Pseudohypericin and Hyperforin in *Hypericum perforatum* from the Northern of Turkey: Variation among Populations, Plant Parts and Phenological Stages

Running title: Pseudohypericin and Hyperforin in *Hypericum perforatum*

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Abstract

*Hypericum perforatum* is a perennial medicinal known as “St. John’s wort” in Western Europe and has been used in the treatment of several diseases for centuries. In the present study, morphologic, phenologic and population variability in pseudohypericin and hyperforin concentrations among *H. perforatum* populations from Northern Turkey was investigated for the first time. The aerial parts of *H. perforatum* plants representing a total of 30 individuals were collected at full flowering from 10 sites of Northern Turkey to search the regional variation in the secondary metabolite concentrations. For morphologic and phenologic sampling, plants from one site were gathered in five phenological stages: vegetative, floral budding, full flowering, fresh fruiting and mature fruiting. The plant materials were air-dried at room temperature and subsequently assayed for chemical concentrations by HPLC. Secondary metabolite concentrations ranged from traces to 2.94 mg g\(^{-1}\) DW for pseudohypericin and traces–6.29 mg g\(^{-1}\) DW for hyperforin. The differences in the secondary metabolite concentrations among populations of *H. perforatum* were found to be significant. The populations varied greatly in hyperforin concentrations whereas they produced similar amount of pseudohypericin. Concentration of both secondary metabolites in all tissues increased with advancing of plant development and higher accumulation levels were reached at flowering. Among different tissues, full opened flowers were found to be superior to stems, leaves and the other reproductive parts with regard to pseudohypericin and hyperforin accumulations. The present findings might be useful to optimize the processing methodology of wild-harvested plant material and obtain increased concentration of these secondary metabolites.
Key words: *Hypericum perforatum*, hyperforin, pseudohypericin, morphologic and phenologic variations, population variability.

*Hypericum perforatum* L. (St. John’s wort) is a well-known traditional medicinal plant that has been used for centuries for the treatment of several diseases, such as skin lesions, eczema, burns and microbial, inflammatory and psychological disorders (Sanchez-Mateo *et al.* 2002). The crude extract of *H. perforatum* is now widely used in Europe as a drug for the treatment of depression. Proven photodynamic, antiviral, antiretroviral, and antitumoral activities of *Hypericum* extracts also suggest using of this plant in Acquired Immuno Deficiency Syndrome (AIDS) and cancer treatments (Vlietinck *et al.* 1998; Guedes and Eriksson 2005).

*H. perforatum* contains a number of secondary metabolites from different classes namely naphthodianthrones, phloroglucinols, flavonoids, phenylpropanes, essential oils, amino acids, xanthenes, tannins, procyanidins and other water-soluble components (Greeson *et al.* 2001). Although hypericin has been paid major attention in pharmaceutical research, the principal naphthodianthrone in *Hypericum* extracts is pseudohypericin. It has been reported to be found two to three times more abundant than hypericin in the species of *Hypericum* containing them (Cameron and Reverty 1976). Hyperforin is the major secondary metabolite occurring in concentrations of 2–4% of the total extract of *H. perforatum* (Medina *et al.* 2006). Results from recent studies have indicated hyperforin as the main secondary metabolite, responsible for antidepressant effects of *Hypericum* extracts (Roz and Rehavi 2004). It also exhibits anti-inflammatory (Feisst and Werz 2004), antitumoral (Schwarz *et al.* 2003) and antiangiogenic (Dona *et al.* 2004) effects.
Variations in the level of secondary metabolites in populations of *Hypericum* plants have an important impact on the pharmacological activity of tested extracts, and these variations in chemistry could indicate different genotypic populations (Bergonzi *et al.* 2001). However, detecting genotypic variation in the concentration of secondary metabolites is not sufficient to make a substantial conclusion unless other sources of variation are clarified (Walker *et al.* 2001). Because, variation in secondary metabolite concentrations among accessions of *H. perforatum* may be influenced not only by variance of genotype but also by other factors such as morphologic composition of plant material and the phenological stage in which plants harvested (Sirvent *et al.* 2002; Martonfi *et al.* 2006). In our previous studies, we documented morphologic, phenologic and population variability of hypericin and several flavonoids (Çirak *et al.* 2007a, b) in *H. perforatum* growing in Turkey. In the present study, the same variability in pseudohypericin and hyperforin concentrations among *H. perforatum* populations from Northern Turkey was investigated for the first time.

