Chemotaxonomic Investigation of Apocynaceae for Retronecine-Type Pyrrolizidine Alkaloids Using HPLC-MS/MS

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ABSTRACT:

Apocynaceae are well-known for diverse specialized metabolites that are distributed in a phylogenetically informative manner. Pyrrolizidine alkaloids (PAs) have been reported sporadically in one lineage in the family, the APSA clade, but few species had been studied to date. We conduct the first systematic survey of Apocynaceae for retronecine-type PAs, sampling leaves from 231 species from 13 of 16 major lineages within the APSA clade using HPLC-MS/MS. We also follow up on preliminary evidence for infra-specific variation of PA detectability in *Echites umbellatus* Jacq. Four precursor ion scans (PREC) were developed for a high-throughput survey for chemicals containing a structural moiety common to many PAs, the retronecine core. We identified with high confidence PAs in 7 of 8 sampled genera of tribe Echiteae, but not in samples from the closely related Odontadenieae and Mesechiteae, confirming the utility of PAs as a taxonomic character in tribal delimitation. The presence of PAs in Malouetieae was confirmed, as we report with high confidence their presence in *Galactophora schomburgkiana* Woodson and *Eucorymbia alba* Stapf, but currently we have low confidence of their presence in *Holarrena pubescens* Wall. ex G. Don (the one Malouetieae species where they were previously reported), as well as in *Kibatalia macrophylla* (Pierre ex Hua) Woodson and in *Holarrena curtisii* King & Gamble. For the first time the presence of PAs in species of *Wrightia* R. Br. (Wrightieae) and *Marsdenia* R. Br. (Marsdenieae) was confirmed. Detectability of PAs was found to vary among samples of *Echites umbellatus* and intra-individual plasticity contributes to this variation. Of toxicological importance, novel potential sources of human exposure to pro-toxic PAs were identified in the medicinal plants, *Wrightia tinctoria* R.Br. and *Marsdenia tinctoria* R.Br., and the food plant, *Echites panduratus* A. DC., warranting immediate further research to elucidate the structures of the candidate PAs identified. Method development and limitations are discussed.

46 Keywords: Apocynaceae, chemotaxonomy, pyrrolizidine alkaloids, retronecine, phenotypic

47 plasticity

1. Introduction

The study of plant specialized metabolites in a phylogenomic framework promises to fulfill the original goal of chemotaxonomy: identification of lineage diagnostic metabolites (Fairbrothers et al., 1975). In combination with other –omic, biochemical, and ecological approaches, chemotaxonomy permits the testing of key hypotheses about the evolutionary assembly and fates of novel metabolic pathways (Scossa and Fernie, 2020) as well as the role of plants' interactions with their environments, including co-evolution with their specialized herbivores, in shaping plant metabolic diversity (Futuyma and Agrawal, 2009). One of the primary impediments to pursuit of this research agenda in large plant families is the fragmentary knowledge of the taxonomic occurrence of specialized metabolites. The published literature on natural products contains multiple biases, with the absence of a class of metabolites in a species being particularly hard to infer from studies that have not specifically targeted a compound class. Systematic surveys for chemicals of interest are often also impeded by availability of tissues across a breadth of species; this can be compounded by the lack of high-throughput, sensitive and selective analytical methods to maximize compound identification from small quantities of test material (Tasca et al., 2018). Infraspecific variation, a ubiquitous feature of plant specialized metabolism (Hartmann, 1996; Moore et al., 2014), can further introduce confusion about the distribution of a metabolite via contradictory reports in the literature.

Apocynaceae are the tenth largest angiosperm plant family, with ca. 5300 species classified in 378 genera (Endress et al., 2018). The family is of particular importance in natural products research because of the occurrence of multiple medicinally important species and compounds including monoterpenoid indole alkaloids, particularly the chemotherapy drugs vincristine and vinblastine in *Catharanthus roseus* (L.) G.Don (Aslam et al., 2010). The expense and difficulty of deriving these valuable molecules from natural sources has motivated the complete biochemical elucidation of their biosynthetic pathway as a steppingstone to its genetic engineering (Caputi et al., 2018; Qu et al., 2019).

The genus *Asclepias* L. and its specialized herbivores are a model system in chemical ecology and evolution of reciprocal adaptations between plants and herbivores, with a particular focus on cardenolides (Agrawal et al., 2012). Pyrrolizidine alkaloids are also implicated in co-evolution between Apocynaceae and one of their specialized herbivore lineages, Lepidoptera subfamily Danainae (milkweed and clearwing butterflies), and the evolution of the first gene of their biosynthetic pathway, homospermidine synthase (*hss*), has been elucidated (Livshultz et al., 2018a). Researchers have detected phylogenetic signals in the distribution of all of these compounds and others, including steroidal and phenanthroindolizidine alkaloids and steroidal glycosides, in Apocynaceae (Endress et al., 2018), but knowledge of their taxonomic distribution still lags progress on the phylogeny of the family (Fishbein et al., 2018).

Pyrrolizidine alkaloids (PAs) are secondary metabolites containing a 1-hydroxymethylated necine core, esterified with a necic acid. The necine base (often referred to as a pyrrolizidine) presents as a heterocycle containing a nitrogen atom positioned at the junction of two fused five-membered rings (Hartmann and Witte, 1995) (Figure 1). Depending on the necine base present, PAs can be classified into five main types: retronecine (155 Da) or its less common diastereomer, heliotridine (155 Da), the monocyclic otonecine (185 Da) (These et al., 2013), the fully saturated platynecine (157 Da) (Lin et al., 1998), or the triol rosmarinecine (173 Da) (De Waal, 1941).

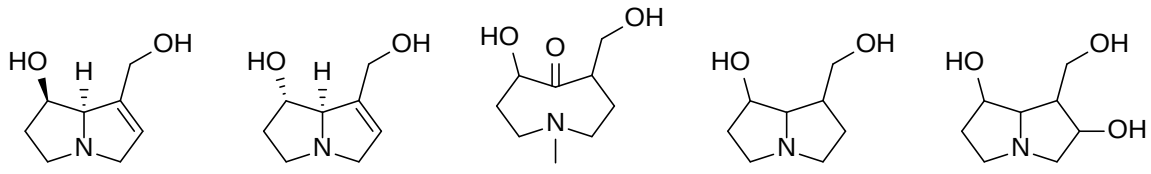

Fig. 1. Varied necine base structures found in pyrrolizidine alkaloids.

Additionally, due to the immense structural diversity imparted by various combinations of necine bases with necic acids, PAs are primarily characterized according to esterification pattern and necic acid type (Hartmann and Witte, 1995) (Fig. 2).

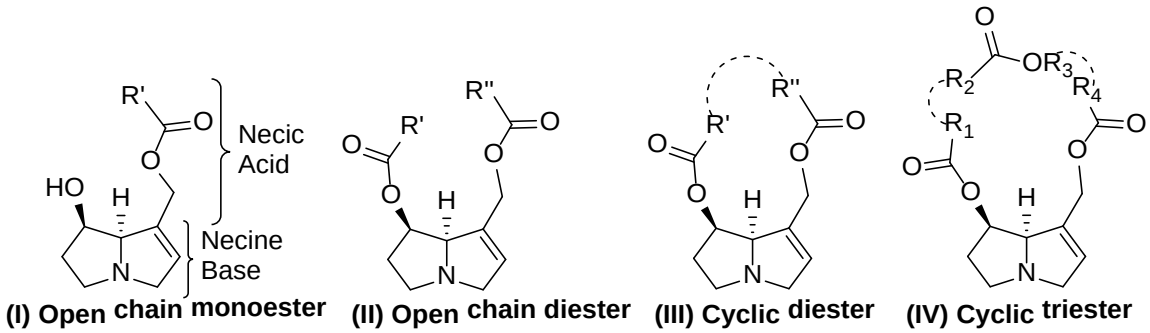

Fig. 2. Common esterification patterns between necic acids and necine bases observed in retronecine-type PAs.

PAs are known in 12 plant families (Hartmann and Witte, 1995; Tamariz et al., 2018) in addition to Apocynaceae. Previous studies exploring the structural diversity of PAs within Apocynaceae have primarily noted the presence of lycopsamine-type PAs, group abbreviation "C" (Hartmann and Witte, 1995), containing various subgroups: lycopsamine, isolycopsamine, latifoline, and parsonsine, referred to as C1, C2, C3, and C4, respectively (Burzynski et al., 2015; Colegate et al., 2016) (Fig. 3). Various other types of PAs are also present within Apocynaceae, including phalaenopsine (E) and miscellaneous (M; comprised of unusual necine esters/ simple necine derivates) (Burzynski et al., 2015). Other PA classes, senecionine (referred to as A), triangularine (B), monocrotaline (D), and loline (L), have never been reported from Apocynaceae (Hartmann and Witte, 1995).

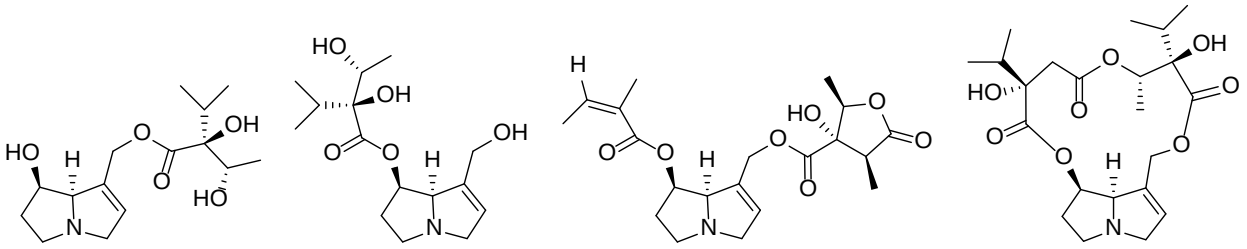

Fig. 3. Subtypes of lycopsamine-type PAs containing a retronecine core.

The chemical ecology of PAs is among the most intensely studied of any group of plant specialized metabolites, with multiple origins of PA-phily among diverse insect lineages (Hartmann and Witte, 1995; Trigo, 2011). PAs are important in agriculture because of their toxicity to livestock (Lucena et al., 2010), and as biocontrol against phytophagous nematodes (Thoden and Boppré, 2010). Their presence in foods and herbal preparations is of concern to public health regulators, given the toxicity of desaturated PAs (Dusemund et al., 2018; Kaltner et al., 2020; Schramm et al., 2019).

To date, only 37 Apocynaceae species have been specifically tested for PAs via chemical means, with 15 PA-positive species discovered in 7 genera (reviewed in Burzynski et al, 2015; Colegate at al., 2016). These genera belong to four distantly related tribes: Nerieae (*Alafia* Thouars), Malouetieae (*Holarrhena* R.Br.), Echiteae (*Echites* P.Browne*, Prestonia* R.Br.*, Parsonsia* R.Br.), Apocyneae (*Anodendron* A.DC., *Amphineurion* (A.DC.) Pichon). Report of PAs in *Macropharynx* Rusby (syn. *Peltastes* Woodson*)*, *Temnadenia* Miers*,* and *Thenardia* Kunth (Echiteae) (Morales et al., 2017) are derived from observed behaviors of PA-philous insects rather than chemical analysis (Brown, 1987; Hernández-Baz et al., 2013). Based on the evolution of *hss*-like genes in Apocynaceae, Livshultz et al. (2018a) proposed that PAs evolved in the common ancestor of these four tribes, early in the diversification of a well-corroborated lineage, the APSA clade, followed by multiple independent losses. This implies that PAs may be more widely distributed within the APSA clade, which includes ca. 4000 species, than reported in the literature. Conflicting reports of presence or absence in a particular species, such as *Prestonia coalita* (Vell.) Woodson (Burzynski et al., 2015), and variation in PA detectability among herbarium

specimens of *Echites umbellatus* Jacq. (Tasca et al., 2018), indicate the potential for infra-specific variation in PA presence.

1.2. Scanning for Retronecine-Type PAs via HPLC-MS/MS

The abundance of compounds classified as PAs, greater than 400 to date, and the immense structural diversity exhibited by PAs, including both isobaric constitutional and stereoisomers (Hartmann and Witte, 1995), in conjunction with a relative lack of available standards, has led to the advancement of broadly useful PA detection methods (Jeong and Lim, 2019; These et al., 2013). These techniques do not require corresponding standards as they target key structural fragments of PAs by subjecting them to mass spectrometry (MS) coupled to either liquid or gas chromatography (Jeong and Lim, 2019; Stelljes et al., 1991). In the case of retronecine/heliotridine-type PAs, previous groups have utilized the m/z 120 and 138 fragment ions to detect cyclic diesters and open-chained diesters (Figure 4). Since retronecine and heliotridine -type PAs only differ by their stereochemical configuration, they present with the same structurally significant fragments upon MS analysis (These et al., 2013). Additionally, m/z 120, 138, and 156 fragment ions have been used to identify open-chained monoesters (Avula et al., 2015; Avula, 2015; Lin et al., 1998; Sixto et al., 2019; These et al., 2013) (Figure 4). Fragments of the retronecine/heliotridine core have also been used as qualifier ions in PA identification via mass spectrometry. The major sub-fragments of the retronecine core include m/z 94 and m/z 96, with m/z 94 being more common in PA characterization (Colegate et al., 2016; Lin et al., 1998; Pedersen and Larsen, 1970).

