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ISOLATION AND CHARACTERIZATION OF AND SOME OBSERVATIONS ON POISONING BY BUFADIENOLIDES FROM *COTYLEDON ORBICULATA* L. VAR. *ORBICULATA*

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ABSTRACT

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The approximate LD_{50} and cumulative effect of 4 bufadienolides, isolated from *Cotyledon orbiculata*, were determined in guinea-pigs. Two of the bufadienolides proved to be mildly cumulative. One of the mildly cumulative bufadienolides induced subacute intoxication with some signs of krimpsiekte when injected intravenously into a sheep over 13 days. In the sheep, the paralysis was accompanied by signs of malaise, such as electrocardiographic changes and ruminal stasis.

INTRODUCTION

Certain species of the genera *Tylecodon* and *Kalanchoe* as well as some of the bufadienolides isolated from them can cause the paretic syndrome, krimpsiekte, in sheep (Henning, 1926; Steyn, 1949; Naudé & Schultz, 1982; Anderson, Joubert, Prozesky, Kellerman, Schultz, Procos & Olivier, 1983a; Anderson, Schultz, Joubert, Prozesky, Kellerman, Erasmus & Procos, 1983b). Suspicion that plants in the *Cotyledon* genus can also cause krimpsiekte was confirmed experimentally by Terblanche & Adelaar (1965) with *C. orbiculata*. Although Sapeika (1936) speculated that the plant contained the same active principle as *T. wallichii*, his hypothesis was never confirmed by any subsequent isolation of bufadienolides from *C. orbiculata*.

After animal losses, supposedly caused by *C. orbiculata*, in the Pretoria–Brits area and in continuation of the research on cardiac glycosides, the toxicity of this plant and its toxic components were investigated.

MATERIALS AND METHODS

Plant material

Cotyledon orbiculata L. var. *orbiculata* plants were collected on the northern slopes of the Magaliesberg range at Hornsnek near Pretoria.

Isolation of the toxins

The fresh plants (212 kg) were minced and extracted 3 times with ethyl acetate in a Waring blender. The solvent was evaporated under reduce pressure and the resultant syrup was partitioned between 95 % methanol (3,5 ℓ) and petroleum ether (3,5 ℓ). Both extracts were evaporated to dryness and the residues tested for toxicity. Only the residue obtained from the methanol extract (89 g) was toxic.

Chromatography of the toxic residue on silica gel (2 kg), using chloroform, chloroform-acetone (7:3 v/v) and chloroform-acetone-methanol (8:2:0,5 v/v/v), gave a yield of 17 g concentrate after recombination of the toxic fractions.

Repeated chromatography of the concentrate on silica gel, using the above solvent systems, yielded 3 colourless crystalline compounds A, B and C. Compound D was obtained in pure form by chromatography on a prepacked Merck Lobar LiChroprep RP-8 column, using methanol water (90:10 v/v), followed by chromatography on silica gel with benzene-methanol (90:10 v/v). The trivial names of Cotyledosides A, B, C and D are suggested for the 4 toxins.

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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 $CDCl_3$ solutions and infra red (IR) to KBr discs. UV absorptions were measured on a Beckman DK-2A spectrophotometer and IR spectra on a Perkin Elmer 257 spectrophotometer. ^1H NMR spectra were recorded at the National Chemical Research Laboratory of the CSIR on a Bruker WM-500 (500 MHz) spectrometer. Merck silica gel (0,063–0,200 mm) was used for column chromatography as well as a prepacked Merck Lobar LiChroprep RP-8 column. Pre-coated silica gel $60F_{254}$ TLC aluminium sheets were used for thin layer chromatography. The plates were developed with chloroform-acetone-methanol (80:19:0,5, v/v/v/), and the spots were visualized by spraying with 85 % sulphuric acid and heating for 1 minute at 120 °C.

Dosing trials

(1) *Guinea-pigs*. The toxicity of the plant extracts and fractions was monitored by dosing them to weaned guinea-pigs. The acute and cumulative effects of the 4 cotyledosides were assayed in young (150–250 g) male albino guinea-pigs as described in Table 1. The approximate subcutaneous 24 h LD_{50} of each bufadienolide was determined (2 animals/dose) and the cumulative effect by the subcutaneous injection of 25 % and 50 % LD_{50}/day, until the guinea-pigs showed clinical signs of intoxication (4 animals/dose).