**Results**

The differences in pseudohypericin and hyperforin concentrations among populations of *H. perforatum* were found to be significant (P<0.01). In general, all populations produced similar amount of pseudohypericin. Higher level of pseudohypericin accumulation was observed in Bafra, Hacıköy and Sarıgazi populations (1.71, 1.69 and 1.67 mg g⁻¹ DW, respectively). On the contrary, the populations varied greatly in hyperforin concentration. Plants from Samsun population produced the highest level of hyperforin (5.72 mg g⁻¹ DW) followed by plants from Bafra and Sarıgazi populations (4.95 and 4.50 mg g⁻¹ DW, respectively) while much
lower of hyperforin accumulation was observed in Merzifon, Karadağ and Yeniköy populations (0.66, 1.36 and 1.81 mg g⁻¹ DW, respectively) (Table 1).

Ontogenetic changes in pseudohypericin and hyperforin concentrations of plant tissues were also found to be significant (P< 0.01). Concentrations of both secondary metabolites increased during the course of ontogenesis and the highest levels were reached during floral development.

Among different tissues, full opened flowers were found to be significantly superior to stems, leaves and the other reproductive parts with regard to pseudohypericin and hyperforin accumulations (P< 0.01). In particular, stems produced much lower amount of pseudohypericin and hyperforin when compared to other tissues. Change in pseudohypericin and hyperforin concentrations of leaves and reproductive parts during the course of plant development followed the same trends. The concentration of both secondary metabolites in those tissues increased with advancing of plant phenology and after reaching the highest level at full flowering stage (2.94 mg g⁻¹ DW pseudohypericin and 6.29 mg g⁻¹ DW hyperforin for flowers; 1.00 mg g⁻¹ DW pseudohypericin and 1.79 mg g⁻¹ DW hyperforin for leaves) their concentrations decreased during fruit development (Fig. 1).

**Discussion**

The populations of *H. perforatum* examined in the present study were located in different parts of Northern Turkey and the growing sites of these populations were differed from each other by climatic and geographic factors (Table 2). As a result of these environmental differences, each site has specific ecological conditions and habitat.
populations may be attributed to the different environmental conditions of sampling sites. Because, environmental factors e.g. climate, topography, insect populations etc. are thought to affect or modulate the production or yield of secondary metabolites from different classes (Upton et al. 1997; Kuth and Spreemann 1998; Sirvent 2001).

However, the present findings also indicate a significant genetic difference/similarity among the populations. For example, Bafra and Sarıgazi populations are separated by a distance of 800 km and have substantially different environment (mean temperature, precipitation, elevation etc.). Hence, they should represent distinct populations. However, both populations produced similar amount of pseudohypericin and hyperforin. On the contrary, although Gümüş and Yeniköy populations are separated by only 4 km and have very similar environment, 1.75 and 2.1 fold differences were detected between those populations in the concentrations of pseudohypericin and hyperforin, respectively. Similar regional differences in the concentrations of hypericins and hyperforin were previously reported for *H. perforatum* populations from USA (Walker et al. 2001; Sirvent et al. 2002), Armenia (Kirakosyan et al. 2003) and Australia (Southwell and Campbell 1991).

