UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

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DIPLOMSKA NALOGA
UNIVERZITETNI ŠTUDIJ FARMACIJE

Ljubljana, 2012

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APPROACHES TO OPTIMIZATION OF HYPERFORIN PRODUCTION IN *HYPERICUM PERFORATUM* SHOOT CULTURES

PRISTOPI K OPTIMIZACIJI PRIDOBIVANJA HIPERFORINA V KULTURAH POGANJKOV *HYPERICUM PERFORATUM*

Ljubljana, 2012

This Diploma thesis was conducted at the Institute for Pharmaceutical Biology, Technical University of Braunschweig, Germany with the collaboration of the Faculty of Pharmacy, University of Ljubljana, Slovenia.

Acknowledgement

I would like to express my gratitude to Prof. Dr. Ludger Beerhues for introducing me to this project and for providing excellent scientific support throughout my work.
I am grateful to Ines Bel Hadj for her constant help and support at experimental and theoretical parts of my thesis. She kindly provided excellent suggestions, which were of great help in the process of making this thesis. My gratitude also goes to Dr. Benye Liu for his time, which he invested in order to give my Diploma thesis a higher scientific quality.
I would like to thank to the entire Institute for Pharmaceutical Biology in Braunschweig for providing friendly atmosphere during my time spent at the institute.
I am also thankful to Hendrik for his valuable advices, help with the use of computer tools and support throughout the entire time.

Profesorju dr. Samotu Kreftu sem hvaležna za podporo tekom nastajanja diplomskega dela ter za čas, ki si ga je vzel za številne nasvete in odgovore na moja vprašanja.
Posebno zahvalo namenjam svojim staršem in bratu, ki so mi brezpogojno stali ob strani, me spodbujali skozi celoten študij ter mi nudili vse, kar sem kadarkoli potrebovala.
Najlepša hvala tudi vsem prijateljem, sostanovalcem in sošolcem, ki so z mano delili prečudovite trenutke študijskih let.

Izjava

Izjavljam, da sem diplomsko nalogo izdelala samostojno pod mentorstvom prof. dr. Samota Krefta in somentorstvom prof. dr. Ludgerja Beerhuesa.

Ljubljana, februar 2012 Ivana Dukovčić

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ABSTRACT

St. John's wort, *Hypericum perforatum* L., is a medicinal plant that has been widely used since ancient history due to its numerous therapeutic effects. One of them is also treatment of psychiatric disorders, such as depression. Aside from being an efficient antidepressant, the plant is interesting because of recently discovered mechanism of action of its main constituent, hyperforin. This promising molecule, with a complex chemical synthesis, has a great potential of becoming a leading compound for a new and improved generation of synthetic antidepressants due to its unique and effective mechanism of action. So far, biotechnology and molecular biology have proven to be the most powerful tools for a scale up process of hyperforin production.

In order to create a stable system for metabolite-oriented cultivation of St. John's wort, we evaluated effects of plant hormones, *myo*-inositol (MI) and a group of B vitamins added to the growth media, on metabolic activity in the plant. The results have shown that a certain combination of nutrients, referred to as the medium A, enhances the biosynthesis of hyperforin and represents an efficient inducing system for production of secondary metabolites in *in vitro* grown plants. Further investigations based on these results, might enable us to set foundations for a subtractive cDNA library, making a step towards genetic elucidation of hyperforin biosynthesis. Sudden decrease of hyperforin amounts in shoots cultivated on hormone-containing growth medium shows its unsuitability for a long-term cultivation of St. John’s wort. Thus, we examined the effects of growth media lacking plant hormones and discovered a stable influence on hyperforin production, as this was constantly increasing during 21 days of cultivation. The combination of media nutrients, which we referred to as the medium F, would be a great choice for cultivation of *H. perforatum* shoots in bioreactors. Our findings about hyperforin accumulation in the shoots correlated with the formation of translucent glands in the examined leaves. Both medium modifications exhibited positive effects on enzyme activity in cell cultures derived from the examined shoots.

Based on our results, we conclude that not only plant hormones, but also the concentration of B vitamins and the presence of *myo*-inositol in the growth media are decisive for alteration of metabolic activity in *H. perforatum in vitro* shoot cultures.

RAZŠIRJEN POVZETEK

UVOD Šentjanževka, *Hypericum perforatum* L., je skoraj na vseh kontinentih razširjena zdravilna rastlina, ki se že od nekdaj uporablja zaradi svojih številnih terapevtskih lastnosti, med drugim tudi za zdravljenje depresivnih motenj. Poleg izrazitega antidepresivnega učinka, pa je ta rastlina dandanes zanimiva predvsem zaradi nedavnih odkritij o mehanizmu antidepresivnega delovanja hiperforina, ki poleg hipericina predstavlja glavni presnovni produkt šentjanževke. Kemijska sinteza hiperforina do danes še ni bila uspešna, le sorodne derivate je mogoče pridobiti z dokaj kompleksno kemijsko sintezo (1). Ta obetavna molekula predstavlja potencialno spojino vodnico za novo in izboljšano generacijo antidepresivov, predvsem zaradi edinstvenega mehanizma delovanja, ki pa je učinkovitejši v primerjavi s sinteznimi antidepresivi. Gojenje šentjanževke in pridobivanje hiperforina, kot ga po svetu poznamo danes, predstavlja nezanesljiv vir učinkovine zaradi številnih vplivov podnebja in okolja. Optimizacija procesa pridobivanja hiperforina bi prinesla mnoge prednosti tako za farmacevtsko industrijo kot za samega potrošnika. Biotehnologija in molekularna biologija sta za enkrat najmočnejši orodji za stopnjevanje pridobivanja hiperforina do večjih meril.

NAMEN Z gojenjem *in vitro* kultur poganjkov šentjanževke na šestih različnih modifikacijah gojišča Murashige in Skoog bomo skušali najti primerno kombinacijo hranil, ki bi zagotovila optimalen donos hiperforina. Ovrednotili bomo vplive rastlinskih hormonov (avksina: NAA in citokinina: BAP), *mio*-inozitola in različnih koncentracij dveh vitaminov B (niacina in piridoksina). Glede na koncentracije hiperforina in njegovih derivatov v različno gojenih poganjkih, bomo izbrali najbolj primerno gojišče za gojenje kultur šentjanževke *in vitro*. Vplive gojišč bomo preverili tudi glede na razvoj prosojnih žlez, v katerih se kopiči hiperforin, ter glede na aktivnost encimov, ki sodelujejo v biosintezi (BUS). Gojišče je lahko koristno za optimizacijo pridobivanja hiperforina iz dveh stališč: 1.) za gojenje poganjkov šentjanževke v večjih merilih, s pomočjo bioreaktorja, potrebujemo takšno kombinacijo hranil v gojišču, ki bi zagotavljala stabilno biosintezo hiperforina v poganjkih; 2.) na ravni molekularne biologije bi z gojenjem na primernih gojiščih lahko ustvarili sistem za izdelavo subtraktivne cDNA knjižnice, saj je bilo ugotovljeno, da je biosintezo hiperforina možno popolnoma zavreti in pod določenimi pogoji ponovno inducirati.

METODE Po 14 in 21 dneh gojenja na različnih modifikacijah gojišča MS smo mikroskopsko analizirali nove poganjke in izmerili premer prosojnih žlez. V enakih časovnih intervalih smo hkrati določili vsebnost hiperforina v metanolnih ekstraktih poganjkov s pomočjo tekočinske kromatografije visoke ločljivosti (HPLC). Kromatograme vzorcev smo primerjali s kromatogramom referenčnega standarda hiperforina, identiteto ostalih frakcij, za katere smo domnevali, da so analogne s hiperforinom, pa smo potrdili z masno spektrometrijo. Za določanje encimske aktivnosti v celičnih kulturah šentjanževke smo pripravili prečiščen ekstrakt znotrajceličnih proteinov ter ga inkubirali z že znanimi substrati izobutirofenonske sintetaze (BUS), z namenom ocenitve aktivnosti te poliketidne sintetaze v celičnih kulturah. Količino encimskih produktov po inkubaciji smo določili s HPLC ter preračunali vsebnost v encimsko aktivnost (nkat/mg proteinov).

REZULTATI IN RAZPRAVA Najbolj očitne spremembe v razvoju prosojnih žlez in v kopičenju hiperforina v le-teh so bile opazne v poganjkih, gojenih na gojiščih MS z in brez rastlinskih hormonov (NAA: 0.05 mg/l; BAP: 1 mg/l). Poganjki šentjanževke, ki smo jih gojili na gojišču z dodatkom rastlinskih hormonov (gojišče A), niso vsebovali hiperforina, v lističih pa zato nismo opazili prosojnih žlez. Po enomesečnem gojenju na gojišču B, ki se je od prej omenjenega razlikovalo le v odsotnosti hormonov, pa smo zasledili močno stimulacijo biosinteze hiperforina ter razvoja prosojnih žlez. V obratnem primeru, kjer smo poganjke z že prisotnim hiperforinom, ki smo jih gojili na gojišču brez hormonov (B), prestavili na gojišče z dodanimi rastlinskimi hormoni (A), pa smo po 21 dneh opazili močno znižanje koncentracije hiperforina ter zmanjšano tkivno diferenciacijo listov. Poleg spremenjene morfologije opazovanih listov in odsotnosti listnih žil, smo ugotovili tudi, da se je razvoj prosojnih žlez popolnoma ustavil. Skupno smo torej preverili vplive treh gojišč brez in treh z dodanimi rastlinskimi hormoni, ki so se med seboj razlikovali še v koncentraciji vitaminov B in v prisotnosti ali odsotnosti *mio*-inozitola. Izmed šestih modifikacij gojišča MS smo izbrali dve z najbolj izrazitim učinkom na presnovno aktivnost v poganjkih. Na osnovi dobljenih rezultatov smo tudi ocenili pomembnost *mio*-inozitola in koncentracij vitaminov v gojišču ter ugotovili, da je *mio*-inozitol pomemben dejavnik za kopičenje hiperforina v poganjkih. Glede koncentracij vitaminov B pa smo ugotovili, da nižje koncentracije niacina in piridoksina, od tistih ki jih vsebujejo splošno uporabljana gojišča MS, ugodneje vplivajo na biosintezo hiperforina v poganjkih šentjanževke gojenih *in vitro* (niacin: 0.1 mg/l; piridoksin: 0.1 mg/l).

Dve gojišči, ki smo ju zaradi njunih različnih vendar enako pomembnih vplivov na presnovno aktivnost v poganjkih uporabljali za nadaljnja opazovanja, sta bili:

Gojišče A:

- Prisotnost rastlinskih hormonov: NAA: 0.05 mg/l; BAP: 1 mg/l
- Prisotnost *mio*-inozitola: 100 mg/l
- Višji koncentraciji vitaminov B: niacin: 0.5 mg/l; piridoksin: 0.5 mg/l

Gojišče F:

- Odsotnost rastlinskih hormonov
- Prisotnost *mio*-inozitola: 100 mg/l
- Nižji koncentraciji vitaminov B: niacin: 0.1 mg/l; piridoksin: 0.1 mg/l

Pozitivne učinke izbranih gojišč smo potrdili tudi z inducirano aktivnostjo encimov (BUS in BPS) v celičnih kulturah šentjanževke, ki smo jih pripravili iz ovrednotenih poganjkov. Rezultati določevanja vsebnosti hiperforina v poganjkih so se pretežno ujemali tudi z razvojem prosojnih žlez v listih opazovanih poganjkov.

Izmerjene vsebnosti hiperforina tekom 21-dnevnega gojenja poganjkov na gojišču A so pokazale sunkovito naraščanje koncentracije tega presnovka, ki pa po devetih dneh doseže vrh in se nato spet hitro znižuje. Gojišče A bi lahko predstavljalo osnovo za izdelavo cDNA knjižnice, s katero bi se približali razjasnitvi genetskega ozadja biosinteze hiperforina. Z dodatnimi raziskavami in optimizacijo gojenja za takšen namen bi z dovolj rastlinskega materiala lahko selektivno izolirali samo tiste molekule mRNA, ki so vpletene v biosintezo hiperforina. Za takšno metodo bi torej potrebovali poganjke s popolno odsotnostjo hiperforina, ki pa omogočajo indukcijo sinteze le-tega z gojenjem na gojišču A.

V primeru gojišča F smo tekom 21-dnevnega opazovanja zabeležili konstantno kopičenje hiperforina v poganjkih in naraščanje njegove koncentracije. Gojišče F bi bilo zato primerno za gojenje poganjkov šentjanževke v bioreaktorjih, z namenom pridobivanja hiperforina v večjih količinah. Nedavne raziskave so namreč pokazale, da je nadzemne dele poganjkov možno gojiti tudi v bioreaktorjih, in sicer ob zagotovljeni ustrezni izmenjavi plinov in mešanju vsebine v tekočem mediju (2). Kombinacija hranil v gojišču, ki bi zagotovila stalno kopičenje hiperforina, pa bi takšen sistem še okrepila.

ZAKLJUČKI Ugotovili smo da ima sestava gojišča za gojenje rastlin *in vitro* odločilen vpliv na presnovno aktivnost v rastlinskih kulturah. Najbolj očitne vplive smo opazili v primeru dodanih rastlinskih hormonov, imenovanih tudi rastni regulatorji, kar ustreza zaznanim makroskopskim in mikroskopskim razlikam v razvoju poganjkov, gojenih na gojiščih z in brez dodanih hormonov. Poleg vpliva na tkivno diferenciacijo pa avksini in citokinini odločilno vplivajo tudi na biosintezo sekundarnih metabolitov v rastlini. Čeprav je bilo že potrjeno, da je določena kombinacija teh dveh vrst hormonov odgovorna za stimulacijo kopičenja hiperforina v *in vitro* poganjkih šentjanževke (3), pa tovrstno dolgotrajno gojenje negativno vpliva na biosintezo te učinkovine. Kljub ugotovitvi, da so gojišča brez dodanih rastnih regulatorjev bolj primerna za gojenje šentjanževke v bioreaktorjih, pa imajo naši rezultati o vplivu hormonov v gojišču na presnovno aktivnost rastlin prav tako veliko vrednost za nadaljnje molekularno-biološke raziskave.

1. LIST OF ABBREVIATIONS

2,4-D: 2,4-dichlorophenoxyacetic acid

5-HT: 5-hydroxytryptamine (serotonin)

BAP: 6-benzylaminopurine

BPS: Benzophenone synthase

BUS: Isobutyrophenone synthase

cDNA: Complementary deoxyribonucleic acid

CHS: Chalchone synthase

CYP: Cytochrome P450

dH_2O: Distilled water

DAD: Diode Array Detector

DAG: Diacylglycerol

DMAPP: Dimethylallyl diphosphate

DTT: Dithiothreitol

EPI: Enhanced product ion (scan)

FW: Fresh weight

GABA: Gamma-amino butyric acid

GPP: Geranyl diphosphate

HAM-D: Hamilton rating scale for depression

HIV: Human Immunodeficiency Virus

HPLC: High performance liquid chromatography

IAA: Indole-3-acetic acid

IPP: Isopentenyl diphosphate

MAOIs: Monoamine oxidase inhibitors

MI: *Myo*-inositol

mRNA: Messenger ribonucleic acid

Ms: Mass spectrometry

MS: Murashige and Skoog (medium)

MS-: Murashige and Skoog hormone-free medium

MSH: Murashige and Skoog hormone-containing medium

NAA: 1-naphtaleneacetic acid

NAD: Nicotinamide adenine dinucleotide

NMR: Nuclear magnetic resonance

PKSs: Polyketide synthases

RIMAs: Reverse inhibitors of monoamine oxidase

SSRIs: Selective serotonin reuptake inhibitors

TCAs: Tricyclic antidepressants

TG: Translucent glands

TRP: Transient receptor potential (channel)

UV: Ultra violet (spectrophotometry)

2. INTRODUCTION

2.1 DEPRESSION

Depression is a common psychiatric disorder with a lifetime prevalence of up to 20% within the world population. This affective disorder is a result of genetic and environmental factors. An important factor in the development of the illness appears to be the exposure to acute or severe stress in early life. Clinical definition describes depression as a group of symptoms that reflect a sad and/or irritable mood exceeding normal sadness or grief. A person suffering from depression is constantly dealing with negative thoughts, loss of self-confidence, inability to feel pleasure, loss of interest for things they usually find exciting, sleeping- and/or eating- disorders, fatigue and poor concentration. Untreated depressive disorder can lead to social isolation, feeling of uselessness, hopelessness and even to suicidal thoughts (4).

Affective disorders have been divided according to their typical symptoms. Unipolar depression is a disorder characterised by mood swings in one direction, resulting in episodes of depressed mood. In case of bipolar affective disorder, depression alternates with manic episodes, characterised by excessive enthusiasm, self-confidence and impulsive actions (5).

The monoamine theory of depression suggests that the patients with major depressive disorders have either low levels of biogenic amines, like serotonin (5-HT) and/or noradrenalin, or an imbalance in the activity of neurotransmitters like serotonin, noradrenaline, dopamine and acetylcholine (6). It has been proposed that depressive disorders are caused by a functional deficit of monoamine transmitters at certain sites in the brain, whereas manic episodes result from their excess (5).

Recent studies have shown that cortisol might be the crucial connection between stressful life experiences and depressive episodes. Elevated cortisol levels, probably caused by stressful events, may lower brain 5-HT function, which in some cases leads to affective disorders like depression (7).

Treatment strategies depend on the type of depressive disorder. Patients suffering from manic-depression are treated with lithium and/or neuroleptics, while those suffering from a major depressive disorder are normally treated with synthetic antidepressants which are

divided into several groups. Tricyclic antidepressants (TCA) inhibit neuronal reuptake of serotonin and noradrenaline, but have a low compliance because of their antagonistic effect on muscarinic, histaminic and alpha-1-adrenergic receptors, thereby causing unpleasant side effects. Another serious disadvantage of TCAs is their cardiotoxicity.

Irreversible inhibitors of monoamine oxidase (MAOIs) inhibit the intra- and interneuronal metabolism of biogenic amine neurotransmitters (noradrenaline, serotonin and dopamine). Their use is limited because of the interaction with the amines present in diet, causing dramatic rise in blood pressure, which occurs due to sudden release of noradrenaline after being displaced by an amine from the diet. Reverse inhibitors of monoamine oxidase (RIMAs) do not have this limitation, but were recognised as less potent as currently available antidepressants.

Selective serotonin reuptake inhibitors (SSRIs) have an antagonistic effect on serotonin transporters. Different classes of SSRIs differ in their specificity towards several neurotransmitter transporters. Other groups of synthetic antidepressants are specific inhibitors of noradrenaline and serotonin reuptake, tetracyclic antidepressants and atypical antidepressants (8).

The Hamilton Rating Scale for Depression (HRSD), abbreviated as HAM-D, is a system for rating the severity of patient's depression. This system includes a questionnaire that rates severity of symptoms such as low mood, insomnia, agitation, anxiety and weight loss. The HAM-D rating is one of the most commonly used scales for rating depression in clinical trials but Max Hamilton, who originally published the scale, warned about its inappropriate use as a diagnostic instrument.

Patients' responses are rated according to their severity and divided into three classes. There are 17 basic questions, but lately additional ones have been added to obtain further information about additional symptoms.

There are also other rating systems that are being used by clinicians in order to give an appropriate diagnose; for example: Montgomery-Åsberg Depression Rating Scale, the Beck Depression Inventory (BDI), the Inventory of Depressive Symptomatology (IDS) and other questionnaires (9).

2.2 ST. JOHN'S WORT (*Hypericum perforatum* L.)

The name of the genus *Hypericum* has an interesting explanation of its origin. It probably originates from the two Greek words: *Hyper* = over and *eikon* = image, which indicates that plants of this genus were believed to ward off evil or bad luck (10). *Hypericum* species belong to the family Hypericaceae. Aside from the genus *Hypericum*, the family includes eight other genera: *Cratoxylum, Eliea, Harungana, Lianthus, Santomasia, Thornea, Triadenum* and *Vismia*. The genus *Hypericum* includes about 450 species of flowering plants and represents approximately 80% of the diversity of the family Hypericaceae. Diverse species of the genus *Hypericum* are either naturally occurring on, or have been introduced to, every continent in the world, except Antarctica. A variety of habitats appears to be suitable for *Hypericum* species; they only avoid zones of extreme aridity, heat and/or salinity. Among all of the species we can find herbs, shrubs and even trees. Many species have been included in traditional medicine systems in countries around the world, or are sold as ornamentals (11).

The most known and investigated species of the genus, commonly used in herbal medicine, is *Hypericum perforatum*. This species has been divided into four subspecies that are distinguished by the size of their sepals: ssp. *angustifolium*, ssp. *latifolium*, ssp. *perforatum* and ssp. *veronense*.

