The Toxicological Properties of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) in Cultured HepG2 Liver Cells

PRISHANIA PILLAY

The Toxicological Properties of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) in Cultured HepG2 Liver Cells

PRISHANIA PILLAY

(BPharm, University of KwaZulu-Natal)

Dissertation submitted for the degree

MPharm

in

Pharmacology

at the

University of KwaZulu-Natal (Westville Campus)

Supervisor: Dr Johannes Bodenstein (Pharmacology)

Co-supervisor 1: Dr Karen du Toit (Pharmaceutical Chemistry)

Co-supervisor 2: Prof Anil A. Chuturgoon (Medical Biochemistry)

Durban

December 2011

“What is a weed? A plant whose virtues have not been discovered.”

Fortune of the Republic (1878), 3

–Ralph Waldo Emerson (1803-1882)

Table of Contents

Declaration ---------- i
Original Work ---------- i
References ---------- i
Ethical Clearance ---------- i
Acknowledgements ---------- ii
Abstract ---------- iii
Abbreviations ---------- v
List of Figures ---------- x
List of Tables ---------- xii

Chapter 1. Introduction ---------- 1
1.1 Dissertation Layout ---------- 1
1.2 Background ---------- 2
1.3 Problem Statement ---------- 3
1.4 Aim ---------- 3
1.5 Research Questions ---------- 3
1.6 Objectives ---------- 4
1.7 Experimental Approach ---------- 4
1.8 Summary and Conclusion ---------- 5

Chapter 2. Literature Review ---------- 6
2.1 *Scilla nervosa* ---------- 6
2.1.1 Botanical Properties ---------- 6
2.1.2 Distribution ---------- 6
2.1.3 Conservation Status ---------- 7
2.1.4 Phytochemistry ---------- 8
2.1.5 Traditional Use ---------- 12
2.1.6 Pharmacological Activity ---------- 13
2.1.7 Toxicology ---------- 13
2.2 The Liver ---------- 14
2.2.1 Structure of the Liver ---------- 14
2.2.2 Functions of the Liver ---------- 15
2.2.3 Hepatotoxicity ---------- 16

2.3 Apoptosis ---16
2.3.1 Caspases ---16
2.3.2 Apoptotic Pathways ---18
2.3.2.1 The Extrinsic Apoptotic Pathway ---19
2.3.2.2 The Intrinsic Apoptotic Pathway ---21
2.4 Oxidative Stress ---24
2.4.1 Free Radicals ---24
2.4.2 Antioxidants ---25
2.4.3 Consequences of Oxidative Damage ---26
2.5 Cytochrome P450 3A4 ---27
2.5.1 Catalytic Cycle ---28
2.5.2 Interactions with CYP3A4 ---29
2.5.3 Modulation of CYP3A4 ---30
2.5.4 Pregnane X Receptor ---31
2.6 Summary and Conclusion ---32

Chapter 3. Materials and Methods ---33
3.1 Preparation of Aqueous Extract ---33
3.1.1 Introduction ---33
3.1.2 Materials and Methods ---33
3.2 HepG2 Liver Cells ---33
3.2.1 Introduction ---33
3.2.2 Maintenance ---34
3.3 Cell Viability Assay ---34
3.3.1 Introduction ---34
3.3.2 Materials and Methods ---35
3.3.3 Data Analysis ---36
3.4 ATP Quantification Assay ---36
3.4.1 Introduction ---36
3.4.2 Materials and Methods ---36
3.4.3 Data Analysis ---37
3.5 Apoptotic Potential Assays ---37
3.5.1 Annexin-V Affinity Assay ---37
3.5.1.1 Introduction ---37
3.5.1.2 Materials and Methods ---38

3.5.1.3 Data Analysis --39
3.5.2 Caspase-8 and -9 Assays --39
3.5.2.1 Introduction --39
3.5.2.2 Materials and Methods --40
3.5.2.3 Data Analysis --40
3.5.3 Mitochondrial Membrane Potential Assay --40
3.5.3.1 Introduction --40
3.5.3.2 Materials and Methods --41
3.5.3.3 Data Analysis --42
3.6 Lipid Peroxidation Assay --42
3.6.1 Introduction --42
3.6.2 Materials and Methods --42
3.6.3 Data Analysis --43
3.7 Single Cell Gel Electrophoresis (Comet) Assay --43
3.7.1 Introduction --43
3.7.2 Materials and Methods --44
3.7.3 Data Analysis --45
3.8 Cytochrome P450 3A4 Assay --45
3.8.1 Introduction --45
3.8.2 Materials and Methods --46
3.8.3 Data Analysis --47
3.9 Summary and Conclusion --47

Chapter 4. Results and Discussion --48
4.1 Cell Viability --48
4.2 Cytotoxicity --49
4.2.1 Metabolic Activity --49
4.2.2 Apoptosis --51
4.2.2.1 PS Externalisation --51
4.2.2.2 Caspase-8 and -9 Activity --52
4.2.2.3 Mitochondrial Membrane Potential --53
4.2.3 Oxidative Damage --54
4.3 Genotoxicity --55
4.3.1 DNA Integrity --55
4.4 Metabolism --58

4.4.1 Cytochrome P450 3A4 Activity --- 58
4.5 Summary and Conclusion --- 59

Chapter 5. Summary, Conclusions and Limitations --- 61
5.1 Summary --- 61
5.2 Conclusions --- 61
5.3 Limitations --- 61
5.4 Future Studies --- 62

References --- 63

Appendix 1 (Full-length Research Article) --- 77
Abstract --- 78
Keywords --- 78
Introduction --- 78
Results and Discussion --- 79
Cell Viability --- 79
Intracellular ATP Concentration --- 79
Phosphatidylserine Externalisation --- 80
Caspase-8 and -9 Activity --- 80
Mitochondrial Membrane Potential --- 80
Lipid Peroxidation --- 81
Genotoxicity --- 81
CYP3A4 Activity --- 82
Experimental --- 82
Chemicals --- 82
Plant Material and Extract Preparation --- 82
Cell Culture --- 83
Cell Viability --- 83
Metabolic Activity --- 83
Phosphatidylserine Externalisation --- 84
Caspase-8 and -9 Activity --- 84
Mitochondrial Membrane Depolarisation --- 84
Oxidative Damage --- 85

DNA Integrity ---85
CYP3A4 Activity ---85
Statistical Analysis --86
Conclusions ---86
Acknowledgements --86
References ---86
Appendix 2 (Abstract – School Research Symposium) ---------------------------------------90
Appendix 3 (Abstract – International Conference)--92

Declaration

Original Work

I hereby declare that this study represents original work that was done by me under the supervision of Dr Johannes Bodenstein (Pharmacology, University of KwaZulu-Natal, Durban, South Africa) and co-supervision of Dr Karen du Toit (Pharmaceutical Chemistry, University of KwaZulu-Natal, Durban, South Africa) and Prof Anil A. Chuturgoon (Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa). It has not been submitted in any form to another university.

References

The use of work by others has been duly acknowledged and referenced in the text. I understand that copywriting or plagiarism could lead to my degree being disqualified.

Ethical Clearance

This study investigated *in vitro* properties and did not involve any animal or human patients. A commercially available cell line was used and it was therefore not necessary to obtain ethical clearance from the relevant university ethical committees.

	Signature	**Date**
Student (Ms P. Pillay) **Student Number 204003492**	---------------------------	--------------------------
Supervisor (Dr J. Bodenstein)	---------------------------	--------------------------

Acknowledgements

I would like to thank:

- My family, both past and present, for their love, support and encouragement. To my parents, this project would not have been possible without you. I am truly grateful for all the sacrifices you have made to help me achieve this.
- Dr J. Bodenstein, for your patience, guidance and advice which made this study a success. Your genuine interest and dedication to this study was an immense source of encouragement for me.
- Dr K. du Toit and Prof A.A. Chuturgoon, for your advice and guidance throughout this study.
- Alisa Phulukdaree, for your assistance with my assays and your continuous help and advice.
- Ruben Moodliar, for your patience and consideration. Your consistent encouragement and faith in me has supported me through all difficulties.
- The National Research Foundation (NRF), for financial assistance.

Abstract

Background and Aims of Study

Scilla nervosa is a member of the Hyacinthaceae plant family that has been naturalised in the grasslands of Southern Africa. The bulbs are traditionally used to treat a variety of ailments. For example, the Zulu people use aqueous decoctions of the bulbs as analgesics in the treatment of rheumatic fever, crushed bulbs are used by the Sotho people as laxatives and the Tswana people use cooked bulbs to treat infertility in women as well as cold aqueous extracts to treat infections. It was recently demonstrated in our laboratory that extracts prepared from the bulbs possess potent anti-inflammatory properties, and this may therefore provide a rationale for the traditional use of the plant as an analgesic.[1] Several studies have demonstrated that the bulbs contain homoisoflavanones and stilbenoids that could be responsible for their therapeutic effects. Although the plant has diverse medicinal applications, and despite it being recognised as a poisonous species particularly in livestock, little is known about its toxicity in human liver cells. The objectives of this study were therefore to investigate the potential toxicity of the bulbs on the liver, a major detoxifying organ. A human liver cell line was treated with an aqueous extract of the bulbs to investigate (1) cell viability, (2) potential mechanisms of cytotoxicity, (3) DNA integrity, and (4) changes in the cytochrome P450 enzyme activity.

Materials and Methods

This study was conducted on the cultured HepG2 human hepatocellular carcinoma cell line, a model system to investigate the cytotoxicity of xenobiotics. The viability of cultured HepG2 liver cells in the presence of varying concentrations of an aqueous extract of the bulbs was determined after 24 hours treatment, and the concentration that reduced viability to 50% (IC_{50}) was derived. Potential mechanisms of cytotoxicity at the IC_{50} were investigated. These included changes in metabolic activity (intracellular adenosine triphosphate (ATP) quantification), apoptosis induction (phosphatidylserine (PS) externalisation, caspase-8 and -9 induction and changes in mitochondrial membrane potential), and oxidative damage via free radical formation (lipid peroxidation). Genotoxicity was investigated by determining changes in DNA integrity (DNA fragmentation). The ability of the extract to stimulate or

---

[1] Du Toit, K., Kweyama, A., Bodenstein, J. 2011. Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae). *South African Journal of Science,* 107:96-100.

inhibit enzymes commonly involved with drug metabolism was investigated by determining cytochrome P450 3A4 (CYP3A4) activity.

Results

Cell-viability decreased in a concentration-dependent manner and the IC_{50} was determined as 0.03 mg/ml. Treating the cells at the IC_{50} resulted in (1) a 1.2-fold increase in intracellular ATP levels, (2) no significant change in PS externalisation, (3) a 1.3-fold increase in caspase-8 activity, (4) a 1.1-fold decrease in caspase-9 activity, (5) no significant change in mitochondrial membrane potential, (6) a 1.9-fold increase in lipid peroxidation, (7) evidence for genotoxicity as demonstrated by DNA fragmentation, and (8) no evidence of changes in CYP3A4 activity.

Conclusion

Results suggest that HepG2 liver cells are sensitive to an aqueous extract of the bulbs of *S. nervosa*. The extract has the potential to (1) induce apoptosis, (2) increase oxidative stress and (3) cause genotoxicity *in vitro*.

Keywords

Scilla nervosa, homoisoflavanones, apoptosis, caspases, lipid peroxidation, genotoxicity

Abbreviations

Abbreviation	Description
$\Delta\Psi m$	Mitochondrial membrane potential
%TL/HL	Percentage tail length/head length
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
APAF-1	Apoptotic protease-activating factor-1
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bok	Bcl-2 related ovarian killer
Bcl-2	B-cell CLL/Lymphoma 2
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Ca^{2+}	Calcium ion
CARD	Caspase recruitment domain
CCM	Complete culture media
cFLIP	Cellular FLICE inhibitory protein
CO_2	Carbon dioxide
Cu^{2+}	Copper ion
CYP	Cytochrome P450
CYP3A4	Cytochrome P450 3A4
DED	Death effector domain
DISC	Death-inducing signalling complex

Abbreviation	Description
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
ED_{50}	Dose that has a therapeutic effect on 50% of the cell population
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain protein
$FADH_2$	Flavin adenine dinucleotide
Fas	Fibroblast associated antigen
FasL	Fas ligand
Fe^{2+}	Iron divalent ion
Fe^{3+}	Iron trivalent iron
FITC	Fluorescein isothiocyanate
FL	Fluorescence
FLICE	FADD-like interleukin-1 beta-converting enzyme
g	Centrifugal force
GPx	Glutathione peroxidise
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
h	Hour(s)
H_2O_2	Hydrogen peroxide

Abbreviation	Description
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HL	Head length
HRMS	High resolution mass spectrometry
IAP	Inhibitor of apoptosis protein
I-κB	Inhibitor of NF-κB
IC_{50}	Concentration of the extract at which cell viability was decreased to 50%
IUCN	International Union for Conservation of Nature and Natural Resources
JC-1	5,5′,6,6′-tetrachloro-l,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide
JNK	Jun NH_2-terminal kinase
LMP	Low melting point
mA	milliampere
MDA	Malondialdehyde
Mg^{2+}	Magnesium ion
min	Minute(s)
Mn-SOD	Manganese superoxide dismutase
MPT	Mitochondrial permeability transition
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide

Abbreviation	Description
NF-κB	Nuclear Factor-kappa B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
O_2	Oxygen
$O_2\cdot^-$	Superoxide
OH·	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OTM	Olive tail moment
p	Statistical probability
PBS	Phosphate buffered saline
PI	Propidium iodide
PRx	Peroxiredoxin
PS	Phosphatidylserine
PTP	Permeability transition pore
PUFA	Polyunsaturated fatty acid
PXR	Pregnane X receptor
RAIDD	RIP-associated ICH-1 homologous protein with death domain
RIP	Receptor-interacting protein
RLU	Relative light unit
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RXRα	Retinoid X receptor alpha
SANBI	South African National Biodiversity Institute

Abbreviation	Description
S. nervosa	*Scilla nervosa*
SEM	Standard error of the mean
Smac/DIABLO	Second mitochondria-derived activator of caspase/direct IAP binding protein of low isoelectric potential
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
tBid	Truncated Bid
TEM	Tail extent moment
TL	Tail length
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TR	Thioredoxin reductase
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor-2
TRAIL	TNF α-related apoptosis inducing ligand
Tris	Tris(hydroxymethyl)aminomethane
TRX	Thioredoxin
UV	Ultraviolet
V	Volts
VDAC	Voltage-dependent anion channel

List of Figures

Figure 1–1. A schematic diagram depicting the experimental approach that was followed to investigate the toxicological properties of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) in cultured HepG2 liver cells.---------- 5

Figure 2–1. The aerial parts of *Scilla nervosa*. ---------- 6

Figure 2–2. Compounds **1-4**, homoisoflavanones of the 3-benzylchroman-4-one type that were isolated by Bangani et al. (1999). ---------- 8

Figure 2–3. Compound **5**, a homoisoflavanone of the 3-benzylidenechroman-4-one type that was isolated by Bangani et al. (1999).---------- 9

Figure 2–4. Compounds **6-7**, stilbenoids that were isolated by Bangani et al. (1999). -------- 9

Figure 2–5. Compounds **1-4**, homoisoflavanones of the 3-benzylidenechroman-4-one type that were isolated by Silayo et al. (1999). ----------10

Figure 2–6. Compounds **5-13**, homoisoflavanones of the 3-benzylchroman-4-one type that were isolated by Silayo et al. (1999).----------11

Figure 2–7. Compounds **14-16**, stilbenoids that were isolated by Silayo et al. (1999). ------12

Figure 2–8. Structure of a liver lobule. ----------15

Figure 2–9. A schematic diagram depicting the effector caspases (caspase-3, -6, and -7) and initiator caspases (caspase-2, -8, -9 and -10). ----------18

Figure 2–10. The extrinsic and intrinsic pathways of apoptosis. ----------21

Figure 2–11. **(a)** Generation of ROS in the mitochondria and **(b)** their inactivation by antioxidants.----------25

Figure 2–12. The catalytic cycle of CYP3A4.----------29

Figure 2–13. Catalytic cycle of CYP3A4 with blue arrows showing ROS-producing branches.----------31

Figure 2–14. A schematic representation of xenobiotic activation of the PXR, causing CYP3A4 induction. ----------32

Figure 3–1. Loss of membrane asymmetry during apoptosis.----------38

Figure 3–2. Scatter plots of JC-1 staining in **(A)** control and **(B)** test cells.----------41

Figure 4–1. Concentration-response curves of % cell viability against varying concentrations of **(A)** *S. nervosa* extract (mg/ml) and **(B)** positive control emetine (μg/ml) in HepG2 cells after 24 h treatment.----------48

Figure 4–2. The annexin-V FITC flow cytometry histograms (number of cells as a function of fluorescence) obtained after treating HepG2 cells with the **(A & B)** negative control and **(C & D)** the extract for 24 h. ----------51

Figure 4–3. The JC-1 flow cytometry scatter plot diagrams obtained after treating HepG2 cells with the **(A & B)** negative control and **(C & D)** the extract for 24 h. ----------------------53

Figure 4–4. Digital images of fluorescent cell DNA after HepG2 cells were treated with **(A)** negative control, **(B)** *S. nervosa* extract, and **(C)** positive control cyclophosphamide after 24 h treatment. --56

Figure 4–5. Random comets were scored after HepG2 cells were treated with the negative control, *S. nervosa* extract, or positive control cyclophosphamide for 24 h. --------------------57

List of Tables

Table 4–1. Caspase-8 and -9 activities that were measured in HepG2 cells after treatment with the negative control and *S. nervosa* extract. ---52

Table 4–2. Changes in the OTM, TEM, TL and %TL/HL of HepG2 cells treated with the *S. nervosa* extract and positive control cyclophosphamide for 24 h when compared to the negative control. ---58

Table 4–3. CYP3A4 activity in HepG2 cells treated with the *S. nervosa* extract and positive control dexamethasone for 24 h when compared to the negative control. -----------------------58

Chapter 1. Introduction

1.1 Dissertation Layout

This dissertation was written as follows:

- *Chapter 1* provides a brief background on traditional medicine; the problem statement and motivation of why the study was necessary and why follow-up studies should be considered; the aim, research questions and objectives of the study; and a brief overview of the experimental approach that was followed in an attempt to answer the research questions posed.
- *Chapter 2* is a literature review on the medicinal plant *Scilla nervosa*, highlighting its botanical, geographical, conservational, chemical and medicinal properties. It also contains a review of the structure and function of the liver and explains hepatotoxicity. In addition, a brief overview of apoptosis, oxidative stress and the cytochrome P450 enzyme (CYP) family is provided.
- *Chapter 3* provides details of the various experimental assays that were conducted.
- *Chapter 4* deals with the results and discussion of the assays.
- *Chapter 5* provides a summary and conclusions of the main findings of the study. It also lists limitations and future studies.
- References that were used throughout the dissertation are listed alphabetically in the *References* section, with the exception of the references for the article in *Appendix 1* where they appear at the end of the chapter instead.
- *Appendix 1* is a full-length research article of the study that was submitted for publication.
- *Appendix 2* contains an abstract summarising additional data on the toxicological properties of the plant in cultured human oesophageal SNO cancer cells. A group of 8 undergraduate pharmacy students was assisted and trained by me to conduct 2 major types of experiments as part of their final-year research project. This abstract was also presented at the Annual Undergraduate Research Symposium of the School of Pharmacy and Pharmacology by the group.

- *Appendix 3* contains an abstract that was accepted for a podium presentation at the 6th International Conference on Pharmaceutical and Pharmacological Sciences (ICPPS) held at Coastlands Umhlanga Hotel, Durban, South Africa from 25-27 September 2011. The abstract was entered for the Young Scientist Competition of the South African Society for Basic and Clinical Pharmacology (SASBCP).

1.2 Background

Traditional plant medicines[2] form an essential part of primary health care in both rural and urban communities in South Africa and other developing countries (Fennell et al., 2004). It is estimated that approximately 27 million South Africans are dependent on traditional medicine for their health care requirements. The extensive reliance on traditional medicine can be attributed to their accessibility and affordability since conventional medicine is largely inaccessible to much of the population residing in rural areas (Light et al., 2005; Street et al., 2008). Although these factors do contribute to the widespread use of traditional medicine, it is however largely the cultural significance of traditional medicine within local communities that maintains their high demand (Light et al., 2005).

South Africa possesses a unique and extensive plant diversity. Of the more than 30 000 South African plant species, approximately 3 000 are used medicinally and up to 500 of these species are informally traded in medicinal plant markets (Light et al., 2005). Despite the widespread reliance on medicinal plants, their use in South Africa is currently not regulated and little scientific information exists regarding their safety and efficacy to support and rationalise their extensive use (Fennell et al., 2004; Van Vuuren, 2008). A common misconception exists that traditional medicines are safe and devoid of adverse effects, because their use is based on the knowledge acquired within local communities during centuries of use. However, studies have shown many medicinal plants to be potentially toxic (Fennell et al., 2004). It is thus of great importance that these plants continue to be evaluated for their safety and efficacy to validate their use (Light et al., 2005).

[2] Traditional medicine is defined according to the World Health Organisation (WHO) as "the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses". Traditional plant medicines contain parts of plants or other plant materials as active ingredients (WHO, 2005).

1.3 Problem Statement

Scilla nervosa (Burch.) Jessop (Hyacinthaceae) [=*Schizocarphus nervosus* (Burch.) Van der Merwe][3] is a perennial plant belonging to the Hyacinthaceae family (Louw et al., 2002) and is widely distributed in the grasslands of Southern Africa (Hutchings et al., 1996). The plant is extensively used medicinally for a variety of ailments such as in the treatment of infections, nervous disorders, fractures, cancers and rheumatic fever (Abegaz, 2002; Bangani et al., 1999; Bisi-Johnson et al., 2010; Louw et al., 2002).

Although it is commercially available from nurseries and is informally traded for its medicinal uses, there are no pharmaceutical formulations available and there is limited scientific information on its pharmacology and toxicity to rationalise its widespread traditional use. It continues to be used medicinally despite being recognised as a poisonous species, as demonstrated previously, particularly in livestock (Van der Walt and Steyn, 1946). Little is known about its toxicity in hepatocytes and the mechanisms by which it exerts cellular death.

1.4 Aim

The aim of this study was to investigate the toxicological properties of an aqueous extract prepared from the bulbs of *S. nervosa* in a cultured human liver cell line (HepG2). The *in vitro* scientific knowledge thus gained could support further *in vivo* studies to eventually develop a safe and effective pharmaceutical formulation. This study on traditional medicine is therefore in line with the policies of the South African government and the United Nations which aim to promote the development, use and regulation of traditional medicine (Draft by the 3rd African Union Conference of Ministers of Health, 2007; United Nations Secretary General Report on Traditional Medicine, 2002).

1.5 Research Questions

This study focussed specifically on the cytotoxic effects of the bulbs of *S. nervosa* in liver cells (HepG2). Since the liver is the main site of metabolism of xenobiotics, it could be particularly prone to the toxic effects of traditional medicines. It is also the major site of drug-drug interactions involving the cytochrome P450 enzyme family which is involved with drug metabolism. Enzyme inducers may increase the metabolism of concurrently

[3] The official current genus and species of the plant is *Scilla nervosa*. Information provided by Dr Christina Potgieter at the Bews Herbarium, University of KwaZulu-Natal (Pietermaritzburg Campus), is acknowledged.

administered drugs and therefore diminish efficacy, while enzyme inhibitors may retard metabolism and cause toxicity and/or pronounced side-effects. The following research questions were therefore asked:

1. Is an aqueous extract hepatotoxic?
2. What are the mechanisms of cytotoxicity?
3. Is there evidence of genotoxicity?
4. Is the activity of CYP3A4 increased or inhibited?

1.6 Objectives

The objectives of this study were to treat a cultured human hepatocyte cell line (HepG2) with an aqueous extract obtained from the bulbs of *S. nervosa* and investigate:

1. Cell viability.
2. Potential mechanisms of cytotoxicity.
3. Evidence of genotoxicity.
4. Changes in the activity of CYP3A4.

1.7 Experimental Approach

The human HepG2 hepatocellular carcinoma cell line was employed as a model to study the toxicological properties of *S. nervosa*. Cells were treated for 24 hours (h) with an aqueous extract previously prepared from the bulbs and viability was determined at varying concentrations of the extract. This resulted in a concentration-dependent decrease in viability and the concentration which caused 50% reduction in viability (IC_{50}) was deduced from the concentration-response curve. After a reduction in cell viability was established, the mechanisms of cytotoxicity, evidence of genotoxicity and changes in activity of cytochrome P450 3A4 were further investigated at the IC_{50} *(Figure 1–1)*.

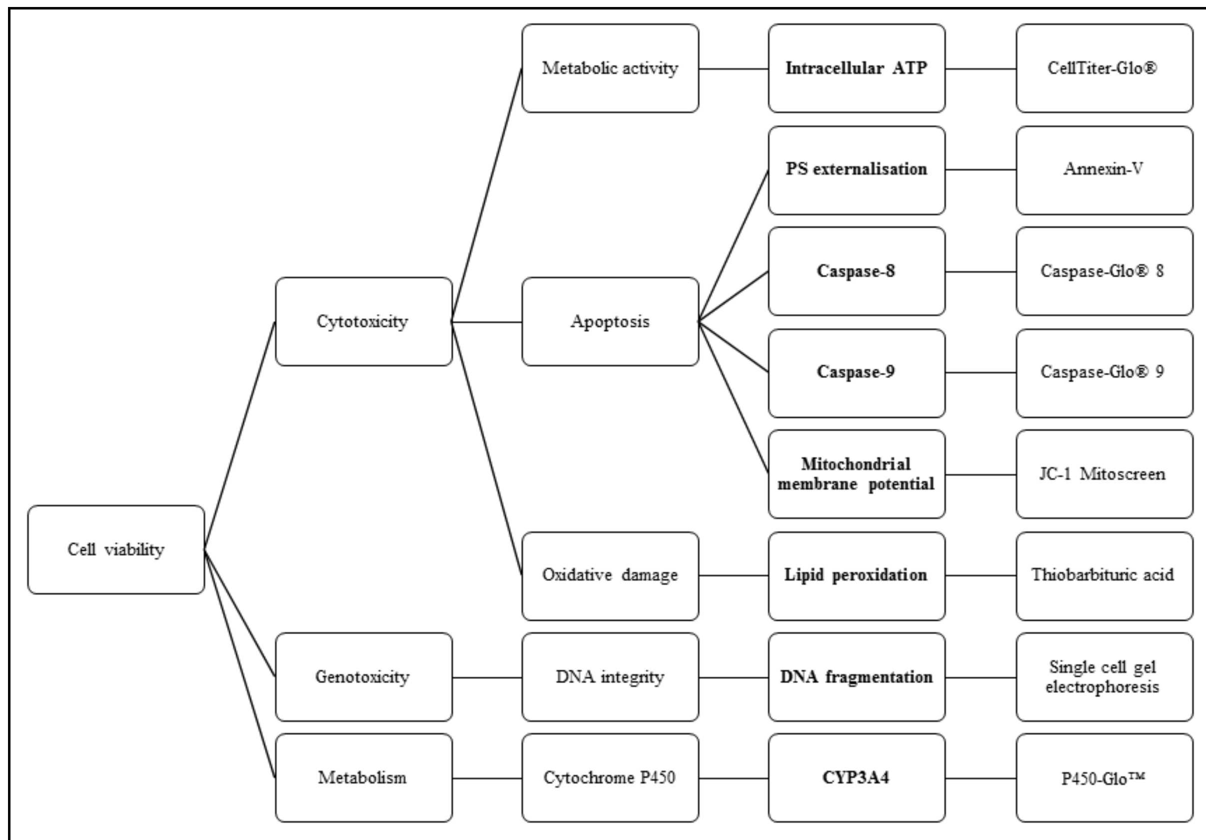


Figure 1–1. A schematic diagram depicting the experimental approach that was followed to investigate the toxicological properties of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) in cultured HepG2 liver cells.

1.8 Summary and Conclusion

Traditional medicines continue to form an integral aspect of primary health care in South Africa. *Scilla nervosa* is an example of a medicinal plant, often used in the treatment of various ailments. There is however limited scientific evidence regarding its toxicological properties to rationalise its extensive use. This study therefore investigated the toxicological properties of this plant in human liver cells (HepG2).

Chapter 2. Literature Review

This chapter provides a brief overview of *Scilla nervosa*, including its botany, distribution, conservation status, chemical constituents, medicinal uses and pharmacological activities. An overview of the structure and function of the liver, and hepatotoxicity is also provided. A brief introduction to apoptosis and apoptotic pathways, oxidative stress, its consequences and antioxidants, and CYP3A4 enzymes and their modulation is presented.

2.1 *Scilla nervosa*

2.1.1 Botanical Properties

Scilla nervosa (Burch.) Jessop (Hyacinthaceae) [=Schizocarphus nervosus (Burch.) Van der Merwe] is a monocotyledonous, perennial plant belonging to the Hyacinthaceae family (Louw et al., 2002). The genus Scilla globally represents 80 taxa. In Southern Africa it is represented by at least 4 species (Silayo et al., 1999) of which *S. nervosa* is the most widespread (Bangani et al., 1999). This herbaceous geophyte possesses a large bulb covered with a sheath of bristles and leaves. It usually has 6-12 leaves which are firm, slightly hairy and bright green. It bears many greenish-yellow flowers in solitary racemes (Sener, 2003) *(Figure 2–1)*.

Figure 2–1. The aerial parts of *Scilla nervosa* (Pacific Bulb Society, 2010).

