PRELIMINARY STUDIES ON THE CHEMICAL PROPERTIES OF THE TOXIC PRINCIPLE FROM *DIAMPHIDIA NIGROORNATA* LARVAE, A SOURCE OF BUSHMAN ARROW POISON

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Summary

The Bushmen of southern Africa use the expressed contents of beetle larvae (*Diamphidia*, *Lebistina* and *Polyclada* species) as arrow poison. An aqueous extract of *Diamphidia nigroornata* larvae was fractionated by gel filtration on Sephadex G-50. Two fractions were obtained: one (I) of high molecular weight which contains a protein of 60 000 daltons, and a low molecular weight fraction (II) of non-protein nature. Both fractions proved to be lethal to mice: an LD_{50} of 0.5 - 0.95 (I) and 3.2 - 3.5 (II) mg/kg (intraperitoneal injection), respectively, was determined. The toxic principle of fraction I could be partly separated from the protein by ammonium sulfate precipitation followed by gel filtration. That of fraction II was further resolved into several subfractions by gel filtration on Sephadex G-10; however, the lethal activity was completely lost during purification. In thin-layer chromatography the low molecular weight toxin(s) did not react with reagents for steroids, alkaloids, sugars or terpenes, but showed a positive ninhydrin reaction. It is concluded that the toxic principle of the Bushman arrow poison is a highly labile, low molecular weight compound which is closely attached or bound to a protein protecting it from inactivation.

Introduction

In the southern part of Africa Bushmen inhabiting the remote areas of the central and northern Kalahari desert make use of a very powerful arrow poison. Livingstone (1865) observed during his expedition to the Zambesi that the Bushmen apply the content of what he erroneously thought to be a caterpillar called *Nga* to the tips of their arrows. However, Baines (1864) correctly identified these "caterpillars" as larvae of a beetle named *Kaa* in the aboriginal language. Similar observations had been made by Schinz

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(1884 - 1887); later on, the beetle species were described by Schinz *et al.* (1894) and Lewin (1894) as *Diamphidia simplex* (presently considered to be a form of *D. nigroornata*) and *Diamphidia locusta*. More recently, Koch (1958) pointed out that the larvae of three phytophagous Chrysomelidae (*Diamphidia nigroornata*, *D. vittatipennis*, *Polyclada flexuosa*) and of three Carabidae beetle species (*Lebistina subcruciata*, *L. holubi*, *L. peringueyi*) which feed parasitically as larvae on those of *Diamphidia* species are used as source for the Bushman's arrow poison (*c.f.* Neuwinger and Scherer, 1976).

Diamphidia beetles lay their eggs on the leaves of shrubby bushes, Burseraceae: *Commiphora angolensis*, *C. africana* and *Sclerocarya caffra* (Koch, 1958). The developing larvae feed on them, drop from the branches and burrow deep in the sandy soil (40 to 100 cm deep) under the bush or the tree, where they form oval cocoons with a hard shell composed of coarse grains of sand glued together. In each cocoon only one larva is found; however, in the case of the parasitic *Lebistina* larvae mostly two are present — one of *Diamphidia* and one of the parasite beetle. The Bushmen dig out the cocoons, remove the shell and squeeze the larvae between thumb and forefinger, putting the extruded contents on the tip of the arrow or mixing them with plant extracts. After drying, the arrow poison is used for hunting game as well as for protection against enemies.

The chemistry and mode of action of the larvae toxic principle are largely unknown. Lewin (1894) and Schinz *et al.* (1894) had observed that the poison can be easily extracted by water or saline from the dry larvae. It could be precipitated by ammonium sulfate and inactivated completely by heating the extract at 80 °C, suggesting that the poison was protein-like in nature. From an alcohol precipitate of the poison, Heubner (1907) obtained by phosphoric acid treatment a protein-free extract which exhibited toxic properties similar to those of the proteinaceous crude aqueous extract. On the other hand, Haendel and Gildemeister (1912) found that the poison has immunogenic properties. An antiserum was produced in rabbits which neutralized even a 200-fold lethal dose. All authors point to the high toxicity of the arrow poison, which causes rapid death due to general paralysis or produces hemorrhage of internal organs in lower doses. Steyn (1957) observed in rabbits which were injected intravenously with an aqueous larvae extract almost immediate miosis, restlessness, accelerated pulse, convulsions, dyspnoea, paresis and paralysis, sometimes associated with pronounced hemolysis.

