SOUTH AFRICAN FISH POISONOUS PLANTS VI.

ISOLATION AND STUDY OF SAPONINS FROM

NEORAUTANENIA EDULIS C.A. SM.

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science of the University of the Orange Free State.

BY

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C O N T E N T S.

C H A P T E R I.

INTRODUCTION.

The work contained in this thesis is a continuation of that done by R.R. Arndt[1] on the Aerial Portions of Neorautanenia Edulis C.A. Sm. and that pertaining to the fish-toxic substances in the tuber of the same plant, carried out by B. van Duuren[2] and A. Brink[3].

The natives using this plant, especially the tuber, for killing fish, made use of the fact that when the plant material is spread on water, the fish died or were stunned. This would suggest that water-soluble compounds, or alternatively, substances which when present in solution possess the property of solubilising insoluble plant material, were responsible for the fish-toxic properties of the plant. It is a fact worth remarking that all the fish-toxic substances isolated by the above authors, were practically insoluble in water.

Since it is known that certain saponins possess the above mentioned properties[5 & 6], and further, since there are definite indications that the plant in question is rich in saponins, the work described in this thesis was undertaken with the object to investigate the extraction, isolation and purification of

the saponin material in Neorautanenia Edulis C.A. Sm. Both the tuber and the leaves were examined.

In the tuber of Neorautanenia Ficifolia the presence of a saponin was established; the isolation however, has not yet been investigated.(4) In the Aerial portion of Neorautanenia Edulis, the work done by R.R. Arndt definitely proved the presence of a saponin-like substance, which however could not be isolated and purified. The impure saponin extracts which were prepared, were very highly fish-toxic(1). This would suggest that the toxicity of the plant was largely due to a fish-poisonous saponin or saponins.

C H A P T E R II.

EXTRACTION OF THE PLANT MATERIAL.

A. Extraction of the Aerial Portion.

Special boiling point benzine (B.Pt. 52 - 72°C), which possesses approximately the same solvent properties as petrol ether, but being less expensive, was used instead of petrol ether for the extraction(1).

The leaves and twigs of the plant, which were collected after the pods had formed, were dried in the sun and then broken up into small pieces in a mortar, ground to a powder in a coffee-mill, and placed into the different percolators.

In all, about 3 kilograms of this fine material were treated with the solvent. The percolate being allowed to run through at a rate of flow of two drops per second.

The first fraction, A, which was green in colour, was collected during the course of a week, and concentrated by distillation to a volume of approximately 300 ml. After standing, a thick yellowish layer of insoluble material separated from the combined concen-

trates. In a similar manner Fraction B, which was collected during the second week and being of a lighter yellow colour, was worked up to a concentrated form.

After three weeks of continuous extraction with benzine, a nearly colourless percolate resulted. No further work was however done on these fractions, since they appeared not to contain any saponins. This extraction had to be done in order to remove material which could interfere with the saponin extraction.

The plant material was then dried and extracted with ether in a similar manner to the above, to remove chlorophyll and fatty material. The darkgreen ether extracts were combined, and after concentration, left to stand. After about three weeks the extraction was discontinued, since the percolate had become more or less colourless.

The extracted plantmaterial was allowed to dry and was then treated with 95% ethyl alcohol, which had previously been purified from aldehydes and other active ingredients by boiling it for 8 hours under reflux with solid potassium hydroxide, followed by distillation. A darkgreen, nearly black percolate, which frothed very strongly on shaking with water,

was obtained. The extracts were again concentrated by distillation and left to stand. After a month and a half the extraction was discontinued, since the extract then was only faintly greenish-yellow or almost colourless.

After the plantmaterial had been exhausted with benzine, ether and alcohol, it was dried for extraction with water. This gave a darkbrown extract, which on shaking formed a strong, stable foam. The extracts were concentrated by vacuumdistillation. A difficulty encountered here, was that the solution foamed so strongly when heated in a vacuum, that it tended to run over with the distillate. This difficulty was overcome by adding one of the higher alcohols viz. methyl-n-hexyl carbinol, which definitely broke the foam(10).

After three weeks of extraction with water, a more or less yellow extract percolated through, which on being left open to the atmosphere, formed a thick white film on the surface. Whether this was due to oxidation by the atmosphere, is unknown. This percolate had a very unpleasant but characteristic odour of decaying plantmaterial. On being allowed to stand

for a considerable time, this concentrate underwent a peculiar colour change, becoming almost entirely black. The white film on the surface of the solution however, was still intact.

B. Extraction of the Tuber.

The moist tubers, were washed with water, cut up into small pieces, and dried in air and sunlight. When quite dry, the material was ground to powder in a coffee-mill and packed into a large copper percolator. The material was then soaked with pure anaesthetic ether, and the tap so adjusted that the ether percolated through slowly(2). The extract was first darkbrown in colour, then brownish-yellow, and eventually colourless. This extract was concentrated and used for other experiments not concerning saponins. Two different batches of ground tuber were subsequently treated by different methods.

