Biotechnological production of hyperforin for pharmaceutical formulation

Mariam Gaid, Eline Biedermann, Jendrik Füller, Paul Haas, Sönke Behrends, Rainer Krull, Stephan Scholl, Ute Wittstock, Christel Müller-Goymann, Ludger Beerhues

PII: S0939-6411(17)30092-9
DOI: http://dx.doi.org/10.1016/j.ejpb.2017.03.024
Reference: EJPB 12479

To appear in: *European Journal of Pharmaceutics and Biopharmaceutics*

Received Date: 18 January 2017
Revised Date: 28 March 2017
Accepted Date: 29 March 2017

Please cite this article as: M. Gaid, E. Biedermann, J. Füller, P. Haas, S. Behrends, R. Krull, S. Scholl, U. Wittstock, C. Müller-Goymann, L. Beerhues, Biotechnological production of hyperforin for pharmaceutical formulation, *European Journal of Pharmaceutics and Biopharmaceutics* (2017), doi: http://dx.doi.org/10.1016/j.ejpb.2017.03.024

Review article

Biotechnological production of hyperforin for pharmaceutical formulation

Mariam Gaid[a,f], Eline Biedermann[a,f], Jendrik Füller[b,f], Paul Haas[c,f], Sönke Behrends[d,f], Rainer Krull[e,f], Stephan Scholl[c,f], Ute Wittstock[a,f], Christel Müller-Goymann[b,f], Ludger Beerhues[a,f,*]

[a] *Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Germany*

[b] *Institute of Pharmaceutical Technology, Technische Universität Braunschweig, Germany*

[c] *Institute of Chemical and Thermal Process Engineering, Technische Universität Braunschweig, Germany*

[d] *Institute of Pharmacology, Toxicology and Clinical Pharmacy, Technische Universität Braunschweig, Germany*

[e] *Institute of Biochemical Engineering, Technische Universität Braunschweig, Germany*

[f] *Center of Pharmaceutical Engineering, Technische Universität Braunschweig, Germany*

***** Corresponding author. E-mail address: l.beerhues@tu-bs.de (L. Beerhues)

Contents

ABSTRACT

Hyperforin is a major active constituent of *Hypericum perforatum* (St. John's wort). It has amazing pharmacological activities, such as antidepressant properties, but it is labile and difficult to synthesize. Its sensitivity and lipophilicity are challenges for processing and formulation. Its chemical complexity provokes approaches of biotechnological production and modification. Dedifferentiated *H. perforatum* cell cultures lack appropriate storage sites and hence appreciable hyperforin levels. Shoot cultures are capable of forming hyperforin but less suitable for biomass up-scaling in bioreactors. Roots commonly lack hyperforin but a recently established adventitious root line has been demonstrated to produce hyperforin and derivatives at promising levels. The roots also contained lupulones, the typical constituents of hop (*Humulus lupulus*). Although shear-sensitive, these root cultures provide a potential production platform for both individual compounds and extracts with novel combinations of constituents and pharmacological activities. Besides *in vitro* cultivation techniques, the reconstruction of hyperforin biosynthesis in microorganisms is a promising alternative for biotechnological production. The biosynthetic pathway is under study, with *omics*-technologies being increasingly implemented. These biotechnological approaches may not only yield hyperforin at reasonable productivity but also allow for modifications of its chemical structure and pharmacological profile.

Keywords: *Hypericum perforatum* • Hyperforin • Formulations • Stabilization • Pharmacological profile • *In vitro* cultures • Root cultures • Gene identification • Heterologous production • Hypericin

1. Introduction

Since its isolation in the 1970s [1,2], hyperforin became the flagship constituent of the medicinal plant *Hypericum perforatum* (Hypericaceae). The common name of the Eurasian perennial species, St. John's wort, originates from the blooming and harvesting period around the St. John's day (June 24th; Fig. 1). According to recent counts [3,4] *H. perforatum* is one of 496 species of the genus *Hypericum*, which is spread worldwide except Antarctica. Its invasiveness was attributed to high genotypic plasticity, paired with facultative apomixis at various levels [5,6]. The ability of the plant to overcome environmental challenges via altering its metabolic profile increases its capacity to adapt to and thrive in various habitats [7]. The verified use of *H. perforatum* as a healing remedy to treat a number of ailments, such as wounds and burns, dates back to 400 BCE, the time of the Greek physicians Galen, Dioscurides, Pliny and Hippocrates [8,9].

Today, the best known and most widely investigated indication of *H. perforatum* is mild to moderate depression, which may be better characterized by the more traditional and broader indication 'psycho-vegetative disorders, depressive disorders, anxiety and/or nervous agitation' [10]. A multitude of randomized controlled clinical trials and meta-analyses demonstrated that *H. perforatum* extracts are more effective than placebo in the acute treatment of depressive disorders and similarly effective as standard antidepressants [11,12,13]. In addition, their tolerability is much better, which is an important factor in the treatment of mild to moderate depression. Patients often fear the adverse effects of psychoactive drugs, which is believed to lead to an overall low rate of compliance of standard pharmacological treatment [14]. The efficacy and tolerability of *H. perforatum* extracts were

validated by a monograph of the *Herbal Medicinal Product Committee* of the *European Medicines Agency* [15,16]. However, hyperforin is responsible for drug–drug interactions upon co-medication with *H. perforatum* extracts [9,17].

2. Commercial formulations

Hyperici herba consists of the dried upper third of the flowering aerial parts of *H. perforatum* [10]. It is used as raw material to derive, besides medicinal teas, a large variety of preparations. The majority of these phytopharmaceuticals are used for the treatment of depression. They are commonly dried hydroalcoholic extracts, which result from complex and sophisticated preparation processes for achieving a high and constant quality. Depending on the manufacturing process, *H. perforatum* preparations vary qualitatively and quantitatively in the profile of their constituents, which exhibit additive and synergetic effects and, in addition, contribute to solubility and bioavailability [18]. The drug-extract ratios are in the range of 3:1 to 7:1. Extracts are available as capsules, tablets and coated tablets. Common daily doses range from 500 to 1,200 mg dry extract.

The topical application is used for skin care and treatment of skin diseases, such as neurodermatitis [19]. Fresh plant material, i.e. either flowers or flowering aerial parts of *H. perforatum*, is commonly used to produce oil-based preparations, which are applied either internally for treatment of dyspeptic complaints or externally for curing lesions and burns. *Hyperici oleum* is the most commonly used topical preparation [19]. A hyperforin-rich oil may be prepared from fruit capsules under light exclusion. The treatment with a hyperforin-rich ointment resulted in an improvement of the stratum corneum moisture, skin surface dryness, skin lipids and the subjective skin parameters, indicating stabilization and improvement of the barrier [20].

In most countries, *H. perforatum* products are marketed as dietary supplements, which are not subject to the strict scrutiny for safety and efficacy as synthetic drugs [10]. Within the *European Community*, they are available as both food supplements and drugs. Herbal pharmaceuticals must fulfil the same requirements of authorization as chemical drugs. Efficacy, safety and quality have to be demonstrated, besides efficiency and sustainability of the production [21]. The *Red List*, the directory of drugs licensed in Germany, lists approximately 40 *H. perforatum* preparations.

3. Extraction and stability

Hyperforin is a mixture of interconverting tautomers (Fig. 2). It is sensitive to oxygen, light, temperature and non-polar solvents. Due to oxidation liability, its contribution to the pharmacological profile was neglected until lipophilic supercritical CO_2 extraction yielded *H. perforatum* extracts that were rich in hyperforin (38.8 %) but devoid of hypericins [22,23]. The choice of proper solvents and extraction conditions are critical for both high yield and good manufacturing process quality [12,24,25,26]. In the *European Union*, the selected extraction solvent should be registered for production of food and food ingredients (council directive 88/344/EEC). Most common solvents are ethanol, n-hexane and carbon dioxide. Carbon dioxide extraction has proven to be most selective for hyperforin recovery (Table 1). While subcritical CO_2 extraction conditions (29.1 mg g^{-1} dry weight [DW], 70 bar) obeyed a typical extraction curve, the extraction rate rapidly decreased under supercritical conditions (22.9 mg g^{-1} DW,

300 bar) [27,28]. Despite comparable hyperforin recoveries, supercritical extraction required less CO_2 consumption. An extraction pressure of 90-120 bar was recommended for a short extraction time (1 h); extraction temperatures > 40°C degraded hyperforin [29,30].

The first strategy of hyperforin stabilization made use of weak salts, dicyclohexylamine (DCHA) being the most popular salt base (Fig. 2). The weak salt retained similar bioactivities by releasing stable hyperforin in solution [31,32]. Recently, a non-polar extract prepared from *H. perforatum in vitro* root cultures was stabilized *via* DCHA salt formation and the resulting hyperforin-rich extract proved to be stable for prolonged periods [26]. The same was reported for DCHA-stabilized octa- and tetrahydrohyperforins, the bioactivities of which were either similar or even superior to hyperforin itself (Fig. 2) [33,34,35].

Hyperforin has recently been complexed with hydroxypropyl-β-cyclodextrin (HP-β-CD), leading to improved photoprotection and increased aqueous solubility [36]. In HaCaT keratinocytes and atopic skin *ex vivo*, this formulation with a hyperforin concentration of 1 µM enhanced the mechanosensitive Ca^{2+} signaling. In addition, hyperforin/ HP-β-CD treatment of atopic skin *ex vivo* led to a recovery of the mechanosensitive ATP/Ca^{2+} signaling whereas stretch-induced ATP/Ca^{2+} signaling was only detectable in normal skin but not in atopic skin *ex vivo*. This suggests the usefulness of hyperforin in the treatment of atopic dermatitis. Very recently, albumin nanoparticles (ANP), prepared by desolvation and hardened by heat, were examined as a stabilizing hyperforin carrier system [37, this issue]. In contrast to hyperforin supplied without ANP, 10 µM hyperforin loaded on ANP strongly decreased the viability of HaCaT cells due to strong cytotoxicity.

Oxidation of hyperforin usually involves the enolized β-dicarbonyl system and the adjacent prenyl group, yielding more stable products which, however, lack antidepressant activity [25,38,39]. Furohyperforin is a hyperforin oxidation product with an interrupted β-dicarbonyl system through a furan-type oxygen bridge and it exhibited only 1/10 of the activity of the parent structure when *in vitro* tested for serotonin synaptosomal accumulation [38]. Regarding the ability of eight hyperforin congeners to inhibit the growth of bovine aorta endothelial cells, visible antiangiogenic activity was only observed for structures with the enolized-β dicarbonyl system, namely hyperforin and the tetra- and octahydrohyperforin derivatives (Fig. 2). Interestingly, human albumin protected the enolized system for approximately one week even in the presence of H_2O_2, while control samples lacking albumin experienced complete oxidation in less than 5 h [40].

An alternative stabilization strategy is chemical saturation of the prenyl residues, leading to the analogue octahydrohyperforin (Fig. 2), whose improved lipophilicity endows an antiproliferative effect superior to that of hyperforin [33]. Aristoforin is an O-(carboxymethyl)-hyperforin derivative, which is more stable and better soluble in aqueous solution than the parental compound [41]. While solubilization in water of at least 170 µM of aristoforin was possible, hyperforin itself reached only 20 µM. While aristoforin was not degraded within a week after exposure to light and air in phosphate-buffered-saline containing 20 % DMSO, hyperforin was not detectable due to instability and precipitation. Hyperforin acetate (Fig. 2) as an active and stable derivative had *in vitro* antitumor activity against various cell lines and caused no change in the pentobarbital sleeping time in rats [42,43].

4. Pharmacological potential

Hyperforin exhibits multiple activities, of which the antidepressant potential is best known and most intensely studied (Fig. 3). It is a broad-band neurotransmitter reuptake inhibitor, based on a novel mode of action. The molecular target is the transient receptor potential channel 6 (TRPC6) in the central nervous system [44,45]. TRPCs constitute a group of non-selective cation channels and hyperforin is the first selective activator of type 6. Stimulation of TRPC6 leads to increased intracellular sodium and calcium. While the calcium entry induces axonal sprouting, the sodium influx inhibits the sodium gradient-driven reuptake of neurotransmitters, such as serotonin, norepinephrine and dopamine. Thus, hyperforin does not directly interact with the transmitter transporters, as e.g. do selective serotonin reuptake inhibitors (SSRIs), which interact competitively at the transmitter binding site. The acute hyperforin effect is followed by adaptive changes in the receptor system [46].

Hyperforin-induced TRPC6 activation also stimulates keratinocyte differentiation, which is impaired in atopic eczema (neurodermatitis) and psoriasis [45]. Influx of calcium through the activated non-selective cation channel results in an enhanced intracellular level, which promotes differentiation but blocks proliferation of keratinocytes in the upper epidermis. Furthermore, a hyperforin-rich cream reduced radical formation, which also plays an important role in the development of atopic eczema and barrier-disrupted skin [47,48]. Thus, hyperforin offers an innovative medicine for treatment of skin diseases, mediated by altered keratinocyte differentiation [20,45,49]. In addition, human dermal fibroblasts have recently been shown to undergo anti-proliferative and anti-migratory effects stimulated by hyperforin [50, this issue]. This finding may provide a new option for the treatment of hypertrophic scars.

The anti-proliferative activity of hyperforin on HaCaT keratinocytes *in vitro* is established [45,51]. Recently, this effect has been found to be affected by the fetal calf serum (FCS) content of the medium [37, this issue]. At least 5 % FCS was required to recover ~100 % of loaded hyperforin (10 µM). In the absence of the serum and most likely due to nanoprecipitation and oxidation of hyperforin the live-dead cell staining showed a dramatic cytotoxicity, which was confirmed by a drastic decrease of cell viability. The anti-proliferative effect was confirmed in medium with 10 % FCS using extracts prepared from *H. perforatum* flower buds and *in vitro* root cultures, both standardized to 10 µM hyperforin [Gaid et al., unpublished]. Besides the morphological evaluation, the viability of the HaCaT keratinocytes and the cytotoxicity of hyperforin were studied using the microculture tetrazolium [52] and Celltox Green assays, respectively. Relative to control cells faced with 1 % Triton X-100, the treatment with the extracts was not toxic to HaCaT cells, however, around 60 % loss of viability was observed. Taking the total cell number as a confirmatory parameter for keratinocyte proliferation, no appreciable change was observed after treatment with either pure hyperforin or root culture extract over 48 h, confirming that the loss of viability of the HaCaT cells was primarily due to the anti-proliferative rather than the cytotoxic effect of hyperforin. Similar observations were recorded for extracts containing 15 µM hyperforin, whereas a visible loss of viability with clear cytotoxicity was found for extracts standardized to 30 µM. Very recently, root culture extracts standardized to concentrations even less than 10 µM hyperforin have also been found to exhibit antiproliferative activity, which may be due to additive and/or synergetic effects (Gaid et al., unpublished).

Hyperforin also possesses anti-inflammatory properties by acting as a dual inhibitor of the key enzymes of the eicosanoid metabolism, i.e. cyclooxygenase and 5-lipoxygenase [53,54]. An acylphloroglucinol-rich fraction from *H. gentianoides* also exhibited anti-inflammatory activity without affecting cell viability [55]. In addition, hyperforin has antibacterial properties. The antibiotic potential of *H. perforatum* extract, first reported in 1959, was attributed to hyperforin [1,56,57]. The growth of Gram-positive bacteria, such as *Corynebacterium diphtheriae*, is inhibited at concentrations as low as 0.1 μg ml^{-1} [57]. Multiresistant strains of *Staphylococcus aureus* are also inhibited by hyperforin and its chemically stabilized congener, octahydrohyperforin (MICs = 1 μg ml^{-1}) [34,57,58]. No growth inhibition was observed for Gram-negative bacteria and *Candida albicans*. Taken together, the above hyperforin activities explain the traditional use of *H. perforatum* preparations for the topical treatment of infected wounds as well as inflamed and atopic skin [57,59,60].

Hyperforin and its stable derivative tetrahydrohyperforin (Fig. 2) also generate neuroprotective effects and prevent memory loss in a double transgenic mouse model of Alzheimer's disease. The compounds hamper deposition of beta-amyloid (Aβ) peptides, prevent Aβ-induced spatial memory impairments and alleviate Aβ neurotoxicity in hippocampal neurons [61]. The Aβ plaque size and the oxidative damage in cortex and hippocampus are reduced and adult neurogenesis is increased [62,63]. Like hyperforin, tetrahydrohyperforin most likely targets TRPC6, the activation of which plays a key role in neuronal survival by transmitting brain-derived neurotrophic factor signalling and promoting gene expression *via* the cAMP response element-binding protein [44,64,65]. The brain level of tetrahydrohyperforin in the mouse model reached ~700 nM after repeated administration, rationalizing the positive effects on memory and cognitive performance [35]. In another study, however, the hyperforin content of *H. perforatum* extracts did not necessarily correlate with the therapeutic effects in Alzheimer's disease [66]. A hyperforin-poor extract was able to activate the ABCC1 transporter, resulting in a significant reduction of intracerebral Aβ42 levels and a decrease in both the number and the size of amyloid plaques in the mouse model.

