Seasonal variation in cotyledoside concentration of *Tylecodon wallichii* (Harv.) Tölken subsp. *wallichii* sampled in a krimpsiekte-prevalent region

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ABSTRACT

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Krimpsiekte, an economically important neuromuscular affliction of small stock, follows upon ingestion of certain members of the Crassulaceae (plakkies) containing cumulative neurotoxic bufadienolides. *Tylecodon wallichii* (Harv.) Tölken subsp. *wallichii* is probably the most important species of the group of plants causing krimpsiekte. The growing tip of the stem and various other plant parts of *T. wallichii*, when available, were collected monthly. The seasonal variation in cotyledoside content of the plant was measured. Cotyledoside concentration was determined by high performance liquid chromatographic-electrospray mass spectrometry analysis (HPLC-ESMS). The cotyledoside concentration in the plant stems fluctuated substantially during the year, but tended to be higher in the cold winter months and increased again in the spring and early summer. Elevated plant stem concentrations corresponded with natural field outbreaks of krimpsiekte, which usually occur during the winter to early summer. The highest cotyledoside concentrations were detected in the flowering stalk. Cotyledoside was not the only component of this type in the plant, as mass spectrometry revealed the presence of other, possibly related, compounds.

Keywords: Cardiac glycoside, Crassulaceae, cotyledoside, goat, krimpsiekte, sheep, *Tylecodon wallichii* subsp. *wallichii*

INTRODUCTION

Poisoning of livestock caused by cardiac glycoside-containing plants is collectively the most important plant-associated poisoning in southern Africa (Kellerman, Naudé & Fourie 1996). The annual impact of mortalities from cardiac glycoside-containing plants on the livestock industry in the Republic of South Africa exceeds 23.5 million South African Rand (ZAR), approximately US$ 4 million (in 1995 monetary terms) (Kellerman *et al.* 1996). Chemically, two main groups of cardiac glycosides *viz.* the cardenolides and bufadienolides are recognized. Poisoning by bufadienolide-containing plants, which surpasses cardenolide-induced poisonings in importance, may be either acute or chronic. Only acute toxicity occurs when livestock ingests tulp (induced by various *Homeria* and *Moraea* species) and slangkop (caused by various *Urginea* species), as these species contain non-cumulative bufadienolides (Kellerman, Coetzer & Naudé 1988). However, both acute and chronic toxicity may occur when animals graze on three different genera of the Crassulaceae (*Cotyledon*, *Tylecodon* and *Kalanchoe* species), colloquially referred

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to as "plakkies", as the bufadienolides found in these plants are cumulative.

Plakkies are succulents which grow in the dry, arid regions of South Africa. The Succulent Karoo Biome stretches through parts of the Northern and Western Cape provinces and is characterized by low rainfall, ranging from 20–290 mm, and extreme summer aridity (Low & Rebelo 1998). During summer, temperatures exceeding 40 °C are common (Low & Rebelo 1998). The vegetation is dominated by dwarf succulent shrubs of which the Crassulaceae is conspicuous (Low & Rebelo 1998). Ingestion of *Tylecodon wallichii* (Harv.) Tölken subsp. *wallichii*, the most important Crassulaceae species associated with poisoning, causes krimpsiekte which predominantly is a disease of small stock (Kellerman *et al.* 1996).

Tylecodon wallichii occurs on the lower, southern slopes of hills and mountains or on gravelly or sandy soils (Tölken 1978; Kellerman *et al.* 1996). The leaves appear during the autumn/winter months and the flowering time is usually from November to February (Vahrmeijer 1981). Active growth of the plant is very variable and depends on climatic conditions and rainfall. Being a deciduous plant the leaves fall off before flowering and only a protuberant scar remains on the stem (Vahrmeijer 1981).

Krimpsiekte, a chronic neuromuscular disease, is caused by cumulative bufadienolides with neurotoxic properties which are unique to the compounds encountered in these members of the Crassulaceae (Kellerman *et al.* 1988; Botha, Van der Lugt, Erasmus, Kellerman, Schultz & Vleggaar 1997). The term *krimpsiekte* refers to the shrunken, tucked-in posture of the affected animal. Krimpsiekte is a paretic/paralytic syndrome. Affected stock tire easily; lag behind the flock; assume the characteristic posture, with feet together and back arched; display torticollis; become recumbent and suffer protracted paralysis. Paralyzed sheep lie fully conscious on their sides, sometimes for weeks, until they die or are euthanased (Kellerman *et al.* 1988).

