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THE ANALYSIS OF COUMARINS FROM *SCOPOLIA CARNIOLICA* JACQ. (*SOLANACEAE*) OF ROMANIAN SPONTANEOUS FLORA

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ABSTRACT. Coumarins from *Scopolia carniolica* Jacq. (*Solanaceae*), henbane bell, have been analysed. The qualitative analysis was performed by thin layer chromatography (TLC), scopoletin and two of its glycosides being emphasized. Scopoletin (free and total) was quantitatively determined by LC/MS techniques. The dynamics of coumarin accumulation was analysed in different vegetative organs harvested at 2-4 weeks interval during the vegetation period. The highest amount of free scopoletin was found in the underground organs before fruit maturation, after that period being identified in the aerial parts. The total scopoletin amount, determined after hydrolysis, was up to 70 times higher than the free scopoletin one, indicating that it is preferentially accumulated as glycosides, mostly in the rhizomes and roots.

Keywords: *scopoletin, LC/MS, Scopolia carniolica*

INTRODUCTION

Scopolia carniolica Jacq. like other *Solanaceae* members as *Atropa belladonna*, *Hyosciamus niger*, *Datura stramonium*, is known and used for its tropane alkaloids, atropine and scopolamine, substances with anticholinergic activity upon acetylcholine and muscarinic receptors. The main source of active principles is the underground part – the rhizomes, harvested for industrial extraction purposes. The isolated alkaloids are utilized as muscle relaxants, in preoperative medication or eye exams [**1**]. The plant also contains coumarins (scopoletin), flavonoids, choline, and polyphenols [**2,3**].

Pharmacological studies performed on the coumarin scopoletin (Figure 1) showed antinociceptive, antioxidant, antispasmodic, hypouricemic, hepatoprotective, antiproliferative and antidepressant-like activity [**4-10**]. Scopoletin also showed acetylcholinesterase inhibitory activity which can be used in treating Alzheimer disease [**11**].

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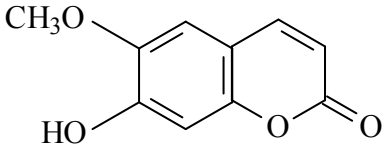

Figure 1. Chemical structure of scopoletin

Considering this promising pharmacological properties for scopoletin, the purpose of the present work was to analyze the vegetative organs of *S. carniolica* harvested from the spontaneous flora of Romania as possible sources for scopoletin extraction.

Literature reports about *Scopolia* genus concern mainly the alkaloid studies and there are few reports concerning the non-alkaloid constituents (coumarins, polyphenolic compounds), especially on *S. lurida* and *S. japonica* [**2**]. One former study on *S. carniolica* presented in literature only indicates the amount of scopoletin and total coumarins in leaves and underground parts of the cultivated species from Poland, quantified by RP-HPLC analysis [**2**].

We aimed to study the dynamics of accumulation of scopoletin in rhizomes, roots, stems and leaves of *S. carniolica*, in order to identify the organ and period when the maximum amount of it could be obtained. The plant samples were harvested at an interval of 2-4 weeks during the vegetation period, from early May to early August.

For the qualitative analysis of scopoletin and other coumarins we have used thin layer chromatography and for the quantitative determination of scopoletin, before and after the sample hydrolysis, high performance liquid chromatography coupled with mass spectrometry was used.

RESULTS AND DISCUSSION

The first step was the TLC identification of scopoletin and coumarin analysis in the plant samples. The samples were rhizomes (Srh), roots (Sra), stems (Sc) and leaves (Sf) harvested in early May (1), middle May (2), early June (3), end of June (4), middle July (5) and early August (6). The samples 2 and 2', 3 and 3', 4 and 4' represented respectively lower and upper stems and leaves. The compounds separated from these samples were compared to standards of scopoletin and umbeliferon (Figure 2 a-d).

By this technique, scopoletin was identified in all samples, except Sf1, Sf2 and Sf2' (afterwards it was identified and quantified in those samples by LC/MS/MS), other three substances (compounds 1, 2 and 3) being also separated.

