# Evaluating the Content of Active Principles from Wild *Hypericum perforatum* L. in Various Harvesting Seasons

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*Hypericum perforatum L., traditionally called St. John's Wort has been used for decades as a phyto-therapeutic herb due to antidepressant, antimicrobial and antiseptic properties. As a result, the presented study evaluates the influence of the harvesting season for the Hypericum perforatum L. plant material, which is crucial for obtaining significant amounts of active principles.*

*Keywords: Hypericum perforatum L., Harvesting season, Active principles*

*Hypericum perforatum* L. (St. John's wort) is a perennial plant, native from Europe and western Asia. St. John's wort is one of the oldest and most popular medicinal species; wild flora is prevalent in many countries [1]. It is much used in folk medicine as balsamic, anti-inflammatory for bronchial pathway and the genitourinary tract, as anti-haemorrhagic, anti-haemorrhoidal, anti-neuralgic, antiulcer and healing agent [2]. Farmore, *Hypericum perforatum* L. was recently categorised as being one of the 24 herbal medicines treating Parkinson's disease [3]. The lipophilic fraction (hyperforin and analogues) are responsible for their antioxidant, anti-inflammatory and antidepressant healing properties [4, 5]. Enhanced antioxidant activity also arises from the considerable content of polyphenols and flavones [6]. The *H. Perforatum* L. has a practical sedative effect overlapping with an actual antidepressant action. Hence, it is used as a psychotropic drug, not as *Rauwolfia* type of phytotranquilizers but milder [7]. Antiviral and antibacterial substances contained in extracts of St. John's wort were studied likewise [8, 9]. The first studies appeared in the literature regarding the effect of naphtodianthrones (NTs [10]) inhibitor action for a wide spectrum of viruses [11, 12]. Conclusively, giving the multitude of known and unknown effects of *H. perforatum* L., studying this herb is still topical [2].

Several pharmaceutical-grade preparations of *H. perforatum* L. are commercially available, typically extracted from dried aerial parts and quantified in hypericin (or NT) equivalents [13]. In this context, the paper describes the influence of the harvesting season (spring and autumn) upon the content of active principles from *H. perforatum* L., to deliver rich NTs primary extracts.

## Experimental part

### *Materials*

The two types of plant material (*H. perforatum* aerial part that included the flowers, the leaves and the stem) were collected from the same place but in two different seasons (late spring-P1 batch and beginning of autumn-P2 batch) to evaluate the influence of the harvesting time upon the content of active principles, particularly upon NTs. Extraction solvents: methylic alcohol (MetOH) p.a., ethylic alcohol 96% (EtOH) (Chimopar), purified water (W). Developer solvents: ethyl acetate p.a, formic acid p.a., acetic acid p.a. (Chimopar). HPTLC Materials: HPTLC silicagel plates (G60F254) from Merck KGaA (200x100 cm; 100x100 cm), cellulose Merck (200x100 cm); reference compounds: rutine, hyperoside, caffeic acid, hypericin, and chlorogenic acid (Fluka Chemie or Sigma-Aldrich); identification reagents (derivatisation): β-ethylamine biphenyl borate (NP), polyethylene glycol 400 (PEG) (Merck KGaA). HPLC-DAD reagents: hypericin standard in ethanol (1 mg·mL$^{-1}$, HPLC grade 95%, Sigma-Aldrich).

### *Instruments and methods*

High Performance Thin Layer Chromatography (HPTLC) was approached to identify the main classes of active ingredients from the *H. perforatum* L. extracts. *Apparatus:* CAMAG System with an LINOMAT IV band-type applicator, CANON digital camera and a CAMAG data base. Another digital photo camera with 16 mm lens, CAMAG-REPROSTAR III- DIGISTORE II + WinCATS Planar Chromatography Manager Software having Image Comparison Viewer permitted visualization of multiple samples and the retention times of specific compounds compared with adequate references, at a high resolution. *Conditions:* Developer -ethyl acetate: formic acid: acetic acid: water = 20 : 2.2 : 2.2 : 5.4 v/v. *Reagents:* (NP) (0.1 g in 10 mL methanol) and PEG 400 (0.5 g in 10 m L methanol) for compounds identification. *Reference:* rutine, chlorogenic acid, caffeic acid, as 0.01% in methanol, and hyperoside and hypericin as 0.02% in methanol. Examination of plates at 254 and at 366 nm is performed before and after spraying the reagents homogenously (process called derivatisation), followed by air drying.

