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Tentative Characterisation of Iridoids, Phenylethanoid Glycosides and Flavonoid Derivatives from *Globularia alypum* L. (Globulariaceae) Leaves by LC-ESI-QTOF-MS

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ABSTRACT:

Introduction – *Globularia alypum* L., belonging to the Globulariaceae family, is a perennial wild shrub found throughout the Mediterranean area, Europe, and Africa. This plant is widely used to treat many diseases, but no previous work on the phytochemical composition of the Algerian *G. alypum* species has yet been reported.

Objective – To investigate the phytoconstituents of the methanolic extract of *G. alypum* using an LC-ESI-QTOF-MS method.

Methods – Ground air-dried leaves of *G. alypum* were macerated with methanol at room temperature for 24 h. The supernatant was filtered and concentrated to dryness under reduced pressure in a rotary evaporator, and extracts were recovered with methanol and filtered. Afterwards, the *G. alypum* extract was injected into the LC-ESI-QTOF-MS system.

Results – The combined LC–MS/MS led to the tentative characterisation of 63 phytochemicals. In this work, a large number of compounds have been characterised in the leaf-extract analysis of this plant. Among others, 24 iridoids and secoiridoids were found, of which nine compounds have not previously been recorded in *G. alypum*. Also, nine unusual phenylethanoid glycosides were characterised for the first time in this species.

Conclusion – The method used has proved to be a valued tool for the characterisation of a wide range of compounds from *G. alypum* leaves. This work constitutes a detailed investigation of the chemical composition of *G. alypum* leaves, which are widely used in different traditional systems of medicine. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: LC–MS/MS; iridoids; phenylethanoid glycosides; *Globularia alypum*; Plantaginaceae (Globulariaceae)

Introduction

Globularia alypum L., of the family Plantaginaceae (formerly Globulariaceae), is a wild perennial shrub found throughout the Mediterranean area, Europe and northeastern Africa (Elbetieha *et al*., 2000; Es-Safi *et al*., 2005). In the Mediterranean region, there are two *Globularia* species: *G. eriocephala* (Pomel), which is endemic, and *G. alypum* L. (Boutiti *et al*., 2008), which is the subject of this study.

Known locally as 'taselgha', *G. alypum* is one of the most prominent plants in the Algerian folk medicine. Its leaves have been used traditionally as a hypoglycaemic, laxative, diuretic, cholagogue, stomachic, tonic, purgative and sudorific agent, and even as an aphrodisiac (Calis *et al*., 2002a; Jouad *et al*., 2002; Es-Safi *et al*., 2005). Moreover, this plant has been used to treat haemorrhoids and cardiovascular diseases. More recently, it has been found that the extracts from the whole plant reduced histamine and serotonin contraction *in vitro* and were active against lymphocytic leukaemia P-388 and neoplastic cell culture (Fehri *et al*., 1996; Bello *et al*., 2002; Es-Safi *et al*., 2006). It was also found to have anti-viral activity against polio (Soltan and Zaki, 2009). Concentrated decoctions of young branches and leaves are used in the treatment of boils and intermittent fever. Leaves are used to treat rheumatism and arthritis, and they have been found to have anti-tumour effects as well as phytotoxic potential (Fehri *et al*., 1996; Elbetieha *et al*., 2000).

The main biological activities described in *G. alypum* can be attributed to the different bioactive compounds previously reported in this plant, such as phenolic compounds and iridoid glycosides (Es-Safi *et al*., 2005; Taskova *et al*., 2006). The wide use of this plant to treat many diseases in addition to the fact

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that no phytochemical study has been reported on the Algerian *G. alypum* strain prompted us to investigate the chemical composition of this plant matrix.

Reversed-phase high-performance liquid chromatography (RP-HPLC) combined with mass spectrometry (MS) detection is one of the most important techniques used to analyse phenolic compounds. Recently, chromatographic performance has been improved by using columns packed with small particles (smaller than 2 μm) and by operating at a pressure of up to 600 bar, thus offering high resolution (Verardo *et al*., 2010). The on-line coupling of HPLC–MS using electrospray ionisation (ESI) as an interface yields a powerful analytical platform because of its highly efficient resolution and enables the characterisation of a wide range of polar compounds. Electrospray ionisation is one of the most versatile ionisation techniques, as well as being preferred for detecting polar compounds separated by liquid chromatography. The advantages of MS detection include the ability to determine the molecular weight and to gain structural information. Quadrupole time-of-flight (QTOF) MS can provide excellent mass accuracy over a wide dynamic range, allowing measurements of the isotopic pattern, and may be used in MS/MS experiments to provide the elemental composition of the parent and fragment ions. Therefore, QTOF/MS is a powerful detection system for identifying target compounds in highly complex matrices (Gómez-Romero *et al*., 2011).

Searching for new sources from natural plants or resources is of practical interest, as pharmaceutical drugs applied to several diseases are often too expensive and the exploration of traditional remedies constitutes the first resort for a deprived population. In this context, the present work uses the LC–ESI/QTOF/MS method to characterise the constituents of *G. alypum*, one of the most widely utilised herbal remedies in Algeria.

Experimental

Chemicals

All chemicals were of analytical reagent grade and used as received. Sodium hydroxide was from Fluka (Buchs, Switzerland), and acetic acid from Merck (Darmstadt, Germany). The organic solvents, methanol, acetonitrile and 2-propanol were from Sigma Aldrich (St Louis, MO, USA). Distilled water with a resistance of 18.2 MΩ was deionised by using a Milli-Q system (Millipore, Bedford, MA, USA). Filtering the sample prior to injection into the HPLC system utilized Millex filters (0.20 μm pore size; Millipore).

