THE ANALYSIS OF ALCOHOLIC EXTRACTS OF *Hypericum* SPECIES BY UV/VIS SPECTROPHOTOMETRY

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Abstract: In this report, the UV/VIS spectrophotometric method was employed in order to identify and dose the active principles contained in the alcoholic extracts of *Hypericum perforatum* L., *Hypericum maculatum* Crantz, with two subspecies, *Hypericum tetrapterum* Fries and *Hypericum hirsutum* L. The most important active principles are hypericin and flavones. For the identification of flavones an officinal method in FR X was used, whereas for the hypericin - an officinal method in the European Pharmacopoeia. The obtained results led to the conclusion that *H. maculatum* has the highest content of total hypericins (0.496g%) as well as flavonoids (6.418g%). In the European Pharmacopoeia the *St. John's wort* monograph specifies the exact content of hypericin of the vegetable product (determined through the spectophotometric method), of 0.08g% total hypericin. Given this fact, all the studied *Hypericum* species fit within the limits imposed by the E. Ph. There is no stipulation in FR X regarding the limits of hypericin concentrations or of other active principles that can be found in *Hyperici herba* extracts.

Keywords: *Hypericum*, UV/VIS spectrophotometry, flavones, hypericins.

INTRODUCTION

UV and visible absorption spectrophotometry is considered one of the classical methods of analyzing medicines, as its importance has not diminished in time. Moreover, by combining it with other methods of analysis, the range of applications of spectrophotometry continues to expand, and its popularity can be explained by the fact that it is a simple, quick and relatively cheap method of analysis, while the impressive number of papers published in recent years demonstrates the researchers' interest in improving the performances of this technique [2].

The absorption of light in UV/VIS takes place when the energy of the former is enough to produce an electronic transition which is associated with changes in the molecule's vibrational and rotational states. The spectra obtained through the absorption of these radiations are called electronic spectra [12].

The analysis and control of medicine frequently uses the concept of specific absorption also.This is noted with $A_{1cm}^{\%}$, which represents the absorbance of a 1 cm thick layer of solution that contains 1 g of substance in 100 ml. Specific absorbance is a constant specific to every substance (in a given wavelength) and is used in calculating the concentration of an analyte [2, 12].

The recording of spectra can be performed either by means of the graphic representation of absorbance depending on the wavelength or of the transmittance depending on the wavelength. This recording is performed automatically, the experimental data being recorded in a computer. Correlation of UV/VIS absorbance with structure is far more empirical, as many constituents have similar electronic spectra, consequently, for a complete identification, UV/VIS measurements have to be correlated with other types of spectral data and/or chemical tests [2, 12].

In Romanian flora, 11 species are present, of which *Hypericum perforatum*, St. John's wort, is the most famous [5, 15]. The others are: *H. acutum, H. hirsutum, H. elegans, H. maculatum*, the last three not being harvested. They differ from each other in: size, degree of lignification, stem, shape of leaves, size of flowers, presence or absence of secretor bags on leaves and so on [18].

Out of all *Hypericum* species, the most used and appreciated one in both traditional therapy and current phytotherapy is St. John's wort. The other species have been studied as well, but the purpose of this report is to determine whether the *Hypericum* species present in the spontaneous flora of the Bihor County meet the expectations of the European Pharmacopoeia regarding the content of total hypericin. In the European Pharmacopoeia the *St. John's wort* monograph specifies the exact content of hypericin of the vegetable product (determined through the spectophotometric method), of 0.08g% total hypericin [20]. This concentration is a minimal condition that must be met in order for the plant product to be used in therapy.

Nowadays *Hyperici herba* is used in phytotherapy, represented by the blossoming tops, dried after harvesting, of the *Hypericum perforatum* species. St. John's wort contains numerous active components, some of which have not been thoroughly researched in recent years. The following are among its most famous active principles: flavones, hypericin and hyperforin [7].

On the one hand, St. John's wort has been traditionally used for its cicatrizing, anti-ulcerous, anti-inflammatory properties, as well as for its choleretic and cholagogic effects. On the other hand, it is important to know that the most scientifically documented of its features is its anti-depressive action. Hypericin, flavones and especially hyperforin interact in the brain with the neurotransmitters that are related to depressive behavior: serotonin, dopamine, monoamines, GABA and so on [14, 18].

