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SOUTH AFRICAN FISH POISONOUS PLANTS III.

PRELIMINARY INVESTIGATION OF THE TUBER.

OF NEORAUTANERIA PICIFOLIA. C.A. SM.

T H E S I S

submitted by

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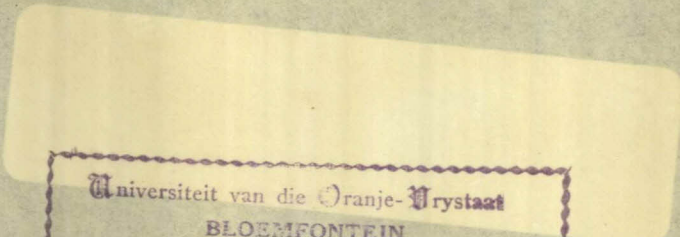


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CHAPTER 1.

INTRODUCTION.

Neorautaneria ficifolia is a leguminous plant and like Derris belongs to the family Papilionaceae. Like Derris, too, *Neorautanenia ficifolia* also has fish poisoning properties. It is for the latter reason that this plant is known to the natives, being designated by the natives in the district of Lusaka as "Kaumbwa".(1) The species is common in the Lusaka district of Northern Rhodesia. R.H. Ashton states that although other localities cannot be definitely described it probably occurs elsewhere, as the root is known to the native immigrants from Portuguese East Africa. (The plant material used in this investigation was not obtained from this locality (vide infra).

It may be mentioned that preliminary investigation of the Tuber of a different species of this plant viz. *Neorautanenia edulis*, which also has fish poisoning properties, has already been carried out in this department.(2)

Neorautanenia ficifolia has a tuberous root which is the part used by the natives for poisoning the fish and therefore this investigation refers only to the roots of the plant. It has however also been established that the aerial parts are toxic to fish, though much less so. The live roots appear to be free from any serious attack from insect pests, although it is thought that termites have worked small cavities in some roots. (This fact is important for the use of this plant as an insecticide). No information is available regarding the month of maximum toxicity. Natives dig and use the root from July to October when the rivers have a minimum flow and water can be confined into channels for their fish poisoning operations.

A reference to the root is to be found in the Literature, (3) which records:

"Another leguminous plant, *Neorautanenia ficifolia* (Benth) C.A.Smith, which is prevalent in various parts of eastern S.A. where the large tuberous root is a native piscicide, has been found to possess marked insecticidal properties".

It is understood that Prof. V. Watt of the Witwatersrand University has carried out experimental work on this plant, and although the presence of saponins in the Tubers was established, it had not been possible to isolate the active principle due to the mass of starch material present.

The material used in the present investigation was supplied by the Government Department of Botany from Pretoria. It was collected near Rietvlei about 12 miles south-east of Pretoria, about the month of February.

Preliminary tests were carried out on goldfish by Dr. Hey, who found that in the case of the Aerial parts (dried), a concentration of 1.0 gm/litre killed the fish in 35 hours, while a concentration of the dried Tuber, one tenth of the above viz 0.1 gm/litre killed the fish in 32 hours, in both cases the symptoms shown by the fish were the same as for *Neorautanenia Edulis*.

As regards the toxicity of the plant to humans and animals there is evidence that unless taken in large doses the root is no more toxic than Derris and allied materials. It is considered safe by the natives for cattle or humans to drink from pools about 24 hours after material has been thrown into them for fish poisoning.

CHAPTER 11.EXTRACTION OF THE TUBERS.

In the present investigation only the tubers of the *H. ficifolia* were used, as this is the only part used for poisoning fish although toxicity of the aerial portions has been established (vide supra). The tubers were chopped into small pieces, allowed to dry and crushed to powder. Preliminary investigation had to determine the best method of extraction.

Preliminary Extraction. 200 gms. portions were extracted in a Soxhlet, using the following Solvents:

(a) Petrol Ether.

This gave a yellow extract, from which a yellow material crystallised on cooling. Evaporation of the extract to dryness gave 2.7 gms of a waxy, yellowish brown residue with a strong revolting plant odour.

(b) Chloroform.

This solvent readily extracted a yellowish brown material, from which a brown residue was obtained on evaporation. (5 gms) This was partially soluble in ether and acetone leaving an insoluble residue. No crystalline material could be isolated from the chloroform extract.

(c) Alcohol.

Extraction with this solvent was slow and unsatisfactory, as it deposited only resinous material but no crystals.

The best method of extraction was found to be that by cold ether, the method used being cold ether percolation in a large copper percolater. This method was used by van Duuren & Groenewoud (2) in the investigation of *H. Edulis*.

LARGE SCALE EXTRACTION.

In this way 12 kilograms of the dried, powdered material were packed into the large percolater. Pure ether was then poured onto the material, the solvent allowed to soak in, and the ether was poured onto the material so that the latter was covered by the solvent. The lid was shut tight and the ether percolate allowed to drip slowly from a tap at the bottom of the percolater, into an appropriate bottle. Ether was continually added to the top so that the material remained covered by the solvent. The percolate was initially dark brown, but overnight became much lighter in colour. The percolate obtained initially, i.e. during the first few hours, rapidly deposited some crystalline material on standing. Percolation was thus continued until the ethereal extract was practically colourless, the actual period being approximately 6 weeks. The greater part of the ether was then distilled off so that only a small volume of solution remained (about 1½ litres). The ethereal solution was then set aside in a corked flask for 6 weeks to enable crystallisation to take place. The solution was then filtered, and the residue washed with a small quantity of ether, when a yield of crude crystalline material (34.5 gms) was obtained. The crude crystalline material will be designated as Fraction A., when dry ^{it} had the appearance of a mustard coloured powder. A further amount of ether was evaporated off, the solution again set aside to crystallise, when a further crop of crystalline material was obtained - yield 2 gms. The second crop of crystals so obtained was added to Fraction A. Finally, evaporation of the ethereal solution to dryness gave a dark brown viscous mass -(Fraction B.) - Yield 220 gms.

Percentage Yield of crude crystalline material = $\pm 0.3\%$

Percentage Yield of resin = $\pm 1.8\%$

Total Percentage Yield = $\pm 2.2\%$

CHAPTER III.THE ISOLATION, SEPARATION AND PURIFICATION
OF CRYSTALLINE SUBSTANCES.Introduction:

In many of the common solvents, the solubilities of all the constituents are essentially the same, rendering the purification of any one of the substances difficult.

Experimental.I Fraction A:- Crude crystalline Material.Preliminary Experiments:

The crude crystalline material was readily soluble in solvents such as chloroform, dichloroethane, fairly soluble in ether, ethyl acetate and acetone; sparingly soluble in methyl and ethyl alcohol and insoluble in the hydrocarbon solvents benzene and petrol ether.

(In this investigation frequent mention is made of two solvents viz. ethylene dichloride and dichloroethane. In order to avoid any confusion with the nomenclature, the molecular formulae and b.p. of the compounds will be given.)

Ethylene dichloride (1,2 dichlorethylene) B.P. 48°C .
 $\text{C}_2\text{H}_2\text{Cl}_2$

Dichloroethane (Sym. 1,2)-B.p. 83°C .- $\text{C}_2\text{H}_4\text{Cl}_2$.)

Different methods were tried in order to effect some separation of the mixture constituting the crude crystalline material. These are:-

(a) Fractional Crystallisation. This was the first method attempted. Since the material was on the one hand either too soluble in a given solvent, or too insoluble in another, a mixture of solvents was resorted to. The best solvents for this purpose were a mixture of dichloroethane and ethyl alcohol, or chloroform and alcohol. Ethyl acetate and petrol ether could also be used. Aqueous acetone was not very satisfactory.

Experimental. 0.5 gm. of the crude crystalline material was dissolved in chloroform, alcohol added until a faint turbidity formed^{and} which was redissolved by adding a little more chloroform. The solution was allowed to crystallise overnight, when a crop of crystals was obtained. Yield 0.15 gm. However this was an impure mixture of fine yellow needles and lighter, broad, rod-shaped needles. M.p.[±] 200° C (uncorr) over a five degree range. Repeated crystallisation failed to give a pure product. This method was eventually abandoned.

(b) Extraction.

Extraction of the material with different solvents was then tried. Thus 0.5 gm. of the crude crystalline material was extracted a few times with small amounts of ether. In this way two fractions were obtained viz:-

Fraction I The ether soluble fraction.

Fraction II The ether insoluble fraction.

Fraction I.

The ethereal extract was divided into two further fractions:-

- (i) A fraction which separated out from the ethereal extract as a light yellow mass; only a very small amount was obtained and from which no crystals could be isolated.
- (ii) The more soluble fraction of the ethereal extract, obtained by pouring off the liquid from (i) and which likewise yielded no crystals.

Fraction II.

The ether insoluble fraction was extracted with hot methyl alcohol. Fraction II could thus also be divided into two fractions, viz. Fraction (i) soluble in the methyl alcohol and Fraction (ii) insoluble in methyl alcohol.

Fraction (i) The methyl alcohol soluble fraction was obtained as an orange coloured liquid which on evaporation yielded a dark

orange residue, together with some white material. On fractional crystallisation of the residue from a mixture of chloroform and alcohol, yellow needles, m.p. $195^{\circ}\text{--}199^{\circ}\text{C}$ (uncorr.) were obtained.

Fraction (11) The methyl alcohol insoluble fraction was dissolved in hot chloroform; hot ethyl alcohol was added to the solution, causing crystallisation to take place. On standing overnight, fine white to pale yellow needles had crystallised out, together with a yellow mass. Recrystallisation of the crystals from chloroform and alcohol gave an impure mixture of yellow needles and some prisms with an approx. m.p. of 200°C . (uncorr)

The above method of extraction with different solvents was also found unsatisfactory and was abandoned for the more suitable method of chromatography.

(c) Chromatography.

It was soon apparent that the most suitable adsorbent in this case was Al_2O_3 . It was shown that the use of MgO or CaCO_3 led to weak adsorption and thus not to clear cut separations. (4) This also proved to be the case in the present investigation. The Al_2O_3 used in this work was that supplied by the British Drug House for Chromatographic use. Before use it was dried by heating in an oven at 300°C for 3 hours. To ensure uniform particle size the Al_2O_3 was sieved through a 80 and 200 mesh sieve. The Al_2O_3 after use was readily reactivated by heating at 300°C for 6 hours, followed by several washings with tap water and finally dried at 300°C for 6 hours and then sieved.

Chromatography being a method of trial and error, various solvents had to be tested. By virtue of the insolubility of the crystalline material in Benzene, petroleum ether, methyl and ethyl alcohol, these solvents were naturally eliminated

For the following tests, small columns of Al_2O_3 were used, 0.05 gm of the material, dissolved in 5 ml of solvent, being used in each case. Size of column 1 x 32 cmS

(1) Acetone

Here a good adsorption of the resin took place but no chromatogram developed, the remainder of the material running through the column and thereby effecting a clean separation from the resin.

(2) Ether.

With this solvent the solution ran through very easily, also effecting a good separation of the resin. The chromatogram itself however was not very useful.

(3) Dichloroethane.

The resin was readily retained at the top of the column, but at the same time a very well developed chromatogram, with clear distinct bands, was also formed. Except for the lower fraction, which readily runs through, the remainder moves rather slowly. Addition of alcohol readily eluted part of the resin, and the chromatogram was spoilt.

(4) Ethyl Acetate.

The resin was strongly adsorbed, the remainder of the material running through as in the case of acetone, and therefore no visual development of a chromatogram took place.

(5) Chloroform.

The solvent gave an identical chromatogram as in the case of dichloroethane, though the bands were not as clearly defined as with the latter solvent.

(6) Ethylene Dichloride.

The resin as well as the crystalline materials are very strongly adsorbed. The well developed bands could not be washed down with the same solvent, while washing with a mixture of dichloroethane and ethylene dichloride gave a slight separation. Since ethylene dichloride caused such strong adsorption, a mixture of ethylene dichloride and ethyl acetate was tried, the latter solvent causes elution

rather than adsorption.

0.05 gm. of crude crystalline material was dissolved in a mixture of ethylene dichloride and ethyl acetate(4:1) On development first with a (5 ; 1) mixture of ethylene dichloride and ethyl acetate, followed by a (10 : 1) mixture of the same solvents, the substances were washed through, but no real separation resulted. 10:1 and 20:1 mixtures of ethylene dichloride and ethyl acetate were also tried with the same results.

From the above experiment it was clear that dichloroethane or chloroform were the best solvents for chromatographic work, although later it was found that ethylene dichloride was an even better solvent in those cases where preliminary separation of the crystalline material had been effected. Why, as in the above preliminary experiment, such powerful adsorption of the crude crystalline material took place, could not be accounted for.

As eluents the following solvents can be used, given in decreasing order of effectiveness:- alcohol, acetone, ethyl acetate and ether. This is in agreement with the observations of van Duuren (4). In the following work, cold acetone was used for the lower bands, which are readily eluted by it. For the higher and more strongly ^{adsorbed} bands, a mixture of alcohol and acetone was used, in some cases extraction in a Soxhlet being necessary.

Experiment on a larger Scale.

4.5 gms of the crude crystalline material was dissolved in 70 m l. of ethyl acetate (redistilled); a portion was insoluble in the ethyl acetate and was filtered off. -
(Fraction I) The filtrate was light brown in colour. -
(Fraction II).

Fraction I.

The fraction insoluble in ethyl acetate was crystallised from chloroform and alcohol, when yellow needles contaminated with other material, the crystalline form of which could not be distinguished, were obtained. The m.p. of the yellow needles was $\pm 200^{\circ}\text{C}(\text{uncorr})$. 10/.....

Fraction II.

The ethyl acetate solution was put onto a column of Al_2O_3 (1.5 x 50 cm) and washed through with 350 ml of the same solvent. The percolate was yellow in colour and appeared to be free from resin. Distillation of the excess ethyl acetate and cooling of the remainder of the solution caused crystallisation to take place rapidly, a pale yellow to white mass separating out - fraction (a); the orange - coloured mother liquor being poured off - fraction (b)

Fraction (a) Fraction (a) which weighed 3.5 gms. was extracted with hot methyl alcohol. Thus fraction (a) could be divided into:-

fraction (i) That fraction soluble in hot methyl alcohol.

fraction (ii) The fraction insoluble in hot methyl alcohol.

fraction (i) On cooling the fraction soluble in hot methyl alcohol, yellow needles, contaminated with other substances ^{were obtained} - Yield 1 gm. The crystals were impure as revealed by the m.p. viz $190^\circ - 202^\circ\text{C}$ (uncorr). The crystals were recrystallised three times from a mixture of chloroform and alcohol, and eventually bright yellow needles m.p. $199^\circ - 200^\circ\text{C}$ (uncorr) were obtained. This substance, although not quite pure will henceforth be called Substance A. When pure it melted at 210°C (corr) and from a mixed m.p. with substance (i). Isolated by B.L.van Duuren from *H. edulis* (4.2) it was concluded that the two substances were identical since there was no m.p. depression.