Secondary metabolite composition of a medicinal plant may vary substantially with the developmental stage of the plants. This is especially true for the genus *Hypericum* (Seidler-Lozykowska, 2003). Therefore, investigations on ontogenetic variation of secondary metabolites from different classes have received considerable interest from plant scientists over several decades. In particular, growth and development of the reproductive parts in *Hypericum* plants is generally followed by acceleration of secondary metabolism resulting in enhanced accumulation of different secondary metabolites such as hypericin, rutin, quercetin, isoquercetin, hyperoside in *H.*
perforatum (Kazlauskas and Bagdonaitė 2004; Çirak et al. 2007b), H. brasiiliense (Abreu et al. 2004), H. maculatum (Martonfi et al. 2006), total phenolics in H. perforatum, H. pruinatum and H. aviculariifolium (Ayan et al. 2006) and hyperforin in H. perforatum (Büter and Büter 2002; Couceiro et al. 2006). Our findings in the present study confirmed this phenomenon. Pseudohypericin and hyperforin contents in stems, leaves and reproductive parts of H. perforatum increased during plant growth and the highest level of both secondary metabolites were reached at full flowering. Similar results regarding the variation of secondary metabolites in H. perforatum were also reported by Repcak and Martonfi (1997).

Tissue-dependence of secondary metabolites is very common among medicinal plants. In the present study, floral parts had the highest level of pseudohypericin and hyperforin. Likewise, in all earlier reports the floral parts had the highest concentration of hypericins in H. perforatum (Southwell and Campbell 1991; Sirvent et al. 2002) as well as H. maculatum (Radusiene et al. 2004). Reproductive parts were also reported as the main storage organs for hyperforin accumulation in H. perforatum (Büter and Büter 2002). H. perforatum is characterized by the presence of secretory structures, including light glands, dark glands and secretory canals, in which biologically active secondary metabolites are synthesized and/or accumulated (Ciccarelli et al. 2001). For example, hypericins are produced in the dark glands, and the occurrence of dark glands in an organ is regarded as a reliable indicator of the presence of hypericins in a given species (Robson 1981). The localisation of the various types of secretory structures varies among plant tissues and flowers with leaves are the main organs for dark glands and secretory canals. The levels of secondary metabolites present in a particular Hypericum tissue depend on the relative abundance of these secretory structures on the harvested
material. Hence, high level of pseudohypericin and hyperforin accumulations in reproductive parts of *H. perforatum* observed in the present study may be attributed to relative abundance of the secretory structures on those tissues.

Previous literature reported pseudohypericin concentrations in *H. perforatum* between 0.07 and 4.27 mg g\(^{-1}\) DW from different accessions of the world (Walker *et al.* 2001; Sirvent *et al.* 2002). Hyperforin concentrations in *H. perforatum* were reported as 8.35-150 mg g\(^{-1}\) DW by Kirakosyan *et al.* (2003) and Maggi *et al.* (2004). For our plant material, pseudohypericin concentration ranged from traces to 2.94 mg g\(^{-1}\) DW and hyperforin from traces to 6.29 mg g\(^{-1}\) DW depending on the populations and ontogenetic and morphogenetic sampling. Thus, comparing the results obtained for our plant material with those reported by named authors it was revealed that relatively low concentrations of the secondary metabolites partly corresponded.

To conclude, results of the present study indicate a significant variation in the content of pseudohypericin and hyperforin in *H. perforatum* from Turkish populations. Regional distribution of this medicinal plant may be an important source of chemical variation and should be considered while optimizing the processing methodology of wild-harvested plant material. The present results also indicate a close relationship between pseudohypericin and hyperforin content of plant parts and development stage during phenological cycle of this species. Thus, the present findings might also be useful to obtain increased concentration of these secondary metabolites.

**Materials and Methods**

**Experimental Procedures**
The aerial parts of *H. perforatum* plants representing a total of 30 individuals were collected at full flowering from 10 sites of Northern Turkey to search the regional variation of pseudohypericin and hyperforin (Table 2). For morphologic and phenologic sampling, *H. perforatum* plants were collected from Samsun site in April-September period of 2005. The plant material including stem, leaf and reproductive parts represented 30 randomly gathered plants in five phenological stages: vegetative, floral budding full flowering, fresh fruiting and mature fruiting. The sampling sites were not grazed or mown during the period when the plants were gathered. The top of 2/3 plants was harvested between 11:00 am and 13:00 pm. Conditions on the day of collection were clear and sunny at all sites. Temperatures ranged from 24 to 35 °C. The plant materials were air-dried at room temperature (20 ±2 °C), and subsequently assayed for secondary metabolite concentrations by HPLC.