2.1.2 Distribution

The plant is indigenous to Botswana but has been naturalised in the grasslands of Southern Africa (Hutchings et al., 1996). It is distributed in the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga and North-West Provinces of South Africa (South African National Biodiversity Institute (SANBI), 2004).

2.1.3 Conservation Status

An estimated 27 million South Africans are reliant on medicinal plants for their health care needs (Light et al., 2005; Mander, 1998; Street et al., 2008). The demand for traditional medicine continues to increase due to the population growth. A report by Mander (1998) more than 10 years ago estimated that the value of the medicinal plant trade was R500 million per year and that the demand for medicinal plants exceeded supply. Most medicinal plants are gathered from wild populations which are steadily declining in numbers due to their exploitation. For example, the medicinal plant *Siphonochilus aethiopicus* ('African ginger'), which is traditionally used for coughs, colds, asthma, headache, candida infection and malaria (van Wyk, 2008), has been harvested to the extent that wild populations have almost been completely depleted (Mander, 1998). The conservation of medicinal plants in South Africa is thus a growing concern as their over-utilisation presents a threat to the survival of many species.

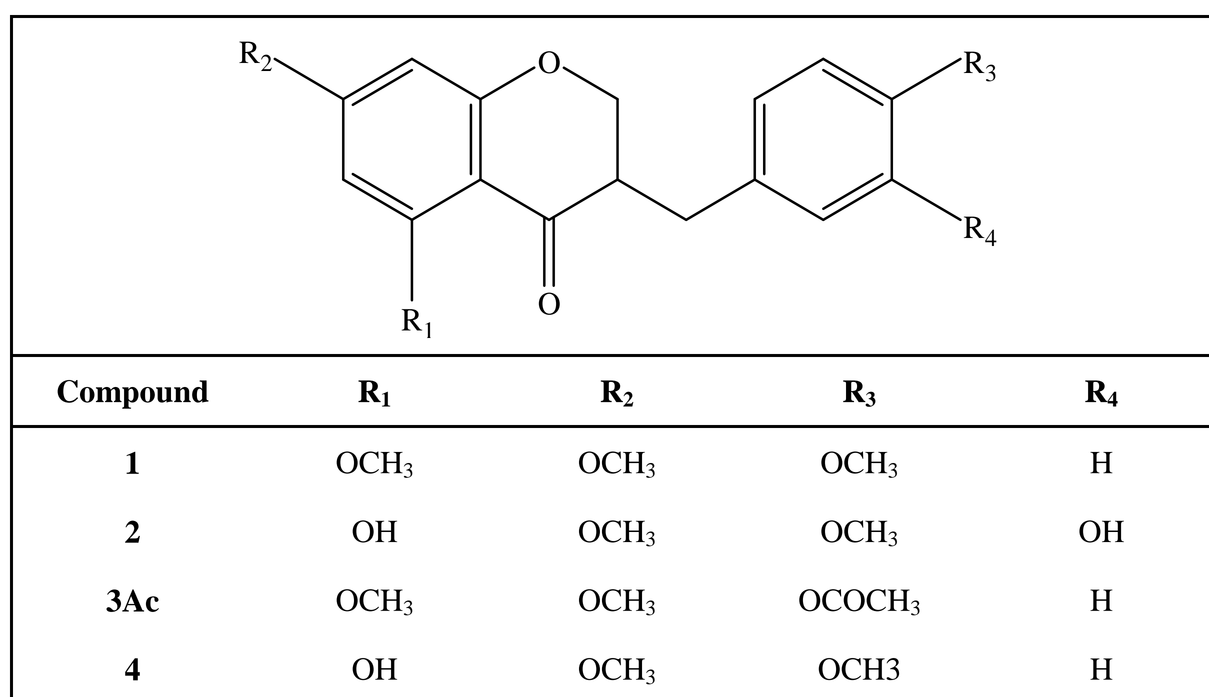
According to the Threatened Species Programme of South Africa the conservation status of *S. nervosa* is that of 'Least Concern'. The species was evaluated against the five International Union for Conservation of Nature and Natural Resources (IUCN) criteria and did not meet the requirements for a Critically Endangered, Endangered, Vulnerable or Near Threatened species as it is not rare, nor is its population diminishing (SANBI, 2004). Although the conservation of *S. nervosa* is currently not under threat, the increasing demand and unsustainable harvesting from the wild may eventually result in its demise.

Even though the wild population of *S. nervosa* has not declined to a level where it is considered threatened, the plant is used extensively in traditional medicine. It would thus be of value to consider diverting harvesting of wild populations to controlled cultivation systems instead since the demise of this plant would have significant ecological and social consequences. Cultivation of *S. nervosa* would assist in reducing the strain on wild populations and provide a sustainable means by which to harvest this plant.

2.1.4 Phytochemistry

Previous studies on the bulbs of *Scilla* species have shown them to contain cardiac glycosides, homoisoflavanones,[4] stilbenoids (Crouch et al., 1999) and triterpenoids (Mimaki et al., 1993). Published reports on the chemical constituents of *S. nervosa* are however limited and the phytochemical profile of the whole plant is not fully known. Previous studies have investigated the constituents of the bulbs.

Phytochemical studies on the bulbs of *S. nervosa* by Bangani et al. (1999) yielded five homoisoflavanones (compounds **1-5**) *(Figure 2–2 and Figure 2–3)*. Compounds **1-4** were of the 3-benzylchroman-4-one type *(Figure 2–2)* while compound **5** was a 3-benzylidenechroman-4-one *(Figure 2–3)*. Compound **3** was isolated as its acetate (3Ac). Two known stilbenoids, (*E*)-3,4′,5-trihydroxystilbene ((*E*)-resveratrol) and (*E*)-3,3′,5-trihydroxy-4′-methoxystilbene (rhapontigenin) were also isolated (compounds **6-7** respectively) *(Figure 2–4)*. Structures were elucidated using nuclear magnetic resonance (NMR), ultraviolet (UV) and mass spectrometry (MS) techniques.



Compound	R_1	R_2	R_3	R_4
1	OCH_3	OCH_3	OCH_3	H
2	OH	OCH_3	OCH_3	OH
3Ac	OCH_3	OCH_3	$OCOCH_3$	H
4	OH	OCH_3	OCH3	H

Figure 2–2. Compounds **1-4**, homoisoflavanones of the 3-benzylchroman-4-one type that were isolated by Bangani et al. (1999).

[4] For a comprehensive review on homoisoflavanones, refer to: Du Toit, K., Drewes, S.E., Bodenstein, J. 2010. The chemical structures, plant origins, ethnobotany and biological activities of homoisoflavanones. *Natural Product Research,* 24:457-490.

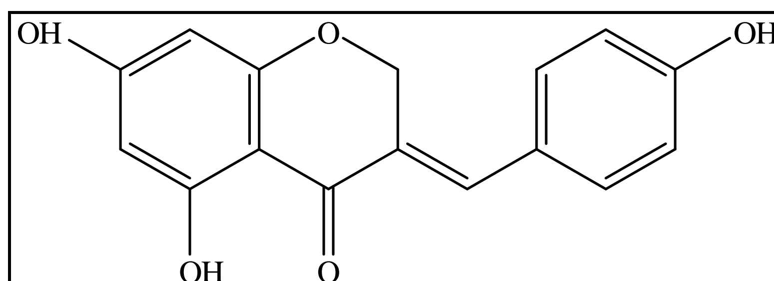
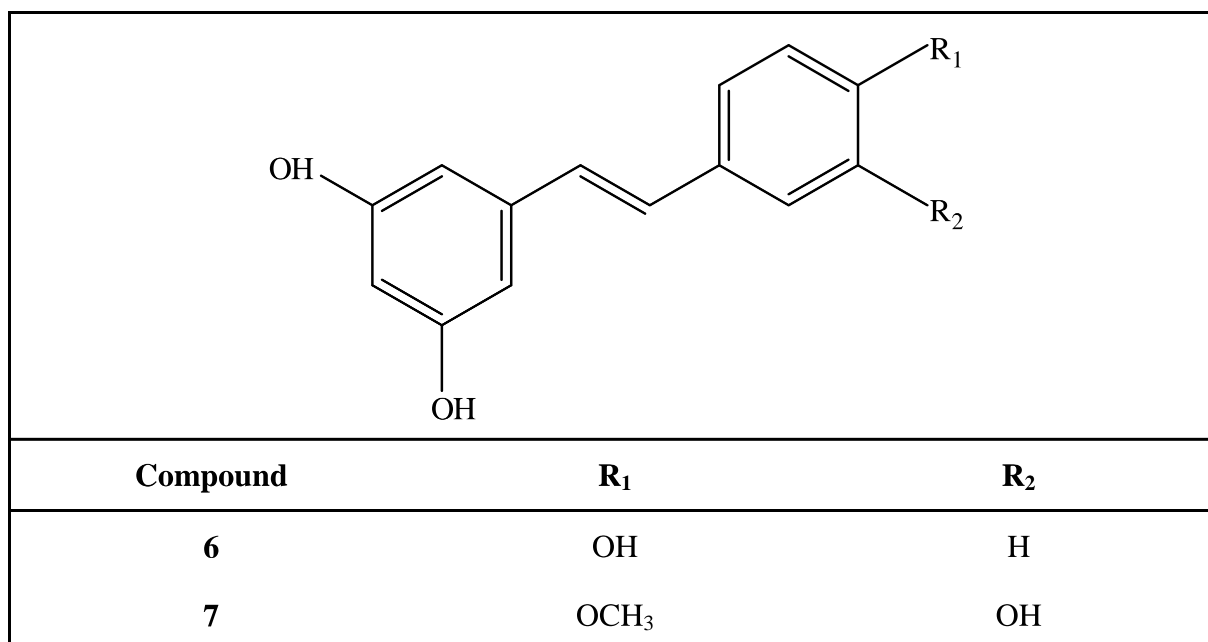


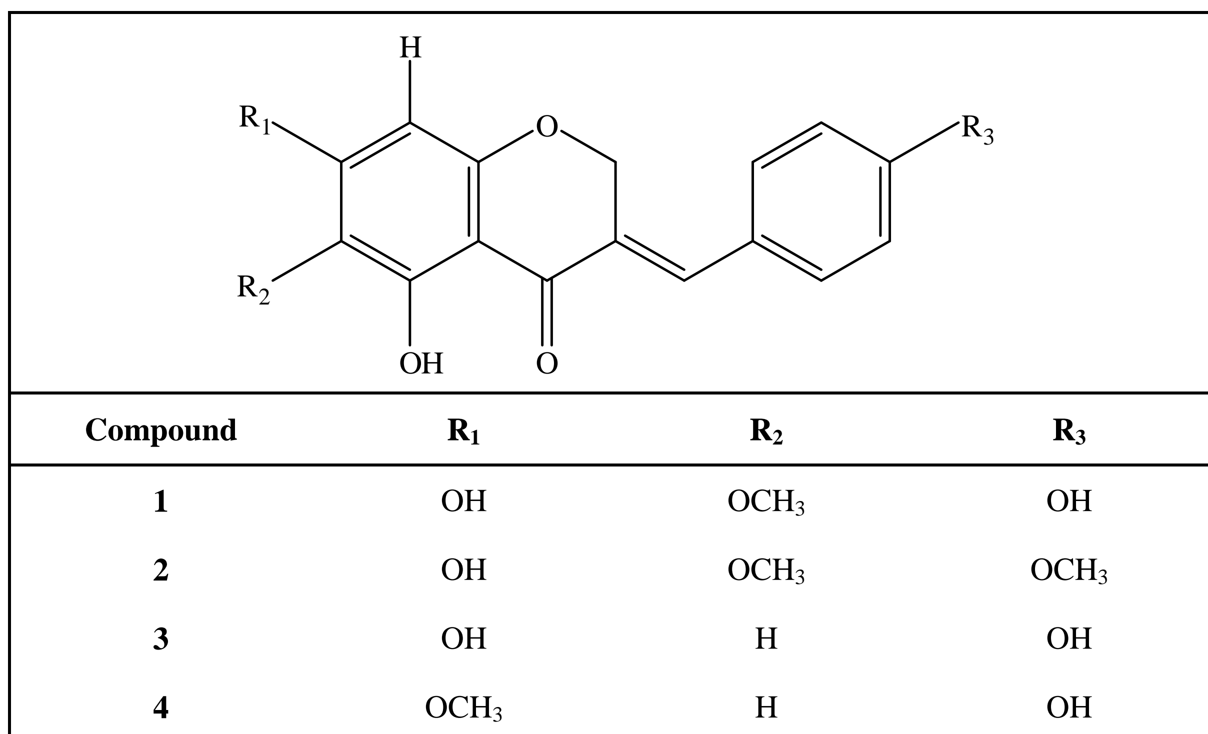
Figure 2–3. Compound **5**, a homoisoflavanone of the 3-benzylidenechroman-4-one type that was isolated by Bangani et al. (1999).



Compound	R_1	R_2
6	OH	H
7	OCH_3	OH

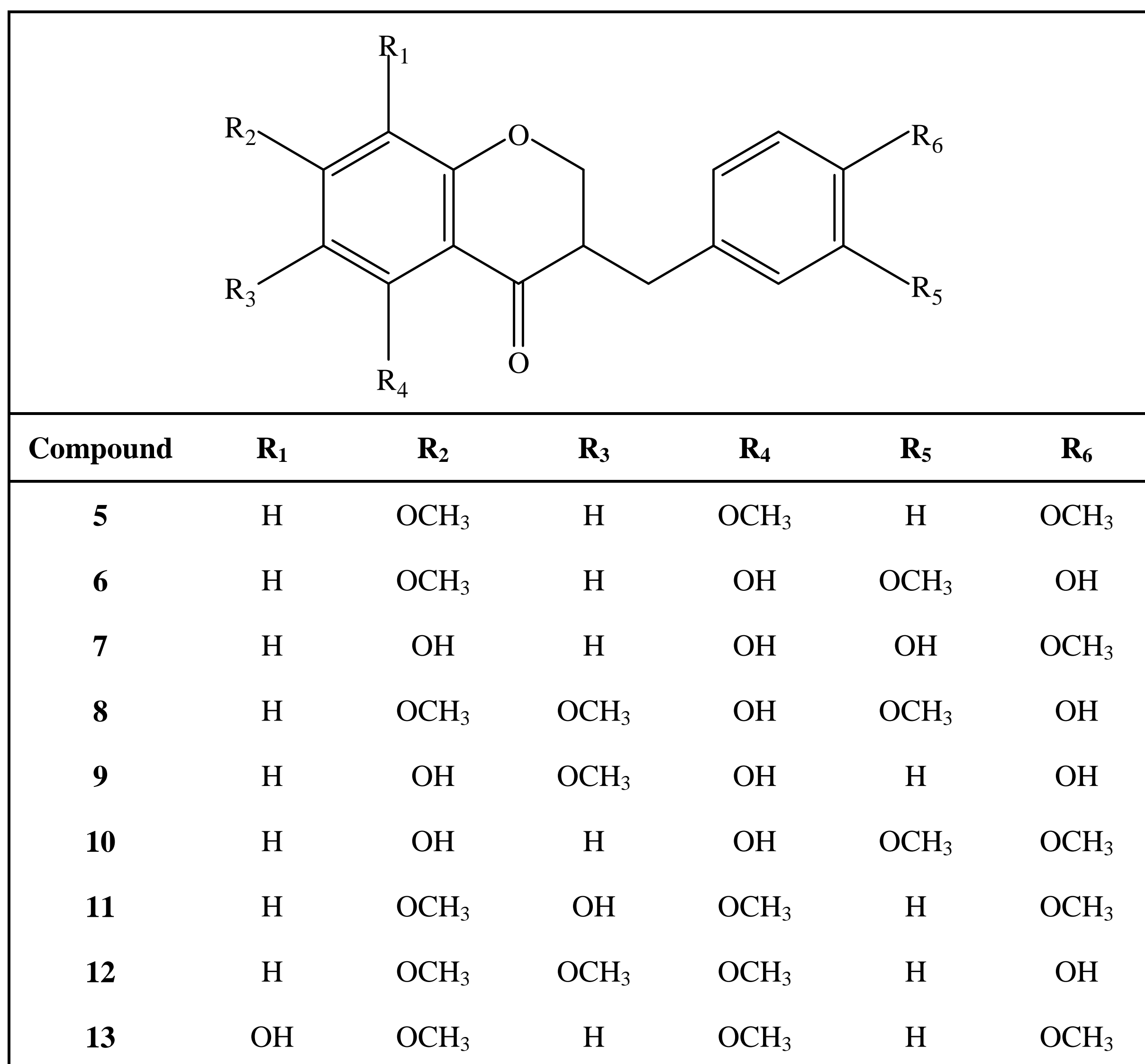
Figure 2–4. Compounds **6-7**, stilbenoids that were isolated by Bangani et al. (1999).

Studies by Silayo et al. (1999) yielded thirteen homoisoflavanones, nine of which were previously unreported (compounds **1-13**) *(Figure 2–5 and Figure 2–6).* Compounds **1-4** comprised a group of 3-benzylidenechroman-4-ones *(Figure 2–5)*, while compounds **5-13** were found to be 3-benzylchroman-4-ones *(Figure 2–6).* Three known stilbenoids, 3′,4-dihyroxy-3,5′-dimethoxystilbene; 3,3′,5′-trihydroxy-4-methoxystilbene (rhapontigenin) and 4,3′,5′-trihydroxy-3-methoxystilbene (isorhapontigenin) were also identified (compounds **14-16** respectively) *(Figure 2–7).* Structural elucidation was performed using high resolution mass spectrometry (HRMS) and NMR.



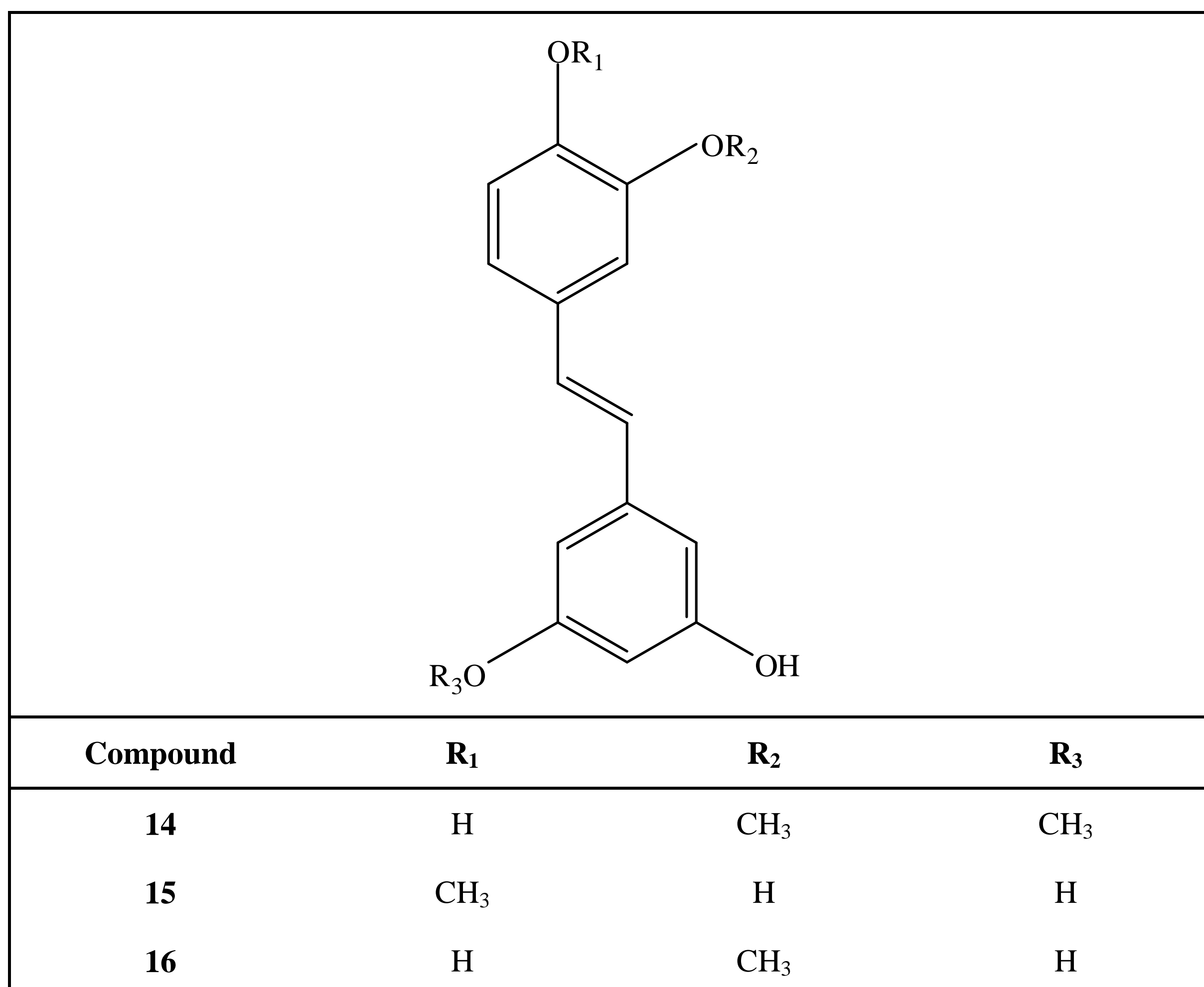
Compound	R_1	R_2	R_3
1	OH	OCH_3	OH
2	OH	OCH_3	OCH_3
3	OH	H	OH
4	OCH_3	H	OH

Figure 2–5. Compounds **1-4**, homoisoflavanones of the 3-benzylidenechroman-4-one type that were isolated by Silayo et al. (1999).



Compound	R_1	R_2	R_3	R_4	R_5	R_6
5	H	OCH_3	H	OCH_3	H	OCH_3
6	H	OCH_3	H	OH	OCH_3	OH
7	H	OH	H	OH	OH	OCH_3
8	H	OCH_3	OCH_3	OH	OCH_3	OH
9	H	OH	OCH_3	OH	H	OH
10	H	OH	H	OH	OCH_3	OCH_3
11	H	OCH_3	OH	OCH_3	H	OCH_3
12	H	OCH_3	OCH_3	OCH_3	H	OH
13	OH	OCH_3	H	OCH_3	H	OCH_3

Figure 2–6. Compounds **5-13**, homoisoflavanones of the 3-benzylchroman-4-one type that were isolated by Silayo et al. (1999).



Compound	R_1	R_2	R_3
14	H	CH_3	CH_3
15	CH_3	H	H
16	H	CH_3	H

Figure 2–7. Compounds **14-16**, stilbenoids that were isolated by Silayo et al. (1999).

Compound **7** (Bangani et al., 1999) and compound **15** (Silayo et al., 1999) were identical amongst the stilbenoids (rhapontigenin). Amongst the homoisoflavanones, compound **3** (Bangani et al., 1999) and compound **5** (Silayo et al., 1999) were similar 3-benzylchroman-4-ones since compound **3** was isolated as the acetate. In addition, compound **5** (Bangani et al., 1999) and compound **3** (Silayo et al., 1999) were identical 3-benzylidenechroman-4-ones.

2.1.5 Traditional Use

The plant has been traditionally used for a variety of ailments.[5] The bulbs of *S. nervosa* are used by the Zulu people in the treatment of nervous disorders in children, dysentery (Bangani et al., 1999), diarrhoea, sprains, fractures and cancers (Bisi-Johnson et al., 2010). Diluted bulb decoctions are used to treat pain associated with rheumatic fever. Crushed bulbs are used by the Sotho people as laxatives in children (Bangani et al., 1999). The Tswana

[5] For a comprehensive review on the claimed medicinal properties of traditional plants, refer to: Watt, J., Breyer-Brandwijk, M. 1962. The medicinal and poisonous plants of southern and eastern Africa. *Livingston,* Edinburgh.

people use cooked bulbs in porridge to treat infertility in women (Bangani et al., 1999; Silayo et al., 1999) and cold water extracts of the bulbs are used to treat infections (Abegaz, 2002).

2.1.6 Pharmacological Activity

A study by Du Toit et al. (2011) confirmed the anti-inflammatory properties and also demonstrated the antimicrobial activity of *S. nervosa*. In a mouse model of acute croton oil-induced auricular dermatitis, oedema was inhibited in a dose-dependent manner by 46% at 0.1 mg and 66% at 0.5 mg of a crude methanol extract. Differences in antimicrobial specificity were also observed with 2 different extracts. Results suggested that the compounds present in the bulbs are individually or in combination responsible for the potent anti-inflammatory and antimicrobial activities.

The pharmacological properties of the constituents present in *S. nervosa* have not been studied in detail, although studies on homoisoflavanones and stilbenoids demonstrated biological activity. Previous studies have shown homoisoflavanones to possess anti-inflammatory (Della Loggia et al., 1989; Du Toit et al., 2005), antimutagenic (Nguyen et al., 2006; Wall et al., 1989), antihistaminic (Amschler et al., 1996), anti-angiogenic (Shim et al., 2004) and antibacterial (Du Toit et al., 2007) activities. Out of the 13 homoisoflavanones identified by Silayo et al. (1999), compounds **1**, **5**, **7** and **9** *(Figure 2–5 and Figure 2–6)* were sent to the National Cancer Institute, USA to be evaluated for cytotoxicity in breast and colon cancer cell lines. Compound **7** was found to be the most active (ED_{50} = 2.24 μg/ml) against the whole range of cell lines, while compounds **5** (6.85 μg/ml) and **9** (8.06 μg/ml) had intermediate activity and compound **1** (21.86 μg/ml) was the least active (Abegaz, 2002). Studies on resveratrol have demonstrated its anti-inflammatory, antiviral, anticoagulant and cardioprotective effects, its potent anti-cancer potential (Athar et al., 2009) and its anti-hyperglycaemic potential (Palsamy and Subramanian, 2008, 2009; Sharma et al., 2011). Rhapontigenin has been shown to possess cancer chemopreventative properties (Chun et al., 2001) as well as anti-inflammatory properties through inhibition of NF-κB (Kageura et al., 2001).

2.1.7 Toxicology

Although *S. nervosa* is considered a plant of medicinal value, it has also been recognised as a poisonous species possibly due to the production of glycosides (Mimaki et al., 1993). However, no glycosides were isolated from the bulbs during phytochemical studies conducted by Bangani et al. (1999) and Silayo et al. (1999). Thus it is likely that other parts

of the plant may contain glycosides. The plant is considered to be particularly poisonous to livestock. A study by Van der Walt et al. (1946) investigated its poisonous effects on sheep. A 34.1 kg and 26 kg sheep were given 1 kg and 500 g respectively of the whole plant in a fresh flowering state in a single dose. Symptoms of dyspnoea, irregular pulse, apathy and an inactive rumen were observed before the sheep died. A post-mortem revealed cyanosis, hyperaemia and oedema of the lungs as well as degenerative changes in the liver and myocardium. Despite its poisonous nature, the plant is still used to treat anaplasmosis in cattle by the Sotho people (Bangani et al., 1999) and as a purgative for calves (McGaw and Eloff, 2008). Based on the toxic effects previously observed in sheep, it was suspected that the plant may also be toxic to humans and was thought to cause cardiac, hepatic and renal toxicity (Hutchings and Terblanche, 1989; Scott, 2003). However, there has been no conclusive data on the toxicity profile of the plant in humans.

2.2 The Liver

2.2.1 Structure of the Liver

The liver is one of the vital organs in the body (Mitra and Metcalf, 2009). At the cellular level the liver is arranged into functional units, the hepatic lobules consisting of hepatocytes. Blood enters the lobules through branches of the portal vein and hepatic artery and flows through venous sinusoids which drain into the central vein (Cunningham and Van Horn, 2003; Planinsic and Nicolau-Raducu, 2006) *(Figure 2–8)*.

Hepatocytes are the major functional cells of the liver and are organised in plates. The surfaces of the polygonal cells are either in contact with neighbouring cells or with the sinusoids. The sinusoidal side of hepatocytes contains numerous microvilli which extend into bile canaliculi. Due to the complex functions of hepatocytes, the cytoplasm is rich in organelles, particularly the endoplasmic reticulum which is the major site of xenobiotic metabolism by CYP enzymes. Glycogen and lipid particles are also commonly present (Phillips et al., 1974; Zangar et al., 2004).

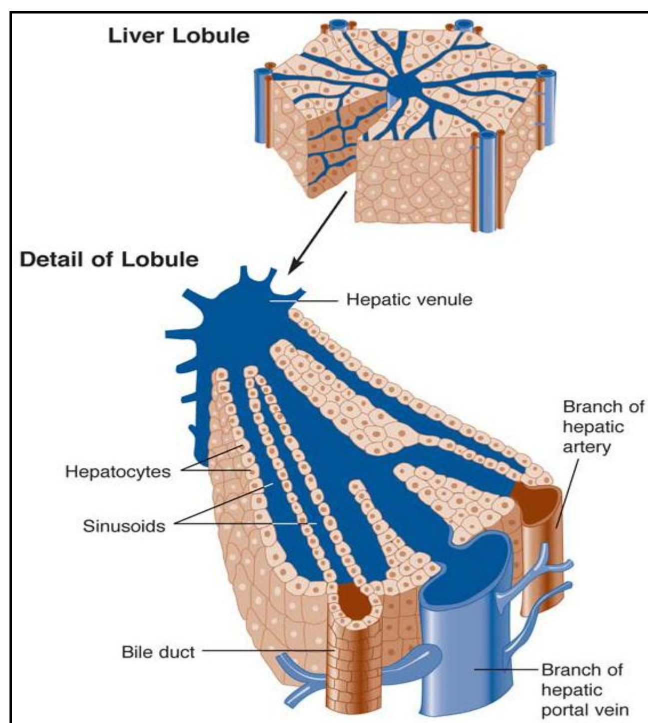


Figure 2–8. Structure of a liver lobule (obtained with permission from Cunningham and Van Horn, 2003).

