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Determination of hypericin and hyperforin content in selected Jordanian *Hypericum* species

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ABSTRACT

A reversed-phase HPLC method with photodiode array detection was established for the simultaneous analysis of hypericin and hyperforin in the methanolic extracts of three widely growing *Hypericum* species of Jordanian origin, namely: *Hypericum empetrifolium* Willd., *Hypericum sinaicum* Hochst. & Steud. ex Boiss, and *Hypericum triquetrifolium* Turra. The samples were extracted with methanol under reflux at 50 °C. The components were separated by RP-C_{18} chromatography column using gradient mobile phase consisting of 20 mM ammonium acetate–acetonitrile over 45-min with 1 mL/min flow rate, wavelength range 250–600 nm (at 287 nm for hyperforin and 590 nm for hypericin). Quantification of hypericin and hyperforin was performed using external reference standards. The standard curves were linear over the concentration ranges, 10–100 ppm for both hypericin and hyperforin. Wide-ranging differences were observed in hypericin and hyperforin content among the investigated *Hypericum* species. Their hypericin content varied from 0.002% to 0.020%, while their hyperforin content varied from 0.013% to 0.989%. *H. sinaicum* showed the highest hyperforin content of 0.989% (w/w); while *H. triquetrifolium* and *H. sinaicum* showed the highest hypericin content of ~0.020% (w/w). This method can be utilized as an invaluable tool for standardization and quality evaluation of St. John's wort herbal preparations simultaneously against hypericin and hyperforin, which are the generally accepted marker compounds.

1. Introduction

The genus *Hypericum*, which comprises about 450 species of herbs, shrubs and trees, belongs to the family Clusiaceae (Guttiferae), formerly Hypericaceae (Robson, 2003). One of the topmost and commercially renowned species of the genus *Hypericum* is *Hypericum perforatum*, commonly known as St. John's wort. It is among the top selling herbal preparations compromising a worldwide market value of about 600 million US dollars (Ernst, 2003). The antidepressant activity of this species has caused the widespread interest in the study of the *Hypericum* genus (Hu and Sim, 2000). Although *Hypericum* has been used by traditional folk medicine for its anti-inflammatory, hepato- and gastroprotecting, as well as generally healing effects, its use for the treatment of mild to moderate depression has become dominant. *H. perforatum* L. is well known for its profound pharmacological activities as mild antidepressant, anxiolytic, antiviral, wound healing and antimicrobial (Sakar and Tamer, 1990; Barnes et al., 2001; Butterweck et al., 2002).

The *Hypericum* genus is known to contain several constituents such as naphthodianthrones (hypericin, pseudohypericin, protohypericin, and protopseudohypericin), phloroglucinols (hyperforin, adhyperforin, hyperfirin, and adhyperfirin), and a broad range of flavonoids (e.g., hyperoside and rutin) (Nahrstedt and Butterweck, 1997). For a long time the antidepressant activity of *H. perforatum* was attributed primarily to naphthodianthrones, particularly hypericin. However, recent experimental and clinical studies showed that the phloroglucinol hyperforin is the major compound responsible for the antidepressant activity (Meruelo et al., 1988; Chatterjee et al., 1998; Laakmann et al., 1998; Muller et al., 1998, 2001; Butterweck et al., 2001).

Hypericin, a dianthrone, is dark green, photosensitive, polycyclic quinine which was first isolated by Brockmann and his co-workers in 1939 from *H. perforatum* L. (Brockmann et al., 1939) and its chemical structure was published in 1950 (Fig. 1) (Brockmann et al., 1950). Hypericin is hydrophobic and soluble in organic bases, aqueous alkaline solutions, and polar organic solvents yielding red solutions with a fluorescence emission maximum of about 600 nm (Kubin et al., 2005). Hypericins (hypericin and pseudohypericin) have been found only in *Hypericum* species, hence, they are important as chemotaxonomic markers for the genus *Hypericum* (Kitanov,

