wild plant material for use in traditional medicine.

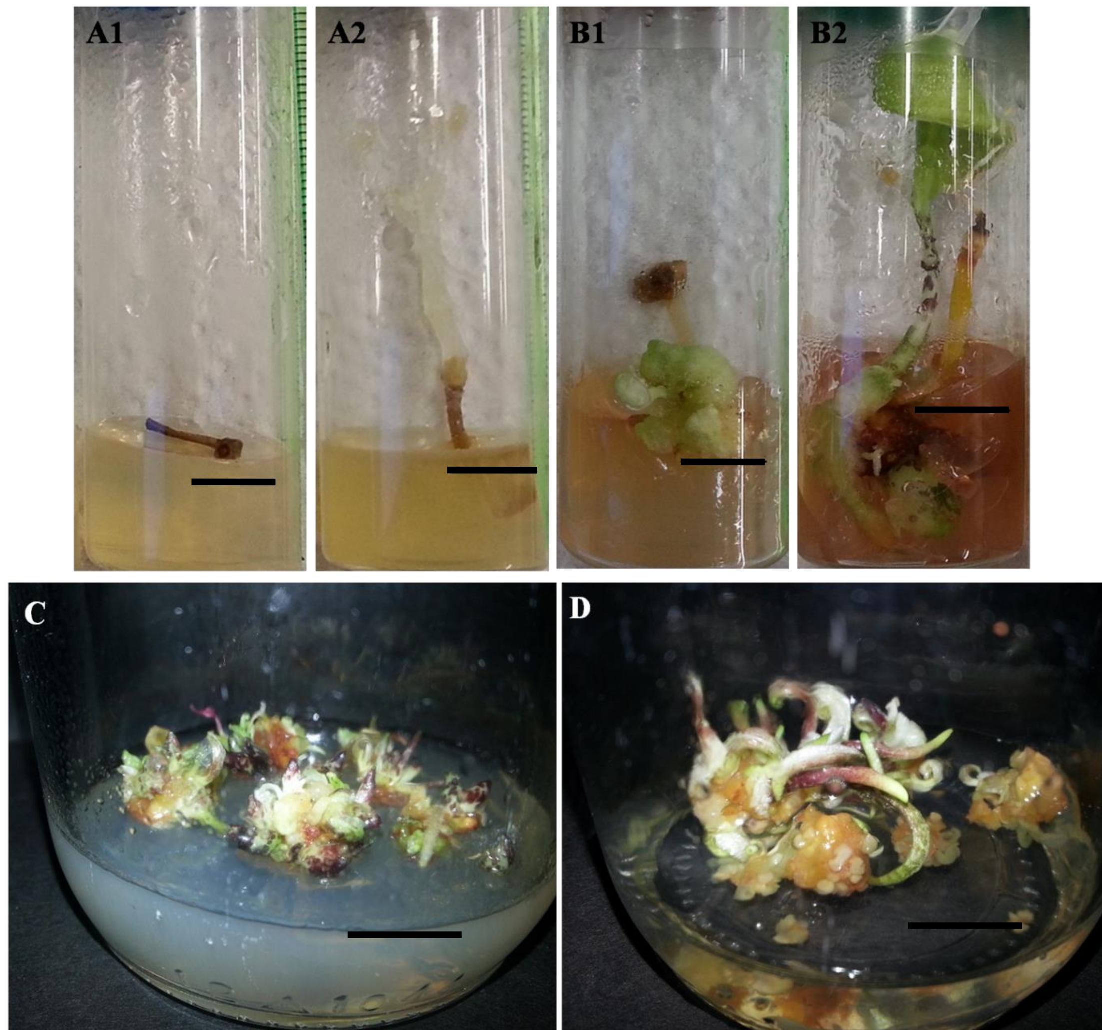


Figure 3.4: Regeneration of *Scadoxus puniceus* from inflorescence explants; pedicel without (**1**) and with (**2**) the attachment to the ovary, anthers and filament on plant growth regulator-free medium (**A**) or Murashige and Skoog (MS) nutrient medium supplemented with benzyladenine and naphthaleneacetic acid (5µM; **B**). Shoot clusters further developed on solid (**C**) or in liquid (**D**) MS medium supplemented with 6% sucrose. Scale bar = 1 cm.

Table 3.5: The development of *Scadoxus puniceus* maintained in liquid media supplemented with different combinations of benzyladenine (BA) and naphthaleneacetic acid (NAA).

Supplements	Shoot length (mm)	Bulb diameter (mm)	Fresh weight (g)
MS + 6% sucrose	10.00 ± 0.00 [a]	1.65 ± 0.21 [a]	0.46 ± 0.08 [a]
5 μM BA : 1 μM NAA	86.50 ± 14.87 [b]	10.20 ± 1.26 [bc]	2.37 ± 0.42 [b]
5 μM BA : 5 μM NAA	67.50 ± 7.31 [b]	6.40 ± 1.07 [b]	1.92 ± 0.37 [b]

Values represent the mean (± SE) of 10 replicates. Different letters represent significant differences at the 5% significance level according to Duncan's Multiple Range Tests.

Figure 3.5: Healthy plantlets produced in liquid media supplemented with benzyladenine and naphthaleneacetic acid (5: 1 μM). Bar = 10 mm.

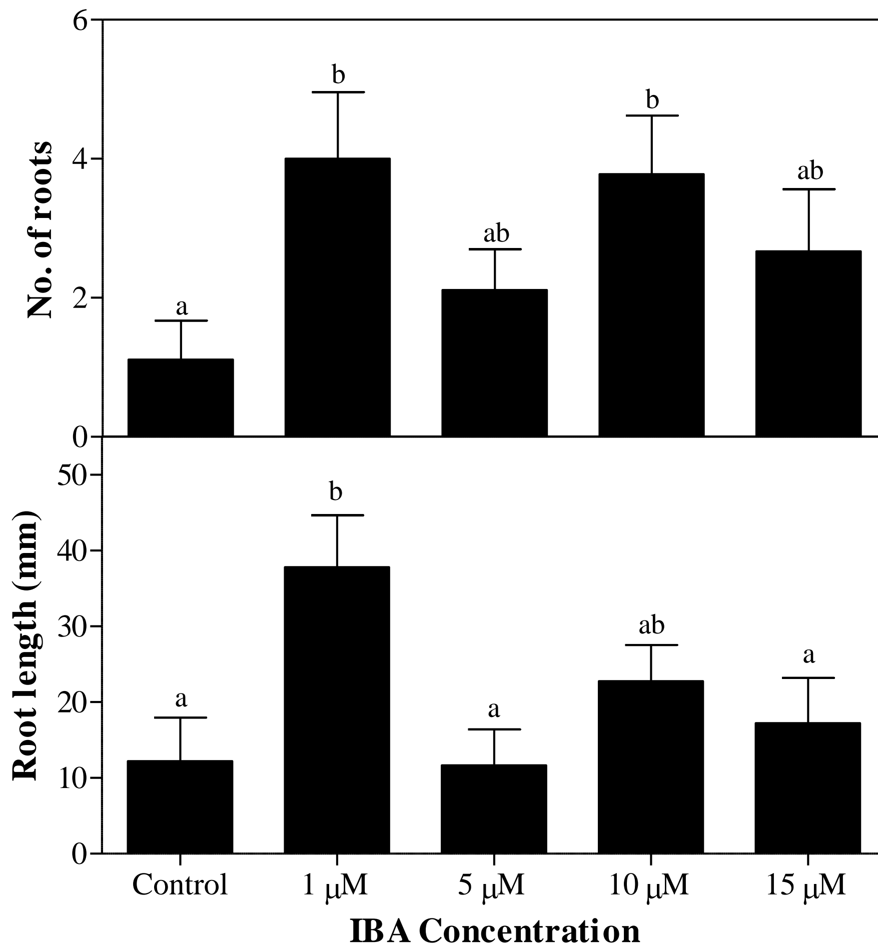


Figure 3.6: Root development of liquid culture-derived *Scadoxus puniceus* plantlets on various concentrations of indole-3-butyric acid. In each group, different letters represent significant differences between samples at the 5% significance level. n = 9.

Figure 3.7: Root development of liquid culture-derived *Scadoxus puniceus* plantlets on various concentrations of indole-3-butyric acid (IBA). A: PGR-free, B: 1 µM IBA, C: 5 µM IBA, D: 10 µM IBA, E: 15 µM IBA. Bar = 10 mm.

3.4. Conclusions

The present **Chapter** aimed at developing an improved *in vitro* propagation protocol for *S. puniceus* by limiting oxidative browning, hyperhydricity and recalcitrance. Based on the data collected, several approaches may be taken to deal with these problems. Improved proliferation rates were achieved with the use of *in vitro*-derived plant material when cultured on MS medium containing BA. Further development of plantlets on MS media or BA supplemented media led to hyperhydricity, a problem that was alleviated by using topolins. Topolins (*m*T specifically) improved the morphological health of plantlets of *S. puniceus* while also maintaining fresh weight and overall plant development.

Phenolic exudation and oxidative browning of twin-scales hindered propagation with the most frequently used explant for Amaryllidaceae *in vitro* culture. The study evaluated the role of two organic phenolic compounds, PG and GCA, and their interaction with PGRs, to improve plant development by reducing oxidative browning. GCA, a competitive inhibitor of PAL, significantly improved the development of both *M. plumbea* (a model species) and *S. puniceus*. Gallic acid improved development by reducing oxidative browning. Explants exhibited reduced red-brown pigmentation which is characteristic of oxidative browning. No significant reductions in phenolic content were noted suggesting that the exudation, rather than the production, of phenolics was inhibited. Given the importance of phenolics in plant development, the results of the current study are of significant importance. Furthermore, the study highlighted the synergistic relationship of GCA with BA and its antagonistic relationship with *m*T. In general, PG seemed to be synergistic with cytokinins and auxins, a relationship that has been documented by several authors. The relationship of PG with an auxin (NAA) was particularly strong, producing a six-fold improvement in plant development. As such, the study highlights the potential of GCA and PG for the alleviation or reduction of oxidative browning, an important challenge associated with the *in vitro* propagation of plants.

The recalcitrance of Amaryllid explants has been demonstrated by several authors; similarly, the recalcitrance of explants of *Scadoxus puniceus* was described in **Chapter 2** of the current thesis. A liquid culture system was therefore established for the production of *S. puniceus* with initial material consisting of highly regenerative inflorescence explants. Although liquid culture may not be the most effective system for bulblet production (which can be achieved with semisolid medium), significantly larger, healthier plantlets (6-8 fold improvement) were

produced when shoot clusters were maintained in a liquid culture system with the addition of BA and NAA.

Scadoxus puniceus is amongst the most highly traded medicinal plant species for use in South African traditional medicine. The frequent overharvesting of mature, slow-growing bulbs will ultimately lead to the decimation of wild populations. The use of *in vitro* propagation techniques may therefore be useful in reducing the strain placed on wild populations. The protocols established in the current **Chapter** are of significant importance, based on the ability to improve or limit the effect of numerous important challenges (hyperhydricity, oxidative browning, and recalcitrance) associated with the *in vitro* propagation of *S. puniceus*.

CHAPTER 4: PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF *SCADOXUS PUNICEUS*

4.1. Introduction

Oxygen is an essential component of life for all aerobic organisms including human beings. The molecule undergoes oxidation and releases energy in the form of adenosine triphosphate, an important energy source for several biochemical processes (**DAVIES, 1995b**). The process of oxidation is thus necessary for survival, however, when unpaired electrons are transferred to the oxygen molecule; the process generates free radicals which have been implicated in the cause of several health complications (**RAMARATHNAM *et al*., 1995; GÜLCIN, 2012**). Free radicals are molecules that possess one or more unpaired electron(s) attached to the orbital. In addition to oxygen which produces reactive oxygen species (ROS), free radicals are also formed from nitrogen (reactive nitrogen species) and sulphur (reactive sulphur species) (**CAROCHO and FERREIRA, 2013**). The ROS include the superoxide anion, hydroxyl radical, hydroperoxyl radical, alkoxyl radical and nitric oxide (**GÜLCIN, 2012; CAROCHO and FERREIRA, 2013**). The reactivity of these radicals vary and they are highly unstable (**GÜLCIN, 2012; CAROCHO and FERREIRA, 2013**). The radicals are also termed oxidants or pro-oxidants given their ability to oxidize several molecules (**HALLIWELL and GUTTERIDGE, 1989; SIES, 1991**).

Free radicals are produced via several metabolic processes including inflammation, phagocytosis, arachidonate pathways, ischemia and physical exercise. The molecules are also produced from the natural metabolism of the mitochondria, and peroxisomes (**CAROCHO and FERREIRA, 2013**). Smoking, the use of drugs and exposure to pollutants, radiation, pesticides, industrial solvents and ozone also promotes the formation of these molecules. Under these conditions, the human body produces more ROS than antioxidants that neutralize the ROS by inhibiting oxidation (**KRISHNAIAH *et al*., 2011; GÜLCIN, 2012**). The imbalance results in oxidative damage to lipids, nucleic acids, proteins and carbohydrates (**ARUOMA, 1998; LEFER and GRANGER, 2000; SMITH *et al*., 2000; LÜ *et al*., 2010; CRAFT *et al*., 2012**). As such, free radicals have been involved in the onset of a significant number of diseases including degenerative diseases (**SHAHIDI *et al*., 1992**), Alzheimer's disease (**DI MATTEO and ESPOSITO, 2003**), cancers (**GERBER *et al*., 2002**), and cardiovascular disorders (**HERTOG *et al*., 1993**). In addition to human health problems, free radicals also have implications in the food and pharmaceutical industries. Lipid peroxidation

by free radicals reduces the quality and shelf life of many food and pharmaceutical products **(KNEKT *et al*., 1996; HALLIWELL, 1997)**.

To combat the impact of free radicals, aerobic organisms possess several antioxidant defences. The most accurate definition of an antioxidant as described by **KHLEBNIKOV *et al*. (2007)** is "any substance that directly scavenges ROS or indirectly acts by up-regulating antioxidant defences or inhibits ROS production". Evolution has allowed for the improvement of the endogenous antioxidant system in humans which is divided into two groups including the enzymatic antioxidants (superoxide dismutase, glutathione peroxidase and catalase, glutathione reductase and glucose-6-phosphate dehydrogenase) and non-enzymatic antioxidants (vitamins, enzyme cofactors, nitrogen compounds and glutathione) **(RAHMAN, 2007; KRISHNAIAH *et al*., 2011; CAROCHO and FERREIRA, 2013)**. However, endogenous antioxidant systems do not suffice and humans often depend on dietary supplements to maintain the balance between ROS and antioxidants **(PIETTA, 2000)**. Several synthetic antioxidants have been developed and incorporated into food products to retard lipid peroxidation and prolong shelf life **(KNEKT *et al*., 1996; GÜLCIN, 2012)**. Of these synthetic compounds, butylated hydroxytolulene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ) and propyl gallate are the most frequently used antioxidants **(GÜLCIN, 2012; CAROCHO and FERREIRA, 2013)**. Concerns over the toxicity and carcinogenic effect of BHT and BHA have prompted a restriction on the allowed daily intake (0.25 mg/kg bw/day and 1.0 mg/kg bw/day, respectively) of these antioxidants **(EFSA, 2011; EFSA, 2012)**. As such, there has been a surge in the search for naturally occurring antioxidants from plant resources **(GÜLCIN, 2007)**.

The bioactivity of natural resources such as plants is attributed to their production of phenolics and flavonoids, two major classes of secondary metabolites **(BOCCO *et al*., 1998)**. Thousands of phenolic compounds have been identified to date, exhibiting varying chemical structures, all of which are characterised by single (hydroxybenzoic and hydroxycinnamic acids) or multiple (flavonoids) hydroxylated aromatic rings **(MUZAFFAR *et al*., 2012)**. The antioxidant activity of phenolics has been attributed to their ability to act as metal chelators and free radical scavengers substantiating the use of plant material in traditional medicine **(KRISHNAIAH *et al*., 2011; CAROCHO and FERREIRA, 2013)**. Phenolics have been labelled as potential natural occurring replacements to BHT and BHA **(BOTTERWECK *et al*., 2000; MOYO *et al*., 2010; LUI *et al*., 2011)**. As such, phenolic compounds are rapidly becoming the preferred alternative to synthetic antioxidants **(BECKER *et al*., 2004)**.

The evaluation and quantification of phenolic acids in plants is vital for the identification of alternate sources of antioxidants for commercial uses. Conventionally, analytical methods such as reverse-phase high-performance liquid chromatography (HPLC), gas chromatography, or capillary electrophoresis are used to determine phenolic components of plants **(AMAKURA *et al*., 2000; AYAZ *et al*., 2005; JIROVSKY *et al*., 2003)**. However, the often multifaceted procedures involved in these methods lead to oxidation and degradation of phenolics rendering the endeavour futile **(GRÚZ *et al*., 2008)**. Ultra high performance liquid chromatography (UHPLC) coupled with electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) has been used successfully in several recent publications for the determination of polyphenols in plants and fruits **(LI *et al*., 2006; GRÚZ *et al*., 2008, AREMU *et al*., 2013)** with the advantage of improved resolution, shorter retention times and higher sensitivity **(YU *et al*., 2006)**.

Given the extensive use of *S. puniceus* in traditional medicine, the current study aimed at determining and quantifying the phenolic acid constituents in different organs of the species using UHPLC-MS/MS. In addition, in light of the fact that phytochemical studies alone cannot replace pharmacological evaluations, the antioxidant activity of different parts of *S. puniceus* was also examined.

4.2. Materials and Methods

4.2.1. Preparation of plant extracts

Whole plant samples of *Scadoxus puniceus* were collected during September 2013 from the botanical garden at the University of KwaZulu-Natal and separated into different organs (leaves, stems, bulbs and roots) and dried in an oven at 50 °C for seven days. Dried plant material were ground into fine powders and used for UHPLC and antioxidant assays. For UHPLC, ground material were homogenized with 80% methanol using an oscillation ball mill (MM 301, Retsch, Haan, Germany) at a frequency of 27 Hz for 3 min. The extracts were centrifuged at 20, 000 rpm for 10 min and the supernatant used for UHPLC. For antioxidant activity, dried plant material was extracted with 50% methanol (RADCHEM Lab Supplies) in a sonication bath containing ice for 40 min. The extracts were filtered through Whatman No. 1 filter paper and concentrated *in vacuo* at 30 °C using a Buchi Rotary evaporator. The extracts were then dried under a stream of cold air at room temperature and used once a constant weight was obtained.

4.2.2. Ultra high performance liquid chromatographic (UHPLC) analysis of phenolic acids

4.2.2.1. Chemicals

Phenolic acid standards namely; gallic acid, 3,5-dihydroxybenzoic acid, protocatechuic acid, chlorogenic acid, gentisic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, 3-hydroxybenzoic acid, 4-coumaric acid, sinapic acid, ferulic acid, 3-coumaric acid, 2-courmaric acid, salicylic acid and trans-cinnamic acid were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Deuterium-labelled standards of 4-hydroxybenzoic acid (2,3,5,6-D4) and salicylic acid (3,4,5,6-D4) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid and methanol were purchased from MERCK (Darmstadt, Germany).

4.2.2.2. Instrumentation and conditions

Samples were analysed using an ACQUITY UHPLC™ system (Waters, Milford, MA, USA) coupled with a PDA 2996 photo diode array detector (PDA, Waters, Milford, MA, USA) and a Micromass Quattro *micro*™ API benchtop triple quadrupole mass spectrometer (Waters, MS Technologies, Manchester, UK), equipped with a Z-spray ESI source operating in negative mode. Instrumentation control, data collection and processing were completed using MassLynx ™ software (version 4.0, Waters, Milford, MA, USA).

Chromatographic conditions of UHPLC and MS/MS settings are as described by **GRÚZ *et al.* (2008)**. Briefly, sample extracts (supernatants) were filtered through 0.45 µm nylon membrane filters (Alltech, Breda, Netherlands) and injected into a reversed phase column (BEH C_8, 1.7 µm, 2.1 x 150 mm, Waters, Milford, MA) maintained at 30 °C. The sequence of linear gradients and isocratic flows in the mobile phase are presented in **Table 4.1**. The column was subsequently equilibrated under initial conditions for 2.5 min. The chromatographic run was completed under pressure ranging from 4000 to 8000 psi. The effluent was inserted into a PDA detector scanning at a range of between 210 to 600 nm with a resolution of 1.2 nm. The effluent was then passed through an electrospray source exhibiting a source block temperature of 100 °C, desolvation temperature of 350 °C, capillary voltage of 2.5 kV and cone voltage of 16 eV. Argon was used as the collision gas (16 eV) with nitrogen the desolvation gas (500 l/h).

Table 4.1: The sequence of linear gradients and isocratic flows of solvents in the mobile phase of the reversed phase UHPLC.

Sequence	Solvent	Duration (min)
1	5% B	0.8
2	5-10% B	0.4
3	Isocratic 10% B	0.7
4	10-15% B	0.5
5	Isocratic 15% B	1.3
6	15-21% B	0.3
7	Isocratic 21% B	1.2
8	21-27% B	0.5
9	27-50% B	2.3
10	50-100% B	1.0
11	100-5%	0.5

Solvent B: Acetonitrile, balanced with 7.5 mM formic acid at a flow rate of 250 µl min^{-1}

4.2.3. Antioxidant activity of plant extracts

Structure-activity relationship studies have discovered several factors that influence the antioxidant potential of phenolics. These include the number and position of hydroxyl groups and other substituents as well as the glycosylation of flavonoid molecules **(CAI *et al.*, 2006)**. The variability and complexity of their activity has prompted studies that include at least two assays for the determination of antioxidant activity **(MOON and SHIBAMOTO, 2009)**.

4.2.3.1. 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity

Determination of antioxidant activity with the use of the DPPH assay is based on the ability of the extract to scavenge the radical anion $DPPH^{\cdot}$. $DPPH^{\cdot}$ is able to accept an electron or hydrogen atom creating the diamagnetic DPPH molecule. $DPPH^{\cdot}$ absorbs visible light at 515 nm appearing red-violet in colour, the reduced form (DPPH) loses the violet colour and appears pale yellow in the presence of a protic solution **(LITESCU and RADU, 2010)**. Antioxidants are electron or hydrogen donors, which allows for their evaluation by use of the DPPH assay.

The free radical scavenging activity of *S. puniceus* extracts was determined using the DPPH assay as described by **KARIOTI *et al.* (2004)**. Dried plant extracts were redissolved to 50 mg/ml in 50% aqueous methanol and used immediately. In a test tube (under dim light), 15 µl of each plant extract was diluted with methanol (735 µl) and added to a freshly prepared methanolic DPPH solution (750 µl, 0.1 mM, Sigma-Aldrich). The reaction mixture was incubated in the dark for 30 min at room temperature after which the absorbance was read at 517 nm with an ultra violet-visible spectrophotometer (Varian Cary 50). Ascorbic acid (ASC) and BHT (50 mg/ml, BDH Biochemicals Ltd. Poole, England) were used as positive controls while a reaction mixture containing 50% methanol instead of the sample was used as a negative control. To correct for the colour of the extract, the absorbance reading of the samples without DPPH was subtracted from readings of samples in the presence of DPPH. Each sample extract was evaluated in triplicate. The free radical scavenging activity (RSA) of plant extracts was determined by the decolouration of the DPPH solution and was calculated according to the following formula:

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

where A_E represents the absorbance of the reaction mixture containing the plant extract, ASC or BHT and A_D represents the absorbance of the DPPH solution only.

4.2.3.2. *β*-Carotene/ linoleic acid (BCA) model system

Antioxidant evaluations based on the BCA model relies on the ability of an antioxidant to slow the bleaching of *β*-carotene caused by radicals formed from the oxidation of linoleic acid. Antioxidant activity of plant extracts was determined with the BCA model system as described by **AMAROWICZ *et al.* (2004)** with modifications. Dried plant extracts were redissolved to 50 mg/ml in 50% methanol and used immediately in the assay. *β*-Carotene (20 mg, Sigma-Aldrich) was dissolved in chloroform (1 ml, MERCK, Darmstadt, Germany) in a Schott bottle covered with foil. Thereafter, linoleic acid (200 µl, FLUKAR) and Tween 20 (2 ml, BDH Biochemicals Ltd. Poole England) were added to the *β*-carotene. The solution was adjusted to 500 ml with aerated distilled water bringing the final concentration of *β*-carotene to 40 µg/ml. The mixture was agitated vigorously to form an orange-coloured emulsion which was used immediately in the assay. The reaction mixture contained the emulsion (2.4 ml) and each of the plant extracts (50 mg/ml, 100 µl). The initial absorbance of the mixture was measured immediately after the addition of the sample extract or BHT (positive control) at 470 nm. The mixture was further incubated at 50 °C in a water bath and the absorbance

measured every 30 min for 2 h. A negative control consisting of 50% methanol was used instead of the sample extract. The rate of β-carotene bleaching was calculated according to the following formula:

Rate of β-carotene bleaching = ln ($A_{t=0}/A_{t=t}$) x 1/t

where $A_{t=0}$ indicates the absorbance of the emulsion at 0 min and $A_{t=t}$ indicates the absorbance at time after 30 min intervals (30, 60, 90 min). The rate of β-carotene bleaching was determined by the average of the rates at 30, 60 and 90 min which were then used to determine the antioxidant activity (ANT). The ANT of plant extracts was determined and expressed as a percentage according to the following formula:

% ANT = ($R_{control}$ - R_{sample})/$R_{control}$ x 100

where $R_{control}$ and R_{sample} represent the average β-carotene bleaching rates for the negative control and plant extracts, respectively.

4.2.3.3. Ferric-reducing antioxidant power (FRAP) assay

Antioxidant evaluations based on the FRAP assay relies on the reduction of the complex ferric tripyridyl triazine at low pH. Antioxidants act as reducing agents, reducing ferric ions to ferrous ions which produce an intense Perl's Prussian blue, absorbing light at 630 nm **(LAI *et al.*, 2001; LITESCU and RADU, 2010)**. The ferric reducing power of plant extracts can be determined by the difference of absorbance between a reaction mixture containing ferrous ions of known concentration and the reaction mixture containing the plant extract **(LITESCU and RADU, 2010)**.

The ferric reducing power of *S. puniceus* extracts was evaluated according to **LIM *et al.* (2009)** with modifications as per **MOYO *et al.* 2010**. Dried plant extracts, ASC and BHT were dissolved in 50% aqueous methanol to a concentration of 50 mg/ml. In a 96-well microplate, 30 µl of each extract were serially diluted two-fold with 30 µl of distilled water. Fourty microlitres each of potassium phosphate buffer (0.2 M, pH 7.2) and potassium ferricyanide (1% w/v, BDH Biochemicals Ltd. Poole, England) were then added to the wells. The reaction mixture was incubated at 50 °C for 20 min in the dark after which, 40 µl of trichloroacetic acid (10% w/v), 150 µl of distilled water and 30 µl ferric chloride (0.1% w/v, MERCK, Darmstadt, Germany) were added to each well. The plate was incubated for a

further 30 min at room temperature in the dark and the absorbance measured at 630 nm using an Opsys MR™ micro-plate reader (Dynex Technologies Inc., Chantilly VA).

4.2.4. Data analysis

All data were subjected to a One-Way-Analysis of variance using SPSS software version 21 for Windows. Where there were significant differences ($p = 0.05$), the means were separated using Duncan's Multiple Range Tests. Calculation of EC_{50} values were conducted on GraphPad Prism Version 4.00 for Windows (GraphPad, Software Inc.).

4.3. Results and Discussion

4.3.1. Phenolic acid profile

The evaluation of *S. puniceus* lead to the identification and quantification of 13 phenolic acids, the concentrations of which differed significantly amongst leaf, stem, bulb and root extracts (**Table 4.2**). A greater profusion of hydroxycinnamic acids (HCAs) accumulated in *S. puniceus* accounting for approximately 69.59% of the total phenolic acids present in plant tissues. Hydroxybenzoic acids (HBAs) make up a further 30.38% while the single isoflavone accounted for a scanty 0.02%. Hydroxycinnamic acid concentrations were higher in leaf extracts as compared to stems, bulbs and roots, with chlorogenic acid (CGA; 5-caffeoyl-$_{D}$-quinic acid) being the most concentrated. The distribution of HBAs amongst plant tissues varied; leaf extracts revealed an accumulation of protocatechuic acid while syringic and vanillic acids were concentrated in root extracts. The concentration of both syringic and vanillic acids increased gradually from aerial to underground parts, with the greatest accumulation occurring in the roots (25.29; 52.90 μg/g DW). The hydroxybenzoate, *m*-hydroxybenzoic acid was identified only in stem extracts while genistein (isoflavone) was quantified only in leaves (chemical structures presented in Appendix 3).