Hyperforin induced apoptosis in a number of tumor cell lines, inhibited angiogenesis and suppressed metastasis formation and lymphangiogenesis *in vivo* [67,68,69,70,71,72]. The semi-synthetic derivative aristoforin exhibited activity against the MT-450 tumor *in vivo* and DNA protective properties, which were comparable to those of hyperforin [41,73]. The compounds lacked signs of acute toxicity *in vivo* [41,67].

Finally, hyperforin activates the nuclear pregnane-X receptor (PXR), a transcriptional regulator for the genes that encode CYP3A4 and P-glycoprotein (MDR1, multidrug resistance 1), which are involved in hepatic metabolism and intestinal efflux of drugs, respectively [74,75,76]. These processes can massively affect the level of co-administered drugs, such as immunosuppressant ciclosporin, cardioactive digoxin, and antineoplastic irinotecan [9,17]. Some diacylphloroglucinols activated TRPC6 but lacked PXR activation [77,78]. Thus, hyperforin derivatization aiming at elimination of the interaction potential while retaining the pharmacological activity is desirable [79].

5. Localization in plants

Hypericum species contain an amazing array of constituents [80]. Besides flavonoids, which ubiquitously occur in plants, the most prominent constituents of *H. perforatum* herb extract involve naphthodianthrones, such as hypericin and pseudohypericin, and acylphloroglucinol derivatives, represented mainly by hyperforin. Hypericins (0.3 – 1 %) are crimson pigments and accumulate in dark cell clusters distributed in leaves and flowers (Fig. 4 A,C,E) [81,82,83]. Not all *Hypericum* species develop these dark nodules in their leaves. *H. pulchrum* and *H. bupleuroides* contain dark nodules only on the reproductive organs [84]. While the upper leaves of *H. erectum* lack dark dots, the third pair of leaves starts to form them throughout the leaf blade. *H. tomentosum* either lacks dark dots or has a single pair of them, symmetrically placed on the leaf apex. Hypericins naturally occur as protopigments (protohypericin and -pseudohypericin), which are photoconverted upon exposure to light. The resulting hypericins are photosensitizers and contribute to the antiretroviral activity [85]. Hypericin is useful in photodynamic diagnosis and therapy of precancerous cells [86]. Although topical application of hypericin-containing creams to irritated skin or fair-skinned individuals causes phototoxicity after extended solar irradiation, the clinical relevance is limited for usual dose ranges [87,88].

The perforated appearance of the leaves is due to schizogenous translucent glands, the storage organelles of hyperforins (Fig. 4 D, F) [89,90]. Flowers, especially pistils and unripe fruits (6 – 8 %) show highest hyperforin content when compared to leaves [81,91]. Occurrence of translucent glands in the genus *Hypericum* does not necessarily correlate with the accumulation of hyperforins [55,92]. The presence of dimeric phloroglucinols rather than monomeric hyperforin is the chemotaxonomic marker of members of the Brathys and Trigynobrathys sections [93]. The contents of the biomarkers hyperforin and hypericin in some *Hypericum* species either resemble or even exceed those in *H. perforatum*. While *H. maculatum*, the diploid sister of *H. perforatum*, owes a similar glandular density and a comparable metabolic profile [94,95], *H. montanum* houses ~1.6, 1.5 and 2-fold higher hypericin, pseudohypericin and emodin (hypericin precursor) contents, respectively [96]. Among seven field-grown *Hypericum* species screened for their total amount of hyperforins in flowers, *H. perforatum* showed the highest level (44 mg g^{-1} DW), followed by *H. polyphyllum* (38.5 mg g^{-1} DW) and *H. kouytchense* (26.6 mg g^{-1} DW) [97]. However, *in vitro* plantlets of *H. perforatum* and *H. androsaemum* housed comparable amounts of hyperforin (16.7 and 12.4 mg g^{-1} DW, respectively) [98]. Some species with high pharmaceutical value might be useful alternatives to *H. perforatum*, which, however, has to be approved by further studies.

6. Cell suspension and shoot cultures

The growing number of consumers of *H. perforatum* medications continuously elevates the worldwide market demands for raw plant material. However, the production of high quality plant material at large quantities is adversely affected by non-controllable factors, such as climatic fluctuations, variable soil properties, plant diseases and genotypic variation [99,100]. In addition, the

post-harvest processes, such as drying, storage and extraction, may cause batch-to-batch variability [101]. For example, the hyperforin and hypericin contents were negatively affected by the decrease in light intensity and temperature [102]. Similarly, plants grown under water stress showed significant reduction in the content of hypericins [103]. Conversely, feeding by insect herbivores caused 30 – 100 % increases in the hyperforin and hypericin levels, compared to non-fed control plants [104]. Among different plant lines, metabolic fluctuations were observed especially for the hyperforin content (0.3 - 1.3 %) [105]. Finally, the risk of contamination represents a great challenge. Recently, *H. perforatum* preparations appeared to be contaminated with liver-toxic pyrrolizidine alkaloids due to unintended harvest of co-occurring weeds, causing supply shortage and batch recall [106].

The drawbacks of field cultivation have for a long time stimulated researchers to strive for stable *in vitro* systems, which produce a desired active pharmaceutical ingredient (API) under proper maintenance and rational biodiversity utilization. A number of *in vitro* systems of *Hypericum* species were established, including cell suspension, shoot and root cultures [107]. The majority of *in vitro* systems were derived from *H. perforatum* (Table 2). Due to the high medicinal potential, factors especially influencing the *in vitro* contents of hyperforins and hypericins were studied. *In vitro* propagation affected the development of translucent glands and the acylphloroglucinol content. For example, *in vitro* shoots of *H. tomentosum* and *H. pulchrum* lacked hyperforin and its analogues, whereas hyperfirin analogues lacking the C_5 unit at C-7 were localized in the translucent glands of *H. rumeliacum* and *H. bupleuroides* [84]. Leaves of *H. tetrapterum in vitro* seedlings developed small translucent cavities, in which hyperfirin and other unidentified acylphloroglucinols were co-localized. However, only negligible amounts of acylphloroglucinols were detected in intact plants despite the presence of translucent glands [96,97,108]. Ultrastructural comparison of the dark glands of two *H. perforatum in vitro* shoot lines indicated a clear difference in the size of the peripheral cells, which correlated to variations in the total hypericin content (0.53 vs. 6.8 mg g^{-1} DW) [109]. Recently, consistent ploidy of *H. perforatum in vitro* shoots has been confirmed, thereby offering a pivotal biotechnological tool for long-term supply of stable elite clones [110]. *In vitro* selection combined with propagation under controlled environmental conditions are essential sequences for germplasm improvement [7]. Furthermore, the cryopreservation technology allows for long-term maintenance of clonally propagated germplasm [111,112].

Variations in the *in vitro* system and the culture medium foster the yields of hyperforins and hypericins (Table 2). Accumulation of hyperforins and hypericins is related to leaf morphogenesis and the occurrence of accumulation sites. Dedifferentiated cell cultures (Figs. 5 A, B) are therefore a poor source of hyperforins [113]. Similarly, their capability of hypericin accumulation correlates to a certain degree of differentiation [114,115]. The best records of hyperforin yield during normal growth were reported for regenerated plantlets with 74 mg g^{-1} DW [116]. However, abnormalities like hyperhydricity and fasciated shoots were detected upon cultivation in liquid medium, making large-scale cultivation of shoot cultures an undesirable application [117,118]. Transplanting *H. perforatum* plantlets from *in vitro* bioreactors to a closed controlled environmental system supported with a CO_2-rich atmosphere provided good biomass and stable constituent content [119]. Agar cubes and cork pieces were applied to shoot cultures cultivated in liquid medium to study the mechanical interaction (massage effect) and

to mimic the shear stress in bioreactors. Visible changes in hyperforin and hypericin production were recorded with five-fold (30 - 35 mg g^{-1} DW) and three-fold increases (10 - 21 mg g^{-1} DW), respectively, compared to non-supplemented control cultivation [120].

A common and often effective strategy of increasing the API yield is the use of elicitors, which mimic a microbial infection and stimulate formation of secondary products as defense compounds [107,121]. However, the reproducibility of the results is still a matter of concern. While ozone treatment (3 h, 90 nL L^{-1}) was an effective elicitor leading to a 4-fold increase in hypericin yield (~29 mg L^{-1}) [122], methyl jasmonate appeared to be an efficient elicitor of hyperforin formation. In numerous cell cultures of *Hypericum* species, elicitors caused accumulation of xanthones, which appear to be the inducible defense compounds of Hypericaceae. The induction provides useful systems for studying the xanthone biosynthetic pathway [123,124,125,126,127].

Feeding of biosynthetic precursors is an alternative biotechnological tool to enhance the *in vitro* yield. Valine is the precursor amino acid of hyperforin, while isoleucine and its precursor threonine lead to adhyperforin formation. Feeding these adhyperforin precursors to shoot cultures stimulated 2 to 3.7-fold the production of adhyperforin [128]. In contrast, feeding of valine failed to enhance the accumulation of hyperforin, indicating that its endogenous pool is not a limiting factor in hyperforin biosynthesis.

7. Root cultures and bioreactor scale

In contrast to shoot cultures, root cultures can be readily subjected to biomass up-scaling in bioreactors (Table 3) [129,130,131,132]. Roots can be grown as submerged cultures [133]. In addition, they lack chlorophylls, which are typically present in the aerial plant parts and known to interfere with downstream processes, such as API extraction and isolation [134]. However, roots are filamentous biological systems and very sensitive to hydromechanical shear stress. Therefore, process equipment requires special design and it needs to be operated at specific conditions to maximize productivity and to achieve constant product quality. These approaches have to be complemented by corresponding strategies for downstream processing [135].

Upon selection of highly productive elite material for further regeneration and propagation, root cultures are attractive production systems for interesting API [109,136,137]. *H. perforatum* cultures consisting of auxin-induced adventitious roots can be used to produce both xanthones and hypericins [127,138,139,140,141,142]. Extracts from *O*-carboxymethylchitosan-treated roots exhibited high antifungal activity against *Candida* spp., *Cryptococcus neoformans* and dermatophytes [139]. When grown in a mist bioreactor, leading to biomass doubling within 28 d, a xanthone-rich extract exhibited activity against planktonic cells and biofilm of *Malassezia furfur* [143]. Similarly, *H. perforatum* hairy roots, which resulted from transformation with *Agrobacterium rhizogenes* and were independent of growth regulators, were reported to be xanthone-producing systems, the hyperforin content remaining open [127,144].

An important step towards biotechnological production is replacement of shake flasks with bioreactor devices for scale-up. When growth conditions have been adapted to obtain sufficient biomass, variation of the cultivation medium, treatment with elicitors and feeding of precursors may

enhance API accumulation. A number of bioreactor systems were successfully employed for cultivation of differentiated plant *in vitro* systems, however, the choice of the appropriate construction is a challenge [145]. Recently, a huge batch of *H. perforatum* root culture biomass was grown in a pilot-scale cultivation (500 L bioreactor) for producing hypericin at a level of 0.03 - 0.04 mg g^{-1} DW [131]. Treatment with methyl jasmonate enhanced the production 6 and 11 times, compared to 1-year-old field-grown plants and *in vitro* plantlets, respectively [132]. The maximum hypericin yield (1.68 mg g^{-1} DW) and productivity (15.57 mg L^{-1}) were obtained at a 350 µmol L^{-1} elicitor concentration. The surface of the hypericin-containing roots was covered by gland-like structures, which were proposed to be the place of hypericin accumulation [141].

As to hyperforin production *in vitro*, a breakthrough has recently been reported for auxin-induced *H. perforatum* root cultures [26]. The selection of adventitious root lines for hyperforin formation resulted in root cultures with a hyperforin extraction yield of 5 mg g^{-1} DW. The productivity at the flask scale was ~50 mg L^{-1} culture after 6 weeks (Figs. 5C and 6). Importantly, hyperforins are barely detectable in roots of field-grown plants but readily found in aerial parts. A single publication reports hyperforin detection in roots of field-grown plants (1.34 mg/g) [96]. Thus, the root cultures recently established represent a unique *in vitro* system for hyperforin production and its up-scaling. Using petroleum ether and *n*-hexane, various extraction procedures, such as stirring and rotation, and evaporation techniques, such as vacuum and N_2 stripping, were studied [26,146, this issue]. Gland-like structures previously observed on the surface of the hypericin-containing roots [141] were absent from the hyperforin-producing roots (Fig. 5D). Consistently, methanolic extracts from the hyperforin-forming root cultures were devoid of hypericins, which offers the chance of producing hypericin-free extracts without photosensitization upon topical use.

Furthermore, some hyperforin analogues were detected in the cultivated adventitious roots [26]. Seco- and adsecohyperforins, which lack the 8-prenyl side chain, were previously detected in shoot cultures but they are absent from field-grown plants and that is true for both aerial and underground parts [147]. This additional finding promotes the root cultures as attractive bio-factories for discovering the unexploited pharmacological activities of the detected hyperforin derivatives. Another observation likewise unprecedented for *Hypericum* species is the production of colupulone and adlupulone, which are biosynthetically related to hyperforins. The lupulones are the typical constituents of hop (*Humulus lupulus*) cones, used for sedation. The compounds continuously accumulated during root culture growth, offering the chance of product recovery by *in situ* extraction to avoid feedback inhibition of product biosynthesis and its degradation. Current studies focus on treating the root cultures with elicitors, feeding them with precursors and varying their cultivation medium to increase the production of hyperforins and to tailor the metabolite profile. The unprecedented combination of constituents, i.e. hyperforins, lupulones and xanthones, bundles interesting pharmacological activities, such as anti-bacterial and anti-Alzheimer properties [148,149]. Both hyperforin and colupulone activate the PXR, which controls the level of the P-glycoprotein efflux pump. In Alzheimer's disease, this transporter is related to the clearance of the beta-amyloid peptide [150].

8. Towards production in heterologous systems

Hyperforin is the prototype molecule of type A polycyclic polyprenylated acylphloroglucinols (PPAP). Its *de novo* chemical synthesis is complicated and the yield is low [151]. In the most recent approach, synthesis of the substituted bicyclo[3.3.1]nonane-1,3,5-trione skeleton took six steps starting from 2-methylcyclopent-2-en-1-one, followed by four more steps to integrate the acyl, prenyl and hydroxyl groups at C-1, C-3 and C-4, respectively [152]. The substituted core would be an attractive starting material for the preparation of highly diverse hyperforins and related analogues. Till now, naturally occurring hyperforin remains the only source to satisfy the commercial demand. However, the extraction and isolation yield is not always sustainable and varies depending on biological and environmental factors as well as downstream processes [102,153,154]. Thus, reassembling the biosynthetic pathway of hyperforin in microorganisms might offer an alternative production platform.

All major constituents accumulating in *H. perforatum*, including hyperforins, hypericins, flavonoids and xanthones, are polyketide derivatives [79,97,155]. Their aromatic cores are biosynthesized by type III polyketide synthases from an acyl-CoA starter and malonyl-CoA extenders [79,113,156]. The hyperforin nucleus, phlorisobutyrophenone, results from condensation of isobutyryl-CoA and three molecules of malonyl-CoA (Fig. 7). Modifications of the aromatic core, especially by prenylation and cyclization reactions, explain the astounding complexity of the acylphloroglucinols [113,157,158,159,160,161,162]. Thus, proper chemical and biochemical characterization of *Hypericum* species is a challenge [55,84,97].

Prenylations of phlorisobutyrophenone are catalyzed by aromatic prenyltransferase (PT) enzymes. Generally, PTs catalyze the addition of isoprenoid side chains to an aromatic core (Fig. 8). Although cytosolic and soluble PTs occur in fungi and bacteria [163,164], PTs isolated from plants are membrane-spanning proteins (Fig. 8 A). They share common characters including plastid localization, dependency on divalent cations, and relatively narrow substrate specificities [165]. An exception is *Lithospermum erythrorhizon* p-hydroxybenzoate:geranyltransferase (*Le*PGT1), which was localized to the endoplasmic reticulum [166]. The occurrence of multiple transmembrane α-helices makes prokaryotes a challenging expression system for PTs. As an alternative, yeast cells (*Saccharomyces cerevisiae*), insect cells (*Spodoptera frugiperda*, *Sf*9) and plants like *Nicotiana benthamiana* were explored as heterologous expression systems for PT characterization (Fig. 8 B). For example, the *Le*PGT1 expression level in *Sf*9 cells was 1000-fold that in yeast [166,167]. While functional expression of *Petroselinum crispum* PT failed in yeast, transient expression in *N. benthamiana* leaves disclosed its coumarin-specific prenylation activity [168]. Functional expression of *H. calycinum* xanthone-specific PT was successful in *Sf*9 but failed in yeast [126]. Conversely, yeast expression succeeded with *Morus alba* isoliquiritigenin 3'-dimethylallyltransferase, which promoted the regiospecific C-2 prenylation of 1,3,7-trihydroxyxanthone [169].

