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ARTICLE

Analysis of Daphnane Orthoesters in Poisonous Australian *Pimelea* Species by Liquid Chromatography−Tandem Mass Spectrometry

Sharon Chow, Mary T. Fletcher,* and Ross A. Mckenzie

Queensland Primary Industries and Fisheries, Animal Research Institute, Locked Mail Bag No. 4, Moorooka QLD 4105, Australia

Cattle grazing in arid rangelands of Australia suffer periodic extensive and serious poisoning by the plant species *Pimelea trichostachya*, *P. simplex*, and *P. elongata*. *Pimelea* poisoning (also known as St. George disease and Marree disease) has been attributed to the presence of the diterpenoid orthoester simplexin in these species. However, literature relating to previous studies is complicated by taxonomic revisions, and the presence of simplexin has not previously been verified in all currently recognized taxa capable of inducing pimelea poisoning syndrome, with no previous chemical studies of *P. trichostachya* (as currently classified) or *P. simplex* subsp. *continua*. We report here the isolation of simplexin from *P. trichostachya* and the development of a liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS) method to measure simplexin concentrations in pimelea plant material. Simplexin was quantified by positive-ion atmospheric pressure chemical ionization (APCI) LC-MS/MS with selected reaction monitoring (SRM) of the *m*/*z* 533.3 > 253.3 transition. LC-MS/MS analysis of the four poisonous taxa *P. trichostachya*, *P. elongata*, *P. simplex* subsp. *continua*, and *P. simplex* subsp. *simplex* showed similar profiles with simplexin as the major diterpenoid ester component in all four taxa accompanied by varying amounts of related orthoesters. Similar analyses of *P. decora*, *P. haematostachya*, and *P. microcephala* also demonstrated the presence of simplexin in these species but at far lower concentrations, consistent with the limited reports of stock poisoning associated with these species. The less common, shrubby species *P. penicillaris* contained simplexin at up to 55 mg/kg dry weight and would be expected to cause poisoning if animals consumed sufficient plant material.

KEYWORDS: Liquid chromatography−mass spectrometry; simplexin; daphnane orthoester; *Pimelea*

INTRODUCTION

Cattle grazing in arid rangelands of Australia suffer periodic extensive and serious poisoning by the plant species *Pimelea trichostachya*, *P. simplex* and *P. elongata* (*1*). These are winter-growing annual herbs, endemic to Australia and occurring across much of its inland grazing regions (*2*, *3*). Their local abundance depends on variations in climatic and environmental conditions (*4*). Pimelea poisoning of cattle, known as St. George disease or Marree disease, is a unique syndrome combining pulmonary hypertension causing right ventricular dilation and circulatory failure with subsequent edema, anemia from hypervolemia, chronic diarrhea, and dilation of hepatic sinusoids (*peliosis hepatis*) (*5*−*8*). Horses are rarely affected (*9*). The impacts of pimelea poisoning vary with seasonal conditions (*4*), but in severe years, the disease has been estimated to cost the Australian beef industry $AUD50 million AUD in terms of production losses, stock deaths, control measures, and extra management work for cattle producers.

*To whom correspondence should be addressed. Tel: 61 7 3362 9426. Fax: 61 7 3362 9429. E-mail: mary.fletcher@deedi.qld.gov.au.

Feeding trials with cattle in the 1960s and 1970s established *Pimelea* species as the cause of this syndrome (*8*, *10*−*16*). Subsequent taxonomic revisions of *Pimelea* species (*2*, *17*, *18*) have caused us to re-examine the lodged herbarium voucher specimens to determine the current botanical identity of plant material employed in these experimental studies. Before revision, the taxon *P. trichostachya* broadly included *P. elongata*, *P. simplex* subsp. *continua*, and the current *P. trichostachya* in its restricted sense. For example, five voucher specimens lodged by Clark and cited in the literature as *P. trichostachya* (*8*), have now been determined by Queensland Herbarium to be individual collections of all three of these taxa (**Table 1**). On the basis of the redetermination of vouchers, currently recognized taxa that have previously experimentally induced pimelea poisoning are *P. trichostachya* (*8*, *13*, *19*), *P. elongata* (*8*, *13*, *15*, *16*), *P. simplex* subsp. *continua* (*8*), and *P. simplex* subsp. *simplex* (*14*). Full details of taxa reassignments relating to these earlier investigations are shown in **Table 1** and demonstrate the value in lodging herbarium voucher specimens and citing corresponding voucher numbers in scientific publications. Voucher numbers were not cited for several investigations conducted in New South Wales, and the relationship with existing