H. perforatum is a perennial plant, which means that it lives more than just one year. The herb dies in fall and returns in the spring from its own root-stock, rather than seeding itself like an annual plant (12). Its stems are erect and branched in the upper section. The plant reaches the height of 60 to 80 cm (Fig. 1). The leaves of St. John's wort are oblong and about 12 mm long.

The species name *perforatum* is related to the perforated appearance of the leaves when held against a light (Fig. 2). The leaves appear to be perforated because of translucent glands in the sub-epidermal layer of the leaf. These cavities are delimited by two layers of cells; the internal one consists of flattened thin-walled secretory cells (13), (14).

St. John's wort is the common name of this plant, probably arising from the fact that this plant is flowering around 24th of June, which is a day dedicated to St. John the Baptist (13). Its flowers have five petals, measure up to 2.5 cm across and are coloured bright yellow with dark spots. The flowers appear on the upper branches, forming broad cymes. Pointed sepals also have glandular dots in the tissue. When the plant is blooming, we can see many

stamens, which are united at the base into three bundles. When crushing flower buds or seeds, reddish liquid is produced. The coloration is due to phenolic compounds present in the dark glands (15).

Figure 1: St. John's wort (*Hypericum perforatum* L.) (16).

Figure 2: Perforated appearance of the leaf of St. John's wort (13).

2.2.1 CONSTITUENTS

St. John's wort is known for its complex mixture of secondary metabolites, many of which are still being investigated.

Leaves and flower buds contain visible secretory structures, known as translucent and dark glands. These two types of glands have been established to selectively accumulate two main constituents of the herb: a naphtodianthrone hypericin in dark glands and a prenylated acylphloroglucinol hyperforin in translucent glands (17).

H. perforatum is the only species of the genus that contains hyperforin as a quantitavely major constituent in the flowers (18). Field grown plants of St. John's wort have been established to additionally contain a hyperforin-related polyprenylated acylphloroglucinol derivative adhyperforin, with an additional methyl group on position 13 (Fig. 3) (19).

On the other hand, extracts of *in vitro* plants have been established to accumulate additional two hyperforin derivatives: secohyperforin, an acylphloroglucinol with a prenyl chain missing on position 15 (Fig. 3) and adsecohyperforin, a hyperforin-related structure with both structural differences of adhyperforin and secohyperforin. The accumulation of these derivatives depends on shoot regeneration, with secohyperforin being the main constituent of morphogenic structures (3).

In Greek *H. perforatum* two different hyperforin derivatives were found, the hyperfirin and the adhyperfirin, which have previously been recognised as precursors in the biosynthesis of hyperforin. The two phloroglucinols also lack a prenyl side chain, though not on position 15 as in case of their homologues, secohyperforin and adsecohyperforin, but actually on position 4 (20).

The main naphtodiantrone derivatives in the crude drug, next to hypericin, are protohypericin, pseudohypericin and protopseudohypericin. Protohypericin and protopseudohypericin are converted into hypericin and pseudohypericin, under the influence of light. Emodinanthrone was recognised as a precursor of naphtodianthrones. Recently, cyclopseudohypericin was detected and suggested to be an oxidation product of pseudohypericin.

Aside from derivatives of acylphloroglucinol and naphtodianthrone, St. John's wort contains other active substances including phenylpropanes, flavonol derivatives, biflavones, tannins, xanthones, some amino acids and essential oil constituents.

Flavonol glycosides with quercetin as the aglycone, represent the major group (2 - 4 %) of plant natural products in *H. perforatum*. Hyperoside is a 3-O-galactoside of quercetin and rutin is a 3-O-rutinoside of quercetin; the two of them usually dominate in the glycoside fraction of St. John's wort extracts.

Two biflavones, which are also worth mentioning, because of their potential in antidepressant therapy, are 13,II8-biapigenin and amentoflavone. They occur exclusively in buds and blossoms of the plant.

The tannin fraction, which reaches its maximum during the flowering stage, was found to make up to 15 % of the crude drug. The tannins in *H. perforatum* appear to be oligomeric procyanidines, which tend to convert into red coloured cyanidines.

Xanthones are typical constituents of the Hypericaceae family and have been found in all anatomical parts of several *Hypericum* species. A xanthone compound found in leaves and

stems, the 1,3,6,7-tetrahydroxyxanthone, has been thoroughly investigated, although it is only present in traces (0.0004 % in the crude drug).

The same transparent secretory cavities, which contain hyperforin, also accumulate some typical essential oil constituents, for example monoterpenes α-pinene and β-pinene, mycrene and limonene and also sesquiterpenes (17), (19).

Free amino acid fraction is present in St. John's wort in a concentration of about 10 mg/g of the crude drug. Among these compounds a sedative neurotransmitter, γ-aminobutyric acid, has also been detected at about 0.7 mg/g of the crude drug. Considering the small amount of this amino acid there is a very low possibility for a significant contribution of this compound to the therapeutic activity of the crude drug (19).

2.2.2 THERAPEUTIC PROPERTIES

Even though physiological functions of St. John's wort's constituents have yet to be elucidated, their pharmaceutical benefits have been known since ancient history. Moreover, St. John's wort was earlier believed to have mystical properties such as protection from evil and bad luck. Thus, extracts of this herb were used as an apotropaic agent. This explanation indicates that it has been known since antique times that *H. perforatum* has psychoactive properties (21).

Back in the first century St. John's wort was recommended by Greek physicians Galen and Dioscorides as a diuretic, a wound-healing agent and as a treatment for menstrual disorders. In the sixteenth century Paracelsus used St. John's wort externally for treating wounds and for alleviating the pain of contusions (10).

The plant was also used all over Europe for treatment of neuralgia, neurosis, anxiety, depression and as a nerve tonic. Nowadays ethanolic extracts of the upper third of the herb are applied against mild to moderate depression.

In Germany these preparations are among the most prescribed medicines. The number of prescriptions approximately tripled since 1993. In the US, the use of St. John's wort extracts against mild to moderate depression is also rapidly increasing (22).

The antidepressant efficiency of *H. perforatum* has been confirmed in several clinical studies. To date, 25 controlled therapy studies have been done in order to investigate the effectiveness of St. John's wort extracts. The given dosage was usually between 300 and 900 mg of total extract per day with a therapy-duration of 2 to 6 weeks. A total number of

1592 treated cases have been investigated. Several placebo-controlled, as well as comparative studies with synthetic antidepressants like amitriptyline, imipramine and maprotiline were performed. Clinical effectiveness of *H. perforatum* extracts was confirmed in three of four placebo-controlled studies. Moreover, the effect was comparable to that achieved with standard synthetic antidepressants. In both types of therapies, i.e. with St. Johns wort extract and with synthetic antidepressant, a period of 4 weeks appears to be necessary for detecting evident efficiency.

The efficiency of *H. perforatum* preparations is at the highest level in cases of outpatients, suffering from mild to moderate depression. These preparations are very well accepted also because of their excellent tolerability. In a study including 3250 patients, the side effects were observed only in 2.5 % of all cases (23).

Other clinical studies have been preformed to establish the effectiveness of St. John's wort in case of major depressions. In a randomized, double-blind, placebo-controlled study, *H. perforatum* extracts were used to treat two hundred adult outpatients, suffering from major depression (HAM-D score of at least 20). No progress due to treatment was observed in this study (24).

Aside from the antidepressant activity, the extracts of St. John's wort have been recognised to have an antimicrobial efficiency against Gramm-positive bacteria and multiresistant *Staphylococcus aureus*. This property, together with the fact that preparations of *H. perforatum* also have anti-inflammatory, anti-oxidative and wound-healing effects, could be a good explanation why these extracts have been traditionally used to treat infected wounds and burns of the skin (25).

Furthermore, hyperforin, the main constituent of *H. perforatum*, is a promising novel anticancer agent. It inhibits the growth of a wide range of human and rat tumour cell lines by induction of apoptosis. This compound has been shown to have antiproliferative activity *in vivo*, which is comparable to that exerted by paclitaxel, but without any signs of acute toxicity. Hyperforin prevents cancer spread and metastatic growth and inhibits angiogenesis *in vivo*, as well as several key steps of this process *in vitro* (26).

Table I shows different therapeutic effects of the plant, grouped according to its constituents.

Table I: Pharmacological properties of St. John's wort's compounds (27).

Constituent	Percentage of dry weight	Activity
Naphtodiantrones Hypericin, Pseudohypericin Protohypericin, Pseudoprotohypericin Cyclopseudohypericin, Emodinanthrone	0.1 - 0.15 % in flowers and leaves	• antiproliferative • antiretroviral • anti-inflammatory • antidepressant • photodynamic
Phloroglucinols Hyperforin Adhyperforin	2 % flowers 4.4 % ripe fruits 4.5 % unripe fruits	• antidepressant • antibacterial (against G+ bacteria) • wound-healing • anticarcinogenic • antimalarial
Flavonoids Flavonol aglycones: kaempferol luteolin myricetin quercetin Glycosides: hyperoside rutin quercitrin isoquercitrin	2 - 4 %	• MAO A inhibition • COMT inhibition *(but too small amount for antidepressant efficacy)* • spasmolytic • antidepressant
Biflavones 3′,8′′-biapigenin Amentoflavone 6′,8′′-diquercetin	0.1 - 0.5 % 0.01 - 0.05 %	• anti-inflammatory • analgesic • binds to benzodiazepine receptor
Proanthocyanidines condensed tannins	2 - 4 %	• anti-oxidative • antiviral

Due to numerous compounds with antibacterial properties, St. John's wort exerts a very good defence mechanism against soil pathogens, which is the reason for the recalcitrance of the plant during the process of developing a stable *Agrobacterium*-mediated transformation system for it (28). A successful genetic transformation of *H. perforatum* would be a major step towards the elucidation of biotechnological aspects involved in regulating the production of hyperforin, as modifications and consequentially improved understanding of genetic background would be possible (29).

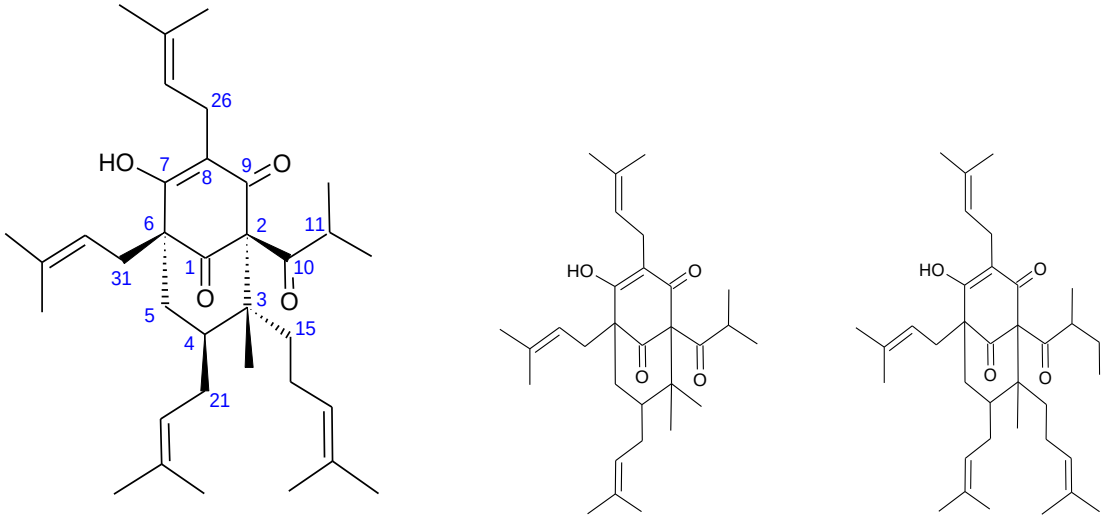
2.2.2.1 Drug interactions

Clinical studies have shown that *H. perforatum* extracts interfere with the cytochrome-dependant metabolism of several drugs. It has been established that hyperforin plays an important role in the induction of cytochrome P450 enzymes. It activates the pregnane X receptor, an orphan nuclear receptor that regulates the expression of the cytochrome P450 (CYP) 3A4 monooxygenase in human hepatocytes. CYP 3A4 monooxygenase is involved in the oxidative metabolism of more than 50 % of all known drugs, which can lead to severe drug-drug interactions. Therefore, the co-medication with preparations from St. John's wort can cause reduction of plasma concentrations of several drugs like HIV protease inhibitors, immunosuppressants and oral contraceptives, leading to reduction of their clinical efficiencies (30).

2.2.2.2 Adverse effects

Considering that *H. perforatum* extracts have been used to treat infected wounds and skin burns, it is interesting that its major unwanted effect is causing photosensibility of the skin, leading to skin inflammation, rashes and lesions of the tissue. This photodynamic effect is probably appearing only after oral administration of the extracts (31). It is due to hypericin, the second most important constituent of St. John's wort. Hypericin has the ability of absorbing light because of its extended π electron system, which is responsible for electron transferring properties of the molecule. Consequently the molecule is building radicals, which have antiviral and antitumoral properties, but also cause damages to the skin (32).

2.3 HYPERFORIN



(a) (b) (c)

Figure 3: Structural formulas of hyperforin (a) and its derivatives secohyperforin (b) and adhyperforin (c).

Since its isolation in 1975 by Bystrov and co-workers, hyperforin has become the best characterised constituent of St. John's wort.

The sites of hyperforin accumulation in the plant are mainly buds, flowers and capsules, though recent studies have confirmed its accumulation in leaves and stems as well (33). In *H. perforatum*, the highest hyperforin concentrations were found in flowers and fruits. Different stages of flower ontogenesis have been examined for contents of hyperforin and the results showed that its amounts continuously increase from 2.5 % in young buds (3 - 4 mm) to 8.5 % in unripe fruits, being the last developmental stage studied. These findings correlate with the discovery that hyperforin accumulates primarily in the pistil. Similar accumulation process as in case of hyperforin has also been found for its homologue adhyperforin. The content of this hyperforin-derivative increased approximately tenfold during the fruit ripening, i.e. from 0.2 % in flowers to 1.9 % in capsules. The concentration of hyperforin in leaves of *H. perforatum* is about 1.5 % and is relatively constant. On the other hand, a great intraspecific variation of hyperforin concentrations was observed in seedlings.

Lately some authors have reported about hyperforin being found in cell and callus cultures, although in very low concentrations. In cell cultures and in callus tissue of *H. perforatum*

the concentration of hyperforin reaches 0.14 % and in case of *in vitro* shoot cultures, which have a higher tissue differentiation, its content is about 0.4 % (34).

Hyperforin is a bicyclic polyprenylated acylphloroglucinol derivative whose complex structure has been determined by extensive degradation and derivatisation, as well as by spectroscopic methods. The compound is available as a mixture of interconverting tautomers. The fact that hyperforin is constantly converting from one tautomer to another (keto - enol) was confirmed by the broad shape of most ^{1}H NMR signals. Only after covalently blocking the tautomeric equilibrium of enolized β-dicarbonyl system, the derivatives show sharp NMR signals.

The activity of hyperforin appears to be strongly affected by oxidative degradation, unless it is stabilised in a form of a salt. Recently the dicyclohexylammonium salt was found to be stable both at room temperature and under the influence of air. The compound shows chemical instability in the presence of light and oxygen. This is the reason why hyperforin has long been neglected as a pharmacologically important substance. Its chemical instability is supposed to be due to the numerous double bonds, which can react with singlet oxygen to produce oxidation products. In the extracts of *H. perforatum*, hyperforin instability was proposed to be at least partly due to the photochemical properties of hypericin, which can generate singlet oxygen under the influence of light (26), (33).

Several studies revealed the presence of additional oxidised phloroglucinols in the herb, which are either genuine plant compounds or are formed during the process of extraction. Furohyperforin, also referred to as orthoforin, was proposed to be the main degradation product of hyperforin (34).

2.3.1 BIOSYNTHESIS OF HYPERFORIN

Biosynthetic pathways leading to formation of hyperforin are still poorly understood, yet some important facts have already been discovered. It has been established that the biosynthesis of hyperforin in *H. perforatum* is divided into two parts. As illustrated in figure 4, the first one is the formation of acylphloroglucinol moiety, which is followed by the attachment of prenyl side chains to it (35).

By performing NMR analysis, the utilisation of ^{13}C-labeled glucose from the growth media by *H. perforatum* shoots was observed. The results suggested a polyketide origin of the phloroglucinol moiety of hyperforin. Further analysis of isotopomer patterns showed that

isobutyril-CoA is a starter unit, which is subsequently lengthened by three malony-CoA units. The following cyclisation of a linear precursor leads to acylphloroglucinol. The enzyme responsible for this cascade of reactions is a type III polyketide synthase, i.e. the isobutyrophenone synthase (BUS).

The same method, based on the utilisation of ^{13}C-labeled exogenous glucose, was applied to establish the origin of prenyl side chains. After comparing the dimethylallyl diphosphate (DMAPP) moieties obtained in the biosynthetic pathway by introducing the ^{13}C-labeled glucose to the predicted isotopomer patterns of DMAPP, obtained via the mevalonate and the nonmevalonate pathway, the results have shown that the prenyl donors, DMAPP, are derived via the nonmevalonate pathway. Namely, the biosynthetic pathway that generates DMAPP is the deoxyxylulose phosphate pathway (36).

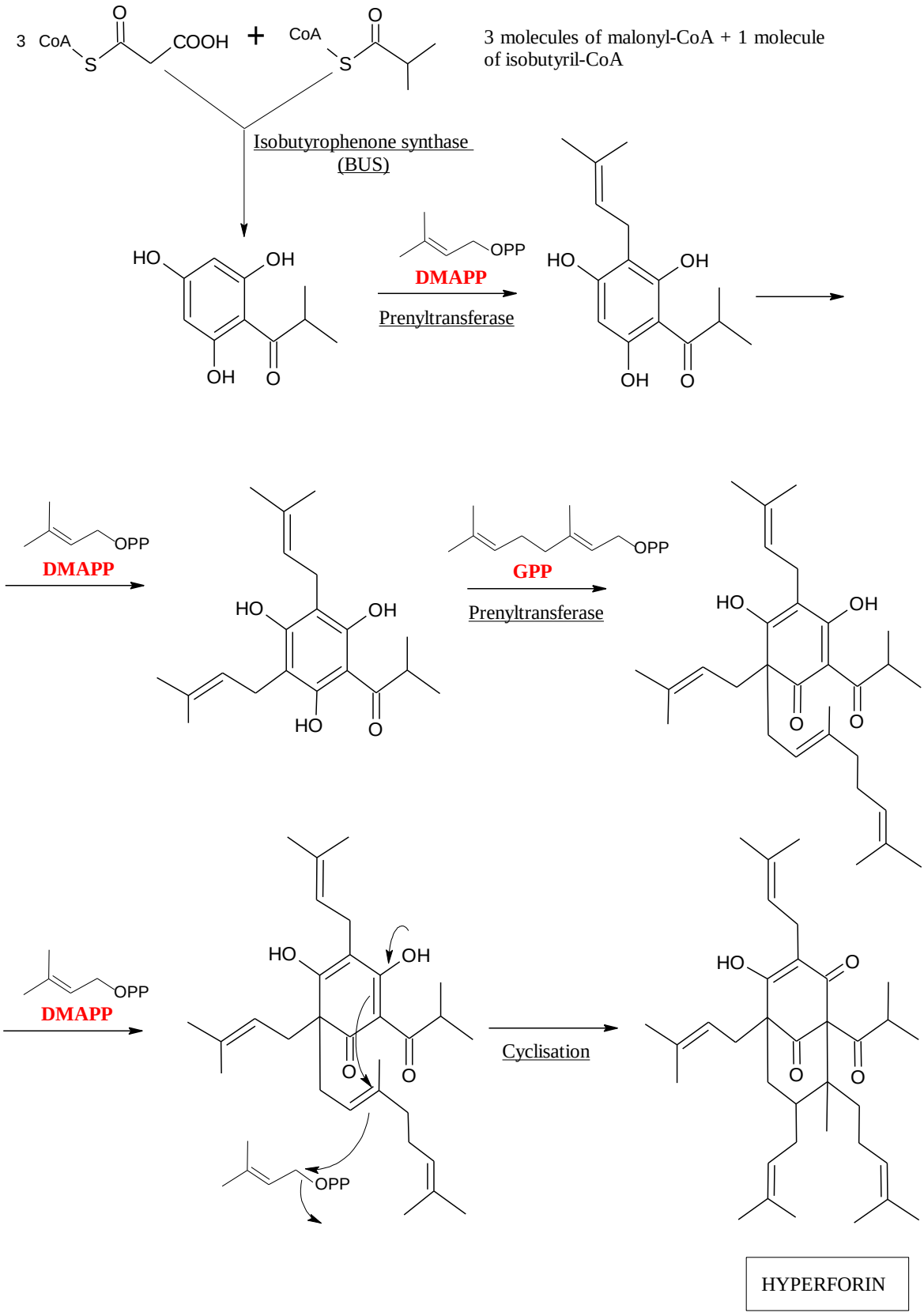


Figure 4: The proposed biosynthetic pathway of hyperforin (26).