Extraction I.

After the ether extraction, the material was allowed to dry, and then extracted directly with distilled water. This procedure however, did not prove very promising. The material swelled up in the perco-

lator probably as a result of fermentation, and eventually the extract had to be drawn off on a Buchner, much trouble being experienced due to excessive foaming. The extracts were concentrated by vacuum-distillation to a volume of about 500 ml., again using methyl-n-hexyl carbinol to break the foam.

On standing, an unknown fungus started to grow on the surface of the concentrate. This property was later made use of, as will be shown.

Extraction II.

After the ether extraction, the material was left to dry and then extracted with hot, 95% ethyl alcohol[11 & 12]. The extract was at first brownish in colour, but after repeated extraction, light-yellow solutions were obtained. The extracts were concentrated by vacuumdistillation and left to stand for further experiments.

Subsequently the ground tuber was treated with 45% ethyl alcohol[13]. The percolate was much darker in colour than that from the 95% alcohol, being very nearly black. These extracts were also concentrated by vacuumdistillation, but when the alcohol was dis-

tilled off, the water solution tended to foam so strongly that the distillation had to be stopped. The extraction was discontinued after a week, the ground tuber exposed to sunlight to dry, and a final extraction with water carried out.

Due to the fermentation encountered in Extraction I, the procedure in this case was ammended. Part of the dried tuber was heated in a beaker with distilled water on a waterbath for 1 hour. The solution was immediately drawn off on a Buchner funnel. No difficulty as a result of foaming was encountered during filtration. Toxicity tests subsequently proved that this extract was only slightly fish-toxic. It was therefore assumed that most of the saponins had been removed in the alcohol extractions.

C H A P T E R III.

Methods of Isolation and Attempted hydrolysis of Saponin-like Substance.

For purposes of comparison, most of the important methods suggested in the chemical literature for the isolation of saponin-like substances, were applied to the extracts prepared by the methods described in the previous chapter.

Thus Rochleder and also Christophson, precipitated saponins from aqueous solutions by addition of hot saturated bariumhydroxide. The resulting precipitate was decomposed with carbondioxide and the glucoside precipitated with ether-alcohol mixture (14).

Kobert and Pachorukow used an excess of neutral lead acetate added to the aqueous saponin solution, whereby acid saponins such as quillaic acid were precipitated. Basic lead acetate was then added to the filtrate to precipitate the neutral saponins. Both precipitates were washed with alcohol, decomposed with sulphuric acid and the lead removed (14 & 15).

F.I. Brownley in his thesis, "Chemistry of Saponins" suggests dialysis in cellophane casing

after the liquid had been saturated with ammonium sulphate[9].

Various other methods of precipitation were tried, including etherate formation as suggested by Conforth and Earl[16].

A. From the Aqueous Extracts.

1.) Precipitation of Saponin by means of hot saturated Barium hydroxide Solution.[14]

(a) Aerial Portion.

About 10 ml. of the darkbrown aqueous concentrate was diluted to 50 ml. with distilled water. To this solution, a hot saturated bariumhydroxide solution was added until no further precipitate was formed, the volume thus being increased to about 100 ml.

A yellowish-brown precipitate was obtained. This precipitate was filtered off, suspended in water and decomposed by passing through carbondioxide gas for 10 minutes. The precipitate of bariumcarbonate was removed by filtration. The saponin was precipitated from the clear solution by adding a mixture of 35 : 50 volumes of ether-

alcohol. A yellowish-brown precipitate was obtained.

Attempted Hydrolysis of product obtained.

All the hydrolytic experiments described, were performed with the aid of concentrated hydrochloric acid.

A portion of the yellowish-brown precipitate obtained above, was washed into a 250 ml. roundbottom flask with water. It was boiled under reflux with 10 ml. concentrated hydrochloric acid for 10 hours. The bulk of the liquid was then evaporated and attempts were made to recrystallise the sapogenin from 50% ethyl alcohol[11]. A small amount of crystalline material was obtained, which however was not pure, since when burnt on a Pt-foil, it melted and an incombustible residue was left behind. This shows that all the Barium had not been removed. A further portion therefore was purified thoroughly, hydrolised and evaporated as before. A crystalline mixture of needles and plates was obtained, which was insoluble in boiling methanol, but dissolved readily in hot

water. From the water solution a crystalline product separated, which was only partly organic, and probably still contained Barium.

The barium was then precipitated with very dilute sulphuric acid. The white barium sulphate was filtered off and the filtrate evaporated to a small volume. Shiny white crystals separated from this solution; inorganic material was however still present and could not be removed by crystallization.

(b) Tuber.

The same procedure as described above was used for precipitation of the saponins from the concentrates of the aqueous extracts prepared from the tuber. A yellowish precipitate was obtained and a definite smell of ammonia observed.

Hydrolysis of Precipitate.