Although the alkaloids within the Amaryllidaceae have been extensively investigated, very little is known of their phenolic acid composition. **BATE-SMITH (1968)** initially revealed the presence of phenolics in the Amaryllidaceae with the use of thin-layer chromatography. Later, **NIKOLOVA and GEVRENOVA (2005)** quantified the phenolic acids in *Pancratium maritimum*, *Galanthus elwesii* (initially reported by **TÜZEN and ÖZDEMİR, 2003**), *Sternbergia colchiciflora*, *Galanthus nivalis*, and *Leucojum aestivum* with the use of HPLC. Apart from these studies, knowledge of the metabolism of phenolic acids in the Amaryllidaceae has been limited. Of the 13 phenolic acids identified in *S. puniceus*, seven

including caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, syringic and vanillic acids have been reported to be present in the Amaryllidaceae **(TÜZEN and ÖZDEMİR, 2003; NIKOLOVA and GEVRENOVA, 2005)**. The current study is the first to identify chlorogenic, sinapic, gallic and *m*-hydroxybenzoic acids within an Amaryllid species.

In general, benzoic and cinnamic acids (phenylpropanoids) are derived from the shikimate pathway from L-phenylalanine or L-tyrosine, mediated by either phenylalanine ammonia-lyase or tyrosine ammonia-lyase respectively **(Figure 4.1) (HERRMANN, 1995; RICE-EVANS *et al.*, 1996; ROBBINS, 2003)**. The production of HCAs involves the deamination of phenylalanine or tyrosine which creates the *trans*-double bond in the cinnamic acid backbone. Hydroxylation of the aromatic ring at position 4 yields *p*-coumaric acid, while further hydroxylation, methylation and dehydration reactions yield several other cinnamic acid derivates including caffeic, ferulic and sinapic acid **(Figure 4.1)**. Chlorogenic acid forms from an ester bond between quinic and caffeic acids. There exist two hypotheses that describe the formation of benzoic acids; one suggests that they form as a result of side chain degradation in hydroxycinnamic acids while the other suggests that an intermediate in the shikimate pathway mediates their formation (**HERRMANN, 1995**).

SIKORSKA *et al.* (2000) suggested that phenolic acids are not distributed uniformly across plant organs, being physically dispersed in seeds, leaves, roots and stems **(MACHEIX *et al.*, 1990; SHAHIDI and NACSK, 1995)**. Similarly, HCA and HBA concentrations varied between organs of *S. puniceus*. However, it is clear that HCAs are the main phenolic acid constituents in *S. puniceus*. Hydroxycinnamic acids accumulated more intensely in aerial organs, 49.6% of which was accounted for by CGA. The production of CGA is often induced by stress when it is produced in greater concentrations **(DIXON and PAIVA, 1995; TORRES-CONTRERAS *et al.*, 2014)**. Furthermore, when CGA is oxidized it forms chlorogenoquinone which binds to free amino acids and proteins. Amino acids become less available and proteins become less digestible by herbivores **(FELTON *et al.*, 1989)**. As such, CGA is synthesised as a functional chemical defence against herbivory. The toxicity of CGA to various insect species including caterpillars (**BERNAYS *et al.*, 2000; MALLIKARJUNA *et al.*, 2004**), beetles **(FULCHER *et al.*, 1998)**, leafhoppers **(DOWD and VEGA, 1996)** and aphids **(MILES and OERTLI, 1993)** had been reported previously. Upon collection of *S. puniceus* for investigation, it had been noted that several of the plants were infested with the Amaryllis borer (*Brithys crini*) which mines the leaves and seeds (fruit) of species within the

Amaryllidaceae. As such, CGA may have been produced at greater concentrations as a direct result of the stress caused by the leaf miner which would in turn explain the selective accumulation in the leaves and stems of the plant only.

Another major phenolic acid present in leaves was *p*-coumaric acid which has been found to be a constituent of cutin. Cutin is responsible for providing a structural basis for the plant cuticle and comprises a polymer of hydroxyl fatty acids **(RILEY and KOLATTUKUDY, 1975)**. The leaves of *S. puniceus* have a waxy cuticle, which may account for the accumulation of *p*-coumaric acid in the leaves.

Table 4.2: The composition of phenolic compounds in leaves, stems, roots and bulbs of *Scadoxus puniceus*

Derivative	Compound	Hydroxyl (OH) constituents		Concentration/ plant part (µg/g DW)				
		OH position	Number of OH	Leaf	Stem	Bulb	Root	Total
Hydroxycinnamic acids	Caffeic acid	3,4-OH	2	23.66 ± 2.30 [c]	10.51 ± 2.36 [b]	1.52 ± 1.60 [a]	0.53 ± 0.08 [a]	**36.22**
	Chlorogenic acid	3,4-OH	2	137.99 ± 2.80 [b]	117.56 ± 48.89 [b]	1.32 ± 0.29 [a]	0.08 ± 0.01 [a]	**256.95**
	Ferulic acid	4-OH	1	16.96 ± 1.24 [c]	16.86 ± 1.99 [c]	3.96 ± 0.45 [a]	10.43 ± 0.26 [b]	**48.21**
	p-Coumaric acid	4-OH	1	70.35 ± 4.45 [c]	8.47 ± 3.03 [a]	2.70 ± 0.28 [a]	34.52 ±0.70 [b]	**116.04**
	Trans-cinnamic acid	None	0	37.53 ± 1.71 [b]	3.30 ± 0.21 [a]	0.65 ± 0.01 [a]	0.61 ± 0.03 [a]	**42.09**
	Sinapic acid	4-OH	1	12.66 ± 0.61 [b]	1.99 ± 0.20 [a]	1.78 ± 0.55 [a]	1.73 ± 0.71 [a]	**18.16**
Total HCA				**299.15**	**158.69**	**11.93**	**47.90**	**517.67**
Hydroxybenzoic acids	Gallic acid	3,4,5-OH	3	5.53 ± 0.07 [d]	2.80 ± 0.11 [c]	0.49 ± 0.02 [b]	0.27 ± 0.02 [a]	**9.09**
	m-Hydroxybenzoic acid	3-OH	1	0.00 ± 0.00 [a]	0.18 ± 0.02 [b]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	**0.18**
	p-Hydroxybenzoic acid	4-OH	1	15.03 ± 0.30 [d]	3.94 ± 0.14 [b]	2.95 ± 0.76 [a]	12.50 ± 0.12 [c]	**34.42**
	Protocatechuic acid	3,4-OH	2	61.83 ± 1.19 [d]	17.93 ± 0.66 [c]	4.68 ± 0.11 [b]	1.96 ± 0.06 [a]	**86.4**
	Syringic acid	4-OH	1	0.99 ± 0.00 [a]	1.04 ± 0.12 [a]	1.76 ± 0.14 [b]	25.29 ± 0.10 [c]	**29.08**
	Vanillic acid	4-OH	1	3.67 ± 0.08 [a]	4.91 ± 0.32 [b]	5.38 ± 0.33 [b]	52.90 ± 0.69 [c]	**66.86**
Total HBA				**87.05**	**30.80**	**15.26**	**92.92**	**226.03**
Isoflavone	Genistein	5,7,4'-OH	3	0.15 ± 0.01 [b]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.15
Total HCA & HBA				**386.35**	**189.49**	**27.19**	**140.80**	**743.83**

Values represent mean ± SE of three replicates. Different letters in the same row indicate significant differences in concentrations between plant parts at the 5% significance level (Duncan's Multiple Range Test). Values in bold represent totals for their respective columns or rows.

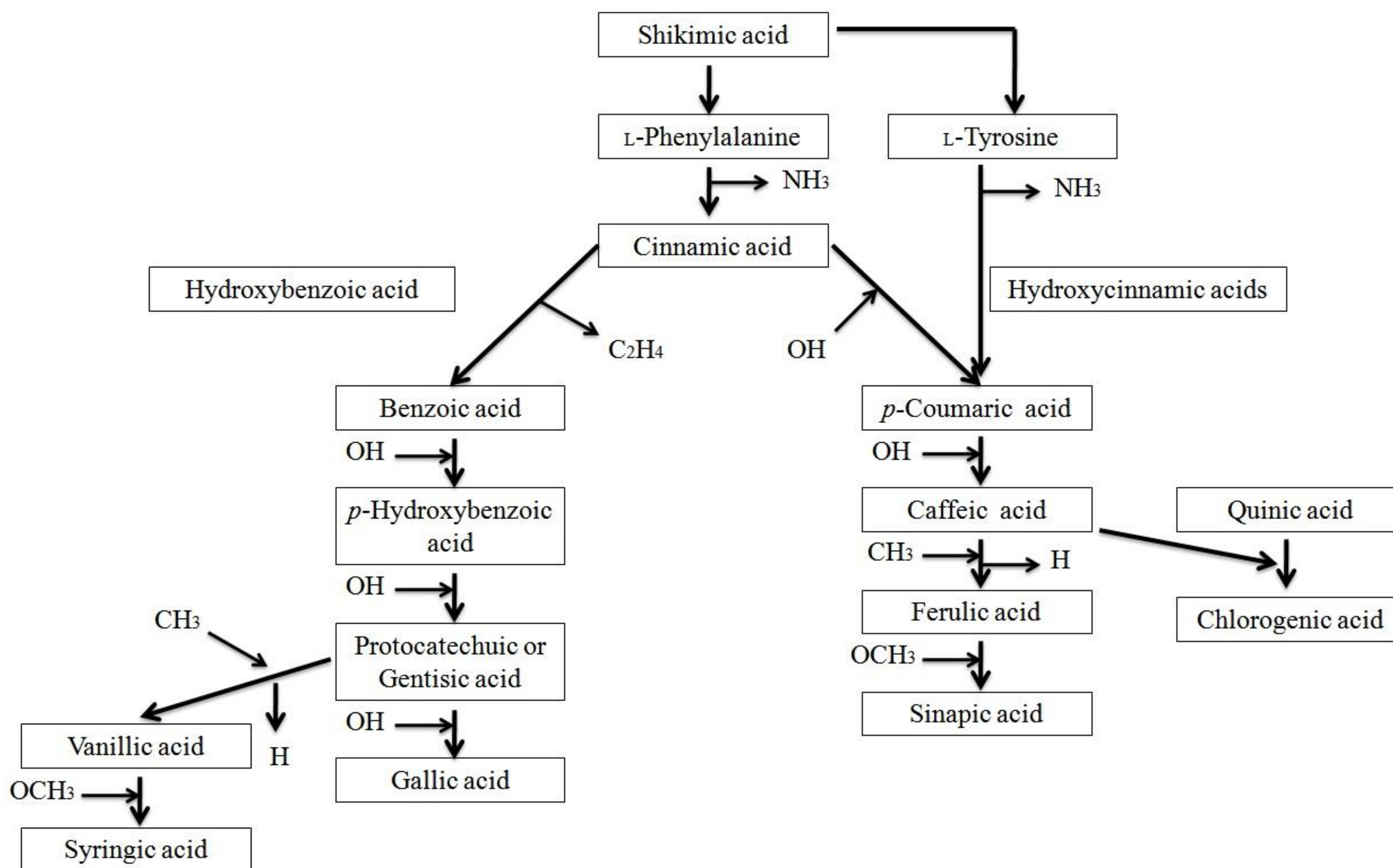


Figure 4.1: The proposed biosynthesis of phenolic acids which are present in *Scadoxus puniceus* via the shikimate pathway (adapted and modified from: **HELENO *et al*., 2015**).

4.3.2. Antioxidant activity

The antioxidant activity of *S. puniceus* plant extracts as determined by the DPPH radical scavenging assay is illustrated in **Table 4.3**. All extracts possessed the ability to donate an electron or hydrogen atom leading to a decrease in the absorbance of the reaction mixture. However, the proportion of activity differed significantly between the organs. Leaf, root and stem extracts presented the strongest radical scavenging activity (91.61 & 89.20, 85.80%, respectively), significantly greater than bulb extracts. Furthermore, leaf extracts possessed a significantly lower EC_{50} value (0.07 mg/ml) than the other organs tested. The antioxidant activity of bulb extracts was extremely low, exhibiting maximum inhibition below 50% at the highest concentration tested.

Table 4.3: 2,2-Diphenyl-1-picryhydrazyl radical scavenging activity (% RSA) of different parts of *Scadoxus puniceus* methanolic extracts.

Sample extract	Plant part	% Yield	% RSA	EC_{50} Values
Scadoxus puniceus	Leaf	35.55	**91.61 ± 0.42** ^{d}	**0.07** ^{b}
	Stem	45.65	**85.80 ± 0.15** ^{b}	0.30 ^{c}
	Bulb	32.75	38.64 ± 0.84 ^{a}	ND
	Root	22.30	**89.20 ± 0.31** ^{c}	0.30 ^{c}
Ascorbic acid	-		98.06 ± 0.55 ^{e}	0.03 ^{a}
BHT	-		101.00 ± 0.12 ^{f}	0.01 ^{a}

Values indicate mean ± SE of three replicates. Different letters associated with% RSA indicate significant differences at the 5% level of significance. ND: extracts exhibiting maximum inhibition below 50% at the highest concentration tested.

The ferric reducing antioxidant power based on the ability of plant extracts and BHT to reduce ferricyanide (Fe^{3+}) to its ferrous form (Fe^{2+}) is presented in **Figure 4.2**. Antioxidant activity differed significantly between extracts as well as the concentration at which they were tested. Leaf extracts exhibited the highest reducing power, not significantly different to that of BHT at the highest concentrations tested (4 and 5 µg/ml). Bulb extracts possessed the weakest reducing power correlating to the lowest antioxidant activity.

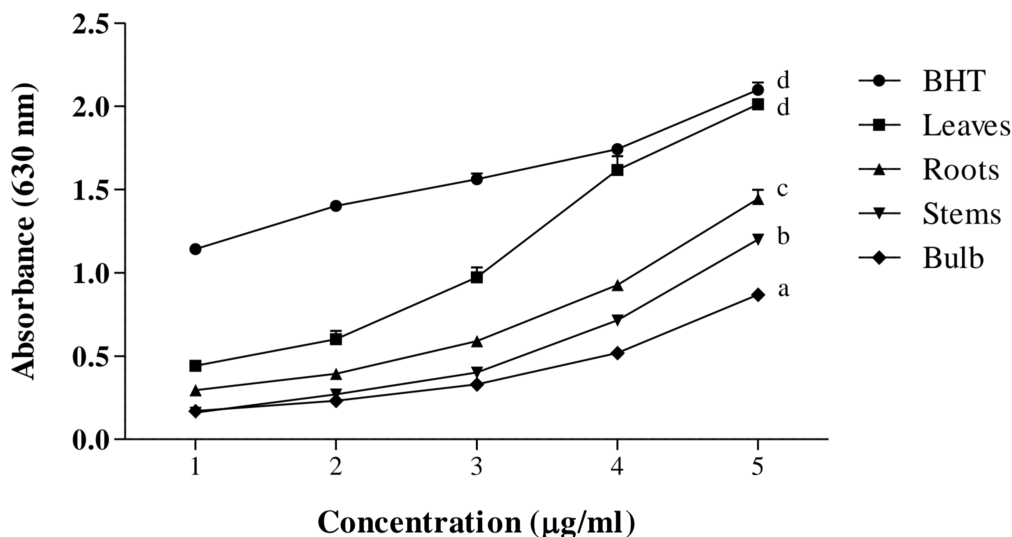


Figure 4.2: The dose dependent ferric ion-reducing power of extracts of *Scadoxus puniceus*. BHT; butylated hydroxyltolulene. Different letters indicate significant differences between samples at the highest concentration tested (5 µg/ml). n = 3.

The ability of plant extracts to prevent the coupled oxidation of *β*-carotene and linoleic acid is presented in **Table 4.4**. The antioxidant activity which was calculated based on the average rate of heat induced *β*-carotene bleaching was high for root and leaf extracts relative to BHT (80.22 & 76.25 and 86.2% respectively). The lowest activity was recorded for bulb extracts (60.70%). The oxidation rate ratio of extracts ranged from the most potent being 0.19 for root extracts to the least potent being 0.39 for bulb extracts. The antioxidant activity based on the inhibition of the oxidation of *β*-carotene was further determined at t = 60, 90 and 120 min. The highest activity was recorded for bulb extracts, increasing from 91.20 to 95.89% at 60 and 120 min. However, the antioxidant activity of all *S. puniceus* extracts was similar and sometimes significantly superior to that of BHT.

Table 4.4: Prevention of the coupled oxidation of *β*-carotene and linoleic acid by extracts from different parts of *Scadoxus puniceus*.

Sample extract	Plant part	% ANT	ORR	AA_{60}	AA_{90}	AA_{120}
Scadoxus puniceus	Leaf	76.25 ± 0.37 [b]	0.24 ± 0.00 [c]	80.10 ± 1.76 [a]	76.67 ± 3.01 [a]	79.88 ± 1.81 [a]
	Stem	74.54 ± 0.29 [b]	0.25 ± 0.00 [d]	78.58 ± 0.48 [a]	79.34 ± 0.94 [ab]	88.45 ± 6.19 [ab]
	Bulb	60.70 ± 0.84 [a]	0.39 ± 0.01 [e]	91.20 ± 0.93 [b]	93.53 ± 1.06 [c]	95.89 ± 1.36 [b]
	Root	80.22 ± 0.57 [c]	0.19 ± 0.01 [b]	80.05 ± 3.91 [a]	84.29 ± 0.53 [b]	83.78 ± 1.65 [a]
BHT	-	86.2 ± 1.86 [d]	0.17 ± 0.00 [a]	73.38 ±1.45 [a]	76.12 ± 1.83 [a]	79.27 ± 1.30 [a]

Values indicate mean ± SE of three replicates. BHT = butylated hydroxytoluene. % ANT = Antioxidant activity calculated according to the rate of *β*-carotene bleaching at t = 60, 90 and 120 min. ORR: oxidation rate ratio, the lower the value the stronger the activity. AA_{60}, AA_{90}, AA_{120} = % antioxidant activity of the extract or BHT at t = 60, 90 or 120 min. Different letters in the same column indicate significant differences at the 5% level of significance.

Researchers have suggested that the chemical structure of polyphenols make them ideal compounds for free-radical scavenging activities. As such, leaf extracts which constituted the highest amount of HCAs possessed the highest antioxidant activity based on the DPPH and FRAP assays. However, root extracts possessed comparable activity in the aforementioned assays and exhibited the strongest affinity to prevent the oxidation of β-carotene despite containing significantly lower concentrations of all HCAs. The degree of activity of polyphenols varies and is not necessarily associated with the quantity at which it occurs in the plant **(MATKOWSKI, 2008)** but is rather influenced by several factors including the number and position of hydroxyl groups in the molecule, the compounds reactivity as a hydrogen or electron donor, its reactivity with other antioxidants, its metal-chelating potential and the fate of the antioxidant derived radical **(RICE-EVANS *et al.*, 1996)**.

The proposed biochemical pathway for the development of phenolic acids is presented in **Figure 4.1.** Monohydroxybenzoic acids that possess a hydroxyl group in a *meta* position have been shown to be effective hydroxyl radical scavengers as a result of the improved reactivity of the hydroxyl radical and their propensity to hydroxylation **(GROOTVELD and HALLIWELL, 1986; RICE-EVANS *et al.*, 1996)**. The antioxidant activity of dihydroxybenzoic acids is dependent on the positions of the hydroxyl group in the benzene ring **(RICE-EVANS *et al.*, 1996)**. The dihydroxybenzoate protocatechuic acid which exhibits the *meta*, *para* (3,4-dihydroxybenzoic) distribution of hydroxyl groups has a slightly improved antioxidant activity than the monobenzoates **(RICE-EVANS *et al.*, 1996)**. However, methylation of monohydroxybenzoic acids as in vanillic or homoprotocatechuic acids reduces the impact of the carboxyl group which improves the antioxidant activity seen in dihydroxybenzoic acids. Despite the substitution of hydroxyl groups with methoxy groups as in syringic acid which reduces the antioxidant activity, the compounds antioxidant activity is still greater than that of monohydroxy acids. The trihydroxybenzoate, gallic acid (3,4,5-trihydroxy benzoic acid) possesses the strongest antioxidant activity of the benzoic acids given the availability of three hydroxyl groups. The current study revealed the presence of gallic acid in all organs however; leaf extracts contained a significantly higher concentration of this compound. Hydroxybenzoic acids are characterised by the presence of a carboxylate group, which drastically reduces their ability to donate an electron or hydrogen atom. For this reason, hydroxycinnamic acids are generally more effective antioxidants than the hydroxybenzoic acids **(RICE-EVANS *et al.*, 1996)**. In *p*-coumaric acid, the presence of an ethylenic group which carries a *p*-hydroxyl and carboxylate group improves the antioxidant

activity of this compound. Further dihydroxlyation of *p*-coumaric acid reduces the antioxidant activity as seen in caffeic acid that possesses similar activity as protocatechuic acid. Similarly, glysosylation of the carboxyl group in caffeic acid has no effect on the antioxidant activity, rendering CGA which possesses the same activity as protocatechuic and caffeic.

As such, in addition to the significantly higher concentrations of CGA in the leaves of *S. puniceus*, the presence of significant amounts of *p*-coumaric, gallic and protocatechuic acids which possess improved antioxidant activity, accounts for the superior activity of leaves over the other organs. However, despite the high concentration of CGA in stem extracts, their lower concentrations of *p*-coumaric and protocatechuic acids and the presence of significantly higher amounts of *p*-coumaric and vanillic acids in root extracts substantiates the fact that the latter organ possesses a higher antioxidant activity than the former.

4.4. Conclusions

The phenolic acid metabolism in the Amaryllidaceae has been largely overlooked due to the plant's production of pharmaceutically important alkaloids. UHPLC-MS/MS yielded 13 phenolic compounds which were distributed in a non-uniform pattern spread throughout different organs of the plant. The current study is the first to document the presence of chlorogenic, sinapic, gallic and *m*-hydroxybenzoic acids within an Amaryllid species. HCAs were the major group of phenolic acids present in the species accounting for almost 70% of the total phenolic acids. The accumulation of CGA in the leaves and stems of *S. puniceus* may indicate a functional role of chlorogenic acid as a natural defence against the Amaryllis leaf borer.

The accumulation of phenolic acids such as CGA, vanillic, protocatechuic and *p*-coumaric acids in various parts of the plant may play a role in the observed antioxidant activity either as single compounds or a combination of several. As such, the biosynthetic capabilities of *S. puniceus* to accumulate phenolic compounds with medicinal potential may provide a means for obtaining useful products for drug development.

CHAPTER 5: *IN VITRO* ANTIMICROBIAL AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF *SCADOXUS PUNICEUS* EXTRACTS

5.1. Introduction

5.1.1. Infectious diseases and human health

Throughout the history of mankind, infectious diseases caused by microorganisms such as bacteria, fungi, parasites and viruses have been a major cause of human mortality (**HEMAISWARYA *et al*., 2008**). Ironically, for the treatment of microbial infections, humans have exploited a trait that allows microbes to survive and thrive. In the same way that flora and mega-fauna compete for resources within an ecosystem, bacterial and fungal colonies produce several chemicals to overcome competition within their respective ecosystems (**NIGAM *et al*., 2014**). The observation made by Alexander Fleming in 1928 that the fungal species *Penicillium notatum* inhibited microbial development by producing a substance now known as penicillin has paved the way for the development of numerous antibiotics that eradicated infections that once claimed hundreds of thousands of human lives (**BENNETT and CHUNG, 2001**). However, the haphazard overuse of antibiotics coupled with the genetic capacity of bacteria to develop resistance to drugs, have resulted in large-scale multi-drug resistance in human pathogenic microorganisms (**SINGH and BARRETT, 2006; MOHANASUNDARI *et al*., 2007**). For instance, it is estimated that approximately 90-95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (**CASAL *et al*., 2005**) while in Asian countries; 70-80% of the strains are resistant to methicillin (**CHAMBERS, 2001**). Furthermore, the resistance of *S. aureus* to vancomycin, the last line of defence for antibiotics has also been demonstrated (**NEU, 1992; TRAVIS, 1994**). *Staphylococcus aureus* is a normal component of the natural flora of human skin and mucous membranes, the bacterium facilitates the development of minor skin infections (pimples, boils and abscesses) as well as serious, life-threatening diseases (septic arthritis, meningitis and pneumonia) (**SLEIGH & TIMBURG, 1998; HEYMANN, 2004**). Considering the threat that microbial resistance to antibiotics poses, coupled with the development of multidrug-resistant *Mycobacterium tuberculosis* and the current AIDS epidemic, the situation has been accepted globally as a serious health issue (**DANCER, 2001; BERGER, 2002; LIVERMORE, 2004**).

The Food and Drug Administration (FDA) has since reduced the number of antibiotics that have been approved. Of the 22 new antimicrobial drugs that the FDA did approve, 12 are derived from natural products (**GRAUL and PROUS, 2005; BUTLER and BUSS, 2006**). Natural products such as those obtained from plants have been used for therapeutic purposes since the existence of mankind (**SAMUELSSON, 2004; RATES, 2001**) and today, comprise an interesting, yet largely unexploited source of medicines (**GIRISH and SATISH, 2008**). The production of drugs from natural resources is not only cost effective but also reduces the potential for the development of resistance (**McGAW *et al*., 2000**). As such, a surge in ethnopharmacological studies validating the medicinal properties of plants including, but not limited to, their antimicrobial, anti-inflammatory and anti-tumor activities have been noted (**JÄGER *et al*., 1996; KELMANSON *et al*., 2000; MASOKO *et al*., 2007; LAMORAL-THEYS *et al*., 2009**). The evaluation of medicinal plants for their proposed bioactivity is thus an essential component of drug development from natural products.

5.1.2. Neurodegenerative disorders

Alzheimer's disease (AD) is a frequently occurring central nervous system (CNS) disorder which is associated with a progressive degeneration of memory and cognitive function, the build-up of amyloid plaques and the formation of neurofibrillary tangles (**LÓPEZ *et al*., 2002; DALL'ACQUA, 2013**). Alzheimer's disease exerts an influence on the elderly population of developed countries and whilst being the most frequent cause of dementia, creates a major concern for the healthcare sector and society as a whole (**DALL'ACQUA, 2013**). The development of AD is said to be multi-factorial, and dependent on a person's genetics, diet, lifestyle and the environment in which he/she lives (**STAFFORD *et al*., 2008; CITRON *et al*., 2002; WILLIAMS *et al*., 2011**).

The most widely accepted hypothesis that describes the pathological development of the disease is the "cholinergic" hypothesis which suggests that the degeneration of a patient's memory is a direct result of a reduction in cholinergic function in the brain (**PERRY, 1986; DALL'ACQUA, 2013**). Cholinergic function is reduced when the activity of acetylcholine (ACh) is suppressed by acetylcholinesterase (AChE), an enzyme that hydrolyses the ester bond in ACh rendering the molecule less effective (**HOUGHTON *et al*., 2005**). For the treatment of AD, studies have focused on identifying drugs such as acetylcholinesterase (AChE) inhibitors that improve acetylcholine levels. Several plant-derived alkaloids have

been associated with effective AChE inhibition; these compounds have become important drugs for the treatment of AD.

The first molecule that was investigated for the treatment of AD was physostigmine, an alkaloid which was isolated from seeds of *Physostigma venenosum*. The compound was associated with several side effects and a narrow therapeutic index and was thus not approved. In 1993, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine; derived from the structure of alkaloids) became the first AChE inhibitor that was approved and introduced for the treatment of AD **(TUMIATTI *et al*, 2010; DECKER, 2005)**. The compound exhibited potent inhibitory activity against AChE and butyrylcholinesterase (BChE; hydrolyses butyrycholine a synthetic compound which is used to differentiate between AChE and BChE receptors) **(FERNANDEZ-BACHILLER *et al.*, 2010)**. Since then, several other compounds including donepezil, rivastigmine, galanthamine, and metrifonate have been introduced to the market. Currently three drugs exhibiting AChE inhibitory activity including galanthamine (Amaryllidaceae alkaloid), rivastigmine (alkaloid-related, synthetic compound), and donepezil are available commercially for the treatment of AD **(MEHTA *et al.* 2011)**. However, in addition to side effects including gastrointestinal disturbances, these compounds occur at very low concentrations in plants leading to reduced availability **(SCHULZ, 2003; MELZER, 1998)**. For these reasons, there still exists a need for the identification and development of compounds that possess lower toxicity and improved CNS penetration **(DALL'ACQUA, 2013)**.