2.3.1.1 Polyketide synthases

Polyketide synthases (PKSs) are a family of enzymes found in bacteria, fungi, plants and also in some animal species. They are catalysing synthesis of a large group of secondary metabolites (37). PKSs are structurally and functionally related to fatty acid synthases, as both enzyme classes catalyze the condensation of activated primary metabolites (acetyl-CoA and malonyl-CoA) to form β-ketoacetyl polymers linked to the enzyme by thioesther bonds. In contrast to fatty acid synthesis, where this condensation is followed by the β-ketoreduction, in case of the polyketide synthesis this reduction is completely or partly omitted, resulting in a diversity of polyketide chains, considering the occurrence of β-ketone, β-hidroxyl and alkyl groups in the structure of polyketide.

PKSs have been categorised into four groups according to the number of their subunits and the mode of synthesis, which can be linear or iterative. All types of PKSs contain eleven different catalytic domains (38).

Type III PKSs are relatively small dimeric proteins with an average subunit size of 40 - 45 kDa. They usually catalyze iterative condensation reactions and generate a diversity of secondary metabolites by passing acetyl units derived from malonyl-CoA to a specific starter molecule (39). Thus, these enzymes orchestrate a number of acyltansferase, decarboxylation, condensation, cyclization and aromatization reactions at two functionally independent active sites.

Recent discoveries have shown that there is a specific type III polyketide synthase activity present in *Hypericum* species. Incubation of crude protein extracts from *Hypericum calycinum* with isobutyryl-CoA and malonyl CoA resulted in an enzymatic product, the phlorisobutyrophenone, which is a central moiety of hyperforin. The polyketide synthase found in *Hypericum* is the isobutyrophenone synthase (BUS).

Aside from BUS, two additional related PKSs have been found in *Hypericum* species: the benzophenone synthase (BPS) with benzoyl-CoA as a preferred starter substrate and the chalchone synthase (CHS) with a preferred starter substrate being the 4-coumaroyl-CoA. The product of BPS is phlorbenzophenone, the xanthone precursor, and that of CHS naringenin chalchone, the flavonoid precursor (40).

2.3.1.2 Prenyltransferases

Lately, discoveries have shown that cell cultures of *H. perforatum* related species, *H. calycinum*, contain a specific prenyltransferase activity, which could be responsible for catalysing the first prenylation step in the hyperforin biosynthesis. Prenyltransferase in *H. calycinum* was found to be dependant on a divalent cation as the presence of Fe^{2+} ions led to its maximal activity. In such cell cultures, dimethylallyl group (DMAPP) was found to be a preferred prenyl donor and the phlorisobutyrophenone a preferred acceptor. The enzyme was shown to have a broad pH optimum (from 6.5 - 8.5) and a temperature optimum from 35 to 40 ˚C (41). Recent studies have suggested that other prenyltransferase types can be present in *Hypericum* species. Their substrate specificities to either DMAPP or GPP might be determinative for the formation of various hyperforin derivatives (3).

2.3.2 PHARMACOLOGY OF HYPERFORIN

Hyperforin, the substance responsible for the antidepressant activity of St. John's wort, has a unique mechanism of action. Although it also exhibits inhibitory effect on neuronal reuptake of neurotransmitters and its efficiency as antidepressant is comparable to that of synthetic antidepressants, hyperforin does not directly block the reuptake, but increases synaptic amine concentrations by an indirect mechanism.

The extract of *H. perforatum* increases synaptic concentrations of serotonin, noradrenaline, dopamine, gamma-amino butyric acid (GABA) and L-glutamate by inhibiting their neuronal reuptake. No other antidepressant has such a wide spectrum of neurotransmitter reuptake inhibition. However, hyperforin does not act as a competitive inhibitor of the respective transport proteins. Recent discoveries have shown that transient receptor potential (TRP) channel is the actual molecular target of hyperforin.

Activation of TRPC6 channels results in immediate increase of intracellular sodium and calcium concentrations. Increased intracellular sodium concentrations lower the sodium gradient and inhibit the gradient-driven reuptake of neuronal amines from the synapse.

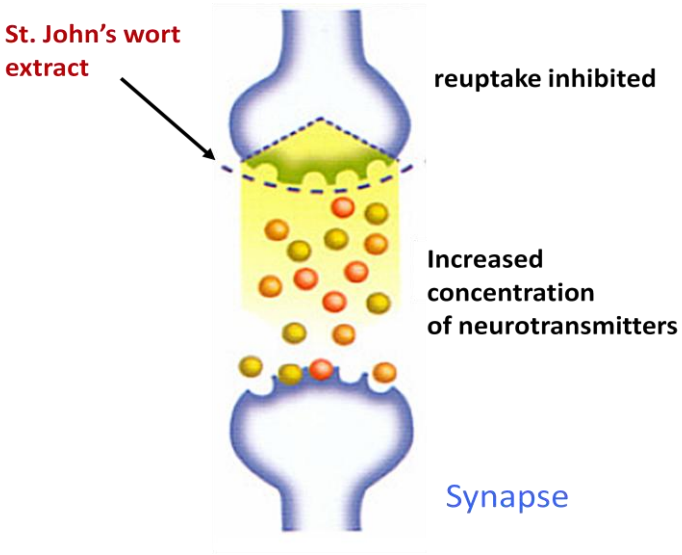


Figure 5: The mechanism of neurotransmitter reuptake inhibition via hyperforin.

TRP channels belong to a superfamily of functionally versatile nonselective cation channels that are present in most mammalian cell types. According to their functional features they can be classified into three subfamilies: the classic or canonical TRPs (TRPC), the vanilloid receptor-related TRPs (TRPV) and the melastatin-related TRPs (TRPM). Seven members of the TRPC subfamily are characterised by their activation via stimulation of G-protein-coupled receptors and phospholipase C (PLC). TRPC channels have shown to be widely expressed in the brain and the role of hyperforin as a nerve growth factor has also been investigated. The results have proven that hyperforin-induced TRPC6-mediated calcium entry mimics neurotrophic effect of the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). The combination of this effect and that on the intracellular sodium concentration is a proposed molecular mechanism for the pharmacological profile of hyperforin.

According to these findings, hyperforin was recognised as the first selective pharmacological tool to study the TRPC6 function. Moreover, it has a great potential of becoming the leading compound for a new class of antidepressants, due to its unique mechanism of action (42).

Newer investigations suggested that phloroglucinol moiety of hyperforin could be the essential pharmacophore responsible for its action. Namely, the phloroglucinol moiety has structural similarities with diacylglycerol (DAG) which is an endogenous nonselective activator of TRPC3, TRPC6 and TRPC7. In contrast to DAG, symmetric 2,4-diacylphloroglucinol derivatives have been identified as potent and selective hyperforin-

like TRPC6 activators. Studies based on the TRPC6 models showed that the natural TRPC6 activator, DAG, hyperforin and symmetric 2,4-diacylphloroglucinols share common interaction points with the target protein. The reason for the non-selectivity of DAG is probably its flexible structure. Further analysis of the crystal structure of TRPC6 and the identification of the binding pocket will probably explain more about the structure-activity relationship of hyperforin (43).

2.4 APPROACHES TO HYPERFORIN PRODUCTION

So far, field grown plants of *Hypericum perforatum* have been used as a source for commercial products, but increased demands of pharmaceutical companies for this herb have brought up ideas for alternative means of its cultivation. The great influence of environment on field grown plants reduces their quality and represents an unstable source of secondary metabolites. Possibilities to avoid the unpredictable effects of environment are cultivation of plant cell and tissue cultures, but their disadvantage is lack of cellular organisation and tissue differentiation. *In vitro* shoot cultures can, to some extent, avoid both disadvantages, but the optimisation of the cultivation process is still a point of extensive research. *In vitro* cultivation of *H. perforatum* shoot cultures in bioreactors is a plausible possibility for a stable scale-up process of hyperforin production (2).

On the other hand, investigations of influences of culture media components on metabolic activity of the cultured tissue could also reveal important facts about the optimal cultivation conditions. Thereby, we will eventually be able to create a system, in which some of the numerous methods of molecular biology could be applied; for example gene cloning or creation of a subtractive cDNA library, in order to genetically elucidate the complexity of biosynthetic pathways of hyperforin (29).

2.4.1 *IN VITRO* SHOOT CULTURES AS A SOURCE OF HYPERFORIN

In order to enhance the production of St. John's wort for commercial purposes and to avoid environmental effects that alter the concentration of active constituents, the ideas about in *vitro* propagation of cell and shoot cultures have been proposed. Finding optimal growth conditions for maximal production of hyperforin is a crucial factor in developing such large-scale propagation systems.

In the growth medium for plants, the presence of essential nutrients is indispensible for a proper crop. Plant tissue culture media are usually made up of the following constituents: macronutrients, micronutrients, vitamins, nitrogen supplements, sugar(s), solidifying agent, growth regulators and other organic supplements. There are several media formulations, which are commonly used for plant tissue cultivation. One of them has been described by Murashige and Skoog (MS medium), already in 1962.

Macronutrients provide six major elements necessary for plant development: nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S). Optimum concentration of each element depends on the plant species.

Micronutrients are required in smaller concentrations as macronutrients, although they are equally essential. They include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Because of the dissolving difficulties, Zn and Fe are used in a form of chelats. Iron is added as a salt with ethylene diaminetetraacetic acid (EDTA).

As a common carbon source for plant tissue cultivation, sucrose is used. Other carbohydrates that have been tested include glucose, fructose, lactose, galactose, rafinose, maltose and starch.

Vitamins are normally synthesised by plants, as they are required by plants as catalysts of several metabolic reactions. Vitamins most frequently used in culture media and their influences on plant development are extensively described in the following chapters.

Myo-inositol (MI) is commonly included in many vitamin stock solutions, although it is a carbohydrate and not a vitamin. There are several evidences about its positive influence on

the plant development (44). A brief description of nutrients which could be decisive for biosynthesis of secondary metabolites, including MI, vitamins and growth regulators, follows.

2.4.1.1 The role of plant growth regulators (phytohormones)

Phytohormones or plant hormones are chemical messengers which are produced by plants and occur in them in very low concentrations. Plant hormones effect plant growth, time of flowering, the sex of flowers, life time, formation, fruit ripening and even plant death. Lacking phytohormones, plants would be a mass of undifferentiated tissue. Plant hormones that are produced in one cell or tissue type can modulate cellular processes in other cells by interacting with specific protein receptors. Endocrine hormones are synthesised in one type of tissue and act on specific targets in another tissue, whereas paracrine hormones can only act on cells adjacent to their source (45), (46).

If plant hormones are applied to plants by humans, they are rather referred to as plant growth regulators. They may be synthetic compounds that mimic naturally occurring plant hormones or natural hormones, extracted from plant tissue (47).

Plant development is regulated by six major types of hormones: auxins, gibberellins, cytokinins, ethylene, abscisic acid and brassinosteroids. Aside from these main hormone groups, a variety of signalling molecules that play different roles in resistance to pathogens and defence against herbivores, have also been identified: jasmonic acid, salicylic acid and the polypeptide systemin.

Auxins were the first growth hormones to be studied in plants. The fact that they are synthesised in one part of the plant and transported to the site of action, confirms that auxins are authentic plant hormones. Auxins and cytokinins differ from other hormone groups in the fact that they are required for viability of plants. The principal and most abundant natural auxin in plants is indole-3-acetic acid (IAA).

Although they are chemically diverse, a common feature of all active auxins is the distance of about 0.5 nm between the positive charge present on the aromatic ring and the negatively charged carboxyl group within the molecule.

Auxin biosynthesis is associated with rapidly growing and dividing plant tissues, especially in shoots, thus, the primary sites of auxin synthesis are shoot apical meristems and young

leaves. As the roots elongate, the root apical meristems become important sites of auxin synthesis.

The polarised apical-basal transport of auxins is necessary for their action, which is primarily the cell elongation. Consequently, auxins affect the bending of coleoptiles toward the light. A steady supply of auxins arriving to the subapical region of the stem is required for the elongation of these specific cells. Thus, these hormones promote growth of stems and coleoptiles. On the other hand, they inhibit growth in roots, probably because of the auxin-induced ethylene synthesis, which appears beyond of their optimal concentrations. Ethylene is a gaseous hormone that inhibits stem elongation in many species. Auxins also play a role in the apical dominance, fruit ripening and tissue regeneration (45).

The simplicity of the auxin structure enabled scientists to synthesize a variety of molecules with auxin-like activity that can be classified in five major categories: indole acids, naphthalene acids, chlorophenoxy acids, benzoic acid and picolinic acid derivatives. A well known 1-naphtaleneacetic acid (NAA) has been frequently used for plant tissue propagation (48).

Cytokinins were discovered in search for factors that could stimulate cell division. The first cytokinin discovered was kinetin, a synthetic analogue of a naturally occurring zeatin. Both kinetin and zeatin are derivatives of 6-aminopurine with different side chains, although both of them are attached to the N^6 of the aminopurine. As most compounds with cytokinin activity are N^6-substituated aminopurines, this seems to be the structural requirement for their activity. They are usually found in higher concentrations in meristematic regions and growing tissues. It has been proposed that cytokinins are synthesised in roots and transported via xylem to the shoots.

When applied to higher plants, cytokinins can stimulate or inhibit a variety of physiological, metabolic, biochemical and developmental processes. They induce cell division in callus cells, promote root or bud formation, delay senescence of leaves and promote expansion of dicot cotyledons. It has also been established that cytokinins promote nutrient mobilization, reduce apical dominance and have a positive effect on seed germination and chloroplast differentiation.

A synthetic cytokinin, 6-benzylaminopurine (BAP or BA), is routinely added to growth media for plant tissue cultivation, as it is stable to autoclaving and therefore suitable for these purposes. Researches have shown that many species response optimally to defined ratios of NAA and BAP (45).

Figure 6: Structural formulas of 1-naphtaleneacetic acid (NAA) (left) (49) and 6-benzylaminopurine (BAP) (right) (50).

2.4.1.2 The role of vitamins

Unlike animals, plants are able to synthesise vitamins essential for their normal development. However, in cultured plant tissues a deficiency of certain factors can occur. In order to improve growth and survival of *in vitro* cultured plants, vitamins are usually added to the media. Most frequently used vitamins are those of the B-complex: thiamine (Vit. B_1), nicotinic acid (niacin; Vit. B_3) and pyridoxine (Vit. B_6). Requirements of plant tissues for vitamins vary according to the nature of the plant. Several experiments showed that vitamins in the media can also inhibit plant development. For some species the combination of all three vitamins of the B-complex, which is commonly present in the Murashige and Skoog (MS) medium, is optimal for their cultivation. On the other hand, researches have shown that only thiamine is unavoidable for *in vitro* plant tissue cultivation (51).

Thiamine is a transport form of the vitamin. Namely, its active forms are phosphorylated thiamine derivatives. Thiamine diphosphate (ThDP) is a coenzyme of several important enzymes in different species, for example: pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain α-keto acid dehydrogenase (52).

Nicotinic acid or niacin is a precursor of NAD^+ / NADH (nicotinamid adenine dinucleotide) and $NADP^+$ / NADPH (nicotinamide adenine dinucleotide phosphate),

coenzymes found in all living cells which are involved in redox reactions, carrying electrons in life-essential biochemical reactions (53).

Pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives are interconvertable substances, all being called the vitamin B_6. It has been shown that pyridoxal-5'-phosphate plays an important role as a coenzyme in animal as well as in plant cells. Phosphatase enzymes in tomato lose their catalytic ability after removing pyridoxal-5'-phosphate. On the other hand, vitamin B_6 also reduces plant cell death induced by reactive oxygen species. Recently it has even been established that pyridoxine has a function in the *in vivo* antioxidative defense of plants (54), (55).

2.4.1.3 The role of *myo*-inositol (MI)

Myo-inositol is a sixfold alcohol of cyclohexane. It is a carbohydrate, but not a classic sugar. It exists in nine possible stereoisomers, but the MI (cis-1,2,3,5-trans-4,6-cyclohexanehexol) is occurring most frequently. Medically it has been classified as a member of the Vitamin B complex as it is required for cell growth of many species. MI is considered as a plant vitamin because it does not normally contribute to carbohydrate utilization as an energy source.

In eukaryotic cells MI plays an important role as a structural basis for numerous secondary messengers. Several studies have shown that it also has a positive influence on the rate of callus growth and induction of morphogenesis. By interacting with cytokinin, it also promotes cell division in certain species. This stimulatory effect in plants probably arises partly from its participation in biosynthetic pathways leading to formation of pectin, which is a basic compound needed for the formation of plant cell wall.

As MI is a basic compound of secondary messengers, it also plays a role in gene expression. Moreover, its phosphates, for example phytic acid, act as secondary messengers to primary actions of auxins (51). Several studies have identified MI as an important factor for regulation of physiological activity of auxins. Namely, some auxins are present in the plant in form of an ester with MI. These esters were proposed to represent auxin storage in case of excessive auxin production in plants. On the other hand, these esters might be responsible for auxin transport within the plant. Researches have shown that MI can even enhance the inhibitory effect of auxins on plant development (56),

(57). Several other important roles of MI in plant physiology have been discovered. For example phosphorylated inositol phosphates were found to play a crucial role in the transduction of intracellular signals, O-methyl esters of inositol were proposed to be involved in stress responses of the plant and glycosyl-inositolphosphorylceramides were identified as essential structures, which anchor proteins to hydrophobic cell membrane (56).

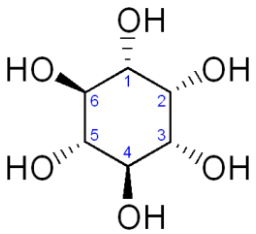


Figure 7: Structural formula of *myo*-inositol (58).

3. WORK PLAN

In order to find an improved and more efficient method for large-scale production of hyperforin, we will cultivate *in vitro* shoots of *H. perforatum* on different modifications of Murashige and Skoog (MS) solid medium. We will study the influences of plant growth regulators (auxin: NAA and cytokinin: BAP), different concentrations of vitamins (nicotinic acid and pyridoxine) and *myo*-inositol in the used growth media, on metabolic activity of the cultured shoots. Aside from optical observation of morphological changes in *in vitro* shoots, young leaves of cultivated *H. perforatum* will be microscopically examined to estimate the influence of growth media components on the formation of translucent glands. *In vitro* shoots will be used for preparation of methanolic extracts, which will be examined for hyperforin contents by means of high performance liquid chromatography (HPLC). The amounts of hyperforin will be determined after 14 and 21 days of cultivation on six different media, containing various combinations of the above mentioned nutrients. We will select two media with the highest stimulatory effects on metabolic activity in the shoots and monitor hyperforin production throughout 21 days of cultivation. We will try to explain the connection between translucent gland formation and hyperforin production in the shoots. The efficiency of optimal medium modification will also be evaluated by determination of enzyme activity in *H. perforatum* cell cultures, derived from the examined shoots. The activity of enzymes participating in the biosynthesis of hyperforin will be compared to the production of hyperforin in the shoots, cultured under the same conditions. After exploring both the hyperforin production and the related enzyme activity, we will try to propose the medium, which would potentially be most favourable for the isolation of mRNA molecules, associated with enzymes, participating in the biosynthesis of hyperforin and its derivatives. By means of mass spectrometry (Ms) we will elucidate the structures of hyperforin derivatives and try to estimate their relationship in the biosynthetic pathway of these particular phloroglucinols.

The purpose of our experiments is to establish the fundamentals for a large-scale bioreactor hyperforin production, possibly using *in vitro* shoots of *H. perforatum*. On the other hand, we will also try to optimize a method for cultivation of specific shoot types, which could be used for the establishment of a cDNA library in the future.

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 *HYPERICUM PERFORATUM IN VITRO* SHOOT CULTURES

In All experiments, in which the influence of different growth media on hyperforin production was studied, *Hypericum perforatum in vitro* shoot cultures were used, established according to Charchoglyan and colleges in 2007. The shoots were established from callus, derived from anthers of *H. perforatum* flowers, which were collected in the national park of Idjevan in Armenia, in July 1995. Shoot organs were initiated on a solid Murashige and Skoog (MS) medium supplemented with 0.05 mg/l NAA and 0.5 mg/l BAP. After being established by Charchoglyan and colleges that hormonal stimulation affects hyperforin and secohyperforin accumulation, the shoots have been cultivated on a MS medium containing 0.05 mg/l NAA and 1 mg/l BAP (MSH) ever since, under the following conditions: 25 ˚C, 16 h light / 8 h dark cycle (3).

H. perforatum type A *in vitro* shoots

We transferred the established shoots onto the fresh MSH medium every 3 to 5 weeks and they retained their reduced form during the entire time of cultivation (Fig. 8). The shoots cultivated on a hormone-containing medium (MSH) are referred to as the type A shoots.