Fraction (ii) i.e. the fraction insoluble in methyl alcohol, was crystallised from a mixture of chloroform and ethyl alcohol. Although crystallisation was rapid, the crystals were impure and consisted of a mixture of

yellow needles and prisms. Yield 2.3 gms. Since fractional crystallisation could not effect a separation of the needle and prisms chromatography was attempted. The mixture of needles and prisms was dissolved in dichloroethane and put onto a column Al_2O_3 . On development with the same solvent three fractions were obtained:

- I. A narrow orange band on top of the column.
- II. A pale orange to yellow band.
- III A wide yellow band which moved down rapidly on development.

Only Fraction III represented by the lower, wide yellow band yielded crystalline material on elution with acetone. The crystalline material when crystallised from a 20% mixture of dichloroethane and ethyl alcohol gave yellow needles m.p. 210°C . (corr) i.e. substance A. Yield 1.3 gm.

Fraction (b). i.e. the orange coloured mother liquor from fraction (a) only deposited a resinous residue on evaporation.

The mother liquors from the crystallisation of substance A. were collected, the solution evaporated to dryness when an amorphous residue was obtained. Crystallisation of the residue from a 20% mixture of dichloroethane and ethyl alcohol gave minute, pale yellow needles, the product however was impure. This particular substance crystallises in a most peculiar manner, remaining in solution for a considerable length of time, when suddenly crystallisation is initiated by the formation of small, round fluffy balls, which rapidly increase in size, until they eventually unite to form a solid mass. The substance is definitely impure and melts at a temperature of approximately 180°C (uncorr). It is suspected that this is the same substance isolated by Prof. Greenewald of the U.O.F.S. from this same plant, and consists of bright yellow, microscopic needles, melting at 193.5°C (corr). The

The substance which crystallised in this peculiar manner was dissolved in dichloroethane and put onto a column of Al_2O_3 (1.5 x 50 cm). On development with dichloroethane three bands were obtained viz:-

- I A light brown band.
- II. A white band.
- III. A broad yellow band.

The development of the chromatogram was continued until the three bands were widely separated. The column was then extruded and cut into three fractions; the fractions corresponding to the three bands of the chromatogram. Fractions I and II yielded negligible residues on elution with acetone and alcohol, followed by evaporation of the solvents. Fraction III, corresponding to the broad yellow band was eluted with acetone and on evaporation of the solvent a crystalline residue was obtained. Crystallisation of the residue from a 10% mixture of chloroform and ethyl alcohol again resulted in the crystallisation of minute, yellow needles in aggregates of round, fluffy balls as described above; the m.p. of which had dropped to $\pm 153^\circ\text{C}$ (uncorr). The mother liquor on slight evaporation and allowing the more concentrated solution to stand for several days, deposited a mixture of very pale yellow, rod-like needles; m.p. 197°C (corr.) - Substance F; and yellow prisms m.p. 180°C (corr.) ^{- Substance B} which were separated by hand. Substance F. was recrystallised from a mixture of the minimum amount of chloroform and a large volume of alcohol, while substance B. was crystallised from a large volume of boiling ethyl alcohol. The mother liquors from the other yellow fractions were also collected in a similar manner, when invariably, Substance B. crystallised out on long standing in relatively large prisms, together with other substances, which could be separated by hand.

The physical properties of substance B. seem to indicate that it is identical to Substance (iii) (yellow prisms m.p. $180^{\circ}\text{C}(\text{corr})$) isolated by B.L. van Duuren and P.W.G. Groenewoud from *N. edulis* (2) Unfortunately crystals of the latter substance were not available to do a mixed m.p. with Substance B.

The above method of separation was very tedious and effected no real separation, the different fractions in some cases gave rise to the same substances. It was therefore decided to try a straight forward chromatogram.

Experimental.

2.5 gms. of the crude crystalline material was dissolved in 50 ml dichloroethane and put onto a column of Al_2O_3 (1.5 x 67 cm). On development with dichloroethane the following bands were obtained:-

Top of Column.

- I. A dark brown band.
- II. A cream band.
- III. A light yellow band.
- IV. A dark orange band.
- V. A light yellow band.
- VI. A dark yellow band.

Band VI was eluted while still on the column. After development of the chromatogram with dichloroethane the column was extruded and the six bands cut out, thereby giving rise to six different fractions viz.

Fraction I.

i.e. the fraction represented by the dark brown band on the upper portion of the chromatogram was eluted by extraction with a mixture of methyl alcohol and acetone for six hours in a Soxhlet. On standing overnight, some material separated out from solution and was filtered off. - Yield 0.14 gm. The filtrate was boiled twice with norite, the solution filtered and the excess solvent boiled off, when, on allowing the remainder of the solution to stand in an Erlenmeyer flask, some more product, which was added to that obtained above, was obtained.

The product so obtained was crystallised from boiling methyl alcohol to give a white substance which readily separated out. M.p. 135° C. (corr) - Substance C. Examination of Substance C. under the microscope revealed no crystalline form but an amorphous white powder. The substance was crystallised from different solvents in an attempt to obtain it in a crystalline form, but all the attempts yielded a white powder. On repeated crystallisation from acetone, the m.p. remained unchanged. The fact that Substance C. melted sharply and the m.p. was unchanged on repeated crystallisation would seem to indicate that the substance is pure.

Fraction II. represented by the Cream band was also eluted by extraction in a Soxhlet with a mixture of acetone and alcohol. On evaporation of the solvents a resinous residue from which no crystals could be isolated was obtained.

Fraction III. (The light yellow band.) The band was eluted with a mixture of alcohol and acetone, but on evaporation of the solvents, it gave only a very small amount of material which could not be worked up further.

Fraction IV. (The dark orange band) The band was eluted in the same way as the three fractions above, a dark yellow solution being obtained. On evaporation of the solvent, an orange coloured residue which was too negligible an amount to purify was obtained.

The last traces of material adsorbed in these four fractions were not removed from the Al_2O_3 by extraction in the Soxhlet.

Fraction V. (The light yellow band) Acetone in the cold readily eluted band V. to give a yellow solution, which deposited a yellow crystalline mass, in which needles could be detected, on evaporation. The yield of the crude product/.....

product was 0.39 gm. which was crystallised from a mixture of 50% dichloroethane and ethyl alcohol. This gave a mixture of substances which could not be purified on repeated crystallisation.

Fraction VI. (The dark yellow band) Band VI was eluted while still on the column. On evaporation of the solvent a residue of yellow needles was obtained. This was crystallised from a 20% mixture of dichloroethane and ethyl alcohol which gave yellow needles of Substance A. m.p. $210^{\circ}\text{C}(\text{corr.})$

It was at this stage in an attempt to effect a more satisfactory method of purification, it was found that chromatography, using dichloroethylene as the solvent gave a much more satisfactory separation of the substances. The actual procedure employed was to effect a preliminary separation of the substances by chromatography with dichloroethane as the solvent, followed by the use of ethylene dichloride. This was the method preferred to any of the others described above.

Large Scale Separation.

2gm. portions of the crude crystalline material were dissolved in 30 ml. dichloroethane and put onto a column of Al_2O_3 (67 x 1.5 cm) 6 gms. were so treated. The chromatogram was developed with dichloroethane until a maximum separation of the bands was obtained. After extrusion the column was divided into four fractions:-

Fraction I. was obtained by the elution of the dark brown band on the upper portion of the column as already described above in the same type of chromatogram. From fraction I Substance C. was obtained. Fractions II, III and IV represented by an orange, a light yellow and a dark yellow band respectively, were rechromatographed, using ethylene dichloride as the solvent.

With the ethylene ^{dichloride} as solvent, fractions II, III and IV gave the following chromatograms:-

Fraction II. Chromatography with thylene dichloride gave two bands viz, an upper orange band and a lower yellow band. The latter, when worked up in the same way as the other yellow bands above, gave a small amount of Substance A. The orange band yielded some orange material which could not be purified.

Fraction III. This fraction which weighed 0.35 gm. on chromatography in ethylene dichloride gave three fractions:-

Fraction (i) A fraction obtained by washing through a lower yellow band to give a light yellow filtrate with a faint green fluorescence. The solution, on evaporation, gave light yellow needles, which when crystallised from a 20% mixture of dichloroethane and alcohol proved to be Substance A. m.p. 210° C. (Corr.) Yield 0.1 gm.

Fractions (ii) and (iii) were discarded as the amounts obtained from them were negligible.

Fraction IV. The fraction on chromatography in ethylene dichloride was also divided into three fractions, by arbitrarily dividing the broad yellow band obtained into three portions. All three fractions however yielded the identical substance.

Fraction (i) was obtained as a yellow solution with a strong green fluorescence, by washing through the lower portion of the yellow band until the solution became lighter in colour. Evaporation of the solution to dryness, gave a residue consisting of light yellow needles and weighing 1 gm. Crystallisation of the yellow needles from a 20% mixture of dichloroethane and ethyl alcohol yielded Substance A. m.p. 210° C. (corr.)

Fraction (ii) was also eluted while still on the column, the solution also being yellow in colour and deposited a pale yellow amorphous substance (0.54 gm) on evaporation. When crystallised twice from a 20% mixture

of dichloroethane and ethyl alcohol, pure Substance A was obtained. M.p. $210^{\circ}\text{C}(\text{Corr})$.

Fraction (iii) was also obtained as a yellow solution yielding minute needles on evaporation (0.2 gm) This was crystallised three times from 20% dichloroethane and alcohol to give 0.11 gm. of Substance A.

It will be observed that although fractions (i) (ii) and (iii) all yielded Substance A, the purity of each fraction steadily decreases, fraction (i) only requiring one crystallisation to obtain a pure product, while fraction (iii) requires at least three crystallisations to reach the same degree of purity.

The mother liquors were all collected, the solution evaporated somewhat and then set aside to allow crystallisation of Substance B. to take place. Small amounts were thereby obtained by separation by hand from other contaminating material, and finally purified by crystallisation from a large volume of ethyl alcohol from which it crystallises out in the form of yellow prisms, m.p. $180^{\circ}\text{C}.$ (corr.) Invariably, Substance B. in this way crystallised together with rod-like needles, which on crystallisation from the minimum amount of chloroform and large volume of alcohol gave pale yellow, almost white rod-like needles of m.p. $197^{\circ}\text{C}.$ (corr.) The yield of these two substances is very small.

After obtaining as much of Substance B. as possible, the mother liquors were evaporated to dryness, the residue dissolved in dichloro ethylene again and put onto Al_2O_3 in order to ^{recover} obtain as much of Substance A. as possible.

FRACTION B. The Resin.

Fraction B, was obtained by the evaporation of the ethereal extract of the plant material after the crystalline material had been filtered off. The residue was dark brown in colour, and the yield 220 gns.

The resin dissolved well in chloroform, dichloroethane

ethylene dichloride, ether, acetone and ethyl acetate. In contrast to the crude crystalline material, methyl alcohol was a very satisfactory solvent, readily dissolving the greater part of the resin and only leaving a very small residue. Like the crude crystalline material, the resin was not dissolved by the hydrocarbon solvents.

Numerous references were found in the literature (5-7, 35, 36) in which work on fish poisonous plants of the Derris type had revealed that certain toxic substances, present in the plant, could only be isolated from the non-crystalline material by preliminary treatment with alkali. These substances evidently existed in some form of combination ^{and} the action of the Alkali caused racemisation of the substances, the racemised products then crystallising out from the solution. Deguelin, for example, could only be isolated in this manner from four species of Derris. Due to the fact that *N. ficifolia* is known to contain rotenone (3) and the Derris type of fish poisonous plants is characterised for the presence of rotenone, it was considered worth while to try the method of alkali treatment on the resin.

Alkali Treatment.

30 gms. of the resin were dissolved in 250 ml. methyl alcohol by heating on a waterbath, the solution was cooled and filtered from a small amount of insoluble material (fraction I). The filtrate which was light brown in colour, constituted fraction II.

Fraction I.

The methyl alcohol insoluble portion readily dissolved in dichloroethane, but not acetone. The physical properties of the solution in dichloroethane seemed to indicate the presence of ^asaponin. The dichloroethane solution was put onto a column of Al_2O_3 (45 x 1.0 cm), and could thereby be divided into three fractions; a, b, and c, on development with the same solvent.

Fraction a. 4. light yellow fraction which ran through

the column very quickly, and gave an oily residue, with an odour of cotton seed oil, on evaporation. No crystalline material was obtained from fraction a. fraction (b). A darker yellow fraction which came through the column more slowly, and deposited, on evaporation, crystalline material consisting of fine balls of needles, together with an oil. The latter was removed by washing the residue with cold petroleum ether, the balls of needles crystallised from a 20% mixture of dichloroethane and alcohol as yellow needles m.p. 210°C . (corr) - Substance A.

fraction C. Extraction of the Al_2O_3 , after fractions a and b had been washed through with ethyl alcohol and acetone gave a light yellow solution, which only yielded a very small residue on evaporation. The residue could not be crystallised.

No trace of the saponin was found in any of these three fractions which would seem to indicate that the saponin is either too strongly adsorbed by the Al_2O_3 or is decomposed by it.

Fraction II.

The methyl alcohol soluble fraction was rendered alkaline by the slow addition of a small amount of strong caustic soda solution. The alcoholic solution assumed a dark reddish brown colour and some precipitate formed (fraction (i)). The solution was allowed to stand for a few days and then filtered. On addition of water to the filtrate some resin was precipitated, and was allowed to coagulate by standing for a few days. The coagulated resin which constituted fraction (ii) was separated from the brownish liquor by pouring off the latter. The methyl alcohol was evaporated from the brownish liquor by heating on the water bath and the aqueous solution so obtained was made slightly

acid with acetic acid and extracted with ether. (fraction(iii)) After extraction with ether, the acid solution was rendered ammoniacal and again extracted with ether-fraction(iv) on evaporation of the ammoniacal solution to dryness, fraction (v) was obtained.

fraction (i) (The NaOH insoluble fraction)

The precipitate, obtained by treating the methyl alcohol solution of the resin with NaOH, was dissolved in acetone, by heating when a brown solution, with only a small amount of insoluble material, was obtained. CO_2 was passed into the solution for some time, and then filtered to remove insoluble material. The dark brown filtrate was put onto a column of Al_2O_3 and washed through with acetone to form an orange eluate (a). When the acetone eluate had become colourless, the Al_2O_3 was cleared by washing through with alcohol which gave a brown coloured filtrate. (b)

(a) On evaporation of the acetone from the orange eluate, a brown crystalline mass was obtained. This was dissolved in dichloroethylene and put onto Al_2O_3 , when three fractions were obtained.

(1) An orange coloured fraction which ran through the column very rapidly and which on evaporation gave crystalline material consisting of prisms, and a yellowish brown oil. The latter was removed by washing with ether and the residue crystallised once or twice from n - butyl alcohol when colourless, short prisms, with a m.p. of 181.6°C . (corr.) were obtained. - Yield 0.47 gm.-

Substance G.

