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Profiling Glucosinolates, Flavonoids, Alkaloids, and Other Secondary Metabolites in Tissues of *Azima tetracantha* L. (Salvadoraceae)

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Azima tetracantha L. (needle bush; bee sting bush; Salvadoraceae) is used as a food and for various herbal medicines in Africa, India, and Madagascar, but there is very little information on the secondary metabolites in this species. High concentrations of *N*-methoxy-3-indolylmethyl-glucosinolate, a common glucosinolate of *Brassica* crops such as Brussels sprouts and broccoli, were found in the roots and seeds of *A. tetracantha*. Lower concentrations were detected in the stems and young leaves. The roots also contained another indole glucosinolate that was provisionally identified, from MS data and comparison with indole glucosinolate standards, as *N*-hydroxy-3-indolymethyl-glucosinolate. The roots, stems, and leaves contained neoascorbigen (the condensation product of *N*-methoxy-indole-3-carbinol and ascorbic acid). The seeds of *A. tetracantha* contained a complex mixture of 26 flavonoids predominantly as glycosides and acyl-glycosides, with traces of aglycones. The core aglycones of these flavonoids were identified as quercetin, isorhamnetin (3′-*O*-methylquercetin), rhamnetin (7-*O*-methylquercetin), and rhamnazin (7, 3′-di-*O*-methylquercetin). No flavonoids or anthocyanins were detected in other tissues, and procyanidins were undetectable. The dimeric piperidine alkaloids azimine, azcarpine, and carpaine were found in all tissues of *A. tetracantha*.

KEYWORDS: Salvadoraceae; *Azima tetracantha*; indole glucosinolates; neoascorbigen; acylated flavonoids; rhamnazin; piperidine alkaloids; LC-MS

INTRODUCTION

Azima tetracantha L. (Order, Capparales; Family, Salvadoraceae) also known as the needle bush or bee sting bush is a xerophytic species found in Africa, India, and Madagascar (*1*). The young leaves of *A. tetracantha* are pickled and used as an appetizer, and there have also been reports on the antiinflammatory and diuretic activities of *A. tetracantha* extracts (*1*, *2*). There are records from Sanskrit manuscripts and also many anecdotal reports of its use in Ayurvedic medicine, but there are very few confirmed pharmacological studies. Other members of the Salvadoraceae family have been listed as famine foods, for example, *Dobera roxburghii* (Maikah; fruit pulp, seeds postboiling to debitter for making porridge), *Salvadora oleoides* (fresh and dried fruit), and *Salvadora persica* (Miswak; fresh ripe fruit) (*3*, *4*). There is considerable data on the agricultural development and medicinal applications of *S. persica*, for example, in dental hygiene, as an anticonvulsant, and as a sedative, and also, the root can be steam-distilled to produce an oil with a high concentration of benzyl isothiocyanate that has been used for multiple applications including insect control (*5*−*8*).

Glucosinolate-containing species are present throughout the world in a wide range of habitats and include salt tolerant and xerophytic forms. The most commonly consumed species are those from the Brassicaceae (Crucifereae), for example, *Brassica*, *Lepidium*, *Sinapis*, and *Raphanus* (*9*−*11*). Many other species are used as herbs or traditional medicines but have rarely been investigated for their active principles such as glucosinolates, flavonoids, or other secondary metabolites. Detailed glucosinolate analyses have been restricted to common vegetable and herb Capparales species (*9*−*12*). The Capparales order is currently subdivided into 15 families including the Salvadoraceae (*13*). There is minimal information on the secondary metabolite composition of the three species constituting the Salvadoraceae family: *Azima*, *Dobera*, and *Salvadora*. There are a few reports on *A. tetracantha* (needle bush) such as seed oil composition, the presence of isorhamnetin 3-*O*-rutinoside in leaves, triterpenoids in leaves and roots, and dimeric piperidine alkaloids in leaves (*14*−*18*). The *S. persica* seed has

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been shown to contain benzylglucosinolate (*18*). There are no data on the phytochemical composition of *Dobera* species.

This study forms part of an ongoing investigation into Capparales species with the aim of identifying the major secondary metabolites in tissues, and specifically those with potential human health-promoting activities, using previously developed liquid chromatography−mass spectrometry (LC-MS) methods (*19*−*22*). The majority of species have been selected on the basis of ethnobotanical data, pharmacological reports, and the fact that these species are known to be consumed by humans. As a secondary aim, the investigation of species related to food plants and from families that are rarely investigated is also part of this research program. This approach of LC-MS and gas chromatography (GC)-MS screening of targeted medicinal and food plants has produced useful data on the phytochemical composition of various plant species and has been useful in identifying novel plant bioactives and sources for their purification.

MATERIALS AND METHODS

Plant Material. The seeds of *A. tetracantha* L. were obtained from B & T World Seeds (Paguignan, Olonzac, France). The seeds were initially sown in loam-free compost (Shamrock Potting Compost), but this was found to be unsuitable because of its poor nutrient content and poor drainage. At 21 days, the plants were transferred to a loam-based compost (John Innes No. 1) with 20% Sharps sand to increase the drainage. The plants were grown at 20−25/18 °C day/night with natural lighting between February and August. The plant tissues were harvested 183 days after sowing: old leaves (leaves 1−5 from the base of the plant); young leaves (leaves 14−17 and leaf primordia); stem [tissue remaining (including thorns) after all leaves were removed]; and root (all root tissue).

