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IDENTIFICATION AND DETERMINATION OF ALKALOIDS IN *FUMARIA* SPECIES FROM ROMANIA

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Four *Fumaria* species (*F. vaillantii* Loisel, *F. parviflora* Lam., *F. rostellata* Knaf and *F. jankae* Hausskn.) were analysed in order to determine the presence of the isoquinoline alkaloids allocryptopine, chelidonine, protopine, bicuculline, sanguinarine, cheleritrine, stylopine, and hydrastine through an HPLC-DAD method. Protopine and sanguinarine were present in all extracts. Bicuculline and stylopine were found in *F. vaillantii* and *F. parviflora*, whilst chelidonine was identified only in *F. vaillantii* and hydrastine in *F. jankae*, so they represent potential taxonomic markers that differentiate the four plants. The richest species in isoquinoline alkaloids was *F. parviflora*. Our study showed significant differences between the four *Fumaria* species, both qualitative and quantitative.

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1. Introduction

The families *Fumariaceae* and *Papaveraceae* are closely related and are both very rich in isoquinoline alkaloids, especially of the aporphine, benzophenanthridine, protoberberine and protopine types. In Europe, the family *Fumariaceae* is represented by the genera *Fumaria, Corydalis, Rupicapnos, Platycapnos, Sarcocapnos* and *Ceratocapnos*. The genus *Fumaria* comprises 60 species, most of which grow around the Mediterranean region. Because the identification of these plants is frequently vague or imprecise owing to their highly similar morphological characteristics, the results of chemotaxonomic investigations could be valuable for the systematic evaluation of this genus [1-3].

The ethnobotanical data from Romania mention nine *Fumaria* species: *F. kralikii* Jord., *F. thuretii* Boiss., *F. schleicheri* Soy.-Willem., *F. parviflora* Lam., *F. vaillantii* Loisel, *F. jankae* Hausskn., *F. officinalis* L., *F. rostellata* Knaf, and *F. densiflora* DC. [4].

Plants of the genus *Fumaria* have been used in traditional medicine for their anti-hypertensive, diuretic, hepatoprotective and laxative properties, as well as in the treatment of some skin diseases, such as rashes or conjuctivites [1,5]. The biological activity is mostly associated with the presence of isoquinoline alkaloids in the plant [3].

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F. parviflora is a small branched annual herb found in many parts of the world, including Europe, Middle East and South Asia [4,6,7]. In traditional medicine, the plant is used as diuretic, expectorant, anti-pyretic, anti-scabies, anti-scorbite, diaphoretic, anti-neoplastic, for management of liver and skin disorders [8-10]. Phytochemical analysis of *F. parviflora* has shown the presence of isoquinoline alkaloids, such as protopine, cryptopine, sinactine, coptisine, fumariline, fumaritine, fumarophycine, O-methylfumarophycine, palmatine, parfumine, parfumidine, dihydrosanguinarine, stylopine, N-methylstylopine [1,3]. Antinociceptive effects [11], acetylcholinesterase and butyrylcholinesterase inhibitory activities [12], antioxidant and hepatoprotective properties [13,14], prokinetic and laxative properties [7], antidiarrheal, antispasmodic and bronchodilatator properties *in vivo* and *in vitro* [6], the antioxidant efficacy and protective role of the *F. parviflora* extract against nimesulide induced apoptosis [8,9] have been reported.

F. vaillantii is an annual plant that grows in Europe and Asia [2,4]. It has been reported to be used traditionally in the folk medicine for treatment of hepatobiliary disorders, dermatological diseases, dysfunction and gastrointestinal disorders and as a blood purifier [2,15]. The antimicrobial and antioxidant properties are due to alkaloids, tannins, fumaric acid and glucosinolates [2,3]. The isoquinoline alkaloids adlumiceine, adlumidiceine, coptisine, cryptopine, fumaricine, fumariline, fumaritine, fumarophycine, O-methylfumarophycine, parfumine, protopine, sinactine, N-methylstylopine were determined in *F. vaillantii* [3]. The major alkaloids of *F. parviflora* and *F. vaillantii* were protopine and fumarophycine [1]. Previous studies showed for protopine antithrombotic, anti-inflammatory, antinociceptive, inhibitory effect on K_{ATP} channel in human embryonic kidney cells [16,17], hepatoprotective properties equally effective to silymarin [18] and anticancer activity on human hormone-refractory prostate cancer cells [19].