In the regions of South Africa where krimpsiekte occurs, farming is extensive and flocks are only inspected occasionally which contributes to the mortalities.

Most cases of krimpsiekte in the Karoo occur between July and November (Henning 1926; Kellerman *et al.* 1988). It is not known whether the seasonal difference in incidence of krimpsiekte is solely due to reduced availability of alternative non-toxic grazing or whether seasonal differences in toxin concentration in the plants may exacerbate the problem. Thus the present study was undertaken to examine the seasonal variations in cotyledoside concentration of the well-known bufadienolide contained by *T. wallichii* (Steyn, Van Heerden & Van Wyk 1984).

MATERIALS AND METHODS

Collection of plant material

Plant samples, which always included the soft growing tip of the stem and leaves, inflorescences and seed heads, when available, were collected monthly (January to December 1999) from three different *T. wallichii* plants (plant A, B and C) growing *c.* 10 m apart from each other, near Springbok (29,40°S; 17,66°E) in the Northern Cape Province, Republic of South Africa (Fig. 1). Plant material was also submitted for verification and positive identification to the Botanical Research Institute, Pretoria. When plant A died during May 1999, samples were collected from an additional plant (D).

The condition, growth stage and reproductive status of plants A–D and the situation of the surrounding vegetation were recorded when plant material was collected. The plant samples were extracted between 2–10 days following collection. The mean moisture content of plant parts collected from *T. wallichii* (n = 4) was determined. Stems and leaves were air dried at room temperature for 5 and 10 days and then in an oven (Victor) at 50–60 °C until the mass remained constant.

Climatological data

Precipitation was measured at the weather station located at Steinkopf (29,25°S; 17,74°E) and daily maximum and minimum temperatures were recorded at Henkries (28,97°S; 18,1°E) (Fig. 1). This data was supplied by the Institute for Soil, Climate and Water, Pretoria.

Extraction of plant material

The plant material was extracted using an adaptation of the methodology of Bourdon & Mercier (1969).

Plant material (2 g) was weighed into a 250-mℓ glass reagent bottle with a screw top and after adding 40 mℓ filtered, purified water (Milli-Q^{50} Ultra-pure water system, Millipore) the sample material was homogenized (Heidolph Diax 6000, Germany). Concentrated hydrochloric acid (10 drops) and dichloromethane (80 mℓ) were added and the mixture shaken for 30 min. The extract (comprised of organic and aqueous phases) was centrifuged for 30 min at 3 000 r.p.m. and the aqueous phase (including the emulsion) was discarded. Filtered, purified water (40 mℓ) and concentrated ammonia (10 drops) were added to the organic phase which was shaken for 30 min and filtered through a Whatman phase separating paper (1PS, silicone treated, 12.5 cm). The organic phase (filtrate) was evaporated off at 50 °C under a nitrogen stream (Turbovap LV, Zymark). Crude extracts were stored in a freezer at –20 °C until they were further processed.

Extract clean-up

The extracts derived from 2 g of plant material were cleaned-up (defatted) for high performance liquid chromatographic-electrospray mass spectrometry (HPLC-ESMS) analysis. Methanol (900 µℓ) was added to the crude extract, which was solubilized with the assistance of vortexing (15 s) and by the application of ultra-sound (*c.* 1–2 min) in a water bath. Distilled water (100 µℓ) and *n*-hexane (*c.* 3 mℓ) were added and the resulting suspension was shaken (1 min) and then centrifuged (1 min at 2 000 r.p.m.). A portion of the methanol phase (*c.* 500 µℓ) was transferred to a Spin-ex filter and spun (Co-star) at maximum speed for 2 min, after which a portion of the filtrate (*c.* 300 µℓ) was transferred to a vial and capped. All chemicals used were either analytical or HPLC grade.

