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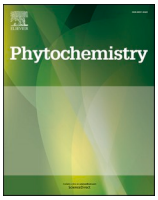

Seed tannin composition of tropical plants

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ABSTRACT

Seeds collected from trees, shrubs and lianas growing on Barro Colorado Island, Panama, were analyzed for their content of phenolic compounds, oxidative activities and protein precipitation capacities. Proanthocyanidins and hydrolysable tannins were detected in one-third of 189 studied species. The most oxidatively active group of species were the ones containing prodelphinidins and ellagitannins whereas the species that had the highest protein precipitation capacity in relation to their total phenolics were the ones containing punicalagin. In addition, the oxidative activity and relative protein precipitation capacity were exceptionally high in the proanthocyanidin-rich genus *Psychotria*. This study offers a comprehensive overview on the tannin composition and the alkaline oxidative activities and protein precipitation capacities of the seeds of tropical plants.

1. Introduction

Tropical plants have been studied extensively for their various classes of phytochemicals, such as steroids, terpenoids, alkaloids, and volatile compounds (for example, Chaverri and Cicció, 2010; Lizcano et al., 2010; Marini et al., 2018; Scalvenzi et al., 2017; Travasarou et al., 2019). This also includes polyphenols, one of the most common and ubiquitous class of specialized metabolites in plants with an important role in plant defenses against e.g. herbivores and pathogens. Most of the accumulated studies on the phenolic compounds of tropical plants utilize "total" methods, such as total tannins and total flavonoids, and only a few studies (e.g. Guldbrandsen et al., 2015; Pereira et al., 2020) focus on their detailed polyphenol composition.

Hydrolysable tannins (HTs) consist of gallic acid (GA) or its derivatives centered around a polyol core and are synthesized via the shikimate pathway in plant cells (Ossipov et al., 2003; Salminen and Karonen, 2011). HTs can be divided into three subgroups: simple GA derivatives, including galloyl glucoses (GGs) and galloyl quinic acids (GQAs); gallotannins (GTs), containing at least one digalloyl moiety where two galloyls are linked with a depside bond; and ellagitannins (ETs), containing at least one hexahydroxydiphenoyl (HHDP) group or its derivative, such as a dehydro-HHDP group or chebuloyl group (Moilanen et al., 2016). Simple GA derivatives and GGs are formed from GA and a polyol, whereas GTs and ETs are produced from pentagalloyl glucose, unless the polyol is not a glucose (Salminen and Karonen, 2011).

Proanthocyanidins (PAs) are a large and diverse class of polyphenols, typically consisting of at least two flavan-3-ol monomer units produced via the acetate/malonate and shikimate pathways in plant cells. PAs are divided into subgroups based on their monomer units. For instance, compounds consisting of (epi)catechin subunits are called procyanidins (PCs), whereas compounds that contain (epi)gallocatechin subunits are called prodelphinidins (PDs). In this paper, (epi)catechins and (epi)gallocatechins in PAs are referred to as PC subunits and PD subunits, respectively. PCs and PDs are the most common PA types found in the plant kingdom. Other, less common main classes of PAs are propelargonidins, profisetinidins, proguibourtinidins and prorobinetinidins (Hemingway, 1989a; Porter, 1989). Even rarer minor classes are prodistenidins, consisting of flavan-3-ol subunits as do all the other classes mentioned so far, as well as proapigeninidins, probutinidins, procassinidins and proluteolinidins that consist of flavan subunits instead of flavan-3-ols (Coetzee et al, 1999, 2000; Gurni and Wagner, 1984; Hori et al., 1988; Krueger et al., 2003; Ricco et al., 2004). PA classes other than PCs can include compounds with a heterogenous composition, as they can contain one or more PC subunits in addition to their characteristic subunits (Hemingway, 1989b; Porter, 1988). PA oligomers can form via C4→C8 or C4→C6 linkages (B-type proanthocyanidins), or with an additional C2→O→C7 or C2→O→C5 linkages (A-type proanthocyanidins). The two most common subunits, (+)-catechin and (−)-epicatechin, have 2*R*,3*S* and 2*R*,3*R* stereochemistry, respectively. As an

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example, the dimeric procyanidin B1 is a (−)-epicatechin-(4β→8)-(+)-catechin. The structural diversity can be further complicated by possible galloylation at 3-OH. If the number of subunits increases, the number of possible configurations expands rapidly (Dixon et al., 2005; Salminen and Karonen, 2011). PAs are usually detected as an unresolved hump in reversed-phase high-performance liquid chromatography (HPLC) due to many plant species producing a large variety of compounds with partially overlapping retention times (Leppä et al., 2018).

Two major modes of action against herbivores have been proposed for phenolics. Phenolic compounds can be oxidized at the alkaline pH of the midgut of many insect herbivores, forming highly reactive quinones. These oxidation products can damage the gut epithelial cells of the herbivore, causing oxidative stress (Appel, 1993; Thiboldeaux et al., 1998). In mammalian herbivores, the phenolics can bind to plant proteins with non-covalent interactions at neutral or slightly acidic pH, which lowers the absorption of the proteins during digestion (Hagerman, 1992).

Many species in the woody plant community of Barro Colorado Island, Panama (BCI) experience high levels of mortality at the seed and early seedling stages, and spatial patterns of mortality suggest that host-specific natural enemies such as insect seed predators and plant pathogens are responsible for at least some of this mortality (Harms et al., 2000; Marchand et al., 2019). In our previous work (Gripenberg et al., 2019) we have shown that approximately two thirds of the woody plant species on BCI are attacked by internally feeding insect seed predators. Others have shown that pathogenic fungi can be an important source of mortality of small-seeded pioneer species in the seed bank (e.g. Dalling et al., 1998; Sarmiento et al., 2017). These findings suggest that natural enemies have strong potential to contribute to the evolution of seed chemistry.

We have tested several hypotheses predicting the investment vs. hypothetical need for the chemical defenses of woody plant species growing on BCI by comparing the phenolic content of their seeds to e.g. the apparency, relative growth rate and life history of maternal plants (Gripenberg et al., 2018). In this follow-up study, which builds on the same seed material, we wanted to gain deeper insight into the relationship between the detailed phenolic composition and *in vitro* activities of the raw seed extracts at the species level using the analytical data collected in our previous study. Flavonoid glycosides and cinnamic acid derivatives (including caffeoylquinic acids) were only detected in a few species (4% of all analyzed species) and in low concentrations, so their role in seed chemistry is likely less significant than that of tannins. Consequently, in this study we only focused on the chemical composition and activities of the species that were rich in HTs and PAs. In accordance with our previous research and existing knowledge on activities of phenolic compounds, we hypothesized that high oxidative activities would be observed in species containing ETs and PDs (Kim et al, 2018, 2020; Vihakas et al., 2014), while high protein precipitation capacities (PPC) would be observed on species containing PDs (Marsh et al., 2020). The contribution of PCs to either type of activity was not expected to be very high.

