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Chemical Investigations of the "Gifblaar" *Dichapetalum Cymosum* (Hook) Engl. I.

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The Plant as a Stock Poison.

The plant *Dichapetalum cymosum* (*Chailletia cymosa*) or "Gifblaar" is one of the most well-known poisonous plants of the Transvaal. Belonging to the family Dichapetalaceae, a small tropical family of trees or woody shrubs, it is the sole representative which has developed a subterranean habit. It is an underground shrub possessing branches, many of which attain great length and from which small shoots ascend to the surface, there giving rise to the familiar tufts of green lanceolate-ovate leaves and inconspicuous flowers.

A good description of the plant is to be found in the publications of Phillips (1927) and of Mogg (1930). Leeman (personal communication) has recently distinguished several varieties. The Gifblaar has a very limited distribution in South Africa, being practically confined to the Transvaal and not occurring south of the delimitation Mafeking, Lichtenburg, Pretoria, Middelburg, nor east of Middelburg-Pietersburg, being thus confined to the lower altitudes. The problems of its distribution have been discussed by Burtt-Davy (1922).

As a stock poison, the Gifblaar appears to have been known to the early settlers, and a fairly extensive literature exists, referring to its toxic properties. A certain seasonal variation in the incidence of poisoning, prompted the studies, extending over two years, undertaken by Sir Arnold Theiler and his collaborators at this Laboratory, a report of which is to be found in the article by Steyn (1928). It was shown that periods of maximum toxicity fall approximately in the seasons September-October, and March-April, respectively, but depending to some extent upon climatic conditions, times during which the plant is producing new young leaves. The bearing of soil temperatures upon this cycle has been investigated by Mogg (1930). From a stock-owner's point of view, this variation in toxicity is of importance since it is just at these two seasons that fresh, green grazing is apt to be scarce.

It has also been observed that the onset of toxic symptoms appears to be accelerated when the animal partakes of water immediately after feeding on Gifblaar. The ready solubility of the toxic principle in water has been adduced in explanation of this phenomenon.

Previous Chemical and Toxicological Investigations.

Comparatively little work has been published upon the chemistry of the " Gifblaar ", and nothing is known concerning the nature of the toxic principle. At one time, the plant was thought to contain a cyanogenetic glucoside, following upon the report by the Imperial Institute that prussic acid had been detected in the leaves, but this was not substantiated by further investigation.

Green, as stated by Steyn (1928), found the toxic principle to be thermostable, water-soluble and insoluble in chloroform or ether; furthermore, it was not precipitated by basic lead acetate nor by the usual alkaloidal reagents. Green finally obtained a pale yellow syrup which was still contaminated by sugars but toxic to rabbits in doses of 0·1 gm. per kilo when given subcutaneously.

Power and Tutin (1906) have recorded a chemical study of the fruits of the closely allied plant *Chailletia toxicaria* from Sierra Leone. They came to the conclusion that the fruit contained at least two active principles, the one causing depression or narcosis, the other, excitation leading to epileptiform convulsions. Some evidence was adduced suggesting a cumulative effect by the latter. The main part of the toxic material was soluble in water, only.

More recently, Steyn (1934) has demonstrated that an acid environment (dilute acetic acid at room temperature) has no effect upon extracts containing the active principle of Gifblaar.

Mode of Action of the Poison.

With regard to the mode of action of the toxic constituent, little is known with certainty. A characteristic post-mortem appearance in animals poisoned by the plant is the flabby dilated state of the ventricular walls of the heart. Power and Tutin, in *Chailletia toxicaria* poisoning, report post-mortem cerebral congestion and thrombosis of the superior longitudinal sinus.

An interesting case of human poisoning (not fatal) by *Chailletia toxicaria* is reported by Renner (1904). A native of Sierra Leone had eaten some fish dusted with the powdered fruits of the plant as a rat poison and within half an hour became ill. The symptoms were vomiting, diarrhoea, muscular twitching, weakness and loss of power to use the limbs. The tendon reflexes became abolished and some hyperaesthesia developed. Vision was slightly impaired. The man made a slow recovery during the course of two weeks but, when discharged, he still had difficulty in walking.

