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DEVELOPMENT AND EVALUATION OF IMMUNOGENS FOR A YELLOW TULP (*MORAEA PALLIDA*) VACCINE

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SUMMARY

DEVELOPMENT AND EVALUATION OF IMMUNOGENS FOR A YELLOW TULP (*MORAEA PALLIDA*) VACCINE

By

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The aim of this study was to investigate if a vaccine can be developed against epoxyscillirosidine, to prevent yellow tulp poisoning in livestock. *Moraea pallida* Bak. (yellow tulp) poisoning is the most important cardiac glycoside toxicosis in ruminants in South Africa. Cardiac glycoside poisonings collectively account for about 33 and 10% mortalities due to plants, in large and small ruminants, respectively. The toxic principle contained by yellow tulp 1α, 2α-epoxyscillirosidine, is a bufadienolide.

Epoxyscillirosidine, proscillaridin and bufalin, were conjugated to [hen ovalbumin (OVA), bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH)]. Adult male New Zealand White rabbits were vaccinated in 3 trials. In trial 1 (T1) and 2 (T2), experimental (n=7) and control (n=5) animals were vaccinated with epoxyscillirosidine-OVA (0.4 mg/rabbit) and OVA (0.8 mg/rabbit), respectively. In T1 Freund's (complete and incomplete) and in T2 Montanide was used as adjuvant, respectively. In Trial 3 (T3), five equal groups of 3 animals each, were vaccinated with proscillaridin-BSA (group 1), bufalin-BSA (group 2),

epoxyscillirosidine-KLH (group 3), epoxyscillirosidine-BSA (group 4) and BSA (group 5), with Montanide as adjuvant, on days (D) 0, 21 and 42 (0.8 mg/rabbit, intradermally). Blood was collected before each vaccination and at 3 weeks after the last vaccination. Antibody response was determined using an indirect ELISA. There was a poor immune response associated with the dose and/or adjuvant in T1. However, after increasing the dose of the immunogen to 0.8 mg (per rabbit) and changing the adjuvant to Montanide, in T2 and T3, antibodies against the conjugates were successfully raised. In T3, epoxyscillirosidine-KLH (group 3) induced the highest immune response. Furthermore, proscillaridin and bufalin antibodies cross-reacted with epoxyscillirosidine and its OVA conjugate in the ELISAs.

Preparatory to *in vitro* studies to assess the efficacy of the raised antibodies to neutralize epoxyscillirosidine, a rat embryonic cardiomyocyte (H9c2) cell line was established and the cytotoxic effect of epoxyscillirosidine was determined. Cells (10 000/well) exposed to epoxyscillirosidine (10–200 µM) for 24, 48 and 72 h were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the lactate dehydrogenase (LDH) assays. Cells (100 000/well) exposed to epoxyscillirosidine (40–200 µM, for 24, 48 and 72 h), were processed and viewed with a transmission electron microscope (TEM). Cell viability indicated a hormetic dose/concentration response, characterized by higher viability (relative to control) at low doses (10–40, 10 and 10–20 µM for 24, 48 and 72 h, respectively) and decreased viability at higher doses. The cytotoxic effect and ultra-structural changes were dose and time dependent. Numerous cytoplasmic vacuoles, karyolysis and damage to the cell membrane, indicative of necrosis, were observed.

The animal vaccination trial was scaled up, to generate more antibodies, for the *in vitro* neutralization studies. Six, adult Mutton Merino whethers were vaccinated with

epoxyscillirosidine-KLH (2 mg subcutaneously), on D0, 21 and 42. Immune response was determined with an indirect ELISA. Antibodies were concentrated and purified using ammonium sulphate precipitation, before evaluation of *in vitro* neutralization efficacy. There was no significant ($p > 0.05$) difference in viability, between cells exposed to a pre-incubated solution of antibodies and epoxyscillirosidine and the epoxyscillirosidine exposed control cells. The antibodies failed to neutralize the toxic effect of epoxyscillirosidine.

In conclusion, conjugated epoxyscillirosidine was an effective immunogen following conjugation to carrier proteins and antibodies were raised in vaccinated animals. Although antibodies against epoxyscillirosidine-KLH were raised in sheep, they failed to neutralize the toxin in the *in vitro* H9c2 cell model. This is possibly because higher ratios of antibodies to toxin are needed to effectively neutralize epoxyscillirosidine than those used in this study. Since antibodies failed to neutralize epoxyscillirosidine in the current study, further studies could optimize the vaccine to produce more specific antibodies with stronger affinity and avidity to be able to neutralize epoxyscillirosidine. Furthermore, the antibody purification method could be adjusted or changed for optimal results in the future. Antibodies against the related commercially available bufadienolides, namely proscillaridin and bufalin, cross-reacted with epoxyscillirosidine and could be investigated in future studies to prevent yellow tulp poisoning by vaccination.

Keywords:

Antibodies, apoptosis, bufalin, BSA, conjugation, cross-reactivity, cytotoxicity, epoxyscillirosidine, Freund's adjuvant, hormesis, H9c2 cells, immunogen, KLH, lactate dehydrogenase, Montanide, *Moraea pallida*, MTT, necrosis, OVA, proscillaridin, TEM, vaccine, yellow tulp.

DECLARATION

The experimental work reported in this thesis was carried out by me at the Section of Pharmacology and Toxicology of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Professor Christoffel Jacobus Botha.

The thesis has not previously been submitted at this University or any other institution of learning for consideration for award of any certificate. Citations from literature and other people's work have been duly acknowledged.

I, Dr Hamza Ibrahim Isa, declare the statements above to be correct.

HI Isa

Prof. CJ Botha (Supervisor)

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

PAPERS EMANATING FROM THIS THESIS

A paper was presented at the 2nd World Congress on Pharmacology and Toxicology, held at Hotel Rome Pisana, Via della Pisana, Roma, Italy, August 16 – 18, 2018.

Hamza I Isa, Catharina H Ferreira, Jan E Crafford, Christoffel J Botha. Epoxyscillirosidine induced cytotoxicity and ultrastructural changes in a rat embryonic cardiomyocyte (H9c2) cell line.

Manuscripts for publication

Hamza Ibrahim Isa, Gezina Catharina Helena Ferreira, Louis Gabriël Jozua Ackerman, Jan Ernst Crafford, Christoffel Jacobus Botha. Raising antibodies against epoxyscillirosidine, the toxic principle contained in *Moraea pallida* Bak. (Iridaceae), in rabbits.

Under review in BMC Veterinary Research

Hamza I Isa, Catharina H Ferreira, Jan E Crafford, Christoffel J Botha. Epoxyscillirosidine induced cytotoxicity and ultrastructural changes in a rat embryonic cardiomyocyte (H9c2) cell line.

Under 2nd review (after minor revision, was resubmitted) in Toxicology Reports (Elsevier).

Hamza I Isa, Catharina H Ferreira, Jan E Crafford, Christoffel J Botha. Evaluation of *in vitro* neutralization of epoxyscillirosidine by antibodies raised in sheep.

Under review in Toxicon (Elsevier).

LIST OF ABBREVIATIONS

AKP	Alkaline phosphatase
Akt	Protein kinase B
ALT	Alanine aminotransferase
AMA	Alpha mannosidase
Ank	Ankyrin
ANOVA	Analysis of variance
AP2	Adaptor protein 2
Apaf 1	Apoptotic protease activating factor 1
APCs	Antigen presenting cells
AST	Aspartate aminotransferase
Atg	Autophagy
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bak	Bcl-2 associated kinase
Bax	Bcl-2 associated X
BCR	B-cell receptor
Bid	BH3 interacting domain
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
Ca^{2+}	Calcium ions
CBM1	Caveolin binding motif 1

CFA	Conditioned feed aversion
Da	Dalton
DCM	Dichloromethane
DMEM	Dulbeccos's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DISC	Death-inducing signaling complex
DLC	Digitalis-like compounds
EDTA	Ethylene di-amine tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
E 1	Ubiquitin activating enzyme
E 2	Ubiquitin conjugation protein
E 3	Ubiquitin protein ligase
EIA	Enzyme immunoassay
ER	Endoplasmic reticulum
ESI	Electron spray ionization
FADD	Fas associated death domain
FBS	Foetal bovine serum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
H_2O_2	Hydrogen peroxide
HRPO	Horse radish peroxidase
HSA	Human serum albumin

Ig	Immunoglobulin
IP3	Inositol 1,4,5-triphosphate
K^+	Potassium ions
kDa	Kilo Dalton
KLH	Keyhole limpet haemocyanin
LC3	Light chain 3
LC_{50}	Lethal concentration 50
LD_{50}	Lethal dose 50
LDH	Lactate dehydrogenase
LSD	Least significant difference
MALDI-TOF	Matrix assisted laser desorption time-of-flight
mIgM	Membrane immunoglobulin M
MS	Mass spectrometry
mTORC	Mammalian target of rapamycin complex
MTT	Methyl blue thiazol tetrazolium
NADH	Nicotinamide adenine dinucleotide (+ Hydrogen)
NADPH	Nicotinamide adenine dinucleotide phosphate (+ Hydrogen)
NaOH	Sodium hydroxide
NK	Natural killer
NEMBA	National Environmental Management: Biodiversity Act
NUNC	Nottingham University Ningbo China
OVA	Ovalbumin
OD	Optical Density

OPD	Ortho-phenylenediamine
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTK	Protein tyrosine kinase
RIPK 1	Receptor-interacting serine/threonine-protein kinase 1
ROS	Reactive oxygen species
SD	Standard deviation
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SR	Sarcoplasmic reticulum
SW	Swainsonine
TBA	Tributyl amine
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF	TNF receptor-associated factor
TRAIL	TNF–related apoptosis-inducing ligand
ULK	UNC 51-like kinase
UV	Ultraviolet
VMP	Vesicle membrane protein

CHAPTER ONE

INTRODUCTION

1.1 Yellow Tulp Poisoning

Poisoning of livestock by plants is a major problem of both veterinary and economic importance in South Africa (Penrith *et al*., 2015). About 600 species of plants have been reported to be poisonous to livestock in southern Africa. Economic losses in the region of 105 million Rand, were reported to be incurred, due to phytotoxins and mycotoxicoses annually in South Africa (Kellerman *et al*., 1996). This was a conservative estimate, as the study did not take into account losses from poor production, reproduction, veterinary costs incurred, abortions, non-utilisation of noxious fields and the decrease in value of infested camps. Among all poisonings due to plants and fungi in South Africa, cardiac glycoside toxicosis causes 33% and 10% mortality in cattle and small stock, respectively. Tulp (Iridaceae) poisoning is the most important (Kellerman *et al*., 1996), followed by slangkop (Hyacinthaceae) poisoning and intoxication with succulent plakkies (Crassulaceae) (Kellerman *et al*., 2005).

Moraea pallida Bak. (Fig. 1.1) also known as yellow tulp, is considered the most significant (Steyn, 1928), among other poisonous tulp species, such as *Moraea miniata* (Fig. 1.3), *M. flaccida*, *M. polystachya* (Fig.1.4) and *M. bipartita*. Yellow tulp is most incriminated in poisoning, as it has an extensive distribution and is found under different conditions, in nearly all provinces in South Africa (Vahrmeijer, 1981). Except for the Western Cape Province, yellow tulp occurs in all other provinces of South Africa, in addition to the neighbouring countries, Lesotho and Namibia (Fig. 1.2). Yellow tulp also colonizes disturbed areas, such as trampled camps and in cultivated lands. Among livestock, cattle is the most frequently affected species (Kellerman *et al*., 1996). All tulp species harbour non-cumulative bufadienolides causing acute intoxication (Kellerman *et al*., 2005). *Moraea polystachya* has a relatively wide distribution (Fig. 1.5) which makes it very important also.

Figure 1.1: *Moraea pallida* in the field (courtesy of the Department of Paraclinical Sciences, University of Pretoria).

Affected animals show involvement of the respiratory, cardiovascular, gastrointestinal and nervous systems. There may be general apathy, weakness of the hindquarters (Fig. 1.7), tremor, respiratory distress and occasionally bruxism and groaning. Tachycardia, various arrhythmias, rumen atony, bloat and diarrhoea may also be observed. Death may occur due to cardiac arrest, within 24-48 h following exposure or animals may recover within three to four days (Steyn, 1928; Naudé, 1977; Strydom and Joubert, 1983). At necropsy, no pathognomonic lesions are observed. Hyperaemia of the intestinal mucosa, subepi- and subendocardial haemorrhages and pulmonary congestion and oedema and congestion of viscera may be observed (Naudé and Potgieter, 1971).

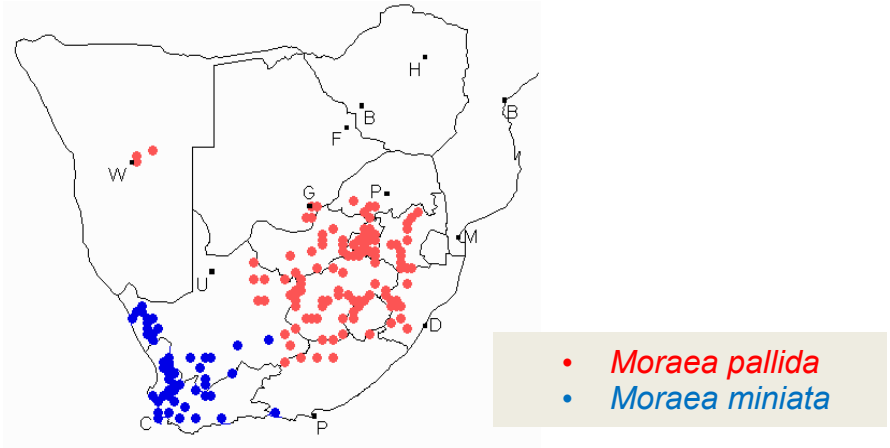


Figure 1.2: Distribution of *Moraea pallida* and *Moraea miniata*

Figure 1.3: *Moraea miniata* in the field (courtesy of the Department of Paraclinical Sciences, University of Pretoria).

Figure 1.4: *Moraea polystachya* in the field (courtesy of the Department of Paraclinical Sciences, University of Pretoria).

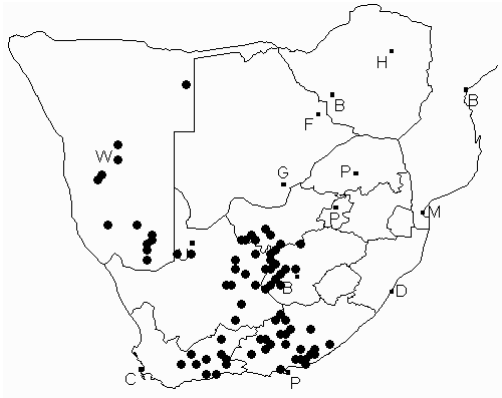


Figure 1.5: Distribution of *Moraea polystachya*

Despite their importance, relatively little has been reported recently, on the toxicity of plants in the genus *Moraea* (Kellerman *et al*., 2005). Most of the reports were from early workers, such as Steyn (1928, 1929), Van der Walt and Steyn (1939, 1940), Enslin *et al*., (1966), Naudé and Potgieter (1966, 1971), where they isolated and characterized the toxic principles from the plants (Kellerman *et al*., 2005). Joubert and Schultz (1982) evaluated activated charcoal in the treatment of sheep intoxicated with *Moraea polystachya*. More recently, conditioned feed aversion (Snyman *et al*., 2003), treatment of poisoning in cattle with activated charcoal (Snyman *et al*., 2009), variability of yellow tulp toxicity (Snyman *et al*., 2011) and spatial variation of epoxyscillirosidine in *Moraea pallida* from different locations (Botha *et al*., 2013) were also reported.

The major toxic principle incriminated in yellow tulp poisoning is 1α, 2α-epoxyscillirosidine (Fig. 1.6), a bufadienolide type of cardiac glycoside. The toxin was isolated, characterized and chemically identified together with another compound 1α, 2α-epoxy-12β-hydroxyscillirosidin (Van Wyk and Enslin, 1969). The molecular formula of the compound is $C_{26}H_{32}O_8.1/2C_6H_6$ and is chemically closely related to scillirosidine, contained by *Urginea maritime* var *rubra* (Enslin *et al*., 1966).

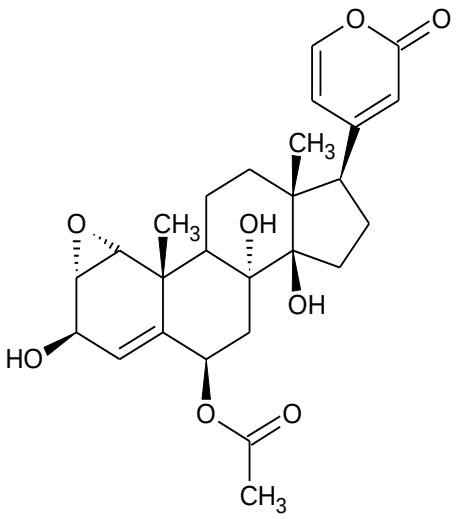


Figure 1.6: Structure of 1α, 2α-epoxyscillirosidine.

Figure 1.7: A heifer in a tulp-infested maize field (A) and in a pen (B) showing weakness of the hindquarters (courtesy of the Department of Paraclinical Sciences, University of Pretoria).

1.2 Justification for the study

Tulp poisoning is a serious problem affecting livestock in South Africa (Naudé and Potgieter, 1971; Kellerman *et al*., 2005). Despite the efficacy of activated charcoal, treatment is nonetheless associated with challenges. Prevention of tulp poisoning usually involves strategic grazing and keeping animals off infested fields, which is not always practical. It is probably easier to manipulate animals to resist the effect of poisoning than other management options (Kellerman *et al*., 2009). Thus it was considered a worthy venture to explore the potential of vaccination as a means of preventing yellow tulp poisoning.

1.3 Aim

The goal of this study was to investigate if a vaccine can be developed against epoxyscillirosidine, to prevent yellow tulp poisoning in livestock.

1.4 Objectives

This study has the following objectives.

To:

1. synthesize epoxyscillirosidine-, bufalin- and proscillaridin-protein conjugates to be able to immunize animals.
2. appraise the effectiveness of the different conjugates in evoking an immunological reaction in rabbits by evaluating antibody titres with an indirect ELISA.
3. determine the degree of cross-reactivity between the antibodies raised against bufalin and proscillaridin with epoxyscillirosidine using an indirect ELISA.
4. scale up the vaccination using the epoxyscillirosidine-KLH conjugate in sheep, to generate more antibodies.
5. conduct *in vitro* cytotoxicity studies on a H9c2 cell line exposed to epoxyscillirosidine and to determine the median lethal dose (LD_{50}).
6. examine the ultra-structural changes induced following exposure to epoxyscillirosidine on H9c2 cells.
7. evaluate the neutralizing and cross-neutralizing efficacy of the raised antibodies against epoxyscillirosidine *in vitro* using the H9c2 cell line.

CHAPTER TWO

LITERATURE REVIEW

2.1 Poisonous plants of veterinary and economic importance in southern Africa

Southern Africa is an area blessed with a flush and varying flora, among which are found a large number of species, capable of inducing toxicosis in humans and most frequently animals (Botha and Penrith, 2008). In South Africa alone, about 600 species of poisonous plants have been reported to abound (Vahrmeijer, 1981). The area has recorded serious livestock losses throughout history, which resulted in an enormous amount of investigations and a corresponding great body of knowledge (Botha and Penrith, 2008). Many of the plants are unique to the southern African region (Kellerman, 2009). Some of the most significant plants causing intoxication in South Africa have been reviewed (Kellerman *et al*., 1996; Kellerman *et al*., 2005; Botha and Penrith, 2008; Penrith *et al*., 2015).

Globally, poisoning by plants is of major economic importance to the livestock farmer. The losses are either direct, such as mortalities, emaciation, loss of unborn foetuses, increased calving intervals, declined productivity and further effects on the animals or indirect losses due to fencing, herding, extra nourishing, veterinary expenditures, husbandry adjustments, and loss of forage (James *et al*., 1992). In the Republic of South Africa, several plant species have been reported to be poisonous, with cardiac glycoside-containing plants being by far the most important (Kellerman *et al*., 1996; Kellerman *et al*., 2005; Botha and Penrith 2008; Kellerman, 2009). Cardiac glycoside toxicoses contribute roughly 33% and 10% of all deaths due to plants and mycotoxicoses in cattle and small stock, respectively. The mortality per year, in cattle due to plant /mycotoxin-induced intoxication in South Africa was estimated to be 37 665 head while that in small stock was about 264 851 head, with an annual total cost to the livestock industry cautiously valued at R104,506,077.00 two decades ago (Kellerman *et al*., 1996). The cost, however excludes hidden losses from diminished production, reproductive failure, wastage of toxic pasture fields and the fall in price of infested land.

2.1.1 Important cardiac glycoside containing plants

Cardiac glycosides are present in an extensive range of native and exotic plants, but the only plants which cause toxicosis of economic importance in South Africa are tulp (Iridaceae), slangkop (Hyacinthaceae) and the succulent plakkies (Crassulaceae) (Kellerman *et al*., 2005). These glycosides exist as secondary metabolites in numerous floras and in certain animals, such as toads (*Bufo* spp) the milkweed butterflies and insects such as *Danaus chryssipus* (Vetter, 2000). Cardiac glycosides affect an inclusive variety of species, among which are humans, however, poisoning in southern Africa is mostly encountered in cattle and sheep (Botha and Penrith, 2008).

2.1.2 Cardenolides and bufadienolides from plant sources

Chemically, cardenolides and bufadienolides are the two major classes of cardiac glycosides elaborated by plants (Krenn and Kopp, 1998). They act through inhibition of the membrane enzyme, sodium potassium adenosine triphosphatase (Na-K-ATPase), which is the only receptor for cardiac glycosides (Steyn and Van Heerden, 1998). More information has been published on cardenolides due to their use in clinical applications, compared to bufadienolides. Digoxin and digitoxin are the two most commonly used cardenolides in clinical medicine for their positive inotropic effect (Steyn and Van Heerden, 1998). Plants that contain cardenolides comprise *Nerium oleander* (oleander), *Thevetia peruviana* (yellow oleander), *Strophanthus* spp. (poison rope), *Acokanthera spp*. (bushman's poison bush), *Gomphocarpus* spp. (milkweeds), *Cryptostegia grandiflora* (rubber vine) and *Adenium multiflorum* (impala lily). Because they have an unpleasant taste, they are rarely eaten by livestock and as such, are not of veterinary significance. However, serious poisoning with severe consequences is frequently encountered after consumption of plants containing bufadienolides (Botha and Penrith, 2008). Bufadienolides cause either acute poisoning (by non-cumulative bufadienolides) or chronic poisoning (by cumulative bufadienolides) (Kellerman *et al*., 2005)

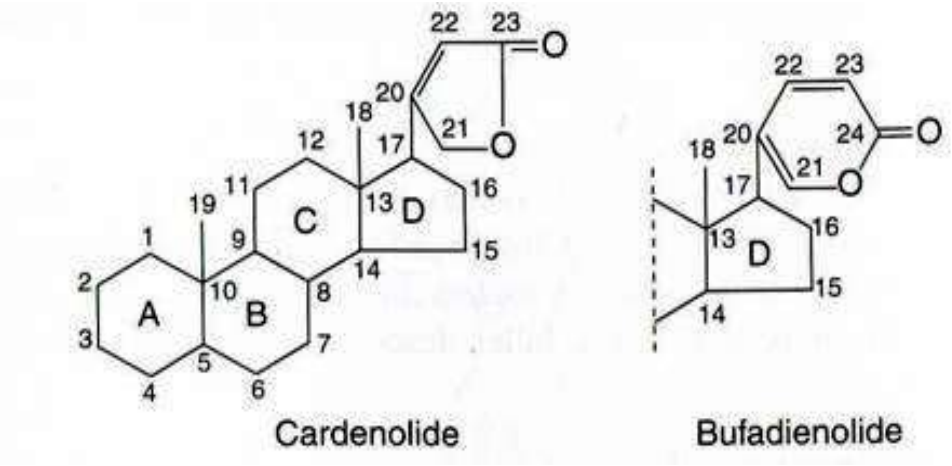


Figure 2.1: Steroid backbone structure of cardenolides and bufadienolides depicting the arrangement and numbering of the carbon atoms.

In cardenolides (Fig. 2.1), the genin is characterized by having a five membered singly unsaturated α, β lactone ring derived from 4-hydroxybutenoic acid (a butenolide) on C-17 of the perhydrophenanthrene nucleus (Naudé 1977; Steyn and Van Heerden 1998; Kellerman, 2009). Bufadienolides (Fig. 2.1) are however characterized by having a doubly unsaturated six membered lactone ring derived from 5-hydroxypentadienoic acid (a pentadienolide) on the C-17 position in the steroid backbone (Naudé, 1977; Schoner, 2013).

Bufadienolides are the toxic principles found in all the significant cardiac glycoside-containing plants in South Africa (Kellerman *et al*., 2005). Acute poisoning usually involves the respiratory, cardiovascular, gastrointestinal and nervous systems, resulting in death or recovery within days, depending on the circumstances surrounding the poisoning (Botha and Penrith, 2008). Cumulative or krimpsiekte-causing bufadienolides, however, involve principally the nervous system occasionally culminating in abrupt demise or more frequently, prolonged paresis or paralysis (Kellerman, 2009). Poisoned animals classically adopt a distinctive stance (with the feet together and back arched), lay down frequently and develop protracted paresis/paralysis. A small number may exhibit persistent torticollis (Kellerman *et al*. 2005).

2.2 Bufadienolide-containing plants causing acute intoxication

Among the species causing acute intoxication, is a number of plants belonging to the family Iridaceae, including *Moraea pallida*, *M. miniata*, *M. polystachya*, *M. bipartita* and *M. carsonii*. In the Hyacinthaceae are *Drimia sanguinea*, *D. macrocentra*, *D. depressa*, *D. altissima*, *D. physodes*, *Pseudogaltonia clavata*, *Merwilla plumbea*, and *Bowiea volubilis*. *Thesium lineatum* and *T. namaquense* belong to the Santalaceae while *Melianthus comosus* and *Melianthus major* belong to the Melianthaceae (Kellerman *et al*., 2005).

2.2.1 *Moraea pallida*

Yellow tulp, scientifically known as *Moraea pallida* is the most important, however, all species of tulp occurring in South Africa are potentially toxic (Kellerman *et al*., 2005). *Moraea pallida* is often incriminated in the poisoning of livestock, with cattle being the most commonly affected species, followed by sheep, goats and donkeys (Kellerman *et al*., 1996).

The corm is white, approximately 30 mm in diameter, covered by a dark-brown fibrous tunic and is deeply buried (up to 200 mm) in the ground. The plant mostly bears a single leaf, at the base of the stem. The leaf emerging from the ground is protected by a spear-like tip, is long (c 600 mm) and narrow (c 20 mm wide), tough and strongly ribbed. The stem may be branched or un-branched and bears 6 – 10, yellow, flowers. The seeds are numerous, angled and contained within club-shaped, three-celled capsules (Stent and Curson, 1922; Phillips, 1926; Vahrmeijer, 1981).

2.2.2 Toxic principle of *Moraea pallida*

The bufadienolide, 1α-2α-epoxyscillirosidine, is contained by *Moraea pallida* (yellow tulp) (Enslin *et al.* 1966; Kellerman *et al.* 2005). The molecular formula of the compound is $C_{26}H_{32}O_8$ and is chemically closely related to scillirosidine (Enslin *et al*., 1966). The concentration of the toxic principle was found to vary in the plant in different geographic locations in South Africa (Snyman *et al*., 2011). In another study, a great difference in average epoxyscillirosidine concentrations amongst different geographic areas was similarly reported. The epoxyscillirosidine concentrations also differed greatly amid distinct plants in the same location, although no general association between epoxyscillirosidine concentrations and soil elemental concentrations was identified (Botha *et al*., 2013). The toxic principle is also responsible for the conditioned feed aversion in livestock (Snyman *et al*., 2004; Kellerman *et al*., 2005; Snyman *et al*., 2009).

2.2.3 Mechanism of action of cardiac glycosides

Cardiotonic glycosides act by impeding the sodium pump (Na^+K^+-ATPase), the ubiquitous enzyme present on the membrane of cells (Klimanova *et al*., 2015). Normally, the Na^+-K^+ pump, of the cardiac myocytes, pumps potassium ions in to and sodium ions out of the cell, with a coupling ratio of 3/2, where it extrudes 3 Na^+ out for every 2 K^+ imported into the cell, at the expense of one molecule of the energy rich ATP (Fontana *et al*., 2013). Cardiac glycosides hinder the ATPase, via steadying it in the E_2-P transition state; occasioning an inability to expel sodium ions, causing the sodium ion concentration to surge, intracellularly over and above physiological levels. There exists a 2nd membrane ion exchanger, the Na^+/Ca^{2+} exchanger, to control the "pumping" of Ca^{2+} out of the cell and Na^+ in ($3Na^+$:$1Ca^{2+}$). Raised intracellular sodium levels, hinder this pump, culminating in non-expulsion of calcium ions, which increase in concentration inside the cell (Fameli *et al*., 2007). Higher than normal intracellular calcium, induces augmented calcium absorption into the sarcoplasmic reticulum (SR), via the sarco/endoplasmic reticulum Ca^{2+}-ATPase carrier. Elevated

calcium reserves in the SR, sanction more calcium release on excitation, so as to produce greater contractions by myocytes (positive inotropic effect). The refractory period of the atrio-ventricular node is elongated and eventually cardiotonic steroids act to mediate cardiac rate (negative chronotropic and dromotropic effects) (Fontana *et al*., 2013).