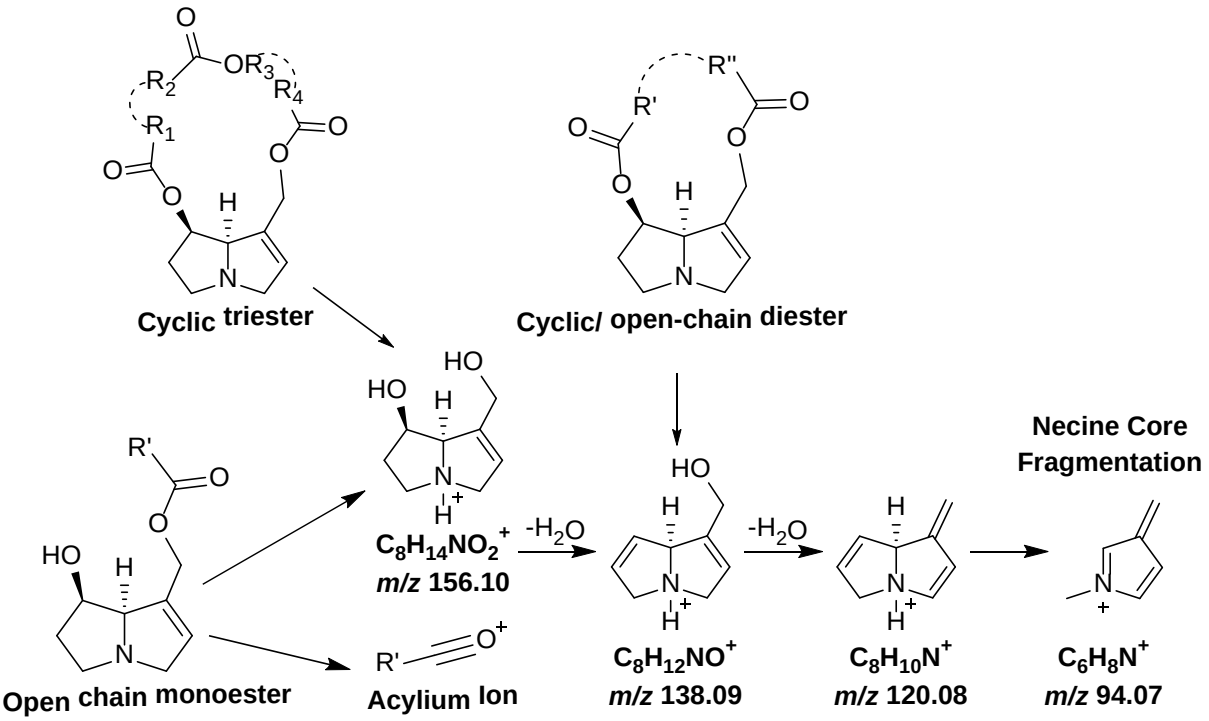

Fig. 4. Fragmentation patterns according to esterification pattern in retronecine-type PAs.

Burzynski et al. (2015) used multiple types of MS scans (Q1, MRM, PREC) to tentatively identify specific PAs, as both free bases and N-oxides, known to be part of the parsonsine (C4) subgroup of lycopsamine-type PAs in Apocynaceae species (Hartmann and Witte, 1995). MRM (multiple reaction monitoring) and PREC (precursor ion) scans were generated to identify the m/z 120 fragment, typical of retronecine-type PAs. Leaf, seed, nectar, and sap samples of *Echites umbellatus* and *Parsonsia alboflavescens* (Dennst.) Mab. (Echiteae) were all found to contain PAs with parsonsine isomers likely present.

Alternatively, to determine total PA content in honey samples, Sixto et al. (2019) utilized MS precursor, product, and multi-reaction monitoring (MRM) scans, similar in experimental design to These et al. (2013). Precursor scans were designed to locate evenly massed molecular ions $[M+H]^+$ with either m/z 120 (open chain mono/di-esters, cyclic diesters, or cyclic diester N-oxides) or m/z 138 fragments (monoester N-oxides). Once molecular ion precursors associated with retronecine/heliotridine-type PAs

were established, these molecular ions were then subject to product scans to evaluate overall fragments, with MRMs being utilized for quantification.

In the current study, to survey for the presence of retronecine/heliotridine-type PAs and avoid the need to generate multiple product scans for the proposed PA molecular ions, PREC scans for the major fragments of retronecine/heliotridine-type PAs were used to identify potential PA molecular ions in positive ion scanning mode. Since nearly all PAs contain a single nitrogen atom within their pyrrolizidine ring, and because we exploited positive electrospray ionization, we selectively targeted species with even mass to charge (m/z) ratio. MCA (multichannel analysis) was used in PREC 120 scans for initial sample screening. This function produced an additive spectrum with increased sensitivity, reducing the chance of falsely identifying samples as negative. However, MCA was not used when samples were subject to a secondary analysis, involving the use of three PREC scans (m/z 120, 138, and 156) to allow for evaluation of the mass spectrum throughout the entirety of the chromatographic run. Our LC-MS methods were validated on a set of purchased PA standards, as well as on positive and negative control species.

We report the largest survey to date of PA occurrence in Apocynaceae, leveraging the long-term stability of PAs in dried leaves (Tasca et al., 2018), the taxonomically diverse collection of leaf samples that one of us (T.L.) has assembled for phylogenetic analysis, and advances in PA identification using HPLC-MS/MS to overcome the barriers that had previously precluded systematic study of their occurrence. We also follow up on preliminary evidence of infraspecific polymorphism of PA presence in *Echites umbellatus* (Tasca et al., 2018) by increasing infraspecific sampling and re-sampling individual plants over time to infer the presence of intra-individual plasticity. Finally, we apply our methods to the analysis of seeds of two medicinal species: *Holarrhena pubescens* Wall. & G.Don and *Wrightia tinctoria* R.Br.

2. Results and discussion

2.1. Method validation

All purchased PA standards (heliotrine, jacobine, retrorsine, europine, lycopsamine and monocrotaline), representing cyclic diesters and open-chained monoesters, resulted in chromatographic separation in the TIC (total ion chromatograph) of PREC 120 and 138 scans. Scans of the open-chained monoesters (europine, lycopsamine, and heliotrine) also resulted in distinct features in the TIC of the PREC 156 scan. All six standards appeared in the TIC (total ion chromatogram) between 9- 12 minutes (45-60% ACN), using the chromatographic conditions outlined below.

Apocynum cannabinum L. was used as a negative control because it is a chemically well-studied species from which PAs have not been reported (Abe and Yamauchi, 1994), and because it has a homospermidine synthase pseudogene and hence should not be able to produce PAs (unpublished data). Subjecting the negative control leaf sample to the four PREC scans resulted in a TIC with a signal to noise ratio lower than three to one (<3:1 S/N).

Parsonsia alboflavescens served as the positive control for method validation of the PREC scans because its PAs have been previously characterized by NMR/ MS (Colegate et al., 2016). We identified 18 candidate PAs in *P. alboflavescens* (Table 1) by targeting common structural fragments of retronecine-type PAs via multiple PREC scans (m/z 120, 138, and 156). Fourteen (14) of these are identical in mass to PAs previously identified in *Parsonsia* spp. via NMR (Abe et al., 1991a; Abe et al., 1990; Abe and Yamauchi, 1987; Abe et al., 1991b; Edgar et al., 1980; Eggers and Gainsford, 1979; Nishida et al., 1991), and 11 match masses previously identified by PREC 120 (Burzynski et al., 2015). However, without HRMS analysis and NMR structural elucidation, which is difficult on the sample scale utilized, molecular ion structural determinations and identifications remain tentative.

208

209 **Table 1.** Candidate PAs identified in *Parsonsia alboflavescens*. Voucher in Table S1.

Candidate PA associated Molecular ion $[M+H]^+$	Uncorrected Retention Time (mins)	Compound Tentative Identification	PREC Scan
300.4	9.199	Unidentified PA	120/138/156
442.4**	9.499	Parsonsianine or Ideamine B (N-oxide)*	120/138/156
302.4	9.566	Ideamine A (N-oxide) or Isoideamine A (N-oxide)	120/138/156
456.5 **	10.359	Parsonsianidine or Spiraline or Parsonsine (N-oxide) or 14-deoxyparsonsianidine (N-oxide)	120/138/156
328.5	10.467	Unidentified PA	120/138/156
456.5 **	10.771	Parsonsianidine or Spiraline or Parsonsine (N-oxide) or 14-deoxyparsonsianidine (N-oxide) or their respective isomers	120/138/156
474.4	10.872	12-seco-14-deoxyparsonsianine-13-methylester (N-oxide)	120/138/156
444.5	10.957	Unidentified PA	120/138
426.3**	11.246	Ideamine B*	120/138/156
470.5**	11.397	17-methyl parsonsianidine or Heterophylline (N-oxide) or Spiranine	120/138/156
442.3**	11.415	Parsonsianine or Ideamine B (N-oxide) or their respective isomers	120/138
488.4	11.531	Unidentified PA	120/138/156
458.5**	11.614	Parsonsianine (N-oxide) or 12-seco-14-deoxyparsonsianine-13-methylester	120/138/156
474.5	12.047	12-seco-14-deoxyparsonsianine-13-methylester isomer (N-oxide)	120/138/156
440.5**	12.206	Parsonsine or 14-deoxyparsonsianidine	120/138/156
472.6 **	12.364	Parsonsianidine (N-oxide) or Spiraline (N-oxide)	120/138/156
454.5 **	12.921	Heterophylline	120/138/156
486.5 **	13.102	17-methyl parsonsianidine (N-oxide) isomer or Spiranine (N-oxide) isomer	120/138

210 *Ideamine B is the common name for 14-deoxyparsonsianine.

211 **Candidate PAs of identical mass previously identified in *Parsonsia alboflavescens* via PREC 120

212 (Burzynski et al., 2015).

213 At the declustering potentials and collision energies utilized by the mass spectrometer, $[M+H]^+$
214 ions corresponding to the masses of predicted cyclic triesters largely produced all three fragments
215 characteristic of retronecine-type PAs, m/z 120, 138, and 156. While retronecine-type PAs are
216 structurally similar, with fragments understood to involve the necine base and the hydroxymethyl
217 moiety positioned at carbon one of the pyrrolizidine ring, the subtle differences in chemical structures
218 and analytical instrumentation prevents absolute optimization of collision energy and declustering
219 potential values utilized in PREC scans; these instrumental parameters are still largely compound
220 dependent, and therefore can result in inadequate sensitivity for the detection of a particular fragment,
221 resulting in the possibility of false negatives.

222 *2.2. Survey for PA distribution*

223 A total of 319 samples from 231 species [representing 16 of 28 major Apocynaceae lineages
224 classified as subfamilies or tribes (Endress et al., 2018)] were screened for PAs utilizing a PREC 120 scan
225 with MCA (vouchers and results in the supporting Information Table S1). Samples containing a molecular
226 ion with a m/z 120 fragment were determined to be positive, with low confidence, for retronecine-type
227 PAs based on following criteria: (1) the TIC (total ion chromatogram) signal, associated with an evenly
228 massed molecular ion, was greater than 3:1 S/N with column retention between 9 and 15 minutes, and
229 (2) the evenly massed molecular ion of interest was greater than or equal to $1x10^4$ intensity (cps; counts
230 per second). Select samples were then evaluated on PREC 120/ 138/ 156 methods to confirm additional
231 fragments of evenly massed molecular ions suspected to be PAs (Table 2).

232 Samples were deemed as "positive with high confidence" if both the PREC 120 and PREC 138
233 scans indicated the same ion at the same retention time. As previously indicated, molecular ions that
234 fragment to m/z 120 and 138 are typically associated with cyclic- diesters and open- chain diesters, and
235 PA compounds whose fragments include m/z 120, 138, and 156 are likely to be open-chained
236 monoesters and cyclic triesters (Fig. 4). Samples that showed no strong features on PREC 138 or 156

237 scans were deemed "positive with low confidence" for retronecine-type PAs. Of the 78 leaf samples
238 analyzed via all PREC methods (m/z 120, 138, 156; without MCA), 45 were determined to be negative or
239 contain PAs below the limit of detection of the method (Table 2). Ten (10) samples from species of
240 Apocyneae, Asclepiadeae, Periplocoideae, and *Holarrhena pubescens* (Malouetieae) were "positive with
241 low confidence" since they yielded only ions with an m/z 120 fragment. Of the "positive with high
242 confidence" samples (23), those from species of Malouetieae, Wrightieae, and Marsdenieae contained
243 likely PAs with only m/z 120 and 138 fragments, while all positive samples from Echiteae species
244 contained one or more candidate PAs with m/z 120, 138, and 156 fragments.
245
246 **Table 2.** Samples analyzed on all PREC scans (m/z 120, 138, 156). Samples determined as N: no
247 candidate ions detected, Y (low): low confidence, candidate ions detected only in PREC 120, and Y (high):
248 high confidence, candidate ions detected in PREC 120 and PREC 138. ND: not detected. Vouchers in
249 Table S1.