Sample preparation and extraction

The leaves of *G. alypum* were harvested in April 2009, in remote areas near the suburbs of Souk Elbatel, 2 km from Seddouk (city of Bajaia, Algeria). The sample was identified at the Botany Laboratory (University of Bajaia). Voucher specimen (D-PH-2013-37-7) was deposited at the Herbarium of the Natural History Museum of Aix-en-Provence, France. Fresh leaves were air-dried in shade at room temperature. After drying, the plant material was ground to a fine powder (diameter < 250 μm) using an electric mill (IKA^R A11 basic, Staufen, Germany) and 4 g of this powder was exhaustively extracted by maceration with 50 mL of methanol, at room temperature for 24 h.

In all cases the solutions were filtered and concentrated to dryness under reduced pressure in a rotary evaporator (40°C). Stock solutions with concentrations of 1 mg/mL were prepared and filtered through 0.20-μm micropore membranes before analysis.

Liquid chromatography–mass spectrometry

Analyses were performed using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), including a standard autosampler and a diode array detector (DAD). The LC column used was a Zorbax Eclipse Plus C_{18} (150 × 4.6 mm, 1.8 μm).

Separation was performed using as mobile phases aqueous acetic acid 0.5% (v/v) (A) and acetonitrile (B). A gradient programme was used as follows: from 5 to 15% B (0–5 min), from 15 to 30% B (5–25 min), from 30 to 95% B (25–35 min), from 95 to 5% B (35–40 min) and to hold 5% B (40–45 min). The flow rate was established at 0.5 mL/min and column temperature was controlled at 25°C. The LC system was coupled to a microTOF-Q II mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with an ESI interface. The effluent from the RP-HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio 1:2). The MS instrument was operated in the negative ion mode with spectra acquired over a mass range from 50 to 1000 *m/z*. The optimum values of the ESI/MS parameters were: capillary voltage, +4.5/kV; drying gas temperature, 190°C; drying gas flow, 9.0 L/min; and nebulising gas pressure, 2 bars.

During the analyses, external mass-spectrometer calibration was performed using a Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, passing a solution of sodium acetate 5 mM. This external calibration provided accurate mass values for a complete run without the need for a dual sprayer set-up for internal mass calibration.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltoniks), which provided a list of possible elemental formulae by using the Smart Formula Editor. The Editor lists and rates possible molecular formulae consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulae, the TIP adds a second dimension to the analyses, using the masses and intensities of each isotope to make a sophisticated comparison of the theoretical with the measured isotope pattern (mSigma value). The smaller the sigma value and the error, the better the fit, and therefore for routine screening an error of 5 ppm and a threshold sigma value of 0.05 are generally considered appropriate (Abu-Reidah *et al*., 2013).

Results and discussion

The base peak chromatogram (BPC) that resulted for the *G. alypum* extract is depicted in Fig. 1, where the peaks are numbered according to their elution order. A large number of metabolites present in *G. alypum* were identified by interpretation of their MS and MS/MS spectra found by QTOF/MS combined with the data provided in the literature. The MS data of the identified compounds, including experimental and calculated *m/z* for the molecular formulae provided, error, mSigma value and the main fragments shown by MS/MS, as well as the proposed compound for each peak, are summarised in Table 1. The analysis of the methanolic extract by LC–ESI/QTOF/MS revealed that iridoids, secoiridoids and phenylethanoid glycosides were the major classes of secondary metabolites in *G. alypum*.

Iridoids and secoiridoids

Several iridoids and secoiridoids were tentatively identified in *G. alypum* leaves. Peaks **4** (*m/z* 361), **10** (*m/z* 375) and **12** (*m/z* 375) were tentatively identified as catalpol, mussaenosidic acid and (epi)loganic acid, respectively. These compounds were characterised by the common fragments at *m/z* 169 and 151 corresponding to $[(M-H)-162-CH_2O]^-$ and $[(M-H)-162-CH_2O-H_2O]^-$, respectively.

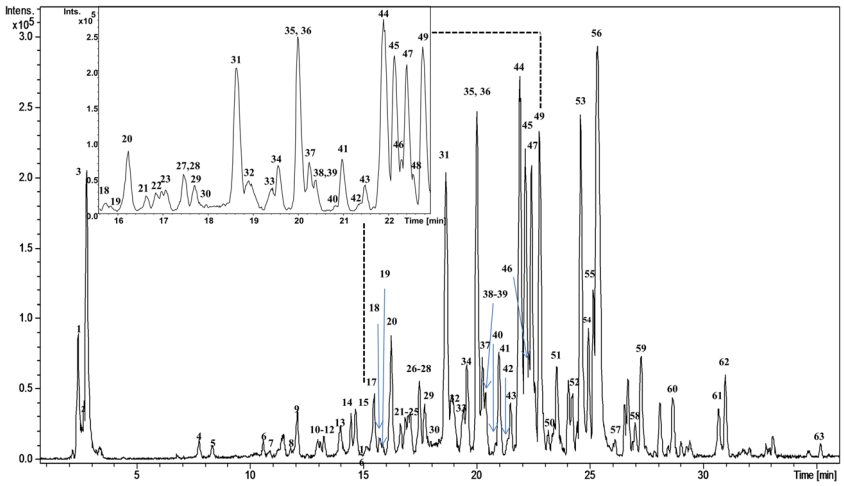

Figure 1. Base peak chromatogram (BPC) of *Globularia alypum* by LC–ESI/QTOF/MS in the negative ion mode. Peak labelling represents the compounds identified.

Compounds **16** (*m/z* 445), 33 (*m/z* 371) and **38** (*m/z* 415) were tentatively proposed as asperulosidic acid methyl ester, deacetylasperuloside and alpinoside, respectively, according to their MS data and information reported in the literature (Calis *et al*., 2001; Ren *et al*., 2007; Hong *et al*., 2010).

