Isolation of the toxin responsible for gousiekte, a plant-induced cardiomyopathy of ruminants in southern Africa

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ABSTRACT

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Gousiekte was induced in sheep and goats by the oral and intravenous (i/v) administration of methanolic and ethanolic precipitates of aqueous extracts of *Pavetta harborii*. Further purification of the toxic principle was carried out by dialysis and ion-exchange chromatography. The toxin was eluted from the cation-exchange resin with 1 mol/ℓ of ammonium acetate and finally purified on Sephadex G-10 and silica gel. Spots on thin-layer chromatographic plates (TLC) were visualized with ninhydrin. Gousiekte was induced with the pure toxin after i/v administration in goats. The yield of pure toxin was about 10 mg/kg of dried leaves.

A compound, indistinguishable from the above on TLC, was also isolated from the gousiekte-inducing plants *Pavetta schumanniana, Fadogia homblei* and *Pachystigma pygmaeum*.

Keywords: Gousiekte, cardiomyopathy, cardiac failure, plant poisoning, cardiotoxin

INTRODUCTION

Gousiekte ("quick" disease) is a cardiac syndrome of ruminants, caused by six species of three genera of the Rubiaceae: *Pachystigma pygmaeum, P. thamnus, P. latifolium, Pavetta harborii, P. schumanniana* and *Fadogia homblei*. The disease is characterized by sudden death following a latent period of 3–6 weeks after initial ingestion of the toxic plants (Kellerman, Coetzer & Naudé 1988; Fourie, Erasmus, Prozesky & Schultz 1994).

Gousiekte is regarded as one of the six most important plant toxicoses of livestock in South Africa. Although outbreaks occur irregularly, severe stock losses have been recorded from this disease. In one documented outbreak a farmer lost more than 1 000 sheep out of a flock of 1 700 (Theiler, Du Toit & Mitchell 1923). Gousiekte is the last of the major plant poisonings in southern Africa of which the causal toxin has not been identified. The toxic principles of the other five are: cardiac glycosides in members of the Iridaceae, Liliaceae and Crassulaceae; monofluoroacetate in *Dichapetalum cymosum*; sesquiterpene lactones in *Geigeria* spp.; pyrrolizidine alkaloids in *Senecio* spp. (Kellerman *et al.* 1988); and steroidal saponins—which have recently been incriminated in the aetiology of geeldikkop, a photosensitization of

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sheep and goats caused by *Tribulus terrestris* (Kellerman, Erasmus, Coetzer, Brown & Maartens 1991).

Despite considerable effort by several workers, gousiekte-inducing toxins have not been isolated from any of the incriminated plants. Veldsman (1952) dosed four crystalline substances from *Pachystigma pygmaeum* to rabbits, with negative results. Anderson & Naudé [Onderstepoort Veterinary Institute (OVI), unpublished data 1962] failed to elicit gousiekte in sheep with ursolic acid isolated from several plants belonging to the Rubiaceae, including non-toxic *Pygmaeothamnus zeyheri* (Anderson & De Kock 1959). Crude extracts of *P. pygmaeum* with cardiotoxic properties (Patterton 1985) were found to be acutely toxic to guinea-pigs (Verschoor & Potgieter 1984); however, since small laboratory animals are not known to be susceptible to the disease (Kellerman *et al.* 1988), these findings might be unrelated to gousiekte. Van der Walt, Van Rooyen & Lötter (1990) also attempted to induce gousiekte with a toxic fraction from *P. pygmaeum,* with negative results.

The aim of this study was to isolate a toxic principle from *Pavetta harborii* that is capable of inducing gousiekte. As a first step, an attempt was made to confirm the unpublished results of Anderson & Naudé (OVI 1959–1962). Their findings can be summarized as follows:

- Ethanolic extracts of *P. harborii* were non-toxic.
- The plant residue, after exhaustive extraction with boiling ethanol, was dosed to three goats. All three died after a latent period, and gousiekte was confirmed by histopathological examination in two of the animals.
- Aqueous extracts of the above-mentioned plant residue proved to be toxic when dosed to six goats. All died, with two of the six having typical lesions of gousiekte.
- The plant residue was not toxic after extraction with water.
- A precipitate obtained by adding ethanol to the water extract was dosed to a goat for 14 d. The goat died on day 29, after showing signs of tachycardia and dyspnoea. However, the histological lesions regarded as being pathognomonic for gousiekte at that time (particularly endocardial fibrosis) could not be detected.

MATERIALS AND METHODS

Experimental animals

Sheep or goats were used for testing the toxicity of extracts, as only ruminants contract gousiekte. Some evidence exists that goats are more susceptible to gousiekte than sheep, but breed, sex and age apparently have no influence in the susceptibility of these animals to the disease (Kellerman *et al.* 1988). Both species have been used successfully in gousiekte experiments (Anderson & Naudé, OVI, unpublished data 1959–1962). The isolation of the toxic principle from *P. harborii* was carried out over a period of 4 years in a series of experiments involving about 30 animals of different breed, sex, age and mass.

