Onderstepoort J. vet. Res., 51, 245–248 (1984)

PREPARATION AND PROPERTIES OF A CRUDE EXTRACT TOXIC TO GUINEA-PIGS FROM *PACHYSTIGMA PYGMAEUM*, A PLANT CAUSING HEART FAILURE IN RUMINANTS

J. A. VERSCHOOR and D. J. J. POTGIETER, Department of Biochemistry, University of Pretoria, Pretoria 0002

ABSTRACT

VERSCHOOR, J. A. & POTGIETER, D. J. J., 1984 Preparation and properties of a crude extract toxic to guinea-pigs from *Pachystigma pygmaeum*, a plant causing heart failure in ruminants. *Onderstepoort Journal of Veterinary Science*, 51, 245–248 (1984)

Pachystigma pygmaeum is a rubiaceous plant which causes heart failure after ingestion by ruminants. Isolation or characterization of a toxic entity has hitherto been unsuccessful. Extraction of the plant material with water or organic solvents gave poor yields of toxicity in guinea-pigs because of some unusual properties of the toxic entity. We present a method whereby liberation of a toxic entity from the plant material was achieved by fermentation with baker's yeast to give a crude extract with sufficient yield of toxicity for use in further isolation work.

INTRODUCTION

"Gousiekte" (literally translated as "quick disease") is a plant poisoning syndrome of ruminants observed only in southern Africa. Sudden death due to heart failure occurs 4–8 weeks after ingestion of a lethal amount of toxic plant material (Walker, 1908; Theiler, Du Toit & Mitchell, 1923). There are 5 known species of rubiaceous plants (*Pachystigma, Pavetta* and *Fadogia* genera) which can cause the disease (Codd & Voorendyk, 1966), all of them indigenous to the Transvaal and bordering areas. Economically, "gousiekte" is regarded as one of the most important plant poisonings of southern Africa because of the heavy stock losses associated with it in cattle, sheep and goats.

The pathology of the disease, as described by Walker (1908), Theiler *et al.* (1923) and Hurter, Naudé, Adelaar, Smit & Codd (1972), indicates that the heart is the target organ. Abnormalities observed in other organs and tissues apparently result from an impaired heart function. Histopathological observations revealed degenerated heart muscle cells and intrusion by connective tissue, especially in the region of the apex (Smit, 1959). The physiological studies undertaken by Pretorius & Terblanche (1967) and Pretorius, Terblanche, Van der Walt & Van Ryssen (1973) led to the conclusion that the toxin impairs the contractility of the heart muscle either directly, by binding to the contractile proteins, or indirectly, by inhibiting the normal energy metabolism of the heart. The isolation of the toxic compound would be useful to facilitate the elucidation of the molecular mechanism of the poisoning and, hopefully, to develop preventive agents to limit stock losses.

Isolation of the toxic entity from the plant was unsuccessfully pursued by Veldman (1952), Anderson & De Kock (1959) and Potgieter, Jordaan, Cronje & Meij (1975) after Theiler (1923) had first related the ingestion of the plant *Pachystigma pygmaeum* to the disease. The crucial problem was how to extract "gousiekte" toxicity from the plant material. The work of Anderson & De Kock (1959) indicated that toxicity (tested in goats) could not be extracted from *Pavetta* species with organic solvents. In the past, our group succeeded in obtaining a macromolecular fraction toxic to guinea-pigs by means of ammonium sulphate precipitation of the plant juice (Potgieter *et al.*, 1975). The yield in terms of kg LD in guinea-pigs per unit mass of plant material was, however, negligible, compared to the toxicity of the plant in ruminants.

In this paper we present a method whereby an extract toxic to guinea-pigs could be prepared by fermenting the plant material with baker's yeast. This method gave reproducible results in terms of toxicity and yield, although seasonal variations were observed.

Received 20 July 1984—Editor

MATERIALS AND METHODS

All reagents were of chemically pure grade except those used for analysis, which were of analytical grade.

