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Isolation of the Toxic Principle of *Senecio latifolius* by Means of the Sensory Receptors of Sheep

Abstract

The aversive substance of *Senecio latifolius* was isolated by means of the sensory receptors of sheep averted to *S. latifolius*. Chemical fractions refused due to the presence of the aversive substance sensed by the sheep were fractionated until a purified substance had been isolated. Nuclear magnetic resonance (NMR) analysis of the purified substance showed it to be sceleratine nitrogen oxide, the toxic principle of *S. latifolius*.

Keywords

Senecio latifolius, isolation, aversive substance, toxic principle, sensory receptors, sheep

Cover Page Footnote

Footnote LD Snyman: Retired veterinary research scientist (snymanleendert@gmail.com) Professor CJ Botha of the Faculty Veterinary Science, University of Pretoria, is acknowledged for reviewing the veterinary aspects of the manuscript

INTRODUCTION

Senecio latifolius (Fig. 1), one of approximately 270 *Senecio* spp. identified in South Africa (South African National Biodiversity Institute) causes major cattle losses due to hepatotoxic pyrrolizidine alkaloids contained by this species (Kellerman *et al*. 1988).

Figure 1. *Senecio latifolius* (with the courtesy of Kellerman *et al*. 1988)

Outbreaks of seneciosis most commonly occur when livestock feed on newly sprouted *Senecio latifolius* on fields denuded by droughts, overstocking or fire (Henning 1932). Seneciosis may result from acute or chronic poisoning, depending on the toxicity of the plant, amount of plant material ingested and the duration of exposure (Kellerman *et al*. 1988).

Similar to *Moraea pallida* (Snyman and Kellerman 2023), *S. latifolius* is an aversive poisonous plant. It is commonly accepted among South African farmers that livestock familiar with *S. latifolius* will not be poisoned when grazing on infested pastures. Kellerman and Schultz (1987) reported that sheep prophylactically treated with activated charcoal refused to eat *S. latifolius* three days later and also when re-exposed to the established *S. latifolius* pasture on several occasions afterwards.

The objective of this research was to isolate the substance present in *S. latifolius* that causes aversion when eaten by livestock. The aversive substance present in *M. pallida* was previously isolated by using the sensory receptors of a sheep to detect the presence of the aversive substance in chemical fractions made from the plant (Snyman and Kellerman 2023). It was the aim of this investigation to replicate this approach in isolating the aversive substance of *S. latifolius*.

MATERIALS

Plant material. *Senecio latifolius* collected in the eMkhondo (=Piet Retief) (27.0245^0S, 30.7925^0E) district and established at a nearby site on the premises of the Onderstepoort Veterinary Institute was used for this study. Harvesting was performed at the apparently more toxic and palatable pre-bloom stage (Kellerman *et al.* 1988). Some of the plant material used was fresh while other was lyophilized and ground to pass a 1 mm sieve. The ground plant material was sealed in plastic bags and stored in a conventional freezer at –20 °C.

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. Naïve Dorper wethers of approximately 50 kg BW were used in these trials. The sheep were housed individually in pens (1 x 3.5 metre) and fed on *Eragrostis curvula* hay and water (*ad lib*).

Apparatus. Plant material was lyophilized with a Christ laboratory freeze drier (Martin Christ, Germany), and ground with a Wiley cutting mill (Arthur H. Thomas Co., Philadelphia). Centrifugation was performed with a Sorvall RC–3B centrifuge (Thermo Fisher Scientific, Germany) and evaporation of the solvents carried out with a Büchi R–100 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).

Chemicals. 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) and RP8 (LiCroprep, 0.040-0.063 mm) (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was carried out with ALUGRAM SIL G/UV_{254} plates (Macherey-Nagel, Düren, Germany).

METHODS AND RESULTS

Isolation of the aversive substance

Investigation of conditions necessary for establishing aversion. In order to isolate the aversive substance of *S. latifolius*, aversion had to be established to *S. latifolius* in the sheep used to sense the aversive substance present in the first chemical fraction made from *S. latifolius*. Establishing aversion to *S. latifolius*,

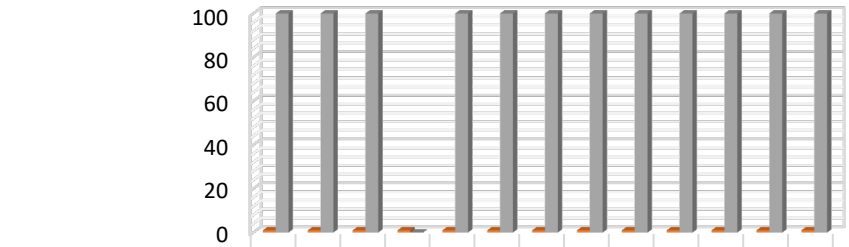
however, was found to be challenging and therefore had to be investigated beforehand as described in the trials below.