2.2.2 Functions of the Liver

The liver performs a multitude of functions. The major metabolic and synthetic functions carried out by the liver, and in particular the hepatocytes, include carbohydrate, protein and lipid metabolism. It synthesises plasma proteins, primarily albumin (Mitra and Metcalf, 2009; Planinsic and Nicolau-Raducu, 2006). It is responsible for the excretion of endogenous substances such as bilirubin, steroid hormones and exogenous substances such as drug metabolites. In addition, the liver secretes bile, required for the absorption of fat and fat-soluble vitamins in the small intestine (Mitra and Metcalf, 2009). The immunological functions of the liver are exerted by the hepatic macrophages, Küpffer cells (Campbell, 2006).

The liver is also the primary site of metabolism of xenobiotics. Metabolism involves biotransformation of the substance, resulting in its inactivation and the creation of more polar molecules thus allowing their excretion in the bile or urine (Campbell, 2006; Planinsic and Nicolau-Raducu, 2006). Most metabolism occurs in the smooth endoplasmic reticulum via phase I and II reactions. Phase I reactions inactivate drugs via oxidation, reduction or hydrolysis and are carried out primarily by the cytochrome P450 system. Phase II reactions involve increasing the water solubility of the compounds by conjugating them with polar molecules such as glucuronide (Campbell, 2006).

2.2.3 Hepatotoxicity

The liver is particularly prone to xenobiotic-induced hepatotoxicity due to its central role in xenobiotic metabolism as well as its portal circulation (Sturgill and Lambert, 1997). Injury to hepatocytes may be cytotoxic (resulting in morphological changes in hepatocytes), genotoxic (resulting in damage to genetic material), or metabolic (causing alterations in cellular metabolism and mitochondrial dysfunction) (Castell et al., 1997).

Following damage that cells are unable to recover from, hepatocytes may either undergo necrosis, a passive form of cell death often resulting from acute cellular injury or apoptosis, a highly regulated, active form of cell death (Robertson and Orrenius, 2000).

2.3 Apoptosis

Apoptosis or 'programmed cell death' is a controlled, energy-dependent, genetically regulated form of cell death. It plays a crucial role in the survival of organisms and is essential in regulating the size of cell populations, functioning and development of the immune system, removal of abnormal cells and maintaining tissue homeostasis (Kam and Ferch, 2000; Rastogi et al., 2009). Disordered apoptosis has however been implicated in the pathogenesis of various diseases. Excessive apoptosis is associated with conditions such as neurodegenerative diseases, immune deficiency and cardiovascular disease; conversely the suppression of apoptosis has been linked to cancer and autoimmune disorders (Kam and Ferch, 2000; Kannan and Jain, 2000; Kiechle and Zhang, 2002).

Apoptosis occurs in a characteristic sequence of biochemical and morphological events. During the process, individual cells undergo a decrease in volume, externalisation of phosphatidylserine (PS) on the outer membrane leaflet, cell membrane blebbing, cytoskeleton collapse, cytoplasmic and chromatin condensation and DNA fragmentation. The process culminates in the fragmentation of the cell into membrane bound apoptotic bodies which are phagocytosed and degraded by macrophages and other neighbouring cells without causing damage to the surrounding tissue. Apoptosis does not elicit an inflammatory response because the cell membrane integrity of apoptotic cells is maintained and leakage of cellular contents is prevented (Graf et al., 2007; Kannan and Jain, 2000; Kiechle and Zhang, 2002; Robertson and Orrenius, 2000).

2.3.1 Caspases

The caspases, which represent a family of cysteine-aspartate proteases, are considered the central effectors of apoptosis and play essential roles in its initiation and execution. Caspases

are synthesised in cells as inactive precursors, or so-called procaspases, consisting of an N-terminal prodomain and a C-terminal protease domain (Kantari and Walczak, 2011; Lossi and Merighi, 2003). Stimuli that induce apoptosis stimulate the proteolytic cleavage of procaspases at aspartate residues to produce activated enzymes. Caspases that are initially activated trigger the activation of other downstream caspases resulting in a proteolytic cascade that ultimately culminates in cell death (Ashe and Berry, 2003; Ghavami et al., 2009).

Caspases are divided into two subsets based on their apoptotic functions. The first group, initiator or so-called upstream caspases (caspase-2, -8, -9, -10), can be further divided into intrinsic pathway activators (caspase-9) and extrinsic pathway activators (caspase-2, -8, -10). These caspases undergo autoactivation prior to activating the second group, the effector or so-called downstream caspases (caspase-3, -6, -7), by proteolytic cleavage of the procaspases (Ashe and Berry, 2003; Ghavami et al., 2009; Graf et al., 2007). Effector caspases are directly involved in the destruction of the cell through the cleavage of diverse cellular substrates (Brunelle and Zhang, 2010).

The length and amino acid sequence of the N-terminal prodomain is highly variable among caspases, with initiator caspases possessing much longer prodomains compared to effector caspases (Ghavami et al., 2009). Long prodomains contain specific motifs (domains) which are fundamental to caspase activity. The death effector domain (DED) is found in caspase-8 and -10 while caspase-2 and -9 contain a caspase recruitment domain (CARD) (Ashe and Berry, 2003). These domains are required to mediate interactions with adaptor proteins involved in cell signalling, and effector caspases (Liou et al., 2003) *(Figure 2–9).*

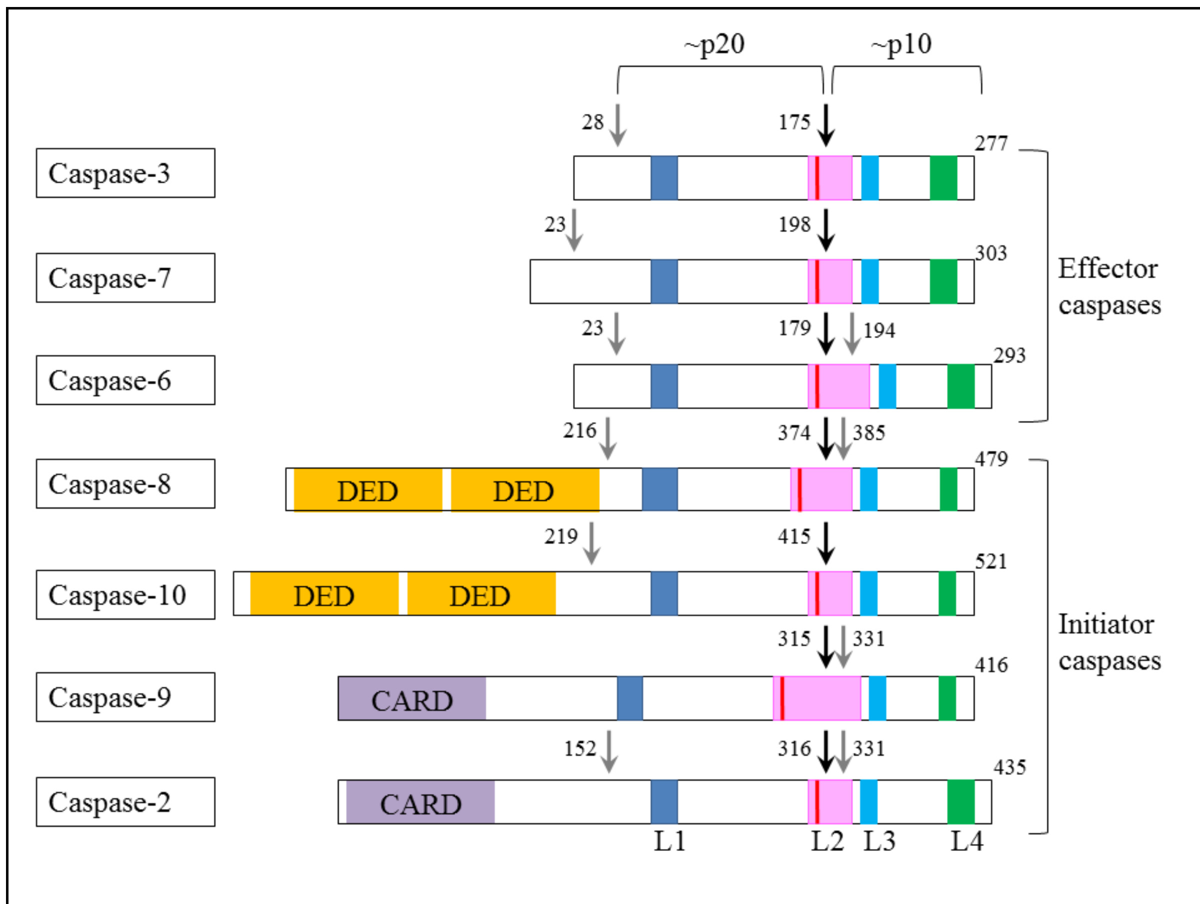


Figure 2–9. A schematic diagram depicting the effector caspases (caspase-3, -6, and -7) and initiator caspases (caspase-2, -8, -9 and -10). The initiator caspases contain relatively longer N-terminal prodomains, such as caspase-8 and -10 that contain DEDs while caspase-2 and -9 contain CARDs. Arrows represent cleavage sites, and the number of amino acids is indicated. The four surface loops (L1-L4) that shape the catalytic groove are shown (adapted and redrawn with permission from Riedl and Shi, 2004).

Caspases are instrumental in producing the morphological and biochemical changes characteristic of apoptosis. These enzymes are responsible for abolishing contact of apoptotic cells with surrounding cells, proteolysis of cytoskeleton proteins, DNA fragmentation, destruction of the nuclear lamina, inducing changes on the membrane surface to facilitate recognition by macrophages, and disintegrating apoptotic cells into apoptotic bodies (Kannan and Jain, 2000; Lamkanfi and Kanneganti, 2010).

2.3.2 Apoptotic Pathways

Apoptosis can occur via various interrelated pathways, however apoptosis can be broadly divided into two major cell signalling cascades (Ashe and Berry, 2003). The pathway that is initiated is dependent on the nature of the inducing stimuli. The extrinsic pathway is generally activated by the binding of ligands to death receptors. The intrinsic pathway may be triggered by DNA damage induced by ionising radiation and anti-cancer agents, withdrawal of growth factors or oxidative stress (Brunelle and Zhang, 2010; Kannan and

Jain, 2000; Lamkanfi and Kanneganti, 2010). Both pathways ultimately merge at a common execution pathway to produce the characteristic cellular changes associated with apoptosis (Ghavami et al., 2009).

2.3.2.1 The Extrinsic Apoptotic Pathway

The extrinsic pathway, also referred to as the death receptor pathway, is activated by the binding of extracellular ligands to cell surface death receptors (Brunelle and Zhang, 2010) *(Figure 2–10)*. Death receptors (DR) belong to the tumour necrosis factor receptor (TNF-R) gene superfamily and are characterised by an intracellular death domain of 80 amino acids that is necessary for transmitting apoptotic signals from the cell surface to intracellular signalling pathways (Fulda and Debatin, 2006). Well-characterised death receptors include Fas (fibroblast associated antigen (CD95)), TNF-R1, death receptor 4 (DR4) and 5 (DR5) (Brunelle and Zhang, 2010) which are activated via ligation of the respective ligands, namely Fas ligand (FasL), tumour necrosis factor (TNF) and TNF α-related apoptosis inducing ligand (TRAIL) (Ashe and Berry, 2003; Kantari and Walczak, 2011).

The interaction of Fas with FasL results in the recruitment of, and association with the adaptor protein, Fas-associated death domain protein (FADD) (Ashe and Berry, 2003). In addition to its death domain, FADD also possesses a DED which facilitates its interactions with the DED-containing procaspases, 8 and 10 and mediates their association with the Fas receptor (Hengartner, 2000). The complex that is subsequently formed is referred to as the death-inducing signalling complex (DISC). Procaspase-8 and -10 undergo self-cleavage and activation at the DISC to form caspase-8 and -10 (Brunelle and Zhang, 2010). Caspase-8 activation is regulated by cellular FLICE inhibitory protein (cFLIP) which competes with caspase-8 for binding to FADD at the DISC. High concentrations of cFLIP can therefore diminish caspase-8 activation (Kantari and Walczak, 2011).

If sufficient quantities of caspase-8 are activated, it is able to directly activate the downstream effector caspases-3, -6, and -7 (Ashe and Berry, 2003; Brunelle and Zhang, 2010). However, if the amount of caspase-8 activated at the DISC is insufficient to cause the direct activation of effector caspases, mitochondrial amplification of death signalling occurs through the intrinsic pathway to mediate full caspase activation (Brunelle and Zhang, 2010; Fulda and Debatin, 2006). This type of Fas signalling requires caspase-8-mediated cleavage of the BH3 interacting domain death agonist (Bid), a member of the pro-apoptotic B-cell CLL/Lymphoma 2 (Bcl-2) family, to truncated Bid (tBid). The resulting tBid translocates to

the mitochondria where it induces the release of pro-apoptotic factors such as cytochrome c, second mitochondria-derived activator of caspase/direct IAP binding protein of low isoelectric potential (Smac/DIABLO), apoptosis inducing factor (AIF) and endonuclease G, causing caspase-9 activation which subsequently activates caspase-3 (Ashe and Berry, 2003; Kantari and Walczak, 2011).

Ligation of TNF-R1 by TNF stimulates the recruitment of the adaptor molecule, TNF receptor-associated death domain (TRADD). FADD is subsequently recruited to the death receptor by TRADD and the indirect association of TNF-R1 with FADD induces apoptosis by a similar mechanism as Fas-mediated signalling (Ashe and Berry, 2003). The secondary signalling proteins, TNF receptor-associated factor-2 (TRAF2) and receptor-interacting protein (RIP) are also recruited to the complex by TRADD. TRAF2 plays an essential role in activating the Jun NH_2-terminal kinase (JNK) pathways and NF-κB (Liu et al., 1996). While TNF-R1-mediated activation of JNK does not appear to be involved in the induction of apoptosis in most cells, the activation of NF-κB has been shown to protect cells from TNF-induced apoptosis by removal of its inhibitor I-κB (inhibitor of NF-κB), allowing it to initiate the transcription of inhibitors of apoptosis proteins (IAPs). The association of RIP with TNF-R1 induces apoptosis via the recruitment of RIP-associated ICH-1 homologous protein with death domain (RAIDD) which contains a CARD with which it associates with caspase-2 (Ashe and Berry, 2003).

TRAIL-induced apoptosis via ligation of DR4 or DR5 occurs through the interaction with FADD and caspase-8 in a manner similar to Fas-mediated signalling. Thus, caspase-8 activation via TRAIL signalling also activates the intrinsic apoptotic pathway and mitochondrial amplification by a pathway similar to the one stimulated by FasL ligation. This mediates full caspase activation and the release of pro-apoptotic factors from the mitochondria (Fulda et al., 2002).

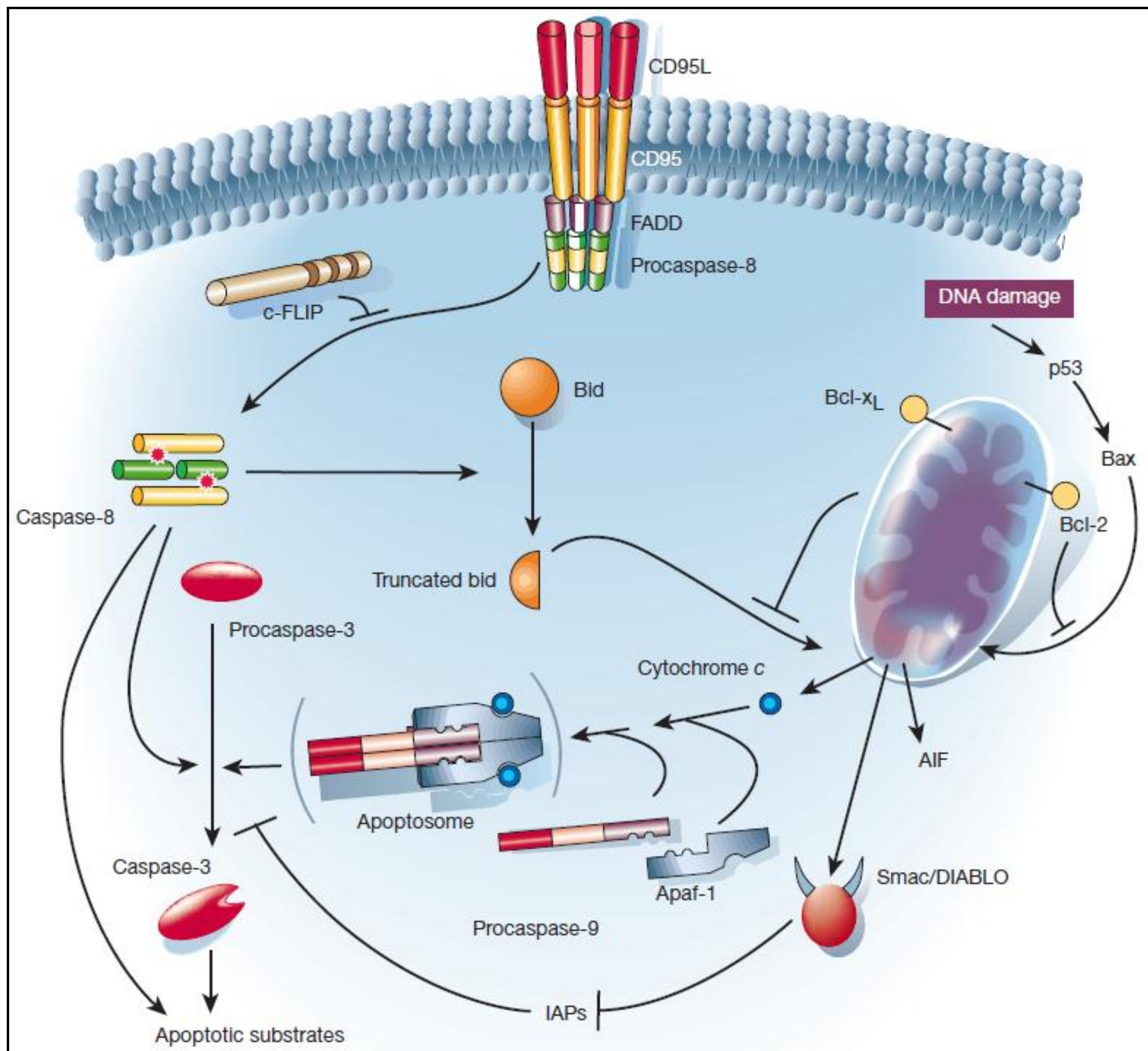


Figure 2–10. The extrinsic and intrinsic pathways of apoptosis (Hengartner, 2000; obtained with permission from Nature Publishing Group).

2.3.2.2 The Intrinsic Apoptotic Pathway

The intrinsic or mitochondrial pathway is activated in response to an apoptotic signal such as DNA damage induced by cytotoxic drugs and oxidative stress. The mitochondria are the major initiation sites of apoptosis via this pathway and play an important role in its regulation (Brunelle and Zhang, 2010; Hengartner, 2000) *(Figure 2–10).* The intrinsic pathway is mediated by the release of pro-apoptotic factors from the mitochondria into the cytosol. It is hypothesised that induction of mitochondrial permeability transition (MPT) and opening of mitochondrial permeability transition pores (PTPs) facilitates this release. Although the complete structure and composition of the PTP has yet to be elucidated, it is thought to be composed of mitochondrial inner membrane proteins such as adenine nucleotide translocator (ANT) and outer membrane proteins such as voltage-dependent anion channel (VDAC).

These proteins act at contact sites in the inner and outer membrane to create the pores (Green and Reed, 1998). Opening of these pores results in the loss of mitochondrial membrane potential (ΔΨm) and uncoupling of oxidative phosphorylation. An influx of fluid into the mitochondria has also been associated with the opening of PTPs, resulting in mitochondrial swelling, rupture of the mitochondrial outer membrane and the release of pro-apoptotic factors (Robertson and Orrenius, 2000).

Mitochondrial apoptosis is primarily regulated by the Bcl-2 family of proteins (Kantari and Walczak, 2011). It has been proposed that the release of pro-apoptotic factors from channels on the mitochondrial outer membrane is facilitated by these proteins (Ashe and Berry, 2003; Hengartner, 2000; Lossi and Merighi, 2003). All members of this family contain at least one of four Bcl-2 homology (BH) domains and can be divided into three groups based on the number of BH domains they contain and their functions. Bcl-2 family members can be first divided into anti- and pro-apoptotic members. The anti-apoptotic proteins, Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1, contain all four BH domains. Pro-apoptotic proteins can be further divided into two subgroups (Kantari and Walczak, 2011). The first subgroup comprising Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak) and Bcl-2 related ovarian killer (Bok) contain BH1 to BH3 domains while the second subgroup contains only the BH3 domain and includes the proteins Bid, Bim, Bik, Bad, Bmf, Hrk, NOXA and PUMA (Ashe and Berry, 2003; Kantari and Walczak, 2011).

In response to intrinsic signals or caspase-8 in type II Fas signalling, BH3-only pro-apoptotic proteins migrate to the mitochondria where they antagonise anti-apoptotic Bcl-2 proteins and activate the multi-domain pro-apoptotic proteins, Bax and Bak (Launay et al., 2005). The release of pro-apoptotic factors into the cytosol has been attributed to various mechanisms. It is proposed that Bax and Bak oligomerise and insert into the mitochondrial outer membrane forming pores in the membrane. It is also thought that these pro-apoptotic proteins bind to the outer membrane protein, VDAC, to stimulate its opening (Ashe and Berry, 2003; Ghavami et al., 2009; Huerta et al., 2007; Robertson and Orrenius, 2000). Both mechanisms result in the permeabilisation of the mitochondrial outer membrane and loss of ΔΨm. Caspase-8 cleavage of Bid to tBid also promotes permeabilisation of the mitochondrial outer membrane (Kantari and Walczak, 2011).

Upon disruption of the mitochondrial outer membrane, apoptogenic proteins including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G, which are normally

found in the intermembrane space of the mitochondria, are released into the cytosol (Fulda and Debatin, 2006). The exact mechanism of cytochrome c is however largely unknown.

Cytochrome c binds to the cytosolic protein, apoptotic protease-activating factor-1 (APAF-1), and in the presence of ATP, triggers its oligomerisation and aggregation of several APAF-1 units (Fulda and Debatin, 2006; Ghavami et al., 2009). The resultant effect is exposure of the APAF-1 CARD that induces the recruitment of procaspase-9, which is subsequently activated through CARD-CARD interactions (Fulda and Debatin). The multi-protein complex formed (consisting of cytochrome c, APAF-1, ATP and caspase-9) is termed the apoptosome. Activated caspase-9 directly activates the downstream effector caspases-3, -6 and -7 and in doing so, initiates the proteolytic cascade leading to apoptotic cell death (Buehrlen et al., 2007).

Smac/DIABLO and Omi/HtrA2 enhance caspase activation by interacting with IAPs (Ghavami et al., 2009). Caspase-9 activity is highly controlled by IAPs such as XIAP, cIAP1, cIAP2 which prevent its activation by directly binding to procaspase-9 and blocking its binding sites (Brunelle and Zhang, 2010; Du et al., 2000). Smac/DIABLO and Omi/HtrA2 bind directly to and sequester IAPs thus antagonising their inhibitory effects (Ashe and Berry, 2003).

AIF and endonuclease G induce apoptosis in a caspase-independent manner. Both proteins translocate to the nucleus following permeabilisation of the mitochondrial outer membrane where they induce DNA fragmentation and chromatin condensation (Fulda and Debatin, 2006).

The ability of medicinal plants to induce apoptosis has been extensively studied. *Sutherlandia frutescens*, which is endemic to southern Africa, is used traditionally to treat fever, poor appetite, wasting diseases, indigestion, gastritis, oesophagitis, peptic ulcers, dysentery, cancer, diabetes, colds, influenza, asthma, chronic bronchitis, kidney and liver conditions, rheumatism, heart failure, urinary tract infections and stress and anxiety (Van Wyk, 2008). A study by Chinkwo (2005) demonstrated that an aqueous extract induced apoptosis in Chinese hamster ovary and cervical carcinoma cell lines. It was hypothesised that apoptotic induction occurred via caspase-3 activation.

The medicinal plant *Tulbachia violacea*, indigenous to the Eastern Cape, is used to treat fever, colds, asthma, tuberculosis and stomach problems. In a study by Bungu et al. (2006) methanol extracts of the leaves and bulbs were shown to inhibit growth and induce apoptosis

in MCF-7, WHCO3, HT29 and HeLa cancer cell lines, although the molecular mechanisms involved in its action remain to be elucidated.

2.4 Oxidative Stress

Oxidative stress denotes an imbalance between the production of free radicals or their reactive metabolites (oxidants) and the antioxidant defence mechanisms in organisms (Reuter et al., 2010). Free radicals are molecules containing one or more unpaired electrons that are capable of independent existence (Halliwell and Chirico, 1993). They include, but are not limited to, reactive oxygen species (ROS), reactive nitrogen species (RNS) and sulphur-centred radicals (Abuja and Albertini, 2001).

Traditional plant medicines have been shown to possess both antioxidant properties as well as having the ability to induce ROS generation. An aqueous extract from *Sutherlandia frutescens* was shown to possess potent antioxidant properties due to its superoxide and hydrogen peroxide scavenging properties (Fernandes et al., 2004).

Conversely, Steenkamp et al. (2005) demonstrated the pro-oxidant effect of the methanol extract of *Callilepis laureola*, used traditionally to treat stomach aches, tapeworm infections, impotence and induce fertility, possibly due to its ability to deplete cellular glutathione.

2.4.1 Free Radicals

Oxygen-derived radicals have both beneficial and adverse roles in cell functioning. ROS, which include superoxide ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2) and the hydroxyl ($OH\cdot$) free radical (Liu et al., 2002), are physiologically important in cell signalling mechanisms where they act as secondary messengers at low concentrations (Kannan and Jain, 2000). However, increased production of ROS can be genotoxic or cytotoxic, capable of inducing apoptosis and necrosis (Shvedova et al., 2002) and has been implicated in various pathological conditions such as aging, neurodegenerative diseases, type II diabetes, atherosclerosis and ischaemia/reperfusion injury (Abuja and Albertini, 2001; Ott et al., 2007).

The mitochondrial electron transport chain (ETC) is recognised as an important source of ROS generation (Chen et al., 2003; Liu et al., 2002). The mitochondrial ETC is composed of a series of electron carriers arranged into 4 complexes according to their redox potentials. Electrons from the reducing equivalents NADH and $FADH_2$, are transported through the ETC from complex I or complex II and ultimately pass to molecular oxygen (O_2), generating water at complex IV. However, during electron transfer some electrons leak out to O_2, causing the one-electron reduction of O_2 to produce $O_2 \cdot^-$ (Liu et al., 2002). The major sites of ROS

generation are thought to be at complex I and complex III of the ETC (Chen et al., 2003; Liu et al., 2002; Ott et al., 2007) *(Figure 2–11)*. The superoxide anion is a precursor of most ROS and is quickly dismutated by manganese superoxide dismutase (Mn-SOD) to produce H_2O_2. The subsequent interaction of H_2O_2 with $O_2\cdot^-$ or the Fe^{2+}- or Cu^{2+}-mediated cleavage of H_2O_2 in a Fenton reaction produces the very reactive OH· (Ott et al., 2007). $O_2\cdot^-$ may also react with the nitric oxide (NO) radical to produce peroxynitrite ($ONOO^-$), a potent oxidant (Madesh and Hajnóczky, 2001).

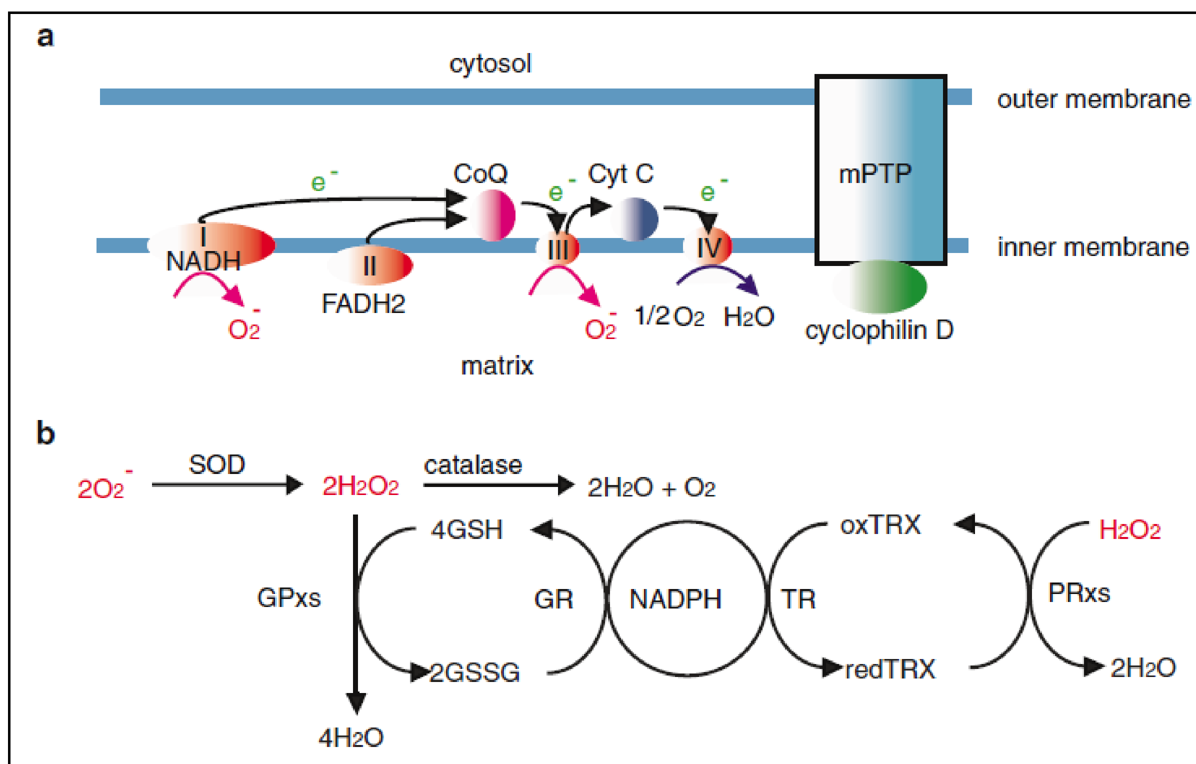


Figure 2–11. **(a)** Generation of ROS in the mitochondria and **(b)** their inactivation by antioxidants (obtained with permission from Nakano et al., 2005).