HPLC-ESMS analysis

HPLC-ESMS was performed using a 5-micron, 4.6 mm x 150 cm, Waters C-8 symmetry column interfaced to a Finnagin LC-Q electrospray mass spectrometer. A gradient solvent system, comprised of methanol and water-0.1 % formic acid mixtures, was used, commencing with 50 % methanol, rising to 75 % methanol (over 14 min) then to 95 % methanol (1 min) and held at this level for 9 min. The flow rate was 0.7 mℓ/min. The ESMS was operated in positive ion mode, with selected ion mode (SIM) detection of cotyledoside (retention time window 10–11.5 min), and total ion chromatogram (TIC) mode detection (*m/z* 300–900) of other components before and after this time window. An authentic specimen of cotyledoside ($MH^+ = m/z$ 575) (Fig. 2), previously isolated from *T. wallichii* (Steyn *et al.* 1984; Botha *et al.* 1997),

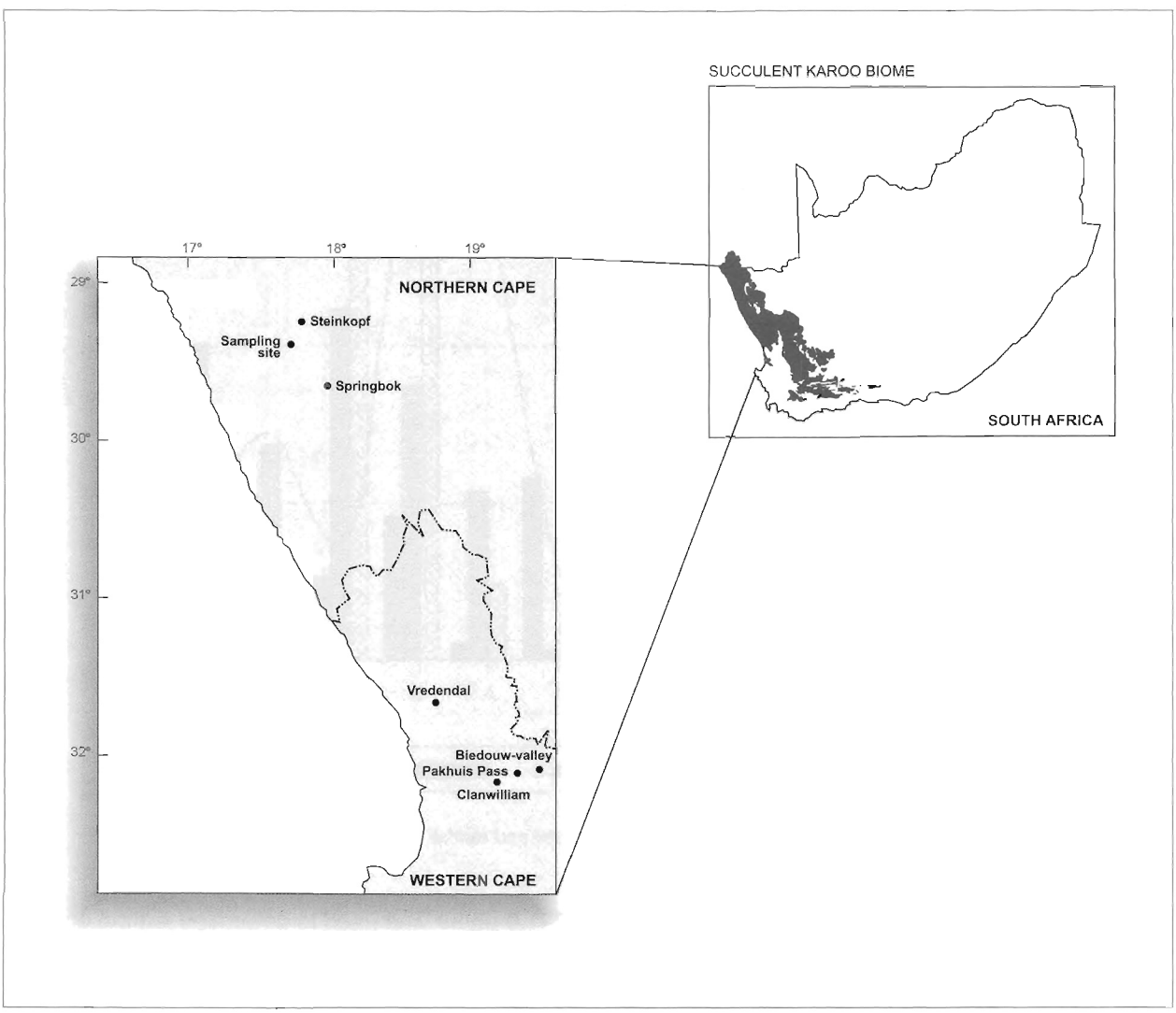

FIG. 1 The Succulent Karoo Biome in the Northern and Western Cape Provinces (reproduced with permission from Tony Rebelo)