Sanguinarine is a benzophenanthridine alkaloid with antimicrobial, antioxidant, anti-inflammatory, antiplatelet properties [20-22]. Sanguinarine also exhibits anticancer potentials and is currently receiving attention from researchers [23]. Sanguinarine induces cell cycle arrest at different phases or apoptosis in a variety of cancer cells [24-28]. Sanguinarine also shows antiangiogenic effects in mice presents anti-invasive effects, and overcomes P-gp-mediated MDR phenotype [29-31].

F. rostellata is an annual species that occurs in Central Europe and the Balkans. *F. jankae* is a very rare endemic species which is found in Romania in a single location, in Sacuieni (Bihor County) [4]. To the best of our knowledge, there are no available reports on chemical composition and biological activities of these two *Fumaria* species.

This paper describes a HPLC-DAD method for identification and quantification of isoquinoline alkaloids in four species of *Fumaria*. The method is based on a previous published paper [3], with some modifications. Because the chemical composition of *Fumaria* species from Romania has been insufficiently studied, the aim of this work was to bring new data on isoquinoline alkaloids in aerial parts of *F. vaillantii*, *F. parviflora*, *F. rostellata* and *F. jankae*.

2. Experimental

General Apparatus and Chromatographic Conditions: an Agilent 1100 HPLC Series system was used (Agilent, Santa Clara, CA, USA) consisting of a degasser (G1379A), a high pressure Quaternary pump (G1311A), a Autosampler (G1313A), a Thermostatic Compartment (G1316A) and a Diode Array Detector (G1315A).

2.1. HPLC-DAD Qualitative Conditions

The separation of alkaloids from *F. vaillantii* was carried out using an Hypersil (Runcorn, UK) ODS C_{18} column (250 × 4.6 mm i.d., 5 μm particle), which was maintained at 25 °C. Solvents for the preparation of the mobile phases were: I acetonitrile and II 0.1M triethylamine and 0.01M sodium heptane sulfonate, adjusted with H_3PO_4 to pH 2.5 [3]. Mobile phases consisted of A 25% of I and 75% of II (v/v); and B 60% of I and 40% of II (v/v). The gradient elution was: 0-2 min 80% A, 2-10 min 50% A, 10-26 min 100% B.

In order to obtain a better separation of alkaloids from *F. rostellata*, an Inertsil (Phenomenex, Torrance, CA, USA) Phenyl RP column (250 × 4.6 mm i.d., 5 μm particle) was used. The mobile phase program was: 0-3 min 90% II, 3-10 min 60% II, 10-25 min 40% II, 25-26 min 90% II. The best separation was achieved using the above column and the gradient elution as follows: 0-1 min 85% II, 1-20 min 70% II, 20-30 min 50% II, 30-35 min 40% II, 35-40 min 85% II.

The flow rate was 1 mL/min and the injection volume was 10 μL. The UV detection was performed at 290 nm.

The UV spectra of each separated compound from extracts were compared to spectra of standards, which allow positive identification of compounds, based on spectral match [32]. Using the chromatographic conditions described above, the isoquinoline alkaloids eluted in less than 40 min.

2.2. HPLC-DAD Quantitative Conditions

Because the best separation of isoquinoline derivatives was achieved using an Inertsil (Phenomenex, Torrance, CA, USA) Phenyl RP column (250 × 4.6 mm i.d., 5 μm particle) and the gradient elution: 0-1 min 85% II, 1-20 min 70% II, 20-30 min 50% II, 30-35 min 40% II, 35-40 min 85% II, the same chromatographic conditions were maintained for quantitative analyses. The isoquinoline alkaloids from *Fumaria* species extracts eluted in less than 30 min (Table 1).

Table 1. Retention times (R_T) of isoquinoline alkaloids (min).

Peak no.	Isoquinoline alkaloid	R_T ± SD
1.	Cheleritrine	18.00 ± 0.05
2.	Hydrastine	21.76 ± 0.03
3.	Bicuculline	22.40 ± 0.04
4.	Protopine	24.79 ± 0.14
5.	Chelidonine	26.29 ± 0.08
6.	Allocryptopine	27.16 ± 0.10
7.	Stylopine	27.25 ± 0.10
8.	Sanguinarine	29.13 ± 0.03

Note: SD, standard deviation.

For all compounds, the limit of quantification was 0.5 μg/mL, and the limit of detection was 0.1 μg/mL. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method [3,33]. The Agilent ChemStation (vA09.03) and DataAnalysis (v5.3) software were used for the acquisition and analysis of chromatographic data.

