

KRIMPSIEKTE AND ACUTE CARDIAC GLYCOSIDE POISONING IN SHEEP CAUSED BY BUFADIENOLIDES FROM THE PLANT *KALANCHOE LANCEOLATA* FORSK

L. A. P. ANDERSON, R. ANITRA SCHULTZ, J. P. J. JOUBERT, L. PROZESKY, T. S. KELLERMAN, G. L. ERASMUS and J. PROCOS, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

ANDERSON, L. A. P., SCHULTZ, R. ANITRA, JOUBERT, J. P. J., PROZESKY, L., KELLERMAN, T. S., ERASMUS, G. L. & PROCOS, J., 1983. Krimpsiekte and acute cardiac glycoside poisoning in sheep caused by bufadienolides from the plant *Kalanchoe lanceolata* Forsk. *Onderstepoort Journal of Veterinary Research*, 50, 295-300 (1983)

Three toxic bufadienolides, one characterized as hellibrigenin 3-acetate, have been isolated from *Kalanchoe lanceolata* Forsk.

Typical signs of cardiac glycoside poisoning, involving the gastro-intestinal, neuromuscular and cardiovascular systems, could be induced by drenching the milled plant to sheep. Such signs could also be induced by dosing the guinea-pigs to sheep or by injecting them into both guinea-pigs (subcutaneously) and sheep (intravenously).

The specific paretic syndrome, krimpsiekte, on the other hand, was reproduced only by the repeated intravenous administration of smaller doses of the 2 unknown bufadienolides to sheep.

Histopathological examination revealed a mild to severe multifocal cardiomyopathy in sheep receiving plant material or bufadienolides.

INTRODUCTION

The plant families Iridaceae, Liliaceae, Melianthaceae and Crassulaceae contain many plants with bufadienolides (cardiac glycosides) as their active toxic principles. Members of the genera *Cotyledon*, *Tylecodon* and *Kalanchoe* (Crassulaceae), however, also cause krimpsiekte, a neurological syndrome differing from typical cardiac glycoside intoxication, which is brought about by repeated ingestion of small quantities of the plants (Naudé, 1977; Vahrmeijer, 1981). Affected animals lag behind the flock. When forced to move, they tire quickly and either lie down or stand with muscles trembling. The neck may be held in a peculiar twisted way (torticollis) and the head often dangles loosely as the animal walks. Animal that recover can suffer from torticollis for months (Henning, 1926).

Steyn (1949) concluded that *Kalanchoe paniculata* Harv., *K. rotundifolia* Harv. and *K. thyrsiflora* Harv., contain a toxin that causes krimpsiekte. Subsequently, *K. lanceolata* Forsk. was also suspected of being poisonous, but this suspicion was never confirmed. A bufadienolide (cotyledoside) from *Tylecodon wallichii* (Harv.) Toelken, subsp. *wallichii* (= *Cotyledon wallichii* Harv., Toelken, 1978) was isolated by Van Rooyen & Pieterse (1968) and characterized by Van Wyk (1975). Krimpsiekte was manifested in sheep given intravenously repeated small doses of cotyledoside, while typical cardiac glycoside poisoning symptoms occurred after a single high dose (Naudé & Schultz, 1982). To date, however, bufadienolides have never been isolated from members of the *Kalanchoe* genus.

MATERIALS AND METHODS

Plant material

Kalanchoe lanceolata Forsk. (Vahrmeijer, 1981) plants for extraction and dosing were collected during spring to autumn in the Waterberg district. Sometimes dead or senescent plants that had overwintered in the veld were given to sheep, while in other instances freshly picked plants, dried in the shade before being milled, were dosed (Table 2).

Isolation of the toxic principles

The fresh plants (120 kg) were minced and extracted 3 times in a Waring blender with ethyl acetate and the solvent evaporated under reduced pressure. The resultant syrup was partitioned between 95% methanol (3.5 l) and petroleum ether. Both extracts were evaporated to dryness and the residues tested for toxicity. Only the residue obtained from the methanol extract (88 g) was toxic.

