

## FULL PAPERS

DETERMINATION OF TOTAL HYPERICINS IN ST. JOHN'S WORT  
AND HERBAL MEDICINAL PRODUCTSMAŁGORZATA ANYŻEWSKA<sup>1\*</sup>, ANNA KOWALCZUK<sup>1</sup>, ANNA ŁOZAK<sup>1</sup>, RENATA  
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**Abstract:** The work aimed to determine the levels of hypericins expressed as hypericin in the herbal substance of St. John's wort, in capsules and tablets containing the extract of St. John's wort, tablets containing powdered herb and in tincture and juice from fresh St. John's wort, by HPLC method with spectrophotometric detection. In addition, the amount of hypericins in the infusion prepared from St. John's wort was determined by HPLC and spectrophotometry methods. According to traditional indications aqueous infusions from St. John's wort containing mainly hydrophilic components are used in gastrointestinal diseases. On the other hand, ethanolic extracts containing hypericin and hyperforin affect the CNS and are indicated for the treatment of episodes of mild depressive disorders. The results obtained in the work indicate that the daily dose of hypericins taken by a patient as infusions is 0.328 mg on average for herbs in sachets and in bulk form. It can be compared to the daily dose of hypericins contained in tablets and capsules based on the alcoholic extract of St. John's wort and tablets containing powdered St. John's wort herb. For solid dosage forms, this dose ranges from 0.288 mg to 0.636 mg. The assays were performed using consistent analytical methods for all tested pharmaceutical products and consequently it was possible to compare doses taken by patients and their strength of action.

**Keywords:** *Hypericum perforatum*, St. John's wort, hypericin, determination, HPLC, herbal medicinal products, safety of administration.

Natural medicinal products are commonly used in medicine and their popularity and availability may significantly affect the health of patients. Traditionally, herbs for infusion or dosage forms containing extracts, oils, juices and recently solid dosage forms, tablets and capsules with powdered or micronised herbs are used. Similarly to synthetic medicinal products, they are subject to extensive assessments on their quality, safety and therapeutic efficacy. However, unlike synthetic medicinal products, herbal medicinal products are a complex mixture of active substances and excipients. In a large number of cases it is not possible to isolate and determine a chemical structure of an ingredient responsible for therapeutic effects (1, 2). The St. John's wort is an example of a herbal medicinal product where an unambiguous indication of the active substance is hardly possible. Whole or cut, dried flowering tops of *Hypericum perforatum* L., harvested during flowering time are the pharma-

copoeial herbal substance. The herb contains the following flavonoids (2–4%): mainly hyperoside (0.3–0.7%), rutoside, quercetin, isoquercetin; naphthodianthrones (0.05–0.3%); hypericin and derivatives: pseudohypericin and protohypericin, isohypericin that are considered to be hypericin precursors; biflavonoids: amentoflavone and biapigenine; fluoroglucinol derivatives: hyperforin (2–4.5%) and adhyperforin (0.2–1.8%); caffeic and chlorogenic acid; catechine tanning agents; xantons, as well as essential oil containing monoterpenes, aliphatic hydrocarbons and aldehydes and sesquiterpenes (3, 4). St. John's wort has been used for centuries to treat wounds and ulcers, metabolism disturbances, and especially as an agent with anti-inflammatory and diuretic properties. Currently, especially important are its supporting of emotional balance properties. Numerous publications indicate that therapeutic effects vary significantly depending on the preparation method of an intermediate product or a pharma-

