PHOTOSENSITIVITY IN SOUTH AFRICA. VI. THE EXPERIMENTAL INDUCTION OF GEELDIKKOP IN SHEEP WITH CRUDE STEROIDAL SAPONINS FROM *TRIBULUS TERRESTRIS*

T. S. KELLERMAN[1], G. L. ERASMUS[1], J. A. W. COETZER[2], J. M. M. BROWN[3] and B. P. MAARTENS[1]

ABSTRACT

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Geeldikkop was induced in sheep by the oral administration of crude steroidal saponins from *Tribulus terrestris*. Two of the sheep developed typical lesions of geeldikkop, including birefringent crystalloid material in bile ducts and concentric periductal lamellar fibrosis. The clinical pathological changes in these sheep were also consistent with those of geeldikkop: aspartate transaminase and gamma-glutamyl transferase activities in the sera of both were elevated, and one had bilirubinaemia. A third sheep became photosensitive without typical lesions of geeldikkop in the liver or changes in the activities of liver enzymes before euthanasia. The findings of these trials are consistent with reports from abroad that ovine hepatogenous photosensitization, caused by *Agave lechuguilla* and *Narthecium ossifragum*, can be induced with crude saponins from the respective plants.

INTRODUCTION

The potential importance of the saponins of *Tribulus terrestris* was first recognized by Henrici (1952), who suggested that they, acting together with other factors, might be involved in the aetiology of geeldikkop. Enslin & Wells (1956) subsequently isolated crude saponins in yields of 0,5–2,0 % of dry mass of the plant. Two years later, De Kock & Enslin (1958) characterized 4 sapogenins from crude extracts of *T. terrestris,* namely, diosgenin, ruscogenin, gitogenin and 25D-spirosta-3:5-diene. Since 20 g of crude *T. terrestris* saponins failed to induce geeldikkop in a sheep, interest in these glycosides waned. Brown (1968) concluded that, although saponins and their aglycones where shown to be hepatotoxic, nephrotoxic, haemolytic (Brown, 1959a, 1959b, 1963) and capable of paralysing smooth muscle (Enslin & Wells, 1956), none of the characteristic features of geeldikkop had been produced by administering these compounds to animals. Saponins were consequently relegated to a position of minor importance in geeldikkop research (Brown, 1962, 1963, 1964, 1966a, 1966b; Brown & De Boom, 1966; Brown & De Kock, 1959; Brown & De Wet, 1962; Brown, 1968).

This study, in which the role of saponins in the aetiology of geeldikkop is re-evaluated, was prompted by reports from abroad that 2 ovine photosensitizations, namely, *Agave lechuguilla* poisoning in the United States of America (Patamalai, 1988) and alveld caused by *Narthecium ossifragum* in Scandinavia (Abdelkader, Ceh, Dishington & Hauge, 1984) could be reproduced by dosing crude saponins from the causal plants to sheep.

MATERIALS AND METHODS

Plant material: Succulent, well grown-out, flowering and fruiting *T. terrestris* was collected during December 1989–January 1990 in the vicinity of the Veterinary Research Institute, Onderstepoort (VRI), and at Northam in the Northern Transvaal, both localities being well outside the endemic geeldikkop area (Fig. 1). Extracts of the plants were prepared within hours of collection or after refrigeration at -10 °C for 1–6 weeks.

FIG. 1 *Tribulus terrestris* from which crude steroidal saponins were extracted

Extraction of crude saponins: This was done according to the method described by Wall, Krider, Rothman & Eddy (1952). Macerated *T. terrestris* was extracted once with 70 %, and twice with 50 % boiling aqueous ethanol on a steam bath. The ethanolic extract was evaporated to approximately ⅛ of the original volume, 50 g/ℓ of sodium chloride was added, and the pH adjusted to 4,0–4,5 with concentrated hydrochloric acid. The resultant solution was extracted 4 times with butanol. The butanolic extracts were combined and evaporated to dryness.

Preparation of sapogenins: Crude saponins, dissolved in 25 % aqueous ethanol, were defatted by gentle shaking with benzene. Concentrated hydrochloric acid was added to bring the aqueous ethanolic solution to 4 moles HC1/ℓ (concentrated HC1: solution=2:3). The solution was refluxed for 6–8 h, allowed to cool to room temperature and filtered through glass wool. The tarry precipitate so obtained was suspended in a mixture of 3 ℓ benzene, 1 ℓ methanol and 200 g sodium hydroxide, and refluxed for about 1 h. After cooling, the mixture was filtered through Whatman No. 1 paper. The residue was washed with a little hot benzene to which 10 % etha-

[1] Veterinary Research Institute, Onderstepoort 0110, Republic of South Africa

[2] Department of Infectious Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria 0002, Republic of South Africa

[3] Department of Physiology, Medunsa 0204, Republic of South Africa

nol had been added. The filtrate plus washings was shaken up in water and the water phase was drawn off. The benzene phase, which contained the crude sapogenins, was evaporated to dryness (Wall *et al.*, 1952).