2.2.4 The sodium potassium adenosine triphosphatase (Na^+K^+-ATPase)

The Na^+K^+-ATPase, a central membrane protein that plays a critical function in ion homeostasis, exists in all tissues and is firmly controlled by several systems (Silva and Soares-da-Silva, 2012). Discovered by Skou (1957), the enzyme catalyzes an ATP-dependent extrusion of three sodium ions (Na^+) out, for two potassium ions (K^+) intruded, into the cell, per pumping cycle, thereby creating a Na^+ gradient across the cell (Silva and Soares-da-Silva, 2012). The resultant Na^+ gradient, powers various cellular processes, including the transport of glucose by intestinal and renal epithelial cells, through a Na^+-glucose co-transporter, in addition to the conveyance of amino acids and additional nutrients (Aperia, 2001; Feraille and Doucet, 2001). The Na^+K^+ pump, also creates the resting potential of cells, vital for neuronal and muscle functions (Therien and Blostein, 2000).

Na^+K^+-ATPase, is a member of a clan of integral membrane proteins, termed P-type ATPases. Members of this class of proteins, symbolize the creation of a transiently phosphorylated aspartate residue, in the course of each catalytic cycle (Silva and Soares-da-Silva, 2012). The pump consist of two major non-covalently bound subunits, the α- and β-subunits (Figs. 2.2 and 2.3) (Feraille and Doucet, 2001; Xie and Cai, 2003). The α-subunit (about 112 kDa), a multispanning membrane protein, is the catalytic subunit which houses the binding site for Na^+, K^+, ATP, steroidal glycosides, and phosphorylation sites for protein kinases A (PKA) and C (PKC) (Schwartz *et al*., 1988; Bertorello *et al*., 1991; Aperia, 2001). Four isoforms of the α-subunit: α1, α2, α3, and α4, have been described, with each having unique tissue distribution (Silva and Soares-da-Silva, 2012).

The sugar-rich ancillary β-subunit, with a molecular weight about 55 kDa, spans the membrane only once (Suhail, 2010). The glycosylated β-subunit functions in enzyme maturation, localization to the plasma membrane, and stabilization of the K^+-occluded intermediary (Geering, 2008). The β-subunit has three known isoforms: β1, β2 and β3. The different isoforms are distributed uniquely, in various tissues of different species of animals (Silva and Soares-da-Silva, 2012).

Lately, a third subunit has been defined, which bind the α/β complex in certain tissues, including the heart, kidney, and brain (Fig. 2.3) (Sweadner and Rael, 2000). This third subunit, belongs in the FXYD family of proteins, a class of structurally similar hydrophobic type I polypeptides, expressed in a tissue-specific fashion (Cornelius and Mahmmoud, 2003; Geering, 2006, 2008). Not a central part of Na^+-K^+-ATPase protein, the FXYD proteins moderate the kinetic properties of the pump, via molecular interfaces with specific Na^+K^+-ATPase domains (Silva and Soares-da-Silva, 2012).

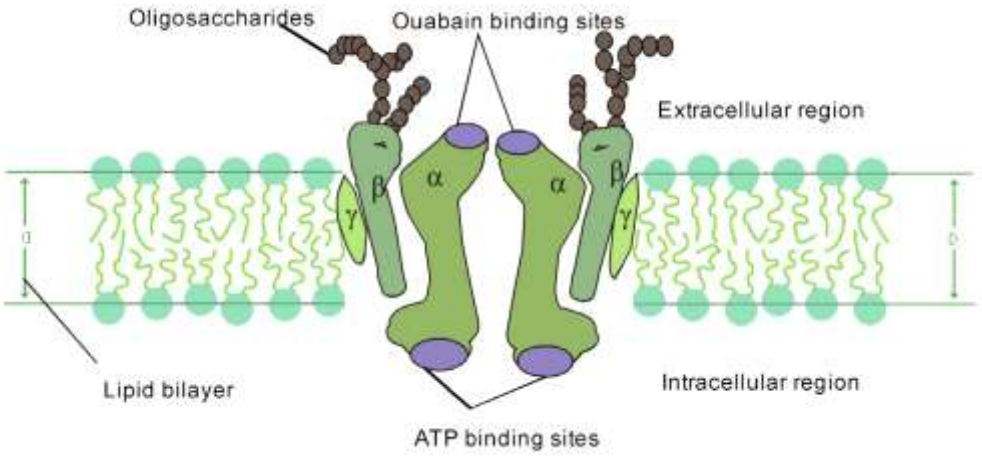


Figure 2.2: Diagrammatic representation of Na^+K^+-ATPase containing the 2α, 2β, 2γ-subunits within the plasma membrane (courtesy Suhail, 2010).

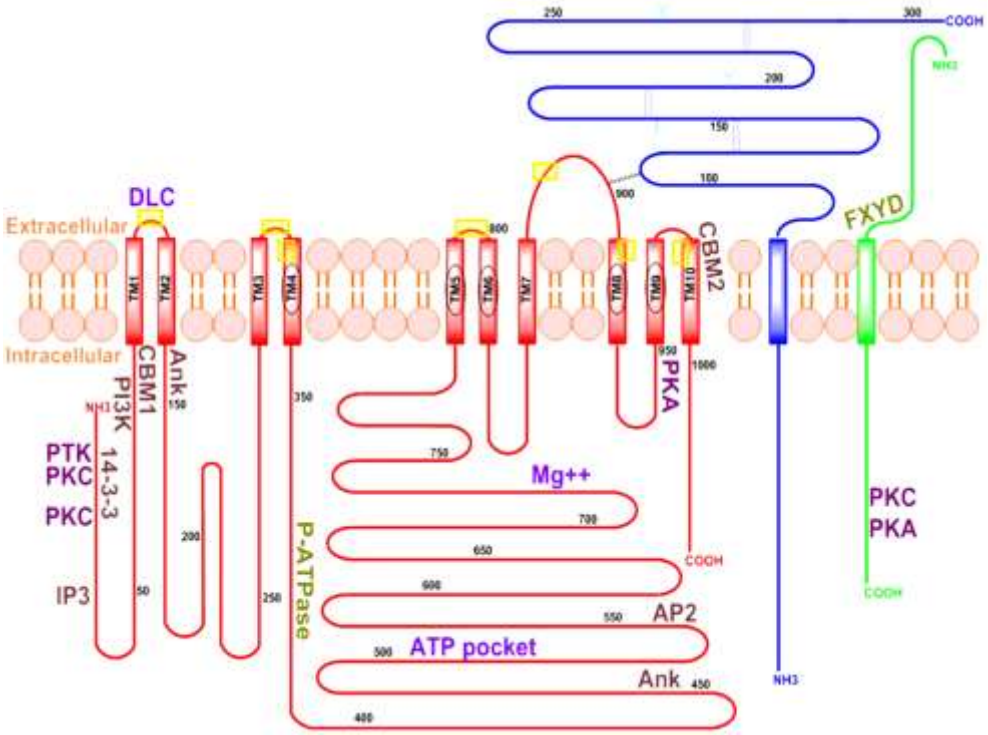


Figure 2.3: Diagrammatic sketch of the Na+, K+-ATPase showing the different subunits. The main ligand binding and active sites on the α1 (red), β1 (blue) and FXYD1 (green) subunits are depicted. Ellipses in the transmembrane domains designate areas associated with Na^+ and/or K^+ binding. DLC, denote the main binding locus for cardiac glycosides. Yellow rectangles designate additional loci associated with cardiac glycoside binding. PTK, PKC and PKA represent phosphorylation sites for protein tyrosine kinase (PTK), protein kinase C (PKC) and protein kinase A (PKA) respectively. Binding site for phospho-inositol-3 kinase, inositol 1,4,5-trisphosphate, 14-3-3 protein, Caveolin 1 and Caveolin 2 are depicted by PI3K, IP3, 14-3-3, CBM1 and CBM2, respectively. Ank, AP2, and Mg^{2+} represent Ankyrin, Adaptor protein 2 and magnesium binding sites, respectively. ATP pocket designates the part involved in the binding of ATP. P-ATPase — denotes the conserved motif of the P-ATPases. FXYD — indicates the conserved motif of the FXYD protein family (courtesy Nesher *et al.*, 2007; figure and legend reproduced with permission from Elsevier).

2.3 Management of cardiac glycoside plant poisoning in domestic stock

Acute poisoning in animals is usually an emergency, requiring immediate therapeutic intervention with specific antidotes or palliative agents. This can be a serious challenge, especially in the field, with possibly severe consequences including death. Furthermore, treatment of already poisoned animals is unsuccessful in most cases. Several therapeutic agents had been tested against cardiac glycoside poisoning in animals without significant success (Kellerman, 2009). However,

Joubert & Schultz (1982) concluded that drenching poisoned animals with activated charcoal is very effective. Despite the efficacy of activated charcoal, it is essential to explore ways to prevent rather than treat poisoning in livestock.

Strategies to minimize effects of poisonous plants, generally, include avoiding conditions such as overgrazing, mineral deficiency and disease which predispose animals to poisoning. It is not always feasible to avoid these conditions due to economic and environmental factors beyond the control of the farmer. Tulp poisoning is usually prevented by herding animals, fencing off infested areas or eradicating the plants (Kellerman *et al*., 2005). Chemical control using herbicides is, alternatively employed, where eradicating the plants physically is not practicable (Steyn, 1928). But this is not without associated hazards and risk of environmental pollution with dangerous chemicals.

Current approaches at controlling plant poisonings, such as by mechanical or chemical eradication of the plants, fencing off weed ridden areas, etc., all include altering the environment in a way, which is not always feasible, due to factors such as capital cost, cash turn over and labour constraints, especially under extensive farming conditions. Thus, there is the need for finding a means of manipulating animals to resist or avoid poisoning (Kellerman, 2009). Methods of exploiting and augmenting an animal's natural ability to avoid toxic plants as well as developing a vaccine against the toxic principle incriminated have been suggested (Kellerman *et al*., 2005).

2.3.1 Conditioned feed aversion (CFA)

Naturally, animals could learn to avoid certain feeds that are associated with toxic (unpleasant) experiences via a phenomenon known as cognitive feed aversion (Bures *et al*., 1998). This aversion may be caused by a specific substance present in the feed and which is usually responsible for the intoxication. Aversion to feed could be induced in an animal through conditioning, whereby animals are gradually exposed to low levels of the harmful substance which

the animals “learn” and try to avoid subsequently. Natural conditioning against yellow tulp poisoning has been reported to be effective where animals raised on tulp infested fields rarely consume the plant. The animals only consume the plant and are poisoned when newly introduced into the infested fields (Kellerman *et al*., 2005). Farmers, historically and traditionally, had thought that CFA could be induced in naïve animals, by pre-dosing them with various concoctions of the plant. To investigate this, Strydom and Joubert (1983), in a limited field trial, utilized different preparations of the plant, but they found no evidence the traditional practice could induce CFA (Kellerman *et al*., 2005). Further investigation found the aversive substance to be epoxyscillirosidine, the toxic principle contained in *Moraea pallida* (Snyman *et al*., 2004). CFA is a potentially useful preventative strategy against yellow tulp poisoning, as it is a natural mechanism, environmentally friendly and could have application in commercial and communal farming system (Kellerman *et al*., 2005).

2.3.2 Potential of vaccines in prevention of plant poisoning

Interest in developing vaccines against phytotoxins has been on the increase in recent times. Scientists are trying to explore the potential of vaccines to prevent plant poisonings in animals, in different parts of the world. This is especially important, as one of the most contemporary challenges facing researchers, is the control and prevention of plant poisonings in the field. Furthermore, earlier studies on cardiac glycoside poisoning revealed that manipulating animals to evade or resist poisoning, is more realizable than attempting to change the environment (Kellerman, 2009).

Several active immunization studies to control plant induced toxicosis in animals have shown promise. The toxic effects of poisonous plants could also be minimized by vaccination, where antibodies will neutralize the toxic compounds (Fonseca *et al*., 2013). Vaccines have been evaluated to prevent poisoning of animals by several plant species. In a study to evaluate the effect of anti-ergot alkaloid vaccination on rabbit performance and protection against fescue toxicosis, it was

reported that following feeding of endophyte-infected tall fescue seed, vaccination had beneficial protective effects (Filipov *et al*., 1998).

In another study to determine if larkspur toxin-protein conjugates could facilitate active immunity in mice, it was concluded that immunization diminished methyllycaconitine toxicity in mice and thus immunization could be beneficial in alleviating the effects of *Delphinium* spp (larkspur) toxins in animals. However, further studies are needed to develop a larkspur vaccine in animals (Lee *et al*., 2003).

A study investigated immunization of goats with the locoweed toxin, swainsonine (SW), and human serum albumin (HSA) conjugate (SW–HAS). High anti-SW antibodies were produced which significantly ($p < 0.01$) lowered the blood SW levels thus decreasing the toxicity of locoweed (Tong *et al*., 2007). Furthermore, analysis of the serum enzymes, namely alkaline phosphatase (AKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), α-mannosidase (AMA) and lactate dehydrogenase (LDH), as well as blood urea nitrogen (BUN) showed vaccination to have protective effects for the immunized goats compared to the control goats (Tong *et al*., 2008).

Botha *et al*., (2007) obtained promising results in developing a krimpsiekte vaccine after immunizing sheep with cotyledoside, the toxic principle contained by *Tylecodon wallichii*. Vaccinated sheep were clinically unaffected, following challenge with daily intravenous doses of cotyledoside, for up to six days.

2.4 Cytotoxicity testing

Evaluating the effects of various agents on the viability of cells *in vitro*, is extensively used, to predict the potential toxicity in animals exposed to such compounds (Riss *et al*., 2011). There are several assays or methods to study cellular toxicity *in vitro* (Kim *et al*., 2009). Techniques used to evaluate cytotoxicity, usually relate cellular activity and/or integrity in control cells with those exposed to a toxicant, stress or disease. The activity being quantified, is determined by the cell type,

the injurious agent used, and the type of cell death being evaluated (Cummings *et al*., 2004). Such methods rely on detecting changes, in various aspects of cellular function, such as metabolism, dye uptake and membrane integrity. It was formerly reported that, different cytotoxicity assays can give dissimilar outcomes, based on the test agent utilized and the cytotoxicity assay used (Weyermann *et al*., 2005).

Among the numerous assays, quantifying the level of ATP is reputed to be the most sensitive, dependable, and appropriate method for evaluating active cell metabolism (Riss *et al*., 2011). It has been recommended, to use additional assay(s), to measure cell viability, in *in vitro* studies, in order to improve the soundness of the outcomes (Fotakis and Timbrell, 2006). Furthermore, newly established combinations of approaches have made it promising, to generate additional data, from *in vitro* cytotoxicity tests, with standard fluorescence and luminescence plate readers (Riss *et al*., 2011).

2.4.1 Methyl blue thiazole tetrazolium bromide (MTT) assay

The methyl blue thiazol tetrazolium (MTT) assay is one of the most commonly utilized in cell viability assays (Berridge and Tan, 1993). It has been reported in numerous scientific publications. The assay is based on a protocol originally developed by Mossman (1983). The method is a quantitative colorimetric assay that spectrophotometrically measures the amount of purple formazan crystals formed by dehydrogenases (in living viable cells) from the yellow tetrazolium salt (MTT). Thus, the assay measures the conversion of MTT into purple-coloured formazan by the redox activity of living cells and a decrease in cellular MTT reduction can be a manifestation of cellular impairment (Abe and Matsuki, 2000)

It was initially believed that mitochondrial dehydrogenases solely metabolize MTT (Mossman, 1983), which was originally derived from the studies of Slater *et al*., (1963). However, later evidence indicated microsomal fractions also reduce MTT to a larger extent (Berridge and Tan,

1993; Berridge *et al*., 2005). It is currently recognized that the mitochondrion is just one of the MTT reduction locations, while non-mitochondrial, cytosolic and microsomal MTT reduction contributes to the greater overall reduction. In fact, MTT reduction takes place all through a cell, which utilizes NADH/NADPH, by oxidoreductases. Furthermore, superoxide might likewise play a role in intracellular MTT reduction, while the superficial oxidoreductases account for extracellular MTT reduction (Berridge *et al*., 2005).

Alterations in the function of lots of the mitochondrial and non-mitochondrial oxidoreductases, cellular metabolic and energy perturbations, and oxidative stress could seriously influence the MTT assay data (Stepanenko and Dmitrenko, 2015). It was shown that the MTT assay, does not truly quantify cell growth or number of viable cells, however, it measures a coordinated set of enzyme functions which are interconnected to cellular metabolism in numerous ways (Berridge *et al*., 2005).

Despite the robustness of the assay, it has some limitations. Manipulations of the experimental conditions, can lead to exaggeration or underestimation of viability. Various inhibitors and proteins could significantly alter MTT assay data by over/underestimating viability (Stepanenko and Dmitrenko, 2015). In addition, different tested compounds may interact directly with, and reduce MTT (Chakrabarti *et al*., 2000; Peng *et al*., 2005; Ulukaya *et al*., 2008), interfere with mitochondrial dehydrogenases (Weyermann *et al*., 2005; Ulukaya *et al*., 2008) or they could induce generation of free radicals, resulting in greater reduction of MTT (Collier and Pristos, 2003). Thus each experiment must be carefully designed and care taken, in interpreting the results. This notwithstanding, the MTT assay remains one of the most popular assays, and it has attained a somewhat gold standard stature, having been utilized in countless studies (van Tonder *et al*., 2015).

2.4.2 Lactate dehydrogenase (LDH) assay

Cytotoxicity is caused by the adverse effects of various chemical and physical agents on cells (Cummings *et al*., 2004). The lactate dehydrogenase (LDH) release assay is another commonly utilized assay to determine cytotoxic effects of compounds in cell culture. LDH is a metabolic enzyme found exclusively in the cytoplasm. However, upon injury or death of cells LDH will be released into the cell culture medium. Thus, by evaluating LDH release cell death can be measured directly (Larner *et al*., 2016). The assay is reputed to be reliable, speedy, with simplicity of evaluation (Decker and Lohmann-Matthes, 1988).

However, major shortcomings relating to the use of the LDH assay can arise due to a range of medium supplements regularly utilized in cell culture studies. Primary amongst these is the use of foetal bovine serum (FBS) (Thomas *et al*., 2015). The substantial quantity of LDH in FBS surpasses that released by test cells, hence decreasing the sensitivity of the LDH assay (Thomas *et al*., 2015). Reducing serum content of cell culture media has been recommended to optimize the sensitivity of the assay (Decker and Lohmann-Matthes 1988; Bopp and Lettieri 2008). This should be done with caution, however, to avoid serious consequences, especially in cell lines requiring high amounts of serum supplementation (Parsons *et al*., 2011). There is thus the need to carefully design the experiment and the resultant data interpreted with caution.

2.4.3 Transmission electron microscopy

Transmission electron microscopy (TEM) is a very valuable tool in studying cells exposed to different experimental conditions. There are several reports, on the use of TEM, to study morphological changes induced in cells, following exposure to different conditions *in vitro* (Li *et al*., 2015; Fujita *et al*., 2018).

2.5 Response to lethal cell injury

Eradicating unwanted or damaged cells by way of cell death is crucial for major biological processes, including animal growth and development, tissue homoeostasis and stress response (Chen *et al*., 2016). In multicellular organisms, cell demise remains an indispensable biological phenomenon (Chen *et al*., 2018). Depending on the morphological and biochemical features, cell death could be categorized into several major classes, including apoptosis, necrosis, autophagic death and mitotic catastrophe (Galluzi *et al*., 2012). However, apoptosis and necrosis remain the most distinct and best characterized cell death pathways (Chen *et al*., 2016). These together with other classical cell death phenomena present divergent morphological features by initiating unique signalling pathways (Kroemer *et al*., 2005) Apoptosis, known as programmed cell death, is a highly controlled process while necrosis was formerly assumed to be an accidental and passive process, not well coordinated. Recently however, this notion has changed following studies in the recent years that resulted in the documentation of a synchronized form of necrosis (Chen *et al*., 2016).

Following lethal injury, any of the cell death modalities may prevail as determined by the nature of the insult, its duration and the intracellular metabolic capability of the cell to maintain favourable cellular environment, since all the pathways are intertwined (Loos and Engelbrecht, 2009). There exist associations between the three major types of cell death (Chen *et al*., 2018). The frontier between apoptosis and autophagy is unclear and perhaps non-existent due to many intrinsic factors in different cell types and cross-talk between organelles within each type. Thus, the decision, when cells embark on a given death pathway, is a complex process (Lockshin and Zakeri, 2004). It seems that autophagy promotes cell survival by avoiding a metabolic crisis and delays the onset of apoptosis and necrosis (Loos and Engelbrecht, 2009). However, ATP is required for the active execution of the final phase of apoptosis and intracellular ATP concentrations determine the eventual cell death modality (Leist *et al*., 1997).

2.5.1 Apoptosis

Apoptosis is in general a caspase-facilitated programmed cell death (Garrido and Kroemer, 2004, Galluzi *et al*., 2008). Apoptotic cells show discrete morphological features, comprising cell shrinkage, chromosome condensation, nuclear fragmentation (late stage), plasma membrane blebbing and the development of apoptotic bodies, and demonstrate biochemical alterations, including the exteriorization of phosphatidyl-l-serine outside the plasma membrane (early stage) (Degterev *et al*., 2003; Danial and Korsmeyer, 2004). Apoptosis can be initiated by way of either the death receptor facilitated, mitochondria-dependent or endoplasmic reticulum (ER) stress induced pathways (Nakagawa *et al*., 2000). A schematic description of apoptosis is presented in Figure 2.4.

The death receptor-facilitated apoptosis mode is initiated following the binding of the Fas ligand, TNF-α (tumour necrosis factor α) or TNF-related apoptosis-inducing ligand (TRAIL) to the matching death receptors (Wang and El-Deiry, 2003; Lavrik *et al*., 2005). The adaptor protein Fas-associated death domain (FADD) (Lavrik *et al*., 2005; Duprez *et al*., 2009) and the procaspase-8 protein unite, forming a complex, viz. death-inducing signaling complex (DISC) (Chen *et al*., 2018). In DISC, procaspase-8 is activated by autohydrolysis (Wang and Tjandra, 2013). The activated caspase-8 converts the apoptosis signal via the instigation of caspase-3 or slicing of BH3-interacting domain (Bid) to truncated Bid (tBid). tBid then transfers to the mitochondria, which leads to configurational modifications of Bax and Bak and their oligomerization for pore formation in the external mitochondrial membrane (Fan *et al*., 2005; Wang and Tjandra, 2013).

The mitochondrial-dependent pathway could be triggered by a number of stress inducers, including DNA damage, growth factor removal and oxidative stress (Favaloro *et al*., 2012; McIlwain *et al*., 2013). The Bcl-2 clan of proteins regulates this intrinsic pathway by controlling the porosity of the mitochondrial external membrane (Özören and El-Deiry, 2002; Ricci *et al*., 2004). Following release from the mitochondria into the cytoplasm, cytochrome *c* combines with Apaf-1 to bring forth

activation of caspase-9, which now stimulates effector caspases (Yuan and Akey, 2013) to initiate a torrent of proteolytic events (Chen *et al*., 2018).

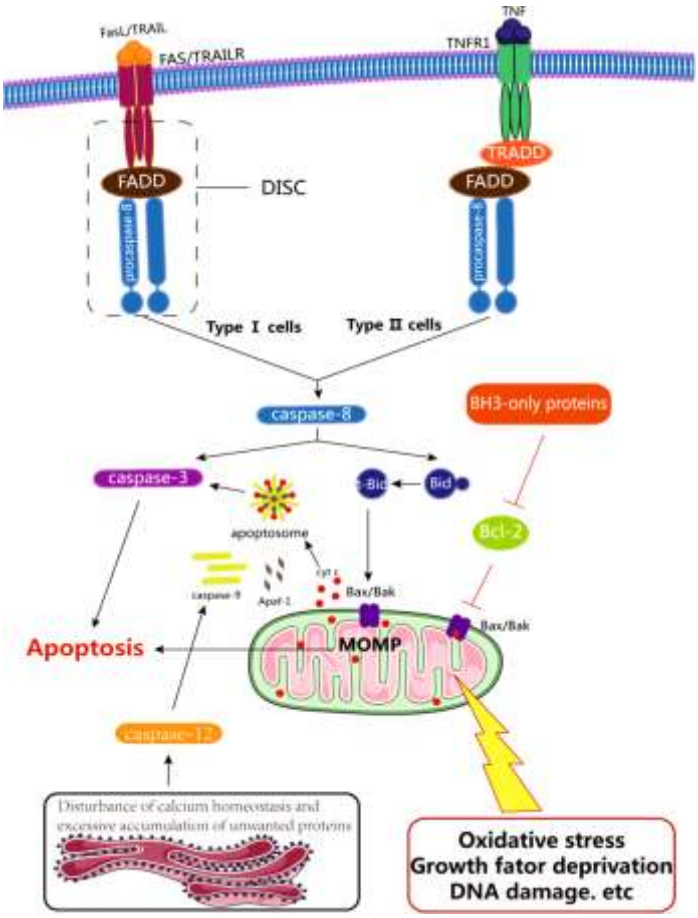


Figure 2.4: Mechanisms of apoptosis. In the exogenous pathway, the binding of FASL, TNF-α, or TRAIL to their analogous receptors transmutes procaspase-8 to caspase-8 via autohydrolysis. In type I cells, activated caspase-8 can trigger caspase-3, and subsequently apoptosis. In type II cells, stimulated caspase-8 could hydrolyze Bid to tBid, subsequently tBid interacts with Bax/Bak, which is found in the mitochondria, to prompt apoptosis. In the intrinsic apoptosis pathway, injury to DNA, growth factor removal, oxidative stress, or noxious injury can disrupt the homeostasis of the mitochondria, normally regulated by the Bcl-2 family members, which could result in increased mitochondrial membrane permeability, to cause the release of cytochrome *c* from the intermembrane space of the mitochondria. Furthermore, the released cytochrome *c* can interrelate with Apaf-1 and caspase-9, to activate caspase-3 and bring forth apoptosis. In the ER stress-induced apoptosis pathway, disruption of Ca^{2+} homeostasis and excessive build-up of unwanted proteins in the ER facilitate caspase-12-mediated apoptosis, wherein activated caspase-12 relocates from the ER into the cytosol, to slice caspase-9 and then activate caspase-3 (courtesy Chen *et al*., 2018).

Furthermore, ER stresses, for example calcium homeostasis perturbation, excessive unfolding, or misfolded protein build-up in the ER, nutrient deficiency, and hypoxia, can prompt

apoptosis. This apoptosis is interceded by caspase-12, an ER-resistant caspase (Nakagawa *et al*., 2000). Activated caspase-12 directly slices caspase-9 after moving from the ER into the cytosol, followed by caspase-3 activation (Morishima *et al*., 2002). The molecular mechanisms of activation of caspase-12 during ER stress include forming a complex with the inositol requiring enzyme-1α-TNF receptor-associated factor 2 (TRAF2) complex (Yoneda *et al*., 2001) or by calpains, a class of Ca^{2+}-dependent intracellular cysteine proteases (Ellis and Horvitz, 1986).

2.5.2 Autophagy

Autophagy is an auto-degradative phenomenon in reaction to different stresses, such as nutrient deficit, injury to subcellular units, oxygen hypoxic states, reactive oxygen species (ROS), ER stress, and drugs. The autophagy phenomenon comprises four fundamental stages—initiation, nucleation, fusion of autophagosome and lysosome and hydrolyzation. Insights into the molecular processes leading to autophagy have been derived from studies on yeast (Chen *et al*., 2018). A group of autophagy controlling molecules was recognized by genetic screening in yeast, principally autophagy (Atg)-related proteins, being the major actors in autophagy. The coming together and accumulation of the Atg1 network, comprising Atg1, Atg13, Atg17, Atg29, and Atg31 (Cheong *et al*., 2008; Kawamata *et al*., 2008), are vital for the creation of the phagophore at the initiation step (Hamasaki *et al*., 2013). But in mammals, the UNC-51-like kinase 1 (ULK)-mAtg13-FIP200 complex, including the corresponding analogues to yeast Atg1, Atg13 and Atg17, is formed (Jung *et al*., 2009). At the nucleation stage, phagophore formation in the ER and other membranes is governed by a network of the class III PI-3 kinase VPS34, Atg6 (known as Beclin1 in mammals), Atg14 and Vps15. Atg9 and vesicle membrane protein 1 (VMP1), that circulate in the Golgi complex, autophagosomes and endosomes, can play a role in carrying lipids to the isolation membrane (He and Klionsky, 2007; Molejon *et al*., 2013). The enlargement and shutting of the autophagosome need two ubiquitin-like protein-conjugated schemes, namely, Atg12 and Atg8 (Atg8

is also known as LC3 in mammals) (Ohsumi, 2001). The Atg12 system is composed of five Atg proteins, Atg5, Atg7, Atg10, Atg12, and Atg16 (Mizushima *et al*., 1999). Atg12 is triggered by Atg7, which is an E1-like enzyme (Mizushima *et al*., 1998) and is thereafter relocated to the E2-like enzyme Atg10 (Shintani *et al*., 1999).