Animals were housed individually or grouped in small pens, they received the standard OVI sheep ration and had free access to water. They were examined daily and their temperature and cardiac and respiratory rates recorded. Blood was collected twice a week for clinical pathological examination. Haematological and clinical pathological determinations were performed on the blood, plasma and serum as described previously (Fourie, Schultz, Prozesky, Kellerman & Labuschagne 1989). In particular, the activities of two enzymes, aspartate transaminase (AST) and γ-glutamyltransferase (γGT), were measured. Cardiac function was monitored once a week by means of electrocardiogram (ECG) and cardiopulmonary-flow-index (CPFI) recordings. The electrical activity was monitored by Lead II of the ECG (Schultz, Pretorius & Terblanche 1972). The CPFI can be defined as the ratio of the cardiopulmonary blood volume to stroke volume, and is equivalent to the number of heart beats necessary to pump blood from the right to the left side of the heart through the lungs (Van der Walt & Van Rooyen 1977; Van der Walt, Van Rooyen, Cilliers, Van Ryssen & Van Aarde 1981).

All animals that died were necropsied and specimens of various organs were collected in 10% buffered formalin for histopathological examination, processed, and stained with haematoxylin and eosin. The Masson's trichrome stain for collagen was also applied to certain myocardial sections (Anon. 1968).

Plant material

Pavetta harborii was collected near Ellisras (23° 32′S, 27°42′E) in the Northern Transvaal, on a farm with a high prevalence of gousiekte. This particular plant was selected for extraction of the toxin because it was the most readily obtainable of the gousiekte bushes and the dried material reportedly maintained its toxicity during storage (Adelaar, Naudé, Terblanche & Anderson, OVI, unpublished data 1959–1962). The identity of the plant was verified by the National Botanical Institute.

The plants were dried in the shade, mechanically defoliated, and the leaf material stored at –10 °C. To confirm the toxicity of the plant material, three Merino wethers were dosed per rumen fistula at different dosage rates (Table 1).

Isolation procedure

In this study, a variety of different procedures were explored to isolate the toxic principle responsible for

gousiekte. Only the final successful procedure, as summarized in the flow diagram, is given.

The extraction and precipitation of the toxin was carried out with the use of tap water and laboratory-grade ethanol and methanol. All further procedures were performed with deionized, double-distilled water and analytical-grade reagents and solvents.

Extraction

Four-kilogram batches of coarsely milled *P. harborii* leaves were extracted twice with 10 ℓ of 96% ethanol at *c.* 70 °C for 24 h. The ethanolic extract was squeezed from the plant residue and discarded; the residue was dried and then extracted with 20 ℓ of hot water for 24 h. The watery extract was expressed and concentrated to 2 ℓ on a rotary evaporator (Büchi, Switzerland) at 80 °C. From this 2 ℓ of watery extract, a precipitate of *c.* 150 g (designated the ethanol precipitate) was obtained by adding 4 ℓ of 96% ethanol. To test whether the toxin could be precipitated with methanol, 3 ℓ of methanol was added to a solution of 150 g of ethanol precipitate from another batch in 1,5 ℓ of water. The yield of the methanol precipitate was 125 g.

INDUCTION OF GOUSIEKTE BY DOSING ALCOHOL PRECIPITATES

The ethanol precipitate (EP) was dissolved in water and, after any remaining ethanol had been evaporated off, dosed per stomach tube to a Merino wether (Table 2). The methanol precipitate (MP) was also redissolved in water and after all traces of methanol had been removed, dosed per stomach tube to a Boer goat doe (Table 2).

INDUCTION OF GOUSIEKTE BY INTRAVENOUS ADMINISTRATION OF THE METHANOL PRECIPITATE

Four kilograms of plant material yielded *c.* 125 g of crude fraction. Although 32-fold enrichment was thus achieved, the mass of toxic extract needed to induce gousiekte *per os* was still high. Compounds are generally more toxic when administered intravenously (Loomis 1978), hence, if gousiekte could be induced by this route, a smaller dosage would be required and purification of the toxin by column chromatography would be facilitated. The crude toxin was prepared for intravenous administration by dissolving 20 g of MP in 50 mℓ of distilled water. This solution was centrifuged at 10 000 rpm for 10 min and the mass of the toxic fraction in the supernatant was computed by evaporating an aliquot to dryness. The supernatant was administered by slow intravenous injection to five animals as set out in Table 3.

In addition, 100 g of MP dissolved in 500 mℓ of water was dialysed against distilled water for 2 d, and the water was changed regularly. The dialysis was carried out with the use of Visking Tubing (Labretoria, Pretoria, RSA), 4,5 cm in diameter, in a 2 ℓ measuring cylinder, while the water was agitated with a magnetic stirrer. Ninety per cent of the MP passed through the membrane. The diffusate (DMP), concentrated on a rotary evaporator at 80 °C and treated similarly to the MP, was administered intravenously to a goat (Table 4). The retentate, amounting to only *c.* 10%, contained interfering black, tarry, phenolic compounds.

