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Original Article

Optimization of HPLC method for the isolation of *Hypericum perforatum* L. methanol extract

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***Abstract*:**
Stamenković, J., Radojković, I., Đorđević, A., Jovanović, O., Petrović, G., Stojanović, G.: ***Optimization of HPLC method for the isolation of Hypericum perforatum L. methanol extract. Biologica Nyssana, 4 (1-2), December 2013: 81-85.***

St. John's Wort (*Hypericum perforatum* L.) is one of the most studied plant species in the family Hypericaceae. The aim of this study was the identification of the constituents of methanol extract of *H. perforatum* and optimization of conditions for their isolation. The main components of the methanol extract were isolated on preparative ZORBAX Eclipse XDB C18 column with solvent system consisting of methanol and $1x10^{-2}$ M ammonium acetate in water. Constituents of the extract were identified by comparing their retention times with the retention times of the standards, with the literature data and the UV spectra. By varying the conditions of chromatography, the optimal conditions for isolation of the methanol extract constituents were determined: mobile phase consisting of methanol and $1x10^{-2}$ M ammonium acetate in water in ratio 1 : 1, sample concentration 100 mg/mL, sample volume 30 µL, flow 2 mL/min. Under these conditions 7 components of the methanol extract were isolated.

Key words: *Hypericum perforatum* L., methanol extract, isolation, preparative HPLC
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Introduction

St. John's Wort (*Hypericum perforatum*) is a herbaceous perennial plant of the Hypericaceae family, which is distributed in Europe, Asia, and Northern Africa, and naturalized in the US. The plant grows approximately one metre high with opposite and paired branches. The leaves are opposite and sessile, up to 2 cm long, oblong and contain numerous translucent glandular dots, which are visible against light. The yellow flowers contain 5 petals with many stamens protruding. These flowers contain a group of reddish fluorescent dianthrone pigments with biological activity (Vattikuti & Ciddi, 2005).

Hypericum species are medicinal plants known as healing herbs due to their various medicinal properties for the last two hundred years. Oily *Hypericum* preparations may be applied externally to treat minor burns, wounds, inflammation of the skin, and nerve pain (Blumenthal et al., 1998). Internally, the herbal preparation is indicated for the treatment of anxiety and depression.

St John's Wort (*Hypericum perforatum* L.) has a chemical composition well studied: anthraquinone derivatives naphthodianthrones – hypericin, pseudohypericin and isohypericin, protohypericin and protopseudohypericin (biosynthetic precursors of hypericin and pseudohypericin, respectively); flavonoids – flavonols (kaempferol, quercetin), flavones (luteolin) and glycosides (hyperoside, isoquercitrin, quercitrin, and rutin), biflavonoids including biapigenin (a flavone) and amentoflavone (a biapigenin derivative) and catechins (flavonoids

often associated with condensed tannins); prenylated phloroglucinols – hyperforin and adhyperforin; tannins; procyanidins (condensed type); other phenols – caffeic, chlorogenic, *p*-coumaric, ferulic, *p*-hydroxybenzoic and vanillic acids; volatile oils, carotenoids, choline, β-sitosterol etc. (Cretu et al., 2011).

Naphthodianthrones, namely hypericin and pseudohypericin, are found in the flowering portions of the plant. Until recently, these two compounds were considered responsible for the purported antidepressant effect of *Hypericum*. Numerous flavonoid compounds, including hyperoside, quercitrin, isoquercitrin, rutin, quercetin, campferol, luteolin, and myricetin, as well as biflavonoids I3,II8-biapigenin and I3′,II8-biapigenin (amentoflavone) are found in the aboveground portions of the plant, including the leaves, stalk, flowers, and buds. Phloroglucinol compounds, including hyperforin and adhyperforin, are present in the flowers and buds (Greeson et al., 2001).

Several methods for the analysis of *H. perforatum* by TLC, photometry, HPLC, HPLC-MS and HPLC-NMR have been reported, but most of these focus on the determination of naphthodianthrones and/or hyperforin only (Gray et al., 2000; Liu et al., 2000; Mulinacci et al., 1999).

The aim of this study was to develop the new simple and efficient method for isolation and identification of the *Hypericum perforatum* L. methanol extract main constituents. For this purpose, the following methods were used:

- Maceration of dried plant material with methanol to obtain the extract;
- High-pressure chromatography (HPLC) method for the isolation, identification and determination of purity of isolated compounds.