Chemicals. All solvents used were high-performance liquid chromatography (HPLC) grade, and all water was ultrapure. All other chemicals were obtained from commercial sources (Sigma/Aldrich). Intact indole glucosinolate standards were purified from *Brassica oleracea* florets (3-indolylmethyl, 4-methoxy-3-indolylmethyl, and *N*-methoxy-3-indolylmethyl glucosinolates) and from *B. oleracea* seed (4-hydroxy-3-indolyl-methylglucosinolate) using methods previously described (*22*). Sinigrin (2-propenyl glucosinolate) was from Sigma. Cheirolin (3-methylsulfinyl-propyl isothiocyanate) was obtained from LKT Labs (St. Paul, MN). dl-Pipecolinic acid was obtained from Sigma/Aldrich. HPLC grade quercetin, quercetin 3-*O*-glucoside (isoquercitrin), quercetin 3-*O*-rhamnoglucoside (rutin), quercetin 3-*O*-rhamnoside (quercitrin), isorhamnetin (3′-*O*-methylquercetin), isorhamnetin 3-*O*-glucoside, isorhamnetin 3-*O*-rutinoside (narcisin), tamarixetin (4′-*O*-methylquercetin), rhamnetin (7-*O*-methylquercetin), (+)-catechin, (−)-epicatehcin, procyanidin B1, and procyanidin B2 were obtained from Extrasynthese (Genay, France). An enriched grape seed procyanidin powder was provided by Dr. Dario Zanichelli (University of Bologna). Polyamide (MN Polyamide CC 60) was obtained from Kinesis (Bedfordshire, United Kingdom). Ascorbigen was synthesized using a previously described method (*23*). Freeze-dried samples of young leaves of *Carica papaya* (for carpaine standard) were from a previous study (*24*).

Partial Purification of Rhamnazin. Rhamnazin (7,3′-di-*O*-methylquercetin) was partially purified from young leaves of *Rhamnus alaternus* (evergreen buckthorn) obtained from a local plant nursery. Freeze-dried powdered leaves (50 g) were extracted with 500 mL of 70% v/v methanol at 70 °C for 30 min. The extract was filtered through four layers of muslin cloth. The protein was removed from the sample by addition of 5 mL of 0.1 M 1:1 barium and lead acetates and was stored overnight at 4 °C. The sample was centrifuged to pellet the precipitated protein, and the supernatant was filtered using a vacuum filtration unit (Millipore Ltd., Watford, United Kingdom) with a 0.45 μm filter. The combined supernatant was rotary evaporated to 100 mL, and 10 mL was loaded onto a preconditioned polyamide solid phase extraction column (1 g column, sequentially prewashed with 20 mL of methanol and 60 mL of ultrapure water). After it was loaded, the column was washed with 20 mL of ultrapure water, dried under vacuum for 30 s, and then eluted with 10 mL of 100% methanol. The methanol fraction was dried under nitrogen and hydrolyzed using the method described below. This posthydrolysis fraction contained a mixture of quercetin, kaempferol, rhamnetin, and rhamnocitrin (kaempferol 7-*O*-methyl ether) and rhamnazin.

Preparation of *A. tetracantha* Tissues and Extraction Methods. The seeds were dried at 100 °C for 24 h prior to extraction. All other tissues were collected on dry ice, weighed, and freeze-dried. After they were freeze-dried, the samples were weighed and milled to a fine powder using a commercial blender. For general secondary metabolite analyses (glucosinolates, flavonoids, cinnamates, phenolics, etc.), subsamples (3 × 40 mg) were extracted in 2 mL screw-top tubes with either 1 (nonseed tissues) or 1.5 mL (seeds) of 70% v/v methanol at 70 °C for 30 min, with vortex mixing every 5 min to improve extraction. To one, replicate sinigrin and quercetin 3-*O*-rhamnoside (quercitrin) were added as glucosinolate and phenolic internal standards, respectively; in both cases, the recovery was always >95% irrespective of the tissue extracted. The samples were centrifuged and processed as previously described (*19*−*22*).

Secondary Metabolite LC-Electrospray Ionization (ESI)-MS Analyses. Phenomenex (Macclesfield, Cheshire, United Kingdom) Luna C_{18} (2) 250 mm × 4.6 mm, 5 μm columns with Securityguard precolumns were used for all reverse phase LC and LC-MS analyses. The glucosinolates were separated using a water (solvent A):methanol (solvent B) gradient, with 0.1% trifluoroacetic acid (TFA) in both solvents, as previously described (*21*−*23*). The glucosinolate hydrolysis products were separated using a linear gradient of solvent A (0.1% v/v TFA in water) vs solvent B (0.1% v/v TFA in methanol): 0 (100% A), 10 (80% A, 20% B), 25 (50% A, 50% B), 30 (100% B), 40 (100% B), 41 (100% A), and 51 min (100% A). The flavonoids and piperidine alkaloids were separated using a gradient of solvent A (water: tetrahydrofuran:TFA [98:2:0.1]) vs solvent B (acetonitrile): 0 (83% A, 17% B), 2 (83% A, 17% B), 7 (75% A, 25% B), 15 (65% A, 35% B), 20 (50% A, 50% B), 25 (100% B), 30 (100% B), 35 (83% A, 17% B), and 45 min (83% A, 17% B).