Ultraviolet-visible (UV-Vis Spectroscopy) spectral analyses in the 200-800 wavelength range were performed solely for identification of some classes of active principles (NTs, PPC and Flv) and their quality. *Apparatus:* CARY 50 and CINTRA 101 Spectrophotometers, 1 cm quartz cuvettes; wavelength range: 200-400 nm and UV-vis Spectrometer Thermo Nicolet Evolution 500, 1 cm quartz cuvettes, wavelength range: 400-800 nm.

High Performance Liquid Chromatography (HPLC,Varian Prostar, Prostar 350 autosampler) equipped with a Mediterranea SEA C18, 5μm 15 x 0.46 cm column

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(Teknokroma) with diode array detector (DAD) set at 593 nm was used as a complementary method to identify the NTs in the extracts. Conditions: mobile phase: methanol/ 20mM K2HPO4 (pH 7.0) 90:10 vol.% adjusted to pH 2.5 with $H_3PO_4$; rate 1 mg·mL$^{-1}$, injection volume 10μL.

*Preparation Methods*

Preparation of H. perforatum L. primary extracts for chromatographic determinations

Plant samples from the two batches of H. perforatum L were weighed and extracted by solvent. For verifying the quality of plant material the experiments were performed on both plant batches (P1, P2). Extraction parameters: extraction solvent 50 and 70% v/v ethanol/ water or methanol alone; plant/solvent wt. ratio = 1/10, extraction time = 30 min, temperature = 55-60°C. Extraction was performed on the heated water bath at reflux and the following extracts were obtained: hydro-alcoholic 50% v/v (AE 50%) and 70% v/v (AE 70%), and methanolic (MeOH), respectively. The obtained solutions were filtered on paper to remove the ballast and analysed by HPTLC, HPLC-DAD and UV-Vis.

Preparation of H. perforatum L. primary extracts for phyto-chemical determinations

These studies consisted of separating the main natural classes (active ingredients) by successive and selective extractions of the plant product with solvents of different polarities: (i) non-polar (diethyl ether/ dichloromethane, 1:1 v/v), (ii) polar (ethanol / methanol, 1:1 v/v) and (iii) water. In the diethyl ether/ dichloromethane extract we can find the lipophilic chemical compounds, and in the other two extracts, hydrophilic chemical compounds. To identify the chemical compounds, specific methods corresponding to the main physical-chemical properties of each class of active ingredients were approached [14], as follows: (A) Etheric extract-The plant powder (10-20 g) was extracted successively with ethyl ether at ambient temperature in a suitable container. The combined etheric extracts were filtered and concentrated to 50 mL in a rotavap. This particular extract contains fat-soluble chemical compounds; (B&B') Alcoholic extracts-The vegetable product left over from extraction with ether was mixed with 100-150 mL of methanol or ethanol and hot reflux extraction on the heated water bath was performed for about 30 min. The obtained extract was separated by filtration and the plant product was washed with small amounts of warm alcohol followed by filtration. The collected extract was then concentrated to 50 mL and analyzed; and (C&C') Aqueous extracts-The remaining product from alcohol extraction was dried and extracted with distilled hot water (50-100 mL) for 15 to 20 min. The obtained extract is filtered and concentrated to a 50 mL volume, than tested.

*Quantitative determination of active substances*

Extractible substances

For this determination, 5 g of vegetal product is lyophilized and immersed in 100 mL of solvent (70 vol.% alcohol/ water), followed by stirring for 24h at room temperature. The extract is evaporated to dryness and the residue is further dried at 105°C until constant weight. The ultimate dry extract relative to the initial weight of plant product (calculated for 100g) represents the extractible substances, ES [eq. 1].

$$ES\ (\%) = \frac{m2 - m1}{m2} \cdot 100 \qquad (1)$$

where *ES %*= soluble substance percent, *m1* and *m2* (g) the weight of resulted residue and that of the crude plant product, respectively

Determination of flavones (as rutoside)