H. perforatum type B *in vitro* shoots

When the type A shoots were transferred onto a hormone-free MS medium (medium B), their appearance significantly changed after 28 days of cultivation. They became elongated and were not as robust as those of the type A shoots (Fig 9). These shoots, which are referred to as the type B shoots, were cultivated under same conditions as those of the type A and subcultivated onto fresh medium B every 3 to 5 weeks.

Figure 8: Type A shoots*: H. perforatum* shoots cultivated on the medium A (MSH).

Figure 9: Type B shoots*: H. perforatum* shoots cultivated on the medium B (MS-).

4.1.2 *HYPERICUM PERFORATUM* CELL SUSPENSION CULTURES

We established the cell cultures of *H. perforatum* from formerly prepared callus cultures. Callus was induced by transferring the type A shoots on to a solid MS medium, supplemented with 1.0 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l of kinetin (59). After approximately one month of cultivation in the dark, during which the callus cultures were created, we dispersed one or two calli into 50 ml of hormone free liquid MS medium (liquid medium B). The cells were subsequently cultivated in the dark at 25 ˚C, on a rotary shaker at 160 rpm. The established cell cultures were transferred into a fresh liquid MS medium every 12 - 14 days.

4.1.3 NUTRIENT MEDIA

4.1.3.1 Murashige and Skoog (MS) medium for *in vitro* plant cultivation

Table II: Composition of Murashige and Skoog basic medium (60) and its modifications.

Medium	Component / concentration		Producer
MS liquid medium; regular medium (60)	NH_4NO_3	1650 mg/l	ROTH, Germany
	KNO_3	1900 mg/l	ROTH, Germany
	$MgSO_4$ x $7H_2O$	370 mg/l	ROTH, Germany
	KH_2PO_4	170 mg/l	ROTH, Germany
	$CaCl_2$ x $2H_2O$	440 mg/l	ROTH, Germany
	H_3BO_3	6.2 mg/l	ROTH, Germany
	$MnSO_4$ x $4H_2O$	22.3 mg/l	Riedel de Haën, Germany
	$ZnSO_4$ x $7H_2O$	8.6 mg/l	Riedel de Haën, Germany
	KJ	0.83 mg/l	Riedel de Haën, Germany
	$NaMoO_4$ x $2H_2O$	0.25 mg/l	MERCK, Germany
	$CuSO_4$ x $5H_2O$	0.025 mg/l	Riedel de Haën, Germany
	$CoCl_2$ x $6H_2O$	0.025 mg/l	Riedel de Haën, Germany
	Glycin	2.0 mg/l	ROTH, Germany
	Na_2EDTA x $2H_2O$	41.3 mg/l	ROTH, Germany
	$FeSO_4$ x $7H_2O$	27.8 mg/l	MERCK, Germany
	Saccharose	20 g/l	Pfeifer&Langen, Germany
MS solid medium	Agar	6 g/l	AppliChem, Germany
MS modifications with hormones	NAA	0.05 mg/l	Fluka, Switzerland
	BAP	1 mg/l	Acros Organics, Germany
MS modifications with vitamins **a) LESS (↓)** **b) MORE (↑)**	a)		
	Thiamine HCl	0.1 mg/l	Sigma, Germany
	Pyridoxal HCl	0.1 mg/l	Calbiochem
	Nicotinic acid	0.1 mg/l	Fluka, Switzerland
	b)		
	Thiamine HCl	0.1 mg/l	
	Pyridoxal HCl	0.5 mg/l	
	Nicotinic acid	0.5 mg/l	
MS modifications with *myo*-inositol	*Myo*-inositol	100 mg/l	ROTH, Germany

4.1.3.2 The examined modifications of Murashige and Skoog *in vitro* plant growth media

Table III: Modifications of the MS medium (A - F) regarding the presence and content of plant hormones, vitamins and *myo*-inositol.

Medium modification	Component variation
A	+ hormones + *myo*-inositol ↑ vitamins
B	− hormones + *myo*-inositol ↑ vitamins
C	+ hormones − *myo*-inositol ↓ vitamins
D	− hormones − *myo*-inositol ↓ vitamins
E	+ hormones − *myo*-inositol ↑ vitamins
F	− hormones + *myo*-inositol ↓ vitamins

All media were prepared in distilled water and autoclaved at 120 ˚C for 20 minutes before use.

4.1.4 CHEMICALS

- **Acetic acid (50 %)** was used for stopping the enzymatic reaction in the enzyme assay (ROTH, Germany).

- **Ascorbic acid** (10 mM) was added to methanol during the process of hyperforin extraction from *in vitro* shoots. Ascorbic acid decreases oxidative degradation of hyperforin and thereby enables optimal extraction (MERCK, Germany).

- **Bradford solution** was used for Bradford protein assay. It was stored at 4 ˚C.

Components	Concentration
Coomassie-Brilliant blue G250	100 mg
Ethanol 96 %	50 ml
Phosphoric acid 85 %	100 ml
Distilled water	Ad 1000 ml

- **DTT** (dithiothreitol) was used as a reductive agent. It reduces intra- and intermolecular disulfide bonds in protein structures because of its conformational propensity allowing it to form a six-membered ring with an internal disulfide bond (AppliChem, Germany).

- **Ethyl acetate** was used for extraction of the enzymatic products, obtained after the incubation of crude protein extract with the malonyl-CoA and the starter substrates.

- **Glycerin (20 %)** was used for storing the extracted protein fraction (ROTH, Germany).

- **HEPES buffer** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); pH 8.0; is a zwitterionic organic buffering agent. It was used in the process of protein isolation from plant material. It maintains physiological pH despite changes in carbon dioxide concentration, caused by cellular respiration (Sigma-Aldrich).

- **Hyperforin standard solution** was used as a reference for qualitative determination of hyperforin in *H. perforatum* shoots (HWI Analytik, Germany).

- **Isobutyryl-CoA, benzoyl-CoA and 4-Cumaroyl-CoA** were used as starter substrates in the enzyme assay (Sigma, Germany).

- **Malonyl-CoA** was used as a proposed elongation substrate of the polyketide synthases in the enzyme assay (Sigma, Germany).

- **Methanol HPLC grade** was used both as a solvent for extraction of hyperforin from the shoots and as a component of the mobile phase in HPLC separation.

- **PD_{10} column regeneration solution** (NaOH 0.16 M) was prepared from 3.2 g NaOH dissolved in 500 ml dH_2O. The column was first washed with 25 ml of the washing solution and subsequently rinsed by water, until the pH was neutral.

- **Phytohormones** for callus induction:

  2,4-D (2,4-Dichlorophenoxyacetic acid) is a synthetic auxin (Fluka)

  Kinetin (N^6-furfuryladenine) is a natural cytokinin that promotes cell division (AppliChem).

- **Polyclar AT** (Polyvinylpolypyrrolidone) was used to adsorb the impurities from the plant material in the process of preparing the crude protein extract (Serva, Germany).

- **Potassium phosphate buffer** (KH_2PO_4), pH 7.5; was used to separate the desired protein fraction from the crude protein extract by using gel filtration chromatography.

- **Sea sand** was used to mechanically break plant cells in order to extract intracellular proteins (ROTH, Germany).

4.2 EQUIPMENTS

Table IV: List of equipments.

Equipment	Type	Manufacturer
Autoclave	Vx-120	Systec GmbH Laborsystemtechnik
Automatic pipettes	Various volumes	Eppendorf research Finnpipette Biohit
Balances	Large and small scale	Sartorius
Centrifuge	Universal 32R Biofuge 13 Sigma 1-15K	Hettich Heraeus Sepatech Sigma
Clean bench	LaminAir HLB 2472	Heraeus
Freezer	-20 ˚C -80 ˚C	Privileg SANYO
Gel filtration column	PD-10 desalting column packed with Sephadex G-25 medium	Amersam Biosciences
HPLC	1260 Infinity VWR Elite LaChrom	Agilent Techologies Hitachi
Incubator-shaker	Minitron	Infors
Optical microscope	Axiostar plus	Zeiss
pH meter	Digital pH meter 325	WTW
Refrigerator	4 ˚C	AEG Privileg
Spectrophotometer	Ultrospec 1000	Pharmacia Biotech
Speed vac	RVC 2-18	Christ
Thermo block	Dri-Block DB-3D	Techne
Vortex	VF2	IKA Labortechnik

4.3 METHODS

4.3.1 CULTIVATION OF *HYPERICUM PERFORATUM* PLANT MATERIAL

H. perforatum in vitro shoot cultures were obtained and cultivated as described in chapters 4.1.1 and 4.1.2. We transferred the two starting types of shoots (A and B), each on five other variations of the MS media (Table V) and examined them qualitatively and quantitatively after 14 and 21 days.

Table V: System of evaluating the influence of media components on metabolic activity in *H. perforatum* shoots.

Type of starting shoots	**MS medium modifications**					
	A	B	C	D	E	F
A		✓	✓	✓	✓	✓
B	✓		✓	✓	✓	✓

✓ … The respective starting type of shoots, A or B, transferred on different media

4.3.2 QUALITATIVE AND QUANTITATIVE ANALYSIS OF METABOLIC ACTIVITY IN PLANT MATERIALS

4.3.2.1 Microscopic analysis of the leaves

H. perforatum shoots of types A and B were cultivated on five modified media and were microscopically examined for the presence of translucent glands after 14 and 21 days of cultivation. We assessed the morphology of leaves and the size and the frequency of translucent glands, by using AxioVision software of the Axiostar microscope. Enlarged photos of the leaves were captured with a microscope-coupled photo camera.

4.3.2.2 The extraction of hyperforin from *Hypericum perforatum* shoots

The shoots of *H. perforatum* were harvested from tissue culture flasks, subsequently frozen with liquid nitrogen and powdered in a mortar. 100 mg of pulverized plant material was transferred into a light protected centrifuge tube and extracted with 1 ml of HPLC grade methanol and 10 mM solution of ascorbic acid. The extraction was carried out in an ultrasonic bath for 60 minutes. The resulting material was centrifuged at 4 ˚C for 10 minutes at 9000 rpm. The supernatant was first filtered trough a paper filter and then by membrane filtration (pore size 0.20 μm), before it was subjected to HPLC analysis.

4.3.2.3 High performance liquid chromatography (HPLC)

For separation and quantification of hyperforin derivatives present in *H. perforatum* shoot extracts, as well as for the analysis of enzymatic products, we used HPLC with a gradient elution. The mobile phase was a mixture of HPLC grade methanol and dH_2O containing 0.1 % phosphoric acid. The composition of mobile phase changed during the separation process within the specified time intervals. For each examined component the composition and the gradient were adjusted. Throughout the entire analysis the flow rate was 0.5 ml/min and the C18 column HyperClone 5 μm (150 mm x 4.60 mm) was used (Phenomenex). The HPLC separation process and the evaluation of chromatograms were performed with Agilent Chemstation, equipped with a DAD detector.

a) Hyperforin

Table VI: Mobile phase gradients used for the separation of hyperforin and its derivatives and the optimal wavelength for their detection.

Time (min)	Water (%)	Methanol (%)	Wavelength (nm)
0	90	10	
10	70	30	
20	40	60	
30	20	80	
40	15	85	272
45	10	90	
50	5	95	
55	2	98	
65	0	100	
75	90	10	

a) Phlorisobutyrophenone (BUS product), phlorbenzophenone (BPS product), naringenin chalchone (CHS product)

Table VII: Mobile phase gradients used for the separation of enzymatic products and the optimal wavelengths for their detection.

Compound	Time (min)	Water (%)	Methanol (%)	Wavelength (nm)
	0	70	30	
	5	70	30	
BUS product	17	40	60	286
BPS product	25	40	60	306
CHS product	27	30	70	290
	30	0	100	
	35	70	30	

4.3.2.4 Mass spectrometry (Ms)

As there is only hyperforin standard available on the market currently, we applied mass spectrometry in order to identify other substances detected in the *H. perforatum* extracts, which also showed UV spectra significant for phloroglucinol derivatives.

- Sample preparation

Methanolic extracts of *H. perforatum in vitro* shoots, grown on MS- medium, were prepared as described in 4.3.2.2. For the preparative analysis we used HPLC from Hitachi, equipped with a C18 column (3 μm pore size, 150 x 4.6 mm). The mobile phase constituency and gradient adjustment were the same as in case of hyperforin detection with the only difference being the added pH modulator. For Ms samples, the pH modulator has to be a volatile substance, in our case this was 0.1 % formic acid, which was added to both organic and inorganic component of the mobile phase. The flow rate was 0.3 ml/min.
After determining specific retention times for three different phloroglucinol derivatives, we were able to capture them separately into light protected vials, thereby enabling the Ms analysis of each fraction.

- Ms analysis

The Ms Analysis was performed by using the Applied Biosystems 3200 Q TRAP, Turbo V Ion Source mass spectrometer with the Analyst 1.4.2 software. The electrospray ionisation (ESI) was applied. N_2 was used as the collision gas for fragmentation of parent structures. For each of the three HPLC fragments, Ms spectra of the parent ion and the product ions, were generated. Detailed conditions of Ms analysis are listed in Table VIII. In order to confirm the structural identity of hyperforin, we compared product ions of the isolated HPLC fraction to product ions of the reference substance.

Table VIII: Experimental parameters for mass spectrometry.

MS-parameter	Detection of parent ion	EPI
Curtain gas	10 ml/min	10 ml/min
Collision gas	-	High
Ion spray voltage	4500 V	4500 V
Temperature		
Ion source gas	10 ml/min	10 ml/min
Declustering potential	85 V	85 V
Entrance potential	3 V	3 V
Collision energy	-	10 V

4.3.3 ENZYME ASSAY

We tested the activity of BUS, BPS and CHS in cell cultures of *H. perforatum* cultivated in liquid media A and F, in order to confirm the positive effect of these two media, which we previously found to be optimal for hyperforin production in the shoots. On the fourth day following each subcultivation in the liquid MS- medium, we transferred 3 g of cultivated cells into liquid media A and F and measured the enzyme activity after 12 hours, 1, 3, 6 and 9 days of cultivation. The preparation of crude protein extract was followed by the incubation with each of the three proposed starting substrates and malonyl-CoA (Table IX). The enzymatic products were separated by HPLC, by applying the conditions described in table VII. Their identity was confirmed with formerly obtained HPLC chromatograms of reference substances.

Table IX: A list of target PKSs, their proposed substrates, products and secondary metabolites.

Starting substrate		Active enzyme	Product	Secondary metabolite
Isobutyryl-CoA	+ 3 Malonyl-CoA	BUS	Phlorisobutyrophenone	Hyperforins
Benzoyl-CoA		BPS	Benzophenone	Xanthones
4-coumaroyl-CoA		CHS	Naringenin chalchone	Flavonoids

4.3.3.1 Extraction of proteins from cell cultures

The entire process of enzyme extraction was carried out at 4 ˚C. The cells were collected by filtration with a Büchner funnel and treated as described in the following steps. First 0.3 g of Polyclar AT, 3 ml of HEPES buffer containing 10 mM dithiothreitol (DTT) and one spatula of sea sand were added to the cells. The resulting mixture was homogenized for 10 minutes and the homogenate centrifuged for 15 minutes at 9000 rpm.

- Gel filtration chromatography

The target protein fraction was separated from the rest by using a gel filtration chromatography column (PD-10 desalting column). Actually, this is a size exclusion chromatography which enabled the separation of molecules, contained in one solution, according to their size and was used to remove the low molecular mass substances from the proteins. First the PD-10 column was equilibrated with 25 ml KH_2PO_4 buffer (pH 7), then 2.5 ml of the extracted supernatant were added and then following the first elution the protein fraction was eluted with 3.5 ml KH_2PO_4 buffer. Finally the column was washed with 25 ml of 0.16 M NaOH solution in order to be regenerated. The column was rinsed with distilled water until the pH was neutral.

- Determination of protein concentration (Bradford assay)

The concentration of extracted proteins was determined with a Bradford assay (Bradford, 1976), by using Coomassie-Brilliant Blue G 250. After mixing the proteins with the dye, the absorbance maximum of the resulting coloured solution shifts from 465 nm to 595 nm, which is measured by UV/Vis spectrophotometry. In order to determine the concentration of protein extract, 900 µl of Bradford reagent was mixed with 95 µl KH_2PO_4 buffer and 5 µl of the crude protein extract. As a blank a mixture of 900 µl Bradford reagent and 100 µl KH_2PO_4 buffer was used. After 5 minutes of incubation the absorbance was measured at 595 nm. The protein concentration was determined from the calibration curve established by using 1 to 10 µg/ml bovine serum albumin (BSA) as a standard. The mass extinction coefficient was 0.0442.

- Protein storage

By adding autoclaved glycerin to the protein extract, in a concentration of 50 %, the proteins can be stored for approximately 3 weeks at -20 ˚C without significant loss of their activity.

4.3.3.2 *In vitro* enzyme activity assay

To estimate enzymatic activity of the extracted protein, 20 µl of each starting substrate was mixed with 20 µl malonyl-CoA and 100 µg of extracted proteins. KH_2PO_4 buffer pH 7.5 was added to the mixture to 250 µl. Three variations of enzyme assays were carried out, as follows:

- Active enzymes

We incubated the substrates and enzymes for 60 minutes at 37 ˚C, stopped the reaction by adding 25 µl of 50 % acetic acid and extracted the enzymatic products with 400 µl of ethyl acetate after 7 minutes of centrifugation at 13000 rpm. The hydrophobic phase was collected and the extracted sample was dried in a Speed vac. For HPLC analysis, the samples were dissolved in 50 µl methanol. 20 µl of each solution were injected on the separation column.

- Zero time

After mixing the proteins with the substrates and buffer, the reaction was stopped with 25 µl of 50 % acetic acid before the incubation had started. All subsequent steps were carried out as described above.

- Denaturised protein

Before the addition of 100 µg of proteins, they were denaturised at 99 ˚C for 15 minutes. All subsequent steps were carried out as described above.

5. RESULTS AND DISCUSSION

5.1 PHYTOCHEMICAL ANALYSIS OF *H. PERFORATUM* STARTING PLANT MATERIAL

The two types of *H. perforatum* shoots, which were used in the experiments, differ in their macroscopic (4.1.1) and microscopic appearances as well as in the contents of hyperforin.

5.1.1 MICROSCOPIC APPEARANCE

H. perforatum in vitro shoots, types A and B

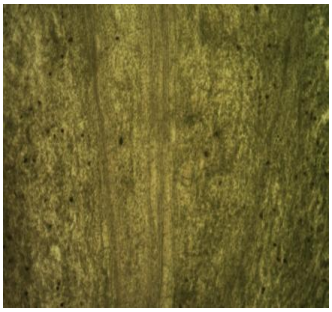


Figure 10: A leaf of *H. perforatum* cultivated on the MSH medium (type A); 10x zoom.

Elimination of hormones (NAA, BAP) from the growth medium (28 days of cultivation).

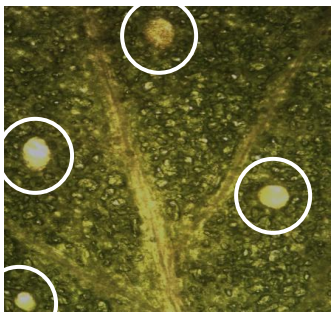


Figure 11: A leaf of *H. perforatum* cultivated on the MS- medium (type B); 10x zoom.

5.1.2 CONTENT OF HYPERFORIN

For qualitative determination of hyperforin, its HPLC chromatograms were compared to those obtained with the hyperforin standard (Fig. 12). The amount of hyperforin in differently treated *H. perforatum* shoots was determined by using the calibration curve obtained with the use of different concentrations of hyperforin standard substance (Fig. 13).