(2) *Sheep*. Fresh, mascerated plant material was dosed per stomach tube to a 2-tooth Merino ram (49 kg) which was clinically examined daily (Table 2).

Cotyledoside A, the only toxin to be administered to a sheep, was repeatedly injected intravenously into a milk-tooth Merino wether (22 kg) as described in Table 2. The sheep was examined daily, and electrocardiograms were periodically recorded until it was slaughtered.

Pathology

Eight guinea-pigs and 1 sheep were autopsied (Tables 1 & 2). Specimens from various organs, including the brain, spinal cord, heart, lungs, liver, spleen, kidneys and skeletal muscles, were collected in 10 % formalin. The tissue blocks were routinely processed and stained with haematoxylin and eosin (HE).

RESULTS

Toxic principles

Cotyledoside A. This component (390 mg) was obtained as white crystals from acetone-ether, m.p. 300–302 °C; λ_{max} (MeOH) 299 nm; ν_{max} (KBr) 3520, 3440, 2920, 1720, 1690, 1635, 1535, 1245 and 830

TABLE 1 Observations on guinea-pigs intoxicated by subcutaneous injection of bufadienolides isolated from *C. orbiculata*

Bufadienolide	24h LD_{50} mg/kg	Cumulative effect		Histopathology of cumalative cases
		25 % LD_{50}	50 % LD_{50}	
Cotyledoside A	0,1	N/u after 5 doses	Clinical signs after 4 doses. Destroyed	Various grades of fatty livers
Cotyledoside B	0,2	N/u after 3 doses	Clinical signs after 4 doses. Destroyed	Mild liver degeneration
Cotyledoside C	0,25	N/u after 4 doses	N/u after 4 doses	—
Cotyledoside D	0,25	N/u after 4 doses	N/u after 4 doses	—

N/u—nothing unusual

cm^{-1}. It gave a pink Liebermann colour reaction changing to purple after 1 minute.

Cotyledoside B. This component (180 mg) was obtained as white crystals from acetone-ether, m.p. 216–220 °C; λ_{max} (MeOH) 299 nm; υ_{max} (KBr) 3510, 3460, 3420, 2940, 1760, 1720(br), 1630, 1535, 1250 and 830 cm^{-1}. It gave a light-green Liebermann colour reaction.

Cotyledoside C. The component (58 mg) was obtained as white crystals (acetone), m.p. 307–309 °C; λ_{max} (MeOH) 299 nm; υ_{max} (KBr) 3460(br), 2930, 1715, 1630, 1535, 1245 and 835 cm^{-1}. It gave a light-green Liebermann colour reaction, changing to light-yellow after 1 minute.

Cotyledoside D. The component was crystallized from acetone-ether as white crystals (48 mg), m.p. 268–272 °C; λ_{max} (MeOH) 299 nm; υ_{max} 3520, 3940, 2920, 1710, 1635, 1535, 1250 and 830 cm^{-1}. It gave a pink Liebermann colour reaction, changing to orange after 1 minute and purple after heating.

All 4 components were identified as bufadienolides by virtue of their positive Liebermann colour reactions, characteristic IR absorptions at *c*. 1710, 1630, 1535, 1240 and 840 cm^{-1}, ultraviolet absorptions at 299 nm and signals in the ^1H NMR spectra characteristic of the 3 protons of the α-pirone ring (Anderson & Koekemoer, 1968).

Clinical signs

Plant material. The findings are summarized in Table 2. An attempt to intoxicate the sheep with varying doses administered over several days resulted only in inappetence.

Bufadienolides. The results are summarized in Tables 1 & 2.

(1) *Guinea-pigs*. The occurrence of mild clinical signs after the administration of 4 × 50 % LD_{50} of 2 bufadienolides (Cotyledoside A and Cotyledoside B) indicated that they were mildly cumulative. The animals dosed with Cotyledoside C and Cotyledoside D recovered before the next dose was given, showing that these particular bufadienolides were not cumulative.