Little is known about phlorisobutyrophenone-specific PTs. Three genes were cloned from hop and shown to be involved in colupulone biosynthesis [170,171]. Although the prenylation sequence of colupulone has recently been resolved and reconstructed in yeast [171], the prenylation and cyclization steps leading to bridged PPAPs, such as hyperforin, remain open [79,172]. The proposed hyperforin biosynthetic sequence involves stepwise prenylation of the unsubstituted

phlorisobutyrophenone core using one geranyl (C_{10}) and two dimethylallyl (C_5) units, followed by ring closure that is triggered by electrophilic attack of a third C_5 unit (Fig. 7) [79,172,173]. Before heterologous production in one of the above-mentioned systems can be attempted, the complete biosynthetic pathway has to be established at the gene level.

9. Discovery of biosynthetic genes

Besides hyperforin, more than 300 PPAP compounds have been identified in the past decades, half of them in the genus *Hypericum* [174,175]. Forty PPAPs have recently been found in a single species, *H. henryi* [176]. In light of this diversity, the genus *Hypericum* represents a great reservoir of new genes to be discovered by next-generation-sequencing. This tool has recently been used to identify genes related to hypericin biosynthesis [98,177]. New transcriptomic resources have been assembled for four *Hypericum* species, which were selected by the existence of dark nodules and the ability to synthesize hypericins [84]. This capability ranged from well-detectable hypericin in *H. perforatum* and *H. annulatum* (5.41 and 2.06 µg mg^{-1} DW, respectively) to *H. tomentosum* (0.27 µg mg^{-1} DW) [98], in which up to two dark dots are distributed on the leaf apex, while *H. kalmianum* and *H. androsaemum* accumulate neither hypericin nor its precursor emodin. In analogy, comparative analysis of sequences in the publicly available *NCBI Sequence Read Archive* may help to approach the molecular basis of hyperforin biosynthesis. In addition, sequence information can be derived from a transcriptomic analysis of a hyperforin-forming but hypericin-free species (*H. androsaemum;* SRX1528157), a hyperforin-free but hypericin-forming species (*H. tomentosum;* SRX1528963, SRX1528964), and finally a species (*H. pulchrum*), the leaves of which accumulate neither hypericins nor hyperforins [84]. Spatio-temporal expression analysis in combination with enzymatic characterization after heterologous expression would provide information about possible involvement of the differentially expressed genes in distinct metabolic pathways. Genes gleaned from these studies are promising targets for *de novo* assembly of PPAP scaffolds in heterologous hosts, which would extend the research direction toward synthetic biology and would provide promising perspectives for future applications.

10. Impact of gene identification on drug discovery

In the past years, researchers focused on polyketide-specific PTs due to their significant role in generating structural diversity, which offers new frontiers in drug discovery. Impressive changes in the pharmacological activity after prenylation were mostly attributed to increased lipophilicity accompanied by enhanced affinity for biological membranes and proteins. In nature, carbo- and oxy- modifications with dimethylallyl and geranyl residues are more abundant than attachments of farnesyl and geranylgeranyl chains [165,178]. Licoflavone C and isobavachin are two prenylated flavonoids with cytotoxic activity against rat H4IIE hepatoma (IC_{50} = 42 and 96 µmol L^{-1}, respectively) and C6 glioma cells (IC_{50} = 37 and 69 µmol L^{-1}, respectively). In contrast, increasing concentrations of the non-prenylated precursors up to 250 µmol L^{-1} failed to exhibit cytotoxicity [179]. Three prenylated

chalcones, namely, 2′,4′,6′,4-tetrahydroxy-3′-C-geranylchalcone, 2',4',4-trihydroxy-6'-methoxy-3'-C-prenylchalcone (xanthohumol) and 2′,4′,6′,4-tetrahydroxy-3′-C-prenylchalcone (desmethylxanthohumol), inhibited the copper-mediated oxidation of LDL at concentrations of 5 and 25 µmol L^{-1} by 70 % after 5 h of incubation, compared to non-prenylated naringenin chalcone that even showed pro-oxidant effects [180]. Due to its effectiveness as a cytotoxic agent against HT-29 (human colon cancer), the diprenylated xanthone α-mangostin was used as a lead compound to synthesize eight derivatives, of which 3,6-di-*O*-acetyl-α-mangostin was most potent (ED_{50} = 1.0 µmol L^{-1}) [181]. γ-Mangostin, a 1,3,6,7-tetrahydroxy-2,8-diprenylxanthone, was previously reported to be a cholinesterase inhibitor [182]. Genes for xanthone-specific PTs were cloned and characterized from *H. calycinum* and *Morus alba* cell cultures. The recombinant enzymes were able to catalyze regiospecific C-8 and C-2 prenylations of 1,3,6,7-tetrahydroxyxanthone and 1,3,7-trihydroxyxanthone to form 1,3,6,7-tetrahydroxy-8-prenylxanthone and 1,3,7-trihydroxy-2-prenylxanthone, respectively [126,169]. Due to the properties of these compounds, both genes may offer the chance of biotechnological synthesis of new API for the treatment of Alzheimer's disease.

11. Conclusions

H. perforatum preparations have a well-established market position and hyperforin is a valuable constituent, which has a challenging chemical structure and an amazing pharmacological potential. However, the chemical composition of extracts derived from *H. perforatum*, including the hyperforin content, is affected by environmental conditions, ontogenetic changes and genotypic variation. Moreover, the bicyclic β-triketone core of hyperforin is oxidation-sensitive posing challenges to extraction and downstream processing. *In vitro* cultivation of *H. perforatum* under controlled conditions is feasible; however, the hyperforin content in derived plant material is commonly poor. *In vitro* selection of elite clones and their controlled propagation *in vivo* may offer a new technology for mass propagation while keeping genotypic stability. As an alternative, a recently established root culture system provides a controllable *in vitro* production platform for hyperforin itself as well as its derivatives and intermediates. Efforts will be directed towards increasing the productivity of these cultures and expanding their chemical profile. Biomass production and processing will be transferred to larger scales, thereby advancing the development of equipment and operation know-how for shear-sensitive biological systems. Synthetic biology is playing an ever increasing role for sustainable supply of APIs. Therefore, the hyperforin biosynthetic pathway and its regulation need to be elucidated. This is also true for hyperforin-related PPAPs, which occur in other *Hypericum* species. In the future, reconstitution of their biosynthetic routes in heterologous hosts such as microbes may be possible. This would not only provide a sustainable route of hyperforin production independent of field cultivation, but also contribute to exploiting the huge biosynthetic potential of *Hypericum* species for pharmaceutical applications.

Acknowledgment

The authors thank the Lower Saxony Ministry for Science and Culture for supporting the joint research project *Novel synthesis and formulation methods for poorly soluble drugs and sensitive biopharmaceuticals* (*SynFoBiA*) of the Center of Pharmaceutical Engineering (PVZ) at the Technische Universität Braunschweig, Germany.

References

[1] A.I. Gurevich, V.N. Dobrynin, M.N. Kolosov, S.A. Popravko, I.D. Ryabova, B.K. Chernov, N.A. Derbentseva, B.E. Aizenman, A.D. Garagulya, Antibiotic hyperforin from *Hypericum perforatum* L., Antibiotiki 16 (1971) 510–513.

[2] N.S. Bystrov, B.K. Chernov, V.N. Dobrynin, M.N. Kolosov, The structure of hyperforin, Tetrahedron Lett. 32 (1975) 2791–2794. DOI: 10.1016/S0040-4039(00)75241-5

[3] N.M. Nürk, F.R. Blattner, Cladistic analysis of morphological characters in *Hypericum* (Hypericaceae), Taxon 59 (2010) 1495–1507.

[4] N.M. Nürk, S. Uribe-Convers, B. Gehrke, D.C. Tank, F.R. Blattner, Molecular phylogenetics and morphological evolution of St.John's wort (*Hypericum*; Hypericaceae), Mol. Phylogenet. Evol. 66 (2013) 1–16. DOI: 10.1016/j.ympev.2012.08.022

[5] F. Matzk, A. Meister, R. Brutovská, I. Schubert, Reconstruction of reproductive diversity in *Hypericum perforatum* L. opens novel strategies to manage apomixes, Plant J. 26 (2001) 275-282. DOI: 10.1046/j.1365-313X.2001.01026.x

[6] M.A. Koch, C. Scheriau, A. Betzin, N. Hohmann, T.F. Sharbel, Evolution of cryptic gene pools in *Hypericum perforatum*: the influence of reproductive system and gene flow, Ann. Bot. 111 (2013) 1083-1094. DOI:10.1093/aob/mct065

[7] S.J. Murch, P.K. Saxena, A melatonin-rich germplasm line of St. John's wort (*Hypericum perforatum* L.), J. Pineal. Res. 41 (2006) 284-287. DOI: 10.1111/j.1600-079X.2006.00367.x

[8] F.C. Czygan, Kulturgeschichte und Mystik des Johanniskrauts: Vom 2500 Jahre alten Apotropaikum zum aktuellen Antidepressivum, Pharm. Unserer Zeit *32* (2003) 184-190. DOI: 10.1002/pauz.200390062

[9] K.M. Klemow, A. Bartlow, J. Crawford, N. Kocher, J. Shah, M. Ritsick, Medical attributes of St. John's wort (*Hypericum perforatum*), in: I.F.F. Benzie, S. Wachtel-Galor (Eds.), Herbal Medicine: Biomolecular and Clinical Aspects, 2nd edition, CRC Press/Taylor&Francis, Boca Raton (FL), 2011, chapter 11. DOI: 10.1201/b10787-12

[10] K. Linde, St. John's Wort – an Overview, Forsch. Komplementmed. 16 (2009) 146-155. DOI: 10.1159/000209290

[11] S. Kasper, F. Caraci, B. Forti, F. Drago, E. Aguglia, Efficacy and tolerability of Hypericum extract for the treatment of mild to moderate depression, Europ. Neuropsychopharmacol. 20 (2010) 747–765. DOI: 10.1016/j.euroneuro.2010.07.005

[12] A. Nahrstedt, V. Butterweck, Lessons learned from herbal medicinal products: the example of St. John's Wort, J. Nat. Prod. 73 (2010) 1015–1021. DOI: 10.1021/np1000329

[13] E.A. Apaydin, A.R. Maher, R. Shanman, M.S. Booth, J.N.V. Miles, M.E. Sorbero, S. Hempel, A systematic review of St. John's wort for major depressive disorder, Syst. Rev. 5 (2016) 148. DOI 10.1186/s13643-016-0325-2

[14] C. Röder, M. Schaefer, S. Leucht, Meta-analysis of effectiveness and tolerability of treatment of mild to moderate depression with St. John's wort, Fortschr. Neurol. Psychiatr. 72 (2004) 330–343. DOI:10.1055/s-2003-812513

[15] Anon., Community Herbal Monograph on *Hypericum perforatum* L. Herba (Well-established Medicinal Use), EMA/HMPC/101304/2008, European Medicines Agency, London, 2009.

[16] Anon., Assessment Report on *Hypericum perforatum* L. Herba, EMA/HMPC/101303/2008, European Medicines Agency, London, 2009.

[17] D.L. Whitten, S.P. Myers, J.A. Hawrelak, H. Wohlmuth, The effect of St. John's wort extracts on CYP3A: a systematic review of prospective clinical trials, Br. J. Clin. Pharmacol. 62 (2006) 512–526. DOI: 10.1111/j.1365-2125.2006.02755.x

[18] M. Schmidt, V. Butterweck, The mechanisms of action of St. John's wort: an update, Wien. Med. Wochenschr. 165 (2015) 229–235. DOI: 10.1007/s10354-015-0372-7

[19] U. Wölfle, G. Seelinger, C.M. Schempp, Topical application of St. John's Wort (*Hypericum perforatum*), Planta Med. 80 (2014) 109–120. DOI: 10.1055/s-0033-1351019

[20] M.C. Meinke, H. Richter, A. Kleemann, J. Lademann, K. Tscherch, S. Rohn, C.M. Schempp, Characterization of atopic skin and the effect of a hyperforin-rich cream by laser scanning microscopy, J. Biomed. Opt. 20 (2015) 051013-1-8. DOI: 10.1117/1.JBO.20.5.051013

[21] M. Miroddi, C. Mannucci, F. Mancari, M. Navarra, G. Calapai, Research and development for botanical products in medicinals and food supplements market, Evid. Based Complement. Alternat. Med. (2013) 649720. DOI: 10.1155/2013/649720

[22] S.S. Chatterjee, S.K. Bhattacharya, M. Wonnemann, A. Singer, M.E. Müller, Hyperforin as a possible antidepressant component of Hypericum extracts, Life Sci. 63 (1998) 499-510. DOI: 10.1016/S0024-3205(98)00299-9

[23] S.S. Chatterjee, M. Nöldner, E. Koch, C. Erdelmeier, Antidepressant Activity of *Hypericum perforatum* and hyperforin: the neglected possibility, Pharmacopsychiatry 31 (1998) 7-15. DOI: 10.1055/s-2007-979340

[24] S. Trifunović, V. Vajs, S. Macura, N. Juranić, Z. Djarmati, R. Jankov, S. Milosavljević, Oxidation products of hyperforin from *Hypericum perforatum*, Phytochemistry 49 (1998) 1305-1310. DOI: 10.1016/S0031-9422(97)00903-5

[25] V. Vajs, S. Vugdelija, S. Trifunovic, I. Karadzić, N. Juranić, S. Macura, S. Milosavljević, Further degradation product of hyperforin from *Hypericum perforatum* (St. John's Wort), Fitoterapia 74 (2003) 439–444**.** DOI: 10.1016/S0367-326X(03)00114-X

[26] M. Gaid, P. Haas, T. Beuerle, S. Scholl, L. Beerhues, Hyperforin production in *Hypericum perforatum* root cultures, J. Biotechnol. 222 (2016) 47–55. DOI: 10.1016/j.jbiotec.2016.02.016

[27] O.J. Catchpole, N.B. Perry, B.M.T. Da Silva, J.B. Grey, B.M. Smallfield, Supercritical extraction of herbs I: Saw palmetto, St John's wort, Kava root, and Echinacea, J. Supercrit. Fluids. 22 (2002) 129-138. DOI: 10.1016/S0896-8446(01)00110-3

[28] S. Glisic, A. Smelcerovic, S. Zuehlke, M. Spiteller, D. Skala, Extraction of hyperforin and adhyperforin from St. John's Wort (*Hypericum perforatum* L.) by supercritical carbon dioxide, J. Supercrit. Fluids. 45 (2008) 332-337. DOI: 10.1016/j.supflu.2008.01.003

[29] M. Mannila, H. Kim, C. Isaacson, C.M. Wai, Optimisation of supercritical fluid extraction for the separation of hyperforin and adhyperforin in St. John's wort (*Hypericum perforatum* L.), Green Chem. 4 (2002) 331-336. DOI: 10.1039/B201363K

[30] H. Römpp, C. Seger, C.S. Kaiser, E. Haslinger, P.C. Schmidt, Enrichment of hyperforin from St. John's Wort (*Hypericum perforatum*) by pilot-scale supercritical carbon dioxide extraction, Eur. J. Pharm. Sci. 21 (2004) 443-451. DOI: 10.1016/j.ejps.2003.10.026

[31] C.A.J. Erdelmeier, K. Klessing, S. Renzl, H. Hauer, New hyperforin analogues from *Hypericum perforatum* and a stable dicyclohexylammonium salt of hyperforin, in: T.J.C. Luijendijk, R. Verpoorte (Eds.), 2000 Years of Natural Products Research – Past, Present and Future, Thieme, 1999, 432.

[32] S.S. Chatterjee, C. Erdelmeier, K. Klessing, D. Marme, C. Schachtele, Stable hyperforin salts, method for producing same and their use in the treatment of Alzheimer's disease, United States Patent (2002) 6,444,662 B1.

[33] B. Martínez-Poveda, L. Verotta, E. Bombardelli, A.R. Quesada, M.A. Medina, Tetrahydrohyperforin and octahydrohyperforin are two new potent inhibitors of angiogenesis, PLoS One (2010) 5, e 9558, 1-9. DOI: 10.1371/journal.pone.0009558

[34] B.I.P. Schiavone, A. Rosato, M. Marilena, S. Gibbons, E. Bombardelli, L. Verotta, C. Franchini, F. Corbo, Biological evaluation of hyperforin and its hydrogenated analogue on bacterial growth and biofilm production, J. Nat. Prod. 76 (2013) 1819−1823. DOI: 10.1021/np400394c

[35] C. Fracasso, R. Bagnati, A. Passoni, G. Guiso, L. Cantoni, A. Riva, P. Morazzoni, M. Gobbi, Brain uptake of tetrahydrohyperforin and potential metabolites after repeated dosing in mice, J. Nat. Prod. 78 (2015) 2029-2035. DOI: 10.1021/acs.jnatprod.5b00302

[36] H. Takada, J. Yonekawa, M. Matsumoto, K. Furuya, M. Sokabe, Hyperforin/HP-β-cyclodextrin enhances mechanosensitive Ca^{2+} signaling in HaCaT keratinocytes and in atopic skin *ex vivo* which accelerates wound healing, Biomed. Res. Int. (2017) 8701801. DOI: 10.1155/2017/8701801

[37] J. Füller, T. Kellner, M. Gaid, L. Beerhues, C.C. Müller-Goymann, Stabilization of hyperforin dicyclohexylammonium salt with dissolved albumin and albumin nanoparticles for studying hyperforin effects on 2D cultivation of keratinocytes *in vitro*, Eur. J. Pharm. Biopharm., submitted (2017), this issue.