Repeated chromatography of the toxic residue on silica gel, using chloroform-acetone (70:30, v/v), chloroform-acetone-methanol (60:40:1,5-5, v/v/v) and benzene-methanol (78:22, v/v), yielded 3 colourless, crystalline, toxic components, K4A (485 mg), K28A (570 mg) and K28 B (1,28 g).

Reagents and apparatus

Melting points (m.p.) are uncorrected and were done on a Büchi melting point apparatus. Ultraviolet (UV) absorption refers to methanol, nuclear magnetic resonance (NMR) to CDC₃ solutions and infra-red (IR) to KBr discs. UV absorptions were measured with a Beckman DK-2A spectrometer, IR spectra with a Perkin Elmer 257 spectrometer, mass spectra with an AEI MS-9 mass spectrometer and NMR spectra with a Varian XL100 spectrometer. Merck silica gel 60, particle size 0,06-0,20 mm, was used for column chromatography.

Dosing trials

(1) *Guinea-pigs*. The toxicity of the plant extract was monitored by dosing orally the various fractions to weaned guinea-pigs.

The toxicity and cumulative effect of the isolated bufadienolides were assayed in young (150-250 g) male albino guinea-pigs (Table 1). The subcutaneous (s.c.) LD₅₀ of these 3 bufadienolides was roughly estimated (2 animals/dose). The cumulative effects were determined by s.c. injections of 25% and 50% LD₅₀/day, until they died (4 animals/dose).

(2) *Sheep*. Ten Merino sheep (milk-tooth-full mouth) of both sexes and with live masses varying between 21 and 56 kg were dosed per stomach tube or by intravenous.

TABLE 1 Observations on guinea-pigs intoxicated by subcutaneous injection of bufadienolides isolated from *K. lanceolata* Forsk.

Bufadienolide	LD ₅₀ mg/kg	Cumulative effect	Histopathology
K 28 A	c. 0,20	*	Not examined
K 28 B	c. 0,10	*	Heart + Brain +
Hellibrigenin 3-acetate	0,36	0	Heart + Brain +++

0 = Not cumulative
 * = Cumulative effect demonstrated
 + = Mild lesions
 ++ = Moderate lesions
 +++ = Prominent lesions
 ++++ = Extensive lesions

injection as set out in Tables 2 & 3. Daily clinical examination and registration of electrocardiograms (Schultz, Pretorius & Terblanche, 1972) were carried out.

Pathology

Two guinea-pigs were killed for necropsy by intraperitoneal administration of pentobarbitone sodium* (Table 1). All the sheep that died were necropsied (Table 2 & 3).

The brains and spinal cords were fixed *in toto* in 10 % formalin before coronal sectioning and selection of blocks for histopathological examination. Specimens from various organs, including the heart, lungs, liver, spleen and kidneys, were collected in 10 % buffered formalin. Tissue blocks were routinely processed and stained with haematoxylin and eosin (HE). The special staining techniques employed include Masson's trichrome technique and Mallory's phosphotungstic acid haematoxylin (MPAH) stain (Lee, 1968).

RESULTS

Toxic principles

K4A. Component K4A had a m.p. of 236–238 °C (from chloroform-ether); λ_{\max} (MeOH) 299 nm (26000); ν_{\max} 2860, 2745, 1720–1700 (broad), 1630, 1535, 1240 and 830 cm^{-1} ; δ 7,85 (dd, J = 10 and 2,5 Hz), 7,28 (dd, J = 2,5 and 1 Hz), 6,28 (dd, J = 9,5 and 1,5 Hz); M^+ , 458. It gave a pink to blue Liebermann colour reaction in 0–60 s.

