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HPLC DETERMINATION OF SOME PHENOLIC COMPOUNDS OF *Scrophularia nodosa* AND *S. scopolii*

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Scrophularia nodosa L. (Scrophulariaceae), also known as figwort, is a perennial herb that grows in Central Europe, Central Asia, and North America and has been used in traditional medicine to treat eczema, wounds, ulcers, fistulae, cancer, and also as a diuretic and anthelminthic agent. In modern herbal medicine *Scrophularia nodosa* L. is used to treat eruptive skin diseases, eczemas, psoriasis, pruritus, ulcers and also as a purgative agent [1–3].

The main active components from *Scrophularia nodosa* L. are iridoid glycosides (aucubine, catalpol, and harpagoside), flavonoids, phenolic acids, and cardio-active glycosides. Phenolic and polyphenolic compounds represent one of the most widespread groups of secondary plant metabolites. They are divided into several structural classes, as follows: phenol carboxylic acids and derivatives, flavonoids, tannins, coumarins, anthranoids, and lignans [4, 5]. Some of the polyphenols show therapeutic properties such as antioxidant, antibacterial, antiviral, analgesic, antispasmodic, neuroprotective, cytostatic, and anti-inflammatory activity [6–11].

In Romanian flora there are only five species of *Scrophularia* genus from the 300 species of the genus growing all over the world. *Scrophularia scopolii* L. is an herb with red-brown flowers that grows in all regions of Romania. This herb is not recognized as a medicinal plant.

The main objective of this work was the characterization and quantification of phenolic compounds in *Scrophularia nodosa* L. and *Scrophularia scopolii* L. as part of a phytochemical study of these species. In order to obtain more accurate data regarding the flavonol and flavone glycosides and aglycones content, the plant material was analyzed with and without acid hydrolysis. The separation of the isolated phenolic compounds was achieved by HPLC (Table 1), previously elaborated at our university [12, 13]. In order to perform the qualitative and quantitative analysis, 18 polyphenolic standards were used, as follows: seven cinnamic acid derivatives (*p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, caftaric acid, and gentisic acid), four quercetin glycosides (hyperoside, isoquercetin, quercitrin, and rutoside), five flavonol aglycones (quercetin, kaempferol, patuletin, myricetol, and fisetin) and two flavone aglycones (apigenin and luteolin). The polyphenols were identified by comparison with authentic standards and additionally by liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS or MS/MS), followed by their quantification based on a HPLC-UV analysis.

The HPLC-UV and HPLC-MS (MS/MS) analysis of the plant materials revealed varying concentrations for some of the analyzed polyphenols, while a few of the chosen 18 polyphenol standards were absent in the different plant tissues (Table 2).

The pattern of phenol carboxylic acids showed the absence of gentisic acid and sinapic acid in all plant materials that were analyzed from both *Scrophularia* species. Also the tartaric ester of caftaric acid could not be detected in these samples. *p*-Coumaric acid appears to be the most abundant constituent in the leaves of *Scrophularia nodosa* L. and the herba of *Scrophularia scopolii* L., mainly in esteric forms but also as free acid in much smaller quantities. These two samples contained also significant quantities of ferulic acid in free and esteric form. In the stem of *Scrophularia nodosa* L. these two acids (*p*-coumaric and ferulic acid) could be detected and quantified only after a previous acid hydrolysis, indicating their presence only as esters.

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TABLE 1. Retention times (t_R) and Mass Spectral Data for the Investigated Polyphenols

Compound	t_R±SD, min	MS (*m/z* precursor ion> *m/z* product ion)	Compound	t_R±SD, min	MS (*m/z* precursor ion> *m/z* product ion)
Caftaric acid	2.10±0.06	311>148.6, 178.6	Rutoside	20.20±0.15	609.1>254.9, 270.9, 300.7
Gentisic acid	2.15±0.07	153>108.7	Myricetol	20.70±0.06	317.1>136.6, 150.6, 178.6
Caffeic acid	5.60±0.04	179.4>134.7	Fisetin	22.60±0.15	285>134.6, 162.6, 240.7
Chlorogenic acid	5.62±0.05	353.5>178.7, 190.7	Quercitrin	23.00±0.13	447.1>178.8, 300.7
p-Coumaric acid	8.7±0.08	163>118.7	Quercetol	26.80±0.15	301>150.6, 178.6, 272.7
Ferulic acid	12.2±0.10	193.2>133.7, 148.7, 177.6	Patuletin	28.70±0.12	331>315.7
Sinapic acid	14.3±0.10	223.4>148.6, 163.6, 178.7	Luteolin	29.10±0.19	285>150.6, 174.6, 198.6
Hyperoside	18.60±0.12	463>254.9, 270.9, 300.7	Kaempferol	31.60±0.17	285>150.6, 256.7
Isoquercitrin	19.60±0.10	463>254.9, 270.9, 300.7	Apigenin	33.10±0.15	269.2>148.6, 150.6, 224.7

SD: standard deviation.

