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Glucosinolate Profile of Croatian Stenoendemic Plant *Fibigia triquetra* (DC.) Boiss. ex Prantl.

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Abstract: As part of our ongoing investigation of the stenoendemic plants belonging to the Brassicaceae family, we report on the chemistry of *Fibigia triquetra* (DC.) Boiss. ex Prantl for the first time. Different plant parts (flower, leaf, stem, and seed) of *F. triquetra* were characterized and quantified for glucosinolates (GLs) according to the ISO 9167-1 EU official method based on the HPLC analysis of desulfo-GLs. A taxonomic screening showed that *F. triquetra* contained relatively high levels of C-4 GLs, namely but-3-enyl GL (gluconapin, **1a**), 4-methylsulfanylbutyl GL (glucoerucin, **3a**), and 4-methylsulfinylbutyl GL (glucoraphanin, **5a**). GC-MS analysis of the volatile fractions obtained after enzyme hydrolysis and/or HPLC-ESI-MS of intact GLs confirmed the GL profile. Four minor GLs, namely isopropyl GL (glucoputranjivin, **6a**), *sec*-butyl GL (glucocochlearin, **7a**), pent-4-enyl GL (glucobrassicanapin, **2a**), and 5-methylsulfanylpentyl GL (glucoberteroin, **4a**) were also identified and quantified while 4-methylpentyl GL, 5-methylhexyl GL, and *n*-heptyl GL, were tentatively identified by GC-MS of their degradation products. Based on the major, as well as the minor GLs, this study shows differences in chemotaxonomy between *F. triquetra* and the related *Degenia velebitica* (Degen) Hayek as well as other investigated species of the genus *Fibigia*.

Keywords: *Fibigia triquetra*, Brassicaceae, glucosinolates, desulfo-glucosinolates, isothiocyanates.

Abbreviations: GL: glucosinolate; DS-GL: desulfo-glucosinolate; NT: near threatened; ITC: isothiocyanate; GC-MS: gas chromatography-mass spectrometry; HPLC-DAD: high-performance liquid chromatography - diode array detector; HPLC-ESI-MS: high-performance liquid chromatography – electrospray mass spectrometry; RPF: relative proportionality factor; GRA: glucoraphanin; GPU: glucoputranjivin; GNA: gluconapin; GCC: glucocochlearin; GBN: glucobrassicanapin; GER: glucoerucin; GBE: glucoberteroin; GAL: glucoalyssin; Met: methionine; Leu: leucine; Tyr: tyrosine; Val: valine; DNA: deoxyribonucleic acid; ITS: internal transcribed spacer; a.s.l.: above sea level.

INTRODUCTION

CROATIA is hosting significant populations of many plant species that are threatened at the European level. One of the reasons for the large number of endemics in Croatia, and specially tertiary relics, is the fact that this area was not greatly affected by glaciation. Among the 13 known species in the genus *Fibigia* (Brassicaceae),[1] two wild-growing are known in the Flora of Croatia, namely *Fibigia clypeata* (L.) Medik. and *Fibigia triquetra* (DC.) Boiss. ex Prantl. The latter species is a rare Croatian paleostenoendemic plant species included in the Croatia Red Book in the category of near threatened (NT) plants.[2,3] *F. triquetra* is a perennial plant, characterized by sub-shrubby growth (up to 5–20 cm in height), large yellow flowers, and compact rosettes of hairy, and grey leaves. The fruit forms an elliptical or elongated ellipsoidal silique. The natural area of distribution is restricted to the rocky grounds of Dalmatia[4,5] on altitudes ranging from 10 to 1000 m above sea level (a.s.l.). There is a striking similarity in morphology, anatomy, and taxonomy between *F. triquetra* and *Degenia velebitica* (Degen) Hayek, which was reported as a Croatian stenoendemic chasmophytic herbaceous plant.[6–9] According to a phylogenetic relationship study, *F. triquetra* seems to be closer to *D. velebitica* than any other *Fibigia* species.[10] The restriction fragment length PCR amplified ribosomal DNA (ITS regions),

as well as the size of the genome types of *F. triquetra* and *D. velebitica* were determined and compared. These analyses indicated that *F. triquetra* varied from *D. velebitica.*