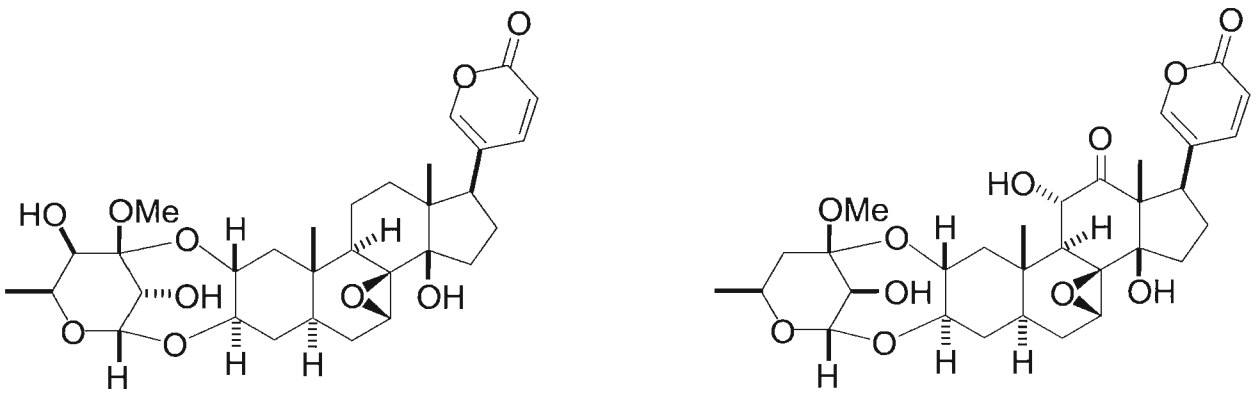

FIG. 2 Cotyledoside and tyledoside D

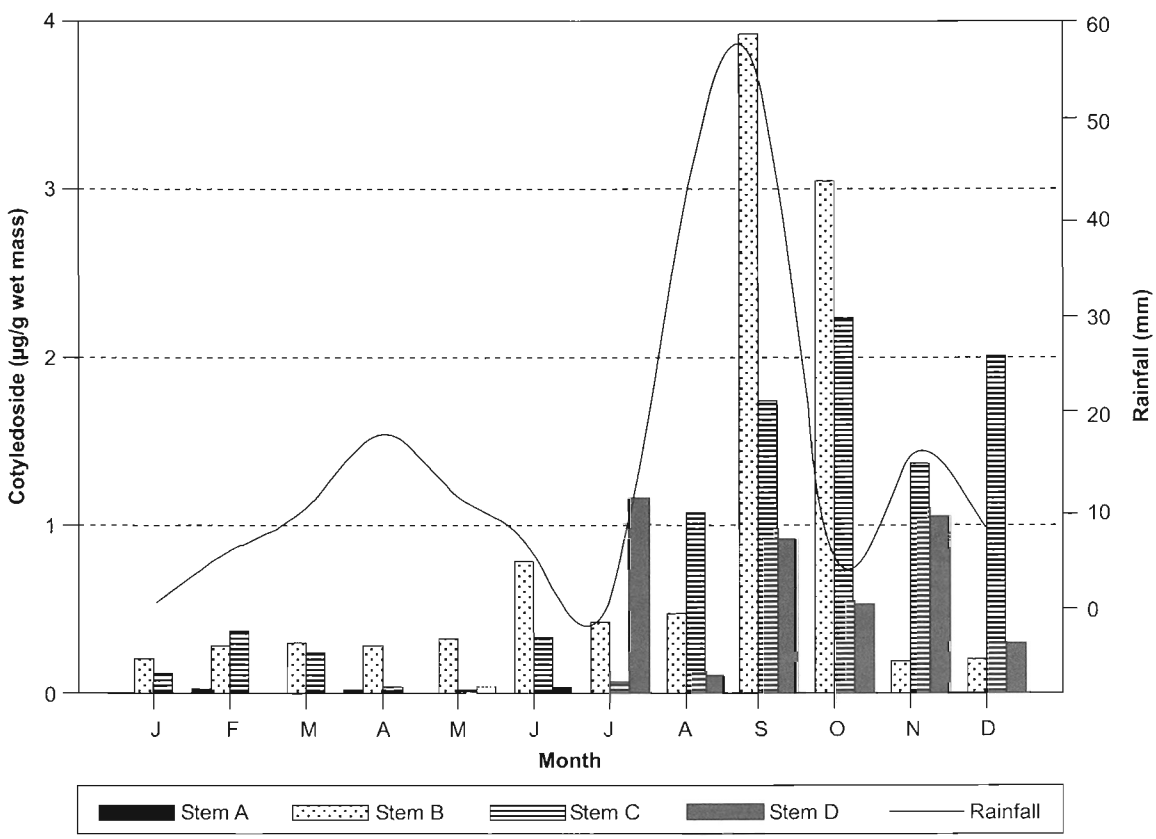

FIG. 3 Monthly cotyledoside concentrations in the stems of *T. wallichii* and rainfall