Flavones in the presence of $Al^{+3}$ cation form bright yellow complexes, observable at λ= 430 nm. Reagents: ethyl alcohol 50% v/v; sodium acetate solution 100 g/L; aluminium chloride solution 25 g/L; rutoside 0.1 g/L in ethanol 50% v/v. For this determination, 3 mL of each extract were brought in 10 mL flasks and completed with 8 mL of ethyl alcohol 50% v/v (solution A). 0.5 mL of solution A are distributed in 25 mL volumetric flasks, and mixed with 5 mL of sodium acetate 100 g/L and 3 mL of aluminium chloride 25 g/L, shaking after each reagent addition and completed up to 25 mL with ethyl alcohol 50% v/v (solution B). Calibration of rutoside was determined in the 0.004-0.012 g/L concentration range using a similar protocol with the sample preparation. The absorbance of samples was verified after 15 min. at 430 nm, using 10 mm quartz cuvettes, against a blank ethylic alcohol 50% v/v solution. Quantification of flavonoids content was performed according to eq.[2] :

$$\textit{Total flavones (in rutoside equivalents)} = \frac{Ap \cdot V \cdot f}{B} \cdot 100 \qquad (2)$$

where *Ap*= sample absorbance (a.u.); *f*= calibration factor (0.850x $10^{-3}$ g/a.u.); *V*=volume of solution A (ml); *B*= volume of solution used for the colorimetric reaction (ml).

Determination of polyphenolcarboxylic acids (as caffeic acid)

Polyphenolcarboxylic acids give a brick-red colour with Arnow reagent detectable at 500 nm. Reagents: ethyl alcohol 50% v/v; Arnow reagent: 10 g of sodium nitrite (R) and 10 g of sodium molybdate (R) was dissolved in 100 mL of distilled water; hydrochloric acid 0.5 mol/L; caffeic acid 0.01% in ethyl alcohol 50% v/v; sodium hydroxide 1 mol/L. For this measurement, 3 mL of each extract was mixed with ethyl alcohol 50% v/v (solution A) in a 10 mL volumetric flask. From each solution, 0.5 mL were taken and contacted with 2.5 mL of hydrochloric acid 0.5 mol/L, 2.5 mL sodium hydroxide 1 mol/L and 2.5 mL reagent Arnow in 25 mL flasks. After each reagent addition the vials were shacked followed by complexation up to the mark with distilled water (solution B). The absorbance of solution B was read after 10 min at 500 nm and compared to the control sample obtained under the same conditions without Arnow reagent. In parallel, the absorbance of a solution C (obtained using 2 mL of caffeic acid 0.01% in ethyl alcohol 50% v/v, 2.5 mL of hydrochloric acid 0.5 mol/L, 2.5 mL Arnow reagent and 2.5 mL of sodium hydroxide 0.1 mol/L and distilled water up to the mark) was measured as standard. The calculation of *Total Polyphenolcarboxylic acids* content expressed in caffeic acid equivalents was made using eq.[3].

$$\textit{Total Polyphenolcarboxylic acids (caffeic acid equivalents)} = \frac{Ap \cdot V \cdot f}{B} \cdot 100 \qquad (3)$$

where *Ap*= sample absorbance (a.u.); *f*= calibration factor (0.8789x $10^{-3}$ g/a.u.); *V*=volume of solution A (mL); *B*= volume of solution used for the colorimetric reaction (mL).

Determination of naphtodianthrones as hypericin

The extracts containing naphtodianthrones is red coloured and quantification is possible by measuring the absorbance at 590 nm. Reagents: dichloromethane; methanol p.a.

*Analysis of plant product*

1 g of grounded plant material was brought to into a Soxhlet separator with dichloro-methane, to remove chlorophyll. Afterwards, the dried plant material was extracted with 100 mL of methanol p.a. for 60 min at reflux on the heated water bath. The sample was filtered after cooling and the filtrate was adjusted to 100 mL with methanol p.a. in a graded flask. Calculation of *total naphtodianthrone content in expressed in hypericin* was done according to eq.[4]:

$$\text{Total naphtodianthrone content in plant (as hypericin equivalents) \%} \frac{A \cdot V}{a \cdot 870 \cdot S} \cdot 100 \qquad (4)$$

where *Ap*= sample absorbance (a.u.); 870= absorbance of 1 mg/mL hypericin standard; *V*=total volume of solution (mL); *a*= the content of plant material (g); *S*= percentage of dry content in the plant material (100- U/100); *U*=humidity of plant material.

**Results and discussions**

*Identification of active principles in H. Perforatum L.-Phytochemical studies*

The analysed plant products contained polyphenols, especially tannins, flavonoid glycosides, triterpenic glycosides and active principles with lipophilic character like grease, volatile oil, fatty substances and resins (including chlorophyll) (table 1), being in line with the newest reported values [15]. Yet, no significant differences in terms of presence or absence of these particular classes of active ingredients were observed between the two batches of interest (P1, P2).