Gifblaar is a very serious menace to stock in those parts of the Transvaal in which it grows. Its eradication presents great difficulty on account of the underground stems, many of which may exceed 40 feet in length and be found at depths of 6 feet or more. No reliable remedy for poisoning by the plant is known.

Scope of the Present Experiments.

The object of this communication is to report certain results obtained in a systematic study of the plant and to describe attempts which have been made to isolate the toxic principle by new methods

which have not previously been employed in this case. A good deal of indirect information has been obtained concerning the nature of the toxic principle.

The plant material used in these experiments was all collected in the Pretoria District at various times. The M.L.D. for rabbits was usually ± 2·5 gm. dry material per kilo body weight.

(a) Extraction Experiments.

A variety of organic solvents was employed in the hope that one could be found capable of removing the toxic principle. All extractions were made in the Soxhlet apparatus and both extract and plant residue subsequently tested for toxicity. No anhydrous solvent was found capable of removing the toxin. The following were tried with negative results: chloroform, ether, petroleum ether, carbon tetra-chloride, nitrobenzene, aniline, formamide, anhydrous acetone, absolute alcohol. The toxin was dissolved out readily by cold water.

(b) Precipitation Experiments.

None of the alkaloidal reagents precipitated the toxic material, neither was it removed by basic lead acetate alone or followed by ammonia, by phosphotungstic acid, mercuric chloride or acetate, flavianic acid or by any other substance tested. This would point to a neutral or amphoteric molecule possessing neither acidic nor basic characters.

(c) Systematic Examination of an Aqueous Extract.

An attempt was made to remove as much inert material as possible from an aqueous extract of the plant, hoping to obtain, finally, a toxic material of sufficient purity to carry out further specialised tests. The plant constituents removed at each stage were subjected to a preliminary examination and yielded certain interesting results. There were thus isolated: a tannin, a glycosidal pigment (methylpentoside), two bases, one of which proved to be the alkaloid trigonelline, a histidine-like material, and a complex polysaccharide of the uronic acid type. The detailed procedure is described below. 500 gm. of dried, ground Gifblaar leaves (M.L.D. ± 2·5 gm. per kilo) was thoroughly extracted in a percolator by water at 60-65°. To the combined extracts measuring seven litres in volume, sufficient basic lead acetate was added to produce complete precipitation. The orange-yellow precipitate was removed, washed with water and then ground with dilute acetic acid, thereby affording an orange coloured solution of an acetic acid soluble portion and a greyish insoluble residue. The soluble fraction was reprecipitated by addition of ammonia and both precipitates were decomposed by hydrogen sulphide.

Tannin Fraction.

The neutral lead precipitate yielded a tannin which was thrown out of solution by addition of sodium chloride. When collected on the centrifuge, washed and dried, it formed a dark brown amorphous powder.

Microanalysis: Ash 0·35 per cent.

C	H
49·60	5·42

In order to gain further insight into the nature of this substance, a portion was refluxed for one hour with 25 c.c. of a 2 per cent. solution of hydrochloric acid. The solution acquired an intense deep crimson colour and a considerable quantity of a red amorphous substance, " tannin red " or phlobaphene, separated. After cooling, the filtered hydrolysate was extracted with ether, the solvent dehydrated and allowed to evaporate. There remained a small quantity of a crystalline substance which was identified as catechol.

Crystalline material from hydrolysed tannin.

With $FeCl_3$ green colour turning to mauve on adding sodium acetate.

Ammoniacal $AgNO_3$ reduced in the cold.

M.P. after recrystallisation from benzene 103°.

The acid liquid from which the catechol had been removed was neutralised by sodium hydroxide, extraneous material removed by lead acetate and lead by hydrogen sulphide. After concentration it was tested and found to reduce Fehlings solution on boiling.

The tannin belongs therefore to the class of catechol tannins.

Glycosidal Pigment Fraction.

The solution remaining after removing lead from the basic lead precipitate had a deep orange-yellow colour. It was reprecipitated by basic lead acetate and the precipitate decomposed by grinding with dilute sulphuric acid. A slight excess of acid was removed from the filtrate by barium carbonate and the filtered, deep yellow solution concentrated on the water bath to the consistency of a syrup. Alcohol was then added to produce complete precipitation and the material centrifuged off and washed with ether.

