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

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ABSTRACT


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 hair-firing 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 toxic hepatogenous photosensitizations, 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, 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 were 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, 1965a, 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 was 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 alveol 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 1980-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.

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 1/4 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 HCl/l (concentrated HCl: 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-

FIG. 1. Tribulus terrestris from which crude steroidal saponins were extracted.
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 aluminum sheets coated with silica gel 60 F$_{254}$. 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 separately. 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) and γ-glutamyl transferase (GGT), and the levels of total bilirubin (TBl) 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 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 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. Later 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
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 eodematosus. 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. 3 Sheep 2: Note swelling of ears, lips and face, and coro­nitis

![Fig. 3 Sheep 2: Note swelling of ears, lips and face, and coro­nitis](image)

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

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FIG. 5 Sheep 1: Marked periductal lamellar fibrosis

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FIG. 6 Sheep 1: Large bile duct occluded by crystallloid material

Note the small eosinophilic globules attached to the material

![Fig. 6 Sheep 1: Large bile duct occluded by crystallloid material](image)
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.

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 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.

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 photosensitzations 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
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FIG. 12 Crystalloid material composed of plate-like structures

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 birefringent crystalloid material (Kellerman, Van der Westhuizen, Coetzer, Roux, Marasas, Minne, 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. schinzzii 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 e­ta!, 1984) in Scandinavia. The experimental reproduction of N. ossifragum photosensitization (Abdel­kader e­ta!, 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 e­ta!, 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...
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thin layer chromatography as smilagenin, the chief saponin present in the plant (Camp et al., 1988).

In the current study, saponins could not be demonstrated in a limited thin layer chromatographic investigation of the crystallloid 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 is caused by the ingestion of the bog asphodel, N. ossifragum, was the first photosensitization in which saponins could be implicated 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 Phyomyces chartarum, a fungal isolate obtained from 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, Bason & 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 that posted 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 partially 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.

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