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Hypericins in *Hypericum montbretii*: Variation among Plant Parts and Phenological Stages

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ABSTRACT

In the present study, morphogenetic and phenological variations of hypericin and pseudohypericin were investigated in *Hypericum montbreti*, a perennial herbaceous plant from Turkish flora for the first time. Wild growing plants were harvested at the vegetative, floral budding, full flowering, fresh fruiting and mature fruiting stages and dissected into stem, leaf and reproductive tissues and assayed for hypericin and pseudohypericin by HPLC. Phenological fluctuation in hypericin and pseudohypericin content of plant material including whole shoots, stems, leaves and reproductive parts was found to be significant ($P<0.01$). Hypericin and pseudohypericin content in whole shoots, leaves and reproductive parts increased during the course of ontogenesis. The highest level of both compounds was reached at full flowering. In contrast, hypericin and pseudohypericin content in stems decreased with an advancement of plant development and stems from newly emerged shoots at the vegetative stage produced the highest level of both compounds. Among different plant tissues, reproductive parts were found to be superior than leaves and stems with regard to both hypericin and pseudohypericin accumulation.

Keywords: high performance liquid chromatography, infrageneric classification, morphogenetic variation, plant phenology, pseudohypericin

INTRODUCTION

Hypericum species have been of great interest to mankind for many centuries and have been used for medicinal purposes due to their various medicinal properties for hundred of years (Demirci *et al*. 2005). *Hypericum* species are also used as sedatives, antiseptics, and antispasmodics in Turkish folk medicine (Baytop 1999). *Hypericum* genus of the family Guttiferae is represented in Turkey by 89 species of which 43 are endemic. This genus is widespread throughout Turkey and the most abundant and well known species is *H. perforatum* L. (Davis 1988).

H. montbretii Spach is a perennial herbaceous plant which grows in damp or shady places among rocks. This plant is widely distributed in Northern Turkey as well as in Balkans, Syria and Georgia. This plant has a great pharmaceutical potential with its well documented hypericins and flavonoids content (Çırak *et al*. 2007).

The methanolic extract from the aerial parts of several *Hypericum* species has been reported to contain many of bioactive compounds from at least ten different classes namely the naphthodianthrones hypericin and pseudohypericin (Kitanov 2001), the phloroglucinol derivatives hyperforin and adhyperforin (Maggi *et al*. 2004; Smelcerovic *et al*. 2006), flavonoids (Radušienė *et al*. 2004), phenylpropanes (Chandrasekera *et al*. 2005), essential oils (Bertoli *et al*. 2003), amino acids, xanthones (Tanaka and Takaishi 2006), tannins (Dall'Agnol *et al*. 2003), procyanidins and other water-soluble components (Greeson *et al*. 2001) which possess a wide array of biological properties.

Many pharmacological activities of *Hypericum* extracts appear to be attributable to their hypericins and hyperforin content (Barnes *et al*. 2001). The naturally occurring red pigments hypericin and pseudoyhpericin have been reported to exhibit important biological activities, namely photodynamic, antiviral, antiretroviral, antibacterial, antipsoriatic, antidepressant and antitumoral activities (Gadzovska *et al*. 2005; Guedes and Eriksson 2005). The photodynamic and photocytotoxic properties of hypericins allow them to acts as antiviral agents indicating their possible use in the treatment of human immunodeficiency virus type 1 (HIV-1) (Meruelo *et al*. 1988) and cancer (Agostinis *et al*. 2002). Hypericins have been found only in *Hypericum* species, thus, are chemotaxonomically important for the infrageneric classification of *Hypericum* genus (Robson 1981). Although hyperforin is a major component occurring in concentrations of 2-4% of the total extract of *H. perforatum*, hypericins remain the popular marker substances for the standardization of *Hypericum* products because of instability of hyperforin in the presence of oxygen and light (Gerlie and Koda 2001). Thus, hypericins have importance from quality control point of view. Due to these reasons, many individual or groups of species of *Hypericum* have been investigated for the presence of hypericins to date (Martonfi and Repcak 1994; Ferraz *et al*. 2002; Alali *et al*. 2004; Ayan *et al*. 2004; Piovan *et al*. 2004; Radušienė *et al*. 2004; Çırak 2006; Çırak *et al*. 2006). In previous studies, *H. montbretii* was reported to contain either abundant (Kitanov 2001; Çırak *et al*. 2007) or trace (Crockett *et al*. 2005) amount of hypericin and pseudohypericin. To our knowledge, no phytochemical investigation was performed on the variability of those natural compounds in *H. montbretii*. In the present study, we report variation of both hypericin forms among plant parts and phenological stages in this species for the first time.