5.1.3. Drug discovery from plants

Drug discovery from plants is an intricate process which frequently involves the collection of species based on Indigenous Knowledge Systems (IKS). The identification and an understanding of plants used in traditional medicine are essential factors in determining the potential pharmacological activity of the species as well as the extraction method that is most suited. After a plant of interest is collected, the plant material is air dried, dried by incubating it in an oven or by lyophilisation and further ground into fine powders **(WILLIAMSON *et al.*, 1996; RATES, 2001)**. To validate the medicinal activity of the identified species, extracts of the plant are prepared and subjected to a series of pharmacological assays **(BALUNAS and KINGHORN, 2005)**. **TIWARI *et al.* (2011)** defined the process of extraction as "the separation of medicinally active portions of plant tissue using selective solvents through standard procedures". Inert solvents such as water, alcohol, acetone, dichloromethane (DCM)

and ether are used to extract plant material by solubilising compounds with similar polarity **(NCUBE *et al.*, 2008)**. The use of different solvents allows for the extraction of different chemicals such as alkaloids, glycosides, phenolics or a mixture of several chemical groups **(HANDA *et al.*, 2008)**. The techniques frequently employed to obtain an extract include maceration, infusion, hot continuous extraction or ultrasound (in a sonicator) extraction in the presence of a solvent **(TIWARI *et al.*, 2011)**. Plant extracts are then subjected to a number of *in vitro* bioassays depending on the plants proposed activity based on IKS. Bioassay-guided fractionation allows for the isolation and characterisation of the active compounds in promising extracts **(BALUNAS and KINGHORN, 2005)**.

The current study employed a similar approach to determine the antimicrobial activity and AChE inhibition of extracts of *Scadoxus puniceus*; a species used frequently in South African traditional medicine as an antiseptic on sores and ulcers and is known to cause CNS excitation or depression and hallucinations.

5.2. Materials and Methods

5.2.1. Preparation of extracts

Whole plant samples of *S. puniceus* were collected during September 2013 and a voucher specimen (Naidoo 02) was prepared and deposited at the Bews Herbarium (UKZN, Pietermaritzburg). The plant was separated into different parts including leaves, stems, bulbs, basal plate and roots and dried in an oven at 50 °C for seven days. Dried material was then ground into fine powders and stored at room temperature in the dark until use. The ground samples were extracted with 20 ml/g of DCM, petroleum ether (PE) or ethanol (MERCK, Darmstadt, Germany) separately in a sonication bath containing ice for 1 h. The extracts were filtered through Whatman No. 1 filter paper and concentrated *in vacuo* at 35 °C using a Buchi Rotary evaporator. The concentrated solvent extracts were dried at room temperature under a stream of cold air.

5.2.2. Antibacterial microdilution assay

The minimum inhibitory concentrations (MIC) of extracts for antibacterial activity were determined using the microdilution bioassay as described by **ELOFF (1998)**. Overnight cultures (incubated at 37 °C in a water bath with an orbital shaker) of two Gram-positive (*Micrococcus luteus* ATCC 4698 and *Staphylococcus aureus* ATCC 12600) and two Gram-

negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacterial strains were diluted with 20 ml sterile Mueller-Hinton (MH) broth (200 µl in 19.8 ml). The dried crude extracts were resuspended in 50% dimethylsulfoxide (DMSO) to a final concentration of 25 mg/ml. The dried water extracts were dissolved in water to give a final concentration of 25 mg/ml.

In a 96-well microplate, 100 µl of each extract were serially diluted two-fold with 100 µl of sterile distilled water for each bacterial strain. One hundred microlitres of each bacterial culture were added to each well. A two-fold serial dilution of neomycin (Sigma-Aldrich, Steinheim, Germany) (0.1 mg/ml) was used as a positive control against each bacterial strain while water was used as the negative control and 50% DMSO, the solvent control. The microplates were covered with parafilm and incubated at 37 °C for 24 h. Subsequently, 50 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) was added to each well to determine bacterial growth. Plates were covered with parafilm and incubated for a further 1 h. The presence of biologically active organisms reduces the colourless tetrazolium salt to a red product, thus allowing for the MIC values to be observed as the concentrations in the wells in which no colour change was observed after addition of the INT indicator. Inhibition of bacterial growth by the extracts was indicated by clear wells while reddish-pink wells indicated bacterial growth. The bioassay was repeated twice with three replicates each. Total activity of extracts was calculated as per **ELOFF (2004)**.

5.2.3. Antifungal microdilution assay

In order to determine the antifungal activity of the extracts of *S. puniceus*, a microdilution method as described by **ELOFF (1998)** and modified for fungi by **MASOKO *et al.* (2007)** was used. An overnight culture of *Candida albicans* (ATCC 10231) (incubated at 37 °C in a water bath with an orbital shaker) in yeast malt (YM) broth was prepared. Four hundred microlitres of the fungal culture were added to 4 ml of sterile saline and the absorbance was read at 530 nm. The absorbance was adjusted to match that of the 0.5 M McFarland standard solution. From this solution, a 1:1000 dilution with sterile YM broth was prepared producing a final inoculum of approximately 10^6 CFU/ml. The dried crude extracts were resuspended in 50% DMSO to a final concentration of 25 mg/ml. The dried water extracts were dissolved in water to give a final concentration of 25 mg/ml.

In a 96-well microplate, 100 µl of each extract were serially diluted two-fold with 100 µl of sterile distilled water. One hundred microlitres of the dilute fungal culture were added to each well. As a positive control, a similar two-fold serial dilution of 2.5 mg/ml Amphotericin B (Sigma-Aldrich, Germany) was used while water and 50% DMSO were the negative and solvent controls, respectively. The microplates were covered with parafilm and incubated at 37 °C for 24 h. Fifty microlitres of 0.2 mg/ml INT were added and incubated for another 24 h at 37 °C. Clear wells indicate inhibition of fungal activity while reddish-pink wells indicate fungal growth. The lowest concentrations of extracts that inhibited fungal growth were recorded as the MIC values. The bioassay was repeated with two replicates each. Total activity of extracts was calculated as per **ELOFF (2004)**.

5.2.4. Microplate assay for determination of acetylcholinesterase (AChE) inhibitory activity

The microplate assay described by **ELDEEN *et al.* (2005)**, and outlined by **MOYO *et al.* (2010)** was used to determine the AChE inhibitory activity. Chemicals used in the study including acetylthiocholine iodide (ATCI), 5,5'-dithiobio-(2-nitro-benzoic acid) (DTNB), acetylcholinesterase (AChE; from electric eel, type VI-S) were acquired from Sigma-Aldrich (Steinheim, Germany). The buffers used in the assay were as follows; buffer A - 50 mM Tris-HCl (pH 8.0), buffer B – 50 mM Tris-HCl, (pH 8.0) which contained 0.1% bovine serum albumin (0.5 g), buffer – C; 50 mM Tris-HCl (pH 8.0) which contained 0.1 M NaCl, and 0.02 M $MgCl_2.6H_2O$.

In a 96-well microplate, 25 µl of plant extracts (diluted to 25 mg/ml with 100% methanol) were added to sterile distilled water and serially diluted. Following this, 25 µl of ATCI, (15 mM), 125 µl of 3 mM DTNB in buffer C, and 50 µl of buffer B were added. The absorbance of the reaction mixture was read at 405 nm using an Opsys MR microplate reader three times every 45 sec. Subsequently, 25 µl of 0.2 U/ml AChE was added and the absorbance read once again every 45 sec (five times). The rate of enzyme reaction was calculated based on the absorbance readings. To determine the inhibition of AChE, the rates of enzyme reaction of the plant extracts was compared to a blank containing water. Galanthamine (20 µM in the assay) was used as a positive control. Inhibition of AChE was calculated as a percentage based on the following equation:

$$\% \text{ Inhibition} = 1 - [RR_{sample}/RR_{control}] \times 100$$

Where RR_{sample} is the rate of enzyme reaction of the plant extracts and $RR_{control}$ is that of the blank.

5.2.5. Data analysis

Data pertaining to the AChE inhibitory activity of plant extracts were subjected to a One-Way-Analysis of variance using SPSS software version 21 for Windows. Where there were significant differences ($p = 0.05$), the means were separated using Duncan's Multiple Range Tests. Calculation of IC_{50} values were conducted on GraphPad Prism Version 4.00 for Windows (GraphPad, Software Inc.).

5.3. Results and Discussion

5.3.1. Antimicrobial activity

The antibacterial MIC values of extracts of *S. puniceus* are presented in **Table 5.1**. The most susceptible bacterium was *K. pneumoniae* while the bacteria that were most resistant to the plant extracts were *M. luteus* and *E. coli*. The strongest MIC recorded which was also the minimum concentration required for the inhibition of *K. pneumoniae* and *S. aureus* growth was 0.39 mg/ml which was achieved by PE extracts of bulbs of *S. puniceus*. DCM extracts of the bulb and leaves also produced MIC values which were less than 1 mg/ml for these two strains. Ethanolic extracts of the bulb also showed strong activity against *K. pneumoniae* only. PE extracts of the bulb and DCM extracts of leaves exhibited good activity (1.56 mg/ml) against *E. coli* and *M. luteus*.

The antifungal MIC values of extracts of *S. puniceus* are presented in **Table 5.2**. The leaf, stem and basal plate extracts revealed excellent activity against *C. albicans*. All extracts of the leaves and basal plate and the DCM extract of the stem of *S. puniceus* produced MIC values of less than 1 mg/ml.

Table 5.1: *In vitro* antibacterial activity (expressed as MIC mg/ml) of different extracts of organs of *Scadoxus puniceus* determined using the microdilution technique.

Plant Part	**Solvent**	**% Yield**	**Antibacterial MIC (mg/ml)**				**Total Activity (ml/g)**			
			Bacteria							
			M. l	*S. a*	*E. c*	*K. p*	*M. l*	*S. a*	*E. c*	*K. p*
Leaves	Ethanol	6.11	3.14	1.56	1.56	1.56	194.58	391.66	391.66	391.66
	Dichloromethane	3.53	1.56	**0.78**	1.56	**0.78**	226.28	452.56	226.28	452.56
	Petroleum Ether	2.07	1.56	1.56	3.14	3.14	132.69	132.69	65.92	65.92
Stems	Ethanol	W	3.14	3.14	3.14	3.14	W	W	W	W
	Dichloromethane	1.88	3.14	3.14	3.14	3.14	59.87	59.87	59.87	59.87
	Petroleum Ether	2.42	>6.25	3.14	>6.25	3.14	38.72	77.07	38.72	77.07
Bulbs	Ethanol	7.85	3.14	1.56	3.14	**0.78**	250.00	503.20	250.00	**1006.41**
	Dichloromethane	0.82	3.14	**0.78**	3.14	**0.78**	26.11	105.12	26.11	105.12
	Petroleum Ether	8.23	1.56	**0.39**	1.56	**0.39**	527.56	**2110.25**	527.56	**2110.25**
Basal plate	Ethanol	12.17	>6.25	> 6.25	>6.25	1.56	194.72	194.72	194.72	780.12
	Dichloromethane	8.04	>6.25	>6.25	>6.25	1.56	128.64	128.64	128.64	515.38
	Petroleum Ether	4.72	ND	ND	ND	ND	ND	ND	ND	ND
Roots	Ethanol	W	>6.25	>6.25	>6.25	3.14	W	W	W	W
	Dichloromethane	1.10	1.56	1.56	3.14	3.14	70.51	70.51	35.03	35.03
	Petroleum Ether	0.43	>6.25	3.14	>6.25	3.14	6.88	13.69	6.88	13.69

Values represent the mean of three replicates. MIC, minimum inhibitory concentration; *E. c.*, *Escherichia coli*; *S. a.*, *Staphylococcus aureus*; *K. p., Klebsiella pneumoniae*; *M. l., Micrococcus luteus*; Values written in bold are considered very active (≤ 1mg/ml); ND., Not determined. W., crude extracts contained oils as such the dry weight could not be determined.

Table 5.2: *In vitro* antifungal activity (expressed as MIC mg/ml) of different extracts of organs of *Scadoxus puniceus* against *Candida albicans* determined using the microdilution technique

Plant Part	Antifungal MIC (mg/ml) against *C. albicans*			Total activity (ml/g)		
	Solvent			Solvent		
	EtOH	DCM	PE	EtOH	DCM	PE
Leaves	**0.20**	**0.05**	**0.20**	**6110**	**7060**	**1089.474**
Stems	>6.25	**0.78**	>6.25	W	241.0256	38.72
Bulbs	>6.25	nd	nd	125.6	nd	nd
Basal plate	**0.10**	**0.20**	**0.39**	**12418.37**	**4123.077**	**1210.256**
Roots	1.56	nd	nd	W	nd	nd
Amphotericin B	0.039					

Values represent the mean of three replicates. MIC, minimum inhibitory concentration; EtOH., ethanol; DCM., Dichloromethane; PE., Petroleum ether. Values written in bold are considered very active (≤ 1mg/ml); ND., Not determined. W., crude extracts contained oils as such the dry weight could not be determined.

Species within the Amaryllidaceae are frequently used traditionally for treating wounds and infections, including *S. puniceus*. Bulbs of the plant are used to treat wounds while the leaves are used as an antiseptic on sores and ulcers **(VAN WYK *et al.*, 1997)**. The results of the current study thus validate the use of this species in traditional medicines. However, it is worth mentioning that the results of the current study are contradictory to a recent study evaluating the pharmacological activity of *S. puniceus* bulbs that were purchased from a *muthi* store. **NDHLALA *et al.* (2011)** revealed poor antimicrobial activity of bulb PE, DCM, ethanol and water extracts against the same range of bacteria and the fungus used in the current study (except *M. luteus*). The difference in activity between the two studies accentuates the role of storage **(STAFFORD *et al.*, 2005)**, geographical distribution and environmental stress **(RATES, 2001)** in the maintenance and diversity of biological activity.

Crude plant extracts are considered potentially useful if they possess MIC values of less than 8 mg/ml while the MIC of isolated compounds is recommended to be lower than 1 mg/ml **(FABRY *et al.*, 1998; GIBBONS, 2005)**. The observed activity of crude extracts of *S. puniceus* that possessed MIC values of less than 1 or 0.1 mg/ml against bacteria and fungi suggests the potential for antibiotic drug development from the species. The activity of *S.*

puniceus may be due to the plant's production of secondary metabolites as a natural defence against pathogens. The preparation of extracts for traditional medicinal purposes is usually achieved by producing aqueous infusions or decoctions of the plant material. However, in *in vitro* bioassays, water extracts often exhibit poor biological activity **(RABE and VAN STADEN, 1997; CHEESMAN, 2013; NDHLALA *et al*., 2011)**. As such, the current investigation made use of ethanol, DCM and PE in order to acquire an assortment of secondary compounds contained by crude extracts that are present in the plant.

Bulbs extracted with either polar (ethanol) or non-polar (DCM and PE) solvents possessed activity against both Gram-negative and Gram-positive bacteria suggesting that bulbs of the species contain several active components that confer antibacterial activity. The use of PE as a solvent is employed frequently for the extraction of fatty acids **(COWAN, 1999)**. Fatty acids (FAs) occur as ubiquitous molecules, attached to other compounds including glycerol, sugars or phosphate head-groups **(DESBOIS and SMITH, 2010).** In nature, FAs function as antimicrobial defences in plants and animals **(HEMSWORTH and KOCHAN 1978; WEBER, 2002; RICKRODE, 1986)**. Several researchers have demonstrated the antimicrobial activity of FAs **(RUSSELL, 1991; OUATTARA *et al*., 1997)**. Furthermore, **WILLE and KYDONIEUS (2003)** revealed that FAs are the most potent antimicrobial agents present on the skin of human beings. **LACEY and LORD (1981)** demonstrated the sensitivity of *S. aureus* to linolenic acid, an essential omega-3 fatty acid which occurs naturally in seeds, nuts and vegetables. The potent activity of *S. puniceus* PE extracts of bulbs against bacteria and leaves against fungi may thus be as a result of the presence of fatty acids that possess strong antibacterial activity.

The current study also revealed broad spectrum antibacterial activity of DCM leaf extracts which ranged from good (1.56 mg/ml) to strong (0.78 mg/ml) and potent antifungal activity (< 0.1 mg/ml) against *C. albicans*. DCM is used for the selective extraction of terpenoids from plant material **(TIWARI *et al*., 2011)**. Structurally, terpenoids are acetate units attached to FAs and exhibit extensive branches and cycles. Bioactivity guided fractionation has allowed for the isolation and investigation of several terpenoids that possess antimicrobial activity **(GRIFFIN *et al*., 1999; ANKLI *et al*., 2000; MARIAJANCYRANI *et al*., 2013)**. Terpenoids are components of essential oils which have shown strong antibacterial activity against *E. coli*, *S. aureus* as well as methicillin-resistant *S. aureus* **(WILLIAMS *et al*., 1989; CARSON *et al*., 1995)**. The activity of terpenoids has been suggested to be a result of the

lipophilic property of the terpenes which are present in the molecule, as well as the functional groups and their aqueous solubility **(KNOBLOCH *et al.*, 1986 & 1988)**. In addition, as previously discussed, chlorogenic acid (CGA) was concentrated in the leaves of *S. puniceus* **(CHAPTER 4)**. **SUNG and LEE (2010)** demonstrated the antifungal activity of CGA against *C. albicans* which was attributed to the compounds ability to disrupt the structure of the cell membranes. The excellent activity of leaf extracts of *S. puniceus* may thus be due to the presence and activity of CGA.

Ethanolic extracts of *S. puniceus* organs exhibited moderate to low activity against both Gram-positive and Gram-negative bacteria. The polarity of ethanol allows for the extraction of several groups of compounds in plants which include tannins, polyphenols, polyacetyllenes, flavonols, terpenoids, sterols and alkaloids **(TIWARI *et al.*, 2011)**. However, in the current study, the polarity of ethanol was increased by adding water (20%) to bring the final concentration to 80%. The change in polarity may have altered the composition of compounds that were extracted which resulted in a reduction of the antimicrobial activity. *Scadoxus puniceus* has been reported to contain potent antibacterial alkaloids and thus the lack of activity is unexpected. Similarly, upon investigation of the bulbs of *Boophone disticha* (Amaryllidaceae), **CHEESMAN (2013)** found that 80% ethanol extracts possessed poor antibacterial and antifungal activity. Although the antibacterial activity was poor, the current study revealed the potent antifungal activity of leaf and basal plate ethanol extracts.

5.3.2. Acetylcholinesterase inhibitory activity

The AChE inhibitory activity of extracts of *S. puniceus* is presented in **Figure 5.1**. AChE inhibitory activity was influenced significantly by the specific organ (F = 115.34; df = 4; p = 0.001), the concentration (F = 544.70; df = 4; p = 0.001) at which they were tested and the interaction (F =5.67; df = 16; p = 0.001) between the two factors. Ethanolic extracts in general possessed the highest inhibitory activity compared to DCM and PE extracts all of which were highly dose dependent. As such, ethanolic extracts of the bulb of *S. puniceus* exhibited the highest inhibitory activity (96.6 ± 0.3%) which was obtained at 1 mg/ml. This was however not significantly different to bulb (0.5 mg/ml) leaf (1 mg/ml) and basal plate extracts (1 and 0.5 mg/ml). Again, bulbs produced the highest inhibition when extracted with DCM (85.2%) while the basal plate produced the highest AChE inhibitory activity when extracted with PE (79.4%).

The IC_{50} values representing the AChE inhibitory activity of plant extracts are presented in **Table 5.3**. Generally, the values were all less than 1 mg/ml. The IC_{50} values of ethanol extracts ranged from 0.07 to 0.18 mg/ml. Bulb and basal plate extracts possessed the strongest activity, although this was significantly different to leaf extracts, their activity was comparable. The IC_{50} values for DCM and PE extracts were higher ranging from 0.18 to 0.40 mg/ml. Leaves, stems and roots extracted with PE exhibited maximum inhibition below 50% at the highest concentration tested and thus IC_{50} values could not be calculated.

Several species within the Amaryllidaceae have been documented for their use in traditional medicine to treat mental illnesses **(NEUWINGER, 2000)**. As such, the family has been extensively investigated for their effects on the CNS. In addition to galanthamine, several pharmaceutically important alkaloids for AChE inhibition have been isolated from the family including sanguinine, and huperzine **(HEINRICH and TEOH, 2004; BAI *et al.*, 2000)**. The South African grouping of the genus *Scadoxus* consists of three species, *S. puniceus*, *S. multiflorus* and *S. membranaceous*. Similar to the effects of *S. puniceus* on the CNS which has been outlined earlier, *S. multiflorus* is also used in Tanzania to treat mental illnesses **(CHHABRA *et al.*, 1987)**. The reason for their activity has been said to be largely due to their production of alkaloids, either as single or combinations of several compounds **(VILADOMAT *et al.*, 1997; BASTIDA *et al.*, 2006)**.

The AChE inhibitory activity of *S. puniceus* bulbs has been investigated **(NDHLALA *et al.*, 2011; ADEWUSI and STEENKAMP, 2011)** however, the current study is the first to document similar activity of ethanolic leaf extracts. Similar to their antimicrobial activity, the AChE activity of bulbs was grossly underestimated (IC_{50} = 271.90 µg/ml) by **NDHLALA *et al.* (2011)** for the reasons mentioned earlier. As previously discussed, ethanol extracts several groups of compounds including alkaloids and polyphenols **(TIWARI *et al.*, 2011)**. The polyphenolic content of bulbs is low (27.19 µg/g DW, **CHAPTER 4**) while **ORHAN *et al.* (2007)** revealed weak AChE activity of phenols including chlorogenic, caffeic and gallic acids, which are major phenolic acid components of leaf extracts. Thus the similar activity between the two organs suggests that leaves of *S. puniceus* contain similar groups of alkaloids although at different concentrations. **BAY-SMIDT *et al.* (2011)** identified alkaloids of the lycorine, montanine, crinine and tazettine types **(JIN, 2009)** in the bulbs of *S. puniceus*. Various studies have suggested that the most potent alkaloids for AChE inhibitors are those that belong to the galanthamine and lycorine types. In addition, the alkaloid, 6-β-

hydroxycrinamine extracted from bulbs of *B. disticha* recently revealed an IC_{50} value of 445 μM (**ADEWUSI *et al*., 2012**). Although galanthamine alkaloids do not occur within *S. puniceus*, the activity of this species may be due to the presence of lycorine and crinine type alkaloids. The isolation and biological activity of compounds from *S. puniceus* will be discussed in detail in the subsequesnt chapter (**Chapter 6**).

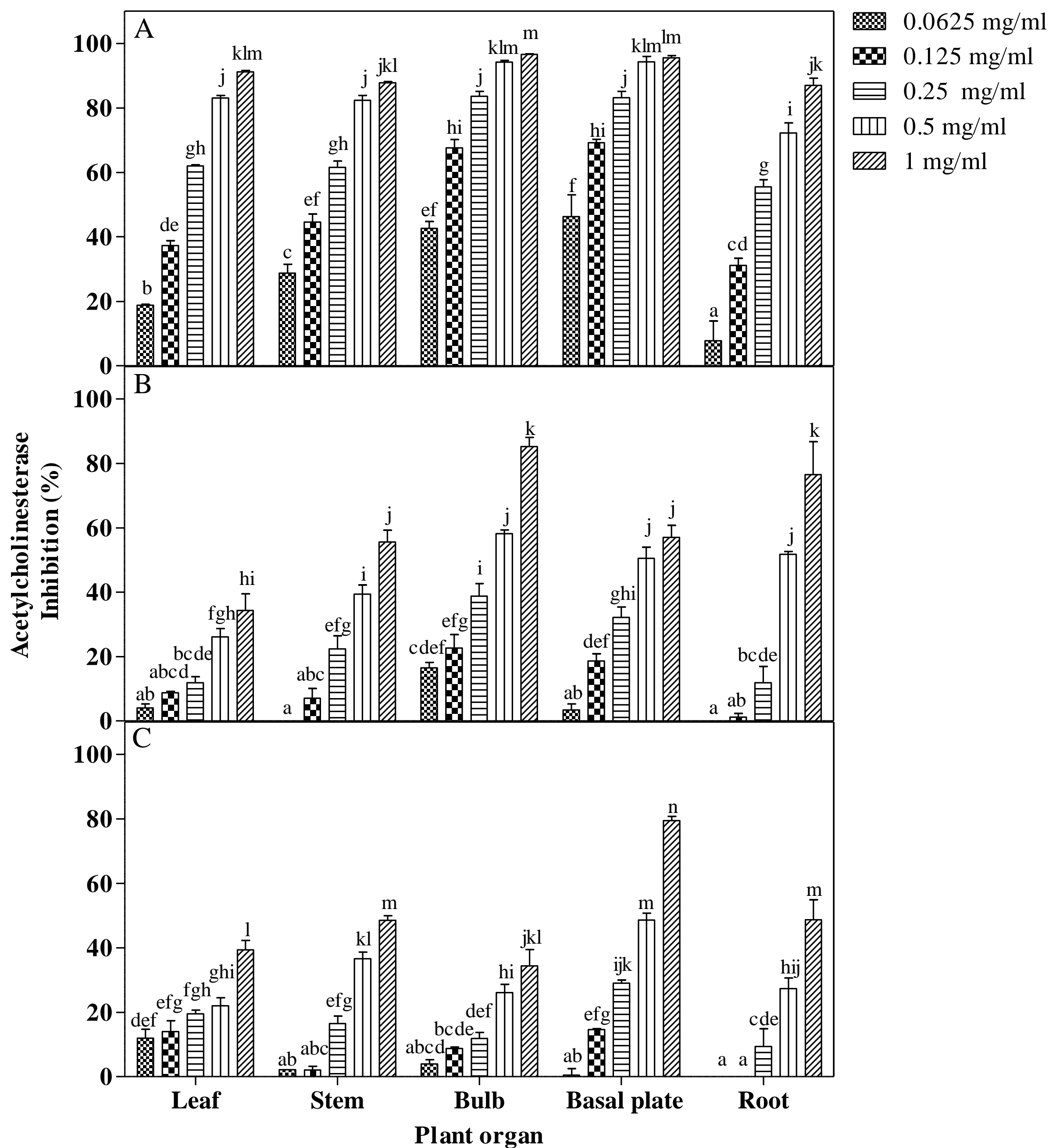


Figure 5.1: The dose dependent acetylcholinesterase inhibitory activity of different organs of *Scadoxus puniceus*. (A) 80% ethanol extracts, (B) dichloromethane extracts, (C) petroleum ether extracts. In each graph, different letters indicate significant differences among samples (within each solvent) at the 5% significance level according to Duncan's Multiple Range Tests. n = 3.

Table 5.3: The IC_{50} (mg/ml) acetylcholinesterase inhibitory values of extracts of *Scadoxus puniceus*.

Solvent	Leaves	Stems	Bulbs	Basal Plate	Roots
Ethanol	0.15 ± 0.00 [c]	0.12 ± 0.01[b]	0.07 ± 0.00[a]	0.07 ± 0.01 [a]	0.18 ± 0.01 [d]
DCM	0.32 ± 0.05 [bc]	0.31 ± 0.04 [abc]	0.25 ± 0.02 [ab]	0.20 ± 0.01 [a]	0.40 ± 0.02 [c]
PE	ND	ND	0.18 ± 0.01 [a]	0.32 ± 0.02 [b]	ND

Values represent the mean ± SE of three replicates. Different letters represent significant differences at the 5% significance level. ND: extracts exhibiting maximum inhibition below 50% at the highest concentration tested. IC_{50} for Galanthamine = 0.27 μM.

5.4. Conclusions

Infectious diseases caused by bacteria and fungi, and age-related disorders such as AD still remain a significant burden on the health and well-being of populations worldwide. The former being the second-leading cause of human mortality, while the latter affects 36 million elderly people worldwide. Despite the success of drug development, the side effects, abuse or incorrect use and resistance associated with synthetic drugs has advocated the search for new, less harmful and more effective drugs. Natural resources such as plants, being termed 'chemical warehouses', offer a platform for the development of new effective drugs.

Organs of *S. puniceus* were evaluated for their proposed antimicrobial and AChE inhibitory activities based on IKS. Bulbs of *S. puniceus* exhibited potent activity against both Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria with the lowest MIC value of 0.39 mg/ml. Leaf extracts also exhibited similar activity against the same bacterial strains (0.78 mg/ml). The presence of CGA in leaf extracts conferred potent activity against *C. albicans* producing excellent MIC values ranging between 0.050 and 0.20 mg/ml, thus validating the species common name 'King of Candida' in traditional medicine **(VAN WYK *et al.*, 1997)**.

Organs of *S. puniceus* exhibited a dose dependent AChE inhibitory activity the strongest of which was presented by bulbs, exhibiting an IC_{50} value of 0.07 mg/ml (96.58%). Although it required a higher concentration, leaf extracts also inhibited AChE up to 91.30%. The activity of ethanolic extracts was attributed to the presence of alkaloids which have been investigated extensively and used commercially for the treatment of AD as AChE inhibitors.

The activities of *S. puniceus* highlighted in the current study may provide a platform for the development of drugs for the treatment of microbial infections as well as age-related disorders such as AD.