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Table 1. Current Determinations of Voucher Specimens Lodged for Previous *Pimelea* Toxicity and Toxin Investigations

reference	collection site	species as cited in original reference text	herbarium voucher[a]	current determination of voucher specimens[b]
		Feeding Trial Research		
16[c]	Bourke NSW	*P. simplex*	(NSW412853)	*P. elongata*
15[c]	Bourke NSW	*P. simplex*	(NSW590111 or NSW412853)	*P. elongata*
14	'Kulkyne' NSW	*P. simplex*	(NSW651609)	*P. simplex* subsp. *simplex*
8	3 localities in western Qld	*P. trichostachya*	BRI099670 = AQ86121	*P. trichostachya*
		P. trichostachya	BRI101503 = AQ97842	*P. trichostachya*
		P. trichostachya	BRI101606 = AQ170241	*P. simplex* subsp. *continua*
		P. trichostachya	BRI101502 = AQ86134	*P. elongata*
		P. trichostachya	BRI109643 = AQ170221	*P. elongata*
13	'Macwood' Qld	*P. trichostachya*	BRI129152 = AQ1682	*P. trichostachya*
		P. trichostachya	BRI129169 = AQ1670[d]	*P. trichostachya*
		P. trichostachya	BRI129170 = AQ1673	*P. trichostachya*
		P. continua	BRI129168 = AQ1653	*P. elongata*
19	St. George Qld	*P. trichostachya*	BRI129169 = AQ1670	*P. trichostachya*
		Simplexin Isolation Reports		
20[e], *24*[e], *22*	'Kulkyne' NSW	*P. simplex*	(NSW651609)	*P. simplex* subsp. *simplex*
	'Willoring' NSW	*P. simplex*	(NSW651733)	*P. simplex* subsp. *simplex*
	'Belvedere' NSW	*P. simplex*	(NSW590111)	*P. elongata*
23[f]	'Belvedere' NSW	*P. simplex*	(NSW590111)	*P. elongata*
	'Kulkyne' NSW	*P. simplex*	(NSW651609)	*P. simplex* subsp. *simplex*
	'Belvedere' NSW	*P. trichostachya* Form B	(NSW251889)	*P. elongata*

[a] Voucher numbers are as cited in original text, and numbers in parentheses were submitted from collection property/author or co-workers around the appropriate time period (information from NSW Herbarium), but actual voucher numbers are not cited in publications. [b] Current determination from re-examination of herbarium voucher specimens. [c] J. E. Cantello lodged NSW412853 and NSW590111 from 'Belvedere' in 1967 and 1971 for feeding trials/experiments at Glenfield Veterinary Research Station, and H. B. Roberts continued this work. [d] Collection listed by Kelly (*13*) as "BRI129160" is believed to be a typographical error as this number is not consistent with the Queensland Herbarium collection, which shows "BRI129169" as a feeding trial sample from W. R. Kelly. [e] Isolation of simplexin from *P. simplex* collected by H. B. Roberts in the Bourke area is presumed to relate to herbarium samples from 'Kulkyne', 'Willoring' and/or 'Belvedere' as cited by Hafez et al. (*22*). [f] Authors acknowledged plant collections by H. B. Roberts and T. J. McClure.

herbarium specimens (**Table 1**) could only be construed from interrogation of details recorded by New South Wales Herbarium (such as name of collector, original determination, collection date, and reason for collection).

The toxin responsible for this poisoning syndrome has been identified as the novel daphnane orthoester simplexin (*20*). It was reported that intravenous inoculation of cattle with isolated pure simplexin (4 μg/kg body weight) produced a 3-fold increase in pulmonary arterial pressure within 100 s, while oral dosing reproduced characteristic signs of pimelea poisoning, including inappetence, weight loss, jugular distention, and submandibular edema (*20*). Daphnane orthoesters are an unusual class of compounds found only in the plant families of Thymelaeaceae and Euphorbiaceae, and the occurrence and biological activity of such compounds have recently been reviewed (*21*).