All mass spectra were obtained using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom) coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, a CO-1560 column oven, and a UV-1575 UV detector [Jasco (UK) Ltd., Great Dunmow, United Kingdom]. The HPLC column temperature was maintained at 25 °C, and the autoinjector was maintained at 4 °C. The 1 mL/min mobile phase flow exiting the LC column was split using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, United Kingdom) so that approximately 200 μL/min entered the mass spectrometer; the remainder of the flow was diverted to the UV detector [either 227 nm for all compounds (glucosinolates, glucosinolate hydrolysis products, phenolics, and flavonoids) or 370 nm specifically or flavonoids]. The flow split was monitored using a Humonics Optiflow 1000 flowmeter (Sigma-Aldrich, Dorset, United Kingdom) coupled to the outflow of the UV cell. The LC conditions were as described above for the specific compounds. The mass spectra were obtained in both positive and negative ion electrospray mode using a Micromass Z-spray ion source. The electrospray probe was operated at 2.5 kV with a programmed ramped cone voltage for intact glucosinolates (*21*), or 3.5 kV with a cone voltage of 34 V (for glucosinolate hydrolysis products; positive ion mode electrospray MS only) and 25 V for flavonoid glycosides and piperidine alkaloids (both positive and negative ion mode electrospray MS) and aglycones (positive ion mode electrospray MS). The source and desolvation temperatures were 140 and 350 °C, respectively. The nitrogen nebulizing and drying gas flow rates were optimized at 15 and 500 L/h, respectively. The spectra were recorded (in centroid mode) between *m/z* 50 and 1500 with a scan duration of 1.5 s/scan and an interscan time of 0.1 s. MS1 was set to unit mass resolution or better (LM and HM resolution parameters both set to 15.0). The spectra were processed using MassLynx 3.4 software (Micromass).

Generation of Glucosinolate Hydrolysis Products and Their Analysis by LC-MS. Subsamples (3 × 200 mg) of fresh root tissue were rapidly ground with a pestle and mortar with 1.5 mL of distilled

Table 1. Concentrations of Glucosinolates, Glucosinolate-Derived Compounds, and Dimeric Piperidine Alkaloids in Tissues of *A. tetracantha*[a]

compound	seed FW (DW)	root FW (DW)	stem and thorns FW (DW)	old leaves FW (DW)	young leaves FW (DW)
glucosinolates and glucosinolate-derived compounds					
3-indolylmethylglucosinolate	1930 ± 9 (2150 ± 10)	25 ± 2 (210 ± 20)	130 ± 10 (490 ± 50)	ND	20 ± 2 (120 ± 10)
N-hydroxy-3-indolylmethyl-glucosinolate	ND	55 ± 4 (469 ± 33)	ND	ND	ND
N-methoxy-3-indolylmethyl-glucosinolate	21700 ± 9 (24170 ± 10)	2590 ± 120 (22190 ± 1010)	1820 ± 200 (7090 ± 800)	50 ± 2 (210 ± 10)	380 ± 10 (2160 ± 70)
neoascorbigen	ND	7 ± 1 (58 ± 6)	72 ± 5 (271 ± 19)	167 ± 6 (702 ± 24)	130 ± 3 (779 ± 19)
dimeric piperidine alkaloids					
azimine	1028 ± 13 (1145 ± 15)	0.89 ± 0.04 (7.50 ± 0.3)	396 ± 2.9 (1493 ± 11)	333 ± 5 (1399 ± 21)	327 ± 3 (1860 ± 17)
azcarpaine	633 ± 6 (705 ± 7)	0.19 ± 0.01 (1.61 ± 0.1)	241 ± 0.8 (911 ± 3)	193 ± 0.5 (812 ± 2)	183 ± 0.9 (1037 ± 5)
carpaine	479 ± 5 (533 ± 6)	0.03 ± 0.01 (0.28 ± 0.05)	99 ± 0.8 (375 ± 3)	55 ± 1.7 (230 ± 7)	58 ± 0.7 (332 ± 4)

[a] ND = not detected in this tissue. Glucosinolate concentrations are expressed as means ± σ_{n-1} μg K^+ salt intact glucosinolate/g FW and DW. Neoascorbigen concentrations are expressed as means ± σ_{n-1} μg ascorbigen/g FW and DW. Alkaloid concentrations are expressed as means ± σ_{n-1} μg pipecolinic acid equivalents/g FW and DW.

water with 10 mg of acid-washed sand. The homogenates were transferred to 2 mL screw-top microtubes and incubated at 37 °C for 3 h. The samples were centrifuged (17000*g*, 30 min, 4 °C), and the supernatants were filtered through Target 0.2 μm PTFE (Chromos Express, Macclesfield, United Kingdom). Cheirolin (3-methylsulfonylpropyl isothiocyanate) was added as an external standard to give a final concentration of 100 μM. The samples were analyzed by LC positive ion mode electrospray MS using the method detailed above.