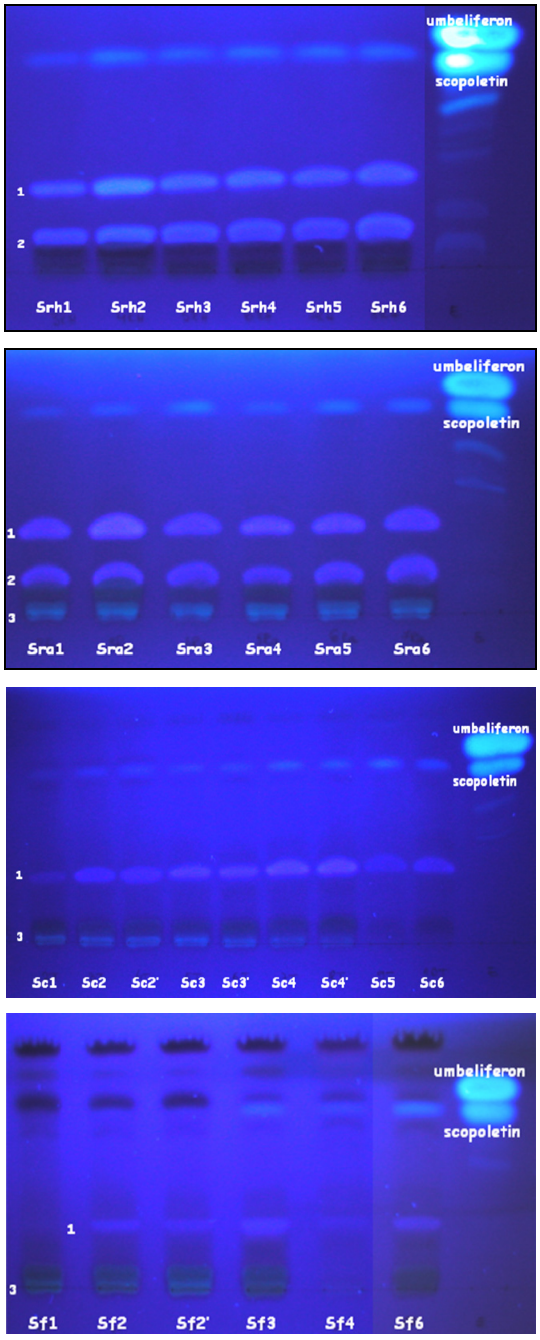

Figure 2. TLC chromatogram of coumarins in *S. carniolica* rhizomes (a), roots (b), stems (c), and leaves (d)

The TLC analysis of compounds 1 and 2 separated by preparative TLC in same analytical conditions as qualitative TLC was performed. The samples were analyzed before and after hydrolysis (H), and compared to the standard of scopoletin and scopoletin isolated by preparative TLC (Sp), the TLC chromatogram being showed in Figure 3. The compounds 1 and 2 are present in samples before hydrolysis and absent in samples after hydrolysis. Instead, in the samples after hydrolysis, scopoletin was identified; therefore we considered them scopoletin glycosides.

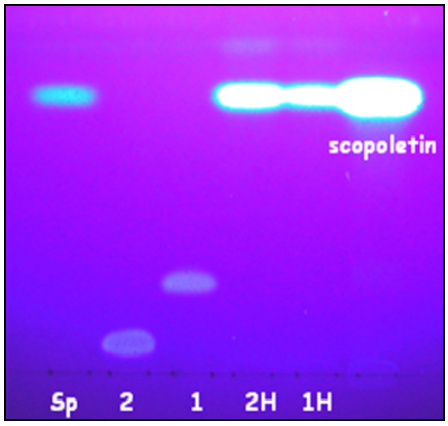

Figure 3. TLC chromatogram of compounds 1 and 2 before and after hydrolysis

Scopoletin (free and total) was quantitatively determined by LC/MS/MS. The plant samples were the same as described for the TLC analysis. The total scopoletin amount was determined in the same samples, but after acid hydrolysis.

The mass spectra of scopoletin is presented in Figure 4. The parent ion, with m/z 193, was fragmented by collision induced dissociations to daughter ions with m/z 133, 137, 149, 165 and 178 (Figure 5); the former ion was chosen for quantification, being the most intense.

The calibration curve of scopoletin was made between 12.8-256 ng/ml (8 concentration levels). For each concentration, the precision and inaccuracy were less than ±5%.