Simplexin has been isolated previously from *P. simplex* subsp. *simplex* (cited originally as *P. simplex*) (*20*, *22*−*24*) and also from *P. elongata* (cited originally as *P. trichostachya* Form B and *P. simplex*) (*23*) (**Table 1**). A number of compounds of related structure have also been identified from these species (*22*, *23*, *25*). *P. trichostachya* as currently classified and *P. simplex* subsp. *continua* have not been chemically examined previously, although these species have been assumed to contain simplexin due to their major contributions to the occurrence of pimelea poisoning of cattle in the field. Additionally, *P. trichostachya* has recently been reported to cause this syndrome in horses (*9*).

This study was undertaken to develop a liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS) method to determine whether simplexin was indeed present in *P. trichostachya* and *P. simplex* subsp. *continua*, and to determine concentrations of simplexin in each of the four poisonous *Pimelea* taxa that commonly induce the syndrome in the field. Several other *Pimelea* species also occur in inland grazing areas of Australia, and some have on occasion been suspected of inducing the pimelea poisoning syndrome (*1*). In this study, simplexin concentrations have also been determined in samples of suspect species, *P. decora*, *P. haematostachya*, and *P. microcephala*, and also in *P. penicillaris*. These four species have not previously been chemically examined.

MATERIALS AND METHODS

Plant Material. Collections of *P. trichostachya*, *P. elongata*, *P. simplex* subsp. *continua*, and *P. simplex* subsp. *simplex* were made of new growth and flowering tops (those parts of plants considered most likely to be eaten by grazing livestock), and were a composite of 10 or more plants collected at each site at various locations in western Queensland and New South Wales. Individual samples of *P. decora*, *P. haematostachya, P. microcephala*, and *P. penicillaris* were also collected for comparison. The nature of collection sites and global positioning system (GPS) coordinates were recorded. Samples (200 g−1 kg) were placed in paper bags. A separate sample was pressed between absorbent paper for botanical identification. Plant identifications were confirmed by the Queensland Herbarium, and botanical specimens from all batches collected were incorporated into their permanent collection as vouchers against any future taxonomic changes. Field-collected plant material was transported to the laboratory, air-drying completed, milled, and stored frozen prior to analysis.

Isolation of Simplexin from *P. trichostachya*. Simplexin standard was obtained by extraction of a milled bulk stem and root sample of *P. trichostachya* (AQ751555, collected November 2006) from a property near Mitchell, Queensland. The plant sample (40 g) was soaked in 90%

Table 2. Molecular Ions and Selected MS/MS Fragment Ions of Daphnane Orthoesters **1**−**7**

compound	$[M + H]^+$	major fragment ions (relative abundance, %)
dehydrosimplexin (**1**)	531	531 (85), 361 (7), 343 (14), 325 (27), 307 (42), 297 (25), 279 (47), 267 (61), 253 (100), 153 (53, R_1CO^+)[a]
acetoxysimplexin (**2**)	591	591 (16), 401 (9), 383 (11), 365 (17), 359 (19), 341 (55), 323 (100), 295 (69), 277 (29), 269 (60), 155 (12, R_1CO^+)[a]
dodecenyl analogue (**3**)	559	559 (100), 361 (8), 343 (20), 325 (30), 307 (48), 297 (25), 279 (27), 267 (74), 253 (83), 181 (29, R_1CO^+)[a]
simplexin (**4**)	533	533 (32), 361 (5), 343 (14), 325 (29), 307 (29), 297 (26), 279 (26), 267 (57), 253 (100), 155 (10, R_1CO^+)[a]
dehydrohuratoxin (**5**)	583	583 (100), 361 (3), 343 (3), 325 (8), 307 (7), 279 (7), 267 (23), 253 (32), 205 (56, R_1CO^+)[a]
acetoxyhuratoxin (**6**)	643	643 (59), 401 (10), 365 (6), 359 (7), 341 (18), 323 (19), 295 (21), 277 (21), 269 (12), 207 (100, R_1CO^+)[a]
huratoxin (**7**)	585	585 (100), 361 (3), 343 (7), 325 (15), 307 (12), 297 (9), 279 (15), 267 (32), 253 (41), 207 (40, R_1CO^+)[a]

