Raising antibodies against epoxyscillirosidine, the toxic principle contained in *Moraea pallida* Bak. (Iridaceae), in rabbits

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Abstract:

Moraea pallida Bak. (yellow tulp) poisoning is the most important plant cardiac glycoside toxicosis in South Africa. The toxic principle, a bufadienolide, is 1α, 2α-epoxyscillirosidine. The aim was to investigate the potential to develop a vaccine against epoxyscillirosidine. Epoxyscillirosidine, proscillaridin and bufalin, were successfully conjugated to hen ovalbumin (OVA), bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH).

There was a low immune response following vaccination of adult male New Zealand White rabbits with epoxyscillirosidine-OVA (n=3) and OVA (n=3) using Freund's adjuvant in Trial (T) 1. The immune response improved significantly in T2 following doubling of the dose to 0.8 mg/rabbit and changing the adjuvant to Montanide. In T3, the rabbits (n=15), allocated into 5 equal groups, vaccinated with proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH, epoxyscillirosidine-BSA and BSA respectively, using Montanide adjuvant, developed antibodies against the administered immunogens, with epoxyscillirosidine-KLH inducing the highest immune response. Proscillaridin and bufalin antibodies cross-reacted with epoxyscillirosidine in an enzyme linked immunosorbent assay.

The conjugation methodology will be adjusted in the future to target optimal conjugation efficiency. Additional vaccination will be conducted in search of neutralizing antibodies against the yellow tulp toxin. The cross-reactivity of proscillaridin and bufalin antibodies with epoxyscillirosidine could be studied in future to explore the potential to prevent yellow tulp poisoning.

Highlights

- Epoxyscillirosidine and the commercially available proscillaridin and bufalin were conjugated to carrier proteins rendering them immunogenic
- Antibodies against the bufadienolide haptens epoxyscillirosidine, proscillaridin and bufalin were raised in rabbits
- KLH conjugate of epoxyscillirosidine produced the highest immune response relative to epoxyscillirosidine-BSA, proscillaridin-BSA and bufalin-BSA
- Proscillaridin and bufalin antibodies cross-reacted with epoxyscillirosidine as antigen in an ELISA

1. Introduction

Poisoning by *Moraea pallida* Bak. (Iridaceae) or yellow tulp, one of the most important cardiac glycoside containing plants, constitutes a serious economic problem as it causes 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; Naude, 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 and second trials evaluated the efficacy of a hen ovalbumin (OVA) conjugate of epoxyscillirosidine, using two different adjuvant systems, to induce an immunological response. The third trial 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 proscillaridin and bufalin with epoxyscillirosidine.

2. Materials and Methods

2.1. Chemicals and reagents

Purified epoxyscillirosidine (isolated according to the method of Naudé and Potgieter, 1971) was available in the plant toxin collection of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, cryodesiccated and stored in the refrigerator at 4°C. 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 (Darmstadt, Germany). Silica gel (1.09385.1000), sodium bicarbonate ($NaHCO_3$), sulphuric acid (H_2SO_4), and chloroform were sourced from Merck (Darmstadt, Germany). Montanide^{TM} ISA (50 V2, Seppic,) was from Puteaux, SEDEX (France). Foetal bovine serum (FBS, cat. No: 10270 Gibco) was from Life Technologies (Grand Island, New York, USA). 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. Conjugation of bufadienolides to proteins

Epoxyscillirosidine was conjugated to OVA, BSA and KLH as described by Isa *et al*., (2019). Bufalin and proscillaridin were conjugated to BSA by mixed anhydride (Erlanger, 1973) and periodate oxidation (Butler and Chen 1967) methods, respectively. Samples were scanned (280 – 320 nm) using a spectrophotometer (Helios β, Thermo Electron Corporation, Waltham, Massachusetts, USA).

The conjugation was evaluated using ultraviolet (UV) spectroscopy and using the equation:

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

where, OD is optical density at absorption maxima
1 is cell path length of 1cm
c is concentration
ε is extinction coefficient of the hapten.

The hapten density was estimated utilizing the molar extinction coefficient ε, (Szurdoki *et al*., 2002).