The phenolic fingerprints and compositions of the seeds were determined using ultrahigh-performance liquid chromatography coupled with a diode array detector and triple quadrupole mass spectrometer (UHPLC-DAD-QqQ-MS). Various phenolic subgroups (e.g. HTs, PAs and flavonol glycosides) were quantified using mass spectrometric multiple reaction monitoring (MRM) and full scan methods (Engström et al, 2014, 2015). Total phenolics were estimated with the Folin-Ciocalteu (FC) redox assay, and oxidative activity was estimated with the Salminen and Karonen (2011). PPC of the samples was determined using radial diffusion assay with bovine serum albumin (BSA) as the model protein. This study offers a comprehensive overview on the tannin composition and activities of the seeds of selected tropical tree, shrub and liana species. To date, only few of these species have been studied in detail for their phenolic constituents (Table S1).

2. Results and discussion

Our sample set consisted of 634 samples, covering 195 species, 149 genera, 57 families and 27 orders (Table S1; one sample was identified only on genus level). 62 seed samples were too hard to grind and were not analyzed, so our final number of samples was 572 (189 species). 178 samples (31%) resulted in greasy granules or thick oily liquid instead of fine dry powder after grinding. Conclusive differences in phenolic content between dry and oily samples could not be determined due to the small number of species having both dry and oily samples for comparison, so the results of the chemical analyses of oily samples have been included as they are, even though their true phenolic content per dry weight of plant material could be higher than now reported.

For quantitation, we used the following limits to roughly categorize the samples before further inspections, as described in more detail under *Tannin-rich samples* in the Experimental section: If the FC assay result was at least 20 mg/g, the sample had a high content of phenolics, and low otherwise. If the mass spectrometric MRM quantitation of HTs (GA and HHDP moieties) and PAs (PC and PD subunits) was at least 10 mg/g in total, the sample had a high concentration of tannins, and low otherwise.

The content of phenolic compounds was very low in the majority of the analyzed species (118 species out of 189, Table S1). Of those that did contain phenolics, the vast majority of species (63 out of 71) contained hydrolysable tannins or proanthocyanidins or both in high quantities (at least 10 mg/g; Table 1, Figures S1 and S2). Most of the remaining 8 species contained cinnamic acid derivatives in similarly high quantities. Caffeic acid derivatives were detected in 21 species altogether. Flavonoids and coumaric acid derivatives were the least common classes of phenolics, being detected in only 8 and 5 species in total, respectively. The results of the chemical analyses have been summarized at species level in Table 1 and at family level in Figure S1. Chromatographic, mass spectrometric and oxidative activity results as well as protein precipitation capacity of an exemplar sample of each tannin-rich species are presented in Figures S2.1–63, and compound identification data is presented in Table S2. Several interesting observations regarding HTs and PAs are highlighted below.

2.1. Hydrolysable tannins

HTs were detected in 30 out of 63 tannin-rich species (Table 1; 17 out of 28 tannin-rich families, Figure S1). Of these species, 14 were HT-rich, i.e. they had at least one sample with the concentration of HTs being 10 mg/g or more based on group-specific MRM quantitation. The species containing HTs can be roughly divided into four groups based on their main compound or type of the main compound: the species primarily contained either simple gallic acid derivatives, punicalagin, geraniin, or simple HHDP esters. ETs were the main compound class in species that had the highest concentration of total phenolics (around 120 mg/g or more based on the FC assay). The order Myrtales was particularly rich in ETs (28 mg/g of HHDPs based on MRM).

The species *Doliocarpus multiflorus*, *D. olivaceus*, *Anacardium excelsum*, *Talisia nervosa* and *Canavalia campylocarpa* contained various simple gallic acid derivatives, e.g. galloyl glucoses or galloylquinic acids or both, in different quantities (Fig. 1). Interestingly, *D. multiflorus* contained high amounts of mono- and trigalloyl glucose, but negligible amounts of the intermediate digalloyl glucose (Fig. 1a). Based on their UV spectrum (λ_{max} 220, 275sh nm), the digalloylquinic acid of *Talisia nervosa*, the digalloyl glucose of *Mabea occidentalis* and trigalloyl glucose of *C. campylocarpa* were of the gallotannin-type, i.e. they contained a digalloyl group (Fig. 1c, S2.31, S2.62 and S2.36). *Tetrapterys macrocarpa* contained two unidentified gallic acid derivatives with the MWs of 648 and 800 Da, with one compound containing one galloyl moiety less than the other (Figure S2.56). Monogalloyl glucose was detected in several species also containing galloylated PAs, e.g. *Coccoloba acuminata* and *Triplaris cumingiana* (Figures S2.15 and S2.16).

Table 1
The list of 63 tannin-rich species in this study, together with the number of analyzed samples and detected phenolics. The species have been arranged according to The Angiosperm Phylogeny Group (2016). The plant nomenclature follows Condit et al. (2017) if the species appears in the 50-ha ForestGEO BCI forest plot (Smithsonian Tropical Research Institute, 2020), and http://www.tropicos.org (accessed Sep 3, 2019) otherwise. Some of the samples resulted in thick oily liquid or greasy granules instead of fine dry powder after grinding. The number of these oily samples from each species is indicated in the table. **PT (mg/g):** Quantitation of selected polyphenols by mass spectrometric MRM methods, in mg/g dry mass. The sum of HT and PA (see following) as well as flavonol glycosides (kaempferols, quercetins and myricetins) and quinic acid derivatives. **HT (%):** Quantitation of galloyl and HHDP moieties, characteristic to hydrolysable tannins. Reported as the portion of PT. **PA (%):** Quantitation of terminal- and extension-type catechin and gallocatechin units, characteristic to procyanidins and prodelphinidins. Reported as the portion of PT. **FC (mg/g):** Quantitation of total phenolics in mg/g dry mass, measured by Folin-Ciocalteu assay. Reported in gallic acid equivalents. **pH 10 (%):** The portion of total phenolics that were oxidized at pH 10 during the 30-min incubation period, i.e. the portion of easily oxidized phenolics. **PPC (%):** The protein precipitation capacity, measured by radial diffusion assay in pentagalloyl glucose equivalents. Reported in relation to the total phenolics. Compound abbreviations: CA: cinnamic acid derivative, CAF: caffeic acid derivative, CoQA: coumaroylquinic acid, CQA: caffeoylquinic acid, ET: ellagitannin, FL: flavonoid, G: galloyl group, GA: gallic acid derivative, GG: galloyl glucose, GQA: galloylquinic acid, GT: gallotannin, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, QA: quinic acid.