The effect of ingestion of various amounts of freeze-dried *S. latifolius* mixed with maize meal on continued intake of the mixtures was investigated. High intake of *S. latifolius* was enabled by administering additional plant material *via* a stomach tube as high concentrations of *S. latifolius* in the meal mixtures became unpalatable and were not eaten. In cases where the additional plant material was dosed in fresh form, the plant material was homogenized in water with a Vevor high speed laboratory homogenizer before dosage. Before the trial commenced the sheep were accustomed to maize meal by feeding 200 g meal daily for 14 consecutive days.

The maize meal and *Senecio* containing maize meal mixtures were presented in the same feeding trough but separate from that used for hay. Treatment and results for sheep 1, 2, 3, 4 and 5 are described below and illustrated in figures 2, 3, 4, 5, and 6, respectively.

Sheep 1 (Fig. 2) was offered 1 g *S. latifolius* mixed with 199 g maize meal for 13 consecutive days. The mixture was totally consumed on days 1, 2 and 3, totally refused on Day 4 and then totally consumed on days 5 to 13 again.

Figure 2. Effect of *S. latifolius* ingestion on continued intake of a *S. latifolius* containing meal mixture

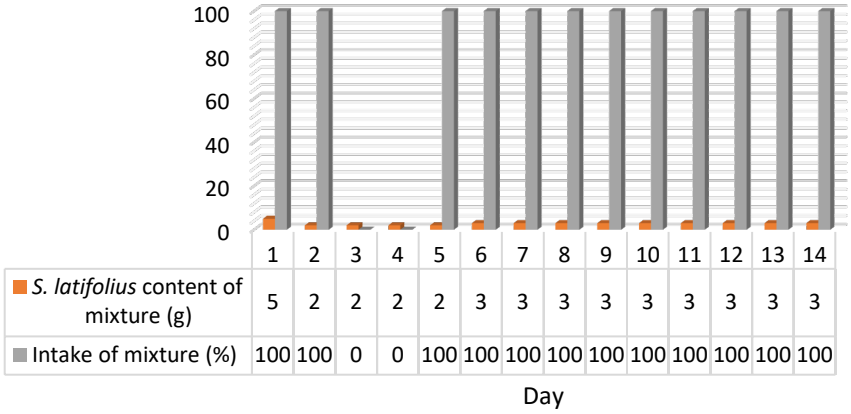


Day	1	2	3	4	5	6	7	8	9	10	11	12	13
S. latifolius content of mixture (g)	1	1	1	1	1	1	1	1	1	1	1	1	1
Intake of mixture (%)	100	100	100	0	100	100	100	100	100	100	100	100	100

Sheep 2 (Fig. 3) was offered 5 g *S. latifolius* mixed with 195 g maize meal on Day 1 and 2 g *S. latifolius* mixed with 198 g maize meal on days 2 to 5. From days 6 to 14 the sheep was offered 3 g *S. latifolius* mixed with 197 g maize meal. The sheep totally consumed the mixtures presented on days 1 and 2, then totally

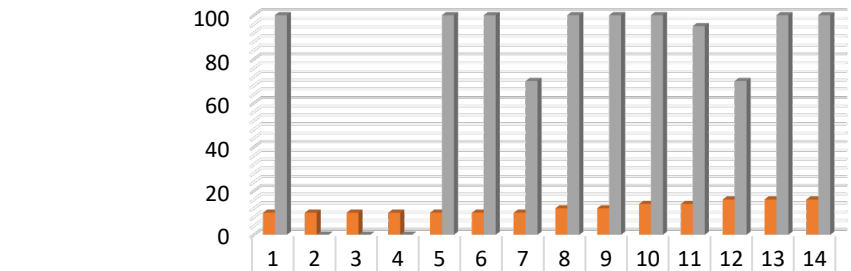
refused the mixtures presented on days 3 and 4 where after the mixtures were totally consumed again until Day 14.