*High Performance Thin Layer Chromatography (HPTLC) for primary extracts*

Further studies of the extracts were performed by HPTLC, which revealed that the alcoholic and hydro-alcoholic extracts presented various phenolic substances (Flv and PPC) and NTs (hypericin and pseudohypericin). Identification was accomplished in two ways: **a)** with no chemical treatment- in ultraviolet light at 254 nm all flavones stop their fluorescence and appear as black areas on the chromatographic plate and at 366 nm, depending on the structural type, specific areas appear fluorescent yellow, blue or green; and, **b)** with chemical treatment (derivatisation) of plates using identification reagents, an intense fluorescence appears at 366 nm, which is specific for each structure.

**Table 1**
SUMMARY OF *H. PERFORATUM* L. COMPOSITION (PLANT BATCHES P1&P2)

| | |
|---|---|
| ***A. Etheric extract*** | |
| **Active compounds in extracts** | **"+"= presence "-"=absence** |
| volatile oil, grease and chlorophyll | (+) |
| fatty substances and resins | (+++) |
| sterols, triterpens | (++++) (green colour) |
| carotenoids, alkaloid bases, flavonoid, cumarines | (-) |
| emodols | (+++++ )(red) |
| ***B. Methanolic extract*** | |
| **Active compounds in extracts** | **"+"= presence "-"=absence** |
| tannins, alkaloid salts, aminoacids | (-) |
| reducing compounds | (+++++) |
| ***B'. Hydrolyzed Methanolic extract*** | |
| **Active compounds in extracts** | **"+"= presence "-"=absence** |
| antracianosides | (+++++) |
| cumarines | (+++) |
| heterosides, antocianosides | (-) |
| flavonoids | (++) |
| ***C. Aqueous Extract*** | |
| **Active compounds in extracts** | **"+"= presence "-"=absence** |
| mucilage, pectin and gums | (+++) |
| saponins, alkaloid salts, aminoacids | (-) |
| tannins, reducing compounds | (+++++) |
| ***C'.Hydrolyzed Aqueous Extract*** | |
| **Active compounds in extracts** | **"+"= presence "-"=absence** |
| antocianosides | (+++ )(red) |
| cumarines, saponins, flavonoids | (-) |
| hyperosides | (+) Brown ring |

The appearance of dark gray spots on the chromatographic plates at 254 nm for the examined samples (before spraying with the identification reagent) indicated the presence of Flv, PPC or NTs (fig. 1a). The HPTLC images also highlighted the separation of constituents in samples and of compounds used as reference (i.e. caffeic acid, rutine, hyperoside, chlorogenic acid, hypericin). Reference substances have been identified in the following ranges of retention times, *Rf*: 0.45 - 0.46 (rutine), 0.55-0.56 (chlorogenic acid), 0.67-0.68 (hyperoside), 0.96-0.97 (caffeic acid), 0.93-0.94 (hypericin). By reference to these substances, specific constituents like rutine, hyperoside, hypericin, chlorogenic and neochlorogenic acids, and caffeic acid were identified in the analyzed extracts, at 366 nm after spraying with the specific identification reagents (fig. 1b). According to the spot intensity, the hydro-alcoholic extracts with ethanol/water 70% v/v from plant batch P2, meaning AE 70% contained the highest amounts of NTs. Using HPTLC digital scan of chromatoplates, fractions of active compounds found in various amounts were transposed as peaks and visually detected on the plate board (fig. 2). The peaks were consistent with the characteristic bands and the brightness levels, registered for the reference compounds, and used further to identify the flavones, the polyphenolcarboxylic acids (fig.2a) and the naphto-dianthrones (fig. 2b).

Quantitative assessment of targeted active principles (i.e. Flv expressed in rutoside, PPC expressed in caffeic acid and NTs expressed in hypericin) indicated that all the three extracts (AE 50% AE 70% and MeOH), corresponding to batch P2, contained higher amounts of active principles (table 2). As suggested by HPTLC as well, a richer phase in active compounds was found for the hydro-alcoholic extract 70% v/v and not for the MeOH as reported by other