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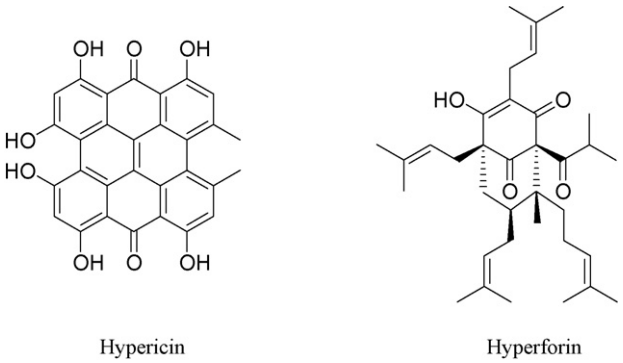

Fig. 1. Chemical structures of the generally accepted markers of *Hypericum*: hypericin and hyperforin.

2001). Hypericins are mainly formed in sepals, petals, and stamens (Repcak and Martonfi, 1997), and have been reported to possess photodynamic, antiviral, antiretroviral, antibacterial, antipsoriatic, antidepressant and antitumoral activities (Agostinis et al., 2002; Guedes and Eriksson, 2005).

On the other hand hyperforin is a bicylclic polyprenylated acylphloroglucinol derivative that was first isolated and characterized by Bystrov and co-workers in 1975 from *H. perforatum* L., (Fig. 1) (Bystrov et al., 1975). It is an easily degradable compound which is poorly stable when exposed to light and oxygen and unstable in most organic solvents (Liu et al., 2005). However, its dicyclohexylammonium salt was found to be stable at room temperature and under the influence of air (Erdelmeier et al., 1999). *H. perforatum* is the only species which contains hyperforin in abundant amounts (Umek et al., 1999; Smelcerovic and Spiteller, 2006). Hyperforin exhibits antidepressant activity by acting as a wide range neurotransmitter reuptake inhibitor of serotonin, dopamine, noradrenalin, glutamate and gamma-aminobutyric acid (GABA) (Singer et al., 1999; Muller, 2003; Treiber et al., 2005). It has also been observed to have antibiotic activity against gram-positive bacteria (Schempp et al., 1999) and antitumoral activity *in vivo* (Schempp et al., 2002). However, it also produces drug–drug interactions by activation of the pregnan X receptor (Moore et al., 2000). Hyperforin mainly accumulates in pistils and fruits where it probably serves as defensive compound to protect the developing seeds against herbivores and microbes (Gronquist et al., 2001; Beerhues, 2006).

The plant specimen records at NCARE, National Center for Agricultural Research and Extension, Ministry of Agriculture, Baq'a, Jordan, showed the presence of seven species of *Hypericum* in Palestine flora at 1900s–1910s. Recently, in his list of wild plants in Jordan, Al-Eisawi reported the presence of 5 species of *Hypericum*: *Hypericum hyssopifolium* Chaix, *Hypericum languinosum* Lam., *Hypericum olivieri* (Spach) Boiss, *Hypericum serpyllifolium* Lam., and *Hypericum triquetrifolium* Turra. (Al-Eisawi, 1998). Danin (1997) discovered a population of *H. sinaicum* Hochst. & Steud. ex Boiss. in Jordan at Dana Nature Reserve (Danin, 1997).

Several analytical methods have been reported for the quantitative analysis of the major active constituents in St. John's wort: LC-UV/Vis (Mulinacci et al., 1999; Pellati et al., 2005), LC-PDA (Liu et al., 2000; Li and Fitzloff, 2001), LC-FL (Draves and Walker, 2000; Jager et al., 2004), CE (Dogrukol-Ak et al., 2001; Peng et al., 2005), LC–API–ES–MS (Mulinacci et al., 1999; Tolonen et al., 2002; Jager et al., 2004), LC–MS–MS (Riedel et al., 2004), and LC–NMR–MS (Holt et al., 1997; Wolfender et al., 1998).