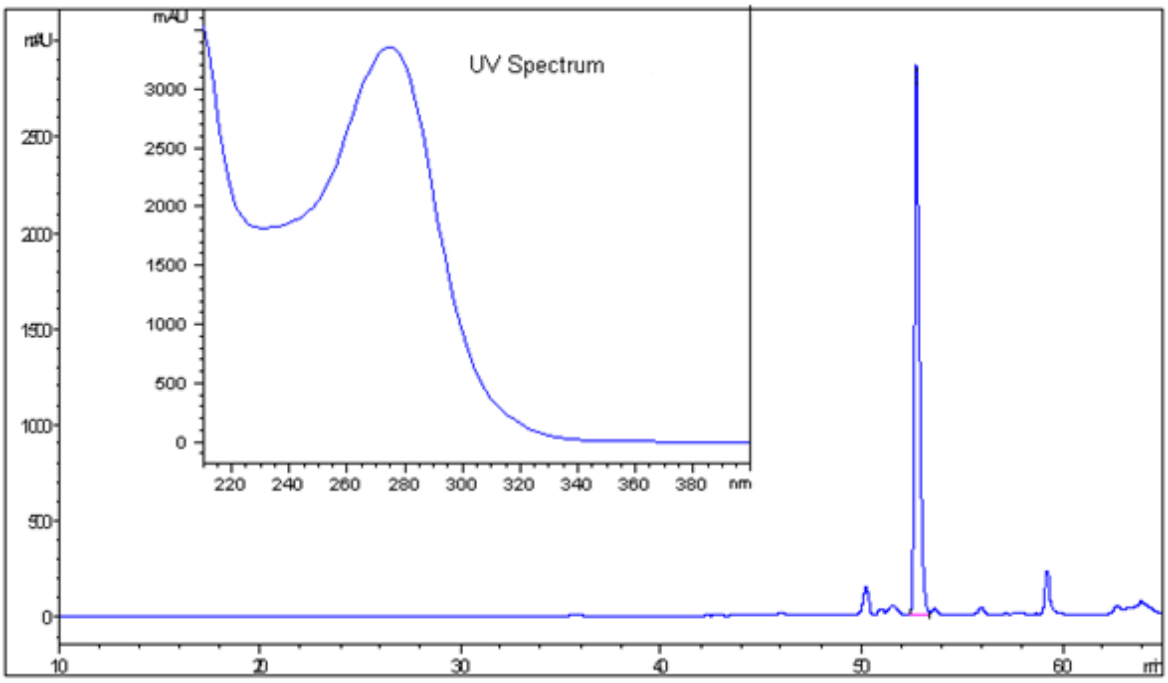


Figure 12: HPLC chromatogram of hyperforin standard substance ($t_R \approx 52.8$ min) with a corresponding UV spectrum (maximal absorbance at 272 nm).

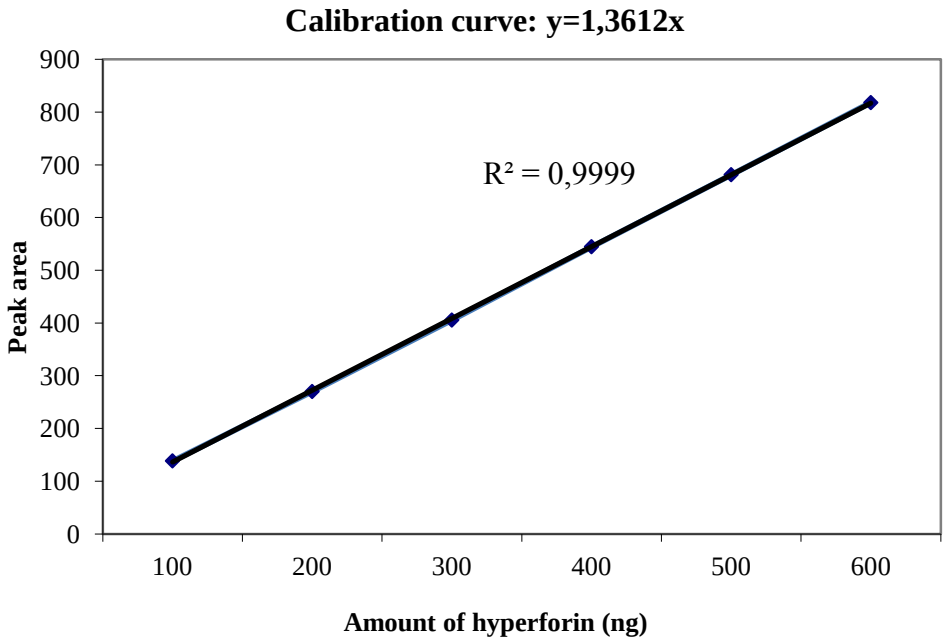


Figure 13: Calibration curve of hyperforin reference substance.

H. perforatum shoots type A contained no trace of hyperforin (Fig. 14). Type B shoots, derived from type A shoots, contained 0.18 mg hyperforin per 1 g of their fresh weight (Fig. 15).

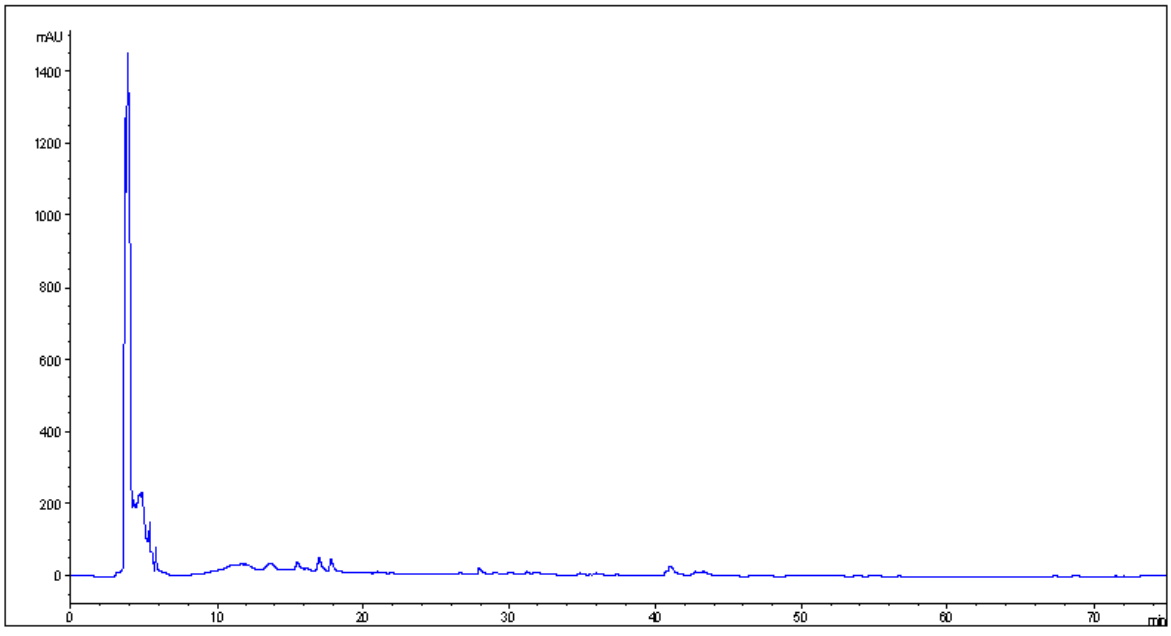


Figure 14: HPLC chromatogram of methanolic extract of *H. perforatum* type A shoots. Around the retention time, significant for hyperforin, there is no visible peak.

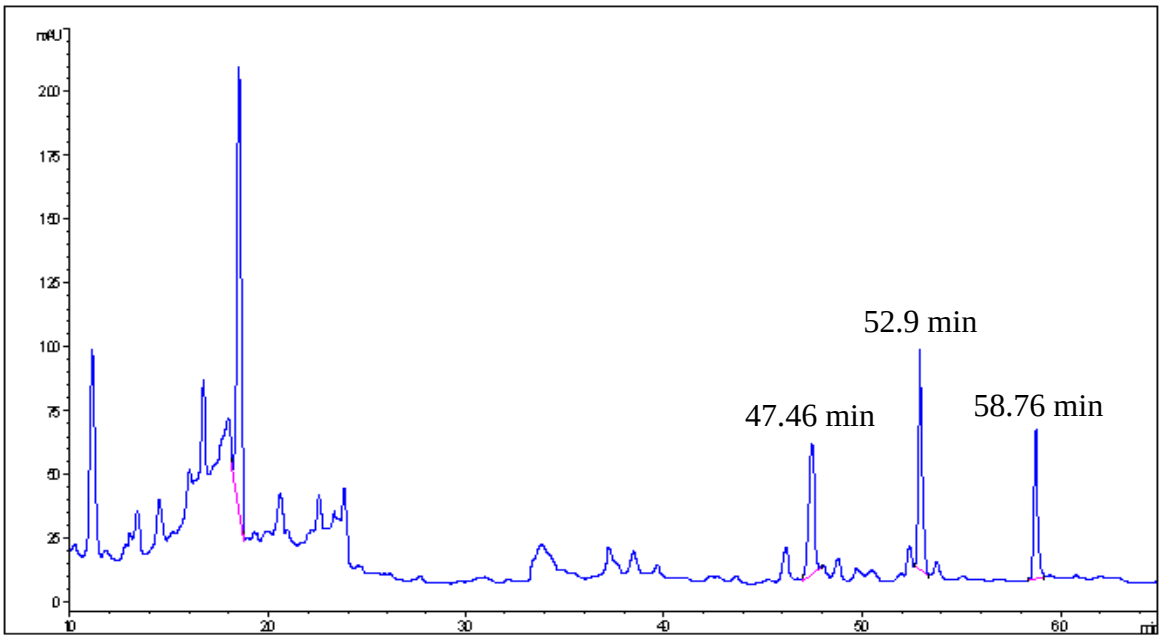


Figure 15: HPLC chromatogram of methanolic extracts of *H. perforatum* type B shoots. Three peaks with hyperforin-like UV spectra were detected.

The HPLC chromatogram of *H. perforatum* type B shoots revealed two additional peaks with UV spectra significant for the hyperforin structure (Fig. 16). Their retention times were approximately 47.46 and 58.76 min, respectively. Based on the Ms analysis and the published data, they were identified as secohyperforin (47 min) and adhyperforin (58 min), respectively.

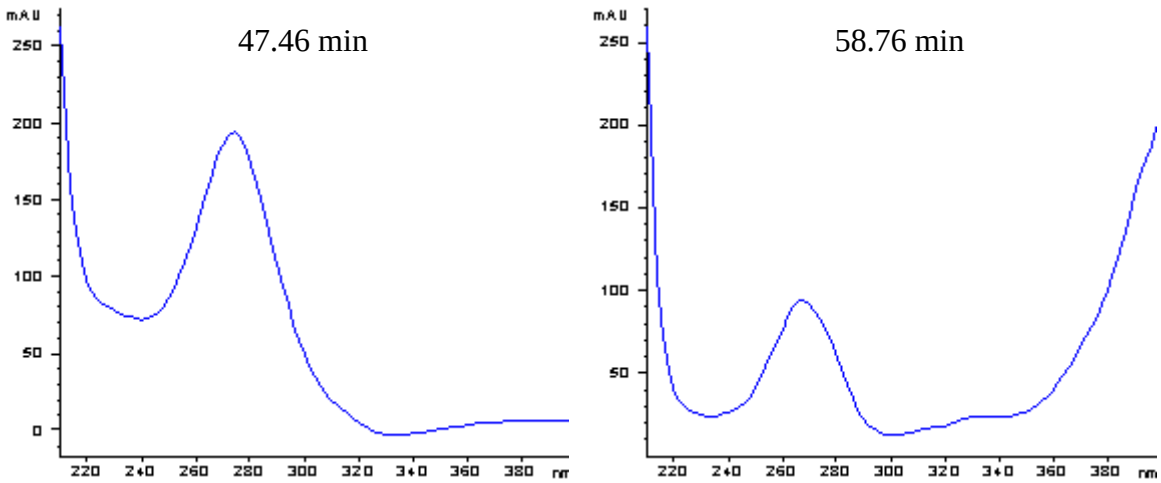


Figure 16: UV spectra of HPLC fractions of *H. perforatum* shoot (type B) extracts at retention times of 47 and 58 min. The maximal absorbance was at 272 nm.

5.2 MASS SPECTRA OF HYPERFORIN DERIVATIVES

Before performing mass spectrometry we assumed that the three HPLC fractions with specific UV spectra, similar to that of hyperforin, might actually be hyperforin derivatives. In previous studies four phloroglucinol derivatives were detected in methanolic extracts of *H. perforatum* type A shoots. Hyperforin derivatives were always eluted in the following chronological order, when analysed by HPLC: secohyperforin, adsecohyperforin, hyperforin, adhyperforin, which correlates with their molecular weight (3). According to this, we were able to assume which of the derivatives is present in our extracts.

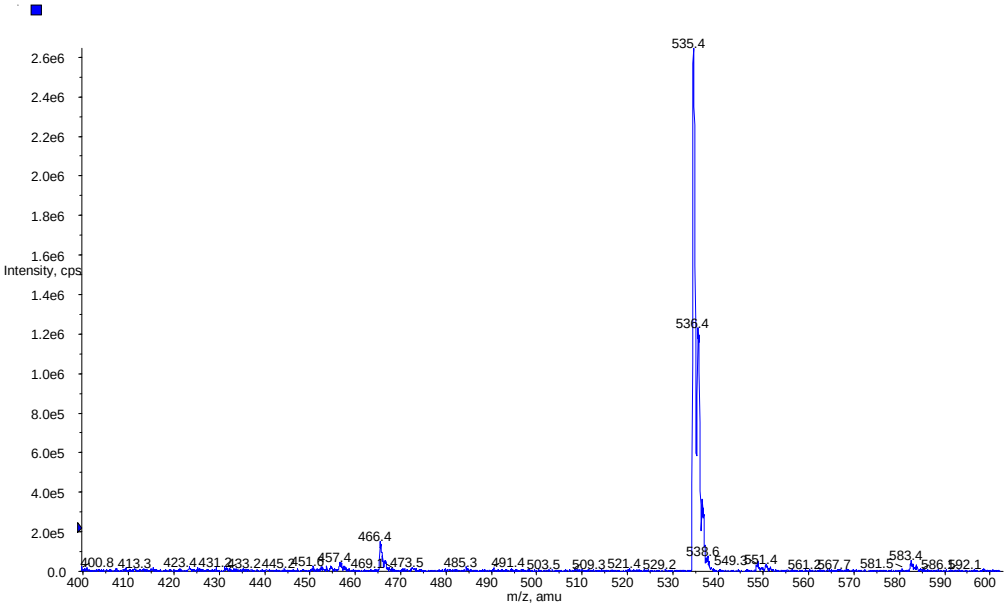


Figure 17: Ms spectrum of hyperforin reference substance.

The peak eluted at 52.9 min was identified as hyperforin by comparing the Ms spectrum of the reference substance (Fig. 17) to those of the three peaks (Fig. 18, 19). The product ions of the peak at 52.9 min were identical to those obtained with the reference substance. The peak eluted at 47.46 min had a molecular mass of 468.3 (Fig. 18), which corresponds to that of secohyperforin. However, we could not unambiguously confirm the presence of secohyperforin, as recently discovered hyperfirin, which also lacks a prenyl side chain, has the same molecular weight. Finally, by considering recent phytochemical studies on *H.*

perforatum type A shoots, we propose that the substance, which was eluted at 47.46 min, is most likely secohyperforin (3).

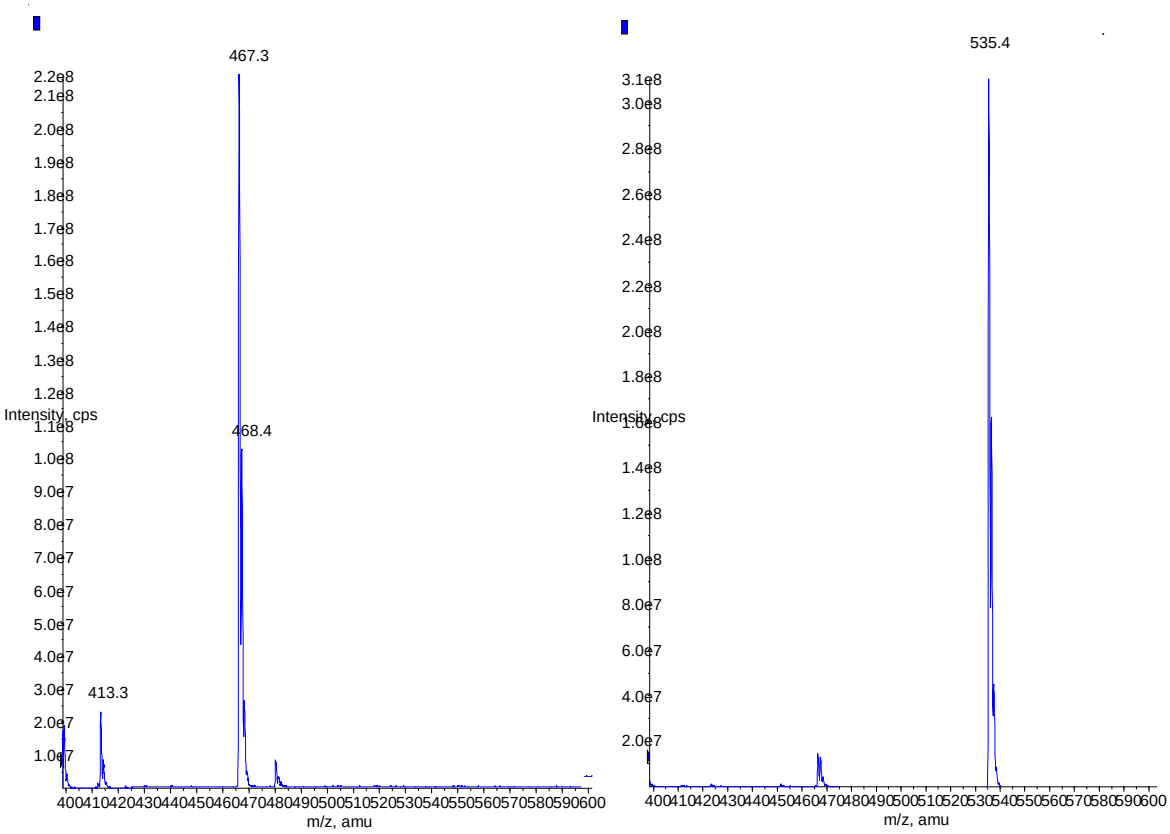


Figure 18: Ms spectra of HPLC fractions eluted at 47.46 min (left) and 52.9 min (right).

We expected that the last eluted derivative should be adhyperforin, based on previous findings about its decreased polarity and higher molecular weight (61). Thus, we focused on a specific peak with molecular weight of 550.81. According to the Ms spectrum of the peak eluted at 58.76 min, we assume that this fraction was actually adhyperforin (Fig. 19). Additionally, by taking in consideration the fact that the UV spectrum of this peak was nearly identical to that of hyperforin reference substance (Fig. 16), we identified it as adhyperforin.

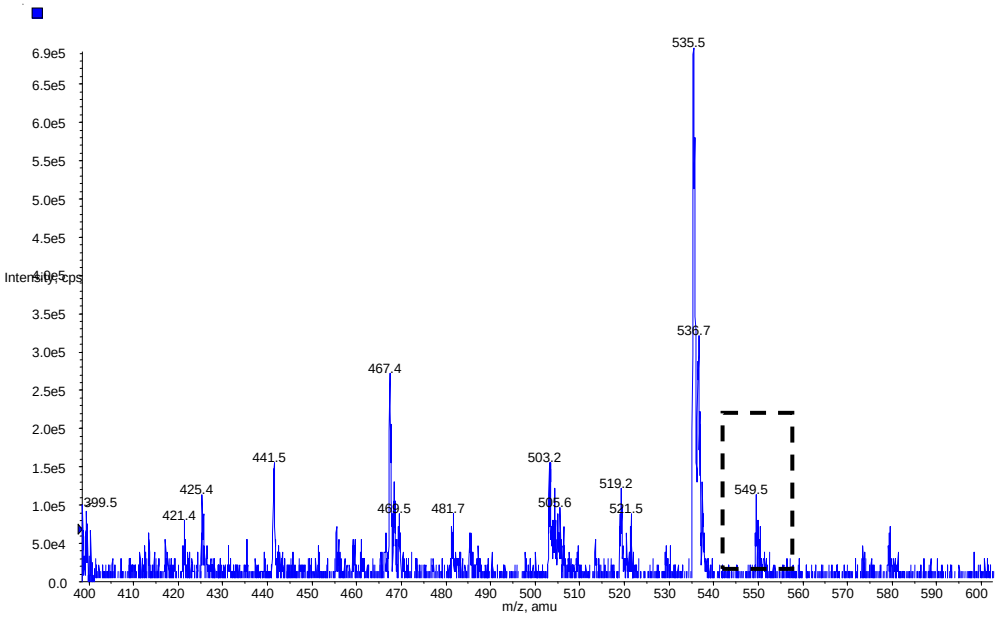


Figure 19: Ms spectrum of the fraction eluted at 58.76 min. Concentration of the fraction appears to be low, as witnessed by the weakness of the targeted peak.

5.3 EFFECTS OF MEDIA COMPOSITION ON METABOLIC ACTIVITY IN *H. PERFORATUM IN VITRO* CULTURES

Components of the growth media used for shoot cultures, such as hormones and vitamins, appear to have a significant influence on biosynthetic pathways of plant's secondary metabolites. Not only that the presence or absence of certain nutrients is important, but also the concentrations of these nutrients affect the accumulation of secondary metabolites, such as hyperforin. We found out that different media components influence the rate of hyperforin production and accumulation, as well as the morphological properties of plant tissue, for example the formation of translucent glands. A certain correlation between metabolic activity leading to the production of hyperforin and formation of translucent glands (TG) in the leaves of *H. perforatum* shoots was established following 2 and 3 weeks of cultivation on different modifications of the MS medium.