Thin-layer chromatography

Sapogenins: The sapogenins obtained from hydrolysis of the crude saponins from *T. terrestris* were chromatographed together with standards of diosgenin, tigogenin, hecogenin, ruscogenin and gitogenin on aluminium sheets coated with silica gel 60 F_{254}[1]. A mobile phase, consisting of ethyl acetate:hexane (30:70), gave the best results. The spots were visualized by spraying the plates with 25 % antimony trichloride in chloroform, followed by heating for 5 min at 110 °C.

Crystalloid material: The crystalloid material, obtained by centrifuging 18 mℓ of bile from a sheep dosed with the crude saponins, was washed twice with distilled water. The resultant deposit was divided into a densely-packed lower and more loosely-packed upper layer, each of which were then processed seperately. Both were refluxed with 20 mℓ of benzene:methanol (3:1) plus a pellet of sodium hydroxide, filtered, evaporated to dryness under negative pressure at 60 °C and chromatographed with standards as described above. n-Hexane:tetrahydrofuran:ethanol (75:20:5) was the most successful of the various mobile phases tested.

Dosing trial: The crude saponins suspended in water was administered per stomach tube to 4–7-month-old Merino lambs fed on green lucerne and kept in the sun. For details of the dosing regimen refer to Results.

Chemical pathology: The activities of aspartate transaminase (AST)[2] plus γ-glutamyl transferase (GGT)[2] and the levels of total bilirubin (TBr)[3] in the serum were regularly recorded (Fig. 4).

Pathological examination: Necropsies were done on the lambs immediately after euthanasia was performed by intravenous administration of an overdose of pentobarbitone sodium. Specimens of various organs were collected in buffered 10 % formalin, processed in a routine manner, and sections were stained with haematoxylin and eosin (HE).

Scanning electron microscopy: A small quantity of crystalloid material was air-dried, mounted on a viewing stub and examined with a Hitachi S-2500.

RESULTS

Extraction of crude saponins and preparation of sapogenins: The yields of crude saponin from *T. terrestris* varied between 0,3 and 0,55 % and that of sapogenins from 0,06 to 0,08 %, on a wet basis.

Thin-layer chromatography: The principal sapogenin detected in *T. terrestris* material was diosgenin. Ruscogenin and gitogenin could also be discerned (Fig. 2). No diosgenin or other sapogenin was identified in the crystalloid material from the sheep's bile.

Dosing trial

Sheep 1, a ram of 22 kg live mass was dosed on Day 0 with ½ the crude saponin extracted from *c.* 27 kg of fresh *T. terrestris*. Dosing was then interrupted for 2 days as the lamb developed diarrhoea. It was
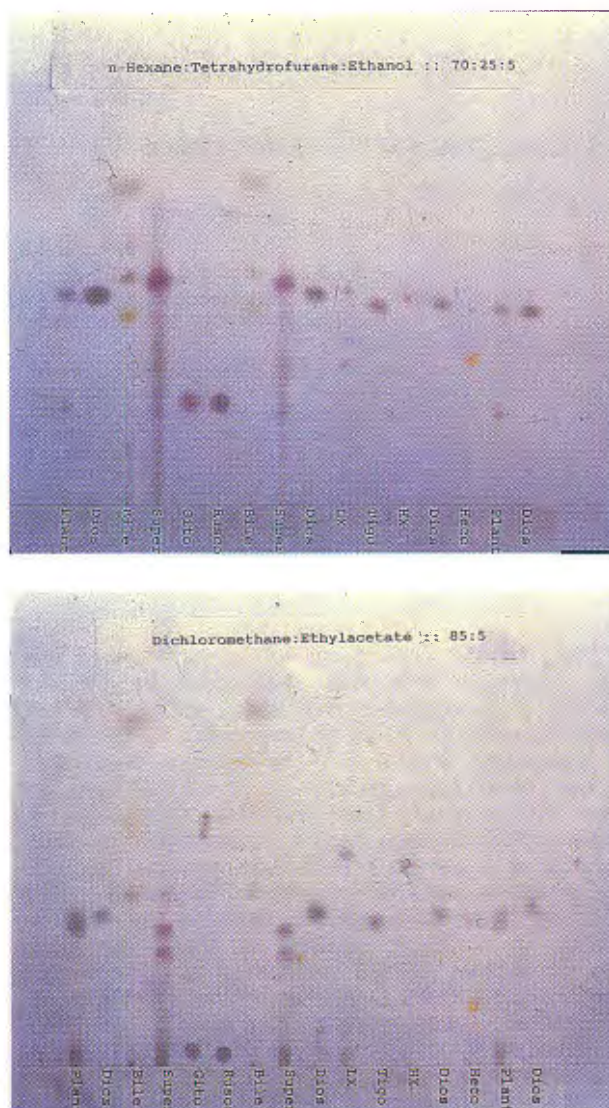
FIG. 2 Thin layer chromatographic investigation for sapogenins in *Tribulus terrestris* and the crystalloid material from bile of a sheep fed crude saponins. Dios = diosgenin standard; Plant = sapogenin fraction from *T. terrestris*; Heco = hecogenin standard; HX = densely packed lower layer of crystalloid material; Tigo=tigogenin standard; LX = losely packed upper layer of crystalloid material; Super = hydrolysate obtained from the supernatant of bile of an affected animal; Bile = hydrolysate obtained from the bile of a normal sheep on the same diet as the experimental animals; Rusco = ruscogenin standard; Gito = gitogenin standard

