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Phenological changes in the chemical content of wild and greenhouse-grown *Hypericum pruinatum*: flavonoids

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Abstract: The present study was conducted to determine the phenological changes in the content of main flavonoids, namely amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin in different tissues of *Hypericum pruinatum*, a promising medicinal herb of the Turkish flora. The wild-growing and greenhouse-grown plants were harvested at vegetative, floral budding, full flowering, fresh fruiting, and mature fruiting stages and dissected into stem, leaf, and reproductive tissues, which were dried separately and subsequently assayed for flavonoid contents by high performance liquid chromatography (HPLC). Chemical contents in whole plants increased during plant phenology and were similar quantitatively for both wild and greenhouse-grown plants. Depending on development stages, reproductive parts produced higher amounts of rutin, quercetin, (+)-catechin, (–)-epicatectin, and amentoflavone; however, leaves accumulated the highest level of hyperoside, isoquercitrin, quercitrin, and avicularin. The present results indicated a close relationship between flavonoid content in plant parts and phenological development of plants. It is suggested that the raw plant should be harvested during flowering for medicinal purposes.

Key words: Phytochemical variation, HPLC, *Hypericum pruinatum*, flavonoids, plant phenology

1. Introduction

The genus *Hypericum* L. (St. John's wort, Hypericaceae) includes 484 species, naturally occurring on every continent in the world except Antarctica (Crockett and Robson, 2011). Turkey is an important center for the genus *Hypericum* with the presence of 89 species, of which 43 are endemic (Bingöl et al., 2011). Members of this genus are very important in pharmacology; in particular, *Hypericum perforatum* L. has been studied extensively for its abundant secondary metabolites and for the bioactivities exhibited by its phytochemicals.

Hypericum pruinatum Boiss. and Bal. is a perennial herbaceous plant of Turkish flora that grows naturally on igneous slopes at high altitudes. In previous studies, *H. pruinatum* was reported to have great pharmaceutical potential with its well documented contents of hypericin (Çırak et al., 2006), hyperforin, organic acids, and flavonoids (Smelcerovic et al., 2008). Because of the similarity in the chemical composition of *H. pruinatum* to *H. perforatum*, the former could be considered a potential cultivated plant being used as an alternative crop to *H. perforatum*.

Various bioactivities of *Hypericum* plants have been associated with several phytochemicals from different classes. The bioactive compounds are the phloroglucinol derivatives hyperforin and adhyperforin; the naphthodianthrones hypericin and pseudohypericin; flavonoids such as hyperoside, rutin, quercitrin, quercetin, and biapigenin; the phenylpropanes caffeic and chlorogenic acids; and essential oils (Kasper et al., 2010). Among the chemicals, phenolic compounds are well known for their significant contribution to the nutritional and antioxidant properties of plants. Flavonoids in particular have received considerable interest as dietary constituents because the results from clinical studies indicate their possible role in preventing cardiovascular diseases and several kinds of cancer (Chu et al., 2000). Although hypericins and hyperforin have been reported to contribute to the antidepressant activity of *Hypericum* extracts (Medina et al., 2006), flavonoids also have an important influence on the pharmacological effects of phytomedicines (Gastpar and Zeller, 2005). Chemotaxonomic significance is also attributed to several flavonoids such as hyperoside, quercetin, quercitrin (Çırak et al., 2010), rutin, and mangiferin (Nunes et al., 2010).