Figure 3. Effect of *S. latifolius* ingestion on continued intake of *S. latifolius* containing meal mixtures



Sheep 3 (Fig. 4) was offered 10 g *S. latifolius* mixed with 190 g maize meal on days 1 to 7, twelve grams of *S. latifolius* mixed with 188 g maize meal on days 8 and 9, fourteen grams of *S. latifolius* mixed with 186 g maize meal on days 10 and 11 and 16 g *S. latifolius* mixed with 184 g maize meal on days 12 to 14. The mixture was totally consumed on Day 1 and then totally refused for the next 3 days, where after it was totally consumed on days 5, 6, 8, 9, 10, 13 and 14. Partial intake of the mixtures took place on days 7, 11 and 12. Total consumption of the mixture on days 13 and 14 took place despite a further increase in *S. latifolius* content. No clinical signs reminiscent of seneciocis were noticeable at the end of the trial. Serum aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) activities of 49 and 38 units per liter (U/L), respectively, determined on Day 14, were in the reference range, thus indicating no hepatic involvement.

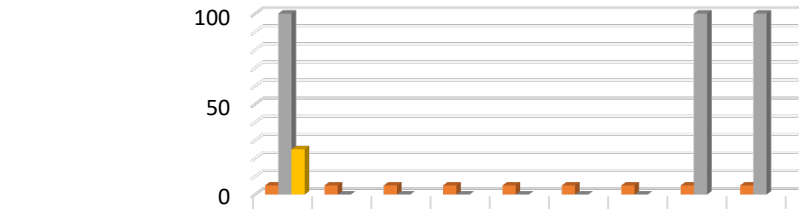
Figure 4. Effect of *S. latifolius* ingestion on continued intake of *S. latifolius* containing meal mixtures



Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
S. latifolius content of mixture (g)	10	10	10	10	10	10	10	12	12	14	14	16	16	16
Intake of mixture (%)	100	0	0	0	100	100	70	100	100	100	95	70	100	100

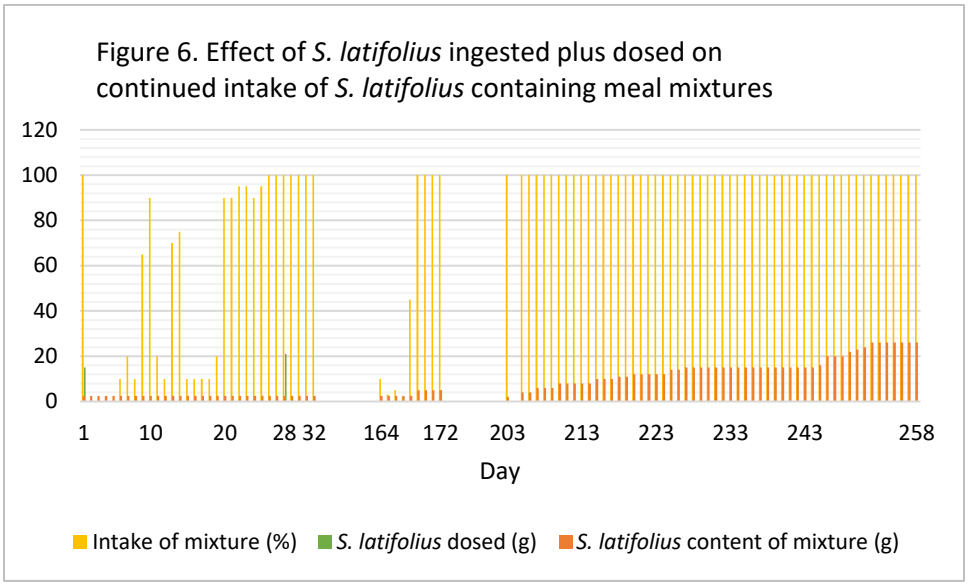
Sheep 4 (Fig. 5) was offered 5 g *S. latifolius* mixed with 195 g maize meal on Day 1. Immediately after total consumption of the mixture the sheep was dosed with 25 g *S. latifolius via* a stomach tube. This was followed by offering 5 g *S. latifolius* mixed with 195 g maize meal on Days 2 to 9. The mixture was totally refused on Days 2 to 7 and then totally consumed on Days 8 and 9.

