The toxicity of *Senecio inaequidens* DC.

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ABSTRACT

This study was designed to confirm the toxicity of a plant implicated in an outbreak of poisoning of stock in Frankfort, Free State Province, South Africa. Cows died acutely after being introduced into a camp, where an abundant, green shrublet was noted to be heavily grazed. This plant was subsequently identified as *Senecio inaequidens* DC. (Asteraceae) by the South African National Biodiversity Institute (SANBI). Extraction and chemical analyses for pyrrolizidine alkaloids (PAs) in *Senecio inaequidens* revealed the presence of 4 different compounds, namely retrorsine and senecionine (known to be hepatotoxic) and 2 unidentified compounds. The average total PA (free base plus *N*-oxide) concentration in plant parts of *S. inaequidens* collected at Frankfort during the outbreak was 0.81 %, compared with the total alkaloid content in the dried, milled *S. inaequidens* plant material, collected 7 weeks after the outbreak, of only 0.18 %. Male Sprague-Dawley rats ($n = 4$), aged 8–9 weeks, were dosed *per os*. Each rat received a different dose of the crude *Senecio inaequidens* extract, ranging from 0.049 mg/g body weight (b.w.) to 0.25 mg/g b.w. No clinical signs were observed in the rat receiving the lowest dose. Rats receiving higher doses showed depression, an unsteady gait, pilo-erection and jaundice, which was particularly noticeable in the ears. Clinical chemistry evaluation revealed an increase in the activities of ALP (except Rat 4), AST and GGT in all animals. Total serum bilirubin, creatinine and urea concentrations were also elevated. All rats had low serum globulin concentrations with an A/G ratio above 1.2. *Post mortem* examination of the rats revealed marked hepatic lesions. Histopathologically, these changes were characterised by necrosis (variable in extent) of the centrilobular and midzonal hepatocytes (but sparing the portal hepatocytes), with extensive haemorrhage and congestion. Proliferation of the bile ducts, fibrosis and oedema were also present. Ultrastructural changes in affected rats were characterised by margination of chromatin, the presence of numerous autolysosomes in necrotic hepatocytes, intramitochondrial woolly inclusions and changes in the endoplasmic reticulum. A sheep, also dosed with the crude extract, failed to exhibit clinical signs, clinical chemistry aberrations or macroscopic lesions; however, examination of the liver of this sheep revealed histopathological and ultrastructural changes similar, though milder, to those displayed by the rats. Pyrrolizidine alkaloids were extracted from the liver and kidneys of the rats and the sheep. In the case of the sheep, retrorsine was also detected in the lungs, urine and bile.

Key words: cattle, hepatotoxicity, pyrrolizidine alkaloids, rats, *Senecio inaequidens*, sheep.

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INTRODUCTION

Seneciosis is one of the most important plant poisonings in South Africa[13]. Certain *Senecio* species contain toxic pyrrolizidine alkaloids (PAs) which, besides other toxicological effects, induce acute or chronic hepatotoxicity in livestock and man[2,3,6]. In the genus *Senecio* the occurrence of the PAs senecionine, retrorsine and integerrimine is well known[7,9]. *Senecio latifolius,* the most important *Senecio* species responsible for poisoning of livestock in South Africa, contains the alkaloids retrorsine, seneciphylline and platyphylline and both, *S. retrorsus* and *S. isatideus* contain retrorsine[16,23]. According to Röder and co-workers[20], *S. inaequidens* contains senecionine and retrorsine; later senecivernine, integerrimine and a retrorsine analogue was added by Bicchi *et al.*[2] to the list of toxic principles in this species.

Senecio species are usually unpalatable and not readily eaten by livestock. Animals may ingest *Senecio* plant material when other forage is scarce or when the stand of the plant is so dense that it cannot be avoided or differentiated from edible forage[18]. Young plants may also be cropped with grass and poisoning has been described as a result of contamination of hay and silage[3,6,18].

Large quantities of *Senecio* species ingested over a short period induce acute poisoning with death ensuing within a few days of exposure; while a large single non-lethal dose, or multiple lower doses ingested over a longer period, may cause chronic disease[14,24]. Acutely affected cattle are anorexic, may display abdominal pain and sometimes diarrhoea[14]. Nervous signs characterised by incoordination of the hind limbs, circling and apparent blindness may be present and in these cases death is usually preceded by tremors[1]. The carcass of acutely affected cattle may exhibit icterus, effusion into the body cavities and visceral oedema, pronounced in the abomasal folds, omentum and large intestinal walls[10,14,17]. Haemorrhages occur in serosal, visceral and subcutaneous tissue[14,17]. The liver is typically swollen, with rounded edges and a mottled surface[10,11,14,17]. The gall bladder wall is usually oedematous and the gall bladder is enlarged with excess bile, which may be blood-tinged[14,17].

Characteristic histopathological features in the liver of acutely affected animals are centrilobular necrosis, which may extend to the midzonal area, with haemorrhaging into the affected areas[14]. Bile duct proliferation and focal accumulation of inflammatory cells are common[17].

During September 2004 a private practitioner from Frankfort (Free State Province, South Africa) reported mortalities in cattle. Cows died acutely after being moved to a camp with a marshy area where a small green shrublet grew abundantly. It was noted that this shrublet was heavily grazed. The plant was collected and submitted for botanical identification. The plant was later identified as *Senecio inaequidens* DC. by the South African National Biodiversity Institute (SANBI).

Necropsy examinations indicated severe hepatic necrosis, multiple haemorrhages and icterus in the longer surviving cases. Histologically, diffuse centrilobular to submassive necrosis and haemorrhage of the liver was reported.

Although all *Senecio* species must be

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regarded as potentially toxic[14], as far as could be ascertained, there are no reports of poisoning having been induced by *S. inaequidens* in South Africa. To assess the toxicity of this *Senecio* species, dosing trials were conducted and specific pyrrolizidine alkaloids (PAs) were extracted from the plant.

MATERIALS AND METHODS

Extraction of plant material and isolation of pyrrolizidine alkaloids

Plant material

Plant material was collected in the toxic camp during the outbreak on the farm Makoupan (27°19′S, 28°32′E) near Frankfort, Free State Province, South Africa, in September 2004. Additional plant material, for a confirmatory dosing trial, was also collected at the same site in November 2004. A voucher specimen of the *S. inaequidens* material has been retained at the Section of Pharmacology and Toxicology, Faculty of Veterinary Science, University of Pretoria. In addition, preserved botanical specimens of *S. inaequidens* (verified by SANBI), collected during December 2003 near Ermelo (26°48′S, 29°48′E), Mpumalanga Province, and in April 2005 near Queenstown, Eastern Cape Province (grid reference unknown), were also used in the experiment.

