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Variations in Total Phenolics during Ontogenetic, Morphogenetic, and Diurnal Cycles in *Hypericum* Species from Turkey

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Several species of *Hypericum* are used in traditional Turkish folk medicine. Their most medicinally important secondary metabolites are the hypericins, hyperforins, and phenolics. Here, we determined the ontogenetic, morphogenetic, and diurnal variations in total phenolics contents from *H. aviculariifolium* subsp. *depilatum* var. *depilatum* (endemic), *H. perforatum*, and *H. pruinatum*. Plants of wild-growing *H. aviculariifolium* subsp. *depilatum* var. *depilatum* and *H. perforatum*, and greenhouse-grown *H. pruinatum* were harvested four times per day during their vegetative, floral-budding, full-flowering, fresh-fruiting, and mature-fruiting stages. They were then dissected into stem, leaf, and reproductive tissues to be dried separately and assayed. The highest level of phenolics in *H. aviculariifolium* subsp. *depilatum* var. *depilatum* and *H. pruinatum* was found in the leaves, whereas the floral buds produced the greatest amount in *H. perforatum*. Variations in contents from whole plants fluctuated diurnally, differing among species over the course of ontogenesis, reaching the highest level at floral-budding and tending to increase at mid-day in *H. aviculariifolium* subsp. *depilatum* var. *depilatum*. For *H. perforatum* and *H. pruinatum*, contents also were the highest during floral development, although no diurnal fluctuations were observed in those species.

Keywords: diurnal variation, *Hypericum* spp., ontogenesis, phenolics, plant defense

Over many centuries, plants of several *Hypericum* species have been of great interest to mankind for medicinal purposes (Dias et al., 1998). Their pharmaceutical importance includes well-documented antioxidant (El-Sherbiny et al., 2003), antitumor (Ferraz et al., 2005), antimutagenic (Barnes et al., 2001), and antibacterial (Gibbons et al., 2002) properties. The most common are the extracts from *Hypericum perforatum* L., which are now widely used in Europe for treating depression (Patocka, 2003). Approximately 400 *Hypericum* species grow in the temperate regions of the world; in Turkey, this genus includes 89 species, 43 of which are endemic (Davis, 1988).

The methanolic extract from the aerial portions of *Hypericum* plants typically contains hypericins, hyperforins, and phenolic compounds. It is especially rich in phenolics, caffeic acids, chlorogenic acid, prenylated derivatives of phloroglucinol and flavonoids, hyperin, rutin, quercitrin, isoquercitrin, and *bis*-apigenins (Mojca et al., 2005). Phenolic compounds are important contributors to the color, sensory attributes, and nutritional and antioxidant properties of plants (Christel et al., 2000). They reportedly have multiple biological effects, including activities against oxidants, tumors, mutagens, and bacteria (Shui and Leong, 2002).

Increased market demand for *Hyperici herba* has led to several global investigations on the levels of secondary metabolites accumulated in plants. These previous studies, conducted with *H. perforatum*, did not produce homogenous results. Furthermore, their entire focus was on hypericins (Sirvent et al., 2002) or hyperforin (Kirakosyan et al., 2003), and the ontogenetic stages of the plants usually were not considered. Little effort has yet been dedicated to the study of variations in phenolic compounds such as total phenols (Ayan et al., 2006), quercitrine, isoquercitrine (Marfonti and Repcak, 1994), and quercetin (Tekel'ova et al., 2000). To our knowledge, no research has been reported on such fluctuations in *H. perforatum* L. or *H. aviculariifolium* Jaup. and Spach subsp. *depilatum* (Freyn and Bornm.) Robson var. *depilatum* (endemic).

Morphologically, *Hypericum* plants are characterized by the presence of different types of secretory structures, including the light and dark glands, and secretory canals. These structures are sites for the synthesis and/or accumulation of biologically active substances, with their localizations depending upon tissue type (Ciccarelli et al., 2001). Therefore, the levels of secondary metabolites obtained may vary according to the amount of those structures being harvested in proportion to the whole plant.

The concentration of plant secondary metabolites, such as saponins in *Phytolacca dodecandra* (Ndamba et al., 1993), alkaloids in *Papaver somniferum* (Itenov et al., 1999), essential oils in *Laurus nobilis* (Kevseroğlu et al., 2003) and hypericins in some *Hypericum* species (Çırak et al., 2006) can fluctuate diurnally, but no information has yet been available concerning those variations in phenolics content from species of *Hypericum*.

