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Isolation of the Poisonous Principle of *Dimorphotheca cuneata* Less.

By

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In a recent report from this Laboratory, the isolation from *Dimorphotheca spectabilis* Schltr. and *Dimorphotheca Zeyheri*, Sond. was described (Rimington, 1932) of the cyanogenetic glucoside which renders these plants poisonous to stock. The active principle was identified with acetonecyanhydrin glucose ether or "linamarin", first obtained from *Linum usitatissimum*.

In the present instance, *Dimorphotheca cuneata*, Less. has been studied and the same glucoside shown to be present.

Dimorphotheca cuneata grows fairly profusely in certain districts in the vicinity of Grahamstown and has been recognized by Curson (see Steyn, 1932) as one of the plants causing death from prussic acid poisoning (geilsiekte) in those areas. No other reference to its toxicity could be found.

Collection of Material.

The material used in the present investigation was obtained, through the kindness of the Principal Botanist of the Division of Plant Industry, from plants growing in the grounds surrounding the National Herbarium, Pretoria. Reference specimens were deposited, Nos. 15720, 15725.

Determinations of the total hydrogen cyanide yielded by the various parts of the plant were made as previously described. In the case of *Dimorphotheca cuneata*, it was found advisable to allow maceration to proceed for 36 hours since enzymatic hydrolysis of the glucoside is not so rapid as in the other two species which have been studied. For comparison, analyses were also made of plants growing in the Onderstepoort poison garden. The results are recorded below:

Material from National Herbarium Grounds.

	Moisture percentage.	*Mgm. HCN per 100 gm. of fresh material*	*Mgm. HCN per 100 gm. calculated dry weight.*
Whole plant	55·7	124·8	281·7
Green stems	37·6	74·3	119·0
Leaves	58·3	164·9	399·5
Flowers	67·6	46·8	144·5
Seeds	61·1	24·5	63·0

Material from Onderstepoort Poison Garden Gathered in the Early Morning (Onderstepoort Herbarium No. 7411).

	Moisture percentage.	*Mgm. HCN per 100 gm. of fresh material*	*Mgm. HCN per 100 gm. calculated dry weight.*
Whole plant	66·2	17·1	50·6
Green stems	44·9	8·7	15·7
Leaves	64·2	47·1	131·4
Flowers	70·7	46·2	157·8
Seeds	66·7	19·3	57·9

Specimens of the same Material Gathered at noon on a Hot Day.

	Moisture percentage.	*Mgm. HCN per 100 gm. of fresh material.*	*Mgm. HCN per 100 gm. calculated dry weight.*
Stems	40·0	13·4	22·3
Leaves	63·0	130·3	352·2
Roots	37·0	183·5	291·4

The above results would suggest a diurnal variation in hydrogen cyanide content and possibly a correlation with intense photosynthetic activity. These and other physiological aspects of cyanogenesis it is hoped to study more closely at a later date.

Proximal analysis of the material in the seeding stage afforded the following figures:—

Moisture content of plant powder ... ... 4·2 per cent.
Crude protein (on dry weight basis) ... 11·6 per cent.
Ash ... ... 7·3 per cent.

Determination of Acetone.

Determinations of the quantity of acetone liberated simultaneously with hydrogen cyanide when the plant is macerated in buffer solution were carried out as follows, since it was deemed of interest to ascertain whether this reaction could also be used for the quantitative determination of glucoside.

100 gm. of plant powder (post-flowering stage) was allowed to macerate for 36 hours, excess of a suspension of lead hydroxide was then added and the mixture steam distilled, the receiving flask being surrounded by ice. The distillate was treated with a slight excess of silver nitrate solution, the volume measured and the mixture filtered. To an aliquot (approximately 60 mgm. acetone), 50 c.c. of N sodium hydroxide followed by 100 c.c. N/10 iodine solution were then added and the mixture shaken for 10 minutes. The acetone is converted into iodoform. 50 c.c. of normal sulphuric acid was added and the excess of iodine titrated by N/10 sodium thiosulphate.

Acetone found = 0·1606 gm. per 100 gm. plant powder.
calculated from
HCN determination = 0·1433 gm. per 100 gm. plant powder.