Recently, Delaharpe and Dowdle (1980) reported on a hemolytic and lethal protein, called diamphotoxin, isolated from larvae by ammonium sulfate fractionation and ion-exchange chromatography, having a molecular weight of 60 000 and an isoelectric point of 9.4. It caused atrioventricular block in the isolated perfused rat heart and abolished the response of the guinea-pig ileum longitudinal muscle to electrical stimulation.

The present paper describes the attempts to isolate and characterize chemically the physiologically active principle of the Bushman arrow poison. This was extracted from beetle larvae that had been collected by the !Ko

Bushmen in the area between Ghanzi and Bere in south-western Botswana (Fig. 1). Two beetles hatched from these cocoons and could be indentified as *Diamphidia nigroornata* ab. *lesnei.*

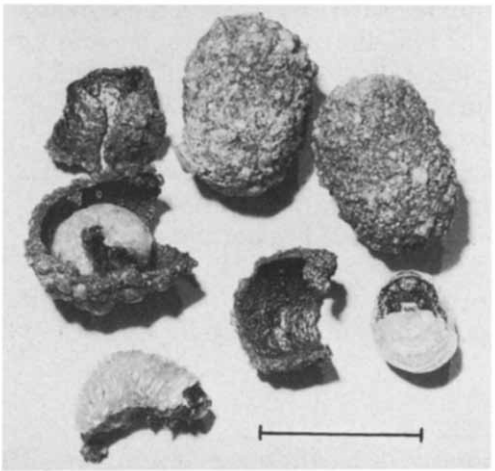

Fig. 1. Cocoons, partly opened to expose the larvae of *Diamphidia nigroornata.* The expressed contents of the larvae are used by Bushmen for the preparation of arrow poison. The scale represents 1 cm.

Methods and material

Poison extraction

The larvae were removed from the cocoons, suspended in distilled water (70 larvae in 100 ml) and extracted for 24 h at 4 °C. Extraction was repeated twice and the combined extracts were lyophilized (about 4.5 g dry material). The same procedure was performed using the earth shells of the cocoons. The extraction has been kindly performed by Dr. H. C. Krebs, Technical University, Darmstadt, F.R.G.

Fractionation

The lyophilized larvae extract was dissolved in 3 ml of distilled water, the insoluble part (about 20%) was removed by centrifugation and the supernatant applied to a Sephadex G-50 (fine) column (120 × 1.5 cm) which was eluted with distilled water. Fractions of 6 ml were collected at a flow rate of 30 ml per h and monitored via their absorbance at 254 nm. The combined active fractions (*i.e.* those lethal to mice) were lyophilized and the low molecular weight constituents further fractionated by gel filtration on a Sephadex G-10 column (83 × 1.5 cm). The high molecular weight fraction was applied to a Sephacryl S-200 column (135 × 2 cm) which had been equilibrated with 0.05 M Tris–HCl buffer (pH 6.8). Elution was carried out using the same buffer. All fractions were lyophilized.

Thin-layer chromatography

Analysis of the purified material was performed by thin-layer chromatography on silica gel plates (HPTLC plates, 10 × 10 cm, silica gel 60 F_{254}, Merck, Darmstadt, F.R.G.) using the following solvent system: methanol–ammonia (99:1, v/v), ethyl acetate–pyridine–water (50:10:40), methylene chloride–methanol–water (87:12:1). For two-dimensional chromatography the solvent systems methanol–ammonia (99:1, first direction) and n-butanol–acetic acid–water (80:20:20, second direction) were used. The following reagents were used. (1) Detection of glycosides, sugars, steroids, terpenes: anisaldehyde–sulfuric acid (to a fresh solution of 0.5 ml of anisaldehyde in 50 ml of acetic acid, 1.0 ml of concentrated sulfuric acid was added; after spraying the plates were heated at 100–105 °C). (2) For cardiac-glycoside aglycones: Kedde reagent. (3) For the detection of alkaloids: Dragendorff reagent and potassium iodide–hexachloroplatinate. (4) For the detection of amino acids and primary amines: ninhydrin reagent. All reagents as given in Stahl (1967).