CHAPTER 6: ISOLATION AND IDENTIFICATION OF THE BIOACTIVE COMPOUNDS FROM *SCADOXUS PUNICEUS*

6.1. Introduction

The global human population is currently estimated at 7.4 billion people, growing at an approximate rate of 1.13% per annum. A continually growing population demands water, food, shelter, energy and suitable healthcare infrastructure from an already resource limited planet. It has been predicted that the proportion of the land surface subject to severe drought is set to increase from 1% currently to 30% by the end of the 21st century, aggravating food and water limitations **(BURKE *et al.*, 2006)**. To make matters worse, the brunt of anthropogenic climate change is expected to have devastating impacts on human health over the next 50 years. Climatic fluctuations are linked to the inception of hundreds of thousands of human fatalities expedited by an increased prevalence of cardiovascular, mental and respiratory illnesses, increased transmission of infectious and vector-borne diseases and malnutrition **(PATZ *et al.*, 2005)**.

REDSHAW *et al.*, (2013) quantifiably predicted that climatic changes will be associated with an increased frequency of infections transmitted by several fungal and bacterial species including *Cryptococcus*, *Cryptosporidium*, *Vibrio*, *Escherichia coli*, *Salmonella*, *Staphylococcus* and *Streptococcus* spp. Furthermore, in addition to the extended distribution of common vector-borne diseases like malaria, dengue, and encephalitis, there has been a recent surge in new parasitic infections including those from liver and lung helminths such as *Schistosoma* spp. **(GRYSEELS *et al.*, 2006)**. To this end, the World Health Organisation quantitatively determined that global warming and climatic changes that occurred since mid-1970 claim over 150 000 lives annually since 2000 **(WORLD HEALTH ORGANISATION, 2005)**. As a result of the increased incidence of human health issues, shifts in the use of pharmaceuticals will become apparent, elevating medicinal drug usage globally **(DEPLEDGE, 2011; REDSHAW *et al.*, 2013)**. This growing health concern compounds the challenges experienced by the current pharmaceutical field. The development of drug resistance by many pathogenic bacterial and fungal strains consequently renders many antibiotic drugs ineffectual. Moreover, the topic of environmental contamination by medication has recently picked up speed amongst ecotoxicologists. Medicinal drugs hold harmful consequences for animal health as evidenced by the death of vultures in India following exposure to Diclofenac, a nonsteroidal anti-inflammatory drug **(TAGGART *et al.*,**

2007), and the feminization of river fish in the United Kingdom, America, Europe and Japan as a result of potent estrogenic components in domestic sewage (**GROSS-SOROKIN *et al*., 2006**). These factors aggravate the need for development of new cost effective, sustainable and potent drugs.

For millennia, human healthcare benefited immensely from a myriad of natural products derived from plants and marine organisms. Historical evidence suggests that since as early as 2600 B.C., indigenous societies in Ancient Mesopotamia utilized approximately 1000 plant derived substances to treat coughs, colds and inflammation (**CRAGG and NEWMAN, 2013**). Since then, many civilisations including the Egyptians, Chinese, Indians, Greeks and Romans have taken advantage of natural products obtained from plants which still remain a major source of chemically diverse pharmaceutical products. The importance of plants as a source of potent therapeutics has been discussed by numerous authors (**BALANDRIN *et al*., 1985; CORDELL, 1987; BALUNAS and KINGHORN, 2005; MISHRA and TIWARI, 2011; DIAS *et* al., 2012; CRAGG and NEWMAN, 2013**) and investigations elucidating the bioactive properties of these plants have identified several types of compounds including, but not limited to; the alkaloids, coumarins, cucuritacins, diarylheptanoids, fatty acids, flavonoids, iridoids, lignans, limonoids, sequesterpenoids and triterpenoids. Although representatives of each group are valued drug leads, alkaloids in particular are of increasing interest to the pharmaceutical industry in that they possess abundant therapeutic promise. Despite accounting for only 15.6% of all known natural products, alkaloids constitute approximately 50% of plant-derived natural products of pharmaceutical importance (**CORDELL *et al*., 2001**).

Amaryllidaceae alkaloids are among the most diverse of secondary metabolites encompassing a cascade of structural types, biosynthetic pathways and pharmacological activities. To date, more than 500 Amaryllidaceae alkaloids have been isolated and classified into 11 ring types, represented by norbelladine, ryllistine, lycorine, hippeastrine, narwedine, galanthamine, haemanthamine, pancratistatin, pretazettine, montanine, indole, cheryline, and ismine (**HE *et al*., 2015**). Pharmacological evaluations have revealed the numerous biological activities of alkaloids including their anticancer, antiviral, antimicrobial, antimalarial, analgesic, acetylcholinesterase inhibitory and cytotoxic activities (**HEINRICH and TEOH, 2004; KORNIENKO and EVIDENTE, 2008; BASTIDA *et al*., 2006; NAIR and VAN STADEN, 2013**). For example, galanthamine from *Galanthus* spp. has become an important

drug for the treatment of Alzheimer's disease as an acetylcholinesterase inhibitor **(HEINRICH and TEOH, 2004)**. Narciclasine has shown antitumor activity against a range of lung cell lines while lycorine and its derivatives have displayed promising activity against cancers and viruses **(KORNIENKO and EVIDENTE, 2008; EVIDENTE and KORNIENKO, 2009)**. However, despite their historical significance and potent activity, alkaloids are underrepresented in the pharmaceutical industry as "marketed drugs" when compared with synthetic, semi-synthetic and other natural products **(AMIRKIA and HEINRICH, 2014)**. This creates a discrepancy between their proposed activity and their occurrence in modern medicine and drug development **(AMIRKIA and HEINRICH, 2014)**.

Amaryllidaceae alkaloids are unique since they are produced exclusively by members of the Amaryllidaceae family **(UNVER, 2007)**. What's more is that, while certain alkaloids are widespread within the family, several are restricted to a particular species. For example, galanthamine has been detected in several species including *Galanthus* spp., *Leucojum* spp., *Lycoris* spp., and *Narcissus* spp. Lycorine, being the most common alkaloid, has been detected in many species including *Sternbergia* spp., *Pancratium* spp., and *Lycoris* spp., while distichamine has only been detected and isolated from *Boophone disticha* **(NAIR *et al.*, 2012)**. Despite their convincing medicinal value, less than half of the Amaryllidaceae genera (34/85) and a fraction of the total number of described species (192/1100) have been evaluated for alkaloid content and biological activity **(CORDELL *et al.*, 2001; HE *et al.*, 2015)**. The activity of rare alkaloids such as narciprimine advocates the search for new structures that possess improved biological activities, from endemic Amaryllidaceae species.

Considering the potential of alkaloids for drug discovery, the aim of the current study was to isolate and identify the alkaloid constituents in the endemic *Scadoxus puniceus*. Pure compounds were also evaluated for their antimicrobial and acetylcholinesterase inhibitory activities.

6.2. Materials and Methods

6.2.1. General

Electron impact mass spectrometry (EIMS) was run on a Micromass Quattro Ultima spectrometer fitted with a direct injection probe (DIP) with ionization energy set at 70 eV. High resolution mass spectrometry (EI) was performed with a Micromass Q-Tof Ultima spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV500 spectrometer (^{1}H

at 500 MHz and ^{13}C at 125.7 MHz) in CD_3OD (referenced to the solvent signal δ = 3.31 and 49.00 ppm, respectively). The complete assignment is based on 2D-NMR correlation experiments including COSY, HSQC, HMBC and NOESY. Chemical shifts (δ) are reported in ppm and coupling constants (J) are expressed in Hz. Silica gel MERCK KGaA (70-230 mesh) was used for initial column chromatography, followed by further purification on a Sephadex LH20 column. TLC silica gel 60 F254 (MERCK KGaA) plates were used for analytical and preparative TLC with ethyl acetate and methanol as the developing solvent system. Spots on chromatograms were detected under UV light (254 and 365 nm) and by Dragendorff's reagent stain.

6.2.2. Collection of plant material

Whole plants of *Scadoxus puniceus* were collected during the flowering season from the Botanical Garden situated at the University of KwaZulu-Natal (UKZN), Scottsville Pietermaritzburg. A voucher specimen (Naidoo 02), authenticated by Dr. Christina Potgieter, was deposited at the BEWS Herbarium, UKZN.

6.2.3. Extract preparation and isolation of alkaloids

After discarding the roots, the remaining plant material were separated into above (stem and leaves) and below ground (bulb and basal plate) parts and dried at 45˚C for seven days. The dry plant material (110 and 130g, respectively) were ground into fine powder and extracted for 24 h, with absolute ethanol on an orbital shaker. The resultant extract was evaporated under reduced pressure in a rotary evaporator. The residue (3.98 and 8.91 g, respectively) were subjected to gravity column chromatography with silica gel by gradient elution with ethyl acetate (EtOAc), /methanol (MeOH) and MeOH. The bulb fraction eluted with 10% methanol (MeOH) in ethyl acetate (EtOAc) was purified on Sephadex LH-20 and preparative TLC (EtOAc:MeOH; 3:1) to yield compound **1**, haemanthamine (65 mg) as a white amorphous powder. The bulb fraction eluted with 5% methanol in ethyl acetate was subjected to column chromatography on Sephadex LH-20 and further purification of preparative TLC (EtOAc:MeOH; 4:1) yielded compound **2**, haemanthidine (115 mg) as a white, amorphous powder. Metolachlor **3** (compound 3) (14 mg) was obtained from a 90% ethyl acetate fraction of the leaves in petroleum ether and was purified by preparative TLC (Petroleum ether:ethyl acetate; 4:1) yielding a colourless liquid.

6.2.4. Physical and spectroscopic data for haemanthamine

***Haemanthamine*,** (11S,13beta,19S)-1,2-Didehydro-3beta-methoxycrinan-11-ol

^{13}C NMR (125.7 MHz, MeOD): 29.04 (CH_2-4); 51.47 (C-10b); 56.71 (CH_3O); 61.70 (CH_2-6); 63.85 (CH_2-12); 64.05 (CH-4a); 74.27 (CH-3); 81.07 (CH-11); 102.24 (OCH_2O); 104.26 (CH-10); 107.84 (CH-7); 126.89 (C-6a); 129.41 (CH-1); 130.59 (CH-2); 137.04 (C-10a); 147.75 (C-8); 148.21 (C-9).

^{1}H NMR (400.2 MHz, MeOD): 1.97 (dddd, 1H, J_{gem} = 13.5 Hz, $J_{4\beta,4a}$ = 4.6 Hz, $J_{4\beta,3}$ = 1.8 Hz, $J_{4\beta,2}$ = 1.3 Hz, H-4β); 2.15 (td, 1H, J_{gem} = $J_{4\alpha,4a}$ = 13.5 Hz, $J_{4\alpha,3}$ = 4.3 Hz, H-4α); 3.13 (dd, 1H, J_{gem} = 13.8 Hz, $J_{12endo,11}$ = 3.5 Hz, H-12*endo*); 3.28 (dd, 1H, $J_{4a,4\alpha}$ = 13.4 Hz, $J_{4a,4\beta}$ = 4.6 Hz, H-4a); 3.36 (s, 3H, CH_3O); 3.43 (dd, 1H, J_{gem} = 13.8 Hz, $J_{12exo,11}$ = 7.0 Hz, H-12*exo*); 3.75 (d, 1H, J_{gem} = 16.8 Hz, H-6α); 3.87 (btd, 1H, $J_{3,4\alpha}$ = $J_{3,2}$ = 4.7 Hz, $J_{3,4\beta}$ = 1.8 Hz, H-3); 3.95 (ddd, 1H, $J_{11,12exo}$ = 7.0 Hz, $J_{11,12endo}$ = 3.5 Hz, $J_{11,LR}$ = 1.2 Hz, H-11); 4.28 (d, 1H, J_{gem} = 16.8 Hz, H-6β); 5.88 (m, 2H, OCH_2O); 6.24 (ddd, 1H, $J_{2,1}$ = 10.1 Hz, $J_{2,3}$ = 5.1 Hz, $J_{2,4\beta}$ = 1.1 Hz, H-2); 6.47 (d, 1H, $J_{1,2}$ = 10.1 Hz, H-1); 6.54 (s, 1H, H-7); 6.90 (s, 1H, H-10).

6.2.5. Physical and spectroscopic data for haemanthidine

Haemanthidine, (3β,6β,11R,13β,19α)-3-Methoxy-1,2-didehydrocrinan-6,11-diol

Epimer A (β-OH) major isomer

^{13}C NMR (100.6 MHz, MeOD): 28.63 (CH_2-4); 51.53 (C-10b); 56.63 (CH_3O); 57.92 (CH-4a); 59.29 (CH_2-12); 74.45 (CH-3); 79.67 (CH-11); 89.23 (CH-6); 102.42 (OCH_2O); 103.75 (CH-10); 110.32 (CH-7); 129.21 (CH-1); 129.46 (C-6a); 130.86 (CH-2); 138.38 (C-10a); 147.68 (C-8); 149.28 (C-9).

^{1}H NMR (400 MHz, MeOD): 1.93 (dddd, 1H, J_{gem} = 13.6 Hz, $J_{4\beta,4a}$ = 4.7 Hz, $J_{4\beta,3}$ = 1.8 Hz, $J_{4\beta,2}$ = 1.3 Hz, H-4β); 2.15 (td, 1H, J_{gem} = $J_{4\alpha,4a}$ = 13.6 Hz, $J_{4\alpha,3}$ = 4.3 Hz, H-4α); 3.07 (dd, 1H, J_{gem} = 14.0 Hz, $J_{12endo,11}$ = 3.6 Hz, H-12*endo*); 3.36 (s, 3H, CH_3O); 3.36 (dd, 1H, J_{gem} = 14.0 Hz, $J_{12exo,11}$ = 7.0 Hz, H-12*exo*); 3.62 (dd, 1H, $J_{4a,4\alpha}$ = 13.6 Hz, $J_{4a,4\beta}$ = 4.6 Hz, H-4a); 3.86 (ddd, 1H, $J_{11,12exo}$ = 7.0 Hz, $J_{11,12endo}$ = 3.6 Hz, $J_{11,LR}$ = 1.3 Hz, H-11); 3.89 (btd, 1H, $J_{3,4\alpha}$ = $J_{3,2}$ = 4.6 Hz, $J_{3,4\beta}$ = 1.9 Hz, H-3); 4.92 (s, 1H, H-6); 5.90 and 5.91 (2×d, 2×1H, J_{gem} = 1.1 Hz, OCH_2O); 6.24 (ddd, 1H, $J_{2,1}$ = 10.1 Hz, $J_{2,3}$ = 5.1 Hz, $J_{2,4\beta}$ = 1.2 Hz, H-2); 6.46 (d, 1H, $J_{1,2}$ = 10.1 Hz, H-1); 6.77 (s, 1H, H-7); 6.89 (s, 1H, H-10).

Epimer B (α-OH) minor isomer

^{13}C NMR (100.6 MHz, MeOD): 29.00 (CH_2-4); 52.06 (C-10b); 53.6 (CH_2-12); 56.72 (CH_3O); 63.53 (CH-4a); 74.25 (CH-3); 80.47 (CH-11); 87.0 (CH-6); 102.4 (OCH_2O); 103.75 (CH-10); 109.09 (CH-7); 129.42 (CH-1); 130.68 (CH-2); 130.99 (C-6a); 137.45 (C-10a); 147.78 (C-8); 149.02 (C-9).

^{1}H NMR (400 MHz, MeOD): 1.99 (dddd, 1H, J_{gem} = 13.5 Hz, $J_{4\beta,4a}$ = 4.8 Hz, $J_{4\beta,3}$ = 1.9 Hz, $J_{4\beta,2}$ = 1.2 Hz, H-4β); 2.28 (td, 1H, J_{gem} = $J_{4\alpha,4a}$ = 13.5 Hz, $J_{4\alpha,3}$ = 4.3 Hz, H-4α); 2.76 (dd, 1H, J_{gem} = 14.3 Hz, $J_{12endo,11}$ = 3.0 Hz, H-12*endo*); 3.36 (s, 3H, CH_3O); 3.40 (bdd, 1H, $J_{4a,4\alpha}$ = 13.5 Hz, $J_{4a,4\beta}$ = 4.7 Hz, H-4a); 3.81 (ddd, 1H, $J_{11,12exo}$ = 7.1 Hz, $J_{11,12endo}$ = 3.1 Hz, $J_{11,LR}$ = 1.2 Hz, H-11); 3.89 (m, 1H, H-3); 4.00 (dd, 1H, J_{gem} = 14.3 Hz, $J_{12exo,11}$ = 7.0 Hz, H-12*exo*); 5.46 (s, 1H, H-6); 5.89 - 5.92 (m, 2H, OCH_2O); 6.23 (ddd, 1H, $J_{2,1}$ = 10.1 Hz, $J_{2,3}$ = 5.1 Hz, H-2); 6.44 (d, 1H, $J_{1,2}$ = 10.1 Hz, H-1); 6.87 (s, 1H, H-7); 6.87 (bs, 1H, H-10).

6.2.6. Physical and spectroscopic data for metolachlor

Metolachlor, *2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide.*

Rotamers (63:37) were observed in NMR spectra at 29°C.

^{13}C NMR (100.6 MHz, MeOD): 14.32, 14.48 (CH_3-10); 15.75, 15.96 (CH_3-15); 19.11, 19.15 (CH_3-11); 24.73, 25.03 (CH_2-9); 43.26, 43.30 (CH_2-8); 56.46, 56.70 (CH-12); 58.83, 58.89 (CH_3O-14); 75.47, 75.50 (CH_2-13); 128.06, 128.14 (CH-3); 130.15, 130.20 (CH-5); 130.38 (CH-4); 138.21 (C-6); 138.36, 138.44 (C-1); 143.90, 143.98 (C-2); 169.34, 169.39 (CO-7).

^{1}H NMR (400 MHz, MeOD): 1.14 and 1.17 (2×d, 2×3H, $J_{15,12}$ = 6.9 Hz, H-15); 2.26 (2×t, 2×3H, $J_{10,9}$ = 7.6 Hz, H-10); 2.23 and 2.25 (2×s, 2×3H, H-11); 2.49 – 2.70 (m, 2×2H, H-9); 3.19 and 3.23 (2×s, 2×3H, H-14); 3.43 and 3.44 (2×dd, 2×1H, J_{gem} = 9.6 and 9.5 Hz, $J_{13a,12}$ = 6.2 and 6.3 Hz, H-13a); 3.61 and 3.67 (2×dd, 2×1H, J_{gem} = 9.6 and 9.5 Hz, $J_{13b,12}$ = 4.6 and 4.5 Hz, H-13b); 3.68 and 3.70 (2×bs, 2×2H, H-8); 4.25 (2×pentd, 2×1H, $J_{12,15}$ = $J_{12.13a}$ = 6.8 Hz, $J_{12,13b}$ = 4.5 Hz, H-12); 7.20 (2×dd, 2×1H, $J_{5,4}$ = 7.1 Hz, $J_{5,3}$ = 2.1 Hz, H-5); 7.26 – 7.30 (m, 2×1H, H-3); 7.32 (2×bt, 2×1H, $J_{4,3}$ = $J_{4,5}$ = 7.4, H-4).

TOF MS ES+: $\mathbf{C_{15}H_{22}NO_2Cl}$, $(M+Na)^+$ calc. 306.1237, experim. 306.1219

6.2.7. Antibacterial microdilution assay

The minimum inhibitory concentrations (MIC) of extracts and pure compounds for antibacterial activity were determined using the microdilution bioassay as described by **ELOFF (1998)**. Overnight cultures (incubated at 37 °C in a water bath with an orbital shaker) of two Gram-positive (*Micrococcus luteus* ATCC 4698 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacterial strains were diluted with 20 ml sterile Mueller-Hinton (MH) broth (200 µl in 19.8 ml). Pure compounds were dissolved in 50% dimethylsulfoxide (DMSO) to a final concentration of 1 mg/ml. The specific methodology of the microplate assay is described in section **5.2.2**.

6.2.8. Antifungal microdilution assay

In order to determine the antifungal activity of isolated compounds and extracts of *S. puniceus*, a microdilution method as described by **ELOFF (1998)** and modified for fungi by **MASOKO *et al*., (2007)** was used. An overnight culture of *Candida albicans* (ATCC 10231) (incubated at 37 °C in a water bath with an orbital shaker) in yeast malt (YM) broth was prepared. Four hundred microlitres of the fungal culture were added to 4 ml of sterile saline and the absorbance was read at 530 nm. The absorbance was adjusted to match that of the 0.5 M McFarland standard solution. From this solution, a 1:1000 dilution with sterile YM broth was prepared producing a final inoculum of approximately 10^6 CFU/ml. Pure compounds were dissolved in 50% DMSO to a final concentration of 1 mg/ml. The specific methodology of the microplate assay is described in section **5.2.3**.

6.2.9. Microplate assay for determination of acetylcholinesterase (AChE) inhibitory activity

The microplate assay described by **ELDEEN *et al*. (2005)**, and outlined by **MOYO *et al*., (2010)** was used to determine the AChE inhibitory activity. The buffers used in the assay are as follows; buffer A - 50 mM Tris-HCl (pH 8.0), buffer B – 50 mM Tris-HCl, (pH 8.0) which contained 0.1 % bovine serum albumin (0.5 g), buffer – C; 50 mM Tris-HCl (pH 8.0) which contained 0.1 M NaCl, and 0.02 M $MgCl_2.6H_2O$.

The specific methodology of the microplate assay is described in section **5.2.4**. Inhibition of AChE was calculated as a percentage based on the following equation:

% Inhibition = 1 – [$RR_{sample}/RR_{control}$] x 100

Where RR_{sample} is the rate of enzyme reaction of the plant extracts and $RR_{control}$ is that of the blank.

6.3. Results and Discussion

6.3.1. Isolation of the bioactive compounds

Chromatographic separation of an ethanolic extract of *S. puniceus* led to the isolation of two alkaloids, haemanthamine (**1**), haemanthidine (**2**) and a chlorinated amide, metolachlor (**3**). The electron impact mass spectrum (EIMS) analysis of haemanthamine had a molecular ion $(M)^+$ peak as the base peak at *m/z* 301 which is correct for the formula $C_{17}H_{19}NO_4$. High resolution mass spectrometry (HRMS) further substantiated the molecular mass correct for $C_{17}H_{19}NO_4$ as 301.3056 g/mol. The ^{1}H NMR spectrum of haemanthamine (**1**), tabulated in **Table 6.1**, was well-resolved for all signals. The molecule consists of three rings, a planar ring A and two non-planar rings B and C. A methylenedioxy group is attached to the aromatic A ring at position C(8) and C(9) while a methoxy group is fused to the C(3) position of ring C. A hydroxyl group is attached to the C(11) position also possessing an ethylidene bridge between C(12) (**Figure 6.1**).

Haemanthidine possessed a molecular ion $(M)^+$ peak as the base peak at *m/z* 318 which is correct for the formula $C_{17}H_{19}NO_5$. HRMS further substantiated the molecular mass correct for $C_{17}H_{19}NO_5$ as 318.1332 g/mol. The ^{1}H NMR spectrum of haemanthidine (**2**), tabulated in **Table 6.1**, was well-resolved for all signals of epimers A (β-OH) and B (α-OH). The compound, similar to haemanthamine possesses a methoxy and methylenedioxy group. However, haemanthidine possesses a hydroxyl substituent at position C(6), unique only to its structure and 6-hydroxycrinamine derived from the 5,10b-ethanophenanthridine nucleus (**Figure 6.1**).

Amaryllidaceae alkaloids are formed biogenetically by intramolecular oxidative coupling of norbelladine **(UNVER, 2007)**, which is produced from a reduction of a Schiff base formed from the combination of 3,4-dihydroxybenzoic acid (formed from phenylpropanoid metabolism) and tryamine (decarboxylation of tyrosine) **(SINGH and DESGAGNÉ-PENIX, 2014)**. Haemanthamine and haemanthidine are derived from 5,10b-ethanophenanthridine formed by *para-para* phenol oxidative coupling. The biological

activities of haemanthamine and haemanthidine are numerous. Haemanthamine has demonstrated potent antiviral activity in model studies against the Herpes simplex virus **(BASTIDA *et al*., 2006)**. The compound also possesses cytotoxic effects against several cancer cell lines including NIH/3T3 cells in animal models, as well as human cancer cells such as HT-1080 fibrosarcoma cells **(NAIR *et al*., 2012)**. Haemanthidine on the other hand, has demonstrated potent anti-inflammatory properties via the carrageenan-induced oedema test **(CITOGLU *et al*., 1998)**.

Table 6.1. ^{1}H and ^{13}C NMR data for haemanthamine (**1**) and haemanthidine (**2**)[a] in Methanol-d_4

	Compound					
	1		**2A**		**2B**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	6.47 d (10.1)	129.4	6.46 d (10.1)	129.2	6.44 d (10.1)	129.4
2	6.24 ddd (10.1, 5.1, 1.1)	130.6	6.24 ddd (10.1, 5.1, 1.2)	130.9	6.23 dd (10.1, 5.1)	130.7
3	3.87 btd (4.7, 1.8)	74.3	3.89 btd (4.6, 1.9)	74.5	3.89 m	74.3
4	2.15 td (13.5, 4.3)	29.0	2.15 td (13.6, 4.3)	28.6	2.28 td (13.5, 4.3)	29.0
	1.97 dddd (13.5, 4.6, 1.8, 1.2)		1.93 dddd (13.6, 4.7, 1.8, 1.3)		1.99 dddd (13.5, 4.8, 1.9, 1.2)	
4a	3.28 dd (13.4, 4.6)	64.1	3.62 dd (13.6, 4.6)	57.9	3.40 bdd (13.5, 4.7)	63.5
6	4.28 d (16.8)	61.7	4.92 s	89.2	5.46 s	87.0
	3.75 d (16.8)		-		-	
6a		126.9		129.5		131.0
7	6.54 s	107.8	6.77 s	110.3	6.87 s	109.1
8		147.8		147.7		147.8
9		148.2		149.3		149.0
10	6.90 s	104.3	6.89 s	103.8	6.87 s	103.8
10a		137.0		138.4		137.5
10b		51.5		51.5		52.1
11	3.95 ddd (7.0, 3.5, 1.2)	81.1	3.86 ddd (7.0, 3.6, 1.3)	79.7	3.81 ddd (7.1, 3.1, 1.2)	80.5
12	3.43 dd (13.8, 7.0)	63.9	3.36 dd (14.0, 7.0)	59.3	4.00 dd (14.3, 7.0)	53.6
	3.13 dd (13.8, 3.5)		3.07 dd (14.0, 3.6)		2.76 dd (14.3, 3.0)	
OCH_2O	5.88 m	102.2	5.90 d (1.1)	102.4	5.89 – 5.92 m	102.4
			5.91 d (1.1)			
OCH_3	3.36 s	56.7	3.36 s	56.6	3.36 s	56.7

[a] Haemanthidine contains two epimers: **2A** (β-OH) as a major isomer and **2B** (α-OH) as a minor isomer

The compound metolachlor (*2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide*), displaying the molecular formula $C_{15}H_{22}NO_2Cl$, is a complex chlorinated amide possessing a molecular ion $(M+Na)^+$ peak at the base 306, further substantiated by the calculated molecular mass 306.1237. The ^{1}H NMR spectrum of two rotamers of metolachlor (**3**), tabulated in **Table 6.2**, was well-resolved for all signals. The structure is represented by the presence of three methyl groups located at C(10), C(11) and C(14) and a chlorine molecule at position C(8) (**Figure 6.1**).

Table 6.2. ^{1}H and ^{13}C NMR data for **3**[a] in Methanol-d_4.