It formed a bright yellow amorphous powder, moderately soluble in water and corresponding in quantity to about 1·7 per cent. of the weight of dry plant taken. It was non-toxic. With ferric chloride solution it gave a greenish coloration. On acidification of a solution the yellow colour became very much less intense.

It did not reduce Fehlings' solution.

That the substance was a glycosidal pigment was demonstrated by acid hydrolysis.

0·05 gm. was refluxed for one hour with 25 c.c. of 2 per cent. HCl. and the resulting solution extracted with ether, which removed a material giving a green colour with $FeCl_3$ solution. The acid liquid was neutralised, cleared with lead acetate and found to give an intense Molisch reaction and to reduce hot Fehlings' solution. The osazone was prepared by addition of 0·1 gm. phenylhydrazine hydrochloride, 0·2 gm. of sodium acetate and heating in the usual way. It was recrystallised from alcohol and had M.P. 180°.

Mixed with Rhamnoseosazone of M.P. 181° the mixture melted at 180°.

Microanalysis:	N.
	Found 16·37.

Rhamnosephenylasazone $C_{18}H_{22}O_3N_4$ requires 16·27.

The substance is therefore a glycoside of the methylpentose rhamnose. The aglycone was not further investigated.

Main Filtrate from the Basic Lead Acetate Precipitation.

This was concentrated, under reduced pressure, to 500 c.c. Of the pale yellow liquid, 10 c.c. killed a 2·3 kilo rabbit in three hours with symptoms of typical Gifblaar poisoning. A carbohydrate material was removed by the addition of 20 per cent. sodium hydroxide solution until further precipitation ceased. This occurred when the mixture was about normal in sodium hydroxide. The buff-coloured, somewhat gelatinous precipitate was centrifuged off and washed, then dissolved in warm, dilute sulphuric acid, using such a quantity that the final reaction was approximately neutral. A small quantity of insoluble material was filtered off and on the pale, yellow-brown solution certain qualitative tests performed as follows:—

Tests upon Polysaccharide Material.

Molisch test	+ intense.
Biuret test	–
Millon's test	–
Ferric chloride	faint green colour.
Fehling's solution	not reduced; reduced after acid hydrolysis.
Ammoniacal silver nitrate	reduced on warming.
Orcinol test	±
Naphthoresorcin test	+
Lime water	small white precipitate.

After hydrolysis by dilute sulphuric acid, a considerable quantity of calcium sulphate separated and the hydrolysate gave positive reactions for sugars. The polysaccharide material which was not soluble in absolute alcohol was not further investigated as tests proved it to be non-toxic. It is probably a complex of the uronic acid type.

To the alkaline main filtrate from the carbohydrate precipitate, sufficient acid was added to bring to neutrality. Since tests for free reducing sugars were strongly positive and these materials, as recorded by Green (see Steyn, 1928), always accompanied the toxic principle in every attempt he made to isolate it, it was decided to remove them completely at this stage of the general investigation.

The exact quantity of reducing sugar, calculated as glucose, was determined upon an aliquot of the main solution using Benedict's quantitative solution and his method of determination.

Thus, in one instance, 25 c.c. of reagent (=0·05 gm. Glucose) required 33·5 c.c. of 1 in 10 dilution of the main solution to effect complete reduction. Since the total volume was at this stage 750 c.c. there was therefore present 11·19 gm. of glucose in all.

For precipitation, basic lead acetate solution (accurately titrated by means of ammonium molybdate) and sodium hydroxide were added in the proportions—

$$2\ C_6H_{12}O_6 : 5\ PbAc_2,\ Pb(OH)_2 : 10\ NaOH$$

that is to say, 156·6 c.c. of basic lead acetate solution containing 56·14 gm. basic salt per 100 c.c. and 12·43 gm. of NaOH dissolved in about 100 c.c. water.

The dense precipitate was removed by suction, the filtrate neutralised and excess of lead removed by hydrogen sulphide. The pale straw-coloured filtrate (1,020 c.c.) contained no reducing sugars but retained its original toxicity to rabbits.

Tests performed at this stage indicated the presence of basic materials which could be precipitated by mercuric chloride and phosphotungstic acid respectively.

Accordingly, the bulk of the liquid was reduced by vacuum distillation to 200 c.c. and a fair excess of saturated mercuric chloride solution added. After four days, the precipitate was removed and mercury eliminated from both fractions.