2.3. Chemicals

Standards: stylopine and allocryptopine were purchased from ChromaDex Inc. (Irvine, CA, USA), hydrastine from Brückner Lampe & Co (Berlin, Germany), sanguinarine chloride hydrate from Sigma-Aldrich (St. Louis, MO, USA), cheleritrine chloride from Extrasynthese (Lyon, France), protopine, bicuculline and chelidonine were a gift from Institute for Drug Research Center, Department of Pharmacognosy, University of Liege, Belgium. All references had a purity ≥98.0% by HPLC-DAD. Methanol gradient grade for liquid chromatography LiChrosolv, acetonitrile gradient grade for liquid chromatography LiChrosolv, sodium heptane sulfonate of HPLC analytical-grade, sulphuric acid of HPLC analytical-grade, ammonia of HPLC analytical-grade and ethyl acetate of HPLC analytical-grade were purchased from Merck (Darmstadt, Germany), trifluoroacetic acid of HPLC analytical-grade, triethylamine of HPLC analytical-grade and phosphoric acid of HPLC analytical-grade from Sigma-Aldrich (St. Louis, MO, USA). Methanolic stock solutions (1mg/mL) of the isoquinoline alkaloid standards were prepared and stored at 4 °C, protected from daylight. They were appropriately diluted with double distilled

water before being used as working solutions. The ultra pure water used for all determinations was obtained from a Milli-Q RG (Millipore SA, Molsheim, France) water system.

2.4. Plant material and preparation of extracts

The aerial parts of *Fumaria vaillantii* Loisel were collected in June 2009 from Bistrita-Nasaud (Bistrita County), *F. parviflora* Lam. and F. *jankae* Hausskn. in May 2010 from Sacuieni (Bihor County) and *F. rostellata* Knaf in May 2010 from Varsolt (Salaj County). These samples were authenticated by the coauthor Professor Mircea Tamas. Voucher specimens are deposited in the Herbarium, Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Medicine and Pharmacy Cluj-Napoca, Romania.

Plant materials were dried in the shade at room temperature and then finely ground. For qualitative analysis, fifty grams of each plant were extracted with 70% ethanol (Merck, Darmstadt, Germany) by a modified Squibb repercolation method [34]. Briefly, three successive applications of the same solvent were repercolated to the plant material. In each percolator, plant material was moistened with the solvent, macerated for two days and then percolated at a rate of about 4 to 6 drops per min for 50 g of raw material. The first percolated fractions from each percolator were saved and the next fractions were poured in the next percolator. Then, saved fractions (60 mL from the first one, 90 mL from the second one and 150 mL from the third one) were mixed and the resulting extract was 1:1 (w:v) [35]. The extract was concentrated in vacuum at temperature below 40 °C using a rotary evaporator (Buchi, Flawil, Switzerland). The obtained residue was extracted with sulphuric acid (15 mL, 0.05M H_2SO_4) in an ultrasonic bath for 15 min. The solution is filtered using filter paper, is completed at 20 mL with 0.05 M sulphuric acid and filtered again. Then, ammonia (1 mL, 30% $NH_3 \cdot H_2O$) and 10 mL ethyl acetate was added. The alkaloids were extracted successively by phase separation in a separating funnel three times. The organic phases were dried over magnesium sulphate and the solvent evaporated under reduced pressure. The residue was dissolved in 10 mL of methanol in a volumetric flask; the mixtures were centrifuged at 4,000 rpm and filtered through a 0.45 μm filter before injection.

For quantitative determination, extraction method described in European Pharmacopoeia 7.0 [36] in *Fumariae herba* monograph was used. Briefly, the powders of *Fumaria* herbs are weighed accurately (approximately 1.0g) in a stopper conical flask, followed by the addition of ammonia (5 mL, 10% $NH_3 \cdot H_2O$), soaking it completely. Then, each sample is extracted using ethyl acetate (50 mL) in a separating funnel. The organic phases were evaporated under reduced pressure, followed by the addition of 0.05M sulphuric acid, filtered and completed with the same solvent in a 100 mL volumetric flask. Subsequently, the pH of each solution was adjusted with 30% ammonia to 9-10. The alkaloids are extracted successively by phase separation in a separating funnel three times with 10 mL ethyl acetate. The organic phases were dried over magnesium sulphate and the solvent evaporated under reduced pressure. The residue was dissolved in 10 mL of methanol in a volumetric flask; the mixtures were centrifuged at 4,000 rpm and filtered through a 0.45 μm filter before injection.

