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Changes in phenolic content of wild and greenhouse-grown *Hypericum triquetrifolium* during plant development

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Abstract: *Hypericum triquetrifolium* is a promising medicinal plant from eastern Europe and the Mediterranean area. The present study was conducted to determine ontogenetic and morphogenetic variation of chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin, and quercetin content in this species. The wild-growing and greenhouse-grown plants were harvested at vegetative, floral budding, full flowering, fresh fruiting, and mature fruiting stages and were dissected into stem, leaf, and reproductive tissues, which were dried separately and subsequently assayed for bioactive compounds by high performance liquid chromatography. Chemical contents in whole plants increased during plant phenology and, generally, higher accumulation levels were observed in wild plants. Depending on the development stages, the reproductive parts accumulated the highest level of hyperoside and quercetin, but the leaves produced a higher amount of chlorogenic acid, rutin, isoquercetin, and quercitrin. According to the results, there is a close relationship between the chemical content in plant parts and development stages during the phenological cycle. The raw material of *H. triquetrifolium* should be harvested during flower ontogenesis for medicinal purposes.

Key words: Chemical variation, high performance liquid chromatography, *Hypericum triquetrifolium*, phenolics, plant phenology

1. Introduction

The genus *Hypericum* L. encompasses approximately 460 species accommodated in 36 sections and has a nearly worldwide distribution, absent only from tropical lowlands, deserts, and Arctic regions (Robson 1977). The genus is represented in Turkey by 89 species, of which 43 are endemic (Bingol et al. 2011). The genus *Hypericum* has been receiving much publicity recently because it is a source of the most important biologically active constituents, namely the phloroglucinol derivatives hyperforin and adhyperforin; the naphthodianthrones hypericin and pseudohypericin; several phenolics such as the flavonoids hyperoside, rutin, quercitrin, quercetin, and biapigenin; and the phenylpropanes caffeic acid and chlorogenic acid, which possess a wide array of biological properties (Kasper et al. 2010). Among the chemicals, phenolic compounds are important for their contribution to the color, sensory attributes, and nutritional and antioxidant properties of plants. Flavonoids especially have attracted considerable interest as dietary constituents, and results from clinical studies indicated their possible role in preventing cardiovascular diseases and several kinds of cancer (Chu et al. 2000). Although hypericins and hyperforin have been reported to mainly contribute to the pharmacological effects of *Hypericum* extracts, flavonoids have also made an important contribution to the antidepressant activity (Gastpar and Zeller 2005).

Hypericum triquetrifolium Turra, native to eastern Europe and the Mediterranean area, is an herbaceous perennial plant from the family Hypericaceae family and grows in open, dry, stony, sandy ground and cultivated fields in Turkey. It has traditionally been used in the treatment of burns and gastrointestinal disease in Turkish folk medicine (Baytop 1999). Results from recent studies reporting the antinociceptive (Apaydın et al. 1999), antiinflammatory (Ozturk et al. 2002), antioxidant (Conforti et al. 2002), antibacterial (Pistelli et al. 2005), antifungal (Fraternale et al. 2006), and cytotoxic (Conforti et al. 2007) activities of *H. triquetrifolium* signal the great potential of this species as a promising medicinal plant.

Increased market demand for *Hypericum*-derived products has led to many investigations on secondary metabolite variations in several *Hypericum* species. These previous investigations were generally carried out on *Hypericum perforatum* L. and did not give homogeneous results. Furthermore, they were limited by only studying

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hypericins (Sirvent et al. 2002; Çırak et al. 2006), hyperforins (Kirakosyan et al. 2002), or essential oils (Radusiene et al. 2005; Çırak et al. 2010), and plant developmental stages were ignored in most of them. To date, little effort has been dedicated to the study of variation of flavonoids such as quercitrin, isoquercitrin (Martonfi and Repcak 1994), and quercetin (Tekeľová et al. 2000). In particular, phenological changes in the content of volatile components and several phenolics in wild-growing *H. triquetrifolium* plants were reported previously from Tunisia by Hosni et al. (2011). However, no study, to our knowledge, has been done on the variation of chemical contents in greenhouse-grown *H. triquetrifolium* plants. Thus, the present study was conducted to determine the relationships between the content of main flavonoids, namely chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, and isoquercetin, in plant materials and development stages in both wild and greenhouse-grown *H. triquetrifolium*. This study also describes the attempt to compare chemical contents of the same species from wild and modified culture conditions.

