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Hyperforin production in *Hypericum perforatum* root cultures

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ABSTRACT

Extracts of the medicinal plant *Hypericum perforatum* are used to treat depression and skin irritation. A major API is hyperforin, characterized by sensitivity to light, oxygen and temperature. Total synthesis of hyperforin is challenging and its content in field-grown plants is variable. We have established *in vitro* cultures of auxin-induced roots, which are capable of producing hyperforin, as indicated by HPLC-DAD and ESI–MS analyses. The extraction yield and the productivity upon use of petroleum ether after solvent screening were ~5 mg/g DW and ~50 mg/L culture after six weeks of cultivation. The root cultures also contained secohyperforin and lupulones, which were not yet detected in intact plants. In contrast, they lacked another class of typical *H. perforatum* constituents, hypericins, as indicated by the analysis of methanolic extracts. Hyperforins and lupulones were stabilized and enriched as dicyclohexylammonium salts. Upon up-scaling of biomass production and downstream processing, *H. perforatum* root cultures may provide an alternative platform for the preparation of medicinal extracts and the isolation of APIs.

1. Introduction

Hypericum perforatum (St. John's wort) is a perennial herb, which is commonly used for the treatment of mild to moderate depressions and skin irritations. The herb is the main source of hyperforins and hypericins, which are interesting APIs. Hypericins are naphthodianthrones, which occur in dark nodules of leaves and flowers (Fig. S1 A and C) (Hölscher et al., 2009). Due to the photosensitizing properties, hypericin is under study for the application in photodynamic diagnosis and therapy (Karioti and Bilia, 2010; Ritz et al., 2012). Hypericin-free but hyperforin-rich dosage forms are in topic use against inflammatory skin diseases, such as atopic dermatitis (Wölfle et al., 2014). Hyperforin is the major constituent of lipophilic extracts and present in the translucent glands of leaves and flowers (Fig. S1A and B) (Hölscher et al., 2009; Soelberg et al., 2007). Among the numerous species examined, *H. perforatum* is the outstanding source of hyperforin (Stojanović et al., 2013). The compound exhibits a novel mechanism of action by binding to the nonselective cation channel TRPC6 (Leuner et al., 2007; Müller et al., 2008). Beside the antidepressant and skin-healing properties, hyperforin has a number of pharmacological activities, including antitumoral and antibacterial properties (Russo et al., 2014; Schiavone et al., 2014). In addition, analogues of hyperforin were isolated from either field-grown plants or *in vitro* cultures. Seco- and adsecohyperforins, which lack the prenyl side chain at C-8 (Fig. S1B), were only detected in *in vitro* cultures (Charchoglyan et al., 2007).

Abbreviations: API, active pharmaceutical ingredient; AUC, area under the curve; DCHA, dicyclohexylamine; EPI, enhanced product ion scan; ESI, electrospray ionization; HPLC-DAD, high performance liquid-chromatography-diode array detection; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ESI–MS, electrospray ionization–mass spectrometry; ESI–MS/MS, electrospray ionization–tandem mass spectrometry; MS, Murashige-Skoog medium; PVDF, polyvinylidenfluoride; RP, reverse phase; TRPC6, transient receptor potential channel 6; Y_e, extraction yield.

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Hyperforin is sensitive to oxidation and unstable in solution when exposed to air (Verotta et al., 2002; Wolfender et al., 2003). The choice of proper solvents and extraction conditions is therefore critical for high yields. Oxidative degradation creates problems in the quality control of extracts and affects the pharmacological efficacy (Nahrstedt and Butterweck, 2010). A well-known oxidized hyperforin derivative, furohyperforin, lacks the enolized β-dicarbonyl moiety (Fig. S1B) and hence the antidepressant activity when tested in rat brain cortical synaptosomes (Verotta et al., 2002). On the other hand, an oxidized form of secohyperforin, garsubellin A, increased choline acetyltransferase activity in rat septal neurons and may be useful for treatment of Alzheimer's disease (Fukuyama et al., 1997).

Total synthesis of hyperforin is complex and expensive. The most recent approach started from 2-methylcyclopent-2-en-1-one comprising 10 steps (Ting and Maimone, 2015). Thus, alternative routes of hyperforin production would be desirable. Since environmental, ecological, and genetic factors greatly affect the hyperforin content in field-grown plants (Bruni and Sacchetti, 2009), *in vitro*-grown tissue cultures may provide an alternative system for bioprocessing. Here we report the establishment of *H. perforatum* root cultures, which are capable of hyperforin production, and demonstrate an efficient way of extraction and stabilization of this API.

2. Materials and methods

2.1. Chemicals

The following solvents (either HPLC or analytical grade) were used: ethyl acetate, *n*-hexane, *n*-heptane, methyl-*tert*-butylether (Sigma-Aldrich, Steinheim, Germany), chloroform (Acros Organics, Geel, Belgium), petrol ether, methanol, ethanol, acetonitrile, acetone (VWR Chemicals, Darmstadt, Germany). The following chemicals were used: dicyclohexylamine base and L-ascorbyl palmitate (Sigma-Aldrich), hyperforin (salt-free; Cayman Chemical, Biomol, Hamburg, Germany), hyperforin dicyclohexylammonium salt (HWI Analytik, Rheinzabern, Germany), indole-3-acetic acid (IAA; Fluka, Buchs, Switzerland), indole-3-butyric acid (IBA; Acros Organics, Geel, Belgium), ascorbic acid and silica gel K60 (Merck, Darmstadt, Germany), citric acid (Carl Roth, Karlsruhe, Germany).