Clade Order	Family	Species (samples, of which oily)	Main phenolics	PT (mg/g)	HT (%)	PA (%)	FC (mg/g)	pH 10 (%)	PPC (%)
Magnoliids									
Laurales	Lauraceae	*Beilschmiedia tovarensis* (3, 0)	PC, CQA	22 ± 11	0 ± 0	95 ± 3	30 ± 11	0 ± 0	19 ± 15
Monocots									
Arecales	Arecaceae	*Astrocaryum standleyanum* (3, 3)	PC, CAF	7 ± 5	0 ± 0	100 ± 0	16 ± 10	10 ± 9	15 ± 17
		Oenocarpus mapora (6, 0)	PC	13 ± 6	0 ± 0	100 ± 0	25 ± 3	0 ± 0	12 ± 9
Core eudicots									
Dilleniales	Dilleniaceae	*Davilla nitida* (5, 1)	PC, FL, CAF	7 ± 4	11 ± 4	89 ± 4	16 ± 11	1 ± 2	0 ± 1
		Doliocarpus multiflorus (5, 0)	GG	5 ± 2	82 ± 6	18 ± 6	13 ± 8	4 ± 5	0 ± 0
		Doliocarpus olivaceus (2, 1)	GG, PC	17 ± 4	31 ± 11	69 ± 11	28 ± 9	10 ± 3	20 ± 20
Superasterids									
Santalales	Olacaceae	*Heisteria concinna* (9, 6)	GG, ET	25 ± 19	97 ± 2	2 ± 1	20 ± 10	25 ± 27	2 ± 5
Gentianales	Rubiaceae	*Guettarda foliaceae* (3, 0)	PC, CQA	11 ± 9	0 ± 0	58 ± 41	26 ± 17	0 ± 0	4 ± 5
		Palicourea acuminata (3, 0)	PC, PD	7 ± 10	9 ± 13	24 ± 34	17 ± 20	8 ± 11	15 ± 21
		Psychotria chagrensis (2, 0)	Other PA	2 ± 0	0 ± 0	100 ± 0	33 ± 1	40 ± 2	98 ± 11
		Psychotria grandis (5, 0)	Other PA	14 ± 1	0 ± 0	100 ± 0	33 ± 2	18 ± 3	80 ± 11
		Psychotria horizontalis (2, 0)	Other PA	2 ± 1	0 ± 0	100 ± 0	30 ± 1	31 ± 0	114 ± 10
		Psychotria limonensis (2, 0)	Other PA	2 ± 1	0 ± 0	100 ± 0	40 ± 7	36 ± 2	100 ± 4
		Psychotria marginata (2, 0)	Other PA	3 ± 1	0 ± 0	100 ± 0	58 ± 4	38 ± 1	105 ± 0
Caryophyllales	Polygalaceae	*Coccoloba acuminata* (1, 0)	PD	19	22	78	61	22	49
		Triplaris cumingiana (3, 0)	PC	9 ± 6	48 ± 6	51 ± 5	16 ± 8	7 ± 5	16 ± 12
Malvids									
Myrtales	Combretaceae	*Combretum fruticosum* (1, 0)	ET	68	63	37	212	21	91
		Combretum laxum (2, 1)	ET	67 ± 44	74 ± 24	26 ± 24	205 ± 62	24 ± 4	103 ± 26
	Lythraceae	*Lafoensia punicifolia* (4, 0)	ET	55 ± 8	70 ± 4	30 ± 4	174 ± 29	30 ± 4	93 ± 4
	Melastomataceae	*Mouriri myrtilloides* (4, 4)	ET, PC	15 ± 8	59 ± 32	41 ± 32	20 ± 10	12 ± 6	39 ± 27
	Myrtaceae	*Eugenia coloradoensis* (1, 0)	ET	68	98	2	62	44	42
		Eugenia nesiotica (2, 0)	ET, PC, PD	18 ± 0	58 ± 3	42 ± 3	30 ± 3	20 ± 6	22 ± 5
		Eugenia venezuelensis (1, 0)	ET	75	95	4	53	29	52
		Myrcia splendens (2, 0)	ET	56 ± 11	84 ± 2	14 ± 2	132 ± 0	20 ± 1	56 ± 1
Malvales	Malvaceae	*Pseudobombax septenatum* (9, 9)	PC, CQA, CA	6 ± 5	0 ± 0	78 ± 42	8 ± 5	0 ± 0	0 ± 0
		Theobroma cacao (1, 0)	PC, CAF	40	0	99	57	8	29
Sapindales	Anacardiaceae	*Anacardium excelsum* (6, 0)	GG, FL	45 ± 11	70 ± 10	30 ± 10	144 ± 40	12 ± 2	64 ± 19
	Sapindaceae	*Matayba apetala* (1, 0)	PC, PD	11	0	100	20	23	0
		Paullinia rugosa (2, 0)	PC	18 ± 1	0 ± 0	100 ± 0	20 ± 1	4 ± 4	21 ± 7
		Serjania mexicana (2, 1)	PC, PD	6 ± 6	51 ± 49	49 ± 49	34 ± 34	8 ± 8	12 ± 12
		Talisia nervosa (1, 0)	GG, CQA	33	42	42	47	12	31
		Thinouia myriantha (6, 6)	PC	4 ± 5	0 ± 0	95 ± 7	7 ± 5	1 ± 3	0 ± 0
	Meliaceae	*Trichilia tuberculata* (5, 0)	PC, FL, CQA, CoQA	50 ± 18	0 ± 0	80 ± 4	70 ± 20	3 ± 2	35 ± 5
Fabids									
Fabales	Fabaceae	*Andira inermis* (6, 0)	PC, FL	11 ± 7	0 ± 0	30 ± 32	23 ± 10	5 ± 4	5 ± 12
		Bauhinia guianensis (3, 3)	PC	21 ± 5	34 ± 3	66 ± 3	28 ± 5	8 ± 3	17 ± 12
		Canavalia campylocarpa (1, 0)	GG	11	88	12	23	18	0
		Courbaril hymenaea (1, 0)	PD, coumarins	8	0	100	59	42	21
		Inga nobilis (2, 0)	PC	13 ± 0	0 ± 0	99 ± 1	15 ± 0	0 ± 0	0 ± 0
		Machaerium milleflorum (4, 3)	PC	21 ± 17	0 ± 0	75 ± 43	72 ± 33	1 ± 1	25 ± 14
		Platypodium elegans (9, 1)	PC, CAF	12 ± 8	1 ± 2	77 ± 41	25 ± 16	7 ± 6	2 ± 5
		Prioria copaifera (7, 0)	PC, CQA	65 ± 23	0 ± 0	98 ± 0	70 ± 11	2 ± 3	49 ± 7
		Pterocarpus hayesii (2, 0)	PC	9 ± 2	1 ± 1	99 ± 1	14 ± 1	0 ± 0	1 ± 1
		Swartzia simplex (2, 0)	PC	16 ± 16	0 ± 0	50 ± 50	25 ± 25	6 ± 6	17 ± 17
		Tachigali panamensis (1, 0)	PC, PD	17	0	100	18	34	15
Rosales	Moraceae	*Ficus citrifolia* (1, 0)	PC	54	0	99	61	0	49
		Ficus obtusifolia (2, 0)	PC, CQA	11 ± 2	0 ± 0	96 ± 1	31 ± 1	2 ± 2	0 ± 0
	Urticaceae	*Pourouma bicolor* (1, 0)	PC, CQA	37	0	87	48	2	24
Celastrales	Celastraceae	*Hippocratea volubilis* (8, 8)	PC, PD	21 ± 22	0 ± 0	100 ± 0	38 ± 16	11 ± 4	22 ± 14
		Maytenus schippii (1, 1)	PC	30	0	100	36	7	17
		Tontelea richardii (4, 4)	PC, PD	72 ± 37	0 ± 0	100 ± 0	77 ± 14	26 ± 4	64 ± 11
Oxalidales	Connaraceae	*Cnestidium rufescens* (4, 0)	PC, CQA	19 ± 6	0 ± 0	97 ± 1	62 ± 6	1 ± 2	21 ± 3
		Connarus panamensis (1, 1)	PC	46	11	89	54	10	41
		Rourea glabra (1, 1)	PC, CQA, CoQA	44	0	96	49	0	14
Malpighiales	Chrysobalanaceae	*Hirtella triandra* (1, 1)	PD	9	0	100	37	44	21
		Licania platypus (2, 0)	PD, FL	25 ± 3	1 ± 0	98 ± 0	92 ± 7	53 ± 0	12 ± 2

