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Isolation of the Toxic Principle of *Moraea pallida* by Means of the Sensory Receptors of Sheep

Leendert D. Snyman
Toxicology, Agricultural Research Council, Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa, snymanleendert@gmail.com

T. Stephanus Kellerman
Toxicology, Agricultural Research Council, Onderstepoort Veterinary Institute, South Africa

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Abstract

Chemical fractions of *Moraea pallida* were offered to a sheep in which aversion to the plant had previously been established. Fractions refused due to the presence of the aversive substance sensed by the sheep were further purified until a single substance had been isolated. The purified substance was characterized as epoxyscillirosidin, the toxic principle of *M. pallida*.

Keywords

Moraea pallida, isolation, aversive substance, toxic principle, sensory receptors, sheep

Cover Page Footnote

Footnotes LD Snyman: Retired veterinary research scientist (snymanleendert@gmail.com) TS Kellerman: Deceased

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INTRODUCTION

Moraea pallida, colloquial named yellow tulp (Fig. 1), is one of the cardiac glycoside-containing plants that collectively are the most important cause of plant associated poisoning of livestock in southern Africa (Kellerman *et al*. 1996).

Figure 1. *Moraea pallida* (yellow tulp)

M. pallida is strongly aversive when ingested by livestock. It is generally accepted among farmers in South Africa that stock raised on *M. pallida* infested pastures will not be poisoned. Kellerman *et al*. (1988) reported that recently weaned calves with no previous exposure to *M. pallida* no longer became sick four days after being introduced to heavily infested pastures.

Aversion is induced when the taste and odor of ingested plants are associated with an aversive post-ingestive feedback elicited in the animals (Provenza *et al*. 1992). The sensory receptors of the animals, which functions at a molecular level (Arnold and Hill 1972, Provenza and Balph 1987), play a key role in the establishment of aversion and subsequent avoidance of the plants (Provenza *et al*. 1992).

The hypothesis made was that the aversive post-ingestive feedback elicited after ingestion of *M. pallida* might also be related to the flavor of the aversive substance. If so, this might enable avoidance/refusal of chemical fractions containing the aversive substance, enabling its isolation.

The objective of this study was to isolate the aversive substance of *M. pallida* by using the sensory receptors of a sheep to sense the presence of the aversive substance in chemical fractions of the plant.

MATERIALS

Plant Material. *M. pallida* in the pre-bloom stage was collected in the Mbombela (=Nelspruit) (25.4753 °S, 30.9694 °E) district. The plant material was air dried in the shade, milled to pass a 1 mm sieve and stored at room temperature.

Experimental Animals. All procedures with animals were carried out according to the South African National Standard (*The Care and Use of Animals for Scientific Purposes* [*SANS 10386:200X*]). Trials with animals were approved by the animal ethics committee of the Agricultural Research Council–Onderstepoort Veterinary Institute.

A naïve Merino whether (60 kg), individually housed in a pen and receiving *Eragrostis curvula* hay and water *ad lib*., was used for this study.

Apparatus. The dried plant material was ground with a Wiley cutting mill (Arthur H. Thomas Co., Philadelphia). Centrifugation of plant extracts was performed with a Beckman Coulter benchtop centrifuge (Beckman Coulter Inc.) and evaporation of the solvents was carried out under vacuum with a Büchi R–100 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).

Chemicals and Reagents. Solvents used for extraction and chromatographic fractionation were analytical grade products (Merck, Darmstadt, Germany), redistilled to ensure absolute purity. Column chromatography was performed with silica gel 60 (0.040-0.063 mm, 230-400 mesh ASTM) (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed with ALUGRAM SIL G/UV_{254} and ALUGRAM RP-18/UV_{254} plates (Macherey-Nagel, Düren, Germany). Reagent 22 in Merck dying reagents manual (Merck, E. 1971) was used as anisaldehyde spraying reagent.

METHODS AND RESULTS

Isolation of the aversive substance. The procedure used was briefly described by Kellerman *et al*. (2005). In order to enable the sheep used to sense the presence of the aversive substance in chemical fractions made from *M. pallida*, aversion to *M. pallida* had to be established in the sheep first.

Procedure for establishing aversion. Aversion in the sheep, used for sensing the aversive substance, was established as follows. Firstly, the sheep was made accustomed to maize meal by presenting him 150 g of the meal in a separate feeding trough not used for hay, each morning for 14 consecutive days. The sheep hereby acquired a so-called “learned safety status” to the meal. During this period the sheep also became accustomed to the environment whereby negative associations with the environment during acquirement of aversion was minimized. Following this treatment, the sheep was offered 1.5 g milled *M. pallida* mixed with 150 g maize meal each morning, after being fasted overnight. The maize meal served to increase the attractiveness of the mixture. The sheep completely

consumed the mixture on days 1 and 2, then partially refused the mixture on days 3 and 4 followed by total refusal from Day 5 onwards, which was as an indication that the sheep became averted to *M. pallida* and thus might be able to sense the aversive substance in chemical fractions of *M. pallida*.

