BIOCHEMICAL AND HPTLC FINGERPRINTING IDENTIFICATION OF THE *HYPERICUM PERFORATUM* L. – FINISHED PRODUCTS

Carmen Elena ŢEBRENCU[1,3*], Elena IONESCU[1,3], Oana Teodora CIUPERCĂ[1], Ruxandra Mihaela CREŢU[2], Maria CHIRIAC[1], Elena IACOB[1]

Abstract: *Based on the data obtained from the instrumental and biochemical fingerprinting of Hypericum perforatum L., an experimental program has been designed in order to test some intermediate products and final products as plant food supplements. Herbal supplements based on H. perforatum L., as single ingredient or in complex mixtures with other medicinal plant species have been selected and tests correlated with extensive phytochemical characterization of the plant species H. perforatum L. have been performed. Based on the results achieved, a extensive phytochemical and chromatographic characterization diagram was obtained which takes into account the correct identification of medicinal plant species and derived complex herbal food supplements.*

Keywords: *Hypericum perforatum* L., herbal supplements, biochemical fingerprinting, HPTLC fingerprinting, authentification

1. Introduction

Hypericum perforatum L. (common St. John's wort, *Hypericaceae*), originally native to southern Europe, is commonly found throughout temperate regions. This species is an important medicinal herb, and extracts are widely recognized as valuable phytopharmaceutical agent [6]. *Hypericum perforatum* contains at least ten classes of biologically active compounds, of which two of the more important bioactive compounds, hypericin and hyperforin.[1,3] Research indicates that these compounds vary in concentration and or constituency depending on species origin, tissue type, genetics, and environmental factors. In addition, concentrations of these compounds can vary widely between accessions derived from the same species. *Hypericum perforatum* L. is the official source of *Hyperici herba* as accepted by both the Romanian Pharmacopoeia and the European Pharmacopoeia. According to European Medicines Agency /Committee on Herbal Medicinal Products/Assessment. report on *Hypericum perforatum L.* herba, preparations of St. John's Wort/*Hypericum perforatum* (flowering aerial parts) are accepted for medicinal and traditional use [9]. The products of St. John's wort are

[1] Medicinal Plants Research and Processing "PLANTAVOREL" S.A., Cuza Voda Street, no. 46, 610019, Piatra Neamț, Romania
[2] NIRDBS / "Stejarul" Research Centre for Biological Sciences, Alexandru cel Bun St., no. 6, Piatra Neamț, 610004, Romania
[3] Academy of Romanian Scientists, Splaiul Independenței , no.54, Bucharest, Romania
[*]Corresponding author. E-mail adrress: carmen@plantavorel.ro

popular for treating mild depression. Naphthodianthrone (hypericin, hyperforine) were identified as the major compounda that contribute to the pharmacological activity. With synergic antidepressant activity it is a flavonoid (rutin); it was demonstrated that the extract containing about 3% of rutin showed positive effects. The research progress on the phytochemistry and pharmacology of St. John's wort suggest that compounds work synergistically to achieve the antidepressant effects [4,8].

Hypericum perforatum L.due to its importance in the dietary supplement industry based on medicinal plants, and its many traditional uses**.** In terms of constituents and authentication of *Hypericum* species there are many assessments and studies. Because of the significance of *H. perforatum* to the herbal supplement industry, it is important to develop a reliable system that can be used to affordably and accurately identify plant material purported to be *H. perforatum* in order to aid producers while protecting consumers from potentially adulterated products. Species substitution in *Hypericum* can lead to products lacking the intended concentrations of active compounds, and/or threaten wild populations of other *Hypericum* species due to unsustainable harvesting.

The current authentification of the products is completed by the high performance biochemical fingerprinting allowing the identification of metabolic markers for the authentification of herbal ingredients. In according to the World Health Organization (WHO) the current screening of the steps during the herbal medicine manufacturing process and DNA barcoding authentification of the herbal ingredients and products must be completed by the extensive phytochemical analyses [2,10]. Chemical fingerprinting has been demonstrated to be a powerful technique for the quality control of herbal medicines.The utilization of HPTLC it is considered as an useful tool in the analysis of complex mixtures of natural products. Adoption of HPTLC by USP, Ph.Eur., AHP and PhPRC (Chinese Pharmacopoeia, 2009) constitute a recognition of the importance of this technique as the method of choice for handling complex analytical task involving herbal drugs and botanicals [5,7].

The objective of this study is to identify the *Hypericum perforatum* L. in finished products (food supplements) based on this species by specific biomarkers obtained with biochemical and HPTLC chromatography .