Tribe	Species	Author	sample #	organ	Determination	PREC 120	PREC 138	PREC 156	Uncorrected Retention Time (min)
Apocyneae	*Amphineurion marginatum*	(Roxb.) G. Don	23	leaf	Y (low)	320.4	ND	ND	15.16
	Amphineurion marginatum	(Roxb.) G. Don	52	leaf	N	ND	ND	ND	
	Amphineurion marginatum	(Roxb.) G. Don	270	leaf	Y (low)	320.4	ND	ND	15.16
	Apocynum cannabinum	L.	28918	leaf	N	ND	ND	ND	
	Beaumontia murtonii	Craib	267	leaf	N	ND	ND	ND	
	Beaumontia murtonii	Craib	268	leaf	N	ND	ND	ND	
	Trachelospermum asiaticum	Nakai	81	leaf	N	ND	ND	ND	
	Vallaris solanacea	(Roth) O. Kuntze	90	leaf	N	ND	ND	ND	
Echiteae	*Bahiella infundibuliflora*	J.F.Morales	28143	leaf	Y (high)	300.1	300.1	300.1	10.22
	Echites panduratus	A. DC.	28459	leaf	N	ND	ND	ND	
	Echites umbellatus	Jacq.	28516	leaf	Y (high)	318.4	318.4	318.4	10.43
						300.5	300.5	300.5	10.73
						456.2	456.2	456.2	11.93
						470.5	470.5	470.5	12.57
						444.4	444.4	444.4	12.73
						458.3	458.3	ND	12.87
						440.5	440.5	440.5	13.26
						472.4	472.4	472.4	13.27

						486.3	486.3	ND	14.15
	Laubertia boissieri	A. DC.	220	leaf	Y (high)	328.4	328.4	328.4	11.59
						356.1	ND	ND	11.8
	Macropharynx isthmica	Woodson	117	leaf	Y (high)	456.2	456.2	456.2	11.81
						498.2	498.2	498.2	12.72
	Macropharynx peltata	(Vell.) J.F.Morales, M.E.Endress & Liede	28964	leaf	Y (high)	498.4	498.4	498.4	12.71
	Rhodocalyx rotundifolius	Müll. Arg.	125	leaf	Y (high)	300.4	300.4	300.4	10.01
						302.4	302.4	ND	10.326
						456.6	456.6	456.6	11.46
						444.5	444.5	444.5	12.13
						470.5	470.5	470.5	12.19
						472.4	472.4	472.4	12.66
						498.3	498.3	498.3	12.92
						486.7	486.7	486.7	13.61
						318.3	318.3	318.3	9.71
	Rhodocalyx rotundifolius	Müll.Arg.	28239	leaf	Y (high)	456.6	456.6	456.6	11.45
						470.5	470.5	470.5	12.18
						498.3	498.3	498.3	12.83
	Temnadenia odorifera	(Vell.) J. F. Morales	213	leaf	Y (high)	498.3	498.3	498.3	13.1
	Temnadenia stellaris	Miers	28957	leaf	Y (high)	484.4	ND	ND	12.34
						498.3	498.3	498.3	13.1
	Temnadenia violacea	(Vell.) Miers	185	leaf	Y (high)	456.5	456.5	456.5	10.97
						456.5	456.5	456.5	11.34
						484.4	ND	ND	12.57
						498.5	498.5	498.5	13.2
Malouetieae	*Eucorymbia alba*	Stapf	28111	leaf	Y (high)	238.3	238.3	ND	11.79
						354.4	354.4	ND	11.93
						396.4	ND	ND	12.96
						436.5	ND	ND	14.72
	Eucorymbia alba	Stapf	28112	leaf	Y (high)	238.3	238.3	ND	11.798
						354.4	354.4	ND	11.932
						436.5	ND	ND	14.67
	Galactophora schomburgkiana	Woodson	116	leaf	Y (high)	270.4	270.4	ND	9.76
						238.2	238.2	ND	11.92
	Holarrhena pubescens	Wall. ex G. Don	231	leaf	N	ND	ND	ND	
	Holarrhena pubescens	Wall. ex G. Don	233	leaf	N	ND	ND	ND	
	Holarrhena pubescens	Wall. ex G. Don	234	leaf	N	ND	ND	ND	
	Holarrhena pubescens	Wall. ex G.Don	29372	seed	Y (low)	474.4	ND	ND	9.52
						498.4	ND	ND	9.83
	Malouetia calva	Markgr.	28965	leaf	N	ND	ND	ND	
	Malouetia cestroides	Müll.Arg.	28966	leaf	N	ND	ND	ND	
Mesechiteae	*Mandevilla boliviensis*	(Hook. f.) Woodson	76	leaf	Y (low)	444.2	ND	ND	9.76
						458.5	ND	ND	9.96

Odontadenieae	*Pentalinon luteum*	(L.) B.F.Hansen & Wunderlin	28673	leaf	N	ND	ND	ND	
	Pentalinon luteum	(L.) B.F.Hansen & Wunderlin	28676	leaf	N	ND	ND	ND	
Wrightieae	*Wrightia antidysenterica*	(L.) R. Br.	28422	leaf	Y (high)	432.5	432.5	ND	11.43
	Wrightia dubia	(Sims) Spreng.	28418	leaf	Y (high)	432.5	432.5	ND	11.4
	Wrightia dubia	(Sims) Spreng.	28421	leaf	Y (high)	432.5	432.5	ND	11.39
	Wrightia karaketii	D.J. Middleton	28451	leaf	Y (high)	432.5	432.5	ND	11.48
	Wrightia tinctoria	R.Br.	29370	seed	Y (high)	210.2	ND	ND	7.56
						328.5	ND	ND	9.99
						460	ND	ND	10.16
						300.2	300.2	ND	~10.36
						432.4	432.4	ND	~10.36
						328.5	328.5	ND	~10.36
						270.3	270.3	ND	~10.36
						352.1	ND	ND	11.4
	Wrightia tinctoria	R.Br.	29371	seed	Y (high)	210.2	ND	ND	7.32
						328.5	ND	ND	9.96
						460	ND	ND	10.19
						300.2	300.2	ND	~10.36
						328.5	328.5	ND	~10.36
						270.3	270.3	ND	~10.36
						432.4	432.4	ND	~10.36
						352.1	ND	ND	11.36
	Wrightia versicolor	S.T. Blake	28423	leaf	Y (high)	432.4	432.4	ND	11.33
						300.2	300.2	ND	11.66
Asclepiadeae	*Barjonia erecta*	(Vell.) K.Schum.	28244	leaf	N	ND	ND	ND	
	Barjonia new spp		28246	leaf	N	ND	ND	ND	
	Blepharodon costae	Fontella & Morillo	28403	leaf	N	ND	ND	ND	
	Blepharodon mucronatum	(Schltdl.) Decne.	28331	leaf	N	ND	ND	ND	
	Blepharodon salicinum	Decne.	28335	leaf	N	ND	ND	ND	
	Calotropis gigantea	(L.) W.T.Aiton	28709	leaf	N	ND	ND	ND	
	Ditassa auriflora	Rapini	28338	leaf	N	ND	ND	ND	
	Ditassa eximia	Decne.	28174	leaf	Y (low)	252.3	ND	ND	13.22
	Ditassa gracilipes	Schltr.	28343	leaf	N	ND	ND	ND	
	Ditassa insignis	Farinaccio	28407	leaf	N	ND	ND	ND	
	Ditassa lanceolata	Decne.	28345	leaf	N	ND	ND	ND	
	Ditassa lenheirensis	Silveira	28409	leaf	Y (low)	252.2	ND	ND	13.01
	Ditassa obcordata	Mart.	28190	leaf	N	ND	ND	ND	
	Ditassa oxyphylla	Turcz.	28411	leaf	N	ND	ND	ND	
	Gonolobus rostratus	(Vahl) Schult.	28201	leaf	N	ND	ND	ND	
	Hemipogon abietoides	E.Fourn.	28351	leaf	N	ND	ND	ND	
	Hemipogon acerosus	Decne.	28269	leaf	N	ND	ND	ND	
	Hemipogon acerosus	Decne.	28352	leaf	N	ND	ND	ND	
	Hemipogon acerosus	Decne.	28354	leaf	N	ND	ND	ND	

	Hemipogon hemipogonoides	(Malme) Rapini	28276	leaf	N	ND	ND	ND	
	Metastelma giuliettianum	Fontella	28291	leaf	N	ND	ND	ND	
	Metastelma harleyi	Fontella	28293	leaf	Y (low)	444.4	ND	ND	9.625
	Metastelma tubatum	Griseb.	28391	leaf	N	ND	ND	ND	
	Minaria magisteriana	(Rapini) T.U.P.Konno & Rapini	28303	leaf	Y (low)	486.4	ND	ND	9.124
						490.4	ND	ND	9.22
	Oxypetalum appendiculatum	Mart.	28397	leaf	N	ND	ND	ND	
	Peplonia adnata	(E. Fourn.) U.C.S. Silva & Rapini	28233	leaf	Y (low)	444.4	ND	ND	9.325
	Peplonia macrophylla	(Malme) U.C.S. Silva & Rapini	28316	leaf	N	ND	ND	ND	
	Petalostelma bracteolatum	(E. Fourn.) Fontella	28234	leaf	N	ND	ND	ND	
	Petalostelma martianum	(Decne.) E. Fourn.	28400	leaf	N	ND	ND	ND	
	Seutera angustifolia	(Pers.) Fishbein & W.D. Stevens	28136	leaf	Y (low)	318.4	ND	ND	13.92
Marsdenieae	*Campestigma purpureum*	Pierre ex Costantin	94	leaf	N	ND	ND	ND	
	Marsdenia glabra	Costantin	99	leaf	Y (high)	432.5	432.5	ND	10.99
						388.2	388.2	ND	11.5
	Marsdenia tinctoria	R. Br.	27354	leaf	Y (high)	432.5	432.5	ND	11.71
	Marsdenia tinctoria	R. Br.	28105	leaf	Y (high)	432.5	432.5	ND	11.09
Periplocoideae	*Petopentia natalensis*	(Schltr.) Bullock	78	leaf	Y (low)	252.2	ND	ND	13.14
	Petopentia natalensis	(Schltr.) Bullock	28447	leaf	N	ND	ND	ND	
Melodineae	*Craspidospermum verticillatum*	Bojer ex Decne.	630	leaf	N	ND	ND	ND	
Vinceae	*Ochrosia poweri*	F.M.Bailey	28097	leaf	N	ND	ND	ND	
	Rauvolfia balansae	(Baill.) Boiteau	28131	leaf	N	ND	ND	ND	
	Rauvolfia verticillata	(Lour.) Baill.	28099	leaf	N	ND	ND	ND	
	Rauvolfia vomitoria	Afzel.	28116	leaf	N	ND	ND	ND	

250

251 *2.3. Phylogenetic distribution of PAs*

252 None of the Rauvolfioid taxa sampled (11 species from 5 genera in 3 of 11 tribes,
253 Tabernaemontaneae, Melodineae, and Vinceae) had any detectable PAs (Table S1, Table 2). These taxa
254 lack a homospermidine synthase-like gene (Livshultz et al., 2018a) and are therefore predicted to lack

255 the capacity for PA biosynthesis. They are known for the production of monoterpenoid indole alkaloids
256 (MIAs) (Endress et al., 1990), which our methods would be unlikely to detect.

257 All ions identified as PAs with high confidence (Table 2) occur in species of tribes of the APSA
258 clade: two previously reported (Echiteae, Malouetieae) (Burzynski et al., 2015) and two newly reported
259 here (Wrightieae, Marsdenieae). Ions identified as PAs with low confidence were detected in species of
260 tribes Echiteae, Malouetieae, and Apocyneae (*Amphineurion*, previously reported) (Colegate et al.,
261 2016) and Wrightieae, Asclepiadeae, Mesechiteae, and Periplocoideae (newly reported). None of seven
262 sampled species from the five (of six total) genera in tribe Nerieae had detectable ions in the PREC 120
263 (with MCA) scans (Table S1). *Alafia* cf. *caudata* Stapf (Nerieae) had been previously reported to produce
264 retronecine-type PAs (Colegate et al., 2016), but we did not detect any candidate compounds in samples
265 of *Alafia barteri* Oliv. or *A. thouarsii* Roem. & Schult. *Alafia* includes 22 species (Leeuwenberg, 1997;
266 Wieringa, 2011) and further sampling may identify chemotaxonomically useful variation in PA profiles.
267 All samples from other major APSA clade lineages (Rhabdadenieae, Odontadenieae, Baisseeae,
268 Secamonoideae) lacked any candidate PA ions in the PREC 120 (with MCA) scans (Table S1). Tribes
269 Fockeeae, Eustegieae, and Ceropegieae were not sampled.