TABLE 2. Concentration of Polyphenols in *Scrophularia nodosa* L. and *Scrophularia scopolii* L. (mg/100 g vegetal sample)

Compound	*S. nodosa* leaves		*S. nodosa* stem		*S. scopolii* herb	
	NH	H	NH	H	NH	H
Caffeic acid	X	X	–	X	–	–
Chlorogenic acid	X	X	–	–	X	–
p-Coumaric acid	17.04	218.68	–	15.84	11.02	110.34
Ferulic acid	14.16	79.38	–	16.18	18.72	31.36
Isoquercitrin	320.96	–	20.46	–	22.00	–
Rutoside	428.68	–	29.36	–	42.72	–
Quercitrin	11.12	–	–	–	7.38	–
Quercetol	–	307.30	–	12.20	–	22.10
Luteolin	6.74	–	–	–	–	1.90
Kaempferol	–	12.78	–	–	–	14.1

NH: non hydrolysed sample; H: hydrolysed sample; X: compounds that were only identified, without quantitative analysis.

The HPLC-MS screening showed the occurrence of caffeic acid and chlorogenic acid, but their quantification through HPLC-UV was not possible since these two phenol carboxylic acids were not separated in the presented chromatographic conditions (see Apparatus and Chromatographic Conditions). The stem of *Scrophularia nodosa* L. contained only caffeic acid in esteric form, while the leaves of the plant showed both acids, before and after hydrolysis. Since quantification of the two acids was not performed, it was impossible to determine if these two compounds were present only as free acids or as esters as well. In the case of *Scrophularia scopolii* L. herba only chlorogenic acid was observed without hydrolysis (as free acid).

In the case of flavone aglycones the two *Scrophularia* species studied contained no trace of apigenin. Minor quantities of luteolin were detected in *Scrophularia nodosa* L. leaves and *Scrophularia scopolii* L. herba only in esteric form.

Regarding the flavonols myricetol, fisetin, and patuletin, they were absent in all samples before and after hydrolysis. Quercetol proved to be the main flavonol in all plant materials, followed by kaempferol. These two aglycones seemed to be present only as glycosides, since they were detected only after hydrolysis.

All samples contained two quercetin glycosides, isoquercitrin and rutoside, with significantly higher quantities in leaves of *Scrophularia nodosa* L. The isoquercitrin/rutoside ratio indicated that in all samples the preferred sugar for glycosylation of quercetin is rutinoside, as the major glycoside was rutoside. Small amounts of quercitrin were found in *Scrophularia nodosa* L. leaves and *Scrophularia scopolii* L. herba. The glycoside hyperoside was absent in all plant materials.

Apparatus and Chromatographic Conditions. The experiment was carried out using an Agilent 1100 Series HPLC system (Agilent USA) consisting of a G1322A degasser, G1311A quaternary gradient pump, and a G1313A autosampler. The chromatographic separation was achieved using a reversed-phase analytical column (Zorbax SB-C18 100 mm × 3.0 mm i.d., 3.5 µm particle) maintained at 48°C. The mobile phase consisted of a binary gradient prepared from methanol (solvent A) and

a 0.1% solution of acetic acid (v/v) (solvent B). The elution started with a linear gradient from 5 to 42% solvent A over the first 35 min, followed by isocratic elution with 42% solvent A for the next 3 min. The final step consisted in the return to the initial conditions (5% solvent A) over the next 3 min, and the system was equilibrated with this mobile phase. The mobile phase was delivered with a flow rate of 1 mL/min, and the injection volume was 5 µL. All solvents were filtered through 0.5 µm (Sartorius) filters and degassed through ultrasonication.

The detection of the compounds was performed on both UV and MS mode. The G1315A diode array detector was set at 330 nm for the first 17.5 min of the sample runtime, then at 370 nm until the end of the chromatographic analysis. The Agilent Ion Trap 1100VL mass spectrometer, coupled to the HPLC system, operated using an electrospray ionization source in the negative ion mode. Nitrogen was used as nebulizing and drying gas. The instrument was set to the following tune parameters: nebulizing gas pressure of 60 psi, drying gas flow of 12 L/min, drying gas temperature 350°C, capillary voltage +3000 V. The chromatographic data were processed using Chemstation and Data Analysis software from Agilent, USA.