Figure 5. Effect of *S. latifolius* ingested plus dosed on continued intake of a *S. latifolius* containing meal mixture



Day	1	2	3	4	5	6	7	8	9
S. latifolius content of mixture (g)	5	5	5	5	5	5	5	5	5
Intake of mixture (%)	100	0	0	0	0	0	0	100	100
S. latifolius dosed (g)	25								

Sheep 5 (Fig. 6) was offered 2.5 g *S. latifolius* mixed with 97.5 g maize meal on Day 1. After consumption of the mixture the sheep was dosed, using a stomach tube, with 90 g fresh (=15 g DM) *S. latifolius* homogenized in water. From Day 2 the sheep was offered 2.5 g *S. latifolius* mixed with 97.5 g maize meal daily until Day 32. The mixture was totally refused on days 2, 3, 4 and 5 and thereafter inconsistently but increasingly consumed with total consumption on days 26 to 28. After consumption of the mixture on Day 28, the sheep was dosed with 125 g fresh (= 21 g DM) *S. latifolius* after which the sheep continued eating the total mixture until Day 32. Exposure to *S. latifolius* from here on was discontinued until Day 164 when the sheep was re-exposed to 2.5 g *S. latifolius* in 97.5 g maize meal until Day 172. The sheep almost totally refused the presentations on the first 4 days (days 164 – 167) but then totally consumed it again on days 169 to 172. Presentation of the mixture was then discontinued for a second time until Day 203. After Day 203 the sheep was exposed to increasing concentrations of *S. latifolius* in 200 g maize meal, starting with 2 g on Day 203 and ending with 26 g on Day 258 when the trial was terminated. The mixtures were totally consumed during this period. Serum AST and GGT activities (U/L) were 40 and 33 on Day 214, 50 and 44 on Day 227, 51 and 40 on Day 252 and 50 and 45 on Day 258, respectively, with all values being within the normal reference range. The sheep also exhibited no clinical signs of seneciosis.



The following observations made from these trials were regarded important for establishing aversion to *S. latifolius*:

All five sheep after initial ingestion of *S. latifolius* refused further intake for a period of time, indicating that experimental induction of aversion to *S. latifolius* is feasible and thus a means to isolate the aversive substance.

Refusal of the *S. latifolius* containing mixtures lasted for a relatively short period. However, the aversion period was sooner and tended to increase after ingestion of greater amounts of *S. latifolius*. These observations suggest that high doses of *S. latifolius* should be administered to establish the most effective aversion.

When sheep started eating the *S. latifolius* containing mixtures after total refusal for some period of time, none of the sheep refused the mixture again despite increasing amounts of *S. latifolius* in some cases (save for Sheep 5 that refused presentations on the first 4 days after re-exposure to *S. latifolius* containing mixtures on Day 164). This observation demonstrates that once aversion has disappeared, the once averted sheep becomes unreliable for further usage.

Procedure for establishing aversion. The procedure followed for establishing aversion in sheep to be used for sensing the aversive substance in *S. latifolius* was as briefly described by Kellerman *et al*. (2005). To induce aversion to *S. latifolius*, sheep were presented 100 g 2% *S. latifolius* in maize meal after being fasted overnight. After consumption of the mixture, the sheep was dosed *via* a stomach tube with a homogenate in water of freshly collected *S. latifolius* equivalent to 18 g dry matter (DM). Refusal of a similar presentation (2% *S. latifolius*) the next day while eaten by a naïve sheep indicated that the sheep became averted to *S. latifolius*, provided that the averted sheep would eat 100 g pure maize meal presented in the same trough. The sheep was then regarded capable to sense the presence of the aversive substance in chemical fractions of *S. latifolius* for as long as the induced aversion lasted where after aversion was established in another sheep as described above.

Establishing aversion to chemical fractions was performed in the same manner as described above for *S. latifolius*, except that chemical fractions were presented at an amount equivalent to 2% *S. latifolius* (mixed with maize meal) followed by dosing of the chemical fraction (shaken up in water) *via* a stomach tube at an amount equivalent to 18 g *S. latifolius*. When aversion of the sheep to a chemical fraction disappeared, aversion in another sheep was established to the last chemical fraction in which the aversive substance was sensed. This process continued until the pure aversive substance was isolated. Prior to presentation and dosing the solvents of the chemical fractions were evaporated at 60 ˚C.