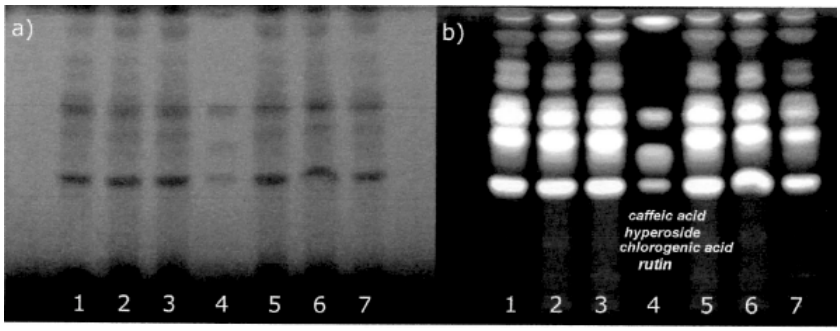
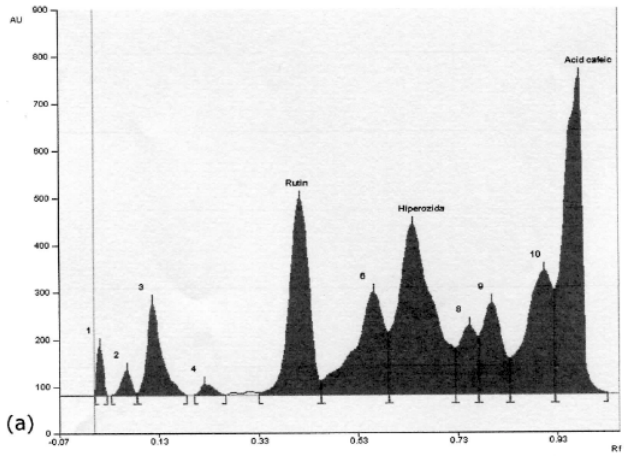



Fig. 1 Identification Chromatograms for flavones, polyphenolcarboxylic acids and naphtodianthrones: a) at 254 nm before derivatisation; b) at 366 nm after derivatisation. *Tracks*: 1-MeOH-P2; 2-AE 50%-P2; 3-AE 70%-P2; 4-reference substances; 5-AE 70%-P1; 6-AE 50%-P1; 7-MeOH-P1



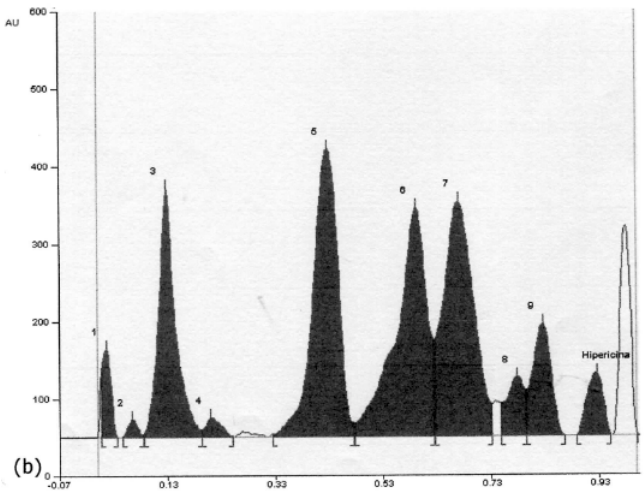




Fig. 2 Identification of rutine, hyperoside, and caffeic acid (a) and of hypericin (NTs) (b) in the AE 70% extract

| Class / Batch | AE 50% v/v | | AE 70% v/v | | MeOH | |
|---|---|---|---|---|---|---|
| | P1 | P2 | P1 | P2 | P1 | P2 |
| NTs in the ES (in hypericin) (wt.%) | 0.26 | **0.61** | 0.41 | **0.60** | 0.23 | 0.29 |
| Flv in the ES (in rutoside) (wt.%) | 24.1 | 38.2 | 27.3 | 35.1 | 24.1 | 34.9 |
| PPC in the ES (in caffeic acid) (wt.%) | 26.1 | 42.2 | 26.8 | 37.1 | 14.1 | 19.5 |

**Table 2**
ACTIVE PRINCIPLES FROM ALCOHOLIC AND HYDRO-ALCOHOLIC *H. PERFORATUM* L. EXTRACTS

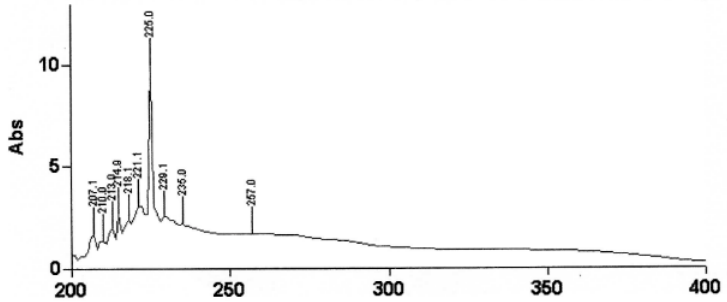



Fig. 3 UV spectrum of the 70% v/v hydro-alcoholic extract of plant batch P2 [200-400 nm].The extract was diluted as follows: 0,1mL extract with 20 mL of ethylic alcohol 70%, v/v for the 200-400 nm range.