Due to the difficulties experienced with the removal of the barium and accompanying inorganic material, no hydrolysis was carried out on the barium hydroxide fraction obtained from the tuber.

2.) Precipitation with Lead Acetate.[8,15,14]

(a) Aerial Portion.

A portion of about 20 ml. of the aqeous concentrate was taken and the saponin precipitated by the addition of excess neutral lead acetate, i.e. ordinary bench reagent. A brown to darkbrown precipitate settled out and was filtered off.

Basic lead acetate was prepared by boiling 115 gms. basic acetate with 250 ml. distilled water for 15 minutes. Any neutral saponins still present in the filtrate, should have been precipitated by adding this basic acetate to the solution. However, no precipitate was obtained. From this it would appear that only acid saponins were present in the plant extract. The precipitate obtained by addition of neutral lead acetate was washed on the filter with ethyl alcohol, and then transferred to a beaker with water and decomposed by addition of dilute sulphuric acid. After boiling to coagulate the precipitate, the lead sulphate was removed by filtration. In order to make sure

that all the lead was removed, hydrogen sulphide gas was bubbled through the solution for 5 minutes; no further precipitation took place.

The saponin in the filtrate was precipitated by addition of ether-alcohol mixture as before, and formed a brownish-yellow precipitate(14).

Attempted hydrolysis of Precipitate.

The precipitate obtained after removal of lead as above, was washed with ether, washed into a roundbottom flask with 20 ml. ethyl alcohol and boiled under reflux for 6 hours with 5 ml. concentrated hydrochloric acid. On evaporation, white needle-shaped crystals separated, although there were still some impurities present. Fraction A.

(b) Tuber.

The same procedure was followed in the precipitation of the saponin from the aqueous extracts obtained from the tuber. The brown precipitate which was obtained, was purified, and the lead removed as the sulphate as before.

Attempted hydrolysis of Precipitate.

Same procedure as above also gave white needle-shaped crystals. Fraction B.

Purification of Fractions A and B.

Both Fraction A and Fraction B were only partly organic in nature and were mixed with inorganic material. Since, when a melting-point determination was carried out, the crystals did not melt, but stars sublimed in the tube, it was attempted to separate the organic portion by subjecting the crystalline products A and B to high vacuumsublimation.

Fraction A.

In this case no sublimate was obtained, even when taking the temperature up to 200°C with a vacuum of 2×10^{-5}cms. The crystals only turned brown.

Fraction B.

The vacuum obtained was 2×10^{-5}cms. At about 135°C a white sublimate was obtained lower in the tube and at 145°C a fatty material subli-

med to the top of the tube. Taking the temperature up to 200°C, the first sublimate tended to melt, but again solidified after removing the tube from the oilbath. This sublimate consisted of white starlike crystals, which on standing in the atmosphere were found to be hygroscopic.

Another portion of the original preparation from the tuber was taken and hydrolised as before. On evaporation, a brown impure residue was left behind, from which the resinious material was removed by extracting with methanol, leaving a white material soluble in water. From the water solution white needle-shaped crystals separated. Again the crystals proved to contain inorganic material which could not be separated from the organic material.

It must be noted, that this method of isolation apparently suffers from several disadvantages, the most important being that many other plant materials are readily precipitated from aqueous solution on the additon of lead

acetate[7]. It may therefore only be applied with success to comparitively pure saponin solutions.

3.) Precipitation of saponin by direct addition of Ether-alcohol mixture.[16]

(a) Aerial Portion.

A 10 ml. portion of the concentrated aqueous extract from the aerial portion of the plant was fractionally precipitated by the direct addition of an ether-alcohol mixture (35 : 50 portions by volume). Yellowish-brown precipitates settled out. These were filtered off. The clear filtrate was eventually evaporated to dryness, leaving a residue which did not form any definite foam when shaken with water. The precipitates obtained by addition of ether-alcohol mixture were however readily soluble in water and frothed strongly on shaking in aqueous solution. It was thus assumed that the saponin was completely removed by the precipitation.

Attempted hydrolysis of precipitate.

In this case only the precipitate obtained above was investigated. The precipitate, after being washed with ether, was washed into a roundbottom flask with 20 ml. ethyl alcohol and hydrolised by boiling with 5 ml. concentrated hydrochloric acid. The mixture was boiled under reflux for 4½ hours and concentrated to a small volume. A small amount of crystals, having the form of rosettes or clusters of needles were seen under the microscope. No effective way of separating or purifying these crystals could be found on account of the small amount, and the large quantity of accompanying resin precipitated together with the saponin.

(b) Tuber.

The same procedure as above was followed to precipitate the saponin from the concentrated aqueous extracts of the tuber. The precipitates which settled out were lighter in colour than those from the aerial portion. In this case however, the same difficulty as above was

encountered. The amount of crystals obtained after hydrolysis was too small in comparison with the resin which was co-precipitated, to work on.