2.4.2 Antioxidants

Both enzymatic and non-enzymatic antioxidant systems work to protect cells from the deleterious effects of oxidants (Reuter et al., 2010). Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidise (GPx), glutathione reductase, peroxiredoxin (PRx) and catalase, while non-enzymatic antioxidants include glutathione (GSH), vitamin C and vitamin E. Following the conversion of $O_2\cdot^-$ to H_2O_2 by SOD, other enzymatic antioxidants catalyse its elimination (Nakano et al., 2005; Reuter et al., 2010).

An important source of antioxidant defence comprises GSH and the various GSH-linked enzymes which provide protection from free radical and peroxide damage. GPx1 and GPx4 catalyse the reduction of H_2O_2 and lipid hydroperoxides to water or the appropriate alcohol

using GSH as an electron donor (Ott et al., 2007; Reuter et al., 2010). GSH is then converted to the oxidised form, glutathione disulphide (GSSG) which is recycled to GSH by glutathione reductase (GR) to prevent its depletion (Nakano et al., 2005; Reuter et al., 2010) *(Figure 2–11).*

Another antioxidant defence system is the mitochondrial thioredoxin (TRX) system (Ott et al., 2007). PRx is also capable of catalysing the reduction of H_2O_2 to water and the reduction of alkyl hydroperoxides to the appropriate alcohol using TRX. Oxidised TRX is then recycled to TRX by TRX reductase (TR). Both recycling processes occur in the presence of NADPH (Nakano et al., 2005; Reuter et al., 2010) *(Figure 2–11).*

Vitamins C and E are located in extracellular fluids (Abuja and Albertini, 2001). Vitamin E, a lipophilic antioxidant, is localised in the lipophilic compartment of membranes and lipoproteins. It scavenges lipid peroxyl radicals to halt chain propagation during lipid peroxidation, and a vitamin E radical is subsequently formed. Vitamin C is a hydrophilic antioxidant that scavenges aqueous radicals in the aqueous phase. Vitamin C is also able to reduce the vitamin E radical and thus regenerates vitamin E, and inhibits the oxidant effects of the vitamin E radical (Niki et al., 2005).

2.4.3 Consequences of Oxidative Damage

Depletion of the antioxidant defence systems or an increase in ROS production results in oxidative stress (Halliwell and Chirico, 1993). ROS may cause irreversible oxidative damage to proteins, nucleic acids, phospholipids and numerous other macromolecules (Grivennikova and Vinogradov, 2006). Mitochondria are thought to be the major targets for the harmful effects of ROS as the concentration of $O_2\cdot^-$ in the mitochondria appears to be higher compared to the cytosol and nucleus of the cell (Ott et al., 2007). Mitochondrial membrane depolarisation and disruption of the ETC are prominent features leading to ROS-mediated cell death, particularly via the apoptotic pathway (Madesh and Hajnóczky, 2001).

Oxidative damage to DNA may cause modification of bases, DNA strand breaks, abnormal DNA cross linking and mutations of proto-oncogenes and tumour suppressor genes, thus promoting mutagenesis (Ott et al., 2007; Reuter et al., 2010). A particularly significant target of oxidative damage is the mitochondrial DNA (mtDNA), possibly due to its close proximity to the ROS generated via the ETC and the lack of histone proteins that protect DNA from injury (Wiseman and Halliwell, 1996).

$O_2\cdot^-$ can also oxidise and inactivate iron-sulphur proteins, such as aconitases and NADH dehydrogenase. The inactivation of mitochondrial aconitases would cause the release of Fe^{2+} and H_2O_2. The release of these molecules could also result in the generation of the highly reactive OH·. This in turn would amplify $O_2\cdot^-$-mediated oxidative damage by attacking mitochondrial proteins, lipids and DNA. Mitochondrial aconitase is also essential to the Krebs cycle, where it is responsible for converting citrate to isocitrate. Inactivation of this enzyme would cause disruption of the Krebs cycle, and as a result, negatively affect ATP production and cell viability (Ott et al., 2007)..

Lipid peroxidation occurs as a result of free radicals readily attacking polyunsaturated fatty acids (PUFAs) such as those incorporated into cellular membrane lipids. The highly reactive OH·, formed by the Fenton reaction, reacts with the single electrons contained in the hydrogen atoms of PUFAs. Removal of this electron leaves behind an unpaired electron on the carbon atom to which it was attached. The resulting carbon-centred lipid radical then undergoes molecular rearrangement prior to reacting with O_2 to form peroxyl radicals (Halliwell and Chirico, 1993; Niki et al., 2005). The peroxynitrite radical, formed by the reaction of $O_2\cdot^-$ with NO, is also capable of causing lipid peroxidation possibly due to the decomposition of $ONOO^-$ to OH· (Halliwell and Chirico, 1993). Lipid peroxidation yields a variety of complex products, such as hydroperoxides and aldehydes, which may be both cytotoxic and genotoxic (Niki et al., 2005).

Lipid peroxidation has numerous pathological implications. Lipid peroxidation at the mitochondrial membranes affects mitochondrial functions such as oxidative phosphorylation, maintenance of the $\Delta\Psi m$, and the Ca^{2+} buffering capability of mitochondria (Ott et al., 2007). Intermediate and end-products formed during lipid peroxidation are also able to attack and damage DNA and mtDNA (Wiseman and Halliwell, 1996). Lipid peroxidation is thus capable of inducing apoptotic and necrotic cell death as a result of the cellular damage it imparts (Tyurina et al., 2000).

2.5 Cytochrome P450 3A4

CYP enzymes comprise a superfamily of haeme-containing mono-oxygenases. In humans, CYP enzymes which are localised in the endoplasmic reticulum (Zangar et al., 2004) of most tissues catalyse the oxidative metabolism of exogenous substances such as drugs, carcinogens and plant products as well as endogenous compounds including steroids, fatty acids, bile acids and prostaglandins (Mukherjee et al., 2011; Pan et al., 2010; Subehan et al.,

2006). In particular, they catalyse phase I reactions involved in the detoxification mechanisms in humans, where they introduce new functional groups such as hydroxyl groups, or modify existing functional groups on xenobiotics to create more polar compounds to assist phase II reactions (Westerink and Schoonen, 2007). Although CYPs are distributed ubiquitously such as in the lung, nasal mucosa and vascular smooth muscle tissues, hepatic and intestinal CYPs play pivotal roles in xenobiotic oxidation (Mukherjee et al., 2011; Zangar et al., 2004).

The CYP3A subfamily is the most abundantly expressed CYP and consists of three isoforms, namely CYP3A4 and CYP3A5, expressed in human adults and CYP3A7 which appears to be expressed primarily in foetal livers (Guengerich, 1999). CYP3A4 is expressed in several extrahepatic tissues such as the lungs and small intestine, however its expression is most abundant in the liver where it represents approximately 50% of the total CYP content (Ding and Kaminsky, 2003; Pan et al., 2010).

2.5.1 Catalytic Cycle

The catalytic cycle of CYP3A4 and other CYPs involves the reduction and activation of O_2. Electrons are provided by the enzyme NADPH-P450 reductase which then oxidises NADPH. The generalised cycle consists of substrate (RH) binding to the active haeme centre of the enzyme. Fe^{3+} is then reduced, followed by oxygen binding, the reduction of O_2, cleavage of the oxygen at the O-O bond which subsequently forms water, the reaction with the substrate at the active perferryl FeO species (FeO^{3+}), and finally the release of the product (Guengerich, 1999; Isin and Guengerich, 2007) *(Figure 2–12).*

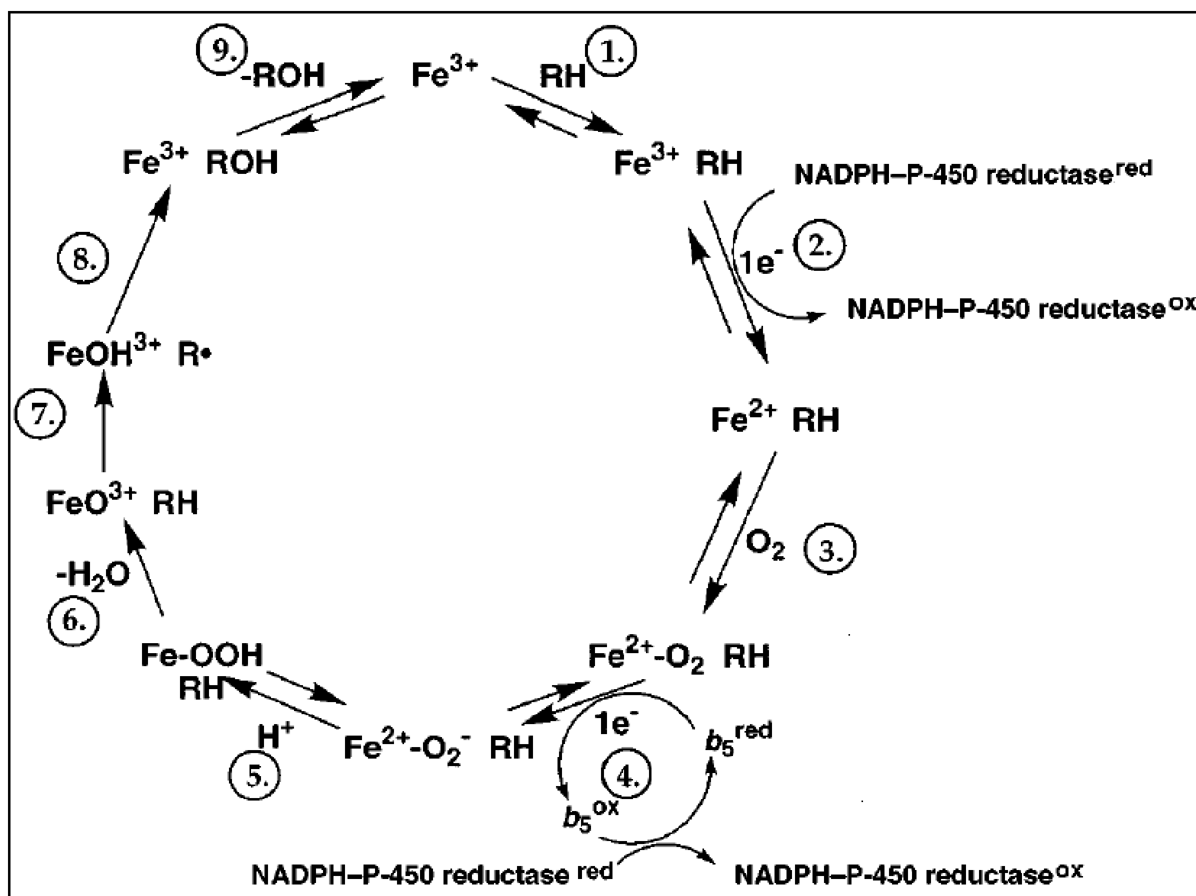


Figure 2–12. The catalytic cycle of CYP3A4 (obtained with permission from Guengerich, 1999).

2.5.2 Interactions with CYP3A4

The substrates for CYP3A4 are structurally diverse. Carcinogens such as aflatoxin B_1 (Hasler et al., 1999) and approximately 60% of pharmaceutical drugs (Raucy, 2003) metabolised by CYPs are metabolised by CYP3A4, thus interactions with this enzyme have both pharmacological and toxicological implications. Drug-drug interactions that alter CYP3A4 activity have been well documented. When drugs are co-administered, one drug may be capable of modulating the metabolism of the second drug by either inducing or inhibiting CYP3A4. This could result in sub-therapeutic or toxic drug levels respectively. Similarly, chemical constituents from medicinal plants may also be substrates, inducers or inhibitors of CYP3A4 and in the same way are susceptible to changes in metabolism and may affect the metabolism of co-administered drugs, thereby increasing the risk of serious adverse reactions (Mukherjee et al., 2011). Several medicinal plant extracts have been shown to interact with CYP3A4.

The interactions of St. John's wort (*Hypericum perforatum*), a herbal preparation used in the treatment of mild anxiety and depression, with CYP3A4 have been extensively studied.

Interestingly, some components in St John's wort appear to inhibit CYP3A4 while others induce the enzyme (Moon et al., 2006; Obach, 2000). Studies have shown the extract to decrease the plasma levels of drugs such as the antiretroviral drug indinavir (Piscitelli et al., 2000) and the immunosuppressant drug cyclosporine A (Ahmed et al., 2001) due to its activation of CYP3A4. The study by Obach (2000) demonstrated the ability of St. John's wort to inhibit CYP3A4 activity but no drug levels were measured.

Several studies have also demonstrated grapefruit juice to be an inhibitor of CYP3A4, and have reported increased plasma levels of many drugs such as the sedative-hypnotic drug midazolam, the antiretroviral drug saquinavir and the calcium channel blocker verapamil when co-administered with grapefruit juice (Bailey et al., 1998).

2.5.3 Modulation of CYP3A4

CYP3A4 is prone to induction and inhibition due to its extensive substrate specificity. Modulation of CYP3A4 has various implications for the pharmacokinetics of drugs metabolised by these enzymes.

Inhibition of CYP3A4 can lead to reduced drug clearance, prolonged effects and drug-induced toxicity (Hasler et al., 1999; Subehan et al., 2006). It can be broadly classified into reversible and irreversible inhibition. Reversible inhibition, the more common type of inhibition, occurs when an inhibitor competes with the substrate for the same enzyme binding site and concentrations of the inhibitor are sufficient to impair enzyme activity. Inhibition of the enzyme is transient and activity is restored upon diffusion of the inhibitor away from the enzyme. On the other hand, irreversible inhibition or mechanism-based inhibition is caused by the conversion of the inhibitor to a reactive metabolite that binds covalently to the haeme or apoprotein of the enzyme (Hasler et al., 1999; Mukherjee et al., 2011). Irreversible binding generally results in prolonged enzyme inhibition and can therefore cause severe, potentially fatal, adverse effects (Zhou et al., 2005).

Conversely, induction of CYP3A4 can result in increased drug elimination, causing a decrease in the plasma concentration of the drug, decreased efficacy and ineffective therapy or treatment failure (Mukherjee et al., 2011; Tompkins and Wallace, 2007). Alternatively, the increased metabolism of certain xenobiotics, including carcinogens, may lead to their bioactivation and the formation of reactive or toxic metabolites (Tompkins and Wallace, 2007; Westerink and Schoonen, 2007).

ROS generation is a known consequence of the catalytic cycle of CYP3A4 and other CYPs, which involves one-electron transfer reactions. During this cycle electrons leak out at various branches, causing the continuous formation of small amounts of ROS such as $O_2{\cdot}^-$ and H_2O_2 (Puntarulo and Cederbaum, 1998; Zangar et al., 2004) *(Figure 2–13)*. Generation of ROS has the potential to cause oxidative stress, lipid peroxidation and cell death. A study by Puntarulo and Cederbaum (1998) investigated the ability of various CYP isoforms to produce ROS. It was found that CYP3A4 was the most active in catalysing the oxidation of NADPH and producing $O_2{\cdot}^-$. Drugs that have inductive capabilities towards CYP3A4 thus also have the potential to increase ROS generation and their deleterious effects.

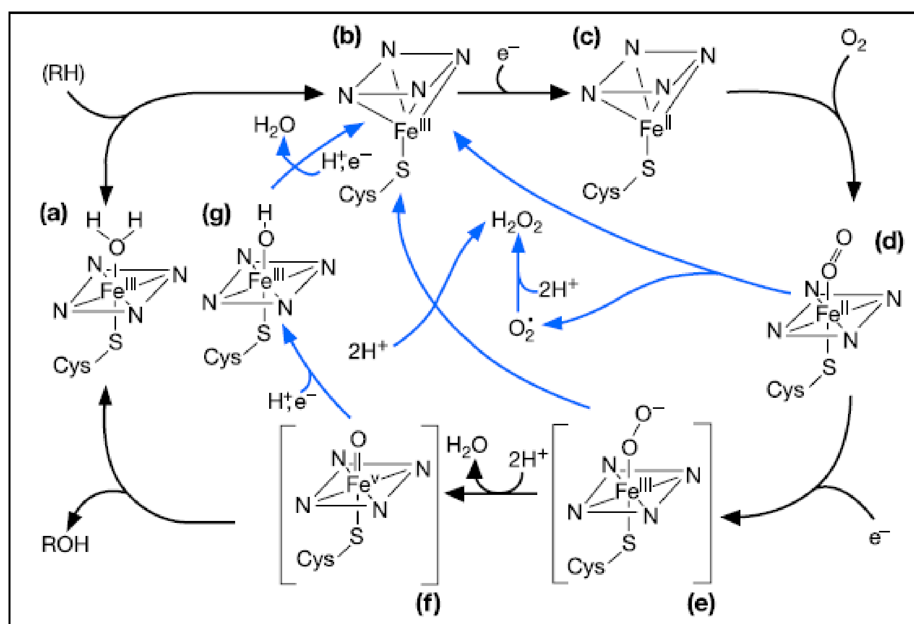


Figure 2–13. Catalytic cycle of CYP3A4 with blue arrows showing ROS-producing branches (obtained with permission from Zangar et al., 2004).

2.5.4 Pregnane X Receptor

The pregnane X receptor (PXR), an orphan nuclear receptor belonging to the nuclear receptor superfamily, is now thought to mediate CYP3A4 gene expression in response to xenobiotics. PXR ligands include a wide variety of endogenous chemicals, such as steroid hormones and xenobiotics, including the tuberculocidal drug rifampicin and antifungal drug clotrimazole (Pascussi et al., 2001; Tompkins and Wallace, 2007). A study by Moore et al. (2000) showed that hyperforin, a chemical constituent of St. John's wort, was a potent ligand of the PXR, thus providing a mechanism by which St. John's wort induces CYP3A4, and demonstrating that the PXR can also be activated by components present in plants. Upon ligation, PXR translocates from the cytosol to the nucleus where it forms a heterodimer with the retinoid X receptor alpha (RXRα) (Komoroski et al., 2004). This complex then binds to

PXR response elements in the promoter region of the CYP3A4 gene, resulting in its activation and an increase in CYP3A4 expression (Pascussi et al., 2001; Tompkins and Wallace, 2007) *(Figure 2–14).*

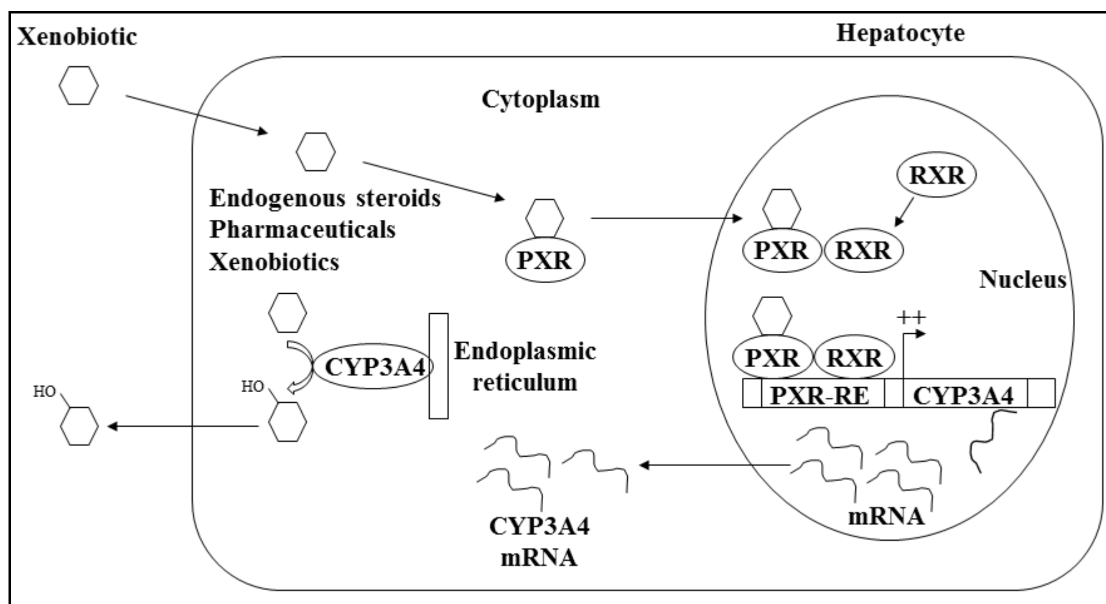


Figure 2–14. A schematic representation of xenobiotic activation of the PXR, causing CYP3A4 induction (adapted and redrawn from Tompkins and Wallace, 2007; obtained with permission from John Wiley and Sons).

2.6 Summary and Conclusion

Traditional medicines are extensively used in South Africa and around the world. *S. nervosa* is used traditionally to treat many ailments although there are no conventional pharmaceutical formulations available. Scientific evidence supports its anti-inflammatory and antimicrobial use. The bulbs contain several homoisoflavanones and stilbenoids which may be individually or in combination responsible for its anecdotal and observed therapeutic effects. Little information is available to elucidate its potential toxic effects. The liver performs a multitude of functions, but it is the primary site of metabolism of xenobiotics, thus assessing the toxicity of the extract in the liver is of particular importance. Apoptosis and oxidative stress are implicated in the pathology of multiple diseases, while xenobiotics capable of modulating CYP3A4 are associated with drug interactions, thus investigating how the extract influences apoptosis, oxidative stress and CYP3A4 enzymes is significant in determining how the extract exerts its toxic effects.

Chapter 3. Materials and Methods

This chapter provides a brief background into the various assays and describes the methodology and materials that were used in the preparation of the aqueous extract of *S. nervosa*, and the procedure and materials used to assess the cytotoxicity of *S. nervosa*, possible mechanisms of toxicity and its effect on CYP3A4 enzymes.

3.1 Preparation of Aqueous Extract

3.1.1 Introduction

S. nervosa is primarily used traditionally as an aqueous extract, either as bulb decoctions or cold water extracts (Abegaz, 2002; Bangani et al., 1999). An aqueous extract of *S. nervosa* was thus prepared to replicate the methods of the herbal practitioner and mimic the effects of its traditional use as close as possible.

3.1.2 Materials and Methods

Briefly, *S. nervosa* was purchased from the Fig Tree Indigenous Nursery (Westville, Durban, South Africa), identified, and a voucher (Du Toit2, NU) was lodged at the University of KwaZulu-Natal Herbarium (Pietermaritzburg, South Africa) (Du Toit et al., 2011). All the plants were of the same developmental stage and were harvested at the same time (June 2008). This was to minimise seasonal variation and changes in metabolite content. The fresh bulbs were chopped into small pieces and dried overnight. Pieces were soaked in methanol (3 days) to release the polar and non-polar components of the material. The extract thus obtained was filtered and dried in a rotary evaporator (Heidolph, Darmstadt, Germany). The dry material thus obtained was dried a second time in the rotary evaporator at the boiling point of methanol to evaporate all traces of methanol (65 °C; 30 min). Small amounts of the dry material that were obtained were dissolved in double distilled water through agitation (10-15 min). Solubility of the material in water was determined to be 10 mg/ml. No sedimentation was observed.

3.2 HepG2 Liver Cells

3.2.1 Introduction

These cells are an adherent, non-tumourigenic, epithelial cell line (ATCC number HB-8065) and were derived from the liver tissue of a 15-year-old Caucasian male with

hepatocellular carcinoma in 1979. There is no evidence of a Hepatitis B virus genome in this cell line.[6] The human HepG2 hepatocellular carcinoma cell line is considered a model cell line for the investigation of toxicity. It retains many of the specialised functions that are normally lost by primary hepatocytes in culture, such as the secretion of major plasma proteins (Mersch-Sundermann et al., 2004).

3.2.2 Maintenance

HepG2 cells were resuscitated from liquid nitrogen storage by incubation at 37 °C for a few minutes. The cells were then cultured to confluency in complete culture media (CCM) in 25 cm^3 tissue culture flasks at 5% CO_2 and 37 °C. CCM contained Dulbecco's modified Eagle's medium (DMEM) (Whitehead Scientific, Johannesburg, South Africa) supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) Penstrep-Fungizone® solution (Merck, Johannesburg, South Africa). CCM was replaced every two days. Once cells were confluent, they were sub-cultured via trypsinisation according to the type of assay.

3.3 Cell Viability Assay

3.3.1 Introduction

The cytotoxicity of *S. nervosa* in HepG2 cells was assessed using the methylthiazol tetrazolium (MTT) cellular viability assay (Mosmann, 1983). The method is based on the reduction of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), a yellow tetrazolium salt, to purple formazan crystals in viable cells. This colorimetric assay is extensively used as an indicator of cell viability and proliferation, since only metabolically active cells are capable of converting the MTT salt to formazan crystals (Bernhard et al., 2003).

Although the MTT assay is widely used in cell proliferation and cytotoxicity assays, the site of, and the biochemical events implicated in MTT reduction, remain unclear. It was long believed that MTT reduction occurred in the mitochondria of living cells due to interactions with the respiratory chain via the succinate-dehydrogenase system (Bernhard et al., 2003). However, studies have since elucidated that alternative mechanisms may also be involved in cellular MTT reduction. Berridge and Tan (1993) found that most cellular MTT reduction occurred outside the mitochondria and involved NADH- and NADPH-dependent

[6] Information provided by the American Type Culture Collection (ATCC; www.atcc.org).

mechanisms in a bone marrow-derived cell line, while a study by Dhanjal and Fry (1997) demonstrated that MTT reduction in rat hepatocytes was the greatest with NADH and the least with succinate. Bernas and Dobrucki (2002) also showed that only 25-45% of MTT-formazan was associated with mitochondria in HepG2 cells by imaging the formation of formazan deposits. In a study by Liu et al. (1997) it was observed that in a cell line derived from rat brain tumours, MTT was reduced in intracellular vesicles such as endosomes and lysosomes. It was also noted that MTT was absorbed by cells via endocytosis and formazan was transported to the cell surface via exocytosis. Berridge et al. (1996) also showed that microsomal enzymes in the endoplasmic reticulum required NADH and NADPH to reduce MTT.

3.3.2 Materials and Methods

Emetine dihydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA), MTT was purchased from Calbiochem (Johannesburg, South Africa). All other reagents were obtained from Merck.

The extract stock solution (10 mg/ml) was serially diluted in CCM within the range of 0-5 mg/ml.

Emetine, an alkaloid drug which has been reported to enhance cytotoxicity (Lee and Wurster, 1995) and induce apoptosis in tumour cells (Bicknell et al., 1994), was included as a positive control. A stock solution of 1 mg/ml was prepared in CCM. The stock solution was then serially diluted within the range of 0-300 µg/ml.

HepG2 cells were trypsinised and seeded into a 96-well microtitre plate (15 000 cells/well) and treated in duplicate or triplicate with the serial dilutions of the extract or emetine for 24 h. Cells incubated with CCM only were used as a negative control. Thereafter, cells were incubated with the MTT salt solution (5 mg/ml in phosphate buffered saline (PBS)) for a further 4 h. Following this incubation, the culture fluid was aspirated from each well, dimethyl sulphoxide (DMSO) (100 µl/well) was added and the cells were incubated for 1 h. The optical density was then measured at 570 nm with a reference wavelength of 690 nm in an ELISA plate reader (Bio-Tek µQuant). Results were expressed as percentage cell viability.

3.3.3 Data Analysis

The assay was performed twice to establish a concentration-response curve for the extract and for emetine. The concentration at which the extract or emetine decreased cell viability to 50% (IC_{50}) was determined from the non-linear, sigmoidal, least-square, variable slope fits by using GraphPad Prism v. 5 for Windows® (GraphPad Software, San Diego, CA, USA). Data was expressed as the mean ± standard error of the mean (SEM).

HepG2 cells were treated for 24 h in subsequent assays with the extract at the IC_{50} of cell viability. It was decided to perform these assays at the IC_{50} of cell viability since lower concentrations could result in cytotoxic effects being underestimated, while higher concentrations could result in experimental artefacts. An IC_{50} provides a workable midpoint.

3.4 ATP Quantification Assay

3.4.1 Introduction

Metabolic activity in HepG2 cells treated with the extract was investigated by quantifying the intracellular ATP concentration. ATP plays an essential role in the energy exchange required to drive cellular processes, and operates as the primary donor of free energy in all metabolically active cells (Crouch et al., 1993). All living cells require ATP to survive and function, thus ATP quantification assays are useful tools in determining cell viability and cytotoxicity.