Senecio retrorsus material was harvested on the farm Spes Bona (31°33′S, 26°48′E) near Molteno, Eastern Cape Province.

Dried, milled *S. latifolius* and *S. consanguineus* was obtained from the Division of Toxicology, ARC-Onderstepoort Veterinary Institute, where it had been stored in a freezer (–8 °C) for an unspecified period of time.

Sample preparation

The *Senecio inaequidens* and *S. retrorsus* plant material was air dried and the various parts (leaves, seeds/flowers and stems) were separated and milled prior to extraction. The previously stored, dried, milled *S. consanguineus* and *S. latifolius* plant material, obtained from the ARC-OVI, was extracted as is. The dried, milled *S. inaequidens*, used in the dosing trial – which had been collected in November 2004, *i.e.* after the outbreak – was also extracted. The mass of the samples for the extraction and isolation of PAs ranged from 0.21 g (seeds/flowers of *S. inaequidens* from Ermelo – the only available plant material) to a maximum of 5 g.

Chemical extraction

The extraction procedures followed the method described by Rösemann[21]. The chemicals and reagents used for the extractions were purchased from Merck (Darmstadt, Germany), except the zinc powder (90 %, analytical reagent) which was obtained from B.D.H. Laboratory Chemicals Division (The British Drug Houses Ltd, South Africa). The quantity of reagents was adjusted to the mass of sample to be extracted. In general, 5 g milled plant material was homogenised for 10 min with 20 mℓ ethanol plus 2 mℓ of deionised water and then shaken mechanically for about 2 h. The layers were allowed to separate and the sample was centrifuged for 3 min at 2500 rpm. The clear solution was divided into 2 equal fractions, marked A and B, and evaporated at 38 °C under a mild stream of nitrogen. The extracts were reconstituted in 2 mℓ 0.6 M H_2SO_4. The crude extract was dewaxed and chlorophyll was removed with 10 mℓ hexane. The *N*-oxides in sub-samples marked A were reduced to basic alkaloids by adding 500 mg zinc powder and left to stand overnight. Samples A and B were alkalinised (pH > 9) by adding approximately 0.5 mℓ of a 25 % ammonia solution. The alkaloids were extracted 3 times with 3 mℓ ethyl acetate and the solvent was then evaporated at 38 °C under a mild stream of nitrogen in a Turbo Vap® LV Evaporator, Zymark. The extracted alkaloids were stored at –25 °C until analysis.

Pyrrolizidine alkaloid analysis

Pyrrolizidine alkaloid analysis was performed at AMPATH Laboratories, Pretoria, South Africa, after re-dissolving the stored extracts in methanol. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed with a Waters 2795 gradient system, equipped with a Micromass Ultima MS/MS with ESI+Mode. Gas chromatography-mass spectrometry (Hewlett Packard 5973 GC-MS with EI+mode (electron impact positive mode) with a CPsil 5CB (Crompack) 25 m × 0.32 mm × 0.25 μm column installed) was also used. Quantification was achieved with a retrorsine calibration curve. Therefore only retrorsine is reported as μg/g retrorsine. The quantities of the other PAs are reported as μg/g retrorsine equivalents.

Rat pilot study

The initial dose of crude extract administered to 4 male Sprague-Dawley rats (Nos 1–4), aged 8–9 weeks and weighing 115–140.5 g, was intended to be equivalent to 10 g dried plant material per kg body weight (b.w.). The rats were dosed by gavage with a *S. inaequidens* crude extract obtained from 50 g dried, milled plant material, which yielded 0.28 g of crude extract. The doses administered to the rats ranged from 0.049–0.245 mg crude extract/g b.w., which was equivalent to 0.012–0.06 mg retrorsine/g b.w. (Table 4).

The rats were observed at least 3 times a day. Based on their habitus and clinical signs the rats were killed with an overdose of pentobarbitone sodium (Euthanaze, Bayer Animal Health Division) administered intraperitoneally. When deeply anaesthetised, blood samples were collected for clinical pathology by intracardiac puncture. The following parameters in the serum were analysed: total serum protein (TSP), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), total bilirubin (Bil T), bile acids (Bile A), urea and creatinine. To determine enzyme activities and serum protein, urea and creatinine concentrations, an automated chemical analyser (Technicon RA-XT system, Miles Inc. Diagnostics Division, Tarrytown, New York) was used following the manufacturer's methods and reagents. Total bilirubin was determined with the NexCT™ Total Bilirubin Reagent Kit using the NExCT™ clinical chemistry system. Bile acids were detected with the formazan method, an enzymatic colourimetric method developed by Next/Vetex Alfa Wassermann Analyser, the Netherlands.

From all 4 experimental rats and 1 control rat, liver, lung and kidney samples were collected in 10 % buffered formalin, sectioned and stained for light microscopical examination. In addition, small blocks measuring 0.5–1 mm were cut from the middle of the liver (parietal surface) and fixed in 2.5 % gluteraldehyde. Selected blocks were post-fixed in 2 % osmium tetroxide for 1 hour, dehydrated in a graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent and embedded in EMBed 812. Sections 1–2 microns thick were cut for tissue orientation and stained with toluidine blue. Ultrathin sections were viewed with a transmission electron microscope.

Fresh tissue (liver, kidney and lung) samples were collected and stored frozen (–25 °C) to determine PA concentrations. The PAs were extracted and analysed using the same methods as previously described. Save for *N*-oxides, the analytical methods utilised could not detect the pyrrolic and other metabolites.

Sheep dosing trial

A male Dorper sheep, aged 8 months and weighing 41 kg, was used in the trial.

During the trial the sheep received lucerne hay, a pelleted concentrate and water was available *ad libitum*. Following an adaptation period of approximately 4 weeks the animal was dosed with *S. inaequidens* crude extract prepared from 4.66 kg dried, milled plant material which yielded 21.2 g of crude extract. The sheep was dosed by stomach tube with the crude extract on 4 consecutive days. Incremental doses, starting from 49.5 mg/kg body weight on Day 0 and Day 1, 99.0 mg/kg on Day 2 and 198 mg/kg on Day 3 were administered. The extract was mixed with 10 mℓ cellofas and approximately 50 mℓ of water.