4.) Isolation by means of dialysis.(9)

About 20 ml. of the concentrated aqueous extract of the aerial portion was saturated with ammonium sulphate and filtered through filterpaper. The filterpaper was washed with a small amount of distilled water, and the filtrate dialysed for 3 days in cellophane casing. The remaining liquid showed weak positive tests for ammonium and sulphate ions. It was therefore dialysed for two more days, and the resulting solution evaporated to dryness. A brownish residue was left behind, which foamed strongly when shaken with water. This residue however, still contained large amounts of impurities which could not be removed.

Hydrolysis of Residue.

The impure residue was washed into a roundbottom flask with 25 ml. ethyl alcohol, and refluxed for 4½ hours with 5 ml. concentrated hydrochloric acid.

The resulting product was evaporated to dryness, but no organic material could be isolated, due to large amounts of resinious material present.

Since it appeared to be impossible to isolate any crystalline organic material by this method, and due to the large amount of impurities which could not be removed, the method was discarded and no experiments were carried out on the aqueous extracts from the tuber.

5.) The Chloroform Procedure.(17)

(a) Tuber.

About 100 ml. of the concentrate from the aqueous extraction of the tuber was mixed with about twice its own volume of ethyl alcohol. Ether was then added in excess and a brown syrupy deposit resulted. After standing for a time, the supernatant liquid was decanted and the brown deposit rubbed with two or three more quantities of ether.

After heating the syrup gently to remove occluded solvent, about four times its weight of chloroform was added. The mixture was shaken

with half its volume of water and then with sufficient alcohol so as to cause rapid separation of the mixture into two layers. The lower layer was separated.

In the chloroform layer, just about no saponin was found. The other portion consisted of a water-alcohol mixture which apparently contained the saponin. An insoluble material found in this layer, was filtered off and washed several times with boiling water. The combined filtrate obtained was brownish-yellow in colour and produced a definite foam when shaken. It was assumed that this portion contained most of the water-soluble saponin. This will be referred to as Fraction B.

The precipitate remaining on the filterpaper had a greyish colour. On shaking this with water, it still produced a foam. It was therefore assumed that there was still some saponin in the precipitate. This precipitate was called Fraction A.

Attempted Hydrolysis of Fractions.

The different preparations obtained from the chloroform procedure as described above were treated as follows:

Fraction A.

The precipitate was brought partly into solution by adding about 150 ml. 1 : 1 aqueous ethyl alcohol. The solution was hydrolised by boiling under reflux for 8 hours with 10 ml. concentrated hydrochloric acid. At first, strong frothing occured, but after about half an hour it did not foam any more. A portion of the insoluble precipitate, remained in suspension even after this time.

An attempt to extract the sapogenin from this solution by shaking out with ether only led to the production of a fatty residue when the ethereal solution was evaporated.

The original solution, after extraction with ether, was evaporated on a waterbath to near dryness. Needle-shaped crystals were deposited together with a large amount of resinious impurity.

Nearly all the resin could be dissolved by extraction with methanol to leave a white residue. This residue on being dissolved in hot water, gave a small quantity of white crystals on standing, which were mostly inorganic material.

Fraction B.

The brownish-yellow solution was evaporated on a waterbath, leaving a brown syrup with a caramel odour. One half of this residue was taken in 150 ml. 1 : 1 aqueous ethyl alcohol and heated on a waterbath to ensure complete solution. It was hydrolised by boiling under reflux with 5 ml. concentrated hydrochloric acid for 6 hours.

From the product, the organic portion was extracted with ether. However, as before, only a brown fatty product was obtained from the extract. The aqueous portion, after the extraction with ether, left a messy brown residue containing some crystals on evaporation. This residue was repeatedly extracted with methanol until all the soluble material had gone into solution. The white insoluble material was

removed by filtration and examined under the microscope when it appeared to be a mixture of two types of crystals; the one type belonging to the cubic system and the other being needles.

This crystalline residue was dissolved in hot water, filtered and left to crystallise. Initially, needle-like clusters separated from the solution and finally after all the water had evaporated, cubic crystals had also been formed. In a meltingpoint determination on this recrystallised product, it was noticed that material, which could have been organic in nature, had sublimed on the walls of the meltingpoint tube. Accordingly a highvacuum sublimation was carried out at 2×10^{-5}cms. Between 120° and 145°C a white, apparently fatty, material sublimed. The unsublimed residue was transferred to a clean sublimation-tube and at the same vacuum, more fatty substance sublimed at 150°. The temperature was taken up to 200°C, without any more organic material coming off.

The fatty sublimate was dissolved in water, yielding a solution, which on evaporation depo-

sited white dendrilic crystals, which turned out to be an ammonium salt.

(b) Aerial Portion.

The same procedure as above was followed to precipitate the saponin from the concentrated aqueous extracts of the aerial portion. The precipitate was also treated with ether and then separated with the aid of chloroform. The different fractions were obtained, but after hydrolysis again no organic material could be isolated, only an inorganic ammonium salt being obtained as before.