treated for the condition with kaolin, pectin and electrolytes. The other half of the crude saponins was administered in a divided dose of 1 part on Day 3 and 2 parts on Day 4. Late on Day 5, the lamb became photosensitive. It shook its head, sought shade, and the right ear was distinctly swollen. During the course of the next day the signs progressively diminished until by Day 7, when euthanasia was performed for necropsy, little evidence of photosensitization could be seen.

Sheep 2, a 38 kg ram, was dosed with saponins extracted from 38 kg of refrigerated *T. terrestris*. The extract was divided into 20 equal parts, which were administered as follows: 1 part on Day 0 and Day 1; 3 parts on Day 3; 4 parts on Day 4; 9 parts on Day 5. The lamb then developed severe diarrhoea, for which it had to be treated. Early on Day 7 it was photosensitive; showing signs such as severe swelling of the ears, face, lips and lower jaw, reddening of the

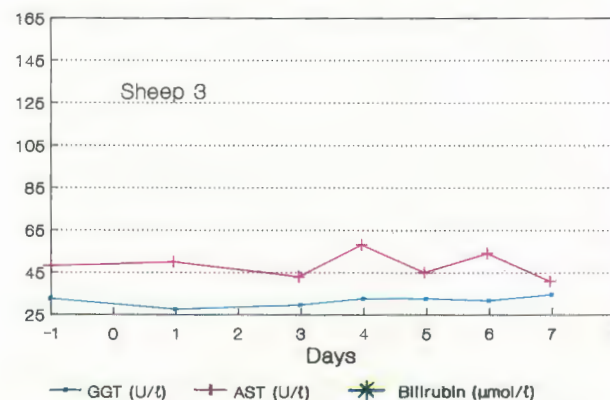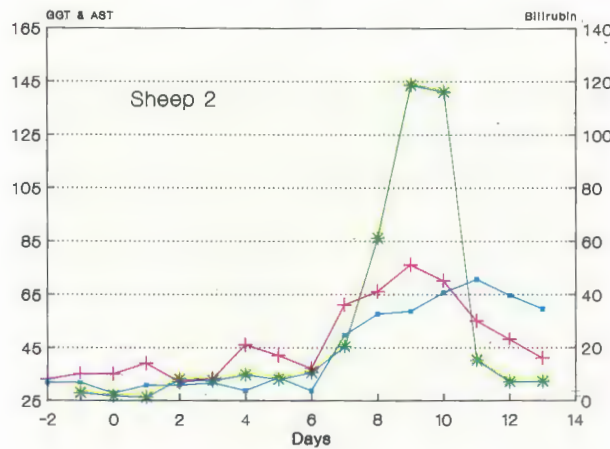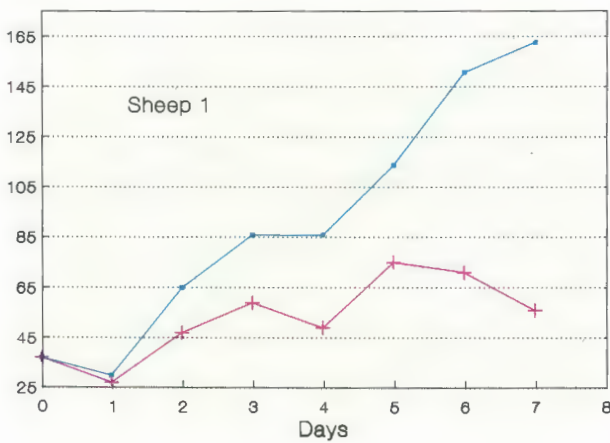FIG. 3 Sheep 2: Note swelling of ears, lips and face, and coronitis

FIG. 4 Chemical pathological changes in sheep dosed with crude steroidal saponins from *Tribulus terrestris*

base of the horns (Fig. 3), and mild icterus. It was immediately stabled. By the time that it was euthanized (Day 13), the signs had greatly diminished and icterus was no longer evident.