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ceutical form. The aqueous extracts rich in hydrophilic agents: flavonoid glycosides, tanning agents and phenol acids have astringent and relaxation properties, and are used in contractions of the bile ducts, gastrointestinal mucous membrane inflammation and as an agent stimulating the production of digestive fluids. Infusions used externally are applied as compresses on poorly healing wounds (5, 6). The extract produced from the herbal drug by a suitable procedure using ethanol (50–80% v/v) or methanol (50–80% v/v) containing mainly hypericin, hyperforin, xanones, flavonoids and procyanamides have effects on the central nervous system (CNS) and are used to treat mild depression-like mood disorders (7–9). The results of the latest studies indicate that the antidepressive effects of hyperforin are more powerful than the ones of hypericin, as it is a more potent inhibitor of the uptake of serotonin, noradrenaline, dopamine, GABA and L-glutamate (10, 11). Tablets and capsules containing a standardized ethanolic extract of St. John's wort at the amount ranging from 60 mg to 425 mg of extract are usually used to treat the episodes of mild depressive disorders. According to manufacturers, these products provide the daily dose ranging from 0.3 mg to 3.0 mg of total hypericins expressed as hypericin. In addition, tablets and capsules containing powdered or micronised St. John's wort are also available on the market and they are used to treat mild depressive disorders. Due to complex composition and incomplete knowledge on the mechanism of its pharmacological effects, St. John's wort raises much interest as well as controversy. Furthermore, there is no unanimous consensus on the choice of chemical compounds that will be used to standardize the plant substance and its preparations. Differences in analytical methods used to determine active substances and in reference materials are the reason why results are presented in a diversified manner, which makes it impossible to compare strengths of products. Different manufacturers use HPLC method with spectrophotometric detection or spectrophotometry method to test the quality of products. The result of hypericin determination in both methods is expressed as the hypericin content; however, in HPLC method it is the sum of hypericin and pseudo-hypericin, whereas in spectrophotometry – it is the sum of all derivatives of hypericin. The differences also regard official methods presented in pharmacopoeia monographs. The European Pharmacopoeia (Ph. Eur.) monograph of St. John's wort (*Hyperici herba*) anticipates hypericin determination expressed as hypericin (minimum 0.08% of total hypericins, expressed as hypericin) using spec-

trophotometry. In 2001, in *Pharmeuropa* Vol. 13, No. 1, there was a recommendation in the monograph of St. John's wort dry extract (*Hyperici herba extractum siccum*), to use the HPLC method for determination of the sum of hypericin and pseudo-hypericin (0.2–0.3%) and hyperforin assay. A modification of this monograph was published in 2004 (*Pharmeuropa* Vol. 16, No. 1). It included the requirement for the sum of hypericins expressed as hypericin (0.15% to 0.30%), the HPLC method was modified and there was a recommendation to leave the tested solution for 2 hours on light exposure. The Ph. Eur. 6.2 edition was supplemented with the monograph of St. John's wort dry extract, quantified. It anticipates to determine with HPLC total hypericins expressed as hypericin (0.10–0.30%), flavonoids expressed as rutin (minimum 6.0%), hyperforin (maximum 6.0%). According to the monograph, the tested solution and the reference solution indicated for hypericin determination should be subject to exposure to a xenon lamp of 765 W/m<sup>2</sup> for 8 min., whereas the solutions should be protected from light during determination of hyperforin and flavonoids. The introduction of the monograph of St. John's wort dry extract, quantified into the Ph. Eur. 6.2 in effect since July 2008 made it possible to standardize the requirements and analytical methods for products containing the extract of St. John's wort herb. However, there is still a problem of inconsistency between analytical methods for the extract and raw material of St. John's wort. Despite using HPLC to determine hypericins in the extract, the Ph. Eur. was not modified in terms of determination of hypericins in St. John's wort. In this work, the possibility of using pharmacopoeial HPLC method for different forms of St. John's wort drugs was checked. The method was validated and the tests to determine hypericins in the plant substance of St. John's wort and in different products containing its preparations were performed. The hypericin content was determined using HPLC according to the Ph. Eur. in *Hyperici herba*, capsules and tablets containing the extract or powdered herb of St. John's wort, in tincture, juice and in infusion from St. John's wort herb. The method to prepare samples of different dosage forms was elaborated.

## EXPERIMENTAL

### Materials

#### Pharmaceutical preparations

Dziurawiec Fix (*Hyperici herba*) herbal tea in sachets (from Herbapol Lublin S.A., Poland). Ziele dziurawca (*Hyperici herba*) herbal tea in bulk form

(from Herbapol Lublin S.A. Poland). Tinctura Hyperici 4.5 g/5 mL (from KZZ Herbapol Kraków S.A., Poland). Succus Hyperici, 2.425 g/2.5 mL (from Phytopharm Kłęka S.A., Poland). Deprim (tablets 60 mg of Hyperici herbae extractum siccum corresponding to 0.05–0.25 mg of hypericin and Deprim forte (capsules 425 mg of Hyperici herbae extractum siccum corresponding to 0.75–1.3 mg of hypericin (from Lek Pharmaceuticals d.d., Slovenia). Hyperherba (tablets 330 mg powdered Hyperici herba corresponding to min. 0.2 mg hypericin (from Labofarm, Poland).