Sodium dodecylsulfate (SDS) electrophoresis

For polyacrylamide gel electrophoresis, in the presence of sodium dodecylsulfate, of the protein components of the poison, the method of Weber and Osborn (1969) was applied. Samples were reduced by adding 2-mercaptoethanol (5% v/v final concentration), and incubated for 2 h at 60 °C. Electrophoresis was performed at room temperature at a current of 7 - 10 mA per tube using 7.5% polyacrylamide gel. Bromophenol blue was applied as the tracking dye; the gels were stained with Coomassie brilliant blue. The molecular weight of the protein was determined from the relative migration on the gels compared with those of standard proteins likewise reduced with 2-mercaptoethanol.

Toxicity determination

Preliminary tests of lethal activity of the various fractions obtained during purification were made by intraperitoneal (i.p.) injection into mice. LD_{50} assay (acute toxicity, the mice died within 24 h) was performed using groups of four mice per dose.

Reagents

Sephadex G-10, G-50 (fine) and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; bovine serum albumin was from Serva, Heidelberg, F.R.G. All other chemicals were of reagent grade.

Results

Isolation of the toxic principle

Two fractions were separated by gel filtration of the crude larval extract on a Sephadex G-50 column (Fig. 2), one (I) which appeared in the

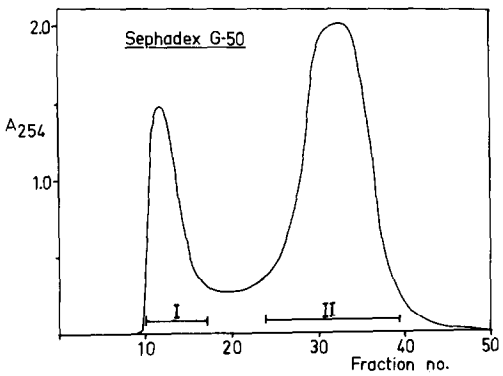

Fig. 2. Gel filtration of 400 mg of lyophilized aqueous larval extract on a Sephadex G-50 column (120 × 1.5 cm) which was eluted with distilled water; fractions of 6 ml were collected at a flow rate of 40 ml/h, combined as indicated and lyophilized.

exclusion volume of the column, thus indicating a high molecular weight, and a second fraction (II) having low molecular weight properties. Both exhibited lethal activity when tested on mice (i.p. injection). Compared to the LD_{50} of the crude extract, the toxicity of fraction I increased 36 times and that of fraction II more than 5 times (Table 1). Moreover, in both cases the yield concerning toxicity was very high, suggesting interacting mechanisms in the crude extract reducing the overall toxicity.

TABLE 1

Purification of the toxic principle from *Diamphidia nigroornata* larvae

	Total material (mg)	Toxicity LD_{50} (mg/kg)	Total toxicity*	Purification**	Yield**
Crude larval extract	400	18.0	22.2	1.0	100
Gel filtration on Sephadex G-50					
Fraction I	70	0.5	140.0	36.0	636.4
Fraction II	120	3.5	34.3	5.4	154.5

*Expressed as number of LD_{50} in the total material.
**Calculated according to toxicity.

Further fractionation of the high molecular weight part (I) on a Sephacryl S-200 column gave only poor separation (Fig. 3). The toxicity, detectable only in the first peak (I-1), was greatly reduced (LD_{50} > 10 mg/kg mouse). In experiments applying ammonium sulfate fractionation, fraction I precipitated mainly at 0.4- to 0.6-fold ammonium sulfate saturation. Whereas the precipitate was highly toxic (LD_{50} about 1.0 mg/kg mouse), the super-

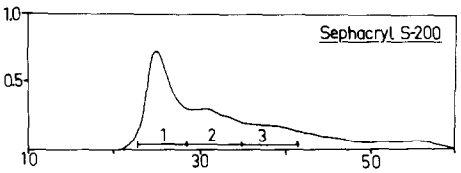

Fig. 3. Gel filtration of fraction I on Sephacryl S-200 (135 × 2 cm column) using 0.05 M Tris–HCl buffer (pH 6.8) as eluant.

natant was devoid of any lethal activity. The ammonium sulfate precipitate was resolved by gel filtration on Sephadex G-50 into two fractions showing essentially the same elution pattern as the crude extract. Both fractions were very much less toxic than the initial material (LD_{50} > 10 mg/kg). However, this separation indicates that the low molecular weight material possessing lethal activity had still been separated from the protein fraction.

