УДК 543.544

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CHROMATOGRAPHIC ANALYSIS AND SEPARATION OF HYPERICIN CONTAINING EXTRACT OF ST. JOHN'S WORT

It has been presented qualitative and quantitative chromatographic analysis of hypericin containing extracts of St. John's Wort which could be used for producing drugs for treating of oncological diseases by the method of photodynamic therapy. The optimal conditions of carrying out of TLC and HPLC analysis of ethanol St. John's Wort extracts have been selected. The chromatographic separation of St. John's Wort extract has been carried out assisted by Diaion HP-20 sorbent. This separation gave the opportunity to enrich the initial extract with hypericins more than in 4 times.

Introduction. At present, the important issue in the Republic of Belarus is separation, identification and determination of biologically active compounds from plants of various agro-climatic zones of Belarus. Based on obtained substances it became possible to create medicines, dietary supplements, herbal remedies. According to the influence on the organism the drugs of vegetable origin have several advantages over synthetic analogs, as they exhibit a mildly expressed therapeutic effect.

Photodynamic therapy is a relatively new and promising method of cancer treatment based on the fact that tumor cells are destroyed by the action of reactive oxygen species, which are formed by a chemical reaction that is activated by light energy. For this reaction the photosensitive substance (photo sensitizer) and also a source of light with a wave length corresponding to the maximum absorption of the substance must be present in the target tissue [1].

As photo sensitizer can act only compounds having in their structure chromophore group of atoms that can absorb light in the visible or near-ultraviolet region of the spectrum. These compounds include hypericin. This substance is one of the main bioactive components of St. John's Wort and represents a condensed derivative of anthraquinone. In addition to hypericin in St. John's Wort has been discovered the second potential photo sensitizer – pseudohypericin.

Main part. The aim of this work is a qualitative and quantitative analysis of hypericins in St. John's Wort using chromatographic methods of analysis.

Hypericins from St. John's Wort were isolated by extraction method using Soxhlet and ethanol as a solvent. The qualitative composition of the resulting extract was determined by TLC. For selection of the optimal conditions for the TLC analysis the ethanol extract was separated in elution solvent systems: I) propanol-2 – hexane (7 : 3); II) 2-propanol – water (9 : 1); III) acetate – n-butanol – formic acid – water (5 : 3 : 1 : 1); IV) chloroform – ethyl alcohol (8 : 2); V) ethyl acetate – formic acid (50 : 6).

The substances were detected by viewing the chromatogram under UV lamp with optical filters with a wave length of 254 and 365 nm. Plates were also developed in iodine vapor. In the course of analysis of the chromatograms obtained in the five proposed eluting systems, it was found that the clearest separation of the components of ethanol extract was observed when using the eluting system V. Typical thin-layer chromatogram of the test extract obtained by using eluting system V, is presented in Fig. 1.

Hypericin (Fig. 1, position 7) in the chromatogram was identified by bright red fluorescence and the value R_f = 0.87, which coincides with the value of R_f standard hypericin sample (Carl Roth GmbH, Germany). When using this eluent system pseudohypericin (Fig. 1, position 6) was identified in the chromatogram by bright red fluorescence (in UV light with a wavelength of 365 nm) and by index R_f = 0.83, the value of which corresponds with the R_f, given in the references [2].

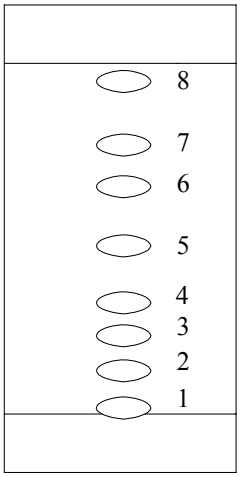

Fig. 1. Typical thin-layer chromatogram of the St. John's Wort extract in eluting system V

Thus, the analysis of hypericin containing extracts by TLC method revealed that the tested extracts consist of eight major components. R_f indicators for all components of ethanol extract are shown in Table 1. The results are presented as arithmetic average of three concurrent experiments with taking into account standard deviation.

