Chemical Industry 'Nevena'[1], Leskovac, Yugoslavia, Department of Organic Chemistry[2], Göttingen, Germany, Faculty of Technology[3], Novi Sad, Faculty of Technology[4], Leskovac, Yugoslavia

The separation of hypericine and pseudohypericine from *Hypericum perforatum* L.

A. Smelcerovic[1], H. Laatsch[2], Z. Lepojevic[3] and S. Djordjevic[4]

The extraction of *Hypericum perforatum* L. was carried out by the method of ultrasonic maceration. The separation of *H. perforatum* L. extract in order to obtain hypericine and pseudohypericine rich fractions was carried out by the following chromatographic methods: flash column chromatography, high speed countercurrent chromatography, XAD solid phase extraction and Sephadex column chromatography. The separation by the Sephadex column chromatography gave the best results. Preparative HPLC was used to isolate hypericine and pseudohypericine from fraction 4 obtained by the Sephadex column chromatography procedure.

1. Introduction

From a pharmacological standpoint, the hypericines are at present the most interesting compounds of *Hypericum perforatum* L. because they are most frequently denominated as the carriers of its medicinal properties. The content of hyperforine and flavonoides is also important. Hypericine (**1**) and pseudohypericine (**2**) were found to possess antiretroviral activity [1, 2]. They inhibit viral adsorption and penetration into cells [3] with effects that are increased by irradiation with daylight [4]. Protein kinase C inhibiting effects [5] and apoptosis inducing activity [6] suggest that these compounds may be useful for the treatment of some types of cancer. Beside hypericine and pseudohypericine, *H. perforatum* L. contains two other photodynamically important substances from the naphthodiantrone group, protohypericine (**3**) and protopseudohypericine (**4**) [7]. Under influence of light and oxygen from the air protohypericine and protopseudohypericine convert to stable products, hypericine and pseudohypericine, and that's why it is not possible to determine their content in *H. perforatum* L. [8].
The aim of this work was to investigate various chromatographic methods for the separation of hypericine and pseudohypericine from *H. perforatum* L.

2. Investigations, results and discussion

The content of hypericines in the ultrasonic extract was 0.39% (hypericine 0.17% and pseudohypericine 0.22%) (Fig.).
In Tables 1 to 4 are given the results of *H. perforatum* L. methanol extract separation by: flash column chromatography, high speed countercurrent chromatography (HSCCC),

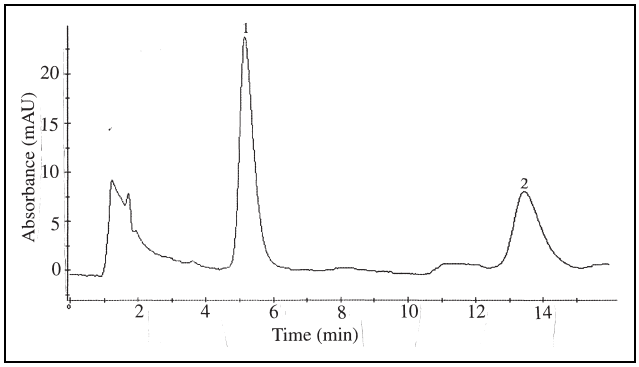
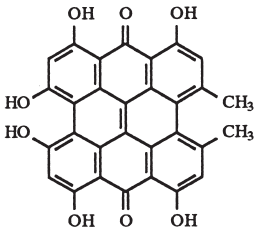
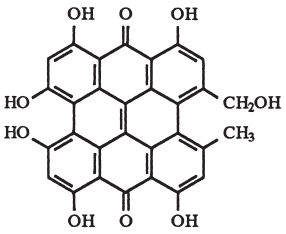


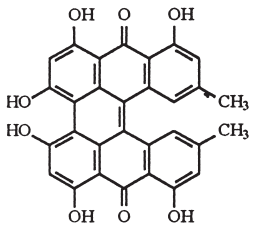
Fig.: HPLC chromatogram of *Hypericum perforatum* L. extract obtained by ultrasonic maceration (1. pseudohypericine; 2. hypericine)



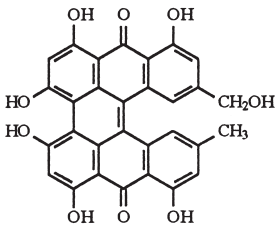
Hypericine **1**



Pseudohypericine **2**



Protohypericine **3**



Protopseudohypericine **4**

XAD solid phase extraction and Sephadex column chromatography methods. The loss of hypericine and pseudohypericine during the methanol extract separation from *H. perforatum* L. by the above chromatographic methods is given in Table 5.

For the separation of hypericine and pseudohypericine from the *H. perforatum* L. extract, as for every separation, it is important to obtain the total content of the substances mentioned in one fraction; to get the highest possible percentage of the substance content in the fraction, and to keep the substance loss at the lowest possible level.