Clinical examination was performed daily and the sheep was observed twice a day for clinical signs. Before dosing, blood and urine samples were collected 5 times and twice, respectively, and every day during the dosing period. Blood was collected from the *Vena jugularis* to determine clinical chemistry and haematological parameters as well as PA concentrations. Urine was obtained for PA determination by placing the sheep in a metabolic crate. Clinical chemistry parameters as listed for the rat pilot study, plus glutamate dehydrogenase (GLDH) activity were determined using the same methodology. Haematological parameters were determined using an automated analyser Cell-dyne 3700 (Abbot Laboratories, South Africa). The following parameters were determined: haemoglobin concentration (Hb), red cell count (RCC), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white cell count (WCC), mature neutrophils (N mat) immature neutophils (N imm), lymphocytes, monocytes, eosinophils, basophils and thrombocyte count (Thr C). The clinical chemistry and haematological parameters were determined by the Clinical Pathology Laboratory, Faculty of Veterinary Science, which also supplied the reference ranges.

The sheep was euthanased on Day 4 by administering an overdose of sodium pentobarbitone (Eutha-Naze, Bayer Animal Health Division) intravenously. A necropsy was conducted and samples were collected in 10 % buffered formalin and processed for microscopical examination. For EM, liver samples were collected from the parietal surface as well as from the middle of the left and right lobes. The samples were prepared and processed as indicated under the rat pilot study.

At necropsy, liver, bile, kidney and lung samples were collected and stored frozen (–25 °C) to determine PA concentrations. The composition and concentration of PAs in the tissues and body fluids of the experimental sheep were determined using the same methods as described previously.

Fig. 1: ***Senecio inaequidens* DC**.

RESULTS

Plant identification and description

The incriminated plant was identified as *Senecio inaequidens* DC., a member of the Asteraceae (=Compositae) family, tribe Senecioneae, by the South African National Biodiversity Institute (SANBI). It is also known by the common names 'narrow leaved-ragwort', 'South African ragwort' and 'canary weed' in English and 'geelopslag' and 'geelgifbossie' in Afrikaans[19,25].

Senecio inaequidens (Fig. 1) is a perennial herbaceous or woody shrub, up to 100 cm tall, spherically shaped, rising from a shallow taproot. The stems and leaves can be described as follows: stems erect, leafy, rising from the woody base, numerously branched and glabrous, but sometimes sparsely hairy; leaves alternate, usually sessile, occasionally petiolate, with the blade bright green, simple and slightly thickened, usually with the base clasping the stems, basal leaves sessile, 3–14 cm long and 0.3–1 cm wide and have linear to elliptic-lanceolate blades with acute apices; the size of the blades is variable, from 3–14 cm long and 0.3–1 cm wide. The name *'inaequidens'* means 'irregular teeth' in Latin and refers to the margins of the leaf blade, which are irregularly-toothed. The upper leaves are shortly petiolate, subsessile or sessile and occasionally pinnately-lobed. The inflorescence is an open, terminal or axillary, corymbose panicle ranging from 80 to 100 per plant. Radiate capitula 18–25 mm in diameter; with about 20 involucral bracts are characteristic of the species. The bracts are narrowly ovate with acute apices, more or less glabrous, keeled, (4–) 5 (–7) mm long and resinous. The calyculus bracts, 8–12, have acute apices, are more or less glabrous and dark tipped. The ray florets, 7–13, are female, with bright yellow ligules, which become revolute. A cypsela (fruit) is 2.0–2.5 mm long, cylindrical, pubescent between ribs with a white pappus, 2–3 times as long as the cypsela and readily detached. *Senecio inaequidens* flowers mainly in spring and autumn, but flowers can occur all year long[7,22].

Distribution of *Senecio inaequidens*

Senecio inaequidens was first described from South Africa and is also found in Mozambique, Namibia, Lesotho and Swaziland[7]. In South Africa the plant occurs in all the provinces. In Mozambique the plant was collected in Guijá (Gaza Province), Inhaca Island, Polana and between Quinta da Pedra and Salamanga in Maputo Province (H. Snyman, SANBI, pers. comm., 2006). Data from the Herbarium of the Department of Botany, University Eduardo Mondlane, also refers to the occurrence of the plant in Namaacha district (Maputo) and in Caniçado (Gaza). The distribution of *S. inaequidens* in southern Africa is plotted in Fig. 2.

Plant extraction and analysis

LC-MS/MS and GC-MS analyses revealed the presence of 4 different PAs in *S. inaequidens* plant material namely:

- retrorsine, molecular mass (MM) 352, confirmed on GC-MS with reference standards;
- senecionine, MM 336, confirmed on GC-MS by library references and
- 2 unidentified compounds, with MM of 338 and 368, respectively, assumed to be

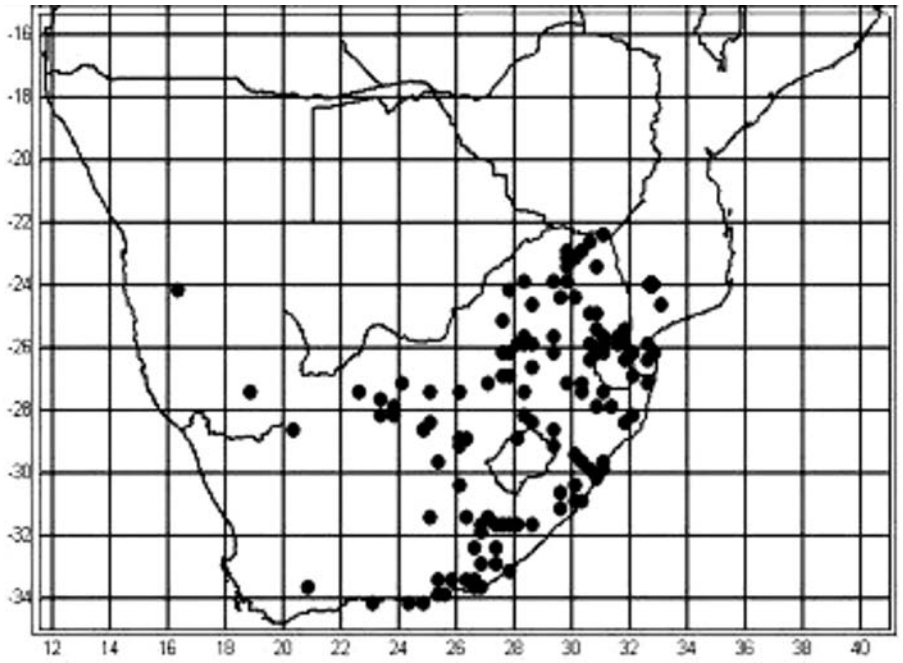

Fig. 2: **Distribution of *Senecio inaequidens* in Southern Africa** (courtesy of H. Snyman, SANBI).