Lastly, there is covalent binding of C-terminal glycine of Atg12 to the Lys149 side chain of Atg5 prior to binding to the dimer protein Atg16 resulting in formation of the E3-like complex (Mizushima *et al*., 1998). The Atg8 system comprising four Atg proteins, Atg3, Atg4, Atg7, and Atg8, embodies additional ubiquitin-like protein-conjugated system (Ichimura *et al*., 2000). Atg8 is sliced by Atg4, a cysteine protease to expose its C-terminal glycine residue (LC3 I in mammals) (Kirisako *et al*., 2000). Atg8 is additionally stimulated by Atg7, an E1-like enzyme which is thereafter conveyed to Atg3, an E2-like enzyme (Ichimura *et al*., 2000), prior to covalent bonding to the amidogen of PE through the E3-like Atg12-Atg5-Atg16 complex (Ichimura *et al*., 2000; Hanada *et al*., 2007). The Atg8-PE covalent structure (LC3 II in mammals) confers Atg8 membrane tethering and hemifusion ability and is a crucial actor in autophagosome formation. LC3 II is associated with both the external and internal membranes of the autophagosome and is a characteristic indicator for autophagy (Chen *et al*., 2018). The Atg8-PE covalent structure can be revocably sliced to Atg8 by Atg4 for the reprocessing of Atg8. Consequently, the merging of autophagosome and lysosome is facilitated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-like proteins (Cheng *et al*., 2017; Diao *et al*., 2017). Lastly, at a low pH, numerous lysosomal enzymes digest different kinds of damaged organelles, proteins, lipids, and nucleic acids (Mizushima *et al*., 2011; Hamasaki *et al*., 2013). The autophagy process is diagrammatically presented in Figure 2.5.

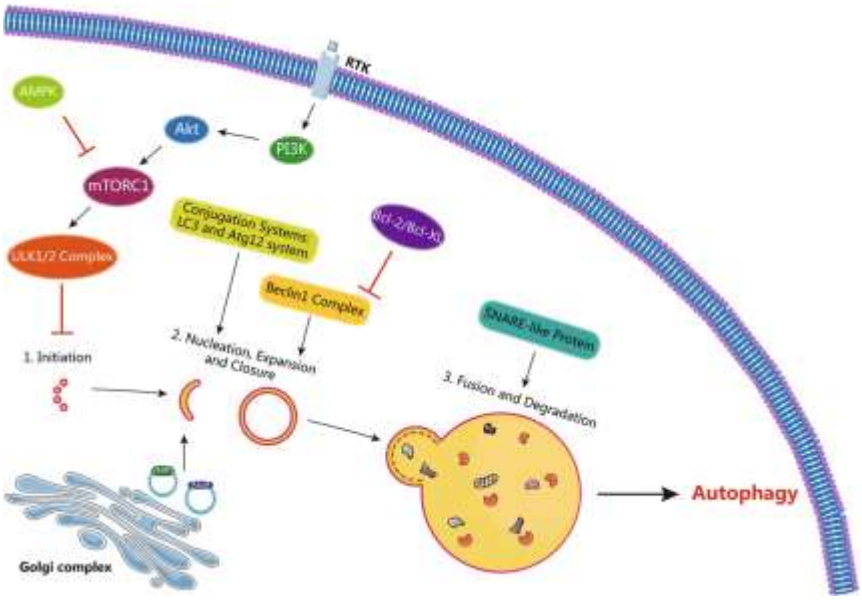


Figure 2.5: Mechanisms of autophagy. The progression of autophagy can be partitioned into four stages, viz. initiation, nucleation, expansion and closure, and fusion and degradation. In mammals, the assembly of the ULK1/2 complex is indispensable for the formation of the phagophore assembly locus, while the ULK1/2 complex is controlled by mTORC1, which is positively regulated by PI3K/AKT and negatively regulated by AMPK. Growth factors trigger the PI3K/Akt pathway via receptor tyrosine kinases (RTKs). The Beclin1 complex, which is typically inhibited by Bcl-2, is triggered and pushes the isolation membrane to nucleation. The transmembrane protein Atg9 and vesicle membrane protein VMP1 may play a role in carrying lipids to the isolation membrane. Furthermore, two ubiquitin-like protein-conjugated systems (Atg12 and LC3 systems) are vital for this process. Consequently, the fusion of the autophagosome with lysosome is facilitated by SNARE-like protein, and, eventually, dissolution of all kinds of damaged organelles, proteins, lipids, and nucleic acids by diverse lysosomal enzymes (courtesy Chen *et al*., 2018).

2.5.3 Necrosis

Originally, the term necrosis was used to define the unregulated and irreversible tissue damage, which seemingly occurs after the injured cells have already died (Majno and Joris, 1995), which was more like the end result and not a process. Necrosis had for long been considered to be an accidental cell death, but later evidence showed it to be an organized cell death pathway (Kroemer *et al*., 2005; Festjens *et al*., 2006).

The discovery of programmed necrosis led credence to the existence of multiple cell death processes (Chen *et al*., 2018). The different types of regulated necrosis that have been reported,

include necroptosis (Galluzi *et al*., 2012), parthanatos (Xu *et al*., 2016), ferroptosis (Shimada *et al*., 2016), pyroptosis (Abe and Morrell, 2016) and NETosis (Dwivedi and Radic, 2014). However, necroptosis, a type of regulated necrotic cell death that shares several key signalling pathways with apoptosis, seems most important (Chen *et al*., 2018). Most of the knowledge on the subject of necroptosis, was derived from studies on TNF signalling.

TNF is a pleiotropic cytokine that features prominently in inflammation (Popa *et al*., 2007). TNF is also a strong cell death inducer under certain conditions, through binding to TNFR (Vandenabeele *et al*., 2010). Although an early study revealed that TNF induced RIPK1-mediated caspase-independent cell death (Vercammen *et al*., 1998), TNF induced non-apoptotic cell death did not attract much attention, until researchers further uncovered that cells executed necrosis-like death when apoptosis was blocked (Degterev *et al*., 2005, 2008).

Morphological features of necrosis include cytoplasmic swelling, dilation of cytoplasmic organelles particularly the mitochondria, permanent plasma membrane destruction, and post-lytic haphazard DNA dissolution (smear pattern on gel electrophoresis) (Grooten *et al*., 1993). The array of intracellular events characterizing necrosis, was defined as follows: early signs of mitochondrial dysfunction, namely production of ROS and mitochondrial swelling, ATP depletion, Ca^{2+} overload, perinuclear assembling of organelles, activation of proteases (in particular calpains and cathepsins), lysosomal rupture and finally plasma membrane disruption (Goldstein and Kroemer, 2007). Necrotic cell demise can be initiated by ligands binding to cell membrane receptors, like tumour necrosis factor α (TNF α), an inflammatory cytokine (Goossens *et al*., 1995). While limited and selective lysosomal permeabilization activates apoptosis, the outcome of substantial breakdown of lysosomes is unregulated necrosis (Bursch, 2001). Necrosis is characterized by ATP depletion, ion disregulation, mitochondrial and cellular swelling (Marx *et al*., 2006), as well as activation of cysteine proteases, Ca^{2+}-activated calpain, cathepsin and caspases (Yamashima, 2000).

A summary of the main features of the three major cell demise pathways is presented in Table 2.1

Table 2.1 Comparison of typical features of cell death by the three programmed cell death pathways

Type of cell death	**Features in cells**	**Reference**
Apoptosis	Activation of the caspase family of serine proteases ATP utilization Cell shrinkage Chromatin condensation (pyknosis) and chromatin fragmentation (karyorrhexis) Cytochrome *c* release from mitochondria into cytosol Exposed phosphatidyl serine Pre-lytic DNA fragmentation (Ladder pattern on gel electrophoresis)	Degterev *et al*., 2003; Danial and Korsmeyer, 2004
Autophagy	Beclin-1 expression Double membraned autophagosome (autophagic vacuoles) Generation of ATP Presence of miocrotubule-associated protein light chain 3 (LC3) Swelling of SR and mitochondria	Diao *et al*., 2017 Cheng *et al*., 2017 Hamasaki *et al*., 2013; Mizushima *et al*., 2011
Necrosis	Cytoplasmic swelling Dilatation of organelles, especially mitochondria LDH leakage Plasma membrane damage	Grooten *et al*., 1993

2.6 Immunogenicity

2.6.1 Antigens and immunogen

The effective production of antibodies hinges upon B-lymphocytes to bind, process and present antigen to T helper lymphocytes, which signal the B cells to synthesize and release antibodies (Janeway *et al*., 2001; Alberts *et al*., 2002). Every molecule that is recognized as non-self, by components of the immune system, is termed an antigen (Alberts *et al*., 2002; Bansal, 2013). An immunogen is any antigen, capable of inducing humoral and/or cell mediated immune response (Bansal, 2013). While all immunogens are antigenic, not all antigenic compounds are immunogenic (Murphy, 2012; Bansal, 2013). This is especially important, in vaccination with substances that are not immunogenic, where successful generation of antibodies against such antigens, requires that they firstly be made immunogenic, by chemically conjugating them to standard immunogens (carriers, usually protein).

2.6.2 Properties determining immunogenicity

Immunogenicity is the ability of a molecule to lobby an immune response (Bansal, 2013). For a substance to be an effective immunogen it needs to have three vital features: foreignness, high molecular weight and chemical complexity (Kuby, 1994). Foreignness is essential so that the vaccinated animal does not get familiar with and disregard the substance as "self." Generally, compounds from an organism are not immunogenic to that same individual and are only poorly immunogenic to others of the same or closely related species.

The second requirement for immunogenicity, is high molecular weight. Small molecular weight compounds (less than 1000 Da), such as penicillin, progesterone and aspirin, as well as many moderately sized molecules (1000 to 6000 Da), are not immunogenic. Compounds having molecular weight larger than 6000 are mostly immunogenic (Benjamini and Leskowitz, 1991). Smaller compounds can often be bound by mIgM (membrane IgM), on the surface of the B-lymphocyte, but

they are not sufficiently large to enable crosslinking of the mIgM molecules. The crosslinking is generally called “capping” and is the indicator for receptor facilitated endocytosis of the antigen (Germain, 1986).

Lastly, certain amount of chemical complexity is essential for a compound to be immunogenic. For instance, even high molecular weight homopolymers of amino acids and simple polysaccharides, rarely make effective immunogens as they lack the chemical complexity required to produce an immune reaction (Benjamini and Leskowitz, 1991).

2.6.3 Haptens vs. Epitopes

Haptens are small chemical compounds unable to act as immunogens in their free soluble form, as they cannot cross-link B-cell receptors and do not recruit T-cell help. They only act as recognition sites for specific antibodies (Gefen *et al*., 2015). However, when coupled to a carrier protein they become immunogenic, as the protein conveys numerous hapten assemblies, which can now cross-link B-cell receptors and stimulate T cells, via peptides originating from the carrier protein (Murphy *et al*., 2012)

Haptens can be made immunogenic by coupling them to a suitable carrier molecule (Lemus and Karol, 2008). An epitope is the specific site on an antigen to which an antibody binds (Frank, 2002). For very small antigens practically the whole compound may function as a single epitope. Alternatively, based on its complexity and size, an antigen may evoke the generation of antibodies directed at various epitopes (Clementi *et al*., 1991). Polyclonal antibodies are assortments of serum immunoglobulins and jointly are likely to bind to multiple epitopes on the antigen. In contrast, monoclonal antibodies contain only a single antibody clone and have binding specificity for one particular epitope (Knapp and Dash, 2016).

Specific antibodies can be generated against nearly any sufficiently unique chemical structure, be it natural or synthetic, as long as the compound is presented to the immune system in a

form that is immunogenic (Bansal, 2013). The resulting antibodies may bind to epitopes composed of entire molecules (e.g., small haptens), particular functional groups of a larger molecule, unique arrangements of several amino acid functional groups in the tertiary structure of proteins, or any other unique structure in lipoproteins, glycoproteins, RNA, DNA or polysaccharides (Bansal, 2013).

2.7 Immunology of vaccination

It is essential to present a brief overview of the major actors, the activation and coordination of the immune system, in order to grasp the immunology of vaccination. The immune system in vertebrates is a complex of various cell types, tissues and organs, which act in a coordinated manner, to protect the body against agents capable of infiltrating its physical and chemical barriers (Saalmüller, 2006; Leo *et al*., 2011). It functions to protect the skin, respiratory passages, intestinal tract and other areas from foreign agents, such as microbes, viruses, cancerous cells and toxins (Warrington *et al*., 2011).

Among the fundamental structures of the immune system, are found the bone marrow and thymus, primary lymphoid organs, where lymphocytes are generated, and the secondary lymphoid organs (peripheral lymph nodes, spleen, tonsils, Peyer's patches), from where immune reactions are instigated and coordinated (Leo *et al*., 2011).

2.7.1 Innate and adaptive immunity

All organisms possess a certain kind of innate protection against the outside world, which could be as simple as a cell wall or waxy coating (Nicholson, 2016). With the evolution of higher organisms, their innate defences became more advanced and the jawed vertebrates established a highly sophisticated system of immunity - acquired (or adaptive) immunity - which probably grew as

a consequence of co-evolution with specialised parasites, increased metabolic rates arising from dietary fluctuations and genomic unpredictability (Leo *et al*., 2011).

The immune system can simplistically be regarded as harbouring two "lines of defence" i.e. innate immunity and adaptive immunity (Warrington *et al*., 2011). The two interlinked systems, function sequentially to establish protective immunity. The innate immune system, composed of both cellular and non-cellular effectors, serves as a first line of defence. It is an antigen-independent (non-specific) mechanism, which suppresses the challenge and defends the organism, during the immediate lag time, before adaptive immune effectors react (Leo *et al*., 2011; Warrington *et al*., 2011). The link between the two systems, is the antigen presenting cells (APCs), which mediate and transmit information from the body tissues and innate immune system, to the adaptive immune component, allowing a coordinated response to a localized threat (Leo *et al*., 2011). The innate immune system pushes and moulds the advancement of adaptive immune responses, by way of chemical and molecular signals, conveyed by APCs, to prompt the most suitable adaptive response. The adaptive immune system, forms the second, antigen-specific line of defence, which is triggered and expanded in response to these signals (Leo *et al*., 2011).

2.7.1.1 The innate immune system

The primary function of innate immunity is the deployment of immune cells, to sites of infection and inflammation, via the production of cytokines (small proteins involved in cell-cell communication). Cytokine production leads to the release of proteins and glycoproteins, which activate the complement system (a biochemical torrent), which acts to recognize and opsonize (coat) foreign antigens, rendering them liable to phagocytosis (process by which cells engulf microbes and remove cell debris) (Warrington *et al*., 2011). Overall, the critical function of the innate immune system is to equip the adaptive immune response, whereby lymphocytes with antigen-specific receptors are generated, to multiply and fight the foreign agent (Leo *et al*., 2011).

Cells of the innate immune system are formed in the bone marrow, from where they transfer to various locations (Leo *et al*., 2011). Various cell types, are involved in the innate immune response, such as phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, and natural killer (NK) cells (Warrington *et al*., 2011). Non-immune system cells at vulnerable locations, including keratinocytes and other epithelial and mucus-producing cells, fibroblasts and endothelial cells, can also exhibit innate defensive behaviours (Leo *et al*., 2011).

2.7.1.2 The adaptive immune system

The adaptive immune component, having been forewarned by the innate immune system, reacts by activating lymphocytes with antigen-specific receptors, which multiply to combat the foreign threat. Their antigen receptors have very diverse characteristics, because of evolutionary advancement, in reaction to the selection pressure of different foreign agents (Leo *et al*., 2011; Warrington *et al*., 2011).

Lymphocytes circulate in the blood/lymph and reside within secondary lymphoid organs, including lymph nodes and spleen. The adaptive immune system basically acts by producing three strategic effector types: antibodies (produced from B cells), cytokines and cytolytic molecules (produced by T cells). T-cytotoxic cells and T-helper cells also function as effectors (Leo *et al*., 2011).

2.7.2 B cells and antibodies

Antibodies symbolize a greatly varied set of soluble proteins, originating from the subclass of lymphocytes known as the B cells (Nicholson, 2016). Antibodies can be regarded as bifunctional molecules, competent to equally identify and eradicate a particular antigen or pathogen (Leo *et al*., 2011). Originating from hematopoietic stem cells in the bone marrow, B cells development and differentiation occurs in primary lymphoid organs. B cells develop and mature in the bone marrow

and Peyer's patches especially in sheep and cattle and in the bursa of Fabricius in birds (Yasuda *et al*., 2006; Parra *et al*., 2013). Similar to T lymphocytes, every B lymphocyte expresses a distinctive antigen receptor (B-cell receptor [BCR]) allowing the cells to respond to a specific antigen (Warrington *et al*., 2011; Leo *et al*., 2011). The antibody receptor, also termed an immunoglobulin (Ig), was the first antigen-specific receptor to be characterized and is universally depicted with a Y-shaped sketch (Nicholson, 2016).

Immunoglobulins (Igs) are members of the eponymous immunoglobulin super-family (IgSF) (Torres *et al*., 2008). They comprise of two heavy and two light chains (Figure 2.6), wherein the light chain can contain either a κ or a λ chain (Schroeder and Cavacini, 2010). Each constituent chain encompasses one amino terminal "variable (V) domain and one or more carboxyl-terminal "constant" (C) domains, with each consisting two sandwiched β folded sheets 'held' together via a disulphide bridge flanked by two conserved cysteine residues (Williams and Barclay, 1988). The C fragment, is a structural feature shared by all antibodies of a given isotype, while the V region embraces the part that offers the antigen specificity (or antigen-binding characteristics) of the antibody (Leo *et al*., 2011). Each variable or constant immunoglobulin domain contains roughly 110–130 amino acids, averaging 12,000–13,000 kDa (Schroeder and Cavacini, 2010). Furthermore, the variable domain can occur in a vast number of molecular configurations. The variability in the variable domain is situated in the 3 hypervariable loops, also known as the complementarity determining regions (CDR), that together form the paratope (antigen binding region). An individual's BCR repertoire is generated to maximise capability to produce antibodies effective against various foreign threats (Leo *et al*., 2011). Both light chains contain only one C domain, whereas the heavy chains contain either three or four such domains.

Heavy chains with three constant regions tend to include a spacer hinge region, between the first (CH1) and second (CH2) domains. A typical light chain is about 25 kDa, and a three constant region Cγ heavy chain with its hinge is approximately 55 kDa (Schroeder and Cavacini, 2010). The

C region of an antibody molecule exists in five different isotypes – IgA, IgD, IgE, IgG and IgM, (Table 2.2) that govern the effector function of an antibody. Different antibody isotypes are restricted to specific locations and target particular types of threat (Leo *et al*., 2011).

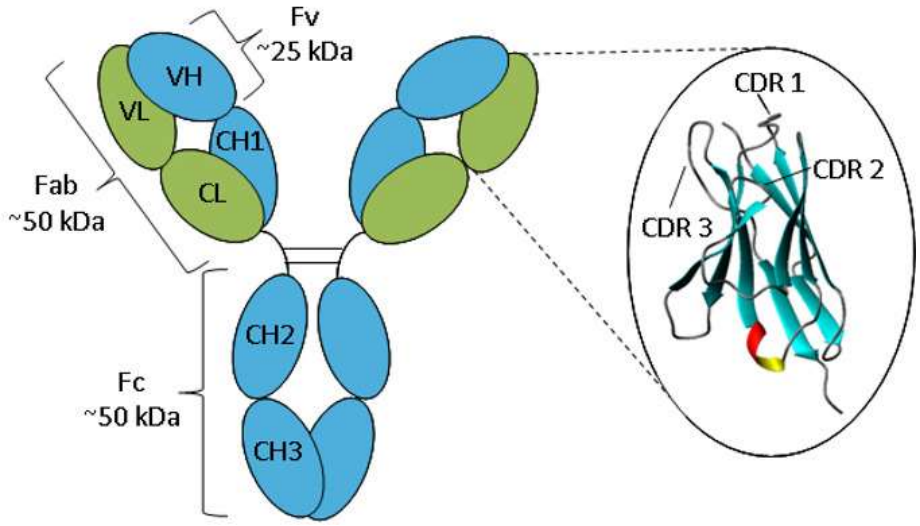


Figure 2.6: Schematic representation of an IgG. An antibody consists of two heavy chains (blue) and two light chains (green) folded into constant and variable domains. The enlargement of the variable domain shows a ribbon representation of the β-sheet framework and complementarity determining regions (CDR) loops (Absolute antibody, 2018).

2.7.3 Characteristics of immunoglobulin classes

2.7.3.1 Immunoglobulin G (IgG)

IgG is the most plentiful antibody in normal human serum, comprising about 70-85% of the entire immunoglobulin pool (Cruse and Lewis, 2010). It is monomeric, with a molecular weight of ±150 kDa. IgGs are the main immunoglobulin type produced during a secondary immune response and is the only antibody with antitoxin activity. In humans, there are four subclasses of IgG – IgG1, 2, 3 and 4, each with slight variations in molecular weight, number and position of the disulphide bonds and differences in their functional properties (Vidarsson *et al*., 2014).

2.7.3.2 Immunoglobulin M (IgM)

This class of antibody accounts for about 5 - 10% of the circulating antibodies in mammals (Cruse and Lewis, 2010). It has a molecular weight of 970 kDa and is a pentamer or hexamer of the four-chain units linked via the CH3 domains by disulphide bonds (Collins *et al*., 2002).

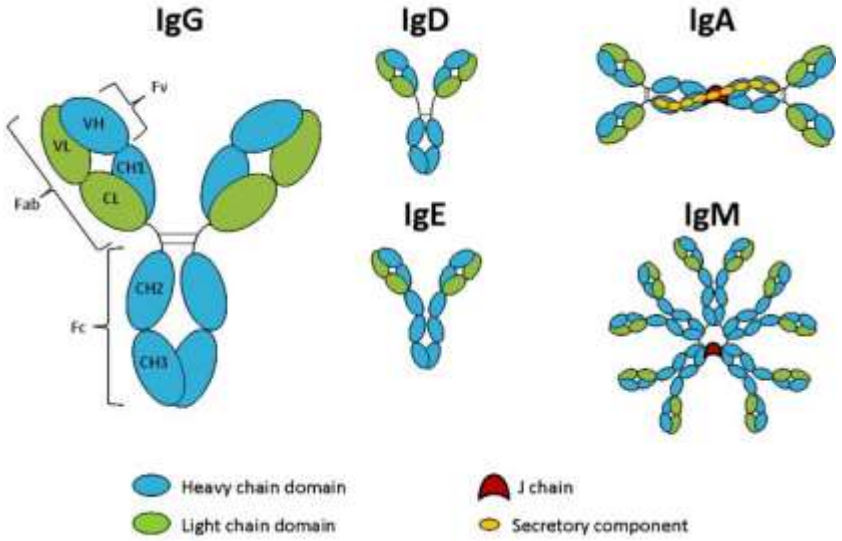


Figure 2.7: Schematic representation of the five immunoglobulin classes or isotypes in mammals (courtesy Absolute antibody, 2018).

2.7.3.3 Immunoglobulin A (IgA)

The IgA constitutes about 5 - 15% of the circulating antibodies in mammals (Cruse and Lewis, 2010). Found in two forms, monomeric (80%) and dimeric form (20%) in humans, while in other mammals the dimeric is the major form. The dimeric form (385 kDa), is the main antibody present in the mucous membranes. There are two subclasses (IgA1 and IgA2) in humans, gorillas, chimpanzees and gibbons, while most other species have one subtype (Kawamura *et al*., 1991).

2.7.3.4 Immunoglobulin D (IgD)

This antibody isoform accounts for ≤1% of the circulating antibodies in mammals. It is expressed on the surface membrane of numerous B-cells and has remained allied with the differentiation of pre-B cells into mature B cells (Cruse and Lewis, 2010).

2.7.3.5 Immunoglobulin E (IgE)

This isotype circulates in only minute amounts and the bulk of these antibodies are found on the surface of mast cells and basophils in mammals. Functionally, it is associated with allergic reactions. Alongside IgM, IgE antibodies have an extra heavy-chain constant domain (Cruse and Lewis, 2010).

2.7.3.6 Immunoglobulin Y – Avian IgE-IgG-like antibodies

The IgY is found in chicken and duck species and corresponds with both IgG and IgE in mammals. Found in the blood and egg yolk, IgY antibodies are not recognised by Fc receptors ("General concepts in antibody production," 2010).

2.7.3.7 Heavy chain - Llama antibodies

This antibody isoform, found in camelid species, exhibits the uniqueness of having heavy chain moieties, without light chains. Comprising 2 constant regions and a lone variable region per heavy chain, Llama antibodies are dimeric ("General concepts in antibody production," 2010).

Table 2.2 Molecular and functional characteristics of mammalian immunoglobulin isotypes (IgG, IgM, IgA, IgD, IgE), chicken IgY and Llama antibodies

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE	IgY	Llama
Heavy chain type	Ƴ1	Ƴ	Ƴ	Ƴ	μ	α	α	δ	ε	N	
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ		None
No. of constant domains	3	3	3	3	4	3	3	3	4	2	2
No. of variable domains	1	1	1	1	1	1	1	1	1	1	-
Molecular weight (kDa)	150	150	165	150	970	160	160	175	190	180	100
Serum concentration (mg/ml)	9	3	1	0.5	1.5	3	0.5	0.04	0.0 003	20 mg/ml egg yolk	
Half-life (days)	23	23	7	23	5	6	6	3	2.5	>30	
Binding to macrophages & phagocytic cells	+	-	-	+	-	-	-	-	+		
Binding to basophils & mast cells	-	-	-	-	-	-	-	-	+		
Complement activation (classical)	++	+	+++	-	+++	-	-	-	-		
Complement activation (alternate)	-	-	+	-	-	+	-	-	-		

Post script

The next chapters (3, 4, 5, 6 and 7) were written as stand-alone independent chapters. Each chapter is preceded by a preface and contains a brief introduction, in which the relevant literature is cited. Furthermore, in the Materials and Method sections there may be similarities between the chapters which may appear repetitive.

CHAPTER THREE

CONJUGATION OF BUFADIENOLIDES TO PROTEIN

Preface

Being small molecular weight compounds, the bufadienolides epoxyscillirosidine, bufalin and proscillaridin are not immunogenic. For the purpose of vaccinating rabbits and sheep, the bufadienolides must be conjugated to protein carriers in order to enhance their immunogenicity. In this chapter, the conjugation of the bufadienolides to OVA, BSA and KLH would be described.

3.1 Introduction

The coupling of a hapten to carrier proteins is a highly efficient and well established method of enhancing the immunogenicity of an otherwise non-immunogenic, usually small molecular weight, compound (Clementi *et al*., 1991; Lemus and Karol, 2008). The compound is chemically linked to a carrier protein such as human serum albumin (HSA), bovine serum albumin (BSA) or hen ovalbumin (OVA). Other commonly utilized carrier proteins include conalbumin, thyroglobulin, immunoglobulin, fibrinogen and keyhole limpet haemocyanin (KLH). However, conjugation with serum albumin is the commonest (Sing *et al*., 2004).

There are several methods of linking the compound of interest to proteins (Lemus and Karol, 2008). Several authors have reported on different methods of conjugation in their effort to generate antibodies against various natural compounds (Yan *et al*., 2017). Toxins, especially those from plants, are usually small compounds and mainly non-proteinaceous in nature and are not effective immunogens (Silbart *et al*., 1997). An effective immunogen must possess high enough molecular weight, foreignness, chemical complexity, and ability to be degraded by macrophage enzymes (Kuby, 1994). Thus, such compounds need to be linked with macromolecules (carrier proteins) before they are immunogenic (Lemus and Karol, 2008).

The method adopted, is usually determined by the functional group of the compound (hapten) to be conjugated (Sing *et al*., 2004). Various chemical reactions, are employed in the synthesis of the immunogen, which may involve introduction of a functional group as side arm, for bonding covalently with the carrier molecule, depending on the structure of the hapten (Yan *et al*., 2017). Butler and Chen (1967) reported a periodate oxidation method, based on a technique originally described by Erlanger and Beiser (1964), where digoxin was conjugated to BSA. Botha *et al*. (2007), in their work to evaluate a potential "Krimpsiekte" vaccine, described a mixed anhydride method, based on a method described by Erlanger *et al*. (1957), to conjugate cotyledoside to OVA and BSA. Fonseca *et al*. (2013) also described an effective method of conjugating gossypol to BSA.

Bufadienolides are natural steroidal compounds, which are characterized by having a doubly unsaturated 6-membered lactone ring (α—pyrone), in the 17β position of the steroid backbone (Krenn and Kopp, 1998). 1α, 2α-Epoxyscillirosidine ($C_{26}H_{32}O_8$), with a molecular mass of 479 Da (Enslin *et al*., 1966), is not immunogenic. Chakraborti *et al*. (2004), as part of method developments, to promote green chemistry, reported an efficient method of epoxide ring opening, by utilizing silica gel as a catalyst. This method, was adopted to open the epoxide ring of epoxyscillirosidine, before subsequent reactions to link carrier proteins to the bufadienolide (Fig. 3.1).

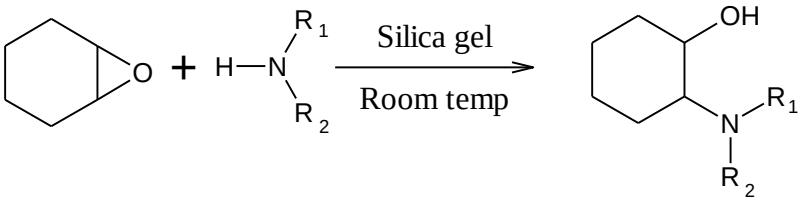


Figure 3.1: Epoxide ring opening using silica gel

Proscillaridin, present in some plant species such as *Scilla maderensis* (Dias *et al*., 2000) and bufalin, obtained from the skin secretions of certain species of toads, such as *Bufo bufo gargarizans*

(Liang *et al*., 2008; Gao *et al*., 2011), are related bufadienolides, that are commercially available in pure form.