was used to prepare a standard solution of cotyledoside containing 200 ng/mℓ. Other reference solutions were prepared by dilution of this solution. Typically 20 µℓ injections of reference solutions and plant extracts were analyzed. A calibration curve corresponding to plant cotyledoside concentrations in the range 0–1.0 µg/mℓ was used to determine extract concentrations.

The presence of tyledoside D (MH^+ = *m/z* 589) (Fig. 2) in the crude extracts was established by comparison of mass spectral and retention time data deter-

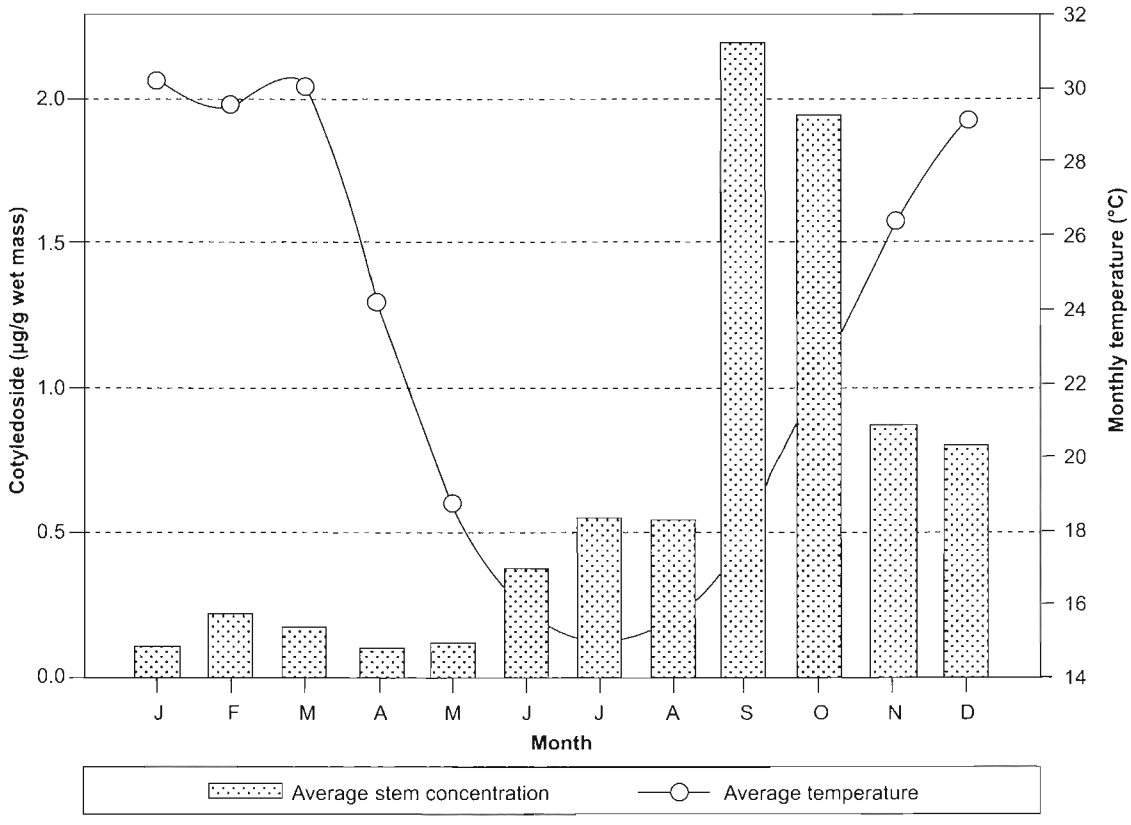

FIG. 4 Average cotyledoside concentrations in the stems of *T. wallichii* and average temperature

mined for an authentic specimen of this compound (Steyn, Van Heerden, Vleggaar & Anderson 1986a).

