Chemodiversity or taxonomy error? A search for the true phytochemical profile of *Ferula arrigonii* Bocchieri

Recherche du vrai profil phytochimique de *Ferula arrigonii* Bocchieri

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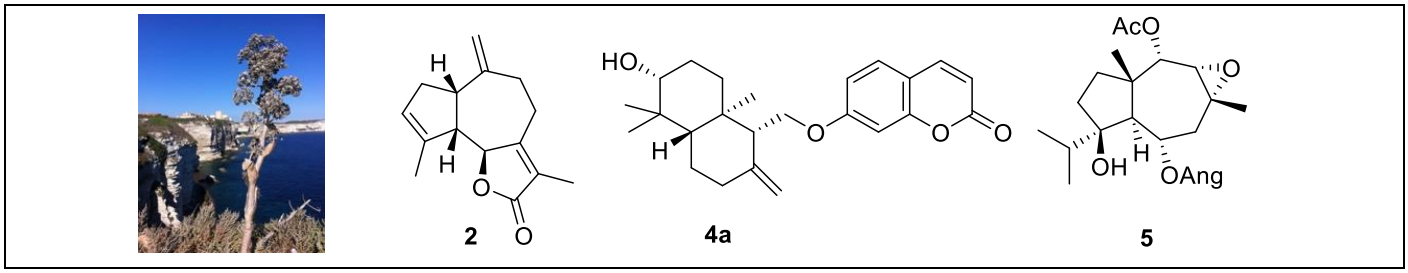
RÉSUMÉ. La guaianolide 11,13-déhydrogrilactone (**2**) a été décrite comme un potentiel marqueur chimiotaxonomique de l'espèce endémique Corso-Sarde *Ferula arrigonii* Bocchieri. Dans le présent travail, la présence de ce constituant n'a pas pu être confirmée dans des extraits obtenus à partir de plantes récoltées en Corse et en Sardaigne ainsi que de matériels végétaux issus des herbiers de référence. Le profil chimique de l'ensemble des échantillons s'est révélé relativement homogène avec une composition caractérisée par les éthers de sesquiterpène-coumarines. Nos résultats suggèrent que l'étude antérieure a probablement été réalisée à partir d'un matériel végétal issu d'un chémotype atypique rare ou bien d'une identification botanique erronée.

ABSTRACT. The guaianolide 11,13-dehydrogrilactone (**2**) was previously described as alleged chemotaxonomic marker of the Sardo-Corsican endemic species *Ferula arrigonii* Bocchieri. In the present work, the presence of this component could not be confirmed in various extracts obtained from plants harvested in Sardinia and Corsica island as well as in its vegetal material from reference herbarium species. All studied samples of *F. arrigonii* showed a homogenous phytochemical profile characterized by sesquiterpene coumarin ethers. These results suggest that the previous investigations have probably been carried out on plant material corresponding to an atypical niche chemotype or, alternatively, that they resulted from an erroneous identification.

MOTS-CLÉS. *Ferula arrigonii* Bocchieri, *F. communis* L., Férulose, lactones sesquiterpéniques, éthers de sesquiterpène coumarine.

KEYWORDS. *Ferula arrigonii* Bocchieri, *F. communis* L., Ferulosis, sesquiterpene lactones, sesquiterpene coumarin ethers.



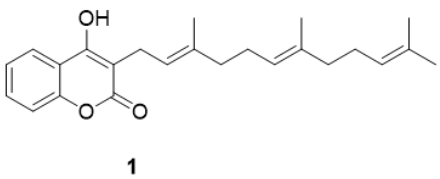
Sesquiterpene coumarin ethers and not sesquiterpene lactones are the phytochemical markers of F.arrigonii Bocchieri.

1. Introduction

The large genus *Ferula* (family Umbelliferae) is typical of dry areas of the Old World, with hotbeds of diversity in Central Asia and in the Mediterranean region.[1] Plants from this genus are a source of perfumes (*F. gummosa* Boiss.),[2] spices (*F. assa-foetida* L.)[3] resins (*F. sumbul* Willd.)[4] and food (*F. communis* L.),[5] but this rich ethnopharmacology also includes poisoning, as exemplified by ferulosis.[6] This hemorrhagic syndrome occurs sporadically in several areas of the Mediterranean region, but is more frequent in the Sardo-Corsican archipelagos, where it was first documented in early 19th century.[6] The nature of ferulosis was long debated, and only in 1925 the syndrome was clearly identified as an intoxication by a poisonous chemotype of *F. communis* abundant in Sardinia and Corsica, but with a point-like distribution in other areas of the Mediterranean region.[6] The toxic principle of *F. communis* is the farnesylated 4-hydroxycoumarin ferulenol (**1**), an inhibitor of the recycling of vitamin K, over 20-fold more potent than Warfarin in terms of affinity for Vitamin K Epoxide Reductase.[7] Ferulenol also potently inhibits oxidative phosphorilation by targeting the mitochondrial enzyme succinate ubiquinone reductase,[8] and was found to contaminate sludge from wastewater treatment plants in areas where the poisonous chemotype grows.[9]