270 *2.4. Wrightieae*

271 Wrightieae are sister to the rest of the APSA clade and include three genera (Livshultz et al.,
272 2007). Likely PAs were detected in leaves of 5 of 11 species of *Wrightia* R.Br. (Table 2) but none of the
273 three species of *Pleiocerас* Baill. and *Stephanostema* K.Schum sampled (Table S1). The most frequent
274 candidate PA present yields an ion of m/z 432.5, containing both m/z 120 and 138 fragments. However,
275 there is some evidence for coelution with an m/z 300.2 compound, as a low intensity m/z 300.2 ion was
276 also associated with the TIC of the m/z 432.5 compound. Seeds of *Wrightia tinctoria* also contain an m/z
277 432.4 compound with fragmentation in both PREC 120 and PREC 138 (Table 2). Within the

chromatography associated with the m/z 432.4 ion, m/z 270.2, 300.2 and 328.3 appeared with lower abundances (counts per second). Alterations to the chromatography method associated with these scans can increase the resolution of these compounds currently co-eluting within the chromatography, thereby increasing the sensitivity to these ions by diminishing ion suppression. Multiple other PREC 120 only ions appeared in these samples including m/z 210.2, 352.1, and 460.0. We suggest with low confidence these molecular ions are associated with PAs.

Five of the *Wrightia* species sampled here were included in a molecular phylogenetic analysis (Livshultz et al., 2007). The distribution of PAs on the *Wrightia* topology is homoplastic, but the potential utility of PAs as a chemotaxonomic character for the infra-generic classification of *Wrightia* warrants more thorough sampling.

Previously reported alkaloids from *Wrightia* species include steroidal (pregnane) alkaloids from *Wrightia pubescens* subsp. *laniti* (Blanco) Ngan (syn. *Wrightia javanica* A.DC.) (Kawamoto et al., 2003). This species is sampled here (Table S1) and determined as PA negative. Three species, *W. tinctoria*, *W. dubia* Spreng., and *W. arborea* (Dennst.) Mabb. (syn. *W. tomentosa* Roem. & Schult.), are used as sources of indigo (Ngan, 1965), and the indole-containing compounds indigotin and indirubin are known from *W. tinctoria*, as well as the quinolizidine, tryptanthrin (Khyade, 2014). Two of these three species, *W. tinctoria* and *W. dubia*, are here reported as likely containing PAs (Table 2) while the third, *W. arborea*, is reported as PA negative (Table S1).

2.5. Malouetieae

Eleven species from nine of 13 genera of Malouetieae were sampled. PAs were detected with high confidence in *Eucorymbia alba* Stapf (two samples) and *Galactophora schomburgkiana* Woodson (one sample) (Table 2). This is the first report of secondary metabolites from these genera. No PAs were

detected in the single sample of *Galactophora crassifolia* (Müll.Arg.) Woodson analyzed (Table S1). Evenly massed ions were detected in the PREC 120 (with MCA) scan in one of two samples of *Holarrhena curtisii* King & Gamble (m/z 284.3, 300.1) (Table S1). *Kibatalia macrophylla* (Pierre) Woodson (one of two samples) contained three possible PAs in its PREC 120 (with MCA) mass spectrum, m/z 284.3, 302.4, and 314.0 (Table S1).

The only previous report of PAs in Malouetieae used the Mattocks' test (Mattocks, 1967), a colorimetric assay with absorption at 565 nm, to detect unsaturated PAs in the seeds of *Holarrhena pubescens* (Arseculeratne et al., 1981). In our study, crude extracts of *Holarrhena pubsescens* leaves were evaluated for PAs via LC-MS/MS, with no strong evidence of PAs in the PREC scans. In seeds of *H. pubescens*, two even-massed ions were identified in the PREC 120 scan only, m/z 476.4 and 498.4 (Table 2). These are considered candidate PAs with low confidence. The specialized metabolites of *H. pubescens* (syn. *H. antidysenterica* (L.) Wall. ex A.DC., *H. floribunda* T.Durand & Schinz) are well studied, with steroidal alkaloids and glycosides of primary interest for their potential bioactivity (Kumar et al., 2007; Sinha et al., 2013). Three steroidal amino-glycosides previously reported from *H. pubescens* (holantosine B, D, and F, MW=475.3 Da) correspond in mass to one of the ions detected in PREC 120 (Afendi et al., 2011, queried 16.07.2020; Janot et al., 1970). Analysis of standards and/or alternative analytical techniques would be required for absolute identification of the compounds detected here.

2.6. Echiteae, Odontadenieae, Mesechiteae

These three tribes form a well-supported monophyletic clade of predominantly New World genera (Fishbein et al., 2018), but the boundaries among them have been redrawn several times (Morales et al., 2017; Simões et al., 2004). Morales et al. (2017) proposed that presence of PAs is a useful chemotaxonomic character for delimiting Echiteae from Odontadenieae and identified their absence in *Pentalinon* Voigt as supportive of its transfer to Odontadenieae. We sampled eight of

fourteen Echiteae, five of nine Odontadenieae, and three of six Mesechiteae genera. PAs were detected in seven of eight sampled Echiteae genera. Only the sampled *Prestonia* species (*P. robusta* Rusby, *P. tomentosa* R.Br.) had no evidence of PAs in the PREC 120 (MCA on) scans (Table S1), although PAs have been previously reported from *P. amabilis* J.F.Morales, *P. quinquangularis* Spreng. [syn. *P. acutifolia* (Benth. ex Müll.Arg) K.Schum], and *P. portobellensis* Woodson (Burzynski et al., 2015). PAs in *Echites* and *Parsonsia* have been previously reported (Burzynski et al., 2015), but PAs in *Bahiella* J.F.Morales, *Macropharynx* (syn. *Peltastes*), *Temnadenia*, *Rhodocalyx* Müll.Arg., and *Laubertia* A.DC. are here reported for the first time, although Brown (1987) had previously suggested their presence in *Macropharynx* and *Temnadenia* based on indirect evidence. Exemplars of these genera had molecular ions appearing at consistent retention times in PREC 120, 138, and 156 scans (Table 2), fragmentation patterns characteristic of the cyclic triesters known from the positive control, *Parsonsia alboflavescens* (Table 1). Among the 22 species sampled from other two tribes (Table S1), only one, *Mandevilla boliviensis* (J.J.Veitch) Woodson (Mesechiteae), yielded an ion detectable in the PREC 120 scan, but not in PREC 138 or 156 (Table 2). We have low confidence in the identification of this compound as a PA, but this result requires further investigation. Overall, this pattern supports (Morales et al., 2017) hypothesis of the chemotaxonomic utility of PA presence for circumscription of Echiteae.

PAs are present in all Echiteae genera studied, but presence of detectable PAs varies both among samples of congeneric species and of conspecific individuals (Table S1, Table 2). Also, the diversity of PAs within a sample varied greatly from 18 candidate compounds in *Parsonsia alboflavescens* (Table 1) to one in *Temnadenia odorifera* (Vell.) J.F.Morales (Table 2). We have evidence that some of this variation is due to plasticity (see Section 2.11), but the role of genetics remains uncertain. Extensive sampling of species and individuals within Echiteae genera will be necessary to determine if there are any species-specific patterns useful for delimiting sub-tribal or infra-generic taxa.

346 *2.7. Apocyneae*

347 We sampled 36 species from 17 of 21 genera of Apocyneae. In this tribe, PAs had been
348 previously reported from *Anodendron affine* Druce (Sasaki and Hirata, 1970) and *Amphineurion*
349 *marginatum* (Roxb.) D.J.Middleton (Colegate et al., 2016). These species belong to two early diverging
350 lineages of Apocyneae (subtribes Papuechitinae and Amphineurinae, respectively) and Livshultz et al.
351 (2018b) suggested that PAs were potentially a chemotaxonomic character for delimiting these early
352 diverging lineages from the rest of the radiation.

353 No PAs were detected with high confidence in any sample of Apocyneae. Two of three
354 accessions of *Amphineurion marginatum* leaves possessed a molecular ion of m/z 320 which appeared
355 only in the PREC 120 scan with associated chromatography at a late retention time of ~15 mins (>3 to 1
356 S/N) (Table 2). Fragmentation of a PA with an m/z 320 via PIS by Colegate et al. (2016) was consistent
357 with our PREC 120 findings, as that compound was found to only result in m/z 120 fragment with no m/z
358 138 or 156 fragments. HRMS was utilized to propose a molecular formula for the m/z 320 ion,
359 $C_{18}H_{26}NO_4$, and therefore it was tentatively identified as an open-chain diester (Colegate et al., 2016).
360 Colegate et al. (2016) reported that leaves of *Amphineurion marginatum* contained the lowest
361 percentage by dry weight of PAs (0.02%) compared to roots (0.13%) and stems (0.09%). This may
362 account for the lack of PA structural diversity and abundance observed in our leaf samples but other
363 factors, environmentally induced plasticity, genetic variation, cannot be ruled out in the present study. A
364 product scan and/ or HRMS would need to be completed on the m/z 320 molecular ion in our
365 *Amphineurion marginatum* leaf samples to fully understand the fragmentation pattern and identity of
366 this compound.

The unusual PAs previously identified in *Anodendron affine* have a platynecine core (Sasaki and Hirata, 1970) that would not be detectable with our strategy. Thus the occurrence and distribution of platynecine-derived PAs in other Apocyneae species also remains an open question.

2.8. Marsdenieae

We sampled 26 species from 10 of 27 genera of tribe Marsdenieae. PAs were identified with high confidence in samples of *Marsdenia tinctoria* R.Br. and *Marsdenia glabra* Constantin (Table 2). The current taxonomic concept of *Marsdenia* R.Br. is highly polyphyletic (Rodda et al., 2020), and the segregation of monophyletic genera from *Marsdenia sensu lato* is ongoing (Espírito Santo et al., 2019). *Marsdenia tinctoria* (the type species) and *M. glabra* belong to the small group of species segregated as *Marsdenia sensu stricto* (Bullock, 1956; Forster, 1995). Presence of PAs may be a useful chemotaxonomic character for recognition of *Marsdenia sensu stricto*, pending further sampling. The most frequently reported natural products from species of *Marsdenia sensu lato* and other genera of Marsdenieae are steroids, steroidal alkaloids, and steroidal glycosides including the antisweet gymnemic acids from the medicinal plant *Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Liu et al., 1992). All three classes of compounds have been previously reported from *Marsdenia tinctoria* (Chowdhury et al., 1994; Gao et al., 2009).

2.9. Asclepiadeae and Periplocoideae

We sampled 60 species from 25 of 105 genera of Asclepiadeae and 8 species from 6 of 33 genera of Periplocoideae. We identified compounds that might be PAs with low confidence in five (5) species of the closely related Asclepiadeae subtribes Metastelmatinae and Oxypetalinae (*Seutera angustifolia* (Pers.) Fishbein & W.D.Stevens, *Ditassa eximia* Decne., *Ditassa lenheirensis* Silveira, *Metastelma harleyi* Fontella, *Minaria magisteriana (Rapini) T.U.P.Konno & Rapini*) and in one of two

389 samples of *Petopentia natalensis* (Schltr.) Bullock (Periplocoideae) (Table 2). The re-circumscription of
390 genera in these species-rich Asclepiadeae subtribes is ongoing (Liede-Schumann et al., 2014; Liede-
391 Schumann et al., 2005; Silva et al., 2012) and a chemotaxonomic approach has not been attempted. The
392 compounds discovered in the present study, once they are identified, can form a nucleus for this
393 research. Asclepiadeae as a whole are known for diverse steroidal compounds, including cardenolides
394 (Endress et al., 2018). Steroidal alkaloids are widely distributed (Lee et al., 2000) and
395 phenanthroindolizidine alkaloids are diagnostic of *Vincetoxicum* Wolf (Endress et al., 2018; Liede-
396 Schumann et al., 2016; Liede, 1996; Staerk et al., 2005).

397 There are no previous reports of specialized metabolites from *Petopentia natalensis*, but a
398 diversity of compounds have been reported from Periplocoideae including steroidal glycosides,
399 cardenolides, steroidal alkaloids and, rarely, indoloquinoline alkaloids from species of *Cryptolepis* R.Br.
400 (Endress et al., 2018; Paulo and Houghton, 2003; Tackie et al., 1991).

401 Rarely do retronecine-type PAs present without other diagnostic fragment ions such as m/z 138
402 and/ or 156 fragments. However, a few other PAs have been documented to contain a base peak of m/z
403 120 without possession of other key fragments. (Jeong and Lim, 2019) noted the tentative identification
404 of two open chained retronecine-type diesters, symviridine and uplandicine, in which a base peak was
405 *m/z* 120 and no other common fragments (m/z 138 or 156) were obtained in PIS scans at the collision
406 energies utilized. Another open-chain diester, 7-acetyllycopsamine, was shown to fragment to a base
407 peak of *m/z* 120 (Colegate et al., 2005; Jeong and Lim, 2019). It is yet to be determined if a common
408 structural feature causes a tendency for particular PAs to result only in a *m/z* 120 fragment, but it does
409 appear open-chained diesters preferentially result in observation of only an *m/z* 120 fragment at
410 particular collision energies. This fragmentation pattern can yield only low confidence identification of

411 retronecine-type PAs and leaves substantial ambiguity about the true identity of compounds that
412 present with this pattern.