Most of the information contained in Romanian specialized literature refer to the chemical composition of the *Hyperici herba* plant product, even though it is mentioned that St. John's Wort can be mistaken for other species of the genus, the chemical composition of which is not yet known and which are not used in

phytotherapy, without indicating the reasons [11]. The *Hyperici herba* plant product has a complex chemical composition [1]. The compounds quantified in this study are:

•**Hypericins,** which are mainly represented by hypericin and pseudohypericin (an oxidized derivative of hypericin) present in dark glands in leaf and petal margins, stamens [3].

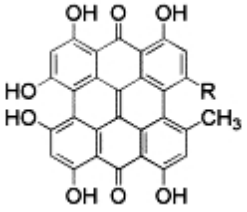


Figure 1. The chemical structure of hypericin (R= CH_3) and pseudohypericin (R= CH_2OH),

The concentration of hypericin in buds and flowers may vary from 0.06% to 0.75%, while concentrations lower than 0.01% may result from harvesting not only the blossomed tops, but also the lower parts of the plant, which is not allowed. A minimal content of 0.04% total hypericin is needed to ensure the pharmaceutical quality of the plant product. [7, 11].

•**Flavonoids** (4%–5%) in glycoside form, represented by: rutoside (rutin), hyperoside (hyperin), isoquercitroside, quercitroside and as free aglycones, such as: quercetol, kaempferol, but also bioflavonoids (I3, II8 biapigenin–0,26%, amentoflavones) [1]. They are localized in floral dehiscent leaves: sepals, stamens, petals, likely accumulation in vacuoles [8].

MATERIALS AND METHODS

The samples: the parts above the ground of 4 species of *Hypericum* harvested from the local spontaneous flora of the Bihor county (NW Romania), harvested between June and July 2008 (Table 2). The materials were dried and grounded to a fine powder (sieve VI-R.Ph) [19].

Table 1. Chemical structure of the main flavonoids present in the chemical composition of the *Hyperici herba* plant product.

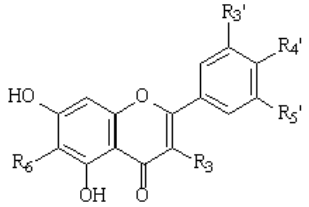
Chemical structure	Flavonols	R_3	R_6	R_3'	R_4'	R_5'
	Quercetol	OH	H	OH	OH	H
	Hyperoside	O-β-D-galactoside	H	OH	OH	H
	Isoquercetin	O-β-D-glucoside	H	OH	OH	H
	Quercitrin	O-β-D-rhamnoside	H	OH	OH	H
	Rutoside	O-β-D-rutinoside	H	OH	OH	H
	Kaempferol	OH	H	OH	H	H
	Miricetol	OH	H	OH	OH	OH

Table 2. The *Hypericum* species, data and stations where they were gathered.

Sample	Species	Data of harvest	Station
1.	*H. perforatum* L.	07.06.2008	Tileagd
2.	*H. tetrapterum* Fr.	13.07.2008	Pietroasa
3.	*H. maculatum* Crantz *ssp. immaculatum*	13.07.2008	Stâna de Vale
4.	*H. maculatum* Crantz *ssp. typicum*	13.07.2008	Stâna de Vale
5.	*H. hirsutum*	07.07.2008	Vadu Crişului

Extractive solutions 1% in methanol were used, obtained through the reflux method in water boiling for 30 minutes, in flasks with reflux coolers.

The quantitative determination of flavonoids was made by spectrophotometric method from three different probe and the total flavonoids were expressed in rutoside (%g/g) [19].

The 10th edition of the Romanian Pharmacopoeia makes official, in the *Cynarae folium* monograph, a spectrophotometric method of dosing flavonoids, following the latter's reaction with aluminum chloride. The reading of the samples is performed at 430 mm. The concentration of flavonoids of the analyzed sample is calculated using a standard curve made at the same time and in the same conditions as the sample solution, while the results are expressed in rutoside (mg %) [19].

Numerous methods have been conceived for dosing flavones, either free or as glycosides, pure or from various vegetable plants or pharmaceutical products. In this respect, all the physicochemical properties of flavones that lend themselves to creating a method of dosing have been employed.