[a] R_1 represents the fatty acid chain of the orthoester in each compound (**Figure 1**).

methanol overnight (200 mL). The mixture was filtered, and the plant material was rinsed with fresh methanol (50 mL). The combined organic extracts were concentrated under reduced pressure and the residue partitioned between 50% saturated brine solution (50 mL) and dichloromethane (3 × 50 mL). After drying over anhydrous sodium sulfate and solvent removal, the green residue was partitioned between hexane (50 mL) and acetonitrile (3 × 20 mL). The acetonitrile layers were combined and concentrated under reduced pressure. The residue was dissolved in minimum amount of dichloromethane and loaded onto four Strata SI-1 Silica SPE cartridges (Phenomenex, Sydney, Australia, 2 g), eluting with dichloromethane and then 1% methanol in dichloromethane. The fractions containing the desired toxin (guided by TLC visualization with ammonium metavanadate reagent (*23*) and confirmed by LC-MS/MS) were concentrated and purified by reverse phase high performance liquid chromatography (HPLC) on a 250 × 15 mm i.d., 5 μm, Luna C18(2) column, with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Sydney, Australia) at 25 °C and eluted isocratically with 95% methanol. The HPLC was equipped with a diode array detector and low temperature evaporative light scattering detector (ELSD). The identity of the isolated simplexin (6 mg from 180 g of dried *P. trichostachya* stems and roots) was confirmed by ^{1}H and ^{13}C NMR data comparisons with literature values (*23*) and shown to be ≥95% in purity by HPLC-ELSD.

Additional simplexin was also isolated by a similar procedure from *P. elongata* (AQ751686, collected July 2007) from Bollon, Queensland.

Analytical Sample Preparation. Milled dried plant samples (0.5 g) were extracted by shaking overnight in methanol (80%, 20 mL). A portion of the centrifuged extract (2 mL) was evaporated under a stream of nitrogen with the residue taken up in dichloromethane (4 mL) and distilled water (1 mL), and washed with brine (5 mL). The organic layer was removed, and the aqueous layer was re-extracted with dichloromethane (2 × 4 mL). After drying the combined organic layers with anhydrous sodium sulfate, the solvent was evaporated. The residue was partitioned between acetonitrile (4 mL) and hexane (10 mL). The hexane layer was washed with fresh acetonitrile (2 mL). Solvent was evaporated from the combined acetonitrile extracts, and the residue was taken up in methanol (8 mL) for LC-MS/MS analysis.

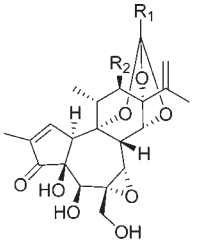
LC-MS/MS Equipment and Conditions. Samples were analyzed using a Waters 2777C Sample Manager and the liquid chromatographic separations were carried out on a Waters 1525μ Binary HPLC pump system. LC separations were performed using a 2.1 × 100 mm i.d., 3.5 μm, Sunfire C18 column (Waters, Sydney, Australia) at 30 °C with a flow rate of 0.2 mL/min. The mobile phase was a mixture of (A) methanol with 0.1% formic acid (v/v) and (B) water with 0.1% formic acid (v/v), with a gradient as follows: t_0, 90% A; t_{15}, 100% A; t_{25}, 100% A; t_{26}, 90% A;. t_{30}, 90% A.

MS/MS detection was made using a Quattro Premier Micromass triple quadrupole mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source used in positive mode. The mass spectrometer response was tuned with a simplexin solution. The capillary voltage was 3.0 kV; the desolvation and cone gases of nitrogen flow were set at 450 L/h and 47 L/h, respectively. The desolvation and source temperatures was set at 450 and 120 °C, respectively. Argon was used as collision gas for MS/MS with a flow rate of 0.3 mL/min, and the collision energy was set at 16 eV and cone voltage at 20 V.