Sheep 3, a 25 kg ewe, was dosed on Day 0 and Day 1 with equal parts of crude saponin from 45 kg of refrigerated *T. terrestris*. This sheep, too, developed diarrhoea, for which it had to be treated. On Day 3 both ears became severely swollen, but the swelling rapidly disappeared after shade was provided. No signs of photosensitivity could be discerned at necropsy on Day 7.

Chemical pathology: The changes are summarized in Fig. 4.

Pathology: At necropsy the liver of *Sheep 1* was moderately enlarged and greyish-brown slightly sunken areas of variable size were scattered throughout the parenchyma. The lobulation in these areas were more distinct than elsewhere in the liver. In addition to mild oedema of the gall bladder wall, the loose connective tissue about the ductus cysticus and extrahepatic bile ducts was oedematous. The gall bladder contained a small amount of dark-green bile in which a fine chalky-white sediment was suspended.

Apart from slight swelling and yellowish-brown discoloration of the kidneys, no other macroscopic lesions were seen in the other organs and tissues.

Microscopically, the liver showed typical lesions of geeldikkop. The portal triads revealed moderate to severe fibroplasia, and, particularly, periductal concentric lamellar fibrosis (Fig. 5); moderate bile duct and bile ductular proliferation; infiltration of moderate numbers of lymphocytes and a few eosino-
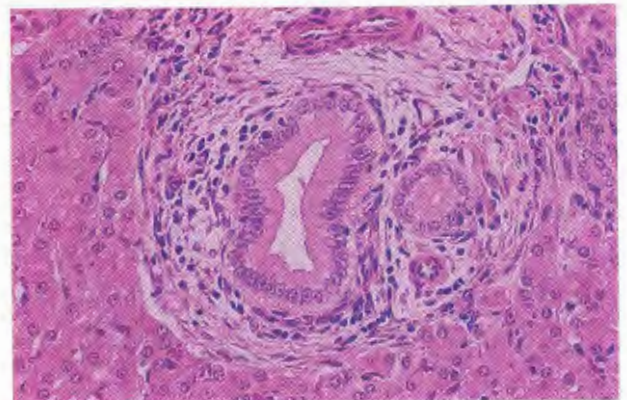

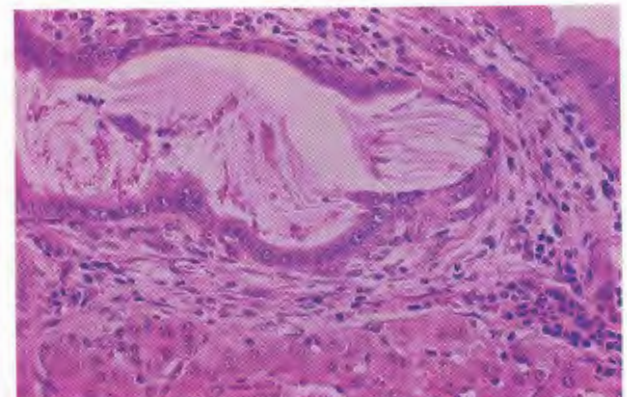
FIG. 5 Sheep 1: Marked periductal lammellar fibrosis

FIG. 6 Sheep 1: Large bile duct occluded by crystalloid material Note the small eosinophilic globules attached to the material

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phils; and crystalloid material in and around bile ducts in some triads. These sometimes occluded or distorted the bile ducts (Fig. 6 & 7). Numerous small eosinophilic globules were attached to some of the crystalloid material (Fig. 6). In the kidneys, only cloudy swelling and hydropic degeneration of the epithelial cells of the convoluted tubules in the cortex were evident.
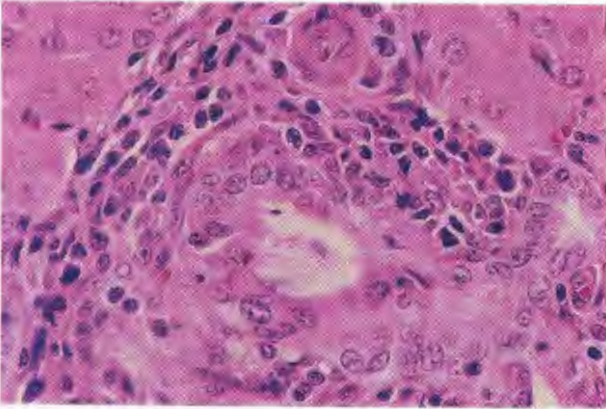