TABLE 1 The toxicity of the *Pavetta harborii* plant material used for extraction of the active principle(s)

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (years)	Dose (g/kg x n)	Days on which dosed	Total dose (g/kg)	
Sheep	1	61	4	10 x 10	0–3 6–10 13	100	Elevated activity of AST (d 17–60). Survived
Sheep	2	59	4	10 x 20	0–3 6–10 13–17 20–24 27	200	Elevated activity of AST (d 38–death). Died on d 50. Histologically positive for gousiekte
Sheep	3	56	4	10 x 34	0–3 6–10 13–17 20–24 27–31 34–38 41–45	340	Elevated activity of AST (d 34–53). Died on d 54. Histologically positive for gousiekte

AST = Aspartate transaminase

TABLE 2 Dosing of the ethanol precipitate from *Pavetta harborii* to a sheep and the methanol precipitate to a goat

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (years)	Dose (g/kg x n)	Days on which dosed	Total dose (g/kg)	
Sheep	4	32,0	1	0,67 x 14	0–4 7–11 14–17	9,4	Elevated activity of AST (d 11–23). Died on d 27. Positive for gousiekte on histopathology
Goat	5	26,5	1	0,94 x 10	0–4 6–9 12–13	9,4	Elevated activity of AST (d 16–death). Euthanased *in extremis* on d 25 after severe tachycardia and increased CPFI of 10,5 (d 19). Positive for gousiekte on histopathology

TABLE 3 Intravenous administration of the methanol precipitate from *Pavetta harborii*

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (years)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Sheep (ewe)	6	50,0	1,5	38,0 x 4	0–3	400	Elevated activity of AST (d 13–death). Increased CPFI of 12 (d 13–death), gallop rhythm d 29, tachycardia d 30–31, forced respiration d 31, euthanased *in extremis* d 31
Goat (ewe)	7	31,5	1	63,5 x 1 47,6 x 1 63,5 x 2 47,6 x 1 63,5 x 2	0 1 2–31 4 7–8	412	Elevated activity of AST (d 8–death). Tachycardia d 12–13, forced respiration d 10–13, euthanased *in extremis* d 13
Goat (ewe)	8	35,5	1	58,0 x 1 43,5 x 1 58,0 x 2 43,5 x 1 58,0 x 2	0 1 2–3 4 7–8	377	Elevated activity of AST (d 14). Tachycardia d 12–14, forced respiration d 12–14, gallop rhythm d 14, died d 14
Goat (ewe)	9	34,0	1	58,8 x 1 44,0 x 1 58,8 x 2 44,0 x 1 58,8 x 2	0 1 2–3 4 7–8	382	Elevated activity of AST (d 15). Mildly increased CPFI of 9 (d 15), tachycardia d 14–17, forced respiration d 13–17, died d 17
Goat (wether)	10	40,0	1	25,0 x 5 25,0 x 3	0–4 7–9	200	Increased CPFI of 15 (d 14–death). Tachycardia d 21–death, forced respiration d 31, died d 32

TABLE 4 Intravenous administration of the diffusate of the methanol precipitate from *Pavetta harborii*

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (years)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (ram)	11	39	0,6	38,5 x 5 38,5 x 3	0–4 7–9	307	Increased CPFI of 17 (d 21–death). Tachycardia d 21–death, forced respiration d 36, died d 36. Positive for gousiekte on histopathology

AST = aspartate transaminase CPFI = cardiopulmonary flow index CPFI for a normal goat = 5–7

TABLE 5 Intravenous administration of an unbound fraction from the anion-exchange column

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (wether)	12	33	12	45,4 x 7	0–3 5–7	318	Elevated activity of AST (d 15–20). Increased CPFI of 10 (d 21). Severe tachycardia from d 26. Died on d 29. Positive for gousiekte on histopathology

TABLE 6 Intravenous administration of an unbound fraction from the cation-exchange column

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (wether)	13	36	8	41,7 x 7	0–6	292	Nothing abnormal. Discharged on d 100

TABLE 7 Intravenous administration of the ninhydrin-positive fraction eluted from Sephadex G-10

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months	Dose (mg/kg xn)	Days on which dosed	Total dose (mg/kg)	
Goat (wether)	14	27	8	1 x 4	0 2 4 6	4	Tachycardia from d 7–16. Increased CPFI of 10 (d 10). Dyspnoea and euthanased *in extremis* on d 16. Positive for gousiekte on histopathology