Here, we investigated the morphogenetic and diurnal variations in total phenolics from *H. perforatum*, *H. pruinatum*, and *H. aviculariifolium* subsp. *depilatum* var. *depilatum* at different stages of their phenology. Our aim was to determine whether phenolics contents were correlated with either tissue type or development stage over diurnal and phenological cycles.

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MATERIALS AND METHODS

Plant Materials

The plant materials used in this study are listed in Figure 1, and were described previously (Çırak et al., 2006).

Experimental Procedures

Plants of *H. aviculariifolium* subsp. *depilatum* var. *depilatum* were collected from the Gümüş district of Amasya Province, Turkey, while the *H. perforatum* plants originated from Samsun Province, Turkey. All sampling occurred between April and October of 2005 to coincide with different stages in their development. These two sites are 145 km apart, and were not grazed or mowed during the experimental period. Geographic and climatic data for the sites and habitat descriptions for both species are shown in Table 1. As the third tested species, seedlings of *H. pruinatum* were established in the greenhouse from 5-month-old seeds collected from plants growing wild in the Gümüş district (Fig. 1b). Seeds were germinated on 2 March 2005 in a float system commonly used for seedling production of broad-leaf tobacco cultivars 'Burley' and 'Flue-Cured-Virginia', under a 16-h photoperiod. Newly emerged seedlings were transferred to 30-cm-diam. pots containing the commercial peat "Tray Substrate", and were then reared in the greenhouse, being watered daily for the first six weeks, then three times per week afterward. The primary chemical and physical properties and the average amount of nutrients added to this peat material are shown in Table 2. During the experimental period, the day length was approximately 12 h, with mean day/night temperatures of 28°C/18°C.

During each developmental stage, 20 individuals each of these wild or greenhouse-grown plants were randomly collected four times per day (0600 h, 1200 h, 1800 h, and 2400 h), as we had reported previously for our diurnal evaluations (Çırak et al., 2006). Clear and sunny weather conditions occurred at both field sites each time the wild-grown

Table 1. Collection sites and habitats for wild-grown *H. perforatum* (Samsun) and *H. aviculariifolium* (Gümüş) in Turkey.

Collection sites	Latitude (N)	Longitude (E)	Elevation (m)	Temperature (°C)	Precipitation (mm)	Habitat
Samsun	41° 35′	35° 56′	195	19.02	435.5	Acorn woodland
Gümüş	40° 52′	35° 14′	785	17.9	262.4	Rocky and open slopes

Note: Climatic data represent April through September 2005.

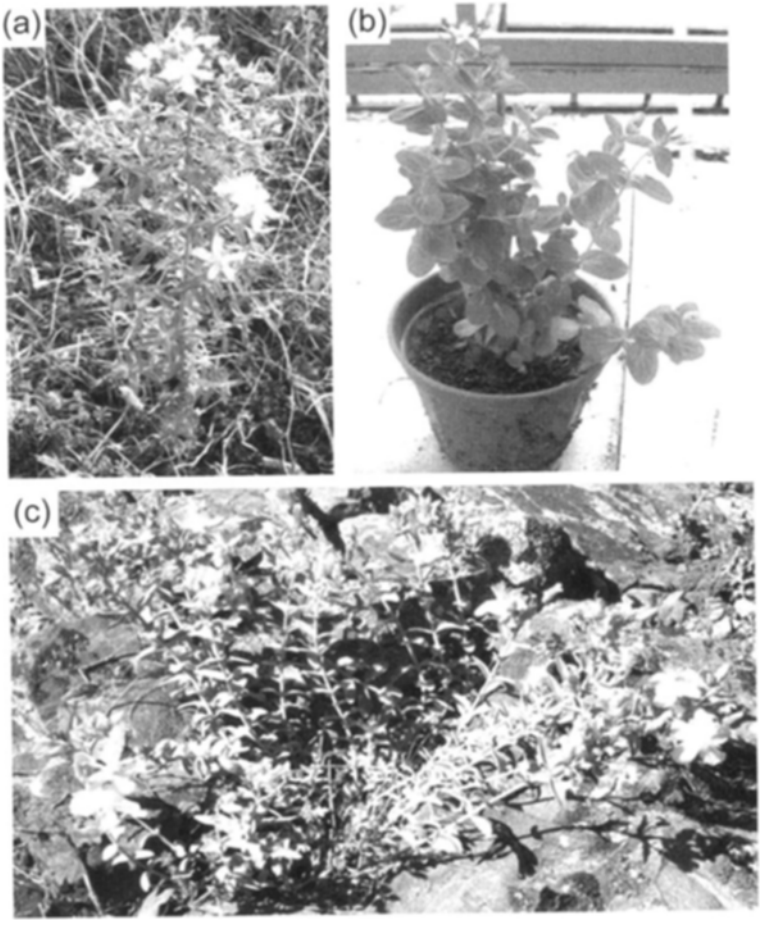

Figure 1. *H. perforatum* (**a**), *H. pruinatum* (**b**), and *H. aviculariifolium* subsp. *depilatum* var. *depilatum* (**c**) plants at flowering stage.