F. communis was long considered the only species of *Ferula* growing in Sardinia and Corsica, but in 1988 a novel species (*Ferula arrigonii* Bocchieri) was reported from a small island offshore Southern Sardinia (Isola dei Cavoli).[10] Further small populations were also identified from a few costal areas of Sardinia, and a large one (over 2500 plants) in Southern Corsica, near Bonifacio.[11] A phytochemical investigation on *F. arrigonii* showed the occurrence of large amounts of a single sesquiterpene lactone (7,11-dehydrogrilactone, **2**), accompanied by the daucane ester siol anisate (**3a**).[12] While sesquiterpene coumarin ethers and sesquiterpenyl-4-hydroxycoumarins, the typical secondary metabolites of the Mediterranean species of *Ferula*,[6] were absent. The structure of **2** attracted attention in the organic synthesis community, and the total synthesis of its enantiomer was reported by Greene in Grenoble in 1994[13] correcting, by comparison with an authentic compound isolated from plant extract, the optical rotation of **2** originally reported.[12] In both investigations, the vegetal material was provided by Emuanuele Bocchieri, the botanist who first described this species.[10] Morever, the essential oil of *F. arrigonii* leaves collected in Bonifacio (Corsica) was investigated by ^{13}C NMR mixture analysis, and the presence of the guaianolide **2** was tentatively reported at the limit of the detection margin (0.3 % of the essential oil).[14]

Surprisingly, a further investigation of *F. arrigonii* from its original location (Isola dei Cavoli) and the nearby island of Serpentara showed a very different phytochemical profile, characterized by the presence of relatively large amounts of the sesquiterpene coumarin ether colladonin (**4a,** 0.90%) and of the daucane polyesters ferutinin (**3b,** 0.26%) and lapiferin (**5**, 0.21%).[15] Various analogues of these compounds were also obtained, including traces amounts of siol anisate (**3a**), but the guaianolide **2** could not be isolated.[12]



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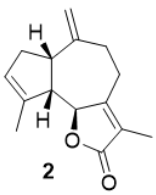

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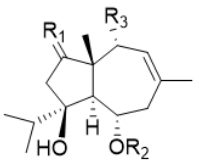
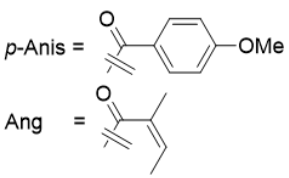


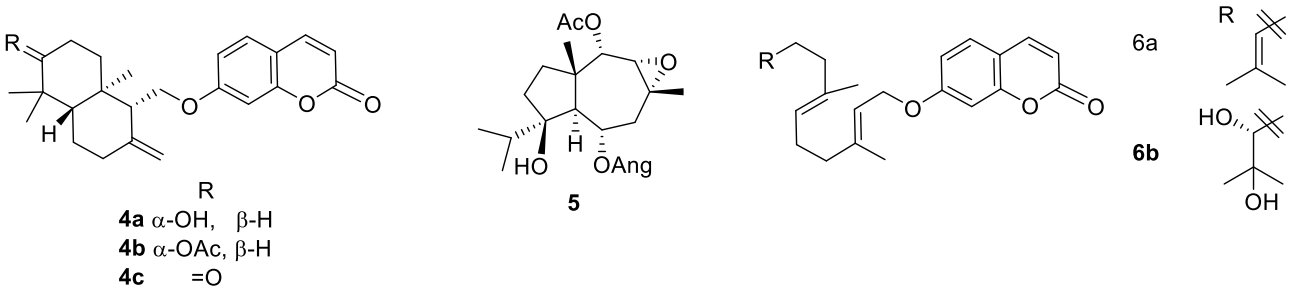
	R_1	R_2	R_3
3a	=O	*p*-Anis	H
3b	H,H	*p*-Anis	H
3c	H,H	p-Anis	OH
3d	H,H	Ang	OH
3e	H,H	Ang	OAng



2. Results and Discussion

The extract of *F. arrigonii* roots collected from the Corsican population of Bonifacio, and from multiple location in Sardinia were investigated in the present work. Two additional leaf samples from

Northern Sardinia (Maddalena and Caprera islands) were also studied. The chemical composition of these samples was found to be very similar (Table 1). In addition to colladonin (**4a**), ferutinin (**3b**) and lapiferin (**5**), eight additional compounds were isolated and identified, all already reported,[15] with differences in concentration well in the range of those expected when distinct populations of wild plants are compared. The inventory of minor constituents included the drimane coumarin ethers colladin (**4b**) and badrakemone (**4c**), the geranylated coumarins umbelliprenin (**6a**) and kataravicinol (**6b**), and various jaeskeanadiol esters (**3c-f**). When analyzed by TLC (hexane-ethyl acetate 8:2), all samples contained the drimane sesquiterpene ethers colladonine (**4a**) and colladine (**4b**). These results, summarized in Table 1, are consistent with the latest phytochemical study of Sardinian specimens,[15] but are inconsistent with previous phytochemical investigations made on plant material directly received from the discoverer of *F. arrigonii*.[12,13] Since this latter plant material was not available, the study was continued by investigating the reference herbarium of *F. arrigonii* deposited by his discoverer at the Botanical Garden of Cagliari. We could have access to small fragments of *F. arrigonii* leaves (ca 100 mg each) from nine herbarium reference samples collected in various Sardinian locations between 1986 and 2006 (Table 2), that we investigated by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).