Isolated simplexin (≥95% pure) was used as an external standard in LC-MS/MS analysis with standard solutions prepared in methanol (0.5−5.5 mg/L).

RESULTS AND DISCUSSION

Isolation of Simplexin from *P. trichostachya*. The crude plant extract of *P. trichostachya* obtained from the liquid−liquid partition



Compound	R_1	R_2
Dehydrosimplexin (**1**)	C_9H_{17}	H
Acetoxysimplexin (**2**)	$(CH_2)_8CH_3$	AcO
Dodecenyl analogue (**3**)	$C_{11}H_{21}$	H
Simplexin (**4**)	$(CH_2)_8CH_3$	H
Dehydrohuratoxin (**5**)	$C_{13}H_{21}$	H
Acetoxyhuratoxin (**6**)	$(CH{=}CH)_2(CH_2)_8CH_3$ -(*E*,*E*)	AcO
Huratoxin (**7**)	$(CH{=}CH)_2(CH_2)_8CH_3$ -(*E*,*E*)	H

Figure 1. Structures of simplexin (**4**) and related compounds detected in the *Pimelea* plant species.

consisted of a complex mixture of components in which simplexin and related diterpenoid esters were present at only low levels. TLC visualization with ammonium metavanadate reagent (*23*) was used to guide SPE fractionation of the extract, and LC-MS/MS analysis was used to confirm the presence of simplexin in the collected fractions. On the basis of the characteristic UV band of simplexin (242 nm), the toxin was separated by repetitive semipreparative HPLC, and the purity of the isolated material was assayed at ≥95% from ELSD and NMR analyses. The structure of the isolated simplexin was confirmed by mass spectrometry and also a comparison of ^{1}H and ^{13}C NMR data with literature values (*23*). This is the first reported isolation of simplexin from *P. trichostachya* (as currently classified) and further confirms the role of simplexin in poisonings of stock by this plant species.

LC-MS/MS Fragmentation of Simplexin and Analogues. LC-MS/MS fragmentation of the protonated molecular ion of simplexin $[M + H]^+$ *m*/*z* 533 in positive-ion APCI mode showed loss of its fatty acid ($C_9H_{19}COOH$) to a *m*/*z* 361 fragment with further sequential losses of 18 Da (H_2O) to *m*/*z* 343, 325, and 307 (**Table 2**). A small $C_9H_{19}CO^+$ ion at *m*/*z* 155 was observed. Using the selected reaction monitoring (SRM) program on the LC-MS/MS, the most dominant daughter ion observed at *m*/*z* 253 was chosen as the transition (*m*/*z* 533.3 > 253.3) for quantification, and the next dominant daughter ion was selected as the transition (*m*/*z* 533.3 > 267.2) for verification (**Table 3**). The efficiency of the ionization and the detection of the precursor and fragments ions were optimized by adjusting collision energy and cone voltage (16 eV and 20 V, respectively).

The initial LC-MS/MS investigations of the crude plant extract of *P. trichostachya*, *P. elongata*, *P. simplex* subsp. *continua*, and *P. simplex* subsp. *simplex* revealed the presence of other minor components as well as simplexin. The mass fragmentation pattern of several of these components reflected a certain degree of