MATERIALS AND METHODS

Plant material

The species was identified by Dr. Hasan Korkmaz, Faculty of Science and Art, Department of Biology, University of 19 Mayis, Samsun-Turkey. A voucher specimen was deposited in the herbarium of Ondokuz Mayis University Agricultural Faculty (OMUZF #100).

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Experimental procedures

The plant material of *H. montbretii* was collected from Çakallı district of Samsun province, Turkey (41° 04′ N; 36° 01′ E; 470 m above sea level) in April-September period of 2005. The sampling site was not grazed or mown during the plant gathering period. The material represented 20 randomly gathered plants in five phenological stages: vegetative, floral budding, full flowering, fresh fruiting and mature fruiting. Newly emerged shoots (4-6 weeks old-age) with leaves were harvested at the vegetative stage (April 27, 2005). For the floral budding stage, only shoots with floral buds were selected (May 20, 2005). At the full flowering stage, only shoots with full opened flowers were harvested (June 14, 2005). At the fresh fruiting stage, the shoots which had green capsules were harvested (July 5, 2005). At the mature fruiting stage, the shoots which had dark brown capsules were harvested (August 10, 2005). The top ⅔ of the plant was harvested between 12:00 am and 13:00 pm. After collected, 10 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 assayed for chemical contents by HPLC. The plant material including leaf, petal and stem were also photographed by using a light microscope to observe the presence of dark glands in aerial parts.

Preparation of plant extracts

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

HPLC analysis

HPLC analysis with UV/PDA detection was performed using a model Waters 2690 chromatography system (Waters, Milford, USA), equipped with a Waters 2487 UV/Vis detector and Waters 996 PDA detector. For separation a Hichrom column Hypersil H5ODS-150A 150×4.6 mm (Hichrom Limited, UK) and a H5ODS-10C guard-precolumn were used.

Hypericin and pseudohypericin were analyzed according to Pierluigi and Piergiorgio (2000) and a modified HPLC method described in Pharmeuropa (2004). The elution program was isocratic. The mobile phase consisted of ethyl acetate/15.6 g/L, solution of sodium dihydrogen phosphate NaH_2PO_4 and methanol (39:41:160). The flow rate: 1.0 ml/min; injection volume: 10 µL. The column temperature was at 20°C. The elution was monitored at 590 nm and the obtained data were compared with standard samples of hypericin and pseudohypericin. The quantity of compounds was calculated from an external standard calibration in the concentration range of 0.5–100.0 µg/mL (r^2 = 0.997). Each sample was analyzed twice and the mean value was used for calculation. Typical HPLC chromatogram of the flower extract is shown in **Fig. 1**.

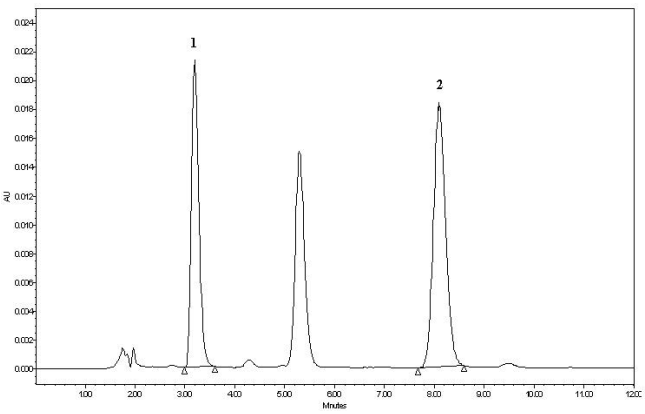

Fig. 1 Typical HPLC chromatogram of *Hypericum montbretii* flowers extract. 1= hypericin; 2= pseudohypericin.