B. From the alcoholic Extracts.

From the alcoholic extracts, the only, and comparatively easy method, used for the isolation of the saponin, was the method of precipitation by ether[13].

Etherate Formation.[12 & 18]

(a) From the Tuber Extracts.

About 25 ml. of the concentrated alcoholic extract was taken and the glycosides precipitated by the addition of ether. These were precipi-

tated in the form of a brown syrup. This syrup was rubbed with successive quantities of ether, until the added ether remained colourless. The ether was decanted. On heating the syrup to expell occluded ether, the residue foamed strongly, even when heating very slowly.

This residue, being ligthbrown in colour, when exposed to the atmosphere, turned to a darkbrown paste, which dissolves easily in water with strong frothing.

From the decanted ethereal solution on standing for a few days, a crystalline material separated, which was partly soluble in ethyl alcohol. The insoluble portion was crystalline and melted at ± 180°C. The alcoholic solution on evaporation on a waterbath, left a yellow sticky material, which bacame nearly solid on standing.

Attempted hydrolysis of Residue.

About 1 gm. of the darkbrown syrup obtained by precipitation of the tuber alcoholic extract with ether, was dissolved in 30 ml.

ethyl alcohol and 5 ml. concentrated hydrochloric acid added. The mixture was boiled under reflux for 3 hours. The solution which was yellow at first, gradually became darker and eventually turned darkbrown in colour.

This hydrolised product was evaporated on a waterbath. It appeared however that entire material had been resinified, since only a black resinious product was obtained, from which no crystalline sapogenin could be isolated.

(b) From the Aerial Extracts.

Again the glycosides were precipitated with ether from the alcoholic aerial extract. These separated as a darkbrown, nearly black residue. It had similar properties to the precipitate obtained under similar conditions from the alcoholic extract of the tuber, but was much darker in colour. Ether-soluble material was removed by washing with successive quantities of ether.

This residue was hydrolised as before, but again only a resinious product was obtained, from which no crystalline organic material could be

isolated.

Purification of the fish-toxic compounds from the alcoholic extracts and attempted hydrolysis of the products.

As will be shown later on in this thesis the toxicity tests carried out with the different extracts prepared from the plant, proved that the above alcoholic extracts, were the only ones which really appeared to be appreciably toxic to fish. They were therefore considered probably to contain the highest concentration of saponin and accordingly, attempts were made to purify them further.

The precipitates from the alcoholic extracts, which had been obtained by the addition of ether, were repeatedly triturated with ether, so as to remove all ether-soluble material. The insoluble residue was then left to stand as to allow the ether to evaporate.

These combined precipitates which had been obtained from the alcoholic extracts were extracted with ethyl alcohol. The yellow syrupy portion dissolved in the alcohol, and an impure white crystalline

material remained.

Toxicity tests carried out with these two fractions proved that the toxic saponin material had been extracted by the ethyl alcohol. The insoluble residue turned out to be cane sugar. The following confirmative tests were carried out:

1.) It formed a blue solution with copper sulphate and sodium hydroxide[19].

2.) Gave a violet colouration on warming with cobalt nitrate and sodium hydroxide[19].

3.) The impure compound did not reduce Fehling's solution, but after it had been hydrolised by dilute hydrochloric acid, it had a definite reducing action.

4.) To 2 ml. of the hydrolised solution of the substance, lead acetate was added and after boiling, 5 ml. dilute ammonium hydroxide added. This mixture was again boiled for 1 minute, when a salmon-pink colour indicated dextrose[20].

5.) To 2 ml. of the hydrolised solution, an

equal volume of concentrated hydrochloric acid and about a ricegrain of Resorcinol were added. The testtube was placed in boiling water, and after 2 minutes a winered colour, which indicates Laevulose, appeared[20].

An attempt was now made to hydrolise this purified compound in two different ways with concentrated hydrochloric acid.[16]

(i) With methyl-alcoholic hydrogenchloride.

About 0.5 gms. of the yellow syrup was dissolved in 25 ml. methanol and 2 ml. concentrated hydrochloric acid added. The solution was boiled under reflux for 2 hours, the colour of the solution changing from a light yellow to nearly black. The solution was freed from excess mineral acid by shaking with silver carbonate, which had been prepared from silver nitrate and sodium carbonate, filtered, and heated on a waterbath until all the alcohol was removed, water being added from time to time to replace it. After cooling, the whole was shaken with ether, a quantity of black tarry

material being deposited. The ethereal layer was evaporated, leaving a very small quantity of needle-shaped crystals in the beaker. The aqueous layer was evaporated, after decolorization with charcoal, leaving a brown syrup, but no crystals separated.

(ii) With aqueous-alcoholic hydrogen chloride.