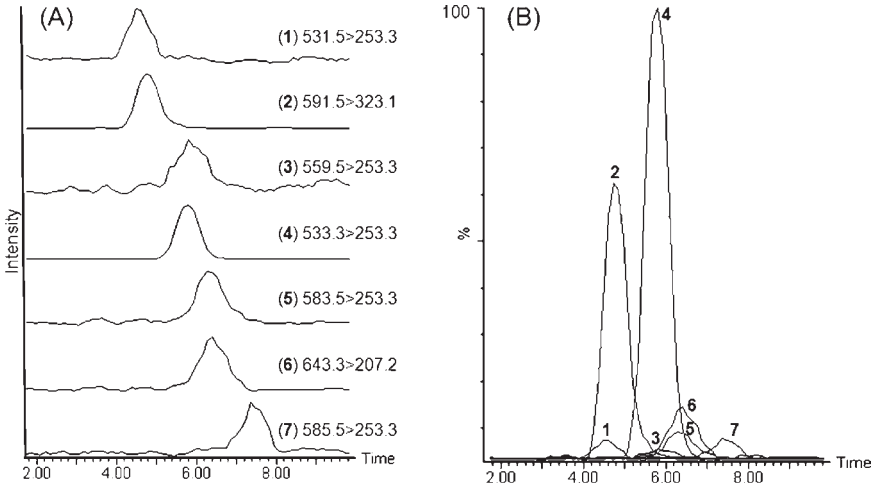


Figure 2. LC-MS/MS analysis of *Pimelea trichostachya* (AQ751829). (**A**) Individual SRM chromatograms for each of the daphnane orthoesters **1**−**7**. (**B**) Overlaid SRM chromatograms.

structural similarity to simplexin, and mass spectral data for all seven compounds (including simplexin) are listed in retention order, **1**−**7**, in **Table 2**. From the mass spectrometric fragmentation, it was presumed that these compounds possessed the same carbon skeleton but with different fatty acid and side chains attached (**Figure 1**). Compounds **1**, **3**, **5**, and **7** have MS/MS spectra very similar to those of simplexin (**4**), including the presence of dominant daughter ions at *m*/*z* 267 and *m*/*z* 253 as shown in **Table 2**. The R_1CO^+ ions, from the fragmentation of the fatty acids, enabled the assignments of the alkyl fatty acids to structures **1**, **3**, **5**, and **7** (**Figure 1**). Two other minor components **2** and **6** had protonated molecular ions $[M + H]^+$ of *m*/*z* 591 and *m*/*z* 643, with mass fragmentation somewhat similar to that seen in simplexin (**4**) and huratoxin (**7**), correspond to acetoxy-derivatives of simplexin and huratoxin (increments of 58 Da., **Table 2**). These compounds were deduced to be acetoxysimplexin (**2**) and acetoxyhuratoxin (**6**). Huratoxin (**7**) and acetoxyhuratoxin (**6**) have previously been isolated from *P. simplex* (*23*) and acetoxysimplexin (also known as gnidiglaucin) (**2**) from *Gnidia glaucus* (*26*).

Development and Validation of the LC-MS/MS Method for Simplexin Analysis. Simplexin (**4**) is unsuitable for GC-MS analysis and does not have a strong UV chromophore for HPLC-UV analysis; thus, the described LC-MS/MS method provides the first sensitive analysis method for this toxin in *Pimelea* taxa. Such a method was required as part of our study to provide a better understanding of the incidence of pimelea poisoning by relating observed field toxicity to measured simplexin concentrations in the various *Pimelea* species.

Simplexin (**4**) is a moderately polar molecule, and a solvent partitioning procedure was developed to provide crude pimelea plant extracts containing simplexin (and related minor components) free from both water-soluble and hydrocarbon components. Reverse phase HPLC on a C18 column eluting with a methanol/water (containing 0.1% formic acid v/v) gradient then provided the separation of components **1**−**7** (**Figure 2**). Concentrations of simplexin (**4**) in plant extracts were determined from the intensity of the positive-ion APCI SRM transition *m*/*z* 533.3 > 253.3 and verified by a secondary transition (*m*/*z* 533.3 > 267.2). The presence of other minor components **1**−**3** and **5**−**7** in plant extracts were determined by measurement of similar SRM transitions (and confirmatory SRM transitions), as listed in **Table 3**. Absolute levels of these compounds were not determined as adequate standards have not been separated for these minor components. Under the described HPLC conditions, all seven components eluted with retention times between 4.4 and 7.3 min (**Table 3**).