Compound **30** with *m/z* 355 was tentatively identified as gentiopicroside (Fig. 2d). Its MS/MS data showed the fragment ions at *m/z* 193 and 175, corresponding to the loss of the hexose moiety and its subsequent dehydration. This constitutes the first report of this secoiridoid in *G. alypum*.

Compounds **6**, **8** and **13** showed the molecular ion at *m/z* 373. In view of the molecular formula provided for their accurate masses and the data given in the literature (Quirantes-Piné *et al*., 2009; Hong *et al*., 2010), they were identified as gardoside and geniposidic acid, although it was not possible to distinguish between them because they showed the same fragmentation pattern. The MS/MS spectra of these compounds showed fragments at *m/z* 211, 167, 149 and 123 corresponding to $[(M-H)-162]^-$ (211) and the loss of CO_2 (167) from the main fragment, as well as the simultaneous elimination of water and CO_2 (149). Another fragment was found at *m/z* 123, corresponding to $[(M-H)-162-88]^-$, which was shown by the loss of a 3-oxopropanic acid molecule. Gardoside and geniposidic acid have been identified in *Globularia* species previously (Calis *et al*., 2001; Taskova *et al*., 2006) but for the first time in *G. alypum*.

Peaks **28** (*m/z* 523) and **42** (*m/z* 537) were tentatively identified as verminoside (Fig. 2f) and minecoside (Fig. 2i), respectively. It was found that fragments obtained from the first compound at *m/z* 179 and 161 corresponded to the caffeic acid moiety and its dehydration product, respectively. Meanwhile, the second compound gave fragments at *m/z* 493, resulting from decarboxylation, and 161 representing the deoxyhexose. Minecoside and verminoside had never been reported in *G. alypum* before.

Furthermore, specioside (peak **34**) was tentatively characterised by comparing its MS data with those reported in the literature (Hong *et al*., 2010). The MS/MS analysis of this compound (Fig. 3a) yielded the fragment ions at *m/z* 163 and 145, corresponding to $[(M-H)-162-182\ Da]$ and $[(M-H)-162-182-H_2O]$, respectively.

Compound **41** was tentatively characterised as globularimin or globularinin, and compound **55** as globularin or globularicisin (Calis *et al*., 2002a,2002b; Kirmizibekmez *et al*., 2004), although it was not possible to assign one specific compound because the data provided by MS was unable to distinguish between stereoisomers. Globularimin and globularinin are two highly oxygenated iridoid glucosides from *G. alypum* (Sudo *et al*., 1998; Kirmizibekmez *et al*., 2008).

Peak **59** gave a molecular ion at *m/z* 527 that was tentatively identified as globularioside or baldaccioside (Es-Safi *et al*., 2006; Kirmizibekmez *et al*., 2009). Peak **5** was identified as shanzhiside. The MS/MS spectrum of this compound showed the fragment ions at *m/z* 229, 211, 167, 149 and 123 corresponding to the successive losses of hexose (229) and water (211) as well as subsequent decarboxylation (167) and dehydration (149) from the fragment ion at *m/z* 211. Another fragment was found at *m/z* 123, corresponding to $[M-H-\text{hexose}-88]^-$, which was obtained by the loss of the 3-oxopropanic acid molecule and a subsequent dehydration. The fragmentation pathway of shanzhiside is shown in Fig. 3b. No available study has reported this iridoid hexoside in *G. alypum*. In addition to shanzhiside, peaks **18** and **20** were assigned as acetylbarlerin isomers. These compounds present a structure based on the shanzhiside methyl ester skeleton (Fig. 2g).

Peak **21** (*m/z* 525) was tentatively identified as 6-*O*-veratroylcatalpol (Saracoglu *et al*., 2011). This compound showed the fragment ions at *m/z* 345, 301, 283, 179 and 161. The fragment ion at *m/z* 345 can be attributed to the aglycone resulting from the loss of hexose, which further formed the fragments at *m/z* 301 and 283 by a neutral loss of CO_2 and subsequent dehydration.

Peaks **51** and **56** were tentatively characterised as decumbeside D isomers. These compounds with the molecular ion at *m/z* 551 presented MS/MS ions at *m/z* 181, 163 and 147 (Fig. 3c). The fragment ions at *m/z* 163 and 147 were referred to the coumaroyl moiety and the formation of an aldehyde from this later, respectively.

Peak **57** was tentatively suggested to be serratoside A. The fragmentation of this iridoid hexoside led to several fragments, among which the fragment ion at *m/z* 193 was formed by the cleavage of a deoxyhexose and a cinnamoyl unit (*m/z* 148), while the fragment ions at *m/z* 147 and 161 could be attributed to cinnamic acid and dehydrated glucose, respectively (Fig. 3d).

Peak **46** presented a molecular ion at *m/z* 519. The ESI/QTOF analysis showed MS/MS fragments at *m/z* 307, 163 and 145. The fragment ion at *m/z* 307 resulted from the loss of the

Table 1. Proposed compounds detected in *Globularia alypum* extract obtained by HPLC–ESI/QTOF/MS