2. Materials and methods

2.1. Plant materials

The species was identified by Dr Hasan Korkmaz, Department of Biology, Faculty of Science and Arts, Ondokuz Mayıs University, Samsun, Turkey. Voucher specimens were deposited in the herbarium of the Ondokuz Mayıs University Agricultural Faculty (OMUZF #134).

2.2. Experimental procedures

Wild *H. triquetrifolium* plants at different stages of their development were collected from the Erbaa district of Tokat Province, Turkey (41°04′N, 36°01′E; 470 m above sea level) between April and October of 2010. The material represented 30 randomly gathered plants in 5 phenological stages: vegetative, floral budding, full flowering, fresh fruiting, and mature fruiting. Newly emerged shoots with leaves were considered to be plants in the vegetative stage. Only shoots with floral buds were selected as plants of floral budding stage. At the full flowering stage, only shoots with fully opened flowers were harvested. For the fresh and mature fruiting stages, the shoots that had green and dark brown capsules, respectively, were harvested.

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

The tops of 2/3 wild and cultured plants, in which many phytochemicals tend to accumulate, were harvested between 0000 and 1300 hours. Ten individuals were kept as whole plants; the remaining were dissected into floral, leaf, and stem tissues, were dried at room temperature (20 ± 2 °C), and were then assayed for chemical contents by high-performance liquid chromatography (HPLC).

2.3. 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 pore size of 0.22 μm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator at 4 °C for no longer than 3 h until analysis.

2.4. HPLC analysis

A Shimadzu Prominence LC-20A (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with 2 LC - 20AD model pumps, an SIL - 20AC autoinjector, a CTO - 20AC thermostat, and an SPD - M20A detector was used for HPLC analysis. Separation of all the compounds was carried out using a YMC Pack

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^{-1}): 210	
Fertilizer (g L^{-1}): 1.5	Phosphorus (mg P_2O_5 I^{-1}): 240	<10 / 80-85 / 5-10
Black sphagnum peat: 30%	Potassium (mg K_2O I^{-1}): 270	0 20 40 60 Vol.-% 100
White sphagnum peat: 70%	Magnesium (mg Mg I^{-1}): 100	■ Dry matter ▫ Water capacity □ Air capacity

Pro - C18 (YMC Europe GmbH, Dinslaken, Germany) column (150 mm × 4 mm i.d.; 3-µm particle sizes) with 10-mm guard precolumn. For the mobile phase, 0.1% aqueous trifluoroacetic acid (TFA) was used as eluent A and acetonitrile containing 0.1% TFA as eluent B. The following binary gradient elution program was used: 0–1 min (B 5% → 5%), 1–14 min (B 5% → 20%), 14–20 min (B 20% → 80%), 20–30 min (B 80% → 100%), 30–39 min (B 100% → 100%), 39–39.5 min (B 100% → 5%), and 39.5–45 min (B 5% → 5%). The mobile phase was delivered with a flow rate of 1.0 mL min^{-1}; the volume of extract injected was 10 µL. Detection was performed at the 210–790 nm wave length range with a constant column temperature of 40 °C. The eluted compounds were identified by comparing their retention time and UV wavelengths with those of reference standards. The maximal absorption in the UV spectra of compounds was obtained as follows: chlorogenic acid, 325 nm; rutin, hyperoside, and isoquercetin, 353 nm; quercitrin, 347 nm; and quercetin, 368 nm. The quantities of compounds were calculated from an external standard calibration curve established from 6 concentrations in the following ranges: 6.0–300 µg mL^{-1} for chlorogenic acid and rutin, 3.0–300 µg mL^{-1} for hyperoside and isoquercetin, and 2.0–200 µg mL^{-1} for quercitrin and quercetin. All calibration curves showed good linear regression ($r^2 \geq 0.999$) within the test range. Each sample was analyzed twice, and the mean value was used for calculation. The concentration of compounds was expressed as mg g^{-1} dry mass (DM). The HPLC chromatogram of *H. triquetrifolium* methanolic extracts at 353 nm is shown in Figure 1.