The ATP quantification assay utilises bioluminescence to ascertain ATP levels in cells. The assay is based on the conversion of a luciferase-inactive derivative by ATP in the presence of Mg^{2+} to D-luciferin. D-luciferin, a luciferase substrate, then reacts with the enzyme luciferase to produce oxyluciferin and release energy in the form of luminescence. This luminescent signal is directly proportional to the concentration of ATP present in the cells (Crouch et al., 1993; Hannah et al., 2001).

3.4.2 Materials and Methods

The CellTiter-Glo® assay kit was obtained from Promega (Madison, WI, USA). All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

The CellTiter-Glo® reagent was prepared according to the manufacturer’s instructions prior to the assay.

HepG2 cells were treated in 25 cm^3 tissue culture flasks with CCM (negative control) or 0.03 mg/ml extract in CCM and incubated (5% CO_2; 37 °C; 24 h). Thereafter, cells were trypsinised and seeded into a white 96-well luminometer plate in duplicate (20 000 cells/well), followed by the addition of 10 µl of the CellTiter-Glo® reagent. The plate was agitated briefly to induce cell lysis and incubated in the dark (RT; 10 minutes (min)). Following incubation, the luminescent signal was measured on a microplate luminometer (Turner Biosystems, USA).

3.4.3 Data Analysis

Data was expressed as a change in relative light units (RLU) compared to the control and expressed as the mean ± SEM. Statistical analysis was performed using the student two-tailed unpaired *t* test in GraphPad Prism. A probability value (p) of less than 0.05 ($p < 0.05$) was considered statistically significant.

3.5 Apoptotic Potential Assays

3.5.1 Annexin-V Affinity Assay

3.5.1.1 Introduction

Phosphatidylserine externalisation in HepG2 cells was quantitatively detected using the annexin-V Affinity assay and fluorescence activated cell sorting (FACS). The distribution of phospholipids in living cells is asymmetric, with the inner membrane leaflet containing anionic phospholipids (including PS) and the outer membrane leaflet containing mainly neutral phospholipids (Vermes et al., 1995). Apoptotic cell death is characterised by several membrane changes, including the loss of asymmetry in cell membrane phospholipids by exposure of PS at the cell surface, which then serves as a signal for phagocytic recognition. The integrity of the cell membrane however, remains unaffected. The externalisation of PS is thus an important indicator of apoptosis (Van Engeland et al., 1998).

Annexin-V, a calcium dependent phospholipid binding protein has a high affinity for PS and provides a useful tool for measuring PS exposure (Vermes et al., 1995). As annexin-V is unable to traverse the undamaged lipid bilayer membranes of viable cells, fluorescein isothiocyanate-labelled (FITC) annexin-V molecules allows the detection of apoptotic cells by binding to PS that is available on their outer membrane leaflet (Buehrlen et al., 2007) *(Figure 3–1)*.

Annexin-V, however, can also attach to PS in the inner membrane of necrotic cells due to the loss of membrane integrity in these cells. To differentiate necrotic cells from apoptotic cells, cells can be additionally stained with DNA-binding dyes such as propidium iodide (PI), which are excluded by cells with intact membranes but can pass through necrotic cells (Darzynkiewicz et al., 1997). This double staining method permits the detection of viable, necrotic and apoptotic cells which can be differentiated by their particular staining patterns (Buehrlen et al., 2007).

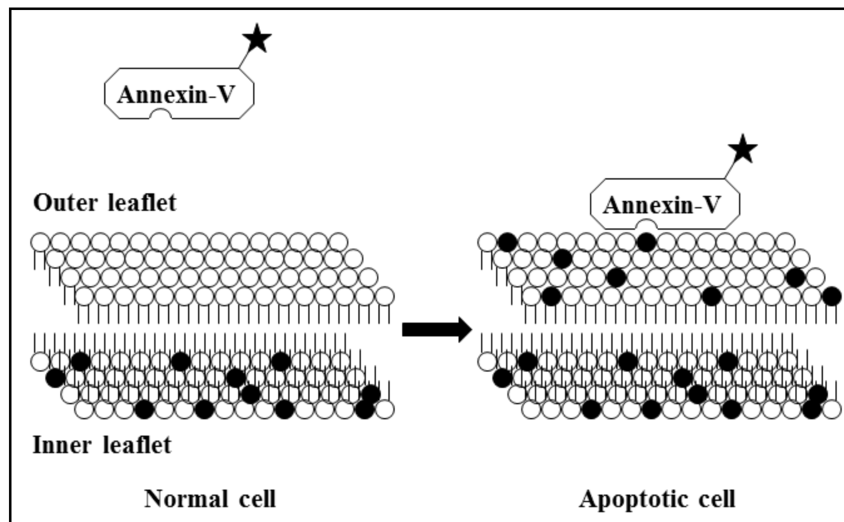


Figure 3–1. Loss of membrane asymmetry during apoptosis. PS molecules (solid black circles) become exposed at the outer membrane leaflet during apoptosis, permitting the binding of FITC-labelled annexin-V (adapted and redrawn from Van Engeland et al., 1998; obtained with permission from John Wiley and Sons).

3.5.1.2 Materials and Methods

The annexin-V-FITC staining kit was obtained from Roche Diagnostics (Penzberg, Germany). All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

The annexin-V-FLUOS labelling solution was prepared prior to the assay by diluting 20 µl of annexin-V-FLUOS labelling reagent in 1 ml of incubation buffer, followed by the addition of 20 µl of PI solution.

HepG2 cells were treated in 25 cm^3 tissue culture flasks with CCM (negative control) or 0.03 mg/ml extract in CCM and incubated (5% CO_2; 37 °C; 24 h). PS externalisation was detected following the incubation using the annexin-V-FLUOS staining kit according to the manufacturer's instructions. Briefly, cells were trypsinised and transferred to polystyrene cytometry tubes (500 000 cells/tube), stained with 100 µl of annexin-V-FLUOS labelling solution and incubated in the dark (RT; 15 min). Flow cytometry analysis was carried out

using a FACS Calibur flow cytometer (BD Biosciences, Johannesburg, South Africa) with CellQuest PRO v. 4.0.2 software (BD Biosciences) at an excitation wavelength of 488 nm.

3.5.1.3 Data Analysis

Data was collected from 2 500 events per sample in duplicate and analysed using FlowJO v. 7.1 software (Tree Star, Inc., USA). Cells were gated on the FL-1 and FL-3 channels to differentiate between apoptotic and necrotic cells. Apoptotic cells were annexin-V-FITC positive and PI negative, while necrotic cells were annexin-V-FITC positive and PI positive. Data was expressed as the mean ± SEM. Statistical analysis was performed using the student two-tailed unpaired t test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.5.2 Caspase-8 and -9 Assays

3.5.2.1 Introduction

Caspase-8 and -9 activities in HepG2 cells treated with the *S. nervosa* extract were investigated using the Caspase-Glo® 8 and Caspase-Glo® 9 assays respectively. Caspases play a fundamental role in the initiation and execution of apoptosis. Caspases are expressed in almost all cell types as inactive precursors (zymogens) with an N-terminal prodomain of variable length (Köhler et al., 2002). Caspase-8 and -9 are classified as initiator caspases and have large prodomains. These, and other initiator caspases are activated by apoptotic stimuli and, in turn, cleave and activate downstream executioner caspases (Lamkanfi and Kanneganti, 2010).

Luminometric evaluation of caspase activity is one method available of quantifying the activation of caspases and thus induction of apoptosis. This assay involves the addition of a luminogenic substrate (caspase-8 cleavage site, Z-IETD; or caspase-9 cleavage site, Z-LEHD), which is attached to aminoluciferin, and the enzyme luciferase to a cell sample. Following cleavage of the substrate by the respective caspases, aminoluciferin, a luciferase substrate, is released and reacts with luciferase in the presence of ATP and O_2 to generate a luminescent signal. This signal is directly proportional to the level of caspase activity present in the cell sample (Brunelle and Zhang, 2010).

3.5.2.2 Materials and Methods

The Caspase-Glo® 8 and Caspase-Glo® 9 assay kits were obtained from Promega. All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

Caspase-Glo® 8 and Caspase-Glo® 9 reagents were prepared according to the manufacturer's instructions.

HepG2 cells were treated in 25 cm^3 tissue culture flasks with CCM (negative control) or 0.03 mg/ml extract in CCM and incubated (5% CO_2; 37 °C; 24 h). Thereafter, cells were trypsinised and seeded into a white 96-well luminometer plate in duplicate (20 000 cells/well) for both caspase assays, followed by the addition of 10 µl of either the Caspase-Glo® 8 or Caspase-Glo® 9 reagent. The plate was agitated (300-500 rpm; 30 seconds) and incubated in the dark (RT; 30 min). Following incubation, the luminescence was measured on a microplate luminometer (Turner Biosystems, USA). Results were expressed as RLU.

3.5.2.3 Data Analysis

Data was represented as a change in RLU compared to the control and expressed as the mean ± SEM. Statistical analysis was performed using the student two-tailed unpaired *t* test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.5.3 Mitochondrial Membrane Potential Assay

3.5.3.1 Introduction

The change in ΔΨm of HepG2 cells was assessed using FACS and the JC-1 Mitoscreen assay (BD Biosciences). ΔΨm, which is essential for mitochondrial functioning, occurs due to the asymmetric distribution of protons and other ions on both sides of the inner mitochondrial membrane. The inner side of the inner mitochondrial membrane is thus negatively charged (Kroemer et al., 1997). Cationic lipophilic fluorochrome dyes, such as 5,5′,6,6′-tetrachloro-l,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) are frequently used to assess changes in ΔΨm. Mitochondria with low levels of depolarisation possess a negatively charged membrane potential which allows accumulation of the positively charged JC-1 dye within the mitochondrial matrix (Smiley et al., 1991). When high concentrations of JC-1 dye are reached, in the presence of a high ΔΨm (normal cells), j-aggregates are formed in the mitochondria. These aggregates are associated with a large shift in its emission

spectra, upon excitation at 490 nm, resulting in mitochondria fluorescing red at 597 nm (Legrand et al., 2001; Salvioli et al., 1997) *(Figure 3–2)*.

Mitochondrial dysfunction was thought to occur early in apoptosis. The disruption of the asymmetric ion distribution across the inner mitochondrial membrane during apoptosis causes a decrease in ΔΨm (Kroemer et al., 1997). As a result, the JC-1 dye is unable to accumulate within the mitochondrial matrix and remains in the cytoplasm (apoptotic cells). When excited at 490 nm, the JC-1 dye fluoresces green in the cytoplasm at 537 nm (Legrand et al., 2001) *(Figure 3–2)*.

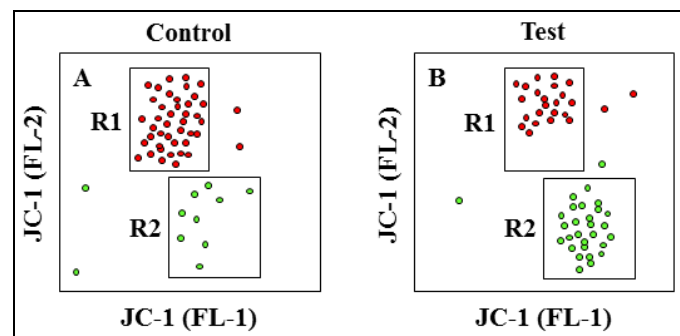


Figure 3–2. Scatter plots of JC-1 staining in **(A)** control and **(B)** test cells. Apoptosis was induced in the test cells. JC-1 fluorescence is seen in both the FL-2 and FL-1 channels (region R1) in the control. Only a small percentage of the population shows decreased fluorescence in the FL-2 channel (R2). In the test, there is a significant increase in the number of cells with lowered red fluorescence in the FL-2 channel (R2), indicative of a change in the ΔΨm and suggesting apoptosis. JC-1 that fluoresces in both the FL-2 and FL-1 channels (red fluorescence) is considered to correspond to mitochondria with polarised ΔΨm, while fluorescence in the FL-1 channel and decreased fluorescence in the FL-2 channel (green fluorescence) is considered to correspond to mitochondria with a depolarised ΔΨm (information obtained from BD Biosciences).

The JC-1 assay provides a qualitative and quantitative method for assessing ΔΨm in intact cells, and can be used for investigating the activities of mitochondria in apoptosis and a range of biological conditions (Salvioli et al., 1997).

3.5.3.2 Materials and Methods

The JC-1 dye was obtained from BD Biosciences. All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

HepG2 cells were treated in 25 cm^3 tissue culture flasks with CCM (negative control) or 0.03 mg/ml extract in CCM and incubated (5% CO_2; 37 °C; 24 h). A change in ΔΨm was detected subsequent to the incubation using the JC-1 Mitoscreen assay according to the manufacturer's instructions. Briefly, cells were trypsinised and transferred to polystyrene cytometry tubes (500 000 cells/tube) and incubated with 150 µl of the JC-1 dye (5% CO_2; 37 °C; 10 min). Cells were then washed in JC-1 wash buffer, centrifuged (400× *g*; 5 min) and

resuspended in 300 μl of flow cytometry sheath fluid. Flow cytometry analysis was carried out using a FACS Calibur flow cytometer (BD Biosciences) with CellQuest PRO v. 4.0.2 software (BD Biosciences) at an excitation wavelength of 488 nm. Results were expressed as a percentage of the depolarised mitochondria contained in the cells.

3.5.3.3 Data Analysis

Data was collected from 2500 events per sample in duplicate and analysed using FlowJO v. 7.1 software (Tree Star, Inc., USA) by scatter plots. Cells were gated to exclude debris in the FL-1 and FL-2 channels where JC-1 monomers and aggregates are fluorescent. FL-1 and FL-2 bright were indicative of healthy, non-apoptotic cells while FL-1 bright and FL-2 dull indicated apoptotic cells with depolarised mitochondrial membranes. Data was expressed as the mean ± SEM. Statistical analysis was performed using the student two-tailed unpaired *t* test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.6 Lipid Peroxidation Assay

3.6.1 Introduction

Oxidative stress caused by *S. nervosa* was assessed by measuring the extent of lipid peroxidation in HepG2 cells using the thiobarbituric acid assay. Lipid peroxidation reactions are generally mediated by free radical chain reactions (Abuja and Albertini, 2001). PUFAs are particularly susceptible substrates for such reactions. Free radicals react with PUFAs to generate a second radical which is then free to react with other PUFAs, thus propagating a chain reaction (Kannan and Jain, 2000). Following peroxidation, unstable lipid peroxides formed as a result of the reactions are degraded into intermediate or end products. Malondialdehyde (MDA) is one of several end products formed by the decomposition of lipid peroxidation products (Meagher and FitzGerald, 2000).

The thiobarbituric acid assay provides a quantitative method for measuring the concentration of MDA and is based on the reaction of MDA with thiobarbituric acid (TBA)-butylated hydroxytoluene. MDA readily reacts with thiobarbituric acid to produce a red, fluorescent 1:2 MDA:TBA compound (Janero, 1990).

3.6.2 Materials and Methods

All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

HepG2 cells were trypsinised and seeded into a 6-well tissue culture plate (150 000 cells/well) and treated in duplicate with CCM (negative control) or 0.03 mg/ml extract (5% CO_2; 37 °C; 24 h). Following the incubation, 200 µl of culture fluid from the wells was dispensed into glass tubes. Then, 200 µl of 2% (v/v) H_3PO_4, 400 µl of 7% (v/v) H_3PO_4 and 400 µl of 1% (w/v) thiobarbituric acid + 0.1 mM butylated hydroxytoluene solution was also added to the tubes. A positive control of 1% MDA was prepared, as well as a blank negative control containing 400 µl of 3 mM HCl in place of the thiobarbituric acid + butylated hydroxytoluene solution. The solutions were adjusted to pH 1.5 and heated in a water bath (100 °C; 15 min). Solutions were then cooled to RT and 1.5 ml of butanol was added to each tube. The samples from each condition were allowed to settle after vortexing, and 500 µl of the butanol phase from each tube was added to Eppendorf tubes and centrifuged (24 °C; 13 200 rpm; 6 min). Subsequent to the centrifugation, 100 µl of the sample and controls was transferred to a 96-well microtitre plate in duplicate. The optical density was measured at 532 nm, with a reference wavelength of 600 nm using an ELISA plate reader (Bio-Tek µQuant).

3.6.3 Data Analysis

The assay was performed twice. Results were expressed as the mean ± SEM of eight replicates divided by the absorption coefficient, 156 mM^{-1}. Statistical analysis was performed using the student two-tailed unpaired *t* test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.7 Single Cell Gel Electrophoresis (Comet) Assay

3.7.1 Introduction

The potential genotoxic effects of *S. nervosa* on HepG2 cells was investigated using the alkaline comet assay (Singh et al., 1988), with minor modifications. The comet assay is a simple, rapid and sensitive technique for measuring DNA damage and repair in mammalian cells (Liao et al., 2009), and is based on the ability of DNA fragments to move out of the cell during electrophoresis (Yusuf et al., 2000). The earliest version of the comet assay, where electrophoresis was performed under neutral conditions, could only detect double-strand breaks in DNA. The protocol was subsequently modified by Singh et al. (1988), to include unwinding of DNA under highly alkaline conditions (pH > 13), which allowed for the detection of double-strand breaks as well as single-strand breaks and alkali-labile sites in DNA (Liao et al., 2009).

In the alkaline comet assay, a small number of cells are embedded in an agarose gel on a microscope slide. Three distinct layers of agarose are used, with the cells contained in the middle layer. Cells are lysed in lysis buffer to liberate DNA, and prior to electrophoresis, cells are incubated in alkaline electrophoresis buffer to facilitate DNA unwinding to produce single-stranded DNA. Thereafter, DNA is subjected to electrophoresis which causes the migration of negatively charged DNA fragments towards the positive pole of the electrophoresis apparatus (Liao et al., 2009; Verschaeve and Van Staden, 2008).

The results are based on the hypothesis that DNA damage caused by a genotoxic agent produces DNA fragmentation, either through DNA breaks, by excision-repair of damaged DNA, or the formation of alkali-labile sites. The broken DNA strands released during cell lysis and DNA unwinding produce the comet tail during electrophoresis, while unharmed DNA does not migrate and remains intact in the comet head (Choucroun et al., 2001). DNA can then be visualised by fluorescence microscopy after staining with a fluorescent DNA-binding dye such as ethidium bromide (Liao et al., 2009).

3.7.2 Materials and Methods

Cyclophosphamide was obtained from Sigma-Aldrich (St. Louis, MO, USA). All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

HepG2 cells were trypsinised and seeded into a 6-well tissue culture plate (150 000 cells/well) and treated in duplicate with CCM (negative control) or 0.03 mg/ml extract (5% CO_2; 37 °C; 24 h). Cyclophosphamide, a known promutagen and DNA alkylating agent (Yusuf et al., 2000), was used as a positive control at a final concentration of 10 µM (equals to 0.003 mg/ml). Thereafter, the culture fluid of the wells was aspirated. The cells were then washed 3 times in PBS, trypsinised and transferred to 15 ml Sterilin tubes. The tubes were centrifuged (24 °C; 400× *g*; 10 min) and the supernatants aspirated. Cells were then resuspended in 100 µl PBS.

Duplicate slides of cells were prepared. Partially frosted microscope slides were covered with 400 µl of 1% (w/v) molten low melting point (LMP) agarose (0.5 g LMP agarose dissolved in 50 ml PBS) toward their frosted ends. The agarose was allowed to solidify under a coverslip at 4 °C for 10 min. Then 25 µl (20 000 cells) of treated cell suspensions and controls in PBS was mixed with 175 µl of 0.5% (w/v) molten LMP agarose (0.5 g LMP agarose dissolved in 100 ml PBS). After removing the coverslips, 200 µl of the respective

cell suspensions was transferred onto the first layer of LMP agarose, the coverslips were replaced and the agarose was allowed to solidify at 4 °C for 10 min. The coverslips were then removed and a third layer of 0.5% (w/v) molten LMP agarose was pipetted onto the second solidified layer. The final layer was allowed to solidify under a coverslip at 4 °C for 10 min. After removal of the coverslips, the slides were submerged in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 1% (v/v) Triton X-100, 10 mM Tris, pH 10, 10% (v/v) DMSO) for 1 h at 4 °C. Following the incubation, the slides were placed in an electrophoresis tank filled with electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) and allowed to equilibrate for 20 min. After the incubation, a current (300 mA; 25V) was applied to the tank for 35 min. Subsequent to the electrophoresis, slides were rinsed 3 times with Tris buffer (0.4 M Tris, pH 7.4) for 15 min to neutralise the alkali. The slides were then stained with 50 µl of the fluorochrome, ethidium bromide (20 µg/ml) and stored overnight at 4 °C. Slides were then viewed, and digital images recorded with an Olympus IX5I fluorescent microscope (Olympus America Inc., PA, USA).

3.7.3 Data Analysis

The assay was performed twice. Image analysis was performed using the Comet Assay IV® software (Perspective Instruments Ltd., Suffolk, UK). Randomly selected comets per slide (40 comets) were scored and 2 slides were used per condition. The evaluation of DNA damage was based on four parameters, namely (1) the comet tail length (TL) (distance over which the DNA has migrated), (2) Olive tail moment (OTM) ((tail mean-head mean) × tail %DNA/100), (3) tail extent moment (TEM) (tail length × tail %DNA/100), and (4) tail length (TL)/head length (HL) (%). Data was expressed as the mean ± SEM. Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) with Bonferroni's Multiple Comparison Test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.8 Cytochrome P450 3A4 Assay

3.8.1 Introduction

The effect of *S. nervosa* on CYP3A4 activity in HepG2 cells was investigated using the P450-Glo™ assay. CYP3A4 is a member of the CYP superfamily of haemoproteins. This subfamily of CYP plays a vital role in the oxidative metabolism of xenobiotics and endogenous compounds in the liver and intestine (Pascussi et al., 2001). Investigations have shown that approximately 60% of pharmaceutical drugs are oxidised by CYP3A4, thus

altered expression and activity of these enzymes by other drugs would have significant effects on the therapeutic outcome of drugs metabolised by them (Raucy, 2003). Consequently, adverse drug interactions frequently occur due to inhibition or induction of CYP3A4. Assessing the effect of medicinal formulations on CYP3A4 thus provides an important tool for predicting drug interactions (Jarukamjorn et al., 2006).

The P450-Glo™ 3A4 assay provides a luminescent method for quantifying CYP3A4 activity. The assay involves the conversion of a luminogenic CYP3A4 substrate by CYP3A4 to luciferin. Each substrate (luciferin-BE, luciferin-PFBE and luciferin-PPXE), a luciferase-inactive D-luciferin derivative, is modified to contain a cleavable group. Following cleavage of the substrate by CYP3A4, D-luciferin, a luciferase substrate, is released and reacts with luciferase to generate a luminescent signal. This luminescent signal is directly proportional to the level of CYP3A4 activity (Cali et al., 2006).

3.8.2 Materials and Methods

The P450-Glo™ assay kit was obtained from Promega. Dexamethasone was obtained from Sigma-Aldrich (St. Louis, MO, USA). All tissue culture reagents were obtained from Whitehead Scientific. All other reagents were obtained from Merck.

Dexamethasone, a known CYP3A4 inducer (Pascussi et al., 2001), was used as a positive control.

The reconstituted luciferin detection reagent and luciferin-PFBE substrate (2 mM stock solution) were prepared prior to the assay according to the manufacturer's instructions. HepG2 cells were trypsinised and seeded into a white 96-well luminometer plate (20 000 cells/well) in triplicate and allowed to attach overnight. Cells were then treated with 0.03 mg/ml of the extract or 50 µM dexamethasone in CCM and incubated (5% CO_2; 37 °C; 24 h). The negative control received CCM only. Following the incubation, the CCM was aspirated and 50 µl of luciferin-PFBE substrate in CCM was added to each well such that a final concentration of 50 µM of substrate was obtained in the wells. Cells were then incubated (5% CO_2; 37 °C; 4 h). Thereafter, 50 µl of luciferin detection reagent was added and allowed to react by briefly agitating the plate. The plate was allowed to equilibrate in the dark (RT; 20 min). Luminescence was measured on a microplate luminometer (Turner Biosystems, USA). Results were expressed as RLU.

3.8.3 Data Analysis

Data was represented as a change in RLU compared to the negative control and expressed as the mean ± SEM. Statistical analysis was performed using ANOVA with Bonferroni's Multiple Comparison Test in GraphPad Prism. A value of $p < 0.05$ was considered statistically significant.

3.9 Summary and Conclusion

The various assays used to investigate the toxicological properties of an aqueous extract of *S. nervosa* in a cultured human HepG2 hepatocellular carcinoma cell line were described. Each assay was described in terms of its background and rationale, the materials and methods that were used, as well as the preparation of the data and statistical analysis. These assays were appropriate in an attempt to answer the scientific questions that were asked in this study.

Chapter 4. Results and Discussion

The toxicological properties of *S. nervosa* in HepG2 cells were investigated in a series of experimental assays. Cell viability was determined and potential mechanisms of cytotoxicity, genotoxicity and CYP3A4 activity were explored. The results are presented and discussed here.

4.1 Cell Viability

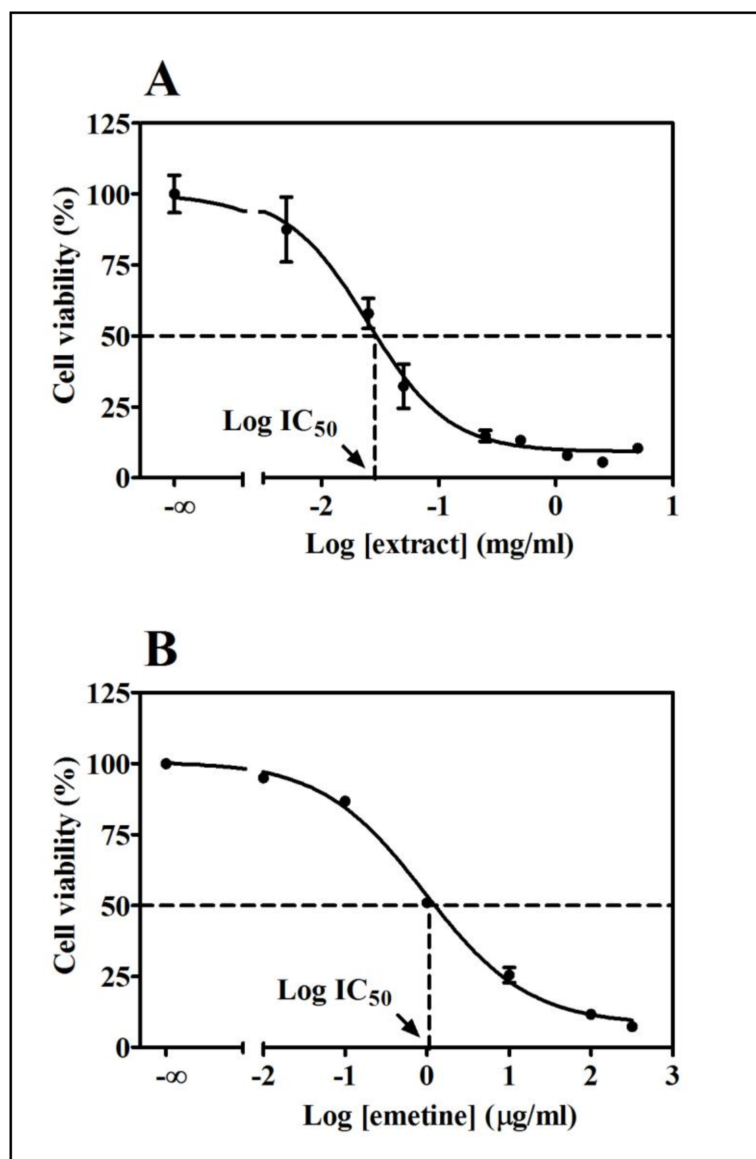


Figure 4–1. Concentration-response curves of % cell viability against varying concentrations of **(A)** *S. nervosa* extract (mg/ml) and **(B)** positive control emetine (µg/ml) in HepG2 cells after 24 h treatment. Arrows indicate the log IC_{50}-values respectively from which the IC_{50}-values were calculated.

The viability of HepG2 cells in the presence of the extract *(Figure 4–1A)* or emetine (positive control) *(Figure 4–1B)* was reduced in a concentration-dependent fashion after 24 h

treatment and the IC_{50}-values were calculated as 0.03 ± 0.005 mg/ml and 1.0 ± 0.026 μg/ml respectively.

At the lowest concentration the extract appeared to conserve metabolic activity, demonstrated by the increase in the conversion of the MTT salt to purple formazan crystals. The reduction of MTT may have been due to an increase in the activity of the succinate-dehydrogenase system in the mitochondria (Berridge and Tan, 1993), but is most likely as a result of NADH- and NADPH-dependent mechanisms (Berridge et al., 1996), suggesting that the production of these reducing equivalents may have been increased. The significantly reduced cell viability at higher concentrations, as evidenced by a decrease in MTT reduction, demonstrates an increase in the cytotoxicity of the extract.

A similar trend was observed when the cells were treated with emetine. This was not surprising since emetine has been reported to enhance cytotoxicity (Lee and Wurster, 1995), and induce apoptosis in tumour cells (Bicknell et al., 1994). However, to the best of knowledge, the effects of emetine on the viability of HepG2 cells have not been reported previously. The 30-fold difference in the IC_{50}-values between the extract and emetine is noteworthy, suggesting that the extract was significantly less toxic to the cells than emetine.

4.2 Cytotoxicity

4.2.1 Metabolic Activity

The intracellular ATP concentration increased significantly ($p < 0.001$) 1.2-fold from 5 837 691 ± 14 219 RLU (negative control) when compared to 6 945 318 ± 5 592 RLU (extract treated cells).