Table 2. Main chemical and physical properties and average amounts of nutrients added to the peat used in greenhouse experiments.

Chemical parameter	Average amount of added nutrients	Physical property
PH range (H_2O): 5.5-6.0	Nitrogen (mg N l^{-1}): 180	
Fertilizer (g l^{-1}): 1.3	Phosphorus (mg P_2O_5 l^{-1}): 210	Dry matter: 10%
Black sphagnum peat: 50%	Potassium (mg K_2O l^{-1}): 240	Air capacity: 15%
White sphagnum peat: 50%	Magnesium (mg Mg l^{-1}): 100	Water capacity: 75%

Table 3. Time course for ontogenetic sampling from each *Hypericum* species.

	Ontogenetic stages				
	Vegetative	Floral-budding	Full-flowering	Fresh-fruiting	Mature-fruiting
H. avicullariifolium	02 May	25 May	07 June	04 July	29 July
H. perforatum	15 April	22 May	24 June	15 July	18 September
H. pruinatum	05 May	15 July	02 August	03 September	05 October

Hypericum tissues were being gathered, and temperatures there ranged from 20 to 38°C, depending on the timing within a particular ontogenetic or diurnal cycle. The dates for ontogenetic sampling varied by species, according to their rate of development (Table 3), and different tissue types were collected depending on the phonological stage. For example, shoots with leaves were harvested at the vegetative stage, while at the floral-budding stage, only shoots with floral buds were selected. At the full-flowering stage, only shoots with fully opened flowers were harvested. During the setting of fresh fruit, shoots with green capsules were harvested, whereas, at the mature-fruiting stage, shoots with dark-brown capsules were gathered. We noted that plants of *H. perforatum* had no leaves remaining at the mature-fruiting stage, whereas, for the other two species, their leaves during that time period were as numerous as they had been at the vegetative stage. After each sampling date, 10 of the 20 individuals collected were kept as whole plants while the rest were dissected into their floral, leaf, and stem portions. All plant materials were first air-dried at room temperature (20 ± 2°C), then accurately weighed so these data could be used in calculating phenolic production per plant as well as the proportion contained within each tissue type.

Total Phenolics Assay (Folin-Denis Assay)

We used the procedure of Swain and Hillis (1959) for determining total phenolics contents from our plant extracts. The basis for this method is the reduction of phosphomolybdic phosphotungstic acid (Folin-Denis) reagent by phenols in an alkaline solution, as represented by the intensity of blue color produced during that reduction. Here, 1 mL of plant extract was mixed with 1 mL of 0.25 M Folin-Denis reagent for 3 min, then 1 mL of 2 M sodium carbonate was added to each test tube. After 30 min at room temperature, precipitation usually occurred. The tubes were then centrifuged for 5 min at ca. 5000 rpm. Absorbency of the resulting solution was measured at 725 nm on a spectrophotometer. A reagent blank, tannic acid blank, and sample blank also were prepared. All samples and tannic acid standard solutions (0.01 to 0.06 mg mL^{-1}) were tested in triplicate, and a mean value was calculated for the three determinations from each sample. These estimations included any phenolics that were soluble in 50% methanol, from simple to polymeric.

Expression of the Results

For each species, data for the content and per-plant production of phenolics were subjected to ANOVA. Significant differences among mean values were tested with a Duncan's Multiple Range Test ($P < 0.01$). Statistical analysis was performed with MSTAT statistical software. However, because generative tissue at the vegetative stage was a missing experimental factor, no statistical analysis was performed for data belonging to the phenolics content from different plant tissues (Steel and Torrie, 1980).