FIG. 7 Distortion of a small bile duct by crystalloid material

The liver of *Sheep 2* was moderately enlarged, friable, light-brown in colour and showed accentuation of the lobulation. The wall of the gall bladder was slightly oedematous. The kidneys were swollen and finely mottled.

Typical microscopic lesions of geeldikkop were observed in the liver. The portal triads showed moderate fibroplasia (arranged particularly in concentric lamellar layers around bile ducts), moderate
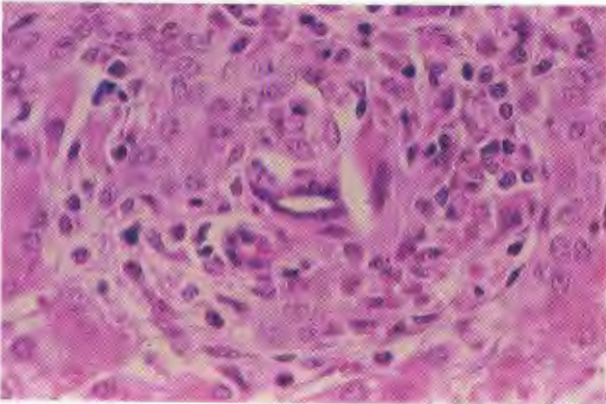

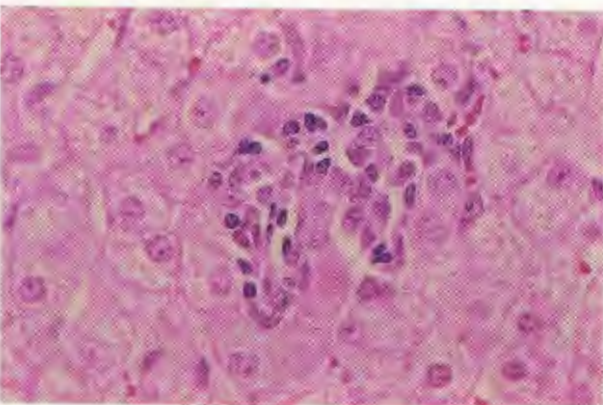FIG. 8 Crystalloid material in the lumen of a bile duct

FIG. 9 Numerous eosinophils in a portal triad

bile ductular and bile duct proliferation, crystalloid material in and around bile ducts, occlusion and distortion of some bile ducts by crystalloid material and infiltration of large numbers of lymphocytes and eosinophils (Fig. 8 & 9). Small eosinophilic globules adhered to the crystalloid material in some bile ducts. Cloudy swelling and hydropic degeneration of the epithelial cells of the convoluted tubules in the cortex of the kidneys were evident.

In *Sheep 3* no noteworthy lesions were seen at necropsy. The microscopic liver lesions comprised cloudy swelling of hepatocytes; sparsely, haphazardly, scattered foci of hepatocytic necrosis which were infiltrated by macrophages (Fig. 10); mild bile ductular proliferation (Fig. 11); and infiltration of small numbers of lymphocytes and eosinophils in some of the portal triads. No crystalloid material could be detected in the liver. The kidneys revealed no noteworthy lesions.
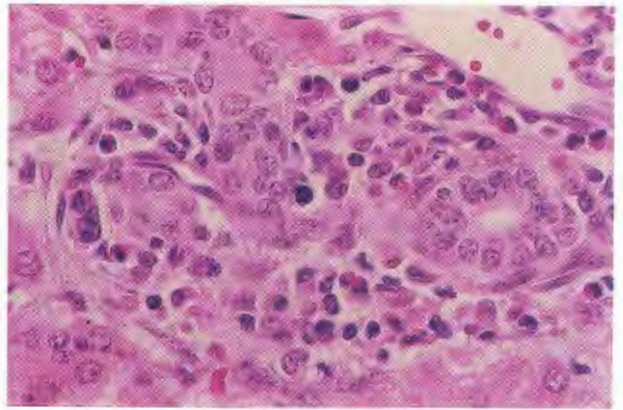