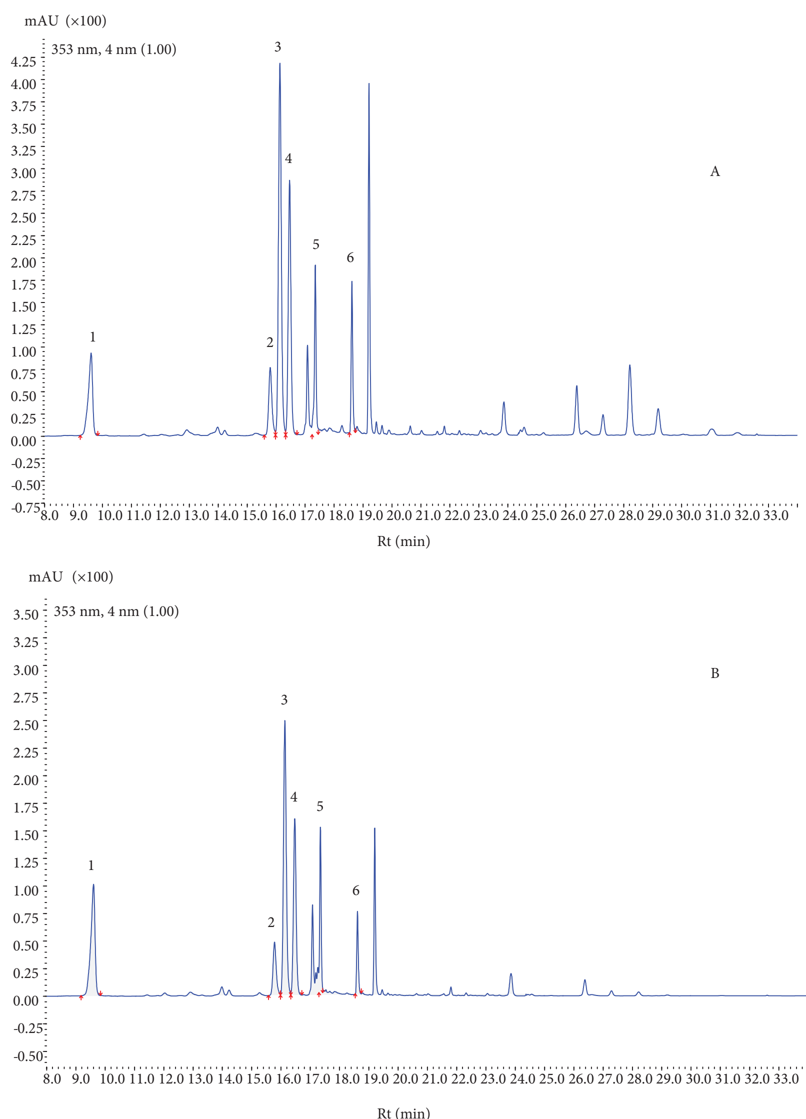

Figure 1. Typical HPLC chromatograms of wild (a) and greenhouse-grown (b) *Hypericum triquetrifolium* flowers extract detected by UV at 353 nm wavelength. Peaks identified: 1 – chlorogenic acid (Rt – 9.61 min), 2 – rutin (Rt – 15.79 min), 3 – hyperoside (Rt – 16.13 min), 4 – isoquercetin (Rt – 16.46 min), 5 – quercitrin (Rt – 17.35 min), and 6 – quercetin (Rt – 18.61 min).

2.5. Chemicals

The reference substances chlorogenic acid (purity: 98.03%), rutin trihydrate (purity: 99.02%), isoquercetin (purity: 99.0%), and quercitrin (purity: 99.0%) were purchased from Karl Roth (Germany). Hyperoside (purity: 98.6%) and quercetin (purity: 95.4%) reference materials were obtained from ChromaDex (USA). Acetonitrile and methanol of HPLC grade were supplied by Karl Roth (Germany).

2.6. Data analysis

Data for chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin, and quercetin contents of the 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 the Duncan multiple range test ($P < 0.01$) by using MSTAT-C statistical software (Russell D Freed, Crop and Soil Sciences Department, Michigan State University, USA).