TABLE 8 Intravenous administration of ninhydrin-positive compound A

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (ewe)	15	10,0	4	0,40 x 1 0,60 x 1 0,60 x 1	0 2 9	1,60	Diarrhoea (d 10–12). Elevated activity of AST (d 14). CPFI increased to 16 (d 14), died (d 15). Histopathologically positive for gousiekte
Goat (ewe)	16	18,5	5	0,54 x 1	0	0,54	Tachycardia (d 9–42); euthanased on d 69. Histopathologically positive for gousiekte
Goat (ram)	17	9,2	3	0,87 x 1	0	0,87	Tachycardia; severe ECG changes; depression and congestion of mucous membranes; euthanased *in extremis* (d 2). Severe myocardial degeneration

AST ≙ aspartate transaminase CPFI = cardiopulmonary flow index CPFI for normal goat = 5–7

TABLE 9 Intravenous administration of ninhydrin-positive compound B

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (ewe)	18	11	4	0,64 1,36 0,36	0 2 17	2,36	Diarrhoea on d 10–12. Discharged on d 60

TABLE 10 *Per os* administration of compound A

Animal				Dosing regimen			Results
Species	No.	Live mass (kg)	Age (months)	Dose (mg/kg x n)	Days on which dosed	Total dose (mg/kg)	
Goat (ewe)	19	14,5	5	4,0 x 1 4,0 x 1 1,0 x 1 4,0 x 1	0 9 11 16	13,00	Configuration of T wave altered (d 13–69). Euthanased on d 70
Goat (ram)	20	8,6	3	15,0 x 1 6,7 x 1 6,1 x 1	0 7 9	27,67	Slightly elevated activity of AST (d 13–16). Configuration of T wave altered (d 12–43). Euthanased on d 43

AST = aspartate transaminase

Ion-exchange chromatography

CATION- AND ANION-EXCHANGE COLUMNS

To determine whether the toxic principle had.cationic or anionic properties, two 30 g DMP aliquots dissolved in 100 mℓ of water, were run through cation- and anion-exchange resins in 25 x 300 mm glass columns at 3 mℓ/min. For the anion-exchange column, Dowex 1–x4, 20–50 dry mesh (J.T. Baker Chemical Co., Phillipsburg, N.J.) in formate form was used. Of the initial 30 g of DMP applied to the column, 20 g passed through. The cation-exchange column consisted of a weakly acidic resin, CM Sephadex C-25 (Pharmacia, Uppsala, Sweden) in the NH_4^+ form. Twenty-seven grams of the 30 g of DMP applied, was collected as the unbound fraction.

The unbound fractions from both columns were tested for toxicity as set out in Tables 5 and 6.

ELUTION FROM THE CATION-EXCHANGE RESIN

Fifty grams (dry mass) of the same weakly acidic cation-exchange resin, CM Sephadex C-25 in the NH_4^+ form, buffered with 0,25 mol/ℓ of ammonium acetate (pH 6), was poured into a 50 x 600 mm glass column and allowed to settle. The pH of 100 g of DMP dissolved in 800 mℓ of distilled water was adjusted to 6 with 25% ammonia solution before it was centrifuged at 3000 rpm for 20 min. The supernatant was slowly poured onto the cation-exchange resin and allowed to run through the column at 5 mℓ/min. Elution was performed firstly with 2 ℓ of water and then successively with ammonium acetate at concentrations of:

(a) 2 ℓ of 0,25 mol/ℓ (pH 7)
(b) 1 ℓ of 0,5 mol/ℓ (pH 7)
(c) 1 ℓ of 1 mol/ℓ (pH 7)

The elutions were collected separately and concentrated on a rotary evaporator. Most of the ammonium acetate was removed in a freeze drier at 30 °C for 48 h. The yield was *c.* 90 g for the fraction that had passed through the column, *c.* 11 g for eluate (a), *c.* 1 g for eluate (b) and *c.* 2 g for eluate (c).

Thin-layer chromatography (TLC)

A sample (*c.* 20 mg) of each of the three eluent fractions was dissolved in 200 µℓ of water and spotted on thin layers (0,25 mm) of silica gel G (Alugram Sil G/UV 254, Macherey-Nagel, Düren). The chromatograms were developed by ascending chromatography to a height of 7–8 cm in a glass tank by use of a mobile phase of water:2-propanol:acetic acid in a ratio of 60:40:4 (v/v/v). After development, the chromatogram was dried, and sprayed with ninhydrin reagent (300 mg of ninhydrin, 100 mℓ of n-butanol, 3 mℓ of acetic acid). After it had been sprayed, the chromatogram was developed in an oven at 110 °C for 2 min.

Gel filtration

Additional batches of eluate (c) (eluted with 1 mol/ℓ of NH_4^+ acetate at pH 7) were further fractionated by gel filtration. Sephadex G-10 (Pharmacia, Uppsala, Sweden) was used in a 25 x 1000 mm column. One to 2 g aliquots of the pH 7 fraction were dissolved in distilled water, applied to the column and eluted with distilled water at a flow rate of 3 mℓ/min. Five-millilitre fractions of the eluent were collected in test tubes on a fraction collector. Aliquots of 5 µℓ from each tube were spotted on TLC, developed and sprayed as described. Before spraying, the UV-visible components (254 nm) were marked with a pencil.