Materials and methods

Plant material was collected in the vicinity of Leskovac in July 2011. Voucher specimen is deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš.

Naphthodianthrones and hyperforin are known to be thermolabile and especially photosensitive; thus, the extraction process was performed at room temperature and protected from the light (Wirz et al., 2001; Orth et al., 1999).

Dry aerial part of the plant was chopped and powdered. 60 g of dry material was transferred in a dark bottle and submeged in 600 mL (1:10, v/v) methanol (Sigma Aldrich, p.a.), and left in the dark for 48 hours. The methanol extract was filtered through filter paper (white bar) into the pre-measured flask. Methanol was evaporated under reduced pressure in a rotary evaporator and 12.10 g of dry extract was obtained.

Re-extraction of the previously treated material was performed with another 500 mL of methanol. After 5 days, the methanol extract was again filtered through filter paper (white bar) into the same flask. The extract was evaporated and 16.28 g of dry extract was obtained in whole after repeated extraction.

The dry extract was extracted with 100 mL of hexane (Sigma-Aldrich, p.a.) in the ultrasonic bath for 30 min. The crude hexane extract was evaporated to dryness at reduced pressure by rotoevaporation and 0.28 g of dry material was obtained.

The respective yields were calculated as percentage using the formula: (crude extract weight/plant material weight) x 100 and were: 20.16%, 27.13% and 1.72% respectively.

For HPLC analysis a weighed amount of extract was dissolved in HPLC grade methanol. All samples were filtered through a 0.45 μm filter before undertaking HPLC analysis. Each sample solution was injected in triplicate and standard deviations were below 2.2 % for all experiments, indicating that the method is precise and reproducible.

HPLC analysis was performed on HPLC chromatography with DAD detector (HPLC system 1200 series, Agilent Technologies, USA). Separation and purification of the extract components was performed on Zorbax Eclipse XDB C18 column with dimensions (5 μm, 150 mm × 4.6 mm, analytical and 5 μm, 250 mm x 9.4 mm, semipreparative). Solvent system consisting of methanol and $1x10^{-2}$ M ammonium acetate in water was used as the mobile phase in isocratic and gradient mode. The ratio of the solvents was varied in order to determine the optimum conditions for separation. All experiments were carried out at room temperature (25±2°C). Chromatograms were recorded at different wavelengths. The constituents of the extract were identified by comparing their retention times with the retention times of standards, based on literature data and comparing the UV spectra. Fractions were isolated and purified using the fraction collector.

Results and Discussion

There are numerous literature data on the HPLC analysis of different extracts *H. perforatum* L. (Brolis et al., 1998; Roempp et al., 2004). The fact common to all of the researched methods is that thay were developed primarily for analytical

purposes in order to achieve a better separation of the constituents of the extract (Li & Fitzloff, 2001). Described methods are based on the usage of the system with different solvents in gradient and isocratic mode. Therefore they are not suitable to be applied in preparative purposes primarily due to long duration of analysis.

In order to determine the optimal conditions for isolating components, preliminary investigations were carried out on the analytical column, varying the amount of injected sample, eluent polarity and flow rate of mobile phase. Work on the analytical column was carried out in isocratic and gradient mode and chromatograms were recorded at different wavelengths.

The methanol extract first was eluated on the analytical column under isocratic conditions. The mobile phase was run at flow rate of 0.5 mL/min and consisted of methanol : water (1 % HCOOH) = 80 : 20.5 µL of sample (concentration 5 mg/mL) was injected in all experiments performed on analytical column.

Since the separation in isocratic mode was not obtained in satisfactory manner, gradient elution was performed by using the following solvent gradient: 0-5 min – 50 % MeOH; 5-10 min – 70 % MeOH; 10-30 min – 90 % MeOH. The flow-rate was kept at 0.5 mL/min.

As gradient conditions did not provide good results, the possibility of using isocratic method with the changed polarity of the mobile phase was tested. A mixture of methanol : water ($1x10^{-2}$ M CH_3COONH_4) = 50 : 50 was used as mobile phase. The resulting chromatogram is shown in Fig. 1.