Table 1

R_f values for components of hypericin containing extract

Substance	R_f
8	0.97 ± 0.01
7 (hypericin)	0.87 ± 0.02
6 (pseudohypericin)	0.83 ± 0.02
5	0.65 ± 0.02
4	0.42 ± 0.02
3	0.34 ± 0.02
2	0.12 ± 0.02
1	0.020 ± 0.006

The quantitative content of hypericin in the extract was determined by HPLC method when using GC-MS "Waters Micro-mass ZQ 2000", and the column BDS HYPERSIL C18 250×4.6 mm. Detection was performed by diode matrix detector with the wave length of 590 nm and mass detector with electrosprayionization.

Elution was performed in a linear gradient using a system consisting of acetonitrile (solution A) and 0,01 M water ammonium acetate solution (solution B) (A : B: 0 min – 15 : 85, 0–5 min – 30 : 70; 5–10 min – 45 : 55, 10–15 min – 60 : 40, 15–20 min – 75 : 25, 20–40 min – 90 : 10) with a flow rate of 1 ml/min.

Hypericin in the extracts was identified in the chromatogram (Fig. 2, *a*) according to the retention time 35.95 min, which coincided with the retention time of the standard hypericin sample.

In the mass spectra of this compound signals were observed corresponding to the molecular hypericin ions with m / z = 503,66 for negative ions and with m / z = 505.64 for positive ions (Fig. 2, *b*).

Pseudohypericin in the extracts was identified by the mass spectrum of negative ions, where there was a signal with m / z = 519.63, corresponding to the ion $[M–H]^-$ for connection with the molecular formula $C_{30}H_{16}O_9$. In the field of positive ions for pseudohypericin was also observed a molecular ion $[M + H]^+$ with m / z = 521.61. It was found that, under given conditions of the HPLC analysis pseudohypericin had a shorter retention time than hypericin – 28.7 min.

Electronic pseudohypericin spectrum was similar to hypericin spectrum, where there was a band with an absorption maximum of 590 nm, which is typical of condensed derivatives of antrohinon.

Quantitative determination of hypericin in St. John's Wort was performed by the calibration curve constructed according to solutions of the standard hypericin sample of (equation of line $y = 328\,775x – 278\,5034$, $R^2 = 0.962068$).

To get hypericin enriched fraction was conducted preparative separation of St John's Wort extract by chromatographic method. The separation of the extract was carried out on the sorbent Diaion HP-20, elution was performed with application of aqueous ethanol in increasing concentrations (0, 20, 40, 60, 80 and 100%) and a mixture of ethanol and chloroform (1 : 1).

