

CHEMICAL INVESTIGATION OF *PACHYSTIGMA PYGMAEUM*
SCHLECHTER (GOUSIEKTEBOSSIE).

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Gousiekte is a disease of sheep in South Africa caused by feeding on the plant *Pachystigma pygmaeum* (figs. 1 and 2) (gousiektebossie). Cases have been encountered only in the Transvaal, the two districts chiefly involved being Pretoria and Ermelo.

It is believed that the plant contains a toxic principle giving rise to "a productive myocarditis and subsequent dilation of the ventricles, ending most frequently with heart failure" (Theiler, du Toit and Mitchell, 1923).

The toxicity of the plant is exceedingly variable. Farmers believe it to be harmless to animals except for short periods, probably associated with climatic factors. The annual rainfall, and its distribution, is held to be a decisive factor.

Theiler and collaborators produced the condition in grazing experiments in the late summer (March to April). In a successful experiment a great variation in the susceptibility of individual animals was revealed and it was found that the immature and mature fruit was non-toxic. It was found that the interval between ingestion of toxic material and death was about 37 days, though cases were recorded where sudden death of an affected sheep did not occur until the 65th day.

For the purpose of the present study leaves and stems of the gousiektebossie were collected in October 1950. The material was dried, finely ground and extracted with ethyl ether. Two crystalline substances, melting at 199° C. and at 253–256° C. respectively, were isolated from this extract. An alcoholic extract of the residual plant material yielded two further crystalline products with melting points of 166° C. and 222–224° C., respectively.

PROPERTIES OF ISOLATED SUBSTANCES.

(a) *Substance with m.p. 199° C.:*

This substance crystallized in glistening needles from 95 per cent. alcohol, m.p. 199° C. It was found to be soluble in alkali but not in acid. The presence of a lactone group was established by refluxation with alkali. Assuming that one lactone group was present, a molecular weight of 325 was obtained. The Rast method gave a value of 324. The combustion analyses suggested a molecular weight of $C_{12}H_{14-16}O_9$. (M.W. 302–304).

When this substance was dissolved in pyridine, acetic anhydride added and the solution left at room temperature for 21 hours, a mono-acetyl derivative,

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m.p. 182° C. (from 95 per cent. alcohol) was obtained. The combustion analyses were in agreement with the formula $C_{14}H_{16-18}O_{10}$. Saponification with alkali showed that two equivalents were required. The acetyltable OH group could be oxidized with Kiliani's chromic acid mixture; the acetyl derivative, however, was quite resistant to oxidation with chromic acid and showed no optical rotation.

(b) *Substance with m.p. 253–256° C.:*

The substance was crystallized from acetone or dilute alcohol with m.p. 253–256° C.

When dissolved in acetic anhydride and a drop of concentrated sulphuric acid added, a red colour was obtained which soon changed to a bluish violet. According to the combustion analyses this substance, which is most probably a phytosterol, has the formula $C_{18}H_{30}O_2$.

On acetylation a mono-acetyl derivative, m.p. 209–212° C., was obtained with probable molecular formula $C_{20}H_{32}O_3$.

(c) *Substance with m.p. 166° C.:**

This crystalline substance has a sweet taste and proved to be identical with mannitol, $C_6H_{14}O_6$. It formed a hexacetyl derivative, $C_{18}H_{26}O_{12}$, which crystallized in prisms from 95 per cent. alcohol, m.p. 124–126° C.

(d) *Substance with m.p. 222–224° C.:**

From water this pale, yellow substance crystallized in needles. It had an optical rotation of 4·82° (in alcohol). A positive Molisch test was obtained and this substance proved to be a glycoside, with molecular formula $C_{21}H_{26}O_{12}$, which belongs to the group of flavone glycosides. This was confirmed by the following colour reactions:

<i>Reagent.</i>	<i>Result.</i>
1. PbAc	Orange yellow precipitate.
2. FeCl ₃ (alcohol)	Dull green.
3. N HCl (Heat)	Dissolved with a yellow colour.
4. 0·1N NaOH	Dissolved with an orange yellow colour which changed to a dark red on heating.
5. Fehlings	Reduction.
6. Boric acid test of Wilson (Wilson, 1939)	Positive.
7. Cyanidin test.	Positive.