Group	Peak	t_R (min)	*m/z* experimental	Molecular formula	*m/z* calculated	Error (ppm)	mSigma value	MS/MS fragments (% relative abundance)	Proposed compound
Iridoids and secoiridoids	4	7.78	361.114	$C_{15}H_{21}O_{10}$	361.1140	0.1	6.7	127.0394 (46), 151.0386 (86), 169.0502 (100)	Catalpol
	5	8.35	391.1253	$C_{16}H_{23}O_{11}$	391.1246	−1.8	13.6	123.0445 (75), 149.0587 (28), 167.0703 (100), 211.0621 (37), 229.0704 (68)	Shanzhiside
	6	10.61	373.115	$C_{16}H_{21}O_{10}$	373.1140	−2.7	8.8	123.0451 (63), 149.0597 (45), 167.0731 (32), 193.0519 (100)	Gardoside/Geniposidic acid
	8	11.81	373.1134	$C_{16}H_{21}O_{10}$	373.1140	1.6	25.8	123.0450 (100), 149.0603 (74), 167.0733 (37), 211.0609 (59)	Gardoside/Geniposidic acid
	10	13.00	375.1292	$C_{16}H_{23}O_{10}$	375.1297	1.2	15.4	125.0591 (55), 151.0797 (30), 169.0851 (26), 213.0775 (100)	Mussaenosidic acid
	12	13.28	375.1314	$C_{16}H_{23}O_{10}$	375.1397	−4.7	2.9	113.0239 (12), 151.0761 (26), 169.0879 (59), 213.0774 (100), 214.0824 (12)	(epi)Loganic acid
	13	13.99	373.1166	$C_{16}H_{21}O_{10}$	373.1140	−7.0	18	123.0444 (55), 149.0620 (100), 167.0717 (44), 193.0501 (31)	Gardoside/Geniposidic acid
	16	15.12	445.1341	$C_{19}H_{25}O_{12}$	445.1351	2.3	28.7	151.0406 (11), 161.0231 (18), 179.0344 (2)	Asperulosidic acid methyl ester
	18	15.74	489.1589	$C_{21}H_{29}O_{13}$	489.1614	4.9	17.7	145.0303 (42), 163.0405 (31), 205.0515 (23), 265.0715 (42)	Acetylbarlerin (isomer 1)
	20	16.23	489.1607	$C_{21}H_{29}O_{13}$	489.1614	1.3	17.2	145.0306 (21), 163.0415 (24), 205.0521 (6), 265.0723 (9), 325.0995 (5)	Acetylbarlerin (isomer 2)
	21	16.61	525.159	$C_{24}H_{29}O_{13}$	525.1614	4.4	29.1	161.0243 (15), 179.0393 (5), 283.0935 (2), 301.1112 (1), 345.0950 (1)	6-*O*-Veratroylcatalpol
	28	17.47	523.1448	$C_{24}H_{27}O_{13}$	523.1457	1.7	25.0	161.0248 (52), 179.0367 (37)	Verminoside
	30	17.93	355.1028	$C_{16}H_{19}O_9$	355.1035	1.	19.7	149.0610 (21), 175.0401 (69), 191.0723 (35), 193.0504 (100), 235.0606 (75), 265.0724 (20), 295.0836 (67)	Gentiopicroside
	33	19.42	371.0978	$C_{16}H_{19}O_{10}$	371.0984	1.6	6.4	113.0241 (22), 121.0321 (91), 249.0638 (100)	Deacetylasperuloside
	34	19.54	507.1498	$C_{24}H_{27}O_{12}$	507.1508	1.9	31.5	145.0297 (82), 163.0400 (100)	Specioside
	38	20.38	415.1239	$C_{18}H_{23}O_{11}$	415.1246	1.8	7.0	113.0233 (11), 149.0604 (100), 163.0757 (27), 175.0407 (22), 191.0702 (44)	Alpinoside
	41	20.95	509.1648	$C_{24}H_{29}O_{12}$	509.1664	3.3	7.0	147.0453 (100)	Globularimin/Globularinin
	42	21.36	537.1584	$C_{25}H_{29}O_{13}$	537.1614	5.6	18.3	161.0222 (8), 493.1686 (10)	Minecoside