Table 3. Selected Reaction Monitoring (SRM) Transitions for Daphnane Orthoesters **1**−**7**

compound	RT (min)	quantification SRM	confirmatory SRM
dehydrosimplexin (**1**)	4.4	531.5 > 253.3	531.5 > 267.2
acetoxysimplexin (**2**)	4.7	591.5 > 323.1	591.5 > 295.3
dodecenyl analogue (**3**)	5.7	559.5 > 253.3	559.5 > 267.2
simplexin (**4**)	5.8	533.3 > 253.3	533.3 > 267.2
dehydrohuratoxin (**5**)	6.3	583.5 > 253.3	583.5 > 267.2
acetoxyhuratoxin (**6**)	6.4	643.3 > 207.2	643.3 > 277.2
huratoxin (**7**)	7.3	585.5 > 253.3	585.5 > 267.2

Simplexin (**4**) in plant extracts from individual field samples was quantitated against a four point calibration curve of simplexin (standards were prepared in methanol) over the concentration range of 0.5−5.5 μg/mL. Plant extracts with simplexin concentrations outside this range were further diluted to bring them back into range. Typically, calibration curves had R^2 values in excess of 0.96. Reproducibility of analysis was demonstrated by replicate analysis. For example, five replicates of a single sample of flowering *P. elongata* (AQ751686) had a mean simplexin concentration of 275.7 mg/kg DW with a standard deviation of 7.6 (RSD 2.76%).

To estimate the recovery efficiency of the extraction method, authenticated (NMR, MS) simplexin (**4**) was spiked into dried milled plant material from *P. simplex* subsp. *continua* (AQ751854). This plant material contained minimal natural simplexin (average analysis of 24.8 mg simplexin/kg dry weight), was spiked at concentrations equivalent to approximately 112, 224, 384, and 880 mg simplexin/kg dry weight, and extracted by the described procedure to afford extracts with theoretical concentrations of 0.7, 1.4, 2.4, and 5.5 μg/mL. When compared with simplexin standard solutions in methanol and allowing for natural simplexin level, these spiking experiments had recoveries of 168, 140, 121, and 92% respectively, and demonstrated that the LC-MS/MS response for simplexin was somewhat enhanced by the presence of matrix components particularly at low simplexin levels. When compared with simplexin standard solution in the plant extract matrix, calculated recoveries were 95, 87, 104, and 92% respectively.

Composition of *Pimelea* Taxa. LC-MS/MS analysis of the four poisonous taxa *P. trichostachya*, *P. elongata*, *P. simplex* subsp. *continua*, and *P. simplex* subsp. *simplex* showed similar composition with simplexin (**4**) as the major diterpenoid ester component

Table 4. Chemical Composition of Green Flowering Samples of *Pimelea* Taxa

Pimelea taxon	location (nearest town)	simplexin content (mg/kg DW)	other diterpenoids identified	Queensland herbarium voucher number
P. decora Domin	Julia Creek	<LOD[a]		AQ751750
P. elongata Threlfall	Cunnamulla	283.6	**2**, **6**, **7**	AQ751191
	Bollon	242.4	**6**, **7**	AQ751688
	Eromanga	125.7	**1**, **3**, **5**, **7**	AQ753721
	Adavale	222.4	**1**, **7**	AQ751906
	Bollon	329.8	**2**, **3**, **5**, **6**, **7**	AQ751823
P. haematostachya F.Muell.	Nebo	<LOD[b]		AQ751882
P. microcephala R.Br.	Roma	2.6	**7**	AQ751828
P. penicillaris F.Muell.	Cunnamulla	53.5	**2**, **6**	AQ751187
	Cunnamulla	2.8		AQ783705
P. simplex subsp. *continua* (J.Black) Threlfall	Cunnamulla	86.4	**6**, **7**[c]	AQ751188
	Cunnamulla	73.3	**6**, **7**[c]	AQ751196
	Cunnamulla	145.2	**2**, **6**, **7**	AQ751195
	Cunnamulla	155.1	**2**, **5**, **6**, **7**	AQ751194
	Cunnamulla	93.9	**2**	AQ751192
P. simplex F.Muell. subsp. *simplex*	Surat	146.3	**2**, **5**, **6**, **7**[c]	AQ751815
	Collarenebri	189.1	**1**, **3**, **5**, **6**, **7**[c]	AQ751853
	Surat	84.8	**5**, **6**, **7**[c]	AQ751850
	Surat	59.8	**5**, **6**, **7**[c]	AQ751799
P. trichostachya Lindley	Mitchell	260.7	**2**, **3**	AQ778050
	Mitchell	315.0	**2**, **3**	AQ734606
	Bollon	275.6	**1**, **2**, **5**, **6**, **7**	AQ762193
	Cunnamulla	250.8	**5**	AQ751193
	Surat	579.6	**1**, **2**, **3**, **5**, **6**, **7**	AQ751829