[38] L. Verotta, G. Appendino, E. Belloro, J. Jakupovic, E. Bombardelli, Furohyperforin, a prenylated phloroglucinol from St. John's Wort (*Hypericum perforatum*), J. Nat. Prod. 62 (1999) 770-772. DOI: 10.1021/np980470v

[39] N. Fuzzati, N. Gabetta, I. Strepponi, F. Villa, High-performance liquid chromatography–electrospray ionisation mass spectrometry and multiple mass spectrometry studies of hyperforin degradation products, J. Chromatogr. A 926 (2001) 187–198. DOI: 10.1016/S0021-9673(01) 01000-7

[40] L. Verotta, E. Lovaglio, O. Sterner, G. Appendino, E. Bombardelli, Modulation of chemoselectivity by protein additives. Remarkable effects in the oxidation of hyperforin, J. Org. Chem. 69 (2004) 7869-7874. DOI: 10.1021/jo048857s

[41] M. Gartner, T. Müller, J.C. Simon, A. Giannis, J.P. Sleeman, Aristoforin, a novel stable derivative of hyperforin, is a potent anticancer agent, Chem. Bio. Chem. 6 (2005) 171-177. DOI:10.1002/cbic.200400195

[42] P. Zanoli, M. Rivasi, C. Baraldi, M. Baraldi, Pharmacological activity of hyperforin acetate in rats, Behav. Pharmacol. 13 (2002) 645–651. DOI: 10.1097/01.fbp.0000047149.28986.2e

[43] F. Sun, J.Y. Liu, F. He, Z. Liu, R. Wang, D.M. Wang, Y.F. Wang, D.P. Yang, In-vitro antitumor activity evaluation of hyperforin derivatives, J. Asian Nat. Prod. Res. 13 (2011) 688–699. DOI: 10.1080/10286020.2011.584532

[44] K. Leuner, V. Kazanski, M. Müller, K. Essin, B. Henke, M. Gollasch, C. Harteneck, W.E. Müller, Hyperforin – a key constituent of St. John's wort specifically activates TRPC6 channels, FASEB J. 21 (2007) 4101–4111. DOI: 10.1096/fj.07-8110com

[45] M. Müller, K. Essin, K. Hill, H. Beschmann, S. Rubant, C.M. Schempp, M. Gollasch, W.H. Boehncke, C. Harteneck, W.E Müller, K. Leuner, Specific TRPC6 channel activation, a novel approach to stimulate keratinocyte differentiation, J. Biol. Chem. 283 (2008) 33942–33954. DOI: 10.1074/jbc.M801844200

[46] W.E. Müller, Current St John's wort research from mode of action to clinical efficacy, Pharmacol. Res. 47 (2003) 101–109. DOI: 10.1016/S1043-6618(02)00266-9

[47] S. Arndt, S.F. Haag, A. Kleemann, J. Lademann, M.C. Meinke, Radical protection in the visible and infrared by a hyperforin-rich cream – in vivo versus ex vivo methods, Exp. Dermatol. 22 (2013) 354–357. DOI: 10.1111/exd.12124

[48] S.F. Haag, K. Tscherch, S. Arndt, A. Kleemann, I. Gersonde, J. Lademann, S. Rohn, M.C. Meinke, Enhancement of skin radical scavenging activity and stratum corneum lipids after the application of a hyperforin-rich cream, Eur. J. Pharm. Biopharm. 86 (2014) 227–233. DOI: 10.1016/j.ejpb.2013.06.016

[49] C.M. Schempp, T. Windeck, S. Hezel, J.C. Simon JC, Topical treatment of atopic dermatitis with St. John's wort cream – a randomized, placebo controlled, double blind half-side comparison, Phytomedicine 10 (2003) 31–37. DOI: 10.1078/1433-187X-00306

[50] J. Füller, C. Müller-Goymann, Anti-proliferative and anti-migratory effects of hyperforin in 2D and 3D artificial constructs of human dermal fibroblasts - a new option for hypertrophic scar treatment? Eur. J. Pharm. Biopharm., in press (2017), this issue.

[51] H. Takada, K. Furuya, M. Sokabe, Mechanosensitive ATP release from hemichannels and Ca^{2+} influx through TRPC6 accelerate wound closure in keratinocytes, J. Cell Sci. 127 (2014) 4159-4171. DOI: 10.1242/jcs.147314

[52] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Meth. 65 (1983) 55-63. DOI: 10.1016/0022-1759(83)90303-4

[53] D. Albert, I. Zündorf, T. Dingermann, W.E. Müller, D. Steinhilber, O. Werz, Hyperforin is a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase, Biochem. Pharmacol. 64 (2002) 1767-1775. DOI: 10.1016/S0006-2952(02)01387-4

[54] C. Feißt, C. Pergola, M. Rakonjac, A. Rossi, A. Koeberle, G. Dodt, M. Hoffmann, C. Hoernig, L. Fischer, D. Steinhilber, L. Franke, G. Schneider, O. Rådmark, L. Sautebin, O. Werz, Hyperforin is a novel type of 5-lipoxygenase inhibitor with high efficacy in vivo, Cell. Mol. Life Sci. 66 (2009) 2759–2771. DOI: 10.1007/s00018-009-0078-3

[55] M.L. Hillwig, K.D.P. Hammer, D.F. Birt, E.S. Wurtele, Characterizing the metabolic fingerprint and anti-inflammatory activity of *Hypericum gentianoide*, J. Agric. Food Chem. 56 (2008) 4359-4366. DOI:10.1021/jf800411v

[56] K.N. Gaind, T.N. Ganjoo, Antibacterial principle of *Hypericum perforatum* L., Ind. J. Pharm. 21 (1959) 172–175.

[57] C.M. Schempp, K. Pelz, A. Wittmer, E. Schopf, J.C. Simon, Antibacterial activity of hyperforin from St. John's wort against multiresistant *Staphylococcus aureus* and gram-positive bacteria, Lancet 353 (1999) 2129. DOI: 10.1016/S0140-6736(99)00214-7

[58] Z. Saddiqe, I. Naeem, A. Maimoona, A review of the antibacterial activity of *Hypericum perforatum* L., J. Ethnopharmacol. 131 (2010) 511–521. DOI: 10.1016/j.jep.2010.07.034

[59] N. Öztürk, S. Korkmaz, Y. Öztürk, Wound-healing activity of St. John's Wort (*Hypericum perforatum* L.) on chicken embryonic fibroblasts, J. Ethnopharmacol. 111 (2007) 33–39. DOI: 10.1016/j.jep.2006.10.029

[60] I. Süntar, E.K. Akkol, H. Keles, A. Oktem, K.H.C. Başer, E. Yeşilada, A novel wound healing ointment: A formulation of *Hypericum perforatum* oil and sage and oregano essential oils based on traditional Turkish knowledge, J. Ethnopharmacol. 134 (2011) 89–96. DOI: 10.1016/j.jep.2010.11.061

[61] M.C. Dinamarca, W. Cerpa, J. Garrido, J.L. Hancke, N.C. Inestrosa, Hyperforin prevents β-amyloid neurotoxicity and spatial memory impairments by disaggregation of Alzheimer's amyloid-β-deposits, Mol. Psychiatry 11 (2006) 1032–1048. DOI: 10.1038/sj.mp.4001866

[62] W. Cerpa, J.L. Hancke, P. Morazzoni, E. Bombardelli, A. Riva, P.P. Marin, N.C. Inestrosa, The hyperforin derivative IDN5706 occludes spatial memory impairments and neuropathological changes in a double transgenic Alzheimer's mouse model, Curr Alzheimer Res 7 (2010) 126-133. DOI: 10.2174/156720510790691218

[63] A.C. Abbott, C. Calderon Toledo, F.C. Aranguiz, N.C. Inestrosa, L. Varela-Nallar, Tetrahydrohyperforin increases adult hippocampal neurogenesis in wild-type and APPswe/PS1ΔE9 mice, J. Alzheimers Dis. 34 (2013) 873-885. DOI: 10.3233/JAD-121714

[64] C. Montecinos-Oliva, A. Schuller, J. Parodi, F. Melo, N.C. Inestrosa, Effects of tetrahydrohyperforin in mouse hippocampal slices: neuroprotection, long-term potentiation and TRPC channels, Curr. Med. Chem. 21 (2014) 3494-506. DOI: 10.2174/0929867321666140716091229

[65] C. Montecinos-Oliva, A. Schüller, N.C. Inestrosa, Tetrahydrohyperforin: a neuroprotective modified natural compound against Alzheimer's disease, Neural Regener. Res. 10 (2015) 552-554. DOI: 10.4103/1673-5374.155420

[66] J. Hofrichter, M. Krohn, T. Schumacher, C. Lange, B. Feistel, B. Walbroel, H.J. Heinze, S. Crockett, T.F. Sharbel, J. Pahnke, Reduced Alzheimer's disease pathology by St. John's Wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice, Curr. Alzheimer Res. 10 (2013) 1057–1069. DOI: 10.2174/15672050113106660171

[67] C.M. Schempp, V. Kirkin, B. Simon-Haarhaus, A. Kersten, J. Kiss, C.C. Termeer, B. Gilb, T. Kaufmann, C. Borner, J.P. Sleeman, J.C. Simon, Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis, Oncogene 21 (2002) 1242–1250. DOI: 10.1038/sj/onc/1205190

[68] M. Donà, I. Dell'Aica, E. Pezzato, L. Sartor, F. Calabrese, M.D. Barbera, A. Donella-Deana, G. Appendino, A. Borsarini, R. Caniato, S. Garbisa, Hyperforin inhibits cancer invasion and metastasis, Cancer Res. 64 (2004) 6225–6232. DOI: 10.1158/0008-5472.CAN-04-0280

[69] B. Martínez-Poveda, A.R. Quesada, M.A. Medina, Hyperforin, a bio-active compound of St. John's Wort, is a new inhibitor of angiogenesis targeting several key steps of the process, Int. J. Cancer 117 (2005) 775–780. DOI: 10.1002/ijc.21246

[70] M. Rothley, A. Schmid, W. Thiele, V. Schacht, D. Plaumann, M. Gartner, A. Yektaoglu, F. Bruyère, A. Noël, A. Giannis, J.P. Sleeman, Hyperforin and aristoforin inhibit lymphatic endothelial cell in vitro and suppress tumor-induced lymphangiogenesis in vivo, Int. J. Cancer 125 (2009) 34–42. DOI: 10.1002/ijc.24295

[71] C. Billard, F. Merhi, B. Bauvois, Mechanistic insights into the antileukemic activity of hyperforin, Curr. Canc. Drug Targ. 13 (2013) 1-10. DOI: 10.2174/1568009138044866601

[72] J. Kwon, K.S. Oh, S.Y. Cho, M.A. Bang, H.S. Kim, B. Vaidy, D. Kim, Estrogenic activity of hyperforin in MCF-7 human breast cancer cells transfected with estrogen receptor, Planta Med. 82 (2016) 1425–1430. DOI: 10.1055/s-0042-112594

[73] A. Ševčovičová, M. Šemeláková, J. Plšíková, D. Loderer, P. Imreová, E. Gálová, M. Kožurková, E. Miadoková, P. Fedoročko, The complex metabolite profile of SJW make excipient compatibility testing essential specially for unstable compounds, Toxicol. In Vitro 29 (2015) 631–637. DOI: 10.1016/j.tiv.2015.01.016

[74] L.B. Moore, B. Goodwin, S.A. Jones, G.B. Wisely, C.J. Serabjit-Singh, T.M. Willson, J.L. Collins, S.A. Kliewer, St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor, PNAS 97 (2000) 7500–7502. DOI: 10.1073/pnas.130155097

[75] I. Mai, S. Bauer, E.S. Perloff, A. Johne, B. Uehleke, B. Frank, K. Budde, I. Roots, Hyperforin content determines the magnitude of the St John's wort-cyclosporine drug interaction, Clin. Pharmacol. Ther. 76 (2004) 330-340. DOI: 10.1016/j.clpt.2004.07.004

[76] S.C. Mueller, B. Uehleke, H. Woehling, M. Petzsch, J. Majcher-Peszynska, E.M. Hehl, H. Sievers, B. Frank, A.K. Riethling, Effect of St John's wort dose and preparations on the pharmacokinetics of digoxin, Clin. Pharmacol. Ther. 75 (2004) 546-557. DOI: 10.1016/j.clpt.2004.01.014

[77] K. Leuner, J.H. Heiser, S. Derksen, M.I. Mladenov, C.J. Fehske, R. Schubert, M. Gollasch, G. Schneider, C. Harteneck, S.S. Chatterjee, W.E. Müller, Simple 2,4-diacylphloroglucinols as classic transient receptor potential-6 activators—identification of a novel pharmacophore, Mol Pharmacol 77 (2010) 368–377. DOI: 10.1124/mol.109.057513

[78] B.A. Kandel, S. Ekins, K. Leuner, W.E. Thasler, C. Harteneck, U.M. Zanger, No activation of human pregnane X receptor by hyperforin-related phloroglucinols, J. Pharmacol. Exp. Ther. 348 (2014) 393–400. DOI: 10.1124/jpet.113.209916

[79] L. Beerhues, Biosynthesis of the active *Hypericum perforatum* constituents, in: M.S. Odabas, C. Cirak (Eds.), Hypericum, Series: Medicinal and Aromatic Plant Science and Biotechnology 5, Special issue 1, Global Science Books, Isleworth UK, 2011, pp. 70-77.

[80] J. Zhao, W. Liu, J.C. Wang, Recent advances regarding constituents and bioactivities of plants from the genus *Hypericum*, Chem. Biodivers. 12 (2015) 309-349. DOI: 10.1002/cbdv.201300304

[81] S.M.A. Zobayed, F. Afreen, E. Goto, T. Kozai, Plant–environment interactions: Accumulation of hypericin in dark glands of *Hypericum perforatum*, Ann. Bot. 98 (2006) 793–804. DOI: 10.1093/aob/mcl169

[82] D. Hölscher, R. Shroff, K. Knop, M. Gottschaldt, A. Crecelius, B. Schneider, D.G. Heckel, U.S. Schubert, A. Svatoš, Matrix-free UV-laser desorption/ionization (LDI) mass spectrometric imaging at the single-cell level: distribution of secondary metabolites in *Arabidopsis thaliana* and *Hypericum* species, Plant J. 60 (2009) 907–918. DOI:10.1111/j.1365-313X.2009.04012.x

[83] S. Kusari, S. Sezgin, K. Nigutova, E. Čellárová, M. Spiteller, Spatial chemo-profiling of hypericin and related phytochemicals in *Hypericum* species using MALDI-HRMS imaging, Anal. Bioanal. Chem. 407 (2015) 4779–4791. DOI: 10.1007/s00216-015-8682-6

[84] A. Kucharíková, K. Kimáková, C. Janfelt, E. Čellárová, Interspecific variation in localization of hypericins and phloroglucinols in the genus *Hypericum* as revealed by desorption electrospray ionization mass spectrometry imaging, Physiol. Plant. 157 (2016) 2-12. DOI: 10.1111/ppl.12422

[85] G.A. Kraus, D. Pratt, J. Tossberg, S. Carpenter, Antiretroviral activity of synthetic hypericin and related analogs, Biochem. Biophys. Res. Commun. 172 (1990) 149-153. DOI: 10.1016/S0006-291X(05)80185-8

[86] A. Barras, L. Boussekey, E. Courtade, R. Boukherroub, Hypericin-loaded lipid nanocapsules for photodynamic cancer therapy in vitro, Nanoscale 5 (2013) 10562–10572. DOI: 10.1039/c3nr02724d

[87] J. Brockmöller, T. Reum, S. Bauer, R. Kerb, W.D. Hübner, I. Roots, Hypericin and pseudohypericin: pharmacokinetics and effects on photosensitivity in humans, Pharmacopsychiatry 30(S2) (1997) 94-101. DOI: 10.1055/s-2007-979527

[88] C.M. Schempp, R. Lüdtke, B. Winghofer, J.C. Simon, Effect of topical application of *Hypericum perforatum* extract (St. John's wort) on skin sensitivity to solar simulated radiation, Photodermatol. Photoimmunol. Photomed. 16 (2000) 125-128. DOI: 10.1034/j.1600-0781.2000.d01-18.x

[89] J. Soelberg, L.B. Jørgensen, A.K. Jäger, Hyperforin accumulates in the translucent glands of *Hypericum perforatum*, Ann. Bot. 99 (2007) 1097–1100. DOI: 10.1093/aob/mcm057

[90] J. Thunig, S.H. Hansen, C. Janfelt, Analysis of secondary plant metabolites by indirect desorption electrospray ionization imaging mass spectrometry, Anal. Chem. 83 (2011) 3256–3259. DOI: 10.1021/ac2004967

[91] D. Tekel'ová, M. Repcák, E. Zemková, J. Tóth, Quantitative changes of dianthrones, hyperforin and flavonoids content in the flower ontogenesis of *Hypericum perforatum*, Planta Med. 66 (2000) 778–780. DOI: 10.1055/s-2000-9779

[92] S.L. Crockett, B. Schaneberg, I.A. Khan, Phytochemical profiling of new and old world *Hypericum* (St. John's Wort) species, Phytochem. Anal. 16 (2005) 479–485. DOI: 10.1002/pca.875

[93] G.V. Ccana-Ccapatinta, F.M.C. Barros, H. Bridi, G.L. von Poser, Dimeric acylphloroglucinols in *Hypericum* species from sections Brathys and Trigynobrathys, Phytochem. Rev. 14 (2013) 25-50. DOI: 10.1007/s11101-013-9332-2

[94] P. Mártonfi, M. Repčák, L. Mártonfiová, Secondary metabolites during ontogenetic phase of reproductive structures in *Hypericum maculatum*, Biologia 61 (2006) 473-478. DOI: 0.2478/s11756-006-0079-8

[95] P.T. Nedialkov, G. Momekov, Z.K. Kokanova-Nedialkova, J. Heilmann, Polyprenylated phloroglucinols from *Hypericum maculatum*, Nat. Prod. Commun. 10 (2015) 1231-1235.