	46	22.27	519.1505	$C_{25}H_{27}O_{12}$	519.1508	0.5	17.5	145.0297 (40), 163.0412 (32), 307.0822 (68)	Coumaroylgeniposidic acid
	51	23.49	551.1774	$C_{26}H_{31}O_{13}$	551.1770	−0.7	15.9	147.0454 (100), 163.0409 (8), 181.0513 (14)	Decumbeside D (isomer1)
	55	25.09	491.1566	$C_{24}H_{27}O_{11}$	491.1559	−1.4	6.9	161.0237 (12), 175.0410 (75), 315.1090 (21)	Globularin/Globularicisin
	56	25.28	551.1791	$C_{26}H_{31}O_{13}$	551.1770	−3.7	21.4	147.0454 (100), 163.0409 (8), 181.0513 (14)	Decumbeside D (isomer 2)
	57	25.98	503.1550	$C_{25}H_{27}O_{11}$	503.1559	1.7	18.9	123.0450 (32), 147.0444 (57), 161.0541 (40), 189.0546 (34), 193.0517 (100)	Serratoside A
	59	27.20	527.1323	$C_{30}H_{23}O_{9}$	527.1348	4.7	157.5	–	Globularioside/ Baldaccioside
Phenylethanoid glycosides	11	13.10	461.166	$C_{20}H_{29}O_{12}$	461.1664	1.1	7.5	113.0255 (15), 135.0446 (14), 397.1152 (19)	Decaffeoylacteoside
	14	14.46	505.1541	$C_{21}H_{29}O_{14}$	505.1563	4.3	18.9	161.0239 (7), 179.0349 (2), 341.0901 (1)	Hebitol II
	24	17.06	519.1721	$C_{22}H_{31}O_{14}$	519.1719	−0.1	16.2	175.0391 (17), 193.0512 (25), 235.0617 (11)	Globularitol
	40	20.81	477.1379	$C_{23}H_{25}O_{11}$	477.1402	4.9	27.9	161.0245 (33), 179.0350 (7)	Calceolarioside A (isomer 1)
	44	21.87	623.2001	$C_{29}H_{35}O_{15}$	623.1981	−3.1	24.6	161.0247 (9), 461.1673 (4)	Verbascoside
	47	22.38	477.1399	$C_{23}H_{25}O_{11}$	477.1402	0.8	3.3	161.0245 (64), 315.1104 (11)	Calceolarioside B
	50	23.14	623.1993	$C_{29}H_{35}O_{15}$	623.1981	−1.9	8.5	161.0243 (7), 461.1636 (3)	Isoverbascoside
	52	24.19	461.1451	$C_{23}H_{25}O_{10}$	461.1453	0.4	17.4	145.0292 (70), 163.0397 (71), 297.0977 (40)	Neosyringalide (isomer 1)
	53	24.54	461.1467	$C_{23}H_{25}O_{10}$	461.1553	−3.1	16.6	145.0297 (100), 315.1084 (19)	Neosyringalide (isomer 2)
	58	26.95	651.2287	$C_{31}H_{39}O_{15}$	651.2294	1.1	28.2	175.0403 (50), 193.0496 (5), 475.1828 (6)	Martynoside
	60	28.59	785.2258	$C_{34}H_{41}O_{21}$	785.2246	−14.3	10.7	–	Rossicaside A
	61	30.60	769.2341	$C_{38}H_{41}O_{17}$	769.2349	1.1	13.7	–	Galypumoside A
	62	30.90	799.2423	$C_{39}H_{43}O_{18}$	799.2455	4	7.9	161.0209 (2), 623.1925 (1)	Galypumoside B
	63	35.06	753.235	$C_{38}H_{41}O_{16}$	753.2400	6.6	21.5	161.0282 (3)	(−)-6′-*O*-(E)-Cinnamoylverbascoside
Flavonoids	25	17.05	401.1442	$C_{18}H_{25}O_{10}$	401.1453	2.7	20.1	269.1024 (100), 161.0464 (75), 113.0243 (25), 101.0230 (22)	Icariside F2
	31	18.62	625.1418	$C_{27}H_{29}O_{17}$	625.1410	−1.2	13.9	301.0358 (2), 461.1429 (1)	Hydroxyluteolin 7-*O*-laminaribioside
	32	18.89	305.0692	$C_{15}H_{13}O_{7}$	305.0667	−8.4	19.4	96.9592 (54), 225.1137 (100)	Gallocatechin/ Epigallocatechin

(Continues)

Table 1. (Continued)

Group	Peak	t_R (min)	*m/z* experimental	Molecular formula	*m/z* calculated	Error (ppm)	mSigma value	MS/MS fragments (% relative abundance)	Proposed compound
	35	19.91	611.1597	$C_{27}H_{31}O_{16}$	611.1618	2.1	3.4	151.0001 (2), 475.1085 (8)	Eriodictiol-*O*-disaccharide
	36	19.98	463.0871	$C_{21}H_{19}O_{12}$	463.0882	2.3	4.6	301.0366 (53)	Quercetin glucoside
	37	20.23	609.1452	$C_{27}H_{29}O_{16}$	609.1461	1.6	10.3	285.0394 (2)	Luteolin disaccharide (isomer 1)
	39	20.38	609.1444	$C_{27}H_{29}O_{16}$	609.1461	2.8	23.2	285.0398 (31)	Luteolin disaccharide (isomer 2)
	45	22.12	447.0924	$C_{21}H_{19}O_{11}$	447.0933	1.9	12.9	151.0037 (5), 285.0414 (49)	Cynaroside
	48	22.52	533.1661	$C_{26}H_{29}O_{12}$	533.1664	0.6	13.6	161.0233 (11), 179.0363 (8), 323.0777 (100)	Amurensin
	49	22.74	477.1051	$C_{22}H_{21}O_{12}$	477.1038	−2.6	3.5	299.0211 (5), 315.0509 (34), 462.0801 (3)	Nepitrin
	54	24.89	517.1717	$C_{26}H_{29}O_{11}$	517.1715	−0.4	11.3	145.0299 (27), 209.0828 (22), 307.0826 (100)	Phellamurin
Other polar compounds	1	2.46	181.0721	$C_6H_{13}O_6$	181.0718	−2.0	7.8	101.0241 (100), 113.0266 (36), 163.0611 (69)	Mannitol
	2	2.73	191.0564	$C_7H_{11}O_6$	191.0561	−1.3	3.4	–	Quinic acid
	3	2.83	341.1091	$C_{12}H_{21}O_{11}$	341.1089	−0.6	13.6	101.0223 (77), 113.0251 (100), 179.0558 (99)	Sucrose
	7	10.86	315.0724	$C_{13}H_{15}O_9$	315.0722	−0.7	27.4	108.0224 (39), 152.0134 (100), 153.0219 (53), 232.9773 (22)	Gentisoyl hexoside
	9	12.08	315.1085	$C_{14}H_{19}O_8$	315.1085	0.2	20.3	101.0267 (34), 113.0257 (32), 119.0376 (15), 135.0439 (100), 153.0564 (16)	Cornoside
	15	14.66	341.0872	$C_{15}H_{17}O_9$	341.0878	1.7	10.6	135.0449 (23), 161.0234 (56), 179.0361 (100), 221.0461 (76), 251.0578 (28), 281.0680 (86)	Caffeoylhexose (isomer 1)
	17	15.48	341.0875	$C_{15}H_{17}O_9$	341.0878	0.8	7.2	135.0460 (23), 161.0250 (48), 179.0346 (99), 221.0470 (71), 251.0579 (32), 281.0675 (100)	Caffeoylhexose (isomer 2)
	19	15.84	163.0413	$C_9H_7O_3$	163.0401	−7.8	9.4	–	*p*-Coumaric acid
	22	16.83	325.0928	$C_{15}H_{17}O_8$	325.0929	0.2	10	119.0510 (24), 145.0295 (100), 161.0602 (31), 163.0405 (70), 205.0517 (79), 235.0633 (20), 265.0744 (79)	*p*-Coumaroylhexose (isomer 1)
	23	16.96	429.1395	$C_{19}H_{25}O_{11}$	429.1402	1.7	12.1	145.0303 (72) ,163.0417 (35)	Neohancoside C
	26	17.37	385.1136	$C_{17}H_{21}O_{10}$	385.1140	1.1	26.7	101.0239 (2), 113.0246 (3), 145.0317 (2), 207.1043 (19)	Sinapic acid *O*-hexoside
	27	17.45	387.165	$C_{18}H_{27}O_9$	387.1661	2.6	4.3	145.0284 (6) , 207.1021 (20)	Tuberonic acid hexoside