RESULTS

The plant was positively identified as *Tylecodon wallichii* (Harv.) Tölken subsp. *wallichii* and a voucher specimen has been lodged at the Botanical Research Institute, Pretoria. Drying of the stems (n = 4) at room temperature for 5 and 10 days resulted in a loss of 11.1 % (± 4.4) and 20.9 % (± 8.2) moisture, respectively. During the same period of air-drying the leaves (n = 4) lost 13.9 % (± 5) and 23.7 % (± 8.2) moisture. The total moisture content contained by the stems and leaves was calculated at 80.9 % (± 0.6) and 91 % (± 1.4), respectively.

The cotyledoside concentrations determined in plant stems and leaves are tabulated in Table 1. The cotyledoside concentration in the stems fluctuated substantially during the year (Fig. 3), but its average suggests a seasonal variation (Fig. 4). Considerable variations in the concentration in the leaves, sampled from May to October, were detected. The highest concentrations were determined in the flowering stalks (n = 3, mean concentration 6.64 µg/g wet mass [WM] ± 1.28). In the fresh flowers (n = 6), concentrations ranged from below the limit of detection (< 0.002 µg/g) to 0.83 µg/g WM. Cotyledoside was also detected in the seed heads (n = 13) in concentrations which varied from below the limit of detection to 5.15 µg/g WM.

Regional climatic data recorded during the 12-month collection period as well as long-term data for the 10-year period from 1990–1999 are given in Table 2. From January to March 1999 prevailing conditions in the area were very hot and dry, with the highest maximum temperatures exceeding 42 °C in each of these months and a 3-monthly precipitation of only 15.5 mm (Table 2). Light rain (17.5 mm) fell during April and new leaves were produced by plants B and C. All the *T. wallichii* plants, including plants B–D, in the vicinity to the sampled plants sprouted after light rain (11 mm) in May. A higher rainfall (40.7 mm) was recorded during August and all three plants showed strong growth. A number of *T. wallichii* plants in the vicinity of the plants being sampled died during October when dry weather conditions were experienced (6 mm rain) and, although retarded growth was observed in all *T. wallichii* plants, inflorescences were nevertheless produced by plants B–D. In November, the *T. wallichii* plants lost their leaves but, in spite of being desiccated, some inflorescences formed. In December, the plants under review as well as other

TABLE 1 Cotyledoside concentration (µg/g wet mass) of stems and leaves of 4 different *Tylecodon wallichii* plants (plants A–D) determined monthly (January to December 1999)

Month	Stem A/D[a]	Stem B	Stem C	Leaves B	Leaves C	Leaves D
January	< LOD	0.19	0.10	–	–	–
February	0.02	0.27	0.36	–	–	–
March	< LOD	0.29	0.23	–	–	–
April	0.003	0.28	0.03	–	–	–
May	0.04	0.32	0.01	0.47	0.01	0.01
June	0.04	0.78	0.33	0.26	0.07	0.02
July	1.16	0.42	0.06	0.09	0.01	0.02
August	0.10	0.47	1.08	0.08	0.02	0.02
September	0.91	3.94	1.75	0.02	0.004	0.02
October	0.53	3.06	2.25	0.03	0.03	0.03
November	1.06	0.20	1.38	–	–	–
December	0.30	0.11	2.03	–	–	–
Mean	0.42	0.86	0.80	0.16	0.024	0.02
(SD)	(0.47)	(1.26)	(0.85)	(0.18)	(0.024)	(0.01)

[a] Plant D replaced plant A, which had died, from May onwards < LOD Below limit of detection (< 0.002 µg/g)

TABLE 2 Monthly and long-term climatic data (precipitation [mm] and temperature [°C]) recorded where the plant material was collected

Month	Temperature (°C)					Rainfall (mm)	
	Average minimum	Average maximum	Lowest to highest	Monthly average	Long-term average[a]	Monthly	Long-term average[a]
January	21.4	38.8	13.3–45.3	30.1	28.9	0.0	5.4
February	20.8	38.0	14.8–43.1	29.4	28.3	5.5	1.6
March	22.3	37.5	17.5–42.4	29.9	26.8	10.0	8.5
April	16.3	31.9	5.5–40.0	24.1	23.0	17.5	22.3
May	11.7	25.4	5.5–30.0	18.6	18.2	11.0	18.7
June	7.7	23.7	4.0–29.5	15.7	13.8	5.6	26.6
July	6.7	23.1	2.2–30.2	14.9	13.6	0.0	25.1
August	7.2	23.7	2.6–32.7	15.5	15.3	40.7	17.3
September	9.8	26.3	5.3–37.0	18.1	19.2	54.0	18.3
October	NR	NR	NR	NR	21.9	6.0	10.5
November	17.8	34.8	11.7–42.4	26.3	24.2	15.5	14.3
December	21.6	36.5	14.9–41.0	29.1	26.8	7.8	4.9
Total						173.6	173.5

NR Not recorded

[a] Long-term average calculated over preceding 10 years

large mature plants at this locality were very desiccated, while the younger plants showed active growth.