FIG. 10 Focus of hepatocellular necrosis infiltrated by macrophages
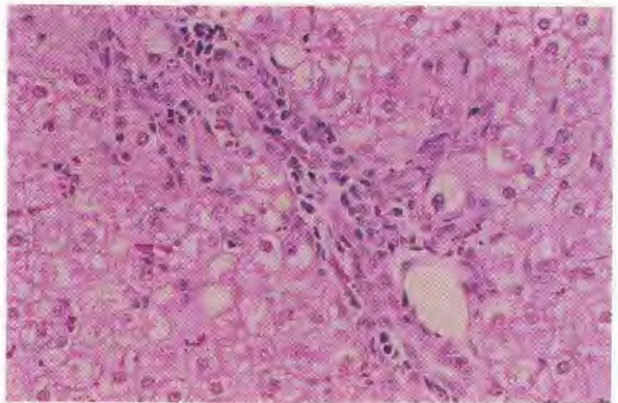
FIG. 11 Mild bile ductular proliferation and mononuclear cell infiltration

Scanning electron microscopy: Examination of the electron micrographs revealed that the crystalloid material was made up of aggregates of plate-like structures, as described for geeldikkop (Coetzer, Kellerman, Sadler & Bath, 1983) (Fig. 12).

DISCUSSION

Ovine hepatogenous photosensitivity is of great economic importance in South Africa. The various photosensitizations may be loosely divided into 2 groups, depending on whether the parenchyma or biliary system is primarily affected. In both instances the liver damage is of a type which results in the

FIG. 12 Crystalloid material composed of plate-like structures

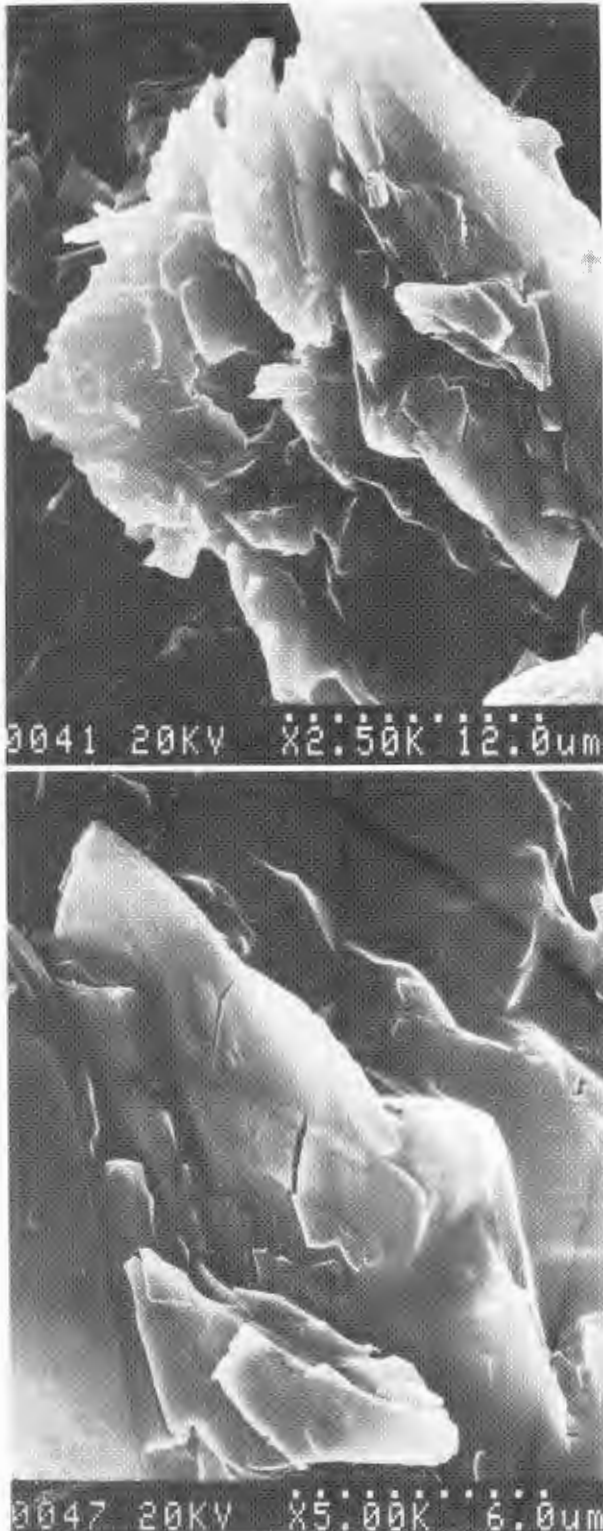retention of phylloerythrin, a photodynamic porphyrin derived from the degradation of chlorophyll by micro-organisms in the rumen (Kellerman & Coetzer, 1985). In South Africa, the most notable photosensitizations involving the biliary system are geeldikkop, *Panicum* photosensitivity (dikoor) and facial exzema. Of these, geeldikkop is the most important: more than half a million sheep may be affected in a single outbreak. Hence, it can rightly be described as the last of the great endemic diseases of South Africa, the aetiology of which has not been completely elucidated.