A typical chromatogram of scopoletin at quantification limit (12.8 ng/ml, determined at a signal-to-noise ratio of 10) is shown in Figure 6.

The quantitative results of the amount of scopoletin in analyzed samples are indicated in the charts below (Figure 7).

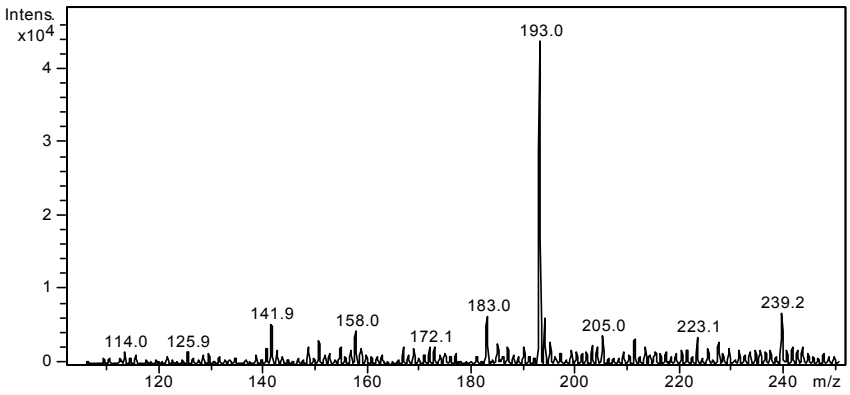

Figure 4. Full scan mass spectra of scopoletin

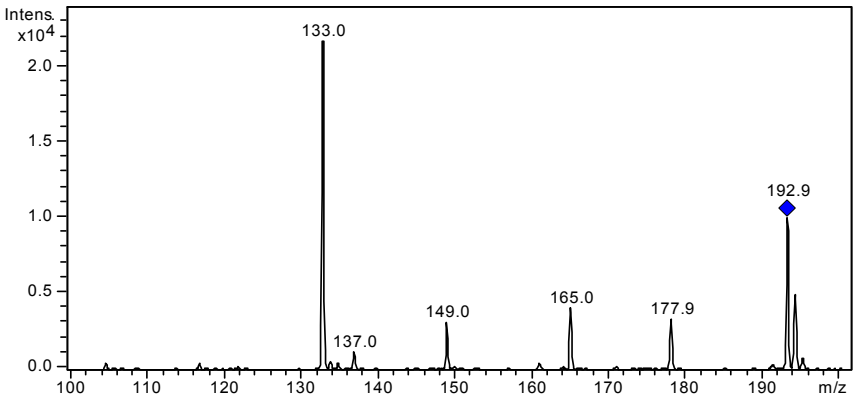

Figure 5. MS/MS fragmentation mass spectra of scopoletin

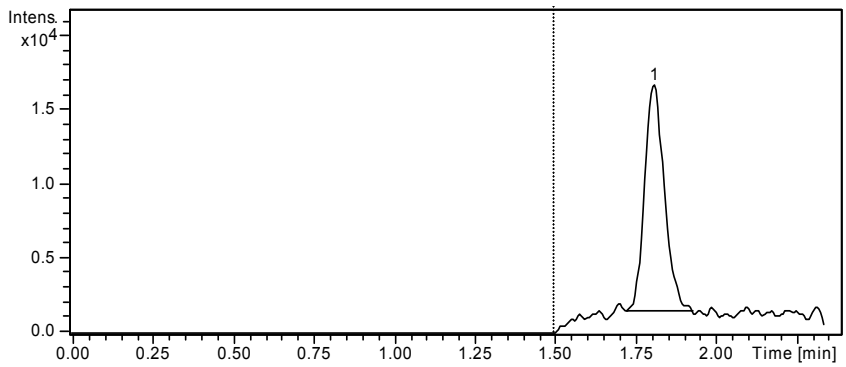

Figure 6. The chromatogram of scopoletin, at quantification limit of the analytical method (12.8 ng/ml)