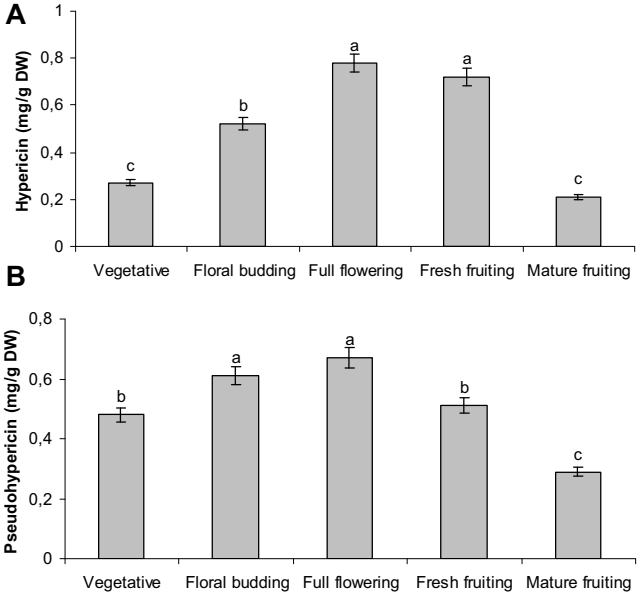

Fig. 2 Hypericin (**A**) and pseudohypericin (**B**) content variation in whole plant of *Hypericum montbretii* at different stages of plant phenology. Values with different small letters (a, b, c) within each development stage differ significantly at P<0.01; Bars are ± SE.

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

Data analysis

Data for hypericin and pseudohypericin content of plant material including whole plant, stem, leaf and reproductive parts were subjected to ANOVA and significant differences among mean values were tested with the Duncan's Multiple Range Test (P<0.01) by using MSTAT statistical software.

RESULTS

Depending on the phenological stage, whole shoots and reproductive parts produced more hypericin while leaves and stems accumulated mainly pseudohypericin. Phenological fluctuation in hypericin and pseudohypericin content of whole shoots was found to be significant (P<0.01). Hypericin and pseudohypericin content in whole shoots increased during the course of ontogenesis. The highest level of both compounds was reached at full flowering (0.78 mg/g DW for hypericin and 0.67 mg/g DW for pseudohypericin). After the development of buds and flowers, the content of those compounds decreased as fruit development advanced (**Fig. 2**).

The difference in hypericin and pseudohypericin content of stem, leaf and reproductive tissues during plant development was also found to be significant (P<0.01). Similar to whole shoots, hypericin and pseudohypericin content in leaves and reproductive parts increased as plant development advanced and the highest accumulation level of both compounds was observed at full flowering (0.79 mg/g DW hypericin and 0.94 mg/g DW pseudohypericin for leaves; 1.80 mg/g DW hypericin and 1.50 mg/g DW pseudohypericin for full opened flowers, respectively). In contrast, hypericin and pseudohypericin content in stems decreased during the course of ontogenesis and stems from newly emerged shoots at vegetative stage produced the highest level of both compounds (0.18 mg/g DW for hypericin and 0.30 mg/g DW for pseudohypericin). Among different plant parts, reproductive tissues were found to be superior over leaves and stems with regard to both hypericin and pseudohypericin accumulation (**Fig. 3**). Besides, morphological observations revealed that all aerial parts including stems, leaves and flowers bear dark glands. The presence of hypericin and