The remarkable similarity between geeldikkop and dikoor has been well documented (Quin, 1928, Steyn, 1928, Kellerman & Coetzer, 1985). Geeldikkop is a seasonal photosensitivity disease of sheep and goats grazing on *T. terrestris* in the Karoo, a semi-arid area covering about a third of South Africa. The plant is a nutritious semi-annual prostrate herb which sporadically becomes toxic under certain conditions, e.g. when young plants become wilted during hot dry spells following summer rains. (Theiler, 1918; Quin, 1928; Kellerman & Coetzer, 1985). Dikoor, on the other hand, occurs sporadically in sheep grazing *Panicum* spp. on disturbed soil in the Orange Free State, Transvaal Highveld and Natal. Like *T. terrestris,* the *Panicum* grasses sometimes become toxic under certain conditions, for instance, when wilted (Steyn, 1928). The clinical signs and lesions of dikoor are indistinguishable from those of geeldikkop. The only difference between the two diseases apparently is that one occurs on *Panicum* and the other on *T. terrestris* grazing (Kellerman & Coetzer, 1985).

In both these diseases, phylloerythin is believed to be retained as a result of the occlusion of bile ducts by birefrigent crystalloid material (Kellerman, Van der Westhuizen, Coetzer, Roux, Marasas, Minné, Bath & Basson, 1980). The factor(s) responsible for the formation of these occlusive microliths are contained by both *T. terrestris* and *Panicum* spp. Since the crystalloid-inducing factor(s) is present in 2 such disparate plants as a dicotyledon and a monocotyledon, it can reasonably be expected to occur in many other species as well. The presence of these factors, however, would not come to light unless the plants are eaten by sheep.

A number of plants abroad, indeed, contain such factor(s). Besides *T. terrestris* in Australia (Glastonbury & Boal, 1985; Jacob & Peet, 1987), crystalloid material in the liver has been associated with ovine photosensitization caused by poisonings with *Nolina texana* (Liliaceae) (Mathews, 1938) *Agave lechuguilla* (Liliaceae) (Mathews, 1940), and *Panicum coloratum* (Poaceae) (Bridges, Camp, Livingstone & Bailey, 1987) in the United States of America; possibly *P. schinzii* in Australia (Button, Paynter, Shiel, Colson, Paterson & Lyford, 1987); *P. meliaceum* in New Zealand (C. S. W. Reid, Applied Biochemistry Division, DSIR, Palmerston North, New Zealand, personal communication, 1973); and *Narthecium ossifragum* (Liliaceae) (Abdelkader *et al.*, 1984) in Scandinavia. The experimental reproduction of *N. ossifragum* photosensitization (Abdelkader *et al.*, 1984), *A. lechuguilla* poisoning (Patamalai, 1988) and now geeldikkop by the administration of crude saponins from the respective plants, provides strong evidence that these compounds are indeed the common crystalloid-inducing factors. This belief is strengthened by the recent extraction of the steroidal sapogenins, diosgenin and yamogenin, from *P. coloratum* by Patamalai, Hejtmancic, Bridges, Hill & Camp (1990) and the induction of ovine photosensitization with diosgenin (Patamalai, 1988).

Little information is available on the chemical nature of the crystalloid material in the liver. According to Anderson, it did not consist of common bile salts, such as cholesterol, cholic acid, sodium glycocholate or sodium taurocholate (Kellerman *et al.*, 1980). Camp Bridges, Hill, Patamalai & Wilson. (1988) demonstrated that the crystalloid material in the bile of sheep poisoned by *A. lechuguilla* was a steroidal sapogenin, tentatively identified by

thin layer chromatography as smilagenin, the chief sapogenin present in the plant (Camp et al., 1988). In the current study, sapogenins could not be demonstrated in a limited thin layer chromatographic investigation of the crystalloid material from the bile of sheep dosed with crude T. terrestris saponins. In particular, no diosgenin, the principal aglycone obtained from hydrolysis of these crude saponins, could be identified.