413 *2.10. PA distribution and homospermidine synthase amino acid motifs*

414 The first step of PA biosynthesis is catalyzed by the enzyme encoded by homospermidine
415 synthase (*hss*) which evolved 7 times independently among angiosperm lineages via duplication and
416 subfunctionalization of deoxyhypusine synthase (*dhs*), a gene of primary metabolism (Livshultz et al.,
417 2018a). Livshultz et al. (2018a) identified a characteristic amino acid motif in HSS (VXXXD) that evolved
418 from the ancestral IXXXN DHS motif each time HSS function and PA-biosynthesis evolved. *In vitro*
419 experiments showed that the DHS of *Ipomoea neei* (Spreng.) O'Donell (wild-type IXXXN) mutagenized to
420 a VXXXD motif had less DHS and slightly more HSS function (Kaltenegger et al., 2013). In Apocynaceae,
421 Livshultz et al. (2018a) placed the gene duplication that gave rise to the functionally characterized HSS of
422 *Parsonsia alboflavescens* early in the diversification of the APSA clade but after the divergence of
423 Wrightieae from the rest of the clade. They hypothesized that species with *hss*-like genes that encode
424 the VXXXD motif have a functional HSS and can produce PAs while species with *hss*-like genes that
425 encode the DHS-like IXXXN motif or an intermediate IXXXD motif have lost PA biosynthesis.

426 Fifteen (15) of the species of from which *hss*-like genes were sequenced are included in the
427 present study (Table 3). Three species with the VXXXD motif are identified with high confidence as
428 producing PAs, three with low confidence, and four have no detectable PAs. One sampled species with
429 the IXXXN motif produces PAs (*Marsdenia glabra*) while the other (*Asclepias syriaca* L.) lacks PAs and has
430 a pseudogenized *hss*-like gene which encodes the IXXXN motif when the ORF is restored (Livshultz et al.,
431 2018a). All three sampled species with the IXXXD motif lack detectable PAs. The relationship between
432 motif and presence of PAs is not dichotomous as predicted by Livshultz et al. (2018) (Table 3). Both
433 species with VXXXD and IXXXN motifs in their HSS-like enzymes can produce PAs. More sequencing of

434 *hss*-like genes from the species included in the present survey is required to determine if there is any
435 correlation between the identified motifs and PA biosynthesis and if any other amino acid substitutions
436 might be functionally important. More infra-specific sampling and scans for PAs with alternative cores
437 (Fig. 1) are required to confirm that species reported here as PA negative truly lack PAs. No *hss*-like gene
438 was found in the transcriptome of *Wrightia natalensis* Stapf (a PA negative species, Table S1). It remains
439 to be determined if PA-producing *Wrightia* species (Table 2) have an *hss* locus in their genomes.

440 **Table 3**. Amino acid motifs of *homospermidine synthase*-like genes (Livshultz et al., 2018a) from
441 Apocynaceae species and PA phenotypes. The VXXXD motif is associated with evolution of a functional
442 HSS in comparative analyses of PA-producing angiosperms.

Tribe/ subfamily	Genus	specific epithet	HSS-like motif	PA phenotype (Table 2, Table S1)	Comment
Nerieae	*Alafia*	*barteri*	IXXXD	not detected (120-)	One sample analyzed. Retronecine-core PAs reported in *Alafia cf. caudata.*
Periplocoideae	*Finlaysonia*	*insularum*	IXXXD	not detected (120-)	
	Raphionacme	*flanaganii*	IXXXD	not detected (120-)	
Asclepiadeae	*Asclepias*	*syriaca*	IXXXN	not detected (120-)	*hss*-like pseudogene
Marsdenieae	*Marsdenia*	*glabra*	IXXXN	high confidence (120+/138+/156-)	
Apocyneae	*Anodendron*	*oblongifolium*	VXXXD	not detected (120-)	Platynecine-core PAs reported in *Anodendron affine*
	Anodendron	*paniculatum*	VXXXD	not detected (120-)	Platynecine-core PAs reported in *Anodendron affine*

Echiteae	*Echites*	*umbellatus*	VXXXD	high confidence (120+/138+/156+)	Infra-specific variation, intra-individual plasticity in PA detectablity.
	Echites	*woodsonianus*	VXXXD	not detected (120-)	One sample analyzed.
	Parsonsia	*alboflavescens*	VXXXD	high confidence (120+/138+/156+)	
	Parsonsia	*eucalyptophylla*	VXXXD	low confidence (120+/138?/156?)	
Malouetieae	*Galactophora*	*schomburgkiana*	VXXXD	high confidence (120+/138+/156-)	
	Holarrhena	*curtisii*	VXXXD	low confidence (120+/138?/156?)	
Mesechiteae	*Mandevilla*	*boliviensis*	VXXXD	low confidence (120+/138-/156-)	
Odontadenieae	*Secondatia*	*densiflora*	VXXXD	not detected (120-)	3 samples analyzed.
Wrightieae	*Wrightia*	*natalensis*		not detected (120-)	No *hss*-like gene identified in transcriptome. Congeneric species have metabolites identified as PAs with high confidence (Table 2).

443

444 *2.11 Infraspecific variation in PA detectability*

445 Tasca et al. (2018) were unable to detect PAs in a subset of *Echites umbellatus* herbarium
446 specimens analyzed with a Q1 scan. Since some of the specimens analyzed in that study were very old,
447 and because a PREC scan can detect PAs with greater specificity than a Q1 scan, we follow up here by
448 analyzing leaf fragments from 19 relatively recent (1935-2006) herbarium specimens with PREC 120
449 (with MCA). Of these, nine (9) had detectable PAs and ten (10) did not (Table S2). We also evaluated 110

450 leaf samples from 96 cultivated plants in seven half-sib families established from wild collected fruits
451 from Florida and the Bahamas (Table S3). Among all samples, 98 had PAs while 31 had no detectable
452 PAs. PA detection was unrelated to extracted leaf mass. On average, less tissue was used for positive
453 samples (mean=11.1 mg, SD=5.5 mg, N=98) than for negative samples (mean= 21.4 mg, SD=16.3 mg,
454 N=31).

455 In five of the seven half sibling families analyzed, we detected PAs in the youngest leaves
456 collected from all individuals sampled (9-14 plants/family). In two families, 5 of 12 and 7 of 19
457 individuals had no detectable PAs when first sampled (Table S3). The youngest leaves from fourteen (14)
458 of the 31 individuals in these two families were re-sampled two years later. Among these plants, the
459 youngest leaves of five (5) individuals maintained the same phenotype (two with detectable PAs, three
460 with no detectable PAs), six (6) shifted from detectable PAs at time one to no detectable PAs at time
461 two, and three (3) shifted from undetectable to detectable. Thus the variation in detectability of PAs
462 observed among samples is, at least in part, due to phenotypic plasticity. Because all plants had already
463 transitioned to flowering when they were sampled at time 1, and because the same approximate
464 developmental stage was sampled at both time points (youngest leaves), environmental factors are
465 implicated. However, the inconsistency in the direction of change, and the difficulty of exactly matching
466 leaf developmental stages, means that ontogenetic variation is not completely excluded based on the
467 current data. Genetic factors may also play a role in determining the presence of plasticity in two of our
468 seed families but not in five others. More tightly controlled experiments are necessary to identify the
469 causal factors.

470 Infraspecific variation is very commonly in distribution of specialized metabolites both among
471 and within conspecific plants (Hartmann, 1996; Moore et al., 2014), included in those that produce PAs.
472 For example, *Senecio vulgaris* L. showed distinction differences in specific PA concentrations depending

on both developmental stage and season (Flade et al., 2019), with the total PAs present increasing with developmental stage.

2.12 Implications for human health

Apocynaceae species are frequently used medicinally and more rarely as food (Endress et al., 2018). Several of the species here reported with high confidence as producing PAs are consumed by people. Notably, commercially available seeds of *Wrightia tinctoria* showed evidence of compounds that are identified with high confidence as PAs (Table 2). Seeds of *Wrightia tinctoria* are consumed in Ayruveda and other medicinal traditions of India, both intentionally (Khyade, 2014), and as contaminants of *Holarrhena pubescens* seeds (Khan, 1987). We found contamination by *Wrightia tinctoria* in our purchased package of *Holarrhena pubescens* seeds. Other organs of *Wrightia tinctoria* (leaves, bark) are also consumed (Reddy et al., 1999), as is *Wrightia antidysenterica* (L.) R.Br. (syn. *Walidda antidysenterica* (L.) Pichon) (Wickramaratne et al., 2015), another species identified with high confidence as PA positive (Table 2). On the other hand, we assign low probability to presence of retronecine-type PAs in seeds of *Holarrhena pubescens*, separated from contaminating *Wrightia tinctoria* seeds. Because specialized metabolites of *Holarrhena pubescens* are so well studied (Sinha et al., 2013) and because neither of the candidate compounds fragmented in the PREC 138 and 156 scans (Table 2), we consider it more likely that the m/z 476 and 498 ions identified in PREC 120 correspond to other primary or secondary metabolites. We suggest that the Arseculeratne et al. (1981) report of PAs in *H. pubescens* seeds derives from either non-specificity of their colorimetric assay or contamination by *Wrightia* spp. But no final conclusion can be reached until the low-confidence compounds that we found are definitively identified.

Marsdenia tinctoria is not as readily available in the global marketplace as *Wrightia tinctoria* and *Wrightia*-contaminated *Holarrhena pubescens*, but *Marsdenia tinctoria* is consumed as a remedy in the

traditional Chinese medicine system and elsewhere in southeast Asia (Chowdhury et al., 1994; Yan-Jiao, 2013). Any potential risk to people from consumption of *Wrightia* and *Marsdenia* products is unknown at this point. Further research, beginning with NMR characterization of the compounds we identified (Table 2), is warranted.

Flowers of *Echites panduratus* A.DC. (syn. *Fernaldia pandurata* Woodson*, Urechites karwinskii* Müll.Arg.) are consumed as a pickled condiment, "loroco," in the cuisines of several Central American countries, and young shoots are eaten as a green vegetable (Morton et al., 1990). A keto dihydro-PA, loroquine, was reported from the roots of *Echites panduratus*, but was not detectable in the shoots (del Castillo et al., 1970). Morton et al. (1990) also reported absence of indole alkaloids in aerial plant organs. Burzynski et al. (2015) did not detect PAs in sampled leaves, but Colegate et al. (2016) commented on unpublished evidence of retronecine-type PA presence in roots. In the present study, we re-analyzed the same specimen sampled by Burzynski et al. (2015) and confirmed absence of detectable PAs in PREC 120 (Table S1) and in a second sample from another plant, confirming no detectable compounds in PREC120/138/156 (Table 2). We also sampled shoot tips, young and mature leaves, and roots from a third plant, and found evidence of PAs with PREC 120 in mature leaves and roots (Table S1). These results need follow up with PREC 138/156 scans for confirmation. Assessment of any human risk from PAs related to consumption of *Echites panduratus* must begin with the identification and quantification of PAs in preparations as consumed. Broad sampling is necessary, given the potential for ontogenetic, environmentally-induced, and genetic variation.

2.13 Limitations of the current approach: sampling strategy

The broad taxonomic sampling that we sought to accomplish in this study, means that most species were represented by only one or a few samples. The variation in PA detectability that we documented in *Echites umbellatus* (ca. 50% of herbarium specimens with no detectable PAs, Table S2),

519 shows that there is a high probability that some of the species here reported as lacking retronecine-type
520 PAs in the leaves, will be reported to have them when more samples are analyzed. Our sampling is
521 overwhelming biased toward young leaves, since most of the samples were acquired for DNA extraction.
522 PA accumulation often has a distinctly organ and developmental stage specific pattern that is variable
523 among species (Hartmann and Zimmer, 1986; Irmer et al., 2015; Stegemann et al., 2018). In
524 *Amphineurion marginatum* and *Alafia cf. caudata* (Apocynaceae), PA concentration and diversity was
525 highest in root and lowest in leaves (Colegate et al., 2016). In *Echites panduratu*s, we detected PAs in old
526 but not in young leaves (Table S1). If these patterns are consistent across Apocynaceae species, our
527 study is biased toward underreporting of PA occurrence.

528 *2.14. Limitations of the current analytical approach: alternative necine cores and N-oxides*

529 The current MS methods exclude the possibly of identifying alternative necine bases, namely
530 otonecine and playtnecine. Identifying these bases would require additionally MS scans to target the
531 diagnostic fragments specific to these structures. Otonecine-type PAs, for example clivorine, readily
532 exhibit m/z 122, 150, and 168 fragments; with m/z 150 and 168 ions being most diagnostic, similar to
533 the m/z 120 and 138 diagnostic ion fragments associated with retronecine-type PAs (Lin et al., 1998).
534 Alternatively, saturated necine bases of playtnecine-type PAs, have a diagnostic fragment ion of m/z 140
535 and a characterization ion of m/z 122 (Lin et al., 1998). Since alternative bases were not pursued, the full
536 diversity of PAs present in our samples cannot be determined with the current methodologies.