RESULTS

The trends for ontogenetic changes in phenolics contents varied among the three species of *Hypericum*. In *H. perforatum* and *H. pruinatum*, whole-plant contents increased over their phenological cycles, reaching the highest levels at floral-budding and full-flowering (4.82 and 3.40 mg g^{-1} DW, respectively), before decreasing. The differences among contents in whole plants at various development stages were significant ($P < 0.01$), but no diurnal fluctuations were seen with these two species (Table 4, 5). In *H. avicullariifolium* subsp. *depilatum* var. *depilatum*, ontogenetic variation in contents was more stable. Similar levels of accumulation in the whole plant were observed at different stages of phenology. After reaching the highest amount during flower ontogenesis, the content declined during fructification. In contrast to *H. perforatum* and *H. pruinatum*, contents for *H. avicullariifolium* fluctuated significantly throughout the day, with the greatest value (3.47 mg g^{-1} DW) being calculated from plants harvested at 1200 h (Table 6).

Significant differences in phenolics production per plant were detected among species during their ontogenetic and diurnal cycles ($P < 0.01$). Those of *H. avicullariifolium* subsp. *depilatum* var. *depilatum* generally produced the greatest amounts at all stages of their phenology, except for the full-flowering stage, when *H. perforatum* was more productive than the others. Nevertheless, for all species examined, the level of phenolics production per plant increased over time, although variations were seen in their diurnal peaks. For example, the highest amount for both *H. avicullariifolium*

Table 4. Diurnal variations in total phenolics content (mg g^{-1} DW) from whole plants of *H. perforatum* over the course of ontogenesis. Plant material was collected from 10 individuals, and included leaves and stems at the vegetative stage; reproductive organs, leaves, and stems at the floral-budding, full-flowering, and fresh-fruiting stages; and the stems and brown capsules with seeds at the mature-fruiting stage.

	Diurnal collecting times				
Development stages	0600 h	1200 h	1800 h	2400 h	Mean
Vegetative	0.90 c*	0.89 c	0.93 c	0.92 c	0.91 C
Floral-budding	4.85 a	4.86 a	4.81 a	4.77 a	4.82 A
Full-flowering	3.13 b	3.16 b	3.11 b	3.12 b	3.13 B
Fresh-fruiting	2.95 b	2.96 b	3.02 b	3.00 b	2.98 B
Mature-fruiting	0.96 c	0.95 c	0.91 c	0.91 c	0.93 C
Mean	2.56	2.56	2.55	2.54	

*Values not followed by the same small letter or capital letter within a column are significantly different ($P < 0.01$) according to Duncan's Multiple Range test.

Table 5. Diurnal variations in total phenolics content (mg g^{-1} DW) from whole plants of *H. pruinatum* over the course of ontogenesis. Plant material was collected from 10 individuals, and included leaves and stems at the vegetative stage; reproductive organs, leaves, and stems at the floral-budding, full-flowering, and fresh-fruiting stages; and the leaves, stems, and brown capsules with seeds at the mature-fruiting stage.

Development stages	Diurnal collecting times				
	0600 h	1200 h	1800 h	2400 h	Mean
Vegetative	0.96 f*	0.95 f	1.01 f	1.01 f	0.98 D
Floral-budding	2.42 b	2.38 bcd	2.41 bc	2.32 b-e	2.38 B
Full-flowering	3.36 a	3.42 a	3.38 a	3.43 a	3.40 A
Fresh-fruiting	1.77 b-f	1.80 b-f	1.74 b-f	1.75 b-f	1.76 C
Mature-fruiting	1.38 ef	1.37 ef	1.44 c-f	1.41 def	1.40 CD
Mean	1.98	1.98	2.00	1.99	

*Values not followed by the same small letter or capital letter within a column are significantly different ($P < 0.01$) according to Duncan's Multiple Range test.

Table 6. Diurnal variations in total phenolics content (mg g^{-1} DW) from whole plants of *H. aviculariifolium* subsp. *depilatum* var. *depilatum* over the course of ontogenesis. Plant material was collected from 10 individuals, and included leaves and stems at the vegetative stage; reproductive organs, leaves, and stems at the floral-budding, full-flowering, and fresh-fruiting stages; and the leaves, stems, and brown capsules with seeds at the mature-fruiting stage.