More than seven fractions were obtained by gel filtration on a Sephadex G-10 column of the low molecular weight part (II) of the extract (Fig. 4).

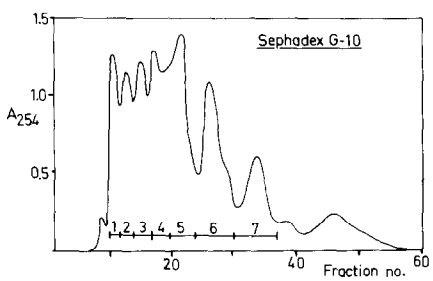

Fig. 4. Gel filtration of fraction II on a Sephadex G-10 column (83 × 1.5 cm).

The separation was accompanied by a considerable loss of lethal activity (LD_{50} of each of the first five fractions > 15 mg/kg); in most cases the toxicity was only detectable when undiluted fractions were injected intraperitoneally into mice. The dramatic loss of toxicity could not be prevented by chromatography at 4 °C or when buffer systems (0.05 M Tris–HCl buffer, pH 7.5) were used for elution instead of distilled water. Lyophilization and repeated gel filtration of the combined fractions 1 and 2 and 4 and 5 (Fig. 5) destroyed nearly all the lethal activity, even though the

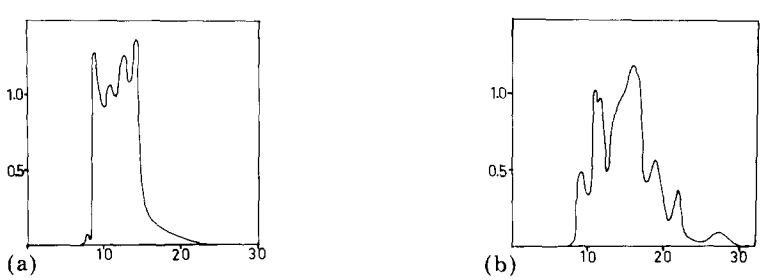

Fig. 5. Repeated gel filtration on Sephadex G-10 of the combined fractions 1 and 2 (a) and 4 and 5 (b) as shown in Fig. 4.

fractions were further resolved into various subfractions. As a result of these procedures an essentially non-toxic, yellowish, slightly hygroscopic product was obtained.

Experiments to isolate the active principle from fraction II by extraction of the brown lyophilizate with methyl alcohol, chloroform, diethyl ether or ethyl acetate failed, because the material proved to be insoluble in the organic solvents.

Essentially the same results were obtained when the lyophilized extract from the shells of the cocoons was fractionated by gel filtration (Table 2).

TABLE 2

Toxicity values of larval extracts and of the isolated fractions

	LD_{50} (mg/kg, i.p.)
Larval homogenate	4.5
Larval homogenate after aqueous extraction	> 200
Aqueous larval extract	18.0
Fraction I	0.5 - 0.95
Fraction II	3.2 - 3.5
Aqueous cocoon extract	40
Fraction I	5.3
Fraction II	9.1

Toxicity tests

Table 2 summarizes the toxicity data of the various extracts and fractions. The results confirm that the toxic principle is easily extracted by suspending the larvae in water, since larval homogenate that had been extracted by this procedure was much less toxic than the original material. It is also interesting to note that even the shells of the cocoons contain a considerable amount of toxic components which are similarly separated into two fractions.

Mice when injected with lethal doses of extracts or of fraction I or II show essentially similar symptoms of acute poisoning: the animals become restless, running and jumping in the cage, and within 30 to 60 minutes, depending on the dose, develop symptoms of severe dyspnoea (shallow breathing, bluish nose). The mice exhibit clonic–tonic convulsions, the hind legs are soon paralysed and the animals die due to respiratory arrest with the mouth wide open. If sublethal doses are applied the symptoms are less pronounced. After a phase of restlessness the mice become calm and retreat to the edges of the cage staying there motionless for hours. Later on, they appear to be asleep and can be readily handled and placed in any position without spontaneous resistance. The animals recover or die after 12 to 48 h. Autopsy did not reveal any severe pathological changes except some inflamed or hemorrhagic areas in the intestines. However, histopathological studies (including studies on rabbits or rats) have still to be done.