Collection and storage of plant material

Most of the plant material was generously supplied by C. J. Sieberhagen from the farm Illmasdale, which is located near Derby in the Western Transvaal, in the midst of an area where severe outbreaks of "gousiekte" occur periodically. Collections of the plant material were made over 14 seasons during 1967–1980. The plants were destemmed, washed with water, distributed into small plastic bags and stored frozen at −10 °C.

Plant juice

Plant juice was prepared by macerating fresh leaves in a blender and pressing out the juice in a Carver press (F. S. Carver Inc. Hydraulic Equipment, N.Y.).

Pach F

Frozen leaves were freeze-dried in 1 kg batches in bags of cheesecloth and ground to a fine powder in a Siebtechnik disc mill (Ferguson Industrial Equipment Co., Johannesburg). This material was denoted *Pach F*.

Fermentation extracts

Magnetically stirred suspensions of *Pach F* (50 g) in water (500 mℓ) were fermented with 1 g dry baker's yeast (N.C.P., Welkom) and sucrose (2 g) at room temperature in stoppered conical flasks of 1 ℓ capacity. The stoppers were provided with carbon dioxide gas outlets. The effectivity of the fermentation was monitored by measuring the volume of carbon dioxide produced and collected over 1 % HCl. After 24 hours, the suspension was centrifuged (1 000 × g, 15 min) and the residue washed twice with water (250 mℓ) before it was discarded. The combined supernatants were then lyophilized. Occasionally, fresh frozen leaves, macerated in a blender with water, were fermented instead of *Pach F*.

Water extracts

Water extracts of *Pach F* were made by reconstitution with water, pressing out in the Carver press, resuspending the residue to 8 % (m/v) in water followed by sonication for 10 minutes in the cold, centrifugation (1 000 × g, 20 min), and a final pressing out of the sediment. The combined, lyophilized, aqueous phases constituted the water extract.

Macromolecular fraction

Plant juice was saturated to 90 % with ammonium sulphate and left overnight in the cold. The precipitate was centrifuged (1 000 × g, 20 min), dialyzed against water and lyophilized. Gel chromatography of this macromolecular fraction was performed at 7 °C in columns of 2,8 × 40 cm dimensions, using a variety of Sephadex and Sepharose gels (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was monitored at 280 nm.

Heat lability

Direct heat lability tests on the plant material were performed by autoclaving fresh, frozen leaves for 15 minutes. The leaves were then macerated in a blender in water to give a 10 % suspension on a dry mass basis. The suspension was then fermented. A control experiment was done likewise, but omitting the autoclaving step. Alternatively, fermentation extract was heat treated by suspending a lethal dose in a minimum amount of water and keeping it in a boiling water-bath for 20 minutes prior to cooling and dosing.

Heat lability tests on aqueous extracts of *Pach F* were performed by boiling the aqueous extract for 20 minutes followed by reconstitution and fermentation with untreated, extracted residue. Heat lability of the extracted residue of *Pach F* was determined by autoclaving the wet residue prior to reconstitution and fermentation with untreated aqueous extract. *Pach F* used in these experiments were extracted with acetone beforehand to remove most of the pigments.

Toxicity determinations

Toxicity determinations were usually done by *per os* dosage to Albino Wistar guinea-pigs of both sexes. The animals were fasted for at least 6 hours prior to dosing. Aqueous suspensions of fractions were made and administered by means of a stomach tube (a shortened, nasal, pediatric feeding tube). Only the macromolecular fractions were administered subcutaneously in Ringer's solution in order to conserve material.

Potassium, sodium and acid determinations

Potassium and sodium contents were determined by flame photometry, according to Basson & Böhmer (1972), but with some modifications. Duplicate samples of 0,5 g of lyophilized fractions were ashed at 600 °C for one hour and extracted with molar HCl at 90–100 °C. The HCl extract was diluted in spectrographic buffer (1 000 μg Li/mℓ) and read in a flame photometer (Gallenkamp Model F H 500) with appropriate filters for potassium and sodium.

Acid contents of extracts were determined by titration with dilute alkali to pH 7,0.