2. Materials and methods

2.1. Plant material, raw material and finished products on *Hypericum perforatum* L.

St. John's wort was used, aerial part (*Hypericum perforatum* L.) was collected in June 2015 from the natural population and conditioned in dry form according to Eur.Ph. 6.0 . The plant was identified and certified by experts according to "St. John's wort –*Hyperici herba* "Monography in European Pharmacopoeia 5.0. St.

John's wort, aerial part (*Hypericum perforatum* L.) is used as raw powder, dry extract or mixed with othet plant species in finished products (**Hp** -St. John's wort, powder; **HEi -**St. John's wort dry extract; **HE-**St John's wort obtained by atomization at Plantavorel; **H-**HEPATOBIL V, tablets (from vegetal powders); **VR-**VITA ROZ, tablets (from extract and powder); **G**- GASTROVIT, tablets (from standardized dry extracts); -Tonic herbs FEMINA, hydroalcoholic solution) produced by Medicinal Plants Research and Processing "PLANTAVOREL" SA Piatra Neamt, Romania.

2.2. Chemicals and reagents

All the reagents were of analytical grade or pure. Hypericin- min. 98% , Quercitrin hydrate~85%, Izoquercitrin- min 90%, Rutin hydrate- min.95%, Chlorogenic acid- min95%, Hyperoside- min 97%, Caffeic acid- min 95% (*Sigma-Aldrich*); Procyanidin B2- min 90%, (+) – Catechin, min 99% (*Fluka*); (-) – Epicatechin, ROTICHROM (*Roth*); ethanol, min. 96 % v/v, and methanol, 99.3 % analytical reagent (*ChemicalCompany, Romania*); formic acidACS, analytical reagent Ph. Eur (*Merck*); ethyl acetate, min. 99.5 %, analytical reagent, (*Chemical Company, Romania*); petroleum ether, analytical reagent (*Chemical Company, Romania*); potassium hydroxide, analytical reagent (*Lach-Ner, Czech Republic*); cobalt chloride (II), analytical reagent (*Chemical Company, Romania)*; Distilled water, prepared in lab. GFL 2001/4. For Thin Layer chromatography HPTLC were used HPTLC plates silica gel, 60 F254, 10 x 10 cm (*Merck*), chromatographic purity solvents and standard substances with chromatographic purity: Natural products – polyethylenglycol (NP/PEG) reagent (*Fluka*).

2.3. Methods

Testing of selected products (table 1) took into account qualitative and quantitative analysis of phytochemicals - *markers* for raw materials, intermediates and finished products containing *Hypericum perforatum* L.. To identify the classes of compounds of interest/specific markers by phytochemical study and phytochemical chromatographic profile, and dosage of the phytochemical compound were used samples of *Hypericum perforatum* L. preliminary evaluated. Classes of compounds/phytochemical compounds selected as markers for identification/authentication of *Hypericum perforatum* L. species are: *flavonoids*: rutin, hyperoside, quercitrin, izoquercitrin; • *phenylpropans*: chlorogenic acid, neoclorogenic acid; *naphtodianthrones*: hypericin.

2.4. The steps of the experimental study were as following

(1) preparation of solutions from raw materials, intermediate and the final products-tablets; **(2)** qualitative analysis by High Performance Thin Layer Chromatography (HPTLC) for the identification of selected markers; **(3)** quantitative analysis of phytochemicals compounds selected as markers.They were used phytochemical methods estabished by experimental protocol. The qualitative

analysis consisted in spectroanalytical profile by HPTLC, equipment CAMAG LINOMAT IV, TLC 3 Scanner, WINCATS Planar Chromatography Manager and stationary phase HPTLC plates G60F254. For each class of compounds a working protocol was prepared based on chromatographic identification sheets/HPTLC densitometric dosage. *HPTLC Analysis for characteristic fingerprint for flavons and poliphenols* : 3-4 µl of the samples ans 1-3 µl of references substances werw loaded as 6 mm band lenght in the plate; the mobile phase was constituted of ethyl acetate: formic ac.: acetic ac.: water 20: 2,2: 2,2: 5,4 (v/v/v/v). After development, plate were dried and derivatized in NP/PEG reagent. The examination and documentation were at 366 nm in fluorescence mod wavelength for quantitative evaluation was 364;329; 368 nm. *HPTLC Analysis for characteristic fingerprint for hypericin*: 5-10 µl of the samples ans 1-3 µl of reference substance werw loaded as 12 mm band lenght in the plate; the mobile phase was constituted of toluene: ethyl acetate: formic acid = 15:12:3 (v/v/v). After development, plate were dried and derivatized in NP/PEG (reagent Heating of the plate (temp./time): 105^0 C/ 5 min). The examination and documentation were at 366 nm in fluorescence mod wavelength for quantitative evaluation was 295 nm. The dosage methods used were either those described in the European Pharmacopoeia, Romanian Pharmacopoeia or the ones developed and validated in the laboratory: flavonoid content (as *rutin*) by following colorimetric aluminum chloride method, at 425 nm, polyphenol content (as *chlorogenic acid*) by Arnow method, at 540 nm and total naphthodianthrones (as *hypericin*) at 590 nm. For each determination, the absorbance of reaction mixture was measured with a CARY 50 UV/VIS spectrophotometer.