The purpose of this study was to induce immunogenicity to bufadienolide toxins, by conjugating them to carrier-proteins, for the purpose of vaccinating rabbits and sheep. The first stage involved extraction, isolation and purification of epoxyscillirosidine according to the method published by Naudé and Potgieter (1971), while proscillaridin and bufalin were sourced commercially. The bufadienolides, were subsequently conjugated, to the different proteins OVA (1α, 2α-Epoxyscillirosidine), BSA (1α, 2α-Epoxyscillirosidine; bufalin; and proscillaridin); and KLH (1α, 2α-Epoxyscillirosidine).

3.2 Materials and Methods

3.2.1 Chemicals and reagents

Bufalin (B-0261), proscillaridin (P2428), bovine serum albumin (BSA - A7638), di-methyl aminopyridine (D-5640), pyridine (360570), succinic anhydride (S7626), isobutylchloroformate (IBC Eastman 1398), sodium sulphate AR, as well as tributylamine (TBA, Eastman 1266) were purchased from Sigma-Aldrich (Germany). Silica gel (1.09385.1000), toluene, chloroform, sodium hydrogen carbonate 0.2 % ($NaHCO_3$), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were sourced from Merck (Germany). Tetrahydrofuran (THF, T–425) was purchased from Fisher Scientific. Thin layer chromatography (TLC) plates (Silica gel 60 F_{254}) were from Merck (Germany).

3.2.2 Extraction of plant material

Yellow tulp (*Moraea pallida*), was collected on the farm Roodewal (GPS: 26°55'57.06"; 30°23'57.67"), in the Lothair district, Mpumalanga Province, Republic of South Africa. The plant material, was air dried by spreading it out in a shed. Epoxyscillirosidine was extracted according to the method by Naudé and Potgieter (1971) with modification. Initially, dried, hammer-milled plant material (1 kg) was soaked overnight at room temperature, in acetic acid solution (≈ 1 N, 7 L). The aqueous phase, was expressed through mutton cloth to obtain an extract. The acetic acid extract (1.4 L batches) was thereafter, extracted using dichloro-methane (DCM) (3 x 150 ml). The emulsion formed during extraction was broken up, by stirring with a glass rod or by repetitive filtrations through absorbent cotton wool wads. The DCM extract was combined and evaporated under reduced pressure (Büchi® Rotavapor® R II, Vacutec, Switzerland) to obtain a crude extract. This was dissolved in DCM (70 ml), washed three times with water, dried over anhydrous calcium chloride, filtered and evaporated under reduced pressure to obtain a dry, crude extract (588.4 mg).

3.2.3 Isolation and purification of 1α, 2α-epoxyscillirosidine

The crude extract was flash chromatographed, on silica gel (Merck cat no. 1.09385.1000) using acetone: *n*-hexane (1:1) as eluent. The eluent was collected as 20 ml fractions and TLC was carried out to identify the toxin. Positive fractions were pooled together and evaporated to obtain a residue. The process was repeated and the column was eluted with acetone and *n*-hexane (ratio 1:1.2). 1α 2α-Epoxyscillirosidine was obtained in pure form (157.2 mg) and the structure was confirmed using Nuclear Magnetic Resonance (NMR).

3.2.4 1α, 2α-Epoxyscillirosidine conjugation to proteins

Conjugation of 1α, 2α-epoxyscillirosidine to OVA, BSA and KLH was achieved by opening of the epoxide ring (Chakraborti *et al*., 2004), before coupling to the proteins. 1α, 2α-Epoxyscillirosidine (10 mg) was dissolved in THF (1 ml) and OVA, BSA, or KLH (50 mg) was dissolved in water (4 ml). While stirring the BSA solution, epoxyscillirosidine solution was added drop wise, to the aqueous solution. Silica gel 60 (Merck) (50 mg), was then added and the mixture stirred overnight (Fig. 3.1). The mixture was thereafter transferred into a centrifuge tube and the solution centrifuged. The reaction flask was rinsed with water, which was added to the centrifuge tube. The aqueous solution was extracted 3 times, with chloroform (shaken on a vortex shaker each time after addition of chloroform). The emulsion formed was broken by centrifuging at 1734 *g*, for 10 min at 20° C. The chloroform was separated and retained to recover unreacted toxin. This was then lyophilized and stored. The degree of conjugation was evaluated using ultraviolet (UV) spectroscopy and using the relationship:

$$OD = 1 \times c \times \varepsilon$$

where, OD is optical density at absorption maxima
1 is cell path length of 1cm
c is concentration (mg/ml)
ε is extinction coefficient of the compound.

3.2.5 Bufalin conjugation to BSA

3.2.5.1 Preparation of bufalin-hemisuccinate

Bufalin was conjugated using the mixed anhydride method (Erlanger *et al*., 1957), with modification. Bufalin (40 mg) was dissolved in pyridine (1 ml) in a sealable tube. Succinic anhydride (50 mg) and 4-dimethylaminopyridine (1 crystal) was added and the closed tube heated at 100 ^{o}C for

at least 4 h. The reaction mixture was transferred to a 50 ml round bottom flask and the solvent evaporated under reduced pressure. Toluene (about 10 ml) was added and evaporated again to dryness and repeated three more times. This was followed by the addition of chloroform (about 10 ml) and evaporation. This was repeated one more time. Chloroform (5 ml) was thereafter added and the chloroform extracted four times with 0.01 N HCl (4 ml). The chloroform phase was extracted with 0.2% $NaHCO_3$ solution (3 ml). The $NaHCO_3$ solution was acidified with 1 N HCl (2 drops) to a pH of about 2. The aqueous phase turned milky. $NaHCO_3$ extraction of the chloroform phase was repeated ten times and all the aqueous phases pooled together. The aqueous phase was centrifuged at 1734 *g* for half an hour. The water was decanted and the precipitate washed once with water and centrifuged again. The water was decanted and the reaction product dried in a vacuum desiccator (Büchi® Rotavapor® R II, Vacutec, Switzerland) to give bufalin-hemisuccinate (14 mg).

3.2.5.2 Reacting BSA with activated bufalin

Ten ml tetrahydrofuran (THF) was dried over sodium sulphate. Approximately 5 mg bufalin-hemisuccinate was weighed and dissolved in THF (0.5 ml) and cooled in an ice bath. Tributylamine (10 µl) was diluted with THF (90 µl) and 20 µl of the resulting mixture added to the bufalin-hemisuccinate solution. After 5 min, 12 µl of a solution of isobutyl chloroformate (10 µl in 90 µl THF) was added to the bufalin-hemisuccinate mixture. The reaction mixture was left for 20 min. Bovine serum albumin (15 mg) was weighed and dissolved in distilled water (1.4 ml). 0.9 ml of THF was added to this solution. The pH of the solution was approximately 6. The pH was adjusted to 9 by the addition of 20 µl of a 1 N sodium hydroxide (NaOH) solution. The previously prepared bufalin-hemisuccinate mixture was then added to the BSA solution. The reaction mixture was stirred gently using a magnetic stirrer. The pH was kept at 9 with the addition of 1 N NaOH solution and the mixture stirred for 2.5 h in an ice bath. The mixture was then dialyzed against water overnight in a cold room. After a day, the product was dialyzed again using two changes of water. To calculate the

incorporation of bufalin into conjugate, the optical densities of conjugate, BSA and bufalin were scanned between 280 and 324 nm on a UV spectrophotometer (Helios β, Thermo Electron Corporation).

3.2.6 Proscillaridin conjugation to BSA

Proscillaridin conjugation to BSA was carried out using the periodate method, as described by Butler and Chen (1967). Proscillaridin (54 mg) was suspended in absolute ethanol (2 ml) at room temperature. Sodium metaperiodate (0.21 g $NaIO_3$ in 15 ml water) was added drop wise during magnetic stirring. BSA (100 mg in 2 ml of water) pH adjusted to 9.5 with 5 % potassium trioxocarbonate (K_2CO_3), was added drop wise, with magnetic stirring. The pH was maintained in the 9.0-9.5 range, by the drop wise addition of 2.0 ml of 5 % K_2CO_3. After 45 min, sodium borohydride (60 mg $NaBH_4$ in 2 ml water freshly dissolved), was added. Three hours later, formic acid (0.76 ml of a 1 M solution) was added to lower the pH to 6.5. After 1 h at pH 6.5, the pH was raised to 8.5 by the addition of 1.5 ml of 1 M ammonium hydroxide (NH_4OH). The entire reaction mixture was then freeze dried. Chromatography on Sephadex G50 was thereafter conducted. The first band (the desired product), which eluted from the column, was retained. This was lyophilized to give 71 mg of the conjugate. To confirm the conjugation, the solution was scanned between 280–324 nm on a UV spectrophotometer (Helios β, Thermo Electron Corporation).

3.3 Results

3.3.1. Conjugation of bufadienolides to protein

The differences in UV absorption spectra (at 280-324 nm) of the conjugates compared with the native proteins were used to confirm the conjugation reaction (Figure 3.3). The conjugation of 1α, 2α-epoxyscillirosidine to OVA was estimated to be 6.696 x 10^1 or about 67 moles of epoxyscillirosidine per mole of OVA, or hapten density.

The degree of conjugation of 1α, 2α-epoxyscillirosidine to OVA, BSA and KLH was estimated as calculated below:

Data needed:

- Molecular mass OVA = 42 700 Daltons
- 1 Mole BSA contains 20 lysine units
- Extinction coefficient of 1α, 2α-epoxyscillirosidine – ε = 5700 at λ 300 nm
- Solution made up to give an optical density (OD) of 1.5 at 280 nm

1. Amount of protein in this solution (toxin level negligible) thus gives a concentration of:

   330 mg/L = 7.7 x 10^{-3} moles

2. Amount of toxin present:

   Using the measured OD value of 0.660 at 300 nm (λ max of toxin)

   $$OD = 1 \times c \times \varepsilon$$

   Where 1 = the cell path length of 1 cm, ε = 5700 and c the concentration in moles/litre

   $$c = \frac{OD}{1} \times \varepsilon$$

   $$c = \frac{0.660}{1} \times 5700$$

   Which gives c = 1.15 x 10^{-4} moles toxin

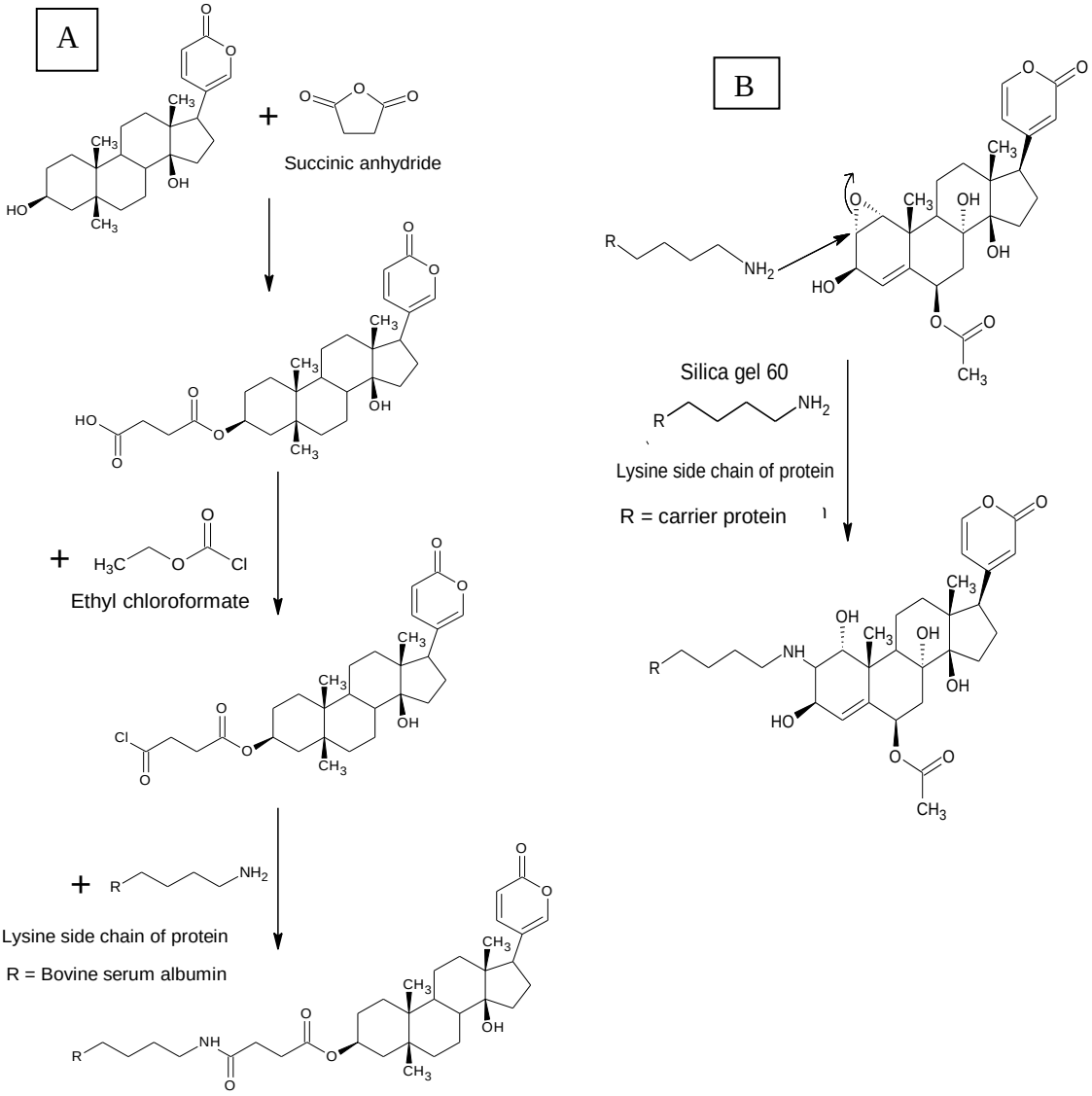
   Thus the hapten density is:

7.7×10^{-3} moles toxin binds to 1.15×10^{-4} moles protein

or $\frac{7.7 \times 10^{-3}}{1.15 \times 10^{-4}}$ toxin molecules per moles protein

This means 6.696×10^{1} moles of epoxyscillirosidine were conjugated per mole of OVA.

The degree of conjugation of epoxyscillirosidine to OVA and KLH were similarly estimated to be 3.3×10^{-3} and 1.54×10^{-3} moles toxin per mole protein respectively.



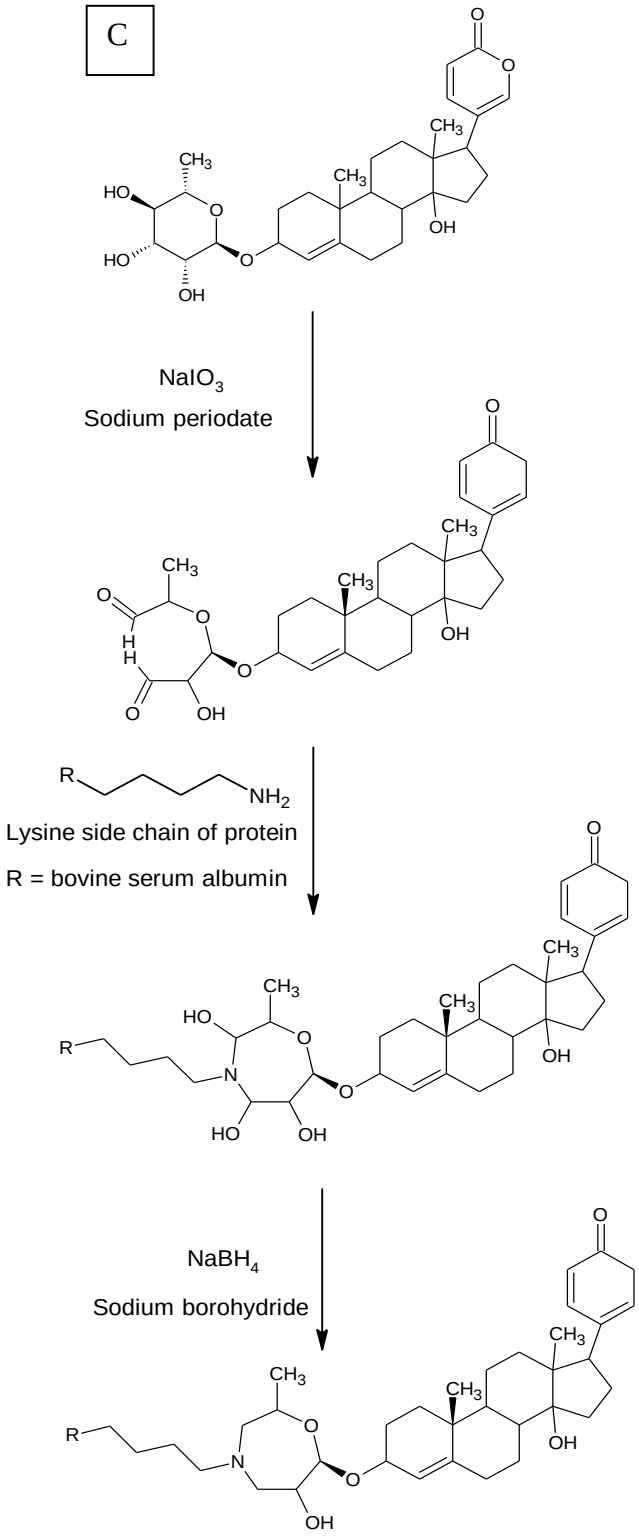
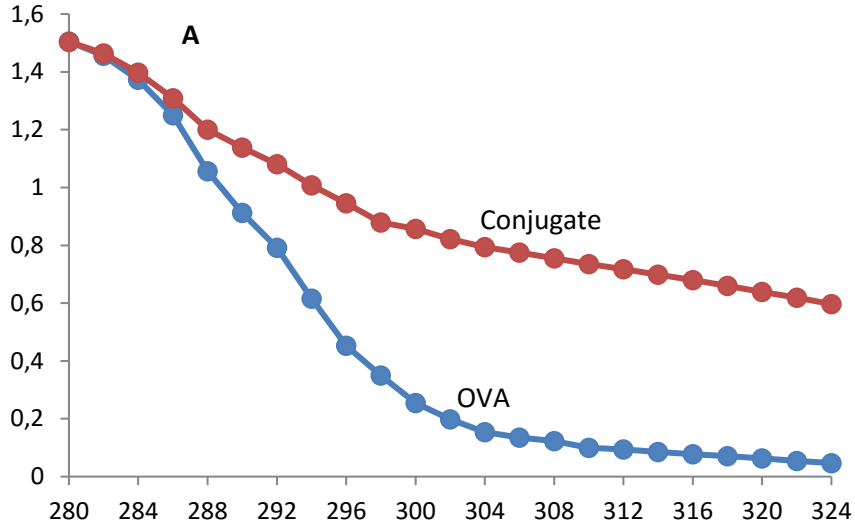
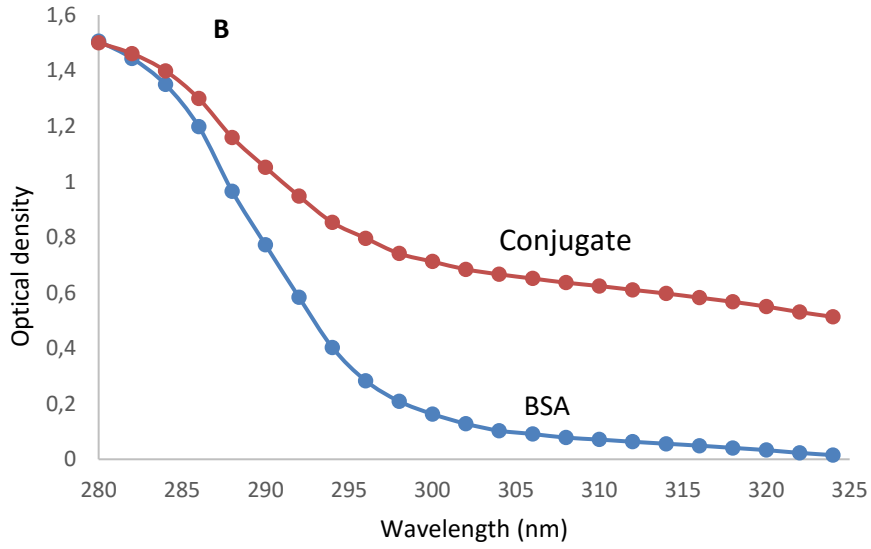
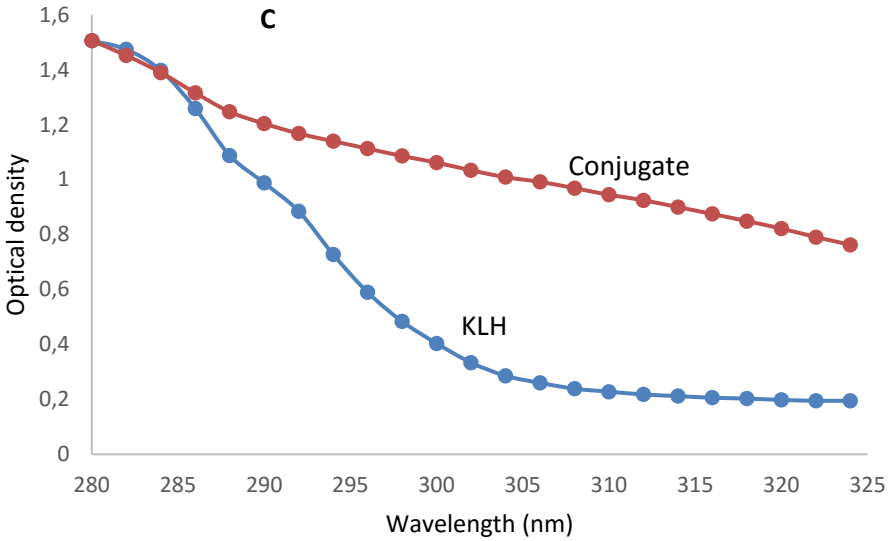


Figure 3.2: Reaction schemes showing the conjugation of bufalin to BSA (A); conjugation of epoxyscillirosidine to OVA, BSA and KLH (B) and conjugation of proscillaridin to BSA (C). R represents carrier protein (OVA, BSA or KLH).

The UV absorption spectra (at 280–324 nm) of the conjugates were compared with the native proteins to confirm the conjugation and are presented in Fig. 3.3.



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1,4
1,2
1
0,8
0,6
0,4
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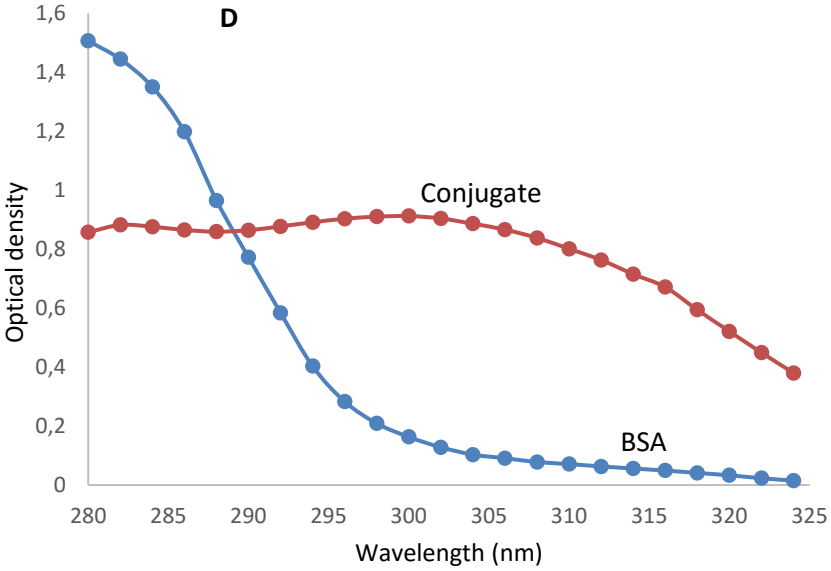
305
310
315
320
325
Wavelength (nm)


D
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Optical density
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BSA
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320
325
Wavelength (nm)

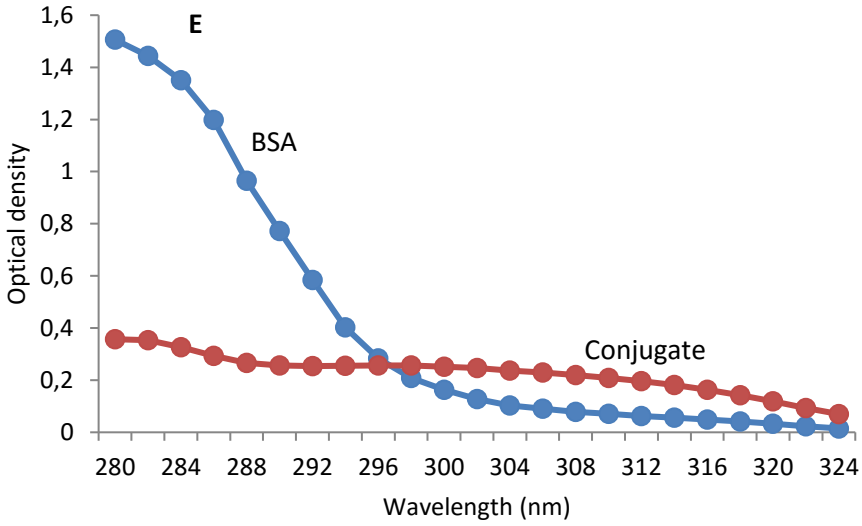


Figure 3.3: Comparative ultraviolet absorption spectra of conjugates and proteins. A 1α, 2α-Epoxyscillirosidine-OVA vs OVA. B Epoxyscillirosidine-BSA vs BSA. C Epoxyscillirosidine-KLH vs KLH. D Proscillaridin-BSA vs BSA. E Bufalin-BSA vs BSA. The respective conjugates and proteins were dissolved (1 mg/ml) in acetonitrile-water (50%, v/v). The solutions (100 µl) were scanned in the range 280-330 nm.

3.4 Discussion

For successful vaccination of animals with small molecular weight plant toxins, the toxins, as haptens, must be conjugated to larger more complex carrier proteins, to render them immunogenic (Goodrow *et al*., 1990). Conjugation of epoxyscillirosidine, proscillaridin and bufalin was successfully carried out, before their use to vaccinate rabbits and sheep. During conjugation reactions, the choice of the method is determined by the functional group of the hapten (Sing *et al*., 2004). Different methods were adopted for each of the different haptens used in this study. However, for 1α, 2α-epoxyscillirosidine, the epoxide ring was utilized to link the compound with the protein carrier (OVA or BSA or KLH) and not the lactone ring functional group. Opening of the epoxide (Chakraborti *et al*., 2004) theoretically makes it available for linking with the surface lysines of the carrier protein (OVA, BSA, KLH), while the lactone ring remains intact, thus not altering the activity of 1α, 2α-epoxyscillirosidine. Bufalin was conjugated by the mixed anhydride method as other steroids have been conjugated to proteins using this method (Erlanger *et al*., 1957; De Goeij *et al*.,

1986; Botha *et al*., 2007). In the conjugation of proscillaridin to BSA, the lactone ring of scillarenin, which is the genin of proscillaridin, reacts with the available lysine residues on BSA.

The linkage of haptens to proteins generally occurs at the most reactive (functional) groups of the proteins (Tijssen 1985). According to Butler and Chen (1967) the lactone ring of the genin could conceivably react with the free amino groups of BSA under the alkaline conditions employed in this reaction. Of the 59 lysine residues in the BSA molecule, only about 30-35 are available for conjugation (Venkataramana *et al*., 2015; Abbas *et al*., 2018). The exact number reacting with the hapten could not be accurately determined with the UV spectroscopy utilized in this study.

The conjugation of a hapten to a carrier, usually a protein has been confirmed using a number of methods including ultraviolet (UV) spectroscopy, by comparing the UV spectrum of the conjugate with that of the native protein (Wang *et al*., 2012; Torres *et al*., 2014). Sing *et al*., (2004) similarly reported spectrophotometry as a valuable tool to evaluate conjugation reactions. Change in structure of the protein confirmed by change in its absorption spectrum is suggestive of a new compound (the conjugate). The UV absorption spectra of the conjugates synthesized in this study were different and higher than those of the native proteins (Fig. 3), the difference in the absorption being contributed by the haptens. In addition, the extinction coefficient of the proteins and conjugates are utilized in estimating the hapten density of the conjugates (Szudorsky *et al*., 2002).

The synthesized conjugates (OVA, BSA and KLH) of epoxyscillirosidine were effective as immunogens, whereby they elicited the generation of epoxyscillirosidine specific antibodies, in vaccinated rabbits (Chapter 4). Evidence from rabbit vaccination trials (Chapter 4), showed that conjugation of epoxyscillirosidine to KLH was most effective. This was probably because it produced the most optimal hapten density, even though KLH is known to be a superior carrier protein, due to its high molecular mass, complexity and foreignness (when used in mammals).

In conclusion, we successfully conjugated the bufadienolide haptens epoxyscillirosidine, proscillaridin and bufalin to carrier proteins, for the purpose of vaccinating rabbits and sheep. The UV spectroscopic method was used, to estimate the conjugation, which was further confirmed by production of antibodies specific to the bufadienolides, following administration in rabbits and sheep. However, the spectrophotometric method does not give an accurate quantification of the hapten-protein conjugation density like other more advanced tools, such as matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Torres *et al*., 2014) and high-resolution mass-spectrometry with electrospray ionization (ESI) (Abbas *et al*., 2018). Furthermore, additional studies are needed, to identify the best conjugation reactions, to produce optimal hapten density, for each of the conjugates. Tools like MALDI-TOF MS can be employed, to characterize the conjugates, in the future. Further animal vaccination trials will then be required, to confirm the efficacy of the conjugates in raising antibodies.