Procedure for sensing the presence of the aversive substance. The various chemical fractions made from *M. pallida* (equivalent to 1.5 g *M. pallida*) were mixed with 150 g maize meal and the solvent evaporated at 60˚C. The chemical fractions obtained with successive purifications were presented to an averted sheep in a separate feeding trough. Presentations were made in the morning after the sheep had been fasted overnight. Total or partial refusal of a chemical fraction after 20 minutes with subsequent ingestion of 150 g unadulterated maize meal presented in the same trough and complete consumption of the chemical fraction by a naïve sheep (indicating that refusal was not due to taste) would indicate the presence of the aversive substance.

Fractionation of M. pallida. Solvent extraction of *M. pallida* followed by further chromatographic fractionation were carried out to isolate the aversive substance. Fractions refused due to the presence of the aversive substance were further fractionated until the pure aversive substance had been isolated. Fractions consumed, indicating the absence of the aversive substance, are not described.

Solvent extraction: Twelve grams of *M. pallida* was extracted with 200 ml ethanol by means of the Soxhlet extraction procedure. This was performed for 3 h. with a flux every 10 min. The ethanol extract was filtered through cotton wool followed by filtering through Whatman No 1 filter paper. The filtrate (refused when tested as formerly described) was evaporated to dryness at 60 °C. The residue was dissolved with the aid of an ultrasonic water bath in 200 ml diethyl ether (saturated with water) and centrifuged for 30 min at 1800 x g. The supernatant (refused) was evaporated to dryness at 40 °C and the residue dissolved in 40 ml ethyl acetate. The insoluble fraction was separated by centrifugation as described above and the supernatant (refused) evaporated to dryness at 40 °C.

Chromatographic fractionation: The residue of the ethyl acetate extract was dissolved in 4 ml ethanol of which 2 ml was fractionated by chromatography on a silica gel column (30 g in a 15 mm x 250 mm glass column), using ethyl acetate:acetone (3:1) as mobile phase. The eluate containing the first 10 ml of the colored front was discarded and the ensuing 80 ml (refused) collected. The solvent of this fraction was evaporated at 60 ^{0}C and the residue dissolved in 1.5 ml ethanol. The solution was thereupon fractionated on a preparative silica gel coated plate with chloroform:methanol (92:8) where after 1 cm broad vertical strips on the edges of the plate was sprayed with anisaldehyde reagent. The horizontal band corresponding with the anisaldehyde coloring bands on the edges was scraped off and shaken with 20 ml ethanol. The silica gel was separated by centrifuging as described for 15 min. and the supernatant (refused) reduced to approximately 1 ml

on a rotary evaporator set at 60 °C. The reduced supernatant was subjected to further fractionation on a preparative silica gel coated plate with chloroform:diethyl ether:methanol (50:42:8). The band corresponding with the anisaldehyde coloring bands on the edges was scraped off and treated as described above. Thin layer chromatography of this fraction (refused) on silica gel and RP18 coated plates developed with chloroform:methanol:water (16:1:1) and chloroform:methanol:water (2:35:15), respectively, showed only one spot with Rf values 0.55 and 0.48, respectively, when sprayed with the anisaldehyde reagent.

The procedure investigated for isolating the aversive substance as described above is schematically represented in Fig. 2.