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Enhanced market demands for *Hypericum,* including medicinal aids and dietary supplements, have resulted in many investigations into variations in the chemical content of *Hypericum* species from different growing localities of the world. Those previous studies generally investigated *Hypericum perforatum* L. and these reports did not produce homogeneous data for several reasons (e.g., difference in species, sampling procedures, chemical analyzing methods, and ecological conditions of plant growing sites). Furthermore, only a specific compound or chemical group was targeted for investigation, such as naphthodianthrones (Kitanov, 2001; Çırak et al., 2006), phloroglucinol derivatives (Kirakosyan et al., 2002), or essential oils (Radusiene et al., 2005; Bertoli et al., 2011) while the influence of plant developmental stages was usually not assessed. So far, only a few studies have been carried out on the variation in flavonoids such as quercitrin, isoquercitrin, quercetin (Hosni et al., 2011) hyperoside, and rutin (Çırak et al., 2013b). In our previous study, we observed the phenological changes in hypericin content of wild-growing *H. pruinatum* plants (Çırak et al., 2006). However, to our knowledge, no report is available on the variation in flavonoid contents in wild or greenhouse-grown *H. pruinatum* plants. In the present study, we investigated the relationships between the content of main flavonoids, namely amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin in plant materials and development stages in both wild and greenhouse-grown *H. pruinatum* for the first time. We also compared the quantitative composition of flavonoids in the same species from wild and modified culture conditions.

2. Materials and methods

2.1. Plant material

The species was identified by Dr Samim Kayikci, Department of Biology, Faculty of Arts and Sciences, Mustafa Kemal University, Turkey. Voucher specimens were deposited in the herbarium of Ondokuz Mayıs University Agricultural Faculty (OMUZF # 107).

Wild *H. pruinatum* plants at different stages of their development were collected from Gümüş District in Amasya Province, Turkey (40°52′N; 35°14′E; 785 m sea level) between April and October 2011. The material represented 30 randomly gathered plants in 5 phenological stages: vegetative, floral budding, full flowering, fresh fruiting, and mature fruiting. Plants with newly emerged shoots with leaves were classified as belonging to the vegetative stage. Only shoots with floral buds were selected to represent the floral budding stage. At the full flowering stage, only shoots with full opened flowers were harvested. For the fresh and mature fruiting stages, the shoots that had green and brown capsules, respectively, were harvested.

For the greenhouse cultivation experiment, seeds were germinated in a float system, commonly used for seedling production of the broad-leaf tobaccos Burley and Flue-Cured-Virginia, under a 16 h light: 8 h dark cycle. Newly emerged seedlings were transplanted into 30-cm-diameter pots filled with the commercial peat tray substrate, whose chemical and physical characteristics are presented in Table 1. The pots with seedlings were moved to greenhouse conditions 16/8 h light/darkness, 25 °C temperature, 75% relative humidity, and 400 µmol m^{-2} s^{-1} parabolic anodized reflector (PAR) and watered daily until they reached maturity; upon reaching maturity, they were then watered 3 times a week. Thirty pots were prepared for each phenological stage; thus plants from a total of 30 × 5 = 150 pots were evaluated (Figure 1).

The top two-thirds of wild and cultured plants, in which many phytochemicals primarily accumulate, were harvested between 1200 and 1300 hours. Ten individuals were kept as whole plants and the rest were dissected into floral, leaf, and stem tissues, dried at room temperature (20 ± 2 °C), and then assayed for phytochemical contents by HPLC.

2.2. Preparation of plant extracts

Air-dried plant material was mechanically ground with a laboratory mill to obtain a homogeneous 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 a pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator (+4 °C) until analysis.

Table 1. Main chemical and physical properties and average amount of added nutrients for peat tested.

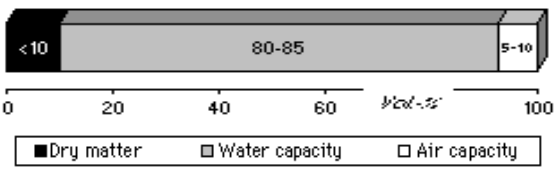
Chemical data	Average amount of added nutrients	Physical properties
pH range (H_2O): 5.5–6.0	Nitrogen (mg N/I): 210	
Fertilizer (g/L): 1.5	Phosphorus (mg P_2O_5/I): 240	
Black sphagnum peat: 30%	Potassium (mg K_2O/I): 270	
White sphagnum peat: 70%	Magnesium (mg Mg/I): 100	

Figure 1. A view of the flowering *Hypericum pruinatum* plant, cultured in a pot under greenhouse conditions.