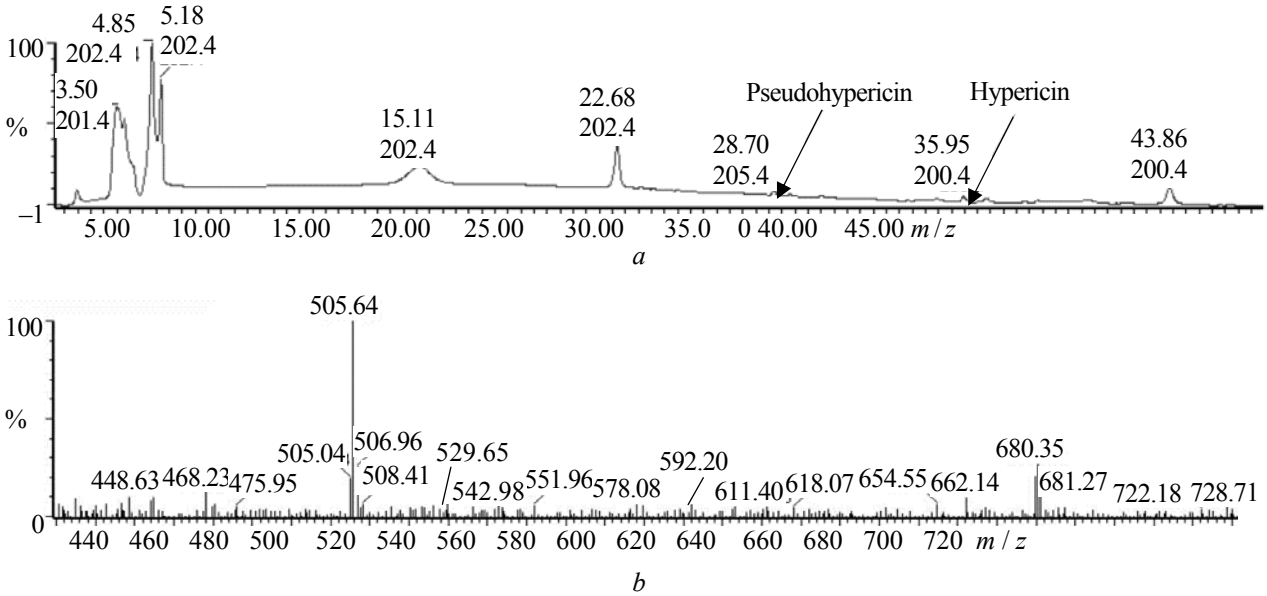

Fig. 2. HPLC chromatogram of St John's Wort extract (*a*), the mass spectrum of hypericin in the field of positive ions (*b*)

Fractions obtained after separation were analyzed on a spectrophotometer resulting in the elution profile as shown in Fig. 3.

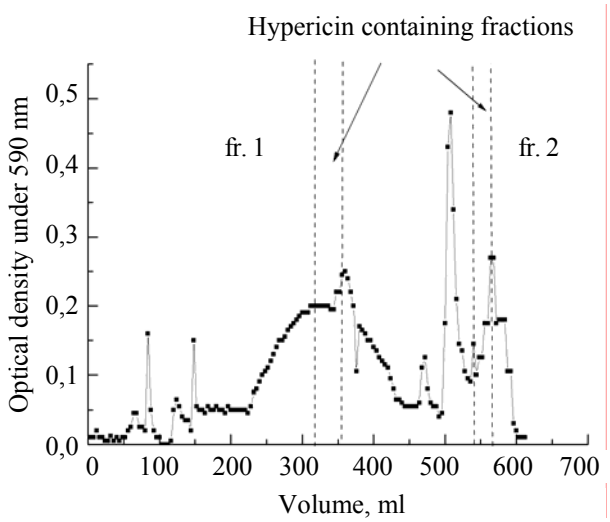

Fig. 3. Elution profile on the sorbent Diaion HP-20

As a result of the TLC analysis of fractions corresponding to peaks in the profile, it was found out that hypericin containing fraction eluted from the column with 80% aqueous ethanol in volume of 304–356 ml (fr. 1), and assisted with a mixture of ethanol and chloroform eluted in volume of 540–564 ml (fr. 2). The results of the HPLC analysis of obtained fractions are shown in Table 2.

As shown in table 2, the separation of St John's Wort on the sorbent Diaion HP-20 provides an opportunity to enrich the original extract by hypericins more than 4 times. This confirms the feasibility of separation of hypericin containing extract on this sorbent.

Table 2

Results of separation of the hypericin containing extract on the sorbent Diaion HP-20

Fraction	Hypericin content in original extract, %	Fraction output towards the inserted extract, %	Hypericin content in the fraction, %
1	0.081	9.98	0.34
2		14.20	0.13

However, the purity of the fractions obtained by chromatographic separation of the ethanol hypericin extract is not fully satisfactory. Therefore, for further use of hypericins as photosensitizer in photodynamic therapy, more research is needed regarding the cleaning and enriching of hypericin containing extracts.

Conclusion. Optimal conditions for HPLC and TLC analyses have been selected. It has been conducted chromatographic separation of St John's Wort on the sorbent Diaion HP-20, which made it possible to enrich the original extract by hypericins in more than 4 times.

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Recieved 19.03.2012