This paper describes the results of simultaneous quantitative determination of hypericin and hyperforin in the methanolic extracts of the aerial parts of three wildly growing *Hypericum* species collected from Jordan flora, namely: *H. triquetrifolium* Turra., *H. empetrifolium* Willd., and *H. sinaicum* Hochst. & Steud. ex Boiss. To the best of our knowledge, this is the first study to quantify hypericin and hyperforin in *H. empetrifolium* and *H. sinaicum* and hyperforin in *H. triquetrifolium* from Jordan. On the other hand, hypericin content of methanolic extracts of dried flowers, leaves, stems, and roots of *H. triquetrifolium* collected from Jordan were determined by HPLC by Alali et al. (2004). Leaves showed the highest hypericin content of 0.36% w/w, while total aerial parts contained 0.11% w/w (Alali et al., 2004).

H. empetrifolium, *H. sinaicum* and *H. triquetrifolium* Turra (Peter's wort, wavyleaf St. John's wort or Tangled *Hypericum*) are wild growing weeds in the northern part of Jordan. *H. sinaicum* is 10–30 cm high, altogether slightly pubescent. Flowers are few in a terminal corymbose panicle. The plant is toxic to livestock (Batanouny, 1999). *H. empetrifolium*, also called *Dwarf Hypericum*, has deep yellow flowers in late spring to early summer (Batanouny, 1999). *H. triquetrifolium* occurs as perennial herb with stiff patent decussate branches, and hence the plant has a more or less pyramidal aspect. Flowers 1–5 together in the summit of leafy branches appear in summer (Al-Eisawi, 1998).

In the current study *H. sinaicum* showed the highest hyperforin content of 0.989% (w/w); while *H. triquetrifolium* and *H. sinaicum* showed the highest hypericin content of ~0.020% (w/w).

2. Experimental

2.1. General experimental procedures

HPLC analysis was performed on a Lachrom® MERCK-HITACHI (Tokyo, Japan) HPLC, with quaternary gradient L-7150 pump, L-7455 Diode-Array Detector, L-7200 auto-sampler, and D-7000 Interface. The analytical HPLC column utilized was Hypersil ODS (125 mm × 4 mm; 5 μm) (Thermo Electron, Auchtermuchty, UK). Rotary evaporator used was RE 200 (Bibby Steriline Ltd, UK), while samples were centrifuged using an EBA 20 centrifuge (Hettich-Zentrifugen GmbH & Co. KG, Tuttlingen, Germany).

Acetonitrile and methanol (HPLC grade) were purchased from Tedia Company, Inc. (Ohio, USA), while ammonium acetate (analytical grade) was obtained from Fluka AG, (Buchs, Switzerland). Hypericin standard was purchased Fluka BioChemika, Fluka Chemie GmbH (Buchs, Switzerland); Sigma-Aldrich, (Steinheim, Germany). Hyperforin standard was obtained from Sigma–Aldrich, Inc. (Montana, USA); Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Plant material

H. empetrifolium and *H. sinaicum* were collected from Ajloun Nature Reserve, Northern part of Jordan during their flowering stage in 2007; lat. 35°86′570″N, long. 07°59′121″E and lat. 35°87′082″N, long. 07°58′999″E, respectively. While *H. triquetrifolium* was collected from Irbid, Northern part of Jordan during it is flowering stage in June 2006; lat. 32°07′116″N, long. 35°50′016″E. The collected materials were identified by Mohammad Gharaibeh. The raw materials were cleaned and air-dried at room temperature and then grounded to fine powder using a blender, Moulinex® (Caen, France), passed through a 24 mesh sieve to generate homogeneous powders, stored at room temperature (22–23 °C), and protected from light until required for analyses.

2.3. Plant's samples preparation

From each finely ground plant material, 1000 mg (±0.1 mg) was weighed and placed into a 250-mL round bottom flask fitted with a reflux condenser, and these were refluxed for 20 min using 80 mL of HPLC-grade methanol. The samples were then filtered, saving the