#### Standards and reference materials

St. John's wort standardized dry extract, Ph. Eur. Standardized Reference Standard (CRS), Assigned value 0.050% of hypericin; Catalogue code Y0001050. Hypericum perforatum (St. John's Wort) Biomass Reference Material reagent grade (BRM-RG), Chromadex INC., CDXA Number CDXA-06-0279. St. John's Wort powdered extract, Finzelberg, ITEM No. 0155325; Assay: Total hypericins calc. with reference to the dried extract (analogous Deutscher Arzneimittel Codex 79/86 mod.): min. 0.32%; Pseudohypericin and hypericin calc. as hypericin (HPLC acc. to Finzelberg method) batch specific determination 0.160%. Hypericin Primary Reference Standard, (98.3%), Chromadex Inc.; Hypericin Reference Standard (99.18%) and Pseudohypericin Reference Standard (95.5%), Roth.

#### Chemicals and reagents

Sodium dihydrogen phosphate cz.d.a. (POCH S.A., Poland), phosphoric acid 85% (Merck, Germany), methanol, ethyl acetate, tetrahydrofuran – HPLC grade (Lab Scan, USA), deionized water.

#### Apparatus and analytical conditions

UV-160A spectrophotometer (Shimadzu); HPLC Ultima 300 System from Dionex, with UV-VIS detector, 2 pumps, autosampler and Chromeleon Datasystem; Moisture Analyzer from Sartorius; halogen lamp LF, 22 V, 1000 W.

#### Chromatographic conditions

Separation was achieved using a Hypersil Gold RP18 column (150 × 4.6 mm, 5 µm). The isocratic phase at a flow 1 mL/min. consisted of 39 volumes of ethyl acetate, 41 volumes of a 15.6 g/L solution of sodium dihydrogen phosphate adjusted to pH 2 with phosphoric acid and 160 volumes of methanol. The injection volume was 20 µL and the wavelength of detection was 590 nm. The separation was performed at 40°C.

#### Standard and reference solutions

Fifty mg of the working standard was dissolved in methanol using an ultrasound bath for 30 min. and filled up with the same solvent to 5 mL. The solution was filtered using Chromafil 0-45/25 filters. The filtrate was exposed to a halogen lamp for 10 min.

#### Determination of hypericin with spectrophotometric method

Samples of St. John's wort for determination of hypericin content using spectrophotometry were prepared according to the Ph. Eur. monograph of St. John's wort (*Hyperici herba*).

#### Determination of hypericin with HPLC method with spectrophotometric detection

Preparation of samples of St. John's wort, tablets and capsules

Samples of St. John's wort doses and in bulk form were ground in a grinder. Samples weighing about 300 mg were placed in measuring flasks of 20 mL. Capsules: the average mass of contents from 10 capsules was determined, and the contents were mixed. Samples weighing about 100 mg were placed in measuring flasks of 25 mL. Tablets: the average mass of 10 tablets was determined, tablets were powdered. Samples weighing about 300 mg of the tablet mass were placed in measuring flasks of 10 mL for tablets with extract and of 20 mL for tablets with powdered herb. Methanol was added to the samples and then the procedure was the same as for the standard and reference solutions. Sample of the tincture and juice was directly exposed to a halogen lamp for 10 min.

#### Preparation of infusions from St. John's wort

Infusions were prepared according to a recipe on the packaging. 2.0 g (1 spoonful) of bulk form of herb or 1 sachet of tea was poured with 200.0 mL of boiling water and infused for 15 min in a covered dish. The infusion was filtered using a filter paper. The solution obtained was exposed to a halogen lamp for 10 min and then the procedure was the same as for the standard solution. The residues after filtering were dried for 24 h in an oven at 50°C. Then, they were ground in a grinder and the following procedure was the same as for herbs.

#### Determination of time period of sample exposure to light

The optimum time period of light exposure of standard solutions and tested solutions was determined. The relationship between the amount of

hypericins expressed as the sum of areas of hypericin and pseudohypericin peaks and the time period of solution exposure was determined. The hypericin content was determined using HPLC method for solutions immediately and after 5, 10 and 15 min of exposure to a halogen lamp.

#### Standardization of working standards

Standardization of working standards of St. John's Wort powdered extract by Finzelberg and *Hypericum perforatum* (St. John's wort) BRM-RG was performed against St. John's wort standardized dry extract, Ph. Eur. Reference Standard. A hundred mg of the CRS standard and standardized working standards: extract and powdered herb of St. John's wort were weighed into measuring flasks of 10.0 mL. Then, the procedure was the same for the standard and reference solutions. *Hypericum perforatum* BRM – RG was used as a working standard during the tests in this work.