Mercuric Chloride Precipitate.

The concentrated Hg-free solution of this fraction was very dark brown in colour and resisted attempts to decolorise it. Some tarry material was, however, removed and the following tests performed upon the filtrate:—

Biuret reaction.....	−
Millon reaction.....	−
Ninhydrine reaction.....	±
Diazobenzenesulphonic acid	+ + intense red primary colour; after reduction, etc., yellow.
Phosphotungstic acid.....	± white ppt. soluble on heating but reappearing on cooling.

The reactions suggest histidine or a histidine-like substance. All attempts to obtain pure histidine hydrochloride from the mixture were, however, unsuccessful.

The main filtrate from the mercuric chloride precipitation was still toxic. Sulphuric acid was added to about 5 per cent. concentration and phosphotungstic acid added so long as a precipitate formed. A very large quantity was required. The bulky white precipitate was filtered off on a Jena glass filter, washed and decomposed in the usual way with hot baryta. The colourless liquid had the following characteristics.

SOLUTION OF BASIC FRACTION PRECIPITATED BY PHOSPHOTUNGSTIC ACID.

Diazobenzenesulphonic acid	–	
Wagner's reagent.....	+	Amorphous precipitate, with fine spear-like prisms appearing but collapsing to oily drops on addition of water.
Dragendorff's reagent.....	+	
Aloxan reaction.....	+ +	red-violet changing to violet blue on addition of sodium hydroxide.
Solution boiled with NaOH		Alkaline, ammoniacal vapours evolved.

It was clear that a mixture of bases was present, amongst which choline was suspected. To a portion, evaporated to dryness and taken up in absolute alcohol, an alcoholic solution of mercuric chloride was added. The precipitate was centrifuged off, washed with 96 per cent. alcohol and decomposed. The solution was found to give the Florence reaction with Iodine in potassium iodide. On evaporation a hygroscopic crystalline residue was left—presumably choline hydrochloride. 0·10 gm. dissolved in 0·5 c.c. water was injected into the dorsal lymph sac of a frog (*Bufo regularis*). Within about ten minutes respiratory distress was noticed; the animal gradually lost all reflexes and died fifteen minutes after dosing.

1·3 gm. was given per os to a rabbit but no untoward symptoms were noticed.

Lack of material prevented a more rigorous identification, but all the evidence pointed to the identity of this base with *choline*.

To the remainder of the phosphotungstic acid fraction, auric chloride was added in excess, the precipitate removed and dissolved in warm water. On cooling, stellate aggregates of a crystalline gold salt separated. These were washed with water and alcohol and had M.P. 185°. On analysis they proved to be the basic gold salt of *trigonelline* which has M.P. 186°.

Microanalysis:

	C	H	N	Au.
Found.....	21·94	2·51	3·63	36·94
$(C_7H_7O_2N)_4 \cdot 3\,HAuCl_4$ requires	21·41	1·98	3·57	37·73

The normal gold salt was also prepared by recrystallisation in excess of acid auric chloride solution. It had M.P. 194°.

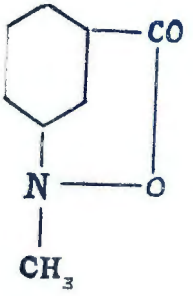
Trigonelline aurichloride melts at 197°.

Microanalysis:

	An
Found.....	40·97
$C_7H_7O_2N_4 . HAuCl_4$ requires.....	41·31

In all about 0·2 gm. of gold salt was isolated.

The alkaloide trigonelline is widely distributed in the vegetable kingdom and is practically non-toxic. Its constitution is—



The main filtrate was freed from phosphotungstic acid by means of baryta and concentrated in vacuo to 200 c.c. It retained its original toxicity, the equivalent of 10 gms. of plant proving fatal to a rabbit.

On evaporation, a large amount of crystalline inorganic material separated. The mass was extracted by hot 98 per cent. alcohol which removed the toxin but eliminated a large quantity of inorganic salts. The presence of sodium acetate proved very troublesome but by repeated extractions, a solution was obtained, still highly toxic in doses of approximately 0·1 gm. per kilo and considerably purified as compared with the original plant extract. Certain tests were carried out to gain further information concerning the nature of the toxic principle.