K28A. Component K28A had a m.p. of 206–208 °C (from chloroform-acetone methanol); λ_{\max} (MeOH) 299 nm; ν_{\max} 3440, 3305, 1714, 1720, 1700, 1630, 1540, 1240 and 830 cm^{-1} . It gave a blue Liebermann colour reaction and a wine-red anthrone colour reaction.

K28B. Component K28B had a m.p. of 190–195 °C; λ_{\max} (MeOH) 299 nm; ν_{\max} 3430, 1740, 1715, 1705, 1635, 1540, 1225 and 830 cm^{-1} . It gave a blue Liebermann colour reaction and a green anthrone colour reaction.

All 3 components were recognized as bufadienolides by their positive Liebermann colour reactions, characteristic IR absorptions at c. 1710, 1630, 1540, 1240 and 840 cm^{-1} , UV absorptions at 299 nm and double doublets in the ^1H NMR spectra at c. δ 6,2, 7,2 and 7,8 (characteristic for the 3 protons of the a-pyrone ring) (Anderson & Koekemoer, 1968).

The pink to blue Liebermann colour reaction in 0–60 s and M^+ peak at 458 strongly suggested component K4A to be hellebrigenin 3-acetate (Anderson, 1969). Its identity was confirmed by an identical IR spectrum with authentic material from *Melianthus comosus* Vahl (Anderson & Koekemoer, 1968).

Although only K28B gave a green anthrone colour reaction (Cheronis & Entikrin, 1958) indicative of a glycoside, there are strong indications in the mass and ^1H NMR spectra of K28A that it is a glycoside as well.

Clinical signs

Plant material. The findings are summarized in Table 2. After receiving a single high dose of the plant, Sheep 1 died during the night without any clinical signs.

Another acutely affected sheep (Sheep 2) manifested diarrhoea, dehydration and apathy. The most chronically intoxicated sheep (Sheep 3) developed diarrhoea, inappetence, ruminal stasis, bradycardia and reduced respiration. This sheep was unaffected by exercise, remained alert throughout and its habitus was normal.

Dried leaves, dosed to Sheep 4, resulted in gastro-intestinal and cardiac changes typical of acute intoxication.

Bufadienolides. The results are summarized in Tables 1 & 3.

(1) *Guinea-pigs.* The occurrence of clinical signs (see below) after administration of about $3 \times 50\%$ or $5 \times 25\%$ LD₅₀ indicated that bufadienolides K28A and K28B had a cumulative effect. The 3rd bufadienolide, hellebrigenin 3-acetate, on the other hand, was not cumulative; the guinea-pigs recovered before the next dose (25 % LD₅₀) was given and it was less toxic than the other 2. The clinical signs noticed after administration of K28A ($4 \times 25\%$ LD₅₀) and K28B ($7 \times 25\%$ LD₅₀) were muscular tremors and neck paresis. In the case of hellebrigenin 3-acetate these signs appeared at $5 \times 50\%$ LD₅₀ and were followed by a sideways twisting of the neck and spine.

(2) *Sheep.* Intravenous injection of K28A and K28B (Sheep 6 & 9) led to the development of typical signs of krimpsiekte, as described in Table 3 and illustrated in Fig 1–6. Oral administration of K28B, however, had only a mild gastro-intestinal effect (Sheep 7 & 8) with terminal signs of acute cardiac glycoside intoxication (Sheep 7).

Macroscopical pathology

(1) *Guinea-pigs.* No macroscopic lesions were evident.

(2) *Sheep.* Apart from a catarrhal enteritis in Sheep 1 and 7 no other lesions were observed.

Microscopical pathology

(1) *Guinea-pigs.* In both animals multifocal myocardial necrosis was present. Affected myofibres stained more eosinophilically with HE and their nuclei were pyknotic. A mild to prominent brain oedema manifested as *status spongiosis* was present in both animals (Table 1). The distribution was comparable with that observed in sheep (*vide infra*).