(*continued on next page*)

Table 1 (*continued*)

Clade Order	Family	Species (samples, of which oily)	Main phenolics	PT (mg/g)	HT (%)	PA (%)	FC (mg/g)	pH 10 (%)	PPC (%)
	Malpighiaceae	*Tetrapterys macrocarpa* (1, 1)	GA	17	43	57	46	11	18
	Ochnaceae	*Ouratea lucens* (1, 0)	PC, CoQA, FL	38	0	74	49	0	12
	Calophyllaceae	*Calophyllum longifolium* (9, 0)	PC	9 ± 3	0 ± 1	100 ± 1	17 ± 5	1 ± 3	10 ± 10
	Clusiaceae	*Garcinia recondita* (3, 0)	PC, FL	7 ± 6	0 ± 1	100 ± 1	18 ± 2	0 ± 0	14 ± 13
	Salicaceae	*Zuelania guidonia* (3, 2)	PC, CQA	23 ± 19	0 ± 0	98 ± 0	25 ± 14	0 ± 0	8 ± 11
	Lacistemataceae	*Lacistema aggregatum* (3, 0)	PC, PD	32 ± 9	20 ± 14	78 ± 11	57 ± 19	8 ± 6	23 ± 9
	Euphorbiaceae	*Mabea occidentalis* (1, 0)	GG, ET	99	90	9	91	28	72
	Phyllanthaceae	*Hieronyma alchorneoides* (5, 0)	ET	5 ± 1	86 ± 4	14 ± 4	30 ± 4	12 ± 2	0 ± 0

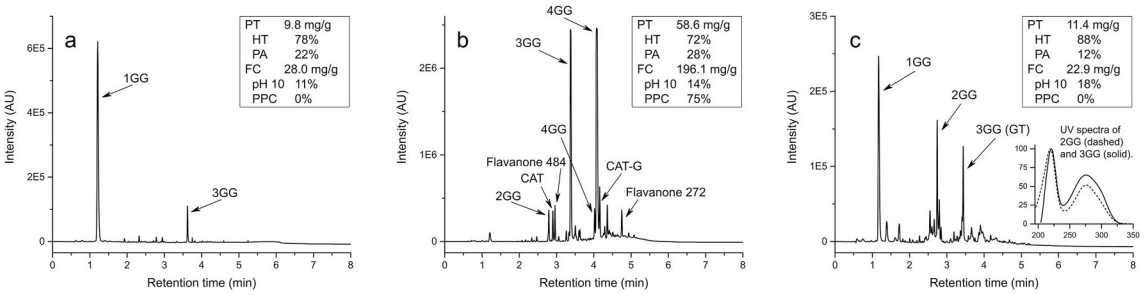

Fig. 1. Examples of the UV chromatograms (280 nm), quantification data and biological activities of the gallic acid derivative-rich samples. a) *Doliocarpus multiflorus*, b) *Anacardium excelsum*, c) *Canavalia campylocarpa*. Abbreviations: 1GG: monogalloyl glucose, 2GG: digalloyl glucose, 3GG: trigalloyl glucose, 3GG (GT): gallotannin-type 3GG, 4GG: tetragalloyl glucose, CAT: catechin, G: galloyl group, PT: mass spectrometric quantitation of total phenolics, HT: portion of hydrolysable tannins, PA: portion of proanthocyanidins, FC: total phenolics quantified using Folin-Ciocalteu assay, pH 10: portion of easily oxidized total phenolics, PPC: protein precipitation capacity reported in relation to the total phenolics.