Table 1: Separation of *Hypericum perforatum* L. methanol extract by flash-column chromatography

Fraction No.	Weight (g)	Hypericine content (%)	Pseudohypericine content (%)
1	0.1903	/	/
2	0.0522	/	/
3	0.0294	/	/
4	0.031	0.035	0.198
5	0.036	1.586	2.103
6	0.2827	0.104	0.084

Table 2: Separation of *Hypericum perforatum* L. methanol extract by high speed countercurrent chromatography method

Fraction No.	Weight (g)	Hypericine content (%)	Pseudohypericine content (%)
1	0.0015	/	/
2	0.0315	/	/
3	0.0806	0.248	0.367
4	0.0158	/	/

Table 3: Separation of *Hypericum perforatum* L. methanol extract by the XAD solid phase extraction

Fraction No.	Weight (g)	Hypericine content (%)	Pseudohypericine content (%)
1	0.1517	0.084	0.125
2	0.0712	0.086	0.131
3	0.0559	0.358	0.486

Table 4: Separation of *Hypericum perforatum* L. methanol extract by the Sephadex column chromatography

Fraction No	Weight (g)	Hypericine content (%)	Pseudohypericine content (%)
1	0.2763	/	/
2	0.3821	/	/
3	0.2725	/	/
4	0.0044	33.69	39.95

Table 5: Hypericine and pseudohypericine loss during chromatographic separation

Chromatography method	Hypericine loss (%)	Pseudohypericine loss (%)
Flash-column chromatography	56.71	58.70
HSCCC	24.27	11.32
XAD solid phase extraction	37.91	29.92
Sephadex column chromatography	18.70	23.72

When separating the extract from *H. perforatum* L. by flash-column chromatography, neither of these conditions were met. Hypericine and pseudohypericine were present in three fractions in small amounts, and the loss of the above substances was the greatest using this chromatographic method. HSCCC gave the least pseudohypericine loss during separation. All the quantity of hypericine and pseudohypericine was found in fraction 3, but their content was low, indicating that good partition of hypericine and pseudohypericine from other components from the extract was not achieved under the above conditions although a number of mobile phases were investigated. HSCCC is based on the liquid-liquid partition of solutes. Separations are carried out without a solid stationary phase, and consequently, problems such as irreversible adsorption or denaturation arising from the use of solid matrices are avoided. Therefore, it is our opinion that further efforts should be made to find the optimal conditions for the separation of hypericine and pseudohypericine from *H. perforatum* L. extract by this method. The separation by the XAD solid phase extraction method gave poor results. All the fractions obtained contained hypericine and pseudohypericine in small percentages, and the loss at partition was great. The separation by Sephadex column chromatography gave the best results. The total content of hypericine (33.7%) and pseudohypericine (40%) was in fraction 4. The loss was admissible.

Preparative HPLC was used to isolate hypericine and pseudohypericine from fraction 4 obtained by the Sephadex column chromatography procedure. The mobile phase used for this separation, beside acetonitrile and water, contained trifluoroacetic acid. This is important for the preparative separation of hypericine and pseudohypericine, because trifluoroacetic acid is very volatile and can be quickly and easily evaporated from the separated products.

MS and ^{1}H NMR spectra of the isolated compounds confirm that they are hypericine and pseudohypericine.

3. Experimental

3.1. Plant material

H. perforatum L. ssp. *angustifolium* picked on the locality of Sobina (Vranje surroundings, South Serbia, Yugoslavia) was used.

3.2. Plant material extraction

The extraction of plant material was carried out by ultrasonic maceration on a Banderlin electronics KG apparatus at plant material to methanol ratio 1 : 10 (m/v) for a period of 15 min.

3.3. Hypericine and pseudohypericine determination

The hypericine and pseudohypericine content in the extract and obtained fractions was determined by HPLC, under the following conditions:

Apparatus: Knauer (two Knauer HPLC pumps 64; Shimadzu SPD-6A UV spectrophotometric detector; Knauer HPLC software). Column: Lichrospher 60 RP-select B (Merck 50829), 5 μm. Eluent: (A) methanol : ethyl acetate : 0.1 M sodium dihydrophosphate = 317 : 90 : 57 (m/m/m), (B) 0.1 M sodium dihydrogen phosphate. Gradient : isocratic: (A) : (B) = 80 : 20. Flow rate: 0.8 ml/min. Task volume: 20 μl. Detection: 590 nm.

The hypericine and pseudohypericine content was determined according to calibration curves of standard substances. Standards of the Institute for Organic Chemistry at Göttingen (Laboratory of Prof. Dr. Hartmut Laatsch) were used.

3.4. Separation of H. perforatum L. extract

3.4.1. Flash column chromatography

Column material: Silica Gel, J. T. Baker B.V. - Deventer, Holland. Column: Glass column h = 50 cm. Filling height: 43 cm. Extract quantity: 1.173 g total methanol extract from *H. perforatum* L. Eluent: (A) 0.5 l chloroform; (B) 0.5 l chloroform : methanol = 99 : 1; (C) 0.5 l chloroform : methanol = 97 : 3; (D) 0.5 l chloroform : methanol = 95 : 5; (E) 0.5 l chloroform : me-

thanol = 90 : 10; (F) 0.5 l chloroform : methanol = 80 : 20; (G) 0.5 l chloroform : methanol = 50 : 50. Flow rate: 25 ml/min. Fraction: (1) 0–1000 ml; (2) 1001–1500 ml; (3) 1501–2000 ml; (4) 2001–2725 ml; (5) 2726–2925 ml; (6) 2926–3500 ml.