2.3. High performance liquid chromatography (HPLC) analysis

A Waters Alliance 2695 (Waters, Milford, MA, USA) separation module system equipped with Waters 2487 UV/Vis and Waters 996 PDA diode-array detectors was used for HPLC analysis. Data were analyzed using Empower Software chromatographic manager system (Waters).

Separation of amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin was carried out on a SunFire C18 column (3.5 µm, 150 mm × 3.0 mm i.d.; Waters) with a 10-mm guard-precolumn. In HPLC analysis, the gradient elution serves to separate the components of a mixture, which are subsequently recorded by detectors. The mobile phase consisted of Milli-Q water acidified with 0.3% phosphoric acid as eluent A and acetonitrile containing 0.3% phosphoric acid as eluent B. Details of this gradient elution are presented in Table 2.

The elution rate was 0.6 mL min^{-1} at a constant 25 °C column temperature; injection volumes were 10 µL. Peaks were detected at a wavelength range of 270–360 nm.

The ACE C18 column (5.0 µm, 250 × 4.6 mm i.d.; MAC-MOD Analytical, Inc) with a guard-precolumn was used for separation of catechin. The mobile phase of gradient elution was composed of eluent A (water acidified with 0.5% glacial acetic acid) and eluent B (acetonitrile). The separation was performed using the following program: 0–30 min (B 5%→35%), 30–36 min (B 35%→90%), and 36–37 min (B 90%→5%). The flow rate was 1.0 mL min^{-1} at 25 °C column temperature. Detection was performed at 277 nm.

Table 2. Gradient elution program for flavonoids and epicatechin.

Time (min)	A (%)	B (%)
0–12	84→47	16→53
18	47→3	53→97
18.1	3	97
29	3→84	97→16
30	84	16

2.4. Quantification

Quantification of compounds was carried out by the external standard method. Standards stock solutions at a concentration of 1.0 mg/mL were freshly prepared in methanol and diluted in appropriate quantities to obtain a set of corresponding concentration ranges for the study of linearity. A calibration curve for each of the compounds was constructed by plotting peak areas against the respective compound concentration. The regression coefficients ($r^2 \geq 0.999$) of all calibration curves indicated that, in the ranges of standard concentrations analyzed, the peak areas were directly proportional to the concentrations and, thus, methods presented adequate linearity. The precision of the method was demonstrated for all analytes, since all the obtained relative standard deviations (RSD) values were lower than 5.0%. The retention time, linear range, regression equation, correlation coefficient, and RSD values of each analysis are summarized in Table 3. The concentration of compounds was expressed as mg/g dry mass (DM).

Solvents used were of HPLC grade and purchased from Roth GmbH (Karlsruhe, Germany). Water was filtered through a Millipore HPLC grade water preparation cartridge (Millipore, Bedford, MA, USA). Reference substances were purchased from ChromaDex (Santa Ana, CA, USA), Sigma-Aldrich (St. Louis, MI, USA), and HWI ANALYTIK GmbH (Germany).

2.5. Data analysis

Data for amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin contents of plant material including whole plant, stem, leaf, and reproductive parts were subjected to ANOVA separately for wild and cultured plants. Significant differences among mean values were tested with Duncan's multiple range test ($P < 0.01$) by using MSTAT-C statistical software (Russell D. Freed, Crop and Soil Sciences Department, Michigan State University).

Table 3. The retention time, linear range, regression equation and correlation coefficient, and precision of each detected analytes of HPLC analysis on evaluated *Hypericum pruinatum*.