Sample preparation: the sample were prepared by extraction with methanol p.a – vegetal material/ solvent ratio -1:10 m/v for 15 minutes at boiling temperature of the solvent. The solution was filtred and cooling until analysis. Samples solutions codes are: PHp (from Hp), PEi (from HEi), PE(from HE), PH(from H) , PVR (from VR), PG (from G) and PF (from F)

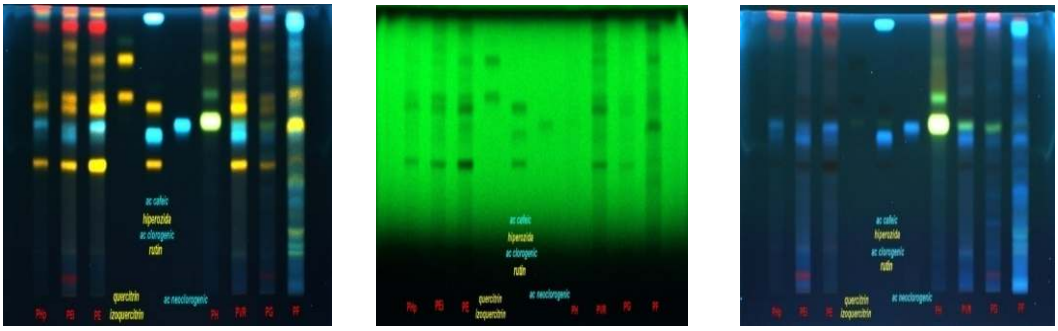
3. Results and discussions

3.1. *Qualitative analytical study/ HPTLC chromatographic fingerprinting*

The chromatographic analysis of the results was done by comparative evaluation and correlation of the content through the marker compounds in raw materials, intermediate and final product whose composition is found.

(Figs 2a,b,c) shows ***chromatographic polyphenols and flavons fingerprint*** of *Hypericum perforatum* L.– finished products and references substances. The appearance of dark-gray spots in the examined samples on the chromatogram viewed at 254 nm after development, indicates the presence of flavonoid compounds and polyphenol acids at different *Rf* values (Fig. 2b). On chromatogram viewed at 366 nm after development (Fig.1a) appearance of blue spots indicates the presence of polyphenol carboxylic acids and black spots indicates flavonoid

compounds. Thus, by UV chromatographic examination at 254 nm after development and in UV at 366 nm after derivation were identified *Rf* values for reference substances (Table 1) and by relating to them, were identified specific constituents in analysed samples at *Rf* distinct values. Qualitative phytochemical study conducted by thin layer chromatography showed that the samples analyzed have a varied content of phenolic substances (flavonoids and polyphenolcarboxylic acids) (Fig.1b).



a) 366 nm after derivatization b) 254 nm after development c) 366 nm after development

Fig. 1a,b,c. Identification cromatograms for flavonoidic compounds and poliphenolcarboxylic acids test solutions: PHp, PEi, PE, PH, PVR, PG, PF).