In 1 g of St. John's wort powder (VI) 100 ml of alcohol water in reflux is added for 30 minutes. The hot solution is filtered through wadding in a volumetric flask and, after cooling, it is filled up to 100 ml, by washing the residue with the same solvent (mixture A) 10 ml mixture A is diluted with methanol (R) in 25 ml in a volumetric flask. It is shaken for 2-3 minutes and left to rest for 10 minutes. It is then filtered and the first portions of filtering are removed. To 5 ml of filtering 5 ml of sodium acetate 100 g/l (R) and 3 ml of aluminum chloride 25 g/l (R) are added, everything is shaken and completed with methanol (R) to 25 ml in a volumetric flask (sample-solution). After 15 minutes, if necessary, it is filled up again with methanol (R) to 25 ml and the absorbance of the solution is determined at 430 mm, using as compensation liquid a solution obtained in the

same conditions as the sample-solution, from 5 ml filtering, 8 ml water and methanol (R) to 25 ml in a volumetric flask [19].

The concentration of flavonoids of the analyzed sample is calculated with a standard curve established at the same time and in the same conditions as the sample-solution, working with: 1, 2, 3, 4 ml of standard-solution of rutoside 0.1 g/l in methanol (R), 5 ml of sodium acetate 100 g/l (R), 3 ml aluminum chloride 25 g/l (R) and methanol (R) to 25 ml, in each volumetric flask. Compensation liquid is used, this solution being obtained in the same conditions as the sample-solution, from 8 ml water and methanol (R) to 25 ml in a volumetric flask [19].

The content of total hypericins was determined by spectrophotometric method, from three different probe and expressed in hypericin (%g/g) [20].

0.100 g of powdered *Hyperici herba* is extracted for 30 minutes in boiling water to reflux with 100 ml of methanol in a flask with reflux coolers. After cooling, it is filtered and brought to 100 ml with methanol in a volumetric flask. The extinction of the solution is read at a 587 nm wavelength, in a 1 cm tub, compared to the methanol. At this amount of absorbantion, in *Hypericum* sp., do not absod the light another substantion, the hypericins are the only active principle that absod the light at 587 nm.

The dosage of hypericin was established with the aid of absorbance measured at 587 nm, the quantity being calculated based on the formula for specific absorbance:

$$Hipg\% = \frac{A}{780} \cdot \frac{100}{m}$$

where: A = the measured absorbance

m = grams of vegetable product in 100 ml of extract.

780= specific absorbance of hypericine at 587nm.

Statistic analysis was performed using Windows2007 Excel, using the functions for the calculation of standard deviation.

RESULTS

The results of the spectrophotometric dosages along with the standard deviation are presented in table 3 and they refer to the total of flavonoids, expressed in rutoside.

Table 3. The content of flavonoids of *Hypericum* species (g% in rutoside).

Sample	Species	Concentration of flavonoids in rutoside g% (x±e.s)
1.	*H. perforatum* L.	3.814±0.25
2.	*H. maculatum* Crantz *ssp. Immaculatum*	5.913±0.37
3.	*H. maculatum* Crantz *ssp. typicum*	6.418±0.26
4.	*H. tetrapterum* L.	2.981±0.16
5.	*H. hirsutum* L.	5.678±0.30

Table 4 presents the concentrations of total hypericins (g%) of the 4 species and 2 subspecies of *Hypericum* chosen for study.

Table 4. Concentrations of total hypericins determined in samples of *Hypericum* sp.

Sample	Species	Concentration of total hypericins g% (x±e.s)
1.	*H. perforatum* L.	0.163±0.012
2.	*H. maculatum* Crantz *ssp. immaculatum*	0.211±0.011
3.	*H. maculatum* Crantz *ssp. typicum*	0.496±0.014
4.	*H. tetrapterum* L.	0.189±0.015
5.	*H. hirsutum* L.	0.096±0.013

DISCUSSIONS

Hypericum species are characterized by a high concentration of flavone compounds. Of all the studied *Hypericum* species, the ones that come from alpine regions (the Bihor Mountains, 1000 m altitude), *H. maculatum* have the highest content of flavonoids. The highest content of flavonoids was determined in *H. maculatum,* both subspecies followed by *H. hirsutum* L. The spectrophotometric method of dosage is advantageous in the case of flavones, because it expresses the global content of flavonoids, including those that cannot be identified through other methods.