(2) A light yellow fraction which washed out very slowly from the Al_2O_3 and gave only a very small amount of needle-shaped crystals which were in-

sufficient to identify.

(3) The third fraction was obtained by elution of the Al_2O_3 with a mixture of acetone and ethyl alcohol. From the eluate white flakes separated out on evaporation of the solvents. The flakes could be crystallised from aqueous acetone to form colourless, shiny flakes - m.p. 160°C . (corr.)

Substance D.

(b) Only a resinous product was obtained from fraction (b).

Fraction (11). i.e. the coagulated resin obtained from the filtrate of fraction (1) was dissolved in acetone and put onto a large column of Al_2O_3 . The Al_2O_3 was washed through with acetone until the filtrate was colourless. The excess acetone distilled off and the orange-brown solution on evaporation to dryness yielded a brown transparent resin. (Yield 10.1. gm.) The resin was dissolved in dichloroethylene and put onto Al_2O_3 , three fractions being obtained on development with dichloroethylene.

fraction 1. which was yellow in colour, rapidly ran through the column of Al_2O_3 . Evaporation of the solvent gave a crystalline residue, consisting of a mixture of yellow substances together with some orange material. Crystallisation of the mixture from a 30% mixture of chloroform and ethyl alcohol yielded yellow needles of Substance A. - m.p. 210°C (corr) Yield 0.18 gm. The mother liquor on evaporation deposited a crystalline residue, which on treatment with 20% chloroform and ethyl alcohol (hot) left a difficultly soluble residue which could be separated by decantation of the solution and proved to be yellow prisms - Substance B. - which were purified by crystallisation from a large volume of ethyl alcohol - yield 20 mg. The decanted solution deposited a mixture of substances which on recrystallisation from chloroform and alcohol 10% gave a crop of colourless prisms

m.p. 181.6° C. (corr). Substance G.

Fraction 2. represented by a very light band on the lower portion of the column, could be readily eluted with a mixture of acetone and alcohol. Evaporation to dryness however, gave only a negligible residue.

Fraction 3. constituted a middle reddish band which was also eluted with alcohol and acetone, but no crystalline material could be obtained from fraction 3.

Fraction (iii) was obtained by the extraction of the acid solution with ether and gave a small amount of a resinous residue on evaporation. The resin was removed by dissolving in acetone, and putting onto a column of Al_2O_3 and washing with acetone when a yellowish brown eluate was obtained. The eluate was evaporated to dryness, dissolved in dichloroethylene and again put onto a column of Al_2O_3 . On development with dichloroethylene a yellowish brown fraction was washed through. On evaporation the yellowish brown fraction gave small balls of needle-shaped crystals together with some resin. The latter being removed by washing with ether, the residue was crystallised from 20% chloroform and alcohol, when yellow needles, m.p. 210° C. (corr.) - Substance A, were obtained.

Fraction IV. was obtained by the extraction of the ammoniacal solution with ether. Only a negligible residue, which could not be worked up further was obtained.

Fraction V. was discarded since it consisted mainly of sodium acetate.

The above treatment proved to be very successful for the complete separation of substances D. and G from other material. Further, since Substance D is strongly adsorbed and remains at the top of the column, one has to watch out for this, since it is easily lost, and wash the column through with alcohol, acetone does not completely remove it.

Bearing in mind that the alkali treatment might have

been too drastic, it was decided to use a milder reagent viz. sodium carbonate, followed by treatment with caustic soda.

Experimental.

28 gms. of the resin were dissolved in 300 ml. hot CH_3OH the insoluble portion being separated as already stated above for the alkali treatment. 3 gms. of solid anhydrous sodium carbonate was added to the solution which was vigorously stirred for about half an hour. The solution was then allowed to stand for a week. Crystallisation was rather slow, so the solution was placed in the refrigerator, when white nodules soon separated out (seen on the sides of the container) This soon stops and then a yellow substance started to separate out. After the week the solution was filtered and the insoluble residue was washed first with methyl alcohol and then water. When dry, the residue was dissolved in acetone (excess sodium carbonate remains undissolved and is filtered off) For clarification the sodium carbonate insoluble portion will be regarded as fraction I; the filtrate from fraction I, as fraction II.

Fraction I. which weighed 3 gm. was dissolved in acetone and put onto Al_2O_3 , acetone being added to the column until the filtrate became colourless (fraction (1)). The column was then washed through with alcohol, which removed those substances strongly adsorbed and not removed by acetone, the washing being continued until the eluate was again colourless (fraction (11))

Fraction (11)

On evaporation of the acetone a mixture of substances, including white, round nodules; some yellow material and a waxy substance, was obtained. The impure mixture was dissolved in 25 ml. dichloroethylene, put onto Al_2O_3 column (67 x 1.5 cm) and

developed with the same solvent. The chromatogram could be divided into four fractions: (a) a fraction which ran through very rapidly, imparting a cream colour to the Al_2O_3 . The band was readily washed through with dichloroethylene and gave a dark yellow solution, the latter on evaporation deposited an oily substance and some needles and prisms. Different solvents viz. ether, petroleum ether and carbon tetrachloride were used to try to remove the oily material from the crystalline, but complete separation could not be effected, thereby rendering crystallisation difficult. In this case it was found advantageous to rechromatograph the fraction and in this way the prisms were obtained as a fraction which readily washed through the column of Al_2O_3 . Crystallisation from a mixture of chloroform and alcohol gave colourless prisms. m.p. $181.6^\circ\text{C}.$ (corr.) Substance G.

(b) Fraction b was obtained by washing through a light yellow band following fraction a. The fraction however was thus also rechromatographed, when in a similar manner to fraction a, Substance G. was obtained.

(c) Fraction C. represented by an upper orange band on the column of Al_2O_3 gave only a very small amount of material which could not be worked up further.

(d) After fractions a, b and c had been washed through by the dichloroethylene, the Al_2O_3 was washed with alcohol until the eluate was colourless. On evaporation of the alcohol, a white crystalline material together with some resin, was obtained, ^{and} which on crystallisation first from a mixture of acetone and methyl alcohol, followed by aqueous acetone yielded

white flakes of substance D. m.p. 160°C (corr) -
yield 0.5 gm.

fraction (11) which was obtained by washing the Al_2O_3 with alcohol after fraction (1) had already been removed by acetone, gave a small amount of white crystalline material and a little resin on the evaporation of the alcohol. When crystallised from aqueous acetone, white flakes of Substance D. m.p. 160°C . (corr) were obtained.

Fraction II i.e. the methyl alcohol filtrate obtained after the filtration of the sodium carbonate insoluble fraction, was treated with 20 ml. 10% caustic soda solution, the methyl alcohol solution rapidly became darker in colour, while some precipitation took place. The alkaline solution was allowed to stand for two days, filtered and the precipitate washed with a little methyl alcohol. The precipitate so obtained was dissolved in acetone, the solution filtered to remove a small amount of undissolved residue and the acetone solution put onto a column of Al_2O_3 . The column was washed with acetone until the eluate was colourless (fraction (14)), followed by washing with alcohol to remove a reddish band on the upper part of the column (fraction 11).

fraction (1) The acetone eluate was evaporated to dryness and yielded a mixture of crystalline material, which was dissolved in dichloroethylene and put onto Al_2O_3 . On development of the chromatogram three fractions were obtained.

- (a) A lower pale yellow band which ran through fairly rapidly and deposited some white crystalline material and oily material on evaporation. The latter was removed by washing with ether and the crystalline material crystallised from aqueous acetone

as white prisms - m.p. 181.6° C. (corr)-

Substance G.

(b) represented by a yellow band which was fairly strongly adsorbed but was readily eluted with acetone, deposited only a small amount of material on evaporation.

(c) was obtained by washing the Al_2O_3 with alcohol after (a) and (b) had been separated. On evaporation of the alcohol, some resin and white crystalline material were deposited. Crystallisation of the residue from aqueous acetone yielded white flakes of Substance D - m.p. 160° C (corr) - Yield 0.08 gm.

fraction (11). which was obtained by elution with alcohol of the reddish band mentioned above also gave on evaporation, followed by crystallisation of the residue from aqueous acetone, white flakes of Substance D.

Since the treatment of the caustic soda soluble fraction has already been described above (see alkali treatment), it will not be discussed here.

In order to ascertain whether Substance G. and D, obtained by the treatment of the resin with alkali, occurred as such in the resin or were precipitated as a result of certain structural changes brought about by the alkali, it was decided to examine the resin without preliminary treatment with either caustic soda or sodium carbonate.

Experimental.

Straight forward Chromatography.

3 gms. of the resin were dissolved in 15 ml dichloroethylene and put onto a column of Al_2O_3 (1.5 x 66cm). On development with the same solvent four fractions were obtained.

fraction (1) was obtained by washing through a lower colourless band and deposited a small amount of prisms on evaporation of the solvent

fraction (ii) was represented by a pale yellow band just above (i) and was also readily washed through. The solution on evaporation deposited a beautiful crop of colourless prisms, identical to those obtained in fraction (i) to which it was added. A small amount of oil which contaminated the prisms could be readily removed by washing with ether, when the prisms were obtained practically pure, m.p. $131.6^{\circ}\text{C. (corr)}$ - Substance G. Yield 0.07 gm.

fraction (iii) A darker yellow band which did not wash through as rapidly as (i) and (ii) and could thus be separated from them, deposited on evaporation of the solvent, a mixture of yellow needles and a small amount of yellow prisms. Crystallisation of the mixture from a mixture of 20% dichloroethane and ethyl alcohol gave yellow needles of m.p. $21.0^{\circ}\text{C (corr.)}$ - Substance A. - Yield 0.18 gm. The mother liquor on evaporation to dryness deposited a mixture of Substance A and some yellow prisms (Probably Substance B.)

fraction(iv). was obtained by washing the Al_2O_3 with alcohol after fractions (i) (ii) and (iii) had been washed through with dichloroethylene. As no preliminary separation of the resin had been carried out, fraction (iv) consisted largely of resinous material, together with a smaller amount of a white substance which was suspected to be Substance D, but this point could not be verified as the substance was exceedingly difficult to purify due to the large amount of resin present. Since the object of this experiment was to try and isolate Substance D from the resin, it was decided to effect a preliminary separation of most of the resin before subjecting it to chromatography with dichloroethylene.

Preliminary purification of the Resin.

Work on the crystalline material had shown that a solution in ethyl acetate on Al_2O_3 readily effected separation of the resin from the crystalline substances. The effectiveness of the method when applied to the resinous fraction was therefore investigated (The effectiveness of acetone for this purpose had already been established).

5 gms. of the resin were dissolved in redistilled ethyl acetate and put onto a column of Al_2O_3 . The resin was readily adsorbed, while the crystalline substances passed through into the filtrate without any visual development of a chromatogram. The column was washed with the ethyl acetate until the filtrate was quite colourless. Excess ethyl acetate was then distilled off and the solution evaporated to dryness to yield a mixture of crystalline substances and a small amount of resin, and was not a clear cut separation as with the acetone. The residue was dissolved in dichloro-ethylene, put onto Al_2O_3 and developed with the same solvent. The chromatogram was exactly the same as that described above for the straight-forward chromatography (see above) with the exception that fraction (iv), obtained by washing the column with alcohol was not so badly contaminated with resin and in this case Substance D. could be isolated after three crystallisations from aqueous acetone, when colourless flakes - m.p. 160°C (corr) were obtained - Yield 0.05 gm. - Substance D. The yield of fraction (1) i.e. Substance G. in the above chromatogram was 0.07 gm. from the 5 gms of resin.

From the above series of experiments it is concluded that Substances D and G. occur as such in the resin and are not substances which have been altered in any way by the treatment with the alkali; the latter merely serves to precipitate them. An advantage of the above method of isolation, i.e. ^{the} alkali treatment of the resin, is that the substances so precipitated are comparatively free from resin

Dr. P. Groenewoud of the U.O.P.S., in a preliminary examination of *N. ficifolia*, isolated from the tuber, in addition to rotenone, a further substance which will be referred to as Substance E. This Substance which has a m.p. of 193.5°C (corr) consists of bright yellow, microscopic needles. (The preliminary examination had been carried out on a different batch of plant material to that used in the present investigation and had been collected about April).

The very fact that Substance E had not been isolated in the present investigation led to the suspicion that the substance might be present in the resin, but had undergone some structural change when the resin was treated with alkali. In order to determine this point 20 mg. of Substance E (isolated by Prof. Groenewoud) in alcohol were treated with 5 ml. 1N caustic soda solution and the solution allowed to stand for a few days. Substance E. showed no change as the result of the action of the alkali i.e. no modified product separated out from the solution. It was concluded that Substance E was evidently not present in the plant material, under investigation since it had not been isolated from the crude crystalline material nor the resin. This was evidently due to a difference in the ages of the tubers used in the present investigation and those used in the preliminary examination. Seasonal variation too undoubtedly has some influence on the nature of the substances isolated at any particular month. This fact has already been established by B.L. van Duuren and P.W.G. Groenewoud when investigating the tubers of *N. edulis* (2).

TABLE I

TABULAR SUMMARY OF METHODS OF ISOLATION ETC.

I. Fraction A. Crude Crystalline Material.

Substance.	Isolation & Separation	Purification.
A.	Chromatography first with di-chloroethane - lower yellow fraction - rechromatographed with dichloroethylene - lower yellow fraction eluted on column.	Crystallisation from 20% dichloroethane and ethyl alcohol.
B.	Recovered from mother liquors by mechanical separation of crystals in many cases.	Crystallisation from a large volume of boiling ethyl alcohol.
C.	Recovered from the strongly adsorbed dark brown band obtained by chromatography with dichloroethane. Eluted by extraction, in Soxhlet, with acetone and alcohol. Separated from resin by allowing soln. to stand when substance C. separated out.	Crystallised twice from methyl alcohol, and then acetone.
F.	Also recovered from mother liquors of yellow fraction	Crystallised from a very dilute chloroform and alcohol solution.

II FRACTION B. Resin.

D.	Methyl alcohol soln. - treat with alkali or Na_2CO_3 - ppt. dissolve in acetone, chromatograph, wash column with alcohol, small amt. of D. recovered. Remainder (obtained by washing with acetone above) chromatographed in ethylene dichloride. Wash column with alcohol, Substance D. recovered.	If contaminated with much resin, crystallise first from a mixture of acetone and methyl alcohol, followed by aqueous acetone.
G.	Precipitate obtained above, chromatographed with acetone to remove resin, followed by use of ethylene dichloride. Fraction I which readily washed through deposits crystals of Substance G. Oily material removed by ether.	Crystallised from 40% dichloroethane and ethyl alcohol, followed by n-butyl alcohol. Aqueous acetone also used.
A, B, F.	Recovered from alkali soluble fraction - coagulating with water, coagulate dissolved in acetone, resin removed by Al_2O_3 . The residue chromatographed in ethylene dichloride, Recovered as in I. above.	

APPROXIMATE PERCENTAGE YIELD OF SUBSTANCES.TABLE II.