[96] S. Kusari, S. Zühlke, T. Borsch, M. Spiteller, Positive correlations between hypericin and putative precursors detected in the quantitative secondary metabolite spectrum of *Hypericum*, Phytochemistry 70 (2009) 1222-1232. DOI: 10.1016/j.phytochem.2009.07.022

[97] A. Porzel, M.A. Farag, J. Mulbradt, L. Wessjohann, Metabolite profiling and fingerprinting of *Hypericum* species: a comparison of MS and NMR metabolomics, Metabolomics 10 (2014) 574–588. DOI: 10.1007/s11306-013-0609-7

[98] M. Soták, O. Czeranková, D. Klein, Z. Jurčacková, L. Li, E. Čellárová, Comparative transcriptome reconstruction of four *Hypericum* species focused on hypericin biosynthesis, Front. Plant Sci. 7 (2016) 1039. DOI.org/10.3389/fpls.2016.01039

[99] R. Bruni, G. Sacchetti, Factors affecting polyphenol biosynthesis in wild and field grown St. John's wort (*Hypericum perforatum L*. Hypericaceae/Guttiferae), Molecules 14 (2009) 682-725. DOI:10.3390/molecules14020682

[100] R. Filippini, A. Piovan, A. Borsarini, R. Caniato, Study of dynamic accumulation of secondary metabolites in three subspecies of *Hypericum perforatum*, Fitoterapia 81 (2010) 115–119. DOI: 10.1016/j.fitote.2009.08.002

[101] H. Böttcher, I. Günther, L. Kabelitz, Physiological postharvest responses of common Saint-John's wort herbs (*Hypericum perforatum* L.), Postharvest Biol. Technol. 29 (2003) 342-350. DOI: 10.1016/S0925-5214(03)00057-7

[102] M.S. Odabas, J. Raduğienë, N. Camas, V. Janulis, L. Ivanauskas, C. Çirak, The quantitative effects of temperature and light intensity on hyperforin and hypericins accumulation in *Hypericum perforatum* L, J. Med. Plants Res. 3 (2009) 519-525.

[103] I.A. Southwell, C.A. Bourke, Seasonal variation in hypericin content in *Hypericum perforatum* L, Phytochemistry 56 (2001) 437-441. DOI: 10.1016/S0031-9422(00)00411-8

[104] T.M. Sirvent, S.B. Krasnoff, D.M. Gibson, Induction of hypericins and hyperforins in *Hypericum perforatum* in response to damage by herbivores, J. Chem. Ecol. 29 (2003) 2667–2681. DOI: 10.1023/B:JOEC.0000008011.77213.64

[105] J. Košuth, J. Koperdakova, A. Tolonen, A. Hohtola, E. Čellárová, The content of hypericins and phloroglucinols in *Hypericum perforatum* L. seedlings at early stage of development, Plant Sci. 165 (2003) 515-521. DOI: 10.1016/S0168-9452(03)00210-3

[106] T. Dingermann, I. Zündorf, Pyrrolizidinalkaloide – Probleme bei Naturheilmitteln, Pharm. Ztg. 13 (2016) 12-14.

[107] E. Čellárová, Effect of exogenous morphogenetic signals on differentiation *in vitro* and secondary metabolite formation in the genus *Hypericum*, in: M.S. Odabas, C. Cirak (Eds.), *Hypericum*, Series: Medicinal and Aromatic Plant Science and Biotechnology 5, Special issue 1, Global Science Books, Isleworth UK, 2011, pp. 62-69.

[108] R. Perrone, P.D. Rosa, C.D. Castro, P. Colombo, Leaf and stem anatomy in eight *Hypericum* species (Clusiaceae), Acta Bot. Croat. 72 (2013) 269–286. DOI: 10.2478/botcro-2013-0008

[109] A. Kornfeld, R.B. Kaufman, C.R. Lu, D.M. Gibson, S.F. Bolling, S.L. Warber, S.C. Chang, A. Kirakosyan, The production of hypericins in two selected *Hypericum perforatum* shoot cultures is related to differences in black gland structure, Plant Physiol. Biochem. 45 (2007) 24-32. DOI: 10.1016/j.plaphy.2006.12.009

[110] A.R. Alan, S.J. Murch, P.K. Saxena, Evaluation of ploidy variations in *Hypericum perforatum* L. (St. John's wort) germplasm from seeds, in vitro germplasm collection, and regenerants from floral cultures, In Vitro Cell. Dev. Biol. Plant 51 (2015) 452–462. DOI 10.1007/s11627-015-9708-7

[111] K. Bruňáková, J. Zámećník, M. Urbanová, E. Čellárová, Dehydration status of ABA-treated and cold-acclimated *Hypericum perforatum* L. shoot tips subjected to cryopreservation, Thermochim. Acta 525 (2012) 62-70. DOI: 10.1016/j.tca.2011.07.022

[112] K. Bruňáková, E. Cellárová, Conservation strategies in the genus *Hypericum* via cryogenic treatment, Front. Plant Sci. 7 (2016) 1-12. DOI: 10.3389/fpls.2016.00558

[113] P. Klingauf, T. Beuerle, A. Mellenthin, S.A.M. El-Moghazy, Z. Boubakir, L. Beerhues, Biosynthesis of the hyperforin skeleton in *Hypericum calycinum* cell cultures, Phytochemistry 66 (2005) 139-145. DOI: 10.1016/j.phytochem.2004.11.003

[114] S. Gadzovska, S. Maury, S. Ounnar, M. Righezza, S. Kascakova, M. Refregiers, M. Spasenoski, C. Joseph, D. Hagège, Identification and quantitation of hypericin and pseudohypericin in different *Hypericum perforatum* L in vitro cultures, Plant Physiol. Biochem. 43 (2005) 591–601. DOI: 10.1016/j.plaphy.2005.05.005

[115] S. Gadzovska, S. Maury, A. Delaunay, M. Spasenoski, D. Hagège, D. Courtois, C. Joseph, The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphtodianthrones and phenylpropanoids in *Hypericum perforatum* L., Plant Cell Tiss. Organ Cult. 113 (2013) 25–39. DOI: 10.1007/s11240-012-0248-0

[116] G. Pasqua, P. Avato, B. Monacellia, A.R. Santamaria, M.P. Argentieri, Metabolites in cell suspension cultures, calli, and in vitro regenerated organs of *Hypericum perforatum* cv. Topas, Plant Sci. 165 (2003) 977–982. DOI: 10.1016/S0168-9452(03)00275-9

[117] M. Ziv, Vitrification: morphological and physiological disorders of in vitro plants, in: P.C. Debergh, R.H. Zimmerman (Eds.), Micropropagation – Technology and Application. Kluwer Academic Publ., Dordrecht, 1991, pp. 45–69. DOI: 10.1007/978-94-009-2075-0_4

[118] S.A. Wilson, S.C. Roberts, Recent advances towards development and commercialization of plant cell culture processes for synthesis of biomolecules, Plant Biotechnol. J. 10 (2012) 249–268. DOI: 10.1111/j.1467-7652.2011.00664.x

[119] S.M.A. Zobayed, P.K. Saxena, Production of St. John's wort plants under controlled environment for maximizing biomass and secondary metabolites, In Vitro Cell. Dev. Biol. Plant 40 (2004) 108–114. DOI: 10.1079/IVP2003498

[120] A. Kirakosyan, D.M. Gibson, P.B. Kaufman, The production of dianthrones and phloroglucinols derivatives in St. John's Wort, in: K.G. Ramawat, J. Merillon (Eds.), Bioactive Molecules and Medicinal Plants, XXIV, Springer, Berlin, 2008, pp. 149-162. DOI: 10.1007/978-3-540-74603-4_7

[121] R.A. Dixon, Natural products and plant disease resistance, Nature 411 (2001) 843-847. DOI: 10.1038/35081178

[122] M. Xu, B. Yang, J. Dong, D. Lu, H. Jin, L. Sun, Y. Zhu, X. Xu, Enhancing hypericin production of *Hypericum perforatum* cell suspension culture by ozone exposure, Biotechnol. Prog. 27 (2011) 1101-1106. DOI: 10.1002/btpr.614

[123] A.M.A. Abd El-Mawla, W. Schmidt, L. Beerhues, Cinnamic acid is a precursor of benzoic acid in cell cultures of *Hypericum androsaemum* L. but not in cell cultures of *Centaurium erythraea* RAFN, Planta 212 (2001) 288-293**.** DOI: 10.1007/s00425000039

[124] M.M. Gaid, D. Sircar, A. Müller, T. Beuerle, B. Liu, L. Ernst, R. Hänsch, L. Beerhues, Cinnamate:CoA ligase initiates biosynthesis of a benzoate-derived xanthone phytoalexin in *Hypericum calycinum* cell cultures, Plant Physiol. 160 (2012) 1267-1280. Doi: 10.1104/pp.112.204180

[125] O. Tusevski, J. Petreska Stanoeva, M. Stefova, S. Gadzovska Simic, Agrobacterium enhances xanthone production in *Hypericum perforatum* cell suspensions, J. Plant Growth Regul. 76 (2015) 199–210. DOI:10.1007/s10725-014-9989-6

[126] T. Fiesel, M. Gaid, A. Müller, J. Bartels, I. El-Awaad, T. Beuerle, L. Ernst, S. Behrends, L. Beerhues, Molecular cloning and characterization of a xanthone prenyltransferase from *Hypericum calycinum* cell cultures, Molecules 20 (2015) 15616-15630. DOI: 10.3390/molecules20091561

[127] D. Zubrická, A. Mišianiková, J. Henzelyová, A. Valletta, G. De Angelis, F.D. D'Auria, G. Simonetti, G. Pasqua, E. Čellárová, Xanthones from roots, hairy roots and cell suspension cultures of selected *Hypericum* species and their antifungal activity against *Candida albicans*, Plant Cell Rep. 34 (2015) 1953–1962. DOI: 10.1007/s00299-015-1842-5

[128] K. Karppinen, J. Hokkanen, A. Tolonen, S. Mattila, A. Hohtola, Biosynthesis of hyperforin and adhyperforin from amino acid precursors in shoot cultures of *Hypericum perforatum*, Phytochemistry, 68 (2007) 1038-1045. DOI: 10.1016/j.phytochem.2007.01.001

[129] S.M.A. Zobayed, S.J. Murch, H.P.V. Rupasinghe, P.K. Saxena, Elevated carbon supply altered hypericin and hyperforin contents of St. John's wort (*Hypericum perforatum*) grown in bioreactors, Plant Cell Tiss. Organ Cult. 75 (2003) 143–149**.** DOI: 10.1023/A:1025053427371

[130] S.M.A. Zobayed, S.J. Murch, H.P.V. Rupasinghe, P.K. Saxena, In vitro production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'), Plant Sci. 166 (2004) 333-340. DOI: 10.1016/j.plantsci.2003.10.005

[131] X.H. Cui, H.N. Murthy, K.Y. Paek, Pilot-scale culture of *Hypericum perforatum* L. adventitious roots in airlift bioreactors for the production of bioactive compounds, Appl. Biochem. Biotechnol. 174 (2014) 784–792. DOI: 10.1007/s12010-014-1123-8

[132] S.Q. Wu, X.K. Yu, M.L. Lian, S.Y. Park, X.C. Piao, Several factors affecting hypericin production of *Hypericum perforatum* during adventitious root culture in airlift bioreactors, Acta Physiol. Plant. 36 (2014) 975–981. DOI: 10.1007/s11738-013-1476-6

[133] H.N. Murthy, E.J. Hahn, K.Y. Paek, Adventitious roots and secondary metabolism, Chin. J. Biotechnol. 24 (2008) 711–716. DOI: 10.1016/S1872-2075(08)60035-7

[134] E. Koch, C. Erdelmeier, J. Herrmann, St. John's wort extracts having reduced chlorophyll and proanthocyanidin contents, useful as topical medicaments, e.g. for treating stomatitis, acne, viral infections or psoriasis, Deutsch. Patent (2000) DE10131641 A1.

[135] M. Gaid, T. Wucherpfennig, S. Scholl, L. Beerhues, R. Krull, Challenges for the cultivation of plant cells on the example of *Hypericum perforatum* and *Taxus chinensis*, in: Bioprocessing of plant in vitro systems, A. Pavlov, T. Bley (Eds), Reference Series in Phytochemistry, Springer, 2017, in press.

[136] A. Bertoli, A. Giovannini, B. Ruffoni, A.D. Guardo, G. Spinelli, M. Mazzetti, L. Pistelli, Bioactive constituent production in St. John's wort in vitro hairy roots. Regenerated plant lines, J. Agric. Food Chem. 56 (2008) 5078-5082. DOI: 10.1021/jf0729107

[137] M.K. Goel, A.K. Kukreja, N.S. Bisht, In vitro manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis, Plant Cell Tiss. Organ Cult. 96 (2008) 1–9. DOI: 10.1007/s11240-008-9453-2

[138] N. Tocci, G. Simonetti, F.D. D'Auria, S. Panella, A.T. Palamara, A. Valletta, G. Pasqua, Root cultures of *Hypericum perforatum* subsp. angustifolium elicited with chitosan and production of xanthone-rich extracts with antifungal activity, Appl. Microbiol. Biotechnol. 91 (2011) 977-987. DOI: 0.1007/s00253-011-3303-6

[139] N. Tocci, F.D. D´Auria, G. Simonetti, S. Panella, A.T. Palamara, A. Debrassi, C.A. Rodrigues, V.C. Filho, F. Sciubba, G. Pasqua, Bioassay-guided fractionation of extracts from *Hypericum perforatum* in vitro roots treated with carboxymethylchitosans and determination of antifungal activity against human fungal pathogens, Plant Physiol. Biochem. 70 (2013) 342–347. DOI: 10.1016/j.plaphy.2013.05.046

[140] N. Tocci, G. Simonetti, F.D. D'Auria, S. Panella, A.T. Palamara, F. Ferrari, G. Pasqua, Chemical composition and antifungal activity of *Hypericum perforatum* subsp angustifolium roots from wild plants and plants grown under controlled conditions, Plant Biosyst. 147 (2013) 557–562. DOI: 10.1080/11263504.2013.806964

[141] H.N. Murthy, Y.S. Kim, S.Y. Park, K.Y. Paek, Hypericins: biotechnological production from cell and organ cultures, Appl. Microbiol. Biotechnol. 98 (2014) 9187–9198. DOI: 10.1007/s00253-014-6119-3

[142] A. Valletta, G. De Angelis, C. Badiali, E. Brasili, A. Miccheli, M.E. Di Cocco, G. Pasqua, Acetic acid acts as an elicitor exerting a chitosan-like effect on xanthone biosynthesis in *Hypericum perforatum* L. root cultures, Plant Cell Rep. 35 (2016) 1009–1020. DOI: 10.1007/s00299-016-1934-x

[143] G. Simonetti, N. Tocci, A. Valletta, E. Brasili, F.D. D'Auria, A. Idoux, G. Pasqua, In vitro antifungal activity of extracts obtained from *Hypericum perforatum* adventitious roots cultured in a mist bioreactor against planktonic cells and biofilm of *Malassezia furfur*, Nat. Prod. Res. 30 (2015) 544-550. DOI:10.1080/14786419.2015.1028059

[144] O. Tusevski, J.P. Stanoeva, M. Stefova, D. Kungulovski, N.A. Pancevska, N. Sekulovski, S. Panov, S. Gadzovska Simic, Hairy roots of *Hypericum perforatum* L.: a promising system for xanthone production, Cent. Eur. J. Biol. 8 (2013) 1010-1022. DOI: 10.2478/s11535-013-0224-7

[145] J. Steingroewer, T. Bley, V. Georgiev, I. Ivanov, F. Lenk, A. Marchev, A. Pavlov, Bioprocessing of differentiated plant *in vitro* systems, Engin. Life Sci. 13 (2013) 26-38. DOI: 10.1002/elsc.201100226

[146] P. Haas, M. Gaid, A. Zarinwall, L. Beerhues, S. Scholl, Downstream processing of hyperforin from *Hypericum perforatum* root cultures, Eur. J. Pharm. Biopharm. (2017) submitted, this issue.