537 Additionally, the N-oxide form of PAs is often more prevalent in plants than the free base, likely
538 due to the higher solubility and lower toxicity of the N-oxide form relative to the free base form of PAs
539 (Hartmann and Witte, 1995; Hartmann and Zimmer, 1986). Free base PAs can be differentiated from
540 their N-oxides via *in situ* reduction, typically via zinc dust (Colegate et al., 2005; Colegate et al., 2016;
541 Nishida et al., 1991). Many examples of the prevalence of N-oxides in Apocynaceae have been

542 documented. In *Alafia cf. caudata,* 10 of 15 PAs isolated in roots were N-oxides (Colegate et al., 2016). In
543 *Parsonia straminea* (R.Br.) F.Muell*.,* 96% of PAs identified via high resolution accurate mass (HRAM) LC-
544 MS/MS in leaves and stems were N-oxides (Hungerford et al., 2019). Therefore, not accounting for the
545 presence of N-oxides in samples can lead to the possibility of underrepresenting the amount of PAs
546 present, in addition to falsely characterizing some species as non-PA producing.

547 N-oxides can be detected without derivatization via MS, resulting in unique diagnostic
548 fragments in addition to those produced by the free base form of PAs. Typically, N-oxide diesters (open
549 and closed) present with m/z 120 and 138 ions, in addition to m/z 136 and 118 fragments (Ruan et al.,
550 2012; These et al., 2013). A PIS (product scan) of lycopsamine N-oxide, under the instrumental
551 parameters utilized, resulted in one ion pair, m/z 138- 136 (Ruan et al., 2012). However, a high intensity
552 m/z 172 fragment was observed, representative of the m/z 156 fragment typical of open-chain
553 monoesters with an associated oxygen atom. These et al. (2013) alternatively observed both ion pairs
554 (m/z 118-120 and m/z 136-138) and the m/z 172 fragment when lycopsamine N-oxide was subject to
555 PIS. Fragmentation of the retronecine core was frequently observed in product scans of N-oxides under
556 the collision energies utilized, with m/z 94 the most prominent ion (Ruan et al., 2012; These et al.,
557 2013).

558 The PREC scans employed in this study were designed to specifically target fragments inherent
559 to pro-toxic, free base, 1,2- unsaturated retronecine-type PAs. Standards of this type were used to
560 ensure proper fragmentation of these PAs. Burzynski et al. (2015) utilized PREC and MRM scans to
561 identify the m/z 120 fragment in masses associated with the N-oxide of parsonsine-type PAs. Our
562 current PREC scans were also able to detect masses associated with tentatively identified N-oxide
563 structures determined by Burzynski et al. (2015) (Table 1). This indicates our methods have some
564 specificity for N-oxides possibly because the N-oxide and free base form of retronecine-type PAs

565 typically present with m/z 120 and 138 fragments. However, more specific structural features must be
566 targeted in our MS protocols to increase the specificity for detection of N-oxide PAs. This is particularly
567 important since (Avula et al., 2015; Avula, 2015) and Colegate et al. (2016) note detection of N-oxide
568 PAs through PIS (utilizing their respective experimental MS parameters) that display either only m/z 120
569 or m/z 138 fragments or neither fragment. With our current method, the m/z 120 fragment had to be
570 present in order for identification of a retronecine-type PA to be considered.

571

572 **3. Conclusion**

573 We have conducted the first systematic survey of Apocynaceae for retronecine-type PAs using
574 high throughput LC-MS methods, PREC 120 (with MCA) and PREC 120/ 138/ 156. We confirm that PAs
575 have a scattered distribution within one lineage of the family, the APSA clade, as predicted from
576 previous reports in the phytochemical literature and from the distribution of *homospermidine synthase*-
577 like genes, which encode the first enzyme of the PA biosynthetic pathway. PAs are confirmed as a useful
578 chemotaxonomic character for delimiting Echiteae (PAs present in at least one species of all genera
579 tested to date) from the closely related Odontadenieae and Mesechiteae (only one species yielded an
580 ion identified with low confidence as a potential PA). In contrast to Echiteae, only a few of the tested
581 species from other previously reported PA positive tribes (Nerieae, Malouetieae, Apocyneae) have
582 detectable PAs. We report likely PAs for the first time in Wrightieae (species of *Wrightia*) and
583 Marsdenieae (species of *Marsdenia sensu stricto*). Discovery of these novel PA sources has potential
584 implications for human health since two of the species that we identify as likely containing PAs, *Wrightia*
585 *tinctoria* and *Marsdenia tinctoria*, are used medicinally. We find clear evidence of infra-specific variation
586 in PA-detectability in young leaves of *Echites umbellatus* and confirm that phenotypic plasticity is one of
587 the causes. Thus it is worth following up with further sampling of genera and species that are reported
588 here as lacking detectable PAs but that are closely related to PA-positive taxa. Targeted studies of organ-

589 specific, developmental stage, environmental, and genetic determinants of the PA phenotype are also
590 necessary. The more complete picture of the taxonomic distribution of PAs in Apocynaceae provided by
591 this report will facilitate research into the evolution of the PA biosynthetic pathway and into co-
592 evolution of Apocynaceae and their PA-adapted herbivores.

593 **4. Experimental**

594 *4.1. Plant materials*

595 Most tissue samples are young leaves, collected into desiccant from the living plant, and stored
596 at room temperature. A minority of samples are young leaves removed from herbarium specimens.
597 Most of these specimens had been dried at ca. 30 °C when collected from the living plant, potentially
598 with alcohol and/or mercuric chloride treatment, and stored at room temperature (Tasca et al., 2018).
599 Shoot tips, young and mature leaves, and young roots collected from a cultivated accession of *Echites*
600 *panduratus* were dried at room temperature. Seeds of *Holarrhena pubescens* and *Wrightia tinctoria*
601 were purchased from retail outlets in the United States and stored at room temperature. Seeds were
602 identified based on morphology, (Khan, 1987) and sorted under magnification to produce pure samples,
603 free of admixture, for analysis. Each analyzed tissue sample is vouchered by a specimen deposited in a
604 publicly accessible herbarium (Table S1). Taxonomy follows Endress et al. (2018) as updates by Espírito
605 Santo et al. (2019) and Rodda et al. (2020).

606 Half-sib families of *Echites umbellatus* were established in fall 2013, spring 2014, and fall 2014,
607 by planting the seeds from individual wild-collected fruits and cultivated in the greenhouse of Florida
608 International University. Seeds were started in small-celled seedling trays, then transplanted into
609 Metromix in 4" pots, and finally into 3 gallon pots, with three bamboo poles attached at the top to
610 support the vines. Plants were grown under ambient light with summer high temperatures usually ca. 29
611 - 35 °C with ca. 75-98% humidity. The youngest leaves from each plant were collected from April-June

2016. The youngest leaves from a subset of these plants were re-sampled in March-April 2018. Leaves were collected immediately into desiccant and stored at room temperature. All plants that were sampled twice had already flowered before the first sampling in 2016.

4.1.2. Chemicals and PA Standards

PA standards (heliotrine, jacobine, retrorsine, and europine) were purchased from Phytolab GMBH (Vestenbergsgreuth, Germany). Lycopsamine and monocrotaline were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Standards were used as received and dissolved in HPLC-grade methanol prior to use. Acetonitrile and water for HPLC use were also HPLC-grade.

4.2. Extraction and isolation- Dried Leaves

The extraction method utilized in this study follows Tasca et al. (2018) to maximize PA recovery. Leaves were placed in 1.7 mL Eppendorf tubes and manually crushed with a microspatula for 15 seconds. Approximately 4-60 mg of dried leaf tissue was used for each extraction. Each specimen was then suspended in 750 µL of methanol, vortexed for 5 seconds, left to rest for 24 hours, and vortexed again for 5 seconds. The crude extracts were then removed and transferred to a second 1.7 mL Eppendorf tube, followed by 2 x 250 µL methanol washes. The methanol was then evaporated from the crude extracts *in vacuo* in a DR 120 SpeedVac with the drying rate on low. The crude extracts were reconstituted in 80 µL of methanol and vortexed for 30 seconds. The reconstituted samples were then transferred to 0.25 mL plastic autosampler vials with 1 mL Norm-Ject® disposable syringes fitted with Acrodisc CR 13 mm, 0.2 µm PTFE membrane filters.

4.2.1. Extraction and isolation- from seeds

To maintain congruency in sample preparation between leaves and seeds; seeds were also extracted according to methods outlined in Tasca et al. (2018) with a few modifications. Between 250-500 mg of seeds were ground with a mortar and pestle until a fine powder was achieved. Ground seeds were then transferred to a 10 ml conical tube and 3 ml of HPLC-grade methanol was added. Samples were then sonicated (not exceeding 60°C) for 12 hours. The sample was then vortexed for 30 seconds and subsequently centrifuged for 10 minutes. The supernatant was then removed and placed into a 1.7 mL Eppendorf tube to be evaporated *in vacuo* on a DR 120 SpeedVac with the drying rate on low. Subsequently, the pellet was washed with another 3 ml of HPLC-grade MeOH. The resuspended pellet was vortexed for 30 seconds, centrifuged for 10 minutes, with the supernatant again dried on the SpeedVac. The dried material in the Eppendorf tube was then resuspended in 250 µL of methanol and vortexed for 30 seconds. The reconstituted sample was then transferred to a 0.25 mL plastic autosampler vial with a 1 mL Norm-Ject® disposable syringe after being passed through an Acrodisc CR 13 mm, 0.2 µm PTFE membrane filter.

4.3. Liquid Chromatography Methods

All samples, regardless of the fragment ion being targeted in the PREC scan, were run on the same liquid chromatography method to allow for comparison of retention times across scans, although retention times were uncorrected. Crude extracts were separated via reverse-phase HPLC (Shimadzu, LC-20AB, Kyoto, Japan) with an ACE C18 column (3 µm particle, 150 x 4.6 mm) fitted with an ACE C18 guard column secured with a guard column holder (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland). A binary mobile phase consisting of acidified water (0.1 % formic acid, v/v; Solvent A) and acidified acetonitrile (0.1 % formic acid, v/v; Solvent B) was utilized at a flow rate of 0.5 mL/min. Chromatography lasted 22 minutes for each run with the following gradient: 10 % B (2 min hold) ramped

654 to a mobile phase concentration of 75 % B over 15 minutes, ramped to 95 % B (3 min hold), then 10 % B
655 for the remainder of the run. 10 µL of sample was injected for each run.

656 To ensure the reproducibility of the HPLC method, a column wash method was utilized every 10-
657 12 samples. This method consisted of a 20-minute hold of solvent B. The frequent use of column washes
658 successfully mitigated retention time shift as the leaf tissue matrix could alter the separation capacity of
659 the column after multiple runs. Prior to separation of the crude extracts, PA standards were analyzed via
660 PREC methods on the washed and equilibrated HPLC column to establish consistency of the
661 chromatography.

662 *4.4. Mass Spectrometry methods and PA positive identification*

663 All mass spectra were collected using an Applied Bio-Systems API 2000 triple quadrupole mass
664 spectrometer (Waltham, Massachusetts) in positive electrospray ionization (ESI+) mode. Preliminary
665 PREC 120 scans utilizing multiple channel acquisition (MCA) were utilized to tentatively identify PAs with
666 a m/z 120 fragment. MS compound dependent parameters were kept the same for PREC 120 scans with
667 and without MCA. Samples containing a molecular ion with a m/z 120 fragment were determined to be
668 positive (with low confidence) when chromatography (greater than 3:1 S/N) present between 9-15 mins
669 (40-75% ACN) in the total ion chromatogram (TIC; PREC 120 ion intensity vs. time) was associated with
670 an evenly-massed molecular ion of intensity (cps; counts per second) greater than or equal to $1x10^4$.
671 Select samples were then run on PREC 120/138/156 methods to elucidate additional fragments
672 associated with even-massed molecular ions appearing in the mass spectrum of 120 MCA scans and to
673 determine the possible presence of PAs in samples containing ambiguous chromatography associated
674 with odd and even molecular ions in their mass spectrum.

675 Samples run on the three precursor scans specific to the fragments of the retronecine core were
676 determined to be PA positive (with high confidence) if evenly massed molecular ions, greater than or
677 equal to $1x10^4$ cps, were observed in the TIC with greater than 3:1 S/N between 9- 15 minutes present
678 within approximately 0.3 mins chromatographically in either the PREC120 and 138 scans or PREC 120,
679 138, and 156 scans.

680 The PREC scan for the m/z 120 fragment was optimized using a monocrotaline commercial
681 standard with a declustering potential of 33 V and a collision energy of 45 V, producing a 120 *m/z*
682 fragment in Q3. Alternatively, to establish the optimal declustering potential and collision energy with
683 which to obtain the m/z 138 and 156 fragments, standards (monocrotaline, retrorsine, jacobine,
684 heliotrine, europine, and lyposamine) were subject to product scans. Compound dependent parameters
685 were manipulated to alter the intensity of the m/z 138 and 156 fragments. Ultimately, a declustering
686 potential of 40 V and collision energy of 40 V was utilized for the PREC138 scan. The PREC156 scan
687 alternatively employed a declustering potential and collision energy of 40 V and 50 V, respectively. All
688 other MS parameters were kept the same across each of the PRECs scans with a scan mass range of m/z
689 200-500. Both system control and data analysis were performed on Analyst 1.6.2 software.