TESTS UPON A HIGHLY PURIFIED CONCENTRATE.

Test.	*Conclusion.*
1. Basic lead acetate..... –	Toxin probably not acidic.
2. Phosphotungstic acid and all alkaloid reagents.... –	Toxin not basic in character.
3. Molisch ..... –	Toxin not carbohydrate.
4. Biuret..... ..... –	No peptones, etc., present.
5. Ninhydrine..... + +	Presence of amino acids or a primary amino group in toxin.
6. Diazobenzene sulphonic acid..... –	Absence of phenolic substances.

The solid residue contained N (Lassaigne's test) but neither S nor halogens. The value of the positive Ninhydrine reaction was difficult to assess on account of the fact that any simple amino-acids derived from the proteins of the plant would have remained in the solution up to this stage. A portion of the residue was treated with nitrous acid, the solution boiled and subsequently tested for toxicity. It proved to be as active as before, from which it might be concluded that the ninhydrine reaction was given by amino-acid impurities or conversely that should a primary amino group be present in the toxin, this can be replaced by hydroxyl without influencing toxicity. In a latter experiment the absence of primary amino groups from the toxin was definitely proved.

Special Methods of Attempted Isolation.

The essential difficulty underlying all attempts at isolation of the Gifblaar toxin is the circumstance that the active material is soluble only in water and it becomes extremely difficult therefore to remove other water-soluble substances such as simple amino-acids, inorganic salts, etc., which are also present in the plant extracts. It may be noted here that, in the present work, toxin concentrates have been obtained for the first time free from all carbohydrate material, by use of the technique described above. This removes all doubt about the possible carbohydrate nature of the toxin itself.

Another embarrassing property of the active material is its remarkable chemical inertness. No substance has been found which will form with it an insoluble compound, neither does it seem to contain any easily identifiable chemically reactive grouping. On account of these difficulties, various specialised methods of approach were investigated in the hope of achieving further purification.

Dialysis and High-pressure Ultrafiltration.

An aqueous solution of a highly puified concentrate was dialysed in a collodion bag (prepared from specially prepared gun-cotton solution) against distilled water. Animal tests showed that the toxin dialysed readily into the surrounding medium.

Attempts were then made to use membranes of very low permeability, employing a steel filtration unit subjected to high pressures of nitrogen gas in order to hasten filtration. Mc.Bain has been able, by similar means, to effect a partial filtration of sucrose molecules from aqueous solutions, so it was hoped that an enrichment, at least, of the toxin would be accomplished. Even with pressures up to 80 atmospheres used in conjunction with the densest filters preparable, no appreciable retention of the toxin occurred. It must therefore be of comparatively small molecular dimensions.

High Vacuum Distillation.

This method is one frequently employed in the purification of materials which do not crystallise readily and which are fairly resistant to heat. The vacuum was obtained by a small Pfeiffer pump. All glass parts of the apparatus were thoroughly dried out before commencing the experiment. About 20 gms. of material was placed in the distilling flask and the following fractions collected.

	Oil bath temperature.	*Vapour temperature.*	*Pressure.*	*Appearance of fraction.*
Fraction I....	90–110°	44–49°	3 m.m.	Water-clear liquid, acid in reaction.
Fraction II....	120–200°	50–125°	3 m.m.	Pale yellow oil in small quantities, slightly acid.
Fraction III....	216–275°	130–4°	3–4 m.m.	Yellow-brown viscous oil, pungent smell, alkaline, not completely soluble in water.

Flask residue.—Carbonaceous mass smelling strongly of ammonia-like bases. Only a negligible quantity soluble in water.

Each fraction was dosed to a test rabbit. The only animal showing any symptoms was that which received fraction III. It lost appetite, was somewhat drowsy and died four days after dosing. The symptoms did not suggest Gifblaar poisoning.

Clearly, then, vacuum distillation and sublimation are of no avail.

Continuous Butyl Alcohol Extraction.

Owing to the strongly positive ninhydrine reaction given by the purest concentrates it was thought possible that the toxin might be a substance of the amino acid or peptone type. Accordingly the continuous butyl alcohol extraction technique of Dakin was tried. A purified concentrate was placed in a Kutscher-Steudel apparatus and the extraction maintained for six days. A deposit of solid material formed in the butyl alcohol extract which was nearly black in colour. After removing butyl alcohol the fractions were tested out upon rabbits as follows:—

M.L.D. of original concentrate ± 5 c.c.