5.3.1 FORMATION OF TRANSLUCENT GLANDS

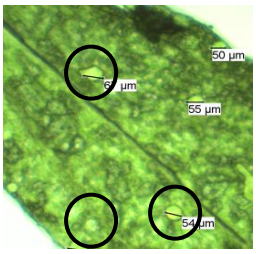
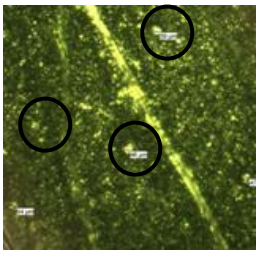
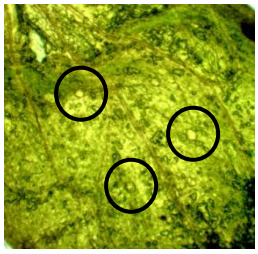
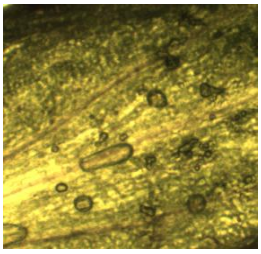
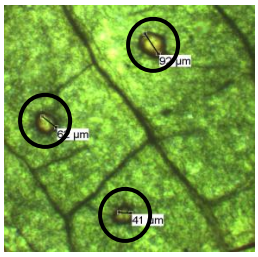
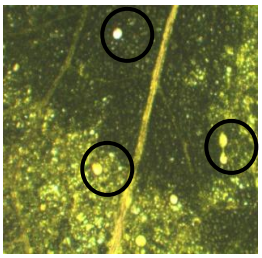
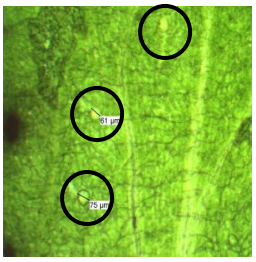
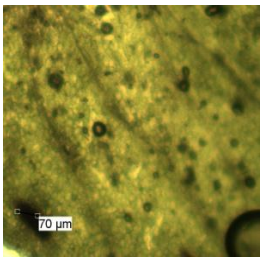
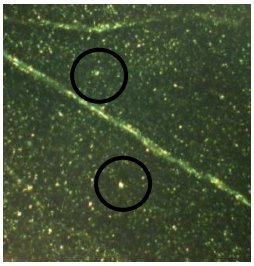
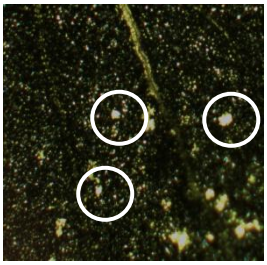
With the AxioStar microscope and a microscope-coupled camera we were able to monitor the development of translucent glands (TG) in the leaves of *H. perforatum* shoots, being cultivated on different modifications of the MS medium (Table III). The lowest optical magnification (4x zoom) was selected, in order to obtain a quality overview of the translucent gland frequency on each individual leaf. Due to various developmental stages of the leaves at the time of examination, deviations of obtained data have to be taken into account. Aside from different diameters and numbers of translucent glands, the examined leaves also exhibited major variations in their morphology and colour (5.3.1.1).

The presence and size of translucent glands, detected in the leaves of differently treated *H. perforatum* shoots, appear to be good indications of the growth media influence on metabolic activity in the shoots, especially the biosynthesis of hyperforin. We have shown that the appearance and the size of TG to some extent correlate with the amounts of hyperforin derivatives produced in the shoots cultivated on different modifications of the MS medium. After first two weeks of cultivation on the medium B (without plant hormones BAP and NAA), *H. perforatum* shoots type A have developed TG with diameters of approximately 55 μm, which is more than the average TG diameter of the wild type *H. perforatum*. Further cultivation on the hormone-free MS medium revealed that the TG formation remains stable, despite slight reduction of their diameters. HPLC analysis of these shoots has confirmed that a removal of plant hormones from the growth medium enhances the biosynthesis of hyperforin derivatives.

MSH (medium A) and MS- (medium B) were the most commonly used modifications of the Murashige and Skoog plant growth medium and their influence on the studied parameters appeared to be stable. Therefore, we prepared two types of *H. perforatum* starting shoots by using these two modifications and monitored their response to additional changes of media composition during cultivation.

5.3.1.1 Effects of different MS media (A - F) on translucent gland formation

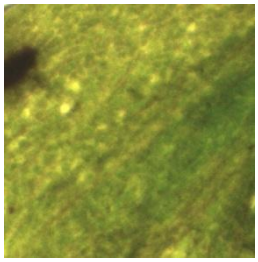
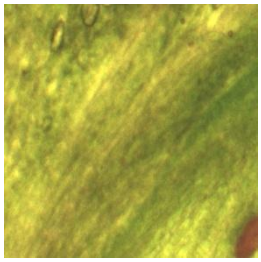
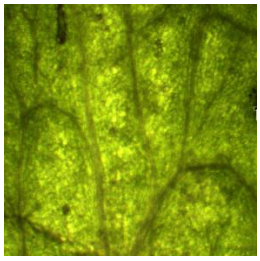
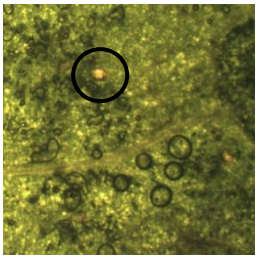
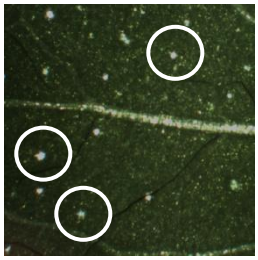
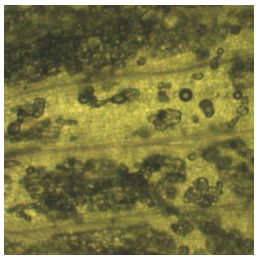
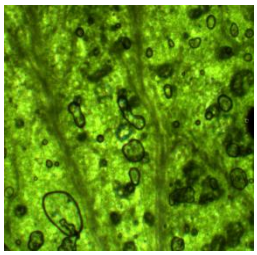
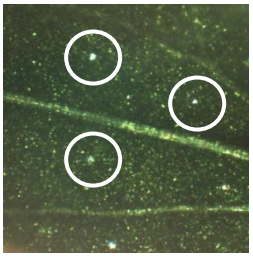
- Subcultivation of *H. perforatum* type A shoots; 4x zoom

MS medium modifications	TG after 14 days of cultivation	Mean TG diameter (µm)	TG after 21 days of cultivation	Mean TG diameter (µm)
B		55		40
C		62		/
D		60		52
E		58		/
F		36		35

According to the formation of TG in case of type A shoots cultivated for two weeks on the other two hormone-containing media C and E (lacking MI), we noticed that the removal of MI induces morphological changes of leaves. Nevertheless, this was not a sufficient trigger for induction of hyperforin production, as it was not detected in the shoots (5.3.2.3). The formation of TG in the leaves might have occurred due to the fact that MI facilitates auxin transport in the plant (56). Thus, after its removal, auxin transport might have been slightly decreased, which resulted in the initiation of tissue differentiation. After three weeks of cultivation on media C and E, the type A shoots exhibited no TG in their leaves, confirming that MI is not a limiting media constituent for the formation of TG and therefore for the accumulation of hyperforin.

Further microscopic examination of the leaves showed significant morphological differences between the shoots cultivated either on hormone-free or hormone-containing media. The leaves of shoots cultivated on the hormone-free media exhibited higher tissue differentiation than those of shoots cultivated on growth media containing plant hormones. Microscopic analysis showed more developed vascular and glandular tissue in case of shoots that were cultivated on the media B, D and F (lacking plant hormones). At this point of investigation we were still not able to estimate the actual influence of vitamin amounts added to the media on metabolic activity in the shoots.

Subcultivation of *H. perforatum* type B shoots; 4x zoom

MS medium modifications	TG after 14 days of cultivation	Mean TG diameter (µm)	TG after 21 days of cultivation	Mean TG diameter (µm)
A		/		/
C		48		/
D		40		42
E		/		/
F		30		32

Probably even more obvious influence of plant hormones on development of TG was detected in these shoots, which contained hyperforin and therefore already had TG present in the leaves (type B shoots). Microscopic observations showed that the transfer of shoots with fully developed TG onto the media containing plant hormones (media A, C and E), negatively affects the formation of TG and the tissue differentiation of leaves in general. Already after 2 weeks of cultivation, macroscopic appearance of the newly developed leaves significantly changed. They were narrower and thinner. TG and vascular tissue of the leaves disappeared, with exception of only few weakly expressed dark glands that were still visible. Even though some underdeveloped TG were present on leaf surfaces of the shoots cultivated for two weeks on the medium C, one additional week of cultivation led to a complete loss of TG. The type B shoots cultivated on media lacking plant hormones (D and F) maintained nearly the same morphologic appearance regarding their vascular and glandular tissue, throughout 21 days of cultivation. The medium F, which differed from the medium D only in the presence of MI, exhibited a stronger influence on TG formation in the shoots. This clearly indicates the importance of MI for metabolic activity in the *in vitro* shoots.

5.3.2 ACCUMULATION OF HYPERFORIN AND ITS DERIVATIVES IN *H. PERFORATUM* SHOOTS

We determined hyperforin concentrations in *H. perforatum* type A and B shoots, cultivated on five different modifications of the MS medium, differing in the presence of plant hormones (NAA and BAP), MI and the concentration of B vitamins (nicotinic acid and pyridoxine). We compared these results to the course of translucent gland development in the leaves of such differently cultivated shoots.

5.3.2.1 *H. perforatum in vitro* shoots type A (hyperforin-free)

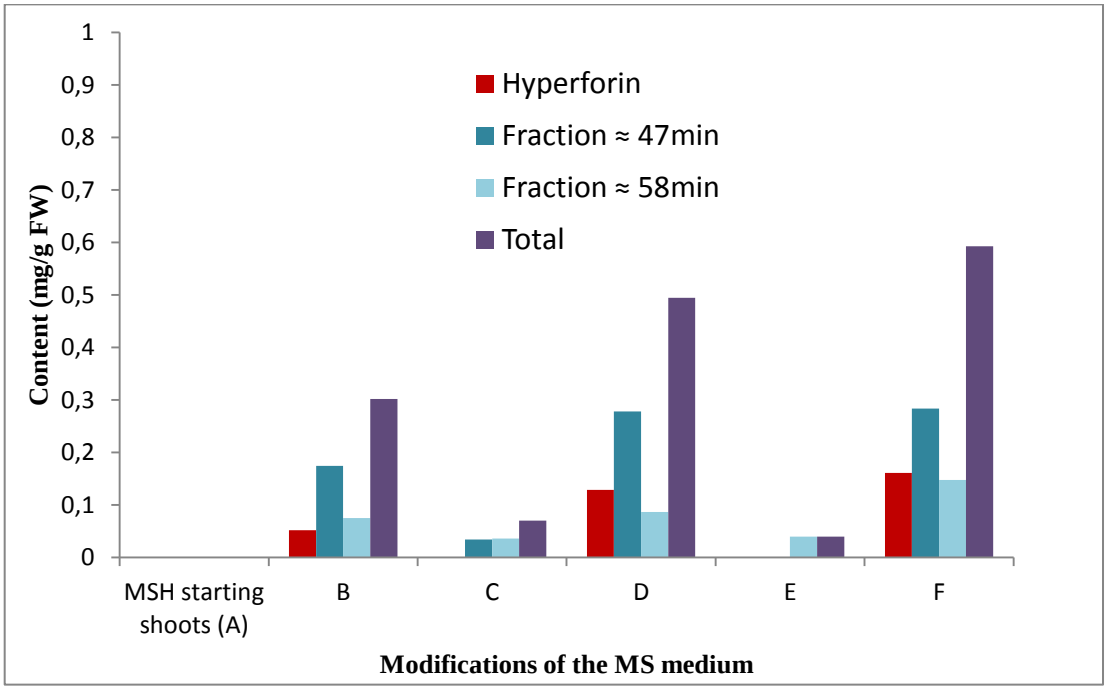


Figure 20: Contents of hyperforin and its two derivatives (secohyperforin and adhyperforin calculated as hyperforin) in *H. perforatum* type A shoots following two weeks of cultivation on 5 different modifications of the MS medium.

H. perforatum type A shoots were transferred from one hormone-containing medium onto two other hormone-containing media (C and E), which differed in the absence of MI and in lower concentration of B vitamins (medium C only). None of these two media induced accumulation of hyperforin in the shoots, though low concentrations of secohyperforin and adhyperforin were detected in the shoots cultivated on media C and E. On the other hand, all three hormone-free MS media (B, D and F) appeared to be excellent stimulators of hyperforin derivatives accumulation in the shoots. Surprisingly, all three hormone-free media induced the production of secohyperforin in greater extent than of hyperforin. The explanation for such response could be a strong stimulation of tissue differentiation after removal of plant hormones and the production of all required factors, resulting in smaller amounts of substrates (GPP) of the specific prenyltransferase, which catalyses hyperforin synthesis. This explanation is based on previously established fact, that the two hyperforin derivatives have a branching point in the part of the biosynthesis where third prenyl chain is attached to the phloroglucinol moiety (3).

More investigations are needed to precisely evaluate the mechanisms of action of these, probably more than two different, enzymes. It also remains to be discovered whether or not adhyperforin is produced by the same mechanism as hyperforin.

Interestingly, the shoots being cultivated on media D and F, both containing lower vitamin concentrations, both produced comparable amounts of hyperforin derivatives, which were higher than in case of the use of medium B with higher vitamin concentrations. Moreover, the amount of total hyperforin derivatives produced in shoots cultivated on the medium F was almost twice as high as in the case of shoots that were transferred onto the medium B. This indicates that lower vitamin concentrations present in growth media have more enhancing influences on accumulation of hyperforin derivatives.

5.3.2.2 *H. perforatum in vitro* shoots type B (hyperforin-containing)

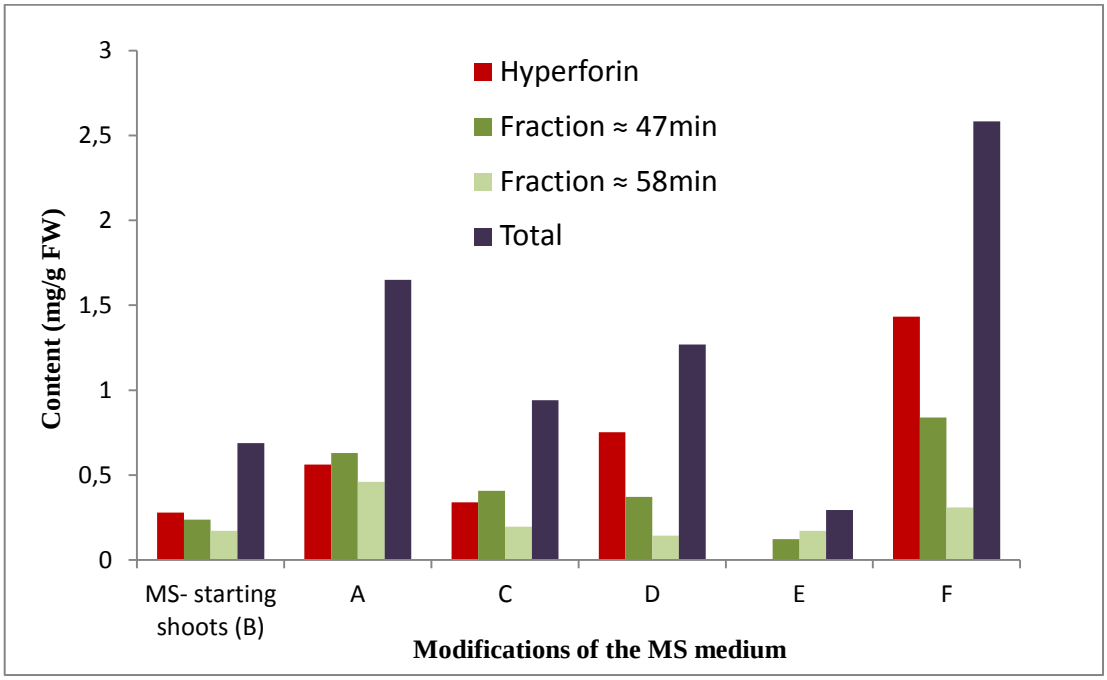


Figure 21: Contents of hyperforin and its two derivatives (secohyperforin and adhyperforin calculated as hyperforin) in *H. perforatum* type B shoots following two weeks of cultivation on 5 different modifications of the MS medium.

Previous results have shown that the removal of plant hormones from growth media most significantly influences metabolic activity in *H. perforatum* shoots, regarding the biosynthesis of hyperforin derivatives. Their quantification in the shoots that were transferred from one hormone-free MS medium modification (medium B) onto other two hormone-free media (D and F), revealed the importance of MI and vitamins B (nicotinic

acid and pyridoxine). The graph in fig. 21 represents the influence of the F medium on the accumulation of hyperforin derivatives, which is stronger comparing to that achieved by the case of the other hormone-free media (D). The amount of hyperforin derivatives in type B shoots cultivated on the medium D decreased after an additional week of cultivation (Fig. 22 and 23). The amount of total hyperforin derivatives in the type B shoots cultivated on the medium F, however, increased from 2.6 mg/g FW to 3.8 mg/g FW in one week. Both D and F media contain lower vitamin concentrations, while medium D is deprived of MI. Therefore, the influence of the presence of MI in the growth medium on the increasing accumulation of hyperforin derivatives is quite evident.

From the amounts of hyperforin derivatives detected in shoots, which were transferred from the medium B onto the medium F, we can, again, observe the importance of vitamin concentration, as this was the only difference between these two media. Only the reduction of vitamin concentration caused total hyperforin to rise from 0.7 mg/g FW to 2.6 mg/g FW in two weeks of cultivation. This finding confirms that hormone-free media with lower vitamin concentrations (0.1 mg/l nicotinic acid and 0.1 mg/l pyridoxine) are favourable to accumulation of hyperforin derivatives in the shoots.

Surprisingly, among hormone-containing media (A, C and E), the highest amounts of hyperforin derivatives were detected in type B shoots cultivated for two weeks on the medium A. This contained higher vitamin concentrations and was the only one among hormone-containing media to cause such stimulus in type B shoots. The explanation for this kind of response might be the positive effect of higher vitamin concentrations in combination with the two hormones used (NAA and BAP), which were previously found to have stimulatory effects on hyperforin biosynthesis (3). An interesting further investigation would be the analysis of hyperforin-free shoots, transferred onto the medium A. To create this kind of *H. perforatum* shoots we would need to cultivate them on the medium E, for example.

In the following charts we compared the accumulation of hyperforin derivatives after two and three weeks of cultivation on different modifications of the MS medium in order to determine whether or not the applied media exhibit stable influences on hyperforin production. The results are presented as hyperforin content in comparison to total amount of hyperforin derivatives (calculated as hyperforin) in *H. perforatum* type A and B shoots.

For easier understanding of the charts, it should be taken into account that the first two columns represent the results of further cultivation of type A shoots on medium A and type B shoots on medium B, thereby presenting the starting amounts of hyperforins.

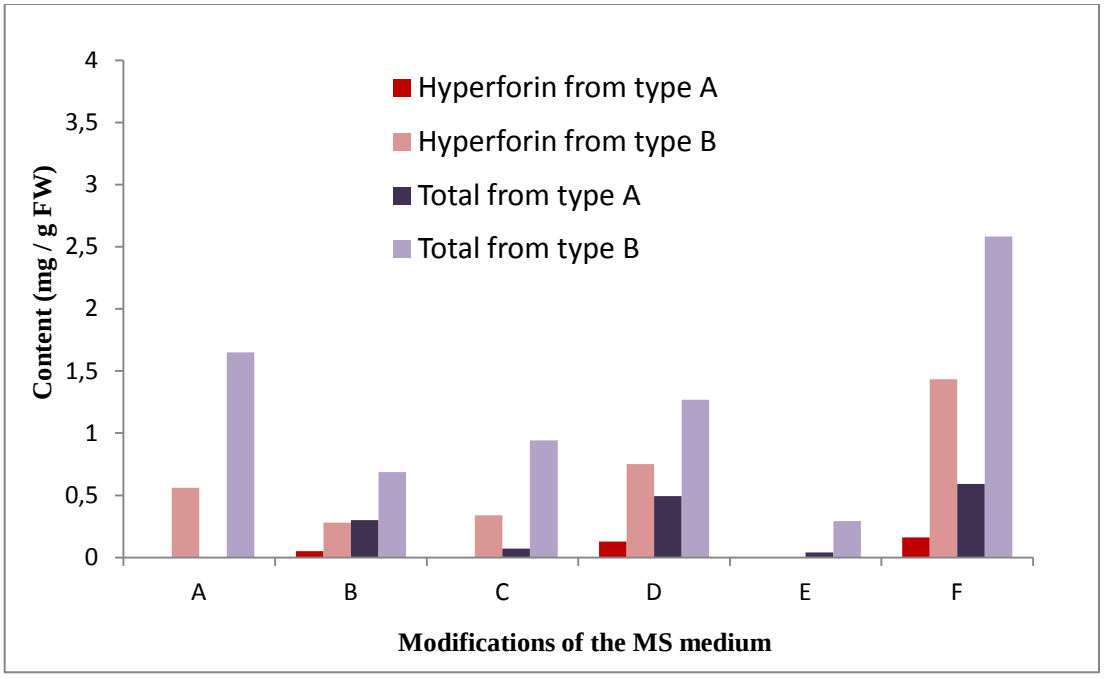


Figure 22: Comparison of hyperforin contents and total contents of hyperforin derivatives (calculated as hyperforin) in *H. perforatum* type A and B shoots following two weeks of cultivation on five different modifications of the MS medium.