The species *Combretum fruticosum*, *C. laxum* and *Lafoensia punicifolia* contained punicalagin, punicalin and pedunculagin, and species in the former genus additionally contained an unidentified punicalagin isomer (Fig. 2a and S2.17–19). *Mouriri myrtilloides*, *Eugenia coloradoensis*, *E. nesiotica*, *E. venezuelensis* and *Myrcia splendens* (Fig. 2b and c and S2.20–24) all contained ETs with cyclic and acyclic glucose cores. *Myrcia splendens* also contained an unresolved ET hump, likely consisting of a complex mixture of ET oligomers or even polymers with simple HHDP ester–type monomer units based on the UV and mass spectra (λ_{max} 221, 270 nm; *m/z* 935, 859, 783, 301, among others; Figure S2.24). The main compound of *Mabea occidentalis* and *Hieronyma alchorneoides* was geraniin, accompanied by a range of unidentified ETs (Figure S2.62 and S2.63).

2.2. Proanthocyanidins

PAs were present in all 63 tannin-rich species (Table 1), with 47 of these species being PA-rich, having at least one sample with the concentration of PAs being 10 mg/g or more based on MRM. The measured mean degrees of polymerization of PAs in this study were relatively low, an average of 6 and a maximum of 19 among the tannin-rich species (data not shown). *Theobroma cacao* (Figure S2.26) and *Platypodium elegans* (Fig. 3c) contained high quantities of a few, well separated flavan-3-ol monomers and oligomers as opposed to a PA hump consisting of large varieties of closely eluting oligo- and polymers.

The shape of the PA humps on UV chromatograms at 280 nm varied from symmetrical to lopsided, with a slow incline followed by a sharp decline (Fig. 3a vs. 3 b). The asymmetrical humps were PCs eluting relatively late, with the hump apex eluting at ca. 5.2 min. These variations of the PA hump shapes likely indicate that the individual PA structures vary between different plant species, even though these structural variations are not perceivable with the data obtained from the assays measuring the total phenolics, PC/PD ratios or mean degree of polymerization in the samples.

The PAs of the genus *Psychotria* differed significantly from the rest of the sample set (Fig. 4). These "Psychotria PAs" consisted of catechin (290 Da) and two types of subunits that are missing one and two hydroxyl groups compared to catechin, with MWs of 274 and 258 Da (e.g.

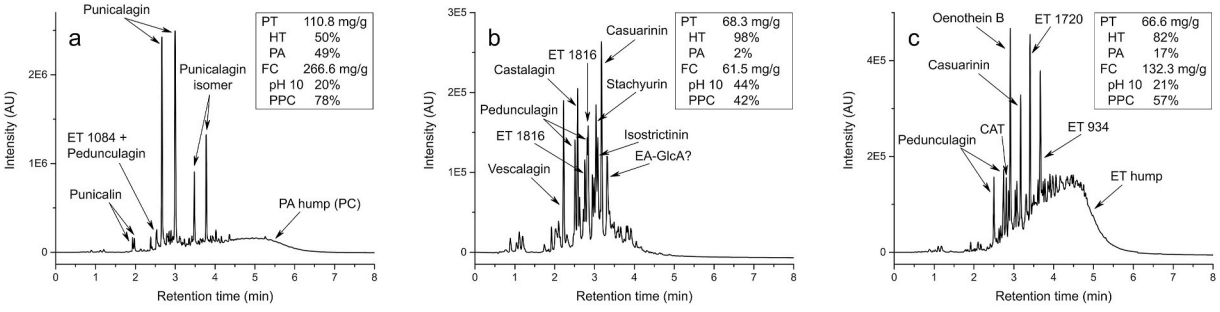

Fig. 2. Examples of the UV chromatograms (280 nm), quantification data and biological activities of the ellagitannin-rich samples. a) *Combretum laxum*, b) *Eugenia coloradoensis*, c) *Myrcia splendens*. Abbreviations: CAT: catechin, EA: ellagic acid, ET: ellagitannin, GlcA: glucuronic acid, PA: proanthocyanidin (in the info box: portion of proanthocyanidins), PC: procyanidin, PT: mass spectrometric quantitation of selected polyphenolics, HT: portion of hydrolysable tannins, FC: total phenolics quantified using Folin-Ciocalteu assay, pH 10: portion of easily oxidized total phenolics, PPC: protein precipitation capacity reported in relation to the total phenolics.

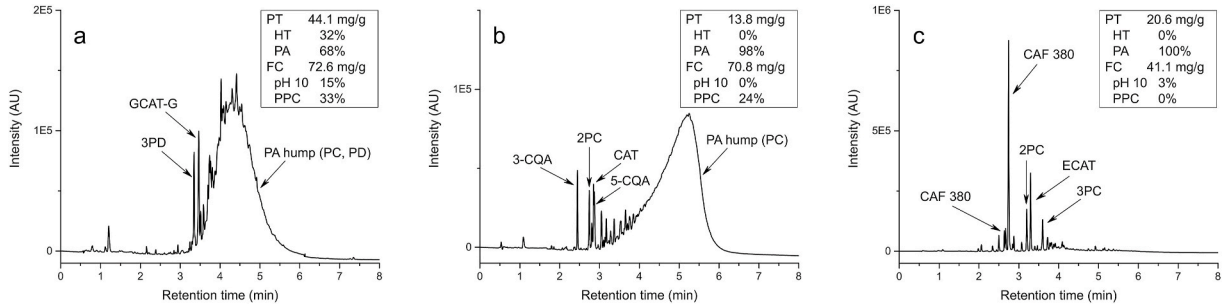

Fig. 3. Examples of the UV chromatograms (280 nm), quantification data and biological activities of the proanthocyanidin-rich samples. a) *Lacistema aggregatum*, b) *Cnestidium rufescens*, c) *Platypodium elegans*. Abbreviations: 3-CQA: 3-*O*-caffeoylquinic acid, 5-CQA: 5-*O*-caffeoylquinic acid, CAF: caffeic acid derivative, CAT: catechin, ECAT: epicatechin, G: galloyl group, GCAT: gallocatechin, PA: proanthocyanidin (in the info box: portion of proanthocyanidins), PC: procyanidin, 2PC: dimeric PC, PD: prodelphinidin, 3PD: trimeric PD, PT: mass spectrometric quantitation of selected polyphenolics, HT: portion of hydrolysable tannins, FC: total phenolics quantified using Folin-Ciocalteu assay, pH 10: portion of easily oxidized total phenolics, PPC: protein precipitation capacity reported in relation to the total phenolics.