	Diurnal collecting times				
Development stages	0600 h	1200 h	1800 h	2400 h	Mean
Vegetative	2.32 e-h*	4.63 a	2.52 d-g	2.54 d-g	3.00 AB
Floral-budding	3.32 bcd	3.68 bc	3.26 b-f	3.09 b-f	3.34 A
Full-flowering	2.82 c-f	3.82 ab	3.07 b-e	3.23 b-e	3.24 AB
Fresh-fruiting	2.71 c-f	3.12 b-e	2.80 c-f	2.70 c-f	2.83 B
Mature-fruiting	1.53 h	2.11 fgh	1.72 gh	1.59 gh	1.74 C
Mean	2.54 B	3.47 A	2.67 B	2.63 B	

*Values not followed by the same small letter or capital letter within a column are significantly different ($P < 0.01$) according to Duncan's Multiple Range test.

subsp. *depilatum* var. *depilatum* and *H. perforatum* was measured in plants harvested at 1200 h during their fresh-fruiting stage (62.4 and 54.36 mg per plant, respectively; Fig. 2d). In contrast, the greatest accumulation of phenolics by *H. pruinatum* was found in plants harvested at 2400 h during their stage of full-flowering (37.73 mg per plant; Fig. 2c).

The percentages of phenolics calculated during ontogenesis in the stem, leaf, and reproductive portions, relative to the whole plant, varied among species (Table 7), as did their individual ontogenetic and diurnal fluctuations. The highest level of phenolics measured in *H. aviculariifolium* subsp. *depilatum* var. *depilatum* and *H. pruinatum* was from leaves collected at 1200 h each day. Moreover, leaves harvested at that time of day during the full-flowering stage had the greatest amount of phenolics, i.e., 5.05 mg g^{-1} DW for *H. aviculariifolium* subsp. *depilatum* var. *depilatum* and 4.67 mg g^{-1} DW for *H. pruinatum* (Fig. 3, 4). For all species, phenolics accumulations increased over the growing season. Although leaf contents tended to be higher than for other tissues of *H. perforatum*, the greatest accumulation was found in floral buds harvested at 0600 h (10.89 mg g^{-1} DW; Fig. 5); phenolics levels in the reproductive organs sharply declined during the stages of flower-