(2) *Sheep.* Mild to severe myocardial lesions were scattered throughout the heart muscle in 7 out of 8 sheep. The early stages of the lesions were characterized by Zenker's hyalin degeneration and necrosis of individual or small to larger groups of myocardial fibres. Affected fibres were occasionally vacuolated and the architecture of the myofibrils was disturbed, as was evident in MPAH stained sections. The lesions were often infiltrated, mainly by macrophages, lymphoblasts and lymphocytes. A mild to moderate interstitial fibrosis was seen during the later stages of the lesions. Multifocal infiltrations of mainly lymphocytes, not associated with myocardial necrosis, often occurred throughout the myocardium, but was particularly conspicuous in Sheep 5, 6 and 7.

A mild to moderate brain oedema was most frequently present in the white matter surrounding the lateral ventricle (extending into the white matter of the cerebral gyri), the brainstem and cerebellum. The white matter of the cerebellar gyri was also occasionally involved. Histopathologically it was confirmed that apart from mild enteritis in Sheep 1 and 7 no other lesions were observed.

* Sagatal V, Maybaker

TABLE 2 Observations on sheep intoxicated with *K. lanceolata* Forsk. plants

No.	Material	Dosing regimen		Clinical signs	ECG changes	Fate	Histopathology
		Dose (g/kg × n)	Period dosed (Day 0–Day n)				
1	Dead/senescent	5 × 1	0	Not observed	—	Died after 24h	Negative
2	Dead/senescent	3,5 × 1	0	Decreased rumination/rumen stasis, inappetence, apathy, forced respiration, weakness followed by diarrhoea and dehydration	Arrhythmia (Day 4), widening of the QRS wave, ST elevation (Day 6)	Slaughtered on Day 6	Heart ++ Brain +
3	Dead/senescent	2,5 × 1	0	Transient forced respiration (Day 1)	No changes seen		
		1 × 2	6–13	Transient slow respiration (20/min), decreased rumination/rumen stasis, inappetence, diarrhoea	Transient bradycardia (60/min), tachycardia (130/min), sinus arrhythmia		
		0,5 × 4	27–37	Transient forced respiration, decreased rumination/rumen stasis, inappetence	Progressive bradycardia (54/min), slow respiration (20/min), sinus arrhythmia and AV block occurring irregularly on Day 38		
		0,25 × 11	41–60	Transient forced respiration, weak rumination, inappetence. No changes were seen after light exercise on Day 57	Bradycardia (40/min), slow respiration (15/min), AV block (Day 49), elevated ST segment (Day 50–60)	Slaughtered on Day 63	Heart +++++
4	Fresh/dried	0,2 × 2 0,1 × 6 0,2 × 2	0–1 5–14 15–19	Recurring, transient ruminal stasis, inappetence and diarrhoea	Recurring transient tachycardia, as short as 10 s periods on Day 16, increased PR time intervals and extra systoles on Day 21	Slaughtered on Day 26	Heart ++++ Brain ++

+ = Mild lesions
 ++ = Moderate lesions
 +++ = Prominent lesions
 ++++ = Extensive lesions
 n = Number of daily doses

TABLE 3 Observations on sheep intoxicated with bufadienolides isolated from *K. lanceolata* Forsk.