PAs given the presence of the fragments 94, 120 and 138 which were also present in the known PAs, retrorsine and senecionine (Tables 1, 2).

Figure 3 shows the spectra of retrorsine as contained in *S. inaequidens.*

The major PA constituent of the dried, milled *S. inaequidens* plant material was retrorsine, with *N*-oxide:free base ratio of 4.12:1, followed by senecionine (*N*-oxide:free base ratio of 3.8:1) (Table 1). The 2 unidentified alkaloids represented only a minor proportion of the total alkaloids in *S. inaequidens*. One of the unidentified PAs with MM 338 (NI1) had a *N*-oxide:free base ratio of 53.6:1 and the other unidentified PA with MM 368 (NI2) a *N*-oxide:free base ratio of 0.1:1. In *Senecio latifolius* the major constituent was another unidentified PA with MM 388 (NI4), followed by retrorsine and by the unidentified PA with MM 370 (NI3). Senecionine and the unidentified PA with MM 338 (NI1) were minor constituents. In *S. consanguineus* only retrorsine was identified at very low concentrations (Table 1). *Senecio retrorsus* had the same PA composition as *S. latifolius.*

The average total PA (free base plus *N*-oxide) concentration in plant parts of *S. inaequidens* collected at Frankfort during the outbreak was 0.81 % (Table 2), compared with the total alkaloid content in the dried, milled *S. inaequidens* plant material, collected 7 weeks after the outbreak, of only 0.18 % (Table 1). The average total PA concentration in plant parts of *S. retrorsus* was 1.62 % (Table 3) and the total alkaloid content in the dried, milled *S. latifolius* plant material was 1.12 % and in *S. consanguineus* merely 0.01 % (Table 1).

The ratio of total *N*-oxides:free bases in *Senecio inaequidens* was 3.7:1. Comparatively, *S. latifolius* had a ratio of total *N*-oxides:free bases of 13.2:1 and that of *S. consanguineus* was 0.15:1 (Table 1).

The pyrrolizidine alkaloid concentrations of the different parts (leaves, flowers/seeds and stems) of the *S. inaequidens* plant material obtained from Frankfort, Ermelo and Queenstown are reflected in Table 2. Flowers/seeds of *S. inaequidens* from the 3 localities as well as *S. retrorsus* from Molteno (Table 3) had higher concentrations than the leaves and stems.

Rat pilot study

Clinical signs

Rat 1 did not exhibit any noticeable clinical signs. The other rats dosed with the *S. inaequidens* crude extract initially became depressed with a decreased habitus. The rats also demonstrated pilo-erection and developed an unsteady gait and icterus, noticeable at the ears. The clinical signs observed are summarised in Table 4.

Table 1: **PA concentrations** (µg/g retrorsine or retrorsine equivalents for S, NI1, NI2, NI3 and NI4) **of dried, milled *Senecio inaequidens*, *S. latifolius* and *S. consanguineus* plant material.**

	S. inaequidens					*S. latifolius*						*S. consanguineus*	
	R	S	NI1	NI2	Total	R	S	NI1	NI3	NI4	Total	R	Total
A	1358.4	359.2	32.8	39.9	**1790.4**	3628	199	7	2120	5321	**11 275**	88.9	**88.9**
B	265.1	74.5	0.6	35.9	**376.1**	243	6	0	103	438	**790**	77.3	**77.3**

R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368); NI3 = not identified (MM 370); NI4 = not identified (MM 388). A = reduced; B = not reduced (free basic alkaloid).

Table 2: **Concentrations of PAs** (µg/g retrorsine or retrorsine equivalents for S, NI1, NI2) **in different parts of *Senecio inaequidens* collected at Frankfort, Ermelo and Queenstown.**

		Frankfort					Ermelo					Queenstown				
		R	S	NI1	NI2	Total	R	S	NI1	NI2	Total	R	S	NI1	NI2	Total
Lv	A	4794.9	1196.1	60.4	48.8	**6 100.2**	448.9	60.9	0	18.3	**528.1**	45.1	4.5	0	4.6	**53.4**
	B	316.4	124.9	1.1	110.9	**553.3**	28.4	0	0	0	**28.4**	1	6.4	0	1.9	**1.3**
F/S	A	13451	1803.5	32.3	0	**15 287.0**	12440	947.7	81.9	142.9	**13 612.5**	72.1	0	0	0.3	**101.8**
	B	1579.7	235.9	0	161.4	**1 977.0**	707.9	26.3	0	142.5	**876.7**	3.5	0	0	1.5	**5.0**
St	A	2556.5	351.5	50	4.9	**2 962.9**	703.9	165.1	52.6	8.2	**929.8**	27.5	3.9	0	0	**31.4**
	B	125.9	33	0.7	42.1	**201.7**	59.57	4.5	0	4.6	**68.7**	0	0	0	0	**0**

Lv = leaves; F/S = flowers and seeds; St = stems. R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368). A = reduced; B = not reduced.

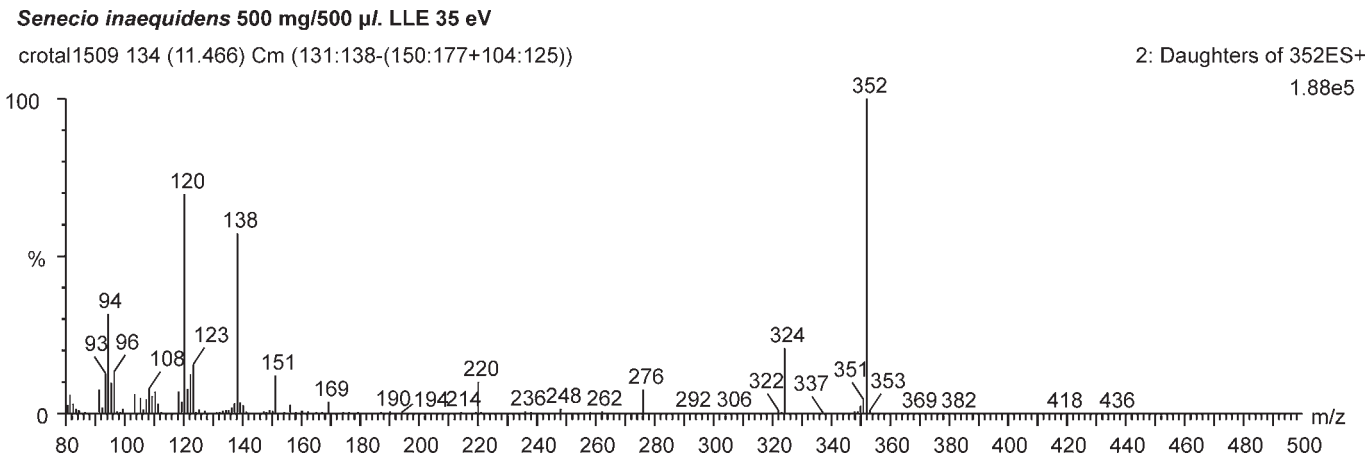

Fig. 3: **MS spectrum of retrorsine in *Senecio inaequidens*** (11.5 min).