By heating a solution of fraction I or II for 5 min at 100 °C, pH 7.0, the toxicity was completely lost. Whereas the solution of fraction II was still clear, that of fraction I exhibited protein flocculations.

Chemical properties of the toxic principle

Thin-layer chromatography of fraction II and of its various subfractions in the solvent system methanol–ammonia (99:1) enabled several spots to be detected using the ninhydrin reagent, which reacted rather rapidly (Fig. 6).

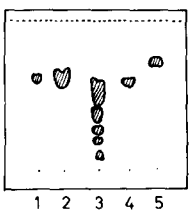

Fig. 6. Thin-layer chromatography of the fraction II, 1 - 5 (Fig. 4) on silica gel using the solvent sytem methanol–ammonia (99:1, v/v) and the ninhydrin reagent.

All othe reagents specific for alkaloids, terpenes, steroids, glycosides, and sugars were negative. In the other solvent systems (methylene chloride–methanol–water, ethyl acetate–pyridine–water) the poison components remained at the point of application. Two-dimensional chromatography of fraction II-3 did not bring about further resolution. After hydrolysis of fraction II-2 (one spot) for 24 h at 110 °C in 6 N HCl in a vacuum-sealed tube, a pattern similar to that of fraction II-3 (four to five spots) was obtained.

The ultraviolet spectrum of fraction II-1 to II-5 is rather uncharacteristic and shows little change under acidic or alkaline conditions.

After combining fraction II with an equal amount of bovine serum albumin dissolved in saline followed by gel filtration of the mixture on a Sephadex G-50 column and elution with saline (Fig. 7), two peaks were separated: the first possessed toxic properties when tested on mice, suggesting that the toxic principle has been partly bound to albumin.

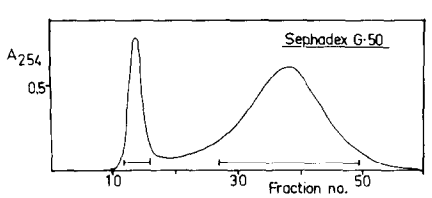

Fig. 7. Gel filtration on a Sephadex G-50 column (120 × 1.5 cm) of a mixture of 20 mg of bovine serum albumin to which had been added 20 mg of fraction II. The first peak represents albumin and bound toxin, the second peak contains unbound toxin.

Sodium dodecylsulfate electrophoresis (Fig. 8) of the reduced protein components of the high molecular weight part of the poison (fraction I) revealed that the main band has a molecular weight of about 60 000, similar to that of bovine serum albumin. On thin-layer chromatography fraction I remained at the origin in all solvent systems; it gave a positive ninhydrin reaction.

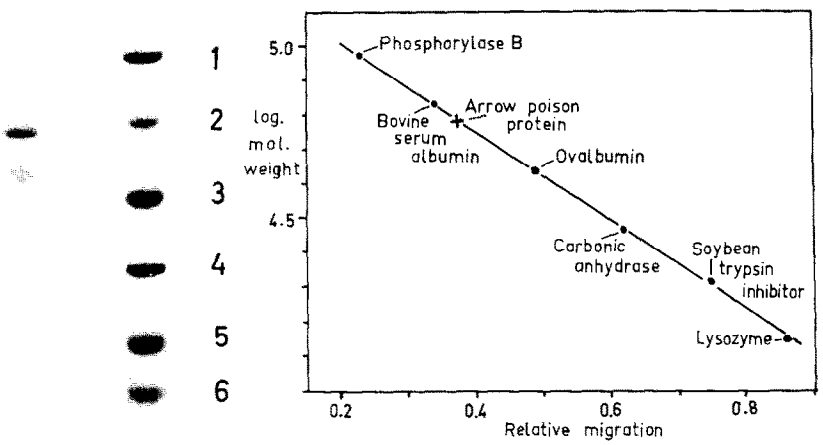

Fig. 8. Sodium dodecylsulfate electrophoresis of the reduced fraction I (left) and standard proteins (right), and plot of log molecular weights vs. the relative migration of the standard proteins: (1) phosphorylase B (94 000 mol. wt.), (2) bovine serum albumin (68 000), (3) ovalbumin (43 000), (4) carbonic anhydrase (29 000), (5) soybean trypsin inhibitor (21 000), (6) lysozyme (14 300). The main protein component of the poison has a molecular weight of about 60 000.