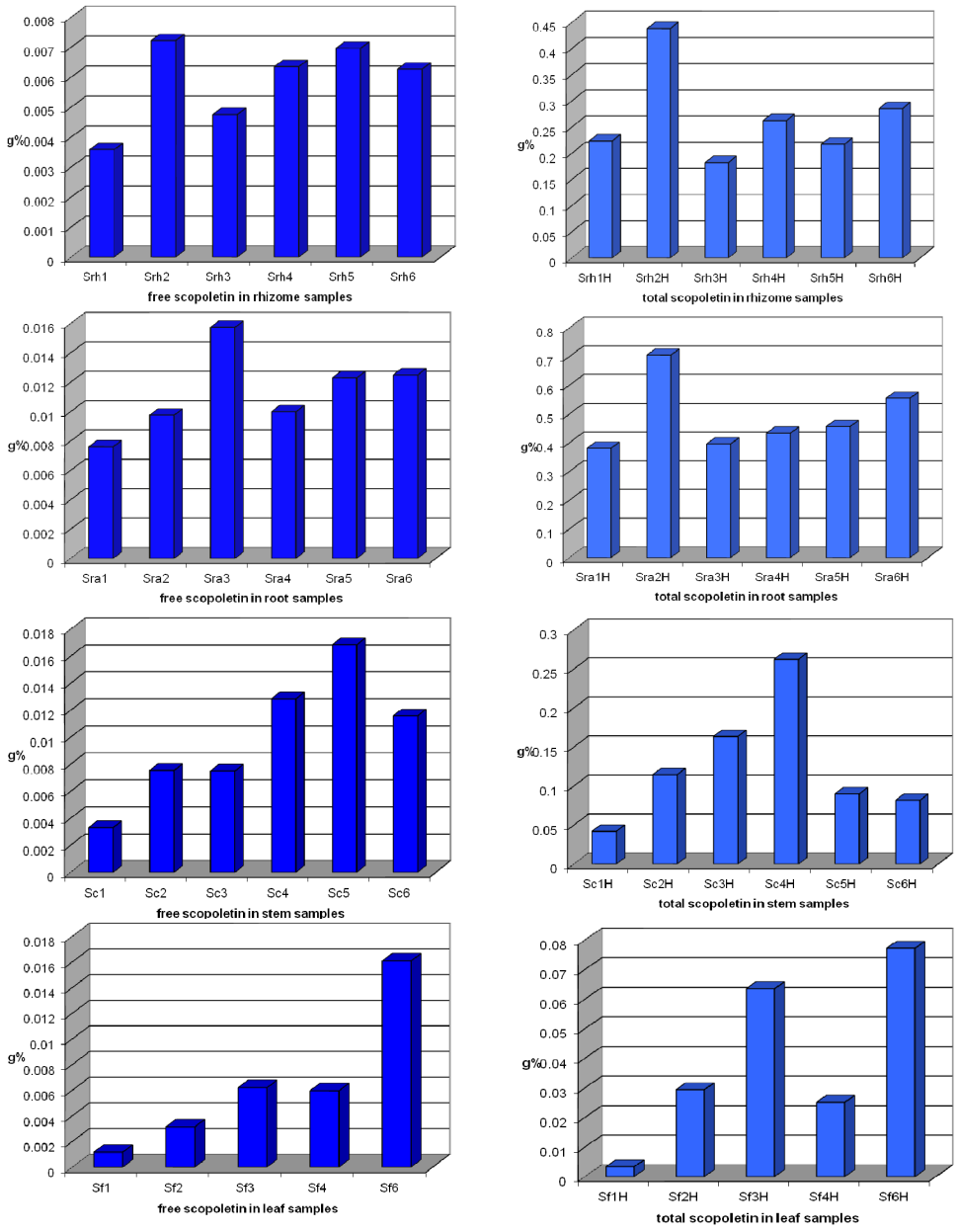

Figure 7. The amount of free and total scopoletin in rhizome, root, stem and leaf samples

The free and total scopoletin amounts determined during the vegetation period were: 0.0035–0.0069% respectively 0.18 - 0.43% in the rhizomes; 0.007 - 0.015% respectively 0.38 - 0.70% in the roots; 0.003 - 0.016% respectively 0.04 - 0.26% in the stems; 0.001 - 0.016% respectively 0.003 - 0.077% in the leaves.

The highest amount of free scopoletin was found for the rhizome in the samples harvested in middle May, for the root in the samples harvested in early June, for the stem in the samples harvested in middle July and for the leaf in the samples harvested in early August. The highest amount of free scopoletin was found in the underground organs before fruit maturation, after that period being identified in the aerial parts.