CHAPTER FOUR

RABBIT VACCINATION TRIALS AND CROSS-REACTIVITY STUDIES

Preface

Following the successful conjugation of bufalin, epoxyscillirosidine and proscillaridin to OVA, BSA and KLH, the next step was to evaluate their efficacy in inducing an immune response to generate antibodies in rabbits. This chapter describes the vaccination of rabbits with the bufadienolide conjugates and evaluation of cross-reactivity between bufalin and proscillaridin with epoxyscillirosidine using an ELISA.

4.1 Introduction

Poisoning by *Moraea pallida* Bak. (Iridaceae) or yellow tulp is of utmost significance, among cardiac glycoside containing plants, as it constitutes a serious economic problem, causing thousands of livestock mortalities annually, in the Republic of South Africa (Kellerman *et al*., 1996). Cardiac glycoside toxicoses account for about 33% and 10% mortality, due to plant poisonings in cattle and small stock, respectively (Kellerman *et al*., 1996). The toxic principle is 1α, 2α-epoxyscillirosidine ($C_{26}H_{32}O_8$), a bufadienolide type of cardiac glycoside (Enslin *et al*., 1966; Snyman *et al*., 2004).

In poisoned animals, there is tachycardia and arrhythmias. Other signs include general apathy, tremors, weakness of hindquarters, respiratory distress and at times bruxism and groaning sounds, rumen atony, bloat and diarrhoea. Death may occur due to cardiac arrest (Steyn, 1928; Naudé, 1977). Treatment of poisoned animals is unsuccessful in most cases (Kellerman, 2009). However, oral administration of activated charcoal was reported to be effective, albeit stressful to the animals, expensive and needs to be instituted soon after ingestion (Joubert and Schultz 1982).

Tulp poisoning is prevented by herding animals, fencing off infested areas or physically eradicating the plants (Kellerman *et al*., 2005). Chemical control using herbicides is sometimes used

(Steyn, 1928), but is expensive. Manipulating animals to resist poisoning is superior to changing the environment, by unnecessary fencing, causing overgrazing and trampling of vegetation or spraying with herbicides, with severe ecological impact (Kellerman 2009).

Prophylaxis by vaccination or immunotherapy by administering hyper-immune serum could potentially be explored, in the management of yellow tulp poisoning, in view of the limitations of the current management strategies. Vaccination against small molecule plant toxins, is a potentially useful preventative strategy. However, for successful vaccination of animals with small molecular weight plant toxins, the toxins, as haptens, must be conjugated to larger more complex carrier proteins, to render them immunogenic (Goodrow *et al*., 1990). Vaccinations against *Delphinium* spp (larkspur) (Lee *et al*., 2003), *Oxytropis kansuensis* (locoweed) (Tong *et al*., 2007), and "Krimpsiekte" (Botha *et al*., 2007) have been reported. Fonseca *et al*., (2013) evaluated vaccination as a possible means of preventing gossypol intoxication.

The aim of this study was to investigate if antibodies, against epoxyscillirosidine and related bufadienolides, will be synthesized by rabbits, and to further ascertain if the antibodies raised against the related bufadienolides will cross-react with epoxyscillirosidine, in an ELISA. The study was conducted in three trials. The first (T1) and second (T2) trials evaluated the efficacy of a hen ovalbumin (OVA) conjugate of epoxyscillirosidine, using 2 different doses and adjuvants (T1 vs. T2), to induce an immunological response. The third trial (T3) evaluated the efficacy of proscillaridin-bovine serum albumin (BSA), bufalin-BSA, epoxyscillirosidine-BSA and epoxyscillirosidine-keyhole limpet haemocyanin (KLH) conjugates to raise antibodies and to determine the degree of cross-reactivity of antibodies raised against the commercially available bufadienolides (proscillaridin and bufalin) with epoxyscillirosidine.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

Epoxyscillirosidine was isolated (Chapter 3). Bufalin (B-0261), proscillaridin (P2428), OVA (A5253), BSA (A7638), KLH (H7017), phosphate buffered saline (PBS, P4417), sodium trioxocarbonate ($Na_2CO_3.10H_2O$), ortho-phenylenediamine dihydrochloride (OPD, P8787), hydrogen peroxide (H1009), Freund's complete adjuvant (FCA, F5881), and Freund's incomplete adjuvant (FIA, F5506) were bought from Sigma-Aldrich (Germany). Sodium bicarbonate ($NaHCO_3$) and sulphuric acid (H_2SO_4) were sourced from Merck (Germany). Montanide^{TM} ISA (50 V2, Seppic,) was from SEDEX (France). Foetal bovine serum (FBS, 10270) was obtained from Gibco, (Life Technologies, Germany). Horse radish-peroxidase recombinant protein G conjugate (EIA Grade) (10-1223) and tetrahydrofuran (THF, T – 425) were from ThermoFisher Scientific (Germany). Skim milk powder for ELISA (LP0031) was purchased from Oxoid Ltd (United Kingdom).

4.2.2 Preparation of immunogens

Five hapten-protein conjugates (immunogens) were prepared. In Trial 1, a stable water-in-oil emulsion was prepared by mixing the hapten-protein conjugate (4 mg) or pure protein (4 mg) dissolved in normal saline (2 ml), with complete Freund's adjuvant (FCA) in a ratio of 1:1 (v/v). For the booster vaccinations, incomplete Freund's adjuvant (FIA) was used at the same ratio as FCA. In Trials 2 and 3, the vaccine was prepared in a similar manner but using a concentration of 4 mg/ml immunogen or protein (4 mg/ml) and Montanide as adjuvant instead, for both initial and booster vaccinations.

4.2.3 Experimental animals

Adult, male, New Zealand White rabbits (*Oryctolagus cuniculus*) were obtained from the University of Witwatersrand, South Africa. The body weight of the animals ranged between 2.33 – 3.83 kg. The rabbits were weighed and randomly assigned into groups. For the entire duration of the study the animals were housed at the University of Pretoria Biomedical Research Centre (UPBRC) in a controlled environment maintained at 22°C under fluorescent light, with a 12 h light and 12 h dark cycle. The animals were kept individually in large floor pens, with free access to commercial rabbit feed (EPOL rabbit pellets) and reverse osmosis water provided *ad lib*. Toys (balls, bottles with bells, teddy bears and wooden toys), hide boxes, fruit (mainly apple) and vegetable treats were provided as enrichment to improve well-being of the animals. Approval for the ethical use and care of laboratory animals (Project No: V079-14; V039-16), was obtained from the Animal Ethics Committee, Faculty of Veterinary Science, University of Pretoria, before the commencement of the experiments. The animals were acclimatized for 2 weeks, prior to the start of each vaccination trial.

4.2.4 Rabbit vaccination

4.2.4.1 Study design

This was done as described by Botha *et al*., (2007) with modification. A summary of the experimental groups and vaccination schedule is presented in Table 4.1. In the first trial, three rabbits (Group I), were vaccinated with epoxyscillirosidine-OVA conjugate (2 mg/ml) in FCA, while the control animals (n=3) were injected with OVA (2 mg/ml) in FCA. Vaccinations were done on days (D) 0, 21, 42, 56, and 63. The two additional vaccinations (D56 and 63) were carried out due to poor immune response. Trial 2 was a modification of Trial 1 wherein the dose of the immunogen was increased 2-fold by doubling the concentration to 4 mg/ml and the adjuvant changed to Montanide. In Trial 2, four rabbits (Group I) were vaccinated with epoxyscillirosidine-OVA in Montanide, while the rabbits (n = 2) in the control group were vaccinated with OVA in Montanide, on D 0, 21, and 42.

For Trial 3, five groups of three animals each were included. Rabbits in groups I to IV were, respectively, vaccinated with proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA conjugates. The rabbits in group V were injected with BSA, which served as control. The vaccinations were done on D 0, 21 and 42 (Table 4.1).

Table 4.1: Vaccination schedule in rabbits

Trial	Experimental Groups	Immunogen (Concentration)	Adjuvant	Day of vaccination (Day of blood collection)
1	Group I (n=3)	Epoxyscillirosidine-OVA (2 mg/ml)	Freund's*	0, 21, 42, 56, 63 (0, 21, 42, 56, 63, 80)
	Group II (n=3) (Control)	OVA (2 mg/ml)	Freund's*	0, 21, 42 (0, 21, 42, 56)
2	Group I (n=4)	Epoxyscillirosidine-OVA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
	Group II (n=2) (Control)	OVA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
3	Group I (n=3)	Proscillaridin-BSA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
	Group II (n=3)	Bufalin-BSA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
	Group III (n=3)	Epoxyscillirosidine-KLH (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
	Group IV (n=3)	Epoxyscillirosidine-BSA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)
	Group V (n=3) (Control)	BSA (4 mg/ml)	Montanide	0, 21, 42 (0, 21, 42, 56)

* Primary vaccination used FCA, while booster vaccinations used FIA.

4.2.4.2 Experimental procedures

A large area on the back of each rabbit was shaved and the vaccine was administered, by intradermal injection of 0.1 ml, at each of four sites, on the back (0.4 mg immunogen per rabbit for trial 1 and 0.8 mg immunogen per rabbit for trials 2 and 3). Before each vaccination, the rabbits were weighed and 2 ml blood collected from an ear vein, to determine pre-vaccination antibody titres. At the end of the animal experiments, on D 80, 67 and 67 for the first, second and third trials respectively, the rabbits were anaesthetized (with a xylazine/ketamine combination [Xylavet 2 % (m/v), Intervet SA (Pty) Ltd and Anaket, Bayer Animal Health Division]) and exsanguinated by cardiac puncture. Collected blood was allowed to stand for 30 min, at room temperature. Serum was subsequently obtained, by centrifugation at 1204 *g* at 20 °C (Allegra^{TM} X-22 Centrifuge, Beckman Coulter), aliquoted and stored at -20 °C.

4.2.5 Evaluation of immunological response using an indirect ELISA

An epoxyscillirosidine ELISA to determine antibody titres was optimized, before use. Maxisorb 96-well plates (NUNC) were coated with 50 µl epoxyscillirosidine-BSA conjugate (Trial 1 and 2) and epoxyscillirosidine-OVA (Trial 3), at a dilution of 100 µg/ml in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6), in alternate rows. The remaining rows, were coated with BSA instead (Trial 1 and 2) or OVA (Trial 3), at the same concentration. The plates were dried by incubation overnight, at room temperature, in a laminar flow cabinet. Plates were blocked with 200 µl PBS supplemented with 5% skimmed milk powder per well and incubated for 30 min, at room temperature. This was followed by washing three times with PBS. Serum dilutions were loaded in duplicate rows (50 µl per well), e.g. row A coated with hapten-protein conjugate and row B coated with only protein. A two-fold serial dilution, starting at 1:50 and using blocking buffer plus 1% foetal bovine serum (FBS), was done down the plate, followed by incubation for 1 h, at room temperature and washing (five times) with wash buffer. After washing, 50 µl horseradish-peroxidase

recombinant protein G (HRPO – r Prot G) conjugate, at a dilution of 1:3 000, was added to all wells. Following further incubation for 1 h and washing five times (with wash buffer), 50 µl substrate (ortho-phenylenediamine (OPD), at a concentration of 0.04 mg/ml and containing 0.05% H_2O_2 30% v/v), was added to all wells and the plates incubated for 15 min, in the dark, after which 50 µl 1N H_2SO_4 was added to all wells, to quench the reaction. The plates were placed in a plate reader (BioTek Synergy HT) and the optical density (OD) was read at 490. The net OD was calculated by subtracting the value obtained for the BSA or OVA coated wells from the corresponding hapten-protein-coated wells. In another assay, only epoxyscillirosidine as antigen and coating buffer were coated on the plate in alternate rows, to confirm the specificity of the generated antibodies against the hapten (epoxyscillirosidine).

4.2.6 Statistical analysis

Data were analysed using the statistical program GenStat® (Payne, 2015). Values were expressed as mean ± SD. ELISA results were analysed using repeated measures ANOVA and Tukey's LSD test at the 1% level for comparing means. P values < 0.01 were considered significant.

4.3 Results

4.3.1 Trial 1

There were no observable signs of intoxication or mortalities in rabbits, following the administration of epoxyscillirosidine-OVA conjugate. However, severe granulomatous ulcerative lesions appeared at the injection sites, as a reaction to the adjuvant (FCA), after vaccination in all rabbits.

The immune response of the rabbits to epoxyscillirosidine, was evaluated with the indirect ELISA. Only one of the three hapten-protein vaccinated rabbits, (Rabbit 4) seroconverted after the

second vaccination, detectable on D 42, followed by a second rabbit (Rabbit 3), 3 weeks after the third vaccination. The antibody levels for Rabbits 3 and 4 peaked at D 56 and remained relatively constant thereafter. Rabbit 6, from the hapten-protein vaccinated group, responded poorly and did not seroconvert. None of the three control rabbits (OVA only) developed detectable antibodies to epoxyscillirosidine, as expected. The optical density values obtained in an epoxyscillirosidine-BSA ELISA for sera from the vaccinated rabbits are indicated in Fig. 4.1.

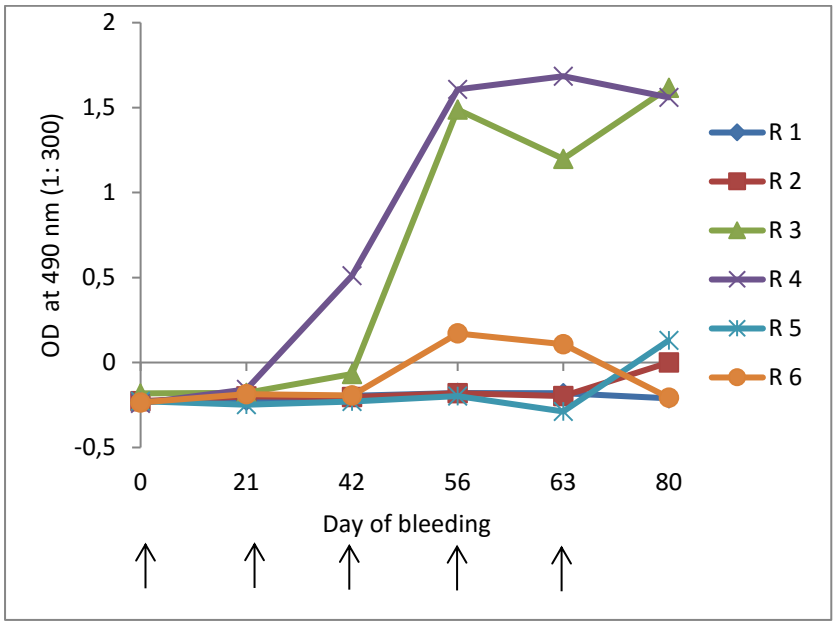


Figure 4.1: Trial 1: Optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA for sera (at 1:300 dilution) from rabbits vaccinated with epoxyscillirosidine-OVA (R3, 4 and 6) or OVA (R 1, 2 and 5). The arrows indicate the days of vaccination. Primary vaccinations were done using Freund's complete adjuvant and subsequent vaccinations included Freund's incomplete adjuvant. The OD of the BSA coated well was subtracted from that of the epoxyscillirosidine-BSA well to obtain the net OD. Wells were treated in duplicate.

4.3.2 Trial 2

Toxic signs due to epoxyscillirosidine were absent in all experimental rabbits. However, all the rabbits developed varying degrees of cutaneous reactions at the injection sites, which were characterized by reddening and slight oedema after vaccine administration. In this trial, all experimental animals seroconverted, detectable three weeks (D 21) after the primary vaccination. The anti-epoxyscillirosidine-OVA antibody concentrations, in all the four experimental rabbits,

increased after each booster vaccination (Fig. 4.2). No rabbit in the control group (OVA only) developed detectable antibodies to epoxyscillirosidine-BSA.

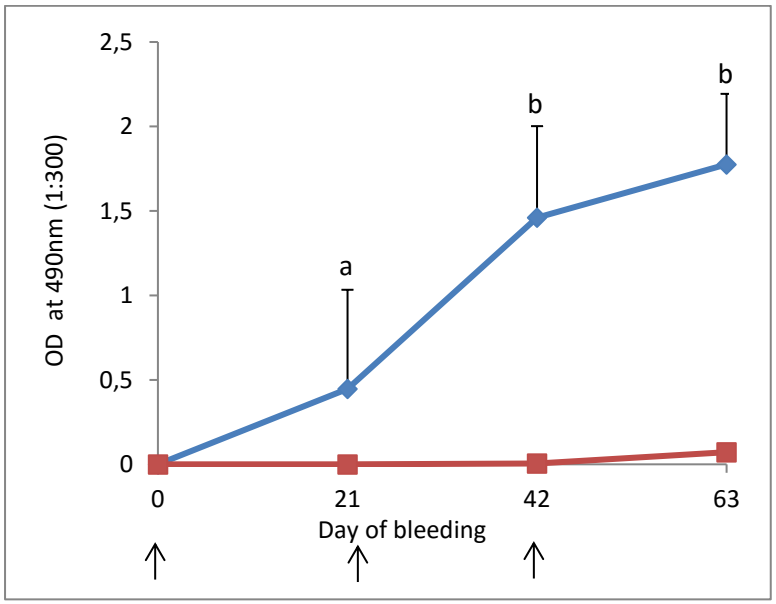


Figure 4.2 : Trial 2: Mean optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA, for sera (at 1:300 dilution) from experimental rabbits (n=4) vaccinated with epoxyscillirosidine-OVA or OVA (control, n=2,). Vaccinations were done using Montanide as adjuvant. Bars indicate standard deviation (SD). *a* - not significantly different from Day 0; *b* indicate significant difference ($p < 0.01$). Blue line indicates experimental animals, while the red line represents the control.

4.3.3 Trial 3

Mild to moderate cutaneous injection site reactions, characterized by erythema and slight oedema, were observed in all rabbits. Antibodies against proscillaridin-, bufalin- and epoxyscillirosidine-conjugates were synthesized by the rabbits. Rabbits vaccinated with hapten-protein conjugates seroconverted, generally, detectable 3 weeks after the first booster vaccination. The epoxyscillirosidine-KLH conjugate produced the highest OD value, which was significantly ($p < 0.01$) higher than the other immunogens, on D 42 and D 63 (Fig. 4.3). The antibodies synthesized against proscillaridin-BSA and bufalin-BSA, also cross-reacted with epoxyscillirosidine-OVA, in the ELISA (Fig. 4.4). Antibodies against the proscillaridin-BSA conjugate, increased with each

vaccination, with the highest level attained on D 67. Antibodies against the bufalin-BSA conjugate that cross-reacted with the epoxyscillirosidine-OVA immunogen only increased after two vaccinations, from D 42, with the same level maintained on D 67.

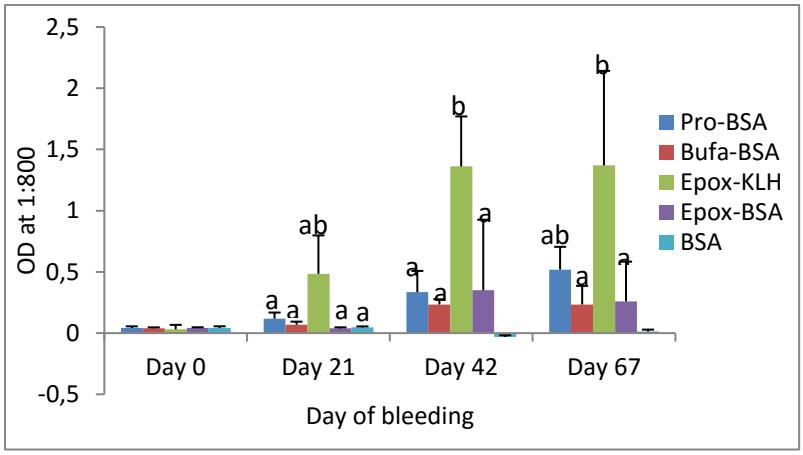


Figure 4.3 : Trial 3: Mean optical density values (490 nm) obtained in an epoxyscillirosidine-OVA ELISA for sera (at 1:800 dilution) from rabbits vaccinated with the hapten-protein conjugates (proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA) or just the protein (BSA). Vaccinations were done using Montanide as adjuvant. Different alphabetical letters for each day are significantly different ($p < 0.01$).

When using epoxyscillirosidine alone as antigen in an ELISA, the antibodies against all the administered conjugates bound to epoxyscillirosidine (Fig. 4.4).

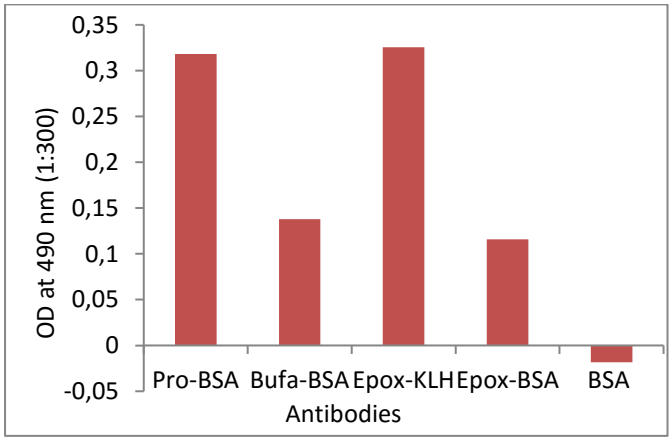


Figure 4.4. Trial 3: Mean optical density values (490 nm) obtained for sera (at 1:300 dilution) from rabbits vaccinated with the hapten-protein conjugates (proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA) or just the protein (BSA) and coating the ELISA plate with only epoxyscillirosidine as antigen. The net OD value was obtained by subtracting the OD of the coating buffer well from that of the epoxyscillirosidine coated well.

4.4 Discussion

In this study, it was unknown if the doses (0.104 – 0.172 mg/kg) of epoxyscillirosidine used in the vaccination fall under toxic dose range, because the median lethal dose in rabbits has not been reported in literature. The toxic reaction to the effect of epoxyscillirosidine was reported to be erratic and unpredictable, where animals may not show any signs at a given dose but may suddenly die, at a slightly higher dose, in a mixed strain of rabbits (Naudé and Potgieter, 1971). However, in the guinea-pig and mice, the subcutaneous LD_{50} values according to the method of Litchfield and Wilcoxen (1949), were reported as 0.194 ± 0.01 and 3.6 ± 0.9 mg/kg, respectively (Naudé and Potgieter, 1966). The absence of any signs of toxicity and or death of rabbits in this study is suggestive that the dose is below the threshold of toxicity and justifies the choice of the doses.

Antibodies against epoxyscillirosidine were raised in rabbits, following the intradermal administration of vaccines containing epoxyscillirosidine-KLH, epoxyscillirosidine-OVA, epoxyscillirosidine-BSA, as well as proscillaridin-BSA and bufalin-BSA conjugates, with a suitable adjuvant. However, only the KLH conjugate of epoxyscillirosidine induced the production of significantly ($p < 0.01$) high levels of antibodies. In Trials 1 and 2, the OVA-conjugate was used in the vaccine. The reasoning was that if the ELISA plates were coated with the BSA-conjugate in alternate wells and the OD from the corresponding wells, coated with BSA only, was subtracted from the OD obtained in BSA-conjugate wells, it should give a net epoxyscillirosidine reaction. Therefore, this ELISA should not detect any antibody from OVA even if there could be cross-reaction between BSA and OVA. It was essential, to use an irrelevant protein to the vaccine carrier protein, to eliminate cross-reaction between the proteins in the assays. The colour reaction due to epoxyscillirosidine was confirmed by coating the plates with only epoxyscillirosidine (Fig. 4.4).

In Trial 1, although antibodies were raised following vaccination with epoxyscillirosidine-OVA the antibody levels were relatively low (Fig 4.1). The dose of the immunogen (0.4 mg per rabbit) administered, adjuvant used or both were most likely responsible for the low immunogenicity

elicited by epoxyscillirosidine-OVA as higher levels of antibodies were synthesized following the doubling of the dose of epoxyscillirosidine-OVA and changing the adjuvant to Montanide in Trial 2. The dose determines the degree of immune response to an administered vaccine (Cahn *et al*., 2004), or to hapten-protein conjugate (Fodey *et al*., 2009). The dose of an antigen delivered to the immune system is determined by the amount of immunogen administered (Cooper *et al*., 2004) or the degree of incorporation of the hapten to the carrier protein (Sing *et al*., 2004). The dose of epoxyscillirosidine-OVA in the first trial, was adjudged too low following the poor immune response generated by the administered immunogen. This necessitated the dose being increased two fold, in Trials 2 and 3. Furthermore, Montanide, the adjuvant used in Trial 2 possibly contributed to the improved immune response compared to Trial 1. Montanide adjuvants have been reported to be superior compared to Freund's (Klimka *et al*., 2015; Lone *et al*., 2017).

In Trial 3, antibodies were raised against proscillaridin-BSA, bufalin-BSA, as well as KLH and BSA conjugates of epoxyscillirosidine. The highest concentration of antibodies was obtained with the KLH conjugate of epoxyscillirosidine. KLH is a superior carrier protein due to its higher molecular weight, complexity and foreignness, which are all properties that determine the degree of immunogenicity of a compound (Kuby, 1994). In addition, it is possible the conjugation with KLH produced a more favourable hapten density, compared with the BSA conjugate of epoxyscillirosidine. This may explain the higher immune response compared with the BSA conjugate of epoxyscillirosidine. The generated antibodies against epoxyscillirosidine conjugates, were specific to epoxyscillirosidine, as we obtained a positive reaction when the epoxyscillirosidine alone (hapten), was coated as antigen in an ELISA (Fig. 4.4). Other studies have reported using the conjugate instead of the hapten, in the ELISA to determine antibodies against the hapten (Fonseca *et al*., 2013; Torres *et al*., 2014). Upon vaccination with a hapten-protein conjugate, antibodies may be hapten-specific or they may express specificity to the link region, depending on the size of the conjugate (Clementi *et al*., 1991). In this trial also, the antibodies raised against proscillaridin-BSA

and bufalin-BSA cross-reacted with both epoxyscillirosidine and its OVA conjugate in an ELISA (Fig. 4.3 and 4.4). Cross-reactivity among cardiac glycosides has been reported previously. Sich *et al*., (1994) raised antibodies against proscillaridin which selectively cross-reacted with bufalin, a related bufadienolide, but not with the cardenolides and other steroid compounds studied.

Proscillaridin and bufalin, 2 commercially available bufadienolides, were utilized in the cross-reactivity studies, in order to circumvent a legislation (NEMBA Act 10; 2004), which prohibits indiscriminate exploitation of the natural environment in South Africa. Furthermore, since they are commercially available, bufalin and proscillaridin could easily be sourced and conjugated, to prepare a vaccine, rather than having to obtain yellow tulp samples in the field and isolating epoxyscillirosidine in a tedious and time consuming manner.

Conjugating proscillaridin and bufalin with KLH might have resulted in synthesis of higher antibodies titres that could cross-react better with epoxyscillirosidine. The degree of specificity and cross-reactivity of antibodies against the bufadienolides could be influenced by the coupling method employed in the preparation of the immunogens (Yan *et al*., 2017). In addition, the hapten density could influence the orientation of the molecules on the carrier protein or cause steric interference when binding to B cell receptors, thus affecting the nature of antibodies produced (Jung *et al*., 2008; Li *et al*., 2012).

In conclusion, the bufadienolide haptens: epoxyscillirosidine; proscillaridin; and bufalin, were successfully conjugated to carrier proteins, thus rendering them immunogenic. Although antibodies against the bufadienolide conjugates were raised in rabbits, the levels were low except for the KLH conjugate of epoxyscillirosidine. Cross-reactivity between the related bufadienolides, proscillaridin and bufalin, with epoxyscillirosidine was demonstrated in an indirect ELISA where antibodies bound unconjugated epoxyscillirosidine. The conjugation methodology for KLH will be adjusted in future studies to identify the optimal hapten density and challenge studies will be conducted in sheep.

CHAPTER FIVE

SHEEP VACCINATION WITH EPOXYSCILLIROSIDINE-KLH

Preface

As antibodies were raised in the three trials where rabbits were vaccinated with the different epoxyscillirosidine conjugates, it was decided to scale up and vaccinate sheep. Higher concentrations of antibodies were raised with the epoxyscillirosidine-KLH conjugate compared to the other conjugates, it was decided to plan a study to vaccinate sheep with this conjugate and evaluate if antibodies would also be raised in sheep.

5.1 Introduction

Poisoning by *Moraea pallida* Bak. (Iridaceae), one of the most important cardiac glycoside plant-induced poisoning, constitutes a serious economic burden. It is estimated that yellow tulp causes thousands of livestock deaths annually, in the Republic of South Africa (Kellerman *et al*., 1996). Yellow tulp poisoning, together with other cardiac glycoside toxicoses, accounts for about 33% and 10% mortalities, due to plant poisonings, in large ruminants and small stock, respectively (Kellerman *et al*., 1996). The plant contains 1α, 2α-epoxyscillirosidine, a bufadienolide, as the major toxic principle, which is chemically closely related to scillirosidine contained by *Drimia maritima* var. *rubra*. Epoxyscillirosidine was identified as being responsible for intoxication of livestock (Enslin *et al*., 1966) as well as being the natural aversive substance resulting in conditioned feed aversion in livestock (Snyman *et al*., 2004; Kellerman *et al*., 2005; Snyman *et al*., 2009).