Studies based on non-morphological characters, such as chemical features together with other biological or genetic informations can help in discriminating species and understanding real relationships among the taxa. The plants of the Brassicaceae family are strikingly chemocharacterized by the presence of thiosaccharidic secondary metabolites called glucosinolates (GLs). GLs are genetically variable within plant species. They share a common structure consisting of a β-D-glucopyrano unit bearing a *NO*-sulfated anomeric thiohydroximate function connected to a variable aglycon side chain derived from an alpha-amino acid. The chemistry of *F. triquetra* has never been investigated, and thus its GL composition is not established. As a matter of fact, information on GLs present in other plants of the genus *Fibigia* are very scarce and only include early approaches regarding the characterization of the GLs present, *i.e.* comparison with the authentic sample on TLC, paper chromatography, and GC-MS analysis of their degradation products - which are mostly isothiocyanates (ITCs). *F. eriocarpa* (DC.) Boiss. seeds were reported to contain 3-methylsulfinyl propyl GL (glucoiberin, GIB), *p*-hydroxybenzyl GL (sinalbin, SNB), and benzyl GL (glucotropaeolin, GTL).[11] Pent-4-enyl GL (glucobrassicanapin, GBN, **2a**) (Figure 1) and 2-hydroxy-3-butenyl GL (progoitrin, PRO or epiprogoitrin, EPRO) were reported in the seeds of *F. clypeata* (L.) Medik.[12] and *F. macrocarpa* (Boiss.) Boiss.[12,13] Next to those GLs, *F. macrocarpa* was reported also to contain but-3-enyl GL (gluconapin, GNA, **1a**) 4-methylsulfanylbutyl GL (glucoerucin, GER, **3a**) and 4-methylsulfinylbutyl GL (glucoraphanin, GRA, **5a**).[12] Bennett *et al.*[14] used ion-pairing LC-MS methodology for the identification of the GLs in *F. clypeata* seeds, which confirmed the previous report of GLs, *i.e.* EPRO (100–125 μmol g^{-1} of dry weight) and PRO (0.1–10 μmol g^{-1} of dry weight). Conversely to GC-MS and other techniques previously mentioned, this latter method ensured accurate measurement of all classes of GLs.[14]

All previous reports dealt with GLs in *Fibigia* seeds. However, the nature of GLs and their relative amounts can vary greatly with plant species and variety, tissue type (seed, root, stem, leaf, flower) and developmental stage of the tissue.[8,15,16]

Thus, the aim of the present study was to investigate the qualitative and quantitative GL profile in the various aerial parts (flower, leaf, stem, seed) of *F. triquetra*. The identification and quantification were performed by HPLC-DAD of the desulfo-glucosinolates (DS-GLs) and comparison with standards. The analyses were confirmed by direct HPLC-ESI-MS analysis of intact GLs and/or indirectly by GC-MS of their breakdown products.

EXPERIMENTAL

General

DS-GLs were analyzed on HPLC Agilent model 1100 (New Castle, Delaware, USA) equipped with a diode array detector (DAD) and an Inertsil ODS-3 column (250 × 3 mm, particle size 5 μm,) thermostated at 30 °C. Intact GLs were analyzed on a HPLC Agilent model 1100 equipped with a quaternary pump, automatic injector, diode-array detector (wavelength range 190–600 nm) degasser, and a Hypersil ODS column (200 × 4.6 mm, particle size 5 μm). The HPLC was interfaced to an Agilent model 6120 mass spectrometer (Toronto, ON) with a Chemstation data system LC-MSD B.03.01. GC analyses were performed with a Varian model 3900 system (Varian Inc., Lake Forest, CA, USA) equipped with a Varian mass spectrometer model 2100T, non-polar capillary column VF-5MS (30 m × 0.25 mm i.d., coating thickness 0.25 μm; Varian Inc.). Homogenization was effected by U-Turrax (IKA T25) homogenizer.