690 **Declaration of competing interest**

691 The authors confirm that this article content has no conflict of interest.

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Appendix A. Supplementary data Table S1: Survey of Apocynaceae samples, voucher specimens and results of PREC 120 (MCA on) scans. Table S2: Survey of *Echites umbellatus* herbarium specimens, voucher specimens and results of PREC 120 (MCA on) scans. Table S3: *Echites umbellatus* half-sib families, voucher specimens and results of PREC 120 (MCA on) scans.

Figure Captions

Title: Necine bases. **Fig. 1.** Varied necine base structures found in pyrrolizidine alkaloids.

Title: PA esterification patterns. **Fig. 2.** Common esterification patterns between necic acids and necine bases observed in retronecine-type PAs.

Title: Lycopsamine-type PAs. **Fig. 3.** Subtypes of lycopsamine-type PAs containing a retronecine core.

Title: PA Fragmentation scheme. **Fig. 4.** Fragmentation patterns according to esterification pattern in retronecine-type PAs.

References

Abe, F., Nagao, T., Okabe, H., Yamauchi, T., 1991a. Studies on *Parsonsia* .4. Macrocyclic pyrrolizidine alkaloids from *Parsonsia laevigata*. Phytochemistry 30, 1737-1739.

Abe, F., Nagao, T., Okabe, H., Yamauchi, T., Marubayashi, N., Ueda, I., 1990. Studies on *Parsonsia* .3. Parsonsianine, a macrocyclic pyrrolizidine alkaloid from the leaves of *Parsonsia laevigata*. Chem Pharm Bull 38, 2127-2129.

Abe, F., Yamauchi, T., 1987. Parsonine, a pyrrolizidine alkaloid from *Parsonsia laevigata*. Chem Pharm Bull 35, 4661-4663.

Abe, F., Yamauchi, T., 1994. Cardenolide glycosides from the roots of *Apocynum cannabinum*. Chem Pharm Bull 42, 2028-2031. 10.1248/cpb.42.2028

721 Abe, F., Yamauchi, T., Yaga, S., Minato, K., 1991b. Studies on *Parsonsia* .5. Pyrrolizidine alkaloids from
722 *Parsonsia laevigata* in Okinawa Island. Chem Pharm Bull 39, 1576-1577.

723 Afendi, F. M., Okada, T., Yamazaki, M., Hirai-Morita, A., Nakamura, Y., Nakamura, K., Ikeda, S.,
724 Takahashi, H., Altaf-Ul-Amin, M., Darusman, L. K., Saito, K., Kanaya, S., 2011. KNApSAcK Family
725 Databases: Integrated Metabolite–Plant Species Databases for Multifaceted Plant Research. Plant Cell
726 Physiol 53, e1-e1. 10.1093/pcp/pcr165

727 Agrawal, A. A., Petschenka, G., Bingham, R. A., Weber, M. G., Rasmann, S., 2012. Toxic cardenolides:
728 chemical ecology and coevolution of specialized plant–herbivore interactions. New Phytol 194, 28-45.
729 10.1111/j.1469-8137.2011.04049.x

730 Arseculeratne, S. N., Gunatilaka, A. A. L., Panabokke, R. G., 1981. Studies on medicinal plants of Sri
731 Lanka: Occurrence of pyrrolizidine alkaloids and hepatotoxic properties in some traditional medicinal
732 herbs. J Ethnopharmacol 4, 159-177. http://dx.doi.org/10.1016/0378-8741(81)90033-7

733 Aslam, J., Khan, S., Siddiqui, Z., Zohra, F., Mehpara, M., Bhat, M., Nasim, S., Ilah, A., Ahmad, I., Khan, S.,
734 Mujib, A., Sharma, M., 2010. *Catharanthus roseus* (L.) G. Don. An important drug: Its applications and
735 production. Pharmacie Globale : International Journal of Comprehensive Pharmacy 4.

736 Avula, B., Sagi, S., Wang, Y.-H., Zweigenbaum, J., Wang, M., Khan, I. A., 2015. Characterization and
737 screening of pyrrolizidine alkaloids and N-oxides from botanicals and dietary supplements using UHPLC-
738 high resolution mass spectrometry. Food Chem 178, 136-148.
739 https://doi.org/10.1016/j.foodchem.2015.01.053

740 Avula, B., Sagi, S., Wang, Y., Wang, M., Khan, I. A., 2015. Pyrrolizidine alkaloids: characterization in
741 botanical and dietary supplements using accurate-mass Q-TOF LC/MS and all ions MS/MS. publication
742 number 5991-6254EN., Application Note. Aligent Technologies, Inc. .

743 Brown, K. S., Jr., 1987. Chemistry at the Solanaceae/Ithomiinae Interface. Ann Mo Bot Gard 74, 359-397.
744 10.2307/2399406

745 Bullock, A. A., 1956. Notes on African Asclepiadaceae: VIII. Kew Bulletin 11, 503-522. 10.2307/4109137

746 Burzynski, E. A., Minbiole, K. P. C., Livshultz, T., 2015. New sources of lycopsamine-type pyrrolizidine
747 alkaloids and their distribution in Apocynaceae. Biochem Syst Ecol 59, 331-339.
748 http://dx.doi.org/10.1016/j.bse.2015.02.006

749 Caputi, L., Franke, J., Farrow, S. C., Chung, K., Payne, R. M. E., Nguyen, T.-D., Dang, T.-T. T., Soares Teto
750 Carqueijeiro, I., Koudounas, K., Dugé de Bernonville, T., Ameyaw, B., Jones, D. M., Vieira, I. J. C.,
751 Courdavault, V., O'Connor, S. E., 2018. Missing enzymes in the biosynthesis of the anticancer drug
752 vinblastine in Madagascar periwinkle. Science 360, 1235-1239. 10.1126/science.aat4100

753 Chowdhury, A. K. A., Hashim, M. F., Sen, B. C., Khan, F., Ahmed, M., 1994. Antifertility principles from
754 *Marsdenia tinctoria* - pharmacological and photochemical studies. Pure Appl Chem 66, 2343-2346. DOI
755 10.1351/pac199466102343

Colegate, S. M., Edgar, J. A., Knill, A. M., Lee, S. T., 2005. Solid-phase extraction and HPLC-MS profiling of pyrrolizidine alkaloids and their N-oxides: a case study of *Echium plantagineum*. Phytochem Analysis 16, 108-119. Doi 10.1002/Pca.828

Colegate, S. M., Gardner, D. R., Betz, J. M., Fischer, O. W., Liede-Schumann, S., Boppré, M., 2016. Pro-toxic 1, 2-dehydropyrrolizidine alkaloid esters, including unprecedented 10-membered macrocyclic diesters, in the medicinally-used *Alafia* cf. *caudata* and *Amphineurion marginatum* (Apocynaceae: Apocynoideae: Nerieae and Apocyneae). Phytochem Analysis 27, 257-276.

De Waal, H., 1941. South African *Senecio* alkaloids. Part 5. Notes on Isatidine, Rosmarinine and Pterophine, and on the structure of their necines and necic acids. Onderstepoort Journal of Veterinary Science and Animal Industry 16, 149-166.

del Castillo, B., Aguirre, A. G. E. d., Bretón, J. L., González, A. G., Trujillo, J., 1970. Loroquin, a new necine isolated from *Urechites karwinsky* Mueller (1-hydroxy-methylene-7-keto-dihydropyrrolizine). Tetrahedron Lett 11, 1219-1220. http://dx.doi.org/10.1016/S0040-4039(01)91592-8

Dusemund, B., Nowak, N., Sommerfeld, C., Lindtner, O., Schäfer, B., Lampen, A., 2018. Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. Food Chem Toxicol 115, 63-72. https://doi.org/10.1016/j.fct.2018.03.005

Edgar, J. A., Eggers, N. J., Jones, A. J., Russell, G. B., 1980. Unusual macrocyclic pyrrolizidine alkaloids from *Parsonsia heterophylla* A. Cunn. and *Parsonsia spiralis* Wall. (Apocynaceae). Tetrahedron Lett 21, 2657-2660.

Eggers, N. J., Gainsford, G. J., 1979. Parsonsine (C22H33NOs): A pyrrolizidine alkaloid from *Parsonsia heterophylla* A. Cunn. Cryst. Struct. Commun 8, 111.

Endress, M. E., Hesse, M., Nilsson, S., Guggisberg, A., Zhu, J. P., 1990. The systematic position of the Holarrheninae (Apocynaceae). Plant Syst Evol 171, 157-185.

Endress, M. E., Meve, U., Middleton, D. J., Liede-Schumann, S., 2018. Apocynaceae. In: Kadereit J., B. V. (Ed.), Flowering Plants. Eudicots. The Families and Genera of Vascular Plants, , vol. 15. Springer, Cham, pp. 207-411.

Espírito Santo, F. d. S. d., Rapini, A., Ribeiro, P. L., Liede-Schumann, S., Goyder, D. J., Fontella-Pereira, J., 2019. Phylogeny of the tribe Marsdenieae (Apocynaceae), reinstatement of *Ruehssia* and the taxonomic treatment of the genus in Brazil. Kew Bulletin 74, 30. 10.1007/s12225-019-9807-4

Fairbrothers, D. E., Mabry, T. J., Scogin, R. L., Turner, B. L., 1975. The bases of angiosperm phylogeny: chemotaxonomy. Ann Mo Bot Gard 62, 765-800. 10.2307/2395273

Fishbein, M., Livshultz, T., Straub, S. C. K., Simões, A. O., Boutte, J., McDonnell, A., Foote, A., 2018. Evolution on the backbone: Apocynaceae phylogenomics and new perspectives on growth forms, flowers, and fruits. Am J Bot 105, 1-19. doi:10.1002/ajb2.1067

Flade, J., Beschow, H., Wensch-Dorendorf, M., Plescher, A., Wätjen, W., 2019. Occurrence of nine pyrrolizidine alkaloids in *Senecio vulgaris* L. depending on developmental stage and season. Plants 8. 10.3390/plants8030054

Forster, P. I., 1995. Circumscription of *Marsdenia* (Asclepiadaceae, Marsdenieae), with a revision of the genus in Australia and Papuasia. Aust Syst Bot 8, 703-933. Doi 10.1071/Sb9950703

Futuyma, D. J., Agrawal, A. A., 2009. Macroevolution and the biological diversity of plants and herbivores. Proc Natl Acad Sci U S A 106, 18054-18061. 10.1073/pnas.0904106106

Gao, Z. L., He, H. P., Di, Y. T., Fang, X., Li, C. S., Zhang, Q., Zhao, P. J., Li, S. L., Hao, X. J., 2009. Three new pregnane glycosides from *Marsdenia tinctoria*. Helv Chim Acta 92, 1775-1781.

Hartmann, T., 1996. Diversity and variability of plant secondary metabolism: A mechanistic view. Entomol Exp Appl 80, 177-188.

Hartmann, T., Witte, L., 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: Pelletier, S. W. (Ed.), Alkaloids: Chemical and Biological Perspectives, vol. 9. Pergamon Press, Oxford, England, Great Britian, pp. 155-233.

Hartmann, T., Zimmer, M., 1986. Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life-history of two annual *Senecio* species. J Plant Physiol 122, 67-80.

Hernández-Baz, F., Coates, R., Teston, J. A., González, J. M., 2013. *Scena propylea* (Druce) (Lepidoptera: Erebidae) an endemic species of Mexico. Neotrop Entomol 42, 246-251. 10.1007/s13744-013-0119-3

Hungerford, N. L., Carter, S. J., Anuj, S. R., Tan, B. L. L., Hnatko, D., Martin, C. L., Sharma, E., Yin, M., Nguyen, T. T. P., Melksham, K. J., Fletcher, M. T., 2019. Analysis of Pyrrolizidine Alkaloids in Queensland Honey: Using Low Temperature Chromatography to Resolve Stereoisomers and Identify Botanical Sources by UHPLC-MS/MS. Toxins (Basel) 11. 10.3390/toxins11120726

Irmer, S., Podzun, N., Langel, D., Heidemann, F., Kaltenegger, E., Schemmerling, B., Geilfus, C.-M., Zörb, C., Ober, D., 2015. New aspect of plant–rhizobia interaction: Alkaloid biosynthesis in *Crotalaria* depends on nodulation. Proceedings of the National Academy of Sciences 112, 4164-4169.

Janot, M., Khuong-Huu, Q., Monneret, C., Kabore, I., Hildesheim, J., Gero, S., Goutarel, R., 1970. Alcaloïdes stéroidïques—C: Les holantosines A et B, nouveaux amino-glyco-stéroïdes isolés des feuilles de l'*Holarrhena antidysenterica* (Roxb.) Wall.(Apocynacées). Tetrahedron 26, 1695-1709.

