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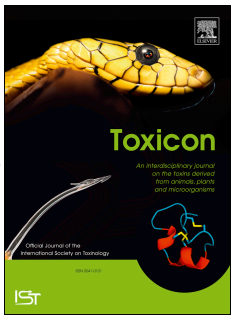

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Evaluation of *in vitro* neutralization of epoxyscillirosidine by antibodies raised in sheep

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Abstract:

Intoxication by *Moraea pallida* Bak. (yellow tulp) in livestock is of great importance in South Africa, ranking top among all plant-induced cardiac glycoside toxicosis. The toxic principle, a bufadienolide, is 1α, 2α-epoxyscillirosidine. Treatment of poisoning is challenging and affected livestock often succumbs due to the stress of handling. Manipulating animals to resist poisoning is a potential management strategy. The goal of this study was to explore the potential to develop a vaccine against epoxyscillirosidine by raising antibodies against epoxyscillirosidine in sheep and to assess the neutralization ability of the antibodies *in vitro*. Epoxyscillirosidine was successfully conjugated to keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA) rendering them immunogenic. The sheep, vaccinated with epoxyscillirosidine-KLH conjugate (n=4) and KLH (n=2) with Montanide, developed antibodies as determined with an indirect enzyme linked immunosorbent assay (ELISA). Total immunoglobulins from sera of vaccinated and control sheep that were purified and concentrated using ammonium sulphate precipitation were 11,940 and 7,850 µg, respectively. The *in vitro* neutralization assay using the methyl blue tetrazolium bromide (MTT) cell viability assay indicated no significant difference ($p>0.05$) between anti-epoxyscillirosidine-KLH and KLH antibodies. Rather, the antibodies seemed to enhance the cytotoxicity of epoxyscillirosidine in H9c2 cells. Thus, it is necessary to develop improved vaccination methods to generate antibodies capable of neutralizing the functional group responsible for epoxyscillirosidine toxicity.

Keywords: Antibodies; epoxyscillirosidine; *in vitro*; keyhole limpet haemocyanin; *Moraea pallida*; neutralization.

1. Introduction

Intoxication by *Moraea pallida* Bak. (yellow tulp) is of utmost significance among cardiac glycoside plant-induced toxicosis in livestock in South Africa. Sudden serious poisoning, with a high mortality rate, occurs, resulting in thousands of livestock deaths annually (Kellerman *et al*., 1996). Signs of yellow tulp poisoning may include general apathy, diarrhoea, tremors, weakness of hindquarters, respiratory distress and at times bruxism and groaning sounds. Other signs include tachycardia, arrhythmia, rumen atony and bloat. 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). Using the LDH release assay and further confirmed by transmission electron microscopy we were able to demonstrate that epoxyscillirosidine causes necrosis (Manuscript under second review in Toxicology Reports).

Remedy for poisoning is most times ineffective (Kellerman, 2009). However, it was shown that drenching poisoned animals with activated charcoal is beneficial, even though traumatic to the animals, costly and must be started quickly after exposure (Joubert and Schultz, 1982). A dire need exists to develop prophylactics and therapeutic agents able to prevent or counter the deleterious effects of toxins, including those from plant sources (Herrera *et al*., 2015). Interest in developing vaccines against phytotoxins has been on the increase in recent times, due to the veterinary and economic importance of poisonous plants. Vaccination has been evaluated to prevent poisoning of animals by plant species, with positive outcomes (Fonseca *et al*., 2013), with the following examples; *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; Tong *et al*., 2008). 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 investigated (Fonseca *et al*., 2013). In addition, it may be possible to use neutralizing antibodies in immunotherapy to reverse clinical intoxications (Lemley *et al*., 1994) in cases of poisoning with yellow tulp. Furthermore, 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 (Lemley *et al*., 1994; Surendranath and Karande, 2008; Herrera *et al*., 2015).

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 investigate if antibodies against epoxyscillirosidine could be raised in sheep and to further evaluate the neutralization effect of the raised antibodies against epoxyscillirosidine using an *in vitro* rat embryonic cardiomyocytes (H9c2) cell model.