Procedure for sensing the presence of the aversive substance. Chemical fractions were presented at the equivalent of 2% *S. latifolius* in 100 g maize meal to sheep averted to *S. latifolius* or successive chemical fractions. Refusal of a chemical fraction while eaten by a naïve sheep would indicate the presence of the aversive substance, provided that the sheep would be willing to eat pure maize meal presented in the same trough.

Fractionation of *S. latifolius.* Solvent extraction of *S. latifolius* followed by further chromatographic fractionation was carried out to isolate the aversive substance (Fig. 7). Chemical fractions refused in addition were also tested for their capability to induce aversion in a naïve sheep, executed according to the procedure described above. Fractions consumed, indicating the absence of the aversive substance, are not described.

Solvent extraction: Two hundred grams of freshly collected *S. latifolius* was homogenized in 1 L water within an hour after being harvested. The homogenized plant material was then stirred for 3 h. by using a magnetic stirrer, filtered through cheesecloth and centrifuged for 30 min. at 900 x g. The supernatant (refused) was subsequently filtered through cotton wool and the filtrate evaporated to dryness at 50˚C. The residue was resuspended using an ultrasonic water bath in 250 ml methanol, shaken, and centrifuged as described. The supernatant (refused) was subsequently filtered through cotton wool and evaporated to dryness at 40 ˚C. The residue was then resuspended with 5 ml methanol after which 250 ml acetone was added. The insoluble fraction was separated by centrifugation and the supernatant (refused) filtered through cotton wool and evaporated to dryness at 40 ˚C.

Chromatographic fractionation: The residue of the methanol:acetone soluble fraction (2.28 g) was dissolved in 10 ml methanol and fractionated by column chromatography on 200 g silica gel 60 with chloroform:methanol (1:2). The eluted fractions were spotted on silica gel coated thin layer plates and developed with chloroform:methanol (1:2). Fractions showing spots that colored brown when sprayed with an acetic anhydride:benzene:hexane (1:4:5) reagent (R_f = 0.25 and 0.34) (refused) were combined and evaporated to dryness on a rotary evaporator at 40 ˚C. The residue (0.771 g) was similarly fractionated by column chromatography on 150 g silica gel where after fractions that contained the acetic anhydride:benzene:hexane (1:4:5) coloring spots (refused) were dried. The residue (0.501 g) was then fractionated under the same conditions on 100 g silica gel. Fractions that contained the two coloring spots with the fore mentioned Rf values (refused) were dried and the residue (0.215 g) fractionated with chloroform:ethanol:water (1:3:1) on 50 g silica gel. Fractions showing similarly colored spots with R_f values 0.32 and 0.43 when developed with chloroform:ethanol:water (1:3:1) on silica gel coated thin layer plates and sprayed with the acetic anhydride:benzene:hexane (1:4:5) reagent (refused) were pooled and dried. The residue (0.161 g) was fractionated by column chromatography on 50 g RP8 with methanol:ethanol (3:1) as eluant. Fractions that contained the less polar acetic anhydride reagent coloring spot (R_f = 0.43) when developed on a silica gel coated thin layer plate with chloroform:ethanol:water (1:3:1) (refused) were

pooled and dried. When acetone was added to a saturated solution of the dried residue (0.041 g) in methanol the content crystallized. The crystallized material induced strong aversion when administered to a naïve sheep.

The procedure investigated for isolating the aversive substance as described above is schematically represented in Fig. 7.