Again about 0.5 gm. of the residue was dissolved in 30 ml. 1 : 1 aqueous alcohol, and 2 ml. of concentrated hydrochloric acid added. The solution was boiled under reflux for 3 hours, and the sample worked up with ether as before. Again no crystals were obtained from the aqueous layer.

This definitely suggests that the aglucone was resinified in the hydrolysis process using concentrated hydrochloric acid. It was therefore attempted to find another way of hydrolising the impure product obtained, in order to find a sapogenin which could be crystallised.

Attempted Hydrolysis by Enzymes.

Certain micro-organisms, when grown in a medium containing saponins produce enzymes which cleave the saponins to sapogenins.(21 & 22) Stoll and co-workers(23) have shown that enzyme preparations from numerous fungi can cleave the carbohydrate-steroid linkage of certain cardiac glycosides. It was noticed however, that a purified saponin substrate contaminated by an actively growing mold was cleaved to the steroidal sapogenin. Of the various fungi tested, several, but not all, species of Aspergillus and Pennicilium gave the best results.

As was previously mentioned in this work, an unknown fungus grew on the aqueous extracts obtained from Neorautanenia Edulis, when these were exposed to the atmosphere. These fungi were isolated and identified to be of the Pennicilium species. A sample of the fungus was cultivated in a pure form and was used for further tests.

About 1 gm. of the purest form of the

saponin available was dissolved in 600 ml. distilled water and a sample of 25 ml. tested on Lebistes reticularus. Two fishes were placed in the solution and they died after 12 minutes immersion, indicating a high toxicity.

The remainder of the saponin solution was divided into 25 ml. portions in 50 ml. conical flasks. These solutions were sterilised in an autoclave for 20 minutes at 121°C and one atmosphere pressure. A subsequent test with live fish proved that this treatment did not seriously diminish the toxicity of the solution.

Sterile Pennicilium fungi were inoculated onto the sterile solutions and allowed to grow before testing again.

Results:

Time (in days)	Control Solution Time required to kill fish.	Inocculated Solution Time required to kill fish.
After 5 days	12 mins.	20 mins.
9 days	12 mins.	35 mins.
14 days	13 mins.	45 mins.
30 days	14 mins.	60 mins.

From the results tabulated above it is clear that the control solution had more or less retained its toxicity. In the case of the solutions however, which had been inocculated with fungi, the toxicity had been markedly decreased. It is therefore, clear that the growing fungi had, in one way or another, broken down the toxic saponin into non-toxic constituents. In the same time a complete change in the appearance of the inocculated solution had been accomplished, whereby they had lost their colloidal nature completely and had become absolutely clear.

One of these solutions was filtered and after evaporation to dryness, attempts were made to crystallise the supposed sapogenin from ethyl acetate, ethyl alcohol, etcetera, but without success. More or less

all the remaining solutions, which had become colourless, were filtered and concentrated to a small volume by vacuumdistillation. No foaming occured, which also points to the possibility that the saponin had been broken down.

The syrupy concentrate was dissolved in 20 ml. ethyl alcohol and after adding an equal volume of freshly distilled acetic anhydride, the mixture was refluxed for 40 minutes. It was allowed to cool and slowly poured into 50 ml. cold water. No acetylation product insoluble in water separated. Since the product could be soluble in water, the solution was evaporated to near dryness, when only a brown syrup was obtained.

The Mannich Hydrolysis.

(a) Tuber.

The hydrolysis method of Mannich was tried next. This consisted of suspending a sample of the purest form of saponin available, in acetone, then passing dry, gaseous hydrogen chloride into the suspension.(28)

For this, a 250 ml. flask was used for the acetone-saponin suspension (150 ml.), and the hydrogen chloride passed from a generator (concentrated hydrochloric acid and concentrated sulphuric acid) through a calcium chloride dryingtower into the suspension.(29) The hydrogen chloride was passed into the suspension for 1 hour, at the end of which time the saponin appeared to have gone nearly completely into solution. The acetone had become yellow in the course of the experiment. The insoluble portion was filtered off.

Half of the acetone-saponin solution was boiled under reflux with 5 ml. concentrated sulphuric acid for 1 hour, the colour of the solution changing to darkbrown. The other half was left to stand onvernight, when it also became nearly darkbrown in colour.

On cooling a white material separated from the portion which was boiled with the sulphuric acid. The solution was placed on a boiling water-bath to expell the acetone, water being added from

time to time to replace it. The solution was cooled, shaken with ether to extract fatty material, the water layer separated and after decolorization with charcoal, evaporated on a water-bath. Small white needles separated on cooling. These were filtered off, washed with cold distilled water and dried.

When this crystalline product was burnt on a Pt-foil, it charred and a white residue was left behind. Therefore, again no organic crystalline material could be obtained by this method of hydrolysis.

Due to a lack of time, no further experiments were carried out using this method, and no crystalline sapogenin could be isolated.

CHAPTER IV.