2. Materials and methods

2.1. Chemicals and reagents

Epoxyscillirosidine was isolated according to the method published by Naudé and Potgieter (1971) previously. Dimethyl sulphoxide (DMSO, cat. no: SAAR1865000LP) and Silica gel 60 (0.040 – 0.063 mm) 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). 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), BSA (A7638), KLH (H7017), ammonium sulphate (cat. no: A4418) and trypan blue (cat. no: T6146) were from Sigma-Aldrich (Darmstadt, Germany). Foetal bovine serum (cat. no: 10499-044, Gibco) was from Life Technologies (Grand Island, New York, USA). Montanide^{TM} ISA (50 V2, Seppic,) was from Puteaux, CEDEX (France). Horse radish-peroxidase recombinant protein G conjugate (EIA Grade) (10-1223) and tetrahydrofuran (THF, T – 425) were from ThermoFisher Scientific (Waltham, Massachusetts, USA). Skim milk powder for ELISA (LP0031) was purchased from Oxoid Ltd (Basingstoke, Hampshire, UK)

2.2. 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 40.1 ± 0.8 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.

2.3. 1α, 2α-Epoxyscillirosidine conjugation to proteins

Conjugation of 1α, 2α-epoxyscillirosidine to BSA and KLH was achieved by opening of the epoxide ring (Chakraborti *et al*., 2004), before coupling to the proteins BSA and KLH, respectively. Epoxyscillirosidine (10 mg) was dissolved in THF (1 ml) and 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 (50 mg), was then added and the mixture stirred overnight. 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. The aqueous fraction was then lyophilized and stored. To confirm the conjugation, the conjugates were scanned between 280–324 nm on a UV spectrophotometer (Helios β, Thermo Electron Corporation, Waltham, Massachusetts, USA)

2.4. Preparation of immunogen

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^{TM} ISA) - 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.

2.5. Sheep vaccination

2.5.1. Study design

Four sheep (group I) were vaccinated with epoxyscillirosidine-KLH conjugate while the sheep in the control group were administered KLH only, on Day (D) 0. Booster vaccinations

were given on D 21 and 42 (Table 1). Two additional vaccinations were administered to maximize antibody response in the sheep at intervals of three weeks each, on D 63 and 84.

Table 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

2.5.2. 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 properly restrained while the vaccination was done by subcutaneous injection of 1 ml (2 mg per sheep) of the prepared vaccine, alternating between the axilla or 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 levels and titres. Blood was allowed to stand for 30 min at room temperature. Serum was subsequently obtained by centrifugation at 1204 *g* at 20 ^{o}C (Allegra^{TM} X-22 Centrifuge, Beckman Coulter Brea, California, United States), aliquoted and stored at -20 ^{o}C.

2.6. Evaluation of immunological response using ELISA

An ELISA to determine antibody titres was conducted. MaxiSorp 96-well plates (NUNC Thermo Fisher Scientific, Waltham, Massachusetts, USA) 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 using 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 wash buffer). After washing, 50 µl horseradish-peroxidase (HRPO) conjugate at a dilution of 1:3,000 was added to all wells. Following further incubation and washing five times, 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 (Winooski, Vermont, USA) 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, 50 µl epoxyscillirosidine (1 mg dissolved in 500 µl acetone + 500 µl coating buffer) as antigen and coating buffer (50 µl) were coated on the plate in alternate rows. The plate was allowed to dry overnight at room temperature in a laminar flow cabinet. An ELISA was conducted as described previously, to confirm the specificity of the generated antibodies against epoxyscillirosidine. The net OD was calculated by subtracting the value for the acetone and coating buffer coated well from the corresponding epoxyscillirosidine coated well.

2.7. 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, Waltham, Massachusetts, USA). 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 (Helios β, Thermo Electron Corporation, Waltham, Massachusetts, USA) at 280 nm.

2.8. 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 previously.

2.9. 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, Waltham, Massachusetts, USA) in a 95% air 5% CO_2 environment at 37 °C. Medium was changed every 3 – 4 days while the cells were sub-cultured after attaining about 70-80% confluency. 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. Cells were seeded in 96-well micro-titre plates for the MTT assay.