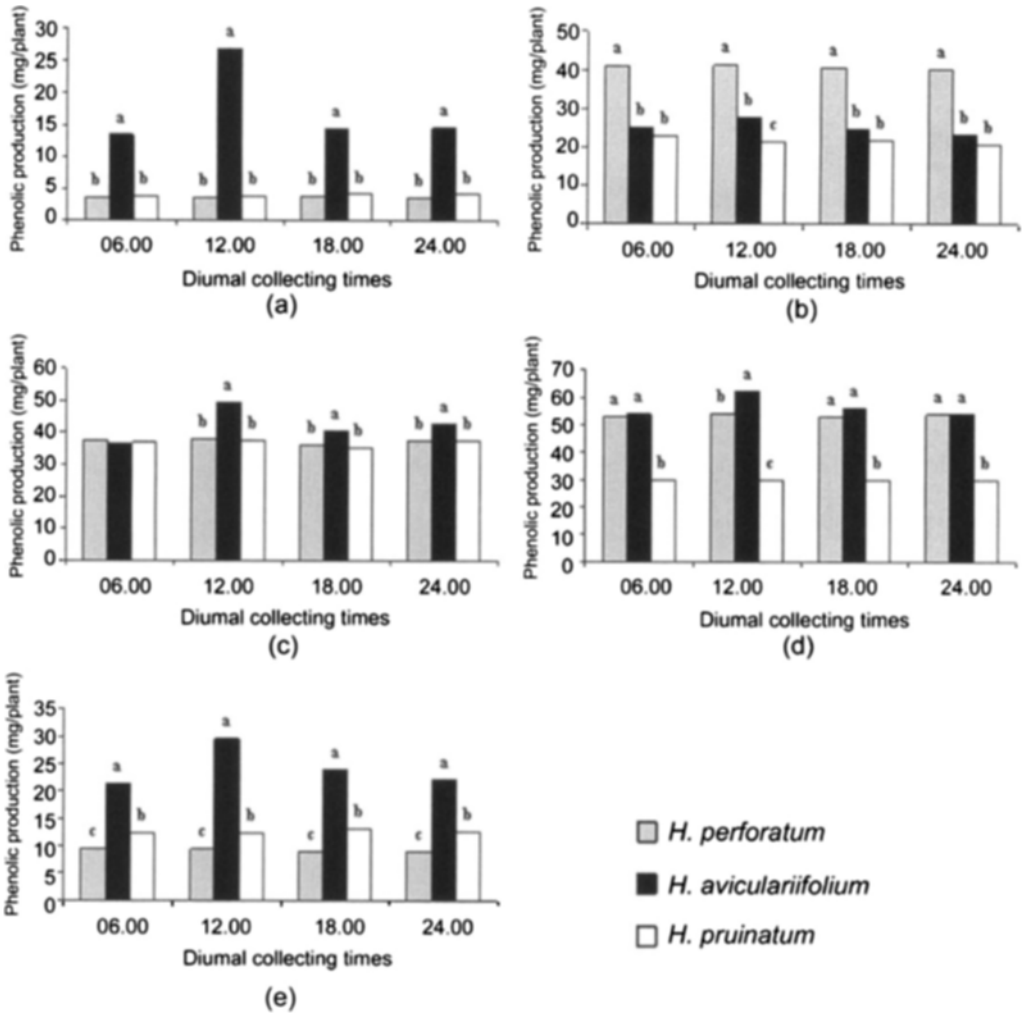

Figure 2. Changes in total phenolics production over diurnal cycle during vegetative (**a**), floral-budding (**b**), full-flowering (**c**), fresh-fruiting (**d**), and mature-fruiting (**e**) stages. Values not followed by same letter within column are significantly different at $P < 0.01$.

Table 7. Proportion (%) of the whole plant represented by stem, leaf, and reproductive tissue samples at different phonological stages.

H. perforatum					
Tissue	Vegetative	Floral-budding	Full-flowering	Fresh-fruiting	Mature-fruiting
Stem	42	36	30	24	51
Leaf	58	39	40	17	-
Reproductive	-	25	40	59	49
H. pruinatum					
Tissue	Vegetative	Floral-budding	Full-flowering	Fresh-fruiting	Mature-fruiting
Stem	40	36	20	18	17
Leaf	60	44	50	40	38
Reproductive	-	19	30	42	44
H. avicularifolium					
Tissue	Vegetative	Floral-budding	Full-flowering	Fresh-fruiting	Mature-fruiting
Stem	25	26	20	14	13
Leaf	75	52	46	41	32
Reproductive	-	22	34	45	55

ing and fructification, the latter response also being true for the other two species. Stems always produced the lowest amount of phenolics, compared with all other tissue types, regardless of the species.

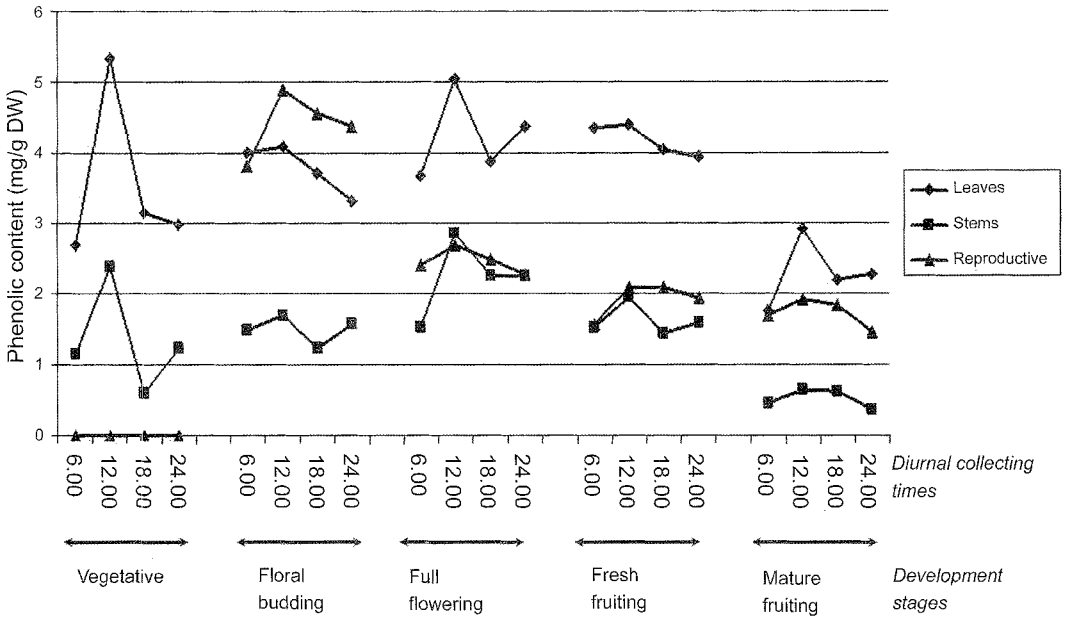

Figure 3. Diurnal and ontogenetic changes in total phenolics content from reproductive, leaf, and stem tissues in *H. aviculariifolium* subsp. *depilatum* var. *depilatum*.