RESULTS AND DISCUSSION

The LD_{50} of fresh *Pachystigma pygmaeum* plant material was found to be in the order of 100 g/kg, the MLD being about 50 g/kg and the LD_{100} about 200 g/kg (T. W. Naude, Veterinary Research Institute, Onderstepoort, personal communication, 1968). With such a relatively high lethal dose and long latent period in ruminants, the determination of the toxicity and the comparison of toxicities and toxicity yields of plant material, extracts and fractions presented serious problems. For practical reasons the experiments could not be done in ruminants, and guinea-pigs had to be resorted to. Even so, concentrated extracts had to be dosed at such high dosage rates, that problems were experienced with factors such as potassium toxicity and metabolic acidosis due to organic acids.

Toxicity of fresh Pachystigma pygmaeum, *plant juice and aqueous extracts of* Pach F

Dosing of suspensions of fresh, macerated leaves of *Pachystigma pygmaeum* to guinea-pigs at up to 220 g/kg did not evoke any symptoms (Cronje, 1976). Plant juice and aqueous extracts of *Pach F* were dosed up to 24 g/kg without any effect other than temporary accelerated breathing, probably due to metabolic acidosis (Verschoor, 1980). At these dose levels, these preparations therefore did not have any permanent adverse effect from either plant toxicity or acidosis. Aqueous extracts from *Pach F* were only found to be lethal at 35 g/kg (Table 2), a dose level at which potassium toxicity and metabolic acidoses due to organic acids can be expected to play the major role in causing mortality.

A toxic, macromolecular fraction from plant juice

Dosing of preparations of a macromolecular fraction (see MATERIALS AND METHODS) evoked a delayed response after 3–4 days, with the animal appearing healthy up until death. This was achieved with either *per os* dosage (10 g/kg) or subcutaneous injection (0,5–0,7 g/kg). This fraction exhibited association-dissociation properties on Sepharose gels as shown in Table 1. The exclusion peak obtained from Sepharose 4B separated into 2 peaks on Sepharose 2B, the 2nd peak of which again appeared as 2 peaks when rechromatographed on Sepharose 4B (Fig. 1). The significant 12–15 times enrichment of toxicity that was achieved this way rendered

TABLE 1 Fractionation of a macromolecular toxic fraction by gel chromatography

Sample	Gel	Exclusion peak		Second peak		Number of peaks
		Toxicity yield[a] (%)	Enrichment[b]	Toxicity yield (%)	Enrichment	
90 % $(NH_4)_2SO_4$ precipitate from plant juice	Sephadex G50	64	3	36	1,3	3
Exclusion peak from Sephadex G 50	Sephadex G 75	85	6	15	0,9	2
Exclusion peak from Sephadex G 50	Sepharose 4B	85	9	15	0,9	2
Exclusion peak from Sepharose 4B	Sepharose 2B	58	15	42	12	2
Second peak from Sepharose 2B	Sepharose 4B	50	12	50	12	2

[a] Toxicity yields were calculated, taking the toxicity of the material put on any column as 100 %

[b] Enrichment of toxicity was calculated relative to the toxicity of the 90 % $(NH_4)_2SO_4$ precipitate from plant juice

TABLE 2 Comparative analytical data of a water extract and fermentation extract from *Pach F* (1.4.1976)

Extract	Acute toxicity (g/kg)	Yield		Potassium (%)	Acid (m.eq/g)
		Mass (g/100 g *Pach F*)	Toxicity (kg LD)		
Water extract	35	35,5	1,06	3,41	0,708
Fermentation extract	16	37	2,22	4,00	1,053