2.10. 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 antibody dilutions (100µl each) were placed into 96 micro-titre plate in triplicates according to a set template. Dilutions (5, 10, 20 and 40%) of the 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.

Percentage cell viability was calculated using the formula:

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

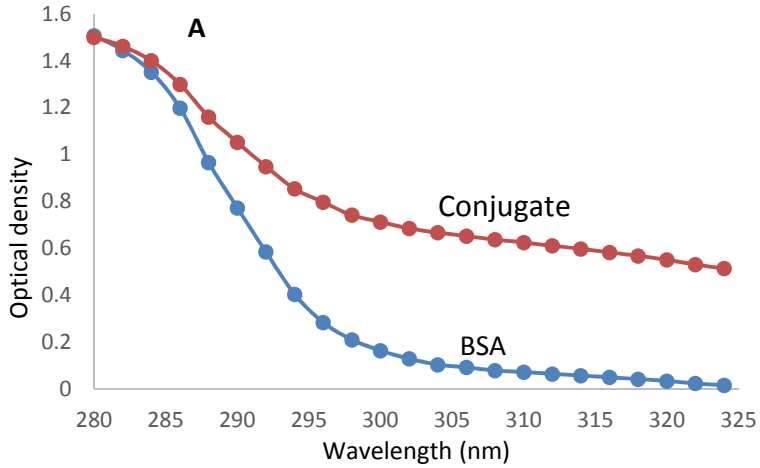
2.11. Statistical analysis

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.

3. Results

3.1. Conjugation of epoxyscillirosidine to BSA and KLH

The UV absorption spectra (at 280–324 nm) of epoxyscillirosidine-BSA and epoxyscillirosidine-KLH conjugates that were compared with the native proteins to confirm the conjugation are presented in Fig. 1.



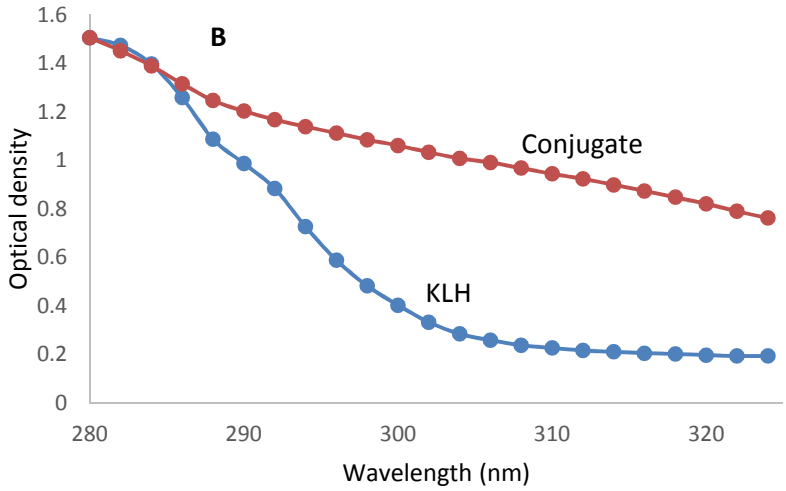


Fig. 1. Comparative ultraviolet absorption spectra of conjugates and proteins. **A** 1α, 2α-Epoxyscillirosidine-BSA vs BSA. **B** Epoxyscillirosidine-KLH vs KLH. The respective conjugates and proteins were dissolved (1 mg/ml) in acetonitrile-water (50%, v/v). The solutions (100 µl) were scanned in the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

3.2. Sheep vaccination with epoxyscillirosidine-KLH

The immune response of the sheep to the administration of epoxyscillirosidine-KLH was evaluated with an indirect ELISA. All the four sheep vaccinated with epoxyscillirosidine-KLH (sheep 363) seroconverted after the first vaccination, detectable on D 21 (Fig. 1). The response of four sheep vaccinated with the KLH conjugate varied after the first booster vaccination, detected on D 42. Immune response of 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.

