

## Regular Article

## Chemical composition of *Hypericum pruinautum* Boiss. and Bal. from wild populations of Northern Turkey

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The present study was conducted to determine the variation in the content of several plant chemicals, namely hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercetine, kaempferol, quercitrine and quercetine among five *Hypericum pruinautum* Boiss. & Bal. populations from Northern Turkey. The aerial parts representing a total of 30 shoots were collected at full flowering. After dried at room temperature, they were assayed for the chemicals by HPLC and the presence of isoquercetine and kaempferol in this species was reported by us for the first time. The populations varied significantly in chemical contents. Plants from Ladik population produced the highest amount of hypericin, chlorogenic acid, hyperoside, isoquercetine, quercitrine and quercetine. Hyperforin and rutin of whole shoots reached the highest level in Cankiri population. The chemical variation among the populations and plant parts was discussed as possible results of different genetic and environmental factors.

**Keywords:** Hyperforin; hypericins; flavonoids, HPLC, *Hypericum pruinautum*; population variability.

The genus *Hypericum* L. comprises more than 450 species in 36 sections with worldwide distribution in warm temperate, subtropical and mountainous tropical regions (Robson, 2001). The genus is represented in Turkey by 89 species of which 43 are endemic. *Hypericum pruinautum* Boiss. & Bal. is one of the species which grows naturally in igneous slopes and rock ledges at high altitudes (1500-2500 m sea level) of Turkey (Davis, 1988). Without specializing any one, all species of *Hypericum* from Turkish flora have traditionally been used as sedatives, antiseptics, and antispasmodics in Turkish folk medicine under the names "kantaron, peygamber çiçeği, kılıçotu, kanotu, kuzukıran and binbirdelik otu".

*Hypericum* extracts contain a complex mixture of bioactive substances, mainly the hyperforins, the hypericins and the flavonoids which possess a wide array of biological properties. The hypericins are the main chemicals of *Hypericum* extracts exhibiting photodynamic, antidepressive and antiviral activities (Bombardelli and Morazzoni, 1995). Hyperforin is a prenylated phloroglucinol derivative that consists of a phloroglucinol skeleton with lipophilic isoprene chains. Results from recent studies have indicated hyperforin as the main chemical, responsible for antidepressant effects of *Hypericum* extracts (Medina *et al.*, 2006). Flavonoids are a group of bioactive compounds present in *Hypericum* plants. They play an important role in preventing cardiovascular diseases and several kinds of cancer (Chu *et al.*, 2000). Due to these reasons, many individual or groups of *Hypericum*

species have been investigated for the presence of these secondary metabolites to date (Kirakosyan et al., 2003; Abreu et al., 2004; Martonfi et al., 2006; Cirak and Radusiene, 2007).

In previous studies, *H. pruinatum* was reported to contain hypericin (Cirak et al., 2006), hyperforin (Smelcerovic et al., 2008) organic acids and several flavonoids (Cirak et al., 2007). However, no report is available on the chemical variation among wild populations of this species. In the present study, we report our phytochemical investigations of *H. pruinatum* populations sampled from five growing localities of Northern Turkey. Besides, isoquercetine and kaempferol have not yet been detected in this species. Here, we also describe the first occurrence of the chemicals in *H. pruinatum*.

## Materials and Methods

### Plant material

The plant materials were described in our previous study (Cirak et al. 2006). The species were identified by Dr. Hasan Korkmaz, Faculty of Science and Art, Department of Biology, University of Ondokuz Mayıs, Samsun-Turkey.

### Experimental procedures

The aerial parts of *H. pruinatum* plants representing a total of 30 shoots were collected at full flowering from four sites of Northern Turkey (Table 1). The sampling sites were not grazed or mown during the plant gathering period. The top of 2/3 plants was harvested between 11:00 am and 01:00 pm. Conditions on the day of collection were clear and sunny at all sites. Temperatures ranged from 26 to 32 °C. After collected, the plant materials were dried at room temperature (20 ±2 °C), and subsequently assayed for chemical contents by HPLC.