All the solvents employed were purchased from Fluka Chemie, Buchs, Switzerland. Anhydrous sodium sulfate was obtained from Kemika, HR-Zagreb, DEAE-Sephadex A-25 anion-exchange resin from GE Healthcare). Enzymes thioglucosidase (myrosinase EC 3.2.1.147; 361 U/g) from *Sinapis alba* seeds and sulfatase Type H-1 from *Helix pomatia* were purchased from Sigma-Aldrich Chemie GmbH, D-Steinheim. Glucoraphanin and gluconapin were purchased from Chromadex, USA, glucoerucin from CFM Oskar Tropitzsch, Germany, while other GLs and DS-GLs were available as pure standards isolated in the laboratory.[17,18]

Plant Material

The aerial parts (leaf-flower, stem, and seed) of *Fibigia triquetra* (DC.) Boiss. ex Prantl were collected on the island of Brač (Mt. Vidova Gora, 770 meters a.s.l.; Gauss-Kruger coordinates *X* = 5631845; *Y* = 4794051) - near Split, during flowering in March (flower, leaf, stem) and June (seed) in 2011 from wild-growing populations. The botanical identity of the plant material was confirmed by the local botanist Dr. Mirko Ruščić, and voucher specimens (no. DBFT001) have been deposited at the Department of Biology, Faculty of Sciences, Split, Croatia.

HPLC of DS-GLs and GLs

EXTRACTION OF GLS AND DESULFATION

GLs were extracted from the different plant parts of *F. triquetra* (leaf-flower, stem, and seed) according to the EU standard procedure,[19] albeit with some modifications.[15] Plant samples were reduced to a fine powder. Samples of *ca* 500 mg were extracted for 5 min at 80 °C in 2 × 5 mL EtOH–H_2O (70 : 30 *v*/*v*), homogenized and then centrifuged.

Supernatants were combined, and the final volume was measured. Each extract (1 mL) was loaded onto a mini-column filled with 0.6 mL of DEAE-Sephadex A-25 anion-exchange resin conditioned with 25 mM acetate buffer (pH 5.6). After washing with 3 mL buffer, 200 μL (0.35 U mL^{-1}) of purified sulfatase[20] was loaded onto the mini-column which was left on the bench overnight. The DS-GLs were then eluted with 3 mL of ultra pure H_2O and were analyzed by HPLC-DAD.

In addition, seeds (540 mg) were frozen in liquid N_2 and ground with a mortar and pestle. The powder was extracted for 5 min at 80 °C in 2 × 5 mL EtOH–H_2O (70:30 *v*/*v*). The solutions were combined and evaporated under reduced pressure and intact GLs were analyzed by HPLC-ESI-MS.

HPLC-DAD ANALYSIS

The chromatography of DS-GLs (20 μL injected solution) was performed with an *Inertsil ODS-3* column at a flow rate of 1 mL min^{-1} eluting with a gradient of H_2O (A) and acetonitrile (B) following the program: 1 min 1 % B; 22 min linear gradient up to 22 % B; 3 min linear gradient down to 1 % B. DS-GLs were detected monitoring the absorbance at 229 nm.[15]

HPLC-ESI-MS ANALYSIS

The extract (121.3 mg) was dissolved in 4 mL EtOH–H_2O (70 : 30 *v* / *v*)and filtered through a plug of cotton prior to HPLC analysis, which was performed by injecting a 5 μL aliquot of the solution of crude extract into HPLC-ESI-MS. The two mobile phase solvents, MeOH and H_2O, were prepared with 0.15 % Et_3N and 0.18 % HCO_2H, added as ion-pairing reagents. Both solutions were filtered using 0.45 μm nylon membranes. The initial mobile phase was 100 % HPLC-grade H_2O. At 10 min, the mobile phase was switched to a linear gradient of 100 % H_2O to 100 % MeOH over 60 min. After each run, the initial mobile phase conditions were set and the system was allowed to equilibrate. The flow rate was kept constant at 1 mL min^{-1}. The column was maintained at room temperature.