Substance.	Percentage Yield %	
	A. of Crude Crystalline material.	B. of Resin.
A.	50%	0.67
B.	2.5	0.16.
C.	5.6.	-
D.	-	1.8.
F.	3.3	0.1
G.	-	2.3

In order to indicate which substances, isolated from *N. ficifolia* in the present investigation, are identical to compounds isolated from *N. edulis* by B.L. van Duuren (2) a comparative table of the substances is given below. Table III. Identical substances are marked with an asterisk.

TABLE III.

M. edulis. (Investigated by B.L. van Duuren.			M. Ficifolia. Investigated by author.		
Substance:	Crystalline form.	M.P.(corr)	Substance:	Crystalline form.	M.P.(corr).
(i) *	yellow needles.	211°C.	* A.	yellow needles.	210°C.
(ii)	colourless needles.	251° - 252° C. (decomp)			
(iii) *	light-yellow prisms.	180°C.	* B.	yellow prisms	180°C.
(iv)	straw-coloured prisms.	149°C.	C.	colourless powder.	136°C.
(v)	yellow needles.	256°C.	D.	colourless flakes.	160°C.
(vi)	colourless needles.	230°C.	E.	bright - yellow microscopic needles.	193.5°C.
			F.	pale-yellow needles.	197°C.
			G.	colourless, short prisms.	181.6° c.

Unless otherwise stated, all melting points given in Chapter III are corrected.

CHAPTER IV.

CHEMICAL INVESTIGATION OF ISOLATED COMPOUNDS.

Before proceeding with this chapter it would be desirable to present and compare the physical properties of the substances, isolated together with their corrected melting points. This data is given in Table IV.

TABLE IV.

Substance.	Colour.	Crystalline form.	Corrected m.p. °C.
A.	Yellow	needles.	210°C.
B.	Yellow to sea green.	prisms or plates.	180°C.
C.	Colourless.	powder.	136°
D.	Colourless: shiny.	flakes.	160°
E.	bright yellow.	microscopic needles.	193.5°
F.	pale yell- ow to colourless.	broad rod-like needles.	197°
G.	Colourless.	short prisms.	181.6°

Part I.

(a) Qualitative Analysis.

Analysis of all seven substances revealed that they were nitrogen, sulphur and halogen free i.e. they were composed of C.H. and O. Only.

(b) Quantitative Analysis.

Percentages carbon and hydrogen were determined by Dr. Weiler of Oxford. Molecular weights were determined by the author by the Micro-Rast (8) using camphor as solvent. The percentages methoxyl were also determined in four of the substances isolated, using the Micro-Zeisel method (9). The results are given in the Table below.

TABLE V.

Subst:	Found.			Molecular Formula.	Calculated.		
	C.%	H.%	M.W.		C.%	H.%	M.W.
A.	68.06	3.56	333	C ₁₉ H ₁₂ O ₆	67.85	3.57	336
B.	65.98	4.25	312	C ₁₈ H ₁₄ O ₆	66.27	4.29	326
C.	72.98	12.27	226	C ₁₃ H ₂₆ O ₂	72.90	12.15	214
D.	82.21	11.63	270	C ₁₈ H ₃₀ O	82.44	11.44	262
E.	65.82	3.64	314	C ₁₇ H ₁₂ O ₆	65.37	3.85	312
F.	67.74	3.97	282	C ₁₆ H ₁₂ O ₅	67.61	4.22	284
G.	67.61	3.72	379	C ₂₂ H ₁₄ O ₇	67.68	3.59	390

Determination of percentages methoxyl in four substances viz. A, D E and G gave the following results:-

Substance A. 8.9% C₁₉ H₁₂ O₆ requires 9.2%
 " D
 " E 15.9% C₁₇ H₁₂ O₆ requires 9.9%
 " G 8.1% C₂₂ H₁₄ O₇ requires 7.95%

Part II

Reactions.

As mentioned above a colourless substance melting at 161° (uncorr) has been isolated by Dr. P. Groenewoud of the U.O.P.S., in a preliminary examination of the tuber of this plant. The suspicion that this substance is rotenone was confirmed by a mixed m.p. determination with a sample of pure rotenone which gave no depression, and also by a series of tests characteristic for rotenone, e.g.

(1) Thus in the Durham test(10) the substance developed a blood red coloration with conc. nitric acid, followed by a bright blue coloration when added to ammonium hydroxide, the colour rapidly fading.

(2) The Jones and Smith Test. (11 - 12)

In this Test the Substance produced a Blue-green colour, similar to the colour produced by a sample of pure rotenone.

(3) The Rogers and Calamari Test.(13)

The Substance gave a yellowish-green colouration identical to a control experiment using pure rotenone.

(4) The Cross Smith Goodhue Test (14 - 15)

In this Test the presence of rotenone was indicated by the development of a red coloration, a reaction given by neither toxicarol, tephrosin, isotephrosin, dehydrorotenone, rotenone, nor dehydrodeguelin.

From the above series of reactions it is concluded that the substance melting at 161°C . (uncorr) is rotenone.

All seven substances gave negative reactions with alcoholic ferric chloride. Of the seven substances, A has been more thoroughly investigated, although Substance A possesses non-toxic properties, it occurs in the tuber in relatively large amounts. Further it is known to occur in *H. edulis* another fish-poisoning species, so that elucidation of it's constitution may throw some light on the nature of the constituents of these plants, especially the active principles.

Preliminary Investigation of Substance A.

From analysis and molecular weight determination this compound has been assigned the formula $\text{C}_{19}\text{H}_{12}\text{O}_6$ which is not in agreement with results obtained by B.L. van Durren(4). No substance represented by this formula and which corresponds with it's physical properties, has been found in the literature.

Substance A is insoluble in water, alcohol, the hydrocarbon solvents; benzene and petrol ether, but readily soluble in chloroform and dichloroethane. It's solution

in ethylene dichloride shows a marked green fluorescence. It is insoluble in both acids and alkalis and gives no ferric chloride reaction. The substance possesses reducing properties, reducing both ammonical silver nitrate and Tollen's reagent.

In attacking the problem of its constitution, the most important point to establish is the nature of the oxygen atoms. From methoxyl determinations (Found 8.9% $\text{-C}_{19}\text{H}_{12}\text{O}_6$ requires 9.2%) although results are a bit low, the presence of one methoxyl group seems to be established. A negative reaction with ferric chloride and its insolubility in alkali would seem to exclude the presence of phenolic hydroxyl groups, this fact being corroborated by an attempted acetylation of the substance, when the original compound was recovered unchanged. Although the substance has reducing properties, attempts to establish the presence of a ketonic group or groups by formation of an oxime and 2,4-dinitro-phenyl hydrazone were also unsuccessful. The presence of a carbonyl group, however is not entirely excluded, since it is known that certain substances containing a carbonyl group yield the above derivatives only with difficulty, Coumarins, for instance only yield the above derivatives by reaction on the corresponding thio derivatives (16). In the methylenedioxy tests (Vide infra) positive reactions were obtained in both cases. An attempt was made to determine the number of methylenedioxy groups quantitatively, the reliability of the method however leaves much to be desired. As colour reactions may also be somewhat misleading, and since no definite proof can be advanced as regards degradation of the substance at this stage, it will be sufficient to say that presence of one or more such groups is suspected, until such degradative or synthetic work has definitely established this point, or otherwise

Experimental.

(1) Attempted acetylation (17)

0.03 Gm. of Substance A was treated with 1 ml. freshly distilled acetic anhydride and 0.03 gm. anhydrous sodium acetate. The solution was boiled under reflux for three and a half hours, and the mixture poured into 15ml. water and vigorously stirred. Substance A was recovered unchanged. (The substance is soluble in the acetic anhydride giving a clear solution.)

(2) Tests for a Carbonyl Group

(1) Oxime formation (18)

0.04 gm. Substance A; 0.03 gm. hydroxylamine hydrochloride and 0.03 gm. sodium acetate in 2ml. alcohol was heated for eight hours on a waterbath. On pouring the product into water the original substance was recovered unchanged.

(11) 2,4 - Dinitrophenylhydrazone. (19)

The reagent is a saturated solution of 2,4-dinitrophenylhydrazine in alcohol (about 1%) containing 1% hydrochloric acid. 5 ml. of this solution was added to a few centigrams of Substance A dissolved in alcohol. No apparent reaction seemed to take place after standing at room temp. for a few hours. Some more hydrochloric acid was therefore added to the solution which was heated for two and a half hours on a waterbath. On pouring the solution into water a red ppt. readily separated out, and which on crystallisation from aqueous acetone proved to be unchanged Substance A contaminated with 2,4-dinitrophenylhydrazine.

(3) Tests for Methylene Dioxy Groups.

(1) Qualitative Tests (20-21)

(a) Method of Labat. (20 - 22)

It was found that a successful positive reaction in the case of Substance A was only obtained by careful control:- Thus a trace of Substance A was treated with 2 ml. conc. H_2SO_4 , followed by a drop of 5% alcoholic

Gallie acid solution, when, after standing for a few hours, or by gentle heating, a green coloration developed, the solution when examined by means of the spectroscope showed an absorption band in the red. This test is characteristic of Substances possessing methylenedioxy groups.

(b) Method of Clowes and Tollens (20-21, 23)

The method has also been applied to the quantitative determination of these groups and depends on the liberation of formaldehyde, which condenses with an appropriate reagent to give the characteristic colour and precipitate.

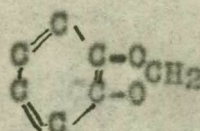
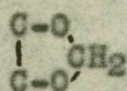
Procedure.

0.02 gm. of substance A was dissolved in 5 ml. Phloroglucin - sulphuric acid mixture by heating. 2 ml. conc. sulphuric acid was added to the solution, stirred and placed in a waterbath for 45 minutes. A red colour was produced, followed by a thick, copious, reddish brown precipitate. In a control experiment with piperonal (Heliotropin) which contains one methylenedioxy group, a red coloration was produced immediately, together with a copious precipitate which resembled that given by substance A. The main difference between the two experiments was the rates of reaction, as will be seen from the above description.

(c) Reaction with Carbazole (24)

E. Votocék and V. Vessely have carried out a series of reactions on substances containing methylene groups, making use of the fact that carbazole, dissolved in hot glacial acetic acid, in presence of hydrochloric or sulphuric acids, readily condenses with formaldehyde to form a white insoluble product. The reaction they found serves to distinguish the nature of the methylene group, since

only certain groups react under these conditions. Thus - a positive reaction is given by the methylene derivatives of the sugars and their acids, containing the grouping:-



ie, methylene groups bound to oxygen are readily yielded to carbazole, in contrast to methylene groups bound to a five membered ring which do not react under these conditions. Further methylene groups bound to nitrogen react under these conditions, while methylene groups bound to carbon atoms do not.

The fact that substance A, when subjected to this test, did not react at all would seem to suggest that the nature of the methylene group is that of one attached through two oxygens to an aromatic ring as shown by the above Formula

(2) Quantitative Determination of Methylenedioxy Groups.
(20, 21, 23)

The method applied to resistant methylene groups was used in this case, since preliminary examination had shown that Substance A is not so readily attacked, and further is relatively insoluble in the reagents used. The method has been extensively investigated by Clowes and Tollens (23).

Procedure.

3 gm. Phloroglucin was warmed with a mixture of 100 gm. sulphuric acid and 100 gm. water. After standing for at least one hour the solution was filtered. 0.1-0.2 gm. of substance A. was weighed into a flask provided with a reflux condenser and a thermometer; 30 ml. of the above solution was added and the solution was heated for three hours at approx. 80°C. A further 10ml. of conc. sulphuric acid was then added and the heating continued for a further three hours. The solution was allowed to stand overnight and the precipitate which had formed was filtered through a

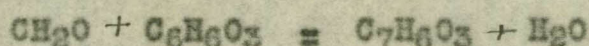
a weighed Gooch crucible, washed with 60 ml. water, dried at 100°C for four hours and finally weighed after cooling for 1 hour.

Weight of Substance A I = 0.1015 gm. II. = 0.1017 gm.

Weight of Formaldehyde - Phloroglucid =

I.	II.
0.1561 gm(23)	0.1546 gm.

Clowes and Tollens on investigation of the nature of the condensation product found that one molecule formaldehyde condenses with one molecule phloroglucin according to the equation.



$$\text{ie. } \text{C}_7\text{H}_6\text{O}_3 : \text{CH}_2\text{O} : 4.6 : 1$$

Therefore weight of Formaldehyde =

$$\text{I } \frac{0.1561}{4.6}$$

$$= 0.03393 \text{ gm.}$$

$$\% = \frac{0.03393 \times 100}{0.1015}$$

$$= 33.4\% \text{ CH}_2\text{O}$$

$$\text{II } \frac{0.1546}{4.6}$$

$$= 0.03360 \text{ gm. Formaldehyde.}$$

$$= \frac{0.03360 \times 100}{0.1017}$$

$$= 33.1\%$$

Theory requires 9.85%

In a control experiment, in which a substance with a known number of methylenedioxy groups was determined, the following results were obtained.

Control Experiment using Heliotropin
(Piperonal).

Weight of Heliotropin = 0.1019 gm.

Weight of Formaldehyde-Phloroglucid = 0.2192 gm.

$$\frac{0.2192}{4.6}$$

=

$$0.04764 \text{ gm. Formaldehyde.}$$

$$\% = \frac{0.04764 \times 100}{0.1019} = 46.77\% \text{ CH}_2\text{O}$$

Theory requires 23.07 %

Thus in the control experiment the amount obtained is exactly double that required by theory; the reliability of the method therefore is to be doubted. Counciler (25) stated that one molecule of Formaldehyde condenses with two molecules of phloroglucin. This was however disputed by Clowes and Tollens. The latter only used this method for the determination of methylene derivatives of different sugars, and only mention that in all probability it could be used for aromatic substances viz. piperonal and safrol, probably with certain modifications. A blank determination, using only the reagents, showed that, either some phloroglucin, or some other impurity is precipitated during the experiment (since this is lighter in colour it would be obscured by the dark, reddish-brown formaldehyde-phloroglucid) which would seem to indicate that some other factor must be taken into account when calculating the % Formaldehyde. Finally, it may be mentioned that substance A is very resistant even to the strong reagents used here. This is evidenced by the length of heating resorted to, so that the reaction might not have gone to completion, in which case unchanged product would have been filtered off with the precipitate.

Since no reliability is placed on the results obtained by this method, it will be sufficient to state that presence of one or more methylenedioxy groups is indicated.

In a further effort to establish the existence of a methylenedioxy group, an attempt was made to split off the methylene group of the substance and thereby isolate the dihydroxy derivative so liberated. This method has been extensively investigated by E. Späth⁽²⁶⁾ and H. Quinetensky⁽²⁶⁾. It was by this method too that E. Späth, P.K. Bose and J. Schlager (27) successfully determined the constitution of Ayapin, a methylenedioxy coumarin.

Experimental.

0.06 ga. of Substance A was treated with 10 ml.