Site of collection and plant part	Date of collection	Constituents identified or isolated
Serpentara (roots)	July 1996	**4a** (0.90%), **3b** (0.26%), **5** (0.12%)
Isola dei Cavoli (roots)	October 1996	**4a** (0.72%), **3b** (0.32%), **5** (0.16%)
Serpentara (leaves)	June 2001	**4a (+++), 3b (++), 5 (++)**
Isola dei Cavoli (leaves)	June 2001	**4a (+++), 3b (++), 5 (++)**
Tharros (leaves)	May 2014	**4a (+++), 3b (++), 5 (++)**
Capo Caccia (leaves)	June 2015	**4a (+++), 3b (++), 5 (++)**
Isola dei Cavoli (roots)	July 2005	**4a (+++), 3b (++), 5 (++)**
Isola dei Cavoli (roots)	July 2008	**4a** (0.54%), **3b** (0.22%), **5** (0.13%)
Isola Maddalena (leaves)	June 2014	**4a (+++), 3b (++), 5 (++)**
Caprera (leaves)	June 2014	**4a (+++), 3b (++), 5 (++)**
Bonifacio (roots)	July 2014	**4a** (0.82%), **3b** (0.26%), **5** (0.19%)

Table 1. *Summary of the collections of F. arrigonii investigated in the present study. When sufficient material was available (collections >100 g), the plant material was extracted and the isolation yields are given in parentheses, (minor constituents are not mentioned). When smaller samples were available, the constituents were identified by spectroscopic (^{1}H NMR) and chromatographic (TLC) analysis, and only their approximate relative concentration is given. A voucher specimen for all the roots collection is kept at the Botanical Garden in Cagliari.*

First, reference sample for the colladonin (**4a**) and colladin (**4b**) were analyzed by direct injection in MS/MS spectrometer fitted with an APCI source and operating in positive ion mode. Both compounds were prone to preeminent in-source fragmentation with the most abundant ions observed resulting from the loss of water for **4a** $[M\text{-}H_2O+H]^+$ (*m*/*z* 365.3), and from the loss the acyl moiety for **4b** $[M\text{-}Ac+H]^+$ (*m*/*z* 383.3). The fragmentation spectra of **4a** and **4b** in-source fragment ions shows an ion for the 7-hydroxycoumarin moiety (*m*/*z* 163.0) and for the sesquiterpene moiety (*m*/*z* 203.0 and 221.2, respectively) [Figure 1]. These reference standards were analyzed by LC-MS/MS, and their retention times, as well as their MS/MS spectra were recorded and uploaded to the GNPS public spectral library.[16]

The herbarium voucher samples of *F. arrigonii* were extracted with acetone and then analyzed by LC-MS/MS in identical chromatographic conditions. The data were first processed using MZmine2,[17] and the output data were uploaded to the GNPS web-platform for molecular networking analysis.[16] Molecular networking is a bioinformatic approach that enables the visualization of MS/MS data, and facilitates the annotation of the detected molecular families.[18] The molecular networks of *F. arrigonii* were composed of three main clusters [Figure 2]. Spectral library annotation and examination of the MS/MS spectra established that: (i) the largest cluster was annotated as sesquiterpene derivative ions and/or in-source fragment ions (i.e. *m*/*z* 221.2); (ii) a second cluster corresponded to in-source fragment ions of the hydroxycoumarin moiety (*m*/*z* 163.0) occuring at different retention times, and thus belonging to different molecules bearing this common sub-structure; and (iii) the last cluster was annotated as sesquiterpene coumarin ether derivative ions, including the compounds **4a** and **4b**. Using the reference MS/MS spectra for **4a** and **4b**, the cluster C was selected for detailed annotation [Figure 3]. It was observed that each MS/MS spectrum in this cluster contained a fragment ion for the hydroxycoumarin moiety (*m*/*z* 163.0), but different fragment ions for sesquiterpene moiety. This indicated that cluster C consisted of sesquiterpene coumarin ether derivatives having a modified sesquiterpene moiety. Those compounds were tentatively annotated by searching into the Dictionary of Natural Products, for candidate structures previously isolated in *Ferula* species having a 7-hydroxycoumarin (umbelliferyl) moiety. Results showed that if those ions are protonated adducts, they could correspond to various known sesquiterpene coumarin ethers such as the fecarpine, ferocaulidin, ferucrinone, and bradakenone (**4c**), or other isomers [Figure 3]. While if those ions are in-source fragments like compounds **4a** and **4b**, they could be either their hydroxylated or their acylated counterparts.