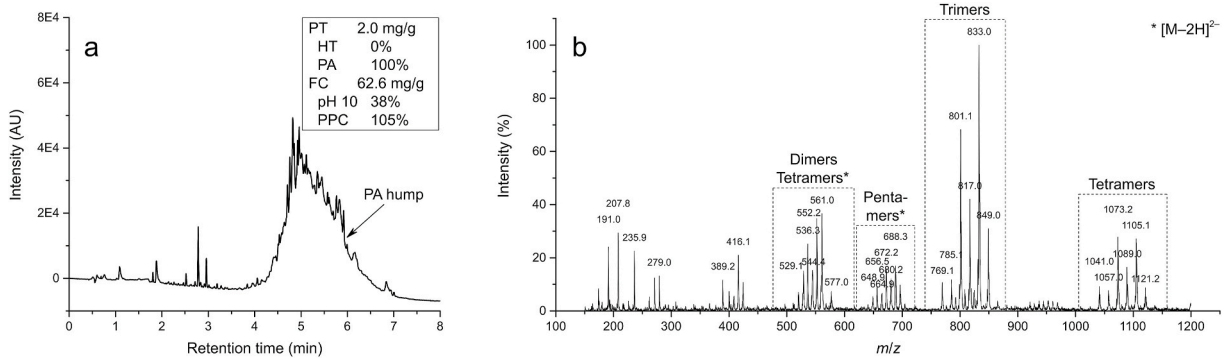

Fig. 4. a) The UV chromatogram and b) total mass spectrum of *Psychotria marginata* containing unidentified proanthocyanidins. Abbreviations: PA: proanthocyanidin (in the info box: portion of proanthocyanidins), PT: mass spectrometric quantitation of selected polyphenolics, HT: portion of hydrolysable tannins, FC: total phenolics quantified using Folin-Ciocalteu assay, pH 10: portion of easily oxidized total phenolics, PPC: protein precipitation capacity reported in relation to the total phenolics.

Figure S2.13). Presumably due to the lower number of hydroxyl groups, these PAs were more hydrophobic than PCs and PDs – the hump of Psychotria PAs began to elute at ca. 4.0 min as opposed to ca. 2.0 min of PDs and ca. 3.0 min of PCs in other species. While fragmentation patterns characteristic to PAs appear in the mass spectra (retro-Diels-Alder fragmentation, heterocyclic ring fission, and quinone-methide fragmentation; Friedrich et al., 2000; Gu et al., 2003), coelution makes the assignation of the fragment ions to the correct corresponding molecular ions impossible. Based on our knowledge on the oxidative activities of phenolics, the high activities of these samples (see the following section) would indicate a presence of pyrogallol moieties (Kim et al, 2018, 2020; Vihakas et al., 2014). However, this would also require highly unusual PA structures, as this would mean that there would be no additional hydroxyl groups in 258 Da subunits, and for 274 Da subunits, there would only be one, likely at positions 3, 5 or 7. Alternatively, the high activity of *Psychotria* extracts was governed by compounds that were not detected or identified in this study, or by an unknown structure/activity relationship.

2.3. Oxidative activity and protein precipitation capacity

The results of the oxidative activity and PPC measurements have been summarized at species level in Table 1 and at family level in Figure S1. Oxidatively the most active species were the ones that contained PAs consisting mainly of PD subunits with few or no PCs subunits – namely, *Coccoloba acuminata*, *Hirtella triandra*, *Licania platypus* and *Courbaril hymenaea* (average: 43% activity; Figures S2.15, S2.54, S2.55 and S2.37). These were followed by the genus *Psychotria* containing PAs (average: 29%; Figures S2.10–S2.14) and the species containing ETs (*Heisteria concinna*, the order Myrtales, *Mabea occidentalis* and *Hieronyma alchorneoides*; average: 22%; Figures S2.7, S2.17–S2.24, S2.62, and S2.63). The oxidative activity of species rich in GA derivatives (*Doliocarpus multiflorus*, *Canavalia campylocarpa*, *Tetrapterys macrocarpa*, *Anacardium excelsum*, *Talisia nervosa*; Figures S2.5, S2.36, S2.56, S2.27 and S2.31) and PCs (the remaining tannin-rich species) tended to be low (average: 10% and 5%, respectively).

We inspected the PPC in relation to the total phenolics of the sample (relative PPC; % of FC), since the PPC partially depends on the total phenolic content of the sample; samples with high total phenolics have a higher PPC than samples with a lower quantity of the same phenolics. This way, the PPC was more comparable between samples with the varying concentrations of total phenolics (e.g. *Coccoloba acuminata* and *Triplaris cumingiana*, Figures S2.15 and S2.16). We would like to emphasize that this was done for the ease of comparison only – the reported values do *not* mean that the said portion of total phenolics could precipitate BSA, or that the said portion of total phenolics are tannins. Furthermore, these two methods quantify phenolics on different principles. In the FC assay, the phenolics are quantified in gallic acid equivalents based on the absorbance at 740 nm of the reduced Folin-Ciocalteu reagent, whereas in radial diffusion assay, the quantitation is based on the size of the visible ring of precipitated proteins in agarose gel, and the standard is pentagalloyl glucose.

Two sample types stood out with their consistently high relative PPC: the genus *Psychotria* rich in PAs, and *Combretum fruticosum*, *C. laxum* and *Lafoensia punicifolia* rich in punicalagin. The average relative PPC of both of these sample types was astonishingly high, around 95%.

Otherwise, there was considerable variation in relative PPC within samples that contained the same tannin subclasses. For example, the relative PPCs of the PC-rich *Connarus panamensis* and *Rourea glabra* were 41% and 14%, respectively (Figures S2.52 and S2.53), whereas *Mabea occidentalis* and *Hieronyma alchorneoides*, both containing geraniin, and the former other ellagitannins in addition, had a relative PPC of 72 and 0%, respectively (Figures S2.62 and S2.63). *Doliocarpus multiflorus* and *Canavalia campylocarpa* contained trigalloyl glucose and smaller galloyl glucoses, and the relative PPC of these samples was 0% (Figures S2.6 and S2.36). On the other hand, *Talisia nervosa* and *Anacardium excelsum* additionally contained tetragalloyl glucose, which is a good protein precipitator unlike trigalloyl glucose and the preceding galloyl glucoses (Engström et al., 2019; Salminen et al., 2011). Their relative PPC was 31 and 75%, respectively (Figures S2.31 and S2.27). The compound class that could be associated with the highest relative PPC was Psychotria PAs (average across all species where present: 95%), followed by ETs (average across all species where present: 42%), PD-containing PAs (average across all species where present: 26%) and finally by PC-containing PAs (average across all species that did not additionally contain ETs or PD-containing PAs: 14%). The PPC of a plant extract depends not only on the structural factors of its protein-binding phenolics (e.g. the ability to form hydrogen bonds, steric hindrance, and the flexibility of the molecule), but also their concentration (Engström et al., 2019; Marsh et al., 2020). The high concentration of hydrolysable tannins may partially explain the high relative PPCs of *Combretum fruticosum*, *C. laxum*, *Lafoensia punicifolia* and *Anacardium excelsum*.