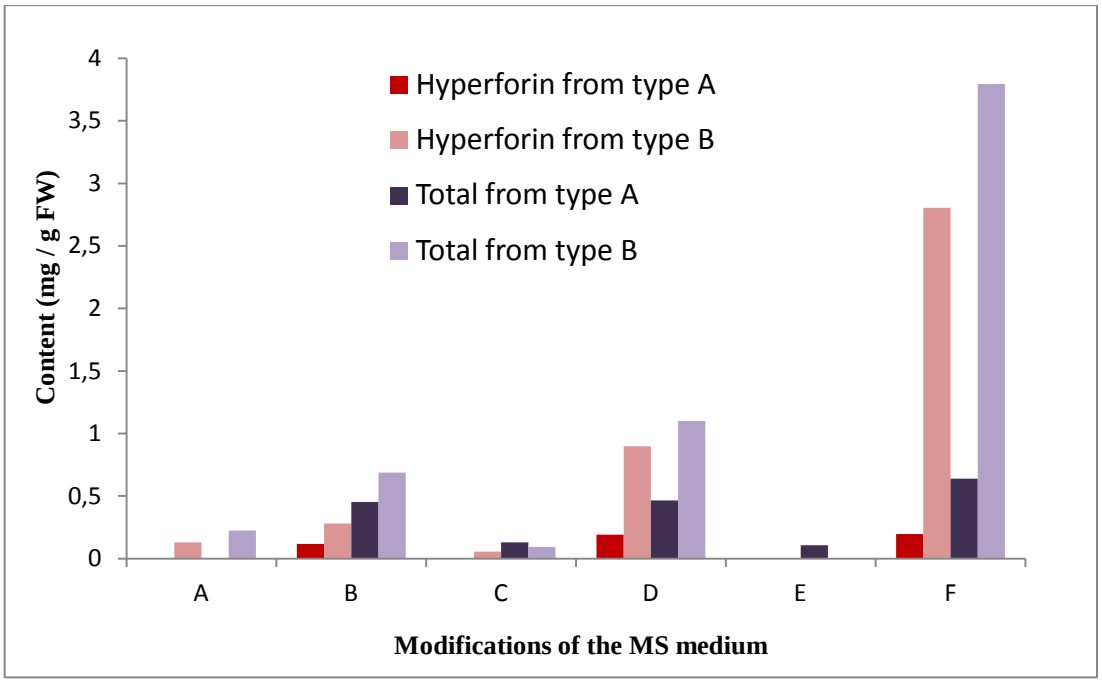


Figure 23: Comparison of hyperforin contents and total contents of hyperforin derivatives (calculated as hyperforin) in *H. perforatum* type A and B shoots following three weeks of cultivation on five different modifications of the MS medium.

5.3.2.3 Correlation of translucent gland formation and hyperforin production in *H. perforatum in vitro* shoots

The quantification of hyperforin derivatives by HPLC analysis revealed a certain correlation with the formation of translucent glands in the leaves of shoots cultivated on different MS medium modifications.

In case of both shoot types, A and B, their cultivation on hormone free media (B, D and F) resulted in a stable TG formation, as well as in higher accumulation of hyperforin derivatives. Although significant differences in hyperforin concentration present in shoots cultivated on different media were detected, we did not observe direct correlation with the formation of TG in the leaves.

H. perforatum type A *in vitro* shoots (hyperforin-free)

In case of the starting shoots without TG (shoots type A), their formation in leaves was induced by all of the hormone-free media used (B, D and F). This phenomenon also correlated with the detected hyperforin concentration in the shoots. Following 14 days of cultivation, the size of newly developed TG was 55 - 62 μm, which is more than in case of *H. perforatum* wild plant. After one additional week of cultivation, their diameter slightly

decreased, though the concentration of hyperforin did not significantly change (Fig. 22, 23). Interestingly, in case of the medium F, which was one of the best used, regarding the accumulation of hyperforin, we observed the smallest average TG diameters (30 - 36 μm). This might be due to the stable development and growing number of glands per leaf. Namely, wild *H. perforatum* plants also have similarly sized TG. The effect of the MI-absence in hormone-containing media (C and E) on the TG formation and hyperforin production was not comparable. Lack of MI in the growth medium appears to have an inducing effect on the formation of TG, although this influence does not remain constant (5.3.1.1). After the plant had regained the omitted MI, this very weak induction of tissue differentiation was probably limited by the inhibitory effect of NAA, which resulted in suppressed TG formation and unsuccessful accumulation of hyperforin in these shoots.

H. perforatum type B *in vitro* shoots (hyperforin-containing)

In case of type B shoots, where TG were already developed, the hormone free media, D and F, both maintained their stable formation, but hyperforin concentrations were significantly different: the cultivation on the medium F resulted in its higher and increasing concentrations, in contrast to the medium D (Fig. 22 and 23). As in the case of type A shoots, the cultivation of type B shoots on the medium F resulted in the smallest TG diameters and the highest hyperforin concentrations. The appearance of leaves of both shoot types cultivated on the medium F suggests that the number of TG per leaf might be higher due to their smaller diameters, which could be connected to the higher activity of hyperforin-related prenyltransferases in the TG delimiting cells. This could explain higher concentrations of hyperforin in the shoots that were cultivated on the medium F.

As discussed in 5.3.1.1, the hormone-containing media (A, C and E) caused the suppression of TG formation in the shoots. The most interesting exception was the cultivation of type B shoots on the medium A, where the concentration of hyperforin increased, although TG completely disappeared during 14 days of cultivation. Altogether, these findings confirm that this specific combination of auxins and cytokinins induces the enzymes participating in hyperforin biosynthesis, as has been proposed previously (3). However, it has to be pointed out that the negative effect on TG formation, which was probably due to both applied phytohormones, limited the accumulation of hyperforin.

5.3.2.4 Selection of the optimal growth media

According to the amount of hyperforin derivatives produced in *H. perforatum* shoots cultivated on growth media containing different nutrient combinations, we were able to select the optimal growth media composition for the induction of hyperforin biosynthesis. Regarding the three groups of components added to the common MS growth medium, i.e. phytohormones, vitamins and *myo*-inositol, we succeeded to prepare two MS medium modifications which were suitable for the optimal hyperforin production. Both exhibited equally relevant effects. We selected the two following combinations of nutrients, based mostly on the results from subcultivation of type B shoots, as they produced higher amounts of hyperforin derivatives and exhibited more significant differences between the media.

1.) Medium A
- Plant hormones: 0.05 mg/l of NAA; 1 mg/ml of BAP
- *Myo*-inositol: 100 mg/l
- 0.1 mg/l of nicotinic acid; 0.1 mg/l of pyridoxine HCl; 0.1 mg/l of thiamine HCl

2.) Medium F
- Plant hormones absent
- *Myo*-inositol: 100 mg/l
- 0.1 mg/l of nicotinic acid; 0.1 mg/l of pyridoxine HCl; 0.1 mg/l of thiamine HCl

Medium A has proven to be a good inducer for production of secondary metabolites in *H. perforatum*, due to the detected comparable increase of all hyperforin derivatives. Based on the results obtained following two weeks of cultivation, it has been recognised as one of the most favourable media for cultivation of the shoots that had been previously cultivated on hormone-free medium (type B) (Fig. 21). After one additional week of cultivation the amount of hyperforin derivatives was considerably lower, which motivated us to further investigate this MS medium modification.

Medium F exhibited positive effects mainly on hyperforin production. Fig. 20 and 21 show that the cultivation of shoots on the medium F resulted in the highest amounts of total phloroglucinols following two weeks of cultivation, regardless of the type of starting shoots. Moreover, this inducing effect remained stable throughout further cultivation periods, as the amounts of hyperforin in the starting type B shoots increased following one additional week of cultivation (Fig. 22, 23). Therefore, we focused on studying *H. perforatum* type B shoots in the following experiments.

5.3.3 KINETICS OF HYPERFORIN-DERIVATIVES ACCUMULATION

As already stated, after 2 weeks of *H. perforatum in vitro* shoot cultivation on different modifications of the MS medium, we identified the A and F media to be optimal for the production of phloroglucinol derivatives (5.3.2.4). Considering similar amounts of all three hyperforin derivatives in type B shoots transferred onto the medium A (Fig. 21), we supposed that the later has an equal influence on their biosynthesis. On the other hand, the medium F was mainly optimal for the biosynthesis of hyperforin. Based on our results, we chose both media to monitor the time response of phloroglucinol derivatives production within 21 days of cultivation. Although the amounts of hyperforin and its derivatives significantly changed following 3 weeks of cultivation (Fig. 23) and the D medium showed the second most favourable influence on their production, we finally decided to observe the effects of the medium A, because it appeared to be the best inductor of the rapid increase of all three hyperforin derivatives in *H. perforatum* shoots.

Fig. 22 and 23 show quantitative comparison of hyperforin derivatives produced in both types of *H. perforatum* starting shoots (A and B). The type B shoots represent better starting material, considering the amount of hyperforin derivatives detected per 1 g of their fresh weight. As the increased accumulation of hyperforin was our goal, we decided to observe the time response of hyperforin derivatives production in the shoots that have already contained hyperforin, i.e. type B shoots, on media A and F.

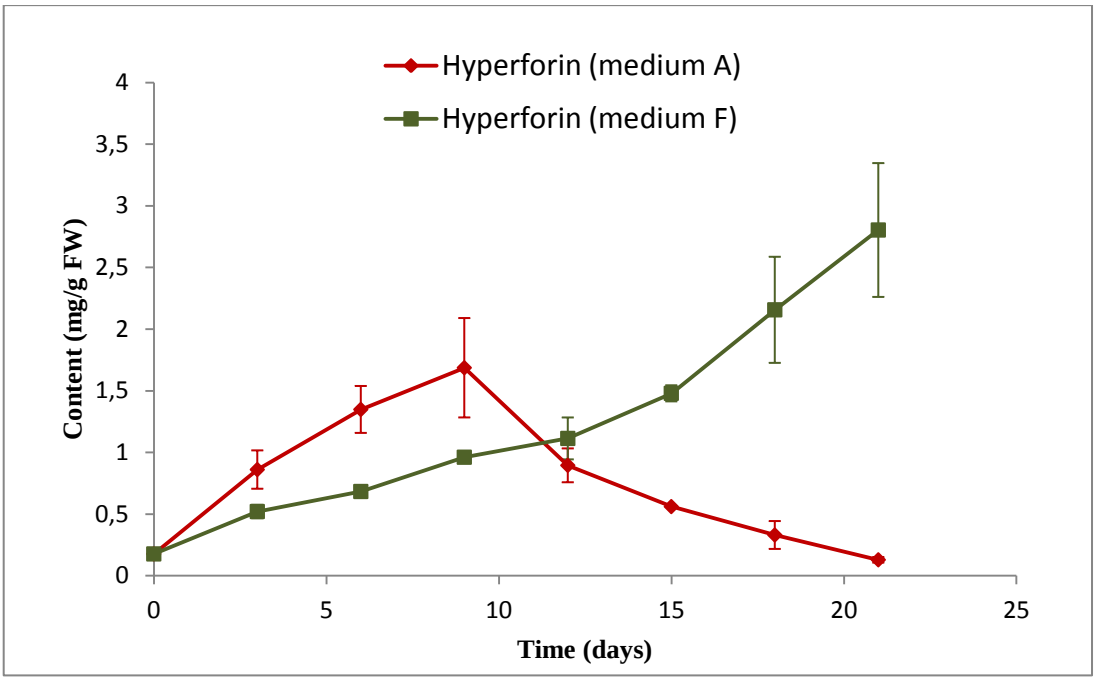


Figure 24: Accumulation of hyperforin in *H. perforatum* type B shoots during 21 days of cultivation on A and F modifications of the MS medium. The results represent mean values of two parallel measurements plus standard deviations. Intervals of sampling: 0, 3, 6, 9, 12, 15, 18 and 21 days.

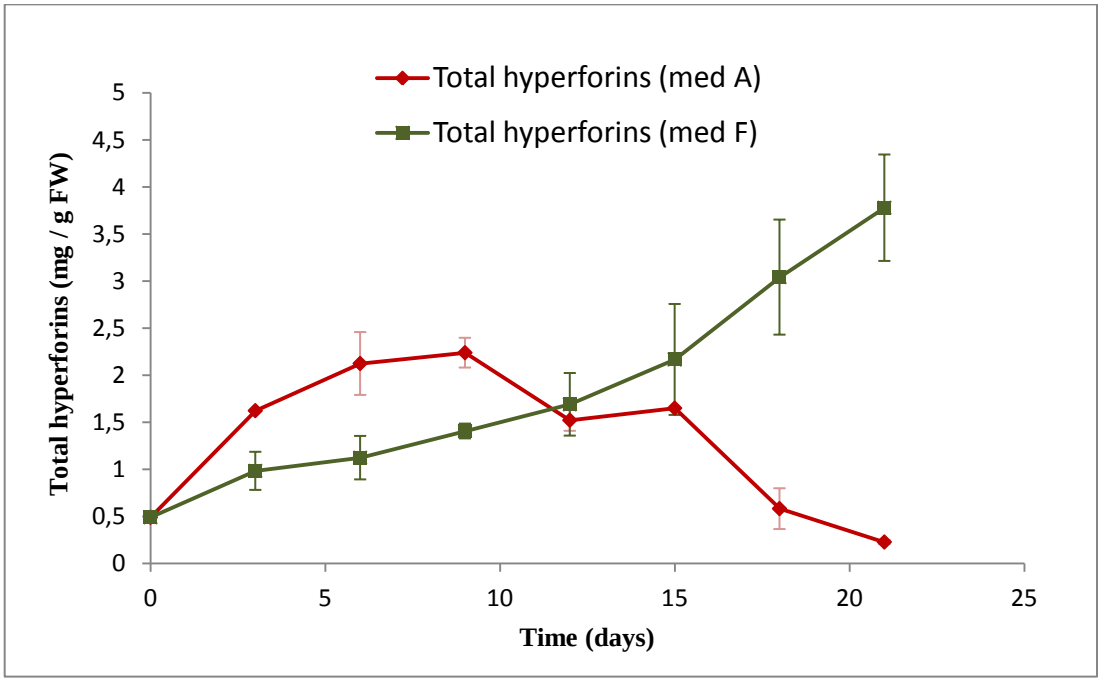


Figure 25: Accumulation of all hyperforin derivatives (calculated as hyperforin) in *H. perforatum* type B shoots during 21 days of cultivation on A and F modifications of the MS medium. The results represent mean values of two parallel measurements plus standard deviations. Intervals of sampling: 0, 3, 6, 9, 12, 15, 18 and 21 days.

Previous results suggested that the medium A equally effects the accumulation of all hyperforin derivatives, but the monitoring of their concentrations in type B shoots during 21 days of cultivation, led to different conclusions. We compared the accumulation of total hyperforin derivatives (calculated as hyperforin) to that of only hyperforin and discovered structural resemblance of time curves during this cultivation period. We assumed that the media composition mostly effects hyperforin production (Fig. 25, 26). After nine days of cultivation on the medium A, only hyperforin content reaches the maximal concentration, while the concentrations of its other two derivatives remain relatively constant (Fig. 26). Similar influence on hyperforin accumulation could also be observed in case of the medium F (Fig. 27).

5.3.3.1 Cultivation of *H. perforatum* shoots type B on medium A

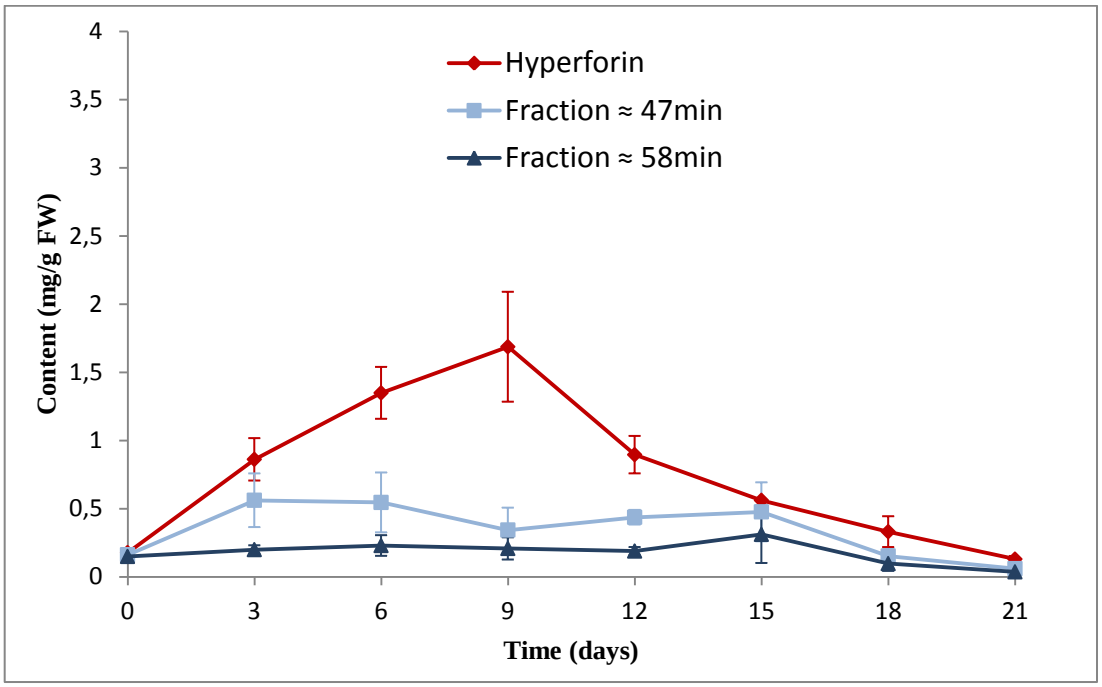


Figure 26: Accumulation of hyperforin and its derivatives (calculated as hyperforin) in *H. perforatum* type B shoots during 21 days of cultivation on the medium A. The results represent mean values of two independent experiments plus standard deviations. Intervals of sampling: 0, 3, 6, 9, 12, 15, 18 and 21 days.

Results presented in fig. 24 confirmed our assumption regarding rapid induction of hyperforin biosynthesis in type B shoots cultivated on the medium A. Namely; two independent experiments have shown that the amount of hyperforin in the shoots reaches its maximum after 9 days of cultivation in these conditions. The decrease rate following its peak appears to be similar as the increase rate. This explains the low concentrations of hyperforin derivatives, which have been detected in the shoots on the medium A after three weeks of cultivation (Fig. 23). Our hypothesis, about equal influences on the production of all three hyperforin derivatives, was thereby declined (Fig. 26). Although the observed amounts of hyperforin in *H. perforatum* type B shoots significantly decreased following three weeks of cultivation, a strong and sudden induction of hyperforin biosynthesis in the shoots is of great importance for further molecular biological studies. Creation of a stable system for rapid enzyme induction in the plant material that shows no activity before treatment could represent a basic concept for setting up a subtractive cDNA library.