Hydrolysis of Flavonoid Glycosides and LC-MS of Aglycones. Essentially, this was done using a previously described method (*25*) with minor modifications. The seeds of *A. tetracantha* were extracted in duplicate using the standard method detailed above. At the resuspension stage, after all of the solvent was removed from the 700 μL supernatant subsamples, one of the duplicate samples was resuspended in water and processed as described above, i.e., a control sample. The duplicate sample was resuspended in 700 μL of 50% v/v methanol containing 10 mg/mL *tert*-butylhydroquinone and 1.2 M hydrochloric acid. The vials were sealed and heated at 85 °C for 2 h in a heating block with vortex mixing every 15 min to ensure efficient hydrolysis. The samples were allowed to cool and were filtered through Target 0.2 μm PVDF (Chromos Express). In addition to the nonhydrolyzed controls, commercial flavonoid aglycone standards (quercetin, tamarixetin, isorhamnetin, and rhamnetin) and a hydrolysate of a methanol extract of young leaves from *R. alaternus* (rhamnazin standard) were also run. During method development, all of the commercial glycosides (listed in the chemicals section of this paper) were tested and hydrolysis under these conditions was complete with <1% loss of aglycones. All samples were analyzed using the LC positive ion mode electrospray MS method for flavonoids described above; the full scan mode was used to confirm complete glycoside hydrolysis.

Procyanidin LC with Fluorescence Detection. Subsamples (3 × 40 mg) were extracted with 1 mL of 100% methanol at 70 °C for 30 min, with vortex mixing every 5 min to improve extraction. The samples were centrifuged (17000*g*, 4 °C, 30 min) and filtered through Target 0.2 μm PVDF (Chromos Express). The samples were analyzed using a previously described LC-MS method with simultaneous fluorescence detection (*26*). (+)-Catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, and a grape seed procyanidin extract were also used for comparative purposes.

RESULTS AND DISCUSSION

High concentrations of *N*-methoxy-3-indolylmethylglucosinolate (**Figure 1**) were found in roots, stems, and seed tissues of *A. tetracantha* (**Table 1**). The roots and seeds were particularly rich sources of this glucosinolate. In addition, the roots contained a hydroxy-3-indolylmethylglucosinolate with a retention time that was later than the 4-hydroxy-3-indolylmethylglucosinolate standard but had the same mass ($[M - H]^- = 463$ and with glucosinolate diagnostic ions of $m/z = 96$ and 97). This new hydroxy-glucosinolate was provisionally identified as *N*-hydroxy-3-indolylmethylglucosinolate, i.e., a logical precursor of *N*-methoxy-3-indolylmethylglucosinolate. MS data are presented for all of the *Azima* glucosinolates and various standards (**Table 3**). Indole glucosinolates (**Figure 1**) are common in many glucosinolate-containing species (*10*−*12*, *27*). In *Brassica* species, 3-indolylmethylglucosinolate, 4-methoxy-3-indolylmethylglucosinolate, and *N*-methoxy-3-indolylmethylglucosinolate are common in the roots and aerial parts (*10*−*12*, *27*). 4-Hydroxy-3-indolylmethylglucosinolate is the major indole glucosinolate in the seeds of most *Brassica* species but has also been found in other tissues of Capparales species (*10*−*12*, *27*, *28*). The conversion of values from previous studies shows that the levels of indole glucosinolates found in *A. tetracantha* leaf tissues are similar to levels in *Brassica* vegetable crops such as white cabbage [*N*-methoxy-3-indolylmethylglucosinolate, 194 μg/g fresh weight (FW)], red cabbage (total indole glucosinolates, 355 μg/g FW), Brussels sprouts (*N*-methoxy-3-indolylmethylglucosinolate, 108 μg/g FW, and 3-indolylmethylglucosinolate, 267 μg/g FW), and broccoli (*N*-methoxy-3-indolylmethylglucosinolate, 46 μg/g FW) (*28*). Root tissues of *A. tetracantha* contained significantly greater concentrations