2.3. 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 2 ml normal saline (2 mg/ml), with complete Freund's adjuvant (FCA) in a ratio of 1:1 (v/v), giving a final vaccine concentration of 1 mg/ml immunogen. 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 with Montanide adjuvant (final concentration 2 mg/ml immunogen), for both initial and booster vaccinations.

2.4. 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.

2.5. Rabbit vaccination

2.5.1. Study design

A summary of the experimental groups and vaccination schedule is presented in Table 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. 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, 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 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 secondary vaccinations used FIA

2.5.2. 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 Brea, California, USA), aliquoted and stored at -20°C

2.6. Evaluation of immunological response using an indirect ELISA

An epoxyscillirosidine ELISA to determine antibody titres was conducted as described by Isa *et al*., (2019), accordingly, for each trial. In Trial 1 and 2, epoxyscillirosidine-BSA conjugate was used to coat the ELISA plates and for the alternate rows, BSA was used. The optical density (OD) values of the BSA coated wells were subtracted from the epoxyscillirosidine-BSA coated wells to obtain the net OD value. In trial 3, epoxyscillirosidine-OVA and OVA

were used to coat the plates in alternate rows, respectively. In another ELISA epoxyscillirosidine was used to coat the plate and for the alternate rows, coating buffer was used, to confirm the specificity of the raised antibodies against epoxyscillirosidine. The conjugates and epoxyscillirosidine (1 mg each) were dissolved in acetone (500 µl) and coating buffer (500 µl) in each case.

2.7. Statistical analysis

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

3. Results

3.1. Conjugation of bufadienolides to protein

The degree of conjugation of epoxyscillirosidine to OVA was estimated to be 6.696×10^1 or about 67 molecules of epoxyscillirosidine per mole of OVA, which is the 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 x c x ε

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

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

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

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

   Thus the hapten density is:

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

   Or 7.7 x 10^{-3}/1.15 x 10^{-4} toxin molecules per moles protein

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

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

3.2. 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.

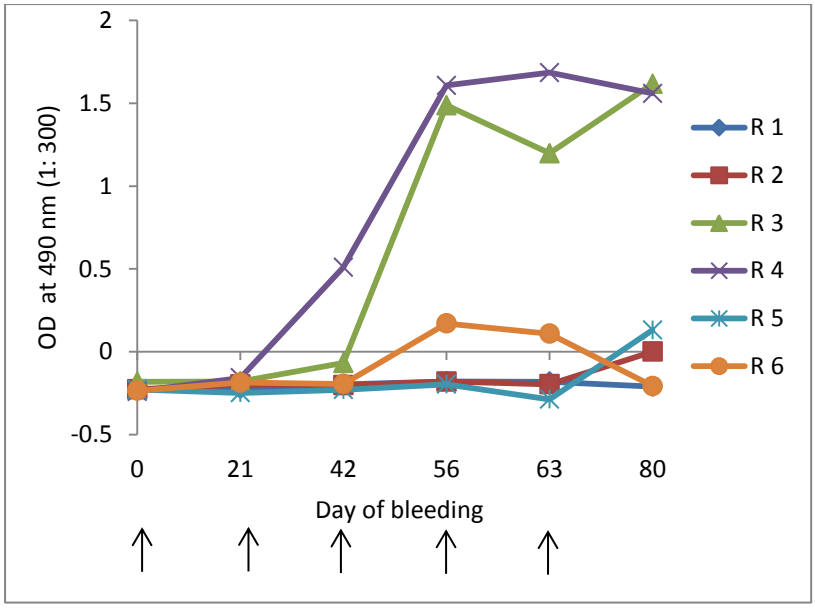

Fig. 1. Trial 1: Optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA for sera (at 1:300 dilution) from rabbits vaccinated with epoxyscillirosidine-OVA (R3, 4 and 6) or OVA (R 1, 2 and 5). The arrows indicate the days of vaccination. Primary vaccinations were done using Freund's 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.

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. 1.

3.3. 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. 2). No rabbit in the control group (OVA only) developed detectable antibodies to epoxyscillirosidine-BSA.