Discussion

The beetle larvae used for the preparation of an arrow poison by the Bushmen contain physiologically very active compounds. The present investigations indicate that toxic activity is associated with a protein of 60 000 molecular weight and confirms the results of Delaharpe and Dowdle (1980). However, the gel filtration experiments suggest that besides this protein component a toxic principle having a molecular weight below 700 is also present. This toxin is closely bound to the protein component; the binding seems to be relatively unspecific, because bovine serum albumin can to some extent serve as a substitute. The protein appears to stabilize the toxin which rapidly loses its lethal properties when separated from the protein and fractionated by several steps of gel filtration. Whether the protein, which has been named diamphotoxin by Delaharpe and Dowdle (1980), represents a true high molecular weight toxin or a component carrying a firmly bound toxin cannot be definitely answered, although there are some indications of the latter

possibility, *i.e.* separation of low molecular weight toxic activity after ammonium sulfate precipitation of the protein and similar toxic symptoms in animals produced by both fractions I and II.

The instability of the low molecular weight toxin in a pure or even semipure state made the exact characterization or identification of one of the various fractions and subfractions as the main toxic principle impossible. However, from thin-layer chromatography and the application of various group-specific reagents it seems to be permissible to conclude that the toxin does not have alkaloid, steroid, glycosidic or terpenoid properties, but reacts rapidly with the ninhydrin reagent for amino acids and primary amines. Resolution of toxin fractions into various subfractions which can be further resolved by repeated gel filtration steps may indicate breakdown accompanied by inactivation of the molecule. Whether there exist isomeric forms or closely related toxins of similar structure is still a matter of speculation. For further studies on the chemical nature of the toxin, methods to keep the molecule in a stable and active conformation are necessary prerequisites.

The present findings can explain some of the contradictory results obtained in earlier studies concerning the protein or non-protein nature of the Bushman arrow poison (Haendel and Gildemeister, 1912; Heubner, 1907; Lewin, 1894; Schinz *et al.*, 1894). Its close binding to a protein from which it can be separated only partly makes it susceptible to ammonium sulfate precipitation. Furthermore, the immunogenic properties of the poison (Haendel and Gildemeister, 1912) are due to its protein binding, enabling the production of an antiserum. On the other hand, protein-free extracts exhibiting toxic properties had also been obtained previously (Heubner, 1907).

The mode of action of the poison is still obscure. Neurotoxic symptoms like paresis and paralysis are observed during the course of poisoning, suggesting that the lethal principle has peripheral as well as central actions. Moreover, hemolysis and hemorrhage in internal organs has been found in rabbits (Haendel and Gildemeister, 1912; Steyn, 1957), and Delaharpe and Dowdle (1980) have drawn attention to the hemolytic activity of the protein component. Furthermore, the diamphotoxin they isolated produced atrioventricular block in the rat-heart preparation and abolished the response to electrical stimulation of the longitudinal muscle of the guinea-pig ileum.

The origin of the poison is not clear. It appears to be secreted also by the beetle larva to its near surroundings, since even the shell of the cocoon contains the poison in a high concentration. Chemical and toxicological analyses of the plants (*Commiphora* species) on which the larvae feed do not exist, but they are not considered to be toxic by either the Bushmen or the farmers in South Africa. The family Burseraceae is not in general noted for the occurrence of species with poisonous properties. Therefore, it is still a matter of discussion whether the poison is a plant product present, perhaps, as an inactive precursor which undergoes chemical modification or transformation by metabolic processes in the larva. Sequestration of plant natural products by insects is a well-known phenomenon (*cf.* Duffey, 1980)

where protein carriers serve in the transport of a great variety of substances by specific or non-specific binding. This seems to be likewise the case with the Bushman arrow poison which is closely associated or bound to a protein exhibiting a molecular weight of the same order of magnitude as serum albumin. However, this protein may also have a stabilizing function, protecting the toxin from inactivation.

A number of questions arise besides the problems concerning the chemical structure and mode of action of the Bushman arrow poison; namely the relationship between the plant and the larvae, between the latter and the parasitic larvae of *Lebistina* species which are considered to be even more poisonous by the Bushmen. In future investigations these particular biological implications should be taken into account when considering the complex chemical and pharmacological entity of the poison.

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