Analytes	Retention time, min	Linearity range (µg/mL)	R^2	Regression equation	Precision, RSD (%)
Rutin	10.1	0.14–90.95	0.9999	$Y = 2.77 \cdot 10^4X - 5.18 \cdot 10^3$	1.02
(–)-Epicatechin	4.5	0.15–194.00	0.9999	$Y = 1.08 \cdot 10^4X + 1.46 \cdot 10^3$	1.36
Hyperoside	11.9	0.16–99.00	0.9999	$Y = 5.27 \cdot 10^4X + 3.24 \cdot 10^2$	0.52
Isoquercitrin	12.8	0.16–99.00	0.9999	$Y = 4.46e \cdot 10^4X - 3.24 \cdot 10^3$	0.66
Avicularin	17.0	0.15–19.16	0.9997	$Y = 3.44 \cdot 10^4X - 1.73 \cdot 10^3$	2.83
Quercitrin	17.2	0.15–98.00	0.9999	$Y = 3.23 \cdot 10^4X - 1.37 \cdot 10^3$	0.31
Quercetin	19.3	0.15–190.00	0.9996	$Y = 3.52 \cdot 10^4X + 4.18 \cdot 10^4$	4.60
(+)-Catechin	19.7	0.30–95.00	0.9997	$Y = 1.20 \cdot 10^4X + 3.85 \cdot 10^3$	3.19
Amentoflavone	20.1	0.14–179.94	0.9999	$Y = 3.48 \cdot 10^4X + 1.27 \cdot 10^4$	1.34

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.

3. Results

Amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, (+)-catechin, and (–)-epicatechin contents of the whole plant varied significantly ($P < 0.01$) during the stages of plant phenology and rutin was detected only during floral development. Chemical contents in whole plants increased as the developmental stages advanced, and some differences in chemical accumulation levels were observed between wild and cultivated plants. Wild plants harvested at full flowering accumulated the highest level of hyperoside, isoquercitrin, quercitrin, quercetin, (+)-catechin, and amentoflavone (6.07, 3.74, 3.42, 6.75, 1.06, and 1.83 mg g^{-1} DM, respectively) while the same wild plants harvested at floral budding produced the highest amounts of avicularin and (–)-epicatectin (1.49 and 1.32 mg g^{-1} DM, respectively) (Table 4). In greenhouse-grown plants, similarly, flavonoid content in whole plants increased during the seasonal development and plants harvested at full flowering produced the highest amount of hyperoside, quercitrin, and quercetin (6.33, 2.78, and 2.46 mg g^{-1} DM, respectively) while isoquercitrin, avicularin, (+)-catechin, (–)-epicatechin, and amentoflavone contents in whole plants were the highest at the floral budding phase (4.81, 0.83, 0.38, 0.96, and 0.77 mg g^{-1} DM, respectively) (Table 5). Content of the flavonoids investigated decreased as fruit development advanced, and the lowest level of their accumulation was detected at the mature fruiting stage in both wild and greenhouse-grown plants.

Significant differences were also observed among flavonoid quantities in different plant parts during the phenological cycle ($P < 0.01$). Depending on the developmental phase, reproductive parts produced higher amount of rutin, quercetin, (+)-catechin, (–)-epicatechin, and amentoflavone; however, leaves accumulated the highest level of hyperoside, isoquercitrin, quercitrin, and avicularin. In wild plants, rutin was accumulated only in floral buds and flowers at similar levels (0.89 and 0.92 mg g^{-1} DM, respectively) (Figure 2a). Quercetin and amentoflavone contents were the highest in fully opened

Table 4. Amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin content (mg g^{-1} DM) variations in wild growing *Hypericum pruinatum* whole plant during its phenological cycle.

Phenological stage	Amentoflavone	Hyperoside	Isoquercitrin	Quercitrin	Quercetin	Avicularin	Rutin	(+)-catechin	(–)-epicatechin
Vegetative	0.01 c*	4.10 b	2.15 b	0.97 c	1.17 b	0.71 b	0	0.22 b	0.35 b
Floral budding	1.48 a	5.78 a	3.39 a	3.41 a	5.47 a	1.49 a	0.77	0.92 a	1.32 a
Full flowering	1.83 a	6.07 a	3.74 a	3.42 a	6.75 a	0.98 a	0.79	1.06 a	1.29 a
Fresh fruiting	0.43 b	1.42 c	0.71 c	1.69 b	1.82 b	0.15 c	0	0.01 c	0.42 b
Mature fruiting	0.11 b	0.46 d	0.06 d	0.12 c	0.98 b	0.03 d	0	0.01 c	0.23 b

*Values followed by different letters in each column are significantly different ($P < 0.01$) according to Duncan's multiple range test.