The first fractions, containing only substances colouring orange with ninhydrin (tubes 46–49, Fig. 1), were combined, concentrated and evaporated to dryness at 70 °C and administered i/v to a goat as described in Table 7.

Separation of the ninhydrin-positive compounds on silica gel

The combined ninhydrin-positive fraction (tubes 46–49, Fig. 1) from Sephadex G-10 column were further fractionated by column chromatography. A 300 × 17 mm glass column was used, packed with 40–63 µm of silica gel (E. Merck No 9385). Eighty milligrams of the sample were applied and eluted with 2-propanol:water:acetic acid (60:40:2,5) at a flow rate of 5 mℓ/min, collecting 7,5 mℓ for the first five tubes and 2,5 mℓ thereafter. An aliquot of 5 µℓ from each tube was spotted on thin layers of silica gel, developed and visualized as described under thin-layer chromatography.

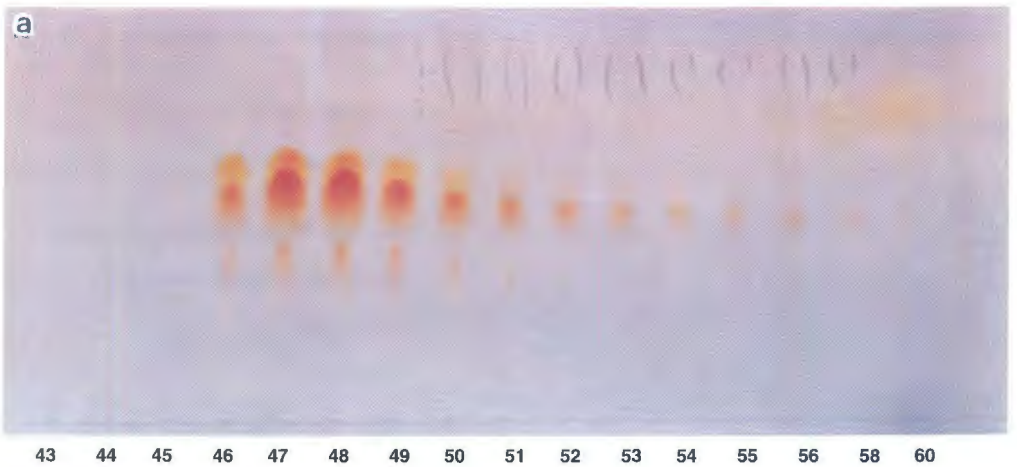

FIG. 1 Thin-layer chromatography plate—Sephadex G-10 elution pattern of the toxic fraction of *Pavetta harborii*

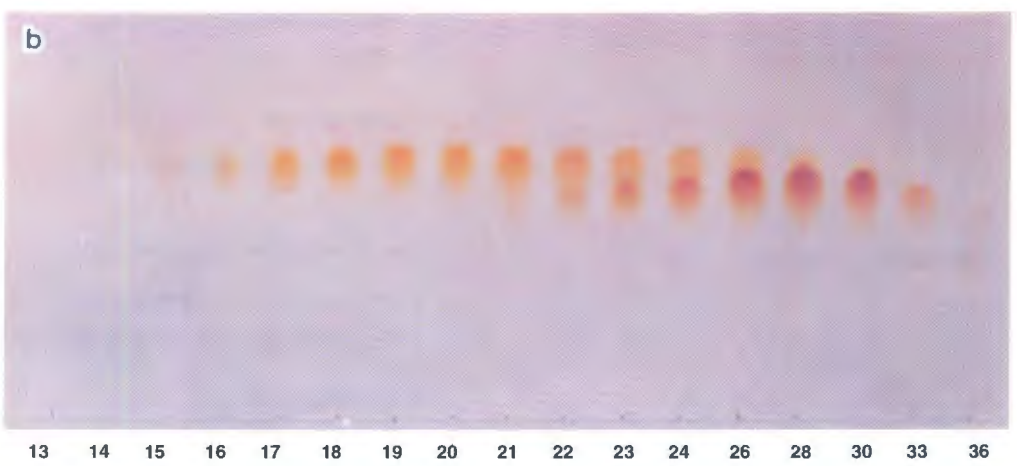

FIG. 2 Thin-layer chromatography plate—Silica-gel elution pattern of the toxic fraction of *Pavetta harborii*