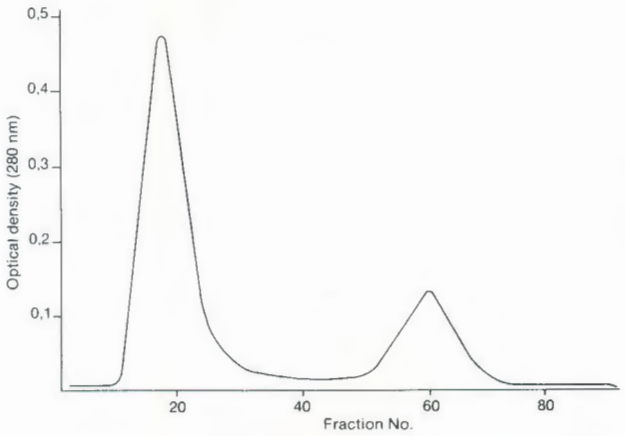

FIG. 1 Chromatogram of the excluded macromolecular toxic fraction from Sepharose 4B on Sepharose 2B

this macromolecular fraction the ideal material for use in further isolation work. Unfortunately, however, the yields never exceeded 2 % as calculated from the original LD_{100} (200 g/kg) to sheep of the plant material. A more effective way of extracting toxicity from the plant material was therefore sought.

Fermentation extracts of Pach F

A simple method for the liberation of toxicity from the plant material was obtained by fermentation of *Pach F* with baker's yeast and sucrose (Cronje, 1976).

At 16 g/kg, the fermentation extracts were twice as toxic as aqueous extracts from *Pach F* (Table 2) and apparently gave quantitative recovery of toxicity. Contrary to the delayed response of 3–4 days evoked in guinea-pigs with the macromolecular fraction, lethal doses of fermentation extracts invariably caused acute death within 6 hours. Moreover, the toxic entity was now dialysable, i.e. of low molecular mass (< 6000 dalton). The potassium and acid content of a lethal dose of fermentation extract was much lower than that of an aqueous extract, and fermentation extracts can therefore be expected to contain a considerable amount of toxicity other than causing metabolic acidosis.

Heat lability

Indications that the toxic entity might possibly be heat labile were first observed by T. W. Naudé (unpublished results, 1968), as drying of the plant material in the shade caused a 50 % decrease in toxicity when administered to sheep. We investigated this further by doing heat lability tests directly on the plant material or on the fermentation extracts. The results of a representative experiment appear in Table 3, and indicate that at least 33 % of the total toxicity of fermentation extracts could thus be inactivated. This would suggest that at least 33 % of the toxicity of the fermentation extract was not due to metabolic acidosis. It is noteworthy that only after fermentation could the heat labile toxic entity be observed in the aqueous phase (see Table 4).

This raised the question as to whether fermentation had its effect on the liberation of toxicity from the plant residue or caused an activation of latent toxicity in the aqueous extract. Exhaustive aqueous extraction of *Pach F*, followed by fermentation of the plant residue with sucrose, did not result in an enriched toxic extract, and this led us to suspect that the toxicity resided in the aqueous extract.

Fermentation experiments were then done on reconstituted mixtures of heated/unheated aqueous extracts and residues of *Pach F*. A typical result is shown in Table 4. It appeared that the heat labile toxicity remained associated with the residue after aqueous extraction.

The similar volumes of CO_2 produced during fermentation eliminate the possibility that a decrease in toxicity resulted from impaired fermentation caused by heat treatment of either the aqueous extract or the residue. The MLD determined for fermentation extracts from reconstituted aqueous extract and autoclaved residue (see Table 5) suggests that up to 50 % of the toxicity of fermentation extracts could be destroyed by heat treatment of the residue.

TABLE 3 Heat lability of the entity of fermentation extracts of *Pachystigma pygmaeum* (23.4.1979) toxic to guinea-pigs

Fraction	Acute toxicity (g/kg)
Fermentation extract	20
Autoclaved fermentation extract	30
Fermentation extract of autoclaved plant material	30 Not lethal

TABLE 4 Effect of heat treatment on the toxicity of the aqueous extract and plant residue of acetone-treated *Pach F* (16.3.1978)

Reconstitution mixture	Amount of aqueous extract (g)	Amount of dry residue (g)	Volume CO_2 produced during fermentation (mℓ)	Yield of dry fermentation extract (g)	Acute toxicity (g/kg)
Control: Untreated aqueous extract and residue	9,5	32	905	8,86	16
1. Boiled aqueous extract and untreated residue	9,5	32	935	8,81	16
2. Autoclaved residue and untreated water extract	9,5	32	1 030	10,13	16-Not toxic