Herbarium voucher [a]	Date	Location	Plant material
#1	21/07/1986	Isola Serpentara, Villasimius, Sardinia (SE)	Stem / seeds
#2	11/06/1988	Buggerru, Sardinia (SW)	Leaf
#3	21/07/1988	Isola Serpentara, Villasimius, Sardinia (SE)	Stem / seeds
#4	17/08/1988	Isola Budelli, Archipelago della Madallena, Sardinia (N)	Stem / seeds
#5	17/07/1988	Isola Budelli, Archipelago della Madallena, Sardinia (N)	Stem / seeds
#6	27/07/1989	Isola Serpentara, Villasimius, Sardinia (SE)	Stem / leaf
#7	02/05/1992	Isola Budelli, Archipelago della Madallena, Sardinia (N)	Stem / seeds / leaf
#8	03/05/1995	Isola di San Stephano, Archipelago della Madallena, Sardinia (N)	Stem / seeds
#9	22/05/2006	Buggerru, Sardinia (SW)	Stem / seeds / leaf

Table 2. *Summary of the Bocchieri's F. arrigonii herbarium vouchers that were sampled at the Botanical Garden in Cagliari. [a] Code 612/A for all samples)*

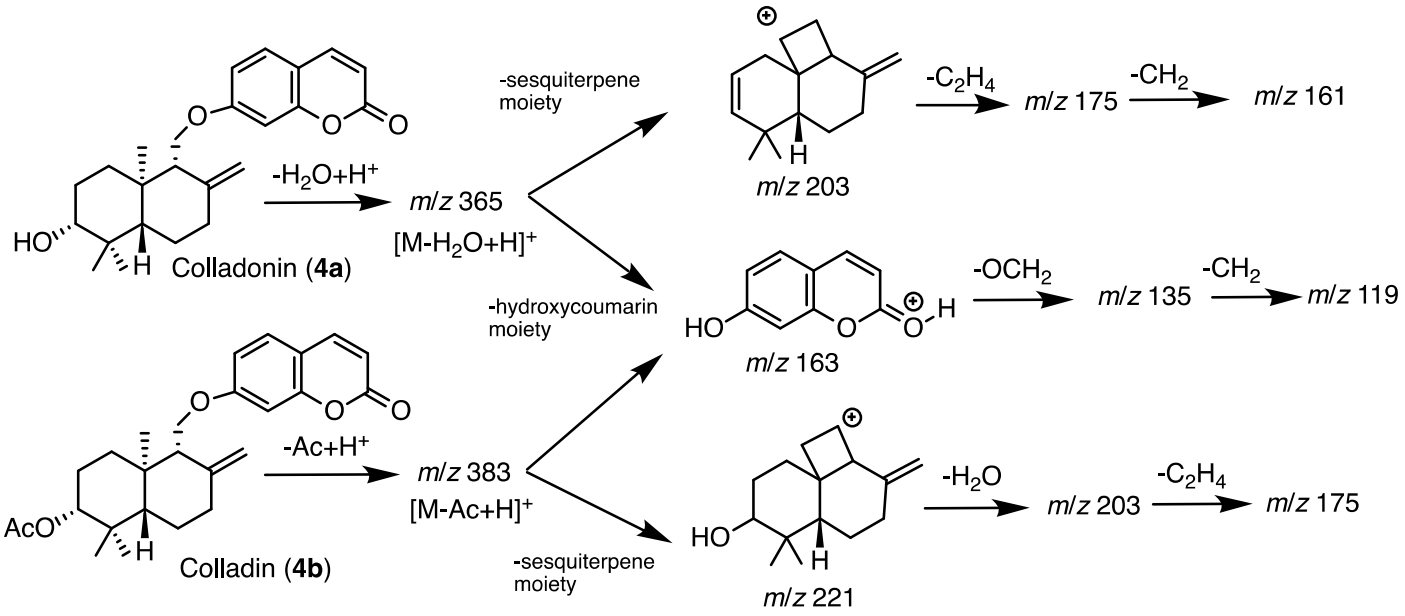


Figure 1. *Fragmentation behavior of colladonin (**4a**) and colladin (**4b**) in positive ionisation.*