Chromatografic fingerprint

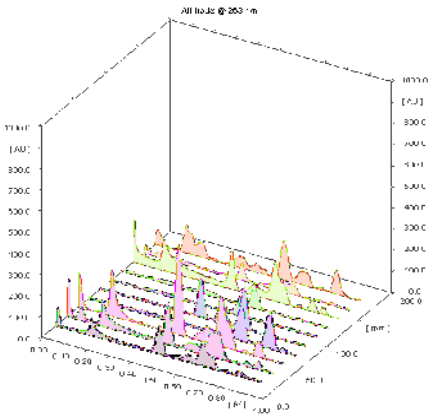
In chromatography after derivatization UV 366 nm (Fig.2a) chromatographic profile for **PVR** sample is very suggestive by the fact it appears as a sum of **PHp** and **PEi** samples, which corresponds with composition. Screening at 263 nm after development, conducted for quantitative assessment of constituents, provided two informations:

• *Assignment of reference substances in samples*: in **PHp** : quercitrin, rutin, hyperoside, chlorogenic acid, neochlorogenic acid; in **PEi** : quercitrin, isoquercitrin, rutin, hyperoside, chlorogenic acid; in **PE** : quercitrin, rutin, hyperoside, neochlorogenic acid; in **PVR** : quercitrin, rutin, hyperoside, chlorogenic acid; in **PG** : rutin, hyperoside, chlorogenic acid; in **PF** and **PH** it cannot assign clear reference substances;

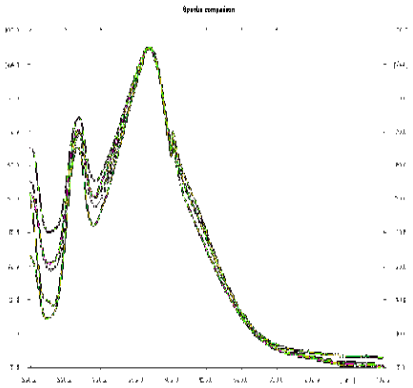
• *Maximum of absorption for reference substances* applied (overlapping of standards spectra and standards attributed samples spectra): it has been highlighted that, only spectra of *rutin* and *hyperoside* have the same allure in standards and samples that were assigned (Figs. 2 a,b,c).

Table 1. *Rf* values for specific constituents/reference substances

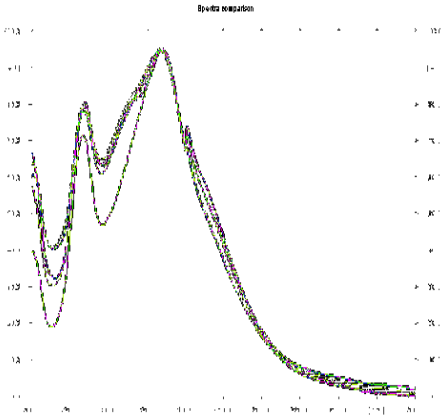
***Rf* values**	***Reference substances***	***Test solutions***
0.45	*Rutin* (orange yellow spot)	**PHp (**very weak**), PEi, PE** (very pronounced), **PVR, PG** (very weak) not identified in **PF, PH**
0.56	*Chlorogenic acid* (fluorescent blue spot)	**PHp, Pei, PVR, PG, PF;** not identified in **PE, PH**
0.59	*Neochlorogenic acid* (fluorescent blue spot)	**PHp, PEi (?), PE, PVR (**very weak**)** not identified in **PF, PH; PG (**at this *Rf* greenish spot**)**
0.61	*Hyperoside* (orange yellow spot)	**PHp, PEi, PE, PVR, PG;** not identified in **PF, PH**
0.69	*Isoquercitrin* (orange yellow spot)	**PHp (**very weak**), PEi, PE**, **PVR, PG (**in **PH** at *Rf* = 0,69 is identified a distinct green spot); not identified in **PF**
0.81	*Quercitrin* (orange yellow spot)	**PHp (**very weak**), PEi, PE**, **PVR, PG (**very weak) (in **PH** la *Rf* = 0,69 is identified a distinct green spot) not identified in in **PF**
0.91	***At this developer was identified hypericin*** (Red spot)	**PHp, PEi, PE** (very pronounced), **PVR** (very pronounced), **PG** (weak) In **PF** is identified a chromatographic profile rich in polyphenols (multiple spots blue) and two spots of flavones la *Rf* = 0,57; 0,68 In **PH** at this *Rf* is identified a yellowish spot
0.95	*Caffeic acid* (fluorescent blue spot)	**There is no evidence to identify**



a) 3D graph -263 nm



b) Rutin and samples spectra



c) Hyperoside and samples spectra

Fig. 2. Spectra of rutin and hyperoside

For further analytical investigation for polyphenols and flavones content, densitometric dosage of compounds assigned to the following reference substances was performed: *rutin and hyperoside* (Table 2).