M.L.D. residual liquid after extraction $\pm 6 \cdot 5$ c.c.

Butyl alcohol extract contained about 3 M.L.D's.

Apparently butyl alcohol removes only traces of the active principle. This result was especially disappointing since Dakin showed that the simple amino-acids, though practically insoluble in butyl alcohol, could be extracted quantitatively from aqueous solutions by use of this solvent, no doubt owing to the more ready solubility of some modification of the molecule, like the " Zwitterion ", which reforms as soon as that originally present in minute quantity has been taken up by the ascending bubbles of the alcohol.

Numerous other methods of isolation were tried but since none proved successful the detailed description of each will be omitted.

It was thought that possibly some derivative might be formed by taking advantage of the reactivity of a group resident in the toxin molecule and inducing combination with a suitable reagent. Such a derivative might reasonably be expected to possess solubilities different from the parent molecule and hence offer a chance of isolation in the pure form. Of such groupings, those most likely to be present in a natural product are $>CO$, $-OH$ and $>NH$.

Test for $>CO$ groups.

To 10 c.c. of a purified concentrate (MLD ± 10 c.c.), was added sufficient hydrochloric acid to render the solution 2 normal and then 8 c.c. of Brady's reagent (0·5 gm. 2:4 dinitrophenylhydrazine in 30 c.c. of warm 2 normal hydrochloric acid). A brick-red precipitate formed which was centrifuged off and washed well with dilute acid and then water. From the mother liquor, excess of reagent was removed by repeated shaking with chloroform, finally from the neutralised solution. This liquid was then administered to a 2·3 kilo rabbit. The animal died, indicating that the toxic principle had not been removed. It is safe to conclude therefore that in all probability no $>CO$ or $-CHO$ group is present in the toxin molecule.

Test for presence of -OH or >NH groups.

Both the hydroxyl -OH and imino >NH groups are capable of being benzoylated by means of benzoyl chloride and the derivatives formed have usually lesser solubility in water and greater solubility in organic solvents than have the parent molecules.

Accordingly, 20 c.c. of the same toxic preparation as was used in the previous experiment (=2 M.L.D.) was benzoylated with benzoyl chloride and sodium hydroxide by the Schotten-Baumann method. A certain amount of a brownish insoluble material separated out during the course of the benzoylation. The alkaline liquid was shaken repeatedly with ether. It was then acidified and again ether extracted. Both extracts and the residual solution were dosed separately to rabbits. The animal receiving the residual solution died in 4½ hours from typical Gifblaar poisoning. There is thus no ether-soluble benzoyl derivative formed and should the toxin molecule possess either an -OH or >NH group one is forced to conclude that this grouping is in no way essential to the manifestation of toxic activity. It seems more probable that these groups are absent from the toxin.

To summarize the evidence available at this point it would appear that the molecule of the Gifblaar toxin contains neither -COOH, -OH, >NH, $-NH_2$ nor >CO groups. It possesses neither acidic nor basic character and appears to be chemically very inert. One is forced to the conclusion that it must be a very peculiarly constructed molecule. The only possibility that to the writer seems open is that it must be a tertiary amine, the grouping $\geqslant N$ having in this case exceedingly feebly basic or neutral characteristics. Unfortunately no possible method of isolation is suggested by this consideration, tertiary bases being comparatively non-reactive.

Experiments upon the stability of the toxin to various agents.

Certain evidence has already been presented, demonstrating that the Gifblaar toxin is thermostable and not readily attacked by acids or alkalis. In order to gain precise information, the following experiments were carried out:—

A concentrate was prepared of which the M.L.D. for rabbits was found to be ±5 c.c. Individual 5 c.c. portions were then treated as follows:—

(*a*) Acid potassium permanganate (0·01 N) solution added so long as decolorisation occurred at room temperature: 30 c..c required in all. The mixture was neutralised and dosed.

(*b*) 2 c.c. of perhydrol added. After one hour at room temperature the mixture was dosed.

(*c*) 1·5 c.c. normal sodium hydroxide added (i.e. to 1 per cent.). Mixture placed in boiling water bath for 1 hour, cooled, neutralised and dosed.