Apoptosis is an energy-driven and ATP-dependent process. It has been demonstrated that ATP is essential for the execution of the final phase of apoptosis, which is characterized by morphological changes in the nucleus. During Fas-mediated apoptosis, the active transport of large molecules across the nuclear membrane is required for the transmission of apoptotic death signals from the cytoplasm to the nucleus (Bradbury et al., 2000; Tsujimoto, 1997).

Given that apoptosis is reliant on ATP for its execution, ATP levels should most likely have decreased in the HepG2 cells, however, slightly higher ATP levels were observed instead. This occurrence suggests that *S. nervosa* may possess hypoglycaemic properties. Resveratrol, a chemical constituent previously isolated from *S. nervosa* has been shown to have anti-hyperglycaemic potential in obese and diabetic rats (Palsamy and Subramanian,

2008, 2009; Sharma et al., 2011). The anti-hyperglycaemic effect is thought to be due to its ability to increase cellular glucose uptake and intracellular glucose transport (Szkudelski and Szkudelska, 2011). This increase in glucose uptake would result in an increase in glycolysis and the Krebs cycle, which is responsible for the production of the reducing equivalents, NADH and $FADH_2$. Electrons from NADH and $FADH_2$ enter the mitochondrial ETC and facilitate ATP production via oxidative phosphorylation. Thus an increase in the concentration of these reducing equivalents would also increase the amount of electrons entering the ETC and subsequent ATP production. It could also be that amino acid and fatty acid metabolism increased pyruvate production which enters the Krebs cycle to increase ATP production (Campbell, 2011). These actions may account for the higher intracellular ATP concentration observed in cells treated with the extract. To the best of knowledge, no information was found on the effects of homoisoflavanones and resveratrol on the intracellular ATP concentration *in vitro*. This hypothesis should also be investigated further *in vivo* in an animal study where blood glucose levels are monitored before and after administration of the extract. It could be that these compounds enhance glucose uptake via increased insulin secretion but this needs to be investigated further.

4.2.2 Apoptosis

4.2.2.1 PS Externalisation

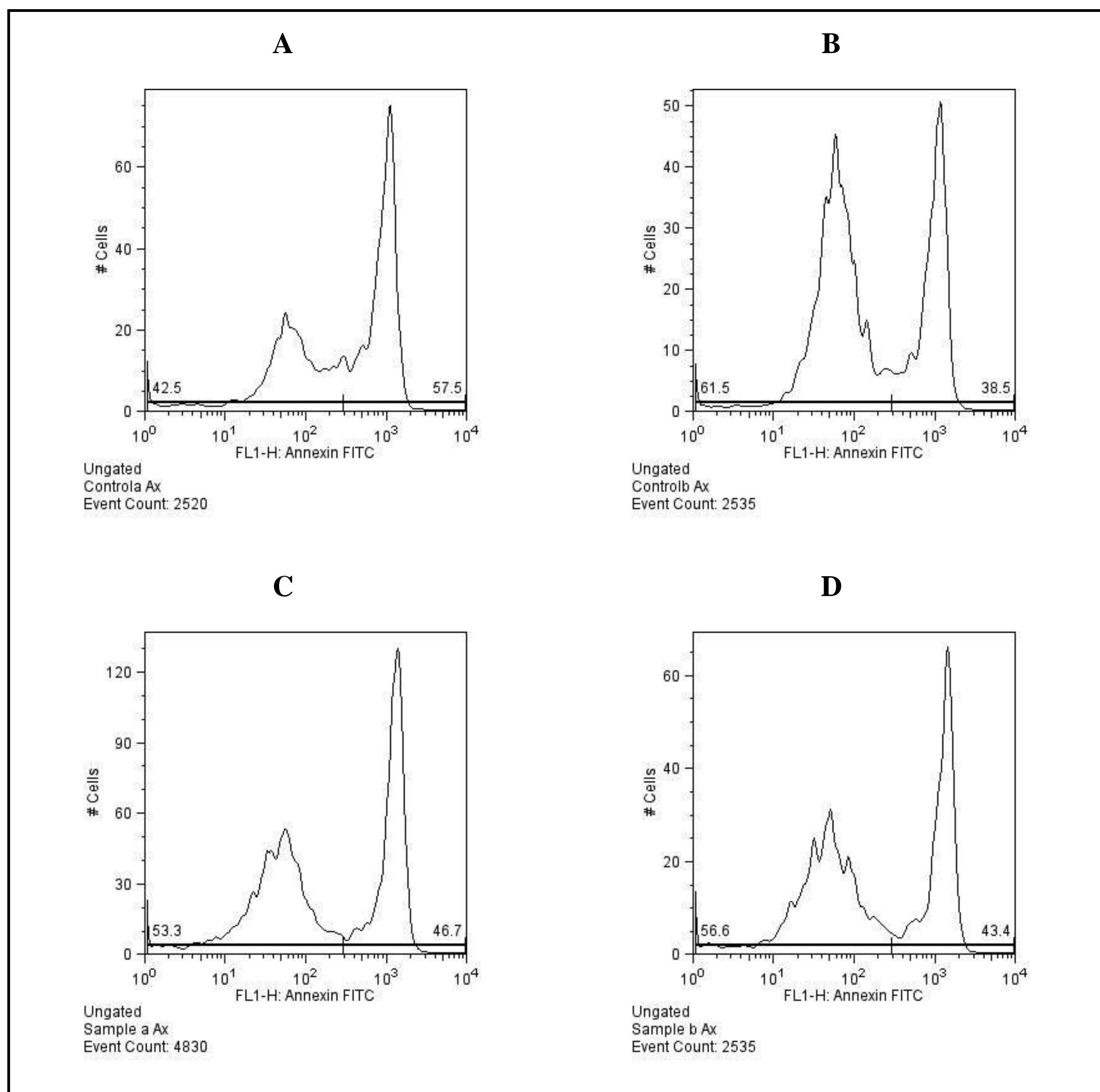


Figure 4–2. The annexin-V FITC flow cytometry histograms (number of cells as a function of fluorescence) obtained after treating HepG2 cells with the **(A & B)** negative control and **(C & D)** the extract for 24 h.

The results generated from the histograms *(Figure 4–2)* were expressed as a percentage of PS binding to annexin-V which was used as an indicator of the percentage PS externalisation. PS externalisation was not significantly different ($p > 0.05$) from 48.0 ± 9.5% (negative control) when compared to 45.1 ± 1.7% (extract treated cells).

Exposure of the aminophospholipid PS occurs early in apoptosis. PS, which is normally localised on the inner leaflet of the plasma membrane becomes externalised on the outer leaflet where it enables phagocytic recognition of the apoptotic cells by macrophages. The

exposure of PS on the cell surface thus serves as a marker of apoptosis (Pelassy et al., 2000). Although the percentage PS externalisation between the control and extract treated cells was not significantly different, levels were relatively high in both conditions. This may suggest that the control cells as well as the extract treated cells were undergoing apoptosis. However, HepG2 cells are a transformed cell line which proliferates at a much faster rate than primary hepatocytes. This may explain the generally high PS externalisation that was observed.

4.2.2.2 Caspase-8 and -9 Activity

Table 4–1. Caspase-8 and -9 activities that were measured in HepG2 cells after treatment with the negative control and *S. nervosa* extract.

Caspase	Negative control (RLU)	Extract (RLU)
8	16 129 ± 68	21 425 ± 39 (↑ 1.3-fold; $p < 0.001$)
9	492 443 ± 2 522	440 190 ± 1 545 (↓ 1.1-fold; $p < 0.01$)

Caspase-8 is the primary initiator caspase of the extrinsic apoptotic pathway or death receptor pathway (Hengartner, 2000). This pathway is initiated by the binding of extracellular ligands to cell surface death receptors that belong to the TNF-R gene superfamily. Procaspase-8 is activated at the DISC subsequent to ligation of death receptors and recruitment of adaptor proteins. Activated caspase-8 then elicits downstream apoptotic events by cleaving and activating the downstream effector caspases-3, -6, and -7; and can also cleave and activate Bid to tBid (Brunelle and Zhang, 2010; Fulda and Debatin, 2006). The significant increase in caspase-8 activation that was observed in the extract treated cells suggests that apoptosis may be modulated via the extrinsic apoptotic pathway *(Table 4–1)*.

Caspase-9 is the key initiator caspase of the intrinsic apoptotic pathway or mitochondrial pathway (Brunelle and Zhang, 2010). Following the release of cytochrome c from the mitochondria into the cytosol, it triggers the oligomerisation and aggregation of several APAF-1 units. Activation of caspase-9 occurs as a result of its association with APAF-1 at the apoptosome, a multi-protein complex formed consisting of cytochrome c, APAF-1, ATP and caspase-9 (Fulda and Debatin, 2006; Ghavami et al., 2009). Activated caspase-9 then directly activates the downstream effector caspases-3, -6 and -7 (Buehrlen et al., 2007). The slightly lower caspase-9 activity observed in the extract treated cells suggests that the intrinsic apoptotic pathway was not affected or induced *(Table 4–1)*.

4.2.2.3 Mitochondrial Membrane Potential

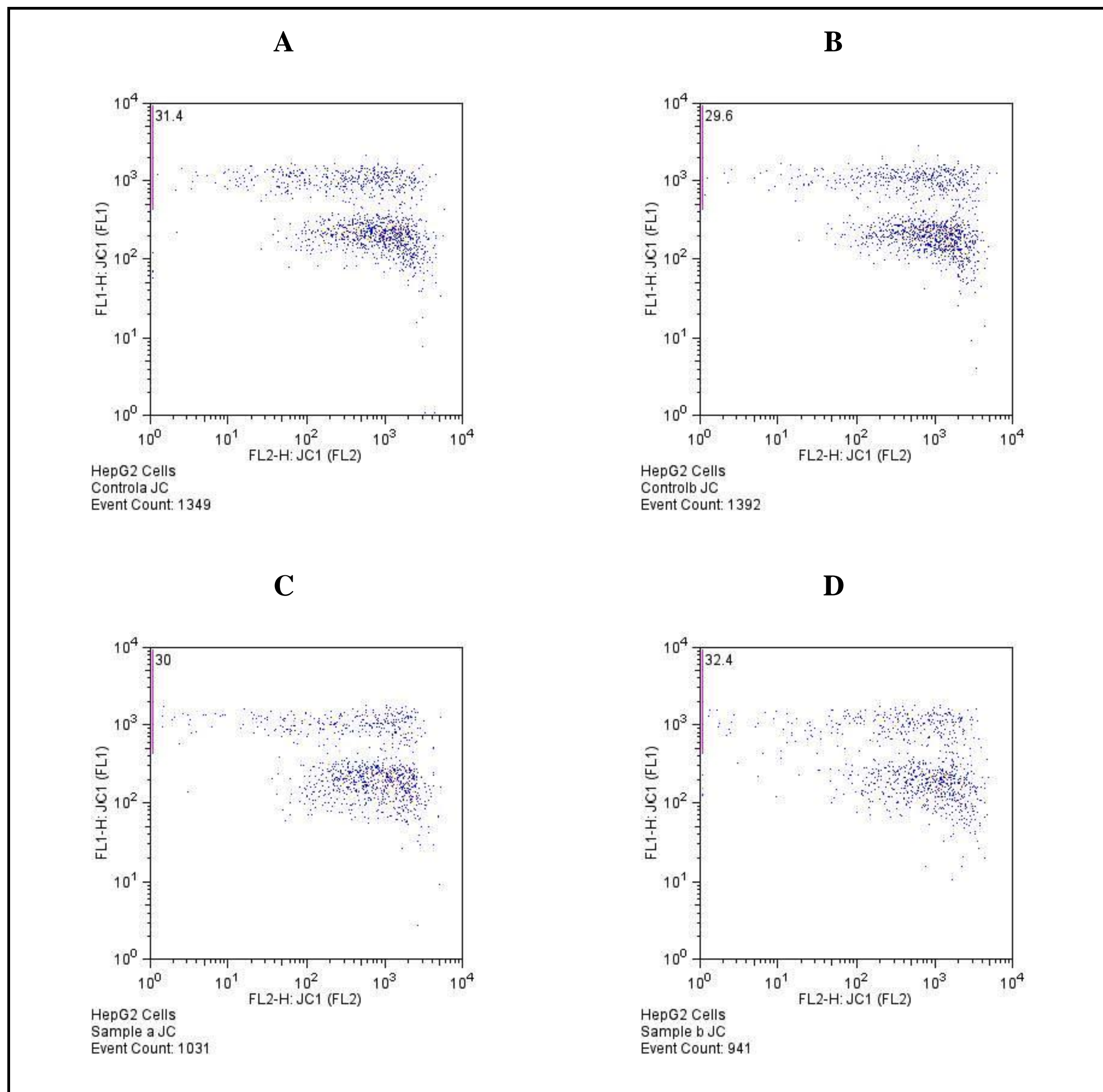


Figure 4–3. The JC-1 flow cytometry scatter plot diagrams obtained after treating HepG2 cells with the **(A & B)** negative control and **(C & D)** the extract for 24 h. For an interpretation of the results, refer to *Figure 3–2*.

The results generated from the scatter plot diagrams *(Figure 4–3)* were expressed as a percentage of depolarised mitochondrial membranes. Depolarised mitochondrial membranes were not significantly different ($p > 0.05$) from 30.5 ± 0.9% (negative control) when compared to 31.2 ± 1.2% (extract treated cells).

Changes in ΔΨm were originally thought to be an early, essential event in apoptosis with many studies demonstrating that changes in ΔΨm precede the nuclear changes associated with apoptosis. There is now, however, accumulating data that suggests that (1) the loss of ΔΨm can be a late event in apoptosis, (2) there is no early requirement for the loss of ΔΨm,

and that (3) the loss of $\Delta\Psi m$ in apoptosis may be a specific feature of the cell type or apoptotic inducer (Ly et al., 2003). It has also been suggested that the release of cytochrome c, and not a loss of $\Delta\Psi m$, is central to apoptosis since $\Delta\Psi m$ has been described as increasing, decreasing, or remaining unchanged during cytochrome c release (Finucane et al., 1999; Scarlett et al., 2000).

Although there was no significant change in mitochondrial membrane depolarisation in extract treated cells, this may not be the most suitable marker for apoptosis. The fact that the extract did not significantly change the mitochondrial membrane depolarisation in HepG2 cells also correlates with the slight increase in ATP production *(see §4.2.1)* since the $\Delta\Psi m$ is the driving force in mitochondrial ATP synthesis. A decrease in $\Delta\Psi m$ (increased depolarisation) would have resulted in less ATP synthesis, which was not observed.

4.2.3 Oxidative Damage

The concentration of the lipid peroxidation product MDA was measured to assess the extent of lipid peroxidation and thus oxidative stress. The MDA concentration increased significantly ($p < 0.01$) 2-fold from 0.2 ± 0.03 μM (negative control) when compared to 0.4 ± 0.03 μM (extract treated cells).

Lipid peroxidation occurs as a result of free radicals, most likely generated from the mitochondrial ETC, attacking PUFAs and causing damage in cellular membranes. The highly reactive OH· reacts with the single electrons contained in the hydrogen atoms of PUFAs and removal of this electron leaves behind an unpaired electron on the carbon atom to which it was attached. The resulting carbon-centred lipid radical then undergoes molecular rearrangement prior to becoming oxidised and forming lipid peroxides. Lipid peroxidation yields a variety of complex products such as hydroperoxides and aldehydes. MDA is one such aldehyde formed as a result of lipid peroxidation (Halliwell and Chirico, 1993; Niki et al., 2005).

The significant 2-fold increase in the MDA concentration in extract treated cells suggests that lipid peroxidation was increased. It is most likely that ROS production was increased as a consequence of the enhanced activity of the mitochondrial ETC, and this in turn increased lipid peroxidation. In addition, the significant increase observed in the intracellular ATP concentration *(see §4.2.1)* correlates with an increase in activity of the mitochondrial ETC. Excessive production of ROS can be genotoxic and is also known to be capable of initiating

apoptosis (Shvedova et al., 2002). Lipid peroxidation following oxidative stress also plays an important role in cytotoxicity via necrosis or disordered apoptosis (Tyurina et al, 2000).

4.3 Genotoxicity

4.3.1 DNA Integrity

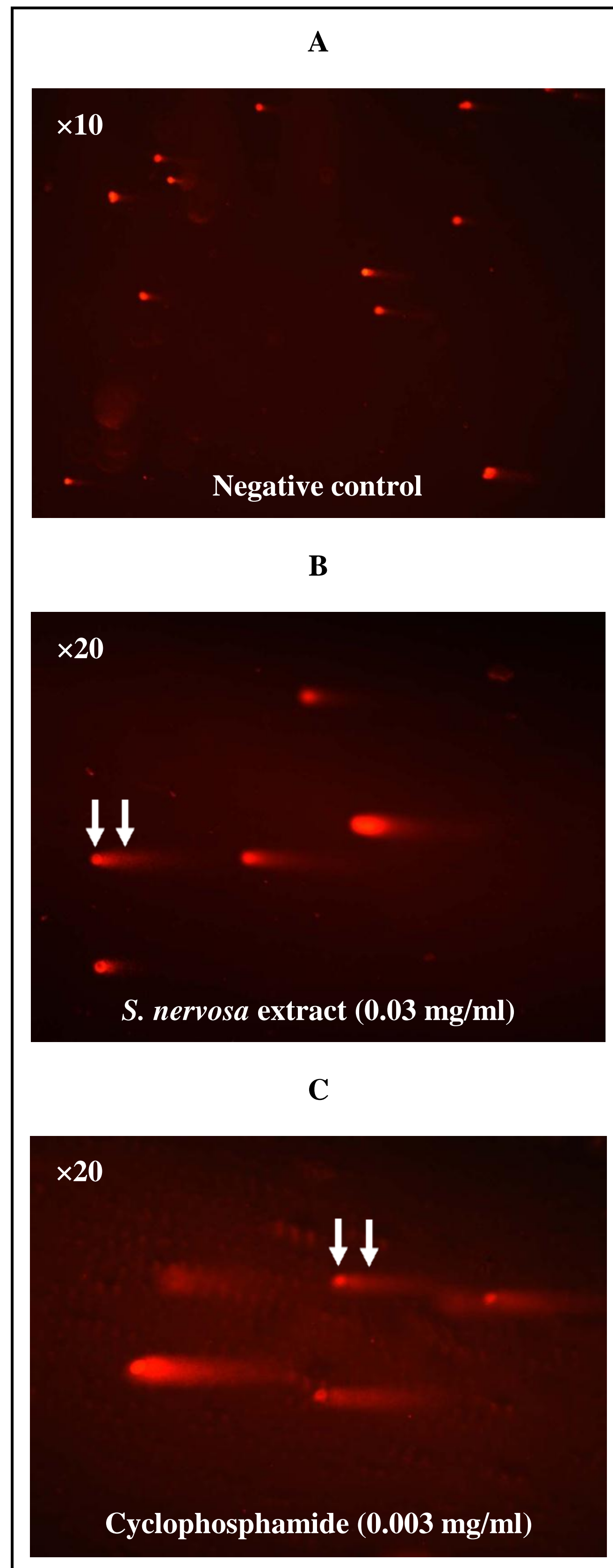


Figure 4–4. Digital images of fluorescent cell DNA after HepG2 cells were treated with **(A)** negative control, **(B)** *S. nervosa* extract, and **(C)** positive control cyclophosphamide after 24 h treatment. Microscopic magnifications are shown. Arrows indicate comet heads and tails respectively. Images are representative of 40 random comets scored per slide, and 2 slides per condition.

Qualitative analysis of the digital images showed that cells treated with the extract *(Figure 4–4B)* or cyclophosphamide *(Figure 4–4C)* exhibited a distinct DNA comet (head and tail) fragmentation pattern as evidenced by the increase in DNA migration compared to the control cells *(Figure 4–4A)*. The results for cyclophosphamide were expected since it is a known genotoxic drug (Yusuf et al., 2000).

The results generated from the scoring of the comets *(Figure 4–4)* were evaluated in terms of different parameters *(Figure 4–5)*. Changes in these parameters of cells treated with the extract or cyclophosphamide when compared to the negative control are summarised *(Table 4–2)*.

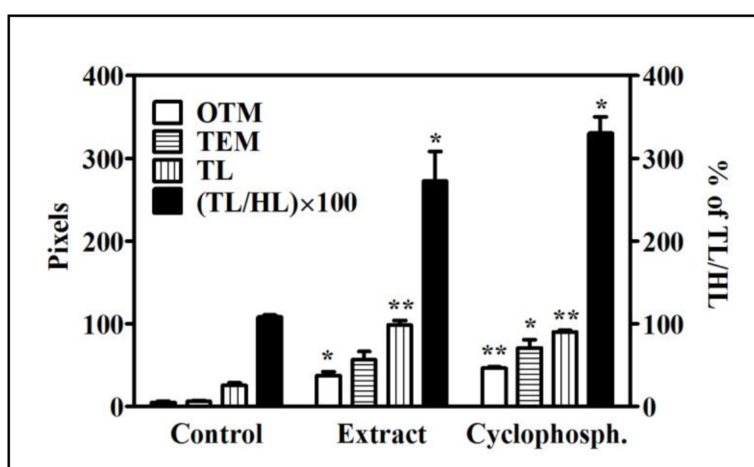


Figure 4–5. Random comets were scored after HepG2 cells were treated with the negative control, *S. nervosa* extract, or positive control cyclophosphamide for 24 h. DNA fragmentation was expressed in terms of the parameters OTM, TEM, TL (pixels) and % TL/HL. Asterisks indicate statistically significant differences when compared with the corresponding parameter of the negative control *(Table 4–2)*.

The methods used for quantifying DNA migration and DNA damage are highly varied. The OTM and TL are the 2 most commonly used parameters in literature (Kumaravel and Jha, 2006). A study by Kumaravel and Jha (2006) found the OTM, TL and TEM to be the most reliable measurements for detecting DNA damage. In a different study, statistical analysis performed by Yusuf et al. (2000) on several known promutagens did not reveal a particular parameter among the OTM, TEM, TL, and %TL/HL that could better indicate the amount of DNA damage caused.

Table 4–2. Changes in the OTM, TEM, TL and %TL/HL of HepG2 cells treated with the *S. nervosa* extract and positive control cyclophosphamide for 24 h when compared to the negative control.

Parameter	Extract	Cyclophosphamide
OTM	↑ 3.7-fold ($p < 0.05$)	↑ 9.6-fold ($p < 0.01$)
TEM	↑ 9.5-fold ($p > 0.05$)	↑ 11.8-fold ($p < 0.05$)
TL	↑ 3.4-fold ($p < 0.01$)	↑ 3.5-fold ($p < 0.01$)
%TL/HL	↑ 2.5-fold ($p < 0.05$)	↑3.1-fold ($p < 0.05$)

The DNA fragmentation observed in extract treated cells suggests that the extract may have induced genotoxicity in HepG2 cells. ROS and ROS-mediated lipid peroxidation are capable of causing both genotoxicity and cytotoxicity via disordered apoptosis (Shvedova et al., 2002). Thus the DNA fragmentation observed could possibly have occurred as a result of oxidative stress, as evidenced by increased lipid peroxidation *(see §4.2.3)*, to DNA. Intermediate and end-products formed during lipid peroxidation attack and damage DNA as well as mtDNA due to their close proximity to this DNA (Ott et al., 2007; Wiseman and Halliwell, 1996). DNA fragmentation is also a hallmark, late-stage event of apoptosis (Kam & Ferch, 2000).

4.4 Metabolism

4.4.1 Cytochrome P450 3A4 Activity

Table 4–3. CYP3A4 activity in HepG2 cells treated with the *S. nervosa* extract and positive control dexamethasone for 24 h when compared to the negative control.

Negative control (RLU)	Extract (RLU)	Dexamethasone (RLU)
2 252 ± 96.5	2 694 ± 193.4 (↑ 1.2-fold; $p > 0.05$)	2 263 ± 21.0 ($p > 0.05$)

Results indicate that the extract and dexamethasone increased the activity of CYP3A4 by 1.2-fold and minimally respectively, and in both cases statistical significance was not reached.

Previous studies have shown that HepG2 cells express low levels of CYPs, including CYP3A4, compared to primary human hepatocytes (Hasler et al., 1999; Westerink and Schoonen, 2007). The possibility thus exists that the ability of compounds to induce CYP3A4 may be underestimated in studies utilising HepG2 cells when compared to primary human hepatocytes.

Primary human hepatocytes are considered the preferred *in vitro* model to study xenobiotic biotransformation due to their resemblance to the human liver compared to HepG2 cells. However, primary human hepatocytes are difficult to obtain due to ethical considerations, isolation can be complex and time consuming, cells may be damaged during isolation and the availability of human livers is limited. In addition, primary hepatocytes do not proliferate and generally lose their metabolic activity after a few passages (splitting and re-seeding). Although HepG2 cells are less suitable to predict xenobiotic metabolism, they are easier to culture and provide a consistently reproducible model for studying xenobiotic toxicity (Brandon et al., 2003; Wilkening et al., 2003) *(see §3.2)*.

The IC_{50} concentration of the aqueous extract was assessed for its ability to induce CYP3A4 activity. This concentration may have been too low to induce CYP3A4 activity in HepG2 cells, bearing in mind that HepG2 cells minimally express this isozyme. Thus although the extract did not significantly induce CYP3A4 at the IC_{50}, this does not exclude the possibility that it is capable of modulating these enzymes.

Dexamethasone induction of CYP3A4 is well documented, however the mechanism by which this induction occurs is not fully understood, with many studies presenting conflicting data (Pascussi et al., 2001). Induction of CYP3A4 by xenobiotics is thought to be largely due to xenobiotic activation of the PXR (Tompkins and Wallace, 2007). A study by Luo et al. (2002) demonstrated that dexamethasone weakly activated the PXR and thus weakly induced CYP3A4 in primary human hepatocytes. The low expression of CYP3A4 in HepG2 cells may thus have also contributed to the negligible induction observed after dexamethasone treatment.

4.5 Summary and Conclusion

The results demonstrate that an aqueous extract of *S. nervosa* was cytotoxic to HepG2 cells and reduced cell viability in a concentration dependent manner (IC_{50} = 0.03 mg/ml). In assessing mechanisms of toxicity, it was found that the extract significantly increased intracellular ATP levels and caspase-8 activity and significantly decreased caspase-9 activity, suggesting the potential for the extract to induce apoptosis, specifically via the extrinsic apoptotic pathway. The extract did not significantly affect PS externalisation, possibly due to the rapid proliferation of HepG2 cells and did not significantly change mitochondrial membrane potential which corresponds to the increased ATP concentration. The significantly higher MDA levels indicated that the extract caused free radical-mediated lipid

peroxidation and oxidative stress and caused genotoxicity as evidenced by DNA fragmentation, possibly as a result of lipid peroxidation. The extract marginally increased CYP3A4 activity although it was not statistically significant. This could be due to the low expression levels of CYP3A4 in HepG2 cells.

Chapter 5. Summary, Conclusions and Limitations

5.1 Summary

1. Traditional medicine continues to form an integral aspect of primary health care in South Africa. *Scilla nervosa* is such a medicinal plant, used in the treatment of various ailments. There is however limited scientific evidence regarding its toxicological properties to rationalise its extensive use.

2. The bulbs of *S. nervosa* contain several homoisoflavanones and stilbenoids which may be individually, or in combination, responsible for its anecdotal and observed therapeutic effects.

3. The liver performs a multitude of functions, but it is the primary site of metabolism of xenobiotics, thus assessing the toxicity of the extract in the liver is of particular importance. Apoptosis and oxidative stress are implicated in the pathology of multiple diseases, while xenobiotics, capable of modulating CYP3A4, are associated with drug interactions. Therefore, investigating how the extract influences apoptosis, oxidative stress and CYP3A4 enzymes is significant in determining how the extract exerts its toxic effects.

4. Various assays were used to investigate the toxicological properties of an aqueous extract of *S. nervosa* in a cultured human HepG2 hepatocellular carcinoma cell line.

5. HepG2 cells were sensitive to the extract.

5.2 Conclusions

1. There was evidence that an aqueous extract was hepatotoxic *in vitro*.

2. It has the potential to induce apoptosis via the extrinsic apoptotic pathway.

3. There was evidence of genotoxicity, possibly as a result of oxidative stress.

4. CYP3A4 activity was slightly elevated. This finding was not statistically significant.

5.3 Limitations

1. The results of this study were obtained from a cancer cell line that is considered a model cell line for the investigation of toxicity. However, results may be different in primary hepatocytes and it could be difficult to extrapolate the effects.

2. HepG2 cells were treated for 24 h and the results could have been different if shorter or longer periods were chosen.

5.4 Future Studies

1. Besides the liver, the kidneys as major excretory organs may also be prone to the toxic effects of traditional medicine. The extract was not tested in kidney cell lines.
2. The immune system may be adversely affected by traditional medicine, for example hypersensitivity reactions and immunosuppression may occur. The extract was not tested in lymphocytes to determine changes in inflammatory mediators for example.
3. The extract was not tested to determine neurotoxicity, carcinogenicity, teratogenicity and haematological effects.
4. The pharmacokinetic parameters of traditional medicine may be complex and various factors could influence the bioavailability, absorption, concentration in the plasma and tissues, distribution, metabolism and excretion. The therapeutic concentration, therapeutic window and therapeutic index are yet unknown. This study only investigated the toxicological properties *in vitro* and there may be differences *in vivo*.

References

Abegaz, B. 2002. Novel phenylanthraquinones, isofuranonaphthoquinones, homoisoflavonoids, and biflavonoids from African plants in the genera *Bulbine*, *Scilla*, *Ledebouria*, and *Rhus*. *Phytochemistry Reviews,* 1:299-310.