Table 5. Amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin content (mg g^{-1} DM) variations in greenhouse-grown *Hypericum pruinatum* whole plant during its phenological cycle.

Phenological stage	Amentoflavone	Hyperoside	Isoquercitrin	Quercitrin	Quercetin	Avicularin	Rutin	(+)-catechin	(–)-epicatechin
Vegetative	0.01 c*	4.83 ab	3.14 b	1.71 b	0.07 c	0.53 ab	0	0.17 b	0.14 c
Floral budding	0.77 a	5.24 b	4.81 a	2.17 a	0.49 b	0.83 a	0.07	0.38 a	0.96 a
Full flowering	0.75 a	6.33 a	4.14 a	2.78 a	2.46 a	0.71 a	0.07	0.21 ab	0.79 a
Fresh fruiting	0.39 b	2.99 c	1.76 c	0.65 c	0.82 b	0.25 b	0	0.06 c	0.35 b
Mature fruiting	0.22 b	1.19 d	0.91 d	0.05 d	0.67 b	0.04 c	0	0.02 c	0.42 b

*Values followed by different letters in each column are significantly different ($P < 0.01$) according to Duncan's multiple range test.

flowers (8.64 and 2.97 mg g^{-1} DM, respectively) (Figures 2b and c), and floral buds produced the highest amount of (+)-catechin and (–)-epicatechin (1.25 and 1.97 mg g^{-1} DM, respectively) (Figures 2d and e). In contrast, hyperoside, isoquercitrin, and quercitrin contents were the highest in leaf tissues of plants harvested at full flowering (9.74, 6.69, and 4.72 mg g^{-1} DM, respectively) and leaves harvested at the floral budding stage produced the highest content of avicularin (1.98 mg g^{-1} DM) (Figures 2f–i). The same chemical accumulation pattern was also observed among different plant tissues in greenhouse-grown plants. Rutin was detected only in floral buds and flowers at the same amount (0.11 mg g^{-1} DM) (Figure 3a). Floral buds accumulated the highest level of (+)-catechin, (–)-epicatechin, and amentoflavone (0.53, 1.20, and 2.79 mg g^{-1} DM, respectively) and quercetin content was the highest in fully opened flowers (3.64 mg g^{-1} DM) (Figures 3b–e). Instead, leaves of plants harvested at flowering yielded the highest amount of hyperoside, isoquercitrin, and quercitrin (9.57, 7.51, and 3.52 mg g^{-1} DM, respectively) and avicularin accumulation reached its highest level in leaves of plants harvested at the floral budding stage (1.14 mg g^{-1} DM) (Figures 3f–i).

4. Discussion

Concentrations of bioactive compounds from different chemical classes can exhibit significant variations during plant developmental stages, depending on plant species as well as tissue, and this phenomenon may be of concern regarding the utilization of a given medicinal plant species. In the present study, all tested flavonoids in whole plants reached their highest level at flowering time in both wild and greenhouse-grown *H. pruinatum* plants. Similarly, hypericin content of wild *H. pruinatum* plants was reported to be the highest at flowering (Çırak et al., 2006). These results are also largely in accordance with those reported for other *Hypericum* species. The data reported for *H. perforatum* (Kazlauskas and Bagdonaite, 2004), the best known and most studied member of the genus *Hypericum*, showed that accumulation of rutin, quercetin, and isoquercetin reached a maximum during flower ontogenesis. The highest content of rutin, hyperoside, and quercitrin in wild *Hypericum triquetrifolium* Turra (Hosni et al., 2011), as well as quercetin, isoquercetin, and chlorogenic acid in greenhouse-grown plants of the same species (Çırak et al., 2013b), was detected at flowering. In *Hypericum orientale* L. and *Hypericum aviculariifolium* subsp. *depilatum* var. *depilatum* (Freyn and Bornm.) Robson var. *depilatum*, an endemic species from Turkish flora, flowering plants produced the highest amount of quercitrin, rutin, hyperoside, and isoquercitrin (Çırak et al., 2013a). The highest concentration of rutin, quercetin, and several phenolics was reported by Abreu et al. (2004) to accumulate during flowering in greenhouse-grown *Hypericum brasiliense* Choisy, a perennial herb native to southern Brazil. Similarly, the contents of hyperoside, chlorogenic acid, quercitrin, and quercetin increased as plant development advanced, and reached their maximum levels at flowering time in *Hypericum perfoliatum* L. (Çırak et al., 2007a), *Hypericum origanifolium* Willd (Çırak et al., 2007b), and *Hypericum montbretii* Spach (Çırak et al., 2008).