Cardiac glycosides generally act by inhibiting the sodium-potassium-ATPase, the ubiquitous enzyme present on the membrane of cells, which is responsible for maintaining sodium and potassium ionic homeostasis (Fontana *et al*., 2013). Yellow tulp poisoning affects the cardiovascular, gastrointestinal, respiratory and nervous systems and signs may include general apathy, diarrhoea,

tremors, weakness of hindquarters, respiratory distress and at times bruxism and groaning sounds. Other clinical signs include tachycardia, arrhythmia, rumen atony and bloat. Death may occur due to cardiac arrest (Hutcheon cited by Kellerman *et al*., 2005; Steyn, 1928; Naudé, 1977).

Treatment of poisoned animals is often unsuccessful (Kellerman, 2009). However, Joubert and Schultz (1982), concluded that drenching poisoned animals with activated charcoal is very effective, albeit stressful to the animals, expensive and needs to be instituted soon following exposure. It is thus imperative to explore other ways to prevent and or treat poisoning by yellow tulp in livestock. Prophylaxis with vaccines or immunotoxicotherapy using immune sera, could be explored in the prevention and management of yellow tulp poisoning (Kellerman, 1996). Tulp poisoning is ordinarily prevented by herding animals, fencing off infested areas or physically eradicating the plants (Kellerman *et al*., 2005). Chemical control using herbicides is, sometimes used, where eradicating the plants physically, is not practicable (Steyn, 1928). Manipulating animals to resist poisoning, has better potential for prevention, than changing the environment (Kellerman, 2009).

Interest in developing vaccines against phytotoxins has been on the increase in recent times, due to the veterinary and economic importance of poisonous plants. Vaccines have been developed to prevent poisoning of animals by plant species such as *Lupinus* spp infected by *Diaporthe toxica* (Edgar *et al*., 1998), *Festuca arundinacea* (tall fescue) infected with *Neotyphydium* endophytes (Filipov *et al*., 1998), *Delphinium* spp (larkspur) (Lee *et al*., 2003), and *Oxytropis kansuensis* (locoweed) (Tong *et al*., 2007). Botha *et al*., (2007), also reported a promising result in their work to develop a krimpsiekte vaccine in sheep. Vaccination as a means of preventing gossypol intoxication was studied (Fonseca *et al*., 2013). The aim of this study was to investigate if antibodies against epoxyscillirosidine could be raised in sheep.

5.2 Materials and Methods

5.2.1 Experimental animals

Adult male Mutton Merino sheep (n=6), were obtained from the experimental farm, University of Pretoria, South Africa. The average body weight of the animals was 53.7 kg. The animals were randomly assigned into two groups of four and two. Group I (n=4) served as experimental while Group II (n=2) served as control. For the entire duration of the study the animals were housed in holding pens of the Onderstepoort campus, University of Pretoria, under natural environment conditions. The animals were kept in one group. Lucerne and teff hay and a pelleted concentrate were provided and municipal water provided *ad lib*. Approval for the ethical use and care of laboratory and other animals was obtained from the Animal Ethics Committee (Project no: V016-16), Faculty of Veterinary Science, University of Pretoria. The animals were acclimatized for 3 weeks, before the commencement of the experiment.

5.2.2 Preparation of immunogen

Epoxyscillirosidine was previously conjugated to KLH (Chapter 3). A vaccine was prepared by adding the epoxyscillirosidine-KLH (4 mg/ml) or KLH, dissolved in normal saline (4 mg/ml), to an adjuvant (Montanide ISA (Sigma)) - in a ratio of 1:1 (v/v). The resulting suspension was continuously mixed, with the aid of a glass syringe, until a stable emulsion was formed.

5.2.3 Sheep vaccination

5.2.3.1 Study design

The 4 test sheep (Group I) and two controls (Group II) were vaccinated with epoxyscillirosidine-KLH conjugate or KLH only respectively on five occasions on days 0, 21, 42, 63 and 84 (Table 5.1).

Table 5.1: Sheep vaccination schedule

Experimental group	Immunogen (Concentration)	Adjuvant	Day of vaccination (Day of blood collection)
Group I (n=4)	Epoxyscillirosidine KLH (4 mg/ml)	Montanide	0, 21, 42, 63*, 84* (0, 21, 42, 63, 84, 105)
Group II (n=2)	KLH (4 mg/ml)	Montanide	0, 21, 42, 63*, 84* (0, 21, 42, 63, 84, 105)

* Additional vaccinations

5.2.3.2 Experimental procedures

An area on the ventro-lateral aspect of the neck of each sheep was shaved to allow easy access to the jugular vein for blood sampling. Animals were restrained and 1 ml (4 mg per sheep) of the vaccine administered subcutaneously, alternating between the axilla and loin. Before each vaccination on D 0, 21, 42, 63, 84 and on D 105 representing the end of the trial, temperature of the sheep was recorded and about 40 ml blood collected from the jugular vein to determine antibody titres. Collected blood was allowed to stand for 30 min, at room temperature. Serum was subsequently obtained by centrifugation at 1204 x *g* at 20 ^{o}C (Allegra^{TM} X-22 Centrifuge, Beckman Coulter), aliquoted and stored at -20 ^{o}C.

5.2.4 Evaluation of immunological response using ELISA

An ELISA to determine antibody titres was conducted. Maxisorb 96-well plates (NUNC) were coated with 50 µl antigen (epoxyscillirosidine-BSA conjugate) at a dilution of 10 µg/ml in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) in alternate rows. The remaining rows were coated with BSA, instead of antigen, at the same concentration. The plates were incubated overnight, in the laminar flow cabinet, to dry. Thereafter, the plates were blocked with 200 µl PBS supplemented with 5% skimmed milk powder per well and incubated for 30 min, at room temperature, while shaking. This was followed by washing three times (with wash buffer). Sera were loaded in duplicate rows (e.g. row A coated with immunogen and row B coated with BSA) and a

two-fold serial dilution (50 µl per well) (using blocking buffer and starting at 1:50 for sheep sera), was done across the plate, followed by incubation for 1 h, at room temperature and washing 5 times, using washing buffer. After washing, 50 µl horseradish-peroxidase (HRPO) protein G conjugate, at a dilution of 1:3,000 was added to all wells. Following further incubation, at room temperature and washing five times, in wash buffer, 50 µl substrate (ortho-phenylene diamine at a concentration of 0.04 mg/ml and containing 0.05% H_2O_2 30% v/v), was added to all wells and the plates incubated for 15 min, in the dark, after which 50 µl stop solution (1N H_2SO_4), was added to all wells. The plates were read in a BioTek EL808 plate reader, at 490 nm and the results recorded, accordingly. The net optical density (OD), was calculated by subtracting the value obtained for the BSA coated well from the corresponding antigen-coated well.

In another assay, only epoxyscillirosidine as antigen and coating buffer were coated on the plate in alternate rows, to confirm the specificity of the generated antibodies against the epoxyscillirosidine.

5.3 Results

5.3.1 Sheep vaccination

Toxic signs were absent in all the sheep following vaccination. The immune response of the sheep, to the administration of epoxyscillirosidine-KLH, was evaluated, with an indirect ELISA. The response of the sheep was variable. All the four sheep vaccinated with epoxyscillirosidine-KLH, seroconverted after the first vaccination, detectable on D 21 (Fig. 5.1). The response of the four sheep, vaccinated with the KLH conjugate, varied after the first booster vaccination, detected on D 42. Immune response of the two experimental sheep (364 and 359) dropped after the fourth and fifth vaccinations, detected on D 63 and 84, respectively. In contrast, Sheep 346 and 363 maintained

relatively the same level of antibodies, three weeks after the fifth vaccination (Fig. 5.1). None of the control sheep developed detectable levels of antibodies against epoxyscillirosidine (Fig. 5.1).

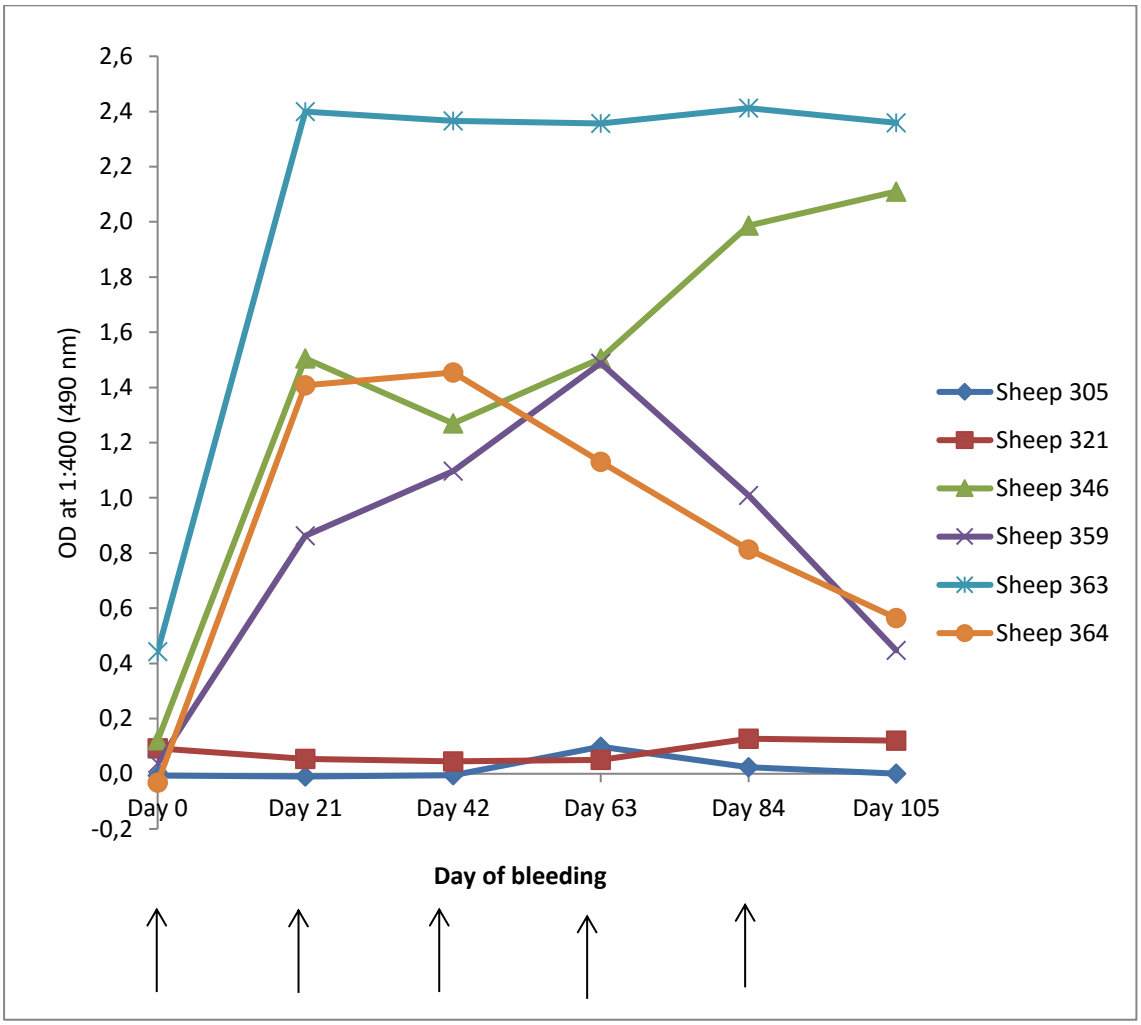


Figure 5.1: Optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA for sera (at 1:400 dilution) from sheep vaccinated with epoxyscillirosidine-KLH (Sheep 346, 359, 363, 364) or KLH (Sheep 305 and 321). The arrows indicate the days of vaccination. Montanide was used as adjuvant. The OD of the BSA coated well was subtracted from that of the epoxyscillirosidine-BSA well to obtain the net OD. Samples were added to the wells in duplicate.

When using unconjugated epoxyscillirosidine as antigen in an ELISA, we were able to demonstrate and confirm antibodies specific to epoxyscillirosidine as hapten (Fig. 5.2).

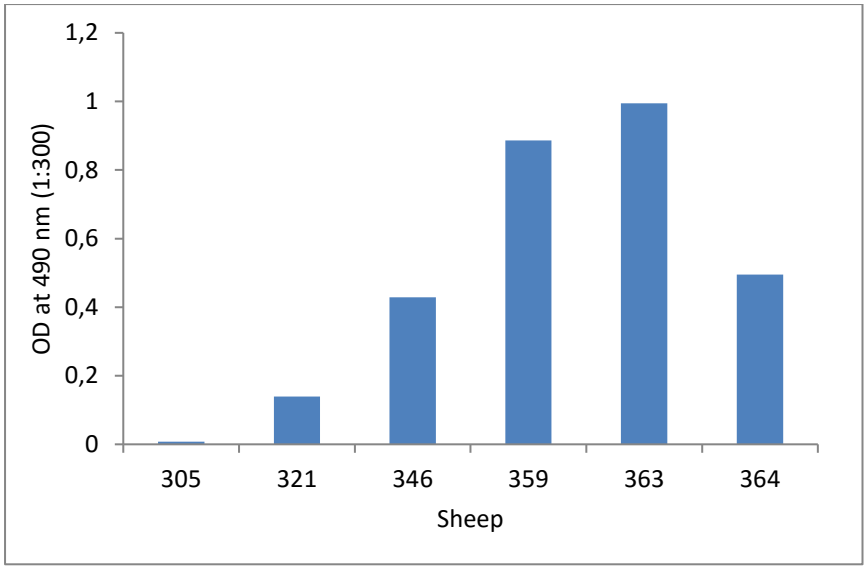


Figure 5.2: Mean optical density values (490 nm) obtained for sera (at 1:300 dilution) from sheep vaccinated with the epoxyscillirosidine-KLH (Sheep 346, 359, 363, 364) or just the protein (KLH; sheep 305 and 321) and coating the ELISA plate with only epoxyscillirosidine as antigen. The net OD value was obtained by subtracting the OD of the coating buffer well from that of the epoxyscillirosidine coated well.

5.4 Discussion

This work was an extension of the previous trials in rabbits (Chapter 4) where antibodies were successfully raised against the administered conjugates. The rationale was to scale up the study to be able to generate larger quantities of antibodies for evaluation of *in vitro* neutralization in a rat embryonic cardiomyocyte model. Antibodies against epoxyscillirosidine were successfully raised in sheep as revealed by the ELISA (Fig. 5.1). The administered conjugate (epoxyscillirosidine-KLH) was selected for the trial in sheep as it produced the best immune response among all conjugates evaluated in rabbits, as reported previously (Chapter 4). The finding from this section has further confirmed the effectiveness of epoxyscillirosidine-KLH as an immunogen. The KLH is a highly efficient carrier protein due to its higher molecular weight, complexity and foreignness which are all properties that determine the degree of immunogenicity of a compound (Kuby, 1994). In addition, the conjugation reaction (Chapter 3) produced a hapten density that was conducive to antibody production.

Since the KLH-conjugate was used in the vaccine, it was essential, to use an irrelevant protein to the vaccine carrier protein, to eliminate cross-reaction between the proteins in the assays. Thus, the ELISA plate was coated with BSA-conjugate. Subtracting the OD from BSA only coated well from the BSA-conjugate wells should give a net epoxyscillirosidine reaction. Therefore, this ELISA should not detect any antibody from KLH or BSA. Following vaccination with a hapten-protein conjugate, the generated antibodies may be hapten-specific or they may express specificity to the link region, depending on the size of the conjugate (Clementi *et al*., 1991). The antibodies raised against epoxyscillirosidine alone (Fig 5.2) further confirmed their specificity.

This study successfully raised antibodies in sheep, against the bufadienolide hapten epoxyscillirosidine. One of the earliest reports of antibodies generated against specific haptens, was by Landsteiner (1945). Butler and Chen (1967), were the first to describe the production of antibodies against cardiac glycosides. They raised and investigated antibodies specific for digoxin for possible use in immunological clinical assays. A year later, digitoxin-specific antibodies were reported by Oliver *et al*., (1968). Smith (1972), reported on antibodies raised against ouabain, while Belz *et al*., (1973) reported on the characterization and specificity of proscillaridin antibodies. More recently, antibodies against cotyledoside, a cumulative neurotoxic bufadienolide, were successfully raised in sheep in a study evaluating the potential for a Krimpsiekte vaccine (Botha *et al*., 2007). The potential for immunizing animals against plant poisonings, had earlier been investigated in South Africa. Joubert, accordingly, attempted developing a vaccine against cardiac glycoside toxicosis by conjugating cardiac glycosides with BSA. Although sheep established robust immunity to a specific cardiac glycoside in the vaccine, there was weak cross-immunity against trials with non-homologous cardiac glycosides or cardiac glycoside-containing plants (Joubert, 1982 cited by Kellerman *et al*., 2005).

In conclusion, antibodies were produced by the administration of epoxyscillirosidine-KLH conjugate together with Montanide as adjuvant in sheep. The generated antibodies will be evaluated for their *in vitro* neutralization efficacy in a suitable model.

CHAPTER SIX

EPOXYSCILLIROSIDINE INDUCED CYTOTOXICITY AND ULTRASTRUCTURAL CHANGES IN A RAT EMBRYONIC CARDIOMYOCYTE (H9c2) CELL LINE

Preface

Approval was received from the Animal Ethics Committee to vaccinate sheep. However, the Committee suggested that before sheep could be challenged in future studies with toxic plant (yellow tulp) material, an *in vitro* toxin neutralization assay must be conducted first, to ascertain if the antibodies raised were able to neutralize the plant toxin (epoxyscillirosidine). Preparatory to the *in vitro* neutralization assay, a rat embryonic cardiomyocytes cell line (H9c2) was grown and maintained to evaluate the cytotoxic effect of epoxyscillirosidine.

6.1 Introduction

Moraea pallida Bak. (yellow tulp) poisoning in livestock is the most important of all cardiac glycoside-associated plant poisonings in the Republic of South Africa. Acute poisoning with severe cardiac rhythm aberrations occurs and the mortality rate is high (Kellerman *et al*., 1996). Microscopic cardiac lesions are myocardial degeneration and necrosis (Kellerman *et al*., 2005). The toxic principle is a bufadienolide, 1α, 2α-epoxyscillirosidine (Enslin *et al*., 1966; Kellerman *et al*., 2005). Bufadienolides, similar to other cardiac glycosides, interfere with the function of the ubiquitous sodium potassium adenosine triphosphatase (Na^+-K^+-ATPase) on the cell membrane (Steyn and van Heerden, 1998). The Na^+-K^+-ATPase acts as the receptor for cardiac glycosides and structurally similar compounds (Steyn and van Heerden, 1998; Kamboj *et al*., 2013).

The H9c2 (2-1) embryonic rat cardiomyocyte cell line is a subclone of the original clonal line which was derived from embryonic BD1X rat cardiac tissue (Kimes and Brandt 1976). This cell line has commonly been utilized in cardiotoxicity studies of novel compounds, mainly anticancer drugs,

to elucidate mechanisms of cell injury in cardiac cells, and to evaluate apoptotic and necrotic lesions in cardiomyocytes (Witek *et al*., 2016).

There has been no *in vitro* study on the effect of epoxyscillirosidine on cardiac cells. The response of these cells following epoxyscillirosidine exposure may reveal important clues as to how cardiac cells and the heart are affected in animals poisoned by yellow tulp. In addition, an *in vitro* tissue culture model can replace the use of sentient animals and circumvent animal ethics concern in future toxicity studies. The aim of this study was to investigate the effect of epoxyscillirosidine on rat embryonic cardiomyocytes of the H9c2 type, by evaluating cell viability, cytotoxicity, as well as characterizing morphological changes, in exposed cells. Cell viability was evaluated using the methyl blue thiazol tetrazolium (MTT) assay, while cytotoxicity was determined using the lactate dehydrogenase (LDH) release assay. Ultrastructural changes were assessed using transmission electron microscopy (TEM).

6.2 Materials and methods

6.2.1 Chemicals and reagents

Purified epoxyscillirosidine was isolated (Chapter 3). Dimethyl sulphoxide (DMSO, Cat. No: SAAR1865000LP) and Triton X-100 were obtained from Merck (Darmstadt, Germany). Trypsin-EDTA (Cat No: BE17-16IF) and L-glutamine (Cat No: BE-17-605E) were acquired from Lonza (Verviers, Belgium). The LDH assay kit (CytoTox-ONE^{TM}, cat. No: G7890) was purchased from Promega Corp., USA. Dulbecco's Modified Eagle's Medium (DMEM, cat. No: D6546), phosphate buffered saline (PBS, Cat. No: P4417), penicillin-streptomycin (Cat. No: P4333), MTT (thiazolyl blue tetrazolium bromide) reagent (Cat. No: M5655), and trypan blue (Cat. No: T6146) were from Sigma-Aldrich (Germany). Foetal bovine serum (Cat. No: 10499-044, Gibco) was from Life Technologies (Grand Island, New York).

6.2.2 Cell culture

Rat embryonic cardiomyocytes [H9c2 (2-1) cells] (Kimes and Brandt, 1976) were purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA, cat no: CRL-1446^{TM}). The cells were cultured in DMEM supplemented with 10% foetal bovine serum, 4 mM L glutamine and penicillin-streptomycin (100 U/ml) in 75 cm^2 tissue culture flasks. Cells were maintained in a humidified incubator (HeraCELL 150^{R}, Thermo-Electron Corporation) in a 95% air 5% CO_2 environment at 37^{o} C. Medium was changed every 3 – 4 days while the cells were sub-cultured after attaining about 70-80% confluency. Cells were detached using trypsin-EDTA. Cells were seeded for cytotoxicity and ultra-structural studies in 96-well micro-titre plates and 12-well plates, respectively.

6.2.3 Cytotoxicity studies

The cells were detached from the cultivation flasks using trypsin-EDTA and counted with a haemocytometer with the aid of trypan blue exclusion to determine viability. Three 96-well plates were seeded with 1 x 10^4 cells per well (final volume 200 µl per well). Wells in each plate containing medium only, with no cells, served as blank. The plates were incubated for 24 h to allow for cell attachment and recovery. Thereafter, 10, 20, 40, 60, 80, 120, 160 and 200 µM concentrations of epoxyscillirosidine were added to the wells in triplicate. Cells cultured in growth medium only, representing 100% viability, were used as negative control in all experiments. In the LDH release assay, Triton X-100 was added to separate cells as positive control. The three plates were incubated for 24, 48 and 72 h, respectively, before evaluation of cell viability and cytotoxicity using MTT and LDH assays, respectively. The assays were validated and optimized before adoption in each case.

The doses selected were identified following a preliminary dose finding study. The experiments were repeated three times, at weekly intervals. All studies were conducted in triplicate.

6.2.3.1 Evaluation of cell viability

Cell viability, following exposure of H9c2 cells to varying concentrations of epoxyscillirosidine, was determined using the MTT assay (Mossman, 1983). This is a quantitative colorimetric assay that spectrophotometrically measures the amount of purple formazan crystals formed by dehydrogenases, in living viable cells, from the yellow tetrazolium salt MTT. In brief, the three plates were treated after 24, 48 and 72 h, respectively, as follows: 100 µl of the medium was transferred to the LDH assay plate and the remaining medium decanted and cells washed with PBS (200 µl per well), followed by the addition of fresh DMEM (200 µl per well). Thereafter, 20 µl MTT reagent was added serially into all wells. The plates were incubated for 2 h. The medium containing MTT reagent was decanted, followed by the addition of 100 µl DMSO and shaking on a microplate shaker for 5 min to dissolve formed formazan. The absorbance was read in a multi-reader (Synergy HT, BioTek^{R}) using the Gen 5 protocol at 570 nm, using a reference wavelength of 630 nm. Percentage cell survival was calculated using the formula:

*Cell viability (%) = (Absorbance of epoxyscillirosidine/Absorbance of cells only) * 100*

The median lethal concentration (LC_{50}) of epoxyscillirosidine was calculated using the straight line equation of the log dose-response curve of cytotoxicity against concentration.

6.2.3.2 Cytotoxicity assay using LDH release

Cytotoxicity in H9c2 cells exposed to epoxyscillirosidine, was evaluated using the lactate dehydrogenase assay, with the aid of a commercial kit CytoTox-ONE™. This is a fluorometric method that estimates the number of dead cells present in multi-well plates. The CytoTox-ONE™ assay rapidly measures the release of LDH from cells with a damaged membrane. LDH released into the culture medium is measured with a 10 min coupled enzymatic assay that results in the conversion of resazurin into a fluorescent resorufin product. The amount of fluorescence produced is proportional to the number of lysed cells. The assay was carried out according to the manufacturer's instructions. Aliquots (100 µl) of medium, from all wells in the plate seeded with H9c2 cells, were transferred into a black Nunc 96 well plate. Reconstituted LDH substrate mixture (100 µl each) was added to the wells in the black plate which was wrapped and shaken for 10 min. Stop solution was added and fluorescence was read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm and at a fluorescence sensitivity setting of 40, in a multi-reader (Synergy HT, BioTek^{R}). Per-cent cytotoxicity was calculated using the formula:

$$Cytotoxicity\ (\%) = (experimental - blank)/(Triton\ X - blank) * 100$$

6.2.4 Evaluation of ultrastructural changes

Three 12-well plates, in which coverslips were placed in each well, were seeded with 1 x 10^5 cells per well (final volume 2 ml per well). The plates were incubated for 24 h for the cells to recover and stabilize. Thereafter, 40, 80, 120, 160 and 200 µM concentrations of epoxyscillirosidine were added to the wells in duplicate. Untreated cells were used as control. The three plates were incubated for 24, 48 and 72 h, respectively, before the cells were further processed for TEM.

Transmission electron microscopy (TEM)

The culture medium was carefully removed from the wells and replaced with 2 ml of 2.5% glutaraldehyde in 0.075 M sodium phosphate ($NaPO_4$) buffer (pH 7.4) and left for 1 h. The

coverslips were removed and placed in a separate holder. The cells were scraped off from the culture coverslips, transferred to 2 ml Eppendorf tubes and centrifuged at 2356 *g* to form pellets. These as well as the coverslips were then rinsed 3 times in 0.075 M phosphate buffer for 10 min and post-fixed for 1 h with 1% osmium tetroxide (OsO_4). Samples were then rinsed again in 0.075 M phosphate buffer for 10 min and then dehydrated serially in 30, 50, 70, 90% and three times with 100% ethanol. The samples were embedded in TAAB 812 epoxy resin (Luft, 1961), followed by ultra-microtome sectioning. The sections were contrasted with a 2% aqueous solution of uranyl acetate, for 10 min and lead citrate (Reynolds, 1963) for 2 min and examined with a Philips CM10 (Philips Electron Optics, Eindhoven, The Netherlands) TEM.

6.2.5 Statistical analysis

Data were analysed using GraphPad Prism 7 for windows (version 7.03). Values were expressed as percentage of untreated control cells. Student's *t*-test or ANOVA was used to evaluate statistical difference for paired or multiple comparison between groups respectively. Statistical significance was set at $p < 0.05$.

6.3 Results

6.3.1 Cell viability

Cell viability increased when the H9c2 cells were exposed to epoxyscillirosidine at low doses (10 - 40, 10 and 10 - 20 µM), for 24, 48 and 72 h respectively. Differences in viability were inversely related to toxin concentration. After 24 h, the highest (relative to control) cell viability (137.68 ± 7.87%) was observed following exposure to 10 µM and the toxic effect started manifesting at 60 µM (95.62 ± 15.68% cell viability). The lowest cell viability (69.42 ± 8.95%) observed was at 160 µM (Fig. 6.1).

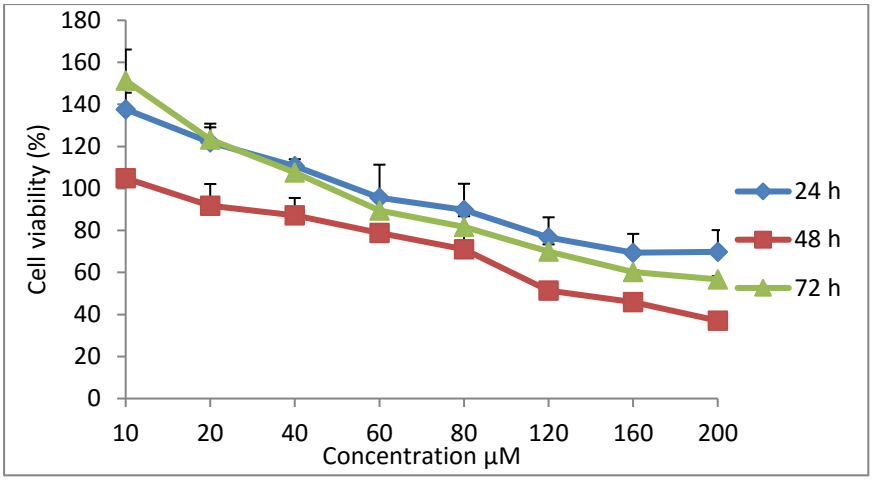


Figure 6.1: Dose-response curve for viability using MTT assay in H9c2 cells exposed to epoxyscillirosidine for 24, 48 and 72 h. Cell viability presented as percentage of control (n = 3). Each value in the curves represents per-cent viability (mean ± SD). The Student's *t*-test (unpaired, two tailed) was used to compare the durations of exposure. Cell viability was not significantly ($p > 0.05$) different between any of the durations (24, 48 and 72 h). The values were the average of three independent experiments.