On hydrolysis of the glycoside with dilute sulphuric acid, the genin was obtained. It crystallized in bright yellow needles from 95 per cent. alcohol with a m.p. of 309–310° C. The genin was optically inactive, had a molecular formula of $C_{15}H_{16}O_7$, and formed a pentacetyl derivative, which crystallized from 95 per cent alcohol in glistening, colourless needles, m.p. 198–200° C.

* These two substances were also isolated by Mr. Immelman, formerly of the Division of Chemical Services, Pretoria (personal communication).

The free sugar proved to be a hexose. The osazone, crystallized from dilute alcohol, melted at 200–202° C. and showed no depression in a mixed melting point determination with authentic D-glucosazone. That this sugar is most probably D-glucose, was confirmed by subjection to paper partition chromatography (Partridge, 1948). An R_F value of 0.38 was obtained (R_F value for D-glucose 0.39).

To prove whether any of these isolated substances are physiologically active they were tested on rabbits by Dr. T. Adelaar, Section of Toxicology, Onderstepoort, to whom my thanks are due. The following report was handed in by him:—

Flavone glucoside.

<i>Animal.</i>	<i>Quantities administered.</i>	<i>Result.</i>
Rabbit (2.59 Kg.)	75 mg. daily subcutaneously for a period of 9 days (total amount injected = 0.675 gm.)	No effect after 54 days.

Mannitol.

Rabbit (2.45 Kg.)	1.46 gm. dosed daily for 21 days.	No effect after 21 days.
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Lactone substance.

Rabbit (2.7 Kg.)	16 mg. daily subcutaneously for 5 days (amount injected = 80 mg.)	No effect after 42 days.
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Phytosterol.

Rabbit (2.64 Kg.)	40 mg. dosed daily for 5 days (amount dosed = 200 mg.)	No effect after 42 days.
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EXPERIMENTAL.

Extraction.

Four hundred grams of the dried leaves and stems were extracted in a Soxhlet with ethyl ether. The hot ether extract was treated with ethyl ether. The hot ether extract was treated with animal charcoal and filtered. The yellowish green solution was concentrated when fine, colourless needles separated. The crystals were filtered off and recrystallized from 95 per cent. alcohol (m.p. 199° C.)

On further concentration of the ether solution, a substance deposited which on recrystallization from dilute alcohol or acetone, melted at 253–256° C.

The plant material was further extracted with chloroform when another small amount of these two substances was obtained.

Subsequently the material was extracted with 95 per cent. alcohol which gave, on concentration, a mass of crystals (yield: 1.8 per cent.). Recrystallization of this substance gave a m.p. of 166° C.

The remainder of the alcoholic filtrate was evaporated to dryness and boiling water added. The solution was filtered and fanned down. A yellowish substance precipitated which was purified by recrystallization from water. The pale yellow needles, thus obtained, sintered at 194–195° C. and finally melted at 222–224° C. (decomp.).

*Properties of substance, m.p. 199° C.**

Molecular weight:

(a) Rast method:

0.0218 gm. was dissolved in 0.5608 gm. pure, resublimed camphor.

Depression of m.p. = 4.8°.

∴ M.W. = 324.

(b) Lactone titration:

0.0781 gm. was dissolved in alcohol and 4 ml. 0.1 N alkali added

After 2 hours the solution was titrated with acid.

2.4 ml. 0.1 N alkali was consumed.

∴ M.W. = 325.5.

Molecular formula:

(Dried over P₂O₅ at 100° C. and 3 mm. pressure for two hours.**

4.030 mg. subst. yielded 7.025 mg. CO₂ and 1.685 mg. H₂O.

4.060 mg. subst. yielded 7.120 mg. CO₂ and 1.740 mg. H₂O.