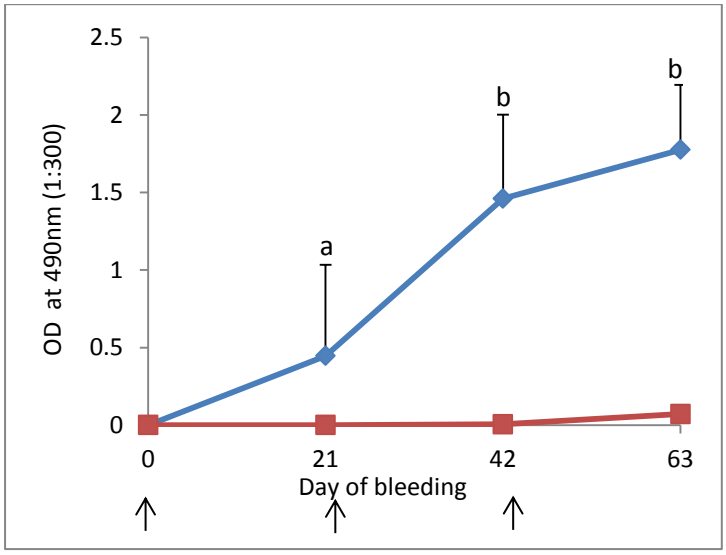

Fig. 2. Trial 2: Mean optical density values (490 nm) obtained in an epoxyscillirosidine-BSA ELISA, for sera (at 1:300 dilution) from experimental rabbits (n=4) vaccinated with epoxyscillirosidine-OVA or OVA (control, n=2,). Vaccinations were done using Montanide as adjuvant. Bars indicate standard deviation (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.

3.4. 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. The rabbits vaccinated with the 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.The

antibodies synthesized against proscillaridin-BSA and bufalin-BSA also cross-reacted with epoxyscillirosidine-OVA in the ELISA (Fig. 3). 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.

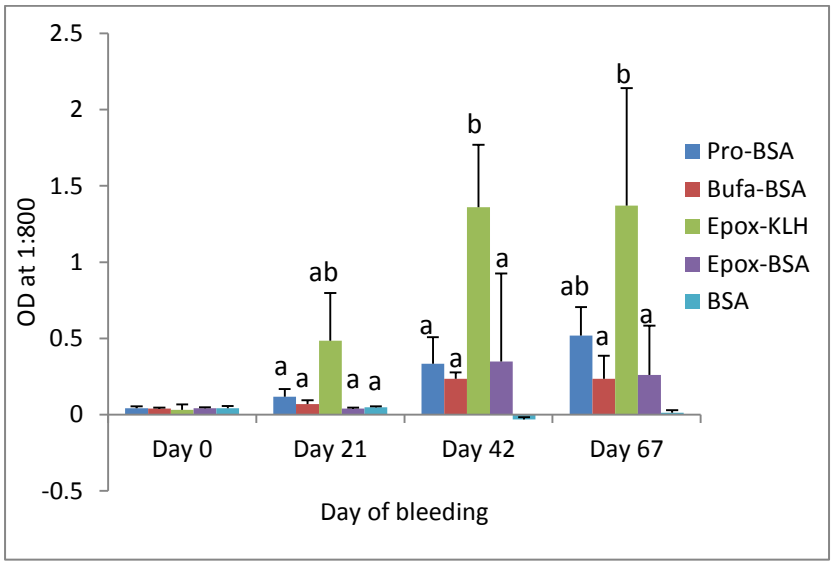

Fig. 3. Trial 3: Mean optical density values (490 nm) obtained in an epoxyscillirosidine-OVA ELISA for sera (at 1:800 dilution) from rabbits vaccinated with the hapten-protein conjugates (proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA) or just the protein (BSA). 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 reacted with epoxyscillirosidine (Fig. 4).