Identification and Quantitative Determination of Polyphenols. The polyphenol carboxylic acids (caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) were detected in UV at 330 nm, while flavonoids (hyperoside, isoquercitrin, and rutoside) and their aglycones (myricetol, fisetin, quercitrin, quercetol, patuletin, luteolin, kaempferol, and apigenin) were monitored at 370 nm.

The MS detection of flavonoids and of their aglycones was carried out using the selective ion monitoring (SIM) mode, while in the case of polyphenol carboxylic acids, the multiple reaction monitoring (MRM) mode was selected. The MS signal was used for qualitative analysis based on specific mass spectra of each polyphenol. The compounds from the analyzed samples were identified by comparing their MS spectra to spectra obtained for standard solutions of polyphenols, based on spectral match. To facilitate this type of qualitative analysis, the mass spectra of the standards were included in a mass spectra library.

The chromatographic conditions described above guaranteed the elution of the 18 polyphenols in less than 35 min. The retention times were determined with a standard deviation ranging from 0.04–0.19 min (Table 1). Under these conditions there were two pairs of substances which coeluted: caftaric acid + gentisic acid and caffeic acid + chlorogenic acid, respectively. For this reason, in the case of these four polyphenol acids, only qualitative analysis was performed based on the recorded MS spectra. The quantitative analysis of the polyphenols, based on the UV detection, was performed using an external standard method. Standard calibration curves in the 0.5–50 µg/mL range with good linearity ($r^2 > 0.999$) for a five point plot were used to determine the concentration of polyphenols in plant samples. The detection limits were calculated as the minimal concentration producing a reproducible peak with a signal-to noise ratio greater than 3.

Plant Material. The plant material, consisting of stems and leaves of *Scrophularia nodosa* L. and *Scrophularia scopolii* L. herba, was collected from the wild flora of Romania and was identified in the Department of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. Voucher specimens were deposited in the Herbarium of this institution.

Sample Preparation. Samples of 100 mg air-dried herb, stem or leaves, were placed in 10 mL glass centrifuge tubes. In order to isolate the phenolic compounds as glycosides and as well as free aglycones, the samples were subjected to two different extraction procedures. For the extraction of phenolic compounds as glycosides, we used 2 mL water and 2 mL methanol, while in case of extraction as free aglycones, 2 mL hydrochloric acid (for acid hydrolysis) and 2 mL methanol were added to the tubes. In both cases, the samples were treated in the same manner. They were extracted at 80°C on a water bath for 30 min, ultrasonicated for 15 min, and then finally heated at 80°C for another 30 min. During the heating, 1 mL of methanol was added, every 10 min, in order to ensure the permanent presence of the solvent in the extraction process. After the extraction, the samples were centrifuged at 4000 rpm, the supernatant was collected, and the residue was extracted two times with 5 mL water using the same procedure. The combined supernatants were diluted with water in a 25 mL volumetric flask and filtered through a 0.45 µm filter before injection.

Chemicals. HPLC gradient grade methanol and analytical grade acetic acid and hydrochloric acid were purchased from Merck (Germany). Distilled deionized water was obtained using a Direct Q-5 Millipore system (Molsheim, France). Chlorogenic acid, *p*-coumaric acid, caffeic acid, rutoside, apigenin, quercetol, quercitrin, isoquercitrin, hyperoside, myricetol, fisetin, and kaempferol standards were obtained from Sigma (Germany), ferulic acid, sinapic acid, gentisic acid, patuletin, and luteolin from Roth (Germany), while caftaric acid was purchased from Dalton (USA). Methanolic stock solutions (100 mg/mL) of the above mentioned standards were prepared and stored at 4°C, protected from light. The working solutions were prepared freshly by diluting the standard stock solutions with distilled deionized water.

The presence of polyphenolic compounds in the leaves and stem of *Scrophularia nodosa* L. and in the herb of *Scrophularia scopolii* L. collected from the wild flora of Romania was investigated by using a previously elaborated HPLC-UV and HPLC-MS/MS method. The present research, as part of a phytochemical study, gives precious information on the composition of *Scrophularia nodosa* L. and *Scrophularia scopolii* L., allowing the comparison of the two *Scrophularia* species from the point of view of polyphenol content. Since these compounds are responsible, at least in part, for the biological activity of this plant material, the present study creates the base for a scientific and rational therapeutic utilization of these species. The *Scrophularia nodosa* L. leaves showed a greater content of polyphenolic compounds than *Scrophularia scopolii* L. herba, but the latter could also be used as a medicinal product, since it contains significant quantities of these types of active principles and so represent an alternative for *Scrophularia nodosa* L.

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