Clinical pathology

Clinical chemistry (Table 5) revealed a marked increase in ALP (except Rat 4), AST and GGT activities. Total bilirubin concentrations were also increased. Decreased TSP, albumin and globulin concentrations were noticed in all 4 rats with especially the globulin fraction in Rats 2, 3 and 4 severely reduced, resulting in an increased A/G ratio in all animals.

Table 3: **Concentrations of PAs** (µg/g retrorsine or retrorsine equivalents for S, NI1, NI3 and NI4) **in *Senecio retrorsus* obtained from Molteno.**

		R	S	NI1	NI3	NI4	Total
Lv	A	932	20	4	1619	4094	**6 669**
	B	86	0	0	131	268	**485**
F/S	A	13 092	229	56	3677	13814	**30 868**
	B	639	0	0	211	1087	**1 934**
St	A	4 728	120	17	1927	4404	**11 196**
	B	146	0	0	97	311	**554**

Lv = leaves; F/S = flowers and seeds; St = stems. R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI3 = not identified (MM 370); NI4 = not identified (MM 388). A = reduced; B = not reduced.

Pathology

Macroscopic lesions

With the exception of Rat 1, which received the lowest dose of the extract, all the rats showed marked hepatic lesions characterised by congestion, accentuated lobulation, multifocal to coalescing pale areas (liver necrosis) and jaundice that ranged in extent from mild to moderate (Table 4).

Histopathology

Rat 1: throughout the liver the hepatocytes were swollen with a loss of cellular detail and the presence of large intracellular empty spaces. No lesions were identified in the other organs examined.

Rat 2: hepatic lesions were characterised by extensive coagulative to lytic necrosis of the centrilobular and midzonal areas, sparing the portal hepatocytes, accompanied by extensive haemorrhage, blood pooling and congestion (Fig. 4a). Hepatocytes in the portal areas that were not necrotic were swollen with a fine granular cytoplasm. Mild bile ductular proliferation (Fig. 4b), portal fibrosis and oedema accompanied by a mild purulent infiltration were also present.

Rat 3: the liver lesions were similar to those reported in Rat 2, but were more severe. Mild nephrosis characterised by swelling of the epithelial cells, mainly in the proximal convoluted tubules, was also present.

Rat 4: extensive hepatic pannecrosis with only 1–2 cell layers of viable hepatocytes bordering the portal triads. The cytoplasm of viable hepatocytes demonstrated an increased basophilia. A mild inflammatory response (scattered neutrophils, Kupffer cell proliferation and fibroblasts) was associated with the necrosis. Lymph vessels in the portal areas were dilated, indicating portal oedema.

Transmission electron microscopy (TEM)

Rat 1: in some hepatocytes the cytoplasm showed greatly increased numbers of smoothly-contoured, single membrane-bound bodies with an electron-dense appearance (lysosomes) compared

Table 4: **Dosing regimen, clinical signs and macroscopic lesions of rats dosed with *Senecio inaequidens* crude extract.**

No.	Rat		Dosing regimen		Clinical signs*	Macroscopic lesions
	Age (weeks)	Body mass (g)	Day	Dose (mg/g)		
1	8	115.5	0; 1	0.049	N/a. Euth (D6)	N/a
2	8	118	0	0.142	Depression (D1)**; pilo-erection, jaundice of ears, unsteady gait (D2); weight loss (D3); Euth (D4)	Liver necrosis
3	9	138.6	0	0.196	Depression, swaying gait (6h); pilo-erection, jaundice of ears (D1); Euth (D1)	Congestion of the liver Liver necrosis
4	9	141.3	0	0.245	Depression, pilo-erection (7h); slow movements (12 h); unsteady gait, jaundice of the ears (D2); Euth (D2)	Jaundice of the skin Liver necrosis

N/a = nothing abnormal noticed; * = clinical signs in order of appearance; ** = time post-dosing; Euth = euthanased.

with the normal cells in the control animal. Furthermore, some hepatocytes were characterised by the presence of large areas of cytoplasm containing medium electron-dense material devoid of organelles (vacuoles).

Rats 2, 3 and 4: nuclear changes ranged from chromatin margination to karyopyknosis and karyorrhexis. A few pyknotic nuclei were visible and were recognised by the shrunken nucleus with diffuse condensation of the chromatin. Chromatin margination representing the early stages of karyolysis was evident as condensation of the chromatin in irregular clumps along the inner membrane of the nuclear envelope, with disappearance of the chromatin from other areas of the nucleus (Figs 5, 6). The cytoplasm of necrotic hepatocytes contained severely morphologically distorted organelles, some of which could not be identified (Fig. 5). The swollen/distorted mitochondria were encircled by free ribosomes dispersed in the cytoplasm. Also present in necrotic hepatocytes were autolysosomes, also known as autophagic vacuoles.

In less severely affected hepatocytes, mitochondria were generally mildly swollen and often contained intramitochondrial inclusions (Fig. 6). These were irregularly shaped, medium to electron-dense and had woolly, filamentous borders, which gave them a flocculant and woolly appearance. Also present was dilatation of the endoplasmic reticulum with vesiculation of the rough endoplasmic reticulum and degranulation of ribosomes. In a few sections dark cells were noted directly adjacent to customary lighter staining cells. This is known as the so-called 'dark cell-light cell phenomenon'.

Extraction of pyrrolizidine alkaloids in tissues

Pyrrolizidine alkaloids were detected in the livers of all experimental rats, ranging from 0.1–214.8 μg/g retrorsine or retrorsine equivalents. The concentration of PAs detected in rat livers was inversely proportional to the amount of the extract dosed to the rats. Thus, Rat 1 had higher PA concentrations in the liver compared to those of Rat 4. Pyrrolizidine alkaloids were also detected in the kidneys of Rat 2 (54.5 μg/g retrorsine or retrorsine equivalents) and Rat 4 (31.2 μg/g retrorsine) and in the lungs of Rat 4 (32.7 μg/g retrorsine *N*-oxide). In these cases, the concentrations of PAs were higher than in the liver. The alkaloids detected in the tissues were the same as those in the extracts of *Senecio inaequidens* (Table 1).