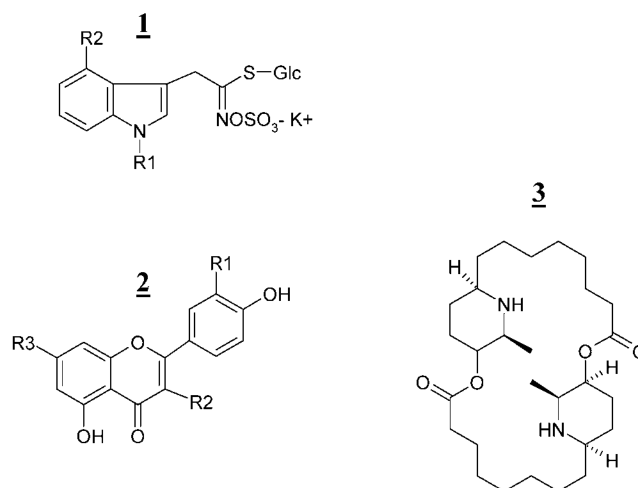

Figure 1. Structures of indole glucosinolates, core flavonoid aglycones, and carpaine. Structure 1: 3-indolylmethylglucosinolate (R1 = R2 = H); *N*-hydroxy-3-indolylmethyl-glucosinolate (R1 = OH, R2 = H); *N*-methoxy-3-indolylmethylglucosinolate (R1 = OCH_3, R2 = H); 4-hydroxy-3-indolylmethylglucosinolate (R1 = H, R2 = OH); and 4-methoxy-3-indolylmethylglucosinolate (R1 = H, R2 = OCH_3). Structure 2: R1 = 3′-position, R2 = 3-position, R3 = 7-position; quercetin (R1 = R2 = R3 = OH); isorhamnetin (R1 = OCH_3, R2 = R3 = OH); rhamnetin (R1 = R2 = OH, R3 = OCH_3); and rhamnazin (R1 = R3 = OCH_3, R2 = OH). Structure 3 is the dimeric piperidine alkaloid carpaine.

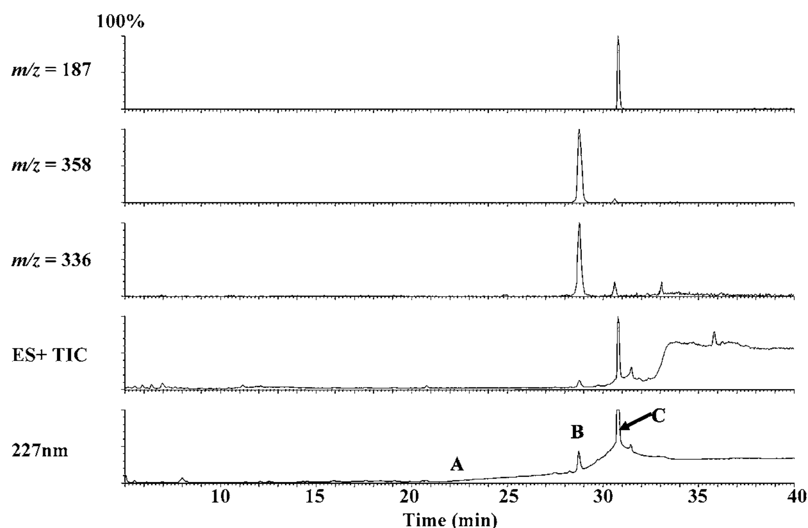

Figure 2. LC-MS data of autolysis products from roots of *A. tetracantha*. The data presented are the analogue trace (227 nm) showing the absence of any intact glucosinolate (**A**), the total ion current trace (ES + TIC), m/z = 336 and 358 {$[M + H]^+$ and $[M + Na]^+$ for neoascorbigen (**B**)} and m/z = 187 {$[M + H]^+$ for *N*-methoxyindole-3-acetonitrile (**C**)}. For all traces, the *Y*-axis is expressed as % relative to the largest peak (100%).

Table 2. Concentrations of Flavonoids in Seeds of *A. tetracantha*[a]

peak	flavonoid	FW (dry weight) concentration
1	quercetin rhamnosylglucoside	24 ± 7 (27 ± 8)
2	quercetin X-glucoside	258 ± 22 (287 ± 24)
3	quercetin 3-*O*-rhamnosylglucoside	972 ± 129 (1083 ± 144)
4	methyl-*O*-quercetin X-glucoside	307 ± 40 (342 ± 44)
5	quercetin 3-*O*-glucoside	371 ± 50 (413 ± 56)
6	isorhamnetin 3-*O*-rhamnosylglucoside	803 ± 105 (894 ± 117)
7	quercetin malonylglucoside	147 ± 26 (164 ± 29)
8	undetermined (quercetin derivative)	50 ± 9 (56 ± 10)
9	isorhamnetin 3-*O*-glucoside	250 ± 61 (278 ± 68)
10	methyl-*O*-quercetin malonylglucoside (1)	178 ± 31 (198 ± 34)
11	methyl-*O*-quercetin rhamnosylglucoside	244 ± 31 (272 ± 34)
12	methyl-*O*-quercetin glucoside (1)	33 ± 8 (37 ± 9)
13	rhamnazin X-glucoside	121 ± 6 (135 ± 7)
14	rhamnazin rhamnosylglucoside	972 ± 21 (1083 ± 23)
15	methyl-*O*-quercetin glucoside (1)	539 ± 36 (600 ± 40)
16	rhamnazin X-(malonylglucoside)	133 ± 22 (149 ± 25)
16	methyl-*O*-quercetin malonylglucoside (2)	606 ± 19 (675 ± 22)
18	rhamnazin glucoside	442 ± 39 (492 ± 43)
19	undetermined (quercetin derivative)	119 ± 21 (132 ± 23)
20	methyl-*O*-quercetin acetylglucoside	94 ± 20 (105 ± 22)
21	rhamnazin malonylglucoside (1)	797 ± 12 (888 ± 13)
22	rhamnazin malonylglucoside (2)	35 ± 2 (39 ± 3)
23	rhamnazin acetylglucoside	186 ± 31 (207 ± 34)
24	isorhamnetin (3′-*O*-methylquercetin)	21 ± 3 (23 ± 4)
25	rhamnetin (7-*O*-methylquercetin	35 ± 1 (39 ± 2)
26	rhamnazin(7,3′-di-*O*-methylquercetin)	22 ± 2 (24 ± 3)
	total flavonoid (FW)	7759 μg