The presence of acetone in the steam distillate may render a strictly quantitative determination of hydrogen cyanide unrealisable by the Liebig titration method, on the other hand it can not be assumed that all of the iodoform-forming material in the above experiment was derived from hydrolysed glucoside.

It will be observed that in the Onderstepoort material the hydrogen cyanide content of the stems was very low, which is possibly to be correlated with the fact that these plants were stunted and very woody whilst those grown in Pretoria were much more luxuriantly developed. The flowers and seeds, however, contained approximately the same amount of hydrogen cyanide in the two cases. Compared with *Dimorphotheca spectabilis*, the yield of hydrogen cyanide from *Dimorphotheca cuneata* is only about one-tenth as great.

Isolation of the Toxic Principle.

The plants were air-dried for some days and then reduced to powder in a mill. Determinations of hydrogen cyanide showed that considerable loss of glucoside had occurred, the powder containing:

	Free HCN mgm. per 100 gm.	*Total HCN mgm. per 100 gm.*
Material in flowering stage	11·1	144·7
Material in post-flowering stage	13·4	66·7

It should be mentioned that the leaves and stems of *Dimorphotheca cuneata* contain a fairly large quantity of a resinous substance which not only retards the drying of the plants but also renders the determination of the hydrogen cyanide content more difficult than in the other species. Volatile constituents of the resin pass over into the steam distillate rendering the solution slightly turbid and thus tending to obscure the end point of the titration. A gradual increase in the weight of the oven-dried material was observed and would suggest the possible oxidation of resinous constituents by atmospheric oxygen. Plants growing in sandy soils were not easily freed from grit adhering to the stems and leaves. 175 gm. of the plant powder was dropped into 1 litre of boiling water containing a little calcium carbonate. After some hours the liquid was pressed off and the plant residue again extracted. The combined extracts were treated with lead acetate, the precipitate filtered off, washed and discarded, and excess of lead removed by hydrogen sulphide. After aerating the filtrate, sodium hydroxide was added to neutrality, and the liquid concentrated under reduced pressure to the consistency of a thick syrup. 96 per cent. alcohol was then stirred in, two volumes of ether added and the resulting precipitate discarded. The filtrate was concentrated, mixed with a fairly large quantity of decolorising charcoal and dried. Water was then added, the mixture filtered and the filtrate again evaporated to dryness. This residue was taken up in 96 per cent. alcohol, two volumes of ether added and the filtrate again concentrated to dryness. The material remaining, which was still of a syrupy consistency was extracted repeatedly with boiling anhydrous ethyl acetate. The combined extracts were concentrated on the water bath to dryness and the residue, after washing with ether, re-extracted with fresh portions of boiling ethyl acetate. This procedure was repeated until no material insoluble in ethyl acetate remained. Since the ethyl acetate solution still had a yellowish colour and contained resinous material which interfered with crystallisation, the evaporated extract was taken up in a little water, boiled with decolorising charcoal until colourless and then evaporated to dryness, the last

traces of water being removed in a vacuum desiccator. The material was recrystallized until pure from boiling ethyl acetate. Yield 12 mgm. The glucoside so obtained had all the properties of " linamarin ". It melted at 139-140°. The optical rotatory power* was determined in a 2 dm. tube using sodium light.

$$[\alpha]_D^{27} = \frac{-0{\cdot}738 \times 14}{2 \times 0{\cdot}2023}$$

$$= -25{\cdot}5°.$$

For synthetic linamarin Fischer and Anger (1919) give M.P. 141-2° and $[\alpha] = -29{\cdot}1°$. Upon micro-analysis it yielded the following figures per cent.:—

	C	H	N
	48·20	7·17	5·72
$C_{10}H_{17}O_6N$ requires	48·54	6·93	5·67

The substance is identical, therefore, with acetonecyanhydrin-glucose ether.

The tetra-acetyl derivative was prepared by acetylation at room temperature with acetic anhydride-pyridine mixture. It crystallised in needles M.P. 139°. Fischer and Anger give M.P. 140-141°.

Direct Ethyl Acetate Extraction Method.

Since the isolation of the glucoside by the methods already described was attended by considerable losses, especially in the present case of *Dimorphotheca cuneata*, a plant containing much more resinous material than does either of the other two species investigated, an attempt was made to extract the glucoside directly from the dried, powdered plant by means of boiling ethyl acetate.