5.3.3.2 Cultivation of *H. perforatum* shoots type B on medium F

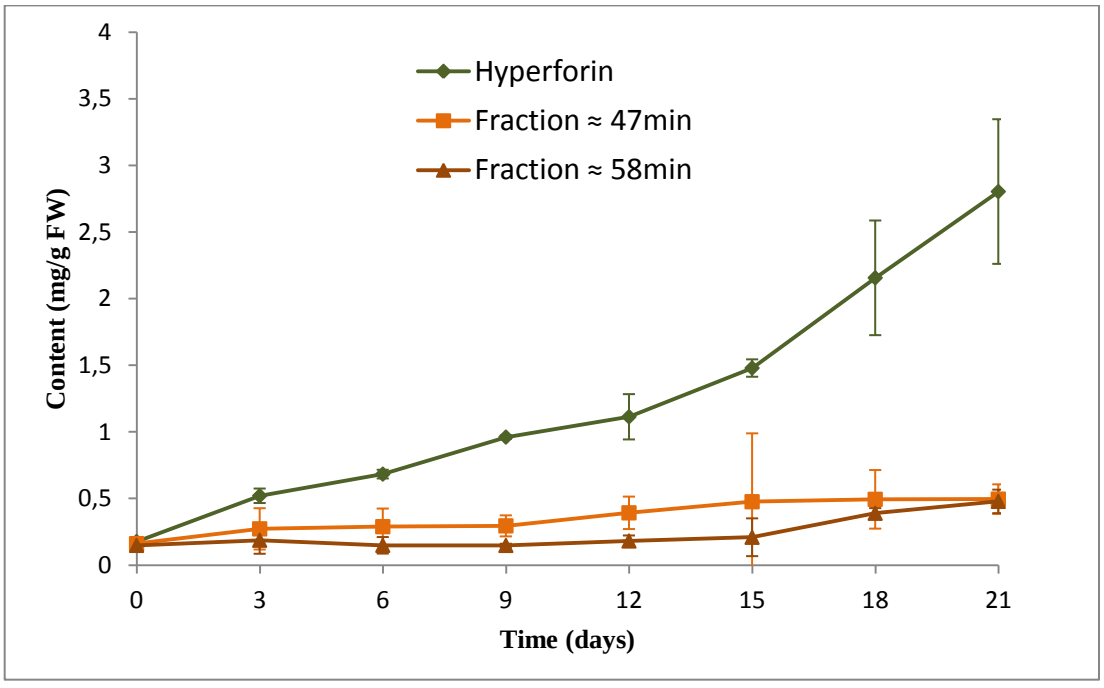


Figure 27: Accumulation of hyperforin and its derivatives (calculated as hyperforin) in *H. perforatum* type B shoots during 21 days of cultivation on the medium F. The results represent mean values of two independent experiments plus standard deviations. Intervals of sampling: 0, 3, 6, 9, 12, 15, 18 and 21 days.

The influence of combination of supplements added to the medium F was found to be accountable for a stable and increasing production of hyperforin, while the amount of its other two related fractions remained relatively constant during 21 days of cultivation. However, the concentration of secohyperforin (fraction eluted at 47 minutes) was slightly lower than that in case of the cultivation on the medium A, although, its increase during further cultivation can not be excluded. The amount of hyperforin in type B shoots, cultivated on the medium F reached the concentration of 0.96 mg/g of their fresh weight at the time when its amount in the shoots on the medium A was at its peak (1.7 mg/g FW). Thus, the medium F can not be considered as an elicitating medium, but rather as an excellent medium for the stable accumulation of hyperforin. The medium F is a considerable choice for scaling up the *H. perforatum in vitro* cultivation in bioreactors. Considering the fact that *H. perforatum* shoots have already been successfully cultivated in bioreactors containing liquid media, the integration of optimal growth media conditions in this process might be of a great profit (2).

Considering the macroscopic and microscopic observations of differently cultivated *H. perforatum* shoots and their correlation with the determined hyperforin production patterns throughout the entire experiment, we were able to form some interesting speculations. It has been established that auxins modulate tissue differentiation in plants and lead to a less differentiated callus-like type of growth, when applied to growing plants. Based on these findings, we can assume that the disappearance of translucent glands in the shoots cultivated on hormone-containing MS media (A, C, E) was due to the effects of NAA. Thereby, we suppose that the rapid decrease of hyperforin concentration in shoots following 9 days of cultivation the medium A is due to improper TG development, which leads to limited accumulation of hyperforin.

The results of studies, which investigated BAP influence on the growth of *H. perforatum* tissue cultures, revealed that this plant hormone also induces morphological changes and results specifically in formation of many auxiliary shoots. This kind of morphological response may also limit TG development (62).

According to the influences of the evaluated media A and F, which is primarily on the biosynthesis of hyperforin in *H. perforatum* shoots, we assume that specific enzymes were activated and their certain substrates were present in such shoots. As it has been previously shown, secohyperforin is not only a by-product of the hyperforin biosynthesis. Namely, the

proposed branching point in the biosynthetic pathway leads to separate formation of the two constituents. Secohyperforin is a product of prenylation with DMAPP as a prenyl donor and hyperforin is formed by prenylation with GPP as a prenyl donor in the third prenylation step of the proposed biosynthetic pathway (Fig. 4). This might be due to different aromatic prenyltransferases involved, with substrate specificities for either DMAPP or GPP (3). Considering our results regarding the induction of metabolic activity in *H. perforatum* shoots caused by different media constituents, we can conclude that our experimental system appears to predominantly induce hyperforin-related prenyltransferase, among other enzymes, which are participating in the earlier stages of hyperforin biosynthesis. The explanation for small amounts of other hyperforin-related HPLC fractions in the shoots, where no hyperforin was actually detected, could be the lack of substrate (GPP) needed for the hyperforin-related prenyltransferase at the time of analysis and/or loss of hyperforin during the extraction process due to its relative chemical instability.

5.3.4 ENZYME ACTIVITY IN *H. PERFORATUM* CELL CULTURES

As stated, the growth media composition appears to have a great influence on the production of secondary metabolites in *H. perforatum in vitro* shoot cultures. To estimate which part of the biosynthesis pathway has been affected by changes in the growth media, we tested the enzyme activity in *H. perforatum* cell cultures, derived from type A shoots. Lack of cellular organisation in cell cultures represents a limiting factor for biosynthesis of secondary metabolites, in spite the fact that the activity of hyperforin-related enzymes can be detected in cells cultivated in liquid media A and F. We applied media A and F as inducing media for *H. perforatum* cell cultures, which have been previously cultivated in liquid hormone-free MS medium (the medium B). As it has previously been documented that enzymes in cell cultures rapidly respond to inducing media, our first time point of enzyme activity determination was after 12 hours of cultivation in both types of media (A and F). In order to evaluate the activity of three different type III polyketide synthases (BUS, BPS, CHS), we used different starting substrates, as described in 4.3.3. Unfortunately, no CHS activity could be detected, but in case of BUS and BPS, we determined a relatively strong enzyme activity together with a similar substrate affinity between the two enzymes.

Recent discoveries have shown that benzophenone synthase (BPS), which has been known for its benzoyl-CoA substrate specificity, also converts one molecule of isobutyril-CoA and three molecules of malonyl-CoA into a phloroglucinol moiety (*Ines Bel Hadj, unpublished results*). Based on these facts, we were only able to assume the impact of the contribution of BUS activity to hyperforin biosynthesis.

5.3.4.1 Activity of type III polyketide synthases in *H. perforatum* cell cultures cultivated in liquid media A and F

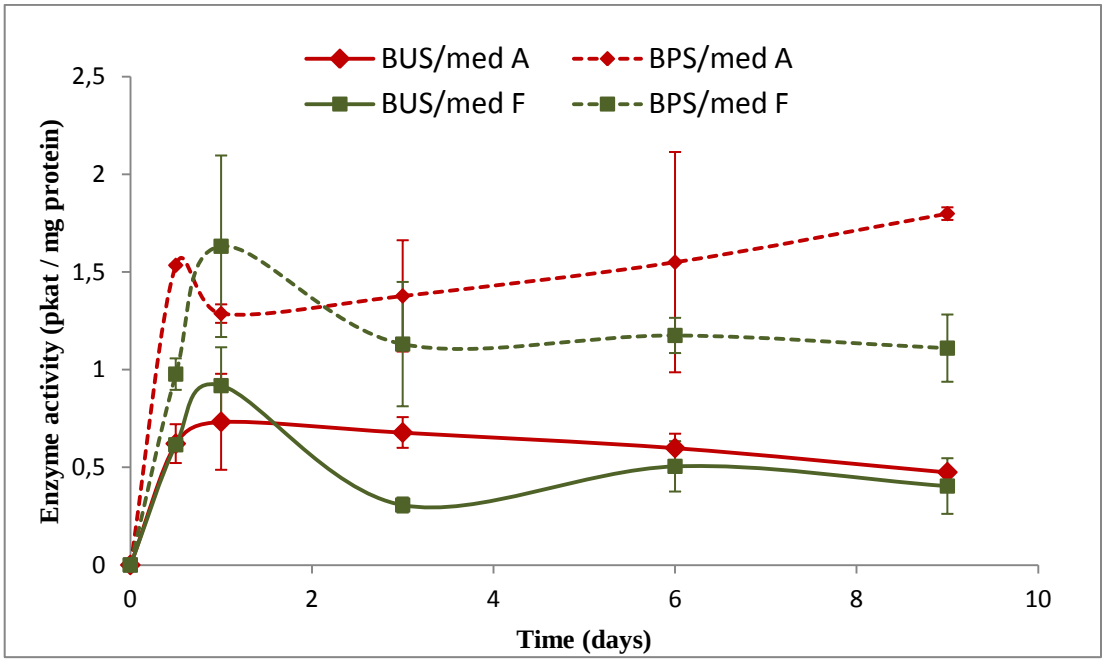


Figure 28: Enzyme activity of isobutyrophenone synthase (BUS) and benzophenone synthase in *H. perforatum* cell cultures during 9 days of cultivation in liquid A and F modifications of the MS media. Continuous lines represent the BUS and dashed lines represent the BPS activity. The results represent mean values of two independent experiments plus standard deviations. Intervals of sampling: 12h, 1 day, 3, 6 and 9 days.

Fig. 28 represents time-responses of BUS and BPS activities in *H. perforatum* cell cultivated in liquid media A and F. It reveals that both media have positive effects on metabolic activity in the cells, as the activity of both enzymes was not detected before the treatment (*Ines Bel Hadj; unpublished results*). The difference in the enzyme activity between both applied media is not significant, therefore, these results only confirm that both formulations might enhance or even initiate the biosynthesis of hyperforin by activating either or both of the enzymes.

5.3.4.2 Correlation between the hyperforin production in *H. perforatum* shoots and the enzyme activity in *H. perforatum* cell cultures

Due to a small quantity of the *H. perforatum in vitro* shoots available, we were not able to prepare crude protein extract from them, to test the activity of enzymes, participating in the biosynthesis of hyperforin. In order to confirm that media A and F induce these enzymes, when applied to plant material, we tested the responses of *H. perforatum* cell cultures cultivated in both media. The cells were derived from type A shoots and were first cultivated in a liquid hormone-free MS medium (the medium B).

Due to differences in tissue differentiation found between both plant materials studied, a direct comparison of metabolic activity in shoots and cell cultures of *H. perforatum* is not the most reliable system. But nevertheless, it gave us a possibility to confirm our findings regarding the effects of selected media on the hyperforin biosynthesis, assessed in the experiments with *in vitro* shoot cultures.

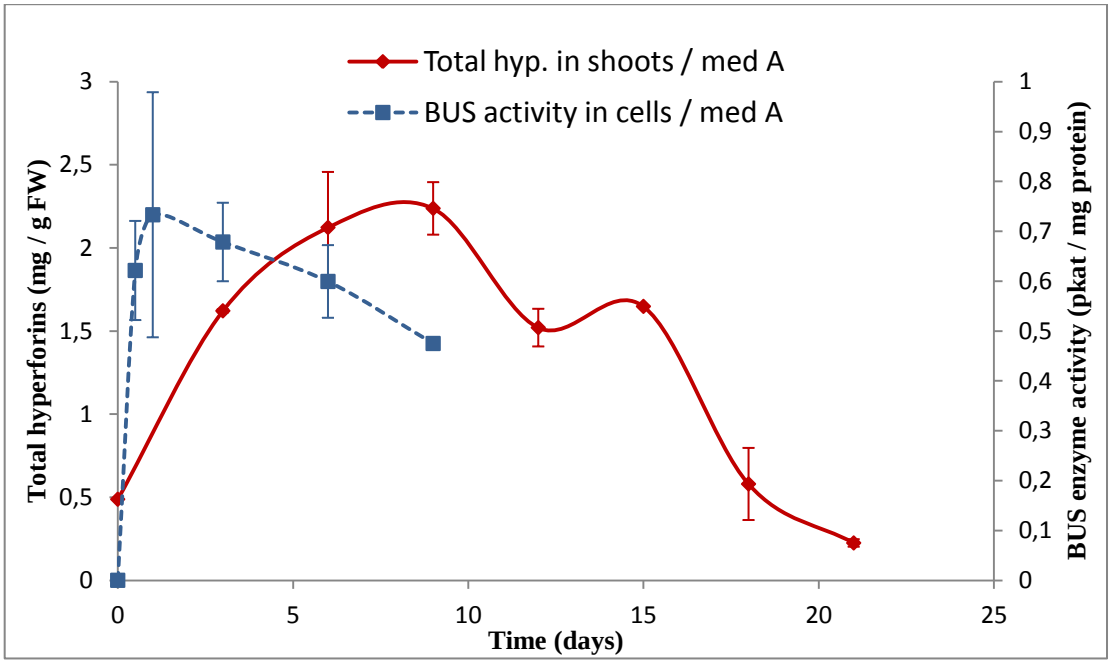


Figure 29: Comparison of the BUS enzyme activity in *H. perforatum* cell cultures during 9 days of cultivation in the liquid medium A and the hyperforin production in *H. perforatum* type B shoots during 21 days of cultivation on the solid medium A.

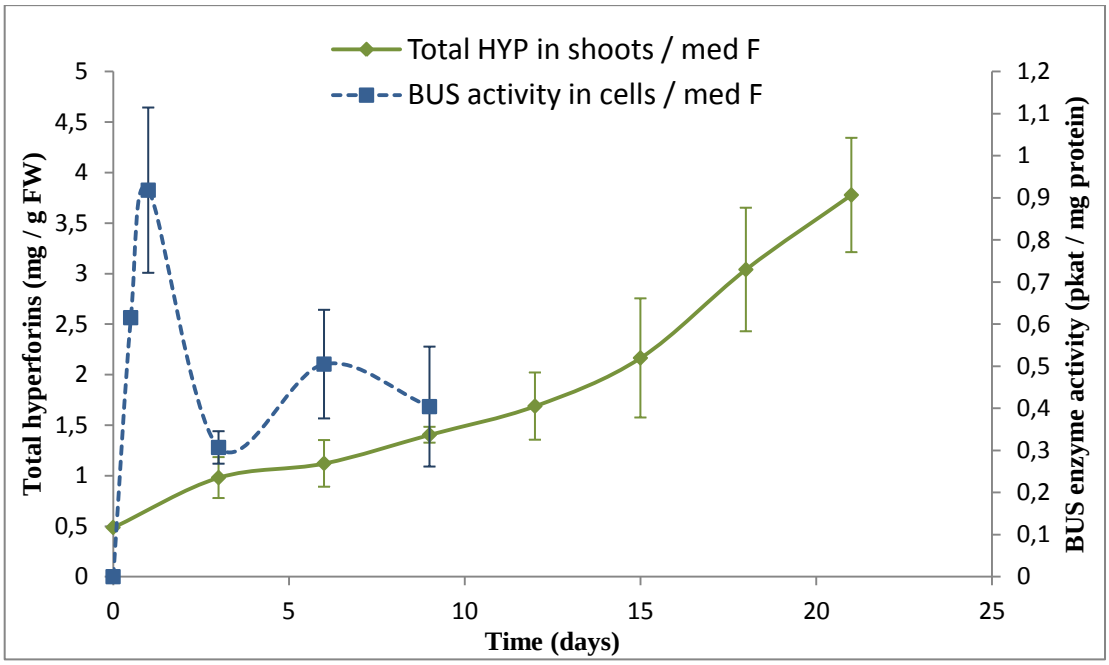


Figure 30: Comparison of the BUS enzyme activity in *H. perforatum* cell cultures during 9 days of cultivation in the liquid medium F and the hyperforin production in *H. perforatum* type B shoots during 21 days of cultivation on the solid medium F.

We were not able to find a direct correlation between the time curves of enzyme activity in cell cultures and the hyperforin production in the shoot cultures after applying media A and F. This was probably due to an incomparable tissue differentiation and substrate diversity between these two plant materials. The time curves of hyperforin production in *H. perforatum* shoots revealed individual effects of each medium. This can be explained by the specific induction of enzymes participating in the final steps of hyperforin biosynthesis, that is, the attachment of prenyl chains onto the phlorisobutyrophenone moiety. These enzymes are prenyltransferases, which were found to be active in the translucent glands of the leaves and in their delimiting cells. In that secretory plant tissue they are also involved in the biosynthesis of monoterpenes, components of the essential oils, which are required for proper accumulation of hydrophobic molecules like hyperforin (17). Translucent glands and thereby prenyltransferases, appear in the leaves in a later developmental stage, which explains why very small amount or no hyperforin was detected in *H. perforatum* cell cultures in earlier studies (34), (33).

We did, however, confirm that the substrates, which we applied, have been enzymatically converted, by performing the same experiments, but this time with the addition of denaturised protein extract. In this case the results were negative.

If enzyme activity in the first part of hyperforin biosynthesis is not the limiting factor for its accumulation, it is probably restricted by insufficient development of TG, due to inhibitory effects of NAA and BAP. Thus, the shoots in our experiments have lost their ability to properly synthesise and accumulate hyperforin. On the other hand, it is also possible that the BUS activity was blocked by the influences of the media and, as a result, there was no need for expression of prenyltransferases nor for the formation of secretory cavities needed to accumulate these enzymes.

Recent findings about substrate specificities of BUS and BPS are adding additional uncertainty to this experiment. The obvious resemblance of BUS and BPS activity time curves in media A and F might be due to the fact that only BPS was active in cell cultures and that only this enzyme utilized isobutyril-CoA as a substrate. Moreover, earlier studies have shown that *H. perforatum* calli and suspended cells produce mainly xanthones, which are end products of the benzophenone synthase activity (33).

Altogether, we were able to determine and confirm that different nutrient combinations differently affect metabolic activity in *H. perforatum in vitro* cultures. Despite high standard deviations, the results represent quite reliable basic data, which can be integrated in the process of further hyperforin production optimization. Even though the *in vitro* cultures have been kept under constant conditions throughout the experimental time, the plant material has been inevitably exposed to various temperatures and surroundings during the process of extraction.

6. CONCLUSION

By optimisation of hyperforin production, with help of biotechnology and molecular biology, we could improve the existing antidepressant therapy. Therefore, we analysed biosynthetic abilities of hyperforin and its derivatives in *H. perforatum in vitro* shoot and cell cultures, cultivated on/in different modifications of the Murashige and Skoog growth media.

The results revealed interesting influences of growth media composition on the accumulation of hyperforin in *in vitro* shoots. Phytohormones, also referred to as plant growth regulators, appear to have the greatest influence on the tissue differentiation, regarding the formation of translucent glands and accumulation of hyperforin. We discovered that a certain combination of phytohormones, *myo*-inositol and higher amounts of vitamins in the solid MS medium, referred to as the medium A, represents an optimal nutrient combination for a strong and sudden induction of biosynthetic activity in the shoots. This kind of increase in metabolite concentration probably correlates with a higher enzyme activity and mRNA concentrations. Thus, the use of the medium A as an inducer might represent a step closer towards enzymatic and genetic elucidation of hyperforin biosynthesis in *Hypericum* species. Even more so, the application of this media modification on *H. perforatum* plants with a complete absence of hyperforin could be used as a basic system for the creation of a subtractive cDNA library. Previous studies have confirmed a branching point in the biosynthetic pathway of secohyperforin and hyperforin, which gives us the possibility to separately modulate their production and isolate mRNA molecules involved in the last steps of only hyperforin biosynthesis (3). For the optimisation of such approach, investigations would have to include stable cultivation of hyperforin-free shoots and the induction of endogenous hyperforin production after their subcultivation on the medium A. We were able to detect hyperforin absence only in the hormone-containing media (C and E). Therefore, the next interesting research would be to study these shoots on the medium A.

In the experiments with cell cultures of *H. perforatum* we showed the induced enzyme activity after applying the media A. It remains uncertain, whether the activity of isobutyrophenone synthase, or the suppressed tissue differentiation is the limiting factor for hyperforin accumulation in the shoots cultivated on this medium.

The second modification of the MS medium that appeared to be favourable to production of hyperforin in the cultivated shoots was a hormone-free combination of nutrients with lower concentrations of B vitamins (0.1 mg/l nicotinic acid and 0.1 mg/l pyridoxine), which we referred to as the medium F. The cultivation of the shoots on this MS medium modification enabled a stable and increasing accumulation of hyperforin in translucent glands. The presence of translucent glands in the leaves correlated with detected amounts of hyperforin. Moreover, the isobutyrophenone synthase activity in *H. perforatum* cell cultures was also induced by the effects of the medium F. Therefore, its liquid formulation might be an optimal medium for a large scale cultivation of *H. perforatum* tissue cultures in bioreactors, although such project would require additional investigations.

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