The electrospray interface was a standard ES source operating with a capillary voltage of 4 kV and temperature of 350 °C. The system was operated in the negative and positive ion electrospray modes. Nitrogen was used as nebulizing and drying gas at a flow rate of 10 L min^{-1} (35 psig). The mass spectrometer was programmed to perform full scans between m/z 100 and 1,000.[21]

GC-MS Analysis of GL Breakdown Products

ENZYMATIC HYDROLYSIS OF GLS AND EXTRACTION

Crushed and dried flower with leaf (10 g), stem (10 g), and seed (1 g), were homogenized separately with deionised H_2O (100 mL, pH ~ 6) and myrosinase (1–2 units, Sigma), then allowed to hydrolyze during 17 h at room temperature (*ca* 30 °C). Sufficient redistilled CH_2Cl_2 (3 × 20 mL) was then added, the mixtures were shaken for 30 min and separated by centrifugation for 5 min at 4,000 rpm. The separated organic layer was dried over anhydrous sodium sulfate and concentrated to 100 μL. All the obtained hydrolyzates were kept (in a tightly closed vial) in a freezer at –20 °C until GC-MS analysis.[22]

GC-MS ANALYSIS

Chromatographic conditions were as follows: helium was the carrier gas at 1 mL min^{-1}, injector temperature was 250 °C. VF-5MS column temperature was programmed at 60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. The injected volume was 1 μL and the split ratio was 1:20. MS conditions were: ionization voltage 70 eV; ion source temperature 200 °C; mass scan range: 40–350 mass units. The analyses were carried out in duplicate.[23]

Identification and Quantification

The identification of DS-GLs was performed on the basis of the retention time and UV spectra of each DS-GL compared with pure standards.[20] The GL amount was quantified by using a calibration curve of pure DS-sinigrin solution (range from 0.14 to 1.4 mM, $y = 36.3 + 5854.3 \cdot x$, $R^2 = 0.9998$, LOD (limit of detection) 0.013 mM, LOQ (limit of quantitation) 0.041 mM. LOD and LOQ were both determined based on the DS-sinigrin calibration curve according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods[24]) and RPFs for each individual DS-GL. The published RPFs for DS-GLs[18,25] were used, with the exception of **5b**, for which an arbitrary RPF value equal to 1 was set.

Peaks of the intact GLs, **1a**, **3a**, and **5a**, were identified by comparison of UV spectra, retention times and mass spectra of commercial standards in the LC-MS library. Glucoberteroin (5-methylsulfanylpentylGL, GBE, **4a**) was identified by comparison of the UV spectrum, retention time and mass spectrum with those of a previously isolated GBE stored in the LC-MS library.[17]

Individual peaks of volatiles were identified by comparing their retention indices and mass spectra to those of authentic samples, as well as by computer matching against the Wiley 275-library spectra database and comparison of the mass spectra with literature data.[26] The percentages in Table 2 and 3 were calculated as the mean value of component percentages on column VF-5MS column for analyses run in duplicate.

RESULTS

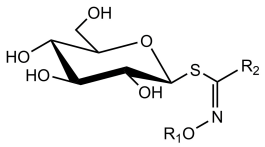
The aerial parts of *Fibigia triquetra* leaf - flower, stem and seed were analysed for GL identification and quantification. The extractions were made according to the EU official method. Each extraction was performed in duplicate. The structures of the major GLs and their DS-counterparts are given in Figure 1. The obtained HPLC chromatograms are given in Figure 2 and the GL contents are summarised in Table 1. The identity of each DS-GL was determined by the comparison of the t_R and UV spectra of each product with those of DS-GL standards.

The leaf - flower extract and the stem extract of *F. triquetra* showed almost the same DS-GL chromatographic qualitative profile. DS-GNA (**1b**) - the major DS-GL in both samples - DS-GER (**3b**) and DS-GRA (**5b**) were identified at t_R 9.4, 5.2, and 14.3 min, respectively. In contrast to other plant parts analysed, the major DS-GL identified in seed extracts was **3b**, followed by **1b** and **5b**.

The other minor DS-GLs present in all the samples were DS-GPU (desulfo isopropyl GL, **6b**) and DS-GCC (desulfo *sec*-butyl GL, **7b**). In addition, DS-GBN (desulfo pent-4-enyl GL, **2b**) was detected in the stem, and DS-GBE (desulfo 5-methylsulfanylpentyl GL, **4b**) in the seed only. The structures of those minor GLs and their DS-counterparts are also given in Figure 1. The HPLC chromatograms showed also unidentified peaks at 20.1 and 20.9 min (seed extract) as well as at 21.6 min (flower - leaf extract) which did not match with any of our available standards.