None of the control sheep developed detectable levels of antibodies against epoxyscillirosidine.

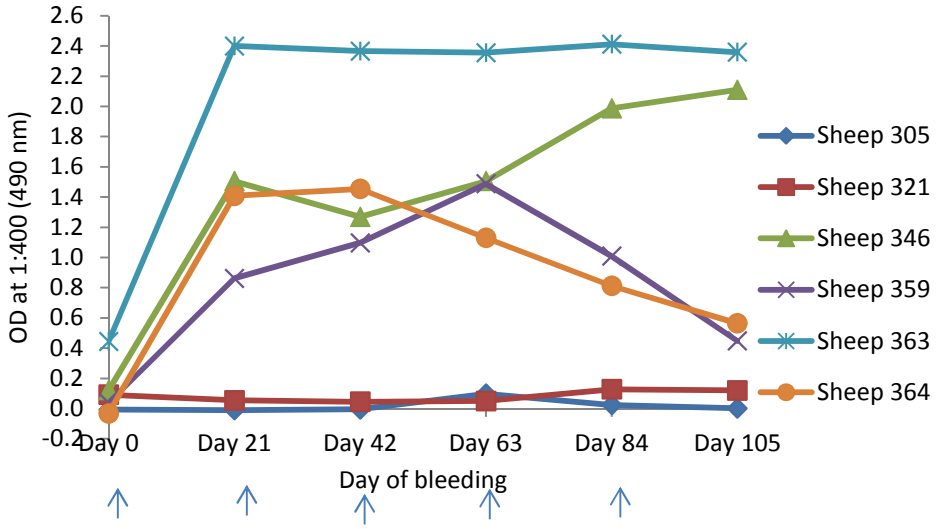


Fig. 2. Optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA for sera (at 1:400 dilution) from sheep vaccinated with epoxyscillirosidine-KLH (Sheep 346, 359, 363, 364) or KLH (Sheep 305 and 321). The arrows indicate the days of vaccination. Montanide was used as adjuvant. The OD of the BSA coated well was subtracted from that of the epoxyscillirosidine-BSA well to obtain the net OD. Wells were treated 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. 3).

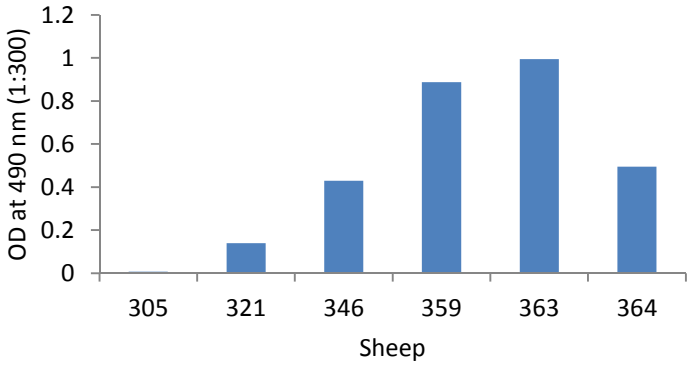


Fig. 3. Mean optical density values (490 nm) obtained for sera (at 1:300 dilution) from sheep vaccinated with the epoxyscillirosidine-KLH (Sheep 346, 359, 363, 364) or just the protein (KLH; sheep 305 and 321) and coating the ELISA plate with only epoxyscillirosidine as antigen. The net OD value was obtained by subtracting the OD of the coating buffer well from that of the epoxyscillirosidine coated well.

3.3. Antibody purification

Antibodies against epoxyscillirosidine-KLH, together with all the other immunoglobulins 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. 3). A total of 11939.67 µg and 7848.95 µg of immunoglobulins, from 20 ml of serum each, were purified for the vaccinated and control sheep respectively.