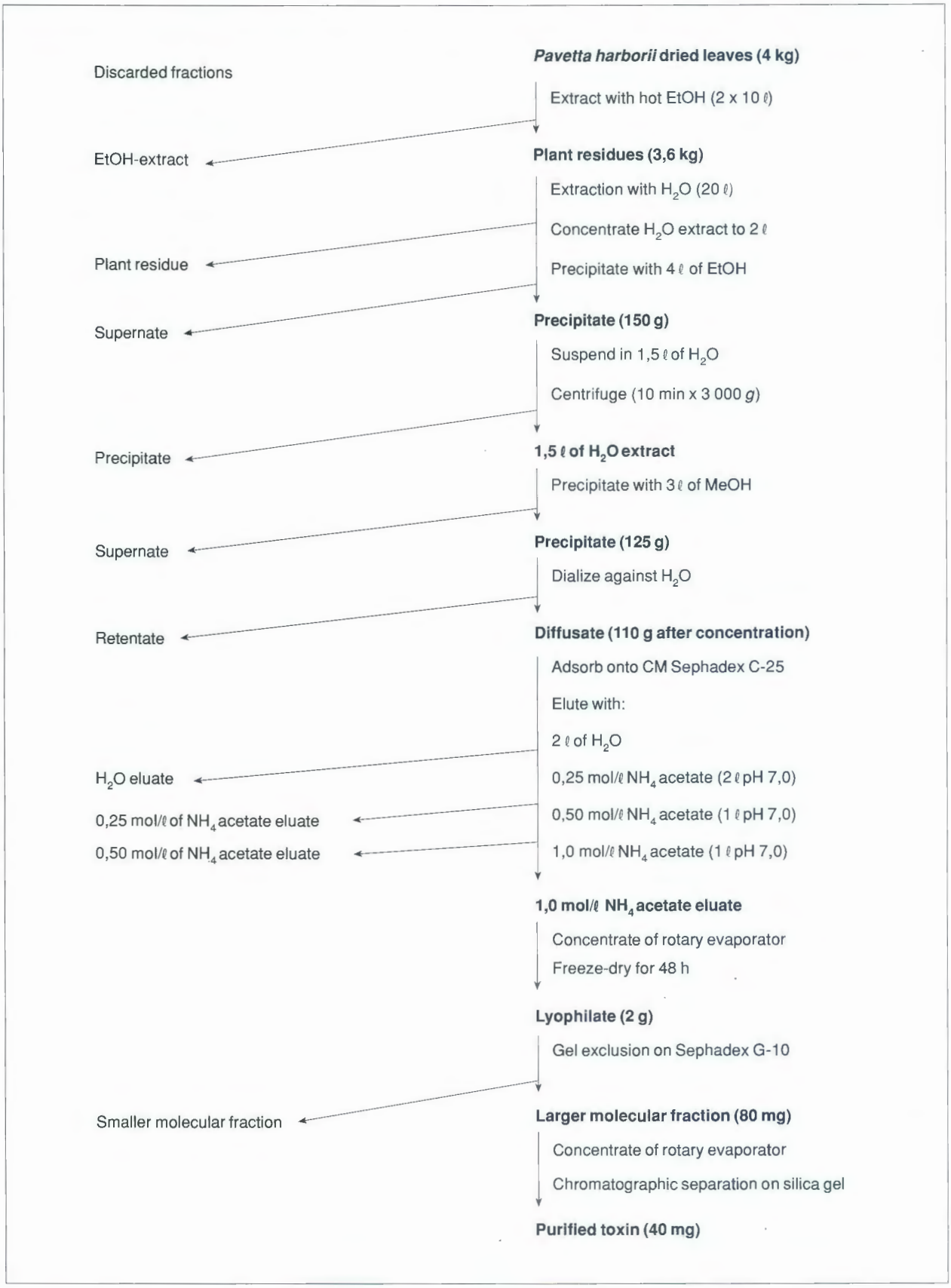

FIG. 3 A diagrammatic representation of the isolation procedure of the toxin of *Pavetta harborii*

Three fractions were obtained: fraction A (tubes 15–20), giving a single spot on TLC (compound A) with a light orange colour after it had been sprayed with ninhydrin, yielded 40 mg; fraction B (tubes 29–35), also giving a single spot (compound B), but with a darker orange colour, yielded 10 mg; fraction C (tubes 21–28), a mixture of A and B, yielded 30 mg (Fig. 2).

INTRAVENOUS ADMINISTRATION OF COMPOUNDS A AND B

Compound A was administered intravenously to three goats and compound B to one goat, as set out in Tables 8 and 9.

PER OS ADMINISTRATION OF COMPOUND A

Compound A was also dosed to two goats, as set out in Table 10.

Isolation of the toxic principle from three other gousiekte plants

To determine whether the same compounds also occurred in other gousiekte plants, *Pachystigma pygmaeum* collected near Ventersdorp (26°17′S, 26°48′E), *Fadogia homblei* collected near Bronkhorstspruit (25°46′S, 28°45′E) and *Pavetta schumanniana* collected near Nelspruit (25°29′S, 30°59′E), were subjected to the same isolation procedure as carried out on *Pavetta harborii*.

A diagrammatic presentation of the isolation procedure is given in Fig. 3.