29	17.70	325.0927	$C_{15}H_{17}O_8$	325.0929	0.6	7.6	119.0493 (36), 145.0301 (73), 161.0620 (30), 163.0403 (86), 205.0510 (100), 235.0616 (19), 265.0734 (85)	*p*-Coumaroylhexose (isomer 2)
43	21.46	569.1892	$C_{26}H_{33}O_{14}$	569.1876	−2.8	30	147.0454 (100), 199.0625 (4), 361.1140 (17), 509.1674 (7)	Columbianin

coumaroylhexose moiety. In addition, the *m/z* ions at 163 and 145 corresponded to the coumaroyl moiety after breakage of the ester bond with the sugar, as well as after an additional loss of an H_2O molecule, respectively. According to these data, compound **46** was tentatively characterised as coumaroylgeniposidic acid.

Phenylethanoid glycosides

Phenylethanoid glycosides represent another important group of metabolites characterised in this study, many of which were previously described in *G. alypum* or in the family Globulariaceae.

Compounds **40** and **47** presented the molecular ion at *m/z* 477 ($C_{23}H_{25}O_{11}$). The MS/MS spectra of both compounds led to the identification of calceolarioside A and calceolarioside B, respectively (Fig. 2b). These compounds are another example of phenyl ethyl glycosides extracted from the genus *Globularia* (Taskova *et al*., 2006; Kirmizibekmez *et al*., 2008). The molecular formula $C_{34}H_{41}O_{21}$ was assigned to compound **60**, although it was not possible to establish a suitable MS/MS spectrum due to its low intensity. This compound was tentatively identified as rossicaside A, on the basis of previous reports for *Globularia* species (Calis *et al*., 1999; Kirmizibekmez *et al*., 2009).

Compounds **61** and **62** were characterised as galypumoside A and galypumoside B, respectively, according to their MS data and the data reported in the literature (Kirmizibekmez *et al*., 2008). These compounds are characterised here for the first time in *G. alypum*.

Compound **44** gave the molecular ion at *m/z* 623 with molecular formula $C_{29}H_{35}O_{15}$. This compound represents the second main peak in the chromatogram of the *G. alypum* extract, according to its MS data; it has been tentatively proposed to be verbascoside (Fig. 2h). Also, another verbascoside isomer was found in the extract (compound **50**) that corresponded to isoverbascoside (Es-Safi *et al*., 2007a).

Besides these known compounds, two new phenylethanoid glycosides were found in *G. alypum* for the first time. Accepted data recorded from MS and MS/MS spectra enabled the tentative identification of peak **11** as decaffeoylacteoside and peak **63** as (−)-6′-*O*-(E)-cinnamoylverbascoside.

Compound **24** with the pseudo-molecular ion at *m/z* **519** and the molecular formula $C_{22}H_{31}O_{14}$ was tentatively identified as globularitol (Fig. 2j). This compound has been previously isolated as a new sugar ester from the methanolic extract of the underground parts of *G. orientalis*, but it has never been reported in *G. alypum*.

Compound **58** gave a $[M-H]^-$ ion at *m/z* 651 with the molecular formula $C_{31}H_{39}O_{15}$. In its MS/MS spectrum, a fragment ion was detected at *m/z* 475, due to the loss of 176 Da, which represented the feruloyl moiety. Other fragment ions were detected at *m/z* 193 and 175, corresponding to ferulic acid and further water loss, respectively. Thus, martynoside was the proposed structure for this compound (Fig. 2a), for the first time in *G. alypum*.

Peak **14** (*m/z* 505) was assigned to hebitol II (Fig. 2c). The fragmentation of this compound yielded the fragment ions at *m/z* 341, 179 and 161. The first fragment ion was extracted after the neutral loss of $C_6H_{12}O_5$ corresponding to the glucityl group, while fragments ions at *m/z* 179 and 161 were attributed to the caffeoyl and hexose moieties, respectively.

Compounds **52** and **53** were found to be neosyringalide isomers (Fig. 2e). Their MS/MS spectrum gave fragments corresponding to the coumaroyl moiety at *m/z* 145 (Es-Safi *et al*., 2007a).