For this purpose, 300 gm. of the plant was used, three large soxhlet extractors being employed and the extraction continued for 24 hours. The bulk of the solvent was distilled off, the extracts combined and evaporated to dryness upon the water bath followed by exsiccation. The dark tarry residue was thoroughly extracted with boiling water, the aqueous solution decolorised by means of charcoal and then evaporated to a syrup which was dried in a vacuum desiccator. The material remaining was extracted exhaustively with boiling ethyl acetate, the extract evaporated and dried, then again taken up in boiling ethyl acetate, this procedure being repeated until the glucoside crystallised out in pure form on cooling the hot liquid. Yield 0·5 gm. M.P. 139-140°. The yield represents only about 14 per cent. of the glucoside actually present in the plant material as shown by HCN determinations, but is superior to that obtained by the water extraction method and the whole procedure is much less laborious. Using a specimen of *Dimorphotheca spectabilis*, a yield of 88·5 per cent. of pure glucoside was easily attained.

The Nature of the Enzyme Present in the Plant.

A preparation of the enzyme present in the plant and normally causing the decomposition of the glucoside was made as follows:—

* Determined upon a sample subsequently isolated by the direct ethyl acetate method.

40 gm. of plant powder were extracted with cold water and the solution centrifuged. Five volumes of 96 per cent. alcohol were then added and the flocculent precipitate collected on the centrifuge, washed with ether and dried rapidly in a vacuum desiccator over sulphuric acid. The powder so obtained was almost completely soluble in water, the residue being centrifuged off before use. Solutions were prepared of linamarin, amygdalin and salicin, enzyme added and these, together with suitable controls, incubated at 37°.

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The results were as follows:—

Enzyme plus	linamarin	amygdalin	salicin
	++ rapid action	slowly +	–

Its action upon α – methylglucoside was investigated by incubating solutions of enzyme and glucoside for 7 days at 37° (in the presence of a trace of chloroform), then adding basic lead acetate, centrifuging and comparing the optical rotatory powers of the filtrates.

	Rotation observed.
Enzyme + α – methylglucoside	+1·41
Enzyme + α – methylglucoside, boiled	+1·43
Enzyme + water	0

It appears that, as in the case of the other two species investigated, *Dimorphotheca cuneata* contains an enzyme specifically adapted to its substrate and having but little action upon other β – glucosides and none upon α – methyl glucoside. The name " linamarase " has been suggested for this enzyme (Rosenthaler, 1922; Rimington,1932). Liberation of HCN from the plant is slower than in the case of the other two *Dimorphotheca* species. An aqueous extract of dried yeast, after purification by alcohol precipitation, was still found to liberate HCN from linamarin.

The Toxicity of the Plant and Determination of the M.L.D.

Feeding tests upon rabbits were carried out using the ground, dried material which was suspended in water and dosed by stomach-tube. The M.L.D. was found to be between 14 and 20 gm. (seeding stage) for approximately 2 Kilo rabbits corresponding to ±14 mgm. HCN per Kilo body weight. The toxic action appeared to be much slower than in the case of *Dimorphotheca spectabilis* and *Dimorphotheca Zeyheri* poisoning, a fact to be correlated, possibly with the more feeble enzymic activity of *Dimorphotheca cuneata.*

As in the case of other *Dimorphotheca* species, it is to be expected that the prophylactic measure advocated by Steyn (1929) of dosing sulphur, incorporated in a lick, should also serve to protect stock animals from the poisonous effects of *Dimorphotheca cuneata.*

Summary.

The toxic substance present in the plant *Dimorphotheca cuneata* has been isolated and identified as the cyanogenetic glucoside " linamarin " or acetonecyanhydrin glucose ether. An improved method of extraction has been described.

The plant contains the enzyme " linamarase " exhibiting a fairly high degree of specificity for its substrate.

Quantitative determination shows that *Dimorphotheca cuneata* in the flowering stage yields about 280 mgm. of HCN per 100 gm. plant, calculated on the dry weight basis. The toxic effects following ingestion of lethal quantities of the plant appear to develop more slowly than is usually the case with *Dimorphotheca* species.

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