3. Results

The differences among chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin, and quercetin contents of the whole plant at different development stages were found to be significant ($P < 0.01$). Chemical contents in whole plants increased during plant phenology and, generally, higher accumulation levels were observed in wild plants than in cultivated ones. The highest levels of chlorogenic acid, hyperoside, and quercitrin were reached at full flowering (7.84, 15.67, and 7.98 mg g^{-1} DM, respectively), and wild plants harvested at floral budding produced the highest amounts of rutin, quercetin, and isoquercetin (5.93, 0.92, and 24.65 mg g^{-1} DM, respectively) (Table 2). In greenhouse-grown plants, similarly, the chemical content in whole plants increased during plant phenology and plants harvested at full flowering produced the highest amounts of rutin, hyperoside, quercitrin, quercetin, and isoquercetin (1.52, 3.96, 1.49, 0.99, and 15.46 mg g^{-1} DM, respectively). However, as an exception, chlorogenic acid content in the whole plant was the highest at the floral budding stage (9.09 mg g^{-1} DM) (Table 3). After the development of buds and flowers, the content of those compounds decreased with the advancing of fruit development, and the lowest level of each compound was detected at the mature fruiting stage in both wild and greenhouse-grown plants.

Table 2. Chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, and isoquercetin content (mg g^{-1} DM) in variations of wild-growing *Hypericum triquetrifolium* whole plant during its phenological cycle.

Phenological stage	Chlorogenic acid	Rutin	Hyperoside	Quercitrin	Quercetin	Isoquercetin
Vegetative	4.45 c	2.47 c	3.22 d	4.52 b	0.36 c	17.49 b
Floral budding	6.86 b	5.93 a	9.32 b	7.64 a	0.92 a	24.65 a
Full flowering	7.84 a	3.61 b	15.67 a	7.98 a	0.64 b	7.33 d
Fresh fruiting	3.48 d	1.22 d	5.93 c	5.68 b	0.62 b	9.10 c
Mature fruiting	0.33 e	0.14 e	0.37 e	0.29 c	0.39 c	1.38 e

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

Table 3. Chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, and isoquercetin content (mg g^{-1} DM) in variations of greenhouse-grown *Hypericum triquetrifolium* whole plant during its phenological cycle.

Phenological stage	Chlorogenic acid	Rutin	Hyperoside	Quercitrin	Quercetin	Isoquercetin
Vegetative	2.20 d	0.36 b	1.31 c	0.61 b	0.66 b	3.27 c
Floral budding	9.07 a	1.03 a	3.31 a	1.05 a	0.36 b	15.01 a
Full flowering	6.06 b	1.52 a	3.96 a	1.49 a	0.99 a	15.46 a
Fresh fruiting	4.58 c	1.19 a	2.89 b	0.51 b	0.35 b	11.03 b
Mature fruiting	0.44 e	0.04 c	0.19 d	0.27 b	0.27 b	0.23 d

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

Significant differences were also observed among stem, leaf, and reproductive tissues with regard to chemical accumulation and phenological fluctuation of each compound ($P < 0.01$). Depending on development stages, reproductive parts accumulated the highest level of hyperoside and quercetin; however, leaves produced higher amounts of chlorogenic acid, rutin, isoquercetin, and quercitrin. In wild plants, hyperoside and quercetin contents were the highest in fully opened flowers and floral buds (20.27 and 2.59 mg g^{-1} DM, respectively) (Figures 2a and 2b). Leaves harvested at the floral budding stage produced the highest contents of rutin and isoquercetin (6.66 and 30.42 mg g^{-1} DM, respectively). Similarly, chlorogenic acid and quercitrin contents were the highest in leaf tissues of plants harvested at full flowering and fresh fruiting stages (10.85 and 9.10 mg g^{-1} DM, respectively) (Figures 2c–2f). The same chemical accumulation manner was also observed among different plant parts in greenhouse-grown plants. Hyperoside and quercetin were accumulated mainly in reproductive parts, and their highest level was detected in fully opened flowers and fresh fruits (8.55 and 1.50 mg g^{-1} DM, respectively) (Figures 3a and 3b). On the contrary, leaves were found to be superior over reproductive parts in terms of rutin, isoquercetin, chlorogenic acid, and quercitrin accumulations (Figures 3c–3f).