Table 2. Results of densitometric determination

Sample code	**Rutoside, 364 nm**		**Hyperoside, 368 nm**	
	µL used (reference standard)	Amount (mg/100 ml sample solution)	µL used (reference standard)	Amount (mg/100 mL sample solution)
PHp	2	28.0	2	21.19
PEi	1	83.5	1	51.98
PE	5	185.3	1	97.74
PVR	4	20.1	4	19.36
PG	4	8.89	4	5.31

Chromatographic fingerprint was obtained for polyphenols and flavones content (Fig. 3). Fig. 4a, b illustrates **identification chromatograms of naphthodianthrones (hypericin)**. HPTLC images show the reference substances separation and constituents separation of analyzed samples.

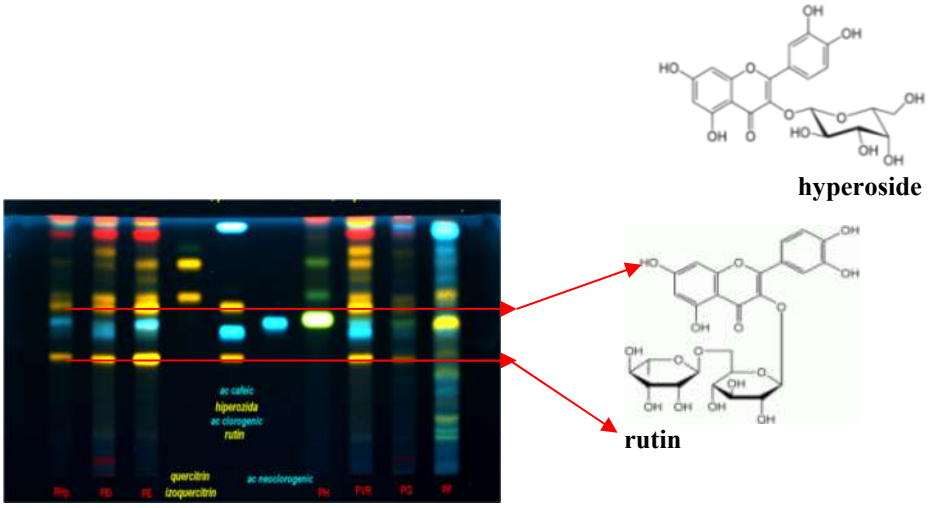
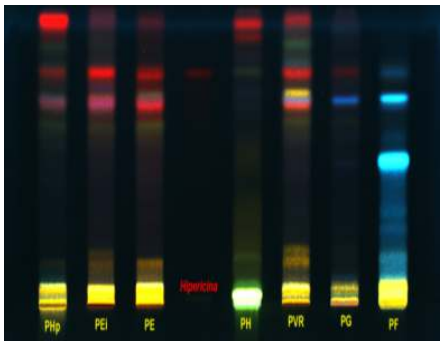
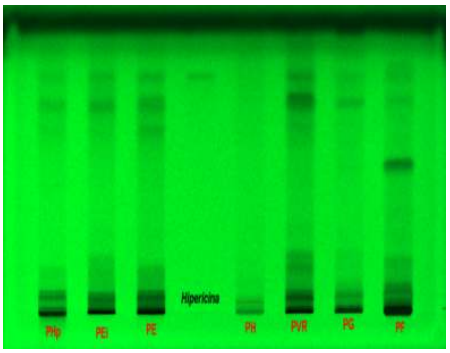


Fig. 3. Flavones and polyphenols HPTLC fingerprint

The reference substance – hypericin - was identified at *Rf*=0,78 (red spot) in reference substance solutin and in the samples **PHp, PEi, PE, PVR, PG** after developing in UV 254 nm (Fig. 4b) and after derivatization in UV 366 nm (Fig. 4a) which is the most relevant. At the same *Rf* (0.78) in the sample pH appears distinctly greenish-yellow spot and in the sample PF appears a blue spot. The evaluation of the compound hypericin al λ= 295 nm, the absorption spectra of hypericin from samples having the allure of the hypericin spectra, but without the their overlapping (Figs. 5a,b,c). The closest spectra as allure is identified in the sample **PE.** In Table 3 there are presented results of densitometric determination.