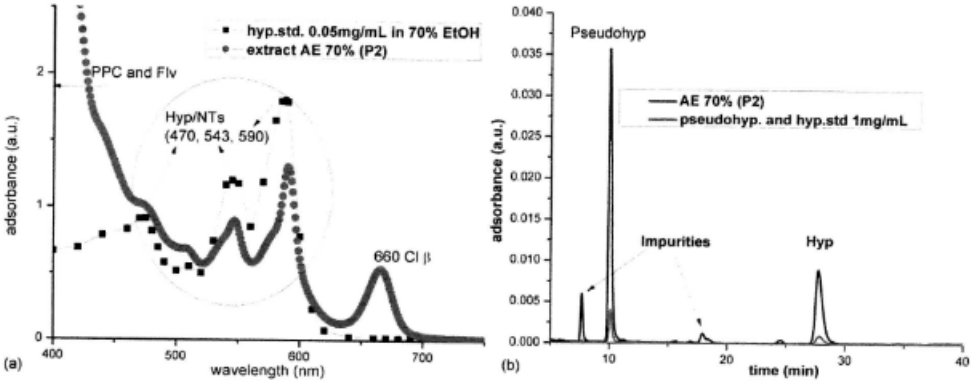



Fig. 4 Qualitative determinations for the 70% v/v hydroalcoholic extract of plant batch P2 (AE 70%): (a) Visible spectrum of the AE 70% (P2) at 400-600 nm wavelength range against hypericin standard 0.05 mg·ml$^{-1}$ (NTs-naphtodianthrones; Cl β-chlorophyll β).; (b) HPLC DAD Chromatogram of the AE 70% (P2), compared to pseudohypericin and hypericin standards of 1 mg·mL$^{-1}$, at 593 nm.

authors [16]. This simple protocol delivered up to 0.6 wt.% NTs relative to the extractible substances (ES was around 14-15 wt.% in dried plant materials when extracting at reflux for 30 min with alcohol or alcohol/water mixtures) which led to values of around 0.1 g of NTs in 100 g of dry plant material. It is also worth mentioning that the 70% v/v solvent was preferred not only for delivering high NTs contents but also for extracting less Flv and PPC, relative to the 50% v/v solvent.

*UV spectral analyses and HPLC-DAD for the primary extracts*

UV qualitative analyses were performed for the hydro-alcoholic 70% v/v extract (AE 70%), considered to be the optimum extract obtained by reflux in a single-step procedure. The AE 70% extract was diluted as follows: 0.3 mL extract with 2.7 mL of ethylic alcohol 70%, v/v. According to the literature [17], the absorption bands in the 210-310 nm range indicated the presence of phenolic groups and those in the 255-280 nm correspond to specific flavones (fig. 3*)*. Also, this 255-270 nm wavelength range is specific for chromophore structures with aromatic groups.

Other information about the presence of NTs is usually found in the visible range, 400-800 nm, as presented in figure 4a. In the analysed extract, the characteristic bands for PPC, for Flv and for NTs were registered at specific wavelengths i.e. 280 nm, 340 nm and 590 nm, respectively. Here, it can be noticed that chlorophyll β (666 nm specific wavelength) was also extracted along with the NTs. Complementary HPLC-DAD analyses (fig. 4b) were also carried-out to highlight the quality and purity of these primary extracts, using as reference standards of pseudohypericin and hypericin (1 mg·mL$^{-1}$, retention time $R_{pseudohyp}$ =11 min and $R_{hyp}$ = 28 min [18]).

Following these results, it can be concluded that the reflux extraction with ethylic alcohol 70% led to primary extracts of high purity and with significant content of NTs (around 10 mg·mL$^{-1}$ or 1 wt.%), which is more than the quantity found in the commercial products (around 0.3 wt.% [13]).

## Conclusions

HPTLC evaluation revealed that the richest in active ingredients are the extracts derived from batch P2 of *Hypericum perforatum* L., harvested in autumn with 50% more NTs, than P1 harvested in spring. It was also found that the hydro-alcoholic solvent 70% v/v ensured a more efficient extraction than the 50% v/v and MeOH. Therefore, our study proved that the content of NTs is closely related to the season of harvesting. This finding is important because it is likely that the plant material to be featuring stronger or weaker actions due to the chemical composition.

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