Jeong, W. T., Lim, H. B., 2019. Determination and Chemical Profiling of Toxic Pyrrolizidine Alkaloids in Botanical Samples with UPLC–Q-TOFMS. Chromatographia 82, 1653-1664. 10.1007/s10337-019-03785-y

Kaltenegger, E., Eich, E., Ober, D., 2013. Evolution of homospermidine synthase in the Convolvulaceae: A story of gene duplication, gene loss, and periods of various selection pressures. Plant Cell 25, 1213-1227. DOI 10.1105/tpc.113.109744

Kaltner, F., Kukula, V., Gottschalk, C., 2020. Screening of food supplements for toxic pyrrolizidine alkaloids. Journal of Consumer Protection and Food Safety. 10.1007/s00003-020-01296-9

Kawamoto, S., Koyano, T., Kowithayakorn, T., Fujimoto, H., Okuyama, E., Hayashi, M., Komiyama, K., Ishibashi, M., 2003. Wrightiamines A and B, two new cytotoxic pregnane alkaloids from *Wrightia javanica*. Chem Pharm Bull 51, 737-739.

828 Khan, P. S. H., 1987. Comparative seed structure of medicinally important *Holarrhena antidysenterica*
829 (Roth.) A.DC. and its adulterant, *Wrightia tinctoria* R. Br. (Apocynaceae). International Journal of Crude
830 Drug Research 25, 81-86. 10.3109/13880208709088131

831 Khyade, M., 2014. *Wrightia tinctoria* R. Br.-a review on its ethnobotany, pharmacognosy and
832 pharmacological profile. Journal of Coastal Life Medicine. 10.12980/jclm.2.2014c1221

833 Kumar, N., Singh, B., Bhandari, P., Gupta, A. P., Kaul, V. K., 2007. Steroidal Alkaloids from *Holarrhena*
834 *antidysenteric*a (L.) Wall. Chemical and Pharmaceutical Bulletin 55, 912-914. 10.1248/cpb.55.912

835 Lee, D. U., Kang, S. I., Yoon, S. H., Budesinsky, M., Kasal, A., Mayer, K. K., Wiegrebe, W., 2000. A new
836 steroidal alkaloid from the roots of *Cynanchum caudatum*. Planta Med 66, 480-482. 10.1055/s-2000-
837 8578

838 Leeuwenberg, A. J. M., 1997. Series of revisions of Apocynaceae XLIII. *Alafia* Thouars. Kew Bulletin 52,
839 769-839.

840 Liede-Schumann, S., Khanum, R., Mumtaz, A. S., Gherghel, I., Pahlevani, A., 2016. Going west – A
841 subtropical lineage (*Vincetoxicum*, Apocynaceae: Asclepiadoideae) expanding into Europe. Mol
842 Phylogenet Evol 94, 436-446. https://doi.org/10.1016/j.ympev.2015.09.021

843 Liede-Schumann, S., Nikolaus, M., Silva, U. C. S. e., Rapini, A., Mangelsdorff, R. D., Meve, U., 2014.
844 Phylogenetics and biogeography of the genus *Metastelma* (Apocynaceae-Asclepiadoideae-Asclepiadeae:
845 Metastelmatinae). Syst Bot 39, 594-612. 10.1600/036364414X680708

846 Liede-Schumann, S., Rapini, A., Goyder, D. J., Chase, M. W., 2005. Phylogenetics of the new world
847 subtribes of Asclepiadeae (Apocynaceae-Asclepiadoideae): Metastelmatinae, Oxypetalinae, and
848 Gonolobinae. Syst Bot 30, 184-195.

849 Liede, S., 1996. *Cynanchum*, *Rhodostegiella*, *Vincetoxicum*, *Tylophora* (Asclepiadaceae): New
850 considerations on an old problem. Taxon 45, 193-211.

851 Lin, G., Zhou, K. Y., Zhao, X. G., Wang, Z. T., But, P. P. H., 1998. Determination of hepatotoxic
852 pyrrolizidine alkaloids by on-line high performance liquid chromatography mass spectrometry with an
853 electrospray interface. Rapid Commun Mass Sp 12, 1445-1456. Doi 10.1002/(Sici)1097-
854 0231(19981030)12:20<1445::Aid-Rcm356>3.0.Co;2-G

855 Liu, H. M., Kiuchi, F., Tsuda, Y., 1992. Isolation and structure elucidation of gymnemic acids, antisweet
856 principles of *Gymnema sylvestre*. Chem Pharm Bull 40, 1366-1375.

857 Livshultz, T., Kaltenegger, E., Straub, S. C. K., Weitemier, K., Hirsch, E., Koval, K., Mema, L., Liston, A.,
858 2018a. Evolution of pyrrolizidine alkaloid biosynthesis in Apocynaceae: revisiting the defence de-
859 escalation hypothesis. New Phytol 218, 762-773. 10.1111/nph.15061

860 Livshultz, T., Middleton, D. J., Endress, M. E., Williams, J. K., 2007. Phylogeny of Apocynoideae and the
861 APSA clade (Apocynaceae s.l.). Ann Mo Bot Gard 94, 324-359.

862 Livshultz, T., Middleton, D. J., van der Ham, R. W. J. M., Khew, G., 2018b. Generic delimitation in
863 Apocyneae (Apocynaceae). Taxon 67, 341-358. 10.12705/672.5

Lucena, R. B., Rissi, D. R., Maia, L. A., Flores, M. M., Dantas, A. F. M., Nobre, V. M. D., Riet-Correa, F., Barros, C. S. L., 2010. Poisoning by pyrrolizidine alkaloids in ruminants and horses in Brazil. Pesquisa Vet Brasil 30, 447-452. Doi 10.1590/S0100-736x2010000500013

Mattocks, A. R., 1967. Spectrometric determination of unsaturated pyrrolizidine alkaloids. Anal Chem 39, 443-447.

Moore, B. D., Andrew, R. L., Külheim, C., Foley, W. J., 2014. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytol 201, 733-750. 10.1111/nph.12526

Morales, J., Endress, M. E., Liede-Schumann, S., 2017. Sex, drugs and pupusas: Disentangling relationships in Echiteae (Apocynaceae). Taxon 66, 623-644.

Morton, J. F., Alvarez, E., Quinonez, C., 1990. *Loroco*, *Fernaldia pandurata* (Apocynaceae) - a popular edible flower of Central America. Economic Botany 44, 301-310.

Ngan, P. T., 1965. A revision of the genus *Wrightia* (Apocynaceae). Ann Mo Bot Gard 52, 114-175.

Nishida, R., Kim, C. S., Fukami, H., Irie, R., 1991. Ideamine n-oxides - pyrrolizidine alkaloids sequestered by the danaine butterfly, *Idea leuconoe*. Agr Biol Chem Tokyo 55, 1787-1792.

Paulo, A., Houghton, P. J., 2003. Chemotaxonomic analysis of the genus *Cryptolepis*. Biochem Syst Ecol 31, 155-166.

Pedersen, E., Larsen, E., 1970. Mass spectrometry of some pyrrolizidine alkaloids. Org Mass Spectrom 4, 249-256. 10.1002/oms.1210040129

Qu, Y., Safonova, O., De Luca, V., 2019. Completion of the canonical pathway for assembly of anticancer drugs vincristine/vinblastine in *Catharanthus roseus*. The Plant Journal 97, 257-266. 10.1111/tpj.14111

Reddy, Y. S. R., Venkatesh, S., Ravichandran, T., Subburaju, T., Suresh, B., 1999. Pharmacognostical studies on *Wrightia tinctoria* bark. Pharm Biol 37, 291-295.

Rodda, M., Simonsson, N., Ercole, E., Khew, G., Niissalo, M., Rahayu, S., Livshultz, T., 2020. Phylogenetic studies in the *Hoya* group (Apocynaceae, Marsdenieae): the position of *Anatropanthus* and *Oreosparte*. Willdenowia 50, 119-138. 10.3372/wi.50.50112

Ruan, J. Q., Li, N., Xia, Q. S., Fu, P. P., Peng, S. Y., Ye, Y., Lin, G., 2012. Characteristic ion clusters as determinants for the identification of pyrrolizidine alkaloid N-oxides in pyrrolizidine alkaloid-containing natural products using HPLC-MS analysis. J Mass Spectrom 47, 331-337. Doi 10.1002/Jms.2969

Sasaki, K., Hirata, Y., 1970. The alkaloids of *Anodendron affine* Druce. Tetrahedron 26, 2119-2126. http://dx.doi.org/10.1016/S0040-4020(01)92789-9

Schramm, S., Köhler, N., Rozhon, W., 2019. Pyrrolizidine Alkaloids: Biosynthesis, Biological Activities and Occurrence in Crop Plants. Molecules 24. 10.3390/molecules24030498

Scossa, F., Fernie, A. R., 2020. The evolution of metabolism: How to test evolutionary hypotheses at the genomic level. Computational and Structural Biotechnology Journal. https://doi.org/10.1016/j.csbj.2020.02.009

Silva, U. C. S. e., Rapini, A., Liede-Schumann, S., Ribeiro, P., cia, L., van den Berg, C., ssio, 2012. Taxonomic Considerations on Metastelmatinae (Apocynaceae) Based on Plastid and Nuclear DNA. Syst Bot 37, 795-806.

Simões, A. O., Endress, M. E., Van der Niet, T., Kinoshita, L. S., Conti, E., 2004. Tribal and intergeneric relationships of Mesechiteae (Apocynoideae, Apocynaceae): Evidence from three noncoding plastid DNA regions and morphology. Am J Bot 91, 1409-1418.

Sinha, S., Sharma, A., Reddy, P. H., Rathi, B., Prasad, N. V. S. R. K., Vashishtha, A., 2013. Evaluation of phytochemical and pharmacological aspects of *Holarrhena antidysenterica* (Wall.): A comprehensive review. Journal of Pharmacy Research 6, 488-492. http://dx.doi.org/10.1016/j.jopr.2013.04.004

Sixto, A., Pérez-Parada, A., Niell, S., Heinzen, H., 2019. GC MS and LC MS/MS workflows for the identification and quantitation of pyrrolizidine alkaloids in plant extracts, a case study: *Echium plantagineum*. Revista Brasileira de Farmacognosia 29, 500-503.

Staerk, D., Nezhad, K. B., Asili, J., Emami, S. A., Ahi, A., Sairafianpour, M., Jaroszewski, J. W., 2005. Phenanthroindolizidine alkaloids from *Vincetoxicum pumilum*. Biochem Syst Ecol 33, 957-960.

Stegemann, T., Kruse, L. H., Brütt, M., Ober, D., 2018. Specific distribution of pyrrolizidine alkaloids in floral parts of comfrey (*Symphytum officinale*) and its Implications for flower ecology. J Chem Ecol. 10.1007/s10886-018-0990-9

Stelljes, M. E., Kelley, R. B., Molyneux, R. J., Seiber, J. N., 1991. GC-MS determination of pyrrolizidine alkaloids in four *Senecio* species. J Nat Prod 54, 759-773. 10.1021/np50075a004

Tackie, A. N., Sharaf, M. H. M., Schiff, P. L., Boye, G. L., Crouch, R. C., Martin, G. E., 1991. Assignment of the proton and carbon NMR-spectra of the indoloquinoline alkaloid cryptolepine. J Heterocyclic Chem 28, 1429-1435.

Tamariz, J., Burgueño-Tapia, E., Vázquez, M. A., Delgado, F., 2018. Pyrrolizidine Alkaloids. Alkaloids Chem Biol 80, 1-314. 10.1016/bs.alkal.2018.03.001

Tasca, J. A., Smith, C. R., Burzynski, E. A., Sundberg, B. N., Lagalante, A. F., Livshultz, T., Minbiole, K. P. C., 2018. HPLC-MS detection of pyrrolizidine alkaloids and their N-oxides in herbarium specimens dating back to the 1850s. Applications in Plant Sciences 6, e1143. doi:10.1002/aps3.1143

These, A., Bodi, D., Ronczka, S., Lahrssen-Wiederholt, M., Preiss-Weigert, A., 2013. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Anal Bioanal Chem 405, 9375-9383. 10.1007/s00216-013-7365-4

Thoden, T. C., Boppré, M., 2010. Plants producing pyrrolizidine alkaloids: sustainable tools for nematode management? Nematology 12, 1-24. Doi 10.1163/138855409x12549869072248

932 Trigo, J. R., 2011. Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10,
933 83-98. DOI 10.1007/s11101-010-9191-z

934 Wickramaratne, M. N., Gunatilake, L., Anuradha, N. D., Godavillathanna, A., Perera, M., Nicholas, I.,
935 2015. Antioxidant activity and antibacterial activity of *Walidda antidysenterica*. Journal of
936 Pharmacognosy and Phytochemistry 4, 121-126.

937 Wieringa, J. J., 2011. Novitates Gabonenses 70. The advantages of a specimen database: *Alafia velutina*
938 is a synonym of *Farquharia elliptica* (Apocynaceae). Blumea - Biodiversity, Evolution and Biogeography
939 of Plants 56, 240-240.

940 Yan-Jiao, D., 2013. Screening of antibacterial activity of 20 Chinese herbal medicines in Yunnan. African
941 Journal of Pharmacy and Pharmacology 7, 2859-2865. 10.5897/ajpp2012.1513

942

943