[147] A. Charchoglyan, A. Abrahamya, I. Fujii, Z. Boubakir, T.A.M. Gulder, T.M. Kutchan, H. Vardapetyan, G. Bringmann, Y. Ebizuka, L. Beerhues, Differential accumulation of hyperforin and secohyperforin in *Hypericum perforatum* tissue cultures, Phytochemistry 68 (2007) 2670–2677. DOI: 10.1016/j.phytochem.2007.06.004

[148] A. Brenn, M. Grube, G. Jedlitschky, A. Fischer, B. Strohmeier, M. Eiden, M. Keller, M.H. Groschup, S. Vogelgesang, St. John's Wort reduces beta-amyloid accumulation in a double transgenic Alzheimer's Disease mouse model – Role of P-glycoprotein, Brain Pathol. 24 (2014) 18–24. DOI: 10.1111/bpa.12069

[149] N. Sasaoka, M. Sakamoto, S. Kanemori, M. Kan, C. Tsukano, Y. Takemoto, A. Kakizuka, Long-term oral administration of hop flower extracts mitigates Alzheimer phenotypes in mice, PLoS One 9 (2014) e87185. DOI: 10.1371/journal.pone.0087185

[150] D.G. Teotico, J.J. Bischof, L. Peng, S.A. Kliewer, M.R. Redinbo, Structural basis of human pregnane X receptor activation by the hops constituent colupulone, Mol. Pharmacol. 74 (2008) 1512–1520. DOI:10.1124/mol.108.050732

[151] B.A. Sparling, D.C. Moebius, M.D. Shair, Enantioselective total synthesis of hyperforin, J. Am. Chem. Soc. 135 (2013) 644-647. DOI: 10.1021/ja312150d

[152] C.P. Ting, T.J. Maimone, Total synthesis of hyperforin, J. Am. Chem. Soc. 137 (2015) 10516-10519. DOI: 10.1021/jacs.5b06939

[153] K. Seidler-Lożykowska, Secondary metabolites content of *Hypericum* sp. in different stages and plant parts, in: E. Ernst (Ed.), Hypericum: The genus *Hypericum*, Taylor & Francis, London, 2003, pp.100-105.

[154] J. Radušienė, B. Karpavičienė, Ž. Stanius, Effect of external and internal factors on secondary metabolites accumulation in St. John's worth, Botan. Lithuan. 18 (2012) 101-108. DOI: 10.2478/v10279-012-0012-8
[155] E. Ernst, Hypericum: The Genus *Hypericum*, Taylor & Francis CRC Press, London, 2003, pp. 196
[156] A.K. Belkheir, M. Gaid, B. Liu, R. Hänsch, L. Beerhues, Benzophenone synthase and chalcone synthase accumulate in the mesophyll of *Hypericum perforatum* leaves at different developmental stages, Front. Plant Sci. 7 (2016) 921. DOI: 10.3389/fpls.2016.00921
[157] J.M. Jez, M.E. Bowman, J.P. Noel, Role of hydrogen bonds in the reaction mechanism of chalcone isomerase, Biochem. 41 (2002) 5168–5176**.** DOI: 10.1074/jbc.M109224200
[158] G. Schröder, E. Wehinger, R. Lukacin, F. Wellmann, W. Seefelder, W. Schwab, J. Schröder, Flavonoid methylation: a novel 4-O-methyltransferase from *Catharanthus roseus*, an evidence that partially methylated flavanones are substrates of four different flavonoid dioxygenases, Phytochemistry 65 (2004) 1085–1094. DOI: 10.1016/j.phytochem.2004.02.010
[159] J.F. Stevens, J.E. Page, Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry 65 (2004) 1317–1330. DOI: 10.1016/j.phytochem.2004.04.025
[160] G. Bringmann, T.F. Noll, T. Gulder, M. Dreyer, M. Grune, D. Moskau, Polyketide folding in higher plants: biosynthesis of the phenylanthraquinone knipholone. J. Org. Chem. 72 (2007) 3247–3252. DOI: 10.1021/jo062566x
[161] K. Karppinen, J. Hokkanen, S. Mattila, P. Neubauer, A. Hohtola, Octaketide-producing type III polyketide synthase from *Hypericum perforatum* is expressed in dark glands accumulating hypericins, FEBS J. 275 (2008) 4329-4342. DOI: 10.1111/j.1742-4658.2008.06576.x
[162] I. El-Awaad, M. Bocola, T. Beuerle, B. Liu, L. Beerhues, Bifunctional CYP81AA proteins catalyse identical hydroxylations but alternative regioselective phenol couplings in plant xanthone biosynthesis, Nat. Commun. 7 (2016) 11472,1-12. DOI: 10.1038/ncomms11472
[163] K. Zhou, L. Ludwig, S.M. Li*,* Friedel-Crafts alkylation of acylphloroglucinols catalyzed by a fungal indole prenyltransferase, J. Nat. Prod. 78 (2015) 929-933. DOI: 10.1021/np5009784
[164] K. Zhou, X. Yu, X. Xie, S.M. Li, Complementaryf flavonoid prenylations by fungal indole prenyltransferases, J. Nat. Prod., 78 (2015) 2229–2235. DOI: 10.1021/acs.jnatprod.5b00422
[165] K. Yazaki, K. Sasaki, Y. Tsurumaru, Prenylation of aromatic compounds, a key diversification of plant secondary metabolites, Phytochemistry 70 (2009) 1739-1745. DOI: 10.1016/j.phytochem.2009.08.023
[166] K. Yazaki, M. Kunihisa, T. Fujisaki, F. Sato, Geranyl diphosphate: 4-hydroxybenzoate geranyltransferase from *Lithospermum erythrorhizon*. Cloning and characterization of a key enzyme in shikonin biosynthesis, J. Biol. Chem. 277 (2002) 6240–6246. DOI: 10.1074/jbc.M106387200
[167] K. Ohara, K. Mito, K. Yazaki, Homogeneous purification and characterization of LePGT1- A membrane-bound aromatic substrate prenyltransferase involved in secondary metabolism of Lithospermum erythrorhizon, FEBS J. 280 (2013) 2572–2580. DOI: 10.1111/febs.12239
[168] F. Karamat, A. Olry, R. Munakata, T. Koeduka, A. Sugiyama, C. Paris, A. Hehn, F. Bourgaud, K. Yazaki, A coumarin-specific prenyltransferase catalyzes the crucial biosynthetic reaction for furanocoumarin formation in parsley, Plant J. 77 (2014) 627–638. DOI: 10.1111/tpj.12409
[169] R. Wang, R. Chen, J. Li, X. Liu, K. Xie, D. Chen, Y. Peng, J. Dai, Regiospecific prenylation of hydroxyxanthones by a plant flavonoid prenyltransferase, J. Nat. Prod. 79 (2016) 2143−2147. DOI:10.1021/acs.jnatprod.6b00417
[170] Y. Tsurumaru, K. Sasaki, T. Miyawaki, Y. Uto, T. Momma, N. Umemoto, M. Momose, K. Yazaki, HlPT-1, a membrane-bound prenyltransferase responsible for the biosynthesis of bitter acids in hops, Biochem. Biophys. Res. Commun. 417 (2012) 393–398. DOI: 10.1016/j.bbrc.2011.11.125
[171] H. Li, Z. Ban, H. Qin, L. Ma, A.J. King, G. Wang, A heteromeric membrane-bound prenyltransferase complex from hop catalyzes three sequential aromatic prenylations in the bitter acid pathway, Plant Physiol. 167 (2015) 650–659. DOI:10.1104/pp.114.253682
[172] L. Beerhues, Hyperforin, Phytochemistry 67 (2006) 2201-2207. DOI: 10.1016/j.phytochem.2006.08.017
[173] P. Adam, D. Arigoni, A. Bacher, W. Eisenreich, Biosynthesis of hyperforin in *Hypericum perforatum*, J. Med. Chem. 45 (2002) 4786-93. DOI: 10.1021/jm0209782
[174] R. Ciochina, R.B. Grossman, Polycyclic polyprenylated acylphloroglucinols, Chem. Rev. 106 (2006) 3963-3986. DOI: 10.1021/cr0500582
[175] J.A. Richard, R.H. Pouwer, D.Y.K. Chen, The chemistry of the polycyclic polyprenylated acylphloroglucinols, Angew. Chem. Int. Ed. 51 (2012) 4536–4561. DOI: 10.1002/anie.201103873

[176] X.W. Yang, M.M. Li, X. Liu, D. Ferreira, Y. Ding, J.J. Zhang, Y. Liao, H.B. Qin, G. Xu, Polycyclic polyprenylated acylphloroglucinol congeners possessing diverse structures from *Hypericum henryi*, J. Nat. Prod. 78 (2015) 885-895. DOI: 10.1021/acs.jnatprod.5b00057

[177] M. Soták, O. Czeranková, D. Klein, K. Nigutová, L. Altschmied, L. Li, A. Jose, E.S. Wurtele, E. Čellárová, Differentially expressed genes in hypericin-containing *Hypericum perforatum* leaf tissues as revealed by de novo assembly of RNA-seq, Plant Mol. Biol. Rep. 34 (2016) 1027–1041. DOI 10.1007/s11105-016-0982-2

[178] A.M. Alhassan, M.I. Abdullahi, A. Uba, A. Umar, Prenylation of aromatic secondary metabolites: a new frontier for development of novel drugs, Trop. J. Pharm. Res. 13 (2014) 307-314. DOI.org/10.4314/tjpr.v13i2.22

[179] W. Watjen, N. Weber, Y.J. Lou, Z.Q. Wang, Y. Chovolou, A. Kampkotter, R. Kahl, P. Proksch, Prenylation enhances cytotoxicity of apigenin and liquiritigenin in rat H4IIE hepatoma and C6 glioma cells, Food Chem. Toxicol. 45 (2007) 119-124. DOI: 10.1016/j.fct.2006.08.008

[180] C.L. Miranda, J.F. Stevens, V. Ivanov, M. McCall, B. Frei, M.L. Deinzer, D.R. Buhler, Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro, J. Agric. Food Chem. 48 (2000) 3876–3884**.** DOI: 10.1021/jf0002995

[181] Y. Ren, S. Matthew, D.D. Lantvit, T.N. Ninh, H. Chai, J.R. Fuchs, D.D. Soejarto, E.J. Carcache de Blanco, S.M. Swanson, D.A. Kinghorn, Cytotoxic and Nf-kB inhibitory constituents of the stem of *Cratoxylum cochinchinense* and their semisynthetic analogues, J. Nat. Prod. 74 (2011) 1117-1125. DOI: 10.1021/np200051j

[182] K.K. Khaw, S.B. Choi, S.C. Tanc, H.A. Wahab, K.L. Chane, V. Murugaiyaha, Prenylated xanthones from mangosteen as promising cholinesterase inhibitors and their molecular docking studies, Phytomed. 21 (2014) 1303–1309. DOI: 10.1016/j.phymed.2014.06.017

[183] A.G. Jensen, S.H. Hansen, E.Q. Nielsen, Adhyperforin as a contributor to the effect of *Hypericum perforatum* L. in biochemical models of antidepressant activity, Life Sci. 68 (2001) 1593-1605. DOI: 10.1016/S0024-3205(01)00946-8

[184] P.J. Nathan, *Hypericum perforatum* (St John's Wort): a non-selective reuptake inhibitor? A review of the recent advances in its pharmacology, J. Psychopharmacol. 15 (2001) 47–54. DOI: 10.1177/026988110101500109

[185] J. Gibon, J.C. Deloulme, T. Chevallie, E. Ladevéze, D.N. Abrous, A. Bouron, The antidepressant hyperforin increases the phosphorylation of CREB and the expression of TrkB in a tissue-specific manner, Int. J. Neuropsychopharmacol. 16 (2013) 189–198. DOI: 10.1017/S146114571100188X

[186] A. Voss, P.E. Verweij, Antibacterial activity of hyperforin from St John's wort, Lancet 354 (1999) 777. DOI: 10.1016/S0140-6736(05)76018-9

[187] P. Avato, F. Raffo, G. Guglielmi, C. Vitali, A. Rosato, Extracts from St John's Wort and their antimicrobial activity, Phytother. Res. 18 (2004) 230–232. DOI: 10.1002/ptr.1430

[188] Y. Lin, J.C. Zhang, J. Fu, F. Chen, J. Wang, Z.L. Wu, S.Y. Yuan, Hyperforin attenuates brain damage induced by transient middle cerebral artery occlusion (MCAO) in rats via inhibition of TRPC6 channels degradation, J. Cereb. Blood Flow Metab. 33 (2013) 253–262. DOI: 10.1038/jcbfm.2012.164

[189] K. Hostanska, J. Reichling, S. Bommer, M. Weber, R. Saller, Hyperforin a constituent of St John's wort (*Hypericum perforatum* L.) extract induces apoptosis by triggering activation of caspases and with hypericin synergistically exerts cytotoxicity towards human malignant cell lines, Eur. J. Pharm. Biopharm. 56 (2003) 121–132. DOI: 10.1016/S0939-6411(03)00046-8

[190] K. Wiechmann, H. Müller, D. Fischer, J. Jauch, O. Werz, The acylphloroglucinols hyperforin and myrtucommulone A cause mitochondrial dysfunctions in leukemic cells by direct interference with mitochondria, Apoptosis 20 (2015) 1508–1517. DOI: 10.1007/s10495-015-1170-9

[191] C. Gey, S. Kyrylenko, L. Hennig, L.H.D. Nguyen, A. Büttner, H.D. Pham, A. Giannis, Phloroglucinol derivatives guttiferone G, aristoforin, and hyperforin: Inhibitors of human sirtuins SIRT1and SIRT2, Angew. Chem. Int. Ed. 46 (2007) 5219–5222. DOI: 10.1002/anie.200605207

[192] J. Hu, H. Jing, H. Lin, Sirtuin inhibitors as anticancer agents, Future Med. Chem. 6 (2014) 945–966. DOI:10.4155/fmc.14.44.

[193] C.M. Schempp, J. Kiss, V. Kirin, M. Averbeck, B. Simon-Haarhaus, B. Kremer, C.C. Termeer, J. Sleeman, J.C. Simon, Hyperforin acts as an angiogenesis inhibitor in vitro and in vivo, Planta Med. 71 (2005) 999-1004. DOI 10.1055/s-2005-871303

[194] L. Verotta, G. Appendino, E. Bombardelli, R. Brun, In vitro antimalarial activity of hyperforin, a prenylated acylphloroglucinol. A structure–activity study, Bioorg. Med. Chem. Lett. 17 (2007) 1544–1548. DOI: 10.1016/j.bmcl.2006.12.100

[195] L. Cantoni, M. Rozio, A. Mangolini, L. Hauri, S. Caccia, Hyperforin contributes to the hepatic CYP3A-inducing effect of *Hypericum perforatum* extract in the mouse, Toxicol. Sci. 75 (2003) 25–30. DOI: 10.1093/toxsci/kfg174

[196] Y. Chen, S.S. Ferguson, M. Negishi, J.A. Goldstein, Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor, J. Pharmacol. Exp. Ther. 308 (2004) 495–501. DOI: 10.1124/jpet.103.058818

[197] M. Ott, G. Fricker, B. Bauer, Pregnane X receptor (PXR) regulates P-glycoprotein at the blood-brain barrier: functional similarities between pig and human PXR, J. Pharmacol. Exp. Ther. 329 (2009) 141-149. DOI: 10.1124/jpet.108.149690

[198] M. Ott, M. Huls, M.G. Cornelius, G. Fricker, St. John's wort constituents modulate P-glycoprotein transport activity at the blood-brain barrier, Pharm. Res. 27 (2010) 811-822. DOI: 10.1007/s11095-010-0074-1

[199] K. Sasaki, K. Mito, K. Ohara, H. Yamamoto, K. Yazaki, Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of *Sophora flavescens*, Plant Physiol. 146 (2008) 1075–1084. DOI: 10.1104/pp.107.110544

[200] K. Sasaki, Y. Tsurumaru, H. Yamamoto, K. Yazaki, Molecular characterization of a membrane-bound prenyltransferase specific for isoflavone from *Sophora flavescens*, J. Biol. Chem. 286 (2011) 24125-24134. DOI: 10.1074/jbc.M111.244426

[201] T. Akashi, K. Sasaki, T. Aoki, S-i Ayabe, K. Yazaki, Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin, Plant Physiol. 149 (2009) 683–693. DOI: 10.1104/pp.108.123679

[202] Y. Tsurumaru, K. Sasaki, T. Miyawaki, T. Momma, N. Umemoto, K. Yazaki, An aromatic prenyltransferase-like gene HlPT-1 preferentially expressed in lupulin glands of hop, Plant Biotechnol. 27 (2010) 199–204. DOI: 10.5511/plantbiotechnology.27.199

[203] R. Munakata, T. Inoue, T. Koeduka, F. Karamat, A. Olry, A. Sugiyama, K. Takanashi, A. Dugrand, Y. Froelicher, R. Tanaka, Molecular cloning and characterization of a geranyl diphosphate-specific aromatic prenyltransferase from lemon, Plant Physiol. 166 (2014) 80–90. DOI: 10.1104/pp.114.246892

[204] S. Morf, B. Debrunner, B. Meier, H. Kurth, Automatische Probenvorbereitung von pflanzlichen Arzneimitteln. Ein Vergleich mit der konventionellen Methode am Beispiel Johanniskraut, Laborpraxis-Würzburg 22 (1998) 56-63.