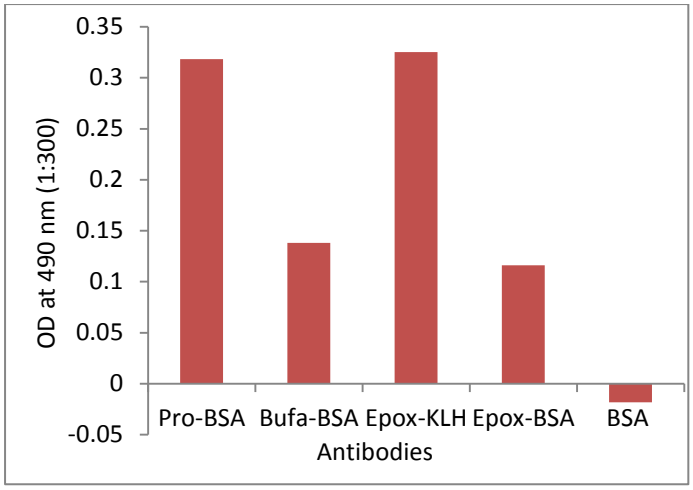

Fig. 4. Trial 3: Mean optical density values (490 nm) obtained for sera (at 1:300 dilution) from rabbits vaccinated with the hapten-protein conjugates (proscillaridin-BSA, bufalin-BSA, epoxyscillirosidine-KLH and epoxyscillirosidine-BSA) or just the protein (BSA) and coating the ELISA plate with only 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. Discussion

Conjugation of epoxyscillirosidine, proscillaridin and bufalin was successfully carried out, before their use for vaccination in rabbits. A variety of techniques, including ultraviolet (UV) spectroscopy, could be utilized to monitor the conjugation of a hapten to a protein carrier, by comparing the UV absorption spectrum of the conjugate with that of the native protein (Wang *et al*., 2012; Torres *et al*., 2014). Sing *et al*., (2004) had likewise reported spectrophotometry as a means of appraising conjugation reactions. Variation in the structure of a protein confirmed by change in UV absorption spectrum is indicative of transformation to a new compound (the conjugate) (Isa *et al*., 2019). The conjugation was further confirmed by the generation of antibodies against the conjugates following administration in the rabbits.

It is interesting to note that Montanide also produces local side effects in rabbits. Although adverse reactions occurred with the Montanide, they were not as severe compared with those observed with Freund's adjuvant used in trial 1. Injection site reactions, pain and other

adverse reactions that ranged from mild to moderate have been reported in humans (van Doorn *et al*., 2016).

In Trial 1, although antibodies were raised following vaccination with epoxyscillirosidine-OVA the antibody levels were relatively low (Fig. 1). The dose of the immunogen (0.4 mg) administered, the adjuvant used (Freund's) or both were likely responsible for the low immunogenicity elicited by epoxyscillirosidine-OVA in trial 1, 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 in turn 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 likely contributed to the improved immune response compared to Trial 1. Although effective, Freund's is an adjuvant whose use is limited to animals and in a regulated manner due to its toxicity. However, 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 epoxyscillirosidine-KLH, epoxyscillirosidine-BSA as well as proscillaridin-BSA and bufalin-BSA conjugates. However, only KLH conjugate of epoxyscillirosidine induced the production of significantly ($p<0.01$) high levels of antibodies.

This was most likely because the conjugation efficiencies for epoxyscillirosidine OVA and BSA conjugates were low. Given the weak immune response to the BSA conjugates, it is

plausible that alternative coupling strategies could have resulted in stronger immune response. Furthermore, 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). 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). 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. 3 and 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. 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 small molecule haptens could be influenced by the coupling method employed in the preparation of the immunogens (Yan *et al*., 2017). In addition, the cross-reactivity could have been improved by the introduction of a spacer molecule during the conjugation of bufalin and proscillaridin to BSA.

In conclusion, the bufadienolide haptens epoxyscillirosidine, proscillaridin and bufalin were successfully conjugated to carrier proteins, 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 related

bufadienolides proscillaridin and bufalin with epoxyscillirosidine was demonstrated in an indirect ELISA where antibodies reacted with unconjugated epoxyscillirosidine.

The conjugation methodology for KLH will be adjusted in the future with emphasis on alternative coupling strategies to identify optimal hapten density and conjugation efficiency. Additional vaccinations will be conducted in search of neutralizing antibodies against epoxyscillirosidine.

Statement of conflict of interest

The authors declare none.

Ethical Statement

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.

Acknowledgements

The authors would like to express their gratitude to Marie Smith, Stats4science, who performed the statistical analysis, Late Dr LJG Ackerman (of blessed memory) who helped with the conjugation reactions and the staff of the University of Pretoria Biomedical Research Center (UPBRC).

This work was supported by the Tshwane Animal Health Innovation Cluster (Grant number TAHC 12-00031).

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