For GL quantification, we used the relative proportionality factors (RPFs) reported in the literature.[18,24] Since the RPFs for DS-GCC and DS-GBE are not reported, we used an arbitrary RPF value of 1 for DS-GCC, whereas for DS-GBE the RPF of DS-GER was used. The results for quantification are given in Table 1. In comparison to the stem extract, the leaf - flower extract showed a similar content of the major GLs, *i.e.* 77.9 and 68.2 % of **1a**, 12.8 and 15.4 % of **5a**, and 5.6 and 8.7 % of **3a**, respectively. The seed contained 56.6 % of **3a**, 24.1 % of **1a**, and 17.3 % of **5a**. The total GL content is strikingly high in all aerial parts, with the highest content in the seed. It is worth mentioning that the upper plant parts (leaf - flower and stem) have a 2–3 fold higher content of **5a** than **3a**, while this content in the seed is *vice versa*. This phenomenon was previously reported for *D. velebitica*[8] and *Raphanus sativus* L.[27] and one could speculate a biological oxidation of **3a** to **5a** during the sprouting of the seeds.

The identification of GLs was confirmed by the GC-MS analysis of the corresponding volatile degradation products resulting from enzymatic hydrolysis. The most common breakdown products are ITCs, which are characterised by odd mass of the molecular ion, and a



	R_1	R_2		R_1	R_2
1a	SO_3^-	CH_2=CH–$(CH_2)_2$	**1b**	H	CH_2=CH–$(CH_2)_2$
2a	SO_3^-	CH_2=CH–$(CH_2)_3$	**2b**	H	CH_2=CH–$(CH_2)_3$
3a	SO_3^-	CH_3–S–$(CH_2)_4$	**3b**	H	CH_3–S–$(CH_2)_4$
4a	SO_3^-	CH_3–S–$(CH_2)_5$	**4b**	H	CH_3–S–$(CH_2)_5$
5a	SO_3^-	CH_3–SO–$(CH_2)_4$	**5b**	H	CH_3–SO–$(CH_2)_4$
6a	SO_3^-	CH_3–CH(CH_3)	**6b**	H	CH_3–CH(CH_3)
7a	SO_3^-	CH_3–CH_2–CH(CH_3)	**7b**	H	CH_3–CH_2–CH(CH_3)

Figure 1. Chemical structures of GLs in *F. triquetra*: gluconapin (**1a**), glucobrassicanapin (**2a**), glucoerucin (**3a**), glucoberteroin (**4a**), glucoraphanin (**5a**), glucoputranjivin (**6a**), glucococchlearin (**7a**), and their desulfo-counterparts (**1b–7b**).

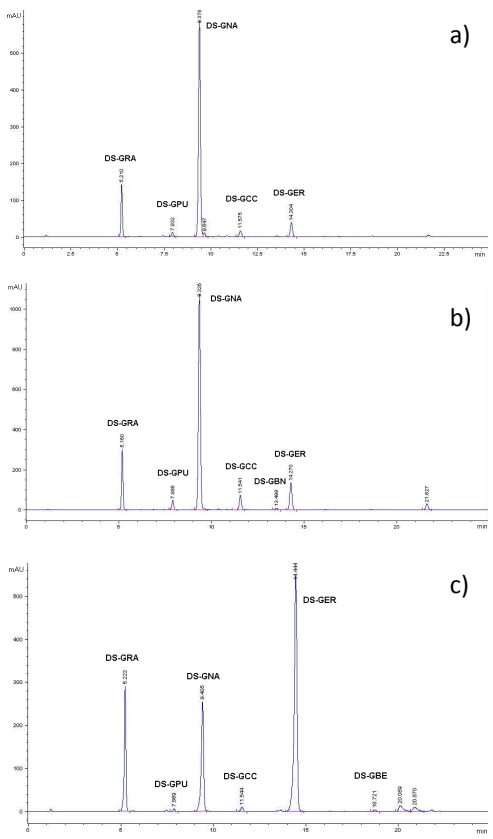


Figure 2. HPLC-DAD chromatograms of DS-GLs isolated from a) leaf and flower; b) stem; c) seed of *F. triquetra*. Peaks correspond to GLs, as follows: DS-GRA, desulfo-glucoraphanin; DS-GPU, desulfo-glucoputranjivin; DS-GNA, desulfo-gluconapin; DS-GCC, desulfo-glucococchlearin; DS-GBN, desulfo-glucobrassicanapin; DS-GER, desulfo-glucoerucin; DS-GBE, desulfo-glucoberteroin.