Compound 3			
Position	δ_H (J in Hz)	δ_C	
		A	B
1		138.44	138.36
2		143.90	143.98
3	7.26 – 7.30 m[b]	128.14	128.06
4	7.32 bt (7.4)[b]	130.38	130.38
5	7.20 dd (7.1, 2.1)[b]	130.15	130.20
6		138.21	138.21
7		169.39	169.34
8	3.68 bs (A), 3.70 bs (B)	43.30	43.26
9	2.49 – 2.70 m[b]	24.73	25.03
10	2.26 t (7.6)[b]	14.48	14.32
11	2.23 s (A), 2.25 s (B)	19.15	19.11
12	4.25 pentd (6.8,4.5)[b]	56.46	56.70
13	3.44 dd (9.5, 6.3, A), 3.43 dd (9.6, 6.2, B)	75.50	75.47
	3.67 dd (9.5, 4.5, A), 3.61 dd (9.6, 4.6, B)		
14	3.23 s (A), 3.19 s (B)	58.89	58.83
15	1.14 d (6.9, A), 1.17 d (6.9, B)	15.96	15.75

[a] Two rotamers (A:B, 63:37) were observed in NMR spectra at T=29°C.
[b] Overlapping resonances for both rotamers in ^{1}H NMR spectrum.
The key COSY and HMBC correlations for **1**, **2**, and **3** are presented in Appendix 4

Natural chlorine containing compounds are very rare in higher plants yet are frequently found in marine algae and fungi **(SCHEUER and DARIAS, 1981; TURNER and ALDRIGDE, 1983)**. The first chlorine-containing-compound sceleratinic acid lactone was isolated from *Senecio sceleratus*, a highly toxic species. Since then, only a few chlorinated amides have been isolated from higher plants. For example, **BACKHEET and SAYED, (2002)** isolated N-(2',6'-diethyl phenyl)-2-chloroacetamide and N-(butyloxymethyl)-N-(2',6'-diethyl phenyl)-2-chloroacetamide from *Nicotiana glauca*. The isolation of N-4'-chlorobutylbutramide from *Aloe sabaea* was the first report of a chlorinated amide from the Aloaceae family **(BLITZKE *et al.*, 2000)**. Similarly, the current study is the first to isolate the naturally occurring structure of a chlorinated amide, metolachlor from the Amaryllidaceae family. However, the structure of metolachlor was synthesised in 1974 by Ciba-Geigy Co. and has since then been used intensively as an herbicide **(PEREIRA *et al.*, 2009)**.

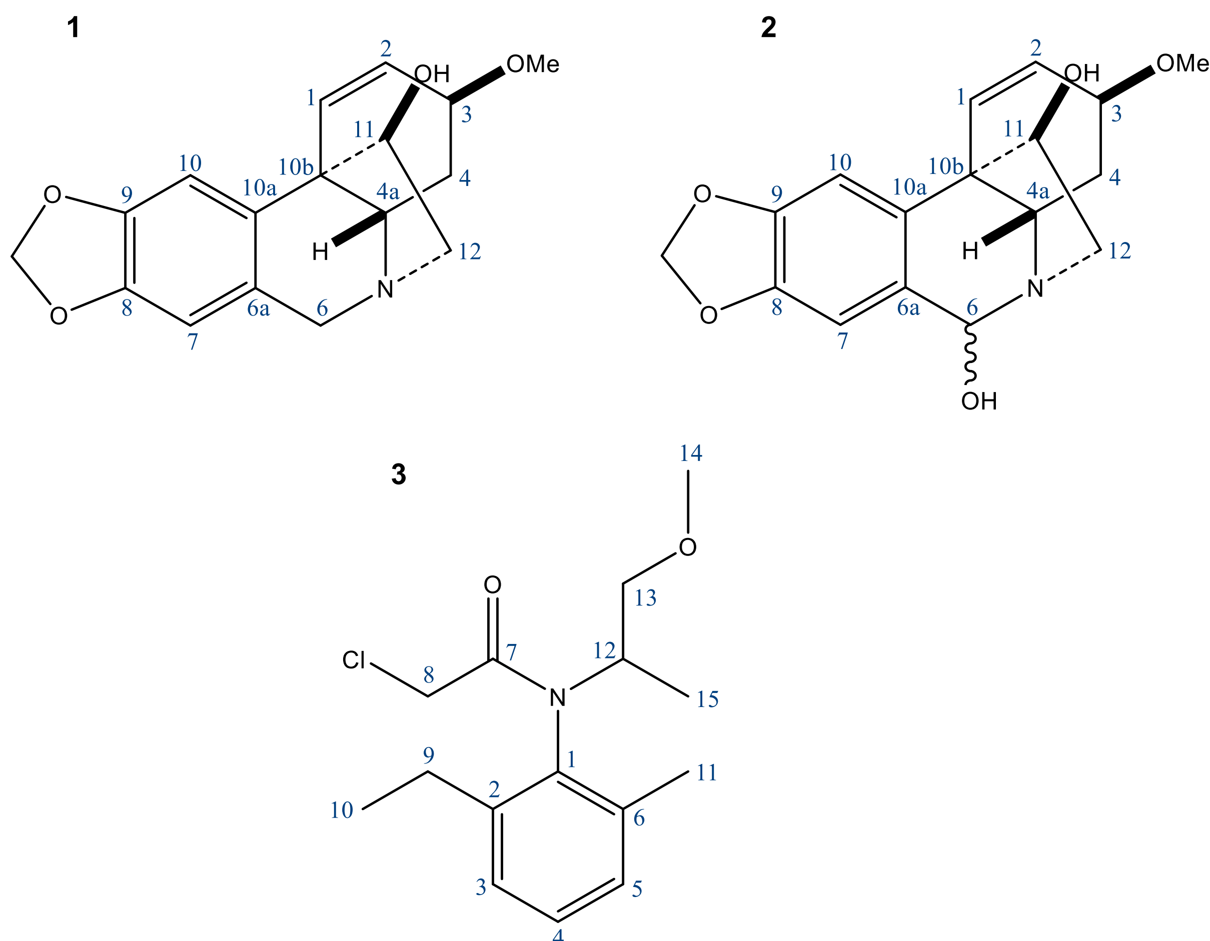


Figure 6.1: The structure of compounds isolated from *Scadoxus puniceus*. Haemanthamine (**1**); Haemanthidine (**2**); Metolachlor (**3**).

6.3.2. Antimicrobial activity of isolated compounds

Scadoxus puniceus is a species endemic to Southern Africa and is used frequently by its people as an antiseptic on sores and ulcers. This warranted investigation into the antimicrobial activity of haemanthamine, haemanthidine and metolachlor. Their antimicrobial activity against two Gram-positive (*Micrococcus luteus* and *Staphylococcus aureus*), two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains and a fungus, *Candida albicans* is presented in **Table 6.3**. The isolated compounds displayed significantly higher activity compared to the crude ethanolic extract. The greatest inhibitory activity (0.062 mg/ml) was recorded for haemanthidine against *S. aureus*. Haemanthamine inhibited bacterial growth at the highest concentration tested (0.250 mg/ml) while *M. luteus* exhibited a level of susceptibility to the chloroacetamide, metolachlor. Similarly, the antifungal activity of the isolated compounds appeared to be stronger than the crude extract, all of which inhibited fungal growth at 0.250 mg/ml.

Table 6.3: The antimicrobial MIC values of isolated compounds from *Scadoxus puniceus*

Antibacterial MIC (mg/ml)					**Antifungal MIC (mg/ml)**
Compound	Bacteria				Fungus
	M. l	*S. a*	*E. c*	*K. p*	*C. a*
Ethanolic extract	3.14	1.56	3.14	0.78	>6.25
Haemanthamine	0.250	0.125	0.250	0.250	0.250
Haemanthidine	0.250	0.062	0.250	0.125	0.250
Metolachlor	0.125	0.250	0.250	0.250	0.250
Neomycin	$1.7x10^{-3}$	$0.8x10^{-3}$	$0.8x10^{-3}$	$1.6x10^{-3}$	-
Amphotericin	-	-	-	-	$9.770x10^{-3}$

Values represent the mean of three replicates. MIC, minimum inhibitory concentration; *E. c.*, *Escherichia coli*; *S. a.*, *Staphylococcus aureus*; *K. p.*, *Klebsiella pneumoniae*; *M. l.*, *Micrococcus luteus*; *C. a.*, *Candida albicans;*

Isolated compounds are considered potentially useful if they possess MIC values of less than 1 mg/ml **(FABRY *et al*., 1998; GIBBONS, 2005)**. The compounds isolated from *S. puniceus* in the current study thus possessed strong broad-spectrum antibacterial activity. Haemanthamine presented antibacterial activity that was, in certain cases, analogous to that of haemanthidine. For example, both alkaloids inhibited the growth of *M. luteus* and *E. coli* at 0.250 mg/ml. However, haemanthidine was twice as active as haemanthamine against *S.*

aureus (0.062 mg/ml < 0.125 mg/ml) and *K. pneumoniae* (0.125 mg/ml < 0.250 mg/ml). These alkaloids are distinguished from one another only by the additional hydroxyl substitution at position C(6) in haemanthidine. It is apparent then that the improvement in antibacterial activity of haemanthidine over haemanthamine is a result of this structural modification. Contrastingly, despite having the same molecular formula, the additional C(6) hydroxyl substitution in crinamine resulting in 6-hydroxycrinamine significantly diminished the compounds antibacterial activity as compared to haemanthidine **(ADESANYA *et al.*, 1992)**. The difference in activity between haemanthidine and 6-hydroxycrinamine may be centred on the biosynthesis of these alkaloids. While the synthesis of haemanthidine involves the loss of a hydrogen molecule at the C-β position in norbelladine and the addition of the molecule at the C(4a) position, 6-hydroxycrinamine retains the hydrogen at the C-β position. These results draw attention to the structure-activity relationship that exists within the 5,10b-ethanophenanthridine series of alkaloids, with regard to their antimicrobial activities.

Several South African Amaryllidaceae species possess potent antibacterial activity against pathogenic bacteria. For example, *Cyrtanthus falcatus*, *C. mackenii*, and *C. sauveolens* exhibited compelling antibacterial activity at concentrations ranging from 10 to 90 ng/ml against six Gram-positive and Gram-negative bacteria **(ELGORASHI and VAN STADEN, 2004)**. More recently, **CHEESMAN *et al.* (2012)** isolated two crinane type alkaloids, buphanadrine and distichamine from *Boophone disticha* which displayed effective inhibition of both Gram-positive and Gram-negative bacteria. Other alkaloids that possess strong antimicrobial activity adding importance to the search for antimicrobials from the Amaryllidaceae are crinamine, vittatine and lycorine **(ADESANYA *et al.*, 1992; EVIDENTE *et al.*, 2004)**. Alkaloids have also inspired the synthesis of several antibiotics from their unique structure **(CUSHNIE *et al.*, 2014)**. For example, the structural modification of quinine produced bedaquiline, a bactericidal drug **(CUSHNIE *et al.*, 2014)**. The alkaloids thus, still remain an interesting avenue for the pursuit of new effective antimicrobials.

6.3.3. Acetylcholinesterase inhibitory activity of isolated compounds

The use of plants as central nervous system (CNS) stimulators by traditional practitioners has prompted the search for inhibitors of AChE from medicinal plants. The ethanolic extract of *S. puniceus* possessed significant activity in terms of AChE inhibition with an IC_{50} value of 70 µg/ml. Isolation of *S. puniceus* yielded three compounds whose ability to inhibit AChE is presented in **Table 6.4**. As can be expected from alkaloids, haemanthamine and

haemanthidine displayed potent activity with IC_{50} values of 23.7 and 23.1 µM/ml, respectively. Their activity was however, approximately 12-fold less active than galanthamine. Interestingly, metolachlor displayed significantly stronger activity, comparable to galanthamine effecting an IC_{50} value of 11.5 µM/ml.

Table 6.4: The inhibition (%) and IC_{50} µM/ml acetylcholinesterase inhibitory values of pure compounds isolated from *Scadoxus puniceus*.

Compound	**% Inhibition (11.25 µg/ml)**	**IC_{50} (µM/ml)**
Haemanthamine	95.68 ± 1.51 [b]	23.7[c]
Haemanthidine	95.15 ± 1.01 [b]	23.1[c]
Metolachlor	114.80 ± 3.05 [a]	11.5[b]
Galanthamine	-	1.9[a]

Central nervous system disorders represented by Alzheimer's and Parkinson's disease exert an overwhelming impact on elderly people in developed countries affecting one in every five over the age of 80. Forty-seven million people are burdened with the effects of dementia worldwide, and this is predicated to increase to 131 million by 2050 **(WORLD ALZHEIMER'S REPORT, 2016)**. As a result, significant efforts have been made towards the pursuit for effective therapeutics for these neurodegenerative disorder. Inhibitors of AChE are currently the only class of drugs that relieve the symptoms of Alzheimer's disease by slowing the biochemical breakdown of acetylcholine, prolonging cholinergic transmission **(HARVEY, 1995; HEINRICH and TEOH, 2004; HOUGHTON *et al.*, 2006)**. As such, drugs that target the enzyme acetylcholinesterase have become the focus of Alzheimer's research. Amaryllidaceae alkaloids boast a diverse collection of structural configurations which influence the compounds ability to inhibit AChE.

Amaryllidaceae alkaloids that possess potent AChE activity commonly belong to the galanthamine and lycorine structural types. Galanthamine, derived from the phenanthrene nucleus is currently a prescription drug used in the treatment of Alzheimer's disease. Galanthamine and several of its derivatives such as sanguinine and 11-hydroxygalanthamine

possess potent AChE inhibitory activity. The structure activity relationship of alkaloids with regard to AChE inhibitory activity has been documented explicitly within the lycorine-type alkaloids belonging to the phenanthridine series. **ELGORASHI *et al*., (2004)** showed that the ethyl-tethered, unsubstituted alkaloids lycorine and crinamine possess weak AChE inhibitory activity. Furthermore, while derivatives of lycorine, 1,2-diacetyllycorine and 2-acetyllycorine also display significantly diminished activity, mono-substitution in ring C forming 1-*O*-acetyllycorine exhibits a two-fold improvement in AChE inhibitory activity as compared to galanthamine **(ELGORASHI *et al*., 2004)**. Haemanthamine and haemanthidine are derived from the 5,10b-ethanophenanthridine nucleus and are structurally classified as haemanthamine-type alkaloids. To date, the AChE inhibitory activity of these compounds have been unspecified despite previous work suggesting that they may be inactive **(LÓPEZ *et al*., 2002)**. Considering the noteworthy activity presented here, the results of the current study contradict those of **LÓPEZ *et al.* (2002)**. The AChE inhibitory activity did not differ significantly between haemanthamine and haemanthidine despite the hydroxyl substitution at position C(6) in haemanthidine. This suggests that the structural configuration of these haemanthamine-type alkaloids consisting of the methoxy and methylenedioxy groups confer potent activity which has gone unrecognised until now.

The current study is the first to propose potent AChE inhibitory activity of a chlorinated amide, metolachlor. The molecular configuration conferred inhibitory activity just 6 fold weaker than galanthamine. Chlorine-containing-compounds often display strong biological activity **(ENGVILD, 1986)**. For example, in 1968, **KUPCHAN *et al.*** isolated eupachlorin acetate, a chlorinated sesquiterpenoid lactone from *Eupatorium rotundiflium* which displayed strong antitumor activity. Furthermore, a group of macrolides known as the maytansinoids that possess a chlorine molecule attached to the benzene ring, *ortho* to a methoxy and amino group also possess potent antitumor activity. A chlorinated xanthone, psorospermin chlorohydrin, isolated from *Psorospermum febrifugum* exhibits potent anti-leukemic activities. Considering the biological activity of these chlorinated compounds, the AChE inhibitory activity reported here is not surprising. However, the toxicity of metolachlor to humans has been well documented. **DIERICKX, (1999)** revealed the toxicity of chloroacetamide herbicides, alachlor, metolachlor and propachlor to human hepatoma-derived cells. In addition, the United States Environmental Protection Agency classified metolachlor as a Group C “possible human carcinogen”. **COLEMAN *et al*., (2000)**, after observing an increase in neoplastic nodules in female rats, suggested that the carcinogenicity

of metolachlor involves a complex metabolic activation pathway which leads to a DNA-reactive dialkylbenzoquinone imine. The observed toxicity and carcinogenicity of metolachlor substantiates the incidences of death after ingestion of *S. puniceus* and may hinder further medicinal evaluation.

6.4. Conclusions

In summary, the current study described the isolation of two known alkaloids, haemanthamine and haemanthidine from the bulbs of *Scadoxus puniceus*. The alkaloids possessed broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacterial strains and a fungus. Haemanthidine, possessing an additional hydroxyl group was twice as effective at inhibiting the growth of bacteria when compared with haemanthamine. These results highlighted the structure-activity relationship that exists within the 5,10b-ethanophenanthridine series of alkaloids. The AChE inhibitory activity of the alkaloids was also investigated. Both haemanthamine and haemanthidine displayed similar, strong inhibitory activity despite the structural difference between the two. The structurally-related AChE inhibitory activity of the alkaloids are of significance considering that the activity of the 5,10b-ethanophenanthiridine series of alkaloids were previously unrecognised. The study also described the isolation of a metolachlor, being the first occurrence of a chlorinated amide in the Amaryllidaceae family. An investigation into the compounds ability to disrupt the action of AChE revealed the potent inhibitory activity of metolachlor. These results further accentuate the importance of drug discovery from natural sources.

CHAPTER 7: GENERAL CONCLUSIONS

7.1. Micropropagation of *Scadoxus puniceus*

In vitro plant culture has been labelled as a collection of techniques that can curb the impact of unsustainable harvesting of medicinal plants for use in traditional medicine. These techniques were employed to develop an efficient *in vitro* propagation protocol for the highly utilised medicinal plant *Scadoxus puniceus*.

The study revealed that the regeneration, growth and development of *S. puniceus* in culture were influenced by several factors. Leaf explants were recalcitrant while bulb scales were highly susceptible to oxidative browning. Regeneration was severely influenced by the photoperiod that explants were exposed to. Continuous incubation in the dark improved plant regeneration and reduced oxidative browning. Benzyladenine improved the regeneration of leaf explants suggesting that the endogenous concentrations of cytokinins were insufficient for regeneration. Despite the efforts, many of the plantlets that were produced using this protocol were hyperhydric.

The initial attempt at the *in vitro* propagation of *S. puniceus* highlighted the need for strategies to alleviate hyperhydricity and oxidative browning. The topolins have been acknowledged widely as plant growth regulators that can alleviate hyperhydricity of micropropagated plants. Several topolins including *meta*-topolin (*m*T), *meta*-topolin riboside (*m*TR), *meta*-Methoxytopolin (me*m*T), *meta*-Methoxy-9-tetrahydropyran-2-yl topolin (me*m*TTHP) were evaluated for their ability to improve the development of *S. puniceus*. All topolins alleviated hyperhydricity; however plant development was influenced by the concentration at which they were applied. In addition to alleviating hyperhydricity, the topolins that induced the best plant development were *m*T and *m*TR.

The exudation and subsequent oxidation of phenolic acids into tissue culture media are a major limiting factors associated with *in vitro* culture. Oxidative browning of twin-scales in the current study limited the success of this important explant. Traditionally, oxidative browning is controlled by adding antioxidants and adsorbents to the medium. However, these methods were not successful for the propagation of *S. puniceus*. Since phenolic acids are produced via the shikimate pathway mediated by phenylalanine ammonia lyase (PAL), recent studies have focused on incorporating PAL inhibitors in the medium to inhibit the production of phenolics. The study thus evaluated the role of gallic acid an organic phenolic compound

and competitive inhibitor of PAL, on oxidative browning and plant development of *S. puniceus* and *M. plumbea* (model species). Gallic acid (GCA) significantly improved the development of both *M. plumbea* and *S. puniceus*. An apparent synergistic relationship existed between gallic acid and the applied cytokinins with regard to the development of *S. puniceus*. While an antagonistic role of GCA with *m*T was noted in the development of *M. plumbea*. Overall, GCA reduced the red pigmentation characteristic of oxidative browning in *S. puniceus* twin scale explants. In addition, GCA did not influence the phenolic acid content of *M. plumbea* suggesting that the exudation rather than the production of phenolics was inhibited, which is significant considering the importance of phenolics in plant development.

A second organic phenolic compound phloroglucinol (PG) was evaluated for its role in alleviating oxidative browning. The compound failed to improve the regeneration of *S. puniceus* when applied singly. However, a synergistic relationship between PG and auxins or cytokinins was highlighted. PG in combination with NAA was able to induce a six-fold improvement in plant development adding to its importance in *in vitro* plant development.

The recalcitrance of leaf explants prompted the search for alternative meristematic tissue for improved regeneration. The regeneration potential of inflorescence explants has been documented for the propagation of several Amaryllids. Inflorescence explants of *S. puniceus* were thus used to develop a liquid culture system to improve the development of this species. Liquid culture was unsuitable for the production of bulblets, however, when shoot clusters which developed from inflorescence explants were maintained in liquid media, significantly larger and healthier plantlets were obtained.

The current study thus describes several methods for alleviating the physiological disorders and challenges encountered when propagating *S. puniceus in vitro*. The results may also have implications on the broader challenges of tissue culture such as oxidative browning, considering the prevalence of this phenomenon.

7.2. Phenolic profile and pharmacology of *Scadoxus puniceus*

From a tissue culturist's perspective, the oxidation of phenolics is an unnecessary challenge that limits the success of several experiments. However, the same oxidative ability makes phenolic compounds highly desirable by the food and therapeutic industries as free radical scavengers. Free radicals are generated when unpaired single electrons are transferred to the oxygen molecule during oxidation **(GÜLCIN, 2012)**. Free radicals such as reactive oxygen

species (oxygen centred free radical) damage lipids, nucleic acids, proteins, and carbohydrates causing ageing, cancer and several other diseases. In aerobic organisms, damaged molecules are removed or repaired by antioxidants by inhibiting the oxidation process. The ability to scavenge free radicals makes antioxidants highly desirable in the food and therapeutic industries as the hindering of lipid peroxidation preserves food and pharmaceutical products for a longer period of time. Amidst growing concerns over the safety of synthetic antioxidants, there has been an increase in interest towards naturally occurring antioxidants such as phenolic compounds from plants and fruits.

Scadoxus puniceus is one amongst the highly traded medicinal plants in South Africa, the current investigation employed UPLC-MS/MS to identify and quantify the phenolic acids present in *S. puniceus*. Thirteen phenolic compounds were identified of which, the presence of chlorogenic (CGA), sinapic, gallic and *m*-hydroxybenzoic acids are reported for the first time in the Amaryllidaceae. CGA was the main phenolic compound, accounting for 49.6% of the total phenols in aerial organs suggesting its active role in protecting the plant from herbivory. The accumulation of significant concentrations of one or more known antioxidants including CGA, vanillic, protocatechuic and *p*-coumaric acids in leaves, stems and roots validated the antioxidant activity of the organs of *S. puniceus*. The stress induced accumulation of certain phenolic acids suggests that the species can be used to harness potent antioxidants for commercial benefit. The results of the investigation further validate the use of *S. puniceus* as an ingredient in a traditional Zulu herbal tonic.

Despite significant improvements to health care systems over the decades, the brunt of infectious diseases still continues to exert tremendous pressure on human health worldwide. The side effects, abuse or incorrect use and widespread microbial resistance associated with antibiotics are a major cause for concern and thus emphasises the intense need for novel solutions to this problem.

The metabolism of secondary compounds in plants has offered the pharmaceutical industry valuable natural products that possess widespread activity and are used for the treatment of various illnesses and disease. As such, the evaluation of plant material for their proposed activity based on indigenous knowledge is an essential component of drug discovery from natural products. For this reason, the study investigated the ability of extracts of *S. puniceus* to inhibit microbial growth. The bulb and leaves of *S. puniceus* possessed broad spectrum antibacterial activity exhibiting strong MIC values (0.39 mg/ml and 0.78 mg/ml, respectively)

against Gram-negative (*S. aureus*) and Gram-positive bacteria (*K. pneumoniae*). Furthermore, the leaf extracts possessed potent (< 0.1 mg/ml) activity against *C. albicans*. Based on these promising results, it is important that future studies elucidate and determine the mode of action of the compounds responsible for the activity.

The Amaryllidaceae are well known for their production of pharmaceutically important alkaloids, several of which have been trialled for the treatment of neurological disorders. The current study evaluated the AChE inhibitory activity of different organs of *S. puniceus* collected from the Botanical Garden (UKZN, Pietermaritzburg) for their ability to inhibit AChE. All ethanolic plant extracts exhibited dose dependent AChE inhibitory activity, inhibiting between 87 and 97% of the enzyme at the highest concentration tested (1 mg/ml). Bulb and basal plate extracts possessed the strongest activity (96.6 and 95.0%, respectively) both with the lowest IC_{50} values (0.07 mg/ml). The results of the study thus reveal the presence of potent compounds that can be harnessed for the treatment of neurodegenerative disorders.

The pharmacological evaluation of *S. puniceus* accentuates the potential that the species holds for the extraction and isolation of potent compounds for the treatment of microbial infections and neurodegenerative disorders. The quantification and the accumulation of phenolic acids that possess strong antimicrobial activity may have implications for the food and pharmaceutical industries. Furthermore, the pharmacological evaluation of different organs of *S. puniceus* may contribute to the sustainable harvesting of the species using plant part substitution in traditional medicine.

7.3. Isolation, identification and biological activity of compounds from *Scadoxus puniceus*

For millennia, human healthcare benefited immensely from a myriad of natural products derived from plants, which until today still remain a major source of chemically diverse pharmaceutical products. Amaryllidaceae alkaloids are among the most diverse of secondary metabolites encompassing a cascade of structural types, biosynthetic pathways and pharmacological activities. However, despite their convincing medicinal value, less than half of the Amaryllidaceae genera (34/85) and a fraction of the total number of described species (192/1100) have been evaluated for alkaloid content and biological activity. Based on the activity of crude extracts of *Scadoxus puniceus*, compounds of interest were isolated from *S.*

puniceus and evaluated for their ability to inhibit microbial growth and inhibit the activity of AChE.

Chromatographic separation of an active ethanolic extract of *S. puniceus* afforded three compounds. From a methanolic fraction of the bulb, two alkaloids, haemanthamine and haemanthidine were isolated, while metolachlor was isolated from an ethyl acetate fraction of the leaves. Haemanthamine and haemanthidine occur naturally in several Amaryllidaceae species while metolachlor is a rare chlorinated amide that possesses a chlorine molecule at position C(8). Based on the traditional use of *S. puniceus* as an antiseptic on sores and ulcers as well as its use as a CNS exciter, the study then focused on investigating the antimicrobial and acetylcholinesterase (AChE) inhibitory activity of the isolated pure compounds.

The isolated compounds exhibited minimum inhibitory concentrations that were at least three-fold lower than that of the crude ethanolic extract. As such, the broad spectrum antimicrobial activity of these compounds against both Gram-positive and Gram-negative bacterial strains and a fungus was described. A structure-activity relationship was proposed within the 5,10b-ethanophenanthridine series of alkaloids with regard to their antimicrobial activity. The C(6) hydroxyl substitution in haemanthidine contributed to improving the antibacterial activity of this compound against *S. aureus* and *K. pneumoniae*.

The alkaloids also displayed strong AChE inhibitory activity. Despite the additional hydroxyl group present in haemanthidine, both compounds exhibited similar activity (23.1, 23.7 μM/ml, respectively). This structurally-related AChE inhibitory activity of the alkaloids were of significance considering that the activity of the 5,10b-ethanophenanthiridine series of alkaloids were previously unrecognised. The study further identified the first occurrence of a chlorinated amide in the Amaryllidaceae family and the first report of the naturally occurring structure of metolachlor. Metolachlor displayed potent AChE inhibitory activity (11.5 μM/ml). This ability to disrupt the action of AChE is unprecedented for a chlorinated amide. Being a compound that has been synthesised before, the presence of metolachlor in a natural, uncontaminated system is not yet understood. Further studies are required to determine the biosynthesis of this compound as well as its effect, if any, on the physiological processes in plants.

These results further highlight the significance of drug discovery from plants based on indigenous knowledge systems.

REFERENCES

ABOEL-NIL, M., 1997. Role of plant tissue culture propagation in the rehabilitation of desert environment. In: Al-awadhi, N., Balba, M.T., Kamizawa, C. (Eds.), Restoration and rehabilitation of the desert environment Elsevier, Amsterdam.

ADELBERG, J., NAYLOR-ADELBERG, J., 2012. Efects of cytokinin on proliferation and rooting of *Aloe barbadensis* during micropropagation on agar and liquid medium. Journal of Medicinally Active Plants 1, 1-5.

ADESANYA, S.A., OLUGBADE, T.A., ODEBIYI, O.O., ALADESANMI, J.A., 1992. Antibacterial alkaloids in *Crinum jagus*. International Journal of Pharmacognosy 30, 303-307.

ADEWUSI, E.A., FOUCHE, G., STEENKAMP, V., 2012. Cytotoxicity and acetylcholinesterase inhibitory activity of an isolated crinine alkaloid from *Boophone disticha* (Amaryllidaceae). Journal of Ethnopharmacology 143, 572-578.

ADEWUSI, E.A., STEENKAMP, V., 2011. *In vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from southern Africa. Asian Pacific Journal of Topical Medicine 4, 829-835.

AFOLAYAN, A.J., ADEBOLA, P.O., 2004. *In vitro* propagation: a biotechnological tool capable of solving the problem of medicinal plants decimation in South Africa. African Journal of Biotechnology 3, 683-687.

ALIYU, O.M., 2005. Applications of tissue culture to cashew (*Anacardium occidentale* p. L.) breeding: An appraisal. African Journal of Biotechnology 4, 1485-1489.