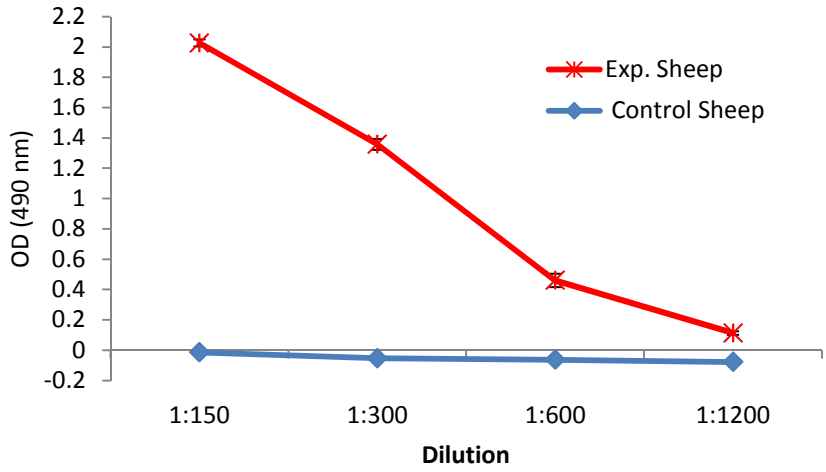


Fig. 4. Mean ±SD (duplicate) optical density values (490 nm) obtained for immunoglobulin fraction obtained from sheep vaccinated with the epoxyscillirosidine-KLH conjugate (S-363) or just the protein (KLH, sheep 305). The ELISA plate was coated with epoxyscillirosidine as antigen. Antibodies were purified 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. The wells in the plate were treated in triplicates.

3.4. Antibody-toxin neutralization assay

In the cells exposed to incubated mixture of antibodies against epoxyscillirosidine, it was observed that increasingly lower concentrations of antibodies (10 to 5%) appear to have some neutralizing activity, compared to higher concentrations (20 – 40%), in the experimental sheep (Table 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 2). In the cells exposed to epoxyscillirosidine (362 µM, which is the LC_{50}), the viability was 52.3%. In contrast, cells exposed to the purified antibodies (40%) from the experimental and control sheep showed 122.49 ± 0.05 and 93.23 ± 0.02% viability, respectively.

Table 2

Determination of *in vitro* neutralization of toxin by purified antibodies

	Cell Viability (%)	
	Experimental sheep	Control sheep
Antibodies (40%)	122.49 ± 0.05	93.23 ± 0.02
40% antibodies + LC_{50}	10.70 ± 0.01	6.51 ± 0.00
20% antibodies + LC_{50}	16.30 ± 0.02	15.02 ± 0.02
10% antibodies + LC_{50}	30.55 ± 0.00	25.60 ± 0.02
5% antibodies + LC_{50}	25.90 ± 0.03	38.91 ± 0.03

Data are mean ± SD (n=2). LC_{50} = Lethal concentration 50.

4. Discussion

For successful vaccination of animals with small molecular weight plant toxins, the toxins, as haptens, must first be conjugated to larger more complex carrier proteins, to render them immunogenic (Goodrow *et al*., 1990). Conjugation of epoxyscillirosidine to KLH was successfully carried out, before its use to vaccinate sheep. During conjugation reactions, the choice of the method is determined by the functional group of the hapten (Sing *et al*., 2004). The linkage of haptens to proteins generally occurs at the most reactive (functional) groups of the proteins (Tijssen 1985). However, the epoxide ring of epoxyscillirosidine was utilized to link it with the protein carrier (BSA/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 (BSA or KLH), while the lactone ring remains intact, thus not altering the activity of 1α, 2α-epoxyscillirosidine. For the BSA molecule, out of the 59 lysine residues, only about 30-35 are available for conjugation (Venkataramana *et al*., 2015; Abbas *et al*., 2018). The exact number of lysine residues of KLH and BSA reacting

with epoxyscillirosidine could not be determined with the UV spectroscopy utilized in this study.

The conjugation of a hapten to a carrier, usually a protein can be confirmed by 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). A change in the structure of the protein confirmed by a 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. 1), the difference in the absorption being contributed by the haptens.

Antibodies against epoxyscillirosidine were successfully raised in sheep as revealed by the ELISA (Fig. 2). This finding has further confirmed the effectiveness of epoxyscillirosidine-KLH as an immunogen following successful conjugation of epoxyscillirosidine to KLH. 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).