Table 1. GL content of leaf-flower, stem, and seed in *F. triquetra*.

Glucosinolates[a]	Leaf-flower	Stem	Seed	LC-MS $[M]^{-}$[b]
Glucoraphanin (**5a**)	8.0 ± 1.7 [c]	15.1 ± 3.0	23.4 ± 1.6	436.0
Glucoputranjivin (**6a**)	0.9 ± 0.1	2.5 ± 0.1	0.7 ± 0.1	–
Gluconapin (**1a**)	48.7 ± 1.7	66.7 ± 3.4	32.6 ± 1.1	372.0
Glucocochlearin (**7a**)	1.4 ± 0.1	4.3 ± 0.1	1.3 ± 0.1	373.8
Glucobrassicanapin (**2a**)	–	0.7 ± 0.0	–	–
Glucoerucin (**3a**)	3.5 ± 0.2	8.5 ± 0.5	76.7 ± 2.4	420.0
Glucoberteroin (**4a**)	–	–	0.7 ± 0.1	434.0
Total content (μmol / g dry weight)	62.5 ± 3.8	97.8 ± 7.1	135.4 ± 5.4	
Yield, (*w* / *w*) / %	2.7	4.2	6.1	

[a] GLs are listed according to elution of their corresponding DS-GL on *Inertsil ODS-3* column. An arbitrary RPF value of DS-GL equal to 1 was used only for quantification of **5a**.

[b] $[M]^{-}$ (%): specific product ion for GL identification in ESI^{-}-MS having 100 % abundance.

[c] Value is the mean ± standard error (n = 2).

fragment ion of m/z = 72. Some GLs form unstable ITCs, such as 2-hydroxyalkenyl ITCs which cyclize to oxazolidine-2-thiones, while 4-hydroxybenzyl ITC and the very reactive indole ITCs are degraded into their corresponding alcohols, releasing the thiocyanate ion.[28] Beside identified ITCs, other breakdown products, mostly nitriles, can be helpful in confirming the parent GL identification. Individual GLs identified by GC-MS analysis of their hydrolysis products in different *F. triquetra* plant parts are shown in Table 2.

GC-MS analysis confirmed the presence of the GLs identified by HPLC analysis of the corresponding DS-GLs, through detection of the following ITCs: i) isopropyl- and *sec*-butyl ITCs, originating from the branched GLs, **6a**, and **7a**, respectively; ii) but-3-enyl- and pent-4-enyl ITCs, from olefinic GLs **1a** and **2a**; and iii) 4-methylsulfanylbutyl- (*erucin*), 5-methylsulfanylpentyl- (*berteroin*) and 4-methylsulfinylbutyl (*sulforaphane*) ITCs confirming **3a**, **4a**, and **5a**, respectively. In addition to the present ITCs, 5-(methylsulfanyl)pentanenitrile confirmed the presence of **3a**. GC-MS analysis was particularly useful for the correct identification of two minor peaks, namely **6a** and **7a**. Three additional minor ITCs, 4-methylpentyl-, 5-methylhexyl-, and *n*-heptyl ITCs were detected via GC-MS analysis. Those GL breakdown products belonging to the saturated C-6 and C-7 aliphatic group were tentatively identified by their t_R and MS spectra.

DS-GLs often bring difficulties in interpreting results of the individual GLs, due to concerns over the impact of pH value, time, and enzyme sulfatase (EC 3.1.6.1) concentration on desulfation products.[18,29] In addition, some GL breakdown products lack volatility or prove unstable in the conditions used during the analysis, and this represents a major drawback of this indirect method.[30] Therefore, a direct analysis of intact GLs present in the seed was performed by LC-MS for more specific and accurate qualitative determination and for better interpretation of analytical results. Specific product ion for GL identification

Table 2. Individual GLs identified by GC-MS analysis of their hydrolysis products in different *F. triquetra* plant parts.