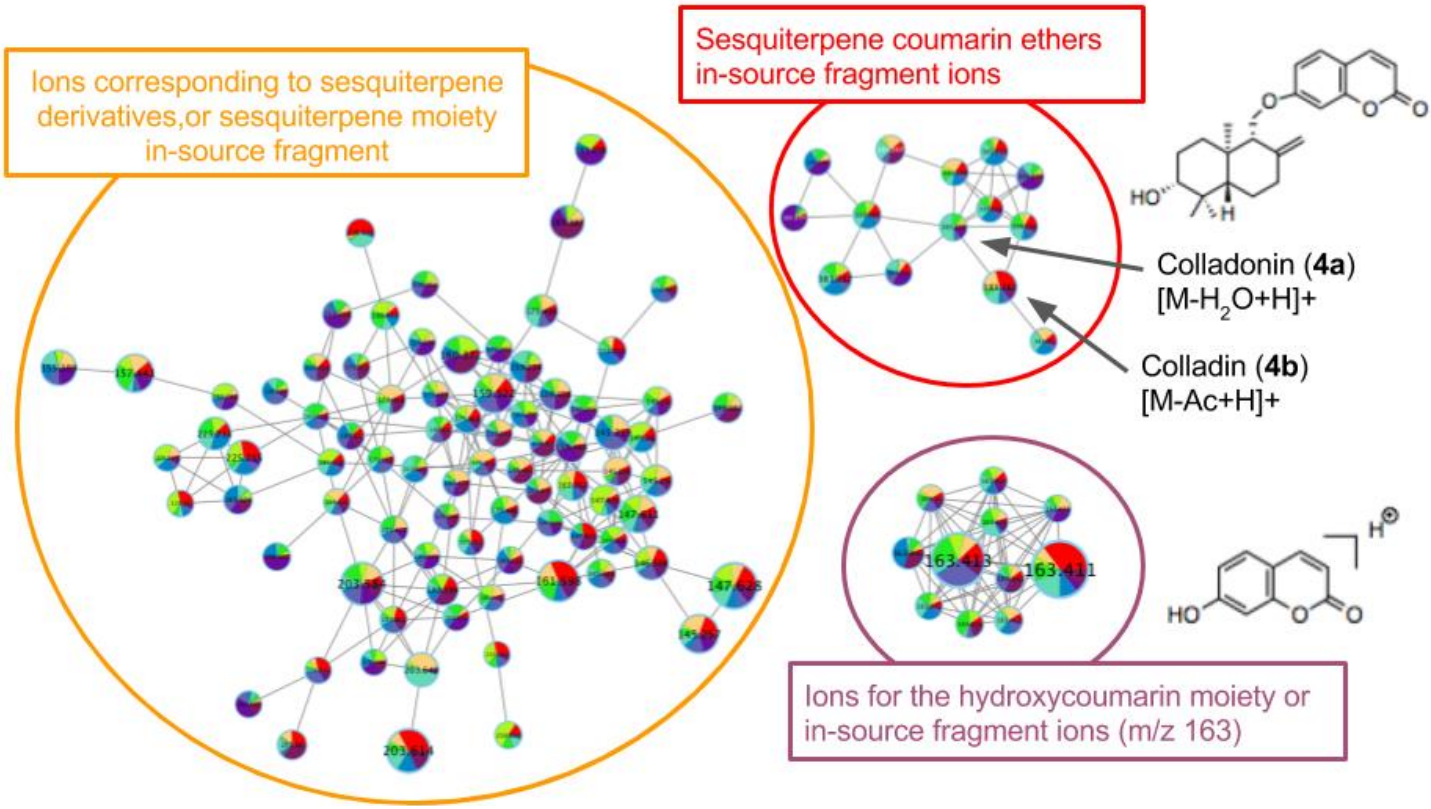


Figure 2. *Overview of the molecular networks of the herbarium voucher samples of F. arrigonii.*