RESULTS

The findings, which have also been published elsewhere (Fourie 1994), are summarized in Tables 1–10. The plant material was toxic at *c.* 200 g/kg when tested initially (Table 1). The results obtained by Anderson & Naudé (OVI, unpublished data 1959–1962) were confirmed when it was shown that the toxic principle of gousiekte was relatively heat-stable and water-soluble, and the toxin could be precipitated with ethanol and further enriched by subsequent methanol precipitation. The 300 g EP and 250 g MP dosed to the two animals were equivalent to *c.* 250 g/kg and 300 g/kg of plant material. Both animals developed cardiac failure (Table 2). All six animals to which the methanol precipitate (sheep 6 and goat 7–10, Table 3) and diffusate of the methanol precipitate (goat 11, Table 4) had been administered intravenously, died or were euthanased *in extremis* after they had shown signs of cardiac failure.

The cationic properties of the toxin was indicated by the death of a goat after it had been injected with the unbound fraction from the anion-exchange resin (Table 5). This finding was confirmed when another animal showed no ill effects after injection with the fraction that had passed through the cation-exchange resin (Table 6). The toxin (compound A) was eluted from the cation-exchange resin with 1 mol/ℓ of ammonium acetate, separated on Sephadex G-10 (goat 14, Table 7) and finally purified on silica gel (goats 15–17, Table 8). Goat 17, which had received the highest single dose, had the most severe myocardial lesions and this probably explains the ECG changes on day 2. According to the definition, however, this animal could not be deemed positive for the disease as it had died just 3 d after administration of the toxin. The dose of compound A, therefore, had to be adjusted to induce gousiekte with an acceptably long, latent period. Goat 15, which had received a total dose of 1,6 mg/kg and had been dosed until day 9, died on day 15 after it had shown signs of severe cardiac failure (CPFI of 16 on day 14 versus the normal value of 5–7). Goat 16, which had received the lowest dose of compound A, survived the experiment and was euthanased on day 69. Histopathological examination of the myocardium of these two goats (goats 15 and 16) was positive for gousiekte.

Cardiac changes in all the animals included nuclear hypertrophy, hyperplasia and granular degeneration sometimes accompanied by early myocytolysis of myocardial fibres. Three of the animals (goats 7–9, Table 3) died after a short, latent period of 13–17 d. Atypical lesions for gousiekte were noted in the lungs of these three animals, i.e. lobar consolidation with scattered, large, emphysematous bullae, often extending subpleurally. Histologically, the lesions were characterized by widespread congestion and focal, extensive atelectasis interspersed by alveolar and interstitial emphysema. Alveolar spaces contained eosinophilic proteinaceous material and scattered macrophages. The terminal bronchiolar and alveolar epitheliums were hyperplastic and the interstitial tissues infiltrated by predominantly round cells and scattered neutrophils. In goats 6 and 10 the lung lesions were milder than those in goats 7–9. A detailed description of the pathology will be reported elsewhere.

Goats 15 and 18 developed diarrhoea which, after identification of coccidia in the faeces, was successfully treated with amprolium (Amprol, Logos Agvet) (6 g/animal/d from day 10–13). The coccidial infection probably resulted from suppression of the immune system similar to that previously recorded under experimental conditions as a result of high doses of gousiekte-inducing plants (Fourie *et al.* 1989).

Compound B was administered at more than 2–4 times the level of compound A, but no signs of cardiac involvement were noticed (goat 18, Table 9).

Since the yield of the toxin was very low (40 mg from 4 kg), two attempts at inducing gousiekte orally with insufficient quantities of toxin failed. Besides altera-

tion in T-wave configuration in both animals, no other abnormalities were observed (goats 19 and 20, Table 10).

A compound indistinguishable from compound A (*P. harborii*) on TLC, was also isolated from *Fadogia homblei*, *Pachystigma pygmaeum* and *Pavetta schumanniana*.

DISCUSSION

Over the past 30 years, several attempts to isolate the toxic principles of gousiekte plants have failed (Kellerman *et al.* 1988), largely for want of a susceptible, small laboratory animal to assay plant fractions for toxicity and the presence of a *c.* 6 week latent period. These two factors, together with the inconsistent toxicity of plants, have made the active principles of gousiekte bushes very difficult to isolate. All the known difficulties in reproducing gousiekte experimentally were encountered in this investigation, one of the most notable being the lack of toxicity in plants. Many time-wasting trials were carried out with low-toxicity plants which never progressed to the extraction phase.

Research on gousiekte commenced in 1908 when Walker, with the aid of field trials, attempted to establish the cause of the disease. From 1916, various experiments were carried out by Sir Arnold Theiler, who in 1920 conclusively proved that gousiekte was caused by *Pachystigma pygmaeum* (Theiler *et al.* 1923). During this 4 year period many negative experiments were carried out, probably owing to the inconsistent toxicity of the plants. Five other species belonging to the Rubiaceae have since been incriminated in the disease (Kellerman *et al.* 1988). Prior to the current study, the most meaningful contribution towards isolation of the active principle was probably the work done by Anderson & Naudé (OVI, unpublished data 1959–1962), who showed that the toxin was water-soluble.