Toxicity of Saponin to Fish and Chemical tests on toxic compounds.

In the work carried out by the previous workers on the tuber and the aerial portions of Neorautanenia Edulis, substances which possess fish-toxic properties were found[1 - 4]. As it was suspected that the toxic properties may be partly due to a saponin present in the plant, the saponin fractions isolated by the different methods described in the previous chapter, were subjected to fish-poisoning tests.

Methods for determining the toxicity of substances with fish-poisoning properties, are found in the work carried out by W.A. Gersdorff[24], and fully discussed by Van Duuren[2]. The tests carried out on fish in the present work were done, not to determine toxicity curves of the crude saponin fractions, but only to determine whether or not these were toxic to fish. As a saponin is a watersoluble substance, there was no necessity to use acetone as a solvent[1].

As was found by previous workers, the fish which gave the best results, were guppies, Lebistes reticularus, and goldfish, Carassius auratus.

Solutions of the substances were made by dissolving about 0.5 gm. of the substance in 20 ml. distilled water, and adding this solution to 250 ml. distilled water in a 600 ml. beaker at 20°C. This temperature was about the same as that of the water in the tanks from which the fish were taken and should be controlled or kept constant within 1°C. Two fishes of 15 - 20 mms. length were added to each test solution as a control.

The following samples of the proposed saponin were tested:

Test solution number.	Description of dissolved material.
I	Saponin from Barium hydroxide precipitation. (Aerial portion).
II	Saponin from Lead Acetate precipitation (Aerial portion).
III	Saponin from Lead Acetate precipitation (Tuber).
IV	Saponin from Chloroform Procedure (Tuber).
V	Precipitate from IV after removal of saponin by boiling water. (Tuber).

Test solution number.	Description of dissolved material.
VI	Saponin soluble in boiling water from IV (Tuber).
VII	Saponin from ether precipitation in alcoholic extract (Aerial).
VIII	Saponin from ether precipitation in alcoholic extract (Tuber).

Solutions number I to VI were non-toxic to the fish after an hour of immersion, but VII caused irritation after 15 minutes and death within 45 minutes. Solution number VIII caused irritation after 5 minutes and death within 20 minutes.

This again proved the fact that the tuber is more toxic to fish than the aerial portion, since fish survived about twice as long in solution number VII than in number VIII. Fresh aqueous extracts of the aerial protion were unfortunately not tested for toxicity, but in the case of the tuber, these tests were carried out in the same manner as described above. In one beaker, two goldfish, Carassius auratus, were placed and in another two of the Lebistes reticularus. After 12 minutes the goldfish were lame and died within

15 minutes. The Lebistes survived longer, mortality setting in only after 20 minutes immersion.

This showed that there are fish-toxic substances present in the aqueous extracts of the plant, but that in the course of the processess used for purification as described in the previous chapter, the toxic substances had been lost in one way or another, except in the case of the etherate formation from the alcoholic extracts.

The same aqueous extract of the tuber, was allowed to stand for one month and toxicity tests again carried out in the same manner as above. After 20 minutes the fish, Lebistes reticularus were still alive, grew lame after about 30 minutes and died only after an immersion of 40 minutes. In a freshly prepared extract as shown above, the fish were defintely dead after 20 minutes had elapsed.

Similar experiments were carried out with the original aqueous extracts prepared from the aerial portion, which had been standing for a few months. Fish placed in these solutions only died after an immersion of nearly three hours.

Unfortunately no tests had been carried out

with the fresh extracts from the aerial portion, but according to the results obtained with extracts from the tuber it must be expected that the saponin in the aerial portion of the plant, would similarly be decomposed or hydrolysed on standing. This is especially so seeing that the same fungus grew on all the aqueous extracts, both from the aerial portion and the tuber when exposed to the atmosphere.

Chemical tests carried out on the toxic substances VII and VIII.

The following characteristic tests for saponins were carried out to prove the identity of the fish-toxic substances VII and VIII.

1. They were readily soluble in cold water, and on shaking produced a strong stable foam. This is the most characteristic property of saponins.

2. They were amorphous and could not be crystallised.

3. Concentrated sulphuric acid on the solid substances gave at first a yellow colour, which darkened on standing, eventually turning to a

dark red or nearly violet coloration.[25]

Saponins produce a yellow colour changing to violet on addition of concentrated sulphuric acid.

4. With ferric chloride and potassium ferricyanide a precipitate of Turnbull's blue was obtained, which indicated reducing properties of the samples.[15]

5. Aqueous solutions of the substances were capable of forming stable suspensions with water-insoluble substances.

6. Salkowski reaction[8] gave a reddish colour i.e. the addition of concentrated sulphuric acid to chloroform solutions of the substances.

C H A P T E R V.

Investigation of Crystalline Compounds Isolated.

Experiments were carried out on the following crystalline compounds which were isolated in the course of this work.

(a) Crystalline deposit from aqueous Extracts.