Phloroglucin-sulphuric acid mixture, (Vide supra). A further 2.5 ml. conc. sulphuric acid was added and the solution heated on a waterbath for one and half hours, when a further 2 ml. sulphuric acid was added. The solution, initially dark orange in colour, gradually assumed a dark red coloration and finally a reddish-brown colour, while later a reddish brown precipitate separated out. A further 2 ml. conc. sulphuric acid was added after three hours heating, and the heating continued for another three hours. The total time of heating was therefore six hours. The solution was then allowed to stand overnight, poured into 90 ml. water, and the solution allowed to stand for another hour. The dark reddish-brown precipitate which had formed was filtered off, the filtrate was extracted with a large amount of ether, the ethereal extract was washed with water and finally dried over sodium sulphate. The excess ether was removed by distillation, while the residue consisted of a resinous product, together with some white flake-like material. In an attempt to separate the latter, the resinous material was dissolved in acetone and put on a small column of Al_2O_3 . A large amount of the resin was adsorbed, the white material passing through the column into the filtrate. On evaporation of the acetone the substance was still contaminated with some resin and the amount of product obtained so small that it has not, at this stage, been possible to isolate it in a pure state. The possibility that this is some unchanged phloroglucin is not excluded. The resinous substance, dissolved in alcohol, gave a positive reaction with ferric chloride, the exact nature of the colour being difficult to determine, as it was obscured by the colour of the resin.

Substance G.

In certain colour reactions this substance showed some

interesting facts viz.

In the Durham test (10), the substance in contact with conc. nitric acid showed a red colour which with ammonium hydroxide developed into a dirty blue to green coloration.

In the Jones-Smith test (11-12) (vide supra) a purple-red coloration developed. (The rotenone group and related substances all give different hues of blue in this test, while toxicarol gives a purple colour(11).

In the Cross-Smith-Goodhue (14-15) no red coloration developed, while finally in the Rogers-Calamarri (13) test, a light orange-green colour was produced.

These facts seem to indicate that substance G. might be related to the rotenone group of fish poisons. This point has however not been investigated. It is definitely not any of the known substances of this group, this being evident from the physical properties of the compound.

Substance G. did not give positive methylenedioxy tests. Further, it is insoluble in caustic soda in the cold. With conc. sulphuric acid alone it develops a green coloration.

CHAPTER V.

TOXICITY DETERMINATIONS.

This chapter deals with the establishment of the fish-poisoning properties of the different substances isolated; the determination of the toxicity curves of the active principles thus established and their comparison with that of rotenone, one of the best known and thoroughly investigated fish poisons. These determinations may not only prove ^{of} the value for constitutional studies(2) but also for comparison of insecticidal properties, fish poisoning properties usually, though not always being associated with insecticidal action. (28)

Methods for determining the toxicity of substances known to possess fish poisoning properties, in which goldfish are used as the test animal, are to be found in the literature(29, 34, 37).

The same method has been used by B.L. van Duuren and F.W.G. Groenewoud(38) for determining the toxicity of the active principle isolated from *Neorautanenia Edulis*, and was the method initiated by Powers(37) subsequently modified by ^{Gersdorff} the author, ^{the method was used here but} certain changes however were introduced, this being due to the fact that the test animal in this case was the fresh-water fish, *Lebistes Reticulatus*.

The activity of the different substances isolated had first of all to be determined. Because all of them were insoluble in water, some organic solvent non-injurious to the fishes had to be used in the tests. Gersdorff(29) and van Duuren (38) found acetone suitable, provided certain concentrations were employed. Since the substances isolated in the present investigation were soluble in acetone at the concentrations employed, this solvent was also employed in these tests. The difficulty

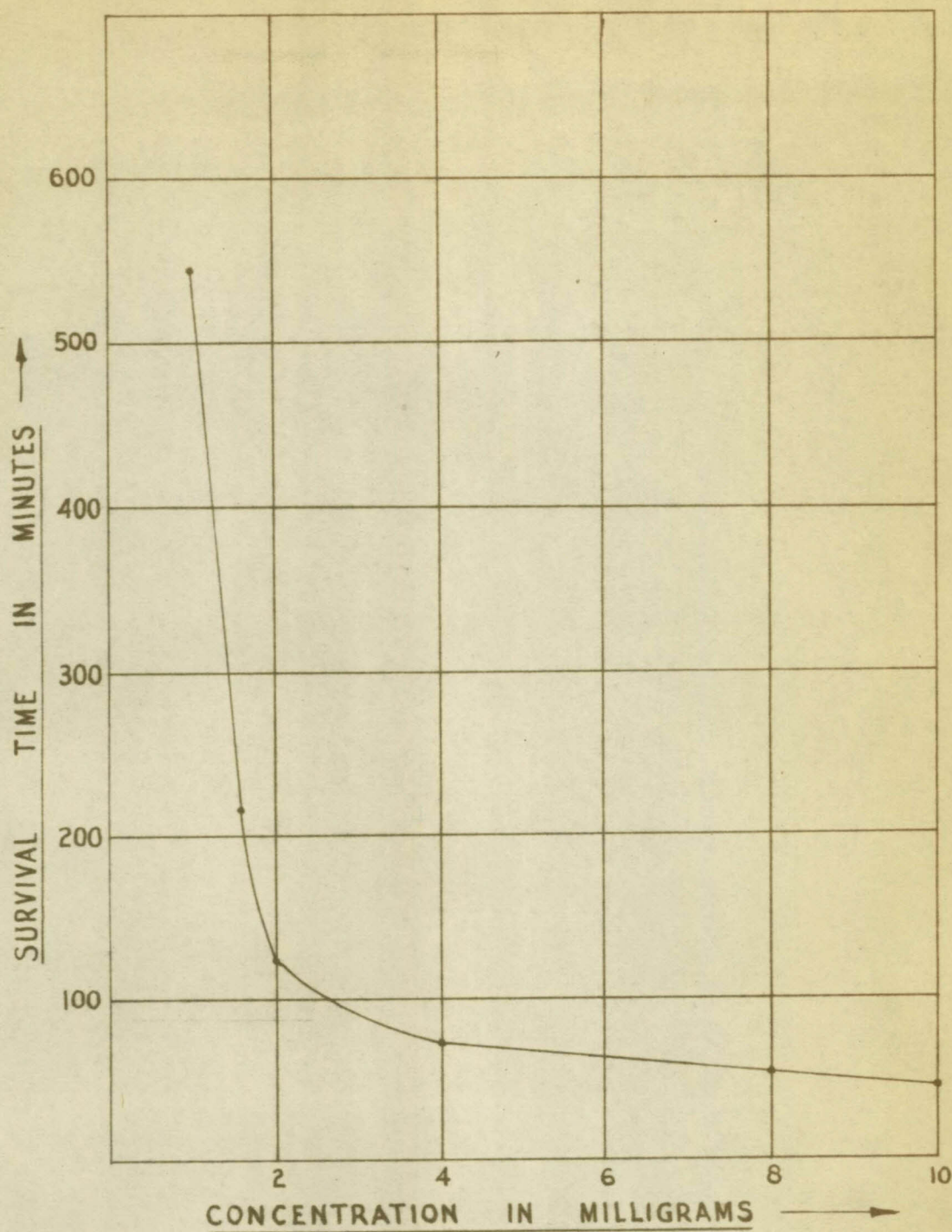


FIGURE 1
TOXICITY CURVE OF SUBSTANCE B
DATA OBTAINED FROM TABLE VI.

experienced by B.L. van Duuren and P.W.G. Groenewoud(38) that these substances in acetone soln. when added to the aqueous test solutions immediately separated out from solution, was also encountered in this investigation. The method used by B.L. van Duuren and P.W.G. Groenewoud(38) of the addition of a protective colloid to the aqueous solution therefore had to be resorted to. They found that for this purpose a solution of gelatine in water proved admirable; this also proved to be the case with the substances isolated from the tuber of *Neorautanenia fleifolia*.

Preliminary experiment had to decide whether these reagents were suitable for carrying out tests on *Lebistes Reticulatis*, and if so, the best concentrations to employ. In all tests, distilled water, which had been aerated by bubbling air through it for five hours, was used.

Experimental.

Since the fish used in these tests were taken from a stock tank in which the water was maintained at a constant temperature, ($\pm 25^{\circ}\text{C}$) the solutions in which the fish were to be tested were initially heated to 25°C and maintained at this level to within $\pm 1^{\circ}\text{C}$. before the fish were placed therein.

Two fishes were tested in each of the following solutions, in order to determine the effect of the concentrations of acetone and gelatine on the fish.

3 parts acetone in 1000 parts 5% gelatine solution.							
2	"	"	"	1000	"	2%	"
1.5	"	"	"	1000	"	1.5%	"
1	"	"	"	1000	"	1%	"
0.5	"	"	"	1000	"	0.5%	"

The fishes showed no sign of distress after half an hour in any of the concentrations employed i.e. the reagents were not detrimental to the fish at these concentrations. It is of interest to note that Gersdorff (29) had to employ a concentration of 1 part acetone to 1000 parts

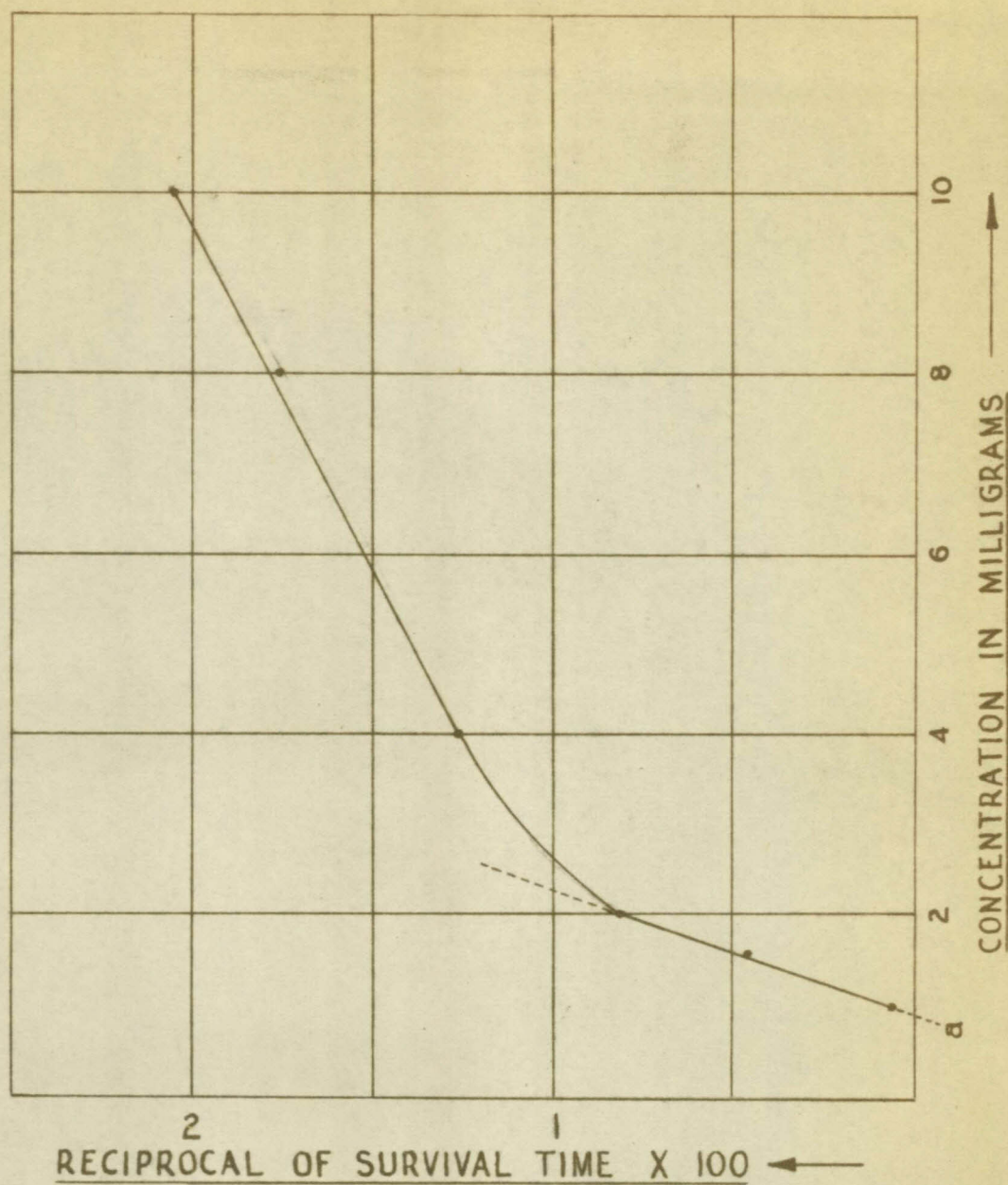


FIGURE 2
VELOCITY OF FATALITY CURVE OF SUBSTANCE B
DATA OBTAINED FROM TABLE VII.

water for tests on the goldfish, for the fish showed signs of distress, within half an hour, at a concentration of 2 parts acetone to, 1000 parts of water.

Since it was found that the 1% gelatin solution served to keep the substances in suspension, this was the concentration employed in the remainder of the experiments, except in those cases in which low concentrations of material were used, when a 0.5% gelatin solution was found suitable. The amount of acetone solution added was such that the concentration was 2 parts acetone per 1000 parts water, i.e. 0.5 ml. per 250 ml. solution.

Determination of Substances Toxic to the fish.

For testing the toxicity, a concentration of 20 mg./L of each substance was initially tried. A 1% gelatin solution was used, as stated above. Tests were carried out in 600 ml. beakers, the volume of gelatine solution employed for each test, being 250 ml. for two fishes.

Of the seven substances tested, three viz. Substances B. E and G were toxic to the fish being effective within two hours. Substance G. showed symptoms different from the other two; thus the fish immediately showed signs of great excitation followed by a period of quiescence in which the fish appeared to be dead but on being touched moved away rapidly. Invariably too with Substances G there was a tendency for the fish to turn over onto their backs.

The other substances viz. A, C, D and F did not appear to cause the fish any amount of discomfort, even a concentration of the substances double that used above viz. 40 mg/Litre did not have any ill-effects on them.