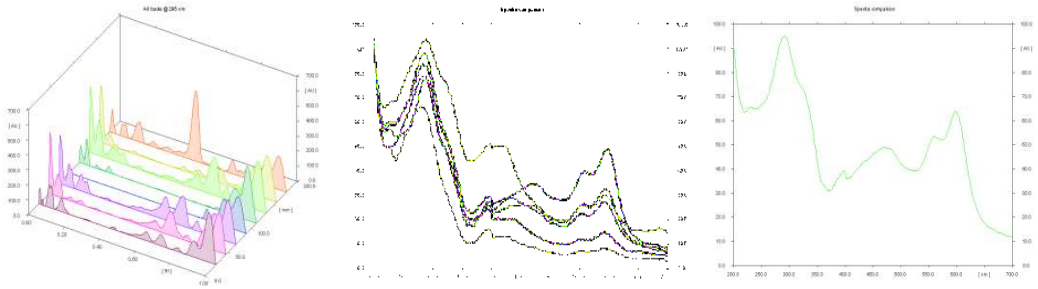


a) 366 nm after derivatization



b) 254 nm after development

Fig. 4. Identification chromatograms of hypericin - PHp, PEi, PE, PH, PVR, PG, PF - samples



a) 3D Chart -295nm

b) The comparison of hypericin spectra of referencesubstance and the samples PHp, PEi, PE, PVR, PG

c) *Hypericin* spectra

Fig. 5. The evaluation of hypericin in the samples PHp, PEi, PE, PH, PVR, PG, PF

Table 3. Results of densitometric determination

Sample code	µl used (reference standard)	Amount (mg/100 ml sample solution)
HYPERICIN, 295 nm		
PHp	10	0.65
PEi	3	3.2
PE	3	5.1
PVR	5	4.1
PG	10	0.66

Chromatographic fingerprint was obtained for hypericin content (Fig.6)

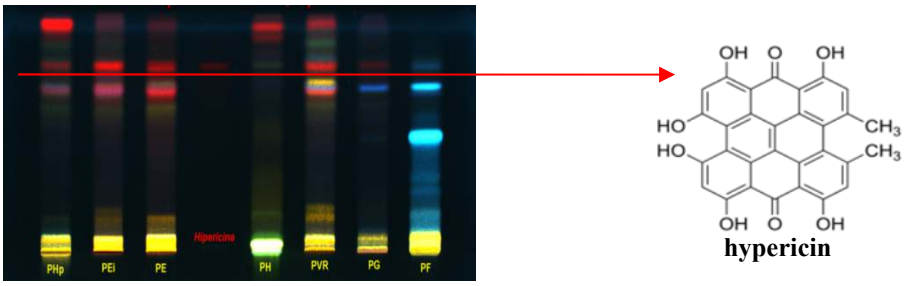


Fig. 6. Hypericin HPTLC fingerprint

Quantitative analysis of the selected samples : The following classes of compounds were dosed: total flavonoid content expressed in rutin and hyperoside, total polyphenol content expressed in chlorogenic acid, total naphtodianthronic content expressed in hypericin. In table 4 are presented the results of analytical determination.

Table 4. Results of the qualitative analysis of selected samples

Phytochemical parameters	**Selected samples/***(% g/g p.v.)*					
	Hp	**HEi**	**H**	**VR**	**G**	**F**
Total polyphenolcarboxylic acids expressed in *chlorogenic acid*	2.7832	5.7092	0.1164	5.1254	1.0248	0.2851
Total flavones expressed in *rutin*	1.6355	7.6253	0.1443	3.6023	1.0636	0.1589
Total flavones expressed in *hyperoside*	1,6122	1.7287	0.1422	1.9454	0.1922	0.3420
Total naphtodianthrones expressed in *hypericine*	0.0489	0.2535	0.0077	0.1233	0.03032	0.02146

Values obtained from phytochemical compounds of interest dosing corresponds exactly to qualitative assessment carried out by chromatographic fingerprinting for flavones, polyphenols compounds and naphtodianthrones. The highest concentration values of phytochemical compounds are found in St. John's wort dry extract (compared to St. John's wort powder as raw material), which also corresponds to higher values in finished products based on extracts (**PVR, PG**). If for solid forms of conditioning (tablets) qualitative and quantitative determinations have been conducted, in fluid form, where phytochemical profile is already selected by extraction solvent, identification of compounds could not be achieved by instrumental methods.

Conclusions

They identified The common “biomarkers” for identifying the species *Hypericum perforatum* L. in raw powder, dry extract or mixed with othet plant species in finished products, respectively *rutin, hyperoside* and *hypericin* through biochemical and HPTLC fingerprinting.

Conclusion (1). In the case of finished products as plant complex compositions (with many plant species / in fluid extract) is required completing investigations with other modern analytical methods for the safety of authentication plant species.

Acknowledgment

The research leading to these results has received funding from the Romanian - EEA Research Programme operated by the MECS-ANCSI PO under the EEA Financial Mechanism 2009-2014 and Project Contract No 2SEE/2014.

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