(*d*) 1·2 c.c. normal sulphuric acid added. Treated as in (*c*).

All four rabbits receiving these preparations died. It is clear therefore that the toxin is stable to mild oxidising agents and not altered appreciably by hot 1 per cent. acid or alkali; it is not therefore a substance suffering hydrolytic fusion.

ELIMINATION OF AMINO-ACIDS AND ABSENCE OF A PRIMARY AMINO GROUP.

Since the most carefully purified extracts always gave a strongly positive ninhydrine reaction, suspicion was entertained that the active principle might contain a primary amino group although the experiments recorded on pages ..... and ..... rendered this somewhat unlikely. Amino acids may be precipitated from complex mixtures by the use of mercuric acetate and sodium carbonate and it was therefore decided to subject a concentrate to this procedure and ascertain in which fraction the toxicity remained.

To 25 c.c. of the same solution as used above (M.L.D. ±5 c.c.) were added 10 per cent. sodium carbonate and 25 per cent. mercuric acetate solutions until precipitation of mercury compounds was completed as shown by the orange-yellow colour of the final turbidity.

The precipitate was removed, suspended in dilute hydrochloric acid and gassed by hydrogen sulphide; mercury was also removed from the filtrate.

One rabbit was given 2/5 of the precipitate fraction, corresponding to 2 M.L.D., but sufffered no ill effects. Another, receiving 3/10 of the filtrate (= $1\frac{1}{2}$ M.L.D.) died $3\frac{1}{2}$ hours after dosing. The toxin is therefore not precipitated by the reagent.

The active filtrate fraction was pale yellow in colour but gave no reaction for amino groups with ninhydrine thus proving that primary amino groups $-NH_2$ are absent from the toxin.

A one M.L.D. equivalent of this amino-acid free solution was evaporated to dryness. The weighed residue, a pale yellow viscous syrup, was ashed and the weight of the ash substracted thus affording the weight of organic matter present in a single lethal dose. A total nitrogen kjeldahl determination was carried out upon a second M.L.D. equivalent. The results were as follows:—

Weight of organic matter = 0·5832 gm.
Total N..... = 21·4 mgm.

This preparation had not been subjected to such a rigorous series of purifications as some of those formerly employed but especial care had been taken to eliminate all nitrogen-containing impurities such as proteins, amino-acids, ammonium salts, etc. The comparatively high nitrogen content of the residue, amounting to 3·7 per cent., would appear to offer evidence strongly presumtive of the presence of nitrogen as an integral constituent of the molecule of the toxin. It is of interest, in this connection, to record that the fumes evolved on ashing the material were found to give a fairly strong pine-splinter test. The formation of pyrrol by pyrolysis is of course no specific

indication of the presence of pyrrollic derivatives in the material but indicates nevertheless that organic, nitrogenous compounds are present.

It is highly probable that the Gifblaar toxin contains cyclically bound nitrogen.

Rapid method for the preparation of concentrates.

The method of purification outlined is very laborious and time-consuming. Two circumstances, in particular, render the procedure troublesome, namely the large amount of certain reagents required and the great mass of inorganic material which accumulates in the final solutions.

In order to obviate these difficulties and provide a rapid method for obtaining a fairly pure concentrate the following method was devised. A watery extract of the plant was cleared with basic lead acetate and the lead-free filtrate concentrated in vacuo, *without neutralisation,* to a small bulk. Drying was continued in front of a fan at room temperature. The syrupy material was then stirred repeatedly with acetone which removed the last traces of acetic acid and served to dehydrate the material. It was then taken up in a suitably large volume of 96 per cent. alcohol rejecting the material remaining undissolved. An equal volume of absolute alcohol was then added and the precipitate discarded. The remaining 98 per cent. alcohol solution contains nearly all of the active principle originally present and in a condition sufficiently pure to serve as the starting point for further experiments. It was found by varying the procedure and extracting the dehydrated mass with absolute alcohol before dissolving in 96 or 97 per cent. that a small quantity of additional inert material could be removed.

The detailed procedure and results obtained in this series of investigations is best illustrated by the following charts:—

Chart I.