Survival time curves of the three toxic substances were then determined and compared with that of rotenone which was also determined in a similar manner. These curves are obtained by carrying out tests on the fish at various concentrations and determining the time necessary to kill the

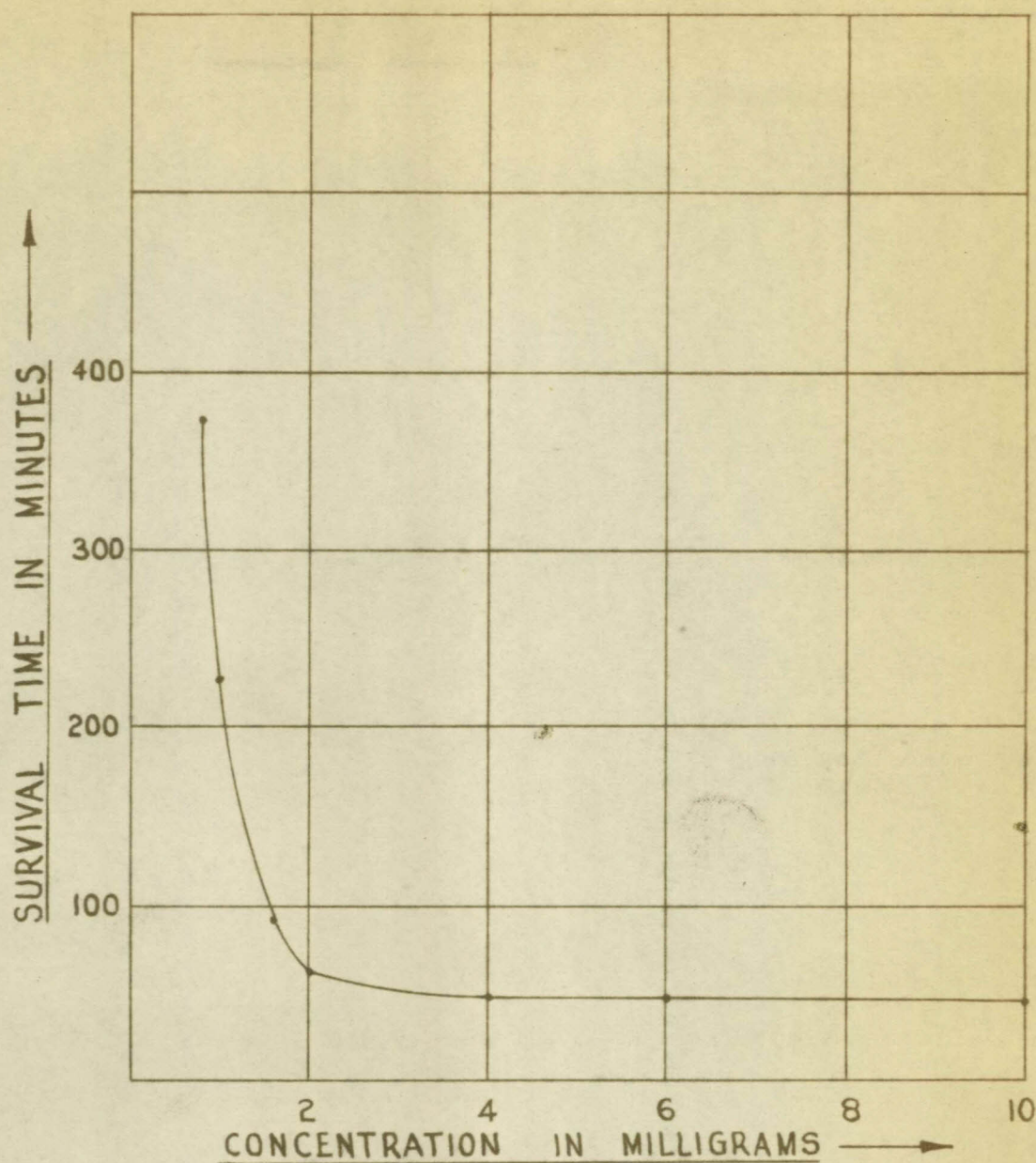


FIGURE 3
TOXICITY CURVE OF SUBSTANCE E
DATA OBTAINED FROM TABLE VIII.

fish (or any other chosen criterion). On plotting the survival time in minutes as ordinates against concentration in mg. as abscissae the so called survival time curves are obtained (29). This was done with the twofold purpose of comparing the toxicities of the three substances with rotenone and for securing data which may throw some light on the constitution of these substances (38). Plotting the reciprocal of the survival time $\times 100$ against concentration in mg., gives the so called velocity of fatality curves. This method of comparing toxicities is the one used by Powers (37) and later by W.A.Gersdorff(29) and to date is the best known method for this purpose.

In determining toxicities experimental conditions are important, further, there is the question of individual variation of the fish, which may however be largely eliminated by the use of relatively large numbers of fish at each concentration. Thus Gersdorff (29) has shown statistically that although the percentage error of a single observation on points on the critical portion of the curve (*vide infra*) may be as high as 20%, by using 10 or twelve fishes for each determination the % error of the mean may be lowered to 7%. In the present investigation only 2 fishes were used at each concentration so that the curves cannot be considered to be so accurate, but at least they serve for a practical comparison of the substances. Since the essential precautions and experimental conditions in the determination of toxicity have already been fully discussed by van Duuren (4) it will suffice to mention the conditions observed in the present investigation.

(a) The weight of Fishes:

In the present toxicity determinations all the fishes were approximately of the same size, viz. 3-10 mm. the actual weight was not ascertained.

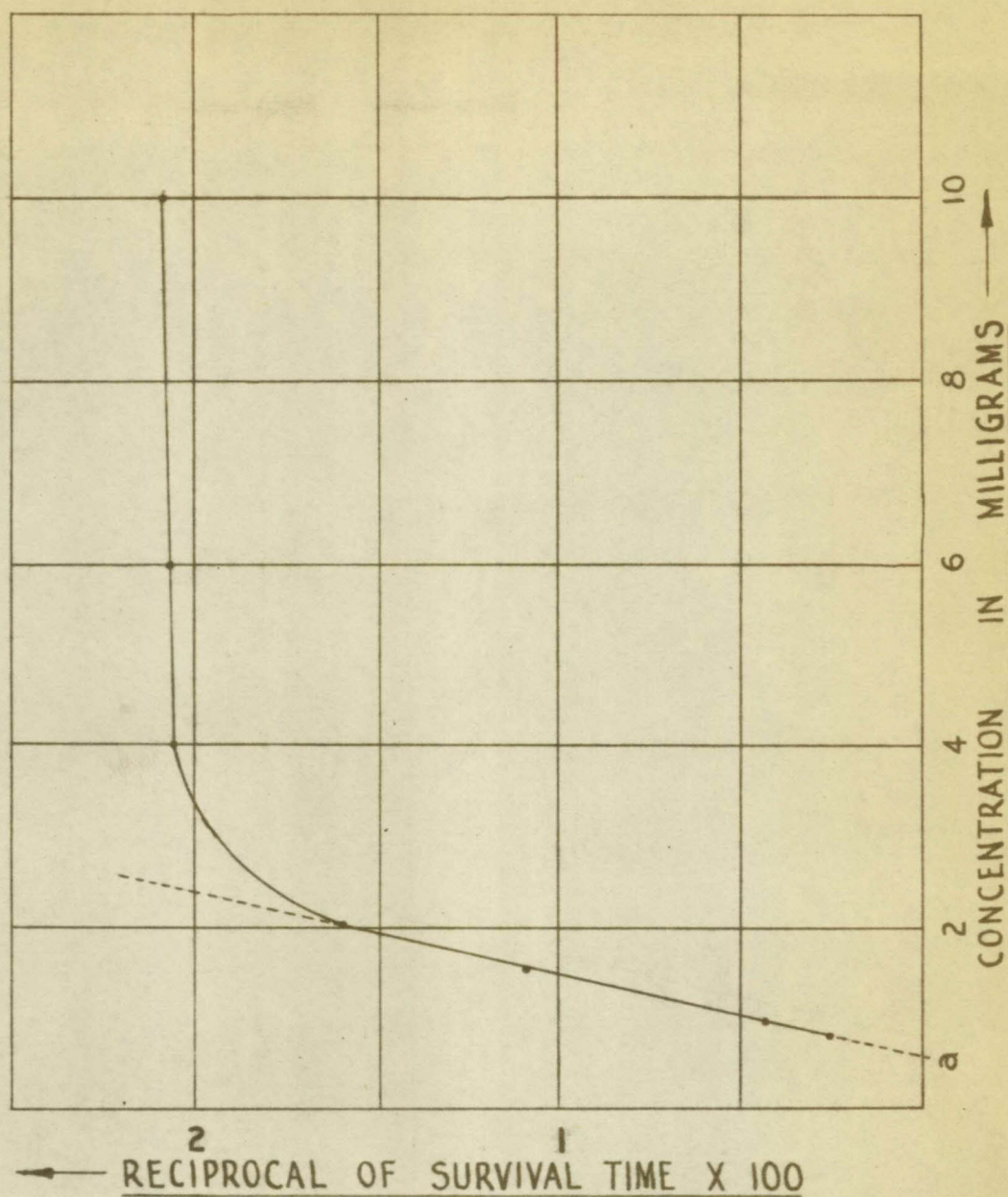


FIGURE 4
VELOCITY OF FATALITY CURVE OF SUBSTANCE E
 DATA OBTAINED FROM TABLE IX.

(b) Species of Fish.

In these experiments the freshwater fish, *Lepistes reticulatus* was used.

(c) Temperature.

Since the fish were kept in water at a constant temperature, and the fish being somewhat sensitive to lower temperatures, the test solutions had to be raised to approximately the same temperature before the fish were placed therein. A temperature of 25°C. was the temperature used, being kept constant to within 1° by means of waterbaths.

(d) Volume of Water.

Due to the small size of the fish a volume of 250ml for two or even three fishes was sufficient.

(e) Number of Fishes.

The accuracy of the determination as regards the number of fish has already been mentioned above. Only two fishes for each concentration were used in the tests.

(f) Howard A Jones and H.H. Hallen (39) have found that rotenone undergoes decomposition in solution yielding yellow compounds which are non-toxic, the change being dependent upon factors such as solvent, access of air etc. Therefore, although the true nature of the substances isolated is not known, it was deemed advisable to use only freshly prepared solutions in all toxicity determinations.

Before comparative toxicities of the substances can be made by interpretation of curves, the general nature of the curve should be known. Thus three distinct characteristics can be recognised (29-34). There is a horizontal portion in which a large increase in concentration corresponds to a very small increase in survival time; a so called middle portion, also known as the critical portion, where the velocity of fatality (as

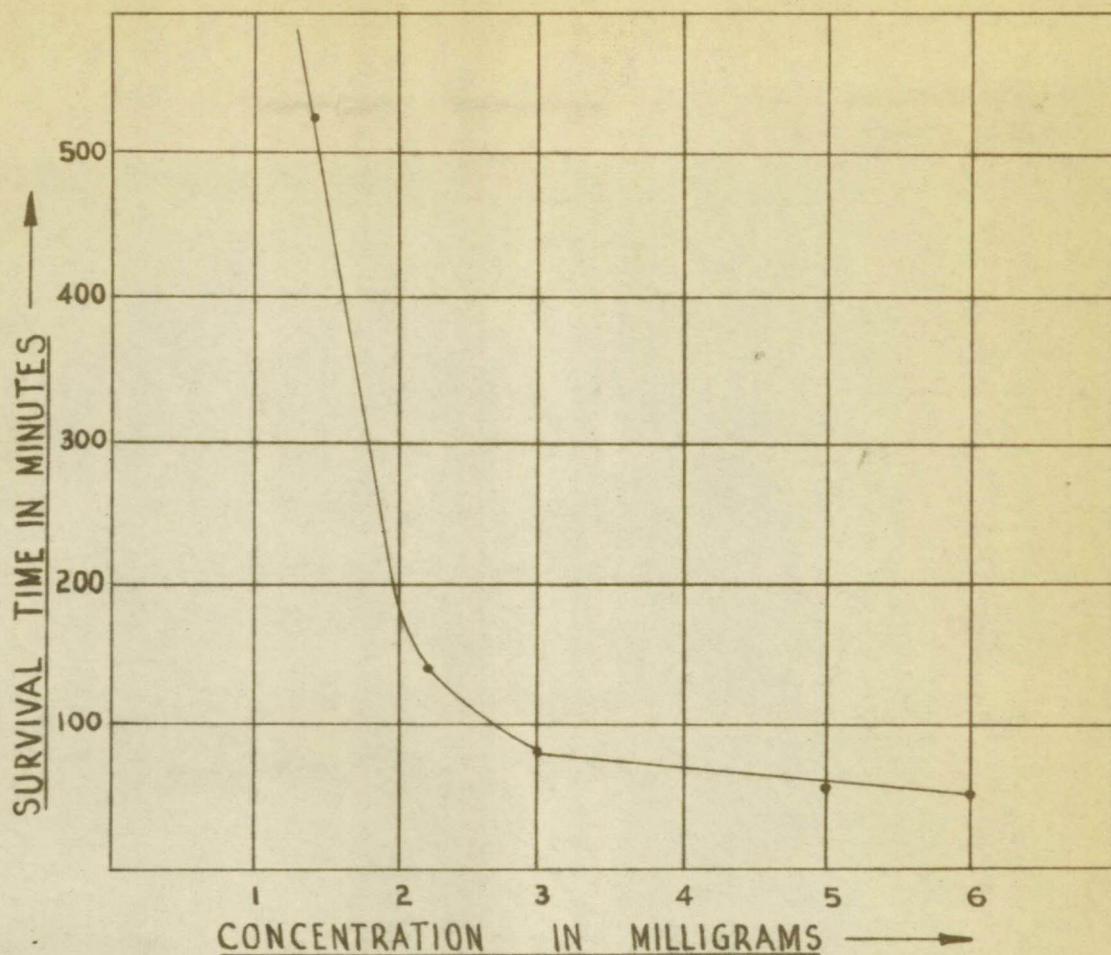


FIGURE 5
TOXICITY CURVE OF SUBSTANCE G
DATA OBTAINED FROM TABLE $\bar{X}$

measured by the reciprocal of the survival time) increases most rapidly with increase in concentration and approaches an equilateral hyperbola, therefore, the corresponding portion in the velocity of fatality curve which is drawn with the reciprocal of the survival time as the ordinates approaches a straight line. This straight line is called the theoretical velocity of fatality curve and when prolonged cuts the X axis at a point which Powers designated as the theoretical threshold of toxicity. Finally we have that portion of the curve approaching the vertical. Although several methods are available for comparison of toxicities, most of them have been found to be unsuitable and as yet a criterion suitable for the expression of relative toxicity has not been developed. The methods used by various workers have been fully discussed by Van Duuren & Groenewoud(38). In addition to these W.A. Geradoff (40) has developed a criterion for the comparison of toxicity with respect to concentration and time, viz. the minimum ct . product (product of concentration and survival time) which he states agrees with results obtained by other methods. He also found that Glaser's equation (41) an equation expressing the relation between concentration and toxicity, is the most nearly applicable of those examined to the concentration - survival time curves obtained in studies with rotenone and related compounds in which the goldfish was the test animal. The equation, however, is too difficult to use in simple comparison of the toxicity of these compounds.

Experimental.