4. Discussion

Secondary metabolite concentrations in a given plant species and/or tissues can vary significantly during the course of ontogenesis, which may be relevant for the utilization of medicinal plants. In the present study, the

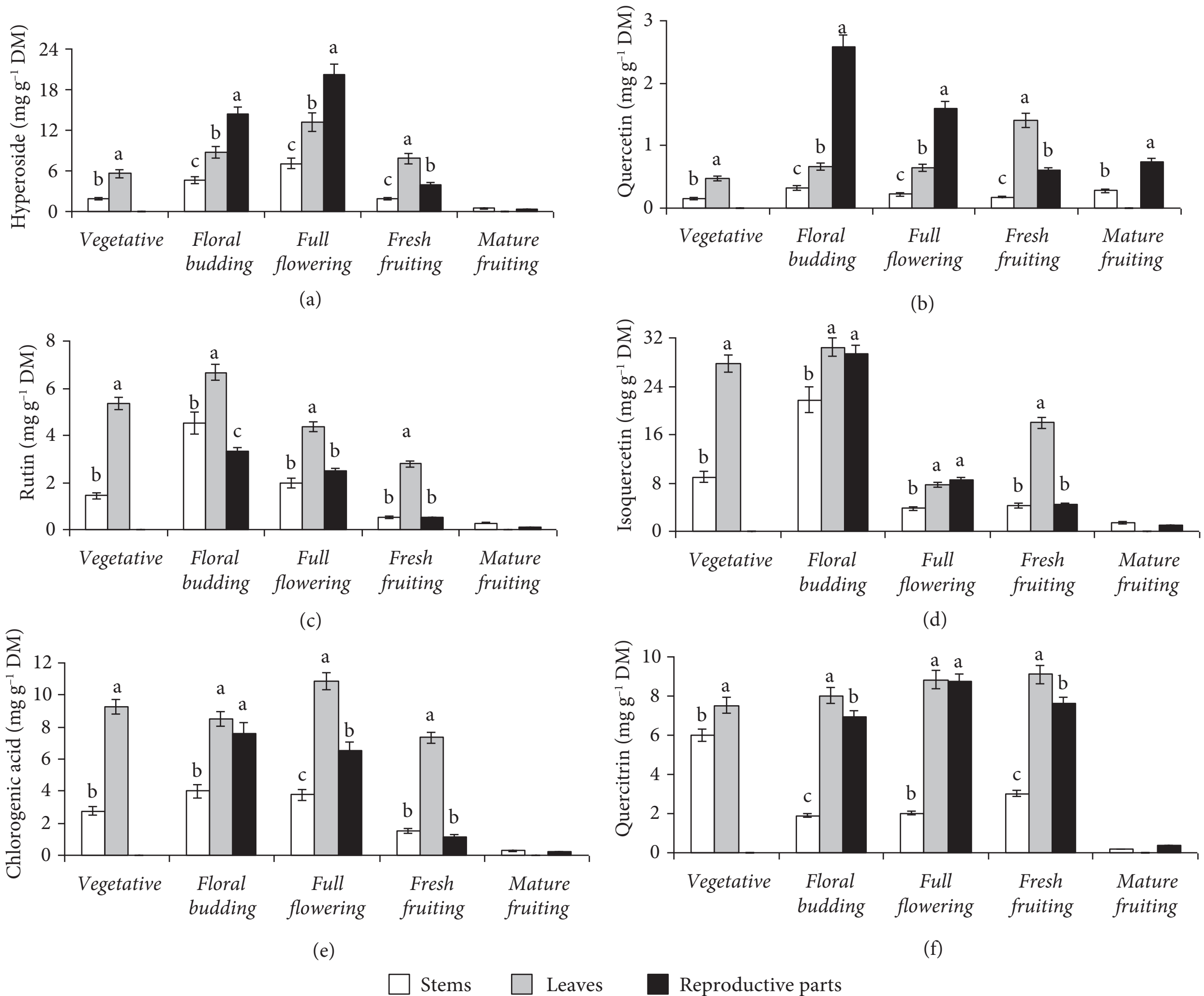

Figure 2. Phenological changes in hyperoside (a), quercetin (b), rutin (c), isoquercetin (d), chlorogenic acid (e), and quercitrin (f) content of stem, leaf, and reproductive tissues in wild-growing *Hypericum triquetrifolium* (values with different letters within columns for each development stage differ significantly at the level of $P < 0.01$).