HPLC-ESMS showed that a number of other cotyledoside-like substances, including tyledoside D, were also present in the cleaned-up plant extracts.

DISCUSSION

Cotyledoside concentrations in the three *T. wallichii* plants sampled fluctuated considerably throughout the year. Kamerman (1926) and Sapeika (1936), as cited by Kellerman *et al.* (1988), reported that the toxicity of plakkies can vary within and between localities. Elevated plant stem concentrations detected during this study corresponded with the period of the year during which natural field outbreaks of krimpsiekte usually occur which is from July to November (Henning 1926; Kellerman *et al.* 1988).

Higher cotyledoside concentrations appear to coincide with seasonal changes. Stem concentrations were slightly higher when the plants were in an active, vigorous growth phase during the cold winter

months of June, July and August (Fig. 4). Plant D exhibited its highest cotyledoside concentration during July when the lowest environmental minimum temperature (2.2 °C) was recorded. During spring (September to October) when average temperatures increased and the plants were growing strongly and producing flowers, the average cotyledoside concentration once again showed a notable increase (Fig. 4). The stem concentration decreased again during late spring to early summer (November to December) when the plants shed their leaves, flowers were produced and the plants became more desiccated. However, the seasonal variation in cotyledoside concentration is based only on that obtained in three plants and in order to obtain results which can be statistically evaluated a greater number of plants in different regions need to be investigated over a longer term.

The average cotyledoside concentration did not always correspond with periods of higher rainfall. No increase in the average cotyledoside concentration was noted following light rain in February, March, April or May. No precipitation was recorded in July when the cotyledoside concentration peaked in plant D, while only 6 mm of rain fell in October when the cotyledoside concentration of plant C was at its highest. On the other hand, the highest cotyledoside concentration of plant B occurred during September which corresponded with the highest monthly rainfall which was 54 mm.

Before the commencement of the experiment, it was conjectured that the cotyledoside concentrations of the plants would be influenced by their moisture content—the drier the plants, the higher the concentrations. This was, however, not the case. In spite of noticeable desiccation of their stems during November and December the cotyledoside concentrations diminished. Cotyledoside concentrations also did not decrease after rain when these succulent plants could have been expected to have a higher moisture content.

The concentration of cotyledoside in the leaves of Plant B was considerably higher during May and June when compared to the other plants (Table 1). The cotyledoside concentrations in the leaves of this plant only once exceeded those of its stem. In May the concentration of its leaves (0.47 µg/g WM) was higher than that of its stem (0.32 µg/g WM).

After feeding trials, Henning (1926) concluded that the flowering shoots of *T. wallichii* are more toxic than the leaves, and Steyn (1949) reported that the flowers and seed heads of *Kalanchoe* species are much more toxic than the leaves. These conclusions are in agreement with the findings in the current study in which it was found that the peduncle contained the highest cotyledoside concentration. The highest cotyledoside concentration detected in the seed heads (5.15 µg/g WM) exceeded the highest leaf concentration (0.47 µg/g WM) by more than ten fold.

Electrospray mass spectral data revealed the presence of other cotyledoside-like compounds in the *T. wallichii* extracts, possibly including tyledoside B (mw = 572 dalton), tyledoside F (mw = 574 dalton), tyledoside G (mw = 574 dalton), orbicuside A (mw = 556 dalton) and orbicuside B (mw = 558 dalton) (Steyn *et al.* 1986a; Steyn, Van Heerden, Vleggaar & Anderson 1986b). The presence of tyledoside D (mw = 588 dalton) was confirmed by comparison with mass spectral and retention time data determined for an authentic specimen of this compound. Tyledoside D has previously been isolated from *T. grandiflorus* and *T. ventricosus* (Steyn *et al.* 1986a; Botha, Kellerman, Schultz, Erasmus, Vleggaar & Retief 1998). The presence of tyledoside D and other cotyledoside-like compounds in *T. wallichii* has not been reported previously. Hitherto seven tyledosides (A–G) have, however, been isolated from *T. grandiflorus* (Anderson, Joubert, Prozesky, Kellerman, Schultz, Procos & Olivier 1983) and three orbicusides (A–C) have been identified from another member of the Crassulaceae, *Cotyledon orbiculata* (Steyn *et al.* 1986b). It seems that other orbicusides, tyledosides and/or, cotyledoside-like compounds are also contained by *T. wallichii*.