The clinical signs noticed after administration of Cotyledoside B, C and D were muscular tremors and neck paresis. In the case of Cotyledoside A, these signs were accompanied by loss of equilibrium and running movements.

(2) *Sheep*. Intravenous injection of Cotyledoside A induced weakness, paresis of the hindquarters and lateral recumbency (Table 2).

Pathology

No notable macroscopic lesions were observed in the guinea-pigs. Mild liver degeneration was observed microscopically in the Cotyledoside B group, and in various grades of fatty livers in the Cotyledoside A group.

No conspicuous lesions were observed in the sheep.

Discussion

The presence of cardiac glycosides in *C. orbiculata* was confirmed by the isolation of 4 toxic bufadienolides. The bufadienolides produced clinical signs in guinea-pigs that were comparable with those of other cardiac glycoside intoxications (Naudé & Schultz, 1982). The disturbance in equilibrium and running movements seen in poisoning with Cotyledoside A have also been reported in hellebrigenin poisoning, a cardiac glycoside isolated from *K. lanceolata* (Anderson *et al.*, 1983b) and *Melianthus comosus* (Anderson & Koekemoer, 1968; R. A. Schultz, unpublished data, 1971). Only 2 of the bufadienolides were slightly cumulative in guinea-pigs, and these were only mildly so.

The fresh plant failed to intoxicate a single sheep to which it was dosed. One of the bufadienolides that was mildly cumulative in guinea-pigs was then repeatedly administered to a sheep in an effort to induce krimpsiekte. Subacute intoxication and some signs suggestive of krimpsiekte were observed.

Cardiac glycoside-containing plants typically affect the gastro-intestinal, cardiovascular and neuromuscular systems (Naudé, 1977). Acute intoxication is characterized by respiratory distress and progressive ECG changes that culminate in excitability and conduction changes, such as firing of ectopic foci and AV dissociation (Naudé & Schultz, 1982). These signs may be accompanied or followed by diarrhoea, hypersensitivity and paresis (Naudé, 1977). On the other hand, chronic intoxication with repeated small doses of cumulative bufadienolides and certain plants such as *K. lanceolata*, can result in krimpsiekte (Anderson *et al.*, 1983b). Sheep affected with krimpsiekte show signs, such as weakness, reluctance to stand, unsteadiness, torticollis and paresis of the hindquarters and neck. Often, too, they assume a typical stance, standing with back arched and feet close together. In the krimpsiekte syndrome the cardiovascular and gastro-intestinal systems are either minimally affected or normal.

Between the 2 extremes of acute intoxication and krimpsiekte, dose-dependent gradations of clinical signs are possible. In chronic intoxication, the cardiac and gastro-intestinal signs usually diminish while the paresis increases. In the case described in this experiment, where prostration was accompanied by signs of malaise, such as ruminal stasis and electrocardiographic changes, either krimpsiekte or subacute poisoning can be diagnosed, depending on the interpretation of these terms by the clinician.

TABLE 2 Observations on sheep intoxicated with *C. orbiculata* and a bufadienolide (Cotyledoside A) that was isolated from it

Dosing material	Dosing regimen		Clinical signs	ECG changes	Fate	Macroscopical pathology
	Dose	Period dosed Day 0–Day n				
Fresh plant	g/kg × n 5 × 4 10 × 3 15 × 10	 0–4 7–9 14–25	Transient recurring inappetence	—	Discharged on Day 25	—
Cotyledoside A	mg/kg × n 0,025 × 1 0,012 × 5	 0 9–13	Initially, ruminal stasis, inappetence and forced respiratory movements. Terminally, the ruminal stasis recurred, accompanied by weakness and paresis of the hindquarters. This was followed by lateral recumbency with the neck bent unphysiologically against wall	Recurring, transient tachycardia, progressively increasing amplitude of the QRS wave in Lead II 3,0–5,2 mV (Days 2–16)	Destroyed *in extremis* on Day 16	Mild general congestion

Fatty livers, such as those seen in guinea-pigs intoxicated by Cotyledoside A, were also reported by Terblanche & Adelaar (1965) in sheep poisoned with semi-dried *C. orbiculata*.

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