ALMOND, J.B., COHEN, G.M., 2002. The proteasome: a novel target for cancer chemotherapy. Leukemia 16, 433-443.

ALTAMURA, M.M., TORRIGIANI, P., FALASCA, G., ROSSINI, P., BAGNI, N., 1993. Morpho-functional gradients in superficial and deep tissues along tobacco stem: polyamine levels, biosynthesis and oxidation and organogenesis *in vitro*. Journal of Plant Physiology 142, 543-551.

AMAKURA, Y., OKADA, M., SUMIKO, T., TONOGAI, Y., 2000. Determination of phenolic acids in fruit juices by isocratic column liquid chromatograpy. Journal of Chromatography A 891, 183-188.

AMAROWICZ, R., PEGG, R.B., RAHIMI-MOGHADDAM, P., BARL, B., WEIL, J.A., 2004. Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry 84, 551-562.

AMIRKIA, V., HEINRICH, M., 2014. Alkaloids as drug leads – A predictive structural and biodiversity-based analysis. Phytochemistry Letters 10, 48-53.

AMOO, S.O., AREMU, A.O., VAN STADEN, J., 2012. *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue and Organ Culture 111, 345-358.

AMOO, S.O., FINNIE, J.F., VAN STADEN, J., 2011. The role of *meta*-topolins in alleviating micropropagation problems. Plant Growth Regulation 63, 197-206.

ANKLI, A., HEILMANN, J., HEINRICH, M., STRICHER, O., 2000. Cytotoxic cardenolides and antibacterial terpenoids from *Crossopetalum gaumeri*. Phytochemistry 54, 531-537.

AREMU, A.O., BAIRU, M., DOLEZAL, K., FINNIE, J.F., VAN STADEN, J., 2012. Topolins: a panaceae to plant tissue culture challenges? Plant Cell Tissue and Organ Culture 108, 1-16.

AREMU, A.O., GRÚZ, J., ŠUBRTOVÁ, M., SZÜČOVÁ, L., DOLEŽAL, K., BAIRU, M.W., FINNIE, J.F., VAN STADEN, J., 2013. Antioxidant and phenolic acid profiles of tissue cultured and acclimatized *Merwilla plumbea* plantlets in relation to the applied cytokinins. Journal of Plant Physiology 170, 1303-1308.

ARIKAT, N.A., JAWAD, F.M., KARAM, N.S., SHIBLI, R.A., 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticose* Mill.). Scientia Horticulturae 100, 193-202.

ARUOMA, O.I., 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists' Society 75, 199-212.

ASCENCIO-CABRAL, A., GUTIERREZ-PULLIDO, H., RODRIGUEZ-GARAY, B., GUTIERREZ-MORA, A., 2008. Plant regeneration of *Carica papaya* L. through somatic embryogenesis in response to light quality, gelling agent and phloridzin. Scientia Horticulturae 118, 155-160.

ASCOUGH, G.D., ERWIN, J.E., VAN STADEN, J., 2008. *In vitro* storage organ formation of ornamental geophytes. Horticultural Reviews 34, 417-445.

AYAZ, F.A., HAYIRLIOGLU-AYAZ, S., GRÚZ, J., NOVAK, O., STRNAD, M., 2005. Separation, characterisation, and quantification of phenolic acids in a little-known

blueberry (*Vaccinium arctostaphylos* L.) fruit by HPLC-MS. Journal of Agricultural and Food Chemistry 53, 8116-8122.

BACKHEET, E.Y., SAYED, H.M., 2002. Two new chlorinated amides from *Nicotiana glauca* R. Graham. Pharmazie 57, 206-208.

BAI, D.L., TANG, X.C., HE, X.C., 2000. Huperzine A, A potential therapeutic agent for treatment of Alzheimer's disease. Current Medicinal Chemistry 7, 355-374.

BAIRU, M.W., JAIN, N., STIRK, W.A., DOLEZAL, K., VAN STADEN, J., 2009. Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. South African Journal of Botany 75, 122-127.

BAIRU, M.W., STIRK, W.A., DOLEZAL, K., VAN STADEN, J., 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can *meta*-topolin and its derivatives serve as a replacement for benzyladenine and zeatin? Plant Cell Tissue and Organ Culture 90, 15-23.

BAJAJ, Y.P.S., FURMANOWA, M., OLSZOWSKA, O., 1988. Biotechnology of the micropropagation of medicinal and aromatic plants. In: Biotechnology in agriculture and forestry, Vol 4, Medicinal and aromatic plants 1, ed. Bajaj, Y.P.S., Springer Verlag, Berlin

BALANDRIN, M.F., KLOCKE, J.A., WURTELE, E.S., BOLLINGER, W.H., 1985. Natural plant chemicals: Sources of industrial and medicinal materials. Science 228, 1154-1160.

BALUNAS, M.J., KINGHORN, A.D., 2005. Drug discovery from medicinal plants. Life Sciences 78, 431-441.

BANDURSKI, R.S., COHEN, J.D., SLOVIN, J.P., REINECKE, D.M., 1995. Auxin biosynthesis and metabolism. In: Davies, P.J. (Ed.), Plant Hormones. Kluwer Academic Publishers, Dordrecht.

BANQUAR, S.R., 1993. The role of traditional medicine in rural medicine. In: Sindinga, I., Nyaigatti-Chacha, C., Kanunah, M.P. (Eds), Traditional medicine in Africa, English Press Ltd, Nairobi.

BASKARAN, P., NCUBE, B., VAN STADEN, J., 2012. *In vitro* propagation and secondary metabolite production by *Merwilla plumbea* (Lindl.) speta. Plant Growth Regulation 67, 235-245.

BASTIDA, J., CODINA., C., VILADOMAT, F., RUBIRALTA, M., QUIRION, J-C., WENIGER, B., 1992. *Narcissus* alkaloids, XIV: (+)-8-*O*-Acetylhomolycorine and vasconine, two novel aklaloids from *Narcissus vasconicus*. Journal of Natural Products 55, 122-125.

BASTIDA, J., LAVILLA, R., VILADOMAT, F., 2006. Chemical and biological aspects of *Narcissus* alkaloids. In Cordell, G.A. (Ed.) The Alkaloids, Vol. 63. Elsevier, Amsterdam. pp. 87-179.

BATE-SMITH, E.C., 1968. The phenolic constituents of plants and their taxonomic significance. Journal of the Linnean Society of London (Botany) 60, 325-383.

BATTEN, A., 1986. Flowers of Southern Africa. Frandsen Publishers, Sandton.

BAY-SMIDT, M.G.K., JÄGER, A.K., KRYDSFELDT, K., MEEROW, A.W., STAFFORD, G.I., VAN STADEN, J., RONSTED, N., 2011. Phylogenetic selection of target species in Amaryllidaceae tribe Haemantheae for acetylcholinesterase inhibition and affinity to the serotonin reuptake transport protein. South African Journal of Botany 77, 175-183.

BECKER, E.M., NISSEN, L., SKIBSTED, L.H., 2004. Antioxidant evaluation protocols: food quality or health effects. European Food Research Technology 219, 561-571.

BECKMAN, C.H., 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiological and Molecular Plant Pathology 57, 101-110.

BENNETT, J.W., CHUNG, K.T., 2001. Alexander Fleming and the discovery of penicillin. Advances in Applied Microbiology 49, 163-84.

BERGER, B.B. 2002. Resistance mechanisms and Gram positive bacteria. International Journal of Microbiology 292, 27-35.

BERGOÑÓN, S., CODINA, C., BASTIDA, J., MELE, E., 1992. The shake liquid culture as an alternative way to the proliferation of *Narcissus* plants. Acta Horticulturae 325, 447-452.

BERNAYS, E.A., OPPENHEIM, S., CHAPMAN, R.F., KWON, H., GOULD, F., 2000. Taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists: a behavioral test of the hypothesis with two closely related caterpillars. Journal of Chemical Ecology 26, 547-563.

BHAT, R.B., JACOBS, T.V., 1995. Traditional medicine in Transkei. Journal of Ethnopharmacology 48, 7-12.

BHAT, S.R., CHANDEL, K.P.S., 1991. A novel technique to overcome browning in tissue culture. Plant Cell Reports 10, 358-361.

BIRMETA, G., WELANDER, M., 2004. Efficient microproagation of *Ensete ventricosum* applying meristem wounding: a three step protocol. Plant Cell Reports, 277-283.

BLITZKE, T., PORZEL, A., MASAOUD, M., SCHMIDT, J., 2000. A chlorinated amide and piperidine alkaloids from *Aloe sabeae*. Phytochemisty 55, 979-982.

BOCCO, A., CUVELEIR, M.E., RICHARD, H., BERSET, C., 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. Journal of Agricultural and Food Chemistry 46, 2123-2129.

BODEKER, G., 2004. Integrating traditional and complementary medicine into national health care: learning from the international experience. In: Packer, L., Ong, N.C., Halliwell, J. (Eds.), Herbal and Traditional Medicine: Molecular Aspects of Health. CRC Press, New York.

BOONEKAMP, P.M., 1997. The role of external factors in growth and development of flower bulbs and bulb-flowers: an update since 1992. Acta Horticulturae 430, 35-43.

BOTHA, E.W., KAHLER, C.P., DU PLOOY, W.J., DU PLOOY, S.H., MATHIBE, L., 2005. Effect of *Boophone disticha* on human neutrophils. Journal of Ethnopharmacology 96, 385-388.

BOTHA, J., WITKOWSKI, E.T.F., SHACKLETON, C.M., 2004. Market profiles and trade in medicinal plants in the Lowveld, South Africa. Environmental Conservation 31, 38-46.

BOTTERWECK, A., VERHAGEN, H., GOLDBOHM, R., KLEINJANS, J., BRANT, P., 2000. Intake of butylated hydroxyanisole and butylated hydroxytolulene and stomach cancer risk: results from analyses in The Netherlands cohort study. Food and Chemical Toxicology 38, 599-605.

BUCHELI, C.S., ROBINSON, S.P., 1994. Contribution of enzymatic browning to color in sugarcane juice. Journal of Agricultural and Food Chemistry 42, 257-261.

BURKE, J.E., BROWN, S.J., CHRISTIDIS, N., 2006. Modeling the recent evolution of global drought and projections for the twenty-first century with the Hadley Centre Climate Model. Journal of Hydrometeorology 7, 1113-1125.

BUTHUC-KEUL A.L., DELIU, C., 2001. Clonal propagation of *Arnica montana* L., a medicinal plant. *In Vitro* Cellular and Developmental Biology-Plant 37, 581-585.

BUTLER, M.S., BUSS, A.D., 2006. Natural Products - The future scaffolds for new antibiotics. Biochemical Pharmacology 71, 919-929.

CAI, Y-Z., SUN, M., XING, J., LUO, Q., CORKE, H., 2006. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sciences 78, 2872-2888.

CAROCHO, M., FERREIRA, I.C.F.R., 2013. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food and Chemical Toxicology 51, 15-25.

CARSON, C.F., COOKSON, B.D., FARRELLY, H.D., RILEY, T.V., 1995. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alterniflora*. Journal of Antimicrobial Chemotherapy 37, 1177-1181.

CASAL, M., VAQUERO, M., RINDER, H., TORTOLI, E., GROSSET, J., RUSCH-GERDES, S., GUTIERREZ, J., JARLIER, J., 2005. A case-control study for multi-drug resistant tuberculosis: risk factors in four European countries. Microbial Drug Resistance 11, 62-67.

CEASAR, S.A., MAXWELL, S.L., PRASAD, K.B., KARTHIGAN, M., IGNACIMUTHU, S., 2010. Highly efficient shoot regeneration of *Bacopa monnieri* (L.) using a two stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. Acta Physiologiae Plantarum 32, 443-452.

CENTENO, M.L., RODRIGUEZ, I., FEITO, I., FERNANDEZ, B., 1996. Relationship between endogenous auxin and cytokinin levels and morphogenic responses in *Actinidia deliciosa* tissue cultures. Plant Cell Reports 16, 58-62.

CHAMBERS, H.F., 2001. The changing epidemiology of *Staphylococcus aureus*. Emerging Infectious Diseases 7, 178-182.

CHANG, C., CHEN, C.T., TSAI, Y.C., CHANG, W.C., 2000. A tissue culture protocol for propagation of a rare plant, *Lilium speciosum* Thunb. var. *gloriosoides* Baker. Botanical Bulletin of Academia Sinica 41, 139-142.

CHEESMAN, L., 2013. Micropropagation and pharmacological evaluation of *Boophone disticha*. PhD Thesis, University of KwaZulu-Natal, Pietermaritzburg.

CHEESMAN, L., NAIR, J., J., VAN STADEN, J., 2012. Antibacterial activity of crinane alkaloids from *Boophone disticha* (Amaryllidaceae). Journal of Ethnopharmacology 140, 405-408.

CHEN, J., ZIV, M., 2005. The effects of storage condition on starch metabolism and regeneration potentials of twin-scales and inflorescence stem explants of *Narcissus tazetta*. *In Vitro* Cellular Developmental Biology-Plant 41, 816-821.

CHHABRA, S.C., MAHUNNAH, B.L.A., MSHIU, E.N., 1987. Plants used in traditional medicine in Eastern Tanzania. Pteridophytes and angiosperms (Acanthaceae to Canellaceae). Journal of Ethnopharmacology 21, 253-277.

CHOW, Y.N., SELBY, C., FRASER, T.W., HARVEY, B.M.R., 1993. Basal plate tissue in *Narcissus* bulbs and in shoot clump cultures: Its structure and role in organogenic potential of single leaf cultures. Annals of Botany 71, 437-443.

CHOW, Y.N., SELBY, C., HARVEY, B.M.R., 1992. A simple method for maintaining high multiplication of *Narcissus* shoot cultures *in vitro*. Plant Cell Tissue and Organ Culture 30, 227-230.

CITOGLU, G., TANKER, M., GAMUSEL, B., 1998. Antiinflammatory effects of lycorine and haemanthidine. Phytotherapy Research 12, 205-206.

CITRON, M., 2002. Alzheimer's disease: treatments in discovery and development. Natural Neuroscience 5, 1055-1057.

COCKS, M.L., 1996. Towards an understanding of *amaYeza esiXhosa* Stores (African chemists). How they operate, and the services they offer in the Eastern Cape. MSc Thesis. Rhodes University, Grahamstown.

COLEMAN, S., LINDERMAN, R., HODGSON, E., ROSE, R.L., 2000. Comparative metabolism of chloroacetamide herbacides and selected metabolites in human and rat liver microsomes. Environmental Health Perspectives 108, 1151-1157.

COLQUE, R., VILADOMAT, F., BASTIDA, J., CODINA, C., 2002. Micropropagation of the rare *Eucrosia stricklandii* (Amaryllidaceae) by twin-scaling and shake liquid culture. Journal of Horticultural Science and Biotechnology 77, 739-743.

CORDELL, G.A., 1987. Pharmacognosy-far from dead. American Drug March 96-98.

CORDELL, G.A., QUINN-BEATTIE, M.L., FARNSWORTH, N.R., 2001. The potential of alkaloids in drug discovery. Phytotherapy Research 15, 183-205.

COWAN, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12, 564-582.

CRAFT, B.D., KERRIHARD, A.L., AMAROWICS, R., PEGG, R.B., 2012. Phenol-based antioxidants and the *in vitro* methods used for their assessment. Comprehensive Reviews in Food Science and Food Safety 11, 148-173.

CRAGG, G.M., NEWMAN, D.J., 2013. Natural products: a continuing source of novel drug leads. Biochimica et Biophysica Acta 1830, 3670-3695.

CUNNINGHAM, A.B., 1993. African medicinal plants, setting priorities at the interface between conservation and primary healthcare, People and Plants Working Paper. UNESCO, Paris.

CUSHNIE, T.T.P., CUSHNIE, B., LAMB, A., 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. International Journal of Antimicrobial Agents 44, 377-386.

CVIKROVÁ, M, HRUBCOVÁ, M., 1999. The role of phenolic substances in the processes of differentiation and morphogenesis. In: Strnad, M., Peć, P., Beck, E. (eds) Advances in regulation of plant development. Peres Publications, Prague.

DA SILVA, E.F., CONTO-CAVALHIERO, M.M., BRAZ, V.R., CYSNE-FINKEL, L., LEON, L.L., ECHEVARRIO, A., 2002. Synthesis and biological evaluation of new 1,3,4-thiadiazolium-2-phenylamine derivatives against *Leishmania amazoniensis* promastigotes and amastigotes. European Journal of Medical Chemistry 37, 979-984.

DAHLGREN, R.M.T. CLIFFORD, H.T., YEO, P.F., 1985. The families of monocotyledons: structure, evolution and taxonomy. Springer Verlag, Berlin.

DALL'ACQUA, S., 2013. Plant-derived acetylcholinesterase inhibitory alkaloids for the treatment of Alzheimer's disease. Botanics: Targets and Therapy 3, 19-28.

DANCER, S.J., 2001. The problems with cephalosporins. Journal of Antimicrobial Chemotherapy 48, 463-478.

DAVIES, K.J., 1995b. Oxidative stress: the paradox of aerobic life. Biochemical Society Symposia 61, 1-31.

DAVIES, P.J., 1995a. Plant hormones: physiology, biochemistry and molecular biology. Kluwer Academics, Dordrecht.

DE BRUYN, M.H., FERREIRA, D.I., SLABBERT, M.M., PRETORIUS, J., 1992. *In vitro* propagation of *Amaryllis belladonna*. Plant Cell Tissue and Organ Culture 31, 179-184.

DE KLERK, G-J., GUAN, H., HUISMAN, P., MARINOVA, S., 2011. Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in *Malus* ‘Jork 9’. Plant Growth Regulation 63, 175-185.

DE SMET, P.A.G.M., 1996. Some ethnopharmacological notes on African hallucinogens. Journal of Ethnopharmacology 50, 141-146.

DECKER, M., 2005. Novel inhibitors of acetyl- and butyrycholinesterase derived from the alkaloids dehyroevodiamine and rutaecarpine. European Journal of Medical Chemistry 40, 305-313.

DEPLEDGE, M., 2011. Pharmaceuticals: Reduce drug waste in the environment. Nature 478, 36.

DESBOIS, A.P., SMITH, V.J., 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Applied Microbiology and Biotechnology 85, 1629-1642.

DI MATEO, V., ESPOSITO, E., 2003. Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Current Drug Targets CNS and Neurological Disorders 2, 95-107.

DIAS, D.A., URBAN, S., ROESSNER, U., 2012. A historical overview of natural products in drug discovery. Metabolites 2,303-336.

DIERICKX, P.J., 1999. Glutathione-dependent cytotoxicity of the chloroacetanilide herbicides alachlor, metolachlor, and propachlor in rat and human hepatoma-derived cultured cells. Cell Biology and Toxicology 15, 325-332.

DIXON, R.A., PAIVA, N.L., 1995. Stress-induced phenylpropanoid metabolism. The Plant Cell 7, 1085-1097.

DOLD, A.P., COCKS, M.L., 1999. Preliminary list of Xhosa plant names from the Eastern Cape, South Africa. Bothalia 29, 267-292.

DOLD, A.P., COCKS, M.L., 2002. The trade in medicinal plants in the Eastern Cape Province, South Africa. South African Journal of Science 98, 589-597.

DOLEŽAL, K., POPA, I., KRYŠTOF, V., SPÍCHAL, L., FOJTÍKOVÁ, M., HOLUB, L., LENOBEL, R., SCHMÜLLING, T., STRNAD, M., 2006. Preparation of biological activity 6-benzylaminopurine derivatives in plants and human cancer cell lines. Bioorganic Medicinal Chemistry 14, 875-884.

DOWD, P.F., VEGA, F.E., 1996. Enzymatic oxidation products of allelochemicals as a basis for resistance against insects: effects on the corn leafhopper *Dalbulus maidis*. Natural Toxins 4, 85-91.

DREWES, F.E., VAN STADEN, J., 1994. *In vitro* propagation of *Gethyllis linearis* L. Bol., a rare indigenous bulb. South African Journal of Botany 60, 295-296.

DU PLESSIS, N., DUNCAN, G., 1989. Bulbous plants of Southern Afrirca - A guide to their cultivation and progatation. Tafelberg Publishers LTD, Cape Town.

DURI, Z.J., SCOVILL, J.P., HUGGINS, J.W., 1994. Activity of a methanolic extract of Zimbabwean *Crinum macowanii* against exotic RNA viruses *in vitro*. Phytotherapy Research 8, 121-122.

DYER, R.A., 1950. A review of the genus *Brunsvigia*. Plant Life 6, 63-83.

EFSA., 2011. Panel of food additives and nutrient sources added to food (ANS); Scientific opinion on the reevaluation of butylated hydroxyanisole-BHA (E 320) as a food additive. EFSA Journal 9, 2392.

EFSA., 2012. Panel of food additives and nutrient sources added to food (ANS); Scientific opinion on the reevaluation of butylated hydroxytolulene-BHT (E 321) as a food additive. EFSA Journal 10, 2588.

ELDEEN, I.M.S., ELGORASHI, E.E., VAN STADEN, J., 2005. Antibacterial, antiinflammatory, anti-acetylcholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. Journal of Ethnopharmacology 102, 457-464.

ELGORASHI, E.E., STAFFORD, G.I., VAN STADEN, J., 2004. Acetylcholinesterase enzyme inhibitory effects of Amaryllidaceae alkaloids. Planta Medica 70, 260-262.

ELGORASHI, E.E., VAN STADEN, J., 2004. Pharmacological screening of six Amaryllidaceae species. Journal of Ethnopharmacology 90, 27-32.

ELOFF, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64, 711-713.

ELOFF, J.N., 2004. Quantification the bioactivity of plant extracts during screening and bioassay guided fractionation. Phytomedicine 11, 370-371.

ENGVILD, K.C., 1986. Chlorine-containing natural compounds in higher plants. Phytochemistry 25, 781-791.

EVIDENTE, A., ANDOLFI, A., ABOU-DONIA, A.H., TOUEMA, S.M., HOMMODA, H.M., SHAWKY, E., MOTTA, A., 2004. (-)-Amarbellisine, a lycorine-type alkaloid from *Amaryllis belladonna* L. growing in Egypt. Phytochemistry 65, 2113-2118.

EVIDENTE, A., KORNIENKO, A., 2009. Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. Phytochemistry Reviews 8, 449-459.

FABRY, W., OKEMO, P., ANSORG, R., 1998. Antibacterial activity of East African medicinal plants. Journal of Ethnopharmacology 60, 79-84.

FAY, M.F., 1994. In what situations is *in vitro* culture appropriate to plant conservation? Biodiversity and Conservation 3, 176-183.

FELTON, G.W., DONATO, K., DEL, V., R.J., DUFFER, S.S., 1989. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. Journal of Chemical Ecology 15, 2667-2694.

FENNELL, C.W., 2002. Micropropagation and secondary metabolite production in *Crinum macowanii*. PhD Thesis, University of Natal, Pietermaritzburg.

FENNELL, C.W., CROUCH, N.R., VAN STADEN, J., 2001. Micropropagation of the River lily, *Crinum variabile* (Amaryllidaceae). South African Journal of Botany 67, 74-77.

FENNELL, C.W., ELGORASHI, E.E., VAN STADEN, J., 2003. Alkaloid production in *Crinum moorei* cultures. Journal of Natural Products 66, 1524-1526.

FENNELL, C.W., VAN STADEN, J., 2004. Biotechnology of southern African bulbs. South African Journal of Botany 70, 37-46.

FERNANDEZ-BACHILLER, M.I., PEREZ, C., GONZALEZ-MUNOZ, G.C., CONDE, S., LOPEZ, M.G., VILLARROYA, M., GARCIA, A.G., ISABEL, M., RODRIQUEZ-FRANCO, M.I., 2010. Novel tacrine-8-hydroxyquinoline hybrids as multifunctional agents for the treatment of Alzheimer's disease. Journal of Medicinal Chemistry 53, 4927-4937.

FOSTER, A.S., GIFFORD, E.M., 1959. Comparative morphology of vascular plants. Freeman, San Francisco, 621-623.

FULCHER, A.F., RANNEY, T.G., BURTON, J.D., WALGENBACH, J.F., DANEHOWER, D.A., 1998. Role of foliar phenolics in host plant resistance of *Malus* taxa to adult Japanese beetle. Horticultural Science 33, 862-865.

GAN, S., AMASINO, R.M., 1995. Inhibition of leaf senercence by autoregulated production of cytokinin. Science 270, 1986-1988.

GASPAR, T., KEVERS, C., PENEL, C., REID, D.M., THORPE, T.A., 1996. Plant hormones and plant growth regulators in plant tissue culture. *In Vitro* Cellular and Developmental Biology-Plant 32, 272-289.

GEORGE, F.E., 1993. Plant propagation by tissue culture. Part 1. The Technology. Exegetics Limited, Edington.

GEORGE, F.E., HALL, A.M., DE KLERK, G., 2008. The components of plant tissue culture media 1: macro and micro-nutrients. Plant Propagation by Tissue Culture, 65-113.

GERBER, M., BOUTRON-RUAULT, M.C., HERCHBERG, S., RIBOLI, E., SCALBERT, A., SIESS, M.H., 2002. Food and cancer: state of the art about the protective effects of fruits and vegetables. Bulletin du Cancer 89, 293-312.

GIBBONS, S., 2005. Plants as a source of bacterial resistance modulators and anti-infective agents. Phytochemical Reviews 4, 63-78.

GIRISH, H.V., SATISH, S., 2008. Antibacterial activity of important medicinal plants on human pathogenic bacteria - a comparative analysis. World Applied Sciences Journal 5, 267-271.

GRAUL, A.I., PROUS, J.R., 2005. The year's new drugs. Drug News Perspectives 18, 21-36.

GRIFFIN, S.G., WYLLIE, S.G., J.L., M., LEACH, D.N., 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour and Fragrance Journal 14, 322-332.

GROOTVELD, M., HALLIWELL, B., 1986. Aromatic hydroxylation as a potential measure of hydroxy radical formation *in vivo*. Biochemistry Journal 237, 499-504.

GROSS-SOROKIN, M.Y., ROAST, S.D., BRIGHTY, G.C., 2006. Assessment of feminization of male fish in English rivers by the environment agency of England and Wales. Environmental Health Perspectives 114, 147-151.

GRÚZ, J., NOVAK, O., STRNAD, M., 2008. Rapid analysis of phenolic acids in beverages by UPLC-MS/MS. Food Chemistry 111, 789-794.

GRYSEELS, B., POLMAN, K., CLERINX, J., KESTENS, L., 2006. Human schistosomiasis. The Lancet 368, 23-29.

GÜLCIN, I., 2007. Comparison of *in vitro* antioxidant and antiradical activities of $_{L}$-tyrosine and $_{L}$-Dopa. Amino Acids 32, 431-438.

GÜLCIN, I., 2012. Antioxidant activity of feed constituents: an overview. Archives of Toxicology 86, 354-391.

GURURAJ, H.B., GIRIDHAR, P., RAVISHANKAR, G.A., 2004. Efficient clonal propagation method for *Decalepis hamiltonii*, an endangered shrub, under the influence of phloroglucinol. Indian Journal of Experimental Biology 42, 424-428.

HALLIWELL, B., 1997. Antioxidants in human health and disease. Annual Reviews in Nutrition 16, 33-50.

HALLIWELL, B., GUTTERIDGE, J.M.C., 1989. Free radicals in biology and medicine, 2nd ed. Clarendon Press, Oxford.

HAMMATT, N., GRANT, N.J., 1997. Micropropagation of mature British wild cherry. Plant Cell Tissue and Organ Culture 47, 103-110.

HANDA, S.S., KHANUJA, S.P.S., LONGO, G., RAKESH, D.D., 2008. Extraction technologies for medicinal and aromatic plants. International Centre for Science and High Technology, Trieste, 21-25.

HANOWER, J., HANOWER, P., 1984. Inhibition et stimulation en culture *in vitro* de l'embryogenesis des souches issues d'explant foliaires de palmier a l'huile. Academy of Sciences Paris 298, 45-48.

HARVEY, A.L., 1995. The pharmacology of galanthamine and its analogues. Pharmaceutical Therapy 68, 113-128.

HE, M., QU, C., GAO, O., HU, X., HONG, X., 2015. Biological and pharmacological activities of Amaryllidaceae alkaloids. Royal Society of Chemistry Advances 5, 16562-16574.

HEINRICH, M., TEOH, H.L., 2004. Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. Journal of Ethnopharmacology 92, 147-162.

HELENO, S.A., MARTINS, A., QUEIROZ, M.A.R.P., FERREIRA, I.C.F.R., 2015. Bioactivity of phenolic acids: metabolites *versus* parent compounds: a review. Food Chemistry 173, 501-513.

HEMAISWARYA, S., KRUTHIVENTI, A.K., DOBLE, M., 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine 15, 639-652.