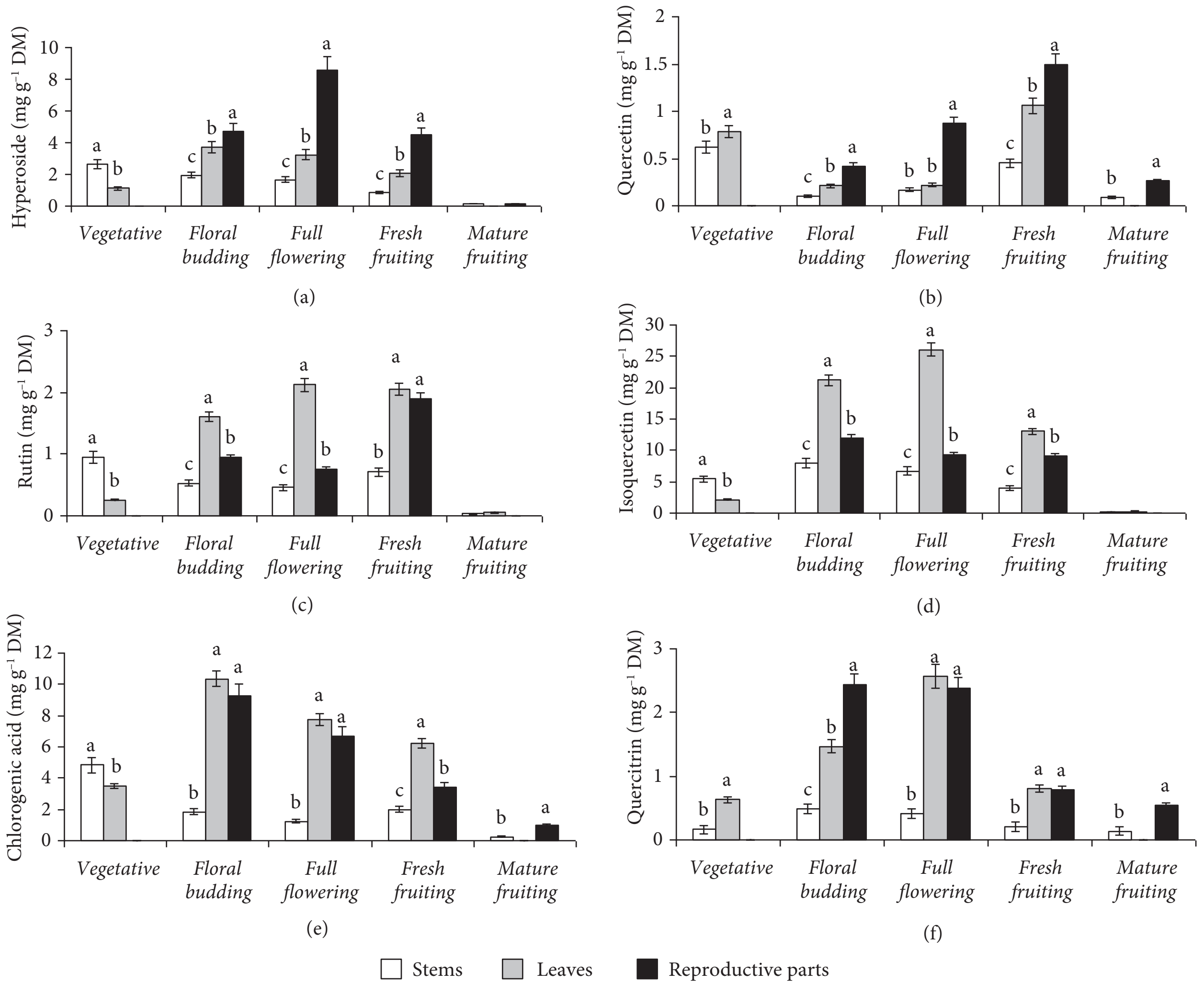

Figure 3. Phenological changes in hyperoside (a), quercetin (b), rutin (c), isoquercetin (d), chlorogenic acid (e), and quercitrin (f) content of stem, leaf, and reproductive tissues in greenhouse-grown *Hypericum triquetrifolium* (values with different letters within columns for each development stage differ significantly at the level of $P < 0.01$).