Certain specific criteria were strictly adhered to during the isolation and biological evaluation of chemical fractions in order to ensure that only gousiekte was induced. Firstly, fractions of the plant extracts were tested for toxicity in sheep and goats, because no evidence could be found that small laboratory animals are susceptible to the disease; secondly, to qualify as gousiekte, the cardiac failure (as measured by the CPFI, Van der Walt *et al.* 1981) had to follow after an appropriate latent period; and thirdly, myocardial lesions consistent with those of gousiekte had to be produced. Elevations in the activity of certain heart enzymes indicating cardiac damage (Fourie *et al.* 1989) were also of great assistance in making positive diagnoses. In the past, much effort was expended on the isolation of chemical fractions from gousiekte plants which caused acute deaths in laboratory rodents; however, without using the above criteria, it is impossible to tell whether true gousiekte was induced in these cases.

Histopathological confirmation of gousiekte was previously based on the presence in the myocardium of varying degrees of fibrosis in the endocardial zone, especially of the left ventrical and apex (Kellerman *et al.* 1988; Fourie *et al.* 1994). Little information on the chronological development of the myocardial lesions in natural cases is available. The picture is complicated by the fact that, within the same animal, acute, subacute and chronic lesions may be present. Under experimental conditions, when abnormally high doses of toxin are administered, the latent period can be shorter than that of natural cases. The initial, most striking lesion in these experimental cases is hypertrophy of the myocardial fibres in the endocardial region, primarily of the left, free ventricular wall. Foci of replacement fibrosis or necrosis and atrophy of myocardial fibres may also be present. The lesions in animals with longer latent periods more closely paralleled those of natural cases and were characterized by diffuse, moderate to severe hypertrophy of endocardial fibres, coalescent to extensive foci of fibrosis, and scattered foci of necrosis.

The investigation revealed that gousiekte can be induced by the intravenous route. Since the toxin was not, as initially suspected, formed from a precursor in the rumen, other reasons for ruminants being the only susceptible animals had to be sought. The result obtained with goat 15, which had received 1,6 mg/kg toxin in a divided dose i/v and died on d 15 after severe cardiac failure, left no doubt that compound A was the active principle responsible for gousiekte. The lung pathology encountered in goats 7–9, which received the methanol precipitate i/v, is not typical of gousiekte. However, this is consistent with the finding of Anderson (OVI, unpublished data 1959–1962) who reported "collapse of the lungs" in a goat dosed with an extract of *P. harborii*. Since animals dosed with a similar fraction (MP) in the current experiment manifested moderate lung consolidation and emphysema, the lung lesions are assumed to be associated with the administration of inordinately high doses of toxin.

Ion-exchange chromatography proved to be the most important fractionation step in the isolation of the toxin. More than 90 % of the extracted crude fraction that passed through the column, tested negatively for gousiekte. The toxin, on the other hand, revealed strong cationic properties and could be separated from other cations by stepwise elution with ammonium acetate. We now know that the gousiekte toxin is:

- water-soluble
- relatively heat-stable
- passes through a dialysis membrane
- has cationic properties
- stains orange with ninhydrin

The structure of this apparently novel toxin is currently being determined. ^{1}H and ^{13}C NMR spectra have shown that the toxin isolated from the four plants has a simple structure and can possibly be synthesized (R. Vleggaar, Department of Chemistry, University of Pretoria, personal communication 1994), which would be a boon for future researchers as the isolation procedure is tedious and the yield of toxin from the plants, low.

As a result of this study, rubiaceous plants can now be chemically assayed for toxicity without resorting to ethically questionable biological trials. Many plants closely related to the six known gousiekte-inducing species occur in South Africa and it would be valuable, even from a chemotaxonomic point of view, to determine which of these contain the toxic principle.

An explanation for the apparent non-susceptibility of monogastric animals to gousiekte possibly lies in the pH of their stomachs. In a pilot trial, a known toxic fraction which had been treated with 1 mol/ℓ HCl at room temperature for 24 h failed to induce gousiekte after its i/v administration to a goat. Changes were also noticed in the appearance of TLC spots produced by the toxin which had been exposed to a pH below 2. The possibility must now be investigated that the low pH in the stomachs of monogastric animals is responsible for the breakdown or deactivation of the gousiekte toxin.

The pathological changes in gousiekte at subcellular level need further investigation. Electron-microscopic studies have shown that the principle lesion is in the contractile proteins (especially myosin) of the heart (Schutte, Els, Booyens & Pienaar 1984). With the toxin now available, it will hopefully be possible to determine whether myosin is broken down by the toxin, or whether the formation of new myosin is inhibited during normal turnover of this protein. A study of the effects of this toxin on contractile protein metabolism might also shed some light on the pathogenesis of certain cardiomyopathies in humans.

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