Parent GL Identified compound	RI[a]	Leaf-flower	Stem	Seed
Glucoputranjivin (6a)				
Isopropyl isothiocyanate	836	0.4	1.6	0.9
Glucocochlearin (7a)				
sec-Butyl isothiocyanate	939	–[b]	–	0.7
Gluconapin (1a)				
But-3-enyl isothiocyanate	998	22.9	48.7	32.0
Glucobrassicanapin (2a)				
Pent-4-enyl isothiocyanate	1090	0.2	0.2	0.2
4-Methylpentyl GL				
4-Methylpentyl isothiocyanate	1169	0.2	0.1	0.1
***n*-Heptyl GL**				
n-Heptyl isothiocyanate	1269	tr	0.4	0.3
5-Methylhexyl GL				
5-Methylhexyl isothiocyanate	1276	–	0.2	0.1
Glucoerucin (3a)				
5-Methylsulfanylpentanenitrile	1213	–	tr[c]	tr
4-Methylsulfanylbutyl isothiocyanate (erucin)	1457	0.3	4.7	6.0
Glucoberteroin (4a)				
5-Methylsulfanylpentyl isothiocyanate (*berteroin*)	1542	–	–	0.3
Glucoraphanin (5a)				
4-Methylsulfinylbutyl isothiocyanate (*sulforaphane*)	1791	4.3	5.5	3.3
Group sum / %		28.3	61.4	43.9

[a] RI: Retention indices determined on a VF-5MS capillary column.

[b] –: not detected.

[c] tr: traces.

in ESI⁻-MS, having abundance 100 %, is given in Table 1. The major intact GLs, **1a**, **3a,** and **5a**, as well as **4a** were clearly identified in chromatograms of crude seed extracts. GCC (**7a**) peak, notwithstanding the low amount (1.0 %), was also observed with specific product ion 373.8 (100 %). On the contrary, LC-MS analysis of intact GLs in the seed did not allow to confirm the presence of either **2a** or **6a**, previously identified by their corresponding DS-GLs and ITCs (Tables 1 and 2). Moreover, it was not possible to confirm the tentatively identified 4-methylpentyl-, 5-methylhexyl- and *n*-heptyl GLs hypothesized by GC-MS analysis of ITCs (Table 2). Hence, the three HPLC peaks of DS-GLs at 20.1, 20.9 and 21.6 min could not be assigned (Figure 1).

With the exception of the above-mentioned molecules, all volatile fractions from the investigated species contained compounds devoid of nitrogen or sulfur (Table 3) - mostly fatty acids, esters, alkanes, phenols, phenylpropanoids and related derivatives. The major products belonging to this class were hexadecanoic acid (12.6–19.9 %) and ethyl linoleate (15.0–25.7 %).

Table 3. GC-MS analysis of miscellaneous volatile compounds from different *F. triquetra* plant parts.

Identified compound	RI[a]	Leaf-flower	Stem	Seed
Alkanes				
1-Phenyl-1-propanone	1178	tr[b]	tr	–[c]
Tricosane	2300	0.9	–	0.5
Pentacosane	2500	1.9	–	–
Heptacosane	2700	12.9	2.6	2.6
Octacosane	2800	3.4	0.7	0.5
Phenols, phenylpropane derivatives and related compounds				
2-Phenylethyl alcohol	1133	tr	0.7	–
Eugenol	1366	0.3	0.3	–
4-Hydroxy-3-methoxy benzaldehyde	1422	tr	–	–
Dihydroactinidiolide	1547	–	0.1	0.1
6,10,14-Trimethyl-2-pentadecanone	1838	0.3	–	0.5
Fatty acids and esters				
Octanoic acid	1216	tr	–	–
Nonanoic acid	1310	tr	–	tr
Decanoic acid	1405	tr	tr	tr
Dibutyl phtalate	1861	0.3	0.4	tr
Pentadecanoic acid	1890	tr	tr	0.2
Hexadecanoic acid	2017	19.9	12.6	19.0
Ethyl linoleate	2195	25.7	15.0	23.7
Other compounds				
Dimethyl trisulfide	981	tr	0.1	0.1
Dimethyl tetrasulfide	1228	tr	0.9	1.0
Phytol	2110	0.2	1.9	–
Group sum / %		65.8	35.3	48.2
Total sum / %		94.2	96.9	92.3

[a] Same as in Table 2.
[b] tr: traces.
[c] –: not detected.