[a] Where pure standards were not available, quercetin flavonoids are expressed as quercetin 3-*O*-Glc equivalents, and isorhamnetin, rhamnetin, and rhamnazin flavonoids are expressed as isorhametin 3-*O*-Glc equivalents. X = arabinose or xylose based on MS data.

of *N*-methoxy-3-indolylmethylglucosinolate as compared with roots from other glucosinolate-containing species, for example, turnip (total indole, 250 μg/g FW) and rutabaga (total indole, 171−255 μg/g FW) (*10*). The content of *N*-methoxy-3-indolyl-methyl-glucosinolate in *A. tetracantha* seeds is also considerably greater than indole glucosinolate levels found in the seeds of broccoli (4-hydroxy-3-indolylmethylglucosinolate, 3514−8233 μg/g FW) and other *Brassica*/crucifer species (*21*, *29*).

Root, stem, and leaf tissues also contained neoascorbigen, the condensation product of *N*-methoxy-indole-3-carbinol and ascorbic acid (**Table 1**). The carbinol precursor was probably derived from catabolism of *N*-methoxy-3-indolylmethylglucosinolate. Ascorbigens are usually formed after cell damage, for example, by autolysis of crucifer tissues containing indole glucosinolates (*23*). However, in *A. tetracantha*, it appears to be a significant component in undamaged leaves. The leaves that were extracted showed no symptoms of gross damage, neither bacterial nor fungal infection, nor insect predation or physical/chemical damage. Because ascorbigens were not detected in extracts prepared in the same way from other species (*21*, *22*, *27*), including those containing *N*-methoxy-3-indolyl-methylglucosinolate, the neoascorbigen in *A. tetracantha* does not appear to be an extraction artifact. Autolysis of fresh *A. tetracantha* roots only generated *N*-methoxy-indole-3-acetonitrile (**Figure 2**). No carbinol or carbinol condensation products, such as 3,3′-di[*N*-methoxy]indolylmethane, other than the neo-ascorbigen already present in the tissue, were detected after autolysis.

The seeds of *A. tetracantha* contained a significant concentration of total flavonoids (7.76 mg/g FW) with a complex profile (**Table 1** and **Figure 3**). Hydrolysis of these flavonoids produced only four aglycones that were identified as quercetin, isorhamnetin (3′-*O*-methylquercetin), rhamnetin (7-*O*-methylquercetin), and rhamnazin (7,3′-di-*O*-methylquercetin) (**Figure 3**). LC-MS analysis of unhydrolyzed extracts showed that the seed flavonoids consisted of various glycosides, malonyl-glycosides, and acetyl-glycosides (**Figure 3**; Supporting Information). The previous report of isorhamnetin 3-*O*-rutinoside in the leaves of *A. tetracantha* could not be confirmed (*15*). Young and old leaves and stem tissues did not contain detectable levels of flavonoids by LC-MS, based on analyses of separate plants of various ages (data not shown). There are no reports on the flavonoid content of seeds of Capparales species, so comparisons with *A. tetracantha* seeds are not possible. Quercetin-derived flavonoids, quercetin methyl-ethers, and their glycosides are found in many plant species, including crucifer species (*30*−*33*). Many of these methylated flavonoids, such as free aglycones, have been found on the surfaces of leaves and flowers of species belonging to the Asteraceae (*34*). Quercetin and isorhamnetin are commonly occurring plant flavonoids (flavonols) and are present in many vegetable crops, herbs, and medicinal plants consumed by humans. However, rhamnetin and rhamnazin are not common in crucifers and are also less