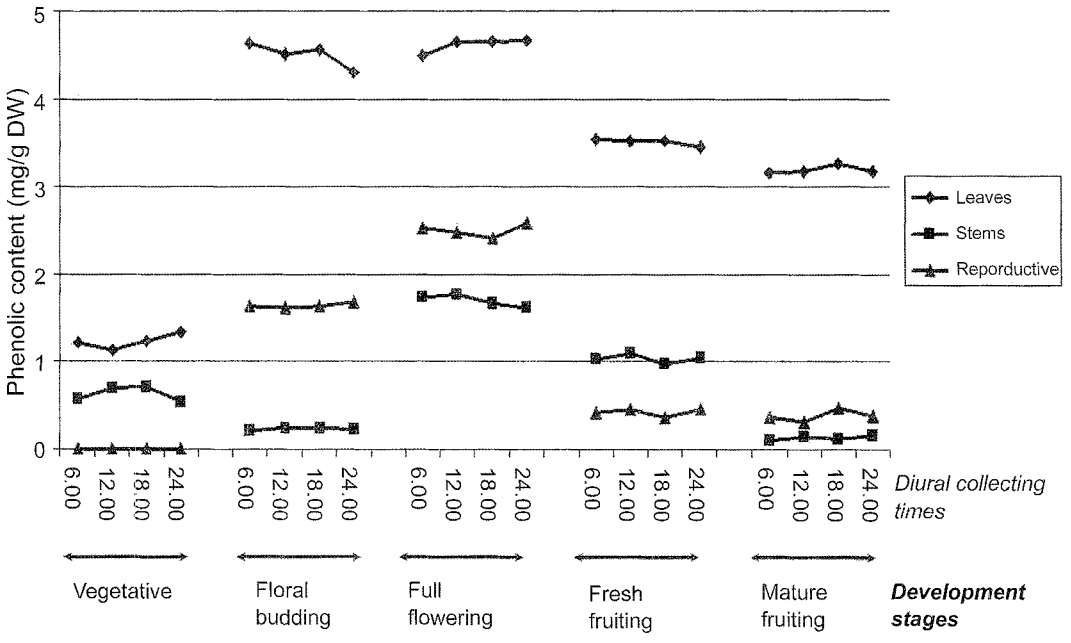

Figure 4. Diurnal and ontogenetic changes in total phenolics content from reproductive, leaf, and stem tissues in *H. pruinatum*.

DISCUSSION

Chemical contents in medicinal plants may vary substantially from one developmental stage to another. Therefore, investigations into ontogenetic fluctuations by secondary metabolites are always of considerable interest to researchers. Here, we determined that, although the total phenolics content in whole plants at various phenological stages differed significantly among our three *Hypericum* species, the highest levels were accumulated during the development and opening of the floral buds. As reported earlier with *H. perforatum*, the highest levels of flavonoids (rutin, quercitrin and isoquercitrin) occur during flower ontogenesis (Kazlauskas and Bagdonaite, 2004). Moreover, Osinska and Weglarz (2003) have found that flavonoid and hypericin contents in 11 Polish *Hypericum* species differ significantly, and according to the developmental stage. A similarly high accumulation of total phenolics has been observed during flower ontogenesis in *H. hyssopifolium*, *H. scabrum*, and *H. pruinatum*. Ontogenetic fluctuations in phenolics content have also been demonstrated with other plant species, such as *Malus domestica* (Treuter, 2001), *Morus alba*, and *M. nigra* (Sivaci and Sökmen, 2004). It is interesting to note that the phenolics content reported previously for wild-grown *H. pruinatum* plants (Ayan et al., 2006) was higher than that from the same species measured here. This difference may