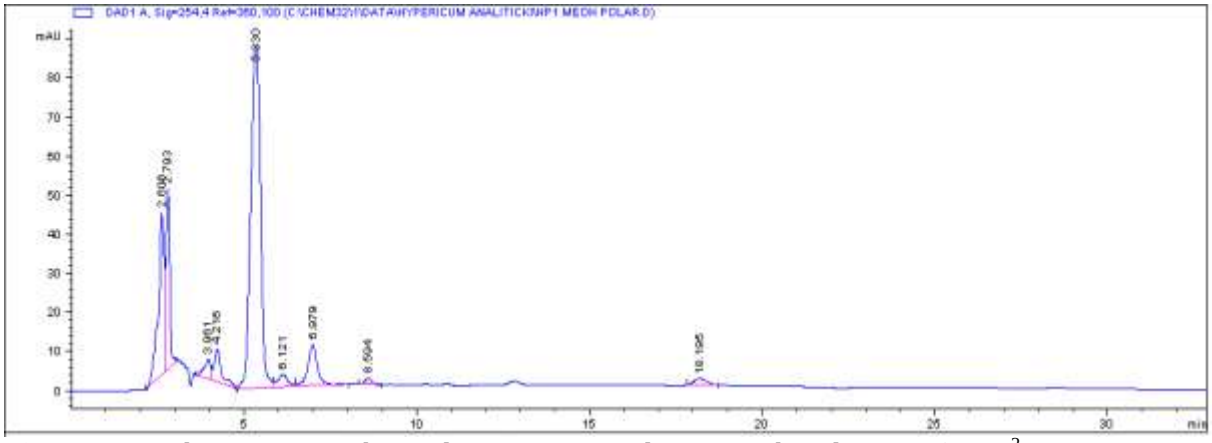

Fig. 1. HPLC chromatogram obtained in isocratic conditions, methanol : water ($1x10^{-2}$ M CH_3COONH_4) = 50 : 50.

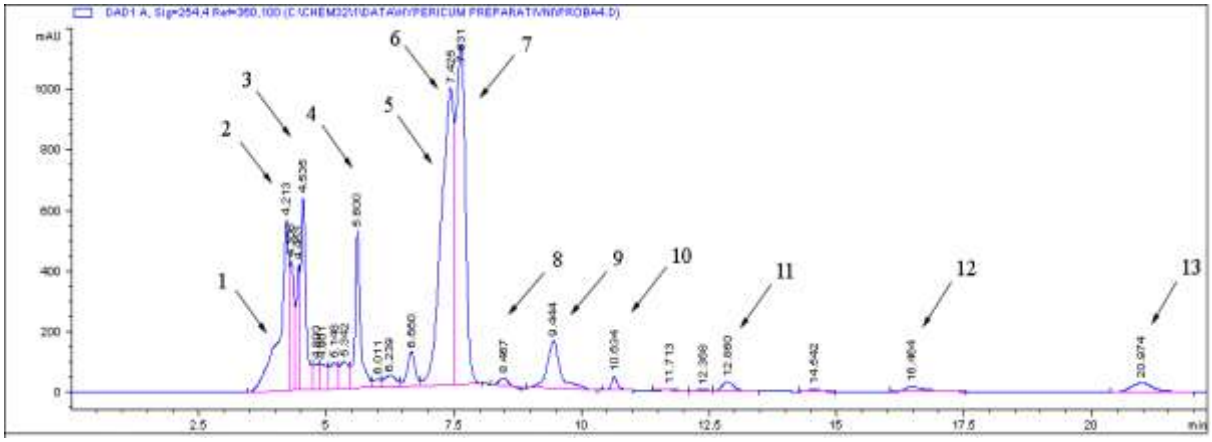

Fig. 2. HPLC chromatogram with the sample volume of 20 µl, at a flow rate of 2 mL/min.

This system was the basis for the transition from the analytical to semipreparative column, by varying the flow rate and injected sample volume, in order to obtain optimal results. All samples that were injected to semipreparative column were significantly more concentrated (100 mg/mL), aimed to increase method efficiency.

Fig. 2 shows the chromatogram of HPLC analysis that was performed on semipreparative column with the optimal composition of mobile

phase (methanol and $1x10^{-2}$ M ammonium acetate in water, ratio (1:1). This system was chosen because of good separation and short runtime. Injection volume of the sample was 20 µl, with a flow rate of 2 mL/min. The peaks in the HPLC chromatogram were identified based on literature data (Hansen et al.,1999), by comparing the retention time and UV spectra (Hillwing et al., 2008) with reference standards.