Validation of the HPLC method to determine hypericins was performed and it regarded the specificity, linearity, precision, repeatability and accuracy. Specificity of the method for *Hyperici herba* raw material was confirmed using St. John's wort standardized dry extract Ph. Eur. Reference Standard, reference material for hypericin and pseudohypericin. The relationship between the peak size and the amount of hypericins expressed as the sum of hypericin and pseudohypericin was tested using seven weighed samples of herb doses. The linearity of a method was tested for the concentrations ranging from 0.0001 to 0.01 mg of hypericin/mL. The equation of a straight line was  $y = 6133.9x - 0.0107$ , the regression coefficient  $r = 0.9999$ . The test of repeatability was performed for the lower range of linearity and relative standard deviation of results for 6 repetitions was RSD 2.6%. Precision of the method (2.5 %) and accuracy (100.4%) of the method were confirmed.

#### DISCUSSION

The work aimed to determine the levels of hypericins expressed as hypericin in the plant substance of St. John's wort, in capsules (Deprim Forte) and tablets (Deprim) containing the extract of St. John's wort, tablets (Hyperherba) containing powdered herb of St. John's wort and in liquid dosage forms such as the tincture and juice and in infusion from St. John's wort herb. The HPLC method presented in the Ph. Eur. monograph of St. John's wort dry extract, quantified and the spectrophotometric method described in the Ph. Eur. monograph of St.

John's wort were used. The first stage of work regarded the determination of the optimum time period of light exposure of solutions prepared for assays using the HPLC method. Chromatograms of samples examined immediately after solutions had been prepared contain peaks of hypericin derivatives that are transformed into hypericin and pseudohypericin with time (Fig. 1). The exposure process significantly accelerates the balance between these compounds (12–14). In the tests, a photo halogen lamp of 1000 W was used for sample exposure instead of a xenon lamp of about 765 W/m<sup>2</sup> recommended by the Ph.Eur. In the case of standard solutions, the plateau of the hypericin content expressed as the sum of areas of hypericin and pseudohypericin peaks was achieved after 5 min, and in the case of tested samples – after 10 min of light exposure (Fig. 2). Standardization of working standards of *Hypericum perforatum* BRM-RG, Chromadex Inc. and St. John's Wort powdered extract by Finzelberg using HPLC with solution exposure according to the Ph. Eur. was performed against St. John's wort standardized dry extract, Ph. Eur. CRS (assigned value 0.050% of hypericin). The level of hypericin assayed in biological material was 0.038%, and in the extract by Finzelberg was 0.042%, and it differs significantly from the values presented in a certificate. The Certificate of St. John's Wort powdered

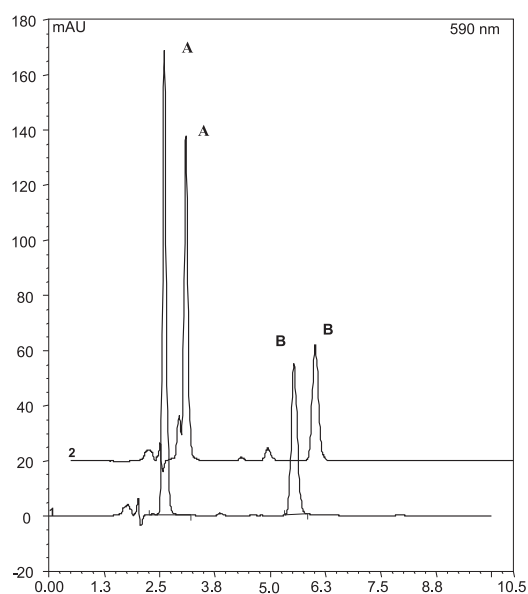


Figure 1. Chromatograms of the working standard of *Hypericum perforatum* Biomass Reference Material: after exposing the solution to a halogen lamp for 10 min (1), immediately after solution preparation (2); A = pseudohypericin, B = hypericin