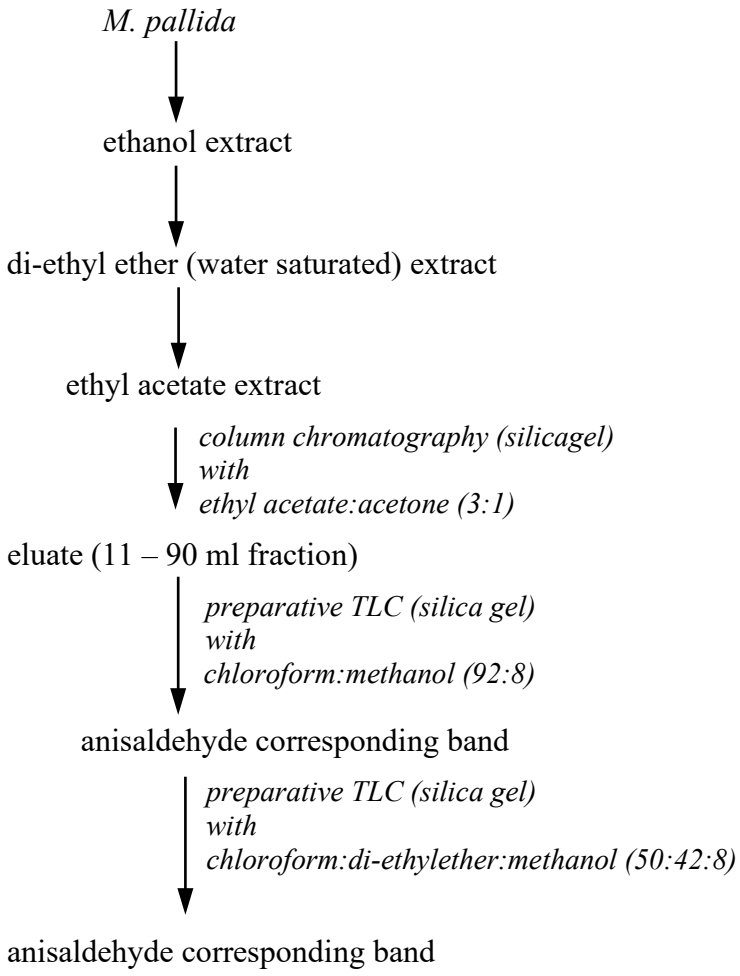

Figure 2. Schematic representation of the procedure investigated to isolate the aversive substance of *M. pallida*.

Characterization of the aversive substance. Nuclear magnetic resonance (NMR) and gas chromatography mass spectrometry (GC-MS) analyses of the purified fraction characterized a single compound with its chemical structure resembling that of epoxyscillirosidin (Fig. 3) (Vleggaar, R., personal communication). Epoxyscillirosidin was previously isolated (Naudé and Potgieter, 1966; Enslin *et al*. 1966) and is the toxic Principle of *M. pallida.*

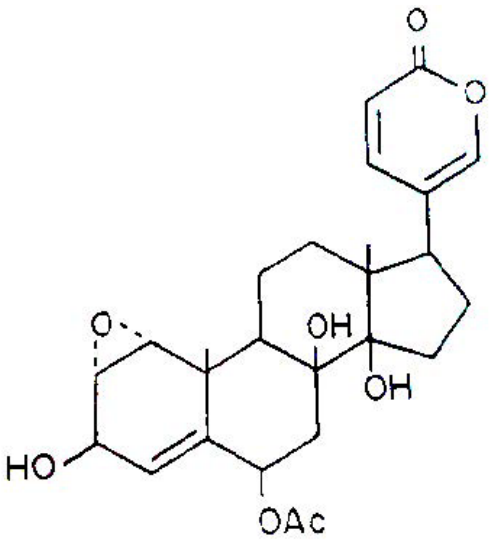

Figure 3. Chemical structure of epoxyscillirosidin

DISCUSSION

The results show that the chemical structure of the aversive substance isolated from *M. pallida* corresponds with that of epoxyscillirosidin, the toxic principle previously isolated from *M. pallida* (Naudé and Potgieter 1966, Enslin *et al*. 1966). This finding supports Riley and Tuck's (1985) description that taste aversions generally corroborate known toxicity. The results confirm the hypothesis made that the aversive substance of *M. pallida* might be isolated by means of the sensory receptors of a sheep. This reflects the enormous sensitivity of the sensory receptors for taste and smell, normally used to select diets in the field from a great variety of plant species differing in nutrient content and secondary metabolites.

The aversive capability of epoxyscillirosidin in conditioning steers to avoid epoxyscillirosidin containing mixtures was previously indicated following the isolation of epoxyscillirosidin provisionally reported by Snyman *et al*. (2004). When tested as aversive agent in conditioning steers to avoid *M. pallida* growing on various pastures it successfully conditioned the animals by significantly limiting the number of animals severely poisoned on the pastures (Snyman *et al*. 2003,

Snyman *et al*. 2007). However, due to its contribution to poisoning of animals when exposed to such pastures (Snyman et al. 2004), epoxyscillirosidin in these trials had to be partly replaced with lithium chloride, an aversive agent previously used for establishing aversion to poisonous plants (Ralphs et al. 2001). For aversion to *M. pallida* occurring on these pastures, however, it also had to be administered together with a hexane extract of *M. pallida*, containing the sensory characteristics of the plant (Snyman *et al*. 2004).

The results of this investigation demonstrated that the aversive substance/toxic principle of *M. pallida* could be isolated by using the sensory receptors of an averted sheep to detect its presence in chemical fractions of the plant.

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