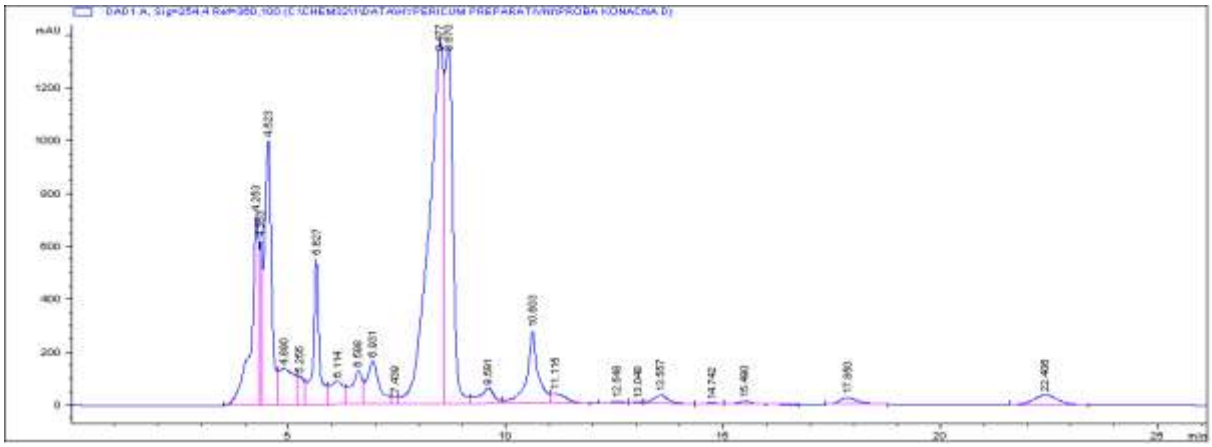

Fig. 3. HPLC chromatogram with the sample volume of 30 µl, at a flow rate of 2 mL/min.

Table 1. Retention time of identified and isolated compounds, mass of isolated compounds and their purity.

No.	Compound	Rt (min)	Isolated mass (mg)	Purity %
1.	Chlorogenic acid	4.033	/	/
2.	Gallic acid	4.213	/	/
3.	Trans- *p*-coumaric acid	4.535	/	/
4.	Unidentified compound 1	5.600	1.4	87.65
5.	Rutin	7.425	/	/
6.	Hyperoside	7.425	/	/
7.	Isoquercitrin	7.631	16.8	79.84
8.	Quercitrin	8.467	/	/
9.	Quercetin	9.444	5.5	92.71
10.	Pseudohypericin	10.634	0.8	96.66
11.	Hypericin	12.860	1.0	97.12
12.	Unidentified compound 2	16.464	0.9	97.43
13.	Hyperforin	20.974	1.4	98.26

Table 1 shows the retention times of identified and isolated components, mass of isolated components and their purity, based on the integral of the peak areas. The purity was determined by the integration of HPLC spectra of the isolated compounds and it was directly proportional to the achieved resolution.

In order to increase method efficiency, 40 µl of sample was injected, but this led to the reduced resolution. As a compromise solution, 30 µl of sample volume was injected at the same flow rate of 2 mL/min (Fig. 3). Based on obtained chromatograms, it was concluded that the best results for the separation and isolation of constituents of *H. perforatum* L. methanol extract on the semipreparative column, were obteined by injecting the volume of 30 µl of the sample (concentration 100 mg/mL) at a flow rate of 2 mL/min.

Conclusion

The results showed that the optimal conditions for isolation of the constituents of *Hypericum perforatum* L. methanol extract on the semipreparative column were: mobile phase methanol : water ($1x10^{-2}$ M CH_3COONH_4) = 50 : 50, sample concentration 100 mg/mL, injection sample volume 30 µl and the flow rate of 2 mL/min. Seven compounds were isolated from the extract with purity : unidentified compound (peak 4) 87.65 %, isoquercitrin (peak 7) 79.84 %, quercetin (peak 9) 92.71 %, pseudohypericin (peak 10) 96.66 %, hypericin (peak 11), 97.12 %, unidentified compound (peak 12) 97.43 %, hyperforin (peak 13) 98.26 %. According to obtained results a new simple isocratic HPLC method was developed, allowing the baseline separation of thirteen main *Hypericum perforatum* standard compounds in less than 25 min, which means an improved separation 25-50 min less than other published systems.

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