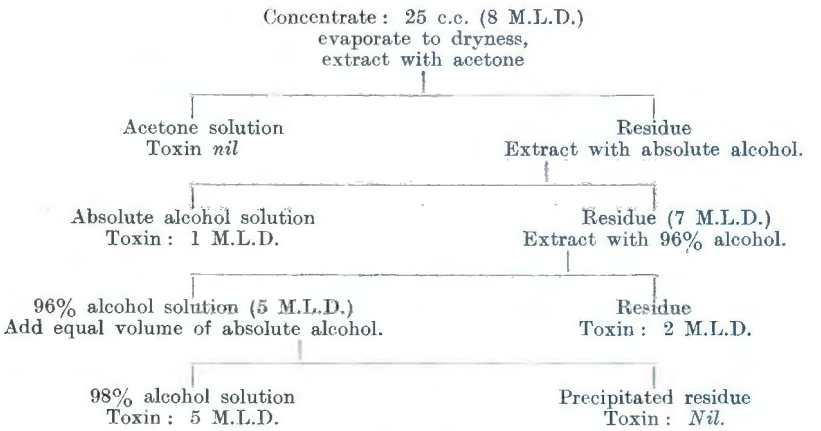
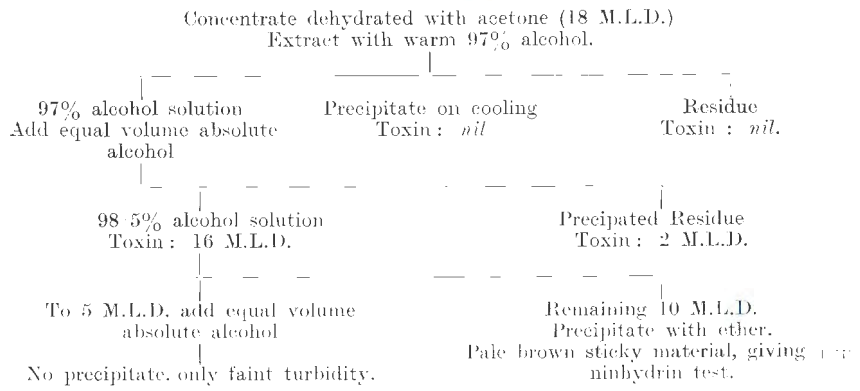


CHART II.



DISCUSSION AND SUMMARY.

Although the investigations recorded in this paper have not resulted in the isolation of the toxic principle of the Gifblaar, *Dichapetalum cymosum* in a state of chemical purity, it is felt that certain valuable information has been obtained relative to the nature of this toxin. In addition, some interesting substances have been isolated from the plant in the course of chemical manipulations. These include:

(1) A catechol tannin.

(2) A yellow colouring matter, shown to be a methylpentoside, yielding rhamnose on hydrolysis.

(3) A histidine-like base.

(4) A base probably identical with choline.

(5) The alkaloid trigonelline, the methyl-betaine of nicotinic acid.

Various specialised methods were tried for the purification of the toxin, including:

(1) Dialysis.

(2) Ultrafiltration under pressures up to 80 atmospheres of nitrogen.

(3) High-vacuum distillation and sublimation.

(4) Continuous extraction with butyl alcohol, etc.

Certain other chemical operations led to the conclusion that the following groups are definitely absent from the toxin molecule:—

Carboxyl	$-COOH$
Ketonic	$>CO$
Aldehydic	$-CHO$
Hydroxyl	$-OH$
Amino	$-NH_2$
Imino	$>NH$

The active principle almost certainly contains nitrogen and it is suggested that this may be included in some cyclic structure. It may possibly occur as in tertiary bases $\geqslant N$ but the molecule, as a whole, does not evince basic characters. Preparations, still highly toxic to rabbits, have been prepared containing no protein, amino-acid, or carbohydrate material, impurities which had not before been successfully removed. The chemical stability or inertness of the toxic substance is very remarkable in view of its pronounced physiological action. Thus, it resists boiling for one hour with 1 per cent. sulphuric acid or 1 per cent. sodium hydroxide solutions; it is not oxidised by potassium permanganate or by hydrogen peroxide in the cold and it is thermostable.

In view of this stability and the absence of any substance known to combine with or precipitate the toxin, the hope of finding any specific prophylactic or curative substance for use in Gifblaar poisoning becomes very remote indeed.

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