3.4.2. High speed countercurrent chromatography (HSCCC)

Apparatus: P. C. INC. High Speed Countercurrent Chromatograph (two Pharmacia LKB HPLC Pumps 2150). Extract quantity: 0.153 g total methanol extract from *H. perforatum* L. Eluent: Methanol : chloroform : water = 5 : 5 : 3 (v/v/v). Flow rate: 1 ml/min. Fraction: (1) 0–150 ml; (2) 151–295 ml; (3) 296–315 ml; (4) 316–365 ml.
The heavier phase was used as the stationary phase. After switching off the apparatus (Fractions (1) and (2)) one more mobile phase was drained from the HSCCC column and the fractions obtained were collected by the fraction collector (Fractions (3) and (4)).

3.4.3. XAD solid phase extraction

Column packing: Amberlite XAD-16, Serva. Column: Glass column h = 50 cm. Filling height: 30 cm. Extract quantity: 0.363 g total methanol extract from *H. perforatum* L. Eluent: (A) 1 l methanol : water = 25 : 75; (B) 1 l methanol : water = 60 : 40; (C) 1 l methanol : water = 100 : 0. Flow rate: 2 ml/min. Fraction: (1) (A); (2) (B); (3) (C).

3.4.4. Sephadex column chromatography

Column filling: Sephadex LH-20. Column: Glass column h = 82 cm. Filling height: 73 cm. Extract quantity: 1.057 total methanol extract from *H. perforatum* L. Eluent: Chloroform : methanol = 60 : 40. Flow rate: 0.33 ml/min. Fraction: (1) 0–270 ml; (2) 271–490 ml; (3) 491–850 ml; (4) 851–1150 ml.

3.5. Fraction preparation

The fractions were separated by the TLC test that was carried out on DC-foil Polygram SIL G/UV_{254} (Macherey Nagel & Co.) by use of mobile phase n-butanol (6) : toluene (3) : formic acid (1).

3.6. Isolation of hypericine and pseudohypericine

The isolation of hypericine and pseudohypericine was carried out by preparative HPLC under the following conditions:
Apparatus: Jasco (two Jasco PU-987 intelligent prep. pumps; Jasco MD-910 Multiwavelength detector; Browin HPLC-software). Column: S3 Lichrosorb RP18, 7 μm, 8 × 250. Eluent: Acetonitril : methanol : trifluoroacetic acid = 59.9 : 40 : 0.1 (v/v/v). Gradient: isocratic. Flow rate: 3 ml/min. Task volume: 500 μl. Detection: Multiwavelength detector.

3.7. ^{1}H NMR spectra and MS

The ^{1}H NMR spectra were recorded on a Bruker WM 300 (300.1 MHz). The ESI-MS were recorded on a Finnigan TSQ 7000 instrument with nano-ESI-API-cons source.

3.7.1. Hypericine (**2**)

^{1}H NMR ([D_6] DMSO): δ = 7.44 (s, 2-H, 5-H); 6.58 (s, 9-H, 12-H); 2.74 (s, CH_3)
(+)-ESI-MS: *m/z* (%) = 505.2 M + H^+ (24), 504.4 M^+ (100)

3.7.2. Pseudohypericine (**3**)

^{1}H NMR ([D_6] DMSO): δ = 7.69 (s, 2-H); 7,44 (s, 5-H); 6.59 (s, 9-H, 12-H); 2.68 (s, CH_3)
(+)-ESI-MS: *m/z* (%) = 521.1 M + H^+ (39), 520.3 M^+ (100)

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References

1 Meruelo, D.; Lavie, G.; Lavie, D.: Proc. Natl. Acad. Sci. USA **85**, 5230 (1988)
2 Lavie, G.; Mazur, D.; Lavie, D.; Meruelo, D.: Med. Res. Rev. **15**, 111 (1995)
3 Weber, N. D.; Murray B. K.; North J. A.; Wood, S. G.: Antiviral Chem. Chemother. **5**, 83 (1994)
4 Hudson, J. B.; Lopez-Bazzocchi, I.; Towers G. N. H.: Antiviral Res. **15**, 101 (1991)
5 Takahashi, I.; Nakanishi, S.; Kobayashi, E.; Nakano, H.; Suzuki, K.; Tamaoki, T.: Biochem. Biophys. Res. Comm. **165**, 1207 (1989)
6 Zhang, W., Lawa, R. E.; Hintona, D. R.; Su, Y. Z.; Couldwell, W. T.: Cancer Lett. **96**, 31 (1995)
7 Brockmann, H.: Fortschr. Chem. Org. Naturst. **14**, 141 (1957)
8 Freytag, W. E.: Dtsch. Apoth. Ztg. **124**, 2383 (1984)

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Andrija Smelcerovic
Chemical Industry 'Nevena'
Djordja Stamenkovica b.b.
16000 Leskovac
Yugoslavia