Table 5: **Clinical chemistry parameters of rats dosed with a crude extract prepared from *Senecio inaequidens*.**

Analytes	Reference values[a]	Rat 1	Rat 2	Rat 3	Rat 4
TSP (g/ℓ)	58.5 (±2.3)	55.2	39.5	40.2	33.2
ALB (g/ℓ)	30.8 (±1.1)	30.5	26.2	29.8	26.0
GLOB (g/ℓ)	31–33[b]	24.7	13.3	10.4	7.2
A/G	0.95–0.96[c]	1.23	1.97	2.87	3.61
ALP (U/ℓ)	290 (±63)	862	1035	690	35
AST (U/ℓ)	78.1 (±13.0)	320	1194	19 170	12 600
GGT (U/ℓ)	5–6[b]	10	14	23	17
Bil T (µmol/ℓ)	1.4 (±0.6)	9.1	49.7	92.3	97.2
Urea (mmol/ℓ)	9.46 (±0.84)	7.7	8.2	24.9	15.1
Creat (µmol/ℓ)	47.6 (±7.4)	50	42	30[d]	25[d]
Bile A (µmol/ℓ)	20–60[e]	58.6	111.3	79.9	88.6

[a]Mean (s.d.) values of male Sprague-Dawley rats (Lillie, Temple, Florence, 1996 Reference values for young normal Sprague-Dawley rats: weight gain, haematology and clinical chemistry. *Human and Experimental Toxicology* 15: 612–616).
[b]Normal range values (I.S.I.S., 1999).
[c]Calculated from the reference values.
[d]Serum icteric, creatinine results may be influenced by colour.
[e]Mean control ranges in CD Rats (Derelanko, Hollinger 2002 *Handbook of toxicology* (2nd edn). CRC Press, Boca Raton, USA).

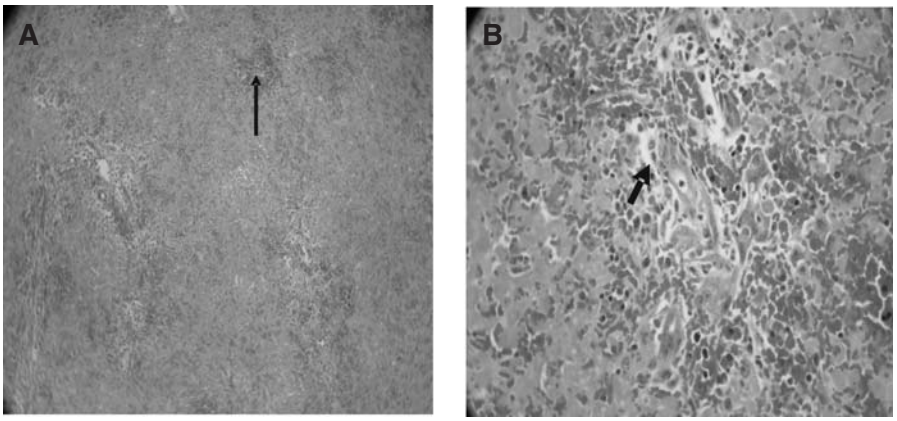

Fig. 4: **Rat 2.** A, **Severe hepatic necrosis with extensive haemorrhages and blood pooling** (arrow; HE ×100); B, **note bile ductular proliferation** (arrow; H&E ×200).

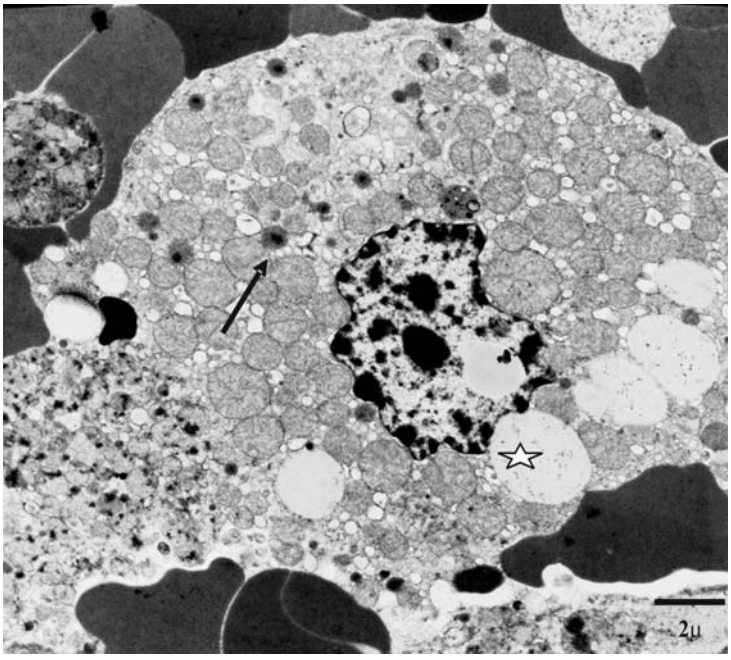

Fig. 5: **Electron micrograph of the liver of Rat 3. The mitochondria are swollen** (arrow) **and intracytoplasmic vacuoles are evident** (star)**. Also note chromatin condensation** (karyopyknosis).

Sheep dosing trial

Clinical signs

On Day 1 of the dosing trial, the sheep's ruminal motility decreased. No other clinical signs were observed until Day 4 when the sheep refused to eat lucerne hay and only ingested 150 g of pellets. The sheep was subsequently euthanased.

Clinical pathology

Haematological values obtained before dosing (Days –18; –17; –14 and Day 0) and during the dosing trial, from Day 1 to Day 4, fluctuated within normal reference ranges. Clinical chemistry analyses revealed that albumin concentrations (22.5–27.3 g/ℓ) and albumin/globulin ratios (0.6–0.7) were below the normal reference ranges of 28–34 g/ℓ and 0.7–1.0, respectively, before and during the dosing trial. Total serum protein was below normal reference values (60–75 g/ℓ) on Day –18, Day –3 and Day 4 and within normal reference ranges on the remaining experimental days. AST activity increased slightly on Day 1 (228 U/ℓ) of the dosing trial and GLDH activity was elevated on Day 3 (53 U/ℓ) and Day 4 (48 U/ℓ). The remaining analytes were within the reference ranges or did not differ much from the values determined during the predosing period.