**Table 1. Geographical data and seasonal climatic conditions of *Hypericum pruinatum* growing localities from Northern Turkey and voucher specimen numbers for the wild populations**

Sites	Collection date	Voucher no.	Latitude (N)	Longitude (E)	Elevation (m)	Mean temperature (°C)	Precipitation (mm)	Habitat
Gumus	July 14, 2010	BMYO # 2/1	40° 52'	35° 14'	785	13.15	435	Rocky and open slopes
Cankiri	July 18, 2010	BMYO # 2/2	40° 59'	34° 47'	750	10.15	343	Rocky and open slopes
Ladik	July 19, 2010	BMYO # 2/3	41° 04'	36° 01'	970	11.10	735	Arid pasturelands
Yenikoy	July 14, 2010	BMYO # 2/4	40° 47'	35° 03'	780	13.11	435	Arid pasturelands
Bafra	July 11, 2010	BMYO # 2/5	41° 34'	35° 57'	200	14.80	782	<i>Quercus</i> woodland

### Preparation of plant extracts

Air-dried plant material was mechanically ground with a laboratory mill to obtain a homogenous drug powder. Samples of about 0.5 g (weighed with 0.0001 g precision) were extracted in 50 ml of 100 % methanol by ultrasonication at 40° C for 30 min. in a Sonorex Super model RK 225H ultrasonic bath. The prepared extracts were filtered through a membrane filter with pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator until analysis no longer than 3 hours. The extracts for naphthodianthrones analysis after ultrasonication were exposure to light for 30 min. due to the photoconversion of protohypericin into hypericin and protopseudohypericin into pseudohypericin.

### HPLC analysis

A Shimadzu Prominence LC-20A (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with two LC-20AD model pumps, a SIL-20AC auto-

injector, a thermostat CTO-20AC and a SPD-M20A detector was used for HPLC analysis. Separation of all compounds was carried out using an YMC Pack Pro-C18 (YMC Europe GmbH, Dinslaken, Germany) column (150 mm x 4 mm i.d.; 3  $\mu$ m particle sizes) with 10 mm guard-precolumn. The mobile phase consists of solvent A (water containing 0.1 % trifluoroacetic acid (TFA)) and solvent B (acetonitrile containing 0.1 % TFA). The following binary gradient elution program was used: 0-1 min (B 5 $\rightarrow$ 5%), 1-14 min (B 5 $\rightarrow$ 20%), 14-20 min (B 20 $\rightarrow$ 80 %), 20-30 min (B 80 $\rightarrow$ 100 %), 30-39 min (B 100 $\rightarrow$ 100 %), 39-39.5 min (B 100 $\rightarrow$ 5 %), 39.5-45 min (B 5-5 %). The mobile phase was delivered with a flow rate of 1.0 mL min<sup>-1</sup>; volume of extract injected was 10  $\mu$ L. Detection was performed at 210-790 nm wave length range with a constant column temperature at 40° C. The eluted compounds were identified on the basis of their retention time by comparison with retention time of reference standards and also confirmed with UV spectra's of reference standards in the wavelength range from 210 to 790 nm.

The hypericin and pseudohypericin elution program was isocratic. The mobile phase consists of acetonitrile containing 0.1% TFA. Flow rate of mobile phase was 1.1 mL·min<sup>-1</sup>. Ten micro liters of extracts were injected. Detection was recorded at 210-790 nm wave length range with a constant column temperature at 40° C.

The quantification of detected compounds was achieved by using external standard method at the maximal absorption on the UV spectra of corresponding compounds: chlorogenic acid - 325 nm, rutin - 353 nm, hyperoside - 353 nm, isoquercetrine - 353, kaempferol, - 346 nm, quercetrine - 347 nm, quercetine - 368 nm, hyperforin - 270 nm, hypericin and pseudohypericin - 580 nm wavelength. A six-point calibration curves were obtained with pure standards dissolved in MeOH in the concentration range of 0.2-110  $\mu$ g/ml. All calibration cures showed good linear regression ( $r^2 > 0.999$ ) within the test range. Typical HPLC chromatograms of *Hypericum pruinatum* extract are shown in Fig. 1 and 2. All solvents and standards of reference substances were of HPLC grade and purchased from Roth Chemical Company (Karlsruhe, Germany).