3. Results and discussion

3.1. The Identification of Isoquinoline Alkaloids

A high performance liquid chromatographic (HPLC) method has been developed for the identification of eight isoquinoline alkaloids from natural products. The simultaneous analysis of alkaloids was performed by a single column pass, and the separation of all examined compounds was carried out in 40 min. In order to obtain a better separation of the compounds, we used several columns and gradient elutions.

The UV spectra of bicuculline and chelidonine from *F. vaillantii* extract are presented in Figure 1 and the UV spectra of protopine and sanguinarine from *F. rostellata* extract are presented in Fig 2.

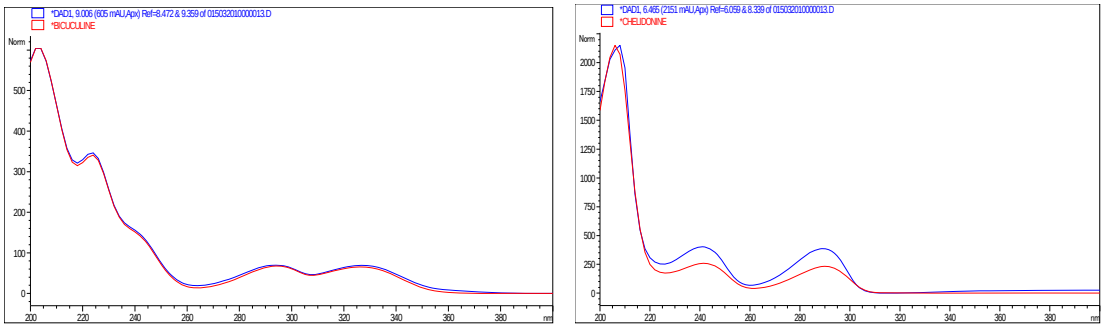

Fig. 1. UV spectra of bicuculline (a) and chelidonine (b) from F. vaillantii extract superposed with references spectra.

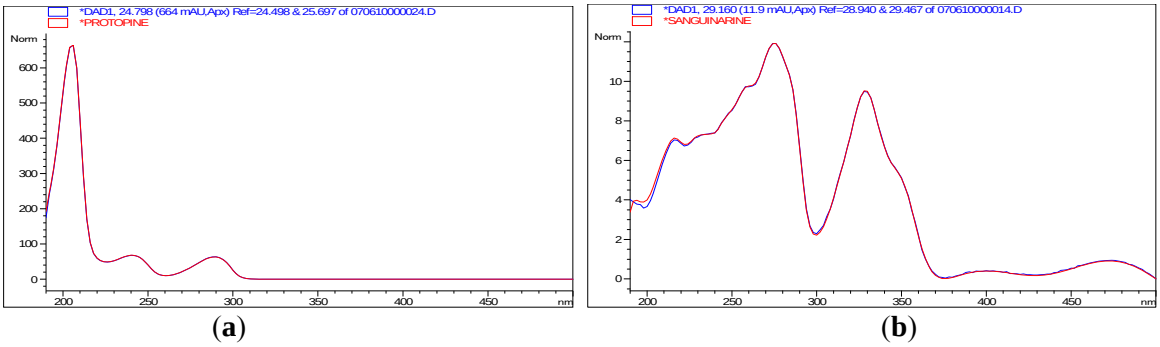

Fig. 2. UV spectra of protopine (a) and sanguinarine (b) from F. rostellata extract superposed with references spectra.

The chemical composition of some *Fumaria* species has been insufficiently studied in the past. Protopine and stylopine were previously identified by GC-MS and by HPLC in *F. vaillantii* and *F. parviflora* [1,3]. The present phytochemical research of four *Fumaria* species from Romania adds information about alkaloid composition: various alkaloids were identified and quantified for the first time in all plants (Table 2).

Table 2. Alkaloids found for the first time in the various Fumaria species investigated.

Species	Alkaloids
F. vaillantii	bicuculline, sanguinarine, chelidonine
F. parviflora	bicuculline, sanguinarine
F. rostellata [a]	protopine, sanguinarine
F. jankae [a]	hydrastine, protopine, sanguinarine

Note: This species has not been previously investigated.

3.2. The Quantitative Determination of Isoquinoline Alkaloids

For quantitative determination, extracts were prepared as described in the European Pharmacopoeia *Fumariae herba* monograph [36]. This method allows a better extraction of isoquinoline alkaloids than the one used for qualitative analysis, some compounds being identified only in these extracts.

The HPLC chromatogram of *F. rostellata* extract is presented in Figure 3.