	% C	% H
Found:	47.55	4.68
	47.85	4.79
Calc. for C ₁₂ H ₁₄ O ₉ (M.W. = 302.2)	47.68	4.67
Calc. for C ₁₂ H ₁₆ O ₉ (M.W. = 304.2)	47.37	5.29

Acetylation:

The substance was dissolved in pyridine, acetic anhydride added and the solution left at room temperature for 21 hours. When poured into cold water a precipitate formed which was filtered off and crystallized from 95 per cent. alcohol. The acetyl derivative crystallized in needles, which became soft at 170° C. and finally melted at 182° C.

Acetyl groups:

0.0618 gm. was dissolved in alcohol and refluxed with 0.1 N alkali for 45 minutes. 3.66 ml. 0.1 N alkali was consumed.

Calc. for one acetyl group (M.W. = 344): 0.0616 gm. needed 3.58 ml. 0.1 N alkali.

* Melting points on Kofler (corrected).

** All samples were dried under these conditions before analysis. Combustion analyses by Drs. Weiler and Strauss, Oxford, England.

*Molecular formula:*3·800 mg. subst. yielded 6·775 mg. CO₂ and 1·750 mg. H₂O.3·440 mg. subst. yielded 6·105 mg. CO₂ and 1·640 mg. H₂O.

	% C	% H
Found:	48·64	5·15
	48·42	5·33
Calc. for C ₁₄ H ₁₆ O ₁₀ (M.W. = 3·442)	48·33	4·68
Calc. for C ₁₄ H ₁₈ O ₁₀ (M.W. = 346·2)	48·55	5·23

*Properties of substance, m.p. 253–254° C.**Molecular formula:*4·147 mg. subst. yielded 11·745 mg. CO₂ and 3·890 mg. H₂O.3·618 mg. subst. yielded 10·275 mg. CO₂ and 3·465 mg. H₂O.3·899 mg. subst. yielded 11·100 mg. CO₂ and 3·895 mg. H₂O.

	% C	% H
Found:	77·32	10·49
	77·34	10·71
	77·67	10·89
Calc. for C ₁₈ H ₃₀ O ₄ :	77·67	10·85

Acetylation:

The substance was acetylated under similar conditions as with the previous substance.

The acetyl derivative crystallized from dilute alcohol, m.p. 209–212° C.

*Molecular formula:*3·508 mg. subst. yielded 9·550 mg. CO₂ and 3·090 mg. H₂O.3·920 mg. subst. yielded 10·690 mg. CO₂ and 3·445 mg. H₂O.

	% C	% H
Found:	74·27	9·85
	74·40	9·83
Calc. for C ₂₀ H ₃₂ O ₃ :	74·99	10·06

Acetyl groups:

0·0788 gm. was dissolved in alcohol and refluxed with 0·1 N alkali for 40 minutes.

2·40 ml. alkali was consumed.

Calc. for C₂₀H₃₂O₃ (one acetyl group): 0·0788 gm. requires 2·46 ml. 0·1 N alkali.

Properties of substance, m.p. 166° C.

Molecular formula:

3.750 mg. subst. yielded 5.520 mg. CO₂ and 2.525 mg. H₂O.

	% C	% H
Found:	40.16	7.53
Calc. for C ₆ H ₁₄ O ₆ :	39.55	7.74

For acetylation the substance was dissolved in hot pyridine, acetic anhydride added and the solution left at room temperature for 20 hours. Then it was evaporated to dryness before a fan. The residue crystallized in prisms, m.p. 124–126° C.

Acetyl groups:

0.0684 gm. was refluxed with 10 ml. 0.1 N alkali for 40 minutes.

9.62 ml. alkali was consumed.

Calc. for C₁₈H₂₆O₁₂ (six acetyl groups): 0.0684 gm. needed 9.46 ml. 0.1 N alkali.

Molecular formula:

3.960 mg. subst. yielded 7.195 mg. CO₂ and 2.250 mg. H₂O.

3.825 mg. subst. yielded 6.960 mg. CO₂ and 2.100 mg. H₂O.

	% C	% H
Found:	49.57	6.36
	49.64	6.14
Calc. for C ₁₈ H ₂₆ O ₁₂ :	49.77	6.03

Properties of substance, m.p. 222–224° C.