No.	Isolated bufadienolide	Dosing regimen		Route	Clinical signs	ECG changes	Fate	Histopathology
		Dose mg/kg × n	Period dosed Day 0–Day n					
5	K 28 B	0.025 × 2 0.01 × 1	0–3	i.v.	Dyspnoea and cyanosis	Sinus arrhythmia occurred before and after dosing	Died on Day 3	Heart + Brain +
6	K 28 B	0.01 × 6 0.01 × 2	0–9 27–28	i.v.	Dyspnoea (Day 2), decreased ruminal movements (Day 10), locomotory/postural changes (Day 8): reluctance to stand, frequently changing position of its feet, muscle tremors, weakness, neck paralysis, back arched and feet close together. After rest tremors disappear. Locomotory changes were exacerbated by exercise but disappeared between doses within c. 12 days	Nothing unusual	Slaughtered on Day 29	Heart + Brain ++
7	K 28 B	0.025 × 2 0.01 × 4 0.025 × 4 0.05 × 4	0–17	per os	Recurring transient inappetence and decreased ruminal movements, grinding of teeth, tremors and apathy (Day 16–death)	Mild sinus arrhythmia, ST depression and complete AV block with different ectopic foci firing (Day 18)	Slaughtered on Day 18	Heart + Brain +
8	K 28 B	0.05 × 7 0.025 × 2 0.05 × 3 0.025 × 9 0.05 × 2 0.13 × 1 0.33 × 1	0–42	per os	Recurring transient inappetence, decreased ruminal movements and inappetence	Nothing unusual	Discharged on Day 47	
9	K 28 A	4 × 0.02 1 × 0.01	0–7 9	i.v.	Dyspnoea (Day 2), decreased ruminal movements, inappetence, reluctance to stand (Day 3), muscle tremors (Day 4), locomotory/postural changes, exacerbated by exercise as in Sheep 6 (Day 10)	Tachycardia (Day 2 & 7), arrhythmia (Day 9)	Discharged on Day 30	
10	K 28 B	0.19 × 1	0	i.v.	Dyspnoea after 3 min	Bradycardia (9 min), tachycardia (11 min) followed by progressive AV dissociation (13 min)	Died after 24 min	Heart ++