After 48 h exposure, at the lowest dose (10 µM) evaluated, cell viability was 104.85 ± 4.32%. Toxic effects of epoxyscillirosidine were noticed from 20 µM with cell viability of 91.75 ± 10.35% (Fig. 6.1). Cell viability was 37.04 ± 2.34% at the highest dose (200 µM) evaluated.

After 72 h incubation at 10 µM epoxyscillirosidine the cell viability was 151.36 ± 14.75%. The toxic effect started manifesting only at 40 µM, where cell viability decreased to 89.47 ± 5.3%. At the highest dose (200 µM) tested the cell viability was 56.71 ± 1.49% (Fig. 6.1).

Overall, cell viability was not significantly ($p > 0.05$) different among the three durations.

6.3.2 Median lethal concentration

The median lethal concentration (LC_{50}) of epoxyscillirosidine after 24 h exposure was extrapolated to be 382.68 µM, while after 48 h it was 132.28 µM and 289.23 µM after 72 h (Fig. 6.2).

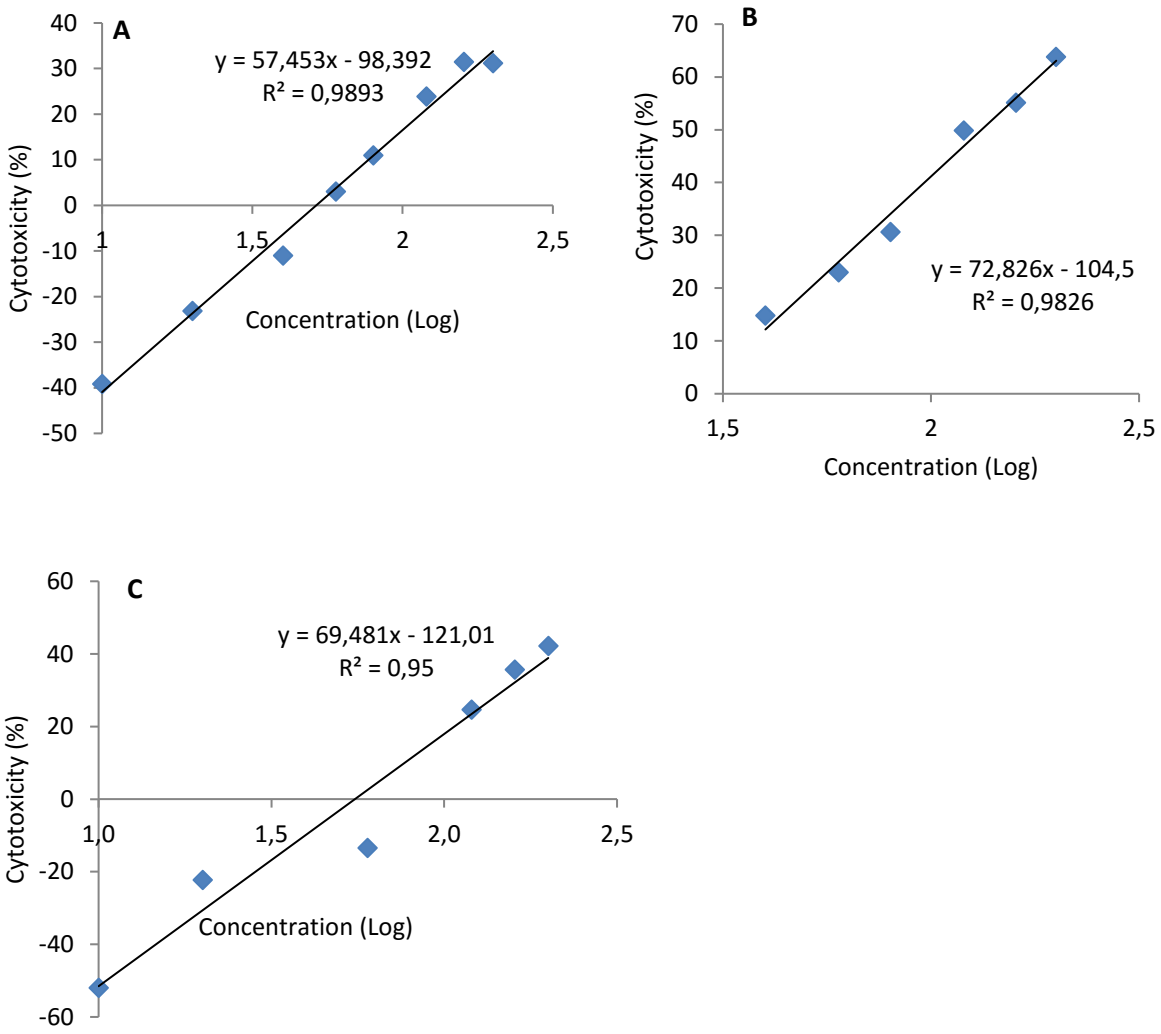


Figure 6.2: Semi-logarithmic concentration-cytotoxicity plots for H9c2 cells exposure to epoxyscillirosidine for 24, 48 and 72 h (A, B, and C, respectively). The MTT assay was used to evaluate cytotoxicity. The LC_{50} was determined by substituting the value for *y* with 50 in the equation $y = mx + c$. The LC_{50} is the antilog of the value obtained for *x*. The result represents the means of 3 independent experiments.

6.3.3 Cytotoxicity

Cytotoxicity following exposure to epoxyscillirosidine as evaluated using the LDH release assay revealed a dose and time dependent effect (Fig. 6.3). Cytotoxicity (expressed as percentage of total lysed cells) increased from 5.69 ± 1.79% after 24 h to 50.73 ± 16.97% after 72 h at the lowest dose (10 um) while it ranged from 40.02 ± 6.82 to 78.25 ± 18.52% at the highest dose (200 µM) evaluated.

Cytotoxicity at the same dose, but at different exposure durations revealed a time dependent effect. After 24 h exposure to epoxyscillirosidine the lowest percentage cytotoxicity across all the different doses occurred (Fig. 6.3). The 48 h duration showed lower percentage cytotoxicity when compared to the 72 h at lower doses up to 60 µM, but the effect was higher from 80 - 200 µM. Cytotoxicity at 72 h was significantly ($p < 0.05$) different from that at 24 h.

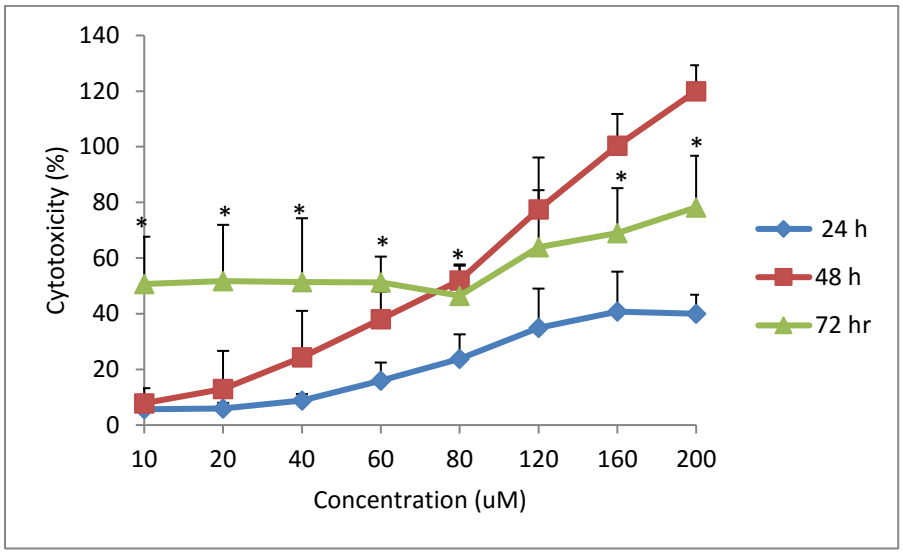


Figure 6.3: Dose-response curve for cytotoxicity (LDH assay) in H9c2 cells exposed to epoxyscillirosidine for 24, 48 and 72 h. Each value in the curve represents cytotoxicity as percentage (mean ± SD) of control. Student's *t*-test (unpaired, two tailed) was used to compare the durations of exposure. *Cytotoxicity at 72 h was significantly ($p < 0.05$) different from than at 24 h. The values are the result of three independent experiments.

Cytotoxicity as revealed by the MTT assay was not significantly ($p > 0.05$) different from the LDH assay at 24 and 48 h (Fig 6.4 A and B). However, there was significant ($p < 0.05$) difference in cytotoxicity revealed by the LDH assay when compared with the MTT assay at 72 h (Fig. 6.4C).

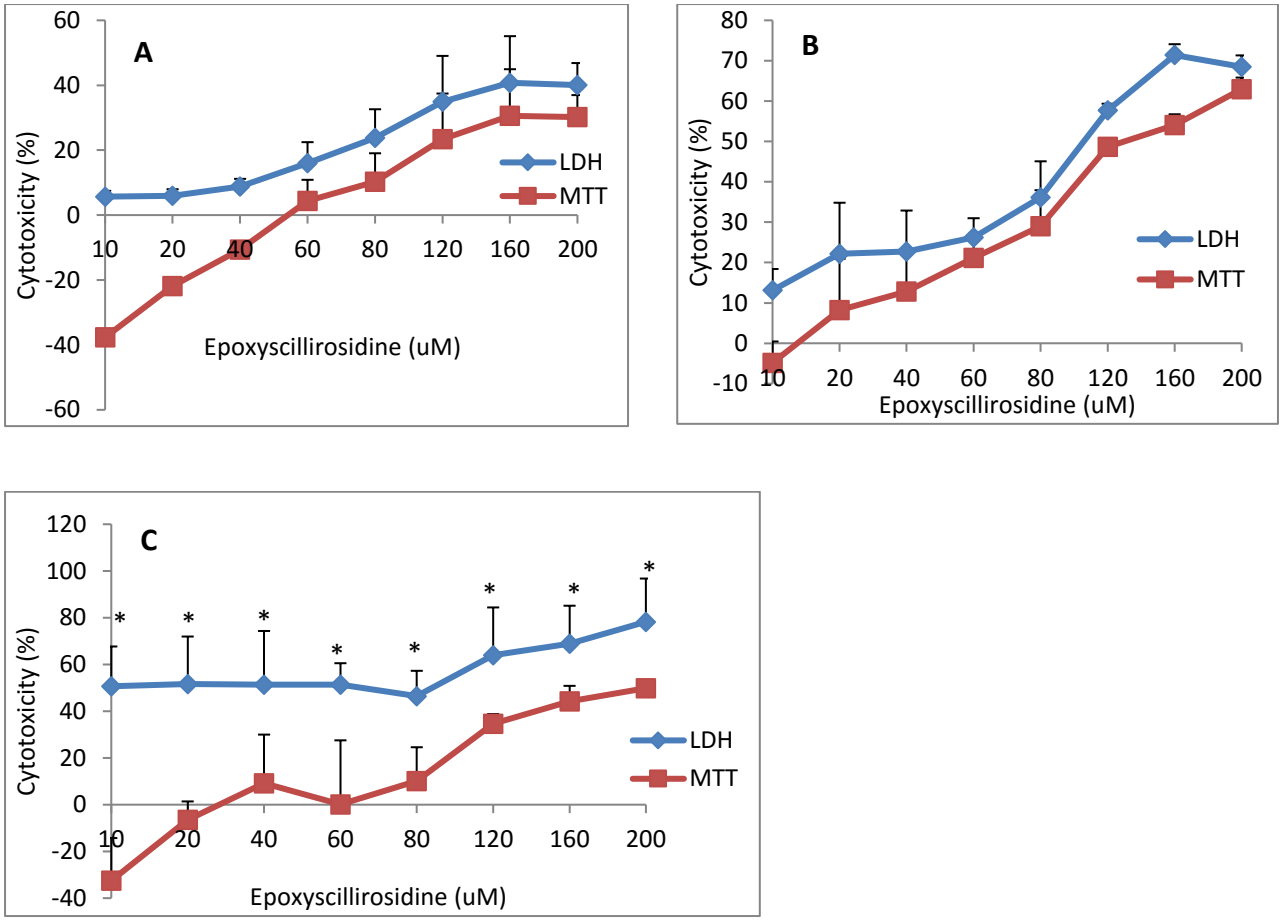


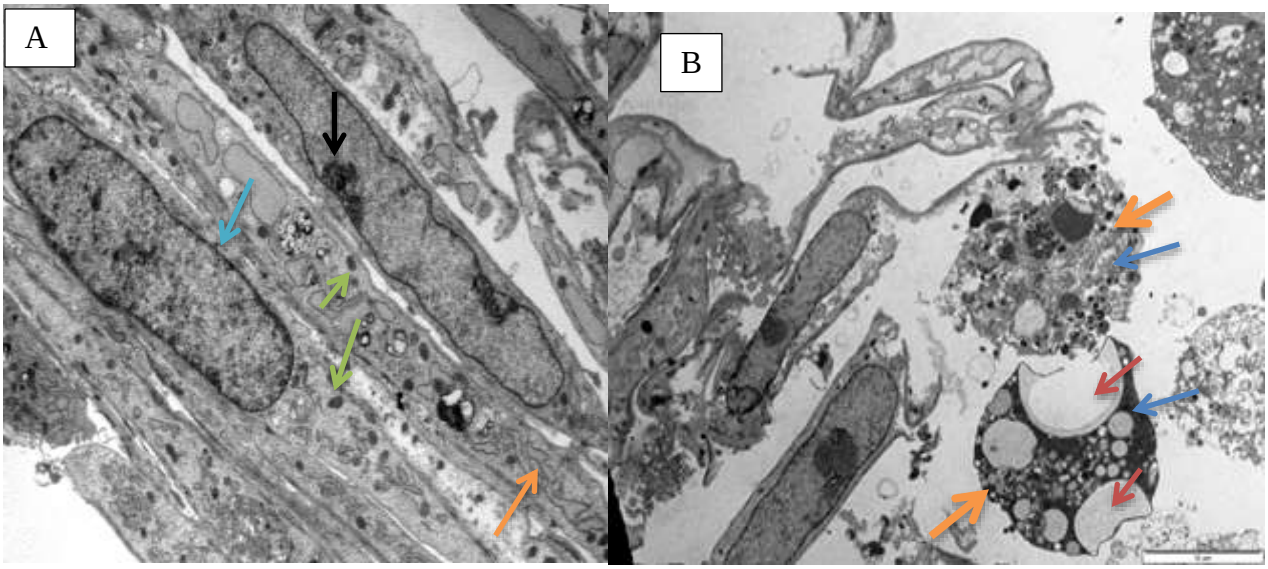
Figure 6.4: Comparison of the MTT and LDH dose-response curves of H9c2 cells exposure to epoxyscillirosidine for 24, 48 and 72 h (A, B and C, respectively). The negative MTT values represent a stimulatory effect and cell proliferation at low doses (hormesis). Each value in the curve represents percentage (mean ± SD) cell viability (relative to control). Student's *t*-test was used to analyse the data. Cytotoxicity using LDH compared with MTT assay was significantly (*$p < 0.05$) higher at 72 h. There was no difference ($p > 0.05$) in cytotoxicity as revealed by LDH and MTT assays at 24 and 48 h. The values are the means of three independent experiments.

6.3.4 Changes in ultrastructure

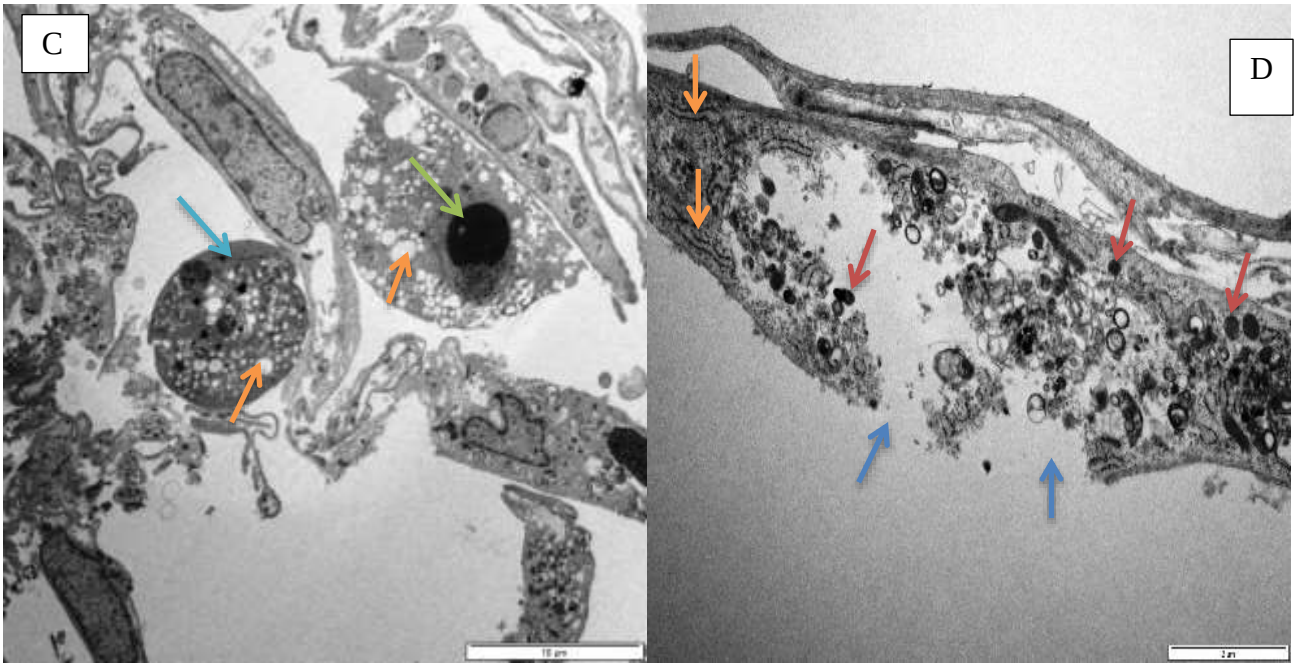
The organelles and cell shape were unaffected in the control cells (Fig 6.5 A). Normally, H9c2 myoblasts are rod-to-star-shaped, uni- or multinucleated cells (Witek *et al.*, 2016). In cells exposed to epoxyscillirosidine over 24, 48 and 72 h, time and dose dependent morphological alterations were observed. Appearance of cytoplasmic vesicles, vacuoles, changes in nuclear morphology, cytoplasm and damage to the cell membrane in H9c2 cells after exposure to epoxyscillirosidine were evident. These changes only became apparent at higher doses (160 – 200

µM) (Figs. 6.5 B, C and F). The cells also changed shape from spindle-like to spherical at higher concentrations (Figs. 6.5 B, C, E and F). Furthermore, varying degree of karyolysis was observed in the different exposures at higher doses (80 - 200 µM) (Figs. 6.5 C and F). Further nuclear changes caused by epoxyscillirosidine exposure were also observed. Chromatin condensation, a classic indicator of apoptosis, was likewise visible in certain cells, most likely as a result of normal physiological function due to senescence and ageing in H9c2 cells which may not be related with epoxyscillirosidine exposure (not shown).

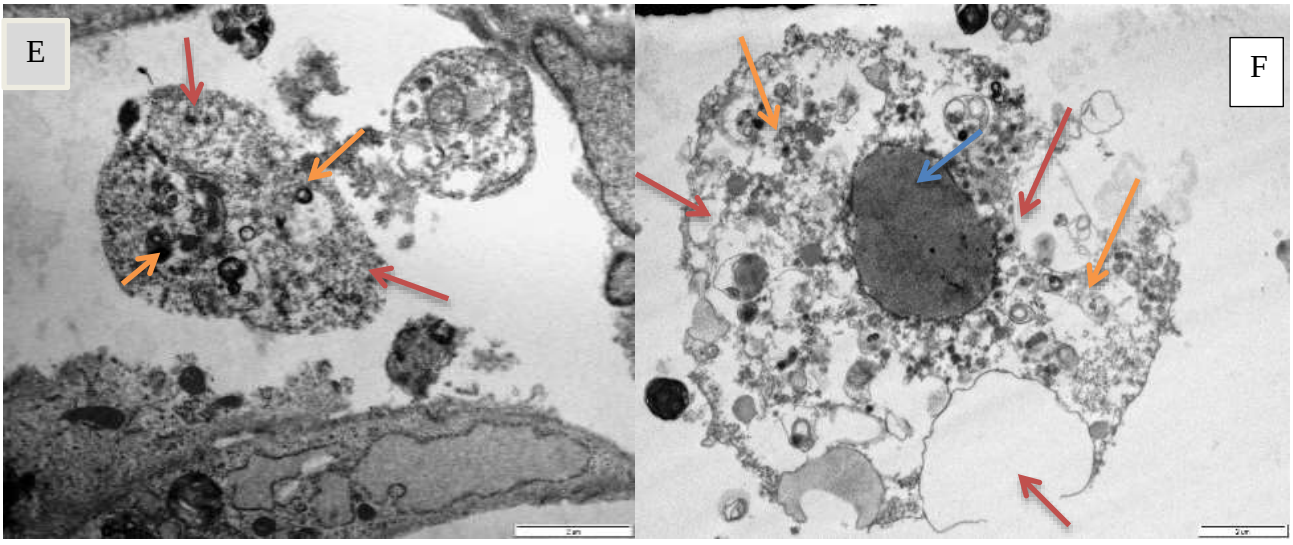
The cell membrane was interrupted, with wide spaces of discontinuity at higher concentrations (120 - 200 µM) at 48 (Fig 6.5 D) and 72 h (not shown). Furthermore, increased cytosolic vacuoles were observed at these exposures (Fig. 5F).



Figures 6.5A and 6.5B: Transmission electron micrograph showing, **A.** nucleus (blue arrow), mitochondria (green arrows) and endoplasmic reticulum (ER, orange arrow) in normal H9c2 cells (72 h control). Scale bar 5 µm. **B.** Micrograph showing vacuolation (brown arrows), diffuse dissolution of the cytoplasm (blue arrows), spherical shaped cells (thick orange arrows) in H9c2 cells exposed to epoxyscillirosidine (160 µM) after 24 h. Scale bar 10 µm.



Figures 6.5 C and 6.5 D: C. Micrograph showing karyolysis (green arrow), vacuolation (orange arrows) and diffuse dissolution of the cytoplasm and organelles (blue arrow) in H9c2 cells (200 µM, 24 h). Scale bar 10 µm. **D.** Micrograph showing damaged plasma membrane (blue arrows), numerous vesicles containing electron dense material (brown arrow) and ER (orange arrows) in H9c2 cells (120 µM, 48 h). Scale bar 2µm.



Figures 6.5E and 6.5F: E. Micrograph showing diffuse dissolution of the cytoplasm (blue arrow) and vesicles containing electron dense material (orange arrows) in amoeboid mis-shaped H9c2 cells (40 µM, 72 h). Scale bar 2 µm. **F.** Micrograph showing karyolysis (blue arrow), vacuolation (brown arrows) and diffuse dissolution of the cytoplasm (orange arrows) in H9c2 cells (200 µM, after 72 h). Scale bar 2 µm.

6.4 Discussion

This is the first report describing the *in vitro* cytotoxic effect of epoxyscillirosidine on H9c2 cells. At 24, 48 and 72 h following exposure to epoxyscillirosidine, a dose and time dependent effect was observed. At low doses, epoxyscillirosidine stimulates cell viability. The toxic effect manifesting as decreased cell viability was observed at higher doses starting from 60 µM at 24 and 48 h and at 120 µM at 72 h. Weyermann *et al*., (2005) reported increased values (above 100%) for viability using MTT assay at low doses, following exposure to sodium azide in mouse fibroblasts L (tk-) cells. This observation could be explained by hormesis, whereby a stress agent that is injurious to a biological system at high doses, produces a stimulatory effect at low doses (Calabrese and Baldwin, 2008; Calabrese, 2013a). Thus, the agent causes low dose stimulation and high dose inhibition of the observed/measured end point. Several *in vitro* studies have reported hormesis in different compounds including the cardiac glycoside, ouabain (Calabrese *et al*., 1999; Calabrese and Baldwin, 2001; Calabrese, 2013b). There are two major mechanisms that have been explained. It has been postulated that hormesis occurs as a compensatory mechanism following exposure to any stress agent and the mechanism is similar regardless of the level of organization (i.e cell, tissue, organ or whole organism), the nature of the stress agent (i.e. chemical, physical or biological) or the endpoint measured (viability, cell proliferation or death). The two pathways reported to mediate hormesis, are the signaling and the receptor mediated pathways (Calabrese, 2013b). It was reported that hormesis causes an increase to a factor less than two-fold and a maximal increase of 30 – 60% relative to control values (Calabrese *et al*., 1999). The mechanism through which hormesis was mediated was however not investigated in this study. Ouabain, a cardenolide-type cardiac glycoside, was reported to induce hormesis through both receptor- (Tian *et al*., 2009) and signalling pathway (Chueh *et al*., 2001) systems.

Viability of H9c2 cells exposed to epoxyscillirosidine at similar concentrations, but at different exposure times varied (Fig. 6.1). In all the three exposure periods evaluated, the highest

sensitivity of the cells to the effect of epoxyscillirosidine was detected after 48 h. At 24h, the time is probably too short to elicit a toxic effect, resulting in higher viability relative to 48 h. Pathological effects following exposure of cells to chemicals/stressors have been reported to be dose and time dependent in *in vitro* studies (Al-Ghamdi, 2008; Ellis *et al*., 2010; Neiva *et al*., 2013; Souza *et al*., 2017). However, at 48 h, more cells were damaged and hence the relatively lower viability. At 72 h, the cells have probably proliferated and increased in number after about 96 h of seeding. In addition, the cells slightly affected by the toxin probably recovered and metabolized MTT at a much higher rate, in form of compensation as explained by hormesis (Calabrese *et al*., 1999). This may explain why the viability at 72 h was higher than that at 48 h.

The median lethal concentrations calculated differed with exposure duration. A higher dose was required to produce death of 50% of the cells at 24 h than at 48 and 72 h. The lowest LC_{50} was observed in the 48 h duration. Thus epoxyscillirosidine requires time to influence the cell metabolism and the optimal effect was reached after 48 hours. At 72 h, the LC_{50} was higher than at 48 h, but lower than at 24 h. This could be explained by the fact at 72 h the cells previously not affected by the toxin have proliferated and increased in number. In addition, cells slightly affected probably were stimulated whereby at 72 h, overcompensation resulted in viability higher than at 48 h, as explained by hormesis (Calabrese and Baldwin, 2008). Values of LC_{50} have been reported to be influenced by exposure times according to the different experimental settings (Zhang *et al*., 2007).

Cytotoxicity using the MTT and LDH assay did not yield similar results in this study. The LDH assay showed a higher degree of variability while the MTT assay produced more consistent values. The MTT assay data was then used to determine the median lethal concentration (LC_{50}). Bopp and Lettieri (2008) reported higher intra- and inter-assay variabilities for LDH compared to the MTT assay and two other fluorometric methods. Cytotoxicity as measured with the LDH assay produced higher values even at low doses (Fig. 6.3), whereas low doses stimulated cell proliferation as determined by the MTT assay. This may be explained by the fact that the LDH assay measures all

dead cells including those that may have died physiologically and not from the action of the toxin. While MTT assay measures cell viability, an active phenomenon which is directly correlated with the effect of the toxin. In addition, recently damaged cells retain residual mitochondrial dehydrogenase activity which could contribute to the metabolism of MTT in non-viable cells (Otto *et al*., 2008). On the other hand, cytotoxicity is not exactly the opposite of cell viability. A cell may be exposed to an injurious agent without the cell dying, but may only experience some compromise in cellular functions. Thus the term '*cell vitality*' is used to refer to physiological capabilities of cells which is different from viability (Kwolek-Mirek and Zadrag-Tecza, 2014). The MTT assay determines viability which is a positive phenomenon in metabolically active and dividing cells (Mossman, 1983). Furthermore, at low doses, cell viability was stimulated (hormesis) and thus the lowered cytotoxicity at lower doses as captured by MTT was possibly due to metabolic activation or resilience to the effect of the toxin resulting in higher than expected values for viability. It was hypothesized that metabolic activation was responsible for increased viability due to chitosan/sulphated locust bean nanoparticle exposure in Caco-2 cells as revealed by MTT assay (Braz *et al*., 2017).

Electron microscopy has provided insight into morphological alterations instigated by exposure to epoxyscillirosidine for 24, 48 and 72 h. At the lowest dose evaluated (40 μM), in all the exposure periods, no noteworthy ultrastructural alterations were observed. The highest dose of epoxyscillirosidine evaluated (200 μM) was characterized by numerous cytoplasmic vacuoles and substantial nuclear changes, which include chromatin dissolution (karyolysis, Fig. 6.5C and F) and interruption of the nuclear membrane (Fig. 6.5D). The end result of the toxic effect of epoxyscillirosidine on H9c2 cells is necrosis, as evidenced by LDH leakage and confirmation by electron microscopy and is consistent with myocardial necrosis observed microscopically in poisoned livestock (Kellerman *et al*., 2005).

Ultra-structural changes induced following exposure of H9c2 cells to epoxyscillirosidine were dose and time dependent. At the lowest dose (40 µM) evaluated there were no significant lesions in subcellular organelles. This is supported by the results of the MTT assay, where decreased viability only started manifesting from 60 µM epoxyscillirosidine (at 24 h). The observed morphological changes at higher doses (160-200 µM) are confirmed by LDH leakage through the damaged cell membranes as demonstrated by the LDH assay findings in this study.