Table 3. LC-MS Data for Glucosinolates, Including Indole Glucosinolate Standards, Glucosinolate-Derived Compounds, and the Dimeric Piperidine Alkaloids[a]

compound	R_T (min)	ion			other ions
glucosinolates (ES$^-$MS)		**[M – H]$^-$**	**[SO$_4$H]$^-$**	**[SO$_4$]$^-$**	
3-indolylmethyl (also confirmed with standard)	15.2	447 (100%)	62	20	
N-hydroxy-3-indolylmethylglucosinolate	13.4	463 (39%)	100	6	
4-hydroxy-3-indolylmethylglucosinolate (standard)	11.0	463 (39%)	100	12	493 (22), 477 (32)
4-methoxy-3-indolylmethylglucosinolate (standard)	19.3	477 (100%)	64	21	
N-methoxy-3-indolylmethylglucosinolate (also confirmed with standard)	22.3	477 (32%)	100	30	446 (10)
glucosinolate-derived compounds (ES$^+$MS)		**[M + Na]$^+$**	**[M + H]$^+$**		
neoascorbigen	28.8	358 (100%)	336 (18)		305 (14, [(M – OCH_3) + H]$^+$) 160 (91, [(M – $C_6H_7O_6$) + H]$^+$) 130 (12, [(305 – $C_6H_7O_6$) + H]$^+$)
N-MeO-indole-3-acetonitrile	30.8	209 (4%)	187 (66)		160 (100, [M – HCN + H]$^+$) 147 (42, [(M – C_2H_2N) + H]$^+$)
dimeric piperidine alkaloids (ES$^+$MS)		**[M + Na]$^+$**	**[M + H]$^+$**		
azimine	23.1	ND	423 (100%)		212 (10, [half molecule + H]$^+$)
azcarpine	24.9	ND	451 (100%)		240 (5, [half molecule1 + H]$^+$) 212 (3, [half molecule2 + H]$^+$)
carpaine (*A. tetracantha*)	28.4	ND	479 (100%)		240 (24, [half molecule + H]$^+$)
carpaine (*C. papaya* young leaves)	28.3	ND	479 (100%)		240 (31, [half molecule + H]$^+$)

[a] ND = not detected in this tissue.

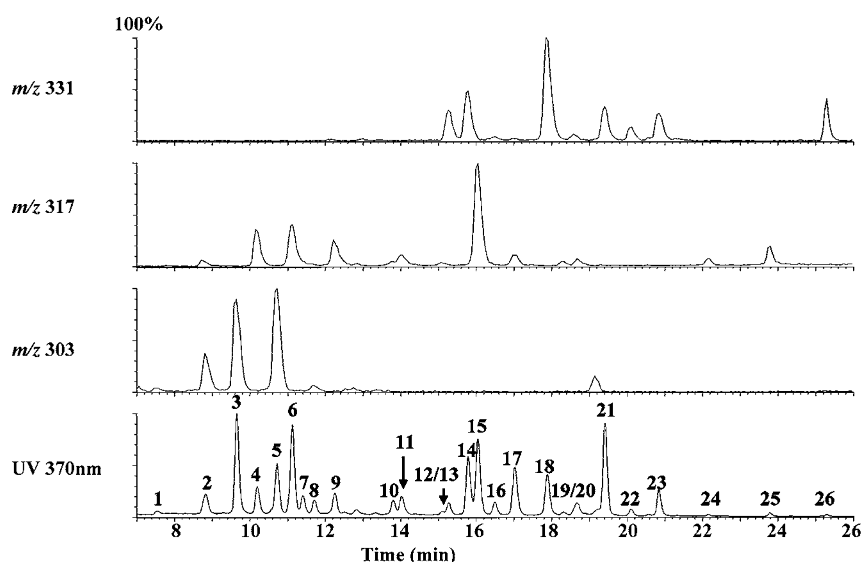

Figure 3. LC-MS of flavonoid glycosides in seeds of *A. tetracantha*. For peak identification, see **Table 2**. The data presented are the analogue trace (370 nm) and selected masses from the full scan data: *m*/*z* = 303 (quercetin flavonoids), *m*/*z* = 317 (methyl-*O*-quercetin flavonoids), and *m*/*z* = 331 (rhamnazin flavonoids).

common in major vegetable crops, but they are present in herbs, medicinal species, and ornamental species from other families (*35*−*38*). The cooccurrence of rhamnetin, isorhamnetin, and rhamnazin, or rhamnetin with rhamnazin, has been reported for several species (*35*−*38*). Other di-*O*-methylquercetin ethers derived from rhamnetin (predominantly 3,7-di-*O*-methylquercetin) are common but are less commonly derived from isorhamnetin (e.g., 3,3′-di-*O*-methylquercetin [rare]; 3′,4′-di-*O*-methylquercetin [rare]) (*32*). Anthocyanins (LC with 520 nm monitoring and LC-MS) and procyanidins (LC with fluorescence detection including comparison with standards) were not detected in any of the *A. tetracantha* tissues using previously described methods (*20*).

A. tetracantha leaves have previously been shown to contain dimeric piperidine alkaloids: azimine, azcarpine, and carpaine (*17*) (**Figure 1**). From the full scan data, no other alkaloids or nitrogenous secondary metabolites were detected in *A. tetracantha* tissues. Carpaine has also been identified in the leaves of *C. papaya*, as well as two structurally related alkaloids dehydrocarpaine I and II (*39*). The three *Azima* alkaloids were found in all tissues, but the highest concentration was in the aerial tissues and seeds (**Table 1**). Alkaloids have been found in several other plants from the Capparales, although their distribution is not uniform nor is it restricted to particular families in the Capparales: macrocyclic polyamine alkaloids in *Capparis* (Capparaceae), *Codonocarpus* (Gyrostemonaceae), and *Lunaria* (Cruciferae) species; piperidine alkaloids in *A. tetracantha* (Salvadoraceae) and *C. papaya* (Caricaceae); monomeric and dimeric imidazole alkaloids in *Lepidium sativum* and *Lepidium meyenii* (Cruciferae); cochlearine (*m*-hydroxybenzoyloxytropane), hygrine, and hygroline in *Cochlearia* species (Cruciferae) (*40*−*43*). Piperidine alkaloids such as coniine from hemlock (*Conium maculatum*) have long been used for the alleviation of pain, and in general, alkaloids have several important pharmaceutical uses, for example, as analgesics, regulating cardiac functions (by interacting with receptors for