The quantitative determination of total hypericin shows a variation in concentration from 0.096 g% for *H. hirsutum* to 0,496 g% for *H. maculatum ssp. typicum.*

The low content of hypericin of *H. hirsutum* could be correlated with the presence of hypericin secreting glands only on the fimbriae of the sepals, not on the petals and also with the low content of flowers in the blossoms of this species. This species lacks hypericin secreting tissue, both on the stems and on the leaves, these organs presenting only simple tector trichomes.

The hypericin content of *H. maculatum ssp. immaculatum* is 57.46 % lower that that of *H. maculatum ssp.typicum*, a result that must be correlated with the histological structure of the petals of these two subspecies. Thus the black secretory channels of *H. maculatum ssp.immaculatum*, are either missing or very few in number, whereas in the case of *H. maculatum ssp. typicum*, these channels that appear in the form of spread out linear black colored spots are very numerous on the petals, being apparent especially on the external side, when the flower is budding.

The high density of such hypericin secretory channels in the *H. maculatum ssp. typicum* species may explain the significantly higher content of hypericin of this species compared to *H. perforatum* (0.496 g% / 0.163 g%), the latter having much smaller hypericin secreting black glands, located only on the superior edge of the petals. Consequently, although the *H. maculatum* species presents a smaller number of flowers/plant compared to *H. perforatum* and therefore the proportion of flowers of the “herba” product is lower, this fact is compensated by the higher density of

secreting tissues located on the petals of the *H. maculatum* species.

The analysis of the obtained results shows that all the studied *Hypericum* species meet the standards of the E. Ph.

The results of some studies performed on various *Hypericum* species gathered from the spontaneous flora and which employed the spectrophotometric method of determining hypericin and total flavones are presented in table 5.

Table 5. Amount of hypericin and flavonoids in the vegetative parts of *Hypericum sp*. harvested from spontaneous flora of different geographical origins.

Place of harvesting / Species	Concentration (%)		Method
	Total hypericins	Flavonoids	
Spontaneous flora Sibiu and Valcea Counties *H. maculatum*	0.083	4.24	UV/VIS [17]
Spontaneous flora Sibiu and Valcea Counties *H. perforatum*	0.025-0.039	1.09-2.72	UV/VIS [17]
Spontaneous flora Alba County *H. maculatum*	0.37	5.30	UV/VIS [13]
Spontaneous flora Brasov County *H. maculatum*	0.46	5.50	UV/VIS [13]
Spontaneous flora Cluj County *H. perforatum*	0.18	6.80	UV/VIS [13]
Spontaneous flora Salaj County *H. perforatum*	0.23	4.20	UV/VIS [13]
Spontaneous flora Bulgaria *H. perforatum*	0.125	-	UV/VIS [9]
Spontaneous flora Turkey *H. perforatum*	0.215-0.246	-	UV/VIS [4]
The minimal content of total hypericin expressed by the E. Ph. 0.08% [20]			

As to the content of hypericin and flavones of the officinal product *Hyperici herba*, there are only two studies in Romania. Quantitative data from different authors vary significantly even if the methods are similar [3, 10]. The study making in Romanian, Bulgaria and Turkey used an officinal method in the European Pharmacopoeia. The study have shown that for *Hypericum sp.* the chemical profile defining the quality of flowering tops is dependent upon the plant development stage upon harvest but it does not seem to be influenced by the soil type or the age of the culture [6, 14, 16].

In the *H. perforatum* harvested from the spontaneous flora of Bulgaria, the content of hypericin is 0.125%, below the value reached by the same species in the Bihor County, that is 0.163%. In the *H. perforatum* harvested from the spontaneous flora found in Turkey, the content of hypericin ranges between 0.215-0.246%, a superior value to the one reached in the Bihor County, that is 0.163%.

The *H. maculatum* and *H. perforatum* species were the most analyzed regarding content of hypericin and flavones, using the spectrophotometric technique. The values obtained in the present study are in accordance with the data found in specialized literature, which show that *H. maculatum* has a higher hypericin content than *H. perforatum*; nevertheless all the analyzed species, including *H. tetrapterum* and *H. hirsutum* fit within the limits imposed by the E.Ph. As to the content of flavones, it is higher in the *H. perforatum* harvested from the spontaneous flora of the Cluj County and in the *H. maculatum* found in the Bihor County.

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