The ovine photosensitivity disease 'alveld' in Scandinavia, caused by the ingestion of the bog asphodel, N. ossifragum, was the first photosensitization in which saponins could be incriminated as possible causal agents (Abdelkader et al., 1984). Considerable difficulties have nevertheless been experienced in reproducing the disease experimentally with either the plant or its saponins. In an attempt to explain these problems, Aas & Ulvund (1989) postulated that outbreaks of alveld might be caused by the ingestion of mycotoxins together with saponins from the bog asphodel. Preliminary mycological observations in Norway have lent some support for the hypothesis that mycotoxins, specifically sporidesmin, may be involved in the pathogenesis of the disease (Aas & Ulvund, 1989). These observations are consistent with findings with regard to geeldikkop in South Africa.

Experimental evidence has been submitted that low levels of sporidesmin can trigger geeldikkop in sheep grazing on wilted T. terrestris in the Karoo (Kellerman et al., 1980). Although Pithomyces chartarum has been isolated many times from T. terrestris during outbreaks of geeldikkop, and although toxic cultures of these isolates, in conjunction with T. terrestris, have induced the disease, the role played by the fungus in the aetiology of natural outbreaks can only be speculated upon.

The sporadic nature of geeldikkop outbreaks has not been explained. Geeldikkop has been experimentally produced by feeding T. terrestris to sheep (Theiler, 1918; Quin, 1928; 1929; Van Tonder, Basson & Van Rensburg, 1972), but many such trials have been unsuccessful (Quin, 1933; Brown, 1959.). There is general consensus amongst farmers and veterinarians that the vast majority of outbreaks occur on young wilted T. terrestris (Quin, 1928; Van Tonder et al., 1972); not all such T. terrestris, however, is toxic (Kellerman, Coetzer & Naudé, 1988). On rare occasions, other growth stages as well might cause the disease (Quin, 1928). Theiler (1918), for instance, induced geeldikkop with green succulent T. terrestris in the late flowering stage before the seeds matured.

This ability of T. terrestris to become sporadically toxic under certain conditions (Theiler, 1918; Quin, 1928; Van Tonder et al., 1972) led to speculation that the plant sometimes produced a hepatotoxin. In the current trials, luxuriant, supposedly non-poisonous T. terrestris from a non-endemic geeldikkop area was shown to contain saponins capable of inducing geeldikkop. This finding raises the possibility that virtually all T. terrestris might contain at least some such saponins. Outbreaks of geeldikkop could thus occur when the saponin content of the plant rises to toxic levels—for instance, when it is wilted. Another explanation for the sporadicity of geeldikkop could be that the precipitation of biliary occlusive microliths is triggered by small amounts of sporidesmin in the pasture (Kellerman et al., 1980). The latter theory is in a measure supported by the findings of Scandinavian workers who suggested that P. chartarum might be involved in the aetiology of al-

veld (Aas & Ulvund, 1989). According to current thinking, therefore, T. terrestris might cause geeldikkop either alone or together with sporidesmin. More research is required to throw light on this aspect, as a clear understanding of many factors involved in the toxicity of T. terrestris (and their interactions) is necessary before rational prophylactic measures can be developed and methods for predicting outbreaks devised.

Typical lesions of geeldikkop were evident in Sheep 1 and 2 (Theiler, 1918; Van Tonder et al., 1972; Coetzer et al., 1983). Sheep 3, on the other hand, did not show typical liver lesions of geeldikkop, but minor hepatic changes similar to those described by Brown, Le Roux & Tustin (1960). These authors reported only mild changes such as bile pigmentation of parenchymal and Kupffer cells, 'necrobiosis' of isolated liver cells, some degeneration of hepatocytes and prominent bile canaliculi in natural cases of geeldikkop. In both Sheep 3 and those described by Brown et al. (1960), birefringent crystalloid material were not observed in the livers. The absence of biliary occlusive microliths in these sheep would suggest that a phylloerythrin retention mechanism other than that postulated by Kellerman et al. (1980) was in operation. It is also possible that the tissue sections of the liver examined were not truly representative of the entire liver; occlusive microliths in the larger intra- and/or extrahepatic bile ducts, especially the ductus cysticus or ductus choledocus, may have been overlooked; or that the microliths may have only transiently occluded the bile ducts before being flushed out. The absence of detectable clinical pathological changes indicating biliary occlusion seems to support this theory.

This study is the culmination of some 7 decades of research into the aetiology of geeldikkop. Since Theiler showed in 1918 that T. terrestris was responsible for the disease, a great deal of effort has been expended on the isolation of the elusive toxic principle of the plant. Identification of crude steroidal saponins as causal agent of geeldikkop must therefore rate as a significant stepping stone towards a better understanding of the disease.

ACKNOWLEDGEMENTS

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