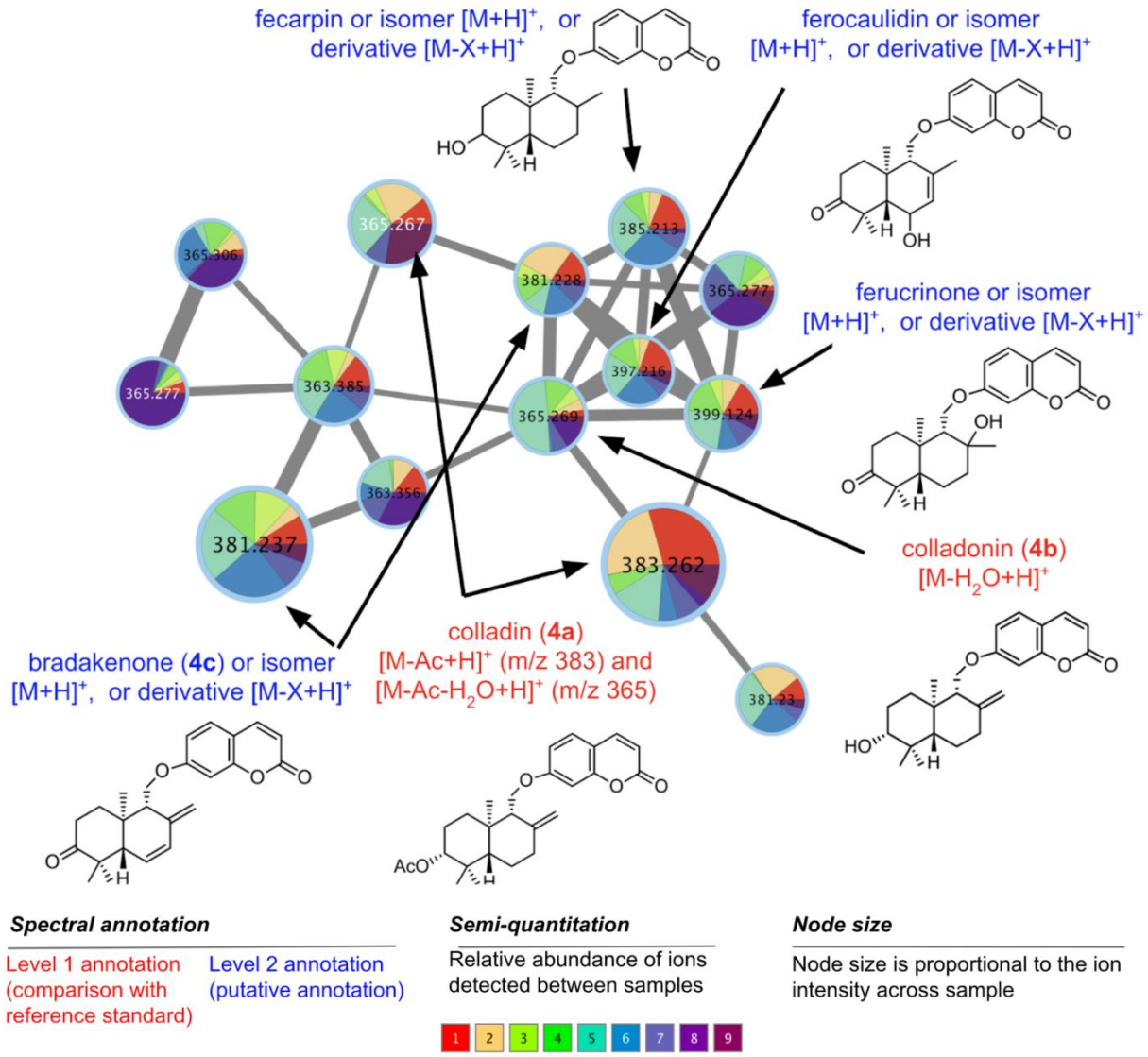


Figure 3. *Zoom on the sesquiterpene coumarin ethers cluster with spectral annotation.*