Abuja, P.M., Albertini, R. 2001. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. *Clinica Chimica Acta,* 306:1-17.

Ahmed, S.M., Banner, N.R., Dubrey, S.W. 2001. Low cyclosporin-A level due to Saint-John's-wort in heart-transplant patients. *The Journal of Heart and Lung Transplantation,* 20:795.

Amschler, G., Frahm, A.W., Hatzelmann, A., Kilian, U., Muller-Doblies, D., Muller-Doblies, U. 1996. Constituents of *Veltheimia viridifolia*. I.Homoisoflavanones of the bulbs. *Planta Medica,* 62:534-539.

Ashe, P.C., Berry, M.D. 2003. Apoptotic signaling cascades. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 27:199-214.

Athar, M., Back, J.H., Kopelovich, L., Bickers, D.R., Kim, A.L. 2009. Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Archives of Biochemistry and Biophysics,* 486:95-102.

Bailey, D.G., Malcolm, J., Arnold, O., David Spence, J. 1998. Grapefruit juice–drug interactions. *British Journal of Clinical Pharmacology,* 46:101-110.

Bangani, V., Crouch, N.R., Mulholland, D.A. 1999. Homoisoflavanones and stilbenoids from *Scilla nervosa*. *Phytochemistry,* 51:947-951.

Bernas, T., Dobrucki, J. 2002. Mitochondrial and nonmitochondrial reduction of MTT: Interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. *Cytometry,* 47:236-242.

Bernhard, D., Schwaiger, W., Crazzolara, R., Tinhofer, I., Kofler, R., Csordas, A. 2003. Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. *Cancer Letters,* 195:193-199.

Berridge, M.V., Tan, A.S. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization,

substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Archives of Biochemistry and Biophysics,* 303:474-482.

Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica,* 4:14-19.

Bicknell, G.R., Snowden, R.T., Cohen, G.M. 1994. Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937. *Journal of Cell Science,* 107:2483-2489.

Bisi-Johnson, M.A., Obi, C.L., Kambizi, L., Nkomo, M. 2010. A survey of indigenous herbal diarrhoeal remedies of O.R. Tambo district, Eastern Cape Province, South Africa. *African Journal of Biotechnology,* 9:1245-1254.

Bradbury, D.A., Simmons, T.D., Slater, K.J., Crouch, S.P.M. 2000. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *Journal of Immunological Methods,* 240:79-92.

Brandon, E.F.A., Raap, C.D., Meijerman, I., Beijnen, J.H., Schellens, J.H.M. 2003. An update on *in vitro* test methods in human hepatic drug biotransformation research: pros and cons. *Toxicology and Applied Pharmacology,* 189:233-246.

Brunelle, J.K., Zhang, B. 2010. Apoptosis assays for quantifying the bioactivity of anticancer drug products. *Drug Resistance Updates,* 13:172-179.

Buehrlen, M., Harréus, U.A., Gamarra, F., Hagen, R., Kleinsasser, N.H. 2007. Cumulative genotoxic and apoptotic effects of xenobiotics in a mini organ culture model of human nasal mucosa as detected by the alkaline single cell microgel electrophoresis assay and the annexin V-affinity assay. *Toxicology Letters,* 169:152-161.

Bungu, L., Frost, C.L., Brauns, S.C., Van de Venter, M. 2006. *Tulbachia violacea* inhibits growth and induced apoptosis in cancer cells *in vitro*. *African Journal of Biotechnology,* 5:1936-1943.

Cali, J.J., Ma, D., Sobol, M., Simpson, D.J., Frackman, S., Good, T.D., Daily, W.J., Liu, D. 2006. Luminogenic cytochrome P450 assays. *Expert Opinion on Drug Metabolism and Toxicology,* 2:629-645.

Campbell, I. 2006. Liver: metabolic functions. *Anaesthesia and Intensive Care Medicine,* 7:51-54.

Campbell, I. 2011. Intermediary metabolism. *Anaesthesia and Intensive Care Medicine,* 12:176-178.

Castell, J.V., Gómez-Lechón, M.J., Ponsoda X., Bort, R. 1997. *In vitro* investigation of the molecular mechanisms of hepatotoxicity. *Archives of Toxicology Supplement,* 19:313-321.

Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.L., Lesnefsky, E.J. 2003. Production of reactive oxygen species by mitochondria: Central role of complex III. *Journal of Biological Chemistry,* 278:36027-36031.

Chinkwo, K.A. 2005. *Sutherlandia frutescens* extracts can induce apoptosis in cultured carcinoma cells. *Journal of Ethnopharmaclogy,* 98:163-170.

Choucroun, P., Gillet, D., Dorange, G., Sawicki, B., Dewitte, J.D. 2001. Comet assay and early apoptosis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 478:89-96.

Chun, Y.J., Ryu, S.Y., Jeong, T.C., Kim, M.Y. 2001. Mechanism-based inhibition of human cytochrome P450 1A1 by rhapontigenin. *Drug Metabolism and Disposition,* 29:389-393.

Crouch, N.R., Bangani, V., Mulholland, D.A. 1999. Homoisoflavanones from three South African *Scilla* species. *Phytochemistry,* 51:943-946.

Crouch, S.P.M., Kozlowski, R., Slater, K.J., Fletcher, J. 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods,* 160:81-88.

Cunningham, C.C., Van Horn, C.G. 2003. Energy availability and alcohol-related liver pathology. *Alcohol Research and Health,* 27:291-299.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., Traganos, F. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry,* 27:1-20.

Della Loggia, R., Del Negro, P., Tubaro, A., Barone, G., Parrilli, M. 1989. Homoisoflavanones as anti-inflammatory principles of *Muscari comosum*. *Planta Medica,* 55:587-588.

Dhanjal, P., Fry, J.R. 1997. Determinants of MTT reduction in rat hepatocytes. *Biomarkers,* 2:111-116.

Ding, X., Kaminsky, L.S. 2003. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annual Review of Pharmacology and Toxicology,* 43:149-173.

Draft 3rd Ordinary Session of the African Union Conference of Ministers of Health (Camh3). 9-13 April 2007. The status of traditional medicine in Africa and review of progress made on the implementation of the plan of action on the African Union Decade of Traditional Medicine (2001-2010).

Du, C., Fang, M., Li, Y., Li, L., Wang, X. 2000. Smac, a mitochondrial protein that promotes cytochrome *c* dependent caspase activation by eliminating IAP inhibition. *Cell,* 102:33-42.

Du Toit, K., Elgorashi, E.E., Malan, S.F., Drewes, S.E., van Staden, J., Crouch, N.R., Mulholland, D.A. 2005. Anti-inflammatory activity and QSAR studies of compounds isolated from Hyacinthaceae species and *Tachiadenus longiflorus* Griseb. (Gentianaceae). *Bioorganic and Medicinal Chemistry,* 13:2561-2568.

Du Toit, K., Elgorashi, E.E., Malan, S.F., Mulholland, D.A., Drewes, S.E., Van Staden, J. 2007. Antibacterial activity and QSAR of homoisoflavanones isolated from six Hyacinthaceae species. *South African Journal of Botany,* 73:236-241.

Du Toit, K., Kweyama, A., Bodenstein, J. 2011. Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae). *South African Journal of Science,* 107:96-100.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J. 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology,* 94:205-217.

Fernandes, A.C., Cromarty, A.D., Albrecht, C., Jansen van Rensburg, C.E. 2004. The antioxidant potential of *Sutherlandia frutescens*. *Journal of Ethnopharmacology,* 95:1-5.

Finucane, D.M., Waterhouse, N.J., Amarante-Mendes, G.P., Cotter, T.G., Green, D.R. 1999. Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. *Experimental Cell Research,* 251:166-174.

Fulda, S., Debatin, K.M. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene,* 25:4798-4811.

Fulda, S., Meyer, E., Debatin, K.M. 2002. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene,* 21:2283-2294.

Ghavami, S., Hashemi, M., Ande, S.R., Yeganeh, B., Xiao, W., Eshraghi, M., Bus, C.J., Kadkhoda, K., Wiechec, E., Halayko, A.J., Los, M. 2009. Apoptosis and cancer: mutations within caspase genes. *Journal of Medical Genetics,* 46:497-510.

Graf, D., Bode, J.G., Häussinger, D. 2007. Caspases and receptor cleavage. *Archives of Biochemistry and Biophysics,* 462:162-170.

Green, D.R., Reed, J.C. 1998. Mitochondria and apoptosis. *Science,* 281:1309-1312.

Grivennikova, V.G., Vinogradov, A.D. 2006. Generation of superoxide by the mitochondrial Complex I. *Biochimica et Biophysica Acta - Bioenergetics,* 1757:553-561.

Guengerich, F.P. 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annual Review of Pharmacology and Toxicology,* 39:1-17.

Halliwell, B., Chirico, S. 1993. Lipid peroxidation: its mechanism, measurement, and significance. *The American Journal of Clinical Nutrition,* 57:715-724.

Hannah, R., Beck, M., Moravec, R., Riss, T. 2001. CellTiter-Glo™ luminescent cell viability assay: A sensitive and rapid method for determining cell viability. *Cell Notes,* 2:11-13.

Hasler, J.A., Estabrook, R., Murray, M., Pikuleva, I., Waterman, M., Capdevila, J., Holla, V., Helvig, C., Falck, J.R., Farrell, G., Kaminsky, L.S., Spivack, S.D., Boitier, E., Beaune, P. 1999. Human cytochromes P450. *Molecular Aspects of Medicine,* 20:1-137.

Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature,* 407:770-776.

Huerta, S., Goulet, E.J., Huerta-Yepez, S., Livingston, E.H. 2007. Screening and detection of apoptosis. *Journal of Surgical Research,* 139:143-156.

Hutchings, A., Terblanche, S.E. 1989. Observations on the use of some known and suspected toxic Liliiflorae in Zulu and Xhosa medicine. *South African Medical Journal,* 75:62-69.

Hutchings, A., Scott, A., Lewis, G., Cunningham, A.B. 1996. Zulu medicinal plants: An inventory. *University of Natal Press,* Pietermaritzburg.

Isin, E.M., Guengerich, F.P. 2007. Complex reactions catalyzed by cytochrome P450 enzymes. *Biochimica et Biophysica Acta - General Subjects,* 1770:314-329.

Janero, D.R. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine,* 9:515-540.

Jarukamjorn, K., Don-in, K., Makejaruskul, C., Laha, T., Daodee, S., Pearaksa, P., Sripanidkulchai, B.-o. 2006. Impact of *Andrographis paniculata* crude extract on mouse hepatic cytochrome P450 enzymes. *Journal of Ethnopharmacology,* 105:464-467.

Kageura, T., Matsuda, H., Morikawa, T., Toguchida, I., Harima, S., Oda, M., Yoshikawa, M. 2001. Inhibitors from rhubarb on lipopolysaccharide-induced nitric oxide production in macrophages: structural requirements of stilbenes for the activity. *Bioorganic and Medicinal Chemistry,* 9:1887-1893.

Kam, P.C.A., Ferch, N.I. 2000. Apoptosis: mechanisms and clinical implications. *Anaesthesia,* 55:1081-1093.

Kannan, K., Jain, S.K. 2000. Oxidative stress and apoptosis. *Pathophysiology,* 7:153-163.

Kantari, C., Walczak, H. 2011. Caspase-8 and Bid: Caught in the act between death receptors and mitochondria. *Biochimica et Biophysica Acta - Molecular Cell Research,* 1813:558-563.

Kiechle, F.L., Zhang, X. 2002. Apoptosis: biochemical aspects and clinical implications. *Clinica Chimica Acta,* 326:27-45.

Köhler, C., Orrenius, S., Zhivotovsky, B. 2002. Evaluation of caspase activity in apoptotic cells. *Journal of Immunological Methods,* 265:97-110.

Komoroski, B.J., Zhang, S., Cai, H., Hutzler, J.M., Frye, R., Tracy, T.S., Strom, S.C., Lehmann, T., Ang, C.Y.W., Cui, Y.Y., Venkataramanan, R. 2004. Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metabolism and Disposition,* 32:512-518.

Kroemer, G., Zamzami, N., Susin, S.A. 1997. Mitochondrial control of apoptosis. *Immunology Today,* 18:44-51.

Kumaravel, T.S., Jha, A.N. 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 605:7-16.

Lamkanfi, M., Kanneganti, T.-D. 2010. Caspase-7: A protease involved in apoptosis and inflammation. *The International Journal of Biochemistry and Cell Biology,* 42:21-24.

Launay, S., Hermine, O., Fontenay, M., Kroemer, G., Solary, E., Garrido, C. 2005. Vital functions for lethal caspases. *Oncogene,* 24:5137-5148.

Lee, Y.S., Wurster, R.D. 1995. Effects of antioxidants on the anti-proliferation induced by protein synthesis inhibitors in human brain tumor cells. *Cancer Letters,* 93:157-163.

Legrand, O., Perrot, J.-Y., Simonin, G., Baudard, M., Marie, J.-P. 2001. JC-1: a very sensitive fluorescent probe to test Pgp activity in adult acute myeloid leukemia. *Blood,* 97:502-508.

Liao, W., McNutt, M.A., Zhu, W.-G. 2009. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Methods,* 48:46-53.

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.

Liou, A.K.F., Clark, R.S., Henshall, D.C., Yin, X.-M., Chen, J. 2003. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. *Progress in Neurobiology,* 69:103-142.

Liu, Y., Fiskum, G., Schubert, D. 2002. Generation of reactive oxygen species by the mitochondrial electron transport chain. *Journal of Neurochemistry,* 80:780-787.

Liu, Y., Peterson, D.A., Kimura, H., Schubert, D. 1997. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry,* 69:581-593.

Liu, Z.-g., Hsu, H., Goeddel, D.V., Karin, M. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell,* 87:565-576.

Lossi, L., Merighi, A. 2003. *In vivo* cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. *Progress in Neurobiology,* 69:287-312.

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.

Luo, G., Cunningham, M., Kim, S., Burn, T., Lin, J., Sinz, M., Hamilton, G., Rizzo, C., Jolley, S., Gilbert, D., Downey, A., Mudra, D., Graham, R., Carroll, K., Xie, J., Madan, A., Parkinson, A., Christ, D., Selling, B., LeCluyse, E., Gan, L.-S. 2002. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metabolism and Disposition,* 30:795-804.

Ly, J.D., Grubb, D.R., Lawen, A. 2003. The mitochondrial membrane potential ($\Delta\Psi m$) in apoptosis; an update. *Apoptosis,* 8:115-128.

Madesh, M., Hajnóczky, G. 2001. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *The Journal of Cell Biology,* 155:1003-1016.

Mander, M. 1998. Marketing of indigenous medicinal plants in South Africa-A case study in KwaZulu-Natal. *Food and Agriculture Organisation of the United Nations,* Rome.

McGaw, L.J., Eloff, J.N. 2008. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. *Journal of Ethnopharmacology,* 119:559-574.

Meagher, E.A., FitzGerald, G.A. 2000. Indices of lipid peroxidation *in vivo*: strengths and limitations. *Free Radical Biology and Medicine,* 28:1745-1750.

Mersch-Sundermann, V., Knasmuller, S., Wu, X.J., Darroudi, F., Kassie, F. 2004. Use of human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology,* 198:329-340.

Mimaki, Y., Ori, K., Sashida, Y., Nakaido, T., Song, L.-G., Ohmoto, T. 1993. Peruvianosides A and B, novel triterpene glycosides from the bulbs of *Scilla peruviana*. *Bulletin of the Chemical Society of Japan,* 66:1182-1186.

Mitra, V., Metcalf, J. 2009. Metabolic functions of the liver. *Anaesthesia and Intensive Care Medicine,* 10:334-335.

Moon, Y.J., Wang, X., Morris, M.E. 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. *Toxicology in Vitro,* 20:187-210.

Moore, L.B., Goodwin, B., Jones, S.A., Wisely, G.B., Serabjit-Singh, C.J., Willson, T.M., Collins, J.L., Kliewer, S.A. 2000. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proceedings of the National Academy of Sciences,* 97:7500-7502.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* 65:55-63.

Mukherjee, P.K., Ponnusankar, S., Pandit, S., Hazam, P.K., Ahmmed, M., Mukherjee, K. 2011. Botanicals as medicinal food and their effects on drug metabolizing enzymes. *Food and Chemical Toxicology,* 49:3142-3153.

Nakano, H., Nakajima, A., Sakon-Komazawa, S., Piao, J.-H., Xue, X., Okumura, K. 2005. Reactive oxygen species mediate crosstalk between NF-κB and JNK. *Cell Death and Differentiation,* 13:730-737.

Nguyen, A.T., Fontaine, J., Malonne, H., Duez, P. 2006. Homoisoflavanones from *Disporopsis aspera*. *Phytochemistry,* 67:2159–2163.

Niki, E., Yoshida, Y., Saito, Y., Noguchi, N. 2005. Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications,* 338:668-676.

Obach, R.S. 2000. Inhibition of human cytochrome P450 enzymes by constituents of St. John's wort, an herbal preparation used in the treatment of depression. *Journal of Pharmacology and Experimental Therapeutics,* 294:88-95.

Ott, M., Gogvadze, V., Orrenius, S., Zhivotovsky, B. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis,* 12:913-922.

Pacific Bulb Society. 2010. Schizocarphus. http://www.pacificbulbsociety.org/pbswiki/index.php/Schizocarphus#nervosus. (Date accessed: 25 July 2011).

Palsamy, P., Subramanian, S. 2008. Resveratrol, a natural phytoalexin, normalizes hyperglycemia in streptozotocin-nicotinamide induced experimental diabetic rats. *Biomedicine and Pharmacotherapy,* 62:598-605.

Palsamy, P., Subramanian, S. 2009. Modulatory effects of resveratrol on attenuating the key enzymes activities of carbohydrate metabolism in streptozotocin–nicotinamide-induced diabetic rats. *Chemico-Biological Interactions,* 179:356-362.

Pan, Y., Abd-Rashid, B.A., Ismail, Z., Ismail, R., Mak, J.W., Pook, P.C.K., Er, H.M., Ong, C.E. 2010. *In vitro* modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of *Centella asiatica*. *Journal of Ethnopharmacology,* 130:275-283.

Pascussi, J.-M., Drocourt, L., Gerbal-Chaloin, S., Fabre, J.-M., Maurel, P., Vilarem, M.-J. 2001. Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. *European Journal of Biochemistry,* 268:6346-6358.

Pelassy, C., Breittmayer, J.-P., Aussel, C. 2000. Regulation of phosphatidylserine exposure at the cell surface by the serine-base exchange enzyme system during CD95-induced apoptosis. *Biochemical Pharmacology,* 59:855-863.

Phillips, M.J., Oda, M., Edwards, V.D., Greenberg, G.R., Jeejeebhoy, K.N. 1974. Ultrastructural and functional studies of cultured hepatocytes. *Laboratory Investigation,* 31:533-542.

Piscitelli, S.C., Burstein, A.H., Chaitt, D., Alfaro, R.M., Falloon, J. 2000. Indinavir concentrations and St John's wort. *The Lancet,* 355:547-548.

Planinsic, R.M., Nicolau-Raducu, R. 2006. Chapter 64 - Physiology and pharmacology of the liver, in: Hugh, C.H., Jr, PhD, M.D., Philip M Hopkins, M.M.F. (Eds.). Foundations of Anesthesia (Second Edition), Mosby, Edinburgh, pp. 773-782.

Puntarulo, S., Cederbaum, A.I. 1998. Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes. *Free Radical Biology and Medicine,* 24:1324-1330.

Rastogi, R.P., Sinha, R., Sinha, R.P. 2009. Apoptosis: molecular mechanisms and pathogenicity. *EXCLI Journal,* 8:155-181.

Raucy, J.L. 2003. Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metabolism and Disposition,* 31:533-539.

Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B. 2010. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine,* 49:1603-1616.

Riedl, S.J., Shi, Y. 2004. Molecular mechanisms of caspase regulation during apoptosis. *Nature Reviews Molecular Cell Biology,* 5:897-907.

Robertson, J.D., Orrenius, S. 2000. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. *Critical Reviews in Toxicology,* 30:609-627.

Salvioli, S., Ardizzoni, A., Franceschi, C., Cossarizza, A. 1997. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Letters,* 411:77-82.

Scarlett, J.L., Sheard, P.W., Hughes, G., Ledgerwood, E.C., Ku, H.-H., Murphy, M.P. 2000. Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jurkat cells. *FEBS Letters,* 475:267-272.

Scott, G. 2003. Acute toxicity associated with the use of South African traditional medicinal herbs. *Transactions of the Royal Society of South Africa,* 58:83-92.

Sener, B. 2003. Biodiversity: biomolecular aspects of biodiversity and innovative utilization. 1st ed. Springer, New York, p. 412.

Sharma, S., Misra, C.S., Arumugam, S., Roy, S., Shah, V., Davis, J.A., Shirumalla, R.K., Ray, A. 2011. Antidiabetic activity of resveratrol, a known SIRT1 activator in a genetic model for type-2 diabetes. *Phytotherapy Research,* 25:67-73.

Shim, J.S., Kim, J.H., Lee, J., Kim, S.N., Kwon, H.J. 2004. Anti-angiogenic activity of a homoisoflavanone from *Cremastra appendiculata*. *Planta Medica,* 70:171–173.

Shvedova, A.A., Tyurina, J.Y., Kawai, K., Tyurin, V.A., Kommineni, C., Castranova, V., Fabisiak, J.P., Kagan, V.E. 2002. Selective peroxidation and externalization of phosphatidylserine in normal human epidermal keratinocytes during oxidative stress induced by cumene hydroperoxide. *The Journal of Investigative Dermatology,* 118:1008-1018.

Silayo, A., Ngadjui, B.T., Abegaz, B.M. 1999. Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa subsp. rigidifolia*. *Phytochemistry,* 52:947-955.

Singh, N.P., McCoy M.T., Tice, R.R., Schnyder, E.L. 1988. A simple technique for quantification of low levels of DNA damage in individual cells. *Experimental Cell Research,* 175:184-191.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D., Chen, L.B. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences,* 88:3671-3675.

South African National Biodiversity Institute. 2004. Hyacinthaceae *Schizocarphus nervosus*. http://sibis.sanbi.org/faces/SearchSpecies/SearchDetails.jsp?1=1. (Date accessed: 29 January 2011).

Steenkamp, V., Grimmer, H., Semano, M., Gulumian, M. 2005. Antioxidant and genotoxic properties of South African herbal extracts. *Mutation Research,* 581:35-42.

Street, R.A., Stirk, W.A., Van Staden, J. 2008. South African traditional medicinal plant trade-Challenges in regulating quality, safety and efficacy. *Journal of Ethnopharmacology,* 119:705-710.

Sturgill, M.G., Lambert, G.H. 1997. Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. *Clinical Chemistry,* 43:1512-1526.

Subehan, Usia, T., Iwata, H., Kadota, S., Tezuka, Y. 2006. Mechanism-based inhibition of CYP3A4 and CYP2D6 by Indonesian medicinal plants. *Journal of Ethnopharmacology,* 105:449-455.

Szkudelski, T., Szkudelska, K. 2011. Anti-diabetic effects of resveratrol. *Annals of the New York Academy of Sciences,* 1215:34-39.

Tompkins, L.M., Wallace, A.D. 2007. Mechanisms of cytochrome P450 induction. *Journal of Biochemical and Molecular Toxicology,* 21:176-181.

Tsujimoto, Y. 1997. Apoptosis and necrosis: Intracellular ATP level as a determinant for cell death modes. *Cell Death and Differentiation,* 4:429-434.

Tyurina, Y.Y., Shvedova, A.A., Kawai, K., Tyurin, V.A., Kommineni, C., Quinn, P.J., Schor, N.F., Fabisiak, J.P., Kagan, V.E. 2000. Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. *Toxicology,* 148:93-101.

United Nations Secretary General Report on Traditional Medicine. 2002. WHO, Executive Board EB111/9, 111th Session, Provisional agenda item 5.7.

Van der Walt, S.J., Steyn, D.G. 1946. Recent investigations into the toxicity of plants etc., XV. *Onderstepoort Journal of Veterinary Science and Animal Industry,* 21:45-55.

Van Engeland, M., Nieland, L.J.W., Ramaekers, F.C.S., Schutte, B., Reutelingsperger, C.P.M. 1998. Annexin V-Affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry,* 31:1-9.

Van Vuuren, S.F. 2008. Antimicrobial activity of South African medicinal plants. *Journal of Ethnopharmacology,* 119:462-472.

Van Wyk, B.-E. 2008. A broad review of commercially important southern African medicinal plants. *Journal of Ethnopharmacology,* 119:342-355.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutellingsperger, C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods,* 184:39-51.

Verschaeve, L., Van Staden, J. 2008. Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants. *Journal of Ethnopharmacology,* 119:575-587.

Wall, M.E., Wani, M.C., Manikumar, G., Taylor, H., McGivney, R. 1989. Plant antimutagens, 6. Intricatin and intricatinol, new antimutagenic homoisoflavanoids from *Hoffmanosseggia intricata*. *Journal of Natural Products,* 52:774-778.

Westerink, W.M.A., Schoonen, W.G.E.J. 2007. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology in Vitro,* 21:1581-1591.

Wilkening, S., Stahl, F., Bader, A. 2003. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *Drug Metabolism and Disposition,* 31:1035-1042.

Wiseman, H., Halliwell, B. 1996. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *The Biochemical Journal,* 313:17-29.

World Health Organisation. 2005. National policy on traditional medicine and regulation of herbal medicines. Report of a WHO global survey, World Health Organisation, Geneva.

Yusuf, A.T., Vian, L., Sabatier, R., Cano, J.-P. 2000. *In vitro* detection of indirect-acting genotoxins in the comet assay using Hep G2 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 468:227-234.

Zangar, R.C., Davydov, D.R., Verma, S. 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicology and Applied Pharmacology,* 199:316-331.

Zhou, S., Chan, E., Li, X., Huang, M. 2005. Clinical outcomes and management of mechanism-based inhibition of cytochrome P450 3A4. *Therapeutics and Clinical Risk Management,* 1:3-13.

Appendix 1 (Full-length Research Article)

The Cytotoxic Effects of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) Aqueous Extract on Cultured HepG2 Cells

Prishania Pillay[a], Alisa Phulukdaree[b], Anil A. Chuturgoon[b], Karen Du Toit[c] and Johannes Bodenstein[a*]

[a]*Department of Pharmacology, University of KwaZulu-Natal, Durban, South Africa*

[b]*Discipline of Medical Biochemistry, University of KwaZulu-Natal, Durban, South Africa*

[c]*Department of Pharmaceutical Chemistry, University of KwaZulu-Natal, Durban, South Africa*

*Corresponding author:

E-mail: bodensteinj@ukzn.ac.za; Tel.: +27 31 260 7500; Fax: +27 31 260 7907

Abstract

Bulbs of *Scilla nervosa*, a medicinal plant indigenous to Southern Africa, are traditionally used in aqueous decoctions to treat a diverse range of illnesses. However, little information is known about the plant's toxicity on the liver, a major detoxifying organ. This study investigated the effects of an aqueous extract of the bulbs in cultured HepG2 liver cells, a model system for investigating the toxicity of xenobiotics. Cell viability was measured to obtain an IC_{50}-value and potential mechanisms of toxicity were investigated as changes in metabolic activity, apoptosis, oxidative damage and DNA fragmentation using the IC_{50}. In addition, cytochrome P450 3A4 (CYP3A4) activity, which is implicated in drug metabolism and interactions was also assayed. Results suggest that liver cells are sensitive to an aqueous extract of the bulbs and at 50% reduction in viability there is an increased potential to induce apoptosis and genotoxicity, and some evidence of increased CYP3A4 activity.

Keywords

Scilla nervosa; traditional medicine; HepG2 liver cells; caspase; apoptosis; genotoxicity; CYP3A4

Introduction

Scilla nervosa (Burch.) Jessop (Hyacinthaceae) [=*Schizocarphus nervosus* (Burch.) Van der Merwe] is a monocotyledonous perennial plant originally endemic to Botswana but has now been naturalised in the grasslands of the eastern parts of Southern Africa (Hutchings, Scott, Lewis, & Cunningham, 1996). The bulbs of the plant have been used by traditional healers of different cultures and the plant is considered to be a valuable medicinal species (Watt & Breyer-Brandwijk, 1962). It has been recently demonstrated in our laboratory that extracts prepared from the bulbs possess potent anti-inflammatory properties and these findings may therefore rationalise the traditional use of the plant as an analgesic for rheumatic fever (Du Toit, Kweyama, & Bodenstein, 2011). Previous studies on the bulbs have revealed that they contain homoisoflavanones and stilbenoids (Bangani, Crouch, & Mulholland, 1999; Silayo, Ngadjui, & Abegaz, 1999). These could individually or in combination be responsible for its therapeutic effects. However, little information is known about the plant's toxicity and only one previous report suggested that 0.5-1 kg of the fresh plant in the flowering stages was toxic to sheep (Van der Walt & Steyn, 1946). Therefore, a current investigation of the potential toxicity of the bulbs on the liver, a major detoxifying organ, is required. The aim of the present study was to investigate the toxic effects of an

aqueous extract prepared from the bulbs in HepG2 liver cells, a model system for investigating the toxicity of xenobiotics. Specific objectives were as follows:

1. The viability of HepG2 cells in the presence of varying concentrations of the extract was investigated to determine the IC_{50}-value.
2. The IC_{50} was used as a reference concentration to investigate the potential mechanisms of toxicity by determining changes in:

- Metabolic activity (intracellular ATP levels).
- Apoptosis (phosphatidylserine externalisation; caspase-8 and -9 activity; and mitochondrial membrane depolarisation).
- Oxidative stress through free radical-mediated lipid peroxidation.
- DNA integrity.