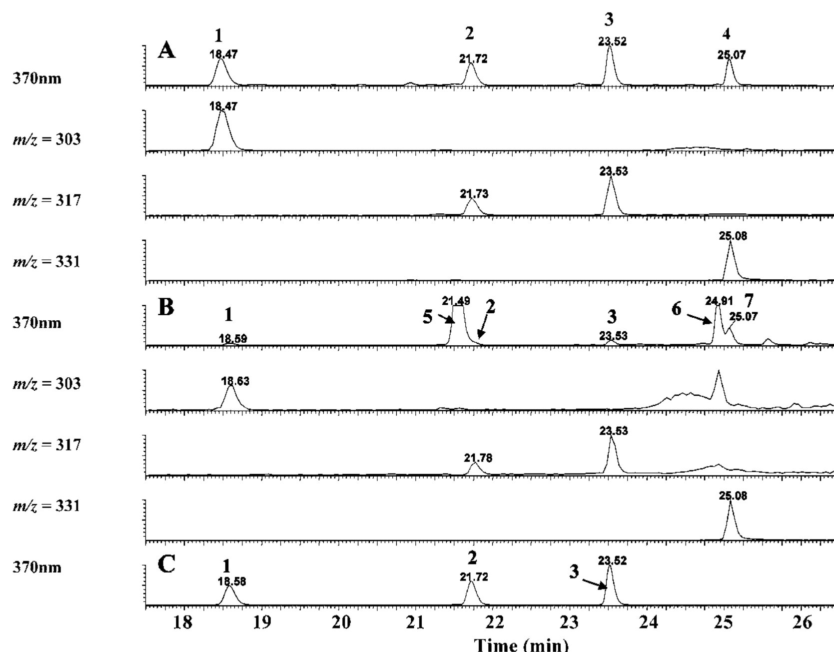

Figure 4. LC-MS of flavonoid aglycones produced from the hydrolysis of the *A. tetracnatha* seed extract (**A**) and comparison with a hydrolyzed extract from *R. alaternus* young leaves (**B**) and commercial standards of quercetin, isorhamnetin, and rhamnetin (**C**). The data presented in panel **A** are the analogue traces (370 nm) for the hydrolyzed seed flavonoids and *m*/*z* = 303 (quercetin), *m*/*z* = 317 (mono-*O*-methylquercetin), and *m*/*z* = 331 (di-*O*-methylquercetin). (**B**) The analogue trace (370 nm) and *m*/*z* = 303, 317, and 331 for the *R. alaternus* extract. (**C**) Only the analogue trace (370 nm) for the standards. Peak ID: 1, quercetin; 2, isorhamnetin; 3, rhamnetin; 4, rhamnazin; 5, kaempferol; and 6, rhamnocitrin (7-methoxy-kaempferol).

dopamine, acetylcholine, serotonin, and cellular calcium transporters), and as therapeutic agents for cancers such as leukaemia (*44*). Others are key flavor components of spices, for example, piperine in pepper (*Piper nigrum* L.) (*45*). Papers reporting alkaloids in Capparales species have rarely presented quantitative data and often do not define the tissue from which the alkaloids were isolated. The carpaine concentration in *C. papaya* leaves was 400 μg/g dry weight (DW) tissue (approximately 4 μg/g FW) (*39*). The highest total piperidine alkaloid content of leaves from *Piper methysticum* was 54.6 mg/g DW (approximately 546 μg/g FW) (*46*).

In conclusion, *A. tetracantha* has a simple glucosinolate profile but has additional complexities with the presence of flavonoids and alkaloids. Although there are several species with a single predominant glucosinolate, they are commonly aliphatic or aromatic glucosinolates rather than substituted indole glucosinolates (*10*−*12*, *21*, *22*). In addition, the high concentration and complexity of the flavonoids, predominant in seed tissues, is unusual. Choline esters of hydroxycinnamic acids, e.g., sinapine, are common in seeds of many glucosinolate-containing species but were absent from *A. tetracantha* seeds. The root tissue of *A. tetracantha* was a rich source of *N*-methoxy-3-indolyl-methylglucosinolate with only negligible concentrations of other secondary metabolites such as flavonoids and alkaloids. This tissue is an ideal source for rapid purification of this indole glucosinolate.

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Supporting Information Available: LC-MS data for flavonoids in seeds of *A. tetracantha*. This material is available free of charge via the Internet at http://pubs.acs.org.

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