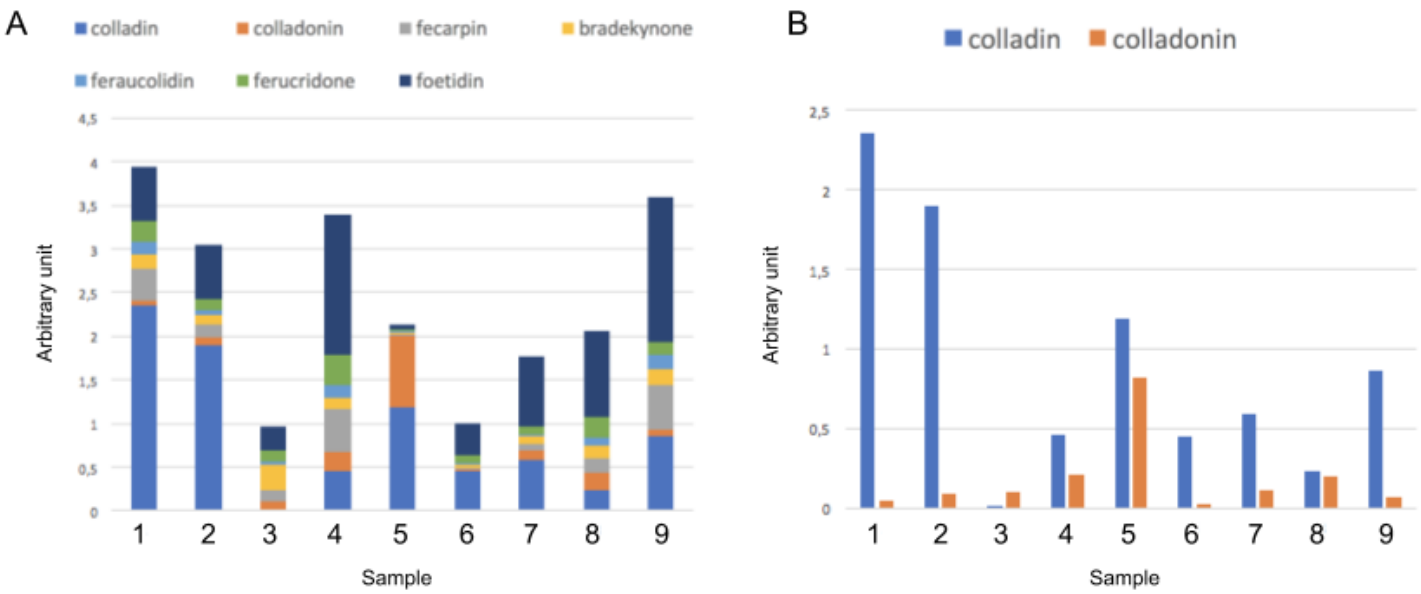


Figure 4. *Relative abundance of the molecules annotated by LC-MS/MS in the herbarium voucher samples. (A) Relative abundance of the annotated sesquiterpene coumarin ethers. (B) Relative abundance of the colladin **4a** and colladonin **4b**.*

A large quantitative variation, which was however expected due to the different plant parts of different age studied, was observed between the herbarium voucher samples [Figure 4A and 4B]. Nevertheless, the LC-MS/MS experiments confirmed that colladonin **4a** and colladine **4b** (Figure 4B), as well as other sesquiterpene coumarin ethers (**Figure 4A**), were present in all herbarium samples. Interestingly, the herbarium voucher sample #3 collected on the island of Serpentara, differed quantitatively from other samples, including those collected on this island (#1 and #6), by having a lower relative content of colladonin **4a**. Regarding the sesquiterpene lactone **2**, no ion corresponding to the protonated component was detected by LC-MS/MS. Nevertheless, the assessment of its presence or absence from these herbarium samples would require the development of a targeted mass spectrometry approach using a reference standard of compound **2**. Taken together, the results of LC-MS/MS analysis showed that the Bocchieri's herbarium voucher samples had a similar phytochemical profile, dominated by sesquiterpene coumarin ethers.

A considerable genetic variation has been observed in *F. arrigonii*,[11] and we cannot rule out that isolated plant specimens containing the guaianolide **2** might exist in Sardinia and/or in Corsica. However, the phytochemical profiles of all collected plants as well as the herbarium reference samples were dominated by sesquiterpene coumarin ethers. Furthermore, *F. arrigonii* is taxonomically related to *Ferula* species from Central and Southern Italy,[11] like *F. glauca*, and known to accumulate compounds of this type rather than sesquiterpene lactones, a trait more typical of *Ferula* species from Caucasus and Central Asia.[19] The possibility also exists that the botanical identification of the plant material containing 7,11 dehydrogrilactone (**2**) was erroneously assigned, but we are unable to propose an alternative chemotaxonomic determination. Indeed, sesquiterpene lactones are very rare in Mediterranean *Ferula* species whereas highly oxygenated and acylated guaianolides are described in various Mediterranean umbellifers, as exemplified by thapsigargins from *T. garganica*.[20]

The general decline in taxonomic training has spurred the proposal to make mandatory in phytochemical journals the adherence to strict rules regarding plant identification, the preparation of voucher specimens, and the identification of the site of collection, introducing voucher digitalization and geolocation.[21] The present investigation on the phytochemical profile of *F. arrigonii* demonstrates how crucial these rules are. In addition, the present work shows that herbarium voucher specimens, even stored for several decades, are still containing chemotaxonomic markers that can be detected by mass spectrometry. Thus, with the progress of analytical methods, such as tandem mass spectrometry and computational mass spectrometry, herbarium voucher specimens should be considered as precious material that can allow future phytochemical (re)investigation.

3. Experimental Section

Plant Material: F. arrigonii was collected in Bonifacio, on July 21st 2014, around the "cimetière marin" near the Citadelle (41°23'14.6"N 9°08'59.8"E). The plant material was collected and idenfied by L.-F. N. and a voucher specimen deposited into the herbarium of the laboratory of natural product chemistry at the Corte University. The plant material was air-dried for a period of three weeks at ambient temperature and was then powdered using a blade miller (PX-MCF 90D Kinematica).

Isolation of the constituents: Dried, powdered roots (200 g) were extracted with acetone (2 x 1.5 L). The pooled extracts were evaporated to leave a gummy residue (21 g, 10.5) that was fractionated by gravity colum chromatography (210 g silica gel, hexane-EtOAc gradient, from 95:5 to 70:30. Fractions were pooled according to their TLC profile (hexane-EtOAc 9:1 or 8:2) into four primary fractions (I-IV). Fraction I, III and IV crystallized spontaneosly to afford, after washing with ether, 420 mg of ferutinin (3b, 0.21%), 380 mg lapiferin (5, 0.19%), and 1.64 g colladonin (4a, 0.82%) respectively. Fraction II was re-chromatographed on neutral alumina to afford umbelliprenin (6a, 79 mg, 0,040%) and colladin (4b, 660 mg, 0.33%). The remaining compounds were obtained from the mother liquors of fractions III and IV by multiple gravity column chromatograp, and were all obtained in yield <0.01%.