[a] Flowerheads analyzed separately had trace amounts of simplexin (9 mg/kg). [b] Flowerheads and roots contained low levels of simplexin (**4**) (24 and 27 mg/kg respectively), **5** and **7**. [c] Levels of huratoxin (**7**) comparable to simplexin (**4**).

in each taxon accompanied by varying amounts of related orthoesters **1**−**3** and **5**−**7** (**Table 4**). The similar toxin composition is consistent with the various feeding trials which demonstrated similar effects on cattle caused by all four taxa (**Table 1**). In both *P. simplex* and *P. trichostachya* taxa, considerable variation in relative amounts of accompanying acetates **2** and **6** was also noted in our analysis, even within samples of the same taxa. Only trace levels of these acetates were detected in *P. elongata* samples.

Simplexin levels were generally the highest in *P. trichostachya* and *P. elongata*, with lower levels seen in *P. simplex* subsp. *continua* and *P. simplex* subsp. *simplex*. Levels of huratoxin (**7**) in several of the *P. simplex* samples (particularly those identified as *P. simplex* subsp. *simplex*) were comparable with levels of simplexin (**4**), in contrast to *P. trichostachya* and *P. elongata*, where huratoxin levels were consistently much lower than simplexin levels (**Table 4**). These results are consistent with the previous isolation of a 1:1 mixture of simplexin (**4**) and huratoxin (**7**) from a sample of *P. simplex* subsp. *simplex* (cited originally as *P. simplex*) from 'Kulkyne' near Bourke, New South Wales, by Freeman et al. (*23*). The same authors isolated simplexin (**4**) without co-occurring huratoxin (**7**) from another *Pimelea* sample also cited as *P. simplex* from 'Belvedere' near Bourke (*23*), but re-examination of lodged herbarium samples suggests this species determination was incorrect. Historical *P. simplex* samples from 'Belvedere' have been redetermined as *P. elongata* (Orme, Andrew, New South Wales Herbarium, personal communication) (**Table 1**), and the reported isolation of simplexin (**4**) alone is then consistent with our detection of only low levels of huratoxin (**7**) in this species. In our toxin isolation work, huratoxin (**7**) was somewhat unstable and decomposed with handling during purification process as noted by earlier authors (*23*). However, huratoxin appeared stable under the mild extraction conditions employed in our LC-MS/MS method, and the differences in composition between *Pimelea* taxa noted in **Table 4** appears to be real rather than induced by decomposition.

The other species analyzed, *P. decora*, *P. haematostachya*, *P. microcephala*, and *P. penicillaris* also contained simplexin (**4**) but at far lower concentrations (**Table 4**), consistent with the limited reports of stock toxicity associated with these species. However, one sample of the shrubby species *P. penicillaris* contained 53.5 mg simplexin/kg dry weight, and although this concentration is less than the levels seen in the four more poisonous taxa (**Table 4**), it would be expected to cause poisoning if animals consumed sufficient plant material.

This study confirms for the first time the presence of the daphnane orthoester simplexin (**4**) in *P. simplex* subsp. *continua* and *P. trichostachya*. This orthoester has been shown to occur with a mixture of related orthoesters **1**−**3** and **5**−**7** in varying proportion in all four poisonous taxa, *P. trichostachya*, *P. elongata*, *P. simplex* subsp. *continua*, and *P. simplex* subsp. *simplex*. The incidence of pimelea poisoning has in the past been difficult to predict due to a lack of understanding of the contributing factors including relative amounts of toxin in plants and its persistence in the environment of susceptible cattle herds after these annual plants have died and disintegrated. It has, for instance, been observed that some properties or animals were affected by pimelea poisoning when others were not despite apparently similar exposure. The LC-MS/MS analysis method developed in this study will enable further investigations of toxin variation in each *Pimelea* species with stage of growth and other environmental factors. Such knowledge will assist in the development of improved management strategies for *Pimelea*-containing pastures.

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