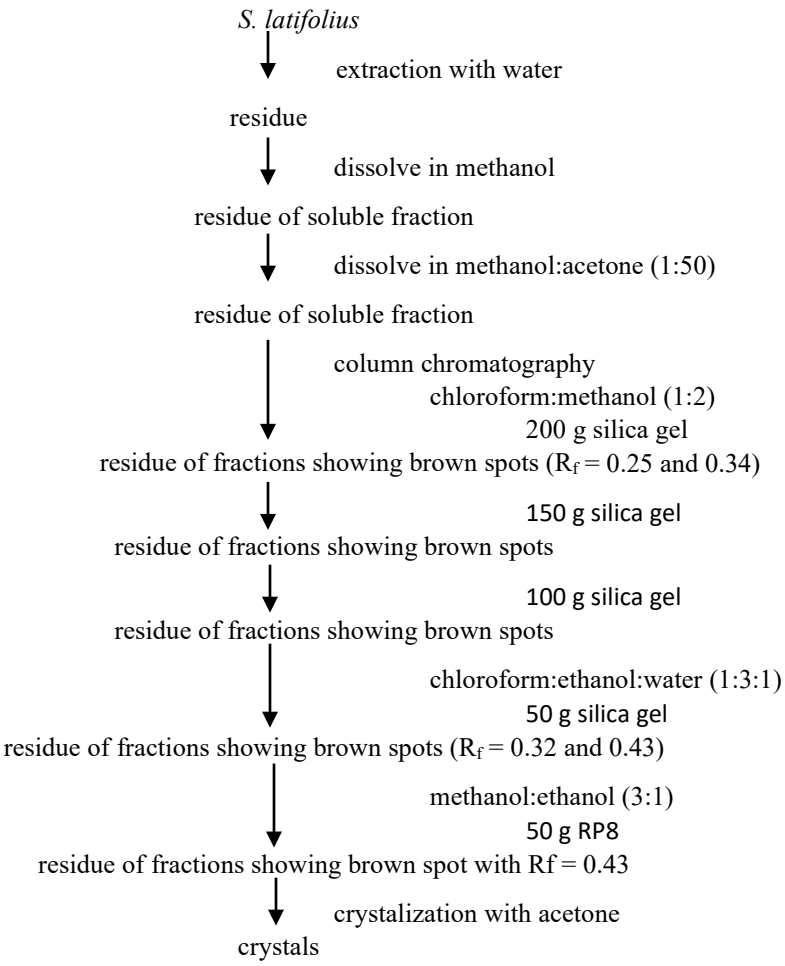


Figure 7. Schematic representation of the procedure investigated to isolate the aversive substance of *S. latifolius*.

Characterization of the aversive substance. A nuclear magnetic resonance (NMR) analysis of the crystallized material characterized the aversive substance as sceleratine nitrogen oxide (sceleratine-NO) (Bode, M; personal communication) a known toxic principle of *S. latifolius*. Sceleratine-NO was first

isolated by Bredenkamp *et al.* (1985) who correctly assigned the chemical structure for sceleratine and thereby for sceleratine-NO as illustrated in Fig. 8

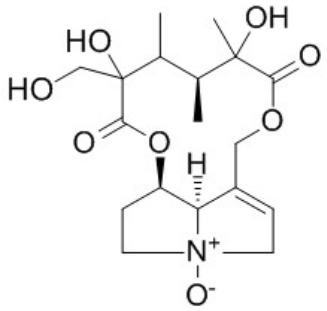


Figure 8. Chemical structure of sceleratine nitrogen oxide

Aversion properties of some chemical fractions of S. latifolius. Intake and refusal of *S. latifolius* and some of the chemical fractions of *S. latifolius* (left overs collected during isolation of the aversive substance) by sheep treated for aversion to *S. latifolius* or to one of the chemical fractions of *S. latifolius,* are shown in Table 1.

The following observations were made from the results in Table 1: The sheep treated for aversion to *S. latifolius* refused intake of the chemical fractions he was tested for, namely the water soluble and acetone soluble fractions, as well as the dichloromethane extract, while sheep treated for aversion to the water soluble fraction, acetone soluble fraction, acetic anhydride coloring spots fraction and to purified sceleratine-NO did not refuse *S. latifolius*. Uncertain results were obtained for the water soluble fraction as one sheep averted to this fraction totally consumed *S. latifolius* after initially refusing it while a second sheep exhibited partial refusal of *S. latifolius*.

Except for the dichloromethane extract, the sheep treated for aversion to specific chemical fractions also refused intake of the other chemical fractions they were tested for.

A sheep treated for aversion to a dichloromethane extract did not refuse intake of *S. latifolius*, the dichloromethane extract itself or the purified sceleratine-NO fraction. A sheep treated for aversion to *S. latifolius*, however, refused intake of the dichloromethane extract.