Survival time, and velocity of fatality curves are plotted from data obtained by determining the survival times in minutes at various concentrations of substances B, E G and rotenone. Preliminary experiments had to determine the correct concentrations to use, since concentration data

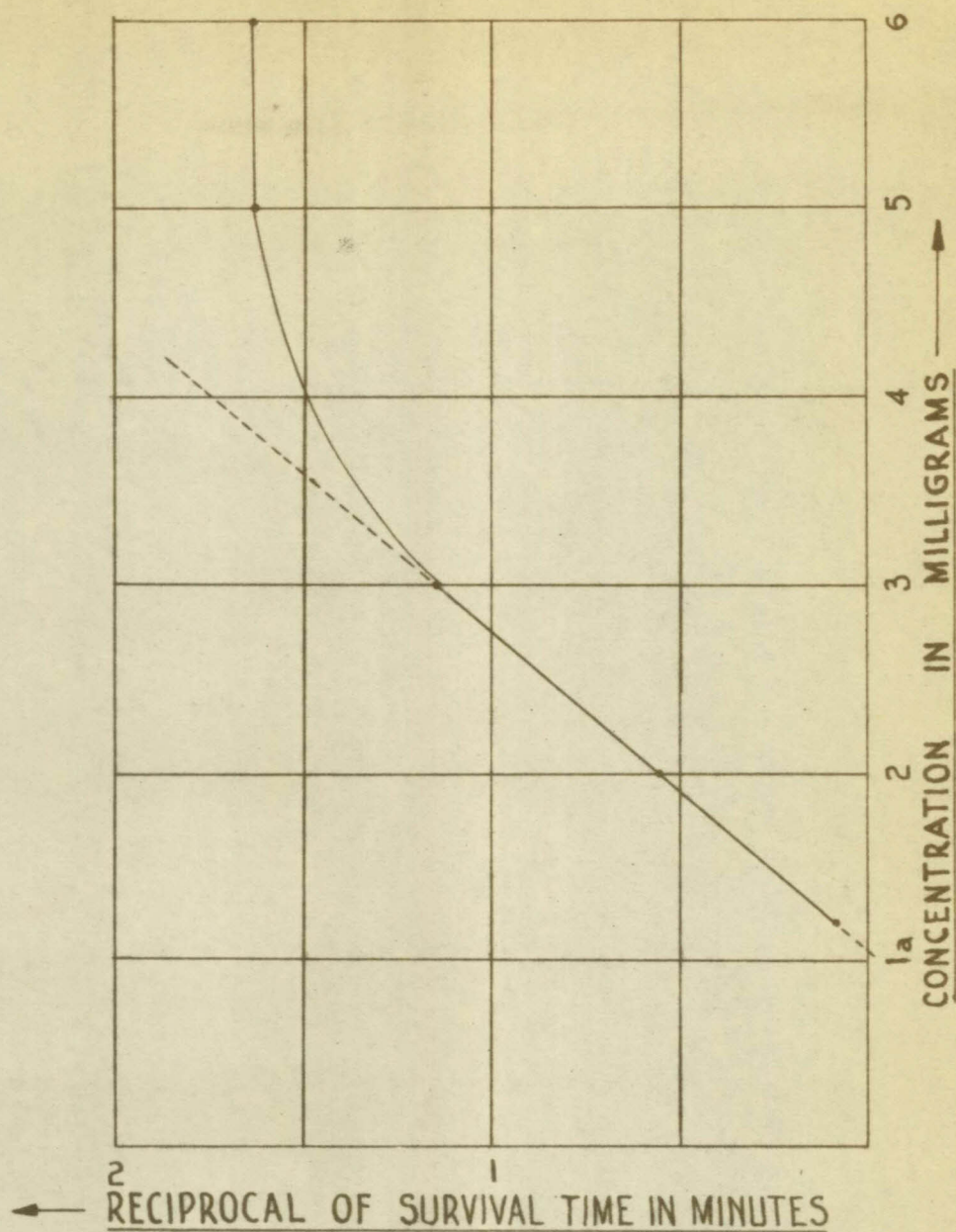


FIGURE 6
VELOCITY OF FATALITY CURVE OF SUBSTANCE G
 DATA OBTAINED FROM TABLE XI

50.

falling outside the critical portion of the curve are useless for comparisons of toxicity. Further in these experiments the weights of the fishes were not determined, the fish however were approximately of the same size ($\pm$ 8-10 mm. in length) and of the same age (approximately two months).

Table VI.

Toxicity to *Lebistes reticulatus* of Substance B. at
 $25^{\circ} \pm 1^{\circ}$ C.

Concentra- tion mg/L.	No. of Fish.	Mean length of Fish in mm.	Medium:	Survival time in mins.	Mean Survival time in mins.
10	2	$\pm$ 8-10 mm.	1% Gelatin.	44 53	48.5
8	2	8-10 mm.	" "	52 62	57
4	2	8-10 mm.	" "	69 87	78
2	2	8-10 mm.	0.5% Gelatin	108 138	123.5
1.6	2	8-10 mm.	" "	169 267	218.
1.0	2	8-10 mm.	" "	543 547	545

TABLE VII.

VELOCITY OF FATALITY OF SUBSTANCE B.

Concent. mg./Litre.	Mean l x 100 S.T.
10	2.06.
8	1.75
4	1.28
2	0.81
1.6	0.46
1.0	0.18.

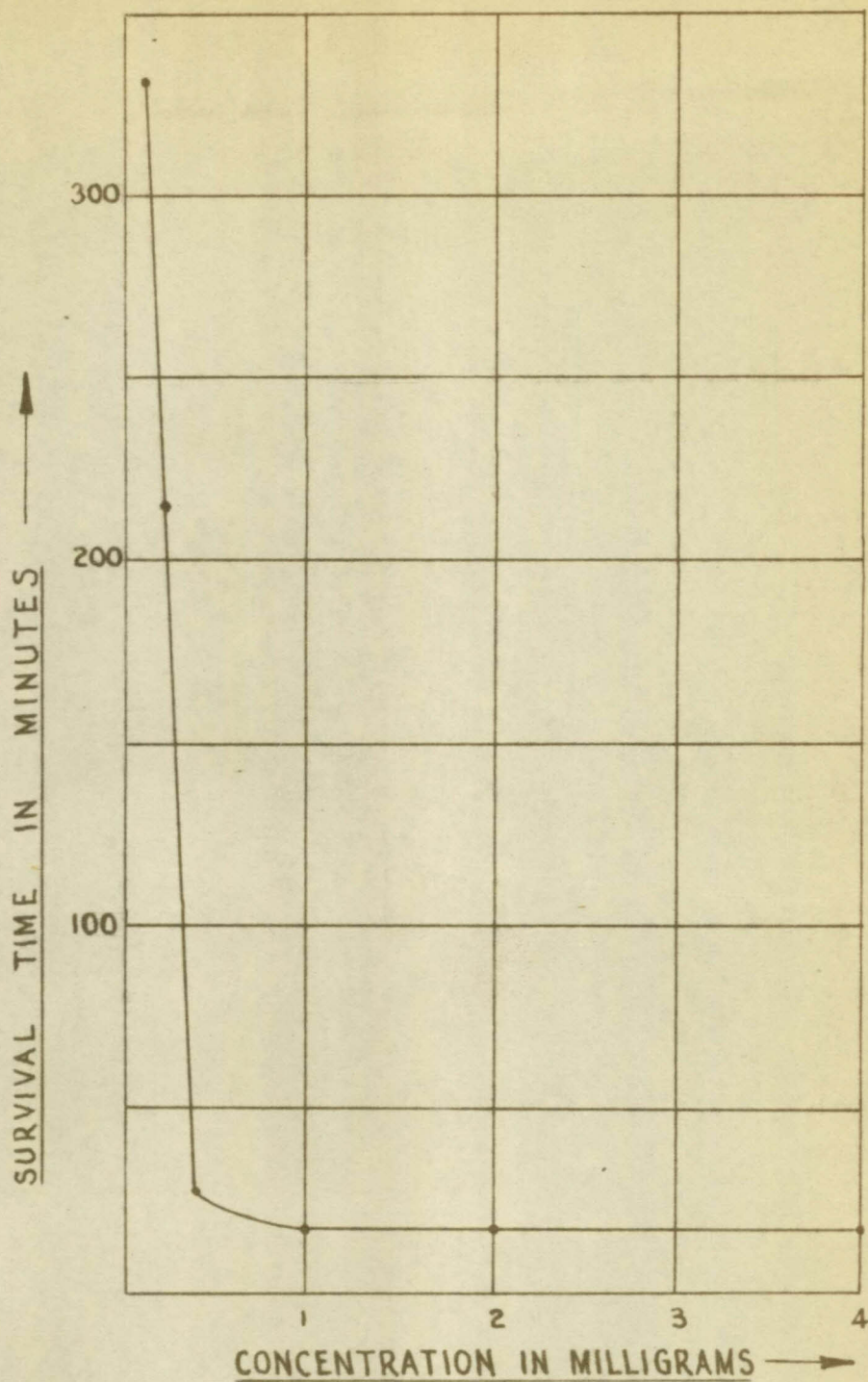


FIGURE 7

TOXICITY CURVE OF ROTENONE

DATA OBTAINED FROM TABLE XII

TABLE VIII.

Toxicity to *Lebistes reticulatus* of Substance E at
25° C $\pm 1^\circ$

Concen- tration mg./L.	No. of Fish.	Mean Length in mm.	Medium.	Survival Time in mins.	Mean Sur- vival Time in mins.
10	2	8-10 mm.	1% Gela- tine.	45 50	47.5
6.0	2	" " "	1% Gela- tine.	49 45	47.0
4.0	2	" " "	1% Gela- tine	37 57	47.0
20	2	" " "	1% Gela- tine	62 63	62.5
1.6	2	" " "	0.5% Gela- tine	72 116	94
1.0	2	" " "	" "	208 245	226.5
0.8	2	" " "	" "	360 382	371.

TABLE IX.

VELOCITY OF FATALITY OF SUBSTANCE E.

Concentration mg./Litre.	Mean l x 100 S.T.
10.	2.1.
6	2.1.
4	2.1.
2	1.6.
1.6	1.08
1.0	0.44
0.8	0.27

52/.....



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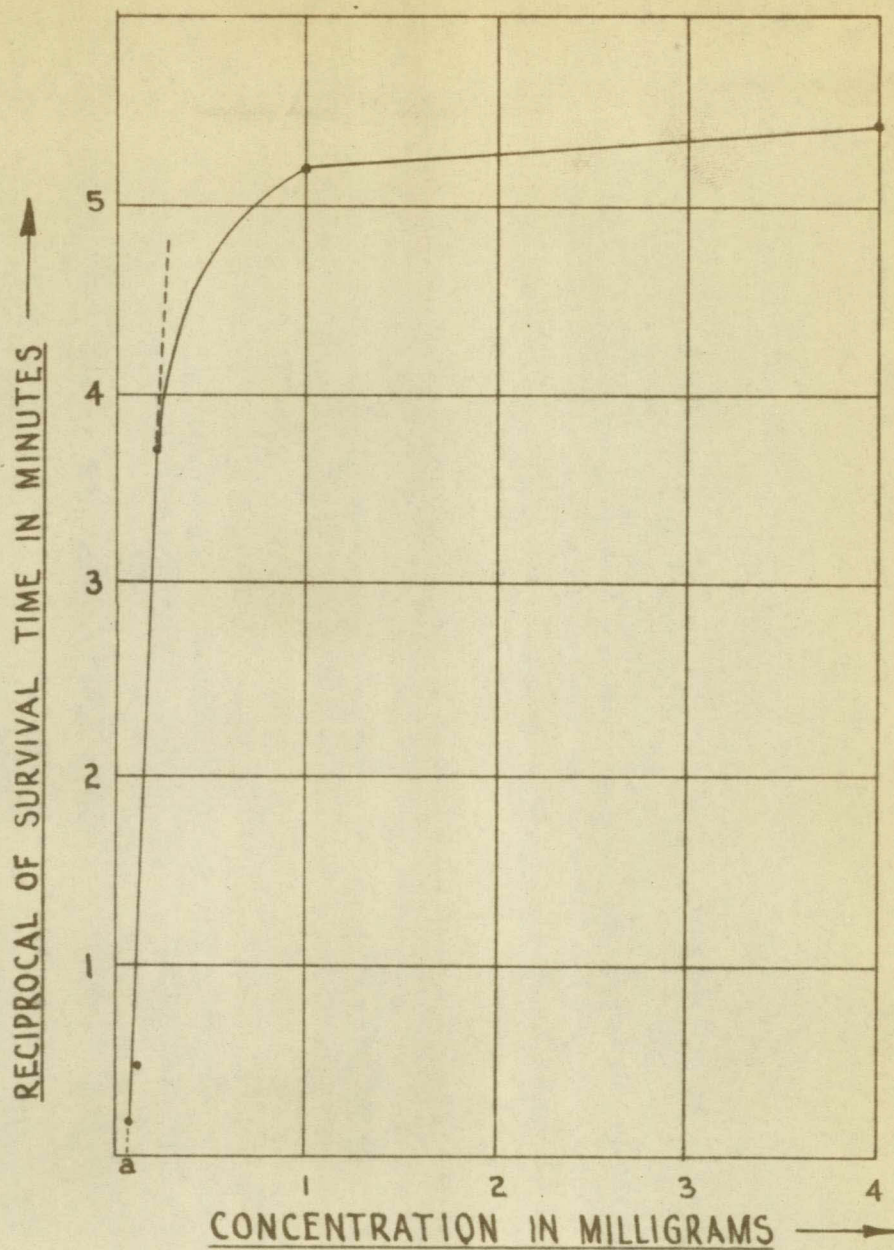


FIGURE 8
VELOCITY OF FATALITY CURVE OF ROTENONE
DATA OBTAINED FROM TABLE XIII

TABLE X.

Toxicity to *Lebistes reticulatus* of Substance G. at
25° C. $\pm$ 1° C.

Concen- tration mg./L.	No. of Fish.	Mean Length in mm.	Medium.	Survival time in mins.	average Survival time in mins.
6	2	$\pm$ 8-10 mm.	1% Gela- tine.	59 61	60
5	2	" " " "	" "	52 62	60.5
3	2	" " " "	" "	74 94	84
2				156 179	173
1.4	2	" " " "	" "	529 519	524.

TABLE XL.

VELOCITY OF FATALITY OF SUBSTANCE G.

Concentration mg./Litre.	Mean l x 100 S.T.
6	1.67
5	1.65
3	1.19.
2	0.58
1.4	0.19.

Difficulty in determining the exact survival time was experienced with Substance G, as the fish although apparently dead, moved when touched. This fact has already been mentioned above.

TABLE XII.

Toxicity to *Lebistes reticulatus* of Rotenone at
25° C. $\pm$ 1° C.

Concen- tration mg/Litre.	No. of Fish.	Mean Length in mm.	Medium.	Survival time in mins.	Mean Sur- vival time in mins.
4	3	$\pm$ 8-10 mm.	Distilled aerated. H ₂ O	20 20	18.6
		" " " "	" "	16	
2	3	" " " "	" "	20 20 18	19.
1.0	3	" " " "	" "	17 20 21	19.
0.2	3	" " " "	" "	20 20 40	26.6.
0.1	2			220 270	215
0.05	2			295 370	333

TABLE XIII.

VELOCITY OF FATALITY OF ROTENONE.