Pathology

Macroscopic lesions

On necropsy a congested carcass and a pale, swollen liver with rounded edges were observed.

Histopathology

Histopathological examination revealed swollen hepatocytes with fine vacuolisation of the cytoplasm. Mild oedema was depicted as dilatation of lymph vessels in the portal area. Necrosis of single cells with mild neutrophil infiltration was also observed. The spleen was congested with white pulp hyperplasia. A mild interstitial infiltration by mononuclear cells and mild neutrophilic leukostasis was noticed in the lungs. Severe accumulation of mononuclear cells in the mucosa and submucosa of the small intestines and a dispersed distribution of coccidian parasites were present.

Transmission electron microscopy

Hepatic lesions in the left lobe of the sheep included chromatin margination, which were comparable to the rats. Compared to the left side, lesions in the hepatocytes of the right lobe were much more pronounced. The morphology of the organelles was distorted and large vacuoles, often with an uneven outline, were scattered throughout the cytoplasm. Some of the vacuoles contained a faint electron translucent material, intermingled with multilaminated structures and small electron-dense granules, interpreted as either glycogen or free ribosomes. Chromatin margination of hepatic nuclei was common (Fig. 7) and identification of organelles in the hepatocytes was problematic. Some of the pyknotic or swollen mitochondria identified in the affected hepatocytes contained electron-dense inclusions, similar to those described in the experimental rats, and the endoplasmic reticulum was dilated. The latter lesion and the presence of large, empty intracytoplasmic spaces were indicative of cellular oedema, confirmed by light microscopy of this case.

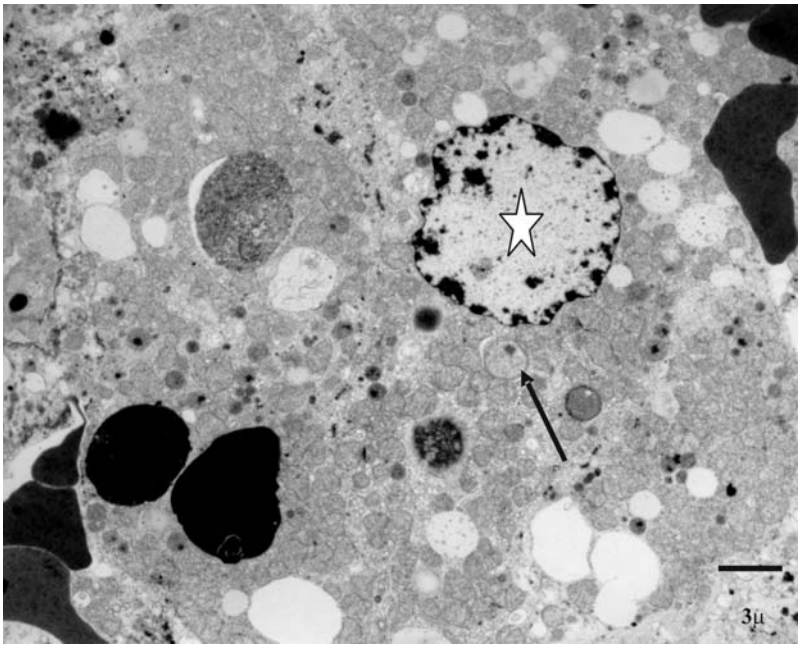

Fig. 6: **Electron micrograph of the liver of Rat 4. Note the electron-dense inclusions in swollen mitochondria** (arrow) **and chromatin margination** (star).

Extraction of PAs in body fluids and tissues of the sheep

In tissues and body fluids, only retrorsine and senecionine were recovered. Pyrrolizidine alkaloids were not detected in urine and serum samples collected before dosing and in the serum samples after dosing. The rumen content collected at necropsy also did not contain any detectable PAs. The highest concentration of retrorsine (82 µg/g) was detected in the urine on Day 4 (one day after the last

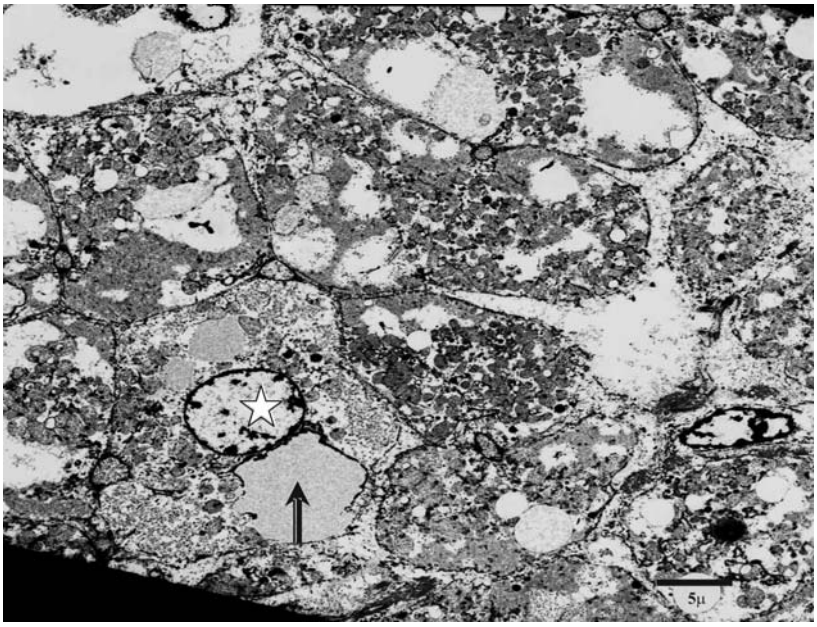

Fig. 7: **Electron micrograph of the right lobe of the sheep liver: the morphology of the organelles is distorted. Note the large intracytoplasmic vacuole** (black arrow) **and chromatin margination** (star).

dose). The liver contained 53.10 μg/g retrorsine or retrorsine equivalents and the kidneys 29.4 μg/g retrorsine or retrorsine equivalents. Traces of PAs were also detected in the bile (6 μg/g retrorsine *N*-oxide) and lungs (11.5 μg/g retrorsine).

DISCUSSION

Four pyrrolizidine alkaloids were isolated and detected by LC-MS/MS and GC-MS from *Senecio inaequidens* DC., namely retrorsine, senecionine and 2 unidentified compounds. The unidentified compounds are assumed to be PAs given the presence of fragments 94, 120 and 138, which were also visible in the spectra of the known PAs retrorsine and senecionine. The difference in the number of PAs identified in the present study compared to the results of Bicchi and co workers[2], who isolated 5 different PAs, can be attributed to the difficulties in identifying unknown PAs with spectral electron impact (EI) libraries (as used in the present study) due to the similar fragments derived from the necine base and the low abundance of the molecular ions.