HEMSWORTH, G.R., KOCHAN, I., 1978. Secretion of Antimycobacterial fatty acids by normal and activated macrophages. Infection and Immunity 19, 170-177.

HERRMANN, K.M., 1995. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 7, 907-919.

HERTOG, M.G.L., FESKENS, E.J.M., HOLLMAN, P.C.H., KATAN, M.B., KROMHOUT, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the zupthen elderly study. The Lancet 342, 1007-1014.

HEYMAN, H.M., HUSSEIN, A.A., MEYER, J.J.M., LALL, N., 2009. Antibacterial activity of South African medicinal plants against methicillin resistant *Staphylococcus aureus*. Pharmaceutical Biology 47, 67-71.

HEYMANN, D.L., 2004. Control of communicable diseases manual, 18th ed. American Public Health Association, Washington DC.

HOAREAU, L., DA SILVA, E.J., 1999. Medicinal plants: a re-emerging health aid. Electronic Journal of Biotechnology 2, 56-70.

HOUGHTON, P.J., HYLANDS, P.J., MENSAH, A.Y., HENSEL, A., DETERS, A.M., 2005. *In vitro* tests and ethnopharmacological investigations: wound healing as an example. Journal of Ethnopharmacology 100, 100-107.

HOUGHTON, P.J., REN, Y., HOWES, M.J., 2006. Acetylcholinesterase inhibitors from plants and fungi. Natural Product Reports 23, 181-199.

HOYLES, M., 1991. The Story of Gardening. Journeyman Press, London.

HUSSEY, G., 1975. Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae, and Amaryllidaceae. Journal of Experimental Botany 26, 253-262.

HUSSEY, G., 1980. Propagation of some members of the Liliaceae, Iridaceae and Amaryllidaceae by tissue culture. In: Brickell, C.D., Cutler, D.F., Gregory, M. (Eds.), Petaloid Monocotyledons. Academic Press, London.

HUSSEY, G., 1982. *In vitro* propagation of monocotylenonous bulbs and corms. In: Fujiwara, A. (Ed.), Plant tissue culture. 5th International Congress of Plant Tissue and Cell Culture. Japanese Association for Plant Tissue Culture, Tokyo.

HUSSON, G.P., VILAGINES, P., SARRETTE, B., VILAGINES, P., 1994. Antiviral effect of the alkaloid extract isolated from *Haemanthus albiflos*. Annales Pharmaceutiques Francaises 52, 311-322.

HUSSON, G.P., VILAGINES, P., SARRETTE, B., VILAGINES, R., 1991. Antiviral effect of *Haemanthus albiflos* leaves extract on herpes virus, adenovirus, vesicular stomatitis virus, rotavirus and poliovirus. Annales Pharmaceutiques Francaises 49, 40-48.

HUTCHINGS, A., SCOTT, A.H., LEWIS, G., CUNNINGHAM, A., 1996. Zulu Medicinal Plants. An inventory. University of Natal Press, Pietermaritburg.

IBAÑEZ, M.R., AMO-MARCO, J.B., 1998. Promotion by phloroglucinol of micropropagation of *Minuartia valentina*, an endangered and endemic Spanish plant. Plant Growth Regulation 26, 49-56.

IWALEWA, E.O., McGAW, L.J., NAIDOO, V., ELOFF, J.N., 2007. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. African Jornal of Biotechnology 6, 2868-2885.

JÄGER, A.K., HUTCHINGS, A., VAN STADEN, J., 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology 52, 95-100.

JIN, Z., 2009. Amaryllidaceae and *Sceletium* alkaloids. Natural Product Reports 26, 363-381.

JIROVSKY, D., HORAKOVA, D., KOTOUCEK, M., VALENTOVA, K., ULRICHOVA, J., 2003. Analysis of phenolic acids in plant materials using HPLC with amperometric detection at a platinum tubular electrode. Journal of Separation Science 26, 739-742.

JONES, A.M.P., SAXENA, P.K., 2013. Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS One 8, DOI: 10.1371/journal.pone.0076802

KAMINEK, M., VANEK, T., MOTYKA, V., 1987. Cytokinin activities of N^6-benzyladenosine derivatives hydroxylated on the side chain phenyl ring. Journal of Plant Growth Regulation 6, 113-120.

KARIOTI, A., HADJIPAVLOU-LITINA, D., MENSAH, M.L.K., FLEISCHER, T.C., SALTSA, H., 2004. Composition and antioxidant activity of the essential oils of *Xylopia aethiopica* (Dun) A. Rich. (Annonaceae) leaves, stem bark, root bark, and fresh and dried friuts, growing in Ghana. Journal of Agriculture and Food Chemistry 52, 8094-8098

KELMANSON, J.E., JÄGER, A.K., VAN STADEN, J., 2000. Zulu medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69, 241-246.

KEVERS, C., FRANCK, T., STRASSER, R.J., DOMMES, J., GASPAR, T., 2004. Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. Plant Cell Tissue and Organ Culture 77, 181-191.

KHLEBNIKOV, A.I., SCHEPETKIN, I.A., DOMINA, N.G., KIRPOTINA, L.N., QUINN, M.T., 2007. Improved quantitative structure-activity relationship models to

predict antioxidant activity of flavonoids in chemical, enzymatic and cellular systems. Bioorganic and Medical Chemistry 15, 1749-1770.

KIM, K.W., DE HERTOGH, A.A., 1997. Tissue culture of ornamental flowering bulbs (geophytes). Horticultural Reviews 18, 87-169.

KIM, K-M., KIM, M-Y., YUN, P-Y, CHANDRASEKHAR, T. LEE, H-Y., SONG, P-S., 2007. Production of multiple shoots and plant regeneration from leaf segments of fig tree (*Ficus carica* L.). Journal of Plant Biology 50, 440-446.

KNEKT, P., JARVINEN, R., REUNANEN, A., MAATELA, J., 1996. Flavonoid intake and coronary mortality in Finland: a cohort study. British Medical Journal 312, 478-481.

KNOBLOCH, K., PAULI, A., IBERL, N., WEIS, H.M., WEIGAND, N., 1988. Modes of action of essential oil components on whole cells of bacteria and fungi in plate tests. In: Schreier, P. (Ed.), Bioflavour. Walter de Gruyther, Berlin.

KNOBLOCH, K., WEIGAND, N., WEIS, H.M., VIGENSCHOW, H., 1986. Progress in essential oil research. Walter de Gruyther, Berlin.

KOMALAVALLI, N., RAO, M.V., 2000. *In vitro* micropropagation of *Gymnema sylvestre* - a multipurpose medicinal plant. Plant Cell Tissue and Organ Culture 61, 97-105.

KOOPOWITZ, H., 1986. Conservation problems in the Amaryllidaceae. Herbertia 42, 21-25.

KOORBANALLY, N.A., RAGHOO, M., CROUCH, N.R., 2006. Amaryllidaceae alkaloids from *Scadoxus puniceus*. American Society of Pharmacognosy Meeting 2006. Poster 114.

KORNIENKO, A., EVIDENTE, A., 2008. Chemistry, biology and medicinal potential of narciclasine and its congeners. Chemistry Reviews 108, 1982-2014.

KOZAI, Y., KUBOTA, C., JEONG, B.R., 1997. Environmental control for the largescale production of plants through *in vitro* techniques. Plant Cell Tissue and Organ Culture 51, 41-56.

KRISHNA, H., SAIRAM, R.K., SINGH, S.K., PATEL, V.B., SHARMA, R.R., 2008. Mango explant browning: effect of ontogenic age, mycorrhization and pre-treatments. Scientia Horticulturae 118, 132-138.

KRISHNAIAH, D., SARBATHY, R., NITHYANANDAM, R., 2011. A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Processing 89, 217-233.

KUBALÁKOVA, M., STRNAD, M., 1992. The effects of aromatic cytokinins (populins) on micropropagation and regeneration of sugar beet *in vitro*. Biologia Plantarum 34, 578-579.

KULKARNI, M.G., SPARG, S.G., VAN STADEN, J., 2005. Enhancing the germination of fresh seeds of *Bowiea volubilis*, a widely used bulbous medicinal plant. South African Journal of Science 101, 491-493.

KUPCHAN, S.M., KELSEY, J.E., MARUYAMA, M., CASSADY, J.M., 1968. Eupachlorin acetate, a novel chloro-sesquiterpeniod lactone tumor inhibitor from *Eupatorium rotundifolium*. Tetrahedron Letters 9, 3517-3520.

LACEY, R.W., LORD, V.L., 1981. Sensitivity of *Staphylococci* to fatty acids: novel inactivation of linolenic acid by serum. Journal of Medical Microbiology 14, 41-49.

LAI, L.S., CHOU, S.T., CHAO, W.W., 2001. Studies on the antioxidative activities of Hsian-tsao (*Mesona procumbens* Hemsl) leaf gum. Journal of Agricultural and Food Chemistry 49, 963-968.

LAMORAL-THEYS, D., ANDOLFI, A., VAN GOIETSENOVEN, G., CIMMINO, A., LE CALVE, B., WAUTHOZ, N., MEGALIZZI, V., GRAS, T., BRUYERE, C., DUBOIS, J., MATHIEU, V., KORNIENKO, A., KISS, R., EVIDENTE, A., 2009. Lycorine, the main phenanthridine amaryllidaceae alkaloid, exhibits significant antitumor activity in cancer cells that display resistance to proapoptotic stimuli: an investigation of structure-activity relationship and mechanistic insight. Journal of Medicinal Chemistry 52, 6244-6256.

LANDRIGAN, M., MORRIS, S.C., McGLASSON, W.B., 1996. Postharvest browning of rambutan is a consequence of water loss. Journal of the American Society of Horticultural Science 121, 730-734.

LAUKKANEN, H., RAUTIAINEN, L., TAULAVUORI, E., HOHTOLA, A., 2000. Changes in cellular structures and enzymatic activities during browning of Scots pine callus derived from mature buds. Tree Physiology 20, 467-475.

LAWSON, C.G.R., ROLFE, B.G., DJORDJEVIC, M.A., 1996. Rhizobium inoculation induces condition dependent changes in the flavonoid composition of root exudates from *Trifolium subterraneum*. Australian Journal of Plant Physiology 23, 93-101.

LEFER, D.J., GRANGER, D.N., 2000. Oxidative stress and cardiac disease. American Journal of Medicine 109, 315-323.

LETHAM, D.S. PALNI, L.M.S., 1983. The biosynthesis and metabolism of cytokinins. Annual Reviews in Plant Physiology 34, 163-197.

LI, X., XIONG, Z., YING, X., CUI, L., ZHU, W., LI, F., 2006. A rapid ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometric method for the qualitative and quantitative analysis of the constituents of the flower of *Trollius ledibouri* Reichb. Analytica Chimica Acta 580, 170-180.

LIGHT, M.E., SPARG, S.G., STAFFORD, G.I., VAN STADEN, J., 2005. Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. Journal of Ethnopharmacology 100, 127-130.

LILIEN-KIPNIS, H., ZIV, M., KAHANY, S., 1992. Proliferation and regeneration of *Nerine* in liquid cultures. Acta Horticulturae 325, 467-473.

LIM, T.Y., LIM, Y.Y., YULE, C.M., 2009. Evaluation of antioxidant, antibacterial, and anti-tyrosinase activities of four *Macaranga* species. Food Chemistry 114, 594-599.

LITESCU, S.C.E., S., RADU, G.L., 2010. Methods for the determination of antioxidant capacity in food and raw materials. Advances in Experimental Medicine and Biology 698, 241-249.

LIVERMORE, D.M., 2004. The need for new antibiotics. Clinical and Microbiological Infections 10, 1-9.

LÓPEZ, S., BASTIDA, J., VILADOMAT, F., CODINA, C., 2002. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Sciences 71, 2521-2529.

LORENZO, J.C., BLANCO, M.A., PALÁEZ, O., GONZÁLEZ, A., CID, M., IGLESIAS, A., GONZÁLEZ, B., ESCALONA, M., ESPINOSA, P., BORROTO, C. 2001. Sugarcane micropropagation and phenolic excretion. Plant Cell Tissue and Organ Culture 65, 1-8.

LOUW, C.A.M., REGNIER, T.J.C., KORSTEN, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. Journal of Ethnopharmacology 82, 147-154.

LÜ, J., LIN, P.H., YAO, Q., CHEN, C., 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. Journal of Cellular and Molecular Medicine 14, 840-860.

LUI, J., WANG, C., WANG, Z., ZHANG, C., LU, S., LIU, J., 2011. The antioxidant and free-radical scavenging activities of extract and fractions from corn silk (*Zea mays* L.) and related flavone glycosides. Food Chemistry 126, 261-269.

MABBERLEY, D.J., 1987. The Plant Book. Cambridge University Press, Cambridge.

MACHEIX, J-J., FLEURIET, A., BILLOT, J., 1990. Fruit phenolics. CRC Press, Florida.

MADHUSUDHANAN, K., RAHIMAN, B.A., 2000. The effect of activated charcoal supplemented media to browning of *in vitro* cultures of *Piper* species. Biologia Plantarum 43, 297-299.

MAKKAR, H.P.S., SIDDHURAJU, P., BECKER, K., 2007. Plant secondary metabolites. Humana Press Inc, New Jersey.

MALÁ, J., PAVLINA, M., HELENA, C., KARADY, M., NOVAK, O., MIKULIK, J., HAUSEROVA, E., GREPLOVA, J., STRNAD, M., DOLEZAL, K. 2009. Micropropagation of wild service tree (*Sorbus torminalis* [L.] Crantz): the regulative role of different aromatic cytokinins during organogenesis. Journal of Plant Physiology 28, 341-348.

MALLIKARJUNA, N., KRANTHI, K.R., JADHAV, D.R., KRANTHI, S., CHANDRA, S., 2004. Influence of foliar chemical compounds on the development of *Spodoptera litura* (Fab.) in interspecific derivatives of groundnut. Journal of Applied Entomology 128, 321-328.

MANDER, M., 1998. Marketing of indigenous medicinal plants in South Africa - a case study in KwaZulu-Natal. FAO - Food and Agricultural Organization of the United Nations, Rome, Italy.

MANDER, M., MCKENZIE, M., 2005. Southern African trade directory of indigenous natural products. Commercial Products from the Wild Group, Matieland.

MANDER, M., NTULI, L., DIEDERICHS, N., MAVUNDLA, K., 2007. Economics of the traditional medicine trade in South Africa. In: Harrison, S., Bhana, R., Ntuli, A. (Eds.), South African Health Review. Health Systems Trust, Pretoria.

MARIAJANCYRANI, J., CHANDRAMOHAN, G., SARAVANAN, ELAYARAJA, A., 2013. Isolation and antibacterial activity of terpenoid from *Bougainvillea glabra* choisy leaves. Asian Journal of Plant Science and Research 3, 70-73.

MARSHALL, N.T., 1998. Searching for a cure: conservation of medicinal wildlife resources in East and Southern Africa. Traffic International, Cambridge.

MASOKO, P., PICARD, J., ELOFF, J.N., 2007. The antifungal activity of twenty-four southern African *Combretum* species (Combretaceae). South African Journal of Botany 73, 173-183.

MATKOWSKI, A., 2008. Plant *in vitro* culture for the production of antioxidants - A review. Biotechnology Advances 26, 548-560.

MAUSETH, J.D., 1991. Botany: an introduction to plant biology. Saunders, Philadelphia.

McGAW, L.J., JÄGER, A.K., VAN STADEN, J., 2000. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72, 247-263.

MEEROW, A.W., CLAYTON, J.R., 2004. Generic relationships among the baccate-fruited Amaryllidaceae (tribe Haemantheae) inferred from plastid and nuclear noncoding DNA sequences. Plant Systematics and Evolution 244, 141-155.

MEEROW, A.W., SNIJMAN, D.A., 1998. Amaryllidaceae. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants. Springer, Berlin.

MEHTA, M., ADEM, A., SABBAGH, M., 2011. New acetylcholinesterase inhibitors for Alzheimer's disease. International Journal of Alzheimer's Disease 2012, 728983-728991.

MELZER, D., 1998. New drug treatment for Alzheimer's disease: lessons for healthcare policy. Biochemical Medical Journal 316, 762-764.

MILES, P.W., OERTLI, J.J., 1993. The significance of antioxidants in the aphid-plant interaction: the redox hypothesis. Entomologia Experimentalis et Applicata 67, 275-283.

MISHRA, B.B., TIWARI, V., 2011. Natural products: An evolving role in future drug discovery. European Journal of Medicinal Chemistry 46, 4769-4807.

MOHANASUNDARI, C., NATARAJAN, D., SRINIVASAN, K., UMAMAHESWARI, S., RAMACHANDRAN, A., 2007. Antibacterial properties of *Passiflora foetida* L. - a common exotic medicinal plant. African Journal of Biotechnology 6, 2650-2653.

MOON, J.K., SHIBAMOTO, T., 2009. Antioxidant assays for plant and food components. Journal of Agricultural and Food Chemistry 57, 1655-1666.

MORAN, G.P., COLQUE, R., VILADOMAT, F., BASTIDA, J., CODINA, C., 2003. Mass propagation of *Cyrtanthus clavatus* and *Cyrtanthus spiralis* using liquid medium culture. Scientia Horticulturae 98, 49-60.

MORRIS, M., 2001. Treating HIV in South Africa - a tale of two systems. The Lancet Diseases 2, 319.

MOYO, M., BAIRU, M.W., AMOO, S.O., VAN STADEN, J., 2011. Plant biotechnology in South Africa: micropropagation research endeavours, prospects and challenges. South African Journal of Botany 77, 996-1011.

MOYO, M., NDHLALA, A.R., FINNIE, J.F., VAN STADEN, J., 2010. Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (Anacardiaceae) extracts. Food Chemistry 123, 69-76.

MUKHTAR, M., ARSHAD, M., AHMAD, M., POMERANTZ, R.J., WIGDAHL, B., PARVEEN, Z., 2008. Antiviral potentials of medicinal plants. Virus Research 131, 111-120.

MULLIN, M., 1970. Tissue culture for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-479.

MURASHIGE, T., SKOOG, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15, 473-497.

MUZAFFAR, S., ALI, B., WANI, N.A., 2012. Effect of catechol, gallic acid and pyrogallic acid on the germination, seedling growth and the level of endogenous phenolics in cucumber (*Cucumis sativus* L.). International Journal of Life Sciences Biotechnology and Pharmaceutical Research 1, 50-55.

NAIR, J.J., CAMPBELL, W.E., GAMMON, D.W., ALBRECHE, C.F., VILADOMAT, F., CODINA, C., BASTIDA, J., 1998. Alkaloids from *Crinum delagoense*. Phytochemistry 49, 2539-2543.

NAIR, J.J., RAROVA, L., STRNAD, M., BASTIDA, J., VAN STADEN, J., 2012. Apoptosis-inducing effects of distichamine and narciprimine, rare alkaloids of the plant family Amaryllidaceae. Bioorganic and Medicinal Chemistry Letters 22, 6195-6199.

NAIR, J.J., VAN STADEN, J., 2012. Acetylcholinesterase inhibition within the lycorine series of Amaryllidaceae alkaloids. Natural Product Communications 7, 959-962.

NAIR, J.J., VAN STADEN, J., 2013. Pharmacological and toxicological insights to the South African Amaryllidaceae. Food and Chemical Toxicology 62, 262-275.

NCUBE, N.S., AFOLAYAN, A.J., OKOH, A.I., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology 7, 1797-1806.

NDHLALA, A.R., FINNIE, J.F., VAN STADEN, J., 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial Zulu herbal mixture: *Imbiza ephuzwato*. Journal of Ethnopharmacology 133, 663-674.

NEELAM, S., CHANDEL, K.P.S., 1992. Effects of ascorbic acid on axillary shoot induction in *Tylophora indica* (Burm. F.) Merr. Plant Cell Tissue and Organ Culture 29, 109-113.

NEERGAARD, J.S., ANDERSEN, J., PEDERSEN, M.E., STAFFORD, G.I., VAN STADEN, J., JÄGER, A.K., 2009. Alkaloids from *Boophone disticha* with affinity to the serotonin transporter. South African Journal of Botany 75, 371-374.

NEU, H.C., 1992. The crisis in antibiotic resistance. Science 257, 1064-1073.

NEUWINGER, H.D., 2000. African traditional medicine. A dictionary of plant use and applications. Medpharm Scientific Publishers, Stuttgart.

NEWMAN, D.J., CRAGG, G.M., SNADER, K.M., 2000. The influence of natural products upon drug discovery. Natural Product Reports 17, 215-234.

NIGAM, A., GUPTA, D., SHARMA, A., 2014. Treatment of infectious disease: beyond antibiotics. Microbiological Research 169, 643-651.

NIKOLOVA, M., GEVRENOVA, R., 2005. Determination of phenolic acids in Amaryllidaceae species by high performance liquid chromatography. Pharmaceutical Biology 43, 289-291.

OKIGBO, R.N., EME, U.E., OGBOGU, S., 2008. Biodiversity and conservation of medicinal and aromatic plants in Africa. Biotechnology and Molecular Biology Reviews 3, 127-134.

OLIVIER, W., 1980. The genus *Cyrtanthus* Ait. Veld Flora (Kirstenbosch) 66, 78-81.

ORHAN, I., KARTAL, M., TOSUN, F., SENER, B., 2007. Screening of various phenolic acids and flavonoid derivatives for their anticholinesterase potential. Zeitschrift fur Naturforschung, 62, 829-832.

OUATTARA, B., SIMARD, R.E., HOLLEY, R.A., PIETTE, G.J-P., BEGIN, A., 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. International Journal of Food Microbiology 37, 155-162.

OZYIGIT, I.I., KAHRAMAN, M.V., ERCAN, O., 2007. Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). African Journal of Biotechnology 6, 3-8.

PÂQUES, M., 1991. Vitrification and micropropagation: causes, remedies and prospects. Acta Horticultuae 289, 283-290.

PARKER, C.W., LETHAM, D.S., GOLLNOW, B.I., SUMMONS, R.E., DUKE, C.C., MACLEOD, J.K., 1978. Regulators of cell division in plant tissues XXV. Metabolism of zeatin by lupin seedlings. Planta 142, 239-251.

PATZ, J.A., CAMPELL-LENDRUM, D., HOLLOWAY, T., FOLEY, J.A., 2005. Impact of regional climate change on human health. Nature 438, 310-317.

PEREIRA, S.P., FERNANDES, M.A, MARTINS, J.D., SANTOS, M.S., MORENO,A.J., VINCENTE, J.A., VIDEIRA, R.A., JURADO, A.S., 2009. Toxicity assessment of the herbicide metolachlor: comparative effects on bacterial and mitochondrial model systems. Toxicology *In Vitro* 23, 1585-1590.

PERRY, E.K., 1986. The cholinergic hypothesis: ten years on. British Medical Bulletin 42, 63-69.

PETTIT, G.R., MELODY, N., HERALD, D.L., 2001. Antineoplastic agents. 99. *Amaryllis belladonna*. Journal of Natural Products 47, 796-801.

PIERIK, R.L.M., 1987. *In vitro* culture of higher plants. Martinus Nijhoff, Dordrecht.

PIERIK, R.L.M., 1991. Micropropagation of ornamental plants. Acta Horticulturae 289, 45-53.

PIERIK, R.L.M., BLOKKER, J.S., DEKKER, M.W.C., DE DOES, H., KUIP, A.C., VAN DER MADE, T.A., MENTEN, Y.M.J., DE VETTEN, N.C.M.H., 1990. Micropropagation of *Hippeastrum* hybrids. In: De Jong, J. (Ed.), Integration of *in vitro* techniques in ornamental plant breeding. Proceedings, Symposium, 10-14 November 1990. CPO Centre for Plant Breeding Research, Wageningen.

PIERIK, R.L.M., IPPEL, B.J., 1977. Plantlet formation from excised bulb scale segments of *Nerine*. Acta Horticulturae 78, 197-202.

PIETTA, P.G., 2000. Flavonoids as antioxidants. Journal of Natural Products 63, 1035-1042.

POOLEY, E. 1998. A field guide to the wild flowers of KwaZulu-Nataland the Eastern Region. Natal Flora Publications Trust, Durban.

PRÈSTAMO, G., MANZANO, P., 1993. Peroxidase of selected fruits and vegetables and the possible use of ascorbic acid as an antioxidant. Horticultural Science28, 48-50.

RABE, T., VAN STADEN, J., 1997. Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56, 81-87.

RAHMAN, K., 2007. Studies on free radicals, antioxidants and co-factors. Journal of Clinical Interventions in Aging 2, 219-236.

RAMARATHNAM, N., OSAWA, T., OCHI, H., KAWAKISHI, S., 1995. The contribution of plant food antioxidants to human health. Trends in Food Science 6, 75-82.

RATES, S.M.K., 2001. Plants as a source of drugs. Toxicon 39, 603-613.

RAVEN, P.H., EVERT, R.F., EICHHORN, S.E., 1992. Biology of plants. Worth, New York.

REDSHAW, C.H., STAHL-TIMMINS, W.M., FLEMING, L.E., DAVIDSON, I., DEPLEDGE, M.H., 2013. Potential changes in disease patterns and pharmaceutical use in response to climate change. Journal of Toxicology and Environmental Health 16, 285-320.

REICHERT, J.M., 2003. A guide to drug discovery: trends in development and approval times for new therapeutics in the United States. Nature Reviews Drug Discovery 2, 695-702.

RICE, L.J., 2009. Micropropagation of *Brunsvigia undulata* F.M. Leight. MSc Thesis, University of KwaZulu-Natal, Pietermaritzburg.

RICE-EVANS, C.A., MILLER, N.J., PAGANGA, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20, 933-956.

RICKRODE, T.E., 1986. Identification and antibiotic activity if fatty acids in dermal secretions of *Plethedon cinereus*. American Midland Naturalist Journal 115, 198-200.

RILEY, R.G., KOLATTUKUDY, P.E., 1975. Evidence for covalently attached *p*-coumaric acid and ferulic acid in cutins and suberins. Plant Physiology 56, 650-654.

ROBBINS, R.J., 2003. Phenolic acids in foods: an overview of analytical methodology. Journal of Agricultural and Food Chemistry 51, 2866-2877.

ROGERS, L.A., CAMPBELL, M.M., 2004. The genetic control of lignin deposition during plant growth and development. New Phytologist 164, 17-30.

ROMAIS, E., TEIXEIRA, C., RIBEIRO, E., LOPEZ, S., 2000. Efeito do floroglucinol na reacao morfogenica *in vitro* de segmentos internodais de *Citrus sinensis* (L,) Osbeck cv. Revista Ceres 47, 113-120.

ROUT, G.R., DAS, P., 1997. *In vitro* organogenesis in ginger (*Zingiber officinale* Rosc.). Journal of Herbs, Spices and Medicinal Plants 4, 41-51.

RUSSELL, A.D., 1991. Mechanisms of bacterial resistance to non-antibiotics: food additives and food pharmaceutical preservatives. Journal of Applied Bacteriology 71, 191-201.

SAHER, S., PIQUERAS, A., HELLIN, E., OLMOS, E., 2004. Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. Physiologia Plantarum 120, 152-161.

SALISBURY, F.B., ROSS, C.W., 1992. Plant Physiology. Wadsworth, Belmont.

SAMUELSSON, G., 2004. Drugs of natural origin: a textbook of pharmacognosy. 5th Swedish Pharmaceutical Press, Stockholm.

SARATHE, S., TRIPATHE, M.K., SANKAR, M.V., SINGH, O.P., 2014. Studies on *in vitro* morphogenesis from bulb scale and leaf disc explants of *Amaryllis belladonna* L. Plant Cell Biotechnology and Molecular Biology 14, 22-32.

SARKAR, D., NAIK, P.S., 2000. Phloroglucinol enhances growth and rate of axillary shoot proliferation in potato shoot tip cultures *in vitro*. Plant Cell Tissue Culture Organ Culture 60, 139-149.

SATHYANARAYANA, B.N., MATHEWS, D., 2007. Plant tissue culture: Practices and new experimental protocols. I.K. International Publishing House Pvt. Ltd., New Delhi.

SATO, T., KIUCH, F., SANKAWA, U., 1982. Inhibition of phenylalenine ammonia-lyase by cinnamic acid derivatives and related compounds. Phytochemistry 21, 845-850.

SCHEUER, P.J., DARIAS, J., (Eds.) 1978-1981. Marine natural products. Vol. 1-5. Academic Press, New York.

SCHULZ, V., 2003. Ginko extract or cholinesterase inhibitors in patients with dementia: what clinical trials and guidelines fail to consider. Phytomedicine 10, 74-79.

SELLÈS, M., VILADOMAT, F., BASTIDA, J., CODINA, C., 1999. Callus induction, somatic embryogenesis and organogenesis in *Narcissus confuses*: correlation between the state of differentiation and the content of galanthamine and related alkaloids. Plant Cell Reports 18, 646-651.