After the concentrated aqueous extracts of the aerial had been left to stand for about four months, crystalline material could be observed on the bottom of the flask.

These were filtered off and dissolved in water. A large portion of the crystals were readily soluble, but some cubic crystals remained undissolved. The aqueous solution was concentrated on a waterbath, when a mixture of needle-shaped and plate-like crystals separated. These were however, very impure and contaminated by a large amount of resin. A chromatographic separation of this mixture was attempted on a column, 1 x 25 cms. of activated aluminium oxide. Acetone which was generally used for chromatographic separations, could not be used as solvent,

as the crystals were insoluble. Water was used as the solvent. A yellow band formed on the aluminium oxide and a brownish-yellow solution passed through. The eluate which had a much lighter colour than the original solution, and appeared to be comparatively free of resin, on evaporation again yielded the organic crystalline mixture.

This mixture could not be separated into its components by crystallisation from various solvents, nor by extraction of their aqueous solution with immiscible solvents. The crystals behaved however as if they were organic in nature, since they charred and burnt away more or less completely when heated on a Pt-foil.

(b) Substance I.

About 1 gm. of the brown syrup, Fraction B, from the chloroform procedure was used in order to prepare an osazone from it. In this way, any sugars present could probably be identified. The following procedure was used:-(26)

1 gm. of the material was Dissolved in 10 ml.

distilled water in a boiling-tube. In another tube, 3.5 ml. glacial acetic acid was dissolved in 10 ml. distilled water, 2.5 ml. phenylhydrazine added, and the mixture shaken until a clear solution of phenylhydrazine acetate was obtained. This solution was added to the proposed sugar solution and gently stirred with a glass rod. The boilingtube was lightly corked, placed in a boiling waterbath, heated for one hour and left in the bath to cool.

Since no crystals separated on cooling, the solution was transferred to a small dish and allowed to evaporate. After standing overnight, yellow crystals, in the form of clusters of long thin plates, separated. These crystals appeared very similar to those of maltosazone. However, after a second crystallization from hot water, yellow plates in the form of clusters were obtained, which had a melting point of 127 - 130°C (uncorrected). The melting point of maltosazone is 190 - 191°C and a freshly prepared sample of this substance also showed distinct differences as far as crystalline form is concerned, so that the product obtained was not malto-

sazone.

The substance, Substance I, was purified by two further crystallizations and after drying in a drying-pistol, it was analysed by Dr. Weiler at Oxford. He obtained to following results:

C : 64.24% H : 6.89% O : 10.27% N : 18.6%

The molecular weight of the substance was determined by the author, using the Micro-Rast method[27], and found to be 151. The formula $C_8H_{11}ON_2$ gives a molecular weight of 151, and the formula was taken as correct. So far however, this compound has not been identified.

(c) Substance (xi).

This substance separated in impure state from the crude alcoholic extract prepared by R.R. Arndt from the aerial portion of the plant.[1]

Since it is practically insoluble in ethyl alcohol, it could be partially purified by extraction with this solvent, which dissolves fatty and other impurities. The crystalline residue was further purified by crystallization from ethyl alcohol con-

taining a small amount of distilled water, from which it separated as colourless needles with a melting point of 335°C.

The compound, even after thorough purification, was found to contain some inorganic material, which proved to be potassium. A small sample of the pure substance was therefore dissolved in a little distilled water and acidified with a few drops of concentrated hydrochloric acid. From this solution only fatty material could be extracted with ether, no crystalline material being obtained.

A larger portion of the substance after heating with concentrated hydrochloric acid on the waterbath for one hour, was extracted with ether to remove fatty material and the aqueous solution evaporated to a small volume. On cooling, yellowish-white crystals separated from the solution. These were collected and subjected to highvacuum sublimation to attempt a separation of the organic portion. Even at a vacuum of 2×10^{-5}cms. and 200°C, only a small amount of a fatty sublimate was formed, but no crystalline material was obtained.

CHAPTER VI.

SUMMARY.

The object of the present investigation was to extract and isolate the fish-toxic saponin present in the tuber and the aerial portions of Neorautanenia Edulis C.A. Sm. Various methods of extraction were attempted, and the best found to be that with hot 95% ethyl alcohol.

Regarding the isolation of the saponin, it was found that the etherate formation with the alcoholic extract, proved to be the most satisfying. Various other methods were also tried, but none of them gave a fish-toxic saponin.

Toxicity tests on fish were carried out, and the most toxic compound found to be that from the etherate formation, mentioned above. The toxic compounds were further purified by extraction with ethyl alcohol, extracting the saponin and leaving an insoluble material which was identified as cane sugar.

Various methods of hydrolysis were attempted, but the saponin was resinified by the concentrated hydrochloric acid.

The purest form of the toxic saponin obtained had a yellow syrupy nature. It was very toxic, to fish, but could not be hydrolised in order to identify it.

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A C K N O W L E D G E M E N T S.

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