Since the KLH-conjugate was used in the vaccine, an irrelevant protein to the vaccine carrier protein was used in the ELISAs, in order 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 gave a net epoxyscillirosidine reaction. Therefore, this ELISA should not detect any antibody against 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 positive reaction to epoxyscillirosidine alone as antigen in the ELISA (Fig. 3) has further confirmed that some the antibodies express specificity to epoxyscillirosidine.

This is the first report on the *in vitro* neutralization study of antibodies against 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 purification was confirmed by assaying for total protein which was read at 280 nm. Using epoxyscillirosidine as antigen against the purified antibodies in the ELISA, the positive reaction further confirmed the specificity of the antibodies to epoxyscillirosidine as shown in figure 4.

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 (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 solely 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 incubated mixture, there was no difference ($p>0.05$) between the treatment and control cells (Table 2). In fact, when compared with the cells exposed to the toxin only (LC_{50}), both the treatment and control antibody dilutions incubated with the toxin seemed to enhance its toxicity as manifested by the lower cell viability (Table 2). Thus it is not just a failure of antibodies to prevent epoxyscillirosidine toxicity; rather the antibodies increased its cytotoxicity. In contrast, cells exposed to the antibodies from the experimental sheep in the absence of the toxin showed increased viability ($122.49 \pm 0.1\%$) over and above the control values (100%). This means that the antibodies stimulated cell viability. Meanwhile, cells exposed to control sheep antibodies showed viability ($93.23 \pm 0.0\%$) approaching that of the control cells (100%).

In the present work, the antibodies failed to prevent the toxic effect of epoxyscillirosidine as determined by the MTT assay. It is to be noted, however, that, the production of antibodies against a toxin does not automatically confer neutralization ability on the toxin (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).

Two assumptions can be made from the antibody neutralization assays presented in Table 2. It is possible the antibodies are toxicity-enhancing, since it appears the toxicity of epoxyscillirosidine was facilitated by the antibodies in H9c2 cells. There have been instances where there was increased susceptibility to the toxin following vaccination as reported by MacDougald *et al*. (1990) and Fonseca *et al*. (2013), where pigs and rats became more susceptible, to zearalenone and gossypol toxicity, respectively.

In addition, the antibodies were 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 ring (Enslin *et al*., 1966; Steyn and van Heerden, 1998). In another study, Filipov *et al*. (1998) could only demonstrate temporary protection against ergot alkaloids toxicity following vaccination with the conjugates in rabbits.

Conclusion

In conclusion, epoxyscillirosidine was successfully conjugated to KLH which was monitored using UV spectroscopy and further confirmed by the generation of antibodies following administration of epoxyscillirosidine-KLH conjugate together with Montanide as adjuvant in sheep. However, following purification and concentrating, the antibodies against epoxyscillirosidine failed to neutralize the toxin, but rather enhanced its cytotoxicity in an *in vitro* assay. This was possibly because the antibodies were non-neutralizing but rather toxicity enhancing.

These findings make it necessary to develop improved methods of animal vaccination that would induce generation of antibodies able to bind and inactivate the functional group responsible for the toxicity of epoxyscillirosidine. The toxin molecule itself could be studied further to identify vulnerable functional groups that could be targeted in vaccine development.

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Highlights

- 1α, 2α-Epoxyscillirosidine was conjugated to KLH before using this conjugate to vaccinate sheep to raise antibodies.
- Anti-epoxyscillirosidine-KLH antibodies raised were concentrated and purified using ammonium sulfate precipitation.
- The neutralization efficacy of antibodies against epoxyscillirosidine was evaluated with the MTT cell viability assay.
- There was no difference ($p>0.05$) in viability between cells exposed to antibodies from experimental and control sheep.
- The antibodies were non-neutralizing but rather enhanced the cytotoxicity of epoxyscillirosidine *in vitro*.

Ethical Statement

Approval for the ethical use and care of laboratory animals (Project No. V016-16) was obtained from the Animal Ethics Committee, Faculty of Veterinary Science, University of Pretoria, before the commencement of the experiment.