SEOPOSENGWE, K., VAN TONDER, J.J., STEENKAMP, V., 2013. *In vitro* neuroprotective potential of four medicinal plants against rotenone-induced toxicity in SH-SY5Y neuroblastoma cells. BMC Complementary and Alternative Medicine 13, 353-364.

SHAHIDI, F., JANITHA, P.K., WANASUNDARA, P.D., 1992. Phenolic antioxidants. Critical Reviews in Food Science and Nutrition 32, 67-103.

SHAHIDI, F., NACSK, M., 1995. Food phenolics: sources, chemistry, effects, and applications. Technomic Publishing Company, Lancaster.

SHARAN, M., TAGUCHI, G., GONDA, K., JOUKE, T., SHIMOSAKA, M., HAYASHIDA, N., OKAZAKI, M., 1998. Effects of methyl jasmonate and elicitor on the activation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco cell culture. Plant Science 132, 13-19.

SIES, H., 1991. Oxidative stress: from basic research to clinical application. American Journal of Medicine 91, 31-39.

SIKORSKA, M., MATLAWSKA, I., GLOWNIAK, K., ZGORKA, G., 2000. Qualitative and quantitative analysis of phenolic acids in *Asclepias syriaca* L. Acta Poliniae Pharmaceutica 57, 69-72.

SINGH, A., DESGAGNÉ-PENIX, I., 2014. Biosynthesis of the Amaryllidaceae alkaloids. Plant Science Today 1, 114-120.

SINGH, S.B., BARRETT, J.F., 2006. Empirical antibacterial drug discovery - foundation in natural products. Biochemical Pharmacology 71, 1006-1015.

SKOOG, F., MILLER, C.O., 1957. Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. The biological action of growth substances. Symposia of the Society of Experimental Biology 11, 118-131.

SLEIGH, D.J., TIMBURG, M.C., 1998. Notes on medical bacteriology. Churchill Livingstone, Edinburgh.

SMITH, M.A. ROTTKAMP, C.A., NUNOMURA, A., RAINA, A.K., PERRY, G., 2000. Oxidative stress in Alzheimer's disease. Biochimica et Biophysica Acta 1502, 139-144.

SNIJMAN, D.A., LINDER, H.P., 1996. Phylogenetic relationships, seed characters, and dispersal system evolution in Amaryllideae (Amaryllidaceae). Annals of the Missouri Botanical Garden 83, 362-368.

STABA, E.J., 1980. Plant tissue culture as a source of biochemicals. CRC Press, Boca Raton, Florida.

STAFFORD, G.I., JÄGER, A.K., VAN STADEN, J., 2005. Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants. Journal of Ethnopharmacology 97, 107-115.

STAFFORD, G.I., PEDERSEN, M.E., VAN STADEN, J., JÄGER, A.K., 2008. Review on plants with CNS-effects used in traditional South African medicine against mental diseases. Journal of Ethnopharmacology 119, 513-537.

STAIKIDOU, I., SELBY, C., HANKS, G., 2006. Stimulation of *in vitro* bulblet growth in *Galanthus* species with sucrose and activated charcoal. Acta Horticulturae 725, 421-426.

STEINITZ, B., YAHEL, H., 1982. *In vitro* propagation of *Narcissus tazetta*. Horticultural Science 17, 333-334.

STRNAD, M., 1997. The aromatic cytokinins. Physiologia Plantarum 101, 674-688.

SULTANA, J., SULTANA, N., SIDDIQUE, M.N.A., ISLAM, A.K.M.A., HOSSAIN, M.M., HOSSAIN, T., 2010. *In vitro* bulb production in *Hippeastrum* (*Hippeastrum hybridum*). Journal of Central European Agriculture 11, 469-474.

SUNG, W.S., LEE, D.G., 2010. Antifungal action of chlorogenic acid against pathogenic fungi, mediated by membrane disruption. Pure and Applied Chemistry 82, 219-226.

TABIYEH, D.T., BERNARD, F., SHACKER, H., 2006. Investigation of glutathione, salicylic acid and GA_3 effects on browning in *Pistacia vera* shoot tip culture. Acta Horticulturae 726, 201.

TAGGART, M.A., SENACHA, K.R., GREEN, R.E., JHALA, Y.V., RAGHAVAN, B., RAHMANI, A.R., CUTHBERT, R., PAIN, J., MEHARG, A.A., 2007. Diclofenac residues in carcases of domestic ungulates available to vultures in India. Environment International 33, 759-765.

TAMAGNONE, L., MERIDA, A., STACEY, N., PLASKITT, K., PARR, A., CHANG, C-F., LYNN, D., DOW, M., ROBERTS, K., MARTIN, C., 1998. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. The Plant Cell 10, 1801-1816.

TANG, W., HARRIS, L.C., OUTHAVONG, V., NEWTON, R.T., 2004a. Antioxidants enhance *in vitro* plant regeneration by inhibiting the accumulation of peroxidase in Virginia pine (*Pinus virginiana* Mill.). Plant Cell Reports 22, 871-877.

TANG, W., NEWTON, R.T., OUTHAVONG, V., 2004b. Exogenously added polyamines recover browning tissues into normal callus cultures and improve plant regeneration in pine. Physiologia Plantarum 122, 386-395.

TEIXEIRA, da SILVA, J.A., DOBRANSZKI, J., ROSS, S., 2013. Phloroglucinol in plant tissue culture. *In Vitro* Cellular Development Biology-Plant 49, 1-16.

TEIXEIRA, da SILVA, J.A., GIANG, D.T., TANAKA, M., 2005. Micropropagation of sweet potatoe (*Ipomoea batatas*) in a novel CO_2-enriched vessel. Journal of Plant Biotechnology 7, 1-8.

THOMAS, T.D., 2008. The role of activated charcoal in plant tissue culture. Biotechnological Advances 26, 618-631.

THORPE, A.T., 2006. History of plant tissue culture. Methods in Molecular Biology 318, 9-32

THRING, T.S.A., WEITZ, F.M., 2006. Medicinal plant use in the Bredasdorp/ Elim region of the southern Overberg in the Western Cape Province of South Africa. Journal of Ethnopharmacology 103, 261-275.

TIPIRDAMAZ, R., 2003., Rooting and acclimatization of *in vitro* micropropagated snowdrop (*Galanthus ikariae* Baker.) bulblets. Akdeniz Ùniversitesi Ziraat Fakùltesi Dergisi 16, 121-126.

TIWARI, P., KUMAR, B., KAUR, M., KAUR, G., KAUR, H., 2011. Phytochemical screening and extraction: a review. International Pharmaceutica Scientia 1, 98-106.

TORRES-CONTRERAS, A.M., NAIR, V., CISNEROS-ZEVALLOS, L., 2014. Plants as biofactories: stress-induced production of chlorogenic acid isomers in potato tubers as affected by wounding intensity and storage time. Industrial Crops and Products 62, 61-66.

TOTH, K., HAAPALA, T., HOHTOLA, A., 1994. Alleviation of browning in oak explants by chemical pretreatments. Biologia Plantarum 36, 511-517.

TRAVIS, J., 1994. Reviving the antibiotic miracle. Science 264, 360-362.

TUMIATTI, V., MINARINI, A., BOLOGNESI, M.L., MILELLI, A., ROSINI, M., MELCHIORRE, C., 2010. Tacrine derivatives and Alzheimer's disease. Current Medicinal Chemistry 17, 1825-1838.

TURNER, S., 2001. *Scadoxus puniceus* (L.) Friis & Nordal. Retrieved from URL: https://www.plantzafrica.com/plantqrs/scadoxuspuniceus.htm. Date accessed: 13-02-2013.

TURNER, W.B., ALDRIDGE, D.C., 1983. Fungal metabolites. 2nd Ed. Academic Press, London.

TÜZEN, M., ÖZDEMİR, M., 2003. Chromatographic determination of phenolic acids in the snowdrop by HPLC. Turkish Journal of Chemistry 27, 49-51.

UCHENDU, E.E.P., G., BROWN, D.C., SAXENA, P.K., 2011. *In vitro* propagation of North American ginseng (*Panax quinquefolius*. p. L.). *In Vitro* Cellular and Developmental Biology-Plant 47, 710-718.

UNVER, N., 2007. New skeletons and new concepts in Amaryllidaceae alkaloids. Phytochemistry Reviews 6, 125-135.

VAN DEN BERGE, D.A., LEVEN, M., MERTENS, F., VLIETINCK, A.J., LAMMENS, E., 1978. Screening of higher plants for biological activities. II. Antiviral Activity. Lloydia 41, 463-471.

VAN STADEN, J., 1999. Medicinal plants in southern Africa: utilization, sustainability, conservation – can we change the mindsets? Outlook on Agriculture 28, 75-76.

VAN WYK, B-E., 2008. A broad review of commercially important southern African medicinal plants. Journal of Ethnopharmacology 119, 342-355.

VAN WYK, B-E., VAN HEERDEN, F., VAN OUDTSHOORN, B., 2005. Poisonous Plants of South Africa. Briza Publications, Pretoria.

VAN WYK, B-E., VAN OUDTSHOORN, B., GERICKE, N., 1997. Medicinal plants of South Africa. Briza Publicationa, Pretoria.

VEALE, D.J.H., FURMAN, K.I., OLIVER, D.W., 1992. South African traditional herbal medicines used during pregnancy and child birth. Journal of Ethnopharmacology 36, 185-191.

VILADOMAT, F., BASTIDA, J., CODINA, C., NAIR, J.J., CAMPBELL, W.E., 1997. Alkaloids of the South African Amaryllidaceae. In: Pandalai, S.G. (Ed.), Recent Research Developments in Phytochemistry. Research Signpost Publishers, Trivandrum

VISHNEVETSKY, J., ZAMSKI, E., ZIV, M., 2003. Enhanced bud and bulblet regeneration from bulbs of *Nerine sarniensis* cultured *in vitro*. Plant Cell Reports 21, 645-650.

WALA, B.B., JASRAI, Y.T., 2003. Micropropagation of an endangered medicinal plant: *Curculigo orcioides* Gaertn. Plant Tissue Culture 13, 13-19.

WATT, J.M., BREYER-BRANDWIJK, M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. Livingston Ltd., Edinburgh.

WEATHERHEAD, M.A., BURDON, T., HENSHAW, G.G., 1979. Effect of activated charcoal as an additive to plant tissue culture media. Zeitscrift fur Pflanzenphysiol 89, 141-147.

WEBB, C. De B., WRIGHT, J.B., 1986. The James Stuart archive of recorded oral evidence relating to the history of the Zulu and neighbouring peoples. Vol 4. University of Natal Press, Pietermaritzburg

WEBER, H., 2002. Fatty acid-derived signals in plants. Trends in Plant Science 7, 217-224.

WERBROUCK, S.P.O., STRNAD, M., VAN ONCKELEN, H.A., DEBERGH, P.C., 1996. The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. Plant Cell Reports 14, 662-665.

WHITE, P.R., 1963. The cultivation of animal and plant cells, 2nd ed. Ronald Press, New York.

WILLE, J.J., KYDONIEUS, A., 2003. Palmitoleic acid isomer (C16: 1Δ6) in human skin sebum is effective against gram-positive bacteria. Skin Pharmacology and Applied Skin Physiology 16, 176-187.

WILLIAMS, L., HOME, V., ZHANG, X., 1989. The composition and bacterial activity of oil of *Malaleuca alternifolia* (tea tree oil). International Journal of Aromatherapy 1, 15-17.

WILLIAMS, P., SORRIBAS, A., HOWES, M.J., 2011. Natural products as a source of Alzheimer's drug leads. Natural Product Reports 28, 47-77.

WILLIAMS, V.L., BALKWILL, K., WITKOWSKI, E.T.F., 2001. A lexicon of plants traded in the Witwatersrand *umuthi* shops. Bothalia 31, 79-98.

WILLIAMS, V.L., VICTOR, J.E., CROUCH, N.R., 2013. Red listed medical plants of South Africa: status, trends and assessment challenges. South African Journal of Botany 86, 23-35.

WILLIAMSON, E., OKPAKO, D.T., EVANS, F.J., 1996. Selection, preparation and pharmacological evaluation of plant material. Wiley, Chichester.

WILSON, P.J., VAN STADEN, J., 1990. Rhizocaline, rooting co-factors, and the concept of promoters and inhibitors of adventitious rooting - a review. Annals of Botany 66, 479-490.

WORLD ALZHEIMER'S REPORT, 2016. Alzheimer's Disease International, London, United Kingdom.

WORLD HEALTH ORGANISATION, 2005. Climate and health fact sheet, July 2005. Retrieved via URL; http://www.who.int/globalchange/news/fsclimandhealth/en/. Date accessed; 25 October 2016.

WU, H-J., YU, X-N., TEIXEIRA da SILVA, J.A., SHEN, M.M., 2011. Micropropagation of *Paeonia lactiflora* 'Zhong Sheng Fen' plantlets and rejuvenation of hyperhydric shoots. New Zealand Journal of Crop Horticultural Sciences 39, 271-278.

YU, K., LITTLE, D., PLUMB, R., SMITH, B., 2006. High-throughput quantification for a drug mixture in rat plasma - a comparison of Ultra Performance liquid chromatography/tandem mass spectrometry with high-performace liquid chromatography/tandem mass spectrometry. Rapid Communication in Mass Spectrometry 20, 544-552.

ZAYED, R., EL-SHAMY, H., BERKOV, S., BASTIDA, J., CODINA, C., 2011. *In vitro* micropropagation and alkaloids of *Hippeastrum vittatum*. *In Vitro* Cellular Development Biology-Plant 47, 695-701.

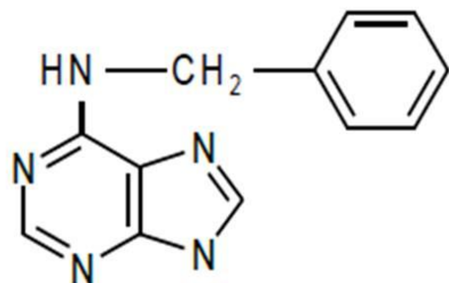
ZIV, M., LILIEN-KIPNIS, H., 2000. Bud regeneration from inflorescence explants for rapid propagation of geophytes *in vitro*. Plant Cell Reports 19, 845-850.

ZSCHOCKE, S., VAN STADEN, J., 2000. *Cryptocarya species* – substitute plants for *Ocotea bullata*? A pharmacological investigation in terms of Cyclooxygenase-1 and -2 inhibition. Journal of Ethnopharmacology 71, 473-478.

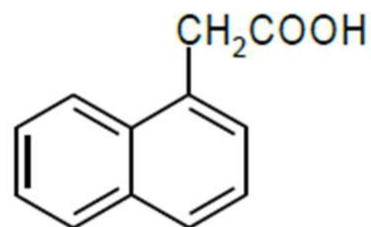
APPENDIX 1 – COMPOSITION OF THE MURASHIGE AND SKOOG (1962) NUTRIENT MEDIUM

Stock	Salt component	Mass/500 ml stock (g)	Mass/1000 ml	Volume stock (ml/l) final medium
1	NH_3NO_3	82.5	165.0	10
2	KNO_3	47.5	95.0	20
3	$CaCl_2.2H_2O$	22.0	44.0	10
4	$MgSO_4.7H_2O$	18.5	37.0	10
5	NaFeEDTA	2.0	4.0	10
6	KH_2PO_4	8.5	17.0	10
7a	H_3BO_4	0.31	0.62	10
	$ZnSO_4.7H_2O$	0.430	0.860	10
	KI	0.0415	0.083	10
7b	$MnSO_4.4H_2O$	1.115	2.230	10
8	$NaMoO_4.2H_2O$	0.0125	0.025	10
	$CuSO_4.5H_2O$	0.00125	0.0025	10
	$CoCl_2.6H_2O$	0.00125	0.0025	10
9	Thiamin HCl (B1/Aneurine)	0.005	0.01	10
	Niacine (Nicotinic acid)	0.025	0.05	10
	Pyridoxine HCl (B_6)	0.025	0.05	10
	Glycine	0.1	0.2	10

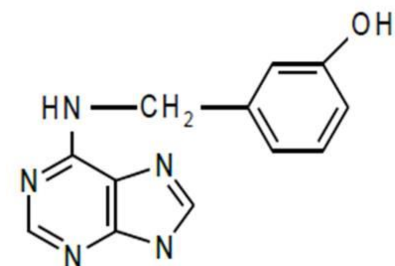
APPENDIX 2 – CHEMICAL STRUCTURES OF GROWTH SUPPLEMENTS USED IN THE CURRENT STUDY



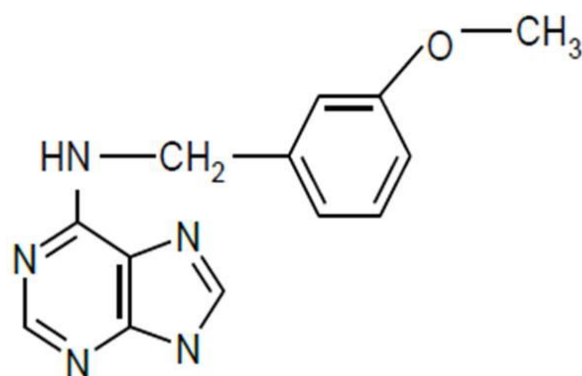
6-Benzyladenine (BA)



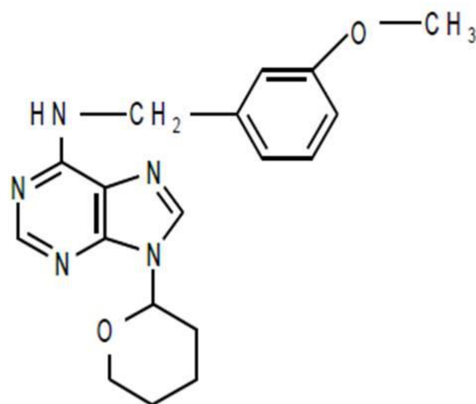
Naphthalene acetic acid (NAA)



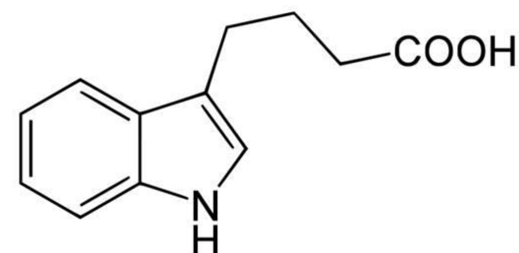
meta-topolin *(m*T*)*



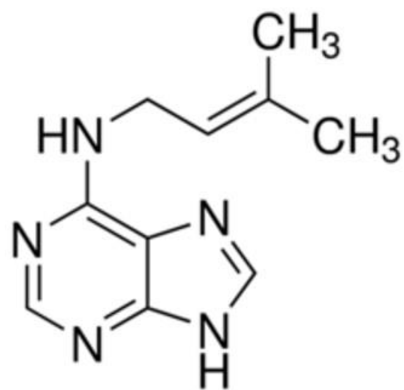
meta-Methoxytopolin (Me*m*T)



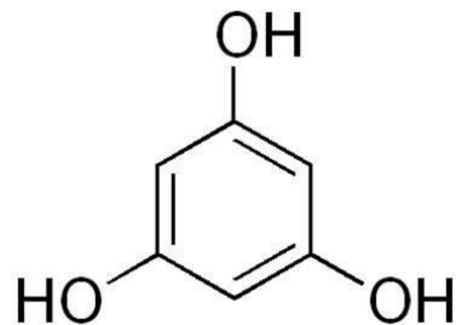
meta-Methoxy-9-tetrahydropyran-2-yl topolin (Me*m*TTHP)



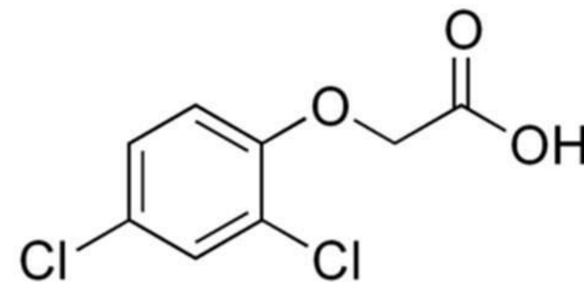
Indole -3-butryic acid (IBA)



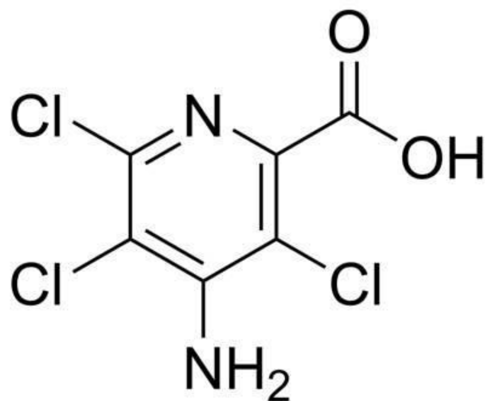
N^6–Isopentenyladenine (2iP)



Phloroglucinol (PG)



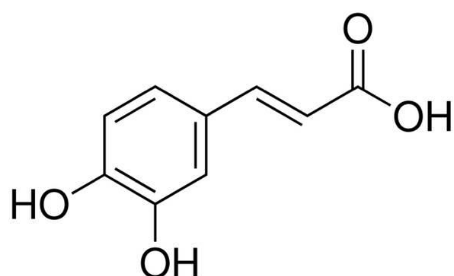
2,4-Dichlorophenoxyacetic acid (2,4-D)



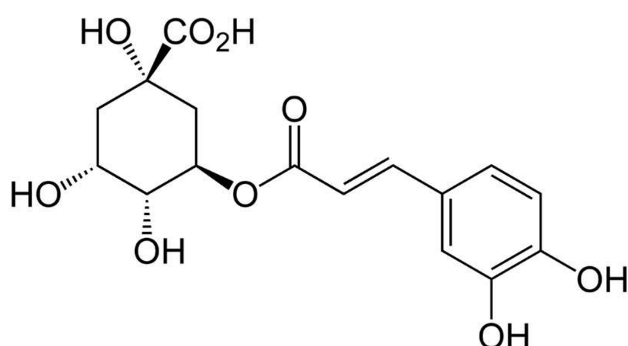
Picloram (PIC)

APPENDIX 3 – CHEMICAL STRUCTURES OF PHENOLIC ACIDS QUANTIFIED IN *SCADOXUS PUNICEUS*

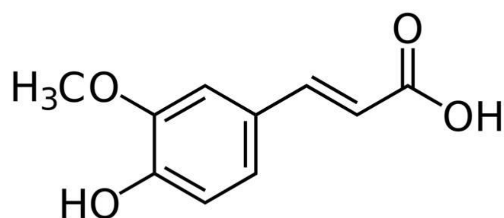
Hydroxycinnamic acids



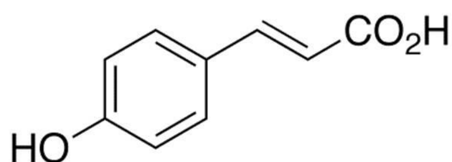
Caffeic Acid



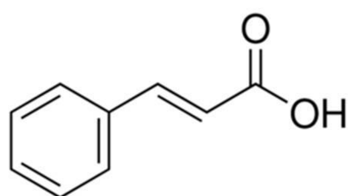
Chlorogenic Acid



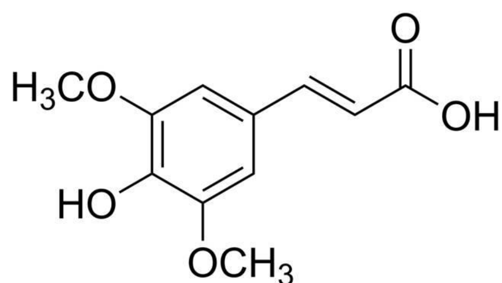
Ferulic Acid



P-Coumaric Acid

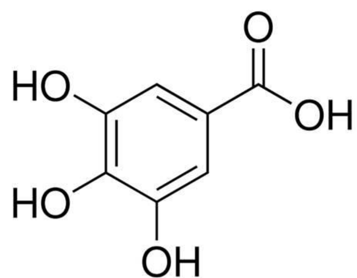


Trans-cinnamic Acid

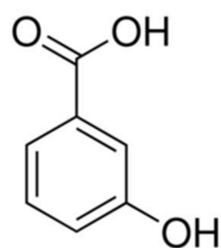


Sinapic Acid

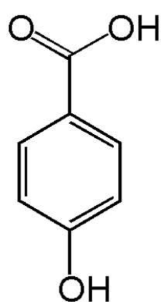
Hydroxybenzoic acids



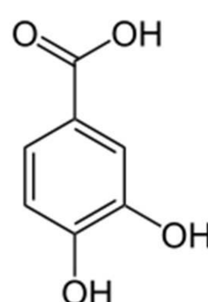
Gallic Acid



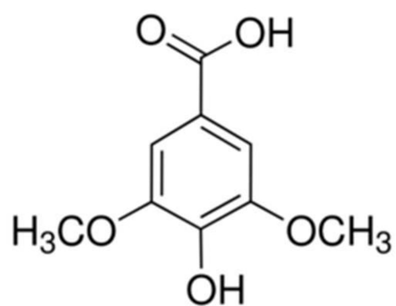
m-Hydroxybenzoic Acid



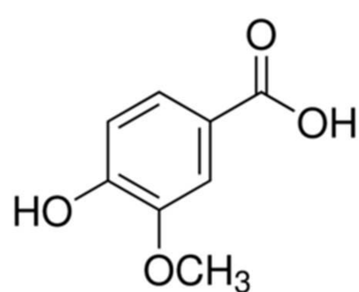
p-hydroxybenzoic Acid



Protocatechuic Acid



Syringic Acid



Vanillic Acid

APPENDIX 4 – KEY COSY AND HMBC CORRELATIONS FOR COMPOUNDS ISOLATED FROM *SCADOXUS PUNICEUS*

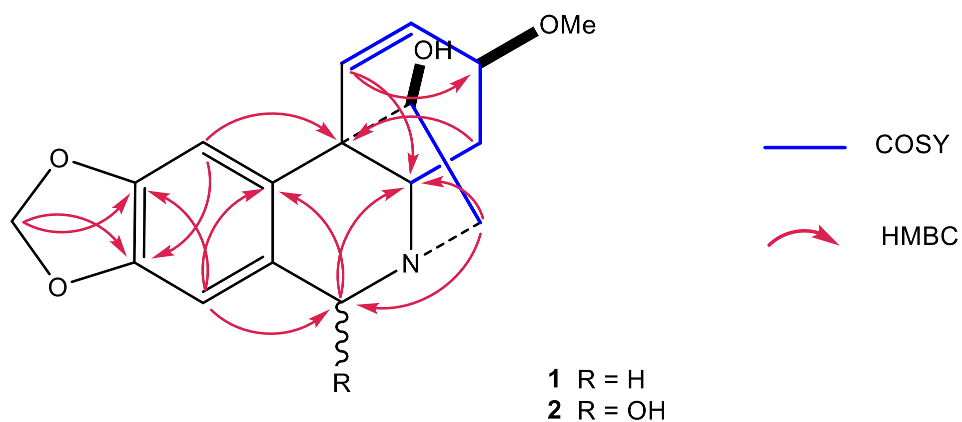


Figure 1. Key COSY and HMBC correlations for haemanthamine (**1**) and haemanthidine (**2**).

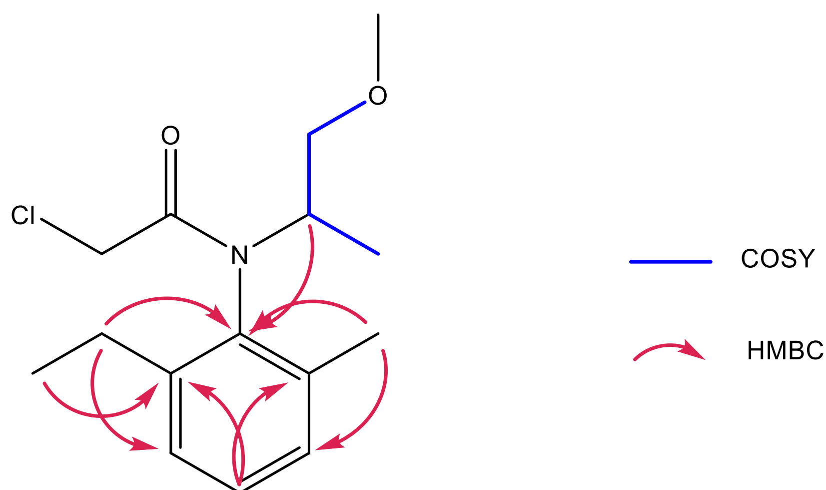


Figure 2. Key COSY and HMBC correlations for metolachlor (**3**).