DISCUSSION

Glucosinolate profile analyses in the diverse plant tissues of *Fibigia triquetra* revealed aliphatic GLs to be the major ones. The C-4 and C-5 GLs **1a–5a** originate from L-methionine (Met) *via* chain elongation by one carbon atom at a time, while C-3 and C-4 GLs **6a** and **7a** are derived from L-valine (Val) and L-leucine (Leu), respectively. The suggested natural occurrence of C-6 and C-7 GLs, 4-methylpentyl-, 5-methylhexyl-, and *n*-heptyl GLs in a plant also accumulating similar Met-, Val-, and Leu-derived GLs seems likely. Reports of alkyl GLs bearing C-6 and longer chains are scarce and analyses revealing their occurrence are mostly based on GC-MS of the derived ITCs.[31] The presence of Leu-derived 4-methylpentyl GL[32] was previously reported in *Alyssoides utriculata* (L.) Medik., *Raphanus sativus* L., *Eruca sativa* Mill by using GC-MS analysis of the ITC[33–36] and LC-MS analysis of intact GLs.[37,38] Natural occurrence of both 5-methylhexyl- and *n*-heptyl GLs was also inferred from GC-MS analysis of their breakdown products. Val-derived 5-methylhexyl GL[32] was previously inferred from 5-methylhexyl ITC analysis, while identification of *n*-heptyl GL was based on degradation to octanenitrile and *n*-heptyl ITC.[16,33,34] It is worth mentioning that until recent years, *n*-heptyl GL has been considered "unnatural" as claimed for example by Botting *et al*.[39]

The present study has shown that C-4 GLs *i.e.* **1a**, **3a**, **5a** are the major ones in *F. triquetra* whereas a C-5 GL, **4a**, is dominant in *D. velebitica*. In addition, minor GLs can be suggested to be important tags which differentiate *F. triquetra* from *D. velebitica*. Our previous investigation of *D. velebitica* has shown the presence of glucoaubrietin (4-methoxybenzyl GL), as one minor GL present in the seed. Thus it seems that, next to the major Met-derived GLs, *F. triquetra* accumulates also Val- and Leu-derived GLs, whereas *D. velebitica* accumulates Tyr-derived GLs, next to the major Met-derived GLs. Earlier reports on other *Fibigia* species showed prevalence of C-4 GLs such as **1a** and **5a**, but also of (*R*)- and (*S*)-2-hydroxy-3-butenyl GLs (PRO and EPRO), which were not identified in *F. triquetra*.[11–14]

Our group has previously focused on the GL profile of endemic plants of the tribe *Alysseae*, which comprises seven genera: *Alyssoides, Alyssum, Aurinia, Berteroa, Clypeola,*

Degenia and *Fibigia*. Those investigations[8,15,16,31,33] of, namely, *D. velebitica*, *Aurinia sinuata* (L.) Griseb. and *A. leucadea* (Guss.) C. Koch., suggest that species in this tribe represent appropriate sources for Met-derived GLs bearing a C-4 and/or C-5 olefinic aglycon chain (**1a**, **2a**) and/or a thiofunctionalized chain (**3a–5a**, GAL). With high GL-contents ranging from 9.9 to 135.4 μmol g^{-1} of dried material in different plant parts - especially in the seed (over 4.0 % *w*/*w* with the highest, 6.1 % *w*/*w* in *F. triquetra*) - those *Alysseae* are found to represent a good GL source.

CONCLUSIONS

The GLs of an ice-age survived plant *F. triquetra* were analyzed by using a multiple method approach, involving HPLC-DAD analysis of DS-GLs and their comparison to standards, HPLC-ESI-MS analysis of intact GLs and GC-MS of the breakdown products obtained by enzymatic degradation of GLs. This approach established the qualitative and quantitative GL profile of *F. triquetra* and the present study revealed differences in the GL chemistry with the most similar paleostenoendemic plant *Degenia velebitica*, the only species in this genus.

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