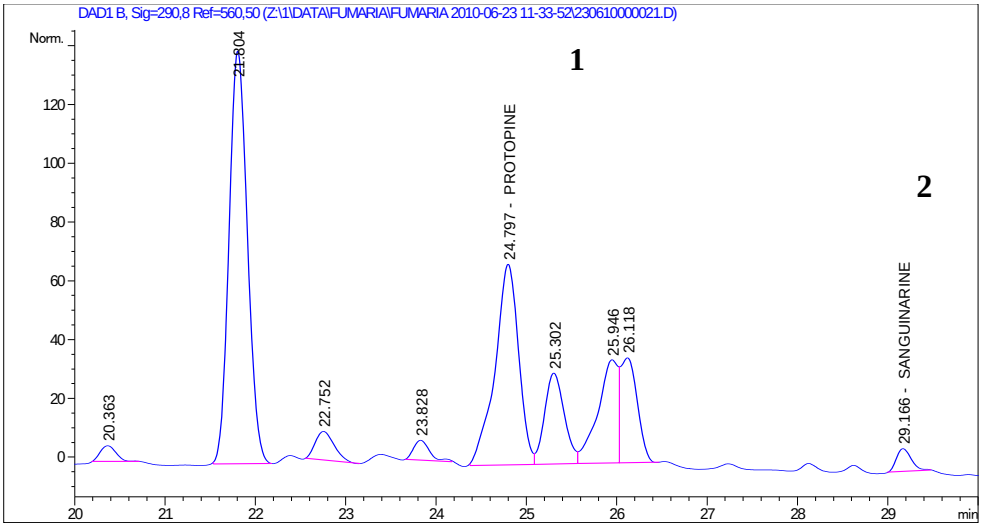

Fig. 3. HPLC chromatogram of F. rostellata.

Notes: Chromatographic conditions were as given in the Experimental section.
The identified compounds: 1, Protopine (R_T=24.79); 2, Sanguinarine (R_T=29.16).

The amount of individual alkaloids in *Fumaria* species extracts as determined by HPLC-DAD is reported in Table 3.

Table 3. The isoquinoline alkaloids content of Fumaria species (mg/100 g vegetal product).

Alkaloid	*F. vaillantii*	*F. parviflora*	*F. rostellata*	*F. jankae*
Hydrastine	-	-	-	6.63 ± 0.07
Bicuculline	23.23 ± 0.41	104.06 ± 1.73	-	-
Protopine	114.56 ± 1.52	288.27 ± 1.81	156.15 ± 1.33	144.32 ± 1.29
Stylopine	5.88 ± 0.12	17.86 ± 0.34	-	-
Sanguinarine	3.23 ± 0.05	-	2.62 ± 0.04	3.41 ± 0.06

Note: Values are the mean ± SD (n = 3); (-) = not determined

Protopine and sanguinarine were identified in all extracts (sanguinarine was present in trace amounts in *F. parviflora* extract). The major alkaloid in all species was protopine, with higher amount in *F. parviflora* (288.27 mg/100 g), followed by *F. rostellata* (156.15 mg/100 g) and *F. jankae* (144.32 mg/100 g). Bicuculline and stylopine were found only in *F. vaillantii* (23.23 mg/100 g, and respectively 5.88 mg/100 g) and *F. parviflora* (104.06 mg/100 g, and respectively 17.86 mg/100 g). Chelidonine was determined only in *F. vaillantii* extract, whereas hydrastine only in *F. jankae*. The pattern of isoquinoline alkaloids indicates large differences between the four *Fumaria* species, so they can be used as potential taxonomic markers in order to distinguish the plants.

We analyzed the isoquinoline alkaloids from four *Fumaria* species: *F. vaillantii*, *F. parviflora*, *F. rostellata* and *F. jankae*. The simultaneous determination of isoquinoline compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by UV detection, and the comparative study showed large differences between the *Fumaria* species. Considering the broad-spectrum therapeutic potential of isoquinoline alkaloids [17-31], further studies are needed to improve medicinal uses of *Fumaria* species from Romania.

4. Conclusions

We analyzed the isoquinoline alkaloids from four *Fumaria* species: *F. vaillantii*, *F. parviflora*, *F. rostellata* and *F. jankae*, and we completed the available data with new information

concerning the alkaloids from *Fumaria* species. The simultaneous determination of isoquinoline compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by UV detection. The analysis of isoquinoline alkaloids from *F. rostellata* and *F. jankae* was performed for the first time. The comparative study showed large differences, both qualitative and quantitative, between the four *Fumaria* species.

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