**Preparation of Plant Extracts**

Samples of 0.5–1.0 g air-dried plant material with moisture content of 10.0% were mechanically ground to obtain a homogenous drug powder and extracted with 96% EtOH (50 mL) for 72 h, at room temperature. The prepared extracts were kept in dark in a refrigerator until used. Before HPLC separation extracts were filtered through a membrane filter with pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany).

**HPLC Analysis**

HPLC analysis with UV/PDA detection was performed using a model Waters 2690 chromatography system (Waters, Milford, USA), equipped with a Waters 2487 UV/Vis detector and Waters 996 PDA detector. For separation a Hichrom column Hypersil H5ODS-150A 150×4.6 mm (Hichrom Limited, UK) and a H5ODS-10C guard-precolumn were used.
The hyperforin elution program was isocratic. Solvent system of mobile phase consists of 25% water containing 0.1% trifluoroacetic acid (TFA) and 75% acetonitrile containing 0.1% TFA. The flow rate was kept at 1.5 mL/min. The detector monitored the eluted components at 270 nm, depending on $\lambda_{\text{max}}$ of PDA spectrum of hyperforin. Ten micro liters of the samples were injected. The eluted hyperforin was identified on the basis of the retention time by comparison with retention time of reference standard. Identity of constituent was also confirmed with PDA detector by comparison with UV spectra’s of reference standard at the wavelength range 190-400 nm. PDA spectra’s of 5 points of hyperforin peak are equal and demonstrate purity of peak. The content of hyperforin was calculated from an external standard calibration in the concentration range of 30.0–150.0 µg/mL. Characteristics of hyperforin calibration curve are linear regression equation ($y=4.69 \times 10^3 X + 2.17 \times 10^2$) and linear correlation ($r^2=0.999$).

Pseudohypericin was analyzed according to a modified HPLC method described in Pharmeuropa (2004). The elution program was isocratic. The mobile phase consisted of ethyl acetate/15.6 g/L, solution of sodium dihydrogen phosphate NaH$_2$PO$_4$ and methanol (39:41:160). The flow rate: 1.0 mL/min; injection volume: 10 µL. The column temperature was at 20 °C. The elution was monitored at 590 nm and the obtained data were compared with pseudohypericin standard sample. The quantity of compound was calculated from an external standard calibration in the concentration range of 0.5–100.0 µg/mL ($r^2=0.997$). Each sample was analyzed twice and the mean value was used for calculation.

All solvents and standards of reference substances were of HPLC grade and purchased from Roth Chemical Company (Karlsruhe, Germany).

Data Analysis
Pseudohypericin and hyperforin content of plant materials was given for each accession and phenological stage. Data for secondary metabolite concentrations of different plant tissues and accessions were objected to one-way analysis of variance (ANOVA) separately and significant differences among mean values were tested with the Duncan Multiple Range Test (P<0.01) using MSTAT statistical software.

Acknowledgement

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References


Büter KB, Büter B (2002). Ontogenetic variation regarding hypericin and hyperforin levels in four accessions of Hypericum perforatum L. J Herbs Spices Med Plants 9, 95-100.


Table 1. Pseudohypericin and hyperforin concentrations of *Hypericum perforatum* populations located at Northern Turkey.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Pseudohypericin (mg/g DW)</th>
<th>Hyperforin (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gümüş</td>
<td>1,47 b*</td>
<td>3,80 c</td>
</tr>
<tr>
<td>Sarıgazi</td>
<td>1,67 a</td>
<td>4,50 b</td>
</tr>
<tr>
<td>Bafra</td>
<td>1,71 a</td>
<td>4,95 b</td>
</tr>
<tr>
<td>Osmancık</td>
<td>0,93 c</td>
<td>2,12 d</td>
</tr>
<tr>
<td>Hacıköy</td>
<td>1,69 a</td>
<td>3,37 c</td>
</tr>
<tr>
<td>Merzifon</td>
<td>0,72 d</td>
<td>0,66 f</td>
</tr>
<tr>
<td>Karadağ</td>
<td>1,30 b</td>
<td>1,36 e</td>
</tr>
<tr>
<td>Havza</td>
<td>1,04 c</td>
<td>2,53 d</td>
</tr>
<tr>
<td>Samsun</td>
<td>1,30 b</td>
<td>5,72 a</td>
</tr>
<tr>
<td>Yeniköy</td>
<td>0,84 c</td>
<td>1,81 e</td>
</tr>
</tbody>
</table>