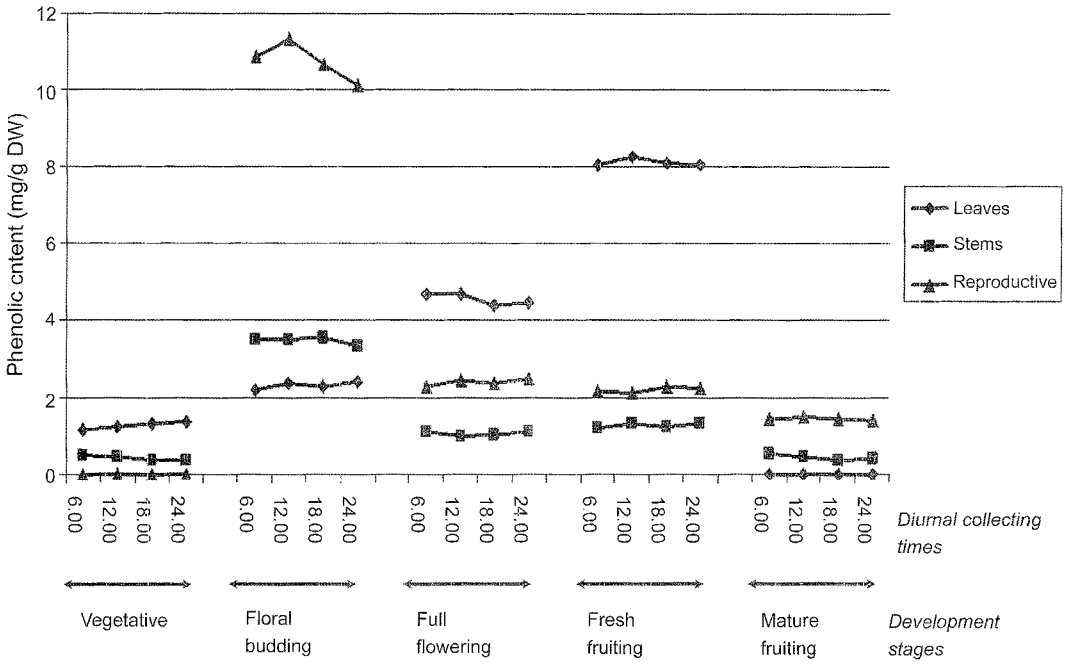

Figure 5. Diurnal and ontogenetic changes in total phenolics content from reproductive, leaf, and stem tissues in *H. perforatum*.

be attributed to the fact that our plants were instead grown in the greenhouse.

Differences in the accumulation of secondary metabolites by various organs is very common among medicinal plants. Leaves and flowers generally contain greater amounts of phenolic acids and terpenoids than do the stems and roots (Hakulinen and Julkunen-Tiitto, 2000). Here, the levels of total phenolics were highest in the leaves of *H. aviculariifolium* subsp. *depilatum* var. *depilatum* and *H. pruinatum* while the floral buds were the greatest producers in *H. perforatum*. These results agree with those of Ayan et al. (2006), who showed that the highest accumulation of phenolics was in the leaves of *H. hyssopifolium*, *H. scabrum*, *H. pruinatum*, and *H. nummularioides*. Similar, significant differences have been detected among the mean concentrations of quercetin, hypericin, rutin, and hyperoside + isoquercetrin in the flowers and leaves of *H. perforatum* and *H. maculatum* (Radusiene et al., 2004). Likewise, the total amount of phenolics in *H. androsaemum* populations depends significantly upon the tissue type and phenological stage (Valentao et al., 2003). Because we demonstrated here that the leaves generally comprised the greatest proportion of all plant material in these *Hypericum* species (Table 7), we suggest that leaf phenolics contents should be used as an important indicator when considering new species for pharmacological or agricultural purposes (Çırak et al., 2006).

Over the course of their ontogenesis, plants accumulate phenolic compounds in various tissues in response to stress factors (Pasqualini et al., 2003), including drought, heat, herbivore/pathogen attacks, and air pollution (Paliyath et al., 1997). Physiological and biochemical damage can result from exposure to less-than-optimal temperatures (Grace et al., 1998), which can then promote the production of those compounds (Christie et al., 1994; Dixon and Paiva, 1995; Sıvacı and Sökmen, 2004). Here, phenolics contents were higher in tissues of wild-grown *H. avicularıifolium* subsp. *depilatum* var. *depilatum* collected at 1200 h, a time normally associated with elevated temperatures, especially out of doors where those plants were sampled. In contrast, plants of *H. pruinatum* were grown in a cooler greenhouse while tissues of *H. perforatum* were collected from plants growing in an acorn woodland. Because those two species were not exposed to higher, mid-day temperatures, we can conclude that ambient conditions influence the accumulation of phenolics.

In summary, phenolics contents are closely correlated with developmental stage during the diurnal and phenological cycles of the *Hypericum* species examined here. Thus, the present results might be useful to research efforts made toward enhancing the concentrations of these compounds. Because their phenolics contents and production capacity were high in all three species, we propose that studies of the endemic *H. avicularıifolium* subsp. *depilatum* var. *depilatum*, in particular, be encouraged with regard to its cultivation and biological evaluation in Turkey.

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