This crystalline substance proved to be a flavone glycoside (colour reactions). It is crystallized from water in pale yellow needles with water of crystallization

Molecular formula:

4.098 mg. subst. yielded 7.685 mg. CO₂ and 1.960 mg. H₂O.

3.795 mg. subst. yielded 7.170 mg. CO₂ and 1.825 mg. H₂O.

	% C	% H
Found:	51.17	5.35
	51.54	5.38
Calc. for C ₂₁ H ₂₀ O ₁₂ · 1.5H ₂ O:	51.31	4.72

Hydrolysis:

0.382 gm. of the glycoside was dissolved in a very small amount of alcohol. Then 25 ml. 0.5 N H₂SO₄ was added and the solution refluxed for 1½ hours.

The solution was concentrated before a fan when a bright yellow substance deposited (0.204 gm.). This substance, which proved to be the genin, crystallized in fine, bright yellow needles from 95 per cent. alcohol, m.p. 309–310° C. No

optical rotation was observed. The acid filtrate, obtained after removal of the genin, was neutralized with BaCO_3 and concentrated on a waterbath for identification of the free sugar.

Molecular formula of genin:

4.110 mg. subst. yielded 8.970 mg. CO_2 and 1.405 mg. H_2O .

3.720 mg. subst. yielded 8.160 mg. CO_2 and 1.210 mg. H_2O .

3.630 mg. subst. yielded 7.990 mg. CO_2 and 1.150 mg. H_2O .

	% C	% H
Found:	59.55	3.82
	59.85	3.64
	60.06	3.54
Calc. for $\text{C}_{15}\text{H}_{10}\text{O}_7$:	59.61	3.31

The genin was dissolved in pyridine, acetic anhydride added and the solution left at room temperature for 20 hours. (The mixture lost its yellow colour within one hour). The acetylated mixture was poured into ice-cold water and the precipitate crystallized from 95 per cent. alcohol.

The acetyl derivative crystallized in glistening, colourless needles, m.p. 198–200° C.

Molecular formula:

3.510 mg. subst. yielded 7.560 mg. CO_2 and 1.190 mg. H_2O .

3.850 mg. subst. yielded 8.290 mg. CO_2 and 1.360 mg. H_2O .

2.956 mg. subst. yielded 6.340 mg. CO_2 and 1.065 mg. H_2O .

	% C	% H
Found:	58.76	3.80
	58.75	3.95
	58.52	4.04
Calc. for $\text{C}_{25}\text{H}_{20}\text{O}_{12}$:	58.59	3.94

Identification of free sugar:

Two ml. of the concentrated sugar solution was treated with phenylhydrazine in acetic acid and left in a boiling waterbath for half an hour. The yellow osazone which separated was recrystallized from dilute alcohol (m.p. 200–202° C.). Mixed with authentic D-glucosazone, no depression in m.p. was observed.

A drop of the sugar solution was analysed by the technique of paper chromatography (phenol-water-ammonia). An R_F value of 0.38 was obtained.

SUMMARY.

Leaves and stems of the plant, *Pachystigma pygmaeum* (gousiektebossie), collected at Kaalfontein on 20th October, 1950, were subjected to a chemical investigation.

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Four crystalline substances were isolated: a lactone, m.p. 199° C.; a phytosterol, m.p. 253–256° C., mannitol, m.p. 166° C., and a flavone glycoside which sintered at 194–195° C. and finally melted at 222–224° C.

All these substances were tested out on rabbits with negative results.

LITERATURE.

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THEILER, DU TOIT AND MITCHELL (1923). Gousiekte in sheep. *Ninth and Tenth Repts. of Dir. Vet Ed. and Res.*, p. 9.

WILSON (1939). A study of the boric acid colour reaction of flavone derivatives. *J. Am. Chem. Soc.*, Vol. 63, No. 2303.

FIG. 1.



Pachystigma pygmaeum (gousiektebossie).

FIG. 2.



Pachystigma pygmaeum (gousiektebossie).