Concentration mg/ Litre	Mean l S.T. x 100
4	5.4
2	5.3
1.0	5.3
0.2	3.8
0.1	0.47
0.05	0.30

The curve for rotenone resembles that given by Gersdorff(30). However whereas he found for (a) a value of 0.0125 mg. in this case (a) was found to have a value of about 0.04 mg.

CONCLUSIONS. Comparative data are obtained from the above curves. As compared with rotenone, Solutions of Substances B, E and G become toxic at higher concentrations. The theoretical thresholds of toxicity i.e. the concentration below which the substance does not cause death are approximately 0.9; 0.6; 1.15 and 0.04 mg./Litre for Substances B, E and G and rotenone respectively i.e. toxicity decreases in the order rotenone > Substance E > Substance B > Substance G. The threshold of toxicity of Substance G is approximately twice; that of Substance B one and a half ($1\frac{1}{2}$) times, that of Substance E. The theoretical velocity of fatality increases with increase in concentration in the order, Rotenone > Substance E > B > G. these comparisons are made by consideration of the velocity of fatality curves.

Minimum survival times (30, 32, 33) of these four substances is in the order, rotenone, Substance B, E and G; is 19 less than 44, 47 and 60 minutes respectively, i.e. at higher concentrations when the rate of increase of velocity of fatality decreases, Substance Band E are nearly equally toxic and Substance G a little less so, while rotenone is considerably more toxic than all three. It must be mentioned that it is not meant that this is the minimum time, in which fish may be killed in solns. of these materials.

As a measure of the relative toxicities of the three substances studied here, use may be made of the Powers formula (37, 38) toxicity = $\sqrt{\frac{\text{Tan } c}{a}}$, although it must be borne in mind that it is not all that could be desired, since it involves only two of the three variables of the velocity of fatality curves (30). Until, however, a more acceptable formula for expressing the course of toxic action is found, use is made of this formula. Data obtained from the velocity of fatality curves are used in the comparative table given below.

TABLE XIV.

Comparative Toxicities at 25° C. of Substances B, E and G to *Lebistes reticulatus*.

Substance.	a, Mg. per Litre.	Tan θ	$\frac{\text{Tan } \theta}{a}$	Relative toxicity with respect to rotenone.
B	0.9	0.77	0.29	0.012.
E	0.6	1.16	1.39	0.058.
G	1.15	0.58	0.23	0.0095

a is based on the expression of concentration in milligrams per litre. a, being the point of intersection of the velocity of fatality curve which approaches a straight line, when prolonged to cut the X-Axis.

Tan θ . Rate of increase of the theoretical velocity of fatality with increase in concentration.

Tests were carried out on the fish using acetone extract of:-

- (a) The dried powdered plant material.
- (b) The crude crystalline material (Fraction A).
- (c) The resin (Fraction B.)

which indicated that Fraction B was the most toxic, followed by the dried powdered plant material, while the crude crystalline material was not as toxic as (a) and (cc). The fish in the case of (a) died within 30 minutes and (c) within 20 min. The rapidity with which these extracts killed the fish led to the immediate suspicion that the really "active" principle had not been isolated, although the above tests could not be regarded as accurate, the actual concentrations of the various substances present not being known. Presence of a trace of rotenone in these extracts was not excluded although qualitative tests

carried out on a, band c failed to reveal it's presence. It was at this stage that it was thought that the presence of Saponins, already indicated in the resin and mentioned by R.H. Aston, (1) might also be responsible for the fish poisoning properties of the plant, Saponins are known to possess this property. This fact is mentioned by Czapek (42) and also by Haas and Hill (43), the fishes being killed by Saponins at a concentration of one part in 100,000 parts of water. Although a little work was done in an attempt to isolate the Saponin, (see addendum) lack of time has prevented any definite conclusions as regards the activity being drawn.

It is of interest to note that E. Späth in his publication on the natural coumarins deals shortly with the physiological action. Of particular interest is the description of the symptoms shown by the fish, *Lebistes reticulatis* in the tests. He describes thus:-

"Anfangs zeigte sich bei diesen Tieren meist erregende, dann aber beruhigende Wirkung. Sie verloren allmählich das Gleichgewicht, lagen oder schwammen auf dem Rücken stellten dann die Fortbewegung ein und schliesslich erfolgte der Tod"

The above symptoms appear to resemble those shown by these fish when tested with Substance G.

Gersdorff, (45-46) who carried out numerous experiments on rotenone and it's derivatives using the goldfish as the test animal, observed that change in chemical composition of the compound effects a characteristic change in toxicity independent of the effect of any other change.

Thus the dihydro derivatives produced by saturation of the double bond in the side chain of rotenone and it's derivatives with hydrogen atoms, have 1.5 times the toxicity of the corresponding unsaturated compounds.

i.e. increased substitution caused an increase in toxicity. The combined effect on the toxicity of more than one change in the constitution is equal to the product of the individual effects. These results are in general agreement with the results observed by Lauger, Martin, and Müller(47) that increasing substitution caused an increase in toxicity.

CHAPTER VI.SUMMARY.

For many years considerable interest has been taken in a number of tropical plants, extracts of which have been used as fish and arrow poisons. There has been considerable commercial development of these plants as sources of insecticides and in this connection the root of the Derris has been widely used. More recently, *Neorautanenia ficifolia*, a fish poisoning plant occurring in Eastern South Africa has been reputed to possess insecticidal activity. The purpose of this investigation therefore was to attempt an isolation of the toxic principle or principles of the tuber of this plant.

The best method of extraction, and the one used in this case, was cold ether percolation. In this way, from twelve kilograms of dried tuber, two fractions were obtained viz. Fraction A - The crude crystalline material and Fraction B- the resin. Both fractions were investigated with a view to separation and isolation of crystalline substances. In the former fraction the method of fractional crystallisation was found unsatisfactory, the final method of separation used was chromatography. In the latter method a preliminary separation was effected by use of dichloroethane as the solvent, followed by ^{re}/chromatography with dichloroethylene. Al_2O_3 was the most satisfactory adsorbent. As eluents acetone, or a mixture of acetone and alcohol, was used. In the treatment of the resin a very satisfactory separation of two of the substances was effected by treatment of the resin in methyl alcohol with alkali, followed by chromatography with dichloroethylene. A further experiment, using only straightforward chromatography, established that these substances occurred as such in the resin and were not racemised products resulting

from the treatment with the alkali.

Seven substances were isolated from the tuber of this plant, while rotenone has been isolated in a small scale preliminary examination of the tuber. Qualitative elementary analysis showed that nitrogen, sulphur and halogen were absent i.e. the compound contained only carbon, hydrogen and oxygen.

Determinations of the percentages carbon and hydrogen were carried out by Dr. Weiler of Oxford. Molecular weights were determined by the author, by means of the Micro-Rast. Percentage methoxyl were determined in four of the substances, three of which were found to possess methoxyl groups, the Micro-Zeisel method was used in this case. Results and) Physical properties indicate that two of the Substances viz. A & B are identical to substance (1) and substance (iii) isolated from *H. edulis* by Van Duren and Groenewald (2). but % C and H do not agree.

A more thorough investigation of Substance A established the ~~presence~~ absence of phenolic hydroxyl groups and apparently also of Ketonic groups. A methoxyl determination seemed to indicate the presence of one methoxyl group. Positive colour reactions were obtained for the presence of a methylenedioxy group. Quantitative determination of the latter did not yield very satisfactory results, while an attempt to isolate the dihydroxy compound, obtained by splitting off the methylene group as Formaldehyde, gave resinous products together with some white material which could not be obtained in a pure state. The latter gave a positive ferric chloride reaction.

From colour reactions it is tentatively suggested that Substance G. might be a derivative of the rotenone group of fish poisons, but no experimental evidence to prove this can be advanced as yet.

Toxicities of the seven substances were then determined,

using as the test animal, *Lebistes riculatus*, a type of fresh water fish. Three of the substances were found to be toxic to the fish, These were Substances B, E and G.

Toxicity curves, obtained by plotting concentration in mg. against survival times in minutes, of the three substances were determined as well as that for rotenone. Velocity of fatality curves were also plotted by taking reciprocals of the survival time multiplied by 100 in place of the survival time. From the latter curves, comparative data regarding the toxicity of the three substances were obtained.

The Substances being insoluble in water, use was made in the experiments of a protective colloid, this method being described by previous authors. (38)

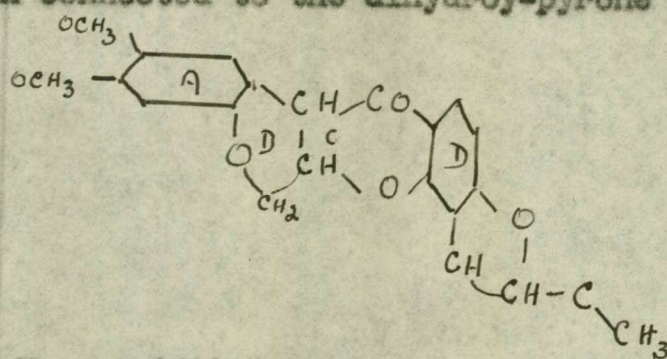
(a) Tests carried out on the fish using an acetone extract of the original plant material (b) Crude crystalline material and (c) Resin, showed that (a) and especially (c) were considerably more toxic than either of the pure substances isolated. This discrepancy was contributed to either the presence of a trace of rotenone, or a saponin, the presence which is definitely indicated, although attempts at the isolation of the substance have as yet, through lack of time, not been successful.

In this preliminary investigation it has not been possible to establish the structure of the toxic principles, nor their associated non-toxic components. It is only to be expected that they are in some way closely related to one another. The toxic substances of various other fish-poisonous plants have been the subject of a very extensive investigation, and thus it has been established that the fish poisons and insecticidal constituents which have been isolated from various tropical Fabaceous (leguminous) plants, (it is too noted that *M. ficifolia* is also a member of this group of plants) are characterised chemically as derivatives of a substituted dihydrobenzopyrone.....

system containing two adjacent asymmetric carbon atoms (48-49). The fact that *N. ficifolia* has been found to contain rotenone, would suggest that the substances isolated from this plant might also be a derivative of such a system. This would be a very interesting point to establish. Further work on the derivatives of the above system has led to the conclusion that they can be classified into three groups viz. (50)

(1) Rotenone Group.

Represented by rotenone itself and being the only natural representative. It has an optically active dihydrobenzofuran system, with an isopropenyl side chain connected to the dihydro-pyrone system.



Harper (51) from earlier work on the active principles of leguminous fish poisonous plants stated that a positive Durham test would seem to be specific for the chromeno-chromanone ring A, B, C and D.

(2) Deguelin Group

Being represented by Deguelin itself, together with tephrosin and isotephrosin. The group possesses an optically inactive dimethyldihydro - benzopyran system in place of the dihydrobenzofuran.

(3) Toxicarol Group.

The only member of the third group, toxicarol was originally thought to contain neither the dihydrobenzofuran nor benzopyran system (50) but Heyer and Robinson(52) have tentatively suggested it to be a 5-hydroxydeguelin.

E. Späth (53-56) has carried out extensive investigations of several other fish poisons, the toxicity of which does not nearly approach that of the above group of fish poisons and none of which is closely related to the rotenone group in structure, but nevertheless it is to be noted that they are also oxygen containing heterocyclics, usually possessing the α -benzopyrone nucleus, instead of the γ -benzopyrone system of rotenone.

A D D E N D U M.

Attempted Isolation of Saponin.

The presence of a saponin in the tuber of *M. ficifolia* was first demonstrated while working on the resinous fraction, but on chromatographic treatment the saponin seem to be lost. When the discrepancy regarding the toxicity of the resin, compared with that of the pure substances, was observed it was thought that this might be due to the presence of the saponin already demonstrated. Many saponins are known to possess the property of stunning and killing fish in very small amounts. (41-42, 57). *Milletia parchcarpa*, also a fish poisonous plant, has been found to contain a saponin and probably rotenone. (58)

A small amount of the original powdered plant extract had been kept and this was used in an attempt to effect isolation of the saponin. The original plant material when shaken with water formed a solution which readily foams, the lather persisting for a long time. In a preliminary experiment, 15 gms. of the plant material was extracted in a small Soxhlet with hot water. The extraction was rather slow, but the solution after one hour readily foamed, the foam sometimes rising right up the flask. The solution was quite clear and not contaminated with any resin. Since saponins are precipitated by lead acetate (57, 59), this reagent was tested on a portion of the solution and

found to give a precipitate. Addition of alcohol to the aqueous solution was also found to give a slimy white precipitate. Alcohol was therefore added to the bulk of the solution and the precipitate allowed to settle on the bottom of the flask when it could be removed from the bulk of the solution by decantation. The solution was evaporated to a smaller volume in vacuum and again precipitated with alcohol. The precipitate so obtained was slightly coloured when dry and was soluble in water to a great extent, (a small amount remained undissolved) the solution yielding a foam when shaken. When, however, the product was tested on the fish, the latter, although initially effected were not killed by the substance. On testing the substance so obtained with iodine in KI a violet coloration was obtained; this would indicate the presence of starch, though not conclusively so, for Saponarin, a saponin gives the same colour reaction with iodine and was originally termed "soluble starch" (60).

Isolation on a larger scale was then attempted, using 80% alcohol as the solvent, 220 gms. of the plant material being extracted in a Soxhlet for 15 hours. Evaporation of the alcohol gave a dark brown resinous product. On dissolving the resinous product in water, a large amount went into solution and yielded a solution which foamed strongly and was coloured light brown. Lead acetate solution was added to the aqueous solution, causing separation of a milky precipitate which was allowed to settle and then filtered. Although there appeared to be a relatively large amount of precipitate, when filtered off the amount obtained was very small.

A few qualitative tests have been carried out on the substance in order to establish the presence of the saponin thus:-

(1) The substance formed a stable foam when the aqueous solution was shaken (A characteristic test for

saponins) (42 -43, 57, 59-60).

(2) Precipitated from solution with lead acetate (acid saponins are precipitated by this reagent as the lead salt. 43, 59).

(3) Precipitated from solution by Barium hydroxide (neutral saponins are precipitated as their Ba compounds by this reagent) 43, 59, 60)

(4) The substance possesses reducing properties.

(a) It reduced ammoniacal silver nitrate(59).

(b) It reduced Fehling's solution after preliminary heating with dil. sulphuric acid.

(c) Blues a solution of Pot.Ferricyanide containing ferric chloride. (Saponins reduce ferric salts to ferrous and so give rise to the formation of Turnbull's blue (59).

(5) With conc. sulphuric acid a yellow coloration was produced which gradually darkens to orange on heating. (Saponins give a yellow, red and finally a violet coloration.) (60).

(6) With conc. sulphuric acid containing a little ferric chloride, a blue coloration was obtained with the substance. (Many Saponins give a blue, or a bluish-green colour or fluorescence in the above test. (57).

The tests seem to indicate that the substance is a saponin, but due to the lack of time, it has not been possible to continue with the investigation of the substance.

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