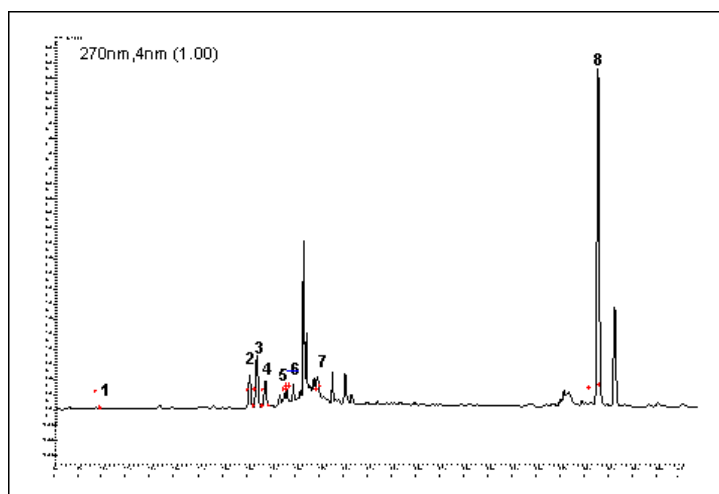


Figure 1. HPLC chromatogram of *Hypericum pruinatum* extract detected by UV at 270 nm wave length. Peak identified: 1 - chlorogenic acid (retation time ( $t_R$ ) - 9.62 min.), 2 - rutin ( $t_R$  -15.84 min.), 3 - hyperoside ( $t_R$  - 16.13 min.), 4 - isoquercetine ( $t_R$  -16.47 min.), 5 - kaempferol ( $t_R$ -17.26 min.), 6 - quercetrine ( $t_R$ -17.35 min.), 7 - quercetine - ( $t_R$ -18.61 min.), 8 - hyperforin ( $t_R$ -29.99 min.).

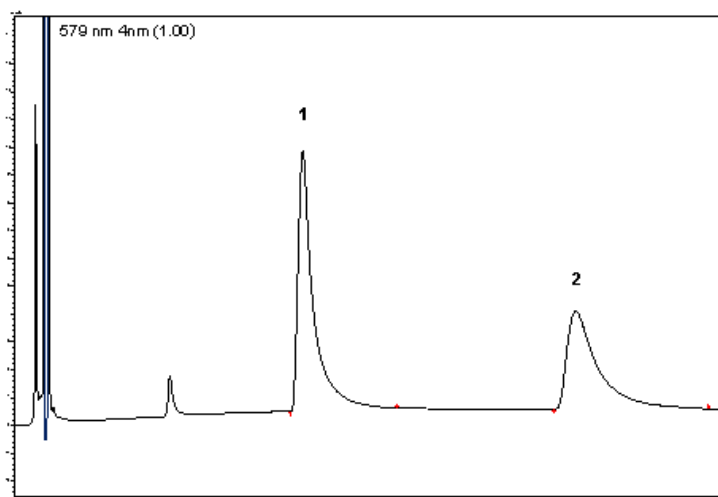


Figure 2. HPLC chromatogram of *Hypericum pruinatum* extract detected by UV at 580 nm wave length. Peak identified: 1 - pseudohypericin ( $t_R$  -12.93 min.), - hypericin ( $t_R$  -25.17 min.).

### Data analysis

Data for hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercetine, kaempferol, quercitrine and quercetine contents of plant were objected to ANOVA and significant differences among mean values were tested with the Duncan Multiple Range Test ( $P < 0.01$ ) by using MSTAT statistical software. Mean values of the chemical contents were normalized using  $x' = \sqrt{x} + 1$  transformation before conducting ANOVA, when necessary, because some chemicals were not detected in several cases.

### Results and Discussion

The contents of hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercetine, quercitrine and quercetine in plant materials varied significantly among populations whereas kaempferol content of whole shoots was similar and the compound was detected only in the populations of Cankiri and Bafra ( $P < 0.01$ ) (Table 2). Plants from Ladik population produced the highest amount of hypericin, chlorogenic acid, hyperoside, isoquercetine, quercitrine and quercetine (1.52, 20.21, 18.92, 20.11, 4.78 and 1.54 mg/g DW for the corresponding compounds, respectively). Hyperforin and rutin of whole shoots reached the highest level in Cankiri population (27.11 and 8.12 mg/g DW respectively). The highest pseudohypericin and quercetine accumulation was observed in plants from Bafra population (2.98 and 1.71 mg/g DW pseudohypericin and quercitrine, respectively). Generally lower accumulation levels were observed in Gumus and Yenikoy populations for all examined compounds.