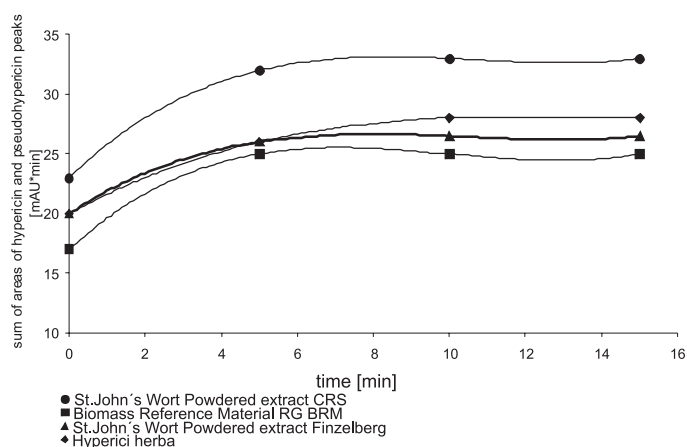


Figure 2. Chart of a relationship between the sum of areas of hypericin and pseudohypericin peaks and time of exposure to a halogen lamp

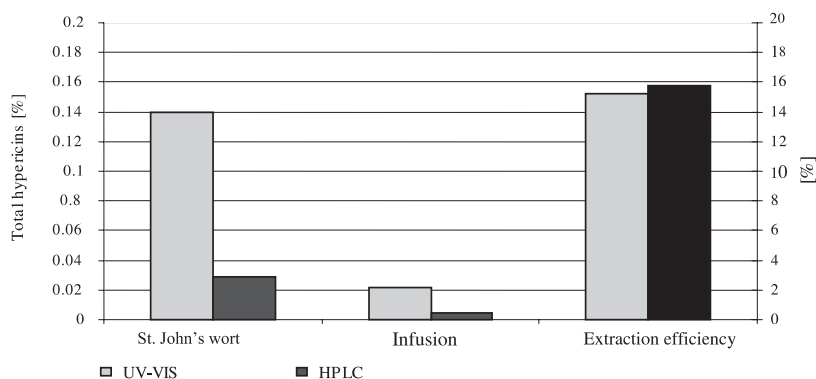


Figure 3. Total hypericins expressed as hypericin (%) in St. John's wort and in infusion from plant substance determined by spectrophotometric and HPLC methods. Extraction efficiency of hypericin from St. John's wort into infusion (%)

extract, Finzelberg contains two values of the hypericin content depending on the determination method used. For spectrophotometry according to DAC 79/86, 0.32% and for HPLC developed by Finzelberg 0.160%. Different results may result from the application of different analytical procedures used to determine the hypericin content in the extract.

The hypericins content in medicinal products containing herbal preparations from St. John's wort was determined. The hypericins content in herb in bulk form was 0.0288%, and in herb doses 0.0231%.

In order to determine the degree of hypericins elution, the hypericins content in infusions prepared according to manufacturer's recipe was determined. The hypericins content in the infusion prepared from bulk form of herb was 0.0044%, and that from herb doses 0.0038%. The degree of hypericins elution into the infusion was 15.2% and 16.5%, respectively. In order to confirm the accuracy of the method, the hypericins content in the residues was determined. Consistency between the hypericins content in St. John's wort and total hypericins in the infusion and the residues was 97% for bulk form of herb and

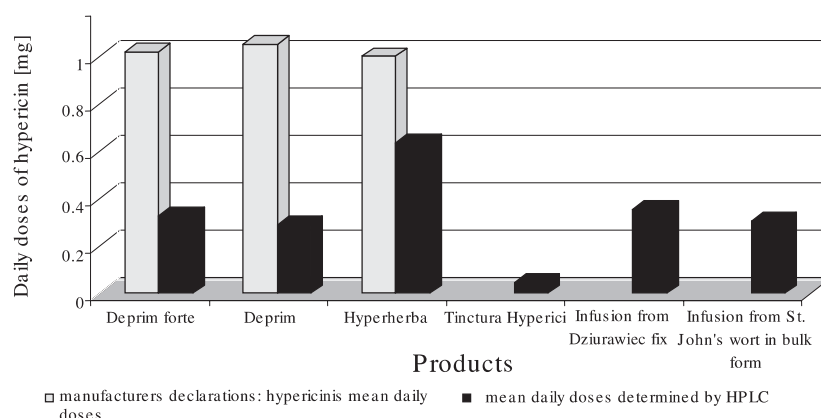


Figure 4. Daily doses of hypericin (mg) in the following products: Deprim Forte, Deprim, Hyperherba, Tinctura Hyperici and in infusions obtained from St. John's wort fix (Dziurawiec fix) and St. John's wort in bulk form

Table 1. Daily doses of hypericins according to manufacturers' declarations and results of assays to determine the content of total hypericins by HPLC method taking into account the recommended dose.