Table 1. Intake and refusal of *S. latifolius* and chemical fractions of *S. latifolius* by sheep averted to *S. latifolius* or to one of the chemical fractions of *S. latifolius*

S. latifolius and chemical fractions of *S. latifolius* to which sheep were treated for aversion[1]	Intake (+) / refusal (-) / intake and refusal (+/-) of *S. latifolius* and chemical fractions of *S. latifolius*						
	S. latifolius	Water soluble fraction	Methanol soluble fraction	Acetone soluble fraction	Acetic anhydride coloring spots	Sceleratine nitrogen oxide	Dichloro -methane extract[2]
S. latifolius	–	–		–			–
Water soluble fraction	+/-	–	–	–			
Methanol soluble fraction			–	–			
Acetone soluble fraction	+	–		–			
Acetic anhydride coloring spots	+	–	–		–	–	
Sceleratine nitrogen oxide	+					–	
Dichloromethane extract[2]	+					+	+

[1]Aversion could not be established in sheep treated with a dichloromethane extract of *S. latifolius*
[2]Dichloromethane extract: prepared from the dried water extracted *S. latifolius* (obtained during isolation of the aversive substance) by extraction with 500 ml dichloromethane on a magnetic stirrer for 3 h.

DISCUSSION

The results of this investigation demonstrated that sheep averted to *S. latifolius* were capable of sensing the aversive substance present in chemical fractions of *S. latifolius* and thereby enabled its isolation. The results furthermore show that the aversive substance isolated was also the toxic principle of the plant. These findings confirm corresponding results previously obtained with *Moraea pallida* (Snyman and Kellerman, 2023).

Contrary to the fact that sheep averted to *S. latifolius* could sense the toxic principle in the various chemical fractions, in the more purified fractions even at a distance from the trough, sheep averted to the toxic principle or chemical fractions containing the toxic principle did not refuse *S. latifolius*. This coincides with a previous finding, namely that a sheep averted to *M. pallida* was able to sense the

toxic principle in the various chemical fractions made from the plant (Snyman and Kellerman 2023) but cattle strongly averted to the toxic principle did not refuse *M. pallida* when exposed to a *M. pallida* infested *Pennisetum clandestinum* pasture (Snyman *et al*. 2004).

Labelling sceleratine-NO as the toxic principle of *S. latifolius* might be disputable as the free base form namely sceleratine was isolated as the toxic pyrrolizidine alkaloid contained by *S. latifolius* (De Waal and Pretorius 1941). Furthermore, the N-oxide form was described as non-toxic (Provenza *et al*. 1992). Indirectly, however, sceleratine-NO may be regarded as the major toxin of *S. latifolius* as the N-oxide form of pyrrolizidine alkaloids mainly occurs in plants, which, following ingestion, are reduced by intestinal or liver microsomal enzymes to the toxic free bases and thus show equal toxicity to that of the free bases (Wiedenfeld and Edgar 2011).

Duration of the induced aversion to *S. latifolius* under the present experimental conditions was relatively short, therefore several sheep had to be used to isolate sceleratine-NO, while only a single sheep was needed to isolate the toxic principle of *M. pallida* (Snyman and Kellerman 2023). Duration of the induced aversion tended to be longer with higher initial doses of *S. latifolius*. It is also noteworthy that aversion could not be re-induced once it had disappeared, despite continued intake of increasing amounts of *S. latifolius*. It appears if sheep might adapt to the aversive effect of *S. latifolius* and chemical fractions containing sceleratine-NO, which might be related to ruminal or hepatic detoxification of pyrrolizidine alkaloids (Cheeke 1994; Wiedenfeld and Edgar 2011). This probability is supported by the observation that no clinical signs of seneciosis and no increase in AST and GGT activities could be demonstrated in the animals used, despite ingestion of *S. latifolius* in amounts much larger than was needed to establish aversion. In the case of epoxyscillirosidin, the aversive substance and toxic principle of *M.pallida*, the minimum toxic dose (0.112 mg/kg BW) was found to be two times the minimum aversive dose (0.06 mg/kg) (Snyman *et al*. 2004).

The inability of a dichloromethane extract of *S. latifolius* to establish aversion in sheep to *S. latifolius* or to itself indicates the absence of an aversive substance. Refusal of a dichloromethane extract when offered to a sheep averted to *S. latifolius* therefore must be ascribed to the sensory characteristics of *S. latifolius* present in the extract. This finding corresponds with that in the previous study (Snyman *et al*. 2004) where aversion to *M. pallida* could not be induced with a hexane extract of *M. pallida* while the hexane extract was strongly refused when offered to cattle averted to *M. pallida*. The results indicate that the dichloromethane extract of *S. latifolius* was sufficiently representative of the sensory characteristics

of the plant material. Administering the dichloromethane extract simultaneously with sceleratine-NO, thus might be a means of conditioning livestock to avoid *S. latifolius*.

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