[205] A.A. Šmelcerović, S.M. Đorđević, Ž.D. Lepojević, D.T. Veličković, The analysis of the kinetics of extraction of resinoids and hypericines from the amber, *Hypericum perforatum* L., J. Serb. Chem. Soc. 67 (2002) 457-463. DOI: 10.2298/JSC0206457S

[206] A. Šmelcerović, M. Spiteller, S. Zuehlke, Comparison of methods for the exhaustive extraction of hypericins, flavonoids, and hyperforin from *Hypericum perforatum* L., J. Agric. Food Chem. 54 (2006) 2750-2753. DOI: 10.1021/jf0527246

[207] T. Kartnig, I. Göbel, B. Heydel, Production of hypericin, pseudohypericin and flavonoids in cell cultures of various *Hypericum* species and their chemotypes, Planta Med. 62 (1996) 51-53. DOI: 10.1055/s-2006-957796

[208] H.P. Bais, T.S. Walker, J.J. McGrew, J.M. Vivanco, Factors affecting growth of cell suspension cultures of *Hypericum perforatum* L. (St. John's wort) and production of hypericin, In Vitro Cell. Dev. Biol. Plant 38 (2002) 58-65. DOI: 10.1079/IVP2001253

[209] T.S. Walker, H.P. Bais, J.M. Vivanco, Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort), Phytochemistry 60 (2002) 289–293. DOI: 10.1016/S0031-9422(02)00074-2

[210] S. Gadzovska, S. Maury, A. Delaunay, M. Spasenoski, C. Joseph, D. Hagège, Jasmonic acid elicitation of *Hypericum perforatum* L. cell suspensions and effects on the production of phenylpropanoids and naphtodianthrones, Plant Cell Tiss. Organ Cult. 89 (2007) 1-13. DOI: 10.1007/s11240-007-9203-x

[211] S. Gadzovska, O. Tusevski, S. Antevskis, N. Atanasova-Pancevsk, J. Petreska, M. Stefova, D. Kungulovski, M. Spasenoski, Secondary metabolite production of *Hypericum perforatum* L. cell suspension upon elicitation with fungal mycelia from *Aspergillus flavus*, Arch. Biol. Sci. 64 (2012) 113-121. DOI:10.2298/ABS1201113G

[212] S.G. Simic, O. Tusevski, S. Maury, C. Hano, A. Delaunay, B. Chabbert, F. Lamblin, E. Lainé, C. Joseph, D. Hagège, Fungal elicitor-mediated enhancement in phenylpropanoid and naphtodianthrone contents of *Hypericum perforatum* L. cell cultures, Plant Cell, Tiss. Organ Cult. 122 (2015) 213-226. DOI: 10.1007/s11240-015-0762-y

[213] T. Sirvent, D. Gibson, Induction of hypericins and hyperforin in *Hypericum perforatum* L. in response to biotic and chemical elicitors, Physiol. Mol. Plant Path. 60 (2002) 311-320. DOI:10.1006/pmpp.2002.0410
[214] A. Kirakosyan, H. Hayashi, K. Inoue, A. Charchoglyan, H. Vardapetyan, Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures, Phytochemistry 53 (2000) 345-348. DOI: 10.1016/S0031-9422(99)00496-3
[215] M. Pavlík, J. Vacek, B. Klejdus, V. Kuban, Hypericin and hyperforin production in St. John's wort in vitro culture: influence of saccharose, polyethylene glycol, methyl jasmonate, and *Agrobacterium tumefaciens*, J. Agric. Food Chem. 55 (2007) 6147-6153. DOI: 10.1021/jf070245w
[216] S. Gadzovska-Simic, O. Tusevski, S. Maury, A. Delaunay, C. Joseph, D. Hagège, Effects of polysaccharide elicitors on secondary metabolite production and antioxidant response in *Hypericum perforatum* L. shoot cultures, Sci. World J. (2014) 609649,10. DOI: 10.1155/2014/609649
[217] J. Koperdáková, H. Komarovská, J. Košuth, A. Giovannini, E. Čellárová, Characterization of hairy root-phenotype in transgenic *Hypericum perforatum* L. clones, Acta Physiol. Plant. 31 (2009) 351-358. DOI: 10.1007/s11738-008-0241-8
[218] X.H. Cui, D. Chakrabarty, E.J. Lee, K.Y. Paek, Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor, Bioresour. Technol. 101 (2010) 4708–4716. DOI: 10.1016/j.biortech.2010. 01.115

Legends

Fig. 1. *Hypericum perforatum*. (A) Entire plant; (B) Blossom.

Fig. 2. Naturally occurring hyperforin and representative examples of stable semi-synthetic derivatives.

Fig. 3. Pharmacological activities of hyperforin and underlying mechanisms. Aβ, amyloid beta; BAEC, bovine aorta endothelial cells; BDNF, brain derived neurotrophic factor; COX-1, cyclooxygenase-1; CYP, cytochrome P-450 enzyme; EC_{50}, half maximal effective concentration; HDMEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; HEK 293, human embryonic kidney cells; IC_{50}, half maximal inhibitory concentration; K562, human erythroleukemia cell line; LEC, lymphatic endothelial cells; LH-60, human leukemia cell line; LN229, glioblastoma human cell line; 5-LO, 5-lipoxygenase; MCF-7, human breast adenocarcinoma expressing estrogen receptors; MDA-MB-468, human breast adenocarcinoma lacking estrogen receptor expression; MIC, minimal inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus*; MT-450, rat mammary carcinoma; PC12, pheochromocytoma of the rat adrenal medulla; PBCEC, porcine brain capillary endothelial cells; PRSA, penicillin resistant *Staphylococcus aureus*; PXR, pregnane-x-receptor; SIRT, sirtuins, homologous to silent information regulator; TRPC6, transient receptor potential cation channel; U937, human leukemia monocyte lymphoma cell line; ΔΨm, mitochondrial membrane potential.

Fig. 4. *H. perforatum in vitro* shoot cultures. (A) Occurrence of dark nodules 3 d after transfer to new medium; (B) Six-day-old culture; (C, E) Dark nodules at the leaf margin, which accumulate hypericins; (D, F) Translucent glands in the leaf lamina, which accumulate hyperforins.

Fig. 5. *In vitro* cultures and products. (A) Cell suspension cultures of *H. calycinum* accumulate hyperxanthone E after elicitor treatment; (B) Dedifferentiated cells of *H. calycinum* under the light

microscope; (C) Root cultures of *H. perforatum* accumulate hyperforin; (D) Electron microscopy of a *H. perforatum* root tip.

Fig. 6. Establishment of *H. perforatum in vitro* root cultures. (A) *In vitro* plantlets; (B,C) Callus and root formation in auxin-containing medium; (D) Freeze-dried root biomass (Bars = 100 µm).

Fig. 7. Hyperforin biosynthesis. PKS, polyketide synthase; PTs, prenyltransferases. Solid arrow, established reaction; broken arrows, reactions under study.

Fig. 8. Topology and reactions of aromatic prenyltransferases. (A) Topology including membrane spanning domains, conserved aspartate-rich motifs located in the non-membrane loops (L1 and L3), and a putative transit peptide (TP); (B) Representative *in vitro* activities reported for recombinant enzymes.

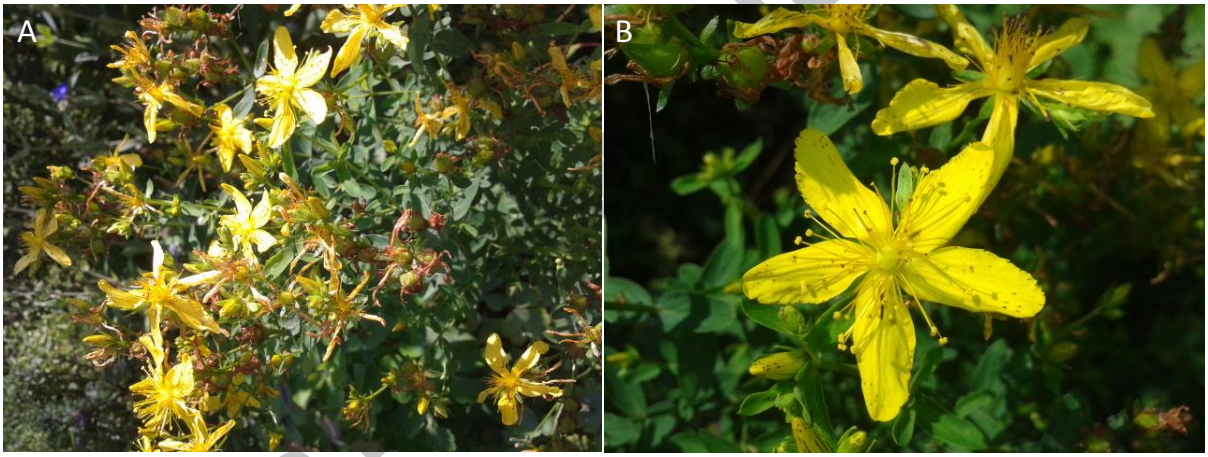


Fig. 1

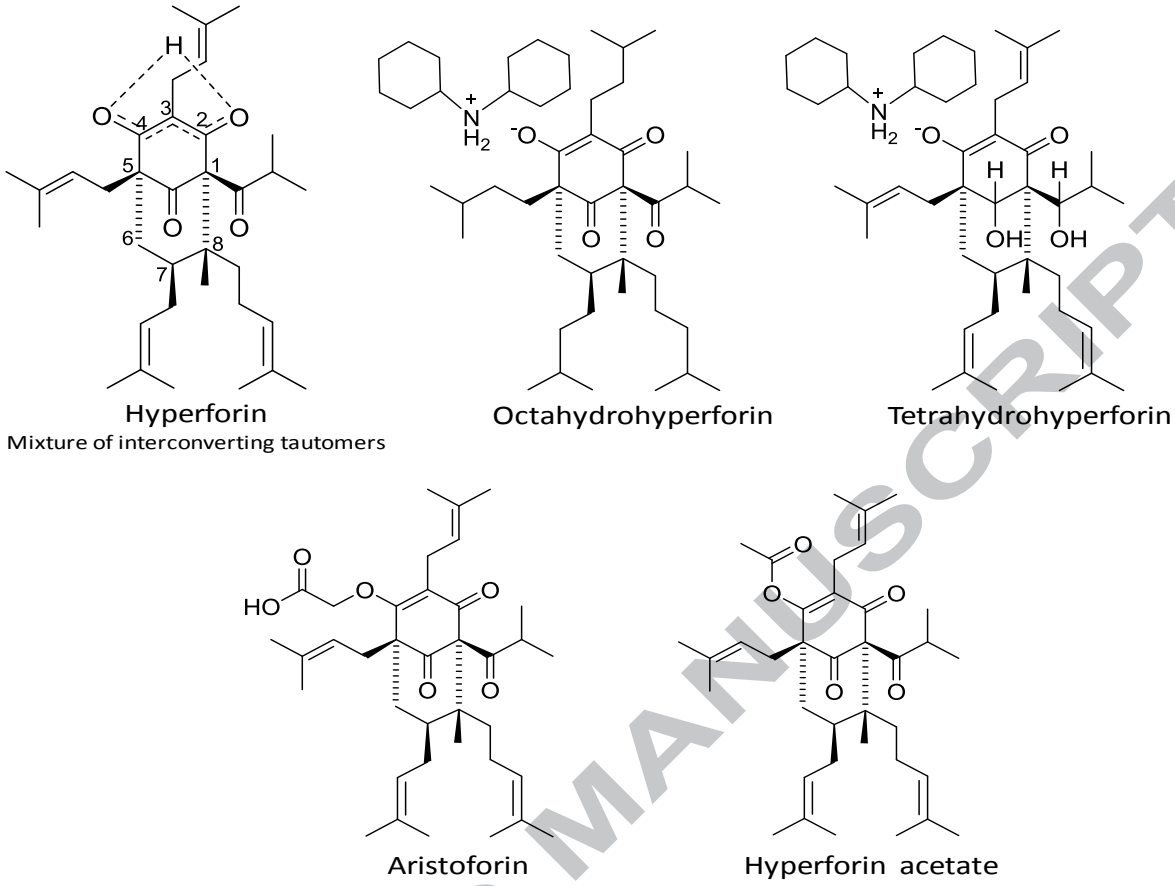
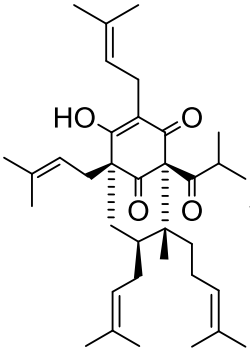


Fig. 2



Hyperforin

Antidepressant

Non-selective neurotransmitter re-uptake inhibitor
-Noradrenaline: IC_{50} = 0.033 -0.123 µM
-Dopamine: IC_{50} = 0.011-0.024 µM
-Serotonin: IC_{50} = 0.067-0.12 µM [22,23,183,184,185]

Anti-inflammatory

Dual inhibitor of key enzymes in eicosanoid biosynthesis
Intact cells or cell-free systems
5-LO: IC_{50} = 1-2 µM
COX-1: IC_{50} = 3 µM [53,54]

Antibacterial

Gram positive bacteria
Multiresistant *Staphylococcus aureus* PRSA, MRSA (planktonic form)
MIC = 1 µg/ml [34,57]
Corynebacterium diphtheriae
MIC = 0.1 µg/ml [57,186,187]
Bacillus subtilis, B. cereus
MIC = 25-50 µg/ml [187]

Neuroprotective

Decreases Aβ aggregation and Aβ-mediated neuropathological changes
In vitro: Primary neuronal hippocampal cell culture [61]
In vivo: male Sprague–Dawley rats
Induces neurite outgrowth: PC12 cells 0.1-0.3 µM [44]
Regulates BDNF expression by TRPC6 activation:
In vitro: HEK293 cells 10 µM [44] and mouse cortical neurons [185]
In vivo: male Sprague–Dawley rats [188]

Antitumoral

Caspase- mediated apoptosis
MT-450 , MDA-MB-468 and MCF-7: IC_{50} < 5 µM [41,67]
K562, U937 and LN229: IC_{50} = 14.9-19.9 µM [189]
Induction of mitochondrial apoptosis in HL-60 cells
Loss of $\Delta\psi m$: EC_{50} = 0.2 µM [190]
Suppression of mitochondrial ATP synthesis: IC_{50} = 0.2 µM [190]
Inhibition of sirtuins in HUVE cells
SIRT1: IC_{50} = 15 µM; SIRT2: IC_{50} = 28 µM [191,192]

Anti-angiogenic

Inhibits proliferation and growth of endothelial cells
HUVEC: IC_{50} = 2.07 µM [70]
Dermal: IC_{50} = 1.32 µM (LEC), IC_{50} = 3.7 µM (HDMEC) [70,193]
Lung LEC: IC_{50} = 1.84 µM [70]
Aortic BAEC: IC_{50} = 2.1 µM [33,69]

Antimalarial

Inhibits growth of *Plasmodium falciparum*
IC_{50} = 1.5-2.1 µM [194]

Pharmacokinetic effects

Activates PXR: In primary cultures of human hepatocytes EC_{50} = 23 nM [74]
Metabolism: Induces CYP3A4 and CYP2C9 [74,195,196]
Excretion: Activates P-glycoprotein in PBCEC [197,198]