The highest amount of total scopoletin for rhizomes and roots was found in the samples harvested in May, for the stem in the samples harvested in June and for the leaves in the samples harvested in August. The total scopoletin amount, determined after hydrolysis, was up to 70 times higher than the free scopoletin one, indicating that it is preferentially accumulated as glycosides, mostly in the rhizomes and roots.

For the underground parts, the total amount of scopoletin is comparable to the total of coumarins found in the cultivated species from Poland [**2**], while the amount of free scopoletin is two times lower than the corresponding one. Concerning the leaves content, the amounts of free scopoletin and total scopoletin were 3 times respectively 6 times lower than the corresponding ones, suggesting that the climate and conditions of growth (spontaneous flora or culture) can influence this content.

CONCLUSION

The dynamics of scopoletin accumulation in vegetative organs of indigenous *S.carniolica* was quantified for the first time, using a rapid, precise and facile LC/MS method. The study revealed that the highest quantity of scopoletin accumulates as glycosides in rhizomes and roots during the month of May, accordingly to which we propose the harvesting of this vegetal medicinal product for extraction purposes in the period specified.

EXPERIMENTAL SECTION

Plant material

The rhizomes, roots, stems and leaves of Scopolia carniolica were collected from the same area in Remeţi, Maramureş County, during the months of May, June, July, and August. Plants were identified at the Pharmaceutical Botany Department, Faculty of Pharmacy, University of Medicine and Pharmacy

Cluj-Napoca, where a voucher specimen (nr. 972) was deposited. The vegetative organs were separated, the rhizomes were cut in round pieces, and all the plant material was dried at room temperature. The vegetal material was finely pulverized (sieve VI, Romanian Pharmacopoea X) [**12**].

TLC analysis

0.5 g powdered plat material was added to 10 mL methanol in a system provided with ascendant refrigerant and maintained at 80^{o}C for 10 min on water bath. The extraction liquid was filtered after cooling and the residue was pressed. Methanol was added to each sample up to 10 mL [**13**].

LC/MS analysis

0.1 g powdered plat material was added to 10 mL methanol in a system provided with ascendant refrigerant and maintained at 80^{o}C for 30 min on water bath. The extraction liquid was filtered after cooling and the residue was pressed. Methanol was added to each sample up to 10 mL (solution A). The hydrolyzed samples were prepared as follows: 0.1 mL solution A was treated with 2 mL of 2 N hydrochloric acid and maintained at 80^{o}C for 10 min on water bath in a system provided with ascendant refrigerant. After cooling distilled water was added up to 3 mL.

Reagents

Methanol of gradient grade for liquid chromatography, formic acid, hydrochloric acid, ethyl acetate, and potassium hydroxide of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water *pro injections* was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca (Romania). Standards: scopoletin from Roth (Germany) ad umbeliferon from Fluka (Germany) were used.

Apparatus and chromatography conditions

The TLC analysis was performed in the following conditions: Adsorbant: TLC Silica gel GF 254 (Merck) plates; Solvent system: ethyl acetate (p.a): methanol (p.a) : water (100/16.5/13.5) (v/v/v); Standards: scopoletin, umbeliferon 0,01% m/v in methanol; 20 μL of samples and standards were applied on 1 cm band with Microcaps TLC spotting capillaries; Detection: after drying, the plate was sprayed with potassium hydroxide 5% in methanol and examined after 30 min in fluorescence at 365 nm [13].

HPLC analysis

HP 1100 Series binary pump, HP 1100 Series auto sampler, HP 1100 Series thermostat, Agilent Ion Trap 1100 VL mass spectrometer; Column: Zorbax SB-C18 100 mm x 3,0 mm i.d., 3,5 μm (Agilent, SUA), on-line filter

0,2 µ (Agilent); Mobile phase: formic acid 0,1% (v/v) - methanol 68/32 (v/v), isocratic elution, 1 ml/min, 40°C, injection volume: 5 µL; Detection: mass spectrometry, m/z 193> m/z 133.

MS detection

Electrospray ionization (ESI) positive ionisation, gas: nitrogen, flow rate 12 l/min, ion source temperature 350°C, nebuliser: nitrogen at 70psi pressure capillary voltage -1500 V, analysis mode MS/MS, m/z 193 > 133.

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