Variations in the levels of bioactive secondary metabolites in populations of *Hypericum* plants have an important impact on the pharmacological activity of tested extracts (Bergonzi *et al.*, 2001). Hence, investigations of population variability in the content of secondary metabolites from different species of *Hypericum* have been made over several decades. In the present study, significant differences were detected in chemical accumulations among wild populations of *H. pruinatum*. The populations were located in different parts of Northern Turkey and growing sites of them 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. The chemical variability in the content of evaluated secondary metabolites among the populations may be attributed to the different environmental conditions of sampling sites. However, the present findings also

indicate a significant genetic difference/similarity among the populations. For example, Ladik and Yenikoy populations are separated by a distance of 200 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 quercetin. On the contrary, although Gumus and Yenikoy populations are separated by only 5 km and have very similar environment, three and five fold differences were detected between the populations in isoquercetin and hyperforin contents, respectively (Table 1). However, it should be noted that it is necessary to perform detailed analyses by using biochemical (i.e. allozymes) and molecular (i.e. RFLP, RAPD, AFLP, ISSR) markers to establish the whole range of genetic diversity of populations. Thus, it would be possible to discriminate exactly the effects of genetic and environmental factors on the observed chemical variability among populations. For example, the genetic diversity within and among seven Tunisian natural populations of *Hypericum humifusum* L., from different geographic regions and bioclimates, was assessed using 11 isozymic polymorphic loci, and 166 RAPD markers by Afef *et al.* (2012). The authors reported a high genetic variation among Tunisian *H. humifusum* populations and pointed out the necessity for further studies including the variation of the chemical composition among populations and its relationship with the genetic diversity.

**Table 2. Hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercetin, kaempferol, quercitrin and quercetin contents of *Hypericum pruinautum* whole shoots from wild populations located in northern Turkey (mg/g DW)**

Populations	Hyperforin	Hypericin	Pseudohypericin	Chlorogenic acid	Rutin	Hyperoside	Isoquercetin	Kaempferol	Quercitrin	Quercetin
Gumus	0.92 c*	0.29 b	0.51 b	8.32 c	1.98 c	9.12 c	15.27 b	0.00	1.57 c	0.61 b
Cankiri	27.11 a	0.00 c	0.00 c	0.67 e	8.12 a	6.17 d	6.11 c	0.11	1.12 c	0.31 b
Ladik	1.22 c	1.52 a	1.21 b	20.21 a	3.11 b	18.92 a	20.11 a	0.00	4.78 a	1.54 a
Yenikoy	5.22 b	0.67 b	1.11 b	5.82 d	1.56 d	6.11 d	5.57 c	0.00	1.11 c	1.11 a
Bafra	0.11 c	1.22 a	2.98 a	11.27 b	2.87 b	12.27 b	16.17 b	0.11	2.51 b	1.71 a

\*Values followed by different small letters in each column are significantly different ( $P < 0.01$ ) according to Duncan Multiple Range test.

In terms of population variability of bioactive substances in other *Hypericum* species, similarly, essential oil components in *Hypericum perforatum*, *Hypericum humifusum*, *Hypericum linarifolium* and *Hypericum pulchrum* were reported to vary significantly with geographic distribution of wild plants (Nogueira *et al.*, 2008). In *Hypericum triquetrifolium*, significant variations were detected in the content of hypericin, pseudohypericin, hyperforin and several flavonoids as rutin, hyperoside, apigenin-7-O-glucoside, kaempferol, quercitrin, quercetin and amentoflavone among four wild populations from Turkey (Camas *et al.*, 2008; Cirak *et al.*, 2011). The previous results indicated the regional distribution of *Hypericum* plants as an important source of chemical variability.

Results from the present study indicate that *H. pruinautum* accumulates lower concentrations of hyperforin, rutin and quercetin, comparable concentrations of hypericin, pseudohypericin, kaempferol and quercitrin, and higher concentrations of chlorogenic acid and hyperoside when compared to *H. perforatum*, a well known and commercial source of the compounds examined (Table 3). The results also indicate that both species contain the similar array of chemical constituents.

*H. pruinautum* belongs to the section Taeniocarpium Jaub. and Spach. (Robson 2001). Several other members of the section had already been investigated for the presence of main secondary metabolites. The comparison of our results with published previous reports revealed that *H. pruinautum* and other members of the section Taeniocarpium, namely *H.*

*confertum* Choisy, *H. hirsutum* L., *H. linarioides* Bosse, *H. nummularioides* Trautv, and *H. venustum* Fenzl have similar array of constituents and all of them contain hypericin, pseudohypericin, hyperforin, hyperoside, rutin, quercetin and quercitrin (Table 4). Among the chemicals, hypericins have a proven taxonomic value for the infrageneric classification of the genus *Hypericum* (Robson, 2001). Because they are not found in species from the primitive sections and seem to be specific only for the taxa of phylogenetically more advanced sections (Kitanov, 2001). Hence, detection of hypericin as well as hyperforin and the phenolics examined in *H. pruinatum* in the present study supports the taxonomic position of the section *Taeniocarpium* within the genus *Hypericum*.