3. CYP isozymes are often implicated in drug-liver-drug interactions. CYP3A4 activity was investigated to determine induction or inhibition.

Results and Discussion

Cell Viability

The viability of HepG2 cells decreased in a concentration-dependent manner with increasing concentrations of extract (IC_{50} = 0.03 ± 0.005 mg/ml) or emetine (IC_{50} = 1.0 ± 0.026 μg/ml).

Results suggest that the extract was significantly less cytotoxic than emetine to the HepG2 cells (IC_{50} ratio = 30).

Intracellular ATP Concentration

The intracellular ATP concentration increased 1.2-fold ($p < 0.001$) from 5 837 691 ± 14 219 RLU (negative control) when compared to 6 945 318 ± 5 592 RLU (extract treated cells).

Given that apoptosis is reliant on ATP for its execution (Tsujimoto, 1997), ATP levels should most likely have decreased in the HepG2 cells, however, slightly higher ATP levels were observed instead. This occurrence suggests that the bulbs may possess hypoglycaemic properties. Resveratrol, a chemical constituent previously isolated has been shown to have anti-hyperglycaemic potential in obese and diabetic rats (Sharma et al., 2011). The anti-hyperglycaemic effect is thought to be due to its ability to increase cellular glucose uptake

and intracellular glucose transport (Szkudelski & Szkudelska, 2011). The increase in glucose uptake would result in an increase in glycolysis and the Krebs cycle, and subsequent increased ATP production via oxidative phosphorylation (Campbell, 2011). This action may account for the higher intracellular ATP concentration observed in the extract treated cells.

Phosphatidylserine Externalisation

Phosphatidylserine externalisation was not significantly different ($p > 0.05$) from 48.0 ± 9.5% (negative control) versus 45.1 ± 1.7% (extract treated cells).

Exposure of this aminophospholipid occurs early in apoptosis. Phosphatidylserine is normally localised on the inner leaflet of the plasma membrane and becomes externalised on the outer leaflet where it enables phagocytic recognition of apoptotic cells by macrophages. Its exposure on the cell surface thus serves as a marker of apoptosis (Pelassy, Breittmayer, & Aussel, 2000). Although the percentage externalisation between the control and extract treated cells was not significantly different, levels were relatively high in both conditions. This may suggest that the control cells as well as the extract treated cells were undergoing apoptosis. However, HepG2 cells are a transformed cell line which proliferates at a much faster rate than primary hepatocytes which may explain the generally high externalisation that was observed.

Caspase-8 and -9 Activity

Caspase-8 is the primary initiator caspase of the extrinsic apoptotic pathway or death receptor pathway (Hengartner, 2000). This pathway is initiated by the binding of extracellular ligands to cell surface death receptors that belong to the tumour necrosis factor TNF-R gene superfamily. The significant increase in caspase-8 activation that was observed in the extract treated cells suggests that apoptosis may be modulated via the extrinsic apoptotic pathway (Table 1).

Caspase-9 is the key initiator caspase of the intrinsic apoptotic pathway or mitochondrial pathway (Brunelle & Zhang, 2010). The slightly lower caspase-9 activity observed in the extract treated cells suggests that the intrinsic apoptotic pathway was not affected or induced (Table 1).

Mitochondrial Membrane Potential

The number of depolarised mitochondria were not significantly different ($p > 0.05$) from 30.5 ± 0.9% (negative control) versus 31.2 ± 1.2% (extract treated cells).

Changes in mitochondrial membrane potential ($\Delta\Psi m$) were originally thought to be an early, essential event in apoptosis with many studies demonstrating that these changes precede the nuclear changes associated with apoptosis. There is now, however, accumulating data that suggests that the $\Delta\Psi m$ may increase, decrease, or remain unchanged during apoptosis (Ly, Grubb, & Lawen, 2003).

Although there was no significant change in mitochondrial membrane depolarisation in extract treated cells, this may not be a suitable marker for apoptosis. The fact that the extract did not significantly change the mitochondrial membrane depolarisation in HepG2 cells also correlates with the slight increase in ATP production, since the mitochondrial membrane potential is the driving force in mitochondrial ATP synthesis.

Lipid Peroxidation

The malondialdehyde concentration increased 2-fold ($p < 0.01$) from 0.2 ± 0.03 μM (negative control) versus 0.4 ± 0.03 μM (extract treated cells).

Lipid peroxidation occurs as a result of free radicals, most likely generated from the mitochondrial electron transport chain, attacking polyunsaturated fatty acids and causing damage in cellular membranes. Malondialdehyde is an aldehyde formed as a result of lipid peroxidation (Halliwell & Chirico, 1993).

The significant 2-fold increase in the MDA concentration in extract treated cells suggests that lipid peroxidation was increased. This could be genotoxic and may initiate apoptosis (Shvedova et al., 2002).

Genotoxicity

Cells treated with the extract or cyclophosphamide exhibited a distinct DNA comet (head and tail) fragmentation pattern as evidenced by the increase in DNA migration compared to the control cells.

The results generated from the scoring of the comets were evaluated for changes in DNA integrity when compared to the negative control (Table 2).

Analysis performed by Yusuf, Vian, Sabatier, and Cano (2000) on several known promutagens did not reveal a particular parameter among the Olive tail moment, tail extent moment, tail length and % tail length/head length that could better indicate the amount of DNA damage caused.

The DNA fragmentation observed in extract treated cells suggests that the extract may have induced genotoxicity in HepG2 cells. Reactive oxygen species-mediated lipid peroxidation is capable of inducing cytotoxicity, genotoxicity and apoptosis (Shvedova et al., 2002). DNA fragmentation is also a hallmark, late-stage event of apoptosis (Kam & Ferch, 2000).

CYP3A4 Activity

Results indicate that the extract and dexamethasone increased the activity of CYP3A4 by 1.2-fold and minimally respectively, and in both cases statistical significance was not reached (Table 3).

Previous studies have shown that HepG2 cells express low levels of CYPs, including CYP3A4, compared to primary human hepatocytes (Westerink & Schoonen, 2007). The concentration at which the extract was assessed for its ability to induce CYP3A4 was at the IC_{50} of cell viability. It may have occurred that this concentration was too low to induce CYP3A4 in HepG2 cells. Thus, although the extract did not significantly induce CYP3A4 at the IC_{50}, this does not exclude the possibility that it is capable of modulating these enzymes.

Dexamethasone induction of CYP3A4 is well documented, however the mechanism by which this induction occurs is not fully understood, with many studies presenting conflicting data (Pascussi et al., 2001). Induction of CYP3A4 by xenobiotics is thought to be largely due to xenobiotic activation of the pregnane X receptor (Tompkins & Wallace, 2007). A study by Luo et al. (2002) demonstrated that dexamethasone weakly activated this receptor and thus weakly induced CYP3A4 in primary human hepatocytes.

Experimental

Chemicals

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Calbiochem (Johannesburg, South Africa) and emetine dihydrochloride from Sigma (St Louis, MO, USA). All tissue culture reagents were obtained from Whitehead Scientific (Johannesburg, South Africa). All other reagents were obtained from Merck (Johannesburg, South Africa).

Plant Material and Extract Preparation

Different extracts of the bulbs were previously prepared from the plant (voucher DuToit2, NU) to investigate anti-inflammatory and antimicrobial activity (Du Toit et al., 2011). An

aqueous extract was prepared by dissolving part of the dried and filtered crude material in water (10 mg/ml) by means of agitation.

Cell Culture

The human HepG2 hepatocellular carcinoma cell line was cultured in a humidified environment (37 °C; 95% O_2, 5% CO_2) to confluency in complete culture medium (CCM) consisting of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) Penstrep-Fungizone® solution. Cells were trypsinised and seeded according to the type of assay.

Cell Viability

Cell viability was measured using the methylthiazol tetrazolium assay. Cells were seeded into a 96-well microtitre plate (15 000 cells/well) and treated in triplicate with varying concentrations of the extract in CCM for 24 hours. The known cytotoxic drug emetine was used as a positive control. Emetine is an alkaloid anti-amoebic drug which has been reported to enhance cytotoxicity (Lee & Wurster, 1995). Thereafter, cells were incubated with a MTT salt solution (5 mg/ml in phosphate buffered saline (PBS)) and incubated (4 h; 37 °C). Culture fluid was then aspirated and dimethyl sulphoxide (DMSO) (100 µl/well) was added to lyse the cells and the plate was incubated (1 h; 37 °C). Optical density was measured (570 nm; reference 690 nm) with an ELISA plate Reader (Bio-Tek µQuant). The concentration of the extract that reduced cell viability to 50% (IC_{50}) was determined and used as a reference concentration to investigate potential mechanisms of cytotoxicity. Cells were treated with the extract (0.03 mg/ml; 24 h; 37 °C) in CCM and seeded according to the type of subsequent assay. The negative control was untreated cells that received CCM only.

Metabolic Activity

Changes in energy exchange required to drive cellular processes were investigated by determining the intracellular ATP concentration with a CellTiter-Glo® kit (Promega, Madison, WI, USA). Treated cells were seeded into a white luminometer plate (20 000 cells/well) in duplicate followed by the addition of 10 µl/well of the supplied reagent. The plate was agitated and incubated in the dark (10 min; room temperature) to lyse the cells. Thereafter, the luminescent signal was measured on a microplate luminometer (Turner Biosystems, USA). The ATP concentration was expressed as Relative Light Units (RLU).

Phosphatidylserine Externalisation

The loss of cell membrane asymmetry and subsequent phosphatidylserine externalisation during apoptosis was determined by measuring the amount of annexin-V bound to phosphatidylserine. An Annexin-V-FITC kit (Roche Diagnostics, Penzberg, Germany) was used according to the manufacturer's instructions. Briefly, 500 000 cells per condition were transferred into polystyrene cytometry tubes. Cells were stained with 100 µl of the Annexin-V-FLUOS labelling solution and incubated in the dark (15 min; room temperature). Flow cytometry analysis was conducted using a FACS Calibur flow cytometer (BD Biosciences, Johannesburg, South Africa) set at an excitation wavelength of 488 nm. Data was collected from 2 500 events per sample in duplicate and cells were gated to exclude necrotic cells.

Caspase-8 and -9 Activity

The activity of the initiator caspases of apoptosis, caspase-8 and -9, were investigated using the Caspase-Glo® 8 and Caspase-Glo® 9 assay kits respectively (Promega). Reagents were prepared according to the manufacturer's instructions. Briefly, cells were seeded into a white luminometer plate (20 000 cells/well) in duplicate followed by the addition of 10 µl/well of either the Caspase-Glo® 8 or Caspase-Glo® 9 reagent. The plate was agitated (300-500 rpm; 30 s) and incubated in the dark (30 minutes; room temperature). Luminescence was measured on the microplate luminometer and activity expressed as RLU.

Mitochondrial Membrane Depolarisation

An increase in mitochondrial membrane depolarisation and subsequent decrease in mitochondrial membrane potential, which contribute to mitochondrial dysfunction in apoptosis was investigated by measuring fluorescence of the cationic lipophilic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1) as part of the JC-1 Mitoscreen assay (BD Biosciences). Briefly, 500 000 cells per condition were transferred into polystyrene cytometry tubes. Cells were incubated with 150 µl/condition of the JC-1 dye (10 min; 37 °C). Thereafter, cells were washed in JC-1 wash buffer, centrifuged (400× *g*; 5 min) and resuspended in 300 µl of flow cytometry sheath fluid. Flow cytometry analysis was conducted using the FACS Calibur flow cytometer set at an excitation wavelength of 488 nm. Results were expressed as a percentage of depolarised mitochondria contained in the cells.

Oxidative Damage

Lipid peroxidation of membrane fatty acids by free radicals caused by the extract was investigated by measuring the concentration of the end product malondialdehyde (MDA) using a thiobarbituric acid-butylated hydroxytoluene solution. Following treatment of the cells, 200 µl culture medium per condition was aspirated and dispensed into glass tubes. Each tube received 200 µl of 2% (v/v) H_3PO_4, 400 µl of 7% (v/v) H_3PO_4 and 400 µl of 1% (v/v) thiobarbituric acid + 0.1 mM butylated hydroxytoluene solution. A positive control of 1% MDA was prepared, as well as a blank negative control containing 400 µl of 3 mM HCl in place of the thiobarbituric acid + butylated hydroxytoluene solution. Solutions were adjusted to pH 1.5 and heated in a water bath (15 min; 100 °C). Once cooled to room temperature, 1.5 ml of butanol was added into each tube. Samples were allowed to settle after vortexing and 500 µl of the butanol phase from each tube was transferred to Eppendorf tubes and centrifuged (13 200 rpm; 6 min; 24 °C). Thereafter, 100 µl of the sample and controls was transferred into a 96-well microtitre plate in duplicate and the optical density was measured (532 nm; reference 600 nm) using the ELISA plate reader. Results were expressed by dividing the values by the absorption coefficient (156 mM^{-1}).

DNA Integrity

The potential genotoxic effects of the extract were investigated using the alkaline comet assay (Singh, McCoy, Tice, & Schnyder, 1988), with minor modifications. The known promutagen cyclophosphamide (10 µM) was used as a positive control (Yusuf et al., 2000). Subsequent to treatment of the cells, the culture medium was aspirated and cells were rinsed three times with PBS, trypsinised and transferred to 15 ml Sterilin tubes. The tubes were centrifuged (400× *g*; 10 min; 24 °C), the supernatants aspirated and cells were resuspended in PBS. Duplicate slides of cells treated with the extract and respective controls were prepared using low melting point agarose and subjected to electrophoresis. Digital pictures were taken with an Olympus IX5I fluorescent microscope (Olympus America Inc., PA, USA). Randomly selected comets (40 comets/slide) from each condition were scored and DNA migration analysed using the Comet Assay IV® software (Perspective Instruments Ltd., Suffolk, UK).

CYP3A4 Activity

The effect of the extract on the activity of the CYP3A4 subfamily of hepatic enzymes was investigated using the P450-Glo™ 3A4 assay kit (Promega) according to the manufacturer's

instructions. The reconstituted luciferin detection reagent and luciferin-PFBE substrate solution were prepared prior to the assay. Cells were seeded into a white luminometer plate (20 000 cells/well) in triplicate and allowed to attach overnight. Cells were then treated with the extract or the known inducer dexamethasone (50 μM), used as a positive control (Pascussi et al., 2001). Following the incubation, the culture medium was aspirated and 50 μl of the luciferin-PFBE substrate (in CCM) was added to each well such that a final concentration of 50 μM was obtained in the wells. Cells were then incubated (4 h; 37 °C). Thereafter, 50 μl of the luciferin detection reagent was added and allowed to react by briefly agitating the plate. The plate was allowed to equilibrate in the dark (20 min; room temperature). Luminescence was measured on the microplate luminometer and activity expressed as RLU.

Statistical Analysis

Data are reported as the mean ± standard error of the mean. GraphPad Prism (version 5.02; GraphPad Software, San Diego, CA, USA) was used to determine the IC_{50} through non-linear least squares regression with variable slope. Statistical comparisons were made by one-way ANOVA followed by Bonferroni's post-test or by Student's two-tailed unpaired *t*-test to determine *p* values. A value of $p < 0.05$ was considered significant.

Conclusions

There was evidence that an aqueous extract prepared from the bulbs of *S. nervosa* was hepatotoxic *in vitro*. It has the potential to induce apoptosis via the extrinsic apoptotic pathway. There was also evidence of genotoxicity, possibly as a result of oxidative stress. CYP3A4 activity was slightly induced although it was not statistically significant. There should be better awareness of the potential toxic effects of traditional medicine.

Acknowledgements

This study was supported by an NRF Masters Freestanding Blockgrant Scholarship.

References

Bangani, V., Crouch, N.R., & Mulholland, D.A. (1999). Homoisoflavanones and stilbenoids from *Scilla nervosa*. *Phytochemistry, 51*, 947-951. doi: 10.1016/S0031-9422(99)00155-7

Brunelle, J.K., & Zhang, B. (2010). Apoptosis assays for quantifying the bioactivity of anticancer drug products. *Drug Resistance Updates, 13*, 172-179. doi: 10.1016/j.drup.2010.09.001

Campbell, I. (2011). Intermediary metabolism. *Anaesthesia and Intensive Care Medicine, 12*, 176-178. doi: 10.1016/j.mpaic.2010.12.006

Du Toit, K., Kweyama, A., & Bodenstein, J. (2011). Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae). *South African Journal of Science, 107*, 96-100. doi: 10.4102/sajs.v107i5/6.259

Halliwell, B., & Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement, and significance. *The American Journal of Clinical Nutrition, 57*, 715-724. Retrieved from: http://www.ajcn.org/

Hengartner, M.O. (2000). The biochemistry of apoptosis. *Nature, 407*, 770-776. doi: 10.1038/35037710

Hutchings, A., Scott, A., Lewis, G., & Cunningham, A.B. 1996. *Zulu medicinal plants: An inventory*. Pietermaritzburg: University of Natal Press.

Kam, P.C.A., & Ferch, N.I. (2000). Apoptosis: Mechanisms and clinical implications. *Anaesthesia, 55*, 1081-1093. doi: 10.1046/j.1365-2044.2000.01554.x

Lee, Y.S., & Wurster, R.D. (1995). Effects of antioxidants on the anti-proliferation induced by protein synthesis inhibitors in human brain tumor cells. *Cancer Letters, 93*, 157-163. doi: 10.1016/0304-3835(95)03796-Y

Luo, G., Cunningham, M., Kim, S., Burn, T., Lin, J., Sinz, M., … Gan, L.-S. (2002). CYP3A4 induction by drugs: Correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metabolism and Disposition, 30*, 795-804. doi: 10.1124/dmd.30.7.795

Ly, J.D., Grubb, D.R., & Lawen, A. (2003). The mitochondrial membrane potential ($\Delta\Psi m$) in apoptosis; an update. *Apoptosis, 8*, 115-128. doi: 10.1023/A:1022945107762

Pascussi, J.-M., Drocourt, L., Gerbal-Chaloin, S., Fabre, J.-M., Maurel, P., & Vilarem, M.-J. (2001). Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. *European Journal of Biochemistry, 268*, 6346-6358. doi: 10.1046/j.0014-2956.2001.02540.x

Pelassy, C., Breittmayer, J.-P., & Aussel, C. (2000). Regulation of phosphatidylserine exposure at the cell surface by the serine-base exchange enzyme system during CD95-induced apoptosis. *Biochemical Pharmacology, 59*, 855-863. doi: 10.1016/S0006-2952(99)00383-4

Sharma, S., Misra, C.S., Arumugam, S., Roy, S., Shah, V., Davis, J.A., … Ray, A. (2011). Antidiabetic activity of resveratrol, a known SIRT1 activator in a genetic model for type-2 diabetes. *Phytotherapy Research, 25*, 67-73. doi: 10.1002/ptr.3221

Shvedova, A.A., Tyurina, J.Y., Kawai, K., Tyurin, V.A., Kommineni, C., Castranova, V., … Kagan, V.E. (2002). Selective peroxidation and externalization of phosphatidylserine in normal human epidermal keratinocytes during oxidative stress induced by cumene hydroperoxide. *The Journal of Investigative Dermatology, 118*, 1008-1018. doi: 10.1046/j.1523-1747.2002.01759.x

Silayo, A., Ngadjui, B.T., & Abegaz, B.M. (1999). Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa subsp. rigidifolia*. *Phytochemistry, 52*, 947-955. doi: 10.1016/S0031-9422(99)00267-8

Singh, N.P., McCoy M.T., Tice, R.R., & Schnyder, E.L. (1988). A simple technique for quantification of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*, 184-191. doi: 10.1016/0014-4827(88)90265-0

Szkudelski, T., & Szkudelska, K. (2011). Anti-diabetic effects of resveratrol. *Annals of the New York Academy of Sciences, 1215*, 34-39. doi: 10.1111/j.1749-6632.2010.05844.x

Tompkins, L.M., & Wallace, A.D. (2007). Mechanisms of cytochrome P450 induction. *Journal of Biochemical and Molecular Toxicology, 21*, 176-181. doi: 10.1002/jbt.20180

Tsujimoto, Y. (1997). Apoptosis and necrosis: Intracellular ATP level as a determinant for cell death modes. *Cell Death and Differentiation, 4*, 429-434. Retrieved from: http://www.nature.com/cdd/index.html

Van der Walt, S.J., & Steyn, D.G. (1946). Recent investigations into the toxicity of plants etc., XV. *Onderstepoort Journal of Veterinary Science and Animal Industry, 21*, 45-55.

Watt, J., & Breyer-Brandwijk, M. (1962). *The medicinal and poisonous plants of southern and eastern Africa*. Edinburgh: Livingston.

Westerink, W.M.A., & Schoonen, W.G.E.J. (2007). Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicology in Vitro, 21*, 1581-1591. doi: 10.1016/j.tiv.2007.05.014

Yusuf, A.T., Vian, L., Sabatier, R., & Cano, J.-P. (2000). *In vitro* detection of indirect-acting genotoxins in the comet assay using Hep G2 cells. *Mutation Research/Genetic*

Toxicology and Environmental Mutagenesis, 468, 227-234. doi: 10.1016/S1383-5718(00)00052-8

Table 1. Caspase-8 and -9 activities (RLU) that were measured in HepG2 cells after treatment with the negative control or extract.

Caspase	Negative control (RLU)	Extract (RLU)
8	16 129 ± 68	21 425 ± 39 (↑ 1.3-fold; $p < 0.001$)
9	492 443 ± 2 522	440 190 ± 1 545 (↓ 1.1-fold; $p < 0.01$)

Table 2. Changes in the Olive tail moment (OTM), tail extent moment (TEM), tail length (TL) and % tail length/head length (%TL/HL) of HepG2 cells treated with the extract or positive control cyclophosphamide for 24 h when compared to the negative control.

Parameter	Extract	Cyclophosphamide
OTM	↑ 3.7-fold ($p < 0.05$)	↑ 9.6-fold ($p < 0.01$)
TEM	↑ 9.5-fold ($p > 0.05$)	↑ 11.8-fold ($p < 0.05$)
TL	↑ 3.4-fold ($p < 0.01$)	↑ 3.5-fold ($p < 0.01$)
%TL/HL	↑ 2.5-fold ($p < 0.05$)	↑3.1-fold ($p < 0.05$)

Table 3. CYP3A4 activity (RLU) in HepG2 cells treated with the extract or positive control dexamethasone for 24 h when compared to the negative control.

Negative control (RLU)	Extract (RLU)	Dexamethasone (RLU)
2 252 ± 96.5	2 694 ± 193.4 (↑ 1.2-fold; $p > 0.05$)	2 263 ± 21.0 ($p > 0.05$)

Appendix 2 (Abstract – School Research Symposium)

Students:

Docrat FS; Ganie F; Mnaheni X; Mohamed S; Mohanlall S; Mseleku S; Mthethwa SF; Naidoo K

Title:

The cytotoxic effects of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) aqueous extract on cultured human oesophageal carcinoma cells

Aims:

Scilla nervosa (Burch.) Jessop (Hyacinthaceae), commonly known in isiZulu as 'ingcino', is widely distributed throughout the eastern parts of Southern Africa. The bulbs have long been used as decoctions in traditional medicine to treat constipation, dysentery, female infertility, nervous conditions in children and pain associated with rheumatic fever. A previous study by our laboratory has shown that extracts prepared from the bulbs exhibited potent antimicrobial and anti-inflammatory activities that would therefore rationalise the traditional use of the plant. Studies have also indicated that the bulbs contain homoisoflavanones and stilbenoids such as resveratrol and rhapontigenin that could be responsible for the therapeutic effects. Currently it is unknown whether the bulbs possess anticancer properties, and if so, what the possible nature of the mechanism(s) is.

Methods:

Cell Culture: The human oesophageal carcinoma cell line SNO was incubated (95% O_2; 5% CO_2; 37 °C) to confluency in DMEM supplemented with 5% foetal calf serum, 1% L-glutamine and 1% Penstrep Fungizone. *Cytotoxicity:* Cells were incubated with an aqueous extract (varying concentrations; 24 h) and viability was measured using the methylthiazol tetrazolium (MTT) assay. Emetine was used as a positive control (1 μg/ml). The IC_{50}-value was calculated and used as a reference to investigate the mechanism. *DNA damage:* After treatment, single cell gel electrophoresis (comet assay) with fluorescent DNA staining was employed to determine double and single strand DNA breaks. Cyclophosphamide was used as a positive control (10 μM). The comets formed were analysed with Comet IV® software and the olive tail moment (OTM), tail extent moment (TEM), tail length (TL) and % tail:head ratio (%T/H) were determined. GraphPad Prism software was used to graph and analyse the data.

Results:

Treatment with an aqueous extract of the bulbs resulted in a concentration-dependent decrease in cell viability and the $IC_{50} = 0.08 \pm 0.014$ mg/ml. Emetine significantly reduced viability to 35.3 ± 2.7% ($p < 0.001$). DNA damage was detected with the extract (OTM increased 4.6-fold, $p > 0.05$; TEM increased 4.1-fold, $p < 0.05$; TL increased 2.4-fold, $p < 0.01$; %T/H increased 1.4-fold, $p > 0.05$) and cyclophosphamide (OTM increased 7-fold, $p < 0.05$; TEM increased 6-fold, $p < 0.01$; TL increased 2.3-fold, $p < 0.01$; %T/H increased 1.7-fold, $p > 0.05$).

Discussion and Conclusion:

These results demonstrate that an aqueous extract from the bulbs of *S. nervosa* exhibits cytotoxicity with DNA damage towards a cancer cell line and suggest that it may have therapeutic potential. Future research should be directed towards determining the specificity of toxicity.

Ethics Number:

Not applicable.

Appendix 3 (Abstract – International Conference)

The Effects of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae) aqueous extract on cultured Hep G2 cells

Prishania Pillay[1], Alisa Phulukdaree[2], Anil A. Chuturgoon[2], Karen du Toit[3] and Johannes Bodenstein[1]*

University of KwaZulu-Natal, Disciplines of [1]Pharmacology and [2]Medical Biochemistry, Durban, South Africa; Tshwane University of Technology, Department of Pharmaceutical Sciences, Pretoria, South Africa

***Correspondence: bodensteinj@ukzn.ac.za**

Purpose

Scilla nervosa, a medicinal plant indigenous to Southern Africa, is traditionally used to treat a diverse range of illnesses. The Zulu people use aqueous decoctions of the bulbs as analgesics in the treatment of rheumatic fever (Bangani et al., 1999). Bulbs have been identified to contain homoisoflavanones and stilbenes (Du Toit et al., 2010). It has been recently demonstrated in our laboratory that extracts prepared from the bulbs possess potent anti-inflammatory properties (Du Toit et al., 2011) and these findings therefore rationalise the traditional use of the plant as an anti-inflammatory agent. However, little information is known about the plant's toxicity and only one historical report suggested 0.5-1 kg of the fresh plant in the flowering stages was toxic to sheep (Van der Walt and Steyn, 1946). Therefore, current research is required into the potential toxicity of the bulbs on the liver as a major detoxifying organ.

In this study we investigated the effects of an aqueous extract of the bulbs in cultured Hep G2 human liver cells, a model system for liver metabolism and toxicity of xenobiotics.

Methods

Cytotoxicity—Hep G2 cells were cultured to confluency, incubated with the extract (varying concentrations; 24 h) and viability was measured using the MTT assay. The IC_{50}-value was determined and used as a reference concentration in the subsequent assays. *DNA Damage*—Single cell gel electrophoresis with fluorescent DNA staining was employed to determine single strand DNA breaks. *Oxidative Damage*—Lipid peroxidation was measured by quantifying the levels of malondialdehyde in a colorimetric assay. *Apoptosis*—The apoptotic potential was determined using fluorescence-activated cell sorting (FACS) and luminometry

with commercial annexin-V-FITC and Caspase-Glo 8/9 assays, respectively. *Mitochondrial Membrane Potential*—FACS and the commercial JC-1 Mitoscreen assay was used.

Results and Discussion

The cell viability of Hep G2 cells showed a dose-dependent decrease and the IC_{50} for the extract was determined as 0.03 mg/ml. DNA fragmentation, as evidenced by an increase in tail length, was more pronounced in the treated cells. Lipid peroxidation increased 2-fold. The percentage of apoptotic cells was higher than the controls, and the intracellular activities of caspase-8 (1.3-fold) and ATP (1.2-fold) increased, while caspase-9 decreased (1.1-fold). There was no change in mitochondrial membrane depolarisation. Results suggest that liver cells are sensitive to an aqueous extract of the bulbs of *S. nervosa*. Future research could be directed towards determining the effects *in vivo*. Our results and future studies would support the policies of the government and United Nations which aim to promote the development, use and regulation of traditional medicine.

References

V. Bangani, N. R. Crouch and D. A. Mulholland, Homoisoflavanones and stilbenoids from *Scilla Nervosa*. Phytochemistry 51, 947-951 (1999).

K. Du Toit, S. E. Drewes and J. Bodenstein, The chemical structures, plant origins, ethnobotany and biological activities of homoisoflavanones. Natural Product Research 24, 457-490 (2010).

K. Du Toit, A. Kweyama and J. Bodenstein, Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae). South African Journal of Science 107, 1-5 (2011).

S. J. Van der Walt and D. G. Steyn, Recent investigations into the toxicity of plants, etc. XV. Onderstepoort Journal of Veterinary Research 21, 45-55 (1946).

Acknowledgements

This study was supported by an NRF Masters Freestanding Blockgrant Scholarship.