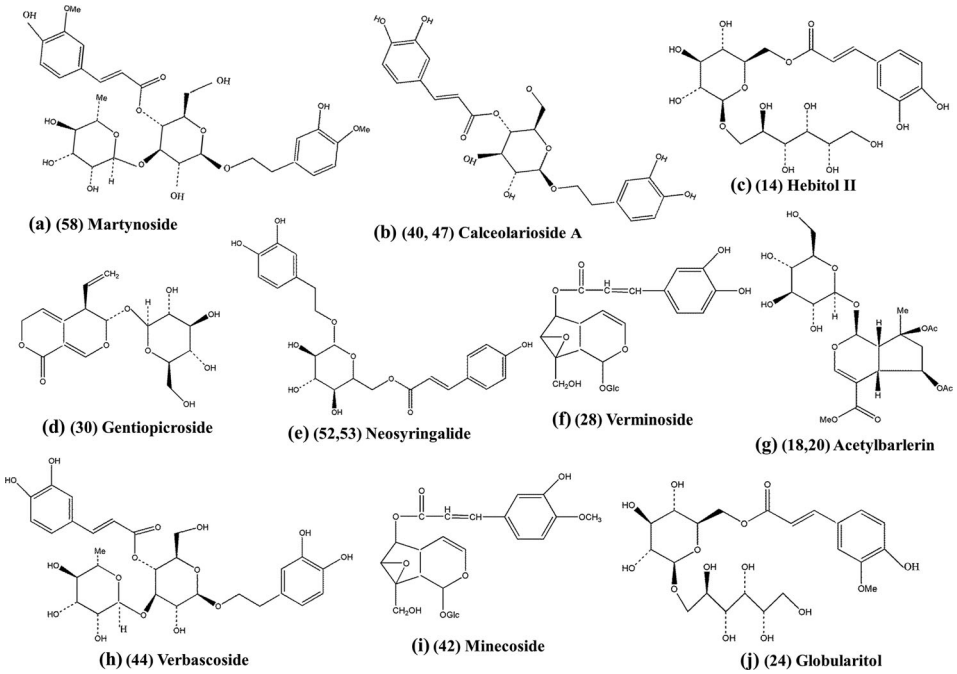

Figure 2. Chemical structures of several proposed compounds in *Globularia alypum* leaves.

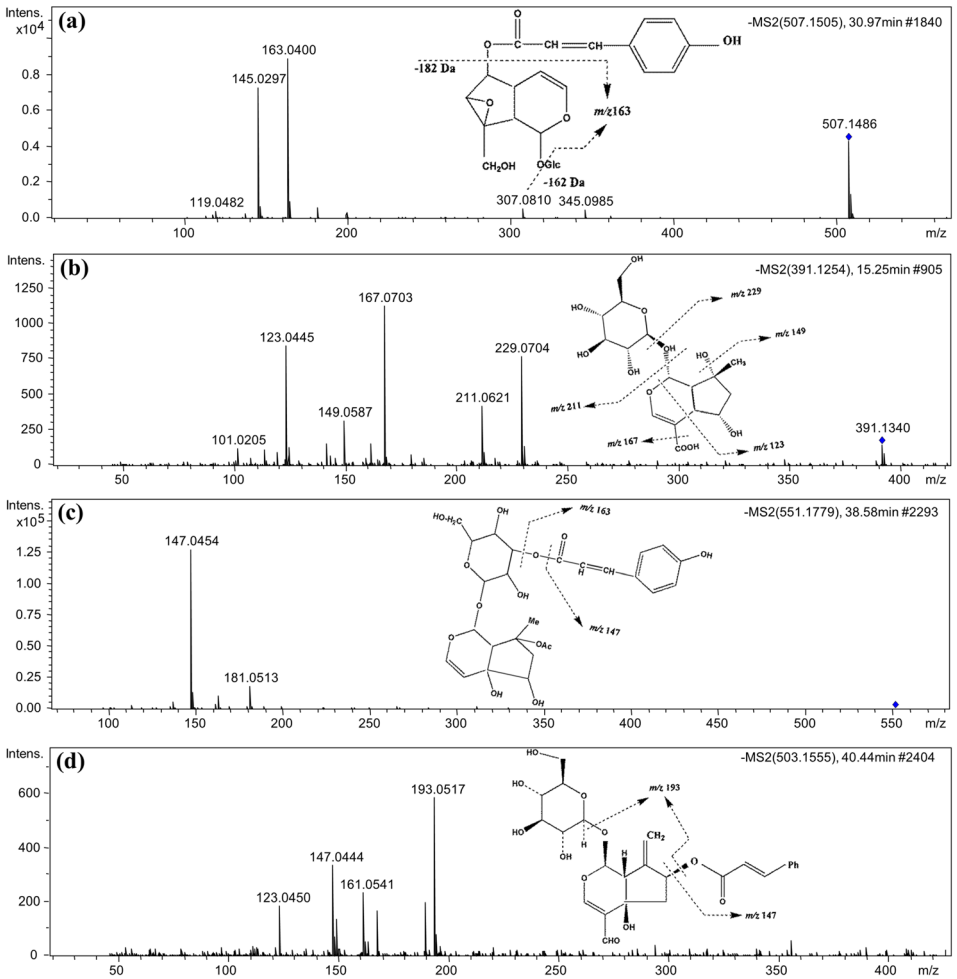

Figure 3. The MS/MS fragmentation pattern of (a) specioside, (b) shanzhiside, (c) decumboside D and (d) serratoside.

Flavonoids

In the present work, five flavone glycosides previously described in *Globularia* were detected. Based on the MS/MS data and on the bibliography (Es-Safi *et al*., 2005; Kirmizibekmez *et al*., 2008), peak **31** was tentatively suggested to be hydroxyluteolin-7-laminaribioside, while peaks **37** and **39** were tentatively attributed to luteolin disaccharide isomers. In addition, peaks **45** and **49** were determined as cynaroside (Ben *et al*., 1982) and nepitrin (Kirmizibekmez *et al*., 2003), respectively. Compound **35** yielded a $[M-H]^-$ ion at *m/z* 611 and its fragmentation resulted in the aglycone ion at *m/z* 151, probably due to the neutral loss (324 Da) from the fragment ion at *m/z* 475, suggesting the presence of two hexose residues. Therefore, based on these data, compound **35** was tentatively characterised as eriodictiol *O*-disaccharide.