Morphologically, 3 kinds of secretory structures, including light glands, dark glands, and secretory canals, are facilitated to characterize *Hypericum* plants (Lu et al., 2001). These structures are accumulation and/or synthesis sites for different kinds of phytochemicals; phenolic compounds, including flavonoids, are thought to be synthesized in secretory canals (Ciccarelli et al., 2001). The localization of the secretory structures varies greatly among plant tissues; for that reason, the levels of phytochemicals in a particular *Hypericum* tissue depend on the relative abundance of these secretory structures in the harvested material (Zobayed et al., 2006). As a result, organ-dependence of a given chemical is common among *Hypericum* species. This phenomenon could explain why a significant variation was observed in the chemical contents of different plant parts of wild and cultivated *H. pruinatum* plants. The differences in chemical composition between leaves and flowers found in the present study

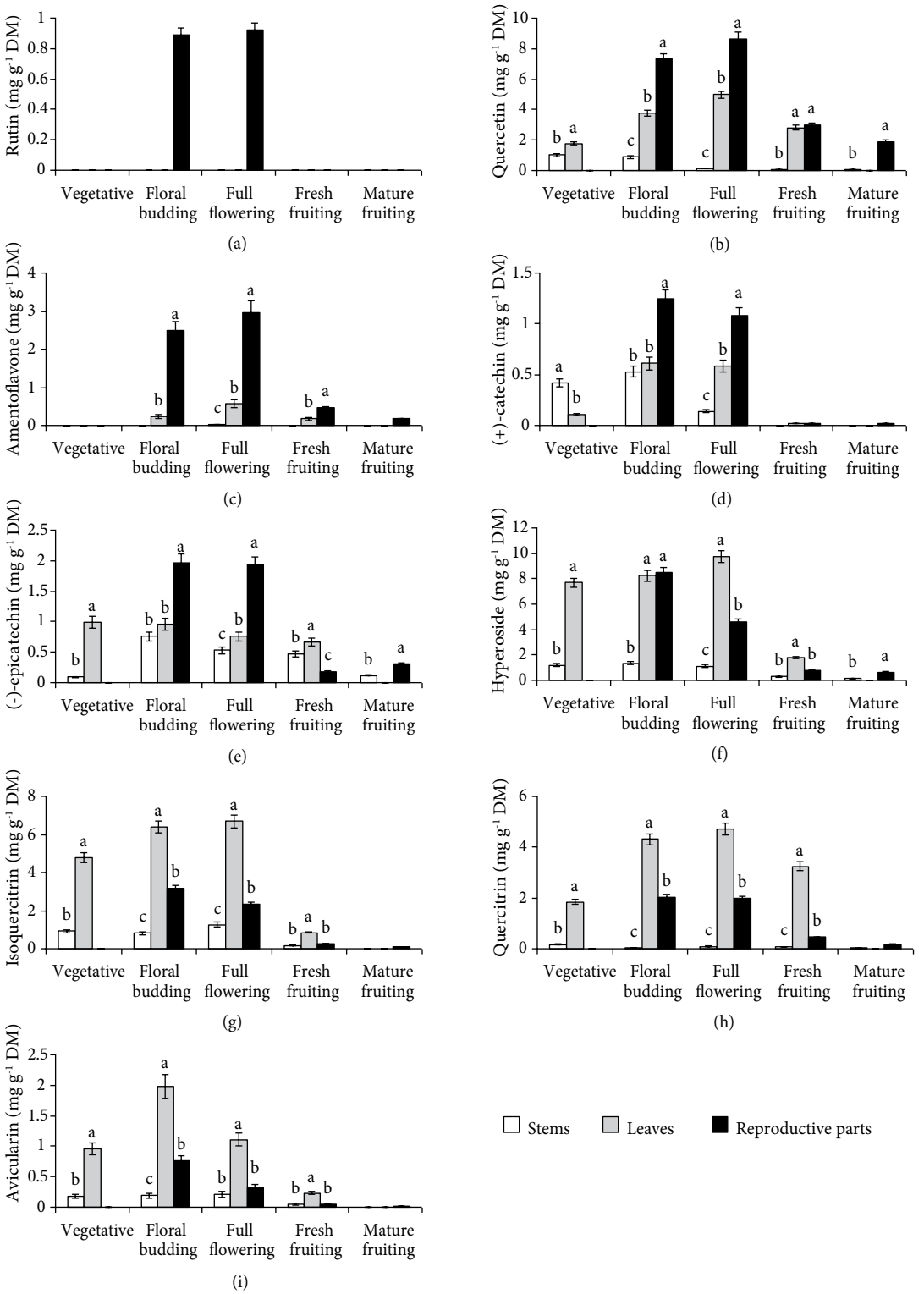


Figure 2. Phenological changes in rutin (a), quercetin (b), amentoflavone (c), (+)-catechin (d), (–)-epicatechin (e), hyperoside (f), isoquercitrin (g), quercitrin (h), and avicularin (i) contents of stem, leaf and reproductive tissues in wild growing *Hypericum pruinatum*. Values with different small letters (a, b, c) within columns for each development stage differ significantly at the level of $P < 0.01$.