3. Conclusions

This study gives a broad overview to the phenolic composition and activities of seeds from a wide variety of tree, shrub and liana species of Central America, with the phenolic constituents of many of these species analyzed for the first time. One-third of the species contained tannins in their seeds in highly varying quantities, with PAs being more common than HTs. The families Combretaceae, Lythraceae and Anacardiaceae, all rich in ETs, had the highest concentrations of total phenolics, an average of 175 mg/g dry mass. In line with our previous large-scale survey on phenolic compounds in plants (Kim et al., 2020), a general trend was that ETs were not as common group of compounds than PAs, but were present in much higher quantities.

Several species had interesting traits in their phenolic profiles. *Doliocarpus multiflorus* mainly contained mono- and trigalloyl glucose, but little digalloyl glucose. GT-type di- or trigalloyl glucose or galloylquinic acid was detected in *Talisia nervosa*, *Mabea occidentalis* and *Canavalia campylocarpa*. An unresolved ET hump was observed on *Myrcia splendens*, likely composed of oligo- and polymeric ETs consisting of simple HHDP ester–type monomer units. The shape of the PA humps ranged from symmetrical to lopsided, indicating variation in their exact PA composition.

High oxidative activities could be associated with species containing ETs (e.g. *Eugenia coloradoensis*) or PDs (e.g. *Courbaril hymenaea*), as we could expect based on the results of our previous study (Kim et al., 2020). However, we were surprised to also observe very high oxidative activities in the genus *Psychotria*, the main compounds of which were PAs of unidentified class, consisting of subunits with a low MW.

Species that had the highest relative PPC either contained punicalagin in large quantities (*Combretum fruticosum*, *C. laxum*, *Lafoensia punicifolia*) or Psychotria PAs (genus *Psychotria*). The relative PPCs of compound classes support the observations we made during our earlier large-scale plant survey (Kim et al., unpublished results). The compound classes could be sorted in the order of decreasing relative PPC as follows: GTs ≈ Psychotria PAs > ETs > PD-rich PAs > PC-rich PAs > flavonoids ≈ hydroxycinnamic acid derivatives. As we could not determine the structure of Psychotria PAs with certainty, the relationship between their structure and the high oxidative activities and protein precipitation capacities of *Psychotria* species could not be assessed.

These results offer a good starting point for further studies in the field of chemical ecology and phytochemistry. To assess the ecological significance of the chemical compounds targeted by our study, an important next step will be to link the chemistry results to patterns of seed mortality in the plant community. Dedicated studies could reveal the variability of structures leading to the asymmetry of PA humps detected in several species, and the composition of the ET hump of *Myrcia splendens*. Structural elucidation on the Psychotria PAs could help assess the relationship between their structure and the high activities in the genus *Psychotria*, possibly revealing new information on the structure/activity patterns of PAs. Differences in investment on chemical defenses throughout the lifespan of plants could be observed by comparing the phenolic content of seeds and the leaves of mature plants.

4. Experimental

4.1. Plant material

Freshly fallen fruits and seeds were collected along the forest trails of BCI from July 2010 to December 2013. Fruit pulp was removed and the counted seeds were freeze-dried in paper envelopes for a minimum of three days. Each sample consisted of whole seeds (embryo, endosperm and coat or testa) of a given species, collected in a given location on the same day. Most of the seeds in a single sample likely come from the same maternal source, as the samples were typically collected under fruiting individual specimen. The samples were stored in plastic ziplock bags in a freezer until they were exported to Finland for chemical analysis under a permit from the *Autoridad Nacional del Ambiente* (ANAM). Seeds were identified to species level by a team of experienced field botanists (see *Acknowledgements*).

4.2. Chemical analyses

The samples were processed and analyzed in the laboratory of Natural Chemistry Research Group at the University of Turku, Finland. The samples were stored in a freezer prior to further processing.

Our objective was to extract the maximum amount of compounds possible, so we aimed to mill the samples to a fine powder. We used various equipment to accommodate the differences in size and toughness of the studied species. We mainly used a ball mill, and small seeds were ground in Eppendorf tubes with two small steel balls (6 mm diameter) whenever possible. Larger seeds were ground in steel capsules with one large steel ball (16 mm diameter). The shaking frequency was 30 s^{-1}, and the milling time was 1 min to keep sample heating to a minimum. Milling was repeated if it resulted in dry but coarse particles. Milling temperature was not controlled, but no significant heating was detected: the Eppendorf tubes and steel capsules were not noticeable warm when removing them from the ball mill immediately after grinding. We therefore consider that thermal degradation is unlikely to have occurred to any significant extent. Seeds that were too large to fit the steel capsules were crushed into millable size using a nutcracker or a plastic cylinder, an aluminum anvil block, an aluminum piston and a hammer. Some of the seed samples ($n = 62$) could not be crushed by any tools available in the laboratory and were excluded from the study. Of 572 samples that could be ground, 178 resulted in thick oily liquid or coarse, sticky granules rather than dry, fine powder. These samples were analyzed normally and their data was included in the results.

Chemical and reagents. Technical grade acetone and analytical grade acetic acid were from VWR (Haasrode, Belgium). Sodium/sodium hydrogen carbonate buffer (pH 10, 50 mM) and analytical grade formic acid were from J. T. Baker (Deventer, the Netherlands). Folin-Ciocalteu phenol reagent, gallic acid (≥97% purity), LC-MS grade acetonitrile, analytical grade sodium hydroxide, bovine serum album (lyophilized powder, ≥96% purity, 66 kDa, pH 7, purified by heat shock fractionation), agarose (type I) and ascorbic acid (≥99% purity) were from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade sodium carbonate

was from Oy FF-Chemicals Ab (Yli-Ii, Finland). Pentagalloyl glucose (≥98% purity) was produced from tannic acid using methanolysis. Water was purified using a Millipore Synergy Water Purification System (Merck KGaA, Darmstadt, Germany).