Compound **25** with the molecular formula $C_{18}H_{26}O_{10}$ yielded the fragments at *m/z* 269, 161, 113 and 101. Where the product ion (*m/z* 269) corresponded to the loss of the [Ph − CH_2 − hexose] group, while the fragments at *m/z* 161, 113 and 101 matched the dehydration of hexose (161) and its fragment ions at *m/z* 113 [hexose − $2H_2O$ − CH_2O] and *m/z* 101 [hexose − H_2O − $2CH_2O$]. This compound was tentatively characterised as icariside F2, and was found in the extract of *G. alypum* for the first time.

Peak **32** had the molecular formula $C_{15}H_{13}O_7$ and showed a fragment ion at *m/z* 225 corresponding to $C_{11}H_{13}O_5$, which resulted from the breaking of the C-ring of gallocatechin or epigallocatechin, but it was not possible to distinguish between these isomers.

Three flavonol glycosides were found in the extract of *G. alypum* leaves. Peak **36** was identified as quercetin glucoside (Kirmizibekmez *et al*., 2009). The characterisation of this compound was based on MS data and the neutral loss of the glucose moiety, which gives rise to the fragment ion at (*m/z* 301) corresponding to quercetin.

Peak **48** at *m/z* 533 showed a MS/MS spectrum with the fragments ions at *m/z* 323, 179 and 161, corresponding to the loss of [M − H − hex − 2(CH_3)], hexose and its dehydration, respectively. Accordingly, compound **48** was tentatively considered to be amurensin.

Peak **54** gave a molecular ion at *m/z* 517. In MS/MS analysis, this compound yielded the daughter fragments at *m/z* 307 and 145. The first fragment resulted from the neutral loss of glucose and two methyl groups from the main ion, while the latter fragment ion at *m/z* 145 corresponded to the loss of $C_9H_5O_2$ (cleavage $^{1,3}A^-$) from the fragment at *m/z* 307. This detected fragmentation pattern is consistent with that proposed by Fernández-Arroyo *et al*. (2010). Therefore, compound **54** was tentatively identified as phellamurin.

Other polar compounds

In addition to the above-mentioned flavonoid derivatives identified in this work, six hydroxycinnamic acids and derivatives were characterised. According to the fragmentation profile of these compounds, peaks **15** and **17** with molecular ions at *m/z* 341 were tentatively characterised as caffeoylhexose isomers, while peaks **22** and **29** at *m/z* 325 were proposed as *p*-coumaroylhexoside isomers. Peak **19** was assigned to *p*-coumaric acid, while peak **26** was attributed to sinapic acid-*O*-hexoside. Most of these compounds were characterised by common losses such as the loss of the hexose and caffeoyl moieties in caffeoylhexose isomers and coumaroyl unit in isomers of coumaroylhexose. Peak **26** presented a fragment at *m/z* 207 found after the loss of hexose and an oxygen. These hydroxycinnamic acid glycosides were found in *G. alypum* for the first time.

Other polar compounds were identified in this extract by using the applied LC–ESI/QTOF/MS method. Compound **2** showed a molecular formula $C_7H_{11}O_6$. This compound was identified as quinic acid on the basis of the main literature reports on quinic acid (Gómez-Romero *et al*., 2010; Gouveia and Castilho, 2010).

Peak **7** with the molecular ion at *m/z* 315.0724 has been tentatively assigned to gentisoyl glycoside. The MS/MS spectrum displayed the fragment ions at *m/z* 153 and 108 corresponding to aglycone and its decarboxylation product, respectively.

The MS/MS spectrum of compound **27** demonstrated the molecular ion at *m/z* 387 and MS/MS fragment ions at *m/z* 207 and 145, which were consistent with the loss of hexose moiety followed by successive dehydration and decarboxylation, respectively. In accordance with these data, peak **27** was proposed as tuberonic acid hexoside.

Compound **9** with the molecular formula $C_{14}H_{19}O_8$, showed the daughter ions at *m/z* 153 and *m/z* 135 corresponding to the loss of hexose and dehydration, respectively. Thus, compound **9** was tentatively characterised as cornoside.

Compound **23** with a molecular ion at *m/z* 429 gave the fragment ions at *m/z* 163 and 145 corresponding to $[M-H-\text{xylose}\ CH_2]^-$ and its dehydration product, respectively. This compound was tentatively proposed as neohancoside C. This phenol glycoside is reported for the first time in this plant. The MS^2 analysis of compound **43** showed the pseudo-molecular ion at *m/z* 569 and the fragment ions at *m/z* 361, 199 and 147. The daughter ion at *m/z* 361 resulted from the loss of hexose and two methyl groups, whereas the fragment at *m/z* 199 arose after the loss of a deoxyhexose group from the fragment ion at *m/z* 361. Moreover, the fragment that appeared at *m/z* 147 was the result of the loss of (CH_2) from the deoxyhexose group. Accordingly, compound **45** was tentatively assigned as columbianin.

Summary

The LC–ESI/QTOF/MS-based metabolite-profiling approach enabled the tentative identification of 63 metabolites in a *G. alypum* extract on the basis of their MS and MS/MS spectra in negative ion mode together with the relevant data from the literature. The method applied combined the advantages of a small-particle-size C_{18}-column (1.8 μm), as such the high resolution made it possible to separate several isomers, with the high selectivity, sensitivity, mass accuracy and measurements of the isotopic pattern associated with QTOF/MS for both parent and fragment ions. The analyses of the leaf extract revealed a larger number of compounds, most being iridoids and phenylethanoid glycosides substituted with acyl groups. Therefore, the described HPLC–ESI/QTOF/MS method has proven to be a valuable tool for simultaneous characterisation of a wide range of bioactive compounds from *G. alypum* leaves. Furthermore, the data compiled may encourage further use of this plant matrix as a folk and alternative medicine in human therapy.

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