*Values followed by different small letters in each column are significantly different (P<0.01) according to Duncan Multiple Range test.*
Table 2. Geographical data and seasonal climatic conditions of the growing localities of *Hypericum perforatum* from Northern Turkey

<table>
<thead>
<tr>
<th>Sites</th>
<th>Collection date</th>
<th>Voucher no.</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Elevation (m)</th>
<th>Mean temperature (°C)</th>
<th>Precipitation (mm)</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gümüş</td>
<td>June 16, 2005</td>
<td>OMUZF # 61/2</td>
<td>40° 52’</td>
<td>35° 14’</td>
<td>785</td>
<td>13.15</td>
<td>435</td>
<td>Rocky and open slopes</td>
</tr>
<tr>
<td>Sarığazi</td>
<td>June 10, 2005</td>
<td>OMUZF # 61/3</td>
<td>41° 04’</td>
<td>20° 06’</td>
<td>150</td>
<td>11.12</td>
<td>819</td>
<td>Pasturelands</td>
</tr>
<tr>
<td>Bafra</td>
<td>June 14, 2005</td>
<td>OMUZF # 61/4</td>
<td>41° 03’</td>
<td>35° 57’</td>
<td>15</td>
<td>12.27</td>
<td>720</td>
<td>Pasturelands</td>
</tr>
<tr>
<td>Osmancık</td>
<td>June 18, 2005</td>
<td>OMUZF # 61/5</td>
<td>40° 58’</td>
<td>34° 48’</td>
<td>340</td>
<td>10.15</td>
<td>343</td>
<td>Pasturelands</td>
</tr>
<tr>
<td>Hacıköy</td>
<td>June 16, 2005</td>
<td>OMUZF # 61/6</td>
<td>40° 53’</td>
<td>35° 14’</td>
<td>750</td>
<td>13.24</td>
<td>450</td>
<td>Wet environment</td>
</tr>
<tr>
<td>Merzifon</td>
<td>June 15, 2005</td>
<td>OMUZF # 61/7</td>
<td>40° 51’</td>
<td>35° 29’</td>
<td>750</td>
<td>14.11</td>
<td>470</td>
<td><em>Pinus</em> woodland</td>
</tr>
<tr>
<td>Karadağ</td>
<td>June 15, 2005</td>
<td>OMUZF # 61/8</td>
<td>41° 04’</td>
<td>36° 01’</td>
<td>970</td>
<td>11.10</td>
<td>735</td>
<td>Arid pasturelands</td>
</tr>
<tr>
<td>Havza</td>
<td>June 15, 2005</td>
<td>OMUZF # 61/9</td>
<td>40° 58’</td>
<td>35° 40’</td>
<td>600</td>
<td>12.11</td>
<td>720</td>
<td>Arid pasturelands</td>
</tr>
<tr>
<td>Samsun</td>
<td>June 20, 2005</td>
<td>OMUZF # 61/10</td>
<td>41° 35’</td>
<td>35° 56’</td>
<td>195</td>
<td>14.80</td>
<td>782</td>
<td><em>Quercus</em> woodland</td>
</tr>
<tr>
<td>Yeniköy</td>
<td>June 16, 2005</td>
<td>OMUZF # 61/11</td>
<td>40° 47’</td>
<td>35° 03’</td>
<td>780</td>
<td>13.11</td>
<td>435</td>
<td>Arid pasturelands</td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1: Pseudohypericin (a) and hyperforin (b) concentrations of stem, leaf and reproductive tissues of *Hypericum perforatum* at different stages of plant development (Values with different small letters-a, b, c-within columns for each development stage differ significantly at the level of $P<0.01$; Bars are ± s.e.).