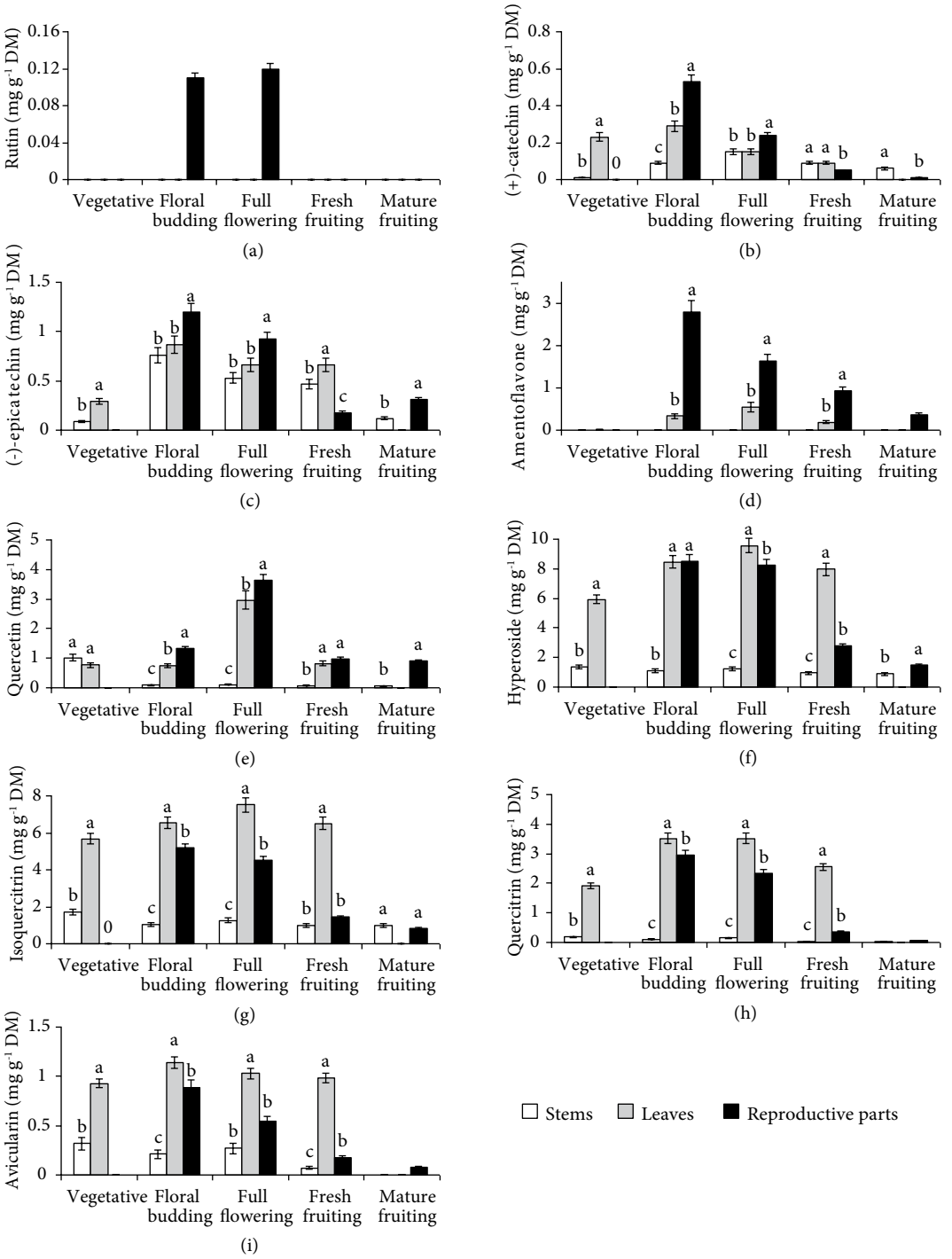


Figure 3. Phenological changes in rutin (a), (+)-catechin (b), (–)-epicatechin (c), amentoflavone (d), quercetin (e), hyperoside (f), isoquercitrin (g), quercitrin (h), and avicularin (i) contents of stem, leaf and reproductive tissues in greenhouse-grown *Hypericum pruinatum*. Values with different small letters (a, b, c) within columns for each development stage differ significantly at the level of $P < 0.01$.

largely corresponded to those described for *H. perforatum*, whose flowers accumulated higher amounts of hypericin, hyperforin, rutin, and quercetin and whose leaves had the highest levels of hyperoside and isoquercitrin (Kazlauskas and Bagdonaite, 2004; Bagdonaite et al., 2010). However, organ-specific accumulation of the tested flavonoids did not correspond to those of other *Hypericum* species. Reproductive parts accumulated the highest level of hyperoside and quercitrin, while leaves produced higher amounts of rutin, isoquercetin, and quercetin in wild and greenhouse-grown *H. triquetrifolium* plants (Çırak et al., 2013b). Similarly, *Hypericum origanifolium* Willd. and *Hypericum perforatum* L. accumulated quercitrin and rutin as well as hypericin, pseudohypericin, and hyperforin (mainly in their floral buds and flowers), while their leaves produced higher amounts of quercetin and chlorogenic acid (Çırak et al., 2007a, 2007b).

In conclusion, the phenological changes in the content of amentoflavone, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (–)-epicatechin in different tissues of wild and greenhouse-grown *H. pruinatum* observed in the present study revealed a close relationship between flavonoid content in plant parts and developmental stages during the phenological cycle. The present results indicated the developmental stages of floral buds and flowering as the most suitable ones for harvesting the plant material, in which the content of the flavonoids investigated reached their highest level. The results from greenhouse experiments indicated that *H. pruinatum* can be cultivated easily and has potential as a medicinal crop. To our knowledge, this is the first report documenting the detailed changes in chemical composition of *H. pruinatum* during its phenological cycle. Thus, the present results might also be useful in obtaining enhanced concentrations of the corresponding compounds. Further studies are currently underway on large-scale production of this promising medicinal plant under field conditions.

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