Retrorsine and senecionine were also detected in *S. latifolius* and *S. retrorsus,* the 2 *Senecio* species most often implicated in livestock poisonings in South Africa. These PAs are known to be hepatotoxic with LD_{50}s of 38 mg/kg and 85 mg/kg for male rats, respectively[3]. Retrorsine was the most abundant PA in *S. inaequidens* and accounted for 75.8 % of the total PAs of the plant, followed by senecionine (20 %) and the 2 unidentified compounds, which represented a minor proportion (<5 %). Pyrrolizidine alkaloids of *S. inaequidens* were mainly in the *N*-oxide form (ratio of *N*-oxide to free bases was 3.71:1). This implies that to exert their toxic effect, the *N*-oxide should first be reduced to the corresponding basic alkaloids, a process suggested to be brought about by enzymes produced by the intestinal flora[16].

The dried, milled *Senecio inaequidens* plant material (subsequently used in dosing trials) yielded a crude extract containing only 0.18 % total alkaloids as opposed to an average total concentration of PAs in plant parts of *S. inaequidens* collected during the field outbreak of 0.81 %. Comparatively, *S. latifolius* and *S. retrorsus* contained 1.12 % and 1.62 % total alkaloids, respectively. On the other hand, the nontoxic *S. consanguineus* yielded only 0.01 % total alkaloids. *Senecio* species with PA concentrations ranging from 0.03 to 0.25 % green material and 1.2 % dry weight, have been reported to cause outbreaks in livestock[5,12].

The toxicity of *Senecio* species to animals depends on the PA composition of the plant species, the total PA content of the plant, the animal's susceptibility and the relative toxicity of the pyrrole metabolites formed in the liver after the animal has ingested the plant[10]. The PA content of *S. inaequidens,* as in other *Senecio* species, varies enormously and depends on the growth stage, season and location of the plant. This was demonstrated by analysing *S. inaequidens* plant material obtained from 3 different localities in South Africa, namely Frankfort, Ermelo and Queenstown and during 3 different seasons. Considering the PA concentration and specific alkaloid composition of *S. inaequidens,* it must be regarded as potentially toxic and dangerous to livestock in areas where it grows.

Pyrrolizidine alkaloid concentrations in the flowers/seeds of *S. inaequidens* from all localities analysed were higher than those in the leaves and stems. This is in agreement with previous observations which report that inflorescences have higher PA concentrations than leaves and stems[5,14].

Using retrorsine as a reference (LD_{50} = 38 mg/kg for male rat[3]), Rat 1 was dosed with the equivalent of 23.4 mg/kg of retrorsine, which is 0.6 times the LD_{50}. Rat 2 received approximately 3 times the initial dose, which was close to, but still below, the LD_{50}. Rat 3 and Rat 4 received 1.2 times and 1.5 times the LD_{50}, respectively. Rats dosed with *S. inaequidens* crude extract equal to or exceeding the LD_{50} of retrorsine exhibited clinical signs of acute pyrrolizidine alkaloid poisoning, while the rat gavaged with the crude extract at a dose below the LD_{50} of retrorsine did not show any clinical signs.

The sheep dosed with *S. inaequidens* crude extract, equivalent in dried plant material to 8 % of body weight, did not present consistent clinical signs that could be related to PAs intoxication. The toxic dose of dried *Senecio* plant material as a percentage of body weight is estimated to be more than 100 % for sheep and goats[5]. This may explain in part why the sheep did not manifest overt clinical signs, even though the appetite decreased and the ruminal movements were reduced on Day 4 of the trial.

Clinical chemistry and gross pathological changes consistent with acute PA poisoning were seen only in the clinically affected rats and were comparable to those previously described in other animal species[4,11,14–16,24]. Liver enzyme activities increase during periods of hepatocyte destruction. Pearson[18] reported that GGT and ALP tend to be consistently elevated as the lesions are mainly in the portal region. In this study, 1 rat (Rat 4) did not develop an increased ALP activity, in fact, ALP activity decreased with no plausible explanation. In the current study all rats presented with decreased TSP, albumin and globulin concentrations, suggesting that impairment of liver protein synthesis occurs early in the course of the disease in rats. In equines, bile acids and bilirubin tend to increase later in the course of the disease[18]. In the present study however, rats that showed clinical signs displayed a dramatic increase in bilirubin concentration.

Macroscopic and histological lesions of acutely affected rats were similar to those described in the cows that died in the Frankfort outbreak and are consistent with those described by several authors[14,16,24]. All experimental animals (rats and a sheep) developed histopathological and ultrastructural lesions comparable to PA poisoning. Microscopic lesions were characterised by centrilobular to midzonal hepatic necrosis and proliferation of bile ducts. The reason why the centrilobular region is particularly affected has been attributed to the high cytochrome P-450 activity in the region[14]. Ultrastructural lesions characterised by margination of chromatin in the nucleus of the hepatocytes and the presence of woolly densities within numerous mitochondria of the experimental rats and the sheep are considered early signs of irreversible cell injury[8].

Retrorsine and senecionine were recovered from the liver of the experimental rats as well as from the liver of the sheep. Retrorsine was also detected in the kidneys of the sheep and 2 of the rats (Rats 2 and 4). In addition, retrorsine was also detected in the lungs, bile and urine of the sheep. Rösemann[21] isolated PAs of *S. inaequidens* from the rumen content of cattle poisoned during the field outbreak. Failure to detect PAs in the sheep's rumen content in the present experiment raises various questions, amongst them the much debated theory of ruminal metabolism and breakdown of PAs by sheep[3,26,27]. Quantification of individual PAs in the organ samples was problematic, probably due to PA losses during preparation and extraction. Although limited dosing trials were conducted, it appears that detection of PAs in tissues of poisoned animals may be useful in confirming a diagnosis of PA intoxication.

In conclusion, the *S. inaequidens* collected at Frankfort, where an outbreak of hepatotoxicity in cattle occurred, contained known hepatotoxic PAs. In addition, the crude extract prepared from plant material of this species was highly toxic when administered to rats. Although the intoxication could not be reproduced in a sheep, this was probably not the ideal species to use in an attempt

to confirm the toxicity in ruminants, in the light of the reported resistance to PA toxicity of sheep. It can be deduced that *S. inaequidens* DC. was most probably responsible for the cattle mortalities.

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