The Succulent Karoo Biome has a limited potential for stock farming as there is a lack of suitable grazing and a shortage of water (Low & Rebelo 1996). The vegetation is dominated by succulents such as plakkies (Crassulaceae) and vygies (Mesembryanthemaceae) (Low & Rebelo 1996). Grasses are rare, except in some sandy soils (Low & Rebelo 1996). Mass flowering of annuals (Asteraceae) occurs in spring (Low & Rebelo 1996). The number of plant species, mostly succulents, is considerable and unequaled elsewhere in the world for an arid area of this size (Low & Rebelo 1996). The paucity of grasses limits grazing and the low carrying capacity requires that substantial supplementary feeding be implemented (Low & Rebelo 1996). In this dry, arid region small stock have to graze the succulent shrubs in order to survive. They also serve as a source of water. Although a seasonal variation in the incidence of krimpsiekte has been reported (Henning 1926; Kellerman *et al.* 1988), the disease may occur at any time of the year when the grazing is sparse and plakkies are available.

It is extremely difficult to obtain reliable prevalence mortality and morbidity rates for plant poisonings. Kellerman *et al.* (1996) furnished the following reasons for this:

- Since intoxications are not notifiable diseases outbreaks are not reported to the authorities.
- Farmers are generally aware of the plant poisonings in their region and tend to stoically accept the

losses induced by them as normal natural hazards, such as drought.

- Stock owners are sometimes reluctant to report plant poisonings for fear that such a disclosure will reduce the value of their land.

Data on annual mortalities of small stock in the Springbok district, Northern Cape Province were obtained from monthly state veterinary reports and surveys during the investigation period. Annual mortalities in small stock in the communal grazing territories south of Springbok is estimated at 3.67% of which 30% is attributed to krimpsiekte. Approximately 250 000 sheep and goats in the Springbok state veterinary area graze in an area abound with *T. wallichii*. If the results of the krimpsiekte mortality rate are extrapolated to include the entire area, it is predicted that 2 750 (1.1%) small stock die annually of krimpsiekte. It is estimated that only 20% of affected stock suffering from krimpsiekte eventually die which implies that a further 11 000 sheep and goats could have contracted krimpsiekte, resulting in considerable production losses.

In the neighbouring state veterinary area of Vredendal, reliable figures are available for the Biedouw-valley and the Clanwilliam District (A. de Kock, personal communication 2000). In the Biedouw-valley (north-east of Clanwilliam) 2% of the sheep and 8% of the goats die annually of krimpsiekte, representing 60 of the 3 000 sheep (mainly Dorpers) and 64 of the 800 goats (predominantly Boer goats). In the Clanwilliam District, 6 700 of the 670 000 (1%) sheep and 225 of the 4 500 (5%) goats die annually of krimpsiekte. In both these areas *T. wallichii* is the most likely causative plant.

These figures provide a valuable indication of the economic importance of this disease in small stock in these regions. Kellerman *et al.* (1996), on the other hand, only estimated the economic importance of cardiac glycoside poisoning in South Africa collectively and did not give an indication of krimpsiekte mortalities. Krimpsiekte mortalities in the Springbok and Clanwilliam districts and Biedouw-valley in the year under review were somewhat higher than those previously reported. Kellerman *et al.* (1996) estimated an annual disease mortality rate from all causes throughout South Africa of 5% for small stock of which 15% of deaths were attributed to poisonous plants. According to their calculations approximately one in 133 small stock die annually of plant poisonings. The higher mortality rate in the Springbok and Vredendal state veterinary areas are attributed to recurrent droughts, relentless overgrazing and extensive trampling of communal veld and the sheer numbers of *T. wallichii* in these districts.

The isolation and structure elucidation of the other cotyledoside-like compounds present in the crude *T. wallichii* extracts should be undertaken and the possibility that some of them are also toxic components should be considered.

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