Sample extraction. The crushed sample (20 mg/g) was extracted twice with 1400 µl of acetone–water (8:2, v/v, 3 h) in 2 ml Eppendorf tubes. The extracts were combined and the acetone was evaporated with an Eppendorf concentrator at room temperature. The aqueous phases were frozen, lyophilized and dissolved in 1 ml of water. The solutions were filtered using a syringe filter (13 mm, 0.2 µm PTFE, VWR International LLC, Radnor, PA, USA) before analyses.

Alkaline oxidation and Folin-Ciocalteu assay. Alkaline oxidation and total phenolics assay were carried out according to Salminen and Karonen (2011). A portion of 20 µl of filtered extract was subjected to alkaline conditions by dissolving it in 180 µl of sodium carbonate buffer (pH 10) in a 96-well plate. The plate was shaken for 10 s every min for 60 min, and the oxidation was stopped by adding 100 µl of 0.6% aq. HCOOH, bringing the pH to 6. The remaining total phenolics were quantified by Folin-Ciocalteu (FC) assay by mixing 50 µl of the analyte/buffer solution with 50 µl of 1 M FC phenol reagent in a new 96-well plate. After shaking the mixture for 60 s, 100 µl of 20% Na_2CO_3 (m/v) was added to activate the FC reagent. The plate was shaken for 10 s every min and the absorbance at 742 nm was read after 30 min. Gallic acid at 0, 10, 25 and 100 µg/ml concentrations was used as the quantitation standard. Control was 20 µl of the filtered extract dissolved in 280 µl of 18:10 (v/v) mixture of pH 10 buffer and 0.6% aq. HCOOH, i.e. the same ratio as in the oxidized sample after neutralizing. As with the oxidized sample, the control sample was shaken for 10 s every min for 60 min, and 50 µl of the solution was mixed with 50 µl of 1 M FC reagent and 100 µl of 20% Na_2CO_3. The oxidized and control samples were measured in triplicates, and the average values, reported in gallic acid equivalents (mg/g), were compared together to obtain the ratio of oxidized phenolics.

UHPLC-DAD-QqQ-MS/MS analyses. The samples were analyzed according to Engström et al. (2015, 2014). In brief, a portion of the filtered extract was diluted to one-fifth of the original concentration with ultrapure water before the analysis. The UHPLC was an Acquity series UPLC® system consisting of a sample manager, a binary solvent manager, a column, and a photodiode array detector coupled to a Xevo TQ triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The column was a 100 mm × 2.1 mm i.d., 1.7 µm Acquity UPLC® BEH Phenyl column (Waters Corporation, Wexford, Ireland). The flow rate of the eluent was 0.5 ml/min and the injection volume was 5 µl. The gradient elution used two solvents, acetonitrile (A) and 0.1% aq. formic acid (B). The first 140 samples were analyzed using a short gradient, which was changed to long gradient for the rest of the samples to ensure the complete elution of PA humps. The first samples were not reanalyzed using the new gradient as no crucial data was missed. The gradients are described below, with percentages referring to % A in B:

Short gradient: 0–0.5 min, 0.1%; 0.5–5.0 min, 0.1–30.0% (linear gradient); 5.0–6.0 min, 30.0–35.0% (linear gradient); 6.0–9.5 min, column wash and stabilization. UV and MS data were collected from 0 to 6 min. Long gradient: 0–0.5 min, 0.1%; 0.5–5.0 min, 0.1–30.0% (linear gradient); 5.0–8.0 min, 30.0–45.0% (linear gradient); 8.0–11.5 min, column wash and stabilization. UV and MS data were collected from 0 to 8 min.

The photodiode array detector was operated at 190–500 nm, with the phenolic compounds being monitored at 280 nm. The mass spectrometer was operated in negative ion mode and the ions were scanned at *m/z* 150–1200. The capillary voltage was 3.4 kV, the desolvation temperature was 650 °C, and the source temperature was 150 °C. The desolvation and cone gas (N_2) flow rates were 1000 l/h and 100 l/h. MRM methods were used to quantify gallic acid derivatives, ETs, PCs, PDs and kaempferol, quercetin and myricetin glycosides as well as quinic acid derivatives. Compounds were identified by comparing their retention time as well as UV and mass spectra to the data available in the spectral library of our research group (Table S2). The UV and total ion chromatograms were processed using Origin software, version 2016 (OriginLab Corporation, Northampton, MA, USA).

Radial diffusion assay. The protein-precipitation capacity of each sample was determined with radial diffusion assay as described by Hagerman (1987), with minor modifications. A portion of 200 µl of filtered extract was freeze-dried and redissolved in 100 µl of water. The model protein (0.1% BSA, w/v) was dissolved in 1% agarose gel (w/v) consisting of 60 µM ascorbic acid and 50 mM acetic acid, buffered to pH 5.0 with 2 M NaOH. 10 ml of gel was dispensed into plastic Petri dishes, and nine 4 mm holes were punched in the set gel. A portion of 24 µl of concentrated extract was applied into a hole, and once the aliquots had dissolved in the gel, the dishes were sealed with parafilm and incubated upside down at 30 °C for 72 h. The standard was pentagalloyl glucose at 1, 2, 3, 4 and 5 mg/ml. All samples and standards were applied in triplicates. The average precipitation ring size was measured using ImageJ (Abràmoff et al., 2004) and transformed into pentagalloyl glucose equivalents (mg/g). The PPC is reported as the portion of total phenolics.

Tannin-rich samples. We wanted to focus on species that were tannin-rich, so we only inspected the tannin content and activities if the concentration of HTs and PAs in at least one of the samples of the species was high (at least 10 mg/g in total, measured by MRM methods). To ensure that samples that contained HTs and PAs not detected by the group-specific MRM methods would also be included in the results, we examined the phenolic profiles of the samples that had a high concentration of total phenolics on FC assay (at least 20 mg/g) but low concentration of HTs and PAs (less than 10 mg/g by MRM). If these samples were found to contain either type of tannin, we included the species to the focus group. Species that had a low content of phenolics in all samples (less than 20 mg/g on FC and less than 10 mg/g of HTs and PAs on MRM) or only contained flavonoid glycosides and quinic acid derivatives were not included in further studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2021.112750.

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