2.2. Plant material

Seeds of *H. perforatum* L. were harvested at the experimental station of the Institute of Pharmaceutical Biology (Braunschweig, Germany) in September 2012. For surface sterilization, air-dried seeds were immersed in ethanol (70%) for 5 min, followed by a 25% aqueous solution of 5.3% sodium hypochlorite (Sigma-Aldrich) for 1 min. Sterilized seeds were washed four times with autoclaved distilled water and germinated on regular strength solid MS medium (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar. The pH of the medium was adjusted to 5.7 prior to autoclaving. Seeds were kept in the dark for 2 weeks until first germinations occurred. Seedlings were grown under a 16/8 h (light/dark) photoperiod at 25 ± 2 °C and transferred to fresh MS medium every 4 weeks. To prepare a reference extract for secohyperforin detection, we used *H. perforatum* shoot cultures, which continuously grew on MS medium supplemented with 0.05 mg/L naphthalene-1-acetic acid and 0.5 mg/L N^6-benzylaminopurine (Charchoglyan et al., 2007). Female flowers (hop cones) of *Humulus lupulus* (Cannabinaceae) were collected at the above-mentioned experimental station, frozen in liquid nitrogen and stored at −80 °C until preparation of extracts containing lupulones.

2.3. Initiation and maintenance of auxin-induced root cultures

Induction of adventitious roots was based on the method of Cui et al. (2010) with the following modifications. Two-week-old leaf explants were excised into small sections with a sterile lancet blade and transferred to MS medium (0.6% agar), supplemented with B5 vitamins (Gamborg et al., 1968) and IAA and IBA (0.5 mg/L each). Leaf sections were kept in the dark at 25 ± 2 °C for 4 weeks. Regenerated roots were isolated from the remaining callus (~2 cm) and cultured in 1/2 MS medium supplemented with 1 mg/L IBA and 15 g/L sucrose. The resulting root cultures were grown on a rotary shaker at 100 rpm and 25 °C in the dark. An inoculum of 0.5 g FW each was transferred to 300 mL flasks containing 100 mL fresh medium every 5 weeks. To study changes in hyperforin content, the cultivation period was extended to 9 weeks.

2.4. Microscopy

Small pieces of 5-week-old root segments were examined using digital microscopy (Keyence, Neu-Isenburg, Germany). Scanning electron microscopy of root surfaces was carried out using a Zeiss EVO LS 25 microscope (Oberkochen, Germany) under high vacuum and with a secondary electron detector and an acceleration voltage of 28 kV at a working distance of 8 mm. Images were digitally recorded at calibrated magnifications (300–400 times) using a Slow-Scan CCD-Camera (ProScan, 1024 × 1024, Scheuring, Germany) and the ITEM software (Olympus Soft Imaging Solutions, Münster, Germany).

2.5. Preparation of extracts from auxin-induced root cultures

Five-week-old *H. perforatum* adventitious roots were harvested by suction filtration, washed with water to remove remaining medium, and freeze-dried. The root biomass was determined by recording the dry weight after 48 h of freeze drying when the biomass was stable. Freeze-dried roots were either stored at −20 °C or immediately ground with a pestle in a mortar using liquid nitrogen. The powdered root material (0.5 g) was mixed with 5 mL petroleum ether pre-flushed with N_2 gas and a spatula of seasand and homogenized at 4 °C in the dark for 5 min. The extract was filtered through filter paper and the root residue was re-extracted with a similar volume of petroleum ether. The combined extract was reduced to dryness under N_2 gas. The dried residue was dissolved into 500 μL methanol, filtered through 0.2 μm PVDF filters (Roth, Karlsruhe, Germany) and kept under N_2 atmosphere in dark glass vials. Similarly, methanolic extracts were prepared to check for the presence of hypericins in the root cultures. The analytes were strictly protected from degradation by light by performing the extraction in a darkened room.

2.6. Preparation of reference extracts

Multiple shoot cultures of *H. perforatum* (Charchoglyan et al., 2007) were used to prepare a reference extract containing hyperforins. Shoots were transferred to MS medium, which lacked benzylaminopurine and naphthalene-1-acetic acid, 3 weeks before extract preparation. Regenerated shoots were freeze-dried and stored at −20 °C. Female flowers of *H. lupulus* (hop), stored at −20 °C, were used to prepare a reference extract containing lupulones (Zhang et al., 2004). Extracts from *H. perforatum* multiple shoot cultures and hop cones were prepared under the same conditions as described under Section 2.5. Unless otherwise stated, all extracts were analyzed immediately after preparation. *H. perforatum* seedlings showing well-developed dark glands were used to prepare a reference extract containing hypericins.