6.5 Conclusion

Following exposure of H9c2 cells to different concentrations of epoxyscillirosidine, the dose-response relationship indicated a hormetic effect, where low doses stimulated cell viability. The median lethal concentration of epoxyscillirosidine in rat embryonic cardiomyocytes (H9c2) was calculated at different exposure times using the MTT assay. The median lethal concentrations varied with 24 h being the highest followed by 72 and 48 h in that order. The LC_{50} after 24 h was 382.68 µM, while after 48 h it was 132.28 µM and 289.23 µM after 72 h. Ultra-structural changes suggested that the cause of H9c2 cell death subsequent to epoxyscillirosidine exposure is necrosis. Increased cell membrane permeability and leakage of content, as expected with necrotic cells, was confirmed with the LDH release assay. Based on the findings from this study, the H9c2 cell line was a suitable *in vitro* model to study the effect of epoxyscillirosidine and could be used to study similar compounds.

CHAPTER SEVEN

IN VITRO NEUTRALIZATION STUDIES IN A RAT EMBRYONIC CARDIOMYOCYTE (H9c2) CELL LINE EXPOSED TO EPOXYSCILLIROSIDINE

Preface

The Animals Ethics Committee suggested that before sheep could be challenged in future studies with toxic plant (yellow tulp) material, an *in vitro* toxin neutralization assay must be conducted first, This study investigated if the antibodies raised in sheep and concentrated and purified using ammonium sulphate precipitation were able to neutralize the yellow tulp toxin (epoxyscillirosidine). If the *in vitro* 'toxin-neutralization' assay demonstrated toxin-antibody binding and inactivation, controlled challenge studies in livestock could follow.

7.1 Introduction

Intoxication by *Moraea pallida* Bak. (yellow tulp) is the most important cardiac glycoside toxicosis in livestock in South Africa. Acute poisoning causes severe cardiac rhythm aberrations, with a high mortality rate (Kellerman *et al*., 1996). Microscopic cardiac lesions are myocardial degeneration and necrosis (Kellerman *et al*., 2005). The toxic principle 1α, 2α-epoxyscillirosidine, is a bufadienolide (Enslin *et al*., 1966; Kellerman *et al*., 2005). Bufadienolides, similar to other cardiac glycosides, interfere with the function of the ubiquitous sodium potassium adenosine triphosphatase (Na^+-K^+-ATPase) on cell membranes (Steyn and van Heerden, 1998). The Na^+-K^+-ATPase acts as the receptor for cardiac glycosides and structurally similar compounds (Steyn and van Heerden, 1998; Kamboj *et al*., 2013). It was shown using the LDH release assay and further confirmed by TEM that epoxyscillirosidine causes necrosis (Chapter 6). A dire need exists to develop prophylactic and therapeutic agents able to prevent or counter the deleterious effects of toxins, including those from plant sources (Herrera *et al*., 2015). Neutralizing antibodies can be used in immunotherapy to reverse clinical intoxications (Lemley *et al*., 1994).

Due to ethical concerns and to reduce animal suffering, i*n vitro* approaches to evaluate the neutralization ability of various agents, including antibodies, against toxins, are becoming important alternatives to *in vivo* testing. There have been a number of reports, including *in vitro* studies, on the neutralizing ability of antibodies against several plant toxins. Monoclonal antibodies (MAbs) specific for ricin A chain produced from two hybridomas were studied. The antibodies, IgG1 type, neutralized ricin *in vitro* using an EL-4 mouse leukemia cell model and in an *in vivo* mouse model. The MAbs similarly recognized ricin toxoid (Lemley *et al*., 1994). Novel antibody constructs specific for ricin were evaluated for their capacity to counteract ricin *in vitro* and *in vivo*. The camelid-type heavy chain heterodimers were capable to fully protect mice against ricin challenge. Furthermore, some of the heterodimers were able to facilitate ricin aggregation and block toxin attachment to cell receptors *in vitro*. The results suggested that the neutralizing antibodies act via the formation of antibody-toxin complexes incapable of accessing host cell receptors (Herrera *et al*., 2015). In another study on abrin, a type II ribosome-inactivating protein, IgG antibodies specific to the recombinant A chain of abrin were generated from hybridomas. A MAb by the name D6F10 protected cells from the toxic effects of abrin. Furthermore, the antibody protected mice from lethal doses of abrin. The protective effects were mediated via inhibition of abrin attachment to cell recepors (Surendranath and Karande, 2008).

The current sensitivities and concerns around animal ethics made it mandatory to conduct *in vitro* studies to evaluate the ability of antibodies to neutralize the toxic effect of epoxyscillirosidine as a pre-condition for future yellow tulp challenge studies in sheep. The aim of this study was to evaluate the neutralization effect of antibodies raised in sheep against epoxyscillirosidine by using an *in vitro* rat embryonic cardiomyocytes (H9c2) cell model.

7.2 Materials and methods

7.2.1 Chemicals and reagents

Epoxyscillirosidine was isolated previously (Chapter 3). Dimethyl sulphoxide (DMSO, Cat. no: SAAR1865000LP) was obtained from Merck (Darmstadt, Germany). Trypsin-EDTA (Cat No: BE17-16IF) and L-glutamine (Cat No: BE-17-605E) were acquired from Lonza (Verviers, Belgium). Dulbecco's Modified Eagle's Medium (DMEM, cat. No: D6546), phosphate buffered saline (PBS, cat. no: P4417), penicillin-streptomycin (Cat. No: P4333), MTT (thiazolyl blue tetrazolium bromide) reagent (Cat. No: M5655), ammonium sulphate (Cat. no: A4418) and trypan blue (Cat. no: T6146) were from Sigma-Aldrich (Germany). Foetal bovine serum (Cat. no: 10499-044, Gibco) was from Life Technologies (Grand Island, New York).

7.2.2 Purification of anti-epoxyscillirosidine antibodies

The immunoglobulin fraction of the vaccinated and control animals was concentrated and purified using ammonium sulphate precipitation. Serum samples from one epoxyscillirosidine-KLH vaccinated (S-363) and one control (S-305) sheep were used. Sera were diluted 1:4 in PBS in a conical flask to a volume V (e.g.: 20 ml serum + 60 ml PBS final volume 80 ml). Saturated ammonium sulphate solution (V/2 = 40 ml) was added dropwise with constant stirring to effect a 33.3% saturation of ammonium sulphate. The pH of the resulting suspension was adjusted to 7.8 by the drop-wise addition of 2 N NaOH. The suspension was stirred for another 2 h. The sample was then centrifuged at room temperature for 30 min at 1400 x *g*. The sample was decanted while the sediment was dissolved in PBS, restoring the volume to the original serum volume (20 ml). The semi-purified sample was re-precipitated for two additional rounds as above. After the third precipitation, the resultant precipitate, after centrifuging, was dissolved in PBS, to a final volume of 5 ml. The sample was dialyzed against PBS for 48 h, to remove the ammonium sulphate, using Slide-A-Lyzer® Dialysis Cassette (Cat no: 87737, Thermo Scientific). The final solution was filtered

through Millipore filters (0.45 and 0.22 μm), and the cloudy solution was preserved at 4 °C before use, in the *in vitro* neutralization assay. The protein concentration was determined using a photo spectrometer at 280 nm.

7.2.3 Determination of antigenicity of purified antibodies

An ELISA was performed to confirm the presence of anti-epoxyscillirosidine antibodies in the purified immunoglobulins. The plate was coated with epoxyscillirosidine and coating buffer, as antigens, in alternate rows. The ELISA was completed as described in Chapter 4.

7.2.4 Cell culture

Rat embryonic cardiomyocytes [H9c2 (2-1) cells] (Kimes and Brandt, 1976) have been established previously (Chapter 6).

7.2.5 Evaluation of neutralization activity

The *in vitro* neutralization of epoxyscillirosidine was determined in H9c2 cells. Antibodies from vaccinated and control animals were prepared in minimum essential medium (DMEM supplemented with FBS). These (100μl each) were placed into 96 micro-titre plate in triplicates according to a set template. Dilutions (20 and 40%) of antibodies in DMEM (100 μl each) were added to previously determined LC_{50} (362 μM) of epoxyscillirosidine in a plate. The plate was incubated for 1 h at room temperature with shaking. The pre-incubated mixture of antibodies and epoxyscillirosidine was added to H9c2 cells seeded on a plate. Cells exposed to epoxyscillirosidine only and cells containing medium only were used as positive and negative controls, respectively. All wells were treated in triplicates. The plates were incubated for 48 h after which neutralization

efficacy of antibodies were evaluated using the MTT cell viability assay. The experiment was repeated after a week. Per cent viability was calculated using the formula:

*Cell viability (%) = (Absorbance of antibodies-epoxyscillirosidine mixture/Absorbance of cells only) * 100*

7.2.6 Statistical analysis

Values are expressed as mean ± SD. Student's *t* test was used for paired comparisons of data. A p value of <0.05 was considered significant.

7.3 Results

7.3.1 Antibody purification

Antibodies against epoxyscillirosidine-KLH, together with all the other immunoglobulins found in the sera of vaccinated sheep, were purified and concentrated. This was confirmed by a titre of 1:1200 to epoxyscillirosidine in the antibody fraction from the vaccinated sheep as compared to no antibody detection in the control sheep using an ELISA (Fig. 7.1). A total of 11939.667 µg and 7848.947 µg of immunoglobulins were purified from the vaccinated and control sheep sera (20 ml each), respectively.

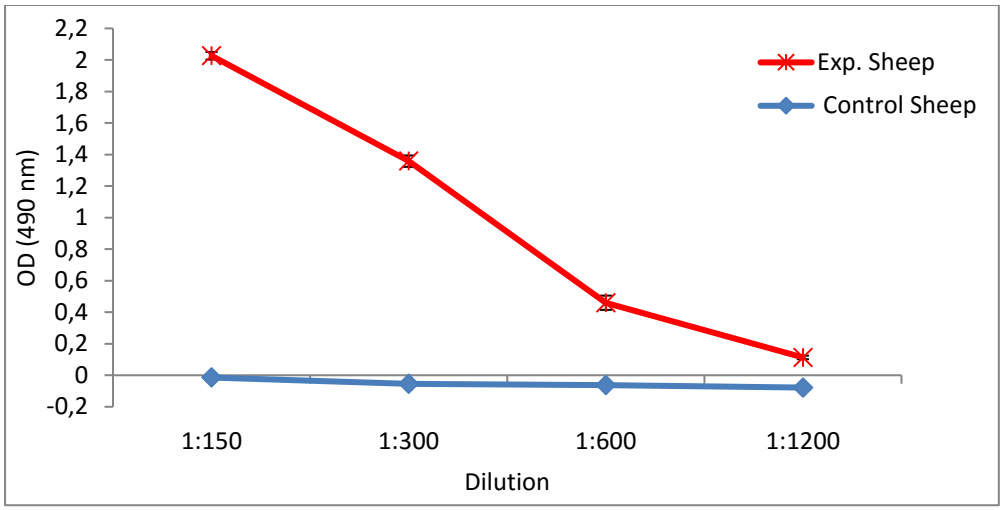


Figure 7.1: Mean (±SD) optical density values (490 nm) obtained for immunoglobulin fraction obtained from sheep vaccinated with the epoxyscillirosidine-KLH conjugate (S-363) or just the protein (KLH, sheep 305). The ELISA plate was coated with epoxyscillirosidine as antigen. Antibodies were purified using ammonium sulphate precipitation. The net OD value was obtained by subtracting the OD of the coating buffer well from that of the epoxyscillirosidine coated well. Samples were added to the wells in triplicate.

7.3.2 Antibody-toxin neutralization assay

In the cells exposed to the incubated mixture of antibodies against epoxyscillirosidine, it was observed that increasingly lower concentrations of antibodies (20, 10, to 5%), appear to have some neutralizing activity in the experimental sheep (Table 7.2). However, a somewhat similar trend was observed with the control sheep harbouring antibodies against KLH. A summary of the viability of cells exposed to varying dilutions of epoxyscillirosidine-KLH and KLH antibodies mixed with a fixed amount (LC_{50}) of epoxyscillirosidine is presented (Table 7.2). In the cells exposed to only epoxyscillirosidine (362 µM, which is the LC_{50}) as a positive control, the viability was 52.3%.

Table 7.1: Determination of in vitro neutralization of toxin by purified antibodies

	Cell Viability (%)	
	Experimental sheep	Control sheep
Antibodies (40%)	122.49 ± 0.0510	93.23 ± 0.0238
40% antibodies + LC_{50}	10.70 ± 0.0104	6.5115 ± 0.0030
20% antibodies + LC_{50}	16.30 ± 0.0155	15.0225 ± 0.0210
10% antibodies + LC_{50}	30.55 ± 0.0035	25.5955 ± 0.0190
5% antibodies + LC_{50}	25.90 ± 0.0250	38.905 ± 0.0250

7.4 Discussion

This is the first report on the *in vitro* neutralization study of antibodies against the cytotoxic effect of epoxyscillirosidine in H9c2 cells. Ammonium sulphate precipitation is a highly efficient and robust method for purifying immunoglobulins (Mariam *et al*., 2015). For *in vitro* and other uses, immunoglobulins need to be refined so as to eradicate probable pathogens and to exclude nonspecific effects of extra serum constituents such as complement, oxidative radicals, cytokines, etc. (Bergmann-Leitner *et al*., 2008). The principle of ammonium sulphate precipitation is linked with the charged interfaces among salt, water and protein. Before the introduction of salt, the dissolved proteins form hydrogen bonds with water molecules via the anionic and cationic species existing superficially on the protein. Following the introduction of ammonium sulphate to the solution, there occurs a struggle amongst the salt ions and the proteins for interface with water molecules. If the salt ions are present in adequately enough amounts, the hydrogen bonds amongst protein and water are ultimately annulled and the solvability of proteins in aqueous solution diminishes. Subsequently, the

protein–protein interfaces become robust and protein precipitation ensues (Mariam *et al*., 2015). The purification was confirmed by assaying for total protein which was read at 280 nm.

The viability of cells was evaluated as a measure of the neutralization ability of the antibodies. Although it appeared as if lower concentrations of antibodies (20, 10 and 5% dilutions) had increasing neutralization activity, a similar effect was also observed using antibodies (anti-KLH) of the control sheep. Thus the observed effect was non-specific and could not have been due to the anti-epoxyscillirosidine antibodies. In summary, following pre-incubation of anti-epoxyscillirosidine antibodies to epoxyscillirosidine with the toxin and then exposing H9c2 cells to the pre-incubated mixture, there was no difference ($P>0.05$) between the treatment and control cells (Table 7.1).

In the present work, the antibodies failed to prevent the toxic effect of epoxyscillirosidine as determined by the MTT assay. Two assumptions can be made from the antibody neutralization assay results presented in Table 7.1. It is possible that the antibodies were non-neutralizing as the production of antibodies against a toxin does not automatically confer neutralization ability to its activity (Fonseca *et al*., 2013). The response to a challenge with a toxin following vaccination against the same toxin can be variable and sometimes unpredictable (Fonseca *et al*., 2013). Filipov *et al*. (1998) could only demonstrate temporary protection against ergot alkaloid toxicity following vaccination with the conjugates in rabbits. In some instances, there could even be increased susceptibility to the toxin following vaccination as reported by MacDougald *et al*. (1990) and Fonseca *et al*. (2013), where pigs and rats were more susceptible, to zearalenone and gossypol toxicity, respectively. It is possible that the antibodies are non-neutralizing probably because they are targeted against epitopes not having any role in the toxicity of the epoxyscillirosidine. Cytotoxicity of epoxyscillirosidine depends mainly on the lactone functional group (Enslin *et al*., 1966; Steyn and van Heerden, 1998). Alternatively, it can be assumed that a very high ratio of antibodies to toxin is required to neutralize the cytotoxic effect of epoxyscillirosidine.

7.5 Conclusion

Antibodies to epoxyscillirosidine were purified and concentrated. ELISA confirmed the specificity of the purified antibodies. However, the antibodies against epoxyscillirosidine failed to neutralize the toxin in an *in vitro* assay. Further studies are required to fully characterize the antibodies, specifically to determine their affinity and avidity. In addition, methods must be developed to purify anti-epoxyscillirosidine antibodies and to concentrate the antibodies. If successful, another *in vitro* neutralization assay may be attempted. The findings also make it necessary, to develop improved methods of animal vaccination, that would induce the production of neutralizing antibodies that are able to effectively bind and inactivate the functional group responsible for the toxicity of epoxyscillirosidine.

CHAPTER EIGHT

GENERAL DISCUSSION – CONCLUSIONS – PROPOSED FUTURE RESEARCH

8.1 General Discussion

Yellow tulp poisoning remains one of the most important, among all cardiac glycoside induced toxicoses. Intoxication is characterized by clinical signs including tachycardia and arrhythmias and death due to cardiac arrest. Other signs may be general apathy, weakness of the hindquarters, tremors, respiratory distress, bruxism, groaning, bloat and diarrhoea (Steyn, 1928; Naudé, 1977). At necropsy, there is myocardial necrosis; other lesions include hyperaemia of the intestinal mucosa, and subepi- and subendocardial haemorrhages (Naudé and Potgieter, 1971).

Treatment of poisoning is unsuccessful in most cases. The only effective treatment currently is oral administration of activated charcoal, which is not without challenges, as it is costly, stressful to the animals and must be instituted as soon as possible following ingestion of yellow tulp. Control of poisoning is done by fencing off infested paddocks, physically uprooting the plants or using herbicides, the latter is costly, somewhat impracticable, or even hazardous. The management strategies all involve changing the environment. It is thus easier to manipulate animals to resist poisoning than changing the environment (Kellerman, 2009).

Several studies have reported vaccination as potential means of countering intoxication of animals by poisonous plants. For successful vaccination of animals with small molecular weight plant toxins, the toxins, as haptens, must be conjugated to larger, more complex, carrier proteins to render them immunogenic (Goodrow *et al*., 1990). Vaccination against yellow tulp could thus be studied, as a strategy to prevent poisoning in animals. The toxic principle, epoxyscillirosidine, being a small weight compound is not immunogenic.

During conjugation reactions, the choice of the method is determined by the functional group of the hapten (Sing *et al*., 2004). Bufalin and proscillaridin were conjugated using established

methods. Bufalin was conjugated by the mixed anhydride method as other steroids have been conjugated to proteins using this method (Erlanger *et al*., 1957; De Goeij *et al*., 1986; Botha *et al*., 2007). In the conjugation of proscillaridin to BSA, the lactone ring of scillarenin, which is the genin of proscillaridin, reacts with the available lysine residues on BSA. The linkage of haptens to proteins generally occurs at the most reactive (functional) groups of the proteins (Tijssen 1985). However, an efficient method of opening the epoxide ring using silica gel (Chakraborti *et al*., 2004) was used in conjugating epoxyscillirosidine. The epoxide ring was utilized to link the compound with the protein carriers (OVA/BSA/KLH) and not the lactone ring, which is the functional group. Opening of the epoxide (Chakraborti *et al*., 2004) theoretically renders it available for linking with the surface lysines of the carrier protein (OVA, BSA, KLH), while the lactone ring remains intact, thus not altering the activity of 1α, 2α-epoxyscillirosidine.

Conjugation of epoxyscillirosidine, proscillaridin and bufalin was successfully carried out, before their use to vaccinate rabbits and sheep. The conjugation of a hapten to a carrier, usually a protein has been confirmed using a number of methods including ultraviolet (UV) spectroscopy, by comparing the UV spectrum of the conjugate with that of the native protein (Wang *et al*., 2012; Torres *et al*., 2014). Change in structure of the protein confirmed by change in its UV absorption spectrum is suggestive of a new compound (the conjugate). The UV absorption spectra of the conjugates synthesized in this study were different and higher than those of the native proteins, the difference in the absorbance being contributed by the haptens. In addition, the extinction coefficient of the proteins and conjugates were utilized in estimating the hapten density of the conjugates (Szudorsky *et al*., 2002).

The synthesized conjugates (OVA, BSA and KLH) of epoxyscillirosidine were effective as immunogens, as they elicited the generation of epoxyscillirosidine specific antibodies, in vaccinated rabbits. Evidence from the rabbit vaccination trials indicated that conjugation of epoxyscillirosidine

to KLH was most effective. This was probably because it produced the most optimal hapten density, even though KLH is known to be a superior carrier protein, due to its inherent properties.

In Trial 1, the vaccinated rabbits responded poorly to the administered immunogens. This was probably due to the dose, the adjuvant or both. The dose of epoxyscillirosidine-OVA in the first trial was adjudged to be too low. This necessitated the dose being increased two fold in Trials 2 and 3. Higher levels of antibodies were generated in Trial 2, following the doubling of the dose and changing the adjuvant to Montanide. Studies have confirmed the role of dose in inducing an optimal immune response following vaccination (Cahn *et al*., 2004; Cooper *et al*., 2004; Sing *et al*., 2004; Fodey *et al*., 2009). Likewise, the role of adjuvant in enhancing the immune response has been reported. Montanide adjuvants have been reported to be superior to Freund's, in inducing an enhanced immune response (Klimka *et al*., 2015; Lone *et al*., 2017). Trial 3 evaluated and compared four different conjugates (bufalin-BSA, proscillaridin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA) in inducing an immune response in rabbits. Although all four immunogens were effective, the KLH conjugate of epoxyscillirosidine was most effective. Keyhole limpet haemocyanin is a highly efficient carrier protein due to its higher molecular weight, complexity and foreignness which are all properties that determine the degree of immunogenicity of a compound (Kuby, 1994). In addition, the conjugation reaction produced a hapten density that was conducive to antibody production.

Proscillaridin and bufalin, two commercially available bufadienolides were utilized in the cross-reactivity study in order to circumvent South African legislation (NEMBA Act. 10; 2004), which prohibits indiscriminate exploitation of the natural environment in South Africa. Being commercially available, bufalin and proscillaridin could easily be sourced and conjugated to prepare a vaccine, rather than having to obtain yellow tulp samples in the field and isolating epoxyscillirosidine in a tedious and time consuming manner.

Following successful vaccination in rabbits, the study was scaled up to sheep. Because the KLH conjugate of epoxyscillirosidine was most effective as immunogen, in the rabbit Trial 3, it was selected for the sheep trial. Antibodies were raised in sheep following vaccination with epoxyscillirosidine-KLH.

The *in vitro* cytotoxicity induced by epoxyscillirosidine, the toxic principle contained by yellow tulp, is the first report of this nature. The dose-response relationship indicated a hormetic effect, where low doses stimulated increased cell viability. Toxic effects of epoxyscillirosidine manifested only at higher doses (starting from 60 µM). Increases in viability above control values has been reported previously (Weyermann *et al*., 2005). This observation could be explained by hormesis, whereby a stress agent causes low dose stimulation and high dose inhibition of the observed/measured end point (Calabrese *et al*., 1999; Calabrese and Baldwin, 2001; Calabrese, 2013b). The median lethal concentration of epoxyscillirosidine in rat embryonic cardiomyocytes (H9c2) was calculated at different exposure times using the MTT assay. The lethal concentrations varied with 24 h being the highest followed by 72 and 48 h in that order. This may indicate a time dependent effect. Several studies have reported on the influence of time in the cytotoxicity of various tested compounds (Al-Ghamdi, 2008; Ellis *et al*., 2010; Neiva *et al*., 2013; Souza *et al*., 2017).

Cytotoxicity using the MTT and LDH assays did not yield similar results in this study. The LDH assay revealed a higher degree of variability, while the MTT assay produced more consistent values. Bopp and Lettieri (2008) reported higher intra- and inter-assay variabilities for LDH compared to the MTT assay and two other fluorometric methods. Cytotoxicity as measured with the LDH assay produced higher values, even at low doses, although low doses stimulated cell viability as determined by the MTT assay. This could partly be explained by the fact that newly damaged cells retain residual mitochondrial dehydrogenase activity (Otto *et al*., 2008), which is partly responsible for the reduction of MTT, resulting in increased viability in the presence of cytotoxicity.

Electron microscopy was utilized to gain insight into morphological alterations caused by exposure to epoxyscillirosidine for 24, 48 and 72 h. At the lowest dose evaluated (40 μM), no noteworthy ultrastructural changes were observed. The highest dose of epoxyscillirosidine evaluated (200 μM), was characterized by numerous cytoplasmic vacuoles and significant nuclear alterations, which include chromatin dissolution (karyolysis) and disruption of the nuclear envelope. Ultrastructural changes suggested that the cause of H9c2 cell death, subsequent to epoxyscillirosidine exposure, is necrosis. Increased cell membrane permeability and leakage of content, as expected with necrotic cells, was confirmed with the LDH release assay.

The viability of cells, as a measure of the neutralization ability of the antibodies, was evaluated. Though lower concentrations of antibodies (20, 10 and 5% dilutions) seemed to show increasing neutralization activity, a similar observation was made with anti-KLH antibodies (control sheep). Thus the effect was non-specific and could not have been due to the anti-epoxyscillirosidine antibodies. In summary, after a 30 min incubation of anti-epoxyscillirosidine antibodies with epoxyscillirosidine and then exposing H9c2 cells to the incubated mixture, there was no difference ($p > 0.05$) between the treatment and control cells.

In this study, the antibodies raised failed to prevent the toxic effect of epoxyscillirosidine, as evaluated using the MTT assay. Two assumptions were made. It is speculated that the antibodies were non-neutralizing, as the production of antibodies against a toxin does not spontaneously confer neutralization of its activity (Fonseca *et al*., 2013). In certain instances, there could even be increased susceptibility to the toxin, following vaccination, as reported by MacDougald *et al*. (1990) and Fonseca *et al*. (2013), where pigs and rats were more susceptible to zearalenone and gossypol toxicity, respectively. It is conceivable, that the antibodies are non-neutralizing, probably because they are targeted against epitopes not having any role in the toxicity of epoxyscillirosidine. Cytotoxicity of epoxyscillirosidine is determined mainly by the functional group (lactone ring)

(Enslin *et al*., 1966; Steyn and van Heerden, 1998). Alternatively, it is presumed that a very high ratio of antibodies to toxin is required, to be able to neutralize the cytotoxic effect of epoxyscillirosidine.

8.2 Conclusions

In conclusion, bufalin, proscillaridin and epoxyscillirosidine were successfully conjugated to protein carriers. The UV spectrophotometric method is an effective tool, to monitor the conjugation of the bufadienolides. The epoxide ring opening (Chakraborti *et al*., 2004), was shown to be a practical method to expose the epoxide ring of epoxyscillirosidine, before coupling to carrier proteins (BSA, OVA and KLH), which is the first report of this nature. The bufadienolides conjugates evaluated in this study were effective as immunogens, as manifested by their ability to induce an immune response, following vaccination in rabbits and sheep. The KLH conjugate proved most effective, compared to OVA and BSA. The cell line (H9c2) used, proved to be a suitable model for conducting *in vitro* cytotoxicity studies and could be employed in future cardiotoxicity studies, as a replacement for *in vivo* testing. Although antibodies against epoxyscillirosidine-KLH were raised in sheep, they failed to neutralize the toxin in this *in vitro* model. This is possibly because higher ratios of antibodies to toxin are needed, to effectively neutralize epoxyscillirosidine, than those used in this study.

8.3 Proposed Future Research

- Based on this conjugation study, it could be speculated that reacting the bufadienolides to KLH, seems to be the most effective immunogen. KLH has inherent properties, which render it superior, compared to other common proteins such as OVA and BSA.

  Studies using KLH as carrier protein linked to epoxyscillirosidine can be conducted, to identify the best conjugation reaction, to produce optimal hapten density for the conjugate,

and which will be suitable for vaccination, in order to generate antibody concentrations with more specificity and higher avidity. Future studies could also deploy more advanced tools, such as MALDI-TOF MS or high-resolution mass-spectrometry with electrospray ionization (ESI), to fully characterize and quantify the hapten density of the conjugated compounds.

- The toxin molecule itself could be studied further, to identify vulnerable functional groups, which could be targeted in vaccine development.

- The cross-reactivity of proscillaridin and bufalin antibodies with epoxyscillirosidine, observed in this study, could be investigated further. Conjugating proscillaridin and bufalin with KLH, might have resulted in the synthesis of higher antibody titres, that could cross-react better, with epoxyscillirosidine. The degree of specificity and cross-reactivity of antibodies, against the bufadienolides, could be influenced by the coupling method employed, in the preparation of the immunogens (Yan *et al*., 2017). In addition, the hapten density that could influence the orientation of the molecules on the carrier protein and can cause steric interference, when binding to B cell receptors, thus affecting the nature of antibodies produced (Jung *et al*., 2008; Li *et al*., 2012).

- The discovery of hormesis in this study was serendipitous. At low doses, epoxyscillirosidine stimulates cell viability. The mechanism by which hormesis is mediated, by epoxyscillirosidine, could be evaluated in future. This could provide more insight into the mechanism of action of epoxyscillirosidine, in addition to inhibition of the sodium potassium pump.

- The H9c2 cell line proved to be a very suitable model, for assessing the cytotoxic effect of epoxyscillirosidine. Future studies of cytotoxic and/or cardiotoxic compounds could be conducted using this cell line.

- The *in vitro* neutralization studies can be investigated further, to confirm the efficacy of generated antibodies against epoxyscillirosidine and similar compounds. The method used to

purify epoxyscillirosidine, concentrated all immunoglobulins in the serum of vaccinated sheep. It is likely that the ratio of antibodies to toxin used in this study was not effective, in neutralizing epoxyscillirosidine, as it was low. Future studies could employ higher ratios of antibodies to toxin.

CHAPTER NINE

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