LC-MS^2 experiments: All samples were extracted with 3 × 10 mL of acetone at ambient for 10 min at ambient temperature. The solutions were evaporated to dryness in vacuo yielding crude extract. The latter were partitioned by liquid–liquid extraction with acetonitrile/water (5/5) and pentane, and the solutions were evaporated to dryness. All extracts were stored at 4 °C until analysis. Solvents and other chemicals were purchased from VWR (France). The samples used for LC-MS^2 analysis were prepared by dissolving the extracts in MeOH at a concentration of 0.7mg/mL, and then filtered on 0.2 µm PTFE filter. General. Solvents and reagents used for sample preparation and chromatography were LC-MS grade: acetonitrile (ACN), methanol (MeOH), were obtained from Fisher Scientific (Illkirch, FR). Experiments were performed using a 3200 QTRAP AB Sciex (Framingham, MA, USA) linear triple quadrupole mass spectrometer fitted with APCI Turbo V^{TM} ion source operating in positive mode. High purity nitrogen was used both as nebulizer and turbo gas. The LC system consists of a Flexar LC PerkinElmer (Waltham, MA, USA) made up of two Flexar FX-10 LC pump, a Flexar solvent manager, a 275-Flexar autosampler, and a Flexar LC PE200 column oven. LC separations were performed on a 100 × 2.1 mm i.d., RP 18, 3 µm, LUNA 3U column (Phenomenex) and the column temperature was set at 25 °C. The injected sample volume was 10 µL using an injection loop of 15 µL in partial loop mode. The mobile phase consisted in milliQ water (solvent A) and ACN (solvent B) each containing 0.1% (v/v) NH_4AcO buffer. During LC analysis, the flow rate was set at 700 µL/min and equilibration of the column was perform by a 50% A-50% B elution (5 min); elution was carried out with the following steps: 50% A-50% B for 1 min, followed by a linear gradient of 50–75% B during 16 min; increased from 75% B to 100% in 4 min; and 100% B during 10 min. The APCI source parameters for LC-MS^n analysis were set as follow: CUR (25 psi); CAD (high); GS1 (31 psi); GS2 (65 psi); IS (5000 V); temperature (450 °C). MS^2 spectra were acquired by an MS^2 scan with the following parameters: Q1 resolution (unit), Q3 resolution (unit); DP (declustering potential) 70 V; EP (entrance potential) 10 V; CE (collision energy) 35 V and CES (± 15 V). LC-MS/MS analyses were performed by using ion trap full scan MS through EMS (Enhanced Mass Spectrometry) followed by an MS^2 (Enhanced Product Ion) scan triggered by IDA (Information Dependent Acquisition) with parameters adapted from a previous publication.[22] MS Range used for EMS and MS^2 experiments were *m*/*z* 100–1000. IDA properties were set to select 1 to 2 peaks above 30,000 counts, and with an exclusion rule after 10 occurrences for 30 s with dynamic background subtraction. MS^2-EPI mass spectra were recorded in the range of *m*/*z* 50–1000 at 4000 Da/s. The most intense fragmentation reaction for the reference compounds 4a and 4b were optimized using the automated component optimization function of the Analyst software. The most intense pseudo-molecular ion was selected as precursor ions, $[M\text{-}H_2O+H]^+$ (*m*/*z* 365) for the colladonine (4a), and $[M\text{-}Ac+H]^+$ (*m*/*z* 383.3) for colladine (4b), and the two most intense fragment ions respectively produced were selected as product ion.

Data processing and molecular networking: Tandem mass spectrometry molecular networks were created using GNPS platform (http://gnps.ucsd.edu).[16] Data were first converted to the .mzML format with MS-Convert. The spectral information (MGF file) was generated with MZmine2[17] and and processed with the GNPS 2.0 workflow (https://bix-lab.ucsd.edu/display/Public/GNPS+data+analysis+workflow+2.0#GNPSdataanalysisworkflow2.0-GNPSdataanalysisworkflow2.0%3AOFFLINEversion). This MGF file was used to generate a MS/MS molecular network using the GNPS Data Analysis workflow, with the MS-Cluster deactivated. The precursor ion mass tolerance was set to 1.0 Da, and to a product ion tolerance of 0.5 Da. The fragment ions below ten counts were removed from MS/MS spectra. MNs networks were generated using 12 minimum matched peaks and a cosine score of 0.7. Data were open and visualized using Cytoscape 3.4.0 software.[23] A force-directed layout modulated by cosine score factor was used for data visualizations. Data of LC-MS/MS analysis were deposited to MassIVE Public GNPS dataset (ftp://massive.ucsd.edu/MSV000081946). The molecular networking job on GNPS can be found here: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bb6baad6557140c5a2c29a708115bbdc.

The corresponding Cytoscape file is available as Supporting Material. The annotated MS/MS spectra were deposited on GNPS spectral library under references: CCMSLIB00004678621, CCMSLIB00004678624 for compounds 4a, 4b, respectively.

Acknowledgement

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