Fig. 3

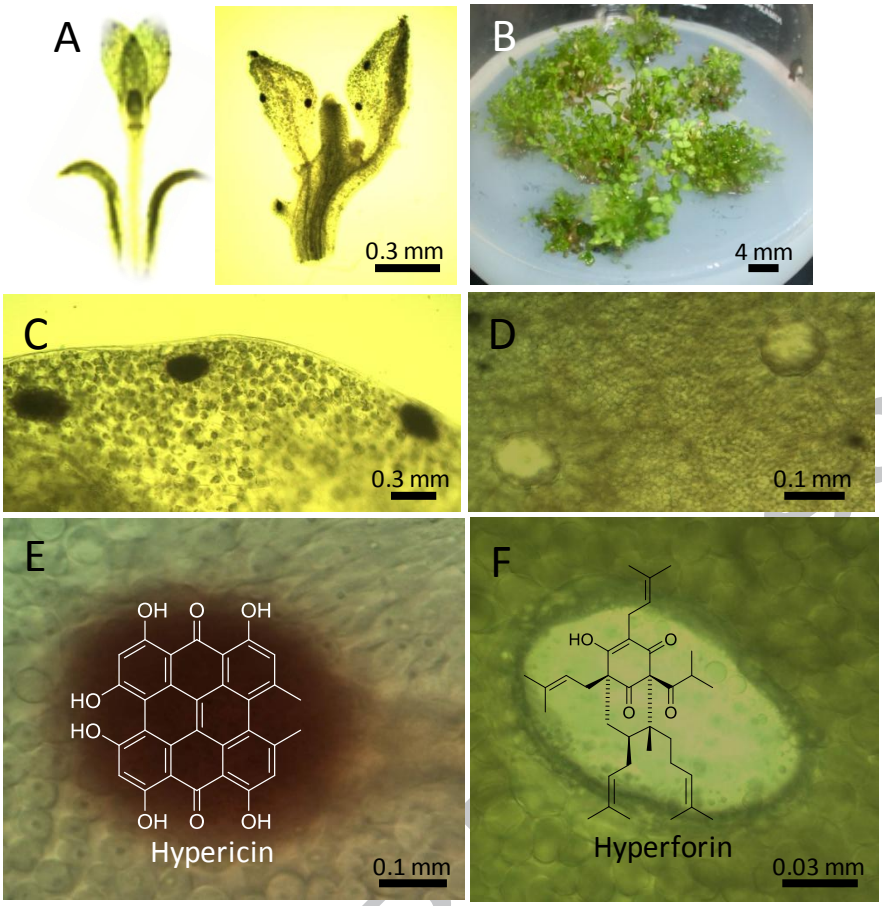


Fig. 4

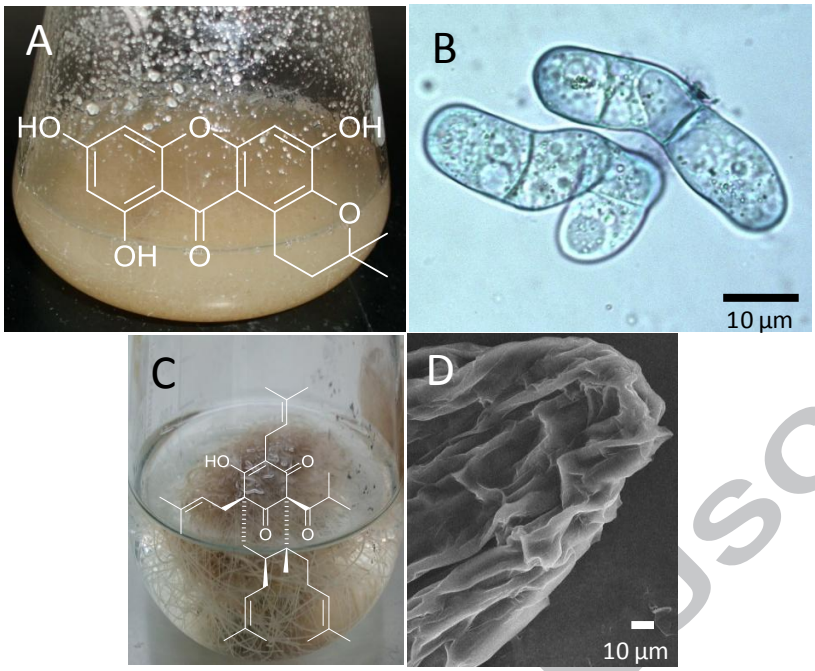


Fig. 5

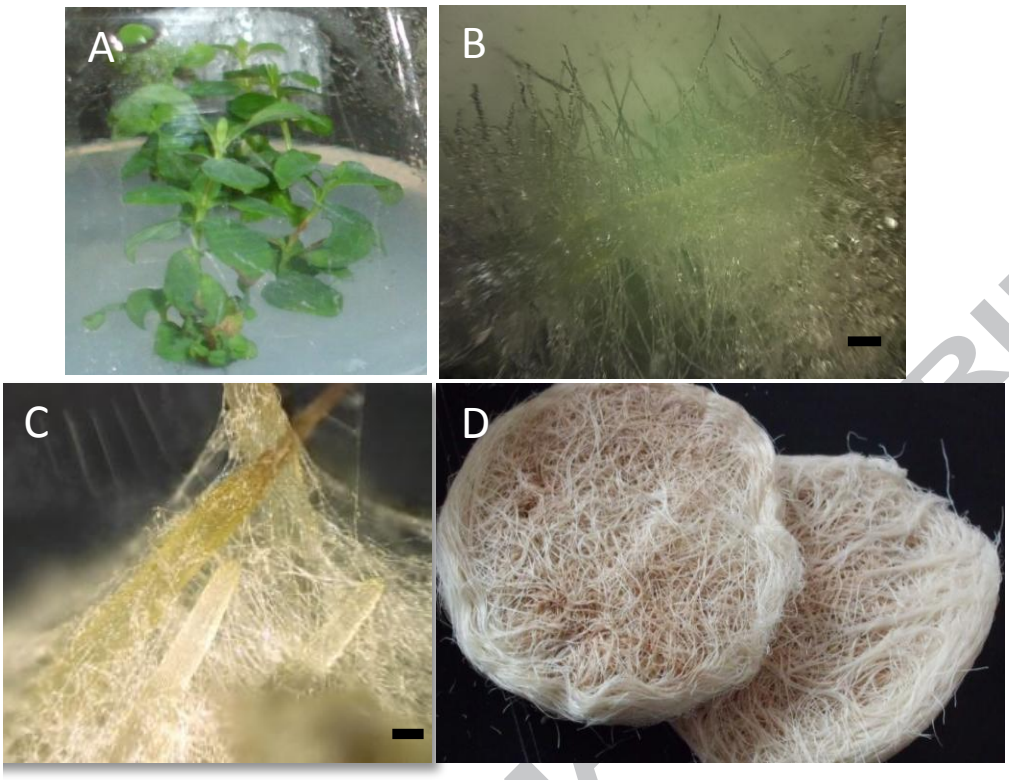


Fig. 6

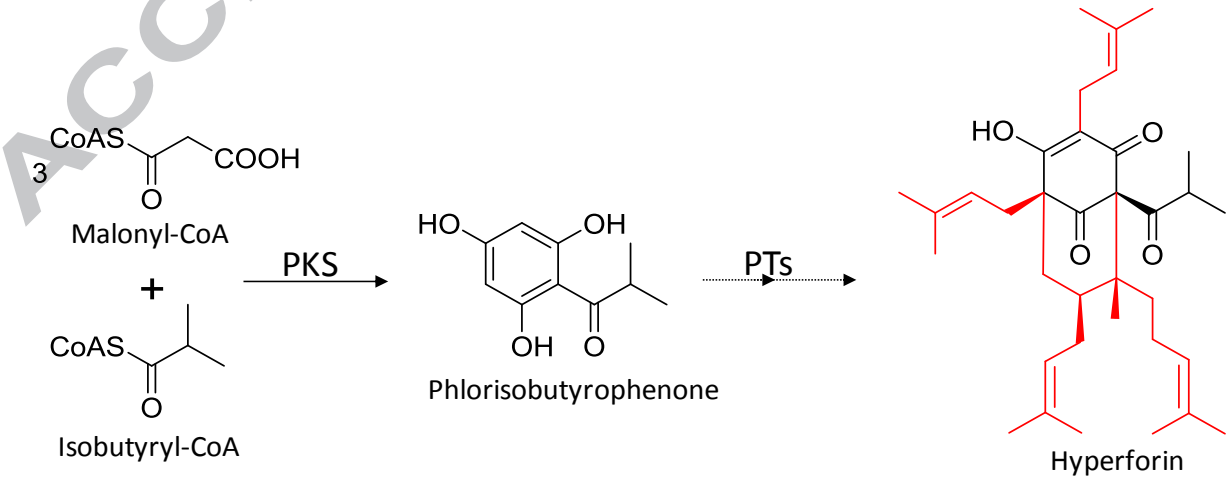
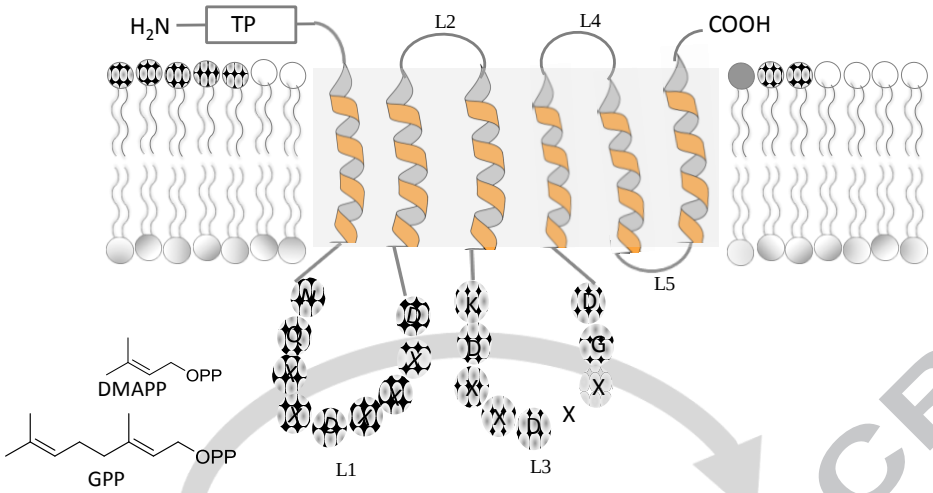
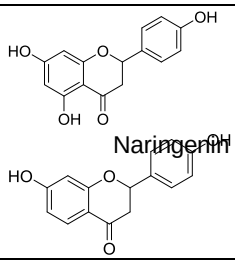
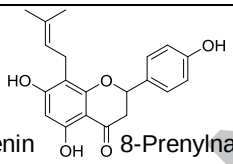
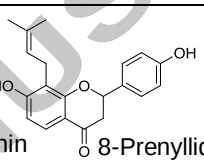
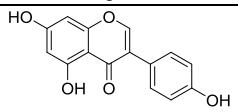
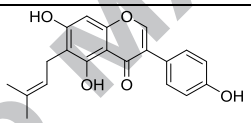
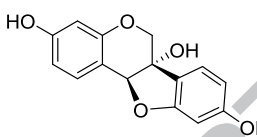
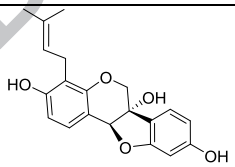
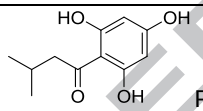
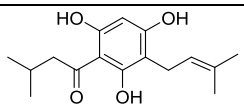
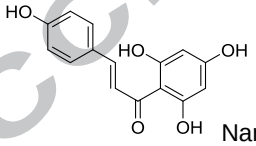
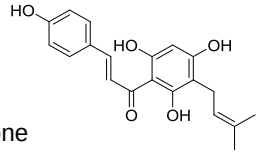
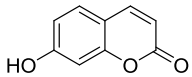
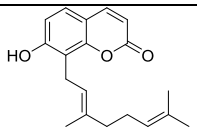
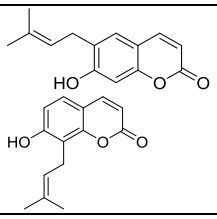


Fig. 7

A



B

		Substrate	Main product	Ref.
Flavonoids	Flavanone	 Naringenin	 Liquritigenin	8-Prenylnaringenin  8-Prenylliquiritigenin	199
	Isoflavone	 Genistein	 6-Prenylgenistein	200
	Petrocarpan	 Glycinol	 4-Prenylglycinol	201
Acylphloroglucinols		 Phlorisovalerophe none	 Prenylphlorisovalerophenone	170, 202
		 Naringenin chalcone	 Desmethylxanthohumol	
Coumarins		 Umbelliferone	 8-Geranylumbelliferone	203
			 Demethylsuberosin Osthenol	168

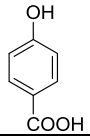
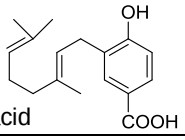
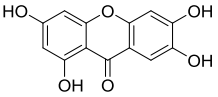
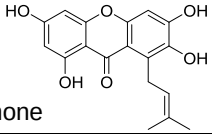
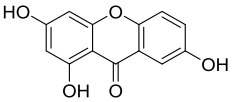
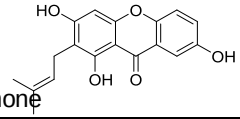
Shikonins	 *p*-Hydroxybenzoic acid	 *m*-Geranyl-*p*-Hydroxybenzoic acid	166, 167
Xanthones	 Tetrahydroxyxanthone	 Tetrahydroxy-8-prenylxanthone	126
	 Trihydroxyxanthone	 Trihydroxy-2-prenylxanthone	169

Fig. 8

Table 1. Methods and yields (mg g^{-1} dry weight) of API extraction from field-grown SJW plants

Extraction method	Extract	Hyperforin	Hypericin	Reference
Maceration without ultrasound	229.6	0.85	0.13	204
Sonication (60 W, 1h)	319	1.52	0.20	205
Soxhlet extraction	253	0.89	0.15	206
Accelerated solvent	240	0.87	0.15	204

extraction					
CO_2 extraction	Subcritical	57.3 (70 bar/37.6°C)	29.1 +5.1*	absent	27
	Supercritical	~14 (120 bar/40°C)	~5.7	absent	30
		25 (100 bar/40°C)	12.4 + 2*	absent	29
		59.9 (300 bar/40°C)	22.9 + 3.9*	absent	27
		35.5 (100 bar/50°C)	14.7–19.3 + 2.1–2.9*	absent	28
*Adhyperforin					

Table 2. Yields of hyperforins and hypericins (mg g^{-1} dry weight) in SJW *in vitro* cultures (shake flask level) in response to various treatments (nd: not determined)

Cultures	Treatment	Hyperforin	Adhyperforin	Hypericin	Pseudohypericin	References
Cell suspension	No treatment	nd	nd	0.15	0.51	207
	No treatment	nd	nd	~ 0.13	nd	208
	No treatment	trace	0.25	nd	nd	113
	Jasmonic acid	nd	nd	~ 0.32	nd	209
	Methyl jasmonate/ jasmonic acid	nd	nd	0.03	0.03	210
	Ozone	nd	nd	~1.2-1.6	nd	122
	Mycelia from *Aspergillus flavus*	nd	nd	0.026	nd	211
	No treatment	nd	nd	0.012-0.013	0.012-0.013	115
	Salicylic acid	nd	nd	0.03	0.03	
	Fungal mycelia (various sources)	nd	nd	0.04-0.05	0.04-0.05	212
Apical meristem cultures	*Colletotrichum gloeosporioides*	0.4	nd	0.018	0.035	213
	Salicylic acid	0.85	nd	0.25	1.65	
	Methyl jasmonate	1.1	nd	0.30	1.3	
Root cultures	No treatment	5	nd	absent	absent	26
Plantlets without roots	No treatment	74.1	3.1	0.15	0.23	116

(continued)

Table 2 (continued)

Cultures	Treatment	Hyperforin	Adhyperforin	Hypericin	Pseudohypericin	References
Shoot cultures	Mannan	nd	nd	0.05	0.82	214
	β-1,3-Glucan (curdlan)	nd	nd	0.03	0.55	
	Pectin (from citrus)	nd	nd	0.03	0.35	
	Methyl jasmonate/ jasmonic acid	4	nd	nd	nd	147
	Mannan	5	nd	nd	nd	
	L-Threonine (2 mM)	~3.6	~0.4	nd	nd	128
	L-Isoleucine (2 mM)	~3	0.67	nd	nd	
	L-Valine (2 mM)	~3.6	~0.26	nd	nd	
	L-Leucine (2 mM)	~3.3	~0.18	nd	nd	
	Methyl jasmonate (at 10 g/L sucrose)	7	nd	0.0078	nd	215
	Methyl jasmonate (at 30 g/L sucrose)	13.5	nd	0.0055	nd	
	Agar cubes	35	nd	7.5	21	120
	Cork pieces	30	nd	5	10	
	Salicylic acid	nd	nd	0.075	0.165	115
	Pectin	nd	nd	0.09	0.21	216
	Dextran	nd	nd	0.08	0.23	
Transgenic shoots regenerated from hairy	No treatment	nd	nd	0.495	6.35	109
		0.01-0.09	nd	0.01-0.25	nd	136

		nd	nd	0.222-1.187	nd	217

Table 3. Yields of hyperforin and hypericins (mg g^{-1} fresh weight) in bioreactor-grown SJW cultures

Cultures	Treatment	Photoperiod	Hyperforin	Hypericin	Pseudohypericin	References
Shoot cultures	No treatment	16 h photoperiod	~0.5[a]	~0.004[a]	~0.05[a]	129, 130
Adventitious roots cultures	No treatment	Dark	nd	1.39*,[b]	nd	218
	No treatment	Dark	nd	0.04*,[c]	nd	131
	Methyl jasmonate (350 µM)	Dark	nd	1.68*,[d]	nd	132

[a] 2 L balloon type bioreactor, [b] 3 L balloon type bubble bioreactor, [c] 500 L pilot-scale airlift bioreactor, [d] 5 L balloon-type airlift bioreactor; *mg g^{-1} dry weight.

Graphical abstract