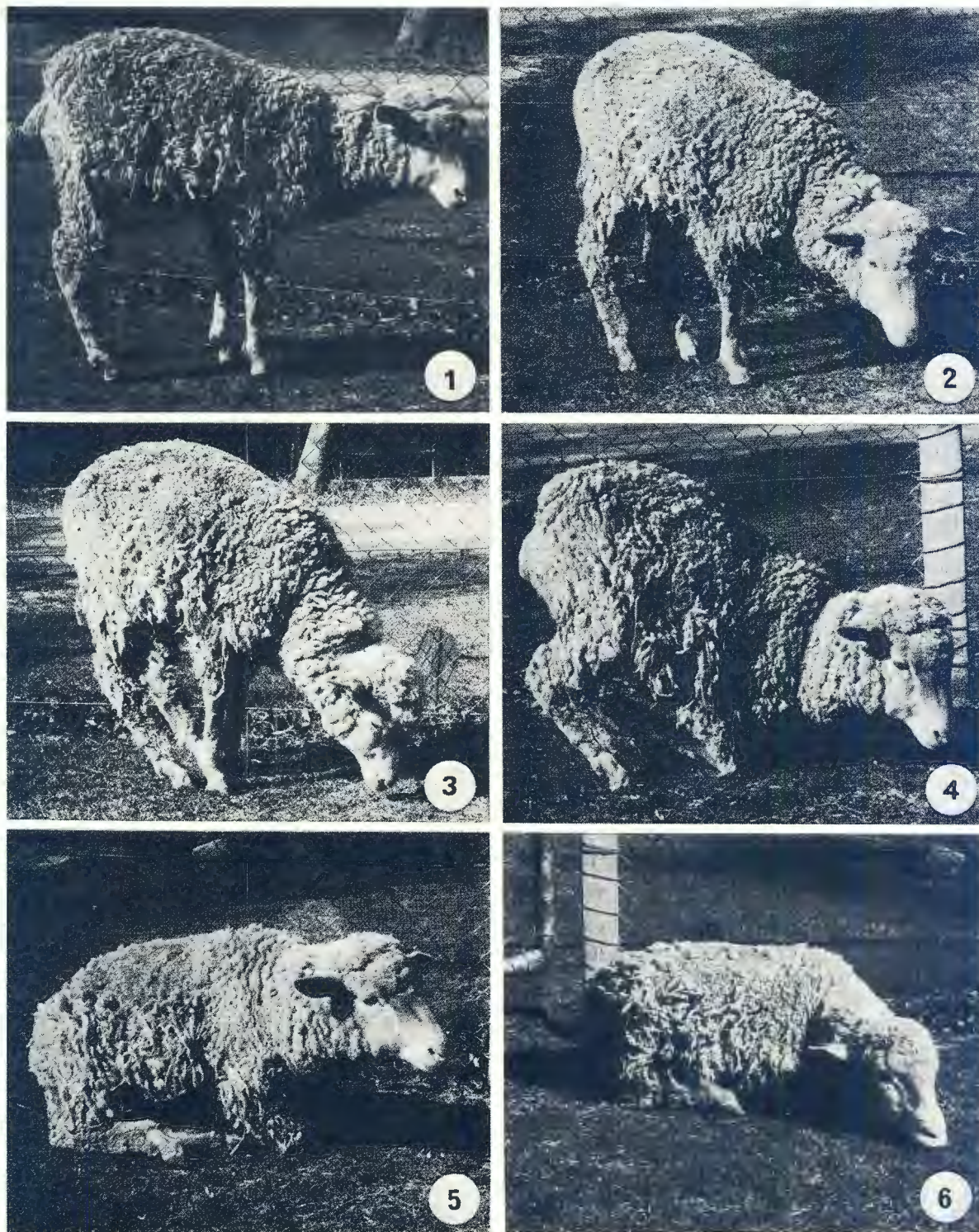


FIG. 1-2 Affected sheep are reluctant to move, they tire easily and often stand with the head held low

FIG. 3 Eventually they assume a typical krimpsiekte posture, with the head down, back arched and feet close together

FIG. 4-6 They tremble, are weak and often lie down, sometimes with the neck stretched out flat on the ground (paresis)

DISCUSSION

The presence of cardiac glycosides in *K. lanceolata* was confirmed by the isolation of 3 toxic bufadienolides. One was identified as hellebrigenin 3-acetate, but the structures of the other 2 are still being investigated.

Typical signs of cardiac glycoside intoxication in sheep (Naudé, 1977), involving the cardiovascular, gastro-intestinal and neuromuscular systems, could be induced by administration of relatively large doses of both the plant and of bufadienolide K28B. The parietic syndrome, krimpssiekte, as described by Henning (1926), only occurred after repeated intravenous injections of small quantities of bufadienolides K28A and K28B. It must be emphasized that krimpssiekte could not be induced by the oral administration of either the plant or K28B, the only bufadienolide given by this route. In fact, repeated administration of small oral doses of the plant over several days seemed only to improve cardiac and respiratory function (Sheep 3), probably because of the positive inotropic effect of cardiac glycosides. The apparent effect of the route of administration on the ability of bufadienolides to cause krimpssiekte should be investigated in greater depth in the future.

Only those bufadienolides (K28A and K28B) that had a cumulative effect in guinea-pigs were tested in sheep. Cumulative bufadienolides were selected because Naudé & Schultz (1982) reported that the cumulative bufadienolide, cotyledoside, administered in this way, could induce krimpssiekte, whereas the non-cumulative bufadienolide, 1 α , 2 α -epoxyscillirosidin (Naudé & Potgieter, 1971) could not. Whether a non-cumulative bufadienolide such as hellebrigenin 3-acetate can cause krimpssiekte is an open question.

The absence of a notable electrocardiographic changes in krimpssiekte (Naudé & Schultz, 1982) was confirmed in this investigation.

Histopathological examination revealed an acute to subacute multifocal cardiomyopathy in sheep that received plant material or bufadienolides. The absence of lesions in acutely intoxicated animals is attributed to the acute course of the disease.

Microscopical lesions observed in sheep and guinea-pigs dosed with plant material or injected with bufadienolides isolated from *Tylecodon grandiflorus* (Burm. F.)

are indistinguishable from the lesions observed in this study (L. Prozesky, unpublished data, 1982).

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