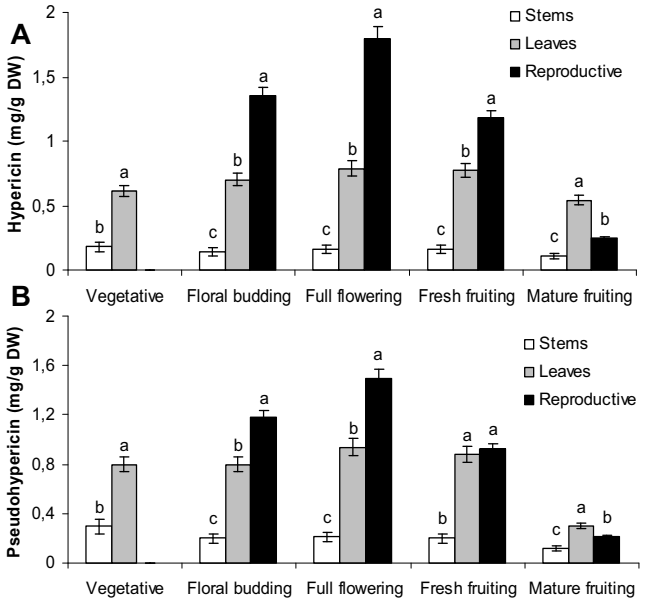

Fig. 3 Ontogenetic changes in hypericin (**A**) and pseudohypericin (**B**) content of stem, leaf and reproductive tissues in *Hypericum montbretii*. Values with different small letters (a, b, c) within each development stage differ significantly at P<0.01; Bars are ± SE.

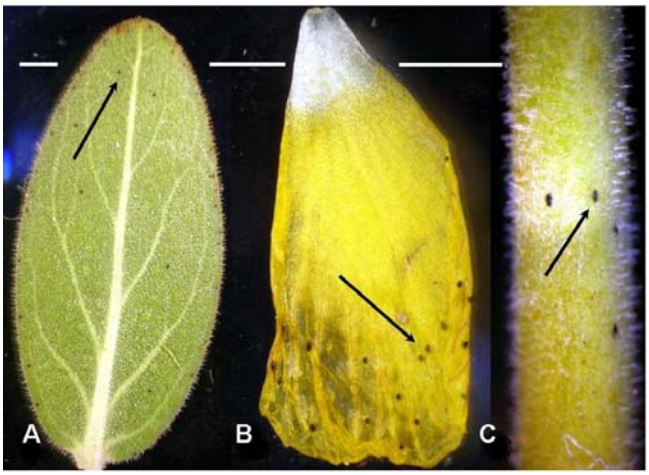

Fig. 4 The dark glands in leaf (**A**) petal (**B**) and stem (**C**) of *Hypericum montbretii*. Arrows indicate the dark glands and bars represent 1 mm.

pseudohypericin in the aforesaid tissues was found to be consistent with the presence of dark glands (**Fig. 4**).

DISCUSSION

Naphthodianthrones are mainly composed of hypericin, pseudohypericin and their precursor namely, protohypericin and protopseudohypericin (Patocka 2003). Although hypericin has been paid major attention in pharmaceutical research, the principal naphthodianthrone in *Hypericum* extracts is pseudohypericin. Generally, it occurs two to three times more abundantly than hypericin in the species of *Hypericum* containing them (Cameron and Raverty 1976). Our results partially confirmed this phenomenon and accumulation of hypericin and pseudohypericin were balanced among different plant parts of *H. montbretii* in the present study.