highest levels of all tested chemicals in whole plants were reached at flowering time in both wild and greenhouse-grown *H. triquetrifolium* plants. Similarly, the highest levels of rutin, hyperoside, quercitrin (Hosni et al. 2011), hypericin, and pseudohypericin (Ayan and Çırak 2008) in wild *H. triquetrifolium* were identified at flowering. These results also largely correspond to those obtained for other *Hypericum* species. Kazlauskas and Bagdonaite (2004) reported that the highest accumulation of hypericin, as well as rutin, quercetin, and isoquercetin, was observed during the development of flowering buds and at flowering time in *Hypericum perforatum* L., a well-known and globally distributed species. Abreu et al. (2004) reported that the highest concentration of rutin, quercetin, and several phenolics was found during flowering in greenhouse-grown *Hypericum brasiliense* Choisy. Similarly, chlorogenic acid, hyperoside, quercitrin, quercetin, and hypericin contents in the whole plant increased during the course of ontogenesis, and the highest levels were reached during flower ontogenesis in *Hypericum origanifolium* Willd. (Çirak et al. 2007), *Hypericum perfoliatum* L. (Çırak et al. 2007), and *Hypericum montbretii* Spach (Çırak et al. 2008).

The differences in chemical composition between leaves and flowers found in the present study did not match those described for *H. perforatum*, whose flowers accumulated larger amounts of hypericin, hyperforin, rutin, quercetin, and quercitrin and whose leaves had the highest level of hyperoside (Büter and Büter 2002; Kazlauskas and Bagdonaite 2004; Bagdonaite et al. 2010). In other species of *Hypericum*, similarly, quercitrin, rutin, hypericin, pseudohypericin, and hyperforin were

accumulated mainly in floral parts, while the leaves produced higher amounts of quercetin, chlorogenic acid, and hyperoside in *H. origanifolium* and *H. perfoliatum* (Çırak et al. 2007, Çirak et al. 2007). The difference is not surprising and probably depends on genetic differences of *Hypericum* species.

In the present study, it is interesting to note that generally higher accumulation levels of the tested compounds were observed in wild plants. Plants in the wild are subjected to many biotic/abiotic stress factors and have evolved different defense systems to avoid the damage caused by these stress factors (Estaban-Carrasco et al. 2001). The first step of defense is based on the activation of preexisting components, resulting in overproduction of toxic compounds. In this sense, most phenolics have been considered to be involved in the chemical defense arsenal of plants against biotic/abiotic stress, i.e. herbivores, plant pathogens, and soil salinity (Close and McArthur 2002). In previous studies on several *Hypericum* species, the content of phenolic compounds, namely rutin, quercetin, and total soluble phenols, was reported to increase significantly in response to water and temperature stress in *H. brasiliense* (Abreu and Mazzafera 2005). *H. perforatum* plants subjected to a brief drought stress showed an increase of quercetin, rutin (Gray et al. 2003), and hyperforin (Zobayed et al. 2007). Germ et al. (2010) pointed out the defensive roles of flavonoids and tannins in plant metabolism by observing the increased leaf concentrations of flavonoids in *H. perforatum* plants exposed to a high level of UV-B radiation. The higher secondary metabolite accumulation in wild *H. triquetrifolium* plants in the present study may be attributed to the biotic/abiotic stress factors, possibly present in the experimental collection site.

Based on ontogenetic and morphogenetic changes in the content of chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin, and quercetin in wild and greenhouse-grown *H. triquetrifolium*, it can be concluded that there is a close relationship between the chemical content of plant parts and developmental stages during the phenological cycle of the plants. For medicinal purposes, plant material should be harvested during flower ontogenesis, during which the content of the tested bioactive substances reached their highest level. The present results also indicated that this medicinal plant can be cultivated easily in greenhouse conditions, but culturing the species in pots resulted in a decrease in the chemical content, possibly due to the controlled environment. Nevertheless, this decrease can be omitted when considering the fact that the main issue in medicinal plant standardization is the huge variability in secondary metabolite profile of a given plant material. Further studies are currently underway on large-scale production of the medicinal plant under field conditions.

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