**Table 3: Comparison of the content (mg/g DW) of hyperforin, hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercetine, kaempferol, quercitrine and quercetine in *H. pruinatum* and *H. perforatum*.**

Compounds	Present results ( <i>H. pruinatum</i> )	Previous results ( <i>H. perforatum</i> )	References
Hyperforin	0.92-27.11	8.35-150	Greeson <i>et al.</i> (2001); Kirakosyan <i>et al.</i> (2002); Maggi <i>et al.</i> (2004);
Hypericin	0-1.52	0.01-2.37	Walker <i>et al.</i> (2001) ; Sirvent <i>et al.</i> (2002); Southwell and Bourke (2001); Cirak <i>et al.</i> (2006)
Pseudohypericin	0-2.98	0.05-1.24	Cirak <i>et al.</i> (2007); Ayan and Cirak (2008)
Chlorogenic acid	0.67-20.21	1.11-2.19	Maggi <i>et al.</i> (2004)
Rutin	1.56-8.12	0.19-34.71	Radusiene <i>et al.</i> (2004); Martonfi and Repçak (1994)
Hyperoside	6.11-18.92	2.07-7.69	Maggi <i>et al.</i> (2004)
Isoquercetine	5.57-20.11	-	No previous report
Kaempferol	0-0.11	0.22-0.26	Silva <i>et al.</i> , 2005
Quercitrine	1.11-4.78	0.05-4.77	Radusiene <i>et al.</i> (2004); Martonfi and Repçak (1994)
Quercetine	0.31-1.71	0.05-24.12	Radusiene <i>et al.</i> (2004); Martonfi and Repçak (1994)

**Table 4: Comparison of the presence of hyperforin (1), hypericin (2), pseudohypericin (3), chlorogenic acid (4), rutin (5), hyperoside (6), quercitrin (7), quercetin (8), kempferol (9), and isoquercetine (10) in *Hypericum pruinatum* (present study) and some other species of *Hypericum* from the section *Taeniocarpium* Jaub. and Spach. (compiled from previous works)**

Compounds	1	2	3	4	5	6	7	8	9	10	References**
<i>H. pruinatum</i>	+	+	+	+	+	+	+	+	+	+	Our study
<i>H. confertum</i>	+	+	+	+	+	+	+	+	+	+	1
<i>H. hirsutum</i>	+	+	+	+	+	+	+	+	NPR*	+	2, 3
<i>H. linarioides</i>	+	+	+	NPR	+	+	+	+	NPR	NPR	3, 5
<i>H. nummularioides</i>	+	+	+	NPR	+	+	+	+	NPR	NPR	3, 6
<i>H. venustum</i>	+	+	+	NPR	+	+	+	+	NPR	NPR	6, 7

\*NPR: no previous report; \*\*References: <sup>1</sup>Cirak *et al.*(2010) <sup>2</sup>Sagrattini *et al.*(2008), <sup>3</sup>Smelcerovic *et al.*(2008), <sup>4</sup>Cirak *et al.*(2007), <sup>5</sup>Smelcerovic *et al.*(2006), <sup>6</sup>Ayan and Cirak (2008), <sup>7</sup>Spiteller *et al.*(2008).

## Conclusions

The main issue in medicinal plant standardization is the huge variability in secondary metabolite profile of a given plant material and screening the wild plant populations for obtaining the improved phytochemical profiles seems to be first step in identifying superior germplasm. Results of the present study indicate a significant variation in the chemical contents of *H. pruinatum* from Turkish populations. Regional distribution of

this medicinal plant may be an important source of chemical variability and should be considered while optimizing the processing methodology of wild-harvested plant material. In the present study, the presence of isoquercetin and kaempferol in this species was reported by us for the first time. Such kind of data could also be useful for elucidation of the chemotaxonomical significance of the corresponding compounds and phytochemical evaluation of *H. pruinatum*.

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