Chemical content composition of a medicinal plant may vary substantially with the developmental stage of the plants. Therefore, investigations on ontogenetic variation of secondary metabolites from different classes have received considerable interest from plant scientists over several decades. In particular, growth and development of the reproductive parts of *Hypericum* plants is generally followed by acceleration of secondary metabolism resulting in enhanced accumulation of different chemical compounds such as flavonoids e.g. rutin, quercetin, isoquercetin, hyperoside in *H. perforatum* (Kazlauskas and Bagdonaite 2004), *H. brasiliense* (Abreu *et al*. 2004), *H. maculatum* (Martonfi *et al*. 2006), total phenolics (Ayan *et al*. 2006) and hypericin (Çırak *et al*. 2006) in *H. perforatum*, *H. pruinatum* and *H. avicullariifolium*, and hyperforin in *H. perforatum* (Büter and Büter 2002; Couceiro *et al*. 2006). Our findings in the present study confirmed this phenomenon. Hypericin and pseudohypericin contents in whole shoots as well as leaves and reproductive parts of *H. montbretii* increased during plant growth and the highest level of both compounds were reached at full flowering. In previous studies, floral parts were reported as main storage organs for hypericin accumulation in several *Hypericum* species such as *H. perforatum* (Kazlauskas and Bagdonaite 2004), *H. maculatum* (Radušienė *et al*. 2004), *H. lydium* (Çırak 2006), *H. pruinatum* and *H. avicullariifolium* (Çırak *et al*. 2006). Similarly, flowers of *H. montbretii* produced more hypericin and pseudohypericin compared to other tissues in the present study.

Morphologically, *Hypericum* plants are characterized by the presence of different kind of secretory structures including light glands, dark glands and secretory canals (Ciccarelli *et al*. 2001). Dark glands are also known as 'nodules' or 'black nodules' (Maggi *et al*. 2004). This gland is the most important secretory structure in *Hypericum* plants. Because, hypericins are produced in the dark glands (Lu *et al*. 2001; von Poser *et al*. 2006; Zobayed *et al*. 2006) and the occurrence of dark glands in an organ is regarded as a reliable indicator of the presence of hypericins in a given species (Robson 1981). In previous studies, we found a close relationship between dark gland density of leaves and hypericin content in several *Hypericum* species from Turkish flora such as *H. avicullariifolium*, *H. perforatum*, *H. pruinatum* (Çırak *et al*. 2006) and *H. lydium* (Çırak 2006). This was also confirmed by the present study for *H. montbretii*. We observed that all aerial parts were covered by the dark glands and the presence of hypericin as well as pseudohypericin in these tissues is consistent with the presence of dark glands (**Fig. 4**). The infrageneric section to which *H. monbretii* belongs is *Drosocarpium* Spach. (Robson 1981) and the members of this section are distinguished by the presence of dark glands in their aerial parts (Davis 1988). It is important to note that several species from this section namely *H. umbellatum*, *H. richeri*, *H. rochelii*, *H. boissieri*, *H. barbatum*, *H. rumeliacum*, *H. bithynicum* and *H. perfoliatum* had already been reported to contain hypericin and pseudohypericin (Kitanov 2001; Ayan *et al*. 2004). Hence, detection of hypericins in different tissues of *H. montbretii* in the present study supports the taxonomic position of the section *Drosocarpium* Spach. within the genus *Hypericum* and indicates the naphthodianthrones as chemical markers of the phylogenetically more advanced sections of the genus *Hypericum*.

Previous literature reports hypericin concentrations ranging from 0.01 to 3.87 mg/g DW from USA (Sirvent *et al*. 2002), Canada (Jensen *et al*. 1995), Australia (Southwell and Bourke 2001) and Turkey (Çırak *et al*. 2006) in *H. perforatum*. Our values for *H. montbretii* ranged from 0.09-1.80 mg/g DW for hypericin and 0.10-1.50 mg/g DW for pseudohypericin depending on ontogenetic and morphogenetic sampling. It can be concluded that *H. montbretii* accumulate moderate quantities of both hypericin forms when compared to *H. perforatum*, a well known and commercial source of hypericins.

In conclusion, results from the present study indicated a close relationship between hypericin and pseudohypericin content of plant tissues and growth stages in *H. montbretii*. This is the first report documenting the variation of hypericins in this species. Considering the significance of hypericins from a pharmacological and taxonomical point of view, the present findings may be useful for phytochemical evaluation of *H. montbretii* and support the rather advanced position given to the section *Drosocarpium* Spach.

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