Product (mean daily doses)	Manufacturers' declarations		HPLC method		
	Total hypericins	Mean daily doses (mg)	Total hypericins	RSD (%)	Mean daily doses (mg)
<b>Deprim forte capsules</b> (1 capsule)	0.75 – 1.3mg/capsule	1.02	0.327 ± 0.0010 mg/capsule	0.30	0.327
<b>Deprim tablets</b> (6 tablets)	0.15 mg/tablet	1.05	0.048 ± 0.0006 mg/tablet	1.25	0.288
<b>Hyperherba tablets</b> (6 tablets)	NLT 0.20 mg /tablet	1	0.106 ± 0.0011 mg/tablet	1.06	0.636
<b>Tinctura Hyperici</b> (5 mL)	NLT 0.003%	0.17	0.942 ± 0.0062 mg/100 mL	0.64	0.047
<b>Hyperici herba</b> (infusion from 8 g)	NLT 0.008%*	0.96**	0.029%	2.84	2.32
			0.0044 %***		0.352***
<b>Hyperici fix</b> (infusion from 8 g)	NLT 0.008%*	1.06**	0.023 %	3.26	1.84 mg
			0.0038 % ***		0.304 ***
<b>Succus hyperici</b> (7.5 mL)	No information	No information	0.057 ± 0.0010 mg/100 mL	1.75	0.004

\* European Pharmacopeia's declaration. \*\* total hypericins in infusion determined by spectrophotometric method, taking into account the degree of hypericins extraction; \*\*\* total hypericins in infusion determined by HPLC method, taking into account the degree of hypericins extraction

99% for herb doses. At the same time, the hypericins content in raw material and infusion was determined using spectrophotometry method according to the monograph of St. John's wort. Spectrophotometry tests confirmed the results of the HPLC analysis regarding the degree of hypericins elution into the infusion, which was 15.8% for herb in bulk form and 16.4% for herb in sachets (Fig. 3).

Determination of the hypericins content was performed using the HPLC method for the following medicinal products: Deprim forte capsules and Deprim tablets containing dry extract, Hyperherba tablets containing powdered plant material, tincture from St. John's wort and juice from fresh herb of St. John's wort (Tab. 1). Differences between the results obtained and values declared by manufactur-

ers are a consequence of differences in analytical methods that had been used and of the fact that there is still no unanimously determined standardized reference material. As a result of the use of standardized extract of St. John's wort CSP and the introduction of the monograph "dry extract quantified of St. John's wort herb 07/2008:1874" into the European Pharmacopoeia, the analytical methods used to determine hypericins in St. John's wort herb and its preparations should be unified. Aqueous infusions are commonly thought to be rich in hydrophilic components, they do not contain hypericin and are traditionally used in gastrointestinal disorders and as anti-inflammatory agents. On the other hand, ethanolic extracts containing hypericin and hyperforin exert effect on the CNS. As a result of our studies, it was possible to determine the degree of elution of hypericin derivatives into aqueous solutions such as infusions from St. John's wort herb. The list of the results obtained (Tab. 1) indicates that the daily dose of hypericins taken by a patient as infusions from St. John's wort herb in bulk form and herb doses is 0.304 mg and 0.352 mg, respectively, and it can be compared to the daily dose of hypericins in tablets and capsules indicated for mild depression-like disorders that is 0.327 mg for Deprim forte, 0.288 mg for Deprim and 0.636 mg for Hyperherba (Fig. 4). It is also important that hypericin derivatives contained in the infusion are present as soluble forms, moreover, their bioavailability is higher than in the case of hypericin contained and released from solid dosage forms (tablets and capsules). With relation to the results confirming the presence of hypericins in infusions that have been obtained, it is still unknown which active substances are responsible for the effect on the CNS and it is necessary to verify the therapeutic indications. As a result of standardization of analytical methods used to test different medicinal products containing St. John's wort herb and its preparations, it will be possible to present strengths of these products and to verify their dosage in a uniform way.

## CONCLUSIONS

As a result of lack of a consistent approach to the presentation of the strengths of herbal medicinal products confirmed by consistent analytical methods, there is a risk that some indications are omitted in patient information leaflet. As a result of the introduction of a consistent compulsory method to determine hypericins in raw material, preparations and products containing St. John's wort herb using certified reference standards, it would be possible to determine the contents of these substances in products and their strengths in a reliable and unambiguous way.

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