TABLE 5 Minimum acute lethal dose of fermentation extracts from reconstituted aqueous extracts and heat treated residue of *Pach F* (30.3.1978)

Reconstitution mixture	Amount of aqueous extract (g)	Volume CO_2 produced during fermentation (mℓ)	Yield of dry fermentation extract (g)	MLD (g/kg)
Control: Untreated aqueous extract and residue	11,6	1 050	8,53	14
Autoclaved residue and untreated water extract	11,6	1 083	9,59	28

These results suggest that the entity toxic to guinea-pigs in *P. pygmaeum* is part of a complex in the plant material from which it can be liberated by fermentation. Should this entity also be the causative agent for "gousiekte" in ruminants, one can postulate that the microbial digestion in the rumen is required for the liberation of toxicity. This might be the reason for the fact that animals other than ruminants are in practice not susceptible to poisoning.

ACKNOWLEDGEMENTS

We are grateful to the Department of Agriculture for their financial support.

REFERENCES

ANDERSON, L. A. P. & DE KOCK, W. T., 1959. The occurrence of ursolic acid in several plants reported to be responsible for "gousiekte" in South Africa. *Journal of the South African Chemical Institute*, 12, 114–115.

BASSON, W. D. & BÖHMER, R. G., 1972. An investigation into more rapid procedures for preparing leaf tissue samples for analysis. *Analyst*, 97, 482–489.

CODD, L. E. & VOORENDYK, S., 1966. Plants which cause "gousiekte" poisoning. *Bothalia*, 8 (Supplement 1), 47–58.

CRONJE, I. J., 1976. Sommige eienskappe van die toksiese komponent van *Pychystigma pygmaeum* (Schltr.) Robyns. MSc thesis, University of Pretoria.

HURTER, L. R., NAUDÉ, T. W., ADELAAR, T. F., SMIT, J. D. & CODD, L. J., 1972. Ingestion of the plant *Fadogia monticola* Robyns as an additional cause of gousiekte in ruminants. *Onderstepoort Journal of Veterinary Research*, 39, 71–82.

POTGIETER, D. J. J., JORDAAN, E., CRONJE, I. & MEIJ, H. S., 1975. Some properties of a plant constituent responsible for heart failure in animals. *Proceedings of the 1st Congress of the South African Biochemical Society*, 74–75.

PRETORIUS, P. J. & TERBLANCHE, M., 1967. A preliminary study on the symptomatology and cardiodynamics of gousiekte in sheep and goats. *Journal of the South African Veterinary Medical Association*, 38, 29–53.

PRETORIUS, P. J., TERBLANCHE, M., VAN DER WALT, J. D. & VAN RYSSEN, J. C. J., 1973. Cardiac failure in ruminants caused by gousiekte. In: BAJUSZ, E. & RONA, G., with BRINK, A. J. & LOCHNER, A. (eds). Cardiomyopathies, 2, 385–397. Baltimore: University Park Press.

SMIT, J. D., 1959. Die histopatologiese diagnose van gousiekte. *Journal of the South African Veterinary Medical Association*, 30, 447.

THEILER, A., DU TOIT, P. J. & MITCHELL, D. T., 1923. Gousiekte in sheep. In: *9th and 10th Reports of the Director of Veterinary Education and Research, Onderstepoort*. 1–106, Pretoria: The Government Printing and Stationery Office.

VELDSMAN, D. P., 1952. Chemical investigation of *Pachystigma pygmaeum* Schlechter (Gousiektebossie). *Onderstepoort Journal of Veterinary Research*, 25, 113–120.

VERSCHOOR, J. A., 1980. Eienskappe van die toksiese entiteit(e) verantwoordelik vir gousiekte. MSc(Agric) thesis, University of Pretoria.

WALKER, J., 1908/9. Gauw ziekte: A disease of sheep. *Reports of the Government Veterinary Bacteriologist of the Transvaal Department of Agriculture*, 74, 74–99.

Printed by the Government Printer, Private Bag X85, Pretoria, 0001